Review

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The yin and yang of evasion and immune activation in HCC

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Summary

Current systemic treatment options for patients with hepatocellular carcinoma (HCC) are limited to sorafenib. With the recent FDA approval of the second PD1-PD-L1 pathway inhibitor, immunotherapy has gained even more interest as a potential novel treatment option for patients with HCC. This is due not only because of the failure of other treatment approaches in the past, but also because immunological mechanisms have been shown to play an important role during tumor development, growth, and treatment. Here we present a review of immunological mechanisms in the liver relevant for tumor progression and treatment. We summarize our current knowledge on immune activating and immune suppressing mechanisms during tumor initiation, development, and treatment. We try to explain the paradox of how inflammatory responses in a setting of chronic infection promote tumor development, while the primary aim of immunotherapy is to activate immunity. Finally we summarize recent advances in addition to providing an outlook for the immunotherapy of HCC.

Introduction

Primary liver cancer, a disease of etiologic and geographic diversity, is now the second most common cause of cancer related death worldwide, with hepatocellular carcinoma (HCC) accounting for the majority of these cases [1]. Globally, the number of deaths from HCC is close to the number of new patients diagnosed. The majority of all HCC incidence is a consequence of chronic viral infection with hepatitis B (HBV) and C (HCV) [2] and develops in the setting of persistent immune modulation by chronic viral inflammation. The HBV vaccine, which is the first adaptive immunity intervention to prevent cancer development, was introduced worldwide in the 1980s. Since that time, a decreased incidence of HCC has been reported in Asia, where HBV-associated HCC is endemic [3]. In recent years, diabetes and obesity (and consecutive non-alcoholic steatohepatitis (NASH)) have been receiving growing attention as risk factors for tumor development in the US [4]. Surgery and local ablative therapies remain the only curative treatment option while intra-arterial treatments (transarterial chemoembolization – TACE- and selective intra-arterial radiotherapy-SIRT-) and systemic chemotherapy are used in the palliative setting in patients with more advanced disease [5]. Currently, the only FDA approved systemic treatment for HCC is sorafenib, a multikinase inhibitor. Sorafenib was established as the standard of care in advanced HCC after a phase III randomized, placebo controlled clinical trial showed a small improvement in overall survival (OS) from 7.9 months to 10.7 months [6]. Thus there is a clear and urgent need for new therapies for this deadly disease.

Chronic hepatic inflammatory responses are the number one risk factor for liver tumor development. At the same time, immune based approaches aimed at enhancing tumor-specific immune responses are currently under investigation as therapeutic strategies in HCC cancer research. A better understanding of how the immune system promotes tumor growth will hopefully lead to the development of more effective treatment options and will be the focus of this review (Fig. 1). In addition we will discuss currently ongoing and recent clinical trials aiming to enhance anti-tumor immunity either by inducing antigen-specific T cells or by blocking immune inhibitory mechanisms.

Tolerance inducing mechanisms in the liver

Under physiological conditions, the liver has to perform multiple tasks, including the uptake of blood borne pathogens, the excretion of toxic waste substances, and the filtration of environmental or bacterial agents from the gastrointestinal tract. These functions result in enormous antigen exposure to the liver [7,8]. To prevent organ autoimmune damage from ongoing immune stimulation by a myriad of antigens, liver evolution resulted in the development of intrinsic liver tolerogenic mechanisms in the innate and adaptive immune response [8–12] (Fig. 2).

Under physiological conditions, cells of the innate immune system such as Kupffer cells, which are resident macrophages of the liver, release interleukin (IL)-10 [10] and, consequently, inhibit immune responses. Furthermore, constitutive release of another active inhibitory cytokine, transforming growth factor

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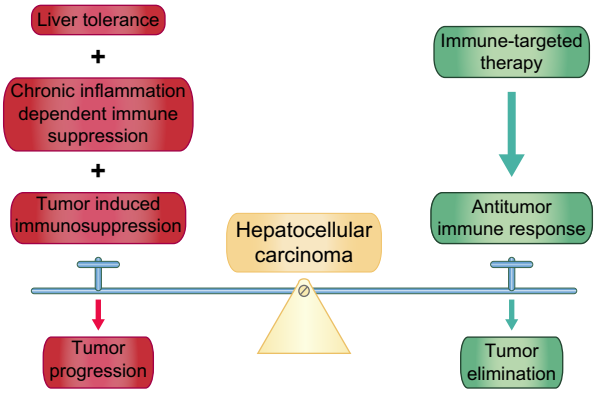

Fig. 1. The counteracting forces regulate the balance between tumor progression and tumor elimination. The forces mediating tumor progression include liver tolerance to antigens, immunosuppression of chronic inflammation and HCC dependent immune tolerance. On the opposite side of the equilibrium is anti-tumor immune response, which can be enhanced by immunotherapy such as immune checkpoint blockade.

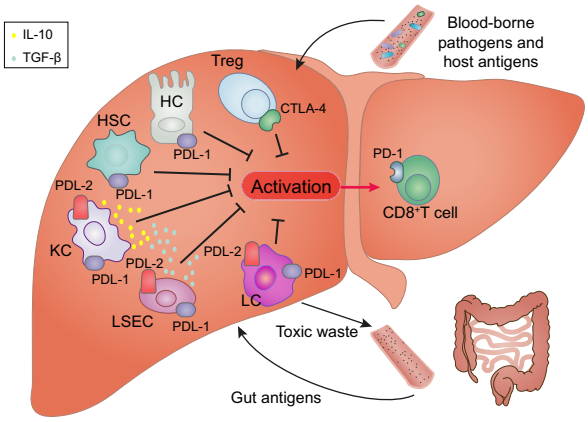

Fig. 2. Under physiological conditions the liver has the capacity to induce tolerance against antigens delivered from the intestine. The identified immune responses promoting liver tolerance to antigens include constitutive inhibitory cytokine release (interleukin 10 (IL-10) by Kupffer cells (KC) and tumor growth factor beta (TGF-β) by KC or liver sinusoidal endothelial cells (LSEC)), and upregulation of immune checkpoints (programmed death-ligand 1 or 2 (PD-L1 or PD-L2) on hepatocytes (HC), hepatic stellate cells (HSC), KC, LSEC and intrahepatic leucocytes (LC), and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) on T regulatory cells (Treg)). All of these immune responses protect the liver from autoimmune damage by blocking activation of effector T cells in the liver microenvironment. While liver immune tolerance is beneficial in case of harmless antigens in cancer-free liver, it may be detrimental with respect to HCC antigen immune escape in cancer-bearing host.

(TGF)-β, by endothelial liver cells and Kupffer cells has been identified [13] as a contributor to local immunosuppression. Although liver sinusoidal endothelial cells (LSECs) can promote differentiation of T cells into memory-like T cells, which give rise to effector T cells after reactivation by antigen presenting cells (APCs) [14] and induces a rapid generation of cytotoxic T cells [15], tolerogenic liver priming by endotoxins leads to defective antigen processing by LSECs, diminishing antigen-specific immune surveillance [11]. Moreover, a decrease in surface expression of immune co-stimulatory molecules, B7-1 (also known as CD80) and B7-2 (a.k.a. CD86) [11], on LSECs limits the ability of these APCs to activate $CD4^+$ T cells, further decreasing adaptive immune system surveillance in liver tissues.

The B7-1 and B7-2 molecules are expressed on various types of APCs and are essential components of the B7-CD28/CTLA-4 immune checkpoint pathways. Interactions of B7-1 and B7-2 transmit co-stimulatory signals to antigen-primed T cells when they are ligated with the CD28 receptor on T cells or co-inhibitory signals when they interact with cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), the inhibitory checkpoint receptor on T cells, therefore enhancing or downregulating T cell activation. With respect to the immunosuppressive role of CTLA-4 in the liver, CTLA-4 expression by $Foxp3^+CD25^+CD4^+$ T regulatory cells (Tregs) has been linked to the induction of host immune tolerance after liver transplantation [16] and, thus represents a potential organ-specific mechanism to regulate immune activation. In addition to the B7-CD28/CTLA-4 pathway, another immune checkpoint pathway, PD-L1/PD-L2/PD-1 (programmed death-ligand 1 or 2/programmed cell death 1 receptor), inhibits immune activation in the liver [17–21]. PD-L1, expressed on hepatocytes [17], hepatic stellate cells (HSCs) [18], LSECs [19], and Kupffer cells [20], contributes to mechanisms of liver immune tolerance via induction of T cell apoptosis [17,18,21] or T cell dysfunction [19]. Furthermore, the physiologic expression of PD-L1 as well as PD-L2 and PD-1 can be increased in chronically inflamed livers [20]. All of these tolerogenic responses can be protective with respect to harmless antigens, yet detrimental in the case of immune tolerance to tumor-associated antigens (TAAs) and HCC progression.

Immunosuppression of chronic inflammation

Chronic inflammation, characterized by the continued expression of different cytokines [22,23] and recruitment of immune cells [20,24,25] to the liver, contributes to HCC carcinogenesis and disease progression by enhancing immune suppression and thus allowing the growth of cancer cells [25,26]. The inhibition of antigen-specific immune surveillance in the chronic inflammatory state is mediated by changes in expression of immune checkpoints [23,27–29], alterations in function of dendritic cells (DCs) [30–32], increases in the frequency of Tregs [25,33], and release of cytokines with immune suppressive functions such as IL-10 and TGF-β [23,33,34].

In recent years, immune inhibitory checkpoints have been increasingly recognized as important players in immunosuppression of chronic inflammation. In chronic viral and autoimmune hepatitis, upregulation of PD-1 expression on – intrahepatic lymphocytes and its ligands PD-L1 (a.k.a. B7-H1) and PD-L2 (a.k.a. B7-H2), on Kupffer cells, LSECs, and leukocytes – have been positively correlated with the degree of liver inflammation [20]. Furthermore, the increased frequency of circulating $CD8^+$ T cells expressing PD-1 has been associated with progression of HBV-related cirrhosis to HCC [35]. In addition to the PD-1 [28,29,36] upregulation, other negative T cell regulatory checkpoint receptors such as CTLA-4 [29] and T cell immunoglobulin domain and mucin domain-3 (Tim-3) [27,37], have been linked to reduced T cell effector function in chronic viral hepatitis. Collectively, these data suggest that immune inhibitory checkpoints contribute to immunosuppression in the chronic inflammatory state, allowing HCC progression.

Interestingly, studies of patients with chronic HCV infection showed that blocking PD-1/PD-L1 or B7-CD28/CTLA-4 signaling restores the capacity for expansion and function of circulating

HCV-specific $CD8^+$ T cells, but not for intrahepatic HCV-specific $CD8^+$ T cells [29,36]. The refractoriness of intrahepatic $CD8^+$ T cells to PD-1/PD-L1 blockage can be explained by Treg-mediated immunosuppression, upregulation of CTLA-4 in HCV-specific hepatic $CD8^+$ $PD\text{-}1^{high}$ T cells [25] and reduced expression of co-stimulatory molecules CD28 and CD127 (IL-7R alpha) by these cells [29,36]. The exhaustion of intrahepatic $CD8^+$ T cells has been reversed by using combined PD-1 and CTLA-4 inhibition [29]. Therefore, this finding suggests that a combination of immune targeting treatments might be necessary for restoration of antigen-specific $CD8^+$ T cell functions in chronically inflamed livers.

HCC immune tolerance

In tumor-bearing hosts, the strategies promoting tolerance to tumor antigens include a decrease in recognition of malignant cells and suppression of immune system function in the tumor microenvironment [38], both of which can lead to cancer progression. Although the pathways involved in cancer-induced immune tolerance have not been fully identified, available data define multiple immune responses promoting HCC progression and thus indicate promising targets for therapy aimed at restoring anti-tumor immunity. These responses include changes in the number or function of immune cells, cytokine level and immune receptor or ligand expression.

On a cellular level, failure of HCC-associated antigen presentation by APCs as a consequence of decreased expression of HLA class-I molecules [39] and ineffective tumor antigen processing [40], contributes to the inability of the immune system to recognize liver cancer. Other immune responses contributing to HCC tolerance include increases in Tregs [41,42], invariant natural killer T cells (iNKT) [43], $CD14^+HLA\text{-}DR^{-/low}$ monocytic-like myeloid-derived suppressor cells (MDSC) [44], and tumor-associated macrophages (TAMs) [45–47], as well as diminished $CD4^+$ T helper cells [48]. With respect to Tregs, an increase in $CD4^+CD25^+$ Tregs within tumor infiltrating lymphocytes (TILs) has been described in association with a decrease in number and function of $CD8^+$ T cells [41,42]. This finding suggests the use of immune treatments aimed at Treg depletion as a possible therapeutic strategy for restoration of anti-tumor immune responses. Furthermore, an increase in Treg numbers either in peripheral circulation or within TILs has been associated with decreased OS [49,50], and thus can be further explored as an immune prognostic marker in biomarker development.

Different cytokines in association with a unique innate immunity signature within the tumor microenvironment have been reported to promote the development of HCC metastases. This poor prognostic signature includes an increase in immunosuppressive cytokines (IL-4, IL-5, IL-8, and IL-10) accompanied by suppression of immune activating cytokines (IL-1, tumor necrosis factor (TNF), and interferon gamma (IFN-γ)) [51]. Furthermore, increased IL-10 levels in patients with advanced HCC have been associated with shorter OS and immune dysfunction, such as diminished activity of lymphokine-activated killer (LAK) and natural killer (NK) cells [52].

On a receptor and ligand level, HCC immune tolerance is mediated by immunosuppression via decreased co-stimulatory or increased inhibitory checkpoint signaling. With respect to co-stimulatory molecules, significant reductions of B7-1 and B7-2 (immune co-stimulatory ligands) expression have been identified on HCC cells [53,54] resulting in a decrease of B7/CD28 mediated activation of effector T cells. Furthermore, several other immune co-stimulatory molecules such as CD244 (2B4) [55], CD28 [56], CD40 [57], CD137 (4-1BB) [58], and OX-40 [58] have been studied in liver cancer and will serve as targets for immune agonist antibodies in further clinical development.

Immune inhibitory receptors and ligands also play a major role in induction and maintenance of HCC immune tolerance [35,59–66]. Several inhibitory checkpoints have been associated with immune dysfunction in HCC including CTLA-4 [62–64], PD-1 and its ligand (PD-L1) [35,59,61], lymphocyte-activation gene 3 (LAG-3) [65], Tim-3 and its ligand (galectin-9) [66], and adenosine A2a receptor (A2aR) [67]. In HCC patients, high CTLA-4 expression on Tregs in peripheral blood has been reported in association with a decrease in cytolytic granzyme B production by $CD8^+$ T cells [63]. In addition, CTLA-4 expression on $CD14^+$DCs was associated with IL-10 and indoleamine-2,3-dioxygenase (IDO)-mediated inhibition of T cell proliferation and induction of T cell apoptosis [62].

Another mechanism of tumor-induced immune tolerance is mediated by changes in the PD-L1/PD-1 immune checkpoint pathway. The increased expression of PD-1 has been reported on $CD8^+$ T cells in patients with HCC [35,59], as well as an increase in tumor infiltrating and circulating $PD\text{-}1^+CD8^+$ T cells associated with disease progression after curative hepatic resection [35]. In addition to the upregulation of PD-1 on T cells, its ligand, PD-L1, is highly expressed on peritumoral stroma cells (Kupffer cells, LSECs, and monocytes) as well as cancer cells, promoting a PD-L1/PD-1 pathway-driven inhibition of anti-tumor T cell responses [59,61,68,69]. This new understanding of increased PD-1/PD-L1 expression provides the rationale for the use of PD-1 and PD-L1 immune checkpoint blocking antibodies in HCC treatment.

However, because HCC-induced immune tolerance in the setting of a tolerogenic liver environment and chronic inflammation is associated with multiple immunosuppressive mechanisms, dual or triple combinations of immune targeting agents along with inhibitory checkpoint blockage as a backbone of therapy are the most promising strategies for clinical development.

Spontaneous tumor-specific immune responses

Despite multiple immunosuppressive alterations, the majority of liver cancer patients mount detectable adaptive immune responses against tumor antigens [64,70]. The detected TAAs against which HCC-specific $CD8^+$ T cells have been identified include alpha-fetoprotein (AFP) [70,71], highly immunogenic cancer-testis antigen NY-ESO-136 [72–74], other cancer-testis antigens such as synovial sarcoma X breakpoint 2 (SSX-2) [72,73], catalytic enzyme telomerase reverse transcriptase (TERT) [64,75], cyclophilin B40 [64], melanoma antigen gene-A (MAGE-A) [72], and fetal oncoprotein glypican-3 (GPC3) [76,77]. Generally, *in vitro* antigen-specific stimulation is required to detect these T cells indicating that T cells only exist at very low frequencies. As a promising treatment strategy, enhancement of TAA-directed immune responses by vaccines and immune checkpoint combinations may result in HCC growth control and tumor regression. In addition to immune responses against classical TAAs, spontaneous immune responses against neo-antigens which are specific for individual tumors and important for immunotherapy have been identified in patients with

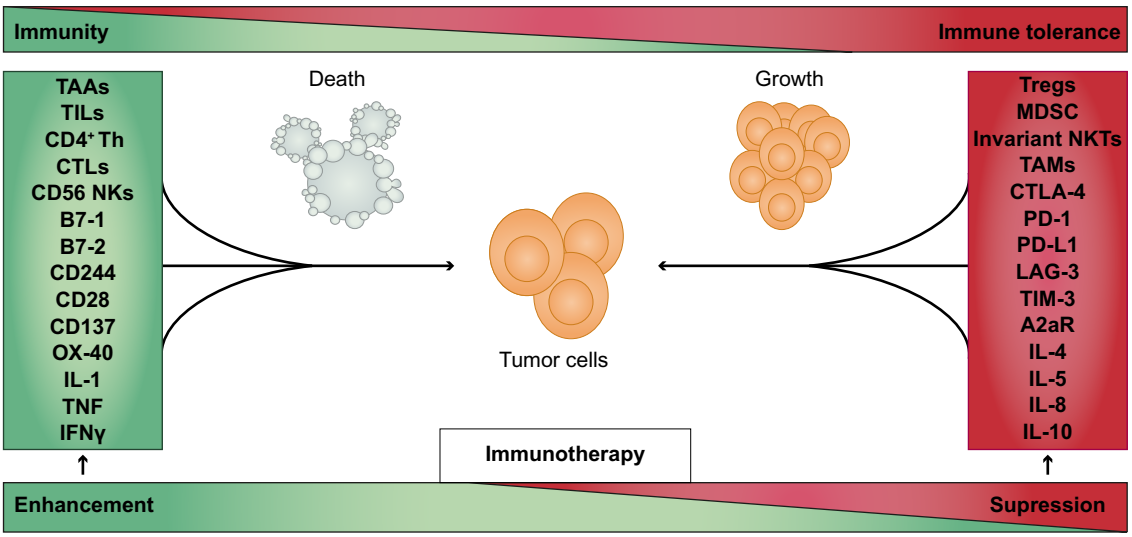

Fig. 3. Immune status of an HCC-bearing host is characterized by different immune responses leading to immunity or immune tolerance and thereby promoting tumor cell death or growth, respectively. Unfortunately, multiple immune alterations associated with carcinogenesis and disease progression shift the immune response towards tumor immune tolerance and disease progression. Treatment approaches such as immune checkpoint blockers can bring the balance back towards immunity and cancer cell death.

melanoma [78,79]. In future, the identification of tumor-specific mutated epitopes inducing immune responses in patients with HCC might provide additional targets for immunotherapy.

Other immune responses, which can serve as targets for enhancement by immunotherapy, include an increase in TILs and cytotoxic NKs. The presence of $CD4^+$ and $CD8^+$ TILs has been associated with a decrease in HCC recurrence after liver transplantation or hepatic resection [43,80]. In addition, after hepatic resection or radiofrequency ablation (RFA), the detection of cytotoxic ($CD56^{dim}$) or activating killer immunoglobulin-like receptor (KIR2DS5) NK cell phenotypes has been correlated with improved OS [81]. Therefore, therapies aimed at increasing TILs or cytotoxic NK cells could improve outcomes after potentially curative surgery.

Collectively, all of these facts suggest that an HCC-bearing host harbors multiple immune regulatory changes that support tumor tolerance and disease progression rather than anti-tumor immunity and cancer elimination. The identification of immune responses that affect the equilibrium between primary liver cancer progression and tumor elimination provides the targets for HCC immunotherapy development (Fig. 3).

Clinical trials

Multiple immune targeted therapeutic strategies aimed at inducing or enhancing anti-tumor immunity against HCC have been evaluated in clinical trials. Most of the agents have been investigated as single agent therapies and are in early phases of clinical trial development (Table 1).

Vaccines

For more than a decade, vaccines targeting HCC TAAs have been investigated in early clinical trials with a mixed history of success and failure [82,71,83–85]. Approximately two thirds of patients with HCC have been reported to have detectable immune responses to one or more TAAs [64]. These findings support vaccine treatments as an attractive strategy for therapeutic development. AFP was the first TAA to be targeted in clinic for HCC treatment. AFP is a self-antigen normally present in fetal development, therefore AFP is well-tolerated by the host immune system. Different modalities have been used to overcome AFP immune tolerance and induce immune responses in clinical vaccination settings. In 2003, the first HCC vaccine clinical trial reported detectable T cell responses to AFP after HLA-A*0201 patients with AFP-positive HCC were immunized with a vaccine to four HLA-A*0201-restricted AFP peptides [71]. Subsequently, a phase I/II clinical trial was completed with only transient immunologic responses detected, possibly due to the limited number of antigens used or deficient $CD4^+$ helper T cell support [71]. In an attempt to overcome these limitations, a phase II clinical trial used DCs pulsed with lysates of a hepatoblastoma cell line containing multiple antigens. Of 25 patients receiving 3 or more DC infusions, disease control rate was 28% (1 partial response, 6 stable disease) and disease stabilization time 6–16 months. However, a short median survival of only 168 days was reported. Notably, detectable antigen-specific T cell immune responses were associated with clinical responses and a fall in AFP expression [85]. Better clinical outcomes were observed in another HCC vaccine trial, whereby DCs pulsed with autologous tumor lysates were administered in two different treatment strategies: 5 courses of DCs weekly or 5 induction treatments followed by monthly boost vaccination maintenance. Reported outcomes among 31 treated patients were: 12.9% partial response, 54.8% stable disease and overall 1 year survival rate of 10.7% (±9.4) *vs.* 63.3% (±12.0) favoring the maintenance treatment schedule [82]. A cell-free vaccine platform was tested in the adjuvant settings when two patients were vaccinated with plasmid DNA followed by AFP-expressing replication-deficient adenovirus injection. AFP-specific T cell responses were observed [86], but further studies are clearly needed to verify the clinical efficacy

Table 1. Clinical trials of immune targeted therapies in hepatocellular carcinoma.

Author, year	Regimen	Disease status	Design	Total number of patients	Results (patients)
Immune checkpoint inhibitors					
Sangro [106], 2013	Tremelimumab (Anti-CTLA-4)	Advanced	Single arm	21	N17: PR (3) 17.6 %, SD (10) 58,8%; OS (21) 8.2 ms
Sangro [108], 2013	Nivolumab (Anti-PD-1)	Advanced	Single arm	n.a.	n.a.
Duffy [107], 2014	Tremelimumab + TACE or RFA	Advanced	Single arm	n.a.	n.a.
HCC vaccines					
Butterfield [71], 2003	4 AFP peptides	Advanced	Single arm	6	AFP-specific T-cell responses detected
Lee [82], 2005	DCs + auto-tumor lysate	Advanced	Single arm, 2 schedules	14 QW×5 17 QW×5 + QM	PR (4) 12.9%, SD (17) 54.8% 1-yr OS 40.1 ms
Butterfield [118], 2006	DCs + 4 AFP peptides	Advanced	Single arm	16, 10 treated	No objective responses
Palmer [85], 2009	DCs + HepG2 lysate	Advanced	Single arm	35	N25 ≥3 DCs: PR (1) 4%, SD (6) 24%, OS (N35) 168 ds
Greten [84], 2010	GV1001 + GM-CSF	Advanced	Single arm	40	SD (17) 45.9%, OS 358 ds
Sawada [87], 2012	GPC3 peptides	Advanced	Single arm	33	PR (1) 3%, SD (19) 57.6% OS 12.2 (G3-CTL ≥50) *vs.* 8.5 ms (G3-CTL <50)
Butterfield [86], 2014	AFP DNA prime + AdV	Adjuvant	Safety	2	AFP-specific T-cell responses detected
Adoptive cell transfer					
Takayama [92], 2000	Activated T cells	Adjuvant	Randomized	150	RFS 37 *vs.* 22 % $p = 0.01$, 3, 5-yr OS-no difference
Hui [119], 2009	Activated T cells	Adjuvant	Randomized	127, 3 groups	1, 3,5 -yr OS-no difference
Shimizu [94], 2014	Activated T cells + DCs vaccine	Adjuvant	Non-randomized	94, 2 groups	RFS 24.5 *vs.* 12.6 ms $p = 0.01$ OS 97.7 *vs.* 41.0 ms $p = 0.029$
Cytokines					
Llovet [96], 2000	IFN-α2b	Advanced	Randomized	58	PR (2) 6.6% 1, 2-yr OS-no difference
Shiratori [97], 2003	IFN-α	Adjuvant	Randomized	74	First RR-no difference 5 yr OS 68% *vs.* 48%
Mazzaferro [98], 2006	IFN-α	Adjuvant	Randomized	150	RFS-no difference
Sun [99], 2006	IFN-α	Adjuvant	Randomized	236	OS 63.8 *vs.* 38.8 ms $p = 0.0003$
Lo [100], 2007	IFN-α2b	Adjuvant	Randomized	80	OS-trend for benefit
Chen [101], 2012	IFN-α2b	Adjuvant	Randomized	268	OS, RFS-no difference
Ishikawa [102], 2012	IFN-α2b/RBV	Adjuvant	Randomized	54	3-yr OS 90.2 *vs.* 61.2%
Sangro [103], 2004	Ad.IL-12	Advanced, GI tumors	Single arm	21	Primary liver cancer N9: 1 PR
Mazzolini [104], 2005	DCs transfected with Ad.IL-12	Advanced, GI tumors	Single arm	17	Primary liver cancer N9: no responses
Sandrine [105], 2014	LY2157299 (TβRI inhibitor)	Advanced	Randomized to 2 doses	109	OS 36 ws
Others					
Safran [109], 2013	Lenalidomide	Advanced	Single arm	40	PR (6) 15%, OS 7.6 ms
Stemmer [67], 2013	CF102 (A(3)AR agonist)	Advanced	Single arm	18	SD (4) 22%, OS 7.8 ms
Yen [110], 2014	GC33 (anti-glypican 3)	Advanced	Randomized	185	PFS, OS-no difference

IFN-interferon (randomized clinical trials with more than 50 patients enrolled), RBV-ribavirin, Ad.IL-12-adenoviral vector encoding human interleukin-12 genes, GI-gastrointestinal, TβRI inhibitor-transforming growth factor-β receptor I inhibitor, TACE-transarterial chemoembolization, RFA-radiofrequency ablation, AdV-AFP-expressing replication-deficient adenovirus, A(3)AR agonist-A3 adenosine receptor agonist, RR-recurrence rate, RFS-recurrence free survival, PR-partial response, SD stable disease, OS-overall survival, ds-days, ws-weeks, ms-months, yr-year, QW-every week, QM-every month, no difference no statistically significant difference, N/A-not available, G3-CTL-glypican-3-specifc cytotoxic T lymphocytes.

of this approach. Lastly, telomerase reverse transcriptase peptide vaccines were tested in patients with advanced HCC. In a phase II trial of GV1001, the immunomodulatory effects of low-dose cyclophosphamide and GM-CSF were used for immune sensitization. In 40 patients treated with this combination, no radiological responses or detectable GV1001-specific immune responses were reported, although an immune response such as a decrease in the number of $CD4^+CD25^+Foxp3^+$ Tregs was detected [84]. In another trial HLA-A∗24:02-restricted $GPC3_{298-306}$ or HLA-A∗02:01-restricted $GPC3_{144-152}$ peptides were tested and demonstrated a

promising outcome: detectable GPC3-specific CTL response in 30 out of 33 patients and a positive correlation of GPC3-specific CTL with survival [87].

These results showed the potential of HCC vaccines to elicit immunological and clinical anti-tumor responses. New and emerging preclinical and clinical evidence can guide the design of effective treatment strategies with the aim of augmenting these responses and translating the serologic anti-tumor immune activation seen into significant clinical benefit. In the recent study, detection of T cell responses against multiple TAA was associated with superior tumor growth control [88]. Therefore, the development of multi-antigen vaccines may have the potential to improve the modest clinical efficacy of vaccination observed in clinical trials evaluating single-antigen vaccines. Importantly, the B7-CD28/CLTA-4 immune checkpoint pathway was described as inhibiting $CD4^+$ T cell proliferation induced by DCs in response to a specific antigen [89]. Furthermore, B7-CD28/CTLA-4 ligation increases IL-10 production by DCs [89], which is the inhibitory cytokine capable of strongly downregulating the antigen presenting function of human monocytes [90]. These antigen-masking capacities of immune checkpoints provide a preclinical rational for vaccine and immune checkpoint inhibitor combinations to achieve enhanced TAA-specific immune activation. Among other future strategies, computer-guided HCC epitope-optimization [91], which increases immunogenicity of known TAAs by biomedical engineering, can be used to enhance anti-tumor immune responses. To further maximize clinical benefit, epitope-optimized vaccines can be combined with immune checkpoints targeting antibodies. These combinations have the potential to achieve a synergistic immunostimulatory effect thereby providing more meaningful clinical benefits of vaccine therapies.

Adoptive cell transfer

Adoptive cell transfer (ACT) is an autologous infusion of ex-vivo selected, activated and expanded TILs, which are extracted from patient's tumor or from peripheral blood. ACT has been investigated in adjuvant settings for HCC treatment. In a randomized clinical trial, 76 out of 150 patients who had undergone curative resection received adjuvant activated autologous lymphocyte infusions. The untreated cohort of 74 patients was considered as the control group. Although an improvement in recurrence-free outcomes was observed, no significant difference in OS was reported between treated and control groups [92]. Different strategies have been applied to augment ACT treatment clinical benefits such as systemic administration of cytokines [93] or vaccination [94]. In a recently published clinical trial, patients with HCC after curative resection were treated with an autologous tumor lysate-pulsed DC vaccine and activated T cell transfer (ATVAC) combination. Patient outcomes after surgery alone (52 patients) were compared with combination treatment (42 patients) and an impressive difference was reported in OS, 41.0 months *vs.* 97.7 months, respectively [94]. This significant clinical benefit provided by a dual immunotherapy combination supports a combined modality approach in the development of new adaptive immunity targeted therapies.

Cytokines

Innate immunity treatments with cytokines have been extensively studied for treatment of HCC. Interferon (IFN), which is used in the treatment of hepatitis C, was a logical choice for HCC treatment development for its possible dual antiviral and anti-tumor action. Multiple randomized clinical trials of different schedules and classes of IFN have been completed with mixed results reported with regard to recurrence-free and survival outcomes [95–102]. Although IFN treatment has the potential to benefit selected patients with HCC, it comes at the expense of a poor side effect profile such as flu-like symptoms (chills, fever, headache, myalgia, malaise), myelosuppression, depression, and hepatotoxicity. One possibility to overcome these effects may be an intratumoral application, which has been achieved using an adenovirus-based approach or transduced dendritic cells [103,104].

Among new promising cytokine targeting approaches, a novel small molecule inhibitor of TGF-β receptor I, LY2157299, is under active investigation for HCC. Preliminary results of LY2157299 therapy were reported in a phase II clinical trial, where 109 were enrolled and a median OS of 36 weeks (93.1 weeks in AFP responders *vs.* 29.6 weeks in non-AFP responders) was observed [105]. Currently, LY2157299 is being evaluated in a phase II, non-randomized trial as a single agent or in combination with sorafenib (NCT01246986).

Immune checkpoint inhibitors

To date, all immune checkpoint targeted therapies consist of monoclonal antibodies developed for a specific immune target. Although several immune checkpoint blocking agents were described in preclinical models, the majority of clinically developed therapies are antibodies that target PD-1, PD-L1 and CTLA-4 molecules. Among those, ipilimumab (anti-CTLA-4) in 2011 as well as pembrolizumab and nivolumab (anti-PD-1) in 2014 were approved by the FDA for the treatment of melanoma. Among CTLA-4 targeted therapies, only tremelimumab, a fully human IgG2 monoclonal antibody, has been clinically evaluated in HCC. A phase II, non-controlled, multicenter tremelimumab clinical trial enrolled patients with HCC and chronic HCV who were not eligible for surgery or locoregional therapy. In this trial, 21 patients with advanced HCC and Child-Pugh scores A (57.1%) or B (42.9%) were enrolled. Each treated patient received 15 mg/kg tremelimumab every 90 days as a single agent therapy. The trial showed a partial response rate of 17.6%, disease control rate of 76.4% and median OS of 8.2 months. Although 45% of patients experienced above grade 3 transaminase toxicity, the transaminitis was transient, only being observed with the first tremelimumab dose and without the need for systemic steroids. Notably, tremelimumab induced a progressive decrease in viral load in most of the patients and a complete viral response in 3 patients [106]. These results indicate a dual effect of anti-tumor and antiviral activity of tremelimumab, suggesting that immune checkpoint treatment can be particularly beneficial in patients with a viral etiology such as HBV or HCV-related HCC. Currently, we are testing tremelimumab in combination with local therapies (TACE or RFA) in a pilot phase I/II clinical trial [107].

Among PD-1/PD-L1 targeted treatments, nivolumab (BMS-936558), which is a fully human IgG4 monoclonal antibody targeting PD-1 receptor, is under active investigation in a phase I dose escalation clinical trial [108]. This trial was designed to evaluate safety and preliminary activity of nivolumab in patients with HCC with or without HBV or HCV infection [108].

Additionally, another anti-PD-1 monoclonal antibody pidilizumab (CT-011) was evaluated in phase I clinical trial (NCT00966251). Unfortunately, the trial was terminated early because of slow accrual without reporting any collected results. Although other immune checkpoint antibodies including LAG3, TIM-3 and NK-inhibitory receptors were described to have antineoplastic activity in preclinical models, their clinical efficacy in patients with HCC has not yet been reported [65,66]. Furthermore, preclinical studies of other immune modulatory molecules such as CD244 (2B4) [55], CD137 (4-1BB) [58], and OX-40 [58] identified another area for further clinical development of immune agonist treatments.

Other immune based approaches

Several other immune targeted agents have been evaluated in phase I/II clinical trials. Treatment with lenalidomide, an immune modulator, was investigated in patients with advanced HCC. A phase II trial testing the efficacy of lenalidomide after sorafenib failure reported a partial response of 15% and OS 7.6 months in patients with advanced HCC [109]. Another phase II trial evaluated the efficacy of a monoclonal antibody against glypican 3, which is an oncofetal protein expressed in liver cancer cells. In this trial, patients with advanced HCC were randomized in a 2:1 ratio to anti-glypican antibody (GC33) *vs.* placebo. A total of 185 patients were treated with no difference in progression free survival or OS observed between the two groups. Notably, analysis of drug exposure revealed that high GC33 exposure had a favorable OS relative to placebo, 9.7 *vs.* 6.7. Therefore, the investigators concluded that a suboptimal dose of GC33 was potentially responsible for the lack of treatment benefit [110]. In addition, safety data has been reported for several immune targeted therapies in HCC treatment, namely mapatumumab, an agonist to tumor necrosis factor-related apoptosis-inducing ligand receptor 1 (TRAIL-R1) [111], tigatuzumab (CS-1008), a death receptor 5 agonist [112], and CF102, an A(3) adenosine receptor agonist [67]. These early investigations reported good tolerability justifying their further development.

Future perspectives: biomarkers, combinations and outcome assessment

Novel opportunities have opened for HCC therapy development after the reported significant clinical benefits of immune checkpoint blockade not only in traditionally highly immunogenic cancers such as melanoma, but also in other solid tumors. As part of effective HCC immune therapeutic strategies, biomarker identification, combination of immune checkpoint targeting treatment with known or new immune modulating therapies, and an effective outcome assessment criteria warrant further investigations.

Detection of biomarkers to guide treatment selection can improve patient care by predicting potential responders to particular immunotherapies. The use of biomarkers offers a promise of personalized immune treatment combinations by defining which immune pathway is the driver in each particular patient and, thus, by identifying the optimal therapeutic target. However, the dynamic expression and heterogeneous geographic distribution of immune molecules present challenges for biomarker development. These characteristics can lead to errors in patient selection if patients are excluded from potentially beneficial treatments based on immune receptors or ligand presence in a specific time and place. Therefore, the identification of immune molecules which are more static in expression or use alterations in specific immune components such as immunomodulatory cytokines (IL-4, IL-10, TGF-β), immunomodulatory cells (TILs, circulating Treg, TAA-specific $CD8^+$ effector T cells) as surrogate biomarkers could be crucial in successful immune biomarker development.

Although immune-directed treatments such as cytokines, vaccines, and activated immune cell infusions have been investigated for HCC treatment, low clinical responses were reported after single immune agent therapy, most likely because multiple immune pathways are involved in HCC progression in the setting of an immune tolerogenic liver microenvironment. Typically, HCC-bearing host immune status is characterized by maintenance of tumor immune tolerance in a setting of chronic inflammation with a major input from several immune inhibitory checkpoints (PD-1, PD-L1, CTLA-4, LAG-3, and TIM-3) [35,59,61,62,65,66,68]. In addition, physiologic liver immune tolerance to antigens, which among other immune alternations is characterized by the upregulation of immune checkpoints, plays a contributory role in HCC progression and resistance to immunotherapy.

To overcome these dual liver and tumor immune tolerance phenomena, the use of combination immunotherapies with inhibitory checkpoint blockage offers the greatest promise. Immune checkpoint pathway-targeting agents combined with the previously investigated immune therapies may provide an additional boost to immune system activation and give a "second chance" to already known immune targeted treatments. Furthermore, the development of immunotherapy in adjuvant settings, i.e. after TACE procedure, can augment conventional treatment benefits. Additionally, double or triple immune checkpoint combinations with new immune targets can be used for anti-tumor immune response induction or enhancement. In future investigations, simultaneous treatments with most clinically developed immune checkpoint targeting therapies (anti-CTLA-4, anti-PD-1, and anti-PD-L1 antibodies), or combinations of checkpoint blockage with antibodies to co-stimulatory (CD-137, OX-40) or co-inhibitory signals (TIM-3, LAG-3, NK-inhibitory receptors), new epitope-optimized vaccines [91], or novel cytokine therapies [113] are potential strategies for successful treatment development. Another strategy to potentiate immune responses is the combination of immunostimulatory drugs with RFA. This rationale is supported by data showing RFA-induced increase in TAAs-specific T cell responses [114]. In contrast to RFA, combining immunotherapy with sorafenib is complicated by the notion that sorafenib has been associated with immunosuppressive effects such as inhibition of NK function [115], increase in MDCS [116], intratumoral accumulation of Tregs and M2-types macrophages, and upregulation of PD-L1 expression [117].

Finally, a unique feature of the immune tumor response that brings additional challenges in immune treatment development is the potential to induce a local immune inflammatory reaction. This reaction can appear as an initial increase in size or enhancement of HCC lesions on magnetic resonance imaging or computed tomography before tumor size regression or resolution of enhancement. This phenomenon dictates usage of the Immune-Related Response Criteria (irRC), which is a criteria based on the total tumor volume changes and confirmatory imaging at

least 4 weeks apart prior to declaring disease progression. To further improve the outcome assessment, the irRC can be used in conjunction with HCC lesion measurements to incorporate the tumor viability features as an additional metric for treatment response in radiologic assessment.

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Conflict of interest

The authors declare no conflicts of interest in relation to this manuscript.

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