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REVIEW

Immune checkpoint inhibition: prospects for prevention and therapy of hepatocellular carcinoma

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The global prevalence of liver cancer is rapidly rising, mostly as a result of the amplified incidence rates of viral hepatitis, alcohol abuse and obesity in recent decades. Treatment options for liver cancer are remarkably limited with sorafenib being the gold standard for advanced, unresectable hepatocellular carcinoma but offering extremely limited improvement of survival time. The immune system is now recognised as a key regulator of cancer development through its ability to protect against infection and chronic inflammation, which promote cancer development, and eliminate tumour cells when present. However, the tolerogenic nature of the liver means that the immune response to infection, chronic inflammation and tumour cells within the hepatic environment is usually ineffective. Here we review the roles that immune cells and cytokines have in the development of the most common primary liver cancer, hepatocellular carcinoma (HCC). We then examine how the immune system may be subverted throughout the stages of HCC development, particularly with respect to immune inhibitory molecules, also known as immune checkpoints, such as programmed cell death protein-1, programmed cell death 1 ligand 1 and cytotoxic T lymphocyte antigen 4, which have become therapeutic targets. Finally, we assess preclinical and clinical studies where immune checkpoint inhibitors have been used to modify disease during the carcinogenic process. In conclusion, inhibitory molecule-based immunotherapy for HCC is in its infancy and further detailed research in relevant *in vivo* models is required before its full potential can be realised.
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INTRODUCTION

Primary liver cancer is the sixth most prevalent cancer globally, but importantly the second most common cause of cancer-related death due to limited treatment options.[1] The risk of adult primary liver cancer is considerably enhanced by cirrhosis resulting from viral hepatitis (hepatitis B virus (HBV) and hepatitis C virus (HCV)), alcohol, obesity, metabolic liver diseases and aflatoxin exposure. Paediatric primary liver cancer generally results from genetic conditions, such as Beckwith–Wiedemann syndrome, hemihypertrophy and familial adenomatous polyposis, and inborn metabolic errors, such as tyrosinaemia, alpha-1 antitrypsin deficiency and glycogen storage disease type 1. Resection and percutaneous local ablation are the only treatment options for early-stage tumours. Repeated transarterial chemoembolisation is used for intermediate stage, while oral sorafenib is the gold-standard treatment for advanced hepatocellular carcinoma (HCC) with only modest improved survival time.[2] Thus it is imperative that new alternatives are developed to limit liver cancer development or to treat advanced liver cancer.

HCC, cholangiocarcinoma (or bile duct cancer), primary hepatic angiosarcoma and hepatoblastoma represent the four main subtypes of primary liver cancer. Rare variants are tumours with combined hepatocellular and cholangiocellular features, referred to as a mixed hepatocellular cholangiocarcinoma.[3] HCC is the most studied subtype and accounts for 85–90% of all primary liver cancers. There is evidence to support its origin from hepatocytes or a liver stem/progenitor cell in both adults and children.[4] Cholangiocarcinoma is a heterogeneous malignancy that develops in the biliary tree of adults and is classified as intrahepatic, perihilar or distal based on the anatomical location.[5] Primary hepatic angiosarcoma is an extremely rare soft tissue sarcoma in which pleomorphic endothelial cells grow into vascular spaces, including sinusoids and terminal hepatic venules.[6] Hepatoblastoma is similarly a very rare paediatric primary liver cancer thought to arise from a hepatocyte precursor known as a hepatoblast, which is present during fetal liver development.[7]

The original six hallmark features of cancer focussed on tumour cell features that enabled survival, proliferation and dissemination.[8] Importantly, the immune system has now also been recognised to be central to tumorigenesis in an expanded roster of hallmarks of cancer.[8] Accordingly, a number of strategies to inhibit carcinogenesis are being developed, which target distinct immunological

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mechanisms.[9] The immune system can (i) suppress viral-induced tumours by protecting the host against infection,[9] (ii) prevent establishment of a chronic inflammatory environment that promotes cancer by inducing genetic instability and mutation in target cells[9,10] and (iii) eliminate tumour cells that often co-express ligands for activating innate immune cell receptors and tumour antigens that are recognised by lymphocyte receptors.[9] However, importantly, the tolerogenic nature of the liver presents unique and specific challenges to suppressing hepatic tumour development.

Oncolytic immunotherapy has been explored in many types of tumours. Immunotherapy for HCC, though, is relatively under-explored. Interleukin-12 (IL-12) cytokine administration and IL-12-based gene and cell-based therapies have been used to treat HCC in preclinical studies.[11–13] Granulocyte macrophage colony-stimulating factor-based gene therapy has been used to successfully reduce tumour burden in HCC patients.[14] However, it is unclear if the efficacy was due to immune- or viral-based oncolysis. Adoptive transfer of chimeric antigen receptor-modified T cells is presently being examined as a possible therapeutic for HCC.[15] There is similarly much excitement with the advent of monoclonal antibody-based therapy to block immune-inhibitory molecules, such as programmed cell death protein-1 (PD-1), programmed cell death 1 ligand 1 (PDL-1) and cytotoxic T lymphocyte antigen 4 (CTLA4), which prevent T cells from killing tumour cells. This therapy is now widely used in the clinic to treat solid tumour melanoma[16] and is in the early stages of evaluation in the setting of HCC. Importantly, the immune response to blocking of inhibitory molecules can be sustained beyond the prescribed treatment duration.

In this review, we will explore how the immune system regulates the development of the most common liver cancer, HCC, with a focus on the inhibitory receptors known as immune checkpoint inhibitors. We will first review the T-cell subsets present in the liver together with basic concepts of T-cell activation, differentiation and exhaustion. We will briefly discuss mechanisms that contribute to hepatic immune tolerance as well as cytokines that regulate the tolerance. Immune inhibitory molecules have primarily been viewed as agents that inhibit immune responses to tumour cells. However, it is now apparent that their expression is also upregulated during viral infection and chronic inflammation. Thus we will focus on how viral hepatitis, chronic inflammation and HCC use the inhibitory molecules to subvert the immune system's ability to prevent hepatic carcinogenesis. Finally, we will explore how the new inhibitory molecule-based immunotherapies may be utilised to prevent HCC development or treat advanced HCC.

IMMUNE CELLS AND CYTOKINES INVOLVED IN HCC DEVELOPMENT

The immune system has a central role in the progression of chronic liver disease and HCC development with the relative contributions and responses of regulatory, helper and cytotoxic effector T cells central to determining whether chronic liver disease progresses to HCC. In particular, cytotoxic T cells are essential for targeting and destroying infected or tumorigenic hepatic cells while regulatory T cells dampen cytotoxic T-cell responses. The T-cell subsets present are determined by contextual information such as the cytokine milieu and strength of antigen stimulation. Below, we examine the key basic concepts of T-cell biology and cytokine regulation of hepatic tolerance that are important for HCC development.

T-cell activation and differentiation

Naive T cells activate and clonally expand in response to antigen-presenting cell (APC)-expressed major histocompatibility complex (MHC)-I or MHC-II binding to the T-cell receptor (TCR) of $CD8^+$ or $CD4^+$ T cells, respectively. In general, the clonally expanded $CD4^+$ T cells differentiate into regulatory and helper T-cell subsets in response to the cytokines they are exposed to and as defined by their transcription factor expression and cytokine secretion. The most common hepatic $CD4^+$ T-cell subsets are T helper type 1 (T_h1), T_h2, T_h9 and T_h17 helper T cell and regulatory T cells (T_{regs}) subsets. $CD8^+$ T cells differentiate into cytotoxic T cells and subsequently into memory T cells. The cytokine combinations that induce each subset *in vitro*, together with the master transcription factor expressed, characteristic cytokines secreted and global function(s) of each T-cell subset, are described below.

T_h1 cells. IL-12 together with interferon (IFN)-γ induce naive $CD4^+$ T cells to differentiate into T_h1 cells, which express the T-bet transcription factor and secrete IFN-γ and tumour necrosis factor (TNF).[17,18] T_h1 cells promote cell-mediated immunity and activate clearance of tumour cells and intracellular pathogens as a result of production of the pro-inflammatory cytokines.[17]

T_h2 cells. T_h2 cells are produced from naive $CD4^+$ T cells in response to IL-4 and IL-33, express GATA-3 transcription factor and secrete IL-4, IL-5, IL-10, IL-13 and IL-25.[17,18] T_h2 cells suppress T_h1-driven inflammation and associated tissue damage and remove extracellular parasites such as helminths and *Schistosoma* as a result of recruitment of eosinophils, mast cells and basophils.[17]

T_h9 cells. T_h9 cells are produced from naive $CD4^+$ T cells by stimulation with transforming growth factor (TGF)-β together with IL-4, express PU.1 transcription factor and secrete IL-9 and IL-10.[18] T_h9 cells promote T_h2 responses in murine atopic diseases, are pro-inflammatory in ulcerative colitis and antitumoral in solid tumour models.[18] Peripheral blood T_h9 cell numbers are elevated in cirrhotic patients and are positively associated with the severity of cirrhosis.[19] However, the nature of the T_h9 cells' role in chronic liver disease remains undetermined.

T_h17 cells. T_h17 cells are produced from naive $CD4^+$ T cells in the presence of TGF-β together with IL-6 and IL-21 in mice or IL-23 in humans.[18] T_h17 cells express retinoic acid–related orphan receptor γt transcription factor and produce IL-17, IL-21, IL-22 and TNF. IL-17 promotes endothelial cell expression of granulocyte macrophage colony-stimulating factor (or colony-stimulating factor-2) and/or granulocyte colony stimulating factor (or colony-stimulating factor-3) and thus inflammatory neutrophil and monocyte recruitment.[20] T_h17 cells may have a critical role in the development of liver diseases such as viral hepatitis and alcoholic liver disease as well as HCC where their numbers correlate with angiogenesis and poor prognosis.[21,22] Indeed, IL-17 mediates the spontaneous HCC that develops by 65 weeks of age in mice expressing human unconventional prefoldin RPB5 interactor (URI) in hepatocytes.[23] However, similar to T_h9 cells, T_h17 cells' role is yet to be determined. T_h17 cells are also important for clearance of extracellular bacteria and fungi.[17]

Regulatory T cells. IL-2 in concert with TGF-β induces naive $CD4^+$ T-cell differentiation to T_{regs} *in vitro*.[17] T_{regs} express the Foxp3 transcription factor and secrete IL-10 and TGF-β, which both suppress the inflammatory functions of monocytes, macrophages, dendritic cells (DCs) and B cells.[24] T_{regs} also promote further differentiation of naive $CD4^+$ T cells to T_{regs} as a result of TGF-β production. Importantly, T_{regs} maintain homeostasis and immune tolerance.[25]

However, an overabundance of T_{regs} promotes an immunosuppressive environment.[24]

T_{regs} may be derived *in vivo* from naive $CD4^+$ T cells either in the thymus (thymus-derived T_{regs}) or in peripheral tissues, such as lymph nodes, spleen and liver (peripheral-derived T_{regs}). The definitive origin of T_{regs} in the peripheral tissues including liver can, however, not be ascertained owing to the current lack of defining markers.[26] Thus, in this review, we will refer to them collectively as T_{regs}.

Cytotoxic and memory $CD8^+$ T cells. Naive $CD8^+$ T cells clonally expand in response to IL-12, or type I IFNs (IFN-α, IFN-β).[27] Cytotoxic T-cell numbers then diminish during clearance of the infection, and the remaining cells mature into memory $CD8^+$ T cells. IL-7 and IL-15 maintain memory T-cell numbers in the absence of antigen.[28] Cytotoxic antigen-specific $CD8^+$ T cells express TNF, IFN-γ, Fas ligand, granzyme B and perforin and kill virus-harbouring or cancer cells expressing a specific antigen. *In vivo*, optimal $CD8^+$ T-cell function usually requires help from $CD4^+$ T cells, including T_h1 cells, in the form of regulatory cytokines (for example, IL-21) and chemokines as well as activating DCs via CD40:CD40 ligand interactions.[27,29] Thus the absence of $CD4^+$ T cells results in defective $CD8^+$ T-cell production, reduced $CD8^+$ memory cells and the inability to mount a vigorous secondary immunogenic response.[30]

Regulation of T-cell activation strength

The strength of T-cell activation is controlled by the amount of antigen presented to the TCR and by positive and negative co-receptors. The most commonly described APC-expressed co-stimulatory receptors are CD80 (or B7-1) and CD86 (or B7-2), which engage T cell-expressed CD28 to induce T-cell activation, as a result of T-cell survival promoted by autocrine IL-2 signalling (Figure 1a).[31] The functions of other co-stimulatory receptors have recently been reviewed extensively by Chen and Flies.[31]

Activation of the TCR is diminished by APC-expressed co-inhibitory receptors such as PD-L1 (or B7-H1 or CD274) and PD-L2 (or B7-DC or CD273), which both interact with T cell-expressed PD-1 (or CD279) and dephosphorylate the TCR (Figure 1b). Further, T-cell activation can be downregulated by APC-expressed CD80 binding to CTLA4 or CD152, which outcompetes costimulatory CD28 for binding to APC-expressed CD80 as a consequence of higher binding affinity (Figure 1c). APC-expressed PD-L1 binding to T-cell-expressed CD80 and APC-expressed CD80 binding to T-cell-expressed PD-L1 also attenuates T-cell activation although the precise mechanism is unknown.[32] Further, APC-expressed galectin 9 and MHC-I/MHC-II can bind to T-cell-expressed T-cell immunoglobulin and mucin-domain containing-3 (Tim-3) and lymphocyte-activation gene 3 (Lag-3/CD223), respectively, to attenuate T-cell activation. Other inhibitory receptors have also recently been reviewed by Chen and Flies.[31]

T-cell exhaustion

T-cell exhaustion has been described for both $CD4^+$ and $CD8^+$ T-cell populations and occurs in response to persistent antigen exposure and/or inflammation. Exhausted T cells have sustained expression of co-inhibitory receptors, reduced effector function and an altered transcriptional state compared with functional effector or memory T cells.[33] Importantly, exhausted T cells cannot mature into memory T cells. T-cell exhaustion is characteristic of chronic viral infection and tumorigenesis.

Induction of T-cell exhaustion. A key feature of T-cell exhaustion is the chronic exposure of the TCR to antigen, with the extent of exhaustion correlating with the level and duration of antigen exposure. Inhibitory receptor signalling through PD-1 also downregulates T-cell activation.[34] Another contributing factor to $CD8^+$ T-cell exhaustion may be $CD4^+$ T_{helper} cell exhaustion with concomitant enhanced IL-10 and IL-21 secretion.[35]

T-cell exhaustion is a stepwise process where the ability of the T cells to produce IL-2 and proliferate is affected first, followed by TNF secretion in the intermediate stages, with IFN-γ secretion most resistant to inactivation.[33] At the same time, there is a progressive increase in the number and expression levels of co-inhibitory receptors such as PD-1, CTLA4 and LAG3, over and above the levels induced during differentiation, in addition to altered transcription factor expression. In the final phase, the antigen-specific T cell can be deleted. Importantly, it is now widely appreciated that exhausted T cells are distinct from memory T cells and require antigen for survival. Exhausted T cells are refractory to IL-7 and IL-15, which promote memory T-cell survival.[33] For further extensive review of the T-cell exhaustion process, please see Wherry and Kurachi.[36]

Reversal of T-cell exhaustion. T-cell exhaustion can be reversed by antibody neutralisation of the co-inhibitory receptors known universally as immune checkpoint inhibitors and that reduce T-cell activation. Neutralising antibody blockade of PD-1 or PD-L1 in the chronic murine lymphocytic choriomeningitis virus (LCMV) infection model established that it was possible to reverse T-cell exhaustion.[37] Specifically, PD-1 blockade increased proliferation, cytokine production and cytolytic activity of hepatic and splenic $CD8^+$ cytotoxic T cells in chronic murine LCMV infection resulting in reduced viral titre. Increased efficacy of treatment can be gained by combining blockade of PD-L1 and other inhibitory molecules, such as Lag-3 and Tim-3.[38,39]

Blockade of inhibitory molecules has also been combined with therapies directed at extrinsic pathways, including IL-2 or IL-10. IL-2 administration synergises with PD-L1 blockade to enhance virus-specific $CD8^+$ T-cell responses and reduce viral titre in the chronic LCMV infection mode despite a concomitant increase in T_{reg} numbers.[40] IL-10 can upregulate PD-L1 expression, and blockade of both IL-10 signalling and PD-L1 can also synergistically enhance cytotoxic $CD8^+$ T-cell expression of IFN-γ and TNF and likewise reduce LCMV viral titre.[41] Additionally, T_{reg}-depletion synergises with PD-1 or Tim-3 blockade to augment $CD8^+$ T-cell responses in the murine Friend Virus model.[42,43] These first successful attempts at combined therapies in preclinical hepatic viral models highlighted their potential clinical value in a patient setting.

Hepatic tolerance and T-cell regulation

The liver is the only non-lymphoid tissue in which naive $CD4^+$ and $CD8^+$ T cells produced in the thymus can be activated independently of the spleen and lymph nodes.[44,45] Naive $CD4^+$ and $CD8^+$ T cells are activated in the liver by non-conventional APCs in addition to DCs.[46] Non-conventional hepatic APCs expressing MHC-I and MHC-II include Kupffer cells (KCs or resident macrophages), liver sinusoidal endothelial cells (LSECs) and hepatic stellate cells.[47] Hepatocytes can also cross-present antigen but only to $CD8^+$ T cells as they express MHC-I and not MHC-II.[47] Hepatic stellate cells, though, may present the antigen via transfer to LSECs in a process akin to trogocytosis, the process of intercellular plasma membrane protein exchange.[48]

Under homeostatic conditions, the liver is continuously exposed to harmless blood-borne non-self antigens originating from dietary

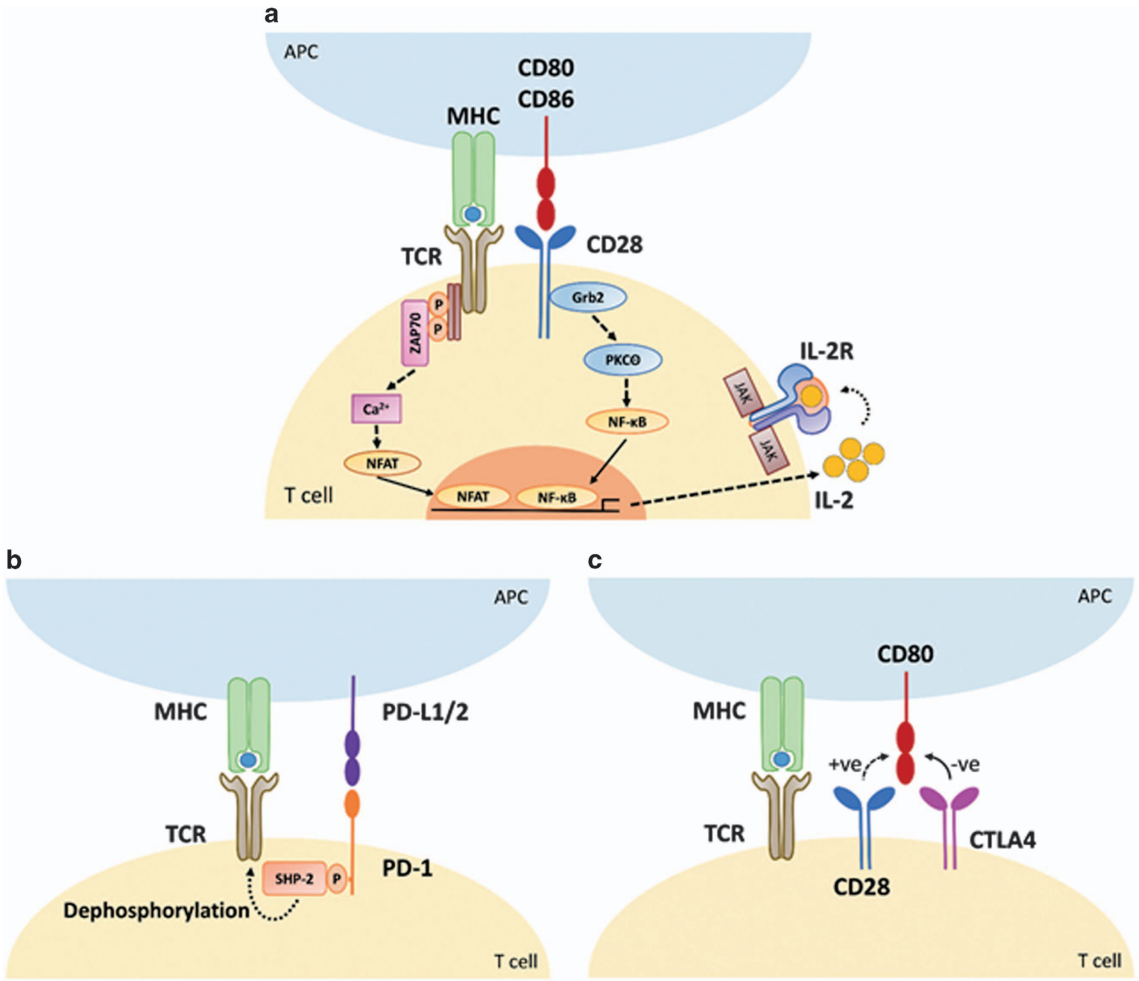

Figure 1 (**a**) T-cell activation is promoted by antigen presented to the TCR by APC-expressed MHC. The strength of T-cell activation is enhanced by APC-expressed CD80 or CD86 binding to T cell-expressed CD28. TCR and CD28 downstream signalling promote nuclear factor (NF)-activated T cells (NFAT) and NF-κB nuclear relocation, respectively, which synergise to promote IL-2 production and T-cell survival by IL-2 autocrine signalling to the IL-2R. (**b**) T-cell activation strength is diminished by APC-expressed PD-L1 or PD-L2 binding to T-cell-expressed PD-1 to promote dephosphorylation of the TCR and reduce TCR downstream signalling and IL-2 production. (**c**) T-cell-expressed CTLA4 binds to APC-expressed CD80 with higher affinity than CD28 and attenuates CD28 downstream signalling and IL-2 production. JAK, Janus-activated kinase.

nutrients, intestinal commensals and cellular debris. Thus specialised mechanisms must be used to maintain hepatic tolerance. Conventional and non-conventional APCs in the liver typically express low levels of MHC molecules as well as CD80 and CD86 and are accordingly weak T-cell activators.[49] KCs are key regulators of tolerance through their ability to produce heightened amounts of IL-10 in response to lipopolysaccharide that then downregulates expression of MHC molecules, CD80 and CD86 by the other APCs.[50,51] Additionally, hepatic DCs differ from other tissue DCs in that they also produce elevated amounts of IL-10.[52] Naive $CD8^+$ T cells also promote PD-L1 expression by LSECs, which in turn suppresses IL-2 production by $CD8^+$ T cells.[53] LSECs indirectly promote tolerance by reducing DC expression of co-stimulatory molecules and IL-12 in a process known as 'vetoeing'.[54] Thus homeostatic hepatic-naive T-cell activation is low, resulting in tolerance.

Cytokine regulation of hepatic tolerance. Hepatic tolerance and breaking of tolerance (or hepatic immunity) is also modulated by microenvironment factors produced by surrounding APCs and T cells themselves, including anti- and pro-inflammatory cytokines, such as IL-10, TGF-β, IFN-γ and TNF. IL-10 induces tolerance via effects on T cells and dampening inflammatory responses. TGF-β is a key cytokine in maintenance of immune homeostasis or tolerance, but elevated levels can promote detrimental tolerance or immunosuppression in the context of tumour cell immune surveillance. IFN-γ and TNF are both thought to break immune tolerance, although chronic exposure to TNF may induce immunosuppression. The effects of these cytokines on hepatic tolerance are briefly discussed below.

Interleukin-10. Hepatic IL-10, produced by KCs, DCs, LSECs, hepatic stellate cells, T cells[55] and tumour-induced myeloid-derived suppressor cells directly and indirectly promotes hepatic tolerance.[56] IL-10 directly inhibits $CD4^+$ T-cell activation and thus reduces effector T-cell numbers[57] and DC-primed cytotoxic $CD8^+$ T-cell function.[29] Hepatic T-cell activation is also indirectly reduced by IL-10 as a result of downregulation of MHC-II and co-stimulatory CD80/CD86 molecules on KCs and LSECs with a subsequent reduction of their T-cell stimulatory capacity.[58] Further, IL-10 downregulates nuclear factor-κB activity with concomitant reduction of inflammatory cytokines, including TNF, IL-6, IL-8 and IL-12, and thus alleviates T-cell activation.[59]

Transforming growth factor-β. Hepatic TGF-β, produced by KCs, DCs, LSECs, hepatic stellate cells, T_{regs}, natural killer (NK) T cells and myeloid-derived suppressor cells also promotes hepatic tolerance.[60] TGF-β is critical for naive $CD4^+$ T-cell differentiation to T_{regs} and inhibits differentiation of naive $CD4^+$ T cells to pro-inflammatory T_h1 and T_h2 T-cell subsets as a result of inhibiting expression of the T_h1 and T_h2 essential transcription factors, T-bet and GATA3, respectively.[61,62] Additionally, TGF-β inhibits differentiation of naive $CD8^+$ T cells to cytotoxic $CD8^+$ T cells independently of Smad3.[63,64] Further, TGF-β inhibits cytotoxic $CD8^+$ T-cell activity as a result of inhibition of perforin and IFN-γ expression.[65]

Interferon-γ. The primary sources of IFN-γ in the liver are NK cells, $CD4^+$ T_h1, cytotoxic $CD8^+$ T cells and NKT cells.[66] IFN-γ secretion by NK and T cells is promoted by IL-12, IL-18 and IFN-γ itself in a feedforward loop, but production in NKT cells is independent of cytokine signalling.

IFN-γ is required for T_h1 immunity as it induces expression of the T_h1 master transcription factor, T-bet, resulting in naive $CD4^+$ T-cell differentiation to T_h1 cells. *In vitro*, IFN-γ inhibits differentiation of T_h2 and T_h17 subsets. However, paradoxically, T_h1 and T_h17 cells are often found in close association in pathology. *In vivo*, T_h1 cell production of IFN-γ can trigger myeloid APC production of IL-1 and IL-23 that promote T_h17 differentiation, at least in psoriasis.[67] IFN-γ also activates innate immune cells such monocytes, macrophages and neutrophils to break tolerance and enhance antiviral effects. IFN-γ promotes M1 macrophage polarisation and boosts innate immune cell production of reactive oxygen species by enhancing the expression of nitric oxide synthase 2, nitric oxide synthase 2 cofactors and NADPH oxidase components.[68] IFN-γ can also promote an immunosuppressive phenotype by synergising with TGF-β to amplify Foxp3 expression by $CD4^+$ T cells and enhance T_{reg} cell differentiation.[68] However, prolonged IFN-γ exposure in IFN-γ-over-expressing mice reduced HCC in DEN-induced HCC model as a result of enhancing hepatocyte apoptosis.[69]

Tumour necrosis factor. Hepatic TNF is produced by KCs, monocytes, DCs, B cells and T_h1 and T_h17 cells. TNF has pleiotropic roles in immune responses and its effects may depend upon the context, amount of TNF present and the involvement of either or both TNF receptors, which often display disparate and opposing roles.[70] The effect of hepatic TNF exposure on immune responses is relatively unexplored. Acute TNF signalling via the TNF receptors 1 and 2 results in apoptosis or inflammation, respectively. Chronic TNF exposure in primary sclerosing cholangitis patients, though, promotes immunosuppression with reduced T-cell proliferation and cytotoxic T-cell activity.[71] The immunosuppression may be due to TNF-induced nuclear factor-κB-mediated upregulation of $CD4^+$ T-cell expression of PD-1 as demonstrated in chronic LCMV infection of neonatal mice.[72]

SUBVERSION OF THE IMMUNE SYSTEM DURING HCC DEVELOPMENT

The immune system can prevent cancer by protecting against viral infection, reducing inflammation and/or eliminating tumour cells as a result of recognition of tumour antigens.[9] Below we will discuss how hepatic immune surveillance is subverted to promote chronic viral infection and inflammation, both of which can amplify liver cancer development. We also examine how hepatic tumorigenic cells can avoid elimination by the immune system, resulting in HCC progression.

Viral-induced chronic liver disease

Globally, HBV and HCV are the leading viral risk factors for liver cancer development. As previously discussed, the tolerogenic nature of the liver invariably means that it does not generate a robust immune response to acute HBV and HCV infections. Thus HBV and HCV trigger persistent and chronic inflammatory infections where $CD4^+$ helper T-cell function is impaired, numbers of exhausted $CD8^+$ cytotoxic T cells with suppressed IL-2, TNF and IFN-γ secretion are reduced and NK cell dysfunction is observed.[73]

The importance of the $CD4^+$ T helper cells in HBV and HCV clearance has been highlighted in the chimpanzee model. In a study by Greyer *et al.*,[29] $CD4^+$ T-cell depletion prior to HBV infection or HCV reinfection gave rise to persistent infection due to reduced responses by the integral $CD8^+$ cytotoxic T cells.[74] Naive $CD8^+$ T cells are 'primed' by $CD4^+$ T cell-induced DC production of IL-15 and differentiate into cytotoxic T cells.[29] Interestingly, though, there are some patients whose HCV-specific T cells are only weakly primed during the acute infection phase.[75] Hepatitis C-infected patients also have increased numbers of $CD4^+$ T_{reg} cells, possibly as a result of the HCV core protein inducing a T_{reg}-like phenotype expressing the T_{reg} transcription factor Foxp3, together with immunosuppressive IL-10.[76]

The mechanisms by which $CD8^+$ T cells become exhausted and/or deleted during viral infection are not completely understood. However, there are several mechanisms in addition to $CD4^+$ T-cell dysfunction that may result in $CD8^+$ T-cell exhaustion. First, prolonged exposure to viral antigen increases TCR signalling. Hepatitis B and C viruses may also downregulate TCR signalling by reducing expression of the TCR-associated signalling molecule, CD3ζ, leading to reduced downstream signalling.[77,78] Expression of the co-stimulatory molecule CD28, is reduced in HBV- and HCV-infected patients, leading to reduced effectiveness of TCR signalling.[77,78] On the contrary, expression of co-inhibitory molecules, including PD-1 and Tim-3, are elevated on virus-specific $CD8^+$ T cells in response to HBV and HCV infection.[74] Treatment of HCV-specific cytotoxic T-cell clones with the Tim-3 ligand, galectin-9, induced apoptosis of the T cells.[79] Tim-3 gene polymorphisms have been linked to enhanced HBV surface antigen seroclearance.[80] Hepatocyte expression levels of PD-1's ligand, PD-L1, are also significantly elevated in response to HBV and HCV infection and may also contribute to the reduced T-cell function. However, as hepatocyte-expressed PD-L1 levels are also increased in autoimmune disease,[81] it has been speculated that this may be a result of elevated inflammation.[74]

Expression of galectin-9, the ligand for Tim-3, is upregulated on KCs and monocytes by HCV and induces expansion of $CD4^+$ T_{regs} in a TGF-β-dependent manner.[79] Hepatitis C virus also downregulates monocyte production of IL-12 as a result of increasing Tim-3 expression.[82] As discussed previously, IL-12 promotes increased $CD4^+$ T_h1 cell numbers.

Non-viral-induced chronic liver disease

The risk of liver cancer is also increased in non-viral pathologies induced by alcohol and non-alcoholic steatohepatitis. Common features of these pathologies are fat deposition, chronic inflammation and elevated TNF, together with persistent injury. Chronic exposure of T cells to TNF downregulates CD3ζ-chain protein expression and results in reduced cell surface TCR complex and associated T-cell hyporesponsiveness as seen in chronic inflammation.[83]

Liver cancer may also be promoted in these pathologies by the free fatty acid, linoleic acid. Linoleic acid induces selective global $CD4^+$ T-cell apoptosis as a result of increased mitochondrial oxidative stress,

and $CD4^+$ T-cell depletion promotes liver cancer in inducible liver-specific MYC oncogenic transgenic mice fed a methionine and choline-deficient diet.[84] $CD4^+$ T cells may attenuate liver cancer development by promoting $CD8^+$ cytotoxic T-cell activity against tumour antigens. Another possible mechanism is that $CD4^+$ T cells are involved in the resolution of inflammation as TGF-β promotes T_h17 T-cell trans-differentiation into T_{reg} cells during inflammation resolution.[85]

The contribution of immune checkpoint signalling to the development of alcohol- and non-alcohol-induced chronic liver disease is relatively unexplored to date. PD-1 protein expression is elevated on $CD4^+$ T cells in alcohol-related cirrhosis and further elevated by chronic systemic endotoxin in acute alcoholic hepatitis patients in the first reported investigation of immune checkpoint molecules in non-viral liver disease.[86] Tim-3 is also upregulated in acute alcoholic hepatitis but not alcohol-related cirrhosis. Ethanol and endotoxin also promote enhanced PD-1 expression on $CD4^+$ and $CD8^+$ T cells, and T_{regs} cells *in vitro*. However, the effect of elevated PD-1 and Tim-3 on T-cell exhaustion was not examined in the study. PD-1, though, promotes inflammation with increased T_h17 cell and neutrophil numbers resulting in enhanced hepatic injury in the murine bile duct ligation model.[87]

Hepatocellular carcinoma

Immune surveillance of HCC is subverted by the hepatic cytokine and immune environment as well as the tumour cells present. Levels of immunosuppressive cytokines IL-10 and TGF-β are upregulated in HCC patients at the same time as pro-inflammatory IFN-γ levels are downregulated.[88] An increased percentage of circulating and tumour-infiltrating $CD8^+$ T cells express PD-1 while tumour cells and tumour-associated monocytes and neutrophils express upregulated PD-L1 and CD47 levels in HCC patients.[89–92] The numbers of myeloid-derived suppressor cells are also elevated in HCC patients.[88,93] Furthermore, activation of the *c-Myc* oncogene switches on PD-L1 and CD47 expression in hepatocytes,[94] raising the possibility that oncogenes may directly regulate PD-L1 levels to subvert surveillance. Nonetheless, the elevated PD-L1:PD-1 interactions reduce $CD4^+$ and $CD8^+$ T-cell numbers and function, which subsequently attenuates immune surveillance. A novel PD-1-expressing B-cell subset whose numbers correlate with disease stage as well as early recurrence has also recently been identified in HCC.[95] The $PD\text{-}1^{hi}$ B cells produce IL-10 in response to PD-1:PD-L1 interaction and so consequently reduce $CD8^+$ cytotoxic T-cell function. Thus the combined actions of cytokines in the tumour environment significantly reduce immune surveillance, allowing HCC to avoid eradication.

IMMUNE CHECKPOINT INHIBITOR THERAPY AND HCC DEVELOPMENT

It is apparent that immune evasion contributes to both early and later stages of HCC development. Immune-based monoclonal antibody therapy has become an attractive alternative to pursue in light of the recent promising outcomes from trials that involve immune checkpoint inhibitors in solid tumour cancer therapy. However, the use of these therapies in treatment at various stages of HCC development is in its infancy. We will discuss how the monoclonal antibody therapy has been used at various stages of HCC development to reduce incidence, from both preclinical and clinical perspectives.

Immunotherapy in viral-induced chronic liver disease

Immunotherapeutic monoclonal antibody treatment of chronic HBV and HCV infection to reverse T-cell exhaustion is relatively unexplored. PD-L1-blockade enhanced IFN-γ production by $CD8^+$ T cells adoptively transferred to HBV transgenic mice and also restored IFN-γ production by $CD8^+$ T cells and viral clearance in the HCV core murine model.[96] *In vitro* blocking of PD-L1 on HBV patient T cells enhanced both $CD4^+$ and $CD8^+$ T-cell production of IFN-γ.[97] Likewise, *in vitro* blockade of Tim-3 on T cells isolated from HBV and HCV patients restores virus-specific $CD8^+$ T-cell cytolytic responses, including IFN-γ expression and T-cell proliferation.[98,99] Despite these promising *in vitro* and *in vivo* results, treating HCV in non-human primates by PD-1-blockade led to mixed results with only one out of three chimpanzees attaining HCV suppression.[100] However, improved outcomes may be achieved by combining the therapy with other immune interventions.

Immunotherapy in non-viral-induced chronic liver disease

Immune checkpoint inhibitors have not been directly used to modify chronic inflammation in non-viral chronic liver disease to date. Instead, they have been used to enhance antibacterial immunity in alcoholic hepatitis. *In vitro* blockade of PD-1 and Tim-3 enhanced IFN-γ and reduced IL-10 production by peripheral blood mononuclear cells and increased neutrophil phagocytic capacity and *Escherichia coli*-stimulated oxidative burst.[86]

Immunotherapy in HCC

Sorafenib, a receptor tyrosine kinase small-molecule inhibitor, is currently the gold standard and the only systemic therapy approved for treatment of unresectable advanced HCC, prolonging survival from 4.2 to 6.5 months in the Asia-Pacific study and from 7.9 to 10.7 months in the SHARP study.[101] The differences in outcomes from the Asia-Pacific and SHARP studies may relate to the disease initiator, with HBV-related HCC accounting for 73% of patients in the Asia-Pacific study compared with 15.5% in the SHARP study. Patients with HCV-related HCC had the greatest benefit with sorafenib treatment prolonging survival from 7.4 to 14.0 months, suggesting its efficacy may depend on the underlying HCC aetiology.[101] The modest survival benefit afforded by sorafenib may be partially derived via beneficial immune modulation, as discussed below, suggesting that combining sorafenib with other immune therapies may be beneficial in the treatment of HCC.

Sorafenib regulates hepatic immunity. Specifically, sorafenib blocks signalling via vascular endothelial growth factor receptor, platelet-derived growth factor receptor and C-Raf.[102–104] Less well known is the ability of sorafenib to block colony-stimulating factor-1 receptor activity, which is required for survival, proliferation and differentiation of monocytes.[105] A number of *in vitro* and *in vivo* studies have now reported that sorafenib also stimulates the immune system. Sorafenib directly upregulates IL-2 secretion by peripheral blood $CD4^+$ T_{regs} isolated from HCC patients.[106] Sorafenib also inhibited the suppressive properties and proliferation of $CD4^+$ T_{regs} as well as enhanced their apoptosis in a preclinical orthotopic HCC model.[107] This was not due to the reduction in tumour burden, as sorafenib also reduced $CD4^+$ T_{regs} numbers in tumour-free mice. Further, sorafenib moderated the number of PD-1-expressing $CD8^+$ T cells. Finally, sorafenib inhibited the activity of the disintegrin and metalloprotease member ADAM-9 to reduce MHC-1-related chain A shedding from tumour cells and enhanced NK cell activity.[108] Hence, it is apparent that sorafenib may increase overall survival via immune effects as well as by the more widely known effects of reducing angiogenesis, arresting cell cycle and inducing apoptosis.

Immune checkpoint inhibitors. A recent study showed that anti-PD-1 therapy was more effective in non-small-cell lung cancer that had a higher nonsynonymous somatic tumour mutation burden.[109] In general, tumour mutation rates are higher when an environmental mutagen is involved such as ultraviolet radiation in melanoma or cigarette smoke in lung cancer. Early studies in HCC suggest that tumour mutation burden is higher in melanoma and lung cancer but may depend upon the aetiology.[110–112] Importantly and similar to melanoma and lung cancer, HCC does not appear to have an 'oncogene addiction'.[113] Thus it is reasonable to suggest immune checkpoint inhibitors as promising HCC treatments. Below, we examine the preclinical and clinical studies that have been reported thus far.

As discussed previously, expression of PD-1 and CD47 are upregulated in HCC. Anti-murine PD-1 antibody monotherapy reduced tumour size of the HCA1 orthotopic tumour model by approximately 50% in preclinical studies. However, the same anti-PD-1 antibody reduced tumour size by only 20% in mammalian sterile 20-like 1 ($Mst1^{-/-}Mst2^{F/-}$) mice in which hepatocarcinogenesis was induced by carbon tetrachloride. Eighteen out of the 41 advanced HCC patients with Child-Pugh score $<$B7 completed a 2-year Phase-I/II clinical safety trial of the PD-1 inhibitor, nivolumab (CheckMate 040; ClinicalTrials.gov number NCT01658878), although the dose cycle was not reported.[114] Two patients had a complete response, while treatment of 23 patients was discontinued as a result of disease progression. Only two patients were discontinued owing to drug-related adverse events. This trial was extended to include a dose expansion arm of 3 mg kg^{-1} every 2 weeks in 214 patients.[115] Complete response was achieved in three patients while partial response was achieved in 39 patients and disease was stable in a further 96 patients.[115]

The expression of CTLA-4 has not been examined in HCC and no preclinical studies involving CTLA4 blockade in HCC have been reported. Notwithstanding, a Phase II clinical trial blocking CTLA4 with a monoclonal antibody has been carried out in 21 patients with unresectable HCC of Child-Pugh class A or B (Clinicaltrials.gov number NCT01008358).[116] Each patient received at least two treatment cycles of 90 days. Seventeen patients were evaluated for tumour response, with no complete responses and three patients with partial response that lasted for up to 15.8 months. Disease was stabilised in a further 10 patients with half stabilised for >6 months.[116]

CD47 is an innate immune checkpoint that binds to signal regulatory protein-α on monocytes, macrophages and DCs. CD47 inhibits phagocytic activity of these APCs by acting as a 'don't eat me' signal. Antibody neutralisation of CD47 enhances phagocytosis of tumour cells and increases the durability of T-cell immune inhibitory therapy. Preclinical studies in various HCC xenograft models have mostly shown that monoclonal antibody inhibition of CD47 suppresses tumour growth.[117] Further, CD47 blockade sensitises Huh7/MHCC-97L tumour cells to the chemotherapeutic drugs doxorubicin and cisplatin by increasing macrophage phagocytosis in *in vivo* murine zenografts.[118] However, to the best of our knowledge, no clinical trials targeting CD47 in HCC have been initiated.

Second-generation immunotherapies against negative regulators of T-cell activation, including Tim-3 and Lag-3, are in the relatively early stages of clinical development for advanced solid tumours. Patients are currently being recruited for Phase I/IIa dose escalation and cohort expansion trials for Tim-3 and Lag-3 (ClinicalTrials.gov NCT02817633 and NCT01968109, respectively). The roles of Tim-3 and Lag-3 are relatively unexplored in HCC development. Tim-3 is expressed on an increased percentage of $CD4^+$ and $CD8^+$ T cells in HBV-induced HCC compared with the surrounding tissue and predicted a poor prognosis.[119] Further, Tim-3 expression on tumour-associated macrophages in HBV-induced HCC stimulated tumour-promoting IL-6 production.[120] Similarly, Lag-3 is also expressed on an increased percentage of $CD8^+$ T cells in HBV-induced HCC and correlates with impaired HBV-specific $CD8^+$ cytotoxicity in HCC patients.[121] However, it remains to be seen how successful these second-generation immunotherapies will be in general.

Immune checkpoint molecules have critical roles in fine-tuning the immune system to maintain self-tolerance. As such, patients may develop immune-related adverse events ranging from common but mild symptoms such as fatique, nausea and skin rash to the severe but much less common such as hypothyroidism and the neurological conditions, Guillain–Barre syndrome and myasthenia gravis.[122] Skin rash (65%), fatigue (55%) and diarrhoea (30%) were the most commonly reported immune-related adverse events in HCC patients receiving anti-CTLA-4 treatment.[116] Hypothyroidism was only observed in 1 out of the 20 patients who underwent the anti-CTLA-4 treatment.[116] Anti-PD-1 treatment of HCC patients resulted in less immune-related adverse events, with skin rash (23%), pruritis (19%) and diarrhoea (10%) most commonly reported.[115]

Another possible immune-related side effect of immune checkpoint inhibitor reactivation of T-cell cytotoxicity is an increase in hepatic damage. Anti-PD-1 (10 mg kg^{-1}) treatment in a proof-of-concept study (NCT00703469) in 10 patients resulted in one Grade 4 elevation (>8 upper limits of normal) of serum alanine transaminase, although other indicators of hepatic function such as bilirubin and the international normalised ratio (that is, blood-clotting test) did not change. The elevated serum alanine transaminase coincided with the onset of a four-log reduction in HCV load and resolved without intervention.[123] Anti-CTLA-4 treatment resulted in a Grade 3 elevation of serum transaminases after the first dose in more than half of the HCV-induced HCC patients but was not associated with a decline in liver function.[116] Moreover, anti-CTLA-4 treatment also reduced the HCV load and tended to increase the numbers of IFN-γ-producing lymphocytes in the study by Sangro *et al.*[116] Regardless, blockade of both CTLA-4 and PD-1 were concluded to be safe in treatment of HCC regardless of viral status.[115,116]

Sorafenib and immune checkpoint inhibitors. As development of resistance is often the long-term response to sorafenib treatment, combination therapy with immune checkpoint inhibitors has been explored in preclinical studies. Sorafenib-resistant tumour cells derived from patient xenografts had elevated CD47 levels and CD47 blockade reduced their proliferation.[118] PD-1 blockade, though, did not enhance sorafenib-reduced tumour growth in the orthotopic HCC implant and Mst-mutant mouse HCC models. However, PD-1 blockade together with inhibition of stromal cell-derived 1α receptor, C-X-C motif chemokine receptor 4, did significantly further reduce tumour growth in these two models.[124] These preclinical data suggest that immune checkpoint inhibitor therapy combined with sorafenib may lead to improved outcomes.[115] We await the outcome of the clinical trial combining sorafenib treatment with the anti-PD-1 monoclonal therapy, PDR001 (ClinicalTrials.gov number NCT02988440). Clinical trials of other currently approved therapies combined with immune checkpoint inhibitors are listed in Table 1.

CONCLUSION

The role of the immune system in liver pathologies leading to HCC is complex and significant. The failure of many new drugs targeting HCC in phase III trials mandates that new and different approaches are more thoroughly explored. Immune checkpoint inhibitor therapy

Table 1 Clinical trials involving immune checkpoint inhibitors

Target	*Antibody*	*Phase*	*Trial ID*	*Sponsor*	*Combination treatment*	*Study population*	*Status*	*Results*
CTLA-4	CP-675,206	2	NCT01008358	Clinica Universidad de Navarra	NA	Patients with HCV-induced HCC not amenable to other therapies	Completed	[115]
	Tremelimumab	1	NCT01853618	National Cancer Institute	TACE, radiofrequency ablation, SBRT or chemoembolisation	Patients with advanced liver cancer	Ongoing/ not recruiting	NA
	Tremelimumab	1/2	NCT02821754	National Cancer Institute	Durvalumab and TACE, radiofrequency ablation, SBRT or chemoembolisation	Patients with advanced liver cancer	Recruiting	NA
PD-1	Nivolumab	1/2	NCT01658878	Bristol-Myers Squibb & Ono Pharmaceutical Co. Ltd.	Part 1—safety; Part 2—comparison with sorafenib; Part 3—combination with Ipillimumab (CTLA-4)	Parts 1, 2—patients with uninfected HCC, HCV-infected HCC patients, HBV-infected HCC patients; Part 3—patients with advanced HCC	Recruiting	[114]
	Nivolumab	3	NCT02576509	Bristol-Myers Squibb & Ono Pharmaceutical Co. Ltd.	Comparison with sorafenib	Patients with advanced HCC	Recruiting	NA
	Nivolumab	1	NCT02837029	Northwestern University, Bristol-Myers Squibb and National Cancer Institute	Y90 glass microspheres	Patients with stages IIIA, IIIB, IIIC, IVA and IVB HCC	Recruiting	NA
	Nivolumab	1/2	NCT02859324	Celgene	CC-122 (pleitropic pathway modifier)	Patients with unresectable HCC	Recruiting	NA
	Nivolumab	2	NCT03033446	National Cancer Centre, Singapore	Y90 radioembolisation	Asian patients with advanced HCC	Recruiting	NA
	Nivolumab	1/2a	NCT03071094	Transgene	Pexa-Vec (JX-594 oncolytic virus)	Patients with advanced liver cancer	Not yet recruiting	NA
	Nivolumab	1	NCT03143270	Memorial Sloane Kettering Cancer Center	Drug eluting bead transarterial chemoembolisation	Patients with advanced HCC	Recruiting	NA
	Pembrolizumab	2	NCT02702414	Merck Sharp & Dohme Corp.	NA	Patients with previously systemically treated HCC	Ongoing/ not recruiting	NA
	PDR001	1/2	NCT02795429	Novartis Pharmaceuticals	INC280 (c-Met)	Adult patients with advanced HCC	Recruiting	NA
	PDR001	1b	NCT02988440	Novartis Pharmaceuticals	Sorafenib	Adult patients with advanced HCC	Recruiting	NA
	SHR-1210	1/2	NCT02942329	The Affiliated Hospital of the Chinese Academy of Military Medical Sciences	Apatinib (VEGFRII)	Patients with HCC or gastric cancer	Recruiting	NA
	SHR-1210	2/3	NCT02989922	Jiangsu HengRui Medicine Co., Ltd	NA	Patients with non-resectable HCC who failed or did not tolerate prior systemic treatment	Recruiting	NA
	Pembrolizumab	1	NCT03099564	Hoosier Cancer Research Network	Y90 radioembolisation	Patients with poor prognosis HCC who are ineligible for liver transplant or surgical resection with well-compensated liver function	Recruiting	NA

Abbreviations: CTLA-4, cytotoxic T lymphocyte antigen 4; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; NA, not applicable; PD-1, programmed cell death protein-1; SBRT, stereotactic body radiation therapy; TACE, transarterial chemoembolization; VEGFR, vascular endothelial growth factor receptor.

to stimulate the immune system has been demonstrated to be safe in HCC patients in the limited clinical data available. While showing promise, efficacy of the checkpoint inhibitors has been somewhat limited. The majority of preclinical studies of immune checkpoint inhibitors in HCC to date have used orthotopic or xenograft models to validate their efficacy. There is currently a profound lack of understanding of how the immune system controls the various stages of HCC development. Comprehensive expression profiling of checkpoint inhibitors together with detailed studies of how the immune system dampens HCC development during disease progression in models of different aetiologies that naturally develop HCC, while mimicking human disease need to be undertaken to realise the clinical potential of immune checkpoint inhibitor therapy. Importantly, these studies would enable the determination of clinical aetiologies and/or stage(s) for which the intervention will be most effective.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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