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

Caryn L. Elsegood
Janina E.E. Tirnitz-Parker
John K. Olynyk

Edith Cowan University

George C.T. Yeoh

Follow this and additional works at: https://ro.ecu.edu.au/ecuworkspost2013

Part of the Medicine and Health Sciences Commons

10.1038/cti.2017.47
Elsegood, C. L., Tirnitz-Parker, J. E., Olynyk, J. K., & Yeoh, G. C. (2017). Immune checkpoint inhibition: Prospects for prevention and therapy of hepatocellular carcinoma. Clinical & Translational Immunology, 6, e161. https://doi.org/10.1038/cti.2017.47
This Journal Article is posted at Research Online. https://ro.ecu.edu.au/ecuworkspost2013/3759
**REVIEW**

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

Caryn L Elsegood1, Janina EE Tirnitz-Parker1, John K Olynyk1,2,3 and George CT Yeoh4,5

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.

Clinical & Translational Immunology (2017) 6, e161; doi:10.1038/cti.2017.47; published online 10 November 2017

**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
mechanisms. The immune system can (i) suppress viral-induced tumours by protecting the host against infection, (ii) prevent establishment of a chronic inflammatory environment that promotes cancer by inducing genetic instability and mutation in target cells, 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. 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. Granulocyte macrophage colony-stimulating factor-based gene therapy has been used to successfully reduce tumour burden in HCC patients. 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. 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) 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 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 (Th1), Th2, Th9 and Th17 helper T cell and regulatory T cells (Tregs) 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.

**Th1 cells.** IL-12 together with interferon (IFN)-γ induce naive CD4+ T cells to differentiate into Th1 cells, which express the T-bet transcription factor and secrete IFN-γ and tumour necrosis factor (TNF). Th1 cells promote cell-mediated immunity and activate clearance of tumour cells and intracellular pathogens as a result of production of the pro-inflammatory cytokines.

**Th2 cells.** Th2 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. Th2 cells suppress Th1-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.

**Th9 cells.** Th9 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. Th9 cells promote Th2 responses in murine atopic diseases, are pro-inflammatory in ulcerative colitis and antitumoral in solid tumour models. Peripheral blood Th9 cell numbers are elevated in cirrhotic patients and are positively associated with the severity of cirrhosis. However, the nature of the Th9 cells’ role in chronic liver disease remains undetermined.

**Th17 cells.** Th17 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. Th17 cells express retinoic acid–related orphan receptor γt transcription factor and produce IL-17, IL-21 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 monocye recruitment. Th17 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. 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. However, similar to Th9 cells, Th17 cells’ role is yet to be determined. Th17 cells are also important for clearance of extracellular bacteria and fungi.

**Regulatory T cells.** IL-2 in concert with TGF-β induces naive CD4+ T-cell differentiation to Tregs in vitro. Tregs 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. Tregs also promote further differentiation of naive CD4+ T cells to Tregs as a result of TGF-β production. Importantly, Tregs maintain homeostasis and immune tolerance.
However, an overabundance of T<sub>reg</sub> promotes an immunosuppressive environment.

T<sub>reg</sub> may be derived in vivo from naïve CD4<sup>+</sup> T cells either in the thymus (thymus-derived T<sub>reg</sub>) or in peripheral tissues, such as lymph nodes, spleen and liver (peripheral-derived T<sub>reg</sub>). The definitive origin of T<sub>reg</sub> in the peripheral tissues including liver can, however, not be ascertained owing to the current lack of defining markers. Thus, in this review, we will refer to them collectively as T<sub>reg</sub>.

**Cytotoxic and memory CD8<sup>+</sup> T cells.** Naïve CD8<sup>+</sup> T cells clonally expand in response to IL-12, or type I IFNs (IFN-α, IFN-β). Cytoxic T-cell numbers then diminish during clearance of the infection, and the remaining cells mature into memory CD8<sup>+</sup> T cells. IL-7 and IL-15 maintain memory T-cell numbers in the absence of antigen. Cytotoxic antigen-specific CD8<sup>+</sup> T cells express TNF, IFN-γ, Fas ligand, granzyme B and perforin and kill virus-harbouring or expanded in response to IL-12, or type I IFNs (IFN-β). Functions of other co-stimulatory receptors have recently been examined, and costimulatory receptors include 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.

**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. Another contributing factor to CD8<sup>+</sup> T-cell exhaustion may be CD4<sup>+</sup> T helper cell exhaustion with concomitant enhanced IL-10 and IL-21 secretion.

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. 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.

**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. Specifically, PD-1 blockade increased proliferation, cytokine production and cytolytic activity of hepatic and splenic CD8<sup>+</sup> 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.

**Hepatic tolerance and T-cell regulation**

The liver is the only non-lymphoid tissue in which naïve CD4<sup>+</sup> and CD8<sup>+</sup> T cells produced in the thymus can be activated independently of the spleen and lymph nodes. Naïve CD4<sup>+</sup> and CD8<sup>+</sup> T cells are activated in the liver by non-conventional APCs in addition to DCs. The liver is the only non-lymphoid tissue in which naïve CD4<sup>+</sup> and CD8<sup>+</sup> T cells produced in the thymus can be activated independently of the spleen and lymph nodes. Naïve CD4<sup>+</sup> and CD8<sup>+</sup> T cells are activated in the liver by non-conventional APCs in addition to DCs. The liver is the only non-lymphoid tissue in which naïve CD4<sup>+</sup> and CD8<sup>+</sup> T cells produced in the thymus can be activated independently of the spleen and lymph nodes. Naïve CD4<sup>+</sup> and CD8<sup>+</sup> T cells are activated in the liver by non-conventional APCs in addition to DCs.
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. 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. Additionally, hepatic DCs differ from other tissue DCs in that they also produce elevated amounts of IL-10 in response to lipopolysaccharide that then downregulates expression of MHC molecules, CD80 and CD86 by the other APCs. Hepatic 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 and tumour-induced myeloid-derived suppressor cells directly and indirectly promotes hepatic tolerance. IL-10 directly inhibits CD4+ T-cell activation and thus reduces effector T-cell numbers and DC-primed cytotoxic CD8+ T-cell function. 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. 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.

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.
Transforming growth factor-β. Hepatic TGF-β, produced by KCs, DCs, LSECs, hepatic stellate cells, Treg, natural killer (NK) T cells and myeloid-derived suppressor cells also promotes hepatic tolerance.60 TGF-β is critical for naïve CD4+ T-cell differentiation to Treg, and inhibits differentiation of naïve CD4+ T cells to pro-inflammatory Th1 and Th2 T-cell subsets as a result of inhibiting expression of the Th1 and Th2 essential transcription factors, T-bet and GATA3, respectively.61,62 Additionally, TGF-β inhibits differentiation of naïve 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+ Treg, 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 Th1 immunity as it induces expression of the Th1 master transcription factor, T-bet, resulting in naive CD4+ T-cell differentiation to Th1 cells. In vitro, IFN-γ inhibits differentiation of Th2 and Th17 subsets. However, paradoxically, Th1 and Th17 cells are often found in close association in pathology. In vivo, Th1 cell production of IFN-γ can trigger myeloid APC production of IL-1 and IL-23 that promote Th17 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 Treg cell differentiation.68 However, prolonged IFN-γ exposure in IFN-γ-overexpressing 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 Th1 and Th17 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

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 Naïve CD8+ T cells are ‘primed’ by CD4+ T-cell-induced DC production of IL-15 and differentiate into cytotoxic T cells.75 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+ Treg cells, possibly as a result of the HCV core protein inducing a Treg-like phenotype expressing the Treg 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+ Treg cells in a TGF-β-dependent manner.77 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-cell apoptosis as a result of increased mitochondrial oxidative stress.

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. 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\textsubscript{reg} T-cell trans-differentiation into T\textsubscript{reg} cells during inflammation resolution.

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. 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\textsubscript{reg} cells \textit{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\textsubscript{reg} cell and neutrophil numbers resulting in enhanced hepatic injury in the murine bile duct ligation model.

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. An increased percentage of circulating and tumour-infiltrating CD8+ T cells express PD-1 while tumour cells and tumour-associated monocyes and neutrophils express upregulated PD-L1 and CD47 levels in HCC patients. The numbers of myeloid-derived suppressor cells are also elevated in HCC patients. Furthermore, activation of the c-Myc oncogene switches on PD-L1 and CD47 expression in hepatocytes, raising the possibility that oncogenes may directly regulate PD-1 expression on 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. The PD-1\textsuperscript{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 adaptively transferred to HBV transgenic mice and also restored IFN-γ production by CD8+ T cells and viral clearance in the HCV core murine model. \textit{In vitro} blocking of PD-L1 on HBV patient T cells enhanced both CD4+ and CD8+ T-cell production of IFN-γ. Likewise, \textit{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. Despite these promising \textit{in vitro} and \textit{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. 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. \textit{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 \textit{Escherichia coli}-stimulated oxidative burst.

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. 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. 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.

\textit{Sorafenib regulates hepatic immunity.} Specifically, sorafenib blocks signalling via vascular endothelial growth factor receptor, platelet-derived growth factor receptor and C-Raf. 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. A number of \textit{in vitro} and \textit{in vivo} studies have now reported that sorafenib also stimulates the immune system. Sorafenib directly upregulates IL-2 secretion by peripheral blood CD4+ T\textsubscript{reg} isolated from HCC patients. Sorafenib also inhibited the suppressive properties and proliferation of CD4+ T\textsubscript{reg} as well as enhanced their apoptosis in a preclinical orthotopic HCC model. This was not due to the reduction in tumour burden, as sorafenib also reduced CD4+ T\textsubscript{reg} 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. 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.\textsuperscript{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.\textsuperscript{110} Importantly and similar to melanoma and lung cancer, HCC does not appear to have an 'oncogene addiction'.\textsuperscript{111} 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\textsuperscript{−/−}Mst2\textsuperscript{−/−}) mice in which hepatocarcinogenesis was induced by carbon tetrachloride. Eighteen out of the 41 advanced HCC patients with Child-Pugh score \textless B7 completed a 2-year Phase-II clinical safety trial of the PD-1 inhibitor, nivolumab (CheckMate 040; ClinicalTrials.gov number NCT01658878), although the dose cycle was not reported.\textsuperscript{112} 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\textsuperscript{−1} every 2 weeks in 214 patients.\textsuperscript{113} Complete response was achieved in three patients while partial response was achieved in 39 patients and disease was stable in a further 96 patients.\textsuperscript{114}

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).\textsuperscript{115} 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.\textsuperscript{116}

CD47 is an innate immune checkpoint that binds to signal regulatory protein-\(\alpha\) 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.\textsuperscript{117} Further, CD47 blockade sensitises HuH7/HMCC-97L tumour cells to the chemotherapeutic drugs doxorubicin and cisplatin by increasing macrophage phagocytosis in \textit{in vivo} murine xenografts.\textsuperscript{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\textsuperscript{+} and CD8\textsuperscript{+} T cells in HBV-induced HCC compared with the surrounding tissue and predicted a poor prognosis.\textsuperscript{119} Further, Tim-3 expression on tumour-associated macrophages in HBV-induced HCC stimulated tumour-promoting IL-6 production.\textsuperscript{120} Similarly, Lag-3 is also expressed on an increased percentage of CD8\textsuperscript{+} T cells in HBV-induced HCC and correlates with impaired HBV-specific CD8\textsuperscript{+} cytotoxicity in HCC patients.\textsuperscript{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 fatigue, nausea and skin rash to the severe but much less common such as hypothyroidism and the neurological conditions, Guillain–Barre syndrome and myasthenia gravis.\textsuperscript{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.\textsuperscript{116} Hypothyroidism was only observed in 1 out of the 20 patients who underwent the anti-CTLA-4 treatment.\textsuperscript{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.\textsuperscript{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\textsuperscript{−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.\textsuperscript{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.\textsuperscript{116} Moreover, anti-CTLA-4 treatment also reduced the HCV load and tended to increase the numbers of IFN-\(\gamma\)-producing lymphocytes in the study by Sangro et al.\textsuperscript{116} Regardless, blockade of both CTLA-4 and PD-1 were concluded to be safe in treatment of HCC regardless of viral status.\textsuperscript{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.\textsuperscript{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 \(\alpha\) receptor, C-X-C motif chemokine receptor 4, did significantly further reduce tumour growth in these two models.\textsuperscript{124} These preclinical data suggest that immune checkpoint inhibitor therapy combined with sorafenib may lead to improved outcomes.\textsuperscript{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.
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.

**ACKNOWLEDGEMENTS**

This work was kindly supported by grants from the Spinnaker Health Research Foundation (to CLE), Cancer Council Western Australia (Grant 1024562) and
Filippi CM, Juedes AE, Oldham JE, Ling E, Togher L, Peng Y et al. Transforming growth factor-beta suppresses the activation of CD8+ T-cells when naive but promotes their survival and function once antigen experienced: a two-faced impact on autoimmunity. *Diabetes 2008; 57: 2684–2692.

McKarns SC, Sair MM. Distinct effects of TGF-beta 1 on CD4+ and CD8+ T cell survival, division, and IL-2 production: a role for T cell intrinsic Smad3. *J Immunol 2005; 174: 2071–2083.

Thomas DA, Massague J. TGF-beta directly targets cytosolic T cell functions during tumor evasion of immune surveillance. *Cancer Cell 2005; 8: 369–386.

Horrás CJ, Lamb CL, Mitchell KA. Regulation of hepatocyte fate by interferon-gamma. *Cytokine Growth Factor Rev 2011; 22: 35–43.

Kryczek I, Bruce AT, Gudjonsson JE, Johnston A, Aphale A, Vatan L et al. Induction of IL-17+ Th1 cell trafficking and development by IFN-gamma, mechanism and pathological relevance in psoriasis. *J Immunol 2008; 181: 4733–4741.

Lees JR. Interferon gamma in autoimmunity: a complicated player on a complex stage. *Cytokine 2015; 74: 18–26.

Luth S, Schrader J, Zander S, Carambia A, Buchkremer J, Huber S et al. Chronic inflammatory IFN-gamma signaling suppresses hepatocarcinogenesis in mice by sensitizing hepatocytes for apoptosis. *Cancer Res 2011; 71: 3763–3771.

Brenner D, Blaser H, Mak TW. Regulation of tumour necrosis factor signaling: live or let die. *Nat Rev Immunol 2015; 15: 362–374.

Bo X, Broome U, Remberger M, Sumirathan-Holgersson S. Tumour necrosis factor alpha impacts function of liver derived T lymphocytes and natural killer cells in patients with primary sclerosing cholangitis. *Gut 2001; 49: 131–141.

Beyer M, Abdulla Z, Chemnitz JM, Maisel D, Sander J, Lehmann C et al. Tumor necrosis factor impacts CD4+ T cell-mediated immunological control in chronic viral infection. *Nat Immunol 2016; 17: 593–603.

Fahey LM, Brooks DM. Opposing positive and negative regulation of T cell activity during viral persistence. *Curr Opin Immunol 2010; 22: 348–354.

Schmidt J, Blum HE, Thimme R. T-cell responses in hepatitis B and C virus infection: similarities and differences. *Emerg Microbes Infect 2013; 2: e15.

Rosen HR. Emerging concepts in immunity to hepatitis C virus infection. *J Clin Invest 2013; 123: 4121–4130.

Fernandez-Ponce C, Dominguez-Villar M, Agudo E, Garcia-Cozar F. CD4+ primary T cells expressing HCV-core protein upregulate Fox3 and IL-10, suppressing CD4 and CD8 T cells. *PLoS ONE 2014; 9: e85197.

Maki A, Matsuda M, Asakawa M, Kono H, Fujii H, Matsumoto Y. Decreased expression of CD28 coincides with the down-modulation of CD3zeta and augmentation of caspase-3 activity in T cells from hepatocarcinoma-bearing patients and hepatitis C virus-infected patients. *J Gastroenterol Hepatol 2004; 19: 1348–1356.

Das A, Hoare M, Davies N, Lopes AR, Dunn C, Kennedy PT et al. Functional skewing of the global CD8+ T cell population in chronic hepatitis B virus infection. *J Exp Med 2008; 205: 2111–2124.

Mengshol JA, Golden-Mason L, Arikawa T, Smith M, Niki T, McWilliams R et al. A crucial role for Kupffer cell-derived galectin-9 in regulation of T cell immunity in hepatitis C virus infection. *PLoS ONE 2010; 5: e9504.

Liao J, Zhang L, Liu Y, Bo C, Chen J, Li L et al. Association of T-cell tumour infiltrating melanobolin and mucin domain-containing molecule 3 (Tim-3) polymorphisms with susceptibility and disease progression of HBV infection. *PLoS ONE 2014; 9: e95390.

Kasel R, Cruise MW, Lasen MJ. Cancer Immunotherapies for liver cancer. *Cln Exp Med 2013; 23: 1050–10506.

63 Gagliani N, Amezcua Vesely MC, Iseppon A, Brockmann L, Xu H, Palm NW et al. Tim-3 negatively regulates IL-12 mediated by Th17 cells and neutrophils. *J Leukoc Biol 2013; 94: 813–823.

Kalathil S, Lugade AA, Miller A, Iyer R, Thanavala Y. Higher frequencies of GARP cause selective CD4+ T lymphocyte loss and promotes hepatocarcinogenesis. *Cancer Immunol Immunother 2012; 61: 1260–1273.

Kohga K, Takehara T, Tatsumi T, Ishida H, Miyagi T, Hosui A et al. Sorafenib inhibits the shedding of major histocompatibility complex class I-related chain A on hepatocellular carcinoma cells by down-regulating a disintegrin and metalloprotei

Rizvi NA, Hellmann MD, Snyder A, Kiswiberg P, Makarov V, Havel JJ et al. Cancer immunology. Mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer. *Science 2015; 348: 124–128.

Alexandrov LB, Nik-Zainal S, Wedge DC, Aparicio SA, Behjati S, Biankin AV et al. Signatures of mutational processes in human cancer. *Nature 2013; 501: 451–452.

Yao S, Johnson C, Hu Q, Yan L, Liu B, Ambrose CB et al. Differences in somatic mutation landscape of hepatocellular carcinoma in Asian American and European American populations. *Oncotarget 2016; 7: 40491–40499.

Fujimoto A, Totoki Y, Abe T, Bonevich KA, Hosoda F, Nagahashi H et al. Whole-genome sequencing of liver cancers identifies etiological influences on mutation patterns and accruing mutations in chronic hepatitis C. *Nat Genet 2012; 44: 760–764.

Llovet JM, Villanueva A, Lichtenhainer A, Finn RS. Advances in targeted therapies for hepatocellular carcinoma in the genomic era. *Nat Rev Clin Oncol 2015; 12: 408–424.

El-Khoueiry AB, Gonen M, Yau TC, Dayal DD, Bhatia S et al. Nivolumab in patients with advanced hepatocellular carcinoma (CheckMate 040): an open-label, non-comparative, phase 1/2 dose escalation and expansion trial. *Lancet 2017; 389: 2492–2502.
116 Sangro B, Gomez-Martín C, de la Mata M, Inarreaegui M, Garralda E, Barrena P et al. A clinical trial of CTLA-4 blockade with tremelimumab in patients with hepatocellular carcinoma and chronic hepatitis C. *J Hepatol* 2013; 59: 81–88.

117 Xiao Z, Chung H, Banan B, Manning PT, Ott KC, Lin S et al. Antibody mediated therapy targeting CD47 inhibits tumor progression of hepatocellular carcinoma. *Cancer Lett* 2015; 360: 302–309.

118 Lo J, Lau EY, Ching RH, Cheng BY, Ma MK, Ng IO et al. Nuclear factor kappa B-mediated CD47 up-regulation promotes sorafenib resistance and its blockade synergizes the effect of sorafenib in hepatocellular carcinoma in mice. *Hepatology* 2015; 62: 534–545.

119 Li H, Wu K, Tao K, Chen L, Zheng Q, Lu X et al. Tim-3 / galectin-9 signaling pathway mediates T-cell dysfunction and predicts poor prognosis in patients with hepatitis B virus-associated hepatocellular carcinoma. *Hepatology* 2012; 56: 1342–1351.

120 Yan W, Liu X, Ma H, Zhang H, Song X, Gao L et al. Tim-3 fosters HCC development by enhancing TGF-beta-mediated alternative activation of macrophages. *Gut* 2015; 64: 1593–1604.

121 Li FJ, Zhang Y, Jin GX, Yao L, Wu DQ. Expression of LAG-3 is coincident with the impaired effector function of HBV-specific CD8+ T cell in HCC patients. *Immunol Lett* 2013; 150: 116–122.

122 Day D, Hansen AR. Immune-related adverse events associated with immune checkpoint inhibitors. *BioDrugs* 2016; 30: 571–584.

123 Gardiner D, Lalezar J, Lawitz E, DiMicco M, Ghalib R, Reddy KR et al. A randomized, double-blind, placebo-controlled assessment of BMS-936558, a fully human monoclonal antibody to programmed death-1 (PD-1), in patients with chronic hepatitis C virus infection. *PLoS ONE* 2013; 8: e63818.

124 Chen Y, Ramjiawan RR, Reiberger T, Ng MR, Hato T, Huang Y et al. CXCR4 inhibition in tumor microenvironment facilitates anti-programmed death receptor-1 immunotherapy in sorafenib-treated hepatocellular carcinoma in mice. *Hepatology* 2015; 61: 1591–1602.

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/