

Specific CD8⁺ T cell response immunotherapy for hepatocellular carcinoma and viral hepatitis

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Abstract

Hepatocellular carcinoma (HCC), chronic hepatitis B (CHB) and chronic hepatitis C (CHC) are characterized by exhaustion of the specific CD8⁺ T cell response. This process involves enhancement of negative co-stimulatory molecules, such as programmed cell death protein-1 (PD-1), cytotoxic T-lymphocyte antigen-4 (CTLA-4), 2B4, Tim-3, CD160 and LAG-3, which is linked to intrahepatic overexpression of some of the cognate ligands, such as PD-L1, on antigen presenting cells and thereby favouring a tolerogenic environment. Therapies that disrupt these negative signalling mechanisms represent promising therapeutic tools with the potential to restore reactivity of the specific CD8⁺ T cell response. In this review we discuss the impressive *in vitro* and *in vivo* results that have been recently achieved in HCC, CHB and CHC by blocking these negative receptors with monoclonal antibodies against these immune checkpoint modulators. The article mainly focuses on the role of CTLA-4 and PD-1 blocking monoclonal antibodies, the first ones to have reached clinical practice. The humanized monoclonal antibodies against CTLA-4 (tremelimumab and ipilimumab) and PD-1 (nivolumab and pembrolizumab) have yielded good results in testing of HCC and chronic viral hepatitis patients. Tremelimumab, in particular, has shown a significant increase in the time to progression in HCC, while nivolumab has shown a remarkable effect on hepatitis C viral load reduction. The research on the role of ipilimumab, nivolumab and pembrolizumab on HCC is currently underway.

Key words: Hepatocellular carcinoma; CD8⁺ T cells;

Immune checkpoint modulation; Chronic viral hepatitis; Cytotoxic T-lymphocyte antigen-4; Programmed cell death protein-1

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Core tip: In certain types of chronic diseases, such as hepatocellular carcinoma and chronic viral hepatitis, disease curation involves restoration of the specific cytotoxic T cell response. Chronic hepatotropic viruses and tumoural cells develop mechanisms to induce exhaustion of the specific CD8⁺ T cells in order to escape immune destruction. One hallmark of this dysfunction is the overexpression of negative co-stimulatory molecules. Blockade of these negative co-stimulatory pathways, a process known as immune checkpoint modulation, is a promising novel therapy that could improve the treatment of liver diseases that feature T cell exhaustion.

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INTRODUCTION

Specific CD8⁺ T cells have a central role in pathogenesis of hepatocellular carcinoma (HCC) as well as control of infection with hepatitis B virus (HBV) and hepatitis C virus (HCV) because these cells are able to recognise infected/tumoural cells and destroy them^[1-11]. Nevertheless, in chronic viral infections and tumoural diseases that feature high-grade and persistent antigenemia, the adaptive immune system has to surrender in order to diminish tissue damage^[12-16]. This is the case for HCC and chronic viral hepatitis, wherein tumoural cells and HBV/HCV viruses modulate common mechanisms to induce specific T cell exhaustion. Among such viral and tumoural strategies, the induction of negative co-stimulatory molecules stands out.

Unfortunately, the on-going lack of effective treatments for HCC^[17], for achieving complete HBV clearance^[18] and for preventing HCV relapse after direct-acting antiviral (DAA) agent failure^[19] has led to an urgent need for developing new therapeutic approaches, such as immunotherapy focused on specific cytotoxic T cell restoration^[20]. Modulation of negative co-stimulatory signalling molecules expressed on these cells could have a substantial impact when developed as a therapeutic tool. In this review we discuss the specific CD8⁺ T cell response during HCC and chronic hepatitis B and C (commonly known as CHB and CHC respectively), focusing on the disease mechanisms used by tumoural cells and hepatotropic

viruses to induce T cell exhaustion and on the potential therapeutic strategies to modulate co-stimulatory pathways in order to restore specific T cell reactivity.

T CELL EXHAUSTION

CD8⁺ T cell activation depends on physical interaction between the T cell receptor and the major histocompatibility complex I (MHC I)/epitope complex, as well as that between co-stimulatory molecules with their ligands in an adequate cytokine milieu^[21]. Upon completion of their effector tasks, primed specific T cells switch-off their effector activity by expressing negative co-stimulatory molecules, generating a sustained memory T cell population^[22]. Thus, the balance between positive and negative co-stimulation determines the status of CD8⁺ T cell activation and the intensity of the accompanying immune response^[23].

During tumoural and persistent viral infections - characterized by high-grade and persistent antigenemia - the adaptive immune system is tuned down in order to avoid host-induced tissue damage. Tumoural cells and persistent viruses have developed mechanisms that induce early expression of negative co-stimulatory molecules so as to favour T cell exhaustion before the effector T cells are able to control the disease^[24-26]. This phenomenon could represent an evolutionary advantage for the chronic persistence of these diseases.

T cell exhaustion is characterized by a lack of effector cell capacity that is linked to overexpression of negative co-stimulatory pathways. Upon binding to their respective ligands, these negative co-stimulatory molecules act to disrupt the processes of T cell proliferation as well as secretion of type- I cytokines and development of cytolytic functions, creating an environment that allows for tumour persistence and virus evasion^[13,27-29]. Among these negative co-stimulatory pathways, the most advanced in the pipeline for clinical use are cytotoxic T-lymphocyte antigen-4 (CTLA-4) and programmed cell death protein-1 (PD-1), as will be discussed later^[30,31].

Exhausted CD8⁺ T cells were first identified during chronic lymphocoriomeningitis virus (LCMV) infection and defined as virus-specific CD8⁺ T cells that did not produce antiviral cytokines and were ineffective at controlling the infection^[32]. Since that time, subsequent research has provided descriptions of T cell exhaustion in different human chronic infections and cancers^[33-35]. Loss of functionality occurs in a hierarchical manner throughout the process of exhaustion. It has been noted that the greater the antigen load or the duration of the disease, the greater the extent of exhaustion. Usually, functions such as interleukin (IL)-2 production, high expansion ability and *ex vivo* killing are lost first; this stage is named "partial exhaustion I". In the next stage of exhaustion, "partial exhaustion II", these cells lose their ability to produce tumour necrosis factor (TNF)- α , and their expansion ability and antigen-induced production of interferon (IFN)- γ .

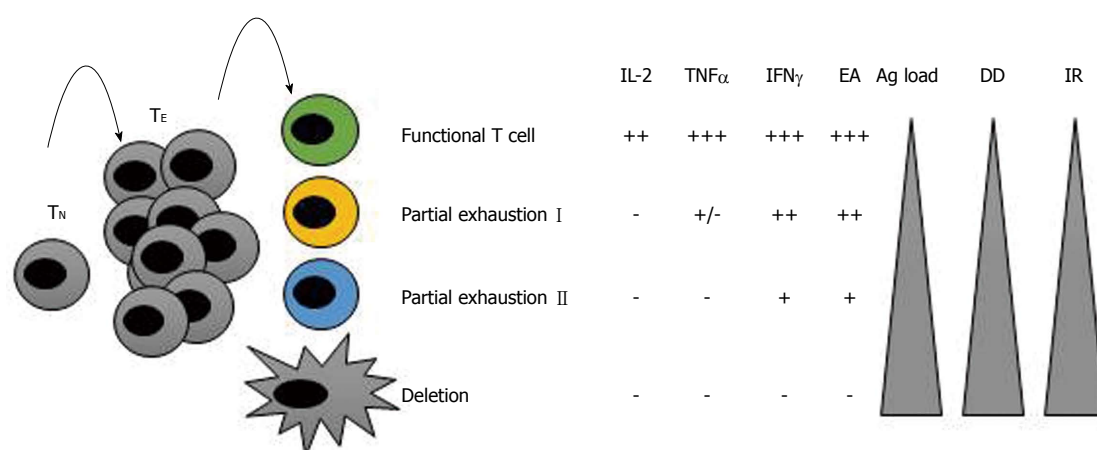


Figure 1 T cell exhaustion during diseases with persistent and high antigenemia. At the beginning of an infection, naïve T cells (T_N) are primed and differentiate into effector T cells (T_E). During acute infections, T_E are completely functional and control the pathogen/tumoural cell. After clearing the antigen, these cells are then deleted by apoptosis and a memory population is generated and maintained. Nevertheless, in conditions of chronic infections or tumours, these cells gradually lose their effector capacity, becoming exhausted. The greater the antigen load or duration of the infection, the more exhausted the cells become. The steps of exhaustion are summarised here. In "partial exhaustion I" IL-2 production, high expansion ability and *ex vivo* killing are lost. In "partial exhaustion II", the more advanced stage of exhaustion, these cells lose their capacity to produce tumour necrosis factor (TNF)- α , produce less interferon- γ and proliferate less. In the final stage of exhaustion, these cells are deleted by apoptosis. Ag: Antigen; DD: Duration of the disease; EA: Expansion ability; IR: Inhibitory receptors' expression.

become impaired. The final stage of exhaustion is the deletion of these cells by apoptosis^[32,36,37] (Figure 1). A detailed understanding of the mechanism underlying this process may aid in development of efficacious therapies that restore the function of these cells and - from a practical point of view - the modulation of negative co-stimulatory pathways.

LIVER AS A TOLEROGENIC ORGAN

As previously described, one reason why specific cytotoxic T cells become exhausted in HCC, CHB and CHC is related to the strategies developed by the pathogen/tumour itself; yet, the host contributes to the exhaustion process as well, due to the particular liver features that are described below.

Bowen *et al.*^[38] elegantly showed that activation of primary CD8⁺ T cells within the lymph nodes leads to an efficient response, whereas activation of primary CD8⁺ T cells within the liver commits T cells to the development of an immunotolerant state. This divergent response is related to the liver's intense tolerogenic properties, which are in line with this organ's role in dealing with a massive load of foreign antigens from the gastrointestinal tract. For this reason, in order to develop new immunotherapeutic approaches to treat viral hepatitis and HCC it is first necessary to understand how intrahepatic immunity is regulated. An important feature to consider is that liver can support primary T cell activation independently of secondary lymphoid tissues and involvement of dendritic cells (DCs). Moreover, the ligands expressed by resident liver cells could favour exhaustion of specific liver-infiltrating T cells after antigen recognition. These two conditions could definitely impair the quality of T cell response^[39,40].

Several liver cell types (listed below) can work as antigen-presenting cells (APCs) to activate naïve CD8⁺ T cells.

Hepatocytes

Hepatocytes represent about two-thirds of the total cell population in the liver. Antigen presentation by hepatocytes is the most relevant mechanism of infection with hepatotropic viruses. Naïve CD8⁺ T cells can directly interact with hepatocytes *via* liver sinusoidal endothelial cell (LSEC) fenestrations^[41]. Although hepatocytes have been demonstrated as capable of promoting rapid activation and proliferation of CD8⁺ T cells *in vivo*^[39,40], they do not express positive co-stimulatory molecules, such as CD80 and CD86; therefore, because of this they could fail to induce functional CD8⁺ T cells in *in vivo* conditions^[42,43]. Besides, one of the ligands of the negative co-stimulatory molecule PD-1 (PD-L1) can be expressed by hepatocytes^[44], and its interaction with PD-1 on the hepatocyte-activated CD8⁺ T cell contributes to its functional suppression^[45].

Kupffer cells

Kupffer cells (KCs) are the resident macrophages in the liver and represent the largest population of resident tissue macrophages in the entire body^[46]. KCs are localized mainly in the periportal area, where they serve to clear endotoxins and phagocytose debris and microorganisms. These cells can also pass through the space of Dissé, coming into contact with hepatocytes and phagocytosing any with apoptotic features^[47,48]. KCs express Fas-ligand^[49] and PD-L1^[50], leading to apoptosis and functional exhaustion of CD8⁺ T cells respectively. In addition, the KCs can secrete immunosuppressive cytokines, such as IL-10

and tumour growth factor (TGF)- β , both of which can contribute to T cell exhaustion^[51].

LSECs

LSECs can express MHC and co-stimulatory molecules and are capable of presenting antigen to CD8⁺ T cells by at least two pathways, thereby promoting tolerance. Firstly, these cells express PD-L1 even at low antigen concentration^[52] and, secondly, they can secrete IL-10 and TGF- β ^[53], which could impair CD8⁺ T cell activation, as previously commented on.

Hepatic stellate cells

Hepatic stellate cells (HSCs), located at the space of Disse, represent the major cell type involved in liver fibrosis, but they are also involved in antigen presentation^[54]. TGF- β secreted by the HSCs contributes both to liver fibrosis and to the exhaustion of CD8⁺ T cells^[55].

DCs

Resident hepatic DCs are predominantly immature cells, prone to capturing and processing of antigens^[56]. Because IL-10 and TGF- β are secreted by KCs and LSECs, the uninfected liver provides a unique cytokine environment that may render a tolerogenic state for the resident DCs^[56-58]. Moreover, resting DCs can induce peripheral CD8⁺ T cell tolerance through up-regulation of PD-1 and CTLA-4^[59].

Consequently, CD8⁺ T cells that are activated by these liver APCs are not optimally primed and fail to exert effector functions, thus promoting tolerance and T cell exhaustion. This situation can represent a survival advantage for hepatotropic viruses and HCC, since the specific cytotoxic T cells that are capable of recognising viral and tumoural antigens can become exhausted easily, due to the tolerogenic liver status. Such an environment features high-level expression of negative co-stimulatory ligands on the resident liver cells as well as induction of negative co-stimulatory receptors on the specific T cells, as related to the liver cytokine milieu.

Following our above introduction of the concept of T cell exhaustion in HCC, CHB and CHC, as well as of the mechanisms involved in this process, we will next highlight the current evidence showing why specific cytotoxic T cell response restoration could impact HCC, HCV and HBV treatment.

HCC

Worldwide rates of liver cancer classify it as the fifth most common cancer in men and the seventh in women. Infection with HBV and HCV, chronic alcoholism and fatty liver disease, among others, are major risk factors for HCC^[60]. Once diagnosed, HCC usually has a poor prognosis, due to lack of efficacy of the available treatments. Therefore, novel effective

therapies are urgently needed to treat patients with this type of tumour, particularly for those in advanced stages for whom the most efficacious of the current treatments are still only suboptimal.

Immune evasion is a general strategy of cancers, but much is still unknown about it. Most of the research on this phenomenon has focused on devising ways to directly destroy the tumoural cell, while the role that immune system restoration may play in resisting or eradicating the tumour formation and its progression has been largely, if not completely, overlooked^[61]. Adaptive immune response, especially the cytotoxic response, is known to play a crucial role in the control of solid tumours^[8]. Several lines of evidence have been reported that support the importance of CD8⁺ T cells during HCC. Firstly, the presence of a high number of tumour-infiltrating T cells in HCC tissue suggests a role in HCC pathogenesis^[9]. Secondly, the quantity of tumour-infiltrating T cells is considered a good prognosis marker of HCC^[10]. Finally, adoptive immunotherapy could protect against HCC, diminishing the recurrence risk after surgical treatment^[11].

Mizukoshi *et al.*^[62] analysed immune responses against various HCC epitopes in peripheral blood mononuclear cells from patients with HCC. After radiofrequency ablation (RFA), the authors noted an improvement of these responses in two-thirds of the patients; interestingly, those patients with a detectable response also experienced longer survival^[62]. Flecken *et al.*^[12] recently described some tumour-associated antigen (TAA)-specific CD8⁺ T cell responses in HCC. In that elegant study, the authors applied overlapping peptides to a large cohort of HCC patients and showed that a variety of TAAs can induce CD8⁺ responses against α -fetoprotein (AFP), glypican-3 (GPC-3), melanoma-associated antigen-1 (MAGE-1) and New York-oesophageal squamous cell carcinoma-1 (NY-ESO-1). The authors also showed a positive correlation between either the quantity of TAA-specific CD8⁺ T cells or the number of TAA targets and the survival of these patients. Finally, they also demonstrated that TAA-specific CD8⁺ T cells were able to proliferate, but not able to produce IFN- γ after antigen encounter^[12]. Therefore, HCC features CD8⁺ T cells that are able to recognise tumoural neo-antigens; although, these cells display an exhausted behaviour. Interestingly, PD-1 was found to be up-regulated in these cells, a feature which could represent a base for immunotherapy by blocking this negative co-stimulatory molecule. Consequently, one possible approach for HCC treatment could be to restore the effector capabilities of these cells and one option towards achieving this end could be the modulation of negative co-stimulatory pathways, such as PD-1, as will be discussed below.

HCV

HCV was first cloned in 1989 as a non-A non-B he-

patitis virus^[63]. Since then, substantial progress has been made in our understanding of both the virus and its interactions with the host system. The final result of this intense research effort has been the generation of DAAs that show curative effect on HCV infection in approximately 95% of the CHC patients^[64-66].

In the last two decades, we have learnt several important lessons about the strategies that the HCV employs to avoid the immune system in order to persist in the host. HCV-specific CD8⁺ T cells play an essential role in controlling HCV during acute infection^[1-3], based upon their abilities to both recognize and destroy the infected cell through cytolytic and non-cytolytic mechanisms^[4,67]. However, in approximately 70% of primo-infections, the virus is able to persist in the host, leading to chronic infection^[68,69]. Viral escape mutations are the first mechanism used by HCV to avoid immune control, exploiting the lack of a proofreading function by the viral polymerase^[70]. The second mechanism involves overwhelming the immune response. Because of the persistent antigenemia that accompanies HCV infection, the HCV-specific CD8⁺ T cell response becomes exhausted and fails to control infection, featuring loss of effector capacities and overexpression of negative-regulation pathways^[13,14].

Thus, although DAAs are very effective treatments, continued research in HCV immunotherapy is still necessary because of the existence of DAA non-responders and to develop it as a strategy to boost other anti-viral treatments (both established and new) and to support development of a therapeutic vaccine. Moreover, since these HCV-specific CD8⁺ T cells up-regulate negative co-stimulatory molecules, blocking the interaction of these receptors with their ligands could be considered as a potential therapeutic strategy.

HBV

HBV is a hepatotropic non-cytopathic DNA virus and member of the family Hepadnaviridae^[71]. Approximately 2 billion people worldwide have been infected by HBV, and it is estimated that more than 350 million of these individuals are persistent carriers of the virus. Most HBV infections occur *via* vertical transmission. Around 5%-10% of patients infected during adulthood develop CHB, with 10%-30% of those patients progressing to liver cirrhosis and/or HCC. Ultimately, however, 1-2 million HBV-related deaths are reported annually^[72].

The primary treatment of CHB is based on two kinds of drugs currently: pegylated IFN- α and nucleoside/nucleotide analogues^[73]. Nevertheless, complete HBV eradication, with clearance of the covalently closed circular DNA, is rarely achieved^[18], making it necessary to develop new effective therapies for this major public health problem. The potential benefit of immunotherapy in HBV is highlighted by several important aspects of the infection itself. Firstly,

in subjects who spontaneously clear the HBV infection, viral control is determined by the development of a strong, polyclonal and multi-specific CD8⁺ T cell response^[5-7]; this usually happens during adulthood, when nearly 90% of the infected subjects are able to control the virus and in contrast to individuals (children) who obtain the virus through vertical transmission and in who the HBV persists due to immunotolerance induction^[74]. Secondly, CHB resolution occurs in some bone marrow transplantation recipients who received tissue from a donor with natural immunity to HBV^[75]. Finally, those individuals with natural immunity to HBV maintain immunological memory through HBV-specific CD8⁺ T cells that can last decades after the primo-infection that is capable of controlling virus at trace amounts^[76]. Nevertheless, in chronically infected patients, the HBV-specific CD8⁺ T cell response is weak (barely detectable) and exhausted in both the peripheral blood and the liver^[15,16], and this feature is accompanied by up-regulated expression of negative co-stimulatory molecules, as will be discussed in the following paragraphs. Thus, taking into account these facts, immunotherapy based on modulation of the co-stimulatory pathway could be a promising approach to improve HBV chronic infection treatment.

IMMUNE CHECKPOINTS

The cytotoxic T cell response is essential to eradication of tumoural and virus-infected cells. In patients with chronic viral hepatitis and HCC this response is impaired and, theoretically, its restoration could help in disease control. As previously commented, one of the strategies used by tumoural and virus-infected cells to induce exhaustion of the CD8⁺ T cells is up-regulation of negative co-stimulatory molecules. Therefore, therapeutic blockade of various inhibitory receptors, a process also referred to as "checkpoint blockade", has begun to provide very promising results in the treatment of different diseases; these will be summarised hereafter.

The first proof of concept of the efficacy of this kind of treatment was reported for "ipilimumab", a human monoclonal antibody against the negative co-stimulatory molecule CTLA-4 that was approved by the United States' Food and Drug Administration in 2011; this drug is currently in clinical use for treating metastatic melanoma^[77]. Since its introduction, this antibody (and others) against different co-stimulatory molecules has entered testing for other malignancies and various viral infections. There are several completed, on-going and planned clinical trials for investigating treatment of chronic hepatitis and HCC with single-agent inhibitors, as well as with combinations of inhibitors targeting multiple checkpoints or adding other therapies to this blockade. In the next lines, we will review the mechanism of action of these immune checkpoints, and the effect of blockade as determined in pre-clinical and clinical studies.

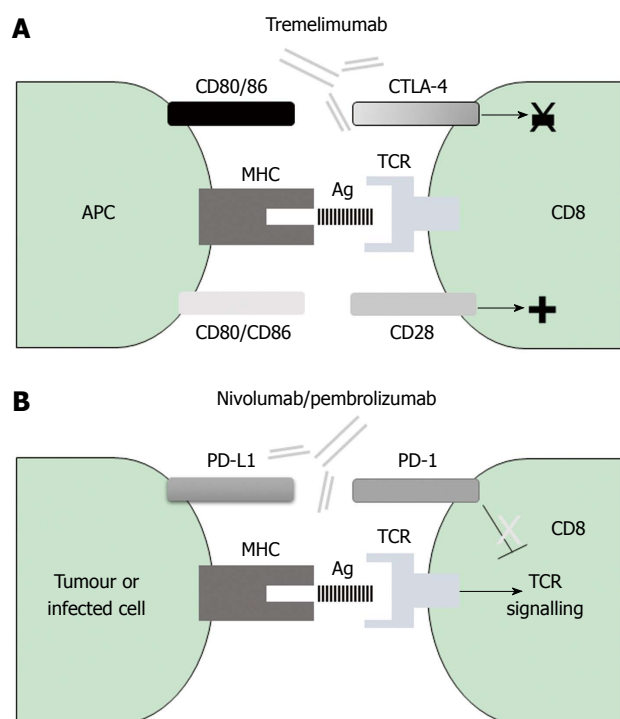


Figure 2 Mechanisms of action of negative co-stimulatory pathways involved in current immunotherapy for cytotoxic T cells. A: The CTLA-4 immune checkpoint impairs early T cell activation; the inhibitory CTLA-4 molecule binds with higher affinity to the CD80/CD86 ligands on antigen presenting cells and prevents their binding to the positive co-stimulatory molecule CD28, with the consequence being a decrease in T cell activation. B: The PD-1 negative receptor on T cells interacts with either PD-L1 or PD-L2 on the surface of the infected or tumoural cell, thereby promoting T cell exhaustion; blockade of either CTLA-4 or PD-1 with humanized monoclonal antibodies releases the brake that is exerted by these molecules and results in T cell activation. CTLA-4: Cytotoxic T-lymphocyte antigen-4; MHC: Major histocompatibility complex; PD-1: Programmed cell death protein-1; TCR: T cell receptor.

CTLA-4

Mechanism of action of CTLA-4: Since its discovery in 1987^[78], research has determined that CTLA-4 is expressed only on T cells, where it regulates early immune activation. This negative co-stimulatory molecule counteracts the activity of the positive co-stimulatory molecule CD28^[79-82]. After antigen encounter, the CD28 co-signal triggers the T cell receptor (TCR) signal that activates T cells^[83]. CTLA-4 and CD28 share the same APC-expressed ligands, namely CD80 and CD86. CTLA-4 displays at least two different ways by which it can inhibit T cell activation; in the first, it inhibits positive signalling of CD28 according to the feature that CTLA-4 has more affinity for CD80 and CD86 than the positive co-stimulatory molecule CD28^[84] and in the second, CTLA-4 directly inhibits TCR signalling^[85,86] (Figure 2).

The central function of CTLA-4 is regulation of the access of CD28 to its shared ligands in order to protect against autoimmunity and to switch-off a normal immune response after antigen control has been achieved. The vital importance of CTLA-4 was demonstrated in two different studies that were

Table 1 Clinical trials of immune checkpoint inhibitors in patients with chronic hepatitis and hepatocellular carcinoma

NCT	Status	Disease	Agent	Result	Ref.
01008358	Completed	HCC and CHC	Tremelimumab	TTP: 6.48 m	[30]
01853618	Recruiting	HCC	Tremelimumab	TTP: 7.4 m	[111]
00703469	Completed	CHC	Nivolumab	15% significant reduction of viral load	[31]
02658019	Not yet recruiting	HCC	Pembrolizumab	NA	[112]
01658878	Recruiting	HCC	Nivolumab Ipilimumab	NA	[113]

Tremelimumab and ipilimumab are humanized monoclonal antibodies against CTLA-4; Nivolumab and pembrolizumab are humanized monoclonal antibodies against PD-1. HCC: Hepatocellular carcinoma; NA: Not available; NCT: Clinical trial number at clinicaltrials.gov; TTP: Time to progression.

based on Ctla-4 knock-out mice. The CTLA-4-deficient mice present a profound immune dysregulation and autoimmune disease that leads to massive lymphoproliferation and fatal multi-organ tissue destruction^[87,88]. Nevertheless, the inadequate induction of CTLA-4 under viral infection and tumour conditions and disrupted effects on specific CD8⁺ T cells could favour early exhaustion of these cells and consequently allow persistence of the disease.

Thus, considering the known mechanism of action of CTLA-4, enhancement of the CD8⁺ T cell response by CTLA-4 blockade could represent a satisfactory approach to treating diseases that feature persistent antigenemia, such as viral hepatitis and HCC.

CTLA-4 blockade (pre-clinical): Nakamoto *et al.*^[27] discovered that CTLA-4 is overexpressed in PD-1⁺ intrahepatic mononuclear cells of patients with CHC (Table 1). In addition, when the authors blocked these inhibitory receptors individually they found no restoration of intrahepatic HCV-specific CD8⁺ T cell response. Surprisingly, however, when they blocked both inhibitory receptors simultaneously, the effector ability of these cells was restored, indicating the existence of a synergic effect between both receptors^[27]. CTLA-4 blockade alone, however, could be sufficient to restore specific cytotoxic T cell response in persistent HBV infection. Schurich *et al.*^[28] showed that CTLA-4 blockade is able to restore the expansion ability of HBV-specific CD8⁺ T cells in both the intrahepatic and peripheral compartments of patients with CHB. It is unfortunate, though, that to date the research in HCC has only involved *in vitro* investigations of the blockade of CTLA-4 in peripheral blood mononuclear cells from HCC patients and that the results have shown no restoration of the ability of IFN- γ secretion by GPC-3-specific CD8⁺ T cells^[29]. However, a study of a mouse model carried out by

Leach *et al.*^[89] showed that *in vivo* administration of antibodies against CTLA-4 can result in regression of certain types of tumours, specifically those that are more immunogenic. These last data are consistent with the results obtained in a recent clinical trial that will be commented on later in this review.

PD-1

Mechanism of action of PD-1: PD-1 was first identified in 1992 by Ishida *et al.*^[90] as a negative co-stimulatory molecule that belongs to the CD28 immunoglobulin superfamily of transmembrane proteins^[91]. PD-1 is inducibly expressed on T cells, B cells and monocytes, upon their activation^[92]. The PD-1 ligands, PD-L1 and PD-L2, are members of the B7 co-stimulatory molecules family^[93,94]. APCs and non-lymphoid tissues, including the liver, express PD-L1, while DCs and macrophages can up-regulate PD-L2 expression^[34,44,94-97] (Table 1). Interaction of PD-1 with its ligands leads to inhibition of proliferation through a cell cycle arrest at G₀/G₁ and also impairs IL-2 secretion by T cells^[98]. In addition, interaction between PD-1 and PD-L1 or PD-L2 promotes apoptosis and secretion of the immunosuppressive cytokine IL-10^[99-101] (Figure 2).

The immunoregulatory properties of PD-1 are reflected by the Pdl-1 knockout mouse model, which presents with severe autoimmune disease^[102]. Thus, PD-1 is considered to play an important role in controlling the cellular immune response and in switching-off cells after they have completed their tasks in order to avoid autoimmune disorders; yet, its early expression can induce T cell exhaustion. Several studies have reported a positive correlation between exhaustion and PD-1 up-regulation^[24,103,104]. Therefore, the blockade of PD-1 and its ligands could represent an efficient therapeutic approach by which to restore an effector T cell response against HCC and viral hepatitis.

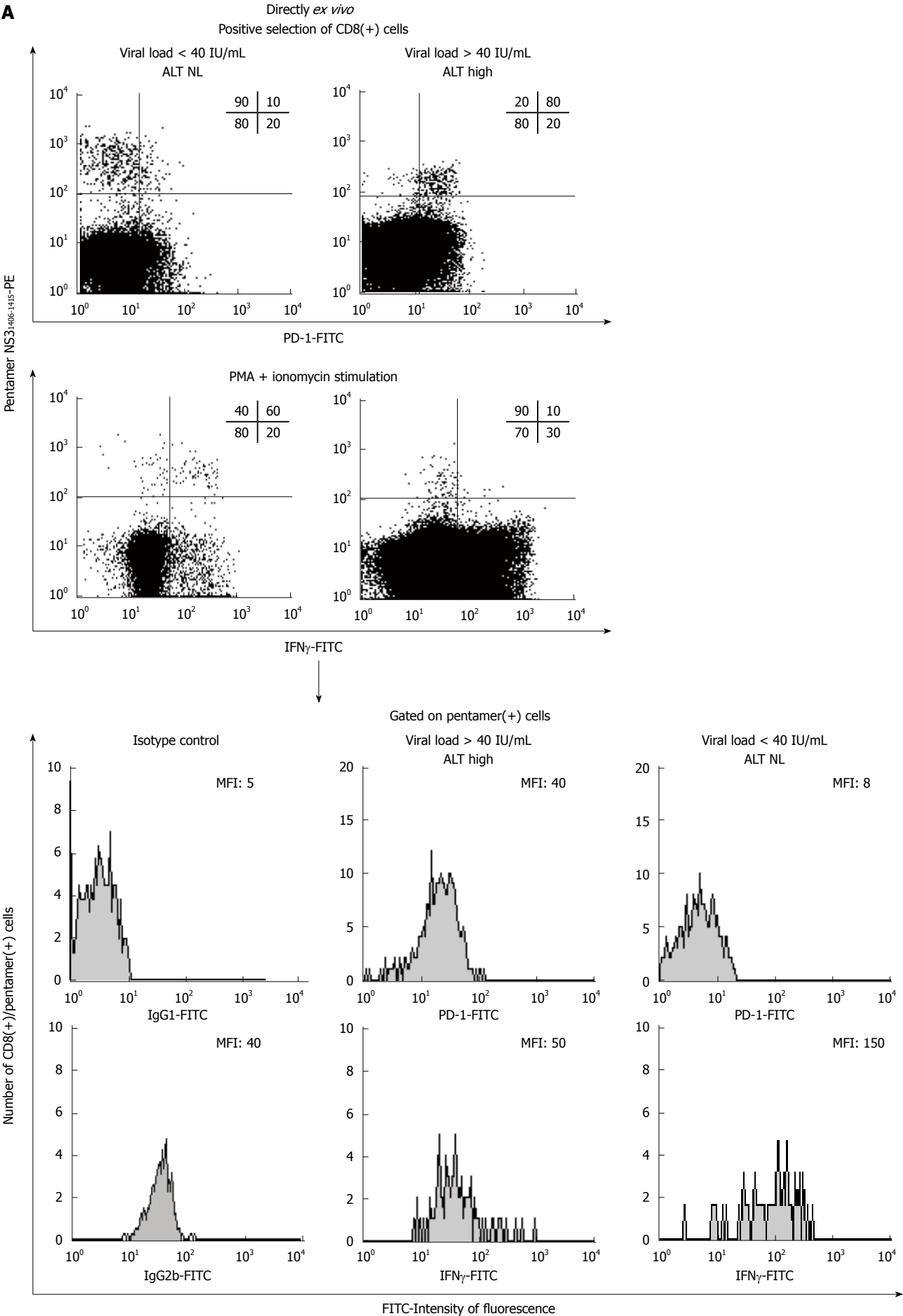
PD-1/PD-L1 blockade (pre-clinical): Several studies have been carried out to evaluate the effect of blocking the PD-1/PD-L1 pathway under conditions of viral hepatitis and HCC. Studies focusing on CHC, by Penna *et al.*^[105] and Radziejewicz *et al.*^[106], along with data obtained by our own group, have shown that up-regulation of PD-1 affects HCV-specific CD8⁺ T cells in peripheral blood and in the intrahepatic compartment during chronic HCV infection (Table 1). Besides, blockade of the PD-1/PD-L1 interaction was shown to improve the expansion ability of and IFN- γ secretion from HCV-specific CD8⁺ T cells^[24,105,106]. Moreover, Fuller *et al.*^[107] elegantly showed how PD-1 blockade could control HCV replication in a chimpanzee model of CHC. Interestingly, the chimpanzee with controlled infection also presented a broader base-line immunity response than the cohort animals that were non-responders, suggesting that anti-PD-1 treatment may be useful in only those cases with a critical threshold of pre-existing HCV-specific CD8⁺ T cells.

Figure 3 shows an example of HCV-specific CD8⁺ T cell restoration achieved by use of anti-PD-1 monoclonal antibody. In a study of CHB infection carried out by Peng *et al.*^[25], PD-1 up-regulation on HBV-specific CD8⁺ T cells was shown in blood samples of patients with chronic infection vs the controls (Table 1); moreover, blockade of the PD-1/PD-L1 pathway was shown to significantly enhance the expansion ability of and the IFN- γ production from HBV-specific CD8⁺ T cells after antigen encounter^[25]. Fiscaro *et al.*^[108] further demonstrated that HBV-specific CD8⁺ T cells from the mononuclear cell population of livers infected with hepatitis virus express higher levels of PD-1 than those in the peripheral compartment; additionally, blockade of the PD-1/PD-L1 pathway was shown to improve the effector capacity of these intrahepatic cells, as evidenced by measure of their expansion ability and production of the Tc1 cytokines, such as IL-2 and IFN- γ ^[108]. Tzeng *et al.*^[109] studied T cell exhaustion affecting the intrahepatic infiltrating T cells, using a mouse model of persistent HBV infection; the authors discovered that PD-1 was up-regulated on the HBV-specific CD8⁺ T cells and that blockade of the interaction between PD-1 and PD-L1 results in restoration of the capacity of these cells to produce IFN- γ ^[29]. Furthermore, Gao *et al.*^[26] showed that overexpression of the PD-1 ligand, PD-L1, in HCC is associated with tumour aggressiveness, providing the rationale for developing a new therapy based on blockade of the PD-1/PD-L1 pathway (Table 1). Finally, Shi *et al.*^[110] demonstrated that the HCC-infiltrating CD8⁺ T cells have a drastic increase in PD-1 expression. Taken together, these data support the rationale to set-up clinical trials to analyse the usefulness of blocking the PD-1/PD-L1 pathway in HCC and HBV/HCV chronic infections.

Checkpoint inhibitors blockade: Clinical trials

Taking into account all the previously discussed known characteristics and features of the specific CD8⁺ T cell response in HCC and chronic viral hepatitis, a number of clinical trials have been designed to analyse the effect of PD-1 and CTLA-4 pathway blocking (Table 2). Here, we will describe the completed, on-going and planned clinical trials investigating checkpoint modulation as a therapeutic approach for treating HCC and chronic viral hepatitis.

Regarding CTLA-4, Sangro *et al.*^[30] performed the first pilot clinical trial to address the potential anti-tumour and anti-viral effects of a monoclonal anti-CTLA-4 antibody in CHC and HCC. For this purpose, the authors used "tremelimumab", a fully humanized IgG2 monoclonal antibody that blocks CTLA-4. The study cohort was a small population of patients with HCC superimposed on CHC. Tremelimumab showed an acceptable safety profile, as well as both anti-tumour and anti-viral activities. The anti-tumour effect was particularly encouraging as it was characterized by a



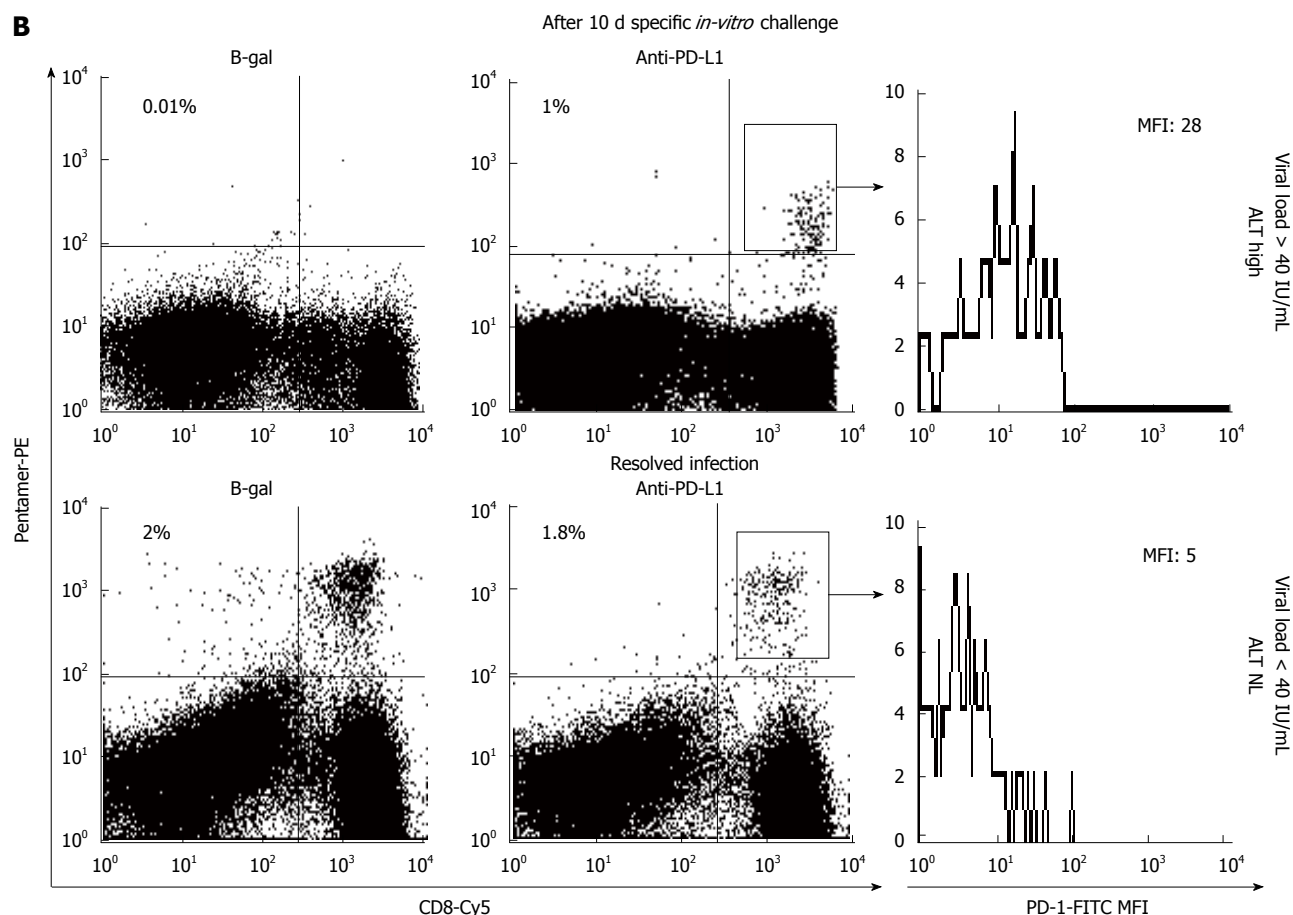


Figure 3 PD-1/PD-L1 blockade-induced restoration *in vitro* of hepatitis C virus-specific CD8⁺ T cells from a patient with chronic hepatitis C virus infection. A: FACSscan[®] dot-plots directly gated on *ex vivo* CD8⁺ T cells showing the PD-1 phenotype and the interferon (IFN)- γ secretion of HCV-NS3₁₄₀₆-pentamer-binding CD8⁺ T cells from a patient with chronic HCV infection and a patient who was able to resolve the infection; B: FACSscan[®] dot-plots showing the frequency of HCV-NS3₁₄₀₆-pentamer-binding CD8⁺ T cells after a 10-d specific *in vitro* challenge in the presence and absence of anti-PD-L1 to block the interaction between PD-1 and its ligand in the patients from (A) with chronic HCV infection and who resolved the infection; PD-1 expression on gated HCV-NS3₁₄₀₆-pentamer-binding CD8⁺ T cells after expansion is also shown. ALT: Alanine aminotransferase; HCV: Hepatitis C virus.

Table 2 Overexpressed negative pathways in liver diseases

Disease	Overexpressed NR	Ligand for the overexpressed NR
HCC	CTLA-4 ^[29] , PD-1 ^[12]	PD-L1 ^[26] , CD48 ^[135]
CHB	CTLA-4 ^[28] , PD-1 ^[25] , LAG-3 ^[117] , Tim-3 ^[129]	PD-L1 ^[136]
CHC	CTLA-4 ^[27] , PD-1 ^[24,105,106] , LAG-3 ^[116] , 2B4 ^[13,122] , Tim-3 ^[128]	PD-L1 ^[44]

Overexpression of negative receptors on specific CD8⁺ T cells in different malignancies and overexpression of their cognate ligands in the liver tissue. CHB: Chronic hepatitis B; CHC: Chronic hepatitis C; HCC: Hepatocellular carcinoma; NR: Negative receptor.

significant increase in the time to progression (TTP), up to 6.48 mo, with respect to the control group. For its anti-viral activity, the drug was shown to induce a significant decrease in viral load, with 15% of the patients achieving sustained virological response without any other anti-HCV treatment. This anti-viral efficacy may appear low, but it could represent a difference in patients who are non-responders to DAA, in who a DAA + anti-PD-1 treatment combination

could improve the sustained virologic response rate. Moreover, these outcomes may be improved by use of a higher dose of the drug^[30].

Other studies with the same anti-CTLA-4 molecule are currently in progress. Greten *et al.*^[111], for example, are currently recruiting patients to participate in their clinical trial to test the safety and effectiveness of tremelimumab in combination with transarterial chemoembolization or RFA in advanced liver cancer. These authors presented their preliminary results during the 2015 American Society of Clinical Oncology Annual Meeting, stating that the combination was safe, feasible and effective, increasing TTP up to 7.4 mo^[18].

Similar to the results of the studies on CTLA-4, the current research data from the studies of PD-1/PD-L1 blockade are encouraging. Gardiner *et al.*^[31] described, for the first time and as proof-of-concept, their findings from an evaluation of anti-PD-1 antibody treatment in patients with CHC, in which the fully human anti-PD-1 monoclonal IgG4 antibody "nivolumab" was administered as a single dose. The treatment did not produce any significant side effects, indicating a suffi-

cient safety, and one-third of the patients experienced significant reductions in viral load. Perhaps, if the authors had combined blockade of the PD-1 pathway with modulation of other co-stimulatory molecules, they could have achieved better results. However, the importance of this pilot clinical trial is that it highlights the significant role of the PD-1 pathway during CHC^[31] and shows that it could represent an adjuvant treatment for DAA regimes.

Feun *et al*^[112] have already designed a clinical trial to determine the anti-tumour effect of anti-PD-1 antibody treatment in patients with advanced, unresectable HCC using another humanized monoclonal IgG4 antibody that binds to PD-1, which is called “pembrolizumab”. The primary objective of this study is to assess therapeutic efficacy, but the researchers also plan to evaluate the expression of PD-L1 in tumour tissue, with the aim of gaining insights into which cases may benefit most from this type of treatment.

After realising that blocking either CTLA-4 or PD-1 pathways could be useful to treat HCC, it becomes apparent that one option to increase anti-tumoural effectiveness could be a treatment combination. A clinical trial is currently in progress to study such an alternative, namely the role of combining the fully humanized anti-CTLA-4 IgG1 antibody called “ipilimumab” in conjunction with the previously introduced anti-PD-1 “nivolumab”^[113]. The first part of that study will evaluate the safety profile of nivolumab in HCC patients, after which the efficacy of nivolumab will be compared with that of sorafenib. Finally, the researchers plan to address the safety and efficacy profiles of the combination nivolumab and ipilimumab for treatment of advanced HCC^[86]. In the near future, hepatologists and oncologists should know if this hopeful combination represents a *bona fide* treatment option for patients with advanced HCC.

Other inhibitory receptors

Although CTLA-4 and PD-1 are the best characterized inhibitory receptors to date, there are other negative co-stimulatory molecules involved in CD8⁺ T cell exhaustion that deserve to be studied. Most of the work carried out on the role of these other co-stimulatory molecules has been done in the conditions of HVB and HCV infections, while information in the condition of HCC remains scarce.

LAG-3

LAG-3 was identified in 1990 by Triebel *et al*^[114]. Even though its main ligand is MHC class II, it also helps PD-1 to maintain CD8⁺ T cell exhaustion during chronic viral infections^[115]. Chen *et al*^[116] demonstrated that LAG-3 was overexpressed on HCV-specific CD8⁺ T cells in patients with CHC and that LAG-3 blockade restored effector capacity of these cells, as evidenced by measure of their expansion ability and cytokine production (Table 1). Kennedy *et al*^[117] demonstrated

different expression patterns of LAG-3 on HBV-specific CD8⁺ T cells during the natural history of CHB, whereby LAG-3 up-regulation occurs progressively, suggesting that HBV infection induces a progressive status of T cell exhaustion over time. Li *et al*^[118] studied LAG-3 expression in patients with HBV-related HCC and discovered an overexpression of LAG-3 on the HBV-specific CD8⁺ T cells from liver tissue, as compared with those in the peripheral blood. Taking these collective data into account, LAG-3 appears to be another immune checkpoint to bear in mind during consideration of diseases affected by T cell exhaustion.

Natural killer cell receptor (2B4)

The 2B4 was first identified in 1993 by Valiante *et al*^[119]. Its ligand, CD48, has 5-10 times stronger affinity for 2B4 than CD2, a molecule necessary for T cell activation^[120,121]. This competitive advantage of 2B4 for binding to CD48 could impair T cell activation if 2B4 is induced early. Bengsch *et al*^[13] and Schlaphoff *et al*^[122] studied 2B4 expression on HCV-specific CD8⁺ T cells in the blood of patients with chronic hepatitis infections and demonstrated overexpression in PD-1⁺-HCV-specific CD8⁺ T cells, which was further linked to an exhausted behaviour. Similarly, Kroy *et al*^[123] showed an up-regulation of 2B4 in HCV-specific CD8⁺ intrahepatic lymphocytes, as compared with peripheral lymphocytes, during CHC. Bengsch *et al*^[13] studied the effect of blockade of several inhibitory receptors, including 2B4, in the functionality of HBV-specific CD8⁺ T cells during CHB and found that the response to blockade was primarily mediated by PD-1 (Table 1). Considering all of these data, 2B4 up-regulation seems to be linked with PD-1 overexpression, and its modulation could have a less robust effect on T cell restoration than other negative co-stimulatory molecules.

T-cell immunoglobulin and mucin-domain containing-3

T-cell immunoglobulin and mucin-domain containing (Tim)-3 was first identified in 2002 by Monney *et al*^[124]. Tim-3 is another immune checkpoint receptor that limits the duration and magnitude of T cell responses^[125-127]. McMahan *et al*^[128] demonstrated that Tim-3 was up-regulated on HCV-specific CD8⁺ T cells in patients with chronic hepatitis infection, as compared to those who had been able to control the infection; in addition, remarkably, Tim-3 blockade restored the expansion ability of these cells. Wu *et al*^[129] studied Tim-3 expression on HBV-specific CD8⁺ T cells during CHB and discovered that Tim-3 overexpression was related to disease progression; the authors also demonstrated that blockade of the Tim-3 pathway restored the effector capacity of the HBV-specific CD8⁺ T cells^[130] (Table 1). Interestingly, a lack of Tim-3 has thus far not been found to be associated with autoimmune diseases^[131], making Tim-3 a very attractive therapeutic target.

CD160

CD160 was discovered in 1998 by Anumanthan *et al.*^[132] and is another negative co-stimulatory molecule associated with T cell exhaustion^[133]. Bengsch *et al.*^[13] and Schlaphoff *et al.*^[122] also studied CD160 expression on HCV-specific CD8⁺ T cells obtained from patients with chronic hepatitis infections and demonstrated an overexpression of this protein that was related with exhaustion. Nevertheless, Kroy *et al.*^[123] showed that there was no overexpression of this molecule in intrahepatic HCV-specific CD8⁺ T cells in CHC, as compared to the peripheral cells. Another study from Viganò *et al.*^[134] investigated the expression of CD160 on HCV-specific CD8⁺ T cells during CHC and found functional impairment in these cells, which was independent of PD-1 expression; moreover, the blockade of CD160/CD160L in this study restored the expansion ability of the HCV-specific CD8⁺ T cells^[134] (Table 1). Nevertheless, due to the current contradictory data more studies are needed to gain an adequate understanding of this pathway before it can be considered as a potential therapeutic target.

CONCLUSION

Immune checkpoint modulation can resolve CD8⁺ T cell exhaustion *in vitro* and *in vivo* in chronic infections with hepatoviruses and in tumoural diseases. This therapy could represent a promising treatment option for patients with advanced HCC and chronic viral hepatitis who are non-responders to the current standard of care. The use of humanized monoclonal antibodies against negative co-stimulatory molecules can reverse the exhaustion state of CD8⁺ T cells, thereby making them able to control either the tumour or the virus. In the upcoming years, we will likely see the results of combining these agents with current HCC and anti-viral therapies. While the current results from clinical studies remain modest, they could be the base for encouraging development and implementation of novel therapeutic strategies. Clearly, we must expand our basic and clinical knowledge about how modulating and finely tuning these receptors will help to avoid adverse events, and how appropriate patient selection will help to define the groups most likely to respond to this kind of treatment. We must also learn how to best use these drugs in combination with other therapies in order to obtain the maximum clinical benefit. Fortunately, the field of immunotherapy highlights the fact that translational medicine can improve the health of our patients, bringing the results obtained by basic research to the bedside in a short period.

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