Costimulatory molecule programmed death-1 in the cytotoxic response during chronic hepatitis C

Juan Ramón Larrubia, Selma Benito-Martínez, Joaquín Miquel, Miryam Calvino, Eduardo Sanz-de-Villalobos, Trinidad Parra-Cid

Abstract

Hepatitis C virus (HCV)-specific CD8^+ T cells play an important role in the resolution of HCV infection. Nevertheless, during chronic hepatitis C these cells lack their effector functions and fail to control the virus. HCV has developed several mechanisms to escape immune control. One of these strategies is the up-regulation of negative co-stimulatory molecules such as PD-1. This molecule is up-regulated on intrahepatic and peripheral HCV-specific cytotoxic T cells during acute and chronic phases of the disease, whereas PD-1 expression is low in resolved infection. PD-1 expressing HCV-specific CD8^+ T cells are exhausted with impairment of several effector mechanisms, such as: type-1 cytokine production, expansion ability after antigen encounter and cytotoxic ability. However, PD-1 associated exhaustion can be restored by blocking the interaction between PD-1 and its ligand (PD-L1). After this blockade, HCV-specific CD8^+ T cells reacquire their functionality. Nevertheless, functional restoration depends on PD-1 expression level. High PD-1-expressing intrahepatic HCV-specific CD8^+ T cells do not restore their effector abilities after PD-1/ PD-L1 blockade. The mechanisms by which HCV is able to induce PD-1 up-regulation to escape immune control are unknown. Persistent TCR stimulation by a high level of HCV antigens could favour early PD-1 induction, but the interaction between HCV core protein and gC1q receptor could also participate in this process. The PD-1/PD-L1 pathway modulation could be a therapeutic strategy, in conjunction with the regulation of others co-stimulatory pathways, in order to restore immune response against HCV to succeed in clearing the infection.

INTRODUCTION

Hepatitis C virus (HCV) is a hepatotropic non-cytopathic positive-strand RNA virus which belongs to the Flaviviridae family. HCV infection is a major public problem, affecting more than 200 million people worldwide. Only around a quarter of acute HCV infections resolve within a few months, while in the majority of cases the virus establishes a persistent infection, and a significant proportion of cases progress to fibrosis, cirrhosis, liver failure or even hepatocellular carcinoma.

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Peer reviewers: Rudi Beyaert, Professor, Department of Molecular Biomedical Research, Flanders Interuniversity Institute for Biotechnology and Ghent University, Technologiepark 927, B-9052 Gent, Belgium; Sabine Mihm, Professor, Department of Gastroenterology, Georg-August-Universität, Robert-Koch-Str-40, Göttingen D-37099, Germany
PD-1 is a 55 kDa glycoprotein which belongs to the CD28 immunoglobulin superfamily of transmembrane proteins. PD-1 shares a 23% homology with CTLA-4, which is another member of this family, although PD-1 has lost the MYPPPY motif for binding to B7 molecules, and the cysteine residue necessary for homodimerization. PD-1 is expressed on activated T cells, B cells and myeloid cells (Table 1). The PD-1 structure consists of two regions; the extracellular region is formed by a single IgV-like domain and its cytoplasmic region contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM). Following antigen stimulation, PD-1 recruits the protein tyrosine phosphatase src homology 2 domain-containing tyrosine phosphatase 2 (SHP-2) to ITSM but not to the ITIM motif, and subsequently, SHP-2 dephosphorylates effector molecules downstream of the TCR-induced nuclear regulatory pathway. The direct result of PD-1 mediated inhibition of T cell proliferation is cell cycle arrest in G0/G1 and the inhibition of IL-2 production (Figure 1). The ligands for PD-1 (PD-L1 and PD-L2) are type I transmembrane proteins with IgV and IgC-like domains in the extracellular region. PD-L1 is expressed on resting and activated B and T cells, and on non-lymphoid cells such as pancreas, placenta and heart, while PD-L2 is induced on dendritic cells (DC) and macrophages (Table 1). Interestingly, PD-L1 can be up-regulated on hepatocytes by α-interferon (IFN) and γ-IFN, and also by activated lymphocytes, and by direct viral infection (perhaps also through IFN pathways). PD-L1 plays an important physiological role in regulating the cellular immune response, tuning-down the effector functions after T cells have developed their tasks. This physiological function of PD-1 can be damaged by persistent viruses inducing a tolerogenic-like status on specific T cells to avoid immune viral control.

**PD-1 EXPRESSION IN THE LIVER**

The liver is characterised by being an immunotolerant organ prepared to deal with intense contact with antigens from the gut, and PD-1/PD-L1 is expressed in resident and infiltrating liver cells to carry out this task. The liver is also the primary site for HCV replication and disease pathogenesis, and HCV can take advantage of the PD-1/PD-L1 pathway to impair the HCV-specific response reaching the infected liver in order to escape immune control. The liver is exposed to antigens and microbiologically-derived molecules which cause a unique microenvironment that requires liver immunological properties to induce tolerance rather than immunity. Hepatic tolerance contributes to the common ineffectiveness of immune response against HCV which often results in chronic viral persistence. When naive T cells reach the liver from the bloodstream they are activated by resident antigen presenting cells and are prone to become anergic, and this process could take part in the interaction between PD-1 and PD-L1 (Figure 2). On the other hand, primed effector HCV-specific T cells, reaching the infected liver, are also conducted through anergy by several mechanisms. One of them is PD-1 up-regulation on T cells in the liver, and the expression of its ligand on resident liver cells, such as hepatocytes, Kupffer cells and sinusoidal endothelial cells (Figure 2). Usually, PD-L1 is constitutively expressed in non-lymphoid tissues such as heart,
lung, placenta, kidney, and liver, but during chronic HCV infection, this molecule is up-regulated on parenchymal liver cells, as previously commented (Figure 3A). The regulation of HCV-specific effector CTLs is also controlled by intrahepatic CD4+CD25+FoxP3+ cells (regulatory T cells, Treg). These cells have an important role in maintaining the balance between tolerance and immunity in HCV infection. The PD-1/PD-L1 pathway is also important in modulating the regulatory activity of these Treg cells. The PD-1/PD-L1 interaction on intrahepatic Treg suppresses their regulatory activity, favouring CTL response. Nevertheless, when it is necessary to down-modulate HCV-specific CTL response in order to avoid liver damage, PD-1/PD-L1 engagement is not produced between Tregs and intrahepatic PD-L1 expressing cells, due to PD-L1 down-regulation on resident liver cells, allowing Tregs to down-modulate HCV-specific CTLs effecter functions (Figure 2). The important role of PD-1 in liver pathogenesis during HCV chronic infection is evident, as it is shown by the high PD-1 expression on total intrahepatic T cells, indicating that some non-specific HCV-dependent stimulus is acting in liver infiltrating T cells to favour PD-1 up-regulation. Previous reports suggest that this factor could be HCV-core protein and this will be discussed later. In addition to this non-specific stimulation on T cells, PD-1 expression is also induced by persistent specific TCR stimulation. PD-1 expression is higher on intrahepatic than in peripheral HCV-specific

Table 1  Summary of CD28 family co-stimulatory and co-inhibitory pathways

| Receptor | Expression | Ligand | Ligand expression | T cell response regulation |
|----------|------------|--------|-------------------|---------------------------|
| CD28     | T cells (naive and some memory) | CD80   | B, T and DC, macrophages | Positive |
| PD-1     | Activate T cells, B cells, macrophages | PD-L1  | B, T and DC, macrophages, non-lymphoid cells (pancreas, placenta, heart) | Negative |
| CTLA-4   | Activate T cells and regulatory T cells | CD80   | B, T and DC, macrophages | Negative |
| BTLA     | B and T cells | PD-L2  | Macrophages, DC | Negative |
| ICOS     | T cells (memory and effector) | ICOS-L | B, T cells, macrophages and DC | Positive |

Figure 1  Programmed death-1 (PD-1) structure and interactions. Interaction between the PD-1 molecule expressed on T cells and its ligand PD-L1 expressed on antigen-presenting cells leads to immunoreceptor tyrosine-based switch motif (ITSM) motif phosphorylation in its cytoplasmic tyrosines which are recognized by src homology 2 domain-containing tyrosine phosphatase 2 (SHP-2). All of these interactions cause T cell anergy due to T cell receptor (TCR)-dependent MAP Kinase-pathway signalling inhibition which avoids interleukin (IL)-2 gene transduction. PD-1 expression is induced by TCR activation but could also be favoured by HCV-core protein through interaction with gC1qR. PD-L1 is up-regulated on antigen presenting cells by the effect of γ-interferon produced during HCV infection by activated lymphocytes.

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Circulating HCV-specific CD8+ T tolerance of virus-specific CD8+ cells caused by PD-1 up-regulation. The exhaustion of virus-specific CTL response could be the exhaustion of the possible mechanisms responsible for impairment highly functional in cases of resolving infection and memory CD8+ when infection becomes chronic outcome of the disease, and this impairment persists CD8+ during persistent infection, and this impairment is produced by TCR stimulation in addition to the interaction between HCV-core protein and the complement receptor (gC1qR). In this micro-environment the regulatory T cells (Treg) also participate, whose activity is also regulated by the PD-1/PD-L1 pathway.

Figure 2 Liver microenvironment. Circulating HCV-specific CD8+ T cells migrating through the hepatic sinusoid interact with resident liver cells [Kupffer cells, dendritic cells (DC), hepatocytes, liver sinusoidal endothelial cells (LSECs), stellate cells] that could act as antigen presenting cells. These cells up-regulate PD-L1 expression during persistent HCV infection and interact with the PD-1 molecule expressed on HCV-specific CD8+ T cells. This interaction leads to T cell anergy. PD-1 up-regulation is produced by TCR stimulation in addition to the interaction between HCV-core protein and the complement receptor (gC1qR). In this micro-environment the regulatory T cells (Treg) also participate, whose activity is also regulated by the PD-1/PD-L1 pathway.

CD8+ cells[41] (Figure 3B). These data suggest that the intense TCR activation produced in the liver in conjunction with the high level of HCV-core protein leads to the highest PD-1 expression on HCV-specific CD8+ cells. This high PD-1 up-regulation on intrahepatic specific CD8+ cells is exquisitely HCV specific, so that PD-1 expression on other virus-specific CD8+ T cells is not up-regulated during chronic HCV infection[40]. Therefore, liver environment conditions produce a huge PD-1 up-regulation on HCV-specific CTLs during persistent infection, and this could impair viral control by the cellular immune response through anergy induction[41].

DIFFERENTIAL PD-1 EXPRESSION IN ACUTE, CHRONIC AND RESOLVED HEPATITIS C VIRUS INFECTION

During the initial phase of acute infection, HCV-specific CD8+ T cells are dysfunctional irrespective of the final outcome of the disease, and this impairment persists when infection becomes chronic[40]. In contrast, effector and memory CD8+ T cells generated after acute onset are highly functional in cases of resolving infection[55,56]. One of the possible mechanisms responsible for impairment of virus-specific CTL response could be the exhaustion of these cells caused by PD-1 up-regulation. The exhaustion of virus-specific CD8+ T cells has been observed in different human infections such as HIV, HBV and HCV infections[55,59-65]. In HCV infection, during the early period of primo-infection irrespective of the final outcome, PD-1 is up-regulated on all HCV-specific CD8+ T cells[53,66]. However, after the acute stage of the disease PD-1 expression is modulated depending on the progression. Therefore, during self-limited infection HCV-specific CD8+ cells down-regulate PD-1 expression, and acquire a CD127+ phenotype which correlates with appropriate effector functions (Figure 4)[67]. CD127 is the IL-7 receptor (IL-7R) which plays an essential role in mature lymphocyte survival through a pathway activated by the interaction with IL-7[67]. However, in persistent infection HCV-specific CD8+ cells remain CD127 negative, and maintain high levels of PD-1 expression[66,68] (Figure 4). Therefore, PD-1+ CD127+ expressing HCV-specific CD8+ cells during persistent infection are not only anergic, but also prone to apoptosis after antigen encounter due to the absence of CD127 expression. Furthermore, PD-1 up-regulation on peripheral and intrahepatic HCV-specific CD8+ cells during the acute and chronic phases of infection is correlated with the apoptosis susceptibility of these cells[56]. As a result, the majority of high PD-1 expressing HCV-specific CD8+ cells could follow an apoptotic process[66], indicating that PD-1 is involved in anergy induction but could also be implicated in specific T cell deletion. Probably, both mechanisms are damaged by HCV infection to escape cellular immune response.
CORRELATION BETWEEN PD-1
EXPRESSION AND EFFECOR FUNCTION
IMPAIRMENT ON HCV-SPECIFIC CTLs

Once differential PD-1 expression on HCV-specific CD8+ T cells between chronic and resolved patients has been described, the next point to address is to analyse whether this difference translates into different quality of HCV-specific CTLs effector functions. Cytotoxic T-cell exhaustion represents a spectrum of effector defects that are correlated with the level of PD-1 expression. Recent reports show that patients with HCV chronic infection, whose CTLs display high PD-1 expression, have impaired CTL capacity to synthesise type-1 cytokines, such as γ-IFN, α-tumor necrosis factor (TNF) and IL-2, in addition to cytolytic molecules, such as perforin and granzyme B, after direct ex-vivo specific in-vitro challenge. One of the variables determining viral control has been suggested to be the ability of virus-specific CD8+ cells to clonally expand after antigen encounter. HCV-specific CD8+ T cells during persistent infection also displayed impaired proliferation ability after specific stimulation, which correlated with PD-1 expression level. Because of the role of the PD-1/PD-L1 pathway in proliferation impairment, subsequent works were aimed at trying to enhance HCV-specific CD8+ T cell proliferation by modulating this pathway. Blocking the interaction between PD-1 and its ligand increased the proliferation ability of peripheral HCV-specific CD8+ cells from some chronic HCV patients, characterised by high PD-1 expression, but did not occur in others, suggesting the presence of another anti-proliferative mechanism not yet described.

During HCV-specific CTL exhaustion, not all effector functions are altered at the same time; proliferative potential and IL-2 production are lost at an early phase, whereas cytokine production and cytolytic function are lost later. This progressive impairment could be related to the level of PD-1 up-regulation. Interestingly, the exhaustion of CTLs during chronic HCV infection is highly antigen-specific and related to the level of antigenemia, not being present in either CTLs against other specificities or HCV-specific CTLs from patients with resolved infection. In these two situations PD-1 is not up-regulated on specific CTLs. As commented before, intrahepatic HCV-specific CD8+ T cells are highly PD-1 positive and they do not expand after antigen encounter and do not produce either γ-IFN or perforin, whereas intrahepatic specific CTLs against other
viruses, such as influenza virus-specific CD8+ T cells, expand efficiently and present a high level of perforin expression, but interestingly they are PD-1 negative [41]. High PD-1 expressing intrahepatic HCV-specific CTLs do not respond to anti-PD-L1 treatment [31]. Therefore, when PD-1 expression is extremely up-regulated, treatment with anti-PD-L1 antibodies can not counteract the HCV-specific CTLs exhaustion, induced by the PD-1/PD-L1 pathway.

**HCV-SPECIFIC CTL FUNCTIONAL RESTORATION AFTER PD-1/PD-L1 INTERACTION BLOCKADE**

Previous studies developed an LCMV infection animal model, and specific CTL function restoration during persistent infection after treatment with anti-PD-L1 monoclonal antibodies was shown [15-23]. This finding could have clinical implications in the treatment of persistent viral infections, as will be discussed later. In HCV infection, the *in-vitro* blockade of the PD-1/PD-L1 pathway with anti-PDL-1 antibodies increases proliferation capacity after antigen encounter in peripheral PD-1 expressing HCV-specific CTLs from chronic patients (Figure 5). *In-vitro* treatment with anti-PD-L1 antibodies also restored γ-IFN, perforin, CD107a, IL-2 and IL-13 production after antigen specific stimulation [41, 54, 74]. However, this PD-1/PD-L1 pathway blockade is not efficient on intrahepatic HCV-specific CD8+ T cells, which are characterised by a higher PD-1 expression, as previously discussed. These cells failed to proliferate and produce perforin, γ-IFN and CD107a after specific stimulation in the presence of anti-PD-L1 antibodies [41]. All these findings suggest that PD-1 expression level correlates inversely with HCV-specific CD8+ T cells functional restoration by PD-1/PD-L1 blockade. PD-1/PD-L1 blockade may increase the functionality of peripheral HCV-specific CD8+ T cells with intermediate PD-1 expression, whereas this blockade did not enhance the effector functions of intrahepatic PD-1 expressing HCV-specific CD8+ T cells. High antigenic stimulation in the liver induces other negative co-stimulatory molecules, such as CTLA-4 [41].

![Graphs showing the effect of PD-1/PD-L1 interaction blockade on HCV-specific CTL functionality.](www.wjgnet.com)
which could maintain the anergic status, despite blockage of the PD-1/PD-L1 pathway. Therefore, the functional restoration of intrahepatic HCV-specific CTLs could be obtained by the combined blocking of different negative co-stimulatory molecules. In fact, a previous report has shown that combined PD-1 and CTLA-4 blockade induces a restoration of intrahepatic HCV-specific CD8$^+$ T cell function in chronically HCV infected patients.\(^7\) Obviously, the modulation of different co-stimulatory molecules on HCV-specific T cells, such as CD137\(^7\), OX40\(^7\) and ICOS\(^8\) should be tested in combination with PD-1/PD-L1 blockade in order to restore HCV-specific CTL effector functions.\(^9,8^0\) Nevertheless, blocking the engagement between PD-1 and PD-L1 is not enough in many cases to restore peripheral HCV-specific CTL functionality in chronic patients, even in combination with the blockade of other negative co-stimulatory molecules. It is reasonable to assume that these cells, exposed to high persistent antigenic stimulus, are prone to apoptosis. Previous data on HBV chronic infection showed an up-regulation of the pro-apoptotic molecule Bim on HBV-specific CTLs.\(^8^1\) In this chronic infection, only CD127\(^+$\) (IL-7R) cells maintained the ability to expand after antigen encounter. These CD127\(^+$\) cells could be protected from apoptosis due to the antiapoptotic molecule Mcl-1, induced by IL-7. Otherwise, CD127\(^-\) HBV-specific CTLs would die due to apoptosis after antigen encounter, mediated by the Bim pathway. Bearing in mind these data on HBV infection, it is possible that the benefit observed by blocking the PD-1/PD-L1 interaction may occur only in specific T cells protected against apoptosis by CD127 expression. This phenotype is quite rare in patients with long-standing HCV infection, and this could explain why not all PD-1 expressing HCV-specific CTLs respond to anti-PD-L1 treatment. This theoretical scenario should be tested in the near future.

**HCV CORE PROTEIN INDUCES PD-1 UP-REGULATION**

The PD-1 up-regulation on intrahepatic total T cells suggests that something other than TCR stimulation is involved in the PD-1 expression regulation during HCV infection. HCV-core protein binding to the complement receptor gC1q (gC1qR) is responsible for impairing T cell proliferation ability\(^8^2\) through down-regulation of the high affinity IL-2 receptor\(^8^3\). A recent report suggests that this process could be mediated by PD-1 expression.

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**Figure 5** Proliferation restoration after PD-1 blockade. FACS$^*$ dot-plots of peripheral blood T cells from two representative patients, one with persistent HCV infection and the other with resolved HCV infection, and a control case, after specific stimulation in the presence or absence of anti-PD-L1 mAb. T cells were stimulated for 10 d with the HCV–specific peptide plus IL-2. After stimulation, T cells were stained with CD8-Cy mAbs and HCV-tetramers-PE. PD-1/PD-L1 pathway blockade by anti-PD-L1 antibodies increases the HCV-specific cell proliferation in the chronic patient that had a high level of PD-1 expression.

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induction. In fact, in an intrahepatic HCV-core protein expressing mouse model, liver infiltration by PD-1 expressing cytotoxic T cells unable to clear the virus has been shown. However, the liver from HCV-core non-expressing mice was infiltrated by non-PD-1 expressing specific-CTLs which could control the viral infection. These data suggest that HCV-core protein could play a role in early PD-1 induction on T cells, mainly in the liver environment where this protein is richly expressed. At least in vitro, PD-1 and PD-L1 expression are up-regulated on activated T cells in the presence of HCV-core protein. PD-1 up-regulation induced by HCV-core protein translated into impairment of T cell proliferation ability. However, this dysfunction could be partially restored by blocking the PD-1/PD-L1 pathway with anti-PD-L1 antibodies (Figures 1 and 6) and by blocking the interaction between HCV-core protein and gC1qR. Probably the interaction between HCV-core protein and gC1qR co-operate with the continuous TCR stimulation to produce an early PD-1 up-regulation in order to induce a premature anergy on HCV-specific CTLs as an efficient HCV escape mechanism.

**PD-1/PD-L1 BLOCKADE AS A THERAPEUTIC TOOL**

As previously commented, a defective virus-specific cytotoxic T cell response is one of the most important causes of host inability to eliminate a persistent viral infection. Several studies have highlighted the role of the PD-1/PD-L1 pathway in the development of anergy on virus-specific CD8+ T cells, and how PD-1/PD-L1 blockade could enhance virus-specific CD8+ T cell functionality in vitro. Recently, several works have been carried out to analyse whether modulation of the PD-1/PD-L1 pathway could improve T cell response against persistent viral infections either directly, using anti PD-L1 antibodies alone, or in combination with a therapeutic vaccine. Therapeutic vaccine usually fails to induce a vigorous T cell response due to the tolerogenic-like status of HCV-specific T cells. This scenario could be positive if negative co-stimulatory molecules, such as PD-1, were blocked when the therapeutic vaccine is administered in order to enhance the specific immune response against the supplied epitopes. In the chronic LCMV infection...
animal model, the administration of a therapeutic vaccine in combination with PD-1/PD-L1 interaction blockade enhances expansion and improves the function of LC-MV-specific CD8⁺ T cells. In addition, this combinatorial therapeutic vaccination accelerates viral control compared with either therapeutic vaccine or PD-1 blockade alone. Moreover, the effect of anti-PD-L1 antibodies alone could also be effective in controlling persistent viral infection by restoring specific CTL response. The administration of anti-PD-L1 monoclonal antibodies during simian immunodeficiency virus (SIV) chronic infection in macaques resulted in a rapid expansion and restoration of SIV-specific CD8⁺ T cells. Although these results seem to be quite promising, the blockade of negative co-stimulatory pathways could lead to the development of autoimmune diseases, which could prevent the use of this strategy as a therapeutic tool in humans. Therefore, more research is necessary in this field before blockade of the PD-1/PD-L1 pathway is suitable for the treatment of chronic HCV infection.

CONCLUSION

In summary, the PD-1/PD-L1 pathway displays an important role in the induction of anergy on HCV-specific cytotoxic T cells, and could be important in the development of HCV persistent infection. Blocking the PD-1/PD-L1 interaction, probably in association with the modulation of other co-stimulatory molecules, could be an interesting strategy to restore HCV-specific CTL response in patients unresponsive to standard anti-HCV treatment.

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