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Impaired hepatitis B vaccine responses during chronic hepatitis C infection: involvement of the PD-1 pathway in regulating CD4⁺ T cell responses

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Abstract

Vaccination for hepatitis B virus (HBV) in the setting of hepatitis C virus (HCV) infection is recommended, but responses to vaccination are blunted when compared to uninfected populations. The mechanism for this failure of immune response in HCV-infected subjects remains unknown but is thought to be a result of lymphocyte dysfunction during chronic viral infection. We have recently demonstrated that PD-1, a novel negative immunomodulator for T cell receptor (TCR) signaling, is involved in T and B lymphocyte dysregulation during chronic HCV infection. In this report, we further investigated the role of the PD-1 pathway in regulation of CD4⁺ T cell responses to HBV vaccination in HCV-infected individuals. In a prospective HCV infected cohort, a poor response rate to HBV vaccination as assayed by seroconversion was observed in HCV-infected subjects (53%), while a high response rate was observed in healthy or spontaneously HCV-resolved individuals (94%). CD4⁺ T cell responses to *ex vivo* stimulations of anti-CD3/CD28 antibodies or hepatitis B surface antigen (HBsAg) were found to be lower in HBV vaccine non-responders compared to those responders in HCV-infected individuals who had received a series of HBV immunizations. PD-1 expression on CD4⁺ T cells were detected at relatively higher levels in these HBV vaccine non-responders than those who responded, and this was inversely associated with the cell activation status. Importantly, blocking the PD-1 pathway improved T cell activation and proliferation in response to *ex vivo* HBsAg or anti-CD3/CD28 stimulation in HBV vaccine non-responders. These results suggest that PD-1 signaling may be involved in impairing CD4⁺ T cell responses to HBV vaccination in subjects with HCV infection, and raise the possibility that blocking this negative signaling pathway might improve success rates of immunization in the setting of chronic viral infection.

Keywords

vaccine; hepatitis C; hepatitis B; PD-1; T cell

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Introduction

Hepatitis C virus (HCV) infection is a common cause of chronic liver disease and is the leading indication for liver transplantation in the United States. Given the shared risk factors for transmission, co-infection of hepatitis B virus (HBV) with HCV is quite common, and may lead to more significant liver disease, including higher rates and more rapid progression to liver cirrhosis and liver cancer¹⁻⁵. As such, HBV vaccine is recommended as the primary means to prevent HBV super-infection and its associated increase in morbidity and mortality in HCV-infected subjects. However, vaccine response (seroconversion with a hepatitis B surface antibody titer >10 IU/L) in this setting is often blunted, with poor response rates to a standard course of HBV vaccinations in chronically HCV-infected individuals when compared to the healthy populations (40~60% versus 90~95%); this is especially noted in the setting of advanced fibrosis and liver cirrhosis⁶⁻⁸. To improve seroconversion rates following HBV immunization, approaches have included different administration routes (intradermal), higher doses of HBV vaccine (40 µg), or adding adjuvants (CPG 7909, levamisole, GM-CSF), but success rates remain disappointing in the setting of chronic HCV infection. Additionally, a poor immune response in HCV-infected subjects is also observed in the clinical setting following other adult immunizations—including hepatitis A, influenza and pneumococcal vaccines—a phenomenon that observed in the setting of human immunodeficiency virus (HIV) infection^{9,10}. This suggests the possibility of a more global immune dysregulation and a shared mechanism in individuals with chronic viral infections.

The mechanism for vaccine-induced immune response is activation and expansion of antigen-specific memory T and B lymphocytes upon encountering antigen. Activation and proliferation of lymphocytes requires two signals: an antigen specific signal and a co-stimulatory signal. Co-stimulatory signaling is pivotal to determining whether recognition of antigen by lymphocytes leads to full cell activation/proliferation or to cell exhaustion/apoptosis. Recently, it has been shown that a virus-induced negative co-stimulatory cell modulator, programmed death-1 (PD-1), contributes significantly to lymphocyte dysregulation^{11, 12}. PD-1 is an inhibitory immune-receptor predominantly expressed on activated T and B lymphocytes. PD-1 ligand, PDL-1, is expressed on both haematopoietic and parenchymal cells. PD-1/PDL-1 engagement induces immune-receptor tyrosine phosphorylation and delivers a negative signal to T/B cell receptor (TCR/BCR) signaling pathways. We have recently demonstrated that HCV core-mediated PD-1 expression is involved in T and B cell dysregulation during chronic HCV infection¹³⁻¹⁵. However, the role of PD-1 in regulating T cell responses to HBV vaccine in HCV-infected individuals has yet to be determined. In this report, we found that patients with chronic HCV infection have poor responses to HBV immunizations, and that PD-1 is involved in regulating CD4⁺ T cell responses to HBV vaccines in chronically HCV-infected individuals.

Materials and Methods

Subjects

The study protocol was approved by an institutional review board at East Tennessee State University and James H. Quillen VA Medical Center (ETSU/VA IRB, Johnson City, TN), which has contributed to a database for the storage of blood samples from HCV-infected individuals for the purpose of viral immunology studies. A total of 61 HCV-infected subjects and 16 controls without serologic evidence of prior exposure to HBV or HAV were recruited into this study to receive either Engerix[®] HBV (if HAV antibody negative), or Twinrix[®] HAV/HBV combination vaccines as appropriate, and peripheral blood was collected at 1~6 months post immunizations to determine seroconversion, with an HBsAb titer >10 IU/L deemed as HBV vaccine response. All subjects gave written informed consent for this study. The subjects were divided into four groups of populations: 1) HBV vaccine

non-responders with HCV infection (HBV-NR, n=29); 2) HBV vaccine responders with HCV infection (HBV-R, n=32); 3) subjects with HCV who spontaneously resolved their infection and were HCV RNA-negative (HCV-resolved individuals, n=6); and 4) healthy subjects who were negative for HBV, HCV, and HIV (n=10). The demographic features of each group, including HCV genotype and viral load (performed by Lexington VAMC) are shown in Table 1. The majority of the study subjects were male. The mean age of the HCV-infected individuals was higher but comparable to healthy subjects.

Cell isolation and culture

Human peripheral blood mononuclear cells (PBMC) were isolated from the peripheral blood of study subjects by Ficoll-density centrifugation with lympho-H (Atlanta biological, Lawrenceville, GA), which were then viably cryopreserved in freezing medium in liquid nitrogen¹⁵. If indicated, CD4⁺ T cells were further purified from isolated PBMC by incubation with a magnetic beads-conjugated with anti-CD4 antibody, followed by positive selection per the manufacturer's instruction (Miltenyi Biotec., Auburn, CA). Purified cells were cultured as described¹⁴.

Flow cytometry

To determine the role of PD-1 in T cell activation, 1×10^6 purified PBMC were stimulated with either anti-CD3/CD28 (1 µg/ml each; BD Pharmingen) or HBsAg (2.5 µg/ml, Biospecific, Emeryville, CA) for 24 hours, followed by double or triple immune-staining with the following antibody conjugates: PE-anti-CD69, APC-anti-CD25, PE-anti-PD-1, PE-anti-PDL-1, and FITC-anti-CD4 (BD Pharmingen or eBioscience). The primary isotype controls were used to determine the level of background staining. 20,000 events were acquired after gating on lymphocyte populations using a BD FACS caliber instrument. Percentages of CD69/CD25 or PD-1/PDL-1 expressions in the CD4⁺ T cells were analyzed using FACS express software.

PD-1/PDL-1 blockade

PBMC isolated from HBV-NR in HCV-infected subjects were incubated with 10 µg/ml anti-PDL-1 (eBioscience, San diego, CA, anti-human CD274 or B7-H1 clone: M1H1) or control IgG antibodies overnight, followed by stimulation with anti-CD3/CD28 or HBsAg for 5 days. T cell activation and proliferation were evaluated by detecting CD69 expression and CFSE dilution as described previously¹³.

Statistical analysis

Results are expressed as means $\pm$ SD with additional confidence intervals of the means included where appropriate. Linear regression models were run predicting percentages of CD69 expression among different groups and beta-estimates of predictor variables generated using the NPARIWAY procedure in SAS software, version 9.1. The difference of CD69/CD25 or PD-1/PDL-1 expression levels between each group was determined using independent samples T test program by SPSS 18 software (IBM, Somers, NY). Correlation between T cell activation markers and PD-1/PDL-1 expression was analyzed using a Pearson Correlation program. Values of *P < 0.05 were considered significant, and **P < 0.01 was considered very significant.

Results

Subjects with chronic HCV infection have a poor response to HBV vaccines

Individuals with chronic HCV infection are at increased risk of severe outcomes upon co-infection with HBV. In this prospective study to evaluate the efficacy of HBV vaccinations

in subjects with HCV infection, those without serologic evidence of prior exposure to HBV were vaccinated with either Engerix® HBV, or Twinrix® HAV/HBV combination vaccine if hepatitis A virus (HAV) nonimmune, and response was defined as the development of anti-HBV surface antibody (HBsAb). A total of 61 HCV-infected subjects received vaccination for HBV: 28% (17/61) received Engerix, and 72% (44/61) Twinrix. Of the 61 Subjects vaccinated, 53% (32/61) of HCV-infected subjects exhibited seroconversion with an HBsAb titer >10 IU/L. The response to HBV vaccination was 47% in subjects receiving Engerix and 55% in those vaccinated with Twinrix. Although the administration of the combination vaccine appeared somewhat superior to HBV vaccine alone, this difference was not statistically significant. In contrast, 94% (15/16) controls (10 healthy subjects and 6 spontaneously HCV-resolved individuals) achieved seroconversion after receiving either Engerix or Twinrix vaccinations, consistent with a large body of data showing excellent rates of HBV vaccine response in healthy individuals^{6,8}. This was higher than HCV subjects who received HBV vaccines ($p=0.002$), and in keeping with data suggesting that subjects with chronic HCV infection may have poorer responses to HBV vaccination⁷.

Specific and non-specific CD4⁺ T cell responses are impaired in HBV vaccine non-responders with HCV infection

CD4⁺ T cells play a critical role in mounting an effective immune response to immunogens, including the provision of help to B cells to facilitate antibody production. To determine whether the poor HBV vaccine responses we observed were associated with poor CD4⁺ T cell responses during HCV infection, we first compared the activation status of CD4⁺ T cells in HCV-infected subjects and healthy subjects who had received a standard series of HBV vaccinations. To this end, PBMC isolated from HCV-infected and healthy subjects were *ex vivo* stimulated with anti-CD3/CD28 or HBsAg for 24 hours, and expression of CD69 (an early T cell activation marker) on the surface of CD4⁺ T cells was examined by flow cytometry. Antigen-specific or non-specific stimuli were employed as these are required to observe CD69 expression, which is quite low in unstimulated cells. No differences in absolute CD4⁺ T cell number were found amongst the groups (data not shown). As shown in Fig. 1 (left panel), CD69 expression on CD4⁺ T cells in response to anti-CD3/CD28 stimulation was detected at significantly lower levels in the grouped subjects with chronic HCV infection (HBV-R + HBV-NR, $47.38\% \pm 17.52\%$, $n=61$) versus the control subjects (HCV-resolved + healthy subjects, $76.01\% \pm 7.81\%$, $n=16$, $p<0.01$). Additionally, the group of HBV-NR exhibited significantly lower CD69 expression on their CD4⁺ T cells than those HBV-R ($37.79\% \pm 18.52\%$, $n=29$ versus $56.06\% \pm 10.98\%$, $n=32$, $p<0.01$); while no significant differences in CD69 expressions were observed between HCV-resolved individuals and healthy subjects ($71.93\% \pm 10.83\%$ versus $78.47\% \pm 4.31\%$, $p>0.05$).

To determine the status of antigen-specific T cell activation, we also examined CD4⁺ T cell responses to HBsAg stimulation *ex vivo*. Lower CD69 expression was detected on CD4⁺ T cells in chronically HCV-infected subjects (Fig. 1, right panel), especially in HBV-NR (HBV-NR 6.61% vs HBV-R 15.42% , $p=0.002$), when compared to those who spontaneously HCV-resolved or healthy subjects (Total HCV-infected subjects 11.23% vs controls 18.66% , $p=0.016$). To account for potential variance, a summary of these data analyzed employing confidence intervals is shown in Table 2, and further linear regression modeling using Wilcoxon testing for non-parametric data found similar results (data not shown). Because a virus-specific response was being assayed in this CD4⁺ T cell population, we expected and observed that the percentages of CD4⁺CD69⁺ T cells stimulated with HBsAg were much lower than those stimulated more generally with anti-CD3/CD28. We found similar expression patterns for the T cell activation marker, CD25 (IL-2 receptor α chain), on CD4⁺ T cells in response to anti-CD3/CD28 (HBV-NR $45.27\% \pm 19.03\%$ $n=29$ vs HBV-R $65.48\% \pm 13.65\%$ $n=32$ $p<0.01$; total HCV-infected subjects $55.87\% \pm 19.21\%$ $n=61$ vs controls

75.49% $\pm$ 7.20% n=16, p<0.01) as well as HBsAg stimulation (HBV-NR 16.62% $\pm$ 7.73% n=29 vs HBV-R 25.48% $\pm$ 11.85% n=32 p=0.001; total HCV-infected subjects 21.27% $\pm$ 10.97% n=61 vs control subjects 25.69% $\pm$ 3.82% n=16, p=0.011). These results suggest an impaired activation status of helper T cells in chronically HCV-infected subjects, especially in those who failed to respond to HBV vaccination, when compared to HCV-negative subjects.

PD-1 and PDL-1 expressions are increased in HBV vaccine non-responders with HCV infection

We have previously shown that the PD-1/PDL-1 pathway is involved in T cell dysfunction during HCV infection¹⁵. To determine the role of this inhibitory pathway in regulation of CD4⁺ T cell responses to HBV vaccinations, we further examined PD-1/PDL-1 expressions on CD4⁺ T cells from chronically HCV-infected and healthy subjects who had received HBV vaccinations, including HBV-NR versus HBV-R. As shown in Figs. 2A and 2B respectively, PD-1 and PDL-1 expressions on CD4⁺ T cells in response to anti-CD3/CD28 stimulation were detected significantly higher in HCV-infected subjects than controls, in contrast to the expression levels of CD69 and CD25. Importantly, HCV-infected HBV-NR exhibited much higher PD-1 and PDL-1 expressions on their CD4⁺ T cells than HBV-R, while no significant differences in PD-1/PDL-1 expressions were observed between HCV-resolved individuals and healthy subjects. In the setting of specific antigen stimulation with HBsAg *ex vivo*, greater expressions of PD-1 (Fig. 2C) and PDL-1 (Fig. 2D) were detected on CD4⁺ T cells in chronically HCV infected subjects once again, especially in HBV-NR, when compared to controls. As expected, the percentages of PD-1⁺ or PDL-1⁺ T cells stimulated in an antigen-specific manner by HBsAg were much lower than following the non-specific TCR stimulation by anti-CD3/CD28.

Because we have previously shown that the intracellular inhibitory molecule, SOCS-1, is up-regulated in T cells from chronically HCV-infected individuals¹⁶, we also compared the level of SOCS-1 expression in CD4⁺ T cells isolated from HCV-infected HBV-NR and HBV-R, *ex vivo* stimulated with HBsAg and assayed by RT-PCR and immunoblotting for SOCS-1 gene and protein expressions (Fig. 3). SOCS-1 was found to be increased by both methods in CD4⁺ T cells from HBV-NR subjects versus HBV-R. The differences were also found using bulk PBMC from HBV-NR and HBV-R when the cells were stimulated by anti-CD3/CD28 antibodies *ex vivo* (data not shown). Data were reproducible in independent experiments using cells isolated from three different HCV-infected subjects per condition.

PD-1 and PDL-1 expressions are inversely associated with the CD4⁺ T cell responses to HBsAg in HCV-infected individuals

We next analyzed the association between the expressions of PD-1/PDL-1 and CD69 on CD4⁺ T cells from chronically HCV-infected as well as healthy subjects who had received HBV vaccination. To this end, PBMC isolated from the subjects who had received HBV immunizations were *ex vivo* stimulated with HBsAg or anti-CD3/CD28 antibodies, followed by flow cytometric analysis as described above. A Pearson correlation program in SPSS 18 software was used to statistically analyze the association between the markers of PD-1 and T cell activation. As shown in Figs. 4A, PD-1 expression was found to be inversely associated with CD69 expression on CD4⁺ T cells stimulated with HBsAg (left panel) or CD3/CD28 (right panel), respectively. Additionally, PDL-1 expression was closely and inversely associated with CD69 expression on CD4⁺ T cells stimulated with HBsAg (Fig. 4B, left panel) but a statistically significant correlation was not found to occur between CD69 and PDL-1 following non-specific stimulation (Fig. 4B, right panel).

Blocking the PD-1 pathway can improve CD4⁺ T cell responses in HBV vaccine non-responders infected with HCV

We have previously shown that blocking the PD-1 pathway can abrogate T cell inhibition and apoptosis in healthy cells exposed to HCV core protein, a viral antigen with immunomodulatory properties¹⁵. To determine whether blocking PD-1 signaling can improve the impaired T cell function in chronically HCV-infected HBV-NR, we examined T cell activation and proliferation by flow cytometric analysis of CD69 expression and CFSE dilution, respectively. We incubated PBMC isolated from HCV-infected subjects who had failed to respond to HBV vaccination with anti-PDL-1 or control antibody overnight, followed by stimulation of the cells with either HBsAg or anti-CD3/CD28 antibodies for 5 days. As shown in Fig. 5A, CD69 expressions on CD4⁺ T cells were found to be significantly improved by pre-incubating the cells with anti-PDL-1 antibody followed by stimulating with HBsAg or anti-CD3/CD28 antibodies. Similarly, T cell proliferation, as represented by CFSE dilutional analysis (left shifting) on gated T cell populations (Fig. 5B), was observed more significantly upon PD-1 pathway blockade versus control treatment. The improvement in proliferative ability varied under the two-stimulating conditions: TCR activation by anti-CD3/CD28 stimulation elicited a more general and significant T cell proliferation as compared to HBsAg specific stimulation, in agreement with our observations that CD69/CD25 and PD-1/PDL-1 expressions on T cells differ following these different stimulations. These data are reproducible in repeated experiments using cells isolated from three different individuals with HBV-NR in the setting of chronic HCV infection.

Discussion

Vaccine responses in the setting of chronic HCV infection appear to be blunted, with poor response rates to a standard course of HBV vaccination in chronically HCV-infected individuals (and especially in those with advanced fibrosis and liver cirrhosis) when compared to healthy populations (40~60% versus 90~95%)⁶⁻⁸. This study confirms this discrepancy and raises the possibility that responses to vaccination may be impaired at least in part by up-regulated expression of the negative immunomodulator, PD-1. Our data consistently demonstrate that HBV vaccine non-responders have up-regulation of PD-1 that correlates with diminished T cell activation in response to both general and virus-specific stimulation, an impairment that can be improved by PDL-1 blockade.

The mechanisms underlying HBV vaccine non-response in 5-10% of healthy subjects and 40-60% of HCV-infected subjects remain poorly understood, although several factors are known to affect this response. These include age, gender, tobacco use, obesity, and certain human leukocyte antigen (HLA) alleles¹⁷⁻²⁰. Given the fact that non-responders also exhibit poor recall responses to tetanus toxoid or *Candida*, it has been suggested that HBV vaccine non-responsiveness may be due to a defect in HBsAg-reactive T cells²¹⁻²⁴, regulatory T cells²⁵, or in APCs^{26, 27}; this has remained controversial²²⁻²³. A number of studies have tried to correct non-response by addition of vaccine adjuvants, altered doses, and different vaccine administration routes or strategies²⁸⁻³¹. These approaches have led to varying degrees of success in healthy subjects, but have been limited in chronically infected individuals. Notably, similar phenomena are observed in chronic HIV infection as well as in other immunosuppressed settings, such as organ transplantation, cancer chemotherapy, and hemodialysis, following routine adult immunizations that include influenza and pneumococcal vaccination; this suggests a possible shared mechanism of non-response in immunocompromised hosts^{9,10}.

The role of PD-1 in viral clearance during HCV (and HIV) infection has been studied extensively by us and others^{11, 15, 32-34}, but a role in vaccine responses during chronic viral

infection is novel in concept. Up-regulation of PD-1 appears to be a marker for T cell anergy, with selective up-regulation observed on “exhausted” T cells; inhibition of its signaling via anti-PD-1 or anti-PDL-1 antibodies has led to improved T cell function and viral clearance in several models³⁴⁻³⁸. PD-1 is an immunoinhibitory receptor predominantly expressed on activated T cells and function through interaction with its natural ligands including PDL-1 (also known as B7-H1, expressed on both haematopoietic and parenchymal cells) and PDL-2 (B7-DC, primarily expressed on monocytes). Activation of the PD-1 pathway induces immunoreceptor tyrosine phosphorylation and recruitment of tyrosine phosphatases, including src-homology proteins (SHP-1/SHP-2), to deliver a negative signal to TCR activation pathways. Evidence is emerging for the involvement of PD-1 inhibitory pathway in normal immune tolerance, autoimmune responses, antitumor and antiviral immune evasion. Our study adds to compendium by suggesting a role in vaccine responses.

Our findings have several limitations worth noting. For example, CD4+ T cell responses were assayed in the presence of other cells that may be contributing to the dysregulation we observed, and thus these other suppressive cells may be playing a role. Future studies using purified CD4+ T cell populations would clarify this issue. We also do not know the role that costimulatory molecules may be playing in this process, although there are data suggesting that such molecules impact the T cell exhaustion associated with PD-1 up-regulation. In addition, we focused on PD-1 expression in the stimulated state only, in part because we and others have already observed up-regulation of PD-1 in HCV-infected individuals at baseline¹⁵. We however do not know if responders differ in baseline PD-1 expression when compared to non-responders prior to stimulation, an area worth exploring. The role of T regulatory cells is also not characterized herein, although their contribution to altering responses is quite possible¹³. Finally, correlation analyses while yielding associations could have more robust co-efficients with a larger sample size.

The finding of vaccine non-response is perhaps not an absolute phenomenon, but one of a range of low responses to vaccine antigen. Pre-existing cellular responses to HBsAg in PBMCs from HBV non-responders have been reported despite a lack of a humoral response²⁵. A more potent vaccination might circumvent this problem, but our data suggest that augmenting CD4+ T cell function/priming may be feasible through blockade of native, negative cellular immunoreceptors such as PD-1. Clinical studies exploring a role for PD-1 blockade in improving viral clearance will be emerging, and it would seem logical to assess vaccine responsiveness in this setting as well. The potential negative consequences of blocking such a key negative modulator as PD-1 are many, including uncontrolled immune responses, autoimmune phenomenon, and malignancy. Nevertheless, multiple investigators are developing agents that inhibit this pathway and their clinical role will likely be explored in the near future. Additional cellular targets almost assuredly exist, including CD150, expression of which has been shown to be linked to decreased CD4+ T cell proliferative responses in healthy vaccine non-responders³⁹; SOCS-1¹⁶, that regulates Jak/STAT signaling; and the recently described negative receptor, Tim-3⁴⁰. Further studies focusing on these potent cell regulators in the setting of vaccine responses are ongoing, studies which might provide a novel approach in to immunization.

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Abbreviations

HCV	hepatitis C virus
PD-1	programmed death-1
PDL-1	programmed death ligand-1
HIV	human immunodeficiency virus
PBMC	peripheral blood mononuclear cells
FACS	fluorescence-activated cell sorting
SD	standard deviation
STAT-1	signal transducer and activator of transcription
JAK	janus-associated kinase-1
SOCS-1	suppressor of cytokine signaling-1

Fig. 1. CD4⁺ T cell activation status in HCV-infected and healthy subjects who received HBV vaccinations

PBMC were isolated from HCV-infected HBV vaccine non-responders (HBV-NR) (n=29), HCV-infected HBV vaccine responders (HBV-R) (n=32), HCV-resolved subjects (n=6), and healthy subjects (n=10), *ex vivo* stimulated with anti-CD3/CD28 (left panel) or HBsAg (right panel) for 24 hrs, and assayed by flow cytometry for CD69 expression on CD4⁺ T cells; representative dot plots are shown above and summary data shown below.

Fig. 2. PD-1 / PDL-1 expressions on CD4⁺ T cells in HCV-infected and healthy subjects who received HBV vaccination

PBMC were isolated from HCV-infected HBV vaccine non-responders (HBV-NR) (n=29), HCV-infected HBV vaccine responders (HBV-R) (n=32), HCV-resolved subjects (n=6), and healthy subjects (n=10), *ex vivo* stimulated with anti-CD3/CD28 or HBsAg for 24 hrs, and assayed by flow cytometric analysis for PD-1 or PDL-1 expressions on CD4⁺ T cells; representative dot plots are shown above and summary data shown below. A) PD-1 expression on CD4⁺ T cells stimulated with anti-CD3/CD28. B) PDL-1 expression on CD4⁺ T cells stimulated with anti-CD3/CD28. C) PD-1 expression on CD4⁺ T cells stimulated with HBsAg. D) PDL-1 expression on CD4⁺ T cells stimulated with HBsAg. Statistical findings are shown between the comparison groups.

Fig. 3. Increase in SOCS-1 expression in CD4⁺ T cells isolated in HCV-infected HBV vaccine non-responders

Purified CD4⁺ T cells were isolated by incubation of PBMCs with magnetic beads-conjugated with anti-CD4 antibody, followed by positive selection per the manufacturer's instruction, from three HBV-R and three HBV-NR. Following stimulation with either HBsAg or anti-CD3/CD28 for 24 h as described above, cells were either lysed for immunoblotting (A) or RNA isolation for RT-PCR (B) as we have described^{13,16,32}. SOCS-1 gene expression was detected by quantitative RT-PCR, and β 2M gene served as an internal control. Densitometric analysis of immunoblots normalized to actin are shown with error bars below. Data were reproducible in independent experiments using cells isolated from three different HCV-infected subjects per condition.

Fig. 4. PD-1 and PDL-1 expressions are inversely associated with CD4⁺ T cell responses to HBV vaccine in HCV-infected individuals

Each symbol represents an individual expressing cell surface marker for PD-1 inhibitory pathway as well as T cell activation in subjects who had received HBV vaccines and *ex vivo* stimulated with HBsAg (left panels) or anti-CD3/CD28 (right panels). The inverse association between PD-1 and CD69 (A) and PDL-1 and CD69 (B) expressions on CD4⁺ T cells was evaluated using Pearson Correlation analysis. Pearson correlation and 2-tailed significance are shown in the upper right corner of each analysis.

Fig. 5. Blocking the PD-1 pathway improves CD4⁺ T cell responses in HBV vaccine non-responders with HCV infection

PBMC isolated from HCV-infected subjects who had received HBV vaccinations and failed to seroconvert were incubated with anti-PDL-1 or control antibody overnight, followed by stimulation with either HBsAg or anti-CD3/CD28 antibodies for 5 days. T cell activation and proliferation were evaluated by detection of CD69 expression (A) and CFSE assay (B) in stimulated T cells. Representative percentages of CD69 expression and gating of CFSE in T cells treated with anti-PDL-1 versus control antibody are shown on the left and summary data on the right. Data are reproducible in repeated experiments using cells isolated from three different individuals with HBV-NR in the setting of chronic HCV infection.

Table 1

Demographic features of the study subjects

Groups	No. of Subjects	Mean age (Range)	% of Males	% of Genotype 1	HCV RNA (IU/ml)
HBV-NR	29	56 (31-69)	93	72	4,890~50,000,000
HBV-R	32	54 (29-71)	96	69	45,800~50,000,000
HCV-resolved	6	51 (37-62)	100	NA	NA
Healthy subjects	10	45 (28-58)	80	NA	NA

NA = not applicable.

Table 2
Comparison of CD69 expression amongst different groups after stimulation with anti-CD3/CD28 or HBsAg

Comparison groups	N	Percentage of CD69 expression			
		Anti-CD3/CD28 stimulated		HBsAg stimulated	
		Mean (95% CI)	p-value	Mean (95% CI)	p-value
HCV infected	61	47.4 (42.9 – 51.9)		11.2 (8.3 – 14.2)	
Controls	16	76.0 (71.9 – 80.2)	<0.0001	18.7 (15.5 – 18.7)	<0.001
HBV-NR	29	37.8 (30.7 – 44.8)		6.6 (2.9 – 10.3)	
HBV-R	32	56.1 (52.1 – 60.9)	<0.0001	15.4 (11.2 – 19.6)	0.002
HCV-resolved	6	71.9 (60.6 – 83.3)		17.7 (10.3 – 25.0)	
Healthy subjects	10	78.5 (75.4 – 81.5)	0.207	19.3 (15.3 – 23.2)	0.619

Abbreviations: CI – confidence interval