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Enhancement of Programmed Death Ligand 2 on Hepatitis C Virus Infected Hepatocytes by Calcineurin Inhibitors

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Background. Post orthotopic liver transplantation (OLT) viral hepatitis is an immunological condition where immune cells induce hepatitis during conditions of immune-suppression. The immune-regulatory programmed death-1 (PD-1)/PD-ligand 1 system is acknowledged to play important roles in immune-mediated diseases. However, the PD-1/PD-L2 interaction is not well characterized, with PD-L2 also exhibiting an immunostimulatory function. We hypothesized that this atypical molecule could affect the recurrence of post-OLT hepatitis. To test this hypothesis, we conducted immunohistochemical staining analysis and in vitro analysis of PD-L2. **Methods.** The expression of PD-L2 was evaluated in liver biopsy specimens from patients with chronic hepatitis B (n = 15), post-OLT hepatitis B (n = 8), chronic hepatitis C (n = 48), and post-OLT hepatitis C (CH-C-OLT) (n = 14). The effect of calcineurin inhibitors (CNIs) and hepatitis C virus (HCV) on PD-L2 expression was investigated in hepatoma cell lines. **Results.** The PD-L2 was highly expressed on CH-C-OLT hepatocytes. Treatment of hepatoma cell lines with CNIs resulted in increased PD-L2 expression, especially in combination with HCV core or NS3 protein. Transfection of cell lines with PD-L2 containing plasmid resulted in high intercellular adhesion molecule-1 (ICAM-1) expression, which might enhance hepatitis activity. **Conclusions.** The PD-L2 is highly expressed on CH-C-OLT hepatocytes, whereas HCV proteins, in combination with CNIs, induce high expression of PD-L2 resulting in elevated expression of ICAM-1. These findings demonstrate the effect of CNIs on inducing PD-L2 and subsequent ICAM-1 expression, effects that may produce inflammatory cell infiltration in post-OLT hepatitis C.

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Orthotopic liver transplantation (OLT) is the established treatment for hepatitis B virus (HBV) and hepatitis C virus (HCV)–related end stage liver disease. Post-OLT HBV recurrence is greater than 90% well controlled with a combination of hepatitis B immunoglobulin and nucleos(t)ide analogue.[1] Post-OLT HCV recurrence remains an unresolved issue that often precipitates severe hepatitis. It is not clear why recipient immune cells are capable of reacting with viral antigens expressed on donor human leukocyte antigen (HLA) and induce hepatitis, even in the presence of immunosuppressive agents, such as calcineurin inhibitors (CNIs) (FK506 [FK] or cyclosporine A [CyA]).[2] Recently, immune regulatory function has become widely accepted as playing important roles in immune-related diseases.

Programmed death-1 (PD-1) is a member of the CD28 family of T cell regulators and is expressed on activated T and B cells. PD-1 has 2 ligands, PD-L1 and PD-L2, which are highly homologous (41% amino acid identity) members of the B7 family.[3] However, the function of PD-L1 differs from that of PD-L2. PD-L1 has been reported to costimulate interleukin (IL)-10 production by T cells, whereas PD-L2 strongly costimulates interferon (IFN)-γ, but not IL-4 or IL-10, production by T cells.[4] The expression of PD-L1, but not PD-L2, on human

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macrophages is upregulated by IL-10.[5] The PD-L1 expression is regulated by IFN-γ, whereas PD-L2 is upregulated in response to IL-4.[6] Programmed death-L1 is broadly expressed on activated T cells, B cells, macrophages, dendritic cells, tumor cells, epithelial cells, and endothelial cells. In contrast, PD-L2 is expressed exclusively on dendritic cells, monocytes, liver cells, placental endothelium, and thymic epithelial cells.[4] PD-1/PD-L1 inhibits T cell proliferation, cytokine production, and cytotoxic T lymphocyte activity; while PD-L2 inhibits lymphocyte activation through PD-1 and also stimulates T-cell responses as well as binding with another unidentified molecule to stimulate Th1 immune responses.[4,7]

We examined immunohistochemical staining of PD-L2 to evaluate the expression pattern of this molecule in the liver of patients with chronic hepatitis B (CH-B), post OLT hepatitis B (CH-B-OLT), chronic hepatitis C (CH-C), and recurrent hepatitis C after OLT (CH-C-OLT). Elevated expression of PD-L2 on hepatocytes was evident in severe hepatitis in CH-C and CH-C-OLT. To reveal whether CNIs and/or HCV protein induce elevated expression of PD-L2, in vitro experiments were conducted. The HCV proteins, in combination with CNIs, were found to induce high expression of PD-L2 in hepatoma cell lines resulting in elevated expression of inflammation related cell adhesion molecule. These findings demonstrate the effect of CNIs on inducing PD-L2, and subsequent adhesion molecule expression, possibly results in inflammatory cell infiltration in post-OLT hepatitis C.

MATERIALS AND METHODS

Patients

The subjects participating in the present study consisted of 84 consecutive patients who received a liver biopsy at Okayama University Hospital from 2003 to 2010 (Table 1). All liver biopsy slides were evaluated by 2 hepatologists (T.Y. and A.T.) who were blinded to the clinical data. Fibrosis staging and inflammation grading in these patients were determined according to their METAVIR score (F0-4, A0-3).[8] Four groups of patients were included in the study: patients with chronic hepatitis B (CH-B, n = 15), patients with hepatitis B after OLT (CH-B-OLT, n = 8), patients with chronic hepatitis C (CH-C, n = 48), and patients with hepatitis C after OLT (CH-C-OLT, n = 14). The CH-B patients were diagnosed as being serum-positive for HBs antigen and HBc antibody. The CH-B-OLT patients showed no serum HBV DNA positivity because the patients were all controlled with nucleos(t)ide analogue and hepatitis B immunoglobulin administration. The CH-C patients were diagnosed as being serum-positive for HCV antibody and HCV RNA. Of the OLT patients, primary immunosuppression treatments included CyA or FK and prednisolone after OLT. The CNI FK was used predominantly in CH-B-OLT, whereas CyA was more commonly used in CH-C-OLT. The patients were followed for a minimum of 6 months post-OLT before being assessed for this study. Informed consent was obtained from each patient, and the study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ethics Committee at the Okayama University Hospital. No donor organs were obtained from executed prisoners or other institutionalized persons.

Immunohistochemical Staining With Anti-PD-L2 and Anti-ICAM-1

Formalin fixed sections were used for immunohistochemical staining. Anti-PD-L2 (diluted 1:100; R&D Systems, Minneapolis, MN) and anti-ICAM-1 (diluted 1:30; Cell Signaling Technology, Danvers, MA) were used.

Evaluation of the Specimens

Immunohistochemical staining was assessed in a blinded manner by 2 hepatologists (T.Y. and A.T.). The PD-L2 expression was evaluated according to staining intensity and scored as follows: 0, negative; 1, weak expression; 2, moderate expression; 3, strong expression.

TABLE 1.

Patients' characteritics

	CH-B	CH-B-OLT	CH-C	CH-C-OLT
n	15	8	48	14
Age	45 (25-64)*$	54 (36-64)	59 (31-75)	59 (53-66)
Male sex (%)	7 (46)	6 (75)	26 (54)	11 (78)
Hb, g/dL	13.6 (11.6-16.4)	13.9 (11.5-18)	14.1 (10.2-16.1)	11.7 (9.1-16.8)*
Platelet ($\times 10^4$/μL)	17.2 (8.1-26.9)	19.6 (6.0-28.9)	17.3 (8.6-28.6)	16.5 (7.7-30.6)
ALT, IU/L	126 (12-459)*	24 (11-83)*#	46 (18-259)	54 (15-446)
Total bilirubin, mg/dL	0.8 (0.3-1.7)	1.0 (0.4-1.3)	0.7 (0.3-1.5)	1.1 (0.4-6.0)*
Albumin, g/dL	4.1 (2.7-4.6)	4.1 (2.7-4.6)	4.2 (3.3-5.1)	4.0 (3.2-4.6)
PT-INR	1.06 (0.90-1.32)*$	0.95 (0.89-1.01)	0.93 (0.84-2.08)	0.95 (0.84-1.07)
HBs antigen positive number, (%)	15 (100)	0 (0)	—	—
HBV DNA, logcopies/mL	6.7 (2.6-8.8)	0#	—	—
HCV serotype-1, (%)	—	—	32 (66%)	13 (92%)
Pre-OLT HCV-RNA, logIU/mL	—	—	—	2.8 (2.7-3.3)
HCV RNA, logIU/mL	—	—	6.2 (2.1-7.5)	6.3 (5.8-6.7)
Fibrosis stage (0/1/2/3)	1/4/5/5	7/1/0/0*#	3/21/11/13	8/5/1/0*#
Necroinflammatory activity (0/1/2/3)	2/6/6/1	7/1/0/0*#	1/29/17/1	7/6/1/0*#
Calcineurin inhibitors (FK/CyA)	—	7 / 1$	—	3 / 11

CyA, cyclosporin A; FK, FK506; Hb, hemoglobin; PT-INR, prothrombin time international ratio.
* $P<0.05$ vs. CH-C, $ $P<0.05$ vs. CH-C-OLT, # $P<0.05$ vs. CH-B, & $P<0.05$ vs. CH-B-OLT.

Cell Lines and Reagents

Hepatoma cell lines (PLC/PRF/5, Huh7, and Hep3B) were used for the in vitro experiments. Human IFN-α2 was kindly provided by Hayashibara Biochemical Laboratories, Inc. (Okayama, Japan). FK506 and CyA were purchased from Calbiochem (San Diego, CA) and Cayman Chemical Co. (Ann Arbor, MI), respectively.

Cells were treated with IFN-α2 (1000 IU/mL) and/or FK (1 μM) or CyA (10 μM). After 48 hours, the cultured cells were collected for reverse transcription polymerase chain reaction (RT-PCR) and Western blotting.

Transfection of HCV or HBV Proteins Into Human Hepatoma Cell Lines

The mammalian expression plasmids pCXN2-core and pCXN2-NS3, which contain the respective HCV genomic regions driven by the β-actin–based CAG promoter,[9] were kindly donated by Dr. Naoya Kato from the University of Tokyo. The hepatoma cell lines were transfected with HCV plasmids using the lipofection method. HCV-transfected hepatoma cell lines were cultured for an additional 48 hours with or without CNIs (FK or CyA) and PD-L2 messenger RNA (mRNA) expression was measured. Using the same method, HBV plasmid (extracted and cloned from the serum of a 36-year-old Japanese woman, genotype C) was transfected into the hepatoma cells as reported before.[1]

Analysis of PD-L2 mRNA Expression by RT-PCR and Quantitative Real-Time PCR

The effects of CNIs and HCV or HBV proteins on cellular PD-L2 expression were quantified using RT-PCR of PD-L2 mRNA. Total RNA was extracted from cultured cell lines using TRIzol reagent (Invitrogen, Carlsbad, CA). Total RNA was reverse transcribed into cDNA and subjected to PCR. The housekeeping gene GAPDH was used as an internal control. For real-time PCR, PD-L2 mRNA and β-actin (housekeeping gene) mRNA were quantified using SYBR Green Master Mix (Roche Diagnostics, Mannheim, Germany) with specific primers on a Light cycler 480-2 (Roche Diagnostics). Each sample was run in triplicate and SYBR Green dye intensity was analyzed using the LightCycler software (Roche Diagnostics). The results were analyzed using the relative standard method.

Analysis of PD-L2 Protein Levels Using Western Blot Analysis

Cultured cells were lysed with lysis buffer and used for Western blotting. The following primary antibodies were used: goat anti-hPD-L2 (R&D Systems) or rabbit anti-beta actin (Sigma-Aldrich, St. Louis, MO), as a control.

Cloning of PD-L2 and Transfection Into Hepatoma Cell Lines

To evaluate the effect of elevated PD-L2 expression, PD-L2 was cloned from activated dendritic cells and transfected into human hepatoma cell lines. Total RNA was extracted using TRIzol reagent from healthy human dendritic cells. The cDNA was synthesized and the forward and reverse primers were 5′-TTTACTTTTGCATCTTTACTTGTG-3′ and 5′-CAGGTGTTGGCTAGTCTTGTTG-3′, respectively. The PCR products were purified and subsequently cloned into the pCRII-TOPO vector (Invitrogen) and putative positive clones were selected. The cloned sequences were validated

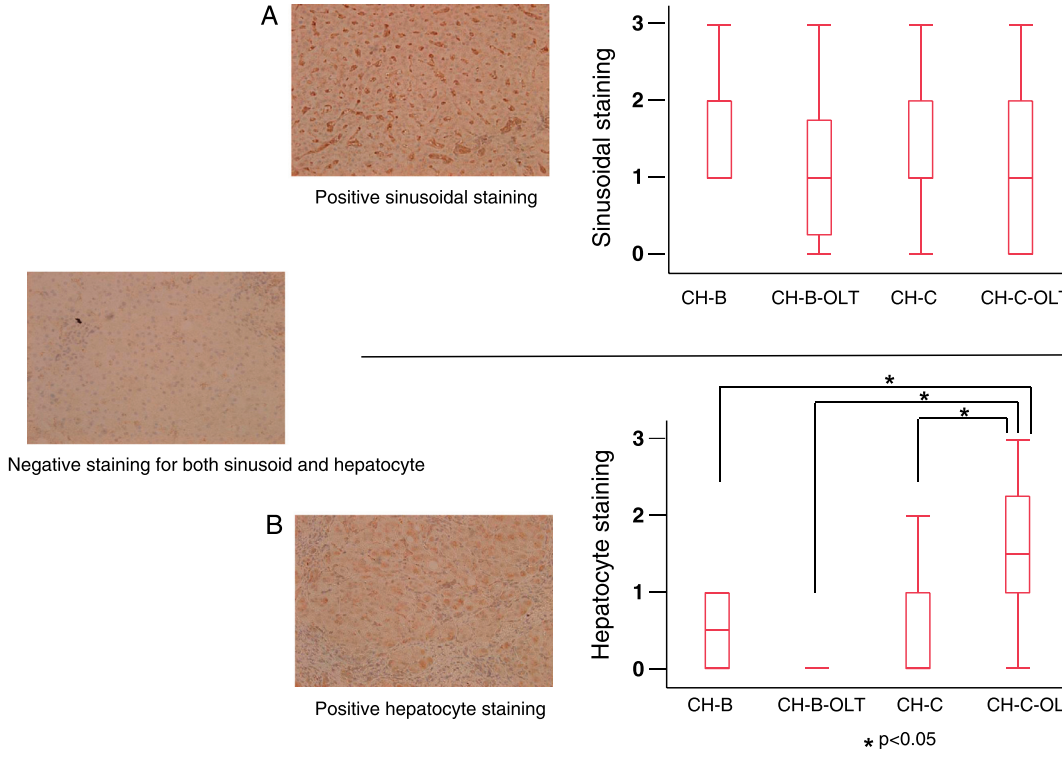

FIGURE 1. Immunohistochemical staining of programmed death ligand 2 (PD-L2) in liver. The negative staining pattern is shown on the left center of the figure. A, Left: representative sinusoidal staining pattern of PD-L2. The expression of PD-L2 was evaluated according to the staining intensity and scored as follows: 0, negative; 1, weak expression; 2, moderate expression; 3, strong expression. Right: sinusoidal staining in different disease states. B, Left: representative hepatocyte staining pattern of PD-L2. Scoring of staining intensity is the same as in sinusoidal tissue (0, 1, 2, 3). Right: hepatocyte staining in different disease states.

by direct sequencing. Thirty μg of PD-L2 containing plasmid were electroporated to hepatoma cell lines and transferred to a plate and cultured for 36 hours. The pCRII-TOPO vector was used as a control.

Flow Cytometric Analysis of Transfected Cell Surface Antigens

Monoclonal antibodies specific for CD95 (Fas)-PE, CD54 (ICAM-1)-PE, CD1d-PE, and HLA-ABC-PE were obtained from BD: Becton, Dickinson and Company (San Jose, CA). The monoclonal antibody specific for CD273 (PL-L2)-PE was obtained from BioLegend (San Diego, CA). The cultured cells were stained for expression of cell-surface antigens and flow cytometry was performed using a FACSCaliber instrument (BD) and analyzed with CellQuest software (BD).

Statistical Analysis

JMP software (Version 9, SAS Institute Inc., NC) was used to perform the statistical analysis. Continuous data were expressed as medians (range) and were analyzed with a nonparametric test (Mann-Whitney or Kruskal-Wallis test). Categorical data were analyzed with Pearson χ^2 test. If a statistical difference was found, we compared the groups using a χ^2 test with Bonferroni's correction. The Tukey-Kramer test was used to compare the mean values of HCV plasmid transfection experiments. Concordance coefficients (κ statistics) were used to

TABLE 2.

Clinical findings differences in hepatitis C virus carrier due to PDL-2 expression

	Sinusoid PDL2					
	CH-C			CH-C-OLT		
	Low (0-1)	High (2-3)	*P*	Low (0-1)	High (2-3)	*P*
n	25	23		10	4	
Age	57 (33-74)	60 (31-75)	0.203	59 (54-66)	59 (53-63)	0.776
Male sex (%)	16 (64)	10 (43)	0.154	7 (70)	4 (100)	0.216
Hb, g/dL	14.2 (10.2-16.1)	14.1 (11.4-15.8)	0.812	11.4 (9.1-14.3)	12.4 (9.9-16.8)	0.536
Platelet $\times 10^4$/μL	18.7 (11.3-28.6)	16.8 (8.6-28)	0.347	18.4 (7.7-30.6)	18.5 (13.2-19.4)	1.000
ALT, IU/L	42 (19-186)	48 (18-259)	0.917	48 (15-157)	20 (18-95)	0.156
Total bilirubin, mg/dL	0.76 (0.40-1.13)	0.65 (0.30-1.54)	0.347	1.09 (0.40-6.09)	1.34 (0.77-5.74)	0.435
Albumin, g/dL	4.2 (3.3-5.1)	4.0 (3.4-4.7)	0.213	4.0 (3.4-4.6)	4.0 (3.2-4.5)	0.831
PT-INR	0.93 (0.85-2.08)	0.93 (0.84-1.19)	0.934	0.94 (0.86-0.97)	0.95 (0.84-1.00)	0.607
HCV serotype-1, (%)	15 (60)	17 (73)	0.585	10 (100)	3 (75)	0.100
HCV-RNA at biopsy, logIU/mL	6.1 (5.6-6.5)	6.2 (5.6-6.5)	0.983	6.3 (6.1-6.7)	6.1 (1.4-6.6)	0.338
Pre-OLT HCV-RNA, logIU/mL	—	—	—	2.8 (2.7-3.2)	3.1 (1.7-3.6)	0.566
Trough levels of FK, ng/mL	—	—	—	6.9 (6.9-14.9)	5.5 (5.5-5.5)	0.150
Trough levels of CyA, ng/mL	—	—	—	108 (91-160)	201 (136-252)	0.052
Previous CMV infection (yes [%])	—	—	—	3 (30)	1 (25)	0.851
Fibrosis stage (0/1/2/3)	2/12/3/8	1/9/8/5	0.301	6/3/1/0	2/2/0/0	0.680
Necroinflammatory activity (0/1/2/3)	1/15/8/1	0/14/9/0	0.569	5/4/1/0	2/2/0/0	0.791
	Hepatocyte PDL2					
	CH-C			CH-C-OLT		
	low (0)	high (1-3)	*P*	low (0-1)	high (2-3)	*P*
n	30	18		7	7	
Age	59 (33-71)	59 (31-75)	0.773	60 (54-66)	59 (53-64)	0.948
Male sex, %	14 (46)	12 (66)	0.175	7 (100)	4 (57)	0.050
Hb, g/dL	14.0 (10.2-16.1)	14.7 (10.8-15.8)	0.462	13.2 (11.4-16.8)	10.6 (9.1-14.3)	0.03
Platelet $\times 10^4$/μL	19.8 (11.3-28.6)	15.5 (8.6-27.9)	0.021	18.2 (11.6-26.2)	19.2 (7.7-30.6)	0.886
ALT, IU/L	40 (18-186)	67 (23-259)	0.037	41 (18-157)	43 (15-132)	0.847
Total Bilirubin, mg/dL	0.72 (0.4-1.1)	0.62 (0.3-1.5)	0.268	1.02 (0.68-1.52)	1.17 (0.40-6.09)	0.556
Albumin, g/dL	4.2 (3.3-5.1)	4.0 (3.4-4.9)	0.142	4.2 (3.6-4.5)	3.8 (3.2-4.6)	0.124
PT-INR	0.93 (0.86-2.08)	0.94 (0.84-1.19)	0.423	0.91 (0.84-0.96)	0.95 (0.92-1.0)	0.124
HCV serotype-1, (%)	23 (76%)	9 (50%)	0.013	6 (85%)	7 (100%)	0.299
HCV-RNA at biopsy, logIU/mL	6.3 (5.8-6.5)	5.9 (5.1-6.5)	0.236	6.4 (2.6-6.7)	6.3 (6.1-6.7)	0.933
Pre-OLT HCV-RNA, logIU/mL	—	—	—	2.9 (2.8-3.3)	2.8 (1.8-3.4)	0.560
Trough levels of FK, ng/mL	—	—	—	5.5 (5.5-5.5)	6.9 (6.9-14.9)	0.157
Trough levels of CyA, ng/mL	—	—	—	120 (95-142)	189 (112-239)	0.134
Previous CMV infection (Yes [%])	—	—	—	2 (28)	2 (28)	1.000
Fibrosis stage (0/1/2/3)	2/14/5/9	1/7/6/4	0.625	3/4/0/0	5/1/1/0	0.192
Necroinflammatory activity (0/1/2/3)	1/19/9/1	0/10/8/0	0.450	2/5/0/0	5/1/1/0	0.084

CMV, cytomegalovirus.

evaluate agreement between PD-L2 expression and ICAM-1 expression on immunohistochemistry.

RESULTS

Clinical Characteristics of the Patients

Patient characteristics are provided in Table 1. The CH-B patients were younger than the CH-C and CH-C-OLT patients, showed higher alanine aminotransferase (ALT) levels than CH-C and higher PT-INR levels than CH-C and CH-C-OLT, representing younger and more active hepatitis than CH-C. Histological stages and activities were lower in CH-B-OLT and CH-C-OLT compared to those of CH-B and CH-C.

PD-L2 Expression by Immunohistochemical Staining

Immunohistochemical analysis revealed PD-L2 staining in sinusoidal cells and/or hepatocytes. Among the 84 tissue samples evaluated, sinusoidal PD-L2 expression levels were scored as 0, 1, 2, and 3 in 11 (13.0%), 35 (41.6%), 31 (36.9%), and 7 (8.3%) cases, respectively (Figure 1A). PD-L2 expression levels in hepatocytes were scored as 0, 1, 2, and 3 in 47 (55.9%), 25 (29.7%), 9 (10.7%), and 3 (3.5%) cases, respectively (Figure 1B). Sinusoidal PD-L2 staining scores were higher than in hepatocytes and showed no significant differences among the disease groups. However, PD-L2 staining in hepatocytes of the CH-C-OLT group was significantly higher than that in the other groups. Additionally, PD-L2 staining in CH-B-OLT hepatocytes was remarkably low.

Clinical Characteristics and Hepatocyte PD-L2 Staining in CH-C and CH-C-OLT Patients

The results of the immunohistochemical staining experiments indicated that HCV infection, especially after OLT, was likely to be involved in regulating hepatic PD-L2 expression. We investigated the relationship between HCV infection and PD-L2 expression by comparing the clinical characteristics of CH-C and CH-C-OLT to PD-L2 staining patterns (Table 2). In CH-C, elevated hepatocyte PD-L2 expression was associated with low platelet counts, high ALT, and lower frequency of HCV serotype 1, representing active and advanced chronic hepatitis. Previous cytomegalovirus infection was not correlated with PD-L2 expression. The trough levels of CyA were relatively higher in patients highly expressing sinusoid PD-L2, although the differences were not significant.

Effect of CNIs and HCV Proteins on PD-L2 Expression in Hepatoma Cell Lines

Because the CH-C-OLT group showed elevated PD-L2 expression on hepatocytes, we surmised that the combination of HCV and CNIs might induce PD-L2 expression. First, we examined the expression levels of PD-L2 mRNA and protein by RT-PCR and Western blotting, respectively, to explore the role of PD-L2 in hepatoma cells treated with CNIs. Treatment of hepatoma cell lines with CNIs resulted in elevated PD-L2 gene and protein expression (Figure 2A). We also examined PD-L2 mRNA expression using real-time PCR in a hepatoma cell line transfected with HCV plasmids and treated with CNIs. PD-L2 mRNA expression was not altered by HCV core protein expression; however, it was enhanced in combination with CNIs (especially with CyA) (Figure 2B). Similarly, transfection with HCV NS3 protein revealed that NS3 protein itself was capable of enhancing PD-L2 mRNA expression and CNI (especially CyA) treatment further elevated this effect. The surface expression of PD-L2 on the hepatoma cell line was assessed using flow cytometric analysis. Surface expression appeared to be increased in the presence of CyA and in combination with HCV proteins, especially with HCV-NS3 (Figure 2C). However, no significant differences were observed following treatment with HCV and/or CNIs (Figure 2C). Next, we investigated whether HBV and CNI treatment altered PD-L2 levels. PD-L2 mRNA expression was not altered with HBV protein (Figure 2D).

Elevated PD-L2 Expression on Hepatoma Cell Lines Upregulates ICAM-1

To determine whether elevated expression of PD-L2 affects other host immune responses, we analyzed the expression of cell surface markers on hepatoma cell lines (Hep3B and Huh7) transfected with PD-L2. Induced PD-L2 mRNA expression was confirmed in Hep3B and Huh7 cells, with the more efficiently transfected Huh7 cells used for protein expression confirmation (Figure 3A). Cell surface expression of ICAM-1, Fas, CD1d, and major histocompatibility complex-class 1 were determined in the transfected cells. Intercellular adhesion molecule-1 was upregulated on PD-L2 transfected cells (Figure 3B and C). As a result, we subsequently evaluated the immunostaining patterns of PD-L2 and ICAM-1 (Figure 3D). Tissues that exhibited strong ICAM-1 staining and strong hepatocyte PD-L2 staining (≥ 2) were strongly correlated in CH-C and CH-C-OLT patients ($\kappa = 0.30$, $P = 0.015$).

DISCUSSION

In this study, we investigated the expression pattern of PD-L2 in liver specimens with CH-B and CH-C, including post-OLT patients. We found that PD-L2 was expressed on hepatocytes and that elevated expression was related to CH-C-OLT and the severe stage of CH-C. Treatment with CNIs, in combination of HCV core or NS3 protein, resulted in an additive effect on PD-L2 expression on hepatocyte cell lines. Induction of PD-L2 expression produced higher expression of the inflammatory adhesion molecule ICAM-1 on hepatocyte cell lines, which might be associated with the severe stages of CH-C and CH-C-OLT.

PD-1 expression is higher on HCV-specific T cells during acute HCV infection and remains high in progression to chronic HCV infection, whereas it decreases in resolving HCV.[10] It was reported that myeloid dendritic cells from CH-C patients expressed upregulated levels of PD-L2, compared to healthy myeloid dendritic cells.[11] Similarly, PD-1 expression on HBV-specific T cells is higher during acute HBV infection and decreases after resolution.[12] PD-L1 and PD-L2 expressions were elevated in liver tissues from HBV-related acute-on-chronic liver failure.[13,14] An immunohistochemical analysis revealed that PD-L2 was highly expressed in the liver of chronic hepatitis C and autoimmune hepatitis patients, while only slightly higher in chronic hepatitis B. Moreover, the hepatitis activity score correlated with increased PD-L2 and PD-1 expression.[15] We observed that the expression of PD-L2 was higher on sinusoidal cells than on hepatocytes. This is consistent with the observation that PD-L2 is expressed on sinusoidal Kupffer cells. No differences between diseases or hepatitis severity were observed in the strength of sinusoidal staining. However, the expression of PD-L2 on hepatocytes was elevated in the CH-C-OLT patient group, whereas the

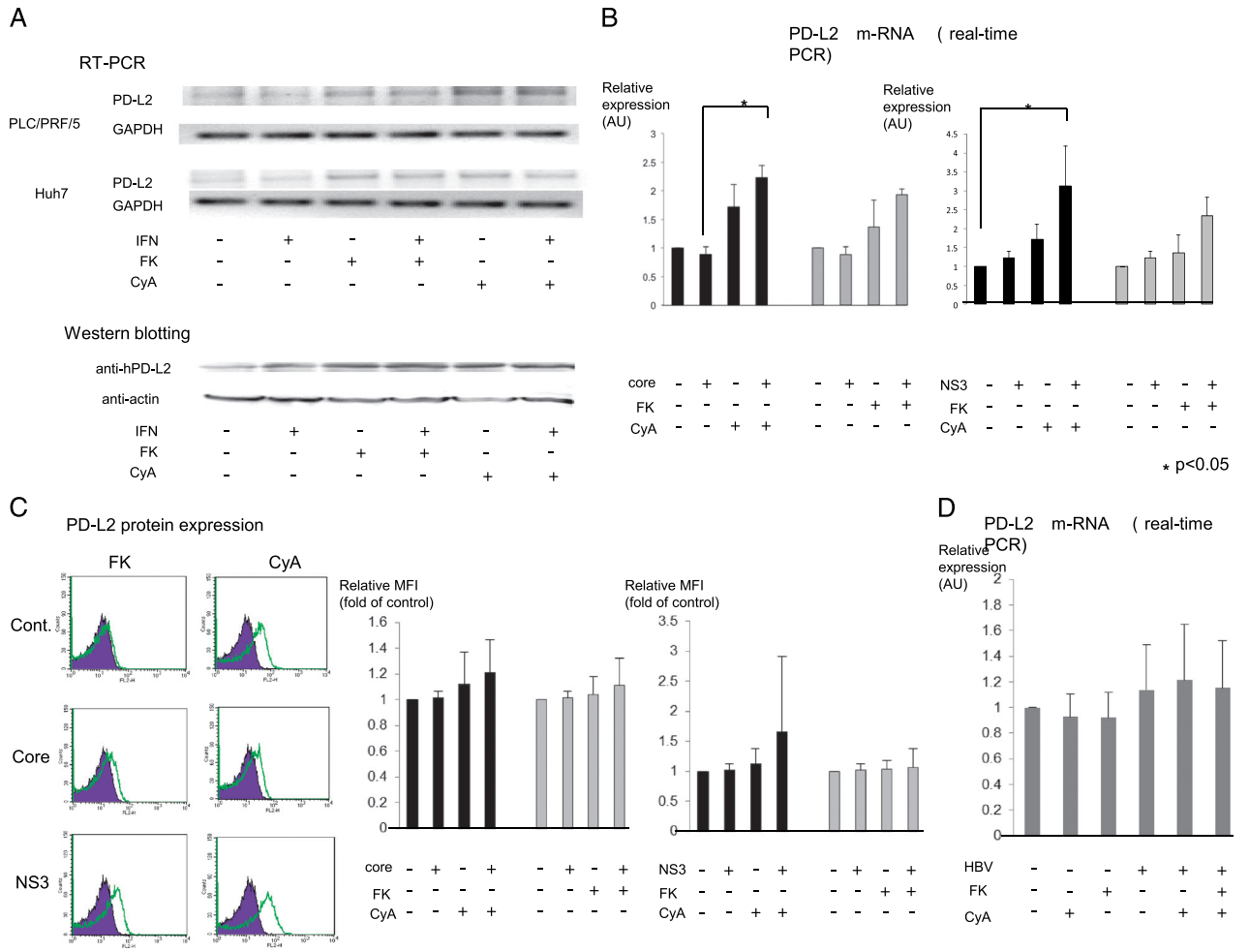

FIGURE 2. Effect of CNI treatment on PD-L2 expression in hepatoma cell lines. A, Expression levels of PD-L2 mRNA determined using RT-PCR (upper section). The PLC/PRF/5 and Huh7 cell lines were treated with IFN-γ2 (1000 IU/mL) and/or FK (1 μM) or CyA (10 μM). Expression levels of PD-L2 protein were determined using Western blotting (lower section). B, Effect of CNI treatment, in combination with HCV proteins, on PD-L2 expression in hepatoma cell lines. Relative PD-L2 mRNA expression levels were determined using real-time PCR in hepatoma cell lines transfected with HCV plasmid and/or treated with CNIs. C, Expression levels of PD-L2 determined using flow cytometric analysis in hepatoma cell lines treated with CNIs in combination with HCV protein expression. The MFI is presented as fold increase relative to control cells. The data represent mean ± SD of triplicate measurements. D, Effect of CNI treatment, in combination with HBV proteins, on PD-L2 expression in hepatoma cell lines. Relative PD-L2 mRNA expression levels were determined using real-time PCR in hepatoma cell lines transfected with HBV plasmid and/or treated with CNIs. MFI, mean fluorescence intensity.

CH-B-OLT group exhibited completely negative expression. This is probably due to the CH-B-OLT patients being serum HBsAg and HBV-DNA negative, as well as the absence of liver inflammation in our present data (Table 1), as previously reported by other institutes.[16,17] The PD-L2 mRNA in the liver of CH-C patients has been found to be higher than normal liver.[18] Hepatitis C virus infection might induce high expression of PD-L2 in the liver; however, the expression pattern is not well characterized. The present results demonstrated that elevated hepatocyte expression of PD-L2 is correlated with elevated ALT and decreased platelet counts. This clinical data of high expressing PD-L2 patients indicates the presence of active hepatitis with elevated ALT and advanced liver fibrosis with low platelet counts. Because PD-L2 has immunostimulatory functions, elevated hepatocyte expression may affect hepatitis activity. However, during immunosuppressive drug administration and HLA mismatch, CH-C-OLT patients often exhibit a severe form of hepatitis.[19,20] The elevated expression of PD-L2 in CH-C-OLT might partly explain this contradiction. The strong PD-L2 staining in hepatocytes of CH-C-OLT patients is indicative of the effect of CNIs and HCV infection. Although there are no previous reports explaining the effects of CNIs on PD-L2 expression, the present report demonstrates that PD-L2 expression is upregulated by CNIs.

Calcineurin inhibitors bind the immunophilin family of cytosolic proteins and the drug-immunophilin complex binds to the calcium/calmodulin-dependent phosphatase calcineurin, leading to the inhibition of nuclear factor of activated T cells (NFAT) activation and nuclear translocation, as well as IL-2 gene transcription.[21] Calcineurin inhibitors have various biological effects on T cells including opposing effects on the immune system.[22] Baan et al[23] showed CNIs inhibit FOXP3 transcription in mixed lymphocyte reactions. Because FOXP3 inhibits NFAT-mediated transcription and acts as a negative regulator of T cell activation, CNIs have immunestimulatory effects in such conditions.[23] Cyclosporine A inhibits the phosphatase activity of calcineurin, resulting in abrogation of nuclear translocation of NFAT. The NFAT family members bind to the PD-1 regulatory element, which

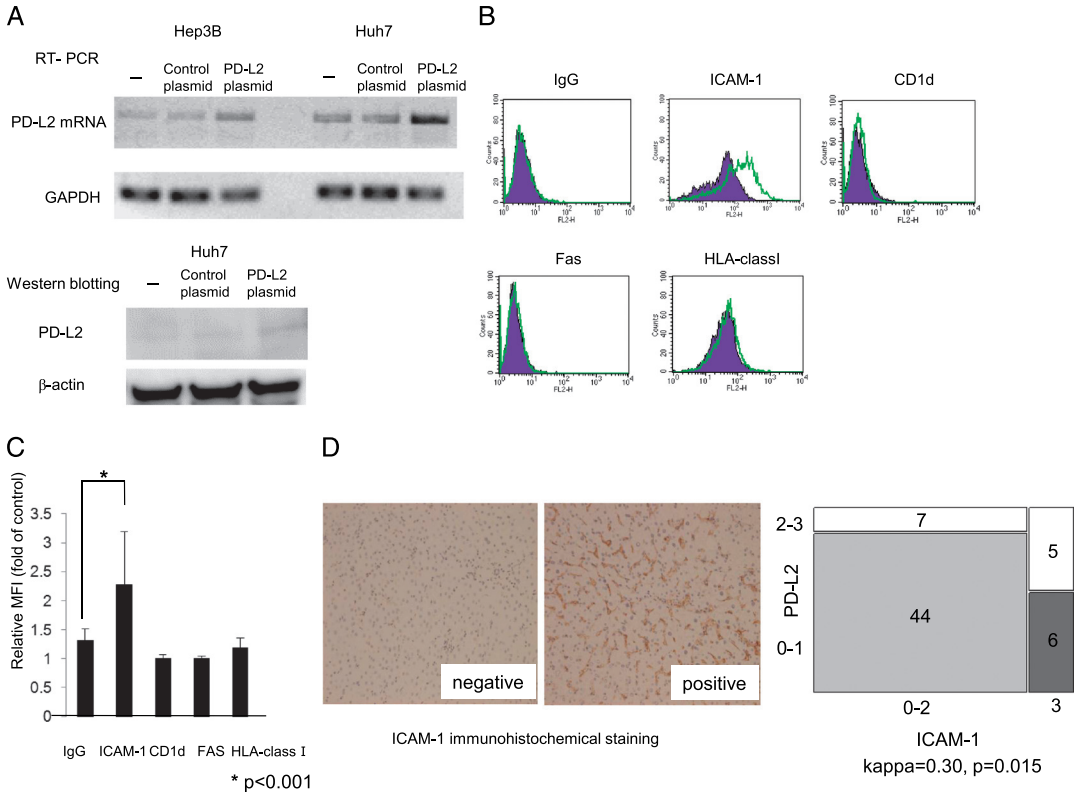

FIGURE 3. The effect of increased PD-L2 expression on immunological parameters. A, RT-PCR and Western blotting were performed to confirm the efficiency of PD-L2 containing plasmid transfection. The ability of the PD-L2 containing plasmid to induce PD-L2 mRNA expression was confirmed in two cell lines, with the more efficiently transfected Huh7 cells used for confirmation of protein expression. B, Representative flow cytometric analysis of Huh7 cells transfected with PD-L2 containing plasmid (green) and control plasmid (purple). Expressions of Fas, CD1d, HLA-class 1 and intercellular adhesion molecule-1 (ICAM-1) are shown. C, The mean fluorescence intensity (MFI) is presented as fold increase relative to control cells. The data represent mean ± SD of triplicate measurements. D, Immunohistochemical staining pattern of ICAM-1. Left panel is the representative staining pattern of ICAM-1 in liver tissue. Expression intensity was scored as follows: 0, negative; 1, weak expression; 2, moderate expression; 3, strong expression. Right: The concordance coefficients (κ statistics) were used to evaluate agreement between elevated PD-L2 expression and elevated ICAM-1 expression.

is critical for PD-1 expression. Thus, CyA induces downregulation of the immune-regulatory molecule PD-1. In our present investigation, CNIs induced expression of PD-L2, which might have an immune stimulatory function, especially in combination with HCV core or NS3.

Hepatitis C virus core protein exerts many biological effects, such as disruption of metabolic pathways, apoptosis, carcinogenesis, and immunomodulation.[24] HCV core-mediated suppression of IFN-γ and IL-2 production results in inhibition of T cell activation.[25] In human macrophage/dendritic cells, HCV core inhibits IL-12 production, which is critical for the induction of IFN-γ synthesis, resulting in dampening Th1 differentiation of CD4+ T cells.[26] Here, we demonstrated that HCV NS3 protein upregulated the expression of PD-L2 in hepatoma cell lines. In addition, the HCV core and NS3 proteins acted synergistically with CNIs to upregulate PD-L2 expression in hepatoma cell lines. Hepatitis C virus core protein has been shown to augment CyA immunosuppression, where combining core with CyA had an additive effect on proliferative suppression of T cells.[27] The present data, along with these previous reports, suggest that upregulation of PD-L2 on CH-C-OLT hepatocytes could be induced by synergy between HCV proteins and CNIs. In recent studies, PD-L2 expression was found to be regulated by the signal transducer and activator of transcription (STAT6) and NF-κB, although other possible regulators cannot be excluded[28] STAT6 is activated by IL-4 as well as other Th2 cytokines and viruses in a janus kinase-independent manner.[29]

Intercellular adhesion molecule-1 is an intercellular adhesion molecule that plays an important role in cellular interactions, including the generation of inflammatory or immune responses between lymphocytes and target cells.[30] We demonstrated that PD-L2 overexpression increased ICAM-1 expression on the hepatocyte cell surface. Intercellular adhesion molecule-1 expression is induced by several inflammatory cytokines, such as IFN-γ and tumor necrosis factor-α ([31]). The PD-L2 expression is induced on monocytes and macrophages by IL-4 and IFN-γ.[6,32] The signaling pathway of ICAM-1 includes several kinase pathways such as protein kinase C, phosphatidylinositol-3-kinase, and mitogen-activated protein kinases.[33] Because of the phosphatidylinositol-3-kinase pathway is essential for the activation of the NFκB pathway, the signaling pathways of PD-L2 and ICAM-1 are likely to modulate each other.

In summary, we report that PD-L2 is highly expressed in hepatocytes of severe CH-C and CH-C-OLT patients. The PD-L2 expression might be induced in hepatocytes by a combination of HCV core or NS3 proteins and CNI treatment, resulting in high ICAM-1 expression. It is difficult to demonstrate a direct correlation between these changes and post-OLT hepatitis C pathogenesis; however, this could account for the occurrence of severe CH-C-OLT during CNI immunosuppression.

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