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High Level of PD-1 Expression on Hepatitis C Virus (HCV)-Specific CD8$^+$ and CD4$^+$ T Cells during Acute HCV Infection, Irrespective of Clinical Outcome$^\nabla$

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We monitored expression of PD-1 (a mediator of T-cell exhaustion and viral persistence) on hepatitis C virus (HCV)-specific CD8$^+$ and CD4$^+$ T cells from blood and liver during acute and chronic infections and after the resolved infection stage. PD-1 expression on HCV-specific T cells was high early in acute infection irrespective of clinical outcome, and most cells continued to express PD-1 in resolved and chronic stages of infection; intrahepatic expression levels were especially high. Our results suggest that an analysis of PD-1 expression alone is not sufficient to predict infection outcome or to determine T-cell functionality in HCV infection.

The up-regulation of the inhibitory receptor programmed death 1 (PD-1; a member of the CD28 costimulatory family) on exhausted T cells has recently been described as a critical determinant of disease outcome in the mouse model of chronic lymphocytic choriomeningitis virus (LCMV) infection (1). For humans, PD-1 has been suggested as a marker of T-cell exhaustion in human immunodeficiency virus type 1 (HIV-1) infection and expression levels have been correlated with disease progression (2, 11, 13). As acute hepatitis C virus infection has two distinct natural outcomes, spontaneous resolution and viral persistence, it is a uniquely pertinent model to define how closely the findings of the role of PD-1 in mice translate into its role in human disease (6).

We performed a comprehensive assessment of PD-1 expression levels on hepatitis C virus (HCV)-specific CD4$^+$ and CD8$^+$ T-cell populations in 16 subjects with acute infections (9 with chronically evolving infections and 7 with self-limiting courses) and in 21 subjects with either chronic infections for more than 2 years or resolved infections. Clinical and virological data for these subjects are listed in Table 1. In addition, we studied intrahepatic lymphocytes from 35 individuals with persistent viremia. Subjects were recruited in Boston (Gastrointestinal Unit, Massachusetts General Hospital, Boston, MA; Lemuel Shattuck Hospital, Jamaica Plain, MA) and Brazil (Departmento de Virologia, Instituto Oswaldo Cruz/Fiocruz, Rio de Janeiro, Brazil). Informed written consent was obtained from each patient, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, as reflected in a priori approval from the local institutional review boards. We used 13 HCV-specific class I HLA multimeric complexes restricted by a wide range of alleles (HLA-A2, A1, B35, B7, B57, and A24) and 6 HCV class II HLA multimeric complexes, including novel DR1101-, DR0701-, and DR0404-restricted tetramers (Table 2). HLA multimeric complexes were obtained from ProImmune (Oxford, United Kingdom), Beckman Coulter (Fullerton, CA), and from William W. Kwok (Benaroya Research Institute at Virginia Mason, Seattle, WA).

PD-1 expression levels on HCV-specific CD8$^+$ and CD4$^+$ T cells do not predict clinical outcomes and remain high in both resolved and chronic infections. We observed high levels of PD-1 expression (60 to 100%) on all HCV-specific CD8$^+$ and CD4$^+$ T cells during the early phase of acute infection, regardless of clinical outcome or viral load (Fig. 1A and B and data not shown). By analyzing on the basis of percent positive PD-1 expression, we observed a decline in the proportion of positive cells after the acute phase of infection (more than 6 months) that reached significance in individuals with spontaneous res-

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TABLE 1. Patient clinical information[a]

Patient code	Age	Sex	Mode(s) of infection	Genotype	Peak viral load (IU/ml)	Peak ALT level (U/ml)	Outcome of infection
05-13	21	Male	Intranasal cocaine, IDU	1a	>700,000	140	Chronic (acute)
05H[d]	37	Male	IDU	1b	>700,000	1,884	
554	34	Female	Sex	1a	147,672	763	Chronic (acute)
02-03	34	Female	IDU	1b	2,260	1,553	Chronic (acute)
04-11[d]	25	Female	IDU	1a (1st) and 1b (2nd)	860,000 (1st), 1,420 (2nd)	2,145 (1st), 374 (2nd)	Chronic (acute)
01-21	30	Female	IDU	1a	>500,000	1,459	Chronic (acute)
00-23[d]	15	Female	Transfusion	1b	599,000	579	Chronic (acute)
01B	43	Female	Unknown	1	530,000	1,732	Chronic (acute)
04-33	22	Female	IDU	1b	>500,000	308	Chronic (acute)
320	35	Female	Sex	1	<600	918	Resolved (acute)
01-40	43	Female	IDU	1a	26,900	995	Resolved (acute)
599	59	Female	Sex	1	53,546	1,694	Resolved (acute)
1144	47	Female	Sex	1	<1,000	1,327	Resolved (acute)
06L	29	Male	IDU	3a	1,150	463	Resolved (acute)
05Y	23	Female	IDU	1	<600	1,624	Resolved (acute)
1816	67	Male	Surgery	ND	32,928	711	Resolved (acute)
00-26	66	Male	Transfusion	1b	288,930		Chronic
99-24	43	Female	Unknown	2a	166,000		Chronic
111	62	Female	Surgery	1a	11,088,387		Chronic
00X	58	Female	IDU	3a	1,213,300		Chronic
O3Q[c]	39	Male	IDU	1a	20,350,000		Chronic
03S[c]	43	Male	Sex	1a	26,600,000		Chronic
02A	29	Female	IDU	1a	2,150		Chronic
01N	55	Male	IDU	1a	468,000		Chronic
03H	20	Female	IDU	1a	200,000		Chronic
01-39	52	Female	Intranasal cocaine	1a	632,000		Chronic
03-45[c]	52	Female	IDU	1a	10,100,000		Chronic
06P	52	Male	IDU	3a	187,000		Chronic
04D	57	Female	Sexual	4	<60		Resolved
01-49[c]	52	Female	IDU	1	<60		Resolved
01-31	45	Male	IDU	1	<60		Resolved
04N	52	Male	IDU	1	<60		Resolved
01E	41	Female	IDU	4	<60		Resolved
98A	42	Male	IDU	1	<60		Resolved
00-10[b]	46	Male	IDU	1	<60		Resolved
O2Z	54	Male	IDU	1	<60		Resolved
99-21	47	Male	Tattoo	1	<60		Resolved

[a] ALT, alanine aminotransferase; IDU, intravenous drug use.
[b] HBV coinfection.
[c] HIV coinfection.
[d] Cleared under therapy.

olution of infection ($P = 0.0313$ [Mann-Whitney U test]) but not in subjects with chronically evolving courses (data not shown). However, the majority of HCV-specific T cells remained PD-1 positive and, in contrast to results from the LCMV model, we observed no normalization of PD-1 levels (i.e., relative to bulk T-cell expression levels).

In order to establish whether distinct differences in PD-1 expression levels in different disease outcomes would be revealed after a longer period of time after acute infection, we analyzed peripheral blood mononuclear cells (PBMC) from subjects that either had resolved HCV infections or had displayed persistent viremia for many years (at least 2 years). An analysis of HCV-specific $CD4^+$ T cells was limited to resolved infection due to the lack of detectable virus-specific $CD4^+$ T cells in the chronic phase (3, 7). We observed elevated PD-1 expression (by both percent positive expression and mean fluorescent intensity [MFI]) on almost all HCV-specific cells relative to the bulk T-cell population, irrespective of the clinical status of the patient (Fig. 1c and d). We conclude that PD-1 expression levels on HCV-specific T cells do not predict the outcome of infection and that continued antigenic stimulation is not required for PD-1 expression. This is clearly different from the original description of PD-1 expression in the LCMV model, where high PD-1 expression during the earliest phase of infection is followed by a rapid decline of PD-1 to normal levels only in mice resolving viremia (1).

Our data are also partially different from the results of a previous HCV study by Urbani et al. that reported lower levels of PD-1 expression in subjects with HCV resolved infections (12). Factors that contributed to this discrepancy may be the use of a more limited number of subjects in the original study than in this one and/or the use of a different PD-1 antibody. The study by Urbani et al. also found significant differences in PD-1 expression on bulk $CD8^+$ T cells during the early phase of infection, whereas we observed similar bulk $CD8^+$ (and $CD4^+$) T-cell expression levels regardless of the stage of infection (data not shown) (12). While these bulk data contrast with what has been observed for HIV infection (2, 9, 11, 13),

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TABLE 2. HLA multimeric complex information

HLA multimeric complex abbreviation	Restricting HLA	Peptide sequence	HCV protein location	HCV sequence location
Class I				
E2 614-626	A2	RLWHYPCTV	E2	614–626
NS3 1406-1415	A2	KLVALGINAV	NS3	1406–1415
NS3 1073-1083	A2	CINGVCWTV	NS3	1073–1084
NS5B 2594-2602	A2	ALYDVVTKL	NS5B	2594–2602
NS3 1273-1282	A2	GIDPNIRTGV	NS3	1273–1282
NS5A 1987-1996	A2	VLSDFKTWKL	NS5A	1987–1996
CORE 41-49	B7	GPRLGVRAT	CORE	41–49
NS5B 2841-2849	B27	ARMILMTHF	NS5B	2841–2849
NS4B 1745-1754	A24	VIAPAVQTNW	NS4B	1745–1754
NS3 1436-1444	A1	ATDALMTGY	NS3	1436–1444
NS3 1359-1367	B35	HPNIEEVAL	NS3	1359–1367
NS5B 2629-2637	B57	KSKKTPMGF	NS5B	2629–2637
Class II				
NS4 1771-1790	DR0401	SGIQYLAGLSTLPGNPAIASL	NS4	1771–1790
NS3 1248-1262	DR0401	GYKVLVLNPSVAATL	NS3	1248–1262
NS5B 2573-2590	DR0404	GRKPARLIVFPDLGVRVC	NS5B	2573–2590
NS4 1773-1790	DR1101	QYLAGLSTLPGNPAIASL	NS4	1773–1790
NS4 1773-1790	DR0404	QYLAGLSTLPGNPAIASL	NS4	1773–1790
NS3 1531-1550	DR0701	TPAETTVRLRAYMNTPGLPV	NS3	1531–1550

we find our observation unsurprising as, typically, the cumulative HCV-specific T-cell response is of a magnitude 10-fold lower than that targeting HIV and, thus, is less likely to significantly influence the bulk population (5).

While we could not determine a clear distinction in PD-1 expression between T cells associated with controlled or uncontrolled infection, we do have data supporting that the PD-1 pathway is relevant in HCV-specific T cells. Blocking the PD-1/PD-L1 inhibitory pathway by an anti-PD-L1-specific antibody results in an increased HCV-specific T-cell proliferation (4, 8, 12). However, we demonstrated this increase even in individuals with resolved infections. For example, in subject 01-49 with a resolved infection, the addition of anti-PDL1 resulted in significant increases of both the NS3 1406-1415-specific and the NS5A 1987-1996-specific T-cell proliferation (Fig. 1e). Interestingly, baseline PD-1 expression levels on these T-cell populations were strikingly different (19% and 76%, respectively) (data not shown). These results highlight the powerful effect of the PD-1 pathway blockade of T-cell proliferation, but they also indicate the additional relevance of this regulatory pathway in the homeostatic control of effective T-cell responses.

PD-1 expression on individual T-cell populations responds to changes in viral load in the acute phase of infection. With almost all HCV-specific T cells expressing PD-1, we could not detect an association between PD-1 expression levels and viral load (cross-sectionally) in our cohort (data not shown). However, when characterizing individual HCV-specific T-cell responses, we saw a close association between viral load and the MFI of PD-1 expression on both $CD4^+$ and $CD8^+$ T-cell responses (Fig. 2). This effect was observed for 10 different $CD4^+$ and $CD8^+$ T-cell populations in six subjects with acute infections, with rapid declines and increases in viremia during spontaneous- or therapy-induced viral resolution or with viral relapse/reinfection after control of viremia. We also observed that the kinetics of PD-1 expression were similar to those of the established activation marker CD38 (data not shown). A similar link between PD-1 upregulation and CD38 can be seen in chronic HIV infection (10), indicating that while the general induction of PD-1 expression is not dependent on the continuous presence of viral antigen, there is an additional component of PD-1 expression that responds to antigen load and resembles a marker of T-cell activation.

Increased PD-1 expression levels on liver-infiltrating lymphocytes. In order to investigate whether PD-1 expression on T cells from the liver, the primary site of HCV infection, was altered in comparison to that on PBMC, we obtained biopsy and explant tissue from 35 chronically infected individuals (with matched blood samples available from 24 of these individuals). Bulk PD-1 expression levels found on liver-infiltrating $CD8^+$ and $CD4^+$ lymphocytes were significantly higher than peripheral blood levels (P values were <0.0001 in both cases) (Fig. 3a and b). The mean PD-1 expression level on bulk $CD8^+$ liver-residing lymphocytes was 71%, whereas the mean expression on $CD8^+$ T cells in blood from the same subjects was 33% (Fig. 3a). For bulk liver-derived $CD4^+$ T lymphocytes, the mean PD-1 expression level was 53%, while the value for cells in the periphery was only 25% (Fig. 3b). A significantly lower level of expression of PD-1 on the $CD4^+$ T-cell population compared to the level on the $CD8^+$ T-cell population was observed in the liver ($P = <0.0001$ [Mann-Whitney U test]) but not the periphery ($P = 0.1313$ [Mann-Whitney U test]).

To examine PD-1 levels on antigen-specific $CD8^+$ T-cell populations, we assessed expression on 10 liver-infiltrating, HCV-specific populations, 2 HBV-specific populations, and 1 Epstein-Barr virus-specific population. Figure 3c shows high PD-1 expression levels on all the liver-infiltrating, virus-specific, $CD8^+$ T-cell populations (93 to 100%). Figure 3d displays increased PD-1 expression levels in liver-residing T cells from individual 00-26 compared to the levels in both a perihepatic lymph node and PBMC for the NS3 1406-1415-specific HCV-

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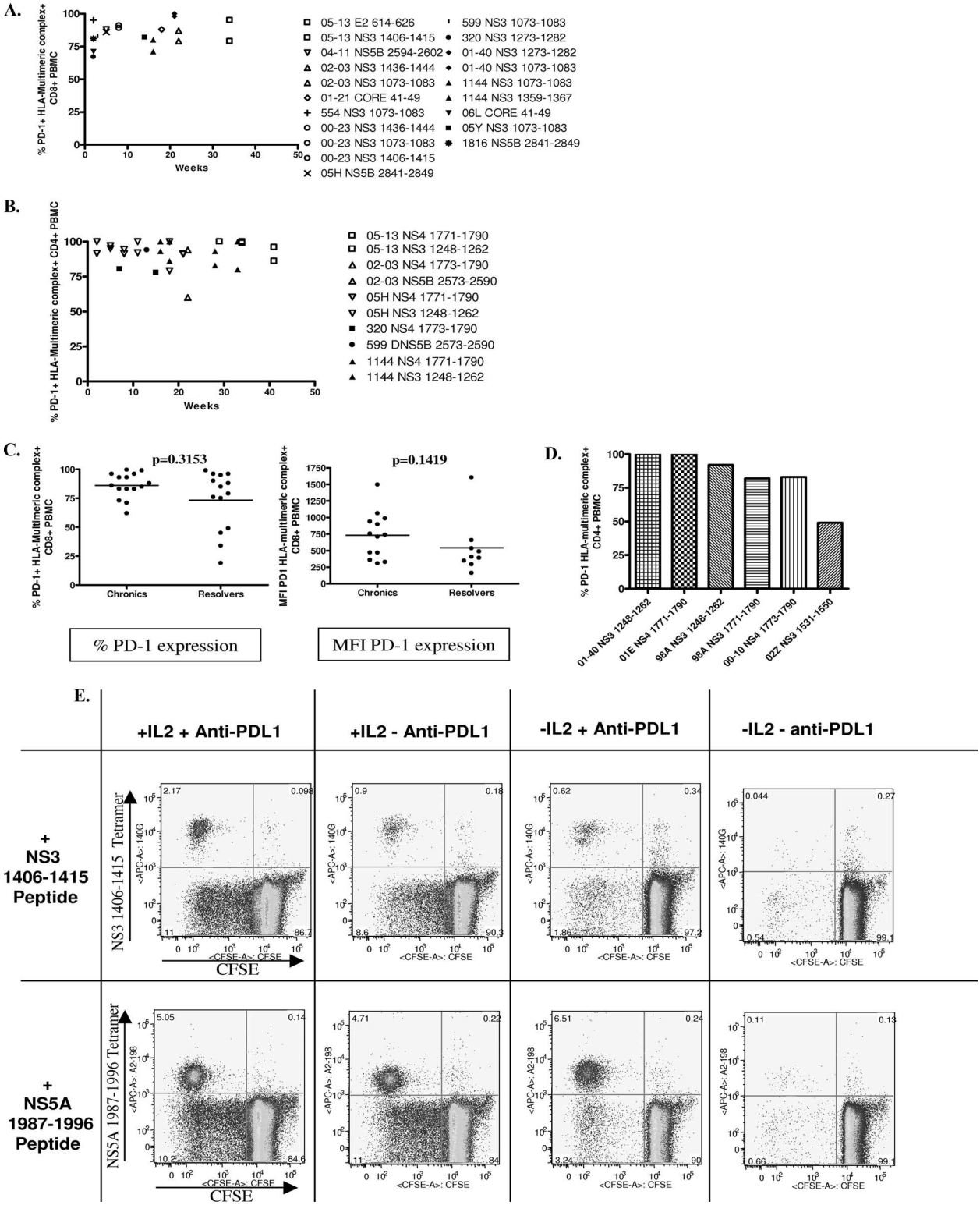

FIG. 1. High PD-1 expression levels in the acute and chronic phases of HCV infection. (A) PD-1 expression levels on HCV-specific $CD8^+$ T-cell populations during the acute stage of HCV infection. For clarity, only data from the first available time point are displayed on the graph. Open symbols indicate individuals with chronically evolving acute infections, while filled symbols indicate individuals with self-limiting infections. (B) PD-1 expression levels on HCV-specific $CD4^+$ T-cell populations during the acute stage of infection. The graph shows data for six HCV-specific $CD4^+$ T-cell responses from three individuals with chronically evolving HCV infections and four HCV-specific $CD4^+$ T-cell responses from three individuals with self-limiting courses of infection. (C) High PD-1 expression levels can be seen on HCV-specific cells from both long-term chronically infected individuals and individuals with documented resolved infections. The left panel shows data as analyzed by percent positive PD-1 expression, and the right panel represents the data as analyzed by MFI. (D) PD-1 expression levels on HCV-specific $CD4^+$ T cells from individuals with resolved infections. (E) Increased proliferation of HCV-specific $CD8^+$ T cells in the presence of anti PD-L1. Individual 01-49 targets two epitopes (NS3 1406-1415 and NS5A 1987-1996). The addition of anti-PDL1 alone resulted in a 14-fold increase in NS3 1406-1415-specific T-cell proliferation and a 60-fold increase in NS5A 1987-1996-specific T-cell proliferation.

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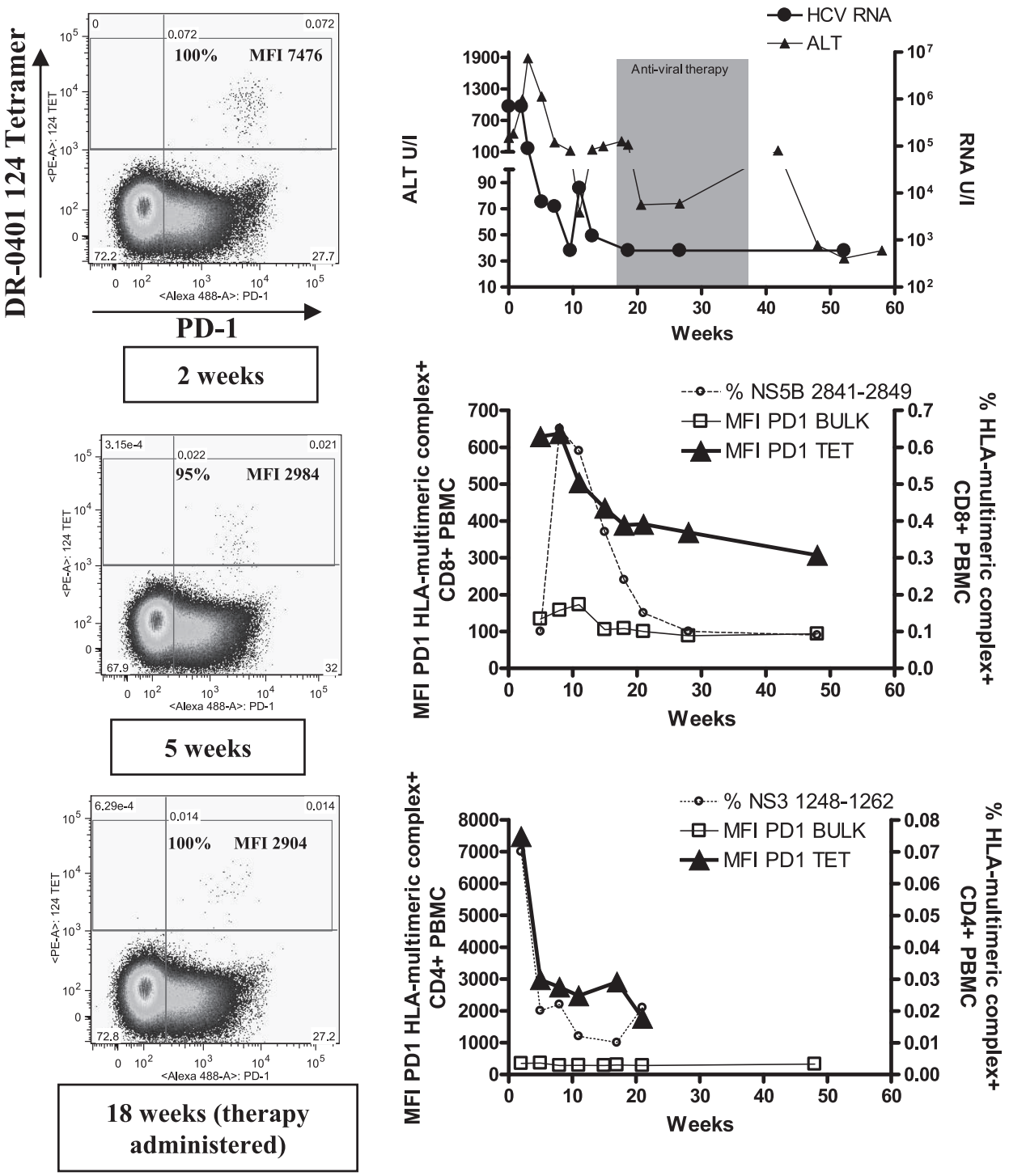

FIG. 2. PD-1 expression on individual T-cell populations is partially associated with viral load in the acute phase of infection. Longitudinal PD-1 expression levels on the DR0401-124 $CD4^+$ T-cell population from individual 05H (left panel). The clinical course of the acute infection is displayed in the right panel. Subject 05H cleared acute HCV infection following the administration of antiviral therapy. Two HCV-specific T-cell populations were tracked in this individual: NS5B 2841-2849 $CD8^+$ T cells and DR0401-124 $CD4^+$ T cells.

specific population and bulk $CD8^+$ T-cell population. Clearly, tissue-dependent differences exist in PD-1 expression (9a) and their significance needs to be further evaluated.

In conclusion, we demonstrate the pervasive and long-term induction of PD-1 expression on the vast majority of HCV-specific $CD8^+$ and $CD4^+$ T cells, irrespective of infection outcome. More subtle effects of viral load fluctuation on individual PD-1 MFI accompany the universal induction of PD-1 expression, suggesting that PD-1 is also partially an activation marker. Finally, PD-1 expression is also dependent on the

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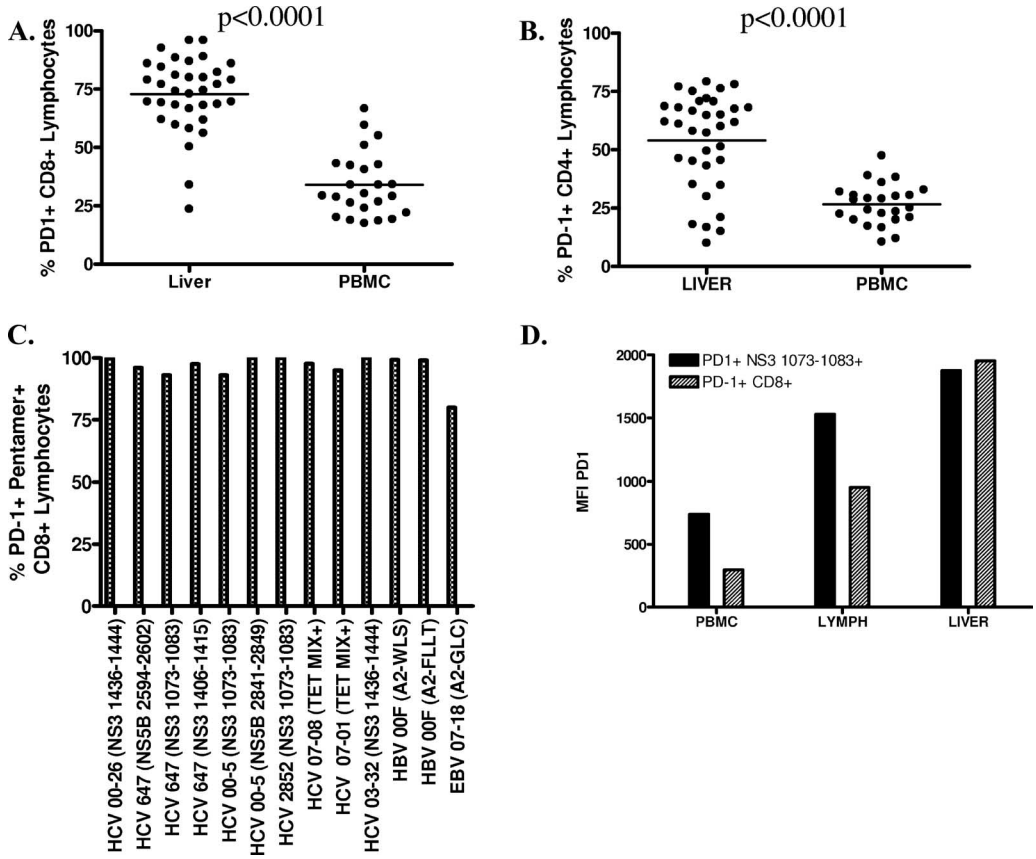

FIG. 3. PD-1 expression levels on bulk and liver-infiltrating $CD8^+$ and $CD4^+$ T cells. PD-1 expression levels on peripheral and liver-infiltrating $CD8^+$ (A) and $CD4^+$ (B) T cells from chronically infected individuals. Significantly higher levels were found on liver-infiltrating $CD8^+$ and $CD4^+$ lymphocytes compared to peripheral blood levels ($P < 0.0001$ and $P < 0.0001$, respectively). (C) PD-1 expression levels on 10 HCV-specific $CD8^+$ liver-infiltrating T-cell populations from seven chronically infected individuals. Also shown are the PD-1 expression levels on two HBV-specific $CD8^+$ T cell populations from one chronically HBV-infected individual and one Epstein-Barr virus-specific population. (D) PD-1 expression levels are displayed for liver, lymph node, and PBMC samples from individual 00-26. PD-1 expression levels were increased in liver compared to that in periphery on both the HCV-specific population and bulk $CD8^+$ T-cell population.

tissue environment where the T cells exercise their antiviral functions. Our data highlight the fact that the expression pattern of PD-1 in human infection differs from what has been described for the LCMV model and that analysis of PD-1 expression on its own is insufficient to explain different clinical outcomes and distinct T-cell functionality for the HCV model. Additional studies on the expression patterns of different splice variants of PD-1 and its ligands, and receptor-ligand interactions in infected tissue, will be important benchmarks for providing a more comprehensive assessment of the level of inhibitory signaling and its impact on the outcome of human infection.

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AUTHOR'S CORRECTION

High Level of PD-1 Expression on Hepatitis C Virus (HCV)-Specific CD8$^+$ and CD4$^+$ T Cells during Acute HCV Infection, Irrespective of Clinical Outcome

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