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Which has been published in final form at:

https://doi.org/10.1016/j.cellimm.2011.03.011

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Bim-mediated apoptosis and PD-1/PD-L1 pathway impair reactivity of $PD1^{+}/CD127$ HCV-specific $CD8^{+}$ cells targeting the virus in chronic hepatitis C virus infection

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ABSTRACT

PD-1 molecule promotes anergy and IL-7 receptor (CD127) induces an anti-apoptotic effect on T cells. Correlation between PD-1/CD127 phenotype and hepatitis C virus (HCV)-specific CD8+ cell reactivity in resolved infection (RI) after treatment and persistent HCV-infection (PI) was analysed. Directly ex vivo, PD-1 and CD127 expression on HCV-specific CD8+ cells displayed a positive and negative correlation, respectively with viraemia. Proliferation after stimulation on PD-1/CD127+ cells from RI cases was preserved, while it was impaired on PD-1+/CD127 cells from PI patients. PD1+/CD127+ population was observed in PI, and these maintained expansion ability but they did not target the virus. Frequency of PI cases with HCV-specific CD8+ cell proliferation increased after anti-PD-L1 and anti-apoptotic treatment. Bim expression on HCV-specific CD8+ cells from PI patients was enhanced. In conclusion, during chronic HCV infection non-reactive HCV-specific CD8+ cells targeting the virus are PD-1+/CD127/Bim+ and, blocking apoptosis and PD-1/PD-L1 pathway on them enhances in vitro reactivity.

KEYWORDS

HCV, Viral persistence, PD-1, CD127 PD-L1, z-VAD-fmk, Bim, Cytotoxic T lymphocytes, Chronic hepatitis, Apoptosis

Introduction

Hepatitis C virus (HCV) infection is often associated with persistent viraemia and progressive liver disease [1]. HCV-specific cytotoxic T lymphocytes (CTL) play a major role in spontaneous infection resolution. Nevertheless, during chronic infection these cells lack adequate effector functions and fail to control HCV [2].

Appropriate activation of experienced virus-specific CTL depends on the engagement between T cell receptor (TCR) and HLA-I/epitope complex [3] plus interaction between positive costimulatory molecules and their ligands [4]. These cells after developing their effector action, express negative co-stimulatory molecules, such as programmed death-1 (PD-1), to switch-off their activity [5]. Engagement of PD-1 and its ligand (PD-L1) delivers a negative signal avoiding proliferation and interleukin (IL)-2 production [6]. On the other hand, IL-7 receptor (CD127) plays an essential role in mature lymphocyte survival by counteracting the induction of apoptosis after antigen encounter through myeloid cell leukemia sequence-1 (Mcl-1) expression and Bcl2interacting mediator (Bim) down-regulation, enhancing IL-2 secretion and life span [7].

HCV infection could modulate these two molecules to impair HCV-specific CTL reactivity through induction of either a virusassociated tolerogenic-like state or apoptosis. PD-1^{+}/CD127 phenotype associated with anergic features and apoptosis in chronic hepatitis C has been shown [8–10]. Nevertheless, HCV-specific CTL reactivity after antigen encounter in a proportion of HCV persistent infection cases are also maintained [11,12]. In this study the effect of PD-1/CD127 phenotype on HCV-specific CTL reactivity after antigen recognition according to viral control was analysed and the effect of modulating these molecules was tested.

Patients and methods

Patients

In this cross-sectional study ninety-four consecutive HLA-$A2^{+}$ subjects with genotype-1 HCV infection were recruited between January-2007 and August-2010 at Guadalajara University Hospital and "Fundación Jiménez Díaz" Hospital, Spain. These patients were divided into two groups according to their serum HCV-RNA and alanine transaminase (ALT) levels at the time of investigation; 76 patients with ALT > 40 IU/mL and HCV-RNA > 1000 IU/mL [hereafter indicated as persistent infection (PI)] (12 relapsers, 10 nonresponders after anti-HCV treatment and 54 naïve) and 18 subjects with

ALT < 40 IU/mL and HCV-RNA < 40 IU/mL, persistently maintained after developing sustained virologic response (SVR) subsequent to anti-HCV treatment [hereafter indicated as resolved infection (RI)] (Table 1). Other causes of chronic liver disease were excluded. 10 HLA-A2 HCV infected patients were taken as controls. In all recruited patients a serum and heparinised blood samples were taken. In treated patients, samples were taken 24 weeks after stopping treatment and in naïve patients at enrollment. All treated patients received treatment with pegylated interferon alfa-2b (1.5 ug/kg/week) and ribavirin (800–1400 mg/day) for 48 weeks following the standard stopping rules [13]. In nineteen PI patients and pre-treatment in all RI cases a liver biopsy was also performed. The study protocol was approved by the Regional Ethical Committee and patients gave written informed consent before enrolment.

Table 1
Clinical features of patients enrolled in the study.

		Resolved infection	Persistent infection	*p*-value
N° of patients (n)		18	76	
SVR (n/%)		18 (100%)	–	
Relapser (n/%)		–	12 (16%)	–
Non responder (n/%)		–	10 (13%)	–
Naïve (n/%)		–	54 (71%)	–
HLA-A2 (%)		100	100	–
Age (years)		41 ± 7	47 ± 9.16	NS
Male sex (%)		70	68	NS
Evolution time (years)		24 ± 9	31 ± 12	NS
ALT (IU/L)		42.1 ± 19.2	92.8 ± 54.5	<0.001
Viral load (IU/mL)		<40	$2.12 \pm 3.7 \times 10^6$	<0.001
Genotype 1a/1b (%)		71/29	70/30	NS
Liver histology*				
	Lobular activity	1.82 ± 0.67†	1.97 ± 0.6	NS
	Portal activity	2.11 ± 0.56†	2.21 ± 0.6	NS
	Fibrosis	1.14 ± 0.45†	1.8 ± 0.7	NS

Data are presented as either percentage or mean ± standard deviation. NS: nonsignificant. SVR: sustained virologic response. *Liver histology is described according to Scheuer index. †Liver histology data just before anti-HCV treatment.

Tissue typing

Screening for the HLA-A2 haplotype was performed by staining PBMC with FITC-conjugated anti-HLA-A2 mAb (Incstar, Stillwater, MN) and flow cytometric analysis.

Virological assessment

Anti-HCV was determined by Ortho HCV Version 3.0 ELISA Test (Ortho Diagnostic System, Raritan, NJ). HCV-RNA load was assessed by a quantitative real-time-PCR assay with a lower detection limit of 40 IU/mL (Cobas Amplicor HCV Monitor 2.0 Roche, Indianapolis, IN). HCV genotype was tested by InnoLiPPA HCV II assay (Innogentics Inc., Alpharetta, GA).

Synthetic peptides and pentamers

A2-restrictedpeptidescorrespondingto the genotype-1 $NS3_{1406–1415}$ region (KLVALGINAV), $NS3_{1073–1081}$ region (CINGVCWTV) and PE-conjugated HLA-A2 pentameric complexes (pentamer) loaded with the same two NS3 peptides and with cytomegalovirus (CMV) $pp65_{495–504}$ (NLVPMVATV) peptide were purchased from ProImmune (Oxford, UK).

$CD8^+$/pentamer$^+$ cell quantification

Peripheral blood (PBMC) and intrahepatic mononuclear cells (IHMC) were purified from heparinised blood samples and biopsies according to method described previously [14]. To quantify HCVspecific $CD8^+$ cells, 0.5–1 10^6 PBMC or a variable number of IHMC were incubated for 10 min at 37 C with 1 lg of PE-labelled pentamers in RPMI 1640 plus 10% FCS. Cells were washed in PBS and then incubated at 4 C for 20 min with saturating concentrations of directly conjugated anti-CD8-Pe-Cy5 (Cy) mAb, (Sigma– Aldrich Inc., St. Louis, MO). Subsequently, stained cells were analysed after washing on a Becton Dickinson FACS using CELLQuest™ software. The cut-off for pentamer assay was determined by staining intrahepatic and peripheral blood mononuclear cells from 10 HLA-A2 PI patients. Using the mean plus 2 standard deviations as the cut-off point, the lowest percentage at which pentamer-binding $CD8^+$ cells could be detected as a separate cell population was 0.018% out of total $CD8^+$ cells.

PD-1/CD127 phenotypic analysis on $CD8^+$/$pentamer^+$ cell

In patients with proven $pentamer^+$ cells, phenotypic analysis was performed. PBMC and IHMC were triple stained with PEpentamers plus anti-CD8-Cy mAb as above and either FITC-conjugated anti-CD127 (Pharmingen BD, San Jose, CA) or anti-PD-1 (eBiosicience Inc., San Diego, CA) mAbs. Subsequently, stained cells were analysed by flow-cytometry. Isotype-matched control mAbs were used to set the markers for the different stainings. Different phenotypes were quantified on $pentamer^+$ cells as the mean fluorescence intensity (MFI) for each staining, and compared between the study groups. Phenotypes were performed directly ex vivo in circulating and liver-infiltrating $pentamer^+$ cells and after specific in vitro challenge in the peripheral compartment. Depending on the MFI, PD-1 and CD127 phenotypes were marked as: low (), intermediate (+) and high (++).

Production of T cell lines

PBMC were re-suspended at a concentration of 1 10^6/ml in RPMI 1640 plus 10% FCS. Cells were stimulated with 1 lM of one of these two NS3 specific peptides, $NS3_{1406–1415}$ and $NS3_{1073–1081}$, in a 96-well plate. Recombinant IL-2 (25 IU/ml) was added on day 2 of culture, and cells were analysed after a total of 10 days of culture. After specific in vitro expansion, $pentamer^+$ cell phenotype was again analysed as above. T cells from selected RI and PI patients were also cultured in presence or absence of either antiPD-L1 blocking mAb (2 lg/mL) (Santa Cruz Biotechnology, Santa Cruz, CA) or the pancaspase inhibitor zVAD-fmk (50 lM) (BD Bioscience, San Jose, CA), using in both cases b-galactosidase (2 lg/ mL) (Santa Cruz Biotechnology, Santa Cruz, CA) as control. Expansion was considered positive when a pentamer-binding $CD8^+$ cell population bigger than base line and higher than 0.04% out of total $CD8^+$ cells was observed after specific stimulation.

HCV-epitope sequencing

In PI patients with directly ex vivo $NS3_{1406–1415}$ or $NS3_{1073–1081}$pentamer binding $CD8^+$ cells, epitope was sequenced in order to test whether PD-1/CD127 phenotype could be affected by HCV sequence variation. Patients' plasma were collected by centrifugation in plasma preparation tubes and frozen immediately at 80 C. Total HCV-RNA was isolated using QIAamp Viral RNA Kit (Qiagen, Germany) and, reverse transcribed. Subsequently, nested-PCR amplification was carried out, using the NS3 primers described in Table 2. A single band of the expected size was obtained. Final PCR products were purified using GeneClean kit (Bio101, La Jolla, CA) and, subsequently sequenced in an ABI PRISM 377 DNA sequencer (Applied Biosystems Inc., Foster City, CA).

Table 2
HCV primers for amplification and sequencing.

		Sense	Antisense
NS31406–1415 (KLVALGINAV)	First PCR	(5^0-ACGTACTCCACCTACGGCAA-3^0)	(5^0-AAGGTAGGGTCAAGGCTGAA-3^0)
	Second PCR	(5^0-CATCCCAACATCGAGGAGGT-3^0)	(5^0-TTGCAGTCTATCACCGAGTC-3^0)
NS31073–1081 (CINGVCWTV)	First PCR	(5^0-GGCYTGCCCGTCTCYGCCCG-3^0)	(5^0-CGGCGCACSGGAATGACATCG -3^0)
	Second PCR	(5^0-CGGCSTACKCCCARCAGACGMGAGGCC-3^0)	(5^0-CCTCGTGACCARGTAAAGGTCC-3^0)

PCR conditions were as follows: 1 cycle (95 C 5 min); 35 cycles (95 C 45 s, 42 C 45 s, 72 C 45 s) and 1 cycle (72 C 10 min).

Intracellular Bim staining

Directly ex vivo and after specific in vitro challenge in presence of zVAD-fmk, HCV-specific $CD8^+$ T cells from selected RI and PI cases were surface stained with PE-pentamers and CD8-Cy mAb as above. After a further wash, cells were subjected to intracellular staining using Cytofix-Cytoperm (BD Bioscience, San Jose, CA) to permeabilise and fix cells according to the manufacturer's instructions, followed by staining with Bim unconjugated Ab (Cell Signaling Technology, Beverly, MA) plus goat anti-rabbit IgG2 Alexa 488 (Invitrogen, Carlsbad, CA) and its isotype-matched control (Pharmingen BD, San Jose, CA). Finally, cells were washed twice and analysed by flow cytometry and expressed as MFI Bim-Alexa 488 on $CD8^+$/pentamer$^+$ cells.

Statistical analysis

Categorical data are presented as either percentage or 95% confidence interval for percentage (CIP) whereas continuous variables are summarised as median plus interquartile range (IQR) or mean ± standard deviation. Wilcoxon, Mann–Whitney-U and Pearson tests were employed where appropriate. Significance was established at $p < 0.05$. Statistical analysis of frequency, phenotype and proliferation of pentamer$^+$ cells was performed pooling the results for the different epitopes tested in a single variable. For correlation tests, HCV viral load and PD-1 MFI were log transformed in order to make these variables follow a normal distribution.

Results

PD-1/CD127 expression on intrahepatic and circulating CD8⁺/ pentamer⁺ cells

Mononuclear cells from PI patients' liver biopsies and peripheral blood samples were studied. A portion of these cells were directly ex vivo double stained with anti-CD8-Cy mAbs and with PE-pentamers to test the presence of HCV-specific $CD8^+$ cells against the two NS3 immunodominant epitopes studied. In 74% and 89% of liver biopsies from PI patients, intrahepatic pentamer$^+$ cells against $NS3_{1406–1415}$ and $NS3_{1073–1081}$ epitopes, respectively were observed with a mean global percentage of 78% (22 out of 28 tests; 95% CIP: 60–91%), while only in 14–28% of PI patients, according to the pentamer tested, there were cells in peripheral blood also, with a mean percentage of 23% (23 out of 115 tests; 95% CIP: 12–25%) (Table 3). Therefore, an intrahepatic sequestration of pentamer-binding $CD8^+$ cells was shown ($p < 0.001$; Table 3) with a median intrahepatic frequency of 0.35% (IQR: 3.7) pentamer$^+$ cells out of total liver-infiltrating $CD8^+$ cells, while median frequency of peripheral pentamer-binding $CD8^+$ cells was 0.046% out of total $CD8^+$ cells (IQR 0.07), in the cases with detectable cells. In patients with RI, the frequency of cases with detectable pentamer$^+$ cells in peripheral blood was also very low, between 20% and 22%, depending on the NS3 epitope tested, with a mean percentage of 21% (7 out of 33; 95% CIP: 7–35%), which was similar to the one observed in PI (p = NS; Table 3). The median frequency of peripheral pentamer-binding $CD8^+$ cells in RI patients with detectable cells was also low (0.056%; IQR: 0.05) and similar to the frequency found in PI cases (p = NS; Table 3). In patients with directly ex vivo detectable pentamer$^+$ cells, mononuclear cells were triple stained with PE-pentamers and anti-CD8-Cy mAb plus either anti-PD1-FITC or anti-CD127-FITC mAbs. The PD-1 FITC MFI on intrahepatic pentamer-binding cells was higher (++) (105; IQR: 94) than the MFI observed in peripheral blood (+) (34; IQR: 25) for PI cases ($p = 0.04$; Fig. 1A and B). PD-1 FITC MFI on peripheral HCV-pentamer$^+$ cells from RI patients was much lower () (8; IQR: 5) than the MFI observed in PI ($p < 0.001$; Fig. 1A and B) and similar to the intensity observed for peripheral CMV-pentamer$^+$ cells from PI patients. On the other hand, CD127-FITC MFI on intrahepatic pentamer$^+$ cells was lower () (5.1; IQR: 1.2) than in the peripheral compartment (+) (13; IQR: 9) in PI ($p = 0.03$; Fig. 1A and B), while CD127 expression on peripheral HCV-pentamer$^+$ cells from RI patients was much higher (++) (32; IQR 15) than in PI ($p < 0.001$; Fig. 1A and B) and similar to the expression on peripheral CMV-pentamer$^+$ cells from PI subjects. In the group of PI patients, these data translated into a significant positive and negative correlation between HCV viral load and PD-1 ($r = 0.443$; $p = 0.03$) and CD127 expression ($r = 0.456$; $p = 0.038$) on peripheral pentamer$^+$ cells respectively (Fig. 2).

Proliferation ability of CD8⁺/pentamer⁺ cells after antigen encounter

The capacity for circulating pentamer⁺/CD8⁺ cells to expand after exposure to viral antigens according to viral control in enrolled patients was investigated. A different clonal expansion potential between PI and RI subjects was observed. Specifically, in 22% (5 out of 22) and 39% (21 out of 53) of PI, and in 82% (9 out of 11) and 72% (13 out of 18) of RI cases an expansion after $NS3_{1073–1081}$ and $NS3_{1406–1415}$ stimulation, respectively was shown ($p < 0.001$ and $p = 0.018$, respectively; Fig. 3A). The proportion of experiments with expansion, taking together the results for both epitopes, was higher in RI (22 out of 29; 76%: 95% CIP [59–92%]) than in PI (26 out of 75; 34%: 95% CIP [23–45%]) ($p < 0.001$; Fig. 3A and B). PD-1/CD127 phenotype was different between PI and RI patients after standard proliferation. PD-1 FITC MFI on pentamer⁺ cells from PI cases was significantly higher (+) (32.3; IQR 32.3) than in RI subjects () (8.3; IQR 8.1) ($p = 0.001$; Fig. 3C). Interestingly, CD127 expression on pentamer⁺ cells from RI patients was low (8.1; IQR 6.4) () after in vitro challenge, as would be expected for effector cells after antigen encounter ($p = 0.03$; Fig. 3C). Nevertheless, pentamer⁺ cells from PI cases with expansion ability without any extra-treatment displayed an intermediate CD127FITC MFI (+) (17.6; IQR 17.25) after proliferation which was higher than in expanded cells from RI subjects () ($p = 0.022$; Fig. 3C).

Table 3
Number of patients tested directly ex vivo for the presence of pentamer+ cells against two different NS3-HCV immunodominant epitopes, and frequency of these cells in patients with detectable pentamer+ cells.

			All epitopes	NS31406–1415	NS31073–1081	p-value
PI	IH	N° of cases tested (n)	28	19	9	<0.001*
		Cases with detectable pentamer + cells (n; %)	22 (78%)	14 (74%)	8 (89%)	
		Frequency of Pent + cells out of total CD8 + cells (%)	0.36 (IQR3.7)	0.21 (IQR3.9)	2.1 (IQR3.8)	
	PB	N° of cases tested (n)	115	76	39	NS*
		Cases with detectable pentamer + cells (n;%)	23(20%)	11(14%)	11(28%)	
		Frequency of Pent + cells out of total CD8 + cells (%)	0.046 (IQR0.07)	0.048 (IQR0.04)	0.07 (IQR0.09)	
RI	PB	N° of cases tested (n)	33	18	15	
		Cases with detectable pentamer + cells (n;%)	7(22%)	4(22%)	3(20%)	
		Frequency of Pent + cells out of total CD8 + cells (%)	0.056 (IQR0.05)	0.064 (IQR0.08)	0.054 (IQR NA)	NA

PI: persistent HCV infection. RI: resolved HCV infection. IH: intrahepatic. PB: peripheral blood. */Pearson Chi-square test. NA: non applicable due to sample size. Pent: pentamer. Median frequency of pentamer+ cells in patients with detectable pentamer+ cells. The lower limit of detection with pentamer staining technique was 0.018% pentamer+ cells out of total CD8+ cells.

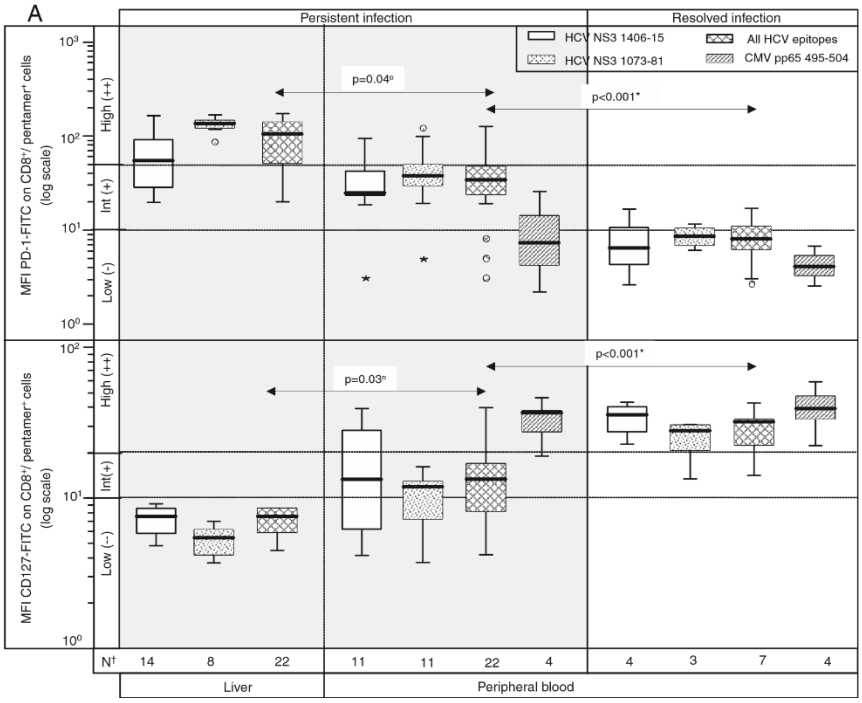

Fig. 1. Directly ex vivo analysis of PD-1/CD127 phenotype on pentamer-binding CD8+ cells in HCV infection according to viral control. (A) Box-plots summarising the PD-1/ CD127 MFI on CD8+/Pentamer+ cells against the different epitopes tested in resolved and persistent HCV patients. Int: intermediate. NS: non significant. O: outlier.⁄: extreme value.⁄Mann–Whitney U test. ¤Wilcoxon test. Number of cases studied for every epitope. (B) FACS dot-plots of peripheral and intrahepatic lymphocytes stained with CD8Cy mAb, Pentamer-PE and either PD-1FITC or CD127-FITC mAbs, and FACS histograms for PD1-FITC and CD127-FITC mAbs intensity of fluorescence gated on CD8+/ Pentamer+ cells. The plots and histograms are representative from one patient with persistent infection and one subject with resolved infection. Upper left figures in each dotplot represent the percentage of positive Pentamer+ cells out of total CD8+ cells. () Low, (+) intermediate and (++) high fluorescence.

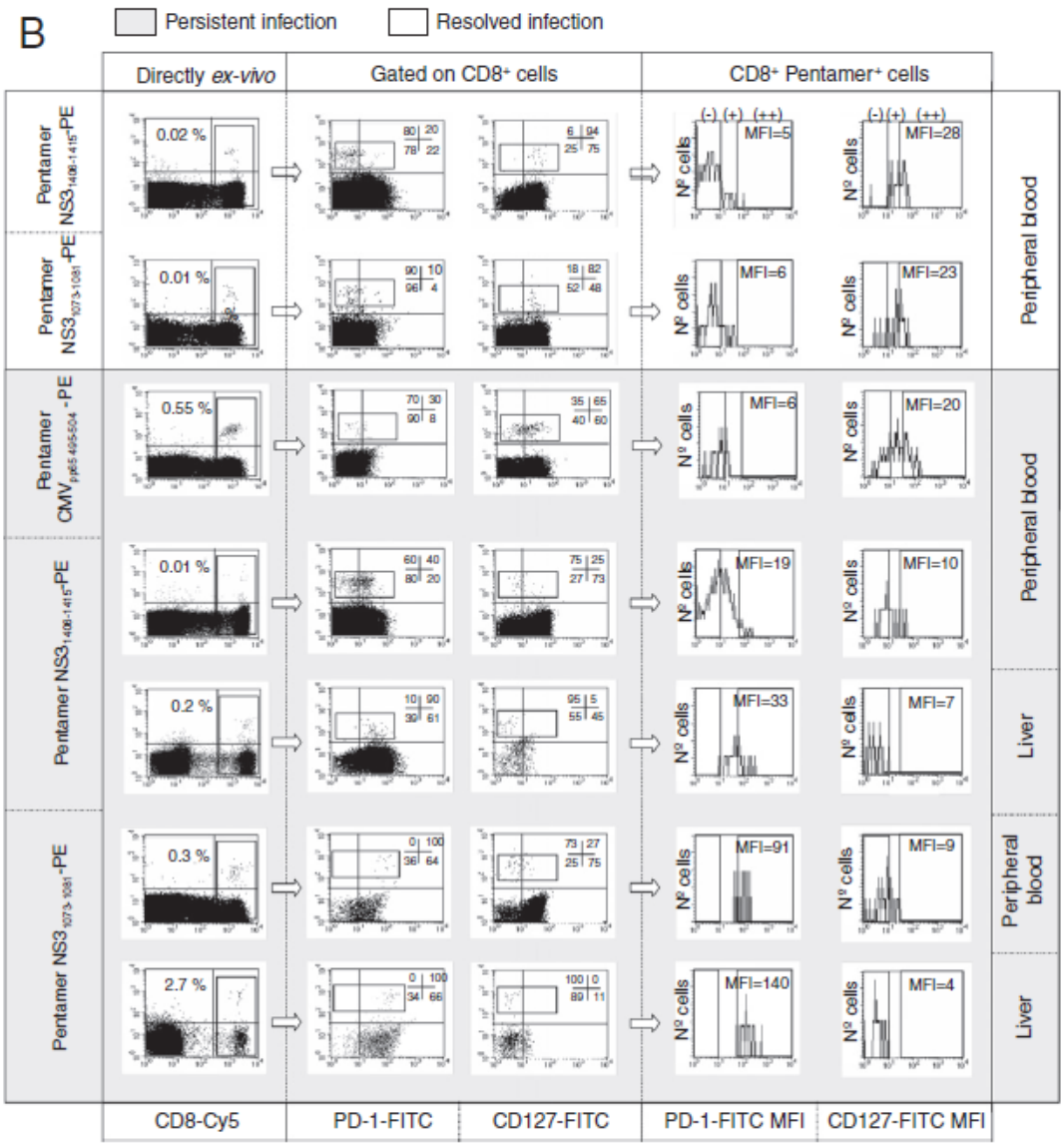

Fig. 1 (continued)

Pentamer-binding CD8+ cell populations according to CD127 expression in PI

In those cases with directly ex vivo detectable pentamer-binding $CD8^+$ cells, PD-1/CD127 phenotype and expansion ability were correlated. The analysis of all the performed experiments together showed that independently of HCV control and PD-1 level, CD127 expression correlated positively with pentamer-binding cell expansion capacity. In fact, 83% of CD127-expressing pentamer$^+$ cells expanded while only 16% of $CD127^-$ did expand (Fig. 4A and B; $p = 0.001$). PD-1 expression also associated with an impaired expansion ability but only in the

$CD127^-$ cell subset (Fig. 4A and B; p = 0.034). After this preliminary study , the experiments were analysed according to viral control. All RI cases with detectable HCV-pentamer binding $CD8^+$ cells displayed a PD-1/$CD127^+$ phenotype (Fig. 4A), while in PI, two different CD127-expressing populations with different expansion potential were observed (Fig. 4A). In PI, $pentamer^+/CD8^+$ cells maintaining expansion ability after antigen encounter expressed a higher CD127 expression (MFI 23.03; IQR 16) than cells without proliferation capacity (MFI 6.04; IQR 3.9) (Fig. 4C and D; p = 0.045). Nevertheless, independently of the pentamer-binding cell proliferative potential, PD-1 expression was high and similar (Fig. 4C and D; p = NS). In eight out of these 13 PI patients with directly ex vivo pentamer-binding $CD8^+$ cells, we were able to sequence HCV-epitopes. The presence of epitope mutations at TCR interaction sites was associated with a higher CD127 expression (Fig. 5A and B; p = 0.048), lower PD-1 expression (Fig. 5A and B; p = 0.034), and a different proliferation ability, although the last one did not reach statistical significance (Fig. 5C; p = 0.112), probably due to the small sample size.

Effect of apoptosis and PD-1/PD-L1 pathway blocking on pentamer-binding $CD8^+$ cell reactivity

The effect of PD-1/PD-L1 interaction blocking on T cell proliferation was tested by paired incubation with either anti-PD-L1 mAb or b-galactosidase as control, during T cell HCV-specific in vitro challenge in selected RI and PI patients. In PI patients, anti-PD-L1 mAb treatment increased significantly from 29% (8 out of 27; 95% CIP [11–48%]) up to 52% (14 out of 27; 95% CIP [31–72%]) the percentage of experiments with specific CTL proliferation after $NS3_{1073\text{-}1081}$ or $NS3_{1406\text{-}1415}$ stimulation (p = 0.023; Fig. 6A and B). However, this treatment did not increase the frequency of cases with expansion on RI patients (13 out of 18: 72%; 95% CIP [49–95%]) (p = NS; Fig. 6A and B), probably due to the low PD-1 expression on these cases. Finally, we tested the effect of blocking apoptosis by pancaspase inhibitor zVAD-fmk on $pentamer^+$ cell proliferation in PI and RI cases. This treatment was performed to counteract the presumable pro-apoptotic effect of IL-7 deprivation on $CD127^-$ $pentamer^+$ cells from PI cases. PBMC were in vitro challenged with either $NS3_{1406–1415}$ or $NS3_{1073–1081}$ in presence of zVAD-fmk or b-galactosidase as control. Blocking apoptosis increased the frequency of experiments with positive proliferation in PI from 23% (3 out of 13; 95% CIP [0–49%]) up to 54% (7 out of 13; 95% CIP [22–85%]) (p = 0.018; Fig. 6C and D). Nevertheless, anti-apoptotic treatment did not affect the proliferation ability of pentamer-binding $CD8^+$ cells after specific in vitro challenge in RI cases (8 out of 11: 72%; 95%CIP [41–100%]) (p

= NS; Fig. 6C and D). Directly ex vivo and after specific in vitro expansion in presence of zVAD-fmk treatment, Bim expression on pentamer-binding $CD8^+$ cells from PI and RI patients was also tested. Directly ex vivo, Bim expression was higher on PI patients (MFI 166; IQR: 151) than in RI cases (MFI 118; IQR: 93), although this difference was not statistically significant, probably due to the sample size. However, pentamer-binding $CD8^+$ cells from PI cases after specific in vitro expansion in presence of zVAD-fmk displayed a significantly higher Bim expression (MFI 221; IQR: 331) than RI patients (MFI 77; IQR: 32) (Fig. 6D and E; $p < 0.001$).

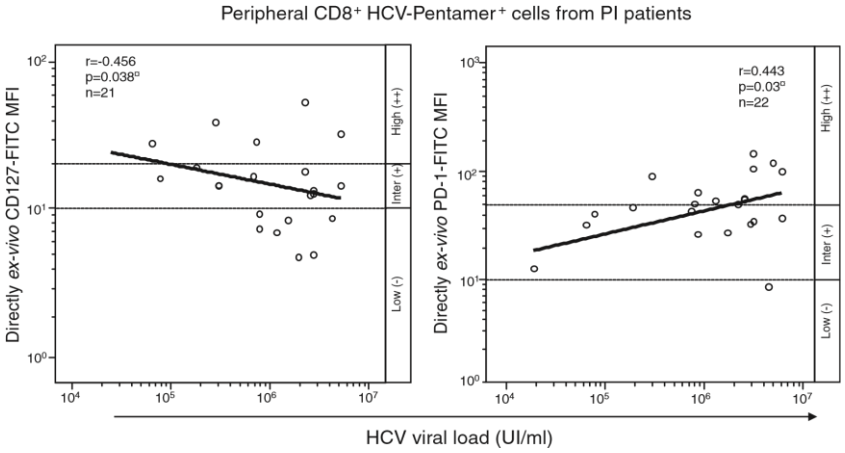

Fig. 2. Correlation between PD-1/CD127 phenotype and viral load in PI patients. Scatter-plot showing the correlation between PD-1/CD127 expression on pentamer binding $CD8^+$ cells and viral load. ¤Pearson's correlation coeficient. Inter: intermediate. MFI: mean fluorescence intensity. PI: persitent infection.

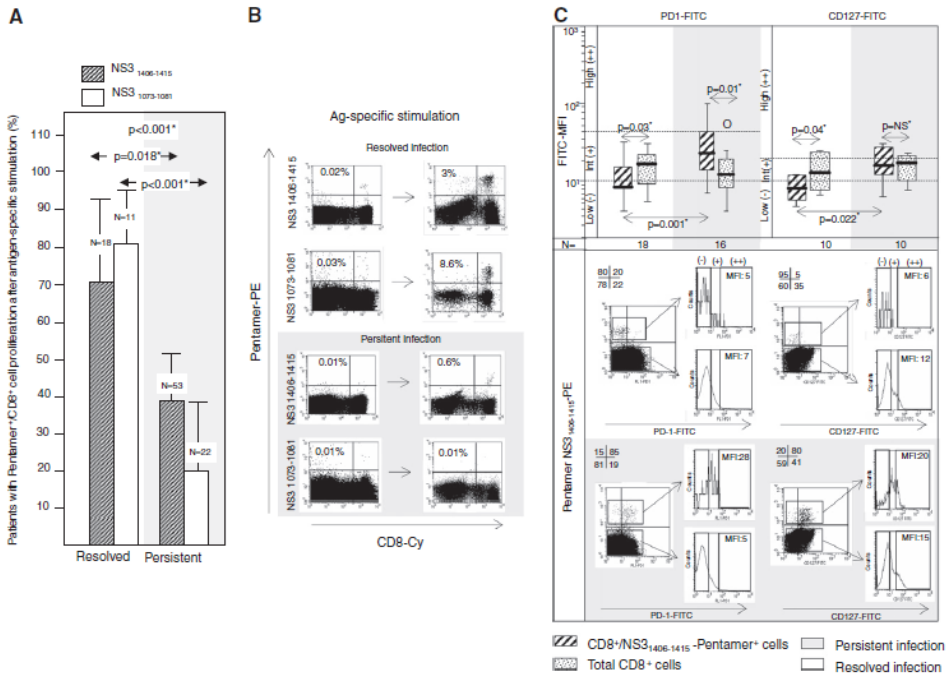

Fig. 3. Pentamer-binding CD8⁺ cells expansion ability after specific in vitro challenge according to viral control. (A). Bar-plots showing the frequency of experiments with pentamer-binding cell expansion after specific in vitro challenge in PI and RI. Whiskers represent 2 standard error of the percentage. ⁄Mann–Whitney-U test. (B). FACS dotplots of peripheral mononuclear cells stained with PE-labelled Pentamers and CD8-Cy mAb directly ex vivo and after specific stimulation from representative PI and RI patients. Upper left figures in each dot-plot represent the percentage of positive Pentamer⁺ cells out of total CD8⁺ cells. (C) Box-plots showing the PD-1 and CD127 FITC-MFI on pentamer binding CD8+ cells and total CD8⁺ cells, as internal control, after specific in vitro challenge in PI and RI. Representative FACS fluorescence histograms of PD-1 and CD127 FITC on total CD8⁺ and pentamer-binding CD8⁺ cells from one RI and one PI patient are shown. ⁄Mann–Whitney U test. Int: intermediate.

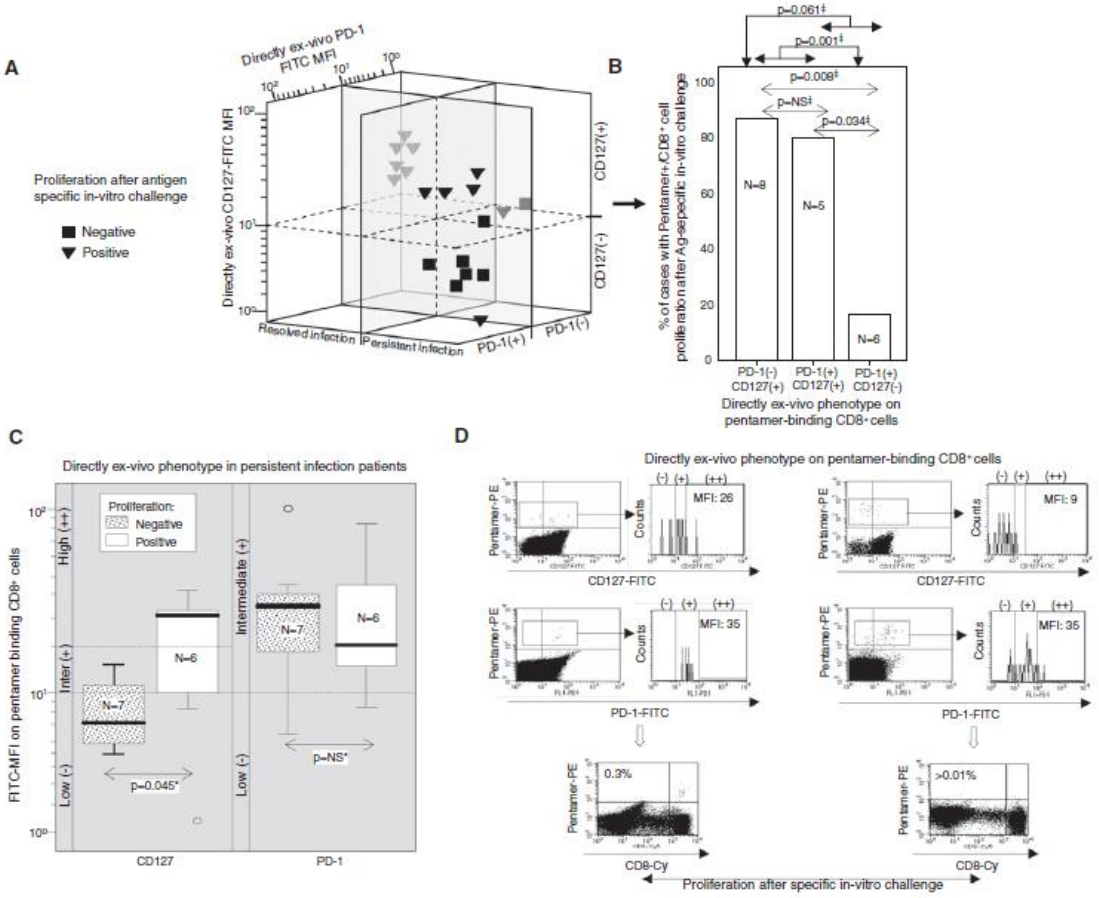

Fig. 4. Association between directly ex vivo PD-1/CD127 phenotype and pentamer-binding cell reactivity after antigen encounter. (A) Three-dimensional scatter plot showing the PD-1 and CD127 FITC MFI on peripheral pentamer-binding cells according to viral control and expansion ability. (B) Bar-plots showing the percentage of experiments with proliferation according to PD-1/CD127 phenotype on $CD8^+$/$Pentamer^+$ cells. The three different PD-1/CD127 subsets are compared between them (small arrows) and also PD1 and CD127 phenotypes are compared independently (big arrows). Man-Whitney U test. (C) Box-plots showing the PD-1 and CD127 FITC-MFI on pentamer-binding cells according to proliferation ability in patients with PI./Mann–Whitney U test. O: outlier. Inter: intermediate. NS: non significant. (D) Representative FACS dot-plots and fluorescence histograms of peripheral pentamer-binding cells stained with PD-1-FITC and CD127-FITC mAbs from two PI patients with different expansion ability after specific in vitro challenge. Upper left figures in each dot-plot represent the percentage of positive $pentamer^+$ cells out of total $CD8^+$ cells. () Low, (+) intermediate and (++) high fluorescence.

A

ID	Genotype	Epitope	Sequence	PD-1 MFI	CD127 MFI	Exp
144	1a	$NS3_{1073\text{-}1081}$	Wild type	38	4	Neg
270	1b	$NS3_{1073\text{-}1081}$	Wild type	31	7	Neg
42	1a	$NS3_{1406\text{-}1415}$	Wild type	39	ND	Neg
184	1b	$NS3_{1073\text{-}1081}$	Wild type	31	8	Pos
292	1b	$NS3_{1073\text{-}1081}$	Wild type	ND	7	Pos
270	1b	$NS3_{1406\text{-}1415}$	L1410M, I1412V	19	12	Pos
300	1a	$NS3_{1406\text{-}1415}$	L1410M, I1412V	26	27	Pos
140	1b	$NS3_{1406\text{-}1415}$	A1409G, I1412V	12	28	Pos

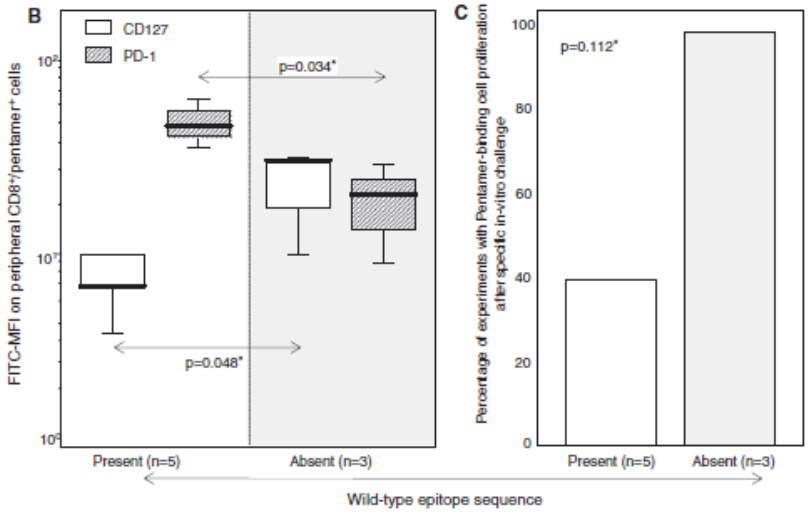

Fig. 5. PD-1/CD127 phenotype on pentamer-binding cells according to epitope sequence. (A) Table showing the PD-1 and CD127 FITC-MFI, the HCV-epitope sequence and the proliferation ability after antigen encounter in eight PI patients with directly ex vivo detectable pentamer$^+$ cells. Exp: expansion. (B) Box-plots displaying the PD-1 and CD127 FITC-MFI on peripheral pentamer-binding cells according to the presence or absence of wild-type HCV-epitope. *Mann–Whitney U test. (C) Bar-plot showing the percentage of experiments with proliferation of pentamer-binding CD8$^+$ cells after specific in vitro challenge according to the HCV-epitope sequence. *Mann–Whitney U test.

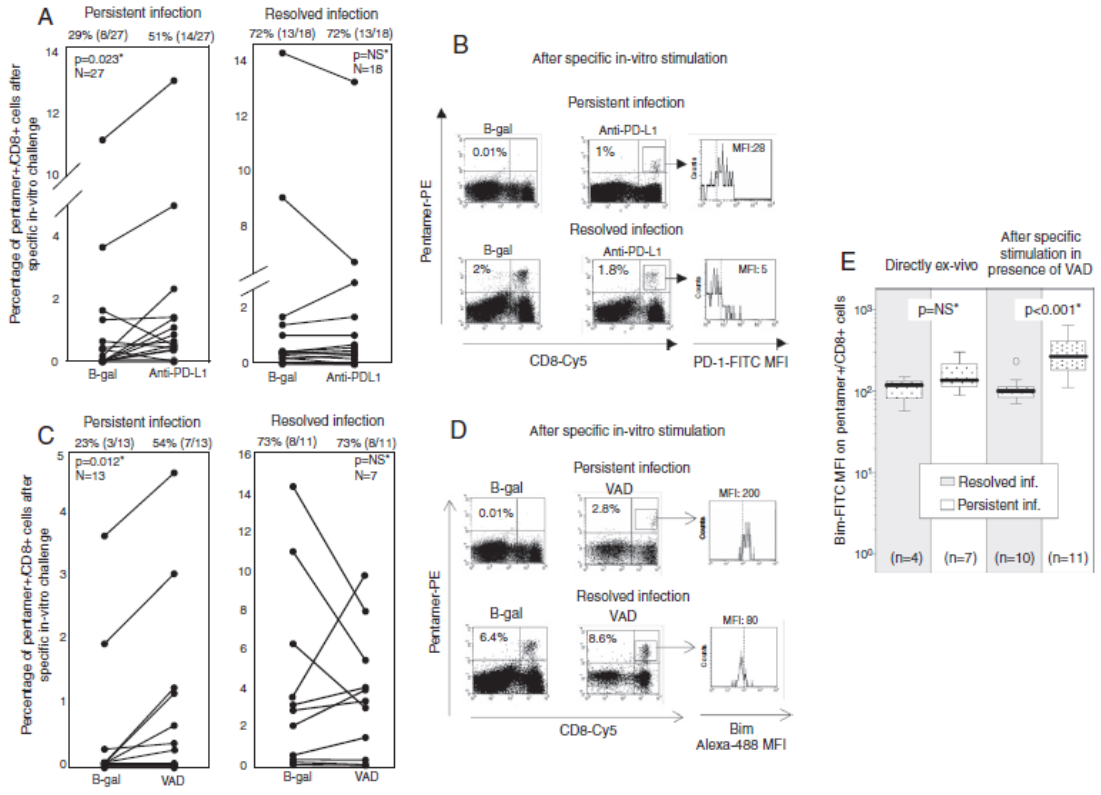

Fig. 6. Pentamer-binding cell proliferation ability restoration after blocking apoptosis and PD-1/PD-L1 pathway. (A) Plots showing the frequency of $CD8^+$/$Pentamer^+$ cells after specific in vitro challenge in presence of anti-PD-L1 mAb or b-galactosidase (b-gal) as control in PI and RI patients. ⁄Wilcoxon test. NS: non significant. (B) Representative FACS dot-plots of PBMC stained with PE-pentamer and CD8-Cy after in vitro stimulation in presence of b-gal or anti-PD-L1 mAb, and PD-1 FITC fluorescence intensity histograms of gated pentamer-binding cells after expansion from one RI and one PI case. Dash-line represents the upper limit of FITC staining for the isotype control. (C). Plots showing the frequency of $CD8^+$/$Pentamer^+$ cells after specific in vitro challenge in presence of z-VAD-fmk or b-gal as control in PI and RI patients. ⁄Wilcoxon test. (D) Representative FACS dot-plots of PBMC stained with PE-pentamer and CD8-Cy after in vitro stimulation in presence of b-gal or z-VAD-fmk, and Bim Alexa-488 fluorescence intensity histograms of gated pentamer-binding cells after expansion from one RI and one PI case. Dash-line represents the upper limit of Alexa-488 staining for the isotype control. (E) Box-plots showing the Bim Alexa-488 MFI in pentamer-binding cells directly ex vivo and after specific in vitro expansion in presence of z-VAD-fmk in RI and PI patients. ⁄Mann–Whitney U test.

Table 4
Summary of the phenotypic and functional features of HCV-specific CD8+ cells according to HCV control.

	PD-1 *ex vivo*	CD127 *ex vivo*	Epitope mutation	Expansion†	Expansion (αPD-L1)§	Expansion (zVAD-fmk)¤
Persistent infection						
PBMC*	(++)	(−)	Absent	Impaired	Increase	Increase
	(+)	(+)	Present	Non-impaired		
IHMC	(++)	(−)				
Resolved infection						
PBMC	(−)	(++)		Non-imparied	No change	No change

In persistent infection two different peripheral HCV-specific CD8 + populations according to PD-1/CD127 phenotype were shown. (-) Low expression; (+) Intermediate expression; (++) High expression. Expansion: proliferation after specific in vitro challenge without any treatment, §in presence of anti-PD-L1 mAb, and after treatment with ¤
z-VAD-fmk. Ag: antigen. PBMC: peripheral blood mononuclear cells. IHMC: intrahepatic mononuclear cells.

Discussion

In persistent HCV infection, specific cytotoxic response is weak and unable to clear the virus [2]. Sustained immunological viral pressure in chronic non-cytopathic viral infection could induce an anergic phase and a subsequent deletion on specific CTL [15,16]. In this process, the expression of the negative co-stimulatory molecule PD-1 [17] and down-regulation of the anti-apoptotic IL-7 receptor [18] could take part. In this study the effect of these two molecules on HCV-specific CTL reactivity according to viral control was analysed (See Table 4).

In the majority of PI patients studied in this work, intrahepatic HCV-specific $CD8^+$ cells were demonstrated and circulating cells were also detectable in a few patients. These data suggested that there did not occur a specific CTL deletion in most chronic patients despite of the intrahepatic apoptotic process previously described [10], but that these cells were sequestered in the liver and below the detection threshold in the peripheral compartment. These cells were characterised by a PD-1/CD127 phenotype associated with anergy and apoptosis [9,10], which could conduct them to die in the liver during chronic infection. Therefore, there probably is a continuous recruitment of newly generated HCV-specific $CD8^+$ cells to maintain the intrahepatic population of specific CTL as it has recently been described in LCMV infection [19]. As a result, the fact that these cells in chronic infection are not completely deleted but dysfunctional is important information, because it could encourage us to search for mechanisms to restore their function in order to clear the HCV infection.

To asses the role of PD-1 and CD127 molecules on HCV-specific CTL reactivity, we analysed deeper the effect of these molecules on peripheral T cell expansion ability. Interestingly, in chronic infection PD-1/CD127 phenotype on

circulating pentamer$^+$ cells correlated with level of viraemia. HCV-specific CTL submitted to high viral load displayed an anergic PD-1$^+$/CD127$^-$ phenotype, probably due to persistent ineffective CTL triggering as it has been shown in other viral infections [16]. The higher the viral load in chronic patients, the more intense the PD-1$^+$/CD127 phenotype is on HCV-specific CTL. The correlation between PD-1 expression and HCV viral load has been described previously [20], but this is the first report showing a negative correlation between CD127 expression and HCV viraemia. Moreover, a gradient for PD-1/CD127 expression between peripheral and intrahepatic compartment in PI was also observed. Consequently, the level of persistent HCV antigenemia was able to regulate the expression of these two molecules on HCV-specific CD8$^+$ cells, being this modulation more intense in the HCV replication site where the antigenemia is higher [21]. Clearly this could be a HCV evolutionary mechanism to escape from immune control.

In the complete cohort of patients enrolled in the study, high specific-CTL reactivity in RI after anti-HCV treatment was observed, suggesting that these cells could be important to obtain a SVR. Whether it is necessary to restore the HCV-specific CTL response to obtain a SVR after treatment is not known yet but our cross-sectional data, and some previous work suggest that this could be required [22]. On the other hand, in chronic infection an impaired reactivity of HCV-specific CD8$^+$ cells after antigen encounter was demonstrated, and this was related with the PD-1/CD127$^+$ phenotype. Nevertheless, some PI cases kept spontaneous HCV-specific CD8$^+$ cell proliferation after specific in vitro challenge. In order to test whether these cells displayed a different PD-1/CD127 phenotype, in patients with directly ex vivo detectable HCV-specific CD8$^+$ cells, PD-1/CD127 phenotype and expansion ability were correlated. Directly ex vivo reactive HCV-specific CTL during PI displayed an unexpected high CD127 and low PD-1 expression. These cells may be able to escape sensitization to apoptosis after antigen encounter by maximizing rescue signals from IL7 such as the anti-apoptotic protein Mcl-1 [23]. This phenotype may be a reflection of the recently primed status of the detectable HCV-specific CD8$^+$ T cells; such continuous recruitment of newly generated T cells to the ongoing response has been previously described in some viral persistent infections [19]. Nevertheless, our HCV-epitope sequencing data suggest that this fact could also be due to absence of immunological pressure, produced by viral variation on the targeted epitope, in agreement with recent previous works [12,24,25]. In this last case, PD-1/CD127 phenotype in PI could be a tool to discover CTL escape mutations. Interestingly, PD-1 expression was slightly increased in CD127$^+$ PI patients with respect to the level that should be expected due to the absence of viral pressure. This detail could be due to a non-TCR dependent PD1 up-regulation induced by HCV infection, as it has been

previously suggested by the effect of HCV-core protein [26]. These reactive cells from PI patients, non-targeting the virus, maintain a higher CD127 expression after expansion than cells from RI patients, probably suggesting that $CD127^+$ cells from RI and PI patients belong to different T cell subsets with distinct degree of differentiation. The status of $CD8^+$ T cells is influenced by the history of antigen stimulation [27,28], therefore in the case of HCV escape mutations the long absence of antigen stimulation could provoke a naïve-early phenotype in peripheral HCV-specific CTL while in RI, these cells would present and early-intermediate phenotype ready to transform into effector-memory cells after antigen encounter. On the other hand, non-reactive HCV-specific CTL targeting the virus expressed a directly ex vivo $CD127^-/PD\text{-}1^+$ phenotype, as was expected. Therefore, therapeutic strategies focused on restoring HCV-specific CTL response should be directed against non-reactive $CD127^-/PD\text{-}1^+$ cells, which are the ones able to recognise the current infecting virus and potentially able to control the infection.

In the second part of our study, we tried to modify HCV-specific CTL reactivity through manipulation of PD-1 and CD127 pathways. To attempt to restore in vitro HCV-specific CTL reactivity in chronic patients, PD-1/PD-L1 pathway and IL-7 deprivation induced apoptosis were blocked. PD-1/PD-L1 interaction conducts to anergy by blocking the TCR signalling stream [6]. In fact, blocking this interaction with anti-PD-L1 mAb treatment resulted in a significant increase of PI cases with HCV-specific $CD8^+$ cell expansion after antigen encounter as it has been previously observed by others [20,29]. Interestingly, not all dysfunctional T cells were rescued by blockade of the PD-1/PD-L1 pathway, suggesting that it is probably necessary to modulate other additional mechanisms in order to restore CTL reactivity, some of which have already been described [24,30,31]. Interestingly, a previous report shows that CD127 expression correlates inversely with the expression of several negative co-stimulatory molecules such as PD-1, suggesting that anergy and apoptosis are processes closely related [24] and, indicating that probably it is necessary to act in both pathways to obtain the HCV-specific CTL response restoration. Therefore, blocking intrinsic apoptosis pathway could be one of the necessary mechanisms to improve HCV-CTL reactivity as it has been shown in other persistent hepatotropic viral infection [23]. In chronic HBV infection, $CD127^-$ specific-CTL are prone to apoptosis due to the down-regulation of the antiapoptotic molecule Mcl-1 and the up-regulation of the apoptosis facilitator Bim, secondary to IL-7 deprivation [7,23]. In our study, by first time to our knowledge, HCV-specific CTL reactivity was significantly restored in chronic HCV infection through an anti-apototic in vitro treatment, using the irreversible pancaspase inhibitor zVAD-fmk. Interestingly, these cells displayed a high Bim

expression, suggesting a similar apoptotic mechanism to the one described in chronic HBV infection [23]. Cytokine withdrawal on T cells results in activation of the mitochondrial apoptosis pathway, regulated by Bcl-2 protein family members. Bim pro-apoptotic activity is counteracted by the binding to Mcl-1[32], but in the case of Bim up-regulation this association would fail and apoptosis could be induced. As a result, our data could suggest that IL-7 deprived $CD127^-$ HCV-specific CTL up-regulate Bim expression during chronic infection, and this could explain the low T cell reactivity after antigen encounter due to apoptosis induction.

Consequently, strategies directed to block the pro-apoptotic effect of IL-7 deprivation should be designed to increase the effectiveness of CTL response restoration, in addition to blocking other negative co-stimulatory molecules. Short-term use of cyclosporine-A or FK506 could block the induction of the pro-apoptotic molecule Bim on $CD127^-$ cells [33]. This strategy could favour HCVCTL restoration during anti-HCV treatment in combination with the standard of care.

Conclusions

In summary, we postulate that in persistent HCV infection, there are two different populations of peripheral HCV-specific CTL; PD-1+/CD127 subset without expansion ability and prone to apoptosis and other one PD-1+/CD127+, characterised by maintaining proliferation capacity, although not targeting the current infecting virus. When HCV-specific CTL reach the liver, they acquire a PD-1++/CD127- phenotype, which could predispose them to apoptosis and anergy. Therefore, strategies designed to block Bim-mediated apoptotic mechanisms on CD127- cells plus blocking of the PD-1/PD-L1 pathway could restore functionality of HCV-specific CD8+ cells targeting the virus. To restore the functionality of these cells could be important to obtain a SVR, since reactive PD1-/CD127+ HCV-specific CTL are found in most of sustained responders.

Acknowledgments

This work was supported by grants from ‘‘Fiscam’’ (PI-2007/32) and ‘‘Fundación de Investigación Médica Mutua Madrileña’’ (2548/ 2008), Spain. Benito S, Calvino M and Lokhande M were supported by research grants from ‘‘Fiscam’’ (MOV-2007_JI/18), from Instituto de Salud Carlos III (CA07/00157) and, Asociación de Hepatología Translacional (AHT10/01) Spain, respectively.

Role of the funding source: Study’s sponsors did not have any role in the collection, analysis, and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

Disclosure statement: The authors who have taken part in this study declared that they do not have anything to disclose regarding funding from industries or conflict of interest with respect to this manuscript.

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