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Polyfunctional HCV-specific T-cell responses are associated with effective control of HCV replication

THESE

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Polyfunctional HCV-specific T cell responses are associated with effective control of HCV replication

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Rapport de synthèse

L'infection par le virus de l'hépatite C (VHC) a une évolution sévère chez les patients co-infectés par VIH/VHC, de même que chez les patients transplantés hépatiques. Toutefois, les mécanismes impliqués dans cette évolution restent peu clairs.

Dans ce travail, nous étudions le profil fonctionnel des cellules T spécifiques dirigées contre le virus de l'hépatite C chez 86 patients mono-infectés par VHC, 48 patients co-infectés par VIH/VHC et 42 patients ayant bénéficié d'une transplantation hépatique. La production d'IFN-γ et d'IL-2 et la capacité de proliférer des cellules T $CD4^+$ et $CD8^+$ sont évaluées après stimulation par des peptides dérivés du VHC.

Chez les patients mono-infectés par le VHC, les cellules T spécifiques au VHC sont polyfonctionnelles du point de vue de la sécrétion de cytokines, avec trois profils de sécrétion pour les cellules T $CD4^+$: sécrétion uniquement de IL-2, sécrétion de IL-2 et IFN-γ et sécrétion uniquement de IFN- γ, et de deux profils pour les cellules T $CD8^+$: sécrétion de IL-2 et IFN-γ et sécrétion uniquement de IFN- γ. En revanche, les cellules T spécifiques au VHC chez les individus co-infectés par VIH/VHC et chez les patients transplantés hépatiques ont un profil de sécrétions de cytokines marqué par l'absence de cellules $CD4^+$ sécrétant uniquement l'Il-2 et l'absence de cellules $CD8^+$ sécrétant à la fois IL-2 et IFN-γ. De plus, la prolifération de cellules T $CD4^+$ et $CD8^+$ spécifiques au VHC est considérablement réduite chez les patients co-infectés par VIH/VHC, comme chez les transplantés hépatiques.

La présence de cellules T effectrices uniquement (définies par l'absence de cellules T $CD4^+$ sécrétants uniquement de l'IL-2 et l'absence de cellules T $CD8^+$ sécrétant à la fois IL-2 et IFN-γ et altération de la capacité prolifératives) est associée avec une charge virale VHC significativement plus élevée et une fibrose hépatique plus sévère. Par conséquent, les présents résultats suggèrent la participation de mécanismes immunitaires dans l'évolution accélérée de l'hépatite C chez les patients co-infectés par VIH-1 et chez les patients greffés hépatiques.

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Polyfunctional HCV-specific T-cell responses are associated with effective control of HCV replication

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HCV infection has a severe course of disease in HIV/HCV co-infection and in liver transplant recipients. However, the mechanisms involved remain unclear. Here, we evaluated functional profiles of HCV-specific T-cell responses in 86 HCV mono-infected patients, 48 HIV/HCV co-infected patients and 42 liver transplant recipients. IFN-γ and IL-2 production and ability of CD4 and CD8 T cells to proliferate were assessed after stimulation with HCV-derived peptides. We observed that HCV-specific T-cell responses were polyfunctional in HCV mono-infected patients, with presence of proliferating single IL-2-, dual IL-2/IFN-γ and single IFN-γ-producing $CD4^+$ and dual IL-2/IFN-γ and single IFN-γ-producing $CD8^+$ cells. In contrast, HCV-specific T-cell responses had an effector profile in HIV/HCV co-infected individuals and liver transplant recipients with absence of single IL-2-producing HCV-specific $CD4^+$ and dual IL-2/IFN-γ-producing $CD8^+$ T cells. In addition, HCV-specific proliferation of $CD4^+$ and $CD8^+$ T cells was severely impaired in HIV/HCV co-infected patients and liver transplant recipients. Importantly, "only effector" T-cell responses were associated with significantly higher HCV viral load and more severe liver fibrosis scores.

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Therefore, the present results suggest that immune-based mechanisms may contribute to explain the accelerated course of HCV infection in conditions of HIV-1 co-infection and liver transplantation.

Key words: Cytokine production · Human · Proliferation · T-cell responses

Introduction

HCV infection is characterized by virus persistence in the majority of cases and by a long course of disease [1]. Indeed, HCV infection usually goes unrecognized for an undetermined period of time until liver disease becomes noticeable. Time from infection to severe liver disease has been estimated to range between 20 and 30 years [1, 2], suggesting that a partial control of viral replication occurs in individuals with established chronic HCV infection. However, HCV-associated disease is highly accelerated and severe liver damage is evident as soon as 5–10 years after infection in HIV-1/HCV co-infection [3, 4]. Similarly, liver transplant recipients in whom re-infection by HCV after transplantation invariably occurs, undergo a fast course of HCV-associated disease, with a mean time to cirrhosis similar to that of HIV/HCV co-infected patients [5–7]. It has been observed that both in conditions of HIV-1 co-infection and liver transplantation, HCV viral load was significantly higher (at least ten fold) than in HCV-infected non-transplanted individuals [8–10]. The immune suppression induced by HIV-1 infection most probably plays a role in the increased HCV viral load and faster progression of disease. In this regard, a negative correlation between CD4 counts and HCV RNA levels [8, 9, 11] has been reported. In liver transplant recipients, increased HCV RNA levels are most probably due to the immunosuppressive regimen [12]. Indeed, it has been shown that HCV RNA levels are related to the levels of immunosuppression and to the severity of liver histopathology [6]. Taken together these observations may indicate that HCV-specific immune responses are less effective in the control of virus replication in both HIV-1/HCV co-infection and liver transplantation. It is, therefore, particularly important to understand whether there are differences in HCV-specific T-cell responses in HCV mono-infected patients compared with HIV/HCV co-infected patients and liver transplant recipients.

Recent studies investigating the presence of HCV-specific T-cell responses in HIV-1 co-infected individuals have shown that their occurrence was not reduced [13–15] but the breadth of the response was limited and they were mostly composed of $CD8^+$ T cells. Similarly, studies assessing the presence of HCV-specific T-cell responses in liver transplant recipients have shown that these were indeed detectable despite immunosuppression [16–20]. However, none of the studies in HIV/HCV co-infected patients and in liver transplant recipients have simultaneously investigated the presence of HCV-specific $CD4^+$ and $CD8^+$ T-cell responses throughout the complete HCV genome. More importantly, there is limited information on the functional profile of HCV-specific T-cell responses in the setting of HIV/HCV co-infection and liver transplantation.

A series of studies have recently investigated the immune correlates of protective T-cell responses in various models of human virus infections [21]. These studies have shown that IFN-γ and IL-2 production and proliferation capacity of $CD4^+$ and $CD8^+$ T cells are key functions to define different levels of protection. In particular, it has been shown that immune responses associated with effective virus clearance or control (low to undetectable viral load) were predominantly composed of polyfunctional $CD4^+$ and $CD8^+$ T cells. Polyfunctionality has been defined by the ability of both $CD4^+$ and $CD8^+$ T cells to produce cytokines such as IL-2 and IFN-γ and to proliferate [22–25]. Other cytokines such as MIP-1β and TNF-α and functions such as degranulation activity and perforin expression are instrumental to better characterize the functional profile of T cells but do not discriminate between polyfunctional and "only effector" T-cell responses. Therefore, polyfunctional $CD4^+$ T-cell responses are defined as being composed of single IL-2-producing, dual IL-2/IFN-γ-producing and single IFN-γ-producing T cells, and $CD8^+$ T-cell responses are composed of dual IL-2/IFN-γ-producing and single IFN-γ-producing T cells. Additionally, both $CD4^+$ and $CD8^+$ T cells are able to proliferate. In contrast, "only effector" T-cell responses are characterized by the absence of single IL-2-producing $CD4^+$ and of dual IL-2/IFN-γ-producing $CD8^+$ T cells and by the lack of proliferation capacity.

In the present study, we have identified and characterized both HCV-specific $CD4^+$ and $CD8^+$ T-cell responses in blood mononuclear cells from HCV mono-infected patients, HIV/HCV co-infected patients and liver transplant recipients using overlapping peptides covering the whole HCV genome. We have then characterized these responses in terms of capacity of $CD4^+$ and $CD8^+$ T cells to produce IFN-γ and IL-2 and their ability to proliferate. We have also analyzed the relationship between the functional T-cell profile and viral load as well as the severity of liver damage.

Results

Identification of HCV-specific $CD4^+$ and $CD8^+$ T-cell responses

In order to characterize and compare the functional profile of HCV-specific T-cell responses in HCV mono-infected patients

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versus HIV/HCV co-infected patients and liver transplant recipients, individuals with detectable HCV-specific $CD4^+$ or $CD8^+$ T-cell responses were identified from a cohort of 176 chronically HCV-infected patients composed of 86 HCV mono-infected patients, 48 HIV/HCV co-infected patients and 42 liver transplant recipients. The patients studied had not been previously treated for HCV infection. HCV-specific T-cell responses were measured using pools of HCV-derived peptides encompassing the whole HCV genome in IFN-γ ELISpot assays as previously described [14]. HCV-specific T-cell responses were detected among 26 HCV mono-infected patients (30%), 16 HIV/HCV co-infected patients (33%) and 14 liver transplant recipients (33%) thus indicating that the overall frequency of HCV-specific T-cell responses was not different between the three study groups. In order to strengthen the present analyses, patients from our initial study that investigated HCV-specific T-cell responses in HCV mono- *versus* HIV/HCV co-infected patients [14] were also included. The patients from that previous study were HCV mono-infected patients M*-1, 2, 12, 23, 31, 36, 46, 47, 49, 50, 55, 59, 60 and 63 (14 patients) and HIV/HCV co-infected patient C*-9 (Tables 1 and 2). Each HCV-specific T-cell response was confirmed using the individual HCV-derived corresponding peptides in a second ELISpot assay (Tables 1 and 2). Characterization of the type of T-cell populations (CD4 *versus* CD8) mediating HCV-specific responses was performed by measuring IFN-γ production in ELISpot assays with CD4- or CD8-depleted populations in patients for whom sufficient blood mononuclear cells were available (34 HCV mono-infected patients, 17 HIV/HCV co-infected patients and 14 liver transplant recipients). The proportion of HCV-specific $CD4^+$ and $CD8^+$ T-cell responses was balanced in HCV mono-infected individuals. Indeed, 17 $CD4^+$ and 17 $CD8^+$ HCV-specific T-cell responses were detected in 34 patients tested. A tendency to a larger proportion of HCV-specific $CD8^+$ T-cell responses was found in HIV/HCV co-infected patients (10 out of 17), as previously observed [14, 15], while a larger proportion of $CD4^+$ T-cell responses was detected in liver transplant recipients (10 out of 14, Tables 1 and 2).

Reduction in IL-2-producing HCV-specific T cells in patients with HIV-1 or liver transplantation

It has been shown that polyfunctional T-cell responses were associated with more effective control of virus replication and lower viral load in chronic virus infections such as CMV, EBV, HCV, HIV-1 and HSV infections [22, 25] whereas "only effector" T-cell responses were associated with higher viral load. As mono/polyfunctionality of T-cell responses are predominantly defined by the ability of CD4 and CD8 T cells to produce IL-2 and to proliferate, we analyzed these parameters in chronic HCV mono-infection, HIV/HCV co-infection and in liver transplant recipients. In order to analyze the profile of cytokine production by HCV-specific T cells, blood mononuclear cells were stimulated with the cognate HCV-derived peptides and IFN-γ and IL-2 production was assessed by intracellular cytokine staining.

Substantial differences were observed in HCV-specific T-cell responses between the HCV mono-infected patients and the HIV/HCV co-infected patients and liver transplant recipients. In HCV mono-infected patients, HCV-specific $CD4^+$ T-cell responses had a typical polyfunctional profile and were composed of single IL-2, dual IL-2/IFN-γ and single IFN-γ-producing cells (Fig. 1A). On the contrary, single IL-2-producing HCV-specific $CD4^+$ T cells were generally not detected in both HIV/HCV co-infected patients and liver transplant recipients in whom HCV-specific $CD4^+$ T cells were predominantly dual IL-2/IFN-γ and single IFN-γ-producing cells (Fig. 1A). The analysis of cumulative data showed that the reduction in single IL-2-producing HCV-specific $CD4^+$ T cells in HIV/HCV co-infected patients and transplant recipients was statistically significant (p=0.0002, Fig. 1B and Table 1). In addition, there was an increase in single IFN-γ-producing cells (p=0.04) in HIV/HCV co-infected patients and liver transplant recipients as compared with patients with HCV mono-infection. The proportion of dual IL-2/IFN-γ-producing cells was similar between the two groups (p=0.09).

A comparable polyfunctional profile was observed in HCV-specific $CD8^+$ T-cell responses in patients with HCV mono-infection (Fig. 1C) with the presence of dual IL-2/IFN-γ and single IFN-γ-producing cells. However, the proportion of dual IL-2/IFN-γ-producing cells was significantly reduced in both HIV/HCV co-infected patients and liver transplant recipients in whom HCV-specific $CD8^+$ T cells were composed almost exclusively of IFN-γ-producing cells (Fig. 1C). The analysis of cumulative data showed that the percentage of dual IL-2/IFN-γ-producing HCV-specific $CD8^+$ T cells was significantly reduced in HIV/HCV co-infected patients and liver transplant recipients as compared with HCV mono-infected individuals (p=0.001, Fig. 1D and Table 2). The differences shown in Fig. 1D for IL-2/IFN-γ-producing cells were not due to the single outlier HCV-infected patient with a very high percentage of IL-2/IFN-γ-producing cells since they were still highly significant even excluding the outlier (p<0.001). No significant differences (p=0.14) were found in the proportion of single IFN-γ-producing cells between HCV mono-infected patients and the HIV/HCV co-infected patients and liver transplant recipients group (Fig. 1D).

In order to rule out the possibility that the reduction in single IL-2-producing $CD4^+$ and IL-2/IFN-γ-producing $CD8^+$ HCV-specific T cells in HIV/HCV co-infected patients and liver transplant recipients was due to defective T-cell activation, we analyzed the up-regulation of the early activation marker CD69 on HCV-specific $CD4^+$ and $CD8^+$ T cells after stimulation with the cognate HCV-derived peptide in five HCV mono-infected patients, five HIV/HCV co-infected patients and five liver transplant recipients. All cytokine-producing $CD4^+$ or $CD8^+$ T cells co-expressed the CD69 marker in HCV mono-infected patients, HIV/HCV co-infected patients or liver transplant recipients (not shown).

Impaired proliferation of HCV-specific T cells in patients with HIV-1 or liver transplantation

We next assessed the proliferation capacity of HCV-specific $CD4^+$ and $CD8^+$ T cells in HCV mono-infected patients, HIV/HCV

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Table 1. Profiles of IL-2, IL-2/IFN-γ and IFN-γ production and proliferation of HCV-specific CD4$^+$ T cells in HCV mono-infected patients, HIV/HCV co-infected patients and liver transplant recipients

Patient code[a)]	infection status	HCV peptide	amino acid sequence	% cytokine-producing cells[b)]			SI[c)]
				IL-2	IL-2/IFN-γ	IFN-γ	
CD4 T cell responses							
M*-2	HCV	E2 688-702	GLIHLHQNIVDVQYL	0.01	0.03	0.03	9.8
M*-12	HCV	NS3 1511-1525	MFDSSVLCECYDAGC	0.12	0.08	0.06	na
M*-23	HCV	NS3 1511-1525	MFDSSVLCECYDAGC	0.01	0.03	0.03	na
M*-31	HCV	NS3 1583-1597	FPYLVAYQATVCARA	0.06	0.04	0.02	na
M*-36	HCV	NS4A 1658-1672	STWVLVGGVLAALAA	0.04	0.07	0.04	na
M*-46	HCV	C 133-147	LMGYIPLVGAPLGGA	0.03	0.02	0.02	6.2
M*-63	HCV	C 145-159	GGAARALAHGVRVLE	0.06	0.12	0.08	na
M-78	HCV	NS3 1511-1525	MFDSSVLCECYDAGC	0.01	0.02	0.04	4.4
M-87	HCV	C 133-147	LMGYIPLVGAPLGGA	0.18	0.12	0.06	3.8
M-113	HCV	E2 329-343	WAIKWEYVVLLFLLL	0.08	0.07	0.06	5.2
M-119	HCV	C 133-147	LMGYIPLVGAPLGGA	0.02	0.07	0.03	10.3
M-120	HCV	E2 720-736	VLLFLLLADARVCSC	0.05	0.02	0.01	na
M-124	HCV	NS3 1219-1233	VPQSFQVAHLHAPTG	0.09	0.10	0.05	7.1
M-128	HCV	NS4A 1658-1672	STWVLVGGVLAALAA	0.02	0.01	0.06	na
M-151	HCV	C 153-167	HGVRVLEDGVNYATG	0.23	0.11	0.11	7.5
M-158	HCV	NS3 1207-1221	SPVFTDNSSPPAVPQ	0.03	0.01	0.02	5.3
M-178	HCV	NS5A 2129-2143	RLHRFAPPCKPLLRE	0.11	0.05	0.02	5.3
			mean	**0.07**	**0.06**	**0.04**	**6.5**
C-4	HIV/HCV	NS3 1511-1525	MFDSSVLCECYDAGC	0	0.19	0.51	na
C-10	HIV/HCV	NS4A 1658-1672	STWVLVGGVLAALAA	0.01	0.05	0.17	1.2
C-12	HIV/HCV	NS3 1511-1525	MFDSSVLCECYDAGC	0	0	0.03	1.1
C-47	HIV/HCV	C 113-127	RRRSRNLGKVIDTLT	0.01	0.02	0.02	4.0
C-48	HIV/HCV	NS3 1511-1525	MFDSSVLCECYDAGC	0.01	0.21	0.15	2.6
C-56	HIV/HCV	NS3 1623-1637	PLLYRLGAVQNEVTL	0.05	0.57	0.28	3.1
C-117	HIV/HCV	C 33-47	GVYLLPRRGPRLGVR	0	0.02	0.02	1.5
T-3	Liver transplant	NS3 1511-1525	MFDSSVLCECYDAGC	0	0.25	0.29	1.0
T-6	Liver transplant	NS4A 1658-1672	STWVLVGGVLAALAA	0	0.02	0.05	1.1
T-9	Liver transplant	C 85-99	LYGNEGCGWAGWLLS	0.02	0.01	0.02	1.1
T-23	Liver transplant	E2 628-642	KVRMYVGGVEHRLEA	0	0.03	0.02	2.3
T-24	Liver transplant	NS4A 1658-1672	STWVLVGGVLAALAA	0	0.06	0.12	1.1
T-25	Liver transplant	NW3 1511-1525	MFDSSVLCECYDAGC	0	0	0.19	1.3
T-28	Liver transplant	C 125-139	TLTCGFADLMGYIPL	0.01	0.01	0.02	0.8
T-34	Liver transplant	NS3 1511-1525	MFDSSVLCECYDAGC	0	0.64	1.6	0.8
T-37	Liver transplant	NS3 1511-1525	MFDSSVLCECYDAGC	0	0.02	0.03	1.3
T-41	Liver transplant	NS3 1511-1525	MFDSSVLCECYDAGC	0	0.02	0.05	na
			mean	**0.01**	**0.12**	**0.21**	**1.6**
			P	**0.0002**	**0.09**	**0.04**	**<0.0001**

[a)] M: HCV mono-infected patient, C: HIV/HCV co-infected patient, T: liver transplant recipient. An asterisk denotes patients from our initial study [14].

[b)] Percentage of cytokine-producing cells among CD4$^+$ T cells. Results are shown with background in absence of peptide substracted.

[c)] SI: stimulation index. SI were calculated by dividing the percentage of CFSE^{low} cells in presence of peptide by that in absence of peptide; na: not available due to high spontaneous proliferation in absence of peptide; nd: not done due to insufficient material.

co-infected patients and liver transplant recipients after antigen-specific stimulation in a 5-day CFSE assay. Recent studies have indeed shown that the proliferation capacity of virus-specific CD4 and CD8 T cells is dependent upon the presence of IL-2-producing cells and that the presence of virus-specific proliferating cells is associated with control of virus replication [22, 24, 25]. Both CD4$^+$ (Fig. 2A) and CD8$^+$ (Fig. 2B) T cells were able to proliferate following stimulation with the HCV-derived cognate peptides in patients with HCV mono-infection. However, the ability to proliferate of both HCV-specific CD4$^+$ and CD8$^+$ T cells after stimulation with HCV-derived peptides was however severely impaired in subjects with HIV/HCV co-infection or in

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Table 2. Profiles of IL-2/IFN-γ and IFN-γ production and proliferation of HCV-specific $CD8^+$ T cells in HCV mono-infected patients, HIV/HCV co-infected patients and liver transplant recipients

Patient code[a)]	infection status	HCV peptide	amino acid sequence	% cytokine-producing cells[b)]		SI[c)]
				IL-2/IFN-γ	IFN-γ	
CD8 T cell responses						
M*-1	HCV	NS5B 2893-2907	SLHSYSPGEINRVAA	0.06	0.02	12.2
M-5	HCV	p7 771-785	FFCFAWYLKGRWVPG	0.07	0.15	11.1
M-8	HCV	NS5A 2121-2135	FFTELDGVRLHRFAP	0.03	0.27	4.2
M-34	HCV	E2 464-478	DFAQGWGPISYANGS	0.06	0.15	6.4
M*-47	HCV	E2 464-478	DFAQGWGPISYANGS	0.05	0.16	nd
M-48	HCV	NS5A 2121-2135	FFTELDGVRLHRFAP	0.03	0.18	na
M*-49	HCV	NS5B 2893-2907	SLHSYSPGEINRVAA	0.06	0.03	7.5
M*-50	HCV	E2 464-478	DFAQGWGPISYANGS	0.02	0.06	7.2
M*-55	HCV	NS3 1355-1359	VTVSHPNIEEVALST	0.1	0.13	nd
M*-59	HCV	E2 692-706	LHQNIVDVQYLYGVG	0.06	0.04	na
M*-60	HCV	NS3 1227-1241	HLHAPTGSGKSTKVP	0.12	0.29	nd
M-64	HCV	E2 464-478	DFAQGWGPISYANGS	0.06	0.06	6.2
M-67	HCV	E2 712-726	WAIKWEYVVLLFLLL	0.01	0.06	9.2
M-107	HCV	E2 692-706	LHQNIVDVQYLYGVG	0.02	0.17	6.3
M-108	HCV	NS5B 2597-2611	DVVSKLPLAVMGSSY	0.07	0.13	14.8
M-121	HCV	NS3 1227-1241	HLHAPTGSGKSTKVP	0.31	0.09	na
M-129	HCV	E2 536-550	VFVLNNTRPPLGNWF	0.01	0.08	4.6
			mean	**0.07**	**0.12**	**8.2**
C-5	HIV/HCV	E2 684-698	ALSTGLIHLHQNIVD	0	0.12	1.1
C-6	HIV/HCV	NS3 1143-1157	RRRGDSRGSLLSPRP	0.01	0.06	na
C-7	HIV/HCV	NS4A 1658-1672	STWVLVGGVLAALAA	0.01	0.09	na
C*-9	HIV/HCV	C 33-47	GVYLLPRRGPRLGVR	0.01	0.03	2.3
C-11	HIV/HCV	C 129-143	GFADLMGYIPLVGAP	0	0.14	1.1
C-17	HIV/HCV	E2 536-550	VFVLNNTRPPLGNWF	0.02	0.17	2.2
C-18	HIV/HCV	NS2 850-864	LTRVEAQLHVWVPPL	0.01	0.11	1.5
C-49	HIV/HCV	NS3 1511-1525	MFDSSVLCECYDAGC	0.02	0.06	3.0
C-83	HIV/HCV	E2 137-151	DRSGAPTYSWGANDT	0	0.05	1.4
C-113	HIV/HCV	NS4A 1662-1676	LVGGVLAALAAYCLS	0	0.11	2.9
T-2	Liver transplant	NS5B 2773-2787	PPQPEYDLELITSCS	0	0.11	1.2
T-30	Liver transplant	NS4B 1712-1726	SQHLPYIEQGMMLAE	0	0.29	1.0
T-31	Liver transplant	NS5A 2137-2151	CKPLLREEVSFRVGL	0	0.38	1.8
T-32	Liver transplant	not determined	not determined	0	0.75	1.3
			mean	**0.01**	**0.18**	**1.7**
			P	**0.001**	**0.14**	**<0.0001**

[a)] M: HCV mono-infected patient, C: HIV/HCV co-infected patient, T: liver transplant recipient. An asterisk denotes patients from our initial study [14].

[b)] Percentage of cytokine-producing cells among $CD8^+$ T cells. Results are shown with background in absence of peptide substracted.

[c)] SI: stimulation index. SI were calculated by dividing the percentage of $CFSE^{low}$ cells in presence of peptide by that in absence of peptide; na: not available due to high spontaneous proliferation in absence of peptide; nd: not done due to insufficient material.

liver transplant recipients (Fig. 2A and B). It is important to underscore that both $CD4^+$ and $CD8^+$ T cells were able to proliferate after stimulation with Staphylococcal enterotoxin B (SEB) in HIV/HCV co-infected individuals and liver transplant recipients (not shown). The analysis of cumulative data showed that the stimulation index (SI) was significantly reduced ($p<0.0001$) for $CD4^+$ and $CD8^+$ T cells in HIV/HCV co-infected patients or liver transplant recipients as compared with HCV mono-infected patients (Fig. 2C and D). However, the impaired proliferation of HCV-specific T cells in HIV/HCV co-infected patients and liver transplant recipients was not the result of an intrinsic defect of T cells as the exogenous addition of IL-2 was able to restore HCV-induced proliferation in both $CD4^+$ and $CD8^+$ T cells. A representative example is shown in Fig. 2E for an HIV/HCV co-infected patient. This result also confirms that the lack of proliferation is linked to the lack of self IL-2 production.

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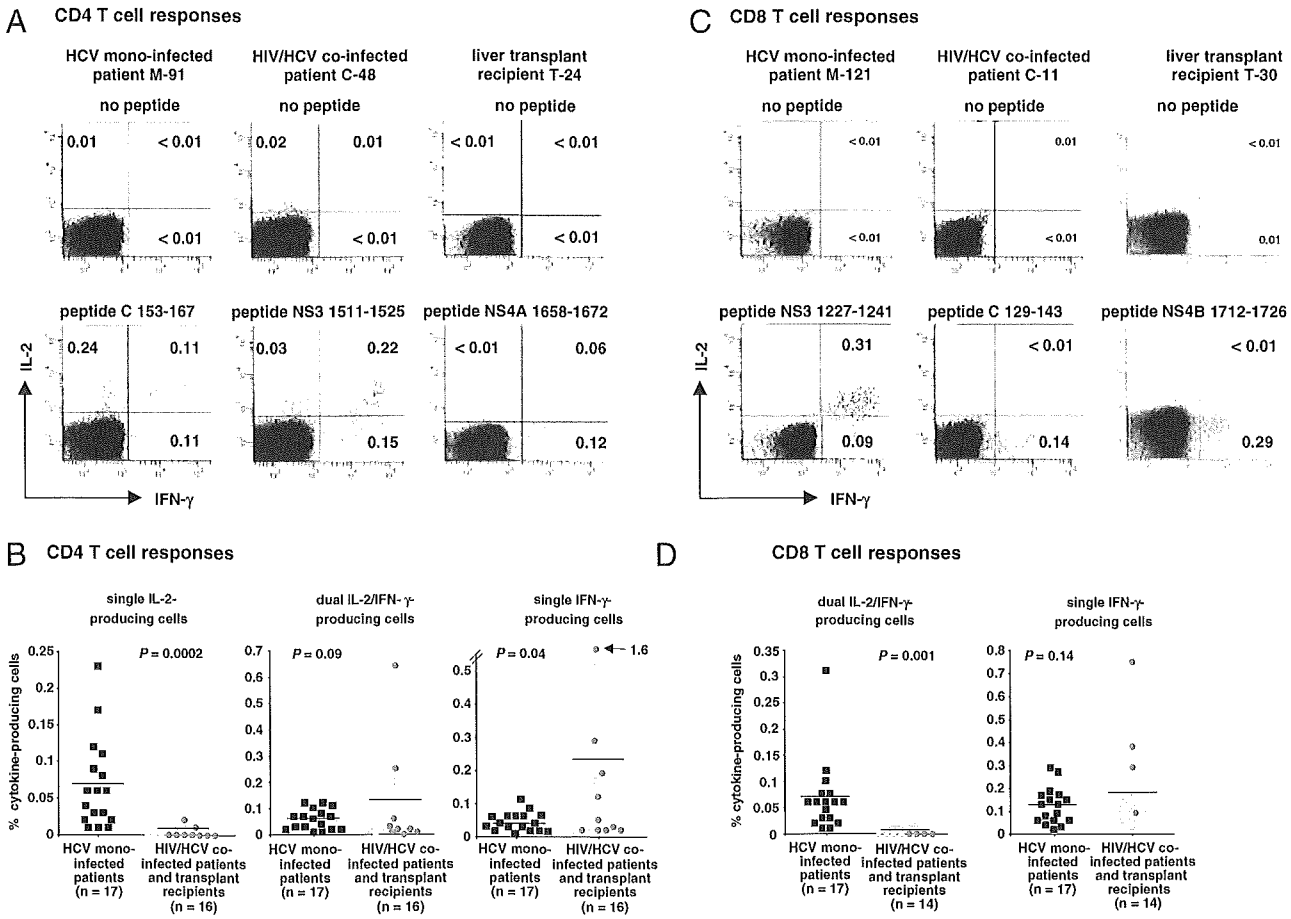

Figure 1. Cytokine-production profiles of HCV-specific T cells in HCV mono-infected patients, HIV/HCV co-infected patients and liver transplant recipients. (A) Profiles of IFN-γ and IL-2 production by HCV-specific $CD4^+$ T cells in HCV mono-infected patients, HIV/HCV co-infected patients and liver transplant recipients after stimulation with the cognate HCV-derived peptide. Spontaneous cytokine production was assessed by incubating cells in absence of peptide. Numbers in quadrants represent the percentage of the corresponding cytokine-producing population among $CD4^+$ T cells. (B) Cumulative data analysis of HCV-specific $CD4^+$ T-cell responses in 17 HCV mono-infected patients, 7 HIV/HCV co-infected patients and 9 liver transplant recipients. Open circles correspond to HIV/HCV co-infected patients and closed circles to liver transplant recipients. (C) Profiles of IFN-γ and IL-2 production by HCV-specific $CD8^+$ T cells in HCV mono-infected patients, HIV/HCV co-infected patients and liver transplant recipients after stimulation with the cognate HCV peptide. (D) Cumulative data analysis of HCV-specific $CD8^+$ T-cell responses in 17 HCV mono-infected patients, 10 HIV/HCV co-infected patients and 4 liver transplant recipients.

Changes in cytokine profiles and proliferation are restricted to HCV-specific T cells

We next investigated whether the reduction in the single IL-2-producing $CD4^+$ and dual IL-2/IFN-γ-producing $CD8^+$ antigen-specific populations as well as their lack of proliferation was a global T-cell dysfunction in HIV/HCV co-infected patients and liver transplant recipients. To this end, we analyzed the profiles of cytokine production and the proliferation capacity in CMV-, EBV- and HSV-specific T cells in these two groups of patients. Virus-specific $CD4^+$ T-cell responses were assessed in HCV mono-infected patients ($n = 19$) and HIV/HCV co-infected patients or liver transplant recipients ($n = 18$) after stimulation with CMV, EBV or HSV lysates. As shown in Fig. 3A in a representative example, single IL-2-producing HSV-specific $CD4^+$ T cells were clearly detectable in HCV mono-infected patients, HIV/HCV co-infected patients and liver transplant recipients. Cumulative data of CMV-, EBV- and HSV-specific $CD4^+$ T-cell responses clearly demonstrated that the proportion of single IL-2-producing cells in response to control antigens was similar in the HCV mono-infected and HIV/HCV co-infected or liver transplant groups (p=0.35, Fig. 3B). These results indicated that the reduction in the single IL-2-producing $CD4^+$ T-cell population was restricted to HCV-specific cells in HIV/HCV co-infected individuals and liver transplant recipients.

Similarly, we investigated whether there were differences in the frequencies of dual IL-2/IFN-γ-producing CMV- and EBV-specific $CD8^+$ T cells in HCV mono-infected patients, HIV/HCV co-infected patients and liver transplant recipients. Cytokine production in response to pools of CMV- and EBV-derived peptides was analyzed in 13 HCV mono-infected patients and 25 HIV/HCV co-infected patients or liver transplant recipients. As shown in Fig. 3C for a representative example, dual

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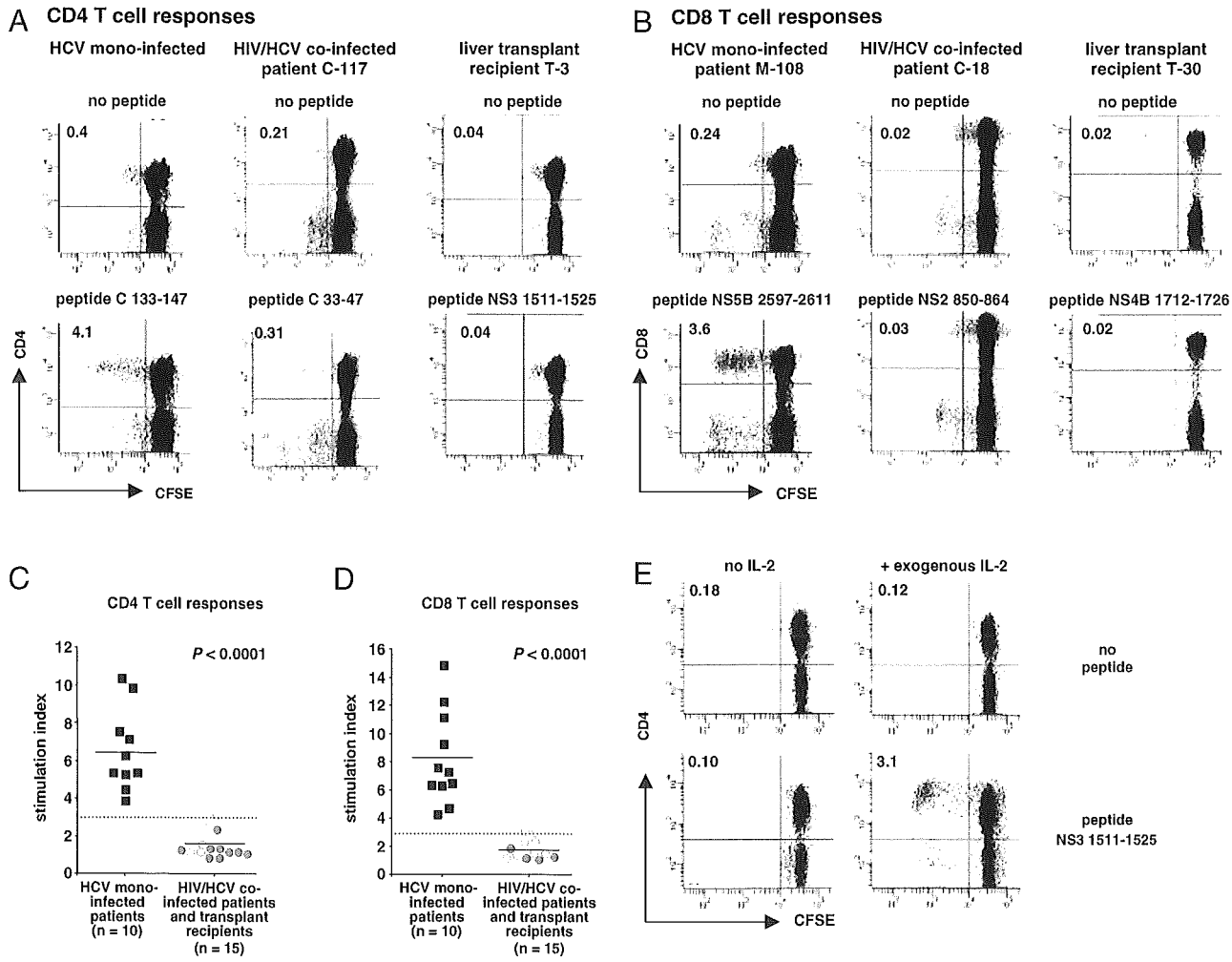

Figure 2. Proliferation of HCV-specific $CD4^+$ and $CD8^+$ T cells. (A) Proliferation of HCV-specific $CD4^+$ T cells after stimulation with HCV-derived peptides in HCV mono-infected patients, HIV/HCV co-infected patients and liver transplant recipients. Spontaneous proliferation was assessed by incubating cells in absence of peptide. Numbers in quadrants represent the percentage of $CFSE^{low}$ cells among $CD4^+$ T cells. (B) Proliferation of HCV-specific $CD8^+$ T cells after stimulation with HCV-derived peptides in HCV mono-infected patients, HIV/HCV co-infected patients and liver transplant recipients. (C) Cumulative data analysis of HCV-specific $CD4^+$ T-cell proliferation in ten HCV mono-infected patients, six HIV/HCV co-infected patients and nine liver transplant recipients. SI was calculated by dividing the number of $CFSE^{low}$ cells in the presence of peptide by that in the absence of peptide. Only SI > 3 were considered to be positive (dotted line). Open circles correspond to HIV/HCV co-infected patients and closed circles to liver transplant recipients. (D) Cumulative data analysis of HCV-specific $CD8^+$ T-cell proliferation in 11 HCV mono-infected patients, 8 HIV/HCV co-infected patients and 4 liver transplant recipients. (E) Proliferation of HCV-specific $CD4^+$ T in the absence or in the presence of exogenously added IL-2 in a representative HIV/HCV co-infected patient.

IL-2/IFN-γ-producing $CD8^+$ T cells were readily detected in HCV mono-infected patients, HIV/HCV co-infected patients and liver transplant recipients after stimulation with a pool of EBV-derived peptides. The analysis of cumulative data showed that there were no significant differences in the proportion of dual IL-2/IFN-γ-producing CMV- and EBV-specific $CD8^+$ T cells between the two patient groups (p=0.29, Fig. 3D). Taken together these results indicated that the defect in the dual IL-2/IFN-γ-producing $CD8^+$ T-cell population was restricted to the HCV-specific $CD8^+$ T-cell population in HIV/HCV co-infected patients and liver transplant recipients.

With regard to proliferative capacity, we observed that, following antigen-specific stimulation, CMV-, EBV- or HSV-specific $CD4^+$ T cells were able to proliferate in both the HCV mono-infected patients ($n = 15$) and HIV/HCV co-infected patients and liver transplant recipients ($n = 21$). A representative example is shown in Fig. 3E for EBV-specific $CD4^+$ T-cell responses. Similarly, $CD8^+$ T cells stimulated with CMV- or EBV-derived peptide pools were able to proliferate in HCV mono-infected patients ($n = 13$) and in HIV/HCV co-infected patients and liver transplant recipients ($n = 24$, Fig. 3F). Cumulative data confirmed that there were no significant differences in the proliferation capacity of both CMV and EBV-specific $CD4^+$ and $CD8^+$ T cells in HCV mono-infected and HIV/HCV co-infected and liver transplant recipient groups (p=0.48 and 0.26, respectively, Fig. 3G).

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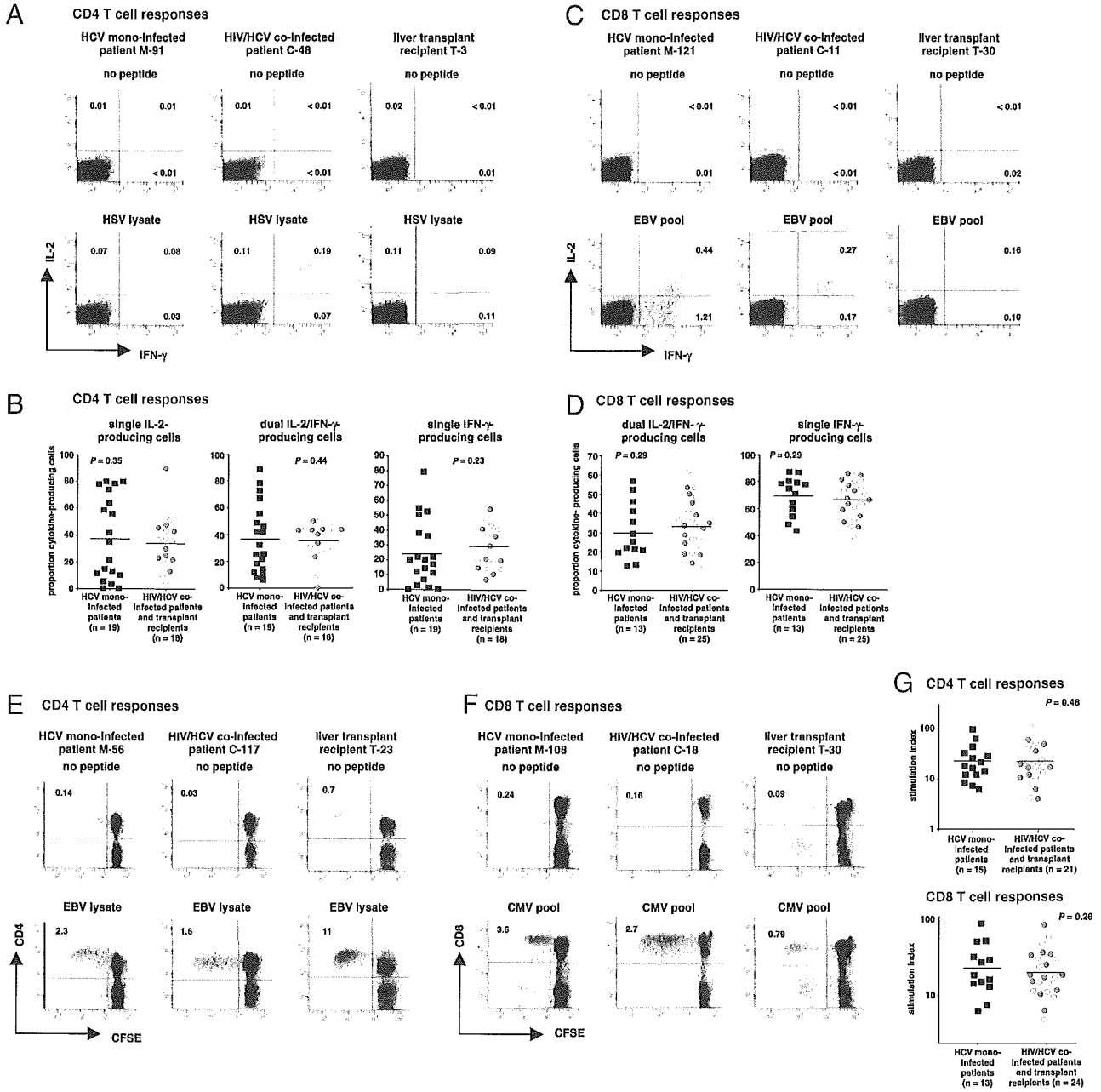

Figure 3. Cytokine production and proliferation of antigen-specific $CD4^+$ and $CD8^+$ T cells in response to control antigens. (A) Profiles of IFN-γ and IL-2 production by $CD4^+$ T cells after stimulation with an HSV lysate. (B) Cumulative data analysis of $CD4^+$ T cells in response to CMV, EBV or HSV lysates in 19 HCV mono-infected patients, 9 HIV/HCV co-infected patients (open circles) and 9 liver transplant recipients (closed circles). (C) Profiles of IFN-γ and IL-2 production by $CD8^+$ T cells after stimulation with a pool of EBV-derived peptides. (D) Cumulative data analysis of $CD8^+$ T cells in response to pools of CMV- or EBV-derived peptides in 13 HCV mono-infected patients, 13 HIV/HCV co-infected patients and 12 liver transplant recipients. (E) Proliferation of $CD4^+$ T cells after stimulation with an EBV lysate. (F) Proliferation of $CD8^+$ T cells after stimulation with a pool of CMV-derived peptides in HCV mono-infected patients, HIV/HCV co-infected patients and liver transplant recipients. (G) Upper panel: cumulative data analysis of $CD4^+$ T-cell proliferation after stimulation with CMV-, EBV- or HSV lysates in 15 HCV mono-infected patients, 11 HIV/HCV co-infected patients (open circles) and 10 liver transplant recipients (closed circles). Lower panel: cumulative data analysis of $CD8^+$ T-cell proliferation after stimulation with pools of CMV- or EBV-derived peptides in 13 HCV mono-infected patients, 12 HIV/HCV co-infected patients and 12 liver transplant recipients.

"Only effector" T cells are associated with higher HCV viral load

Previous studies have shown presence of higher levels of HCV viral load in patients with HIV/HCV co-infection [3, 26, 27] and in patients with liver transplant [9–11]. In addition, it has also been shown that "only effector" and polyfunctional T-cell responses were generally associated with higher and lower levels of viral load in CMV and HIV-1 infection, respectively [22, 25]. Therefore, we investigated whether higher levels of HCV viral

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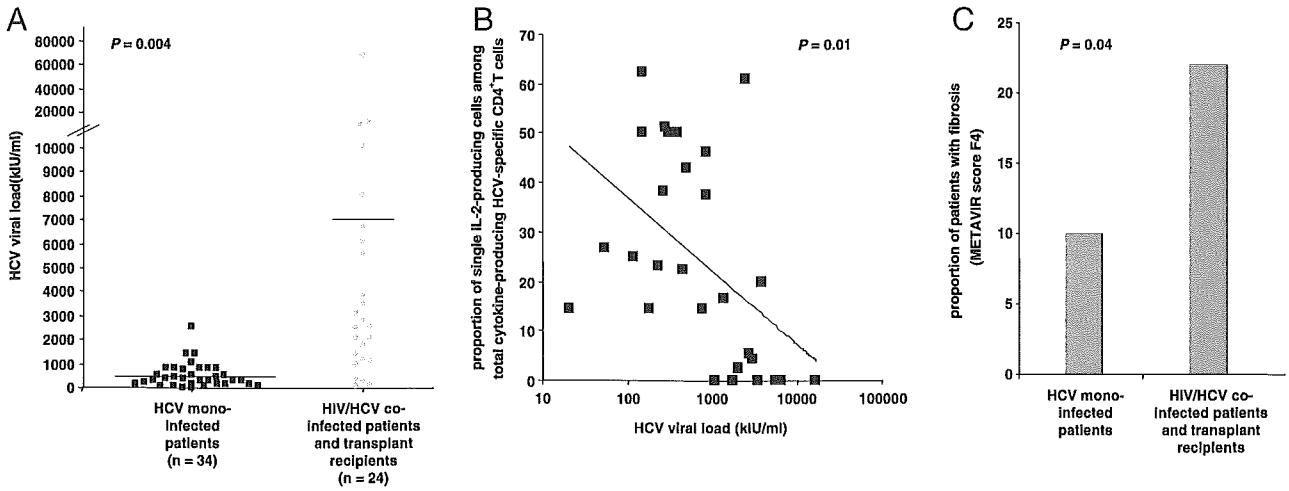

Figure 4. Differences in HCV viral load and liver fibrosis scores in HCV mono-infected patients and HIV/HCV co-infected patients and liver transplant recipients. (A) Distribution of HCV viral load in HCV mono-infected patients ($n = 34$) and HIV/HCV co-infected patients or liver transplant recipients ($n = 24$) is shown. Values are given in kIU/mL. (B) Correlation between the proportion of single IL-2-producing $CD4^+$ T cells among total cytokine-producing HCV-specific $CD4^+$ T cells and HCV viral load. The correlation coefficient is −0.46. (C) Proportion of patients with fibrosis (METAVIR score F4) in the HCV mono-infected and HIV/HCV co-infected and liver transplant recipient groups.

load were present in HIV/HCV co-infected patients and liver transplant recipients compared with HCV mono-infected patients. To this end, we measured HCV viral load in patients with characterized functional profiles of HCV-specific T-cell responses. We indeed observed substantial differences in the plasma viral load in HCV mono-infected patients *versus* HIV/HCV co-infected patients and liver transplant recipients (Fig. 4A). The mean HCV viral load was 485 ± 88 kIU/mL (mean ± SEM) (range: 11–2.512) in the HCV mono-infected patients ($n = 34$) with polyfunctional T-cell responses and 7.015 ± 2.695 kIU/mL (range: 61–69.000) in the HIV/HCV co-infected patients and liver transplant recipients with "only effector" T-cell responses ($n = 24$, Fig. 4A). These differences were statistically significant (p=0.004) and indicated that substantial differences in the functional profile of HCV-specific T cells were associated with different levels of control of virus replication in HCV mono-infected patients *versus* HIV/HCV co-infected patients and liver transplant recipients. Furthermore, a correlation was also found between the functional profile of HCV-specific $CD4^+$ T-cell responses and viral load. Indeed, the proportion of single IL-2-producing $CD4^+$ T cells within the total cytokine-producing HCV-specific $CD4^+$ T-cell population was inversely correlated with viral load (Fig. 4B, $r = -0.46$, p=0.01), supporting a direct link between the functional T-cell profile and control of virus replication.

"Only effector" T cells are associated with more severe liver fibrosis

We further assessed the impact of polyfunctional *versus* "only effector" HCV-specific T-cell responses on the clinical status of patients. Liver biopsies were performed in all HCV mono-infected and HIV/HCV co-infected patients and in 21 out of 42 liver transplant recipients. Among liver transplant recipients with HCV-specific T-cell responses, 8 out of 14 patients underwent liver biopsy. We did not observe differences in the levels of alanine aminotransferase and/or aspartate aminotransferase between the different groups of patients (Table 3). When analyzing levels of liver inflammation (A in METAVIR scores, Table 3), there were again no statistical differences between the HCV mono-infected patients and the HIV/HCV co-infected patients and liver transplant groups. There was, however, a statistically significant increase in the proportion of patients with cirrhosis (score F4) in the HIV/HCV co-infected and liver transplant recipient groups *versus* the HCV mono-infected group (Fig. 4C, p=0.04). Therefore, although the number of patients tested is quite small, these results further support the hypothesis that the "only effector" HCV-specific T-cell response is less efficient and thus is associated with more severe liver disease.

Discussion

In this study, we have characterized the function of HCV-specific T-cell responses under conditions of HCV mono-infection and in the setting in which HCV infection is associated with a variable degree of immunosuppression, namely caused by HIV-1 co-infection or immunosuppressive therapy in liver transplantation. We demonstrate that there are major differences in the HCV-specific T-cell responses between HCV mono-infected patients and HIV/HCV co-infected patients and liver transplant recipients.

In HCV mono-infection, HCV-specific $CD4^+$ and $CD8^+$ T cells had a polyfunctional profile, with $CD4^+$ T cells being composed of proliferating and single IL-2, dual IL-2/IFN-γ and single IFN-γ-producing cells and $CD8^+$ T cells composed of proliferating and dual IL-2/IFN-γ and single IFN-γ-producing cells. In HIV/HCV

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Table 3. Clinical characteristics of HCV mono-infected patients, HIV/HCV co-infected patients and liver transplant recipients displaying HCV-specific T-cell responses

		HCV mono-infected patients	HIV/HCV co-infected patients[a)]	Liver transplant recipients
HCV genotype	1a	7[b)]	5	2
	1b	5	5	7
	2	3	2	0
	3	12	3	3
	4	4	1	0
	uk	3	1	2
CD4 counts (cells/mL)[c)]		1150±84	674±66	na
ALT (U/L)[c)]		90±9	75±8	60±9
AST (U/L)[c)]		55±5	62±6	48±14
Liver inflammation	A0–A1	20[b)]	7	2
	A2–A3	11	8	2
Liver fibrosis	F0–F1	17	7	3
	F2–F3	11	4	4
	F4	3	4	1

[a)] All HIV/HCV co-infected patients with HCV-specific T-cell responses had undetectable HIV viral load except for patient C-113 (viramia: 22 000 copies/μL of serum).
[b)] Expressed as number of patients.
[c)] Mean±SEM. ALT: alanine aminotransferase, AST: aspartate aminotransferase, na: not available, uk: unknown.

co-infected patients and liver transplant recipients, HCV-specific T cells had the "only effector" functional profile with a strong reduction in proliferating and IL-2-producing $CD4^+$ and $CD8^+$ T cells. These results show that HCV-specific T cells in HCV mono-infection have a polyfunctional profile similar to that of virus-specific $CD4^+$ and $CD8^+$ T cells found in normally controlled infections with low or undetectable persistent viral load such as CMV, EBV, HSV, influenza and HIV-1-infected subjects with non-progressive disease [22, 25, 28]. In contrast, HCV-specific T-cell responses in HIV/HCV co-infected individuals and liver transplant recipients have the "only effector" profile and resemble those detected in uncontrolled viral infections such as untreated viremic HIV-1 infection [22, 25]. The reduction in IL-2-producing $CD4^+$ and $CD8^+$ T cells can directly explain the impaired capacity of these cells to proliferate [25]. Therefore, although there were not significant quantitative differences in the frequencies and in the magnitude of HCV-specific T-cell responses in the three groups of patients studied, there were substantial qualitative differences in the functional profile of HCV-specific T-cell responses between HCV mono-infected patients and HIV/HCV co-infected patients or liver transplant recipients.

The "only effector" HCV-specific T-cell responses in HIV/HCV co-infected patients and liver transplant recipients were associated with significantly higher levels of HCV viral load as compared with HCV mono-infected individuals with polyfunctional responses. Therefore, qualitative, and not quantitative, differences of HCV-specific T-cell responses seem to influence the effectiveness of HCV-specific T-cell responses in mediating their antiviral effect and controlling viral replication.

"Only effector" HCV-specific T-cell responses were also associated with a more severe liver disease as the proportion of patients with cirrhosis was higher in the HIV/HCV co-infection and liver transplant group than in the HCV mono-infection group. The HCV genotype has been shown to influence the course of HCV disease and the response to antiviral treatment. It is important to note that there was no significant increase in the proportion of genotypes 1 and 4 in patients with cirrhosis both in HCV mono-infected individuals and in HIV/HCV co-infected individuals and liver transplant recipients. There was also no significant difference in the proportion of favorable genotypes (2 and 3) between HCV mono-infected patients and HIV/HCV co-infected patients or liver transplant recipients (48 *versus.* 31 and 25%, respectively, $P>0.05$ for both comparisons). Therefore, the association between polyfunctional HCV-specific T-cell responses and HCV mono-infection cannot be explained on the basis of a more favorable virus genotype. Considering liver transplant recipients, there were no significant differences in either profiles of IFN-γ and IL-2 production, proliferation index, viral load or histopathological findings when analyzing patients according to immunosuppressive treatment with calcineurin inhibitors (cyclosporin or tacrolimus) *versus* rapamycin. However, one limitation of the histopathological correlation analysis in liver transplant recipients is that not all patients underwent liver biopsies.

The absence of polyfunctional HCV-specific T-cell responses in HIV/HCV co-infection and in liver transplantation may be dependent upon a number of factors. These include a lower $CD4^+$ T-cell counts in co-infected patients [14, 15] and the immunosuppressive therapy in liver transplantation. In this regard, it is worth mentioning that CD4 T-cell counts were significantly lower in HIV/HCV co-infected patients as compared with HCV mono-infected patients (Table 3, $P<0.001$). However, other

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virus-specific (CMV, EBV and HSV) T-cell responses were not affected in HIV/HCV co-infection and liver transplant recipients. Furthermore, the defect was selective for IL-2-producing and proliferating HCV-specific T cells since the frequencies of IFN-γ-producing HCV-specific T cells were comparable in HCV mono-infection, HIV/HCV co-infection and liver transplantation.

The major difference between HCV and control viruses is that HCV continually replicates and it is clearly detectable in the blood whereas CMV, EBV and HSV do not replicate actively and reactivate transiently. CMV and EBV replication can be detected in the blood only in the presence of severe immunosuppression. Therefore, it is not excluded that differences for the other virus-specific T-cell responses can be detected when re-activation of virus replication occurs in the presence of severe immunosuppression.

We have previously shown that the levels of viral load influence the functional profile of T-cell responses in HIV-1 and CMV infections under conditions of uncontrolled virus replication. In particular, high levels of viral load are associated with "only effector" T-cell responses. One attractive explanation for the presence of "only effector" HCV-specific T-cell responses in HIV/HCV co-infected patients and liver transplant recipients, therefore, is that the presence of immunosuppression in these patients may favor more active virus replication. The higher levels of virus load may in turn impair polyfunctional responses while maintaining the generation of "only effector" T-cell responses. Therefore, the presence of the "only effector" HCV-specific T-cell responses is consistent with the higher levels of HCV viral load found in HIV/HCV co-infected patients and liver transplant recipients. In this regard, it would be interesting to investigate whether polyfunctional HCV-specific T cells may be restored after suppression of HCV viral load following antiviral treatment. Indeed, suppression of HIV viral load following antiretroviral treatment in HIV-1-infected individuals was associated with the restoration of polyfunctional HIV-specific $CD4^+$ and $CD8^+$ T-cell responses in a significant proportion of individuals [21, 29].

It is worth mentioning that polyfunctional responses are generated during HCV mono-infection in the presence of levels of viral load much greater than those found in CMV and EBV infections or non-progressive HIV-1 infection. Therefore, these results indicate that only under conditions of immunosuppression associated with HIV/HCV co-infection and liver transplantation, the further increase in viral load levels drives the generation of "only effector" HCV-specific T-cell responses. These, in turn, are associated with poor control of virus replication. The long and relatively benign course of HCV mono-infection before the eventual progression to liver cirrhosis suggests that an efficient although not optimal control is mediated by the observed polyfunctional T-cell responses.

In conclusion, polyfunctional T-cell responses are associated with a more effective control of virus replication during HCV mono-infection. The present results support the concept that immune-based mechanisms are involved in the modulation of the course of HCV infection in humans. Finally, whether functional profiles of T-cell responses may become valuable surrogate markers of HCV disease activity remains to be studied in prospective studies.

Material and methods

Patients and samples

Eighty-six chronically HCV mono-infected, 48 HIV/HCV co-infected patients and 42 HCV-infected patients having undergone liver transplantation for end-stage liver disease due to HCV-related cirrhosis were enrolled in this study that conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Institutional Review Board of all centers concerned. All patients gave written informed consent. All HCV mono-infected patients, HIV/HCV co-infected patients and liver transplant recipients were naïve to antiviral treatment for HCV infection. Thirty-six out of the 48 HIV/HCV co-infected individuals were on antiretroviral treatment for HIV-1 infection. All liver transplant recipients were clinically stable (no evidence of rejection) and received a first cadaveric graft. All were studied more than 12 months after transplantation. Immunosuppressive therapy for liver transplant recipients consisted of cyclosporin monotherapy ($n = 8$), tacrolimus monotherapy ($n = 7$), cyclosporin or tacrolimus in combination with mycophenolate mofetil ($n = 15$), or in combination with prednisone ($n = 3$), or in combination with azathioprine ($n = 5$), rapamycin monotherapy ($n = 2$) and rapamycin in combination with azathioprine ($n = 2$). Peripheral blood (twice 50 mL) was obtained from all patients by venipuncture and lymphocytes were isolated from peripheral blood samples upon centrifugation on a Ficoll gradient (Amersham Biosciences, Otelfingen, Switzerland) and either directly used for ELISpot assays or cryopreserved for further analyses. Liver biopsies were performed in all HCV mono-infected and HIV/HCV co-infected patients at time of HCV infection diagnosis. Biopsies were performed in 21 out of 42 liver transplant recipients at time of elevation of liver enzymes. All biopsies were evaluated for extent of inflammation and fibrosis and scored using the METAVIR system [30].

Synthetic peptides and peptide pools

A total of 728 peptides (15 aa long overlapping by 11 aa) corresponding to the HCV 1a strain and spanning all HCV proteins were reconstituted in sterile DMSO and diluted to prepare peptide pools or used as individual peptides. The 728 HCV-derived peptides were used to generate 54 peptide pools in a matrix setting [14]. Each pool was composed of 27 peptides and each peptide was present in two different pools, allowing for identification of the respective peptide by response in the two corresponding pools. Each peptide was present in the pools at the same concentration. All individual peptides or peptide pools were aliquoted and stored at −80°C. CMV, EBV and HSV lysates were obtained from Applied Biosystems (Rotkreutz, Switzerland).

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Pools of CMV- or EBV-derived peptides consisting of the corresponding peptides from the CEF control peptide pool [31] were obtained from Synpep Corporation (Dublin, CA, USA). All were reconstituted, aliquoted and stored at −80°C.

IFN-γ ELISpot assay

IFN-γ ELISpot assay was performed as previously described [14]. Briefly, 200 000 blood mononuclear cells were incubated in triplicate in the presence of peptide pools or individual peptides (5 μg/mL) for 18 h at 37°C. For ELISpot assays with depleted populations, $CD4^+$ or $CD8^+$ T cells were positively selected using magnetic beads (Miltenyi Biotech, Bergisch Gladbach, Germany) and incubated as described above. Spontaneous IFN-γ production was assessed by incubation of cells in the absence of peptide and incubation of cells with SEB (0.2 μg/mL) (Calbiochem, La Jolla, CA, USA) was used as positive control. Responses were expressed as number of spots per 10^6 blood mononuclear cells. Twenty uninfected healthy donors were used in order to validate the use of the ELISpot assay. The significance of the ELISpot was determined as follows: using peripheral blood mononuclear cells from patients, the assay was considered experimentally valid only if the number of spots in the absence of peptide was below 50 spots per 10^6 blood mononuclear cells and the number of spots in response to the peptides was greater than 55 spots per 10^6 blood mononuclear cells and at least fourfold above the background level. This cut-off was obtained by calculating the mean plus 3 standard deviations of the mean responses observed with blood mononuclear cells of the 20 healthy controls. Pools of HCV-derived peptides were used in the initial screening for presence of HCV-specific T-cell responses in HCV mono-infected patients, HIV/HCV co-infected patients and liver transplant recipient. Individual HCV-derived peptides were then identified and confirmed in a second ELISpot assay. Individual HCV-derived peptides were then used for intracellular cytokine production or proliferation experiments.

Multiparameter flow cytometry and antibodies

All antibodies used were from BD Biosciences (Franklin, NJ, USA) unless stated otherwise. For intracellular IFN-γ and IL-2 detection, cryopreserved peripheral blood mononuclear cells were incubated for 12 h in the presence or absence of the corresponding HCV-derived individual peptides, pools of CMV- or EBV-derived peptides, CMV, EBV or HSV lysates together with anti-CD28 (1 μg/mL) and Brefeldin A (GolgiPlug, 1 μL/mL, BD Biosciences). Cells were then stained for extracellular markers, permeabilized (Perm-2, BD Biosciences) and stained for intracellular markers. Antibodies used were: $IFN\text{-}\gamma^{FITC}$, $IL\text{-}2^{PE}$, $CD4^{PerCP\text{-}Cy5.5}$ and $CD8^{APC}$. Data were acquired on a FACSCalibur and analyzed using CellQuest™ software (BD Biosciences). Twenty uninfected healthy donors were used in order to validate the use of the intracellular cytokine staining assay with the sets of HCV-derived peptides. For intracellular cytokine staining assays to be valid, the background in the unstimulated control (incubation in absence of peptide) had not to exceed 0.01% and the percentage of cytokine-secreting cells had to have a background of less than 20% of the total percentage of cytokine-positive cells in the stimulated samples and be at least threefold above level in unstimulated samples. This cut-off was obtained by calculating the mean plus 3 standard deviations of the responses observed with blood mononuclear cells of 20 healthy controls testing the individual HCV peptides.

Ex vivo proliferation assay

Proliferation assays were performed as previously described [25]. Peptide concentration used for stimulation was 20 ng/mL. SEB (20 ng/mL) was used as positive control. No exogenous IL-2 was added except where specifically stated (rhIL-2, 10 IU/mL, Roche, Mannheim, Germany). Cells were harvested at day 5 and stained with $CD3^{PerCP\text{-}Cy5.5}$ and CD4 or $CD8^{APC}$ (BD Biosciences). Data were acquired on a FACSCalibur and analyzed using CellQuest™ software (BD Biosciences). SI was calculated as the ratio of the number of $CFSE^{low}$ cells in the presence relative to that in the absence of peptide. An $SI > 3$ was considered to be significant.

Statistical analysis

Comparison of two means was calculated by two-sample *t*-test. A p value < 0.05 was considered to be significant.

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Conflict of interest: The authors declare no financial or commercial conflict of interest.

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Abbreviations: **SEB:** staphylococcal enterotoxin B · **SI:** stimulation index

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