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The role of CCR5/CXCR3 expressing CD8+ cells in liver damage q and viral control during persistent hepatitis C virus infection

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ABSTRACT

Background/Aims:CXCR3 and CCR5 play a major role in recruiting cytotoxic T cells (Tc) and secreting secondary type 1 cytokines (Tc1) in the liver. HCV could impair their expression as a survival mechanism. The role of these chemokine receptors on CD8+ cells in chronic hepatitis C is analysed.

Methods:Serum, chemokines, peripheral blood and intrahepatic lymphocytes from chronic hepatitis C patients were studied. CXCR3 / CCR5 expressing CD8+ cells were quantified by flow-cytometry. Serum chemokines concentration (CXCL10/CCL3) was measured by ELISA. Basal data were correlated with liver inflammation. Longitudinal data were obtained during treatment and correlated with virologic response.

Results:CCR5/CXCR3 expressing CD8+ cells were enriched in the liver and correlated with inflammation. Chronic HCV patients presented the same frequency of $CCR5^{high}/CXCR3^{high}$ expressing CD8+ cells in peripheral blood as in healthy controls but higher serum concentration of CXCL10/CCL3. Treatment with PEG-interferon a-2b plus ribavirin increased $CCR5^{high}/CXCR3^{high}$ expressing CD8+ cells frequency in peripheral blood and decreased CXCL10/CCL3 serum concentration. Increase in $CXCR3^{high}$ expressing CD8+ cells after 24 weeks of treatment was correlated with SVR.

Conclusions:In chronic hepatitis C, anti-viral treatment induces an increase in CD8+ cells expressing chemokine receptors associated with Tc1 response and a reduction in their ligands. Achievement of viral control is associated with an increase in $CXCR3^{high}$ expressing CD8+ cells during treatment.

Keywords: Hepatitis C virus; Chemokine receptors; CCR5; CXCR3; Chemokines; CCL3; CXCL10; Chemotaxis; Liver damage; Viral escape mechanism

Introduction

Hepatitis C virus (HCV) is a major cause of chronic liver disease worldwide. Approximately three-quarters of infected subjects develop a chronic infection [1]. Thus HCV is likely to efficiently evade the immune system [2] one hypothetical mechanism being that HCV could suruvive via reduction of hepatic lymphocyte chemotaxis. Chemokine receptors (CRs) play an important role in T cell recruitment into infected sites and are involved in infection control and in tissue damage [3]. A polarisation of CR expression on T cells depending on the cytokine production profile has been demonstrated [4]. CRs such as CCR5 and CXCR3 are associated with type-1 response [5–8]. In an experimental model of influenza virus infection, cytotoxic specific CD8+ cells expressing CRs associated with type-2 response were not able to reach the infected site and clear the virus, while CCR5 expressing specific cytotoxic T cells controlled infection without tissue damage [9]. On the other hand, it has been demonstrated that a massive hepatic infiltration by non-specific T cells, expressing CRs associated with type-1 response, can cause acute liver failure [10]. Bearing this in mind, the impairment of the expression of CR's associated with type-1 response could be of importance in improving the ability of HCV to survive.

The majority of HCV infections in Western Europe are genotype-1 [1]. Treatment with pegylated (PEG) interferon-a plus ribavirin in chronic hepatitis C (CHC) genotype-1 provokes a sustained virologic response (SVR) in approximately 50% of patients [11,12]. A HCV-RNA positivity on week 24 is associated with a very low probability of developing a SVR [13]. It means that important immunological events occur during these first 6 months of treatment which could affect viral clearance. The possible modifications on the frequency of CCR5/CXCR3 expressing CD8+ cells induced by anti-HCV treatment during this period have not yet been fully analysed.

In this paper, the influence of HCV genotype-1 infection on CCR5/CXCR3 expressing CD8+ cells and the roleofanti-HCVtreatmenttoavoidinfectionarestudied.

Patients and methods

Patients

Between March-03 and December-04, 30 consecutive CHC genotype-1 patients were recruited. All patients presented persistently elevated ALT and were HCV-RNA positive. Other causes of chronic liver disease were excluded. In 17 cases, a liver biopsy was performed at the time of recruitment. In 14 patients treatment with PEG-interferon a-2b (1.5 lg/kg) plus ribavirin (600–1200 mg) was administered. Eleven patients completed 48 weeks of treatment and in

three cases treatment was stopped in week 24 due to HCV-RNA positivity. SVR was considered when HCV-RNA was negative and ALT was normal 6 months after finishing treatment. Seven treated patients developed SVR and four relapsed after treatment response. Twenty healthy adults with normal ALT and HCV-RNA negative were taken as controls. Demographic and clinical features are summarised in Table 1. Basal and monthly serum samples were collected to test ALT and HCV-RNA. The protocol was approved by the Regional Ethical Committee.

Table 1
Demographic and baseline clinical features

	Chronic hepatitis C (n = 30)	Healthy controls (n = 21)
Male sex (%)	69%	65%
Age (years)	41 (7.50)	38 (8.89)
Disease duration (years)	17.91 (8.46)	–
ALT (IU/L)	89 (61)	22.50 (10)
Viral load (copies/mL)[a]	$2.29 \cdot 10^6$ ($4.74 \cdot 10^6$)	–
Genotype 1 (%)	100%	–
Treatment outcome (n/treated patients)		
HCV-RNA () at week 24	11/14	–
Relapse after treatment	4/14	–
SVR[b]	7/14	–

Data are expressed as number of patients (n), percentage (%) or mean plus standard deviation (SD) except for viral load and ALT which are expressed as median plus interquartile range (IQR) because these variables did not follow a normal distribution.

[a] HCV-RNA was quantified by VERSANT HCV RNA 3.0 Assay (HCV 3.0 bDNA assay), (Bayer Diagnostics, Berkeley, CA).

[b] SVR, sustained virologic response.

Separation of intrahepatic and peripheral blood lymphocytes

At recruitment and at weeks 12 and 24 of treatment, heparinised blood samples were collected to analyse CRs expression on peripheral blood lymphocytes (PBL). PBL were isolated from heparinised blood samples by density gradient centrifugation on Ficoll–Hypaque (Amersham-Pharmacia Biotech AB, Uppsala, Sweden). At recruitment, a piece of liver biopsy not

needed for diagnostic purposes was used for CRs analysis. Liver infiltrating lymphocytes (LIL) were purified from biopsies according to previous methods [14]. In brief, liver tissue was washed extensively in RPMI 1640 plus 1% FCS (Gibco, Les Ullis, France) and then digested with collagenase-I (1 mg/ml; Sigma Chemical Co, Saint Louis, MO) and DNase (25lg/ml; Sigma Chemical Co, Saint Louis, MO). The cell suspension was washed, and LIL were recovered by centrifugation over a Ficoll–Hypaque density gradient. PBL and LIL were analysed immediately. Some publications reported that the isolation of lymphocytes by Ficoll–Hypaque density gradient method resulted in a loss of CRs staining [15,16]. Pilot experiments, carried out in our laboratory, comparing lymphocyte CRs staining directly and after Ficoll–Hypaque separation, showed a significant 5–10% reduction in CCR5 staining but not in CXCR3. Nevertheless, this fact should not affect the analysis performed in this study and its conclusions since all the lymphocyte samples compared were obtained by the same method.

FACS analysis for CCR5 and CXCR3 expression

$0.2 \cdot 10^6$ PBL or variable numbers of LIL ($>0.05 \cdot 10^6$) were incubated for 30 min at 4 C with saturating concentrations of CD8-Pe (Sigma Chemical Co, Saint Louis, MO) and either CCR5Cy5 IgG_{2a} (Pharmingen BD, San Jose, CA) or CXCR3-FITC IgG_1 (R&D Systems Inc., Minneapolis, MN) mAbs. Cells were washed and then analysed immediately on a Becton–Dickinson FACS using CELLQuest software (Becton–Dickinson, San Jose, CA). Isotype controls (mouse IgG_1-FITC and mouse IgG_{2a}-Cy5 (Becton– Dickinson, San Jose, CA)) were used to setup markers for CCR5-Cy5 and CXCR3-FITC staining. A cell fluorescence higher than 40 U for CCR5-Cy5 and CXCR3-FITC was considered a high expression of these CRs based on isotype control staining (Fig. 1B). Cells with this staining level are called either $CCR5^{high}$ or $CXCR3^{high}$ in this paper. To focus the study on T cells, FACS analysis was carried out in cells gated on side-scatter/forward-scatter ‘‘lymphocyte gate’’ [17,18]. This gate selected a high purity CD3+ population. Thereafter, another gate on $CD8^{high}$ population was performed to exclude NK and NKT cells.

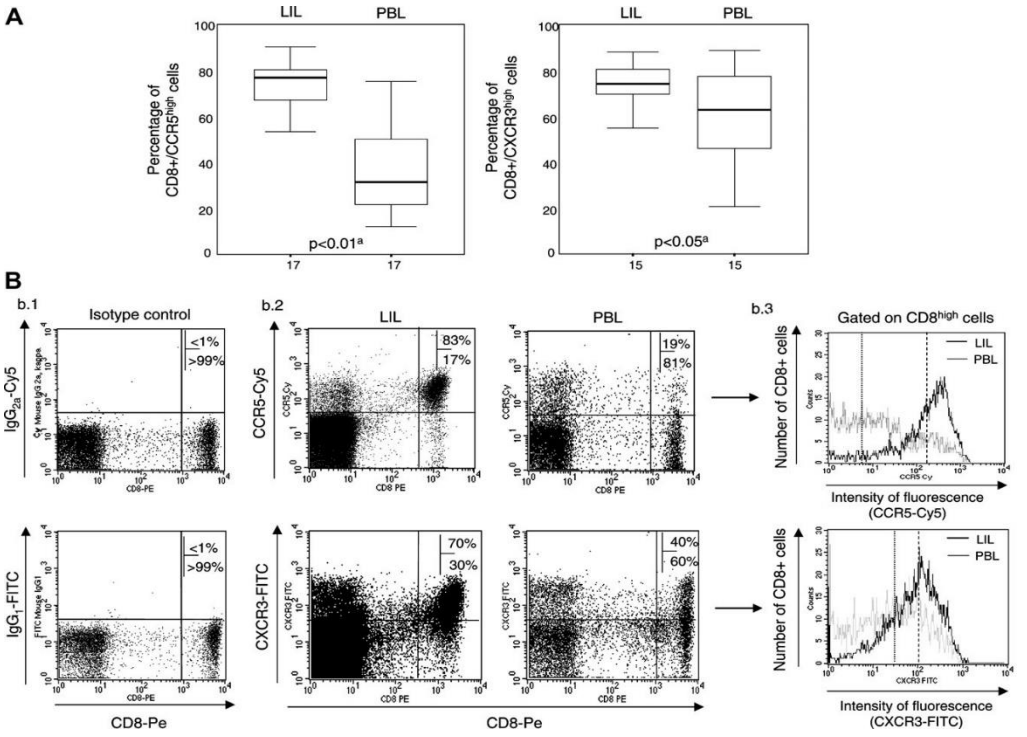

Fig. 1. Intrahepatic enrichment of CCR5^{high} and CXCR3^{high} CD8+ cells in chronic hepatitis C genotype-1. (A) Box-plots showing the percentage of CCR5^{high} and CXCR3^{high} expressing CD8+ cells from peripheral blood and liver from patients with paired samples. (B) (b.1) Isotype controls FACScan dot-plots. PBL were double stained with CD8-Pe mAb and two different isotype controls, either mouse IgG2a-Cy5 or mouse IgG1-FITC, to setup the markers for CCR5 and CXCR3 positive cells. (b.2) FACScan dot-plots of PBL and LIL from a representative chronic hepatitis C patient. PBL and LIL were double stained with CD8-Pe mAb and with either CCR5-Cy5 or CXCR3-FITC mAbs. In the upper right quadrant are represented the double positive cells (CD8+/chemokine receptor^{high}). (b.3) Histogram-plots of fluorescence intensity for CCR5-Cy5 and CXCR3-FITC on CD8+ cells from LIL (grey line) and PBL (black line) of the same chronic hepatitis C patient. ---, median value of fluorescence intensity for CCR5-Cy5 (median: 173 U) and CXCR3-FITC (median: 103 U) on CD8+ cells from LIL. ..., median value of fluorescence intensity for CCR5-Cy5 (median: 6 U) and CXCR3-FITC (median: 33 U) on CD8+ cells from PBL. [a]Wilcoxon test. PBL, peripheral blood lymphocytes; LIL, liver infiltrating lymphocytes.

Measurement of serum CXCL10/CCL3

Serum samples were collected at recruitment, week 12 and week 24 of treatment to measure CXCL10/CCL3 serum concentration by ELISA (Biosource, Camarillo, CA). Collected samples were immediately frozen until analysis was performed. ELISAs were carried out following the manufacturer's instructions.

Histology and immunohistochemistry analysis

Liver biopsies containing at least five portal spaces were studied to assess liver fibrosis and inflammation. Liver sections, paraffin-embedded, formalin-fixed and hematoxylin-stained, were analysed by a single pathologist according to the Scheuer index [19] (Table 2).

The distribution of T cells in the liver was visualised by immunostaining in formalin-fixed, paraffin-embedded liver specimens as previously described [14]. The liver sections were first microwaved in citrate buffer (pH 6.0) for Ag retrieval, followed by incubation with a mAb to human CD8 and CD4 (DAKO, Glostrup, Denmark). Detection was performed using a sensitive immunoperoxidase-kit (EnVision HRP system; DAKO, Glostrup, Denmark) with diaminobenzidine as a substrate, and the sections were counter-stained with hematoxylin. Endogenous peroxidase activity was blocked using sodium azide and hydrogen peroxide [20]. The mean number of CD8+ and CD4+ cells per 400· field in portal tracts and intralobular areas was calculated from three randomly selected fields (Table 2).

Table 2
Histological features of chronic HCV patients

Liver histology[a]	
Activity (1–4)	
Porto-periportal	1.91 (0.85)
Lobular	1.66 (0.84)
Fibrosis (1–4)	
	1.51 (0.95)
Immunohistochemistry: (Intrahepatic CD8+ and CD4+ cells per 400× field)[b]	
Porto-periportal	
CD8+ cells	97.36 (33.82)
CD4+ cells	37.92 (18.46)
Lobular	
CD8+ cells	32.36 (14.79)
CD4+ cells	7.33 (4.19)

[a] Scheuer index.
[b] CD8+ and CD4+ cells in liver sections were counted by immunostaining using a sensitive immunoperoxidase-kit. Data are presented as means + standard deviation.

Statistical analysis

Categorical data are presented as percentages whereas continuous variables are summarised as either median and interquartile range (IQR), or mean and standard deviation (SD). Spearman's coefficient and Wilcoxon, Friedman and Mann–Whitney U tests were employed where appropriate. Receiver Operating Characteristics (ROC) curves were calculated to estimate the validity of CRs expression to predict SVR. It was considered significant at $p < 0.05$.
hepatic enrichment. The data obtained in this study demonstrate a higher frequency of CXCR3^{high}/CD8+ cells in the liver than in PB, which would have been much higher if a different LIL isolation method had been used.

Results

Intrahepatic enrichment of CCR5/CXCR3 expressing CD8+ cells

The majority of portal and lobular T cells in CHC biopsies were CD8+ ($p < 0.001$ and $p < 0.01$, respectively) (Table 2). The frequency of intrahepatic CCR5^{high}/CXCR3^{high} expressing CD8+ cells was compared with the frequency of these cells in peripheral blood (PB) in paired samples. An intrahepatic enrichment of both CD8+/CCR5^{high} and CD8+/CXCR3^{high} cells was demonstrated. The percentage of intrahepatic CD8+/CCR5^{high} cells was 76.79% (IQR 18.19) whereas it was 31.46% (IQR 31.87) in PB ($p < 0.01$; Fig. 1). In the same way, the percentage of CD8+/CXCR3^{high} cells was also higher in the liver (73.30%; IQR 15.05) than in PB (61.03%; IQR 36.73) ($p < 0.05$; Fig. 1).
An earlier publication has shown that using collagenase to isolate LIL produced a significant reduction in mean fluorescence intensity for CXCR3 staining [20]. Nevertheless, other papers have been able to study the expression of CXCR3 on tissue infiltrating T cells by FACS, despite using collagenase to isolate these cells [21–23]. In any case, this fact would not affect the demonstration of a CXCR3^{high} expressing CD8+ cells intra intrahepatic enrichment. The data obtained in this study demonstrate a higher frequency of CXCR3high/CD8+ cells in the liver than in PB, which would have been much higher if a different LIL isolation method had been used.

Correlation between frequency of intrahepatic CCR5high/CXCR3high expressing CD8+ cells and liver inflammation

The correlation between the intrahepatic frequency of CCR5high/CXCR3high expressing CD8+ cells and liver inflammation and fibrosis according to the Scheuer histological score was studied. A significant positive correlation between the percentage of intrahepatic CXCR3high/CCR5high expressing CD8+ cells and both porto-periportal and lobular activity was shown (Fig. 2). On the other hand,

CCR5 expression on intrahepatic CD8+ cells was not correlated with liver fibrosis and neither was CXCR3.

Increase in CCR5^{high}/CXCR3^{high} expressing CD8+ cells after 24 weeks of traten

In CHC patients before treatment and in healthy controls, peripheral blood CCR5^{high}/CXCR3^{high} expressing CD8+ cells were quantified. A lower non-significant frequency of CCR5^{high}/CXCR3^{high} expressing CD8+ cells was found in chronic hepatitis C patients (Table 3).

In patients treated with PEG-interferon a-2b plus ribavirin, the percentage of CCR5^{high}/CXCR3^{high} expressing CD8+ cells at recruitment, week 12 and week 24 was quantified. An increase in CXCR3^{high} expressing CD8+ cells in 11 out of 14 patients was observed after 24 weeks of treatment, with an 11.50% (IQR 25.92) increase in CD8+/CXCR3^{high} cells ($p < 0.01$; Fig. 3A). In 11 out of 13 treated patients an increase in CCR5^{high} expressing CD8+ cells was also shown after 24 weeks of treatment. Specifically, a 10.25% (IQR 13.50) increase in CD8+/CCR5^{high} population was observed ($p < 0.05$; Fig. 3A).

Decrease in CXCL10/CCL3 serum concentrationduring anti-viral treatment

CXCL10/CCL3 serum concentrations were measured at recruitment, week 12 and week 24 of treatment to determine whether there was an association with the expression of their receptors on CD8+ cells. Baseline CXCL10 and CCL3 serum concentrations were higher in CHC patients than in healthy controls ($p < 0.01$ and $p < 0.05$, respectively; Table 3). CXCL10 serum concentration decreased progressively from 378.77 pg/ml (IQR 153.38) at week 0 to 298.52 pg/ml (IQR 154.45) at week 24 of treatment ($p < 0.05$; Fig. 3A). The same observation was made with CCL3. Its serum concentration decreased during the first 24 weeks of treatment from tions occurred together with the increase in CCR5^{high}/ 8.24 pg/ml (IQR 8.02) at recruitment to 6.70 pg/ml CXCR3^{high} expressing CD8+ cells previously shown (IQR 4.51) at week 24 ($p < 0.05$; Fig. 3A). Both reduc- (Fig. 3A). CCL3 decreased by week 12 of treatment while CXCL10 maintained the same level it had before treatment at this point. This difference was due to the varying behaviour of CXCL10 in patients with or without SVR. In responder patients, CXCL10 level decreased 63.01 pg/ml (IQR 233.45) at week 12 while for non-responders, the reduction was only 3.55 pg/ml (IQR 94.80). On the other hand, a significant positive correlation between HCV viral load and CCL3 serum concentration was found ($r = 0.47$; $p < 0.01$). Also, a non-significant positive correlation between CXCL10 and HCV viral load was observed ($r = 0.32$; $p = 0.076$) (Fig. 4).

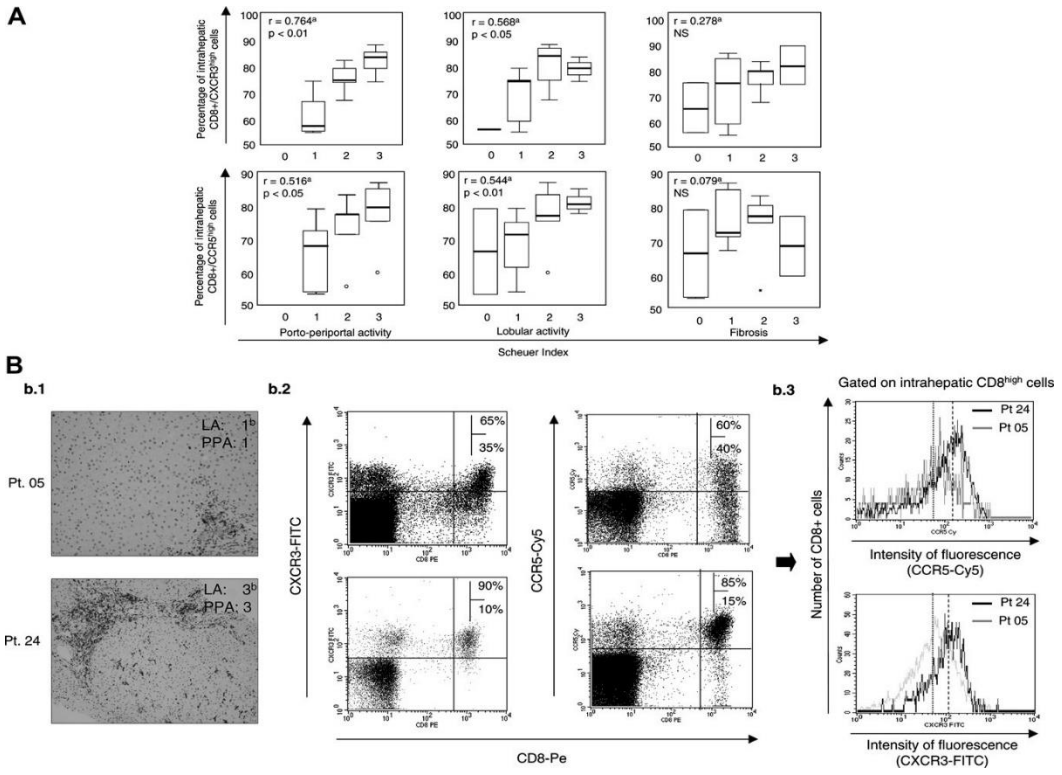

Fig. 2. Correlation between liver inflammation and frequency of intrahepatic CCR5 and CXCR3 expressing CD8+ cells in chronic hepatitis C genotype1. (A) Box-plots showing the percentage of intrahepatic CD8+ cells expressing either CCR5^{high} or CXCR3^{high} according to the degree of liver inflammation and fibrosis. [a]Spearman's correlation coefficient. NS, non-significant; o, outlier value; *, extreme value. (B) (b.1) Representative photomicrographs of liver immunostaining for CD8 from two chronic hepatitis C patients (05 and 24) with different grades of inflammation. CD8 cells are stained in dark by immunoperoxidase technique. Original magnification 400·. Patient 05 with lobular activity (LA) 1 and porto-periportal activity (PPA) 1 showed scarce staining while patient 24 with LA 3 and PPA 3 presented intense CD8 staining. (b.2) FACScan dot-plots of intrahepatic lymphocytes from these two patients after staining with CD8-Pe and with either CCR5-Cy5 or CXCR3-FITC mAbs. In the upper right quadrant are represented the double positive cells (CD8+/chemokine receptor^{high}). [b]Scheuer index. (b.3) Histogram-plots of CCR5-Cy5 and CXCR3-FITC fluorescence intensity on intrahepatic CD8+ cells from patient 05 (grey line) and patient 24 (black line). ---, median value of fluorescence intensity in patient 24 for CCR5-Cy5 (median: 170 U) and CXCR3-FITC (median: 116 U). ..., median value of fluorescence intensity in patient 05 for CCR5-Cy5 (median: 60 U) and CXCR3FITC (median: 55 U). Pt, patient.

Table 3
Baseline percentage of CCR5^{high}/CXCR3^{high} expressing CD8+ cells from peripheral blood and CCL3/CXCL10 serum concentration in chronic hepatitis C and healthy controls

	Healthy controls	Chronic hepatitis C	p value[a]
CD8+/CCR5^{high} cells (%)	28.30 (IQR 21.74)	24.51 (IQR 28.46)	NS
CCL3 (pg/ml)	4.81 (IQR 9.87)	8.24 (IQR 8.02)	<0.01
CD8+/CXCR3^{high} cells (%)	66.06 (IQR 10.88)	60.98 (IQR 27.39)	NS
CXCL10 (pg/ml)	140.50 (IQR 103.10)	378.77 (IQR 153.38)	<0.05

Data are expressed as median plus interquartile range (IQR). NS, non-significant.

[a] Mann–Whitney U test.

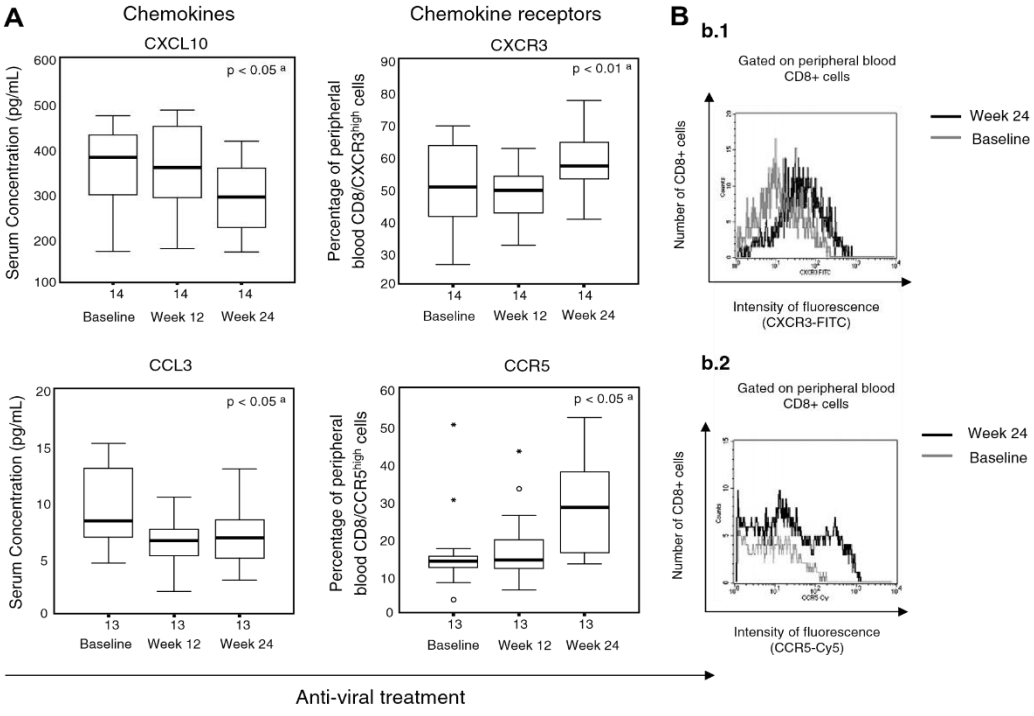

Fig. 3. Chemokines serum concentration and chemokine receptors expression on peripheral blood CD8+ cells during anti-viral treatment in chronic hepatitis C genotype-1. (A) Box-plots representing the CXCL10 and CCL3 serum concentration and the percentage of CD8+ cells expressing either CXCR3^{high} or CCR5^{high} during first 24 weeks of treatment. A decrease in CXCL10 and CCL3 serum concentration during treatment, which is associated with an increase in CCR5 and CXCR3 expressing CD8+ cells, is shown. [a]Friedman test. o, outlier value; *, extreme value. (B) Intensity of fluorescence histograms for CXCR3-FITC (b.1) and CCR5-Cy5 (b.2) gated on CD8+ cells from one representative chronic hepatitis C patient before (grey line) and after 24 weeks of treatment (black line).

Increase in CXCR3^{high} expressing CD8+ T cells and SVR

Once the increase in CCR5^{high}/CXCR3^{high} expressing CD8+ cells on PB after 24 weeks of treatment was demonstrated, the possibility of identifying which patients would develop SVR depending on the degree of CR expression was analysed. Only the increase in CXCR3^{high} expressing CD8+ cells showed the ability to properly predict SVR for a cut-off value of a 14% increase with 100% positive predictive value (Fig. 5). Moreover an increase in CXCR3^{high} expressing CD8+ cells lower than 1% after 24 weeks of treatment was associated with no SVR with 100% negative predictive value (Fig. 5). Increase in CCR5^{high} expressing CD8+ cells did not predict SVR (data not shown).

Discussion

Persistent HCV infection is characterised by a nonspecific inflammatory infiltrate in the liver, mainly of CD8+ cells [24,25], responsible for liver damage [26,14]. The migration of lymphocytes to the liver is provoked by the interaction between CRs and their ligands [3]. In CHC, the expression of different chemokines in the liver has been described [20,27]. These chemokines are associated with type-1 response and recruit T cells expressing specific CRs such as CCR5 and CXCR3 [6]. In this paper, a predominant liver infiltration by CD8+ lymphocytes in CHC genotype-1 is demonstrated. The majority of these CD8+ cells showed a CCR5^{high}/CXCR3^{high} phenotype and were positively correlated with liver inflammation but not with liver fibrosis. These data suggest that CCR5 and CXCR3 could play an important role in chronic liver damage in CHC by means of CD8+ cells recruitment into the liver. Several previous studies agree with these findings [28,22,16,29,30]. Other possibilities, that cannot be rejected could be either the proliferation of these cells in the liver and the death of cells with a different chemokine receptor profile, or up-regulation of these receptors in the intrahepatic CD8+ population.

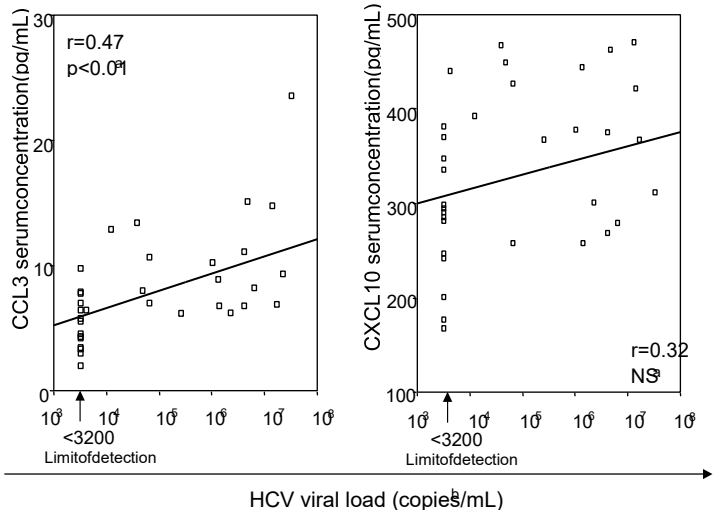

Fig. 4. Correlation between HCV viral load and chemokine serum concentration. Paired data of HCV viral load and CCL3/CXCL10 serum level at baseline, week 12 and week 24 of treatment were analysed. A positive correlation between CCL3 serum level and HCV viral load was found. The black straight line represents the scatter-plot fit line by linear regression. HCV viral load is shown in log scale. [a]Spearman's correlation coefficient. [b]Lower limit of HCV viral load detection using VERSANT HCV RNA 3.0 assay (Bayer Diagnostics, Berkeley, CA) was 3200 copies/mL. NS, non-significant.

The natural history of CHC can take up to three decades to develop liver cirrhosis [31]. This means that the immunologically mediated liver damage must be continuous but very light. For HCV it is essential to extend host survival as much as possible to assure its own viability. One mechanism to achieve this could be to reduce CD8+ lymphocytes migration into the liver through impairment of CRs expression. To maximise the immune system ability to control the infection, a high frequency of CCR5+/CXCR3+ T cells should be expected. Soon after HCV infection, prominent CD8+ cell responses are observed involving transient up-regulation of CCR5 expression [32]. During CHC, however, CD8+ cells show reduced expression of CRs associated with Tc1 response [33]. In this paper, a high serum concentration of CCL3/CXCL10 associated with a normal frequency of PB $CCR5^{high}/CXCR3^{high}$ CD8+ cells in CHC patients is described. The absence of $CCR5^{high}$/ $CXCR3^{high}$ CD8+ cells increase could be due to either an intrahepatic sequestration of $CCR5^{high}/CXCR3^{high}$ expressing CD8+ cells due to

CCL3/CXCL10 attraction, or to a down-regulation of these CRs produced by the observed high serum concentration of their ligands.

In fact, it has been shown that GB virus C, a close relative of HCV [34], is able to reduce CCR5 expression on T cells by inducing CCL5 release [35,36]. One study in HCV infection has also described a CCR5 down-regulation on CD8+ cells by receptor internalisation [33]. It has been shown that the HCV-E2 protein, after binding to CD81 [37], induces CCL5 secretion by CD8+ cells and the ensuing interaction between CCL5 and CCR5 is responsible for CCR5 down-regulation on these cells
[38].

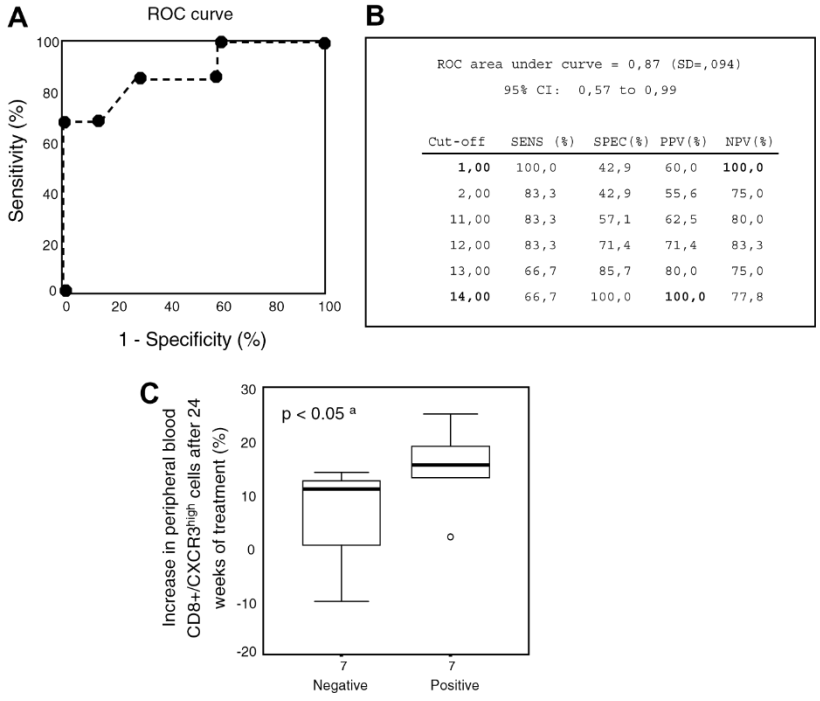

Fig. 5. Correlation between increase in CXCR3 expressing CD8+ cells in peripheral blood after anti-viral treatment and sustained virologic response in chronic hepatitis C genotype-1. (A) Receiver Operating Characteristics (ROC) curve for increase of CXCR3^{high} expressing CD8+ cells from peripheral blood after 24 weeks of treatment. (B) Table showing the sensitivity (SENS), specificity (SPEC), predictive positive value (PPV) and negative predictive value (NPV) for different cut-offs of CXCR3^{high} expressing CD8+ cells increase. The cut-off values for 100% NPV and 100% PPV are shown in bold. CI, confidence interval. (C) Box-plots of CXCR3^{high} expressing CD8+ cells increase after 24 weeks of treatment for patients with positive and negative sustained virologic response. [a]Mann–Whitney U test. o, outlier value.

On the other hand, previous studies have shown a high hepatocyte synthesis of CXCL10, CXCL9 and CCL5 induced by some HCV proteins such as NS5A and core [39]. Subsequently, another explanation for the absence of increase in the PB frequency of $CCR5^{high}/CXCR3^{high}$ expressing CD8+ cells could be an intense intrahepatic T cells migration because of a high concentration of chemokines in the liver. In fact, in this study a $CCR5^{high}/CXCR3^{high}$ expressing CD8+ intrahepatic enrichment was demonstrated. Therefore, the absence of increase in PB $CCR5^{high}/CXCR3^{high}$ expressing CD8+ cells found in CHC patients, could be explained by both mechanisms.

Another investigation was performed to analyse whether HCV infection could influence in vivo on CCR5/CXCR3 expression on CD8+ cells through chemokine synthesis induction. If HCV is able to interfere with CCR5/CXCR3 expression, an increase in T cells expressing these CRs after viral load drop due to antiviral treatment should be expected, together with a CXCL10/CCL3 decrease. To address this issue, a longitudinal analysis of $CCR5^{high}/CXCR3^{high}$ expressing CD8+ cells during treatment was carried out. In the majority of treated patients an increase in $CCR5^{high}$/ $CXCR3^{high}$ expressing CD8+ cells was demonstrated. This finding was associated with a significant decrease in CXCL10 and CCL3 serum levels after 24 weeks of treatment. Nevertheless, the fact that a-interferon can up-regulate in-vitro CCR5 expression on T cells has been published [40]. Therefore, we cannot rule out, at least for CCR5, that the observed finding could be due to an intrinsic a-interferon property. However, another likely explanation for these data is that HCV control during treatment would decrease CXCL10/CCL3 release, allowing CCR5/CXCR3 up-regulation on PB CD8+ cells. In fact, in this study a significant positive correlation between HCV viral load and CCL3 is shown.

Moreover, it has been shown in this paper that at least the increase in $CXCR3^{high}$ expressing CD8+ cells during treatment is associated with SVR. This suggests that for HCV, it is important to modulate the expression of these receptors not only to keep liver viability but also to escape from immunological control. This correlates with previous studies that showed how baseline CXCR3 ligand serum concentration is associated with the outcome of anti-viral therapy [41–44]. Moreover, in this study, a faster reduction in CXCL10 serum concentration in responders than in non-responders during first 12 weeks of treatment is suggested. On the other hand, the absence of increase in $CXCR3^{high}$ expressing CD8+ cells after 24 weeks of treatment shows a 100% negative predictive value of SVR. This information can be clinically important in predicting non-response and allowing the termination of treatment in those patients with no increase in the frequency of $CXCR3^{high}$ expressing CD8+ cells after 24 weeks of treatment. This ROC analysis is very preliminary since it is based on a very small number of patients. Therefore, these data should be re-confirmed by a larger multivariate study.

In summary, this paper suggests a role for CCR5/ CXCR3 expressing CD8+ cells in CHC liver damage and demonstrates an increase in these cells in peripheral blood and a decrease in CCL3/CXCL10 serum concentration during treatment. Viral control after treatment is associated with an increase in $CXCR3^{high}$/CD8+ cells and a decrease in CXCL10. Based on these data, we suggest that chemokine over-production during HCV infection could interfere with CRs expression associated with Tc1 response as a viral escape mechanism.

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