Direct-acting antiviral therapy for chronic hepatitis C

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Upregulation of CXCR3 expression on CD8+ T cells due to the pervasive influence of chronic hepatitis B and C virus infection

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Submitted
ABSTRACT

Background: Chronic systemic ‘latent’ viral infections such as Cytomegalovirus infection (CMV) are known to leave a fingerprint in the total T-cell population, identified by an increased percentage of highly differentiated effector-memory type T-cells.

Methods: In the present study we investigated whether chronic infections with a ‘persistent’ viremia, such as chronic hepatitis B and C (CHB and CHC), that are characterized by local organ-specific inflammation, also impact the total peripheral T-cell population or other virus specific T-cells that do not target hepatitis viruses.

Results: No phenotypic or functional differences were found between CD8+ T-cells in viral hepatitis or healthy controls (HC). Also no clear phenotypic differences were found in CMV- or Epstein Barr virus specific T-cells in viral hepatitis compared to HC. However, expression of the chemokine-receptor CXCR3 was significantly higher on total peripheral CD8+ T-cells of CHB or CHC patients compared to HC (p<0.005). In CHB higher CXCR3 expression was associated with positive HBeAg-status and correlated with the percentage of HbsAg expressing hepatocytes found in liver biopsies, both pointing to a relation between CXCR3 expression and disease activity. The higher expression of CXCR3 on T-cells of viral hepatitis patients may reflect the pervasive influence of a persistent viral infection, even when restricted to the liver, on total peripheral blood CD8+ T-cells. In fact chemokine-receptors such as CXCR3 are important for T-cell recruitment to the liver and chemokine-ligands specific for CXCR3 have been found to be upregulated in chronic hepatitis.

Conclusion: Modulating chemokine (receptor) expression could be a potential target for future therapy to optimize the anti-viral immunologic environment in the liver.

INTRODUCTION

Infection with hepatitis B virus (HBV) and hepatitis C virus (HCV) are the most common causes of liver disease. Worldwide more than 500 million people are chronically infected and annually more than 1 million people die of infection mediated liver cirrhosis and hepatocellular carcinoma. In order to establish a chronic infection, the virus must successfully evade host immune responses. Clear mechanistic insights on viral eradication are not available. The presence of weak and narrowly focused T cells in patients who develop chronic infection is in contrast to the strong and broad virus-specific T cell responses found in most individuals that spontaneously resolve infection, suggesting that the outcome of infection is largely determined by the magnitude and breadth of T cell responses. Why certain viruses, like Influenza are eradicated from the body whereas on the other side of the spectrum viruses such as hepatitis C, B and human immunodeficiency virus (HIV) can persist with high viral loads for years, remains a subject of intensive research. Availability of peptide-MHC tetramers to detect virus-specific CD8+ T cells has boosted the knowledge on T cell function and phenotype. It became clear that with respect to differentiation phenotype T cells directed against viruses that are cleared from the host differ from virus-specific T cells responsive to latent or chronic infections. This indicates that the differentiation phenotypes mostly seem to be determined by the chronicity and level of viral load during infection together with the inflammatory milieu. Latent infection with Cytomegalovirus (CMV) has been shown to leave a phenotypical fingerprint even in the total T cell population, identified by an increased percentage of highly differentiated effector-memory type T cells. This is most prominent in immunosuppressed and older individuals as CMV-specific T cell responses continue to increase throughout life and can come to dominate the entire T cell repertoire in the elderly, perhaps impairing responses to other antigens. Contrary to systemic latent virus infections such as CMV with undetectable viral load in healthy individuals, HBV and HCV together with HIV are characterized by continuous presence of high viral loads and are the three clinically most important persistent viral infections. It is not clear whether viruses like HBV or HCV which cause a localized, organ-specific chronic infection can also impact the total peripheral T-cell population.

Accumulating evidence shows that CD8+ T cell phenotypes also largely depend on the signals they receive from the tissue environment. Recent data in humans show that CMV-specific T cells in peripheral blood differ in phenotype and function from CMV-specific cells in lymph nodes and T cells directed against FLU diverge between lung and blood. As T cells continuously recirculate through organs, lymph nodes and peripheral blood, it can well be envisaged that a local infection will be also reflected in the total peripheral T cell population in affected individuals. Indeed, one study showed that the effect of chronic HCV infection on T cell phenotype extends beyond the T cells that target this virus. CMV-specific CD8+ T cells were shown to have a more immature phenotype in HCV-infected individuals compared with healthy controls.
T cell migration to the inflamed site is induced by chemotactic factors. As such, analysis of chemokine receptor expression is especially interesting since this provides information on the migration properties of the cells. In order to clear acute infection in the liver a strong virus specific T cell recruitment to the liver is required. On the other hand, continuous redirection of T cells to the liver could cause immunopathology. Several reports have described the upregulation of chemokines during chronic viral hepatitis. Under influence of type 1 inflammatory cytokines hepatocytes and liver endothelial sinusoid cells produce CXCL9, 10, and 11, which can ligate to their receptor CXCR3. The way in which T cells are polarized with regard to their chemokine receptor profile depends on the tissue cytokine profile. During chronic hepatitis C infection it has been shown that expression of type 1 associated chemokine receptors, such as CXCR3, has been associated with hepatic infiltrates.

In the present study we investigated whether indeed local chronic HCV or HBV infection can alter the total peripheral CD8+ T-cell population in a similar way as has been described for CMV.

We compared the phenotypic and functional characteristics of the total CD8+ T-cell population of CMV-seropositive individuals with and without chronic hepatitis B or C. Furthermore, to determine the effect of eventual clearance (HCV) or suppression (HBV) of the virus upon therapy, we compared T cells from responders and non-responders before and after therapy.

**MATERIALS AND METHODS**

**Subjects**

Peripheral blood mononuclear cells (PBMC) were collected from patients with chronic hepatitis B, chronic hepatitis C and healthy controls. All subjects were HIV seronegative. Patients co-infected with HBV, HCV or hepatitis D virus were excluded from this study. Chronic hepatitis B patients participated in a clinical study designed to assess markers of response during treatment with peg-IFN and adeovir. The treatment regime consisted of weekly peg-IFN-alfa-2a 180µg and 10mg adeovir daily for 48 weeks, followed by a 24-week treatment-free follow-up. Sustained virological response (SVR) was defined as HBV DNA levels s2.0x10^3 IU/mL and normalization of ALT for HBeAg negative patients. For HBeAg positive patients SVR was defined as HBeAg seroconversion and ALT normalization. For CMV serostatus was determined by anti-CMV IgG in serum using the AxSYM microparticle enzyme immunoassay (Abbott Laboratories, Abbott Park, IL) according to the manufacturer’s instructions. The EBV serostatus was investigated by determination of IgG to EBV viral capsid antigen (Biotest, Dreieich, Germany).

**Immunofluorescent staining and flowcytometry**

PBMC were washed in PBS containing 0.01% (w/v) NaN3, 0.5% (w/v) bovine serum albumin and 2 mM EDTA (PBA). Thawed PBMCs (1.0 x 10^6 cells) were incubated for 30 min in the dark at 4°C with different combinations of fluorescent label-conjugated mouse antibodies. For analysis of expression of surface markers, the following mAbs were used: CD25 FITC, CD45RA FITC, CD38 PE, CD161 PE, CD4 PerCP-Cy5.5, HLA-DR PerCP-Cy5.5, CD8 PerCP-Cy5.5, CD45RA PE-Cy7, CCR7 PE-Cy7 (BD Biosciences, San Jose, USA), CX3CR1 PE (MBL International, Naka-ku Nagoya, Japan), CXCR3 PE (R&D Systems, Minneapolis, USA), CD3 PE-Alexa610, CD27 APC-Alexa750 (Invitrogen, Camarillo, USA), CD127 PerCP-Cy5.5, CD8 Alexa700, PD-1 PE, IL18receptor alpha FITC (eBioscience, San Diego, USA). Cells were labelled according to manufacturer’s instructions and washed and analyzed in PBA and analyzed using BD FACS Canto flow cytometer and FACS Diva Software. Analysis was done using FlowJo MacV8.6.3. Analysis was done on all or a subset of patients (table 1).
PART III - Immune modulating therapy

Table 1. Baseline characteristics of patients and healthy controls.

<table>
<thead>
<tr>
<th></th>
<th>HBV+</th>
<th>HCV+</th>
<th>Healthy controls</th>
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<tbody>
<tr>
<td>Number of subjects</td>
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<td>10</td>
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<td>19/3</td>
<td>3/7</td>
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<tr>
<td>Age, median yrs. (range)</td>
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<td>44 (25-62)</td>
<td>30 (27-60)</td>
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<td>ALT, median IU/L</td>
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<td>103 (53-494)</td>
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<tr>
<td>log viraemia, IU/ml, median (range)</td>
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<td>log HBsAg serum,median (range) IU/ml</td>
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Viral genotype

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Response to therapy SVR/NR*

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Liver biopsy

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<td>E</td>
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Fibrosis stage, median (range)**

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<tr>
<td>C</td>
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N.D. = not determined, *SVR: sustained viral response 24 weeks after therapy for HBV; HBeAg seroconversion HBV DNA<2,000 IU/mL, for HCV; HCV RNA negative. NR: non response after therapy **According to ISHAK ***: HBeAg % of HBsAg positive hepatocytes in liverbiopsy

Antigen-specific T cells

For detection of antigen-specific CD8+ T cells, PBMC were incubated with tetrameric complexes for 30 min in the dark at 4°C and subsequently with other fluorescent antibodies as described above. HLA-peptide tetramer complexes were obtained from Sanquin Reagents (Amsterdam, The Netherlands). For CMV, we used 8 different tetramers loaded with pp65- and IE-derived peptides: for EBV, we used 6 different tetramer loaded with BMLF1-, EBNA3A-, and BZLF1-derived peptides. Ex vivo HCV-specific CD8 T cell responses were analyzed using two APC-labeled tetramers. HLA-A2 tetramers contained the HCV NS3 CINGVCWTV peptide. CMV- and EBV-specific T cell responses were measured at baseline in chronic hepatitis B patients (n=8/8), chronic hepatitis C patients (n=8/7) and healthy controls (n=8/4) respectively.

Five HLA-A*02 chronic hepatitis C patients were analyzed to detect changes in HCV-specific T cell responses between responders and non-responders after antiviral therapy at week 52. HLA class I genotyping for HLA A2 was carried out by PCR.

Intracellular staining

For intracellular perforin, granzyme B, granzyme K and Ki-67 staining, the following technique was used: cells were fixed after surface staining with FACS Lysing Solution (BD). After permeabilization (FACS Permeabilizing Solution 2 (BD) cells were incubated for 30 min in

RESULTS

No changes in peripheral CD8+ T cell differentiation phenotype and function in viral hepatitis

To examine whether chronic liver infection can have a pervasive influence on the immune system we analyzed peripheral blood T cells for differentiation, migratory and activation properties and compared CD8+ T cells of chronic hepatitis patients with healthy controls. The percentage of CD3+ T cells within the PBMCs did not differ between patients infected with HBV or HCV and healthy controls (data not shown). Using a combination of CD27 and CD45RA expression, a phenotypic analysis was performed to discriminate between naïve (CD45RA+CD27-), memory (CD45RA-CD27+) and effector (CD45RA‘CD27’) CD8+ T cells. The distribution of effector, memory and naïve T cells did not significantly differ between HBV or HCV infected individuals and healthy controls (Figure 1a and 1b). In addition, no significant differences were seen in expression of CD28 and CCR7 or activation markers (data not shown).

Functional characteristics of CD8+ T cells were examined by analysis of the cytotoxic molecules granzyme B and perforin, which represent the cell-mediated killing ability. In line with the equal percentages of effector/memory T cells, no differences were seen in the expression of these cytotoxic molecules between HBV- and HCV-infected patients and healthy controls (Figure 1c). In addition, in contrast to other studies on CD8+ T cells of HBV and HCV infected patients, no differences were seen in PD-1 expression in either percentage positive cells or mean fluorescence intensity (MFI) between HBV and HCV infected patients and healthy controls (Figure 1d).

It is largely unclear why some patients with chronic hepatitis infection respond to therapy whereas others do not. CD8+ T cells are crucial in clearing virus-infected cells. Therefore we studied T cell phenotype and function before and after therapy and compared responders to non-responders. In this study phenotypic CD8+ T cells markers associated with differentiation, migration and activation properties were not altered after a favourable treatment response to therapy in HBV (sustained virologic response: HBV DNA<2,000 IU/mL +/- HBeAg seroconversion, or HBeAg seroconversion) or HCV (viral clearance) (data not shown).
Pervasive influence of chronic viral hepatitis infection on CD8+ T cells - CHAPTER 12

Figure 1. (a) In order to discriminate between differentiation of CD8+ T cells, a phenotypic analysis was performed by using a combination of CD27 and CD45RA expression: naïve T cells: CD45RA+CD27+, memory T cells: CD45RA-CD27+, effector T cells: CD45RA+CD27-. Figure shows representative FACS plots. (b) No significant differences were seen in distributions of effector, memory and naïve T cells between HBV or HCV and healthy controls (HC) (two tailed-Mann whitney U). T cells of HBV patients were measured before therapy, T cells of HCV patients were measured after therapy (c,d) perforine, granzyme B (c) and PD-1 (d) expression on total CD8 T cells did not differ between HBV or HCV and HC.

Pervasive influence of chronic hepatitis infection on other virus specific cells
An earlier study showed that in patients with chronic HCV, CMV-specific CD8+ T cells lost markers associated with effector differentiation; they had increased expression of CCR7 and reduced expression of Fas and perforin.17 Another study found that the exhaustion marker PD-1 was not only markedly upregulated on HCV-specific cells in peripheral blood and liver, but also on CMVpp65-specific cytotoxic lymphocytes in chronically infected patients compared to normal controls. These findings suggest a global effect of HCV infection on the phenotype of all T cells.24

We analyzed CMV- and EBV-specific CD8+ T cells from HBV- and HCV-infected patients and healthy controls. No differences were seen in frequencies of CD8+ T cells directed against CMV or EBV (Figure 2a). As to the differentiation status, no differences were seen in CMV- or EBV-specific cells between the groups (Figure 2b). Figure 2b shows similar percentages of effector-phenotype (CD27-CD45RA+), CCR7+ cells and perforin or granzyme B-containing cells within CMV- and EBV-specific CD8-T cells. Also no changes were seen in PD-1 expression on CMV specific cells between HBV- and HCV-infected patients and healthy controls (Figure 2c). Therefore, our data do not show a more exhausted or less mature phenotype of CMV- or EBV-specific CD8+ T cells in chronic hepatitis patients compared to healthy controls. If anything, CMV-specific T cells in HBV have an increased effector-phenotype based upon CD27 and CD45RA expression (Figure 2b) but this was not reflected in i.e. perforin expression.
PART III - Immune modulating therapy

**Figure 2.** CMV- and EBV- specific CD8+ T cells in viral hepatitis.

A) 0.4% of CD8 population

B) 0.8% of CD8 population

% virus specific cells

CMV specific cells

EBV specific cells

EBV cells

CD8

PD1

Granzyme B

Perforine

CD27- CD45RA+

CCR7 +

**Legend:**

- **CMV:** Cytomegalovirus
- **EBV:** Epstein-Barr virus
- **HBV:** Hepatitis B virus
- **HCV:** Hepatitis C virus
- **HC:** Healthy control

**Statistical Significance:**

- ns: Not significant
- p = 0.015
- p = 0.157
- p = 0.542
**Upregulation of inflammatory CXCR3 chemokine receptor expression in viral hepatitis**

Chronic HBV and HCV infections are characterized by T cell mediated hepatic inflammation, eventually leading to hepatic fibrogenesis. As the recruitment of immune cells into the infected liver is orchestrated by chemokines, we investigated the presence of chemokine receptors on peripheral blood T cells in patients with HBV or HCV compared to healthy individuals. As T-helper (Th) 1 inflammatory cells, characterized by interferon (IFN)-gamma and interleukin (IL)-2 secretion, have been shown to predominate in the liver during chronic HCV infection, we focused on Th1 chemokine receptors, in particular CXCR3. As shown in Figure 3, expression of chemokine receptor CXCR3 was significantly higher on CD8+ T cells of patient chronically infected with HBV (p<0.005) compared to healthy controls. The higher expression of CXCR3 was observed on the total CD8+ T cell population and across all T cell subsets (effector, memory and naïve T cells) and expression remained high after therapy (week 52 or 72) irrespective of the outcome. In HCV-infected patients CXCR3 was significantly higher after therapy (p<0.05) compared to healthy controls. Before therapy CXCR3 expression did only differ between naïve T cells of HCV patients and healthy controls.

Next we analyzed CXCR3 expression on HCV specific cells (Figure 3c and d). HCV specific cells were CD45RA-CD27+ indicating that they have a memory-like phenotype. Compared to total memory CD8+ T cells CXCR3 expression on HCV-specific cells was similar (Figure 3d). Detection of HBV specific cells directly ex vivo by HBV tetramers was not possible due to low frequencies. To investigate whether CXCR3 upregulation was also detectable on virus specific T cells directed to viruses not affecting the liver, CXCR3 expression on CMV and EBV specific T cells was analyzed. No differences were found in CXCR3 expression on CMV and EBV specific cells between viral hepatitis patients and healthy controls (Figure 3e).
PART III - Immune modulating therapy

Pervasive influence of chronic viral hepatitis infection on CD8+ T cells - CHAPTER 12

CXCR3 expression HCV patients

E

HBV HCV HC

CXCR3 expression CD8+ T cells correlates with disease activity in chronic HBV infection.

In HBV, we investigated whether CXCR3 expression on CD8+ T cells was associated with disease activity according to HBeAg status. HBeAg, the product of transcription of the HBcore open reading frame is involved in formation of the viral capsid and has been shown to have immunomodulatory properties. A high level of serum HBeAg is a marker of active viral replication and is associated with high viral loads in the serum.7" CXCR3 expression on all T cell subsets was increased in HBeAg positive patients compared to HBeAg negative patients (Figure 4a). Effector CD8+ T cells have the highest capacity for direct cytotoxicity and are likely involved in maintaining a situation of chronic inflammation. Therefore we analyzed whether CXCR3 expression on effector T cells correlated with liver pathology and viral load. Neither in HBV nor HCV infection a correlation was observed between CXCR3 expression on effector T cells and ISHAK fibrosis score in liverbiopsies, ALT levels or viral load in plasma (Figure 4b). However, in HBV infection the percentage of hepatocytes which stained positive for HBSAg,
which is a measure for the amount of infected hepatocytes in the liver biopsy, was associated with CXCR3 expression on effector T cells in peripheral blood (Figure 4c and d). These findings could indicate a relation between CXCR3 expression on peripheral T cells and disease activity.

**Figure 4.** CXCR3+ expression on CD8+ T cells in relation to disease activity in HBV-infected patients.

(a) A difference in CXCR3 expression on total CD8+ T cells was seen between HBeAg+ and HBeAg- patients. (b) CXCR3+ expression on effector CD8+ T cells was not associated with liver fibrosis in both HBV and HCV. (c) Representative immunohistochemistry analysis of HBsAg-staining (black) of hepatocytes in a liver biopsy of a chronic hepatitis B patient taken at start of therapy. (d) CXCR3+ expression on effector CD8+ T cells was significantly correlated with the amount of HBsAg+ hepatocytes (P value for correlation).

**DISCUSSION**

In the present study we investigated whether chronic HCV and HBV infection, both causing a localized organ-specific infection with persistent high viral loads, could have a pervasive influence on the total peripheral CD8+ T-cell population and on other virus specific T cells. We showed that CD8+ T cells in patients with chronic viral hepatitis are not different from healthy controls when focusing on differentiation phenotype. Both the total CD8+ T cell population and CMV- and EBV-specific T cells in patients with chronic HCV or HBV did not show a reduction in effector phenotype or other clear changes in maturation. These characteristics also did not change after antiviral therapy and were independent of the treatment outcome.

However, we did find a pervasive influence of the chronic liver infections with respect to expression of the chemokine receptor CXCR3. CXCR3 expression was increased on peripheral blood CD8+ T cells chronic HBV and HCV patients with a localized liver inflammation compared to healthy individuals infected with a latent CMV, infection (Figure 3). The CXCR3 upregulation was also found on HCV-specific T cells. However, CMV and EBV virus-specific cells that do not specifically target the liver do not seem to be affected by the upregulation of CXCR3. This may indicate that CMV and EBV specific T cells that do not specifically home to the chronic inflammatory milieu in the liver, avoid trigger signals that lead to upregulation of CXCR3. Interestingly, the inflammatory effect seems not to be restricted to T cells as also the total B cell population in patients with HCV and HBV infection has been shown to have increased activation markers and CXCR3 expression. Thus far it is unclear which factors in the inflammatory environment directly regulate CXCR3 expression.

The upregulation of CXCR3 in HBV and HCV infected patients was most prominent on naïve CD8 T cells. It has been shown in mice that naïve phenotype bystander CD8 T cells could indeed
be sensitized by acute viral infections via low affinity MHC-TCR interactions in combination with type I interferon such that they temporarily upregulate cytotoxic molecules prior to cognate antigen stimulation. Perhaps this phenomenon plays a role in the ‘bystander’ CXCR3 upregulation on naive CD8 T cells.

Our in vitro experiments, showed upregulation of CXCR3 upon TCR-mediated activation of the T cells but could not confirm CXCR3 upregulation upon cytokine stimulation. However, the in vivo environment is difficult to simulate and upregulation of CXCR3 may only occur in the context of a “cytokine storm” with an accumulation of inflammatory cytokines. Although hard to address experimentally, it is likely that T cells recirculate from the liver, where they are exposed to an inflammatory milieu, back to the blood while maintaining high expression of CXCR3.

Next to inflammatory cytokines also alterations in chemokine ligands, direct effects of interferon and induction of chemokines by viral proteins could be associated with CXCR3 expression. CXCR3-associated chemokines, CXCL9, CXCL10 (IP10) and CXCL11 have been shown to be overexpressed in hepatic parenchyma of patients with viral or non-viral chronic liver disease. These observations suggest a specific role for CXCR3 and its ligands in the recruitment of T cells into these otherwise restricted sites. Recent studies in mice have revealed that CXCR3 may play a crucial role in directing T cell migration to the proximity of inflammatory cytokines or antigen. These studies suggest that in this way CXCR3 expression can influence cell fate decision that controls effector versus memory commitment early after T cell activation.

It is unclear what determines the feedback loop between the presence of chemokine ligands and expression of their chemokine receptor; both increased and decreased chemokine levels have been associated with in CXCR3 upregulation. In HCV patients we found that CXCR3 expression was increased upon therapy compared to healthy controls. Indeed it has been shown that HCV proteins such as NS5a and core could directly induce synthesis of CXCR3 ligands CXCL10 and CXCL9 in the liver. In another study the increase of CXCR3 during therapy has been associated with favourable response to therapy, which in our study could not be confirmed. As upregulation of CXCR3 on CD8+ T cells is not seen during treatment in HBV-infected patients who also receive exogenous interferon, upregulation due to a direct effect of type I interferon seems less likely.

CXCR3 expression has been associated with liver inflammation, but not with fibrosis. Also in our study no correlation between CXCR3 expression and fibrosis grade was present. This indicates that other factors such as the duration of liver inflammation but also genetic and non-genetic factors are necessary to develop fibrosis. The finding that CXCR3 expression on T cells correlated with the amount of HBV antigen in the liver (Figure 5) is certainly of interest in this respect. Also the higher CXCR3 expression observed in HBeAg positive chronic hepatitis B patients could reflect the higher viral load that is seen in these patients compared to patients without detectable serum HBeAg. It is however plausible that increased antigen expression is accompanied by elevated inflammation, making it difficult to distinguish one from the other.

Summarizing, we have shown in a well defined cohort of patients that chronic HCV infection, just like chronic HBV infection, does have a pervasive influence on the peripheral blood CD8+ T cell pool. Not so much by changes in the maturation or differentiation of the T cells, but by increased expression of the chemokine receptor CXCR3 which is involved in migration to the inflamed liver. The continuous antigen load and inflammation may alter the functional status of both HBV/HCV-specific and non-specific T cells in order to redirect immune cells to the site of infection. Further research is necessary in order to investigate whether modulating chemokine expression could be a potential target for future therapy optimizing the anti-viral immunologic environment in the liver.
REFERENCES