Lymphocytes and liver fibrosis in HIV & HCV coinfection

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(met samenvatting in het Nederlands)

Illustration on the cover: *from healthy liver to liver fibrosis*, by the author of this dissertation, depicting the architecture of the healthy liver (on the left side) and progression towards liver fibrosis (on the right side) including deposition of collagens, infiltration of lymphocytes and loss of hepatocytes.

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Lymphocytes and liver fibrosis in HIV & HCV coinfection

Lymfocyten en leverfibrose in HIV & HCV coinfectie
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door

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Chapter 1

General introduction

Hepatitis C virus epidemiology and perspective

Hepatitis C virus (HCV) is a major cause of chronic liver disease with an estimated prevalence of 2.35% worldwide, affecting around 160 million individuals in 2010, with higher prevalence in low-income countries.[1] In the Netherlands, seroprevalence is presently estimated at 0.22% (28.100 individuals).[2] However, estimations of epidemiology of HCV are hampered by asymptomatic course, often during decades, and lack of appropriate surveillance.[3] The virus is mainly transmitted via blood contact but may also occur via the placenta from mother to child or via sexual intercourse.[4] Injected drug use, blood transfusion before 1992, high lifetime number of sexual partners and dialysis are important risk factors.[5] In the Netherlands, immigrants from high-endemic countries are ten times more likely to be infected with HCV than the native Dutch inhabitants.[2] Notably, in recent years HCV has become a significant problem in MSM.[6]

HCV infection became apparent in the twentieth century due to parenteral transmission routes of medical treatment, blood transfusions and injected drug use. After a long search for the cause of non-A non-B hepatitis, HCV was finally discovered in 1989.[7, 8] Since then, the field of hepatitis C research has rapidly progressed, leading to improved understanding of its pathogenesis and immunology and to new diagnostics and effective therapeutic interventions. Recent major developments in the field of HCV research include the development of a genetically humanized mouse model for HCV-infection,[9] the discovery of the role of microRNAs (miRNAs) in viral replication,[10] recognition of IL28B as a prognostic marker in outcome of HCV infection,[11-14] implementation of non-invasive measurements in evaluation of liver fibrosis[15] and introduction of direct acting antiviral agents (DAA), including protease inhibitors such as boceprevir and telaprevir, which dramatically improved treatment efficiacy.[16, 17] However, HCV still presents a major challenge, since present treatment options are complicated with severe side effects, and due to high costs are not available in many countries with high prevalence. [18]

Infection with HCV

HCV is a single-stranded virus belonging to the family of Flaviviridae and has a genome consisting of 9.5 kB positive stranded RNA encoding for three structural proteins, a viroporin, six nonstructural proteins and the so-called F-protein.[19, 20] Hepatitis C virus consists of at least six different genotypes, of which genotype 1 is the most common in Europe.[5, 21]

Acute infection with HCV is usually asymptomatic or accompanied with minor symptoms.[22] In most cases, HCV can be detected in the serum within 2 weeks after infection.[22] Spontaneous clearance may occur, but HCV often causes chronic infection,

defined by persistent viral RNA six months after transmission.[23]

Several viral and host factors are associated with outcome of infection. For instance, HCV genotypes 1 and 4 are associated with low spontaneous viral clearance and decreased rates of response on treatment with interferon-α - the first line treatment option for chronic HCV infection,[22, 24] in comparison to genotypes 2, 3, and HCV-genotype remains relevant with the introduction of protease inhibitors and direct acting antivirals.[24] Male gender, African ancestry and coinfection with HBV or HIV are other negative predictive factors for spontaneous viral clearance and treatment response in HCV-infection.[24-26] From 2009 on, a number of research groups have independently and consistently reported that single nucleotide polymorphisms (SNP) near the interleukin-28B gene (IL28B) were associated with spontaneous clearance and interferon-based treatment response, especially in HCV genotype 1 and 4.[11-14, 27]

Liver fibrosis

The major consequence of chronic infection with HCV is the development of liver fibrosis, which in turn may lead to hepatic insufficiency, development of hepatocellular carcinoma (HCC) and hemorrhage from esophageal varices.[22, 28] Progression to this end stage of liver disease is slow but variable, and typically develops over decades with usually no or nonspecific symptoms.[29] It is generally accepted that chronic HCV results in liver fibrosis and eventually cirrhosis in 25-30% of patients, 20-30 years after infection, with a wide individual variance.[30] The rate of HCC is 1% to 3% over 30 years in HCV-infected patients.[18] Factors associated with progression of liver fibrosis include HCV genotype 3, age, duration of infection, alcohol consumption, coinfection with HBV or HIV (especially with low CD4 T cell numbers), male gender and the metabolic syndrome.[31-33]

Liver fibrosis is characterized by excessive deposition and changed composition of extracellular matrix (ECM) in the liver, as a reversible wound-healing response to liver injury.[31] Hepatic fibrogenesis results from activation of resident mesenchymal cells, mainly hepatic stellate cells (HSCs). In the healthy liver, quiescent HSCs are found in the subendothelial space of Disse and contain high storage of vitamin A. Activation of HSCs may results in proliferation of fibrogenic myofibroblasts (MFs), which are contractive, release proinflammatory and fibrogenic cytokines and produce ECM components including collagens I, III and IV. Activated HSCs are thought to be the primary contributors to MFs and are therefore essential in liver fibrosis.[29] Furthermore, various studies have shown that HSCs are also involved in reversibility of liver fibrosis (*figure 1*).[34, 35]

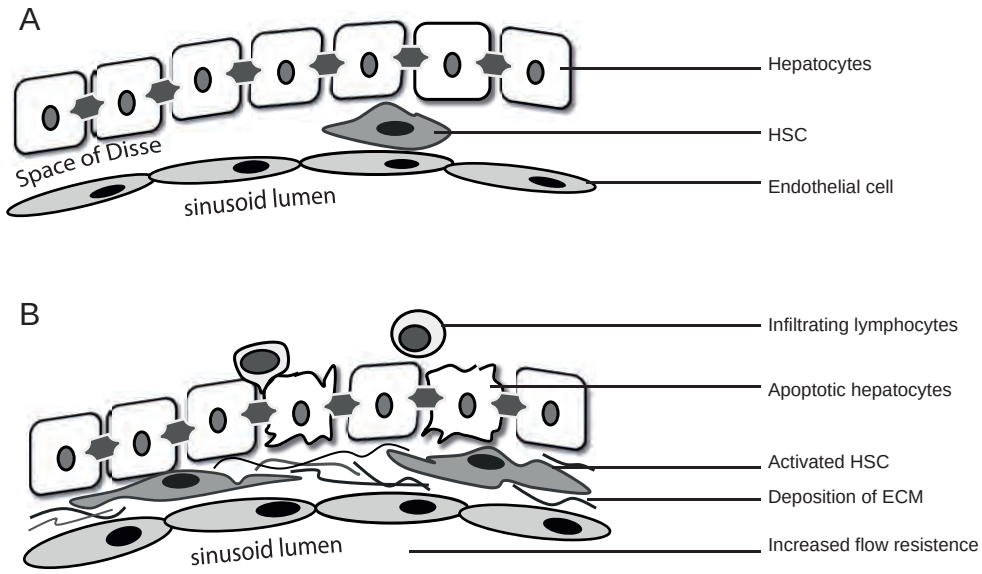

Figure 1: liver fibrosis. A: The healthy liver, with endothelial cells lining the sinusoid lumen and hepatic stellate cells (HSCs) in the space of Disse, between the endothelial linage and hepatocytes. B: Changes associated with liver fibrosis, with infiltrating lymphocytes leading to apoptosis of hepatocytes and activation of HSCs, deposition of excessive extracellular matrix (ECM) and increased flow resistance in the sinusoid lumen.

Immunology of HCV

Chronic infection with HCV is the result of the incapability of the host immune system to clear the virus and is partly due to downregulation of immune responses.[36, 37] For instance, the hepatic immune environment is known to be tolerogenic, as the liver is exposed to a wide range of systemic and gut-derived antigens, including microorganisms. Various cell types contribute to a specific immune environment in which antigens are neutralized but at the same time immune-induced damage, i.e. liver fibrosis, is prevented.[38] Both the innate and adaptive immune systems are involved in this process.[29] In the case of HCV-infection, viral control is mediated by both innate and adaptive immune responses.[39, 40] Innate immunity includes effector cells such as natural killer (NK) cells, and interferon responses, whereas T cells are important players of the adaptive immune arm.[40]

Innate immunity

The innate immune system in the liver comprises cells of different origins, including natural killer (NK) cells, dendritic cells, liver-resident macrophages (Kupffer-cells), but also hepatic stellate cells (HSC) and endocytes.[41] Early innate immune responses to viral infections are activated upon recognition of pathogen associated molecular patterns (PAMPs) by specific pattern recognition receptors.[42, 43] Upon infection with HCV, hepatocytes produce type 1 interferons and upregulate interferon-stimulated genes (ISG) in a RIG-I dependent manner.[43, 44] In turn, interferon-α/β and ISG are able to limit HCV replication and mediate NK cell activation and proliferation.[45-48]

NK cells represent a lymphocytic cell line that is a component of the innate immune system with both cytotoxic and cytokine-producing effector functions.[49] NK cell responses to viral infection are typically rapid, and occur before adaptive immune responses have developed.[50, 51] NK cells can be divided in different subsets, based on relative expression of CD56 and CD16.[52] Various studies have shown that $CD56^{bright}$ NK cells are likely to be precursors of $CD56^{dim}$ NK cells, since the latter can develop from $CD56^{bright}$ NK cells upon activation.[53, 54] $CD56^{dim}$ NK cells make up around 90% of the NK cells in the peripheral blood and are generally considered as the principal cytotoxic subsets, based on the high level of cytoplasmatic granules containing perforin and granzymes.[55, 56] The $CD56^{bright}$ NK cells make up around 5-10% of peripheral NK cells, but are relatively expanded in lymphoid tissues and in the liver.[57] $CD56^{bright}$ cells are thought to merely exert cytokine-producing effects, as early studies have shown a high production of IFN-γ, TNF-α and other cytokines upon stimulation.[58]

Upon activation, which depends on interaction of several activating and inhibitory receptors with their ligands,[59, 60] NK cells can exert their cytotoxic functions by two major mechanisms which are both also employed by cytotoxic T lymphocytes.[60, 61] The first pathway involves secretion of cytoplasmic granule toxins (predominantly perforin and granzymes) by exocytosis. In the second pathway, death receptors (such as Fas) on target cells are engaged by their cognate ligands (such as FasL) on NK cells. Both mechanisms may result in death of the target cells, in a process of programmed cell death which is referred to as apoptosis.[60, 61]

Chronic hepatitis C infection is associated with decreased percentages of peripheral NK cells, but despite this, overall cytotoxicity is normal or even increased, due to increased cytotoxicity per cell.[62, 63] Several studies have shown that the $CD56^{bright}$ NK cell subset is expanded relative to the $CD56^{dim}$ NK cell subset in chronic HCV.[64-66] Furthermore, altered expression of several activating and inhibiting receptors on NK cells is linked to outcome of HCV infection and the development of liver fibrosis and cirrhosis,[50, 67-72] indicating that NK cells play a major role in immunity against HCV. Indeed, NK cells may exert an antiviral role by production of IFN-γ, but may also kill T cells and

attenuate liver fibrosis by killing of activated HSCs.[50, 73] However, due to limited data on NK cells in chronic HCV infection, the precise role of these cells in pathogenesis of viral hepatitis is presently not well defined.

Adaptive immunity in HCV infection

Adaptive immunity involves the antigen-specific immune response of T cells and B cells producing antibodies.[74] Around two months after HCV-infection, specific neutralizing antibodies against HCV can be detected in the peripheral blood.[19] However, except use of these antibodies in diagnosis of HCV infection, the role of these antibodies in viral clearance or control is still controversial.[7, 19, 75]

Several reports have underlined the importance of a vigorous T cell response in the spontaneous clearance of hepatitis C.[76-78] Upon infection with HCV, nearly all individuals develop vigorous specific CD4 T cell responses to a wide range of epitopes, irrespective of clinical outcome, but in most patients these T cell responses become undetectable in time as infection persist, while they remain stable in case of viral clearance.[78-80]

HCV-specific CD8 T cell responses in the peripheral blood and in the liver are strongly associated with viral clearance, and depletion studies in chimpanzees have revealed that virus specific CD8 T cells are essential effector cells in control of HCV replication.[77, 81, 82] In addition, depletion of CD4 T cells from HCV-recovered chimpanzees abolishes CD8+ T cell immunity upon re-infection.[83] These findings suggest that CD4 T cell help is essential for CD8 T cell-mediated suppression of viral replication. However, despite the well-established role of T cells in the outcome of viral infection and the protective role of specific T cell responses in re-infection, the exact features of an effective T-cell response, which should pave the way for an effective vaccine, remain elusive.[84]

In addition to their role in viral control, T cells may also be involved in liver fibrogenesis by modulation of the local cytokine response by CD4 T cells or amplification of tissue injury due to bystander killing by CD8 T cells.[85] However, the role of T cells in liver fibrosis remains debated, since actual evidence is scarce and controversial.[29, 85] For instance, CD8 T cells were found to contribute to liver fibrosis in transgenic mice.[86] On the other hand, in livers of HCV-infected individuals, IL10-producing HCV-specific CD8 T cells were found in relatively healthy areas of the infected liver, suggesting that CD8 T cells may in fact also attenuate liver fibrosis.[87] In addition, CD8 T cells in the liver of HCV infected individuals are hyporesponsive to CD3 triggering, which further questions their contribution to liver fibrosis.[88] Taken together, although a number of pro- and antifibrotic mechanisms have been revealed, the relevance of these pathways remains unclear to date.

Suppression of adaptive immunity through T-cell exhaustion and apoptosis

A number of studies have shown that prolonged viral infection results in a gradual loss of cytotoxic, proliferative and cytokine producing effector functions, in a process which is called 'T cell exhaustion'.[37] Severity of T cell exhaustion is determined by various factors, including levels of stimulatory and suppressive cytokines and receptors, availability of CD4 T cell help and duration and magnitude of antigenic activation.[89] Several cellular markers of T cell exhaustion have been identified, including PD-1 and Tim-3.[37] Indeed, HCV-specific T cells expressing PD-1 and Tim-3 lose cytotoxic and proliferative functions.[90-92] Moreover, HCV-specific T cell cytotoxicity can be restored by blockage of PD-1 and Tim-3.[92-95] Although associated with prolonged infection, it can be hypothesized that T cell exhaustion is also beneficial as a host mechanism to prevent immune mediated damage in the liver, since chronic HCV infection results in decades of asymptomatic liver disease despite massive infection of hepatocytes and high rate of viral replication.

Another host mechanism to prevent immune mediated damage in chronic infection is to maintain T cell homeostasis through activation induced cell death (AICD) of T cells.[96] T cell apoptosis may be induced by two distinct pathways. The extrinsic pathway involves engagement of a death receptor (e.g. Fas / CD95) by its receptor (e.g. FasL / CD178), which results in the formation of a death-inducing signaling complex (DISC), in which Fas-associated death domain (FADD) interacts with the intracellular tail of a death receptor and with procaspase 8.[97-101] Cleavage of procaspase 8 by FADD leads to activated initiator caspase 8, which in turn activates effector caspases 3 and 7 to initiate apoptosis.[97] Alternatively, apoptosis may be initiated via the intrinsic apoptosis pathway by a variety of cellular or mitochondrial stressors. Following the death trigger, mitochondria become partly permeabilized and release cytochrome c into the cytosol, where it binds to Apaf-1 to form a complex known as the apoptosome.[102, 103] Initiator caspase 9 is recruited to the apoptosome to become activated and subsequently it cleaves effector caspases 3 and 7 to induce apoptosis.[102, 103] The anti-apoptotic protein Bcl-2 is a key protein in regulation of the intrinsic apoptosis pathway through controlling the permeabilization of the mitochondrial outer membrane that releases cytochrome c into the cytosol (*figure 2*).[104-106]

Indeed, chronic HCV infection is associated with increased T cell apoptosis.[107] However, it is presently unclear if this is the result of chronic T cell activation and ongoing AICD, or whether this is actually the result of interaction with the inflamed environment of the liver, since expression of HCV structural proteins by hepatocytes can induce T cell apoptosis in vitro.[108] The latter mechanism would imply that progression of liver disease, i.e. liver fibrosis, might be reflected by the level of T cell apoptosis in the peripheral blood.

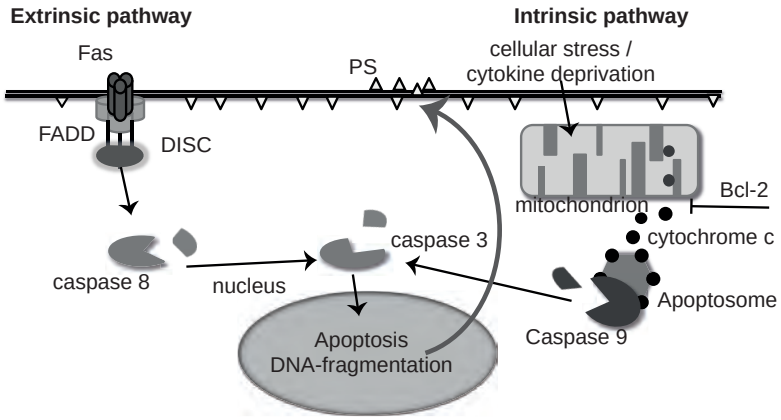

Figure 2: activation of the intrinsic and extrinsic apoptosis pathways. The extrinsic apoptosis pathway is activated upon engagement of a death receptor (e.g. Fas) by its ligand (e.g. FasL), which results in the formation of a death inducing signaling complex (DISC) and subsequently activation of caspase 8 and caspase 3. The intrinsic apoptosis pathway can be activated by various cellular stressors, upon which cytochrome C is released from the mitochondrion into the cytosol, leading to formation of an apoptosome in which caspase 9 is activated which in turn activates caspase 3. Bcl-2 may prevent mitochondrial release of cytochrome c and thus activation of the intrinsic apoptosis pathway. Caspase 3 activation leads to apoptosis as characterized by DNA fragmentation and expression of phosphatidylserine (PS) on the outer membrane of the cell, while PS is normally restricted to the inner cell membrane.

HIV and HCV coinfection

HIV-HCV coinfection is a common clinical condition, due to shared routes of transmission.[3, 109] HCV infection has been found in 25–30% of HIV-positive persons, but this number is highly variable among regions and risk groups.[109] HIV-HCV coinfection is most common in injection drug users, where prevalences of 72% to 95% have been reported.[109] Co-infection with HIV has major clinical consequences, since it is associated with immune dysregulation, resulting in accelerated progression of liver fibrosis and decreased rates of spontaneous viral clearance and treatment response.[110]

Several mechanisms may contribute to accelerated hepatic fibrosis in HIV-HCV coinfected patients.[111, 112] Firstly, HIV may lead to production of TGF-β, which is a key factor in hepatic fibrogenesis, and increased HCV replication.[113] In addition, both HIV and its envelop protein gp120 may induce cell signaling in hepatocytes, HSCs and other immune cells through interactions with chemokine receptors.[114-116] Furthermore, HIV may lead to upregulation of death receptors and enhanced apoptosis of hepatocytes.[117] Alternatively, immune activation may also contribute to hepatic fibrogenesis.[118, 119] However, the exact contribution of these mechanisms to liver fibrosis in HCV-HIV infected individuals is unclear.[111, 112]

Once HIV is adequately treated, liver fibrosis progression becomes comparable to that in HCV-monoinfected patients, but response to treatment with interferon and ribavirin treatment remains poor.[120-122] In addition, antiviral treatment is associated with increased toxicity in HIV-HCV coinfected patients.[123, 124] However, introduction of DAAs for HCV-infection dramatically increases HCV-treatment responses also in HIV-HCV coinfected patients.[125]

The underlying mechanism responsible for immune dysregulation in HIV-HCV coinfection is currently not fully understood.[110, 111] With respect to decreased rates of spontaneous clearance and HCV-treatment responses, several mechanisms have been proposed, including HIV- and HCV interaction with T cell activation, exhaustion and apoptosis, as well as altered activation of NK cells.[111, 126]

Although a number of studies have focused on the impact of HCV on HIV-infection, no definite conclusions could be drawn, since results have been conflicting.[110, 127] A meta-analysis of 37 studies covering over 100,000 patient found no increase in mortality in co-infected patients in comparison to HIV monoinfected patients before the introduction of highly active anti-retroviral treatment (HAART).[127] In the HAART-era, HIV-HCV coinfection was associated with increased mortality, but not with AIDS-defining events.[127] Moreover, a recent study demonstrated that clinical events and death in HIV-HCV coinfected patients were independently associated with stage of hepatic fibrosis, suggesting that the degree of liver disease itself contributes to excess in morbidity and mortality in HIV-HCV coinfected patients.[128]

Scope of this dissertation

The main focus of the studies presented in this dissertation was to find potential biomarkers of disease progression in HCV monoinfection and HIV-HCV coinfection and to gain more insight in immunological patterns associated with these clinical conditions. More specifically, we aimed to identify non-invasive markers of liver fibrosis in HCV infection. To this end, immunological markers related to T cell and NK cell phenotypes were assessed in HCV monoinfected and HIV-HCV coinfected patients in relation to clinical status of liver disease. In addition, clinical impact of transient elastography was assessed in a retrospective cohort of viral hepatitis patients.

Since recent findings by Arends *et al* demonstrated that HCV monoinfected patients have increased levels of peripheral CD4 and CD8 T cell apoptosis,[107] we hypothesized that peripheral T cell apoptosis might reflect liver fibrosis stage. To test this hypothesis, we explored peripheral T cell apoptosis and its relationship with disease parameters in a larger cohort of HCV monoinfected and HIV-HCV-coinfected patients with known level of fibrosis (ACATLIFE-study), as well as in healthy and disease (HIV, HBV, PBC)

controls, as presented in **chapter 2**. Since a sub-analysis was suggestive for a differential effect of combined antiretroviral treatment (cART) regimens for HIV-infection on peripheral T cell apoptosis, this was further addressed in an additional study in HIV monoinfected patients (ARTA-study; **chapter 3**).

From the ACATLIFE study, we also explored T cell and NK cell phenotype, in order to investigate whether liver fibrosis or coinfection with HIV is associated with distinct features of immune activation and exhaustion in HCV infection. The relationship between HIV-HCV disease parameters and level of T cell activation and exhaustion is addressed in **chapter 4**, while **chapter 5** focuses on NK cell phenotype and its changes associated with HCV and HIV infection.

Bridging the gap between immunology and patient care, we investigated whether CD4/CD8 ratio could be used as a marker of liver fibrosis in HCV monoinfection in **chapter 6**. Furthermore, the clinical impact of transient elastography, as another non-invasive estimation of liver fibrosis, was investigated in a different cohort (FibroScan-study) and presented in **chapter 7**.

In the last chapter, the main findings of this dissertation are discussed in the light of the current knowledge in HCV-research and possible implications of the presented studies are highlighted (**chapter 8**).

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Chapter 2

Activation of the extrinsic apoptosis pathway in HCV monoinfected and HIV-HCV coinfected patients, regardless of liver disease severity

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Abstract

Introduction: Chronic HCV infection is associated with increased levels of peripheral T cell apoptosis. We aimed to study whether T cell apoptosis markers indicate pathways that may contribute to clinical progression in HCV monoinfected and HIV-HCV coinfected patients.

Methods: Activation of the extrinsic apoptosis pathways was measured by levels of death receptor Fas, initiator caspase 8 and effector caspases 3&7 activity and Annexin V binding on peripheral CD4 and CD8 T cells of HCV monoinfected and HIV/HCV coinfected patients, as well as healthy controls and HIV-infected, HBV-infected and PBC disease controls. Association with liver fibrosis was assessed by biopsy or by transient elastography.

Results: HCV monoinfected and HIV-HCV coinfected patients displayed enhanced peripheral CD4 and CD8 T cell apoptosis. Caspase 8 activity was highest in HIV-HCV coinfection, without enhanced downstream activity of caspases 3&7. Level of peripheral T cell apoptosis was independent of liver fibrosis or other disease parameters in all disease groups.

Conclusions: the extrinsic apoptosis pathway is upregulated in HCV monoinfection and HIV-HCV coinfection, but this is independent of liver disease severity.

Introduction

HCV leads to chronic infection in the majority of patients.[1] Ongoing viral replication in hepatocytes triggers chronic hepatic inflammation, which may over time result in liver fibrosis and finally cirrhosis.[1, 2] Vigorous CD4 and CD8 T cell responses are essential in viral clearance as can be observed in the first months after infection, but viral persistence is associated with downregulation of T cell immune responses in several ways, including apoptosis of activated T cells.[3-6] Indeed, chronic HCV infected patients display higher levels of peripheral T cell apoptosis than healthy controls.[7] T cell apoptosis may not only allow viral replication, but may also contribute to liver fibrosis, since hepatic stellate cells (HSC) may become activated upon phagocytosis of apoptotic lymphocytes.[8] Activated HSCs are the main producers of extracellular matrix in liver fibrogenesis.[9]

Cellular apoptosis is a major pathway of cell death, and may be initiated either by stimulation of death receptors (extrinsic pathway) or by mitochondrial damage (intrinsic pathway), which are both affected in hepatocytes and PBMCs of HCV- and HIV-infected patients.[7, 10, 11] The extrinsic pathway involves stimulation of a death receptor (e.g. Fas) by its receptor (e.g. FasL), which results in the formation of a death-inducing signaling complex (DISC). In the DISC, Fas-associated death domain (FADD) interacts with the intracellular tail of a death receptor and with procaspase 8.[12-16] Cleavage of procaspase 8 by FADD leads to activated initiator caspase 8, which in turn activates effector caspases 3 and 7 to initiate apoptosis.[12]

Coinfection with human immunodeficiency virus is relatively common in HCV-infected patients due to shared routes of viral transmission.[17] HIV-HCV coinfection is associated with accelerated progression of liver fibrosis and with poor response to HCV treatment.[17-19] Several factors may contribute to this poor prognosis in coinfected patients, including T cell apoptosis.

The present study was performed on freshly isolated peripheral blood mononuclear cells (PBMCs) in order to further elaborate on our previous findings of increased peripheral T cell apoptosis in chronic HCV infected patients.[7] Here, we specifically aimed to evaluate whether level of T cell apoptosis is a reflection of the degree of liver fibrosis. To this end, patients with other liver disease (hepatitis B virus (HBV) infection or primary biliary cirrhosis (PBC)) resulting in liver fibrosis were included as disease controls.

Methods

Patients

Eighteen HCV monoinfected and 14 HIV-HCV coinfected patients with known stages of liver fibrosis were included in this study. Controls comprised of 15 healthy controls and 10 HIV-monoinfected, 18 treated HBV infected and 14 PBC patients as disease controls. All patients were recruited from the Infectious Diseases outpatient clinic

or from the Hepatology outpatient clinic of the University Medical Center Utrecht (UMCU). Data on T cell activation and exhaustion, including Fas-expression, of part of the subjects have recently been published elsewhere.[20] Patients with other clinical conditions possibly interfering with their immune system (e.g. liver disease from other causes, alcohol abuse, use of opioids, auto-immune disease, malignity or any other severe systemic diseases) were excluded from the study. None of the patients received treatment for HCV at the time of inclusion or within the preceding 12 months. All HIV-infected patients were on highly active antiretroviral therapy resulting in CD4+ T-cell-counts >200/mL and undetectable HIV-viral load (<50 copies/mL). Fibrosis stage was assessed by liver biopsy or transient elastography as previously described.[20] All HBV-infected patients were on antiviral nucleoside analog treatment, resulting in low HBV-DNA (<1000 IU/mL).

Disease parameters

Levels of HCV-RNA and HIV viral load were measured with COBAS® AmpliPrep/ COBAS® TaqMan Polymerase Chain Reaction (PCR; lower limit of detection 15 IU/mL for HCV and 50 copies/ml for HIV). HBV-DNA loads were measured with COBAS AmpliPrep/TaqMan quantitative polymerase chain reaction, (qPCR, lower limit of detection 20 IU/ml). Blood from anonymous healthy controls was requested from the bloodbank Mini Donor Dienst of the UMC Utrecht and was tested negative for hepatitis B, hepatitis C and HIV. All patients and healthy controls were between 18 and 65 years old.

Informed consent was obtained in writing from all patients in accordance with the WMA Declaration of Helsinki and in accordance with the ICH guideline for Good Clinical Practice (6th revision, 2008). The medical ethics committee for research in humans (METC) of the University Medical Center Utrecht, The Netherlands, approved the protocol of this study.

Processing of blood for isolation of PBMCs and flow cytometric analysis

Whole blood was collected and processed as previously described.20 Then, PBMCs were incubated with antibodies against CD3 (HorizonTM V500, clone SP34, provided by BD Biosciences, San Diego, US), CD4 (APC-eF780, RPA-T4, eBioscience, San Diego, CA, USA; or PE-Cyanine7, RPA-T4, eBioscience), CD8 (Pacific BlueTM, RPA-T8, BioLegend, San Diego, CA, USA; or APC-eF780, RPA-T4, eBioscience) and Fas (APC, DX2, BD Biosciences), for 20 minutes at 4˚C. After washing, cells were incubated with fluorescent inhibitor of caspases (FLICA) for measuring of activity of extrinsic initiator caspase 8 or effector caspases 3 and 7 (FITC, ImmunoChemistry Technologies, Bloomington, MN, USA) at 37˚C (1x106 cells per sample). For detection of surface phosphatidylserine (PS), cells were incubated with Annexin V (eF450, eBioscience) for 15 minutes at room temperature and then directly analyzed by flow cytometry. Per sample, 100.000 events were acquired. In all patients, laboratory procedures were performed within 16 hours from venipuncture.

Statistical analysis

Medians were compared with Mann Whitney test and, in case of multiple groups, preceded by Kurskal-Wallis. Two-way ANOVA was used for comparing medians with two independent variables. Chi square test was used to test relationships between categorical variables. Dependence of variables was tested using Spearman's one-tailed correlation coefficient. Statistical analysis was performed with IBM SPSS Statistics version 19.0 (SPSS Inc., IBM, USA) and GraphPad Prism 5 for Windows version 5.03 (GraphPad Software, Inc, USA).

Results

Patient characteristics

A total of 89 subjects, including 18 chronic HCV monoinfected patients, 10 HIV-1 monoinfected patients, 14 HIV/HCV coinfected patients, 18 HBV infected patients, 14 PBC patients and 15 healthy controls were included in this study. Patient characteristics are shown in *table 1* and most are comparable between the patient group. As expected, HCV-RNA loads were higher in coinfected (1.9e6 IU/mL) compared to HCV monoinfected patients (3.5e5 IU/mL; p=0.07). PBC patients (median 61 years) and HCV monoinfected patients (median 55 years) were older than other patients (group medians 46 - 48 years), but the degree of liver fibrosis was similar in all patient groups (*table 1*).

Table 1: Patient characteristics

	HCV	HIV-HCV	HIV	HBV	PBC	
	n=18	n=14	n=10	n=18	n=14	p-value[1]
General characteristics						
Age, median (IQR), years	55 (51-60)	48 (40-54)	46 (42-58)	48 (40/56)	61 (54/69)	0.02
Gender, % male	72%	86%	90%	78%	29%	0.43
ALT, median (IQR), IU/L	72 (48-144)	65 (54-134)	30 (19-41)	33 (23/38)	43 (28/56)	0.94
Liver fibrosis / cirrhosis						0.69
F0-F2, number (%)	9 (50%)	8 (57%)		8 (44%)	7 (50%)	
F3-F4, number (%)	9 (50%)	6 (43%)		10 (56%)	7 (50%)	
HCV characteristics						
HCV-RNA, med. IU/mL (IQR)	3.5 e5 (1.5e5-9.5e5)	4.5 e5 (4.0e5-5.2e6)				0.05
Genotype, % genotype 1	77%	83%				0.66

General and disease-specific characteristics of patient groups. [1] p-value for comparison of HCV with HIV-HCV. Abbreviations: ALT, alanine transaminase; IQR, interquartile range; PI, protease inhibitor; ART, highly active antiretroviral treatment.

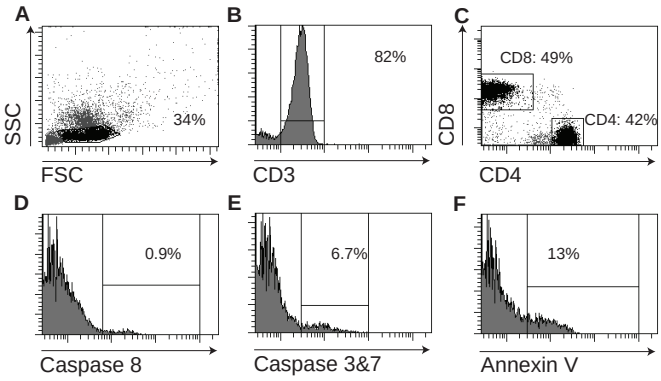

Figure 1: gating strategy and representative flow cytometry plots of apoptosis markers. Representative flow cytometry plots of T cell apoptosis in an HIV-HCV coinfected patient. A-C: gating of T lymphocytes by lymphogate (A), CD3 positive T cells (B) and CD4 and CD8 positive T cells (C); D: activated initiator caspase 8 in CD4 T cells; E: activated effector caspases 3 and 7 in CD4 T cells, F: Annexin V positive CD4 T cells. Percentages of positive events are depicted in each plot.

Increased activation of the extrinsic apoptosis pathway in HCV monoinfected and HIV-HCV coinfected patients

In order to determine levels of peripheral T cell apoptosis, we characterized surface expression of death receptor Fas,[20] intracellular levels of activated initiator caspase 8 and activated effector caspases 3 and 7 as well as Annexin V-binding to phosphadidylserine (PS) on peripheral CD4 and CD8 T cells (*figure 1*).

In line with previous findings from our group,[7] CD4 and CD8 T cell of HCV-infected patients depicted overall higher levels of markers of T cell apoptosis than healthy controls. This was evidenced by higher median percentages of Fas (CD4: 61.6% versus 39.5%, p=0.0012 and CD8: 29.2% versus 16.2%, p=0.0971), and activated caspases 3&7 (CD4: 1.0% versus 0.63%, p=0.07 and CD8: 0.9% versus 0.61%, p=0.01) compared to healthy controls. In contrast, caspase 8 (CD4: 5.5% versus 4.9%, p=0.16 and CD8 4.2% versus 3.6%, p=0.29) and Annexin V-binding (CD4: 7.1% versus 9.2%, p=0.56 and CD8 7.5% versus 10.3%, p=0.58) were similar to healthy controls (*figure 2*). HIV-HCV coinfected patients depicted a similar pattern in comparison to healthy controls, though activated caspase 8 was present at higher levels in coinfected patients in comparison to HCV monoinfected patients (CD4: 8.8% versus 5.5%, p=0.04; CD8: 9.2% versus 4.2%, p=0.02) and healthy controls (CD4: 8.8% versus 4.9%, p=0.0018; CD8: 9.2% versus 3.6%, p=0.0037) (*figure 2*). Correlations between Fas-expression and downstream caspase activation and annexin V-binding further support the hypothesis that the extrinsic apoptosis pathway is upregulated in chronic HCV-infection, similar to infection with HIV (*figure 3*).

CD4 and CD8 T cells of treated HBV patients displayed higher levels of Fas-expression than healthy controls without downstream caspase-activation, while CD4 and CD8 T cells of PBC-patients depicted increased levels of Fas and activated caspases 3&7 in comparison with healthy controls (*figure 2*). This indicates that increased levels of peripheral T cell apoptosis is not specific for viral infection.

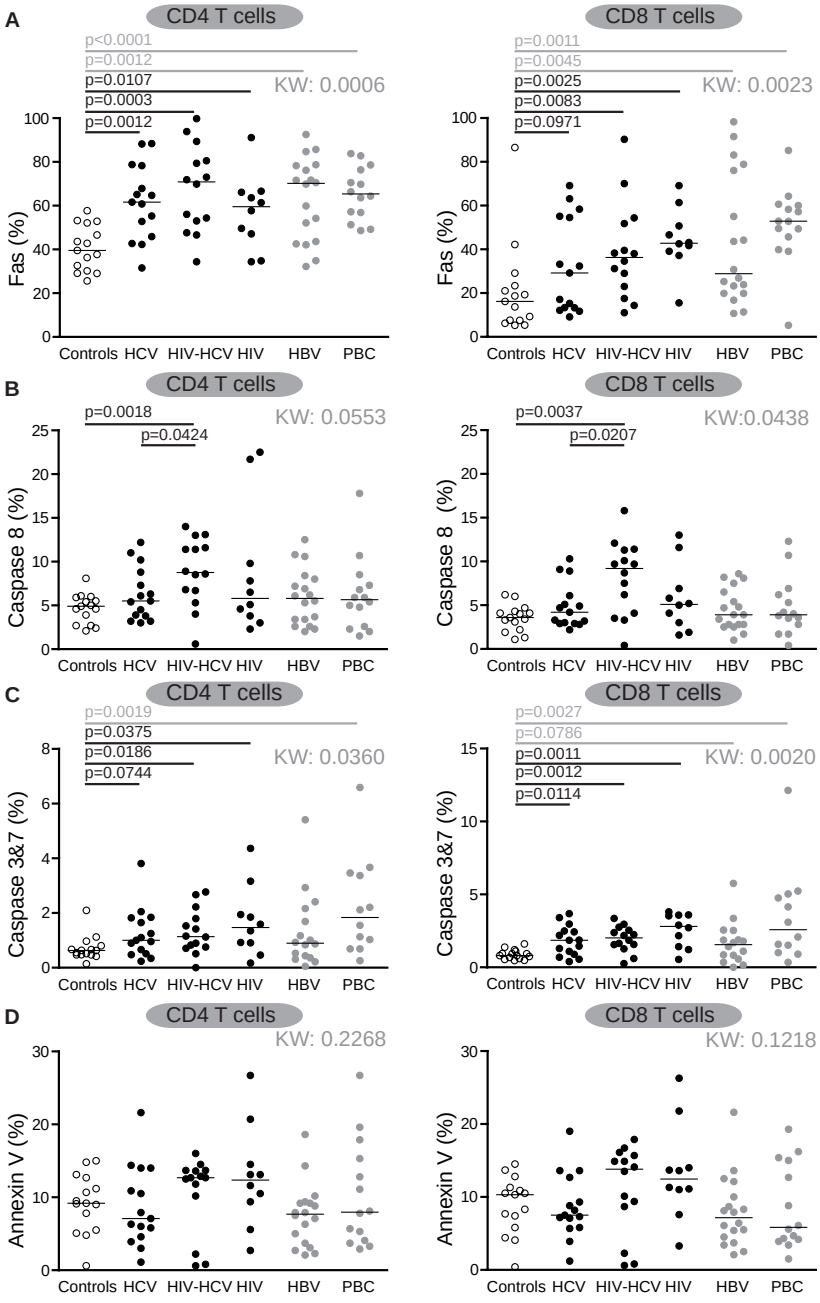

Figure 2: T cell apoptosis affected in patient groups and healthy controls. Measurements of death receptor Fas (A), activated extrinsic initiator caspase 8 (B), activated effector caspases 3 and 7 (C), Annexin V-binding (D) in CD4 (left graph) and CD8 T cells (right graph) in healthy controls, HCV-monoinfected, HIV-HCV coinfected and HIV monoinfected patients, as well as HBV- and PBC- patients as disease controls. **KW:** Kruskal Wallis p-value.

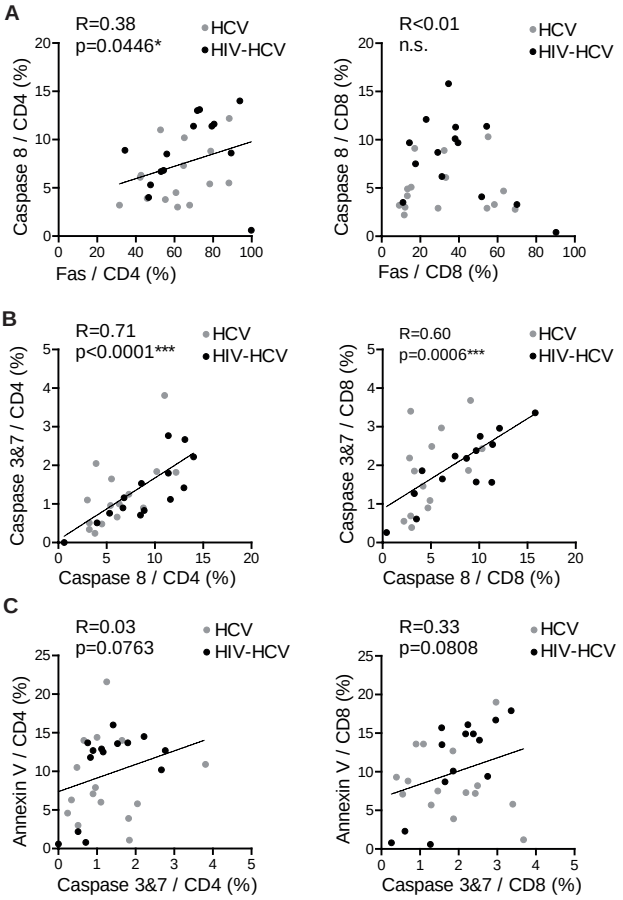

Figure 3: correlation between mutual apoptosis markers. Correlations and linear regression lines between Fas-expression and activated extrinsic initiator caspase 8 (A), caspase 8 and effector caspases 3 and 7 (B), caspases 3 and 7 and Annexin V-binding (C) CD4 T cells (left graph) and CD8 T cells (right graph) of HCV monoinfected (grey dots) and HIV-HCV coinfected patients (black dots). **R:** Spearman R; **p:** p-value.

Peripheral T cell apoptosis does not reflect degree of liver fibrosis

To investigate whether peripheral T cell apoptosis reflects the degree of liver fibrosis, we compared the expression of apoptosis markers on T cells of HCV monoinfected patients and HIV coinfected patients, as well as in disease control groups consisting of patients with treated HBV-infection or PBC with minimal or no fibrosis (F0-F2) versus those with severe fibrosis or cirrhosis (F3-F4). Although median percentages of Fas expression on CD4 T cells were higher in patients with F3-F4 fibrosis in comparison to those with F0-F2 fibrosis, this was only borderline significant (p=0.09) (*figure 4)*. Neither Fas expression on CD8 T cells nor any other apoptosis marker showed a consistent pattern, indicating that peripheral T cell apoptosis does not reflect severity of liver fibrosis. Furthermore, no correlations were found with level of HCV viremia, ALT or CD4 T cell counts or HCV genotype (*data not shown*).

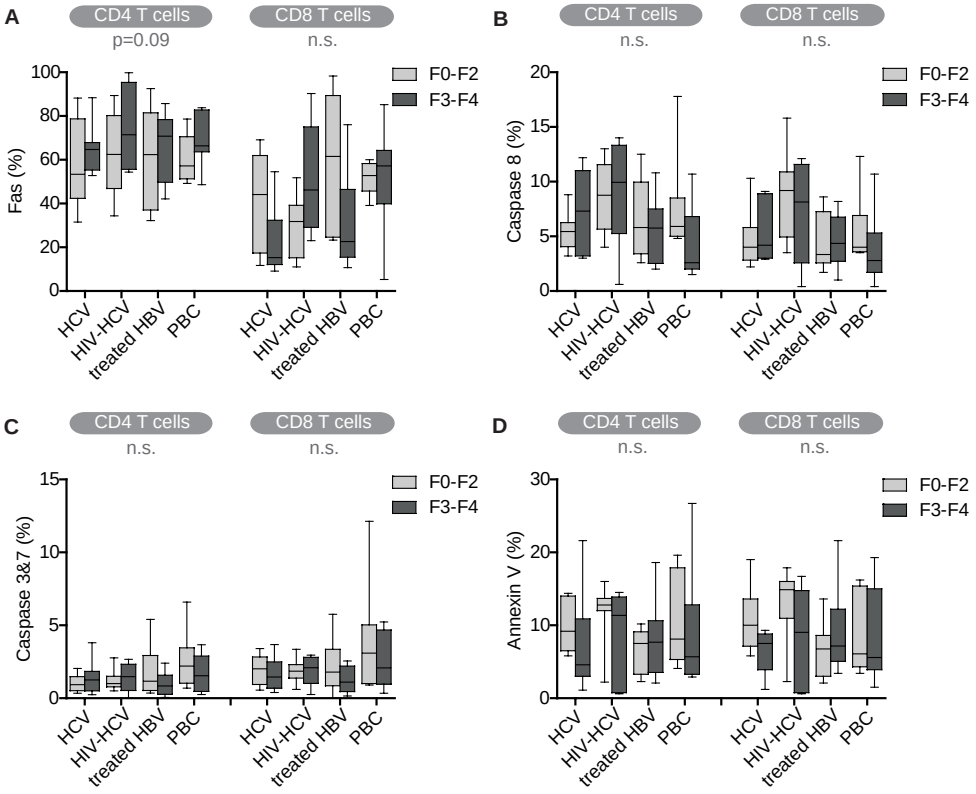

Figure 4: markers of T cell apoptosis in relation to liver fibrosis. Box-plots with median and min-max whiskers of percentages of Fas (A), caspase 8 (B), caspases 3&7 (C) and Annexin V-binding (D) in HCV-monoinfected, HIV-HCV coinfected, HBV-infected, and PBC patients with F0-F2 (light grey) or F3-F4 fibrosis (dark grey) liver fibrosis.

Discussion

In the present study, we demonstrate that markers of the extrinsic apoptosis pathway are upregulated in peripheral T cells of HCV monoinfected and HIV-HCV coinfected patients. Interestingly, CD4 and CD8 T cells of HIV-HCV coinfected patients depict higher levels of initiator caspase 8 compared to HCV monoinfected patients, but the downstream level of effector caspases 3&7 was similar (still higher than in healthy controls). Peripheral T cell apoptosis was not associated with degree of liver fibrosis or with other disease parameters.

Consistent with previous findings, our present data demonstrate that peripheral T cell apoptosis is increased in chronic HCV infection.[7] This may be the result of several mechanisms. For instance, HCV core protein enhanced Fas-mediated apoptosis in a T cell line,[21] but may also inhibit apoptosis via enhanced Bcl-xL expression,[22] or through

upregulation of an inhibitor of caspase-activated DNAse.[23] On the other hand, chronic antigen stimulation is also known to induce T cell death in order to maintain T cell homeostasis, in a process called activation-induced cell death (AICD).[24, 25] AICD may be exerted in a death receptor-dependent or -independent manner, as reviewed in by Brenner *et al*.[26] However, in the present study, increased levels of peripheral T cell apoptosis were not only observed in HCV and HIV infection, but also in a non-viral liver disease like PBC. This may be the result of activation due to reactivity against mitochondrial autoantigen (PDC-E2),[27] or by abnormal cytokine regulation associated with PBC.[28, 29]

Crispe *et al* have proposed two main models to explain intrahepatic T cell apoptosis. First, lymphocyte activation and apoptosis may be induced in the circulation, after which these cells become trapped in the liver, making the liver a 'graveyard'.[30] In this model, T cell apoptosis could contribute to liver fibrosis by activation of HSCs through phagocytosis of apoptotic lymphocytes.[8] In an alternative model, the liver merely represents a 'killing field', with lymphocyte apoptosis being induced in the liver via clonal deletion or upon entering viral antigen presented by hepatocytes, as Iken *et al* proposed based on in vitro experiments.[6, 31] In the 'killing field' model, apoptotic peripheral T cells may re-enter the circulation after apoptosis is induced, providing another theoretical basis for peripheral T cell apoptosis mirroring liver disease severity. However, we found no clear cut association between severity of liver fibrosis and cirrhosis and levels of peripheral T cell apoptosis in HCV-infected patients and HBV infected or PBC disease controls, indicating that T cell apoptosis in the periphery is not likely to play an important role in liver fibrosis. However, it should be noted that observations in peripheral T cells do not allow drawing definite conclusions about intrahepatic events.

Since all measurements were performed on freshly isolated peripheral blood mononuclear cells, to minimize influence of freezing and thawing of lymphocytes which is known to influence apoptosis-related proteins, our data is expected to closely reflect actual T cell apoptosis in the periphery.[32] However, apoptosis is by definition a dynamic process, which makes it difficult to draw definite conclusions from our data. Our findings do provide a basis for future studies to investigate whether T cell apoptosis may be involved in well-controlled coinfection with HIV. Although the clinical relevance of peripheral T cell apoptosis needs to be determined, it may be hypothesized that in HIV-infected patients this reflects ongoing activation and turnover of T cells which contributes to accelerated immune aging and occurrence of diverse late complications in patients with effective cART.[33] Interestingly, initiator caspase 8 activity was significantly higher in T cells of HIV-HCV coinfected patients in comparison to HCV monoinfected patients. This may be caused by increased AICD, as a consequence of higher levels of HCV viraemia in coinfected patients. However, downstream caspase 3&7 activity was similar between HIV-HCV coinfected and HCV monoinfected patients. Caspase 8 is not

only a key player in activation of the extrinsic apoptosis pathway, but is also involved in T cell activation.[34, 35] Thus, increased levels of activated caspase 8 may provide one explanation for T cell activation in HIV-HCV coinfected patients in comparison to HCV monoinfected patients.[20]

In conclusion, our data confirm previous findings that HCV infection is associated with upregulation of the extrinsic apoptosis pathway. Furthermore, peripheral T cell apoptosis was not associated the stage of liver fibrosis, suggesting that T cell apoptosis in the peripheral level does not play a prominent role in liver fibrosis.

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Chapter 3

Peripheral T cell apoptosis is not differentially affected by antiretroviral regimens in HIV-infected patients

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Abstract

Background: HIV-induced CD4+ and CD8+ T cell apoptosis decreases upon start of combination antiretroviral therapy (cART). Although in vitro evidence suggests an anti-apoptotic effect of protease inhibitors (PI) as opposed to non-nucleoside reverse transcriptase inhibitors (NNRTI), in vivo studies are inconclusive about effects of differential cART-regimens on T cell apoptosis.

Methods: Peripheral T cell apoptosis was evaluated in a cross-sectional study including 20 patients on PI- and 19 on NNRTI-based combination antiretroviral therapy (cART), all with backbone therapy of tenofovir and emtricitabine and undetectable viral loads 6 months before inclusion. Spontaneous T cell apoptosis was measured in freshly isolated peripheral blood mononuclear cells (<4 hours after venipuncture) using Annexin V, propidium iodide and staining for caspase activity and levels of the anti-apoptotic protein Bcl-2.

Results: The groups were comparable in general- and HIV-specific characteristics. In addition, T cell activation was similar in both groups. We observed no difference in T cell apoptosis as measured by annexin V, propidium iodide or caspase staining between PI- and NNRTI-treated patients. Interestingly, the level of anti-apoptotic protein Bcl-2 was higher in PI-treated than in NNRTI-treated patients.

Conclusions: In this cross-sectional study on HIV-infected patients, direct ex vivo spontaneous T cell apoptosis rates are not differentially affected by NNRTI- or PI-based cART.

Introduction

Currently, standard combination antiretroviral regimes (cART) for treatment of HIV can be divided in protease inhibitor (PI) based regimens and non-nucleoside reverse transcriptase inhibitor (NNRTI) based regimens, both with a backbone of two nucleoside reverse transcriptase inhibitors (NRTI).[1] Both regimens are known to be similarly effective on suppression of HIV RNA but differ in profile of adverse effects. Therefore, first choice between both treatment options is still under debate.[2, 3]

Treatment of HIV results in gradual reversal of HIV-induced immune distortions including a reduction of excessive HIV-related T cell apoptosis.[4, 5] While PIs may inhibit T cell apoptosis in vitro, even in the absence of HIV, NNRTIs exhibit pro-apoptotic effects in vitro.[6-8] However, only few clinical studies have evaluated the impact of different cART regimes on peripheral T cell apoptosis.[9-12] Two of these studies showed comparable levels of peripheral T cell apoptosis among PI- and non-PI based cART.[10] In contrast, a recently published small but carefully performed longitudinal study revealed a favourable effect of PI-based cART (n=8) on peripheral T cell apoptosis in comparison to NNRTI-based cART (n=8).[11] However, the use of frozen samples, lack of specifying backbone NRTI therapy as well as baseline differences in T cell apoptosis between the patient groups hampered their conclusions.[11, 13]

In the present study, we characterised T cell apoptosis directly ex vivo (<4 hours from venipuncture) in patients with PI- or NNRTI-based cART with identical NRTI-backbone therapy. Only patients with undetectable HIV-viral loads and without concomitant infections or any other medical conditions likely to affect T cell viability were included.

Methods

Patients

A total of 40 HIV-infected patients, of whom 20 on boosted PI- and 20 on NNRTI-based cART, all with identical backbone therapy consisting of tenofovir and emtricitabine, were recruited from the outpatient clinic of the University Medical Center Utrecht based on undetectable viral loads (HIV-RNA <50 copies/ml) for at least 6 months before inclusion, last CD4-counts above 350 cells/mm3 and without concomitant infections or any other medical condition likely to interfere with T cell activation or apoptosis. HIV-viral loads were measured using AmpliPrep/COBAS Taqman PCR (Roche the Netherlands; lower limit of detection 50 copies/ml). Informed consent was obtained from all patients in compliance with the WMA Declaration of Helsinki and in accordance with the ICH guideline for Good Clinical Practice (6th revision, 2008). The local Medical Ethical Committee approved the study protocol.

Blood samples and flow cytometry

Within 4 hours of venous puncture, peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll Hypaque following standard procedures. Cells were incubated with antibodies against CD3 (AF700; Biolegend, San Diego, CA, USA), CD4 (PB; Biolegend), CD8 (V500; BD Biosciences, San Diego, CA, USA), CD38 (PE; Caltag Laboratories, San Francisco, CA, USA) and CD95 (APC; BD) for 20 minutes at 4°C. After washing, cells were incubated with fluorescent inhibitor of caspases (FLICA) for caspase 8, caspase 9 or caspases 3&7 (FITC; ImmunoChemistry Technologies; Bloomington, MN, USA) following product guidelines, at 37 °C (1x106 cells per sample). Another sample was stained for Annexin V (FITC; Biolegend) and propidium iodide (Biolegend) at room temperature for 15 minutes and directly analyzed by flow cytometry. For staining of Bcl-2, samples were incubated with Cytofix/Cytoperm solution (BD), washed with Perm/Wash buffer (BD) and subsequently stained with antibodies directed against Bcl-2 (PE, 4G7, BD) for 30 minutes at room temperature. All samples were washed, fixed with Cellfix (BD) and directly analyzed on a LSRII flow cytometry (BD). Per sample, 250,000 cells were analyzed. Maximally 6 patients were sampled at once; laboratory procedures were started within 4 hours and finished within 8 hours after venipuncture.

Statistics

Data were normally distributed and therefore expressed as mean with standard deviations. Means were compared using student's T test. Chi square test was used to test relationships between categorical variables. Dependence of variables was tested with Pearson's correlation coefficient. Statistical analysis was performed with IBM SPSS Statistics version 20 (SPSS Inc., IBM) and GraphPad Prism 5 for windows version 5.03 (Graphpad Software, Inc).

Table 1: patient characteristcs

	PI	**NNRTI**	**Statistic significance**
	n=20	n=19	p-value
Gender (%male)	18 (90%)	16 (84%)	0.59
Age, years	48 (9)	48 (8)	0.94
Years since diagnosis HIV	10 (7)	9 (5)	0.64
Years since start antiretro-viral therapy	6 (4)	7 (5)	0.34
CD4-count, cells/mL	615 (165)	711 (231)	0.14
Prescription of statins	6 (30%)	4 (21%)	0.52

Characteristics of patients treated with PI- or NNRTI-based cART are given in means and standard deviations or number and percentages.

Results

A total of 40 patients participated in the study. Of those, one was excluded because of detectable viral load in combination with decreased CD4-count at the day of inclusion (CD4 342 cells/mm3 and HIV-VL 161 IU/mL). Patients on PI- (n=20) and NNRTI-based cART (n=19) were comparable in gender, age and HIV-specific parameters (*table 1*). At the time of inclusion, viral loads were undetectable (<50 copies/mL) in all patients. The majority of patients in the PI-group received ritonavir-boosted atazanavir (n=18) while efavirenz (n=18) was the preferred drug in the NNRTI-group. The NRTI-backbone consisted of tenofovir and emtricitabine in all patients, without addition of other antiretrovirals.

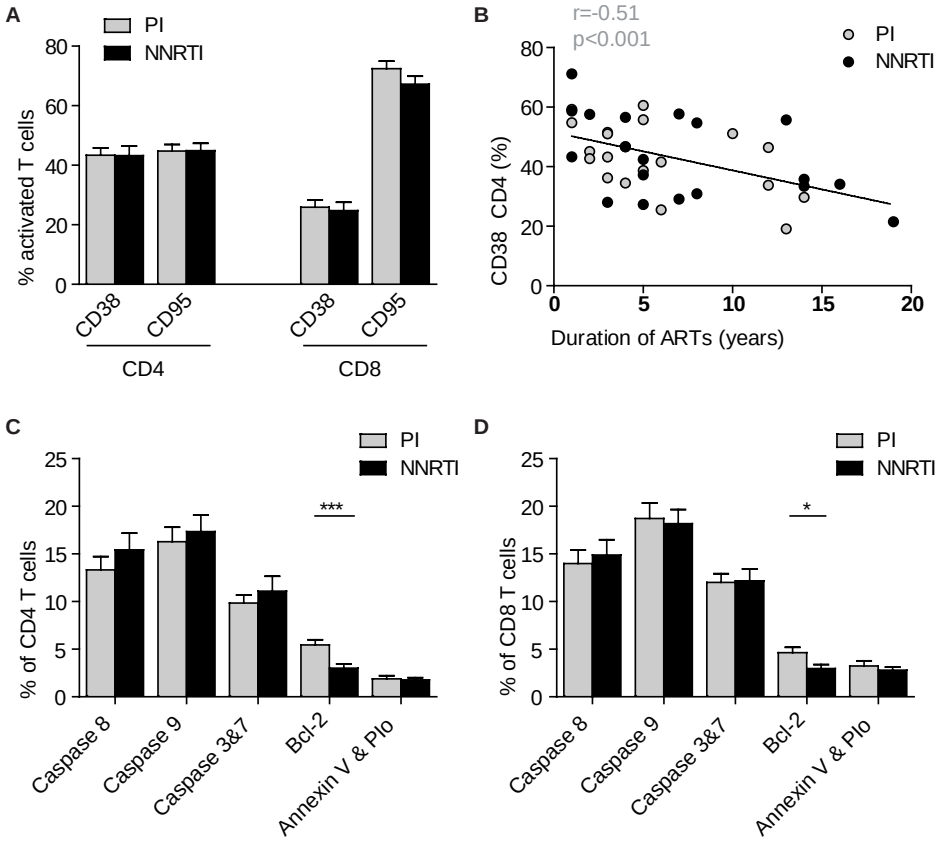

Figure 1: Activation and apoptosis markers in CD4+ and CD8+ T cells. A: percentages and standard error of the mean of CD38+ and CD95+ activated CD4+ (left) and CD8+ (right) T cells in patients with PI- (grey bars) or NNRTI-based (black bars) cART. B: correlation of time since start antiretroviral therapy (X-axis) with percentages of CD38+ activated CD4+ T cells (Y-axis) in patients on PI (grey dots) or NNRTI-based cART (black dots) and regression line. C and D: Mean percentages and standard error of the mean of CD4+ (C) and CD8+ T cells (D) positive for activated extrinsic activator caspases 8, intrinsic activator caspases 9, effector caspases 3 and 7, Bcl-2 and annexin V- propidium iodide in HIV-infected patients on PI (grey bars)- or NNRTI-based (black bars) antiretroviral therapy. **PI:** protease inhibitor. **NNRTI:** non-nucleoside reverse transcriptase inhibitor. **PIo:** propidium iodide.

No differences in CD4 T cell activation were observed between PI- and NNRTI-treated patients (CD38+: 43% versus 43% respectively, p=0.98) (*figure 1A*). In both groups, T cell activation correlated negatively with the duration of cART (CD4: r=-0.51; p=0.0010; CD8: r=-0.51, p=0.0008) (*figure1B*).

PI- and NNRTI-treated patients depicted similar levels of directly ex vivo CD4 T cell apoptosis as measured by Annexin V and propidium iodide (PI: 1.9%, NNRTI: 1.8%; p=0.80). In addition, no differences were observed in activation of the extrinsic apoptosis pathway, indicated by expression of death receptor CD95 (PI: 45%, NNRTI: 45%; p=0.60) and activated initiator caspase 8 (PI: 13%, NNRTI 15%, p=0.35). However, with regard to the intrinsic apoptosis pathway, expression of anti-apoptotic protein B cell lymphoma 2 (Bcl-2) was elevated in CD4 T cells of PI- compared to NNRTI-treated patients (5.4% versus 3.0%, p<0.001), but this was not accompanied by decreased activation of downstream intrinsic apoptosis pathway since activation of initiator caspase 9 (PI: 16%, NNRTI: 17%, p=0.64) or effector caspases 3 and 7 (PI: 10%, NNRTI: 11%, p=0.98) (fig 1C) were similar between both groups. CD8 T cells showed a similar pattern of significantly elevated Bcl-2 (4.6% versus 3.0%, p=0.02) without other differences in expression of apoptosis- or activation-markers (*figure 1D*). Lastly, no correlations were found between levels of apoptosis or caspase-activity and duration of cART, age or CD4-count (*data not shown*).

Discussion

This study shows no differences in directly ex-vivo apoptosis levels between PI- and NNRTI-treated patients, as measured by co-staining of annexin V and propidium iodide. Furthermore, the extrinsic apoptosis pathway (Fas and caspase 8) was not affected. With regard to the intrinsic apoptosis pathway, we found higher levels of the anti-apoptotic protein Bcl-2 in the PI- versus NNRTI-treated patients, but this was not associated with downstream activation of caspases 3&7.

Though excessive HIV-related T cell apoptosis is reduced soon upon start of cART5, [14, 15,] in vitro studies indicate an anti-apoptotic effect of PI and pro-apoptotic effects of NNRTI in chronically treated patients, even in the absence of HIV.[8] However, this has not conclusively been observed in clinical studies due to possible effects of cryopreservation and heterogeneity in cART backbones.[6, 7, 9]

Our study is distinctive since we performed our apoptosis analysis on freshly isolated PBMCs (< 4 hours after venipuncture). Furthermore, by matching for gender, age and CD4-counts, we minimized the potential for biased patient selection. Lastly, only patients without concomitant infections, malignancies, substance abuse or other medical conditions likely to interfere with T cell apoptosis, were included.[16]

Although one study showed that PIs (with the exception of atazanavir) were able to inhibit HIV glycoprotein 120-mediated T cell death in vitro,17 other studies reveal

that, even in the absence of HIV, susceptibility to apoptosis is affected by PIs.[8] Our aim was to evaluate pro- or anti-apoptotic effects of the long-term, aviremic phase of treatment, since we expect that this is most relevant for possible long-term side effects of cART.

T cell activation was similar in both groups, as indicated by the expression of the activation marker CD38. Furthermore, CD38-expression correlated negatively with time since start of cART, independent of cART-regimen, suggesting that T cell activation gradually decreases over years. This finding is in line with findings from a larger cohort, indicating that our study population is representative for treated HIV-patients.[18]

Bcl-2 is an important anti-apoptotic protein that may influence mitochondrial membrane permeabilization, a key event of apoptosis, by interaction with other pro- and anti-apoptotic proteins. This mechanism may explain anti-apoptotic effects of PIs in vitro.9 However, increased Bcl-2 alone does not necessarily result in reduced apoptosis.[19, 20] We hypothesize that increased levels of Bcl-2 results in decreased apoptosis-rates after stressing conditions like ex vivo cryopreservation, rather than affecting apoptosis in physiologic conditions, which may explain the discrepancy between our findings and those of Jung et al., who observed increased apoptosis in cryopreserved T cells of NNRTI- in comparison to PI-treated patients.[11] Our data support another study analysing CD4 and CD8 T cell apoptosis of whole blood and a longitudinal study comparing different cART-regimens in which a combination of PI plus NNRTI showed similar levels of T cell apoptosis as a regimen of NNRTI plus 3 NRTIs.[10, 12]

Based on our findings, we conclude that PI- and NNRTI-based cART-regimens do not differentially affect levels of T cell apoptosis in HIV-infected patients on stable cART regimens with undetectable HIV-RNA and high CD4 cell counts.

Disclosure statement

The authors declare that they have no conflict of interest.

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Chapter 4

Complementary role of HCV and HIV in T cell activation and exhaustion in HIV/HCV coinfection

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Abstract

Objectives: To investigate whether T cell activation and exhaustion is linked to HCV- and HIV disease parameters in HIV/HCV infected individuals, we studied T cell characteristics in HIV/HCV coinfected patients and controls.

Methods: 14 HIV/HCV coinfected, 19 HCV monoinfected, 10 HIV monoinfected patients and 15 healthy controls were included in this cross-sectional study. Differences in expression of activation and exhaustion markers (HLA-DR, CD38, PD-1, Tim-3 and Fas) and phenotypic markers on $CD4^+$ and $CD8^+$ T-cells were analysed by flow cytometry and were related to HCV disease parameters (HCV-viremia, ALT and liver fibrosis).

Results: Frequencies of activated $CD4^+$ and $CD8^+$ T cells were higher in HIV/HCV-coinfected compared to healthy controls and HCV or HIV mono-infected individuals. Coinfected patients also showed high expression of the exhaustion marker PD-1 and death receptor Fas. In contrast, the exhaustion marker Tim-3 was only elevated in HIV-monoinfected patients. T cell activation and exhaustion were correlated with HCV-RNA, suggesting that viral antigen influences T cell activation and exhaustion. Interestingly, increased percentages of effector $CD8^+$ T cells were found in patients with severe (F3-F4) liver fibrosis compared to those with no to minimal fibrosis (F0-F2).

Conclusions: HIV/HCV coinfected patients display a high level of T cell activation and exhaustion in the peripheral blood. Our data suggest that T cell activation and exhaustion are influenced by the level of HCV viremia. Furthermore, high percentages of cytotoxic / effector $CD8^+$ T cells are associated with liver fibrosis in both HCV monoinfected and HIV/HCV coinfected patients.

Introduction

Coinfection with human immunodeficiency virus (HIV) is relatively common in hepatitis C virus (HCV) infected patients because of shared routes of viral transmission.[1] HIV/HCV coinfection is associated with an accelerated course of HCV disease progression and increased HCV viral loads compared to HCV-monoinfection, even when HIV is effectively treated.[1-3] Several factors may contribute to this poor prognosis in coinfected patients. Reduced HCV-specific T cell responses have been demonstrated in coinfected patients in the chronic stage of HCV infection, but these studies were limited by either analyzing data of interferon-γ producing cells only 4 or by describing a rather heterogeneous study population including untreated HIV as well as patients on antiretroviral treatment.[4-6] A recent study, investigating the production of interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α), found similar HCV-specific T-cell responses in HIV/HCV coinfected patients on antiretroviral treatment compared to HCV monoinfected individuals.[7] Other factors possibly contributing to disease progression in HIV/HCV coinfection include reduced $CD4^+$ T cell help in elimination of infected hepatocytes and direct or indirect cytopathic effects of HIV.[8] Increased immune activation has also been proposed as one of the underlying mechanisms of poor clinical outcome of HCV infection in HIV/HCV coinfected patients.[9]

Next to generalised T cell activation, chronic viral infection is associated with loss of effector and proliferative functions of $CD8^+$ T cells, leading to ineffective viral control.[10] Among other markers of this so-called immune exhaustion, an important function of programmed death receptor 1 (PD-1) has been reported in both HIV and HCV infection and blockage of PD-1 has proved to restore immune function in chronic infection.[10-12] Furthermore, dual expression of exhaustion markers Tim-3 and PD-1 on HCV-specific T cells was shown to be correlated with disease progression in HIV/HCV coinfected patients.[13]

We have previously shown increased expression of the death receptor Fas (CD95) on peripheral $CD4^+$ and $CD8^+$ T-cells in chronic HCV infected patients.[14] This could be a sign of immune activation in these patients similar to the observations of increased immune activation in HIV patients on effective HAART.[15, 16] However, little is known about the additive effect of co-infection with HCV on immune activation in HIV-infected individuals on HAART. A few studies have examined T-cell activation and exhaustion in HIV/HCV co-infection, most of them either lacking a HIV-positive control group or being performed on frozen samples.[5, 17, 18] To study the contributions of HIV and HCV on T cell activation and exhaustion, we used freshly obtained blood to characterize T cell phenotypes, activation and exhaustion in HIV/HCV-coinfected patients compared to control groups of healthy individuals, HCV monoinfected and HIV monoinfected patients. Additionally, we investigated correlations of T cell

phenotype with HCV disease parameters including stage of liver fibrosis, level of HCV viremia, level of alanine transaminase (ALT) to unravel the contributions of these factors to immune activation.

In the present study we demonstrate that T cell activation and exhaustion are increased in patients with HIV/HCV coinfection compared to control groups. In addition, T cell activation and exhaustion are correlated with the level of HCV-RNA, suggesting that viral antigen drives T cell activation and exhaustion.

Methods

Ethics statement

Informed consent was obtained in writing from all patients in accordance with the WMA Declaration of Helsinki and in accordance with the ICH guideline for Good Clinical Practice (6th revision, 2008). The medical ethics committee for research in humans (METC) of the University Medical Center Utrecht, The Netherlands, approved the protocol of this study.

Patients

A total of 58 subjects, including 19 chronic HCV monoinfected patients, 10 HIV-1 monoinfected patients, 14 HIV/HCV coinfected patients and 15 healthy controls were included in this study. All patients were recruited from the Infectious Diseases outpatient clinic or from the Gastroenterology outpatient clinic of the University Medical Center Utrecht (UMCU). All patients were negative for hepatitis B surface antigen (HBsAg). None of the patients received treatment for HCV at the time of inclusion or within 12 months before. All HIV-infected patients were on highly active antiretroviral therapy (HAART), resulting in CD4-counts >200/μL and undetectable HIV viral load (<50 copies/mL). Patients with other diseases possibly interfering with their immune system (e.g. liver disease from non-viral causes, auto-immune disease, malignity or any other severe systemic diseases) were excluded from the study, as well as patients with known prior or present alcohol abuse. In the HCV-monoinfected and the HIV/HCV-coinfected group, liver fibrosis was assessed using transient elastography (Fibroscan® (FS), www.echosens.com, Paris, France). Classification of fibrosis was done using the METAVIR scoring system with F0-F2 being no to mild fibrosis (cut-off <9.5 kPa) and METAVIR F3-F4 being sever fibrosis to cirrhosis (cut-off > 9.5 kPa). In patients with recent assessment of fibrosis by liver biopsy (<1 year before inclusion), or known cirrhosis, no fibroscan was required. Levels of HCV-RNA and HIV viral load were measured with COBAS® AmpliPrep/COBAS® TaqMan Polymerase Chain Reaction (PCR; lower limit of detection 15 IU/mL for HCV and 50 copies/ml for HIV). Blood from anonymous healthy controls was requested from the bloodbank Mini Donor Dienst of the UMC

Utrecht and was tested negative for hepatitis B, hepatitis C and HIV. All patients and healthy controls were between 18 and 65 years old.

Processing of blood for isolation of PBMCs and analysis by flow cytometry

From all patients whole blood was collected by vena puncture in sodium heparin tubes (approximately 27mL) for PBMCs. Within 8 hours, peripheral blood mononuclear cells (PBMCs) were isolated by standard density centrifugation using Ficoll Hypaque. Per patient, 5 million freshly isolated PBMCs were washed twice with phosphate buffered saline (PBS) and directly stained for markers of T-cell phenotype, activation and exhaustion. The following antibodies were used: anti-CD3 (label: V500; provided by: BD horizon; clone: SP34-2), CD4 (eFluor780; eBioscience; RPA-T4 and PE-Cy7; BioLegend; L3T4), CD8 (PB, BioLegend; RPA-T8 and eFluor780; eBioscience; RPA-T8), CD27 (eFluor780; eBioscience; O323); CD38 (R-PE; Invitrogen; HIT2); CD45RO (APC; BioLegend; UCHL1); CD95 (APC; BP Pharmigen; DX2); PD-1 (PerCP/Cy5.5; BioLegend; EH12.2H7); Tim-3 (PE, BioLegend; F38-2E2). Cells were incubated with the antibodies for 20 minutes at 4ºC. After washing with PBS/0.5% bovine serum albumin, cells were fixed with Cellfix (BD) and directly analysed by flow cytometry. As an additional marker for effector T-cells, we analysed intracellular perforin expression. To this end, freshly isolated PBMCs were, directly after staining of surface markers, permeabilized and lysed (FACS permeabilizing solution 2 and FACS lysis solution; BD). After permeabilization, cells were incubated with anti-perforin (FITC; δG9; BD). After washing, cells were fixed with Cellfix (BD) and directly analysed on an LSR II FACS machine (BD). Per sample, 100.000 events were acquired. This resulted in exclusion of 2 healthy controls, 4 HCV-monoinfected and 2 HIV/HCV-coinfected patients for the perforin-staining.

Statistical analysis

Medians were compared with Mann Whitney test or, in case of multiple groups, with one-way ANOVA followed by Dunnett's multiple comparison test. Two-way ANOVA was used for comparing medians with two independent variables. Fisher's exact tests were used to test relationship of categorical variables. Dependence of variables was tested using Spearman's one-tailed correlation coefficient. Statistical analysis was performed with IBM SPSS Statistics version 19.0 (SPSS Inc., IBM) and GraphPad Prism 5 for Windows version 5.03 (GraphPad Software, Inc).

Results

Patient characteristics

Peripheral blood was drawn from a total of 58 subjects, consisting of 14 HIV/HCV-coinfected patients, 19 HCV monoinfected patients, 10 HIV monoinfected patients and 15 uninfected healthy controls. Characteristics of the different patient groups are shown in *table 1*. None of the patients received treatment for HCV at the time of inclusion. However, eight out of nineteen HCV monoinfected patients (42%) and three out of thirteen HIV/HCV coinfected patients (21%) had a history of ineffective HCV treatment more than 1 year before inclusion, while all others were treatment-naïve (p=0.07). All HIV infected patients (both monoinfected and HCV coinfected) were on HAART at the time of this study, resulting in $CD4^+$ T cell counts above 200 cells/mm3 and HIV-RNA <50 copies/mL. Median duration of HAART was 8 years in the monoinfected and 13 years in the coinfected group (p=0.35). As expected, the ALT value was lower in HIV mono-infected patients (30 IU/L) compared to the HCV-infected patients (65 IU/L; p<0.05), but ALT levels were similar between HIV/HCV coinfected (65 IU/L) and HCV monoinfected patients (69 IU/L; p=0.92). There was a trend towards higher level of HCV-viremia in coinfected patients (1.9e6 IU/mL) compared to HCV monoinfected patients (4.5e5 IU/mL; p=0.07) (*table 1*).

Table 1: Patient characteristics

	HCV-HIV	HCV	HIV	
	n=14	n=19	n=10	p-value
General characteristics				
Age, median (IQR), years	48 (14)	55 (7)	46 (6)	0.12
Gender, % male	86%	74%	90%	0.27
ALT, median (IQR), IU/L	65 (80)	69 (85)	30 (22)	<0.001
HCV disease characteristics				
HCV-RNA, median (IQR), IU/mL	1.9e6 (4.8e6)	4.5e5 (1.1e6)		0.07
Genotype, % genotype 1	75%	79%		1.00
History of HCV treatment:				0.07
Naïve, number (%)	3 (21%)	11 (58%)		
Previously failed treatment, n (%)	11 (79%)	8 (42%)		
Liver fibrosis / cirrhosis				0.73
F0-F2, number (%)	8 (57%)	9 (47%)		
F3-F4, number (%)	6 (43%)	10 (53%)		

Table 1: patient characteristics *(continued)*

	HCV-HIV	HCV	HIV	
	n=14	n=19	n=10	p-value
HIV disease characteristics				
Viral load, copies/mL	All <50		All <50	-
CD4-count, median (IQR), /mm3	540 (330)		661 (247)	0.11
HAART regimen				0.70
PI-based	7 (50%)		4 (40%)	
Other regimens	7 (50%)		6 (60%)	
Years of HAART, median	13		8	0.35

General and disease-specific characteristics of patient groups. Abbreviations: ***ALT,*** *alanine transaminase;* ***IQR,*** *interquartile range;* ***PI,*** *protease inhibitor;* ***HAART,*** *highly active antiretroviral treatment.*

HIV/HCV coinfection is associated with increased T cell activation and PD-1 expression

To study T cell activation and exhaustion in HIV/HCV-coinfected patients, we measured expression of activation markers CD38 and HLA-DR 19, exhaustion markers Programmed Death Receptor-1 (PD-1) and T cell immunoglobulin domain and mucin domain 3 (Tim-3) and death receptor Fas (CD95) in coinfected patients, HIV- or HCV-monoinfected patients and uninfected healthy controls. Gating strategy and representative plots of a healthy control, HCV-monoinfected, HIV-monoinfected and HIV/HCV-coinfected patient are shown in *figure 1A-E.*

Frequencies of activated $CD4^+$ and $CD8^+$ T cells, defined by CD38/HLA-DR double positivity, were higher in HIV/HCV-coinfected patients (median 1.9% and 5.6% respectively) compared to healthy controls (medians 0.8%; $p<0.001$ and 1.7%; $p<0.01$). Coinfected patients also displayed higher $CD4^+$ T cell activation (1.9%) in comparison to HCV- or HIV-monoinfected patients (1.4%, $p<0.01$ and 1.2%, $p<0.01$) (*figure 2A*). Expression of the T cell exhaustion marker PD-1 was higher on $CD4^+$ T cells of coinfected patients (13.2%) in comparison with HIV-monoinfected patients (7.8%; $p<0.05$) and healthy controls (5.5%; $p<0.001$). A similar pattern of PD-1 expression was observed in $CD8^+$ T cells (HIV/HCV: 13.3%, healthy controls: 8.1%, HCV: 10.3%, HIV 9.5%; not significant) (*figure 2B*). Interestingly, Tim-3 expression was not significantly increased in HIV/HCV co-infected patients. In contrast, HIV monoinfected patients depicted significantly higher levels of Tim-3 on both $CD4^+$ and $CD8^+$ T cells (1.9% and 2.8%) compared to HIV/HCV coinfected patients (0.8%; $p<0.05$ and 1.1%; $p<0.01$) and healthy controls (0.6%; $p<0.01$ and 0.6%; $p<0.001$) (*figure 2C*). Numbers of PD-1 and Tim-3 dual positive T cells were too low for reliable analysis.

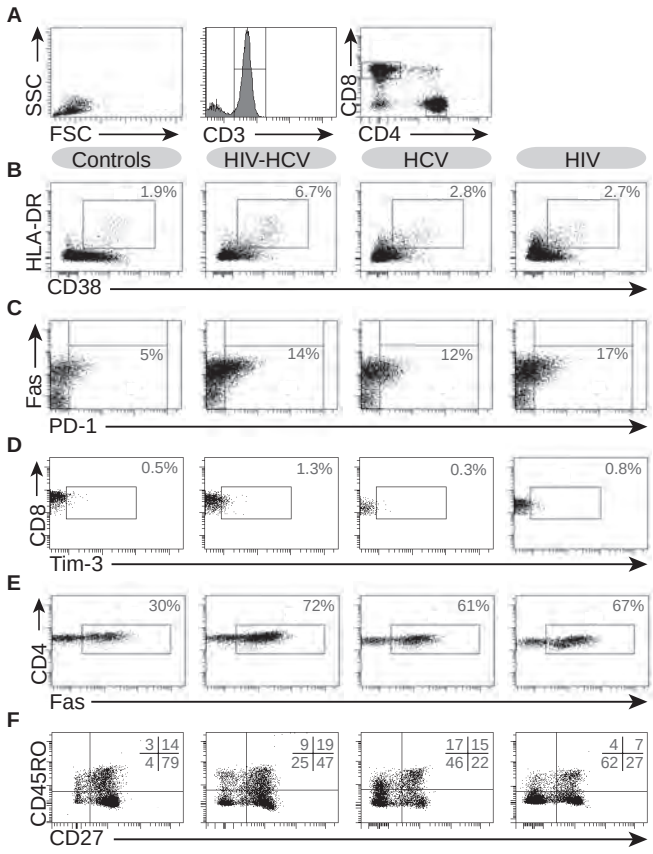

Figure 1: Representative flow cytometry plots. Representative flow cytometry plots of T-cell activation- and exhaustion markers in HIV/ HCV coinfected patients, HCV mono- and HIV mono-infected patients and healthy controls. A: gating of CD4+ and CD8+ T-cells by lymphocyte-gate (left panel), CD3-gate (middle panel) and gates for CD4+ or CD8+ T-cells (right panel). B-E: representative flow cytometry plots of a healthy control (left), HIV/ HCV coinfected (middle left), HCV monoinfected (middle right) and HIV -monoinfected patient (right) showing (B) activated CD8+ T-cells; (C) Fas-positive CD4+ T-cells; (D) PD-1 positive CD4+ T-cells and (E) Tim-3 positive CD8+ T cells. Percentages are depicted in the right upper corner.

Furthermore, with high activation and exhaustion in HIV/ HCV coinfected patients compared to healthy controls, a higher susceptibility to apoptosis was expected.[20] Indeed, expression of the death receptor Fas on $CD4^+$ T cells of coinfected patients (median 71%) was significantly increased compared to healthy controls (40%, $p<0.001$) and HIV infected controls (60%; $p<0.05$). However, in coinfected patients Fas-expression on $CD4^+$ T cells (71%) did not differ in comparison to HCV monoinfected controls (63%). On $CD8^+$ T cells, there were no significant differences of Fas expression in coinfected patients (36%) compared to HCV or HIV monoinfected patients (31% and 43%) or healthy controls (16%) (*figure 2B*).

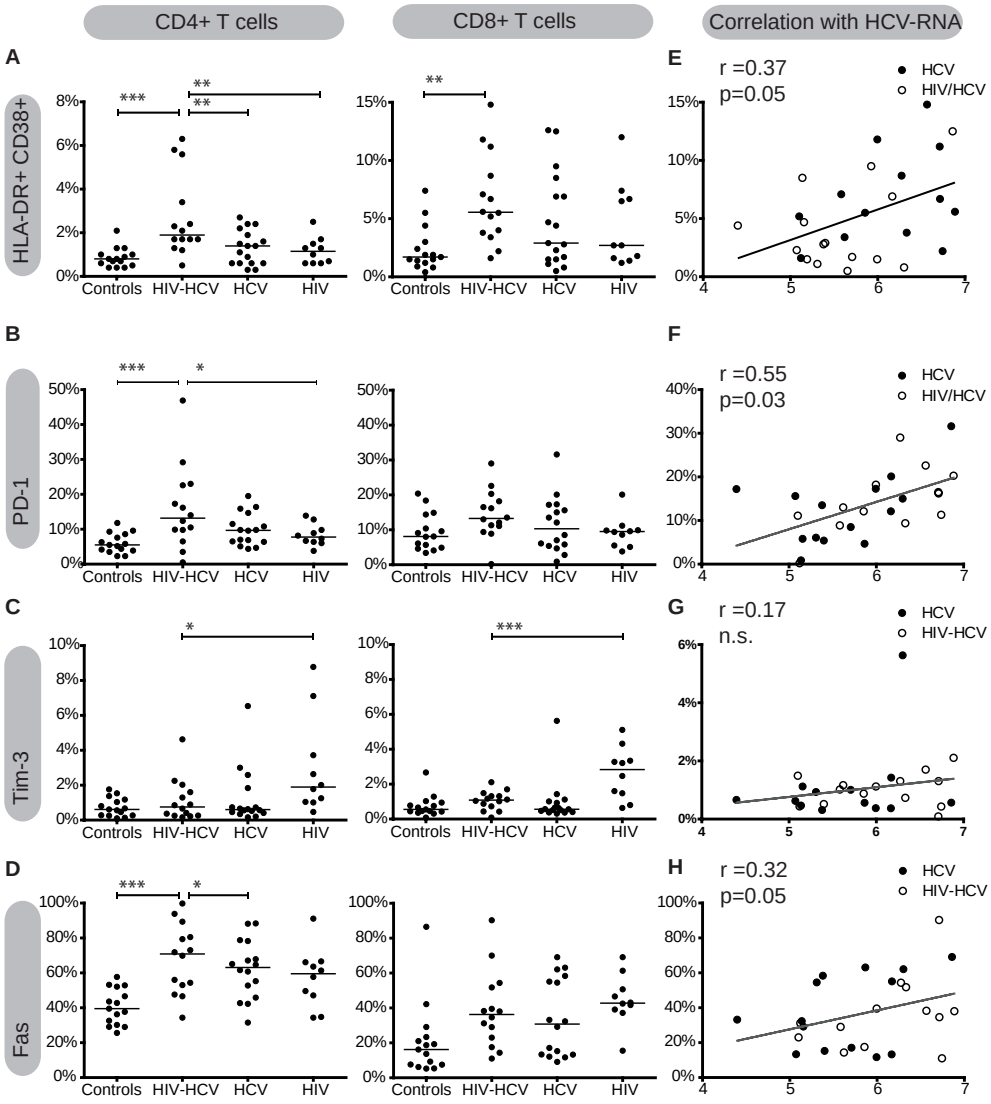

Figure 2: T-cell activation and exhaustion and its correlations with HCV-RNA in HIV/HCV-coinfection and control groups. A: percentages of HLA-DR+CD38+ activated CD4+ (left) and CD8+ T-cells (right). B: percentages of PD-1 positive CD4+ and CD8+ T-cells. C: percentages of Tim3-positive CD4+ and CD8+ T-cells. D: percentage of death receptor Fas positive CD4+ and CD8+ T cells. E-H: correlations of HCV viral load within HIV/HCV coinfected (open dots) and HCV monoinfected patients (closed dots) with percentages of HLA-DR and CD38 positive CD8 T cells (E); PD-1 positive CD8 T cells (F); Tim-3 positive CD8 T cells (G) and Fas positive CD8 T cells (H). HCV viral loads are depicted in IU/mL. P-values are indicated with * ($p<0.05$), ** ($p<0.01$) or *** ($p<0.0001$). Spearman r and p-value of correlations are depicted in the upper left corner of each graph; grey lines represent semilog fit lines.

T cell activation and exhaustion are linked to level of HCV-RNA in HIV/HCV co-infection

To elucidate which clinical parameters may contribute to T cell activation and exhaustion, we investigated correlations of ALT and HCV viremia with expression of HLA-DR/CD38, PD-1, Tim-3, and Fas.

Within all HCV-infected patients, HCV-RNA correlated positively with T cell activation (CD8: r=0.37, p<0.05; CD4: r=0.28, p=0.08) as well as with expression of the exhaustion marker PD-1 ($CD4^+$: r=0.52, p<0.01 and $CD8^+$: r=0.55, p<0.001), whereas there was no correlation with expression of Tim-3. The positive correlation of PD-1 expression was still present when the patients were divided in HCV monoinfected (CD4: r=0.49, p=0.04; CD8: r=0.39, p=0.08) and HIV/HCV coinfected patients (CD4: 0.26, not significant; CD8: 0.53, p=0.03). Additionally, a borderline significant correlation of HCV-RNA and death receptor Fas expression on $CD8^+$ T cell was found (r=0.32, p=0.05) (*figure 2; table 2*).

In contrast, there was no correlation of T cell activation and exhaustion with ALT (*table 2*). Furthermore, T cell activation and exhaustion as well as level of HCV-viremia were similar in patients with F0-F2 fibrosis or F3-F4 fibrosis (*table 2*). Lastly, a history of failed HCV-treatment was not associated with differences in T cell activation or exhaustion.

HCV and HIV are associated with differences in memory and effector phenotype of $CD8^+$ T cells

Chronic viral infections are shown to coincide with changes in T cell memory and effector phenotype.[21] Therefore, we examined whether HIV/HCV-coinfection drives changes in memory and effector phenotype of $CD8^+$ T cells, by studying percentages of naïve ($CD45RO^-CD27^+$), central memory ($CD45RO^+CD27^+$), effector memory ($CD45RO^-CD27^+$) and effector ($CD45RO^-CD27^-$) $CD8^+$ T cells. Representative plots of a healthy control, an HCV monoinfected patient and an HIV/HCV coinfected patient are shown in *figure 3A*.

Mean percentages of T cell phenotypes in patients compared to healthy controls are depicted as pie graphs in *figure 3B*. Due to the small percentages of effector T cells, changes in these subsets are more apparent when depicted as relative increase compared to healthy controls (*figure 3C*). HIV/HCV coinfected patients showed significantly higher frequencies of central memory $CD8^+$ T cells compared to healthy controls (15.1% versus 11.8% p<0.05). The increase of effector $CD8^+$ T cells was significant in HCV and HIV monoinfection but not in HIV/HCV coinfection (*figure 3B-C*). There was no correlation with levels of HCV-RNA or ALT with memory and effector phenotype.

Table 2: Correlations of clinical parameters and T cell markers

	CD4⁺			**CD8⁺**		
	Spearman R		p-value	Spearman R		p-value
HCV-RNA						
HLA-DR+CD38+	0.28		0.08	0.37		0.03
PD1+	0.52		<0.01	0.55		0.03
Tim3+	0.01		n.s.	0.17		n.s.
Fas+	0.20		n.s.	0.32		0.05
ALT						
HLA-DR+CD38+	-0.09		n.s.	0.13		n.s.
PD1+	0.16		n.s.	0.26		n.s.
Tim3+	-0.17		n.s.	0.24		n.s.
Fas+	0.24		n.s.	0.12		n.s.
Liver fibrosis	F0-F2	F3-F4	p-value	F0-F2	F3-F4	p-value
HLA-DR+CD38+	1.6%	1.9%	n.s.	4.4%	5.4%	n.s.
PD1+	10.2%	10.4%	n.s.	14.6%	9.8%	n.s.
Tim3+	0.6%	1.1%	n.s.	0.8%	0.7%	n.s.
Fas+	57%	66%	n.s.	34%	32%	n.s.

Spearman correlations of HCV-RNA and ALT with expression of activation and exhaustion markers and median expression of these markers in patients with F0-F2 versus F3-F4 fibrosis in all HCV-monoinfected and HIV/HCV-coinfected patients.

Liver fibrosis is associated with increased frequencies of effector CD8⁺ T cells

As T cell mediated killing of infected hepatocytes is essential in liver fibrogenesis,[22] we investigated whether liver fibrosis was associated with differences in T cell effector phenotype. Indeed, we found increased effector CD8⁺ T cell frequencies in patients with F3-F4 liver fibrosis in both HCV monoinfected patients (median 41.3% versus 21.7% in patients with F0-F2 liver fibrosis) and HIV/HCV coinfected patients (33.2% versus 25.0%; p<0.01) (*figure 3D*). This finding was further confirmed by a positive correlation of percentages effector CD8⁺ T cells with Fibroscan-score (r=0,57; p=0.0019) (data not shown). Additional analysis of intracellular perforin expression within CD8⁺ T cells, as an indicator of effector T cells with a potency to kill, revealed slightly higher frequencies of perforin-positive CD8⁺ T cells in mono- and coinfected patients with severe fibrosis, albeit not significant (p=0.54; data not shown). However, frequencies of perforin-positive CD8⁺ T cells correlated strongly with effector phenotype (r= 0.48; p<0.001) (*figure 3E*). Altogether, these data suggest a role for perforin-positive / effector phenotype CD8⁺ T-cells in liver fibrosis.

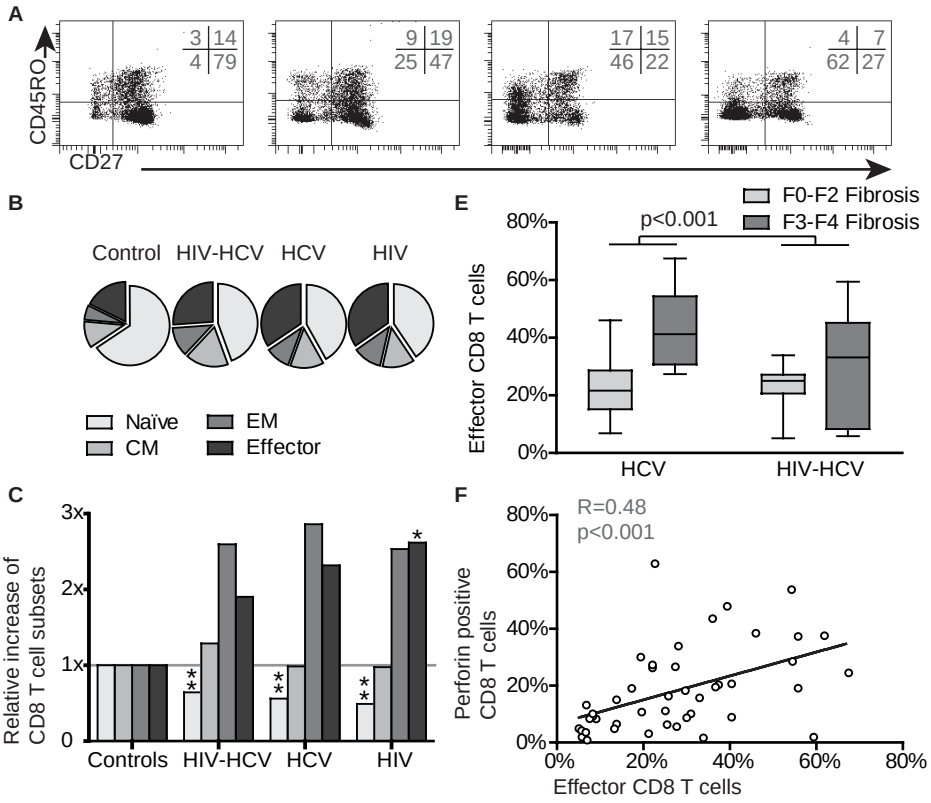

Figure 3: Changes in CD8+ effector and memory phenotype in all patients and healthy controls. **A:** representative plots of a healthy control, HIV/HCV coinfected, HCV monoinfected and HIV- mono patient showing naïve (right lower quadrant), central memory (right upper quadrant), effector memory (left upper quadrant) and effector (left lower quadrant) CD8+ T-cells by CD27 and CD45RO staining. **B:** pie charts of mean percentages of naïve (white), central memory (CM; light grey), effector memory (EM; dark grey) and effector (black) CD8+ T-cells in 42 patients and 3 healthy controls. **C:** relative increase of median percentages of memory subsets compared to healthy controls. **E:** box plot showing percentages of effector CD8+ T-cells in HCV-monoinfected (left) or HIV/HCV-coinfected patients (right) with fibrosis scores F0-F2 (light grey) versus F3-F4 (dark grey). The depicted p-value was calculated with two way ANOVA and indicates statistical significant difference in percentages of effector CD8+ T cells in F0-F2 fibrosis compared to F3-F4 fibrosis, independent of coinfection with HIV. Liver fibrosis was assessed with Fibroscan in 74% of HCV monoinfected patients and 71% of HIV/HCV coinfected patients. **F:** correlation of staining for perforin (Y-axis) with effector phenotype (X-axis) in CD8+ T-cells. Box-plots show median, quartiles and range. Line represents linear regression. P-values are depicted as: * (<0.05) and ** (p-value <0.01).

Discussion

HIV/HCV-coinfection is associated with faster HCV disease progression than HCV-monoinfection.[3] The underlying pathogenic mechanisms for poor clinical outcome in HCV/HIV coinfection remain unclear although various mechanisms have been suggested, including increased immune activation.[9, 23] The present study confirms that HIV/HCV coinfected patients on HAART indeed have higher levels of T cell activation as indicated by CD38/HLA-DR expression. In addition, we show that these T cells are also high in expression of PD-1 and Fas, both linked to T cell exhaustion and apoptosis.[24, 25] Furthermore, this study shows correlations of concentration between HCV-RNA and markers for T cell activation and exhaustion, suggesting a role for HCV viremia in influencing T cell activation and exhaustion in this group of patients.

Recently, it has become clear that HIV-infected patients on antiretroviral treatment still display slightly increased T cell activation,[26, 27] despite its initial decrease upon start of antiretroviral treatment.[28] Interestingly, we observed higher T cell activation in HIV/HCV coinfected patients compared to HIV-monoinfected patients despite longer history of HAART in coinfected patients.[29] Various underlying mechanisms contributing to T cell activation in those patients have been proposed in literature, of which the concept of microbial translocation is a currently widely accepted model.[16] As we show that the level of T cell activation correlates with HCV-RNA, we hypothesize that this concomitant viral infection may well contribute to the observed increase of T cell activation in HIV-infected individuals. Indeed, in a cohort of HCV monoinfected patients[30] we have previously observed a decrease in $CD4^+$ and $CD8^+$ T cell activation after 4 weeks of IFN-α/ribavirin treatment in the subset of patients with rapid viral response (HCV-RNA <50 IU/mL at week 4 of therapy), whereas there was no change in T cell activation in patients without rapid viral response (unpublished data). Since immune activation is thought to play an important role in long-term morbidity and mortality, [31] it can be hypothesized that coinfection with HCV may contribute to long-term extrahepatic morbidity in HIV-infected individuals through increased immune activation. Indeed, a recent study observed a higher prevalence of subclinical carotid plaque formation in HIV-patients coinfected with HCV compared to HIV-monoinfected patients.[32] However, a prospective study is required to examine whether increased peripheral T cell activation in HCV-monoinfected and HIV/HCV-coinfected patients is indeed associated with mortality and long-term morbidity.

In this study, T cell exhaustion, measured by PD-1 expression, correlated with HCV-RNA in HCV monoinfected and HIV/HCV coinfected patients. This is in line with a rather old study showing a correlation between HCV viral load and decreased T cell function in HCV-monoinfection, but at that time a role for PD-1 was in T cell exhaustion was not yet discovered.[33] Thus, to our knowledge, we are the first to show

increased T cell exhaustion indicated by PD-1 expression is linked to HCV viremia. Based on a study evaluating T cell exhaustion upon LCMV-infection in C57BL/6 mice,[34] it can be hypothesised that high level of viremia contributes to increased exhaustion, although it can also be reasoned that T cell exhaustion by itself may lead to higher antigen levels through decreased viral control.11 In contrast, Tim-3 was lower in HIV/HCV coinfected patients compared to HIV-monoinfected controls. This finding is supported by data from a recent study by Vali *et al*, in which Tim-3/PD-1 on total $CD4^+$ and $CD8^+$ T cell pools were highest in HIV-monoinfected patients in comparison with HIV/HCV coinfected and HCV monoinfected patients.[13] Although not significant, our data support the observation by Vali *et al* that Tim-3 expression is increased in HCV-infected patients in comparison to healthy controls.[13] It must be noted that age and gender of healthy volunteers were not characterised do to the anonymous service of the blood bank.

One possible explanation for decreased Tim-3 expression in HIV/HCV coinfected patients compared to HIV-monoinfected patients could be decreased activity of T-bet, a transcription factor classically associated with Th1 phenotype[35] but recently also shown to induce Tim-3 and repress PD-1 expression.[36, 37] Persistent antigen exposure was shown to lead to downregulation of T-bet.[37] This suggests that downregulation of T-bet due to persistent HCV antigen may be involved in decreased Tim-3 expression and increased PD-1 expression, as we observed in the present study, next to decreased Th1/Th2 ratio, observed by others,[38] associated with coinfection of HCV in HAART-treated HIV patients.

A second interesting observation in our cross-sectional study is the high level of effector $CD8^+$ T cells in HCV monoinfected and HIV/HCV-coinfected patients with severe fibrosis. Few other studies investigate changes of memory and effector T cell memory and effector compartments in HIV/HCV-coinfection.[5, 17, 18, 39] However, these studies did not compare to HCV- or HIV mono-infected controls or were heterogeneous in terms of HIV treatment, including both patients treated with HAART and patients with untreated HIV,[5, 17, 18, 39] which may result in HIV-antigen driven differences in memory and effector phenotype. To our knowledge, no studies relating these T cell phenotypes to fibrosis progression have been published. Our data suggests that effector T cells might play a role in liver fibrosis since it has been shown that similar T-cell populations are present in blood as well as in the liver of HCV infected patients.[40] Killing of infected hepatocytes by intrahepatic cytotoxic T cells may be perforin-dependent[41] and apoptosis of hepatocytes has a strong profibrotic effect through activation of hepatic stellate cells.[42] We therefore postulate that high frequencies of effector $CD8^+$ T cells may contribute to liver fibrosis by killing infected hepatocytes which in turn activate hepatic stellate cells and promote fibrogenesis. Alternatively, it may be hypothesized that increased percentages in the peripheral blood would reflect an actual decrease at the

site of infection due to compartmentalization. However, it has recently been shown that CD27 expression by intrahepatic lymphocytes is reflected by CD27 expression in the peripheral blood.[43] Examination of effector T cell phenotypes in liver biopsies in patients with minimal or severe fibrosis are needed to provide a definite conclusion.

The design of the present study was distinctive for several reasons. First, all stainings for activation and exhaustion markers were performed on freshly isolated PBMCs, thereby eliminating any influence of cryopreservation on expression of activation and exhaustion markers and T cell phenotype which hampers other studies.[44, 45] Albeit our sample size is somewhat smaller than other studies, we believe that our data closely reflect the actual phenotype of T cells in the peripheral blood. Furthermore, patients with no or minimal fibrosis as well as patients with severe fibrosis were included enabling us to investigate whether phenotypic differences are correlated to HCV disease progression. The HIV-infected groups were homogenous, including only patients on successful antiretroviral therapy (HIV not detectable, CD4 >200). The frequency of HCV treatment experienced patients was higher in the HCV-monoinfected group in comparison to the HIV/HCV-coinfected group, but a minimum of 1 year interferon free period was required before inclusion and no late effects of interferon are known from literature.

The present study is hampered by the fact that we did not investigate antigen-specific T cells, we cannot be sure whether our findings with respect to T cell memory subsets, activation and exhaustion are directly influencing HCV-specific T cell responses. However, various previous studies have convincingly proved that PD-1 and Tim-3 expression suppress HCV-specific immune responses and thereby contribute to viral persistence in HCV.[46] Furthermore, liver infiltrates are largely composed of HCV-nonspecific T cells, suggesting that disease progression may well be affected by non-specific activation.[40, 47, 48]

From the present study we conclude that T cells in HIV/HCV-coinfected patients show increased activation and exhaustion surface markers compared to healthy controls as well as to HIV- or HCV (co-)infected patients. This suggests that HIV and HCV have a complementary role on T cell activation in HIV/HCV coinfection. This could at least partly be explained by the level of antigen, since T cell activation and exhaustion correlated with the level of HCV viremia. Furthermore, liver fibrosis is associated with increased frequencies of effector $CD8^+$ T cells, based on extracellular surface markers and intracellular perforin expression, suggesting that these effector $CD8^+$ T cells may contribute to liver fibrosis in a perforin dependent manner.

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Chapter 5

Chronic HCV-infection is associated with increased expression of Fas ligand on CD56^{bright} and CD56^{dim} NK cells, regardless of coinfection with HIV

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Submitted

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Abstract:

Background: accumulating evidence supports a major regulating role for NK cells in chronic HCV infection, but the contribution of the two described NK cell populations, the $CD56^{bright}$ NK and $CD56^{dim}$ NK cells is not well understood.

Methods: in 15 healthy controls (HC), 19 HCV monoinfected patients, 14 HIV-HCV coinfected patients and 10 HIV monoinfected patients on cART, we analysed expression of cytotoxicity- and activation associated-markers by $CD56^{bright}$ and $CD56^{dim}$ NK cells.

Results: $CD56^{bright}$ NK cells of HC expressed markedly more FasL and lower levels of activating NK receptors than $CD56^{dim}$ NK cells. Chronic HCV-infection was associated with higher expression of granzyme B and perforin on CD56bright NK cells and higher FasL expression in $CD56^{bright}$ and $CD56^{dim}$ NK cells, independent of coinfection with HIV. Furthermore, HCV-infection was associated with decreased 2B4, NKp30 and NKp46 expression in $CD56^{dim}$ but not in the $CD56^{bright}$ NK cells.

Conclusion: FasL expression is a specific hallmark of the $CD56^{bright}$ NK cell population. Chronic infection with HCV is associated with increased cytotoxic potential as evidenced by enhanced FasL and granzyme B and perforin expression on both $CD56^{bright}$ en $CD56^{dim}$ NK cells, irrespective of coinfection with HIV.

Introduction

Hepatitis C virus (HCV) leads to chronic infection in the majority of patients and is a major cause of liver fibrosis and liver-related deaths throughout the world.[1] Outcome of infection, liver disease progression and clearance upon treatment are the result of a combination of viral and immunological factors.[2] Recent studies indicate that natural killer (NK) cells play an important role in immunity against HCV infections, by influencing viral clearance as well as by modulation of liver fibrogenesis.[3-7] NK cells may exert their antiviral function either directly, by killing infected hepatocytes, or indirectly by production of cytokines and thereby influencing other immune cells such as T cells, hepatic stellate cells (HSCs) or dendritic cells.[5, 8] NK effector functions are tightly regulated by an extensive repertoire of activating and inhibitory receptors and co-receptors.[9, 10]

Based on the relative expression of CD56 and CD16, human NK cells have classically been divided in two major subsets: the $CD16^{+}CD56^{dim}$ and $CD16^{-}CD56^{bright}$ subset.[11-13] $CD56^{bright}$ NK cells are considered precursors of $CD56^{dim}$ NK cells.[13] The latter contain much more perforin, granzymes and cytolytic granules and are regarded as the principal cytotoxic NK cells.[14] The $CD56^{bright}$ NK cells make up only 5-10% of peripheral NK cells, but are much more abundant in secondary lymphoid tissues. They express little or no killer-cell immunoglobulin-like receptors (KIRs) and CD16. CD16 is the low-affinity receptor for the Fc portion of immunoglobulin G, and therefore $CD56^{bright}$ NK cells have much lower potential for antibody-dependent cellular cytotoxicity (ADCC). Moreover, they display a different pattern of activating and inhibiting receptors than $CD56^{dim}$ NK cells.[13] $CD56^{bright}$ NK cells express TNF-receptor apoptosis inducing ligand (TRAIL) on their surface but are suggested to have regulatory rather than cytotoxic functions, since they are the main producers of interferon gamma (IFN-γ) and interleukin 10 (IL-10).[14]

In several studies, chronic infection with HCV was associated with a lower number of total peripheral NK cells but with a higher proportion of $CD56^{bright}$ NK cells, in comparison to resolvers and healthy individuals.[5,15] Furthermore, the HCV-infected liver is enriched in $CD56^{bright}$ NK cells, which make up around 20% of intrahepatic NK cells,[16] suggesting that the $CD56^{bright}$ NK cell subset may be important in the hepatic viro-immunological interplay. Recently, it has become clear that NK cells may suppress the adaptive immune response in chronic HCV-infection.[17] Furthermore, NK cells may influence hepatic fibrogenesis by inducing apoptosis of activated HSCs, which are the main producers of expanded extracellular matrix in liver fibrosis.[18]

Coinfection with human immunodeficiency virus (HIV) is relatively common in HCV infected patients and remains a clinical challenge.[19] Even when adequately treated with combination antiretroviral therapy (cART), coinfection with HIV is associated with poor outcome of HCV treatment with antiviral agents like peginterferon-alfa and ribavirin.[19] Several factors may contribute to this altered immunity in coinfected patients, including

modulation of NK cell populations. A recent case report described a markedly expanded $CD56^{bright}$ NK cell population in a rare case of a HIV-HCV coinfected haemophilic patient with good viral control in the presence of low CD4 counts,[20] suggesting that $CD56^{bright}$ NK cells may contribute to viral control in HIV-HCV coinfection.

Here we aimed to study the role of the $CD56^{bright}$ and $CD56^{dim}$ NK cell populations in chronic HCV monoinfected as well as HIV-HCV coinfected patients in more detail. In order to characterize the cytotoxic potential of the $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets in HIV-HCV coinfected patients, we examined phenotype and receptor expression in coinfected patients as well as in HCV- or HIV-monoinfected and uninfected controls.

Methods

Study population

All patients were recruited from the Infectious Diseases outpatient clinic or from the Gastroenterology outpatient clinic of the University Medical Center Utrecht (UMCU). All patients were negative for hepatitis B surface antigen (HBsAg). None of the patients received treatment for HCV at the time of inclusion or within 12 months before. All HIV-infected patients were on combination antiretroviral therapy (cART), resulting in CD4-counts >200/mm3 and undetectable HIV viral load (<50 copies/mL) in all patients. Patients with other diseases possibly interfering with their immune system (e.g. liver disease from non-viral causes, auto-immune disease, malignity or any other severe systemic diseases) were excluded from the study, as well as patients with known prior or present alcohol abuse. Blood samples of anonymous healthy individuals were obtained from the mini blood donor service of the UMC Utrecht (tested negative for hepatitis B virus (HBV), HCV and HIV).

Informed consent was obtained in writing from all patients in accordance with the WMA Declaration of Helsinki and in accordance with the ICH guideline for Good Clinical Practice (6th version, 2008). The medical ethics committee for research in humans of the University Medical Center Utrecht, the Netherlands, approved the protocol of this study.

Clinical data

In the HCV-monoinfected and the HIV-HCV coinfected group, liver fibrosis was assessed using transient elastography (Fibroscan® (FS),www.echosens.com, Paris, France). Classification of fibrosis was done using the METAVIR scoring system (F0–F2 no to mild fibrosis (cut-off <9.5 kPa) and F3–F4 severe fibrosis to cirrhosis (cut-off >9.5 kPa)). In patients with recent assessment of fibrosis by liver biopsy (<1 year before inclusion), or known cirrhosis based on clinical parameters (increased prothrombin time, low albumin and low platelets), no fibroscan was required. Levels of HCV-RNA and HIV viral load were measured with COBAS® AmpliPrep/COBAS® TaqMan Polymerase

Chain Reaction (PCR; lower limit of detection 15 IU/mL for HCV and 50 copies/ml for HIV). All patients and healthy controls were between 18 and 65 years old.

Flow cytometric analysis of fresh peripheral blood mononuclear cells

Unless otherwise specified, freshly isolated peripheral blood mononuclear cells were used, as described previously.[21] Briefly, whole blood was collected by vena puncture in sodium heparin tubes. Peripheral blood mononuclear cells (PBMCs) were isolated by standard density centrifugation using Ficoll Hypaque. Per patient, 3 million freshly isolated PBMCs were washed twice with phosphate buffered saline (PBS) and with PBA (PBS containing 0.5% bovine serum albumin and 0.1% sodium-azide) and directly stained for markers of NK subsets and cytotoxicity. The following surface antibodies were used: CD3 (HorizonTM V500, clone SP34, provided by BD Biosciences, San Diego, US), CD16 (Pacific BlueTM, 3G8, BD Biosciences), CD56 (PE-Cy7, B159, BD Biosciences), Fas ligand (FasL) (=CD178; AlexaFluor647, 14C2, AbD Serotec, Kidlington, UK). Cells were incubated for 20 minutes at 4˚C and then washed twice with PBA. For analysis of intracellular perforin and granzyme B, freshly isolated PBMCs were, directly after staining of surface markers, permeabilized and lysed (FACS permeabilizing solution 2 and FACS lysis solution; BD Biosciences). After permeabilization, cells were incubated with anti-perforin (FITC; δG9; BD Biosciences) and anti-granzyme B (PE, CLB-GB11, Abcam, Cambridge, UK). After washing, cells were fixed with Cellfix (BD Biosciences) and directly analyzed on an LSR II FACS machine (BD Biosciences). Per sample, a minimum of 100.000 total events was acquired.

Analysis of NK receptors on cryopreserved PBMCs

To characterise expression of activating NK receptors by $CD56^{bright}$ and $CD56^{dim}$ NK cells, PBMCs were cryopreserved before analysis. For this, thawed PBMCs were washed with medium and resuspended in PBA. For staining of surface receptors, cells were incubated for 20 minutes at 4˚C with the following antibodies: CD3 (APCeFluor780, clone: UCHT1, eBioscience, San Diego, USA), CD16 (AlexaFluor700, 3G8, Biolegend, San Diego, CA, USA), CD56 (PE-Cy7, B159, BD Biosciences), Fas (=CD95; APC, DX2, BD Biosciences), NKp30 (=CD337; PE, p30-15, Biolegend), NKp46 (=CD335; Brilliant Violet 421, 9E2, Biolegend) and 2B4 (=CD244; PerCP-Cy5.5, C1.7, BioLegend). Cells were washed and incubated with permeabilization and lysis solution (BD Biosciences) for 10 minutes at room temperature and subsequently stained for expression of effector caspase 3 (FITC, C92-605, BD Biosciences) for 20 minutes at 4˚C. After washing, cells were fixed with Cellfix (BD Biosciences) and analyzed by flow cytometry (LSRII, BD Biosciences). The $CD56^{dim}$ and $CD56^{bright}$ NK cells were divided based on expression of CD56 and CD16.

Statistical analysis

Non-parametric testing was used for all continuous data. Mann Whitney U-test was used for comparison of continuous data from two different groups and Kruskal Wallis one-

way analysis for multiple groups, followed by Mann Whitney U-test. Wilcoxon signed rank test was used for testing differences between paired continuous data. Dependence of continuous variables was tested using Spearman's two-tailed correlation coefficient. Fisher exact test was used to test relationship between categorical variables. Statistical analysis was performed with IBM SPSS statistics version 19 (SPSS Inc., IBM, New York, USA) and Graphpad Prism version 5.03 (Graphpad Software, Inc., San Diego, USA).

Results

Patient characteristics

A total of 58 subjects, including 14 HIV-HCV coinfected patients, 19 chronic HCV monoinfected patients, 10 HIV-monoinfected patients and 15 uninfected healthy controls were included in this study and were described previously.[21] All HIV-infected patients were on cART with undetectable HIV-RNA. None of the patients received treatment for HCV at the time of inclusion. ALT levels were similar between HIV-HCV coinfected (65 IU/L) and HCV monoinfected patients (69 IU/L; p=0.92). There was a trend towards higher level of HCV-viremia in HCV-HIV coinfected patients (1.9e6 IU/mL) compared to HCV monoinfected patients (4.5e5 IU/mL; p=0.07) (*table 1*).

Table 1: patient characteristics

	HCV/HIV n=14	**HCV** n=19	**HIV** n=10	P-value
General characteristics				
Age, median (IQR), years	48 (14)	55 (7)	46 (6)	0.12
Gender, % male	86%	74%	90%	0.27
ALT, median (IQR), U/L	65 (80)	69 (85)	30 (22)	<0.001
HCV disease characteristics				
HCV-RNA, median (IQR), IU/mL	1.9e6 (4.8e6)	4.5e5 (1.1e6)		0.07
Genotype, % genotype 1	75%	79%		1.00
Liver fibrosis / cirrhosis				
F0-F2, number (%)	8 (57%)	9 (47%)		0.73
F3-F4, number (%)	6 (43%)	10 (53%)		
HIV disease characteristics				
Viral load, copies/mL	All <50		All <50	-
CD4-count, median (IQR), /mm3	540 (330)		661 (247)	0.11

Abbreviations: ***ALT,*** *alanine transaminase;* ***IQR,*** *interquartile range*

Chronic HCV is associated with increased FasL expression on CD56bright and CD56dim NK cells

To characterize cytotoxic features of CD56^{bright} and CD56^{dim} NK cells, we first measured expression of cytotoxicity-associated markers granzyme B, perforin and Fas ligand (FasL) on both NK cell subsets of healthy controls. NK cell subsets were defined by expression of CD16 and CD56; CD56^{dim} NK cells included only CD16^{pos} cells, while the CD56^{bright} subset contained CD56^{bright}CD16^{neg} as well as CD56^{dim}CD16^{neg} NK cells (*figure 1A*).

CD56^{bright} NK cells expressed significantly higher levels of FasL (median 11%; IQR 6.5% - 25.6%) in comparison to CD56^{dim} NK cells (median 1.9%, IQR 0.8% to 9.8%; p=0.0005) (*figure 1C*). Furthermore, CD56^{dim} NK cells of healthy controls displayed higher percentages of perforin and granzyme B positive cells (Prf^{+}GrB^{+}) than CD56^{bright} NK cells (median 59%; IQR 32.4% - 94% versus median 3.2%; IQR 1.6% - 5.1%; p=0.0005) (*figure 1B*).

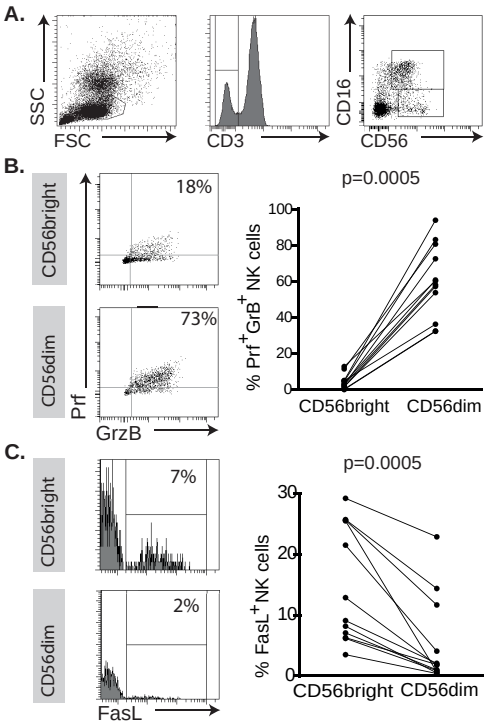

Figure 1: CD56bright and CD56dim NK cells differ in expression of cytotoxic markers in healthy controls. A: Gating strategy of CD56bright and CD56dim NK cells based on expression of CD56 and CD16. Left graph: selection of lymphocytes; middle: selection of CD3-negative NK cells; right: selection of CD56bright and CD56dim NK cells, primarily based on expression of CD16. B: Gating of Prf+GrB+ cells in CD56bright (upper left) and CD56dim (lower left) NK cells. Percentages and p-value are depicted in the right graph. C: Gating of FasL positive cells in CD-56bright (upper left) and CD56dim (lower left) NK cells. Percentages and p-value are depicted in the right graph. FSC: forward scatter. SSC: sideward scatter. **FasL:** Fas ligand (CD178). **Prf:** perforin. **GrB:** granzyme B.

Next, we analysed the impact of HCV infection and coinfection with HIV on NK cell subsets. No significant differences in both the percentages of CD56^{bright} and CD56^{dim} cells were observed between HCV-infected patients and healthy controls (*figure 2A*). However, in comparison to HCV-negative subjects, chronic HCV infection was associated with higher percentages of FasL-positive CD56^{dim} NK cells (median HCV: 10.6% HIV-HCV: 12.3% versus HC: 1.9% and HIV: 1.7%; p=0.003) and CD56^{bright} NK cells (medians HCV: 19.5% HIV-HCV: 20.0% versus HC: 11%, and HIV: 6.9% p=0.01).

CD56^{dim} cells are specialized cytotoxic NK cells as evidenced by high percentage of Granzyme B and perforin expression (median 59% in healthy controls) with the highest percentage of cytotoxic molecules in the HIV-infected patients (median 78%, p=0.03). Interestingly, additional analysis demonstrated that all chronically infected patients (HCV, HIV or HIV-HCV) displayed increased intracellular perforin and granzyme B expression in CD56^{bright} NK cells compared to uninfected controls (p=0.05) (*figure 2C-D*). Percentages of intracellular granzyme B and perforin NK cells did not correlate to CD4 counts in these patients (*data not shown*).

Thus, chronic infection with HCV is associated with increased cytotoxic potential as evidenced by enhanced FasL and granzyme B and perforin expression, irrespective of coinfection with HIV.

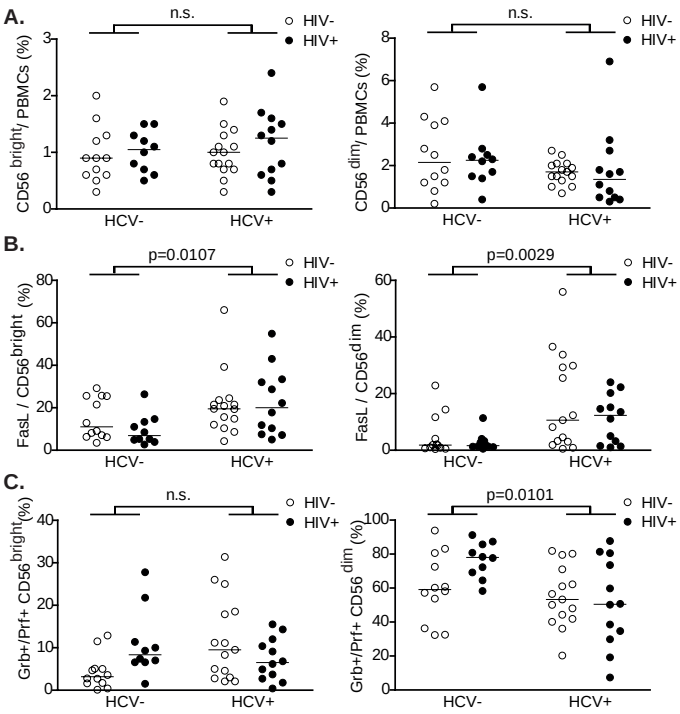

Figure 2: Differences in NK cell subsets distribution and expression of cytotoxic markers of both subsets associated with HCV- and HIV-infection. A: CD56bright (left graph) and CD-56dim (right graph) NK cells as percentages of total lymphocytes. B-C: Percentages of FasL-positive (C) and Perforin and granzyme B-positive (D) CD56bright NK cells (left graph) and CD56dim NK cells (right graph) in HCV-negative (left) and HCV-positive (right) patients with (black dots) or without (open dots) HIV-infection. **KW:** Kruskal Wallis p-value for multiple testing. **PBMC:** Peripheral blood mononuclear cells. **FasL:** Fas ligand (CD178). **Prf:** perforin. **GrB:** Granzyme B.

FasL expression on CD56^{bright} NK cells is not linked to apoptosis

To examine whether FasL-expression on CD56^{bright} NK cells could be linked to auto-apoptosis of these cells, we analysed apoptosis associated markers of the NK subsets in cryopreserved PBMCs by measuring expression of death receptor Fas and activated caspase 3. Interestingly, CD56^{bright} NK cells displayed markedly lower levels of the death receptor Fas (median 42%, IQR 29% - 55% versus median 83%; IQR 73% - 87%; p<0.0001) as well as activated caspase 3 (1.9% IQR 0.6% - 3.1% versus 14.2%; 9.6% - 20.6% p<0.0001) in comparison to CD56^{dim} NK cells of the same subjects. A similar pattern was observed in all patient groups (*table 2*). Although FasL-expression by CD56^{dim} NK cells was clearly associated with increased Fas expression and apoptosis, as indicated by its positive correlations with expression of Fas (R=0.60; p=0.05) and caspase 3 (R=0.74; p=0.0098) in these cells, expression of FasL on CD56^{bright} NK cells was not correlated with expression of Fas (R=-0.03; p=0.95) or caspase 3 (R=0.01, p=0.99) (*figure 3A-B*). In HCV-infected patients, expression of FasL was not correlated to caspase 3 or FasL in neither CD56^{bright} NK cells nor in CD56^{dim} NK cells (*figure 3C-D*). Thus, whereas FasL expression seems to be associated with apoptosis in CD56^{dim} NK cells of healthy controls, this is not the case in the CD56^{bright} NK subset and in HCV-infected patients, who display higher levels of FasL.

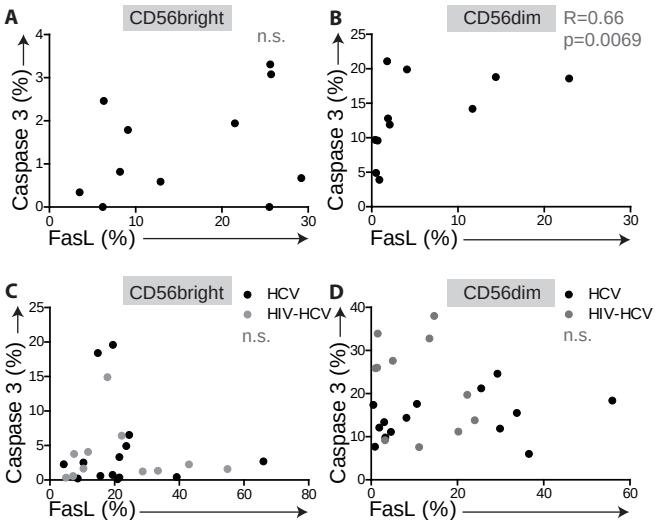

Figure 3: correlations between caspase 3 and FasL in healthy controls and HCV-infected patients. A-B: correlation between percentages of cells positive for FasL (X-axis) and caspase 3 (Y-axis) in CD56bright (A) and CD56dim NK cells (B) of healthy controls; C-D: correlation between percentages of cells positive for FasL (X-axis) and caspase 3 (Y-axis) in CD56bright (C) and CD56dim NK cells (C) of HCV monoinfected (black dots) and HIV-HCV coinfected (grey dots) patients. **R:** Spearman R. **MFI:** mean fluorescence intensity.

Table 2: apoptosis markers in CD56^{bright} and CD56^{dim} NK cells

		CD56bright (%)	CD56dim (%)	p-value
Fas (CD95)	Controls	42 (29-54)	83 (73-87)	<0.0001
	HCV	38 (31-57)	83 (75-87)	0.0003
	HIV-HCV	55 (41-62)	97 (93-97)	0.0001
	HIV	46 (34-56)	92 (81-95)	0.0020
Caspase 3	Controls	1.9 (0.59-3.1)	14 (9.6-21)	<0.0001
	HCV	2.3 (0.44-5.7)	13 (8.8-18)	0.0021
	HIV-HCV	1.6 (0.68-3.9)	21 (14-29)	0.0001
	HIV	0.84 (0.42-3.3)	16 (8.1-42)	0.0020

Median percentages (with IQR) of CD56bright and CD56dim NK cells positive for apoptosis markers Fas (CD95) and activated caspase 3. P-values were calculated by paired t test (Wilcoxon signed-rank test).

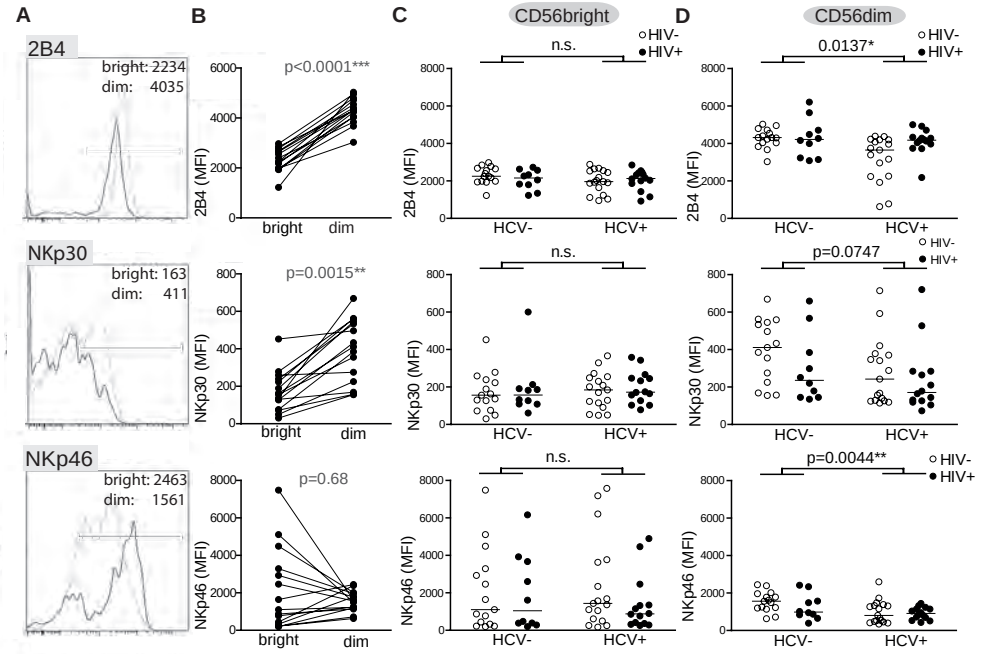

Figure 4: Expression of activation NK markers in CD56bright and CD56dim NK cells in healthy controls and patients. A: representative plots of 2B4 (upper graph, NKp30 (middle graph) and NKp46 (lower graph) in CD56bright (dark line) and CD56dim (light line) NK cells of a healthy control. B: MFI of 2B4 (upper graph), NKp30 (middle graph) and NKp46 (lower graph) in CD56bright and CD56dim NK cells of healthy controls; lines connect both subsets in each patient. C-D: MFI of 2B4- (upper graph), NKp30- (middle) and NKp46-positive (lower) of CD56bright (C) and CD56dim (D) NK cells of HCV positive (left) and HCV-negative (right) subjects with (black dots) or without (open dots) HIV-infection. **MFI:** Mean fluorescence intensity.

Different expression pattern of activating NK receptors on CD56^{bright} and CD56^{dim} NK cells

To further explore features of CD56^{bright} and CD56^{dim} NK cells, we examined the expression of activating NK receptors. Since expression of NKp30, NKp46 and 2B4 were shown to be associated with cytotoxic NK cell function as well as with disease progression in HCV and HIV infection,[6, 10, 22-25] we selected these activating receptors (*figure 3A*). In healthy controls, CD56^{bright} NK cells displayed significantly lower surface expression levels of 2B4 (MFI 2251 versus 4314; p<0.0001) and NKp30 (156 versus 411; p=0.0015) than CD56^{dim} NK cells, whereas expression of NKp46 did not significantly differ between the NK subsets (1105 versus 1561) (*table 2, figure 3B*). Expression of activating NK markers did not correlate with surface FasL or presence of intracellular perforin and granzyme B in CD56bright or CD56dim NK cells (*data not shown*).

We then measured expression of these activating receptors in all patient groups. Infection with HCV and/or HIV was not associated with any statistically significant changes in expression of 2B4, NKp30 or NKp46 on CD56^{bright} NK cells (*figure 3B*). However, expression of 2B4 on CD56^{dim} NK cells was significantly decreased in HCV infected patients (HCV monoinfection MFI: 3647, HIV-HCV coinfection 4177) in comparison to HCV-negative subjects (HC: median MFI: 4314; and HIV monoinfection 4216). Expression levels of NKp30 and NKp46 on CD56^{dim} NK cells were lower in HCV infected patients, irrespective of HIV-infection (NKp30: HCV: median MFI 242, versus HC: 411, p=0.07; NKp46: HCV: median MFI 803 versus HC 1561, p=0.004) and reached a similar expression level as found in CD56^{bright} NK cells in these patients (NKp30 in HCV: median MFI 184 and NKp46: 1435) (*figure 3C*).

Thus, chronic HCV-infection is associated with decreased expression of activation markers 2B4, NKp30 and NKp46 in the CD56^{dim} NK cell subset but not in CD56^{bright} NK cell subset, suggesting that, due to maintenance of activating receptors, the latter subset may be more important in chronic HCV.

Table 3: Activation associated NK cell receptor expression on CD56bright and CD56dim NK cells in healthy controls

	CD56bright	CD56dim	p-value
2B4, MFI	2251 (1986-2677)	4314 (4035-4715)	<0.0001
NKp30, MFI	156 (76-239)	411 (225-545)	0.0015
NKp46, MFI	1105 (333-3287)*	1561 (1196-1966)	0.68 (n.s.)

*Median expression (with IQR) of receptors associated with activation of NK cells in CD56bright and CD56dim NK cells. * MFI of NKp46 on CD56bright NK cells depicted a wide range; additional analysis revealed a strong correlation with percentages of NKp46-positive CD56bright cells (Spearman R=0.96, p<0.0001). P-values were calculated by paired t test (Wilcoxon signed-rank test).* **MFI:** *Mean Fluorescence Intensity.*

Discussion

Coinfection of HIV and HCV is a common and challenging clinical condition.[19] Although HCV and HIV infections are both associated with numeric and functional differences of CD56^{bright} NK cells,[15, 26] the precise functions of these cells remain poorly understood. In the present study, we show that FasL-expression is merely a feature of CD56^{bright} NK cells, as they express FasL typically at much higher levels than CD56^{dim} NK cells. Secondly, we demonstrated that chronic HCV-infection is associated with an altered NK cell phenotype with increased expression of cytotoxic markers by the CD56^{bright} NK population, independent of coinfection with HIV. Furthermore, we observed that chronic infection with HCV is associated with decreased expression of activating receptors in CD56^{dim} NK cells but not in CD56^{bright} NK cells. Together, the numeric and phenotypic differences indicate that the CD56^{bright} NK cell cytotoxic capacity may be of functional importance in chronic HCV. However, it remains elusive whether this is a physiologic immune mechanism in order to handle chronic viremia, for instance by killing of activated T cells,[27] or whether this is merely a pathologic condition induced by HCV in order to establish persistent infection.

To our knowledge, we are the first to demonstrate that CD56^{bright} NK cells display more FasL on the cell surface than CD56^{dim} NK cells. NK cells expressing high levels of functional FasL have previously been found in the uterus of pregnant women.[28, 29] Interestingly, those placental and decidual NK cells resemble CD56^{bright} NK cells also in terms of high expression of CD56 and low or absent CD16.[29] These phenotypic similarities suggest that functional properties may also be comparable. FasL expression by placental and decidual NK cells are thought to contribute to maternal tolerance against the fetus,[30, 31] and thus plays a regulatory role.

The liver of HCV-infected patients displays high number of CD56^{bright} NK cells and high levels of Fas and FasL.[13] Others have shown that activated HSCs may be killed in a Fas/FasL dependent manner.[18, 32, 33] In turn, apoptosis of activated HSCs may prevent liver fibrogenesis.[34, 35] Indeed, an expanded CD56^{bright} NK cell subset was associated with slower disease progression in a recent case report.[20] Therefore, it could be hypothesized that altered balance of NK cell phenotype towards CD56^{bright} NK cells with increased surface expression of FasL may play a regulatory role in development of liver fibrosis in HCV-infected patients. We however found no association with disease parameters (data not shown) in this small set of patients despite an even distribution among F0-F2 and F3-F4 fibrosis.

Alternatively, high expression of FasL may be linked to apoptosis of these cells, since FasL may induce apoptosis in an autocrine propriocidal manner.[36, 37] Interestingly, CD56^{bright} NK cells displayed much lower levels of the Fas receptor, for which FasL has high affinity,[38] than CD56dim NK cells. Similarly, intracellular levels of activated effector

caspase 3, involved in apoptosis,[39] were also lower. Interestingly, surface expression of FasL was correlated with expression of Fas and caspase 3 in $CD56^{dim}$ NK cells of healthy controls, but not in $CD56^{bright}$ NK cells. These findings suggest that FasL expression is indeed associated with apoptosis, possibly in an autocrine propriocidal manner, but only in the $CD56^{dim}$ NK cells. This may be explained by two separate mechanisms. First, FasL may be induced upon Fc receptor binding to activated NK cells, facilitating cytotoxicity and subsequent autocrine propriocidal apoptosis.[36, 40] $CD56^{bright}$ NK cells would naturally be less sensitive for this, since these cells lack expression of CD16, the low-affinity receptor for Fc. Alternatively, oxygen radicals may cause inactivation and apoptosis of NK cells, for which $CD56^{bright}$ NK cells are less sensitive than $CD56^{dim}$ NK cells.[41, 42]

We also observed increased percentages of perforin- and granzyme B-positive $CD56^{bright}$ NK cells in all groups of patients in comparison to healthy controls. Together with the obseration of increased FasL, this is in line with previous findings by others, that chronic HCV-infection is associated with increased cytotoxicity potential of NK cells,[5,43,44] whereas production of IFN-γ and TNF-α is decreased.[45, 46] Increased cytotoxicity of NK cells may also contribute to suppression of adaptive immune response in chronic HCV by killing of activated T cells.[17] As for HIV, reversal of decreased expression of activating NK markers and cytotoxic function upon start of cART have been reported by others,[47] but increase of perforin and granzyme B to levels that are higher than healthy controls have not been reported.

Since expression of activating NK markers has been clearly linked to NK cell activation and cytotoxic activity, we also characterized the expression of a selection of these activating receptors on both peripheral NK cell subsets.[23,25] $CD56^{bright}$ NK cells displayed markedly lower levels of 2B4 and NKp30 in comparison to their $CD56^{dim}$ counterparts. Interestingly, chronic HCV infected patients depicted lower levels of 2B4, NKp30 and NKp46 on $CD56^{dim}$ cells, which suggest that these cells are less activated in chronic HCV-infection.

A limitation in interpretation of our data is that the phenotype of peripheral NK cells does not necessarily reflect the intrahepatic population. However, the $CD56^{bright}$ population is relatively expanded in the liver of HCV-infected patients,[4] thus if our data are reflective for intrahepatic NK cells, FasL might be of much more importance in chronic HCV-infection than generally assumed. Furthermore, we performed stainings for FasL, perforin and granzyme B on freshly isolated peripheral blood mononuclear cells in order to minimize influence of freezing and thawing of lymphocytes, which may affect measurement of apoptosis-related proteins.[48] Thus, our data reflect actual phenotype of NK cells in the peripheral blood.

Taken together, our data show that $CD56^{bright}$ NK cells depict high levels of FasL. Furthermore, chronic HCV infection is associated with an altered balance of NK cell phenotype with increased FasL expression in $CD56^{dim}$, but especially in the $CD56^{bright}$ NK cells. Decreased expression of activating NK receptors on $CD56^{dim}$ NK cells may further indicate that $CD56^{bright}$ NK cells are of relatively greater importance than the $CD56^{dim}$ NK cells in chronic HCV. In our data, coinfection with HIV has no major impact on NK cell phenotype in HCV infected individuals.

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Chapter 6

CD4/CD8 ratio is a promising candidate for non-invasive measurement of liver fibrosis in chronic HCV-monoinfected patients

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Abstract

Purpose: The extent of liver fibrosis is an important factor in prognosis and clinical decision-making in chronic hepatitis C virus (HCV) infection. We investigated CD4/CD8 ratio in HCV-monoinfected and HIV/HCV-coinfected patients, in order to reveal its relation with liver fibrosis.

Methods: CD4/CD8 ratio in the peripheral blood was assessed by flow cytometry in a cohort of 19 HCV-monoinfected, 14 HIV/HCV-coinfected, 10 HIV-monoinfected patients and 15 healthy controls. Liver fibrosis was assessed by transient elastography (n=25) or by liver biopsy (n=8).

Results: Coinfection with HIV was associated with decreased CD4/CD8 ratios in chronic HCV-infected patients, despite adequate antiretroviral treatment. Furthermore, HCV-monoinfected patients with F3-F4 liver fibrosis demonstrated much lower CD4/CD8 ratios than patients with F0-F2 fibrosis (1.4 versus 2.5, p=0.023). Similarly, we observed a strong negative correlation between the CD4/CD8 ratio and liver stiffness measured by transient elastography (R=-0.78, p=0.0006). ROC analysis revealed that CD4/CD8 ratio as a non-invasive marker for fibrosis is very promising (area under the curve 0.8).

Conclusions: CD4/CD8 ratio is a promising candidate for non-invasive evaluation of liver fibrosis in HCV-monoinfected patients.

Introduction

Staging of liver fibrosis is essential in chronic HCV-infection since it has implications for treatment of HCV and screening for hepatocellular carcinoma.[1] Although various non-invasive methods have been developed, the gold standard for assessment of fibrosis still remains liver biopsy.[2] Because of shared routes of transmission, coinfection with HIV is relatively common in HCV-infected patients. Natural history of HIV/HCV-coinfection is characterised by accelerated HCV-disease progression, mainly when HIV is not well suppressed. Even when adequately treated, coinfection with HIV leads to altered immunity against HCV, leading to decreased treatment response to interferon.[3] Microbial translocation and increased immune activation are likely to contribute to this altered immunity against HCV in patients coinfected with HIV on antiretroviral treatment (ART).[3,4]

Peripheral CD4/CD8 T cell ratio is a well established marker of disease progression in untreated HIV, and has lately gained renewed attention as it may also reflect immune activation in treated HIV.[5-7] In addition, CD4/CD8 is associated with immune activation and poor outcome in a number of clinical conditions, either infectious (e.g. Dengue fever) or non-infectious (e.g. myocardial infarction, cervical carcinoma).[8-10]

T cells may be involved in development of liver fibrosis.[11] We previously showed a correlation between differentiated CD8 T cells and liver fibrosis, substantiating the role of CD8 T cells in liver fibrosis.[7] Therefore, we studied CD4/CD8 ratios in chronic HCV patients with or without treated HIV-coinfection, in order to evaluate its possible use as a non-invasive marker for liver fibrosis.

Material and Methods

Patients

19 HCV-monoinfected patients, 14 HIV/HCV-coinfected patients and 10 HIV-monoinfected patients were recruited from the Infectious Diseases outpatient clinic or from the Gastroenterology outpatient clinic of the University Medical Center Utrecht (UMCU), as previously described.[7] All HIV-infected patients were on effective antiretroviral therapy with undetectable HIV-RNA and none of them had another coinfection (TPHA, HBsAg, HEV-IgG negative). Blood samples from 15 anonymous healthy controls were requested from the bloodbank Mini Donor Dienst of the UMC Utrecht, and were tested negative for hepatitis B, hepatitis C and HIV.

Informed consent was obtained in writing from all patients in accordance with the WMA Declaration of Helsinki and in accordance with the ICH guideline for Good Clinical Practice (6th revision, 2008). The medical ethics committee for research in humans (METC) of the University Medical Center Utrecht, The Netherlands, approved the protocol of this study.

Assessment of liver fibrosis

Scoring of liver fibrosis was performed by liver biopsy using the METAVIR scale (n=8), or by using Fibroscan as previously described, with cut-off <9.5 kPa for F0-F2 fibrosis (n=25).[7]

Processing of blood for isolation of PBMCs and flowcytometric analysis

Peripheral blood mononuclear cells (PBMCs) were isolated by standard density centrifugation within 8 hours from venipuncture and were directly incubated with antibodies to CD3 (HorizonTM V500, clone SP34, provided by BD Biosciences, San Diego, US), CD4 (APC-eF780, RPA-T4, eBioscience, San Diego, CA, USA) and CD8 (Pacific BlueTM, RPA-T8, BioLegend, San Diego, CA, USA) for 20 minutes at 4˚C. After washing, cells were directly analyzed by flow cytometry to determine percentages of $CD4^+$ and $CD8^+$ T cells.

Statistical analysis

Medians were compared with Mann Whitney test. Kruskal-Wallis was used for testing differences of continuous data in multiple groups, followed by Mann Whitney test. Chi-square test was used to test relation of categorical variables. Dependence of variables was tested using Spearman's correlation coefficient. Diagnostic performance of the CD4/CD8 ratio in prediction of F3-F4 liver fibrosis was calculated by receiver-operating characteristic (ROC) curves and non-parametric area under the ROC curve. Statistical analysis was performed with IBM SPSS Statistics version 19.0 (SPSS Inc., IBM, USA) and GraphPad Prism 5 for Windows version 5.03 (GraphPad Software, Inc, USA).

Results

None of the patients received treatment for HCV at the time of inclusion or within the preceding year. All HIV-infected patients (both monoinfected and HCV-coinfected) were on stable cART at the time of this study, resulting in CD4+ T-cell counts well above 200 cells/mm3 (median 540/mm3 in HIV/HCV-coinfected and 661 in HIV-monoinfected patients (p=0.07)) and HIV-RNA <50 copies/mL. Patient groups were comparable in terms of age, gender, HCV-RNA level, and duration of antiretroviral treatment (*table 1*). Absolute numbers of lymphocytes were comparable between HCV monoinfected and HIV-HCV coinfected patients (median 2.0 and 2.3; p=0,58)

Both HIV-monoinfected and HIV/HCV-coinfected patients depicted significantly lower CD4/CD8 ratios (median 1.6, p=0.035 and 0.9, p=0.001) than healthy controls (2.0). CD4/CD8 ratios of HIV/HCV-coinfected patients were also lower than those of HCV-monoinfected patients (1.9, p=0.001) while the difference with HIV-monoinfected patients was borderline significant (1.6, p=0.08) (*figure 1A*).

Table 1: patient characteristics

	HCV	HCV/HIV	HIV	P-value
	n=19	n=14	n=10	
General characteristics				
Age, median (IQR), years	55 (7)	48 (14)	46 (6)	0.12
Gender, % male	74%	86%	90%	0.27
HCV disease characteristics				
HCV-RNA, median (IQR), IU/mL	4.5e5 (1.1e6)	1.9e6 (4.8e6)		0.07
HCV genotype, % genotype 1	79%	75%		1.00
ALT, median (IQR), IU/L	69 (85)	65 (80)		0.92
Liver fibrosis / cirrhosis				0.73
F0-F2, number (%)	9 (47%)	8 (57%)		
F3-F4, number (%)	10 (53%)	6 (43%)		
HIV disease characteristics				
Viral load, copies/mL		All <50	All <50	-
CD4-count, median (IQR), /mm3		540 (330)	661 (247)	0.11

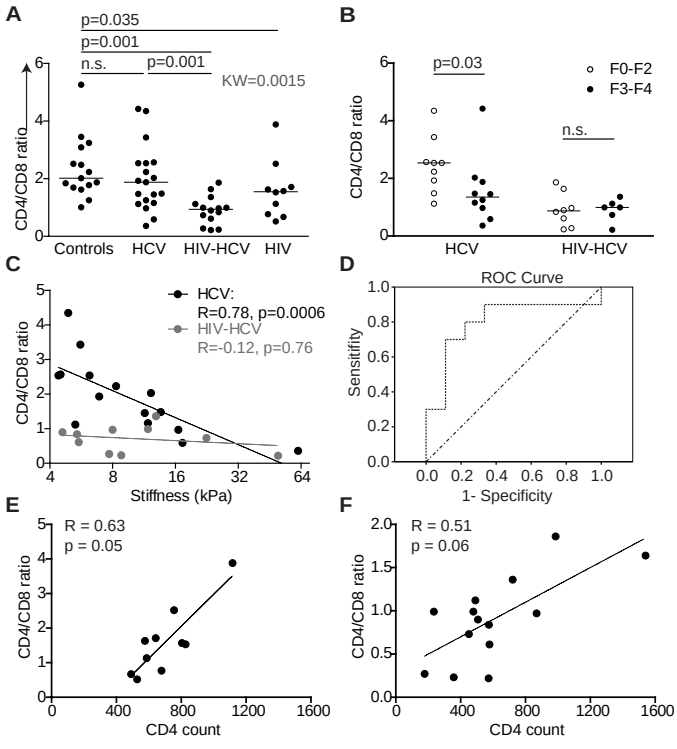

Figure 1: CD4/CD8 ratios in patients and controls. A: CD4/CD8 ratios in healthy controls, HCV-monoinfected, HIV/HCV-coinfected and HIV-monoinfected patients, measured by flow cytometry, lines depict median values. B: CD4/CD8 ratios in HCV-monoinfected (left) and HIV/HCV-coinfected patients (right) with no or minimal fibrosis (F0-F2, open dots) or severe fibrosis or cirrhosis (F3-F4, full dots). C: Correlation and linear regression line of liver stiffness measured by FibroScan and CD4/CD8 ratio in HCV-monoinfected patients (black dots) and HIV/HCV-coinfected patients (grey dots). D: ROC-curve of CD4/CD8 ratio as a diagnostic test for liver fibrosis (F3-F4). E-F: correlations and linear regression analysis of CD4 counts (X-axis) and CD4/CD8 ratios (Y-axis) in HIV-HCV coinfected (E) and HIV-monoinfected (F) patients.

Interestingly, when we compared CD4/CD8 ratios between HCV-monoinfected patients with severe (F3) fibrosis or cirrhosis (/F4) (median 1.4) versus those with moderate or absent (F0-F2) fibrosis (median 2.5; p=0.03), a low CD4/CD8 ratio was associated with enhanced liver fibrosis. Furthermore, there was a strong negative correlation between CD4/CD8 ratio and liver stiffness as measured by transient elastography (Spearman R=-0.78, p=0.0006). This was not the case for HIV/HCV-coinfected patients, as all those patients showed low CD4/CD8 counts (median 0.9), independent of the presence of liver fibrosis (Spearman R=-0.12, p=0.76) (*figure 1B-C*). No correlation existed between CD4/CD8 ratio and HCV viraemia in either HCV monoinfection (Spearman R=-0.08, p=0.75) or HIV/HCV coinfection (Spearman R=0.04, p=0.89) (data not shown). Correlation between CD4/CD8 ratio and routinely clinically measured CD4-counts were borderline significant in HIV-monoinfected (Spearman R=0.63, p=0.05) and HIV/HCV-coinfected patients R=0.51, p=0.06) (*figure 1E-F*).

A receiver operating curve (ROC)-curve was created to evaluate whether the CD4/CD8 ratio would be valuable as a diagnostic test for detection of liver fibrosis in HCV-monoinfected patients. Area under the receiver operating curve (AUROC) was 0.80 (confidence interval 0.58-1.0, p=0.027), confirming that CD4/CD8 ratio in HCV-monoinfected patients is a potential candidate for non-invasive discrimination between F0-F2 and F3-F4 liver fibrosis (*figure 1D*).

Discussion

Since liver fibrosis is an important factor in prognosis and clinical decision-making in HCV-infection, we investigated whether the CD4/CD8 T cell ratio reflects degree of liver fibrosis in HCV-monoinfected and HIV/HCV-coinfected patients. The clinical significance of the peripheral CD4/CD8 T cell ratio is well established in both HIV-infection as well as other clinical conditions.[5,6,8-10] Furthermore, CD8 T cells may be involved in liver fibrosis.[7,11]

Interestingly, CD4/CD8 ratio was associated with HIV-coinfection and with liver fibrosis in HCV-monoinfected patients. Furthermore, our data demonstrate that CD4/CD8 ratio is a promising candidate for non-invasive evaluation of liver fibrosis.

Decreased CD4/CD8 ratio may reflect either low CD4 counts, high CD8 counts or combined changes in both T-cell compartments. Low peripheral CD4/CD8 ratios in untreated HIV-infection may not only reflect HIV-induced loss of infected CD4 T cells, but may also be the result of migration of T cells or altered T cell differentiation.[12] Indeed, a recent study demonstrated that coinfection with HCV was associated with decreased CD4 counts in HIV-1 natural suppressors.[13] This is in line with our results in adequately treated HIV-patients, suggesting that either T cell differentiation or

migration is involved. Since the HCV-infected liver is enriched for CD8 T cells, CD4 T cell migration to the liver seems less likely to be responsible for low peripheral CD4/CD8 counts.[11] Furthermore, HIV/HCV-coinfected patients depict similar intrahepatic CD4 and CD8 T-cell responses compared to those of HCV-monoinfected individuals,[14] suggesting that decreased CD4/CD8 ratio in HIV/HCV-coinfected patients is not the result of migration. Therefore, we hypothesize that low CD4/CD8 ratio in coinfected patients is caused by altered differentiation, which may be the result of increased immune activation in HIV/HCV-coinfected patients,[15] since low CD4/CD8 ratio is associated with increased T cell activation in HIV-monoinfected patients with long-term viral suppression.[6]

However, in the case of HCV-monoinfection it is less obvious which factors contribute more to the low CD4/CD8 ratio. Our recent finding that liver fibrosis is associated with increased percentages of effector CD8 T cells, in the same patient group, is suggestive for changes in the CD8 rather than in the CD4 T cell compartment.[7] Even so, livers of HCV-monoinfected with cirrhosis depict relatively more hepatic CD8 T cells than those without cirrhosis,[11] suggesting that our findings in the peripheral blood may mirror changes in hepatic T-cell populations. No correlation was found between CD4/CD8 ratio and liver stiffness in HIV/HCV-coinfected patients, possibly because HIV-coinfection itself already induces low CD4/CD8 ratios.

Although CD4/CD8 ratio is highly influenced by absolute CD4-counts, the correlation between these routinely clinically measured CD4-counts and CD4/CD8 ratio was only borderline significant in this study. This can be explained by previous findings by others, demonstrating discordance between CD4 T-cell counts and CD4/CD8 ratios in patients with liver disease.[16]

In the present study, liver fibrosis was assessed by transient elastography in the majority of patients. In general, measurement of liver fibrosis by transient elastography corresponds highly with liver biopsies in severe fibrosis and cirrhosis (F3 and F4), but it is less accurate in differentiating F3 from F2 fibrosis, which has major implications in clinical decision-making for initiation of HCV-treatment.[17] To investigate whether CD4/CD8 ratio could bridge this gap in noninvasive measurement of liver fibrosis it would be preferable to measure liver fibrosis by biopsy as the golden standard in order to establish cut-off value for CD4/CD8 T cell ratio.

Since venous blood samples are easily obtained and can be send for analysis of CD4/CD8 ratio to a central laboratory, assessment of CD4/CD8 ratio could be easily implemented even in HCV-endemic countries where resources are often limited.[18] Considering the AUROC of 0.80, CD4/CD8 ratio is a promising candidate for non-invasive measurement of liver fibrosis in HCV-monoinfected patients. However, the number of HCV-monoinfected patients in the present study is small. Although we have

observed similar trends in HCV-infected patients outside this study (*unpublished data*), our findings need to be confirmed in a population-based study and an optimal cut-off value needs to be determined in order to further assess the value of CD4/CD8 T cell ratio alone as a marker for liver fibrosis, or in combination with other variables such as APRI-score or Fibro-test.[19]

Altogether, our data reveal that a low CD4/CD8 ratio is associated with HIV-coinfection and with liver fibrosis in HCV-infected patients, making it a promising candidate for non-invasive evaluation of liver fibrosis in HCV-monoinfection. Implementation of CD4/CD8 ratio for estimation of liver fibrosis could especially be useful in resource-limited settings.

Conflict of interest

The authors declare that they have no conflict of interest.

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Chapter 7

Impact of transient elastography on clinical decision-making in patients with chronic viral hepatitis

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Abstract

Objective: Transient elastography is a non-invasive tool to quantify liver fibrosis by liver stiffness measurements (LSM). Previous studies have extensively evaluated the accuracy of LSM compared to liver biopsy. In this retrospective study we explore potential impact of LSM on clinical decisions in chronic viral hepatitis.

Material and methods: LSM-based medical advice whether to start antiviral treatment and/or surveillance for hepatocellular carcinoma and clinical follow-up after LSM were analysed in 349 patients.

Results: In 20% of 184 HBV-infected patients and 38% of 165 HCV-infected patients, significant fibrosis (≥F2) was detected. In 5% (n=7) of the 129 untreated HBV patients and in 12% (n=19) HCV-infected patients, antiviral treatment was recommended solely based on LSM. Advice for surveillance for hepatocellular carcinoma was in 40 patients based solely on LSM (11% of all patients). Furthermore, 95% of 19 non-viremic HCV-patients (after spontaneous clearance or sustained viral response) could be discharged due to favourable LSM (≤F2). Medical advice was followed by the treating physician in the majority of cases. However, in only 47% of 51 HCV-infected patients with advice to start treatment, this was followed in clinical practice.

Conclusions: Transient elastography has a major impact on clinical practice, both as an indication to start or postpone antiviral treatment, to start surveillance for hepatocellular carcinoma and to discharge HCV-patients from follow-up after viral clearance and favourable LSM. Medical advice to start antiviral treatment is followed in the large majority of HBV-patients, but in only half of HCV-patients.

Introduction

Chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infection may lead to liver cirrhosis.[1] The major clinical consequences of liver cirrhosis are ascites, variceal bleeding and hepatocellular carcinoma (HCC).[2] Assessment of the extent of liver fibrosis is important, considering its relevance for the decision whether to start antiviral treatment or periodical surveillance for HCC.[3, 4] Histological assessment of percutaneous liver biopsies has traditionally played a central role in the evaluation of liver fibrosis, despite its known disadvantages such as sampling error, interobserver variability and a small risk of complications and mortality.[5] Non-invasive strategies to evaluate the extent of liver fibrosis have been introduced recently and are now being implemented in clinical practice.[6] A promising candidate for replacing liver biopsies is transient elastography, which measures liver stiffness as a parameter of liver fibrosis.[7-9] Various studies have demonstrated high concordance between liver stiffness measurements (LSM) obtained by transient elastography and liver biopsies in patients with chronic viral hepatitis or cholestatic liver disease.[10] Since LSM is easily repeated, it may improve monitoring of liver fibrosis and its use in clinical practice is expected to increase. Furthermore, transient elastography has a high intra- and interobserver agreement.[11] Nonetheless, although LSM is quite reliable to distinguish F2 versus F3-4 fibrosis, accuracy to distinguish F0-1 versus F2 is limited.

Only few studies have evaluated impact of LSM on clinical management.[12, 13] We previously reported potential impact of LSM on patient care in viral hepatitis. However, that study was limited by relatively small patient numbers and implementation of fibroscan-based advices in clinical practice was not evaluated.[12] In the current study we analyse the clinical impact of LSM in a large group of patients with chronic viral hepatitis.

Methods

Patient group

Data of all LSMs performed in our center in patients with chronic viral hepatitis between October 2008 and October 2011 were evaluated. No patients from our previous study (time period 2006 – September 2008) were included.[12] In case of multiple LSMs in the same patient during the indicated period, only data of the first LSM were taken into account. A minimum of 6 months follow-up in our hospital was required for evaluation of implementation of clinical advice.

Laboratory parameters

Serum chemistry and haematology were assayed with standard assays. HBV-DNA loads were measured with COBAS AmpliPrep/TaqMan® quantative polymerase chain reaction, (Hoffman- La Roche limited, Basel, Switzerland; lower limit of detection 20

IU/mL). HCV-RNA loads were measured with COBAS AmpliPrep/Taqman® quantative polymerase chain reaction (Hoffman- La Roche limited, Basel, Switzerland; lower limit of detection 15 IU/mL).

Liver stiffness measurement

Three experienced investigators (JA, MM and TF) performed all LSMs, using a Fibroscan® 502 device with M-probe (Echosens, Paris, France), according to standard procedures.[7] Before measurement, physical examination and ultrasound were performed to exclude ascites. Measurements were taken through intercostal spaces on the right lobe of the liver, with the patient in horizontal position and with maximal abduction of the right arm. Depth of measurements was between 25 and 65 mm below the skin surface. Results of LSM were expressed in kilopascals (kPa) corresponding to the median value of at least 10 validated measurements. Successful LSM was defined as a success percentage of at least 60% (i.e. the number of successful measurements divided by total number of measurements) and an interquartile range (IQR) of less than 30% of the median. In case of high IQR, LSM was still considered successful if all measurements fell into the same fibrosis-stage. In case of unsuccessful LSM, the measurement was repeated by a different investigator. Results were categorized according to the criteria of Castera *et al*[14]: <7.1 kPa, no or minimal fibrosis (F0/1); 7.1 – 9.4 kPa, moderate fibrosis (F2); 9.5 – 12.4 kPa, severe fibrosis (F3); >12.4 kPa, cirrhosis (F4).

Medical advice based on LSM

Based on results from LSM and clinical data, an experienced hepatologist (KvE) and an experienced infectious diseases physician (JA) discussed each patient in a multidisciplinary setting providing joint medical advice whether or not to start antiviral therapy and/or HCC surveillance. Antiviral treatment was recommended based on current EASL guidelines.[3, 4] In short, in HBV-infected patients, LSM indicating significant fibrosis (≥F2) or elevated serum ALT levels during at least three months in combination with significant HBV viral load (>2x103 IU/mL or 2x104 IU/mL, depending on HBeAg status) signified antiviral treatment indication.[4] In chronic HCV-infected patients with genotypes 1 or 4, antiviral treatment was recommended in treatment-naïve patients with significant fibrosis (≥F2) and in chronic HCV infection genotype 2 or 3 irrespective the degree of fibrosis.[3] In HBV- or HCV-infected patients with ≥F3 fibrosis, surveillance for HCC was advised.[3, 4] Liver biopsy was advised after LSM in patients with equivocal LSM results, especially in patients with F2 fibrosis considering the low accuracy of LSM to distinguish between F0/1 and F2 fibrosis. From electronic patient files we evaluated retrospectively whether the advice was indeed followed in clinical practice.

Statistical analysis

For continuous variables, medians and interquartile range (IQR) were calculated. Statistical significance of differences in continuous variables was calculated with the Mann-Whitney U test. Categorical variables were reported as percentages. Chi-square

testing was used to test differences in categorical variables. Data were analyzed using IBM SPSS Statistics version 19.0 (SPSS INC., IBM, USA).

Results

Patient characteristics

A total of 506 LSMs were performed in our hospital from October 2008 to October 2011. Of those, 84 were excluded because LSM was performed for other liver diseases than HBV- or HCV-infection and 41 because of repeated assessment (only first LSM was taken into account). Of the remaining 381 patients, 32 (8%) were excluded because LSM failed (mainly because of obesity). The remaining 349 patients were included in the study. Of these, 161 (46%) had HBV monoinfection, 23 (7%) HBV-HIV coinfection, 122 (35%) HCV monoinfection, 39 (11%) HCV-HIV coinfection and 4 (1%) HBV-HCV-HIV coinfection (*figure 1*). For further analysis, patients were divided in HBV-infected and HCV-infected groups. HBV-HCV coinfected patients were included in the HCV-infected group since HCV is usually considered the dominant pathogen in HBV-HCV coinfection (all 4 HBV-HCV coinfected patients were also HIV-positive).[15]

Figure 1: flow-chart of the study

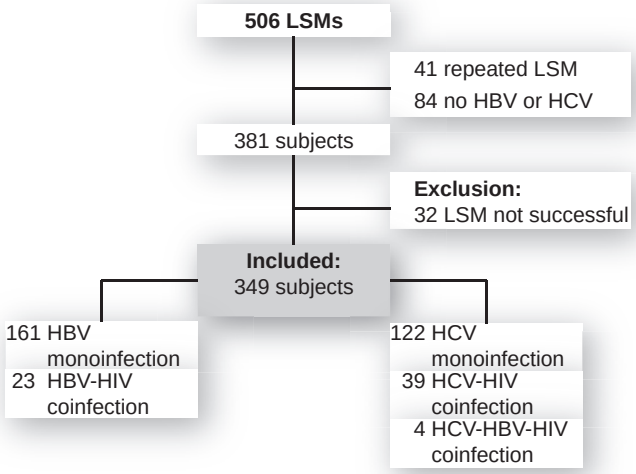

Table 1: Baseline characteristics in patients with chronic HBV or HCV-infection

	HBV-patients n=184		HCV-patients n=165		p-value
General characteristics					
Gender, male	124	(67%)	131	(79%)	<0.001
Age, years	39	(30-46)	46	(38-54)	<0.001
BMI, kg/m2	22.7	(20.7 – 26.2)	23.7	(21.0 – 25.2)	n.s.
HIV coinfection	23	(13%)	43	(26%)	<0.001
HBV disease parameters					
HBV-DNA, IU/mL (IQR)	1.1 x103 (0-4.9x104)				
HBeAg-positive	45	(24%)			
On antiviral therapy	55	(30%)			
HCV disease parameters					
HCV-RNA, IU/mL (IQR)	-	-	4.0 $x10^5$ ($9.9x10^4$-$1.9x10^6$)		-
Genotype 1 /2 /3 /4,%	-	-	61 / 10 / 22 / 7		-
Haemophilia	-	-	29	(18%)	-
IDU	-	-	60	(36%)	-
Laboratory values					
Bilirubin, µmol/L	14	(11 – 18)	13	(10 – 18)	n.s.
ALP, U/L	69	(58 – 86)	82	(64 – 104)	<0.001
γ-GT, U/L	27	(20 – 38)	60	(38 – 96)	<0.001
AST, U/L	21	(6 – 30)	39	(26 – 81)	<0.001
ALT, U/L	32	(24 – 52)	54	(33 – 103)	<0.001
>ULN	62	(34%)	112	(64%)	<0.001
Albumin, g/L	40.9	(38.5 – 42.3)	39.9	(36.7 – 42.6)	n.s.
Thrombocytes, x109/L	226	(183 – 264)	216	(164 – 267)	n.s.
PT, seconds	13.7	(13.2 – 14.2)	13.5	(13.0 – 14.1)	n.s.

Values are numbers (proportion) or medians (interquartile range). ***n.s.:*** *not significant.* ***HBeAg:*** *hepatitis B antigen.* ***IDU:*** *history of intravenous drug use.* ***ALP:*** *alkaline phosphatase.* ***AST:*** *Aspartate transaminase.* ***ALT:*** *Alanine transaminase.* ***ULN:*** *Upper limit of normal (35 IU/mL for females; 45 IU/mL for male patients).* ***γ-GT:*** *γ-glutamyl-transpeptidase.* ***PT:*** *prothrombin time.*

Patient characteristics of the HBV and HCV patient groups are given in *table 1*. In short, 67% of HBV-infected patients and 79% of HCV-infected patients were male and median age was 39 for HBV- and 46 years for HCV-infected patients. Furthermore, HCV-infected patients had significantly higher liver biochemistry values than HBV patients. In addition, HCV-infected patients had higher LSM results (38% versus 20% ≥F2, p<0.001) than HBV-infected patients. Stage F0-1, F2, F3 and F4 were present in 72%, 11%, 5% and 12% of all patients. Median IQR of individual measurements (usually 10 measurements) within each patient, depicted as a percentage relative to median value, was 16.3% and did not significantly differ between HBV- and HCV infected patients (*table 2*).

Clinical impact of LSM in HBV infection

Fifty-five (30%) HBV-infected patients were on antiviral treatment (in all cases tenofovir or entecavir maintenance therapy) at the time of LSM. Of the 129 (70%) untreated HBV-infected patients, 3 (2%) had an indication for treatment based on prolonged elevation of serum ALT values in combination with high viral load, irrespective of extent of liver fibrosis. In 7 other HBV-patients (5%), antiviral treatment was recommended based on LSM (*figure 2; table 2*). In 17 HBV-infected patients (9%), surveillance for HCC was recommended. In 6 (35%) of these patients, ≥F3 fibrosis was already evident before LSM, based on liver biopsy (n=1), ultrasound (n=3) or other investigations (n=2).

Finally, in 16 HBV-infected patients (9%), additional liver biopsy was recommended, generally because of perceived discrepancy between clinical data and LSM results or because of F2 fibrosis with LSM.

Table 2: Results of LSM in patients with chronic HBV or HCV-infection

	All patients		**HBV patients**		**HCV patients**		**Statistical significance**
	n=349		n=184		n=165		p-value
LSM, median (kPa)	5.5		5.1		6.3		0.002
LSM, range (kPa)	2.5 – 72.1		2.5 – 40.3		2.8 - 72.1		
IQR, median (%)	16.3%		16.2%		18.1%		0.218
F0/1	251	(75%)	148	(80%)	103	(62%)	
F2	39	(11%)	16	(9%)	23	(14%)	
F3	18	(5%)	10	(5%)	8	(5%)	
F4	41	(12%)	10	(5%)	31	(19%)	

Median and range of LSM results, IQR of individual measurements as a percentage of the median of each patient and frequency of LSM results per patient group. ***LSM:*** *Liver stiffness measurement.* ***IQR:*** *interquartile range.*

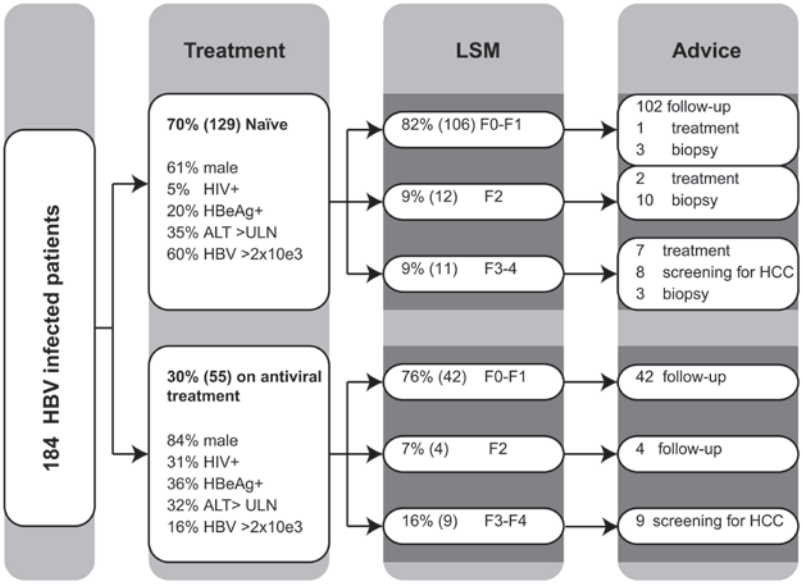

Figure 2: Baseline characteristics, results of LSM and advice based on LSM in patients with chronic HBV-infection. LSM: liver stiffness measurement. **Naïve:** patients without (previous) antiviral treatment. **ALT:** *alanine transferase.* **ULN:** Upper limit of normal. **HCC:** hepatocellular carcinoma.

Clinical impact of LSM in HCV infection

Of 101 treatment-naïve HCV-patients, 32% had an indication for HCV-treatment based on favourable HCV-genotype (i.e. genotype 2 or 3). In 12% (n=19) of all HCV-infected patients, treatment was recommended solely based on LSM (*figure 3; table 3*).

In 22% of HCV-infected patients (n=36), surveillance for HCC was recommended. In 7 (19%) of these patients, F3-4 fibrosis was already evident before LSM, based on previous liver biopsy (n=2), ultrasound (n=2) or other investigations (n=3). Furthermore, LSM was performed in 19 patients who had been cured from their HCV-infection due to spontaneous clearance (SC; n=3) or sustained viral response after antiviral treatment (SVR; n=16). Of these HCV patients, 18 (95%) could be discharged due to favourable LSM (F0-2) without need for HCC surveillance (*Figure 3*).

Finally, in 10 HCV-infected patients (6%), additional liver biopsy was recommended because of F2 fibrosis or discrepancy between LSM result and clinical data.

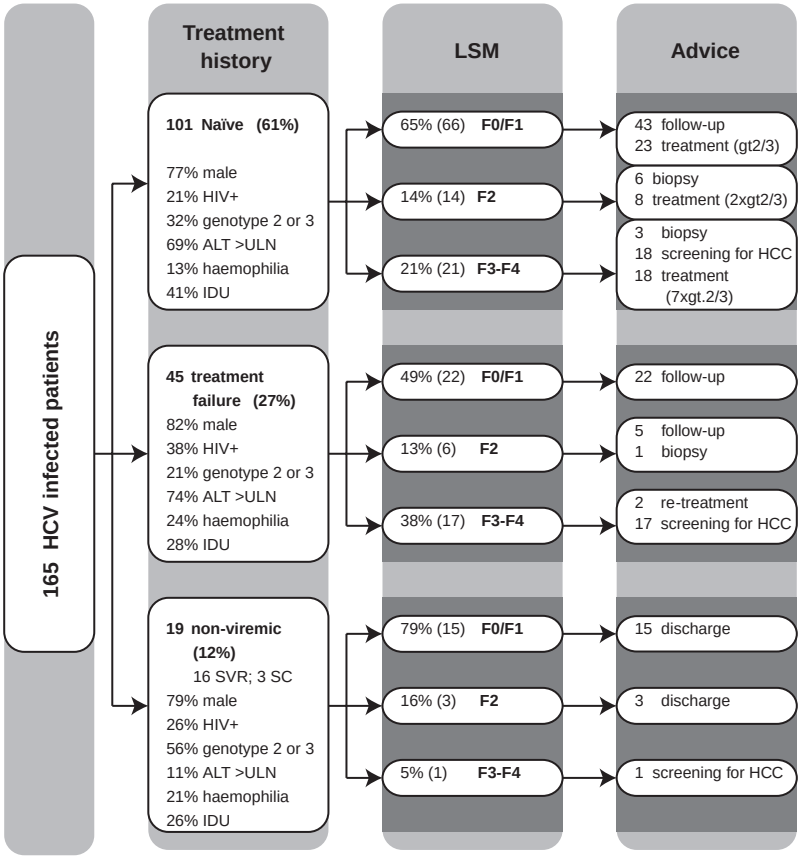

Figure 3: Flow chart of HCV-infected patients. LSM: liver stiffness measurements. **Naive:** no history of treatment for HCV. SVR: sustained viral response to HCV-treatment. **SC:** spontaneous clearance. **ALT:** alanine transferase. **ULN:** Upper limit of normal. **IDU:** (history of) intravenous drug use. **HCC:** hepatocellular carcinoma.

Table 3: Clinical impact of LSM in patients with chronic HBV or HCV-infection

	All patients		HBV-patients		HCV-patients		Statistics
	n=349		n=184		n=165		p-value
Treatment indication	61		10		51		<0.0001
Independent of LSM	35	(57%)	3	(30%)	32	(63%)	
Based on LSM	26	(43%)	7	(70%)	19	(37%)	
Surveillance for HCC	53		17		36		<0.01
Independent of LSM	13	(25%)	6	(35%)	7	(19%)	
Based on LSM	40	(75%)	11	(65%)	29	(81%)	

Advice after LSM, based on clinical and laboratory findings (independent of LSM) or based on LSM. ***LSM:*** *Liver stiffness measurement.* ***HCC:*** *Hepatocellular carcinoma.*

Adherence to medical advice

Surveillance for HCC by abdominal ultrasound was recommended in 53 patients (15% of all patients) and was initiated in 46 patients (87%). Liver biopsy was recommended in 30 patients (9% of all patients) and performed in the majority of them (n=22; 73%) (*figure 4*).

Antiviral treatment was recommended in 10 HBV-infected patients (8% of treatment naïve HBV patients) and 51 HCV-infected patients (35% of viremic patients). In most (80%) HBV-infected patients, treatment was initiated according to advice within 6 months after LSM. Of note, in only 47% (n=24) of 51 HCV-patients with recommendation to start treatment, this advise was followed in practice: 2 patients died before treatment could be started (1 due to sudden cardiac arrest and 1 due to hepatic failure); 6 had significant contra-indications to therapy; 3 did not want to start because of personal circumstances; 12 were lost to follow up and in 4 patients treatment was not started because of other reasons (*figure 4*). Neither of the factors gender, HIV-coinfection, HCV genotype, history of previous HCV treatment, haemophilia or history of intravenous drug use were predictive for adherence to medical advice in those patients. Finally, all recovered HCV-patients with favourable LSM (≤ F2) were discharged from follow-up as recommended (*figure 4*).

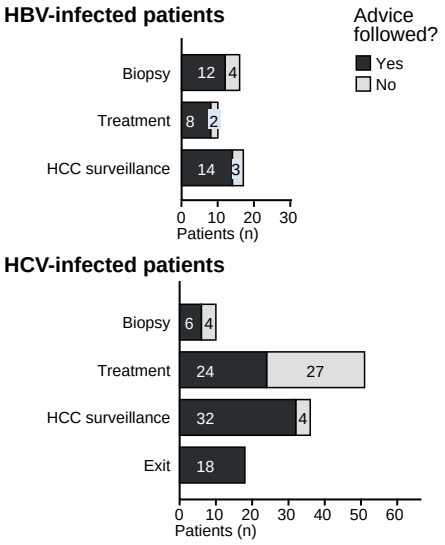

Figure 4: Follow-up on medical advice based on LSM. Bars represent number of HBV-infected (upper graph) and HCV-infected patients (lower graph) in whom medical advice after LSM was followed (black) or not followed (grey) by the requesting clinician. **LSM:** liver stiffness measurement. **HCC:** hepatocellular carcinoma.

Discussion

Evaluation of liver fibrosis is of clinical importance in chronic HBV or HCV-infection since indication for antiviral treatment often depends on the extent of liver fibrosis. Furthermore, in patients with severe liver fibrosis or cirrhosis, surveillance for HCC is required.[3, 4] Although liver biopsy has been considered to be the gold standard to determine the extent of liver fibrosis, its use is limited due to its invasiveness and risk of serious complications.[5] In this paper we describe the impact of transient elastography, as a non-invasive strategy to evaluate liver fibrosis,[10] on clinical decisions in chronic viral hepatitis.

We show that in the majority of untreated HBV- or HCV-infected patients, antiviral treatment could safely be postponed based on favourable (<F2) LSM. However, it must be taken into account that LSM lacks accuracy in differentiating ≥F2 from non-significant (<F2) fibrosis.[10] Therefore, additional biopsies were still recommended in a number of cases when LSM indicated F2 fibrosis. In HBV-patients, clinical advice to start antiviral treatment was generally followed in practice by the physician caring for the patient. However, in half of the patients for whom treatment of HCV was recommended, antiviral treatment was not initiated within 6 months due to a variety of reasons. This can be explained by a relatively high threshold to start treatment due to expected side effects and relatively low success rates.[16] However, we were not able to identify any factor that could predict adherence to medical advice in those patients. Future and improved treatment strategies for HCV-infection with less side effects and higher success rates may improve the rate of followed advice in these patients.[17-20] Nevertheless, we can conclude from our data, that LSM can be helpful to start or postpone treatment in both HBV and HCV infection.

Apart from treatment indication, LSM was also used to detect severe (≥F3) liver fibrosis and need for surveillance for HCC. In most patients with ≥F3 liver fibrosis detected by LSM, there was no evidence of severe liver fibrosis or cirrhosis before LSM. Indeed, in most of these cases surveillance for HCC was initiated within 6 months after LSM was performed.

Patients with severe fibrosis are at increased risk for HCC, even after successful treatment of HCV.[21] However, SVR is associated with lower risk for HCC in comparison to patients with persistent infection.[21] Since necroinflammation and increased transaminases are associated with overestimation of liver fibrosis by transient elastography, reduced LSM values after SVR might either reflect reduction of liver fibrosis or reduced hepatic inflammation.[22] However, SVR is also associated with reversibility of liver fibrosis.[23] In either way, no indication for screening for HCC remains in case no severe fibrosis (<F3) is detected in cured HCV-patients (after SC or SVR), allowing us to discharge these patients from follow-up.

LSM has several limitations. First, LSM may result in failure or indeterminate measurement. Previous studies have identified high body mass index (BMI), limited experience of the operator, and age >50 and type 2 diabetes mellitus as independent factors associated with failure.[24, 25] Since data on failed LSM was often limited, we were not able to measure contribution of these factors in this retrospective study. However, in a number of cases obesity was mentioned as the cause for failed LSM (*data not shown*).

Furthermore, reduced interobserver agreement of LSM has been associated with increased BMI (>25), histological steatosis and low grades of fibrosis.[26] In addition, cholestasis, chronic heart failure and flares of liver inflammation may result in overestimation of fibrosis by LSM.[22, 27, 28] Thus, LSM results must be interpreted with caution in any of these patient groups.

Altogether, we show that in our university medical center, clinical decisions in viral hepatitis now largely depend on LSM. A limitation of this report is the retrospective setting of the study. Prospective studies are needed to evaluate whether full implementation of transient elastography in the care of HBV- and HCV-patients indeed contributes to a decline in the number of treated patients and decrease of occurrence of HCC or liver failure. Furthermore, cost-benefit studies are needed to evaluate the impact of transient elastography on budget costs in viral hepatitis. However, we believe that our study population is representative for most patient populations worldwide, in whom transient elastography could be introduced. Therefore, we conclude that transient elastography has improved the assessment of liver fibrosis, thereby leading to more accurate treatment policy.

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Chapter 8

Discussion and perspectives

Rapidly changing perspectives in the field of HCV research

Since the discovery of HCV in 1989, the field of HCV research has rapidly moved forward. In the period from 2009 to 2013, during which the studies presented in this dissertation were performed, increased understanding of HCV-infection has contributed to major clinical developments. From the patient's point of view, these comprised improved success rates of HCV-treatment with the introduction of protease inhibitors boceprevir or telaprevir in addition to interferon and ribavirin.[1, 2] From the physician's point of view, non-invasive measurements of liver fibrosis, for instance by transient elastography, have now largely replaced liver biopsy in assessment and follow-up of HCV liver disease.[3, 4] From the researcher's point of view, the development of a genetically humanized mouse model,[5, 6] discovery of single nucleotide polymorphisms (SNPs) near the IL28B locus as a prognostic factor in viral clearance,[7-10] and recognition of microRNAs as important factors in HCV replication[11] have led to a better understanding of pathological mechanisms underlying chronic HCV-infection and liver fibrogenesis.

However, patients, physicians and researchers are still facing mayor challenges with respect to HCV infection, including severe side effects and high costs of the present treatment armamentarium, reduced efficacy of HCV treatment depending on patient- and viral factors and, last but not least, limited world-wide availability of diagnostic and treatment options, since the main burden of HCV disease is in developing countries.[12]

The studies presented in this dissertation were performed with the initial aim to find non-invasive methods to measure liver fibrosis. In this process, specific features of T cell activation and exhaustion were uncovered together with new cytotoxic features of NK cells. Below, several of our findings will be highlighted and their implications for immunology and clinical practice will be discussed.

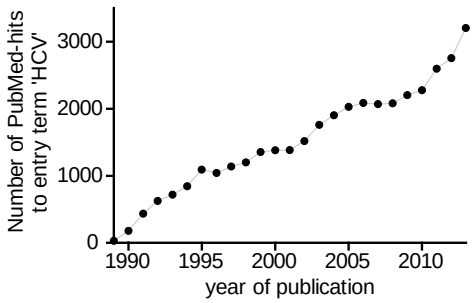


Figure 1: Numbers of publicated research artcles by year from 1989 to 2013.

T cell apoptosis in HCV monoinfection and HIV-HCV coinfection

Previously, our group reported that chronic HCV infected patients depict higher rates of peripheral T cell apoptosis than healthy controls.[13] In the mean time, others have shown that HCV-infected hepatocytes can induce apoptosis of co-cultured T cells in vitro, in a FasL-dependent manner.[14] Based on these findings, we hypothesized that peripheral T cell apoptosis might serve as a marker for liver disease. To this end, we set up a large case-control study with HBV and HCV infected patients and healthy controls and primary biliary cirrhosis (PBC) patients as disease controls. Next to HCV-monoinfected patients, we also included HIV-HCV coinfected patients in order to investigate whether the level of T cell apoptosis was further influenced by coinfection with HIV, presented in **chapter 2**. In addition, T cell activation and exhaustion were characterized, as presented in **chapter 4** and NK cell phenotypic changes associated with HCV and HIV are presented in **chapter 5**.

In **chapter 2**, we confirm that chronic HCV infection is associated with increased peripheral T cell apoptosis, as characterized by increased activation of caspases from the extrinsic apoptosis pathway (i.e. initiator caspase 8 and effector caspases 3&7), but this was independent of degree of liver fibrosis or any other disease parameter in HCV-monoinfected patients. As outlined in **chapter 1** of this thesis, apoptosis can be induced either upon stimulation of a death receptor by its ligand (extrinsic apoptosis pathway) or by mitochondrial stress (intrinsic apoptosis pathway). However, many factors may be involved in activation of the extrinsic apoptosis pathway. In the case of HCV infection, T cell apoptosis may be induced by encountering antigen in the liver,[14] as an immunological mechanism of downregulating the immune response in prolonged infection through activation induced cell death (AICD),[15] or even directly by HCV through infection of T cells.[16, 17] Furthermore, cellular fate upon activation of the apoptosis pathways also depends on a number of intracellular apoptosis-inhibitory mechanisms such as unfolded protein responses and transcriptional regulation of apoptotic genes.[18-20] However, since the level of peripheral T cell apoptosis was not correlated to level of HCV-viraemia in our data, it can be hypothesized that increased level of T cell apoptosis is merely a feature of T cell activation. Indeed, increased levels of T cell apoptosis can be found in many chronic inflammatory conditions associated with ongoing immune activation such as chronic infections, malignant disease and autoimmune disease.[15, 21-23]

T cell apoptosis in HIV-HCV coinfection

Loss of infected and non-infected CD4 T cells is the hallmark of HIV-infection, and is responsible for opportunistic infections and the acquired immune deficiency syndrome (AIDS), but upon antiretroviral treatment, CD4 counts gradually rise to normal values in most of the patients.[24-26] However, HCV treatment with pegylated interferon-alpha and ribavirin remains less effective in coinfected despite adequate HIV suppression,

raising the question whether adaptive immunity is involved.[27]

In **chapter 2** we demonstrate that HIV-HCV coinfected patients on antiretroviral treatment depicted higher levels of activated caspase 8 in comparison to healthy controls and HCV monoinfected patients. Levels of caspase 8 correlated with levels of downstream activated effector caspases 3 and 7 and annexin V, indicating that the extrinsic apoptosis pathway is upregulated in T cells of HIV-HCV coinfected patients. Increased T cell apoptosis might be the result of HIV-infection, despite adequate antiretroviral treatment, since HIV monoinfected patients also depicted higher levels of activated caspases and annexin V (**chapter 2**). These data indicate that both HCV and treated HIV affect T cell apoptosis, thus providing one explanation for altered immunity against HCV and decreased treatment responses in HIV-HCV coinfected patients.

A sub-analysis among HIV monoinfected and HIV-HCV coinfected patients was highly suggestive for differential effect of NNRTI- versus PI-based ART regimen on T cell apoptosis. Some in vitro studies suggested that NNRTIs may enhance apoptosis whereas PIs may exert anti-apoptotic properties.[28-30] However, from previous studies it was unknown whether this potential differential effect on apoptosis would be relevant in vivo, due to inconsistencies in the data possibly influenced by limitations of these studies.[31-34] To investigate the differential effect of cART-regimens on peripheral T cell apoptosis, we performed an additional study on freshly isolated PBMCs from carefully selected patients, which is presented in **chapter 3**. Our data demonstrate that HIV-infected patients treated with PI-based versus NNRTI-based cART (with similar NRTI-backbone therapy) display equal levels of peripheral T cell apoptosis. Interestingly, NNRTI-based treatment was associated with lower levels of Bcl-2, an anti-apoptotic protein involved in the intrinsic apoptosis pathway, in comparison to PI-based treatment. However, downstream activation of caspases 3 and 7 and annexin V did not differ between the groups. Thus, our data indicate that regimen of cART may affect cellular stress, but does not lead to differential levels of peripheral T cell apoptosis.

Whereas T cell apoptosis in untreated HIV is, at least partly, induced by HIV-encoded proteins like gp120,[35] this is unlikely to play an important role in adequately treated HIV infected patients with undetectable viral loads. However, low HIV viral loads which were not quantified by our standard PCR (lower limit of detection <50copies/mL) could possibly still contribute to peripheral T cell apoptosis.[36] New, more sensitive methods to quantify these low levels of viremia may shed light on this issue.[37] Alternatively, it may be hypothesized that increased levels of peripheral T cell apoptosis in HIV monoinfection (as well as HIV-HCV coinfection) may be the result of chronic immune activation, as will be discussed below.[15, 38]

Immune activation

T cell activation in HCV monoinfection

In **chapter 4**, we show that HCV monoinfected patients also depicted decreased percentages of naïve CD8 T cells and relatively increased memory/effector T cells. Furthermore, we identified HCV-RNA as a factor that correlated to T cell activation and exhaustion, suggesting that the level of viral antigen drives T cell activation and exhaustion. Indeed, by injecting different doses of lymphocytic choriomeningitis virus (LCMV) in C57BL/6 mice, Mueller et al. confirmed that the level of viral antigen drives T cell exhaustion in a dose-dependent manner.[39] These findings raise the question whether peripheral T cell exhaustion is induced in the environment of the inflamed liver, or in the periphery, possibly though systemic immune activation related to serum levels of viral antigen. This is even more disputable since level of HCV-RNA in the serum is known to be closely correlated to intrahepatic levels.[40-42]

Systemic immune activation in HIV monoinfected and HIV-HCV coinfected patients

In HCV-infected patients, coinfection with HIV is associated with higher HCV viral load, decreased HCV viral clearance, and accelerated progression of liver fibrosis in chronic HCV infection.[27, 88] Upon start of antiretroviral treatment, liver fibrosis progression in HIV-HCV coinfected patients decreases to a level similar as in HCV monoinfected patients,[89] possibly through reduced intrahepatic expression of pro-inflammatory cytokines such as TNF-alpha.[90] However, spontaneous and treatment-induced HCV clearance rates remain lower in HIV-HCV coinfected patients in comparison to HCV monoinfected controls.[27, 88] Although several mechanisms have been proposed, the underlying immunopathogenesis of HIV-HCV coinfection is currently poorly understood.[47, 91] Gaining insight into these mechanisms can lead to new diagnostic and therapeutic strategies for patients with HCV/HIV coinfection.

Systemic immune activation might provide one explanation for decreased viral control in coinfected patients. Indeed, HIV monoinfected patients are increasingly at risk for age-related diseases such as cancer and cardiovascular disease, which has in recent years been linked to systemic immune activation.[43, 44] Several factors may contribute to systemic immune activation in well treated HIV patients, including microbial translocation and chronic viral coinfections.[43, 45, 46] HIV-mediated destruction of gut barrier integrity may lead to translocation of microbial products into the circulation, which may promote activation of innate immunity via pattern recognition pathways.[47] Interestingly, HCV-infection may also lead to microbial translocation, as measured by sCD14, and subsequent immune activation.[48] HIV and HCV activate T cells via different pathways which may act in a synergistic way, thus providing an explanation for increased immune activation in HIV-HCV coinfected patients in comparison to HIV

or HCV monoinfected patients.[47, 48] Indeed, T cell activation in HIV-HCV coinfection may partly be reversed by HCV treatment.[49]

In **chapter 4**, we demonstrate that HIV-HCV coinfected patients showed significantly higher levels of activated T cells, as defined by expression of HLA-DR and CD38, in comparison to HCV monoinfected patients. Expression of exhaustion markers PD-1 and Tim-3 depicted similar patterns, though not significant. Levels of T cell activation and exhaustion correlated with HCV-RNA, and HIV-HCV coinfected patients depicted borderline significant higher levels of HCV-RNA, providing one explanation for increased levels of immune activation and exhaustion in these patients, since increased levels of viral antigen may cause higher levels of T cell exhaustion.[39]

Furthermore, CD4/CD8 ratios were significantly lower in coinfected patients in comparison to their HCV-monoinfected counterparts, as demonstrated in **chapter 6**. CD4/CD8 ratio has mostly been used as a marker for disease progression of untreated HIV infection, as decreased CD4/CD8 ratios are associated with poor prognosis.[50, 51] Recently, decreased peripheral CD4/CD8 ratio has also been implied in treated HIV infection, as it is associated with T cell activation and exhaustion.[52] Furthermore, low CD4/CD8 ratios are associated with immune activation and poor outcome in a number of clinical conditions, including infectious (e.g. dengue fever) and non-infectious (e.g. myocardial infarction, cervical carcinoma) disease.[53-55] Decreased CD4/CD8 T cell ratios have also been linked to immune activation in HIV natural viral suppressers who were coinfected with HCV.[56] Thus, from these studies it appears that decreased CD4/CD8 ratios reflect an ongoing inflammatory state in many clinical conditions, including HIV-HCV coinfection, as also demonstrated by T cell activation in these patients, presented in **chapter 6**.

Taken together, our findings presented in the **chapters 2, 4, 5 and 6** indicate that despite adequate antiretroviral treatment, HIV-HCV coinfected patients display higher level of immune activation than HCV monoinfected patients, and this may be caused by microbial translocation, as suggested by others, and possibly by HCV viral loads.

Non-invasive measurement of liver fibrosis

For decades, liver fibrosis has been classified by liver biopsy.[57] However, its invasive nature, sampling error and risk of severe complications have prompted research to identify noninvasive methods to estimate liver fibrosis.[4, 57] A number of non-invasive methods to evaluate liver fibrosis have made it to the clinic, including transient elastography.[4] In a retrospective study, presented in **chapter 7**, we demonstrate that transient elastography has a major impact on clinical practice, both as an indication to start or postpone antiviral treatment, to start surveillance for hepatocellular carcinoma

and to discharge HCV-patients from follow-up after viral clearance and favourable liver stiffness measurement (LSM). Despite its potential as a diagnostic tool, transient elastography is only available in a minority of hospitals, due to its costs and the required training of examiners.[4] Therefore, a simple blood test would be preferable in centers where transient elastography is not available.

However, levels of T cell activation, exhaustion and apoptosis were not different between patients with F3-F4 fibrosis and patients with F0-F2 fibrosis as shown in **chapters 2 and 4**. Interestingly though, in **chapter 6** we demonstrate that CD4/CD8 ratio is a potential candidate for a non-invasive test for diagnosing liver fibrosis in chronic HCV-monoinfected patients. CD4 and CD8 counts are relatively stable over time in the chronic phase of viral infections,[58, 59] making CD4/CD8 ratio a reliable marker. Since low CD4/CD8 T cell ratio indicates a shift towards increased numbers of CD8 relatively to CD4 T cells, our data raise the question whether the relatively expanded CD8 T cell subset would also be found in the liver and if this may imply that CD8 T cells are involved in liver fibrogenesis. With respect to the first part of that question, it has been reported that intrahepatic CD8 T lymphoctyes of chronic infected HCV patients were relatively enriched for HCV-specific CD8 T cells in comparison to the peripheral blood.[60, 61] Moreover, it has been demonstrated that in the liver of chronic HCV-infected patients, CD4 but not CD8 cells decrease in parallel with fibrosis.[62] Thus, it is very likely that not only in peripheral blood, but also in the liver CD8 predominate over CD4 T cells in patients with liver fibrosis.

Relatively expanded CD8 T cells can be mechanically linked to liver fibrosis by two major mechanisms. First, since we demonstrated in **chapter 4** that liver fibrosis is also associated with increased effector CD8 T cell phenotype (which correlates with perforin expression), it can be hypothesized that relatively expanded CD8 T cells may lead to enhanced hepatic inflammation, resulting in liver fibrosis. However, this concept is opposed by studies performed by Golden-Mason *et al*, indicating that in chronic HCV-infection, many intrahepatic HCV-specific T cells are of an exhausted phenotype, displaying PD-1 and Tim-3, with decreased proliferative, cytotoxic and cytokine productive functions.[63, 64] Therefore, it is questionable whether increased percentages of CD8 T cells directly contribute to liver fibrogenesis since many of the intrahepatic CD8 T cells are in fact incapable of attacking infected hepatocytes.[63, 64] However, in light of this paradoxical concept, a possible explanation is provided by Radziewicz *et al*, revealing that in the liver of chronic infected HCV-infected patients, intrahepatic CD8 T cells expressing PD-1 massively undergo apoptosis.[65] This is an important finding, since phagocytosis of (apoptotic) lymphocytes by hepatic stellate cells (HSCs), which in turn become activated, is currently believed to be an important factor contributing to liver fibrosis.[66] Thus, liver infiltrating CD8 T cells may be involved in liver fibrogenesis not only by bystander killing of non-infected hepatocytes, but also by intrahepatic

exhaustion and apoptosis, leading to activation of HSCs.

The data presented in **chapter 6** are further supported by the observation of a similar pattern in PBC patients, though not significant, as presented in *figure 2*. With regard to the actual CD4/CD8 ratios in these patients, it should be noted that these are highly influenced by decreased overall numbers of T cells in the peripheral blood associated with PBC.[67]

However, our data in **chapter 6** indicate that CD4/CD8 ratio as a diagnostic tool is not applicable in HIV-HCV coinfection, since coinfected patients typically depict low CD4/CD8 ratio independent of liver fibrosis, possibly due to immune activation in these patients, as explained in the former paragraph.

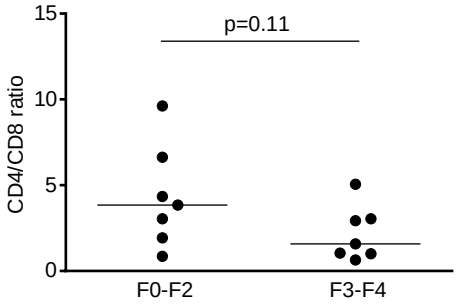


Figure 2: CD4/CD8 T cell ratios in PBC patients with F0-F2 versus F3-F4 fibrosis.

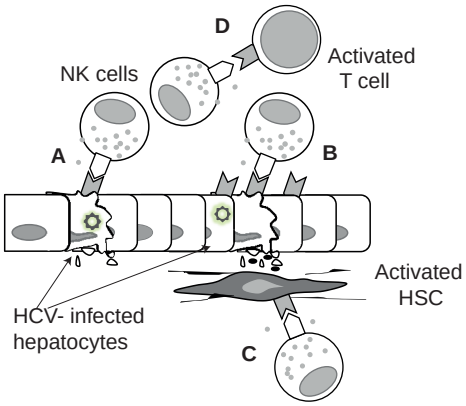
Innate versus adaptive immunity in HCV infection

Recently, the relative expression of a number of activating and inhibiting receptors on NK cells were found to be associated with outcome of HCV infection, thus indicating that NK cells are of major importance in HCV infection.[68] This is in line with our own data on NK cell phenotype in HCV and HIV-HCV infection as presented in **chapter 5**. In our data, $CD56^{bright}CD16^{neg}$ NK cells (further termed $CD56^{bright}$ NK cells) express high levels of the apoptosis-inducing ligand FasL, and that HCV is associated with upregulation of FasL on $CD56^{bright}$ and $CD56^{dim}$ NK cells, independent of coinfection with HIV, as described in **chapter 5**. These findings shed new light on the function of the $CD56^{bright}$ NK cell subset, since they are highly suggestive for increased cytotoxic potential of these cells in chronic HCV-infection, whereas the $CD56^{bright}$ NK cell subset was traditionally regarded as a non-cytolytic NK cell subset, mainly functioning as a producer of IFN-γ, TNF-α and other cytokines.[69, 70] Unfortunately, it was impossible to test in vitro cytotoxicity of the $CD56^{bright}$ NK cells, due to the low frequency of this cell subset in the blood. In contrast to small numbers in the peripheral blood, it must be noted that the HCV-infected liver is enriched for NK cells in general and for $CD56^{bright}$

cells more specifically.[71] Fas/FasL mediated killing has been extensively studied by others[72-74] and it is very likely that CD56^{bright} NK cells expressing FasL are able to induce death of target cells. At least we did show that increased FasL expression on CD56^{bright} NK cells was not associated with autocrine apoptosis since CD56^{bright} cells depicted less Fas and activated caspase 3 in comparison to CD56^{dim} NK cells (**chapter 5**).

Thus, although supplementary experiments are still required to draw definite conclusions, our data suggests that CD56^{bright} NK cells have FasL-mediated cytotoxic potential, which is enhanced in chronic HCV. However, it remains unknown which cells could be target of this mechanism and in which way Fas/FasL mediated killing contributes to the course of HCV liver disease. Possible mechanisms include: (A) Fas/FasL mediated killing of Fas-expressing infected hepatocytes,[75] which may contribute to viral clearance, (B) non-specific 'bystander' killing in the liver,[76] contributing to fibrosis (C) Fas/FasL mediated killing of activated HSCs, resulting in regression of fibrosis,[77] and (D) regulation of the adaptive immune response, through apoptosis induction of Fas-expressing activated T cells[78] (*figure 3*).

Figure 3: model of Fas-FasL mediated NK cell cytotoxicity relevant to HCV-infection and liver fibrosis. A: killing of infected hepatocytes; B: bystander killing of non-infected hepatocytes, resulting in activation of HSCs; C: killing of activated HSCs; D: killing of activated T cells, thereby downregulating adaptive immunity. **HSC:** Hepatic stellate cell.



Noteworthy, in light of the latter mechanism, we observed increased expression of Fas in HCV monoinfected patients in comparison to healthy controls, as described in **chapter 4**, suggesting that T cells may be the target cells of FasL mediated killing by NK cells in chronic HCV infection. Indeed, Waggoner *et al* have recently demonstrated that NK cells downregulate adaptive immunity by killing of activated CD4 T cells, thereby contributing to CD8 T cell exhaustion through reduction of CD4 T cell help.[79] Furthermore, intrahepatic apoptosis of T lymphocytes may also contribute to liver fibrosis through activation of HSCs upon phagocytosis of apoptotic lymphocytes.[66]

Altogether, our data show that chronic HCV infection is associated with distinct differences in NK cell phenotype, including upregulation of cytotoxic markers, suggesting that chronic HCV drives activation of innate immunity. This is in line with the concept that innate immunity predominates over adaptive immunity in chronic HCV, as recently posed by Rehermann in a comprehensive review in Nature Medicine.[68] Indeed, all mediators of innate immunity are involved in chronic HCV infection. One of these mediators, interferon-alpha is still the backbone of HCV treatment.[80] Administration of interferon-alpha leads to a rapid decline of viral load within a few hours, which is caused by blockage of virion production or release.[81, 82] In addition, it exerts its effects by modulation of numerous non-coding microRNAs (miRNAs).[83] Similarly, SNPs near the locus of IL28B are important predictors for spontaneous and treatment-induced clearance of HCV.[7-10, 84] The idea that innate immunity is a main determinant in clearance of a virus which tends to cause chronic infection is somehow in contradiction with the classical concept of innate versus adaptive immunity. In this theory the innate immunity is considered as the first line of defense against infection, before adaptive immunity can kill infected cells in an antigen-specific manner.[85] Rather, it now seems that in chronic viral infection, various mechanisms, including exhaustion and apoptosis, are downregulating T cell responses in order to reduce collateral immune-mediated damage of the liver during chronic viral infection.[42, 68, 86] Indeed, whereas acute infection is associated with hepatic symptoms, the chronic phase of HCV-infection is usually free of symptoms before severe liver fibrosis is established.[87]

Innate immunity in HIV-HCV coinfection

Coinfection with HIV may interfere with innate immunity against HCV in several ways.[47, 91] For instance, HCV and HIV engage TLR7 and TLR9 signaling pathways and may thereby interfere with IFN-α secretion upon recognition of viral antigen by dendritic cells (DCs).[92-94] Furthermore, chronic HCV and HIV infection are associated with similar alterations in NK cell phenotype, including relatively reduced percentage of $CD56^{dim}$ NK cells.[95] Interestingly, HIV-HCV coinfection is associated with expansion of functionally skewed $CD56^{neg}$ NK cells.[96] Although the functional properties of this $CD56^{neg}$ NK cell subset is far from clarified, it has been suggested that these cells influence antiviral immunity by the secretion of cytokines.[97] HCV and HIV may each alter NK cell function through interference with cell activation in several other ways, but underlying mechanisms are currently poorly understood.[47]

As demonstrated in **chapter 5**, we found no clear differences between NK cell phenotype of HCV-monoinfected and HIV-HCV coinfected patients who were on adequate antiretroviral treatment. FasL expression, which was found to be increased on $CD56^{bright}$ and $CD56^{dim}$ NK cells of chronic HCV infection, did not differ between HIV-positive and –negative HCV-infected patients. Likewise, percentages of granzyme

B and perforin positive NK cells were similar between HIV-HCV coinfected and HCV monoinfected patients, even though HIV-monoinfected patients depicted higher levels of granzyme B and perforin-positive $CD56^{bright}$ and $CD56^{dim}$ NK cells than healthy controls. Coinfected patients also displayed similar levels of NK activating markers as HCV monoinfected patients. Taken together, our data did not reveal any alterations in NK cell phenotype in well treated HIV-HCV coinfected patients in comparison to HCV monoinfected patients, and thus suggest that treated HIV coinfection has no major impact on NK cell function.

Innate predominates over adaptive immunity in HCV-infection

In conclusion, our data demonstrate that in chronic HCV infection, NK cells depict a phenotype consistent with upregulation of Fas/FasL mediated cytotoxic potential, while CD4 and CD8 T cells demonstrate an exhausted phenotype and may be more sensitive to apoptosis, suggesting that innate immunity predominates over adaptive immunity in chronic HCV infection. This is consistent with the now widely accepted concept that, though essential in spontaneous clearance in the acute phase of infection, HCV-specific CD4 and CD8 T cell function decreases as viral infection persists, through T cell exhaustion and NK cell-induced T cell apoptosis.[42, 68, 79, 98]

The use of peripheral blood lymphocytes in studying HCV immunology

In most organs, vascular endothelial cells form a physical barrier to prevent access of T lymphocytes to the surrounding tissue.[99] However, electron microscopy revealed that the distinctive architecture of hepatic sinusoids, with fenestrations in the endothelial cell linage, enables T cells to interact with hepatocytes in the liver.[100-102] Furthermore, peripheral and hepatic T cells depict a similar pattern of antigen specific T cell responses,[103, 104] suggesting an active exchange between hepatic and peripheral blood lymphocytes. This concept provides the theoretical basis for the potential of phenotypic properties of peripheral lymphocytes as a marker for liver disease and was therefore the main subject of this thesis.

However, this concept is opposed by two theories. The first is the theory of compartmentalization of T cells to the site of infection which may lead to reverse changes in the peripheral blood.[105] The second theory suggests that development and differentiation of certain resident liver lymphocytes may in fact take place in the liver.[106, 107] Thus, although previous studies revealed that intrahepatic changes in T cell phenotype associated with HCV infection are reflected in the peripheral blood,[63] most of our findings require confirmation by studies on hepatic infiltrates before definite conclusions about pathogenic mechanisms can be made.

Research perspectives

HCV is a significant world-wide health problem.[108] Although new diagnostic and treatment options with improved outcome have come to the market, these are not available in countries with highest prevalence of HCV, due to high costs.[12, 108] Currently, non-governmental organizations like Médecins Sans Frontières (MSF) are considering to start treatment of HCV as part of public health care programs in resource-limited settings (*personal communication*). In these countries, it would be particularly useful to asses liver fibrosis in order to distinguish patients at high risk for hepatic complications. Based on our data in **chapter 7**, transient elastography could be helpful in that aspect but is mostly due to financial restrictions no option. Therefore, in **chapter 6** we identified peripheral CD4/CD8 T cell ratio as a promising candidate for evaluation of liver fibrosis in HCV monoinfection. CD4/CD8 ratios could be incorporated in currently existing health care programs in developing countries if annexed to CD4-count determination for HIV. Therefore, the use of CD4/CD8 ratio as a noninvasive marker for liver fibrosis should be tested in a large cohort, in order to confirm our findings and to determine optimal cut-off in the clinical setting. Furthermore, factors potentially influencing CD4/CD8 ratios should be identified, before this measure can be used in clinical practice. Lastly, it would be worthwhile to whether the CD4/CD8 could also be used in patients with other underlying disease, such as HBV-infection of alcoholic liver disease.

Although a long road is left behind, many more steps are still to be taken towards a full understanding of HCV immunopathogenesis and development of robust and easy screening tools for HCV disease progression. These diagnostic tests will aid in the decision making of therapy use, which may specifically be helpful in resource poor settings which most likely have less access to more powerful anti-HCV treatment options.

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8

Chapter 9

Samenvatting in het Nederlands

Achtergrond

Het hepatitis C virus (HCV) is een virus dat zich vermenigvuldigt in de cellen van de lever. De immuunreactie die gericht is tegen hepatitis C speelt zich daarom voornamelijk af in de lever, en kan op de lange termijn verlittekening veroorzaken, oftewel leverfibrose. Patiënten met ernstige leverfibrose hebben hoge kans op complicaties en op leverkanker en ze hebben een sterk verkorte levensverwachting. Het is daarom van belang om leverfibrose op tijd te ontdekken en de infectie goed te behandelen. De behandeling van HCV, met combinatie van ribavirine en interferon is echter zwaar, langdurig, kostbaar en lang niet altijd effectief. Er zijn inmiddels nieuwe middelen van HCV op de markt (boceprevir en telaprevir) en combinatie van één van deze nieuwe middelen met de standaardtherapie leidt tot sterk verbeterde behandelingsresultaten. Van succesvolle behandeling wordt gesproken als het virus na de behandeling niet meer meetbaar is.

Gelijktijdige infectie (coinfectie) met het humaan immuundeficiëntie virus (HIV, dat we kennen als de veroorzaker van AIDS) komt relatief vaak voor, doordat de virussen op dezelfde manier kunnen worden overgebracht en vaker voorkomen onder bepaalde risicogroepen, waaronder homoseksuelen, intraveneus drugsgebruikers en immigranten uit gebieden waar de infecties vaak voorkomen. Coinfectie met HIV leidt tot een veranderde immuunreactie, waardoor patiënten sneller leverfibrose ontwikkelen en de behandeling voor HCV ook nog eens minder goed aanslaat. Indien HIV echter succesvol wordt behandeld en de productie van nieuwe virusdeeltjes dusdanig wordt onderdrukt dat het virus niet meetbaar is in het bloed, is de fibroseprogressie vergelijkbaar met patiënten met enkel HCV-infectie (monoinfectie). De resultaten van HCV-behandeling met ribavirine en interferon combinatietherapie zijn in die patiënten echter alsnog beduidend slechter dan in HCV monoinfectie. Nieuwe behandelingopties voor HCV zoals toevoeging van boceprevir en telaprevir verbeteren de resultaten wel, maar zijn niet in alle gevallen toepasbaar.

Het doel van het in dit proefschrift samengevat onderzoek was om op zoek te gaan naar nieuwe methoden om fibrose in HCV monoinfectie en HIV-HCV coinfectie te detecteren, en om meer inzicht te verkrijgen in de immunologische bijzonderheden die bij deze ziekten een rol spelen.

T cel apoptose bij HCV monoinfectie en HIV-HCV coinfectie

In het eerste deel van het proefschrift hebben we apoptose onder de loep genomen. Apoptose is een vorm van geprogrammeerde celdood dat dient om op een nette manier overbodige cellen uit de weg te ruimen. Gebaseerd op eerdere observaties door onze onderzoeksgroep, waarbij er meer T cel apoptose werd aangetroffen in het bloed van HCV geïnfecteerde patiënten in vergelijking met gezonde controles, veronderstelden we dat de mate perifere T cel apoptose gerelateerd zou kunnen zijn aan de mate van leverziekte. Om deze hypothese te testen hebben we een grote studie opgezet, waarbij in het bloed van patiënten met verschillende leverziekten onder andere de mate van T cel apoptose werd bestudeerd. Deze studie, waarvoor meer dan honderd mensen werden

geïncludeerd, heette de ACATLIFE-studie. In **hoofdstuk 2** van dit proefschrift zijn de resultaten met betrekking tot T cel apoptose samengevat. De studie bevestigt de eerdere bevinding dat extrinsiek geactiveerde T cel apoptose verhoogd is in het bloed van HCV geïnfecteerde patiënten in vergelijking met gezonde controles, maar dit was onafhankelijk van de mate van fibrose.

Ook de T cellen van patiënten met behandelde HIV monoinfectie lieten verhoogde mate van apoptose zien. Bij verdere analyse van de data leek het erop dat de soort behandeling tegen HIV van invloed was op de mate van T cel apoptose in het bloed. Dit was bovendien redelijk goed te verklaren met bestaande wetenschappelijke literatuur. Om dat beter te onderzoeken hebben we een nieuwe studie opgezet, de zogenaamde ARTA-studie, die weergegeven is in **hoofdstuk 3** van dit proefschrift. Daarin onderzochten we of in het bloed van HIV-patiënten de mate van apoptose van T cellen inderdaad gerelateerd was aan de soort behandeling (protease remmer (PI) versus non-nucleotide reverse transcriptase remmer (NNRTI)). Uit de studie bleek echter dat dit niet het geval was. Vanwege de strikte inclusiecriteria en de nauwkeurige analyse, onder andere door uitsluitend gebruik te maken van vers bloed, hebben we kunnen vaststellen dat er in het verse bloed geen verhoogde apoptose is bij patiënten die met NNRTI worden behandeld, maar dat er mogelijk wel een verhoogde apoptose-gevoeligheid is, gezien de T cellen van NNRTI-behandelde patiënten significant minder Bcl-2 bevatten, een eiwit met anti-apoptotische eigenschappen.

Immuunactivatie

In **hoofdstuk 4** is T cel activatie onder de loep genomen. Niet-specifieke activatie van immuuncellen is een fenomeen dat waarschijnlijk een rol speelt in de veroudering van het immuunsysteem. We ontdekten dat HCV-geïnfecteerde patiënten relatief minder naïeve CD8 T cellen in het bloed hebben. Daarnaast observeerden we dat patiënten met HIV-HCV coinfectie significant hogere T cel activatie kennen dan patiënten met HCV-monoinfectie. Ook de T cel immuun-uitputting (immune exhaustion), wat betekent dat immuuncellen minder functioneel zijn, liet eenzelfde patroon zien, al was dit niet statistisch significant. Bijzonder interessant is onze bevinding dat de mate van T cel activatie en uitputting gecorreleerd was aan het aantal virusdeeltjes in het bloed, wat suggereert dat T cel activatie en uitputting direct viraal geïnduceerd wordt.

In **hoofdstuk 6** beschrijven we bovendien dat ook de CD4/CD8 T cell ratio van HIV-HCV coinfectie patiënten was lager dan die van HCV-monoinfectie patiënten, wat ook gerelateerd is aan immuunactivatie.

Niet-invasieve metingen van leverfibrose

Gedurende vele jaren was de microscopische beoordeling van leverweefsel de enige methode om de mate van leverfibrose vast te stellen. Hiertoe wordt door middel van een bioptie een stukje weefsel uit de lever worden genomen, wat een invasieve ingreep is en risico's met zich meebrengt. De afgelopen jaren zijn verschillende nieuwe methodes

ontwikkeld, zoals transiënte elastografie (Fibroscan). Transiënte elastografie is echter niet in iedere kliniek beschikbaar, dus zou een simpele bloedtest handiger zijn.

Zoals beschreven in **hoofdstuk 2 en 4** zijn de mate van T cel apoptose of T cel activatie en uitputting in het perifere bloed niet gerelateerd aan leverfibrose. In **hoofdstuk 6** demonstreren we echter dat de CD4/CD8 T cel ratio wél een geschikte kandidaat is voor beoordeling van leverfibrose in patiënten met HCV monoinfectie. Eenzelfde patroon zagen we in patiënten met primair biliaire cirrhose (PBC), zij het niet statischs significant, zoals weergegeven in het hoofdstuk **discussion**. Bij HIV-HCV coinfectie bleken alle patiënten een lage CD4/CD8 T cel ratio te hebben, wat mogelijk te maken heeft met verhoogde immuunactivatie in deze patiëntengroep. De CD4/CD8 ratio is derhalve geschikt om de mate van leverfibrose vast te stellen bij patienten met HCVmonoinfectie, maar niet in het geval van HIV-HCV coinfectie.

Daarnaast beschreven we in **hoofdstuk 7** de impact van transiënte elastografie op klinische beslissingen bij HBV en HCV infectie. Uit deze studie is bleek dat de uitkomst van transiënte elastografie veelal beslissend is voor bijvoorbeeld het starten van antivirale behandeling of het screenen voor hepatocellulair carcinoom bij patiënten die ernstige fibrose of cirrhose blijken te hebben.

Aangeboren versus aangepaste immuniteit

Lange tijd werd aangenomen dat aangeboren (niet-specifieke) en aangepaste (specifieke) immuunreacties min of meer onafhankelijk van elkaar opereerden. De afgelopen jaren is er in de wetenschap echter steeds meer aandacht voor de manier waarop aangeboren en aangepaste immuunreacties elkaar beïnvloeden. Tekenend hiervoor is dat o.a. Ralph Steinman in 2011 (vlak na zijn overlijden) de Nobelprijs won voor zijn onderzoek naar dendritische cellen, die gelden als een belangrijke brug tussen het aangeboren en het aangepaste immuunsysteem. T cellen zijn belangrijke spelers van het aangepaste immuunsysteem, terwijl NK cellen tot het aangeboren immuunsysteem worden gerekend. Recent is duidelijk geworden dat NK cellen ook een belangrijke rol spelen in de afweer tegen HCV infectie, zowel door regulatie van T cel reacties alsook in de progressie van leverfibrose.

In **hoofdstuk 5** van dit proefschrift hebben we eigenschappen van twee subsets van natural killer (NK) cellen onderzocht bij patiënten met HIV en HCV infectie: de $CD56^{bright}$ en $CD56^{dim}$ NK cellen. Van deze twee subsets wordt de $CD56^{dim}$ subset als de subset beschouwd die andere cellen kan doden, terwijl de $CD56^{bright}$ subset, die in lagere percentages in het bloed voorkomt maar juist meer in de weefsels, vooral een regulatoire rol wordt toegedicht, vanwege de productie van cytokinen. Wij ontdekten echter dat juist deze $CD56^{bright}$ NK cellen relatief meer FasL tot expressie brengen, een eiwit dat andere cellen kan aanzetten tot apoptose. Daarnaast was chronische infectie met HCV ook nog eens geassocieerd met hogere expressie van FasL maar ook van granzyme B en perforine, twee andere eiwitten die celdood kunnen induceren, onafhankelijk van coinfectie met HIV.

Belangrijkste conclusies uit dit proefschrift:

- Chronische infectie met HCV is geassocieerd met activatie van de extrinsieke apoptose-route, onafhankelijk van de mate van leverfibrose
- Behandeling met PI- of NNRTI-gebaseerde antiretrovirale therapie is niet van invloed op de mate van perifere T cel apoptose
- Bij HCV-infectie is coinfectie met HIV geassocieerd met verhoogde perifere T cel activatie
- $CD56^{bright}$ NK cellen brengen meer FasL tot expressie dan de $CD56^{dim}$ NK cellen, hetgeen in strijd is met de gangbare perceptie dat $CD56^{bright}$ NK cellen niet cytotoxisch zijn.
- CD4/CD8 T cel ratio in het perifere bloed is een kandidaat voor niet-invasieve beoordeling van leverfibrose in HCV monoinfectie.

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Curriculum vitae

De schrijver van dit proefschrift werd op 8 oktober 1981 in Nijmegen geboren. Na het behalen van zijn VWO-diploma in 2000 bij het Kandinsky College te Nijmegen en vervolgens een jaar te hebben gewerkt in Engeland en Ierland, begon hij halverwege 2001 met zijn studie Geneeskunde aan de Universiteit van Amsterdam. Zijn eerste wetenschappelijk onderzoek verrichtte hij in Nepal onder begeleiding van lepraspecialist Wim Brandsma en professor William Faber. Na het afronden van zijn geneeskundestudie in 2008 werkte hij als arts op de afdeling interne geneeskunde van het Medisch Spectrum Twente, waar hij zijn werk als arts zodanig met het hardlopen wist te combineren dat het hem een bronzen medaille opleverde bij de Nederlandse Kampioenschappen marathonlopen. Op zoek naar een promotieonderzoek in de infectieziekten kwam hij terecht in het interessante veld van hepatitis C en de anti-virale immunologie onder begeleiding van professor dr. Andy I.M. Hoepelman, professor dr. Peter D. Siersema, dr. Joop E. Arends en dr. Debbie van Baarle. Nadat het laboratoriumwerk erop zat is hij naar Finland verhuisd en op het moment werkt hij daar bij het universitair ziekenhuis van Turku (TYKS) als arts in opleiding tot internist.

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