The impact of negative regulation on T cell immunity during chronic hepatitis C virus infections

A study on immunity of liver and peripheral blood

Colofon

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The Impact of Negative Regulation on T cell Immunity During Chronic Hepatitis C Virus Infections

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De invloed van negatieve regulatie op de T cel immuniteit tijdens chronische hepatitis C virus infecties

Een studie naar immuniteit van lever en perifeer bloed

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Nullum magnum ingenium sine mixtura dementiae fuit.

Seneca

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Chapter 1

General introduction and outline of the thesis

Chronic hepatitis C virus infection – a global burden

The hepatitis C virus (HCV) is very successful in establishing persistent infections by evading the immune system and predominantly infecting hepatocytes. HCV was known as non-A non-B hepatitis since the 1970's and identified as a unique virus in 1989 [1]. HCV is a single-strand positive sense RNA virus belonging to the *Flaviviridae* family, has six major genotypes (1 to 6) and more than 100 subtypes have been identified. The single ~9600 nucleotide RNA molecule carries one open reading frame encoding for the structural proteins core, envelope 1 (E1), envelope 2 (E2), p7, and 6 non-structural proteins (NS) needed for replication (NS2, NS3, NS4a, NS4b, NS5a and NS5b). The current model of HCV infection suggests that after entering circulation, HCV is transported to the liver via lipoproteins where HCV binds to low density lipoprotein receptors, and possibly DC-sign and other receptors on hepatocytes, followed by viral entry in a clathrin-dependent endocytic process requiring interaction between the viral envelope with cell surface tetraspanin CD81, scavenger receptor type B class I, and the tight junction proteins claudin-1 and occludin (reviewed in [2]).

Controversy exists whether HCV directly impairs immune cell functions by infecting these cells. However, following infection, innate immunity and the HCV-specific immune response mediated via T cells are functionally impaired, and unable to eliminate the HCV in the majority of individuals [3-6]. Only a minority of infected patients are able to clear HCV spontaneously, and about 80% develop a chronic infection with viral replication primarily occurring in the liver [7].

It is estimated that globally 120 to 170 million patients are persistently infected with HCV. However, symptoms are relatively mild in the majority of these patients, and it may take decades before the serious consequences of chronic HCV infection become apparent. In the end however, these patients are at increased risk of developing cirrhosis, and subsequently liver decompensation, liver failure and hepatocellular carcinoma. In the United States, the long-term complications of chronic HCV infection are currently responsible for approximately one third of the 6000 annual liver transplantations, the only definitive therapy for end stage liver disease. However, the number of liver transplantations attributed to chronic HCV infection and HCV related death on the waiting list has stabilized since 2006 [8-10]. This is primarily the consequence of a stabilized incidence of new infections after a peak in the 1980's [11]. Decreased transmission of HCV infection may be largely explained by the implementation of standard blood testing from 1992 onwards, but also changed habits among drug users towards other ways than intravenous drug use as a consequence of oral substitution therapy [12]. Viral eradication by alpha interferon (IFN-α) based therapies and subsequent halting of disease progression may contribute to stabilization in the number of chronic HCV patients with end stage liver disease [13-15].

Nevertheless, global burden of HCV-related disease will rise dramatically for several reasons. First, in developed countries, prevalence of infection is already significant as a consequence of the high incidence of new infections before regular blood testing in 1992. Second, transmission is ongoing, in developing nations possibly at continuously high rate, and a vaccine is not available and also not soon expected to prevent this in the near future. Third, morbidity associated with chronic HCV infections generally takes decades to develop and many patients infected in the eighties will develop end-stage liver disease in the future [16]. Moreover, as life expectancy will slowly increase in developing countries, more

patients will suffer from the complications of chronic HCV infections as well. Fourth, even in the era of replication inhibitors, viral eradication is not expected in all patients. However, more important, most patients will not have access to the expensive and elaborative combination therapies. This is not only an issue in the developing world, but also in the US, the richest country on earth. Fifth, HCV co-infected with the human immunodeficiency virus (HIV) results in accelerated damage to the liver [17-19], which will especially have a significant impact on mortality in developing countries with high HIV/HCV coinfection rates. Taken together, the long-term consequences of chronic HCV infection will remain an enormous global health problem. I foresee that decision makers will fail to make this issue a priority, especially due to the slow disease progression in HCV-infected patients.

Alpha interferon, cornerstone of therapy for chronic HCV infections

Type I interferons (Type I IFNs), alpha interferon (IFN-α) and beta interferon (IFN-β), are proteins central to the natural immune response to HCV infections. Type I IFNs and the role of the immune system in HCV infections will be discussed later on in this introduction. Pharmaceutical pegylated IFN-α-2a or IFN-α-2b (pegIFN-α) in combination with weight based ribavirin (pegIFN-α/ribavirin) is currently the standard treatment for chronic HCV-infected patients. A sustained virological response (SVR), defined as an undetectable plasma HCV RNA 24 weeks after cessation of pegIFN-α/ribavirin therapy, is achieved in around 80% of patients infected with genotypes 2 or 3, however in only 40 to 50% of patients infected with genotype 1 [20-22]. Also in the next decade, the only way to limit disease progression caused by chronic HCV infection will be clearance induced by pegIFN-α/ribavirin in combination with new antiviral agents.

New antiviral agents targeting viral entry or the life cycle of HCV are under clinical evaluation and the NS3/4a protease inhibitors boceprevir® (Schering-Plough/Merck) and telaprevir® (Vertex/Tibotec/JanssenPharmaceutica) are currently available to the first genotype 1 patients in early access programs. In phase III registration trials, these new drugs combined with pegIFN-α/ribavirin showed enhanced SVR rates for IFN-α-based therapy naïve and experienced genotype 1 patients, at present the largest group of hard-to-treat patients. Resistant HCV variants are selected within weeks after start of treatment with NS3/4a protease inhibitors resulting in rebound HCV RNA replication. Monotherapy is therefore not effective [23]. However, resistant variants remain sensitive to IFN-α, and enhanced HCV RNA reductions were observed when a protease inhibitor was combined with pegIFN-α/ribavirin [24-26]. Rapid viral load reduction by the new antivirals may downregulate the highly active endogenous IFN system, which possibly contributes to a nonresponse to exogenous IFN-α [27]. Efficacy of these new combination therapies in clinical practice has to be evaluated, and especially decreased out-of-trial compliance as a consequence of accumulation of known side effects of pegIFN-α/ribavirin, and side effects of the new antivirals, may limit SVR rates [28].

At present, much is known on the predictors of pegIFN-α/ribavirin treatment failure. Importantly, induction of resistance to either IFN-α or ribavirin is not one of them. Several pre-treatment patient characteristics are associated with poor response to standard pegIFN-α/ribavirin therapy, of which upregulated interferon stimulated genes [27, 29], and recently the interleukin (IL)-28B TT-genotype [30] have attracted most attention from immunologists. These

findings suggest that an attenuated innate immune system may partially explain treatment failure. Other predictors of a nonresponse to therapy are liver cirrhosis and steatosis [21, 31-32], insulin resistance and higher weight [20-21, 33-34], coinfection with the human immunodeficiency virus (HIV) [35], but also Afro-American race [31, 36-37], age above 45 years [34] and possibly male gender [38]. In addition, genotype 1 [21] and high baseline HCV RNA load [20-21, 31] are viral characteristics negatively influencing SVR rates. Moreover, lack of a rapid virological response (RVR), defined as undetectable HCV RNA loads at week 4 of therapy, is regarded as the strongest on-treatment predictor for a nonresponse [21]. Finally, suboptimal adherence as a consequence of frequent and sometimes severe side effects may have resulted in lower response rates in every day practice than reported in the original registration studies [39].

IMPAIRED IMMUNITY TO HEPATITIS C VIRUS INFECTIONS

Liver immunology is pivotal to a better understanding of immunity to hepatitis C virus infections

The local immune response in the liver is important for the outcome of HCV infection and the persistence of the virus in the liver, since replication takes place in hepatocytes and T cells clearly must traffic to the liver to recognize and target infected hepatocytes. However, the dynamics of the immune response in the liver of chronic HCV patients is largely unknown. Studies in humans using intrahepatic cells are difficult to perform since liver material for research purposes can only be obtained from biopsies that are collected from patients for diagnostic purposes.

The liver is the first organ that receives blood from the gut via the portal vein. This blood is loaded with pathogen associated molecular patterns (PAMPs) from gut flora and food that are presented to the innate immune system of the liver. This is often suggested to be the reason for the immunotolerant character of the liver under non-inflammatory conditions. Moreover, the liver allows sheer unlimited interactions between antigen presenting cells and T cells, as not only liver DC, but also hepatocytes, liver sinusoidal endothelial cells (LSEC), hepatic stellate cells (HSC) and liver macrophages (Kupffer cells) are able to present antigens [40-44]. However, antigen presentation by LSEC, HSC and hepatocytes may induce T cell tolerance [45-46]. The latter may be as a consequence of incomplete costimulation in the liver and subsequently weak effector functions [47].

Lymphocytes make up approximately 10% of the total cells in a healthy liver. Almost two-third of these is T cells, around one-third is natural killer cells (NK cells) and B cells make up a smaller fraction (5-6%). The T cell compartment is composed of conventional $CD8^+$ (15-30%) and $CD4^+$ T cells (5-15%), $CD4^-CD8^-$ T cells (1-5%), NKT cells (20%), and γδ T cells (5-18%) [48]. The intrahepatic $CD8^+$ and $CD4^+$ T cells primarily reside around the portal tract, are generally activated and differentiated, and display an effector phenotype [40]. During chronic HCV infections, HCV-specific $CD4^+$ and $CD8^+$ T cells are abundantly present in the liver of chronic HCV patients. Some studies propose that baseline intrahepatic $CD8^+$ T cell responses in chronic HCV patients are important for successful response to IFN-α-based therapy [49-51], which is in line with reports on peripheral blood T cells [52-53]. However, this has

not been shown by others [54-58], and it remains unclear how intrahepatic immunity is affected by IFN-α-based therapy and how this contributes to treatment outcome.

Strong effector functions of intrahepatic immune cells resulting in protective cellular immunity to HCV infection has however not yet been demonstrated, either during spontaneous, or therapy-induced resolution. Intrahepatic T cell responses may by be inhibited by several mechanisms, including inhibition mediated by regulatory T cells (Treg), transforming growth factor-beta (TGF-β) and interleukin-10 (IL-10), which will be discussed later on in this introduction.

SUBOPTIMAL INNATE IMMUNITY TO HEPATITIS C VIRUS INFECTIONS

Type I interferon responses are attenuated by the hepatitis C virus

The immune response is pivotal to the control over viral infections, including HCV infection. Pathogen recognition receptors continuously sense the environment and recognize viral products, resulting in rapid induction of Type I IFNs, INF-α and IFN-β. Type I IFNs have direct antiviral effects on infected cells by inducing of the expression of multiple IFN-stimulated genes (ISGs) [59]. A number of these genes, including protein kinase R (PKR), Mx proteins, ISG-15 and 56, IRF-7, RNAseL/2,5-OAS and RNA helicases, have been well-characterised, and via different mechanisms all have potent antiviral activity [60]. In addition, Type I IFNs are immunomodulatory, and are thought to prime multiple immune cells, to efficiently respond to the attack of the host by pathogens.

The combined effect of Type I IFNs to activate direct antiviral mechanisms and to prime the adaptive immune system, makes it a very powerful strategy to efficiently eradicate viral infection. Most likely, one of the most important effects of IFN-α is on natural killer (NK) cells [61]. IFN-α augments the cytotoxicity of NK cells, possibly by enhancing the expression of perforin. In addition, IFN-α induces NK cell proliferation and enhances the production of NK cell-derived cytokines, such as IFN-γ [62-63]. Furthermore, by enhancing the expression of MHC class I, and MHC class II molecules, IFN-α is an important interplay between the innate and adaptive immune system. Enhanced expression of MHC molecules leads to potent activation of $CD8^+$ and $CD4^+$ T cells [64-65]. Especially for $CD8^+$ T cells it has been demonstrated that IFN-α induces clonal expansion and survival, and plays an important role in the preservation of memory $CD8^+$ T cell responses [66-67]. For $CD4^+$ T cell responses, one of the most studied effects of IFN-α is an enhancement of the development of IFN-γ producing type 1 helper T cells (Th1 cells), important antiviral effector cells, which is mediated by augmented expression of the IL-12 receptor β2 chain making $CD4^+$ T cell more responsive to IL-12 [68]. Moreover, direct suppression of IL-4 and IL-13 gene expression has also been demonstrated, which also further promotes Th1 cell development [69].

Indications of the role of Type I IFNs during viral hepatitis infection are mostly based on studies in chimpanzees, since this is the only animal that can be infected with hepatitis B virus (HBV) and HCV. For HCV infections, the first response is thought to be IFN-β production by infected hepatocytes. The pattern recognition receptor retinoic acid–inducible

gene I (RIG-I) recognizes the polyuridine motif of the HCV 3' UTR in the cytoplasm [70], and possibly, toll-like receptor-3 (TLR3) recognizes HCV double strand RNA present in endosomes. IFN-β induces a multitude of ISGs, including 2,5-OAS, ISG-56, IRF-7, and STAT1, which has direct antiviral effects and amplifies the Type I IFNs response, including IFN-α production [60]. At present, it is unclear what cells are the major sources of Type I IFNs that control HCV replication. Hepatocytes are potential candidates, although a recent report suggests that Type I IFNs production in the liver is primarily by plasmacytoid dendritic cells (pDC) triggered by infected hepatocytes without these pDC being infected themselves [71]. The increase in Type I IFNs related genes coincides with an increase in HCV RNA levels, suggesting that the increase in viral load is the trigger for the induction of antiviral response genes. This IFN response occurs in all patients infected with HCV irrespective of whether the final outcome is a self-limiting resolving infection, or whether a chronic HCV infection develops [72-75]. Although HCV is sensitive to Type I IFNs as shown by numerous *in vitro* studies [76], HCV replication is not controlled during the early stages after infection, and in fact the serum HCV RNA levels increase exponentially in the first weeks after infection. This suggests that HCV has developed evasion mechanisms to withstand the potent effect of Type I IFNs. Indeed, in recent years a number of mechanisms have been identified by which HCV proteins attenuate the induction or the activity of the antiviral IFN response, by degradation or inhibition of crucial molecules such as Cardif, and TRIF [77-80]. It is unlikely that HCV is able to completely block this response, since the products of various IFN-induced genes are detected. However, reduced efficiency of the IFN response due to the evolution of evasion mechanisms by HCV may allow HCV to withstand complete eradication during the initial stages of the infection. Four to six weeks after infection, the HCV RNA levels remain relatively stable, suggesting that a balance has been achieved between viral replication and control of the replication by immune pressure. This control is believed to be primarily exerted by the Type I IFNs mediated response. It is thought that this plateau is reached before the adaptive immune system, mediated by HCV-specific T and B cells, becomes activated.

Link innate and adaptive immunity – natural killer & dendritic cells

NK cells may contribute to the immunity against HVC infection. They are known for their aspecific cytotoxicity to cells not recognized as self and are recruited to the liver by Type I IFNs secreted shortly after infection [81]. HCV-infected hepatocytes are therefore potential targets for NK cells. NK cells are normally inactive while dominated by inhibitory signals via self-MHC class I ligands binding to inhibitory receptors and are activated only when signalling via activating receptors overcome the inhibitory signals. It has been reported recently that NK cells show increased expression of the activating receptor NKG2D, and enhanced IFN-γ production, degranulation and cytotoxicity during acute HCV infection [82-83]. In addition, it has been suggested that a genotype encoding for diminished inhibitory signals via NK cell receptor KIR2DL3 and its ligand HLA-C1 confer protection against HCV, especially when patients are infected with low doses of HCV. Possibly, NK cells are only able to deal with HCV effectively if antigen exposure is not to high [84]. On the other hand, the function of NK cells in HCV infection may be directly impaired by the binding of the HCV E2 protein to NK cell surface CD81, although exposure of healthy donor NK cells to *in vitro*

produced infectious hepatitis C virions did not inhibit NK cell activation and interferon gamma (IFN-gamma) production [85-87].

Under normal conditions, NK cells interact with dendritic cells (DC) and this results in the regulation of both innate and adaptive immune responses. Moreover, DC can activate NK cells by binding to the NK cell activating receptor NKp30 on the surface of NK cells and by secreting numerous cytokines such as IL-12. NK cells, in turn, secrete IFN-γ and TNF resulting in DC activation and subsequent triggering of adaptive immune responses. In addition, NK cells can also kill immature DC and inhibit their capacity to prime or tolerize adaptive T cell responses [6].

Dysfunction of DC is suggested to contribute to the insufficient response to HCV infection. Under normal conditions, DC are central to the initiation of adaptive immune responses. After pathogen encounter, DC are activated, which is characterized by the expression of MHC, costimulatory and adhesion molecules, as well as by the production of cytokines and chemokines. Next, DC migrate to lymphoid organs where they present captured antigens to $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells, thus linking innate and adaptive immunity. During HCV infection, DC are recruited to the infected areas of the liver mirrored by reduced numbers in blood (reviewed in [88]). It has been suggested that DC function in HCV infection is hampered as a consequence of decreased antigen presentation to $CD4^+$ T cells directly mediated through direct interference by HCV proteins [89]. Moreover, reports suggest that IL-12, an important cytokine directing T cells towards a Th1 cell IFN-γ producing profile [90], is selectively inhibited by HCV core proteins [91]. As a consequence, priming of HCV-specific $CD4^+$ T cells may be suboptimal, allowing HCV to persist.

A limited role for humoral immunity in hepatitis C virus infections

Neutralizing antibodies are produced during acute HCV infection. However, these antibodies appear relatively late, titers are relatively low, and are not sterilizing, since 80% of patients evolve into chronic infection. However, neutralizing antibodies may aid control over HCV infection by fixing complement, opsonizing particles for phagocytosis, and enhancing antigen presentation to T cells (reviewed in [3]).

Recently, it has been shown that as a consequence of chronic viral infection in mice, T cells differentiate towards T follicular helper (Tfh) cells that promote B cell immunity [92]. Interestingly, intraportal lymphoid follicles containing T and B cells have been observed in HCV-infected livers already in the 1990's [93]. However, it has to be investigated whether real Tfh cells are induced in chronic HCV as well and if they promote protective immunity by the generation of neutralizing antibodies to HCV.

Dysfunctional T cell immunity hampers hepatitis C virus clearance

As a consequence of the insufficient innate immunity, HCV loads increase exponentially over the first few weeks of infection. As a result, HCV is able to infect as much as 10% of all hepatocytes [73] and does not give rise to any liver damage in the vast majority of acutely infected patients. The lack of symptoms in these patients – reflected by an absence of elevated alanine transaminase (ALT) levels or jaundice – highlights the non-cytopathic nature of HCV. Four to eight weeks after infection, HCV-specific T cell responses in blood

can often be detected for the first time, sometimes accompanied with a transient mild rise in ALT, however rarely with jaundice [94-96] (Figure 1). A characteristic feature of patients who resolve acute HCV infections is the presence of HCV-specific $CD4^+$ and $CD8^+$ T cell responses directed against multiple HCV epitopes in blood [94-95, 97-107]. Further, protective T cell memory may result in spontaneous HCV clearance after reinfection [97, 108]. We assume that HCV-specific $CD4^+$ and $CD8^+$ T cell responses are also important for protective immunity in the liver as HCV predominantly infects hepatocytes, and chimpanzee studies are in support of this assumption [95, 98, 101, 105]. However, limited data is available to substantiate this assumption for humans as there are several limitations in obtaining sufficient liver material from chronic HCV patients [109].

In contrast, in patients who are unable to clear the virus and become persistently infected, initially HCV-specific T cell responses are detected, but these responses are often weak, narrowly focused and not sustained [110-113]. This inability to mount strong and lasting T cell responses against HCV is considered crucial for the development and maintenance of the persistent infection. Dysfunctional T cell responses enable prolonged coexistence of HCV with – and possibly prevent accelerated damage to – the host. This delicate balance of silenced protective immunity along mild immunopathology has been described for several other chronic infections before (reviewed by [114-115]). The T cell dysfunction in chronic HCV patients seems limited to HCV-specific responses only, since immunity to other infections is generally normal until end-stage liver disease occurs.

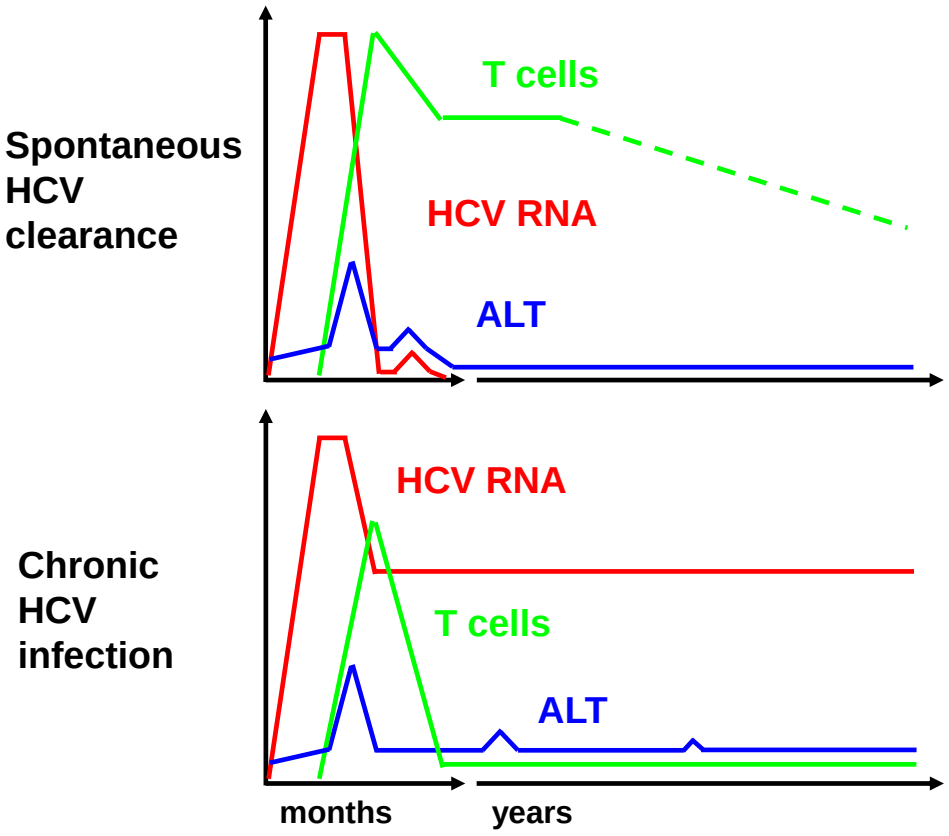

Figure 1. Kinetics of viral replication (HCV RNA), liver damage (ALT) and HCV-specific $CD4^+$ and $CD8^+$ T cell responses (T cells).

Several host and viral mechanisms have been proposed to explain the weak T cell responses to HCV (Figure 2), including the occurrence of HCV immune escape mutations, impaired NK cell function, suboptimal antigen presentation by DC resulting in incomplete differentiation and activation of effector and memory T cell populations, exhaustion of the T cells resulting from persistent high viral loads, anergy, and suppression by negative regulators of HCV-specific immunity (reviewed in [2-3, 116]). Regulation of the weak T cell response to HCV by Treg, IL-10 and TGF-β is the subject of my research and will be discussed further down in this introduction.

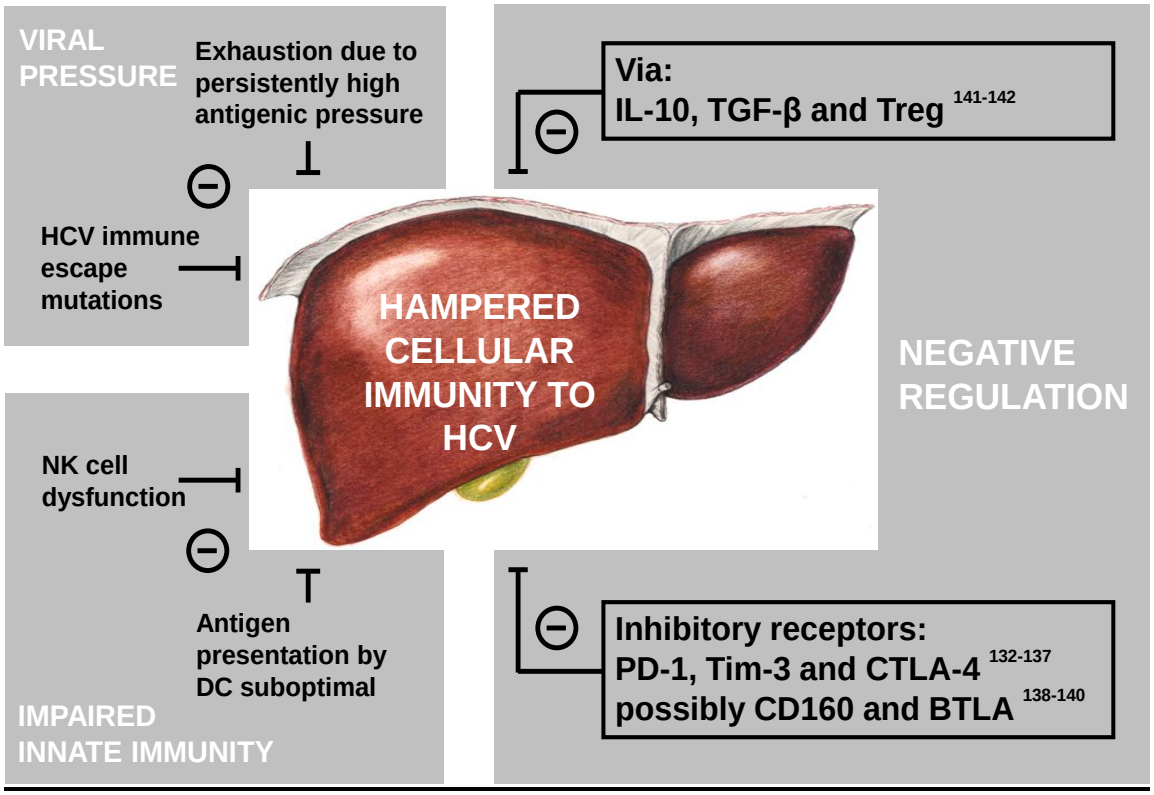

Figure 2. host and viral mechanisms that have been proposed to explain the weak T cell responses to HCV

HCV-specific T cell responses – important for therapy outcome?

The goal of treatment is an SVR, meaning that 6 months after cessation of therapy no HCV RNA is detected in serum. It has been shown that the decline of serum HCV RNA levels during treatment occurs in 2 phases [117-118]. During the first phase, a rapid decline of serum HCV RNA levels may be observed in treatment responders due to the direct inhibition of viral replication in infected cells. The second phase occurs over a period of weeks or months. Although not completely understood, it has been suggested that the slow decline of serum HCV RNA levels during this phase may depend on the activity of the immune system, including the HCV-specific T cell response.

Apart from the direct antiviral effects of therapy (reviewed in [119]), the impact of IFN-α-based therapy on the immune cells may be important in determining the treatment response. It has been suggested that cellular immune responses and modulation of these responses by pegIFN-α and ribavirin, play a role in forced viral eradication. This is based on the

immunological properties attributed to these anti-viral compounds [120-123]. However, the role of HCV-specific T cells before and during pegIFN-α/ribavirin therapy is still controversial.

The limited HCV reactivity of T cells and the fact that blood leukocyte numbers decline dramatically shortly after start of IFN-α-based treatment makes studies focused on changes in HCV-specific T cell responses during treatment extremely difficult to perform. Some studies have shown that achievement of an SVR is associated with high baseline $CD4^+$ and/or $CD8^+$ specific T cell responses [52-53, 124-125], while others have seen no such relationship [54-58, 121, 126-127].

Similar controversy exists on the role of HCV-specific T cells during pegIFN-α/ribavirin combination therapy. Some groups have reported enhanced responses in patients achieving an SVR [54-56, 121, 126, 128-129], whereas in nonresponders to therapy no such enhancement was observed. In contrast, others observed a decline of HCV-specific T cell responses during combination therapy in SVR patients [52, 58, 125, 127].

In summary, at present it is not well understood to what degree and by what mechanisms the HCV-specific immune system contributes to the effectiveness of treatment with pegIFN-α/ribavirin. Several methodological problems limit the progression made in this field of research. Importantly, good small animal models enabling *in vivo* immunological research during acute and chronic infection with HCV are currently unavailable [130]. However, human research on immunity to HCV can be improved as well. At present, a consensus on the optimal ex vivo experimental cell culture protocols is lacking [131]. This may be the reason for conflicting data on the importance of HCV-specific immunity for the efficacy of combination therapy. Moreover, frequencies of circulating HCV-specific T cells are very low [102] and therefore hard to detect, and a robust and sensitive assay able to detect low frequencies of HCV-specific T cell responses is needed to resolve the above mentioned controversies. Finally, only few have investigated HCV-specific responses in the liver, as they are difficult to perform. However, more research on intrahepatic HCV-specific responses rather than responses in peripheral blood need to be conducted, since this better reflects the local immune response during HCV infections.

Negative regulators of HCV-specific immunity

Negative regulation of HCV-specific immunity has been introduced above as one of the mechanisms responsible for the deficient HCV-specific T cell response during chronic infection and the relatively slow progression of liver fibrosis (Figure 2). Suppression of the HCV-specific T cell response is mediated via inhibitory receptors such as PD-1, Tim-3 and CTLA-4 [132-137]. Additional receptors with similar inhibitory functions in other diseases, including CD160 and BTLA [138-140], may turn out to be involved in the attenuation of HCV-specific T cell reactivity as well. In addition, active suppression of virus-specific T cell responses by Treg or by the immunosuppressive cytokines IL-10 or TGF-β [141-142] has been shown to regulate HCV-specific immunity. Although it is generally accepted that regulation via IL-10, TGF-β and Treg is involved in controlling HCV-specific immunity, the relative importance of these regulatory pathways and whether they control different effector activities is unknown. The immunoregulatory properties of IL-10, TGF-β and Treg, and their importance for the immunity to HCV infection, will now be discussed separately.

Immunoregulatory properties of regulatory T cells

The suppressive effect of Treg is suggested to be of importance in controlling HCV-specific immunity, by simultaneously antagonizing protective immunity and excessive immunopathology to the liver [143-147]. Similar findings have been reported for parasitic, bacterial, fungal and chronic virus infections including HBV (reviewed in [114]).

Treg were first identified as suppressors of autoimmune disease in mice [148-149]. Nowadays, Treg are also known to be essential for control over chronic inflammatory diseases and maintenance of peripheral tolerance in men and mice through suppression of a variety of immune cells including $CD4^+$ [150] and $CD8^+$ T cells [151], NKT cells [152], DC [153], monocytes [154], B cells [155] and NK cells [156] via multiple modes of action (reviewed in [157]). Treg represent a stable population of human peripheral blood $CD4^+$ T cells with a frequency between 3-10% of the total $CD4^+$ population [158]. Thymus derived Treg are currently characterized on the basis of the expression of CD25 and the transcription factor Forkhead box P3 (FoxP3) [159-160]. However, Treg can also be induced from $CD4^+$ or $CD8^+$ effector T cells during inflammatory processes in the periphery. Retinoic acid [161-162], TGF-β [163-164] and $CD103^+$DC [165] have been shown to be involved in the conversion of effector T cells to Treg showing different levels of FoxP3 expression. TGF-β alone suffices to induce $FoxP3^+$ Treg [163-164]. Other examples of peripherally generated Treg include $CD4^+$ and $CD8^+$ T cells that mediate suppression through IL-10 and/or TGF-β [166-167]. Thymus derived $FoxP3^+$Treg may also use TGF-β or IL-10 as a mechanism to suppress antigen-specific T cells under certain circumstances [157, 168]. However, this has not been shown for suppression of HCV-specific T cell responses [143-145, 147].

In blood, $CD4^+CD25^+$Treg were able to suppress both HCV-specific proliferation and IFN-gamma production by $CD4^+$ and $CD8^+$ T cells [143-147]. Also, the percentage of circulating $CD4^+CD25^+$Treg may be increased in chronic HCV patients as compared to healthy control subjects, or individuals who resolved the infection [143, 145-146]. It is suggested that $CD4^+CD25^+$Treg, at least partly, control chronic liver inflammation, with a higher suppressive capacity of blood Treg in patients with lower ALT levels [144].

The findings on Treg in blood do not necessarily reflect intrahepatic immunity. More information on liver infiltrating Treg is needed to appreciate whether they are indeed important regulators of immunity to HCV infections. Immunohistochemical studies have demonstrated significant Treg populations in the livers of chronic HCV patients [145, 147, 169-172]. These Treg predominantly resided in the portal tracts, in close proximity to other liver infiltrating effector T cells and correlated with lower liver inflammation. However, control over the outcome of immunopathology, i.e. liver fibrosis, has not yet been shown [171-172].

The *ex vivo* phenotype of intrahepatic Treg in the setting of chronic HCV infections has not been studied. This phenotype is however very instructive, since it can shed light on the functional properties of Treg in the infected liver, while *in vitro* functional experiments are difficult to perform due to the limited number of cells available. Based on several surface markers, antigen experienced T cells can be further divided into functionally distinct subsets at different stages of differentiation. These markers include CD45RO, CD62L and CCR7 ([173] and reviewed in [174-175]). On the basis of the differentiation models proposed, the expression of CD45RO, CD62L and CCR7 on antigen-specific T cells defines cell populations at an early stage of differentiation, that home to and proliferate within secondary

lymphoid organs, and differentiate into CCR7-negative effector cells upon secondary stimulation [173-174, 176]. In contrast, cells lacking the expression of both CD62L and CCR7 define cell populations at advanced stages of differentiation and display immediate effector function [173-175]. Circulating Treg are in majority antigen experienced, which is indicated by the expression of CD45RO. Treg in the peripheral blood of both healthy controls and chronic HCV patients are of a predominantly $CD45RO^{+}CTLA\text{-}4^{+}CCR7^{+}CD62L^{+}$ phenotype [145, 177]. Importantly, a number of studies have indicated that the expression of CCR7 and CD62L on Treg may have functional consequences with respect to their potency to suppress immune responses [178-181].

Immunoregulatory properties of IL-10

Initially presented as a suppressor of the differentiation and function of Th1 cells [182], monocytes and macrophages, IL-10 is now known as a cytokine with broad immunoregulatory effects. IL-10 can be produced by many different immune cells, including monocytes, myeloid DC, macrophages, B cells, Mast cells and eosinophils. The principal function of IL-10 appears to be to limit and ultimately terminate inflammatory responses through the inhibition of pro-inflammatory Th1 cytokine production (for example IFN-γ, TNF and IL-2), suppression of antigen presentation (for example via MHC class II on DC) and costimulatory molecules. Suppression of DC by IL-10 in turn induces innate and adaptive immunity to control effector responses, partially again via IL-10. In addition to these activities, IL-10 enhances B cell survival, proliferation, and antibody production, and regulates growth and/or differentiation NK cells, cytotoxic and helper T cells, mast cells, granulocytes, dendritic cells, keratinocytes, and endothelial cells. It has become clear that almost every T cell is able to produce IL-10, including Th1 cells, first known targets of IL-10. As the circle comes round again, it seems that IL-10 acts as an autocrine or paracrine self-regulator of excessive adaptive immunity [142, 183].

IL-10 is also implicated in the regulation of adaptive immunity to HCV, as several studies showed that neutralization of IL-10 enhances HCV-specific T cell proliferation and IFN-γ production in a subset of patients [184-189]. In addition,studies have found higher IL-10 production in nonresponders to IFN-α-based therapy, as opposed to SVR patients [190-191]. However, this may also be due to long-term effects of IFN-α therapy itself.

Elevated IL-10 concentrations have been detected in serum of chronic HCV patients compared to healthy control subjects. However, enormous differences in IL-10 concentrations have been measured in these studies ranging from a mean of 3 to 3000 pg/mL [191-196]. Moreover, others did not find a difference between chronic HCV patients and healthy controls [197], or detected elevated concentrations only in cirrhotic chronic HCV patients [198]. Serum IL-10 levels have not shown to be directly correlated to the magnitude of IL-10 mediated suppression of HCV-specific responses. Possibly, IL-10 levels are primarily elevated at the site of regulation – in the lymph node where APC and naive T cells may be regulated, or in the liver where further antigen presentation and T cell effector functions may be suppressed.

The initial studies that demonstrated HCV-induced IL-10 production in chronic HCV patients have been performed with whole peripheral blood mononuclear cells [199-200]. Today, more detailed studies have suggested that monocytes and T cells are main sources of IL-10

in chronic HCV patients, and augmented production of IL-10 by these cells has been shown in comparison with healthy control individuals [184-185, 187-189, 201-205]. Moreover, HCV-specific IL-10 production by T cells has been detected in acute infection from the onset of adaptive responses onwards [188]. Importantly, it seems that the intrahepatic counterparts of circulating monocytes, liver macrophages or Kupffer cells, can be a source of IL-10 in chronic HCV infections [206]. In addition, NK cells [207-208] and DC have been identified as sources of elevated IL-10 levels in chronic HCV patients. However, data on DC are conflicting as one report has suggested dendritic cells as a source of IL-10 [209], while another has claimed that there is no difference in IL-10 production by dendritic cells between patients and controls [210].

IL-10 production in HCV patients has been suggested to be dependent on stimulation by HCV core. Indeed, core antigens induced IL-10 production by either full PBMC [199], monocytes/Kupffer cells [190, 206] or T cells [201, 211]. However, non-structural genes have shown to elicit IL-10 production by PBMC [200], monocytes/Kupffer cells [184, 202, 204, 212] and HCV-specific T cells [185] as well. In addition, two studies show similar [185, 202] or higher [212] induction of IL-10 production after stimulation with non-structural antigens as compared to core antigen.

The question arises whether IL-10 has clinical consequences for HCV-infected patients. Does it simultaneously hamper protective immunity and protect against excessive immunopathology? Kaplan and colleagues were able to prospectively study a small group of 8 acutely HCV-infected patients and detected HCV-specific IL-10 production in these patients from the onset of adaptive responses onwards. IL-10 production was not only related to a lower chance of spontaneous clearance of the virus, but also seemed to protect against severe fibrosis [188].

Other reports on the suppression of protective immunity against HCV and subsequent spontaneous clearance are conflicting. However, these studies were limited to a cross-sectional approach and compared chronic HCV patients with seropositive HCV RNA negative subjects. Two studies showed lower HCV-induced IL-10 production by monocytes in patients with a previous spontaneous clearance [190, 202], whereas another study reported the opposite for PBMC [213]. However, the last study only measured IL-10 messenger RNA levels and it cannot be ruled out that this accounts for the differences found. Polymorphisms in the IL-10 promotor have also been associated with outcome of acute infection, albeit that data are confusing and often based on to small sample sizes for a proper genetic analysis. One study found an association between promoter polymorphism -592AA genotype and spontaneous clearance [214] and another study a relation between -592AA genotype and lower IL-10 production in PBMC after stimulation with core [215]. However, another study did not find the same association [216].

In addition to the study by Kaplan and colleagues, others have also presented data supporting the hypothesis that IL-10 limits immunopathology due to chronic HCV infection. Two independent groups presented data that HCV-specific $CD8^+$ IL-10 producing cells may reduce liver inflammation, as they were more abundant in healthier parts of the liver than inflamed areas [203], and were inversely correlated with the hepatic inflammation index [185]. The results of experimental IL-10 therapy provide further support for the positive effect of IL-10 on immunopathology. As a consequence of IL-10 therapy, liver inflammation and fibrosis was reduced in chronic HCV patients with a previous nonresponse to standard IFN-α-based therapy [217-218]. However, after prolonged IL-10 therapy, HCV RNA levels increased with a

mean 0.5 log, resulting in an acute flare in serum ALT in 2 patients out of 30 included and therapy was stopped after 12 months. This was likely a consequence of hampered protective immunity, as the investigators observed a drop in HCV-specific IFN-γ production by $CD4^+$ and $CD8^+$ T cells [219].

The question arises whether regulation by IL-10 affects response to IFN-α-based therapies, as a single dose of IFN-α has shown to increase HCV-specific IL-10 production accompanied by reduced effector T cell functions [220]. Several studies have attempted to link polymorphisms in the IL-10 promotor to treatment outcome, especially at the -1082, -819 and -592 positions. Although two independent studies found an association between response to therapy and a homo- or heterozygosity for A at site -592 of the IL-10 promotor [221-222], others did not find such a relation or correlated SVR or nonresponse to other polymorphisms in the IL-10 promotor [223-225]. In combination therapy, the suggested induction of IL-10 may be countered by the addition of ribavirin [121, 211].

Immunoregulatory properties of TGF-β

TGF-β can be produced by almost every cell and has various biological activities. Initially identified as a growth factor for fibroblasts [226], it is now known to be involved in general cell function, fibrogenesis and wound repair and a multitude of immunological processes as well. TGF-β has three known isoforms (TGF-β1, 2 and 3), of which TGF-β1 is most important to immunity and is relevant to our research [141]. TGF-β has inactive precursors (latent TGF-β binding protein; LTBP, and latency associated peptide; LAP) that are present throughout the human body in enormous concentrations. Only a very small fraction of these precursors is activated into TGF-β *in vivo*, which possibly occurs on the surface of effector cells, and often in small concentrations at short distance from target cells [227]. TGF-β1, from now on TGF-β, has potent suppressive effects on antigen-specific T cells and virtually all other immune cells [141]. However, many scientists ignore that TGF-β regulates T cells in an autocrine or paracrine fashion [228] and draw conclusions based on amounts of LAP detected on effector cells, or concentrations of *in vitro* activated TGF-β, thereby including the likely irrelevant inactive precursors.

TGF-β is involved in controlling T cell immunity to HCV as blocking TGF-β has been shown to enhance HCV-specific T cell proliferation, IFN-γ production and cytotoxicity by T cells in a subset of patients [145, 187, 189, 207, 229]. Moreover, high serum TGF-β levels were associated with a nonresponse to IFN-α/ribavirin therapy, and serum TGF-β decreased in responders, but not nonresponders [195, 230-235]. The TGF-β in these studies originated from PBMC, monocytes [189], NK cells [207], $CD8^+$ T cells [187, 229] or $CD4^+CD25^{hi}$ T cells [145] and may have resulted from a direct targeting of the TGF-β promoter by HCV core proteins or the induction of reactive oxygen species [236-238]. TGF-β alone suffices to induce $FoxP3^+$ Treg [163-164] and TGF-β induced by HCV, may expand HCV-specific Treg, and inhibit HCV-specific immunity in an indirect fashion [239-240]. In addition, TGF-β polymorphisms may be associated with spontaneous clearance of HCV and HCV RNA levels during chronic infection [241-242].

In contrast to a immunoregulatory role of TGF-β during chronic HCV infections, many authors suggest that TGF-β is exclusively involved in the acceleration of liver fibrosis (reviewed in [243]). However, in favour of an alternative hypothesis, data on the immunoregulatory properties of TGF-β will be presented in this thesis.

AIM AND OUTLINE OF THIS THESIS

HCV does not kill the hepatocytes it infects, but triggers chronic immunopathology that is relatively mild in most patients due to dysfunctional T cell responses. Negative regulation of HCV-specific immunity by Treg, IL-10 and TGF-β has been introduced above as possible mechanisms responsible for the deficient HCV-specific T cell response during chronic infection and the relatively slow progression of liver fibrosis.

Aims

The aim of the work presented in this thesis was to get further insight in the impact of negative regulation by IL-10, TGF-β and Treg on immunopathology (principally liver fibrosis) and the strength of adaptive immunity to HCV infections. We also questioned what the importance is of HCV-specific immunity and negative regulation for the outcome of PegIFN-α/ribavirin therapy.

Focus

The work is focussed on two aspects that have received little attention: regulation of HCV-specific immunity in the liver compartment, and the dynamics of immunoregulation to HCV before, during and after IFN-α based therapy.

Outline

In the first part of the thesis, data are presented on the role of intrahepatic regulatory T cells during chronic HCV infection and after PegIFN-α/ribavirin therapy-induced viral eradication. In chapter 2, the frequency and phenotype of $CD4^+FoxP3^+$ Treg and conventional $CD4^+$ T cells, and the distribution of lymphocytes and leukocytes were studied in liver and peripheral blood of chronic HCV patients at different phases of liver disease. The findings were compared with blood and liver of healthy subjects, and correlated with disease parameters. Chapter 3 describes the phenotype of peripheral blood and liver infiltrating regulatory T cells in chronic HBV-infected patients and serve as a comparison to our findings in chronic HCV-infected patients. In chapter 4, we investigated longitudinally how intrahepatic Treg are affected by IFN-α-based therapy, and whether this contributed to treatment outcome.

The second part of this thesis describes two prospective studies before, during and after PegIFN-α/ribavirin therapy. The first deals with the importance of HCV-specific immunity for the outcome of therapy (chapter 5). The second answers how HCV-specific immunity is regulated by IL-10, TGF-β and Treg, and finally, how this regulation is affected by PegIFN-α/ribavirin therapy (chapter 6).

Chapter 7 offers a discussion of our major findings in the context of the present literature.

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Part I:

Role for intrahepatic regulatory T cells during chronic hepatitis C virus infection and after therapy-induced viral eradication

Chapter 2

Abundant numbers of regulatory T cells localize to the liver of chronic hepatitis C infected patients and limit the extent of fibrosis

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ABSTRACT

Background & aims

Weak hepatitis C virus (HCV) specific immunity in peripheral blood has been shown to be partially controlled by regulatory T cells (Treg). However, little is known about Treg present in livers of HCV-infected patients, and their association with clinical parameters and immunopathology resulting in disease progression.

Methods

The frequency and phenotype of $CD4^+FoxP3^+$ Treg and conventional $CD4^+$ T cells, and the distribution of lymphocytes and leukocytes were studied by multi-color flowcytometry in liver and peripheral blood of 43 chronic HCV patients at different phases of liver disease. The comparison with healthy blood and liver, and correlations with disease parameters were made.

Results

An extensive lymphocyte infiltration containing abundant numbers of $CD4^+FoxP3^+$ Treg was present in HCV-infected livers, while absent from the healthy liver. Moreover, in all patients, intrahepatic $CD4^+FoxP3^+$ Treg showed a fully differentiated and highly activated phenotype on the basis of the surface markers CD45RO, CCR7, CTLA-4 and HLA-DR. Furthermore, these Treg were more numerous in those HCV-infected livers showing only limited fibrosis. However, HCV RNA loads or alanine transaminase levels did not correlate with $CD4^+FoxP3^+$ Treg frequencies.

Conclusions

Our data demonstrate that large numbers of highly activated and differentiated $CD4^+FoxP3^+$ Treg localize to the infiltrated chronic HCV-infected liver and may result in limiting the extent of fibrosis. This suggests that $CD4^+FoxP3^+$ Treg play a pivotal role in limiting collateral damage by suppressing excessive HCV-induced immune activation.

INTRODUCTION

Following infection with the hepatitis C virus (HCV), immunity fails to successfully eradicate the virus in the majority of individuals [1-3]. As a consequence, an estimated 120 to 170 million individuals are currently chronically infected worldwide [4]. Due to ongoing immunopathology, these patients are at increased risk of developing cirrhosis, and subsequently liver decompensation and/or hepatocellular carcinoma. However, without accelerating factors, such as coinfections and co-morbidities, disease progression is slow and it typically takes over a decade before serious health problems occur.

Patients chronically infected with HCV generally show a weak peripheral blood T cell response against HCV, which is insufficient to eradicate the virus [5-8]. It has been convincingly shown that peripheral blood regulatory T cells (Treg) from HCV-infected patients suppress both HCV-specific T cell proliferation and IFN-gamma production [9-13]. Thus, these cells may hamper the immune response against HCV during chronic infection, although other mechanisms have been proposed as well (reviewed in [1-3, 14-15]). Especially the role of intrahepatic Treg may be important to understand the chronic nature of the disease, since HCV predominantly infects hepatocytes. Although Treg have been detected before in livers of chronic HCV patients by performing immunohistochemical stainings [16-19], data on the phenotype of Treg at the primary site of infection is still lacking and their role in immunopathology remains unclear.

Regulation of the magnitude of the effector response may result in failure to eliminate the pathogen, and thus in the case of HCV infection, may lead to the establishment of a persistent infection. It has been demonstrated in many experimental infections that Treg act by dampening excessive inflammatory responses, and consequently help to limit tissue damage associated with the inflammatory reaction (reviewed by [20]). In chronic HCV infections, higher suppressive capacity of peripheral blood Treg was observed in patients showing a relatively low level of hepatocyte death, as reflected by alanine transaminase (ALT) levels [10]. However, Treg have not yet been implicated in controlling the outcome of immunopathology, i.e. liver fibrosis.

In this study, we characterized intrahepatic Treg in chronic HCV patients and their impact on disease progression. An extensive lymphocyte infiltration containing abundant numbers of $CD4^+FoxP3^+$ Treg was present in HCV-infected livers, while absent from the healthy liver. Moreover, these intrahepatic $CD4^+FoxP3^+$ Treg of patients at diverse stages of liver disease showed a fully differentiated and highly activated phenotype, and were more numerous in HCV-infected livers with mild fibrosis, suggesting an important role for intrahepatic Treg during chronic HCV infection.

METHODS

Patients and healthy controls

Intrahepatic cells were obtained from 43 chronic HCV-infected patients (Table 1 for clinical characteristics) by fine needle aspiration biopsy (FNAB; n=28) or percutaneous core needle biopsy (n=15). Paired venous blood samples were collected from all patients. All patients had detectable HCV RNA levels in serum.

Patients co-infected with human immunodeficiency virus or hepatitis B virus were excluded from the study. Diagnostic core biopsy specimens from all 43 patients, obtained within 3 months of the retrieval of intrahepatic cells for this study, were scored for fibrosis using the Metavir score by an experienced liver pathologist. A previous non-response to treatment did not affect the parameters assessed in this study. Wedge biopsies from 5 livers and 4 spleen samples were obtained from 8 organ donors. Finally, 31 healthy control subjects donated 10 ml venous blood. The institutional review board of the Erasmus MC approved these protocols, and informed consent was obtained from all individuals.

Table 1. Characteristics of chronic HCV infected patients (n = 43)

Gender*	Age (years)†	ALT (IU/l)†	HCV RNA (IU/ml)¥	Genotype*	Fibrosis (Metavir score)*	Treatment*
Male 29 (67%)	48 (27 – 67)	88 (17 – 228)	$7.7x10^5$ ($6.1x10^2$ – $2.7x10^7$)	1 33 (77 %) 2 3 (7 %) 3 6 (14 %) 4 1 (2 %)	0 7 (16%) 1 10 (23%) 2 7 (16%) 3 11 (26%) 4 8 (19%)	Never 23 (53%)
Female 14 (33%)						Previous 20 (47%)

† mean (range); ¥ median (range); * Group, number per group (percentage of total)

FNAB procedure

Details of the FNAB procedure are described elsewhere [21]. Briefly, a 25-gauge needle (Braun, Melsungen, Germany) containing a mandrin was used to puncture into the intercostal space after sonographic localization of the liver and exclusion of vascular or pathological structures. After removal of the mandarin, a syringe filled with RPMI supplemented with heparin and 0.1% human serum albumin (Sanquin, Amsterdam, the Netherlands) was attached, and liver cells were aspirated by negative syringe pressure. Two FNABs were collected per patient and pooled for further analysis. Generally, less than 50,000 intrahepatic cells were obtained.

Cell isolation and flowcytometry

Liver specimen were collected in RPMI-1640 (Lonza, Verviers, Belgium) and passed through a 70µm nylon cell strainer (BD-Falcon, Bedford, MA) to obtain a single cell suspension. Next, liver cells and peripheral blood were fixed and erythrocytes lysed using FixPerm™ reagent (eBioscience, San Diego, CA). To determine the frequency and phenotype of T cell and Treg subsets, multi-color flowcytometry was performed. Samples were stained with antibodies against CD25-PE-Cy7 (2A3; BD, San Jose, CA), FoxP3-APC (PCH101; eBioscience), CD4-APC-H7 (SK3; BD), CD45-Pacific Blue (HI30; eBioscience) or CD45-FITC (J33; Beckman), and CD3-AmCyan (SK7; BD). Further phenotyping was performed using either antibodies against CCR7-FITC (150503; R&D, Minneapolis, MN) and CD45RO-PE (UCHC1; BD) or CTLA-4-PE (BNI.3; Immunotech, Marseille, France) and HLA-DR-PerCP (L243; BD). For all staining procedures, permeabilization buffer was used (eBioscience). Cell acquisition was performed on a FACSCanto II (BD), and analyzed using FacsDiva™ software (BD). For analysis, gates were set on the basis of isotype antibody controls, where appropriate. Absolute leukocyte concentrations were determined by an automated impedance hematology analyzer (ABX Micros-60, Horiba Medical, Montpellier, France), and used to calculate the absolute numbers of specific lymphocyte populations.

Immunohistochemistry

FoxP3-expressing cells in paraffin-embedded liver tissue were identified using mouse anti-FoxP3 antibody (236A/E7; Abcam, Cambridge, UK). After deparaffinization, antigen retrieval, incubation with rabbit anti-mouse immunoglobulins (RMA, Dako) and alkaline-phosphatase-anti-alkaline-phosphatase complex (Serotec, Kidlington, UK), Fast Blue salt naphtol AS-BI phosphate solution supplemented with levamisole (all from Sigma-Aldrich, Steinheim, Germany) was added to visualize $FoxP3^+$ cells within tissue. Nuclear Fast Red was used as counter stain. Negative control stainings were performed by replacement of the primary antibody with an isotype-matched antibody.

Statistical analysis

Cell frequencies in blood and liver samples from different subjects were compared using the Mann Whitney U test. Correlations between different cell types (e.g. $CD4^+FoxP3^+$ Treg) and clinical parameters (e.g. fibrosis) were calculated using the Spearman correlation test. SPSS 17.0 for Windows (SPSS, Chicago, IL) was used for these analyses. All p-values were two-tailed.

RESULTS

Extensive inflammation of the liver is observed in patients with chronic HCV infections

To determine the degree and nature of inflammation in the liver of patients with chronic HCV infections, flowcytometric analyses were performed. As expected, vast numbers of CD45-expressing inflammatory cells were detected in the liver of patients with chronic HCV infections (Figure 1). The degree of inflammation, defined as the fraction of CD45-positive leukocytes of total liver cells [22], was on average 15% for HCV-infected livers., whereas in control livers, only an average degree of inflammation of 2% was detected. The majority of $CD45^+$ leukocytes infiltrating the infected liver were lymphocytes (mean: 69%), whereas in healthy liver this was only 24%. A large fraction of intrahepatic lymphocytes were $CD4^+$ T cells (mean: 28%) with a predominant $CD45RO^+CCR7^-$ effector phenotype (data not shown). These clear signs of ongoing inflammation were observed in HCV-infected livers irrespective of the stage of fibrosis.

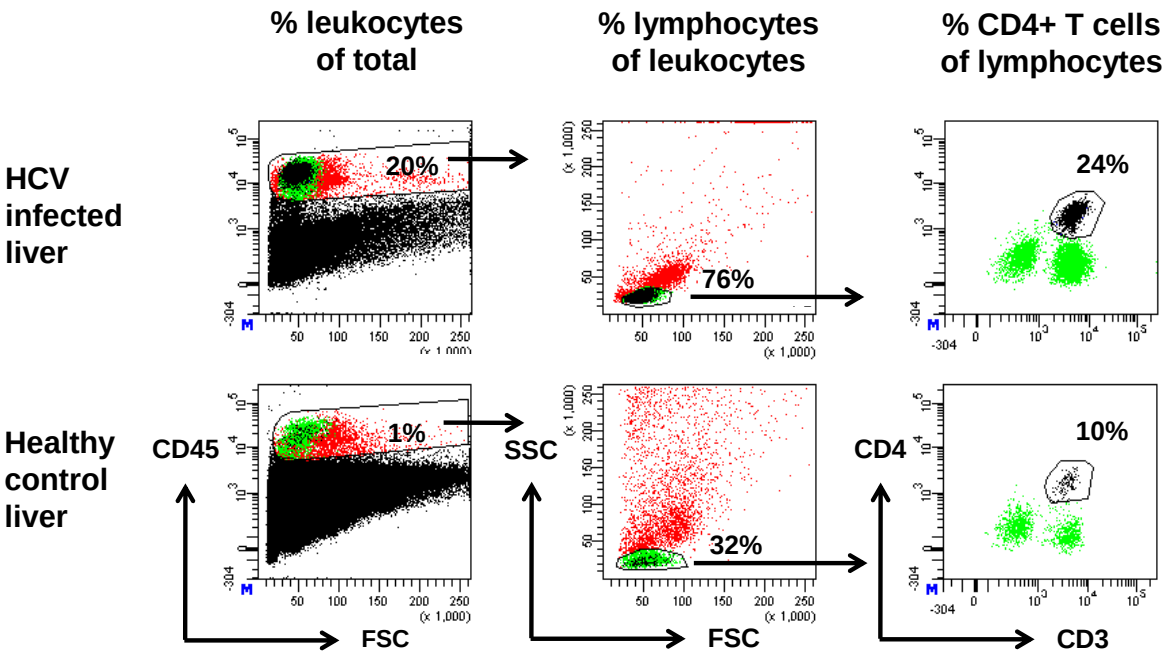

Figure 1. In contrast to healthy control liver, extensive intrahepatic inflammation is observed in patients with chronic HCV infections. Cell suspensions from liver biopsies and PBMC were stained for CD45 to separate leukocytes from parenchymal liver cells. Subsequently, lymphocytes were gated on the FSC/SSC profile, and $CD4^+$ T cells were identified. The proportion of $CD45^+$ cells to total liver cells, lymphocytes to leukocytes and $CD4^+$ T cells to lymphocytes were higher in HCV-infected livers as compared to healthy livers (respective mean values: 15% vs. 2%, p=0.004; 69% vs. 24%, p=0.004, 28% vs. 10%; p=0.0007). Representative dot plots are shown.

CD4$^+$FoxP3$^+$ Treg are abundantly present in the inflamed HCV-infected livers, while almost absent from the healthy liver

Control of intrahepatic inflammatory reactions by Treg may be an important mechanism regulating immunity and preventing immunopathology. Indeed, in contrast to healthy control livers, Figure 2A shows that a relatively high proportion of infiltrating CD4$^+$ T cells in the inflamed, HCV-infected liver were CD4$^+$FoxP3$^+$ Treg. In line with these findings, in HCV patients, CD4$^+$FoxP3$^+$ Treg constituted a significant fraction of intrahepatic lymphocytes and leukocytes (Figure 2B). FoxP3$^+$ Treg were located predominantly within the portal tract areas of chronic HCV-infected livers, whereas healthy livers were almost without Treg (Figure 2A). The low number of intrahepatic CD4$^+$FoxP3$^+$ Treg in healthy subjects was a feature of the liver, since the spleen of healthy controls did contain a significant population of CD4$^+$FoxP3$^+$ Treg (Figure 2A). The low frequency of CD4$^+$FoxP3$^+$ Treg in the healthy liver combined with high numbers of Treg within the HCV-infected liver, suggests that CD4$^+$FoxP3$^+$ Treg are involved in regulating the disease caused by HCV infection, possibly by controlling the strength of the immune response against HCV and preventing excessive immunopathology within the liver of patients with chronic HCV infections.

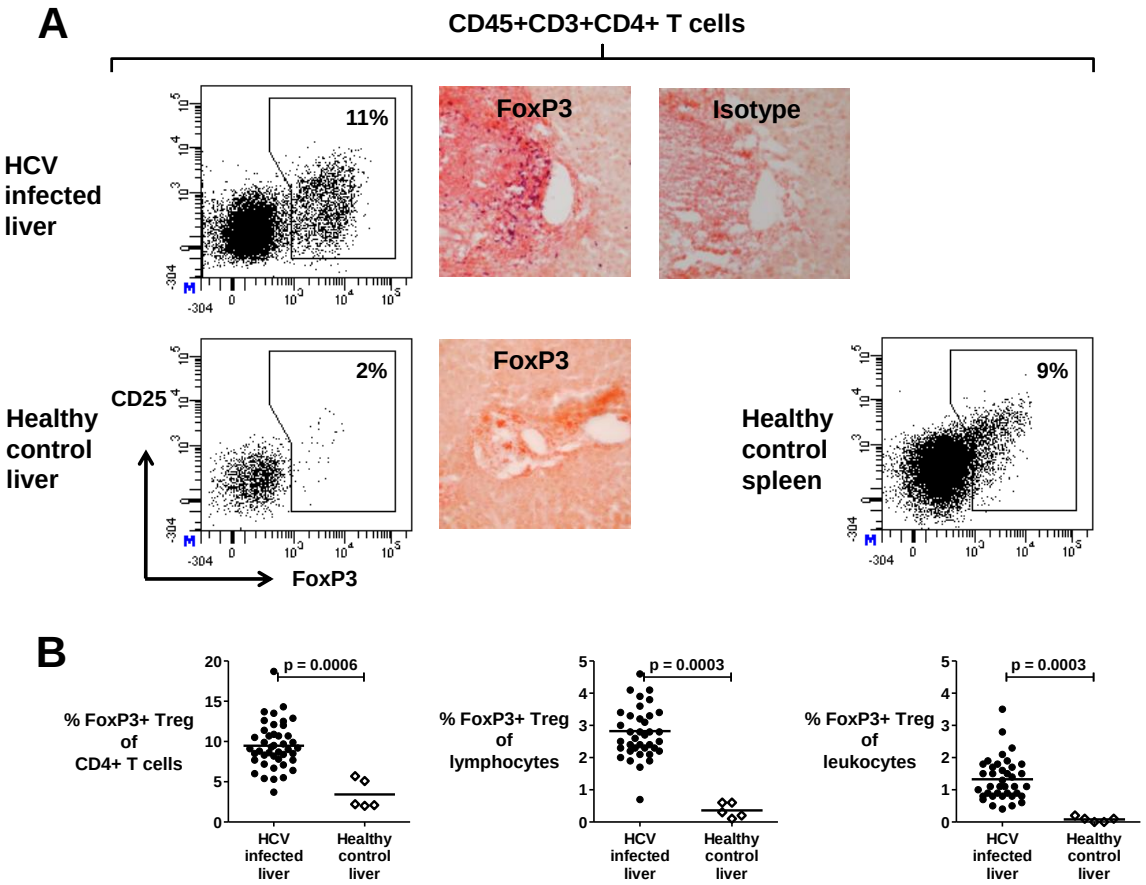

Figure 2. CD4$^+$FoxP3$^+$ Treg with variable CD25 expression are present in high numbers in HCV-infected livers, while almost absent from healthy liver. Treg were demonstrated based on FoxP3 expression within CD4$^+$ T cells, as shown in Figure 1. (A) In HCV-infected livers, CD4$^+$FoxP3$^+$ Treg were abundantly present, predominantly within the portal tract areas, while only few CD4$^+$FoxP3$^+$ Treg were present in uninfected livers. However, healthy control spleen did contain substantial percentages of CD4$^+$FoxP3$^+$ Treg (mean: 7.1% of CD4$^+$ T cells and 0.5% of total spleen leukocytes). Representative dot plots of cells, and immunostainings for FoxP3 (dark purple stain) or the appropriate isotype control antibody of liver tissue are shown. (B) Individual percentages and the mean percentage (horizontal line) of CD4$^+$FoxP3$^+$ Treg relative to CD4$^+$ T cells, lymphocytes or leukocytes are shown.

CD4$^+$FoxP3$^+$ Treg in blood of chronic HCV patients are less frequent than in healthy controls

In blood, the proportion of CD4$^+$FoxP3$^+$ Treg to CD4$^+$ cells was similar in HCV-infected patients and healthy controls (Figure 3A and 3B). However, in contrast to our findings in the liver, absolute CD4$^+$FoxP3$^+$ Treg numbers, and CD4$^+$FoxP3$^+$ Treg to lymphocyte ratios in blood of HCV-infected patients were lower than in healthy subjects (Figure 3B). This was not a result of enhanced migration of peripheral blood leukocytes towards the liver, since absolute numbers of circulating leukocytes and lymphocytes were similar between chronic HCV patients and healthy subjects (data not shown).

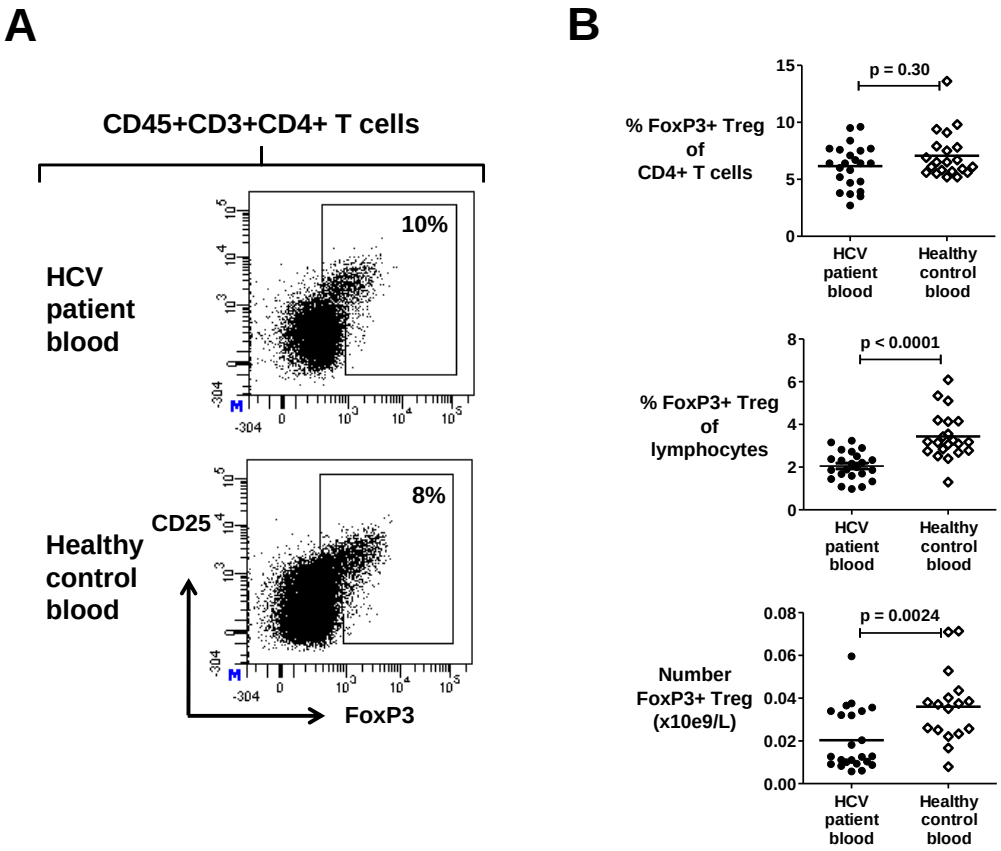

Figure 3. Chronic infection with HCV did not affect cellularity in blood, except for the number of CD4$^+$FoxP3$^+$ Treg, which were reduced as compared to healthy subjects. CD45$^+$ leukocytes, lymphocytes, CD4$^+$ T cells and Treg were demonstrated according to the gating strategies as depicted in Figure 1 and 2. (A, B) Blood of HCV-infected patients and healthy controls showed similar percentages of CD4$^+$FoxP3$^+$ Treg as a proportion of CD4$^+$ T cells. Representative dot plots are shown. (B) The absolute number as well as the proportion of these Treg to lymphocytes was lower in blood of HCV-infected patients than in blood of healthy individuals. The horizontal lines depict the mean values.

Intrahepatic CD4$^+$FoxP3$^+$ Treg from chronic HCV patients show a fully differentiated and highly activated phenotype, however partially downregulate the IL-2 receptor alpha chain

The *ex vivo* phenotype of intrahepatic Treg in the setting of chronic HCV infections has not been studied before. This phenotype is however very instructive, since it can shed light on the functional properties of Treg in the infected liver, which is important since *in vitro* functional experiments are difficult to perform due to limited numbers of cells available. Assessment of CD4$^+$FoxP3$^+$ Treg originating from the HCV-infected liver revealed a

predominant highly differentiated, antigen-experienced $CD45RO^+CCR7^-$ effector/memory phenotype. Intrahepatic $CD4^+FoxP3^+$ Treg were further differentiated than in peripheral blood, with CCR7 expression being lower in the liver than in blood of HCV-infected patients (mean: 6% and 22% respectively, p=0.001), albeit that CD45RO was expressed at similar levels (mean: 80% and 83% respectively, Figure 4A).

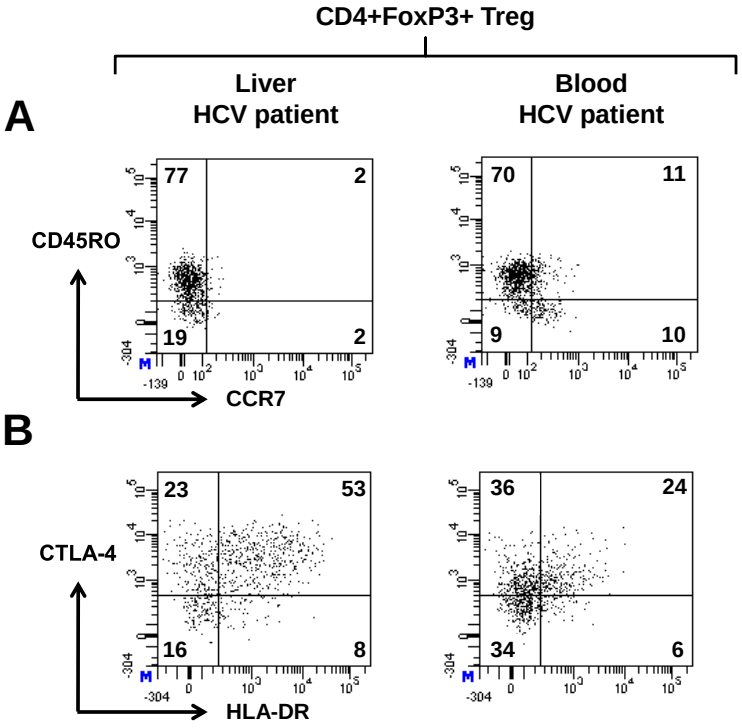

Figure 4. The majority of $CD4^+FoxP3^+$ Treg in HCV-infected livers display a fully differentiated $CD45RO^+CCR7^-$ and activated $HLA\text{-}DR^+CTLA\text{-}4^+$ phenotype. The populations displayed are all $CD4^+FoxP3^+$ Treg from liver (left-hand side) or blood (right-hand side) of a representative chronic HCV-infected patient. Treg were evaluated for expression of CD45RO and CCR7 (A), or HLA-DR and CTLA-4 (B). Gates were set according to their matched isotype controls. Numbers depicted in the plots are percentages.

Intrahepatic $CD4^+FoxP3^+$ Treg of chronically infected HCV patients expressed high levels of HLA-DR, as opposed to their counterparts in peripheral blood, indicating that liver Treg are more activated (mean: 42% and 22% respectively, p=0.008, Figure 4B). This was not only observed for $CD4^+FoxP3^+$ Treg, but was characteristic of all intrahepatic T cells in chronic HCV patients (data not shown). Also, CTLA-4, which is induced upon activation and shown to be important for the suppressive capacity of Treg [23-24], was expressed at higher level by liver than blood $CD4^+FoxP3^+$ Treg (mean = 89% and 78% respectively, p=0.04, Figure 4B).

Interestingly, in most HCV-infected livers, a substantial fraction of $CD4^+FoxP3^+$ Treg expressed low levels of CD25 (Figure 2A). These $CD25^{low}CD4^+FoxP3^+$ Treg displayed a similar differentiation and activation status as their $CD25^+$ counterparts with respect to CD45RO, CCR7, HLA-DR and CTLA-4 expression (data not shown). The presence of $CD4^+FoxP3^+CD25^{low}$ Treg was a feature of the HCV-infected liver, since blood of HCV-infected patients was almost devoid of $CD4^+FoxP3^+CD25^{low}$ Treg (Figure 3A). Hence, the commonly used definition of blood Treg as $CD4^+CD25^+FoxP3^+$ T cells does not apply to the liver.

Involvement of intrahepatic CD4$^+$FoxP3$^+$ Treg in HCV-induced immunopathology

The presence of high numbers of intrahepatic Treg in chronic HCV patients likely hampers effective antiviral immunity as has been shown for peripheral blood Treg in *in vitro* assays [9-13]. However, these intrahepatic Treg may have set a delicate balance resulting in an attenuated protective immunity and a limited immune-mediated liver damage. Therefore, we examined to what extent CD4$^+$FoxP3$^+$ Treg present in the liver of HCV-infected patients are linked to disease parameters. The ALT and HCV RNA levels did not correlate with the number of liver CD4$^+$FoxP3$^+$ Treg. However, a relation between intrahepatic CD4$^+$FoxP3$^+$ Treg and fibrosis was found (Figure 5). The ratio of CD4$^+$FoxP3$^+$ Treg to leukocytes present in non-fibrotic livers was higher as compared to livers with signs of fibrosis. Therefore, variation in the degree of liver fibrosis due to inflammation caused by chronic HCV infection may be partially explained by the frequency of intrahepatic CD4$^+$FoxP3$^+$ Treg.

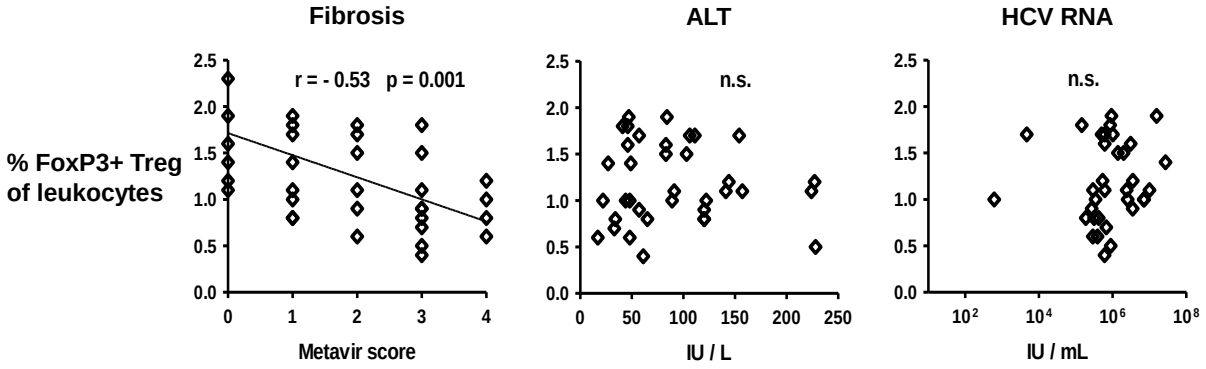

Figure 5. Involvement of intrahepatic CD4$^+$FoxP3$^+$ Treg in the immunopathology of HCV infection. The proportion of CD4$^+$FoxP3$^+$ Treg of leukocytes in livers of HCV-infected patients did not correlate with serum ALT levels or serum HCV RNA levels. However, HCV-infected patients with less CD4$^+$FoxP3$^+$ Treg as a proportion of total liver leukocytes showed milder fibrosis (r: Spearman correlation coefficient). Abbreviation: n.s., not significant.

DISCUSSION

In patients chronically infected with HCV, the virus replicates at high levels within the liver for decades. In order to maintain this viral persistence, regulatory mechanisms are in place that balance an ineffective protective HCV-specific immunity and mild immune-mediated liver damage. The present study shows that high numbers of CD4$^+$FoxP3$^+$ Treg accumulate in the HCV-infected liver. These CD4$^+$FoxP3$^+$ Treg display a highly activated and differentiated effector/memory phenotype and may be involved in limiting HCV-induced fibrogenesis. Importantly, they do not appear to control viral replication, as reflected by serum HCV RNA levels, or hepatocyte death, as assessed by the ALT.

We now show using multi-color flowcytometry that the livers of chronic HCV-infected patients are infiltrated with large numbers of T lymphocytes, a high proportion being CD4$^+$FoxP3$^+$ Treg. In addition, it has been demonstrated by us and others using immunohistochemistry that FoxP3$^+$ Treg reside primarily within the portal tract areas [16-19]. The CD4$^+$FoxP3$^+$ Treg isolated from HCV-infected livers were predominantly CD45RO$^+$HLA-DR$^+$CTLA4$^+$CCR7$^-$, reflecting an antigen-experienced, activated and highly differentiated

effector phenotype. Therefore, these cells likely exhibit immediate effector functions [25-26]. In this study, we were unable to formally prove this point by performing functional assays due to limitations in obtaining sufficient numbers of Treg from the liver. In contrast to the activated status of $CD4^+FoxP3^+$ Treg in the liver, relatively high numbers of circulating Treg from chronic HCV patients and healthy controls showed an early differentiated, weakly activated $CD45RO^+$HLA-DR-CTLA-4^+CCR7^+ phenotype [11, 27].

Interestingly, intrahepatic Treg from chronic HCV patients differed also from peripheral blood Treg in that about half of the intrahepatic $CD4^+FoxP3^+$ Treg displayed a downregulated expression of CD25, the alpha chain of the IL-2 receptor, despite their highly activated status. This downregulation seems specific for chronically infected organs, since similar findings have been shown before by us for livers of chronic HBV-infected patients [22], and by others for *Mycobacterium tuberculosis* infected mouse lungs [28] and tonsils of patients infected with human immunodeficiency virus [29]. It has been suggested that these $CD4^+FoxP3^+$ Treg are equally suppressive as their $CD25^+$ counterparts and that IL-2 is only indispensable for maintaining *in vivo* homeostasis of $FoxP3^+$ Treg. It can not be completely ruled out that FoxP3 is transiently upregulated on intrahepatic $CD25^-$ T cells upon activation as demonstrated by *in vitro* studies (reviewed in [30]). However, to our knowledge there is no information that this occurs *in vivo* on human T cells. Furthermore, the intrahepatic $CD25^-FoxP3^+$ Treg express FoxP3 at similarly high levels as their $CD25^+$ counterparts, which was in contrast to the *in vitro* assays where the transient FoxP3 expression was relatively low [30]. Therefore, based on the literature and our own findings, we define Treg as $CD4^+FoxP3^+$ T cells regardless of their CD25 expression.

In contrast to HCV-infected livers, healthy livers were almost without $CD4^+FoxP3^+$ Treg. This excludes an important role for $CD4^+FoxP3^+$ Treg in healthy livers without inflammation, while in livers of HCV-infected patients, high numbers of $CD4^+FoxP3^+$ Treg likely suppress the activity of infiltrated lymphocytes. Importantly, regulation of intrahepatic immunity by Treg is not unique for HCV infection, since we and others also observed increased numbers of $CD4^+FoxP3^+$ Treg in livers of patients with chronic HBV infections, primary biliary cirrhosis or auto immune hepatitis [16-19, 22]. Therefore, a general consequence of excessive immune activation in the liver is possibly negative regulation by various mechanisms, including $CD4^+FoxP3^+$ Treg. These processes may simultaneously control immunopathology, and hinder viral clearance.

The question rises whether these enhanced numbers of $CD4^+FoxP3^+$ Treg in the inflamed liver are the consequence of accumulation from the periphery, or due to *de novo* generation within the liver. This has not been formally addressed in the present study, although we found that in blood of chronic HCV-infected patients absolute $CD4^+FoxP3^+$ Treg counts were actually lower than in healthy controls, while total numbers circulating leukocytes were unchanged. However, we cannot determine whether this translates into the high number of $CD4^+FoxP3^+$ Treg found in the HCV-infected liver. While one previous report was in line with our findings [31], in most studies, higher circulating Treg frequencies were observed as opposed to healthy subjects [9-11, 13], although they defined Treg as $CD4^+CD25^+$ T cells and did not include FoxP3, the transcription factor specific for Treg.

High numbers of intrahepatic $CD4^+FoxP3^+$ Treg likely affect the liver compartment by balancing between protective immunity and immunopathology. We found that variation in the

degree of liver fibrosis due to inflammation caused by chronic HCV infection may be partially explained by the frequency of intrahepatic $CD4^+FoxP3^+$ Treg, while Treg were more numerous in HCV-infected patients showing only mild disease. These observations were not biased by the age of the HCV patients studied, a surrogate marker for time-since-infection, since age did not correlate with fibrosis stage or with the frequency of $CD4^+FoxP3^+$ Treg (data not shown). Interestingly, the relation between Treg and fibrinogenesis was only found for intrahepatic, but not peripheral Treg (data not shown). This observation is in line with a previous report, by Franceschini and colleagues, describing an inverse correlation between the histological activity index score and the fraction of $CD4^+CD25^+$ T cells expressing FoxP3 [32]. Using immunohistochemistry, Ward and colleagues, did not observe more $FoxP3^+$ cells in the liver of patients with mild liver disease [19]. However, in our study, the correlation between the ratio of Treg relative to infiltrating leukocytes was investigated, while Ward and colleagues assessed the correlation with the absolute number of $FoxP3^+$ cells in the portal tract areas, regardless of the presence of other immune cells. In our hands, other disease parameters, such as the level of viral replication, as determined by serum HCV RNA levels, or hepatocytes death, as assessed by ALT levels, did not appear to be controlled by liver $CD4^+FoxP3^+$ Treg frequencies or a specific Treg phenotype. Importantly, the established stage of fibrosis itself is the best marker for disease progression, rather than the grade of liver inflammation, serum ALT levels or serum viral loads [33]. Of interest is also that the genotype did not appear to affect the ratio of intrahepatic $FoxP3^+$ cells to $CD3^+$ T cells in our patient cohort as opposed to a previous study [17].

It is unclear how intrahepatic $CD4^+FoxP3^+$ Treg may limit fibrogenesis. One possibility is that IL-10 produced by Treg, inhibit collagen matrix deposition by hepatic stellate cells (HSC) [34]. Also, $CD4^+FoxP3^+$ Treg may inhibit effector functions of other intrahepatic T cells thereby indirectly inhibiting activation of HSC [35]. Interestingly, in this respect we observed a relation between the differentiation status of conventional FoxP3-$CD4^+$ T cells and the extent of liver fibrosis, with CD45RO-$CCR7^+$ naïve conventional T cell frequencies being highest in livers without signs of fibrosis (data not shown). Hence, $CD4^+FoxP3^+$ Treg may limit the differentiation of intrahepatic conventional T cells, which may result in reduced cytokine production. Alternatively, TGF-β produced by $CD4^+FoxP3^+$ Treg may worsen fibrosis by activating HSC. However, $CD4^+FoxP3^+$ Treg are likely only a minor source of free active TGF-β in the liver and TGF-β bound to the membrane of Treg only inhibits other immune cells in close proximity [36-37].

In conclusion, our findings clearly show that large numbers of $CD4^+FoxP3^+$ Treg localize to the inflamed liver in chronic HCV patients. These $CD4^+FoxP3^+$ Treg are highly activated and differentiated cells, and may function by preventing collateral damage induced by excessive immune activation. This may explain why in the majority of patients, liver pathology is relatively mild and only slowly progressing. As a consequence, the price to pay is that effective immune control is not achieved, resulting in the maintenance of chronic HCV infection.

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Chapter 3

Intrahepatic regulatory T cells are phenotypically distinct from their peripheral counterparts in chronic HBV patients

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ABSTRACT

Peripheral blood $CD4^+CD25^+$ regulatory T cells (Treg) prevent the development of strong hepatitis B virus (HBV)-specific T cell responses *in vitro*. In this study, we examined the phenotype of $FoxP3^+$ regulatory T cells in the liver of patients with a chronic HBV infection. We showed that the liver contained a population of $CD4^+FoxP3^+$ cells that did not express CD25, while these cells were absent from peripheral blood. Interestingly, intrahepatic $CD25^-FoxP3^+CD4^+$ T cells demonstrated lower expression of HLA-DR and CTLA-4 as compared to their $CD25^+$ counterparts. Patients with a high viral load have a higher proportion of regulatory T cells in the liver, but not in blood, compared to patients with a low viral load. In conclusion, the intrahepatic Treg are phenotypically distinct from peripheral blood Treg. Our data suggest that the higher proportion of intrahepatic Treg observed in patients with a high viral load may explain the lack of control of viral replication.

INTRODUCTION

Worldwide 400 million people suffer from a chronic hepatitis B virus (HBV) infection and approximately 1 million people die annually from HBV-related disease. In the majority of adult patients, infection with HBV manifests itself as a self-limiting acute hepatitis, which confers protective immunity and causes no further disease. However, in 10% of infected adults, HBV infection becomes persistent, which may result in severe liver disease and may lead to premature death as a consequence of decompensated liver failure or hepatocellular carcinoma [1-2]. In patients with an acute self limiting HBV infection, a multispecific $CD4^+$ and $CD8^+$ T cell response with a type 1 cytokine profile is important to control the infection [3-4]. Importantly, patients with a chronic HBV infection lack such a vigorous multispecific T cell response [4-5]. At present, it is still unclear why the immune system fails in chronic HBV infections.

Many mechanisms have been described by which pathogens can escape immune control to ensure conditions that allow their survival. One of the mechanism that is exploited by pathogens, such as *Leishmania major, Mycobacterium tuberculosis, Plasmodium spp., Cytomegalovirus and Human Immunodeficiency Virus*, is by enhancing regulatory mechanisms that normally terminate an immune response (reviewed in [6]). In this, specific T cells with a regulatory function have been shown to suppress virus-specific immune responses, and contribute to the development of persistence. Various populations of these regulatory T cells (Treg) have been described on the basis of the production of immunosuppressive cytokines, such as IL-10 or TGF-β, or on the basis of high expression expression of CD25, and the forkhead family transcription factor 3 (FoxP3) [7-9]. $CD4^+CD25^+$ Treg represent a stable population of human peripheral lymphocytes with a frequency between 3-10% of the total $CD4^+$ population [10].

In recent years, Treg have been implicated in regulating the immune response during HBV infections. We and others showed that patients with a chronic HBV infection have increased percentages of $CD4^+CD25^+FoxP3^+$ Treg in their peripheral blood compared to healthy controls and individuals who have resolved their HBV infection [11-14]. $CD4^+CD25^+$ T cells isolated from peripheral blood of these patients are capable of inhibiting the HBV-specific $CD4^+$ and $CD8^+$ T cell response *in vitro* [11-12, 15]. Furthermore, we showed that reduction of the viral load by potent antiviral therapy resulted in a decrease of the frequency of peripheral blood Treg [16]. Combined, these findings suggest an important role for Treg during either the establishment or the maintenance of chronic HBV infection. However, little information is available on the involvement of intrahepatic Treg in HBV infection. This information is crucial since the liver is the primary site of infection. Gaining more insight in the phenotype and function of intrahepatic Treg may provide valuable information for development of novel treatment strategies. In this study we performed an in depth analysis of Treg populations in the liver in chronic HBV patients, by assessing their phenotype and frequency in relation to disease parameters.

MATERIALS AND METHODS

Patients

32 chronic HBV patients underwent percutaneous needle liver biopsy as part of their diagnostic evaluation (Table 1). All patients had detectable HBV DNA levels in serum. Patients co-infected with human immunodeficiency virus, hepatitis A virus, hepatitis C virus or hepatitis D virus, and patients with a resolved viral hepatitis were excluded from this study. Excess tissue from liver biopsies (not needed for histological examination) was used to isolate intrahepatic leukocytes. In addition, a venous blood sample was collected from each patient shortly before, or within 3 hours after the liver biopsy was taken. Samples from 7 patients were used for detailed phenotypic analysis of Treg. The institutional review board of the Erasmus MC – University Medical Center Rotterdam approved this protocol, and informed consent was obtained from all patients.

Table 1. Patient characteristics

Characteristics	All patients (n=32)
Sex (M/F)	28/4
Age (y) [a]	37 (18–70)
HBV DNA (geq/ml) [b]	$4.6x10^9$ (10^3 –$1.9x10^{10}$)
ALT (U/L) [b]	50 (25–630)
HBeAg (pos/neg)	16/16
Metavir score	
0	6
1	10
2	9
3	4
4	2

[a] mean (range)
[b] median (range)

Virological assessment

Serum HBeAg and anti-HBe were determined quantitatively using the Abbott IMX system (Abbot Laboratories, North Chicago, IL) according to the manufacturer's instructions. Serum HBV DNA was determined using an in-house developed real-time polymerase chain reaction based on the Eurohep standard (detection limit: 373 geq/ml) (Applied Biosystems, Foster City, CA) [17].

Cells

PBMC were obtained by ficoll separation (Ficoll-Paque™ plus, Amersham Biosciences, Buckinghamshire, UK). Liver tissue was collected in RPMI 1640 (Bio Whittaker, Verviers, Belgium) and digested with 0.04% collagenase P (Roche, Mannheim, Germany) for 15 minutes at 37 °C. After digestion, the cells were passed through a 70 μm nylon cell strainer (BD Falcon, Bedford, MA) to obtain a single cell suspension.

Flow cytometry

For analysis of the frequency of different T cell subsets, isolated cells prepared from liver biopsies or blood were stained with anti-CD25-PE (M-A251; BD Pharmingen, San Jose, CA), anti-CD4-PerCP-Cy5.5 (SK3; Becton Dickinson, San Jose, CA), CD8-FITC (DK25, DAKO, Glostrup), and anti-FoxP3-APC (PCH101; eBiosciences, San Diego, CA). The FoxP3 antibody staining was performed according to the manufacturer's instructions. The cells were analyzed using a FACScalibur, and analyzed using CellQuest Pro software, Becton Dickinson). Further phenotyping of intrahepatic and blood leukocytes from 7 patients was done using 8-color cytometry using a FACS Canto II. Antibodies used were PD-1-FITC (MIH4.1; BD Pharmingen), CTLA-4-PE (BNI.3; Immunotech, Marseille, France), HLA-DR-PerCP (L243; Becton Dickinson), CD25-PE-Cy7 (2A3; Becton Dickinson), FoxP3-APC (PCH101; eBioscience), CD4-APC-H7 (SK3; Becton Dickinson), CD8-Pacific Blue, and

CD3-AmCyan (SK7; Becton Dickinson). All gates were set using isotype matched control antibodies. The analysis was performed using FACS Diva software (Becton Dickinson).

Statistical analysis

Data from the different patient groups was compared using a Mann Whitney U test. Flow cytometry data from PBMC and liver cells, and from intrahepatic FoxP3$^+$CD25$^+$ and FoxP3$^+$CD25$^-$ T cells were compared using the Wilcoxon matched pairs signed rank sum test. For these analyses SPSS 11.5 for Windows (SPSS, Chicago, IL) was used.

RESULTS

The liver contains a population of CD4$^+$CD25$^-$FoxP3$^+$ cells which is not detected in peripheral blood

To determine the presence of FoxP3-expressing regulatory T cells in the liver, cell suspensions prepared from diagnostic liver samples were assessed by flow cytometry. On the basis of CD45 expression parenchymal cells were excluded from the analysis, as described before [18]. As shown in Figure 1, CD3$^+$CD4$^+$FoxP3$^+$ Treg are abundantly present in the liver of chronic HBV patients. We consistently found that the fluorescence intensity of the FoxP3 staining on intrahepatic Treg was higher than on peripheral Treg (7.99 ± 1.21 vs. 4.95 ± 0.88; p=0.001). Moreover, a substantial fraction of FoxP3-expressing Treg in the liver did not express CD25. This FoxP3$^+$CD25$^-$ population was consistently found in the liver, but was not detected in blood.

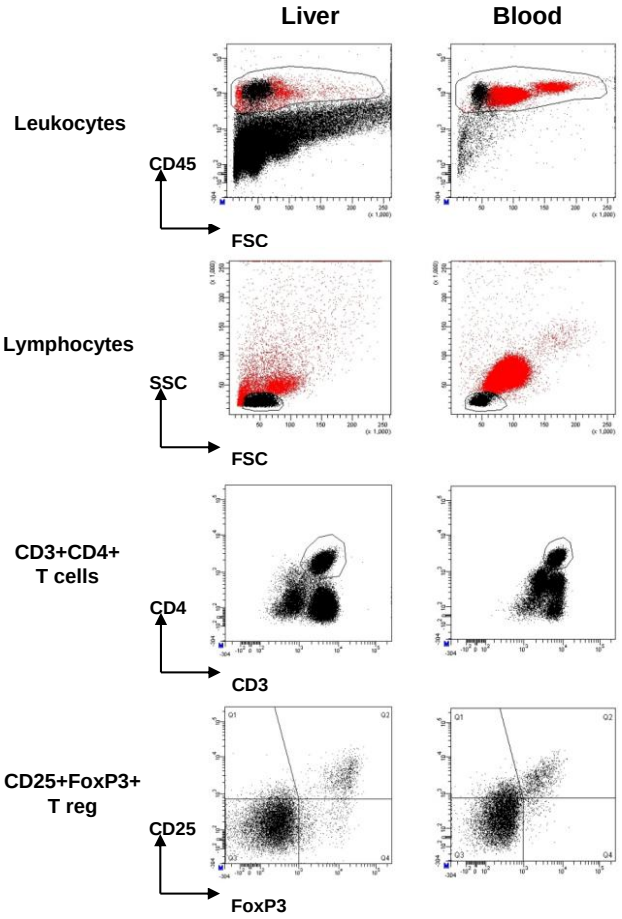

Figure 1. Intrahepatic Treg display higher FoxP3 expression, and lower CD25 expression as compared to peripheral Treg in chronic HBV patients. Cell suspensions from liver biopsies and PBMC were stained for CD45 to separate leukocytes from parenchymal liver cells. Treg were then demonstrated after sequential gating of lymphocytes based on the FSC/SSC profile, CD3+CD4+ T cells within the lymphocyte gate, and finally CD25+FoxP3+Treg.

The proportion of Treg relative to CD3$^+$ T cells in the liver is lower than in blood of chronic HBV patients

To determine the frequency of intrahepatic Treg, a careful analysis was made of the proportion of CD4$^+$ T cells expressing CD25 and FoxP3 in the liver of chronic HBV patients. The patient characteristics of this group are described in Table 1. Conventional Treg, as defined by expression of CD25 and FoxP3, comprised the largest population of FoxP3$^+$ cells in the liver, with an average frequency of 7.7% ± 0.4% relative to the CD4$^+$ T cell population (Figure 2A). The CD25$^-$FoxP3$^+$ population was present in all liver samples tested, albeit with different frequencies. The average frequency of CD25$^-$FoxP3$^+$ cells relative to CD4$^+$ T cells was 2.3% ± 0.2%. In blood samples from all 25 patients tested, this population of CD25$^-$ FoxP3$^+$ T cells was completely absent. Comparison of the frequency of FoxP3$^+$ cells to CD4$^+$ T cells, irrespective of the expression of CD25, showed similar ratios of FoxP3$^+$ T cells in the liver as compared to the blood of chronic HBV patients (Figure 2A).

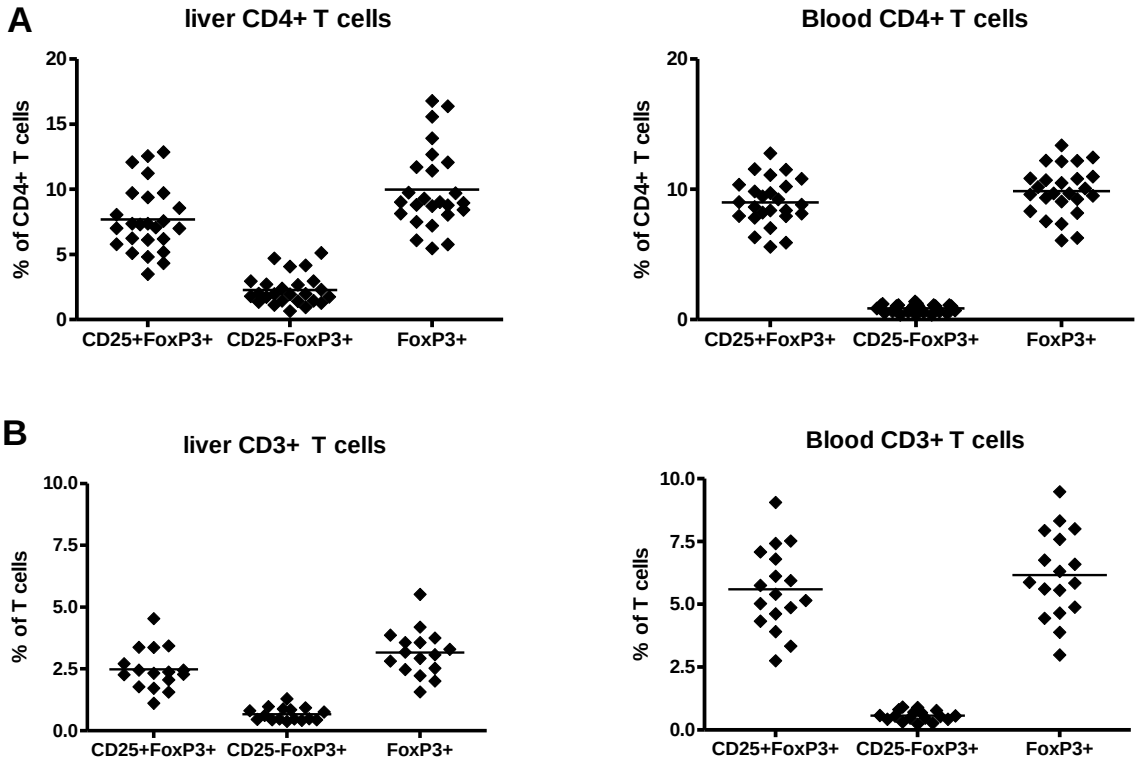

Figure 2. The ratio of FoxP3$^+$ Treg relative to CD3$^+$ T cells was lower in the liver as compared to blood. The frequency of Treg was analysed in paired liver and blood samples from 25 chronic HBV patients as described in the legend of Figure 1. The ratio of CD4$^+$CD25$^+$FoxP3$^+$, CD4$^+$ CD25$^-$FoxP3$^+$ and total CD4$^+$FoxP3$^+$ T cells are shown relative to the total number of CD4$^+$ T cells (A) or all T cells (B).

The frequency of Treg in the liver and blood is commonly expressed relative to the CD4$^+$ T cell population. However, it is known that the ratio of CD4$^+$ to CD8$^+$ T cells is reduced in the liver as compared to blood [19]. When the proportion of Treg relative to the total CD3$^+$ population was determined, the ratio of CD25$^+$FoxP3$^+$ to T cells as well as the ratio of FoxP3$^+$ cells to T cells was significantly lower in the liver as compared to the peripheral compartment (Figure 2B). Recently, Billerbeck et al showed de novo generation of FoxP3$^+$ regulatory CD8$^+$

T cells upon antigen recognition in vitro using blood samples of patients with a chronic hepatitis C virus infection [20]. We also looked for $CD8^+$ regulatory T cells in our study, however we did not detect $CD8^+FoxP3^+$ T cells in blood and liver samples obtained from chronic HBV patients (data not shown).

Conventional liver $CD4^+CD25^+FoxP3^+$ Treg express higher levels of CTLA-4 and HLA-DR as compared to intrahepatic $CD4^+CD25^-FoxP3^+$ cells and conventional peripheral blood Treg

Conventional $CD4^+CD25^+FoxP3^+$ Treg have been extensively studied in many human diseases. In contrast, although previously described in both mice and human, little information is available on $CD4^+CD25^-FoxP3^+$ cells, and these cells have not been described before in HBV patients. Therefore, we examined both FoxP3 expressing $CD4^+$ T cell subsets for expression of the inhibitory receptors CTLA-4 and PD-1, and an activation marker for T cells, HLA-DR, in a subset of 7 chronic HBV patients [21-25]. Conventional $CD4^+CD25^+FoxP3^+$ Treg expressed higher levels of CTLA-4 than $CD4^+CD25^-FoxP3^+$ cells, whereas the expression of PD-1 was lower on the conventional Treg compared to the $CD4^+CD25^-FoxP3^+$ cells (Figure 3A, 3B). Furthermore, the activation marker HLA-DR showed higher expression on conventional Treg as compared to the $CD4^+CD25^-FoxP3^+$ cells. So, conventional Treg consistently express higher levels of CTLA-4 and HLA-DR, but not PD-1 compared to $CD4^+CD25^-FoxP3^+$ cells. However, the relative lower levels of CTLA-4 and HLA-DR expressed on $CD4^+CD25^-FoxP3^+$ cells are still higher than on conventional $CD4^+$ T cells in the liver (data not shown). In addition, as shown in Figure 3C, conventional $CD4^+CD25^+FoxP3^+$ cells in the liver expressed higher levels of CTLA-4 and HLA-DR, but not PD-1, as compared to their counterparts in peripheral blood.

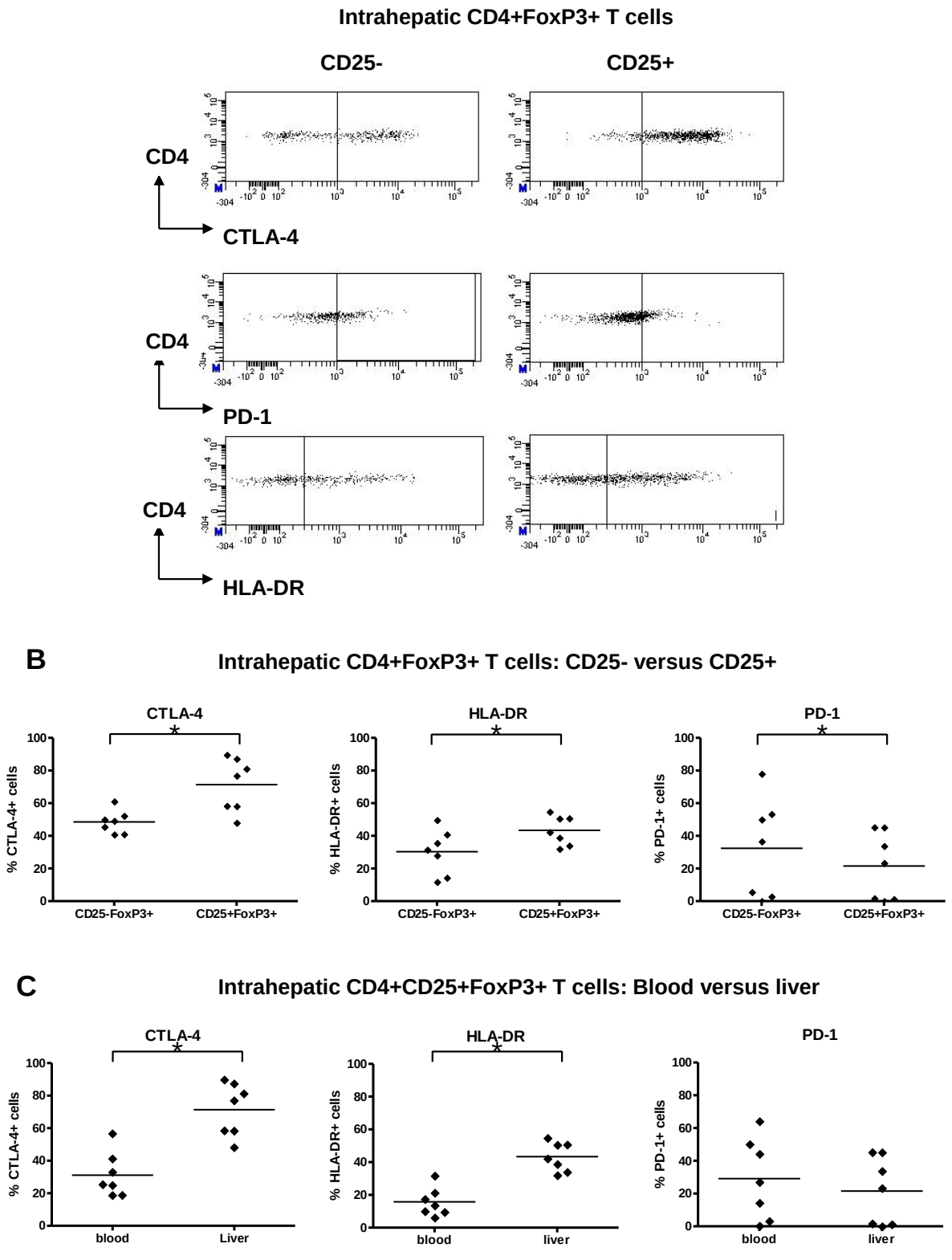

Figure 3. CD25$^+$FoxP3$^+$ Treg are phenotypically distinct from CD25$^-$FoxP3$^+$ Treg. Conventional Treg and CD25$^-$FoxP3$^+$ Treg were stained for intracellular CTLA-4, surface PD-1 and surface HLA-DR. (A) The populations displayed are gated based on the strategy as shown in Figure 1. Representative dot plots are depicted out of 7 individual stainings of liver samples from chronic HBV patients. (B) Percentage of cells staining positive for CTLA-4, PD-1 or HLA-DR from 7 chronic HBV patients. * $p < 0.05$ (C) Percentage of CD25$^+$FoxP3$^+$ cells from liver and peripheral blood staining positive for CTLA-4, PD-1 or HLA-DR from 7 chronic HBV patients. * $p < 0.05$

No correlation was observed between ALT, metavir score and the proportion of Treg

Since Treg play a role in maintaining tolerance to self antigens as reviewed by Sakaguchi [26], the liver damage caused by the inflammation might be a stimulus for Treg induction. Previously, we and others showed that the proportion of Treg in blood did not correlate with liver inflammation or the degree of liver damage [11-14]. Using a different patient cohort, we confirmed these findings (Table 2). However, since the primary site of HBV infection is the liver, it is more likely that correlations with disease scores are found with parameters assessed within the intrahepatic compartment. However, as depicted in Table 2, both the metavir score [27] and serum ALT levels did not show any correlation with the proportion of intrahepatic Treg to $CD4^+$ T cells. Also when the proportion of intrahepatic Treg relative to $CD3^+$ T cells was assessed, no correlation was found with either the metavir score or ALT levels.

Table 2 The frequency of intrahepatic Treg does not correlate with the metavir score or serum ALT levels

		% of $CD4^+$ T cells		% of T cells	
		$CD25^+FoxP3^+$	$FoxP3^+$	$CD25^+FoxP3^+$	$FoxP3^+$
Metavir	0	6.6 ± 0.9	8.6 ± 0.9	1.6 ± 0.2	2.2 ± 0.3
(score 0 to 4)	1	8.6 ± 1.5	10.8 ± 1.6	2.7 ± 0.5	3.4 ± 0.6
	2	7.5 ± 0.7	10.1 ± 1.1	2.3 ± 0.3	2.9 ± 0.3
	3	9.1 ± 3.0	12.6 ± 4.2	2.3 ± 0.1	2.4 ± 0.5
	4	7.2 ± 0.2	8.9 ± 0.1	1.8 ± 0.3	2.0 ± 0.3
ALT	<40	6.9 ± 0.8	9.9 ± 0.8	1.8 ± 0.9	2.6 ± 1.3
(U/L)	40–100	8.0 ± 0.7	10.1 ± 0.9	2.2 ± 0.6	3.1 ± 1.6
	>100	7.6 ± 0.7	9.4 ± 0.9	3.1 ± 0.7	3.8 ± 1.5

Data are depicted as mean ± SEM

Patients with a high viral load have an increased proportion of intrahepatic Treg

To determine whether a correlation exists between the amount of antigen present and the proportion of Treg, we analysed the mean proportion of Treg of two groups based on their viral load. No difference was observed in the proportion of peripheral blood Treg, defined as $CD4^+CD25^+FoxP3^+$ cells, between patients with high viral load and low viral load (9.2% ± 0.3% vs. 8.4% ± 0.3% respectively). In contrast, patients with a high viral load did have a higher proportion of intrahepatic conventional Treg relative to $CD4^+$ T cells compared to patients with a low viral load (8.4% ± 0.4% vs. 5.6% ± 0.3% respectively, $p< 0.05$; Figure 4A). This increased ratio of Treg versus $CD4^+$ T cells was also observed when Treg cells were defined as $CD4^+FoxP3^+$ cells, albeit not significant ($p=0.05$). No difference was observed in the proportion of $CD4^+CD25^-FoxP3^+$ cells between patients with a high and patients with a low viral load (2.33 ± 0.26 vs. 2.12 ± 0.53, respectively). Since Treg also suppress $CD8^+$ T cells we also determined the proportion of Treg relative to the entire T cell population. Also when the frequency of Treg was assessed relative to the $CD3^+$ T cell population, we observed that patients with a high viral load had an increased proportion of intrahepatic Treg (Figure 4B).

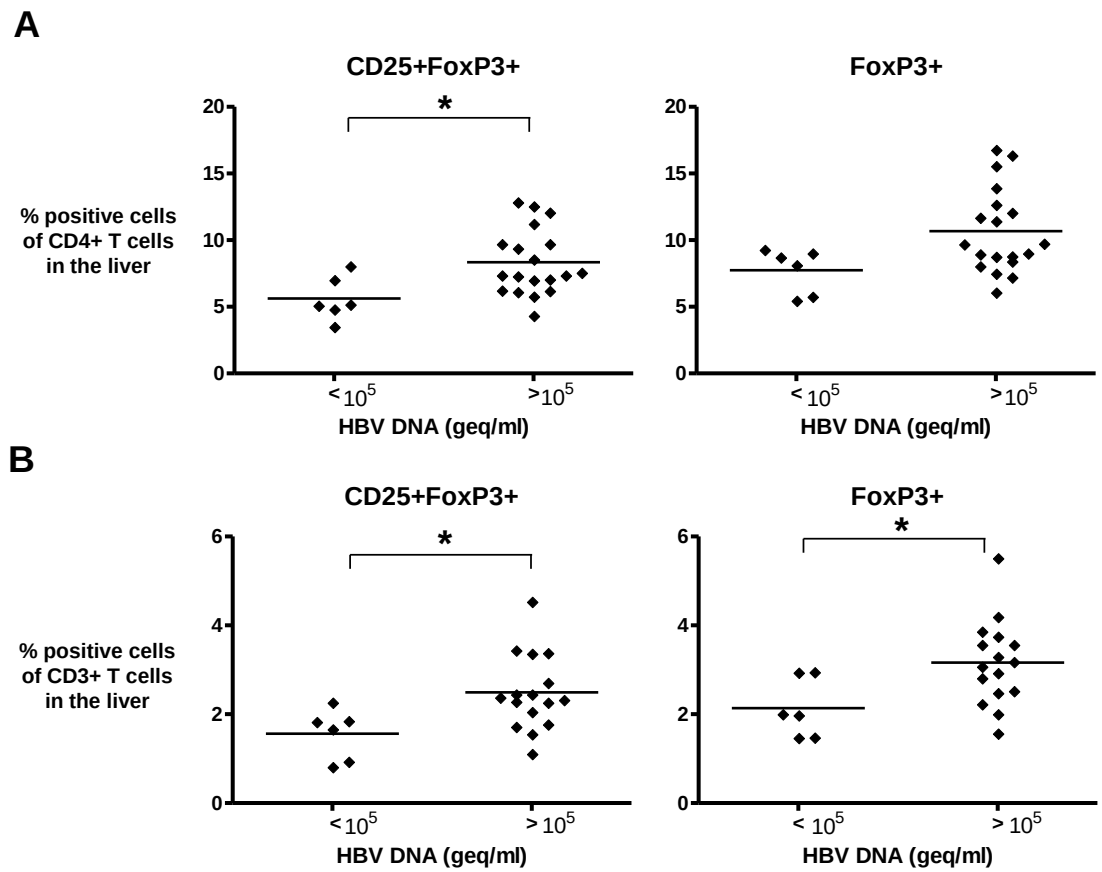

Figure 4. Patients with a high viral load have an increased proportion of intrahepatic Treg. The proportion of intrahepatic or peripheral Treg is defined as the percentage of cells staining positive for CD4, CD25 and FoxP3 relative to the percentage of cells staining positive for CD4 or CD3. The bar represents the mean proportion of Treg. * denotes $p<0.05$. Patients were divided into 2 groups: one group with a viral load of $<1 \times 10^5$ (geq/ml; n=6) and one group with viral load $>1 \times 10^5$ (geq/ml; n=19). (A) Conventional Treg relative to $CD4^+$ T cells or total $FoxP3^+$ Treg. (B) relative to all $CD3^+$ T cells.

DISCUSSION

The present study shows that regulatory T cells in the liver of chronic HBV patients display a different phenotype than their circulating counterparts. Higher FoxP3 expression is observed on intrahepatic Treg from chronic HBV patients and a population of $CD25^-$ $FoxP3^+$Treg is observed in the liver but not in the blood. We could not detect $CD8^+FoxP3^+$ regulatory T cells in liver nor blood from chronic HBV patients (data not shown). Higher levels of serum HBV DNA correlated with a higher frequency of Treg in the liver, but not in blood. These findings underscore the importance to evaluate immune parameters within the intrahepatic compartment for assessment of HBV-specific immune responses.

Using multicolour flowcytometry, we observed striking differences between intrahepatic and peripheral $CD4^+FoxP3^+$ cells in chronic HBV patients. In the liver, these cells expressed higher levels of FoxP3, whereas a significant fraction of $CD4^+FoxP3^+$ cells showed reduced CD25 expression. This population in the liver had lower CTLA-4 and HLA-DR expression compared to their conventional $CD4^+CD25^+FoxP3^+$ Treg.

Our study is the first to assess the phenotype of intrahepatic Treg in chronic HBV patients in detail. Most studies that evaluated human Treg in chronic HBV patients have studied peripheral blood as this is the most accessible compartment [11-15]. However, this may not reflect the situation in all tissues and during organ-specific diseases. Following HBV infection, the virus accumulates in hepatocytes in the liver, and therefore this compartment is the most relevant site to study HBV-specific immunological processes.

The population of $CD4^+CD25^-FoxP3^+$ cells has not been identified before in the liver of HBV patients. The proportion of this population did not correlate with the viral load. $CD4^+CD25^-FoxP3^+$ cells were first described by the group of Rudensky using GFP-FoxP3 knockin mice [28]. By micro-array, they showed that $CD4^+CD25^+FoxP3^+$ cells and $CD4^+CD25^-FoxP3^+$ cells have a largely common gene expression signature, which was quite distinct from $CD4^+CD25^-FoxP3^-$ cells. Furthermore, this gene expression profile was highly characteristic of regulatory T cells with expression of ICOS, GITR, IL-10, CD103 and CTLA-4. Importantly, both FoxP3 expressing populations exhibit strong suppressive activity *in vitro* [28-30]. In addition, $CD4^+CD25^-FoxP3^+$ T cells have also been reported in macaques infected with the simian immunodeficiency virus [31], and in the tonsil of HIV-infected patients [32]. At present it is unknown why CD25 expression is down regulated on these cells. In the liver, down-regulation of CD25 expression caused by specific stimuli (such as cytokines, or as a consequence of growth factor deprivation) is a possible explanation. On the basis of the mouse studies [28-30], it is highly likely that $CD4^+CD25^-FoxP3^+$ cells display regulatory features. However, in our study we were unable to examine whether intrahepatic $CD4^+CD25^-FoxP3^+$ cells from chronic HBV patients have suppressive activity, since this requires functional assays, which are difficult to perform with the limited cell numbers obtained from liver biopsies. We can therefore not exclude the possibility that FoxP3 expression is induced in non-regulatory T cells as a consequence of activation, as has been shown before in human T cells [33-35]. However, the lower expression of activation-associated markers, such as CD25, HLA-DR and CTLA-4 on the $CD4^+CD25^-FoxP3^+$ makes this possibility less likely. Importantly, HLA-DR^+ Treg have previously been described to define a population of highly suppressive Treg, and CTLA-4 has been shown to be very important for the suppressive capacity of Treg *in vivo* in mouse models [22, 36]. Therefore, reduced CTLA-4 expression on $CD25^-FoxP3^+$ Treg might be indicative of a weaker suppressive capacity. The observed augmented PD-1 expression on the $CD25^-FoxP3^+$ Treg however, is surprising for a Treg subset with reduced HLA-DR and CTLA-4 expression. One could envisage differential mechanisms of regulation involving PD-1 as a distinctive feature between the $CD25^+$ and $CD25^+$ $FoxP3^+$ Treg subsets.

Chronic HBV infection is characterized by a weak immune response to HBV. We and others previously showed that $FoxP3^+$ T cells from patients with a chronic HBV infection are capable of inhibiting HBV-specific $CD4^+$ and $CD8^+$ T cell proliferation as well as IFN-γ production [11-12, 15]. Increased numbers or enhanced activity of Treg during chronic HBV infection may contribute to an inadequate immune response against the virus, leading to viral persistence. This hypothesis can only be tested by performing a longitudinal study in which acute patients are studied. Alternatively, Treg can also play a role during the chronic phase of the infection by limiting pathological responses caused by liver inflammation or cirrhosis. Various groups have studied the frequency of Treg in blood, and found no correlation with

serum ALT levels [11-14]. In this study we now show that the frequency of liver Treg did not correlate with liver inflammation, nor with liver pathology, as determined by histological assessment of the degree of fibrosis. This indicates that the increased proportion of Treg observed in patients with a chronic HBV infection is, most likely, not the result of liver damage or the presence of self-antigen caused by liver damage.

In this study, we show a positive correlation between the HBV DNA viral load and the frequency of Treg in the liver. Treg can be induced through repetitive stimulation of T cells by the presence of high concentrations of antigen for longer periods of time [37] which could be an explanation for the higher proportion of intrahepatic Treg observed in the chronic HBV patients with a high viral load. Previously, our group and Franzese et al found no correlation between the viral load and the frequency of Treg in blood [11, 15], whereas two recent studies did find a significant association [13-14]. Differences in the patient cohorts, and differences in the definition of the Treg population may account for these discrepancies. The definition of the Treg population is an important issue, as demonstrated by our phenotypic study of liver Treg. However, also in the peripheral compartment the selection of all CD25 expressing cells, only CD25-high expressing cells, or combined expression of CD25 and FoxP3 to identify Treg, will affect the outcome of the studies. In line with our previous findings [11], we did not find a correlation between HBV DNA levels and peripheral Treg using the $CD4^+CD25^+FoxP3^+$ gating strategy as depicted in Figure 1 (data not shown).

In summary, our findings demonstrate that Treg obtained from the liver of chronic HBV patients are phenotypically distinct from their peripheral counterparts. These findings are crucial for the accurate enumeration of Treg in the liver, but also might have important implications for their function. Furthermore, patients with a high viral load have a higher proportion of intra-hepatic Treg compared to patients with a low viral load, which might be an explanation for the lack of control on viral replication. These results contribute to our understanding of the role of Treg in chronic HBV infections, as well as the mechanisms of viral persistence.

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Chapter 4

Retention of $CD4^+CD25^+FoxP3^+$ regulatory T cells in the liver after therapy-induced hepatitis C virus eradication in humans

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ABSTRACT

Following infection with the hepatitis C virus (HCV), in most cases immunity fails to eradicate the virus, resulting in slowly progressing immunopathology in the HCV-infected liver. We are the first to examine intrahepatic T cells and $CD4^+CD25^+FoxP3^+$ regulatory T cells (Treg) in chronic HCV patients during and after antiviral therapy by collecting multiple aspiration biopsies from the liver at different time-points. We found that intrahepatic Treg frequencies were increased upon interferon-α and ribavirin administration in about 50% of chronic HCV patients, suggesting stronger regulation of intrahepatic immunity by Treg during antiviral therapy. After cessation of antiviral therapy, the frequency of intrahepatic Treg remained increased in the large majority of livers of individuals who successfully cleared the virus. The differentiation stage of these Treg that were retained in the liver months after therapy-induced clearance of HCV RNA, displayed a reduced contribution of effector-memory cells. Our findings, gathered by multiple sampling of the liver, indicate that successful antiviral therapy of chronic HCV patients does not lead to normalization of the local immune response to a resting state comparable to healthy livers. The continuous presence of high numbers of Treg, with a phenotype reflecting a relatively weak suppressive activity, suggests ongoing residual regulation of immunopathology. These findings provide important insight in the dynamics of the immune response to HCV, as well as the effect of therapy on intrahepatic immunity.

INTRODUCTION

Following infection with the hepatitis C virus (HCV), immunity fails to successfully eradicate the virus in the majority of individuals [1-3]. As a consequence, an estimated 120 to 170 million individuals are currently chronically infected worldwide. In the long term, these patients are at increased risk of developing cirrhosis, and subsequently liver decompensation and hepatocellular carcinoma, due to ongoing immunopathology.

The HCV-infected liver displays extensive infiltrates containing mainly $CD8^+$ and $CD4^+$ T cells around the portal tract areas. Functionally these cells have not been characterized in detail, but they are generally activated and differentiated, and display an effector phenotype [4-6]. Despite the fact that the liver of chronic HCV patients contains extensive leukocyte infiltrates, liver pathology progresses slowly and the development of liver fibrosis may take decades. This may be explained by the activity of high numbers of regulatory T cells (Treg), which are present in the liver of chronic HCV-infected patients [6-8], but absent from healthy livers, and the number of intrahepatic Treg negatively correlates with the development of fibrosis [4].

In chronic HCV infection, disease progression can only be impeded after viral clearance induced by interferon-alpha (IFN-α)-based therapy. Unfortunately, the best available treatment, pegylated-IFN-α combined with ribavirin, results in sustained clearance of serum HCV RNA in only about 50% of patients. Apart from the direct antiviral effects of therapy (reviewed in [9]), the impact of IFN-α-based therapy on the size, distribution, and composition of the leukocyte pool may be important in determining the treatment response. While the number of blood leukocytes declines dramatically shortly after start of IFN-α-based treatment, blood lymphocytes seem differently affected. Recently, it was reported that the proportion and phenotype of circulating $CD4^+CD25^{hi}$ Treg remained unchanged during IFN-α and ribavirin therapy of chronic HCV patients [10], whereas IFN-α induced Treg in multiple sclerosis *in vitro* [11].

In contrast to HCV-specific immune responses in blood, no studies have been conducted that examine the kinetics of the immune response in the liver of chronic HCV patients, or the effects of antiviral therapy on the intrahepatic immune response. This is highly relevant since HCV replication takes place within hepatocytes. Studies using intrahepatic cells are difficult to perform since liver biopsies are collected from patients for diagnostic purposes only, and are therefore limited to pre-treatment assessment of liver cells. The findings of these studies propound that baseline intrahepatic $CD8^+$ T cell responses in chronic HCV patients are important for successful response to IFN-α-based therapy [12-14], which is in line with reports on peripheral blood T cells [15-16]. However, it remains unclear how intrahepatic immunity is affected by IFN-α-based therapy and how this contributes to treatment outcome. Also the role of Treg in the liver during therapy has not been investigated before.

By performing multiple aspiration biopsies of the liver at different time-points, we determined the effect of IFN-α-based therapy on recruitment of Treg and other lymphocyte populations towards the liver of HCV-infected patients. In addition, we examined the leukocyte composition in the liver after therapy-induced HCV eradication to determine to what extent viral load reduction normalizes the intrahepatic immune system to a state as

observed in healthy individuals. A better understanding of local immunoregulation in the liver during viral infection and its modulation by antiviral therapy may lead to further improvement of the therapeutic success rate of chronic HCV patients.

METHODS

Patients and antiviral therapy

Twenty-two treatment-naïve chronic HCV-infected patients were included in this study (Table 1); none of them were co-infected with HIV or HBV. Diagnostic core biopsy samples from 18 of 22 patients, obtained within 3 months prior to start of therapy, were assessed for fibrosis stage by an experienced liver pathologist using the Metavir score. All patients received 24 (genotype 2 or 3) or 48 weeks (genotype 1) of antiviral therapy consisting of orally administered ribavirin and subcutaneous infusions with pegylated-IFN-α2b (PegIntron®, Schering-Plough, Houten, the Netherlands), both weight-based. The institutional ethical review board of the Erasmus MC approved the clinical protocols, and written informed consent was obtained from all individuals.

Table 1. Individual patient characteristics (n = 22)

Study number	**Sex** (M/F)	**Age** (years)	**Liver fibrosis** (Metavir)	**Genotype**	**HCV RNA baseline** (IU/mL)	**HCV RNA after therapy†** (IU/mL)	**ALT baseline** (U/L)
1	M	47	2	1	1.41×10^6	undetectable	75
4	F	57	3	1	1.13×10^7	undetectable	150
5	F	37	n.d.	3	3.19×10^3	undetectable	79
6	M	54	1	1	2.72×10^7	$5.95E^{+}05$	49
9	F	27	1	1	3.70×10^2	undetectable	34
10	M	48	2	3	1.09×10^5	undetectable	146
11	M	51	2	1	3.08×10^6	$1.86E^{+}06$	46
12	M	52	2	3	1.46×10^5	undetectable	41
13	M	34	2	1	3.75×10^6	undetectable	103
14	M	56	2	1	6.49×10^6	$1.57E^{+}07$	157
15	F	57	4	1	7.70×10^5	$1.40E^{+}06$	17
17	M	47	1	1	4.46×10^5	undetectable	227
19	M	46	3	2	1.56×10^7	undetectable	47
20*	F	41	1	3	8.56×10^5	undetectable	46
21*	M	40	3	3	3.23×10^6	undetectable	91
22	M	40	2	1	2.20×10^4	$4.14E^{+}03$	58
23	M	49	n.d.	1	2.88×10^5	undetectable	20
24	F	40	3	1	3.14×10^5	$1.47E^{+}06$	120
25	M	45	n.d.	1	1.08×10^7	$1.54E^{+}07$	36
26	F	58	4	1	1.56×10^5	undetectable	179
28	M	42	1	1	1.48×10^6	undetectable	65
30	F	42	n.d.	1	3.33×10^6	undetectable	107

Abbreviation: n.d., not determined within 3 months before start of therapy.
* Patient 20 and 21 finished the complete course of antiviral therapy, but aspiration biopsies from the liver and blood were not collected at all time points.
† Undetectable if HCV RNA negative both at 4 and 24 weeks after end of therapy, otherwise HCV RNA data from 4 weeks after therapy are shown.

Aspiration of liver cells and collection of peripheral blood

Details of the collection of liver cells as fine-needle aspiration biopsies are described elsewhere [17]. Briefly, a 25-gauge needle (Braun, Melsungen, Germany) containing a mandrin was used to puncture into the intercostal space after sonographic localization of the liver and exclusion of vascular or pathological structures. After removal of the mandrin, a syringe filled with RPMI-1640 medium was attached, and liver cells were aspirated by negative syringe pressure. Two aspiration biopsies were collected per patient, checked for low erythrocyte contamination and pooled for further analysis. Aspirate biopsies and paired venous blood samples were collected from all patients at start of therapy (week 0), during therapy (week 4) and after therapy (4 weeks after cessation of treatment). In 3 patients, who successfully cleared the virus, additional aspirate biopsies were collected 24 weeks after the end of treatment.

Cell isolation and flowcytometry

Liver specimen were collected in RPMI-1640 medium (Lonza, Verviers, Belgium) and passed through a 70µm nylon cell strainer (BD-Falcon, Bedford, USA). Liver cells and peripheral blood were fixed and erythrocytes lysed using FixPerm™ reagent (eBioscience, San Diego, USA). To determine the frequency and phenotype of T cells and Treg cells, multi-color flowcytometry was performed [18]. Samples were stained with antibodies against CD25-PE-Cy7 (2A3; BD-Biosciences, San Jose, USA), FoxP3-APC (PCH101; eBioscience), CD4-APC-H7 (SK3; BD), CD45-Pacific Blue (HI30; eBioscience) or CD45-FITC (J33; Beckman, Fullerton, USA), and CD3-AmCyan (SK7; BD-Biosciences). Further phenotyping was performed using either antibodies against CD62L-PE-Cy5 (Dreg56; BD-Biosciences) and CD45RO-PE (UCHC1; BD-Biosciences). For all staining procedures, permeabilization buffer was used (eBioscience). Cells were acquired using a FACS Canto II (BD-Biosciences), and analyzed by FacsDiva™ software (BD-Biosciences). On average 19,000 intrahepatic leukocytes could be acquired. Samples from patients were included in the analysis only if at least 50 $CD25^+FoxP3^+$ cells were acquired within the $CD45^+CD3^+CD4^+$ population. On average 238 $CD25^+FoxP3^+$ cells (range 53 to 826) were acquired within on average 2764 $CD45^+CD3^+CD4^+$ cells (range 504 to 9724). For analysis, gates were set on the basis of isotype antibody controls, where appropriate.

Virological assessment

Serum HCV RNA levels were determined by quantitative PCR (Cobas® Ampliprep/ Cobas® TaqMan® HCV test (limit of detection <15 IU/ml, Roche Diagnostics, the Netherlands). HCV genotypes were determined by an in-house developed sequence analysis assay (Department of Virology, Erasmus MC).

Statistical analysis

Cell frequencies in blood and liver samples from different subjects were compared using the Mann Whitney U test, and paired samples were compared using the Wilcoxon signed-rank test. SPSS17.0 software was used for all analyses. All p-values are two-tailed, and values $p>0.05$ were considered not significant (n.s.).

RESULTS

Fine-needle aspiration biopsies as a method to assess the lymphocyte profile in the liver during the course of antiviral therapy

The local immune response in the liver is considered vital in the control of HCV replication in hepatocytes, and the persistence of the virus in the liver of infected patients [1, 5]. To determine the contribution of conventional T cells and $CD4^+CD25^+FoxP3^+$ Treg to the regulation of immune responses to HCV, multiple minimally invasive and safe liver aspiration biopsies were collected and evaluated at baseline, as well as during and after therapy. Using the total aspirate, on average 19,000 leukocytes were acquired by flowcytometry at each time-point, which allowed us to perform flowcytometric analysis, but no another assays. Lymphocytes constituted a major population of liver infiltrating leukocytes in these patients

(Figure 1; 46% on average); the majority being $CD3^+$ T cells. In line with previous reports, in all HCV-infected patients $CD4^+$ T to $CD8^+$ T cell ratios in the liver were significantly lower than in blood (respective mean ratio: 0.78 in liver and 1.9 in blood, p<0.0001), indicating a higher proportion of $CD8^+$ T cells of the total intrahepatic $CD3^+$ T cell population. Sufficient numbers of $CD4^+CD25^+FoxP3^+$ Treg could be detected in aspiration biopsies obtained from chronic HCV-infected patients to allow reliable evaluation of their frequency and phenotype by flowcytometry-based analysis (Supplementary Figure 1).

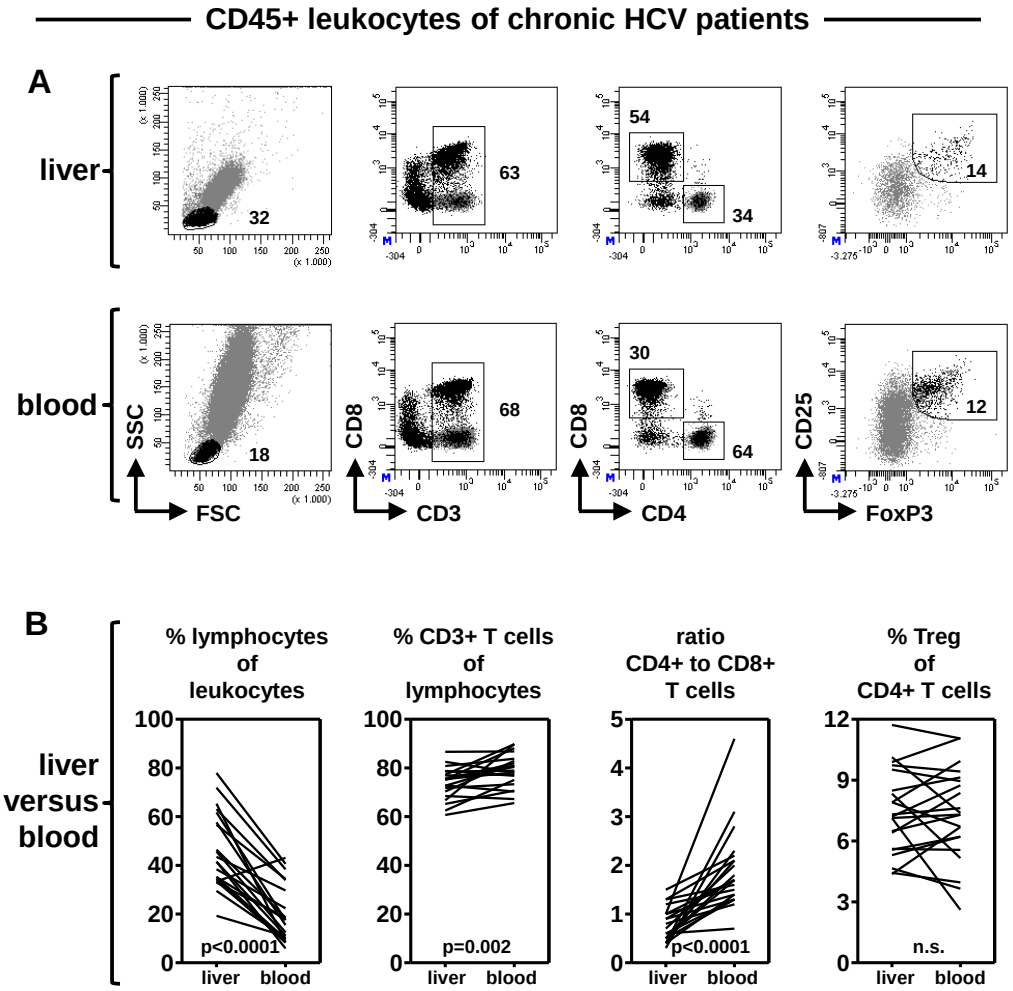

Figure 1. Assessment of the intrahepatic lymphocyte profile by fine-needle aspiration biopsies. (A) Using flowcytometry, $CD45^+$ leukocytes from the liver (upper panels) and peripheral blood (lower panels) were characterized by serial gating on the basis of their forward/sideward scatter profile, and expression of various T cell markers. The dot plots are representative for specimen taken before, during or after antiviral therapy. (B) Pretreatment frequencies and ratios of various lymphocyte populations from paired liver and blood samples are shown.

Administration of IFN-α and ribavirin does not lead to substantial changes in the general composition of the lymphocyte compartment in the liver of chronic HCV patients

As expected, 4 weeks after start of administration of IFN-α and ribavirin, reduction of the viral load and ALT levels accompanied by major losses in circulating leukocytes were observed in most patients (Figure 2A). In peripheral blood, the decrease in blood leukocyte counts was due to a drop in the absolute numbers of circulating granulocytes as well as lymphocytes within the first weeks of treatment (Figure 2B).

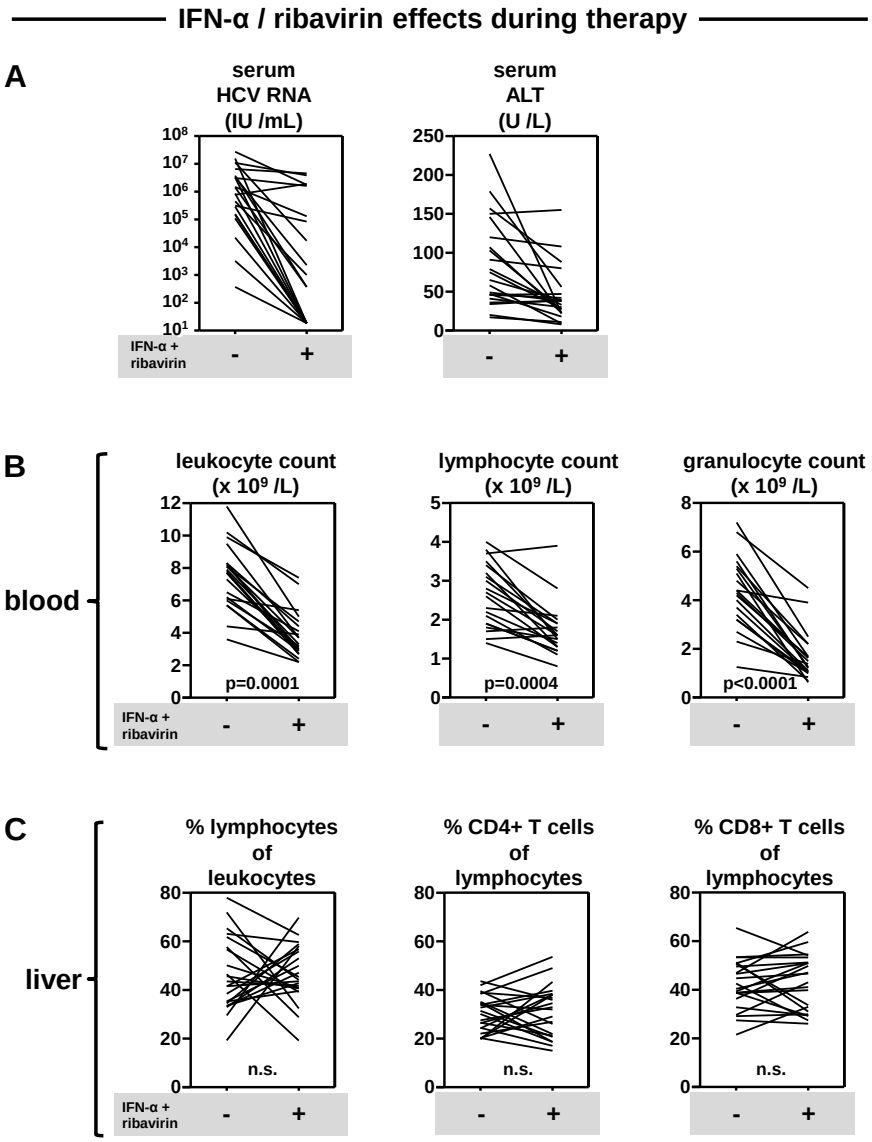

Figure 2. Administration of IFN-α and ribavirin does not lead to dramatic changes in the composition of the intrahepatic lymphocyte compartment of chronic HCV patients. (A) Serum HCV RNA and ALT levels were determined in blood from 22 chronic HCV patients are displayed prior to treatment (-), and 4 weeks after starting therapy ($^{+}$). (B) The absolute numbers of leukocytes, lymphocytes and granulocytes were determined in blood. (C) The effect of antiviral therapy on the frequencies of intrahepatic leukocytes as determined by flowcytometry on aspirate biopsies from the liver is shown.

We now show that the frequency of intrahepatic lymphocytes relative to leukocytes was not significantly changed at the group-level when the composition of the liver at baseline and during IFN-α-based therapy was compared (Figure 2C). Also, the relative contributions of $CD4^{+}$ and $CD8^{+}$ T cells to the lymphocyte population in the liver were not significantly affected at the group-level. However, individual patients showed considerable shifts in the frequency of intrahepatic lymphocytes. Out of 22 patients, 13 showed an increased frequency of lymphocytes, while in 9 patients the frequency of intrahepatic lymphocytes was reduced as a consequence of treatment. Similarly, highly heterogeneous changes in the $CD4^{+}$ and $CD8^{+}$ T cell frequencies in the liver were observed as a consequence of therapy in these patients (Figure 2C). Changes in liver lymphocyte and $CD4^{+}$ and $CD8^{+}$ T cell frequencies were not associated with the magnitude of serum HCV RNA or ALT reductions

during IFN-α and ribavirin therapy. Due to the small volume of the aspiration biopsies, accurate enumeration of the absolute leukocyte and lymphocyte numbers in HCV-infected livers was unreliable for individual patients.

The frequency of intrahepatic $CD4^+CD25^+FoxP3^+$ Treg increases during IFN-α and ribavirin therapy in half of the HCV-infected patients

We previously showed that $CD4^+CD25^+FoxP3^+$ Treg are present at high numbers in the liver of chronic HCV-infected patients, while virtually absent in livers from healthy controls [4]. To determine the effect of IFN-α-based therapy on immunoregulatory mechanisms in the liver of chronic HCV patients, the frequencies of Treg were determined before and 4 weeks after start of treatment. As shown in Figure 3A, during antiviral treatment the number of intrahepatic $CD4^+CD25^+FoxP3^+$ Treg increased in 10 out of 22 patients relative to $CD4^+$ T cells and in 12 out of 22 patients relative to $CD8^+$ T cells. Augmented Treg proportions may be the result of either an increase in the number of Treg as well as a reduction of the number of effector cells. At the group-level, these ratios were significantly enhanced with a mean increase of 1.3 Treg per 100 $CD4^+$ T cells ($p=0.0018$) and 4.3 Treg per 100 $CD8^+$ T cells ($p=0.0086$). In some, but not all, cases enhancement of $CD4^+CD25^+FoxP3^+$ Treg numbers in the liver by IFN-α-based therapy were also observed in peripheral blood (data not shown). However, these changes were not significant at the group-level for Treg relative to $CD4^+$ and $CD8^+$ T cells ($p=0.39$ and $p=0.91$, respectively). Therefore, antiviral therapy appears to modulate the capacity to regulate HCV-specific immune responses by $CD4^+CD25^+FoxP3^+$ Treg to a greater extent locally, in HCV-infected livers, than systemically.

We confirmed our previous findings that liver $CD4^+CD25^+FoxP3^+$ Treg from chronic HCV patients generally express an effector or central-memory phenotype prior to treatment (Figure 3B; mixed $CD45RO^+CD62L^-$ and $CD45RO^+CD62L^+$, and Supplementary Figure 1) [4]. Following exposure to IFN-α and ribavirin no significant changes of the memory phenotype were observed for intrahepatic $CD4^+CD25^+FoxP3^+$ Treg ($p>0.05$). Similarly, comparison of the phenotype of conventional $CD4^+CD25^-FoxP3^-$ T cells in the liver did not demonstrate any effects of therapy (data not shown). These findings suggest that the relative increase in intrahepatic $CD4^+CD25^+FoxP3^+$ Treg in chronic HCV patients as a consequence of antiviral therapy is unlikely to be the result of *de novo* generation of Treg.

$CD4^+CD25^+FoxP3^+$ Treg remain present in the liver of subjects after therapy-induced clearance of HCV

Next, we determined the modulation of intrahepatic Treg frequency after ending antiviral therapy. Out of 20 patients with a complete follow-up, thirteen patients responded successfully to antiviral therapy and remained negative for serum HCV RNA after termination of therapy (Figure 4A). All these individuals were negative early during the 24 to 48 week treatment period, making that HCV RNA was absent or undetectable in serum for at least 6 months. Despite the absence of virus, enhanced intrahepatic $CD4^+CD25^+FoxP3^+$ Treg frequencies were observed in the majority of these individuals, as compared to the Treg frequencies prior to treatment. Four weeks after cessation of treatment, intrahepatic Treg were increased relative to total liver lymphocytes, $CD4^+$ and $CD8^+$ T cells in all but one patient (Figure 4B). Additional sampling of liver cells 24 weeks after successful antiviral

therapy in 3 patients demonstrated that intrahepatic $CD4^+CD25^+FoxP3^+$ Treg remained present (Treg frequency to CD4: 6.9, 11.2 and 4.4), albeit that in some patients their levels were lower as compared to 4 weeks after treatment, but – more importantly – substantially higher than in healthy livers which were almost without Treg.

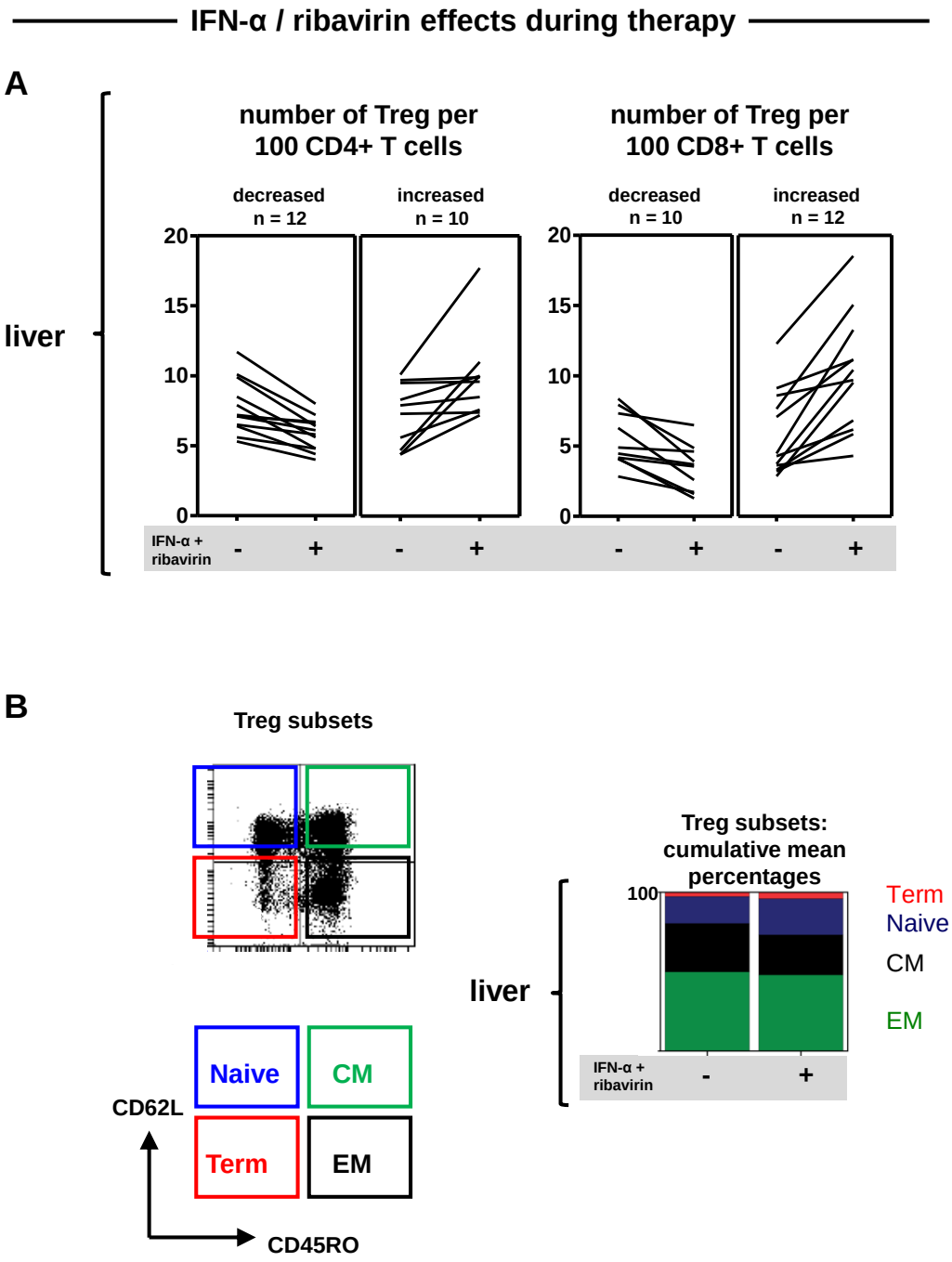

Figure 3. IFN-α and ribavirin therapy rapidly increases the frequency of Treg in the livers of many HCV-infected patients. (A) Liver $CD4^+CD25^+FoxP3^+$ Treg frequencies relative to $CD4^+$ and $CD8^+$ T cells are shown at baseline and 4 weeks after starting antiviral therapy (n=22). The findings are grouped as 'decreased' or 'increased' on the basis of the difference between the Treg frequencies before and during therapy, irrespective of the magnitude of the shifts of liver Treg relative to $CD4^+$ or $CD8^+$ T cells. (B) Treg subsets from 22 chronic HCV patients are phenotyped by flowcytometry and classified as naïve (marked in blue), central-memory (CM; marked in green), effector-memory (EM; marked in black), or terminally differentiated (Term; marked in red) based on CD45RO and CD62L expression. All changes in the frequency of specific memory populations determined prior to and during therapy were not significant.

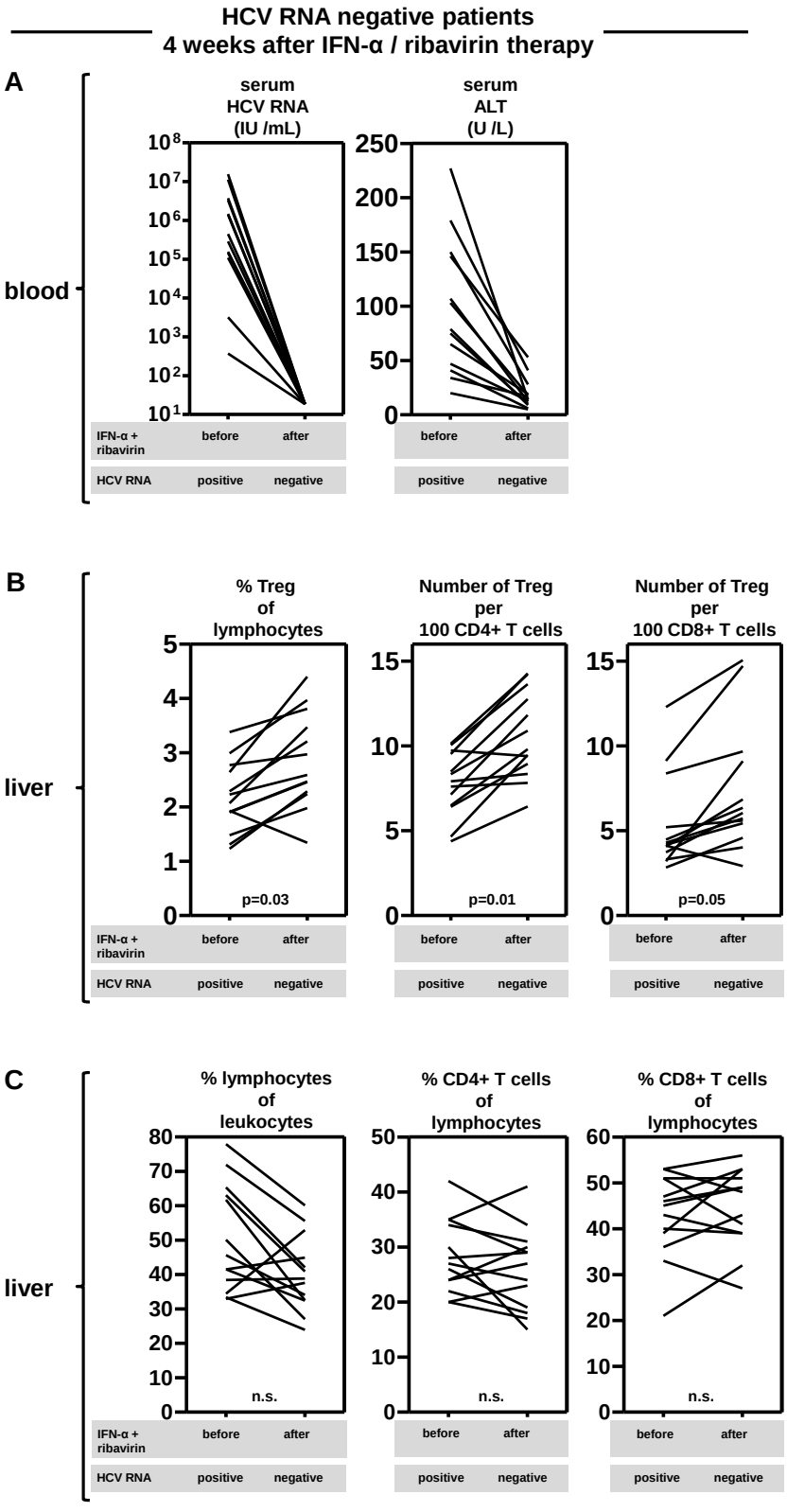

Figure 4. Therapy-induced clearance of HCV RNA does not lead to reduced Treg frequencies in the liver. (A) Serum HCV RNA and ALT levels frequencies are displayed prior to, and 4 weeks after termination of IFN-α and ribavirin treatment. Only patients being HCV RNA negative after therapy (n=13) are depicted. (B) Liver $CD4^+CD25^+FoxP3^+$ Treg frequencies relative to lymphocytes, $CD4^+$ and $CD8^+$ T cells are shown before, and 4 weeks after stopping IFN-α-based therapy. (C) Frequencies of liver lymphocytes relative to leukocytes, and $CD4^+$ and $CD8^+$ T cells relative to lymphocytes are shown before and 4 weeks after ending of IFN-α-based therapy.

In the other 7 patients, treatment failed and chronic infection with HCV persisted. Also in these patients, intrahepatic $CD4^+CD25^+FoxP3^+$ Treg frequencies were increased after cessation of therapy as compared to baseline (data not shown). Changes in the frequency of lymphocytes in responders and non-responders to therapy, both in liver and blood (respectively Figure 4C and data not shown) did not show a clear pattern, and were not related to treatment outcome. We did however find that pretreatment intrahepatic $CD8^+$ T cell frequencies as a proportion of lymphocytes were higher in patients with therapy-induced clearance of HCV than in therapy non-responders (43 vs. 34 %, p=0.03). This is in line with previous reports suggesting that intrahepatic $CD8^+$ T cells may be important for viral clearance [12, 14-15]. This difference in $CD8^+$ T cells between responders and non-responders was not affected by IFN-α and ribavirin, and remained unchanged 4 weeks after therapy (45 vs. 35 %, p=0.03).

During the course of treatment, the differentiation status of intrahepatic Treg was not affected at the group level (Figure 3B). However, as shown in Figure 5, we evaluated the differentiation status of Treg after stopping IFN-α and ribavirin administration in 11 out of 13 individuals who responded to therapy and became negative for HCV RNA. From these 11 patients, 7 showed a decline of the percentage of intrahepatic $CD45RO^+CD62L^-$ effector-memory Treg when comparing the differentiation status at baseline and at 4 weeks after the end of treatment. In sum, these findings suggest that irrespective of the outcome of therapy, intrahepatic Treg are still present in the liver of individuals even after prolonged negativity in serum for HCV RNA. However, although their regulatory function could not be assessed directly because of the limitation in obtaining sufficient material, differences in effector-memory phenotype suggest that liver $CD4^+CD25^+FoxP3^+$ Treg in responders after therapy are weaker than prior to therapy when there is still virus present in the liver.

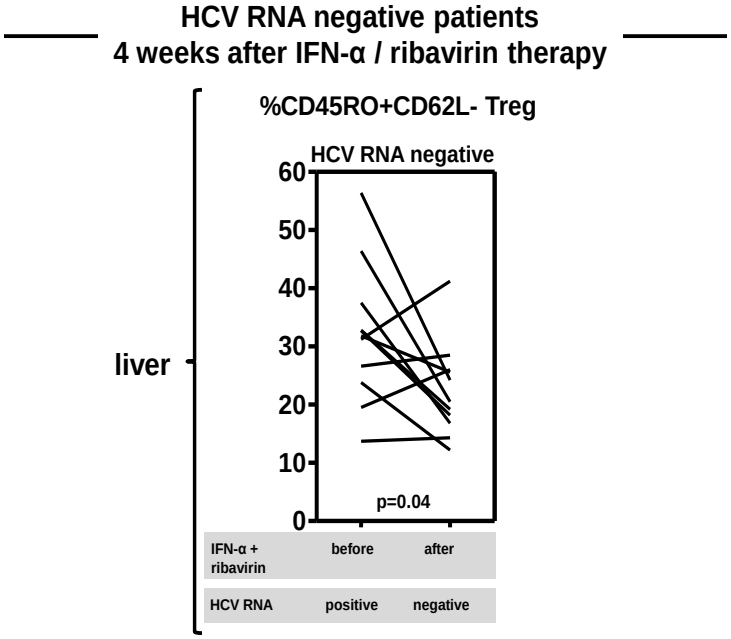

Figure 5. Effector $CD45RO^+CD62L^-$ phenotype of liver Treg is decreased in patients after IFN-α and ribavirin induced viral clearance. The proportion of intrahepatic Treg expressing a $CD45RO^+CD62L^-$ effector phenotype are shown for 11 patients before and 4 weeks after termination of antiviral therapy for patients being HCV RNA negative after therapy.

DISCUSSION

The local immune response in the liver is important for the outcome of HCV infection and the persistence of the virus in the liver, since replication takes place in hepatocytes. However, the dynamics of the immune response in the liver of chronic HCV patients is largely unknown. Repeated aspiration biopsies during the course of IFN-α-based therapy enabled us to study this for the first time. We demonstrated that the consequence of antiviral therapy is an increased contribution of $CD4^+CD25^+FoxP3^+$ Treg in the inflamed liver without alterations in the distribution of intrahepatic $CD4^+$ and $CD8^+$ T cells. In addition, we showed that the livers of individuals who became negative for HCV RNA as a result of treatment were still inflamed, and in fact Treg remained increased after cessation of therapy in the large majority of patients. However, these subjects showed a smaller contribution of intrahepatic $CD45RO^+CD62L^-$ effector-memory Treg, whereas in patients with persistent infections, the phenotype of Treg was more skewed towards effector-memory cells, suggesting important differences in the magnitude of suppressive abilities of intrahepatic $CD4^+CD25^+FoxP3^+$ Treg.

Longitudinal assessment of intrahepatic immunity by core liver biopsies is generally considered unethical for research purposes due to the risk of complications and patient discomfort. In addition, experimental animal models to examine the intrahepatic immune system during chronic HCV infections are not readily available [19]. However, using fine-needle aspiration biopsies we were able to perform such a study without these constraints. To our knowledge, in only one study a liver biopsy was collected before and 4 hours after start of IFN-based treatment in the same patient to examine the early effects of therapy on intrahepatic miR-122 expression, while effects on intrahepatic immunity during therapy were not investigated [20].

The livers of patients with chronic HCV infections show extensive leukocyte infiltration with substantial numbers of $FoxP3^+$ Treg around the portal tract areas [4, 6]. We found that in response to exposure to IFN-α and ribavirin, $CD4^+CD25^+FoxP3^+$ Treg frequencies increased in the livers of chronic HCV-infected patients. It cannot be completely ruled out that FoxP3 is transiently upregulated on a small subset of intrahepatic $CD4^+$ T cells upon activation as demonstrated by *in vitro* studies (reviewed in [21]). However, to our knowledge there is no information that this occurs *in vivo* on human T cells. Importantly, similar to Burton et al., we also found that Treg frequencies measured in blood remained unchanged during IFN-α-based therapy [10]. These findings suggest that the intrahepatic, but not blood, $CD4^+CD25^+FoxP3^+$ Treg compartment may play a more important role in regulating a curative immune response to HCV during IFN-α-based therapy. Evaluation of intrahepatic cells obtained from aspirate biopsies does not allow us to draw conclusions on the change in absolute numbers of these Treg in time. However, in experimental autoimmune encephalomyelitis in mice, it has been shown that the ratio of effector T cells to Treg is of crucial importance in controlling disease severity caused by immunopathology, and not the absolute numbers [22]. Similarly in our experimental setup, it is likely that a high ratio of intrahepatic $CD4^+CD25^+FoxP3^+$ Treg to effector T cells, results in strong negative regulation of HCV-specific immune responses. In line with this, 4 of our patients with the highest Treg to $CD8^+$ T cell ratios all failed to clear the HCV infection (data not shown), and it is tempting to speculate that the delicate balance between intrahepatic $CD4^+CD25^+FoxP3^+$ Treg and

effector T cells explains the response to IFN-α-based treatment in a subset of chronic HCV patients.

We showed that therapy-induced HCV eradication did not lead to normalization of the intrahepatic immune system after therapy. Intrahepatic $CD4^+CD25^+FoxP3^+$ Treg remained increased after cessation of therapy in the large majority of patients, including subjects that showed a therapy-induced viral clearance, and who were became negative for HCV RNA early during the course of treatment. Importantly, in a follow-up evaluation of 3 patients who successfully cleared the virus and who had undetectable virus levels 24 weeks after ending antiviral therapy, the intrahepatic $CD4^+CD25^+FoxP3^+$ Treg still remained increased. These findings were unexpected, since we reported before that Treg were virtually absent from healthy livers not previously exposed to HCV antigens [4], and suggests that inflammation and regulation by intrahepatic $CD4^+CD25^+FoxP3^+$ Treg is ongoing in patients who successfully clear the virus and in whom HCV antigens are not longer detectable. However, we cannot rule out that these Treg will resolve later in time. Recently, it was reported that in plasma samples from 15% of SVR patients trace amounts of HCV RNA were detected years after successful therapy [23]. Importantly, the reappearance of HCV RNA was found to induce HCV-specific T cell responses. Therefore, it may be speculated that the intrahepatic Treg that remain present the first 6 months after successful IFN-α therapy control residual HCV replication in the liver. Furthermore, also other processes than HCV-specific immunity may be controlled, such as bystander T cell activation or ongoing fibrogenesis. The latter is in line with our previous findings suggesting a role for Treg in limiting the extent of immunopathology in patients still infected with HCV [4]. In addition, other inflammatory processes in the liver, for example alcoholic or non-alcoholic steatohepatitis [24], may be modulated by Treg present in the liver after clearance of HCV as well. Despite our finding that Treg frequencies did not differ between non-viremic patients and patients with a non-response to therapy, the continued regulation by Treg in non-viremic patients may be less potent, since their lower proportion of $CD45RO^+CD62L^-$ Treg hints towards a less immediate effector activity. Further characterization of Treg in liver aspiration biopsies in only two patients learnt that the expression of the activation marker HLA-DR was not changed during and after therapy, and we observed that the high level of HLA-DR expression on intrahepatic Treg was maintained (data not shown).

In conclusion, we show for the first time that intrahepatic $CD4^+CD25^+FoxP3^+$ Treg frequencies increase during IFN-α-based therapy, and that intrahepatic Treg ratios remain increased even 6 months after successful clearance of HCV RNA from serum. These findings indicate that immunoregulation by $CD4^+CD25^+FoxP3^+$ Treg is not only important during chronic HCV infection and therapy, but also pivotal in controlling liver immunity in previously HCV-infected patients.

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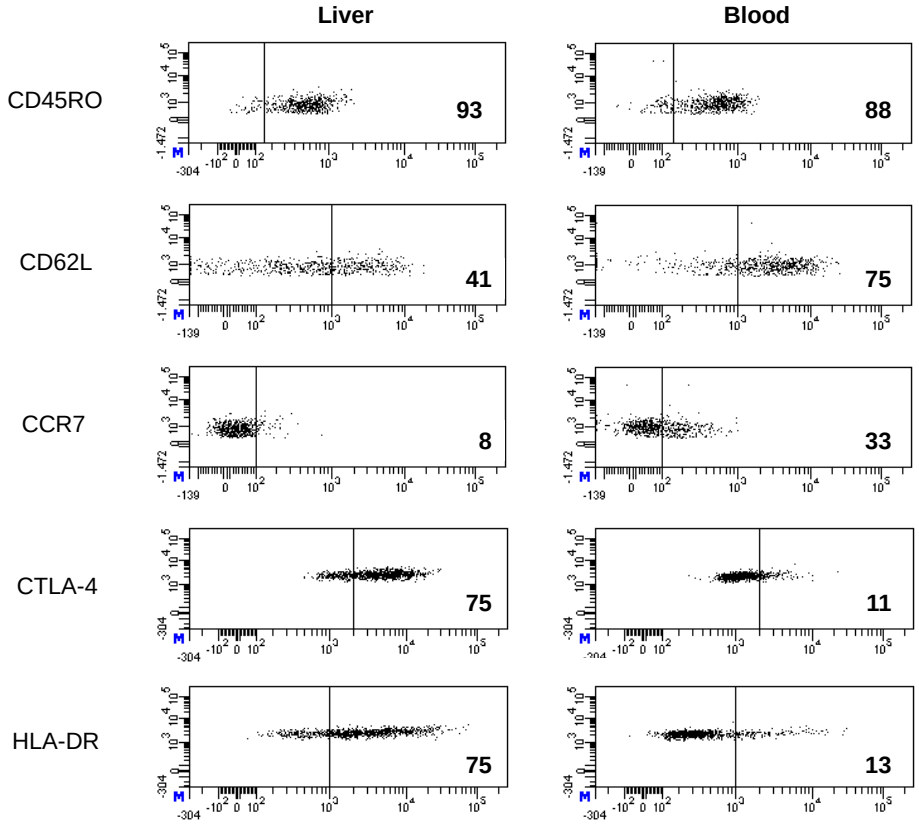
SUPPLEMENTARY FIGURE



Supplementary Figure S1. Assessment of the phenotype of intrahepatic and peripheral blood FoxP3$^+$ Treg. (A) Using flowcytometry, CD4$^+$CD25$^+$FoxP3 Treg were identified as depicted in Figure 1 and further characterized by various differentiation markers (CD45RO, CD62L, CCR7) and activation markers (CTLA-4, HLA-DR). Representative dot plots are shown.

Part II:

Importance of hepatitis C virus-specific T cell immunity and inhibition by regulatory mechanisms during interferon-α-based therapy

Chapter 5

T cell responses at baseline and during therapy with peginterferon-α and ribavirin are not associated with outcome in chronic hepatitis C infected patients

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ABSTRACT

Background

Since the association between Hepatitis C virus (HCV)-specific T cell responses both pre-treatment and during interferon-α based therapy and viral clearance is unresolved, a combined analysis of distinctive T cell characteristics (proliferation and IFN-γ production) is important to clarify this issue.

Methods

Peripheral blood mononuclear cells (PBMC) collected in 22 chronic HCV-infected patients at pretreatment and at week 4 during pegIFN-α/ribavirin therapy, were stimulated with overlapping peptide pools in a [^{3}H]-thymidine assay, an IFN-γ ELISA, and a sensitive 12-day T cell expansion assay.

Results

Compared to the [^{3}H]-thymidine proliferation and interferon-gamma secretion assays, the 12-day T cell expansion assay was more sensitive in detecting T cell responses. No significant association was demonstrated between pre-treatment HCV-specific $CD4^+$ or $CD8^+$ T cell responses and either a sustained virological response (SVR) or a rapid virological response (RVR). However, a skewing of individual responses towards the non-structural antigens was observed. During pegIFN-α and ribavirin therapy, HCV-specific $CD4^+$ and $CD8^+$ T cells declined similarly in both SVR/ RVR and non-SVR/ non-RVR patients.

Conclusion

No correlation was found between the magnitude of pre-treatment HCV-specific T cell responses and the outcome of pegIFN-α/ribavirin therapy in terms of SVR and RVR. Moreover, the magnitude of HCV-specific T cell responses declined in all patients early during treatment.

INTRODUCTION

Pegylated interferon alfa-2a or 2b (pegIFN-α) in combination with weight based ribavirin is currently the standard treatment for chronic hepatitis C virus (HCV) infected patients [1]. A sustained virological response (SVR), defined as an undetectable plasma HCV RNA 24 weeks after cessation of therapy, is achieved in around 50% of patients infected with genotypes 1 and 4 [2]. Pre-treatment characteristics like baseline viral load [3], liver cirrhosis [4], coinfection with the human immunodeficiency virus (HIV) [5] and recently the interleukin-28B cc-genotype [6] are associated with outcome of therapy. Furthermore, achievement of a rapid virolgical response (RVR, i.e. plasma HCV RNA with undetectable i.e. <50 IU/ml at week 4 of therapy) is regarded as a strong on-treatment predictor for SVR [7-8].

It has been suggested that cellular immune responses, modulated by pegIFN-α and ribavirin, play a role in forced viral eradication, based on the immunological properties attributed to these anti-viral compounds [9-12]. However, the role of HCV-specific T cells before and during pegIFN-α/ribavirin therapy is still controversial [13-14]. Some studies have shown that achievement of a SVR is associated with high baseline $CD4^+$ and/ or $CD8^+$ specific T cell responses [15-18], while others have seen no such relationship [19-23]. Similarly, contradictory results have been reported on the role of HCV-specific T cells during pegIFN-α and ribavirin therapy showing either augmentation [10, 21, 24] or decline [15, 17, 19] of HCV-specific T cells in relation to SVR. In patients achieving a RVR, higher percentages of baseline IFN-γ producing $CD8^+$ T cells have been demonstrated compared to non-RVR patients [17].

There is no conclusive evidence whether HCV-specific immunity contributes to therapy-induced viral clearance for several reasons. Good animal models are unavailable [25], frequencies of circulating HCV-specific T cells are very low [26] and therefore hard to detect, and a consensus on the optimal ex vivo experimental cell culture protocols is lacking [27]. A robust and sensitive assay able to detect low frequencies of HCV-specific T cell responses is needed to resolve the above mentioned controversies.

For this purpose we examined the magnitude and breadth of HCV-specific T cell responses at baseline and during therapy in patients with a HCV mono-infection using a sensitive 12-day expansion assay as has been previously reported by our group [28-30]. This assay measures both the IFN-γ production and the proliferative capacity of HCV-specific $CD4^+$ and $CD8^+$ T cells simultaneously, allowing a more comprehensive analysis of the HCV-specific T cell response. Using this assay, we found no relation between the magnitude of pre-treatment HCV-specific $CD4^+$ or $CD8^+$ T cell responses and achievement of either a SVR or a RVR, albeit that skewing of these responses was demonstrated against HCV non-structural antigens. In addition, irrespective of treatment outcome, HCV-specific T cell responses declined in all patients early during treatment.

METHODS

Patients

In this multi-centre cohort study, 22 patients diagnosed with chronic HCV mono-infection were consecutively enrolled [31], and prospectively sampled during standard treatment with either peginterferon alfa-2a (40 KD) (Pegasys® 180 µg/week; Roche, Basel, Switzerland) or peginterferon alfa-2b (12KD) (PegIntron® 1.5 µg/kg/week; Schering-Plough, Kenilworth, USA) in combination with weight based ribavirin (Copegus® or Rebetol®). Only patients with HCV genotypes 1 or 4 were included while patients with a coinfection with either hepatitis B or HIV were excluded. During treatment plasma HCV RNA was measured using the qualitative Roche Amplicor® polymerase chain reaction (PCR) assay with a lower limit of detection of 50 International Units /milliliter (IU/ml). A rapid viral response (RVR) is defined as a qualitative undetectable HCV RNA at week 4 of treatment (<50 IU/ml) whereas an early viral response (EVR) is defined as achieving either a $\geq 2\log_{10}$ viral load decrease from baseline or a qualitative undetectable (<50 IU/ml) HCV RNA at week 12 after initiation of treatment. A sustained virolological response (SVR) was defined as a negative HCV viral load (<50 IU/ml) 24 weeks after discontinuation of therapy. All patients provided written informed consent and institutional ethical review boards at participating centers approved the protocol.

PBMC processing

Peripheral blood (approximately 25 ml) was collected before and at week 4 during therapy. Within 24 hours, peripheral blood mononuclear cells (PBMC) were isolated using a Ficoll-Hypaque density gradient centrifugation. Cells were re-suspended in RPMI 1640 (Gibco Life Technologies, Breda, the Netherlands) to which 20% fetal calf serum and 1% penicillin and streptomycin were added (hereafter called medium). After adding 10% DMSO, PBMC were frozen to -80° C and stored thereafter at -180° C until further use. Except for the proliferation assay, all experiments were performed using frozen PBMC.

T cell assays

In vitro quantification of proliferation and cytokine production

Freshly isolated PBMC at a final concentration of $1x10^6$ cells/ml were cultured in quadruplets in 96-well round bottom plates in 200 µl culture medium in the presence of overlapping peptide pools (spanning the core, NS3, NS4 and NS5a and NS5b HCV genome; clone J4 genotype 1b; BEI Resources, Manassas, USA), anti-CD3 antibody (1 µg/mL; OKT-3; orthoclone), cytomegalovirus lysate or no stimulus. All cultures were performed in the presence of anti-CD28 (1 µg/ml; CD28.2; eBioscience) and anti-CD49d antibody (1 µg/ml; 9F10; eBioscience). After culturing for 3 days, 100 µl supernatant was harvested and replaced by 100 µl fresh culture medium. IFN-γ production was determined by a commercially available ELISA kit (eBioscience, San Diego, USA). After stimulation for 5 days, the cells were pulsed with 0.5 µCi/well of [3H]-thymidine (Radiochemical Centre, Amersham, Little Chalfont, UK), and harvested 16 hours later. Proliferation was determined as counts per minute (cpm) via liquid scintillation counting of the harvested cells. The cut-off value for a positive response was set as the median plus 2x the standard deviation (SD) of the medium.

HCV-specific $CD4^+$ and $CD8^+$ T cell expansion assay

To stimulate both $CD4^+$ and $CD8^+$ T cells, overlapping peptide pools (www.mimotopes.com) with a length of 18 amino acids (aa) and an overlap of 11 aa, spanning the whole HCV genome, were used. The peptides were pooled as follows: core (core/E1/E2/p7, aa 1-805 with a total of 116 peptides), non-structural protein 2 (NS2)(aa 806-1022, with a total of 31 peptides), NS3 (aa 1023-1645, with a total of 90 peptides), NS4 (aa 1646-1967, with a total of 49 peptides), NS5A (aa 1968-2415, with a total of 67 peptides) and NS5B (aa 2416-3011, with a total of 87 peptides). Peptide pools were dissolved in DMSO.

The 12-day expansion assay measured proliferative capacity and IFN-γ production as previously published [30, 32]. Both pre-treatment and week 4 time points were assessed concurrently. PBMC were stimulated at $1x10^6$/ml with 2 µg/ml peptide pool (the DMSO concentration was never more than 1% in the final stimulation). Thereafter, cells were incubated in 96-well round bottom plates at $2x10^5$ cells/well at 37 ºC and 5% CO_2 and Interleukin-2 (IL-2) (20 U/ml) was added at days 3, 6 and 9. HCV peptides at a concentration of 2 µg/ml were

added again on day 6. Cells were pooled, washed and counted on day 12, rested overnight in medium after which re-stimulation with HCV peptide pools for 6 hours in the presence of anti-CD28 (1 ug/ml) and anti-CD49d (1 µg/ml) as co-stimuli was performed. Concurrently, medium alone and PMA (10 ng/ml) with ionomycin (50 ng/ml) were used a negative and positive control respectively. After 1 hour, Brefeldin A (Golgiplug, BD Biosciences, San José, CA, USA) was added, followed by a further incubation period of 5 hours. Thereafter, cells were washed, permeabilized (FACS Permeabilizing Solution, BD) and stained with specific antibodies against CD3 (Pacific Blue, eBioscience), CD4 (PE Cy7, eBioscience), CD8 (APC AlexaFluor750, eBioscience) and IFN-γ (FITC, BD) for 20 minutes at 4 °C. After washing, cells were fixed with cellfix (BD) and at least 100 000 cells were acquired by flow cytometry (FACS, LRSII, BD). Using the forward and sideward scatter, the lymphocyte population was gated and analyzed by FACSDiva software (BD). The HCV-specific T cell response (i.e. IFN-γ production) was calculated by subtracting the IFN-γ production in the unstimulated control and subsequently multiplied with the proliferation ratio (fold increase) of the expansion assay, resulting in a number of HCV-specific T cells per 106 PBMC, which is a combination of the (memory) T cells initially present that survived, proliferated and differentiated into effector T cell phenotype in each individual. A positive HCV-specific T cell response was defined as a response of more than 200 out of 1 million PBMC in since T cell responses in healthy controls after culture with HCV peptide pools never exceeded 200 per million PBMC. Therefore, this was taken as a 'cut-off' for a positive HCV-response.

All HCV-specific T cell responses per patient (core, NS2, NS3, NS4 and NS5) were summed and displayed as the total HCV-specific T cell response. For the analysis, patients were divided into SVR/ non-SVR or RVR/ non-RVR.

Surface marker staining

Direct ex vivo surface marker expression for T cell subsets (CD27/CD45RO) and T cell activation (CD38/HLA-DR) were analyzed pre-treatment and at week 4. After thawing and washing, cells were incubated for 20 minutes at 4 °C with a combination of antibodies against CD3 (PerCP, BD), CD4 (PE Cy7, eBioscience), CD8 (APC Alexa Fluor 750, eBioscience), CD38 (PE Cy7, eBioscience), HLA-DR (FITC, eBioscience), CD27 (FITC, BD) and CD45RO (APC, BD). After washing, cells were fixed with Cellfix and at least 200 000 cells were acquired by FACS (LRSII, BD). Using the forward and sideward scatter the lymphocyte population was gated and analyzed by FACSDiva software (BD).

Statistical analysis

Continuous data are presented as median values (with interquartile range (IQR)) analyzed using a Mann-Witney U-test whereas categorical variables are given as number of cases (percentage) analyzed using a Wilcoxon signed-rank test. Comparison of categorical variables was done using a Fisher's exact test. Spearmann's Rank correlation and linear regression analysis were performed to examine the relationship between continuous variables and immunological parameters. A p-value ≤0.05 was considered as statistically significant and all tests used were two-sided. All data were analyzed using GraphPad Prism (version 5.0 for Windows, GraphPad Software, San Diego, USA).

RESULTS

Of the 22 chronic HCV genotype 1 and 4 infected patients treated with pegIFN-α/ribavirin, 11 patients reached a SVR (50%). Patient characteristics, shown in table 1, are similar when grouped into SVR and non-SVR. Factors possibly influencing the HCV-specific T cell response like age, gender and extent of liver injury were not of significant influence on the outcome of treatment. In patients reaching a SVR, 64% also achieved a RVR and all reached an EVR. In contrast, in the non-SVR group only 18% achieved a RVR (p=0.08) and 55% reached an EVR (p=0.04). Furthermore, the baseline values of activated $CD4^+$ and $CD8^+$ T cells (CD38/ HLA-DR double positive) as well as the percentages of T cell subsets

(defined by CD27 and CD45RO) were not different between SVR patients and non-SVR patients (table 1).

Table 1: Characteristics of patients grouped by clinical outcome

Feature	SVR (n=11)	non-SVR (n=11)	p-value
General characteristics			
male / female	10 / 1	9 / 2	**ns**
Age (years)	48 (40 – 52)	47 (46-55)	**ns**
Weight (Kg)	78 (63 - 86)	78 (62 - 95)	**ns**
Caucasian ethnicity	11 (100)	11 (100)	**ns**
HCV/ liver related characteristics			
Genotype			
1	8 (73)	9 (82)	**ns**
4	3 (27)	2 (18)	
HCV RNA (Log10 IU/ml)	5.82 (5.55 – 6.57)	6.62 (5.50 – 6.68)	**ns**
ALT (IU/ml)	86 (30 – 135)	94 (48 – 120)	**ns**
Liver biopsy			
≤ F2	6 (55)	3 (27)	**ns**
F3-F4	1 (9)	3 (27)	
NP	4 (36)	5 (45)	
Immunological characteristics			
$CD38^+/HLA\text{-}DR^+$			
CD4	0.30 (0.20-0.50)	0.60 (0.20-0.80)	**ns**
CD8	0.40 (0.30-0.70)	0.50 (0.20-1.30)	**ns**
CD8 subsets			
naive ($CD27^+CD45RO$-)	32.2 (20.3-51.8)	39.4 (20.0-42.9)	**ns**
effector ($CD27^-CD45RO^+$)	37.6 (20.4-58.7)	22.3 (17.3-51.9)	**ns**
memory ($CD27^+CD45RO^+$)	27.8 (8.0-39.4)	36.7 (15.8-44.0)	**ns**
Treatment outcome characteristics			
RVR	7 (64)	2 (18)	**0.08**
EVR	11 (100)	6 (55)	**0.04**

Continuous variables are shown as median values (interquartile range) while categorical variables are given as numbers (percentages). ns = not significant; NP = not performed.

Higher HCV-specific T cell responses were detectable using the T cell expansion assay compared to proliferation and cytokine assay

Since direct ex vivo analyses has shown that very low numbers of HCV-specific T cells are present [26], stimulation assays have been used to circumvent this problem [17, 19, 33]. In this study we compared the 12-day expansion assay with the [^{3}H]-thymidine proliferation assay and the IFN-γ ELISA as a more sensitive way to measure HCV-specific T cells. In a randomly selected subgroup of patients the majority (6 out of 8 patients) failed to show HCV-specific T cell proliferation in the [^{3}H]-thymidine assay, although one of the remaining 2 patients displayed a relatively strong response (4304 cpm; figure 1a). Likewise, the IFN-γ ELISA showed low IFN-γ levels in supernatant after HCV peptide stimulation around the cut-off value (35 pg/ml) in 5 out of 8 patients (figure 1b) with a stimulation index (SI) of ≤3. In the remaining 3 patients moderate to good IFN-γ production was detectable with values ranging between 159 and 3773 pg/ml (SI between 8 and 188). Finally, the 12-day expansion

assay showed low background values and T cell responses were detectable in all evaluable patients well above the cut-off value (SI ≥12; figure 1c). The 12-day expansion assay therefore allows for a sensitive detection of both HCV-specific $CD4^+$ and $CD8^+$ T cell responses since it combines both production of IFN-γ and proliferation of (memory) T cells into becoming effector T cells.

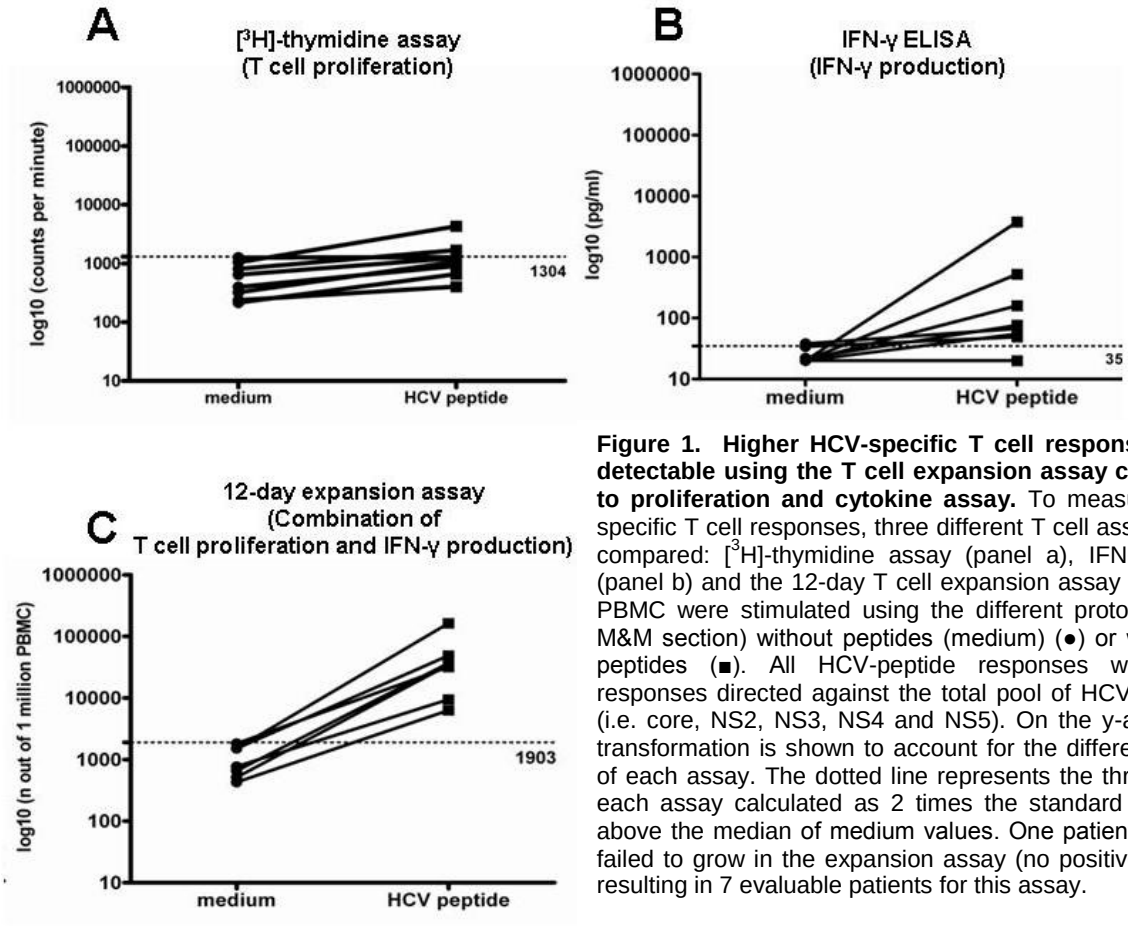

Figure 1. Higher HCV-specific T cell responses were detectable using the T cell expansion assay compared to proliferation and cytokine assay. To measure HCV-specific T cell responses, three different T cell assays were compared: [^{3}H]-thymidine assay (panel a), IFN-Γ ELISA (panel b) and the 12-day T cell expansion assay (panel c). PBMC were stimulated using the different protocols (see M&M section) without peptides (medium) (●) or with HCV peptides (■). All HCV-peptide responses were total responses directed against the total pool of HCV-peptides (i.e. core, NS2, NS3, NS4 and NS5). On the y-axis a log transformation is shown to account for the different scales of each assay. The dotted line represents the threshold of each assay calculated as 2 times the standard deviation above the median of medium values. One patient's PBMC failed to grow in the expansion assay (no positive control) resulting in 7 evaluable patients for this assay.

Pretreatment HCV-specific T cell responses are not associated with outcome of therapy

To address the question whether pre-treatment HCV-specific T cell responses were associated with therapeutic outcome, we measured HCV-specific $CD4^+$ and $CD8^+$ T cells before start of therapy using the 12-day expansion assay in patients achieving a SVR (n=11) and those without a SVR (n=11). The pre-treatment HCV-specific $CD4^+$ T cell response was equally strong in patients reaching a SVR (9 564 with IQR 6 281-13 335) compared to those without a SVR (6 870 with IQR 2 699-14 001; p=0.53; figure 2a). Similarly, no difference in HCV-specific $CD8^+$ T cell responses was noted between SVR and non-SVR patients (27 237 (IQR 10 643-48 021) and 11 491 (IQR 829-26 992); p=0.08). Likewise, when patients were grouped according to achievement of a RVR or non-RVR, HCV-specific T cell responses between the groups were not significantly different (figure 2b). Irrespective of treatment outcome, HCV-specific $CD8^+$ T cells responded better to stimulation with HCV antigens than HCV-specific $CD4^+$ T cells.

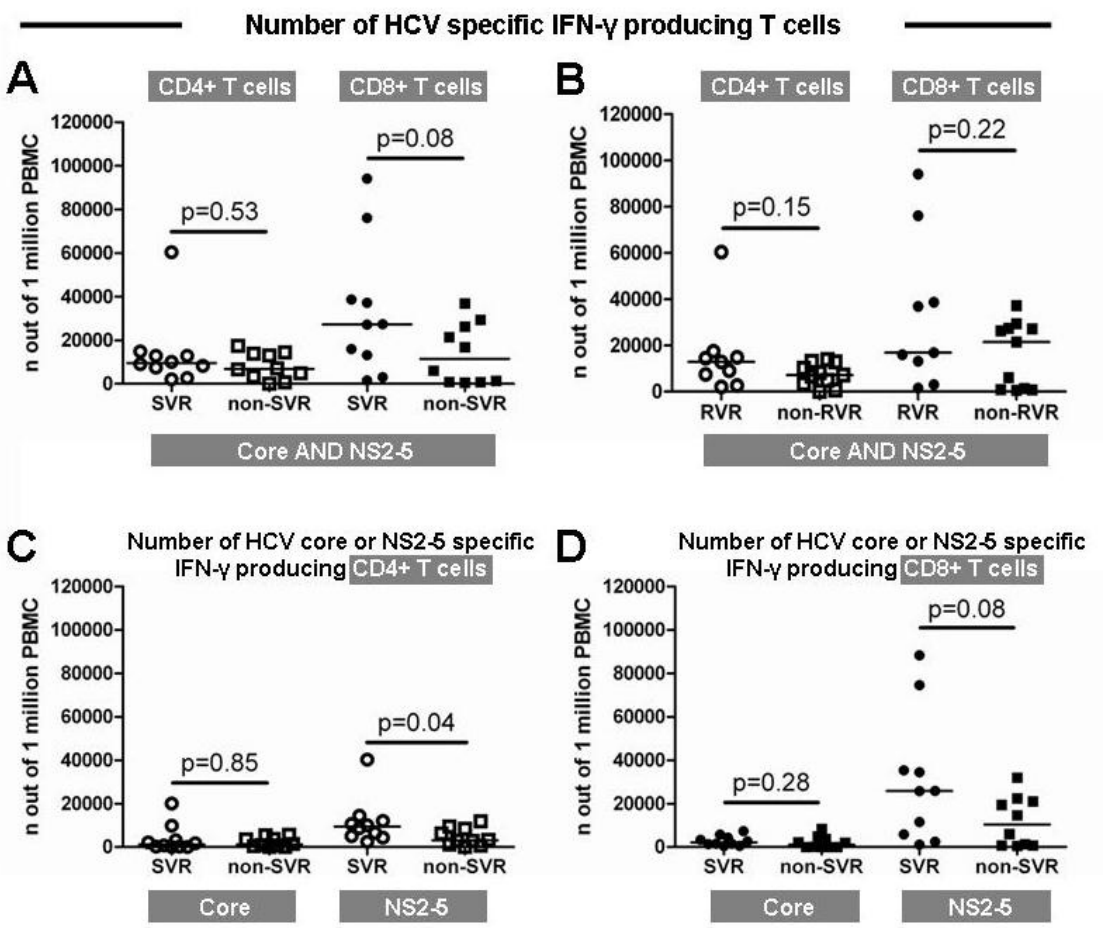

Figure 2. Pretreatment total and individual HCV-specific T cell responses in relation to outcome of therapy. Total pre-treatment HCV-specific $CD4^+$ (o/□) and $CD8^+$ (●/■) T cell responses (sum of core, NS2, NS3, NS4 and NS5A/B), measured with the 12 day T cell expansion assay, are shown in relation to achieving a SVR (panel a) or a RVR (panel b) with pegIFN-α/ribavirin therapy. Responses against individual HCV antigens (core or non-structural (ns, sum of NS2, NS3, NS4 and NS5A/B)) are shown for $CD4^+$ (panel c) and $CD8^+$ T cells (panel d). On the y-axis the number of IFN-γ producing T cells per million PBMC is shown. Of one patient in the SVR group too few pre-treatment PBMC were available for analysis while of 1 patient in the non-SVR group the PBMC failed to grow in the expansion assay (no positive control).

Subsequently, we assessed the focus of the T cell response directed against individual HCV antigens in relation to the outcome of therapy. In patients achieving a SVR, preferential targeting of the non-structural HCV peptides by $CD4^+$ T cells was demonstrated (p=0.04) when compared to those patients not achieving a SVR (figure 2c). NS4 and NS5 being most significant for HCV-specific $CD4^+$ T cells (p=0.009 and p=0.05 respectively).

Several studies have analyzed the interdependence of $CD4^+$ and $CD8^+$ T cell responses in the same individual either via depletion studies [26, 33] or direct ex vivo quantification [20] showing a correlation between both responses. In agreement with these findings, we also found a positive correlation between individual HCV-specific $CD4^+$ and $CD8^+$ T cell responses (r=0.47 with p=0.04; Spearman Rank correlation; data not shown). There was no correlation between the HCV-specific $CD4^+$ or $CD8^+$ T cell responses on the one hand and other baseline characteristics like ALAT and plasma HCV RNA on the other hand (data not shown).

HCV-specific T cell responses decline during pegIFN-α/ribavirin treatment regardless of outcome of therapy

Next, we investigated the role of HCV-specific $CD4^+$ and $CD8^+$ T cells in eradication of HCV during pegIFN-α/ribavirin therapy by quantifying the number of HCV-specific T cells at week 4 of therapy. This time point is used to define RVR which is an important virological parameter determining the chances of SVR [7-8]. Consequently, at this time point patients with detectable and those without detectable HCV RNA were present allowing us to investigate the relationship between antigen presence and HCV-specific T cell responses.

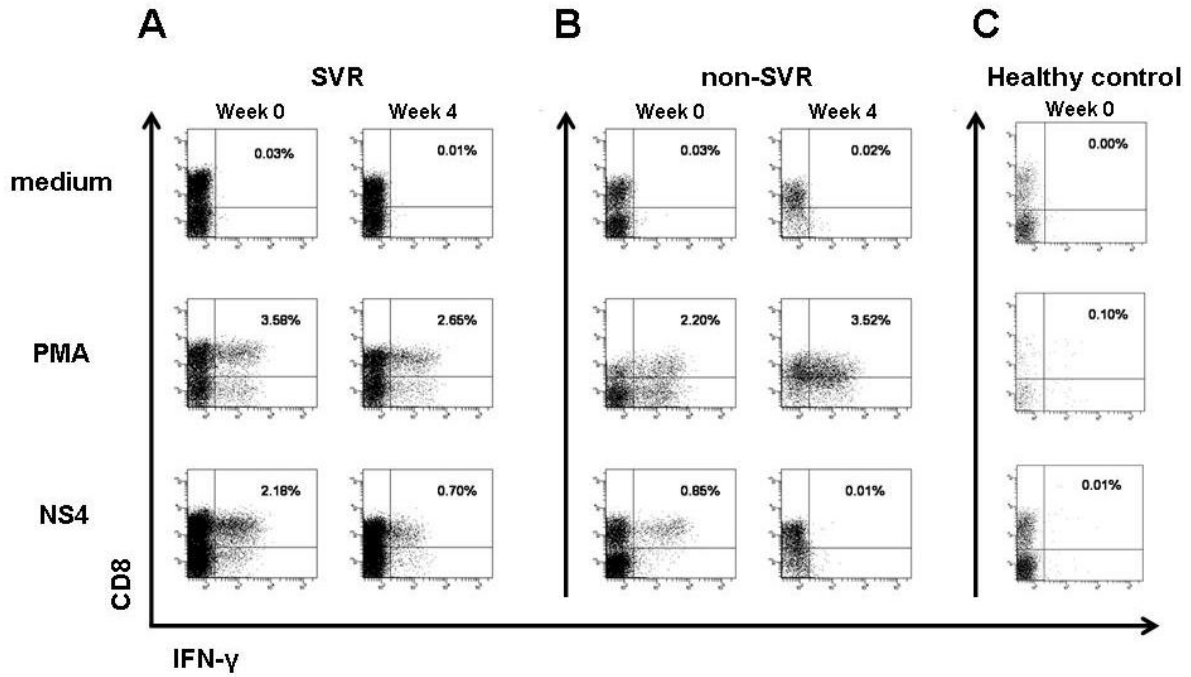

Figure 3. The effect of pegIFN-α/ribavirin therapy on the percentage of IFN-γ producing HCV-specific $CD4^+$ and $CD8^+$ T cell responses against HCV-NS4. Flow cytometry dot plot showing a longitudinal analysis of the percentage of IFN-γ producing HCV-specific $CD8^+$ T cell responses against HCV-NS4 pre-treatment and at week 4 in a representative patient achieving a SVR (panel a), patient without a SVR (panel b) and a healthy control (panel c). On the y-axis, CD8 APC-Cy7 and on the x-axis IFN-γ FITC is shown. The upper row displays the negative control (medium), the middle row is the positive control (PMA) and the bottom row shows the HCV-NS4 peptide.

To illustrate the HCV-specific T cell responses over time, flow cytometry dotplots of IFN-γ producing $CD8^+$ T cells against NS4 are shown (figure 3) for both a SVR and a non-SVR patient. The percentage of IFN-γ producing HCV-specific $CD8^+$ T cells declined during therapy in both the SVR-patient (from 2.18% at baseline to 0.70% at week 4) and the non-SVR patient (from 0.85% at baseline to 0.01% at week 4). To account for the difference in T cell proliferation, total HCV-specific T cell responses were calculated by combining these T cell proliferation ratios with the IFN-γ production. Regardless of the outcome of therapy, a decline in HCV-specific $CD4^+$ and $CD8^+$ T cell responses was observed from baseline to week 4 (figure 4a and 4b). This decrease was more prominent in the $CD8^+$ T cell responses in non-SVR patients (median decline from 11,491 (IQR 829-26 992) to 1 011 (IQR 351-5 904); p=0.02)). The patient who showed a marked increase in HCV-specific $CD8^+$ T cell responses from baseline (27 345) to week 4 (89 956 per million PBMC) had no distinctive

clinical, virological or immunological abnormalities that could explain this sharp increase (figure 4b). Similarly, when patients were grouped according to achievement of a RVR, a decline in HCV-specific $CD4^+$ and $CD8^+$ T cell responses was observed as seen in SVR/ non-SVR patients (data not shown).

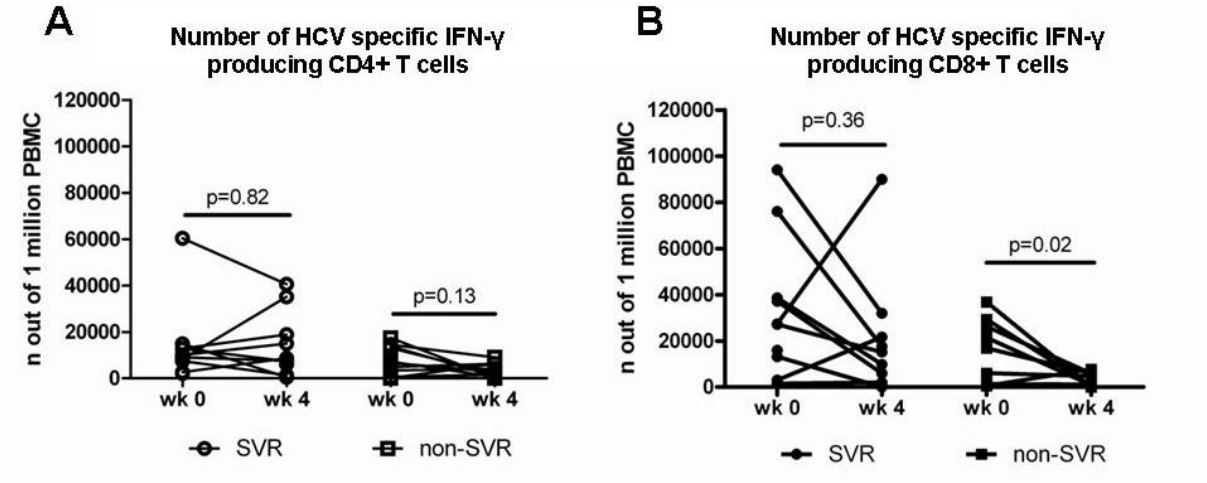

Figure 4. HCV-specific T cell responses decline during treatment regardless of achieving a SVR. Longitudinal total HCV-specific T cell responses, measured with the 12 day T cell expansion assay, are displayed pre-treatment and at week 4 of pegIFN-alfa/ ribavirin therapy for $CD4^+$ (panel a) and $CD8^+$ (panel b) T cells. The y-axis shows the number of IFN-γ producing T cells per million PBMC. On the x-axis the different time point are shown (pre-treatment and week 4) while patients are grouped into SVR (o/●) or non-SVR (□/■). Removing the SVR-patient with the high HCV-specific $CD8^+$ T cell response at week 4 of therapy from the analysis resulted in a trend towards a significant decrease in T cell responses at this time point (p=0.08). Of 2 patients (1 SVR and 1 non-SVR) the PBMC failed to grow in the expansion assay (no positive control).

Since at baseline skewing towards the NS peptides was associated with SVR, we evaluated the breadth of the response by the number of HCV NS-antigens targeted per patient at baseline and at week 4 of therapy. We observed that for both the $CD4^+$ and $CD8^+$ T cell response the median number of targeted NS-antigens remained relatively conserved from baseline to week 4 in both SVR patients (3 to 4 and 4 to 3, respectively) and non-SVR patients (3 to 2 and 3 to 2, respectively; data not shown).

DISCUSSION

This study evaluated the role of HCV-specific T cell responses in PBMC of chronic HCV genotype 1 and 4 patients before and during pegIFN-α/ribavirin therapy using a sensitive 12-day expansion assay. We are able to demonstrate that irrespective of clinical outcome, pre-treatment HCV-specific $CD4^+$ and $CD8^+$ T cell responses exist and that they markedly decline during pegIFN-α/ribavirin therapy in both SVR and non-SVR patients. Moreover, a similar pattern of HCV-specific T cell responses was observed in patients achieving a RVR when compared to those without a RVR. Finally, skewing of the T cell response against the NS-antigens was found to be associated with reaching a SVR.

T cell proliferation and IFN-γ production are important parameters of an effective anti-viral response [34] which was generally analyzed using separate assays [16, 18, 22, 35-36]. In recent

years several groups have used T cell expansion in different assays trying to combine both features [17, 19, 33]. The 12-day T cell expansion used in our study also combines both features of proliferation and IFN- γ production into one assay allowing simultaneous detection of $CD4^+$ and $CD8^+$ T cell responses. The assay, was first described by Reece et al. [37] as an ex vivo expansion assay in which IFN-γ secretion was assessed by ELISPOT. Our group has previously modified this 12-day expansion assay to allow the IFN-γ secretion to be measured by flow cytometry validating this method for Epstein-Barr virus infection [28, 38], HIV [39] and HCV infections [29-30]. It combines the proliferation (and/or survival) of precursor (memory) HCV-specific T cells after HCV-antigen stimulation and IFN-γ secretion upon specific re-stimulation for both $CD4^+$ and $CD8^+$ T cell responses simultaneously. Compared to proliferation and IFN-γ production assays, the 12-day expansion assay showed an improved detection of HCV-specific T cell responses. Although, this assay provides an estimate of the total HCV-specific T cell response in a given patient, irrespective of HLA-type, it does not allow dissecting the specific epitopes which are recognized and results cannot be confirmed using tetramer-staining.

Compared to previous studies showing either no or low pre-treatment HCV-specific T cell responses [10, 16, 21-22, 24, 40] or studies demonstrating strong and multi-specific T cell responses to HCV at baseline associated with or without achievement of SVR [15, 17-20, 23, 41-43], our study was distinctive. First, in this prospective study the use of a sensitive expansion assay greatly improved the detection of HCV-specific T cell responses. Second, no association was found between either pre-treatment or on-treatment HCV-specific $CD4^+$ and $CD8^+$ T cell responses and the outcome of pegIFN-α/ribavirin therapy in terms of achieving a RVR, although the number of included patients was small. Furthermore, T cell responses decline during anti-HCV treatment in all patients irrespective of clinical outcome.

The HCV-specific T cell response in chronic HCV patients is highly regulated. It has become clear that multi-factorial mechanisms negatively regulate the HCV-specific T cell response via mechanisms involving immunosuppressive cytokines and regulators such as PD-1 [44-45]. The enhanced sensitivity of the 12 day T cell expansion assay could rely on lifting the regulation during the culture period, possibly as a result of the addition or production of growth factors, such as IL-2. Furthermore, when analyzing the different HCV-peptides in relation to SVR, a skewing of reactivity towards the non-structural HCV peptides is observed. This is in agreement with previous studies [46-47] showing an association between a vigorous T cell response against NS3 and successful outcome of therapy. Furthermore, albeit not performed in our study, some studies have shown via tetramer analysis that specific epitopes in core and NS3 correlate with SVR [15, 24]. Therefore, rather than looking at the total HCV-specific response, future studies, possibly using tetramers, should focus more on the detailed poly-functionality and quality as well as regulation of HCV-specific T cell responses to identify pre-treatment factors responsible for successful therapy.

By evaluating the role of HCV-specific T cells during pegIFN-α/ribavirin therapy in both SVR and non-SVR patients a similar decline in HCV-specific T cell responses from baseline to week 4 was demonstrated in both patient groups. Immunological parameters like T cell activation or a shift in the numbers of T cell subsets could not explain the therapy-induced decline of the HCV-specific T cell response. This is in accordance with some other studies showing a decline in HCV-specific T cell responses using IFN-γ production as direct

effector function of T cell activity [15, 17, 19]. In contrast, others have suggested augmentation of T cells during therapy using T cell proliferation assays with fresh PBMC and [^{3}H]-thymidine incorporation, thereby mainly describing T cell memory function [10, 21, 24]. A possible explanation is that due to the decrease in viral load and thereby loss of antigen-triggering of HCV-specific T cells, these T cell responses decline. This would suggest that, similar to other infections like HIV [39, 48], loss of antigen leads to a reduction of virus-specific T cells and demonstrates no role for HCV-specific T cell responses in forced viral clearance during pegIFN-α/ribavirin therapy. This argument is supported by the observation that T cell responses recover after treatment discontinuation in patients experiencing a relapse of HCV viremia [19, 24, 41]. One of our patients also experienced a relapse at the end of therapy with an increase in HCV RNA levels subsequently followed by an increase in HCV-specific T cell responses (data not shown).

Homing of HCV-specific T cells to the liver during treatment is mentioned as an explanation for the low or absent T cell responses measured in blood [22, 49]. This hypothesis is supported by the observation that in cross-sectional studies higher percentages of HCV-specific T cells are found in the liver than in peripheral blood [50-51]. However, peripheral T cell responses are easily measurable in patients spontaneously clearing acute HCV [26, 52-53]. Moreover, a recent study in chronic HCV patients demonstrated that several of the measured intra-hepatic epitope-specific T cell responses were also found in PBMC only after ex vivo expansion of these T cells [49, 54]. This indicates that these responses present in the liver can also be measured in peripheral blood albeit at low frequencies. Therefore, the use of a sensitive expansion assay, as used in our study, enabled us to detect these small frequencies of peripheral HCV-specific T cells. However, extrapolation of findings obtained using peripheral blood to the functionality of intra-hepatic T cells should be made with caution, since the microenvironment of the liver may modulate the phenotype and activity of these cells [55].

In conclusion, using a sensitive expansion assay, we found no correlation between pre-treatment HCV-specific T cell responses and outcome (SVR or RVR) of pegIFN-α/ribavirin therapy by assessing the total response to peptides spanning the entire HCV genome. However, skewing of the individual T cell responses towards the NS-antigens was observed. Furthermore, irrespective of treatment outcome, HCV-specific T cell responses declined during treatment in all patient groups. Although this suggests that total HCV-specific T cell responses do not play a role in forced viral eradication, it is possible that HCV-specific T cells at baseline initially maintain (or even increase) their effector function in the first weeks of therapy after which a decline sets in. Further studies to assess HCV-specific T cell poly-functionality, kinetics and regulation in detail are needed.

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Chapter 6

Negative regulation of hepatitis C virus-specific immunity is highly heterogeneous and modulated by peginterferon-α and ribavirin therapy

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ABSTRACT

Background & aims

The mechanisms behind the deficient T cell response against the hepatitis C virus (HCV) in chronic HCV patients are poorly understood. Although several inhibitory mechanisms, including suppression by IL-10, TGF-β or regulatory T cells (Treg), have been identified to be involved, the relative importance of suppression by IL-10, TGF-β and Treg for the dysfunctional HCV-specific immunity and the impact of pegylated interferon-alpha and ribavirin (PegIFN-α/ribavirin) therapy on these inhibitory mechanisms are still unclear.

Methods

The suppressive effects of IL-10, TGF-β and Treg on HCV-specific T cell proliferation and IFN-γ production were examined in blood of 43 chronic HCV-infected patients, before, during and after PegIFN-α/ribavirin therapy, in combination with clinical disease parameters.

Results

Our findings revealed that coregulation by IL-10, TGF-β or Treg of the HCV-specific T cell response in chronic HCV patients varied greatly, showing highly heterogeneous patterns of regulation. IL-10 mediated suppression of HCV-specific IFN-γ production was observed in patients with a relatively high viral load, which suggests that protective antiviral immunity in therapy-naive chronic HCV patients is controlled by IL-10. In addition, negative regulation of especially HCV-specific IFN-γ production by TGF-β and IL-10 changed dramatically as a consequence of PegIFN-α/ribavirin therapy, irrespective of treatment outcome.

Conclusions

Our findings emphasize the importance of negative regulation in the control of HCV-specific T cell proliferation and IFN-γ production. Patient-specific patterns of co-regulation that control T cell reactivity may explain variability in immunopathology between chronic HCV patients. Moreover, antiviral therapy strongly affects the regulatory mechanisms that maintain the dysfunctional HCV-specific immunity in chronic HCV patients.

INTRODUCTION

In the vast majority of patients, the hepatitis C virus (HCV) causes chronic infection with viral replication primarily in the liver. The inability to mount strong, broadly targeted and lasting $CD4^+$ and $CD8^+$ T cell responses against HCV is considered crucial for the development and maintenance of this persistent infection (reviewed in [1-3]). Several host and viral mechanisms have been proposed to contribute to this deficient HCV-specific T cell response, including viral escape, impaired antigen presentation, anergy, exhaustion of the T cell response mediated via inhibitory receptors such as PD-1 and Tim-3 [1-2], and active suppression of virus-specific T cell responses by regulatory T cells (Treg) expressing the transcription factor FoxP3 or by the immunosuppressive cytokines IL-10 or TGF-β [4].

We and others provided evidence for increased numbers of $FoxP3^+$Treg in the liver of chronic HCV patients [5-8]. Treg in blood have been shown to suppress both HCV-specific proliferation and IFN-γ production by $CD4^+$ and $CD8^+$ T cells [9-13]. Moreover, it has been suggested that Treg, at least partially, control chronic liver inflammation, with higher Treg suppressive capacity in patients with lower serum alanine transaminase (ALT) levels [10]. Other studies have shown that blocking IL-10 or TGF-β can enhance HCV-specific T cell proliferation and IFN-γ production [14-18]. In addition, serum IL-10 and TGF-β levels were enhanced in HCV-infected patients as compared to control individuals, and augmented production of these inhibitory cytokines by monocytes and T cells has been described for HCV-infected patients [14, 16-21].

Despite overwhelming evidence on the importance of IL-10, TGF-β and Treg in controlling immunity to HCV, little information is available on the coregulation of these regulatory mechanisms by assessing whether multiple pathways simultaneously contribute to impairment of HCV-specific immunity. Also, the modulation of immunoregulatory pathways by antiviral therapy of chronic HCV patients has received little attention, but is highly relevant since it is still under debate whether pegylated interferon-α and ribavirin (PegIFN-α/ribavirin) therapy restores the dysfunctional HCV-specific T cell response or not [22].

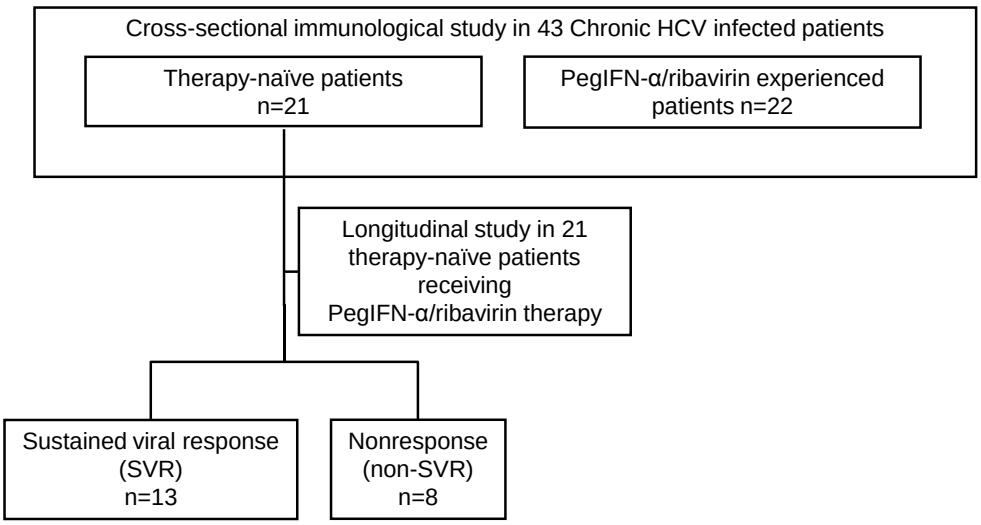
In this study, we therefore investigated the relative contribution of suppression by IL-10, TGF-β and Treg to dysfunctional HCV-specific immunity in peripheral blood from chronic HCV patients. In addition, we studied whether this regulation had clinical implications for these patients. Finally, a detailed examination of coregulation by multiple inhibitory mechanisms was conducted during and after PegIFN-α/ribavirin therapy. Our findings reveal that regulation of the HCV-specific T cell response in chronic HCV patients is highly heterogeneous, as individuals show distinct patterns of suppression by IL-10, TGF-β or Treg. However, relatively high viral loads were observed in therapy-naive chronic HCV patients showing pronounced IL-10 driven suppression of HCV-specific IFN-γ production. In addition, irrespective of viral response to therapy, negative regulation of especially HCV-specific IFN-γ production by TGF-β and IL-10 changed dramatically as a consequence of PegIFN-α/ribavirin therapy.

METHODS

Patients, controls and antiviral therapy

Forty-three chronic HCV-infected patients were included (Table 1 and Supplementary Figure 1 for patient details). Patients were excluded in case of decompensated liver disease, HBsAg positivity, or HIV coinfection. Diagnostic core biopsy samples from 36 of 43 patients, obtained within 3 months prior to start of therapy, were assessed for fibrosis stage by an experienced liver pathologist (Metavir score). Twenty-one patients had never received therapy for HCV before (therapy-naive) and 22 were nonresponders to previous IFN-α and ribavirin therapy (therapy-experienced). A cross-sectional immunological study was performed in all 43 patients. In addition, a longitudinal study followed, in which the 21 therapy-naive patients were investigated at week 4 and 12 during PegIFN-α/ribavirin therapy, and during the follow-up (FU) period at week 4 and 24 after ending treatment. Therapy consisted of twice daily orally administered ribavirin (<65kg;800mg/day, 65-80kg;1000mg/day, 81-105kg;1200mg, >105kg;1400mg, Rebetol®, Schering-Plough now MSD, Houten, the Netherlands) and weekly subcutaneous injections with pegylated interferon-α-2b (1.5 μg/kg, PegIntron®, Schering-Plough now MSD). Furthermore, 18 age and sex matched healthy control subjects were included. The institutional ethical review board approved the clinical protocols, and written informed consent was obtained from all individuals prior to their inclusion.

Flowchart of patients included in the study.



Supplementary Figure 1. Flowchart of patients included in the study. 43 Chronic HCV-infected patients were included: therapy-naive (n=21) and PegIFN-α/ribavirin experienced (n=22). A cross-sectional immunological study was carried out on these 43 patients. Therapy-naive chronic HCV patients received standard PegIFN-α/ribavirin therapy. Thirteen patients achieved an SVR, as they remained HCV RNA negative 6 months after end of therapy. Eight patients showed a nonresponse to therapy, and did not become HCV RNA negative. A longitudinal immunological study was carried out in which the 21 therapy-naive patients were followed up during and up to 24 weeks after therapy.

Table 1. Patient characteristics.

Study number	Sex (M/F)	Age (years)	Liver fibrosis (Metavir)	Genotype	HCV RNA (IU/mL)	ALT (U/L)	Therapy naïve	Sustained Viral Response
1	F	57	3	1	$1.1x10^7$	150	Yes	Yes
2	F	37	n.d.	3	$3.2x10^3$	79	Yes	Yes
3	M	54	1	1	$2.7x10^7$	49	Yes	No
4	F	27	1	1	$3.7x10^2$	34	Yes	Yes
5	M	48	2	3	$1.1x10^5$	146	Yes	Yes
6	M	51	2	1	$3.1x10^6$	46	Yes	No
7	M	52	2	3	$1.5x10^5$	41	Yes	Yes
8	M	34	2	1	$3.8x10^6$	103	Yes	Yes
9	M	56	2	1	$6.5x10^6$	157	Yes	No
10	F	57	4	1	$7.7x10^5$	17	Yes	No
11	M	45	2	1	$4.5x10^6$	55	Yes	Yes
12	M	47	1	1	$4.5x10^5$	227	Yes	Yes
13	F	41	1	3	$8.6x10^5$	46	Yes	Yes
14	M	60	3	3	$3.2x10^6$	164	Yes	Yes
15	M	40	2	1	$2.2x10^4$	58	Yes	No
16	F	40	3	1	$3.1x10^5$	120	Yes	No
17	M	45	n.d.	1	$1.1x10^7$	36	Yes	No
18	F	58	4	1	$1.6x10^5$	179	Yes	Yes
19	M	57	0	1	$7.2x10^6$	48	Yes	No
20	M	42	1	1	$1.5x10^6$	65	Yes	Yes
21	F	42	n.d.	1	$3.3x10^6$	107	Yes	Yes
22	M	47	1	1	$5.3x10^5$	144	No	n.a.
23	M	43	4	1	$3.4x10^5$	89	No	n.a.
24	F	50	3	1	$6.1x10^5$	111	No	n.a.
25	F	55	0	1	$5.5x10^5$	59	No	n.a.
26	M	44	0	1	$6.7x10^5$	33	No	n.a.
27	F	48	1	1	$6.0x10^5$	61	No	n.a.
28	M	45	3	1	$1.6x10^6$	57	No	n.a.
29	M	49	0	1	$3.9x10^5$	48	No	n.a.
30	M	36	4	1	$9.3x10^5$	98	No	n.a.
31	M	48	3	1	$2.6x10^6$	122	No	n.a.
32	M	41	4	1	$1.9x10^5$	120	No	n.a.
33	M	44	n.d.	1	$6.2x10^2$	44	No	n.a.
34	F	53	n.d.	1	$2.4x10^6$	77	No	n.a.
35	M	43	2	1	$6.1x10^5$	224	No	n.a.
36	M	50	n.d.	1	$1.8x10^5$	55	No	n.a.
37	M	41	2	1	$3.4x10^5$	67	No	n.a.
38	M	59	3	1	$9.3x10^5$	84	No	n.a.
39	M	39	2	1	$8.3x10^5$	53	No	n.a.
40	F	46	0	1	$5.2x10^4$	74	No	n.a.
41	M	41	2	1	$4.5x10^5$	57	No	n.a.
42	M	44	n.d.	4	$2.3x10^5$	33	No	n.a.
43	M	48	0	1	$2.4x10^6$	56	No	n.a.

Abbreviations: n.d., not determined within 3 months before start of therapy; n.a., not applicable

In vitro quantification of HCV-specific T cell proliferation and IFN-γ production

Peripheral blood mononuclear cells (PBMC) were isolated from venous blood by ficoll separation (Ficoll-Paque^{TM} plus, Amersham, Buckinghamshire, UK) and immediately cultured in quadruplets in 96-well round-bottom plates ($2x10^5$ cells in 200 µL). Cells were stimulated with overlapping peptide pools (1 µg/mL per individual peptide; spanning the core, NS3, NS4, NS5a and NS5b HCV genome; clone J4, genotype 1b; BEI Resources, Manassas, USA), anti-CD3 antibody (1 µg/mL; OKT-3; Janssen-Cilag, Tilburg, the Netherlands), CMV antigens (34 µg/mL; AD-169; Microbix, Toronto, Canada) or no stimulus. Culture medium was RPMI 1640 supplemented with L-glutamin, Penicillin-Streptomycin, HEPES, and 5% human serum (all from Lonza, Verviers, Belgium), anti-CD28 (1 µg/mL; CD28.2; eBioscience, San Diego, USA) and anti-CD49d antibody (1 µg/mL; 9F10; eBioscience). After culturing for 3 days, 100 µL supernatant was collected, stored at -80 ºC, and replaced by fresh culture medium. IFN-γ levels were determined in these supernatants by ELISA (Ready Set Go; eBioscience). After stimulation for 5 days, cells were pulsed for 16h with [^{3}H]-thymidine (0.5 µCi/well; Amersham, Little Chalfont, UK). Proliferation was determined as counts per minute (cpm) by liquid scintillation. For HCV-specific T cell proliferation and IFN-γ assays, results were considered positive when more than 500 counts or 100 pg/mL were detected above background, which corresponded to 3 SD above any response observed in healthy controls (data not shown).

Blocking and depletion experiments

Neutralizing antibodies against the IL-10 receptor (IL-10R, 5 µg/mL; 3F9; Biolegend, San Diego, USA), or TGF-β (5 µg/mL; 1D11, kindly provided by Dr. Boon, Bioceros, Utrecht, the Netherlands) were added to the cultures and HCV-specific assays were performed as described above. In addition, assays were performed with PBMC from which $CD25^{hi}$ cells were depleted using CD25 magnetic beads (Miltenyi Biotech, Bergisch Gladbach, Germany). This strategy allowed depletion of at least 80% of $CD4^+CD25^+FoxP3^+$ Treg (data not shown). HCV-specific responses were considered regulated by Treg, TGF-β or IL-10 when proliferation or IFN-γ levels increased by at least 500 cpm or 100 pg/mL, respectively.

In vitro quantification of circulating IL-10, TGF-β and Treg levels

Serum IL-10 and latent TGF-β levels were determined by ELISA (IL-10; Quantikine-HS-kit, RnD, Minneapolis, USA and TGF-β; Ready–Set-Go; eBioscience). To determine the frequency and phenotype of circulating $FoxP3^+$Treg cells, venous blood samples were fixed and erythrocytes lysed using FixPerm^{TM} reagent (eBioscience). Samples were stained with antibodies against CD25-PE-Cy7 (2A3; BD), FoxP3-APC (PCH101; eBioscience), CD4-APC-H7 (SK3; BD) and CD3-AmCyan (SK7; BD). Cell acquisition was performed on a FACSCanto II flowcytometer (BD), and analyzed using FacsDiva^{TM} software (BD). For analysis, gates were set on the basis of isotype antibody controls, where appropriate.

Virological assessment

Serum HCV RNA levels were determined by quantitative PCR (Cobas® Ampliprep/ Cobas® TaqMan® HCV test (limit of detection <15 IU/mL, Roche Diagnostics, the Netherlands). HCV genotypes were determined by an in-house developed sequence analysis assay (Department of Virology, Erasmus MC).

Data analysis

Data are depicted as mean ± 1 SD and unpaired data were compared using Student's t-test or the Mann Whitney U test for nonparametric data. Correlations between immunological and clinical parameters were calculated using the Spearman correlation test. SPSS 17.0 for Windows (SPSS, Chicago, USA) was used for these analyses. All p-values were two-tailed and considered significant if <0.05.

RESULTS

HCV-specific T cell proliferation and IFN-γ production are weak or undetectable in therapy-naive and PegIFN-α/ribavirin therapy-experienced chronic HCV patients

The strength of T cell responses to HCV antigens was evaluated in 43 chronic HCV patients. In line with previous reports (reviewed in [1-2]) upon stimulation of PBMC with a cocktail of HCV peptides, proliferative responses were weak in 24 out of 43 chronic HCV patients, and undetectable in the remaining 19 (Figure 1A). Despite the fact that HCV-specific T cell responses were weak or undetectable, up to 100 fold stronger CMV-specific T cell proliferation was detected in PBMC of 40 out of 43 chronic HCV patients (Figure 1B). HCV-specific IFN-γ production was also low to undetectable in most patients, as only 12 out of 43 patients showed substantial IFN-γ production (Figure 1A).

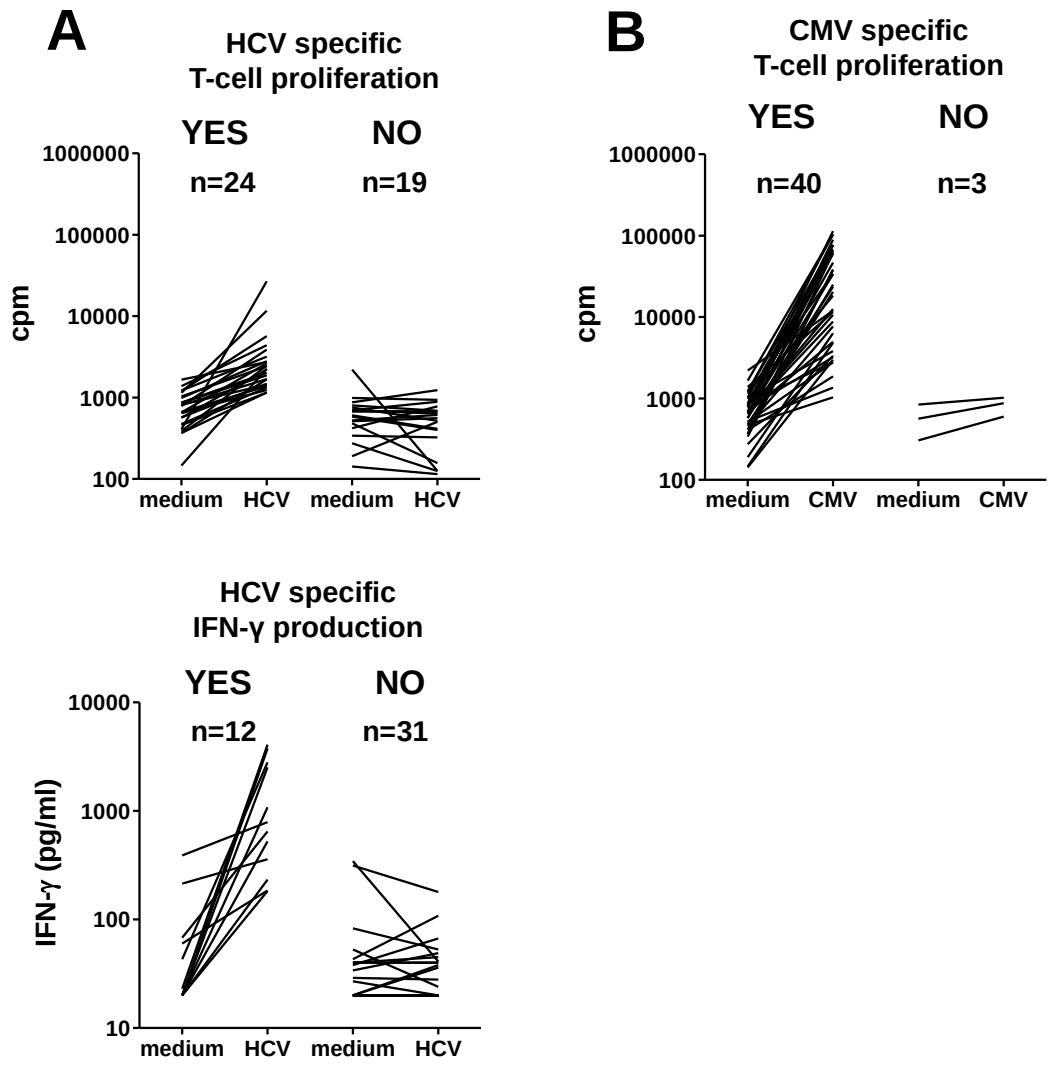


Figure 1. Chronic HCV-infected patients show dysfunctional HCV-specific T cell responses. Forty-three chronic HCV-infected patients were investigated (21 therapy-naive and 22 PegIFN-α/ribavirin experienced). T cell proliferation (cpm) to HCV **(A)** or CMV **(B)** were determined at day 5 upon stimulation of PBMC, and IFN-γ production at day 3. The sensitivity of the IFN-γ ELISA was 20 pg/mL, and therefore data of 17 patients are shown as one single horizontal line in the graph. For all graphs, patients showing significant responses are depicted to the left (YES) and those without are shown to the right (NO).

Multiple regulatory mechanisms suppress HCV-specific T cell proliferation in chronic HCV patients

Next, we evaluated the regulation by IL-10, TGF-β and Treg on the dysfunctional HCV-specific T cell reactivity in the 43 patients. For this purpose, we first determined HCV-specific T cell proliferation with or without *in vitro* blockade of the IL-10R or TGF-β using neutralizing antibodies, or depletion of $CD4^{+}CD25^{hi}$Treg. In this cross-sectional study, suppression by IL-10, TGF-β or Treg of HCV-specific T cell proliferation was operational in respectively 6, 11 and 19 out of 43 patients (Figure 2A). The regulation of HCV-specific proliferation was highly heterogeneous in that each of 8 possible combinations of regulatory mechanisms occurred, albeit some at low frequency. Furthermore, no dominant pattern could be identified, and regulation of HCV-specific proliferation and IFN-γ production were often by different mechanisms. Interestingly, HCV-specific T cell proliferation was not affected by a previous nonresponse to PegIFN-α/ribavirin therapy, as the frequency (Figure 2A) and strength (Supplementary Figure 1) of regulation by IL-10, TGF-β and Treg was comparable to therapy-naive chronic HCV patients.

Regulation of HCV-specific IFN-γ production differs between PegIFN-α/ribavirin experienced and therapy-naive chronic HCV patients

Next, we studied whether HCV-specific IFN-γ production in chronic HCV patients was suppressed by multiple regulatory mechanisms similar as the heterogeneous regulation of HCV-specific T cell proliferation. As shown in Figure 2B, blocking the suppressive effects of IL-10, TGF-β, or Treg enhanced HCV-specific IFN-γ production, albeit that a different pattern of regulation was observed between therapy-naive and PegIFN-α/ribavirin experienced chronic HCV patients. For therapy-naive chronic HCV patients, similar as for T cell proliferation, regulation of HCV-specific IFN-γ production was highly heterogeneous.

In sharp contrast, in PegIFN-α/ribavirin experienced chronic HCV patients IL-10 mediated regulation of IFN-γ production was absent (Figure 2B). Moreover, different from therapy-naive patients, TGF-β suppressed HCV-specific IFN-γ production in the majority of therapy-experienced chronic HCV patients (Figure 2B; respectively 5 out of 21 and 14 out of 22) with strong increments in IFN-γ levels detected after blocking TGF-β (Supplementary Figure 2B). Regulation by Treg was also observed in PegIFN-α/ribavirin experienced patients, however at similar rates and with comparable strength as for therapy-naive patients.

Levels of circulating TGF-β, or Treg were similar for therapy-naive patients, PegIFN-α/ribavirin experienced patients and healthy controls (Figure 2C) and did not correlate with HCV-specific T cell reactivity (data not shown). In contrast to healthy individuals, enhanced serum IL-10 was measured in some, but not all, HCV-infected patients (Figure 2C). However, the presence of serum IL-10 did not correlate with the presence of IL-10 dependent regulation of HCV-specific T cell proliferation or IFN-γ production (data not shown).

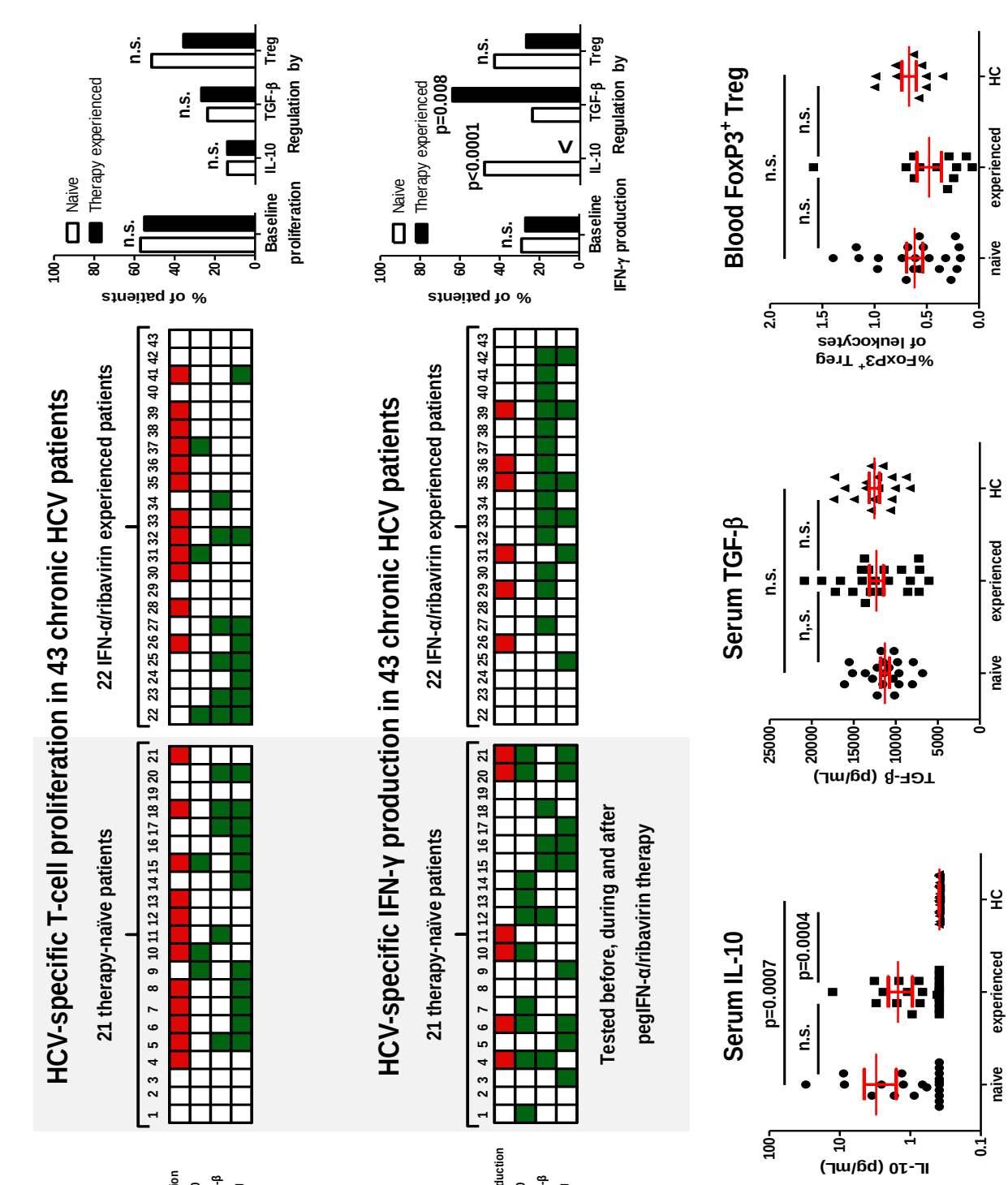


Figure 2. Multiple regulatory mechanisms control HCV-specific T-cell reactivity in PBMC from chronic HCV infected patients. (A) Individual data of 43 patients on regulation of HCV-specific T-cell proliferation and **(B)** IFN-γ production are shown (number 1-21 are therapy-naive; number 22-43 are PegIFN-α/ribavirin experienced patients). Red squares depict HCV-specific T-cell proliferation or IFN-γ production without *in vitro* blockade of any regulatory pathway (baseline response). Green squares reflect patients with a significant increase of HCV-specific responses after IL-10R or TGF-β neutralization, or depletion of Treg. White squares reflect the absence of a baseline response or regulation. The experiments were performed similar as in Figure 1A. Histograms to the right hand side show percentages of patients with HCV-specific responses at baseline, or significant regulation of these responses by respectively IL-10, TGF-β or Treg. **(C)** Serum IL-10 and TGF-β levels and blood CD4+FoxP3+ Treg as a proportion of leukocytes are presented in naïve and treatment experienced patients and healthy controls.

IL-10 mediated suppression of HCV-specific IFN-γ production affects the level of HCV replication in therapy-naive patients

To identify mechanisms explaining clinical differences between patients, we questioned whether regulation of HCV-specific T cell reactivity was related to disease parameters. As shown in Figure 3, HCV RNA levels in therapy-naive patients were significantly higher in case of active IL-10 mediated suppression of HCV-specific IFN-γ production, as opposed to the lower HCV RNA levels observed in therapy-naive patients without IL-10 driven regulation of HCV-specific IFN-γ production ($p<0.004$). Other significant associations between regulation by IL-10, TGF-β, or Treg and disease parameters such as time-since-infection, ALT levels, and liver fibrosis grade were not found, neither for the whole group of 43 chronic HCV patients, nor for therapy-naive or PegIFN-α/ribavirin experienced patients separately (data not shown).

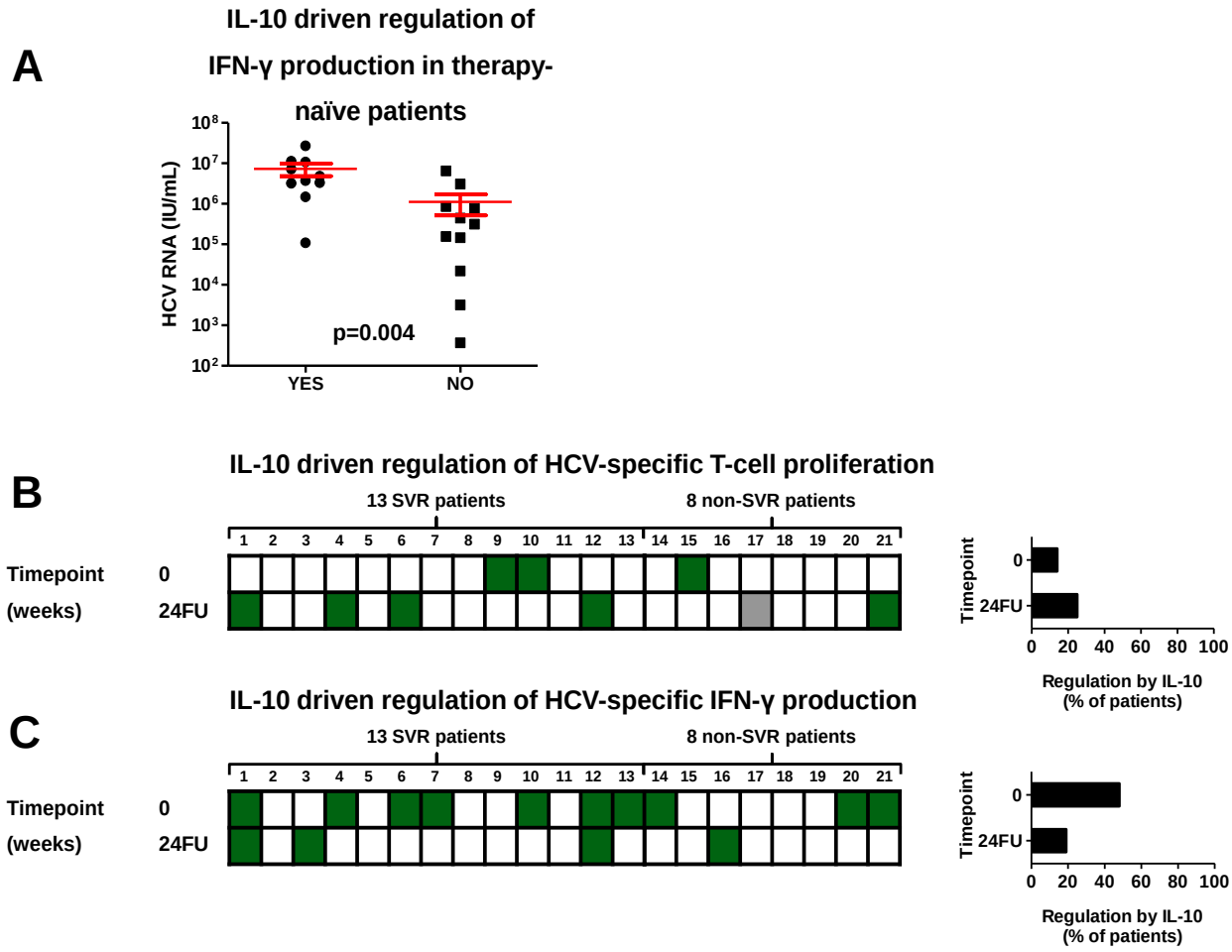


Figure 3. Regulation of HCV-specific immunity by IL-10 is associated with higher HCV RNA levels in therapy-naive patients, however not linked to success of PegIFN-α/ribavirin therapy. (A) HCV RNA levels of 21 therapy-naive chronic HCV patients with or without regulation by IL-10 of HCV-specific IFN-γ production. **(B)** Individual data of 21 previously therapy-naive chronic HCV patients on regulation of HCV-specific T cell proliferation and **(C)** IFN-γ production by IL-10 before and at 24FU after antiviral therapy. Patients 1-13 showed a sustained virological response (SVR), patients 14-21 a viral nonresponse. Dark grey squares reflect patients with a significant increase in either HCV-specific proliferation or IFN-γ production after neutralization of IL-10R. White squares reflect the absence of regulation by IL-10. Light grey squares reflect missing data. Histograms to the right side show percentages of patients with significant IL-10 driven regulation of HCV-specific responses at the indicated timepoints.

Irrespective of viral outcome, regulation of HCV-specific IFN-γ production by TGF-β increases during and up to 24 weeks after PegIFN-α/ribavirin therapy

There is an ongoing debate whether PegIFN-α/ribavirin therapy restores the dysfunctional HCV-specific T cell response due to removal of antigenic pressure on immune cells [22]. If this is the case, the dynamics in activity of regulatory pathways may explain this restoration. To examine this in our cohort, we prospectively examined the type and degree of regulation of HCV-specific T cell responses at various timepoints during, and up to 24 weeks after therapy in 21 therapy-naive patients receiving PegIFN-α/ribavirin therapy for the first time. When pretreatment and 24 weeks after therapy were compared, regulation of HCV-specific T cell proliferation by IL-10 was never observed at both timepoints and only present in a small proportion of patients (Figure 3B). At the same time, IL-10 driven suppression of HCV-specific IFN-γ production decreased substantially after therapy, both in patients achieving a sustained virological response (SVR) and in non-SVR patients (Figure 3C and Supplementary Figure 2B). Regulation of HCV-specific T cell reactivity by IL-10 was not related to the outcome of therapy, as regulation of HCV-specific T cell proliferation and IFN-γ production was equally distributed among the 13 SVR patients and 8 non-SVR patients.

Suppression of HCV-specific T cell proliferation by TGF-β was infrequent during and 24 weeks after therapy as it was before therapy. Only at 4 weeks after end of therapy, a transient increase in regulation of HCV-specific T cell proliferation by TGF-β was observed (Figure 4A).

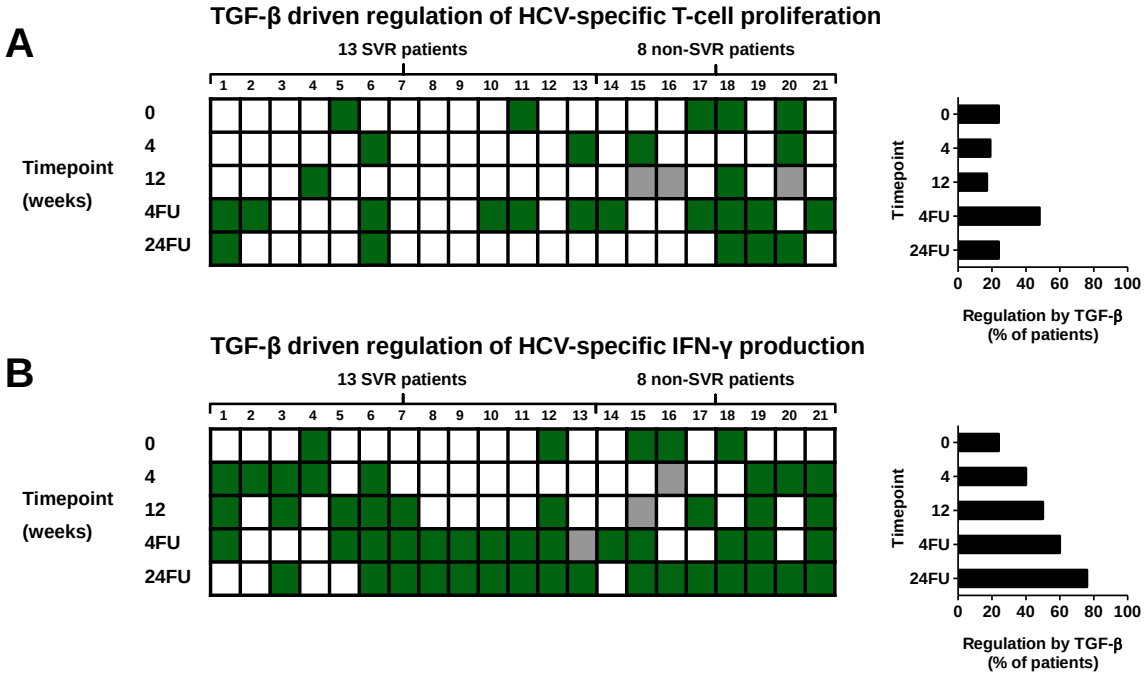


Figure 4. Irrespective of viral outcome, regulation of HCV-specific IFN-γ production by TGF-β increases during and up to 24 weeks after PegIFN-α/ribavirin therapy. Individual data of previously naive chronic HCV patients before, during and after therapy. Patients 1-13 showed an SVR, patients 14-21 did not achieve an SVR (non-SVR). **(A)** Dark grey squares reflect patients with a significant increase in either HCV-specific proliferation or **(B)** IFN-γ production after neutralization of TGF-β. White squares reflect the absence of regulation by TGF-β. Light grey squares reflect missing data. Histograms to the right side show percentages of patients with significant TGF-β driven regulation of HCV-specific responses at the indicated timepoints.

In contrast, although serum TGF-β levels remained unchanged (data not shown), TGF-β driven regulation of HCV-specific IFN-γ production increased gradually during therapy and continued to increase up to 24 weeks after therapy, when 16 out of 21 patients showed regulation by TGF-β (Figure 4B). Twenty-four weeks after therapy, regulation was not only most frequent, but also strong as TGF-β neutralization resulted in a mean increase of HCV-specific IFN-γ production of 3111 pg/mL.

DISCUSSION

This study establishes that the regulation by IL-10, TGF-β and Treg of dysfunctional HCV-specific immunity in chronic HCV patients is highly heterogeneous. However, two clear differences between PegIFN-α/ribavirin experienced patients and therapy-naive patients could be distinguished. In therapy-experienced chronic HCV patients, regulation of HCV-specific IFN-γ production by TGF-β was dominant, while regulation by IL-10 was absent. In contrast, in about half of therapy-naive chronic HCV patients, especially those with a high viral load, IL-10 was involved in the regulation of HCV-specific IFN-γ production. Upon treatment with PegIFN-α/ribavirin of previously therapy-naive patients, regulation by TGF-β of HCV-specific IFN-γ production gradually increased over time and reached a maximum at the end of follow-up at 24 weeks after therapy.

The present study clearly shows that Treg, TGF-β and IL-10 all contribute to the suppression of HCV-specific T cell proliferation in chronic HCV patients. Importantly, also in those patients in whom HCV-specific T cell proliferation or IFN-γ production was not observed upon stimulation with HCV peptides alone, these responses could be revived by blocking one of the inhibitory mechanisms studied. However, we now show that the importance of the specific pathways to inhibit HCV-specific immunity differs between patients and no dominant regulatory mechanism can be identified. This strong heterogeneity of regulation of HCV-specific immunity has not been emphasized before. In contrast, previous reports suggested exclusive suppression of HCV-specific immune responses by Treg, TGF-β or IL-10 [9-13, 15, 17] and to our knowledge only two previous reports showed involvement of both TGF-β and IL-10 in the suppression of HCV-specific immunity [16, 18]. The heterogeneous character of regulation of HCV-specific T cell immunity is reminiscent of the expression of exhaustion markers and their functional synergy to control HCV-specific T cell immunity [23].

One possible explanation for the heterogeneity of regulation is that patients are at different phases of disease, and vary greatly in level of viral replication, liver inflammation, pathology and other disease parameters. We suggest that time-since-infection, and subsequent differences in phase of disease are important factors explaining the heterogeneous regulation of T cell responses in chronic and therapy-induced resolved HCV infection. This has been proposed before as a general model for regulation of immunity to chronic infections [4]. In line with this concept, our study shows that HCV RNA loads were higher in chronic HCV patients showing active IL-10 mediated suppression of HCV-specific IFN-γ production. We cannot explain why individual patients differ with respect to the usage of IL-10 pathways leading to differences in HCV RNA levels, but genetic polymorphisms in

the *IL-10* gene may be important. A larger patient cohort and more detailed information on time-since-infection are needed to establish further relations between disease parameters and regulation of HCV-specific immunity.

This is the first prospective study examining PegIFN-α/ribavirin therapy-induced effects on IL-10 and TGF-β mediated suppression of HCV-specific immune responses. We observed that upon antiviral treatment IL-10 mediated regulation was diminished and TGF-β mediated regulation strongly enhanced. Irrespective of response to therapy, IL-10 driven regulation of HCV-specific IFN-γ production was decreased 24 weeks after therapy, albeit that a small group of patients still showed IL-10 regulation. Possibly, a longer follow-up is needed to loose IL-10 driven regulation of HCV-specific IFN-γ production, as our cohort of 22 chronic HCV-infected patients with a previous nonresponse to therapy showed an absence of IL-10 driven regulation of HCV-specific IFN-γ production. As mentioned above, IL-10 driven regulation may allow higher levels of HCV replication in therapy-naive chronic HCV patients. It seems that exposure to PegIFNα/ribavirin therapy diminishes the importance of this IL-10 mediated suppression of anti-HCV immunity irrespective of virologic response to therapy.

Our prospective data showed that when therapy-naive patients were treated with PegIFN-α/ribavirin, regulation by TGF-β of HCV-specific IFN-γ production gradually increased over time, and reached a maximum at the end of follow-up at 24 weeks after therapy. Thus, exposure to PegIFN-α/ribavirin increases the frequency of TGF-β driven regulation of HCV-specific IFN-γ production. This may be a direct effect of PegIFN-α/ribavirin therapy and not secondary to a decrease in HCV RNA load, since regulation by TGF-β increases gradually in both SVR and non-SVR patients. Our finding that TGF-β driven regulation of HCV-specific IFN-γ production increases induced by PegIFN-α/ribavirin, is supported by our cross-sectional data on previous nonresponders to therapy, who still show frequent regulation by TGF-β. For SVR patients, TGF-β mediated suppression of HCV-specific IFN-γ production by memory T cells was unexpected, since the activity of TGF-β in HCV is often linked to liver fibrogenesis [24] and SVR is associated with a reduction in fibrosis [25]. Importantly, total TGF-β concentrations in serum were similar before and after successful HCV eradication (data not shown), in contrast to older studies reporting reduced serum TGF-β levels in SVR patients [26-27]. Our study indicates a role for TGF-β as a regulatory cytokine suppressing the pro-inflammatory responses after PegIFN-α/ribavirin therapy, irrespective of viral response, rather than a cytokine promoting liver fibrogenesis.

Treg mediated regulation of HCV-specific proliferation gradually decreased during and after PegIFN-α/ribavirin therapy in both SVR and non-SVR patients, while regulation by Treg of HCV-specific IFN-γ production remained relatively stable (Supplementary Figure 4). These data are in line with the only previous study that prospectively examined regulation by Treg during PegIFN-α/ribavirin therapy that did not find a role for peripheral blood Treg in the response to treatment [28]. In contrast to blood, we recently showed that intrahepatic $CD4^+CD25^+FoxP3^+$ Treg increased during therapy, and their numbers remained elevated for more than 6 months after therapy-induced viral clearance in the majority of patients [8].

Additional research on the complex process of heterogeneous coregulation is required to understand the dysfunctional HCV-specific immunity in chronic HCV patients. Further evaluation of multiple regulatory mechanisms may explain variability in

immunopathology between chronic HCV patients, and optimization of immunotherapy should take into account the substantial differences between patients with respect to inhibitory processes preventing protective immunity against HCV.

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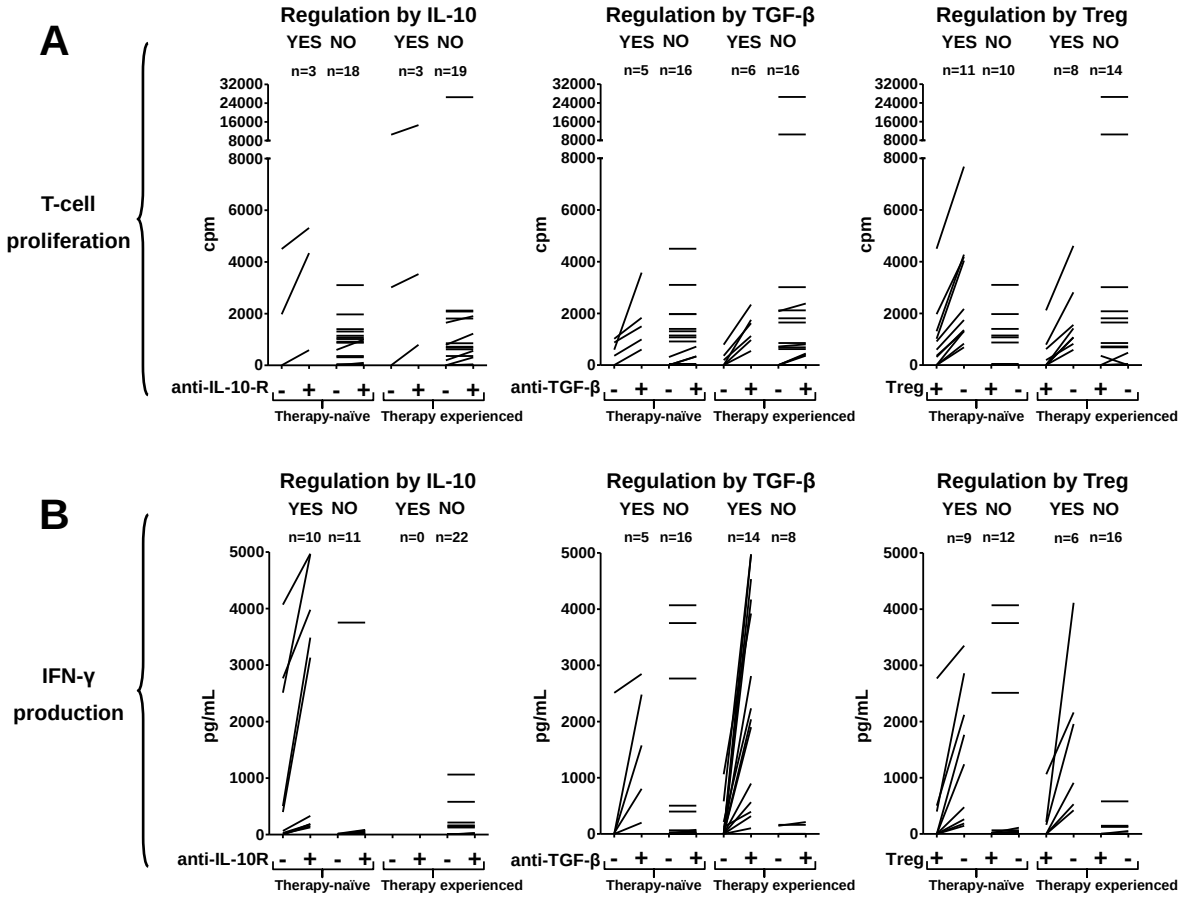
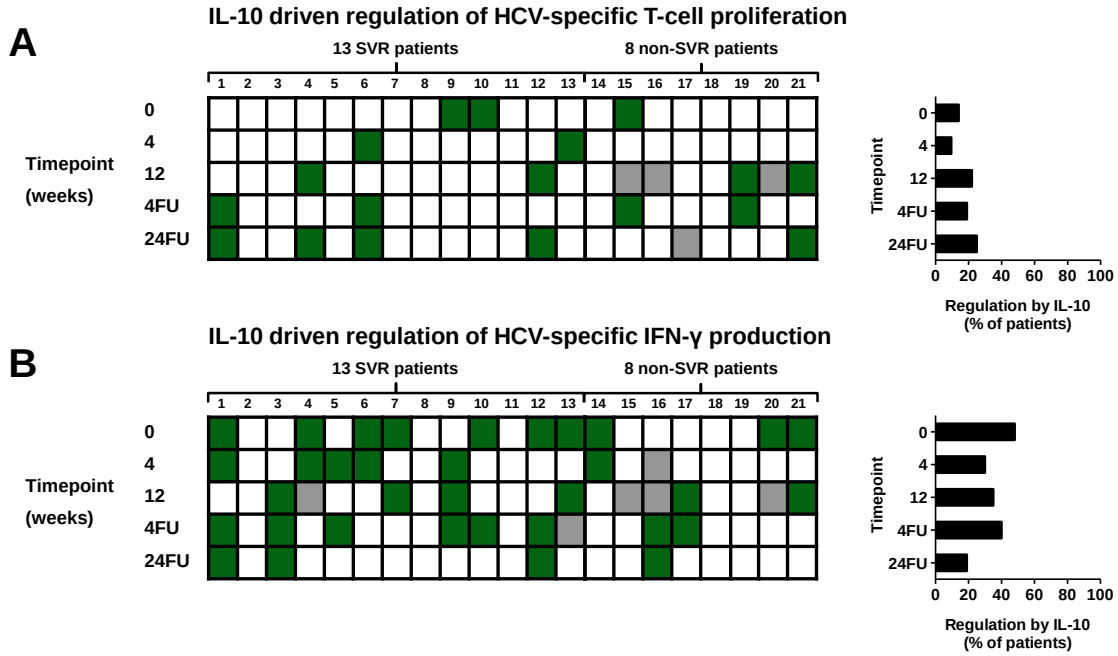
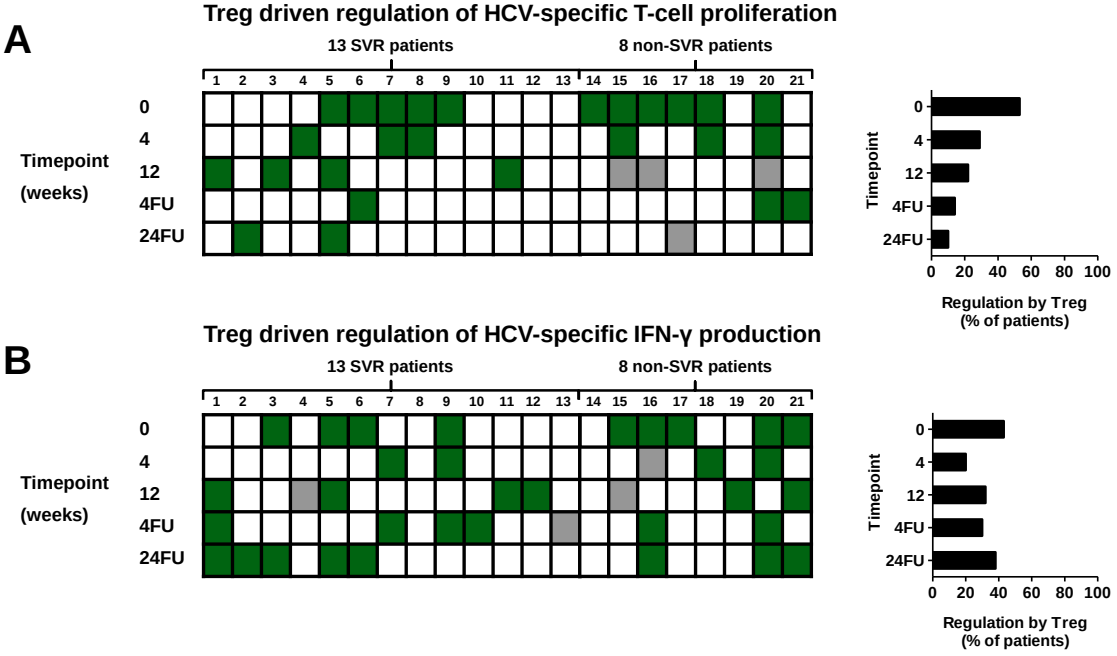
SUPPLEMENTARY FIGURES



Supplementary Figure 2. Multiple regulatory mechanisms control HCV-specific T cell reactivity in PBMC from chronic HCV-infected patients. Quantitative data on **(A)** regulation of HCV-specific T cell proliferation (cpm) and **(B)** IFN-γ production (pg/mL) are shown for 43 chronic HCV patients (21 therapy-naive and 22 PegIFN-α/ribavirin therapy-experienced). Graphs to the left, middle and right, respectively, show the effects of neutralization of the IL-10R or TGF-β, or depletion of Treg. The experiments were performed similar as in Figure 1A and 2. For all graphs, the number of patients with and without regulation is given (YES, n=number and NO, n=number, respectively).



Supplementary Figure 3. Frequency of regulation by IL-10 of HCV-specific T cell proliferation is stable during PegIFN-α/ribavirin therapy and regulation of HCV-specific IFN-γ production by IL-10 is decreased at 24 weeks after PegIFN-α/ribavirin therapy. Individual patient data before (T=0), week 4 or 12 during (T=4 and T=12, respectively) and 4 or 24 weeks after PegIFN-α/ribavirin therapy (T=4FU and T=24FU, respectively) are shown for 21 previously therapy-naive chronic HCV patients. Patients 1 to 13 showed a sustained virological response, patients 14 to 21 a viral nonresponse. **(A)** Dark grey squares reflect patients with a significant increase in either HCV-specific proliferation or **(B)** IFN-γ production after neutralization of IL-10R. White squares reflect the absence of regulation by IL-10. Light grey squares reflect missing data. Histograms to the right side show percentages of patients with significant IL-10 driven regulation of HCV-specific responses at the indicated timepoints.



Supplementary Figure 4. Irrespective of viral outcome, regulation of HCV-specific T cell proliferation by Treg decreases during and up to 24 weeks after PegIFN-α/ribavirin therapy. Individual patient data before (T=0), week 4 or 12 during (T=4 and T=12, respectively) and 4 or 24 weeks after PegIFN-α/ribavirin therapy (T=4FU and T=24FU, respectively) are shown for 21 previously therapy-naive chronic HCV patients. Patients 1 to 13 showed an SVR, patients 14 to 21 a viral nonresponse. **(A)** Dark grey squares reflect patients with a significant increase in either HCV-specific proliferation or **(B)** IFN-γ production after depletion of Treg. White squares reflect the absence of regulation by Treg. Light grey squares reflect missing data. Histograms to the right show percentages of patients with significant Treg driven regulation of HCV-specific responses at the indicated timepoints.

Chapter 7

Summary and general discussion

How do the findings presented in this thesis advance our understanding of the importance of negative regulation by IL-10, TGF-β and Treg for adaptive immunity to HCV infections, the specific immunological environment of the liver, and the dynamics of immunoregulation before, during and after IFN-α based therapy of chronic HCV patients?

Negative regulation by intrahepatic regulatory T cells may limit immunopathology during chronic hepatitis C virus infections

In blood, Treg have been shown to suppress HCV-specific proliferation and IFN-γ production by $CD4^+$ and $CD8^+$ T cells [1-5]. However, implications for immunopathology to the HCV-infected liver remains unclear. It has been suggested that negative regulation by Treg in blood may control chronic liver inflammation to a certain extent, as chronic HCV patients with a higher suppressive capacity of blood Treg showed lower ALT levels [2]. However, a recent study reported that the proportion, phenotype and function of circulating $CD4^+$CD25hi Treg remained unchanged during PegIFN-α/ribavirin therapy. Therefore, blood Treg did not seem involved in HCV eradication or reduced liver inflammation induced by therapy [6].

In the present thesis however, data are presented in favour of an important role of intrahepatic Treg in limiting immunopathology in the liver during chronic viral hepatitis infections. We showed that abundant numbers of $CD4^+$FoxP3$^+$Treg localize to the liver of chronic hepatitis C and B infected patients, whereas Treg were almost absent from healthy control livers (chapter 2 and 3). Elevated Treg frequencies in HCV-infected [3, 5, 7-10] and HBV-infected[8] livers has been shown before. In addition, high frequencies of intrahepatic Treg have also been shown for other chronic liver diseases, such as auto-immune hepatitis and primary biliary cirrhosis [7-9]. However, our study is the first to make a comparison with healthy control specimen, and we can now conclude that our and previous findings on increased intrahepatic Treg are specific for chronic liver disease and is not reflecting normal intrahepatic homeostasis.

Moreover, we suggest that $CD4^+$FoxP3$^+$Treg isolated from HCV-infected livers exhibit immediate effector functions as they were predominantly $CD45RO^+$HLA-$DR^+$$CTLA4^+$$CCR7^-$, reflecting an antigen-experienced, activated and highly differentiated effector phenotype [11-12]. These immediate effector functions may serve to limit immunopathology during chronic hepatitis C virus infections. We found that variation in the degree of liver fibrosis due to inflammation caused by chronic HCV infection may be partially explained by the frequency of intrahepatic $CD4^+$FoxP3$^+$Treg, as these liver infiltrating Treg were more numerous in HCV-infected patients showing only mild disease (chapter 2).

It is highly likely that the delicate balance between intrahepatic Treg and effector T cells affects the level of HBV and HCV replication. In experimental autoimmune encephalomyelitis in mice, it has been shown that the ratio of Treg to effector T cells is of crucial importance in controlling immunopathology, and not the absolute numbers [13]. In line with this, patients with the highest Treg to $CD8^+$ T cell ratios in the liver failed to clear the HCV infection during PegIFN-α/ribavirin therapy (chapter 4), while liver $CD4^+$FoxP3$^+$Treg frequencies or a specific Treg phenotype did not affect the level of HCV virus replication (chapter 2). In addition, in case of higher ratios of intrahepatic Treg to $CD3^+$ T cells, HBV DNA levels were higher during chronic infection (chapter 3).

We provided detailed information on the phenotype of Treg at the primary site of infection and are the first to present data on their inverse relation with fibrosis severity. However, formal proof of the functional impact of intrahepatic Treg on HCV-specific immunity, viral replication, immunopathology to the liver, and the mechanism of suppression remains unclear. $CD4^+FoxP3^+$ Treg may inhibit effector functions of other intrahepatic T cells thereby indirectly inhibiting activation of hepatic stellate cells (HSC), the cells central to liver fibrogenesis [14]. Interestingly, in this respect we observed a relation between the differentiation status of conventional FoxP3-$CD4^+$ T cells and the extent of liver fibrosis, with CD45RO-$CCR7^+$ naïve conventional T cell frequencies being highest in HCV-infected livers without signs of fibrosis (data not shown). Hence, $CD4^+FoxP3^+$ Treg may limit the differentiation of intrahepatic conventional T cells, which may result in reduced proinflammatory cytokine production and subsequent lower activation of HSC.

We showed in chapter 4 that therapy-induced HCV eradication did not lead to normalization of the intrahepatic immune system after therapy, as intrahepatic $CD4^+CD25^+FoxP3^+$ Treg remained increased up to 6 months after cessation of therapy in the large majority of patients. This suggests that regulation, possibly of HCV-specific memory T cells, by intrahepatic $CD4^+CD25^+FoxP3^+$ Treg is ongoing in patients who successfully clear the virus. However, we cannot rule out that these Treg will resolve later in time. Recently, it was reported that in plasma samples from 15% of SVR patients trace amounts of HCV RNA were detected years after successful therapy [15]. Importantly, the reappearance of HCV RNA was found to induce HCV-specific T cell responses. Therefore, it can be speculated that the intrahepatic Treg that remain present the first 6 months after successful PegIFN-α/ribavirin therapy control residual HCV replication in the liver. Furthermore, intrahepatic Treg may continue to protect against fibrogenesis after successful HCV clearance. I speculate that these Treg suppress ongoing activation of bystander T cells and HSC by products of liver damage, for example by reactive oxygen species [16], or suppress other inflammatory processes in the liver, for example due to alcoholic or non-alcoholic steatohepatitis [17].

TGF-β may protect against immunopathology

With respect to liver immunology, TGF-β is generally seen as an exclusively profibrotic cytokine (reviewed in [16]). In line with this paradigm, previous reports on liver fibrosis in HCV-infected patients were generally dogmatic about the profibrotic effect of TGF-β, albeit that data were often not convincing and even conflicting. Indeed many studies have reported a positive association between liver fibrosis and serum TGF-β concentrations, intrahepatic TGF-β mRNA levels or strong immunohistochemical staining for TGF-β [18-28]. However, not all studies did find this association and two recent studies even found an opposite relation suggesting a protective role of TGF-β against liver fibrosis [29-31]. In addition, HCV-specific TGF-β production by circulating $CD4^+$CD25hi cells has been suggested to reduce liver inflammation [2-3].

All these studies are inadequate since they have two major flaws. First, they measured very crude estimates of activated TGF-β *in vivo:* total TGF-β mRNA, total serum TGF-β or positive immunohistochemistry for TGF-β, which all include large quantities of irrelevant precursors of bioactive TGF-β. Future studies should only include measurements of active TGF-β, which is set free from the LAP molecule after binding of LAP to an activating

receptor. Second, since TGF-β only affects cells in close proximity to the cell that produces TGF-β [16, 32]. serum TGF-β levels, and also total intrahepatic TGF-β levels are inadequate to study the role of TGF-β in intrahepatic immunopathology. The preferred method to assess the activity of TGF-β is by functionally blocking its effects with neutralizing antibodies.

I now propose a more subtle dual role for TGF-β on hepatic fibrosis during chronic HCV infections: a profibrotic role via the direct activation of HSC and an antifibrotic role via its inhibitory effects on HCV-specific T cells which indirectly inhibits HSC activation. Importantly, HSC are central to liver fibrogenesis through type 1 collagen deposition and other changes in extracellular matrix deposition [16]. HSC activation occurs via several mechanisms of which two major ones are central to my hypothesis.

First, active TGF-β directly activates HSC via Smad proteins 2 and 3 and enhances collagen transcription [33 34]. Importantly, initial TGF-β activation is by cells in close proximity to HSC, likely LSEC and Kupffer cells. Once HSC have been activated, most TGF-β is produced by HSC themselves [33].

Second, pro-inflammatory effector functions of NK cells and HCV-specific $CD4^+$ and $CD8^+$ T cells induce killing and apoptosis of hepatocytes infected by HCV. Degradation products that come free from these hepatocytes activate HSC [35]. I suggest that intrahepatic HCV-specific effector T cells are suppressed by TGF-β, which results in less HSC activation and subsequently lower levels of fibrosis. Candidate immune cells in close proximity to HCV-specific effector T cells that activate TGF-β by setting it free from the LAP molecule are $CD4^+CD25^+$ T cells, DC and Kupffer cells as they all harbour activating receptors for LAP on their surface [32]. In blood, negative regulation via TGF-β has been shown to suppress these HCV-specific T cell responses during chronic HCV infection (chapter 6 and shown by many others [3, 36-39]). Functional data on inhibition of intrahepatic HCV-specific T cells by TGF-β are at present not available. However, it is highly likely that TGF-β plays a similar role within the liver. So, depending on the source and location of active TGF-β production, TGF-β may either promote or limit fibrogenesis during chronic HCV infections.

In chapter 6, we showed that irrespective of viral outcome, regulation of HCV-specific IFN-γ production by TGF-β increases during and years after PegIFN-α/ribavirin therapy, albeit that we have not studied the impact of intrahepatic TGF-β. This may explain why PegIFN-α/ribavirin therapy not only improves histology in patients that cleared the infection, but possibly also in patients with a nonresponse to IFN-α therapy (reviewed in Bailly et al. 2010).

Balance between negative regulation and effector T cell functions may explain the response to IFN-α-based therapy

At present it is not well understood to what degree and by what mechanisms HCV-specific immunity contributes to the effectiveness of treatment with pegIFN-α/ribavirin. Conflicting data on the role of HCV-specific T cell reactivity in therapy-induced viral clearance have been reported ([6, 40-52] and reviewed in chapter 1 of this thesis). This controversy in literature may be explained by the fact that frequencies of circulating HCV-specific T cells are very low [53] and decline even further after start of IFN-α-based therapy which makes studies focused on changes in HCV-specific T cell responses during treatment extremely difficult to perform. In chapter 5, we used a robust and sensitive assay able to detect low frequencies of

HCV-specific T cell responses. This expansion assay greatly improved the detection of HCV-specific T cell responses. However, we did not find a correlation between pre-treatment HCV-specific T cell responses and outcome of pegIFN-α/ribavirin therapy by assessing the total response to peptides spanning the entire HCV genome. This may be explained by the fact that HCV-specific T cell responses are suppressed by multiple regulatory mechanisms (chapter 6). We did not examine the contribution of the genotype of the infecting virus [54-55], or skewing of the T cell response towards certain HCV genes or epitopes in determining treatment outcome as has been suggested by some studies [44, 48].

I propose that the balance between negative regulation and effector T cell functions explains the response to IFN-α-based therapy. In experimental autoimmune encephalomyelitis in mice, it has been shown that the ratio of effector T cells to Treg is of crucial importance in controlling disease severity caused by immunopathology, and not the absolute numbers [13]. Similarly in our experimental setup, it is likely that a high ratio of intrahepatic $CD4^+CD25^+FoxP3^+$ Treg to effector T cells results in strong negative regulation of HCV-specific immune responses (chapter 4). In line with this, 4 of our patients with the highest Treg to $CD8^+$ T cell ratios all failed to clear the HCV infection (data not shown), and it is tempting to speculate that the delicate balance between intrahepatic $CD4^+CD25^+FoxP3^+$ Treg and effector T cells explains the response to IFN-α-based treatment in a subset of chronic HCV patients.

Likely, the balance between TGF-β or IL-10 and effector T cells also accounts for differences in response to IFN-α-based therapy. For example, upon treatment with PegIFN-α/ribavirin of previously therapy-naive patients, regulation by TGF-β of HCV-specific IFN-γ production gradually increased over time and reached a maximum at the end of follow-up at 24 weeks after therapy. However, we could not associate the strength of regulation by TGF-β or IL-10 of effector T cell responses with the outcome of therapy. Since regulation of HCV-specific T cell responses is highly heterogeneous and different combinations of regulation are operational in individual patients, investigation of larger numbers of patients is required to establish such an association.

Heterogeneity of negative regulation of hepatitis C virus-specific immunity may reflect differences in disease progression between patients

Despite overwhelming evidence on the importance of IL-10, TGF-β and Treg (reviewed in chapter 1), and inhibitory receptors such as PD-1 and TIM-3 in controlling immunity to HCV [56-62], little information is available on the coregulation of these regulatory mechanisms. Data presented in this thesis underline that Treg, TGF-β and IL-10 all contribute to the suppression of HCV-specific T cell proliferation in chronic HCV patients (chapter 6). What we add to the present literature is that the importance of these inhibitory pathways differs between patients. Previously, most reports have suggested exclusive suppression of HCV-specific immune responses by a single mechanism. These studies have claimed important roles for suppression of HCV-specific T cell reactivity mediated by Treg, TGF-β, IL-10,[1-5, 63-64] or inhibitory receptors such as PD-1, Tim-3 and CTLA-4 [56-59]. However, our findings presented in chapter 6 show that there is no dominant mechanism suppressing HCV-specific immunity. This strong heterogeneity of regulation has not been emphasized before. However, recent data are in line with our assumption, as involvement in the

suppression of HCV-specific immunity has been shown for both TGF-β and IL-10,[38-39] TIM-3 and PD-1 [60], PD-1 and CTLA-4 [61], or TGF-β, IL-10 and PD-1 [62]. The heterogeneous character of regulation of HCV-specific T cell immunity is reminiscent of the expression of exhaustion markers and their functional synergy to control HCV-specific T cell immunity [65].

I hypothesize that differences in regulatory profile reflect differences in disease progression between individual chronic HCV-infected patients. Possibly, time-since-infection, and subsequent differences in level of viral replication, liver inflammation, pathology and other disease parameters are important factors explaining the heterogeneous regulation of T cell responses in chronic and therapy-induced resolved HCV infection. This has been proposed before as a general model for regulation of immunity to chronic infections.[66] In line with this we showed in chapter 6 that IL-10 mediated suppression of HCV-specific IFN-γ production affects the level of HCV replication in therapy naive patients, but not in therapy-experienced patients. However, more research should be done to delineate what regulatory mechanisms are important during the different phases of HCV related disease. Possibly differences between patients in the regulatory processes that are active may explain why certain patients spontaneously clear HCV or respond to PegIFN-α/ribavirin therapy and others do not. Moreover, regulation of HCV-specific immunity was modulated during and after PegIFN-α/ribavirin therapy. This may be a direct and persisting effect of the exogenous IFN-α, however may also be an indirect effect of viral clearance, or possibly an effect of lower levels of hepatic damage due to PegIFN-α/ribavirin therapy (reviewed in Bailly et al. 2010).

In conclusion

The findings presented in this thesis advance our understanding of the importance of negative regulation by IL-10, TGF-β and Treg. First, negative regulation by intrahepatic regulatory T cells may limit immunopathology during chronic hepatitis C virus infections. Second, TGF-β may protect against immunopathology. Third, the balance between negative regulation and effector T cell functions may explain the response to IFN-α-based therapy. Fourth, retention of intrahepatic Treg occurs following successful therapy-induced viral clearance of HCV. Finally, the heterogeneity of negative regulation of hepatitis C virus-specific immunity may reflect differences in disease progression between patients.

Last and important, we have also learned that blood data do not necessarily reflect intrahepatic immunity. In chapter 2 we showed that Treg are present in high numbers in HCV-infected livers, while almost absent from healthy liver, whereas Treg frequencies in blood did not differ greatly between HCV patients and healthy control subjects. Moreover, similar to the findings by Burton and colleagues, we described in chapter 4 that Treg frequencies measured in blood remained unchanged during IFN-α-based therapy, while intrahepatic Treg frequencies changed dramatically [6]. These findings suggest that the intrahepatic, but not blood, compartment may play an important role in the negative regulation of curative immune responses to HCV during chronic HCV infection and during PegIFN-α/ribavirin therapy. Therefore, investigators should increase their efforts to study the liver compartment, although there are many technical and ethical problems to it.

In vivo depletion of Treg, or functional blockade of IL-10 or TGF-β in patients are potential strategies tot revive antiviral immunity in patients with chronic HCV infections. However, restoration of protective immunity to HCV may come at the high cost of a severe

hepatitis and may cause severe damage to the liver, severe morbidity and even death of the patient. Therefore, studies should focus on the safety of these strategies first.

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Appendices

Samenvatting voor leken (summary in Dutch for laymen)

INLEIDING

Epidemiologie van hepatitis C virus infecties

Het hepatitis C virus (HCV) is nog maar relatief kortgeleden ontdekt. De leverziekte veroorzaakt door HCV was sinds de jaren zeventig van de twintigste eeuw bekend als non-A non-B hepatitis totdat het virus zelf in 1989 werd geïdentificeerd. De wereldwijd meest voorkomende subtypen van HCV zijn genotype 1 tot en met 4. Besmetting met HCV vindt vrijwel alleen plaats via bloed-bloedcontact en leidt bij 80% van de patiënten tot een chronische infectie van de lever en wordt dus slechts door een minderheid van de patiënten geëlimineerd. Dit komt doordat HCV meestal succesvol het afweersysteem ontwijkt. Hierdoor zijn naar schatting 120 tot 170 miljoen patiënten wereldwijd chronisch geïnfecteerd met HCV (chronische HCV patiënten). In Nederland komt HCV relatief weinig voor en ongeveer 0,1 tot 0,4% van de bevolking is chronisch geïnfecteerd, wat overeenkomt met tussen de 16.000 en 67.000 mensen (bron: Gezondheidsraad, publicatienummer 1997/19). De infectie komt vaker voor bij sommige groepen migranten uit landen waar chronische HCV infecties wel vaak voorkomen, bijvoorbeeld Egypte en veel landen in sub-Sahara Afrika. Ook mensen die ooit drugs in hun bloedbaan injecteerden zijn veel vaker met HCV geïnfecteerd en vormen de overgrote meerderheid van de chronische HCV patiënten in Nederland.

Symptomen en gevolgen van hepatitis C virus infecties

De symptomen van een chronische infectie met HCV zijn meestal relatief mild en het kan tientallen jaren duren voordat de ernstige gevolgen van een chronische HCV infectie duidelijk worden. Na verloop van tijd hebben HCV patiënten echter een sterk verhoogd risico op het ontwikkelen van verlittekening van de lever (fibrose). Op de lange termijn kan deze fibrose leiden tot cirrose (zeer ernstige fibrose) en daardoor een sterke vermindering of volledige uitval van de functie van de lever (decompensatie of leverfalen), of leverkanker (hepatocellulair carcinoom). De lange termijn gevolgen van chronische infecties met HCV zijn op dit moment verantwoordelijk voor ongeveer een derde van de 6000 levertransplantaties die jaarlijks in de Verenigde Staten plaatsvinden. Veel patiënten komen echter niet in aanmerking voor een levertransplantatie en overlijden door de ziekte.

Alpha interferon is de hoeksteen van de huidige behandeling van hepatitis C virus infecties

Interferon-alpha (IFN-α) is een lichaamseigen signaalmolecuul dat onderdeel uitmaakt van het natuurlijke afweersysteem tegen virussen. De huidige standaard behandeling van HCV infecties bestaat uit synthetisch nagemaakt IFN-α met daaraan polyethyleenglycol (peg), een groot molecuul dat ervoor zorgt dat IFN-α langer in de bloedbaan blijft. Met behulp van de combinatie van PegIFN-α en het virus remmende medicijn ribavirine kan 80% van de met genotype 2 en 3 geïnfecteerde patiënten succesvol het virus klaren. Hierdoor worden de lange termijn complicaties van chronische HCV infecties voorkomen. De combinatie van PegIFN-α en ribavirine noem ik hierna kortweg

IFN-α. Deze IFN-α therapie is echter minder succesvol bij de grote groep genotype 1 patiënten, die in slechts 40 tot 50% van de gevallen het virus definitief kwijtraakt. Voor deze moeilijk te behandelen groep komen echter binnenkort nieuwe virusremmende medicijnen op de markt die samen met IFN-α en waarschijnlijk ook ribavirine gegeven zullen worden en in studieverband veelbelovende resultaten hebben laten zien.

De rol van het afweersysteem bij hepatitis C virus infecties

HCV doodt de levercellen (hepatocyten) die het infecteert niet, maar veroorzaakt een afweerreactie die leidt tot een chronische ontsteking van de lever. Bij deze ontsteking spelen specifiek tegen HCV gerichte cellen (T cellen) een grote rol. Deze ontsteking is echter meestal relatief mild doordat de afweerreactie door T cellen slecht functioneert. Onderdrukking, of regulatie van de HCV specifieke T cel afweer door regulatoire T cellen (Treg) en twee signaal moleculen, interleukine-10 (IL-10) en tumor groei factor-bèta (TGF-β), zijn mogelijk mede verantwoordelijk voor deze T cel dysfunctie. De keerzijde van deze zwakke specifieke afweer is dat besmetting met HCV zelden geklaard wordt en zoals hierboven beschreven meestal tot een chronische infectie leidt. Regulatie van de specifieke T cel afweer leidt dus mogelijk tot uitblijven van klaring van het virus met als winst een slechts milde ontsteking met relatief trage verlittekening van de lever.

DIT PROEFSCHRIFT

Doel van het onderzoek

Het doel van het onderzoek dat wordt beschreven in dit proefschrift was om meer inzicht te krijgen in de remmende werking van IL-10, TGF-β en Treg op de specifieke T cel afweer tegen HCV infecties en de verlittekening van de lever. Daarbij was extra aandacht voor twee aspecten die in de eerdere literatuur relatief verwaarloosd werden: remming van de HCV specifieke afweer in de lever en verandering van deze remming onder invloed van behandeling van HCV infecties met IFN-α.

Bevindingen gepresenteerd in dit proefschrift

Rol van Treg in de lever tijdens HCV infecties en invloed van behandeling met IFN-α

In hoofdstuk 2 worden de frequenties en het fenotype van Treg en gewone HCV specifieke T cellen bestudeerd in de lever en het bloed van chronische HCV patiënten in verschillende fasen van leverziekte. De bevindingen werden vergeleken met lever- en bloedmateriaal van gezonde proefpersonen. We vonden dat overvloedige aantallen Treg zich specifiek in de levers van chronische HCV patiënten bevonden en niet in de levers van gezonde proefpersonen. Verder bleek dat deze Treg voor een deel de ernst van de lever verlittekening beperkten.

In overeenstemming met onze bevindingen bij chronische HCV patiënten wordt in hoofdstuk 3 beschreven dat het fenotype van Treg in bloed en lever van chronisch met hepatitis B virus (HBV) geïnfecteerde patiënten sterk van elkaar verschilt. Dit hoofdstuk laat verder zien dat remming door Treg niet exclusief is voor HCV infecties, maar ook van belang is voor andere chronische ontstekingen van de lever. Verder laten we in dit hoofdstuk zien

dat een groter aantal Treg in de lever samenhangt met een groter aantal HBV deeltjes in het bloed, mogelijk door een remmend effect op de afweer tegen HBV.

In hoofdstuk 4 hebben we onderzocht hoe IFN-α therapie het aantal Treg in de lever beïnvloedt. Tot onze verrassing vonden wij dat klaring van HCV door IFN-α therapie niet tot normalisatie van de locale afweerreactie in de lever leidt naar het niveau van gezonde, nooit geinfecteerde levers. Tijdens de IFN-α therapie nam het aantal Treg zelfs toe en dit aantal bleef hoog tot maanden na het einde van de behandeling, ook in patiënten die het virus geklaard hadden. Dit suggereert dat er nog steeds afweerprocessen geremd worden, zelfs als het virus al geruime tijd geklaard is.

De rol van het afweersysteem tijdens behandeling met IFN-α

Het tweede deel van dit proefschrift beschrijft twee prospectieve studies waarin verschillende afweerprocessen voor, tijdens en na IFN-α therapie van chronische HCV patiënten onderzocht worden. De eerste studie laat zien dat een sterke specifieke T cel afweer tegen HCV bij aanvang van de behandeling en tijdens de behandeling niet automatisch leidt tot een goede uitkomst van de therapie (hoofdstuk 5). De tweede studie (hoofdstuk 6) maakt duidelijk dat de remming van de HCV specifieke afweer door IL-10, TGF-β en Treg zeer heterogeen is. Dit wil zeggen dat patiënten onderling een verschillende combinatie van remming laten zien. Waarschijnlijk komt dit doordat de bestudeerde patiënten in verschillende fasen van HCV gerelateerde ziekte verkeren met daardoor variatie in ziekte-activiteit en bijpassende soorten remming van het afweersysteem. Sterke remming van de afweer door IL-10 leidde bijvoorbeeld alleen bij patiënten die nooit eerder behandeld waren met IFN-α tot een lager aantal HCV deeltjes, terwijl dit niet het geval was bij patiënten die al eens met IFN-α behandeld waren. Verder laten wij zien dat de remming van de specifieke afweer door IL-10, TGF-β en Treg wordt beïnvloed door IFN-α therapie. Zo neemt de remming van de specifieke T cel afweer door TGF-β sterk toe onder invloed van de behandeling en houdt aan tot lang na de behandeling.

CONCLUSIE

De bevindingen gepresenteerd in dit proefschrift bevorderen ons begrip van het belang van remming van de specifieke afweer door IL-10, TGF-β en Treg. Ten eerste remmen Treg in de lever en mogelijk ook TGF-β de verlittekening van de lever die optreedt als gevolg van een chronische infectie met HCV. Ten tweede is de balans tussen de specifieke T cel activiteit en remmende effecten van IL-10, TGF-β en Treg waarschijnlijk verantwoordelijk voor de kans op een succesvolle behandeling van HCV met IFN-α. Ten derde is de combinatie van remmende factoren op de T cel respons waarschijnlijk een gevolg van de verschillende fasen van HCV gerelateerde ziekte waar patiënten in verkeren.

We hebben verder ook geleerd dat gegevens over het afweersysteem in het circulerende bloed niet noodzakelijk een weergave zijn van het afweersysteem in de lever, de plaats waar de infectie plaatsvindt en de afweer tegen HCV ook het meest relevant is. Daarom moeten onderzoekers in de toekomst nog meer moeite doen om de lever zelf te

bestuderen, zoals wij dit ook deden, ondanks dat dit veel technische en ethische problemen met zich meebrengt.

Blokkeren van de remmende effecten van Treg, IL-10 of TGF-β in chronische HCV patiënten is een strategie die in de toekomst mogelijk de specifieke afweer tegen HCV kan herstellen, waardoor het virus alsnog door het eigen afweersysteem geklaard kan worden. Herstel van de beschermende afweer tegen HCV kan echter ook een ernstige hepatitis tot gevolg hebben en leidt daardoor mogelijk tot ernstige acute beschadiging van de lever en mogelijk zelfs een fatale afloop voor de patiënt. Daarom moeten wetenschappers die deze aanpak willen onderzoeken zich eerst concentreren op de veiligheidsaspecten.

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Curriculum vitae auctoris

The author of this thesis was born on May 12th, 1977 in Numansdorp, the Netherlands. After graduating from high school at Marnix Gymnasium, Rotterdam in 1995, Mark moved to Belgium to study medicine at the Catholic University Leuven, where he met his wife Kristien De Bruyne. After completing the first two years (kandidaturen) of medicine in Leuven in 1999, he continued his studies at Erasmus MC, part of Erasmus University Rotterdam, the Netherlands. In the years 2000-2002 he was chairman of STOLA, a foundation that organizes internships in developing countries for Erasmus MC medical students. In 2002 he performed a research project together with Brigitte Elbers on *Pneumocystis carinii* and *mycobacterial* infections in HIV positive patients in a rural hospital in Manyemen, Cameroon under supervision of Dr. J.L. Nouwen and Dr. C. Timah, and in November 2002, he received his Master of Science degree in medicine. August 2002 he entered a second Master of Science program on clinical epidemiology at the Netherlands Institute for Health Sciences (NIHES), Rotterdam. As part of this program, he performed a research project on *Staphylococcus aureus* nasal carriage and Vitamin D receptor gene polymorphisms under supervision of Dr. A. Ott and Dr. J.L. Nouwen and attended the annual Harvard School of Public Health summer program, Boston, Massachusetts, USA. After obtaining his Master of Science degree in clinical epidemiology in 2003, and his medical degree in 2005, he shortly worked as a resident (ANIOS) internal medicine at Medisch Centrum Rijnmond Zuid, Rotterdam before starting his PhD study at the department of Gastroenterology and Hepatology of Erasmus MC, Rotterdam, supervised by Dr. A. Boonstra and Prof.dr. H.L.A. Janssen. The research performed at the department of Gastroenterology and Hepatology is presented in this thesis. In January 2010 Mark enrolled in the residency program in internal medicine of Erasmus MC, headed by Prof.dr. J.L.C.M. van Saase and started his training at Maasstad Ziekenhuis, Rotterdam supervised by dr. M.A. van den Dorpel.

In July 2006 Mark married Kristien, now a talented general practitioner. Together they live in Rotterdam and are parents to Sem (2008) and Nora (2010).

List of publications

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T cell responses at baseline and during therapy with peginterferon-α and ribavirin are not associated with outcome in chronic hepatitis C infected patients.
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Mark A.A. Claassen, Robert J. de Knegt, Harry L.A. Janssen, André Boonstra
Retention of $CD4^+CD25^+FoxP3^+$ regulatory T cells in the liver after therapy-induced hepatitis C virus eradication in humans.
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Mark A.A. Claassen, Robert J. de Knegt, Duygu Turgut, Zwier M.A. Groothuismink Harry L.A. Janssen, André Boonstra.
Negative regulation of hepatitis C virus-specific immunity is highly heterogeneous and modulated by pegylated interferon-alpha/ribavirin therapy.
Submitted

Abbreviations

ALT	alanine transaminase
CD	cluster of differentiation
CM	central-memory
CMV	cytomegalovirus
cpm	counts per minute
DC	dendritic cell
DMSO	dimethylsulphoxide
E	envelope
ELISA	enzyme-linked immunosorbent assay
EM	effector-memory
FACS	fluorescence activated cell sorting
FoxP3	forkhead box P3
FNAB	fine needle aspiration biopsy
HBV	hepatitis B virus
HCV	hepatitis C virus
HIV	human immunodeficiency virus
HSC	hepatic stellate cell
IFN-α	alpha interferon
IFN-β	beta interferon
IFN-γ	gamma interferon
IL	interleukin
IL-10R	interleukin-10 receptor
IQR	interquartile range
ISG	interferon stimulated genes
LAP	latency associated peptide
LSEC	liver sinusoidal endothelial cells
MHC	major histocompatability complex
NK	natural killer cell
NS	non-structural
PAMP	pathogen associated molecular pattern
PBMC	peripheral blood mononuclear cell
pDC	plasmacytoid dendritic cell
PD-1	programmed death-1 (receptor)
Peg	pegylated, or: polyethylene glycolated
pegIFN-α	pegylated interferon alfa-2a or 2b
PMA	phorbol myristate acetate
RIG-I	retinoic acid–inducible gene I
RNA	Ribonucleic Acid
RVR	rapid virological response
SI	stimulation index
STAT	Signal Transducers and Activators of Transcription
SVR	sustained virological response
Treg	regulatory T cell
Term	terminally differentiated
TGF-β	tumor growth factor-beta
Th	T helper cell
Tim-3	T cell immunoglobulin domain and mucin domain 3
TNF	Tumor necrosis factor
TLR	toll-like receptor-3

PhD portfolio

Name PhD student	Mark Arthur Alvin Claassen
Erasmus MC Department	Gastroenterology and Hepatology
PhD period	December 2005 – December 2009
Promotor	Prof.dr. H.L.A. Janssen
Copromotor	Dr. P.A. Boonstra

Summary of PhD training activities

General academic and research skills (Erasmus MC)
English biomedical writing and communication
Radioactivity safety course, level 5B
MolMed Annual course on biomedical research techniques

Selection of conferences and presentations

2006

- 41st Annual Meeting of the European Association for the Study of the Liver (EASL), April 26-30, Vienna, Austria.
- Post-EASL symposium, May 9, Utrecht, the Netherlands.
  Invited oral presentation: Overview on HCV immunology
- Monothematic Conference on Clinical Immunology in Viral Hepatitis, sponsored by the European Association for the Study of the Liver EASL) and endorsed by the American Association for the Study of Liver Diseases (AASLD), October 7-8, University College London, United Kingdom.

2007

- 2nd International Conference on the Management of patients with Viral Hepatitis, January 22-23, Paris, France.
- 14th International Symposium on Hepatitis C Virus and Related Viruses, September 9-13, Glasgow, Scotland.
  Oral presentation: Intrahepatic Regulatory T cells differ from their circulating counterparts in chronic HCV patients
- 58th Annual Meeting of the American Association for the Study of Liver Diseases (AASLD), November 2-6, Boston, MA, United States of America.
  Poster presentation: Intrahepatic Regulatory T cells differ phenotypically from their circulating counterparts in chronic therapy naïve HCV patients
  Poster presentation: Quantification of the anti-HCV activity in Chronic Hepatitis C - genotype 1 patients using a replicon-based bioassay.
- Annual meeting of the Dutch Society of Gastroenterology and Hepatology (NVGE), October 4-5, Veldhoven, the Netherlands.

Poster presentation: Intrahepatic Regulatory T cells differ phenotypically from their circulating counterparts in chronic therapy naïve HCV patients.
Poster presentation: Detection of anti-HCV factors in blood plasma of Chronic Hepatitis C patients using a replicon-based bioassay.

- Annual meeting of the Dutch Society for Immunology (NVVI), December 20-21, Noordwijkerhout, the Netherlands.
  Poster presentation: Intrahepatic Regulatory T cells differ phenotypically from their circulating counterparts in chronic HCV-infected patients.

2008

- Annual meeting of the Dutch Society of Gastroenterology and Hepatology (NVGE), March 13-14, Veldhoven, the Netherlands.
  Poster presentation: Conclusive evidence for the importance of intrahepatic regulatory T cells in chronic hepatitis C patients.
- World Immune Regulation Meeting, March 16-20, Davos, Switzerland.
  Poster presentation: Phenotype of peripheral blood and liver regulatory T cells in chronic Hepatitis C infected patients.
- 59th Annual Meeting of the American Association for the Study of Liver Diseases (AASLD), October 31-November 4, San Francisco, CA, United States of America.
  Poster presentation: Abundant numbers of highly activated and differentiated regulatory T cells localize to the liver of chronic hepatitis C infected patients.
- Annual meeting of the Dutch Society for Immunology (NVVI), December 18-19, Noordwijkerhout, the Netherlands.
  Poster presentation: Phenotype of peripheral blood and liver regulatory T cells in chronic Hepatitis C infected patients.

2009

- Annual meeting of the Dutch Society of Gastroenterology and Hepatology (NVGE), March 19-20, Veldhoven, the Netherlands.
  Poster presentation: Role of multiple regulatory T cell populations in controlling peripheral blood and liver immunity to human hepatitis C virus infections.
- Keystone symposium on Regulatory T cells, March 1-5, Keystone, Colorado, United States of America.
  Oral presentation: Role of multiple regulatory T cell populations in controlling peripheral blood and liver immunity to human hepatitis C virus infections.
- 60th Annual Meeting of the American Association for the Study of Liver Diseases (AASLD), October 30-November 3, Boston, MA, United States of America.
  Poster presentation: High numbers of intrahepatic regulatory T cells in chronic HCV patients are negatively linked to severity of disease.

2010

- Keystone symposium on Viral Immunity, March 21-25, Banff, Alberta, Canada.
  Poster presentation: Role of multiple regulatory T cell populations in controlling peripheral blood and liver immunity to human hepatitis C virus infections

Supervising Bachelors theses

2008

- Onderzoek naar het effect van interferon-alpha en ribavirine op het immuunsysteem, Sharine van der Veen.

2009

- HCV-specific T cells with a IL-10^{+}CD4^{+} phenotype express IFN-γ mRNA, while endogenous IL-10 and IL-10/TGF-β suppress the IFN-γ production in peripheral blood derived from chronically HCV-infected patients, Silvie Hansenová Maňásková.