Immune responses of chronic HCV infection. Throughout and long-term after therapy

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Immune Responses of Chronic HCV Infection.
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INTRODUCTION

The hepatitis C virus

The hepatitis C virus (HCV) is a small positive-stranded enveloped RNA virus and member of the flavivirus family. Until 1989, when the virus was discovered, the virus was known as non-A non-B virus [1]. The HCV RNA molecule consists of a single open reading frame that is about 9600 nucleotide bases long. The open reading frame is used to produce a single long polyprotein, which is cut by both host and viral proteases into structural viral proteins (core protein and envelope proteins E1 and E2) and non-structural viral proteins (NS2, NS3, NS4A, NS4B, NS5A, NS5B). NS3-4A is a serine protease that is responsible for cleavage at four sites of the HCV polyprotein and is essential for viral replication. The NS5B protein is the viral RNA polymerase, important for viral replication. Since the NS5B RNA polymerase lacks proofreading capacities, the virus has a high mutation rate which leads to an enormous diversity and various subtypes. The HCV virus has six major genotypes (1 to 6) and more than 100 subtypes have been identified. This natural diversity is one of the challenges that needs to be taken in consideration for the development of an effective HCV vaccine [2].

The global burden of chronic hepatitis C

Approximately 170 million people (about 3% of the world population) have a chronic HCV infection [3]. In the Netherlands, around 30,000 patients have been identified to be chronically infected with HCV, with the highest prevalence among migrants from endemic countries, intravenous drug abusers and men who have sex with men (MSM) [4]. Of the patients that are infected with HCV only a fraction of individuals are able to clear the infection spontaneously, whereas about 80% of the infected individuals develop a chronic infection [5, 6]. In these persistently infected patients the symptoms are relatively mild, and it may take decades before the serious consequences of chronic HCV infection become apparent. These patients are at increased risk for developing liver fibrosis, cirrhosis and/or hepatocellular carcinoma. Furthermore, the long-term complications of liver failure, as a result of chronic HCV infection, are an important indication for liver transplantation [7, 8]. Due to the implementations of routine blood testing before blood transfusions, but also the awareness among drug abusers to use clean needles, the incidence of new infections is decreasing [9]. This is reflected by stabilization of the number of liver transplantations since 2006 [10]. Viral eradication by direct acting antivirals (DAA) may further stabilize disease progression and the need for liver transplantation.

Nevertheless, the global burden for hepatitis C will increase dramatically in the future. The main reason for this is the high infection rate in the 80's and considering the slow disease progression, many patients are expected to be identified with tardy cirrhosis and accompanying liver failure or HCC in the upcoming years [7].

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The immune response in hepatitis C infection

Chronic infections pose a serious health problem worldwide. Diseases like malaria, tuberculosis, HIV and viral hepatitis have infected millions of people, and continue to cause significant morbidity and mortality. These diverse infections, caused by bacteria, parasites or viruses, become chronic when invading pathogens avoid or disrupt elimination by the host immune response in order to allow their survival and to guarantee their successful transmission. HCV is one example of a viral pathogen that is very successful in establishing persistent infections by evading the immune system.

The immune response is of pivotal importance for control of diverse viral infections, also during HCV infection [11-13]. Pathogen recognition receptors continuously sense the environment and recognize viral products resulting in the rapid production of pro-inflammatory cytokines, such as TNF and IL-6, as well as type I interferons (IFN), IFN-α and IFN-β. Type I IFN initiate a nonspecific antiviral immune response by the induction of antiviral products, and they are also immunomodulatory by priming distinct immune cells, including natural killer (NK) cells and T cells, to efficiently respond to the attack of the host by pathogens. In addition, during viral infections, virus-specific CD4$^+$ and CD8$^+$ T cells play an important role, and –in general- these cells can be detected in blood. The helper CD4$^+$ T cells provide help to cytotoxic CD8$^+$ T cells, which are able to eradicate the virus in a highly specific manner.

In this following sections, I will present an overview of my understanding of the immunological mechanisms that are acting on HCV-infected cells in the liver, and that are impaired or insufficient to eliminate the virus in patients chronically infected with HCV.

Innate immunity to HCV

IFN responses induced by HCV

Upon recognition of viral products by pathogen recognition receptors, infected hepatocytes rapidly induce type I IFN. The family of type I IFN include many structurally related members, such as 13 subtypes of IFN-α, IFN-β, and IFN-ω. They are genetically and structurally very similar, and all interact with the same receptor [14]. Type I IFN act by binding to their specific receptor, which leads to activation of the intracellular pathway, involving the Janus-activated kinase (Jak) and signal transducers and activators of transcription (STAT) proteins. Activation of this pathway leads to the induction of multiple interferon-stimulated genes (ISG) [15]. The proteins encoded by a number of these genes, including protein kinase R (PKR), Mx proteins, ISG-15, RnaseL/2,5-OAS pathway and RNA helicases, have been well-characterized, and via different mechanisms all have potent antiviral activity [16]. Upon stimulation with IFN-α, expression of hundreds of different interferon-stimulated genes are induced, suggesting that the host has evolved multiple redundant pathways to make evasion of specific IFN-induced antiviral responses more difficult.

Indications of a role for type I IFN during viral hepatitis infection are based on studies in chimpanzees, since this is the only immunocompetent animal that can be infected with HCV [17]. As early as 2 days after infection a strong upregulation of interferon-stimulated genes was observed in the liver. This response is most likely initiated in hepatocytes by triggering of the pathogen recognition receptor retinoic acid-inducible gene-I (RIG-I) that recognizes the polyuridine motif of the HCV 3' UTR in the cytoplasm [18]. Also, Toll-like receptor 3 (TLR3) may be involved by recognizing HCV double strand RNA in the endosome of HCV-infected hepatocytes [19]. The increase in ISGs 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 outcome is a self-limiting resolving infection, or whether a chronic HCV infection develops. The observation that HCV RNA levels increase despite the induction of type I IFN early after infection demonstrates that evasion mechanisms of the virus have evolved. Indeed, in recent years it has been shown that HCV proteins attenuate the induction or activity of the IFN response by degradation or inhibition of crucial molecules, such as CARD and TRIF that are involved in subsequent signal cascade triggered by HCV [20]. Four to six weeks after infection, the HCV RNA levels remain relatively stable, suggesting that the balance has been achieved between viral replication and control of the replication by immune pressure. Besides being a potent inducer of the antiviral response, IFN-α is also a potent immunomodulatory protein that modulates the activity of numerous leukocytes, including NK cells, macrophages, DC and T cells, thereby making it an important cytokine that influences both the innate and adaptive immune system [15]. The role for IFN in the treatment for HCV will be discussed later on in this introduction.

Role of NK cells
NK cells play an important role in innate immune responses, and contribute to immunity against HCV infection [21, 22]. They are known for their nonspecific cytotoxicity against cells not recognized as self, and are recruited to the liver shortly after infection [23]. HCV-infected hepatocytes are therefore likely targets for NK cells. Activated NK cells are able to kill virus-infected cells via cytotoxic molecules, such as perforin or granzymes. In contrast, cytokines (e.g. IFN-γ, TNF) lead to suppression of viral replication as well as activation of subsequent adaptive immune responses. The activation of NK cells is regulated by the balanced expression of various activating and inhibitory receptors. In a healthy state, the inhibitory signals are dominant, but during viral infection, NK cells express a more activated state [24]. Among the inhibitory receptors are killer immunoglobulin-like receptors (KIRs), like CD158a and CD158b, which recognize MHC class I molecules. To evade specific T cell immunity, tumor or virus infected cells down-regulate MHC-class I molecules and in response to this adaptation, NK cells preferentially kill cells with down-regulated MHC class I. This is called the missing-self hypothesis. Other natural killer cell receptors include C-type lectins for inhibitory (NKG2A) and activation (NKG2C/D) signals, as well as the natural cytotoxicity

receptors (NCRs) NKp30, NKp44 and NKp46 that deliver activation signals. Tumor necrosis factor (TNF)-related apoptosis-induced ligand (TRAIL) can also be expressed on NK cells and upon ligation can induce cell death [25].

By determining the genotype of genes expressing inhibitory and activating receptor in individuals infected with HCV, it was shown that individuals who are homozygous for the inhibitory NK cell receptor HLA-C1 and KIR2DL3 resolve HCV infection more frequently than patients with other genotypes [26]. Besides genetic factors, the function of NK cells may also be directly impaired by binding of the HCV envelope protein E2 to the surface molecule CD81 on NK cells, although exposure of healthy donor NK cells to *in vitro* produced infectious HCV virions did not inhibit NK cell activation and IFN-γ production [27-29]. Furthermore, it has been reported recently that during acute infection, NK cells show increased expression of the activating receptor NKG2D, and enhanced IFN-γ production, degranulation and cytotoxicity [30, 31].

In contrast to the acute phase, NK cells from chronic HCV patients display a mildly augmented cytotoxic potential as compared to healthy individuals, whereas the ability of NK cells to produce IFN-γ is not or only weakly affected [32, 33]. In addition, Oliviero also demonstrated that NK cells from HCV patients display a more activated phenotype, and therefore possibly contribute to the immune responses involved to control viral persistence. However, compared to NK cells from healthy individuals, NK cells from chronic HCV patients show higher expression of the inhibitory receptor CD94/NKG2A and produced higher levels of the immunosuppressive cytokines IL-10 and TGF-β when cultured with hepatic cells [34]. On the contrary there are other groups that report that NK cells are hampered in chronic HCV infection and unable to provide a proper cytotoxic response [33, 35], while others find minimal or no difference in NK cells function between HCV patients and healthy controls [36].

Thus, although we are beginning to understand the complexity of receptor expression on NK cells, a detailed understanding of the implications of the changes in receptor expression require further studies that focus on the simultaneous detection of multiple receptors on NK cells instead of only a single receptor and correlate these to functional properties.

As described before, endogenous IFN-α is secreted by hepatocytes in HCV-infected livers [18, 19]. IFN-α is a potent activator of NK cells, which results in the induction of IFN-γ. Binding of IFN-α to its specific receptor leads to phosphorylation of STAT1, and it has been reported that pSTAT1 levels are higher in chronic HCV patients than in healthy individuals, suggesting a higher sensitivity of NK cells to IFN-α, but lower sensitivity to STAT4 suggesting their weaker ability to respond to IL-12p70. However, this was not reflected by enhanced IFN-α induced cytotoxic parameters in NK cells, such as perforin or granzyme levels or CD107 degranulation [37].

The vast majority of studies examined circulating NK cells in HCV. The few studies that examined the intrahepatic compartment assessed the phenotype of cells in the liver, making functional data very limited, although important observations are reported as correlations with clinical parameters [26, 35, 38-42]. For instance, recently an intra-hepatic accumulation of NKp46^{high} NK cells was observed in HCV patients. These cells display a higher cytolytic activity, and the frequency of intra-hepatic NKp46^{high} NK cells was inversely correlated with HCV-RNA levels and fibrosis stage [43].

Role of NKT cells

Natural Killer T (NKT) cells are CD1d-restricted lymphocytes that express both CD56 and CD3, and are activated by the presentation of lipid antigens to their invariant TCR. NKT cells produce cytokines like IFN-γ and IL-4, within minutes after antigen stimulation. In general, two classes of CD1d-restricted NKT cells are distinguished. The classical type I NKT cell recognises lipid antigens and Type II NKT cells recognise also other hydrophobic and non-lipid molecules by the expression of more diverse TCR Vα chains [44, 45]. While viruses do not express glycolipids, NKT cells are nevertheless involved in antiviral responses. The factors that regulate peripheral NKT cell development are not well understood. In contrast to conventional T cells, which require ongoing interaction with their ligand (MHC class I or II), NKT cells do not require ongoing interactions with CD1d [46]. NKT cell development seems to be predominantly dependent on the cytokines IL-15 and IL-7 [47]. The major paradox is that NKT cells are both able to stimulate and inhibit immune responses. This is probably due to the presence of many subtypes of NKT cells which produce different cytokines at different locations [44]. Different subtypes of NKT cells can produce Th1 and/or Th2 cytokines.

The role of NKT cells in antiviral immunity has been investigated for infection with intravaginal HSV-2 infection. CD1d-deficient mice exhibited reduced survival compared to healthy mice. Both NK cells, CD1d-restricted NKT cells, IFN-γ and especially IL-15 were shown to play a role [48]. The importance of NKT cell in chronic hepatitis infection remains unclear. In LCMV infected mice, which is widely used as a model for chronic HCV and HBV infection, NKT cells were excluded as a major contributor. CD1d-deficient mice exhibited viral titers comparable to wild type mice and virus-specific T-cell responses were generated equally effective [49]. This suggests that NKT cells do not play an active role in the control of these viruses, however they may be important for regulating certain immune responses. In chronic HCV infection, the frequency of NKT cells was found to be higher compared to healthy individuals. Whether the decreased frequency was due to apoptosis of these cells or due to migration to the liver, could was not determined in this study [50, 51]. However, CXCR6, a chemokine that causes NKT cell migration to the liver has shown to be increased in hepatitis infection [52]. Although NKT cells do not seem to play a significant role in the control of some viral infections, their ability to rapidly release immunoregulatory cytokines makes them an interesting cell type worth exploiting to improve antiviral immune responses.

Role of dendritic cells

Besides NK cells, dendritic cells (DC) are also key players in the initiation of immune responses. After pathogen encounter, DC are activated, which is characterized by the upregulation of MHC molecules, costimulatory and adhesion molecules, as well as by the production of chemokines and cytokines. Following migration of activated DC to the lymph nodes, an intimate interaction with CD4+ helper T cells and cytotoxic CD8+ T cells takes place, leading to the activation of the T cell compartment. The strength of the interaction between DC and T cells as well as specific cytokines such as IFN-α or IL-12 may determine the development of T cells towards IFN-γ producing Th1 cells or alternative T cell populations. Two major subsets of human DC have been identified, plasmacytoid DC and conventional myeloid DC, which differ phenotypically and functionally. Myeloid DC are considered classical antigen-presenting cells, and are able to activate naive and effector T cells and facilitate their development into Th1 cells via the secretion of IL-12. Plasmacytoid DC are less efficient in activating T cells, but are specialized in the production of type I and III IFN. In patients with chronic HCV infection, reduced numbers and frequencies of myeloid and plasmacytoid DC have been observed in blood. These effects are not only observed for HCV, but also in HBV and HIV [53-55]. The changes in frequencies may be due to migration to other anatomical compartments in the body, such as the liver for viral hepatitis. Indeed, as demonstrated by immunohistochemistry, myeloid and plasmacytoid DC are enriched in the liver of chronic HCV patients as compared to healthy individuals [56]. Interestingly, it was reported that also type I IFN may influence the number of DC during chronic viral infection, since type I IFN mediates death of plasmacytoid DC via triggering apoptosis [57].

It has been suggested that DC function in HCV infection is hampered as a consequence of decreased antigen presentation to CD4+ T cells mediated through interference by HCV proteins [53, 58]. Moreover, DC-derived IL-12, important for development of IFN-γ-producing Th1 cells, was selectively inhibited by HCV core proteins [59]. IL-12 production by myeloid DC from chronic HCV patients was impaired since less IL-12 was produced in response to pathogen-derived products as compared to healthy controls [53, 60, 61]. Similarly, also reduced production of IFN-α by plasmacytoid DC from chronic HCV patients has been reported upon stimulation *in vitro* with herpes simplex virus or pathogen-derived products [53, 60-63]. Monocyte-derived TNF and IL-10 were shown to be responsible for the reduction of IFN-α production by plasmacytoid DC [63]. Despite being impaired, Takahashi demonstrated a role for plasmacytoid-derived IFN-α in controlling HCV replication. Hepatocyte-to-plasmacytoid DC transfer of RNA led to triggering of plasmacytoid DC and consequently production of endogenous IFN-α [64]. Although numerical and functional modulation of NK and DC has been convincingly demonstrated for chronic HCV patients as compared to healthy individuals, it is not clear if these altered immune parameters contribute and maintain viral persistence, or whether they are the consequence of the continuous presence of high amounts of viral protein. This is also true for monocytes for which we recently showed that the TLR4 signalling pathways was selectively impaired in chronic HCV patients [65]. Recently, a new

subset of myeloid DC, BDCA3^{+} DC, have been identified. BDCA3^{+} DC express CLEG9A, DNGR-1 and XCR1 and produce mainly IFN-λ. BDCA3^{+} DC may be involved in anti-viral immunity, since IFN-λ directly inhibits viral replication [66]. For a long time BDCA3^{+} DC were thought to be superior in the cross-presentation of soluble viral antigen, however recent studies showed that that there are other subsets of DC similarly capable[67]. However, for the cross-presentation of necrotic cells to T cells, BDCA3^{+} DC have shown to be superior [68, 69]. Future studies will have to address the importance of BDCA3^{+} DC, plasmacytoid DC and myeloid DC in the initiation of an effective immune response against HCV.

Monocytes

Monocytes are a subset of circulating white blood cells that can further differentiate into a range of tissue macrophages and dendritic cells (DC). Human blood monocytes are far more abundant than blood DC, representing about 10% of the total peripheral blood mononuclear cells (PBMC) [70]. Monocytes play an important role in homeostasis by scavenging necrotic and toxic compounds and by producing large amounts of cytokines like TNF and IFN-γ in the defence against pathogens [71, 72]. The ability of monocytes to migrate to the inflamed or damaged site is central for their function in promoting immune defence during infection and in driving inflammatory diseases like arthritis and atherosclerosis [73, 74]. Human monocytes are divided into subsets on the basis of CD14 and CD16 expression [75]. CD14^{+}CD16^{-} monocytes are the most abundant subtype which express high levels of CC-chemokine receptor 2 (CCR2), which is an important chemokine for trafficking [76, 77]. CD16^{+} monocytes migrate via patrolling [78]. Functionally, both CD16^{+}CD14^{-} and CD14^{+}CD16^{-} monocytes express pathogen-recognition receptors, such as Toll –like receptors (TLR), which enable monocytes to response to a broad range of bacterial and viral pathogens [79], resulting in the production of cytokines and chemokines [70]. The responsiveness of both CD16^{+}CD14^{-} and CD14^{+}CD16^{-} monocytes to TLR4 is well studied [80, 81]. Interestingly it was demonstrated both CD16^{+}CD14^{-} and CD14^{+}CD16^{-} monocytes were found to strongly respond to TLR8 agonists [65, 82]. This is important since HCV is a single-stranded RNA virus, which is a putative agonist for TLR8.

For chronic HCV infections it is generally accepted that CD14^{+}CD16^{-} monocytes from HCV patients are more activated compared to healthy controls [83]. It is not entirely clear how monocytes in chronic HCV responds to TLR4 ligand LPS, as conflicting results are described [65, 83, 84]. It was described that CD14^{+}CD16^{-} monocytes from patients with HCV produce more TNF, IL12 and IL-10 upon TLR8 stimulation [65]. However, CD16^{+}CD14^{-} monocytes did not seem to be affected by chronic HCV [82]. Interestingly, it was reported that the frequency of a TLR8 polymorphisms was significantly altered in patients with chronic HCV compared to healthy controls [85].

Kupffer cells

Kupffer cells (KC) are liver resident cells and known for their phagocytic function. Together with the sinusoidal endothelial cells, KC are the first barrier for pathogens that enter the liver via the portal vein [86]. Kupffer cells express the classical monocyte markers CD14 and CD16 and are further characterised by the expression of CD68 [87, 88]. These markers are however not unique and are expressed by various immune cells in tissues, making it hard to differentiate these cells from, for instance, inflammatory monocytes. It is preferable to identify KC not only by the expression of markers but also based on their morphology and functions since especially during viral infection, inflammatory monocytes and DC share certain surface markers.

Besides their function as phagocytes, KC express MHC and co-stimulatory molecules, making them potential APC. In HCV infection, KC become MHC I and II [high], express co-stimulatory molecules CD40 and CD80 and form clusters with CD4^{+} T cells, suggesting a possible role for antigen presentation by KC [89].

Three important antiviral functions for KC have been described in HCV infection. Frist, KC are activated in the HCV infected liver and produce IL-1β, IL-6, TNF and IL-10 [86, 90, 91] that were found to inhibit HCV replication [88, 92]. Second, release of KC-produced cytokines causes migration of liver infiltrating leukocytes and DC and activation of NK cells and NKT cells, which may initiate and promote virus-specific T-cell responses. Besides the antiviral effects of KC during HCV infection, HCV can also evade certain immune responses by interfering with TLR pathways, RIG-I signalling and subsequent immune cell activation [93, 94]. Although many studies have described this effect on monocytes, studies on KC are limited [54, 89, 95].

A function for KC in liver tolerance has been suggested by many investigators, since KC have the capacity to present antigens to T cells along with inhibitory molecules like PD-1 and IL-10, which can induce tolerance. Interestingly, it has been shown that HCV core protein induces IL-10 production by KC [90]. In line, HCV patients have higher serum IL-10 levels [65], which may suppress pro-inflammatory cytokine production, KC-NK cell interaction and antigen presentation to T cells [96-98].

MAIT cells

Mucosal-associated invariant T (MAIT) cells are a relatively newly discovered T cell subtype and part of the innate T cells. In 1999, Tilloy *et al.* ascribed a unique TCRα rearrangement to a new subset of T cells [99]. They were found to be enriched in the gut, which led to their name as mucosal-associated invariant T cells. They are characterized by the expression of an invariant T cell receptor (TCR) Vα7.2 and CD161, a tissue homing receptor. MAIT cells account for 1 to 10% of T cells in peripheral blood [100]. MAIT cells were found to recognize antigen-loaded cells in a MR1-dependent manner, an MHC class-I related molecule (HLA-1b) on antigen-presenting cells (APC). Upon MR1 stimulation, MAIT cells can produce pro-

inflammatory cytokines like IL-17, IFN-γ and TNF [100-102] and release cytotoxic granules [103]. It was already known that MHC molecules present peptides to CD4^{+} and CD8^{+} T cells, and that another MHC-like protein called CD1d presents lipid molecules to a class of T cells called NKT cells. With the characterization of MAIT cells, a third type of antigen-presenting molecule, MR1, was discovered which presents metabolites of B vitamins to the MAIT cells [104]. Besides stimulation by MR1-restricted antigen stimulation, MAIT cells can also become fully activated upon stimulation with the innate cytokines IL-12 and IL-18 [105, 106].

Initially, MAIT cells were thought to be especially important during anti-bacterial responses, reacting to APC infected with a wide variety of bacteria and yeasts [102, 107]. The importance of MAIT cells in the immune response against bacterial infections was confirmed in several mouse models in which a lack of MAIT cells correlated with increased bacterial loads of *Escherichia coli, Mycobacterium* and *Klebsiella* [102, 108]. Furthermore, in patients infected with *M. Tuberculosis*, a pulmonary bacterial infection, MAIT cells were found to be decreased in peripheral blood but enriched at the side of infection, suggesting an active role for MAIT cells in the control of bacterial infections [107]. It has now become clear that MAIT cells are involved in numerous diseases, including multiple sclerosis and psoriasis [109, 110]. Interestingly, in patients with HIV infection, MAIT cells were found to be reduced in peripheral blood and in lymph nodes [105, 111] while their overall cytotoxic potential remained intact. Interestingly, the frequency of MAIT cells fails to recover during antiretroviral therapy (cART) for HIV [111, 112]. One possible explanation for the reduction of MAIT cells during HIV infection is the migration towards other tissues, although an increase of MAIT cells was not observed in colon tissue during HIV infection [112].

During HCV infection little is known on the frequency and function of MAIT cells. The population of CD161^{+} cells, which contains a large proportion of MAIT cells, has shown to be increased in the liver of chronic HCV patients compared to peripheral blood [113, 114]. One study showed that HCV liver contained more CD161^{+}IL-17^{+} cells compared to healthy liver and that the frequency of CD161^{+}IL-17^{+} cells negatively correlated with fibrosis levels [115]. Jo *et al.* elegantly showed that MAIT cells, identified as CD161^{bright} cells, together with NKT cells are the main producers of IFN-γ in the liver upon stimulation with ssRNA40 [116]. However, in end-stage liver disease, CD161+ MAIT cells in the liver are decreased in frequency compared to non-end stage HBV infected liver and healthy liver (around 5% vs 19% and 17% of viable T cells respectively). Since in most of the studies MAIT cells were not identified using a marker for the invariant T cell receptor (Vα7.2), frequency and function of MAIT cells in peripheral blood and the liver in chronic HCV infection remains largely unknown.

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Adaptive immunity to HCV

B-cell responses

B cells are bone marrow derived lymphocytes that express a B-cell receptor (BCR) that recognizes specific antigen. The early development of B cells in the bone marrow occurs through different stages with the processes called VDJ recombination[117]. In the periphery, B cells encounter their antigen and become activated. Once a B cells has encountered its antigen and receives an additional signal from a T-helper cell, it can further differentiate into one of the two types of B cells: plasma B cells and memory B cells [117, 118]. Before this differentiation step however, B cells may undergo an intermediate differentiation step, the Germinal Center (GC) reaction. The GC reaction takes place in the secondary lymphoid structures where mature B cells proliferate, differentiate, mature their antibody genes (by somatic hypermutation) and switch their antibodies (class switch recombination). This maturation of B cells is supported by follicular helper T (T_{FH}) cells and follicular dendritic cells (FDC) and is important for the humoral immune response during infections. It generates matured B cells that are specialized in producing improved antibodies that effectively recognize infectious agents and the production of durable memory B cells [117-119].

If B cells do not undergo this differentiation step in the GC, B cells can be activated in a T cell-dependent or independent manner. When a B cell processes and presents the same antigen to the primed T-helper cell, the T cell secretes cytokines that can activate the B cell. B cells can mature into plasma B cells and if isotype switching occurs into IgG, IgA and IgE producing memory B cells. T-cell independent activation occurs after antigen recognition by the B cell and via activation amongst other things by toll-like receptors [120].

In HCV infection it is widely accepted that strong and efficient T-cell responses are needed to clear the infection [121, 122]. The role for B cells in chronic HCV infection has received little attention. However, there are several clues that B cells might be clinically important. First, chronic HCV infection is characterized by the presence of follicle-like lymphoid structures in the HCV livers, which can allow an efficient B-T cell reaction [123, 124]. However, it is still unclear what the function of these follicles is, and if they are important in clearance of HCV infection. Second, HCV patients often suffer from cryoglobulinemia, with disturbed immunoglobulin production by B cells, that induces arthritis and glomerulonephritis [125, 126]. Third, B cells can induce an effective T-cell response and were shown to be required for the generation of memory CD4+ T cells in mice [120]. Fourth, B-T cell interaction has been shown to upregulate PD-1 on the surface of T cells, which is also a marker for T-cell exhaustion (discussed later in the introduction) [127, 128]. Fifth, it has been shown that the appearance of neutralising antibodies after acute HCV infection is correlated with an effective clearance of the disease [129-131]. However, neutralising antibodies to HCV are frequently lacking in the acute phase and only appear in the chronic phase of the infection [12]. Of interest, in some studies, B-cells display a more activated phenotype in chronic HCV infection as compared to

healthy controls [125, 132]. Although it is clear that B cells are capable in modulating the T-cell response, it is not clear if this is relevant in chronic HCV infection [133].

HCV-specific T-cell responses

T cells play a key role in persistence of HCV infection. Acute infection, defined as the period of 6 months after the actual infection, is characterised by the activity of strong T-cell responses to the virus in patients who resolve the infection, but interestingly also in those patients who will become chronic carriers of the virus. In contrast, during the chronic phase of infection HCV-specific T-cell responses are weak. The importance of HCV-specific T cell responses during HCV infection is supported by a number of observations. Firstly, during acute infection the appearance of HCV-specific T cells occurs simultaneously with the decline of viral titers, which suggests the involvement of T-cells in controlling the infection. Moreover, intrahepatic virus-specific CD8$^+$ T-cell responses are related to viral clearance and to liver inflammation [134]. Secondly, strong associations have been found between HLA class I and II alleles and resolution of HCV infection. HLA class I alleles A3 and B27 are protective and associated with viral clearance, which can be linked to dominant CD8$^+$ T cell epitopes [135]. There are also a number of studies that show a similar effect of the specific HLA class II alleles in different populations [136, 137]. Thirdly, the importance of T cells have been demonstrated in experiments in chimpanzees, which provide the most convincing evidence for the role of T cells in persistence of HCV. In these experiments, HCV-infected chimpanzees were depleted of their CD4$^+$ T cells by injecting depleting antibodies. This resulted in persistence of HCV infection and emergence of CD8$^+$ escape variants [138]. In another study, CD8$^+$ T cells were depleted in chimpanzees resulting in HCV persistence until the re-emergence of HCV specific CD8$^+$ T cell response [139]. Thus, both CD4$^+$ as well as CD8$^+$ T cells are involved in the control of HCV infection *in vivo*.

Mechanisms of T-cell failure

During the chronic phase of infection, HCV-specific T cells are detected in blood, but only at low frequencies. The liver contains a higher frequency of HCV-specific T cells than the blood [140, 141]. HCV-specific T cells are enriched in the liver up to 10- to 30-fold for CD8$^+$ T cells, but the average intrahepatic frequencies within the CD8$^+$ T cell compartment were still only 0.087% to 0.142% depending on the specific epitope [142]. In addition to the small numbers of HCV-specific T cells, also the cells are functionally weak, their responses are directed at a narrow range of HCV epitopes, and the responses are not sustained [143-146]. The 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 the host, and likely prevent accelerated liver damage. Indeed, the lack of symptoms in the majority of chronic HCV patients, reflected by the absence of elevated alanine transaminase (ALT) levels or jaundice, and the slow progression of liver disease are the consequence of weak T-cell responses. The delicate balance of the immune

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system between protective immunity and mild immunopathology is observed for other chronic infections as well [147]. The T-cell dysfunction seems limited to responses against HCV, and so no general immunosuppression is observed. Indeed, examination of the function of intrahepatic CD8^{+} T cells specific for influenza virus in individuals chronically infected with HCV showed no functional defect, whereas only HCV-specific T cells exhibited impaired function [142].

Furthermore, it is important to mention that in recent years our knowledge on biology of T cells has increased dramatically. Detailed information is now available on CD4^{+} T cell populations that are characterized by the secretion of IL-17, IL-22, and IL-21. Altered differentiation of CD4^{+} T cells may hamper the strength of the antiviral IFN-γ-producing Th1 response, as was recently described in mouse models [148]. The levels of IL-17 correlate with disease progression in HBV patients, suggesting a role in liver injury [149, 150]. Also during chronic HCV infection, virus-specific T cells are found to produce IL-17 and IL-22 in peripheral blood as well as the liver [115, 151, 152]. These cells may play a role in the pathogenesis of chronic hepatitis.

Several host and viral factors have been proposed to explain the weak T-cell responses to HCV. These factors include the occurrence of HCV immune escape mutations, the interaction of T cells with dysfunctional NK cells and DC, exhaustion of T cells as a consequence of the continuous exposure to high viral load, and active suppression by regulatory T cells or the immunosuppressive cytokines IL-10 and TGF-β [11, 13, 153, 154]. To investigate adaptive immunity, virus-specific T cells are labelled with peptide-MHC multimers like tetramers and dextramers. During chronic HCV infection no immunodominant epitope can be identified and since frequency of HCV-specific T cells is low (0.01% of CD8^{+} T cells) it has been a challenge to functionally characterize these cells. The IL-7 receptor CD127 have shown to be important marker for virus-specific T cell function. The anti-viral function of HCV-specific CD8^{+} T cells was diminished in the absence of CD127 on their surface [155, 156].

Exhaustion of the HCV-specific T cell response
Exposure of mice to the lymphocytic choriomeningitis virus (LCMV) results in a chronic viral infection. Similar to HCV infection in humans, the LCMV infection in mice is characterized by high viral replication rates, and as a consequence high serum levels of the virus are detected for prolonged periods. Using the LCMV model, it was demonstrated by Moskophidis in 1993 that continuous exposure to high levels of viral antigens leads to a gradual loss of effector functions of virus-specific T cells [157]. This phenomenon was called "T cell exhaustion". The loss of function is gradual, meaning that the proliferative potential and IL-2 production are lost early, followed by the loss of TNF production, and in the most extreme stages of exhaustion also IFN-γ production is lost upon continuous antigen exposure [128]. Barber et al. reported that a surface molecule "programmed death 1", PD-1, was upregulated on exhausted CD8^{+} T cells in LCMV infected mice. Blockade of the PD-1/PD-L1 inhibitory pathway with

antibodies restored the effector functions of the virus-specific T cells [158]. Studies in patients with chronic HCV infection demonstrated that HCV-specific CD8^{+} T cells also expressed high levels of PD-1 in blood and liver, and that blockade of the pathway augmented the virus-specific T cell response in cell culture systems [159, 160]. Since then, it has become clear that PD-1 is not the only molecule that determines the state of exhaustion, but numerous other inhibitory receptors have been identified, including CTLA-4, 2B4, BTLA, CD160 and TIM-3. *In vitro* blockade of the individual receptors or multiple receptors simultaneously has been shown to restore the function of HCV-specific CD8^{+} T cells, and similar findings were reported for HBV- or HIV-specific T cells [158, 161-167]. Similar as in cell culture systems, there are indications that the functional impairment of T cells is reversible, as shown after therapy of HBV infection in patients [168] or LCMV infection in mice [169, 170]. The expression patterns of exhaustion markers and their functional consequences need to be further investigated to better understand its correlation to HCV persistence. Blocking of multiple inhibitory receptors to restore impaired HCV-specific T cell functionality is an interesting therapeutic approach that needs to be explored further.

Active suppression by Treg and immunoregulatory cytokines during chronic HCV
Numerous studies have also demonstrated the importance of regulatory T cells (Treg) and immunosuppressive cytokines in suppression of the HCV-specific T cell response. Regulatory T cells and the cytokines IL-10 and TGF-β may limit viral clearance in chronic infections by inhibiting the proliferation and effector function of T cells or other immune cells [171]. During immune responses induced by distinct pathogens, but also during auto-immunity, the crucial role of active regulation in limiting excessive immunopathology has been demonstrated. Treg are CD4^{+} T cells that are characterized by the expression of the surface molecule CD25, absence of CD127 and the expression of the transcription factor FoxP3. They regulate T cell activity indirectly by modulating antigen-presenting cells, or directly. In some, but not all systems, Treg have been shown to secrete IL-10 or TGF-β, but numerous other cell populations can produce these cytokines as well, e.g. IL-10 can be produced by all T cell subsets described to date, including Th1, Th2, and Th17 [172].

Increased numbers of circulating as well as intrahepatic Treg have been reported in chronic HCV patients as compared to healthy individuals [173-176]. In blood, these Treg were able to suppress both HCV-specific proliferation and IFN-γ production by CD4^{+} and CD8^{+} T cells [174, 177, 178]. We previously showed that Treg are present in the liver of chronic HCV patients [175, 179, 180], but virtually absent from the livers of healthy individuals, and that the numbers of intrahepatic Treg negatively correlated with the development of fibrosis [176]. Furthermore, others showed that Treg control chronic liver inflammation, with a higher suppressive capacity of blood Treg in patients with lower ALT levels [174, 177, 178]. Interestingly, by performing fine-needle aspirate biopsies during the course of treatment with pegIFN-α and ribavirin, we observed increased frequencies of Treg in the liver of chronic HCV patients. Moreover, treatment-induced clearance of HCV RNA did not result in normalization of the liver at

4 weeks after end of therapy, but -instead- retention of Treg in the liver was observed, suggesting ongoing residual regulation of immunopathology at week 4 after end of therapy [181]. This situation is reminiscent of the studies by the Abbas group, who showed retention of so-called memory Treg in skin inflammation [182].

Besides Treg, other studies showed that blocking IL-10 or TGF-β can enhance HCV-specific T cell proliferation and IFN-γ production [177, 183-186]. In addition, augmented levels of IL-10 and TGF-β have been detected in serum of chronic HCV patients as compared to healthy individuals, and enhanced production has been described by monocytes and T cells from chronic HCV patients [65, 183, 185, 187, 188]. Nowadays, it is generally accepted that regulation via IL-10, TGF-β and Treg is involved in controlling HCV-specific immunity. However, the relative importance of these regulatory pathways and whether they control different effector activities is still unknown. Also, detailed information on these regulatory pathways in the liver is still scarce, and this should be an important focus for future research since the immunoregulatory mechanisms in blood may not be necessary representative of processes in the liver.

Therapeutic options for chronic HCV

As discussed previously, type I IFN have a role in the natural immune response against hepatitis C. Already soon after the virus was discovered, patients were treated with unconjugated IFN-α [189]. Since viral efficacy of the treatment was poor, ribavirin was added to the treatment in 1995 [190] and in 2001 a pegylated form of interferon was introduced to prevent relapse. A 48-week treatment regime of pegylated-IFN-α (PegIFN-α) resulted in a 60% cure in chronic HCV patients [191]. However, in difficult to treat patients e.g. patients with severe cirrhosis, cure rates remained low. In addition, IFN-based therapy is accompanied by severe side effects like flu-like symptoms, fatigue, depression, rashes, anemia, insomnia and hair loss, often causing suboptimal coherence. Also thrombocytopenia and leukocytopenia frequently occur during IFN-based therapy, and dose reductions are implemented to prevent increased infection rates and bleeding risk. This eventually results in lower response rates in every day practice than reported in the original registration studies.

A common side effect of PegIFN-α/ribavirin therapy for chronic HCV is anemia. Anemia is caused by the direct suppressive effect of PegIFN-α on the bone marrow combined with the haemolytic activity of ribavirin. Due to anemia, patients often experience fatigue and dose reductions have to be implemented to continue treatment. However, dose reductions negatively impact the virological efficacy of antiviral therapy [192]. Erythropoietin (EPO) is a renally secreted hormone that promotes red blood cell production in bone marrow, and makes a potent molecule to treat anemia in various patients groups. Various clinical trials investigated the effect of EPO administration during therapy of chronic HCV [193-196]. Data have shown that the administration of EPO is effective in the treatment for anemia, but in studies with telaprevir and boceprevir in combination with PegIFN-α/ribavirin treatment, ribavirin

dose reduction does not seem to affect virological response rates [197, 198].

In the last decade, direct acting antivirals (DAA) have been developed to eradicate the HCV virus. Different DAA target distinct stages of the HCV lifecycle by inhibiting HCV polymerases and proteases that facilitate critical enzymatic steps for HCV replication. In 2011, NS3/4 protease inhibitors telaprevir and boceprevir were added to PegIFN-α/ribavirin treatment [199, 200]. This triple therapy combination increased sustained viral response (SVR) rates, but was only successful for patients with genotype 1. Both telaprevir and boceprevir require combination treatment since monotherapy causes resistant mutations. Since the introduction of telaprevir and boceprevir, many new DAA's targeting NS3/4A proteases, NS5B polymerases and NS5A proteins have been introduced in various clinical trials and since 2013 also in clinical practice.

One of the first DAA's to be introduced in clinical practice was sofosbuvir. Sofosbuvir is a potent NS5B polymerase inhibitor. Clinical trials showed a SVR rate around 90% in genotype 1 and 96% in genotype 4, 5 and 6 with a 12 week regime combining sofosbuvir with PegIFN-α/ribavirin [201]. Current European Association of the Study of the Liver (EASL) guidelines 2015 state that the first option for patients with genotype 1 HCV infection in an IFN-containing regime consisting of sofosbuvir in combination with PegIFN-α/ribavirin for 12 weeks [202]. However, because of the side effects of treatment with PegIFN-α/ribavirin, IFN-free options are much more interesting. However, not all genotypes respond well to IFN-free therapy and best treatment options must be categorized. Current EASL guidelines state that patients with HCV genotype 1 or 4 infection can be treated with different strategies [202]. The first option is treatment with sofosbuvir in combination with ledipasvir (an NS5A inhibitor) for 12 weeks as one tablet once daily. For genotype 1 patients, treatment may be shortened to 8 weeks in treatment-naïve patients without cirrhosis if their baseline HCV RNA level is below 6 million IU/ml. However, for patients with genotype 1 or 4 and cirrhosis, ribavirin should be added ór duration of therapy should be prolonged to 24 weeks in total. The second option for genotype 1 includes combination of ombitasvir (NS5A inhibitor), paritaprevir (NS4/4A inhibitor) and ritonavir (CYP3A4 inhibitor, all in 1 tablet once daily) and twice daily dasabuvir (NS5B inhibitor) for 12 weeks. In patients with either subtype 1a ór cirrhosis, ribavirin should be added. Patients with subtype 1a and cirrhosis should be treated for 24 weeks. For patients with genotype 4, dasabuvir should be replaced by ribavirin and in patients with genotype 4 and cirrhosis treatment duration should be 24 weeks in total. For option 3, sofosbuvir in combination with simeprevir or in combination with daclatasvir can also be given to genotype 1 patients for 12 weeks. Also for this treatment combination, ribavirin should be added in patients with cirrhosis. Importantly, the presence of Q80K, a naturally occurring NS3 polymorphism, was associated with lower SVR rates in patients treated with simeprevir [203]. Therefore HCV resistance testing is only advised in the patients group with subtype 1a who receive the treatment combination with simeprevir. For patients with genotype 2, treatment with daily sofosbuvir in combination with ribavirin for 12 weeks is

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advised. Treatment for cirrhotic or treatment experienced patients with genotype 2 should be elongated to 20 weeks. For patients with genotype 3 infections, combination therapy with PegIFN-α/ribavirin and sofosbuvir seems the best option. IFN-free treatment with ribavirin and daily sofosbuvir is an IFN-free treatment options but is less potent. For chronic HCV patients in countries where DAA therapy is not yet available, treatment combination with PegIFN-α/ribavirin remains acceptable.

While writing this thesis, potent DAA as sofosbuvir, ledipasvir, paritaprevir, ritonavir and ombitasvir are being evaluated in different combinations and for shorter treatment duration in clinical trials. Worldwide use of different treatment regimens will show if data obtained from clinical trials will also be valid in the real world. By optimizing these treatment regimes, HCV is expected to be a curable disease for most patients in 10 years. In the next decades, the focus for HCV will shift also towards the detection of all patients infected with HCV. Global screening projects will be of upmost importance to be able to eradicate HCV globally.

Vaccine prospects and failures
Although HCV is now a curable disease, the global burden of HCV is unlikely to be diminished without a vaccine.

In contrast to HBV infection, patients do not develop protective immunity after an infection with HCV. However, studies with intravenous drug abusers who spontaneously resolved the infection showed that patients were more likely to clear a subsequent HCV infection [130, 204]. In addition, studies of chimpanzees that have previously spontaneously cleared HCV, showed rapid clearance of a second infection [205]. These studies have shown that protective immunity may be an achievable goal.

Since many HCV genotypes and subtypes of the virus exist, the development of a consensus vaccine is a challange. As discussed in this introduction, broad and efficient HCV-specific T-cell responses [206, 207] and also the early release of effective neutralising antibodies [129, 208] seem to be important to clear the infection.

Humoral immunity in HCV vaccine development
The role of neutralizing antibodies in clearance of HCV infection is not completely understood. Neutralising antibody responses are often absent in patients that are acutely infected with HCV, while they appear later during chronic infection [209, 210]. However, it has also been shown that in a group of young women who were infected with the same HCV strain, early appearance of neutralising antibodies correlated with spontaneous clearance of the virus [129]. Conversely, there are numerous studies that describe spontaneous clearance of the virus often in the absence of neutralizing antibodies [2, 208, 211, 212]. Immunization with neutralising antibodies can mediate protection. The envelope proteins E1E2 are often used as targets for a neutralising antibody response. In one study, healthy volunteers were injected with

a vaccine that contained E1E2 proteins that was immunoprotective in guinea pigs and was able to induce strong T-helper cell responses [213, 214]. Studies have shown that the vaccine induced very broad cross neutralization activity against all seven major HCV genotypes [215]. This supports that a vaccine derived from one single HCV strain can elicit broad neutralising antibodies against multiple genotypes and is promising in the search for a protective vaccine against HCV [2, 216].

The importance of cellular immunity in HCV vaccine development

As discussed before, several studies have shown an important role for both CD4^{+} and CD8^{+} T-cell responses in the clearance of HCV infection [121, 217]. It is however difficult to distinguish if failure of priming or T-cell exhaustion causes the lack of strong and efficient T-cells responses in patients with chronic HCV infection. Both mechanisms need to be addressed when designing a vaccine. Vaccine candidates include different combinations of multiple epitopes like NS3, NS5A, NS4B and core and were shown to induce strong cellular immune responses [216, 218]. Also vaccines containing both T cell epitopes and DC that stimulated effective priming with the release of IL-12 are being tested [219]. However, all these vaccine were able to induce only weak T-cell responses. Novel adjuvants that can enhance priming and/ or reduce exhaustion of T cells are needed to progress the development of these vaccines. Moreover, developing multi–epitope vaccines combining both B-cell and T-cell epitopes might be an additional strategy to find an effective vaccine. In this thesis we investigate possible mechanisms of T-cell priming and T-cell exhaustion that can help to develop an effective vaccine for chronic HCV.

AIMS AND OUTLINE OF THE THESIS

The overall aim of the thesis is to better understand the mechanisms by which HCV is modulating the immune system in humans. Since highly effective DAA-based treatment regimens are now able to cure most of chronically infected patients, the goal for future research should be the design of an effective vaccine.

As previously described, HCV-specific T-cell responses are weak or absent, because of the continuous antigenic pressure resulting from high concentrations of viral antigens. We know that the presence of an efficient T-cell response is important in eliminating the HCV virus. In **Part I** of the thesis, we investigate T cells during chronic HCV infection to get better insight in the mechanisms important for T-cell failure. In **chapter 2** of the thesis, we study the frequency and phenotype of CD4^{+}CXCR5^{+} T-follicular helper (T$_{FH}$)-cells in chronic HCV infection. We investigate if CD4^{+}CXCR5^{+} T cells of chronic HCV patients differ with respect to their capability of producing IL-21 and giving help to B cells compared to healthy controls. Importantly, we examine if CD4^{+}CXCR5^{+} T-cells are present in HCV infected livers and study

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their localization relative to B cells in the liver.

In **chapter 3,** we describe the role for inhibitory receptor molecules like TIM-3, PD-1 and CTLA-4 on T cells in chronic HCV and HBV infection. The importance of the molecules during acute and chronic infection and in the liver is discussed, as well as the possibility to use these inhibitory receptors as targets for immune therapy.

Since patients at high risk for re-infection especially benefit from a vaccine and might be the primary target, it is important to study the immune response after treatment. In **Part II** of the thesis, we study immune responses during treatment for HCV infection to get a better insight in the effects of EPO administration during therapy and the effect of IFN-based and IFN-free therapy on immune cells. During IFN-based therapy numerous studies have examined the clinical effects of EPO to treat anemia and improve response rates in chronic HCV patients. However, its immunological effects have received less attention. In **chapter 4** of this thesis, we study the effect of EPO administration on the immune cells. We define which human leukocyte subpopulations are potential targets for EPO, and explore the functional effects of EPO on these cells *in vitro*. Since monocytes expressed the EPO receptor, we define if administration of EPO during antiviral therapy of chronic HCV patients altered the frequencies of cytokine-producing monocytes. In **chapter 5,** effects of PegIFN-α/ribavirin on the liver is investigated. With the use of fine needle aspiration biopsies (FNA) we study in intrahepatic NK cell phenotype during triple therapy for chronic HCV. In **chapter 6**, we investigate the effect of IFN-free therapy with DAA on T cells, NK cells and on the gene expression profile in blood. We determine HCV-specific T cell frequency and NK cell frequency at various time-points during therapy. Effect of rapid viral load decline on the expression of Interferon Stimulating Genes (ISG) and serum cytokine levels is investigated. In **chapter 7,** MAIT cells are studied in healthy individuals, chronic HCV, HIV patients and patients with HIV/ HCV co-infection. We study frequency and phenotype of MAIT cells and their ability to produce IFN-γ upon different stimuli. We determine the effect of IFN-α on MAIT cells during various treatment regimens for chronic HCV and HIV/ HCV co-infection.

Many HCV patients have been effectively cured in the past but are still at risk to become newly infected. In the **Part III** of the thesis we study immune responses long-term after successful therapy for HCV. This is important to clarify why some patients experience protective immunity after re-infection and why other do not. In **chapter 8,** we investigate the durability of SVR after treatment with the NS3 protease inhibitor narlaprevir, in combination with PegIFN-α/ribavirin. We study the presence of HCV RNA in peripheral blood and the presence of HCV-specific T-cell responses 24 months after end of therapy. During HCV infection, various leukocytes infiltrate the liver to target infected hepatocytes and eliminate the virus. Besides inflammatory T cells, like Th1 cells, also Treg are localized in the liver to dampen the immune response. We previously demonstrated that shortly after therapy, Treg are still detected in the liver of SVR patients. In **chapter 9,** we investigate if 4 years after

therapy induced clearance of HCV, Treg are still present in these livers and we speculate about their potential function.

Finally in **chapter 10**, I summarize and discuss the result and describe future perspectives.

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Part I

HCV infection modulates T-cell responses in liver and peripheral blood

Chapter 2

CD4^{+}CXCR5^{+} T-cells in chronic HCV infection produce less IL-21, yet are efficient at supporting B-cell responses

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ABSTRACT

Introduction: During chronic HCV infection, T-cell dependent virus-specific antibodies are produced. However, the role of B-T-cell interaction in chronic HCV is largely unknown. $CD4^+CXCR5^+$ T-follicular helper (T_{FH})-cells activate B-cells and are important for clearance of various chronic viral infections. We investigated the function of T_{FH}-cells and B-cells in liver and in peripheral blood of chronic HCV patients.

Methods: T-cells from chronic HCV patients and healthy individuals were analysed for expression of CXCR5, PD-1, ICOS and IL-21 and IFN-γ production by flowcytometry. $CD19^+$ B-cell subpopulations were identified on the basis of CD27 and IgD expression. In order to assess the frequency and function of T-cells and B-cells in liver follicles, immunohistochemistry was performed for CD3, CXCR5, Bcl6, IL-21, CD20, IgD, IgM and IgG.

Results: The frequency of IL-21-producing $CXCR5^+CD4^+$ T-cells in blood was lower in HCV patients compared to healthy individuals (p=0.002), which was reflected by lower serum IL-21 levels (p<0.001). Nonetheless, $CXCR5^+CD4^+$ T-cells from HCV patients and healthy individuals were equally capable to stimulate $CD19^+CD27^+$ memory B-cells into IgG and IgM-producing plasmablasts. Importantly, human intrahepatic T_{FH}-cells and their related function were identified by immunohistochemistry on liver biopsies for CD3, Bcl6 and CD20 within portal areas and follicles.

Conclusion: The specific localization of T_{FH}-cells and IgG and IgD/IgM-producing B-cells suggests a functional B-T-cell environment in liver follicles during HCV infection. The decreased frequency of IL-21-producing $CXCR5^+CD4^+$ T-cells and lower serum IL-21 levels in chronic HCV patients did not lead to an altered T_{FH}-B-cell interaction.

INTRODUCTION

An estimated 180 million individuals worldwide are chronically infected with the hepatitis C virus (HCV). Persistence of HCV causes fibrosis, cirrhosis, liver failure and hepatocellular carcinoma, leading to high morbidity and mortality [1]. Since a large proportion of patients fails to respond to antiviral treatment, they remain at risk for accelerated development of liver fibrosis [2].

Patients who spontaneously resolve HCV infection are characterised by an efficient anti-viral immune response. This is in contrast to patients with chronic HCV infection, who have weak or even absent HCV-specific T-cell responses that display an exhausted phenotype and a decreased ability to proliferate and produce cytokines [3-6]. The contribution of the humoral immune response to the clearance of HCV infection is less clear. During chronic lymphocytic choriomeningitis virus (LCMV) infection and HCV infection large amount of T-cell dependent virus specific antibodies are produced, indicating that CD4+ T-cells are able to give help to B-cells in this chronic infection stage [7]. However, virus-specific CD4+ T-cells can also cause polyclonal B-cell activation, resulting in deficient production of specific neutralizing antibodies [8]. Of interest, also some studies in chronic HCV infection, B-cells display a more activated phenotype compared to healthy controls [9-11]. However, the role of CD4+ T-cells in inducing B-cell responses in HCV infection is largely unknown.

CD4+ T-helper-cells that can provide help to B-cells within germinal centers of lymphoid follicles are CD4+ T follicular helper (T_{FH})-cells [12, 13]. Peripheral blood CD4+CXCR5+ T-cells are the counterparts of T_{FH}-cells and are identified by high CXCR5, and low PD-1 and ICOS expression [14, 15]. These CD4+CXCR5+ T-cells induce B-cell proliferation and maturation, which is -at least in part- mediated via the activity of IL-21 [13, 16]. Little is known on the role of CD4+CXCR5+ T-cells in chronic HCV infection [17, 18], however some data is available from studies in other chronic infections like LCMV, HIV and HBV. In LCMV-infected mice CD4+CXCR5+ T-cells sustained LCMV-specific B-cell responses and facilitated viral clearance [19-21]. In HIV infection, a possible role for T_{FH}-cells in skewing of the B-cell compartment was suggested since circulating CD4+CXCR5+ T-cells correlated with B-cell function of HIV patients [22], and expansion of T_{FH}-cells in lymph nodes of HIV patients correlated with an increase in (pre-) germinal center B-cells and plasma cells [23]. Finally, in chronic HBV infection circulating CD4+CXCR5+ T-cells were increased in patients undergoing treatment-induced HBeAg seroconversion compared to patients who did not seroconvert. This was attributed to IL-21 [24, 25]. Thus, CD4+CXCR5+ T-cells and IL-21 modulate the B-cell compartment in various chronic viral infections, which may -directly or indirectly- benefit viral clearance. However, no studies have been conducted that examine the function of intrahepatic T_{FH} and B-cells or their relation with clinical parameters like ALT and fibrosis score. In order to get more insight into the importance of CD4+CXCR5+ T-cells in chronic HCV, the current study was performed

in which the frequency and phenotype of circulating and intrahepatic CD4^{+}CXCR5^{+} T-cells and their relation to B-cells in chronic HCV patients were examined. We hypothesize that circulating CD4^{+}CXCR5^{+} T-cells are dysfunctional in chronic HCV infection and therefore unable to give help to B-cells.

MATERIALS AND METHODS

Patients and healthy subjects

Heparinized blood was collected from 29 chronic HCV patients and 22 healthy subjects. Fibrosis scores were determined using liver biopsies or fibroscan. Table 1 shows their characteristics. Serum IL-21 levels were measured in samples from a different cohort of 75 chronic HCV patients visiting our clinic (mean ALT 111 IU/l (range: 17-444), mean HCV RNA 2.6×10^6 IU/ml (range: 3.7×10^2–2.2×10^7). From 9 patients, serum IL-21 levels were determined 24 weeks after successful IFN-based therapy. 25 age and sex matched healthy subjects were enrolled. For intrahepatic studies, biopsies were collected from chronic HCV patients visiting the Erasmus Medical Center. An experienced pathologist assessed the presence of lymphoid follicular aggregates in 150 liver biopsies. Biopsies were scored for the presence of fibrosis/ cirrhosis (Metavir 1-4) and stages of inflammation (Ishak 1-7). 12 random biopsies containing follicles were used for immunohistochemical staining. Table 2 shows their characteristics. The institutional ethical review board of the Erasmus Medical Center approved the protocols, and informed consent was obtained from all individuals.

Table 1. Patient characteristics

		HCV patients	Healthy Controls	Level of significance
Number		29	22	
Age (years)		47.4	42	p=0.10
Gender	M/F	22/7	12/10	p=0.11
Mean ALT (U/l)		111		
Mean HCV RNA (U/ml)		2.89×10^6		
Fibrosis	F0-2/F3-4	13/16		

Table 2. Patient characteristics intrahepatic studies

		HCV patients
Number		12
Age (years)		51.5
Gender	M/F	8/4
Mean ALT (U/l)		73.2
Mean HCV RNA (U/ml)		4.68×10^6
Fibrosis	F0-2/F3-4	7/5

Analysis of cell surface molecule expression by flow cytometry

Peripheral blood mononuclear cells (PBMC) were isolated from venous blood by ficoll separation (Ficoll-Paque™ plus, Amersham) and frozen at -150°C. Prior to flowcytometry, PBMC were washed in RPMI 1640 culture medium supplemented with 10% FCS, L-glutamine, Pen-Strep and Hepes (Cambrex, Invitrogen/Gibco). Cells were stained with CD3-PacificBlue (UCHT1, BD Pharmingen), CD4-APCeFluor780 (SK3, eBioscience), CD8-APC (SK1, eBioscience), CXCR5-PerCP-Cy5.5 (TG2, Biolegend), PD-1-FITC (M1H4.1, BD Pharmingen), ICOS-PE-Cy7 (ISA-3, eBioscience), CD19-PacificBlue (HIB19, eBioscience), mIgD-PE-Cy7 (Biolegend), CD27-FITC (L128, BD Bioscience), CD69-PE (TP1.55.3, Beckman), or CD86-APC (IT2.2, Biolegend). Cells were washed and marker expression was detected by flow cytometry (Canto-II, BD).

Analysis of intracellular cytokine expression by flow cytometry and immunoassay

The percentages of cells producing IL-21 and IFN-γ were determined by flow cytometric evaluation of intracellular cytokines. PBMC from healthy individuals and HCV patients were cultured in a 24-well plate ($2x10^6$/well) in 1 mL culture medium. Cells were stimulated with PMA (50 ng/ml) and ionomycin (400 ng/ml, both from Sigma) for 1 hour at 37°C. Brefeldin A (10 µg/ml, Sigma) was added and the cells were incubated for another 4 hours. Samples were fixed, permeabilized and stained with CD3-FITC (UCHT1), CD4-APC-eFluor780 (SK3), CD8-APC (SK1), IFN-γ-PE-Cy7 (25723.11, all eBioscience), CXCR5-PerCp-Cy5.5 (TG2, Biolegend), and IL-21-PE (3A3-N2.1, BD Pharmingen). Cytokine-producing cells were detected by flow cytometry (Canto-II, BD). Serum levels of IL-21 were determined using a commercial ELISA kit (Legend Max, Biolegend).

T$_{FH}$-B-cell culture

PBMC stained with CD3-Amcyan (SK7), CD4-APC (SK3), CXCR5-PerCp-Cy5.5 (TG2), CD19-APC-H7 (HIB19), CD27-FITC (L128), IgD-PE-Cy7, CD38-PB (HIT2) and CXCR3-PE (49801). CD4⁺CXCR5⁺, CD4⁺CXCR5⁻, CD19⁺CD27⁺ and CD19⁺CD27-IgD⁺ cells were purified using a FACS ARIA cell sorter (BD). The purity was always more than 98%. 20.000 T-cells in 1:1 ratio with B-cells were stimulated in a total volume of 200 µl with Staphylococcal enterotoxin B (SEB, 1 µg/ml, Sigma). At day 7, supernatant was harvested and stored at -20°C. Cells were washed with 1% FCS in PBS, and stained for surface markers for flow cytometry (Canto-II, BD). IgG and IgM levels in supernatant were determined by ELISA according to the manufacturer's instructions (ITK).

Immunohistochemistry

Sections of paraffin embedded liver biopsies and control tonsil tissue were deparaffinized followed by antigen retrieval. Non-specific staining was prevented by H_2O_2 by adding medium

containing 5% BSA, 10% human serum and 10% normal goat serum, and endogenous biotin was blocked using a biotin blocking system (Dako). Sections were incubated with mouse-anti-human Bcl6 (G1191E/A8, Cell Marque) and rabbit-anti-human CD3 antibodies (F7.2.38, Dako) for 60 min. After washing, the sections were incubated with biotinylated goat-anti-mouse Ig and AP-conjugated swine-anti-rabbit Ig antibodies (both from Dako) followed by incubation with ABC complex labeled with HRPO (Vectastain). Visualization was performed with DAB and fast blue. IgG, IgD and IgM staining was performed routinely by the pathology department using the Benchmark Ultrastainer (Verntana, Roche). The sections were mounted with Imsol mount (Klinipath). Pictures were taken using the Zeiss Axioskop20 microscope, Nikon Digital Sight DS-U1 camera and NIS-Elements 3.00 program.

Statistics

Statistical comparison was tested using the Kruskal-Wallis and Mann-Whitney test for unpaired non-parametric analyses and the Paired student T test for paired observations. The relationship between two variables was evaluated using Spearman's rank correlation test. Correlations between the presence of liver follicles, inflammation and advanced fibrosis were analyzed using Chi-Square test for categorical data. A *p* value ≤ 0.05 was considered significant.

RESULTS

CD4+CXCR5+ T-cell frequency in blood did not differ between healthy controls and HCV patients.

CD4+CXCR5+ T-cells play a role in various chronic viral infections [19, 23, 24], however, little is known on their role during chronic HCV. Therefore, we determined the frequency of CD4+CXCR5+ T-cells in peripheral blood of healthy individuals and chronic HCV patients with different fibrosis scores and ALT levels. The frequency of CD4+CXCR5+ T-cells within the blood CD4+ T-cell population varied from 5-20%, and expression of PD-1 and ICOS was low (Fig. 1A and data not shown). In line with other studies [14, 24], we observed that the expression of PD-1 was higher on CD4+CXCR5+ T-cells compared to CD4+CXCR5- T-cells in both patients and healthy individuals (p<0.001, Fig. 1A). No differences were observed between healthy controls and chronic HCV patients in the frequencies of CXCR5+, CXCR5+PD-1+, CXCR5+ICOS+ and CXCR5+PD-1+ICOS+ CD4+ T-cells (Fig. 1B, and data not shown). Moreover, also stratification of patient groups according to their ALT levels and fibrosis stage showed similar frequencies of CD4+CXCR5+ as well as CXCR5+PD-1+CD4+ T-cells (Fig. 1B).
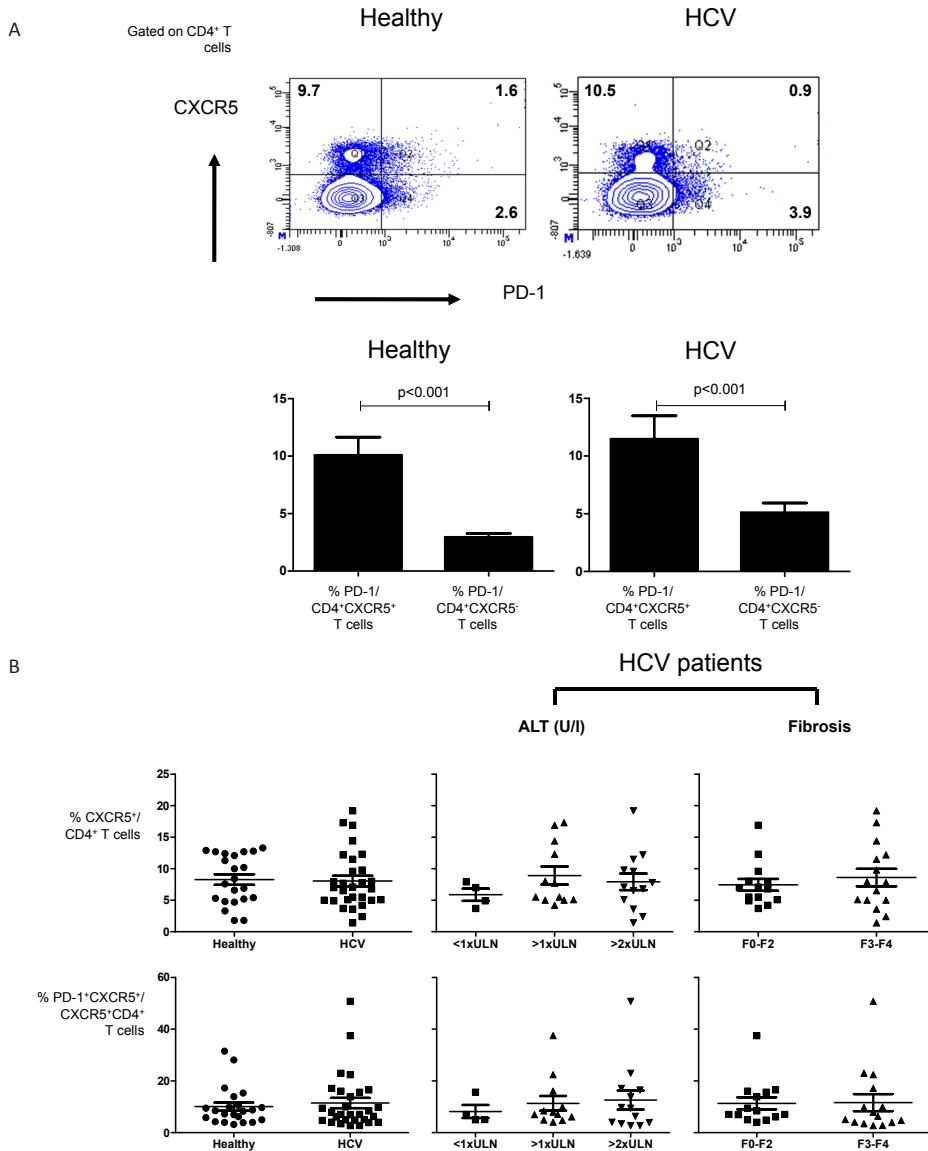
Figure 1. Frequency of CD4+CXCR5+ T-cells in blood did not differ between healthy individuals and chronic HCV patients. (A) Representative dot plots and frequency of CXCR5 and PD-1 expression on CD4+ T-cells of healthy individuals and chronic HCV patients. (B) Frequency of CXCR5+ CD4+ T-cells and CXCR5+PD-1+ CD4+ T-cells in healthy individuals (n=22), and HCV patients with different levels of ALT and fibrosis score (n=29). Levels of ALT were divided in <1, ≥1 and ≥2 times the upper limit of normal (ULN for: female 33 IU/l and for male 44 IU/l). Statistical comparison was tested using Wilcoxon matched pairs test, Kruskal-Wallis and Mann-Whitney test.

IL-21-producing CD4+CXCR5+ T-cells were decreased in HCV patients.

IL-21 is mainly produced by CD4+CXCR5+ T-cells, and has been described to be involved in antiviral responses [19, 26]. Following polyclonal stimulation of cells, the percentages of

CD4$^+$CXCR5$^+$ T-cells expressing cytokines was 1-7% for IL-21 and 5-40% for IFN-γ (Fig. 2A). The frequencies of IFN-γ-producing CD4$^+$CXCR5$^+$ T-cells were comparable between healthy individuals and HCV patients (data not shown). In contrast, we observed that HCV patients have lower frequencies of IL-21-producing CD4$^+$CXCR5$^+$ T-cells compared to healthy controls (p=0.002, Fig. 2A), indicating a decreased function of these cells in HCV patients. CD4$^+$CXCR5$^+$ T-cells are the major producers of IL-21 in healthy individuals (Supplementary Fig. 1). However, in patients with chronic HCV infection, the frequency of IL-21-producing CD4$^+$CXCR5$^-$ T-cells appears to be higher compared to CD4$^+$CXCR5$^+$ T-cells. This illustrates that there is not an overall decrease of IL-21-producing cells, but a specific reduction of the frequency of IL-21-producing CD4$^+$CXCR5$^+$ T-cells (Supplementary Fig. 1).
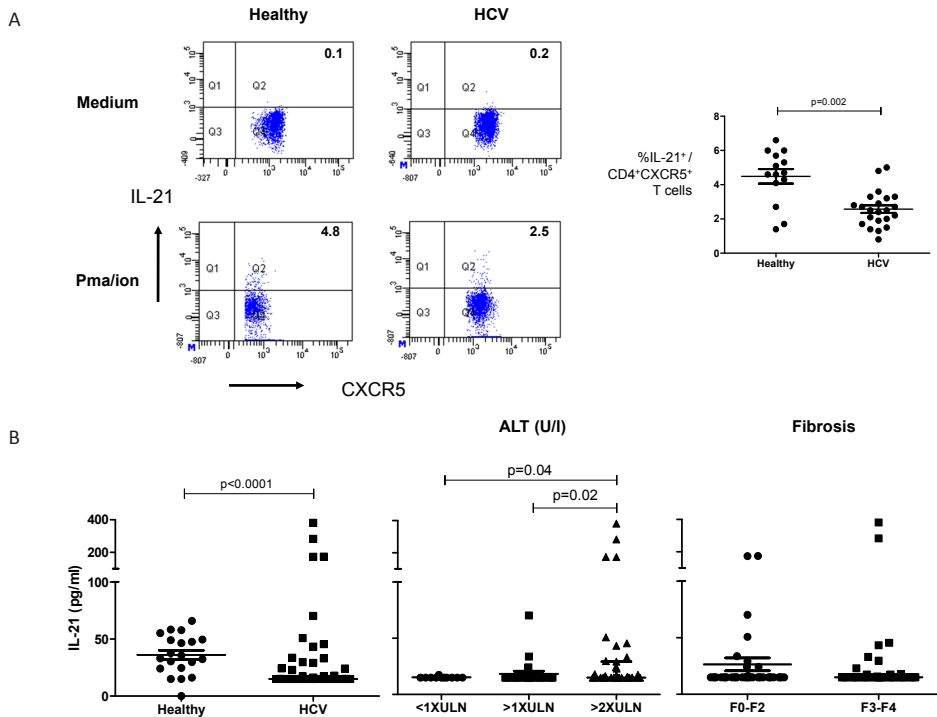
Figure 2. IL-21-producing CD4$^+$CXCR5$^+$ T-cells and serum IL-21 were decreased in HCV patients compared to healthy controls. (A) Dot plots and frequencies of IL-21-producing CXCR5$^+$CD4$^+$ T-cells of healthy individuals and HCV patients after medium and PMA/ionomycin stimulation. (B) Sera of 75 chronic HCV patients and 20 healthy individuals were evaluated for the levels of IL-21 by ELISA, and correlated with their serum ALT levels and fibrosis score. Statistical comparison was tested using Kruskal-Wallis and Mann-Whitney test.

Levels of circulating IL-21 were decreased in chronic HCV patients and correlate with ALT.

To determine whether lower IL-21-producing CD4$^+$CXCR5$^+$ T-cells resulted in lower circulating IL-21 levels in HCV patients, serum samples from 75 chronic HCV patients and

20 healthy individuals were evaluated. As shown in figure 2B, the mean concentration of IL-21 in sera from the infected group was statistically lower than in sera from healthy individuals (p<0.0001), which is in line with the flowcytometry data. In 55 of the 75 chronic HCV patients, the serum IL-21 levels were less than 15 pg/ml. The relatively high serum IL-21 levels in 4 patients did not correlate with clinical parameters, medical history or other patient characteristics. Interestingly, serum IL-21 levels positively correlated with ALT levels (p=0.04, p=0.02, Fig. 2B), but not with HCV RNA load (results not shown) or fibrosis score (Fig. 2B). In addition, baseline serum IL-21 levels in a cohort of 36 HCV patients who were non-responsive to peginterferon and ribavirin treatment were comparable to the levels in 15 responder patients (data not shown). Interestingly, in responder patients serum IL-21 levels did not increase 24 weeks after end of IFN-based therapy as compared to baseline, indicating that viral clearance does not restore IL-21 production (data not shown).

T$_{FH}$-cells were located in close proximity to B-cells in the follicle infiltrates of HCV infected liver.

Since we observed reduced frequencies of IL-21-producing CXCR5⁺CD4⁺ T-cells and reduced serum IL-21 levels, we examined the modulation of the B-cell compartments during chronic HCV infection. T$_{FH}$ derived IL-21 leads to activation and maturation of B-cells, which has been described extensively in lymphoid structures [14, 27]. Since chronic HCV livers are often characterised by lymphoid-like structures or follicle aggregates [28, 29], we investigated follicle aggregates in HCV infected liver for indications of specific B-T-cell interaction. An experienced pathologist assessed 150 liver biopsies and observed follicles aggregates in 30% of livers from chronic HCV patients. The presence of inflammation score > grade 4 correlated strongly with advanced fibrosis (p<0.0001) and the presence of follicles in the liver (p=0.002). The presence of follicles showed a positive trend with advanced fibrosis, albeit not significant (results not shown). Twelve biopsies containing follicles were used for immunohistochemical staining. As shown in figure 3A, B-cells were abundantly present in the intrahepatic follicles as evidenced by IgG, IgD and IgM staining. Interestingly, similar to observations in healthy tonsil, <5% T$_{FH}$-cells expressing Bcl6 of total CD3⁺ T-cells were estimated to be present in the livers of chronic HCV patients in infiltrates and follicles (Fig. 3B). Importantly, these follicles contain distinct B and T-cell areas, which suggests not only that T$_{FH}$-cells are present in the livers of chronic HCV patients, but that they can also physically interact with and potentially modulate the activity of B-cells within the follicle aggregates (Fig. 3C).

Circulating CD19⁺ B-cells were activated and correlated with the frequency of CD4⁺CXCR5⁺ T-cells in chronic HCV patients.

Having demonstrated that T$_{FH}$-cells are in close proximity to B-cells in intrahepatic follicle aggregates, we further examined the possible effects of the altered T$_{FH}$ cell function on B-cells in peripheral blood, since the availability of liver material is limited. In blood of
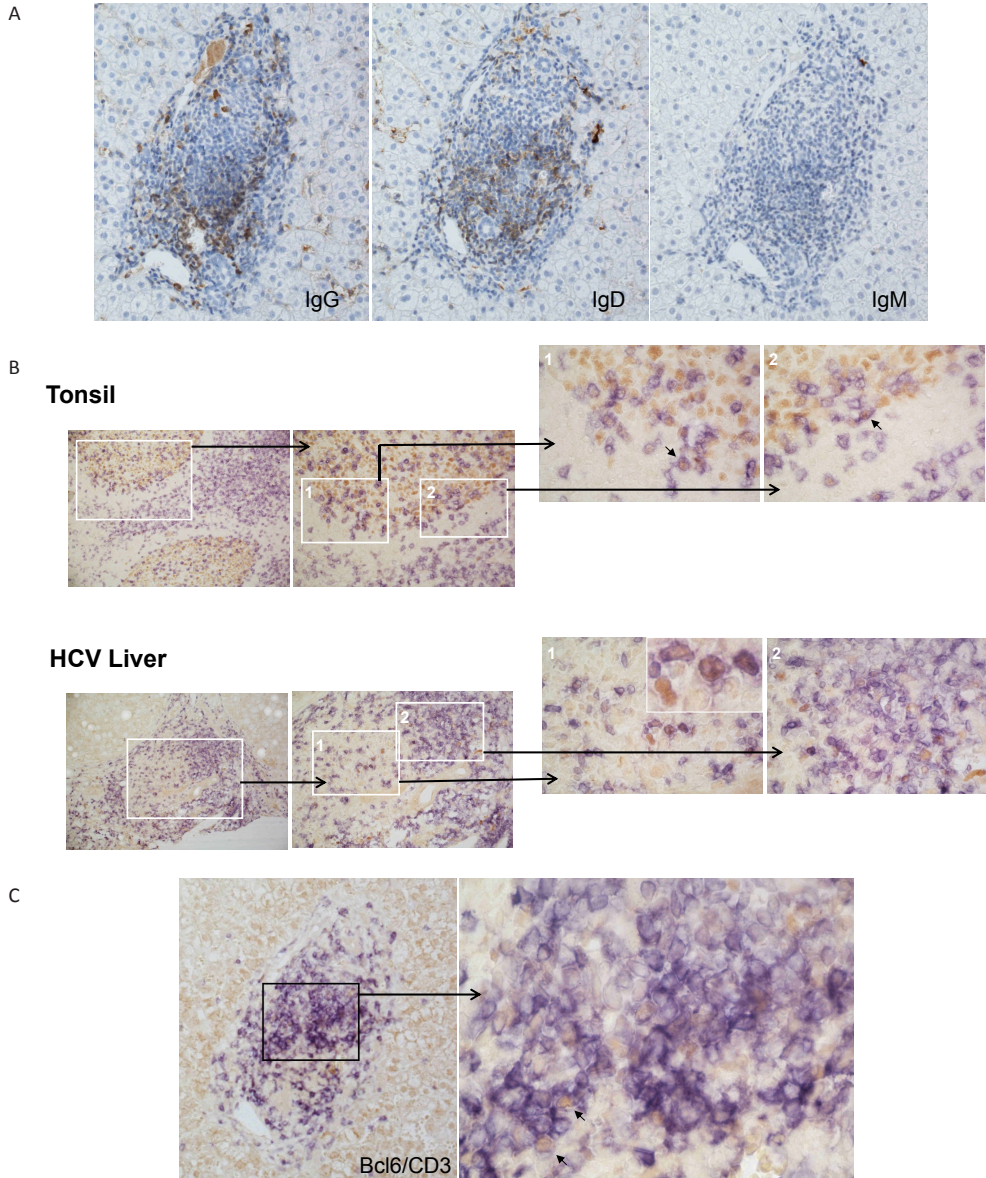
Figure 3. T$_{FH}$-cells were present in follicular infiltrates in HCV infected livers. (A) Liver biopsies containing follicles from HCV patients (n=5) were stained for functional B-cells markers. IgG was located at the outer border and IgD and IgM at the inner border of the follicles. (B) Tonsil from healthy individuals and liver biopsies from chronic HCV patients (n=12) were stained for CD3 (purple) and Bcl6 (brown). Single and double positive cells indicate T-cells (CD3$^+$), B-cells (Bcl6$^+$) and T$_{FH}$-cells (CD3$^+$Bcl6$^+$) in tonsil (upper panels) and a liver biopsy from a chronic HCV patient (lower panels). Black arrows indicate double positive cells. (C) Liver biopsy from figure 4A was stained for Bcl6 and CD3. Clear B and T-cell areas are shown within one follicle and T$_{FH}$-cells are present in close proximity to the B-cell area.

HCV patients, we showed a correlation of the frequency of CD19+ B-cells with CD4+CXCR5+ T-cells (p=0.04, Fig. 4A). Phenotyping of B-cells is generally done on the basis of CD27 and IgD expression with CD27-IgD+ cells being defined as naïve B-cells, CD27+IgD- cells as class-switched memory B-cells and CD27+IgD+ cells as non-class-switched memory B-cells [16]. Evaluation of the frequency of different B-cell populations demonstrated no differences between healthy controls and HCV patients (Fig. 4B). In a more detailed analysis in which the fibrosis stage was taken into account, the frequency of total CD27+ memory B-cells was lower in HCV patients with severe fibrosis than in patients with mild fibrosis (p=0.01, Supplementary Fig 2), which was the result of reduced frequencies of non-class switched as well as class switched memory B-cells (p=0.06, p=0.05 respectively, Supplementary Fig. 2). To determine whether the decrease in IL-21-producing CD4+CXCR5+ T-cells affected the activation status of B-cells, the expression levels of the activation markers CD69 and CD86 on naïve, class-switched memory B-cells and non-class-switched memory B-cells were analysed. Comparing HCV patients with healthy individuals, no alteration in the expression levels of CD69 or CD86 on B cell subtypes could be observed. However, as shown in figure 4C, the expression of CD69 was elevated in patients with high fibrosis scores compared to healthy controls on CD27-IgD+ naïve B-cells and CD27+IgD- B-cells (p=0.03, p=0.01). Expression of CD86 was also elevated in patients with a high fibrosis score compared to healthy controls, and was significantly increased on CD27+IgD+ B-cells (p=0.02) and CD27+IgD- B-cells (p=0.04, Fig. 4C). Thus, depending on the severity of fibrosis, B-cells obtained from chronic HCV patients display a more activated B-cell phenotype.

Peripheral blood CD4+CXCR5+ T-cells of chronic HCV patients were potent inducers of plasmablasts in vitro.

Since enhanced B-cell activation in chronic HCV patients could not be clarified by the frequency of CD4+CXCR5+ T-cells or their ability to produce IL-21, we. examined the functionality of CD4+CXCR5+ T-cells further by assessing their capacity to provide B-cell-help. Sorted CD4+CXCR5+ T-cells were incubated with autologous CD19+CD27+ B-cells from healthy controls and chronic HCV patients and plasmablast formation was evaluated by flowcytometry. Plasmablasts were defined as CD20- cells and CD27+CD38hi cells as shown in figure 4D. In line with findings in literature [15], CD4+CXCR5+ T-cells were more efficient than CD4+CXCR5- T-cells in inducing memory B-cells to become plasmablasts (Supplementary Fig. 3). In addition, co-culture of CD4+CXCR5+ T-cells with memory B-cells overall led to the development of more plasmablasts than co-cultures containing naïve B-cells (Fig. 4D and Supplementary Fig. 3). Comparing the ability of CXCR5+ T-cells to give help to B-cells in becoming plasmablasts, chronic HCV patients and healthy controls showed comparable plasmablast formation with a trend toward an even better maturation of B-cells by HCV patients (p=0.11, Fig. 4D). Functional evaluation showed that IgG and IgM levels in supernatant varied between subjects (0-4.7 μg/ml for IgG and 0-12 μg/ml for IgM) but in line with our flowcytometry data, no differences between HCV patients and healthy controls
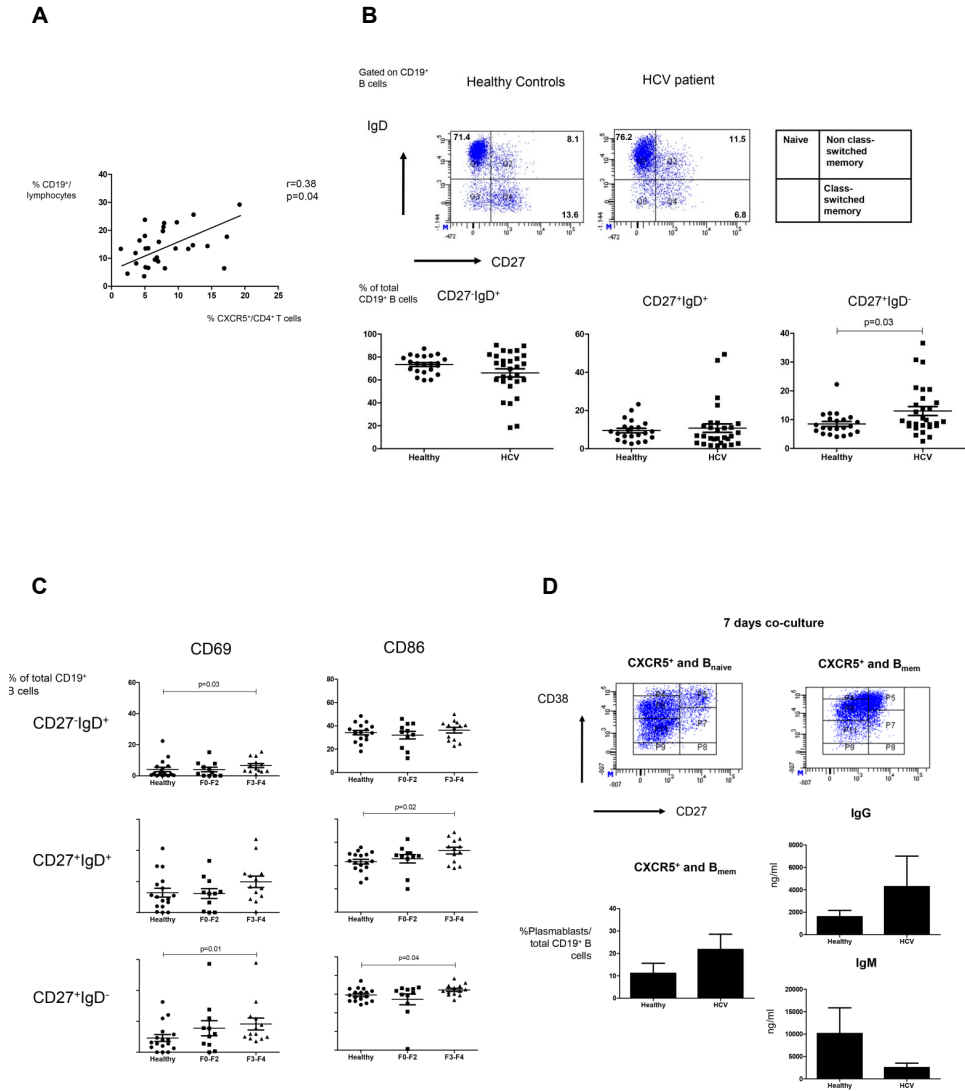
Figure 4. Blood CD4+CXCR5+ T-cells of chronic HCV patients were potent inducers of plasmablasts *in vitro*.
(A) Statistical correlation of the frequencies of CXCR5+ cells within the total CD4+ T-cell population and CD19+ B-cells within the total lymphocyte population. (B) Representative dot plots and frequencies of CD27-IgD+ naïve, IgD-CD27+ unswitched memory and CD27+IgD- switched memory B-cells. To define B-cells, gates were set on lymphocytes and CD19+ cells. (C) CD69 and CD86 expression was determined on B-cell subtypes from 22 healthy controls and 29 chronic HCV patients: 13 mild (F0-F2 and 16 severe fibrosis (F3-F4). Severe fibrosis in chronic HCV is associated with increased activation. All statistical comparisons made using Kruskal-Wallis, Mann-Whitney test, and Spearman correlation tests. (D) Representative dot plots from 7 days co-culture with CXCR5+CD4+ T-cells with CD19+CD27+ memory B-cells or CD19+CD27-IgD+ naïve B-cells. After 7 days, B-cells were defined as CD3-CD4- cells, since plasmablasts can down regulate CD19 and are negative for CD20 [27], and plasmablasts are gated as CD27+CD38hiCD20- cells. The frequency of plasmablasts after 7 days co-culture with CXCR5+CD4+ and CD19+CD27+ B-cells were determined for healthy individuals and HCV patients (n=7 for each group). Statistical comparisons made using Mann-Whitney test.

were observed. Importantly, we did not observe a difference between the HCV patients with high fibrosis grade and low fibrosis grade (results not shown). These results indicate that CD4+CXCR5+ T-cells in chronic HCV patients are able to give help to memory B-cells and induce immunoglobulin-producing plasmablasts.

DISCUSSION

In this study, we performed a detailed analysis of the interaction between T_{FH} and B-cells in chronic HCV. In the liver of patients, T_{FH}-cells were identified in infiltrates and follicular structures in the vicinity of B-cells, and importantly, IL-21-producing T_{FH}-cells were observed. Despite a reduced IL-21-producing capacity of blood T_{FH}-cells and lower serum IL-21, altered T_{FH}-B-cell interaction led to normal B-cell responses in chronic HCV.

The inflamed livers of chronic HCV patients are frequently characterized by prominent lymphoid aggregates whose histological and immune phenotypical features are reminiscent of B-cell follicles. Such aggregates are therefore excellent sites for T_{FH}-cells to exert their function [29, 30]. When these ectopic follicles develop, B and T-cells interact closely, resulting in their mutual activation. We now identified T_{FH}-cells by co-expression of Bcl6 and CD3 in these follicles. The presence of distinct B and T-cell areas within these follicles with clear fields of IgG+, IgD+ and IgM+ B-cells is in line with other reports, and suggests an efficient area for B-T-cell interaction [28, 31]. Notably, immunohistochemistry on chronic HCV livers showed IL-21-producing cells (data not shown), which further indicates that T_{FH}-cells are present in the liver and are functional. Future studies have to establish whether intrahepatic T_{FH}-cells are fully capable to provide B-cell help in chronic HCV infection.

CXCR5+CD4+ T-cells in peripheral blood are the counterparts of germinal center T_{FH}-cells and express ICOS and PD-1 at very low levels [15]. Importantly, no differences in their frequency nor in the expression of ICOS and PD-1 were observed in blood from chronic HCV patients and healthy controls. This is in contrast to studies in HBV where increased frequencies of CXCR5+CD4+ T-cells were observed [24, 32]. Importantly, we observed lower frequencies of IL-21-producing CXCR5+CD4+ T-cells which was accompanied by lower serum IL-21 levels in HCV patients. This is in line with studies showing that CXCR5+CD4+ T-cells are the main producers of IL-21 in serum [14]. Others have reported frequencies of IL-21-producing CXCR5+CD4+ T-cells and the levels of IL-21 to be higher in HBeAg+ chronic hepatitis and inactive carrier HBV patients than in healthy controls [24]. Apparently, the effect of different chronic viral hepatitis infections on T_{FH} and B-cell interaction varies. Additionally, the genetic background of the study populations (Chinese cohorts in the three mentioned studies versus a Caucasian cohort in our study) might be important as well, since IL-21 gene polymorphisms are correlated with serum IL-21 levels in viral hepatitis and systemic lupus erythematosus (SLE) patients [33, 34].

Importantly, despite lower IL-21-producing CXCR5$^+$CD4$^+$ T-cells in HCV patients, we show that CXCR5$^+$CD4$^+$ T-cells from chronic HCV patients are able to stimulate B-cells into IgG and IgM-producing plasmablasts, and no evidence was observed for dysfunctional T$_{FH}$-B-cell interactions in chronic HCV patients. Since IL-21 also plays an important role in CD8$^+$ T-cell survival and NK-cell activity in chronic viral infections, future studies will have to address the impact of lower IL-21-producing CXCR5$^+$CD4$^+$ T-cells on the induction of effective CD8$^+$ T-cell and NK-cell responses. Despite the loss of memory B-cells in F3-F4 fibrosis patients, we show that the expression levels of the activation markers CD69 and CD86 on B-cells were higher in these patients than in healthy individuals. Since we observe impaired IL-21 production in HCV patients, we hypothesize that other factors contribute to the polyclonal B-cell activation observed in HCV patients with advanced fibrosis. Our data show that high inflammation score and advanced fibrosis are correlated to the presence of follicles in the liver. It is tempting to speculate that development of follicles in the fibrotic liver contributes to B-cell activation in HCV patients with advanced fibrosis. Whether the enhanced activation state of B-cells in fibrotic livers is the result of development of tertiary lymphoid structures in the liver needs to be determined in future studies. Since B-cell abnormalities are most prominent in HCV patients with cryoglobulinemia, we assessed the role of T$_{FH}$-cells in this patient group. We included 5 patients with HCV and mixed cryoglobulinemia in our studies and observed equal frequencies of CXCR5$^+$CD4$^+$ T-cells (8.7% in HCV vs. 6.2% in HCV-cryoglobulinemia patients) and expression of PD-1 on CXCR5$^+$CD4$^+$ T-cells (2.9% in HCV vs. 1.2% in HCV-cryoglobulinemia patients), which could not explain their activated B-cell status.

In conclusion, despite decreased IL-21-producing CXCR5$^+$CD4$^+$ T-cells and lower serum IL-21 levels, we observed no alteration in T$_{FH}$-B-cell interactions in chronic HCV patients. Intrahepatic T$_{FH}$-cells were identified primarily in infiltrates and follicular structures in livers of chronic HCV patients. The identification of these cells in the liver opens new possibilities for studying B-T-cell interaction in relation to HCV chronicity. Understanding the alterations in the B-cell compartment and the involvement of T$_{FH}$-cells is crucial for a better understanding of immunopathogenesis during chronic HCV infection.

ACKNOWLEDGEMENTS

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SUPPLEMENTARY MATERIAL
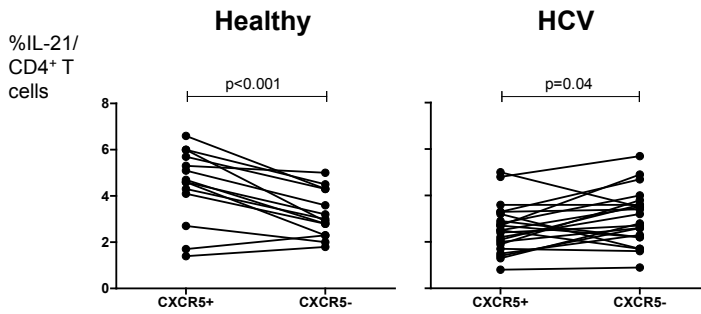
Supplementary figure 1. CD4+CXCR5+ T-cells are the main producers of IL-21 in healthy individuals but not in chronic HCV patients. Frequency of IL-21-producing CD4+CXCR5+ T-cells and CD4+CXCR5- T-cells are compared within healthy individuals and chronic HCV patients after PMA/ionomycin stimulation.
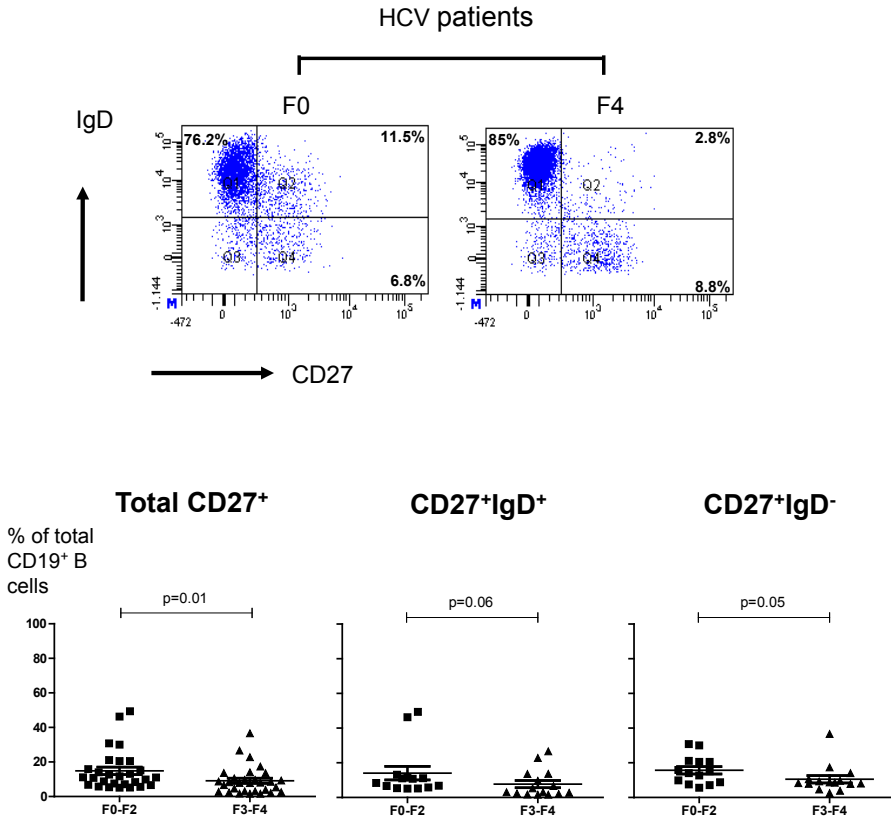
Supplementary figure 2. CD27⁺ memory B-cells are reduced in HCV patients with advanced fibrosis. Representative dot plots are presented. Frequencies of CD27⁺ total memory, IgD⁻CD27⁺ unswitched memory and CD27⁺IgD⁻ switched memory B-cells are presented from chronic HCV patients with low fibrosis score (F0-2) and advanced fibrosis score (F3-4).
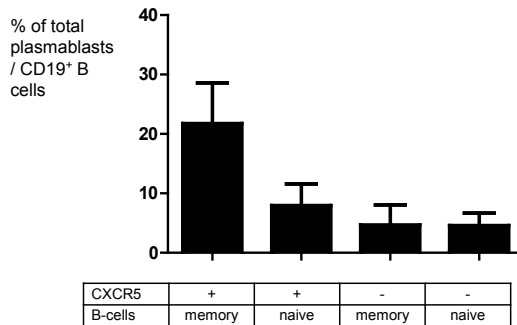
Supplementary figure 3. CD4⁺CXCR5⁺ T-cells are the better inducers of plasmablasts in HCV patients. The findings of a 7 days co-culture experiment are shown for CD4⁺CXCR5⁺ T-cells, CD4⁺CXCR5⁻ T-cells, CD19⁺CD27⁺ memory B-cells and CD19⁺CD27⁻IgD⁺ naïve B-cells. Mean frequencies of plasmablasts of CD19⁺ B-cells are presented.

2

Chapter 3

Inhibitory Receptor Molecules in Chronic Hepatitis B and C Infections: Novel Targets for Immunotherapy?

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ABSTRACT

Chronic HBV and HCV infections are the leading cause of liver-related morbidity and mortality. For effective anti-viral immunity, virus-specific T cells are required, but these cells have been shown to be weak or absent in chronic HBV and HCV patients. One of the mechanisms that underlies the impaired T cell response is the result of the continuously high viral load that causes HBV- and HCV-specific T cells to become exhausted, which is characterized by impaired proliferation, cytokine production and cytotoxic activity of T cells as well as high susceptibility to apoptosis. *In vitro* studies from chronic HBV and HCV patients as well as *in vivo* studies in animal models demonstrated a reversible state of T cell exhaustion, which can be manipulated to reinvigorate the specific anti-viral immune responses. In chronic HCV infection, this concept has been explored in clinical trials by administration of specific antibody to block the inhibitory pathways. The manipulation of inhibitory receptors is a promising and potential strategy for immunotherapeutic interventions in chronic HBV and HCV patients to facilitate complete elimination of the viruses or sustained viral control.

INTRODUCTION

HBV and HCV are the most common causes of liver diseases worldwide [1, 2]. Vertical transmission of HBV is the main infection route and is responsible for 90% of chronic viral hepatitis patients. However, HBV infection during adulthood typically induces the development of protective immunity and thereby results in only 10% progression to a chronic infection. Interestingly, the majority of HCV infected individuals are not able to clear the virus and 80% progress towards persistent infection [3]. Chronic HBV and HCV infections are characterized by relatively mild symptoms in most patients. Progression from mild to severe fibrosis and cirrhosis may take twenty to thirty years, and can eventually lead to liver failure and hepatocellular carcinoma [3].

Currently available treatments for hepatitis B (pegylated interferon-α [PEG-IFN] or nucleos(t)ide analogues [NUC]) and hepatitis C (PEG-IFN, ribavirin and a protease inhibitor for HCV genotype 1) have shown a significant clinical benefit. However, a considerably high percentage of patients fails to respond to the therapy [1, 2, 4, 5]. In addition, the clinical use of PEG-IFN in combination with ribavirin is not optimal due to the high cost, subcutaneous administration and frequent side effects, like anemia, susceptibility to infections, loss of appetite, depression and headaches [5]. A number of limitations also apply to NUC therapy, such as the necessity for life-long therapy, risk of anti-viral resistance development and potential relapse after long-term use of the therapy [5]. Although numerous promising drug candidates are being tested in phase II and III trials, especially for hepatitis C, treatment strategies need to be improved further in order to develop better and more efficient therapies.

T cell exhaustion in chronic viral hepatitis
A crucial role for T cell-mediated anti-viral immunity in HBV and HCV elimination has been demonstrated [6]. However, both viruses possess efficient strategies to silence the adaptive immune system. Virus-specific T cell responses are weak and impaired in chronic HBV and HCV patients [3, 6, 7]. Factors that contribute to the suppression of virus-specific T cell responses are lack of CD4^{+} T cell help, active immunosuppressive regulation by regulatory T cells (Treg) and inhibitory cytokines such as interleukin-10 (IL-10) and transforming growth factor-β (TGF-β), as well as T cell exhaustion due to continuously high viral antigen pressure [8-10]. The contribution of those mechanisms may vary depending on the characteristics of the specific viral infection, such as lack of CD4^{+} T cell help which may be less obvious in HBV and HCV than in HIV infection.

T cell exhaustion, as an important factor determining viral persistence, has been the focus of many studies [11, 12]. T cell exhaustion was first demonstrated in chronic lymphocytic choriomeningitis virus (LCMV)-infected mice where LCMV-specific CD8^{+} T cells were unable

to perform anti-viral effector functions *in vivo* [13]. Further studies showed that the so-called exhaustion of the anti-viral T cells was a gradual process and progressed from mild (reduced cytotoxic activity, decreased IL-2 and TNF production), to moderate (modestly defective IFN-γ production or cytotoxicity and decreased IL-2 or TNF production), to severe exhaustion (lack of IFN-γ, TNF, IL-2 and cytotoxicity) [14]. Ultimately, physical deletion (apoptosis) of T cells may occur [14]. The continuous exposure of immune cells to high amounts of viral antigens is an important factor responsible for T cell exhaustion during chronic LCMV infection [14, 15]. Detailed characterization of exhausted LCMV-specific CD8⁺ T cells showed overexpression of multiple inhibitory receptor genes, such as those encoding programmed death-1 (PD-1), T cell immunoglobulin and mucin domain containing molecule-3 (Tim-3), CD244 (2B4), cytotoxic T lymphocyte antigen-4 (CTLA-4), CD160, lymphocyte-activation gene 3 (LAG-3), Killer cell lectin-like receptor (KLR) G1 (KLRG-1), KLRA-3 and KLRA-9 [16].

T cell exhaustion has not only been reported in chronic LCMV infection, but also in other conditions where sustained antigenic stimulation occurs, such as *Plasmodium* sp.*,* HIV, HBV and HCV, as well as in cancer [17]. In chronic HIV infection, the occurrence of HIV-specific T cell exhaustion leads to failure of viral clearance and is associated with disease progression such as low CD4⁺ T cell count [18]. Similarly, T cell exhaustion plays a prominent role in immune resistance in cancer [19]. Manipulating signaling pathways to reverse T cell exhaustion in HIV and cancer is therefore a promising approach in immunotherapy of these diseases [19, 20]. In fact, anti-CTLA-4 (ipilimumab) was approved as the first immunotherapeutic antibody by the US Food and Drug Administration for melanoma [19]. At the moment, several agents targeting other inhibitory receptors such as PD-1 and LAG-3 are in different clinical phases of their development in cancer therapy [19].

It is important to note that T cell dysfunction as a consequence of exhaustion differs from a state in which the immune system as a whole is immunosuppressed. This was shown in a study of 43 chronic HCV patients, who had been previously exposed to CMV [8]. In this study, low or undetectable HCV-specific T cell proliferation was observed in most patients. In contrast, up to 100-fold higher levels of T cell proliferation were detectable in peripheral blood upon exposure to CMV antigens as compared to HCV antigens *in vitro* [8]. Accordingly, HCV-specific T cells exhibited the lowest activity to suppress viral replication *ex vivo* compared to influenza-, EBV- and CMV-specific T cells from the same patients [21]. These findings indicate, in line with other studies demonstrating that specific anti-viral immune responses to other viruses were preserved [22-24], that T cell exhaustion in these patients is HCV-specific. Similar findings were reported in chronic HBV patients, where impaired HBV-specific IFN-γ responses were observed, whereas IFN-γ production by CD8⁺ T cells specific for influenza viruses was preserved [25]. Noteworthy, although HCV does not lead to a general immunosuppressed state like HIV does, several studies reported that HCV-infected patients responded less potently to HBV vaccination, suggesting that it may affect the induction of certain immune responses [26, 27].

Due to the broad availability of reagents to detect virus-specific CD8^{+} T cells (multimers), many studies focused on T cell exhaustion in virus-specific CD8^{+} T cells. Although a number of multimers has recently become available for virus-specific CD4^{+} T cells, knowledge on the phenotype of exhausted CD4^{+} T cells is still limited [28-32]. Functionally, strong and broad HCV-specific CD4^{+} T cell responses are detected during the initial phase of HCV infection, irrespective of the clinical outcome [29]. Progression towards chronic infection was associated with impairment of these responses and subsequent deletion of HCV-specific CD4^{+} T cells [29]. Similar as virus-specific CD8^{+} T cell exhaustion, inhibitory receptors, such as PD-1 and CTLA-4, may also be involved in modulating the dysfunctional state of virus-specific CD4^{+} T cells [28, 30-32]. However, the expression levels could be different [30, 32]. Several factors may contribute to the differential regulation of inhibitory receptor expression between virus specific CD4^{+} and CD8^{+} T cells, such as viral tropism and the strength of the stimulus at their corresponding epitope [33].

The function of T cells is regulated by several extrinsic (such as Treg and inhibitory cytokines) and intrinsic (such as intracellular signaling and inhibitory receptor molecules expressed on T cells) immunoregulatory pathways [11]. As stated above, continuous antigen stimulation and triggering of the inhibitory receptors can cause T cell exhaustion [11, 34]. However, it is important to mention that in acute infection, the expression levels of many inhibitory receptors, such as CTLA-4, PD-1, and Tim-3, are increased upon activation following antigen-specific T cell receptor (TCR) stimulation [34]. Since these T cells are fully functional, this upregulation likely limits effector function of T cells and therefore pathology. In this review, we will discuss the role of inhibitory receptor molecules during chronic HBV and HCV infections and their potential to be manipulated as a novel therapeutic approach to improve the outcome of current standard therapy.

The role of inhibitory receptors on circulating HBV- and HCV-specific T cells during chronic infections

When patients acutely infected with HBV and HCV cannot resolve the infection spontaneously, they become chronically infected. Chronic HBV patients are highly heterogeneous in their viral load and ALT levels which is used to define distinct clinical phases of the disease [3]. By contrast, chronic HCV patients are characterized by a persistently high viral load, relatively low ALT levels and mild pathology [3, 6], indicating the inability of T cell-mediated cellular immunity to both control viral replication and to kill virus-infected hepatocytes. The exhausted HBV- and HCV-specific T cells observed during chronic infections are phenotypically characterized by high levels of inhibitory receptors which reduce in people who resolve the infection (Fig. 1).

The important role of PD-1 in T cell exhaustion during chronic viral infection was first shown in a study of chronic LCMV-infected mice [35]. During the chronic phase of infection, PD-1 was highly expressed on LCMV-specific CD8^{+} T cells, and displayed an impaired proliferation and
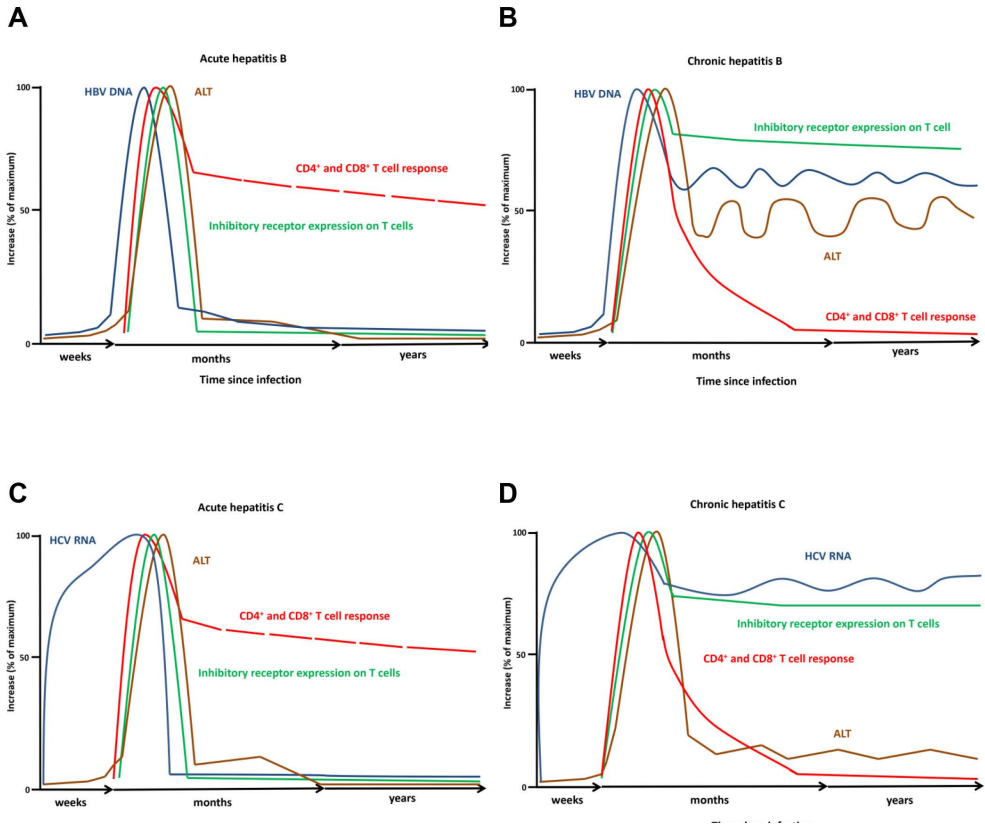
Figure 1. Differential expression of inhibitory receptor molecules during acute-resolved or chronic HBV (1-2) and HCV (3-4) infections. (A) Upon acute HBV infection, viral replication takes place in hepatocytes and peaks within 4-6 weeks of infection, followed by elimination. During the phase of viral clearance, serum ALT levels peak in parallel with the presence of strong and multi-specific HBV-specific T cell responses, indicating immune-mediated liver injury. To limit excessive inflammation and subsequent liver damage, inhibitory receptors are transiently upregulated on HBV-specific T cells. The development of memory HBV-specific T cells (dashed lines) after viral clearance occurs concurrently with downregulation of inhibitory receptor molecules expressed on HBV-specific T cells. (B) In chronic HBV infections, failure of HBV DNA elimination is associated with progressive loss of HBV-specific T cell responses. At this chronic stage, HBV-specific T cells continuously express high levels of inhibitory receptors. Variation of ALT levels is depicted as shown in immune-active patients. **(C)** Upon HCV infection, HCV rapidly replicates and peak levels of HCV RNA are observed within few weeks after infection. In general, 4-8 weeks after infection, HCV-specific T cell responses are observed accompanied by increased ALT levels and transient upregulation of inhibitory receptors. Following HCV elimination, HCV-specific memory T cells have developed (dashed lines) as indicated by normalization of ALT levels and downregulation of inhibitory receptors on T cells. **(D)** In association with the failure to eliminate HCV infection during the acute stage, HCV-specific T cell responses gradually become weak and dysfunctional during chronic HCV infection, which is accompanied by relatively low ALT levels. HCV-specific T cells fail to downregulate inhibitory receptors and maintain high levels of inhibitory receptor expression.

cytokine production. *In vivo* blockade of the PD-1 pathway in mice by blocking one of its ligands (PD-L1) resulted in enhanced virus-specific CD8^{+} T cell activity and reduced viremia [35]. The importance of PD-1 in regulating adaptive immunity was confirmed in chronic simian

immunodeficiency virus (SIV)-infected macaques, where PD-1 blockade rejuvenated both cellular and humoral immune responses and resulted in significantly reduced viral load and prolonged survival [36].

In humans, PD-1 is involved in chronic infections with HIV [18, 37-39], HBV [40-43] and HCV [21, 22, 44-47]. HBV- or HCV-specific CD8+ T cells that expressed PD-1 during chronic HBV and HCV infections were nonfunctional and were not able to proliferate or produce IFN-γ and TNF [21, 22, 42, 43]. In addition, these PD-1-expressing T cells, irrespective of antigen specificity, were more susceptible to undergo apoptosis [37]. Moreover, a recent study demonstrated that PD-1 may impair the motility of exhausted LCMV-specific T cells, thereby reducing their interaction with virus-infected cells [48]. In HIV, *in vitro* blockade of the PD-1 pathway resulted in increased proliferation and cytokine production of HIV-specific CD8+ T cells [18, 37, 38]. *In vitro* blockade of the PD-1 pathway reactivated the functionality of HBV- and HCV-specific T cells obtained from chronically infected patients, as indicated by increased proliferation, cytokine production and cytotoxicity [22, 40, 42, 44, 45, 49]. Further support for a central role of the PD-1 pathway during the persistent state of HBV and HCV infections comes from the observation that its ligand, PD-L1, is present at higher levels on myeloid dendritic cells (DC) and monocytes of chronic HBV- and HCV-infected patients [50, 51] compared to normal individuals.

Prospective analysis of a chronic HCV patient who spontaneously resolved the infection, demonstrated a significant increase of HCV-specific IFN-γ response and downregulation of PD-1 expression on HCV-specific CD8+ T cells during the clearance phase. This finding underlines that T cell exhaustion is a reversible process and its reversion is associated with viral decline [52]. However, it is not clear yet whether this spontaneous restoration of anti-viral immune responses is the cause or the consequence of viral load decline. Interesting is the observation that PD-1 expression levels were decreased on HCV-specific T cells upon the emergence of viral escape variants [47, 53]. This finding suggests a link between mutational escape and T cell exhaustion [54], and indicates that ongoing antigen stimulation of CD8+ T cells of a cognate viral epitope is a prerequisite for virus-specific T cell exhaustion [53]. This link was supported by the finding that the presence of HCV-specific T cells with high CD127 expression in chronic HCV patients was associated with more viral sequence variants that led to loss of antigenic stimulation on HCV-specific T cells [55]. These results suggest that mutations of the autologous virus diminishes recognition and prevent exhaustion of T cells specific for the original epitope, which facilitates their differentiation into long-lived CD127-expressing memory T cells. Therefore, viral escape mutation and T cell exhaustion are likely two distinct mechanisms contributing to virus coexistence in infected hosts [54].

The importance of Tim-3 in T cell exhaustion was first identified in chronic HIV infection. HIV-1-specific CD8+ T cells expressing Tim-3 were identified as a distinct population from HIV-1-specific CD8+ T cells that expressed PD-1, but had similar dysfunctional characteristics [56]. Studies in chronic HBV- and HCV-infected patients further highlighted a notable role for

Tim-3 in chronic human viral infections [57-61]. HBV- and HCV-specific CD8^{+} T cells obtained from chronic viral hepatitis patients contained a significantly higher expression of Tim-3 than CMV-specific CD8^{+} T cells [57-59]. These Tim-3-expressing HBV- and HCV-specific CD8^{+} T cells had a diminished capability to produce TNF and IFN-γ, which could be restored by *in vitro* inhibition of Tim-3 to interact with its ligand, galectin-9 (gal-9) [57, 58]. More importantly, *in vitro* Tim-3 blockade on HCV-specific T cells has been suggested to be more effective in enhancing cytotoxicity than PD-L1/PD-L2 blockade, as demonstrated by enhanced CD107a expression (marker for release of cytotoxic granules), killing of HepG2 cells expressing cognate HCV epitope and higher aspartate transaminase (AST) levels [60]. Similar findings were reported in HBV as assessed by CD107a expression [58].

In contrast to PD-1 and Tim-3, *in vivo* blockade of CTLA-4 in mice chronically infected with LCMV, either alone or in combination with anti-PD-L1, had no (additional) beneficial effect in boosting T cell responses and controlling viremia [35]. However, subsequent studies of chronic human viral infections, such as HIV [32, 62], HBV [63] and HCV [23, 28, 64], did show a role for CTLA-4 in modulating T cell function. In HIV infection, CTLA-4 was specifically upregulated on HIV-specific CD4^{+} T cells, but not on HIV-specific CD8^{+} T cells. *In vitro* blockade of CTLA-4 augmented proliferation and cytokine production of HIV-specific CD4^{+} T cells [32]. The role of CTLA-4 blockade was also investigated in SIV-infected macaques, but no improved SIV-specific CD8^{+} T cell expansion and cytokine production was observed [65].

HCV-specific CD4^{+} T cells from chronic HCV patients displayed higher CTLA-4 levels than HCV resolvers, which supports an inhibitory role for CTLA-4 in chronic hepatitis infections [28]. Indeed, also on HBV-specific CD8^{+} T cells, CTLA-4 expression was higher in chronic HBV patients with viral load >2,000 IU/mL than in those who resolved the infection or showed lower HBV DNA levels [63]. In line with this, blockade of the CTLA-4 pathway *in vitro* increased the expansion of IFN-γ producing HBV- and HCV-specific CD8^{+} T cells [63, 64].

To our knowledge, few studies investigated the importance of CD244 (2B4) in modulating HCV- and HBV-specific CD8^{+} T cells activity [21, 66-68]. The frequency of CD244 on HBV-specific CD8^{+} T cells was increased in patients with chronic HBV compared to HBV resolvers and *in vitro* blockade of CD244 by antibodies against either CD244 or its ligand (CD48) resulted in increased virus-specific T cell proliferation, cytokine production and cytotoxicity [66]. A possible role for other inhibitory receptors, such as CD160 and LAG-3, has not been investigated in depth. However, their involvement in modulating T cell exhaustion was reported by several studies in which the phenotypical and functional consequences were examined of simultaneous expression of multiple inhibitory receptors on exhausted virus-specific T cells instead of individual receptors.

3

Co-expression of multiple inhibitory receptors

Co-expression of multiple inhibitory receptors has been shown in the chronic LCMV infection model with simultaneous expression of PD-1, LAG-3, CD160 and 2B4 on the surface of virus-specific CD8^{+} T cells, which was associated with severity of infection (high viral load and infection duration) and lower T cell function, as assessed by co-production of IFN-γ and TNF [69]. These findings suggest that virus-specific T cells co-expressing more inhibitory receptors are in a deeper state of exhaustion. Functional recovery of exhausted virus-specific T cells observed after simultaneous blockade of inhibitory receptors are therefore more pronounced and consequently provide a better control of viral load *in vivo.*

Chronic HCV-infected patients exhibit increased simultaneous expression of PD-1, 2B4, CD160, and KLRG1 on HCV-specific CD8^{+} T cells. This co-expression of multiple inhibitory receptors inversely correlated with CD127 expression and proliferation capacity [53]. In chronic HCV/HIV co-infected patients, a substantially higher proportion of HCV-specific CD8^{+} T cells co-expressed PD-1 and Tim-3 as compared to HIV- and CMV-specific CD8^{+} T cells [70]. Simultaneous blockade of PD-1 and Tim-3 showed a synergistic effect in some, but not all patients [70]. The frequency of HBV-specific CD8^{+} T cells co-expressing PD-1 and 2B4 was higher in chronic HBV patients as compared to HBV resolvers [66]. Interestingly, about 80% of HBV-specific CD8^{+} T cells in chronic HBV patients co-expressed PD-1 and 2B4 [66]. The fact that PD-1/PD-L1 blockade in several studies could not rejuvenate all T cell functions and had no effect in some patients may be the consequence of other inhibitory receptor pathways that should be blocked to fully restore T cell function [58, 63, 71, 72]. These findings point out the complexity of the inhibitory receptor network that may either have similar or distinct pathways to regulate specific T cell activities [11]. Therefore, extensive studies need to be done to clarify co-expression of various inhibitory receptors in association with different stages of T cell exhaustion. Delineation of downstream signaling pathways of these inhibitory receptors may provide crucial information for the probable additive effects of combination blockade to achieve better reconstitution of T cell function than single blockade alone.

The role of inhibitory receptors in the liver during chronic infections

Our knowledge of T cell immunity during infections with HBV and HCV is predominantly based on studies performed using peripheral blood, since this is easily available. However, both viruses are hepatotropic and infect primarily hepatocytes. Viral replication takes place within hepatocytes at high replication rates, resulting in local accumulation of high viral titers. Although infiltration of T cells in the liver is extensive, liver injury is modest as evidenced by low ALT levels. This indicates the limited cytotoxic activity of these infiltrating T cells and suggests an exhausted state. The continuous presence of high amounts of viral antigens likely leads to increased and possibly differential expression of inhibitory receptors on intrahepatic T cells compared to peripheral blood T cells.

Liver infiltrating T cells in chronic HCV-infected patients exhibited significantly higher expression of PD-1 on total CD8^{+} and CD4^{+} T cell populations compared to peripheral blood T cells [23, 30]. Moreover, intrahepatic total PD-1^{+} CD8^{+} T cells of HCV infected patients expressed higher levels of CTLA-4 but less CD28 and CD127 than peripheral blood PD-1^{+} CD8^{+} T cells [23]. Higher expression of PD-1 and lower expression of CD127 was also observed on HCV-specific T cells in the liver compared to peripheral blood T cells and to total CD8^{+} T cells [23, 30, 45, 49, 68, 73]. Investigation of other inhibitory receptors revealed that intrahepatic HCV-specific CD8^{+} T cells expressed comparable level of Tim-3 [57], but higher levels of CTLA-4 and CD244 [64, 68] than circulating HCV-specific CD8^{+} T cells. In HBV infected patients, liver-derived HBV-specific CD8^{+} T cells exhibited higher levels of PD-1 and LAG-3 but lower levels of CD127 versus their peripheral counterpart [40, 68]. In addition, higher frequencies of HBV-specific T cells co-expressing PD-1 and CD244 were found in the liver as compared to blood, indicating a higher exhausted state [66].

In the inflamed liver, high viral antigen pressure likely results in high expression of inhibitory receptors on virus-specific T cells and various studies have examined whether this severe exhausted state of intrahepatic T cells could be reversed. It has been reported that exhausted intrahepatic PD-1-expressing HCV-specific CD8^{+} T cells could not be rescued by single blockade of PD-1 using anti-PD-L1/PD-L2, in contrast to peripheral blood PD-1-expressing HCV-specific CD8^{+} T cells [23]. Similar findings were reported for CTLA-4 blockade alone or combination of PD-1/PD-L1 blockade and induction of co-stimulatory molecule 4-1BB (CD137) of intrahepatic HCV-specific CD8^{+} T cells [64, 68]. To our knowledge, no other simultaneous blockades to reinvigorate intrahepatic HCV-specific T cells have been investigated so far. Strikingly, in contrast to HCV, intrahepatic HBV-specific CD8^{+} T cells were responsive to single PD-1 or CTLA-4 blockade and CD137 stimulation [40, 63, 68]. This difference may be due to the heterogeneity between the studied patient population (infection duration or disease stage) or differential regulation of co-stimulatory and co-inhibitory pathways in HBV- and HCV-specific T cells.

Inhibitory receptors can only transmit their signal to suppress T cell functions when engaged with their corresponding ligands [34]. It has been shown that proliferation of activated T cells expressing PD-1 can be inhibited *in vitro* by liver nonparenchymal cells, such as liver sinusoidal endothelial cells (LSEC) and Kupffer cells, that constitutively expressed PD-L1 [74].

PD-1 ligands, PD-L1 and PD-L2, were upregulated in inflamed livers, including those infected with HBV and HCV [43, 75]. In HBV- or HCV-infected liver, PD-L1 was highly expressed on hepatocytes, Kupffer cells, LSEC, as well as on DC [41, 43, 75], while Gal-9 was highly expressed on Kupffer cells [58, 76] and also, *in vitro*, on HCV-infected hepatocytes [77]. Therefore, it is tempting to speculate that upregulation of inhibitory receptor ligands is one of the counter-regulatory mechanisms to prevent immune-mediated damage in inflamed tissues, but subsequently negatively impacts the effective T cell responses needed for viral clearance.

Collectively, these results show that liver-infiltrating lymphocytes in chronic HBV and HCV patients display a more exhausted phenotype compared to circulating T cells. Besides intrinsic factors, intrahepatic T cells in inflamed livers are also more subjected to other negative regulatory mechanisms, like the tolerogenic environment [78] and the relatively high number of Treg in the liver compared to blood in HBV- and HCV-infected patients [68, 79]. These factors need to be taken into account to better understand the phenomenon of T cell exhaustion in the liver in order to define novel strategies to reinvigorate virus-specific T cell function.

Inhibitory receptors as potential targets for immunotherapy

Restoration of the impaired T cell response against HBV or HCV is an attractive strategy to eradicate these viruses in a very specific manner. Lifting the inhibitory effect of exhaustion markers on virus-specific T cells might be one alternative approach to reach this goal [10, 80, 81]. Recently, anti-PD-1 treatment was investigated in three chimpanzees with chronic HCV infection. A substantial decline of viral load in association with functional reactivation of HCV-specific CD4+ and CD8+ T cells targeting multiple HCV epitopes was demonstrated in one out of three animals. The animal with a viral response had stronger T cell responses detected in the blood and in the liver before initiation of treatment than the non-responder animals, which may indicate that pre-existing T cell responses influence the success of treatment [82]. Phase-I clinical trials examining the use of antibodies against PD-1 (MDX-1106) and CTLA-4 (tremelimumab) in patients with chronic hepatitis C have been completed [83, 84]. Out of 45 chronic hepatitis C patients who received a single dose of anti-PD-1 antibody, 5 patients showed a decline in their serum HCV RNA level of more than 0.5 log [83]. Interestingly, one of the responders, who showed undetectable HCV RNA for more than one year after study completion, was a previous non-responder to PEG-IFN-based treatment [83]. This study provides important information that HCV replication can be inhibited by anti-PD-1 treatment in some chronic HCV patients. A substantial reduction of viral load in association with enhanced HCV-specific immune responses was also observed in chronic HCV patients treated with tremelimumab, even though there was a heterogeneous response between patients [84]. Changes in viral quasispecies variants were observed during the course of treatment, suggesting that enhanced HCV-specific responses were responsible for the anti-viral effect of tremelimumab [84]. These trials are a first step to translate the concept of T cell exhaustion mediated by inhibitory receptors into the clinical setting. Nevertheless, several considerations have to be taken into account.

First, inhibitory receptors might be involved in the induction of central as well as peripheral tolerance to prevent T cell-mediated immune destruction [85, 86]. It has been shown that infection with LCMV of mice deficient in PD-L1 led to rapid death due to severe immunopathologic reactions [35]. Several viruses apparently exploit this homeostatic mechanism to dampen virus-specific immune responses and consequently facilitate their long-lived persistence

in the infected host. Taking this into account, there is a considerable risk of inducing immunopathology after blockade of those inhibitory receptors [87]. Various immune-related adverse events were reported in phase I clinical trials of anti-PD-1 and anti-PD-L1 neutralizing antibody in patients with cancer, such as pneumonitis, hepatitis, thyroiditis and colitis, which required administration of glucocorticoids [88, 89]. Moreover, during anti-PD-1 antibody treatment in chronic HCV patients, hyperthyroidism and exacerbation of diabetes were reported [83], while chronic HCV patients treated with tremelimumab revealed a good safety profile [84].

Second, chronic HBV- or HCV-infected patients might be in different stages of T cell exhaustion, and single blockade of a specific pathway is likely beneficial in some, but not all patients. At present, it is not well understood what determines the profile of exhaustion markers on virus-specific T cells and how this pattern can be manipulated in patients. It is unknown whether *in vivo* simultaneous blockade of inhibitory receptors would provide a better clinical efficacy without additional toxicities. Therefore, the number of inhibitory receptors that must be blocked to effectively eradicate the virus should be based on the consideration of optimally restoring T cell function to reduce viral load on one hand and reducing risk of immunopathology on the other hand.

Third, besides the phenomenon of T cell exhaustion mediated by inhibitory receptors, Treg and inhibitory cytokines, such as IL-10 and TGF-β can also suppress HBV- and HCV-specific T cell activity (Fig. 2) [8-10]. Interestingly, we have demonstrated that regulation of HCV-specific T cell responses by IL-10, TGF-β and Treg was highly diverse in chronic HCV patients and that no single dominant negative regulatory mechanism was observed [8]. Therefore, blockade of inhibitory receptor molecules in combination with IL-10, TGF-β or Treg blockade might be beneficial in some individual patients to more optimally revive T cell functions, as shown in previous studies in animal models of chronic viral infection which combined PD-1 with IL-10 in chronic LCMV-infected mice (Fig. 3) [90, 91]. It is worth noting that some of the inhibitory receptors such as PD-1 and Tim-3 have been reported to be involved in negatively regulating the proliferation and suppressive capacity of Treg [79, 92]. Consequently, blockade of inhibitory receptors could also reinforce the functionality of Treg and thereby limit the improved functions of effector T cells. Such issues have not been addressed in previous studies.

Fourth, due to the relatively high costs of monoclonal antibody therapy, selection of patients who have a high likelihood to respond to the therapy is an important aspect for clinical decision-making. In cancer patients, it was shown that only PD-L1-expressing tumors responded to the anti-PD-1 treatment [89]. Moreover, careful analysis of the presence of viral epitope mutations is also important, since targeting HBV- or HCV-specific T cells unable to recognize the circulating viral epitope, may be useless [55, 73, 93]. Since all of these studies examined viral epitope escape from virus-specific CD8+ T cells, further studies are required for virus-specific CD4+ T cells. Therefore we need to take into account the possibility to
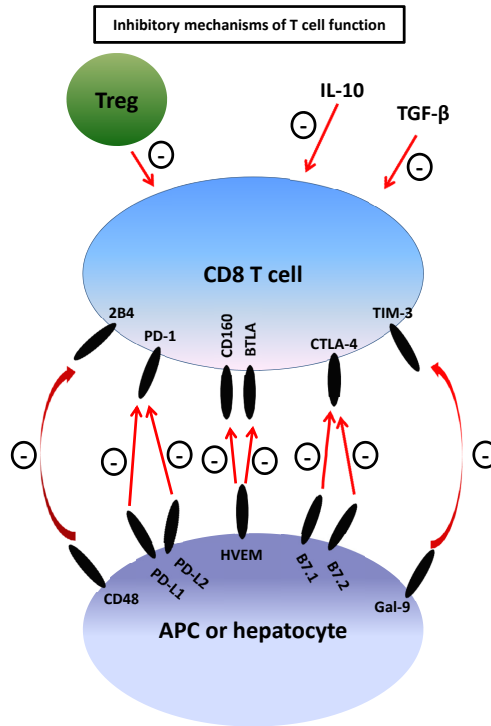
Figure 2. Regulatory mechanisms of T cell function. T cell function is modulated by several regulatory mechanisms, such as expression of (multiple) inhibitory receptor molecules on the surface of T cells, active suppression by Treg and suppression by inhibitory cytokines such as IL-10 and TGF-β.

'screen' patients for optimal personalized immunotherapy.

The expression levels of the inhibitory receptors may represent a relatively simple biomarker to predict the therapeutic efficacy of inhibitory receptor blockade. In an *in vitro* study it was shown that HCV-specific CD8^{+} T cells with high PD-1 expression were unresponsive to PD-L1 blockade, while HCV-specific CD8^{+} T cells with lower PD-1 expression levels exhibited enhanced proliferation and effector functions [23, 28]. Additionally, the responders of *in vitro* CTLA-4, Tim-3 or PD-L1/PD-L2 single blockade in chronic HBV patients were largely non-overlapping [58, 63], indicating a more dominant role of each inhibitory pathway in individual patients. Therefore, assessing the expression profiles of inhibitory receptors may be a valuable tool for therapy decision. Of note, the function of inhibitory receptors is also determined by the interaction with other molecules, which can modulate the signaling pathway of inhibitory receptors, such as the presence of Bat3 which binds to and blocks internal signaling of Tim-3 receptor [94]. It is therefore possible that the presence of the associated-signaling molecules, and not the expression of inhibitory receptors, is more responsible for the observed T cell function. The potential role of PD-1, PD-L1 and other
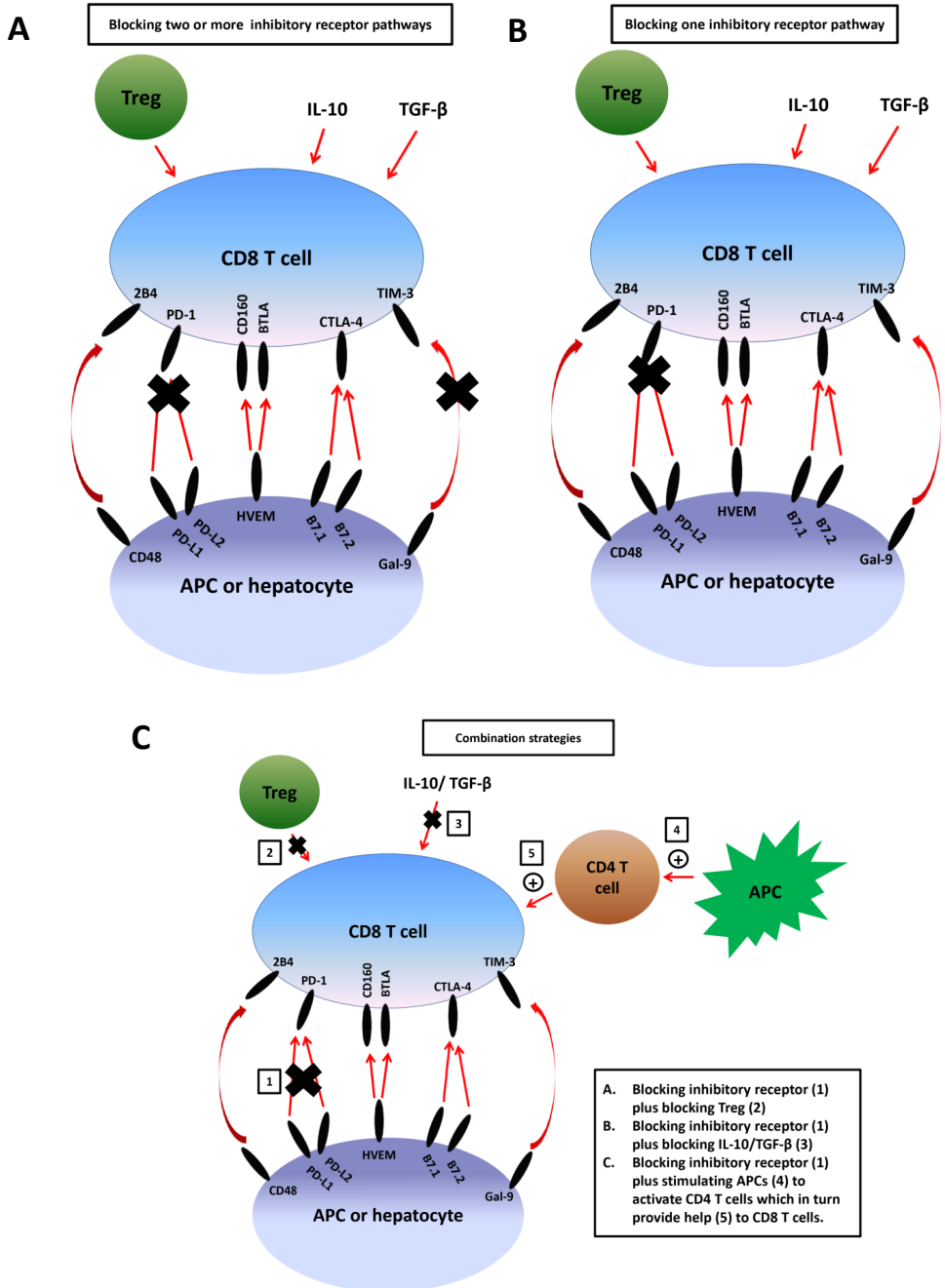
Figure 3. Different possible strategies to reverse T cell exhaustion of HBV- and HCV-specific T cells. To reverse the state of T cell exhaustion, several strategies could be employed such as blockade of single (A) or multiple (B) inhibitory receptor pathways. Combination strategies (C) could be beneficial by blocking inhibitory receptors together with blockade of Treg, IL-10 and/or TGF-β. Moreover, stimulating CD4+ T cell function, for instance via antigen presenting cells (APC), could also provide help to exhausted CD8+ T cells.

molecules as clinically relevant biomarkers to predict therapy outcome requires further investigation.

Clinical trials in chronic HCV with novel direct-acting antivirals (DAA) showed promising results with eradication of HCV RNA in 85-90% of patients depending on the specific inclusion criteria of the trial. These studies raise hope towards future PEG-IFN-free therapy in chronic HCV infections [95]. Therefore, it is unlikely that the effect of inhibitory receptor blockade in chronic HCV patients will be explored in large trials. However, inhibitory receptor blockade could still be considered in chronic HCV to treat those who are not eligible for or do not respond to the combination therapy [83]. In chronic HBV infections, current treatment with NUC is highly effective in suppressing viral replication. However, sustained-off treatment responses are rarely achieved [96] since complete eradication of covalently closed circular DNA (cccDNA) accompanied by loss of HBsAg is infrequent, and consequently viral relapse frequently occurs upon discontinuation of NUC therapy. The optimal approach to eliminate HBV infection may require combination strategies. NUC therapy can suppress viral load to reduce HBV-specific T cell exhaustion to some extent [97]. Therapeutic vaccination to HBV to induce the development of functional effector and memory T cells [81] in combination with blockade of multiple inhibitory receptors and possibly other negative immunoregulatory pathways (IL-10 and TGF-β) may further improve the functionality of HBV-specific T cells, and may lead to sustained viral control.

CONCLUSION

Our understanding of the mechanisms responsible for T cell exhaustion in chronic viral infection has improved dramatically over the last few years [11]. Detailed cellular and molecular characteristics of exhausted virus-specific T cells have been described in LCMV infection in mice, as well as HIV, HBV and HCV in humans. It is now well established that inhibitory molecules, such as PD-1, Tim-3 and CTLA-4 are involved in regulating the state of T cell exhaustion, which allows us to design novel therapeutic interventions to improve the clinical outcome of our current standard treatment. Despite significant achievements in understanding the immunoregulatory role of inhibitory receptors, their regulation and associated-signaling molecule(s) are still poorly understood. Further investigation is highly important since it will help us to comprehend how the immune system directs T cell function via modulation of inhibitory receptors, which may control the progression towards chronic infection or spontaneous clearance of HBV or HCV during acute or chronic stage of infection, as well as immunopathogenesis. In addition, therapeutic strategies involving neutralization of exhaustion markers to restore impaired T cell responses against HBV or HCV is an attractive approach for complete eradication of HBV and HCV.

ACKNOWLEDGEMENTS

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3

Part II

The effect of HCV treatment on peripheral and intrahepatic immune responses

Chapter 4

Erythropoietin administration suppresses human monocyte function *in vitro* and during therapy-induced anemia in HCV patients

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ABSTRACT

Erythropoietin (EPO) is a hormone that controls red blood cell production. Binding of EPO to the EPO-receptor results in increased numbers of red blood cells in the circulation, which makes EPO a potent molecule to treat anemia in various groups of patients. Although numerous studies have examined the clinical effects of EPO, its immunological effects have received less attention.

In this study, we examined the immunological effects of EPO on human monocytes. We show that human monocytes express EPO receptor mRNA, and are responsive to EPO in cell culture. *In vitro* exposure of PBMC from individuals to EPO and the TLR4 ligand LPS showed a significant reduction of monocytes producing IL-6 and TNF, while the frequencies of IL-12p40, IL-10, MIP-1β and IL-8-producing cells did not change upon incubation with EPO. In addition, EPO did increase the phagocytic activity but did not affect the ability to produce ROS by monocytes. Moreover, we studied 8 chronic HCV patients undergoing treatment with peg-IFN and ribavirin, who were administered EPO for treatment-induced anemia. Blood was collected before and 7 days after EPO injection. In 7 patients, we observed a significant decline at day 7 after EPO administration of the frequency of monocytes producing various pro-inflammatory cytokines following stimulation with the TLR4 ligand LPS and the TLR7/8 ligand R848, which is in line with our *in vitro* findings. Our findings demonstrate an inhibitory effect of EPO on the secretion of effector molecules by monocytes and a stimulatory effect on the phagocytic activity by monocytes.

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INTRODUCTION

Erythropoietin (EPO) is a renally secreted hormone that promotes red blood cell production in bone marrow by binding to the EPO-receptor (EPO-R). This interaction results in an increased number of red blood cells in the circulation, which makes EPO a potent molecule to treat anemia in various groups of patients [1]. Although numerous studies have examined the clinical effects of EPO, its immunological effects have received less attention. Immune cells have been shown to bear the EPO-R, making them probable targets [2, 3]. Indeed, in polyclonally stimulated whole blood cell cultures from hemodialysis patients, EPO increased IL-2, IL-10 and IL-12 production, while IL-6 and TNF production was reduced [4-6]. Also granulocytes and neutrophils have been shown to be activated by EPO [7] and incubation of B cells with EPO led to increased IgM production [8]. Recently, it was shown that administration of EPO to mice reduced the production of IL-6 and TNF, as well as nitric oxide. Furthermore, systemic bacterial infection and impaired pathogen clearance was observed in these mice, which resulted in reduced survival [9].

Eighty percent of patients infected with the hepatitis C virus (HCV) are unable to resolve the infection by their own immune system. It has been shown that natural killer (NK) cells as well as dendritic cells (DC) are functionally impaired in chronic HCV patients compared to healthy individuals [10-12]. Besides innate immunity, adaptive immunity is affected as well. The continuous presence of high levels of viral antigens leads to a weaker effector function of HCV-specific T cells, which is a characteristic feature of immunity in chronic HCV patients [13, 14]. Antiviral therapy consisting of pegylated interferon-alpha (peg-IFN) and ribavirin has been the standard of care for chronic HCV patients for many years, with the recent addition of protease inhibitors to this treatment further improving the efficacy [15]. A major side effect of treatment with ribavirin and peg-IFN is anemia, which is even more pronounced by the addition of protease inhibitors [16]. Normalization of hemoglobin (Hb) levels can be achieved by ribavirin dose reductions, but this may lower the treatment efficacy. As an alternative to manage anemia, EPO can be administered to stimulate the generation of erythrocytes [17-20], while reducing the necessity of ribavirin dose adjustments, which may benefit the efficacy of therapy [20, 21]. However, recent data showed no beneficial effect of EPO compared to ribavirin dose reductions on sustained viral response rates in HCV patients treated with boceprevir, peg-IFN and ribavirin [22, 23]. To get more insight into the immunological effects of EPO in humans, we defined which human leukocyte subpopulations are potential targets for EPO, and explored the functional effects of EPO on these cells. We observed that EPO affected monocytes *in vitro,* which was in line with detectable EPO-R mRNA expression by monocytes. Moreover, similar to the *in vitro* effects, administration of EPO during antiviral therapy of chronic HCV patients resulted in reduced frequencies of monocytes producing cytokines.

MATERIALS AND METHODS

Patients

Patients were treated at the Erasmus MC according to a study protocol and were seen at our outpatient clinic (EudraCT 2007-005344-25). Patients were treatment-naive, infected with HCV genotype 1 and were treated for 12-48 weeks with peg-IFN (Pegasys, 180 μg once weekly, Roche) and ribavirin (Copegus, 1200-2400 mg daily, Roche). Hb levels were monitored throughout therapy. Per protocol, at Hb levels below 6.8 mmol/l, EPO (NeoRecormon, Roche) was administered at a dose of 30,000 IU once weekly. When Hb levels increased above 7.5 mmol/l, EPO administration was discontinued. Eight patients were treated with EPO, heparinized blood was collected during antiviral therapy before administration of EPO and 7 days after the first injection of EPO. Furthermore, we included 7 healthy volunteers outside the study protocol who were used as control(s). The institutional review board of the Erasmus MC approved the protocols, and informed consent was obtained from all individuals.

Cell culture

Peripheral blood mononuclear cells (PBMC) were isolated from venous blood by ficoll separation (Ficoll-Paque™ plus, Amersham). For all *in vitro* experiments, PBMC were suspended in serum-free X-VIVO15 medium (BioWhittaker) supplemented with L-glutamin (Cambrex), Pen-Strep (Invitrogen/Gibco) and HEPES (Cambrex) and used for the various assays.

EPO-R mRNA expression

For determination of the expression of EPO-R mRNA, PBMC from healthy volunteers were separated by cell sorting into $CD19^+$ B cells, $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells, $CD3^-CD56^+$ NK cells, $CD14^+$ monocytes and granulocytes (FACS Aria SORP, BD). Granulocytes were sorted from full blood on the basis of the FSC-SSC profile. Cells were stored in RNAlater. Total RNA was extracted using the RNeasy kit (Qiagen) and cDNA was prepared using the iScript cDNA Synthesis Kit (Bio-Rad). Real-time PCR reactions were performed using a MyIQ5 detection system (Bio-Rad). Primers-probes for GAPDH (Hs00959427_m1) and EPO-R (Hs00959427) were obtained from Applied Biosystems. The expression of target genes was normalized to GAPDH using the formula: $2^{-\Delta Ct}$, $\Delta Ct = Ct_{EPOr} - Ct_{GAPDH}$.

ROS production and phagocytosis by monocytes

PBMC were rested for 1h at room temperature, and DHR123 (0.1 μg/ml, Sigma) was added for 10 minutes, followed by pretreatment for 15 minutes with EPO (125 IU/ml, NeoRecormon).

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Next, cells were primed with fMLP (1 mM, F3506, Sigma) and incubated at 37°C for 0, 5, 15 and 30 minutes. ROS was detected by flow cytometry (FACS Calibur 4, BD). EPO-pretreated PBMC were also used for the detection of phagocytosis. *E.coli* FITC (2 µg/ml, Invitrogen) was added and incubated at 37°C for 15 minutes. Cells were washed with trypan blue to remove unbound *E.coli* FITC. Phagocytosis was measured by flowcytometry (FACS Calibur 4, BD).

Expression of intracellular and cell surface molecules by flow cytometry

The frequencies of cytokine producing CD14^{+} monocytes were determined by flow cytometry [24, 25]. PBMC from healthy individuals were first pretreated with or without EPO variant alpha (125 IU/ml, EPREX) or EPO (125 IU/ml, NeoRecormon) for 30 minutes. PBMC from HCV patients on therapy were not pretreated *in vitro* with EPO. For the expression of activation markers, cells were stained with CD80-FITC (MAB104, Beckman) CD86-APC (IT2.2, Biolegend) and HLA-DR-FITC (L243, BD Bioscience). For determining cytokine expression, cells were stimulated overnight with an optimal concentration of LPS (0.8 ng/ml, InvivoGen) or R848 (1 µg/ml, Alexis). Brefeldin A (10 µg/ml, Sigma) was added 2h after the addition of TLR agonists. Samples were fixed, permeabilized and stained with MIP-1β-PE (D21-1351, BD Pharmingen), IL-6-FITC (MQ2-13A5, BD Pharmingen), TNF-PE-Cy7 (MAb11, eBioscience), MCP-1-APC (5D3-F7, eBioscience), IL-8-FITC (6217, R&D), IL-12p40-PE (C11.5, BD Pharmingen), IL-10-APC (JES3-19F7, Biolegend) and CD14-eFluor450 (61D3, eBioscience). Cytokine producing cells were detected by flowcytometry (Canto-II, BD).

Statistics

The Wilcoxon signed-rank test was used for paired non-parametric analyses. The level of significance for all tests was $P \leq 0.05$.

RESULTS

The EPO-R is expressed by human monocytes

To determine if human monocytes are responsive to EPO, we first assessed the expression levels of EPO-R mRNA. As shown in figure 1, using highly purified monocytes, EPO-R mRNA was detected by real-time PCR. The EPO-R mRNA expression levels in monocytes were lower than observed in B cells and granulocytes, but higher than by other lymphocyte subtypes, like T cells and NK cells. These findings demonstrate that monocytes, next to B cells and granulocytes, are putative targets for EPO.
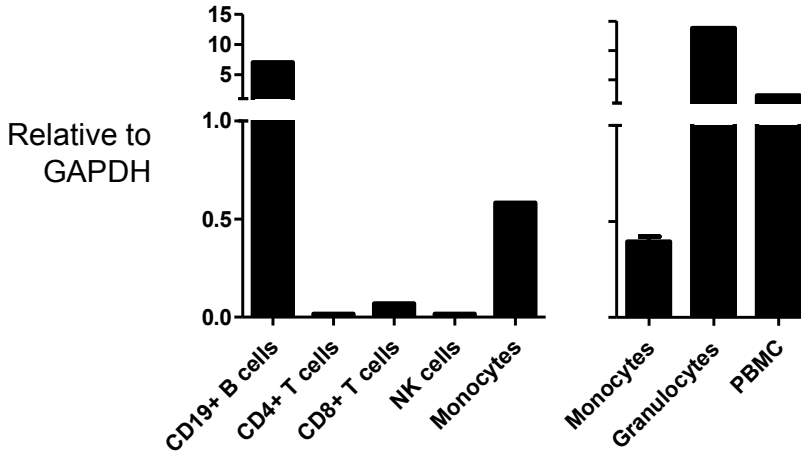
Figure 1. Monocytes express EPO-R mRNA. CD19^{+} B cells, CD4^{+} and CD8^{+} T cells, CD56^{+} NK cells, CD14^{+} monocytes and granulocytes were purified by flow cytometry. EPO-R mRNA was measured by real time PCR, and expression was determined relative to GAPDH mRNA.
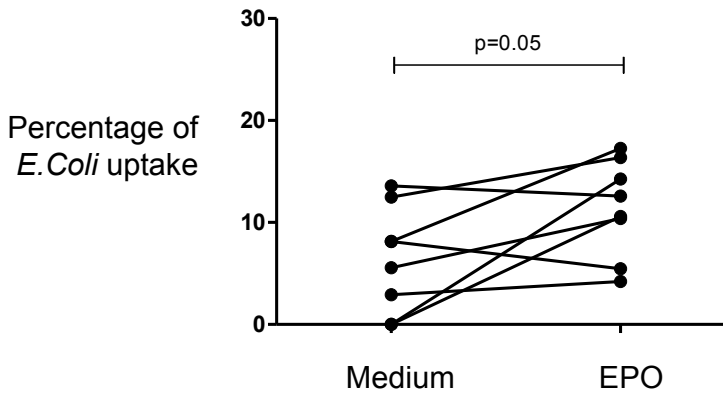
Figure 2. Increased phagocytosis of *E.coli* by monocytes *in vitro*. PBMC from healthy individuals (n=8) were treated with medium or EPO, and subsequently exposed to *E.coli*-FITC. *E.coli* uptake by CD14^{+} monocytes was measured by flow cytometry as the difference between the frequency at 37 °C and at 0 °C.

EPO increases phagocytosis ability of monocytes in vitro.

To study the influence of EPO on monocyte function, we first investigated the ability of EPO-treated monocytes to perform phagocytosis of *E.coli* and to produce reactive oxidative species (ROS) upon stimulation. As shown in figure 2, upon incubation of PBMC from healthy individuals, phagocytosis of *E.coli* by monocytes was observed. Pretreatment of PBMC with EPO for 15 minutes resulted in an increased uptake of *E.coli* in 5 out of 8 cultures from

A

B

C
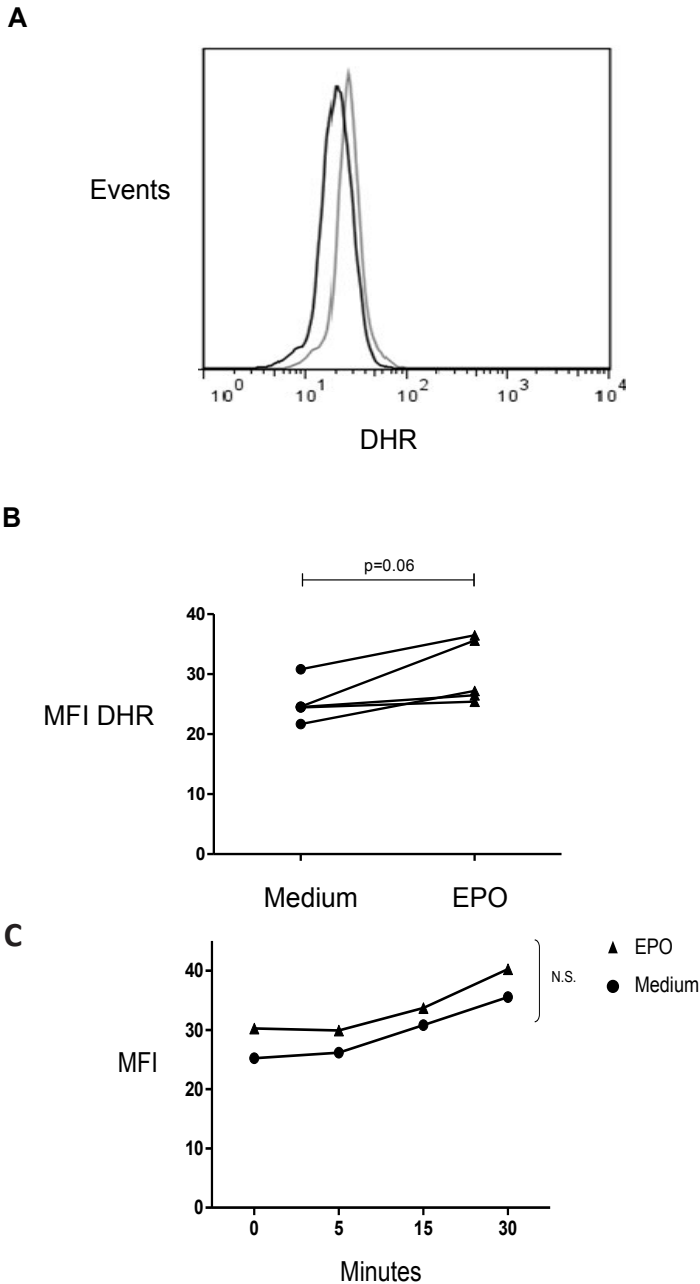
Figure 3. The frequency of ROS producing monocytes is not altered by EPO *in vitro*. (A) A representative histogram showing the DHR expression of monocytes in the absence (black line) or presence of EPO (grey line). (B) PBMC from healthy individuals (n=5) were treated with medium or EPO, and the direct effect of EPO on ROS-producing monocytes was determined by flow cytometry. (C) PBMC from healthy individuals (n=5) were incubated with or without EPO and stimulated with fMLP. ROS was determined in CD14+ monocytes at the indicated time points after stimulation.

healthy volunteers, while the other 3 cultures showed no modulation of phagocytic ability of monocytes (p=0.05).

To study the effect of EPO on the function of monocytes in more detail, we examined their ROS production. As shown in figure 3, unstimulated PBMC induced ROS production by monocytes, which was further enhanced by pretreatment with EPO (Fig. 3A). On average, the mean fluorescence intensity, representing the ROS levels, increased from 25.0 without EPO to 30.2 with EPO, although this result was not statistically significant (p=0.06, Fig. 3B). Importantly, the kinetics of ROS production by fMLP-stimulated monocytes in the presence of EPO were comparable as ROS production in the absence of EPO, and again no significant differences were observed (p=0.12, Fig. 3C).

EPO moderately down regulates cytokine production by monocytes in vitro

Another important function of monocytes is the production of pro-inflammatory as well as anti-inflammatory cytokines. After treatment of PBMC with EPO, the frequencies of IL-6 and TNF producing monocytes were decreased upon stimulation with the TLR4 ligand LPS, compared to PBMC not treated with EPO (Fig. 4A). Using PBMC from 7 healthy volunteers, we showed a significant reduction of monocytes producing IL-6 (average from 23.2% to 17.9%, p=0.03), and TNF (42.2% to 36.8%, p=0.02) upon LPS stimulation (Fig. 4B). No significant effects of EPO were observed on the frequencies of monocytes producing IL-8, IL-12p40, IL-10, and MIP-1β upon LPS stimulation (Fig. 4B and results not shown). Pretreatment of R848-stimulated PBMC did not lead to differences in cytokine production by monocytes (results not shown).

Ribavirin-induced anemia in chronic HCV patients can be treated by EPO as well as EPO-alpha variant. To examine if these two different EPO variants have comparable effects on the functionality of monocytes, PBMC were also incubated with EPO-alpha variant *in vitro*. As presented in figure 4C, we showed that incubation of PBMC with EPO-alpha variant significantly reduced the frequency of monocytes that produced IL-6 (p=0.01), TNF (p=0.01) and IL-8 (p=0.03) upon LPS stimulation. Thus, the inhibitory effect on cytokine production was observed for both EPO variants.

EPO administration to patients with HCV-treatment induced anemia mildly reduces cytokine production by monocytes

Next, we assessed whether the immunomodulatory effect of EPO on monocytes *in vitro* is also observed *in vivo*. Cytokine production by monocytes was measured at baseline and 7 days after EPO injection in eight patients with HCV treatment-induced anemia (Fig. 5A). The patient characteristics are shown in Table 1. Upon stimulation of PBMC with the TLR4 ligand LPS and the TLR7/8 ligand R848, a decreased frequency of cytokine producing monocytes at day 7 compared to baseline was observed in 7 out of 8 patients (Fig. 5B). Upon LPS
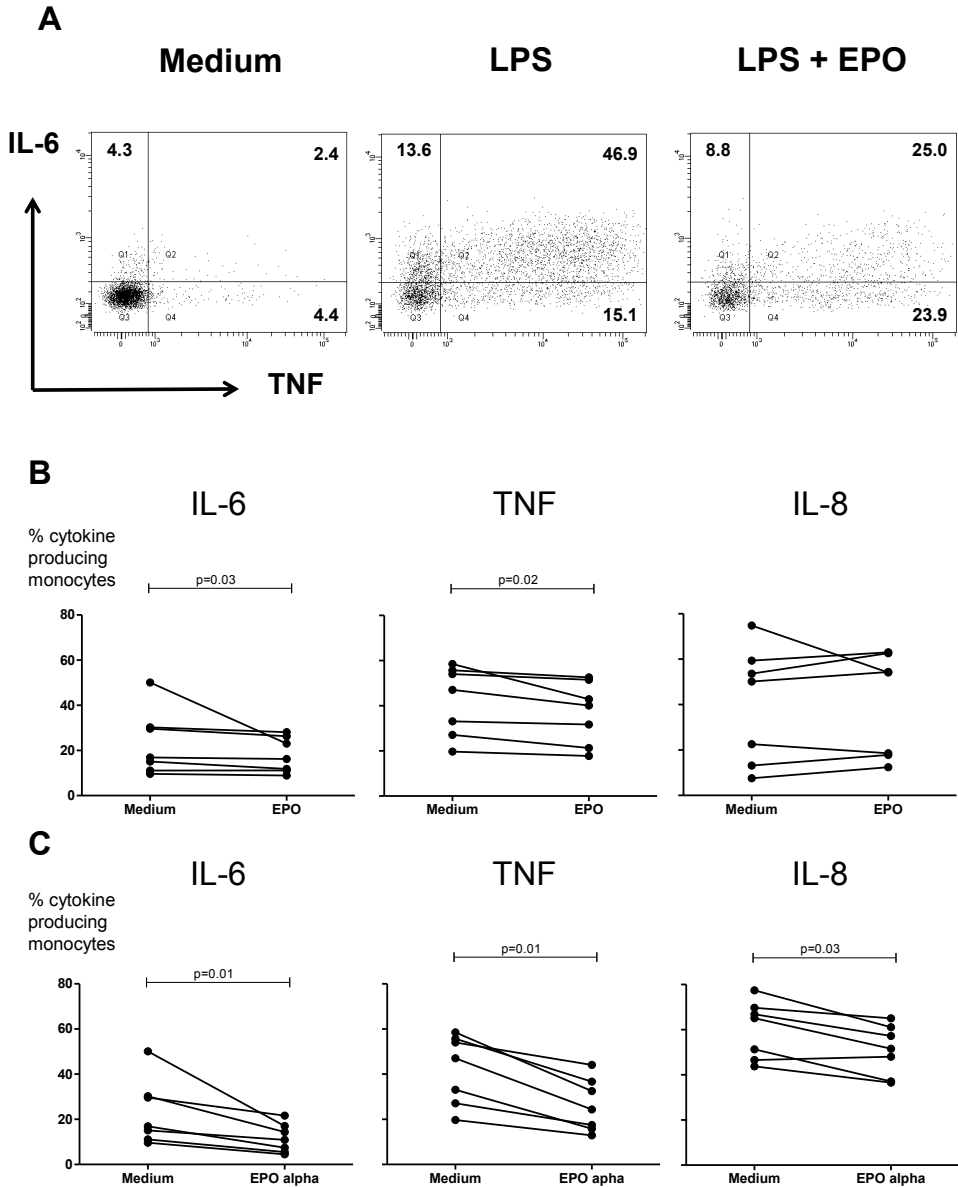
Figure 4. Pretreatment with EPO of LPS stimulated PBMC shows decreased frequencies of IL-6 and TNF producing monocytes *in vitro*. (A). Representative dot-plots showing IL-6 and TNF producing CD14⁺ monocytes upon LPS stimulation of PBMC after incubation with medium or EPO. (B) PBMC from healthy individuals (n=7) were pretreated with EPO and stimulated with LPS. The frequency of cytokine producing monocytes was determined by flow cytometry. (C) Similar as in *B*, except that PBMC were pretreated with EPO-alpha variant.

A

B
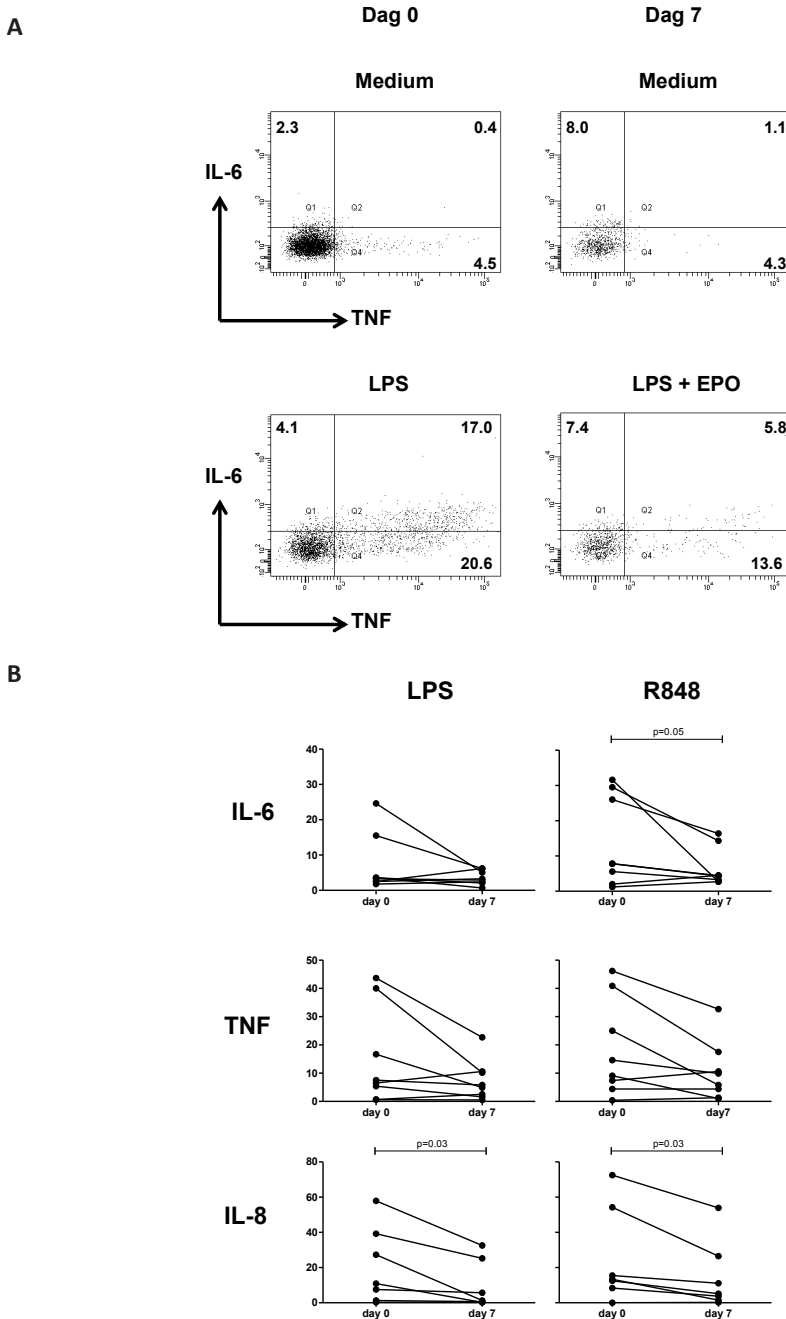
Figure 5. EPO administration to chronic HCV patients with ribavirin-induced anemia mildly reduces cytokine production by monocytes. (A) Representative dot plots of IL-6 and TNF producing monocytes before and 7 days after injection of EPO from a chronic HCV patient on peg-IFN/ribavirin therapy who is being treated for anemia. (B) The frequency of IL-6, TNF, IL-8 producing monocytes upon LPS stimulation (left panels) or R848 stimulation (right panels) of PBMC collected from HCV patients (n=8) before and 7 days after administration of EPO.

stimulation, a decreased percentage of monocytes producing MIP-1β (average from 20.3% to 11.7%; p=0.02, results not shown) and IL-8 (3.6% to 1.6%, p=0.03) was found. Upon R848 stimulation, lower percentages of monocytes producing IL-6 (13.9 to 6.6%, p=0.05), MCP-1 (13.0% till 8.5%, p=0.05, results not shown) and IL-8 (25.2% to 14.6%, p=0.03) were observed.

Despite the fact that we observed a trend towards lower cytokine levels, no significant differences were found for the frequency of TNF (Fig. 5B), IL-12p40 and IL-10 production (results not shown). Furthermore, the activation status of monocytes as reflected by their expression of CD80, CD86 and HLA-DR was determined at baseline and 7 days after administration of EPO. No differences were observed as a consequence of EPO administration in the frequency or mean fluorescence intensity (MFI) of monocytes expressing these markers (results not shown). Management of therapy-induced anemia in chronic HCV patients by administration of EPO resulted in reduced frequencies of cytokine producing monocytes, which may have consequences for inflammatory responses by monocytes upon challenge by pathogens.

Table 1. Patient characteristics of HCV patients.

Patient ID	Gender	Genotype	Hb (mmol/l)	Bilirubin (umol/l)	Albumin (g/l)	Platelet count (E9/l)	AST (U/l)	ALT (U/l)	Viral load (U/ml)
1	F	1	9.3	11	45	183	59	91	6.82E+06
2	M	1	9.0	11	45	130	60	65	4.24E+06
3	M	1	9.8	8	42	226	55	90	6.79E+06
4	M	1	10.4	11	46	213	34	51	4.42E+06
5	F	1	7.9	12	44	99	110	113	9.14E+05
6	M	1	9.8	17	43	179	52	68	1.07E+06
7	M	1	9.9	7	44	202	87	74	4.09E+06
8	F	1	9.1	6	45	217	80	81	1.93E+06

Data presented are at start of therapy (n=8).

DISCUSSION

Although the stimulatory effect of EPO on the erythrocyte lineage in bone marrow is well examined, the immunomodulatory activity of EPO is not well understood. In this study, we investigated the *in vitro* and *in vivo* effects of EPO on human leukocytes, with a focus on monocytes, which express the EPO-R as we demonstrated in this study.

We show that *in vitro* exposure of PBMC to EPO resulted in decreased frequencies of IL-6 and IL-8 producing monocytes upon TLR ligation. Although differences were seen in potency, both EPO-alpha variant and EPO (EPO-beta, which was used throughout this study) showed comparable immunomodulatory effects on monocytes *in vitro*. In addition, we found EPO-induced modulation of phagocytic ability by monocytes *in vitro,* but no effect on ROS production. This demonstrates a shift in the function of EPO-exposed monocytes towards a more potent anti-microbial activity, and a weaker ability to produce effector cytokines. It is important to mention that we have no indications that the viability of monocytes was affected by short-term exposure to EPO as determined by annexin-V staining *in vitro*. Similar effects on inhibition of cytokine production were seen in the *ex vivo* model where monocytes were obtained from chronic HCV patients undergoing antiviral therapy. Decreased frequencies of cytokine producing monocytes upon TLR ligation were observed following administration of EPO to patients. This inhibitory effect was not restricted to TLR4 ligation, but also observed upon TLR8 ligation, leading to lower frequencies of monocytes producing pro-inflammatory cytokines. Although we observed significant modulation of monocyte function *in vivo* at day 7, we cannot exclude that more pronounced effects can be demonstrated at different time points following EPO administration, since we and others did not perform kinetics studies.

Our observations in humans are in line with a recently published study in mice that showed that EPO inhibited LPS-induced pro-inflammatory cytokines and nitric oxide production in peritoneal macrophages *in vitro*. *S. typhimurium*-infected mice treated with EPO demonstrated a higher bacterial load and reduced expression of IL-6, TNF and Nos2 as compared to control mice treated with PBS [9]. Besides the studies in mice, the effect of EPO in hemodialysis patients has also been examined. In these patients, neutrophils were significantly more activated after hemodialysis and EPO therapy, which is in line with our results, where we show that neutrophils express the EPO-R and are likely responsive to EPO [7]. In other studies on the direct effect of EPO, decreased TNF and increased IL-10 production were produced by phytohemagglutimin (PHA)-stimulated whole blood cell cultures of this patient population during therapy with EPO [4, 5]. Lower TNF production as a result of EPO treatment is in line with our results, but we did not observe an increase of IL-10 producing monocytes. The discrepancy is likely the result of the use of different stimuli, with PHA also activating T cells, whereas TLR ligands do not. In this respect, it is important to note that we did not observe EPO-R mRNA expression by CD4$^+$ and CD8$^+$ T cells, and the activity of T cells

upon stimulation with anti-CD3 antibodies was not affected by EPO, as reflected by their production of IL-2 or IFN-γ *in vitro* and *in vivo* (data not shown).

Chronic HCV patients undergoing antiviral therapy consisting of peg-IFN and ribavirin are more susceptible to develop bacterial infections [26]. The enhanced occurrence of infection does not correlate with neutropenia [26, 27], while the involvement of monocytes is unclear. Monocytes are important players in the first-line defense against numerous pathogens [28], and are functionally modulated in chronic HCV patients as compared to control monocytes. We previously showed that TLR4 ligation of monocytes from chronic HCV patients induced lower TNF and IL-12p40 production as compared with healthy individuals [24]. Little information is available on the effects of EPO administration on the clinical course of infections. In critically ill patients who are treated with antimicrobial therapy, it has been shown that administration of EPO is safe [29]. Our data suggest, however, that EPO reduces the pro-inflammatory function of monocytes, which might be relevant in patients not treated with antimicrobial therapy but who are at risk for these infections. Since our observed effects of EPO are influenced by the simultaneous presence and modulatory effect of IFN-alpha during the course of antiviral therapy, future studies that examine in more detail the effect of IFN-based therapy as well as of EPO on monocytes in relation to the increased infection risk in HCV patients are needed. It has been reported that with the introduction of protease inhibitors to antiviral therapy of chronic HCV patients, the beneficial effects of EPO in managing anemia during therapy are limited. The high costs of EPO and reported side effects argue against the use of EPO in these patients [22, 23].

In conclusion, although we were unable to demonstrate an effect of EPO on human NK cells and T cells, EPO inhibited TLR-induced cytokine production by monocytes *in vitro* as well as *in vivo*. The inhibitory effect of EPO on cytokine producing monocytes may hamper efficient immune responses by monocytes against bacterial infection in patients undergoing treatment for anemia. The clinical implications for patients using EPO to treat anemia needs to be further determined.

ACKNOWLEGDEMENTS

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4

Chapter 5

Longitudinal analysis of peripheral and intrahepatic NK cells in chronic HCV patients during antiviral therapy

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ABSTRACT

Introduction: A strong immune response is integral to the clearance of HCV infection. NK cells are specialized cells that are able to inhibit replication of HCV in infected hepatocytes. Previous studies have correlated therapy outcome in HCV to the expression of various markers on NK cells. However, the effect of viral load reduction on NK cell function during therapy is still largely unknown, particularly in the liver. Therefore we investigated NK cell phenotype and effector function in both the peripheral and intrahepatic compartments during the course of antiviral therapy in chronic HCV patients.

Methods: Chronic HCV patients were treated for 24 or 48 weeks with triple therapy consisting of telaprevir, pegIFN-α and ribavirin. Blood and fine needle aspiration (FNA) biopsies of the liver were collected at start and 6 hr, 1 wk and 12 wks during therapy. Flowcytometry was performed for expression of different markers (NKG2A, NKG2D, NKp46, and CD69).

Results: Our results demonstrate a highly activated phenotype of NK cells in liver compared to blood in chronic HCV patients. Six hours after start of triple therapy, no activation of intrahepatic NK cells was observed in the liver as compared to baseline. At 1 week after start of triple therapy, the frequency of NK cells with the activating receptor NKp46 was increased in blood, whereas at week 12, the frequencies of the inhibitory receptor NKG2A was increased. No alterations were observed during therapy in liver NK cell phenotype.

Conclusion: IFN-based therapy for chronic HCV affects NK cell phenotype in peripheral blood more than in the liver.

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INTRODUCTION

Natural killer (NK) cells are crucial in the control and elimination of virally infected cells. Activated NK cells are able to kill virus-infected cells via cytotoxic molecules, such as perforin or granzymes. In addition, cytokines produced by NK cells (e.g. IFN-γ, TNF) lead to suppression of viral replication as well as activation of subsequent adaptive immune responses. The activation of NK cells results from expression of various activating and inhibitory receptors. NK cell receptors include C-type lectins for inhibitory (NKG2A) and activation (NKG2C/D) signals, as well as the natural cytotoxicity receptors (NCRs) NKp30 and NKp46 that deliver activation signals.

During chronic viral infections in human, such as hepatitis C virus (HCV) infection, blood NK cells have been shown to be altered as compared to NK cells from healthy individuals. These modulations may hamper efficient antiviral immune responses capable of eliminating the virus, and consequently may lead to viral persistence. NK cells from chronic HCV patients display a mildly augmented cytotoxic potential as compared to healthy individuals, whereas the ability of NK cells to produce IFN-γ is not or only weakly affected [1, 2]. Oliviero demonstrated that NK cells from HCV patients display a more activated phenotype, and therefore possibly contribute to the immune responses involved to control viral persistence. However, compared to NK cells from healthy individuals, NK cells from chronic HCV patients show higher expression of the inhibitory receptor CD94/NKG2A and produce higher levels of the immunosuppressive cytokines IL-10 and TGF-β when cultured with hepatic cells [3], suggesting an inhibitory role. Interestingly, other groups reported that NK cells are severely hampered in chronic HCV infection and inefficient in providing a proper cytotoxic response [2, 4, 5].

Upon infection with HCV, hepatocytes rapidly induce type I interferons (IFN), such as IFN-α and IFN-β. IFN-α has shown to be a potent inducer of NK cell activation *in vitro* [6, 7] thereby inhibiting HCV replication [8]. During IFN-α-based therapy *in vivo* NK cells were shown to become activated and to mediate viral clearance in HCV [9-12], which was independent from the effect of ribavirin [13]. Treatment response correlated with the expression of the degranulation marker CD107, but also NKp46 and the activating receptor CD69 [9, 10, 14].

Despite the vast number of studies that examined the effect of IFN-α-based therapy on circulating NK cells, few studies have investigated the effects on NK cells in the liver of chronic HCV patients. Since the control of HCV replication by NK cells takes place in the liver where HCV infected hepatocytes reside, it is essential to obtain detailed information on intrahepatic NK cells in order to evaluate whether intrahepatic NK cells are modulated by IFN-α-based therapy, and whether their activity is mirrored by their circulating counterparts. To get more insight, we collected peripheral blood and aspiration biopsies of the liver from a cohort of 10 chronic HCV patients during IFN-α-based triple therapy and analysed the NK cell compartment.

MATERIALS AND METHODS

Patients and viral load measurements

Ten chronic HCV patients, genotype 1, were treated with triple therapy consisting of pegylated-IFN-α2a (pegIFN-α, Pegasys®), weight based ribavirin (Copegus®) and telaprevir (Incivo®). All patients received 12 weeks of triple therapy, and continued their treatment for 12 or 36 weeks with pegIFN-α and ribavirin according to fibrosis stage and treatment history. All patients were between 18-70 years old, patients were naïve to treatment or previous non-responder to pegylated-IFN-α and ribavirin therapy, and none of them were co-infected with HIV or HBV. Patients were excluded if they had decompensated liver cirrhosis (Child Pugh B or C) or hepatocellular carcinoma. The institutional ethical board of the Erasmus MC approved the clinical protocol, and written informed consent was obtained from all individuals. Patient characteristics are listed in Table 1. HCV RNA levels were measured using Cobas Taqman real-time PCR (Roche Diagnostics, Palo Alto, CA) with a lower limit of detection of 15 IU/ml.

Table 1: Individual patient information

	HCV-RNA (IU/ml)			ALT (IU/ml)	Treatment response	Treatment history	Fibrosis
	baseline	week 4	week 12	baseline			
E008	$1.5*10^6$	15	$1.0*10^6$	35	Non-response	Experienced	F0-F1
E010	$1.2*10^6$	15	15	42	Non-response	Experienced	F0-F1
E011	$3.2*10^6$	$2.9*10^4$	15	251	Non-response	Experienced	F0-F1
E014	$0.7*10^6$	15	15	39	SVR	Experienced	F0-F1
E016	$0.9*10^6$	15	15	51	SVR	Experienced	F4
E017	$5.0*10^6$	15	15	32	SVR	Naive	F2
E018	$3.8*10^6$	15	15	201	SVR	Experienced	F4
E019	$2.3*10^6$	15	15	85	SVR	Experienced	F0-F1
E020	$1.9*10^6$	$1.0*10^4$	-	40	Non-response	Naive	F0-F1
E022	$0.7*10^6$	15	15	131	SVR	Naive	F4

Aspiration of liver cells and collection of peripheral blood,
Aspirate biopsies of the liver were collected at baseline, 6-hours, 1-week, and 12-weeks of treatment. Details of the collection of intrahepatic leukocytes via fine needle aspirate (FNA) biopsies are described elsewhere [15, 16]. Briefly, a 25-gauge needle (Braun, Melsungen, Germany) was inserted in the intercostal space after ultrasound localization of the liver. A syringe filled with RPMI-1640 medium was attached to the needle and liver cells were aspirated into the medium under negative pressure. Three aspirate biopsies were collected per time-point. Only aspirate biopsies containing low amounts of erythrocytes were pooled

for further analyses. At multiple time-points, peripheral blood mononuclear cells (PBMC) were isolated from venous blood by ficoll separation (Ficoll-Paque™ plus, Amersham).

Analysis of liver and peripheral blood cells by flow cytometry

Liver cells and peripheral blood were lysed using ammonium chloride. To determine the frequency and phenotype of intrahepatic and peripheral NK cells, multi-colour flowcytometry was performed. Cells were stained for 20 min at 4°C with CD3-Alexa-Fluor700 (OKT-3), CD56-APC-eFluor780 (CMSSB), CD45-eFluor605NC (HI30; all eBioscience), NK2GA-PE (Z199), NKp46-PE-Cy5 (BAB281, all Beckman), NK2GD-PerCp-Cy5.5 (1D11; BD-Biosciences), CD69-Pacific Blue (FN50; Biolegend) and Live/Dead Aqua® (Life Technologies). Cells were acquired using a FACS ARIA cell sorter (BD-Biosciences). On average 13,000 intrahepatic leukocytes were acquired per pooled aspirate biopsy.

Statistics

Statistical comparison was tested using the Wilcoxon matched t test for paired non-parametric analyses. A p value ≤ 0.05 was considered significant.

RESULTS

Treatment efficacy

Out of 10 patients, 7 were cured (sustained virological response, HCV RNA <15 IU/ml); 2 patients were non-compliant (E008 and E020) and 1 patient had to stop therapy prematurely due to side-effects (E011). Patient characteristics are shown in Table 1.

The frequency of NK cells in the liver and blood of chronic HCV patients is not altered during triple therapy

Aspirate biopsies of the liver of chronic HCV patients during therapy have shown to be a valuable tool to monitor intrahepatic immune responses during treatment [15, 16]. Therefore, we first examined the effect of IFN-based triple therapy on the frequencies of CD3$^-$CD56$^+$ NK cells in liver and blood by flow cytometry (Fig. 1A). The frequency of NK cells within the lymphocyte population at baseline was not significantly different between liver and blood (Fig. 1B). In addition, frequency of NK cells in the liver, as well as in peripheral blood was not modulated during various time-points of treatment.

Activation of NK cells from chronic HCV patients is higher in liver than in blood, but not affected by triple antiviral therapy

Besides modulation of the number of NK cells, their functionality is important for evaluation of their biological relevance in viral hepatitis. One of the membrane markers known to be expressed on activated NK cells is CD69 [17]. As shown in figure 2A, the majority of CD56$^+$ NK cells from the liver express CD69, whereas only a minority of blood NK cells express this activation marker (p<0.001). The higher frequencies of activated CD69$^+$ NK cells found in
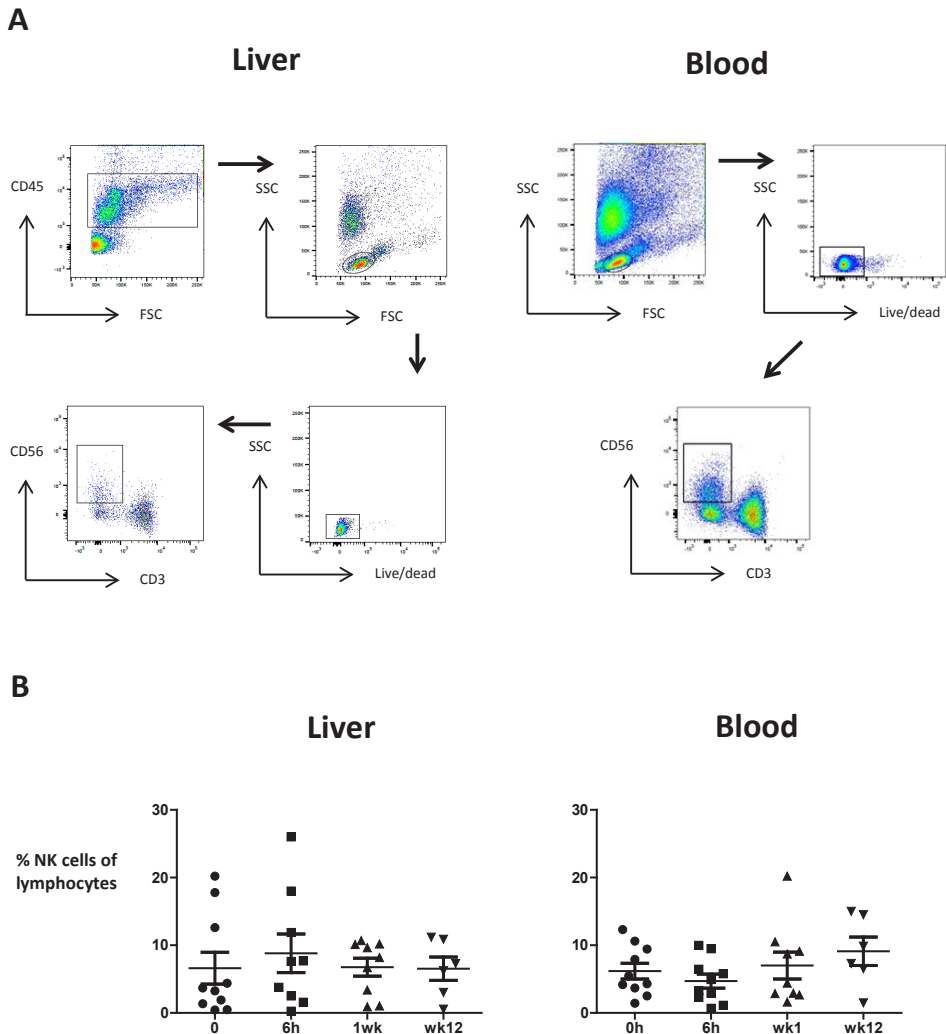
Figure 1. The frequency of NK cells in the liver and blood of chronic HCV patients is not altered during triple therapy. The frequency of CD56$^+$CD3$^-$ NK cells was determined by flow cytometry within the population of CD45$^+$ leukocytes from fine needle aspirate biopsies of 10 patients. (A) Representative dot plots of liver and blood samples showing the gating strategy are depicted. (B) Data show the percentage of NK cells in each individual patient and the mean within the total lymphocyte population in liver and whole blood during triple therapy.

liver were not enhanced during triple therapy at the specified time-point (Fig. 2B). However, albeit not significant, blood NK cells showed a trend towards increased numbers of CD69+ NK cells during the course of therapy.

A

B
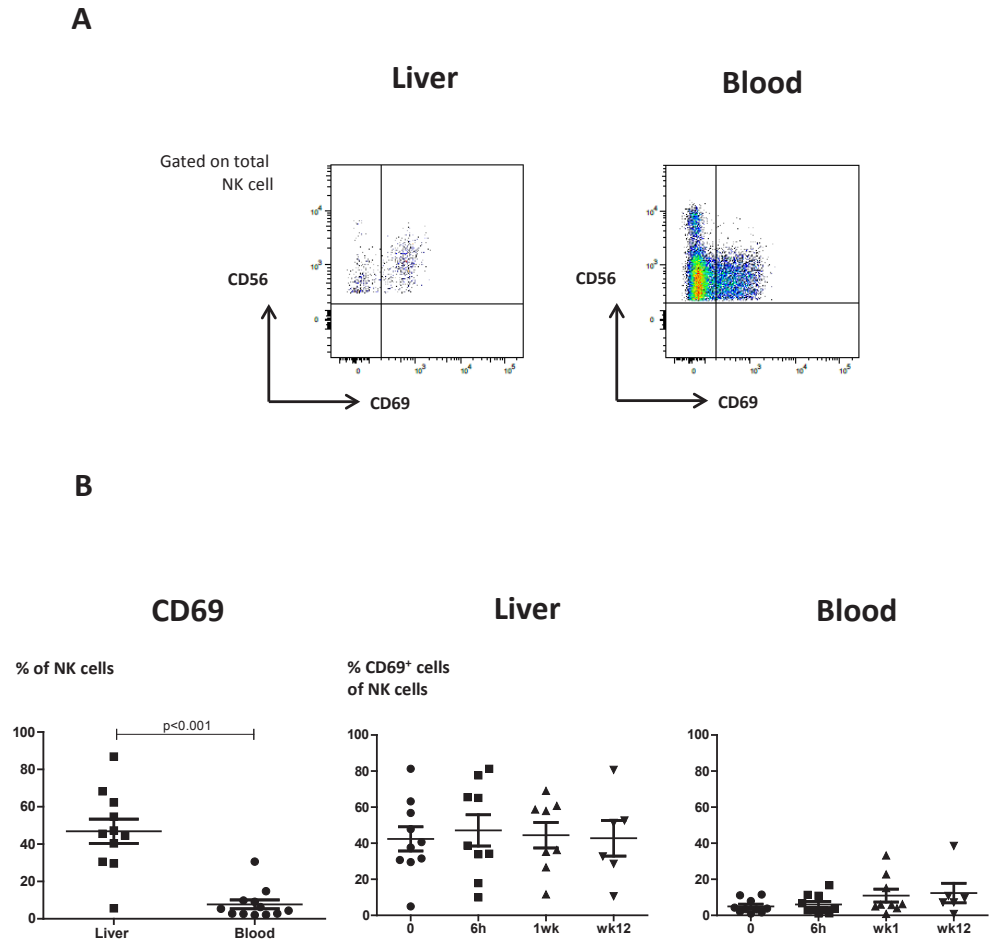
Figure 2. Activation of NK cells from chronic HCV patients is higher in liver than in blood, but not affected by triple antiviral therapy. (A) Representative dot plots are depicted of CD69 expression NK cells in liver and blood. (B) Data shown represent the percentage of CD69 expressing cells in total NK cells from liver and blood of individual HCV patients during the course of triple therapy.

Expression levels of NKG2A and NKp46 on liver and blood NK cells are modulated during IFN-based therapy

Having demonstrated that both the frequency of NK cells and the expression of CD69 were not affected by antiviral treatment in HCV patients, we further evaluated the expression of NKp46, NKG2D and NKG2A on total NK cells in liver aspirate biopsies and blood during the

A

B **Liver** **C** **Blood**
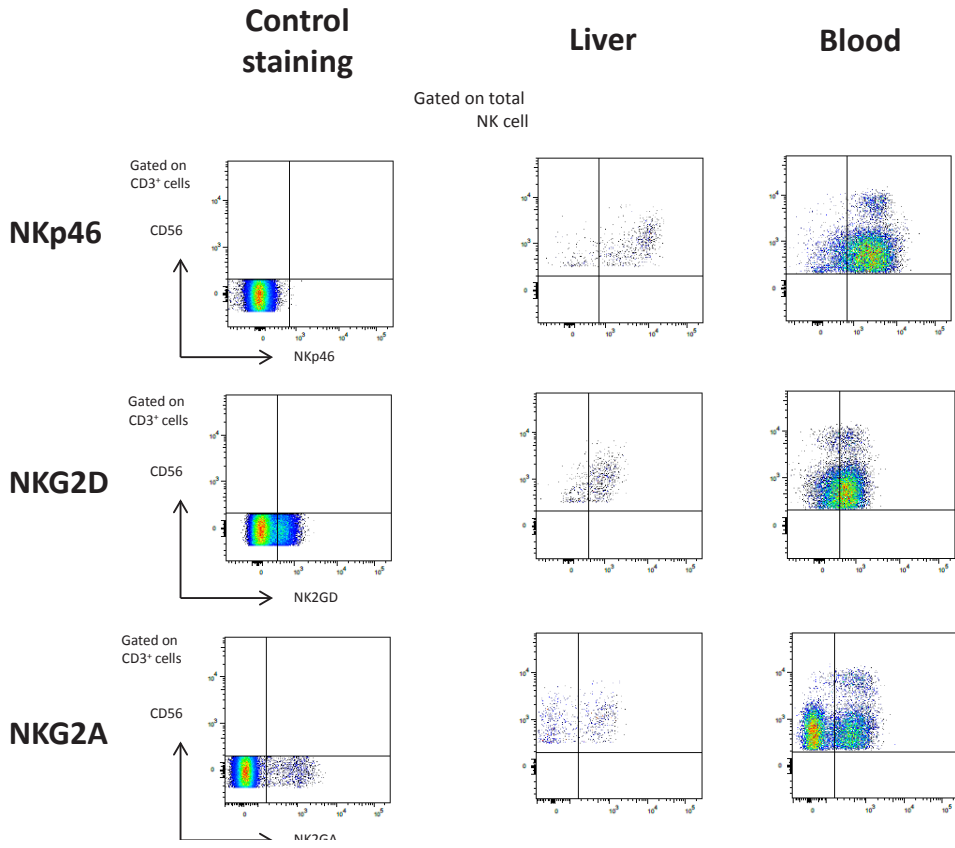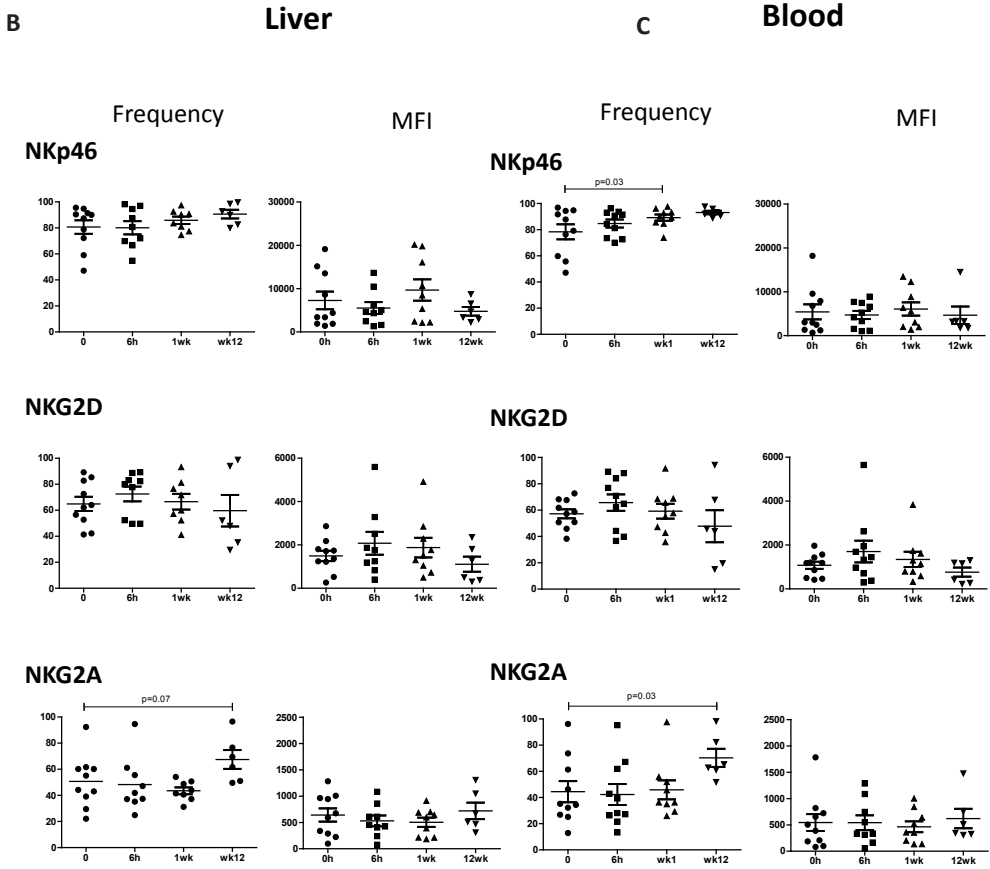
Figure 3. Early during therapy, blood NK cells have higher frequencies of the activating receptor NKp46, whereas at later phases during therapy the frequency of inhibitory cells (NKG2A) increases. (A) Representative dotplots of CD45+CD3-CD56+ NK cells obtained from liver aspirate biopsies or blood stained with antibodies against NKp46, NKG2D, NKG2A. The gates were set on internal controls, i.e. negative CD3+ T cell populations. The frequency and mean fluorescence intensity (MFI) was determined of NK cells expressing NKp46, NKG2D and NKG2A at baseline as well as during triple therapy at 6 hours, 1 week and 12 weeks after start of therapy. Results from individual patients and the mean value are depicted of (B) the liver and (C) the blood.

course of treatment (Fig. 3A). Albeit not significant, the activating markers NKp46 and NKG2D both show modulation of expression following 6 hours or 1 week after start of treatment, but not at 12 weeks of treatment (Fig. 3B and 3C). For NKp46, increased frequencies were observed at 1 week after start of treatment on blood NK cells (p=0.03), but not on liver NK cells. For NKG2D, no changes in the frequencies of NKG2D+ NK cells were detected, but on NK cells from liver aspirates and blood the fluorescence intensities slightly increased 6 hours after start of treatment, and slightly decreased at 1 week, however, this did not reach statistical significance. The inhibitory marker NKG2A was also examined, and this receptor was found not to be modulated during the early phases of therapy on either liver or blood NK cells. However, the frequencies of NKG2A+ NK cells in peripheral blood were significantly

increased at week 12 during the course of therapy (p=0.03). Also, in paired liver samples from the same patients, NKG2A+ NK cell frequencies showed a trend toward increased frequencies at week 12 compared to baseline (p=0.07).

DISCUSSSION

In this study we assessed the intrahepatic NK cell compartment in chronic HCV patients undergoing IFN-based therapy, and compared the findings with their circulating counterparts. By repeated sampling of the liver and blood, we demonstrated that frequencies of NK cells were not altered during IFN-based therapy. Assessment of a selected panel of inhibitory and activating markers on NK cells showed no alterations in their frequencies in the liver. In contrast, in blood, increased frequencies of activating NKp46+ NK cells were observed at week 1, and of the inhibitory NKG2A+ NK cells at week 12 after start of therapy.

Repeated sampling of the liver using fine needle aspiration is a valuable tool to assess the dynamics of immune responses in the liver of patients during antiviral therapy. Since standard core needle biopsies routinely used for assessment of liver pathology carry the risk of discomfort and complications, these core needle biopsies cannot be used to answer certain research questions that require repeated sampling. Previously, we successfully used this method to monitor regulatory T cells in chronic HCV patients [15, 16]. We now show that the consequences of IFN-based therapy on modulation of the numbers and phenotype of liver NK cells of chronic HCV are limited, despite the observation that all patients showed significant viral load decline at week 4 and most patients were HCV RNA negative at this time point during therapy (Table 1). Furthermore, it is well known that IFN-α has a strong effect on the activation state and phenotype of NK cells *in vitro* as we and others reported before [6, 7]. One of the explanations for the limited effects of IFN-based therapy on the intrahepatic NK cell compartment could relate to the observation that NK cells in the liver are already in an enhanced state of activation as evidenced by higher CD69 expression levels compared to blood NK cells. As a consequence they may be less susceptible to modulation by exogenous IFN-α. This needs to be examined in future studies in which isolated NK cells from paired blood and liver samples should be compared. This is particularly relevant for HBV infection, where IFN-α is still a cornerstone of antiviral therapy, since the enhanced activation state of liver NK cells as compared to blood was also observed in patients with chronic HBV infections [18]. Interestingly, our data are in line with Pembroke et al, who also showed minor, non-significant changes in the expression of CD16, NKp46 and CD107 during IFN-based therapy of chronic HCV patients [14]. It is important to mention that using the technique of fine needle aspiration biopsies the number of CD56^{bright} NK cells in the liver is too low to allow reliable assessment, and the analysis is therefore based on the total CD56 population, which consists primarily of CD56^{dim} cells. In contrast to liver NK cells, we did

observe modulation of the phenotype of blood NK cells during the course of IFN-based therapy. Similar as in the liver, no changes were observed in the frequency of total or CD69-expressing NK cells at 6h, 1 week or 12 weeks after start of antiviral therapy. However, the increased frequencies of NKp46-expressing NK cells in blood at week 1 during therapy suggests that the NK cell compartment was modulated as a consequence of exposure to IFN-α. Since NKp46 is considered an activating receptor for NK cells, these findings suggest that NK cells are primed and might become involved in the antiviral immune response during the early stages of IFN-based therapy. Of note: although many studies have reported on NKp46 in HCV as a biomarker for treatment outcome and involved in fibrosis progression [14, 19], the biology of these cells is still not clear, which is partly due to the fact that the natural ligand has not been identified yet. Interestingly, we observed that 12 weeks after start of therapy when the majority of patients were HCV RNA negative, a significant increase of the frequency of blood NK cells expressing NKG2A was observed. Since NKG2A is considered an inhibitory receptor for NK cells these findings suggest that at the later stages of therapy when the HCV RNA levels have declined and patients are exposed to IFN-based therapy for a considerable amount of time, that blood NK cells become functionally less active. The increase in NKG2A expressing NK cells could be important to shift the balance to a less active NK cell compartment, thus representing a compensatory mechanism to avoid hyper-activation of this compartment. The experimental setup of our study does not allow us to determine whether viral load or the continuous exposure to IFN-α is responsible for the increase in NKG2A expression.

In conclusion, our results demonstrate that blood, but not liver NK cells, exhibit dynamic changes in the expression of activating and inhibitory receptors. More specifically, higher frequencies of NK cells expressing the activating receptor NKp46 appeared early after start of IFN-based therapy, whereas the expression of the inhibitory NKG2A receptor was increased relatively late during the course of therapy.

ACKNOWLEDGEMENTS

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Chapter 6

Immunological analysis during IFN-free therapy for chronic HCV reveals modulation of the NK cell compartment

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ABSTRACT

Background: Chronic hepatitis C is a global health problem, resulting in liver failure, hepatocellular carcinoma and liver-related death. Natural Killer (NK) cells are innate immune cells and their activity is known to correlate to viral treatment response of HCV. In this study we investigate the immune effects of viral load decline with IFN-free direct acting antivirals (DAA) in blood.

Methods: Twelve patients with chronic HCV were treated with asunaprevir and daclatasvir (NCT02282709), and peripheral blood was analyzed at various time-points during therapy. HCV-specific T cells, and NK cell frequency, phenotype and function and serum was analyzed by multiplex cytokine assays.

Results: In line with previous studies, we confirmed restoration of HCV-specific T cell frequency upon viral load decline. In addition, we show that serum IP-10, IL-12p40 and IL-18 levels decreased early after start of therapy. Surface expression of activation receptors NKp30, NKp46 and inhibitory receptor NKG2A on blood NK cells reduced during therapy. In addition, the expression of TRAIL on NK cells was reduced during IFN-free therapy, suggesting a decrease in TRAIL mediated killing by NK cells.

Conclusions: We show that viral load decline as a consequence of treatment with novel DAA in chronic HCV patients reduces serum levels of NK cell stimulating cytokines and causes correction of the altered NK cell phenotype observed in chronic HCV patients.

INTRODUCTION

Chronic hepatitis C infection (HCV) is a disease that affects about 170 million people worldwide. Chronic infection of HCV can lead to mortality from hepatic as well as extra-hepatic causes [1]. Until recently, the standard of care therapy of chronic HCV patients was based on the use of pegylated-IFN-α and ribavirin. The recent development of highly potent, IFN-free direct acting antiviral (DAA) compounds that specifically inhibit NS3 protease activity or NS5 polymerase activity has demonstrated superior antiviral activity as compared to IFN-based therapy and has less adverse events [2]. In a phase III clinical trial, combined treatment with the NS3B protease inhibitor asunaprevir and the NS5A polymerase inhibitor daclatasvir resulted in sustained viral response (SVR) rates of 80-85% in chronic HCV patients with genotype 1 [3].

In blood as well as the liver of chronic HCV patients, the HCV-specific immune responses are weak or absent, because of the continuous antigenic pressure resulting from high concentrations of viral antigens [4]. Viral load decline as a consequence of therapy of chronic HCV patients offers a unique opportunity to determine whether the reported impaired immunity to HCV can be restored. However, this could not be investigated before during IFN-based therapy, since pegylated-IFN-α directly modulates immune parameters. The IFN-induced effects include: leukopenia, inhibition of CD4^{+} and CD8^{+} T cell proliferation, direct activation of NK cells, and suppression of IL-12 production by monocytes. These are considered direct immunomodulatory effects of pegylated-IFN-α, and not related to viral load decline. Recently, two studies have examined immune effects during treatment of chronic HCV patients with DAA in the absence of pegylated-IFN-α. These studies observed a rapid downregulation of IFN-stimulated gene (ISG) expression in liver and blood [5], and enhanced HCV-specific CD8^{+} T cell responses in blood of successfully treated patients [6].

Besides T cells, also natural killer (NK) cells are important mediators of the antiviral immune response to HCV. Although conflicting data have been reported in HCV regarding the quality of the NK cell response during the chronic HCV infection [7-9], their roles during IFN-based therapy as a predictor of treatment efficacy has been well-documented [10, 11], and are likely related to the direct effect of IFN-α on NK cells [12]. NK cell activity is controlled by various inhibitory receptors like NKG2A and killer immunoglobulin-like receptors (KIRs), which sense down-regulation of MHC class I on virus-infected cells. Other receptors like NKG2C/D and NKp30, NKp44 and NKp46 deliver activation signals and shift the balance to an activated state of NK cells during chronic HCV infection [13, 14]. Importantly, variations in haplotype of KIR and HLA have been associated with spontaneous and treatment induced HCV clearance, indicating an important role for NK cells in viral elimination [15, 16].

In the present study, we evaluate the functionality of circulating NK cells in chronic HCV patients during viral decline induced by IFN-free DAA therapy using asunaprevir and

daclatasvir. This information is important for a better understanding of the effect of HCV virus eradication on the immune response and to determine the factors that influence protection against reinfection.

MATERIALS AND METHODS

Patients and healthy subjects

12 chronic HCV patients, genotype 1b received daclatasvir (60 mg, orally, once daily), asunaprevir (100 mg, orally, twice daily [BID]) for 24 weeks. The study was an investigator-initiated single center study at the department of Gastroenterology and Hepatology of the Erasmus MC, the Netherlands. Clinical Trail registration number: NCT02282709. Patients were previous non-responders to peginterferon and ribavirin therapy and had no signs of co-infection with HBV and/or HIV. Patients with evidence of hepatocellular carcinoma or cirrhosis child Pugh B or C were excluded. Patients' blood samples were obtained at screening, baseline, 3 days and 1, 2, 4, 8 and 12 weeks after start of therapy. HCV RNA was determined with COBAS Taqman assay. Blood samples from 12 age and sex matched healthy subjects were used as controls for this study. The institutional ethical review board of the Erasmus MC approved the protocols, and informed consent was obtained from all individuals.

Analysis of cell surface molecule expression by flow cytometry

Peripheral blood mononuclear cells (PBMC) were isolated from venous blood by ficoll separation (Ficoll-Paque™ plus, Amersham). For NK cell phenotype, cells were stained for 20 min with CD56-APC-eFluor780 (CMSSB), CD16-PerCp-eFluor710 (eBioCB16), HLA-DR-PerCp-Cy5.5 (LN3), CD69-PE-Cy7 (TP1.55.3), CD3-FITC (UCHT1), and Streptavidin-eFluor450 (all eBioscience), NKp46-APC (185314), NKG2A-PE (Z199), NKp30-PE (Z25, all Beckman), CD3-PacificBlue (UCHT1), NKG2D-PerCp-Cy5.5 (ID11, all BD Bioscience), NKp44-Biotin (P44-8, Biolegend), NKG2C-Alexa-488 (134591, R&D) and Aqua life/dead marker (Life Technologies). In addition, for granzyme B, perforin and TRAIL staining, frozen PBMC were thawed, fixed with 2% formaldehyde, permeabilized with 0.5% saponin and stained with perforin-PerCp-Cy5.5, granzyme B-PE (GB-11), CD56-APC-eFluor780 (CMSSB), CD8-APC (SK1), CD69-PE-Cy7 (FN50, all eBioscience), CD3-PB (UCHT), CD4-PE-Cy7 (SK3, both BD Bioscience), TRAIL-AlexaFluor488 (75402) and NKp46-APC (195314, both R&D).

For the determination of frequency and function of HCV-specific T cells, $3x10^6$ cells from HLA-A2 positive patients were used per staining. Cells were washed with 5% FCS/PBS and stained with dextramers for the HLA-A2 restricted epitopes HCV-NS3 peptide 1073–1081, CINGVCWTV, HCV-NS4 peptide 1406–1415, KLVALGINAV and HCV-NS5 peptide 2594-2602

ALYDVVTKL (all Immudex). Cells were washed with 5% FCS/PBS and stained with CD3-PacificBlue (UCHT1), CD8-APC-H7 (SK1, all BD Biosciences), CD45RO-PerCp-Cy5.5 (UCHL1, eBioscience) and Aqua life/dead marker (Life technologies). Expression was detected by flow cytometry (Canto-II, BD).

Assessment of the cytokine levels using multiplex immunoassays

Serum was collected and cytokine levels were determined using Procarta Plex human cytokine/chemokines multiplex immunoassays (eBioscience, Vienna, Austria). The concentrations of 39 distinct analytes were determined measured using the microsphere-based multiplex LUMINEX-100 (supplementary table 1).

Gene expression analysis

For gene expression analysis using microarray, blood was collected in Tempus RNA tubes at week 0, 1, 2, 4, 8 and 12. RNA was isolated from whole blood. Gene expression was evaluated using GeneChip Human Genome U133 plus 2.0 arrays (Affymetrix). Sample preparation, microarray and processing of array data are described elsewhere [17]. For each gene, the expression at baseline was compared to its expression during therapy using significant analysis of microarray for two class paired samples. To correct multiple testing, false discovery rate approach (FDR) was controlled by randomly permutating the class of samples 1000 times. The differentially expressed genes were identified by a change factor >=2. Different cut-offs of FDR adjusted p-value (q-value) were used for different SAM (significance analysis of microarrays) comparisons to get comparable number of differentially expressed genes. All analysis was performed in R.

Statistics

Statistical comparison used for microarray analyses is described above. Normal distribution was tested with Kolmogorov-Smirnov. If values were normally distributed, paired t-test was used for paired analyses. If values were not normally distributed, Wilcoxon matched pairs test was used. For comparison between patients and healthy controls, unpaired t-test or Mann-Whitney test was used. Overall linear behaviors of the markers NKp30, NKp46 and NKG2A during treatment were estimated with linear regression analyses allowing a random intercept and slope for each patient. Proc Mixed in SAS 9.3 and SPSS 21.0 were used for analyses. A p-value ≤ 0.05 was considered significant.

RESULTS

Recovery of HCV-specific T cells and down-regulation of ISGs during IFN-free therapy

Combination therapy with asunaprevir and daclatasvir has shown to be an effective treatment for chronic HCV[3]. 12 patients were treated in a clinical trial, which was designed to evaluate in detail the functionality of immune cells in blood in chronic HCV patients before, during and after treatment with ASV and DCV, in an IFN-free regimen (NCT02282709). Patient characteristics are shown in table 1. 11 out of 12 patient in our treatment cohort obtained an initial treatment response, defined as undetectable HCV RNA (<15 U/ml) at week 8. One patient developed a viral breakthrough at week 4 during therapy and stopped treatment immediately. HCV RNA and ALT levels during therapy are depicted in figure 1A.

Table 1: Patient characteristics

	HCV patients	Healthy Controls
Number	12	12
Age (years)	54.1	53.1
Gender (M/F)	9/3	9/3
Cirrhosis (%)	42%	
Hemoglobin (mmol/l)	9.3 (8.2-10-6)	
Mean ALT (U/l)	108 (42-173)	
Mean HCV RNA (U/ml)	9.97×10^6 $(1.64 \times 10^5 - 2.68 \times 10^6)$	
Thrombocytes ($\times 10^9$/l)	136 (71-333)	

We first examined the effect of IFN-free therapy on the frequency of HCV-specific CD8^{+} T cells at various time-points in 5 HLA-A2^{+} patients. In line with literature, baseline *ex vivo* frequencies of HCV-specific CD8^{+} T cells were low in chronic HCV patients (varying from 0-0.35% of total CD8^{+} T cells, Supplementary Fig. 1). Importantly, HCV-specific CD8^{+} T cell frequencies increased at week 12 during therapy (p=0.041, Fig. 1B). In 2 patients, CMV-specific CD8^{+} T cells were detected, however, their frequency remained unchanged during therapy (data not shown). Moreover, multiplex cytokine assays demonstrated that serum IP-10 as well as serum MIP-1β levels decreased steadily during IFN-free therapy (Fig. 1B and Supplementary Fig. 2).

Microarray analyses on whole blood demonstrated that predominantly ISGs were significantly upregulated in chronic HCV patients compared to healthy controls (supplementary table 2). Gene expression levels were compared at week 1, 2, 4, 8, and 12 during therapy with baseline. Genes with a more than 2-fold modulation in expression are presented in table 2. During treatment, a downregulation of ISGs was observed, including members involved in anti-viral activity (*RSAD2, OAS3, IFIT1*, and *ISG15*) and ligands for chemokine receptor CXCR3 (CXCL10 = IP-10) (Fig 2 and Table 2).

A

HCV RNA

ALT

B

HCV-spec CD8⁺

serum IP-10

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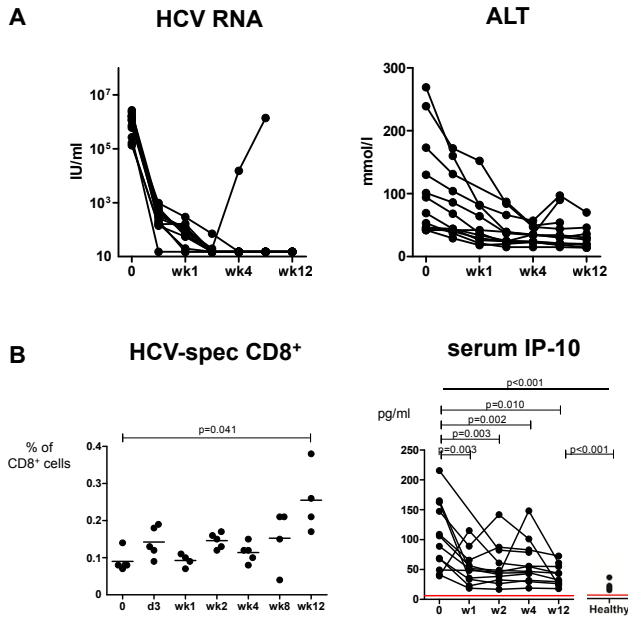
Figure 1. Recovery of HCV-specific CD8⁺ T cells during IFN-free therapy. (A) HCV RNA and ALT levels for individual patents during asunaprevir/daclatasvir therapy (n=12). (B) Frequencies of HCV-specific CD8⁺ T cells during IFN-free therapy of individual HLA-A2⁺ patients (n=5) (left panel). Serum IP-10 levels detected by multiplex cytokine analysis are presented of individual patients (n=12, right panel). The red line represents the cut-off value of the assay as presented in Supplementary Table 1. A paired T-test was used for statistical comparisons.

RSAD2

ISG15

OAS3

IFIT1
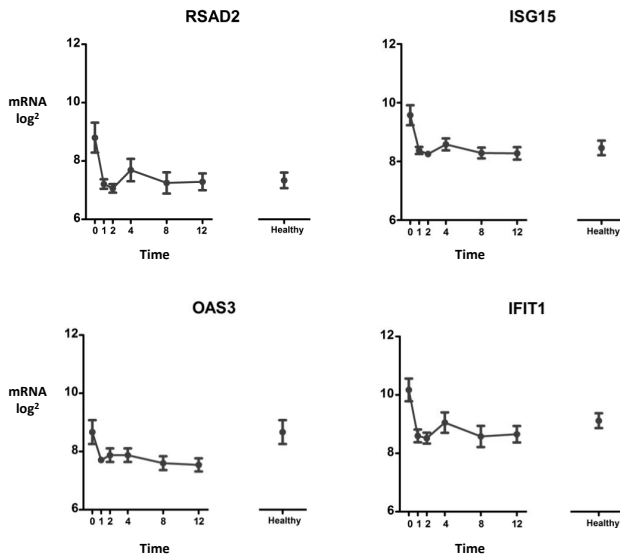
Figure 2. Viral load decline in chronic HCV causes down-regulation of ISGs in blood leukocytes. Microarray analyses of blood were performed at baseline, week 1, week 4, week 8 and week 12 during therapy. Expression levels of 4 ISGs known to be important in chronic HCV are depicted in the graphs. Graphs represent mean expression and standard error of the mean (SEM) of 12 patients and 12 healthy controls.

Table 2: Differentially expressed genes during IFN-free therapy for chronic HCV compared to baseline.

Week 1			Week 4			Week 8			Week 12		
Gene name	Ratio	q-value (%)	Gene name	Ratio	q-value (%)	Gene name	Ratio	q-value (%)	Gene name	Ratio	q-value (%)
IFI44L	0.48	20.82	IFI44L	0.37	9.329	IFI44L	0.30	4.02	IFI44L	0.30	19.94
IFIT1	0.47	17.07	IFIT1	0.46	9.956	IFIT1	0.33	5.00	HINT3	0.42	6.90
RSAD2	0.49	20.82	RSAD2	0.46	11.174	RSAD2	0.34	7.70	STK4	2.23	8.54
CXCL10	0.49	17.07	ASMTL-AS1	2.24	3.575	ISG15	0.41	4.02	ISG15	0.46	23.46
SEGLEC1	0.48	12.13	SLC4A1	2.07	2.262	OAS3	0.48	6.33	LOC100505812	2.32	6.54
			LOC441461	3.19	<0.001	LOC441461	3.18	<0.001	LOC441461	3.11	8.54
			STRN	2.34	<0.001	STRN	2.21	<0.001	STRN	2.44	<0.001
			IFI44	0.39	9.626	IFI44	0.40	7.70			
			APOBR	2.70	0.895	APOBR	2.60	<0.001			
			MALAT1	2.46	3.575	MALAT1	3.00	6.32			
						ASMTL-AS1	2.22	7.70			
						CMPK2	0.49	6.33			
						FTX	2.22	2.48			
						IFI6	0.42	4.02			
						IFIT3	0.45	7.70			

For each gene, the expression at baseline was compared to its expression at various time-points during therapy. The differentially expressed genes were identified with a fold change of ≥ 2.

DAA combination therapy increases CD56^{dim} and decreases CD56^{bright} NK cells in chronic HCV

Since we observed a downregulation of ISGs and IFN-α related cytokines, an investigation on the effects of viral load decline on NK cells is highly interesting. CD3-negative NK cells can be divided into CD56^{high} NK cells (NK^{bright}) and CD56^{low} NK cells (NK^{dim}) (Fig. 3). We observed that chronic HCV patients show higher NK^{bright} and lower NK^{dim} frequencies compared to healthy controls (p=0.02, p=0.02, respectively). Interestingly, during the first 12 weeks of IFN-free therapy, NK cell frequencies altered to levels comparable to healthy controls (Fig. 3), suggesting a direct effect of viral load decline on the composition of the NK cell compartment.

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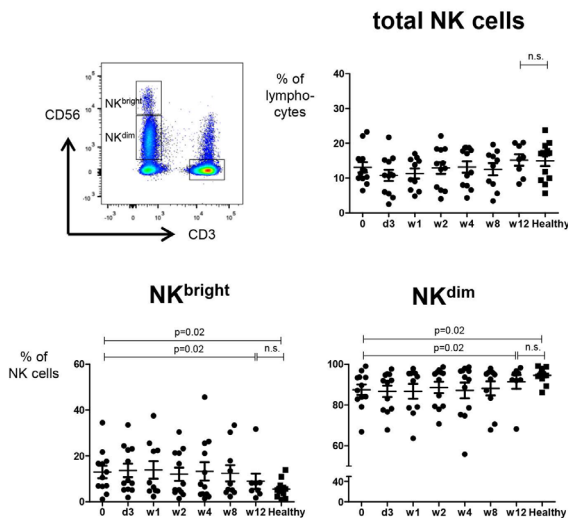
Figure 3. DAA therapy enhances the frequency of NK^{dim} and decreases NK^{bright} cells in chronic HCV patients. Representative dot plot and frequencies of total NK (CD3$^-$CD56$^+$), NK^{bright} (CD3$^-$CD56^{high}) and NK^{dim} (CD3$^-$CD56^{low}) cells during therapy with asunaprevir and daclatasvir. NK cell frequencies were analyzed for fresh PBMC at baseline, day 3, week 1, 2, 4, 8 and 12 by flowcytometry (n=12). Statistical comparison was tested using Wilcoxon matched pairs test and Mann-Whitney test.

DAA therapy downregulates surface NKp30, NKp46 and NKG2A expression on NK cells to a phenotype resembling healthy controls

To investigate whether the decrease in activation was reflected by their NK cell phenotype, we determined activation and inhibitory receptors on the surface of NK cells. Our results showed that the activation receptor NKp30 was downregulated on total NK cells. Since the decrease in expression was linear, we could determine a slope (β) and corresponding p-value (Fig. 4). Expression of the activation receptor NKp46 was also decreasing upon IFN-free therapy, suggesting a lower activation state (p<0.0001, Fig. 4 and Supplementary Fig. 3).

Another important group of surface molecules are C-type lectins, which include inhibitory as well as activating receptors. Expression of the activating receptors CD69, CD16, NKG2C, and NKG2D on NK cells was not affected by therapy (Supplementary Fig. 4). However, there was a clear decrease of the frequency of the inhibitory receptor NKG2A on total NK and NK^{dim} cells (p=0.001 and p=0.012, respectively) (Fig. 4 and Supplementary Fig. 3). Importantly, no differences, except for the expression of NKp46 on NK^{bright} and NK^{dim} cells, were observed for NK cell markers comparing the expression between HCV patients at week 12 and healthy controls. This suggests that the NK cell phenotype is already normalized at week 12._
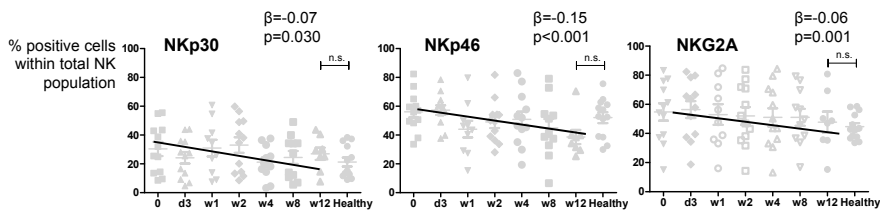
Figure 4. IFN-free DAA therapy modulates NKp30, NKp46 and NKG2A on NK cells. Flowcytometric analyses of activation receptors NKp30 and NKp46 and inhibitory receptor NKG2A on total NK cells. The graphs display the frequencies during therapy of patients and in healthy controls (n=12). Linear decrease over all time-points is presented as a line, matching p-value is shown, β=slope.

Viral load reduction decreases NK cell related cytokines in serum and TRAIL expression

Since we observed an effect of IFN-free therapy on NK cell frequencies and NK surface molecules, we next determined if certain NK cell functions were modulated. IFN-γ production by NK cells was determined upon IL-12/IL-18 stimulation but showed no difference between all time-points during therapy (Supplementary Fig. 5A). In addition, IFN-free therapy did not alter the frequencies of total NK cells, or NK^{dim} and NK^{bright} cells producing perforin or granzyme B (Supplementary Fig. 5B, and data not shown) or specific killing of K562 target cells or suppression of viral replication by NK cells (Supplementary Fig. 5C).

As shown in figure 2 and supplementary figure 2, we demonstrated significant downregulation of ISG mRNA expression, as well as reduced serum levels of eotaxin, MIP-1β and IP-10, which act as chemoattractants. Other cytokines, including IL-12 and IL-18, promote NK cell activation [18]. Comparison of serum levels of these cytokines at baseline showed that IL-12p40 and IL-18 levels were lower in healthy individuals compared to chronic HCV patients (p<0.001, p=0.002, respectively). Importantly, both serum IL-12p40 and IL-18 levels decreased significantly during treatment induced viral load decline, however, this was not sustained at week 12 (Fig. 5A). Of interest, serum soluble TNF apoptosis induced ligand
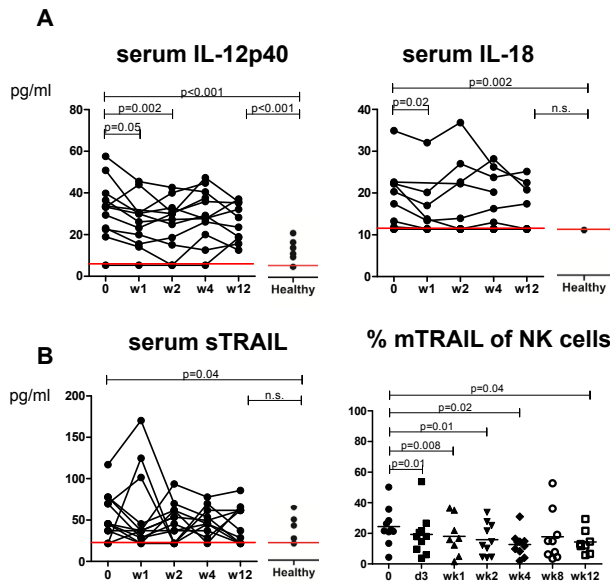
Figure 5. Viral load reduction decreases NK cell related cytokines in serum and TRAIL expression on NK cells.
(A) Graphs show serum IL-12p40 and IL-18 levels of 12 chronic HCV patients during IFN-free therapy and matched healthy individuals (n=12). Horizontal lines represent detection limit of the assay. (B) Serum TRAIL levels during therapy and in healthy individuals (left panel). TRAIL expression on total NK cells during therapy determined by flowcytometry (right panel). The red lines represent the cut-off values of the assays as presented in Supplementary Table 1. Statistical comparison was tested using Wilcoxon matched pairs test and Mann-Whitney test.

(TRAIL) levels were lower in healthy controls compared to HCV patients (p=0.04) and showed a modest reduction during DAA therapy, however, this was not significant. Since TRAIL is expressed on the surface of NK cells and important for killing of hepatocytes, we determined TRAIL expression on NK cells by flow cytometry, and observed reduced frequencies of TRAIL expressing NK cells during therapy (Fig. 5B). This decline of mTRAIL expression was only observed for NK^{dim} cells (data not shown). Combined, therapy-induced reduction of serum cytokines levels important for NK cell activation are indicative of a less activated state of NK cells as shown by a decrease in expression of activating receptors as well as TRAIL.

DISCUSSION

Our study evaluated the immune effects during therapy-induced viral load decline in chronic HCV patients treated with daclatasvir and asunaprevir. We show that reduced HCV RNA load causes correction of the altered NK cell phenotype, including TRAIL, NKp30 and NKp46, whose expression diminished during DAA therapy to levels observed in healthy individuals. Furthermore, we confirmed a recent study by showing that viral load decline following successful DAA therapy led to reduction of blood ISG mRNA and restoration of the HCV-

specific CD8^{+} T cell compartment.

Since NK cells directly respond to IFN-α *in vitro* [14, 19] previous studies conducted in HCV patients during treatment with pegylated-IFN-α and ribavirin combination therapy demonstrated higher levels of activation markers compared to baseline [2, 8, 20]. Also, an increase in TRAIL expression on the surface was observed [21]. However, due to the direct effect of IFN-α on NK cells, the consequences of viral load decline on NK cells could not be examined, as we did in our study. In contrast to IFN-α therapy, DAA therapy led to a lowering of the activation state of blood NK cells, as reflected by reduced levels of TRAIL, NKp30 and NKp46 on NK cells and reduced serum levels of IL-12 and IL-18, likely due to the removal of the viral stimulus. Importantly, comparison of TRAIL expression on NK cells obtained from healthy individuals and HCV patients showed higher frequencies of TRAIL-expressing NK cells in patients prior to therapy (15.7% versus 3.8%; p<0.0001). The simultaneous DAA-induced reduction of NKG2A levels on NK cells was unexpected, but might be the consequence of compensatory mechanisms exerted upon declining activating signals.

We performed a sub-analysis for the 4 cirrhotic patients and did not observe that any of the parameters were differentially modulated during DAA therapy in cirrhotics as compared to non-cirrhotics (data not shown). The sub-analysis was conducted at baseline for serum IP-10, IL-18, IL-12p40, IL-12/IL-18-induced IFN-γ, perforin, granzyme B and mTRAIL expression as well as the modulation during the course of therapy.

In line with the reversal of the activation state of blood NK cells, we observed a rapid downregulation of ISG mRNA, including *RSAD2, OAS3*, *ISG15* and *IFIT1* during DAA therapy. Since many ISG mRNAs are expressed at higher levels in blood and liver during chronic infection [22, 23], lowering the endogenous IFN system to a less active state by DAA therapy might enhance the responsiveness to IFN-based treatment, and be important for sustained eradication of the virus.

In our study, 11 patients were successfully treated with DAA and had undetectable serum HCV RNA at week 24. However, one patient experienced a viral breakthrough at week 4 during DAA therapy. Careful examination of the study parameters of this patient demonstrated no differences in gene expression levels, HCV-specific T cell frequency or serum cytokine levels compared to responder patients. The NK cell phenotype resembled that of the other patients, except for a lower expression of CD57 on NK^{dim} cells during all time-points of DAA therapy (28.7% vs 44.6% at baseline). While IFN-free therapy did not affect NKG2D or CD16 expression on NK^{dim} cells, the patient with viral breakthrough showed an increased frequency of NKG2D expressing NK cells and a decrease of CD16 expressing NK cells during therapy (Supplementary Fig. 6). Our present study provides a better understanding on the immune consequences of HCV eradication by IFN-free therapy.

ACKNOWLEDGEMENTS

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SUPPLEMENTARY MATERIAL

SUPPLEMENTARY METHODS

IFN-γ production by NK cells upon IL-12/IL-18 stimulation
PBMC were thawed and 500,000 cells were seeded in a 24 wells plate and stimulated with IL-12 (0.5 ng/ml; 0.25 ng/ml; 0.12 ng/ml) in combination with IL-18 (50 ng/ml, MBL). Cells were incubated for 24 hours, with brefeldin A (10 µg/ml, Sigma Aldrich) for the last 3 hours. Next, cells were fixed, permeabilized and stained for CD3-PacificBlue, CD56-PE, CD69-APC and IFN-γ-FITC in 0.5% saponin for 20 minutes. Cells were washed and cytokine producing cells were detected by flow cytometry (Canto-II, BD).

Cytotoxicity and inhibition of viral replication by NK cells
For determination of cytotoxicity, sorted NK cells were stimulated with IL-2 (25 U/ml; Miltenyi) for 18 hours and co-cultured with 50,000 CFSE-labeled K562 cells in effector: target ratios of 1:1, 1:5 and 1:10 for 4 hours. Cells were washed and specific killing was measured by 7-AAD staining using flow cytometry. For determination of viral replication, 50,000 HuH7$_{A2}$HCV replicon cells were seeded in DMEM medium supplemented with 10% FCS in a 96 wells plate for 3 hours. After medium was removed, sorted NK cells were added at different effector to target ratios (1:1, 1:5, 1:10) IL-2 was added (50 U/ml, Miltenyi) and cells were incubated overnight at 37°C. Luciferase (Promega) was added and incubated for 30 minutes at 37°C. Luciferase activity was measured by LUMIstar OPTIMA (BMG Labech).

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Supplementary Table 1: Cytokines tested by multiplex assay in serum

Cytokine	Cut-off (pg/ml)	Cytokine	Cut-off (pg/ml)	Cytokine	Cut-off (pg/ml)
Eotaxin	21.7-3319	IL-1RA	37.6-10237	IL-9	10.5-2667
GM-CSF	13.7-11353	IL-2	4.6-308	IL-10	1.3-5300
GRO-α	1.55-332	IL-4	8.7-31778	IL-12p40	7.2-11770
IFN-α	0.5-2250	IL-5	6.8-6325	IL-13	1.9-7800
IFN-γ	3.1-12700	IL-6	10-11724	IL-15	3.9-2398
IL-1α	0.5-2363	IL-7	0.75-485	IL-17α	2.6-3138
IL-1β	3.4-860	IL-8	1.7-1877	IL-18	11.4-13418
IL-21	12.7-50631	TNFα	3.6-14900	IL-17F	1.7-2190
IL-22	34.3-7844	TNFβ	4.1-16058	MIG	8.6-2157
IL-23	18.2-32481	IL2RA	122.1-50000	TRAIL	21.7-3319
IL-27	24-98300	TNFα	3.6-14900	RANTES	0.8-213
IL-31	18.4-17419	TNFβ	4.1-16058	CXCL12	9.5-39000
IP-10	2-8200	MIP-1α	1.7-1388		
MCP-1	0.8-893	MIP-1β	4.7-3992		

Supplementary Table 2: Differentially expressed genes between chronic HCV patients and healthy controls

Gene Name	Ratio	q-value (%)
LOC441461	0.29	<0.001
NID1	2.49	<0.001
SERPINB2	2.05	0.42
OAS1	2.64	0.19
PTX3	2.40	<0.001
PLA2G7	2.02	0.08
IFI35	2.06	0.26
C3AR1	2.17	0.19
APOBR	0.40	<0.001
PDCD6	0.43	<0.001
VPS35	2.21	<0.001
ERGIC1	2.12	<0.001
ALYREF	2.58	<0.001
OAS2	2.00	0.42
STRN	0.47	<0.001
FUS	0.49	<0.001
ASMTL-AS1	0.40	<0.001
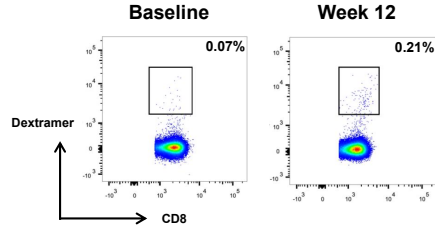
Supplementary figure 1. Recovery of HCV-specific CD8+ T cells during IFN-free therapy. Representative dot plots of HCV-specific CD8+ T cells at baseline and at week 12 during IFN-free therapy.
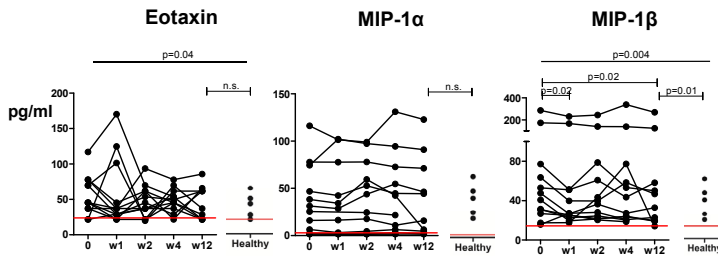
Supplementary figure 2. Multiplex cytokine assay was performed on serum samples at baseline, week 1, 2, 4 and 12 during therapy (n=12) and on 12 matched healthy controls. Serum levels of eotaxin, MIP-1α, and MIP-1β are presented. For multiplex cytokine assays, statistical comparison was tested using Wilcoxon matched pairs test and Mann-Whitney test. The red lines represent the cut-off value of the assay as presented in Supplementary Table 1.
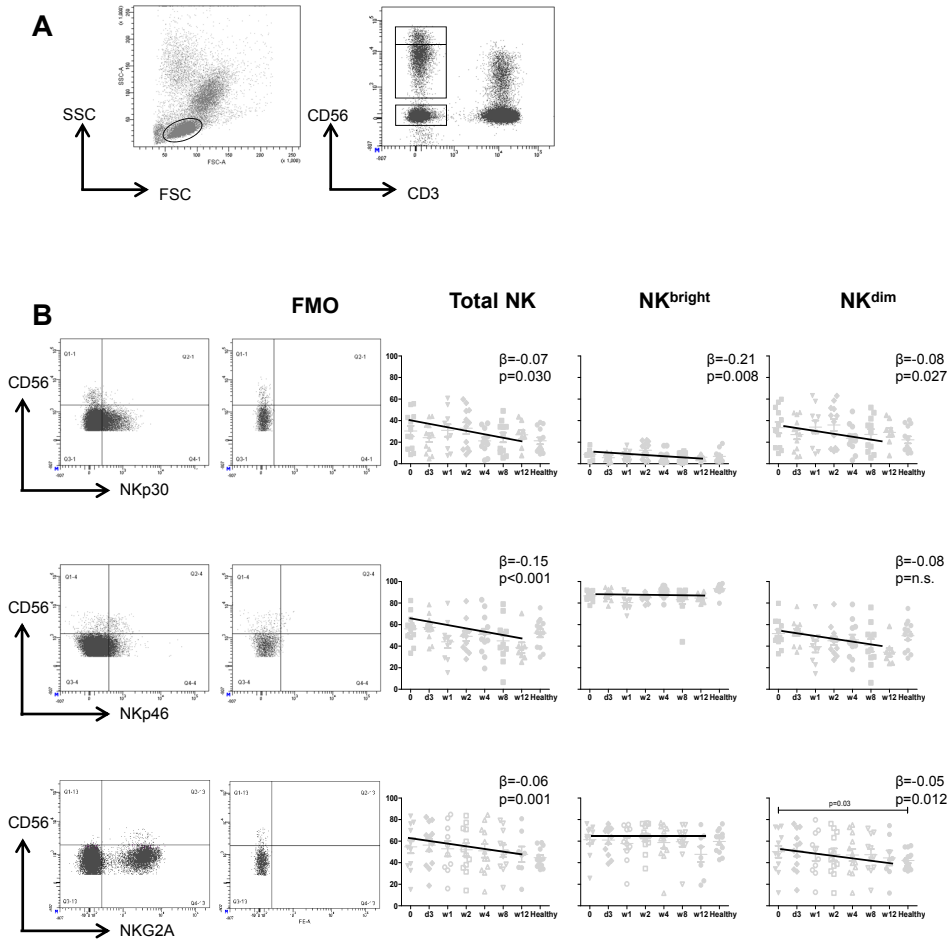
Supplementary figure 3. IFN-free DAA therapy modulates NKp30, NKp46 and NKG2A on NK cells. Flowcytometry analyses of activation receptors NKp30 and NKp46 and inhibitory receptor NKG2A on total NK, NK[bright] and NK^{dim} cells. Representative dot plots of the indicated markers and FMO (fluorescence minus one) control stainings are presented, as well as and graphs of frequencies during therapy of patients and in healthy controls (n=12). Linear decrease over all time-points is presented as a line, matching p-value is shown, β=slope.
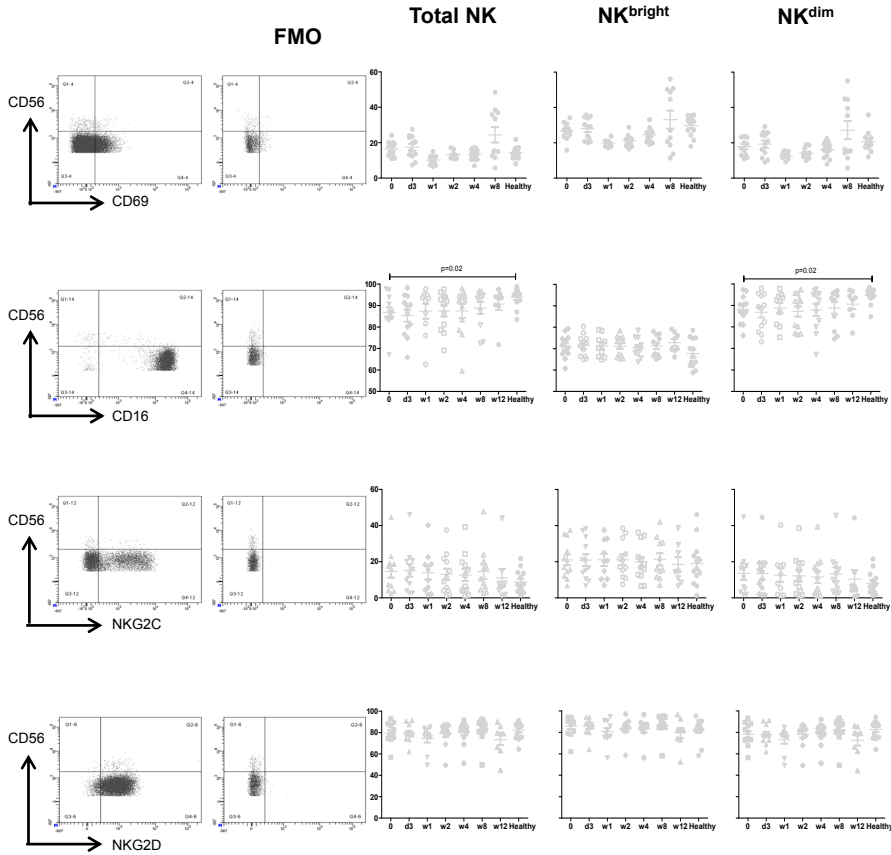
Supplementary figure 4. Flowcytometry analyses of CD69, CD16, NKG2C, NKG2D on total NK, NK^bright and NK^dim cells. Representative dot plots of the indicated markers and FMO (fluorescence minus one) control stainings are presented and graphs of frequencies during therapy and in healthy controls (n=12). CD69 staining on NK cells at week 12 failed. Statistical comparison was tested using Wilcoxon matched pairs test and Mann-Whitney test.

A

IFN-γ production upon IL-12/18 stimulation

B

C
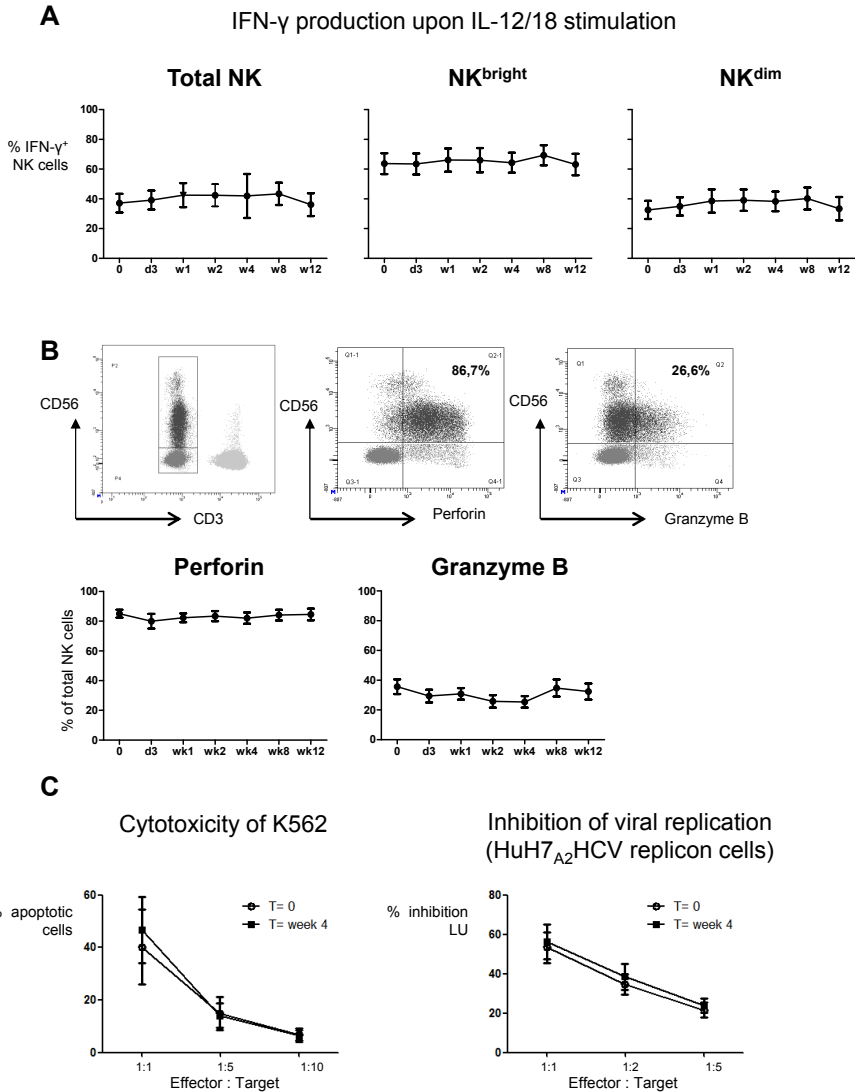
Supplementary figure 5. IFN-free therapy does not modulate NK cell mediated cytotoxicity and IFN-γ production in chronic HCV. (A) PBMC were thawed and stimulated with 0.12 ng/ml IL-12 and 50 ng/ml IL-18 for 18 hours. Frequency of IFN-γ positive NK cells was determined by flowcytometry at baseline, day 3, week 1, 2, 4, 8, and 12 during therapy. Graphs represent mean frequency and standard error of the mean (SEM) of 12 patients. (B) Representative dot plot of perforin/ granzyme producing NK cells *ex vivo*. Light grey clouds represent negative control stainings. Graphs present mean and standard error of the mean (SEM) of perforin and granzyme positive NK cells during IFN-free therapy (n=12). (C) Sorted NK cells were co-cultured with K562 target cells at different effector to target ratios (1:1, 1:5 and 1:10). Cytotoxicity was measured by 7-AAD at baseline (circles) and at 4 weeks (squares) during therapy (n=5). Y-axis represents mean percentage of apoptotic cells and standard error of the mean (SEM). Inhibition of viral replication was determined by co-culturing of sorted NK cells and HuH7$_{A2}$HCV replicon cells at 1:1, 1:2 and 1:5 effector to target ratios. Graph shows inhibition of luciferase activity (LU) in relation to HuH7$_{A2}$HCV replicon cells cultured in medium alone. NK cell mediated inhibition of HCV replication at baseline (t=0, circles) is compared to week 4 during therapy (squares) (n=10). Statistical comparison was tested using Wilcoxon matched pairs test and Mann-Whitney test.

A

B

6
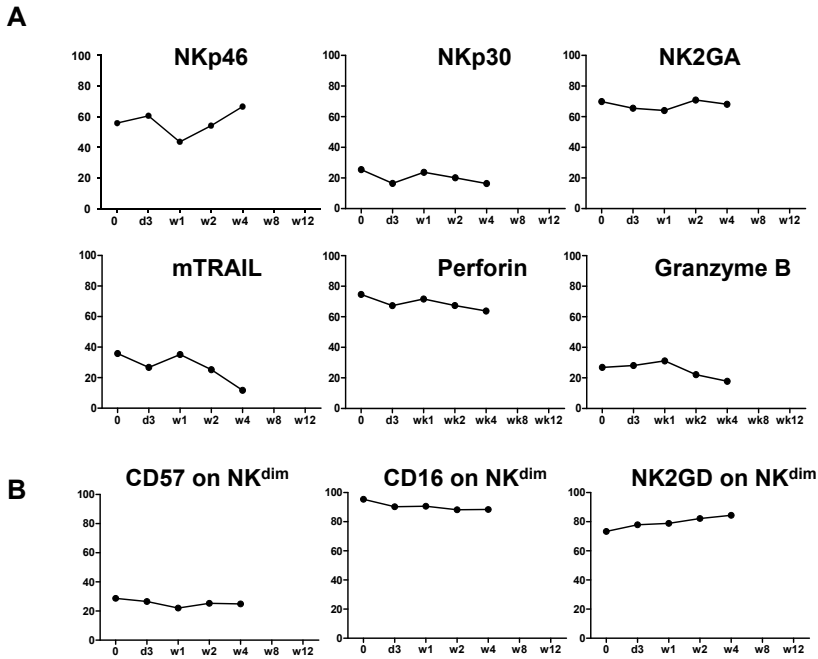
Supplementary figure 6. Flowcytometric analyses of NK cells of the patient with a viral breakthrough during DAA therapy. The frequency of cells expressing the marker of interest is presented within the total NK cell population (A) or within the CD56dim population (B). DAA therapy was stopped at week 4.

Chapter **7**

Frequencies of circulating CD3^{+}CD161^{+}TCR Vα7.2^{+} MAIT cells are diminished in chronic HCV, HIV and HCV/ HIV co-infection and do not recover during therapy.

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ABSTRACT

Introduction: Mucosal invariant T (MAIT) cells comprise a subpopulation of T cells that can be activated by a broad range of bacterial products and cytokines to produce IFN-γ and express cytolytic enzymes. Loss of MAIT cells is observed in HIV infection, which is thought to compromise anti-bacterial immunity and microbial translocation in the gut. Since little is known on MAIT cells during HCV infection, we compared their phenotype and function in comparison to HIV and HCV/HIV co-infected patients, and determined the effect of IFN-α-based and direct acting antiviral (DAA) therapy on MAIT cells of HCV patients.

Methods: Peripheral blood was collected from patients with chronic HCV before, during and 24 weeks after HCV therapy (CHCV, n=27), virologically suppressed HIV (n=10), acute HCV/HIV co-infection (AHCV/HIV, n=9) and healthy individuals (n=12). Patients with CHCV were treated with PegIFN-α/ribavirin with (n=11) and without (n=11) telaprevir or with IFN-free therapy (n=5). Patients with AHCV/HIV co-infection were treated with PegIFN-α/ribavirin and boceprevir. MAIT cells were identified by surface expression of CD161 and Vα7.2, and were stimulated with *E.coli*, IL-12/IL-18 or IFN-α/IL-18 for 19 hours. NK cells and MAIT cells were analyzed for the expression of CD38, CD69 and IFN-γ by flowcytometry.

Results: Compared to healthy individuals, the frequency of MAIT cells was significantly decreased in patients with CHCV, HIV and AHCV/HIV co-infection. Expression of CD38 on MAIT cells was comparable in CHCV, HIV and healthy individuals, but was significantly increased in patients with AHCV/HIV co-infection. MAIT cells were responsive to IFN-α *in vitro* as evidenced by enhanced frequencies of IFN-γ producing cells. IFN-α-based therapy for CHCV decreased the frequency of IFN-γ$^+$ MAIT cells and this remained visible even until 24 weeks after successful therapy. Importantly, even after successful IFN-α-based as well as IFN-α-free therapy for CHCV, decreased frequencies of MAIT cells persisted.

Conclusion: We show that the frequencies of MAIT cells are reduced in blood of patients with CHCV, HIV and in AHCV/HIV co-infection compared to healthy individuals. Successful therapy for CHCV did not normalize MAIT cell frequencies. The major impact of HIV and HCV-infection on MAIT cells, and potent effects of exposure to IFN-α, both *in vitro* and *in vivo*, warrant further studies on this specialized T cell subpopulation.

INTRODUCTION

The hepatitis C virus (HCV) is a positive-sense single stranded RNA virus that primarily infects hepatocytes. Due to an ineffective immune response, the virus is able to chronically infect the liver in about 80% of the adult patients [1, 2]. In acute HCV infection, viral particles bind to pathogen recognition receptors, which trigger hepatocytes to produce various subtypes of type I interferons (IFN) responsible for the induction of hundreds of IFN stimulating genes (ISG) which possess anti-viral activity [3-5]. From studies in chimpanzees, it is known that during acute HCV infection, HCV RNA titers increase despite the induction of type I IFN, suggesting that the virus has evolved evasive mechanisms. Besides inducing ISGs, IFN-α can activate natural killer (NK) cells, T cells and dendritic cells (DCs) and is therefore an important immune modulator [4, 6-9]. Comparable to HCV infection, type I IFNs are produced in large amounts after infection with human immunodeficiency virus (HIV) [9]. During the acute phase of infection, HIV stimulates DCs to produce large amount of type I IFNs causing induction of antiviral responses that target every step of the HIV life cycle [9].

In recent years, our understanding of Mucosal Invariant T (MAIT) cells has increased substantially. MAIT cells are a subtype of T cells that express CD8, and are characterized by the expression of CD161 and the invariant T cell receptor (TCR) Vα7.2 that recognizes vitamin metabolites produced by bacteria and yeast presented by MR1, a MHC class I related protein on the surface of antigen-presenting cells (APC) [10, 11]. MAIT cells are also activated in an MR1-independent manner in the presence of IL-12 and IL-18 [12]. MAIT cells are abundant in human blood (1-10% of $CD8^+$ T cells) and are known for their antimicrobial activity to bacteria and yeast in the gut and lungs [13, 14] via release of cytokines and cytotoxic enzymes [10]. Interestingly, MAIT cells are reduced in peripheral blood and lymph nodes of patients with chronic HIV infection, and unable to recover after successful combination antiretroviral therapy (cART) therapy [15-18]. It has been suggested that this decrease of MAIT cell numbers contributes to the high incidence of bacterial infections observed in HIV patients [18]. At the moment it is unclear what causes the depletion of MAIT cells in HIV infection. To our knowledge, up till now no data on MAIT cells in AHCV/HIV co-infection are available and the role for MAIT cells in HCV infection is largely unknown. In this study, we investigate the impact of HCV, HIV and AHCV/HIV-infection and on MAIT cells and their response to type I IFNs. In addition, NK cells and MAIT cells were investigated during IFN-α based therapy for CHCV and AHCV/HIV co-infection.

MATERIALS AND METHODS

Patients and healthy subjects

Heparinized blood was collected from 37 patients with chronic HCV (CHCV) infection, 9 patients with virologically suppressed HIV (HIV RNA < 20 geq/mL) that were acutely infected with HCV (AHCV/HIV), 10 patients with virologically suppressed HIV mono-infection and 12 healthy subjects. Patients' characteristics are listed in table 1. From 10 HCV patients, blood samples were collected at one time-point and used for our analyses. Out of 37 CHCV patients, 27 patients were treated in three different historical treatment regimens. In cohort 1, 11 patients were treated with pegylated-IFN-alpha-2a (PegIFN-α) and ribavirin for 24 or 48 weeks, according to HCV genotype. The results of the study have been published and the treatment regime has been extensively described (NCT00422838, [19]). Patients in cohort 2 (n=11) were treated with telaprevir, PegIFN-α and ribavirin for 24 or 48 weeks, according to their fibrosis level and previous treatment response to PegIFN-α and ribavirin. Consistent with international guidelines [20], patients were treated with telaprevir, PegIFN-α and ribavirin for the first 12 weeks. Thereafter, patients continued treatment consisting of PegIFN-α and ribavirin only (NCT01641094). In this treatment cohort, naïve patients, patients with a partial response (>2log drop in viral load) to previous IFN-based therapy, and patients without cirrhosis were treated for 24 weeks when HCV RNA was undetectable (<15 IU/l) at week 4 and 12 during therapy. Medication was stopped in patients with detectable HCV RNA (>15 IU/l) at week 12 during therapy. In cohort 3, 5 patients were treated with daily daclatasvir and twice daily asunaprevir according to international guidelines for 24 weeks (NCT02282709, [21]). For patients with CHCV, blood was collected at baseline, week 12 during therapy and 24 weeks after end of therapy in all three treatment cohorts. Patients with cART-suppressed HIV that were acutely infected with HCV, were treated in cohort 4 (n=9). Patients were treated within 26 weeks after HCV infection with 12-weeks boceprevir, PegIFN-α and ribavirin (NCT01912495). Blood was collected at baseline and week 4 during therapy. Virologically suppressed HIV mono-infected patients (n=10) and 12 healthy subjects were selected as controls. The institutional ethical review board of the Erasmus Medical Center approved the protocols, and informed consent was obtained from all individuals.

Analysis of cell surface molecule expression by flow cytometry

Peripheral blood mononuclear cells (PBMC) were isolated from venous blood by (Ficoll-Paque™ plus, Amersham) and frozen at -150°C. PBMC were thawed and washed with RPMI 1640 (Lonza) with 10% FCS. For flow cytometry, 500,000 PBMC were used for each staining. Cells were stained with CD8-FITC (RPA-T8), CD38-PerCp-eFluor710 (HB7), CD3-PE-Cy7 (UCHT1), CD161-Pacific Blue (HP-3G10, all eBiosciences), CD4-APC-H7 (SK3, BD Biosciences), TCR Vα7.2-PE (3C10, Biolegend), CD56-APC (N901, Beckman) and Live/ dead

marker (Aqua, Life technologies) for 20 minutes at 4°C in the dark. Cells were washed and marker expression was detected by flow cytometry (Canto-II, BD). MAIT cells were defined as $CD3^+CD8^+CD161^+V\alpha7.2^+$ cells within lymphocyte gate and NK cells were defined as $CD3^-CD56^+$ cells within the lymphocyte gate. For stimulation assays MAIT cells were defined as $CD3^+CD4^-CD161^+V\alpha7.2^+$.

Analysis of intracellular cytokine expression by flow cytometry.

The percentage of cells producing IFN-γ was measured by flowcytometry using various stimuli. For each condition, 500,000 cells were stimulated in a 24 wells plate with IL-12 (0.025 ng/ml, Miltenyi), IL-18 (50 ng/ml, MBL) and IFN-α (2500 IU/ml, INTRON-A, Merck). For stimulation with *E. Coli,* 25 million formaldehyde-fixed *E. Coli* in medium with 10% FCS were used. For all conditions, cells were stimulated for 16 hours at 37°C at 5% CO_2. Brefeldin A (10 μg/ml, Sigma) was added and the cells were incubated for another 3 hours. Cells were stained with CD3-PerCp-Cy5.5 (UCHT1), CD4-APC-H7 (SK3, both BD biosciences), CD69-PE-Cy7 (TPI.55.3), CD161-PB (HP-3G10, both eBiosciences), TCR Vα7.2-PE (3C10, Biolegend), CD56-APC (N901, Beckman) and Live/ dead marker (Aqua, Life technologies). Cells were fixed, permeabilized and stained with IFN-γ-FITC (25723.11, BD Biosciences). Cytokine-producing cells were detected by flow cytometry (Canto-II, BD). Quadrants were set on negative expression of CD69 on monocytes and the absence of IFN-y production by B cells.

Statistics

Statistical comparison was tested using the Kruskal-Wallis and Mann-Whitney test for unpaired non-parametric analyses and the Paired student T test for paired observations. A *p* value ≤ 0.05 was considered significant.

RESULTS

MAIT cells are decreased in patients with chronic HCV, HIV and AHCV/HIV co-infection compared to healthy controls.

We investigated MAIT cells and compared them to NK cells to examine whether they are differentially affected during chronic viral infections. We evaluated the frequencies of $CD3^-CD56^+$ NK cells and MAIT cells, which were identified as $CD3^+CD8^+CD161^+V\alpha7.2^+$ T cells (Fig. 1A). The frequencies of MAIT cells and NK cells were determined in peripheral blood of patients with CHCV, HIV, AHCV/HIV and heathy individuals (Fig. 1B). The frequency of MAIT cells in CHCV, HIV and AHCV/HIV patients was significantly decreased compared to healthy controls (p=0.01, p=0.04 and p=0.01, respectively, Fig. 1B). Interestingly, patients with AHCV/HIV co-infection appeared to have lower MAIT cell frequencies compared to patients

Table 1: Patient characteristics

		All CHCV patients	Cohort 1 (CHCV)	Cohort 2 (CHCV)	Cohort 3 (CHCV)	Cohort 4 (AHCV/HIV)	Patients with HIV mono-infection	Heathy individuals
Number		37	11 (30%)	11 (30%)	5 (14%)	9 (24%)	10	12
Gender (% male)		76	73	82	80	100	100	67
Age (mean, yrs)		50	45	50	52	40	49	54
HCV RNA (mean, IU/ml)		6.79E+06	4.60E+06	2.90E+06	1.20E+06	2.60E+05		
Fibrosis (%)	0-1	35	30	27	80			
	2	22	50	18	0			
	3	16	20	36	0			
	4	22	0	18	20			
	n.d.	5	0	0	0			
ALT (mean, U/l)		92	79	70	188	324		
SVR (%)		85	82	82	100	100		
HCV genotype (%)	1	81	36	100	100	100		
	2	5	18	0	0	0		
	3	14	46	0	0	0		
	4		0	0	0	0		
cART (%)						100	100	
HIV Load <20 geq/mL (%)						100	80	
CD4 (mean, x10^{E9}/ml)						0.87	0.67	

Cohort 1: CHCV patients that received PegIFN-α/ribavirin. Cohort 2: CHCV patients that received PegIFN-α/ribavirin and telaprevir. Cohort 3: CHCV patients that received asunaprevir and daclatasvir. Cohort 4: AHCV/HIV patients on cART treatment that received PegIFN-α/ribavirin and boceprevir.

with HCV or HIV mono-infection although this did not reach statistical significance (Fig. 1B). As described previously, no differences were observed in frequencies of NK cells in patients with CHCV compared to healthy controls [22-25]. NK cells were found to be decreased in HIV mono-infected patients compared to healthy controls (p=0.002) and CHCV patients (p=0.05) (Fig. 1B). Next, we determined the expression of the activation marker CD38 on MAIT cells and observed that patients with AHCV/HIV co-infection had increased frequencies of activated MAIT cells in peripheral blood compared to HIV, CHCV and healthy individuals (p=0.01, p<0.001, p=0.002 respectively, Fig. 1B). Stratification of patients with CHCV of their fibrosis stage showed similar frequencies of MAIT cells (Fig. 1C).

Frequency of IFN-γ producing MAIT cells does not differ between patients with CHCV, HIV, AHCV/HIV co-infection or healthy individuals.

The cytokines IL-12/18 and IFN-α are able to activate NK cells directly. In contrast to NK cells, MAIT cells have the unique property to respond to bacterial stimuli like *E. Coli*, making them important for anti-bacterial immunity [11]. In this study, we determined the ability of MAIT cells from the various patient subgroups to respond to *E. Coli,* IL-12/18 as well as to IFN-α/IL-18 and evaluated the expression of CD69 and IFN-γ. A representative dot plot and gating strategy after stimulation of CHCV peripheral blood with IL-12/18 is shown in figure 2A. As shown in figure 2B, CD69 expression on both MAIT cells and NK cells was upregulated upon stimulation with *E. Coli*, IL-12/18 and IFN-α/IL-18, as compared to medium control conditions. Unexpectedly, stimulation with *E. Coli* also increased CD69 expression on NK cells (p<0.001). The frequency of IFN-γ producing MAIT cells was significantly increased upon stimulation with *E. Coli* and IL-12/18 (p<0.001 and p<0.001). Importantly, IFN-α was also found to be a potent activator of MAIT cells (Fig. 2B). Of note, *E. Coli* was able to induce IFN-γ production by MAIT cells (p<0.001) but not by NK cells (Fig. 2B). Next, we determined the function of MAIT cells in different viral infections and observed that MAIT cells are equally capable to become activated and produce IFN-γ upon stimulation with *E. Coli*, IL-12/18 and IFN-α/IL-18 in CHCV, HIV, AHCV/HIV co-infection and healthy individuals (Fig. 2C).

IFN-based therapy for chronic HCV reduces the frequency of IFN-γ producing MAIT cells upon IL-12/IL-18 stimulation

Figure 2B shows that IFN-α is a potent stimulator of MAIT cells *in vitro*. Since IFN-α is a well-known therapy for HCV infections, we determined whether IFN-α-based therapy for CHCV activates MAIT cells and affects MAIT cell frequencies in CHCV and AHCV/HIV co-infection. Twenty-two CHCV patients were treated with IFN-based therapy with (n=11) or without (n=11) telaprevir. All patients were HCV RNA negative at week 12 during therapy. We observed that IFN-α-based therapy did not alter MAIT cell frequencies, but increased CD38 expression on MAIT cells (p<0.001, Fig. 3A, upper panels). No difference was observed between treatment with or without addition of telaprevir (data not shown). The NK cell
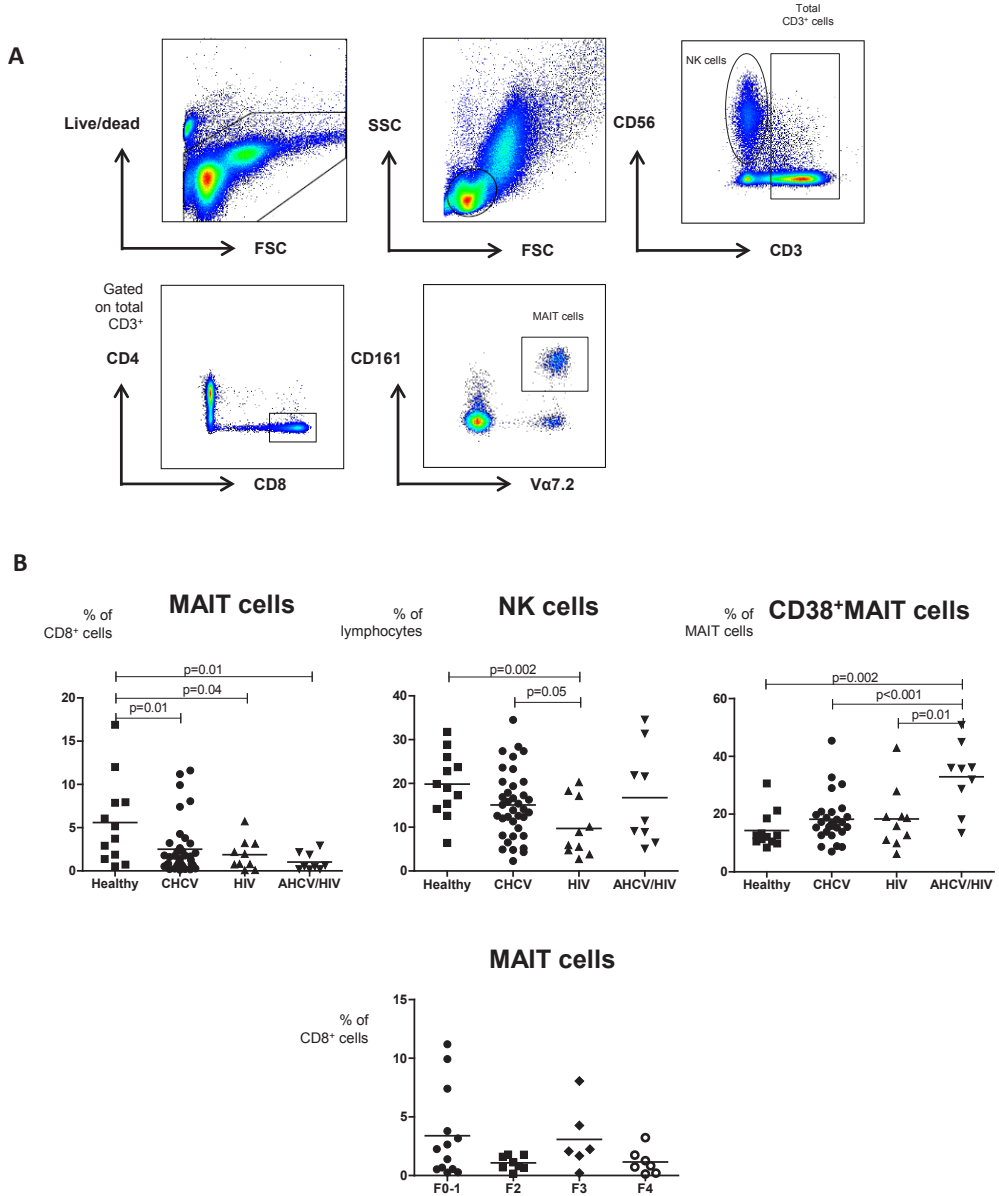
Figure 1. Frequencies of MAIT cells are decreased in patients with CHCV, HIV and AHCV/HIV co-infections compared to heathy individuals. (A) Representative dot plots and gating strategy of CD3⁺CD8⁺CD161⁺Vα7.2⁺ MAIT cells and CD3⁻CD56⁺ NK cells. (B) Frequency of MAIT cells, NK cells and CD38⁺MAIT cells in healthy individuals (n=12), chronic HCV (CHCV) patients (n=37), virologically suppressed HIV (HIV) patients (n=10) and patients with acute HCV/HIV co-infection (AHCV/HIV) (n=9). (C) Frequency of MAIT cells within the CD8⁺ T cell population in CHCV patients with different levels of fibrosis score. Statistical comparison was tested using Kruskal-Wallis and Mann-Whitney test.

frequency was reduced early during IFN-based therapy (p=0.02), but this was not sustained at week 12 (Fig. 3A, upper panels). Recently, IFN-free therapy became widely available in the clinic and has substituted IFN-α-based therapy in many high-income settings because of higher SVR rates and reduced side-effects. To determine if the observed effects on MAIT
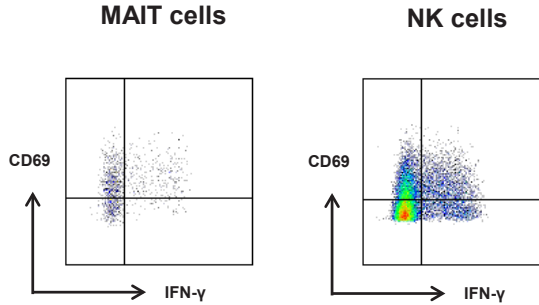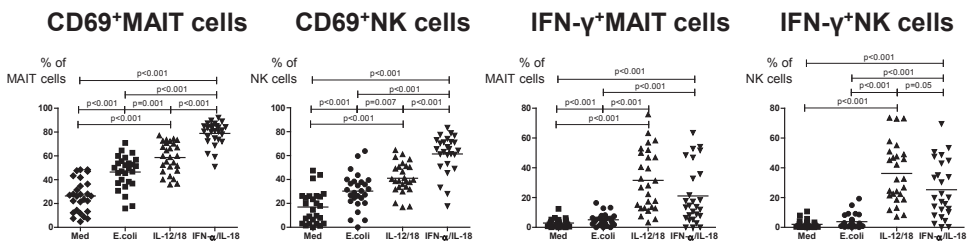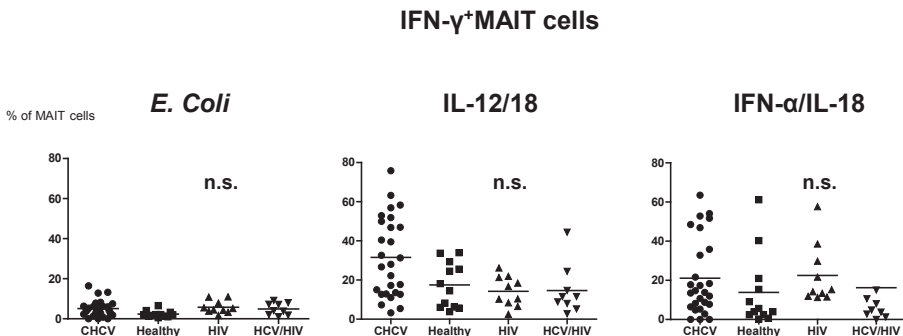
Figure 2. MAIT cells function does not differ between patients with chronic HCV, HIV and AHCV/HIV co-infection. (A) Representative dot plots of IFN-γ production and CD69 expression on MAIT cells in CHCV after stimulation with IL-12/18. (B) Frequency of CD69 expression and IFN-γ producing MAIT cells and NK cells in CHCV patients after 19 hours stimulation with medium, *E. Coli*, IL-12/18 and IFN-α/IL-18. (C) Frequency of IFN-γ producing MAIT cells after various stimuli between healthy individuals, patients with CHCV, HIV and AHCV/HIV co-infection. Statistical comparison was tested using paired T test, Kruskal-Wallis and Mann-Whitney test.

cells were due to a direct effect of IFN-α or to viral load decline, 5 CHCV patients were treated with an IFN-free regimen. In all patients HCV RNA titers were undetectable after 4 weeks of treatment, however, no effect was observed on MAIT cell frequency or activation (Fig. 3A, middle row). In addition, no effect was observed on NK cell frequencies (Fig. 3A, middle panels). These data suggest that the increased frequencies of CD38-expressing MAIT cells in CHCV patients is the consequence of exposure to IFN-α, rather than of viral load decline. Importantly, the absolute numbers of MAIT cells in blood of CHCV patients did not change during IFN-α-based therapy (Supplementary Fig. 1).

Patients with AHCV/HIV co-infection were treated with a combination of boceprevir, PegIFN-α and ribavirin; all patients were HCV RNA negative at week 4 during therapy. IFN-α-based therapy did not alter MAIT cell or NK cell frequencies in this patient population (Fig. 3A, lower panels). The enhanced expression of CD38 on MAIT cells of patients with AHCV/HIV co-infection (Fig. 1) was not further increased during therapy (Fig. 3A, lower panels).

A

IFN-γ⁺ MAIT cells

B
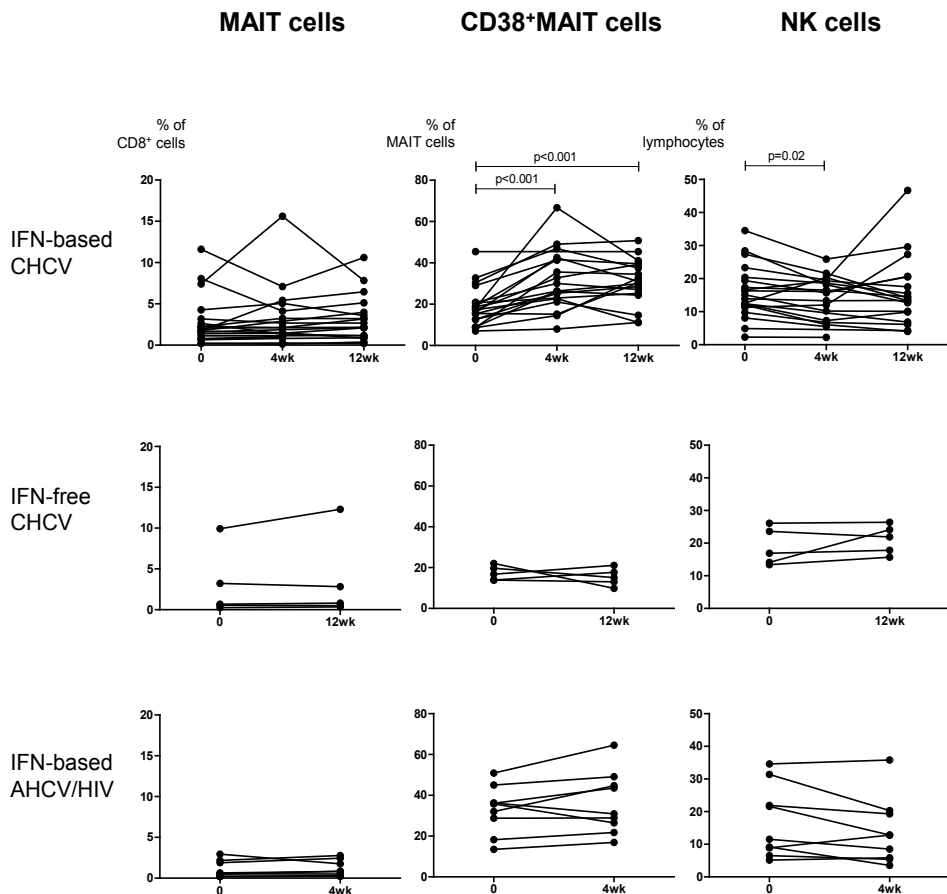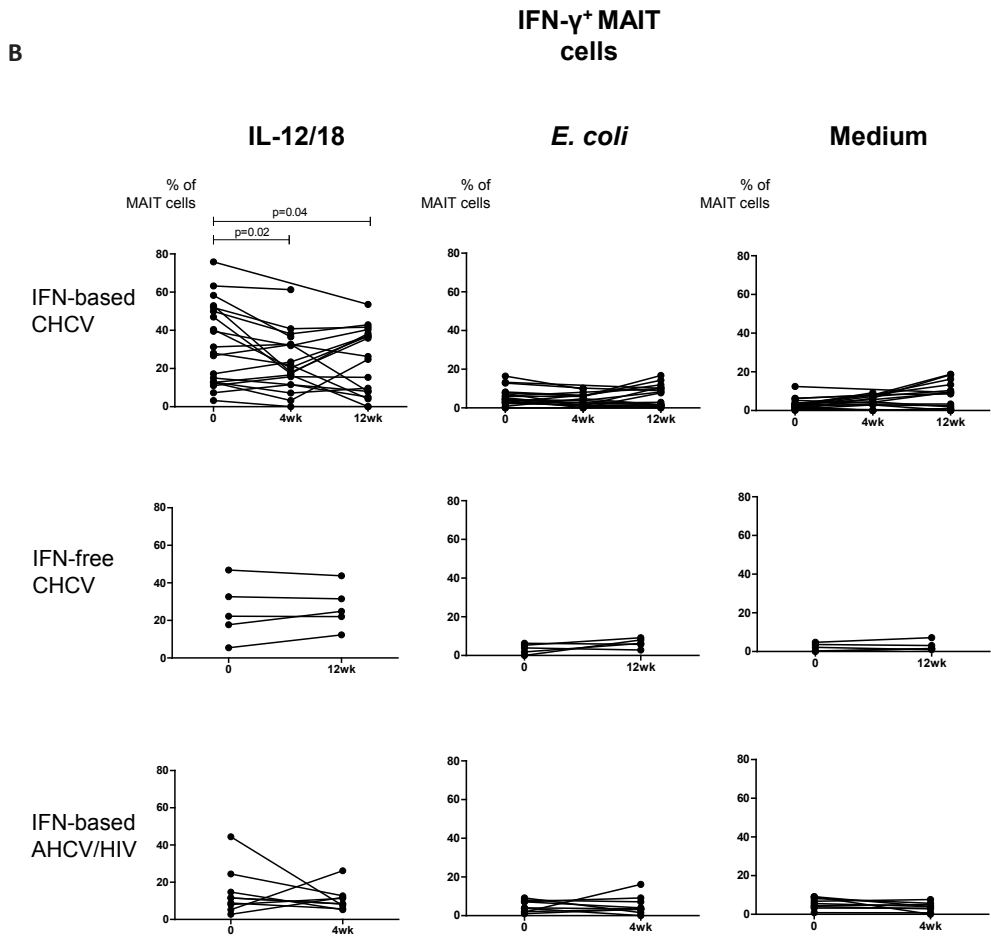
Figure 3. IFN-based therapy for chronic HCV increases expression of CD38 on MAIT cell but reduces MAIT cell function. (A) Frequency of MAIT, CD38⁺MAIT cells and NK cells during IFN-based therapy for CHCV (cohorts 1 and 2, n=22), IFN-free therapy for CHCV (cohort 3, n=5) and IFN-based therapy for AHCV/HIV co-infection (cohort 4, n=9). At week 12 during therapy for CHCV and week 4 therapy for AHCV/HIV, all patients were HCV RNA negative. (B) Frequency of IFN-γ producing MAIT cells after stimulation with IL-12/18, *E. Coli* and medium during IFN-based and IFN-free therapy for CHCV infection. The lower panels show frequency of IFN-γ producing MAIT cells at baseline and week 4 during IFN-based therapy for AHCV/HIV. All AHCV patients were HCV RNA negative at week 4. Statistical comparison was tested using paired T test.

To investigate whether MAIT cell function was altered during therapy for HCV, MAIT cells were stimulated with IL-12/18, and *E. Coli*. Surprisingly, although IFN-α-based therapy for CHCV caused increased expression of activation marker CD38 on MAIT cells as shown in figure 3A, a decrease in IFN-γ producing MAIT cells was observed upon stimulation with IL-12/18 during therapy (p=0.02 at week 4, and p=0.04 at week 12; Fig. 3B, upper panels). No alterations were observed during therapy upon stimulation with *E. Coli* or medium (Fig. 3B, upper panels). In CHCV patients treated with IFN-free therapy nor in patients with AHCV/
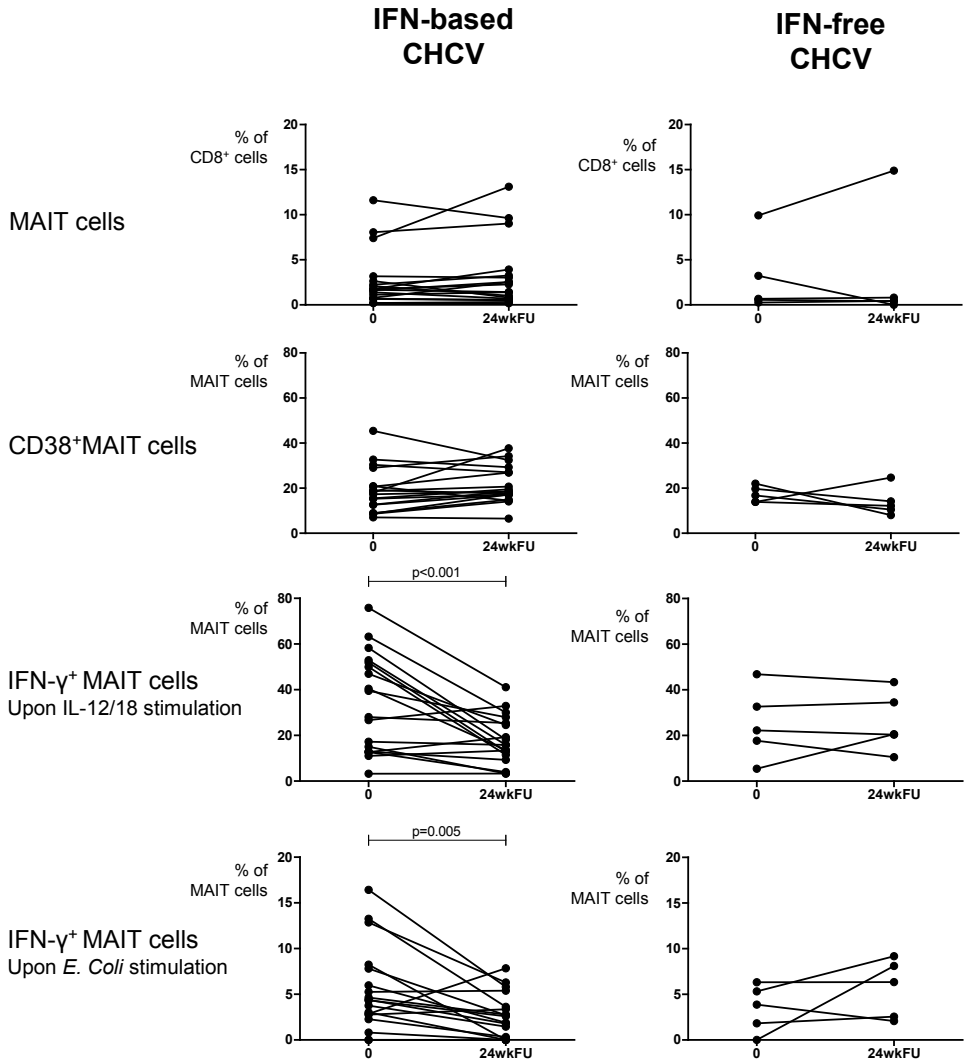
Figure 4. Frequency of MAIT cells in peripheral blood of chronic HCV patients does not recover after successful therapy. Frequency of MAIT, CD38+MAIT and IFN-γ+ MAIT cells before and 24 weeks after successful IFN-based (left, n=22) and IFN-free (right, n=5) therapy for CHCV. Frequencies of IFN-γ+ MAIT cells were measured after 19 hours stimulation with IL-12/18 and *E. Coli*.

HIV co-infection, therapy altered the frequencies of IFN-γ producing MAIT cells (Fig. 3B, middle and lower panels). In addition, no effect was observed after stimulation with IL-12/18, *E. Coli* and IFN-α/IL-18 on CD69 expression on MAIT cells during therapy for CHCV or AHCV/HIV co-infection (Supplementary Fig. 2).

Successful therapy for chronic HCV does not restore MAIT cell frequency

Besides evaluation of the effect of therapy on phenotype and function of MAIT cell, it is especially relevant to determine effect of viral clearance. Blood was collected 24 weeks after successful therapy-induced HCV eradication. No samples from patients with AHCV/HIV co-infection were available after therapy. Figure 4 shows that 24 weeks after cessation of IFN-based therapy as well as IFN-free therapy, the MAIT cell frequencies were not restored in CHCV patients and remained low (2.7% at baseline vs 3.0% at week 24 after therapy (wk24FU), figure 4, upper panels). The increased expression of CD38 on MAIT cells during IFN-based therapy for CHCV (Fig. 3A) was not maintained 24 weeks after therapy and returned to pre-treatment levels (Fig. 4). Interestingly, the suppressive effect of IFN-based therapy on IFN-y producing MAIT cells as shown in figure 3, was still observed 24 weeks after cessation of therapy upon IL-12/IL-18 as well as upon *E. Coli* stimulation (p<0.001, p=0.005, respectively, Fig. 4). Therefore, it appears that IFN-based therapy for CHCV affects IFN-y producing MAIT cells long-term after therapy. No differences in frequencies of MAIT cells, CD38$^+$ MAIT cells or IFN-γ producing MAIT cells were observed before and 24 weeks after IFN-free therapy for CHCV (Fig. 4, right panels).

DISCUSSION

In this study we performed a detailed analysis on MAIT cells in CHCV, HIV and AHCV/HIV co-infections. We observed that the frequency of MAIT cells is decreased in all three groups of infected patients compared to healthy individuals, and that no normalization was observed following successful anti-HCV therapy. Moreover, the frequency of IFN-γ producing MAIT cells was reduced in blood from HCV patients receiving IFN-α-based therapy, but not in blood from patients receiving IFN-free therapy.

Our findings show that the frequencies of MAIT cells within the population of CD8$^+$ T cells are significantly reduced in CHCV patients, virologically suppressed HIV patients and AHCV/HIV patients. Although similar findings have been reported in HIV patients before [16-18], to our knowledge, we are the first that show reduced frequencies of CD161$^+$Vα7.2$^+$MAIT cells in peripheral blood of CHCV patients, irrespective of fibrosis scores. From our studies, we cannot conclude whether the reduced frequencies of MAIT cells are due to depletion of cells via apoptosis, migration of MAIT cells from blood to peripheral organs or skin, or due to down-regulation of characteristic markers, such as CD161.

Comparison of the 3 patient groups shows a trend towards a strong reduction of MAIT cells in individuals infected with AHCV/HIV as compared to HCV mono-infected patients, but increased activation by the expression of CD38. Activation-induced cell dead is can be a possible explanation that causes the depletion of MAIT cells from the periphery in this patient population. Besides a lower frequency of MAIT cells, we also observed a lower frequency of NK cells in HIV patients compared to CHCV and healthy individuals (p=0.02 and p=0.02). Decreased NK cell frequencies and function in HIV patients has been described before to be associated with a more rapid progression to AIDS in untreated patients [26]. Importantly, virus eradication by IFN-based therapy as well as by IFN-free therapy did not lead to normalization of the reduced MAIT cell frequencies since assessment of their numbers 24 weeks after cessation of therapy did not lead to an increase in their frequencies. This observation is reminiscent of the findings reported in HIV where long-term suppression of viral replication by cART does not result in normalization of MAIT numbers in blood [15, 17, 18]. Although cART controlled HIV infection leads to undetectable HIV RNA levels, it is well-known that the immune system in these patients remains in a higher activation status as compared to control healthy individuals. It needs to be determined whether enhanced immune activation results in depletion of MAIT cells, both in successfully treated HIV and HCV patients. In this respect it is important to mention that we previously showed that months to years after CHCV eradication regulatory T cells are retained in the liver [27, 28] and that blood gene expression profiles do not return to pre-infection levels [29].

Besides their numbers, the function of MAIT cells is also an important determinant of the contribution of MAIT cells to the overall response to HIV or CHCV. As shown in this paper and reported by others, MAIT cells are highly responsive to IL-12/IL-18 and to a lesser extent to *E. Coli* [12, 16]. The response of MAIT cells to *E. Coli* likely requires additional co-stimulation provided by molecules such as CD80, CD86 or CD40 or other cytokines [30]. In this study we show that MAIT cells are also responsive to the combination of IFN-α and IL-18, leading to the production of IFN-γ. The addition of IL-18 was required since stimulation with IFN-α alone resulted in undetectable to low IFN-γ. The ability of MAIT cells to respond to IFN-α is shared with NK cells. At baseline, frequency of IFN-y producing MAIT cells obtained from patients with CHVC, HIV or AHCV/HIV upon *E. Coli*, IL-12/IL-18 or IFN-α/IL-18 stimulation was not altered as compared to healthy individuals. However, since MAIT cells are decreased in these patients groups, the total amount of IFN-y by these MAIT cells is likely to be decreased.

Since we observed that IFN-α can stimulate MAIT cells *in vitro*, we examined whether IFN-α based therapy for CHCV affects MAIT cells *in vivo.* Our findings show that although IFN-based therapy for HCV did not alter MAIT cell frequencies, frequencies of IFN-y producing MAIT cells were decreased during therapy, which is the opposite of the effect observed *in vitro*. Since IFN-based therapy enhances the expression of the activation marker CD38 on MAIT cells, the inhibitory effect of IFN-α administration is unlikely the result of an overall inhibitory effect on the MAIT cells. Future studies will need to be conducted to better

understand these apparent contradictory effects of IFN-α. In these studies processes like apoptosis induction following activation, as well as the complex regulation by IFN-α of the IL-12/IL-10 axis should be examined [31]. Since no effect was observed of IFN-free therapy for CHCV on MAIT cells, we can conclude that the decrease of IFN-γ⁺ MAIT cells during IFN-based therapy was due to a direct effect of IFN-α, and not to viral load decline.

In conclusion, we show that MAIT cells are decreased in patients with chronic HCV, HIV and AHCV/HIV co-infection compared to healthy controls. We show that IFN-α modulates MAIT cells *in vitro*, and *in vivo* during IFN-based therapy for HCV but importantly, a sustained viral response for HCV does not rescue MAIT cell frequency.

7

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7

SUPPLEMENTARY MATERIAL

Absolute numbers MAIT cells
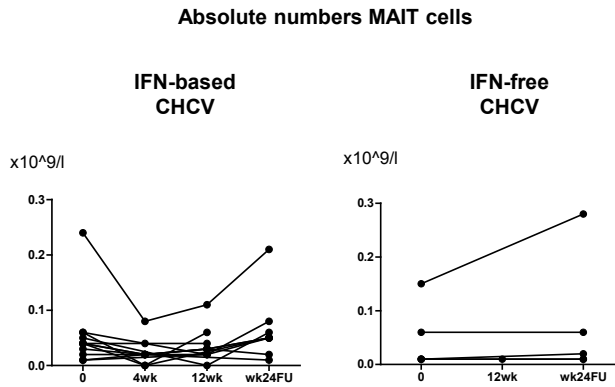
Supplementary Figure 1. Absolute numbers of MAIT cells in chronic HCV do not recover after successful therapy. Absolute numbers of MAIT cells during IFN-based (n=22) and IFN-free therapy (n=5) for CHCV. Absolute numbers of MAIT cells in peripheral blood are shown at baseline, at week 4 and 12 during therapy, and at 24 weeks after successful therapy.
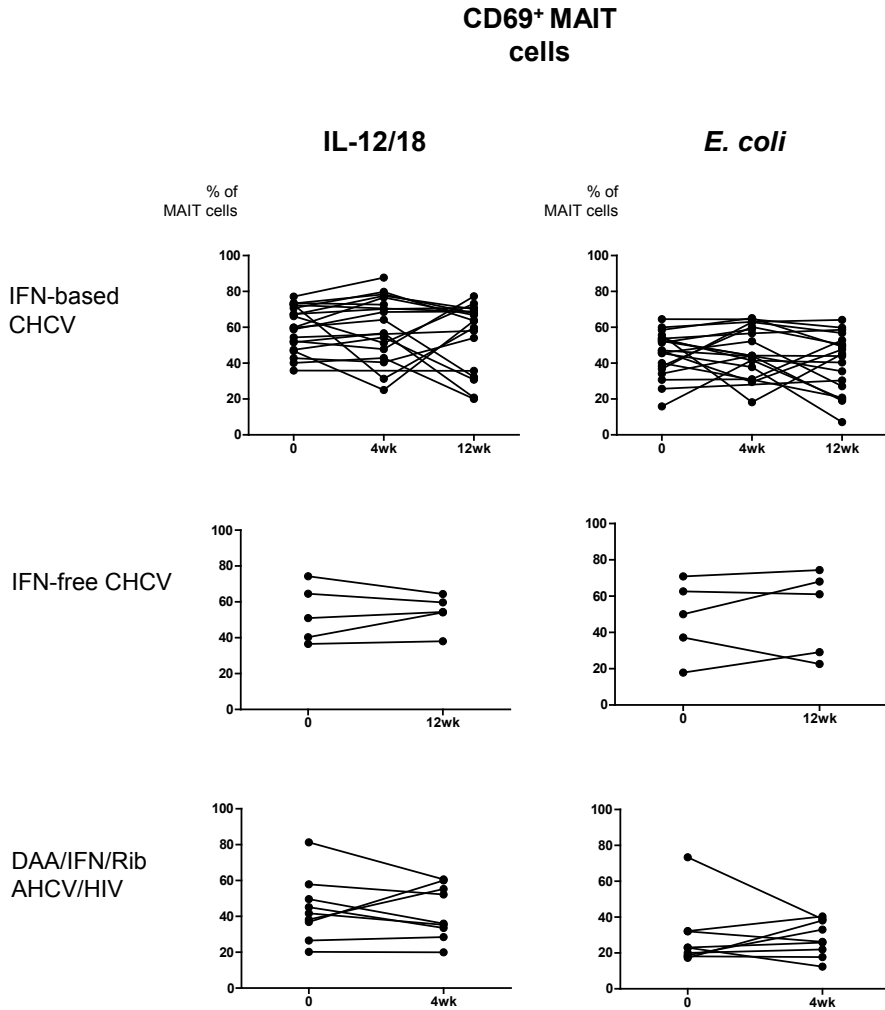
CD69+ MAIT cells

Supplementary Figure 2. Therapy for CHCV or AHCV/HIV co-infection does not modulate CD69 expression on MAIT cells. Frequency of CD69+MAIT cells after stimulation with IL-12/18 and *E. Coli* during IFN-based and IFN-free therapy for HCV infection. All CHCV patients were HCV RNA negative (<15 IU/ml) at week 12. The lower panels show frequency of CD69+ MAIT cells at baseline and week 4 during IFN-based therapy for AHCV/HIV. All AHCV patients were HCV RNA negative at week 4. Statistical comparison was tested using paired T test.

Part III

Long-term outcome of treatment induced viral clearance of HCV infection.

Chapter 8

Sustained virologic response after therapy with the HCV protease inhibitor narlaprevir in combination with peginterferon and ribavirin is durable through long-term follow-up

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ABSTRACT

Introduction: Achievement of a sustained virologic response (SVR) after peginterferon (PEG-IFN) and ribavirin (RBV) treatment is considered to be a marker for cure of chronic hepatitis C virus (HCV) infection. Long-term follow-up of patients with SVR after treatment with a direct acting antiviral has not yet been described.

Methods: We used a randomized placebo-controlled, double-blind, two-period phase 1b trial that was conducted in 40 HCV genotype 1 (treatment-naïve and treatment-experienced)-infected patients.

Results: Nineteen patients achieved SVR after treatment with the HCV protease inhibitor narlaprevir followed by PEG-IFN / RBV. In these patients, HCV-RNA tests were scheduled at three, six, 12 and 24 months after end of treatment. Patients were followed for a median of 27 months (range 15-32) after end of treatment with a median number of follow-up visits of 4 (range 3-8). All patients remained HCV-RNA negative over time.

Conclusion: SVR achieved following narlaprevir and PEG-IFN / RBV therapy was durable up to 32 months end of treatment.

INTRODUCTION

The standard of care antiviral treatment (AVT) for chronic hepatitis C virus (HCV) infection, peginterferon (PEG-IFN) and ribavirin (RBV) has been modified to include a direct acting antiviral (DAA), either telaprevir or boceprevir [1]. Recently, boceprevir and telaprevir (direct acting antiviral agents (DAA)) have been added to peginterferon (PEG-IFN) and ribavirin (RBV) in the treatment of chronic hepatitis C [1] .

Successful HCV treatment has been defined as achievement of sustained virologic response (SVR), undetectable HCV-RNA 24 weeks after end of treatment (EoT). SVR has been shown to be durable up to 18 years post-treatment and is therefore considered a marker for successful HCV antiviral therapy [2]. Clinical studies investigating triple therapy regimens have used the same end-point. To date, undetectable HCV-RNA 24 weeks after DAA-based therapy has not yet been described to be a marker of long-term sustained viral eradication. In contrast to PEG-IFN/RBV, DAAs select for resistant HCV strains and their immune modifying capacities are limited [3-6]. Trace amounts of HCV-RNA have been detected in different compartments of the human body years after SVR following PEG-IFN / RBV [7,8]. Veerapu et al. showed that, with long-term follow-up after achieving SVR, HCV-RNA reappearances in plasma induced HCV-specific T cell responses [7]. It is not known if traces of HCV-RNA also persist after successful DAA-based therapy, and whether these traces are subject to immune control or could result in a late virologic relapse.

We studied durability of SVR after treatment with the protease inhibitor narlaprevir and PEG-IFN with narlaprevir, followed by PEG-IFN / RBV for 24-48 weeks [9].

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MATERIALS AND METHODS

We used a randomized, placebo-controlled, double-blind, two-period phase 1b trial that was conducted in 40 HCV genotype (GT) 1-infected (naïve and treatment-experienced) patients [9]. Narlaprevir was administered for seven days as 800 mg thrice daily without ritonavir or 400 mg twice daily with 200 mg ritonavir twice daily. After a 4-week washout, the same dose and regimen of narlaprevir was administered in combination with PEG-IFN-α-2b for 14 days. Thereafter, all patients initiated PEG-IFN-α-2b and RBV treatment for 24 weeks if a rapid virologic response was achieved, otherwise 48 weeks. Six of 20 (30%) treatment-experienced and 13 of 20 (65%) treatment-naïve patients achieved SVR and these were all randomized narlaprevir [9]. Patients were followed up at 3, 6, 12 and 24 months after EoT. At each follow-up visit, blood was collected and tested for HCV-RNA using Roche Ampliprep/ Cobas Taqman HCV/HPS assay (Roche Molecular Systems Inc., Branchburg, NJ) with lower limit of detection (LLOD) of 15 IU)/ mL or the VERSANT® TMA HCV RNA qualitative assay (Siemens Medical Solutions Diagnostics, Eindhoven, the Netherlands) with LLOD of 5.3 IU/ mL. At the last follow-up visit, which was at least 24 months after EoT, the VERSANT® TMA HCV RNA qualitative assay was used.

At the final follow-up visit, *in vitro* HCV-specific T cell proliferation and HCV-RNA levels were determined in peripheral blood mononuclear cells (PBMC). Fresh PBMC were cultured in quadruplets in 96-well round-bottom plates ($2x10^5$ cells in 200 μL), and stimulated with overlapping peptide pools (1 μg)/ mL) per individual peptide; spanning the core, NS3, NS4, NS5a and NS5b HCV genome; clone J4, GT 1b; BEI Resources, Manassas, USA), CMV antigens (34 μg/mL; AD-169; Microbix, Toronto, Canada) or no stimulus. RPMI 1640 medium supplemented with 5% human serum (Lonza, Verviers, Belgium) and anti-CD28 and anti-CD49d antibodies (both 1 μg/mL; eBioscience, San Diego, USA) were used. At day 5, cells were pulsed for 16h with [³H]-thymidine (0.5 μCi/well; Amersham, Little Chalfont, UK). Proliferation was determined as counts per minute by liquid scintillation. For HCV-RNA determination in PBMC, cells were lysed by repeated freeze-thaw cycles and tested for HCV-RNA using the VERSANT® TMA HCV RNA qualitative assay. A parallel study was conducted in 22 treatment-naïve chronic HCV-infected patients treated with PEG-IFN-alfa2b and RBV for 24 weeks (GT 2 and 3) or 48 weeks (GT 1 and 4) [10]. Six of these patients achieved SVR. The results of identical immunologic assays performed with cells from these six patients were compared to those from the patients receiving narlaprevir treatment.

RESULTS

Of the 19 patients that achieved SVR with the narlaprevir-based regimen, 14 (75%) were male, 15 (79%) of Caucasian race, median age was 56 years (range 33-65 years) and six patients (32%) were previously treated with an IFN-based regimen. At follow-up, three patients (16%) had signs of cirrhosis which was already present before narlaprevir treatment, and median alanine transaminase (ALT) was 23 (range: 11-89). Only the three cirrhotic patients had ALT levels above the upper limit of normal. After EoT, the median number of follow-up visits was 4 (range 3-8) and the median follow-up time in months was 27 (range 15-32) months. To be more specific regarding patients with a higher chance of relapse; number of follow-up visits and median follow-up time in patients with cirrhosis was 3 and 27 (range 24-30) months, respectively. One patient did not respond to the call for his final visit; his final follow-up visit was 12 months after EoT. All patients had undetectable HCV-RNA at all follow-up visits. In addition, no clinically relevant abnormalities were observed for any patient during follow-up.

In vitro quantification of HCV-specific T cell proliferation in blood was performed in six patients (one patient with cirrhosis) with SVR after narlaprevir-based therapy at the final follow-up visit, which was at least 24 months after EoT (median 25 months, range 23-30 months). HCV-specific T cell proliferation was low compared to the positive control CMV and comparable to the negative control of culture medium alone. Also, the six patients who achieved SVR after PEG-IFN / RBV- therapy showed no HCV-specific T cell proliferation at follow-up when compared to medium (Fig. 1A/1B). Regarding PBMC, five out of six patients tested HCV-RNA negative; the remaining patient tested positive for HCV-RNA. This patient did not suffer cirrhosis and/or changes in ALT.
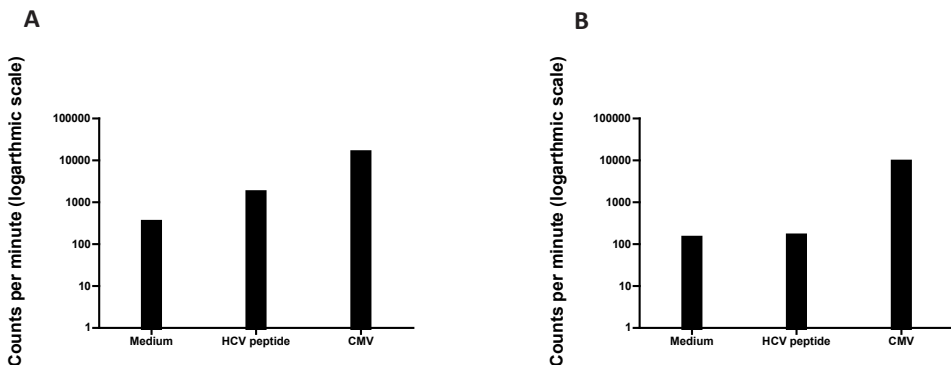
Figure 1. (A) HCV-specific T cell proliferation in patients with SVR after peginterferon and ribavirin. (B) HCV-specific T cell proliferation in patients that achieved SVR with the narlaprevir-based regimen followed by 24-48 weeks of peginterferon and ribavirin.

DISCUSSION

In the present long-term follow-up study in HCV GT-1-infected patients, we demonstrated durability of SVR antiviral treatment with the protease inhibitor narlaprevir (+/- ritonavir) followed by narlaprevir/PEG-IFN-2b (+/- ritonavir) and 24-48 weeks of PEG-IFN/RBV. In addition, no detectable HCV-specific T cell responses were measured in blood, as expected in the absence of HCV-RNA in the circulation.

SVR is considered a marker for cure because of its durability and associated improved clinical outcomes [2, 11, 12]. Recently, the protease inhibitors telaprevir/boceprevir have been added to standard of care AVT. Therefore, knowledge of durability and reliability of SVR as surrogate marker with these new regimens is of major importance.

In our study, no reappearances of HCV RNA during follow-up were observed, and, at final follow-up, T cell proliferation against HCV in blood was low. In a subset of patients who achieved SVR and were only treated with PEG-IFN/RBV, HCV-specific T cell proliferation was low as well. These T cell proliferation findings are in line with the inability to detect HCV-RNA at follow-up; both findings are indicative of viral eradication. These findings are important since some studies, in which HCV patients achieved SVR with PEG-IFN/RBV, minute amounts of HCV-RNA were detected in the circulation at long-term follow-up [7, 8]. Veerapu et al demonstrated HCV-specific T cell responses with detection of small amounts of HCV-RNA [7]. Consequently, the absence of HCV-specific T cell response supports the undetectable HCV-RNA test results over time, suggesting viral eradication. Because we used a highly sensitive test to detect HCV-RNA (LLOD 5.3 IU/mL), performed frequent and long-term follow-up visits and because a virologic relapse would be expected to result in persistent viremia, we consider SVR durable in these narlaprevir-treated patients. In 1 of 6 patients, PBMC tested positive for HCV-RNA, while other tests, HCV-RNA in the peripheral circulation, HCV-specific T cell responses and ALT, were not suggestive of viral relapse. With long-term follow-up of patients that achieved SVR after PEG-IFN/RBV, HCV-RNA has been demonstrated in PBMC as well, even in the absence of viral relapse [13]. Our study is limited by the small number of patients. Recently, similar results have been presented in a larger cohort of telaprevir-treated patients, although this has not been published yet [14]. With the introduction of DAAs to PEG-IFN/RBV as standard of care for HCV infection, patients should be monitored long-term until our important clinical finding is validated in larger cohorts and in cohorts treated with other DAA-based regimens.

In conclusion, SVR achieved with narlaprevir in combination with PEG-IFN and RBV was durable, suggesting SVR to be a valid surrogate marker for successful DAA-based treatment for chronic HCV infection.

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8

Chapter 9

The intrahepatic T cell compartment does not normalize years after therapy-induced hepatitis C virus eradication

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ABSTRACT

Little is known on the immune status in liver and blood of chronic HCV patients long after therapy-induced viral clearance. In this study, we demonstrate that 4 years after clearance, regulation of HCV-specific immunity in blood by regulatory T-cells (Treg) and immunosuppressive cytokines IL-10 and TGF-β is still ongoing. Importantly, sampling of the liver 4 years after clearance shows that intrahepatic Treg are still present in all patients, suggesting that liver T-cells remain regulated. Identifying mechanisms that regulate HCV-specific memory T-cell responses after clearance is highly relevant for the development of protective vaccines, especially in patients at high-risk of reinfection.

INTRODUCTION

Virus-specific CD4^{+} and CD8^{+} T cell responses are key players in controlling infection with the hepatitis C virus (HCV). During chronic HCV infections these T cells are present at low frequencies and are dysfunctional [1, 2]. Critical factors that were shown to contribute to the weak T cell responses include active suppressive regulation by FoxP3^{+}CD25^{+}CD4^{+} regulatory T cells (Treg) and inhibitory cytokines, such as IL-10 and TGF-β, as well as T cell exhaustion due to continuously high viral antigen levels [1, 3]. In a previous study, we showed that 4 weeks after therapy-induced viral clearance, negative regulatory mechanisms like IL-10, TGF-β and Treg are still functional in controlling HCV–specific T cell responses in peripheral blood [4].

Although HCV replication takes place in the liver, little information is available in humans on intrahepatic T cell immunity after therapy-induced viral clearance. We showed that prior to therapy, but also 4 weeks after the end of IFN-based antiviral treatment, Treg are present in the liver of chronic HCV patients [5]. This is in contrast to livers never exposed to HCV, which contain virtually no Treg [6], and this suggests that virus-specific T cell responses in the liver remain regulated by Treg shortly after viral clearance. Retention of Treg has also been demonstrated in mouse skin after clearance of the stimulus following primary antigen exposure [7]. These skin Treg were shown to possess memory cell characteristics since antigen re-expression led to more efficient suppression of CD4^{+} and CD8^{+} T cell responses by these memory Treg and less severe skin disease as compared to the situation following primary antigen exposure [7, 8].

Identification of mechanisms responsible for the failure of the HCV-specific T cell response to create long-term protective immunity are important especially for the development of protective vaccines and for patients at high risk of reinfection. At present, it is unknown whether intrahepatic Treg are retained in the liver after therapy-induced viral clearance and whether these cells have similarities to memory Treg observed in mouse skin and in human. In this study, we evaluate patients with a sustained viral response following IFN-based therapy 4 years earlier by sampling the liver. In addition, HCV–specific T cell responses and mechanisms for regulation in peripheral blood are studied.

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MATERIALS AND METHODS

Patients

In the present study, chronic HCV patients were included from our previous cohort of 21 treatment-naïve patients who were treated with pegylated-IFN-alpha-2a (PegIntron®, Schering-Plough now MSD, Houten, the Netherlands) and ribavirin (Rebetol®, Schering-Plough now MSD) and who were evaluated longitudinally [5]. Thirteen out of 21 patients obtained a sustained virological response and were eligible for inclusion in the present study. Five patients were lost in follow-up, 1 patient was unable to participate because of interfering medication (methotrexate) and 1 patient because of immobility. Five patients volunteered to participate in this study and donated blood, and 4 of them underwent additional sampling of the liver by fine needle aspiration. The institutional ethical board of the Erasmus MC approved the clinical protocol for this follow-up study, and written informed consent was obtained from all individuals. Patients' characteristics are listed in Supplementary Table 1, and patient's study numbers coincide with those in our previous paper to allow full transparency of the clinical and immunological data from the individual data at all time-points [5].

Aspiration of liver cells and collection of peripheral blood

Intrahepatic leukocytes were obtained via fine needle aspiration as described elsewhere [5]. Peripheral blood mononuclear cells (PBMC) were isolated from venous blood by ficoll separation (Ficoll-Paque™ plus, Amersham, Buckinghamshire, UK). For microarray analyses, blood was collected in Tempus Blood RNA tubes (ABI, Foster City, USA). Samples were collected 4 years after ending of therapy and compared with historical data at baseline and at week 4 and 24 after ending HCV therapy. Flowcytometry to analyze cell surface molecule expression was done on fresh whole blood or liver aspirate biopsies similarly as described previously [4], Also functional assays using fresh PBMC to quantify HCV-specific T cell proliferation by ^{3}H-thymidine incorporation on day 6, and IFN-γ production by ELISA on day 3 are described elsewhere [3-5]. As described before, cultures were stimulated with HCV overlapping peptide pools, supplemented with antibodies against the IL-10 receptor (IL-10R) or TGF-β or following depletion of Treg from PBMC. Polyclonal stimulations using soluble anti-CD3 antibodies or CMV lysate were used as positive controls.

Gene expression analysis

Sample preparation, microarray and processing of array data are described elsewhere [9]. PubMed and Ensemble databases were used to identify genes involved in T cell activation and regulation. Eventually, 244 genes were included in our analyses, which are listed in Supplementary Table 2. Gene expression data was evaluated using GeneChip Human

Genome U133 plus 2.0 arrays (Affymetrix). For each gene, the expression at 24 weeks or 4 years follow-up was compared to baseline using an adjusted unpaired t-test for high throughput data. The test assumed normal distribution and performed a standard two-tailed and two-sample t-test for each gene. The output of the test was a p-value for each gene. A two-tailed p-value of 0.05 was considered statistically significant. All analyses were performed with MatLab (v2013a, Natick).

RESULTS

Negative regulation of HCV-specific T cells is still present after long-term viral clearance

Besides Treg, IL-10 and TGF-β are well-described negative regulators of virus-specific T cell proliferation and IFN-γ production in various viral infections [6, 10] as well as during therapy of chronic HCV patients [10]. We evaluated whether these mechanisms continue to regulate HCV-specific T cell responses in PBMC from 5 patients 4 years after therapy-induced HCV eradication. Patient characteristics are described in Supplementary Table 1. Although HCV RNA was undetectable in serum of all patients, HCV-specific T cell proliferation was observed after stimulating PBMC with an overlapping HCV peptide pool (Fig. 1, left column). Importantly, depletion of Treg or neutralization of TGF-β or IL-10-receptor in these cultures resulted in enhanced virus-specific T cell proliferation (Fig. 1). In contrast to T cell proliferation against HCV, no virus-specific IFN-γ production was detected in culture supernatant of PBMC stimulated with peptides at 4 years follow-up (Fig. 1, right column). However, when blocking the IL-10-receptor or TGF-β, IFN-γ levels were enhanced in 4 out of 5 patients. Altogether, we show here that HCV-specific memory T cells persist in peripheral blood 4 years after therapy-induced viral eradication, are functionally responsive to HCV antigens, and continue to be regulated by various inhibitory mechanisms. To determine the status of the T cell compartment of patients 4 years after viral clearance at the transcript level, we evaluated the expression of 244 probe sets (corresponding to 121 genes) that are important for T cell function and immunoregulation. The list of genes is presented in Supplementary Table 2. 21 genes were still modulated 4 years after therapy-induced viral clearance in blood (fold change =1.5, and p-value <0.05) (Supplementary Table 3). The genes with the highest fold increase encoded for *TGFBR1* and *TGFB1*, which may indicate a stronger involvement of TGF-β-mediated regulation 4 years after viral clearance as compared to the baseline situation.

Retention of Treg in the liver 4 years after therapy-induced viral clearance of HCV

By repeated sampling of the liver, we previously demonstrated retention of intrahepatic Treg in patients 4 weeks after end of treatment [5]. To evaluate whether intrahepatic Treg
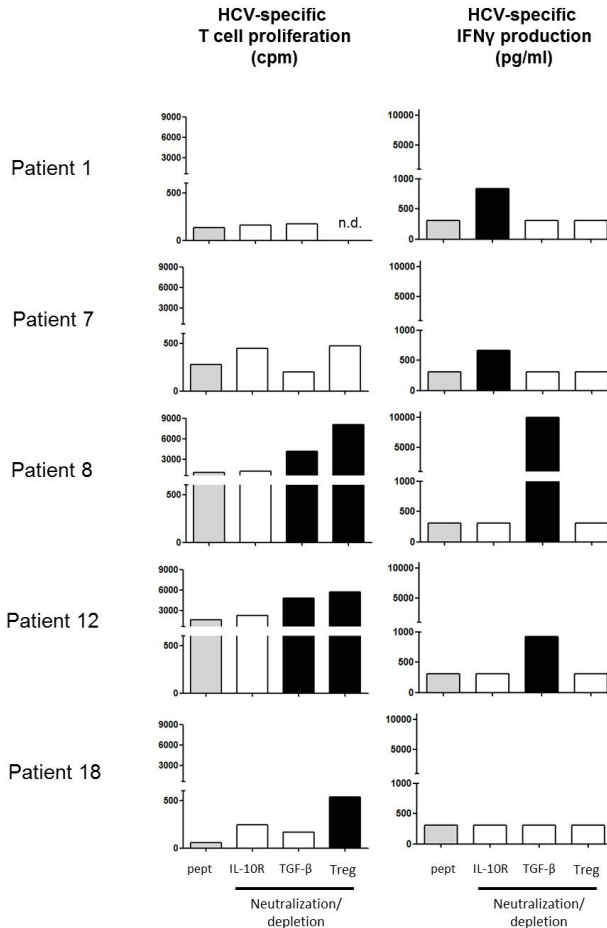
Figure 1. Negative regulatory mechanisms control HCV-specific T cell responses in the absence of viral antigen. Individual data of 5 patients 4 years after viral clearance are shown for HCV-specific T cell proliferation and IFN-γ production. Grey bars illustrate baseline response, white bars indicate no regulation, and black bars indicate regulation of HCV-specific T cell responses via TGF-β, IL-10 and/or Treg. Similar as in our previous studies [4], results on HCV-specific T cell proliferation and IFN-γ assays were considered positive when more than 500 counts or 100 pg/ml were detected above background.

continue to be present in the liver long-term after viral clearance, the livers of 4 patients from our previous cohort were sampled 4 years after sustained viral response. As shown in figure 2A, Treg were defined as CD45+CD3+CD4+ lymphocytes with high expression of CD25 and FoxP3. Four years after sustained viral response, we observed Treg in the liver of 4 out of 4 patients with frequencies varying from 3.0% - 10.7% of total CD4+ T cells. Comparison of these Treg frequencies with those detected at 4 weeks and 24 weeks follow-up showed that their numbers were retained at relatively high levels. Phenotypic characterization demonstrated that on average 23% of Treg displayed an effector memory phenotype (CD45RO+CD62L- cells), which was in line with their phenotype 4 weeks after the
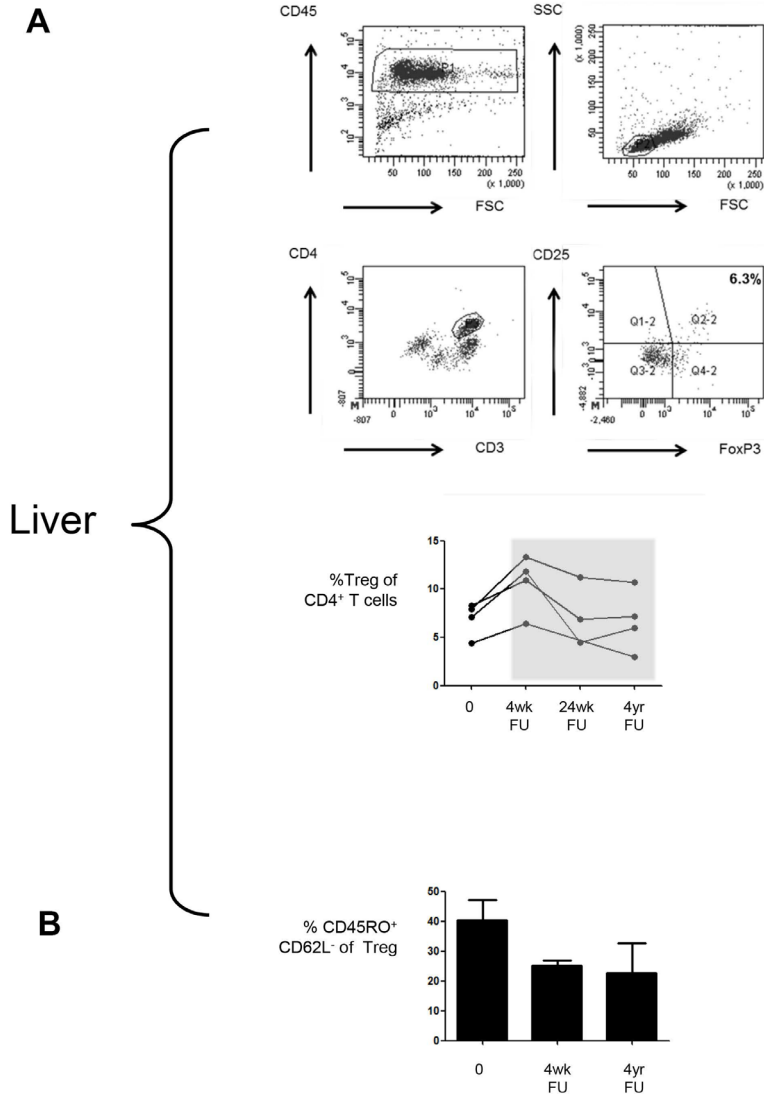
Figure 2. Treg remain present in the liver 4 years after therapy-induced viral clearance. (A) Representative dot plots and frequencies of aspirate biopsies of the liver showing intrahepatic CD45$^+$CD3$^+$CD4$^+$FoxP3$^+$CD25$^+$ Treg of a HCV patient 4 years after viral clearance. The graph shows Treg frequencies in the liver of 4 patients at baseline before therapy, and after successful treatment when patients are followed up for 4 weeks, 24 weeks and 4 years. (B) The proportion of Treg in the liver expressing CD45RO and lacking CD62L are presented of 4 patients. The graph shows the average values at baseline, 4 weeks follow-up and 4 years follow-up.

end of treatment (Fig. 2B). Frequencies and phenotype of Treg in peripheral blood were comparable to healthy individuals or at various time-points during therapy as presented in our previous study [4]. Overall, these results indicate that even 4 years after HCV eradication intrahepatic Treg with a memory phenotype remain present in the liver despite the absence of detectable antigen for an extended period of time.

DISCUSSION

This study shows that years after therapy-induced HCV clearance, Treg remain present in the liver despite the absence of detectable serum HCV RNA. Besides regulation via Treg in the liver, we also observed in peripheral blood that after virus eradication HCV-specific T cell responses remain suppressed by Treg, TGF-β and IL-10. In addition, a role for these mechanisms was supported by their gene expression profile, which demonstrated abundant transcripts of the TGF-β pathway after therapy-induced HCV clearance.

During the chronic phase of HCV infection, Treg are present at relatively high numbers in the liver, which is in contrast to healthy livers that harbor virtually no Treg, i.e. only 2% in our previous study [6]. It is generally accepted that during the chronic phase, these intrahepatic Treg control HCV-specific T cell responses and likely prevent immunopathology [6]. Interestingly, our data demonstrate that Treg are still present in the liver 4 years after successful IFN-based therapy. An interesting explanation for the presence of Treg in the liver after viral clearance could be that these regulatory mechanisms are maintained to limit fibrogenesis, since Treg and IL-10 have been shown to limit pathology in HCV infected patients [11]. Interestingly, at the transcript level we demonstrated that, among other regulatory genes, genes involved in TGF-β regulation (*TGFBR1* and *TGFB1*) are up-regulated 4 years after viral clearance. A role for TGF-β to promote as well as inhibit fibrogenesis via hepatic stellate cells has been extensively described [12]. A better understanding on the role for Treg, TGF-β and IL-10 in the immunopathogenesis of fibrosis can lead to development of therapeutic targets to delay this process.

Phenotypic characterization of intrahepatic Treg 4 years after cessation of therapy showed predominantly a CD45RO$^+$CD62L^{high} central memory phenotype, indicating a less active state as during the chronic phase of HCV infection. This phenotype is in line with memory Treg observed in mouse and human skin [7, 13]. Due to low numbers of cells that can be isolated from a fine needle aspiration biopsy we were unable to further investigate the putative memory function of Treg in our patients, and to demonstrate whether the proliferative capacity and cytokine profile resembles that of mouse memory Treg [13].

Another explanation for the retention of Treg long after viral eradication, is the detection of low amounts of HCV RNA in the liver and PBMC of patients after viral clearance. Interestingly,

these low amounts of HCV RNA are able to stimulate cellular immune responses [14]. It is tempting to speculate that the active regulatory mechanisms observed in peripheral blood as well as the intrahepatic Treg long-term after viral clearance are present to control or attenuate sterilizing HCV-specific T cell responses targeted against residual HCV RNA. This way, local and low-level viral persistence can be established that may benefit the host by maintaining a functional HCV-specific memory T cell pool. On the other side, these regulatory mechanisms may, on the contrary, dampen HCV-specific recall responses and might therefore prevent protection against reinfection. One study showed the presence of HCV-specific T cells after successful IFN-free therapy in a chimpanzee with chronic HCV. These CD8+ T cells had a narrow antigenic specificity, were weak, and unable to prevent persistence of a secondary infection [15].

Vaccination after cure to broaden otherwise narrowly focused CD8+ T cell memory may provide protection from reinfection, especially in patients who are successfully treated but remain at risk for exposure to the virus, for example men-who-have-sex-with-men or intravenous drug users. The presence of negative regulatory mechanisms in peripheral blood and liver after viral clearance needs to be taken into account when designing such a protective vaccine.

ACKNOWLEDGEMENTS

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SUPPLEMENTARY MATERIAL

Supplementary Table 1. Characteristics of patients 4 years after therapy-induced HCV clearance

	Patient 1	Patient 7	Patient 8	Patient 12	Patient 18
Age (years)	63	58	40	54	63
Gender (M/F)	F	M	M	M	F
ALT (U/l)	42	12	23	33	31
HCV RNA (U/ml)	<15	<15	<15	<15	<15
Fibrosis (Metavir)	1	0	1	1	1

Supplementary Table 2. Full list of genes important for T cell function and immunoregulation and used for microarray analysis

CAMP	cathelicidin antimicrobial peptide
CBFB	core-binding factor, beta subunit
CCL1	chemokine (C-C motif) ligand 1
CCL17	chemokine (C-C motif) ligand 17
CCL19	chemokine (C-C motif) ligand 19
CCL20	chemokine (C-C motif) ligand 20
CCL22	chemokine (C-C motif) ligand 22
CCL4	chemokine (C-C motif) ligand 4
CCR2	chemokine (C-C motif) receptor 2
CCR4	chemokine (C-C motif) receptor 4
CCR5	chemokine (C-C motif) receptor 5 (gene/pseudogene)
CCR6	chemokine (C-C motif) receptor 6
CCR7	chemokine (C-C motif) receptor 7
CCR8	chemokine (C-C motif) receptor 8
CD101	CD101 molecule
CD109	CD109 molecule
CD27	CD27 molecule
CD274	CD274 molecule
CD28	CD28 molecule
CD34	CD34 molecule
CD38	CD38 molecule
CD3E	CD3e molecule, epsilon (CD3-TCR complex)
CD4	CD4 molecule
CD40	CD40 molecule, TNF receptor superfamily member 5
CD40LG	CD40 ligand
CD44	CD44 molecule (Indian blood group)
CD5	CD5 molecule
CD69	CD69 molecule
CD70	CD70 molecule
CD72	CD72 molecule
CD80	CD80 molecule
CD83	CD83 molecule
CD86	CD86 molecule
CD8A	CD8a molecule
CREB1	cAMP responsive element binding protein 1
CRTC2	CREB regulated transcription coactivator 2
CRTC3	CREB regulated transcription coactivator 3
CTLA4	cytotoxic T-lymphocyte-associated protein 4
CXCL10	chemokine (C-X-C motif) ligand 10
CXCL12	chemokine (C-X-C motif) ligand 12
CXCL9	chemokine (C-X-C motif) ligand 9
CXCR3	chemokine (C-X-C motif) receptor 3
CXCR4	chemokine (C-X-C motif) receptor 4
EBI3	Epstein-Barr virus induced 3
ENTPD1	ectonucleoside triphosphate diphosphohydrolase 1

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FAS	Fas cell surface death receptor
FOXP3	forkhead box P3
GZMB	granzyme B
HLA-DRA	major histocompatibility complex, class II, DR alpha
ICAM1	intercellular adhesion molecule 1
ICOS	inducible T-cell co-stimulator
IFNG	interferon, gamma
IFNGR1	interferon gamma receptor 1
IFNGR2	interferon gamma receptor 2
IKZF2	IKAROS family zinc finger 2 (Helios)
IL10	interleukin 10
IL10RA	interleukin 10 receptor, alpha
IL10RB	interleukin 10 receptor, beta
IL12A	interleukin 12A (p35)
IL12B	interleukin 12B (p40)
IL1A	interleukin 1, alpha
IL1B	interleukin 1, beta
IL1R1	interleukin 1 receptor, type I
IL1R2	interleukin 1 receptor, type II
IL2	interleukin 2
IL27	interleukin 27
IL2RA	interleukin 2 receptor, alpha
IL2RB	interleukin 2 receptor, beta
IL2RG	interleukin 2 receptor, gamma
IL4	interleukin 4
IL4R	interleukin 4 receptor
IL7R	interleukin 7 receptor
ITGA7	integrin, alpha 7
ITGAE	integrin, alpha E (antigen CD103)
ITGAL	integrin, alpha L (antigen CD11A (p180), LFA-1)
ITGAV	integrin, alpha V
ITGB2	integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)
JAK1	Janus kinase 1
JAK3	Janus kinase 3
LAG3	lymphocyte-activation gene 3
LGALS1	lectin, galactoside-binding, soluble, 1
LRRC32	leucine rich repeat containing 32
MADCAM1	mucosal vascular addressin cell adhesion molecule 1
NRP1	neuropilin 1
NT5E	5'-nucleotidase, ecto (CD73)
PDCD1	programmed cell death 1
PDCD1LG2	programmed cell death 1 ligand 2
PDCD1LG2	programmed cell death 1 ligand 2
PDCD6	programmed cell death 6
PTPRC	protein tyrosine phosphatase, receptor type, C
RARA	retinoic acid receptor, alpha
REL	v-rel reticuloendotheliosis viral oncogene homolog (avian)
RNF128	ring finger protein 128, E3 ubiquitin protein ligase
RORC	RAR-related orphan receptor C
RXRA	retinoid X receptor, alpha
SELE	selectin E
SELL	selectin L
SELP	selectin P (granule membrane protein 140kDa, antigen CD62)
SLAMF1	signaling lymphocytic activation molecule family member 1
SMAD3	SMAD family member 3
STAT5A	signal transducer and activator of transcription 5A
STAT5B	signal transducer and activator of transcription 5B
T	T, brachyury homolog (mouse)
TGFB1	transforming growth factor, beta 1
TGFBR1	transforming growth factor, beta receptor 1
TGFBR2	transforming growth factor, beta receptor II (70/80kDa)
TIGIT	T cell immunoreceptor with Ig and ITIM domains
TLR4	toll-like receptor 4
TLR7	toll-like receptor 7

TNF	tumor necrosis factor
TNFRSF11A	tumor necrosis factor receptor superfamily, member 11a, NFKB activator
TNFRSF18	tumor necrosis factor receptor superfamily, member 18
TNFRSF1B	tumor necrosis factor receptor superfamily, member 1B
TNFRSF4	tumor necrosis factor receptor superfamily, member 4
TNFRSF8	tumor necrosis factor receptor superfamily, member 8
TNFRSF9	tumor necrosis factor receptor superfamily, member 9
TNFSF10	tumor necrosis factor (ligand) superfamily, member 10
TNFSF11	tumor necrosis factor (ligand) superfamily, member 11
TNFSF18	tumor necrosis factor (ligand) superfamily, member 18
TNFSF4	tumor necrosis factor (ligand) superfamily, member 4
TNFSF8	tumor necrosis factor (ligand) superfamily, member 8
TSLP	thymic stromal lymphopoietin

Supplementary Table 3. TGF-β related genes are upregulated 4 years after viral clearance

	Fold change	p-values
TGFBR1	2.1235	0.0028
TGFB1	1.9827	0.0010
STAT5B	1.6793	0.0077
ENTPD1	1.6175	0.0309
CAMP	1.5858	0.0235
PDCD6	1.5515	0.0035
RARA	1.5346	0.0159
ICOS	0.6731	0.0128
CCR6	0.6132	0.0340
GZMB	0.5957	0.0408
TLR4	0.5869	0.0065
CCR2	0.5609	0.0025
IFNGR1	0.5460	0.0048
PTPRC	0.5399	0.0000
TNFSF8	0.5367	0.0004
FAS	0.4992	0.0020
IL7R	0.4948	0.0002
CD69	0.4662	0.0006
CREB1	0.4237	0.0010
JAK1	0.3219	0.0006
CXCR4	0.3004	0.0001

Expression of genes involved in T cell function and regulation at 4 years follow-up. The fold-change represents the increase of 7 genes at 4 years follow-up compared to baseline, and the reduction of the expression of 14 genes at 4 years follow-up compared to baseline.

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Chapter 10

SUMMARY AND GENERAL DISCUSSION

Chronic hepatitis C infection is a global burden that affects around 170 million people worldwide [1-3]. In recent decades, a better understanding on the pathogenesis of the disease has led to our current knowledge on how to prevent infection. Screening blood products for hepatitis C and distribution of free needles to intravenous drug abusers have led to a substantial decrease in incidence rates [4]. Since the discovery of the virus in 1989 treatment for chronic HCV has improved tremendously, first by adding ribavirin to interferon-α therapy and in the recent years by the discovery of very potent DAA's that target HCV proteases and polymerases needed for viral replication [4-6]. The immune response is of pivotal importance for control of diverse viral infections, also during HCV infection. The overall aim of this thesis was to provide insight in the modulating role of HCV on immune cells in peripheral blood and in the liver. DAA-based therapy can cure most of infected patients and is well tolerated. Since no protected immunity is created after an infection with HCV and re-infection often occurs in specific patients groups, the design of a protective vaccine is of upmost importance to diminish the global burden of HCV. By studying the effect of HCV (**Part I**) but also the effect of therapy (**Part II**) on immune cells is of importance because re-infection often occurs in patients who were successfully treated before. In the last part of the thesis (**Part III**) we studied durability of HCV treatment and the presence and regulation of HCV-specific immune responses 4 years after clearance.

In this chapter we report the main findings of our studies and subsequently we discuss the results and propose suggestions for future research. We end the chapter with concluding remarks.

MAIN FINDINGS

Part I - HCV infection modulates T-cell responses in liver and peripheral blood

For effective anti-viral immunity, strong virus-specific T cells are required, which are often weak or absent in chronic HBV or HCV infection [7-9]. Various mechanism have been described that can explain the weak CD8$^+$ T cell response in chronic HBV and HCV infection [10, 11]. In part I of the thesis we study the effects of chronic HCV infection on T-cell responses in peripheral blood and in the liver. Two mechanisms that are suggested to contribute to T-cell failure are lack of CD4$^+$ T cell help, which is investigated in **chapter 2** and T cell exhaustion, which is reviewed in **chapter 3** of the thesis [11-15].

In **chapter 2,** we performed a detailed analysis on the interaction between T$_{FH}$ and B-cells in chronic HCV. CXCR5$^+$CD4$^+$ T-cells are the peripheral counterparts of T$_{FH}$ cells that produce IL-21 and are important for B cell maturation and activation [16, 17]. Since IL-21 was found to be important for the elimination of viral infections in mice, we investigated the role of IL-21 and CXCR5$^+$CD4$^+$ T-cells in HCV [18, 19]. Serum levels of IL-21 and frequency of IL-21-producing

CXCR5^{+}CD4^{+} T-cells were found to be decreased in chronic HCV patients compared to healthy controls [20]. Interestingly, we could also identify T[FH] cells in the liver, in infiltr ates and follicular structures in the vicinity of B cells [20]. Distinct B and T-cell areas were found in liver follicles with clear fields of IgG[+], IgD[+] and IgM[+] B-cells, suggesting an efficient area for B-T cell interaction. Since follicles were found to be more present in patients with cirrhosis, it is interesting to speculate that influx of infiltrating leukocytes and development of fibrosis is correlated to follicle formation and B cell help. This in in line with the increased activation of B cells in patients with HCV in peripheral blood, observed by us and other groups [21, 22]. However, the exact function of B cells in hepatitis C infection is not clear. Interestingly, we clearly observe a lower level of IL-21-producing CXCR5^{+}CD4^{+} T-cells in HCV patients, suggesting that IL-21 is not causing the maturation of B cells. Possibly direct T[FH]-B cell interaction is more important for the B cell activation in liver follicles. Future studies will have to investigate if T[FH]-B cell interaction in liver follicles causes the increased B cell activation found in peripheral blood in HCV patients. Importantly, despite lower frequency of IL-21-producing CXCR5^{+}CD4^{+} T-cells in HCV patients, we show that CXCR5^{+}CD4^{+} T-cells from chronic HCV patients are able to stimulate B-cells into IgG and IgM-producing plasmablasts. Since IL-21 also plays an important role in CD8^{+} T-cell survival and NK-cell activity in chronic viral infections, future studies will have to address the impact of lower IL-21-producing CXCR5^{+}CD4^{+} T-cells on the induction of effective CD8^{+} T-cell and NK-cell responses. The appearance of neutralising antibodies in peripheral blood upon HCV infection is correlated to viral control [23]. In our study we were unable to investigate if the plasmablasts produced by B cells upon stimulation with CXCR5^{+}CD4^{+} T-cells *in vitro* were able to neutralise HCV infection. Future studies will have to investigate if lower frequency of IL-21-producing T[FH] cells in chronic observed in HCV patients has an effect on the production of neutralising antibody production and possible elimination of HCV infection.

In **chapter 3** we describe that T cell exhaustion as a mechanism of T-cell failure during chronic HBV and HCV infection [24-26]. T cell exhaustion is characterized by impaired proliferation, cytokine production and the expression of inhibitory receptors like PD-1, TIM-3 and CTLA-4 on the surface of T cells [13, 15, 27]. Studies have shown that T cell exhaustion is a gradual process, which starts with the loss of T cells to produce IFN-y and continues with the loss of producing IL-2, TNF and ability to proliferate. Eventually T cells induce an apoptosis signal and disappear from the periphery [13]. Increased expression of inhibitory receptors is correlated to a decrease in function [28]. In our review we discuss that blocking inhibitory receptors has been shown to reinvigorate T cells which is a potential strategy in chronic HBV and HCV infection to facilitate complete elimination [15, 29, 30]. Four important considerations have to be taken into account. First, Inhibitory receptors like PD-1 are widely spread among various immune cells to dampen virus-specific immune responses. By blocking the receptor there is a risk of inducing immunopathology. Second, blocking only one receptor has not always been effective. Pathways underlying inhibitory receptors have not been well understood and various stages of T cell exhaustion or co-expression pattern variations might lead to inefficient

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stimulation [31]. Third, besides T cell exhaustion, inhibitory mechanisms like Treg and cytokines IL-10 and TGF-b can inhibit T-cell responses and need to be taken into account and possibly combined with inhibitory receptor blocking to create a proper immune response [32]. Fourth, recent studies have shown a clear hierarchy of inhibitory receptor expression in chronic HCV and HBV and dominant inhibitory receptor expression varies widely between patients. Individualized therapy has to be taken into account when choosing proper inhibitory receptor blocking. Up till now one randomized controlled trial has shown decrease in viral load upon treatment for chronic HCV with anti-PD-1. The manipulation of inhibitory receptors is a promising strategy for immunotherapeutic interventions in chronic viral infections. Blocking antibodies targeting regulatory molecules on T cells such as CTLA-4 and PD-1 have renewed the field of cancer immunotherapy. These agents have demonstrated clinical activity across a variety of tumor types. Now that safety and clinical activity has been demonstrated in the monotherapy setting, the field is moving in the direction of testing novel combinations [33]. In the field for chronic HCV therapy, inhibitory receptor blockade will be no longer be part of therapy since treatment with DAA's can cure about 95% of patients [4, 5, 34]. However in chronic HBV infection, there is still need for optimizing treatment regimes. In contrast to hepatitis C infection, patients with hepatitis B do not completely clear the infection, since covalently closed circular DNA (cccDNA) persist in the form of a minichromosome in the host. Consequently, even in spontaneously recovered patients a re-activation of the virus can occur when using immunosuppression e.g. during chemotherapy of organ transplants [35]. Limited amounts of HBV DNA can circulate in the body and stimulate HBV-specific T-cell responses which in turn control viremia [36-38]. Ultimate anti-viral therapy for HBV would both stimulate adaptive immunity and eliminate cccDNA. Current therapy for chronic HBV consist long-term use of nucleotide analogues (NA) like entecavir, tenofovir and telbivudine which inhibit viral DNA polymerases and suppress viral replication. Nucleotide analogues are well tolerated and improve liver histology [39-41]. However, long-term use of lamivudine and tenofovir is associated with moderate adherence, appearance of resistant mutation and has a huge impact on medical costs [42]. Moreover, NA does not seem to enhance immune control since only small a proportion of patients maintain HBeAg loss after discontinuing treatment [43, 44]. PegIFN-α therapy for HBV can cause HBeAg loss, HBsAg loss and seroconversion to anti-HBeAg and anti-HBsAg. However, after 1 year of therapy, HBsAg loss is achieved in only 30% of patients and similar to treatment for HCV, this is accompanied with major side effects [45, 46]. NA analogues are known to restore HBV-specific T cell proliferation and therefore enhance adaptive immunity [47]. Interestingly, PegIFN-α therapy for chronic HBV does not restore HBV-specific T cell frequency [48, 49]. In addition, PegIFN-α therapy does not restore IFN-γ production or cause downregulation of exhaustion markers on HBV-specific T cells [49]. Recent research has shown a possible advantage of combining both PegIFN-α and NA therapy causing an inhibition of replication of cccDNA but also boost the adaptive immune system [47, 50-52]. However seroconversion rates are still not optimal. Adding blocking antibodies like anti-PD-1 and anti-CTLA-4 might enhance adaptive immunity. Future studies

will have to define if immune modulatory therapy can also be used to facilitate complete elimination of viruses in NUC treated patients.

Part II - The effect of HCV treatment on peripheral and intrahepatic immune responses

For proper anti-viral immunity, strong and efficient immune responses are needed [53]. However, we and others have shown that HCV infection modulates peripheral and intrahepatic immune responses allowing to evade anti-viral immunity [7]. Treatment for HCV with PegIFN-α/ribavirin cures around 50% of the patients [54]. Advances in treatment strategies with the discovery and implementation of DAA's we can now obtain around 95% response rates even in the difficult to treat patient groups [34, 55]. In part II of the thesis we investigated the effect of HCV therapy on immune responses and showed the effect of treatment in the liver environment.

Anemia is an important side effect of therapy with PegIFN-α/ ribavirin for the treatment of chronic HCV [56]. EPO can be administered to reduce side effects during treatment [57, 58]. In **chapter 4** we investigated the effect of EPO on human leukocytes *in vitro* and during HCV therapy. Although we did not observe an effect of EPO on T and NK cell phenotype or function, we did observe a clear effect of EPO on the cytokine production by monocytes, *in vitro* and *in vivo* during HCV therapy [59]. Various studies have shown that although EPO administration reduces anemia, ribavirin dose reductions were equally effective and did not decrease response rates for therapy for HCV [60, 61]. Therefore EPO was no longer used in the treatment for HCV. It is interesting to speculate that the inhibiting effect of EPO on monocytes potentially causes increased bacterial infections which might eventually lead to less coherence and early treatment discontinuations. Whether the effect of EPO on monocytes indeed enhances the susceptibility for bacterial infections has to be studied in more detail. Since current treatment regimens with PegIFN-α for chronic HCV will still be important in developing countries and other type I IFNs are still used in the treatment for multiple sclerosis (MS) [62] and other viruses [63], this issue is still relevant.

T cells and NK cells are crucial in the control and elimination of virally infected cells [7, 64-66]. NK but not T cells in peripheral blood have been correlated to treatment response with IFN-α [49, 67-71]. However, few studies have investigated these cells in the liver. With the technique of fine needle aspiration (FNA) biopsies, we were able to longitudinally study immune cells in the liver during therapy for HCV or HBV [72-74]. However, due to the low amount of virus-specific T cells in liver and the on average 13,000 intrahepatic leukocytes that were acquired per pooled aspirate biopsy, no analyses on virus specific T cell could be obtained. In **chapter 5** we studied NK cells in the liver and in blood of chronic HCV patients during IFN-based therapy. We show that in peripheral blood, increased frequencies of activating NKp46+ NK cells were observed at week 1, and of the inhibitory NKG2A+ NK cells at week 12 after start of therapy. However, frequencies of intrahepatic NK cells as well as expression of NKp46, NKG2A and NKG2D on NK cells was not altered at 6 hours, 1 week or 12 weeks during IFN-

based therapy compared to baseline. As shown by us and others, liver NK cells are highly activated as shown by the expression of CD69. This highly activated state could potentially make them less susceptible to IFN-α. Interestingly, it had been shown before by Cosgrove et al that HCV infection affects NK cell in blood more than in the liver [75]. From our study we can conclude that also IFN-α therapy for HCV affects NK cell phenotype in blood more than in the liver.

Recent works has shown that NK cells are especially relevant in the acute phase of viral infection, where NK cells can acts as regulators of the anti-viral T cell response. NK cell mediated lysis of CD4+ T cells dampened virus specific T cell response, possibly by the upregulation of TNF-related – apoptosis induced ligand (TRAIL) on NK cells [65, 76-79]. In line it has been shown in acute HCV infection that a reduced expression of activating receptors NKp30, NKp46 and NKG2D on NK cells predicts viral clearance [80]. Future studies will have to address if intrahepatic NK cells during acute HCV and HBV infection are predictive of viral clearance and can possibly be modulated to improve outcome.

As discussed before during chronic HCV infection, HCV-specific T-cell responses are weak because of continuous high antigen pressure. Besides T-cell responses, NK cells are also important in the anti-viral immunity to HCV. Although it is still unclear how NK cells are modulated during HCV infection, their role during IFN-based therapy as predictors of therapy outcome is well established [68, 81]. The direct effect of viral load decline on NK cells and T cells has not been investigated before since viral load decline was mediated through therapy with PegIFN-α which directly causes NK cell activation and inhibition of CD4+ and CD8+ T cell proliferation. In patients with chronic HBV infection, treatment with nucleotide analogues NK cells became more activated and function improved [82]. However, these finding are controversial since With the introduction of DAA-based therapy we were able to evaluate the effect of viral load decline on immune responses in chronic HCV patients. In **chapter 6**, we conducted an investigator-initiated study where 12 patients were treated with daclatasvir and asunaprevir for 24 weeks. We showed that reduced HCV RNA load causes a lower activation state of blood NK cells as shown by the expression of TRAIL, NKp30 and NKp46 on NK cells and serum IL-12 and IL-18 levels. Furthermore, we confirmed recent studies showing that successful DAA therapy led to reduction of blood ISG mRNA [83] and restoration of the HCV-specific CD8+ T cell compartment [84]. In chronic HCV, as well as in chronic HBV, ISG mRNAs are expressed at high levels in blood and liver. If DAA therapy can lower the endogenous IFN system to a less activated state this could be interesting for the development of treatment strategies of HBV. Clinical trials are now investigating if therapy based on nucleotide analogues prior to PegIFN-α therapy results in better response rates and the chance of obtaining HBsAg loss [85]. This could be supported by our findings and be explored in the future. Besides their direct cytotoxic capabilities, NK cells have an important immunoregulatory role by targeting T cells [77]. We show that during IFN-free therapy, HCV-specific T cells are restored, while the frequency of TRAIL-expressing NK cells decreases.

Although it is unclear whether the effects of DAA on T cells and NK cells are related, it has been convincingly shown in chronic HBV infection that low T cell numbers are -at least in part- caused by depletion by NK cells [79]. Interestingly, the key molecule involved in NK cell mediated killing of T cells was found to be TRAIL. Future studies will have to clarify if the DAA induced restoration of T cells in chronic HCV is due to a decrease in TRAIL mediated killing by NK cells. These results could be important for the development of new targets for treatment for HBV and the development of a vaccine for HCV.

Besides NK cells, MAIT cells are also potentially important in chronic viral infections. MAIT cells are a subtype of T cells that express CD8, and are characterized by the expression of CD161 and the invariant T cell receptor (TCR) Vα7.2 that recognize bacterial ligands in an MR1 (MHC class I related protein) dependent manner [86, 87]. Interestingly, the number of MAIT cells has found to be decreased in patients with bacterial infections [88] and correlated with the susceptibility of hospital admissions due to infections [89]. But apparently, MAIT cells are also involved in inflammatory disorders like auto-immune hepatitis, primary biliary cirrhosis and steatohepatitis, where MAIT cells were found to be enriched in the livers of these patients [90, 91]. Also patients with inflammatory bowel disease have reduced frequencies of MAIT cells in peripheral blood and increased frequency of MAIT cells in the inflamed bowel [92]. In chronic HIV patients we know that MAIT cells are reduced in peripheral blood and lymph nodes and unable to recover after successful antiretroviral therapy (cART) therapy [93-96]. However, the role for MAIT cells in HCV and HCV/HIV co-infection is largely unknown. In **chapter 7** we studied MAIT cells in patients with chronic HCV (CHCV) infection, patients with virologically suppressed HIV that were acutely infected with HCV (AHCV/HIV), patients with HIV mono-infection and healthy subjects. We observed that the frequency of MAIT cells is decreased in all three groups compared to healthy individuals. Similar findings have been reported in HIV patients before [94-96] but to our knowledge, we are the first to show reduced frequencies of CD161$^+$Vα7.2$^+$MAIT cells in peripheral blood of CHCV patients and AHCV/HIV patients. From our studies, we cannot conclude whether the reduced frequencies of MAIT cells are due to migration of MAIT cells to peripheral tissue like the liver or due to down-regulation of e.g. CD161.

Besides the decrease of MAIT cells in HIV and CHCV we observed a trend towards an even lower frequency of CD161$^+$Vα7.2$^+$MAIT cells in AHCV/HIV co-infection. Of interest, patients with AHCV/HIV co-infection had increased expression of CD38 on MAIT cells, suggesting an activated population. Till now we cannot explain this discrepancy. A possible explanation could be that activation-induced cell dead is causing the depletion of MAIT cells from the periphery in this patient population [97].

Since patients with HCV/HIV co-infection are known to have faster development of fibrosis [98], it is interesting to speculate that a decreased frequency of MAIT cells may cause a decrease in anti-bacterial barrier and therefore a higher load of bacterial product to the liver, possibly

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causing a faster development of fibrosis. Future studies will have to address if MAIT cells are part of this process.

In these inflammatory diseases it is not obvious that bacterial derived products activate MAIT cells through their TCR. More likely, cytokines like IL-12/18, which have been shown to directly stimulate MAIT cells, activate MAIT cells in these conditions. In **chapter 7** we show that also IFN-α is a potent stimulator of MAIT cells *in vitro*. Since IFN-α is largely produced upon infection with HIV and HCV and many other viruses, MAIT cells could potentially be involved in a much broader range of diseases. Besides the effect of IFN-α *in vitro*, we investigated if IFN-based therapy for CHCV affects MAIT cells *in vivo*. Interestingly, rather than a stimulating effect of IFN-α therapy we observed a reduction in IFN-y producing MAIT cells during IFN-based therapy. We concluded that this effect was directly due to IFN since IFN-free therapy for CHCV did not cause this defect. From our studies, we cannot conclude whether the reduced frequencies of MAIT cells are due to depletion of cells via apoptosis, migration of MAIT cells from blood to peripheral organs or skin, or due to down-regulation of characteristic markers, such as CD161. Future studies will need to be conducted to better understand the causes and consequences of the reduced MAIT cell frequency in CHCV and HIV.

Part III - Long-term outcome of treatment induced viral clearance of HCV infection.

Various studies have investigated immune responses during chronic HCV infection and therapy-induced viral load decline. Much less attention is given to immune responses long-term after successful therapy. In part III we determined if therapy induced sustained viral responses are durable and aimed to determine factors that could influence this durability. In **chapter 8** HCV patients with genotype 1 were treated with narlaprevir (NS3 protease inhibitor) in combination with PegIFN-α/ribavirin and ritonavir (CYP3A4 inhibitor) for 24-48 weeks [99]. At 2 years follow- up, in patients that were successfully treated, no HCV RNA could be detected in serum of patients. Moreover, no HCV-specific T cell proliferation against HCV could be detected, which was in line with the absence of HCV RNA in peripheral blood [99]. These data suggest durability of viral response, which was confirmed by multiple clinical trials including different DAA regimens [5, 34]. Interestingly, in larger patient cohorts, some groups were able to detect minimal amounts of HCV RNA and the presence of HCV-specific T cell proliferation at long term follow-up, suggesting an ongoing T cell response [100, 101]. However, similar as during chronic HCV infection, these T-cell responses were weak and unable to proliferate or produce large amount of IFN-y upon stimulation with HCV peptides.

New DAA drugs are now used worldwide or are in phase II and III clinical trials and show improved SVR rates, safety and convenience compared to IFN-based regimens. The

potential wide-spread use of the treatment regimens will inhibit further development of fibrosis in chronic HCV patients and decrease the pool of HCV infected persons, preventing new infections. However, in this new area of DAA therapy there is still need for a vaccine. Since a large proportion of HCV patients are intravenous drug abusers and men-who-have-sex-with-men, a chance for HCV re-infection is substantial. Studies in cohorts of intravenous drug users have shown that individuals who spontaneously resolve an episode of HCV infection were more likely to clear a subsequent infection [102-104]. Also chimpanzee who have previously cleared HCV, rapidly clear a second infection [102, 104]. Since DAA-therapy does not create protective immunity, a vaccine after treatment with expensive DAAs might be an inexpensive way to prevent reinfection. Moreover, DAA therapy will not be available for developing countries in the upcoming years, while vaccination programs can be available much cheaper. A detailed understanding of immune mechanisms that contribute to protective immunity and determining factors that could influence a proper immune response during reinfection is therefore of upmost importance. In **chapter 9** we tried to discover mechanisms that could potentially inhibit HCV–specific T-cell responses long-term after therapy induced viral clearance. As discussed in part I of the thesis, the absence of proper CD4$^+$ T cell help (chapter 2) and T cell exhaustion (chapter 3) are mechanisms suggested to cause T-cell failure. For factors inhibiting a proper T cell response long-term after therapy, we were interested in a third mechanism, the inhibition by Treg and inhibitory cytokines IL-10 and TGF-β. We investigated this mechanisms since recently it was demonstrated that infusion of plasma from patients with trace amounts of HCV RNA in chimpanzee càn generate HCV-specific T-cell responses and can even cause high level viremia [105]. However, upon repeated exposure to trace amount of HCV, it was found that no effective recall responses were induced upon subsequent HCV infection as a result of more pronounced suppression by Treg [106]. It is known that Treg, IL-10 and TGF-β suppress HCV-specific T-cell responses in peripheral blood and that Treg are present at relatively high numbers in the HCV infected liver, which is in contrast to healthy livers that harbor virtually no Treg [32, 72]. It is generally accepted that during the chronic phase, these intrahepatic Treg control HCV-specific T-cell responses and likely prevent immunopathology [72]. We now show that years after therapy-induced HCV clearance, HCV-specific T-cell responses remain suppressed by Treg, TGF-β and IL-10 and Treg remain present in the liver. It is important to note that it still unclear how Treg are maintained in the liver. Possible explanation could be the minute amounts of HCV RNA that induces HCV-specific T cells and attracts Treg to dampen the immune response. Besides new migration from peripheral blood after viral clearance, there is a possibility that Treg are maintained in the liver after clearance of chronic HCV infection and have a memory like function as been previously described in human and mice skin [107-109]. This coincides with the memory phenotype of the Tregs found in our study. Following question remain to be answered: Do Treg in the liver have a memory function? How are liver Treg maintained at the site or do they migrate from peripheral blood? Can liver Treg be safely inhibited to reinforce local HCV-specific T cells and create proper anti-viral immunity?

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Since it was previously shown that no effective recall responses could be induced because of the presence of Treg, this should be considered in the design of vaccination strategies, especially in patients previously treated for HCV infection. Understanding the pathogenesis of weak virus-specific immune responses will help us to protect the patients who are at high risk for reinfection.

IN CONCLUSION

With the discovery of HCV in 1989, hundreds of thousands of patients with CHCV have been treated with PegIFN-α. Over the last 10 years many new drugs have been tested leading to the availability of all-oral IFN-free combination therapies. It is important to realize that cure is not the most important challenge facing HCV infection. Instead, discovering patients who are unaware of their HCV infection, making a cure for HCV available in developing countries and the development of a vaccine are three important issues that need to be addressed in the future. In this thesis we gained knowledge on adaptive and innate immunity throughout and long-term after therapy of chronic HCV infection. Understanding the pathogenesis of factors that influence a proper immune response aid the development of a vaccine to create protective immunity. Moreover, the knowledge obtained from chronic HCV is highly relevant in the battle against other chronic viral infections like HBV and HIV. Keeping in mind at what speed research has unraveled the secrets of HCV infection in the last decades, global eradication of HCV will be possible.

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Chapter 11

NEDERLANDSE SAMENVATTING

Het hepatitis C (HCV) virus is een klein RNA virus en was tot zijn ontdekking in 1989 beter bekend was als het non-A non-B virus. Ongeveer 170 miljoen mensen zijn wereldwijd besmet met dit virus. In de afgelopen decennia, heeft een beter inzicht in de pathogenese van de ziekte ertoe geleid dat we nu beter weten hoe we de ziekte moeten voorkomen. Zo worden bloedproducten nu gescreend op de aanwezigheid van het virus en worden gratis schone naalden verstrekt aan intraveneus drugs gebruikers, wat heeft geleid tot en aanzienlijke daling in de incidentie [1]. Sinds de ontdekking van het virus in 1989 is de behandeling van chronische HCV enorm verbeterd, eerst door toevoeging van ribavirine aan interferon-α therapie en in de afgelopen jaren door de ontdekking van zeer krachtige middelen die direct het virus aanvallen (de direct acting antivirals of DAA). Deze DAA's remmen HCV proteasen en polymerasen die nodig zijn voor virale replicatie [1-3].

In dit proefschrift hebben we als doel gehad een beter inzicht te geven in de modulerende rol van HCV op immuun cellen in het perifere bloed en in de lever. Vele patiënten kunnen door effectieve behandel regimes genezen worden van het HCV virus. Het belangrijkste doel van toekomstig onderzoek is het zoeken naar een effectieve vaccinatie. In het eerste deel van de thesis hebben we gekeken naar T cel responsen en mechanismen die kunnen verklaren waarom de T cel responsen in chronische HCV patiënten zwak of zelfs afwezig zijn. Omdat veel patiënten die hoog risico lopen op een her infectie, als eens behandeld zijn, hebben we in het tweede deel van de thesis het effect van PegIFN-α therapie en virale load daling op immunologische mechanismen bestudeerd. In het laatste deel van de thesis onderzochten we duurzaamheid van de behandeling van HCV en de aanwezigheid en regulering van HCV-specifieke immuun responsen lang na klaring van het virus. In dit hoofdstuk rapporteren we de belangrijkste bevindingen van onze studies, bespreken we de resultaten en maken we suggesties voor toekomstig onderzoek. We eindigen het hoofdstuk met een conclusie.

Deel I – Chronische HCV-infectie moduleert T-cellen in lever en perifere bloed

Voor een effectief antiviraal immuun systeem, zijn sterke virus-specifieke T-cellen nodig, maar deze zijn vaak zwak of afwezig bij chronische HBV of HCV-infectie [4-6]. Verschillende mechanismen zijn geopperd die de zwakke CD8+ T-cel respons in chronische HBV en HCV-infectie kunnen verklaren [7, 8]. In het eerste deel van het proefschrift onderzoeken we de effecten van chronische HCV-infectie op de T-cel response in perifeer bloed en in de lever. Twee mechanismen die worden voorgesteld om bij te dragen aan de zwakke T-cel respons zijn van een gebrek aan CD4+ T-cel hulp, dat wordt onderzocht in **hoofdstuk 2** en T-cel uitputting, dat wordt beschreven **hoofdstuk 3** van het proefschrift [8-12].

In **hoofdstuk 2** hebben we een gedetailleerde analyse van de interactie tussen T_{FH} en B-cellen in chronische HCV verricht. CXCR5+CD4+ T-cellen zijn de perifere tegenhangers van T_{FH} cellen die IL-21 produceren en zijn belangrijk voor B-cel activatie en maturatie [13, 14]. Aangezien IL-21 belangrijk is gebleken voor de eliminatie van virale infecties bij muizen, onderzochten we de rol van IL-21 en CXCR5+CD4+ T-cellen in HCV [15, 16]. De hoeveelheid IL-21 in het serum

en de frequentie van IL-21-producerende CXCR5^{+}CD4^{+} T-cellen bleken te zijn afgenomen bij chronische HCV patiënten vergeleken met gezonde controles [17]. In dit hoofdstuk hebben we T$_{FH}$ cellen geïdentificeerd in de lever, waarin ze in de nabijheid lagen van infiltraten en folliculaire structuren met onder andere B-cellen [17]. Duidelijke B- en T -cel gebieden werden gezien in de lever follikels met gebieden van IgG[+], IgD[+] en IgM[+] B-cellen, wat suggereert dat de follikel een effectief en efficiënt gebied voor B-T-cel interactie kan zijn.

Omdat follikels in toenemende mate voorkomen in levers van patiënten met cirrose, is het interessant om te speculeren dat de ontwikkeling van fibrose is gecorreleerd met follikel vorming en B-cel hulp. Dit is in lijn met de verhoogde activatie van B-cellen in patiënten met HCV in perifeer bloed, waargenomen door ons en andere groepen [18, 19]. De exacte functie van B-cellen in hepatitis C infectie is niet duidelijk. We zien duidelijk lagere IL-21-producerende CXCR5^{+} CD4^{+} T-cellen bij patiënten met HCV, wat suggereert dat IL-21 niet alleen verantwoordelijk is voor de maturatie en activatie van B-cellen. Mogelijkerwijs is direct T$_{FH}$-B-cel interactie belangrijker voor de activering van B-cellen in de lever follikels. Toekomstige studies moeten onderzoeken of T$_{FH}$-B-cel interactie in lever follikels de verhoogde activatie van B-cellen in perifeer bloed veroorzaakt in patiënten met HCV. Belangrijk is, dat ondanks de lagere frequentie van IL-21-producerende CXCR5^{+}CD4^{+} T-cellen in patiënten met HCV we laten zien dat CXCR5^{+}CD4^{+} T-cellen van chronische HCV patiënten goed in staat zijn om B-cellen te stimuleren in IgG- en IgM-producerende plasmablasten. Aangezien IL-21 een belangrijke rol speelt in de overleving van CD8^{+} T-celen en NK-cel activiteit bij chronische virale infecties zullen toekomstige studies het effect van de lagere frequentie IL-21-producerende CXCR5^{+}CD4^{+} T-cellen op de inductie van een effectieve CD8^{+} T-cel response moeten onderzoeken. Het verschijnen van neutraliserende antilichamen in perifeer bloed gedurende een HCV-infectie is gecorreleerd aan virale klaring [20]. In ons onderzoek konden we niet onderzoeken of de door B-cellen gevormde antilichamen in staat waren de HCV-infectie te neutraliseren. Toekomstige studies moeten onderzoeken of lagere frequentie van IL-21-producerende T$_{FH}$ cellen in chronische waargenomen bij HCV patiënten een effect hebben op de productie van neutraliserende antilichaamproductie en mogelijke klaring van HCV-infectie.

In **hoofdstuk 3** beschrijven we dat T-cel uitputting een mechanisme is dat het falen van T-cellen tijdens chronische HBV en HCV-infectie kan verklaren [21-23]. T-cel uitputting wordt gekenmerkt door een verminderde proliferatie, cytokine productie en de expressie van remmende receptoren zoals PD-1, TIM-3 en CTLA-4 op het oppervlak van T-cellen [10, 12, 24]. Studies hebben aangetoond dat T-cel uitputting een geleidelijk proces is dat begint met het verlies van IFN-y productie en daarna met het verlies van de productie van IL-2, TNF en de mogelijkheid tot proliferatie. Uiteindelijk induceren T-cellen een apoptose signaal en verdwijnen van de circulatie [10]. De verhoogde expressie van de remmende receptoren op het oppervlak van T-cellen correleert met een afname van functie [25]. In onze review bespreken we dat dat het blokkeren van de remmende receptoren, T-cellen een nieuwe impuls kunnen

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krijgen. Dit is een potentiële strategie om volledige eliminatie te bereiken in chronische HBV en HCV-infectie [12, 26, 27]. Echter vier belangrijke dingen dienen in overwegingen te worden genomen. Ten eerste komen remmende receptoren zoals PD-1 gespreid voor op verschillende immuuncellen om te sterke reacties te temperen. Door het blokkeren van de receptor is er een risico op het induceren van immunopathologie. Ten tweede is het blokkeren van slechts één receptor is niet altijd effectief gebleken. De signaal cascades die onderliggend zijn aan de remmende receptoren zijn niet goed begrepen en verschillende stadia van T-cel uitputting of co-expressiepatronen kunnen leiden tot inefficiënte stimulatie [28]. Ten derde moet er rekening worden gehouden met andere remmende factoren van de T-cel response zoals Treg en cytokinen IL-10 en TGF-β. Ten vierde hebben recente studies aangetoond dat er een duidelijk verschil is in de expressie van de verschillende remmende receptoren in chronische HCV en HBV en dat deze hiërarchie verschilt tussen patiënten. Het is mogelijk dat geïndividualiseerde therapie een rol gaat spelen bij het kiezen van de juiste remmende receptor blokkade.

Tot nu toe is er 1 gerandomiseerd studie geweest die heeft aangetoond dat behandeling van chronische HCV met anti-PD-1 zorgde voor een afname in de virale load. De manipulatie van de remmende receptoren is een veelbelovende strategie voor immunotherapeutische interventies bij chronische virale infecties. Blokkerende antilichamen gericht tegen regulerende moleculen op T-cellen, zoals CTLA-4 en PD-1 hebben het gebied van kanker immunotherapie vernieuwd. Nu dat de veiligheid en klinische werkzaamheid is aangetoond in de monotherapie wordt er nu gewerkt aan het testen nieuwe combinaties [29]. In chronische HCV-infectie, zal de receptor blokkade niet meer worden geïmplementeerd in de therapie gezien behandeling met DAA's een klaring kunnen bewerkstelligen in ongeveer 95% van de patiënten [1, 11, 30]. Echter blijft het interessant in het ontwikkelen van een vaccin. In chronische HBV-infectie is er nog steeds grote behoefte om bestaande behandelingsregimes te vernieuwen. In tegenstelling tot hepatitis C-infectie, kunnen patiënten met hepatitis B het virus niet volledig klaren, aangezien covalent gesloten circulair DNA (cccDNA) blijft bestaan in de vorm van een minichromosoom in de gastheer. Ook bij spontaan klarende HBV patiënten kan er een reactivering van het virus plaatsvinden bij het gebruik van bijvoorbeeld immunosuppressiva als chemotherapie voor orgaantransplantaties [31]. De ultieme antivirale therapie voor HBV zou zowel het adaptieve immuun response stimuleren en het cccDNA te elimineren. De huidige therapie voor chronische HBV bestaat uit nucleoside analogen (NA) zoals entecavir, tenofovir en telbivudine die virale DNA-polymerasen remmen en op lange termijn de virale replicatie onderdrukken. Nucleotide-analogen worden goed verdragen en ze lijken de de lever histologie te verbeteren [32, 33]. Echter, het gebruik van lamivudine en tenofovir gaan op lange termijn samen met een matige therapietrouw, het ontwikkelen van resistente mutaties en het heeft een enorme impact op de medische kosten [34]. Bovendien lijken NA niet de eigen immuun controle te verbeteren aangezien slechts een klein deel van de patiënten HBeAg negatief blijven na het staken van de behandeling [35, 36]. PegIFN-α therapie voor HBV kan zorgen voor HBeAg verlies, HBsAg verlies en seroconversie naar anti-

HBeAg en anti-HBsAg. Echter, na 1 jaar behandeling wordt HBsAg verlies gerealiseerd in slechts 30% van de patiënten en net als in de behandeling voor HCV, gaat dat gepaard met ernstige bijwerkingen [37, 38]. NA analogen kunnen HBV-specifieke T-celproliferatie herstellen en kunnen dus het adaptieve immuun systeem verbeteren [39].

PegIFN-α therapie voor chronische HBV kan de HBV-specifieke T-cel frequentie niet herstellen [40, 41]. Bovendien heeft PegIFN-α therapie geen effect op de IFN-γ productie of de expressie van remmende receptoren op HBV-specifieke T-cellen [41]. Recent onderzoek heeft aangetoond dat er een mogelijk voordeel is van het combineren van zowel PegIFN-α en NA therapie dat een remming van zowel de replicatie van cccDNA als ook het adaptieve immuunsysteem zouden stimuleren [39, 42]. Toekomstige studies moet bepalen of immuun modulerende therapie kan ook worden gebruikt om volledige klaring te krijgen van HBV in NUC behandelde patiënten.

Deel II - Het effect van de behandeling van HCV op perifere en intra-hepatische immuun responsen

Voor een goede antivirale immuniteit is een sterke en efficiënte immuunrespons nodig [43]. Wij en anderen hebben aangetoond dat HCV-infectie perifere en intra-hepatische immuun responsen moduleert en om daarmee antivirale immuniteit te ontwijken [4]. Behandeling van HCV-infectie met PegIFN-α/ribavirine resulteert in ongeveer 50% van de patiënten tot genezing [44]. Door de vooruitgang in de behandelingsstrategieën door de ontdekking en implementatie van DAA's kunnen we nu rond de 95% van de patiënten genezen, zelfs binnen de moeilijk behandelbare patiëntengroepen [45]. Voor het tweede deel van het proefschrift onderzochten we het effect van HCV-therapie op de immune respons.

Anemie is een belangrijke bijwerking van de behandeling met PegIFN-α/ribavirine voor de behandeling van chronische HCV [46]. EPO kan worden toegediend tijdens de behandeling om bijwerkingen te verminderen [47, 48]. In **hoofdstuk 4** is het effect van EPO op humane leukocyten onderzocht *in vitro* en tijdens de behandeling voor HCV. Hoewel we geen effect van EPO op T- en NK-cel fenotype of functie konden waarnemen, was er een duidelijk remmend effect te zien van EPO op de cytokineproductie van monocyten, *in vitro* en *in vivo* tijdens HCV-therapie [49]. Verschillende studies hebben aangetoond dat hoewel het geven van EPO anemie behandeld, een dosis reductie ribavirine even effectief was en geen effect had op het slagingspercentages [50, 51]. Derhalve werd EPO niet meer gebruikt bij de behandeling van HCV. Het is interessant om te speculeren dat het remmende effect van EPO op monocyten potentieel leidt tot een verhoogd aantal bacteriële infecties, die uiteindelijk kunnen resulteren in minder therapietrouw en het eerder stoppen van de behandeling. Of het effect van EPO op monocyten inderdaad de gevoeligheid verhoogt voor bacteriële infecties, dienst in meer detail te worden bestudeerd. Daar de behandelingsregimes met PegIFN-α voor chronische HCV nog steeds belangrijk zijn in ontwikkelingslanden en andere type I IFN's worden gebruikt bij de behandeling van multiple sclerose (MS) [52] en andere

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virussen [53], is dit effect nog steeds relevant.

T-cellen en NK-cellen zijn cruciaal voor de controle en verwijdering van viraal geïnfecteerde cellen [54, 55]. Fenotype en functie van NK-cellen in perifeer bloed zijn gecorreleerd aan de behandelingsrespons op IFN-α therapie [56-59]. Echter, slechts enkele studies hebben NK-cellen onderzocht in de lever. Met behulp van fijne naald aspiratie (FNA) biopten, konden we longitudinaal immuun cellen in de lever bestuderen tijdens de behandeling van HCV of HBV. Vanwege de geringe hoeveelheid virus-specifieke T-cellen in de lever en met een gemiddelde van 13.000 intra-hepatische leukocyten die werden verkregen per naald biopsie, konen we geen analyses doen naar virus-specifieke T-cellen. In **hoofdstuk 5** bestudeerden we NK-cellen in de lever en het bloed van chronische HCV patiënten tijdens IFN-gebaseerde therapie. We onderzochten de frequentie en fenotype van NK-cellen op 6 uur, 1 week, 2 weken en 12 weken na starten van de behandeling. De expressie van CD69, NKp46 en NK2GD die een activatie signaal doorgeven en NK2GA en NK2GC die een remmend signaal doorgeven werden bepaald op NK-cellen. We toonden aan dat in perifeer bloed er een verhoogde frequentie van NKp46+ NK-cellen aanwezig waren na week 1, en er meer NKG2A+ NK-cellen aanwezig waren op week 12 in vergelijking met voor de behandeling. Geen veranderingen in de frequenties van intra-hepatische NK-cellen of de expressie van NKp46, NKG2A en NKG2D werden geobserveerd tijdens IFN-gebaseerde therapie. Zoals eerder beschreven door ons en anderen, waren lever NK-cellen sterk geactiveerd, zoals beek uit de hoge expressie van CD69 in vergelijking met bloed NK-cellen. De zeer geactiveerde toestand van intra-hepatische NK-cellen zou ze minder gevoelig kunnen maken voor IFN-α. Dit is in lijn met een studie door Cosgrove et al. waarin bleek dat een infectie met HCV NK-cellen in het bloed meer beïnvloedde dan NK-cellen in de lever [60]. Uit ons onderzoek kunnen we concluderen dat ook IFN-α therapie voor HCV het NK-cel fenotype in het bloed meer beïnvloedt dan in de lever.

Zoals eerder besproken tijdens chronische HCV-infectie, zijn HCV-specifieke T-cel responsen zwak vanwege een voortdurende hoge druk van antigenen. Naast T-cellen, zijn ook NK-cellen belangrijk in de antivirale immuniteit tegen HCV. Hoewel het nog onduidelijk is hoe NK-cellen worden gemoduleerd tijdens een HCV-infectie, is hun rol tijdens IFN-gebaseerde therapie als voorspellers van therapie uitkomst beter bekend [57, 61]. De rechtstreekse werking van de viral load daling op NK-cellen en T-cellen is niet onderzocht gezien virale load daling gedurende lange tijd werd bewerkstelligd door de behandeling met PegIFN-α die rechtstreekse activatie geeft van NK-cellen en remming geeft van CD4+ en CD8+ T-cel proliferatie. Bij patiënten met chronische HBV-infectie, zorgde de behandeling met nucleoside analogen voor een activatie van NK-cellen [62]. Door de introductie van DAA-gebaseerde therapie konden we het directe effect van virale load daling op immuun responsen evalueren bij chronische HCV patiënten. In **hoofdstuk 6** hebben we een studie opgezet waarin 12 patiënten werden behandeld met daclatasvir en asunaprevir gedurende 24 weken. We toonden aan dat met een daling van de HCV-RNA in perifeer bloed, NK-cellen minder geactiveerd werden zoals aangetoond door

de expressie van TRAIL, NKp30 en NKp46 op NK-cellen en de serum IL-12 en IL-18 niveaus. Tevens bevestigden we recent werk dat liet zien dat succesvolle DAA therapie leidde tot een afname van bloed ISG mRNA [63] en herstel van de HCV-specifieke CD8+ T-cel compartiment [64]. In chronische HCV, evenals in chronische HBV komt ISG mRNA tot hoge expressie in zowel het bloed als in de lever. Als DAA therapie in staat is het endogene IFN systeem te verlagen tot een minder geactiveerde toestand kan dit interessant voor de ontwikkeling van behandelingsstrategieën voor HBV-infectie. Klinische trials worden op dit moment gedaan om te onderzoeken of therapie op basis van nucleotide-analogen vóór PegIFN-α therapie resulteert in een betere respons en de kans op het verkrijgen van HBsAg verlies [65]. Dit potentieel gunstige effect zou verklaard kunnen worden door onze bevindingen. Naast hun directe cytotoxisch vermogen, spelen NK-cellen een belangrijke immunoregulatoire rol voor T-cellen [66]. In **hoofdstuk 6** laten we zien dat tijdens IFN-vrije therapie, HCV-specifieke T-cellen in frequentie toenemen, terwijl de expressie van TRAIL op NK-cellen afneemt. Hoewel het onduidelijk is of de effecten van DAA op T-cellen en NK-cellen zijn gerelateerd, is er overtuigend aangetoond dat bij chronische HBV-infectie de lage T-cel frequentie -in ieder geval voor een deel- wordt veroorzaakt door depletie van T-cellen door NK-cellen [67]. Interessant is dat TRAIL de receptor is die deze NK-cel gemedieerde doding van T-cellen induceert. Toekomstige studies moeten verduidelijken of de DAA geïnduceerde herstel van T-cellen bij chronische HCV wordt veroorzaakt door een afname van TRAIL gemedieerde doding door NK-cellen. Deze resultaten zijn belangrijk voor het ontwikkelen van nieuwe behandelstrategieën voor HBV en het ontwikkelen van een vaccinatie voor HCV.

Naast NK-cellen zijn MAIT-cellen mogelijk ook van belang bij chronische virale infecties. MAIT-cellen zijn een subtype van T-cellen die CD8 tot expressie brengen en worden gekenmerkt door de expressie van CD161 en de T-cel receptor (TCR) Vα7.2 die bacteriële producten herkent in een MR1 (MHC klasse I-gerelateerd eiwit) afhankelijke manier [68, 69]. Het aantal MAIT-cellen is verlaagd in patiënten met bacteriële infecties [70]. Daarnaast zijn MAIT-cellen ook betrokken bij inflammatoire ziekten zoals auto-immune hepatitis, primaire biliaire cirrose en steatohepatitis, waarbij MAIT-cellen bleken te zijn toegenomen in de lever van deze patiënten [71, 72]. Bij chronische HIV patiënten weten we dat het aantal MAIT-cellen in het perifere bloed en de lymfeklieren zijn verlaagd en dat ze niet in staat zijn om te herstellen na succesvolle highly active antiretroviral therapy (HAART) [73-76]. Echter, de rol van MAIT-cellen in HIV maar ook in HCV en HCV/HIV co-infecties is grotendeels onbekend. In **hoofdstuk 7** bestudeerden we MAIT-cellen bij patiënten met chronische HCV (CHCV) infectie, patiënten met HAART onderdrukte HIV, patiënten met HIV die acuut waren geïnfecteerd met HCV (AHCV/HIV), patiënten met HIV mono-infectie en gezonde proefpersonen. We observeerden dat de frequentie van MAIT-cellen verminderd was in alle drie de groepen vergeleken met gezonde individuen. In deze virale infectie ziekten is het niet vanzelfsprekend dat bacteriële afgeleide producten MAIT-cellen activeren. Waarschijnlijker is het dat cytokines zoals IL-12/18, waarvan is aangetoond dat ze rechtstreeks MAIT-cellen stimuleren, MAIT-cellen activeren in deze omstandigheden. In **hoofdstuk 7** laten we zien

dat ook IFN-α MAIT-cellen kan stimuleren *in vitro*. Aangezien IFN-α geproduceerd wordt tijdens een infectie met HIV en HCV maar ook bij vele andere virus infecties, zouden MAIT-cellen een rol kunnen spelen bij een veel breder scala aan infectie ziekten. Naast het effect van IFN-α *in vitro*, onderzochten we of IFN-therapie voor CHCV van invloed was op MAIT-cellen *in vivo*. Interessant is dat we in plaats van een stimulerend effect van IFN-α therapie op MAIT-cellen, we een verlaging van IFN-y producerende cellen vonden tijdens therapie. We concludeerden dat dit effect direct was te wijten aan IFN sinds IFN-vrije therapie voor CHCV dit remmende effect niet veroorzaakte. Uit onze studies kunnen we niet concluderen of de verlaging van de frequentie van MAIT-cellen het gevolg is van uitputting van de cellen via apoptose, migratie van cellen uit het bloed naar de perifere organen of huid, of door neerwaartse regulering van kenmerkende markers, zoals CD161. Toekomstige studies moeten worden uitgevoerd om beter inzicht te krijgen in de oorzaken en gevolgen van de verminderde MAIT-cel frequentie in CHCV en HIV.

Deel III - Lange-termijn uitkomst van de behandeling geïnduceerde virale klaring van HCV-infectie.

Verschillende studies hebben de immuunrespons tijdens chronische HCV-infectie en therapie geïnduceerde virale load daling onderzocht. Veel minder aandacht is er geweest voor immuunreacties op lange termijn na een succesvolle therapie. In deel III hebben we onderzocht of de door therapie geïnduceerde virale respons duurzaam was en hebben we bekeken of HCV-specifieke immuun reacties nog altijd aanwezig waren 3 jaar na klaring van de infectie. In hoofdstuk 8 werden HCV patiënten met genotype 1 behandeld met narlaprevir (NS3 protease inhibitor) met PegIFN-α/ribavirine en ritonavir (CYP3A4-remmer) gedurende 24-48 weken [77]. 2 jaar na succesvolle behandeling, kon HCV-RNA niet worden gedetecteerd in het serum van de patiënten. Bovendien kond geen HCV-specifieke T-cel proliferatie tegen HCV worden gedetecteerd, wat in overeenstemming was met de afwezigheid van HCV-RNA in perifeer bloed [77]. Deze gegevens suggereren duurzaamheid van de virale respons, wat werd bevestigd door meerdere klinische studies met verschillende DAA regimes [3, 30]. Interessant is dat in grote patiënten cohorten, minimale hoeveelheden HCV-RNA en de aanwezigheid van HCV-specifieke T-cel proliferatie werden gedetecteerd op lange termijn na behandeling, wat een voortdurende T-cel respons suggereert [78, 79]. Net als bij chronische HCV-infectie, waren deze T-cel responsen zwak en niet in staat te prolifereren of grote hoeveelheden IFN-y te produceren na stimulatie met HCV-peptiden.

Nieuw ontwikkelde DAA drugs worden wereldwijd bebruikt of zijn in verschillende fases van klinische studies en beloven betere sustained viral response rates, veiligheid en gemak ten opzichte van de regimes met IFN. Het potentiële wereldwijde gebruik van de nieuwe succesvolle behandel regimes zullen verdere ontwikkeling van fibrose bij chronische HCV patiënten remmen en de pool van HCV besmette personen verminderen, waardoor

de incidentie zal dalen. Echter ondanks de nieuwe behandelingen met DAA´s, is er nog steeds behoefte aan het ontwikkelen van een vaccin. Aangezien een groot deel van de HCV patiënten intraveneuze drugsgebruikers zijn en homoseksuelen mannen, is de kans op re-infectie met HCV aanzienlijk. Cohort studies naar intraveneuze drugsgebruikers hebben aangetoond dat patiënten die spontaan een HCV-infectie hebben geklaard, meer kans hebben op klaring van een daaropvolgende infectie [80-82]. Ook chimpansees die eerder zelf een HCV-infectie klaarden, klaarden ook snel een tweede infectie [80, 82]. Omdat DAA-therapie geen beschermende immuniteit creëert, is behandeling met een vaccin na zo´n dure DAA behandeling misschien een goedkope manier om re-infectie te voorkomen. Bovendien zullen veel van de DAA behandel regimes niet beschikbaar komen in ontwikkelingslanden in de komende jaren vanwege de kosten, terwijl het implementeren van vaccinatieprogramma's waarschijnlijk veel goedkoper is. Een gedetailleerd inzicht in de mechanismen die bijdragen aan de beschermende immuniteit en het bepalen van factoren die een goede immuunrespons tijdens re-infectie kunnen beïnvloeden is daarom van groot belang. In **hoofdstuk 9** hebben we onderzocht of de factoren die tijdens HCV-infectie de HCV-specifieke T-cel responses remmen, nog steeds aanwezig zijn op vier jaar na therapie geïnduceerde virale klaring. Zoals besproken in deel I van het proefschrift, zijn het uitputten van T-cellen door hoge virale antigeen druk (hoofdstuk 3) en de afwezigheid van goede CD4$^+$ T-cel hulp (hoofdstuk 2) belangrijke mechanismen die bij dragen aan de zwakke T-cel response in HCV-infectie. Uit eerdere studies van onze groep is gebleken dat een derde mechanisme, namelijk directe inhibitie van de T-cel response door Treg en cytokinen IL-10 en TGF-β ook een rol spelen tijdens HCV-infectie [83]. In dit hoofdstuk onderzochten we of deze factoren ook een rol speelden op lange termijn na een succesvolle behandeling. We waren geïnteresseerd in specifiek dit mechanisme omdat recent is aangetoond dat het HCV-RNA dat geïsoleerd wordt uit plasma van patiënten die nog sporen van HCV-RNA hadden na klaring, een nieuwe infectie kan genereren in chimpansees. Infusie van de HCV-RNA leidde zelfs tot hoge viremie en induceerde daarbij HCV-specifieke T-cel responsen [84]. Bij herhaalde blootstelling aan deze kleine hoeveelheden HCV-RNA bleek dat er geen effectieve T-cel responsen werden geïnduceerd door sterkere onderdrukking van de T-cellen door Treg [85]. Het is bekend dat Treg, IL-10 en TGF-β HCV-specifieke T-cel responses in perifeer bloed onderdrukken en dat Treg aanwezig zijn in relatief hoge aantallen in de HCV-geïnfecteerde lever, wat in tegenstelling is tot gezonde levers die nauwelijks Treg bevatten [83, 86]. Algemeen wordt aangenomen dat tijdens de chronische fase van HCV, die intra-hepatische Treg HCV-specifieke T-cel responses en daarmee immunopathologie controleren [86]. In **hoofdstuk 9** tonen we nu aan dat vier jaar na klaring, HCV-specifieke T-cel responsen aanwezig zijn maar nog steeds onderdrukt worden door Treg, TGF-β en IL-10 en dat Treg, in lage frequenties, nog steeds aanwezig zijnin de lever. Het is belangrijk om op te merken dat het nog onduidelijk is hoe Treg in de lever komen. Een mogelijke verklaring hiervoor zouden de kleine hoeveelheden HCV-RNA zijn die HCV-specifieke T-cellen induceren en Treg aantrekken om de immuunreactie te temperen. Naast nieuwe migratie uit perifeer bloed

na virale klaring is er ook een mogelijkheid dat Treg in de lever aanwezig blijven en een geheugen functie hebben zoals eerder beschreven in de huid van mensen en muizen [87, 88]. Omdat eerder bleek dat er geen effectieve T-cel respons kon worden geïnduceerd in de aanwezigheid van Treg, zal hiermee rekening gehouden moeten worden bij het ontwerpen van vaccinaties, vooral bij patiënten die eerder behandeld zijn voor een HCV-infectie. Inzicht in de pathogenese van de zwakke virus-specifieke immuunrespons naar HCV zal ons helpen om bescherming te creëren van de patiënten met een hoog risico op re-infectie.

CONCLUDEREND

Met de ontdekking van HCV in 1989 zijn honderdduizenden CHCV patiënten behandeld met PegIFN-α. In de afgelopen 10 jaar zijn vele nieuw middelen getest en zijn inmiddels zeer effectieve gebleken waardoor geheel IFN-vrije combinatie therapieën met DAA nu beschikbaar zijn. Het is belangrijk om te beseffen dat genezing niet de belangrijkste uitdaging meer is in de strijd tegen HCV-infectie. Het ontdekken van patiënten met HCV-infectie, het beschikbaar maken van een effectieve behandeling voor HCV in ontwikkelingslanden en de ontwikkeling van een vaccin zijn drie belangrijke doelen die moeten worden nagestreefd in de toekomst. Met dit proefschrift hebben we meer kennis verworven over het adaptieve en het aspecifieke immuun systeem gedurende chronische HCV infectie, de behandeling en daarna. Inzicht in de pathogenese van factoren die van belang zijn voor een goede immuunrespons zullen helpen in de ontwikkeling van een vaccin om beschermende immuniteit te creëren. Bovendien is de kennis die we verwerven over chronische HCV-infectie zeer relevant in de strijd tegen andere chronische virale infecties zoals HBV en HIV. In het achterhoofd houdend met welke snelheid de geheimen van chronische HCV-infectie zijn ontrafeld, zal een wereld vrij van HCV zeker mogelijk worden.

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APPENDIX

DANKWOORD

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LIST OF PUBLICATIONS

1. **Spaan M**, van Oord G, Kreefft K, Hou J, Hansen BE, Janssen HL, de Knegt RJ, Boonstra A. Immunological analysis during IFN-free therapy for chronic HCV reveals modulation of the NK cell compartment. J Infect Dis. 2015 Jul 28.

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3. **M. Spaan**, K. Kreefft, G.N. de Graav, W.P. Brouwer, R.J. de Knegt, F.J.W. ten Kate, C.C. Baan, T. Vanwolleghem, H.L.A. Janssen, A. Boonstra. CD4$^+$CXCR5$^+$ T-cells in chronic HCV infection produce less IL-21, yet are efficient at supporting B-cell responses. Journal of Hepatology. J Hepatol. 2015 Feb;62(2):303-10

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6. **Spaan M**, Groothuismink ZM, Koning L, Roomer R, Janssen HL, De Knegt RJ, Boonstra A. Erythropoietin administration suppresses human monocyte function in vitro and during therapy-induced anemia in HCV patients. Antiviral Res. 2013 Jun;98(3):469-75.

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9. **Spaan M**, de Man R. Coeliakie als oorzaak van levertestafwijkingen. (English title: Coeliakie as cause of elevated liver enzymes). Ned Tijdschr Geneeskd. 2011;155:A3579

10. **Michelle Spaan**, Pauliina Porola, Mikael Laine, Blaz Rozman, Masayuki Azuma, Yrjö T. Konttinen. Healthy human salivary glands contain a DHEA-S processing intracrine machinery, which is deranged in primary Sjögren's syndrome. J Cell Mol Med. 2009 Jul;13(7):1261-70.

11. Konttinen YT, **Spaan M**, Stegaev V, Porola P, Lorés M, Vivó A, Koskenpato K, Przybyla BD: Female dominance in Sjögren's syndrome – A paradox and a new paradigm. In: Sjögren's syndrome and associated disorders. Editors: Margit Zeher and Péter Szodoray. Transworld Research Network, Kerala, India, pp. 59-77, 2009

PHD PORTPOLIO

Name of PhD student: Michelle Spaan
PhD period: January 2011 – October 2015
Department: Gastroenterology and Hepatology, Erasmus Medical
                              Center, Rotterdam
Promotor: Prof.dr. H.L.A. Janssen
Co-promotor: Dr. A. Boonstra

General courses

2011   Course 'ultrasound of the abdomen', Dutch Liver Retreat
2011   Course clinical hepatology, Dutch Liver Retreat
2012   Basic introduction course SPSS
2012   Postgraduate course: Personalized medicine and the practice of
          hepatology, AASLD, Boston, USA
2013   Course on research ethics, Erasmus University
2013   Immunology course, Erasmus University
2014   Statistical course at Netherlands Institute of Health Sciences (NIHES)
2014   English biomedical writing course, Erasmus University
2014   Course for Photoshop and Illustrator, Erasmus University

Attended scientific conferences

- American Association for the Study of Liver Diseases (AASLD), 2011 (San Francisco, USA), 2012 (Boston, USA), 2014 (Boston, USA)
- European Association for the Study of the Liver (EASL), 2013 (Amsterdam, the Netherlands), 2014 (London, UK), 2015 (Vienna, Austria)
- European Association for the Study of the Liver (EASL) Monothematic Conference: Immune Mediated Liver Injury, Birmingham, UK, 2012
- European Association for the Study of the Liver (EASL) Monothematic Conference: Translational Research in Chronic Viral Hepatitis, Lyon, France, 2013
- Dutch society for Immunology (NVVI), Velthoven, the Netherlands, 2011, 2012.
- Dutch Liver Retreat (DLR), Spier, the Netherlands, 2013
- Molecular Medicine Day, Rotterdam, the Netherlands, 2013, 2014

Poster presentations

Erythropoietin administration suppresses innate immunity during therapy-induced anemia in HCV patients. AASLD, 2011, San Francisco, USA.

Erythropoietin used for treatment of HCV infection inhibits cytokine production, but not ROS production and phagocytosis by monocytes. AASLD, 2012, Boston, USA.

T follicular helper cells: do they play a role in chronic HCV? EASL, 2013, Amsterdam, the Netherlands,

Abstract also presented at NVH (Dutch society of hepatology), 2013, Velthoven, the Netherlands, Abstract also presented at Molecular Medicine day, Rotterdam, the Netherlands. Poster awarded as: <u>best poster presentation.</u>

Assessment of T follicular helper cells in peripheral blood and liver follicles in chronic HCV. EASL Monothematic Conference, 2013, Lyon, France.

Longitudinal analysis of TRAIL and CD158b expression on peripheral and intrahepatic NK-cells in HCV patients during triple therapy. NVH (Dutch society for hepatology), 2014, Velthoven, the Netherlands
Abstract also presented at Molecular Medicine Day, Rotterdam, The Netherlands.

Triple therapy for chronic HCV patients induces early activation of intrahepatic NK cells. EASL, 2015, Vienna, Austria.

IFN-free therapy for chronic HCV: transcriptomics and NK cell analyses. EASL, 2015, Vienna, Austria. <u>Awarded with Young Investigator full bursary.</u>

Oral Presentations

The intrahepatic T-cell compartment does not normalize years after therapy-induced hepatitis C virus eradication. NVGE, 2015, Velthoven, the Netherlands

The intrahepatic T-cell compartment does not normalize years after therapy-induced hepatitis C virus eradication. AASLD, the Liver Meeting 2014, Boston, USA.

Evaluation of peripheral and intrahepatic T follicular helper cells in chronic HCV patients. Dutch Liver Retreat, 2013, Spier, the Netherlands.

Grants

Travel grant	Erasmus Trustfonds	2011, 2012, 2014
Travel grant	Nederlandse Vereniging voor Immunologie	2014
Travel grant	Nederlandse Vereniging voor Hepatology	2014

Supervising activities

February – August 2013 Supervising master student: master in infection and Immunity

ABOUT THE AUTHOR

Michelle Spaan was born on July 2nd 1986 in Gouda, the Netherlands, where she grew up. In 2004 she completed her secondary school at Antonius College in Gouda and started her medical training at the Erasmus University in Rotterdam. In 2008 she performed her graduation research on "The role of DHAE-S in salivary glands of patients with primary Sjögren's syndrome" at the Biomedicum in Helsinki, Finland. She got interested in basic science and decided to perform a PhD. After obtaining her medical degree in 2011, she started her PhD project under supervision of Prof.dr. H.L.A. Janssen and Dr. A. Boonstra at the department of Gastroenterology and Hepatology. The results of this project are presented in this thesis. From January 2015, she worked as a resident not-in-training at the Internal Medicine department at the Sint Fransiscus Gasthuis under supervision of dr. A.P. Rietveld. After her PhD defence she will continue working at the Hepatology department at King's College Hospital, London, under the supervision of Prof.dr. K. Agarwal and Prof.dr. M.A. Heneghan.