Determinants of HCV-specific CD8+ T cell expansion

Inaugural-Dissertation

zur

Erlangung des Doktorgrades

Dr. rer. nat.

der Fakultät für

Biologie

an der

Universität Duisburg-Essen

vorgelegt von

Susanne Maria Magdalena Ziegler

aus Euskirchen

Juni 2013

Für meine Familie

Da es sehr förderlich für die Gesundheit ist,
habe ich beschlossen glücklich zu sein.

Voltaire

Die der vorliegenden Arbeit zugrunde liegenden Experimente wurden am Institut für Virologie der Universität Duisburg-Essen durchgeführt.

1. Gutachter: Prof. Dr. Jörg Timm

2. Gutachter: Prof. Dr. Astrid Westendorf

3. Gutachter:

Vorsitzender des Prüfungsausschusses: Prof. Dr. Bernd Sures

Tag der mündlichen Prüfung: 10. September 2013

CONTENTS

1 Introduction

Infections with the hepatitis C virus (HCV) are one of the leading causes of acute and chronic liver disease. HCV is distributed worldwide with an estimated 150 million people chronically infected, representing approximately 2-3 % of the world population. Every year 3-4 million people are newly infected with HCV, in developed countries mostly resulting from injection drug use. Following infection, approximately 85-90 % of patients do not exhibit any symptoms. Though, in the majority of newly infected people the virus persist in the liver, leading to chronic HCV infection. People with persistent infection often show histological signs of liver inflammation associated with progressive liver disease, resulting in an increased risk of developing hepatocellular carcinomas. Hence, HCV infection is one of the main causes for liver transplantations and in addition, responsible for more than 350 000 deaths per year due to HCV-related liver diseases (WHO, 2012).

The current treatment regimen for chronic HCV infections is based on a combination of pegylated interferon (pegIFN)-α and the nucleoside analogue ribavirin. Unfortunately, sustained virological response (SVR) is only reached in 50 % of treated patients, which largely depends on the HCV genotype. More recently, two protease inhibitors, telaprevir and boceprevir have been approved. These novel directly acting antiviral (DAA) drugs will clearly improve the sustained response rates, however, such novel therapies with protease inhibitors or other DAA can lead to the selection of resistant viral variants. In addition, HCV treatment is accompanied by severe side effects and extremely high costs. The novel treatment options are therefore restricted to areas with highly developed public health systems. Thus, development of an effective vaccine is still a major goal, but the high genetic variability of the virus makes vaccine development a challenging task.

1.1 Hepatitis C virus

Initially identified in 1989 by immunoscreening of patient sera suffering from post-transfusional non A-, non B-hepatitis (Choo *et al* 1989), the hepatitis C virus was classified as a member of the family *Flaviviridae* in the genus *Hepacivirus*. The enveloped virus is composed of an icosahedral particle of about 40-50 nm in diameter. The lipid envelope contains the envelope proteins E1 and the dimerized E2. The viral envelope surrounds the capsid containing the small ribonucleic acid (RNA) genome.

1.1.1 The genome organization

The HCV genome is composed of one single stranded RNA molecule of 9.6 kilo bases (kb) in length. The small, positive-sense RNA genome encodes a single open reading frame (ORF) of 3000 amino acids (Bartenschlager *et al* 1993) that is proteolytically processed by host and viral proteases into ten single proteins: core, E1, E2, p7, NS2, NS3, NS4A and NS4B and NS5A and NS5B (see figure 1.1). The ORF is uncapped and flanked by untranslated regions (UTR). Of those highly conserved regions, the 5'-UTR of 340 nucleotides contains the internal ribosome entry site (IRES), which initiates translation of the polyprotein (Tsukiyama-Kohara *et al* 1992). The polyprotein encoding region is followed at the 3'-end by a second UTR approximately 200 nucleotides in length. It contains a variable region and a highly conserved region necessary for replication (Yanagi *et al* 1999).

1.1.2 HCV proteins

After cleavage of the polyprotein by host and viral protease, the resulting proteins can be divided into structural proteins, i.e. core, envelope 1 and envelope 2, and non-structural proteins (NS), i.e. NS2, NS3, NS4A, NS4B ,NS5A and NS5B (see figure 1.1). Between structural and non-structural, an additional membrane protein p7 is located. The structural proteins and the p7 protein are processed by the endoplasmatic reticulum (ER) signal peptidases, whereas the non-structural proteins are cleaved by viral proteases, i.e. the NS2/ 3 protease and the NS3/ 4A serine protease (see figure 1.1).

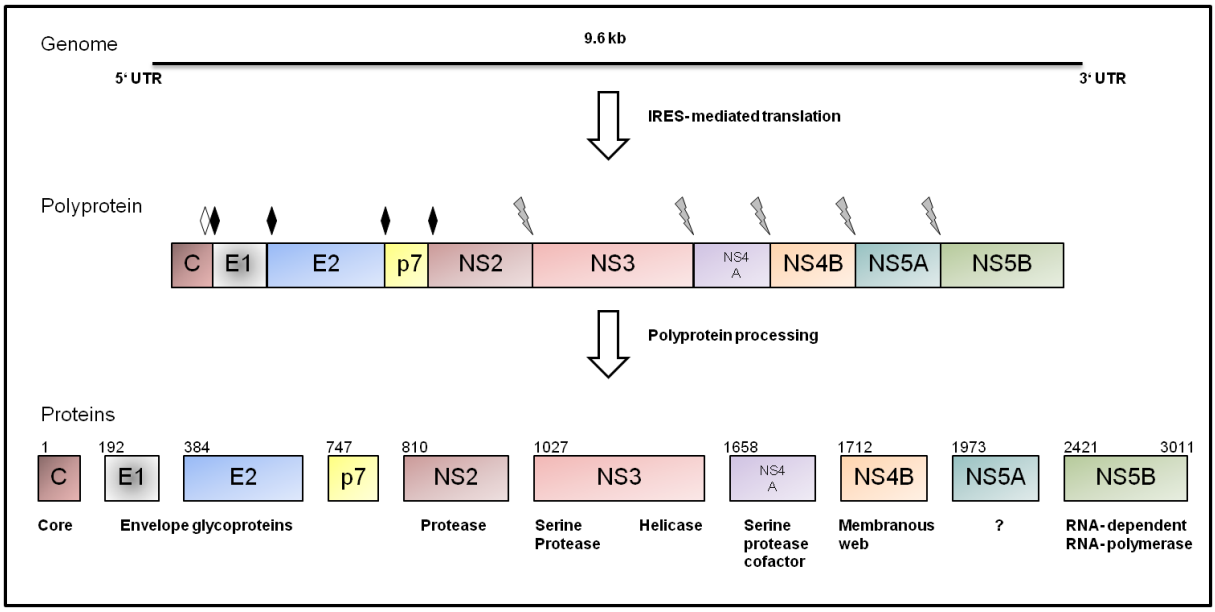

Figure 1.1 Organization of the HCV genome.
The HCV genome is flanked by 5'- and 3'-UTR. The 5'-UTR contains the IRES, initiating translation. The resulting polyprotein is cleaved into structural and non-structural proteins by host endoplasmatic reticulum signal peptidase (diamonds) or by viral NS2/ 3 and NS3/ 4A serine protease (flash). Modified from (Moradpour *et al* 2007).

The N-terminal core protein is highly conserved and makes up the viral nucleocapsid. Core protein can bind viral RNA (Santolini *et al* 1994), in addition, it is assumed that core protein plays a role in hepatocarcinogenesis and steatosis hepatitis (Hope *et al* 2002, Lerat *et al* 2002).

Downstream of core protein, the two envelope proteins E1 and E2 are encoded. Envelope proteins are highly glycosylated, which differs among genotypes and play an important role in cell entry. In addition, envelope protein E2 contains two hypervariable regions HVR-1 and -2. Several studies analyzed the role of HVR-1 and -2 as a target for neutralizing antibodies. However, the constant selection for mutations in this region enables the virus to evade the immune system (Polyak *et al* 1998, von Hahn *et al* 2007). Furthermore, it is assumed that HVR-1 regulates the binding of E2 to cluster of differentiation 81 (CD81) (Roccasecca *et al* 2003, Sklan *et al* 2009, Zeisel and Baumert 2009).

P7 is a membrane-spanning protein located in the endoplasmatic reticulum. It is assigned to a group of proteins called viroporine, which are essential for ion channel formation that play an important role in virus infection (Griffin *et al* 2003), as well as particle assembly and release of infectious virions (Steinmann *et al* 2007).

Non-structural proteins NS2, NS3, NS4A, NS4B, NS5A and NS5B are located in the C-terminal region of the polyprotein. They are responsible for several processes including viral replication and processing of the polyprotein.

NS2 is a transmembrane protein and essential for the completion of the viral replication cycle. Together with the N-terminal domain of NS3, NS2 forms an auto protease that cleaves between NS2 and NS3 (Grakoui *et al* 1993, Hijikata *et al* 1993). The NS3 genome region encodes for a protein with multifunctional activity. The NS3 N-terminal part has a serine protease activity, whereas the C-terminal part has NTPase/ helicase activity (Gallinari *et al* 1998). The NS4A polypeptide functions as a co-factor for the NS3 serine protease and the resulting complex is responsible for the cleavage of all remaining C-terminal proteins. In addition, the NS3/ 4A protease is the target for recently approved drugs (Lamarre *et al* 2003). The 3'-encoded helicase is indispensable for cytoplasmic RNA replication (Gallinari *et al* 1999, Moradpour *et al* 2003), although its exact function is not fully defined yet.

One of the functions of NS4B is the formation of a membranous web that acts as a scaffold for the replication complex (Egger *et al* 2002). NS5A plays an important role in viral replication, modulation of cell signaling pathways (Reyes 2002) and interferon response (Macdonald *et al* 2004). The ability of NS5A to bind to RNA defined a new functional target for development of agents to treat HCV infection and a new structural class of RNA-binding proteins (Huang *et al* 2005).

Among the essential non-structural proteins, the most important is the tail-anchored NS5B, the viral RNA-dependent RNA polymerase, crucial for the transcription of viral RNA. Due to the lack of proof-reading activity of the error prone RNA-dependent RNA polymerase, HCV mutates with high rates leading to the significant generation of viral variants or 'quasispecies' [reviewed in (Bowen and Walker 2005b), see 1.2]. Resulting from a ribosomal frameshift +1 in the N-terminal domain of core, the F-protein or alternative reading frame protein (ARFP) is synthesized. Its function and antigenic properties are largely unknown, though the presence of anti-F antibodies was shown in sera of chronically infected patients indicating an antigenic relevance in chronic infections (Komurian-Pradel *et al* 2004).

1.1.3 Viral replication

Analysis of the HCV replication cycle became possible when *in vitro* model systems were developed. The invention of subgenomic replicons and later the full-length HCVcc system offer adequate *in vitro* model systems to study molecular mechanisms of HCV replication and the development of antiviral drugs (Lohmann *et al* 1999). These replication systems were complemented by the HCV pseudoparticle system (HCVpp) that enables monitoring of HCV entry (Bartosch *et al* 2003, Hsu *et al* 2003).
With these models, various receptors have been identified to interact with HCV cellular membrane receptors. It is assumed that binding to hepatocytes requires the interaction of E1 and E2 proteins with glycosaminoglycanes (GAG), dendritic cell-specific intracellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), liver/ lymph node-specific intracellular adhesion molecules-3-grabbing non-integrin (L-SIGN) as well as the binding to (very)-low-density lipoprotein ((V)LDL) receptor. After binding to the cell surface, viral entry requires binding to further molecules such as tetraspanin, CD81, scavenger receptor class B type 1 (SR-B1), and both tight junction components Claudin (CLDN)-1 and Occludin (OCLDN)-1 (Sabahi 2009). It is assumed that CLDN-1 and OCLDN-1 are both essential for viral entry (Chevaliez and Pawlotsky 2012, Pietschmann 2009).
A hallmark of HCV is its narrow host range that is in part due to species determinants residing in the entry molecules CD81 and OCLDN-1 (Ploss *et al* 2009). This is one of the reasons why small animal models were not yet developed.

HCV internalizes via pH-depended clathrin-mediated endocytosis followed by fusion within an acidic endosomal compartment [see figure 1.2, (Blanchard *et al* 2006, Coller *et al* 2009)]. Decapsidated genomic RNA serves as messenger RNA for the IRES-initiated translation into the polyprotein by recruiting viral, as well as cellular proteins (Honda *et al* 1999). At the ER membrane the polyprotein is processed into the three structural proteins, the viroporin p7 and the six non-structural proteins [(Moradpour *et al* 2007), see 1.1.2]. Replication is mediated by the NS5B polymerase (Gwack *et al* 1997). The replication complex is build by NS4B protein membranous web formation and associates viral proteins, host cellular components and nascent RNA (Egger *et al* 2002, Elazar *et al* 2004). The positive-sense RNA molecule serves as template for a negative-sense RNA intermediate form that is used as template for

the numerous production of positive-sense RNA strands that are used for protein translation or packing into virions (Bartenschlager *et al* 2004, Moradpour *et al* 2007). For infectious HCV particle formation, the newly synthesized RNA interacts with core protein (Shimoike *et al* 1999, Tanaka *et al* 2000), cytoplasmic lipid droplets and VLDL machinery (Bartenschlager *et al* 2011). However, the export and transfer of mature virions to neighboring cells still needs to be elucidated (figure 1.2).

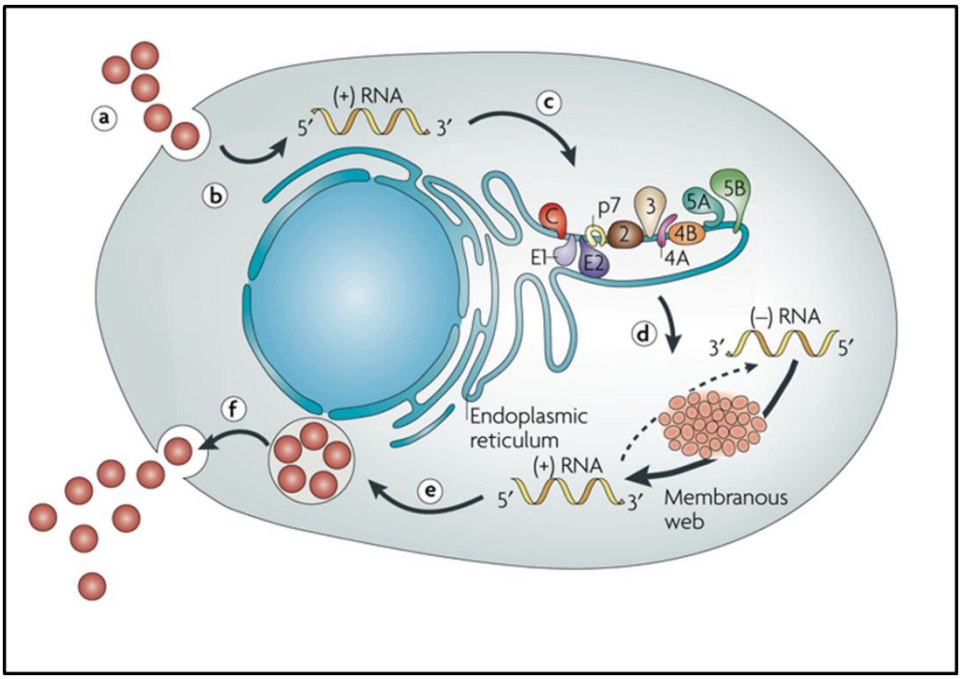

Figure 1.2 Schematic lifecycle of HCV.
(a) Virus binding and internalization, (b) cytoplasmic release and uncoating, (c) IRES-mediated translation and polyprotein processing, (d) RNA replication, (e) packaging and assembly, (f) virion maturation and release (Moradpour *et al* 2007).

1.2 HCV genomes and the origin of quasispecies

Analysis of the HCV RNA genome resulted in the classification of HCV into seven genotypes, which can be further classified into subtypes (Murphy *et al* 2007). These genotypes are characterized by at least 30 % divergence in nucleotide sequence. HCV genotypes (and subtypes) have evolved separately in different geographical regions and are distributed worldwide. The most common variants found in Western countries are genotype 1a, 1b, 2a-c and 3a. There is much greater diversity of HCV in certain regions of Sub-Saharan Africa and South Asia (Simmonds *et al* 2005). Genotype 4 is predominant in the Middle East and Africa. Genotype identification is clinically important, as for the current treatment regimen of pegIFN-α and ribavirin

genotypes 2 and 3 have increased SVR rates in comparison to genotypes 1 and 4 (Hnatyszyn 2005).

HCV replicates at very high rates, with about 10^{12} copies per day (Neumann *et al* 1998). Due to the lack of proofreading activity of the RNA-dependent RNA polymerase, HCV and other RNA viruses mutate frequently (Holland *et al* 1982) with a mutation rate of approximately $1.4x10^{-4}$ base substitutions per genome site per infected cell (Bukh *et al* 1995, Sanjuan *et al* 2004). Given the high replication capacity, it is likely that at least 10^9 viral variants arise in each infected person multiple times daily (Guedj *et al* 2010), resulting in a quasispecies of multiple closely related but distinct variants. Variants harboring substitutions that are deleterious for viral replication are negatively selected and removed from the quasispecies. Upon transmission of HCV only few members of the quasispecies establish infection of the next host (Bull *et al* 2011). This so called bottleneck may be the consequence of selection of the most fit variant for viral entry and consequently early infection. In addition, there is increasing evidence that immune selection pressure by B and T cells results in the positive selection of immune escape mutations in the viral genome [reviewed in (Bowen and Walker 2005b)]. After transmission of quasispecies, a second bottleneck has been described that might arise from host immune selection pressure driven by B and T cells (Bull *et al* 2011). This phenomenon has also been described for other RNA viruses, like human immunodeficiency virus (HIV) (Bar *et al* 2010, Boutwell *et al* 2010, Keele *et al* 2008).

1.3 HCV pathogenesis

1.3.1 Transmission of HCV

HCV is most commonly acquired through exposure to infectious blood. Before the introduction of diagnostic screening methods, HCV was mainly transmitted through contaminated blood transfusion products. Approximately 10-20 % of patients who received multiple blood products seroconverted to anti-HCV positive. Nowadays, blood products in Germany can be considered as safe with only a minimal remaining risk for HCV infection. Unfortunately, screening methods are not the standard of care in all countries. Blood transfusions from unscreened donors and therapeutic

procedures remain the major cause of HCV transmission in developing countries. In developed countries, newly diagnosed HCV infections mainly result from needle sharing of injection drug users (IDU) (Alter 2002). In addition, the effect of other modes of transmission, like tattooing or acupuncture has been discussed (Shepard *et al* 2005). Perinatal transmission is the major cause of HCV acquisition in children, with a rate of mother-to-child transmission of 4-7 % in infected women with detectable viremia [reviewed in (Maheshwari *et al* 2008)]. Infection via breast feeding seems to be very unlikely.

1.3.2 Course of disease

In approximately 85-90 % of newly HCV infected patients the acute infection processes asymptomatic (figure 1.3) and is thus frequently not diagnosed. Interestingly, asymptomatic infection more frequently results in the establishment of a chronic infection (85-90 %), whereas spontaneous clearance is more frequently observed in patients with severe clinical signs of hepatitis and jaundice (Gerlach *et al* 2003) in comparison to asymptomatic infections. The presence of jaundice might be an indicator for a successful host immune response by cytotoxic cells that lead to clearance of the virus. Acute HCV infection is often accompanied by symptoms of fatigue, myalgia, low-grade fever, nausea and vomiting.

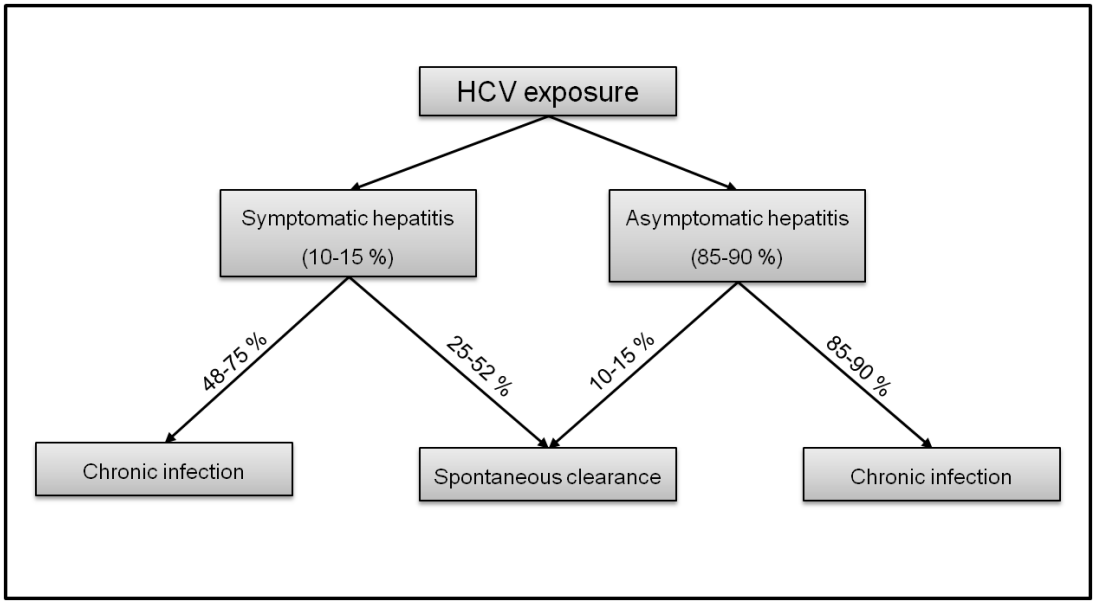

Figure 1.3 Outcome of HCV infection.
Adapted from (Maheshwari *et al* 2008).

A chronic HCV infection is characterized by the detection of anti-HCV antibodies and HCV RNA in serum of infected patients for more than six months. Risk factors for developing a chronic infection include age at the time of infection, male gender, alcoholism and co-infection with hepatitis B virus (HBV) or HIV. During chronic HCV infection about 15-30 % of patients have progression to liver cirrhosis, of which 1-5 % are at risk to develop hepatocellular carcinomas (Afdhal 2004, Fattovich *et al* 1997, Poynard *et al* 1997, Thein *et al* 2008).

1.3.3 Diagnosis

HCV infection can be diagnosed via detection of antibodies against HCV by immunoassays (e.g. ELISA). Of note, seroconversion can be delayed in approximately 30 % at the onset of symptoms (Farci *et al* 1991), especially in immunocompromised patients. In addition, approximately 10 % of acutely infected might lose HCV-specific serological markers (Alter and Seeff 2000). Detecting viral nucleic acid represents the most sensitive method for the diagnosis of HCV infection. The detection limits vary between the different polymerase-chain reaction (PCR)-based test and nowadays reach lower limits of detection of 10 IU/ mL with a specificity of 95 %.

1.3.4 Antiviral therapy

Over the past decade, the dual therapy using pegIFN-α plus ribavirin became the standard treatment for all genotypes in acute infections, resulting in a decline of liver transaminase levels to normal levels, as well as a decrease in viremia in about 90 % of treated patients. However, in chronic infections success and duration of this treatment regimen largely depends on the viral genotype. Upon treatment of genotype 2 and 3 infected patients, SVR is reached in about 80 %, in contrast to genotype 1 and 4 infected, where SVR is achieved in less than 40 % of patients.

Recently, considerable progress has been made for the treatment of HCV infections. The increasing knowledge of the HCV lifecycle led to the development of direct acting antivirals. These include the new protease inhibitiors boceprevir and telaprevir, which have been approved in 2011. The addition of telaprevir or boceprevir to the

existing dual therapy resulted in a shorter time to achieve undetectable HCV RNA levels and markedly increased therapy success in genotype 1 infected patients (Berman and Kwo 2009, Jacobson *et al* 2011, Kwo *et al* 2010, McHutchison *et al* 2009, Poordad *et al* 2011, Zeuzem *et al* 2011).

1.4 The immune response during an HCV infection

Effective immune responses, including the innate and adaptive immunity are essential to control an HCV infection. Though, in the majority of infections, the immune system is not able to control the virus, leading to persistent infections. This is mainly due to several strategies of HCV to evade innate and adaptive immune responses.

1.4.1 The innate immune response

The innate immune response represents the first line of defense in viral infections. An important role of the innate immune response in viral clearance is suggested by the genomic association between a single-nucleotide polymorphism (rs12979860) 3 kilobases upstream of the *IL28B* gene, which encodes the type III interferon IFN-λ3 and sponatenous control of HCV infection (Thomas *et al* 2009, Tillmann *et al* 2010). Studies by Thomas and colleagues revealed that the C/ C genotype strongly enhances resolution of HCV infection among individuals of both European and African ancestry, whereas studies by Tillmann and colleagues showed that women with the C/ T or T/ T genotype who did not develop jaundice had a lower chance of spontaneous clearance of HCV infection.

HCV-infected hepatocytes trigger the earliest immune responses via recognition of viral constituents by cells of the innate immunity. Infected cells and cells of the innate immunity recognize pathogen-associated molecular patterns (PAMP) via pattern recognition receptors (PRR), like toll-like receptors (TLR) and intracellular helicases of the retinoic acid-inducible gene-I (RIG-I)-like receptor family, which lead to the subsequent production of type I interferons (IFN-α and -β) and possibly type III interferons (IFN-λ) by hepatocytes and plasmacytoid dendritic cells (pDC). Binding to

PRR lead to the induction of several signaling cascades, including the downstream activation of IFN-regulatory factor-3 (IRF-3) and NF-κB, which results in the subsequent production of type I IFN. Binding of newly synthesized IFN-α/ β to the common type I IFN receptor on the surface of the infected (autocrine) or neighbouring (paracrine) cells induces signaling via the Jak/ Stat pathway [reviewed in (Schindler and Plumlee 2008)] and transcription of interferon stimulated genes (ISG). Newly produced IFN induce an antiviral state in the liver and by this contributes to viral clearance (Li and Lemon 2013, Rehermann 2009).

Notwithstanding, HCV has evolved numerous mechanisms to circumvent RIG-I- and TLR- signaling and ISG expression. Several contradicting pathways have been suggested by which HCV dysregulates or block the Jak/ Stat pathway [reviewed in (Thimme *et al* 2012)]. These include HCV core protein interference with suppressor of cytokine signaling 3 (SOC3) that lead to the inhibition of tyrosine phosphorylation of STAT-1 (Bode *et al* 2003) or an inhibition of ISG transcription by upregulation of the protein phosphatase PP2Ac (Duong *et al* 2004). Evidence suggest that HCV NS3/ 4A protease functions as an antagonist of virus-induced IRF-3 activation and IFN expression [reviewed in (Gale and Foy 2005)], which lead to an impairment of type I IFN production. Furthermore, it is assumed that HCV blocks interferon effector functions by inducing protein kinase R phosphorylation, which inhibits eukaryotic translation initiation factor eIF2α and attenuates ISG protein expression (Garaigorta and Chisari 2009).

Despite the induction of type I interferons by hepatocytes and pDC, other cell types of the innate immunity play a central role in the control of HCV infections in the liver. The liver contains an unusual population of resident lymphocytes, among which natural killer (NK) cells and natural killer T (NKT) cells are found in increased numbers (30 % NK cells, up to 50 % NKT cells) relative to their proportions in peripheral blood and other organs (Crispe and Mehal 1996, Norris *et al* 1998), which can even increase in HCV infected livers [up to 90 % NK cells (Doherty and O'Farrelly 2000)].

NK cells are granular lymphocytes that play a central role in viral infections. The main function of NK cells is to lyse malignant or virally infected cells by secretion of cytoplasmic granules, Fas ligand-mediated apoptosis and antibody-dependent cellular cytotoxicity. NK cells (and NKT cells) are thought to release IFN-γ, which lead

to the noncytopathic inhibition of HCV replication (Guidotti and Chisari 2006). Genetic studies revealed an association between homozygosity for the inhibitory NK cell receptor killer immunoglobulin-like receptor 2DL3 (KIR2DL3) and its ligand the human leukocyte antigen-C1 (HLA-C1) group and viral clearance (Khakoo *et al* 2004, Knapp *et al* 2010, Romero *et al* 2008). HCV might escape NK cell mediated killing by cross-linking of CD81 on the surface of NK cells by HCV E2 protein, which lead to NK cell inhibition (Crotta *et al* 2002, Tseng and Klimpel 2002).

1.4.2 The adaptive immune response

There are numerous lines of evidence that the adaptive immunity plays an important role in shaping the outcome of an HCV infection. CD4+ T cells play a prominent role in viral clearance during the acute phase of infection and are associated with long-term control of HCV infection (Gerlach *et al* 1999). In addition, CD8+ T cells are important in immune control of the virus, as accumulation of HCV-specific CD8+ T cells in the livers of infected chimpanzees was shown to be associated with the onset of hepatitis and a decrease in viral load (Thimme *et al* 2002). The importance of neutralizing antibodies in controlling HCV infection gain increasing interest, as evidence suggest that neutralizing antibodies contribute to HCV clearance and protect from reinfection [reviewed in (Fafi-Kremer *et al* 2012)].

1.4.2.1 Humoral immunity

During acute infection, the humoral immune response is directed against epitopes within structural and non-structural components of the virus. However, the role of antibodies during HCV infection is not completely understood. Studies in immunocompromised, antibody-deficient patients showed that HCV can be cleared without humoral immune responses (Semmo *et al* 2006). Antibodies titers increase 8-20 weeks after infection (Thimme *et al* 2002), though, the largest fraction of the produced antibodies has no antiviral activity. Just a small fraction of antibodies has neutralizing capabilities. These neutralizing antibodies are in principle able to inhibit virus binding, entry and uncoating. During chronic infection, the amount of cross-reactive neutralizing antibodies, targeting multiple genotypes, increase, yet they fail

to clear the virus (Logvinoff *et al* 2004). Studies by Pestka and colleagues suggest a central role for neutralizing antibodies in the early phase of infection. In a homogenous cohort, development of neutralizing antibodies in the majority of patients who cleared the virus was described, whereas delayed induction was observed in patients that processed to chronic infection (Pestka *et al* 2007). The main target for neutralizing antibodies has been identified in the envelope proteins E1 and E2, especially in HVR-1, which is important in virus binding and entry (Sabo *et al* 2011). The high degree of genetic variability of this short region in the viral envelope protein is the product of humoral immune pressure by neutralizing antibodies. Continuous selection of escape mutations in targeted B cell epitopes is a key feature of chronic infection and one reason for the lack of sterilizing immunity against HCV (Osburn *et al* 2010, Ray *et al* 1999, von Hahn *et al* 2007).

1.4.2.2 Cellular immunity

CD4+ and CD8+ T cells recognize infected cells when they encounter antigen-presenting cells (APC) displaying their cognate peptide antigen on major histocompatibility complex (MHC) class I or II molecules, respectively. T cell activation requires the interaction of T cell receptors (TCR) and peptide-MHC complexes and the interaction of ligand-receptor pairs providing costimulatory signals mediated by CD80 and CD86. Upon recognition of the cognate antigen complexed with MHC molecules CD4+ and CD8+ T cells start to produce proinflammatory cytokines interleukin (IL)-2, IFN-γ and tumor necrosis factor-α (TNF-α), if co-stimulation is provided, which in turn activates cells of the immune system including macrophages. Activated CD8+ T cells can lyse infected cells via secretion of cytotoxic granules containing granzymes and perforin, resulting in Fas-mediated apoptosis of target cells. CD4+ T cells function to help B and T cell responses by promoting the activation of APC and by secreting cytokines and chemokines.

While effector CD8+ T cells are particularly important in viral clearance, CD4+ T cells are absolutely necessary to provide CD8+ T cell help and play a role in long-term control of the virus. In a chimpanzee model, it was shown that antibody-mediated depletion of CD4+ T cells resulted in the inability of CD8+ T cells to control HCV

replication followed by the emergence of escape mutations in MHC class I-restricted epitopes (Grakoui *et al* 2003). Broadly directed HCV-specific CD4+ T cells are universally detectable during early stages of infection with a similar repertoire of targeted epitopes, regardless of outcome. Nevertheless, in chronically evolving HCV infection, HCV-specific CD4+ T cells show early functional defects and becomes completely undetectable within months (Schulze zur Wiesch *et al* 2012). In chronic HCV infection, CD4+ T cell responses are impaired, in the context of weak or even absent, dysfunctional CD4+ T cells, which might be associated with decreased IL-2 amounts (Semmo *et al* 2005). In addition, increasing evidence suggest that regulatory T cells play a prominent role in HCV infections, by suppressing virus-specific T cells in a contact-depended manner. It was shown that the amount of CD4+ CD25+ T cells is increased in chronic HCV infection, in comparison to patients that resolved the infection (Cabrera *et al* 2004).

It is well accepted that the cell-mediated immunity, in particular, strong and sustained CD4+ and CD8+ T cells responses targeting multiple epitopes, is critical for HCV resolution [reviewed in (Bowen and Walker 2005a, Rehermann 2009, Thimme *et al* 2012, Walker 2010)]. In contrast to other viral infections, the onset of cellular immune responses is delayed in HCV infections, starting 8-12 weeks after infection (Bowen and Walker 2005a, Rehermann 2009). Interestingly, the frequencies of HCV-specific CD8+ T cells are generally lower than those observed in other chronic infections, like Epstein-Barr virus (EBV) or HIV infection (Callan *et al* 1998, Ogg *et al* 1998). The increase in HCV-specific T cell responses coincides with the increase of transaminases and the onset of hepatitis.

CD8+ T cells recognize their cognate antigens on peptide-MHC class I complexes and respond by killing the infected cells by cytolytic elimination and the secretion of antiviral cytokines to create an antiviral state. The importance of functional CD8+ T cell responses was shown in a chimpanzee model, where infected animals that lack CD8+ T cell responses were unable to control viremia until HCV-specific CD8+ T cell responses recovered (Shoukry *et al* 2003).

Peptides for endogenous MHC class I-presentation to CD8+ T cells are generated in the cytosol of APC. When viral RNA or DNA enters the cell and replicates within it, the originating messenger RNA is transcribed into proteins in the cytosol. Part of the

proteins is subsequently degraded in proteasomes and transported through the transporter associated with antigen processing (TAP) complex into the endoplasmatic reticulum, where they are further trimmed to produce peptides of 8-10 amino acids in length. The short peptides are assembled with MHC class I molecules and transported via the Golgi apparatus to the cell surface. The HLA class I molecule is composed of a β_2-microgloblin light chain and the α heavy chain, which contains the two peptide-binding domains α1 and α2 and an immunoglobulin-like domain α3. The peptide binding groove of HLA class I molecules is, in contrast to HLA class II molecules, closed by the ends of the α helixes of the α1 and α2 domains. Consequently, the closed class I groove enchases shorter peptides. Naturally, an HLA class I molecule has six pockets, of which the second and the last pocket are particularly important in determining which peptide is bound [figure 1.4; reviewed in (Klein and Sato 2000)]. This results in a restriction in the amount of peptides that can bind with high affinity to particular HLA alleles. Antigen presentation is thus a sensitive mechanism with respect to variation in the peptide sequence. It was shown that mutations in HLA-restricted epitopes can result in the abrogation of antigen presentation of a particular epitope (Timm *et al* 2007).

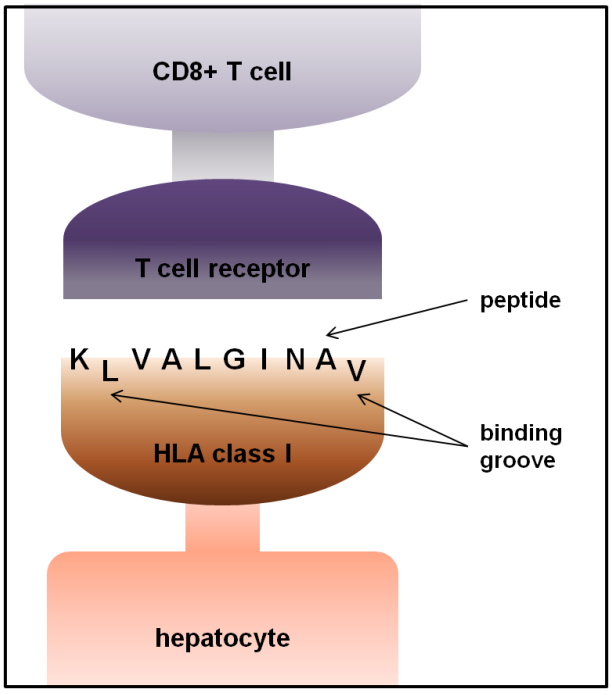

Figure 1.4 Schematic drawing of antigen-presentation to CD8+ T cells via HLA class I.

It has been highlighted in numerous studies that the CD8+ T cell response during HCV infection is relatively weak and dysfunctional.

Dysfunctional or exhausted CD8+ T cells are not able to secrete antiviral cytokines or to proliferate in response to recognition of viral antigens. CD8+ T cell exhaustion is mainly due to the up-regulation of inhibitory receptors and ligands on the cell surface of CD8+ T cells or infected cells, respectively. Among those are the programmed cell death-1 (PD-1), which was shown to be up-regulated on HCV-specific CD8+ T cells of chronically infected patients (Golden-Mason *et al* 2007, Penna *et al* 2007, Radziewicz *et al* 2007). Dual up-regulation of PD-1 and cytotoxic T lymphocyte associated antigen-4 (CTLA-4), which structurally resembles and thus competitively inhibits the co-stimulatory CD28, on HCV-specific CD8+ T cells was observed in liver infiltrating lymphocytes, which were profoundly dysfunctional (Nakamoto *et al* 2008). Furthermore, T cell immunoglobin domain and mucin domain containing molecule-3 (Tim-3) (Golden-Mason *et al* 2009), as well as the NK cell receptor 2B4 (CD244) (Schlaphoff *et al* 2011) has also been found to be up-regulated on HCV-specific dysfunctional CD8+ T cells. These finding strongly suggest that blockage of inhibitory receptor pathways represents a novel therapeutic strategy to restore HCV-specific CD8+ T cell function.

Beyond progressive dysfunction of HCV-specific CD8+ T cells mutational escape also plays a role in viral immune evasion. The combination of the strong immune pressure exerted by cells of the adaptive immunity and the low fidelity viral RNA polymerase, results in the emergence of viral escape mutations during acute phase of infection, which is associated with viral persistence (Erickson *et al* 2001, Timm *et al* 2004). However, the emergence of escape mutations is associated with a decrease in viral fitness costs and a reduced replicative capacity of the virus, which is supported by studies of Timm and colleagues showing that the transmission of an HLA-B*08-associated escape mutation to an HLA-B*08-negative patient resulted in reversion of the mutation to the consensus sequence (Timm *et al* 2004). Nevertheless, factors regulating viral escape mutations are not fully identified. As already mentioned, CD4+ T cell help during acute phase of infection might play a role (Grakoui *et al* 2003). In addition, limited diversity of T cell receptors is discussed to be associated with the emergence of escape mutations (Meyer-Olson *et al* 2004).

HCV is characterized by high levels of genetic variation between genotypes and subtypes, but also between isolates of the same subtype. On the context of HLA-allelic restriction, this high sequence diversity thus represents the main barrier for immune control. There is strong evidence for the association between particular HLA class I alleles and the outcome of infection. In a well-defined homogeneous cohort of women accidentally infected with HCV, HLA-A*03, -B*27 and -Cw*01 were associated with viral clearance, whereas HLA-B*08 was significantly associated with viral persistence (McKiernan *et al* 2004). Indeed, the protective effect associated with the presence of particular alleles can be limited to a certain HCV genotype. Studies indicate that the protective effect of HLA-B*27 is limited to HCV genotype 1 infection and does not expand to other genotypes, which was due to intergenotype sequence diversity of the immunodominant HLA-B*27-restricted epitope (Neumann-Haefelin *et al* 2010). Along with these findings, studies by Giugliano and colleagues analyzed the impact of genotype-specific sequence difference on the CD8+ T cell response in a cohort of injection drug users infected with genotype 1 and 3 and showed that the majority of CD8+ T cell responses were genotype-specific, indicating that sequence differences between genotypes are relevant at the epitope level (Giugliano *et al* 2009). In line with this, HLA-B*57 was associated with viral clearance in a cohort where genotype 1a was the predominating circulating virus. In contrast, in another cohort where genotype 1b was the predominating circulating virus HLA-B*57 was not protective (Kim *et al* 2011). The same study provided evidence, suggesting that transmission of viral strains with pre-existing mutations in HLA-B*57 epitopes attenuates viral control and that epitope variants that are less recognized abrogate the protective effect of HLA-B*57. Thus even minor sequence variations in the same subtype can abrogate CD8+ T cell responses.

1.4.3 Liver immunology

As being a hepatotropic virus, the major site of HCV replication is the liver or more precisely hepatocytes. The immunology of the liver is unique as antigen-presentation in the liver can induce immunological tolerance. This is advantageous in case of liver transplantation [(Calne *et al* 1967), reviewed in (Calne 2000)], though it remains a

major problem in hepatotropic viral infections, like HCV or hepatitis B virus (HBV) infections.

The microenvironment of the liver is characterized by the release of immunosuppressive mediators produced by both parenchymal and non-parenchymal cells, including IL-10, transforming growth factor-β (TGF-β) and prostaglandin E_2 (PGE_2), which contribute to the tolerogenic environment.

Liver-resident antigen-presenting cells include myeloid DC (mDC), pDC, liver sinusoidal endothelial cells (LSEC), hepatic stellate cells and hepatocytes. Each of those cell types contribute to tolerance induction by different strategies, which include the release of inhibitory cytokines, the expression of inhibitory molecules, like B7-H1 by pDC, LSEC, hepatocytes and stellate cells, or the failure to provide co-stimulation [reviewed in (Thomson and Knolle 2010)].

Hepatocytes function as efficient antigen-presenting cells *in vitro* (Bertolino *et al* 1998, Bertolino *et al* 1999), however, their role in antigen presentation *in vivo* has long been ambiguous. Hepatocytes are separated from the lumen of the sinusoid by a layer of fenestrated LSEC, making hepatocytes not easily accessible for circulating lymphocytes. Meanwhile, it was shown in mouse models that the combination of slow blood flow, the fenestrated endothelium and the lack of a basal membrane provide circulating lymphocytes access to hepatocytes through cytoplasmic extensions penetrating the endothelial fenestrations (Warren *et al* 2006). Nevertheless, CD8+ T cells primed by hepatocytes only undergo initial expansion, which is followed by clonal deletion due to BCL-2-interacting mediator of cell death (Bim) and caspase-dependent apoptosis (Holz *et al* 2008). Furthermore, it was demonstrated that CD8+ T cells can enter hepatocytes by a mechanism of so called suicidal emperipolesis. This might represent a unique mechanism of CD8+ T cell deletion and a process that is critical for maintenance of tolerance (Benseler *et al* 2011). Taken together, hepatocytes, which represent the primary site for HCV replication and virus-specific CD8+ T cell recognition, might play a prominent role in the failure to control HCV infection. Though, the role of hepatocytes in tolerance induction in humans is not clarified yet. Unfortunately, the lack of a suitable small animal model hampers studies for the influence of hepatocytes *in vivo*.

1.5 Vaccination trials in HCV

The development of a suitable vaccine to prevent chronic HCV infections still remains a major goal. Unfortunately, the high genetic variability of the virus makes vaccine design challenging. Indeed, 10-30 % of patients clear the virus and upon reinfection these patients have significantly increased rates of viral clearance in comparison with primary infections (83 % *vs*. 25 % in primary infection) (Osburn *et al* 2010). These results indicate that the induction of HCV-specific memory responses is possible and vaccine development reasonable.

The current vaccination studies are mainly designed to elicit an adaptive immune response, as HCV clearance require immune responses mounted by HCV-specific cellular and humoral immunity. The effort that had been made in vaccine development so far can be divided in studies that aimed the induction of T cell responses targeting non-structural proteins, and those that target the induction of neutralizing antibodies against the envelope proteins E1 and E2. The lack of a suitable animal model additionally hampered the development of suitable vaccines. Most data that study the efficacy of therapeutic vaccination have to be obtained from the chimpanzee model.

Examples for vaccine candidates that are in preclinical or early clinical development are shortly summarized in the following [reviewed in (Fauvelle *et al* 2013)]. Drane and colleagues developed a prototype vaccine with the HCV core protein and ISCOMATRIX adjuvant (HCV Core ISCOMATRIX vaccine), which has been shown to induce CD4+ and CD8+ T cell responses specific for the HCV core protein in animal models, as well as human studies. In a phase I placebo controlled study, antibody responses were detected in 23 out of 24 patients receiving different concentrations of the vaccine. CD8+ T cell responses specific for core peptides were detectable in 2 out of 8 patients (Drane *et al* 2009).

Another phase I placebo controlled study investigated the efficacy and safety of the HCV E1E2/MF59C.1 vaccine, containing the envelope proteins E1 and E2 and the adjuvant MF59C.1. Experiments revealed neutralizing antibodies, as well as CD4+ T cell responses specific for E1 and E2 but neutralizing antibody titers declined already 16 weeks after the last vaccination (Frey *et al* 2010).

Barnes and colleagues aimed to elicit HCV-specific T cells using recombinant adenoviral vectors expressing HCV non-structural proteins to achieve potential

protection in a phase I study of healthy human volunteers. Immunization with these vectors resulted in the induction of polyfunctional CD4+ and CD8+ T cells targeting multiple epitopes also across genotypes. Memory responses were detectable even one year after immunization (Barnes *et al* 2012). The latter study underlines the importance of the induction of broad and polyfunctional CD8+ T cell responses.

Creating vaccines that elicit CD8+ T cell responses covering the genetic diversity of circulating isolates, as well as infecting quasispecies still seems to be a major obstacle. Indeed, it is not clear, whether a vaccine aimed to elicit a broad cross-reactive response should contain several immunogens representing a bunch of viral targets and variants, or if it rather should contain less immunogens that are itself able to prime CD8+ T cells that are cross-reactive among viral quasispecies.

1.6 Aim of the study

It has been highlighted in numerous studies that HCV-specific CD8+ T cells play a central role in viral containment, however, CD8+ T cell responses during HCV infection are relatively weak and dysfunctional. Activation of virus-specific CD8+ T cells is a complex process, in which, the sequence of the presented antigen, the site of initial antigen-encounter by CD8+ T cells has important consequences for the quality of the CD8+ T cell response. Thus, the aim of this study was to analyze determinants of HCV-specific CD8+ T cell expansion.

HCV frequently evades adaptive immune responses by selection of escape mutations in MHC class I-restricted epitopes. These high variations in the viral genome make the development of a suitable vaccine challenging. An effective vaccine should induce CD8+ T cell responses that i) are polyfunctional with respect to effector mechanisms and ii) highly cross-reactive. Thus, it is of great importance to study the global sequence diversity of immunodominant CD8+ T cell epitopes and its impact on primary T cell responses.

To study the impact of sequence differences in immunodominant epitopes on *de novo* immune responses, one aim of this dissertation was to establish a protocol for the priming and expansion of HCV-specific CD8+ T cells from the naïve T cell repertoire. The impact of the immunodominant HLA-A*02-restricted HCV $NS3_{1406-1415}$ CD8+ T cell epitope on CD8+ T cell priming was analyzed. For this, the most frequent HCV $NS3_{1406-1415}$ sequence variants were selected from the HCV sequence database. The present study included in total five $NS3_{1406-1415}$ genotype 1b variants KLSGLGLNAV, KLSSLGLNAV, KLSALGLNAV, KLSALGVNAV and QLSGLGINAV, as well as two genotype 1a variants KLVALGINAV and KLVALGVNAV. In addition to the analysis of the priming potential, $NS3_{1406-1415}$-specific CD8+ T cells were analyzed for their potential to cross-react with other epitope variants and their polyfunctionality. To understand the nature of T cell receptors specific for the variants the individual TCR variable beta (Vβ) chain profiles used were assessed using a comprehensive antibody panel.

A hallmark of chronic infections with hepatotropic viruses, such as HCV is the notoriously weak antiviral immune response by CD8+ T cells. As a hepatotropic virus, the main target and site of replication of HCV are hepatocytes. It is believed that the

liver-specific environment can induce tolerance of CD8+ T cells. Direct antigen presentation by virally infected hepatocytes thus might inhibit the CD8+ T cell response. Hence, the second aim of the study was to analyze the role of primary human hepatocytes on the activation of virus-specific CD8+ T cells, which may give important hints for the establishment of chronic infections by hepatotropic viruses.

2 Materials

2.1 Chemicals and reagents

Biocoll Separating Solution	Biochrome
CD14 MicroBeads	Miltenyi Biotech
CD45RO MicroBeads	Miltenyi Biotech
CD57 MicroBeads	Miltenyi Biotech
Dimethyl sulfoxide (DMSO)	Roth
Dulbecco's phosphate-buffered saline, 1X (PBS)	Gibco
Ethanol	Sigma-Aldrich
FACS-Clean	Becton Dickinson
FACS-Flow	Becton Dickinson
FACS-Rinse	Becton Dickinson
Intracellular (IC) Fixation Buffer	eBioscience
Isopropanol	Sigma-Aldrich
Permeabilization Buffer (10X)	eBioscience

2.2 Cell culture media and additives

anti-CD28/ CD49d	Becton Dickinson
anti-TGF-β antibody	Becton Dickinson
activin receptor-like kinase-5 (ALK-5) inhibitor (SB-431542)	Sigma Aldrich
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 1M	PAA
Brefeldin A	Sigma Aldrich
DNase I	Roche
Fetal bovine serum (FBS)	Biochrome
GMP CellGro® DC	CellGenix™
Granulocyte-macrophage colony-stimulating factor, recombinant human	Peprotech
Ham´s F12 + L-glutamine	PAA
human serum (HS), off the clot, type AB	PAA

Interferon-γ, recombinant human	Promokine
Interleukin-1β, recombinant human	Promokine
Interleukin-15, recombinant human	Peprotech
Interleukin-2, recombinant human	Roche
Interleukin-21, recombinant human	Peprotech
Interleukin-4, recombinant human	Peprotech
Interleukin-7, recombinant human	Peprotech
Interleukin-6, recombinant human	Peprotech
Lipopolysaccharide (LPS)	Sigma Aldrich
Medium 200 + LSGS kit	Invitrogen
Non-essential amino acids (100X)	PAA
Penicillin/ streptomycin (100X)	PAA
Prostaglandin E_2	Sigma Aldrich
RPMI 1640 + L-glutamin	PAA
Tryphan Blue	Gibco BRL/Invitrogen
Trypsin/ EDTA (100X)	PAA
TNF-alpha, recombinant human	Promokine

2.3 Cell culture media composition

Table 2.1 Cell Culture media composition.

Media	Composition
DC medium	GMP Serum-free Dendritic Cell Medium 1 % HS 40 U/ mL penicillin 40 µg/ mL streptomycin
Freezing medium	FBS 10 % DMSO
human umbilical vein endothelial cells (HUVEC)	Medium 200 + LSGS Kit (commercial); Invitrogen
primary human hepatocytes	DMEM/ Ham´s F12 10 % FBS 100 U/ mL penicillin 100 µg/ mL streptomycin
R10 Peripheral blood mononuclear cells (PBMC)	RPMI 1640 10 % FBS 10 mM HEPES 100 U/ mL penicillin 100 µg/ mL streptomycin
T cell medium	GMP Serum-free Dendritic Cell Medium 5 % HS 40 U/ mL penicillin 40 µg/ mL streptomycin

2.4 Commercial kits

CD8+ T Cell isolation kit, human	Miltenyi Biotech
Human IL-10 ELISA Kit	eBioscience
Human/ mouse TGF-β1 ELISA Kit	eBioscience
Human TGF-β1 ELISA Kit	Promokine
Monocyte isolation kit, human	Miltenyi Biotech
Qiamp DNA Blood Mini Kit	Qiagen

2.5 Peptides and MHC class I-multimers

All peptides were synthesized by EMC microcollections. Peptides used for T cell priming had a purity of ≥ 95 %. CEF pool, Influenza A virus (Flu) and cytomegalovirus (CMV) peptide had a purity of ≥70 %.The lyophilized peptides were dissolved in DMSO to 20 mg/ mL and stored at -80 °C. Working solutions were further diluted with medium without supplements to a concentration of 1 mg/ mL and stored at -20 °C.
All fluorescently labeled phycoerythrin (PE) MHC class I-dextramers were purchased from Immudex (Denmark) and were stored at 4 °C in the dark (peptides and dextramer are listed in table 2.2 and table 2.3, respectively).

Table 2.2 Peptides.

Name	Sequence	Protein	Location	Variant	HLA-allele restriction
p11	KLVALGINAV	HCV NS3	1406-1415	gt 1a prototype	HLA-A*02
p42	KLSGLGLNAV	HCV NS3	1406-1415	gt 1b consensus	HLA-A*02
p43	KLSALGLNAV	HCV NS3	1406-1415	gt 1b Japan	HLA-A*02
p45	QLSGLGINAV	HCV NS3	1406-1415	gt 1b Anti-D	HLA-A*02
p295	KLVALGVNAV	HCV NS3	1406-1415	gt 1a escape	HLA-A*02
p301	KLSALGLNAV	HCV NS3	1406-1415	gt 1b escape	HLA-A*02
p302	KLSALGVNAV	HCV NS3	1406-1415	gt 1b Japan	HLA-A*02
p296	ALYDVVSKL	HCV NS5B	2594-2602	gt 1a	HLA-A*02
p339	ALYDVVSTL	HCV NS5B	2594-2602	gt 1b	HLA-A*02
CMV	NLVPMVATV	matrix protein	495-503		HLA-A*02
Flu	GILGFVFTL	Influenza A	58-66		HLA-A*02
CEF pool	32 peptides	CMV, EBV, Influenza	---	---	defined HLA types

Table 2.3 MHC class I-dextramers.

Name	Sequence	Protein	Location	Variant	HLA-allele restriction
p11	KLVALGINAV	HCV NS3	1406-1415	gt 1a consensus	HLA-A*02
p42	KLSGLGLNAV	HCV NS3	1406-1415	gt 1b consensus	HLA-A*02
p43	KLSALGLNAV	HCV NS3	1406-1415	gt 1b Japan	HLA-A*02
p45	QLSGLGINAV	HCV NS3	1406-1415	gt 1b Anti-D	HLA-A*02
p295	KLVALGVNAV	HCV NS3	1406-1415	gt 1a escape	HLA-A*02
p301	KLSALGLNAV	HCV NS3	1406-1415	gt 1b escape	HLA-A*02
p302	KLSALGVNAV	HCV NS3	1406-1415	gt 1b Japan	HLA-A*02
p296	ALYDVVSKL	HCV NS5B	2594-2602	gt 1a	HLA-A*02
p339	ALYDVVSTL	HCV NS5B	2594-2602	gt 1b	HLA-A*02
CMV	NLVPMVATV	matrix protein	495-503		HLA-A*02
Flu	GILGFVFTL	Influenza A	58-66		HLA-A*02

2.6 Antibodies for flow cytometry

All antibodies used in this study were anti-human antibodies and stored at 4 °C. The concentrations applied for the antibodies utilized in this study can be seen in table 2.4.

Table 2.4 Fluorochrome conjugated antibodies for flow cytometry.
Phycoerythrin (PE), allophycocyanin (APC), fluorescein isothiocyanat (FITC), peridinin chlorophyll protein (PerCP), cyanine (Cy), 7-Aminoactinomycin D (7-AAD).

Antigen	Conjugate	Concentration; Dilution	Company
CD4	PE	0.2 mg/ mL; 1:1000	BD/ eBioscience
	FITC	1:100	BD
	PE-CF594	1:500	BD
CD8	APC	1:1000	BD/ eBioscience
	PerCP Cy5	1:400	eBioscience
	Alexa700	0.2 mg/ mL; 1:100	eBioscience
CD45RA	PE	1:200	Miltenyi Biotech
CD56	APC	1:100	BD
CD107a	PE	1:20	BD
IFN-γ	FITC	0.5 mg/ mL; 1:1000	eBioscience
	PE-Cy7	1:500	eBioscience
Interleukin-2	PerCP-eFluor710	0.012 mg/ mL; 1:50	eBioscience
MIP-1β	PE-Cy7	1:50	BD
TNF-α	APC	0.2 mg/ mL; 1:1000	eBioscience
viability dye, fixable	eFluor780	1:5000	eBioscience
7-AAD	PerCP	1:100	eBioscience
V alpha 24	FITC		Beckman Coulter
V beta 1	PE	1:200	Beckman Coulter

V beta 2	FITC	1:100	Beckman Coulter
V beta 3	FITC	1:20	Beckman Coulter
V beta 4	FITC	1:20	Beckman Coulter
V beta 5.1	APC	1:100	eBioscience
V beta 5.2	PE	1:10	Beckman Coulter
V beta 5.3	APC	1:100	eBioscience
V beta 7.1	PE	1:20	Beckman Coulter
V beta 7.2	PE	1:100	Beckman Coulter
V beta 8	eFluor450	1:500	eBioscience
V beta 9	APC	1:100	eBioscience
V beta 11	APC	1:20	Beckman Coulter
V beta 12	FITC	1:20	Beckman Coulter
V beta 13.1	FITC	1:100	eBioscience
V beta 13.2	PE	1:100	eBioscience
V beta 13.6	PE	1:20	Beckman Coulter
V beta 14	PE FITC	1:5	Beckman Coulter
V beta 16	FITC	1:100	Beckman Coulter
V beta 17	PE	1:20	Beckman Coulter
V beta 18	PE	1:20	Beckman Coulter
V beta 20	FITC	1:10	Beckman Coulter
V beta 21.3	FITC	1:10	Beckman Coulter
V beta 22	FITC	1:50	Beckman Coulter
V beta 23	PerCP-eFluor710	1:50 0.1 mg/ mL	eBioscience

2.7 Consumables and equipment

Cell culture flasks, T25, T75	Greiner Bio-One
Cell culture inserts for 24-well plates, 0.4 µm, translucent PET membrane	BD Falcon™
Cell culture plates (6-, 12-, 24-, 48-, 96-well)	Greiner Bio-One
Centrifuge 5415C	Eppendorf
Centrifuge 5415R	Eppendorf
Collagen I 24-well multiwell plates	BD BioCoat™
Cryo tubes, 2.0 mL	Greiner Bio-One
MidiMACS separator	Miltenyi Biotech
MACS MultiStand	Miltenyi Biotech
Polystyrene round-bottom tubes, 5 mL	BD Falcon™
Leucosep™ tubes, 50 mL	Greiner Bio-One
LS-and LD-columns	Miltenyi Biotech
Megafuge 1.0R	Heraeus
Megafuge 40R	Thermo Scientific
Microscope TMS	Nikon
Microscope Primo Vert	Zeiss
Mr. Frosty freezing container	Thermo Scientific

Flow cytometer

FACS Calibur	Becton Dickinson
FACS Canto II	Becton Dickinson
Navios flow cytometer	Beckman Coulter

2.8 Software and webpages

FlowJo 7.6	Tree Star, Inc. 1997-2008
GraphPad Prism	GraphPad Software, Inc
NCBI Homepage	http://www.ncbi.nlm.nih.gov/
Microsoft Office	Microsoft
Pestle	VCR, NIH
SPICE	Nozzi/ Roederer

3 Methods

3.1 Peripheral blood mononuclear cell isolation from buffy coats of healthy volunteer blood donors

Buffy coats from healthy blood donors were obtained from the institute of transfusion medicine, university hospital Essen. For the isolation of peripheral blood mononuclear cells (PBMC) from buffy coats, first Leukosep^{TM} tubes were equilibrated with 15 mL Percoll, a silica colloid density gradient medium with a density of 1.077 kg/ L and spun down for one minute at 470xg. On the top of each filter, 30 mL of the blood product was carefully applied and centrifuged for 10 minutes at 1000xg. After centrifugation, the solution above the filter membrane containing the leukocytes was transferred to a new 50 mL Falcon tube. The Leukosep^{TM} tube containing the remaining red blood cells and cell debris was discarded. PBMC were washed with 45 mL PBS and additionally centrifuged for 8 minutes at 830xg. After discarding the supernatant the cell pellet was pooled in PBS and again centrifuged for 8 minutes at 830xg. After these three purification and washing steps the cells could directly be used for cell culture or be dissolved in freezing medium, i.e. FBS containing 10 % DMSO for freezing in liquid nitrogen. For freezing, the cells were placed in a Mr. Frosty freezing container containing 100 % isopropyl alcohol to gently cool the cells down by ~1 °C/ minute in a -80 °C freezer. After overnight freezing of the cells, the cells could now be placed in liquid nitrogen tanks.

For these studies mainly PBMC from HLA-A*02-positive donors were required. Testing for the presence of HLA-A*02 molecules on the cell surface was performed using a fluorochrome-conjugated HLA-A*02-specific antibody. In brief, PBMC were resuspended in 10 µL PBS/ 1 % FBS containing 2 µL of anti-HLA-A*02 FITC antibody and incubated for 15 minutes at 4 °C. After washing, the cells were analyzed for the presence of HLA-A*02 by flow cytometry.

3.2 Thawing of PBMC for primary cell culture

One vial of frozen PBMC was taken out of liquid nitrogen and quickly thawed in a 37 °C water bath. Cells were taken up in 10 mL medium containing 1 mg DNase I. After three washing- and centrifugation steps (530xg, 7 minutes), the cell number was determined by counting in a Neubauer hemocytometer. Aliquots of the cell suspension were diluted with Tryphan blue stain and 10 µL of the diluted aliquot solution were transferred onto a Neubauer hemocytometer. Viable cells were counted and the number of cells per mL was calculated as follows:

$$\textit{Number of cells/ mL} = \textit{number of cells in the large square} \times \textit{dilution factor} \times 10^4$$

After the last washing step, the cell pellet was resuspended in medium supplemented with the required cytokines or peptides and placed in cell culture plates or flasks at a concentration of 1.5×10^6 cells/ mL. Cells were cultured at 37 °C in humidified atmosphere containing 5 % CO_2. Every 2-3 days the cells were fed with fresh medium containing cytokines and/ or growth factors.

3.3 Intracellular cytokine staining for the detection of IFN-γ secretion

For intracellular cytokine staining (ICS) 2×10^5 cells were seeded in a round bottom 96-well plate, washed once with 200 µL medium without supplements and centrifuged for 5 minutes at 470xg. For the stimulation of IFN-γ secretion 100 µL medium (plus antibiotics and serum) containing 10 µg/ mL peptide were added to each well and incubated for 5 hours at 37 °C/ 5 % CO_2 in the presence of 10 ng/ mL Brefeldin A. After incubation, the cells were washed with PBS and centrifuged for 5 minutes at 470xg. All following centrifugation steps were performed with these parameters. For the staining of dead cells, fixable viability dye eFluor780 was diluted 1:5000 in 100 µL PBS and applied to the cells for 20 minutes at 4 °C. After washing, fluorochrome-conjugated antibodies directed against CD4 and CD8 were added and incubated for another 15 minutes at 4 °C. After incubation the cells were washed and fixed using fixating solution for 20 minutes at 4 °C. Afterwards, the cells were washed once with 200 µL Permeabilisation buffer and centrifuged. Cells were stored overnight at 4 °C or directly stained with antibodies targeting intracellular structures

and molecules, like IFN-γ, which is trapped in the endoplasmatic reticulum. For intracellular staining of IFN-γ the cells were incubated with 0.5 mg/ mL anti-human IFN-γ FITC in 100 µL Perm buffer for 30 minutes at 4 °C. After washing, the cells were transferred in 5 mL Polystyrene tubes suitable for the application to flow cytometers. After fluorescent cytometry, data were evaluated using FlowJo software. For analysis, following gating strategy was applied: cells positive for the viability dye (dead cells) as well as CD4 positive cells were excluded by gating on negative cells, which are then further analyzed for the presence of CD8 and IFN-γ. Further statistical analysis was performed using GraphPad Prism software.

3.4 Experimental setup for the priming of CD8+ T cells using monocyte-derived dendritic cells

3.4.1 Generation of monocyte-derived dendritic cells

3.4.1.1 Isolation of CD14+ monocytes from PBMC

For the enrichment of monocytes several technical capabilities are possible. In the present study, monocytes were isolated either by their ability to adhere to plastic or by magnetic cell separation (MACS), i.e. CD14+ MicroBeads or untouched isolation. The purity of the enriched cell fraction was determined by flow cytometry according to their location in a forward/ sideward scattered dot blot.

3.4.1.1.1 Enrichment of monocytes by adherence to plastic

For the isolation of monocytes, ~$2x10^8$ PBMC were thawed in 20 mL CellGro (0.4 % penicillin/ streptomycin). Cells were centrifuged for 4 minutes at 530xg and resuspended in 10 mL CellGro. Cell number was determined and the cells were incubated at a concentration of $2x10^7$ cells/ well in 2 mL CellGro on a 6-well cell culture plate for 2-3 hours at 37 °C/ 5 % CO_2. Monocytes were isolated by their ability to adhere to plastic. After incubation, the upper cell layer was carefully removed, while trying not to scratch the plastic surface. The remaining adhered cells represented the enriched monocyte fraction.

3.4.1.1.2 Isolation of CD14+ monocytes by magnetic cell separation

For the isolation of monocytes by magnetic cell separation, two possible methods were applied. First, monocytes were isolated by direct labeling of CD14+ cells with a MicroBead-conjugated CD14-antibody.
In brief, PBMC were thawed, washed and cell number was determined. 80 µL PBS/ 1 % human serum (HS) and 10 µL CD14-MicroBeads per 10^7 were added to the cells and incubated for 15 minutes on ice. Afterwards, the suspension was filled up to 5 mL and centrifuged for 5 minutes at 530xg. One LS column was rinsed with 3 mL PBS/ 1 % HS. The cell pellet was resuspended in 0.5 mL PBS/ 1 % HS and applied onto the column. The column was washed three times with 3 mL PBS/ 1 % HS. The flow-through representing the unlabeled cells was discarded. To elute the bound CD14+ cells, the column was removed from the separator and 5 mL PBS/ 1 % HS were applied. Quickly, the plunger was pressed into the column and the CD14+ monocytes were collected.

In addition, highly enriched unlabeled monocytes were obtained by depletion of the magnetically labeled cells using a cocktail of biotin-conjugated antibodies (i.e. anti-CD3-, anti-CD7-, anti-CD16-, anti-CD19-, anti-CD56-, anti-CD123- and anti-glycophorinA-antibodies, representing T-, NK-, B- and dendritic cells and basophils) as well as anti-Biotin MicroBeads. In brief, 30 µL PBS/ 1 % HS, 10 µL FcR Blocking reagent and 10 µL Biotin-antibody cocktail per 10^7 cells were added to the cells, mixed well and incubated for 10 minutes at 4 °C. 30 µL PBS/ 1 % HS and 20 µL anti-Biotin MicroBeads per 10^7 cells were added and incubated for additional 15 minutes at 4 °C. The cells were washed by adding 2 mL PBS/ 1 % HS and centrifuged. The cell pellet was resuspended in 500 µL PBS/ 1 % HS and isolation of unlabeled monocytes was achieved by depletion of the magnetically labeled cells as described above.

3.4.1.2 Maturation of immature monocyte-derived dendritic cells

After isolation the monocytes were differentiated into monocytes-derived DC (moDC) by the addition of 1000 U/ mL IL-4 and 800 U/ mL GM-CSF. After 48 hours, 1.5 mL CellGro containing 1 % HS, 1000 U/ mL IL-4 and 1600 U/ mL GM-CSF were

added. The next day, non-adherent cells, representing immature moDC can be harvested and seeded at a concentration of 1-2x10^6 cells/ mL in 1 mL or 2 mL DC medium in a 12- or 6-well plate, respectively.

In the present study, dendritic cells were either matured by a cytokine cocktail, i.e. 1 µg/ mL PGE_2, 10 ng/ mL IL-1β, 1000 U/ µL IL-6 and 10 ng/ mL TNF-α or a combination of 100 ng/ mL LPS and 100 U/ mL IFN-γ. Dendritic cells were matured overnight either in the presence or absence of the corresponding peptides, i.e. variants of the HCV $NS3_{1406-1415}$ epitope at a concentration of 1 µg/ mL.

Successful maturation of the dendritic cells was assessed by upregulation of the costimulatory molecules CD80 and CD86, as well as by microscopical appearance of dendritic cells, i.e. adherence, enlongated cell shape and the presence of dendrites.

After overnight maturation, non-adherent cells representing immature dendritic cells were carefully removed and 2 mL cold PBS/ 1 % HS were added to the 6-well plate. After incubation for 30 minutes at 4 °C, dendritic cells were detached by resuspending. Large cells consisting of mature dendritic cells were counted. Because of few possible contaminating other cell types, e.g. NK cells and memory cells, the dendritic cells were irradiated with 30 Gy to avoid outgrowth. Irradiation was done at the institute of medical radiation biology at the university hospital Essen.

3.4.2 Isolation of naïve CD8+ T cells from PBMC

For CD8+ T cell isolation, CD8+ T Cell isolation kit was used according to manufactures instructions. In brief, PBMC were thawed as previously described (see 3.2). After determining the cell number, 40 µL PBS/ 1 % HS and 10 µL CD8+ T cells biotin-antibody cocktail (containing biotin-labeled antibodies directed against CD4+ T cells, γ/ δ T cells, B cells, NK cells, dendritic cells, monocytes, granulocytes, and erythroid cells) were added per 10^7 cells and incubated for 5 minutes on ice. 30 µL PBS/ 1 % HS and 20 µL CD8+ T cells MicroBead-cocktail per 10^7 cells were added and incubated for 15 minutes on ice. Per 10^8 cells, one LS column was prepared by rinsing with 3 mL PBS/ 1 % HS. The cell suspension was applied onto the column and the unlabeled cell-containing flow-through, representing the enriched CD8+ T cells, was collected. The column was washed twice with 3 mL PBS/ 1 % HS. The

cells that attached to the magnetic column, representing the non-CD8+ T cells were discarded.

After isolation of CD8+ T cells, CD57 positive cells, representing NK cell subsets and late stage effector CD8+ T cells, as well as CD45RO positive cells, representing activated and memory T cells were removed by depletion with specific antibodies. 40 µL PBS/ 1 % HS and 10 µL CD57 and CD45RO MicroBeads per 10^7 cells were mixed with enriched CD8+ T cells and incubated for 15 minutes on ice. Afterwards, the cells were centrifuged for 4 minutes at 470xg. 500 µL of the cell suspension were applied onto the LD-column, which was previously rinsed with 3 mL PBS/ 1 % HS. After flow through of the cell suspension the column was washed 3 times with 3 mL PBS/ 1 % HS. The flow through representing the naïve CD8+ T cell population was collected, washed and the cell number was determined. Naïve CD8+ T cells were incubated overnight in 3 mL CellGro containing 5 % HS and 5 ng/ mL IL-7.

3.4.3 Co-culture of irradiated peptide-pulsed dendritic cells plus naïve CD8+ T cells

Overnight cultures of naïve CD8+ T cells were harvested and counted. CD8+ T cells were adjusted to a concentration of $1x10^6$ cells/ mL in CellGro.

Irradiated dendritic cells were added at a ratio of 4:1 CD8+ T cells:DC with $4x10^5$ CD8+ T cells. The CD8+ T cell-DC suspension was centrifuged and resuspended in 0.5 mL CellGro supplemented with 5 % HS and 30 ng/ mL IL-21 and placed in a 48-well plate. For each peptide variant the co-culture was initiated in duplicate, if possible. After 72 hours, the cells were fed with 0.5 mL CellGro supplemented with 5 % HS, 5 ng/ mL IL-7 and IL-15 and cells were subsequently fed every 2-3 days. To support cellular expansion, the cells were transferred to 12-well plates on day 5 of co-culture and to 6-well plates on day 7.

3.4.4 Restimulation of co-culture

After 10 and 19 days of co-culture, the cells were restimulated with either homologous peptide-pulsed PBMC or monocytes. When using peptide-loaded PBMC for restimulation, the cells were irradiated prior restimulation to avoid outgrowth of contaminating and inhibiting cells types.

3.4.4.1 Restimulation of CD8+ T cell/ DC co-culture with autologous irradiated peptide-pulsed PBMC

A maximum of $1x10^7$ PBMC/ per well of co-culture was used for restimulation of the co-culture. PBMC were quickly thawed in 20 mL CellGro. The cells were centrifuged for 4 minutes at 530xg and resuspended in 10 mL CellGro. $1x10^7$ cells were incubated at a concentration of $2x10^6$ cells/ mL in T cell medium supplemented with the corresponding peptide at a final concentration of 1 mg/ mL. After incubation for 2-3 hours at 37 °C/ 5 % CO_2, the cells were washed twice and irradiated with 30 Gy. The co-culture was harvested and mixed with the irradiated, peptide-pulsed PBMC. After centrifugation, the cells were resuspended in 3 mL T cell medium. After 48 hours, the cells were fed with T cell medium supplemented with 100 U/ mL IL-2 and 5 ng/ mL IL-7 and IL-15 and were subsequently fed every 2-3 days.

3.4.4.2 Restimulation of co-culture with autologous peptide-pulsed CD14+ monocytes

For the restimulation with peptide-loaded monocytes, CD14+ monocytes were isolated by positive selection as described (see 3.4.1.1.2). $0.5x10^6$ monocytes were pulsed with 1 µg/ mL of the corresponding peptide in 2 mL CellGro supplemented with 1 % HS, 1000 U/ mL IL-4 and 800 U/ mL GM-CSF at 37 °C/ 5 % CO_2 overnight. The next day, the monocytes were harvested and mixed with the 10 or 19 day co-culture, respectively and continued as described in 3.4.4.1.

3.4.5 Analysis of peptide-specific CD8+ T cells by MHC class I-multimer staining

After 26 days of co-culture, cultured cells were analyzed for the presence of peptide-specific CD8+ T cells, as well as their ability to cross-recognize the other variants. For dextramer staining, primed CD8+ T cells were harvested and the cell number was determined. $2x10^5$ cells were transferred to a 96-well plate, washed with 200 µL PBS/ 1 % HS and centrifuged for 5 minutes at 470xg. 10 µL PBS/ 1 % HS containing 1 µL of the corresponding dextramer were applied and cells were incubated for 30 minutes at room temperature (RT) in the dark. After incubation, cells were washed with 200 µL PBS/ 1 % HS and centrifuged for 5 minutes at 470xg. For the surface staining, anti-CD8 APC antibody was diluted in PBS/ 1 % HS and incubated for 20 minutes at 4 °C. After another washing step, 7-AAD PerCP was applied for 10 minutes at 4 °C. The cells were transferred in 5 mL polystyrene tubes and analyzed by flow cytometry.

3.4.6 Analysis of effector molecule production by intracellular cytokine staining for IL-2, TNF-α, IFN-γ, MIP-1β and the degranulation marker CD107a

For intracellular cytokine staining, first CD14+ monocytes were isolated as described in 3.4.1.1.2 and pulsed with the corresponding peptides. For each well, $1x10^5$ monocytes were seeded and incubated with the peptides at a final concentration of 1 µg/ mL in 2 mL CellGro supplemented with 1 % HS, 1000 U/ mL IL-4 and 800 U/ mL GM-CSF at 37 °C/ 5 % CO_2 overnight. The peptide-pulsed monocytes were used for the restimulation of CD8+ T cells the next day.

The DC-primed CD8+ T cells were harvested and the cell number was determined. A total of $2x10^5$ cells were transferred to the required number of wells on a 96-well plate. The cells were washed and supplemented with T cell medium containing IL-2, IL-7 and IL-15 as already described (see 3.4.4.1).

The next day, peptide-pulsed monocytes were mixed with corresponding CD8+ T cells in T cell medium and incubated in the presence of 10 ng/ mL Brefeldin A and 50 µL/ mL CD107a-antibody for 5 hours at 37 °C/ 5 % CO_2. Table 3.1 shows an example of a 96-well plate used for ICS.

Table 3.1 Pipetting scheme for intracellular cytokine staining.

monocytes +peptides / Co-culture	1 unstim (-)	2 p11	3 p42	4 p43	5 p45	6 p295	7 p301	8 p302	9	10	11	12
A p11												
B p42												
C p43												
D p45												
E p295												
F p301												
G p302												
H												

After 5 hours of incubation, cells were centrifuged and washed with 200 µL PBS. For viability staining, cells were incubated for 20 minutes at 4 °C in 100 µL of fixable viability stain (-eFluor780). For surface staining, cells were incubated with anti-CD8 Alexa700 and anti-CD4 PE-CF594 for 20 minutes at 4 °C. After incubation, cells were washed and centrifuged. For fixation, 100 µL IC Fixation Buffer was added and incubated for another 20 minutes at 4 °C. After fixation, the cells can be stored at 4 °C overnight. For the staining of intracellular trapped cytokines, the following antibodies were used in 100 µL PBS/ 1 % HS for 30 minutes at 4°C: anti-IL-2 PerCP-eFlour710, anti-TNF-a APC, anti-MIP-1β PE-Cy7 and anti-IFN-γ FITC. After intracellular staining, the cells were washed and resuspended in 200 µL PBS, transferred to 5 mL polystyrene tubes and analyzed by flow cytometry. Boolean gating was used to create a full array of all possible response patterns. Data were further analyzed using SPICE and Pestle software programs (by Mario Roederer and Joshua Nozzi, NIAID, NIH).

3.4.7 Analysis of the peptide-specific T cell receptor V beta repertoire

To further analyze the antigen specific T cell responses, we implemented a comprehensive antibody panel directed against individual variable beta (Vβ) chain TCR. The panel contained 27 different monoclonal antibodies which span approximately 70 % of the Vβ repertoire.

For this purpose, $2x10^5$ cells were stained simultaneously with antibodies against CD8 and IFN-γ and a set of 2-3 antibodies directed against TCR Vβ families as listed

in table 2.4. Most of the antibodies directed against TCR families were labeled with FITC, PE, or APC. CD8+ T cells responding to peptide restimulation were selected based on their IFN-γ secretion and further analyzed for their Vβ use using FACS Canto II.

3.4.8 Analysis of the variant-specific avidities to the HLA-A*02 molecule

Peptide binding studies were performed at the Sanquin Company in Amsterdam, the Netherlands. In brief, MHC class I-molecules are loaded with conditional ligands to form stable complexes. These ligands degrade during exposure to UV light, making the resulting empty MHC molecules suitable to be loaded with arrays of peptide ligands to determine MHC binding properties (Toebes *et al* 2006).

3.5 Primary human hepatocyte cultures

Primary human hepatocyte (PHH) cultures were a kind regular gift from the department of gastroenterology and hepatology at the university hospital in Essen. The hepatocyte isolation procedure was carried out by a modified two-step collagenase perfusion technique and several Percoll centrifugation steps. The obtained hepatocytes were seeded on a collagen-I-coated 24-well plate and further purified by washing. Afterwards, the cells reached a ~90 % confluence with approximately $4x10^5$ cells per well. The data of the patients used in this study are summarized in the appendix (see chapter 9).

Before use, every hepatocyte culture was examined for their microscopically appearance, i.e. viability, number, polygonal shape, round prominent nuclei, contamination of other cell types and proliferation of fibroblastic cells. Before application, the cells were washed with medium. For all experiments DMEM/ Ham´s F12 medium, supplemented with 10 % FBS, 100 U/ mL penicillin and 100 µg/ mL streptomycin was used.

3.5.1 Co-culture of PHH and PBMC

Co-cultures of PHH and PBMC where accomplished in different compositions. If not stated different, $1.5x10^6$ PBMC from healthy HLA-A*02 positive donors where used for the experiments. As viral model antigens, either the HLA-A*02-restricted Influenza M1 epitope (Flu) or CMVpp65 epitope (CMV) was used at a final concentration of 1 µg/ mL. After 72 hours of co-culture, the cells were initially fed with fresh medium supplemented with 100 U/ mL IL-2 and 5 ng/ mL IL-7 and IL-15. Feeding was subsequently repeated every 2-3 days. After 7 to 10 days of co-culture, PBMC were harvested from the co-culture and used for MHC class I-multimer staining and/ or ICS for IFN-γ as already described in 3.4.5 and 3.3, respectively.

4 Results

4.1 *In vitro* T cell priming of HCV-specific CD8+ T cells

HCV is characterized by high levels of genetic variation between genotypes and subtypes but also between isolates of the same subtype. This enormous sequence diversity represents a major obstacle to vaccine design. Thus, it is of great importance to study the global sequence diversity of immunodominant CD8+ T cell epitopes and its impact on primary T cell responses. The obtained results will give important insights in the choice of effective sequences for vaccine design.

In contrast to the expansion of memory T cell responses, the induction of primary T cell responses from naïve precursors *in vitro* requires contact between T cells and dendritic cells. As naïve T cells have more stringent activation and costimulatory requirements, the initial encounter of a peptide-MHC complex that is recognized by a specific T cell receptor will only result in full T cell activation (characterized by induction of activation markers, cytokine secretion, and proliferation) upon co-stimulation provided by mature DC.

4.1.1 Monocytes isolated by adherence to plastic are differentiated to dendritic cells

To study the impact of sequence differences in immunodominant epitopes on *de novo* immune responses, one aim of this dissertation was to establish a protocol for the priming and expansion of HCV-specific CD8+ T cells from the naïve T cell repertoire.

For *in vitro* CD8+ T cell priming, monocyte-derived dendritic cells (moDC) were used for the priming of naïve CD8+ T cells. Therefore, monocytes were enriched from PBMC from healthy HLA-A*02 blood donors (see 3.4.1.1). To analyze which strategy is optimal to enrich monocytes from PBMC, three different strategies were applied. The first approach was the isolation of CD14+ monocytes using CD14 MicroBeads followed by magnetic separation. The second magnetic separation approach included negative selection of CD14+ cells with MicroBeads directed against, e.g. CD4, CD8 and CD19 positive cells (monocyte isolation kit). The last method took

advantage of the ability of monocytes to adhere to plastic. The latter method represents a good way to isolate monocytes from PBMC, as the cells are untouched and are less stressed by staining and isolation protocols. Figure 4.1 shows representative dot plots comparing the three strategies.

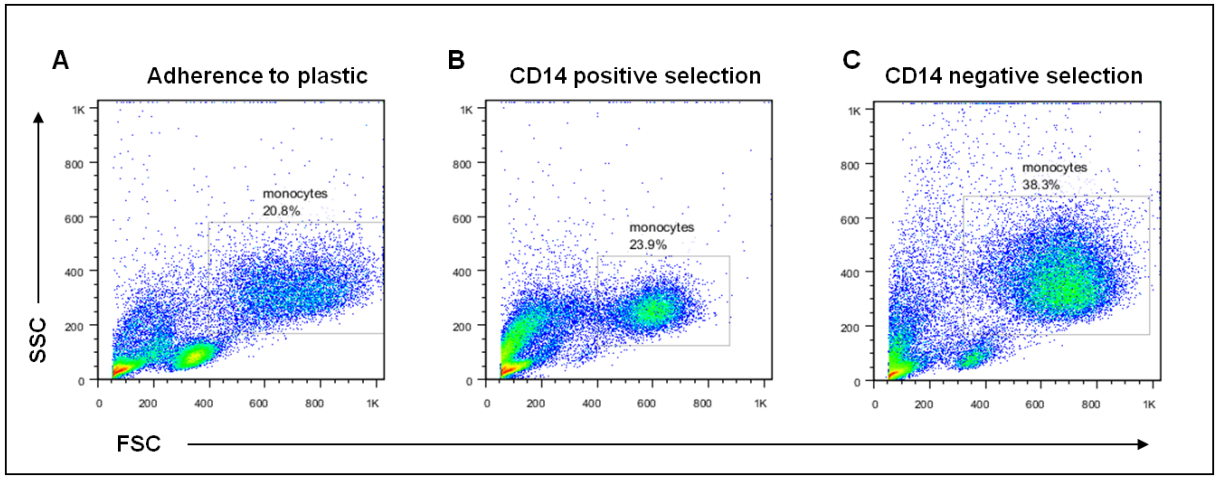

Figure 4.1 Different methods to isolate CD14+ monocytes.
Monocytes were enriched by (A) adherence to plastic, (B) CD14+ MicroBeads, or (C) depletion of non-CD14+ cells using MicroBeads. Sideward scatter (SSC), forward scatter (FSC).

Magnetic separation resulted in purities of 23.9 % monocytes when using positive selection and 38.3 % monocytes by depleting non-CD14+ cells (figure 4.1B and C). Monocytes isolated by adherence to plastic resulted in a purity of 20.8 % monocytes from total PBMC. Monocytes isolated by negative selection using the monocyte isolation kit (figure 4.1C) gave the highest purity in comparison to the other strategies. Monocytes isolated by positive section using CD14 MicroBeads were comparable in terms of purity (figure 4.1B), however, those cells have been coupled to antibodies/ MicroBeads, which might interfere with subsequent experiments. Monocytes isolated by their ability to adhere to plastic had lesser purities (figure 4.1A) but are untouched and will be further purified in the following differentiation process to monocyte-derived dendritic cells. In addition, the latter approach represented the most sensitive and cost-effective isolation procedure and will be used for the generation of moDC throughout the study.

4.1.2 LPS/ IFN-γ-maturated dendritic cells upregulate the costimulatory molecules CD80 and CD86

Primary stimulation with autologous peptide-pulsed dendritic cells is the most essential step in successful priming of CD8+ T cells. This implies that DC used for the initial stimulus have to be fully functional antigen-presenting cells with respect to upregulation of co-stimulatory molecules. In the current study, after differentiation of monocytes into dendritic cells with IL-4 and GM-CSF for 72 hours, the resulting immature monocyte-derived dendritic cells were matured either by the application of a cytokine cocktail containing PGE_2, IL-1β, IL-6 and TNF-α, or by a combination of IFN-γ and LPS both for 24 hours (see 3.4.1.2). Figure 4.2A and B depict representative histograms showing the mean fluorescent intensity (MFI) of the CD80 FITC antibody stained monocytes after maturation in comparison to immature cells. Figure 4.2C and D show examples of dot plots depicting the dual upregulation of CD80 and CD86 in LPS/ IFN-γ-matured moDC. Figure 4.2E and F show microscopic pictures of LPS/ IFN-γ matured DC.

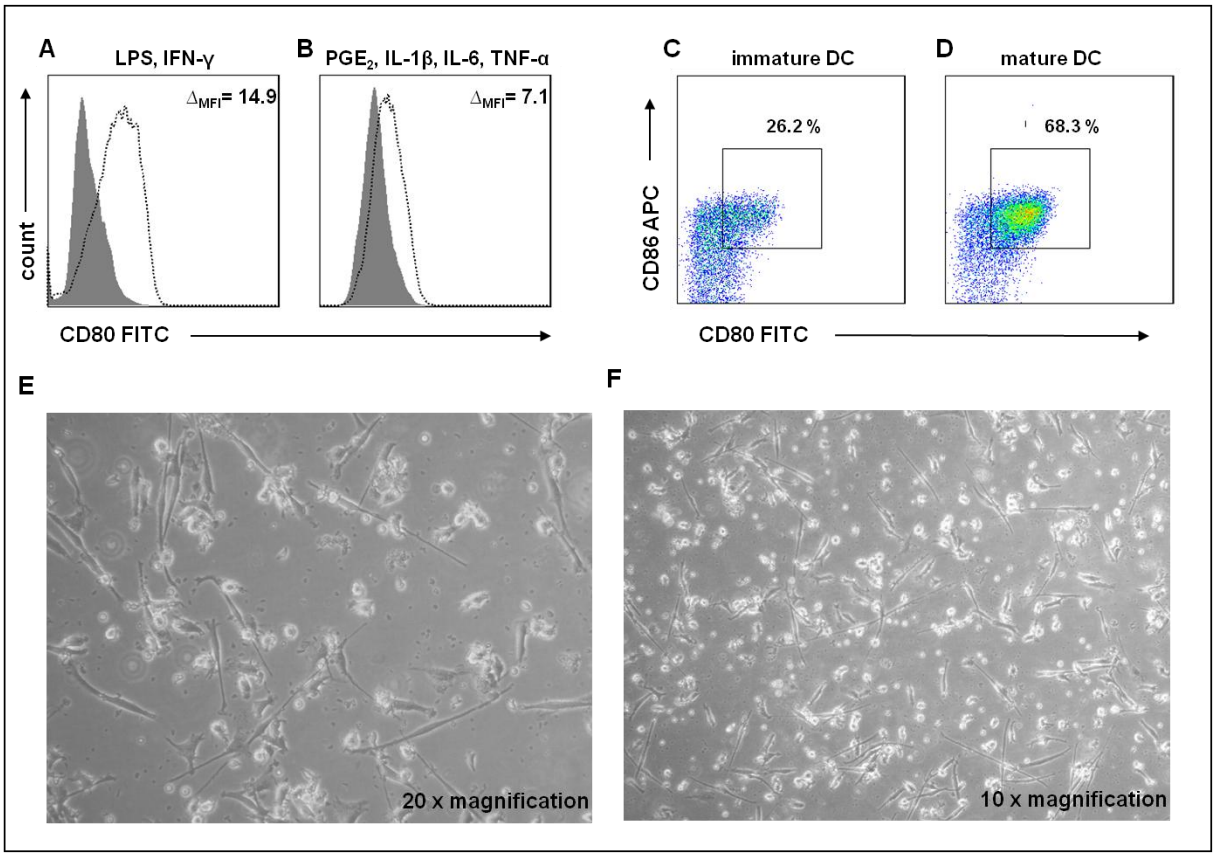

Figure 4.2 LPS/ IFN-γ matured moDC upregulate CD80 and CD86.
MFI of CD80 FITC expression of (A) LPS/ IFN-γ-matured DC or (B) PGE_2, IL-1β, IL-6 and TNF-α-matured DC. (C+D) Dual upregulation of CD80/ CD86 on LPS/ IFN-γ-matured DC. (E+F) Microscopic pictures of LPS/ IFN-γ-matured DC at (E) 20x and (F) 10x magnification.

As can be seen from figure 4.2A the stimulation with LPS/ IFN-γ for the maturation of DC led to a stronger upregulation of CD80 expression on DC ($\Delta_{MFI,\ CD80\text{-}FITC}$=14.9, figure 4.2A) in comparison to stimulation with the cytokine cocktail ($\Delta_{MFI,\ CD80\text{-}FITC}$=7.1, figure 4.2B). More important, LPS/ IFN-γ-matured moDC showed dual upregulation of CD80 and CD86 expression (68.3 % CD80+ CD86+ cells; figure 4.2D) in comparison to immature cells (26.2 % CD80+ CD86+ cells; figure 4.2C). In addition, LPS/ IFN-γ matured DC showed a typical morphology with extensive long dendrites when examined by light microscopy (figure 4.2E and F).

Therefore, the LPS/ IFN-γ matured moDC resembled mature DCs with regard to immunophenotypic markers and microscopic appearance and were thus considered as being fully effective functional antigen-presenting cells suitable for the priming of naïve CD8+ T cells.

4.1.3 Isolated naïve CD8+ T cells have a high purity

For the expansion of peptide-specific CD8+ T cells using mature moDC, isolated naïve CD8+ T cells were used. As the percentages of naïve CD8+ T cells vary significantly between donors, I chose to use normalized numbers of isolated naïve CD8+ T cells to keep the experimental conditions more similar. In addition, memory CD8+ T cells have a lower activation threshold, which can result in non-specific, cytokine-driven expansion of those cells.

For the isolation of naïve CD8+ T cells, the CD8+ T cell isolation kit was used, followed by positive depletion of CD45RO (memory cells) and CD57 (NK-, activated cells) positive cells using MicroBeads for the respective antigens (see 3.4.2). In order to determine the purity of the enriched cell fraction, samples were taken after each isolation step and stained with antibodies directed against the following antigens: CD8, CD4, CD56 (NK cells) and CD45RA (naïve T cells). The dot plots depicted in figure 4.3 display the results of one representative experiment. After purification, only a few contaminating CD4+, CD56+ and CD45RA+ cells were present, resulting in a purity of CD8+ CD4- CD56- CD45RA+ T cells of ~71 %. Overall, the purity of the isolated naïve CD8+ T cells was sufficient for the application in the following experiments.

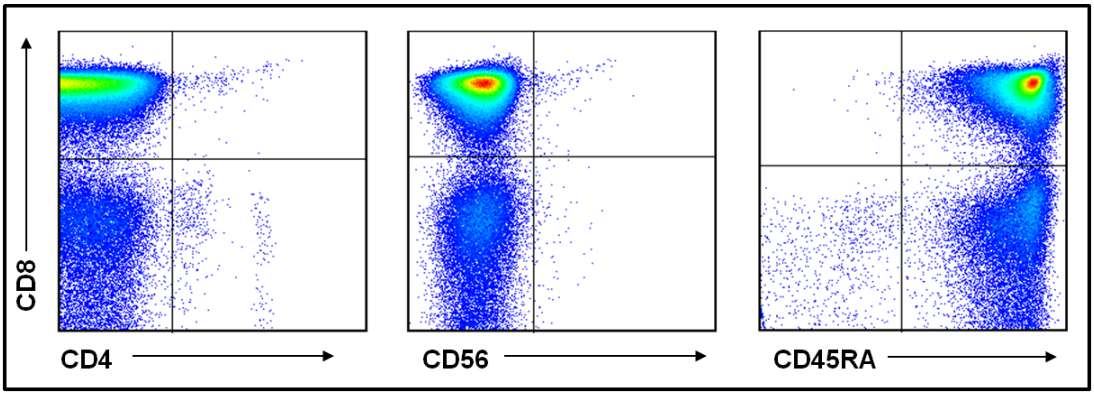

Figure 4.3 Isolated naïve CD8+ T cells have a high purity.
CD8+ T cells were isolated by depletion of non-CD8+ T cells followed by positive depletion of CD45RO+ and CD57+ cells. To determine the purity of the isolated CD8+ T cells, cells were stained with anti-CD8, anti-CD4, anti-CD56 and anti-CD45RA antibodies and analyzed by flow cytometry.

4.1.4 Restimulation of *in vitro* primed CD8+ T cells with irradiated, peptide-pulsed PBMC results in increased expansion of specific CD8+ T cells

For the initial priming, naïve CD8+ T cells were incubated with peptide-loaded mature moDC in a ratio of 4:1 in the presence of IL-21. IL-7 and IL-15 were added after 72 hours (see 3.4.3). The expansion of antigen-specific CD8+ T cells requires multiple cycles of *in vitro* restimulation, due to the exceedingly low precursor frequency of T cells specific for a given antigen. For this purpose, cultures were restimulated using either homologous, peptide-pulsed monocytes isolated by magnetic separation or homologous, peptide-pulsed PBMC at day 10 and day 19 of co-culture (see 3.4.4). When PBMC were used for restimulation, cells had to be irradiated prior to restimulation in order to avoid outgrowth of e.g. NK cells. As positive control, a well-described HLA-A*02-restricted melanoma epitope was used. Those Melan-A-specific CD8+ T cells are readily detectable in the majority of HLA-A*02-positive healthy individuals and in melanoma patients. Figure 4.4 depicts CD8+ dextramer+ T cells expanded with either the control peptide (HLA-A*02-restricted melanoma epitope; A and D) or the HCV-specific epitopes $NS3_{1406-1415}$ (B and E) or $NS3_{1073-1082}$ (C and F) and restimulated with peptide-loaded monocytes (A-C) or irradiated peptide-loaded PBMC (D-F).

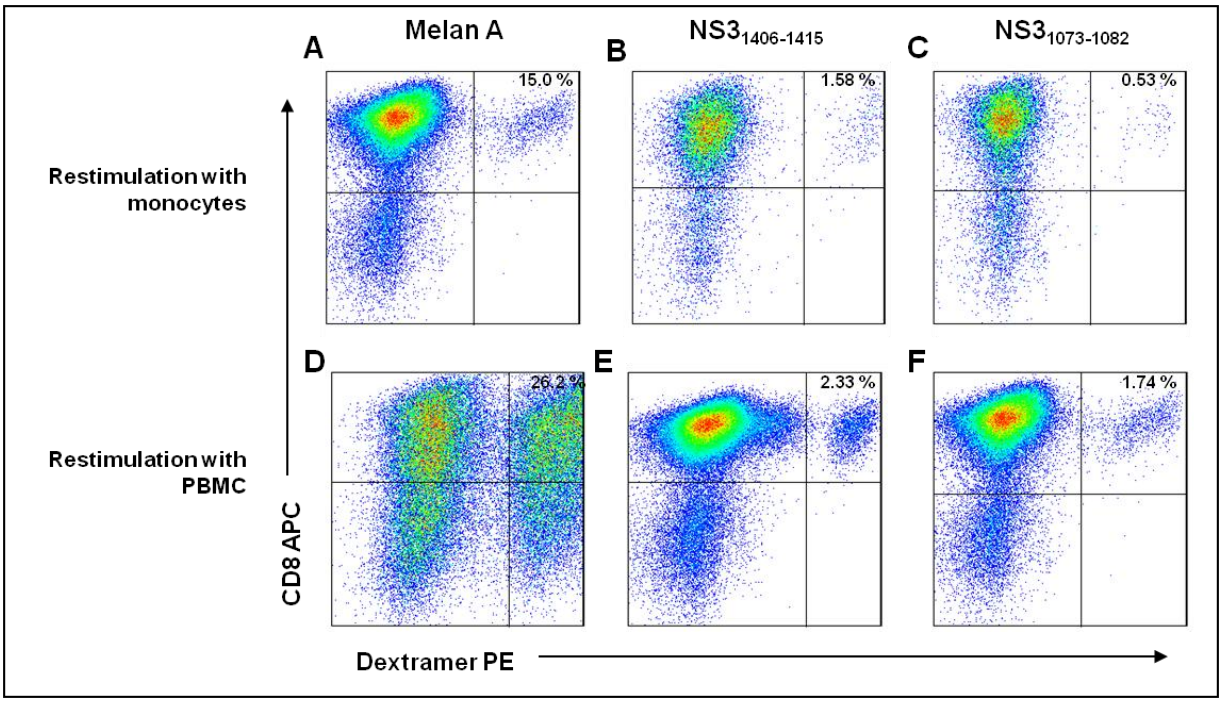

Figure 4.4 Restimulation using peptide-pulsed PBMC resulted in increased expansion of peptide-specific CD8+ T cells.
Expanded peptide-specific CD8+ T cells are shown after two rounds of restimulation using either (A-C) peptide-pulsed CD14+ monocytes or (D-F) irradiated peptide-pulsed PBMC.

Analysis of the dextramer positive cells after two rounds of restimulation revealed increased expansion of peptide-specific CD8+ T cells when using homologous, irradiated, peptide-pulsed PBMC (figure 4.4D-F) for the restimulation of the primed CD8+ T cells in comparison to the restimulation using peptide-pulsed monocytes (figure 4.4A-C). Using PBMC for the restimulation gave approximately 2-fold more peptide-specific CD8+ T cells in comparison to restimulation using monocytes (26.2 %, when using PBMS vs. 15.0 % when using monocytes for Melan-A peptide, 2.33 % vs. 1.58 % and 1.74 % vs. 0.53 % for HCV peptides). Additionally, using PBMC for restimulation does not require monocyte isolation and thus represents a cost effective and time preserving method.

To analyze the reproducibility of CD8+ T cell priming, naïve CD8+ T cells from five patients were primed with moDC and incubated in the presence of IL-21, IL-7 and IL-15 for 10 days (see 3.4.2). After restimulation with homologous, irradiated, peptide-pulsed PBMC the expansion of peptide-specific CD8+ T cells was analyzed using MHC class I-multimer staining (see figure 4.5).

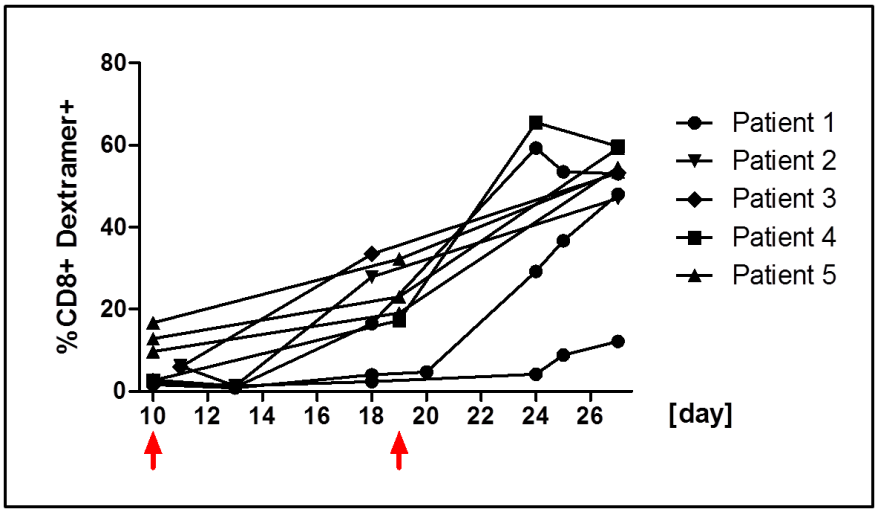

Figure 4.5 Reproducible expansion of Melan-A-specific CD8+ T cells from five healthy blood donors throughout the culture period.

Naïve CD8+ T cells were primed with moDC and incubated in the presence of IL-21, IL-7 and IL-15 for 10 days. Cultures were restimulated at day 10 and day 19 (red arrows) using homologous, irradiated, peptide-pulsed PBMC in the presence of IL-2. The expansion of peptide-specific CD8+ T cells was analyzed using MHC class I-multimer staining.

Figure 4.5 summarizes the results of five patients (in duplicate) used for the expansion of Melan-A-specific CD8+ T cells. The expansion of peptide-specific CD8+ T cells with two rounds of restimulation using peptide-loaded PBMC resulted in a continuous increase of peptide-specific CD8+ T cells throughout the culture period, yielding in average more than 50 % Melan-A-specific CD8+ T cells after 28 days.

4.1.5 Experimental setup for the priming of virus-specific CD8+ T cells

In vitro priming of *de novo* HCV-specific T cell responses to viral antigens in healthy donors require the expansion of specific T cells from the naïve T cells repertoire, as the healthy blood donors were not exposed to the HCV antigens. In this study, we established a protocol for the expansion of antigen-specific CD8+ T cells from the naïve CD8+ T cell repertoire using an initial stimulation with peptide-pulsed IFN-γ/ LPS-matured dendritic cells, followed by two rounds of restimulation with homologous, peptide-pulsed, irradiated PBMC with the sequential use of IL-21, IL-7, IL-15 and IL-2 (see 3.4.3). The following figure (4.6) schematically outlines the established workflow for the generation of virus- (and tumor-) specific CD8+ T cells from the naïve CD8+ T cell repertoire. The established procedure was highly reproducible as demonstrated for the expansion of tumor-specific CD8+ T cells from healthy donors (figure 4.5).

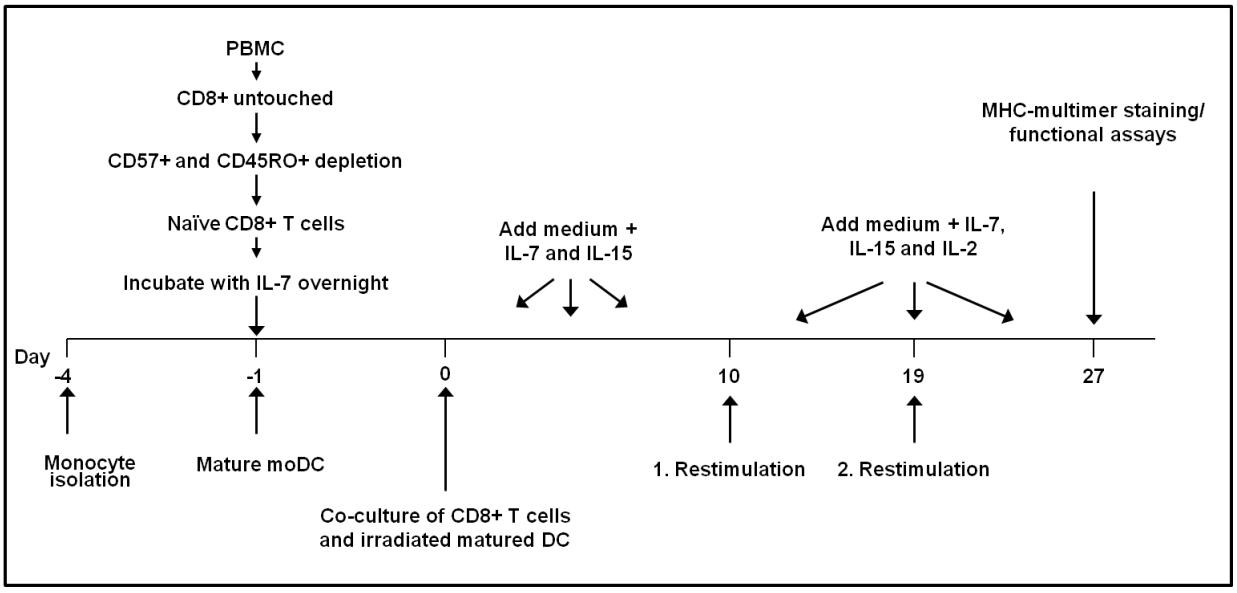

Figure 4.6 Experimental setup for the priming of virus-specific CD8+ T cells.
Adapted from (Wölfl *et al* 2011).

4.1.6 Most frequent HCV $NS3_{1406-1415}$ sequences

After successfully establishing a protocol for the expansion of virus-specific CD8+ T cells from the naïve CD8+ T cell repertoire, the impact of the immunodominant HLA-A*02-restricted HCV $NS3_{1406-1415}$ CD8+ T cell epitope on CD8+ T cell priming was analyzed.

For this, the most frequent HCV $NS3_{1406-1415}$ sequence variants were selected from the HCV sequence database (www.hcv.lanl.gov). The present study included in total five $NS3_{1406-1415}$ genotype (gt) 1b variants predominately circulating in Europe (KLSGLGLNAV and KLSSLGLNAV) and Japan (KLSALGLNAV and KLSALGVNAV), as well as an unique epitope sequence (QLSGLGINAV) originating from a large single-source outbreak (Wiese *et al* 2000). Viral sequence analyses in this cohort suggested strong selection pressure resulting in consistent mutational escape (Ruhl *et al* 2011). In addition, the most frequent genotype 1a sequence variants were studied (prototype: KLVALGINAV and variant: KLVALGVNAV). The genotype 1a prototype sequence is the most commonly used in human immunology studies of HCV infection for identification of HCV-specific CD8+ T cells. Table 4.1 summarizes the $NS3_{1406-1415}$ viral variants used in the present study.

Table 4.1 Most frequent HCV $NS3_{1406-1415}$ sequence variants used for CD8+ T cell priming.

Variant	Genotype	Sequence	Frequency [%] [http://hcv.lanl.gov, (Yusim *et al* 2005)]
gt 1a prototype	gt 1a	**K**L**VA**LG**I**NAV	43.3
gt 1a variant	gt 1a	**K**L**VA**LG**V**NAV	31.8
Europe I	gt 1b	**K**L**SG**LG**L**NAV	42.8
Europe II	gt 1b	**K**L**SS**LG**L**NAV	18.4
Japan I	gt 1b	**K**L**SA**LG**L**NAV	12.5
Japan II	gt 1b	**K**L**SA**LG**V**NAV	4.3
Anti-D	gt 1b	**Q**L**SG**LG**I**NAV	Anti-D cohort

4.1.7 Variant peptide-binding to HLA-A*02:01 molecule

Peptide-MHC class I-complex recognition by cognate TCR is the molecular basis of antigen-recognition by CD8+ T cells. Each MHC allele has a distinct peptide-binding motif which favors certain amino acids in particular sequence positions. Amino acid substitutions in T cell epitopes thus can result in decreased peptide binding avidities to the HLA-A*02 molecule. To test potential differences in the binding avidity of the peptide variants used in this study to the HLA-A*02 molecule, peptide-binding assays to HLA-A*02:01 were performed at the Saquin company in the Netherlands, as already described elsewhere (Toebes *et al* 2006).

Analysis of the peptide-binding to HLA-A*02:01 revealed no differences in binding avidities (figure 4.7). All peptides equally bind with high avidities to HLA-A*02:01.

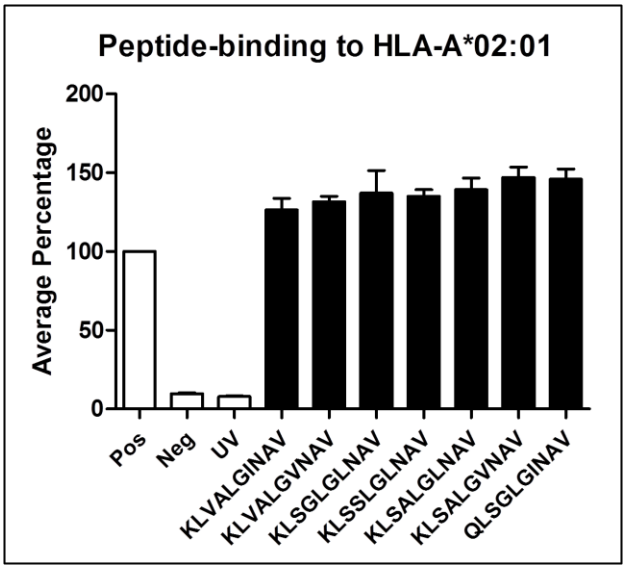

Figure 4.7 Peptide-binding avidities to HLA-A*02:01.
All variants used in this study were analyzed for their specific binding avidity to the HLA-A*02:01 molecule as described in (Toebes *et al* 2006).

4.1.8 HCV $NS3_{1406-1415}$ sequence variants show distinct priming potentials

The most frequent HCV $NS3_{1406-1415}$ sequence variants (see table 4.1) were selected and used for the priming of naïve CD8+ T cells from healthy donors as summarized in 4.1.5, to study the impact of sequence differences on the priming capacity, cross-reactivity, polyfunctionality and Vβ usage of CD8+ T cells.

After 27 days of culture, $NS3_{1406-1415}$ expansion of peptide-specific CD8+ T cells was analyzed by MHC class I-dextramer staining (see 3.4.5) and IFN-γ production as described in 3.3. Figure 4.8 summarizes the results of eleven patients used for the expansion of HCV $NS3_{1406-1415}$ variant-specific CD8+ T cells.

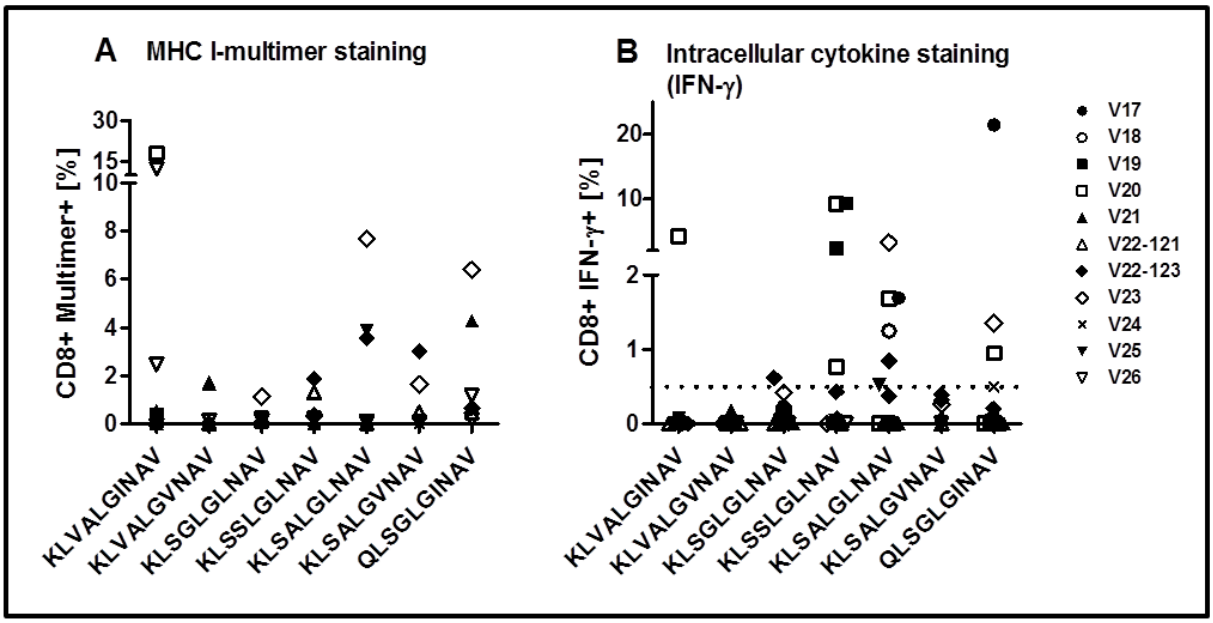

Figure 4.8 Different epitope sequences show distinct priming potential.
Primed virus-specific CD8+ T cells were expanded for 28 days and peptide-specific cells were analyzed by (A) peptide-specific MHC class I-multimers and (B) intracellular cytokine staining for IFN-γ.

Primed $NS3_{1406-1415}$-specific CD8+ T cells were first analyzed using MHC class I-multimer staining specific for the utilized $NS3_{1406-1415}$ variants (figure 4.8A). In addition, cells were analyzed for their effector function by staining for intracellular IFN-γ production (figure 4.8B). For analysis, cells were restimulated using monocytes pulsed with peptides representing the sequence of the epitope that was used for priming.

Figure 4.8 shows that the different variants used for priming of CD8+ T cells exhibited distinct priming potentials. Overall one can conclude that genotype 1b variants exhibited better priming potential than genotype 1a variants. Interestingly, CD8+ T cells specific for the genotype 1a consensus sequence, as shown by MHC class I-multimer staining (figure 4.8A), were not functional as they were not able to produce IFN-γ (figure 4.8B) and other cytokines (see figure 4.11). Importantly, the Japanese $NS3_{1406-1415}$ KLSALGLNAV variant rarely circulating in Europe had the best priming capacity (6 out of 11).

4.1.9 CD8+ T cells primed for the $NS3_{1406-1415}$ KLSALGLNAV variant have a high degree of cross-reactivity

In addition to the analysis of the priming potential, $NS3_{1406-1415}$-specific CD8+ T cells were analyzed for their potential to cross-react with other epitope variants. For this purpose, monocytes were pulsed separately with all epitope variants and used for restimulation as described in 3.4.6. Cells that secrete IFN-γ above the threshold of 0.5 % after background subtraction were considered as being cross-reactive. Figure 4.9 shows an example of a cross-reactive CD8+ T cell response (upper panel) and an example of a CD8+ T cell response without cross-reactivity (lower panel).

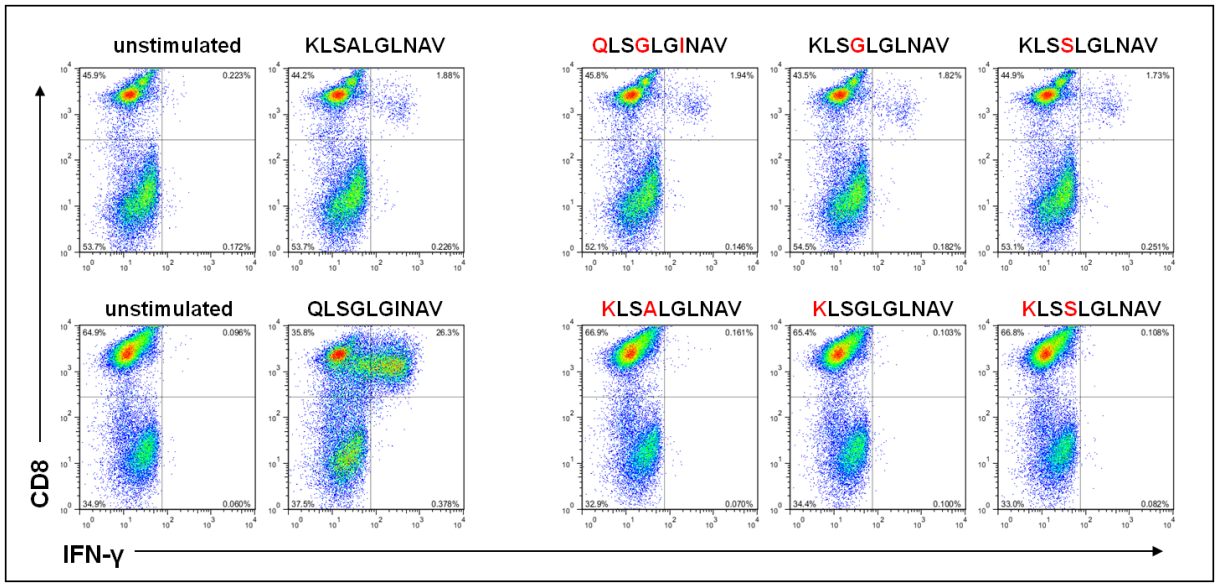

Figure 4.9 Primed peptide-specific CD8+ T cells show distinct cross-reactivity patterns.
The expanded variant-specific CD8+ T cells (Upper panel: $NS3_{1406-1415}$ KLSALGLNAV, Lower panel: QLSGLGINAV) were analyzed by intracellular cytokine staining. Peptide variant-pulsed monocytes were used for the restimulation of virus-specific CD8+ T cells.

CD8+ T cells expanded with the $NS3_{1406-1415}$ KLSALGLNAV peptide (1.88 % CD8+ IFN-γ+ cells) showed high degrees of cross-reactivity, as primed CD8+ T cells responded also to restimulation with other viral variants (i.e. KLSGLGLNAV 1.82 % CD8+ IFN-γ+, KLSSLGLNAV 1.73 % and QLSGLGINAV 1.94 %, figure 4.9, upper panel). In contrast, although CD8+ T cells primed against the anti-D variant (QLSGLGINAV) proliferated vigorously upon restimulation with the homologous variant (26.3 % CD8+ IFN-γ+ cells), they were not cross-reactive with other peptide variants (figure 4.9, lower panel).

Figure 4.10 summarizes the results of cross-reactivity testing of 11 patients used for the priming of HCV $NS3_{1406-1415}$-specific CD8+ T cells. It is important to mention that the $NS3_{1406-1415}$ KLSALGLNAV variant-primed CD8+ T cells were the most cross-reactive T cells (figure 4.10C). CD8+ T cells primed against this peptide-variant frequently recognized other epitope variants. In contrast, CD8+ T cells primed with the $NS3_{1406-1415}$ QLSGLGINAV variant expanded in 4 out of 9 experiments, but were not cross-reactive with other variants (figure 4.10G). Despite the $NS3_{1406-1415}$ KLSALGLNAV variant (figure 4.10C), the $NS3_{1406-1415}$ KLSSLGLNAV variant also was cross-reactive with other viral variants in 2 of 11 experiments and was thus considered as being cross-reactive as well (figure 4.10E). Genotype 1a sequences showed neither good priming potential nor cross-reactivity (figure 4.10A and D).

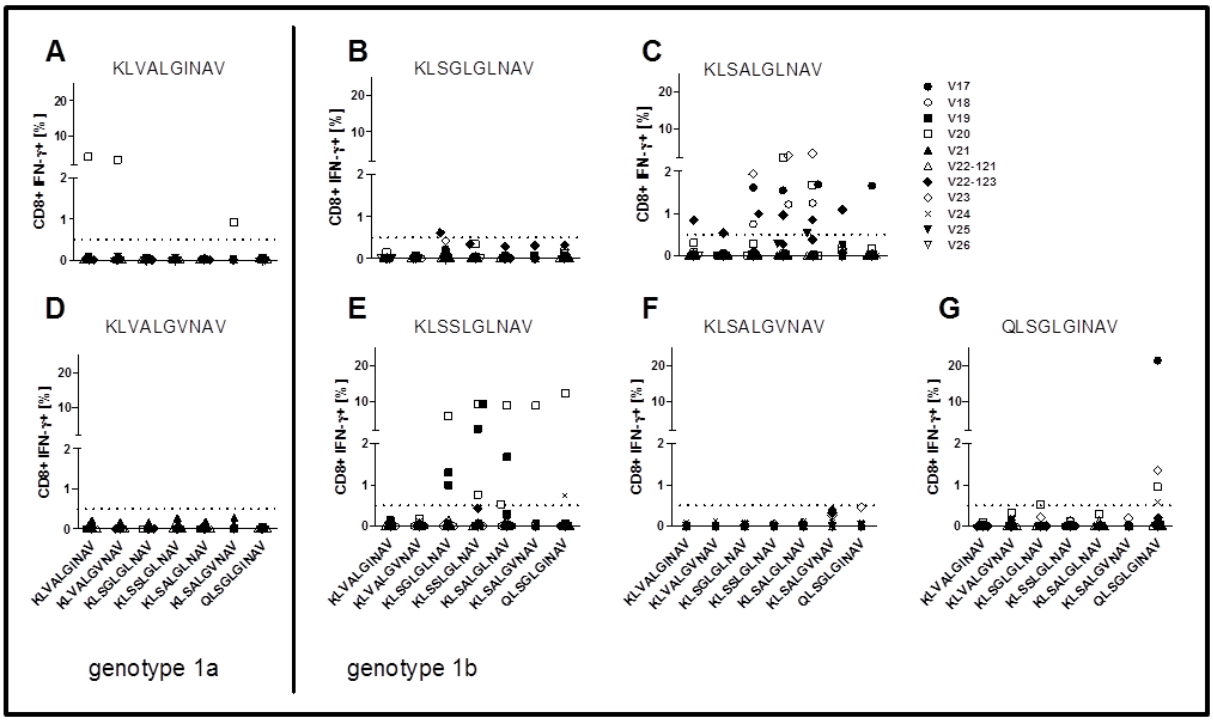

Figure 4.10 Primed virus specific CD8+ T cells show distinct cross-reactivity patterns.
After 28 days, the expanded virus-specific CD8+ T cells were analyzed by intracellular cytokine staining. Peptide variant-pulsed monocytes were used for the restimulation of virus-specific CD8+ T cells for 5 hours. IFN-γ secretion was analyzed by ICS. IFN-γ response above the set cut-off of 0.5 % CD8+ IFN-γ+ cells after background subtraction were considered as being cross-reactive. (A and D) Genotype 1a peptides, (B and C, E-F) genotype 1b peptides.

4.1.10 $NS3_{1406-1415}$ KLSALGLNAV variant-primed CD8+ T cells are polyfunctional

In addition to IFN-γ secretion, we examined polyfunctionality of primed CD8+ T cells in selected experiments, including the ability of variant-specific CD8+ T cells to simultaneously produce the effector cytokines and chemokines IFN-γ, IL-2, TNF-α and MIP-1β and to release cytotoxic factors by monitoring the expression of the degranulation marker CD107a after restimulation with homologous peptides (as described in 3.4.6). Peptide-specific CD8+ T cells were simultaneously stained with specific antibodies and analyzed by flow cytometry. Data were further analyzed using Pestle and SPICE software. Figure 4.11 (upper panel) depicts dot plots of one representative experiment showing examples CD8+ T cells producing these cytokines/ markers. Figure 4.11 (lower panel) displays pie-charts, illustrating the polyfunctionality of variant-specific CD8+ T cells, with respect to the number of different cytokines secreted.

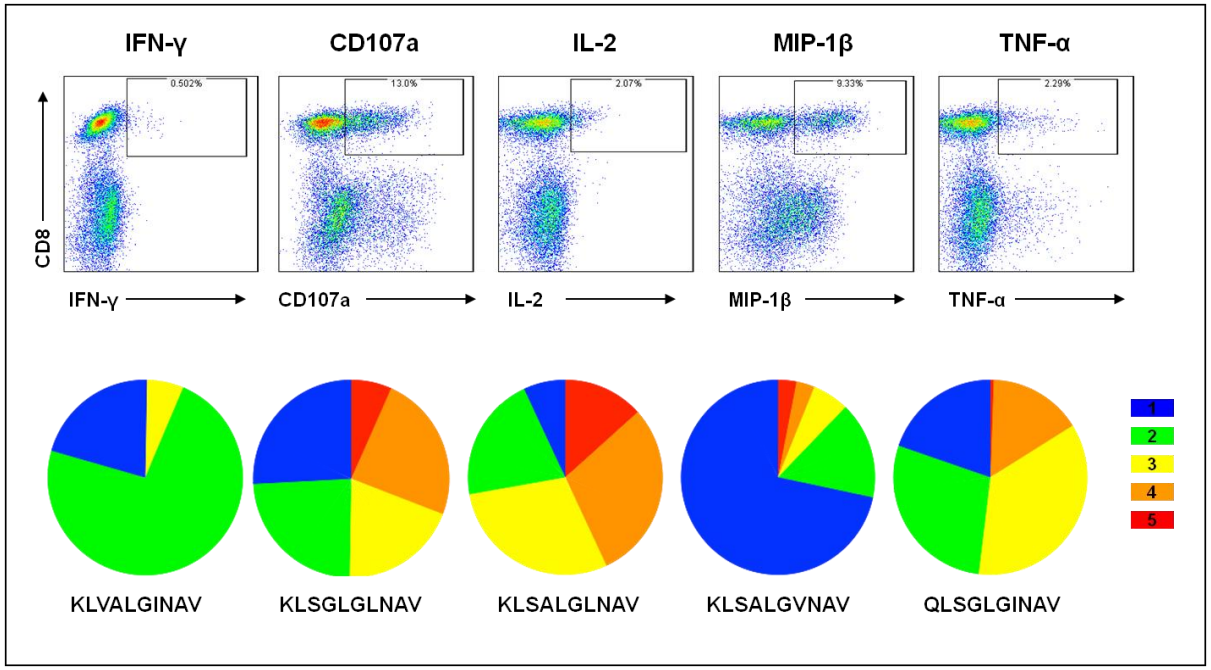

Figure 4.11 $NS3_{1406-1415}$ KLSALGLNAV variant primed CD8+ T cells are polyfunctional.
After expansion of primed virus-specific CD8+ T cells for 28 days, cells were analyzed by intracellular cytokine staining for IFN-γ, TNF-α, IL-2, MIP-1β and CD107a. (Upper panel) Representative dot plots of the different stainings. (Lower panel) Pie charts of primed CD8+ T cells. Pie charts were generated using Pestle and SPICE software (blue= one function, green= two functions, yellow= three functions, orange= four functions and red= five functions).

CD8+ T cells specific for the $NS3_{1406-1415}$ KLSALGLNAV variant were the most polyfunctional, as almost ¾ of the cells were able to produce more than three effector molecules. In contrast, cells specific for the $NS3_{1406-1415}$ KLSALGVNAV and the

genotype 1a $NS3_{1406-1415}$ KLVALGINAV variant had less polyfunctional CD8+ T cell responses. Most cells were able to secrete just one or two effector molecules. Thus, $NS3_{1406-1415}$ KLSALGLNAV primed CD8+ T cells had the best effector properties in comparison to CD8+ T cells specific for the other variants (see figure 4.11, lower panel).

4.1.11 Limited TCR Vβs elicted by the $NS3_{1406-1415}$ QLSGLGINAV variant in contrast to the cross-reactive $NS3_{1406-1415}$ KLSALGLNAV variant

To understand the nature of T cell receptors specific for the variant $NS3_{1406-1415}$ QLSGLGINAV (good priming capacity; no cross-reactivity) versus the $NS3_{1406-1415}$ KLSALGLNAV variant (very good priming capacity; good cross-reactivity), the individual TCR Vβ chain profiles used were assessed using a comprehensive antibody panel. Therefore, primed CD8+ T cells were restimulated with $NS3_{1406-1415}$ QLSGLGINAV, KLSALGLNAV or KLSGLGLNAV variants. CD8+ T cells specific for the NS3 variants were selected based on IFN-γ secretion after restimulation and used for Vβ chain analysis (see figure 4.13). Figure 4.12 depict dot plots showing the percentages of IFN-γ secreting CD8+ T cells after restimulation.

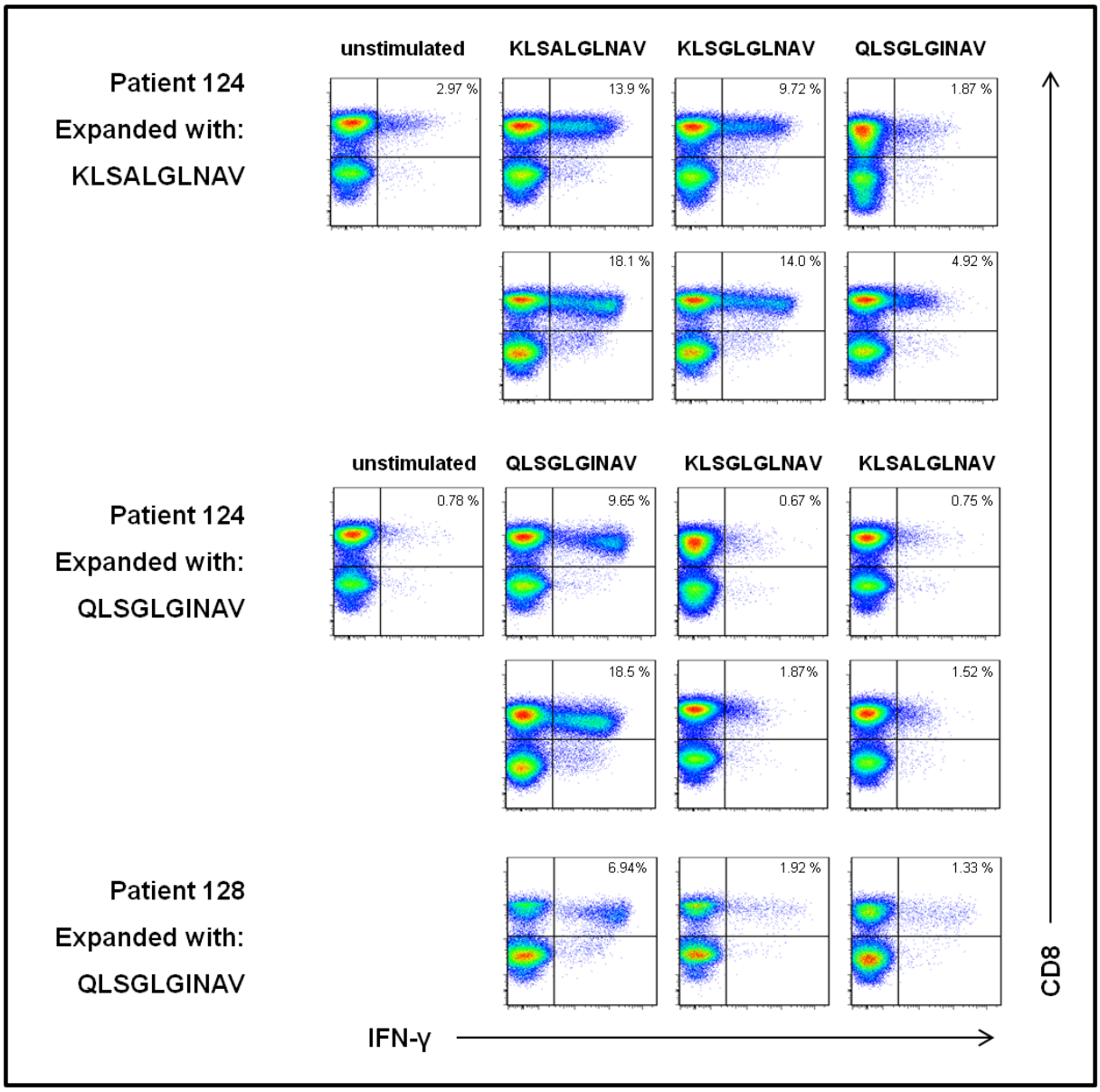

Figure 4.12 Dot plots of expanded CD8+ IFN-γ+ virus-specific T cells used for Vβ chain analysis.

After expansion of primed virus-specific CD8+ T cells for 28 days, cells were restimulated with $NS3_{1406-1415}$ KLSALGLNAV, QLSGLGINAV and KLSGLGLNAV variants and analyzed by intracellular cytokine staining for IFN-γ. Non-restimulated expanded cells were used as background control. Responding cells were further analyzed for their Vβ chain repertoire (see figure 4.13).

Figure 4.12 shows the results of the two patients used for the Vβ chain analysis. Patient 124 was either expanded with $NS3_{1406-1415}$ KLSALGLNAV or QLSGLGINAV (in duplicate) and patient 128 was expanded with $NS3_{1406-1415}$ QLSGLGINAV. As can be seen in figure 4.12, $NS3_{1406-1415}$ KLSALGLNAV primed CD8+ T cells were cross-reactive with the KLSGLGLNAV variant, but (as expected) only to a minor extend with the QLSGLGINAV variant (Patient 124, first and second row). $NS3_{1406-1415}$ QLSGLGINAV primed CD8+ T cells were not cross reactive at all, as expected (see

figure 4.10 and figure 4.12, Patient 124 expanded with QLSGLGINAV and Patient 128). Figure 4.13 shows the Vβ chain analysis of the $NS3_{1406-1415}$ variant expanded cells.

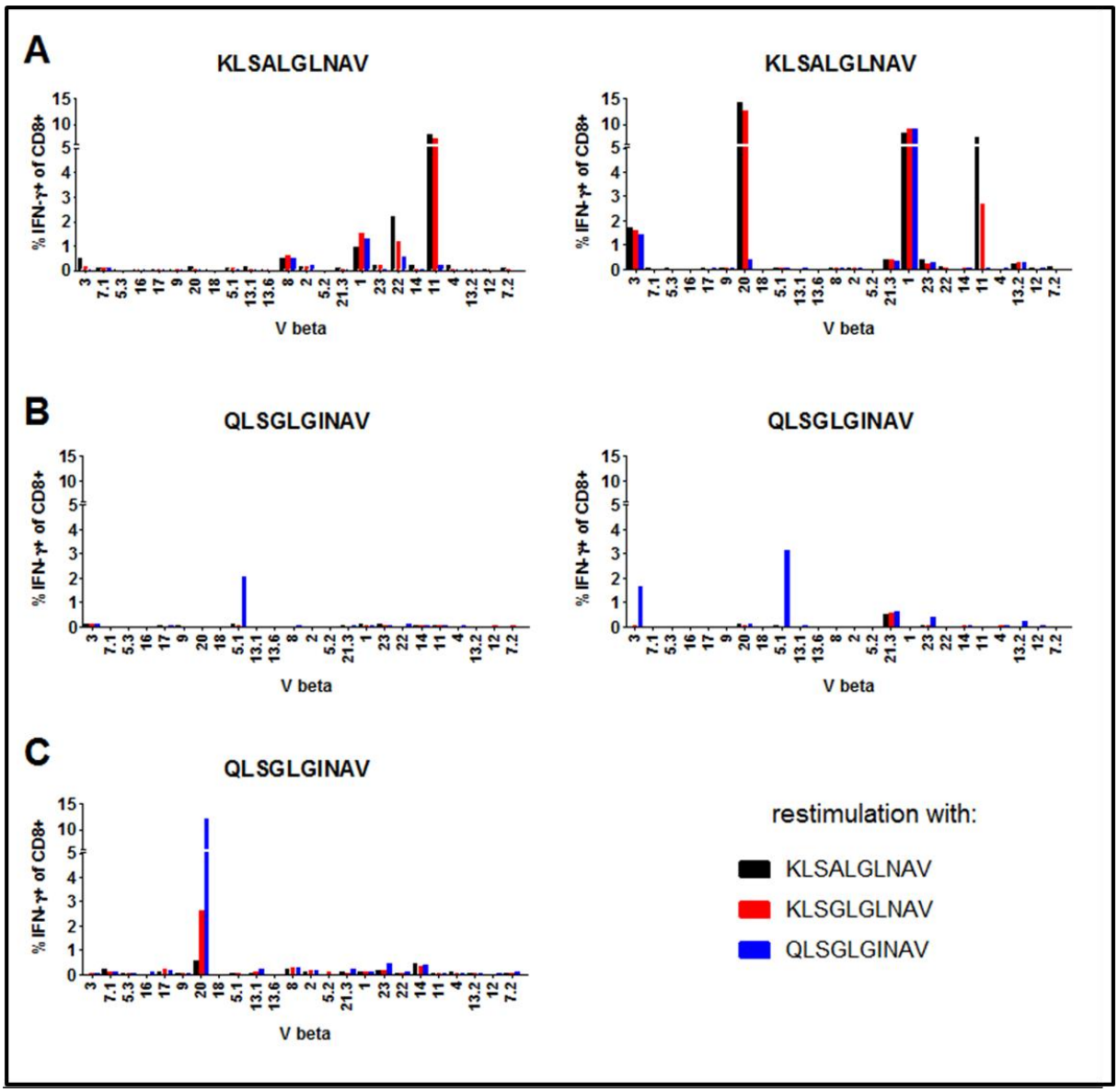

Figure 4.13 Limited T cell receptor Vβ use for the QLSGLGINAV-primed CD8+ T cells.
(A and B) Patient 124 in duplicate, (C) Patient 128. CD8+ T cells were restimulated with homologous or other $NS3_{1406-1415}$ peptide variants. Cells that were 7-AAD- CD4- CD8+ IFN-γ+ were analyzed with a panel of 24 antibodies covering approximately 70 % of the TCR Vβ repertoire.

After restimulation of primed CD8+ T cells with their homologous epitope variants, the TCRVβ profiles for $NS3_{1406-1415}$ KLSALGLNAV and QLSGLGINAV-specific CD8+ T cells showed variable profiles of TCR Vβ bias. CD8+ IFN-γ+ T cells primed with the KLSALGLNAV variant were characterized by a broader TCR Vβ use, namely Vβ1, -3,

-11, -20 and -22 (figure 4.13 panel A, black bars). In comparison, CD8+ IFN-γ+ T cells primed with the QLSGLGINAV variant were characterized by a more restricted use of TCR Vβ chains, i.e. solely Vβ3 and -5.1 for Patient 124 (figure 4.13, panel B, blue bars) and Vβ20 for Patient 128 (figure 4.13C, blue bars). When restimulating KLSALGLNAV-primed CD8+ T cells with the KLSGLGLNAV variant of $NS3_{1406-1415}$ epitope, the same Vβ chains as for the homologous variant were utilized (figure 4.13, panel A, red bars). As expected, KLSALGLNAV-primed CD8+ T cells showed less cross-reactivity with the QLSGLGINAV sequence (see figure 4.9 and 4.10; figure 4.13, blue bars).

4.2 Influence of primary human hepatocytes on the expansion of virus-specific CD8+ T cells

A hallmark of chronic infections with hepatotropic viruses such as HBV and HCV is the notoriously weak antiviral immune response by CD8+ T cells. It is believed that the liver-specific environment can induce tolerance of CD8+ T cells. Direct antigen presentation by virally infected hepatocytes thus might inhibit the CD8+ T cell response. Studying the role of primary human hepatocytes (PHH) on the activation of virus-specific CD8+ T cells may give important hints for the establishment of chronic infections by hepatotropic viruses.

4.2.1 Characteristics of hepatocyte donors

To study the influence of primary human hepatocytes on CD8+ T activation, liver tissue was dissected from patients undergoing surgery and PHH were isolated at the department of gastroenterology and hepatology, university hospital Essen. The patients included in this study represent a heterogeneous population with respect to age and gender (figure 4.14A), the presence of viral hepatitis (HCV or HBV, figure 4.14B) and the type of surgery they underwent (figure 4.14C).

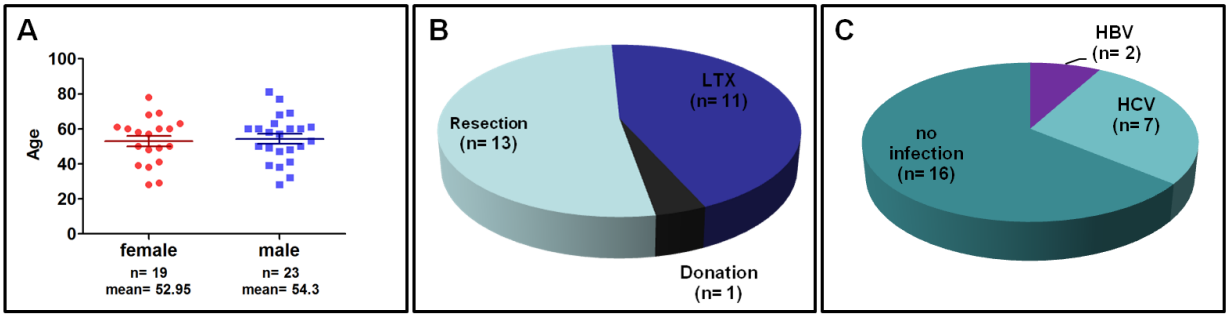

Figure 4.14 Patient characteristics.
(A) Age and gender, (B) type of surgery, (C) presence of viral infections.

4.2.2 PHH inhibit expansion of virus-specific CD8+ T cells in a contact-dependent manner

Before each experiment, the quality and purity of the PHH cultures was examined based on their microscopical appearance. Figure 4.15A shows an example of a freshly isolated PHH culture. Fresh hepatocytes had a polygonal shape, round prominent nuclei and showed little proliferation of fibroblastic cells.

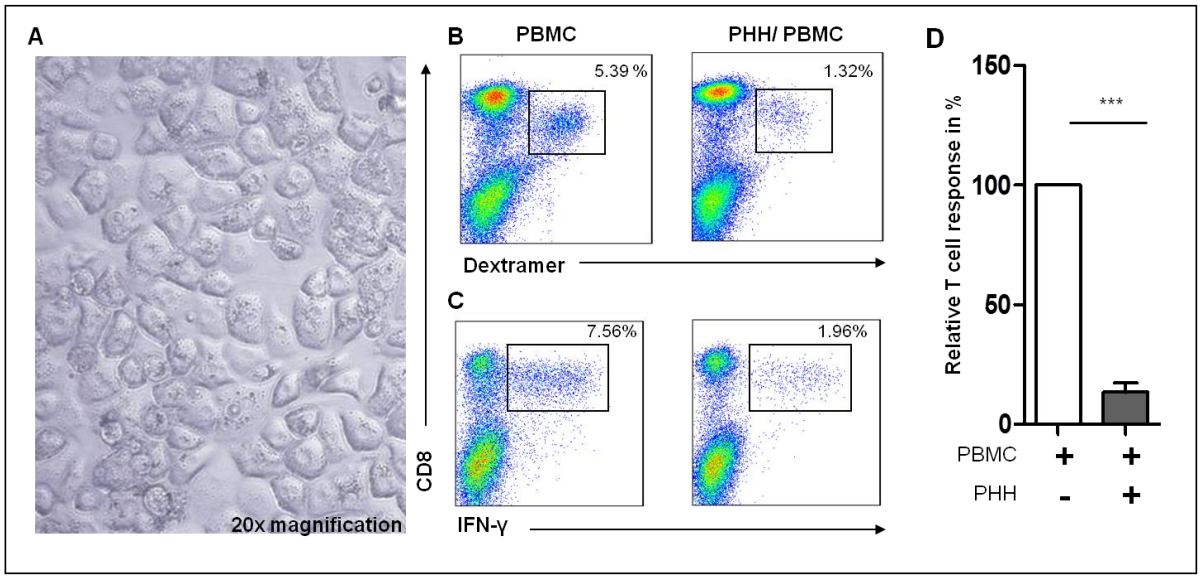

Figure 4.15 PHH inhibit the expansion of virus-specific CD8+T cells *in vitro*.
(A) Light microscopic picture of a freshly isolated PHH monoculture. Expansion of virus-specific CD8+ T cells in the presence of PHH is assessed using MHC class I-multimer staining (B) or intracellular IFN-γ staining (C+D) after 7-10 days. Normalized mean, *** p<0.001.

In the first set of experiments, the influence of PHH on the expansion of virus-specific CD8+ T cells was examined. For this purpose, co-cultures of PHH and PBMC were established. $1.5x10^6$ PBMC were incubated with approximately $0.4x10^6$ PHH in the presence of viral peptides (see 3.5). Next, PBMC were harvested from co-cultures and analyzed by MHC class I-multimer staining. Analysis of the dextramer positive CD8+ T cells after 7-10 days of co-culture revealed a reduced expansion of antigen-specific CD8+ T cells in the presence of PHH (figure 4.15B).

In addition, the number of IFN-γ secreting cells was significantly reduced as shown in figure 4.15C and D. The mean reduction of IFN-γ secretion after co-culture was approximately 75 % in several independent experiments.

It was first addressed, if the inhibitory effect required the contact between MHC-peptide complexes on the hepatocytes with their specific TCR. For this purpose, we used hepatocytes from an HLA-A*02 negative donor, which were not able to present the HLA-A*02-restricted peptides. Figure 4.16A summarizes the results of two independent experiments. There was no difference in inhibition between HLA-A*02 positive and negative donors, indicating that an antigen-specific contact was not necessary to mediate inhibition.

In further experiments, we analyzed the role of soluble factors secreted by the hepatocytes *per se* in mediating inhibitory effects. For this, medium transfer experiments were performed. The supernatant of a 24 hour PHH or PBMC mono- or co-culture was used in increasing concentrations for the culture of freshly thawed PBMC in the presence of viral antigens. Figure 4.16B depict the normalized mean of three independent experiments performed. The supernatant of a PHH monoculture had a mild inhibitory effect on the expansion of virus-specific CD8+ T cells (60 % response compared to control). The supernatant of PBMC monocultures or PBMC/ PHH co-cultures similarly showed a mild inhibitory effect (minimum 75 % response compared to control).

The results in figure 4.16B indicate that soluble factors released by hepatocytes *per se* or exhaustion of nutrients only marginally played a role and did not completely explain the drastic reduction in the number of antigen-specific T cells in the presence of hepatocytes seen in figure 4.15D.

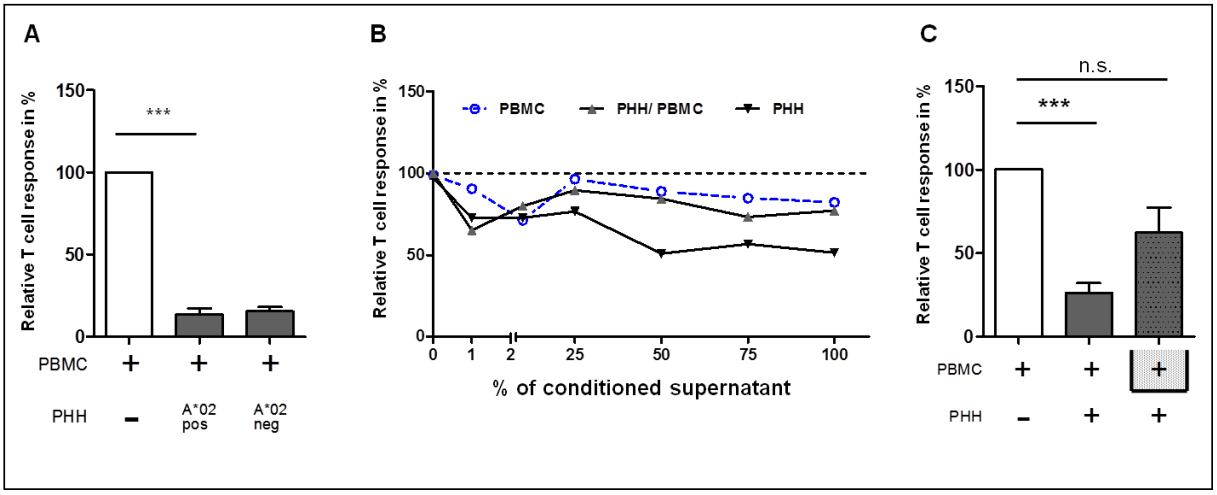

Figure 4.16 PHH inhibit in a contact dependent manner.
(A) PHH from HLA-A*02 negative donors were co-cultured with PBMC in the presence of HLA-A*02-restricted viral peptides. (B) The supernatant of a 24 hour PBMC, or PHH mono- or co-cultures was used in increasing concentrations for the culture of freshly thawed PBMC. (C) PBMC were grown in a membrane insert. (A-C) After 7-10 days of culture, PBMC were harvested and analyzed by intracellular IFN-γ staining. Bars represent the normalized means in comparison to PBMC monocultures (ANOVA; $p<0.001$).

Membrane inserts are commonly used in co-cultures to evaluate cell behavior in the absence of cell-cell contact. In order to determine if the inhibitory effect of PHH on CD8+ T cells depends on contact between PBMC and hepatocytes, transwell experiments were performed. On top of the hepatocytes a membrane insert with 0.4 µm pore size was placed and $1x10^6$ PBMC were seeded inside the membrane insert in the presence of viral antigens. In this experimental setup, PBMC and PHH share the same medium, but are not able to make physical contact. The results of the transwell experiments are depicted in figure 4.16C. The reduction in IFN-γ secreting cells was less pronounced in the transwell system (mean: 62.3 % response compared to control) and was similar to the effect when PHH-conditioned medium was used (figure 4.16B). In line with previous experiments the inhibitory effect was more pronounced when PBMC and PHH had physical contact (mean: 26.3 % response compared to control), indicating that physical contact between hepatocytes and PBMC was required to mediate the full inhibitory effect.

4.2.3 The inhibitory effect is hepatocyte specific

To confirm that the inhibitory effect was specific for PHH and did not represent a culture artifact, human umbilical vein endothelial cells (HUVEC) were purchased as a control and incubated with peptide-pulsed PBMC. After 7 days of co-culture, virus-specific CD8+ T cell expansion was verified by intracellular cytokine staining for IFN-γ and compared to PBMC monocultures.

Indeed, the presence of HUVEC in the co-culture system did not lead to a decrease in the expansion on virus-specific CD8+ T cells (figure 4.17A).

In addition, the role of a potential influence of the allogenic reaction, as PBMC and PHH are isolated from different patients, was excluded by a mixed-lymphocyte reaction. PBMC from two different patients were co-incubated with viral antigens for 7 days and the frequency of IFN-γ producing cells was assessed. As control, PBMC from both patients were incubated separately. From figure 4.17B we can conclude that the allogenic background has no additional influence on the PHH/ PBMC co-culture.

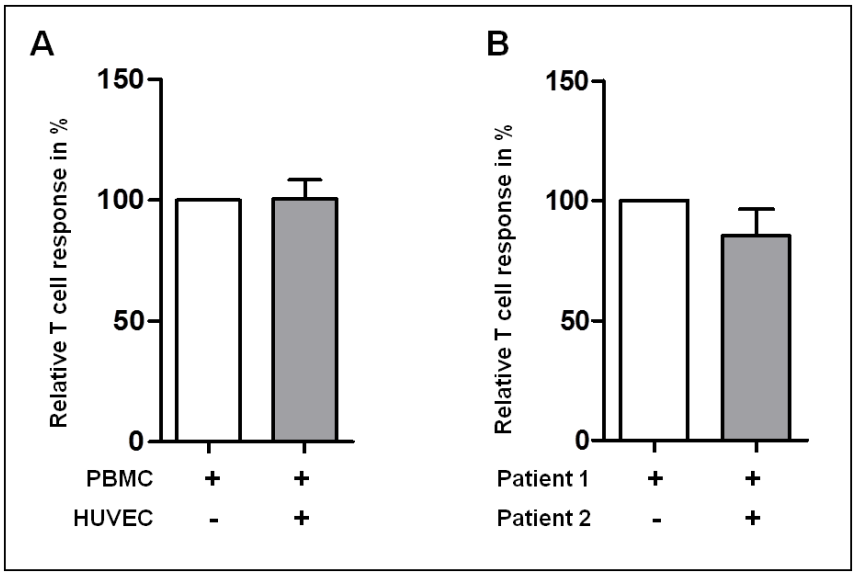

Figure 4.17 Inhibitory effect is hepatocyte specific.
(A) Human umbilical vein endothelial cells were co-cultured with PBMC and viral peptides for 7 days. (B) PBMC from two different patients were co-cultured in the presence of viral antigens. After 7 days of culture, PBMC were harvested and analyzed by intracellular IFN-γ staining. Bars represent the normalized means in comparison to PBMC monocultures.

4.2.4 Increased TGF-β level in the supernatant of PHH/ PBMC co-cultures

To verify the influence of potential proinflammatory or inhibitory cytokines in the supernatant, after 72 hours of co-culture, the supernatant of the PHH and PBMC mono- and co-cultures was harvested and analyzed for the presence of different inhibitory or pro-inflammatory cytokines. The concentration of IL-2, TNF-α, IFN-γ and IL-10 was determined by Luminex technology at the institute of medical microbiology, university hospital Essen. The amount of TGF-β in the supernatant was determined by ELISA. Figure 4.18 depicts the results of the Luminex data. As can be seen, there was a slight increase in the cytokines IL-2 and IFN-γ in the presence of hepatocytes which might be due to the allogenic activation of the PBMC. Unexpectedly, no difference in the level of the inhibitory cytokine IL-10 could be detected (figure 4.18).

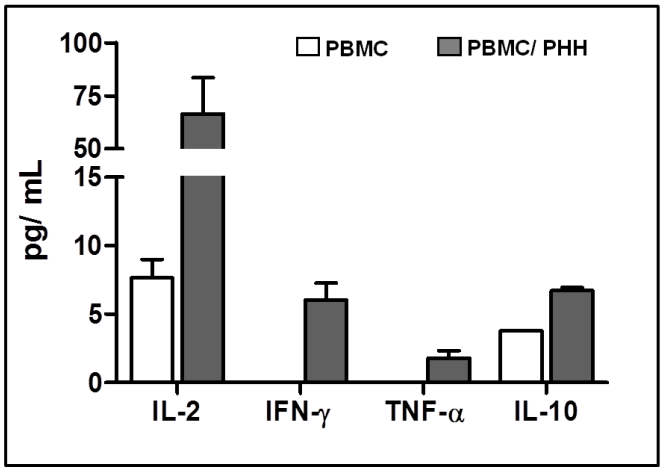

Figure 4.18 Cytokine profile in the supernatant of a PHH/ PBMC co-culture.
After 72 hours, the supernatant from PBMC monocultures or PHH/ PBMC co-cultures was analyzed for the presence of IL-2, IFN-γ, TNF-α and IL-10 using Luminex technology.

TGF-β is an important immunomodulatory cytokine that can inhibit differentiation of effector T cells. TGF-β is produced by various liver cell populations, including hepatocytes and thus might play a role in CD8+ T cell tolerance induction.

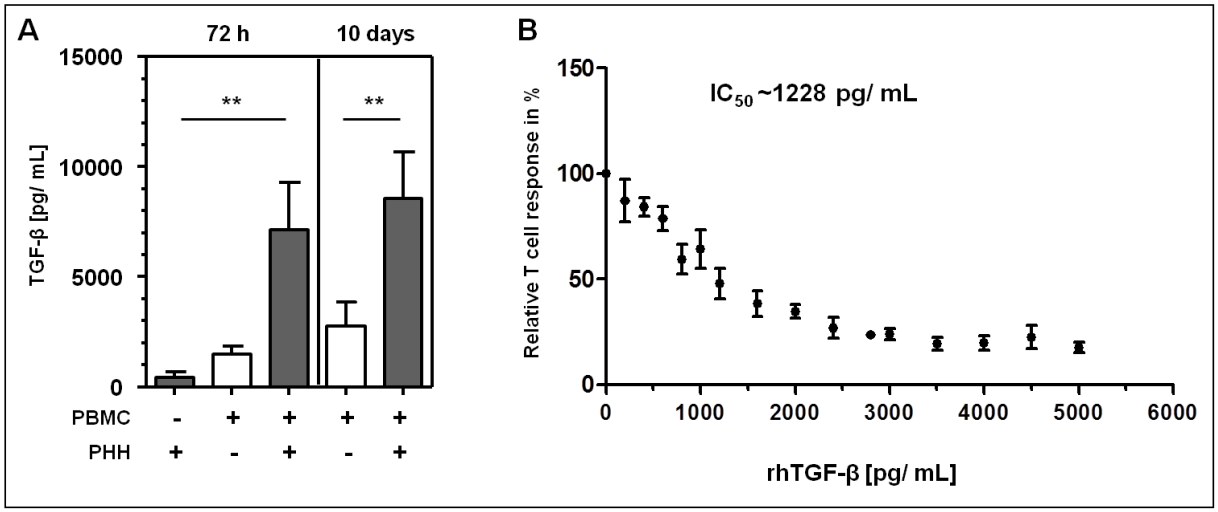

Figure 4.19 Increased TGF-β concentrations in PHH/ PBMC co-cultures.
(A) The concentration of TGF-β was verified in the supernatant of a 3 or 10 days co-culture. (B) RhTGF-β was added in increasing concentrations to PBMC. After 7 days virus-specific CD8+ T cell expansion was analyzed.

Soluble TGF-β in the supernatant was about 7-fold increased in PHH/ PBMC co-cultures in comparison to TGF-β secretion by hepatocytes or PBMC monocultures after 3 days and about 4-fold increased after 10 days of co-cultures (figure 4.19A).
To confirm that the concentrations of TGF-β determined by ELISA are indeed able to cause inhibition (see figure 4.13 and 4.14) we artificially added rhTGF-β in increasing concentration to PBMC cultures.
Figure 4.19B depicts the results of four independent experiments. We could observe increasing inhibition of the CD8+ T cell response by increasing rhTGF-β concentrations and with an IC_{50} of approximately 1200 pg/ mL, the inhibitory effect caused by TGF-β fits to the concentration determined by ELISA.

4.2.4.1 TGF-β blocking/ neutralization

In order to confirm the participation of TGF-β in the induction of inhibition in PHH/ PBMC co-cultures, several strategies were applied to block or neutralize TGF-β-mediated effects. The first approach included repeated application of an anti-TGF-β neutralizing antibody. A second approach was to block TGF-β in the co-cultures by

the application of Follistatin. Follistatin is a compound that blocks the mode of action of members of the TGF-β superfamily by binding and neutralizing them.

Last, the activin receptor-like kinase (ALK)-5 inhibitor (SB-431542) was applied to the co-cultures. ALK-5 is a part of the TGF-β type 1 receptor, thus blocking ALK-5 inhibits TGF-β mediated signaling (Inman *et al* 2002). Hepatocytes where pre-incubated with different concentrations of ALK-5 inhibitor ranging from 1 to 1000 µM/ mL, before adding $1.5x10^6$ PBMC plus 1 µg/ mL peptide. Figure 4.20 summarizes the results.

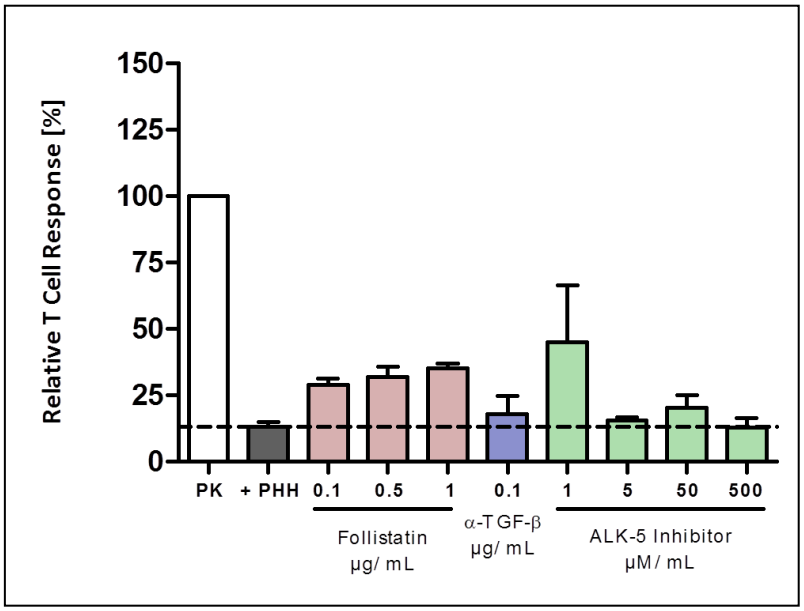

Figure 4.20 TGF-β blocking.
TGF-β blocking/ neutralizing was assessed using the following reagents in PHH/ PBMC co-cultures: 0.1, 0.5 or 1 µg/ mL Follistatin, 0.1 µg/ mL anti-TGF-β antibody, 1, 5, 50, 500 µM/ mL ALK-5 inhibitor. After 7 days of culture, PBMC were harvested and analyzed by intracellular IFN-γ staining. Bars represent the normalized means in comparison to PBMC monocultures.

Unfortunately, none of the molecules applied to the PHH/ PBMC co-cultures were able to reverse the PHH-mediated inhibition of CD8+ T cell expansion. In one experiment the application of 1 µM/ mL ALK-5 inhibitor could reverse the inhibitory effect, however, this was not reproducible.

Thus, the possible contribution of TGF-β in this system could not be confirmed and needs to be further elucidated.

5 Discussion

5.1 *In vitro* priming of HCV-specific CD8+ T cells

Chronic viral infections, like those caused by the Hepatitis C virus still remain a major health problem. An estimated 150 million people worldwide are chronically infected with HCV and are at high risk to develop steatosis and liver cirrhosis, resulting in an increased risk of developing hepatocellular carcinomas. The high rate of chronicity in HCV infected patients is mainly due to the impairment of the host immune system to control the viral infection. Due to the error prone viral polymerase, HCV frequently evades CD8+ T cell immune responses by substitutions in MHC class I-restricted epitopes. Mutations located in the HLA-binding anchors of an epitope (position two and the C-terminal amino acid for most HLA class I alleles) attenuate peptide binding, whereas mutations in the centre of the epitope more often result in impaired TCR recognition of the HLA class I/ peptide complex (Soderholm *et al* 2006). Moreover, beyond impairment of peptide affinity or TCR avidity substitutions inside epitopes or in the epitope flanking region can alter processing of the viral antigens. The high mutation rate of the virus is the key aspect in the failure to develop an effective vaccine. Due to the fact that 20-25 % can spontaneously clear the virus associated with a vigorous T cell response and importantly associated with a lower likelihood of persistent infection upon reexposure (Osburn *et al* 2010), the induction of memory T cell responses that provide at least partial protection should in principle be feasible. Unfortunately, due to the lack of a suitable model, trials to study the efficacy of different vaccines in humans are difficult. Thus, surrogate measures for vaccine efficacy are needed. *In vitro* T cell priming for the study of *de novo* T cell responses represents a tool to study the impact of antigen sequence variations on vaccination efficacy. Moreover, such *in vitro* generated HCV-specific T cells could also be utilized to treat HCV-reinfection after liver transplantation.

In this study, we analyzed the role of minor sequence differences in an HLA-A*02-restricted immunodominant CD8+ T cell epitope located in NS3 and the outcome of sequence differences on priming capacity and the induction of polyfunctional, cross-reactive CD8+ T cell responses. We identified one epitope variant ($NS3_{1406\text{-}1415}$ KLSALGLNAV) mainly circulating in Japan, which was more potent in the induction of effective CD8+ T cell responses in comparison to the other variants analyzed.

5.1.1 *In vitro* T cell priming

To study the impact of sequence substitutions in the immunodominant HLA-A*02-restricted $NS3_{1406-1515}$ epitope on the *de novo* induction of $NS3_{1406-1515}$-specific CD8+ T cells, we established an *in vitro* CD8+ T cell priming model. For this purpose, naïve CD8+ T cells were enriched from PBMC and stimulated with monocyte-derived dendritic cells. Models to reliably generate antigen-specific T cell responses *in vitro* are useful to screen candidate epitopes for vaccination trials. However, in contrast to the expansion of effector memory CD8+ T cells, the expansion of virus-specific CD8+ T cells from the naïve repertoire is elaborate.

In this study, monocytes were isolated by adherence to plastic and differentiated in the presence of IL-4 and GM-CSF. Resulting moDC were matured with LPS/ IFN-γ, loaded with the epitope variants and used for the stimulation of naïve CD8+ T cells. Numerous studies have aimed at optimizing cytokine combinations and the duration of the stimulation for the maturation of DC including TNF-α and IL-1β, LPS and CD40 ligand (Cella *et al* 1999), PGE_2, IL-1β, IL-6 and TNF-α (Jonuleit *et al* 1997) and LPS/ IFN-γ (Vieira *et al* 2000, Wölfl *et al* 2011). In the present study, LPS/ IFN-γ-matured DC showed in contrast to PGE_2, IL-1β, IL-6 and TNF-α-matured DC an upregulation of the costimulatory molecules CD80 and CD86 (see figure 4.2).

The reason to use purified naïve CD8+ T cells relies on the lower activation threshold of memory CD8+ T cells, which might result in the unspecific expansion of those cell types. In addition, the amount of naïve T cells decreases with age and thus varies significantly between individuals (Ginaldi *et al* 1999). Due to the exceedingly low numbers of HCV-specific T cells in the naïve repertoire (Schmidt *et al* 2011), HCV-specific CD8+ T cells need to be restimulated for expansion, using either peptide-pulsed monocytes or peptide-pulsed, irradiated PBMC on day 11 and day 19. Both strategies were suitable for the expansion of antigen-specific CD8+ T cells, though, the use of irradiated PBMC seemed to be more effective, especially for the expansion of virus-specific CD8+ T cells. In addition, the use of PBMC represents the more time and cost effective strategy.

5.1.2 $NS3_{1406-1415}$-specific CD8+ T cells have distinct priming potential

To analyze the impact of minor sequence differences in immunodominant CD8+ T cell epitopes, seven variants of the HLA-A*02-restricted $NS3_{1406-1415}$ epitope were selected [see table 4.1, HCV database, http://hcv.lanl.gov (Yusim *et al* 2005)]. These included the five most frequent genotype 1b variants KLSGLGLNAV (42.8 % of genotype 1b sequences), KLSSLGLNAV (18.4 %), which predominantly circulates in Europe, KLSALGLNAV (12.5 %) and KLSALGVNAV (4.3 %), which predominantly circulates in Japan, as well as a unique epitope sequence (QLSGLGINAV) originating from a large single-source outbreak (Wiese *et al* 2000). In addition, a well described genotype 1a sequence and its variant were studied (prototype: KLVALGINAV, 43.3 % and variant: KLVALGVNAV, 31.3 %).
Several studies indicated that HLA-binding shape CD8+ T cell immunodominance (Kotturi *et al* 2008, Sette *et al* 1994). Analysis of the peptide-binding to HLA-A*02 revealed that all seven variants show equally high binding affinity (see figure 4.7) and therefore did not have any impact on priming capacity in this study.

After expansion for 28 days, the presence of variant-specific CD8+ T cells was analyzed by dextramer staining (see figure 4.8A). There were substantial differences in the priming capacities of the different epitope variants. Interestingly, the variant that is mainly circulating in Japan $NS3_{1406-1415}$ KLSALGLNAV showed the best priming potential in comparison to the other variants. Furthermore, this epitope variant elicited functional effector CD8+ T cells by means of IFN-γ production (see figure 4.8B). Moreover, CD8+ T cells primed with the KLSALGLNAV variant were polyfunctional with respect to the secretion of effector cytokines and chemokines, i.e. TNF-α, MIP-1β, IL-2, IFN-γ and the expression of the degranulation marker CD107a. Almost ¾ of KLSALGLNAV-specific CD8+ T cells were able to produce more than three effector molecules. Several studies suggest that effective vaccination should induce robust CD4+ and CD8+ T cell responses for long-term immunity (Cooper *et al* 1999, Gruner *et al* 2000, Lechner *et al* 2000). In addition, vaccination has to overcome the enormous viral sequence variation and therefore has to induce a broad, polyfunctional and cross-reactive immune response, capable to respond to viral sequence variants. Analysis of the cross-reactivity of the peptide-specific CD8+

T cells revealed a high degree of cross- reactivity for KLSALGLNAV-primed CD8+ T cells (see figure 4.10).

Immune resposes are initiated via T cell receptors that make contact to peptide-MHC complexes. The TCR consists of four polypeptides (α, β, γ, δ) that merge to two forms of heterodimers (αβ and γδ). Each polypeptide has a variable (V), joining (J) and constant (C) region. TCR diversity is generated upon rearrangement of this regions. Additional diversity is mediated by the random insertion of non-germline-encoded nucleotides at the junctions of these rearranged segments, which provides the main site of antigen recognition (Rubtsova *et al* 2009). Several methods to analyze T cell receptor repertoires are available, including the TCR-variable segment analysis by implementation of a comprehensive antibody panel directed against individual TCR Vβ chains. Vβ chain analysis defines whether a peptide-specific T cell response is polyclonal or dominated by a few TCR clonotypes. In the present study, the TCR Vβ chain repertoire was analyzed in $NS3_{1406-1415}$ QLSGLGINAV-primed CD8+ T cells (good priming capacity, no cross-reactivity) and compared to $NS3_{1406-1415}$ KLSALGLNAV-primed CD8+ T cells (very good priming capacity, good cross-reactivity), using an antibody panel. Analysis of the individual Vβ chains showed a clear difference in profiles of TCR Vβ bias. CD8+ IFN-γ+ T cells primed with the KLSALGLNAV-variant were characterized by a broader TCR Vβ use (see figure 4.13 panel A, black bars), whereas CD8+ IFN-γ+ T cells primed with the QLSGLGINAV variant that were characterized by a more restricted use of TCR Vβ chains (see figure 4.13, panel B and C, blue bars). Importantly, upon restimulation with other epitope variants the same Vβ chains as for the homologous variant were utilized (see figure 4.13, red bars) suggesting that the individual Vβ were cross-reactive. It is unclear, if cross-reactivity upon priming with the KLSALGLNAV-variant was defined at the TCR clonotype level or if a complex mixture of multiple clonotypes with distinct fine specificities was primed. To address this in more detail, TCR clonotype analysis by cloning of individual TCR would be required. Of note, prior studies suggest that the number of individual TCR expressed on CD8+ T cells is essential in determining the fine specificity and effector functions in antigen-specific CD8+ T cells (Lim *et al* 2000). Thus, usage of multiple Vβ chains as observed upon priming with the KLSALGLNAV-variant strongly suggests a benefit for the CD8+ T cells.

In the present study, PBMC isolated from HLA-A*02 positive buffy coats from healthy volunteer blood donors were used. HLA-typing revealed that all patients had the HLA-A*02:01 allelic variant (data not shown). Among the HLA-A*02 allelic variants HLA-A*02:01 is the most prevalent variant, hence commonly used to study HLA-A*02-restricted CD8+ T cell responses. HLA-A*02 allelic variants are unevenly distributed worldwide, with certain alleles predominating among different ethnicities. HLA-A*02:01 is predominantly present in the Caucasian and Native American population, with almost >90 % of HLA-A*02 positive carrying this allelic variant. In contrast, in the Japanese population, only 47 % of the HLA-A*02 population express this allelic variant and 36 % carry HLA-A*02:06 (Chen *et al* 2012). Evidence suggests that polymorphisms in the peptide-binding groove affect binding of peptide-antigens (Matsui *et al* 1994) and thus influences the repertoire of peptides presented by this allelic variant. Taking this into account, the blood donors used in this study are somewhat biased towards the Caucasian allelic variant. It can be hypothesized that the results of the priming capacity for the different variants of the present study (see figure 4.8) is influenced by this allelic restriction. Studying the epitope variants in the context of other allelic variants, e.g. HLA-A*02:06, might influence the outcome significantly and has to be taken into consideration.

HCV is characterized by high levels of genetic variation between genotypes and subtypes but also between isolates of the same subtype. In the context of HLA-allelic restriction, this high sequence diversity thus represents the main barrier for immune control. There is strong evidence that the protective effect of certain HLA alleles on infection outcome is limited to certain HCV genotypes. Studies indicate that the protective effect of HLA class I alleles can be abrogated due to sequence differences between genotypes (Giugliano *et al* 2009, Neumann-Haefelin *et al* 2010) indicating that sequence differences are relevant at the epitope level. Evidence suggests that transmission of viral strains with pre-existing mutations in immunodominant epitopes attenuates viral control and epitope variants that are less recognized abrogate protective effects (Kim *et al* 2011). In line with that we could recently show that pre-existing substitutions in the infection source influence the impact of corresponding HLA alleles. In two large HCV genotype 1b outbreaks, from the late ‘70s in Ireland and the former German Democratic Republic, conflicting results have been observed

concerning the effect of particular HLA class I alleles on disease outcome. We compared sequences in immunodominant CD8+ T cell epitopes from the infection source of both cohorts and the consequences for immune selection pressure and viral evolution in the East-German cohort. We found that sequence differences in key epitopes in the infection source between the Irish and the East-German cohort have profound effects on the ability to clear HCV infection in the presence of particular HLA class I alleles (Ziegler *et al* 2013).

Taken together, these findings show that even minor sequence variations in the same subtype can abrogate CD8+ T cell responses, which is of great importance in choosing suitable sequences for vaccine design.

Over the last decades numerous vaccine approaches have been assessed, including those that have focused on inducing T cell responses to the non-structural HCV antigens. NS antigens are genetically more conserved compared to envelope proteins and were shown to contain multiple CD4+ and CD8+ T cell epitopes. Four main strategies to induce T cell responses upon vaccination have been studied in humans so far. These include recombinant protein vaccines, peptide vaccines, DNA vaccines and vector vaccines [reviewed in (Halliday *et al* 2011)]. Peptide vaccines are well tolerated, however, peptide vaccines are restricted to defined epitopes and may not cover the whole variety of HLA alleles, although the number of epitopes required to achieve immune control remains elusive for HCV. If only a few epitopes are included in a vaccine, T cell response to individual epitopes may be maximized, but there is the risk that the induced immune response is insufficient to control the virus, e.g. because the genetic barrier to escape is too low. The therapeutic peptide vaccine IC41 contains five synthetic peptides from core, NS3 and NS4 proteins combined with the adjuvant poly-l-arginine (Klade *et al* 2008). Biweekly IC41 administration was found to induce T cell responses in chronically HCV infected patients (Firbas *et al* 2010), but there was no impact on viral load. In contrast to this peptide approach, mosaic vaccines contain fragments of natural sequences, which were assembled using an algorithmic approach to maximize the coverage of potential T cell epitopes (Yusim *et al* 2010). This approach may theoretically be beneficial to allow T cell priming against the majority of circulating viral variants, though, the huge antigen diversity in a vaccine may also be disadvantageous when the T cell response

is biased towards irrelevant epitopes. Both extremes in ongoing vaccine research show that the seemingly simple task to select an antigen for vaccination is rather complex and not defined yet for HCV. It is clearly important to induce broad and polyfunctional CD8+ T cell responses covering the genetic diversity of circulating isolates, as well as infecting quasispecies. The data obtained in this thesis suggest that it may be necessary to address this experimentally for individual epitopes. Importantly, in contrast to our expectation, the optimal epitope sequence for priming of a CD8+ T cell against the predominant sequence in Europe was not this European sequence itself. We could show that a Japanese sequence (KLSALGLNAV) rarely observed in Europe showed the highest priming capacity and induced CD8+ T cells that were cross-reactive even with European sequences. This has important consequences for vaccine design against variable pathogens. The data imply that superior antigen sequences for activation of T cells may exist and that these “optimal” antigens are not necessarily the most frequent in circulating variants of the pathogen. In fact, it is possible that the “optimal” antigen sequence is even absent from circulating pathogen variants, which makes selection of the optimal antigen sequence for vaccine design rather difficult.

5.2 PHH inhibit the expansion of virus-specific CD8+ T cells

One possible mechanism that might explain the impaired immune responses in HCV infection represents the site of primary T cell activation, which in HCV infection occurs in the liver. In fact, the liver displays distinct immunological properties, as primary T cell activation in the liver frequently results in immunological tolerance. This advantage in settings of tolerance induction to liver grafts, but is detrimental to immune responses generated against HCV. In this study, we analyzed the influence of primary human hepatocytes (PHH) on the expansion of virus-specific CD8+ T cells. We could show that PHH inhibit the expansion of CD8+ T cells in a contact depended manner, which was accompanied by increased levels of TGF-β.

5.2.1 Role of hepatocytes on primary intrahepatic T cell activation

Hepatocytes comprises 70-80 % of hepatic cells (Wick *et al* 2002) and are thus the main cell type in the liver. Hepatocytes are involved in the regulation of metabolisms, including detoxification and processing of lipids, amino acids and proteins. In early HCV infection, hepatocytes are the predominant liver cell expressing HCV antigens to virus-specific T cells. Studies by Warren and colleagues showed that circulating naïve CD8+ T cells and hepatocytes occur, although it was long thought to be impossible due to physical separation (Warren *et al* 2006). In this study, we show that primary human hepatocytes inhibit the expansion of CMV- or Flu-specific CD8+ T cells in a contact depended manner (see figure 4.17). Former studies in mice models already indicated that purified hepatocytes are able to activate naïve CD8+ T cells *in vitro*, even in the absence of costimulatory molecules. The induced proliferation was comparable in magnitude to that seen in response to DC stimulation, but in contrast to CD8+ T cells activated by splenocytes, hepatocyte activated CD8+ T cells lost their proliferative capacity after 3 days of co-culture (Bertolino *et al* 1998). Similar to that, *in vivo* studies of mice indicated that activation of CD8+ T cells by antigen-bearing hepatocytes lead to the premature death, which was assigned to limited IL-2 production and low expression of the antiapoptotic bcl-xL and bcl-2 survival genes. In addition, T cell activation by hepatocytes resulted in the upregulation of the proapoptotic proteins Bim and caspase 3, which triggers apoptosis of those cells (Holz *et al* 2010). Premature death could be prevented by supplying exogenous IL-2

or by providing CD28 co-stimulation, suggesting that the failure of hepatocytes to express CD28 ligands may have been responsible for premature death of activated CD8+ T cells (Bertolino *et al* 1999). In the present study, PHH/ PBMC were repeatedly fed with exogenous IL-2, which could not overcome the hepatocyte mediated inhibition.

Recently it has been shown that hepatocytes up-regulate PD-L1 upon viral infections. PD-L1 is the ligand for the co-inhibitory molecule PD-1, which is additionally upregulated on HCV-specific CD8+ T cells (Golden-Mason *et al* 2007, Penna *et al* 2007, Radziewicz *et al* 2007) (see 1.4.2.2). Blocking the physical contact of co-inhibitory molecules by the insertion of a transwell in the co-culture system, might explain the reversion of the inhibition in the transwell setting (see figure 4.17C). This was additionally confirmed by medium transfer experiments. Soluble factors play only a minor role in this setting, as the transfer of conditioned medium did only mediate minor inhibitory effects, which can be explained by the exhaustion of nutrients.

5.2.2 Role of TGF-β in PHH/ PBMC co-cultures

One main factor despite liver-resident APC in the induction of tolerance is the local presence of immunoregulatory mediators IL-10, PGE_2 and TGF-β. These immunosuppressive mediators are secreted by various cell types, including LSEC, Kupffer cells and hepatocytes (Bissell *et al* 1995, Knolle *et al* 1995, Rieder *et al* 1990). To verify the influence of potential proinflammatory or inhibitory cytokines in the present study, the cytokine profile in the supernatant was analyzed (see figures 4.18 and 4.19). We could not detect any increase in IL-10 (see figure 4.18) nor in PGE_2 concentrations (data not shown). Importantly, increased concentrations of TGF-β were detectable in PHH/ PBMC co-cultures (see figure 4.19). In this setting, regulatory T cells might play a role. It was shown that CD4+ CD25- naïve T cells are converted to CD4+ CD25+ regulatory T cells by TGF-β-mediated induction of the transcription factor *Foxp3* (Chen *et al* 2003). CD4+ CD25+ regulatory T cells have emerged as a unique population of suppressor cells that mediate peripheral tolerance (Sakaguchi 2000, Shevach 2002). Evidence suggest that compared with normal livers, the livers of HCV or HBV infected patients contain increased numbers

of *Foxp3*-positive regulatory T cells, which suppress effector T cells (Franceschini *et al* 2009, Manigold and Racanelli 2007). Unfortunately, we were not able to block the mode of action of TGF-β in this setting and could thus not confirm a role of TGF- β. Studies in mice models performed in collaboration with Jia Liu at the institute of virology in Essen supported the role of TGF-β-mediated suppression. These data confirmed on the one hand that the hepatocyte mediated inhibition is contact-dependent (figure 5.1A and B), as the production of pro-inflammatory cytokines IL-2, TNF-α and IFN-γ is reconstituted in transwell experiments (figure 5.1B, blue edge), but also shows that neutralization of TGF-β using an anti-TGF-β neutralizing antibody can restore splenocyte proliferation (Jia Liu *et al* 2012, unpublished data).

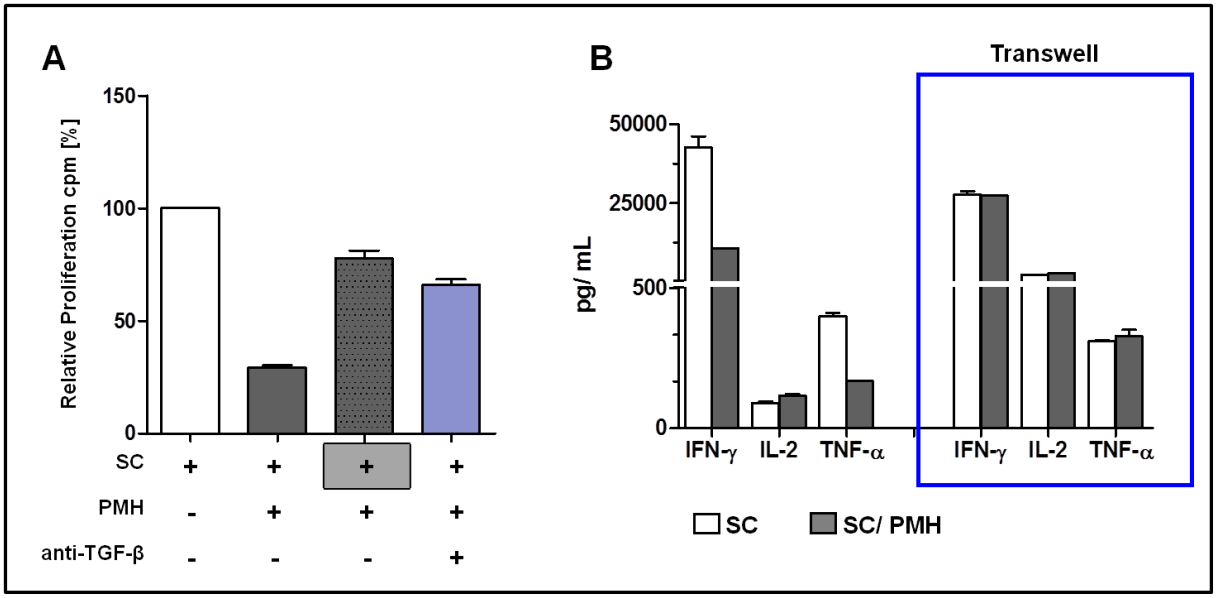

Figure 5.1 Primary murine hepatocytes (PMH) inhibit the proliferation of splenocytes (SC) in a contact-dependent manner.
(A) SC were isolated and incubated with anti-CD3/ CD28 antibodies in the presence or absence of PMH and anti-TGF-β. (B) The cytokine profile of contact-dependent or independent (blue edge) PMH/ SC co-cultures was analyzed after 72 hours by ELISA (Jia Liu *et al* 2012, unpublished data).

Long-term culture of primary hepatocytes is a challenging task, as primary hepatocytes rapidly lose their cuboidal morphology and liver-specific functions over a few days of culture. The cells accumulate actin stress fibers and become fibroblast-like. One common technique to culture primary hepatocytes is to seed the cells on a single layer of collagen gel. Under these conditions, hepatocytes keep liver-specific functions for a short period, notwithstanding, liver-specific functions decline within the first week (Shulman and Nahmias 2013).

Primary human hepatocytes are mainly isolated from fibrotic livers, hepatocellular carcinomas or those rejected from transplantation (see appendix and figure 4.14) resulting in a huge variability in quality. Indeed, human hepatocytes are isolated in most cases from diseased livers, which results in differences in the isolation procedure, as well as the quality of PHH, leading to variations concerning the purity of the hepatocyte culture. Furthermore, PHH are very rarely available, which lead to a limitation in experimental recapitulation. In contrast to that primary murine hepatocytes are available in a physiological, as well as genetically more defined background. Thus, primary murine hepatocyte cultures are in contrast to human hepatocytes better reproducible, which might explain the differences obtained between the primary human and murine hepatocyte cultures. In addition, technical differences between the isolation procedure of murine and human hepatocytes might influence the purity of primary hepatocyte cultures.

Bowen and colleagues discussed a two-site model of T cell activation, which suggest that the site of initial antigen encounter by CD8+ T cells determines the outcome of an immune response mounted in the liver. If the majority of T cells are activated in the liver in contrast to the lymph nodes, tolerance will occur. Effective immune responses would than require that the majority of antigen-specific cells are activated in the lymph nodes (Bowen *et al* 2005). In early HCV infection, HCV-specific antigen presentation to resident lymphocytes occurs solely in the liver and might thus represent a mechanism of the failure of the host immune response to hepatotropic viral infections.

6 Summary

Infection with the hepatitis C virus still remains a global health problem. The antiviral immune response against HCV by CD8+ T cells plays a central role in viral containment, however, it has been highlighted in numerous studies that the CD8+ T cell responses during HCV infection are relatively weak and dysfunctional. Activation of virus-specific CD8+ T cells is a multi-step process, in which the sequence of the presented antigen, as well as the site of antigen presentation has important consequences for the quality of the CD8+ T cell response. Hence, in the present thesis, determinants of HCV-specific CD8+ T cell expansion were analyzed.

HCV is characterized by high levels of genetic variation between genotypes and subtypes but also between isolates of the same subtype. This high sequence variation in immunodominant epitopes precludes protective immunity and represents a major obstacle to vaccine design.
In this study, we analyzed the global sequence diversity of the immunodominant HLA-A*02-restricted CD8+ T cell epitope $NS3_{1406-1415}$ in circulating genotype 1a and 1b isolates and its impact on CD8+ T cell priming and induction of cross-reactive T cell responses. Circulating sequence variants of the $NS3_{1406-1415}$ epitope showed distinct priming potential and cross-reactivity patterns. Importantly, the highest degree of cross-reactivity was induced by a sequence that is rarely observed in the European population. Upon vaccination, the elicited CD8+ T cells should be polyfunctional and target multiple epitope variants. This study highlights the relevance of antigen sequence variation for subsequent CD8+ T cell responses and provides a rational for the selection of the optimal epitope sequence for a vaccine against HCV, associated with the induction of a broad cross-reactive and polyfunctional CD8+ T cell response.

A hallmark of chronic infections with hepatotropic viruses such as HCV is the notoriously weak antiviral immune response by CD8+ T cells. Primary antigen encounter in HCV infections happens in the liver. It is believed that the liver-specific environment can induce tolerance either by induction of dysfunctional CD8+ T cells

or by premature death of activated CD8+ T cells. Recent reports from mouse models indicate that hepatocytes can act as antigen-presenting. Direct antigen presentation by virally infected hepatocytes thus might inhibit the CD8+ T cell response.

This study showed that the frequency of virus-specific CD8+ T cells after 10 days expansion was dramatically reduced in the presence of hepatocytes. This suppressive effect was independent of the cell-type that was used for antigen-presentation (hepatocyte or PBMC) and of antigen-specific contact. Expansion of CD8+ T cells in the presence of hepatocytes was almost completely restored in transwell experiments indicating that the suppressive effect is cell-contact dependent. Analysis of the cytokine profile from culture supernatants revealed increased concentrations of TGF-β in the presence of hepatocytes compared to their absence.

This study gives important hints for the establishment of chronic infections by hepatotropic viruses.

7 Zusammenfassung

Infektionen mit dem Hepatitis C Virus (HCV) sind global ein enormes Problem. Verschiedene Studien belegen, dass während einer akuten HCV-Infektion CD8+ T Zellen eine wichtige Rolle bei der Kontrolle der Virusinfektion spielen. Allerdings sind HCV-spezifische T Zellantworten oft schwach und dysfunktional. Die Aktivierung virusspezifischer T Zellen ist ein sensitiver, komplexer Prozess, bei dem sowohl die Sequenz des präsentierten Antigens, als auch das Umfeld in dem der primäre Kontakt zwischen antigenpräsentierender und virusspezifischer Zelle stattfindet, Einfluss auf die resultierende T Zellantwort ausübt. Daher wurden in der vorliegenden Studie Faktoren der HCV-spezifischen T Zellantwort untersucht.

Das Hepatitis C Virus zeichnet sich durch ein hohes Maß an genetischer Variabilität aus. Es werden sieben verschiedene Genotypen und multiple Subtypen unterschieden, aber selbst zwischen Varianten des gleichen Subtyps gibt es wichtige Sequenzunterschiede. Diese hohe Sequenzvariabilität ist ein entscheidendes Hindernis in der Entwicklung eines geeigneten Impfstoffs gegen HCV. Allerdings wird selbst nach spontaner Ausheilung einer Infektion eine protektive Immunantwort häufig nicht erreicht, da sich immundominante Epitope zwischen verschiedenen HCV Isolaten unterscheiden und diese Sequenzvarianten häufig nicht von CD8+ T Zellen erkannt werden.

Im ersten Teil dieser Arbeit wurde die globale Sequenzdiversität der Genotypen 1a und 1b in einem immundominanten HLA-A*02-assoziierten CD8+ T Zell-Epitop $NS3_{1406-1415}$ analysiert. Dabei wurde der Einfluss der Sequenzunterschiede auf die ‚Priming'-Kapazität von CD8+ T Zellen sowie die Induktion von kreuzreaktiven CD8+ T Zellen untersucht. Es konnte gezeigt werden, dass sich die global zirkulierenden Varianten in ihrer ‚Priming'-Kapazität von CD8+ T Zellen unterscheiden. Diese *in vitro* generierten T Zellantworten unterschieden sich maßgeblich in ihrer Fähigkeit der Kreuzreaktivität. Interessanterweise stammt die Sequenzvariante mit der breitesten Kreuzreaktivität aus Isolaten, die hauptsächlich in Japan zirkulieren und somit nur selten in Europa zu finden sind. Ein Impfstoff gegen HCV sollte eine polyfunktionelle CD8+ T Zellantwort hervorrufen und ebenso kreuzreaktiv mit Sequenzvarianten des Epitops sein. In dieser Studie konnte die entscheidende Bedeutung von Sequenz-

variationen in einem Antigen für die folgende CD8+ T Zellantwort gezeigt werden. Die Daten unterstreichen, wie wichtig die Auswahl der optimalen Epitopsequenz für eine Vakzine gegen HCV ist, indem eine polyfunktionelle sowie auch kreuzreaktive CD8+ T Zellantwort aktiviert wird.

Im Falle einer Infektion mit dem Hepatitis C Virus findet der erste Kontakt zwischen antigenpräsentierender und virusspezifischer Zelle in der Leber statt. Die Leber spielt eine wichtige Rolle im adaptiven Immunsystem, da hier die Präsentation von Antigenen an CD8+ T Zellen Toleranz induzieren kann, indem dysfunktionale CD8+ T Zellen induziert werden oder deren Aktivierung in der Leber zu früher Apoptose führt. Ein Kennzeichen chronischer Infektion mit hepatotropen Viren ist somit die schwache Immunantwort durch CD8+ T Zellen. Jüngere Studien an Mausmodellen konnten belegen, dass Hepatozyten ebenfalls als antigenpräsentierende Zellen fungieren und demnach möglicherweise eine Rolle in der Toleranzinduktion von CD8+ T Zellen einnehmen.
Im zweiten Teil der vorliegenden Arbeit wurde gezeigt, dass die Expansion virus-spezifischer CD8+ T Zellen in der Anwesenheit von primären humanen Hepatozyten drastisch reduziert ist. Die Hemmung der virusspezifischen CD8+ T Zellexpansion war unabhängig vom antigenpräsentierenden Zelltyp und war unabhängig vom antigenspezifischen Kontakt. Die Hemmung der virusspezifischen CD8+ T Zell Expansion war allerdings aufgehoben, wenn direkter Kontakt zwischen Hepatozyten und PBMC durch den Gebrauch eines Membraneinsatzes in der Kultur verhindert wurde. Dies deutet an, dass der hemmende Effekt von Hepatozyten kontaktabhängig ist. Eine Analyse der Zellkulturüberstände erwies eine erhöhte Konzentration von TGF-β in der Anwesenheit von Hepatozyten.
In der vorliegenden Studie wurde demnach gezeigt, dass eine Hemmung der CD8+ T Zellantwort durch Hepatozyten möglicherweise zur Chronifizierung von Infektionen mit hepatotropen Viren beitragen kann.

8 References

Afdhal NH (2004). The natural history of hepatitis C. *Semin Liver Dis* 24 Suppl 2**:** 3-8.

Alter HJ, Seeff LB (2000). Recovery, persistence, and sequelae in hepatitis C virus infection: a perspective on long-term outcome. *Semin Liver Dis* 20**:** 17-35.

Alter MJ (2002). Prevention of spread of hepatitis C. *Hepatology* 36**:** S93-98.

Bar KJ, Li H, Chamberland A, Tremblay C, Routy JP, Grayson T *et al* (2010). Wide variation in the multiplicity of HIV-1 infection among injection drug users. *J Virol* 84**:** 6241-6247.

Barnes E, Folgori A, Capone S, Swadling L, Aston S, Kurioka A *et al* (2012). Novel adenovirus-based vaccines induce broad and sustained T cell responses to HCV in man. *Sci Transl Med* 4**:** 115ra111.

Bartenschlager R, Ahlborn-Laake L, Mous J, Jacobsen H (1993). Nonstructural protein 3 of the hepatitis C virus encodes a serine-type proteinase required for cleavage at the NS3/4 and NS4/5 junctions. *J Virol* 67**:** 3835-3844.

Bartenschlager R, Frese M, Pietschmann T (2004). Novel insights into hepatitis C virus replication and persistence. *Adv Virus Res* 63**:** 71-180.

Bartenschlager R, Penin F, Lohmann V, Andre P (2011). Assembly of infectious hepatitis C virus particles. *Trends Microbiol* 19**:** 95-103.

Bartosch B, Dubuisson J, Cosset FL (2003). Infectious hepatitis C virus pseudo-particles containing functional E1-E2 envelope protein complexes. *J Exp Med* 197**:** 633-642.

Benseler V, Warren A, Vo M, Holz LE, Tay SS, Le Couteur DG *et al* (2011). Hepatocyte entry leads to degradation of autoreactive CD8 T cells. *Proc Natl Acad Sci U S A* 108**:** 16735-16740.

Berman K, Kwo PY (2009). Boceprevir, an NS3 protease inhibitor of HCV. *Clin Liver Dis* 13**:** 429-439.

Bertolino P, Trescol-Biemont MC, Rabourdin-Combe C (1998). Hepatocytes induce functional activation of naive CD8+ T lymphocytes but fail to promote survival. *Eur J Immunol* 28**:** 221-236.

Bertolino P, Trescol-Biemont MC, Thomas J, Fazekas de St Groth B, Pihlgren M, Marvel J *et al* (1999). Death by neglect as a deletional mechanism of peripheral tolerance. *Int Immunol* 11**:** 1225-1238.

Bissell DM, Wang SS, Jarnagin WR, Roll FJ (1995). Cell-specific expression of transforming growth factor-beta in rat liver. Evidence for autocrine regulation of hepatocyte proliferation. *The Journal of clinical investigation* 96**:** 447-455.

Blanchard E, Belouzard S, Goueslain L, Wakita T, Dubuisson J, Wychowski C *et al* (2006). Hepatitis C virus entry depends on clathrin-mediated endocytosis. *J Virol* 80**:** 6964-6972.

Bode JG, Ludwig S, Ehrhardt C, Albrecht U, Erhardt A, Schaper F *et al* (2003). IFN-alpha antagonistic activity of HCV core protein involves induction of suppressor of cytokine signaling-3. *Faseb J* 17**:** 488-490.

Boutwell CL, Rolland MM, Herbeck JT, Mullins JI, Allen TM (2010). Viral evolution and escape during acute HIV-1 infection. *J Infect Dis* 202 Suppl 2**:** S309-314.

Bowen DG, McCaughan GW, Bertolino P (2005). Intrahepatic immunity: a tale of two sites? *Trends Immunol* 26**:** 512-517.

Bowen DG, Walker CM (2005a). Adaptive immune responses in acute and chronic hepatitis C virus infection. *Nature* 436**:** 946-952.

Bowen DG, Walker CM (2005b). The origin of quasispecies: cause or consequence of chronic hepatitis C viral infection? *J Hepatol* 42**:** 408-417.

Bukh J, Miller RH, Purcell RH (1995). Genetic heterogeneity of hepatitis C virus: quasispecies and genotypes. *Semin Liver Dis* 15**:** 41-63.

Bull RA, Luciani F, McElroy K, Gaudieri S, Pham ST, Chopra A *et al* (2011). Sequential bottlenecks drive viral evolution in early acute hepatitis C virus infection. *PLoS Pathog* 7**:** e1002243.

Cabrera R, Tu Z, Xu Y, Firpi RJ, Rosen HR, Liu C *et al* (2004). An immunomodulatory role for CD4(+)CD25(+) regulatory T lymphocytes in hepatitis C virus infection. *Hepatology* 40**:** 1062-1071.

Callan MF, Tan L, Annels N, Ogg GS, Wilson JD, O'Callaghan CA *et al* (1998). Direct visualization of antigen-specific CD8+ T cells during the primary immune response to Epstein-Barr virus In vivo. *J Exp Med* 187**:** 1395-1402.

Calne RY, White HJ, Yoffa DE, Binns RM, Maginn RR, Herbertson RM *et al* (1967). Prolonged survival of liver transplants in the pig. *Br Med J* 4**:** 645-648.

Calne RY (2000). Immunological tolerance--the liver effect. *Immunol Rev* 174**:** 280-282.

Cella M, Salio M, Sakakibara Y, Langen H, Julkunen I, Lanzavecchia A (1999). Maturation, activation, and protection of dendritic cells induced by double-stranded RNA. *J Exp Med* 189**:** 821-829.

Chen KY, Liu J, Ren EC (2012). Structural and functional distinctiveness of HLA-A2 allelic variants. *Immunol Res* 53**:** 182-190.

Chen W, Jin W, Hardegen N, Lei KJ, Li L, Marinos N *et al* (2003). Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med* 198**:** 1875-1886.

Chevaliez S, Pawlotsky JM (2012). Virology of hepatitis C virus infection. *Best Pract Res Clin Gastroenterol* 26**:** 381-389.

Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M (1989). Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* 244**:** 359-362.

Coller KE, Berger KL, Heaton NS, Cooper JD, Yoon R, Randall G (2009). RNA interference and single particle tracking analysis of hepatitis C virus endocytosis. *PLoS Pathog* 5**:** e1000702.

Cooper S, Erickson AL, Adams EJ, Kansopon J, Weiner AJ, Chien DY *et al* (1999). Analysis of a successful immune response against hepatitis C virus. *Immunity* 10**:** 439-449.

Crispe IN, Mehal WZ (1996). Strange brew: T cells in the liver. *Immunol Today* 17**:** 522-525.

Crotta S, Stilla A, Wack A, D'Andrea A, Nuti S, D'Oro U *et al* (2002). Inhibition of natural killer cells through engagement of CD81 by the major hepatitis C virus envelope protein. *J Exp Med* 195**:** 35-41.

Doherty DG, O'Farrelly C (2000). Innate and adaptive lymphoid cells in the human liver. *Immunol Rev* 174**:** 5-20.

Drane D, Maraskovsky E, Gibson R, Mitchell S, Barnden M, Moskwa A *et al* (2009). Priming of CD4+ and CD8+ T cell responses using a HCV core ISCOMATRIX vaccine: a phase I study in healthy volunteers. *Hum Vaccin* 5**:** 151-157.

Duong FH, Filipowicz M, Tripodi M, La Monica N, Heim MH (2004). Hepatitis C virus inhibits interferon signaling through up-regulation of protein phosphatase 2A. *Gastroenterology* 126**:** 263-277.

Egger D, Wolk B, Gosert R, Bianchi L, Blum HE, Moradpour D *et al* (2002). Expression of hepatitis C virus proteins induces distinct membrane alterations including a candidate viral replication complex. *J Virol* 76**:** 5974-5984.

Elazar M, Liu P, Rice CM, Glenn JS (2004). An N-terminal amphipathic helix in hepatitis C virus (HCV) NS4B mediates membrane association, correct localization of replication complex proteins, and HCV RNA replication. *J Virol* 78**:** 11393-11400.

Erickson AL, Kimura Y, Igarashi S, Eichelberger J, Houghton M, Sidney J *et al* (2001). The outcome of hepatitis C virus infection is predicted by escape mutations in epitopes targeted by cytotoxic T lymphocytes. *Immunity* 15**:** 883-895.

Fafi-Kremer S, Fauvelle C, Felmlee DJ, Zeisel MB, Lepiller Q, Fofana I *et al* (2012). Neutralizing antibodies and pathogenesis of hepatitis C virus infection. *Viruses* 4**:** 2016-2030.

Farci P, Alter HJ, Wong D, Miller RH, Shih JW, Jett B *et al* (1991). A long-term study of hepatitis C virus replication in non-A, non-B hepatitis. *N Engl J Med* 325**:** 98-104.

Fattovich G, Giustina G, Degos F, Tremolada F, Diodati G, Almasio P *et al* (1997). Morbidity and mortality in compensated cirrhosis type C: a retrospective follow-up study of 384 patients. *Gastroenterology* 112**:** 463-472.

Fauvelle C, Lepiller Q, Felmlee DJ, Fofana I, Habersetzer F, Stoll-Keller F *et al* (2013). Hepatitis C virus vaccines - Progress and perspectives. *Microb Pathog* 58**:** 66-72.

Firbas C, Boehm T, Buerger V, Schuller E, Sabarth N, Jilma B *et al* (2010). Immunogenicity and safety of different injection routes and schedules of IC41, a Hepatitis C virus (HCV) peptide vaccine. *Vaccine* 28**:** 2397-2407.

Franceschini D, Paroli M, Francavilla V, Videtta M, Morrone S, Labbadia G *et al* (2009). PD-L1 negatively regulates CD4+CD25+Foxp3+ Tregs by limiting STAT-5 phosphorylation in patients chronically infected with HCV. *The Journal of clinical investigation* 119**:** 551-564.

Frey SE, Houghton M, Coates S, Abrignani S, Chien D, Rosa D *et al* (2010). Safety and immunogenicity of HCV E1E2 vaccine adjuvanted with MF59 administered to healthy adults. *Vaccine* 28**:** 6367-6373.

Gale M, Jr., Foy EM (2005). Evasion of intracellular host defence by hepatitis C virus. *Nature* 436**:** 939-945.

Gallinari P, Brennan D, Nardi C, Brunetti M, Tomei L, Steinkuhler C *et al* (1998). Multiple enzymatic activities associated with recombinant NS3 protein of hepatitis C virus. *J Virol* 72**:** 6758-6769.

Gallinari P, Paolini C, Brennan D, Nardi C, Steinkuhler C, De Francesco R (1999). Modulation of hepatitis C virus NS3 protease and helicase activities through the interaction with NS4A. *Biochemistry* 38**:** 5620-5632.

Garaigorta U, Chisari FV (2009). Hepatitis C virus blocks interferon effector function by inducing protein kinase R phosphorylation. *Cell Host Microbe* 6**:** 513-522.

Gerlach JT, Diepolder HM, Jung MC, Gruener NH, Schraut WW, Zachoval R *et al* (1999). Recurrence of hepatitis C virus after loss of virus-specific CD4(+) T-cell response in acute hepatitis C. *Gastroenterology* 117**:** 933-941.

Gerlach JT, Diepolder HM, Zachoval R, Gruener NH, Jung MC, Ulsenheimer A *et al* (2003). Acute hepatitis C: high rate of both spontaneous and treatment-induced viral clearance. *Gastroenterology* 125**:** 80-88.

Ginaldi L, De Martinis M, D'Ostilio A, Marini L, Loreto MF, Martorelli V *et al* (1999). The immune system in the elderly: II. Specific cellular immunity. *Immunol Res* 20**:** 109-115.

Giugliano S, Oezkan F, Bedrejowski M, Kudla M, Reiser M, Viazov S *et al* (2009). Degree of cross-genotype reactivity of hepatitis C virus-specific CD8+ T cells directed against NS3. *Hepatology* 50**:** 707-716.

Golden-Mason L, Palmer B, Klarquist J, Mengshol JA, Castelblanco N, Rosen HR (2007). Upregulation of PD-1 expression on circulating and intrahepatic hepatitis C virus-specific CD8+ T cells associated with reversible immune dysfunction. *J Virol* 81**:** 9249-9258.

Golden-Mason L, Palmer BE, Kassam N, Townshend-Bulson L, Livingston S, McMahon BJ *et al* (2009). Negative immune regulator Tim-3 is overexpressed on T cells in hepatitis C virus infection and its blockade rescues dysfunctional CD4+ and CD8+ T cells. *J Virol* 83**:** 9122-9130.

Grakoui A, Wychowski C, Lin C, Feinstone SM, Rice CM (1993). Expression and identification of hepatitis C virus polyprotein cleavage products. *J Virol* 67**:** 1385-1395.

Grakoui A, Shoukry NH, Woollard DJ, Han JH, Hanson HL, Ghrayeb J *et al* (2003). HCV persistence and immune evasion in the absence of memory T cell help. *Science* 302**:** 659-662.

Griffin SD, Beales LP, Clarke DS, Worsfold O, Evans SD, Jaeger J *et al* (2003). The p7 protein of hepatitis C virus forms an ion channel that is blocked by the antiviral drug, Amantadine. *FEBS Lett* 535**:** 34-38.

Gruner NH, Gerlach TJ, Jung MC, Diepolder HM, Schirren CA, Schraut WW *et al* (2000). Association of hepatitis C virus-specific CD8+ T cells with viral clearance in acute hepatitis C. *J Infect Dis* 181**:** 1528-1536.

Guedj J, Rong L, Dahari H, Perelson AS (2010). A perspective on modelling hepatitis C virus infection. *J Viral Hepat* 17**:** 825-833.

Guidotti LG, Chisari FV (2006). Immunobiology and pathogenesis of viral hepatitis. *Annu Rev Pathol* 1**:** 23-61.

Gwack Y, Kim DW, Han JH, Choe J (1997). DNA helicase activity of the hepatitis C virus nonstructural protein 3. *Eur J Biochem* 250**:** 47-54.

Halliday J, Klenerman P, Barnes E (2011). Vaccination for hepatitis C virus: closing in on an evasive target. *Expert Rev Vaccines* 10**:** 659-672.

Hijikata M, Mizushima H, Akagi T, Mori S, Kakiuchi N, Kato N *et al* (1993). Two distinct proteinase activities required for the processing of a putative nonstructural precursor protein of hepatitis C virus. *J Virol* 67**:** 4665-4675.

Hnatyszyn HJ (2005). Chronic hepatitis C and genotyping: the clinical significance of determining HCV genotypes. *Antivir Ther* 10**:** 1-11.

Holland J, Spindler K, Horodyski F, Grabau E, Nichol S, VandePol S (1982). Rapid evolution of RNA genomes. *Science* 215**:** 1577-1585.

Holz LE, Benseler V, Bowen DG, Bouillet P, Strasser A, O'Reilly L *et al* (2008). Intrahepatic murine CD8 T-cell activation associates with a distinct phenotype leading to Bim-dependent death. *Gastroenterology* 135**:** 989-997.

Holz LE, Bowen DG, Bertolino P (2010). Mechanisms of T cell death in the liver: to Bim or not to Bim? *Dig Dis* 28**:** 14-24.

Honda M, Beard MR, Ping LH, Lemon SM (1999). A phylogenetically conserved stem-loop structure at the 5' border of the internal ribosome entry site of hepatitis C virus is required for cap-independent viral translation. *J Virol* 73**:** 1165-1174.

Hope RG, Murphy DJ, McLauchlan J (2002). The domains required to direct core proteins of hepatitis C virus and GB virus-B to lipid droplets share common features with plant oleosin proteins. *J Biol Chem* 277**:** 4261-4270.

Hsu M, Zhang J, Flint M, Logvinoff C, Cheng-Mayer C, Rice CM *et al* (2003). Hepatitis C virus glycoproteins mediate pH-dependent cell entry of pseudotyped retroviral particles. *Proc Natl Acad Sci U S A* 100**:** 7271-7276.

Huang L, Hwang J, Sharma SD, Hargittai MR, Chen Y, Arnold JJ *et al* (2005). Hepatitis C virus nonstructural protein 5A (NS5A) is an RNA-binding protein. *J Biol Chem* 280**:** 36417-36428.

Inman GJ, Nicolas FJ, Callahan JF, Harling JD, Gaster LM, Reith AD *et al* (2002). SB-431542 is a potent and specific inhibitor of transforming growth factor-beta superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7. *Mol Pharmacol* 62**:** 65-74.

Jacobson IM, McHutchison JG, Dusheiko G, Di Bisceglie AM, Reddy KR, Bzowej NH *et al* (2011). Telaprevir for previously untreated chronic hepatitis C virus infection. *N Engl J Med* 364**:** 2405-2416.

Jonuleit H, Kuhn U, Muller G, Steinbrink K, Paragnik L, Schmitt E *et al* (1997). Pro-inflammatory cytokines and prostaglandins induce maturation of potent immunostimulatory dendritic cells under fetal calf serum-free conditions. *Eur J Immunol* 27**:** 3135-3142.

Keele BF, Giorgi EE, Salazar-Gonzalez JF, Decker JM, Pham KT, Salazar MG *et al* (2008). Identification and characterization of transmitted and early founder virus envelopes in primary HIV-1 infection. *Proc Natl Acad Sci U S A* 105**:** 7552-7557.

Khakoo SI, Thio CL, Martin MP, Brooks CR, Gao X, Astemborski J *et al* (2004). HLA and NK cell inhibitory receptor genes in resolving hepatitis C virus infection. *Science* 305**:** 872-874.

Kim AY, Kuntzen T, Timm J, Nolan BE, Baca MA, Reyor LL *et al* (2011). Spontaneous control of HCV is associated with expression of HLA-B 57 and preservation of targeted epitopes. *Gastroenterology* 140**:** 686-696 e681.

Klade CS, Wedemeyer H, Berg T, Hinrichsen H, Cholewinska G, Zeuzem S *et al* (2008). Therapeutic vaccination of chronic hepatitis C nonresponder patients with the peptide vaccine IC41. *Gastroenterology* 134**:** 1385-1395.

Klein J, Sato A (2000). The HLA system. First of two parts. *N Engl J Med* 343**:** 702-709.

Knapp S, Warshow U, Hegazy D, Brackenbury L, Guha IN, Fowell A *et al* (2010). Consistent beneficial effects of killer cell immunoglobulin-like receptor 2DL3 and group 1 human leukocyte antigen-C following exposure to hepatitis C virus. *Hepatology* 51**:** 1168-1175.

Knolle P, Schlaak J, Uhrig A, Kempf P, Meyer zum Buschenfelde KH, Gerken G (1995). Human Kupffer cells secrete IL-10 in response to lipopolysaccharide (LPS) challenge. *J Hepatol* 22**:** 226-229.

Komurian-Pradel F, Rajoharison A, Berland JL, Khouri V, Perret M, Van Roosmalen M *et al* (2004). Antigenic relevance of F protein in chronic hepatitis C virus infection. *Hepatology* 40**:** 900-909.

Kotturi MF, Scott I, Wolfe T, Peters B, Sidney J, Cheroutre H *et al* (2008). Naive precursor frequencies and MHC binding rather than the degree of epitope diversity shape CD8+ T cell immunodominance. *J Immunol* 181**:** 2124-2133.

Kwo PY, Lawitz EJ, McCone J, Schiff ER, Vierling JM, Pound D *et al* (2010). Efficacy of boceprevir, an NS3 protease inhibitor, in combination with peginterferon alfa-2b and ribavirin in treatment-naive patients with genotype 1 hepatitis C infection (SPRINT-1): an open-label, randomised, multicentre phase 2 trial. *Lancet* 376**:** 705-716.

Lamarre D, Anderson PC, Bailey M, Beaulieu P, Bolger G, Bonneau P *et al* (2003). An NS3 protease inhibitor with antiviral effects in humans infected with hepatitis C virus. *Nature* 426**:** 186-189.

Lechner F, Wong DK, Dunbar PR, Chapman R, Chung RT, Dohrenwend P *et al* (2000). Analysis of successful immune responses in persons infected with hepatitis C virus. *J Exp Med* 191**:** 1499-1512.

Lerat H, Honda M, Beard MR, Loesch K, Sun J, Yang Y *et al* (2002). Steatosis and liver cancer in transgenic mice expressing the structural and nonstructural proteins of hepatitis C virus. *Gastroenterology* 122**:** 352-365.

Li K, Lemon SM (2013). Innate immune responses in hepatitis C virus infection. *Semin Immunopathol* 35**:** 53-72.

Lim DG, Bieganowska Bourcier K, Freeman GJ, Hafler DA (2000). Examination of CD8+ T cell function in humans using MHC class I tetramers: similar cytotoxicity but variable proliferation and cytokine production among different clonal CD8+ T cells specific to a single viral epitope. *J Immunol* 165**:** 6214-6220.

Logvinoff C, Major ME, Oldach D, Heyward S, Talal A, Balfe P *et al* (2004). Neutralizing antibody response during acute and chronic hepatitis C virus infection. *Proc Natl Acad Sci U S A* 101**:** 10149-10154.

Lohmann V, Korner F, Koch J, Herian U, Theilmann L, Bartenschlager R (1999). Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* 285**:** 110-113.

Macdonald A, Crowder K, Street A, McCormick C, Harris M (2004). The hepatitis C virus NS5A protein binds to members of the Src family of tyrosine kinases and regulates kinase activity. *J Gen Virol* 85**:** 721-729.

Maheshwari A, Ray S, Thuluvath PJ (2008). Acute hepatitis C. *Lancet* 372**:** 321-332.

Manigold T, Racanelli V (2007). T-cell regulation by CD4 regulatory T cells during hepatitis B and C virus infections: facts and controversies. *Lancet Infect Dis* 7**:** 804-813.

Matsui M, Moots RJ, McMichael AJ, Frelinger JA (1994). Significance of the six peptide-binding pockets of HLA-A2.1 in influenza A matrix peptide-specific cytotoxic T-lymphocyte reactivity. *Hum Immunol* 41**:** 160-166.

McHutchison JG, Everson GT, Gordon SC, Jacobson IM, Sulkowski M, Kauffman R *et al* (2009). Telaprevir with peginterferon and ribavirin for chronic HCV genotype 1 infection. *N Engl J Med* 360**:** 1827-1838.

McKiernan SM, Hagan R, Curry M, McDonald GS, Kelly A, Nolan N *et al* (2004). Distinct MHC class I and II alleles are associated with hepatitis C viral clearance, originating from a single source. *Hepatology* 40**:** 108-114.

Meyer-Olson D, Shoukry NH, Brady KW, Kim H, Olson DP, Hartman K *et al* (2004). Limited T cell receptor diversity of HCV-specific T cell responses is associated with CTL escape. *J Exp Med* 200**:** 307-319.

Moradpour D, Gosert R, Egger D, Penin F, Blum HE, Bienz K (2003). Membrane association of hepatitis C virus nonstructural proteins and identification of the membrane alteration that harbors the viral replication complex. *Antiviral Res* 60**:** 103-109.

Moradpour D, Penin F, Rice CM (2007). Replication of hepatitis C virus. *Nat Rev Microbiol* 5**:** 453-463.

Murphy DG, Willems B, Deschenes M, Hilzenrat N, Mousseau R, Sabbah S (2007). Use of sequence analysis of the NS5B region for routine genotyping of hepatitis C virus with reference to C/E1 and 5' untranslated region sequences. *J Clin Microbiol* 45**:** 1102-1112.

Nakamoto N, Kaplan DE, Coleclough J, Li Y, Valiga ME, Kaminski M *et al* (2008). Functional restoration of HCV-specific CD8 T cells by PD-1 blockade is defined by PD-1 expression and compartmentalization. *Gastroenterology* 134**:** 1927-1937, 1937 e1921-1922.

Neumann-Haefelin C, Timm J, Schmidt J, Kersting N, Fitzmaurice K, Oniangue-Ndza C *et al* (2010). Protective effect of human leukocyte antigen B27 in hepatitis C virus infection requires the presence of a genotype-specific immunodominant CD8+ T-cell epitope. *Hepatology* 51**:** 54-62.

Neumann AU, Lam NP, Dahari H, Gretch DR, Wiley TE, Layden TJ *et al* (1998). Hepatitis C viral dynamics in vivo and the antiviral efficacy of interferon-alpha therapy. *Science* 282**:** 103-107.

Norris S, Collins C, Doherty DG, Smith F, McEntee G, Traynor O *et al* (1998). Resident human hepatic lymphocytes are phenotypically different from circulating lymphocytes. *J Hepatol* 28**:** 84-90.

Ogg GS, Jin X, Bonhoeffer S, Dunbar PR, Nowak MA, Monard S *et al* (1998). Quantitation of HIV-1-specific cytotoxic T lymphocytes and plasma load of viral RNA. *Science* 279**:** 2103-2106.

Osburn WO, Fisher BE, Dowd KA, Urban G, Liu L, Ray SC *et al* (2010). Spontaneous control of primary hepatitis C virus infection and immunity against persistent reinfection. *Gastroenterology* 138**:** 315-324.

Penna A, Pilli M, Zerbini A, Orlandini A, Mezzadri S, Sacchelli L *et al* (2007). Dysfunction and functional restoration of HCV-specific CD8 responses in chronic hepatitis C virus infection. *Hepatology* 45**:** 588-601.

Pestka JM, Zeisel MB, Blaser E, Schurmann P, Bartosch B, Cosset FL *et al* (2007). Rapid induction of virus-neutralizing antibodies and viral clearance in a single-source outbreak of hepatitis C. *Proc Natl Acad Sci U S A* 104**:** 6025-6030.

Pietschmann T (2009). Virology: Final entry key for hepatitis C. *Nature* 457**:** 797-798.

Ploss A, Evans MJ, Gaysinskaya VA, Panis M, You H, de Jong YP *et al* (2009). Human occludin is a hepatitis C virus entry factor required for infection of mouse cells. *Nature* 457**:** 882-886.

Polyak SJ, McArdle S, Liu SL, Sullivan DG, Chung M, Hofgartner WT *et al* (1998). Evolution of hepatitis C virus quasispecies in hypervariable region 1 and the putative interferon sensitivity-determining region during interferon therapy and natural infection. *J Virol* 72**:** 4288-4296.

Poordad F, McCone J, Jr., Bacon BR, Bruno S, Manns MP, Sulkowski MS *et al* (2011). Boceprevir for untreated chronic HCV genotype 1 infection. *N Engl J Med* 364**:** 1195-1206.

Poynard T, Bedossa P, Opolon P (1997). Natural history of liver fibrosis progression in patients with chronic hepatitis C. The OBSVIRC, METAVIR, CLINIVIR, and DOSVIRC groups. *Lancet* 349**:** 825-832.

Radziewicz H, Ibegbu CC, Fernandez ML, Workowski KA, Obideen K, Wehbi M *et al* (2007). Liver-infiltrating lymphocytes in chronic human hepatitis C virus infection display an exhausted phenotype with high levels of PD-1 and low levels of CD127 expression. *J Virol* 81**:** 2545-2553.

Ray SC, Wang YM, Laeyendecker O, Ticehurst JR, Villano SA, Thomas DL (1999). Acute hepatitis C virus structural gene sequences as predictors of persistent viremia: hypervariable region 1 as a decoy. *J Virol* 73**:** 2938-2946.

Rehermann B (2009). Hepatitis C virus versus innate and adaptive immune responses: a tale of coevolution and coexistence. *The Journal of clinical investigation* 119**:** 1745-1754.

Reyes GR (2002). The nonstructural NS5A protein of hepatitis C virus: an expanding, multifunctional role in enhancing hepatitis C virus pathogenesis. *J Biomed Sci* 9**:** 187-197.

Rieder H, Ramadori G, Allmann KH, Meyer zum Buschenfelde KH (1990). Prostanoid release of cultured liver sinusoidal endothelial cells in response to endotoxin and tumor necrosis factor. Comparison with umbilical vein endothelial cells. *J Hepatol* 11**:** 359-366.

Roccasecca R, Ansuini H, Vitelli A, Meola A, Scarselli E, Acali S *et al* (2003). Binding of the hepatitis C virus E2 glycoprotein to CD81 is strain specific and is modulated by a complex interplay between hypervariable regions 1 and 2. *J Virol* 77**:** 1856-1867.

Romero V, Azocar J, Zuniga J, Clavijo OP, Terreros D, Gu X *et al* (2008). Interaction of NK inhibitory receptor genes with HLA-C and MHC class II alleles in Hepatitis C virus infection outcome. *Mol Immunol* 45**:** 2429-2436.

Rubtsova K, Scott-Browne JP, Crawford F, Dai S, Marrack P, Kappler JW (2009). Many different Vbeta CDR3s can reveal the inherent MHC reactivity of germline-encoded TCR V regions. *Proc Natl Acad Sci U S A* 106**:** 7951-7956.

Ruhl M, Knuschke T, Schewior K, Glavinic L, Neumann-Haefelin C, Chang DI *et al* (2011). CD8+ T-cell response promotes evolution of hepatitis C virus nonstructural proteins. *Gastroenterology* 140**:** 2064-2073.

Sabahi A (2009). Hepatitis C Virus entry: the early steps in the viral replication cycle. *Virol J* 6**:** 117.

Sabo MC, Luca VC, Prentoe J, Hopcraft SE, Blight KJ, Yi M *et al* (2011). Neutralizing monoclonal antibodies against hepatitis C virus E2 protein bind discontinuous epitopes and inhibit infection at a postattachment step. *J Virol* 85**:** 7005-7019.

Sakaguchi S (2000). Regulatory T cells: key controllers of immunologic self-tolerance. *Cell* 101**:** 455-458.

Sanjuan R, Moya A, Elena SF (2004). The distribution of fitness effects caused by single-nucleotide substitutions in an RNA virus. *Proc Natl Acad Sci U S A* 101**:** 8396-8401.

Santolini E, Migliaccio G, La Monica N (1994). Biosynthesis and biochemical properties of the hepatitis C virus core protein. *J Virol* 68**:** 3631-3641.

Schindler C, Plumlee C (2008). Inteferons pen the JAK-STAT pathway. *Semin Cell Dev Biol* 19**:** 311-318.

Schlaphoff V, Lunemann S, Suneetha PV, Jaroszewicz J, Grabowski J, Dietz J *et al* (2011). Dual function of the NK cell receptor 2B4 (CD244) in the regulation of HCV-specific CD8+ T cells. *PLoS Pathog* 7**:** e1002045.

Schmidt J, Neumann-Haefelin C, Altay T, Gostick E, Price DA, Lohmann V *et al* (2011). Immunodominance of HLA-A2-restricted hepatitis C virus-specific CD8+ T cell responses is linked to naive-precursor frequency. *J Virol* 85**:** 5232-5236.

Schulze zur Wiesch J, Ciuffreda D, Lewis-Ximenez L, Kasprowicz V, Nolan BE, Streeck H *et al* (2012). Broadly directed virus-specific CD4+ T cell responses are

primed during acute hepatitis C infection, but rapidly disappear from human blood with viral persistence. *J Exp Med* 209**:** 61-75.

Semmo N, Day CL, Ward SM, Lucas M, Harcourt G, Loughry A *et al* (2005). Preferential loss of IL-2-secreting CD4+ T helper cells in chronic HCV infection. *Hepatology* 41**:** 1019-1028.

Semmo N, Lucas M, Krashias G, Lauer G, Chapel H, Klenerman P (2006). Maintenance of HCV-specific T-cell responses in antibody-deficient patients a decade after early therapy. *Blood* 107**:** 4570-4571.

Sette A, Vitiello A, Reherman B, Fowler P, Nayersina R, Kast WM *et al* (1994). The relationship between class I binding affinity and immunogenicity of potential cytotoxic T cell epitopes. *J Immunol* 153**:** 5586-5592.

Shepard CW, Finelli L, Alter MJ (2005). Global epidemiology of hepatitis C virus infection. *Lancet Infect Dis* 5**:** 558-567.

Shevach EM (2002). CD4+ CD25+ suppressor T cells: more questions than answers. *Nat Rev Immunol* 2**:** 389-400.

Shimoike T, Mimori S, Tani H, Matsuura Y, Miyamura T (1999). Interaction of hepatitis C virus core protein with viral sense RNA and suppression of its translation. *J Virol* 73**:** 9718-9725.

Shoukry NH, Grakoui A, Houghton M, Chien DY, Ghrayeb J, Reimann KA *et al* (2003). Memory CD8+ T cells are required for protection from persistent hepatitis C virus infection. *J Exp Med* 197**:** 1645-1655.

Shulman M, Nahmias Y (2013). Long-term culture and coculture of primary rat and human hepatocytes. *Methods Mol Biol* 945**:** 287-302.

Simmonds P, Bukh J, Combet C, Deleage G, Enomoto N, Feinstone S *et al* (2005). Consensus proposals for a unified system of nomenclature of hepatitis C virus genotypes. *Hepatology* 42**:** 962-973.

Sklan EH, Charuworn P, Pang PS, Glenn JS (2009). Mechanisms of HCV survival in the host. *Nat Rev Gastroenterol Hepatol* 6**:** 217-227.

Soderholm J, Ahlen G, Kaul A, Frelin L, Alheim M, Barnfield C *et al* (2006). Relation between viral fitness and immune escape within the hepatitis C virus protease. *Gut* 55**:** 266-274.

Steinmann E, Penin F, Kallis S, Patel AH, Bartenschlager R, Pietschmann T (2007). Hepatitis C virus p7 protein is crucial for assembly and release of infectious virions. *PLoS Pathog* 3**:** e103.

Tanaka Y, Shimoike T, Ishii K, Suzuki R, Suzuki T, Ushijima H *et al* (2000). Selective binding of hepatitis C virus core protein to synthetic oligonucleotides

corresponding to the 5' untranslated region of the viral genome. *Virology* 270**:** 229-236.

Thein HH, Yi Q, Dore GJ, Krahn MD (2008). Estimation of stage-specific fibrosis progression rates in chronic hepatitis C virus infection: a meta-analysis and meta-regression. *Hepatology* 48**:** 418-431.

Thimme R, Bukh J, Spangenberg HC, Wieland S, Pemberton J, Steiger C *et al* (2002). Viral and immunological determinants of hepatitis C virus clearance, persistence, and disease. *Proc Natl Acad Sci U S A* 99**:** 15661-15668.

Thimme R, Binder M, Bartenschlager R (2012). Failure of innate and adaptive immune responses in controlling hepatitis C virus infection. *FEMS Microbiol Rev* 36**:** 663-683.

Thomas DL, Thio CL, Martin MP, Qi Y, Ge D, O'hUigin C *et al* (2009). Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. *Nature* 461**:** 798-U752.

Thomson AW, Knolle PA (2010). Antigen-presenting cell function in the tolerogenic liver environment. *Nat Rev Immunol* 10**:** 753-766.

Tillmann HL, Thompson AJ, Patel K, Wiese M, Tenckhoff H, Nischalke HD *et al* (2010). A Polymorphism Near IL28B Is Associated With Spontaneous Clearance of Acute Hepatitis C Virus and Jaundice. *Gastroenterology* 139**:** 1586-+.

Timm J, Lauer GM, Kavanagh DG, Sheridan I, Kim AY, Lucas M *et al* (2004). CD8 epitope escape and reversion in acute HCV infection. *J Exp Med* 200**:** 1593-1604.

Timm J, Li B, Daniels MG, Bhattacharya T, Reyor LL, Allgaier R *et al* (2007). Human leukocyte antigen-associated sequence polymorphisms in hepatitis C virus reveal reproducible immune responses and constraints on viral evolution. *Hepatology* 46**:** 339-349.

Toebes M, Coccoris M, Bins A, Rodenko B, Gomez R, Nieuwkoop NJ *et al* (2006). Design and use of conditional MHC class I ligands. *Nat Med* 12**:** 246-251.

Tseng CT, Klimpel GR (2002). Binding of the hepatitis C virus envelope protein E2 to CD81 inhibits natural killer cell functions. *J Exp Med* 195**:** 43-49.

Tsukiyama-Kohara K, Iizuka N, Kohara M, Nomoto A (1992). Internal ribosome entry site within hepatitis C virus RNA. *J Virol* 66**:** 1476-1483.

Vieira PL, de Jong EC, Wierenga EA, Kapsenberg ML, Kalinski P (2000). Development of Th1-inducing capacity in myeloid dendritic cells requires environmental instruction. *J Immunol* 164**:** 4507-4512.

von Hahn T, Yoon JC, Alter H, Rice CM, Rehermann B, Balfe P *et al* (2007). Hepatitis C virus continuously escapes from neutralizing antibody and T-cell responses during chronic infection in vivo. *Gastroenterology* 132**:** 667-678.

Walker CM (2010). Adaptive immunity to the hepatitis C virus. *Adv Virus Res* 78**:** 43-86.

Warren A, Le Couteur DG, Fraser R, Bowen DG, McCaughan GW, Bertolino P (2006). T lymphocytes interact with hepatocytes through fenestrations in murine liver sinusoidal endothelial cells. *Hepatology* 44**:** 1182-1190.

Wick MJ, Leithauser F, Reimann J (2002). The hepatic immune system. *Crit Rev Immunol* 22**:** 47-103.

Wiese M, Berr F, Lafrenz M, Porst H, Oesen U (2000). Low frequency of cirrhosis in a hepatitis C (genotype 1b) single-source outbreak in germany: a 20-year multicenter study. *Hepatology* 32**:** 91-96.

Wölfl M, Merker K, Morbach H, Van Gool SW, Eyrich M, Greenberg PD *et al* (2011). Primed tumor-reactive multifunctional CD62L+ human CD8+ T cells for immunotherapy. *Cancer immunology, immunotherapy : CII*, 2010/10/26 edn. pp 173-186.

Yanagi M, St Claire M, Emerson SU, Purcell RH, Bukh J (1999). In vivo analysis of the 3' untranslated region of the hepatitis C virus after in vitro mutagenesis of an infectious cDNA clone. *Proc Natl Acad Sci U S A* 96**:** 2291-2295.

Yusim K, Richardson R, Tao N, Dalwani A, Agrawal A, Szinger J *et al* (2005). Los alamos hepatitis C immunology database. *Appl Bioinformatics* 4**:** 217-225.

Yusim K, Fischer W, Yoon H, Thurmond J, Fenimore PW, Lauer G *et al* (2010). Genotype 1 and global hepatitis C T-cell vaccines designed to optimize coverage of genetic diversity. *J Gen Virol* 91**:** 1194-1206.

Zeisel MB, Baumert TF (2009). HCV entry and neutralizing antibodies: lessons from viral variants. *Future Microbiol* 4**:** 511-517.

Zeuzem S, Andreone P, Pol S, Lawitz E, Diago M, Roberts S *et al* (2011). Telaprevir for retreatment of HCV infection. *N Engl J Med* 364**:** 2417-2428.

Ziegler S, Ruhl M, Tenckhoff H, Wiese M, Heinemann FM, Horn PA *et al* (2013). Susceptibility to chronic hepatitis C virus infection is influenced by sequence differences in immunodominant CD8+ T cell epitopes. *J Hepatol* 58**:** 24-30.

Webpages

Hepatitis C virus database Retrieved 20.05.2013 from http://hcv.lanl.gov

WHO (2012) Retrieved 08.05.2013 from http://www.who.int/mediacentre/factsheets/fs164/en/

9

10 Appendix

Table 10.1 Patient data.

#	Gender	Age	Disease	Surgery
1	w	78	liver metastasis	Resection
2	w	29	healthy donor	donation
3	m	60	hepatocellular carcinoma	resection
4	w	60	metastasis	resection
5	m	68	HCV, hepatocellular carcinoma	LTX
6	m	60	HCV infection	LTX
7	w	41	Adenoma	resection
8	w	49	HCV infection	resection
9	m	48	HCV infection	LTX
10	w	28	Healthy	resection
11	w	61	HCV infection	LTX
12	m	50	HCV infection	LTX
13	w	69	bile duct carcinoma	resection
14	m	63	HBV infection	LTX
15	w	57	Carcinoma	resection
16	m	58	hepatocellular carcinoma	LTX
17	m	50	HBV,hepatocellular carcinoma	LTX
18	w	38	Nonalcoholic Steatohepatitis	resection
19	w	39	acute liver failure due to bei Budd-Chiari syndrome	LTX
20	m	47	Primary sclerosing cholangitis	LTX
21	m	77	cholangiocellular carcinoma	resection
22	m	32	Metastasis	resection
23	m	53	HCV infection	LTX
24	m	60	hepatocellular carcinoma	resection
25	m	81	Klatskin Tumor	resection

11 Abbreviations

α	anti
7-AAD	7-Aminoactinomycin D
ALK-5	Activin receptor-like kinase-5
APC	Allophycocyanin
APC	Antigen-presenting cell
ARFP	Alternative reading frame protein
approx	approximately
BCL-2	B cell lymphoma-2
Bim	BCL-2-interacting mediatior of death
C	Constant
C	Cytosine
°C	Degree celsius
CD	Cluster of differentiation
CLDN	Claudin
CMV	Cytomegalovirus
CO_2	Carbon dioxide
C-terminal	Carboxy terminal
CTLA-4	Cytotoxic T lymphocyte associated antigen-4
Cy	Cyanine
DAA	Direct acting antivirals
DC	Dendritic cell
DC-SIGN	Dendritic cell-specific intracellular adhesion molecule-3-grabbin non-integrin

DMEM	Dulbecco's Modified Eagles' Medium
DMSO	Dimethyl sulfoxide
DNA	Desoxyribonucleic acid
EBV	Epstein-Barr Virus
EDTA	Ethylenediaminetetraacetic acid
e.g.	for example (lat. *exempli gratia)*
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmatic reticulum
et al	and others (lat*. et alii*)
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
g	Gram
g (x g)	Gravity; times gravity
GAG	Glycosaminoglycanes
GM-CSF	Granulocyte macrophage colony-stimulating factor
Gy	Gray
h	Hour
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HCVcc	Hepatitis C virus cell culture system
HCVpp	Hepatitis C virus pseudo particle
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HS	Human serum
HUVEC	Human umbilical vein endothelial cells
HVR	Hypervariable region
IC	Intracellular
ICS	Intracellular cytokine staining
IDU	Injection drug user
i.e.	that is (lat. *id est*)
IFN	Interferon
IL	Interleukine
IPS	IFN-β-promotor stimulator protein-I
IRES	Internal ribosomal entry site
IRF-3	Interferon-regulatory factor-3
ISG	Interferon-stimulated genes
IU	International units
J	Joining
kb	Kilo base
KIR	Killer immunoglobulin-like receptor
L	Liter
LPS	Lipopolysaccharide
LSEC	Liver sinusoidal endothelial cells
L-SIGN	Liver/ lymph node-specific intracellular adhesion molecule-3-grabbin non-integrin
LTX	Liver transplantation

M	Molar
µ	Micro
m	Milli
MACS	Magnetic cell separation
MAVS	Mitochondrial antiviral signaling molecule
mDC	Myeloid dendritic cells
MFI	Mean fluorescent intensity
MHC	Major Histocompatibility Complex
MIP-1β	Macrophage inflammatory protein -1beta
moDC	Monocyte-derived dendritic cell
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer cell
NKT	Natural killer T cell
n(m)	Nano(meter)
NS	Non-structural
n.s.	not significant
N-terminal	amino terminal
OCLDN	Occludin
ORF	Open reading frame
p	Pico
PAMP	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed cell death-1

pDC	Plasmacytoid dendritic cells
PE	Phycoerythrin
peg	Pegylated
PerCP	Peridinin chlorophyll protein
PET	Polyethylene terephthalate
PHH	Primary human hepatocytes
PMH	Primary murine hepatocytes
PP2AC	Protein phosphatase 2AC
PGE_2	Prostaglandin E_2
PRR	Pattern recognition receptors
rh	Recombinant human
RIG-I	Retinoic acid-inducible gene-I
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
RT	Room temperature
SC	Splenocytes
SSC	sideward scatter
SOC3	Suppressor of cytokine signaling 3
SR-B1	Scavenger receptor class B type 1
SVR	Sustained virological response
T	Thymine
TAP	Transporter associated with antigen processing
TCR	T cell receptor
TGF	Transforming growth factor
Tim-3	T cell immunoglobulin domain and mucin domain protein-3

TLR	Toll-like receptors
TNF	Tumor necrosis factor
TRIF	TIR domain-containing adapter-inducting interferon-β
U	Unit
UTR	Untranslated region
UV	Ultra violet
V	Variable
(V)LDL	(very)-low-density lipoprotein receptor
vs.	towards (lat. *versus)*
WHO	The World Health Organization

12 List of Figures

13 List of Tables

14 Publications

Ziegler S, Ruhl M, Tenckhoff H, Wiese M, Heinemann FM, Horn PA *et al* (2013). Susceptibility to chronic hepatitis C virus infection is influenced by sequence differences in immunodominant CD8+ T cell epitopes. *J Hepatol* 58**:** 24-30.

Schildgen V, Ziegler S, Tillmann RL, Schildgen O (2010). Novel mutation in YMDD motif and direct neighbourhood in a child with chronic HBV-infection and clinical lamivudine and adefovir resistance - a scholarly case. *Virol J* 7**:** 167.

Bohmer A, Schildgen V, Lusebrink J, Ziegler S, Tillmann RL, Kleines M *et al* (2009). Novel application for isothermal nucleic acid sequence-based amplification (NASBA). *J Virol Methods* 158**:** 199-201.

Ziegler S, Tillmann RL, Muller A, Simon A, Schildgen V, Schildgen O (2008). No gastroenteric Bocavirus in high risk patients stool samples. J Clin Virol 43: 349-350.

15 Presentations

- „23rd Annual Meeting of the Society for Virology“, Kiel; poster presentation
- „International Symposium on Viral Hepatitis and Liver Disease (ISVHLD)“, Shanghai, China; oral presentation
- „22nd Annual Meeting of the Society for Virology“, Essen, Germany; oral presentation
- „German Association for the Study of the Liver (GASL)“, Hamburg; poster presentation
- „18th International Symposium on Hepatitis C Virus and Related Viruses“, Seattle, USA; poster presentation
- „21st Annual Meeting of the Society for Virology“, Freiburg; poster presentation
- Graduate school BIOME, regular research progress reports
- Institute of Virology, regular research progress reports and participation in the “Journal Club”.

16 Acknowledgements/ Danksagung

An erster Stelle möchte ich mich bei meinem Doktorvater Prof. Jörg Timm bedanken. Seine hervorragende Betreuung und stete Gesprächsbereitschaft haben die Jahre meiner Doktorarbeit zu einer angenehmen und erfolgreichen Zeit gemacht. Insbesondere möchte ich mich für seine Unterstützung und sein Interesse an meiner zukünftigen wissenschaftlichen Karriere bedanken.

Ebenfalls möchte ich mich bei Prof. Ulf Dittmer und dem ehemaligen Leiter der Virologie, Prof. Michael Roggendorf für die Möglichkeit bedanken, an ihrem Institut an interessanten Themen zu arbeiten.

Ich möchte mich bei meiner Arbeitsgruppe Lejla Timmer, Svenja Groten, Kathrin Skibbe, Christine Thöns und Dr. Andreas Walker bedanken. Es herrschte immer eine sehr kollegiale und hilfsbereite Atmosphäre, auch außerhalb des Labors. Meine netten Kollegen haben den größten Anteil daran, dass ich immer mit Freude meiner Arbeit nachgegangen bin, auch wenn die Kuchenwünsche oft sehr speziell waren.

Ein großer Dank gilt ‘den Top-Mädels’ Dr. Simone Abel, Dr. Marina Babranyi, Dr. Kathrin Gibbert, Dr. Milena Lange und Dr. Marianne Wammers, für fabelhafte Donnerstagabende. Ich bedanke mich für anregende wissenschaftliche Ratschläge und Gespräche, aber speziell für die Gespräche neben der Wissenschaft.

Mein besonderer Dank gilt Mirco Schmidt. Er hat mich während der gesamten Phase meiner Doktorarbeit mit seiner Liebe und seinem Glauben an meine Fähigkeiten unterstützt und bestärkt. Auch in schwierigen Zeiten konnte er ‚na gut‘ sagen, mich aufbauen und stand immer an meiner Seite.

Mein größter Dank gilt meiner Familie. Ich bedanke mich bei meinen Eltern Johannes Ulrich und Marianne Ziegler für ihren Rat, ihre Bekräftigung, ihre Unterstützung, ihr volles Vertrauen und ihre Liebe. Ich bedanke mich bei meinen Geschwistern Kerstin, mit Matthias und Sophia, Christian und Julia für einen starken Zusammenhalt in schweren Zeiten und dafür, dass sie immer für mich da waren. Daher möchte ich diese Arbeit meiner Familie widmen.

17 Curriculum Vitae

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.

18 Declaration (Erklärung)

Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. 2, f der Promotionsordnung der Math.-Nat. Fakultäten zur Erlangung der Dr. rer. nat., dass ich das Arbeitsgebiet, dem das Thema „Determinants of HCV-specific CD8+ T cell expansion“ zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von Susanne Ziegler befürworte.

Essen, den _____ _____

Prof. Dr. med. Jörg Timm

Erklärung:

Hiermit erkläre ich, gem. § 7 Abs. 2, c und e der Promotionsordnung der Math.-Nat. Fakultäten zur Erlangung des Dr. rer. nat., dass ich die vorliegende Dissertation selbständig verfasst und mich keiner anderen als der angegebenen Hilfsmittel bedient habe und alle wörtlich oder inhaltlich übernommenen Stellen als solche gekennzeichnet habe.

Essen, den _____ _____

Susanne Ziegler

Erklärung:

Hiermit erkläre ich, gem. § 7 Abs. 2, d und f der Promotionsordnung der Math.-Nat. Fakultäten zur Erlangung des Dr. rer. nat., dass ich keine anderen Promotionen bzw. Promotionsversuche in der Vergangenheit durchgeführt habe, dass diese Arbeit von keiner anderen Fakultät abgelehnt worden ist, und dass ich die Dissertation nur in diesem Verfahren einreiche.

Essen, den _____ _____

Susanne Ziegler