Analysis of the CD8+ T cell response against hepatitis C virus in intravenous drug users

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1 Introduction

Hepatitis C virus (HCV) infects approximately 170 million people worldwide (World Health Organization, 1999). The virus was discovered after specific diagnostic tests for both hepatitis A virus (HAV) and hepatitis B virus (HBV) which revealed that most cases of post-transfusion hepatitis were not linked to HAV or HBV (Bradley, 1999). However, it took more than 10 years to isolate the agent responsible for this so-called post-transfusion non-A, non-B hepatitis (NANBH). In 1989 a new ribonucleic acid (RNA) virus termed HCV was isolated with the aid of modern techniques of molecular cloning and phage display (Choo *et al.*, 1989). HCV causes a persistent infection in the majority of infected people and can lead to cirrhosis of the liver and hepatocellular carcinoma (HCC) (Shimotohno, 2000). For this reason, and the high prevalence of infection worldwide, HCV is considered a major human pathogen.

1.1 Clinical aspects of HCV infection

The course of disease varies widely among infected persons. HCV infection is hardly ever diagnosed during the acute phase. The majority of persons have either none or only mild and unspecific symptoms after exposure to HCV. Nevertheless, clinical manifestations of acute hepatitis consisting of jaundice, malaise, and nausea can occur in some patients, usually within 7 to 8 weeks after infection (Lauer and Walker, 2001). Progression to chronic disease occurs in about 50-80% of infected persons, whereas 20-50% show spontaneous recovery (Fig. 1.1). The early stage of chronic infection is typically characterized by a prolonged asymptomatic period. Once chronic infection has been established, spontaneous clearance of viremia is rare. Most chronic infections will lead to hepatitis and to some degree of fibrosis. 10-20% of those infected chronically develop liver cirrhosis. At this stage of the disease, the risk of developing HCC is 1-4% per year (Tsukuma *et al.*, 1993). Factors that increase the risk of clinical disease progression include alcohol intake, co-infection with HBV (Zarski *et al.*, 1998) or human immunodeficiency virus type 1 (HIV-1) (Sanchez-Quijano *et al.*, 1995), male sex and older age at infection (Fig. 1.2). In this high-risk group, cirrhosis can develop within 20 years or less after infection. In contrary, low risk patients often do not have progressive liver disease until 30 or more years after infection. In HCV-infected patients, a superinfection with HAV can lead to fulminant hepatitis (Vento *et al.*, 1998).

In addition to hepatic disease, HCV infection is associated with important extrahepatic manifestations. A number of B cell-mediated disorders are associated with HCV infection. For example, HCV is the major cause of essential mixed cryoglobulinemia. Cryoglobulins can be found in about 50% of HCV patients and 10-15% of infected individuals develop a symptomatic disease consisting of weakness, arthralgia and purpura (Horcajada *et al.*, 1999). Since cryoglobulins accumulate in kidney, membranoproliferative glomerulonephritis can occur as a serious complication (Segerer *et al.*, 2002). A higher incidence of B cell non-Hodgkin's lymphoma (Pozzato *et al.*, 1994), Sjögren's syndrome (Haddad *et al.*, 1992), autoimmune thyroiditis, idiopathic pulmonary fibrosis and dermatologic manifestations like *Lichen planus* and *Porphyria cutanea tarda* in association with HCV infection has been observed (Zignego and Brechot, 1999).

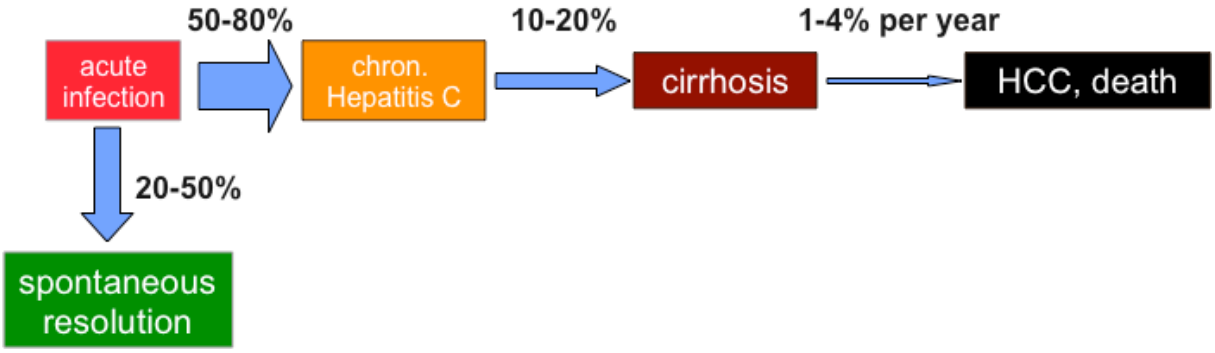


Fig. 1.1: Disease progression

1.2 Treatment

There is currently no vaccine for HCV but treatment is available for patients with chronic infection. Both viral [genotype (GT), quasispecies diversity, viral load] and host (gender, age, fibrosis etc.) factors (Fig. 1.2), influence the outcome of treatment (Feld and Hoofnagle, 2005). The current recommended treatment for HCV is a combination therapy of pegylated interferon alpha (peg-IFN-α) and ribavirin (Reichard, 1998). Two peg-IFNs are approved for treatment, peg-IFN-α-2a (Pegasys; Roche) and peg-IFN-α-2b (peg-Intron; Schering-Plough). The current recommendations are therapy with peg-IFN-α and ribavirin for 48 weeks in patients infected with GT1 and a shorter 24-week treatment for patients infected with GT2 and 3. In clinical trials, sustained virological response (SVR) can be achieved in 70-80% of non-GT1 patients whereas only 45% of GT1 patients have a SVR (Fig. 1.2). SVR is defined

as persistent absence of serum HCV RNA for at least 6 months after cessation of therapy. Treatment is recommended for patients with chronic HCV infection with detectable HCV RNA in serum, elevations in alanine aminotransferase (ALT) levels, histological evidence of progressive liver disease and no other severe co-existing conditions (NIH Consensus Statement on Management of Hepatitis C, 2002). Even if today is available treatment cures many chronically infected individuals, the treatment has some significant drawbacks. Many patients experience severe side effects, such as haematological toxicity, depression, and haemolytic anemia, and therapy is expensive (35.000 United State Dollars for 48 weeks treatment). Interestingly, both drugs - IFN-α and ribavirin - were not specifically developed for the treatment of HCV infection. More recently, novel targets for antiviral drugs have been identified in the replication cycle of HCV leading to the development of specifically targeted antiviral therapy for HCV (STAT-C). However, this development is still in process and these drugs are not yet available on the market. Most importantly, a prophylactic vaccine, which would be the most efficient way to prevent HCV-associated liver disease, is not available.

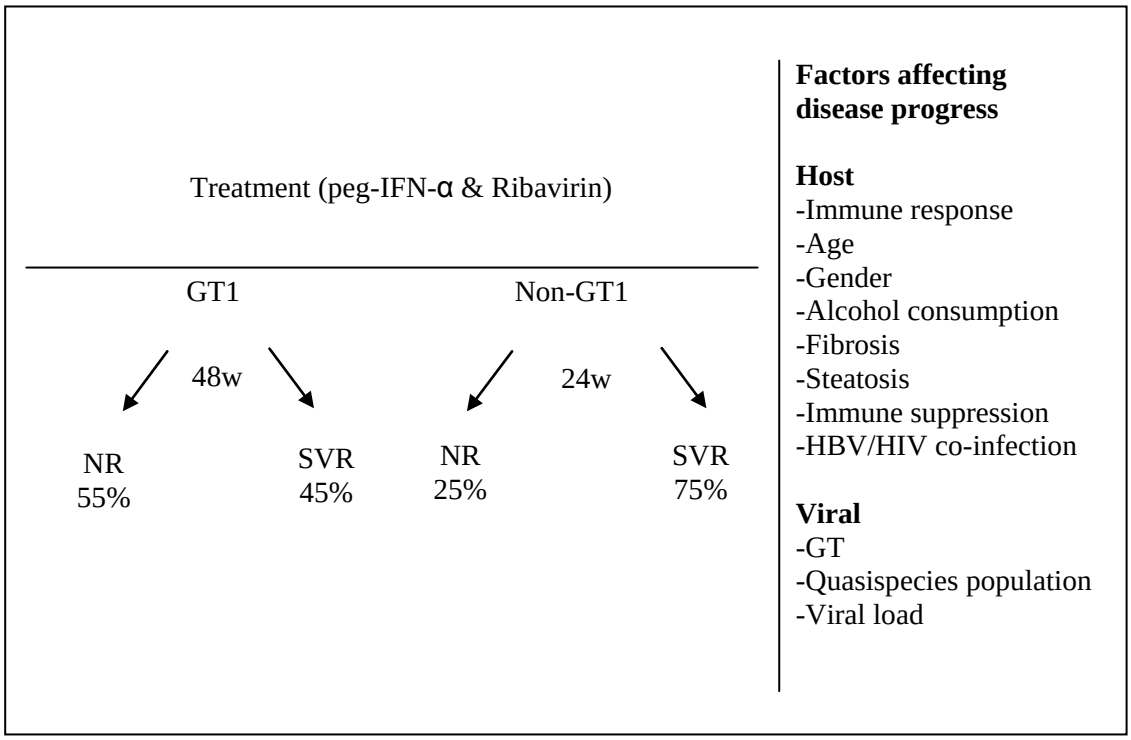


Fig. 1.2: Factors affecting the disease progression and response rates to peg-IFN-α and ribavirin treatment.

1.3 Molecular virology of HCV

1.3.1 Classification

HCV is classified as a member of the *Flaviviridae* family of viruses, which are associated with both human and animal diseases (Miller and Purcell, 1990). The *Flaviviridae* family comprises at least three distinct genera: *pestiviruses*, which cause disease in the cattle and pigs; *flaviviruses*, which are the most important cause of diseases such as dengue fever and yellow fever; and *hepaciviruses*, which currently includes HCV and GB virus A, B and C (Thiel *et al.*, 2005).

1.3.2 HCV replication cycle

HCV virions are enveloped with spherical symmetry and estimated to be 40-50 nm in diameter. The envelope contains viral glycoproteins that mediate attachment and entry into its host cell. Hepatocytes are the natural targets of HCV. The cell surface receptors that mediate viral attachment and entry have been identified. The cluster of differentiation 81 (CD81), the scavenger receptor class B member 1 (SR-B1), the tight junction (TJ) proteins Claudin-1 (CLDN1) and Occludin (OCLN) are sufficient to mediate endocytosis of HCV particles (Pietschmann, 2009). Within the cell the nucleopcapsid is released into the cytoplasm. The nucleocapsid is round, exhibits polyhedral symmetry (diameter of 25-30 nm) (Büchen-Osmond, 2003) and contains the HCV-genome. HCV has an RNA genome of positive polarity that is approximately 9.6 kb in length. The genome encodes a large polyprotein of roughly 3,000 amino acids. The HCV open reading frame (ORF) is flanked by 5´-and 3´-untranslated regions (UTRs), which are 341 and approximately 230 nucleotides in length, respectively (Choo *et al.*, 1991). The 5´and 3´-UTRs are considered important for viral translation and replication (Kim *et al.*, 2002). The 5´-UTR consists of four highly structured domains and contains the internal ribosome entry site (IRES), which can recruit host factors such as the 40S ribosomal subunit and thus initiate the translation of viral proteins (Tsukiyama-Kohara *et al.*, 1992). The 3´-UTR consists of stable stem-loop structures and an internal poly uracile (U)/polypyrimidine tract. The role that the 3´-UTR have in the regulation of the IRES-mediated translation of HCV RNA is not well defined (Murakami *et al.*, 2001;

Wang *et al.*, 2005). Several signals essential for RNA replication have been mapped within both UTRs (Friebe *et al.*, 2005).

The polyprotein precursor is co- and posttranslationally processed by both cellular and viral proteases at the endoplasmatic reticulum (ER) membrane to yield 10 mature proteins, including the structural proteins Core, the envelope proteins E1 and E2, the hydrophobic peptide p7 and non-structural proteins (NS2, NS3, NS4B, NS5A and NS5B) (Tellinghuisen and Rice, 2002) (Fig. 1.3). The major part of the NS2 protein and the amino-terminal (N-terminal) part of the NS3 protein form a viral metalloprotease (NS2-3 protease) that cleaves at the NS2/3 junction. The cleavage of the remaining non-structural proteins is catalyzed by a serine protease (NS3-4A protease) that is a part of the NS3 protein (Hijikata *et al.*, 1993). In addition, the carboxy-terminal (C-terminal) domain of NS3 contains sequences for a nucleoside triphosphatase (NTPase) and RNA helicases that are considered to regulate the replication of viral RNA (Kim *et al.*, 1995). The NS4A polypeptide acts as a cofactor for the NS3 serine protease. All HCV proteins are associated to intracellular membranes. The processing of the polyprotein occurs at the ER. Moreover, it is currently hypothesized that the formation of the membrane-associated viral replication complex occurs at altered cellular membranes possibly derived from the ER. NS4B was reported to induce the formation of these so-called membranous webs (Gosert *et al.*, 2003). The NS5A varies in size depending on GT. This multifunctional protein is highly phosphorylated and associates with membranes (Brass *et al.*, 2002). NS5A is also involved in resistance of HCV-infected cells to the antiviral activity of IFN-α through repression of the double stranded RNA-dependent protein kinase (Gale *et al.*, 1998). A protein binding function for NS5A may have also significant implications on roles for NS5A in the switch from genome translation to genome replication (Huang *et al.*, 2005). The most C-terminal cleavage product of the viral polyprotein is NS5b, the RNA-dependent RNA polymerase (RdRp) responsible for the replication of the HCV virus (Behrens *et al.*, 1996).

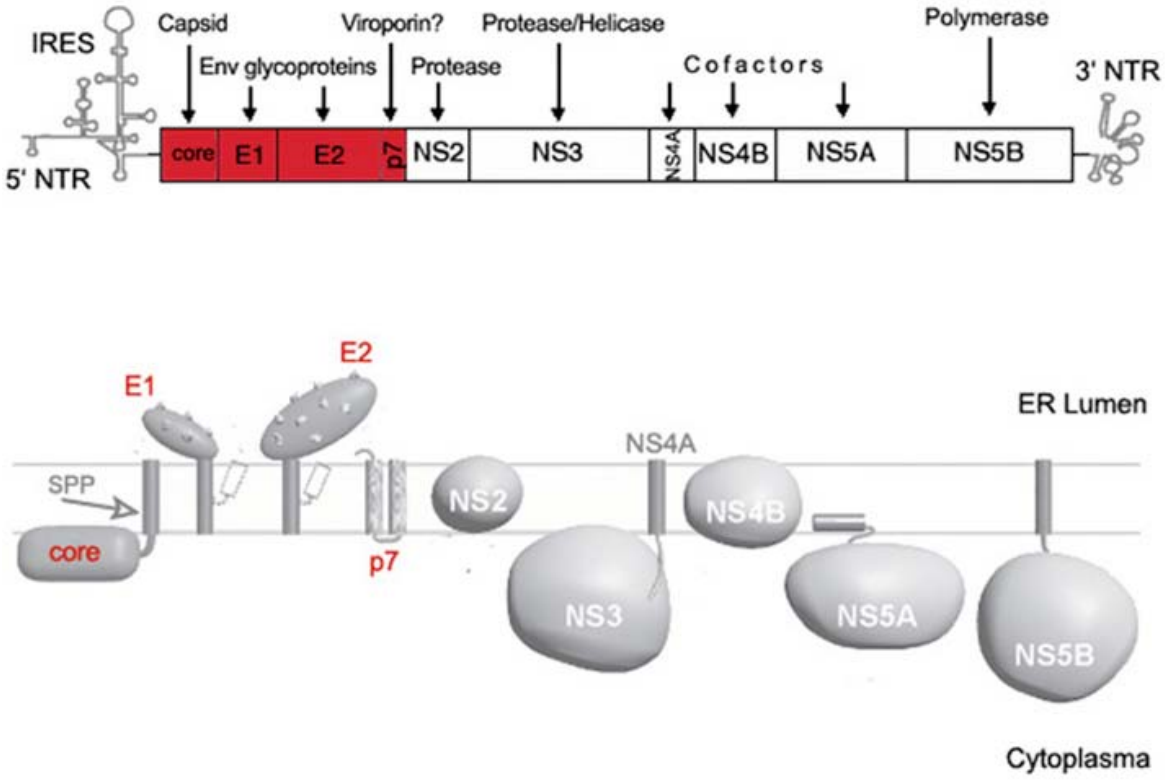


Fig. 1.3: Genome organization of HCV (Chevaliez and Pawlotsky, 2006).

1.4 Genetic variability of HCV

HCV exhibits high genetic diversity due to lack a proofreading function of its RdRp. As a result, randomly introduced errors in the genome remain uncorrected (Major and Feinstone, 1997). Many of these mutations are lethal for the virus. Others are silent and therefore neutral and some lead to amino acid changes with less prominent effects on viral replication. Thus, variants of the HCV genome can evolve rapidly establishing co-existence of several closely related but distinct viruses within the same host. This type of viral population is termed quasispecies. Viruses within the same quasispecies show 91-99% similarity in the conserved genomic regions, but can be distinguished within the more variable regions such as hypervariable region 1 (HVR1) and HVR2. The quasispecies nature of HCV enables rapid adaptation to changes in the replication environment. In the presence of selection pressure, imposed by drugs or by the host immune response, viral variants - that escape from this pressure - are selected and outcompete the originally predominant variant.

Beyond this sequence diversity between viral variants in the same or between different individuals there are more substantial differences between HCV variants at the population level. Based on the sequence variability HCV is classified into 7 major genotypes designated 1 to 7, and so far round 80 subtypes (Fig. 1.4) (Simmonds *et al.*, 2005). Complete genomes from different genotypes differ at about 30-35% of nucleotides. Subtypes (of the same GT) typically differ at about 20-25% of nucleotides (Simmonds, 2000). Although the clinical course of HCV infection does not seem to differ between genotypes, it is important to differentiate them as the response to treatment strongly differs. Interestingly, recombinations are less frequently observed compared with RNA viruses such as HIV. A few recombinants have been described. The first recombination form of HCV containing structural genes from subtype 2k and non-structural genes from subtype 1b was found in intravenous drug users (IVDUs) in St. Petersburg, Russia (Kalinina *et al.*, 2002). The same recombinant form was reported from unrelated individuals originating from Russia and Georgia but resident in Ireland at the time of sample collection (Moreau *et al.*, 2006), from HCV chronically infected patient in Estonia (Tallo *et al.*, 2007) and from IVDUs in Uzbekistan (Kurbanov *et al.*, 2008). Recently other naturally occurring recombinants have been identified, 2i/6p from blood donors in Vietnam (Noppornpanth *et al.*, 2006), 2b/1b from patient in Philippines (Kageyama *et al.*, 2006) and a 2/5 recombinant from a patient with history of medical interventations in France (Legrand-Abravanel *et al.*, 2007). An intragenotype 1a/1b recombinant has also been described in patients with chronic hepatic disease in Peru (Colina *et al.*, 2004). However, overall these recombination forms seem to be rare. Superinfection or co-infection with two different isolates which is the prerequisite for such recombination events are rare even in high-risk groups (Viazov *et al.*, 2010).

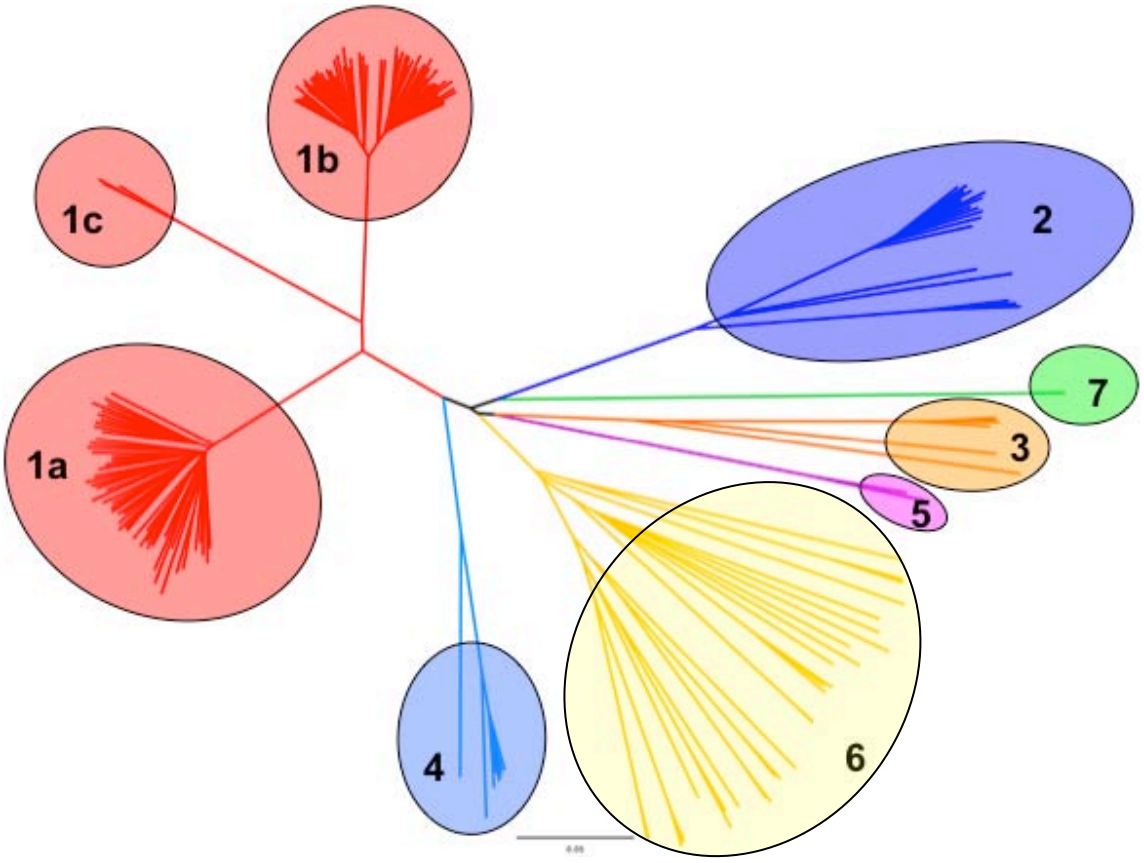


Fig. 1.4: Phylogenetic tree newly classified in seven HCV genotypes.

1.5 Epidemiology

Blood transfusions represented the highest risk factor for HCV infection before its discovery in 1989. The development of diagnostic tests and establishment of an efficient blood-screening system has then drastically decreased the incidence of post-transfusion hepatitis in developed countries (McHutchison *et al.*, 1998). Blood-screening measures based on the detection of HCV antibodies carry a residual risk resulting from a delay of approximately 8-12 weeks between a possible infection and the generation of antibodies. This diagnostic gap after primary HCV infection can be decreased by more sensitive screening methods based on polymerase chain reaction (PCR) of HCV-RNA (Fried *et al.*, 2002). Indeed, the residual risk of transmitting HCV by blood products is at present estimated at 1/1.000.000 units distributed. New cases of HCV infection continue to occur mainly because of IVDU. Maternal-fetal transmission is a rare event and mainly seen in mothers who are co-infected with HIV-1 (Manns *et al.*, 2001). Current guidelines for pregnant women recommend normal childbirth and breast-feeding, both associated with only minimal risk of HCV transmission.

Likewise, the sexual transmission of HCV is infrequent but its risk increases upon co-infection with HIV-1 (Thomas *et al.*, 1998). Nosocomial transmission of the virus is possible and includes needle-stick injuries among health care workers, infection during surgery, colonoscopy or dialysis. In one third of cases, however, the transmission route of the disease remains unclear.

The distribution of the various genotypes differs by geographic region (Smith and Simmonds, 1997). Generally, genotypes 1, 2 and 3 are predominantly found in Europe, West-Africa, Japan, China, Australia United States and Brazil (Campiotto *et al.*, 2005), while others have a more restricted distribution, such as GT4 in the Middle East and Central Africa, GT5a in South Africa, and GT6 in Southeast Asia (Bukh *et al.*, 1993). The geographical distribution and diversity of HCV genotypes may provide clues regarding the historical origin of HCV (Smith and Simmonds, 1997). The high diversity in subtype distribution in some regions of the world, as Africa and South-Eastern Asia suggests that HCV has been endemic there since long. On the other hand, the lower diversity of subtypes observed in United States and Europe may be related to more recent introductions of these subtypes from endemic areas (Pybus *et al.*, 2001). Importantly, the GT distribution does not only differ between geographic regions but also between risk groups. HCV infection acquired by infusion of contaminated blood products are predominantly GT1b whereas GT1a and 3a are more frequently observed in IVDUs (Esteban *et al.*, 2008). Consequently, epidemiological data are consistent with a decline in circulation of subtype 1b and increase of 1a and 3a infection in Germany, France, Norway and United States (Ross *et al.*, 2000). Shift in predominating subtype from 1b to 3a mediated by increase in IVDU has been reported (Kalinina *et al.*, 2001).

1.6 Risk group of IVDUs

IVDU has become up to now the predominant mode of HCV transmission mechanism of HCV in the developed world (Quer and Esteban, 2005). The European Monitoring Centre for Drugs and Drug Addiction (EMCDDA 2006 report update, available at: http://www.emcdda.europa.eu/) and Eurosurveillance (Wiessing, 2005) have estimated a median rate of 5.3 IVDUs per 1.000 population aged 16 to 64 (range: 1.1 to 17). IVDU is one of the most efficient routes for HCV transmission, which is acquired more rapidly after initiation of IVDU than other viral infections, especially during the first year (Sutton *et al.*, 2006). The pooled prevalence of anti-HCV among IVDUs during the late 1980s and early

1990s in Western Europe was 79% (Mathei *et al.*, 2002). Recent estimates have shown a wide variation in anti-HCV prevalence among IVDUs in the European Union (EU), with prevalence over 60%. Factors associated with an increased risk of HCV infection include age, duration and frequency of IVDU, sharing equipment, polydrug use, HCV prevalence among experienced IVDUs, homelessness and having served a prison sentence (Hickman *et al.*, 2007). Differences in the relative contribution of these factors likely explain geographical disparities in HCV prevalence. Although harm reduction interventions (i.e. needle-exchange and methadone substitution programs) decreased both incidence and prevalence of HCV among IVDUs during the 1990s (Roy *et al.*, 2002), HCV transmission among IVDUs remains uncontrolled with prevalence increasing among young IVDUs and incidence rates ranging between 11 and 42 per 100 person/years (Judd *et al.*, 2005). The worst situation is in Eastern Europe where the dramatic IVDU-related HCV epidemic started in the early 1990s and harm reduction interventions remain limited (Kalinina *et al.*, 2001; Tallo *et al.*, 2007). Phylogenetic analysis of HCV isolates from IVDUs in different European cities shows a typical epidemic profile (large number of isolates per subtype, with a short genetic distance) and lack of site-specific segregation of isolates (Cochrane *et al.*, 2002) suggesting that HCV exchange between European IVDUs has occurred on a large scale.

The IVDU-associated HCV epidemic is having several epidemiological consequences. First, most new HCV infections occur in young IVDUs and the proportion of patients with IVDU-related chronic HCV has surpassed those infected by other routes. Second, IVDU-related HCV genotypes (1a, 3a, and 4) have replaced genotypes 1b and 2 among blood donors and young patients (Payan *et al.*, 2005). Even among IVDUs, relative GT distribution is rapidly changing (Schröter *et al.*, 2004), with GT4 spreading into Central and Northern Europe (Payan *et al.*, 2005), and increasing in Southern Europe (Katsoulidou *et al.*, 2006). Third, at least one of the recently described intergenotypic HCV recombinants (a 1b/2k) appears to have arisen during high-risk IVDU (Kalinina *et al.*, 2002), and is rapidly spreading (Tallo *et al.*, 2007). Despite the limited number of recombinants thus far described, the rapid spread of the 2k/1b and the generation of further hybrids amidst the IVDU epidemic might limit the accuracy of genotyping assays and their predictive value for treatment response (Simmonds *et al.*, 2005). Fourth, active HCV infection continues to increase among HIV-infected IVDUs (>70%) whose liver disease progresses faster (Martinez-Sierra *et al.*, 2003) and appear more resistant to current therapy (Brau, 2005), warranting every effort to provide them with optimised treatment strategies (Crespo *et al.*, 2007). Finally, although there is no reliable data on the extent to which IVDUs spread HCV over to the general population, strong evidence supports that such spread occurs. In Southern Spain, 31% of HCV GT4-infected individuals

have no known risk factor (Fernandez-Arcas *et al.*, 2006). In cohorts of Greek patients with chronic HCV, genotypes 3 and 4 were detected in a significant proportion of patients with nosocomial or community acquired infection (Katsoulidou *et al.*, 2006). In a French study GT3a infection among transfusion recipients increased almost threefold between 1979 and 1990 (Bourliere *et al.*, 2002). IVDU related subtypes 1a and 3a have been found in a substantial proportion of non-IVDU blood donors in the Netherlands and France (van de Laar *et al.*, 2006).

1.7 Immune response against HCV

Despite a high rate of viral persistence, there is a minority of patients with acute HCV infection able to contain virus replication and to achieve sustained eradication of HCV. The differences between those patients who achieve control and those who fail to control the infection are of utmost interest. It is believed that the virus-specific immune response plays an important role in containment of viral replication raising hopes that a vaccine might be possible. HCV enters a host either directly through transfusion of contaminated blood products or less efficient by perinatal or sexual transmission. The virus reaches the liver via the hepatic artery or the portal vein and enters the hepatocytes, its preferred site of replication. Once HCV has entered the hepatocyte, the immune response is activated.

1.7.1 Innate immune response

When HCV enters the liver and the infection starts the innate immunity is activated. The activation of early type I interferons (IFNs), IFN-α and IFN-β, is an important part in the early intracellular defence system against the viral infection. The production of double-stranded RNA (dsRNA) intermediates during HCV replication activate type I IFN genes. In Chimpanzees already two days after HCV infection large amounts of type I IFNs are produced in the liver (Bigger *et al.* 2001). The host cell recognizes dsRNA either via the Toll like receptor 3 (TLR3) (Alexopoulou *et al.*, 2001), or in a TLR-3 independent way. The latter pathway represented by the cytoplasmatic sensors of viral RNA, the retinoic acid inducible gene-I (RIG-I) (Yoneyama *et al.*, 2004) seems to be particularly relevant in HCV infection.

Upon RIG-I activation the IFN-β promoter stimulator 1 (IPS-1) (Kawai *et al.*, 2005) binds to the caspase activation and recruitment (CARD) domain of RIG-I and thereby initiates activation of IFN regulatory factor 3 (IRF-3) which translocates into the nucleus where it induces production of IFN-α/β. The viral dsRNA can also be recognized by TLR3. Activated TLR3 recruits Toll/interleukin-1 receptor (TIR) domain (containing adaptor-inducing IFN-β also named TRIF), which will lead to downstream activation of nuclear factor kappa B (NFκB) and ultimately leading to production of IFN-α/β. IFN-α/β is recognized by type I IFN receptor (IFNAR) resulting in a positive feedback loop and activation of multiple IFN-inducible genes (Hiscott *et al.*, 1999). Examples of these are, protein kinase R (PKR) production which leads to block of messenger RNA (mRNA) translation in the infected cell (Taylor *et al.*, 1999), and 2'-5' oligoadenylate synthetases (OAS) that activate the endoribonuclease L (RNase L) which in turn degrades RNA in the cell (Taguchi *et al.*, 2004). Interestingly, HCV has developed escape strategies that interfere with the activation pathway of the innate immune response. For example, the virally encoded NS3/4A protease is able to cleave the adapter molecules IPS-1 in the RIG-I pathway and TRIF in the TLR3 pathway, thereby preventing the production of type I IFNs within an infected cell.

At a cellular level, natural killer (NK) and natural killer T (NKT) cells are important players in the early response against a viral infection. However, the role of NK cells in control of HCV infection is less clear. Khakoo *et al.* in 2004 described an association between specific NK cell receptors and clearance of HCV suggesting that NK cells are important for viral containment. NK and NKT cells recognize infected cells in an antigen independent manner controlled by activating and inhibiting receptors on the NK cell, or by the interaction between CD1 (or other yet unknown ligands) and the T cell receptor on NKT cells, and perform cytotoxic lysis of infected cells by releasing granules containing perforin and proteases (granzymes). The antigen independent recognition of abnormalities in infected cells, such as down regulation of the major histocompatibility complex (MHC) class I molecules, trigger the cytolytic activity by the NK cell. NK and NKT cells also produce large amounts of the interferon-γ (IFN-γ) cytokine, a potent antiviral and immune regulatory cytokine promoting recruitment of inflammatory cells. The production of IFN-γ and tumor necrosis factor-α (TNF-α) from NK and NKT cells function as a link between the innate and the adaptive immunity by the stimulation of dendritic cell (DC) maturation. This is of vital importance for antigen presenting cells (APCs) towards activation of the specific immunity.

1.7.2 Adaptive immune response

1.7.2.1 Humoral immune response

Despite the early increase in HCV titer and the induction of IFN-stimulated genes (ISGs), delayed immune responses are one of the keys characteristics of HCV infection. HCV-specific antibodies are detected 8–12 weeks after infection, and HCV-specific T cells are typically detectable 8 weeks after infection (Thimme *et al.*, 2002). The role of the humoral immune response in HCV infection is still unclear. HCV develops an effective B cell response, which is responsible for high titers of specific antibodies. However, in contrast to other diseases in which antibodies play an important role to clear the virus by neutralization and degradation, in HCV-infection it has been highlighted that most antibodies are unable to eliminate the virus (Thimme *et al.*, 2002). Recently tools that are more sophisticated have been developed to study the humoral immune response including pseudotyped HCV-like particles. These studies concluded that the kinetics of the development of truly neutralizing antibodies upon infection plays an important role. The early appearance of antibodies able to neutralize the autologous virus seems to be associated with spontaneous control (Pestka *et al.*, 2007). One important mechanism for viral evasion from a neutralizing antibody response is mutational escape. Indeed, the HVR1, which is contained in the N-terminal part of the E2 protein, is characterized by enormous sequence diversity. This sequence diversity is the result of continuous selection of variants that escape from the humoral immune response (von Hahn *et al.*, 2007). Interestingly, this region is conserved in patients with agammaglobulinemia supporting that immune selection is the major driving force for evolution within this region (Booth *et al.*, 1998).

1.7.2.2 Cellular immune response by CD4+ and CD8+ T cells

The decrease in HCV viral load during acute infection coincides precisely with the appearance of HCV-specific T cells and IFN-γ expression in the liver (Shin, 2006), which suggests that viral clearance is T cell mediated. The importance of both T cell subsets - CD4+ and CD8+ T cells - has been elegantly shown by selective depletion experiments in HCV infected chimpanzees. Depletion of CD4+ helper T cells led to prolonged viremia associated with blunted CD8+ effector T cell responses (Grakoui *et al.*, 2003). Similarly, depletion of CD8+ T cells resulted in high-level viremia that was not cleared before CD8+ T cells reappeared in the liver at the site of infection (Shoukry *et al.*, 2003). It has not been

determined, whether IFN-γ is directly involved in HCV clearance, or whether it is just a marker for other T cell functions. Direct antiviral functions would be consistent with the observation that IFN-γ-mediated inhibition of subgenomic and genomic HCV RNAs is about 100 to 1.000 fold more effective than cytotoxicity (Jo *et al.*, 2009).

In humans, during the acute phase of a HCV infection, it has been shown that strong CD4+ and CD8+ T cell responses to HCV antigens are detected in patients who clear the infection as compared to those who develop chronic infection (Cucchiarini *et al.*, 2000). When analyzing the proliferative CD4+ T cell response in patients with self-limited infection, the immune response shows much more vigor compared to the response in chronically infected individuals (Missale *et al.*, 1996). Therefore, the induction and maintenance of an HCV-specific CD4+ T cell response may be an important factor in the defence against HCV infections. Patients who spontaneously cleared HCV infection show a strong and multispecific CD8+ T cell response. In contrast, CD8+ T cell responses are typically weak or even absent in patients with chronic HCV infection (Lauer *et al.*, 2004). Early studies in patients with acute HCV reported that a strong and vigorous CD8+ T cell response is associated with viral clearance (Thimme *et al.*, 2001). Recent studies suggested that CD8+ T cells are activated during acute HCV infection but that these responses are not maintained in those patients who develop chronic infection (Bowen and Walker, 2005). The mechanism of CD8+ T cell failure has been extensively investigated over the past 10 years. To understand these mechanisms the activation of CD8+ T cells needs to be discussed.

1.8 Activation of CD8+ T cells

As outlined earlier CD8+ T cells seem to play an important role in clearance of HCV infection. This requires activation and recruitment of CD8+ T cells to the site of infection in the liver. This activation process is complex and can be dissected into the following steps.

1.8.1 Antigen processing and presentation

The individual cell in the organism necessitates an efficient way to show the surrounding immune system whether it has been infected with an intracellular pathogen. The process starts with the degradation of the foreign proteins into smaller fragments. This is mediated by the

proteasome complex (Seemuller *et al.*, 1996). In order to get recruited to the proteasome proteins need to be ubiquitinated (Michalek *et al.*, 1993). Targeted proteins are then unfolded and processed in the proteasome to peptides. Proteasome degradation generates peptide fragments, which have to be actively transported into the ER where they can associate with the relevant MHC class I molecule. This process is mediated by the transporter associated with antigen processing (TAP) protein located in the ER membrane (Spies *et al.*, 1990). The TAP complex prefers peptides of 8-16 amino acids in length, which is similar to the size of peptides binding to MHC class I molecules. There is also a selective translocation determined by the peptide sequence and, in particular, the character of its C-terminal residue (Heemels and Ploegh, 1994). The MHC class I molecules are assembled in the ER. Upon binding of peptide, the peptide/MHC class I complex is released and transported through the Golgi apparatus to the cell surface. MHC class I molecules which cannot bind an appropriate peptide are unstable and will eventually be translocated into the cytoplasm and degraded by the proteasome. The MHC class I molecules are composed of a single membrane-spanning heavy chain paired with the soluble light-chain β2m. The heavy chain has a highly polymorphic region that forms a cleft where a peptide of 8-10 amino acids in length can bind. The most variable residues of the MHC class I molecules point into this groove, conferring unique peptide-binding and T cell receptor (TCR)-binding specificity of each MHC class I molecule (Maenaka and Jones, 1999).MHC class I molecules are composed by 6 pockets. The first and the last are conserved while the intermediates are more varying in size and character between different MHC class I alleles, thereby imposing different sequence requirements and constraints on the peptide that is bound. One consequence of this is that MHC class I binding peptides contain allele-specific sequence motifs (Rothbard and Taylor, 1988). Predictably, this system of antigen presentations is highly sensitive to variations of the peptide antigen. By introducing mutations in an immunogenic peptide or in the flanking region of an epitope, the antigen processing and presentation pathway can be affected.

1.8.2 Interaction between CD8+ T cells and peptide/MHC class I complex

CD8+ T cells can interact with the peptide/MHC class I complex via the highly diverse TCR. Beyond the characteristics of a peptide that determine the binding affinity to the MHC class I molecule the interaction between the peptide/MHC class I complex and the TCR on CD8+ T cells is also strongly influenced by the peptide sequence. The TCR contacts the MHC class I complex through the TCR variable domains. The affinity between the specific TCR and

peptide/MHC class I complex in orders of magnitude is weaker than that of a specific antibody–antigen interaction (Matsui *et al.*, 1991). However, because the MHC class I molecules serve not only as ligands for the TCR, but also as non-antigen-specific ligands for the TCR co-receptors (Gao *et al.*, 1997) - for example, MHC class I molecules for CD8+ T cells - even these weak interactions are sufficient to initiate signal transduction in cytotoxic T lymphocytes (CTLs).

The activation of CD8+ T lymphocytes can be divided into two phases, reflecting different aspects of the response. In the first phase, naïve cells become activated and differentiate into effector cells. In the second phase, the effector cells recognize antigen on specific target cells at the site of antigen entry or replication resulting in the destruction of the target cells. Depending on the differentiation stage, CTLs may respond with different efficiencies to signals mediated by the TCR and, therefore they require different levels of co-stimulatory signals for activation. Naïve cells require the signal via the TCR as well as via CD28 and additional co-stimulatory signals, while mature effector CTLs only need stimulation through the TCR (Schwartz, 1990). CD8+ T cells can be divided into at least 4 different groups, naïve, effector, effector memory and central memory. The different states of T cell differentiation are associated with distinct functional and phenotypic characteristics. The naïve cells, which have yet to encounter their antigen, are continuously recirculating between the secondary lymph nodes and the blood stream while the effector and memory cells modulate their expression of different homing molecules changing their paths of circulation. The effectors are found in inflamed tissues, the spleen, and the liver together with the effector memory cells. The circulating pattern of central memory cells more closely resembles that of naïve cells, which circulate between the blood stream and the secondary lymph organs (Sallusto *et al.*, 1999).

The ability of naïve T cells to expand and acquire effector functions depends not only on the strength of the stimulus through the TCR but also on an array of co-stimulatory receptors present on the T cells, such as CD28 (Acuto and Michel, 2003) as well as on soluble factors referred to as cytokines (Ahlers *et al.*, 2003). The membrane receptor CD28 has been shown to enhance TCR induced proliferation and differentiation of naïve T cells (Shahinian *et al.*, 1993). These properties have given the receptor the reputation of being the molecule responsible for “signal two” in T cells. The two CD28 ligands CD80 and CD86 are expressed at high levels on professional APCs (Sharpe and Freeman, 2002). Considerable evidence has accumulated to demonstrate that signaling through the TCR and CD28 is not sufficient to fully activate naïve CD8+ T cells and support the establishment of a memory population and that a third signal is also required. The presence or the absence of IL-12, IFNs, and possibly

IL-21 is a major determinant in whether encounter with foreign antigen leads to CD8+ T cell tolerance or immunity. In the absence of this third signal, the antigen-stimulated cells fail to develop effector functions, and those that survive long term are tolerant (Mescher *et al.*, 2006).

1.9 Mechanisms of CD8+ T cell failure in HCV infection

As outlined earlier, there is growing evidence that strong and broad HCV-specific CD4+ and CD8+ T cell responses are required for viral clearance and protective immunity. For the majority of acutely infected patients the infection becomes chronic and CD8+ T cell failure is observed (Post *et al.*, 2004). Various mechanisms of CD8+ T cell failure have been described (Fig. 1.6). The potential failure mechanisms of CD8+ T cells are described here in more detail.

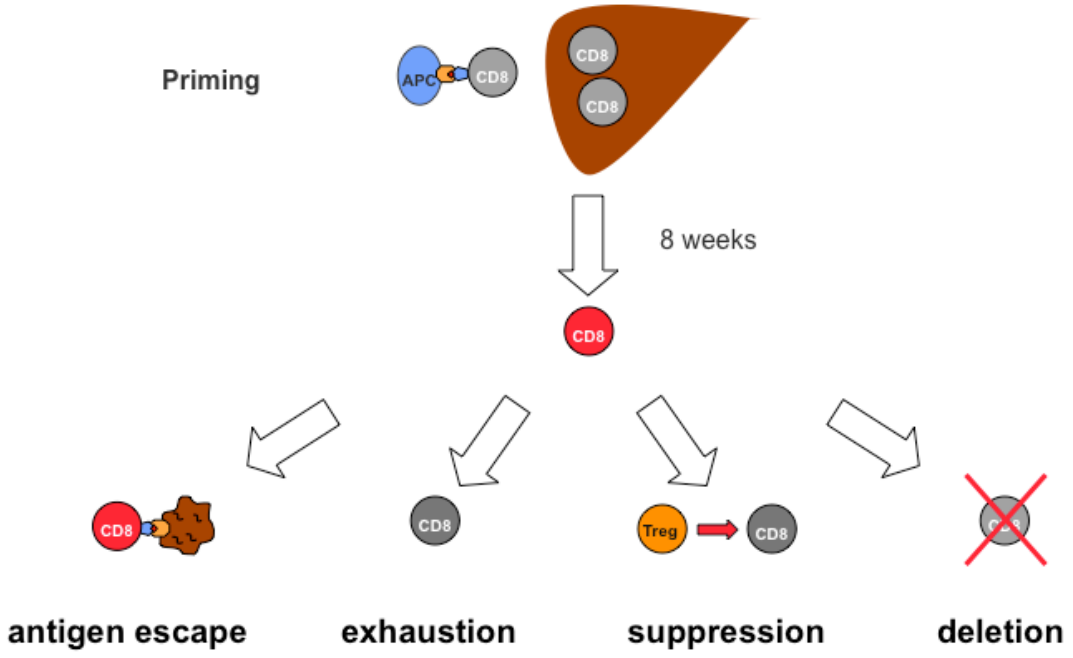


Fig. 1.5: Mechanisms of CD8+ T cell failure.

1.9.1 Primary CD8+ T cell failure

The T cell response in subjects who spontaneously cleared HCV infection is generally strong and multispecific whereas the response is weak or even absent in subjects with chronic infection (Lauer *et al.*, 2004). These data were derived in cross-sectional analyses. It is therefore not clear whether there is primary failure of CD8+ T cells or secondary failure in the chronic phase of infection. One striking feature of the CD8+ T cell response upon acute HCV infection is its delayed onset. HCV-specific CD8+ T cells are not detectable until 8 weeks after infection (Thimme *et al.*, 2001). It was speculated that DCs as the most important professional APCs for CD8+ T cell priming are impaired. However, controversial data have been published on this topic including reports showing DCs dysfunction in patients with chronic HCV infection (Nettemann *et al.*, 2006) as well as reports showing that DCs function is unchanged (Larsson *et al.*, 2004). However, recent data from patients with acute HCV infection suggest that the magnitude of the CD8+ T cell response does not differ between patients who spontaneously clear the infection and those who continue to viral persistence (Cox *et al.*, 2005). In both cases the CD8+ T cells response is strong and multispecific suggesting that secondary failure of CD8+ T cells strongly contributes to viral persistence.

1.9.2 The role of the liver as a tolerogenic organ

The liver is the primary replication site of HCV; analyses of liver infiltrating lymphocytes (LILs) reveal distinct features from those obtained with peripheral blood mononuclear cells PBMCs (Grabowska *et al.*, 2001). The immunological environment in the liver is potentially tolerogenic to LILs (Crispe, 2003). Liver sinusoidal endothelial cells, Kupffer cells, stellate cells, and liver DCs may mediate this tolerogenic effect (Lau and Thomson, 2003). This has not been specifically addressed in the context of HCV infection. However, activation of CD8+ T cells in the liver was analyzed in more detail in mouse models. Priming of CD8+ T cells in the liver resulted in dysfunctional antigen-specific CD8+ T cells (Diehl *et al.*, 2008). Moreover, priming of CD8+ T cells by hepatocytes results in upregulation of pro-apoptotic proteins and consequently premature deletion of antigen-specific CD8+ T cells (Holz *et al.*, 2010).

1.9.3 Antigen escape by selection of mutations

Mutations in the epitopes of MHC class I-restricted HCV-specific CD8+ T cells may be one important mechanism for failure of a T cell response (Timm and Roggendorf, 2007). The presence of quasispecies - a striking feature of HCV - enables the viruses to rapidly adapt to immune pressure. It has been observed that mutations in CD8+ T cell epitope regions can occur within weeks of acute infection both in chimpanzees and in men (Erickson *et al.*, 2001; Timm *et al.*, 2004; Tester *et al.*, 2005; Cox *et al.*, 2005). T cell escape mutations predominantly occur during early infection, and no additional mutations are observed during several years of subsequent follow-up (Chang *et al.*, 1997). Adaptation of HCV to T cell immune pressure was also observed at a population level data (Timm *et al.*, 2007; Ray *et al.*, 2005). Lack of CD4+ T cell help was associated with emergence of viral escape mutations (Grakoui *et al.*, 2003). Mechanistically escape mutations can be classified into those that cause (1) lower binding affinity of epitope peptide to MHC class I molecule (Tester *et al.*, 2005); (2) decreased TCR recognition of mutant peptides (Timm *et al.*, 2004); (3) impaired antigen processing by proteasomes (Seifert *et al.*, 2004). It is unclear to what extent selection of escape mutations contributes to viral persistence. In an analysis of the intrahepatic CD8+ T cell response about 50% of the targeted epitopes show evidence for escape. The remaining 50% are intact and recognized by circulating CD8+ T cells. Therefore, selection of escape mutations is clearly not the only factor involved in HCV persistence and T cell failure.

1.9.4 T cell dysfunction and exhaustion

It was described in several reports that effector functions of CD8+ T cells are impaired during acute and chronic HCV infection (Lechner *et al.*, 2000; Wedemyer, 2002). By means of tetramer, Elispot, ICS, and FACS technologies, defective HCV-specific CD8+ T cells in frequency, proliferation, cytotoxicity, and secretion of TNF-a and IFN-γ have been observed. The anergic CD8+ T cells are referred as stunned phenotype (Wedemeyer, 2002). T cell dysfunction was identified not only in chronic (Spangenberg *et al.*, 2005), but also in acute HCV infection (Thimme *et al.*, 2001), including both CD8+ (Wedemeyer, 2002) and CD4+ T cells (Cucchiarini *et al.*, 2000) from PBMCs (Penna *et al.*, 2007) and the liver (Wieland and Chisari, 2005). This T cell exhaustion can now be addressed since specific markers became available. Programmed death 1 (PD-1) is a strong marker for exhausted virus-specific CD8+ T cells in the lymphocytic choriomeningitis virus (LCMV) mouse model, and a similar role

for PD-1 has been shown in HCV infection. During the acute phase of an HCV infection, similar to an LCMV infection, PD-1 is upregulated in HCV-specific CD8+ T cells, independent of the outcome (Radziewicz *et al.*, 2007). However, in individuals with resolving infection, the PD-1 expression decreases rapidly, while in patients with a chronic infection, HCV-specific CD8+ T cells remain PD-1 positive (Urbani *et al.*, 2006). This finding is in parallel with the ‘‘stunned’’ phenotype of HCV-specific CD8+ T cells in the early acute phase of infection, which is restored in resolving infection but remains in persisting infection. In chronic HCV infection, HCV-specific CD8+ T cells in the peripheral blood and in the liver have been shown to express high levels of PD-1 (Wieland and Chisari, 2005). Inhibition of the PD-1/PD ligand 1 (PD-L1) interaction by antibodies restored cytokine production and proliferation of the exhausted CD8+ T cells from both acute and chronic infection in vitro (Golden-Mason *et al.*, 2007). In contrast to those in the periphery, intrahepatic HCV-specific T cells compartmentalized to liver, express not only high levels of PD-1 but also decreased CD127, were extremely refractory to PD-1/PD-L1 blockade (Nakamoto *et al.*, 2008).

1.9.5 Suppression by regulatory T Cells

Different subsets of regulatory T (Treg) cells contribute to the dysfunction of virus-specific T cells and thus to the HCV persistence. The frequencies of CD4+CD25+FoxP3+Treg and CD8+CD25+FoxP3+Treg in the periphery of HCV-infected patients are reported to be higher than those in resolved patients or uninfected controls (Billerbeck and Thimme, 2008). In addition, these cells inhibit HCV-specific CD8+ and CD4+ T cells by secreting IL-10 and transforming factor beta (TGF-β) (Sugimoto *et al.*, 2003). It was found that the frequency of CD4+CD25+ T cells and the HCV RNA titer is positively correlated. CD8+CD197- regulatory T cells are present in the HCV-infected liver, these cells are capable of suppressing HCV-specific CD8+ T cells by secreting IL-10, thus favouring HCV persistence and limited liver injury (Accapezzato *et al.*, 2004). Rosen’s group in 2008 demonstrated that FoxP3 expression levels and Treg-mediated suppression in the acute phase of HCV infection do not differ between patients who subsequently clear HCV and those who develop chronic infection (Smyk Pearson *et al.*, 2008), suggesting that Tregs are induced as a result of acute inflammation.

1.10 Evidence for protective immunity against HCV

The degree of possible HCV cross-protective immunity against homologous or heterologous HCV strains has been addressed in a number of observational and experimental studies both in humans (Proust *et al.*, 2000; Mehta *et al.*, 2002) and chimpanzees (Landfrod *et al.*, 2004). In chimpanzees, repetitive infections with HCV are possible. This was mostly evident when animals were infected with different HCV genotypes raising concerns that protective immunity will be difficult to achieve in the context of a pathogen with such dramatic sequence variability. However, observational studies in high-risk groups for HCV infection suggested that previous exposure to HCV at least provides partial protection from re-infection. In 2002, Mehta *et al.* reported that previously infected IVDUs were significantly less likely to be reinfected, even after accounting for risk behavior. Further reports supported this finding: Grebely *et al.* in 2006 reported a significantly lower incidence of HCV reinfection in IVDUs than in naïve individuals; and Dove *et al* in 2005 detected no reinfections in a small group of ongoing IVDUs with previous HCV clearance. These findings suggest that human beings can acquire protective immunity as has been shown in chimpanzees (Basset *et al.*, 2001). This degree of protection is unclear after previous HCV infection is unclear. Other studies in chimpanzees have instead demonstrated a relative lack of protection against rechallenge with heterologous virus strains, indicating poor cross-GT recognition (Prince *et al.*, 2005). Schulze Zur Wiesch *et al.*, in 2007 showed that persistent HCV-specific CD4+ T cell responses detected in a subset of chronically infected patients are unable to recognize the contemporary circulating viruses, likely representing immunologic scars from a previously resolved HCV infection. Only few data on the role of preexisting T cell responses on the outcome of a subsequent HCV infection are available. Interesting insights in this direction came in 2006 from Folgori *et al.*, they showed that vaccination with adenoviral vectors in combination with electroporated deoxyribonucleic acid (DNA) coding for the HCV non-structural region is able to protect chimpanzee from acute hepatitis. The challenge in this case was performed with a heterologous virus differing from the vaccine sequence by more than 13% at the amino acid level, and all vaccinated chimpanzees that developed a cross-reactive T cell response against the challenge virus (4 of 5) were capable of resolving the infection (Folgori *et al.*, 2006). No data on this topic are available in humans. Importantly, the relevance of GT-specific sequence differences on the CD8+ T cell response and the level of protection in humans are completely unknown. IVDUs being highly susceptible to reinfection represent a perfect model for studying the impact that multiple exposures to different genotypes may have on the immune system. In the present work, the

CD8+ T cell responses to HCV were therefore studied in more detail recruiting IVDUs from the Department of Addictive Behaviour and Addiction Medicine, Rhine State Hospital Essen.

2 Aims of the study

The overall aim of this study is to characterize the CD8+ T cell response in IVDUs representing a high-risk group for HCV infection that is possibly exposed multiple times to different HCV genotypes.

The following questions are specifically addressed:

1. What is the extent of sequence differences between the most common HCV genotypes in IVDUs (GT1 and 3) at the CD8 epitope level?
2. What is magnitude and breadth of the CD8+ T cell response against HCV GT1 and GT3 in this high-risk group?
3. What are the consequences of sequence differences between HCV GT1 and 3 for the CD8+ T cell response at the epitope level?
4. Is there immunological evidence for exposure to different HCV genotypes in individual patients?
5. Are there phenotypic differences in the CD8+ T cell epitope repertoire of patients who control virus replication compared to patients with ongoing virus replication?
6. Are there qualitative differences between CD8+ T cells from patients who control virus replication compared to patients with ongoing virus replication?
7. Is it possible to prime naïve CD8+ T cells against immunodominant HCV epitopes?

3 Materials

3.1 Equipment

Table 3.1: Equipment

Item	Manufacturer
AutoMACS Pro Separator	Miltenyi Biotec (Ger)
Binders CB Series CO_2 Incubators	Binder USA
Centrifuge 5415 D	Eppendorf (Ger)
Elisa Reader A Sys Expert Plus	Asys Hiteck Gmbh (Aus)
FACS calibur flow cytometer	BD (Ger)
Freezer	Liebherr (Ger)
GeneAmp PCR System 2400	Applayed Biosystems (Ger)
Inverted Microscope TMS Nikon	Nikon (Ger)
Laminar flow	Herasafe (Ger)
Mastercycler Personal	Eppendorf (Ger)
Megafuge 1,0 R	Heraeus (UK)
Metall-Block-Thermostate MBT 250	Kleinfeld Labortechnik (GER)
Neubauer cell counting chamber	BD (Ger)
Photometer Gene Quant	Amersham Bioscience Europe Gmbh (Ger)
Refrigerator	Premium (Ger)
Ultra-low Temperature Freezer	Sanyo (Ger)
UV transluminator FLX-20M	MWG-Bio Tech (Ger)

3.2 Materials

Table 3.2: **Materials**

Material	Manufacturer
Beakers	Beakers Schott (Ger)
Cell culture plates, sterile (6; 24 and 96 well)	Greiner bio-one (Ger)
FACS tubes	BD (Ger)
Plastic pipettes (sterile; 1 ml; 5 ml; 10 ml; 25 ml)	Greiner bio-one (Ger)
PP screw-cap tubes (15 ml; 50 ml)	Greiner bio-one (Ger)
Reaction tubes (1,5 ml; 2 ml)	Eppendorf AG (Ger)
U-shaped microplates (96 wells)	Greiner bio-one (Ger)
2 ml, 1.5 ml and 0.2 ml tubes	Eppendorf AG (Ger)

3.3 Chemicals and Media

Table 3.3: Chemicals and Media

Chemicals and Media	Manufacturer
Biocoll separating solution 1.10	Biochrom (Ger)
Brefeldin A	BD (Ger)
CellGro Medium	CellGenix (Ger)
Cytofix/Cytoperm	BD Pharmingen (Ger)
DMSO (Dimethyl sulfoxide)	ROTH GmbH (Ger)
EDTA	Ambion (UK)
Ethanol	ROTH GmbH (Ger)
Ethidium bromide	ROTH GmbH (Ger)
FCS (Fetal calf serum)	Biochrom
HEPES	PAA Laboratories (Ger)
Isopropanol	ROTH GmbH (Ger)
Lipopolysaccharides (LPS)	Sigma Aldrich Chemie GmbH (Ger)
RPMI 1640 Medium	Gibco (Ger)
Smart ladder	Eurogentec (Ger)
Sodium Azide	Applichem (Ger)

Chemicals and Media	Manufacturer
Tris-Borate-EDTA (TBE)	Invitrogen GmbH (Ger)
Via-Probe Cell Viability solution	BD (Ger)

3.4 Enzimes

Table 3.4: Enzymes

Enzymes	Supplier
Protease (protienase K)	QIAgen (Ger)
Go Taq Polymerase	Promega (Ger)
Dnase I	Roche (Ger)

3.5 Cytokines

Table 3.5: Cytokines

Cytokines	Manufacturer
Recombinant Human Interleukine-2 (rHu IL-2)	Promokine (Ger)
Recombinant Human Interleukine-4 (rHu IL-4)	Promokine (Ger)
Recombinant Human Interleukine-7 (rHu IL-7)	Promikine (Ger)
Recombinant Human Interleukine-15 (rHu IL-15)	Promokine (Ger)
Recombinant Human Granulocyte and Macrophage Colony Stimulating Factor (rHu GM-CSF)	Promokine (Ger)
Recombinat Human Intereferon-gamma (rHu IFN-γ)	Promokine (Ger)

3.6 Commercial Kits

Table 3.6: Commercial Kits

Cytokines	Manufacturer
QIAmp Viral RNA Mini Kit 250	QIAgen GmbH (UK)
QIAGEN OneStep RT-PCR Kit	QIAgen GmbH (UK)
QIAQuick Gel extraction Kit	QIAgen GmbH (UK)
QIAamp DNA Blood Mini KIT50	QIAgen GmbH (UK)
Murex HCV Serotyping	Abbott Murex (UK)

3.7 Antibiotics

Gentamicin PAA Laboratories GmbH (Ger)

Penicillin / Streptomycin PAA Laboratories GmbH (Ger)

3.8 Buffers and Media

Table 3.7: Buffers and Media

Description	Composition
DCs culture medium	500 ml 10% Human serum 1% Gentamicin 2000 IU/ml IL-4 2000 IU/ml GM-CSF
Co-culture medium	500 ml RPMI 1640 10% FCS 1% Penicillin/Streptomycin mixture 1% Hepes

Description	Composition
Freezing medium	90% FCS 10% DMSO
PBMCs culture medium	500 ml RPMI 1640 10% FCS 1% Penicillin/Streptomycin mixture 1% Hepes IL-2 2.5 µl/ml CD28 µl/ml
FACS buffer	1 l PBS 0.02% Na-azide 0.5% FCS
MACS buffer	1 l PBS 2mM EDTA 0.5% FCS

3.9 Oligonucleotides

All the oligonucleotides used in this work have been synthesized by Biomers net GmbH (Ulm). In the following list are mentioned only primers used for the amplification of different HCV genome's regions.

Table 3.8: Oligonucleotides

Genotype	Primer	Sequences	Targeted region
	p439s	5'-GAGTWTACBTGYTGCCGCGCAG-3'	Core
GT1b	SV963s_neu	5'-CTACCCGTCTCCGCCCGVAGR-3'	NS3
GT1b	NS3-1113-R	5'-CTTGGAGTGRCAGAAAATGAG-3'	NS3
GT1b	SV694as_neu	5'-ATCCTRCCCACAATGACCACR-3'	NS3
GT1b	HCV3404s_neu	5'-AGGGTGGCGACTCCTYGCK-3'	NS3
GT1b	Anti-D_NS3-913-F	5'-ACRGTCCTGGACCAAGCR-3'	NS3
GT1b	NS3-1058-R	5'-GCYTTRCCATAGAAGGGRA-3'	NS3
GT1b	SV696as	5'-GCCCACAATGACCACGCTGCC-3'	NS3
GT1b	GT1b-3c-R	5'-CCAARTAAAGGTCCGAGCTGCC-3'	NS3
GT1b	GT1b-3e-R	5'-CGCCCGTGGTGATGGTCC-3'	NS3
GT1b	GT1b-4c-F	5'-CTATGGCAAAGCCATCCC-3'	NS3
GT1b	GT1b-4b-R	5'-CATTAGAGCGTCTGTTGC-3'	NS3
GT1b	GT1b-4d-F	5'-GCATMTACAGGTTTGTGACTCC-3'	NS3
GT1a	3b-F	5'-TGGRTTCCCCCCCTCAACG-3'	NS3
GT1a	4a-R	5'-TCTCCGGTGGTGGACAGAGC-3'	NS3
GT1a	4a-F	5'-AGTGCCCCAGAGCTTCCAGG-3'	NS3
GT1a	4e-R	5'-GGGCCCTTCTGCTTGAACTGC-3'	NS3
GT1a	3c-F	5'-TCCRAATGGAGACCAAGC-3'	NS3
GT1a	3e-R	5'-CGCCCGTGGTGATGGTCC-3'	NS3
GT1a	4b-F_new	5'-GTAARAGCACCAAGGTYCC-3'	NS3
GT1a	4d-R	5'-ACCCAGGTGCTCGTGACG-3'	NS3
GT1a	4b-F	5`-AAGGACCATCACCACGGG -3`	NS3
GT3a	3256-F	5'-CTAGCAGTGGCCACTGAACC-3'	NS3
GT3a	4577-R	5'-GTTGAGCCCCATRCCTCTRA-3'	NS3

Genotype	Primer	Sequences	Targeted region
GT3a	4294-F	5’-ATTATCTGTGATGARTGYCATGC-3’	NS3
GT3a	5452-R	5’-TATTGTTGRTACARCACCTC-3’	NS3
GT3a	3341-F	5’-ATATTCTTTGCGGGCTGC-3’	NS3
GT3a	4460-R	5’-GGGATCTCACCTTCRGARC-3’	NS3
GT3a	4328-F	5’-CTAGCATAYTGGGYATAGGCAC-3’	NS3
GT3a	5439-R	5’-CACCTCYTTRTCTGGAACG-3’	NS3
GT3a	3341-F	5’-ATATTCTTTGCGGGCTGC-3’	NS3

3.10 Peptides

a. List of overlapping and optimal peptides used in the preliminary screening of the immune responses of the IVDUs (from Filiz Oezkan, Department of Dermatology, University Hospital St. Josef, Bochum, Germany).

Table 3.9: Peptides

Group	Name	GT1b	GT3a
Pool 1	1	APITAYSQQTRGLLGCII	APITAYAQQTRGLLGTIV
	2	SQQTRGLLGCIITSLTGR	AQQTRGLLGTIVTSLTGR
	3	GCIITSLTGRDKNQV	GTIVTSLTGRDKNVV
	4	SLTGRDKNQVEGEVQVV	SLTGRDKNVVTGEVQVL
	5	NQVEGEVQVVSTATQSFL	NVVTGEVQVLSTATQTFL
	6	VVSTATQSFLATCVNGVC	VLSTATQTFLGTTVGGVM
	7	FLATCVNGVCWTVYHGA	FLGTTVGGVMWTVYHGA
	8	GVCWTVYHGAGSKTLAGP	GVMWTVYHGAGSRTLAGA
	9	GAGSKTLAGPKGPITQMY	GAGSRTLAGAKHPALQMY
Pool 2	10	GPKGPITQMYTNVDQDLV	GAKHPALQMYTNVDQDLV
	11	MYTNVDQDLVGWQAPPGA	MYTNVDQDLVGWPAPPGA
	12	LVGWQAPPGARSLTPCT	LVGWPAPPGAKSLEPCA
	13	PGARSLTPCTCGSSDLYL	PGAKSLEPCACGSADLYL
	14	CTCGSSDLYLVTRHADVI	CACGSADLYLVTRDADVI

Group	Name	GT1b	GT3a
	15	YLVTRHADVIPVRRR	YLVTRDADVIPARRR
	16	HADVIPVRRRGDSRGSLL	DADVIPARRRGDSTASLL
	17	RRGDSRGSLLSPRPVSYL	RRGDSTASLLSPRPLACL
	18	LLSPRPVSYLKGSSGGPL	LLSPRPLACLKGSSGGPV
Pool 3	19	YLKGSSGGPLLCPSGHAV	CLKGSSGGPVMCPSGHVA
	20	PLLCPSGHAVGIFRAAV	PVMCPSGHVAGIFRAAV
	21	HAVGIFRAAVCTRGVAKA	HVAGIFRAAVCTRGVAKA
	22	AVCTRGVAKAVDFVPV	AVCTRGVAKALQFIPV
	23	VAKAVDFVPVESMETTMR	VAKALQFIPVETLSTQAR
	24	PVESMETTMRSPVFTDNS	PVETLSTQARSPSFSDNS
	25	MRSPVFTDNSSPPAV	ARSPSFSDNSTPPAV
	26	FTDNSSPPAVPQTFQVAH	FSDNSTPPAVPQSYQVGY
	27	AVPQTFQVAHLHAPTGSGK	AVPQSYQVGYLHAPTGSGK
Pool 4	28	HLHAPTGSGKSTKVPAAY	YLHAPTGSGKSTKVPAAY
	29	GKSTKVPAAYAAQGYKVL	GKSTKVPAAYVAQGYNVL
	30	AYAAQGYKVLVLNPSVAA	AYVAQGYNVLVLNPSVAA
	31	VLVLNPSVAATLGFGAYM	VLVLNPSVAATLGFGSFM
	32	AATLGFGAYMSKAHGV	AATLGFGSFMSRAYGI
	33	GAYMSKAHGVDPNIR	GSFMSRAYGIDPNIR
	34	KAHGVDPNIRTGVRTI	RAYGIDPNIRTGNRTV
	35	PNIRTGVRTITTGAPITY	PNIRTGNRTVTTGAKLTY
	36	TITTGAPITYSTYGKFLA	TVTTGAKLTYSTYGKFLA
Pool 5	37	TYSTYGKFLADGGCSGGA	TYSTYGKFLADGGCSGGA
	38	LADGGCSGGAYDIII	LADGGCSGGAYDVII
	39	CSGGAYDIIICDECHS	CSGGAYDVIICDECHA
	40	DIIICDECHSTDSTTIL	DVIICDECHAQDATSIL
	41	CHSTDSTTILGIGTVL	CHAQDATSILGIGTVL
	42	TTILGIGTVLDQAETAGA	TSILGIGTVLDQAETAGV
	43	VLDQAETAGARLVVLATA	VLDQAETAGVRLTVLATA
	44	GARLVVLATATPPGSVTV	GVRLTVLATATPPGSITV
	45	TATPPGSVTVPHPNIEEV	TATPPGSITVPHSNIEEV
Pool 6	46	TVPHPNIEEVALSNTGEI	TVPHSNIEEVALGSEGEI
	47	EVALSNTGEIPFYGKAI	EVALGSEGEIPFYGKAI
	48	GEIPFYGKAIPIEVIK	GEIPFYGKAIPIALLK

Group	Name	GT1b	GT3a
	49	GKAIPIEVIKGGRHLIF	GKAIPIALLKGGRHLIF
	50	VIKGGRHLIFCHSKKK	LLKGGRHLIFCHSKKK
	51	HLIFCHSKKKCDELAAKL	HLIFCHSKKKCDEIASKL
	52	KKCDELAAKLSALGLNAV	KKCDEIASKLRGMGLNAV
	53	KLSALGLNAVAYYRGLDV	KLRGMGLNAVAYYRGLDV
	54	AVAYYRGLDVSVIPTSGDV	AVAYYRGLDVSVIPTTGDV
	55	VSVIPTSGDVVVVATDAL	VSVIPTTGDVVVCATDAL
Pool 7	56	DVVVVATDALMTGYTGDF	DVVVCATDALMTGFTGDF
	57	ALMTGYTGDFDSVIDCNT	ALMTGFTGDFDSVIDCNV
	58	DFDSVIDCNTCVTQTVDF	DFDSVIDCNVAVEQYVDF
	59	NTCVTQTVDFSLDPTFTI	NVAVEQYVDFSLDPTFSI
	60	DFSLDPTFTIETTTV	DFSLDPTFSIETRTA
	61	PTFTIETTTVPQDAVSR	PTFSIETRTAPQDAVSR
	62	TTVPQDAVSRSQRRGR	RTAPQDAVSRSQRRGR
	63	AVSRSQRRGRTGRGRR	AVSRSQRRGRTGRGRL
	64	RRGRTGRGRRGIYRFVT	RRGRTGRGRLGTYRYVA
	65	GRRGIYRFVTPGERPSGM	GRLGTYRYVAPGERPSGM
Pool 8	66	VTPGERPSGMFDSSVL	VAPGERPSGMFDSVVL
	67	PSGMFDSSVLCECYDA	PSGMFDSVVLCECYDA
	68	SSVLCECYDAGCAWYEL	SVVLCECYDAGCSWYDL
	69	YDAGCAWYELTPAETSVR	YDAGCSWYDLQPAETTVR
	70	ELTPAETSVRLRAYL	DLQPAETTVRLRAYL
	71	ETSVRLRAYLNTPGLPV	ETTVRLRAYLSTPGLPV
	72	AYLNTPGLPVCQDHLEFW	AYLSTPGLPVCQDHLDFW
	73	PVCQDHLEFWESVFTGL	PVCQDHLDFWESVFTGL
	74	EFWESVFTGLTHIDAHFL	DFWESVFTGLTHIDAHFL
	75	GLTHIDAHFLSQTKQAGD	GLTHIDAHFLSQTKQQGL
Pool 9	76	FLSQTKQAGDNFPYLVAY	FLSQTKQQGLNFSYLTAY
	77	GDNFPYLVAYQATVCARA	GLNFSYLTAYQATVCARA
	78	AYQATVCARAQAPPPSW	AYQATVCARAQAPPPSW
	79	ARAQAPPPSWDQMWKCLI	ARAQAPPPSWDETWKCLV
	80	SWDQMWKCLIRLKPTLH	SWDETWKCLVRLKPTLH
	81	CLIRLKPTLHGPTPLLYR	CLVRLKPTLHGPTPLLYR
	82	LHGPTPLLYRLGAVQNEV	LHGPTPLLYRLGPVQNEI

Group	Name	GT1b	GT3a
	83	YRLGAVQNEVTLTHPITK	YRLGPVQNEICLTHPVTK
	84	EVTLTHPITKYIMACMSA	EICLTHPVTKYIMACMSA
	85	TKYIMACMSADLEVVT	TKYIMACMSADLEVTT
OPT 1	1	ATDALMTGY	
	2	HSKKKCDEL	
	3	HPNIEEVAL	
	4		CVNGVCWTV
	5	KLVALGINAV	
OPT 2	1	AYSQQTRGL	
	2	MYTNVDQDL	
	3	YLVTRHADV	
	4	HAVGLFRAA	
	5	TLGFGAYMSK	
	6	ALYDVVTKL	
OPT 1	1	GAYMSKAHGV	
	2	GRGKPGIYR	
	3	KSKKTPMGF	
	4	ARMILMTHF	
	5	NTRPPLGNW	
Control	Flu	GILGFVFTL	

b. List of peptides identified during the MHC class I binding prediction analyses and used to identify the epitope specific responses (EMC, Ger).

Name	Sequence	Genotype	HLA restriction
p23	HSNIEEVAL	3a	B35
p24	CINGVCWTV	1b prototype	A2
p25	TVGGVMWTV	3a	A2
p26	KAVDFVPVE	1b	A26
p27	FVPVESMET	1b/3a	A26
p28	VDFVPVESM	1b/3a	A3
p29	VESMETTMR	1b/3a	B40
p30	ETTVRLRAY	1b/3a	A26

Group	Name	GT1b	GT3a
p31	ETSVRLRAY	1b/3a	A26
p32	RLGAVQNEV	1b	A2
p33	RLGPVQNEI	3a	A2
p34	VTLTHPITK	1b	A3
p35	VQNEICLTH	3a	B15
p36	NEICLTHPV	3a	A3
p37	KAHGVDPNI	3a	A2
p38	HLHAPTGSGK	1b	A3
p39	GSFMSRAYGI	3a	A3
p40	GLNAVAYYR	3a	A3
p41	KLSGLGINAV	1b variant	A2
p42	KLSGLGLNAV	1b variant	A2
p43	KLSALGLNAV	1b	A2
p44	KLRGMGLNAV	3a	A2
p46	HSRRKCDEL	3a	B8
p47	ALYDVVTKL	1b	A2
p48	RLGAVQNEA	1b	B13
p49	RLGPVQNEV	3a	B13
p50	RLGAVQNEI	1b variant	B13
p51	HSKKKCDDL	1b variant	B8
p48 1073	CINGVCWSV	1a variant	A2
p49 1073	CINGVCWAV	1a variant	A2
p50	HAVGIFRAAV	1a/b	A68
p51	HAVGIFKAAV	1a/b variant	A68
p52	HAVGIFQAAV	1a/b variant	A68
p53	HAVGIFRTAV	1a/b variant	A68
p54	RRGRTGRGRL	3a	B27
p55	RRGRTGRGRR	1b	B27
p56	GRGRLGTYRY	3a	B27
p57	GRGRRGIYRF	1b	B27
p58	GRGKPGIYRF	1a	B27
p59	YLHAPTGSGK	3a	A3
p60	GEIPFYGKAI	1b/3a	B51
p61	LIRLKPTL	1b	B8

Name	Sequence	Genotype	HLA restriction
p62	LVRLKPTL	3a	B8
p63	HSKKKCDEI	3a	B8
p64	HVAGIFRAAV	3a	A68
p65	TYSTYGKFL	1b/3a	A24
p66	GIDPNIRTGV	described	A2
p67	GVDPNIRTGV	1b	A2
p68	GIDPNIRTGN	3a	A2

3.11 Antibodies

Unless otherwise noted, the antibodies were procured from BD, Germany.

Table 3.10: Antibodies

Antibodies	Clone
$Bim_{S/EL/L}$: monoclonal rat anti-human antibody (Rat IgG2a) [Alexis]	3C5
CD8-PE: monoclonal mouse anti-human antibody (IgG1, k)	HIT8a
CD8-APC: monoclonal mouse anti-human antibody (IgG1, k)	RPA-T8
CD14-PE: monoclonal mouse anti-human antibody (IgG2a, k)	M5E2
CD14 monoclonal mouse anti-human antibody (IgG2a) conjugated MicroBeads [Miltenyi Biotec]	TÜK4
CD28 / CD49d: monoclonal mouse anti-human antibodies	L293, L25
CD80(B7-1)-FITC: monoclonal mouse anti-human antibody (IgM, k)	BB1
CD127(IL-7Ra)-FITC: monoclonal mouse anti-human antibody (IgG1) [eBioscience]	eBioRDR5
HLA-A2-FITC: monoclonal mouse anti-human antibody (IgG2b, k)	BB7.2
IFNγ-FITC: monoclonal mouse anti-human antibody (IgG1, k)	4S.B3
IFN-γ APC: monoclonal mouse anti-human antibody (IgG1, k)	B27
Rat IgG2a-FITC: polyclonal goat anti rat antibody [Bethyl]	

3.12 Fluorochromes

The antibody-coupled fluorochromes and their absorption- and emission maxima are listed below.

Table 3.11: Fluorochromes

Flurophore	Abbreviation	Absorption (nm)	Emission (nm)
Fluorescein isothiocyanate	FITC	488	518
Phycoerythrin	PE	488	575
Peridinin-chlorophyll-protein complex	PerCP	488	675
Allophycocyanin	APC	633	660

3.13 MHC class I-Pentamers

Table 3.12: **MHC class I-Pentamers**

HLA type	Sequence	Epitope Origin	Virus	Label
A2	GILGFVFTL	Influenza A MP 58-66	Influenza	PE
A2	CINGVCWTV	HCV NS3 1073-1081	HCV	PE
A2	KLVALGINAV	HCV NS3 1406-1415	HCV	PE

3.14 Computer programs

Table 3.13: Computer programs

Software	Manufacturer
Cell quest	BD
CodonCode Aligner	CodonCode Corporation
Flowjo	TreeStar Inc.
Graphpad Prism	Graphpad
Microsoft Office	Microsoft

3.15 Websites

HCV Sequence Database (http://hcv.lanl.gov/)

Immune Epitope Database and Analysis resource (http://www.iedb.org/)

4 Methods

4.1 Patients

Patients with a history of IVDU who reported to be HCV-positive were recruited in the ward for inpatient detoxification treatment of drug addicts as well as in the clinic for opiate substitution treatment (OST) at the Department of Addictive Behaviour and Addiction Medicine, Rhine State Hospital Essen, University of Duisburg-Essen after written informed consent was obtained. By this approach 53 anti-HCV positive patients (identified by chemiluminescent microparticle immunoassay, CMIA) were identified including 17 infected with GT1, 22 infected with GT3 and 14 HCV-RNA negative subjects. The Glutamic-Pyruvic Transaminase (GPT) levels, the viral load and the serotype of the autologous virus were analyzed in all occasions. The study was approved by the local ethics committee according to the guidelines of Helsinki.

4.2 Extraction of viral RNA

HCV-RNA was extracted from patient's serum by using a QIAamp Viral RNA mini Kit. As shown in the manufacturer's instructions, 140 µl of serum were added to the lysis buffer (200 µl AVL buffer) into a 1.5 ml microcentrifuge tube then incubated for 10 min at room temperature (RT) (15–25°C). The lysate was mixed with 560 µl of 100% ethyl alcohol (EtOH), then transferred to the QIAamp Mini column and centrifuged for 1 min at 8000 rpm. The column was washed with 500 µl of AW1 and 500 µl of AW2 buffers. The viral RNA was eluted in 50 µl of water in new 1.5 ml microcentrifuge tube and directly used in the reverse transcription reaction or kept at - 80°C.

4.3 Reverse transcriptase reaction

HCV-RNA isolated from patient's serum was reverse transcribed into DNA using OneStep reverse transcriptase (RT-PCR), 5 µl extracted RNA was added to the following:

One Step RT-PCR Mix

5x Buffer	5 µl
Water (H_2O)	11 µl
RT-PCR Enzyme Mix	1 µl
Forward-Primer1 (10 pmol/µl)	1 µl
Reverse-Primer1 (10 pmol/µl)	1 µl
Deoxynucleosides (dNTPs, 10 mM)	1 µl
Total	20 µl

Thermal cycling

45 min	50º
15 min	94º
1 min	94º
1 min	54º
1 min/kb	72º
Cycles	35
10 min	72º
Unlimited	4º

And these amplificats were used for second-round PCR

PCR 2 Master Mix

H_2O	27.75 µl
5x Puffer	10 µl
dNTP (10mM)	1 µl
Forward-Primer2 (10 pmol/µl)	5 µl
Reverse-Primer2 (10 pmol/µl)	5 µl
DNA Polymerase (Go Taq)	0.25 µl
Total	49 µl

1 µl of the PCR 1 productions were added to PCR 2 Master Mix.

Thermal cycling

2 min	94º
1 min	94º
1 min	58º
2 min	72º
Cycles	35
20 min	72º
For ever	4º

PCR products were sequenced on an ABI 3730 XL automated sequencer. Sequences were aligned, edited and analyzed with CodonCode Aligner (Dedham, MA, USA) and Se-Al (available from http://evolve.zoo.ox.ac.uk). All sequences were submitted to GenBank and are available under accession numbers FJ864731- FJ864816.

4.4 Purification of restricted DNA by gel extraction

The amplified DNA was separated by electrophoresis on 1.2% agarose gel. The fragments of interest were cut from the gel and minimized in size removing extra agarose. Gel slices were weighed and 3 volumes of Buffer QG were added to 1 volume of gel. The slices were incubated at 50°C for 10 min and 1 volume of isopropanol was added to the sample and mixed. The samples were applied to the QIAquick column, and centrifuged for 1 min. 0.75 ml of Buffer PE were added to QIAquick column and centrifuged for 1 min (washing step), then the DNA was eluted with 50 µl of water.

4.5 PBMCs extraction from peripheral blood

PBMCs were isolated by centrifugation gradient using Biocoll separating solution (10 min at 2100 rpm) and washed three times for 7 min at 2000 rpm with sterile RPM 1640. The cells were than resuspended in complete PBMCs culture media for direct analysis or in the freezing medium to be cryporeserved and subsequently used for further investigations.

4.6 Flow cytometry

Flow cytometry is a method to differentiate and count cells and microparticles that are tagged with fluorescent antibodies. Until a decade ago, the flow cytometer was seen only in research laboratories. Recently, it has evolved from its highly specialized research tool status to a commonplace clinical assay. The impetus for this change has been that flow cytometry is the only technique capable of quantitative measurements of multiple features of individual cells in a rapid manner (McCoy and Overton, 1994). Flow cytometry is a generic term, while FACS (Fluorescence activated cell sorter) is a trademark of the Becton-Dickinson Corporation.

4.7 Methodology of flow cytometry

A flow cytometer is made up of three main systems: fluidics, optics, and electronics (http://www.bdbiosciences.com) (Fig. 4.1).

• The fluidics system hydrodynamically focuses the cell stream to the laser beam for interrogation.

• The optics system consists of lasers to illuminate the particles in the sample stream and optical filters to direct the resulting light signals to the appropriate detectors.

• The electronics system converts the detected light signals into electronic signals that can be processed by the computer.

For some instruments equipped with a sorting feature, the electronics system is also capable of initiating sorting decisions to charge and deflect particles. Any suspended particle or cell from 0.2–150 micrometers in size is suitable for analysis. Cells from solid tissue must be disaggregated before analysis.

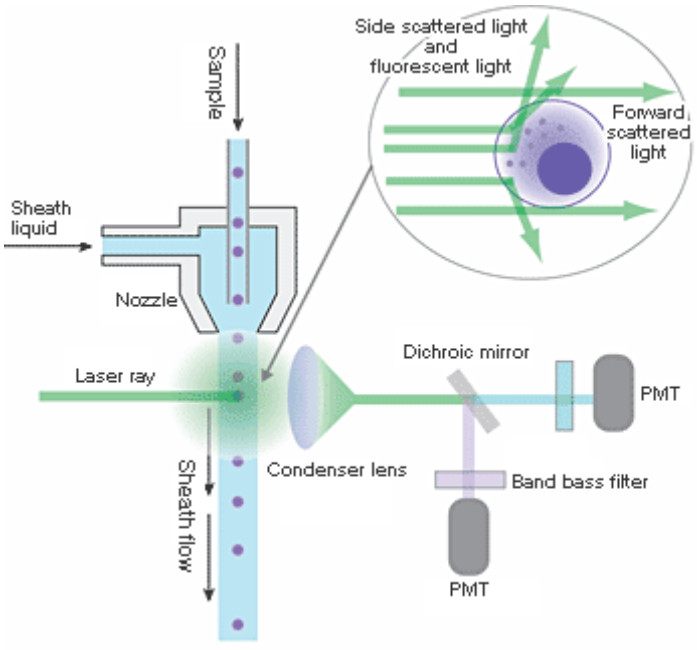


Fig. 4.1: Principle of flow cytometry.
(http://www.bdbiosciences.com)

Light scattering

A flow cytometer operates by causing a fluid stream to pass single file through a beam of light generated by a laser. The photons of light, which are disrupted and scattered and emitted by the cells following their interaction with the laser beam, are separated into constituent wavelengths by a series of filters and mirrors (McCoy and Overton, 1994). The deflection of the beams depends upon impact of the laser beams on the cells and is divided into two categories. The light which detects the relative size of the single cells is called forward scatter (FSC) light, and the light which is deflected by 90° and thus reveals data about the inner granularity of the cells is called sideward scatter (SSC) light (http://www.bdbiosciences.com) (Fig. 4.2).

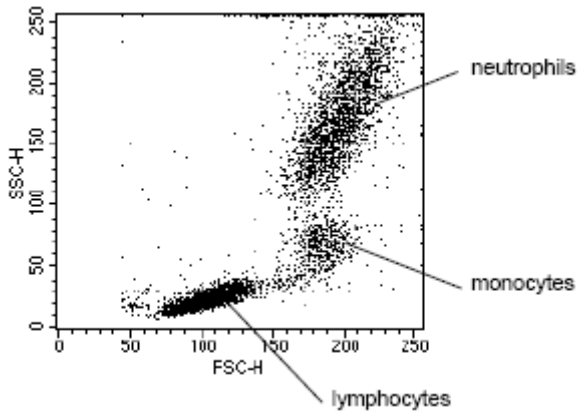


Fig. 4.2: Preliminary identification of cells based on FSC v/s SSC.
(http://www.bdbiosciences.com)

Fluorochromes

A fluorescent compound (fluorochrome) absorbs light energy over a range of wavelengths that is characteristic for that compound. This absorption of light causes an electron in the fluorescent compound to be raised to a higher energy level. The excited electron quickly decays to its ground state, emitting the excess energy as a photon of light. This transition of energy is called fluorescence (http://www.bdbiosciences.com). These methods enable, amongst other things, a quantitative investigation of the surface molecules. The basis for this is an antigen-antibody reaction conducted with fluorescently-marked antibodies which are aimed at particular surface molecules. The single cell suspensions are analyzed by flow cytometry (Fig. 4.3).

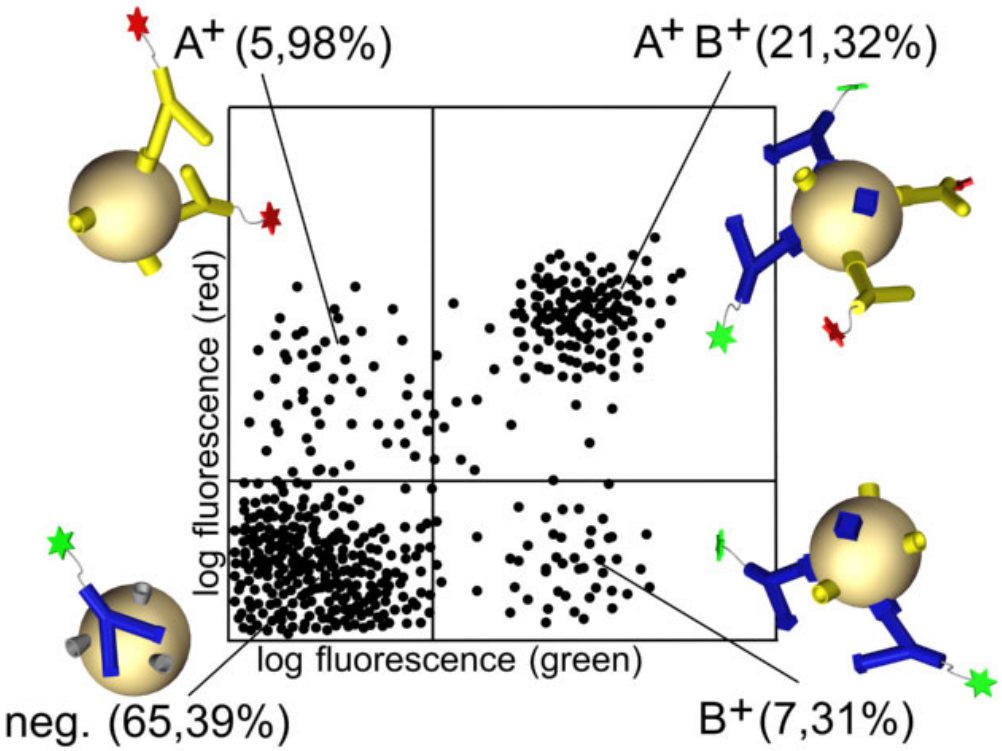


Fig. 4.3: Specific binding of fluorochrome-labeled antibody to cell surface antigens. (http://www.bdbiosciences.com)

Signal detection

Light signals are generated as particles pass through the laser beam in a fluid stream. These light signals are converted to electronic signals (voltages) by photodetectors and then assigned a channel number on a data plot. A voltage pulse is created when a particle enters the laser beam and starts to scatter light or fluoresce. Once the light signals, or photons, strike one side of the PMT or the photodiode, they are converted into a proportional number of electrons that are multiplied, creating a greater electrical current. The electrical current travels to the amplifier and is converted to a voltage pulse. The highest point of the pulse occurs when the particle is in the centre of the beam and the maximum amount of scatter or fluorescence is achieved. As the particle leaves the beam, the pulse comes back down to the baseline (http://www.bdbiosciences.com) (Fig. 4.4).

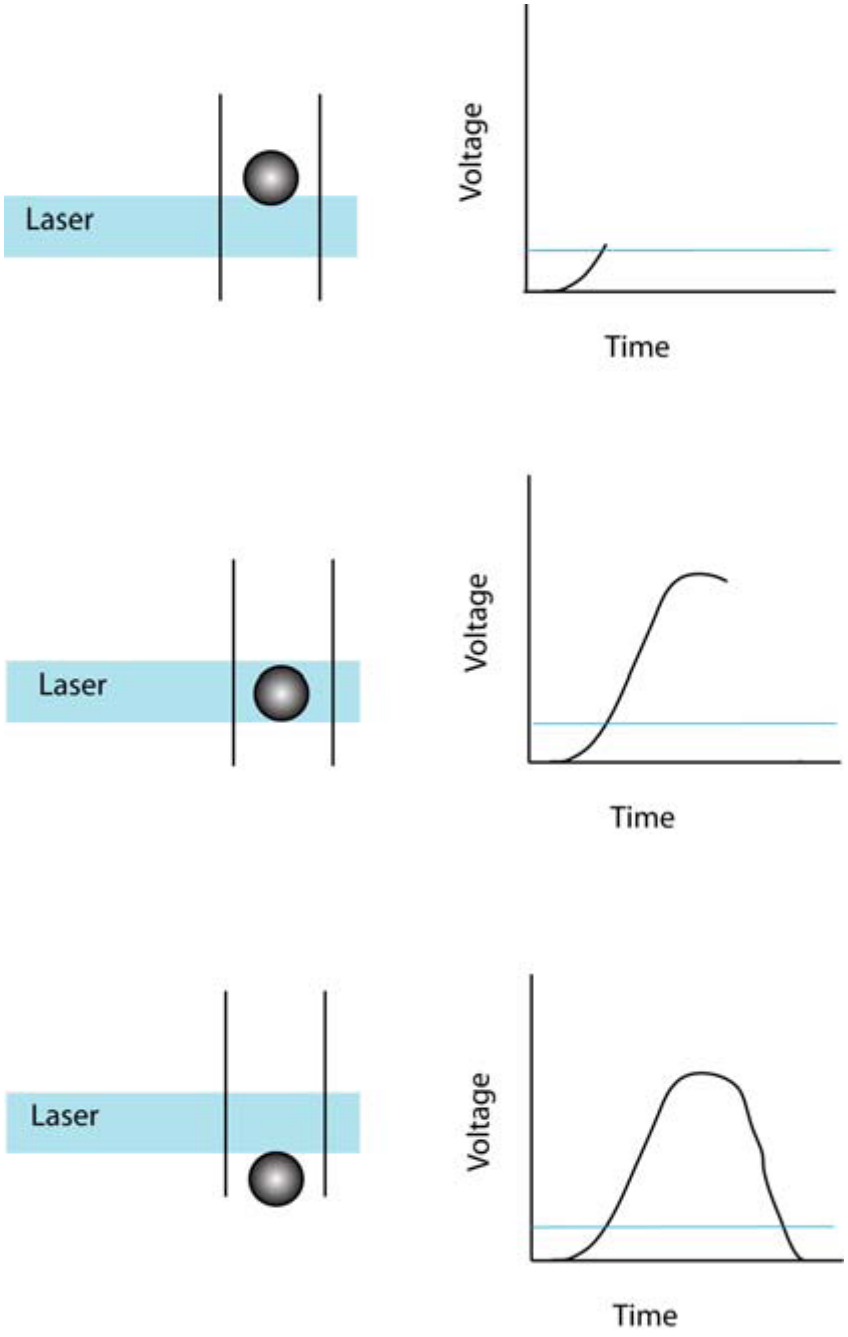


Fig. 4.4: Formation of a voltage pulse.

(http://www.bdbiosciences.com)

4.8 Polyclonal antigen-specific expansion of T cells and FACS analyses

Two overlapping peptide sets (each 85 peptides 15-18 amino acids in length) covering local consensus sequences from HCV GT1b and 3a were generated with PeptGen (available at http://hcv.lanl.gov/) and synthesized (≥ 70 % purity) together with additional twelve previously defined optimal epitopes (Table 3.9). The overlapping peptides were combined in 9 pools for each GT (9-10 peptides/pool). Fresh or crypreserved PBMCs (2 x 10^6) were count using the Trypan blue exclusion assay. The reactivity of trypan blue is based on the fact that the chromopore is negatively charged and does not interact with the cell unless the membrane is damaged. Therefore, all the cells that exclude the dye are viable (Fig 4.5). Aliquot was diluted with 0.4 % Trypan blue stain and 10 µl of the diluted aliquot solution is transferred onto the Neubauer cell counting chamber and covers lipped to be examined under microscope (at 10x) for cell counting.

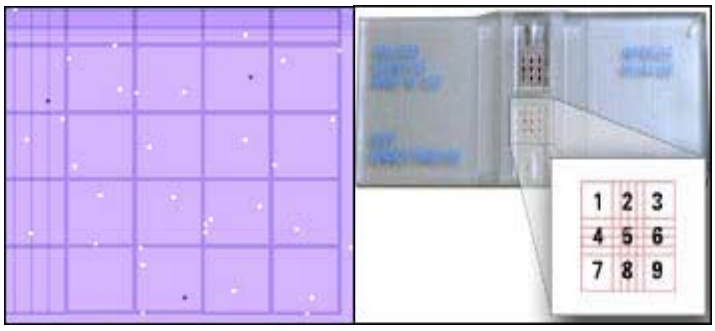


Fig. 4.5: Illustration of viable cell counting using Trypan Blue exclusion assay.

Grid with viable cells appears white and dead cells appear blue from the Trypan Blue dye. (http://www.bme.gatech.edu/vcl/Tissue_Engineering/Background/6_cell_passaging.htm)

Formula for counting number of viable cells for the total cell concentration: **Number of cells/ml = number of cells over a large square x dilution factor x 10^4**. Meanwhile, the suspensions in the tubes were centrifuged at 1700 rpm at 20°C for 7 min. The cell pellet was resuspended in 1 ml of PBMCs culture medium on a 24-well plate and stimulated with peptide pools (each peptide 1 µg/ml) or optimal defined peptides (1 µg/ml). On day 3 and 7, 1 ml of complete medium and recombinant IL-2 (2.5 µl/ml) were added. On day 10, the cells were tested for IFNγ secretion after stimulation with the same peptide pool by intracellular cytokine staining. For this purpose an aliquot of 100 µl of each cell suspension was incubated in the presence or absence of the peptide pool (10 µg/ml) and single optimal peptides (1

μg/ml) on a 96-well plate at 37°C. After 60 min 5 μl Brefeldin A were added. After 5 hours cells were washed twice in PBS containing 1% FCS and stained with 1 μl CD4-PE, 1 μl CD8-APC and 4 μl Viaprobe for 30 min at 4°C. Cells were washed once in cold PBS containing 1% FCS and incubated in Fixation/Permeabilization solution for 20 min at 4°C. Cells were then washed three times in BD Perm/Wash buffer before adding 0.25 μl IFNγ-FITC. Cells were then analyzed on the FACS Calibur. Pools were considered positive when the frequency of CD8+IFNγ+ cells in the presence of peptides was at least three times higher as the frequency of CD8+IFNγ+ cells in the absence of peptides. The individual reactive peptide in positive pools was identified the next day by re-stimulation with single peptides followed by IFNγ staining. Data were acquired from 30,000 to 60,000 lymphocyte-gated events per sample. The fluorescently-stained characteristics of the cells were measured on FACS Calibur and evaluated either with Cell quest software or FlowJo software.

4.9 DNA extraction from PBMCs

HCV-DNA was extracted from patient's PBMCs by using a QIAamp DNA Blood Mini Kit 50. As shown in the manufacturer's instructions, 200 μl of PBMCs suspension (up to 5 x 10^6 lymphocytes) were pipetted in a 1.5 ml microcentrifuge tube containing 20 μl of QIAGEN Protease. 200 μl of Buffer AL were added to the sample and incubate at 56°C for 10 min. The lysate was mixed with 200 μl of EtOH (96–100%) then transferred to the QIAamp Mini column and centrifuged for 1 min at 8000 rpm. This mixture was applied to the QIAamp Mini spin column (in a 2 ml collection tube), and centrifuged at 8000 rpm for 1 min. The column was washed with 500 μl AW1 and 500 μl AW2 buffers. The viral DNA was eluted in 200 μl Buffer AE and quantified by spectrophotometry. An aliquot of the DNA was HLA-typed using standard molecular techniques, while the rest was kept at -20°C.

4.10 Identification of cross-reactive peptides

The prediction of the optimal epitopes from each reactive response previously detected was performed utilizing the MHC class I binding predictions tool from the Immune Epitope Database and Analysis Resource (http://www.iedb.org/). The degree of cross-GT reactivity was determined by cultivating PBMCs in the presence of the GT1b and GT3a sequence of

every reactive peptide. The cells were thawed in a water bath of 37°C, transferred into a medium containing 1 ml of FBS and 10 ml of RPM 1640 ml and centrifuged for 7 min at 1700 rpm and 4°C. The pellet was rinsed again to remove the DMSO from the medium completely. As previously described (see Methods, paragraph 4.8) after 10 days both cultures were re-simulated with the GT1b and the GT3a peptide before intracellular IFNγ-staining. Lack of cross-reactivity between genotypes was assumed when no specific T cells were expanded in the presence of one peptide in at least two attempts or when the frequency of CD8+IFNγ+ T cells upon re-stimulation of the non-reactive peptide was more than 10-fold below the frequency of the reactive peptide. Full cross-GT reactivity was assumed when specific T cells were expanded in the presence of both peptides and when the frequency of CD8+IFNγ+ T cells of one peptide was less than 10-fold reduced and there was a less then 10-fold difference in the peptide concentration needed to stimulate 50% of the maximum response (SD50). Partial cross-GT reactivity was instead assumed when specific T cells were expanded in the presence of both peptides and when the frequency of CD8+IFNγ+ T cells in the presence of the highest concentration (10 μg/ml) of one peptide was less than 10-fold reduced but there was a more then 10-fold difference in the SD50. The results were than acquired by FACS Calibur and evaluated with either Cell quest software or FlowJo software.

4.11 Statistical analysis

Nonparametric Kruskal-Wallis tests, Fisher's Exact tests and *t* tests were performed using GraphPad Prism 4.0 software (GraphPad Software, San Diego, CA).

4.12 HCV serotyping

The enzyme immunoassay has been performed to distinguish antibodies to serotypes 1-6 of HCV in human plasma by using Murex HCV Serotiping kit (Abbott, Murex). As shown in the manifacturer's instructions, the plates made up of 96-well coated with HCV peptides were incubated with 10 μl of competing solution containing different peptides mixes specific for 1 to 6 serotypes. 80 μl of sample's diluents and 10 μl of sample were added to each well and incubated for 30 min at 37°C. After two washing steps 100 μl of conjugate and substrate were

added respectively and incubated for 30 min at 37°C. In order to identify the specific serotype the stop solution was added to each well and within 15 min, the plate was read at absorbance at 450 nm (A_{450}) using 690 nm as the reference wavelength.

4.13 MHC class I-Pentamers and MHC class I-Pentamer staining

ProImmune has developed Pro5^{TM} MHC Class I Pentamenrs for detecting and enumerating CD8+ single antigen-specific T cells using flow cytometry. Pro5^{TM} MHC class I-Pentamenrs bind to T cell receptors of a particular specificity, as determined by the MHC class I allele and peptide combination. MHC class I-Pentamers can readily be used to detect and separate antigen-specific T cell populations as rare as 0.02 % of lymphocytes. Pro5^{TM} MHC class I-Pentamers comprise five peptide/MHC class I complexes assembled through a coiled-coil domain. Due to their planar configuration, all five peptide/MHC class I complexes in the MHC class I-Pentamer are available for binding to complementary TCRs. Each Pro5^{TM} MHC class I-Pentamer also comprises a PE fluorescent tag for bright and efficient labelling (Fig.4.6). Pentamers bind to T cells that express T cell receptors specific for the cognate peptide/MHC class I complex and can therefore be used to track antigen-specific T cells by flow cytometry. For our purpose an aliquot of 100 µl of each cell suspension was washed with FACS buffer incubated with the HLA-A2 peptide-specific MHC class I-Pentamer PE labeled on a 96-well plate at RT for 10 min. Cells were washed twice again in FCS buffer and stained with 1µl anti-CD8-APC and 2 µl Viaprobe for 30 min at 4°C. The cells were then acquired and analyzed by flow cytometry.

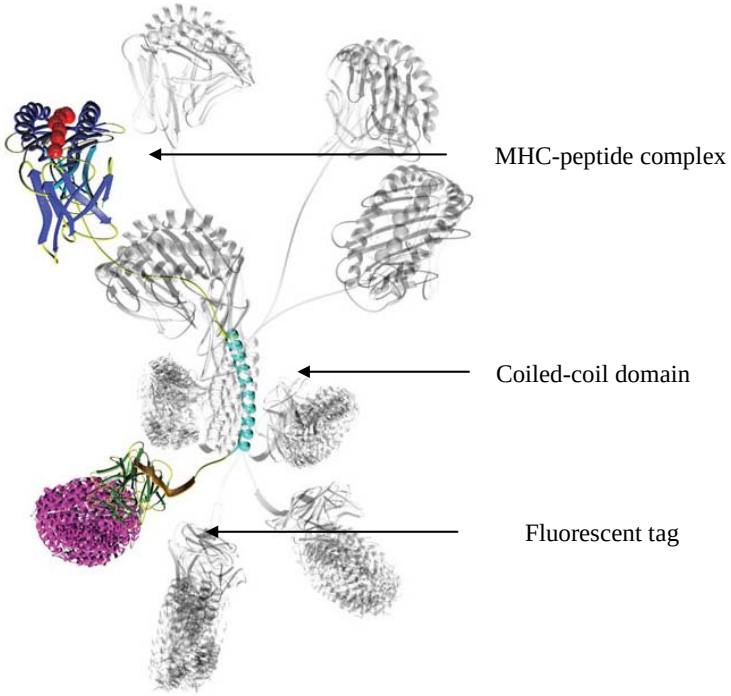


Fig. 4.6: Schematic representation of MHC class I-Pentamer components.
(http://www.proimmune.com/ecommerce/htlm/pentamer_info/wbs%201_size6.jpg)

4.14 Phenotypical analysis of antigen-specific T cells

Total PBMCs were thawed (see Methods, paragraph 4.10) and stained with MHC class I-Pentamer as described in the previous paragraph. The *ex vivo* CD127 expression on peptide-specific CD8+ T cells was determined staining total PBMCs with CD127-FITC antibody (30 min at 4°C). The expression of the pro-apoptotic protein Bim was determined *ex vivo* and after 7 days of peptide-specific stimulation (see Methods, paragraph 4.8) staining peptide-specific CD8+ T cells with the rat anti-human $Bim_{S/EL/L}$ antibody (1h at RT) and with the anti-Rat IgG2-FITC antibody (30 min at RT). To evaluate the proliferation capacity of peptide-specific CD8+ T cells the percentage of MHC class I-Pentamer positive cells obtained after 7 days of peptide-specific stimulation was compared to the percentage of cells detected directly *ex vivo*.

4.15 AutoMACS Pro technology for enrichment of monocytes

The AutoMACS Separation system was used for the purification of monocytes from PBMCs of healthy HLA-A2 buffy coats recruited from the Transfusion Department, Rhine State Hospital Essen, University of Duisburg-Essen. Using the magnetic activated cell sorting (MACS) technology, monocytes were labeled with super paramagnetic Micro Beads (Miltenyi Biotec) coupled with CD14 and purified by immunomagentic separation on autoMACS columns.

Principle

MACS® Technology is based on MACS Micro Beads, MACS Separators, and MACS Columns. MACS Micro Beads are super paramagnetic particles of approximately 50 nm in diameter. They are composed of a biodegradable matrix, and it is therefore not necessary to remove them from cells after the separation process. Usually, MACS Micro Beads do not alter structure, function, or activity status of labeled cells and are not known to interfere with subsequent experiments. Separation of cells using MACS technology takes place inside the columns. When a MACS Column is placed in a MACS Separator, a strong permanent magnet, a high-gradient magnetic field is induced on the column matrix, which is enough to retain cells labeled with minimal amounts of MACS Micro Beads. Unlabelled cells (negative fraction) are eluted out while labeled cells (positive fraction) bound to the column are released after removal of the column from the magnet. Thus, with MACS Technology both labeled and unlabeled cell fractions can easily be isolated with high purity. The entire procedure of positive selection or depletion takes less than 20 min, and cells can immediately be used for further experiments (http://www.miltenyibiotec.com) (Fig 4.7).

Cryopreserved PBMCs isolated from a buffy coat of a healthy HLA-A2 donor were washed three times with R1640 and counted in order to have $2x10^7$ cells in 160 µl; these cells were then incubated with 40 µl of CD14 Microbeads for 15 min at 4°C. After a washing step the cells were resuspended in 500 µl of MACS buffer and the monocytes were separated using the autoMACS Pro facilities. The negative and positive fractions harvested and aliquots of both samples analyzed by flow cytometry to evaluate the purity of the fractions.

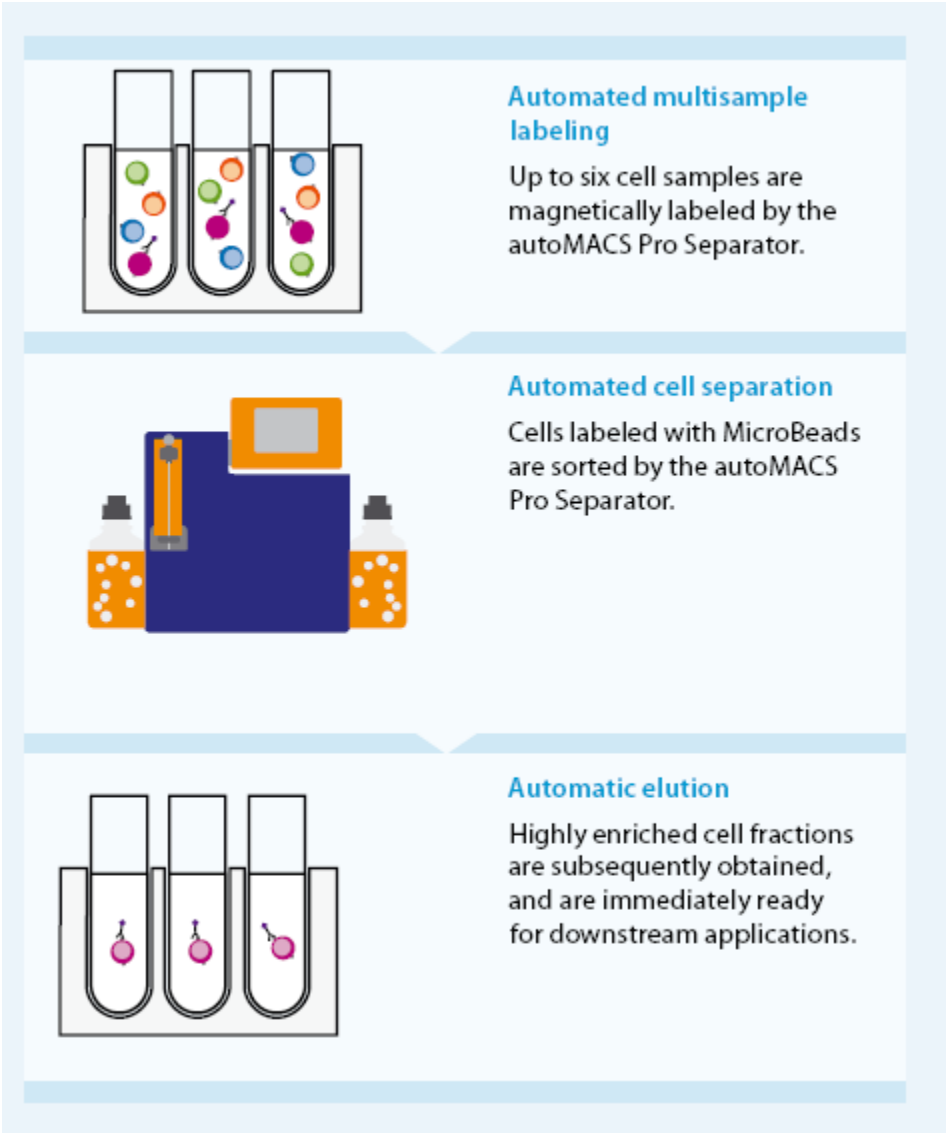


Fig. 4.7: Purification of positive fraction of cells by direct magnetic labeling using autoMACS technology.
(http://www.miltenyibiotec.com)

4.16 Monocyte maturation

1.5 cells/ml were cultured in DCs maturation medium for two days in a 6-well plate at 37°C in a 5% CO_2 incubator. An additional 2000/ml of IL-4 and GM-CSF were added on day 2. To induce DCs maturation at day 3 a maturation mixture was added containing LPS 100 ng/ml and IFNγ 1000 UI/ml. After 48 hours the cells were harvested, washed once with R1640 and a sample of 100 ml was stained with CD80-FITC 30 min 4°C to verify the expression of the co-stimulatory molecule specifically up-regulated in matured DCs. Flow cytometry acquisition

was performed using FACS Calibur and evaluated either with Cell quest software or FlowJo software.

4.17 Activation of memory T cells and priming of naïve peptide-specific T cell lines

Two sets of triplicate round-bottom microtiter 96-well were used to activate memory peptide-specific CD8+ T cells. In one set of wells we plated 200,000 PBMCs of a healthy HLA-A2 donor, in the other one we co-cultured (200,000) PBMCs with 20,000 autologous DCs to verify the activation capacity of monocyte-derived DCs. Both cultures were incubated with or without 1 μg/ml of the HLA-A2 peptide of interest to have a positive and negative control of the peptide-specific response. The cells were cultured in 150 μl of co-culture medium containing IL-2 (50 UI/ml), IL-7 (5 ng/ml) and IL-15 (5 ng/ml). At day 4 fresh co-culture medium (100 μl) and cytokines (IL-2 50 UI/ml, IL-7 5 ng/ml, IL-15 5 ng/ml) were added to both cultures using the same concentrations previously described. The cultures were maintained for a total of 10 days adding each two days new IL-2, IL-7 and IL-15 always at the same concentrations. After this time period new DCs (20,000 cells) were added only to the co-culture following the same procedure previously described. After a total of 20 days of culture all cells were harvested and tested by IFNγ to identify the activation of memory peptide-specific response. The priming of naïve peptide-specific CD8+ T cells was performed culturing PBMCs of a healthy HLA-A2 donor with autologous monocytes-derived DCs as previously described. The priming of naïve CD8+ T cell lines was analysed by IFNγ and MHC class I-Pentamer staining. All the data were than acquired by FACS Calibur and the results evaluated by FlowJo software.

5 Results:

5.1 Identification of the sequence differences between HCV GT1 and GT3

IVDUs are the population subgroup at greatest risk of HCV infection in more developed countries, with prevalence rates of 45% (Judd *et al.*, 2005) and incidence rates of 25% per year (Maher *et al.*, 2006). One characteristic of European IVDUs cohorts is the heterogenic GT distribution. In the present study we recruited anti-HCV positive individuals (by CMIA) in collaboration with the Department of Addictive Behaviour and Addiction Medicine, Rhine State Hospital Essen. In all patients the GT was determined by PCR and sequence analysis. After analysis of 211 patients we detected that 32% were infected with GT1 (50 GT1a and 12 GT1b) and 22% were infected with GT3a. Seven additional patients were infected with GT4 and one with GT2c. In 41% of the patients no HCV-RNA was detectable indicating that these patients spontaneously control viral replication (Fig. 5.1). Being highly susceptible to reinfection, this subgroup represents a perfect model for studying the impact that multiple exposures to different genotypes may have on the immune system.

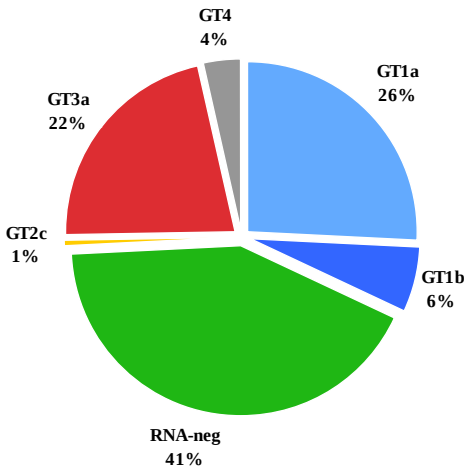


Fig. 5.1: GT distribution in IVDUs cohort from Essen.

The inherent sequence diversity of HCV possibly represents one of the major obstacles for immune control (Simmonds, 2004). Complete genomes from different genotypes differ in about 30-35% of the nucleotide sites over the complete genome (Simmonds, 2000). Hence, the first purpose of this study was to determine the sequence differences between GT1 and 3

which represent the predominant genotypes in IVDUs from Essen. The work was performed focusing on the NS3 protein of HCV. NS3 is the protease/helicase protein of the virus and is recognised as an important target for CD8+ T cell response during acute and chronic HCV infection (Diepolder *et al.*, 1995).

Complete NS3 of a total of 42 HCV GT3a isolates and a total of 44 HCV GT1b isolates was amplified and sequenced. A GT1b and a GT3a consensus sequence was constructed based on the most frequent amino acid in each position of the protein (Fig. 5.2). In this comparison, 108 of all 631 (17.1%) residues of NS3 differed between the GT1b and GT3a consensus sequence.

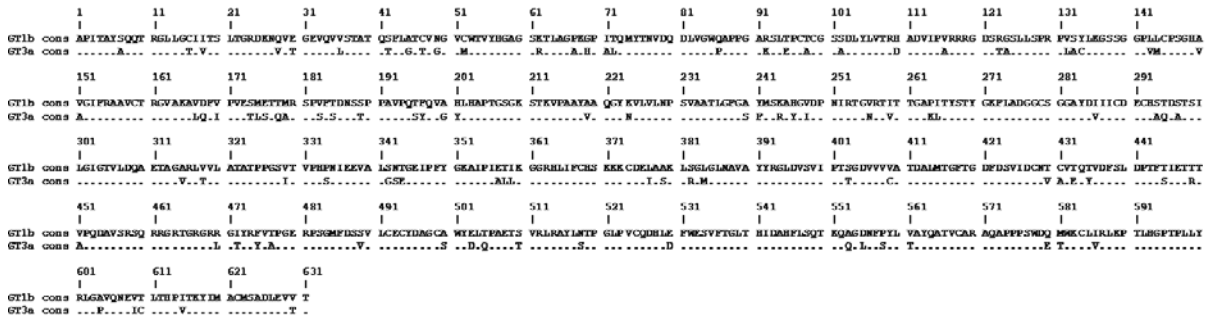


Fig. 5.2: NS3 GT1b consensus sequence aligned to NS3 GT3a consensus sequence.

To better figure out how this GT-specific sequences could affect the CD8+ T cell responses the amino acids differences were analyzed in the range of 9mers, considering that as the optimal length for CD8+ T cell epitopes. Only 22% of all 9mers were identical between both consensus sequences, 78% of these 9 amino acid windows harboured at least one amino acid difference and 48% at least two amino acids differences (Fig. 5.3), indicating that differences in targeted epitopes between HCV GT1b and GT3a may be common.

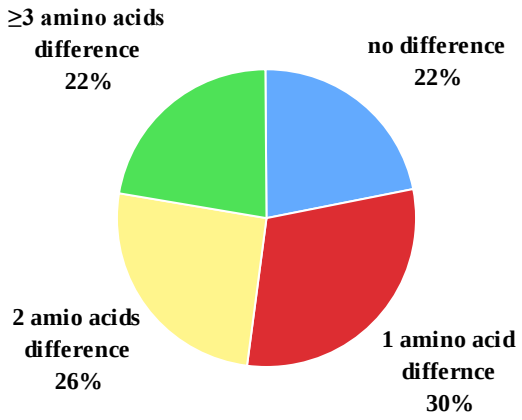


Fig. 5.3: Comparison of a GT1b and a GT3a consensus sequence.

Comparison of a GT1b (based on 44 isolates) and a GT3a consensus sequence (based on 42 isolates). The frequency of 9 amino acid windows with the indicated number of differences is given in percent.

An example of the GT-specific sequence variability is shown in figure 5.4. The epitopes here shown are two previously described HLA-A2 restricted CD8 epitopes which are frequently targeted in patients with acute and chronic HCV-infection. The prototype sequence of the epitope is shown on top. GT1a sequences randomly taken from a public database and the GT1b and GT3a sequences amplified from our cohort were aligned to the prototype epitope sequence to address the polymorphisms in these two regions. Both epitopes were overall highly conserved within each subtype indicating that the sequence differences observed are GT-specific and not the consequence of escape-mutation. In both cases the GT3a and the GT1b sequences differed from the GT1a reference sequence (H77) suggesting that even subtype-specific differences are potentially relevant.

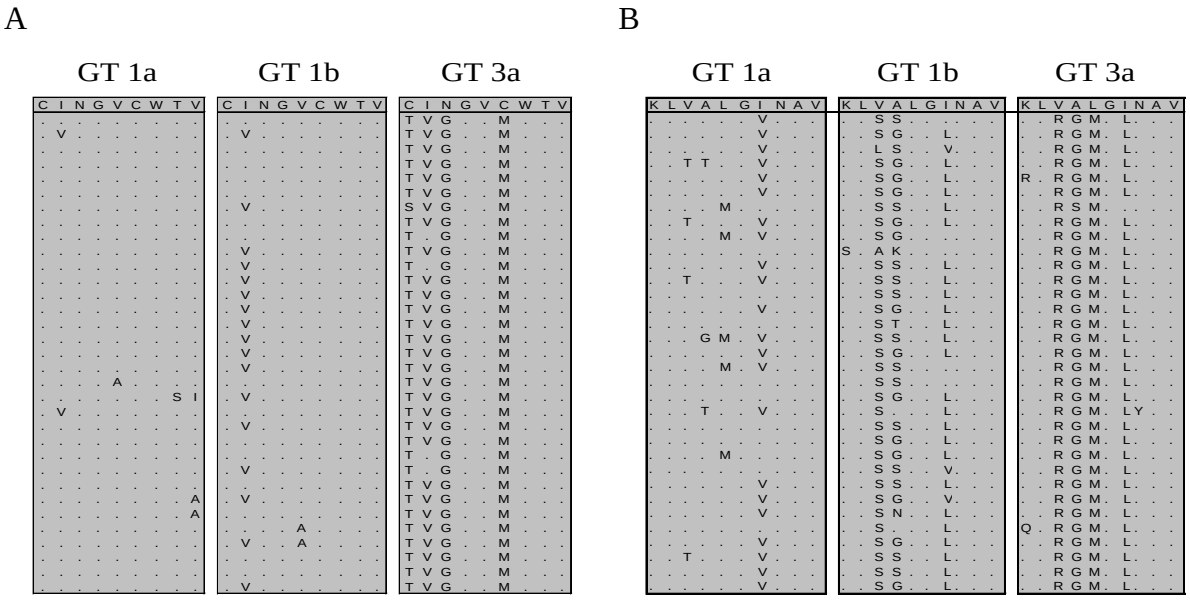


Fig. 5.4: Example of GT-specific sequence variability.

The reference sequence H77 of two immunodominant HLA-A2 epitopes (A: 1073-1081 and B: 1406-1423) was aligned to GT1a sequences taken from a public database and to GT1b and 3a sequences taken from our IVDUs cohort. Differing amino acids from the reference sequence are highlighted.

In this work we focused on the relevance that high sequence variability between GT1 and GT3 have in T cell immunology.

5.2 Impact of GT-specific sequence differences on the CD8+ T cell response

There are substantial sequence differences between GT1 and GT3 even in described CD8 epitopes. The relevance of these sequence differences on CD8+ T cells is unknown. We decided to focus the analysis on a subgroup of 53 patients from our cohort. Patients infected with GT1 and GT3 as well as HCV-RNA negative patients were included. Patients infected with GT2a were excluded from the analysis. To address this we utilized overlapping peptide sets (each 85 peptides 15-18 amino acids in length) covering our local consensus sequences from HCV GT1b and 3a generated with PeptGen (available at http://hcv.lanl.gov/) and synthesized (≥ 70% purity) together with additional 12 previously defined optimal epitopes (see: Materials, Table 3.9). The subgroup of 53 subjects included 14 subjects with undetectable viremia, 17 subjects with HCV GT1 infection and 22 subjects with HCV GT3

infection (Table 5.1). Although seven of 14 (50%) of the HCV-RNA negative subjects were not typeable with a HCV serotyping assay, serotype 1 was detected in four subjects, serotype 3 in two subjects and a mixed GT1/GT3 serotype in 1 additional subject, indicating heterogeneous GT exposure also in this group (Table 5.1).

Table 5.1: Cohort characteristics.

	GT1	GT3	HCV-RNA-neg°	
N	17	22	14	
male/female	12/5	15/7	10/4	
age in years, mean (SD)	39.5 (8.3)	38.6 (8.6)	43.9 (6.0)	n.s.
median HCV VL in IU/mol (interquartile range)	132.783 (52.739-1.215.000)	262.400 (56.095-1.302.000)	-	n.s.
GPT in IU/ml, mean (SD)	129.3 (35.6)	113.5 (21.2)	44.8 (17.9)	$p < 0.05$[1]
Serotype[2]	14 x GT1	15 x GT3	4 x GT1	
	1 x GT4	2 x GT1	2 x GT3	
	2 x NT[3]	1 x GT2	1 x mixed GT1/GT3	
		1 x mixed GT1/GT3	7 x NT[3]	
		3 x NT[3]		

°Versant Transcription-mediated amplfication-Test negative
[1]GT1 vs. HCV-RNA negative and GT3 vs. HCV-RNA negative (Kruskal-Wallis-Test)
[2]Serotyping was performed with the Murex HCV Serotyping Assay
[3]NT: not typable

To analyse the immune responses of the cohort PBMCs were isolated from each subject, cultured and stimulated with peptide pools (each peptide 1 μg/ml) or optimal defined peptides (1 μg/ml). On day 10, the cells were tested for IFNγ secretion after stimulation with the same peptide pool by intracellular cytokine staining. An aliquot of each cell suspension was incubated in the presence or absence of the peptide pool (10 μg/ml). Pools were considered positive when the frequency of CD8+IFNγ+ cells in the presence of peptides was at least three times higher as the frequency of CD8+IFNγ+ cells in the absence of peptides. The individual reactive peptide in positive pools was identified the next day by restimulation with single peptides (10 μg/ml) followed by IFNγ staining. An example is shown in figure 5.5, after 10 days of in vitro culture the chronically infected patient C73 was tested positive for pool 9 with 9.30% of CD8+ T cells secreting IFNγ upon restimulation. In order to identify the single reactive peptide, T cells were restimulated the next day with each single peptide included in pool 9. Peptide 82 was identified as the one responsible for the IFNγ production with a 14.54% of CD8+ T cells secreting IFNγ. This approach was applied for the screening of each response in order to identify all the reactive peptides. Step necessary for the further identification of the single optimal epitopes.

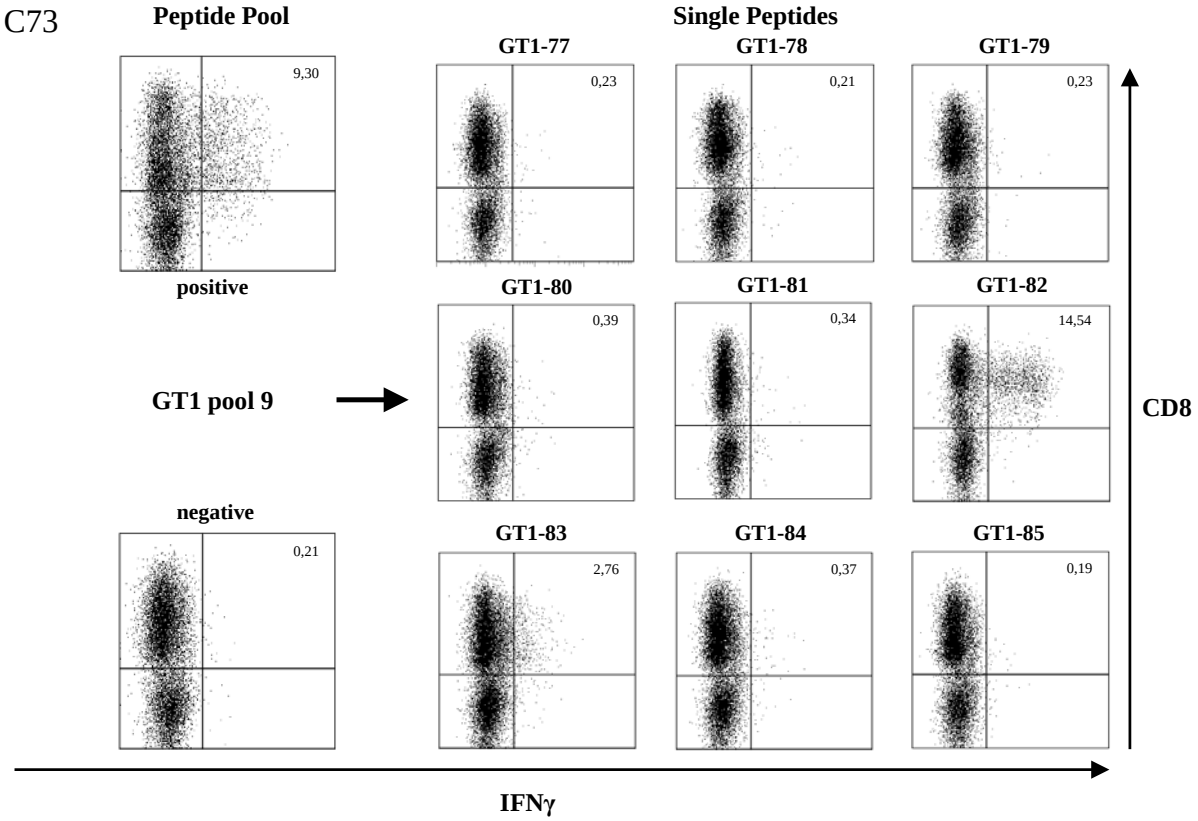


Fig. 5.5: Example of the approach used for the detection CD8+ T cells responses.

Antigen-specific T cells were expanded *in vitro* in the presence of peptide pools for 10 days before restimulation with the same peptide pool and staining for IFNγ. The reactive peptide in positive pools was determined the next day by restimulation with individual peptides. Here peptide 82 was considered as being reactive.

After screening with the overlapping peptide sets a total of 27 individual epitopes was identified (one additional epitope was only detected with an optimal peptide) corresponding to a total of 57 CD8 responses (Table 5.2).

Table 5.2: List of the identified responses.

									CD8 epitopes detected with overlapping peptides				additional optimal			
	ID	age	gender	genotype	serotype	VL*	ALT	HLA	GT1b cons sequence	strength	GT3a cons sequence	strength	prototype sequence	strength	GT3a sequence	strength
GT1	21	41	male	1a	1	1,278,890		A1,3 B7,8					ATDALMTGY .....F	3,98	ATDALMTGF	-
	23	45	male	1b	1	59	27	A2,29 B15,44								
	24	43	male	1a	1	206	337	A1,3 B8,27								
	30	46	female	1b	NT	1,777,000	23	A1,3 B8,35								
	34	38	male	1a	1	133		A2, B40,51								
	43	47	female	1a	4	3	69	A1,3 B7,8								
	46	50	male	1a	1	1,674,380	187	A2,25 B7,39								
	57	29	male	1a	1	1,580,680		A3,30 B7,13								
	68	28	female	1a	1	1,064	448	A2,32 B15,44					CVNGVCWTV .I..... KLSGLGLNAV ..VA..V...	8,95 0,68	TVGGVMWTV CIG..M... KLRGMGLNAV ..ALG.....	- -
	73	43	male	1b	NT	2	18	A24,30 B13,55	RLGAVQNEV .....A	8,3	RLGPVQNEI ...A....A	23,42				
	74	45	male	1b	1	179		A1, 24 B8					ATDALMTGY .....F	5,47	ATDALMTGF	-
	78	29	male	1b	1	83	105	A2,32 B35,41	VTPGERPSGMFDSSVL	-	VAPGERPSGMFDSVVL .T.....S..	1,29	HPNIEEVAL .S..... KLSGLGLNAV	- 2,56	HSNIEEVAL ..... KLRGMGLNAV ..S.L.....	3,8 -
	83	33	female	1b	1	47	46	A2,25 B7,44								
	84	49	female	1b	1	62	31	A1,2 B40,51					KLSGLGLNAV .....I...	12,91	KLRGMGLNAV ..S.L.....	-
	85	33	male	1a	1	499	122	A2 B7,15	GPKGPITQMYTNVDQDLV SS...VI.....	1,03	GAKHPALQMYTNVDQDLV SS.H.VI.....	-	KLSGLGLNAV .....V...	1.41	KLRGMGLNAV ..VAL.V...	-
	86	48	male	1b	1	1,150,150	118	A29,32 B40,55								
	89	25	male	1a	NT	65	22	A3,30 B15,39								
GT3	11	35	male	3a	3	<615	45	A3,26 B39,40	VDFVPVESM LQ.I...TL ELTPAETSVRLRAYL D.Q....T.....	5,2 0,5	LQFIPVETL ..... DLQPAETTVRLRAYL	- -				
	18	32	male	3a	3	440	48	A3,24 B35								
	19	52	male	3a	2	779	132	A2 B39,52					CVNGVCWTV T.G..M...	5,62	TVGGVMWTV	-
	26	34	male	3a	NT	<615	135	A1,11 B57								
	28	38	male	3a	3	95		A1,3 B7,44					ATDALMTGY .....F	0,31	ATDALMTGF	-
	32	56	male	3a	3	3,507,000	261	A2 B18								
	36	51	male	3a	3	1,284,000	44	A3,24 B15,55								
	39	33	female	3a	3	1	69	A2,11 B15,35								
	44	43	female	3a	3	1,536,000	80	A2,24 B40,51								
	59	40	male	3a	3	601	329	A3,24 B7,35								
	60	53	female	3a	NT	85		A3,33 B35,38								
	63	42	male	3a	1	28	30	A2 B7,40					CVNGVCWTV T.G..M... KLSGLGLNAV ..RGM.....	11,01 1,75	TVGGVMWTV ..... KLRGMGLNAV	- -
	64	32	male	3a	3;1	2,387,000		A3,24 B15,35								
	65	36	female	3a	3	231		A2,24 B39,40								
	67	33	female	3a	3	20		A2,68 B38,51	GKSTKVPAAYAAQGYKVL .....V...N.. IPFYGKAI V.....	3,23 9,64	GKSTKVPAAYVAQGYNVL .....A..... IPFYGKAI V.....	- 9,98	CVNGVCWTV T.G..M...	4,05	TVGGVMWTV	-
	70	35	male	3a	3	293,7		A2,24 B18,37	FLATCVNGVCWTVYHGA ..G.T.G..M.....	5,35	FLGTTVGGVMWTVYHGA	-				
	71	40	male	3a	3	1,320,000		A2,3 B7,15								
	76	40	female	3a	3	2,009,000	240	A2,31 B39,40	EFWESVFTGLTHIDAHFL D.....	2,88	DFWESVFTGLTHIDAHFL	4,09	CVNGVCWTV T.G..M...	8,11	TVGGVMWTV	-
	77	27	female	3a	1	222		A2 B44					CVNGVCWTV T.G..M...	6,83	TVGGVMWTV	-
	79	28	male	3a	NT	341		A2 B8,13								
	88	25	male	3a	3	93		A2,32 B51,55					CVNGVCWTV T.G..M...	5,18	TVGGVMWTV	-
	90	43	male	3a	3	189		A2 B15,40								
RNA neg	9	44	male	neg	3;1	-	7	A23 B15,40								
	10	37	male	neg	3	-	21	A30,31 B8,35	GKSTKVPAAYAAQGYKVL AATLGFGSFMSRAYGI	- -	GKSTKVPAAYVAQGYNVL AATLGFGSFMSRAYGI	0,45 8,42	HPNIEEVAL	0,41	HSNIEEVAL	-
	12	44	female	neg	NT	-	68	A2,3 B13,15	RLGAVQNEV VTLTHPITK	31,92 11,51	RLGPVQNEI ICLTHPVTK	52,22 -				
	13	41	female	neg	NT	-	236	A1,3 B7,13	HLHAPTGSGK GFGAYMSKAH KLSALGLNAVAYYRGLDV	12,68 - -	YLHAPTGSGK GFGSFMSRAY KLRGMGLNAVAYYRGLDV	- 20,12 5,2				
	14	42	female	neg	NT	-	19	A2,30 B13,44	GVDPNIRTGV SWDQMWKCLIRLKPTLH RLGAVQNEV	12,38 1,12 51,85	GIDPNIRTGN SWDETWKCLVRLKPTLH RLGPVQNEI	5,66 - 38,95	KLSGLGLNAV	0,44	KLRGMGLNAV	-
	29	47	male	neg	1	-	33	A2,11 B27,44	TLGFGAYMSK VTLTHPITK GRGRRGIYRF	3,73 5,43 -	TLGFGSFMSR ICLTHPVTK GRGRLGTYRY	- - 14,99	CVNGVCWTV	28,87	TVGGVMWTV	-
	37	38	male	neg	NT	-	19	A33,68 B40,44	HAVGIFRAAV	13,38	HVAGIFRAAV	7,56				
	40	49	male	neg	1	-	30	A2 B27,44					CVNGVCWTV	0,79	TVGGVMWTV	-
	42	48	male	neg	1	-	14	A2 B14,51	GVDPNIRTGV IPFYGKAI	2,05 11,9	GIDPNIRTGN IPFYGKAI	- 10,83	CVNGVCWTV	14,06	TVGGVMWTV	-
	58	50	male	neg	NT	-		A3,26 B7,38	GKAIPIEVIKGGRHLIF VTLTHPITK	1,27 2,88	GKAIPIALLKGGRHLIF ICLTHPVTK	- -				
	61	39	female	neg	NT	-	31	A2,33 B7,14								
	62	43	male	neg	NT	-		A1,2 B40,51	IPFYGKAI	11,95	IPFYGKAI	18,52	CVNGVCWTV	8,24	TVGGVMWTV	-
	66	57	male	neg	3	-	33	A1,3 B8,35	LIRLKPTL	39,51	LVRLKPTL	43,89	ATDALMTGY HSKKKCDEL HPNIEEVAL	29,26 62,5 31,47	ATDALMTGF HSKKKCDEI HSNIEEVAL	- 59,38 11,95
	82	35	male	neg	1	-	27	A2 B35,39					CVNGVCWTV KLSGLGLNAV	2,64 1,69	TVGGVMWTV KLRGMGLNAV	- -

ınt HCV-RNA 3.0 assay (bDNA) with a limit of detection of 615IU/ml

HCV-specific T cells were substantially more frequent in HCV-RNA negative subjects compared to HCV-RNA positive subjects. HCV-specific CD8+ T cells were detected in seven of 17 (41.2%) subjects infected with GT1, in nine of 22 (40.9%) subjects infected with GT3 and 12 of 14 (85.7%) subjects with undetectable viremia. The total strength and number of HCV-specific T cell responses identified with overlapping peptides was significantly higher in the HCV-RNA negative group when compared to the GT1 or GT3 infected groups ($p<0.01$). What we also observed was the lack of difference between the strength and magnitude of the T cell response directed against GT1 or GT3 peptides in HCV-RNA negative subjects. In the subjects with GT1 or GT3 infection there was a trend towards preferential targeting of the heterologous GT, although this trend was statistically not significant (Fig. 5.6).

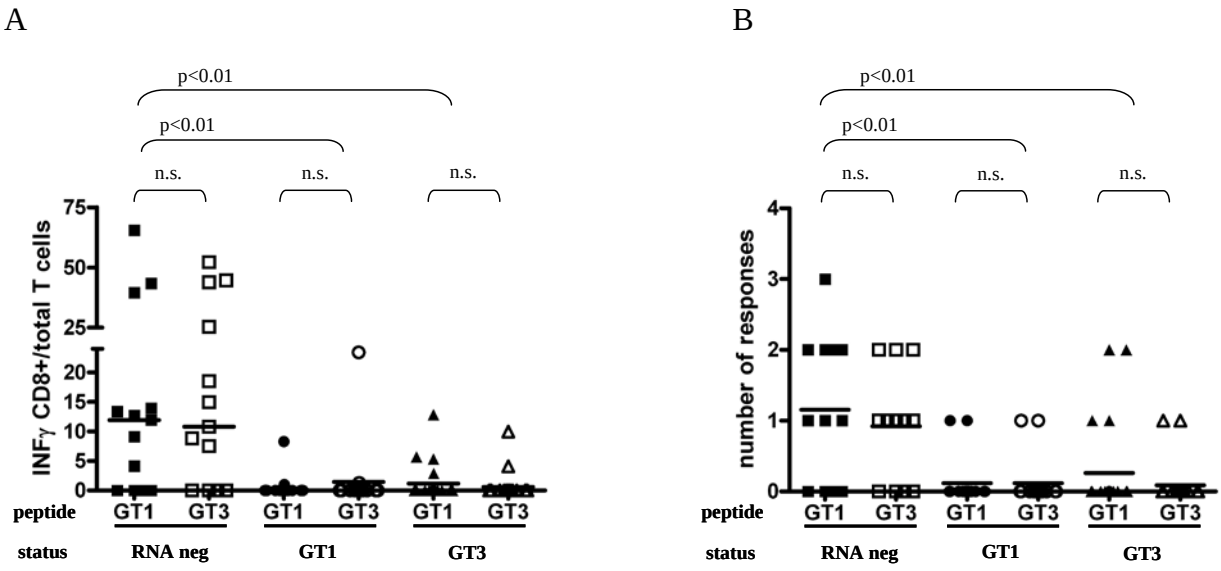


Fig. 5.6: Strength and magnitude of the CD8 response in each subject of the IVDUs cohort.

(A) The relative frequency of HCV-specific T cells and (B) the number of CD8 epitopes detected with overlapping peptides (GT1, closed symbols; GT3, open symbols) after 10 days of *in vitro* expansion in the presence of peptide pools is shown. Horizontal lines represent the median. P values for the comparison between GT1 and GT3 peptides are indicated (Kruskal-Wallas test with Dunn's post test).

5.3 Analysis of the degree of cross-GT reactivity of CD8+ T cell epitopes

One of the first goals of this work was to identify cross-reactive peptides between GT1 and GT3. The idea was to identify interesting targets that could be used in the development of a broad protective vaccine. For this purpose all individual responses were confirmed in a second series of cultures utilizing cryopreserved PBMCs and fine-mapped if possible based on predicted motifs within the reactive peptide matching the subject's HLA-type. The prediction of the optimal epitopes was performed utilizing the MHC class I binding predictions tool from the Immune Epitope Database and Analysis Resource (http://www.iedb.org/). The degree of cross-GT reactivity was determined by cultivating PBMCs in the presence of the GT1b and GT3a sequence of every reactive peptide as described in the previous paragraph. After 10 days both cultures were re-simulated with the GT1b and the GT3a peptide before intracellular IFNγ-staining.

Lack of cross-reactivity between genotypes was assumed when no specific T cells were expanded in the presence of one peptide in at least two attempts or when the frequency of CD8+IFNγ+ T cells upon restimulation of the non-reactive peptide was more than 10-fold below the frequency of the reactive peptide. Full cross-GT reactivity was assumed when specific T cells were expanded in the presence of both peptides and when the frequency of CD8+IFNγ+ T cells of one peptide was less than 10-fold reduced and there was a less then 10-fold difference in the SD50. Partial cross-GT reactivity was instead assumed when specific T cells were expanded in the presence of both peptides and when the frequency of CD8+IFNγ+ T cells in the presence of the highest concentration (10 μg/ml) of one peptide was less than 10-fold reduced but there was a more then 10-fold difference in the SD50.

Among the 28 epitopes that have been identified in our CD8+ T cell response analysis, only three showed an identical consensus sequence between the two genotypes (Table 5.3).

Table 5.3: List of peptides detected in this study.

position	restriction	GT1b consensus sequence	GT3a consensus sequence	times detected	reactive sequence
1069-1085	n.d.	FLATCVNGVCWTVYHGA	FLGTTVGGVMWTVYHGA	1	1b
1073-1081	A2	CVNGVCWTV	TVGGVMWTV	12	1b
1092-1109	n.d.	GPKGPITQMYTNVDQDLV	GAKHPALQMYTNVDQDLV	1	1b
1175-1184	A68	HAVGIFRAAV	HVAGIFRAAV	1	1b, 3a
1193-1201	B40	VDFVPVESM	LQFIPVETL	1	1b
1227-1236	A3	HLHAPTGSGK	YLHAPTGSGK	1	1b
1235-1252	n.d.	GKSTKVPAAYAAQGYKVL	GKSTKVPAAYVAQGYNVL	1	3a
1235-1252	n.d.	GKSTKVPAAYAAQGYKVL	GKSTKVPAAYVAQGYNVL	1	1b
1259-1274	n.d.	AATLGFGSFMSRAYGI	AATLGFGSFMSRAYGI	1	3a
1261-1270	A11	TLGFGAYMSK	TLGFGSFMSR	1	1b
1265-1274	n.d.	GAYMSKAHGV	GSFMSRAYGI	1	3a
1273-1282	A2	GVDPNIRTGV	GIDPNIRTGN	3	1b (3a)
1359-1367	B35	HPNIEEVAL	HSNIEEVAL	3	1b (3a)
1373-1380	B51	IPFYGKAI	IPFYGKAI	3	1b, 3a
1377-1393	n.d.	GKAIPIEVIKGGRHLIF	GKAIPIALLKGGRHLIF	1	1b
1395-1403	B8	HSKKKCDEL	HSKKKCDEI	1	1b, 3a
1406-1415	A2	KLSGLGLNAV	KLRGMGLNAV	7	1b
1406-1423	n.d.	KLSALGLNAVAYYRGLDV	KLRGMGLNAVAYYRGLDV	1	3a
1436-1404	A1	ATDALMTGF	ATDALMTGF	4	1b, 3a [a]
1487-1501	B27	GRGRRGIYRF	GRGRLGTYRY	1	3a
1502-1517	n.d.	VTPGERPSGMFDSSVL	VAPGERPSGMFDSVVL	1	3a
1529-1543	n.d.	ELTPAETSVRLRAYL	DLQPAETTVRLRAYL	1	1b
1556-1573	n.d.	EFWESVFTGLTHIDAHFL	DFWESVFTGLTHIDAHFL	1	1b, 3a [b]
1603-1619	n.d.	SWDQMWKCLIRLKPTLH	SWDETWKCLVRLKPTLH	1	1b
1611-1618	B8	LIRLKPTL	LVRLKPTL	1	1b, 3a
1627-1635	B13	RLGAVQNEV	RLGPVQNEI	3	1b, 3a
1635-1643	A3	VTLTHPITK	ICLTHPVTK	2	1b
1636-1643	A11	TLTHPITK	CLTHPVTK	1	1b

[a] consensus sequence in both genotypes is not reactive, this epitope was only detected with described optimal peptide (ATDALMTGY)

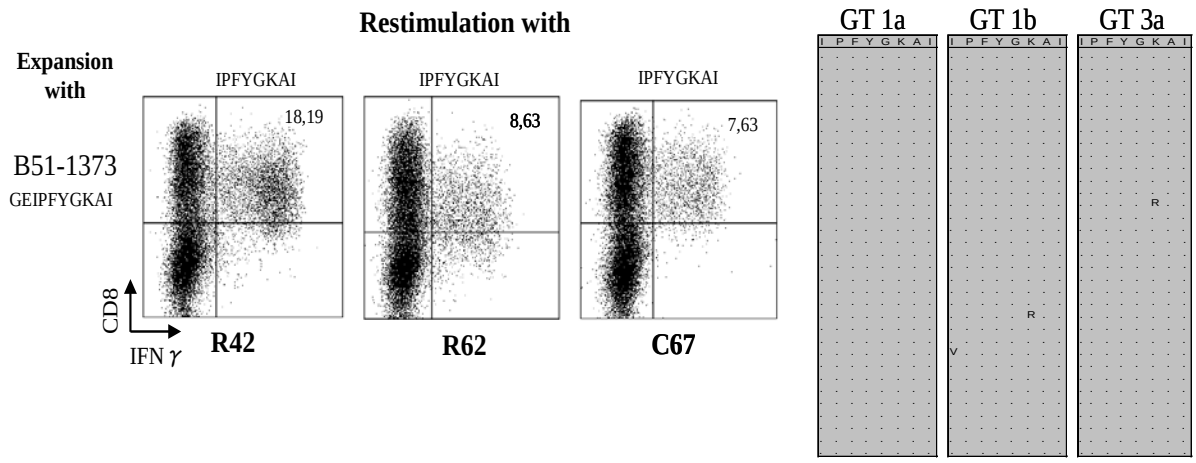
[b] the reactive 18mer has one amino acid difference between GT1b and GT3a at the N-terminal position, the peptide was considered identical in the analysis

5.3.1 Fully cross-GT reactive CD8+ T cell responses

Two of the three epitopes that showed an identical consensus sequence between GT1 and GT3 induced cross-reactive responses: the optimal peptide targeting the region 1373-1380 (this epitope has been described as HLA-B51 restricted epitope by Yerly *et al.*, 2008) and the peptide sequence targeting the amino acids region 1556-1573 (Fig. 5.7 and 5.8).

Three subjects of the cohort were able to target the amino acids region 1373-1380 (subjects 42, 62 and 67). The alignment of GT1 and GT3 sequences indicates that this region is highly conserved in both genotypes, pointing out a potential attractive target for immune therapies (Fig. 5.7A). As expected, all these subjects were HLA-B51-positive. Two of the subjects targeting this epitope were HCV-RNA negative (R42 and R62) and the single subject with chronic infection (C67) harboured an autologous sequence variant (VPFYGKAI; Table 5.2). T cells directed against the sequence variant VPFYGKAI even if successfully expanded after *in vitro* culture showed to be only partially cross-reactive with the prototype-specific response indicating that it may represent an escape variant (Fig. 5.7B).

A



B

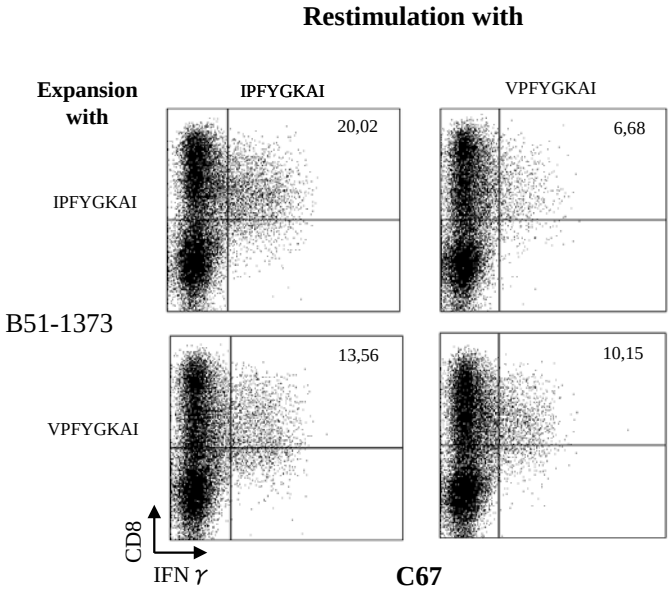


Fig. 5.7: CD8 epitope with identical consensus sequence in HCV GT1 and GT3.

(A) T cells were expanded for 10 days from PBMCs of subject R42, R62 and C67 in the presence of an individual peptide that contains the previously described HLA-B51-restricted epitope B51-1373. After *in vitro* expansion cells were restimulated with 10 µg/ml of the peptide B51-1373 before intracellular IFNγ staining. On the right side, HCV GT1a sequences taken from the database and local sequences from HCV GT1b and GT3a were aligned to the H77 (NC_004102) reference sequence of the epitope region. (B) T cells from PBMCs of the chronically infected subject C67 were expanded for 10 days with the HLA-B51 prototype and with the autologous variant sequence VPFYGKAI. After *in vitro* expansion cells were restimulated with both peptides (10 µg/ml) before intracellular IFNγ staining. The number of IFNγ+CD8+ T cells is indicated in the upper right quadrant of each dot plot (relative to all CD8+ T cells in percent).

The second response mentioned before was not fine mapped and was directed against two GT-specific peptides that differed in one amino acid in the first position (D→E). Although the exact location of the optimal epitope sequence within the 18mer is unclear we assume that the targeted region is probably identical in GT1 and 3 (Table 5.3). The cross-reactivity of the GT-specific CD8+ T cells targeting the region 1556-1573 was evaluated based on the number of IFNγ producing cells detected after restimulation. The strength of the CD8+ T cell responses after heterologous restimulation was comparable to the one obtained after autologous restimulation indicating a full cross-reactive response (Fig. 5.8).

To address the polymorphism of the GT1 and the GT3 targeted regions the H77 GT1a reference was aligned with the GT1a sequences from the public database and the GT1b and GT3a sequences of our cohort. The peptide sequence resulted highly conserved between the GT1b and 3a suggesting that the targeted region was with high probability included in the identical region (Fig. 5.8).

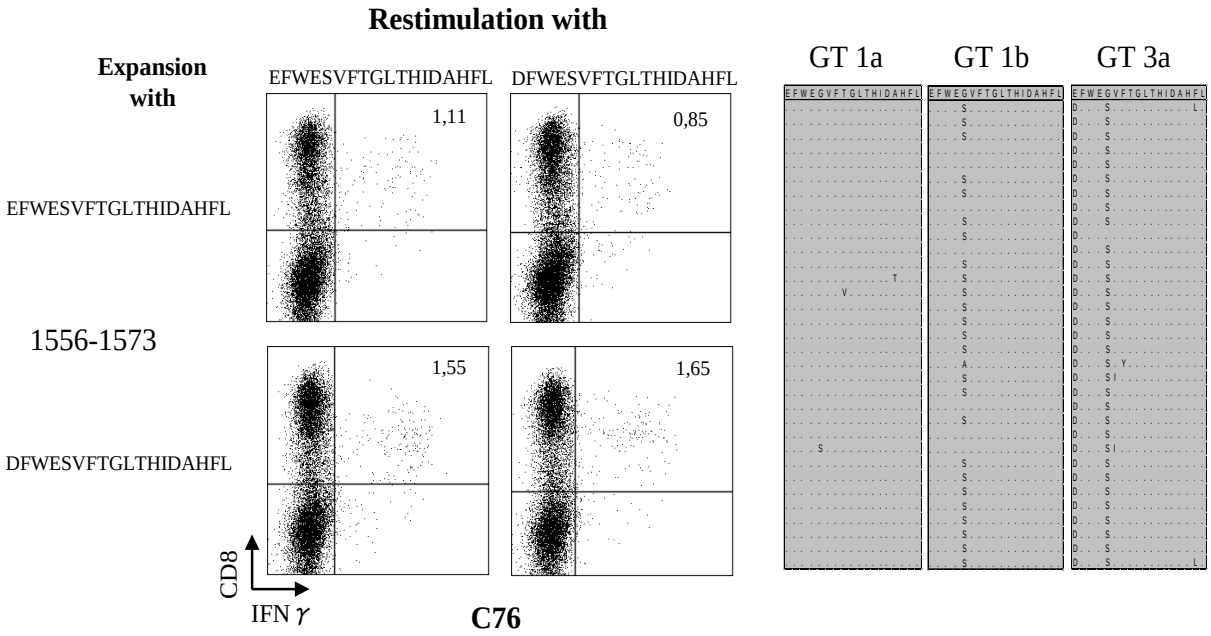
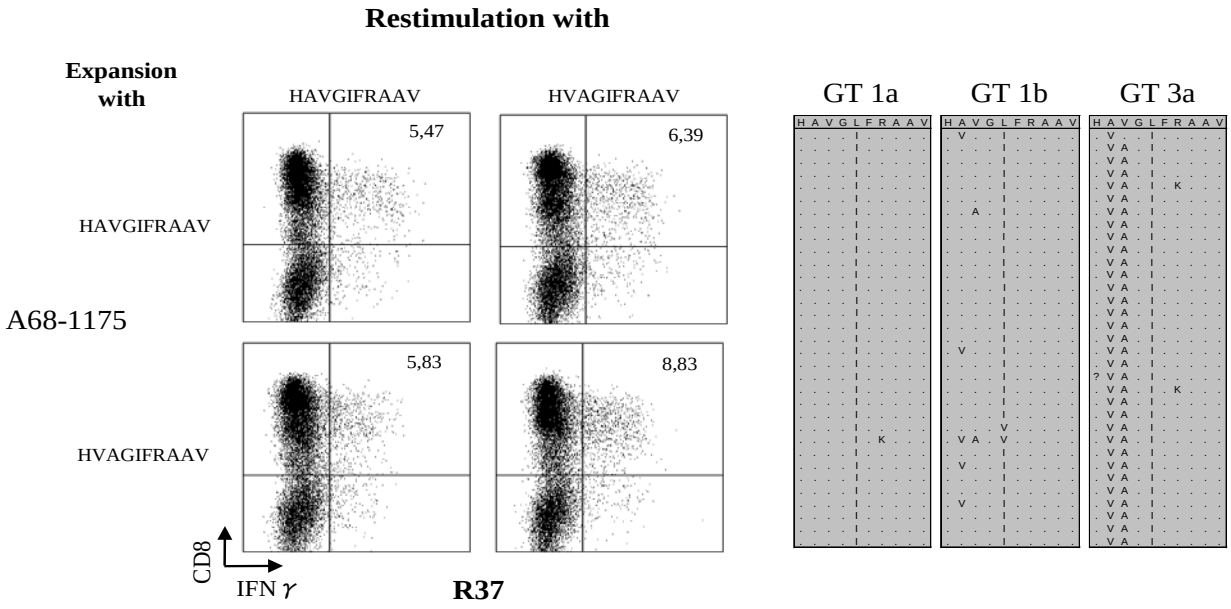


Fig. 5.8: CD8 epitope with highly conserved consensus sequence in HCV GT1 and GT3.

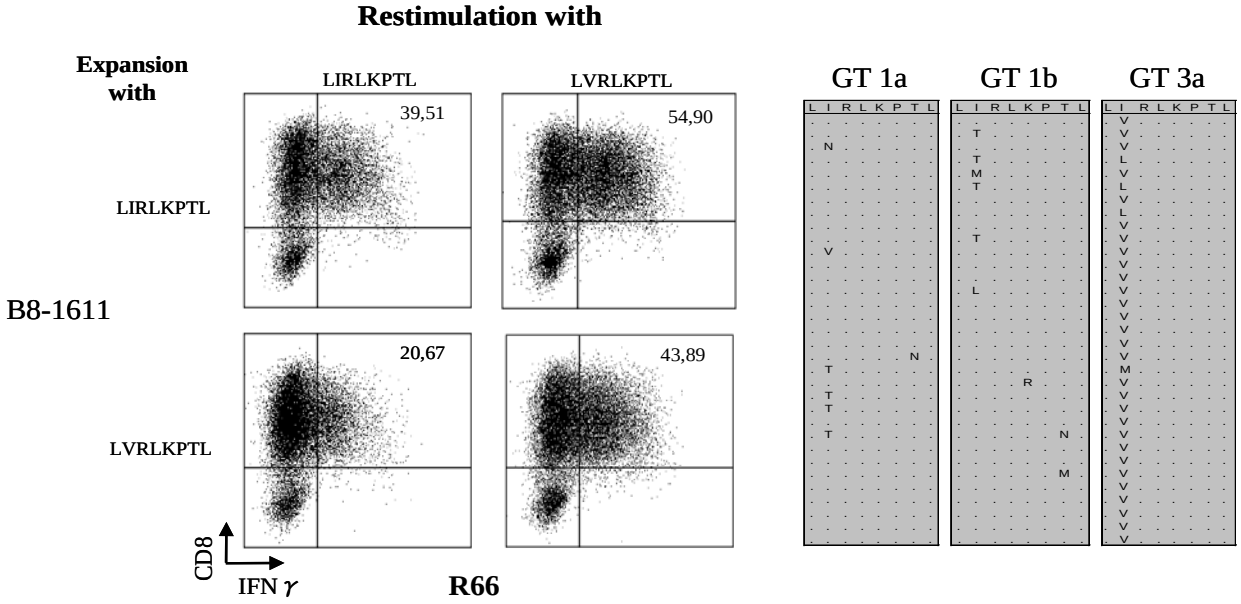
T cells were expanded for 10 days from PBMCs of subject C76 in the presence of the peptide targeting the amino acids region 1556-1573. After *in vitro* expansion cells were restimulated with both GT-specific peptide variants (10 μg/ml) as indicated before intracellular IFNγ staining. The number of IFNγ+CD8+ T cells is indicated in the upper right quadrant of each dot plot (relative to all CD8+ T cells in percent). On the right side, HCV GT1a sequences taken from the database and local sequences from HCV GT1b and GT3a were aligned to the H77 (NC_004102) reference sequence of the epitope region.

Of the total of 28 epitopes the remaining 25 harboured at least one amino acid difference between the consensus sequences of GT1b and GT3a. Examples of fully cross-GT reactive CD8+ T cell response characterized by targeting of a region that differs between GT1 and GT3 are shown in figure 5.9. The RNA negative subject R37 had a CD8 response directed against the HLA-A68-restricted epitope. The GT1 and GT3 sequence in the HLA-A68-restricted epitope differed in position 2 and 3 of the epitope (Fig. 5.9A). The RNA negative subject R66 instead had two CD8 responses directed against the B8-1611 and the B8-1395-restricted epitopes that differed respectively in the second and last position of the epitope (Fig. 5.9B and C).

A



B



C

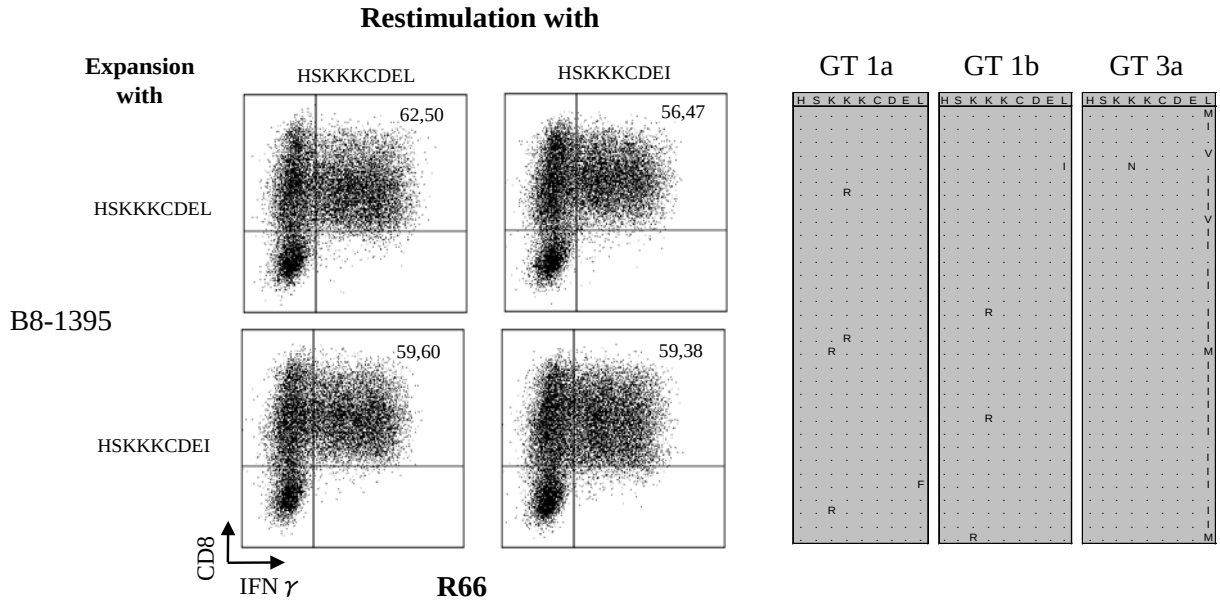


Fig. 5.9: Examples of three non-identical CD8 epitopes with full cross-GT reactivity.

T cells were expanded for 10 days from PBMCs of subject (A) R37 in the presence of the individual peptide A68-1175 and of subject (B) R66 in the presence of the individual peptides B8-1611 and (C) B8-1395. After *in vitro* expansion cells were restimulated with both GT-specific peptide variants (10 µg/ml) as indicated before intracellular IFNγ staining. The number of IFNγ+CD8+ T cells is indicated in the upper right quadrant of each dot plot (relative to all CD8+ T cells in percent). On the right side, HCV GT1a sequences taken from the database and local sequences from HCV GT1b and GT3a were aligned to the H77 (NC_004102) reference sequence of the epitope region.

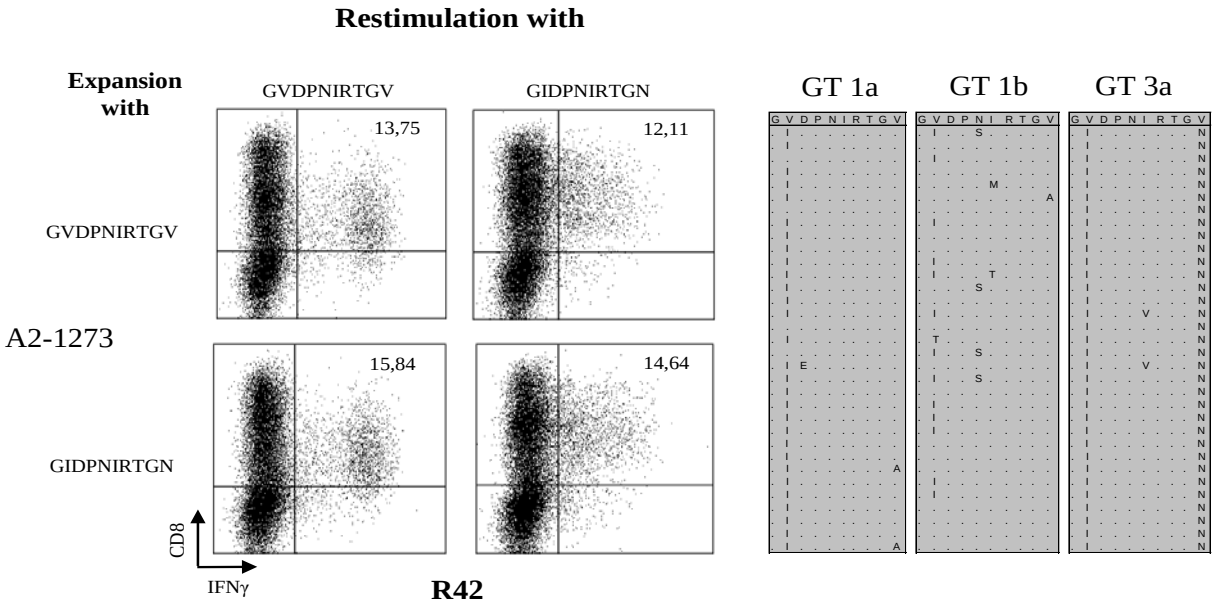
In all the three different cases T cells were successfully expanded in the presence of the GT1 and the GT3 sequence and the two cultures reacted with both peptide variants suggesting full cross-GT reactivity.

5.3.2 Partial cross-GT reactive CD8+ T cell responses

Examples of CD8 response with partial cross-recognition are shown in figure 5.10 and 5.11. As already mentioned CD8+ T cell responses were classified as partial cross-reactive when there was more then a 10-fold difference in the SD50. Two RNA negative individuals of our cohort showed these characteristic responses: subject R42 and subject R66. T cells from R42 directed against the epitope A2-1273 were successfully expanded in the presence of the GT1b and the GT3a sequence and showed cross-reactivity in the presence of high peptide

concentrations (Fig. 5.10A). However, additional peptide titrations revealed preferential targeting of the GT1b sequence (Fig. 5.10B).

A



B

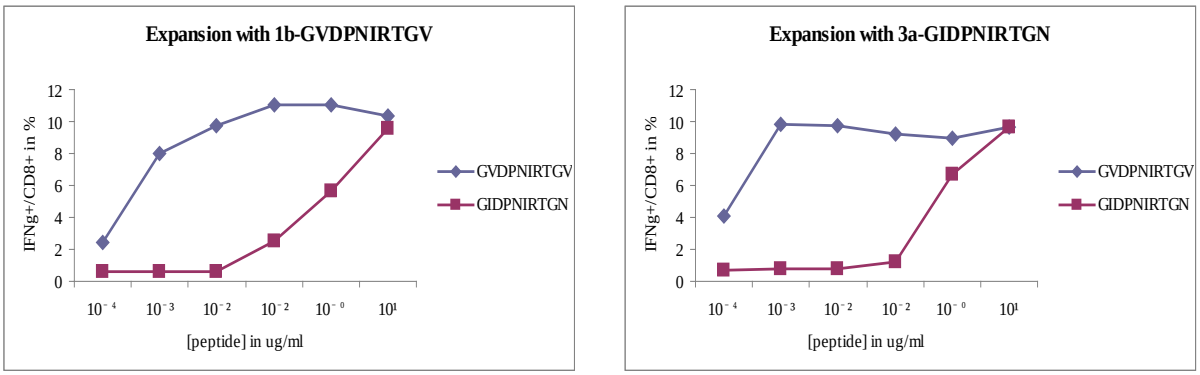


Fig. 5.10: First example of a CD8 epitope with partial cross-GT reactivity.

(A) T cells were expanded for 10 days from PMBCs in the presence of GT1b and GT3a peptides as indicated. After *in vitro* expansion cells were restimulated with both GT-specific peptide variants (10 µg/ml) as indicated before intracellular IFNγ staining. On the right side, HCV GT1a sequences taken from the database and local sequences from HCV GT1b and GT3a were aligned to the H77 (NC_004102) reference sequence of the epitope region. The number of IFNγ+CD8+ T cells is indicated in the upper right quadrant of each dot plot (relative to all CD8+ T cells in percent). (B) Serial peptide dilutions of the GT1b (rhombus) and GT3a (squares) sequence of the A2-1273 peptide were tested.

Patient R66 had instead a response targeting the B35-1359 restricted epitope that at the highest peptide concentrations already showed evidence of a GT1b sequence preferential targeting (Fig. 5.11).

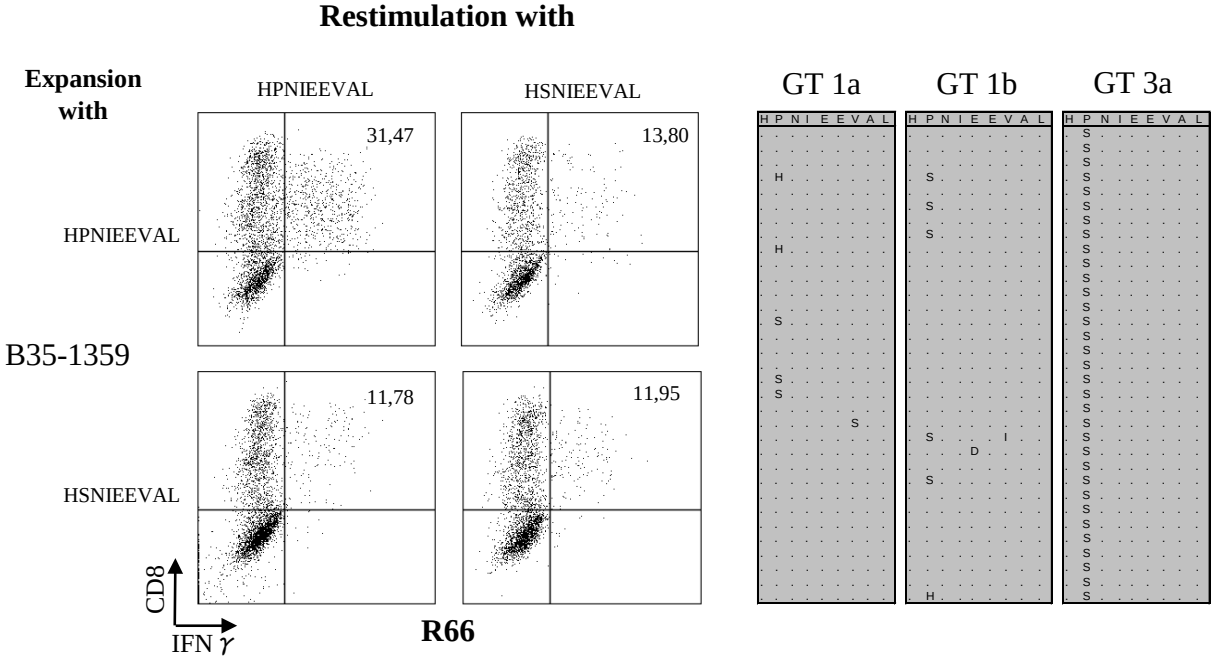


Fig. 5.11: Second example of a CD8 epitope with partial cross-GT reactivity.

T cells were expanded for 10 days from PMBCs in the presence of GT1b and GT3a peptides as indicated. After *in vitro* expansion cells were restimulated with both GT-specific peptide variants (10 µg/ml) as indicated before intracellular IFNγ staining. The number of IFNγ+CD8+ T cells is indicated in the upper right quadrant of each dot plot (relative to all CD8+ T cells in percent). On the right side, HCV GT1a sequences taken from the database and local sequences from HCV GT1b and GT3a were aligned to the H77 (NC_004102) reference sequence of the epitope region.

5.3.3 Non cross-GT reactive CD8+ T cell responses

A clear example of a CD8 response without cross-GT reactivity could be obtained from subjects C63 and C11 chronically infected with GT3 (Fig. 5.12A and B).

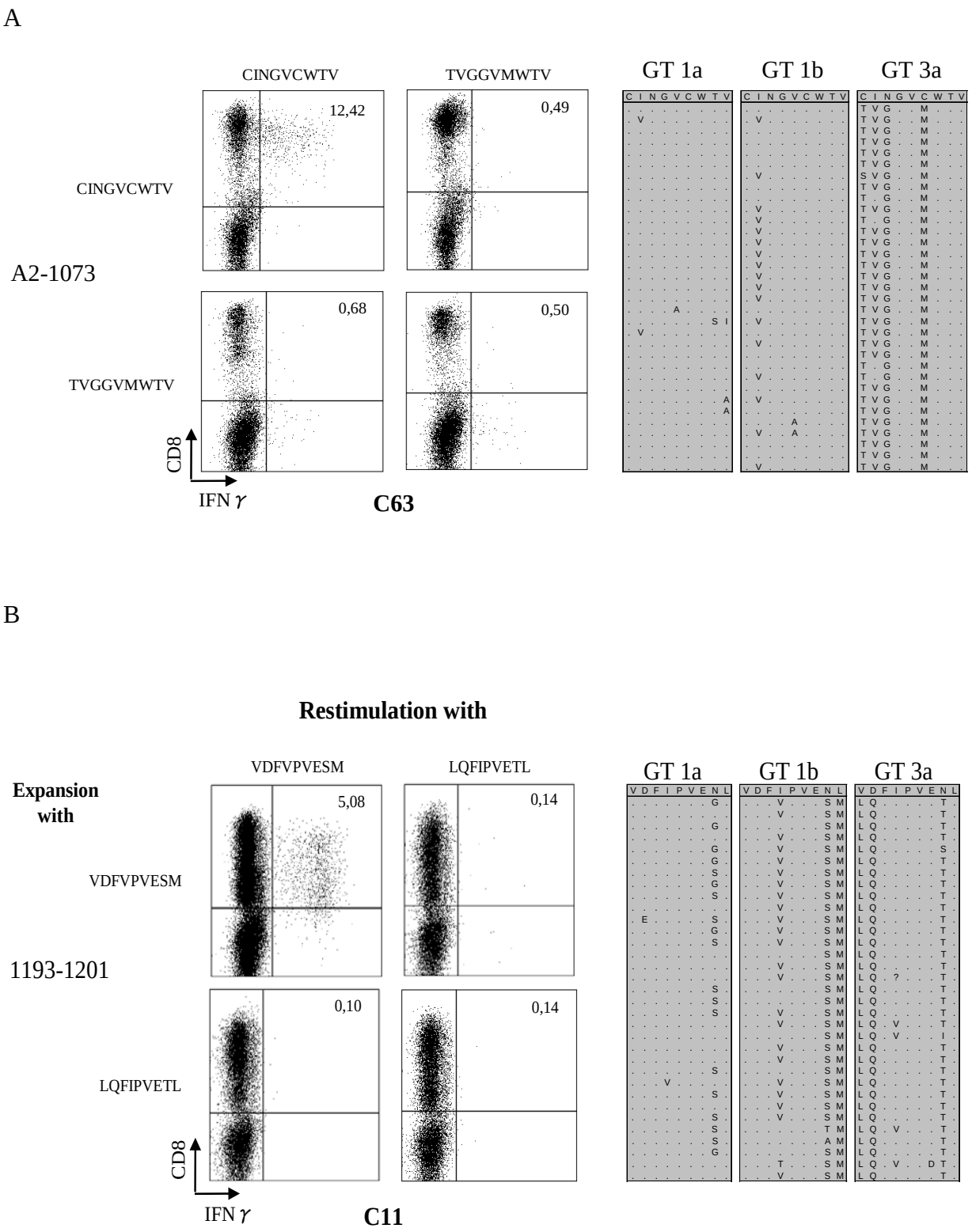


Fig. 5.12: Examples of CD8 epitopes without cross-GT reactivity.

T cells were expanded for 10 days from PMBCs of subjects (A) C63 and (B) C11 in the presence of GT1b and GT3a peptides as indicated. After *in vitro* expansion cells were restimulated with both GT-specific peptide variants (10 µg/ml) as indicated before intracellular IFNγ staining. The number of IFNγ+CD8+ T cells is indicated in the upper right quadrant of each dot plot (relative to all CD8+ T cells in percent). On the right side, HCV GT1a sequences taken from the database and local sequences from HCV GT1b and GT3a were aligned to the H77 (NC_004102) reference sequence of the epitope region.

In summary, the majority of epitopes (19 of 28; 67.9%) detected in this study did not show any cross-GT reactivity between GT1 and GT3. This includes 13 epitopes (46%) that were detected in GT1 only and six epitopes (21%) that were detected in GT3 only. Six of the total of 28 epitopes (22%) showed cross-GT reactivity. This includes three epitopes, where the targeted sequence is identical in GT1 and GT3 and three additional epitopes that targeted both GT-specific variants. Two additional epitopes showed partial cross-reactivity between both genotypes. One additional epitope ATDALMTGF could not be classified into any of these categories because different patterns of cross-reactivity were observed. The consensus sequence of this epitope was not reactive in both genotypes and the responses were only detected with the optimal ATDALMTGY described epitope, as direct consequence of an escape mutation event (Fig. 5.13).

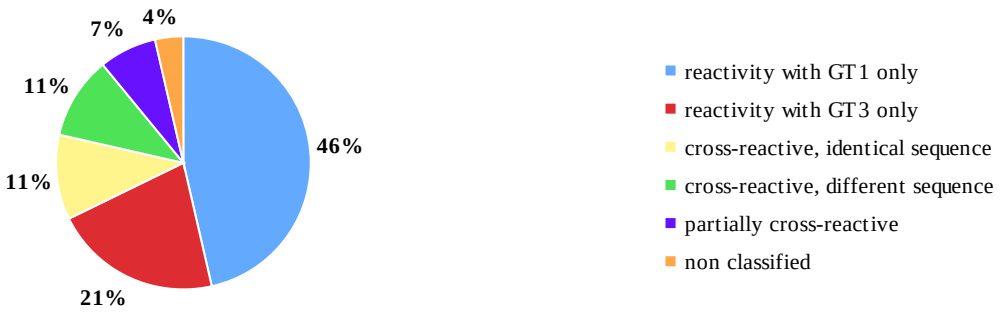


Fig. 5.13: Degree of cross-GT reactivity of the identified CD8 epitopes.

A total of 28 epitopes identified in this study was categorized as indicated in the figure into those epitopes reactive in GT1 or GT3 only, epitopes reactive in both genotypes and identical consensus sequence of the targeted region, epitopes reactive in both genotypes and different consensus sequence and epitopes with partial cross-GT reactivity. One additional epitope was not classified.

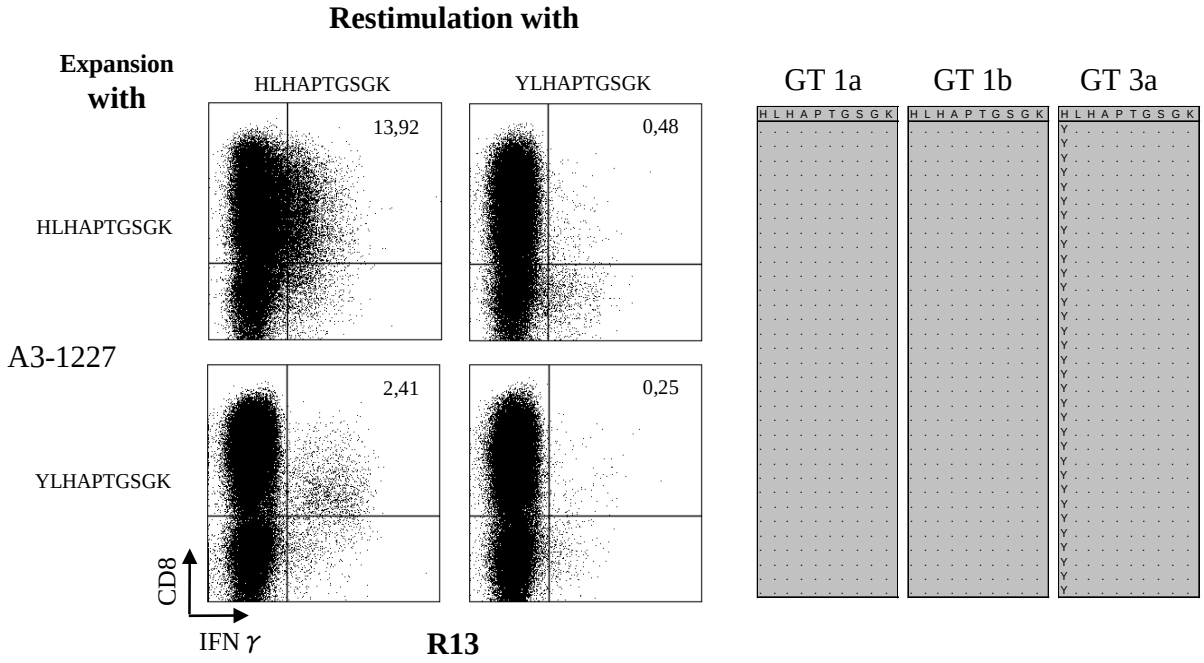
5.4 Immunological evidence for exposure to different HCV genotypes

The risk profile and heterogeneous GT distribution of IVDUs raises the possibility that repetitive exposures to different genotypes may not be uncommon in this cohort. In order to address the possible rate of multiple infections we performed serological analyses. We assumed that a discrepancy in serotyping and genotyping patterns might be indicative of the frequency of re-exposure to different HCV types. HCV serotype determination was performed using the Murex HCV serotyping kit, which is based on the detection of antibodies to type specific peptides derived from the NS4 proteins of different HCV genotypes. Two GT3-infected subjects were identified positive for serotype 1 and a discrepancy between serotype

and GT was detected in four of 39 subjects with chronic HCV infection. One additional subject had a mixed GT1/GT3 serotype, and five subjects were not typable (Table 5.1). Overall in most cases, the serotyping data confirmed the previous HCV-type attribution based on genotyping results.

On a cellular level exposure to both genotypes can be suspected if CD8 responses directed against both genotypes and without cross-reactivity are detected in the same individual or if the detected CD8 response is not reactive with the autologous GT. One example for CD8 responses directed against both genotypes and without cross-reactivity in the same individual is shown in figure 5.14. Subject R13 with undetectable HCV-RNA targets three epitopes in NS3 including the HLA-A3 restricted epitope A3-1227 and a novel epitope in GT3 spanning amino acids 1265-1274. In the presence of the GT1 sequence of the A3-1227 epitope, T cells were expanded that did not cross-react with the GT3 sequence of the epitope. In this case there is only one amino acid difference between both genotypes (position 1 of the epitope; Fig. 5.14A). In contrast, T cells targeting the novel epitope (1265-1274) were only expanded in the presence of the GT3 peptide sequence (Fig. 5.14B). After a close look at the epitopes already described in literature emerged that this region entirely overlaps with an HLA-A11-restricted epitope (A11-1265) previously described in GT1. However, this subject is HLA-A11-negative and five of ten residues differ in the non-reactive GT1 peptide sequence supporting that this is truly a novel GT3-specific epitope.

A



B

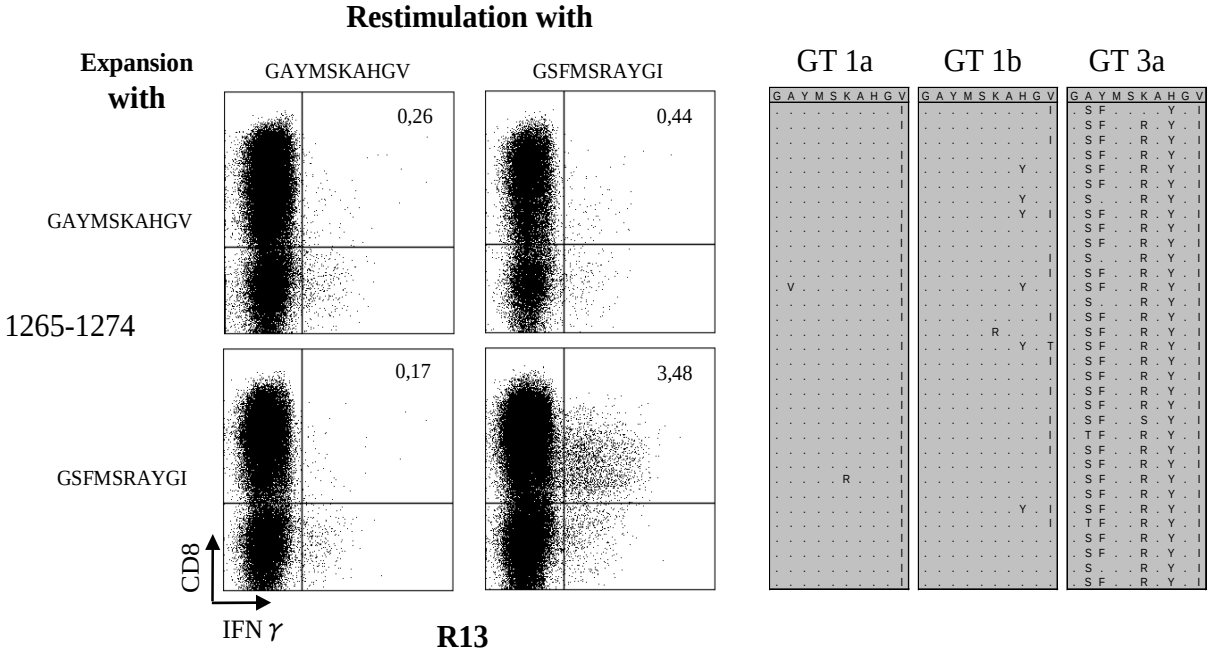
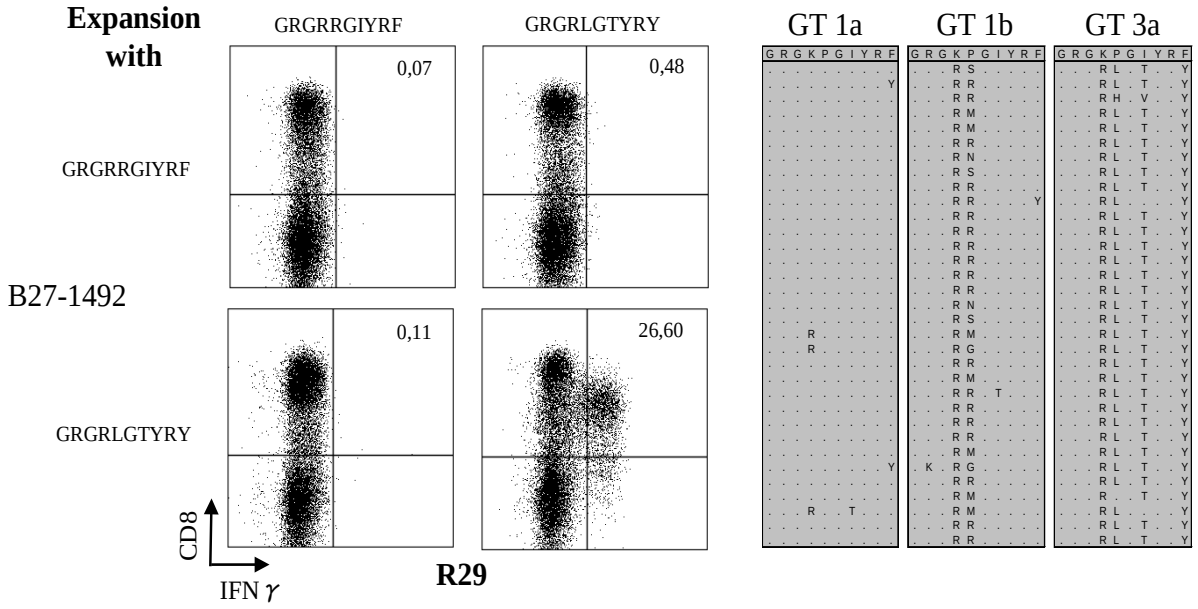


Fig. 5.14: Firt example of co-existence of two distinct T cell populations targeting different NS3 regions in both genotypes.

T cells were expanded for 10 days from PBMCs of subject R13 in the presence of GT1 and GT3 peptides targeting (A) the A3-1227 epitope and (B) the amino acids region 1265-1274. After *in vitro* expansion, cells were restimulated with the same peptides (10 µg/ml) before intracellular IFNγ staining. The number of IFNγ+CD8+ T cells is indicated in the upper right quadrant of each dot plot (relative to all CD8+ T cells in percent). On the right side, HCV GT1a sequences taken from the database and local sequences from HCV GT1b and GT3a were aligned to the H77 (NC_004102) reference sequence of the epitope region.

A second example for exclusive GT1- and GT3-specific T cells in the same individual is shown in figure 5.15. Subject R29 with undetectable HCV-RNA targets two NS3 epitopes, the HLA-B27-restricted epitope B27-1492 and the HLA-A2-restricted epitope A2-1073. Although the B27-restricted epitope was previously reported in GT1a, only the GT3a sequence was targeted in this subject (Fig. 5.15A). In contrast, T cells targeting the HLA-A2-restricted epitope were only expanded in the presence of the GT1 peptide sequence (Fig. 5.15B).

A



B

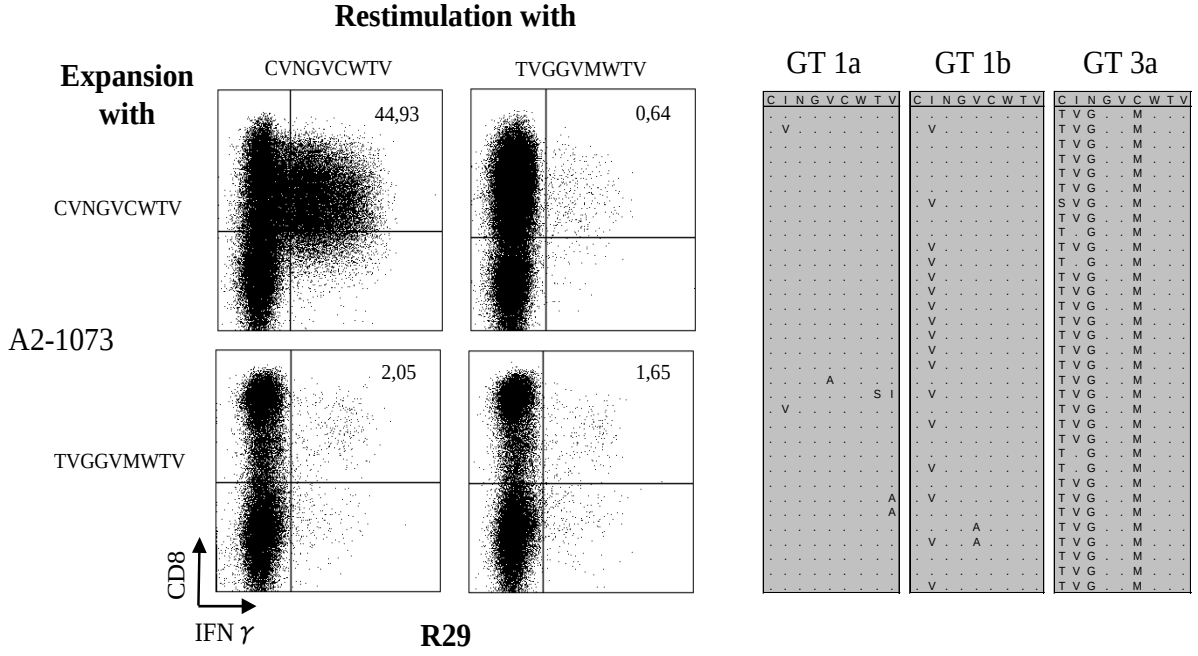


Fig. 5.15: Second example of co-existence of two distinct T cell populations targeting different regions in both genotypes.

T cells were expanded for 10 days from PBMCs of subject R29 in the presence of GT1 and GT3 peptides targeting (A) the B27-1492 and (B) the A2-1073 epitopes. After *in vitro* expansion, cells were restimulated with the same peptides (10 µg/ml) before intracellular IFNγ staining. The number of IFNγ+CD8+ T cells is indicated in the upper right quadrant of each dot plot (relative to all CD8+ T cells in percent). On the right side, HCV GT1a sequences taken from the database and local sequences from HCV GT1b and GT3a were aligned to the H77 (NC_004102) reference sequence of the epitope region.

Based on this comparison we found two subjects infected with GT1 and immunological evidence for exposure to GT3 and eight subjects infected with GT3 and immunological evidence for exposure to GT1. Two T cell populations specific for each GT in the same individual were detected in additional five subjects with undetectable viremia.

The detailed analysis of the GT-specific responses gave the opportunity to identify one epitope that was predominantly recognised in a GT-specific manner. Three subjects of the cohort showed responses against amino acids 1627-1635, and all of them displayed two T cell populations reactive against one or the other GT. Two of these subjects were HCV-RNA negative (Fig. 5.16A and B) instead the third was chronically infected with GT1 with a viral load (1.785 IU/ml) (Fig. 5.17). The targeted epitope is a novel HLA-B13-restricted epitope with a two amino acids difference between the GT consensus sequences. In the three patients was found the co-existence of GT specific cell populations after peptide-specific expansion. One T cell population was expanded in the presence of the GT1 sequence of the epitope (RLGAVQNEV) and did not target the GT3 sequence (RLGPVQNEI). The other T cell population was expanded in the presence of the GT3 sequence and did not target the GT1 sequence (Fig. 5.16).

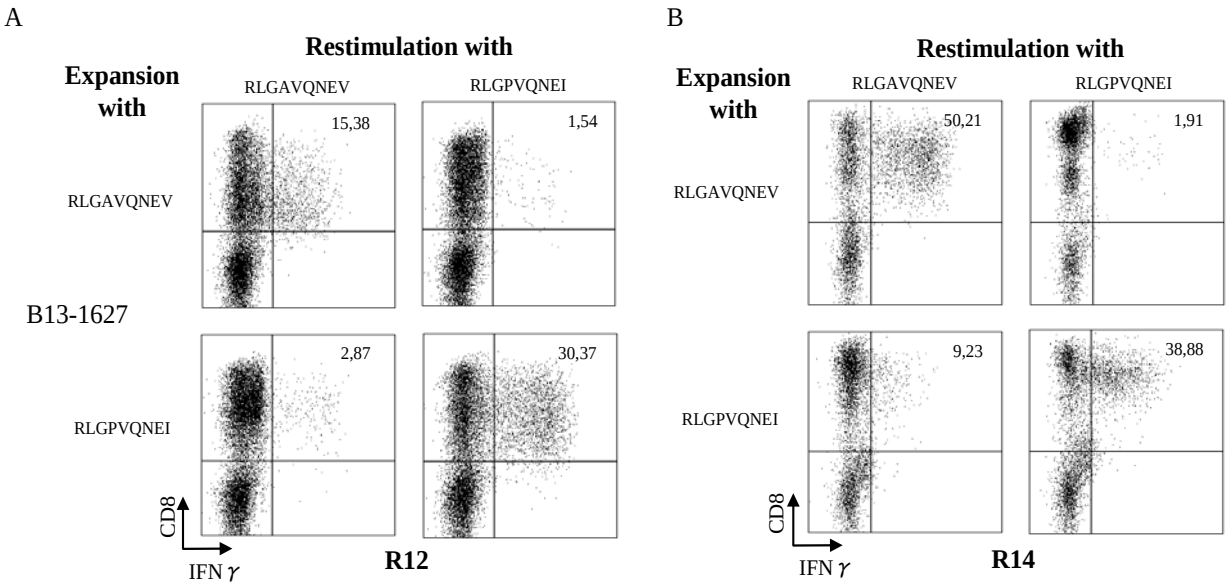


Fig. 5.16: Co-existence of two distinct T cell populations targeting the same region in both genotypes in two HCV-RNA negative individuals.

T cells were expanded for 10 days from PBMCs of subjects (A) R12 and (B) R14 in the presence of GT1 and GT3 peptides of the epitope B13-1627. After *in vitro* expansion, cells were restimulated with the same peptides (10 µg/ml) before intracellular IFNγ staining. The number of IFNγ+CD8+ T cells is indicated in the upper right quadrant of each dot plot (relative to all CD8+ T cells in percent).

To support the fact that these amino acids substitutions were GT-specific the sequences present in the HCV database were analysed. The data observed that the A to V substitution in position four of the GT3a sequence of the epitope is completely absent from all 44 GT1b sequences and present in all 42 GT3a sequences (Fig. 5.17A).

The same pattern was observed in the third chronically infected subject indicating that they all have been exposed to both genotypes and that two distinct T cell populations have been primed. This subject with detectable HCV GT1a viremia harbours a variant (RLGAVQNEA) that has been shown to be similarly targeted when compared to the prototype (Fig. 5.17B).

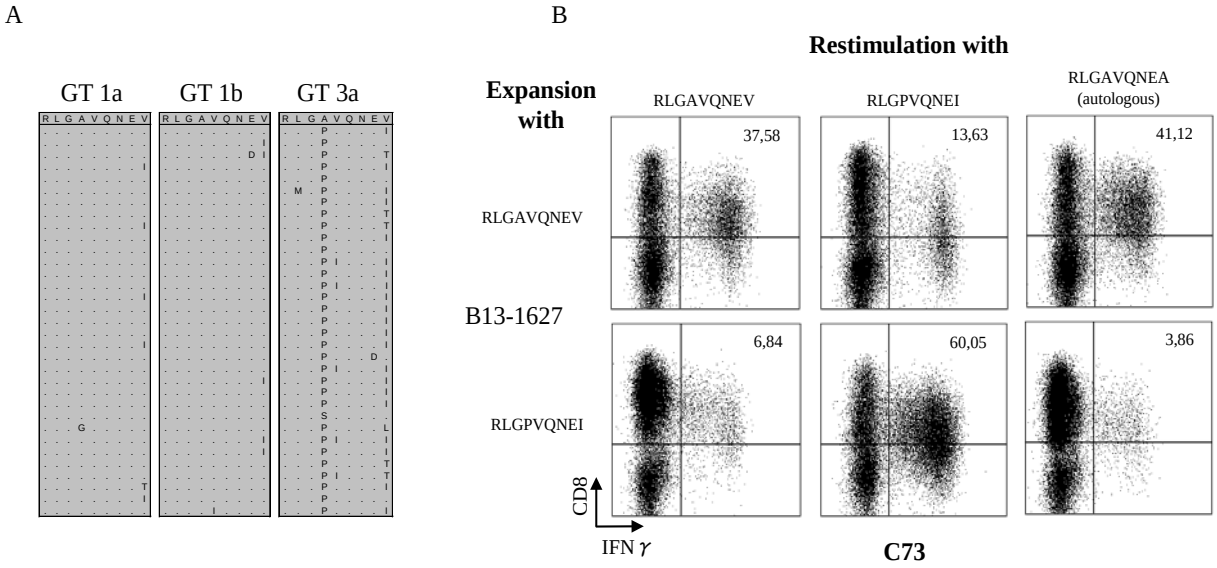


Fig. 5.17: Co-existence of two distinct T cell populations targeting the same region in both genotypes in a chronically infected individual.

T cells were expanded for 10 days from PBMCs of subject C73 in the presence of GT1 and GT3 peptides of the epitope B13-1627. After *in vitro* expansion, cells were restimulated with both peptides and with the autologous detected variant (RLGAVQNEA) (10 μg/ml) before intracellular IFNγ staining. The number of IFNγ+CD8+ T cells is indicated in the upper right quadrant of each dot plot (relative to all CD8+ T cells in percent). On the left side, HCV GT1a sequences taken from the database and local sequences from HCV GT1b and GT3a were aligned to the H77 (NC_004102) reference sequence of the epitope region.

Summarizing the data obtained within this close analysis of the cross-reactive immune responses emerged that we identified five subjects with undetectable viremia with co-existence of two GT-specific T cell populations. Suggesting that immune control of two different genotypes can be obtained in presence of broad reactive responses.

5.5 Broad reactive CD8 responses against both genotypes are mainly identified in HCV-RNA negative subjects

Subjects with multiple responses against both genotypes were analysed here to identify if cross-GT responses provide some degree of protection from chronic HCV infection. These analyses showed that in our cohort CD8 responses active against both genotypes were predominantly found in HCV-RNA negative subjects. Nine of 14 (64.3%) subjects with undetectable viremia harboured T cells active against GT1b and GT3a. This includes T cells that were directed against fully cross-reactive epitopes (four subjects) and T cells that were GT-specific, but two T cell populations specific for each GT co-existed (five subjects). T cells active against both genotypes instead were only detected in six of 39 (15.3%) subjects with detectable HCV-RNA (Fig. 5.18).

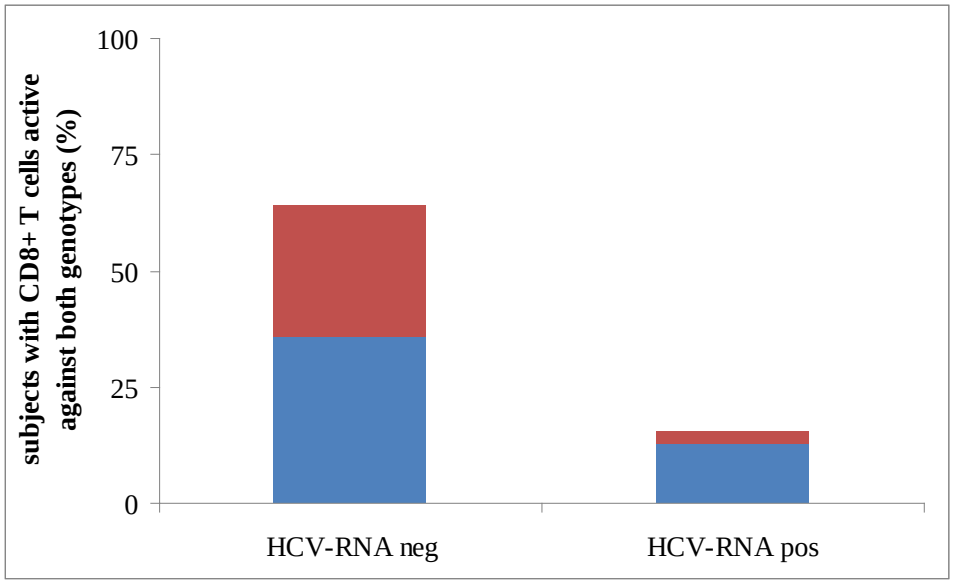


Fig. 5.18: CD8 responses active against GT1b and GT3a.

The frequency of subjects with fully cross-reactive CD8+ T cells (red region) or with co-existence of GT-specific CD8+ T cells against GT1b and GT3a (light blue region) is significantly higher in the HCV-RNA negative group compared with the HCV-RNA positive group (Fisher's exact test).

5.6 Analysis of the memory phenotype and of the pro-apoptotic state of HCV peptide-specific cells

Phenotypic and functional studies of virus-specific T cells have attempted to define the determinants of a successful versus an unsuccessful T cell response in viral infections (Lauer *et al.*, 2004). It was described that the number and the strength of responses in chronically infected subjects were significantly less strong and frequent compared to RNA-negative individuals. This phenomenon has also been observed in the IVDUs cohort previously analysed (Fig. 5.6). Until now, the identification of consistent features distinguishing between T cell responses that result in self limiting versus chronic HCV infection is still not completely understood. For this reason we performed here an analysis of the phenotype of peptide-specific CD8+ T cells with the aim to discriminate between T cells of individuals that cleared the infection from the ones that got into a chronic state. The analysis performed focused on the CD8+ T cells of four individuals targeting two HLA-A2 immunodominat epitopes: CINGVCWTV (NS3-10739) and KLVALGINAV (NS3-1406) (Table 5.4). The low number of responses presented here is a direct consequence of the difficulties that we encountered in the detection of sustained peptide-specific responses necessary for detailed phenotypical analyses.

Table 5.4: Epitope sequence analysis correlated with the percentages of Pentamer+CD8+ T cells detected *ex vivo*.

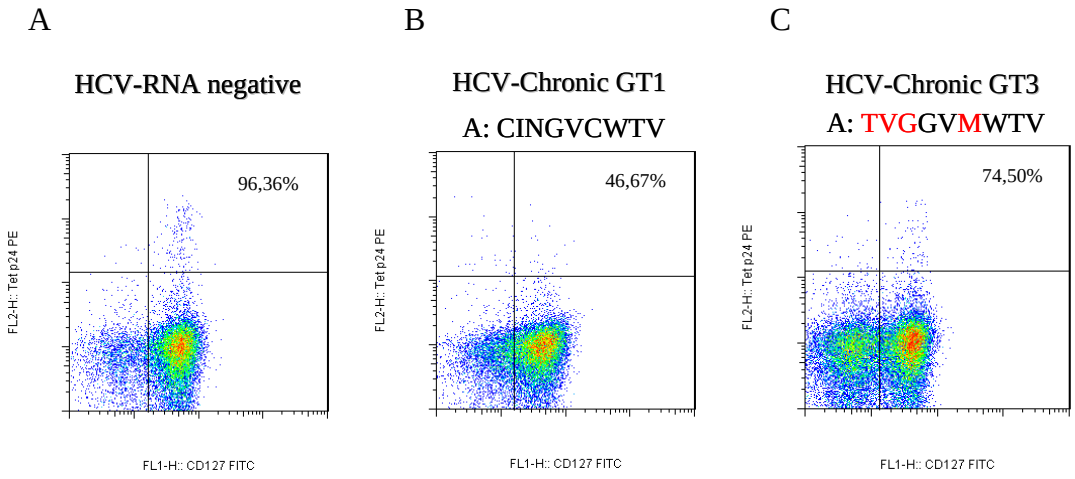
ID	GT	Sequence CINGVCWTV	% of Pentamer+CD8+T cells	Sequence KLVALGINAV	% of Pentamer+CD8+T cells
124	1a	----------	0.15	----------	58.33
125	negative	N.A.[a]	0.21	N.A.[a]	0.08
126	1a	----------	0.02		
127	3a	TVG--M---	0.10		

[a]N.A.: not applicable

CD127 is a key molecule associated with the maintenance of memory T cell populations (Lang *et al.*, 2005). Therefore we decided to analyse the *ex vivo* expression of CD127 in IVDUs individuals with different clinical outcomes to identify the memory phenotype of

peptide-specific CD8+ T cells. PBMCs of each subject were thawed (see Methods, paragraph 4.10) and analysed directly *ex vivo*. The cells were stained with MHC class I-Pentamer, with the CD8 antibody (see Methods, paragraph 4.13) and with the CD127 antibody. CD8+ T cells specific for CINGVCWTV were identified in four subjects and two subjects showed CD8+ T cells targeting the KLVALGINAV epitope.

In figure 5.19 are shown CINGVCWTV peptide-specific CD8+ T cells of three individuals with different clinical outcomes. One HCV-RNA negative individual (Fig. 5.19A) and two chronically infected subjects with GT1 and 3 respectively (Fig. 5.19B and C). Peptide-specific CD8+ T cells of the GT1 infected individual (Fig. 5.19B) showed a significant lower CD127 expression compared to the peptide-specific cells of the other two individuals. GT1 and GT3 isolates were amplified and sequenced as previously described (see Methods, paragraph 4.3) to correlate the sequence of the circulating antigen to the level of CD127 expression in peptide-specific CD8+ T cells. Contrary to the autologous sequence detected in the GT3a infected individual that had four GT-specific mutations (A: TVGGVMWTV), the autologous sequence detected in the GT1 infected patient showed a full match with the epitope targeted by the CD8+ T cells (A: CINGVCWTV). This analysis gave us the proof of the influence that the continuous triggering of the TCR have on the memory phenotype of peptide-specific CD8+ T cells. Lower CD127 expression on epitope-specific CD8+ T cells was also observed in other two chronically infected individuals in whom the sequence of the infecting virus fully matched the targeted epitope (Fig. 5.19D).



D

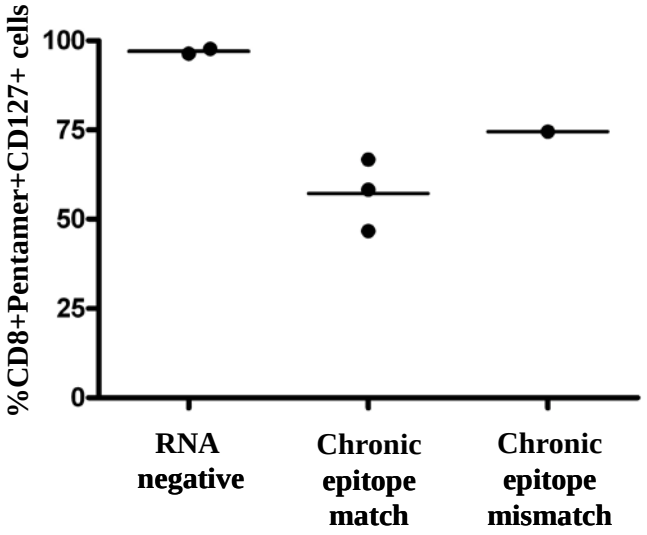


Fig. 5.19: Representative example of *ex vivo* CD127 expression in peptide-specific CD8+ T cells.

The CD127 expression in HCV-specific CD8+ T cells was analysed in (A) an HCV-RNA negative individual, (B) a chronically GT1a infected subject and (C) a chronically GT3a infected individual. The percentage of epitope-specific CD8+CD127+ T cells is indicated in the upper right quadrant of each plot (relative to all CD8+ T cells in percent). (D) Frequency of CD127 expression on peptide-specific CD8+ T cells. Each dot represents CD127 expression on epitope-specific CD8+ T cells. Horizontal lines represent the mean.

To address if the differential expression of the CD127 marker could have an impact on the proliferation capacity of peptide-specific CD8+ T cells, PBMCs of each individual were put in culture with the corresponding reactive peptide for 7 days (see Methods, paragraph 4.8). The frequency of peptide-specific CD8+ T cells obtained after *in vitro* culture was compared to the one detected directly *ex vivo* to estimate the proliferation index. Peptide-specific CD8+ T cells continuously triggered by the antigen had a significantly lower proliferation capacity, showing an impaired phenotype *ex vivo* (Fig. 5.20).

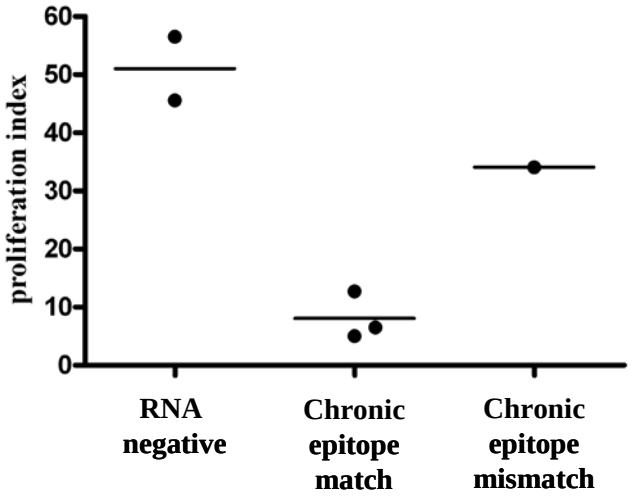
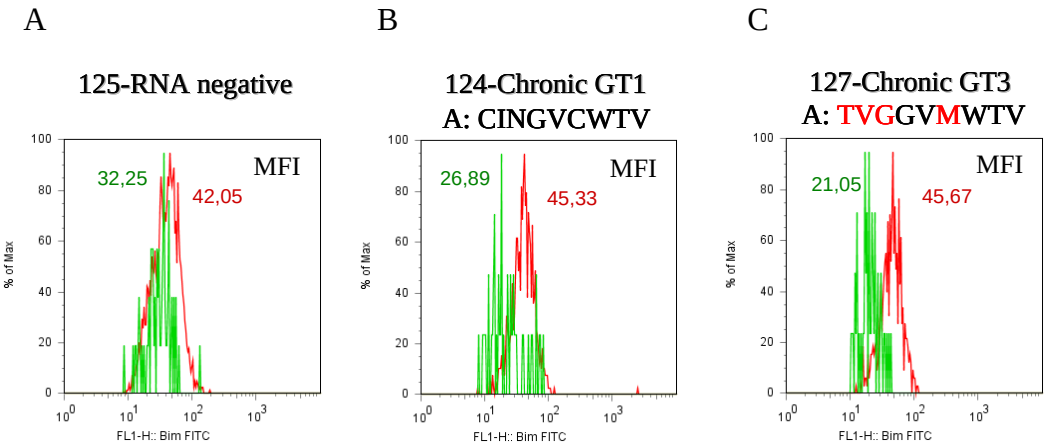


Fig. 5.20: Proliferation capacity of HCV-specific CD8+ T cells upon 7 days of peptide-specific stimulation.

The proliferation index was calculated by the formula (frequency of HCV-specific CD8+ T cells after culture/frequency of HCV-specific CD8+ T cells *ex vivo*). Horizontal lines represent the mean.

A recent study conducted in HBV infection suggested that a higher expression of the pro-apoptotic protein Bim in virus-specific CD8+ T cells seemed to be associated with chronic infection (Lopes *et al.*, 2008). We decided to define if similarly a peripheral deletion of CD8+ T cells could be responsible for the paucity of HCV-specific CD8+ T cell responses in chronically infected subjects. PBMCs of each individual were thawed and analysed *ex vivo* or after 7 days of *in vitro* culture (see Methods, paragraph 4.8-4.10). The cells were stained with MHC class I-Pentamer and with the $Bim_{S/EL/L}$ antibody (see Methods, paragraph 4.13-4.14). In each subject we identified a significant increase in the levels of Bim expression after *in vitro* culture even if there was no significant statistical difference between the levels of expression comparing RNA negative patients with chronically infected individuals (Fig. 5.21).



D E

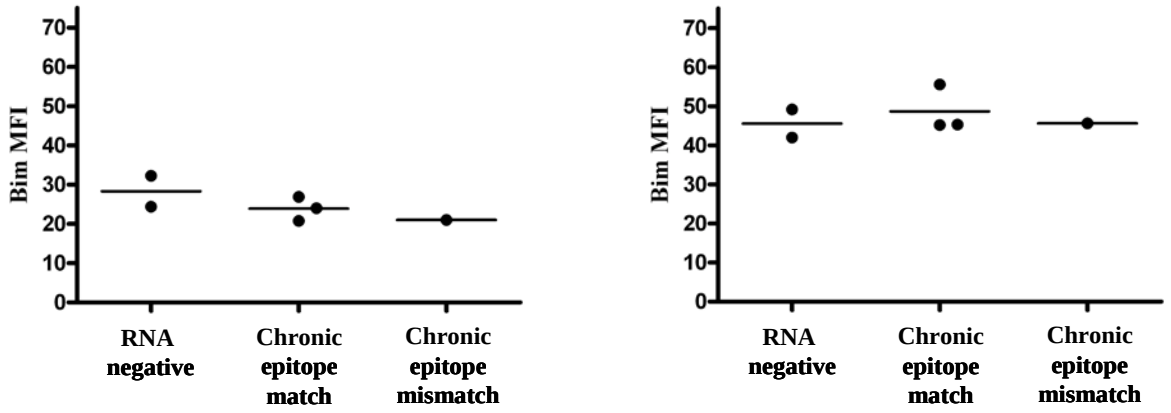


Fig. 5.21: Mean fluorescence intensity of Bim expression in CINGVCWTV-specific CD8+ T cells *ex vivo* and after 7 days of peptide-specific stimulation.

We analyzed the level of Bim expression in peptide-specific CD8+ T cells of (A) an HCV-RNA negative subject, (B) a chronically GT1a infected individual and (C) a chronically GT3a infected subject. The analyses were performed *ex vivo* (green) and after 7 days of peptide-specific stimulation (red). (C and D) Frequency of Bim expression in HCV-specific CD8+ T cells, each dot represent Bim expression on epitope-specific CD8+ T cells (D) *ex vivo* and (E) after 7 days of peptide-specific stimulation. Horizontal lines represent the mean.

Even if the number of the responses until now detected was not sufficient to make a strong conclusion the level of Bim expression did not seem to differ between the groups analyzed here.

5.7 DC priming of naïve CD8+ T cells and induction of HCV peptide-specific T cell lines

The last aim of this work was to establish an assay that could potentially help the generation of antigen-specific CD8+ T cells to successfully employ in clinical trials. Studies illustrating the role of CD8+ T cells in viral clearance of HCV infection support the notion that an adoptive immunity holds promise as a treatment (Li *et al.*, 2008). Therefore we aimed to establish a technique for the priming of naïve CD8+ T cells against immunodominant HCV epitopes. This assay included the maturation of monocytes into DCs and the co-culture of these professional APCs with autologous PBMCs. Addition of the peptide to the co-culture allowed than the priming of naïve peptide-specific CD8+ T cell lines. At first we decided to

activate a HLA-A2 CD8+ T cell memory response against the HLA-A2 FLU-peptide: GILGFVFTL to verify the efficiency of the assay. PBMCs were extracted form the buffy coat of a healthy HLA-A2 donor and monocytes were separated from a total of $2x10^7$ cells. The isolation procedure was performed with the autoMACS Pro facilities using CD14 Microbeads (see Methods, paragraph 4.15). After the separation, aliquots of the positive and negative fractions were collected and analysed by flow cytometry in order to verify the purity of the positive fraction (Fig. 5.22A and B). Monocytes were than counted by Trypan Blue exclusion assay (see Methods, paragraph 4.8) and 1.5 cells/ml were put in culture in a 6-well plate for 5 days. GM-CSF and IL-4 were added at day 0 and day 2 to promote cell proliferation, instead IFNγ and LPS were added at day 3 to obtain monocytes-derived DCs (see Methods, paragraph 4.16). The maturation of monocytes into DCs was verified checking the up-regulation of the co-stimulatory molecule CD80 (Fig. 5.22C).

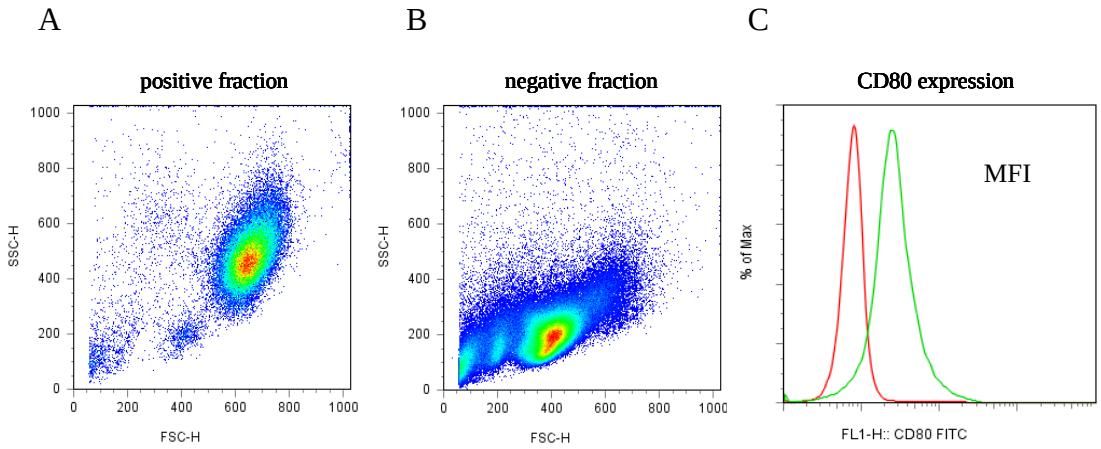


Fig. 5.22: Purification of monocytes from total PBMCs and maturation into DCs.

(A) Positive fraction: monocytes isolated from total PBMCs using CD14 MicroBeads. (B) Negative fraction: monocyte-depleted PBMCs. (C) Maturation of monocytes (red) into DCs (green) was checked based on the up-regulation of the co-stimulatory molecule CD80 after *in vitro* culture with IFNγ and LPS.

In a 96-well plate, 20.000 DCs were co-cultured with 200.000 PBMCs. The cells were obtained from the buffy coat of a healthy HLA-A2 subject which had CD8+T cell responses against the FLU-peptide: GILGFVFTL. This experiment was conducted in triplicate in a final volume of 250 µl, adding to each well 1 µg/ml of the peptide. We also exogenously added cytokines to promote the expansion of CD8+ T cells (IL-2, IL-7 and IL-15) (see Methods, paragraph 4.17). To verify the efficacy of mature DCs in activating antigen-specific memory CD8+ T cells we established in parallel three cultures using only PBMCs containing non

professional antigen presenting cells (the experiment was conducted with the same parameters described above). After 20 days of culture the cells were harvested and tested for IFNγ production. The analysis of the results showed that the DCs were able to induce a significant higher activation of HLA-A2 FLU memory CD8+ T cells compared to the non-professional APCs (Fig. 5.23).

A B

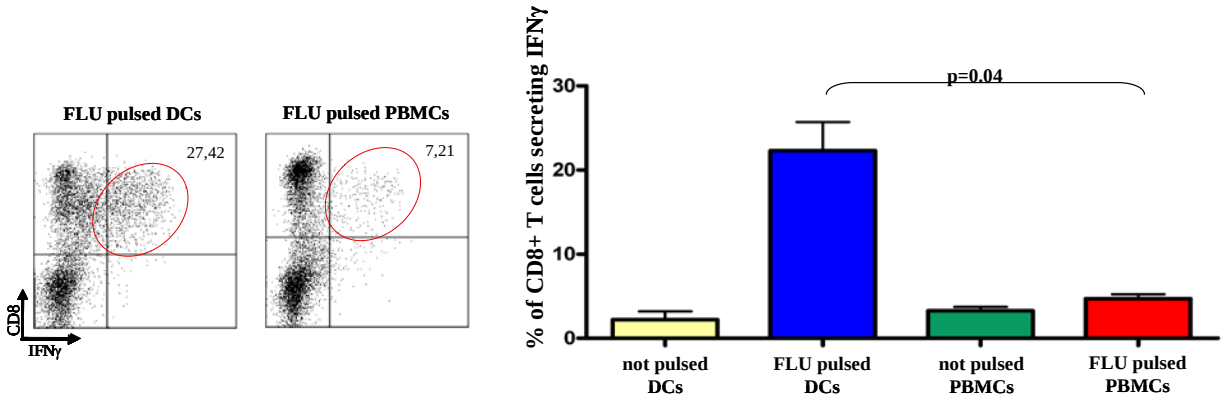


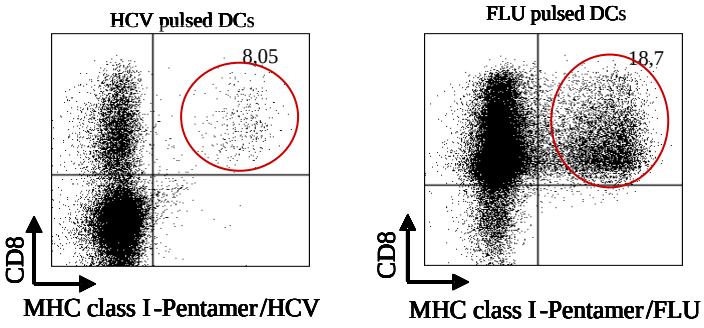
Fig. 5.23: Activation of FLU-specific memory CD8+ T cells using monocyte-derived DCs and PBMCs loaded with the FLU peptide.

Peptide-loaded DCs and PBMCs were co-cultured with autologous PBMCs and used to activate a FLU-specific memory CD8+ T cells response *in vitro*. (A) After 20 days of *in vitro* co-culture the cells were harvested and analyzed by IFNγ staining. The number of activated IFNγ+CD8+ T cells is indicated in the upper right quadrant of each plot (relative to all CD8+ T cells in percent). (B) The experiment was performed in triplicate. P value of the comparison between the peptide-specific CD8+ T cells activated by peptide loaded DCs (blue) and peptide loaded PBMCs (red) is indicated (Fisher's exact test).

After the successful activation of a memory HLA-A2 FLU-specific response the next step was to prime naïve CD8+ T cells in healthy individuals. For this purpose we used the HLA-A2 HCV-peptide: CINGVCWTV (region 1073-1081), the epitope mostly recognized during the analysis of the immune responses of the IVDUs cohort previously described (Table 5.3). Monocytes were matured into DCs, loaded with the HCV-peptide and put in co-culture with PBMCs as mentioned before. The activation of the HLA-A2 FLU memory response (peptide: GILGFVFTL) was used as control of the assay's efficacy. After 20 days of co-culture the cells were harvested and analysed by MHC class I-Pentamer/HCV and MHC class I-Pentamer/FLU staining to verify the proliferation of peptide-specific cells (Fig. 5.24A). An ICS was also performed to have a look at the functionality of the expanded cells (Fig. 5.24B).

Analyses of the results showed the successful priming of functional naïve CD8+ T cells against the HLA-A2 HCV-peptide: CINGVCWTV (Fig. 5.24).

A



B

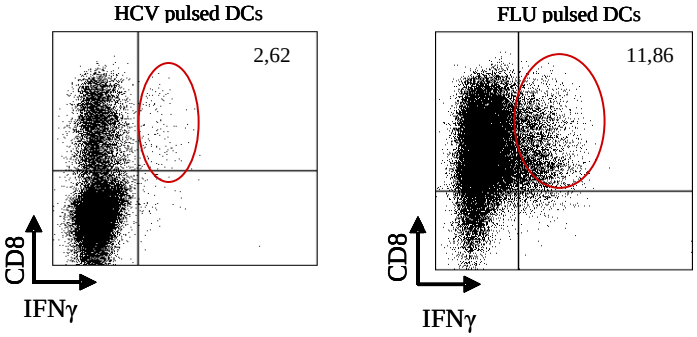


Fig. 5.24: Priming of HCV-specific naïve CD8+ T cells and activation of FLU-specific memory CD8+ T cells using monocyte-derived DCs.

Peptide-loaded APCs were used to stimulate autologous CD8+ T cells *in vitro*. After 20 days of culture each cell line was analyzed by (A) MHC class I-Pentamer/peptide and (B) intracellular IFNγ staining. The number of (A) MHC class I-Pentamer+CD8+ T cells and (B) IFNγ+CD8+ T cells is indicated in the upper right quadrant of each plot (relative to all CD8+ T cells in percent).

6 Discussion

IVDUs are the population subgroup at greatest risk of HCV infection in more developed countries, with prevalence rates of 45% or greater (Miller *et al.*, 2002) and incidence rates of 25% per year or higher being common (Hahn *et al.*, 2002). Most patients are young male adults and it is unclear weather their heroin abuse has an impact on the ability to control viral or bacterial infections. In the cohort analyzed here the frequency of patients who seemingly control HCV replication (41% Anti-HCV positive and RNA negative) is in the normal range, suggesting that the immune response is not substantially impaired per se in these patients. Of note, the high risk behaviour in this patient group suggests that repetitive exposures to HCV are possible. In a recent survey in local drug consumption facilities in Essen (DCF) 63% of drug users reported to be HCV positive and 22% reported needle sharing within the previous month (Scherbaum *et al.*, 2009). HCV differs from the HBV and many other infectious viral agents for the generation of an immune response that does not necessarily induce protective immunity (Aitken *et al.*, 2008). Importantly, in 2002 Mehta *et al.* reported that previously infected IVDUs were significantly less likely to be reinfected, even after accounting for risk behaviour. Similarly, Grebely *et al.* reported in 2006 a significantly lower incidence of HCV reinfection in IVDUs than in naïve individuals. These findings indicate that some level of protective immunity is induced after a previous infection with HCV. The sequence diversity of HCV is believed to represent one of the major obstacles to immune control. Of note, the protective effect in the studies by Mehta and Grebely were observed in IVDUs cohorts with a very homogeneous GT distribution. In North America HCV GT1 by far predominates also in the group of IVDUs (Alter *et al.*, 1999). Therefore, immune control may be easier to achieve if re-exposure occurs with the same subtype. Clearance after rechallenge with homologous strains has also been observed in chimpanzees (Basset *et al.*, 2001) even if Bukh *et al.*, in 2008 have shown that it doesn't seem to be always the case.

In the present study we analyzed the impact of GT-specific sequence differences on the CD8+ T cell immune responses in a subgroup of IVDUs cohort (53 anti-HCV positive IVDUs). The cohort was composed of 17 infected with GT1, 22 infected with GT3 and 14 HCV-RNA negative subjects. Of note, we found that the majority of CD8 responses were GT-specific. Considering that in our study the majority of T cell responses were directed against one GT only and did not cross-react with the other GT, we were able to show that sequence differences are relevant in T cell immunology. Over recent years a great effort was made to

improve vectors for antigen delivery or define more efficient vaccine strategies. Interestingly, in HCV vaccine design only few studies addressed which protein or peptide sequences are optimal for immunization (Yerly *et al.*, 2008). This aspect is relevant considering that HCV, like most RNA viruses, circulates *in vivo* as a complex population of different but closely related viral variants, commonly referred to as quasispecies (Martell *et al.*, 1992). The importance of sequence differences within the host at the level of quasispecies has been previously highlighted. The quasispecies serve as a pool from which variants that harbour beneficial mutations are selected. Accordingly, rapid selection of immune escape and drug resistance mutations has been observed (Cox *et al.*, 2005). Notably, the sequence differences between HCV genotypes are not the result of selection within individuals. Confirmation of that can be taken looking at the sequences present in the HCV database (http://hcv.lanl.gov/). Most differences are specific for the GT and reproducibly observed across all other isolates of the same type.

In this cross-sectional study, the importance of pre-existing immunity on control of viral replication was not addressed. For this purpose, it would be necessary to perform a longitudinal study in which the CD8+ T cell response is analyzed before re-exposure to HCV. However, we identified a number of subjects with undetectable viremia and evidence for immune control of two different genotypes indicating that in some patients re-infection can be controlled even with a different HCV GT. As already reported, spontaneous clearance of HCV infection occurs in 20-50% of acutely infected individuals and is associated with a broadly specific and vigorous cellular immune response (Takaki *et al.*, 2000). Therefore, the high number of HCV-RNA negative patients (41%) in our cohort maybe unexpected, taking into account that they are potentially repeatedly exposed.

Cross-reactive CD8+ T cells represent a high precious repertoire for HCV protection against heterologous infections. One of the goals in this work was to identify such cross-reactive responses. Cross-reactive T cell responses either target an epitope that is conserved across genotypes or different GT-specific variants of the epitope that are equally recognized. In this study we identified both kinds of CD8+ T cell responses. Notably, of the 28 epitopes identified only three conserved identical consensus sequence between the two genotypes. One of the conserved epitopes (HLA-B51-restricted epitope IPFYGKAI$_{1373\text{-}1380}$) was targeted by three different individuals. The HLA-B51 is not only highly conserved across all genotypes but also within different isolates of the same subtype indicating that sequence variation is not well tolerated in this region. A pre-existing T cell response against this epitope would likely

be beneficial upon infection with both GT1 and 3. In this study 25 of the identified epitopes harboured at least one amino acid difference between the consensus sequences of GT1b and GT3a. Based on the different degrees of cross-GT reactivity the identified epitopes were classified in cross-reactive, partial cross-reactive and non-cross-reactive epitopes. Three CD8 responses were cross-reactive against GT1 and 3 despite sequence differences in the targeted region. We identified three different patients with such a response, all three targeting different epitopes. Because these responses were unique it is unclear whether this degree of cross-reactivity is characteristic for all CD8+ T cell responses against these epitopes or this characteristic belongs only to these particular patients. It was previously suggested that existence of high-avidity CD8+ T cells that are able to cross-react with different sequence variants is associated with control of viremia (Yerly *et al.,* 2008). Although the total number of such responses is too small to draw solid conclusions in this study, it is striking that CD8+ T cells cross-reactive with both GT-specific variants were only observed in HCV-RNA negative subjects.

The remaining 22 epitopes were partially cross-reactive or not cross-reactive. One epitope was not classified. Responses against the HLA-A1-restricted epitope were detected only in cells expanded with the optimal described epitope ATDALMTGY but not with the consensus sequence ATDALMTGF. The Y to F mutation was previously described as an escape mutation (Neumann-Haefelin *et al.*, 2008).

One epitope (restricted by HLA-B13) particularly caught our interest for the following reason: we identified three subjects with the co-existence of two GT-specific T cell populations targeting the same region. One directed against the GT1 variant while a second was directed against the GT3 variant. The co-existence of two GT specific CD8+ T cell populations suggested that there had been an exposure to both antigens and priming of two distinct T cell populations. However, it is unclear whether this is a consequence of a simultaneous exposure to either genotypes or of two subsequent distinct infections with different genotypes. Although mixed infections with two HCV genotypes have been reported (Bowden *et al.,* 2005), they are overall rarely detected even in high-risk groups (Viazov *et al.*, 2000) arguing against simultaneous exposure to two HCV genotypes as a common event. It is unclear why co-existence of two T cell populations was only observed for the B13-epitope in our study. Interestingly, HLA-B13 is associated with delayed disease progression in HIV and this was mechanistically linked to T cell responses targeting HLA-B13-restricted epitopes in HIV gag (Honeyborne *et al.,* 2007). Of note, two of the three subjects here targeting the HLA-B13-

restricted epitope were HCV-RNA negative instead the third one chronically infected with GT1, was identified as a low carrier (1,785 IU/ml). In this case the autologous sequence of the infecting virus had a mutation in the last position of the epitope (RLGAVQNEA) (Fig. 5.17B). The HLA-B13-response described in this study is the first in HCV; however, the impact of HLA-B13 on disease outcome is unclear, as the low frequency of this allele in many populations may have precluded a solid analysis.

Although the risk profile was not specifically addressed, in the present study the previously reported risk behaviour suggests that multiple exposures to HCV may occur. The comparison of GT-specific T cell responses in this study allows conclusions about exposure to different HCV genotypes. Immunological evidence for exposure to different genotypes was previously reported for both CD4+ (Schulze Zur Wiesch *et al.*, 2007) and CD8+ T cells (Sugimoto *et al.*, 2005). Multiple studies have shown that a subgroup of chronically infected patients - typically those infected with genotypes other than GT1 - can display rather broad and vigorous T helper cell responses targeting GT1-derived antigens (Kaplan *et al.*, 2005). The role of these responses, (Diepolder *et al.*, 2004) and their contribution to viral control remained unclear. Our data suggest that it is unlikely that responses targeting non-autologous sequences contribute to viral containment as the autologous sequences are frequently not cross-recognized. According to these previous studies, multiple exposures could take place taking note of the discrepancy between T cell responses and the GT-specific sequence of the autologous virus in the targeted region (Sugimoto *et al.*, 2005). We similarly detected a mismatch between the GT of the autologous virus and the CD8 response in 10 of 39 subjects with chronic infection. In two cases this was supported by serotyping results through the detection of serotype 1 in GT3 infected subjects. However, a discrepancy between serotype and GT was detected in only four of 39 subjects with chronic HCV infection. One additional subject had a mixed GT1/GT3 serotype and five subjects were not typeable (Table 5.1). Recent studies suggest that the response against the autologous virus may preferentially be impaired and responses against a previously resolved heterologous GT persist (Sugimoto *et al.*, 2005). In line with this, there was a trend towards a stronger CD8 response against a heterologous GT in the present study, although this trend was not statistically significant. Importantly, the strength of these putative memory responses in subjects with chronic HCV infection did not reach the same magnitude as it has been observed in HCV-RNA negative subjects (Fig. 5.6). Of note, we detected only three epitopes where the autologous sequence fully matched with the mapped response. In the majority of cases where the CD8 response corresponded to the GT of the isolated virus a divergent epitope sequence was encoded by the

autologous virus. In the majority of these variants (five of eight; 62.5%) T cell recognition was decreased compared to the prototype consistent with previous studies (Neumann-Haefelin *et al.*, 2008).

In the present study we also identified five subjects with undetectable viremia and co-existence of distinct CD8 responses against each GT suggesting immune control of two different genotypes. Importantly, T cells active against both genotypes were more frequent in subjects with undetectable viremia (64.3% in HCV-RNA negative versus 15.3% in HCV-RNA positive). Although we certainly underestimate the true number of CD8 responses - as any epitope outside NS3 would have been missed - this raises the possibility that broad responses directed to different genotypes characterize a subgroup of individuals among IVDUs that are seemingly protected from chronic HCV infection. Prospective studies in high risk groups will be needed to confirm this observation.

Despite the presence of an activated immune system most subjects show continued viral replication. Survival of HCV, despite virus-specific CD8+ CTLs might be explained by several mechanisms: impaired priming by DC dysfunction (Sarobe *et al.*, 2003), selection of escape mutations (Hiroishi *et al.*, 2002), impaired cellular effector functions (proliferation, cytokine secretion, cytolytic activity) (Wedemeyer *et al.*, 2002) or suppression by CD8+Treg cells (Boettler *et al.*, 2005). In mouse models T cells activated in the liver are reported to have a dysfunctional phenotype. *In vitro* experiments revealed that naive T cells are activated by liver sinusoidal endothelial cells but do not differentiate into effector T cells. These T cells show a cytokine profile and a functional phenotype that is compatible with the induction of tolerance. Besides sinusoidal endothelial cells, other cell populations of the liver, such as DCs, Kupffer cells and perhaps also hepatocytes, may contribute to tolerance induction by deletion of T cells through induction of apoptosis (Knolle and Gerken, 2000). Upregulation of the pro-apoptotic molecule Bim has been described in virus-specific cells in HBV infection. Bim is one of the pro-apoptotic BH3-only group of proteins from the Bcl-2 family that plays a central role in the initiation of apoptosis signalling in lymphocytes (Bouillet *et al.*, 1999). Bim has been shown in murine models to be required for the shutdown of the CD8+ T cell response in the setting of a superantigenic stimulus (Hildeman *et al.*, 2002) or an acute viral infection (Pellegrini *et al.*, 2003). Conversely, downregulation of Bim is critical for CD8+ T cell memory survival in the absence of antigen (Sabbagh *et al.*, 2006). A comparison of chronically HBV-infected patients and patients who spontaneously resolved HBV-infection found a higher expression of Bim in the former group.

We therefore tried to address the role of Bim in HCV infection and aimed to characterize the phenotype of HCV-specific CD8+ T cells in more detail. During our work it became evident that the autologous sequence of the circulating virus had a strong influence on the phenotype of specific CD8+ T cells. Upon spontaneous clearance of an infection, virus-specific cells typically acquire a memory phenotype with upregulation of the memory marker CD127. In line with this specific CD8+ T cells from HCV-RNA negative IVDUs showed high CD127 expression. Interestingly, patients with an autologous sequence - that differed from the prototype epitope sequence and that was not recognized by the CD8+ T cells - also showed upregulation of CD127. A recent study that systematically evaluated this observation demonstrated that by analysis of CD127 we can distinguish two groups: 1.) A functional CD8+ memory population that is directed against a sequence which is absent in the patient either by antigen escape or by re-infection with a different GT. 2.) A fully exhausted CD8+ T cell population that targets the autologous virus although it is dysfunctional and has poor effector functions (Bengsch *et al.*, 2010). We expected that the latter group of CD8+ T cells would be continuously activated in the liver and potentially also have upregulation of the pro-apoptotic molecule Bim. This would also explain the difference not only in the quality of HCV-specific CD8+ T cells but also in the quantity of CD8+ T cells between patients with chronic HCV infection and those who spontaneously resolved infection. Recent studies suggest that lack of vigorous CD8 responses in patients with chronic infection is the result of secondary rather than primary CD8+ T cell failure. Cox *et al.* in 2005 reported that CD8+ T cells are primed during acute infection in HCV-RNA positive as well as in HCV-RNA negative subjects but these responses are not maintained in those who continue to viral persistence. Deletion of these virus-specific CD8+ T cells by apoptosis would be an attractive explanation for this event, however, we were unable to identify any differences in the expression levels of Bim between the different patient groups here even when we controlled for the presence of the correct antigen.

The number of CD8 responses that have been analyzed is still rather low. Unfortunately, the challenge to find patients with strong CD8+ T cell responses is relatively high, especially in chronically infected individuals. HCV-specific CD8+ T cells were detected and analyzed on cryo-preserved PBMCs by staining with MHC class I-Pentamers. It will therefore be necessary to increase the number of patients for this analysis before coming to any final conclusions. Moreover, future studies should also include analyses of other members from the Bcl-2 family that could either promote or block cell survival to better define the role that deletion of peptide-specific CD8+ T cells has in HCV persistence.

It is estimated that the incidence of complications from chronic hepatitis, namely, liver cirrhosis and hepatocellular carcinoma, will increase in the next few decades (Armstrong *et al.*, 2000). At present, chronic liver disease associated with HCV is the leading indication for liver transplantation (LT) in many Western countries (Willems *et al.*, 2002). Recurrence of HCV infection is (almost) universal after orthotropic LT (Böker *et al.*, 1997). Although a small proportion of the patients may progress to a ‘‘healthy’’ carrier state with viremia but normal aminotransferase concentrations and normal liver and minimal histological changes (Gane *et al.*, 1996), progression to histological chronic hepatitis occurs in the majority of HCV-positive transplant patients (Feray *et al.*, 1999).

Different studies have demonstrated the safety and relative efficiency of adoptive-transfer of specific T cell clones to treat viral infections (Pahl-Seibert *et al.*, 2005). Injections of cytomegalovirus CMV and Epstein-Barr virus EBV-specific T cell clones or lines into patients who received an allogenic bone marrow transplant have a protective effect against CMV and EBV infection (Walter *et al.*, 1995; Rooney *et al.*, 1998). Injections of HIV-specific CTL clones to HIV patients lead to a temporary decrease of the number of CD4+ T cells infected by HIV (Brodie *et al.*, 1999). Therefore adoptive immunotherapy with antigen-specific CD8+ T cells seemed to represent a promising application against recurrence of HCV infection after LT. The ultimate goal of T cell generation for adoptive therapy is to generate T cells that exhibit optimal qualities in terms of function, expansion, survival, homing and persistence that can help the eradication of virus infection (Sadelain *et al.*, 2003). There are different strategies for the generation of epitope-specific CD8+ T cell line. Virtually any cell expressing HLA molecules can be used as a potential APC. However, APCs are not equal in their ability to stimulate T cells. The professional APCs, which include DCs, macrophages and B cells, are better equipped than other cell types to maximize T cell stimulation.

In this study we decided to induce the priming of naïve CD8+ T cells using the immunodomint HLA-A2 HCV-peptide: CINGVCWTV, highly recognised in our IVDUs cohort. Monocyte-derived DCs from a healthy patient were co-cultured with autologous PBMCs. The expansion of specific T cells was obtained after two rounds of stimulation with peptide pulsed DCs and exogenous addiction of cytokines (IL-2, IL-7 and IL-15). The successful detection of HCV-specific T cells in polyclonal cultures through IFN-γ intracellular staining and MHC class I-Pentamer staining represented a positive achievement to further apply for the investigation and development of prophylactic and immunotherapeutic vaccines.

The future experiments will therefore focus on the establishment of long-lasting and functional CD8+ T cell lineages targeting multiple immunodominant HCV epitopes. To elucidate the role that various HCV peptides have in the induction of T cell responses and to identify the epitopes involved in virus control or clearance.

7 Summary

GT-specific sequence differences are certainly important in the antiviral immune response against HCV. The present study for the first time systematically analyzed the degree of cross-GT reactivity of HCV-specific T cells at the epitope level. To determine the impact that these GT-specific mutations have on the CD8+ T cell responses we analyzed a group of IVDUs including subjects with undetectable viremia and individuals with HCV GT1 and HCV GT3 infection. Overlapping peptide sets covering local consensus sequences from HCV GT1b and 3a were used to analyze the immune responses of each individual. The total strength and number of HCV-specific T cell responses detected were higher in the HCV-RNA negative group when compared to the GT1 or GT3 infected groups. Interestingly in the chronically infected subjects CD8+ T cells preferentially targeted the heterologous GT. These responses more possibly are memory CD8+ T cells of a previously resolved heterologous infection.

Cross-reactive CD8+ T cells are a high precious repertoire for HCV protection against heterologous infections. In this work we wanted to identify cross-reactive peptides that could be useful for the development of a broad-protective vaccine. 22% of the 28 epitopes identified was cross-reactive. This includes epitopes where the targeted sequence was identical in GT1 and GT3 and epitopes that targeted both GT-specific variants. Although the majority of these cells showed only limited cross-reactivity, we were able to identify a number of subjects with T cells active against both genotypes. Interestingly, T cells active against both genotypes were preferentially detected in HCV-RNA negative subjects. This demonstrates that CD8 responses targeting different HCV genotypes can be primed in the same individual and that these responses are potentially linked to protection from chronic infection. In the face of a heterogeneous genotype distribution in many areas of the world, this has important implications for vaccine design.

To define the determinants of a successful versus an unsuccessful CD8+ T-cell response in HCV infection we decided to perform phenotypic studies on memory HCV-specific CD8+ T cells. We evaluated the expression level of the memory marker CD127 and of the pro-apoptotic protein Bim in individuals with different clinical outcomes to highlight the role that peripheral deletion of peptide-specific CD8+ T cells could have on the paucity of CD8+ T cell responses in chronically infected subjects. During our work it became evident that the

autologous sequence of the circulating virus had a strong influence on the phenotype of specific CD8+ T cells. Upon spontaneous clearance of an infection, virus-specific cells typically acquire a memory phenotype with upregulation of the memory marker CD127. In line with this specific CD8+ T cells from HCV-RNA negative IVDUs showed high CD127 expression. Interestingly, patients with an autologous sequence - that differed from the prototype epitope sequence and that was not recognized by the CD8+ T cells - also showed upregulation of CD127. We expected that the latter group of CD8+ T cells would be continuously activated in the liver and potentially also have upregulation of the pro-apoptotic molecule Bim. This would also explain the difference not only in the quality of HCV-specific CD8+ T cells but also in the quantity of CD8+ T cells between patients with chronic HCV infection and those who spontaneously resolved the infection. Unfortunately we were unable to identify any differences in the expression levels of Bim between the different patients groups here analyzed even when we controlled for the presence of the correct antigen. The number of CD8 responses that have been analyzed is still rather low. It will therefore be necessary to increase the number of patients for this analysis before coming to any final conclusions.

The last part of this study was focused on the establishment of an assay for the priming of naïve CD8+ T cells with HCV immunodominant epitopes. For this purpose monocytes from healthy individuals were matured into DCs and co-cultured with autologous PBMCs in presence of the immunodominant HCV-A2-peptide 1073. The successful detection of HCV-specific T cells in polyclonal cultures was analyzed through IFN-γ staining and MHC class I-Pentamer staining. CD8+ T cells are thought to be one of the major responsible for clearance in HCV infection, therefore the establishment of a peptide-specific functional CD8+ T cell line is an interesting tool that could be applied as adoptive immune transfer for example after liver transplantation to prevent reinfection of the allograft.

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9 Appendix

9.1 Abbreviations

Abbreviations	Full name
aa	Amino acid
ALT	Alanine aminotransferase
APC	Allophycocyanin
APC	Antigen presenting cell
°C	Degree Celsius
CARD	Caspase activation and recruitment domain
CD	Cluster of differentiation
CLDN1	Claudin-1
CMIA	Chemiluminescent microparticle immunoassay
CMV	Cytomegalovirus
C-terminal	Carboxy-terminal
CTL	Cytotoxic T cell
DC	Dendritic cell
DCF	Drug consumption facilities

DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic
DNase	Deoxyribonuclease
dNTPs	Deoxynucleotide triphosphates
dsRNA	Double-stranded RNA
EBV	Epstein-Barr virus
EMCDDA	European Monitoring Centre for Drugs and Drug Addiction
ER	Endoplasmatic reticulum
EtOH	Ethyl alcohol
FACS	Fluorescence Activated Cells Scanner (Flow cytometer)
FCS	Fetal Calf Serum
Fig	Figure
FITC	Fluorescein isothiocyanate
Foxp3	Forkhead box P3
FSC	Forward scatter
g	Gram
GM-CSF	Granulocyte-Macrophage colony-stimulating factor
GPT	Glutamic-Pyruvic Tansaminase

GT	Genotype
h	Hour
H_2O	Water
HAV	Hepatitis A virus
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HCC	Hepatocellular carcinoma
HIV	Human Immunodeficiency Virus
HVR	Hyper variable region
HLA	Human leucocyte antigen
IFN	Type I interferon
IFNAR	Type I interferon receptor
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
IPS-1	Interferon-β promoter stimulator 1
IRES	Internal ribosome entry site
IRF-3	Interferon regulatory factor 3

ISG	Interferon-stimulated gene
IU	International unit
IVDUs	Intravenous Drug Users
Kb	Kilo base
kDa	Kilodalton
l	Liter
LCMV	Lymphocytic choriomeningitis virus
LIL	Liver infiltrating lymphocyte
LT	Liver transplantation
M	Molar
MACS	Magnetic activated cell sorting
mg	Milligram
MHC	Major histocompatibility complex
min	Minute
ml	Millilitre
µl	Microlitre
mM	Millimolar
mol	Mole

mRNA	messenger RNA
N.A.	Not applicable
NANBH	Non-A, non-B hepatitis
NFκB	Nuclear factor κB
NK	Natural killer cell
NKT	Natural killer T cell
nm	Nanometer
NR	Non-responder
NT	Not typable
N-terminal	Amino-terminal
NTPase	Nucleoside triphosphatase
OAS	2’-5’ oligoadenylate synthetases
OCLN	Occludin
ORF	Open reading frame
OST	Opiate substitution treatment
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction

PD-1	Programmed death 1
PD-L1	Programmed death ligand 1
PE	Phycoerythrin
peg-IFN-α	Pegylated interferon alpha
PKR	Protein kinase R
RdRp	RNA-dependent RNA polymerase
RIG-I	Retinoic acid inducible gene-I
RNA	Ribonucleic acid
RNase L	Ribonuclease L
rpm	Revolutions per minute
RPMI-1640	Roswell Park Memorial Institute Medium 1640
RT	Room temperature
RT-PCR	Reverse transcription-polymerase chain reaction
SD	Sensitizing dose
SR-B1	Scavenger receptor class B member 1
SSC	Sideward scatter
STAT	Signal Transducer and Activator of Transcription
STAT-C	Specifically targeted antiviral therapy for HCV

SVR	Sustained virological response
TAP	Transporter associated with antigen processing
TCR	T cell receptor
TGF-β	Transforming Growth Factor-beta
TIR	Toll/Interleukin-1 receptor domain
TJ	Tight junction
TLR	Toll-Like Receptor
TNF-α	Tumor necrosis factor-alpha
Treg	Regulatory T cells
U	Unit
U	Uracile
UTR	Untranslated region
VL	Viral load
WHO	World Health Organisation

9.2 List of figures

9.3 List of tables

10 Publications

1. Schulte I, Hitziger T, **Giugliano S**, Timm J, Gold H, Heinemann F, Yuri Khudyakov Y, König C, Castermans E, Mok JY, van Esch WJE, Bertoletti A, Schumacher TN, Roggendorf M. Acute hepatitis A is associated with a multispecific CD8+ T cell response in HLA-A2 positive patients. In press ***J Hepatol*** 2010.

2. **Giugliano S**, Oezkan F, Bedrejowski M, Kudla M., Reiser M, Viazov S, Scherbaum N, Roggendorf M, and Timm J. Degree of Cross-Genotype Reactivity of Hepatitis C Virus–Specific CD8+ T Cells Directed Against NS3. ***Hepatology*** 2009; 50(3):707-16.

3. Salloum S, Oniangue-Ndza C,Neumann-Haefelin C, Hudson L, **Giugliano S**, aus dem Siepen M, Nattermann J, Spengler U, Lauer G.M, Wiese M, Klenerman P, Bright H, Scherbaum N, Thimme R, Roggendorf M, Viazov S, and Timm J. Escape from HLA-B*08-Restricted CD8 T Cells by Hepatitis C Virus Is Associated with Fitness Costs. ***J Virol*** 2008; 82(23): 11803–11812.

11 Curriculum Vitae

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12 Declaration (Erklärungen)

Erkärung:

Hiermit erkläre ich, gem. § 6 Abs. (2) f) der Promotionsordnung der Fakultäten für Biologie und Geografie, Chemie und Mathematik zur Erlangung der Dr. rer. nat., dass ich das Arbeitgebiet, dem das Thema ,,Analysis of the CD8+ T cell response against hepatitis C virus in intravenous drug users“ zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von Silvia Giugliano befürworte.

Essen, den 08.12.10 _____

Priv.-Doz. Dr. med. Jörg Timm

Erklärung:

Hiermit erkläre ich, gem. § 7 Abs. (2) c) + e) der Promotionsordnung Fakultäten für Biologie und Geografie, Chemie und Mathematik zur Erlangung des Dr. rer. nat., dass ich die vorliegende Dissertation selbständig verfasst und mich keiner anderen als der angegebenen Hilfsmittel bedient habe.

Essen, den 08.12.10 _____

Silvia Giugliano

Erklärung:

Hiermit erkläre ich, gem. § 7 Abs. (2) d) + f) der Promotionsordnung der Fakultäten für Biologie und Geografie, Chemie und Mathematik zur Erlangung des Dr. rer. nat., dass ich keine anderen Promotionen bzw. Promotionsversuche in der Vergangenheit durchgeführt habe und dass diese Arbeit von keiner anderen Fakultät/Fachbereich abgelehnt worden ist.

Essen, den 08.12.2010 _____

Silvia Giugliano