Genetic heterogeneity of virus isolates in chronic hepatitis C patients infected in a single-source HCV outbreak - special structural features and evidences that both humoral and cellular immune responses contribute to evolution of the envelope 2 viral protein

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Content

1 Introduction

The hepatitis C virus (HCV) was identified in 1989, although the existence of the non-A, non-B hepatitis virus, as a causative agent of post-transfusion hepatitis, was postulated much earlier [1,2]. Currently, about 3 % of the world population (~ 160 million people) is infected with HCV. Only in 20 to 30 % of cases of acute HCV infections the virus is cleared spontaneously [3]. In other 70 to 80 % of cases the initial infection leads to a persistent viremia accompanied by hepatic inflammation and fibrosis of variable degrees; these patients have an increased risk to develop liver cirrhosis and hepatocellular carcinoma (HCC) [4].

The standard therapy of chronic HCV infection at the moment consists of pegylated alpha interferon (IFN-α) in combination with ribavirin and is effective only in 40-60 % of infected individuals depending on the HCV genotype. Recently, two protease inhibitors, boceprevir and telaprevir, that target the viral NS3/4A protease have been introduced into medical practice to treat patients with chronic hepatitis C. Application of these inhibitors in combination with the standard IFN-α and ribavirin therapy increased the sustained virological response (SVR) against HCV genotype 1 strains up to 75 % [5,6].

1.1 HCV genome organization

The hepatitis C virus is a member of the Flaviviridae family, which is divided into three genera. The flavivirus group includes yellow fever virus, dengue fever virus, tick-borne encephalitis virus and Japanese encephalitis virus, whereas classical swine fever virus, bovine viral diarrhea virus, and Border disease virus belong to the pestiviruses. HCV is a member of the hepacivirus genus (also includes the GB virus B and GB virus C) and is divided into seven major genotypes and numerous subtypes [7,8].

HCV is an icosahedral virus with a diameter of 50 to 70 nm. The viral particles have an envelope formed by a host-derived lipid bilayer that anchors the viral envelope glycoproteins E1 and E2 [9]. The viral envelope encloses the spherical nucleocapsid, which consists of multimerized core protein (C), and contains a single positively oriented RNA genome molecule of 9.6 kilobases (kb). The single open reading frame (ORF) encodes a polyprotein of 3000 amino acids (aa) and is flanked by 5‘ and 3‘ untranslated regions (UTR), which are essential for RNA replication and polyprotein translation [10]. The most conserved region of the HCV genome is the 5‘UTR with a size of 341 nucleotides (nt). It is located upstream of the codon for initiation of translation, and contains four highly conserved structured domains (I-IV) with a number of stem-loops and one pseudoknot [11,12]. The IRES (internal ribosome entry site) is formed by the domains II, III and IV of the 5‘-UTR in combination with the first 12 to 30 nt of the core-coding region [13]. The

canonical translation initiation factors are not needed for the translation of the HCV polyprotein due to a capability of the IRES to form a stable pre-initiation complex with the ribosomal 40S subunit. The 3‘UTR consist of approximately 225 nt and is separated into three regions, starting with a so-called variable region of 30 - 40 nt, followed by a long poly-(UC) tract, and a highly conserved 3‘-terminal region of 98 nt (3‘X region) that contains three stem-loop structures SL1, SL2 and SL3 [14-16]. The 3‘X region and 52 nt located upstream of the poly-(U/C) tract are essential for viral RNA replication, and the remaining part of the 3‘UTR functions as enhancer sequence for viral replication [17-20].

The viral polyprotein is processed by virus and host derived proteases and cleaved into 10 polypeptides: core (C), envelope 1 (E1), envelope 2 (E2), p7 (nonstructural protein 1, NS1), nonstructural protein 2 (NS2), nonstructural protein 3 (NS3), nonstructural proteins 4A and 4B (NS4A and NS4B), and nonstructural proteins 5A and 5B (NS5A and NS5B) (Fig.1.1)

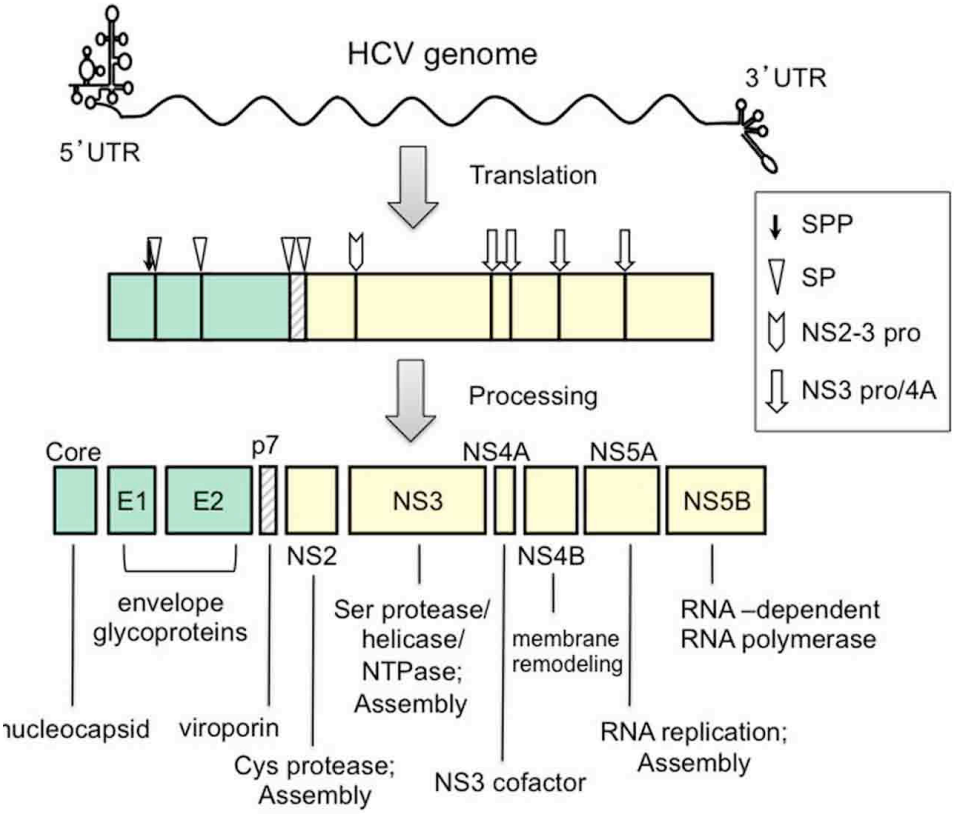

Fig. 1.1 The HCV genome and polyprotein.

The RNA genome comprises a 9.6-kb RNA of plus strand polarity. Post-translational cleavages by SPP (signal peptide peptidase), SP (signal peptidase), NS2-3 pro (NS2–NS3 cysteine protease), and NS3 pro/4A (NS3 serine protease and NS4A complex) lead to the production of functional HCV proteins. Functions of each protein in the viral lifecycle are indicated below the open reading frame [21].

HCV is characterized by high genome variability and is classified into the seven major genotypes or genetic groups [22]. Most of the genotypes are further divided into a number of subtypes. In HCV infected individuals the HCV pool is present as a population of closely related but slightly different genetic variants or quasi-species. Such a population of distinct genetic variants of the same virus is formed due to a combination of several factors. One of them is an inability of the HCV RNA-dependent RNA-polymerase (NS5B protein) to perform a proofreading function, which results in a high mutation rate of approximately $1\text{-}3x10^{-3}$ substitutions/site/year [23]. Another factor is the high viral production rate (up to 1012 virions/day) in the infected host [24]. The viral quasi-species pool is subject of permanent change during both acute and persistent infection and this process seams to play a very important role in HCV biology and pathogenesis [24-26]. In particular, it should be mentioned that the existence of a swarm of quasi-species provides a basis for mechanisms by which HCV avoids the host immune surveillance.

1.2 Characteristics and functions of HCV proteins

Depending on the genotype of HCV the ORF consists of 9024 to 9111 nt and encodes at least 10 proteins, including three structural proteins (C, E1, and E2), and seven nonstructural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A. NS5B). The HCV polyprotein is processed by host cellular and viral proteases (Fig.1.1.).

1.2.1 Core

The first structural protein, the HCV core, is highly basic protein with RNA-binding capacity, which forms the viral capsid by multimerization. It is released as a precursor of 191 aa (23 kDa, P23), whereas the predominant mature core protein has a size of 21 kDa [27] and can be divided into three functional domains: the 120 aa N-terminal hydrophilic domain D1 is involved in RNA binding and mediates nuclear localization by three predicted nuclear localization signals (NLS) [28-30] 50 aa C-terminal hydrophobic domain D2 is responsible for the association of core protein with endoplasmic reticulum (ER) membranes, outer mitochondria membranes and lipid droplets [30,31]. The last ca. 20 aa of the core protein serve as a signal peptide for the downstream located E1 protein [32-34]. Besides being involved in particle formation, the core protein interacts with multiple host cellular pathways and proteins with potentially important roles in the viral life cycle [35]. In particular, the core protein transcriptionally up-regulates growth-related genes in Huh-7 cell [36]. It also demonstrates pro- and anti-apoptotic effects [37-39], and was suggested to be involved in tissue-damage and progression of liver fibrosis [40]. Furthermore, the core protein

might regulate the activity of cellular genes like c-myc and c-fos, and influence transcription of other viral promotors [41,42]. In transgenic mice the expression of core induces the development of HCC, and by the induction of lipid droplet formation it may play a direct role in steatosis development [43-45].

1.2.2 ARFP

The alternate reading frame protein (ARFP) of approximately 160 aa., which seems to be a product of a frame shift in the N-terminal core region during polyprotein synthesis, has been described [46]. Presence of this protein in natural HCV infection was confirmed by the detection of antibodies against ARFP in chronically infected patients. The role of ARFP during HCV lifecycle is still unknown, although, some indications of its contribution to viral persistence have been reported [47].

1.2.3 E1/E2

E1 and E2 are glycoproteins of the HCV viral envelope with sizes of 33 - 35 and 70 - 72 kDa, respectively, form noncovalent heterodimers [48] that are essential for viral fusion and entry [49,50]. Classified as type I transmembrane glycoproteins, the E1 and E2 have N-terminal ectodomains of 160 and 334 aa, respectively, and contain up to 5 and 11 glycosylation sites, respectively, as well as numerous cysteine and proline residues, and short C-terminal transmembrane domains of approximately 30 aa [51,52].

The E2 protein bears three hyper-variable regions with differences in the aa sequence of up to 80 % between different HCV genotypes and even sometimes between isolates of the same virus strain [53]. The hypervariable region 1 (HVRI) consists of the first N-terminal 27 aa of E2 and presumably contains a major HCV neutralizing epitope [54,55]. Despite a high sequence variability of the HVRI, there is a conservation of aa at particular positions, as well as a preservation of physiochemical properties of aa residues at other positions [56]. Overall, these findings suggest that both variability of the aa content and conservation of the molecular conformation of HVRI are important and reflect an essential role of HVRI in the viral life cycle [57,58]. Thus, the variability of the HVRI seems to be a basis for the virus to escape from the host humoral immune response [57]. The HVRI as a region with positively charged residues at particular sequence positions might interact with negatively charged molecules found on surfaces of host cells and thereby play a role in host cell recognition, attachment, tissue and cell compartment localization [49,57-59]. The HVRI interacts with high-density lipoproteins (HDL) and scavenger receptor B type I (SR-BI) [60,61]. Furthermore, the HVRI was found to modulate the binding of E2

to the CD81 receptor [62-64]. E2 contains two further hypervariable regions: the hypervariable region 2 (HVR2) and the intergenotypic variable region (igVR). These regions are suggested to be involved in heterodimerization of E1 and E2, as well as in viral infectivity [65,66].

Not much is known about functions of E1, except that it's folding is dependent on the co-expression of E2 [67,68] and that the E1/E2 heterodimer is responsible for the first steps of HCV interaction with the host cell [69,70]. Some data suggest that E1 might play a role in fusion of the virus with membranes inside the cytoplasm [69].

1.2.4 P7

The first nonstructural protein p7 with a size of 63 aa belongs to the viroporin family and might function as a calcium ion channel [71,72]. It was shown to be essential for assembly and release of infectious particles, as mutations in the cytoplasmic loop of p7 suppressed infectivity in experiments on chimpanzee [73,74].

1.2.5 NS2

The NS2 protein with a size of 21 - 23 kDa is a non-glycosylated transmembrane protein bearing two internal signal sequences (aa 839 - 883 and aa 928 - 960) that mediate association with the ER membrane [75,76]. In combination with the N-terminal part of the NS3 protein, NS2 forms the NS2-3 protease, which acts as a zinc-dependent metalloprotease and cleaves between NS2 and NS3 [32,77,78]. After auto-cleavage from NS3 the NS2 protein looses its catalytic activity and is directed to the proteasome for degradation. It was shown that NS2 is essential for the production of infectious virus particles [79].

1.2.6 NS3/NS4A

The NS3 protein with a size of 67 kDa is multifunctional protein with an N-terminal located serine protease domain and a C-terminal helicase/NTPase domain. The NS4A protein (54 aa) acts as an activity enhancing cofactor and forms a heterodimer with NS3, the NS3-NS4A protease, which is essential for the viral life cycle. The NS3-NS4A protease catalyzes the cleavage between NS3/NS4A, NS4A/NS4B, NS4B/NS5A and NS5A/NS5B [80-82]. It was shown that the NS3-NS4A protease interacts with different host cell pathway, like antagonizing the dsRNA-dependent interferon regulatory factor 3 (IRF-3) [83] or hampering the toll-like receptor 3 signaling upstream of IRF-3 [84]. Thus, the NS3-NS4A protease is an important factor for the virus to counteract the host innate immune response. The C-terminal helicase/NTPase domain of NS3 belongs to the helicase superfamily-2 and possesses several functions, like RNA binding, RNA

unwinding and RNA-stimulated NTPase activity [85,86]. It was also suggested that the NS3 protein might be involved in development of hepatocellular carcinoma [87,88].

1.2.7 NS4B

The NS4B integral membrane protein with a size of 261 aa (27 kDa) is located at the ER membrane [89,90]. As a part of the replicase complex the NS4B protein functions as an ER membrane anchor for the replication complex, thereby creating a site for viral genome synthesis [91-93]. Furthermore, NS4B is involved in the process of virus particle assembly and release [94].

1.2.8 NS5A

The phosphorylated zinc-metalloprotein NS5A with a size of 56 - 58 kDa is membrane associated and is essential for viral replication. It plays a role in formation of the viral replication complex and regulates host cellular pathways [95]. Additionally, the NS5A protein is able to interact directly with the RNA-dependent RNA-polymerase NS5B and influence its activity by a mechanism that still needs to be clarified [96].

1.2.9 NS5B

The NS5B protein of 65 kDa is a tail-anchored membrane protein functioning as an RNA-dependent RNA-polymerase (RdRp) [97-100]. NS5B can interact with cellular factors as cyclophilin B, which is likely to regulate viral replication by influencing the RNA binding capacity of NS5B [101]. Many anti-HCV drugs target the RdRp-activity of NS5B [102-105].

1.3 HCV life cycle

1.3.1 Viral attachment and entry

The interaction of HCV envelope proteins with host cellular heparan sulfate proteoglycans (HSPG) is thought to be the initial step in attachment of HCV to target followed by binding to another receptor/receptor complex with high affinity that mediates viral entry via clathrin-dependent endocytosis [59,106]. Several cellular molecules were identified as members of the receptor complex: the low density lipoprotein (LDL) receptor (LDLR), dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), and liver/lymph node-specific intracellular adhesion molecule-3-grabbing non-integrin (L-SIGN), CD81, Scavenger receptor class B member 1 (SR-BI), claudin-1 and occludin-1 [107,108]. Figure 1.2 shows a model of cell receptors needed for successful infection of cells.

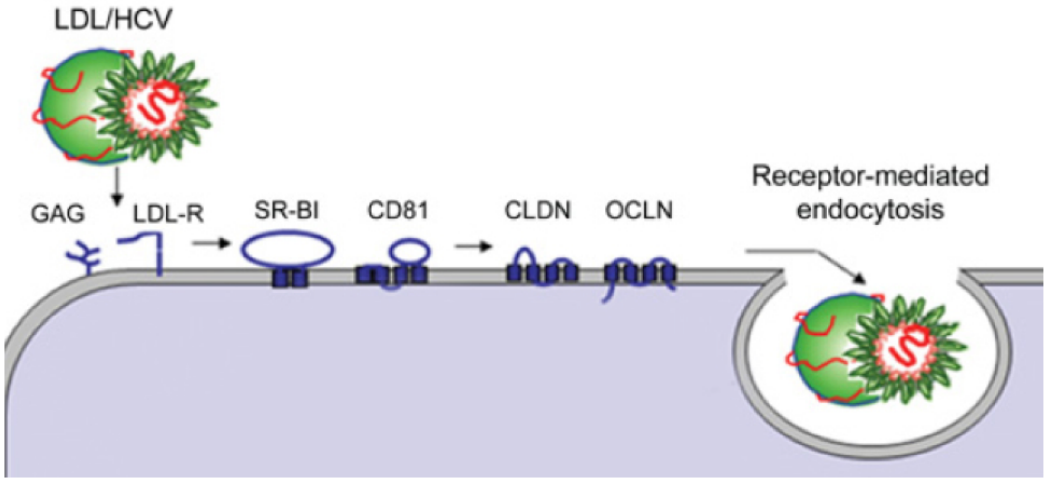

Fig. 1.2 Model of cell surface molecules essential for HCV entry [109].

1.3.1.1 CD81

The human CD81 molecule with a size of 25 kDa belongs to the tetraspanin or transmembrane 4 superfamily. It is found at the surface of numerous cell types and is involved in pleiotropic activities such as cell adhesion, motility, metastasis, cell activation, and signal transduction [110]. CD81 contains four hydrophobic transmembrane regions (TM1 - TM4) and two extracellular loop domains of 28 and 80 aa, respectively. The small extracellular loop (SEL) between TM1 and TM2 is needed for optimal surface expression of the large extracellular loop (LEL) that is located between TM3 and TM4 and mediates binding of HCV by interactions with E2 [111,112]. Among different species the intracellular and transmembrane domains of CD81 are highly conserved, whereas the LEL is highly variable. The only species that can be infected by HCV are humans and chimpanzees, due to their similarities in the LEL of CD81 [113,114]. The human hepatoma cell lines HH29 and HepG2 lack the expression of CD81 and do not support HCV infection in cell culture. Their permissiveness to infection with HCV pseudo-particles (HVCpp) could be restored by transfection with and expression of human CD81. A reduced rate of viral infection was achieved through silencing of CD81 by CD81-specific small interfering RNA (siRNA) [115,116]. The expression of human CD81 in murine fibroblasts was shown to be insufficient to support HCV entry and mice transgenic for human CD81 remained resistant to HCV infection [117,118]. Inhibition of viral entry by anti-CD81 antibodies was suggested to occur at the post-binding step [119]. These findings support the essential role of CD81 in the process of viral entry, but also indicate that other host cellular factors are needed to establish HCV infection.

1.3.1.2 SR-BI

The scavenger receptor B type I (SR-BI) with a size of 82 kDa is a glycoprotein bearing a large extracellular loop that is anchored by C- and N-terminal transmembrane domains with short cytoplasmic tails, was shown to serve as another receptor for HCV E2 [120,121]. SR-BI is expressed at high levels in hepatocytes, in steroidogenic cells, as well as in human monocyte derived dendritic cells [120,122,123]. Its physiological ligands are high-density lipoproteins (HDL) and low-density lipoproteins (LDL), which are internalized after binding by clathrin-independent endocytosis that mediates the uptake of cholesterol and recycling [124]. HDL was shown to enhance viral entry by a mechanism that depends on the physiological function of SR-BI in terms of lipid transport. The LEL was suggested to be responsible for binding of HCV through interaction with HVRI of HCV E2 and deletion of HVRI significantly reduced HCVpp infectivity [49,61,121,125]. In a chimpanzee model, different HCV clones with deletion of the HVRI showed attenuated infectivity [126]. Antibodies specific for SR-BI were shown to reduce but not completely block the binding of HCV to hepatocytes, indicating that other molecules are also involved in this process [127].

1.3.1.3 Claudin-1 and occludin

Recently, two tight-junction proteins claudin-1 (CLDN1) and occludin (OCLN) have been reported as two additional essential HCV entry factors [107]. These proteins seem not to interact directly with HCV virions and, most probably, are involved in a late post-binding step. It was suggested that CLDN1 can interact with CD81 and that this interaction might be a crucial step in formation of the HCV receptor complex [128]. Several cell lines were rendered susceptible to HCVpp and HCVcc entry through transient expression of CLDN1, whereas silencing of CLDN1 by specific siRNA in permissive cells caused a significant reduction of HCV entry [129]. Specific antibodies against CLDN1 were able to inhibit HCV entry by constraining the interaction between HCV E2 and CLDN1, as well as between CD81 and CLDN1 [130]. Two other tight junction proteins, claudin-6 and claudin-9, are possibly also functioning as co-receptors for HCV [131]. The exact role of OCLN in the HCV entry mechanisms is not completely clear.

1.3.1.4 EGFR and ephrin receptor A2

More recently, two receptor tyrosine kinases, the epidermal growth factor receptor (EGFR) and the ephrin receptor A2, were identified to be required for HCV entry. These proteins may function as modulators of interactions between CD81 and CLDN1 [132].

1.3.1.5 DC-SIGN and L-SIGN

Different studies suggest that the dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) and the liver/lymph node-specific intercellular adhesion molecule-3 (I-CAM-3)-grabbing integrin (L-SIGN) could determine the tissue tropism of HCV and might play a role in viral pathogenesis [133-136]. The type II integral membrane protein DC-SIGN with a size of 44 kDa is highly expressed on myeloid dendritic cells and its interaction with ICAM-3 leads to activation of T-cells [137]. L-SIGN with a 77 % sequence identity to DC-SIGN is highly expressed at the cell surface of liver and lymph node endothelial cells, but not on DCs [138]. It also shares functional similarities with DC-SIGN in its interaction with ICAM-3. One study with HCV pseudo-particles (HCVpp) showed rapid internalization upon interaction of HCV E2 with DC-SIGN and L-SIGN [139], whereas another did not report this [135].

1.3.1.6 LDLR

The low-density lipoprotein (LDL) receptor (LDLR) is a glycoprotein with a single transmembrane domain that is involved in the endocytic transport of lipoproteins, mainly cholesterol-rich LDLs [140]. It was demonstrated that HCV virions complexed with LDL enter their target cells by utilizing the LDLR [141,142]. Specific antibodies against LDLR or the addition of high levels of LDL were shown to inhibit HCV infection of hepatocytes in cell culture [143].

1.3.2 HCV replication, particle assembly and release

Cellular attachment and interaction of HCV with the receptor complex leads to endocytosis of the virus particles, and is followed by fusion of viral and endosomal membranes, in a pH-dependent process, leading to the release of the viral genome to the cytoplasm of host cells [8,144,145]. The released viral RNA serves as mRNA for synthesis of the HCV polyprotein, as well as template for production of the negative RNA strand. The negative strand then acts itself as a template for synthesis of new positive-oriented viral RNAs. The new positive RNAs are subsequently used for synthesis of negative strands of RNA, which are either used for protein expression or for packaging into virions [97]. HCV polyprotein synthesis is mediated by the HCV IRES in cap-independent manner through recruitment of several cellular proteins, like the eukaryotic initiation factors (eIF) 2, 3, and 5B, and viral proteins [146-148]. Subsequently, a functional 80S ribosome complex is formed that initiates viral protein production. The processed HCV proteins are retained at the membrane of the endoplasmic reticulum (ER). As a consequence of HCV protein expression, the intracellular membranes are altered to form the „membranous web“, which is derived from ER membranes and represents the site of viral

replication [97,149]. The viral particle assembly is initiated by interactions of HCV core protein with viral genomic RNA and seems to occur on lipid droplets (LDs), as core protein was shown to coat the surface of LDs [150]. The HCV structural proteins were detected in the ER and Golgi apparatus, suggesting an important role of these cell organelles in the later maturation process [151]. The viral progeny virions might be released from host cells via the constitutive VLDL secretory pathway [152](Fig.1.3).

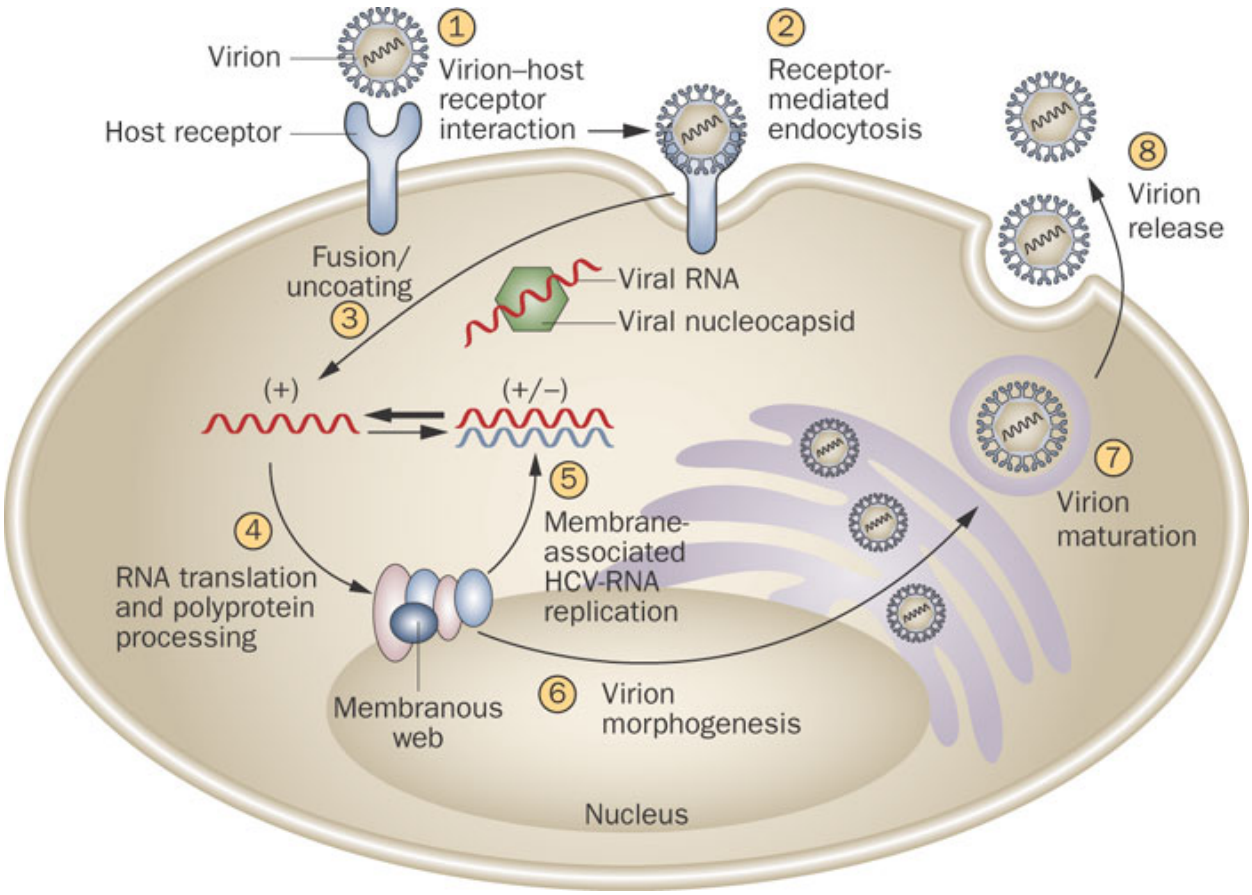

Fig. 1.3 Extracellular HCV virions interact with receptor molecules, at the cell surface (1) and undergo receptor-mediated endocytosis (2). Following HCV glycoprotein-mediated membrane fusion, the virion's nucleocapsid (which contains the viral RNA) is released into the cytoplasm (3). The genomic RNA is translated to generate a single large polyprotein that is processed into the 10 mature HCV proteins. The endoplasmic reticulum is modified by viral and cellular factors to form a membraneous web, which is the major site of viral RNA amplification (4). Six of the mature HCV proteins assist the replication of viral RNA via synthesis of positive strands (+) from a replicative intermediate negative RNA strand (-) template (5).A portion of this newly synthesized RNA is packaged into nucleocapsids and associated with the HCV glycoproteins, a process that leads to virion budding into the endoplasmic reticulum (6). During processing through the cellular secretory pathway the virions achieve maturation (7). Mature virions are released from the cell to complete the life cycle (8). [153]

1.4 Antiviral immunity

1.4.1 The innate immune response

For HCV, as for many viruses, the innate immune responses, especially the induction of type I and III interferons (IFNs) are the first line of defense limiting viral replication and spread, thus contributing to control of viral infection [154,155]. In the very early phase of infection the host innate immune response is triggered when infected cells recognize molecular signatures within the virus [155-157]. These signatures, known as pathogen-associated molecular patterns (PAMPs), are recognized as „non-self" by host pattern recognition receptors (PRRs). Engagement of the different PRRs, divided into the cytoplasmic retinoic inducible gene-I (RIG-I)-like receptors (RLRs) and Toll-like receptors (TLRs) depending on their sub-cellular localization, initiates a cascade of events that induces the expression of antiviral effector genes and production of interferons (IFNs), as well as a spectrum of inflammatory cytokines and chemokines [154,155]. IFN-α/β and IFN-λ induce the expression of more than 300 interferon-stimulated genes (ISGs) and their products have antiviral, anti-proliferative, and immuno-modulatory functions [155,158]. The IFN-induced proteins include the protein kinase R (PKR), the dsRNA-specific adenosine deaminase I (ADARI), and the 2',5'-oligoadenylate synthetase (OAS). PKR reduces the rate of HCV RNA translation by phosphorylation of the eukaryotic initiation factor 2 (eIF2). ADARI catalyzes the transformation of adenosine to inosine, thereby causing the accumulation of mutations inside the replicating viral RNA. OAS activates the latently expressed RNAse L, which subsequently catalyzes the degradation of cellular and viral RNA molecules. The list of identified products of the ISGs also includes several known broadly active regulators of antiviral signaling pathways such as the retinoic acid-inducible gene-I (RIG-I), the melanoma differentiation–associated protein 5 (Mda5), interferon regulatory factor 1 (IRF1), 2 (IRF2), and 7 (IRF7), as well as a number of proteins with not yet well defined functions [155,158]. In addition to the expression of ISGs type I IFNs influence the regulation of miRNAs, including those inhibiting HCV RNA replication and the proviral miR-122 [155]. Overall, the available data suggest that not a single but multiple factors participate in IFN-mediated inhibition of HCV replication and that only a well-concerted activity of all IFN-induced proteins and miRNAs is able to establish an antiviral state of the host cell. In the majority of infected individuals, however, the innate immune responses do not eliminate the invading virus, suggesting that type I IFNs alone are insufficient to completely control HCV replication in infected hepatocytes [155].

1.4.2 The humoral immune response

For many years the progress towards understanding the role of the humoral immune response in HCV infection was hampered due to the absence of adequate experimental techniques for virus binding, entry and virus neutralization measurement [159]. A breakthrough occurred after the development of the HCV pseudotype (HCVpp) system based on the usage of rhabdo- or retrovirus particles pseudotyped with HCV E1 and E2 glycoproteins [49,160,161]. The most recent introduction of virus strains and recombinant viral genomes capable to replicate and form infectious particles in cell culture (HCVcc) provided a major new approach to study HCV interaction with human cells and neutralization potentials of antiviral antibodies, including human monoclonal antibodies directed against envelope proteins [159,162,163].

Antibodies to both structural and nonstructural HCV proteins appear 7-8 weeks after onset of an acute infection and are preserved during the whole period of viral persistence [163-169]. Most of the neutralizing antibodies (nAbs) seem to be directed against linear and conformational epitopes of E2 and, to a lesser extent, E1. Available data clearly indicate that the virus-specific humoral immune response plays an essential role in the control of HCV infection [159,162,163]. This is supported by an experiment where passive transfer of an HCV-specific immunoglobulin to chimpanzees significantly delayed the super-infection of the animals, although complete prevention of super-infection was not achieved [170]. Another study demonstrated a passive protection against HCV in a cohort of patients who received immunoglobulin preparations derived from HCV RNA-positive plasma [171]. In immune-compromised patients, e.g. hypogammaglobulinemic patients, a rapid disease progression was observed, showing the potential important role for the humoral immune response of controlling the virus in the chronic phase [172]. One should note, however, that some of these patients were able to eliminate the virus without the support of humoral response, suggesting a contribution of the cellular immune response to the control of the virus [173]. Experiments with the HCVpp system have shown that a rapid induction of nAbs in the early phase of infection is associated with spontaneous viral clearance and that these antibodies demonstrate a broader cross-neutralization capacity. In contrast, patients, who developed a chronic infection, had only very low titers of nAbs or no antibodies during the acute phase of infection [174,175]. Patients in the chronic stage of HCV infection usually show high titers of anti-E2 antibodies, but these are unable to clear the virus [175]. An impaired ability to cross-neutralize viral variants that rapidly emerged during acute infection may thus contribute to viral evasion from the neutralizing response in persistent HCV infection [175,176]

Antibodies directed against the HVRI seem to play a special role, as they exhibit a high virus isolate specificity. High concentrations of these antibodies are needed for effective neutralization

[49,165,177-179,58]. Some studies of HCV viral evolution in the acute phase of infection revealed an association between resolved infection and stability of the HVRI, whereas viral persistence was accompanied by remarkable sequence changes in the HVRI over time [57,180]. Most recent data have shown that antibodies to HVRI play the major role in neutralization of virus spreading by cell-to-cell route but not of cell-free virus [181].

1.4.3 The adaptive cellular immune response

The successful clearance of HCV by the infected individual is associated with strong, multi-specific and sustained CD4+ and CD8+ T-cell responses targeting the different HCV proteins [182-189]. Generally, T-cell responses can be detected 6 - 8 weeks after initial infection and correlate with hepatic inflammation. This delay of detectable T-cell response is rather due to a delayed induction than to an impaired recruitment of specific CD8+ T-cells to the site of infection, namely the liver [190]. Once HCV infection is cleared, the specific CD4+ and CD8+ T-cell responses can be detected even after decades and sometimes they also outlast the specific humoral immune response [191].

As a strong, multi-specific CD4+ T-cell response in acutely HCV infected patients is associated with a self-limiting course of infection the HCV-specific CD4+ T-cells contribute to viral clearance [184-186,192-194]. Thus, CD4+ T-cell depletion in chimpanzees led to viral persistence after HCV infection and the appearance of CD8+ T-cell escape variants of the infecting strain [195]. Additionally, particular HLA type II alleles were observed to be associated with the outcome of HCV infection. In heterogeneous cohorts, the HLA type II alleles DRB1*1101 and DQB1*0301 seem to be protective, whereas in the well described Irish anti-D cohort the alleles DRB1*01, DRB1*0401, and DRB1*15 seem be associated with viral clearance [196,197]. Epitopes of CD4+ T-cells can be recognized by more than one HLA type II molecule, thus, these epitopes are restricted by several HLA type II molecules [159]. Additionally, CD4+ T-cells producing IL-17 have been detected in chronically infected patients, but their role during HCV infection is still unclear [198].

In addition to CD4+ T-cells, HCV- specific CD8+ T-cells are recognized as a key contributor to HCV clearance [155]. Thus, a robust CD8+ T-cells multi-specific response against HCV in the liver and peripheral blood was found to coincide with the onset of liver disease and viral clearance [182,183,187-189,199]. Several HLA type I molecules, including the A03, A11, B27, B57 and Cw*01, were shown to be protective and associated with viral clearance, whereas the presence of allels B08 and Cw*04 correlated with HCV persistence [197,200-204]. Recent experiments with the HCV replicons in vitro have demonstrated that the virus-specific CD8+ T-cells possess non-

cytolytic effector functions mediated by IFN-γ and to a lesser extend cytolytic effector functions [205]. Furthermore, CD8+ T-cells restrained HCV replication in vivo, indicating them as the key effector cells for controlling HCV infection: the experimental depletion of CD8+ T-cells in HCV infected chimpanzees led to viral persistence until the CD8+ T-cell population was restored and HCV-specific CD8+ T-cell response became detectable [155,206].

Taken together, the available data clearly indicate that HCV-specific CD4+ T-cells play an important role as key regulator cells, whereas virus-specific CD8+ T-cells play the essential role of the key effector cells during HCV infection [155].

1.5 Mechanisms of immune evasion and viral persistence

The majority of HCV infections (70-80 %) result in a virus persistence that might lead to such life-threatening sequelae as liver cirrhosis and hepatocellular carcinoma. The fact that HCV persistence is often observed in immuno-competent adults, perfectly capable of combating other infections, including those caused by hepatotropic pathogens, suggests that HCV developed several ways to circumvent the antiviral responses of the infected individual during coevolution with its host. The mechanisms by which HCV escapes from the immune surveillance are not fully understood. It is assumed that HCV has evolved several strategies to evade both the innate and adaptive immunity. As a result, HCV infected cells are not recognized and eliminated, and conditions for a long lasting persistent infection are created.

1.5.1 Counteraction of the innate immune responses

In cells infected with HCV the innate antiviral immune responses exerted through a complex multi-level network of cytokines and transcription factors play an essential role in elimination of the virus. There is accumulating evidence that HCV encoded proteins are engaged in pleiotropic interactions with many cellular macromolecules, thus interfering with the establishment of an IFN-induced antiviral state of the host cells [154,155,157,158]. Indeed, the HCV NS3/4A protease was shown to cleave MAVS and TRIF, the central adaptor molecules in two IRF3 activating antiviral pathways, thereby interfering with the induction of the IFN response [207,208]. Additionally, the NS3 protein can influence the RIG-1 and TLR3 signaling by competing with IRF3 for interaction with TBK1 [206]. Furthermore, HCV NS5A and core proteins have been reported to impair Jak-Stat signaling by affecting the phosphorylation step, which led to inhibition of the transcription of downstream ISGs [209,210]. In addition, the expression of NS5A has been shown to induce IL8, which in turn attenuates the antiviral action of IFNs. The HCV proteins might also demonstrate their inhibitory activity by blocking the function of antiviral effector ISGs [155,157,158].

This is supported by the observation that both E2 and NS5A proteins bind to PKR and thereby block its effector function [155].

Collectively, all available data provide evidence that several proteins encoded by HCV are involved in a wide variety of interactions with host cellular macromolecules causing deregulation of intracellular pathways. Most of these interactions are not yet recognized or fully characterized and it is still not clear, which of them are directly or indirectly related to the evasion from innate immune responses. Considering the complex interplay among multiple intracellular pathways it can be suggested that many viral proteins are implicated in the development of persistent infection.

1.5.2 Evasion from the humoral immune response

In the plasma of chronically infected patients high-titer cross-neutralizing antibodies are present [169,171,175,176,211]. Paradoxically, these antibodies are not able to control the infection, suggesting that HCV evolved some mechanisms to circumvent or to minimize the effects of the antiviral humoral immune response.

It is well known that due to the fact that the virus-encoded RNA-polymerase lacks proofreading functions, the HCV population in every infected individual exists as a pool of quasispecies, closely related but phylogenetically different variants [26]. These variants are changed over time due to a selective immune pressure driven by the host and this, in turn, might lead to the appearance of those virus variants that escape from antibody-mediated neutralization [49,169,211]. Several studies on HCV sequence evolution in infected chimpanzees and humans support this hypothesis [162,169,180,212]. Interestingly, the HVRI evolves more rapidly than other parts of the envelope proteins in vivo [162]. It has been suggested that HVRI contains major neutralizing epitopes, and the ongoing variations of this fragment plays a major role in maintenance of persistent HCV infection [159,162]. Of note, the HVRI was shown not to be essential for the HCV life cycle and HCV variants with deleted HVRI demonstrated infectivity, but in a diminished manner [213]. Furthermore, HVRI deletion mutants showed a greatly increased susceptibility to nAbs in the HCVcc system [213,214]. These data indicate that the HVRI shields the key nAb epitopes. Therefore, HVRI has been suggested to serve as an immunological decoy during infection by masking other functionally important but more conserved neutralizing epitopes [159,162].

In addition to HVRI, neutralizing epitopes have been identified in other regions of the E2 protein, including those involved in interaction with the host cell receptors [162,215]. Neutralizing antibodies to these epitopes seem to exert a continuous selective immune pressure on the

circulating virus variants, thereby contributing to HCV envelope gene evolution and emergence of viral escape variants [26,216,217]. Of note is the fact that some of the viral mutations driven by nAbs are located outside the receptor binding sites. Nevertheless, due to allosteric effects these mutations might lead to complete escape from nAbs [162].

Recently, one longitudinal study has provided an experimental proof of the viral escape hypothesis [169]. The authors used a series of HCVpp bearing E1/E2 heterodimers of the HCV H77 strain obtained from a single patient at different time-points of infection. At each of the time-points patient-derived serum was able to cross-neutralize a wide spectrum of HCVpp and to neutralize those HCVpp variants with E1/E2 heterodimers from the preceding sampling dates but not the particles bearing the autologous E1/E2 heterodimer. Thus, this longitudinal analysis provided evidences for the continuous viral escape from emerging antibodies during the course of infection and suggested that humoral immune pressure is the major force for the sequence diversity of HVRI. However, it remains to be proven that this mechanism of escape from the humoral immune response is operative in all patients, including those with a long history of HCV persistence.

Additionally, physical complexing of HCV virions with lipoproteins or their components, such as lipids and apolipoproteins provides another mechanism that might be involved in attenuation of the recognition of E2 neutralizing epitopes by nAb [162]. Thus, it was shown that high-density lipoproteins (HDL) facilitate HCVpp infectivity and reduce the sensitivity of the particles to nAbs [60,159,218]. The accessibility of the virus to neutralization might also be limited by the presence of a number of glycosylation sites in the envelope proteins [162].

Recently, another mechanism supporting viral persistence has been presented [219,220]. It was proposed that binding of antibodies to neutralizing epitopes located within the E2 region encompassing residues 412 to 423 can be inhibited by antibodies to non-neutralizing epitopes located in a region encompassing residues 434 to 446. However, such interference between neutralizing and non-neutralizing antibodies could not be confirmed in another report [221].

Finally, it seems that the mode of infection of hepatocytes may influence the sensitivity of HCV to nAbs. HCV has recently been shown to spread not only by cell-free virions but also by cell-to-cell transmission [181,222,223]. At that, the cell-to-cell route seems to be resistant to both polyclonal and most monoclonal anti-HCV glycoprotein antibodies. Among the tested antiviral nAbs, those antibodies directed against HVRI demonstrated the highest efficacy in interfering with cell-to-cell transmission [181].

1.5.3 Evasion from the cellular immune response

Although HCV-specific T-cell responses are detectable and virus-specific T-cells are enriched in the liver chronically infected patients, the virus cannot be eliminated from most of these individuals [159,224,225]. Two mechanisms are suggested to be mainly responsible for the failure of virus-specific CD4+ and CD8+ T-cell responses during chronic HCV infection.

1.5.3.1 Inhibition by cell surface molecules

First, CD8+ T-cell dysfunction, e.g. the impairment to secrete IFNγ and/or to proliferate following a contact with antigen, is considered by many researchers as a major cause of viral persistence [226-228]. Several pathways have been implicated in mechanisms of CD8+ T-cell exhaustion, although, the relative contribution of different inhibitory receptors for such dysfunction remain unclear [155,159]. In chronically infected patients up-regulation of inhibitory molecules like PD-1 on HCV-specific CD8+ T-cells can be detected and intrahepatic CD8+ T-cells expressing high amounts of PD-1 are prone to cell death [229-232]. Antibody blockage of PDL-1 lead to a partly restored proliferation capacity of these HCV-specific CD8+ T-cells after antigen contact, but not to the restoration of full functionality [229-232]. The pathways other than PD-1 were also implicated. Thus, the application of antibodies directed against CTLA-4, another inhibitory molecule, lead to an increased antiviral CD8+ T-cell function [226]. Additionally, another immune suppressive receptor, Tim-3, was detected on virus-specific CD8+ T-cells in chronic HCV infection and its blockade with specific antibodies also caused a restoration of CD8+ T-cell function [233-235]. Recently, co-expression of the inhibitory receptors 2B4, KLRG1, and CD160 in addition to PD-1 was observed on virus-specific CD127-low CD8+ T-cells in chronic infection [236,237], suggesting that the cumulative or simultaneous effect of different pathways to HCV-specific CD8+ T-cell exhaustion is probable. Another factor leading to an exhausted phenotype of HCV-specific CD8+ T-cells might be the lack of CD4+ T-cell help. During chronic HCV infection CD4+ T-cells were shown to be functionally impaired, but not deleted, indicating an important role for CD4+ T-cell help in CD8+ T-cell dysfunction [155,238]. Another possible cause of CD8+ T-cell dysfunction is the suppression of virus-specific CD8+ T-cells by different subsets of regulatory T-cells. CD4+CD25+ regulatory T-cells have been found to be upregulated in chronically infected patients compared to those, who cleared the infection or uninfected controls. These cells suppress proliferation and IFN-γ secretion by HCV-specific CD8+ T-cells in vitro [209,239,240]. Additionally, virus-specific regulatory CD8+ T-cells producing high amounts of IL-10 can also be detected in the liver during HCV infection [241]. The suppressive effect of these cells on HCV-specific CD8+ T-cells could be abolished by antibodies

directed against IL-10 [242]. Myeloid suppressor cells might contribute to dysfunction of HCV-specific CD8+ T-cells by production of reactive oxygen species [243]. Finally, the specific microenvironment of the liver directs the immune response in the liver rather towards tolerance than to activation and liver dendritic cells, hepatic stellate cells, as well as liver sinusoidal endothelial cells play an important role in the induction of immune suppression [244].

1.5.3.2 Immune escape by mutation of epitopes

Another means of HCV evasion from T-cell responses is based on the immune escape mechanism [155,159]. As has already been described in the current text, in each infected host the HCV population consists of permanently renewable pool of quasispecies. This pool provides a basis for a selection of virus variants under T-cell immune pressure. As a result, cells bearing the viral genomes with these escape mutations within the epitopes are not eliminated. Viral escape from CD8+ T-cell responses was detected in the acute phase of infection in individuals that developed a chronic course of infection, whereas this phenomenon could not be observed in patients that resolved HCV infection [183,245-247]. Thus, the appearance of viral escape variants in the acute phase of HCV infection seems to correlate with viral persistence. After transmission of the mutated virus to a new host with different genetic background (different HLA types) a reversion of the escape mutation to the original one or to the consensus residue occurred in a large proportion of cases. Indeed, several studies showed that HCV reverts to the fittest sequence, probably the consensus one, if no sufficient selection pressure by T-cells is present [245]. For example, an HLA-B08 associated escape mutation within the epitope reverted after transmission of the virus to an HLA-B08 negative subject [247]. Furthermore, in the Irish anti-D cohort in the presence of the particular HLA-allele most of the amino acid substitutions inside known CD8+ T-cell epitopes were forward mutations, whereas in individuals lacking the particular HLA-allele reversions toward the consensus sequence could be observed [248]. These data allowed to formulate the concept of a fitness cost and suggested that viral escape mutations often could reduce the virus fitness and lead to a reduced ability of the virus to replicate [249]. Several recent studies on HLA allele-dependent HCV polymorphism allowed to identify putative new CD8+ T-cell epitopes on the assumption that CD8+ T-cell mediated pressure would select virus variants with escape mutation, thus leaving the corresponding specific footprints in viral sequences [203,250-253]. Taken together, these data indicated that the CD8+ T-cell-mediated viral escape seems to be one of the major mechanisms of HCV persistence [155,159,254].

In conclusion, it should be noted that many important questions regarding the escape mechanism are not completely answered yet. Thus, it looks like that so far, not all CD8+ T-cell epitopes

have been identified. The frequencies of the escape mutations in many epitopes as well as their fitness cost remain to be determined. Additionally, the contribution of the CD8+ T-cell-mediated immune pressure to evolution of the structural HCV proteins, first of all, of the envelope proteins, is not clear. One may expect, however, that further application of such approaches as population-based studies of special cohorts of HCV infected individuals (e.g. the single-source hepatitis C outbreaks) and their complementation with new experimental tools such as HCVcc system would allow to clarify these and many other unresolved issues

1.7 The goals and aims of the study

As has been already shown in the previous chapter, intensive efforts by the scientific community during the last years led to a tremendous progress in our understanding of HCV biology and major characteristics of HCV infection. Nevertheless, many aspects of HCV research still remain far from being investigated in full details. The current study aims to fulfill some gaps in our knowledge, dealing with the evolution of viral genome at a population level, causal relationship between immune responses and viral evolution, and structural characteristics of viral proteins.

The major goals of the current project were:

- Analysis of the genetic heterogeneity of HCV isolates found in individuals infected by the HCV AD78 strain in a single source hepatitis C outbreak, which occurred in 1977-1978 in the former German Democratic Republic.
- Analysis of the HCV evolution at a population level and assessment of the contribution of CD8+ T-cell and humoral immune responses to evolution of HCV envelope protein E2 in a cohort of chronic hepatitis C patients infected in a single-source HCV outbreak.
- Analysis of the isolate-specific features of the envelope protein E2 sequences of the HCV AD78 strain and of their influence on virus infectivity and replication.

To reach these goals the following aims were formulated:

- To create a sequence database (core-NS2 and NS4-NS5 genomic regions) for multiple isolates of the HCV AD78 strain obtained from individuals infected in a single-source outbreak of hepatitis C and from contaminated immunoglobulin batches. To characterize the heterogeneity of the obtained sequences.
- To apply this database in a special statistical approach to identify CD8+ T-cell epitopes under selective pressure associated with HLA class I.
- To perform an experimental confirmation of putative novel epitopes.
- To use the created consensus sequences of the HCV AD78 strain for generation of the AD78/JFH1 chimeric virus and to apply this newly developed HCVcc system for analysis of the fitness cost of the putative escape mutations identified in the current study.

- To analyze the frequency of synonymous and nonsynonymous mutations inside and outside of known and putative B- and T-cell epitope regions within the E2 protein from AD78 isolates obtained from the AD-cohort.
- To apply the HCVcc system as well as the HCVpp–based approach for analysis of the influence of the additional amino acid tracks at the N-terminus of the E2 on virus infectivity and replication.

2 Material and Methods

2.1 Material

2.1.1 Human sera from chronically infected HCV patients

Sera of patients infected with the AD78 strain were kindly provided by Prof. M. Wiese, St. Georg Hospital, Leipzig, Germany. Between August 1978 and March 1979, 14 HCV1b contaminated batches of anti-D immunoglobulin had been administered to 2867 women throughout East Germany for prophylaxis of Rhesus factor incompatibility. After application of the contaminated globulin, 93 % of the recipients developed an acute hepatitis C. After 25 years, 86 % of the 1833 affected women still tested positive for hepatitis C virus antibodies and 46 % for HCV RNA [255].

2.1.2 Chemicals

Product	Supplier
β-mercaptoethanol	Sigma-Aldrich Chemie GmbH
Acrylamide 2 K solution	AppliChem
Agarose	Eurogentec
Ampicillin (50 µg/mL)	Roche Diagnostics
APS	Roth
Aqua Roti Phenol	Roth
Bromphenole-blue	Sigma-Aldrich Chemie GmbH
D-PBS	Invitrogen
Dimethyl sulfoxide	Roth
DMEM (4.5 g/L glucose) with L-glutamine	Gibco
EDTA solution (0.5 M), pH 8.0	AppliChem
ECL Western Blotting Reagents	GE Healthcare
Ethanol	Sigma-Aldrich Chemie GmbH

Product	Supplier
Ethidiumbromide	Roth
FACS-Clean	Becton Dickinson
FACS-Flow	Becton Dickinson
FACS-Rinse	Becton Dickinson
Fix Perm	Becton Dickinson
Fetal Calf Serum	Biochrome
Glycerol	Sigma-Aldrich Chemie GmbH
HEPES	PAA Laboratories
Isopropanol	Roth
Interleukin-2, human (10.000U)	Roche Diagnostics
Sodium-acetate (3 M), pH 5.5	Ambion
Sodium-chloride	Merck
Orange G	Sigma-Aldrich Chemie GmbH
Penicillin/Streptomycin	PAA Laboratories
Perm Wash	Becton Dickinson
RPMI 1640 with L-glutamine	PAA Laboratories
Sodium dodecyl sulfate (SDS)	AppliChem
TBE ultra pure 10x	Invitrogen
TEMED	Roth
Triton X-100	Invitrogen
Trypan Blue	Gibco
Trypsin-EDTA	Gibco

Product	Supplier
X-Gal	Bio Solve LTD

2.1.3 Equipment

Device	Supplier
Automatic incubator CO_2	Kendro GmbH
Balnce	Vibra
Bioimaging System (Gene genius)	Syngene
Centrifuge 3 LR	Kendro GmbH
Centrifuge 5804	Eppendorf
Centrifuge 5414 R	Eppendorf
Centrifuge 5415 D	Eppendorf
Freezer	Liebherr
Fridge	Liebherr
FACS Calibur	Becton Dickinson
GeneAmp PCRSys 2400	Roche
Gene Pulser II	BioRad Laboratories
Hera Safe Flow	Kendro GmbH
Heraeus CO2 Incubator	Kendro GmbH
Heraeus Megafuge 1.0 R	Kendro GmbH
Heraeus Multifuge 3 LR	Kendro GmbH
Inverted Microscope	Carl Zeis
Luminometer	Promega

Device	Supplier
Microwave	Panasonic
Mini-Protean	BioRad Laboratories
Mr. Frosty	Nalgene
Pipette-Boy	Eppendorf
Pipettes	Eppendorf
Photometer Gene Quant	Amersham Bioscience
pH meter MP 220	Toledo
Power-Pack	BioRad Laboratories
Rotary Mixer	Oehmen
Thermocycler	Eppendorf
Trans-Blot SD Transfer cell	BioRad Laboratories
Neubauer counting chamber	Superior Marienfeld Germany
Ultracentrifuge	Beckman Coulter
UV transluminator FLX-20M	MWG-Bio Tech

2.1.4 Commercial kits

Kit	Supplier
Bio-Rad Protein Assay Kit	BioRad Laboratories
CalPhos Mammalian Transfection Kit	Clontech
Expand High Fidelity PCR System	Roche
GoTag PCR System	Promega
GloLysis Buffer	Promega

Kit	Supplier
One-step RT-PCR Kit	Qiagen
Qiagen Plasmid Maxi Kit	Qiagen
Qiagen Plasmid Mini Kit	Qiagen
RNeasy Kit	Qiagen
Qiaquick Gel Extraction Kit	Qiagen
Qiaquick PCR Purification Kit	Qiagen
QuikChange Lightning Site-Directed Mutagenesis Kit	Stratagene
TopoTA Cloning Kit	Invitrogen
MegaClear Kit	Ambion
MegaSkript T7 in vitro transcription Kit	Ambion

2.1.5 Plastic products

Product	Supplier
FACS tubes	Falcon/Becton Dickinson
Dishes	Greiner Bio-One
Disposable syringes (10 mL)	Sarstedt
Gene Pulser Cuvette (0.4 cm)	BioRad Laboratories
Pipettes (2 mL, 5 mL, 10 mL, 25 mL)	Greiner Bio-One
Pipette tips (10 µL, 100 µL, 200 µL, 1000 µL)	Starlab
Reaction tubes (0.2 mL, 1.5 mL, 2 mL)	Eppendorf
V-bottom tubes (15 mL, 50 mL)	Falcon/Becton Dickinson
Cell-culture flasks	Greiner Bio-One

Product	Supplier
Cell-culture plates	Greiner Bio-One

2.1.6 Membranes

PVDF membrane	Millipore, Germany

2.1.7 Enzymes

Enzyme	Supplier
GoTaq Polymerase	Promega
High Fidelity Polymerase	Roche
T4 DNA Ligase	Roche
Sal1 FastDigest (FD)	Thermo Scientific
Psi1 FD	Thermo Scientific
EcoRV FD	Thermo Scientific
Bcl1 FD	Thermo Scientific
Age1 FD	Thermo Scientific
Not1	Thermo Scientific
Ssp1	Thermo Scientific
Shrimp Alkaline Phosphatase	Thermo Scientific

2.1.8 Oligonucleotides

Primer designation	Sequence	Usage
sv655s	5'-TAT AGA TAT CAT GGG GTA CAT TCC GCT CGT C-3'	Amplification of AD78 E1 (+ HVRI), with sv690as (57 °C), PCR II
sv687s	5'-TTC GCC GAC CTC ATG GGG TAC-3'	Amplification of AD78 E1 (+ HVRI), with sv828s (58 °C), PCR I
sv690as	5'-GTT TTG AGA GAG TCA TTG CAG-3'	Amplification of AD78 E1 (+ HVRI), with sv655s (57 °C), PCR II
sv692s	5'-GCC TCG CCT ACT ATT CCA TG-3'	Amplification of AD78 E2, with sv1104as (57 °C), PCR II
sv828as	5'-AGA ACA GMG CGG CAA KRA ACC-3'	Amplification of AD78 E1 (+ HVRI), with sv687s (58 °C), RT and PCR I
sv980as	5'-GCT GCC GTT GGT GTT TAT AAG CTG-3'	Anti-sense primer for insertion of additional aa tracks N-terminal of HVR1 (AD78 variants A and B), HCVpp
sv1019s	5'-TT GCT GGC GTC GAC GGC CCC ACC CGC ACG ATA GG-3'	Deletion of additional aa tracks N-terminal of HVR1 (AD78 variant B) with sv980as, HCVpp
sv1020s	5'-TT GCT GGC GTC GAC GGC CGT GGA GGT GGA CCC ACC CGC ACG ATA-3'	Insertion of additional aa track "RGGG" N-terminal of HVR1 (AD78 variant B) with sv980as, HCVpp
sv1021s	5'-TT GCT GGC GTC GAC GGC TCA CCC ACC CGC ACG ATA GG-3'	Insertion of additional aa track "S" N-terminal of HVR1 (AD78 variant B) with sv980as, HCVpp
sv1022s	5'-TT GCT GCC GTC GAC GGC ACG TCA TGGG CCC ACC CGC ACG ATA GG-3'	Insertion of additional aa track "TSW" N-terminal of HVR1 (AD78 variant B) with sv980as, HCVpp
sv1023s	5'- TT GCT GCC GTC GAC GGC ATG TCA GGA CCC ACC CGC ACG ATA GG-3'	Insertion of additional aa track "MSG" N-terminal of HVR1 (AD78 variant B), HCVpp

Primer designation	Sequence	Usage
sv1024	5'- TT GCT GCC GTC GAC GGC GCT TCA GCA CCC ACC CGC ACG ATA GG-3'	Insertion of additional aa track "ASA" N-terminal of HVR1 (AD78 variant B) with sv980as, HCVpp
sv1025s	5'- TT GCT GCC GTC GAC GGC GAC AAC GAC AAC CCC ACC CGC ACG ATA GG-3'	Insertion of additional aa track "DNDN" N-terminal of HVR1 (AD78 variant B) with sv980as, HCVpp
sv1026s	5'- TT GCT GCC GTC GAC GGC CGT GGA GGT GGC GGA GGT GGT GGC GGC GGA CCC ACC CGC ACG ATA GG-3'	Insertion of additional aa track "RGGGGGGGGG" N-terminal of HVR1 (AD78 variant A) with sv980as, HCVpp
sv1027s	5'- TT GCT GCC GTC GAC GGC CGT GGA GGT GGC GGA GGC GGA GGC CCC ACC CGC ACG ATA GG-3'	Insertion of additional aa track "RGGGGGG" N-terminal of HVR1 (AD78 variant B) with sv980as, HCVpp
sv1032s	5'-CTG CCG TTG GTG TTG ATC AGT TGG ATG TTC-3'	Insertion of additional aa track "RGGG" N-terminal of HVR1 (H77/JFH1) with sv980as, HCVcc
sv1033as	5'-CTG CCG TTG GTG TTG ATC AGT TGG ATG TTG-3'	Anti-sense primer for insertion of additional aa track "RGGG" N-terminal of HVR1 (H77/JFH1) with sv1043s, HCVcc and sequencing of E1/E2 junction
sv1043s	5'-GCC GGC GTC GAC GCG CGT GGA GGT GGA GAA ACC CAC GTC ACC-3'	Insertion of additional aa track "RGGG" N-terminal of HVR1 (H77/JFH1) WITH sv1033as, HCVcc
sv1050s	5'-CTT TGC TGG CGT CGA CGG CAC CAC CCG CAC GAT AGG G-3'	SDM of 1st position of HVR1, AD78 variant B, HCVpp
sv1051as	5'-CCC TAT CGT GCG GGT GGT GCC GTC GAC GCC AGC AAA G-3'	SDM of 1st position of HVR1, AD78 variant B, HCVpp
sv1055s	5'-TAT CCT RTT GCT CTT CCT TCT C-3'	Amplification of AD78 p7-NS2, with sv1056as, PCR II and sequencing
sv1056as	5'-TGA TGA TAC AGC CAA GYA GGC-3'	Amplification of AD78 p7-NS2, with sv1055s, PCR II and sequencing

Primer designation	Sequence	Usage
sv1057s	5’-GGT RTA GGG TCA GTG CTC GTC TC-3’	Amplification of AD78 p7-NS2, with sv1058as, PCR I
sv1058as	5’-GAC TRG TGA TGA TAC AGC CAA G-3’	Amplification of AD78 p7-NS2, with sv1057s, RT and PCR I
sv1059as	5’-CAG CAA AGC CAA GAG GAA GAT AG-3’	Amplification of AD78 core, with sv1060s, RT and PCR I
sv1060s	5’-GGG GCG ACA CTC CAC CAT AGA TC-3’	Amplification of AD78 core, with sv1059as, PCR I
sv1061s	5’-TCC ACC ATA GAT CAC TCC CCT GTG-3’	Amplification of AD78 core, with sv1062as, PCR II
sv1062as	5’-AGA GGA AGA TAG AGA AAG AGC AAC C-3’	Amplification of AD78 core, with sv1061s, PCR II and sequencing
sv1067s	5’-GAT ACT GCC CTG TTC CTT CAC-3’	Amplification of AD78 E2-p7 from globulins with sv1068as, PCR II
sv1068as	5’-CTT TAT AGT ATG GTG ACA AGG TC-3’	Amplification of AD78 E2-p7 from globulins with sv1067s, PCR II
sv1069s	5’-CTA CAA CAG AGT GGC AGA TAC-3’	Amplification of AD78 E2-p7 from globulins with sv1070as, PCR I
sv1070as	5’-ATA TGA GCT TAG CGA GGA ACA-3’	Amplification of AD78 E2-p7 from globulins with sv1069s, PCR I
sv1071as	5’-GCC GCA TTG AGG ACC ACC AGG-3’	Specific amplification of AD78 variant C E2 with sv1072s, PCR I
sv1072s	5’-TGA TGA ATT GGT CAC CTA CAA-3’	Specific amplification of AD78 variant C E2 with sv1071as, PCR I
sv1073as	5’-ATC AGC AGC ATC ATC CAC AAG-3’	Specific amplification of AD78 variant C E2 with sv1074s, PCR II
sv1074s	5’-GTG GTA TCG CAG TTA CTC CGG-3’	Specific amplification of AD78 variant C E2 with sv1073as, PCR II
sv1075s	5’-AAG CTG TCG RGG ACA TGG TG-3’	Amplification of AD78 E2-p7 from patients with sv1070as, PCR I
sv1076s	5’-TAT TCC ATG GTG GGG AAC TGG-3’	Amplification of AD78 E2-p7 from patients with sv1068as, PCR II
sv1080as	5’-GCC GCG AGC GTG GGG GTG AGC-3’	Amplification of AD78 core from patients with sv1081s, PCR I

Primer designation	Sequence	Usage
sv1081s	5'-GAT CAA CCC GCT CAA TGC CTG-3'	Amplification of AD78 core from patients with sv1080as, PCR I
sv1082as	5'-CCA GCA GCG AGA GGA GTT GTC-3'	Amplification of AD78 core from patients with sv1083s, PCR II
sv1083s	5'-TAG CCG AGT AGT GTT GGG TC-3'	Amplification of AD78 core from patients with sv1082as, PCR II
sv1102s	5'-AAG CTG TCR TGG ACA TGG TGG-3'	AD78 E2, with sv1103as (59 °C), PCR I
sv1103as	5'-GCA GCA CAG AAG AAC ACA AGG-3'	AD78 E2, with sv1102s (59 °C), RT and PCR I
sv1104as	5'-CAG AAG AAC ACA AGG AAG GAG-3'	AD78 E2, with sv692s (57 °C), PCR II
sv1153s	5'-CGG AAC CGG TGA GTA CAC CG-3'	Amplification of core-E2 from H77/JFH1 with sv1154as
sv1154as	5'-CAT CGC GGC CGC CGC G-3'	Amplification of core-E2 from H77/JFH1 with sv1153s
sv1155s	5'-ARC AGG CAG GAG ACA ACT TC-3'	Amplification of AD78 NS3-NS5 fragment A, with sv1164as (58 °C), PCR II
sv1159s	5'-ACT GGA TAT GCA CGG TGT TG-3'	Amplification of AD78 NS3-NS5 fragment B, with sv1160as (58 °C), RT
sv1160as	5'-TCC CCC GGC TCY CCC TCA AG-3'	Amplification of AD78 NS3-NS5 fragment B, with sv1159s (58 °C), RT
sv1161s	5'-CAG TCC AAR CTC YTG CCG CG-3'	Amplification of AD78 NS3-NS5 fragment B, with sv1162as (58 °C), PCR II
sv1162as	5'-CTC AAG RGG RGG CAT GGA GG-3'	Amplification of AD78 NS3-NS5 fragment B, with sv1161s (58 °C), PCR II
sv1163s	5'-CCC AGA CYA ARC AGG CAG GR-3'	Amplification of AD78 NS3-NS5 fragment A, with sv1164as (58 °C), RT
sv1164as	5'-CCT CAT GGA ACC RTT YTT GAC-3'	Amplification of AD78 NS3-NS5 fragment A, with sv1163s (58 °C), RT

Primer designation	Sequence	Usage
sv1182s	5’-CCG CGC AGG GGC CCT AAA TTG GGT GTG CGC-3’	SDM H77/JFH1: core R43K, with sv1183as
sv1183as	5’-GCG CAC ACC CAA TTT AGG GCC CCT GCG CGG-3’	SDM H77/JFH1: core R43K, with sv1182s
sv1184s	5’-CCT CTT GGA GGC GTT GCC AGG GCC CTG-3’	SDM H77/JFH1: core H153Q, with sv1185as
sv1185as	5’-CAG GGC CCT GGC AAC GCC TCC AAG AGG-3’	SDM H77/JFH1: core H153Q, with sv1184s
sv1202s	5’-CCG CGC AGG GGC CCC AAG TTG GGT GTG CGC-3’	SDM AD78/JFH1 variant A: core R43K, with sv1203as
sv1203as	5’-GCG CAC ACC CAA CTT GGG GCC CCT GCG CGG-3’	SDM AD78/JFH1 variant A: core R43K, with sv1202s
sv1204s	5’-CCT CTA GGG GGT GTT GCC AGG GCC CTG-3’	SDM AD78/JFH1 variant A: core H153Q, with sv1205as
sv1205as	5’-CAG GGC CCT GGC AAC ACC CCC TAG AGG-3’	SDM AD78/JFH1 variant A: core H153Q, with sv1204s
sv1206s	5’-C AGG CCG CCG GGG GGC AAC TGG TT-3’	SDM AD78/JFH1 variant A: E2 Q546G, with sv1207as
sv1207as	5’-AA CCA GTT GCC CCC CGG CGG CCT G-3’	SDM AD78/JFH1 variant A: E2 Q546G, with sv1206s
sv1208s	5’-C AGG CCG CCG GGC AAC TGG TT-3’	SDM AD78/JFH1 variant A: E2 Q546A, with sv1209as
sv1209as	5’-AA CCA GTT GCC CCG CGG CGG CCT G-3’	SDM AD78/JFH1 variant A: E2 Q546A, with sv1208s
sv1210s	5’-C AGG CCG GCG GGC AAC TGG TT-3’	SDM AD78/JFH1 variant A: E2 Q546R, with sv1211as
sv1211as	5’-AA CCA GTT GCC CGC CGG CGG CCT G-3’	SDM AD78/JFH1 variant A: E2 Q546R, with sv1210s
sv1214s	5’-CA GAG TGG CAG GTA CTG CCC TGT TC-3’	SDM AD78/JFH1 variant A: E2 I674V, with sv1215as
sv1215as	5’-GA ACA GGG CAG TAC CTG CCA CTC TG-3’	SDM AD78/JFH1 variant A: E2 I674V, with sv1214s

2.1.9 Plasmids

2.1.9.1 Commercial plasmids

The pCR4 Topo plasmid (Invitrogen, Germany) was routinely used for cloning of PCR products.

2.1.9.2 Plasmid containing the envelope genes of HCV AD78 infected patients

The phCMV-IRES plasmid (5520 bp) was used for expression of the E1 and E2 proteins of HCV. This plasmid contains the IRES of EMCV that drives the translation of the HCV envelope genes. The inserted envelope protein genes were obtained during the amplification of HCV sequences from serum samples of AD78 patients collected at different time points. The generated set of plasmids was used for transfection of 293T cells in order to produce the HCV E1/E2 heterodimer for the generation of HCVpp.

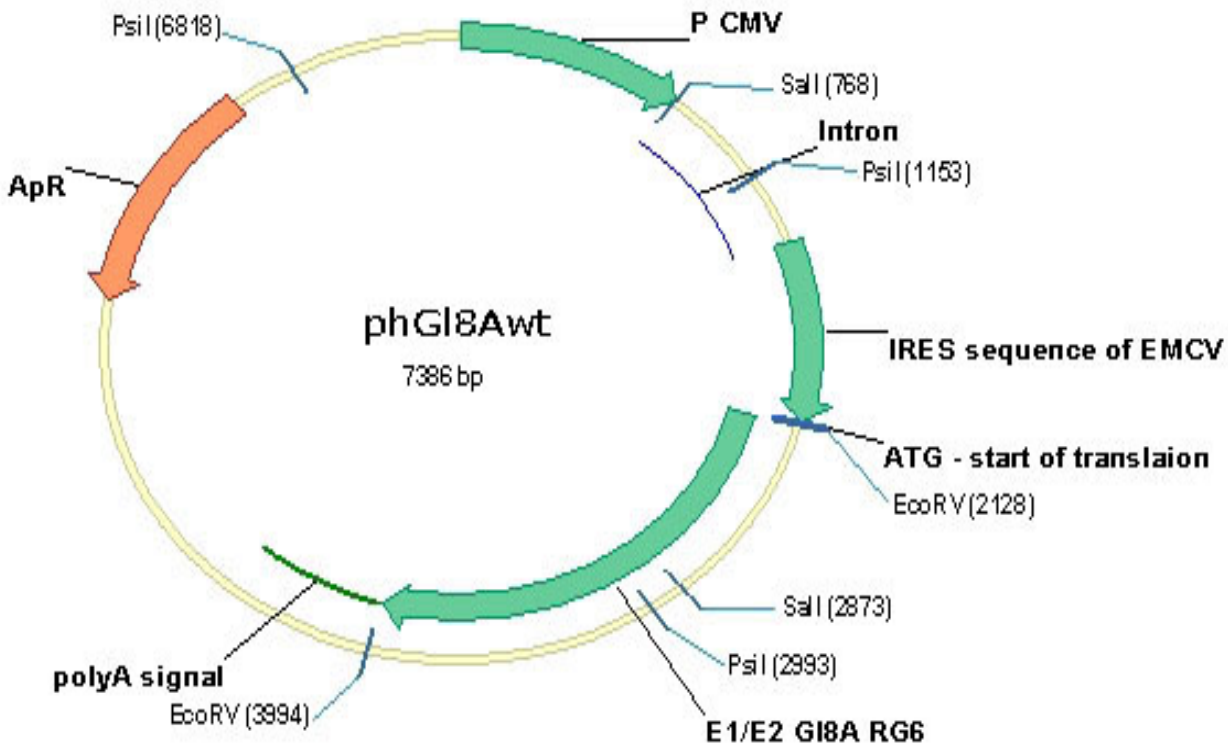

Fig. 2.1 Plasmid containing the envelope genes of HCV AD78 infected patients (var. A)

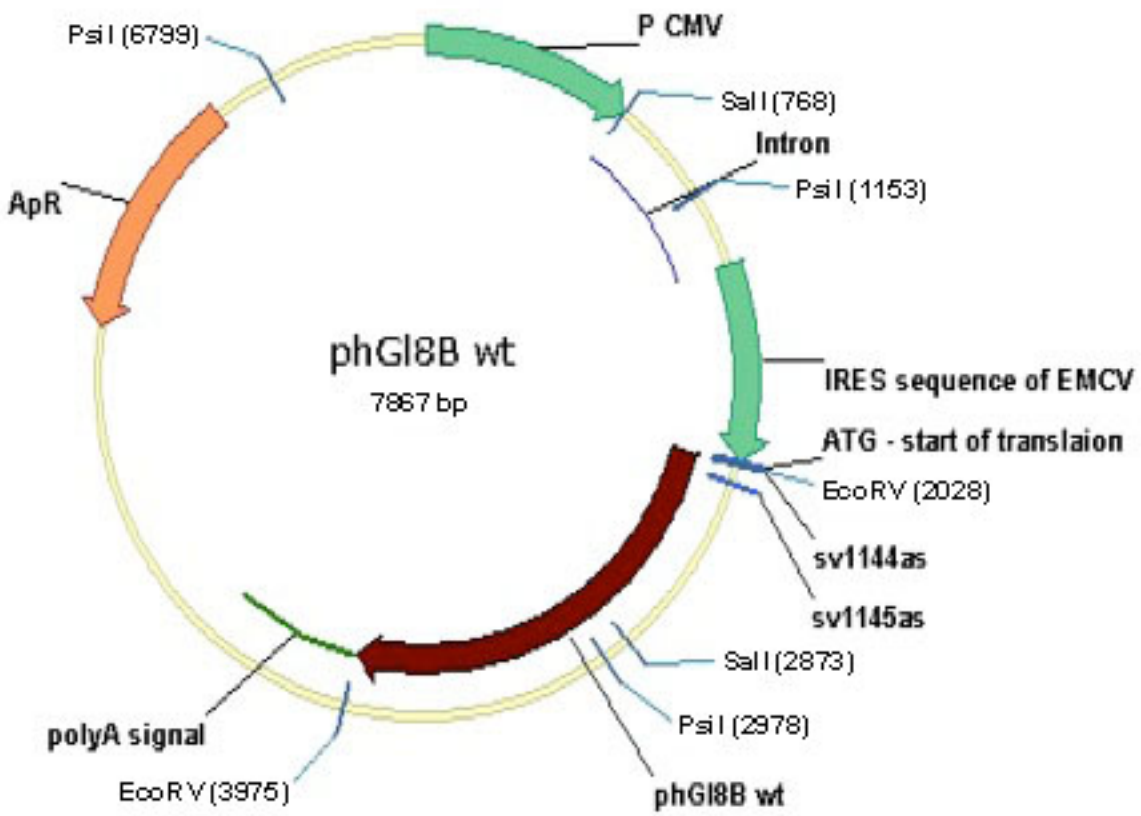

Fig. 2.2 Plasmid containing the envelope genes of HCV AD78 infected patients (var. B)

2.1.9.3 Plasmid containing HIV-Gag, Pol genes

The lentiviral vector pCMV delta R8.2 (8128) has been obtained from Addgene (USA). This plasmid expresses the HIV Gag and Pol proteins in 293T cells and was used for production of HCVpp.

2.1.9.4 Plasmid containing the luciferase reporter gene

The HPPT-EF1α- Luc HIV-1-based transfer vector contains the EF1 internal promoter, which drives the expression of Luc (luciferase reporter gene) gene. The plasmid was used for generation of HCVpp in 293T cells.

2.1.9.5 Plasmids for production of HCVcc

The AD78/JFH1 and H77/JFH1 plasmids were linearized and subsequently used for in vitro RNA transcription to produce full length HCV RNA for electroporation.

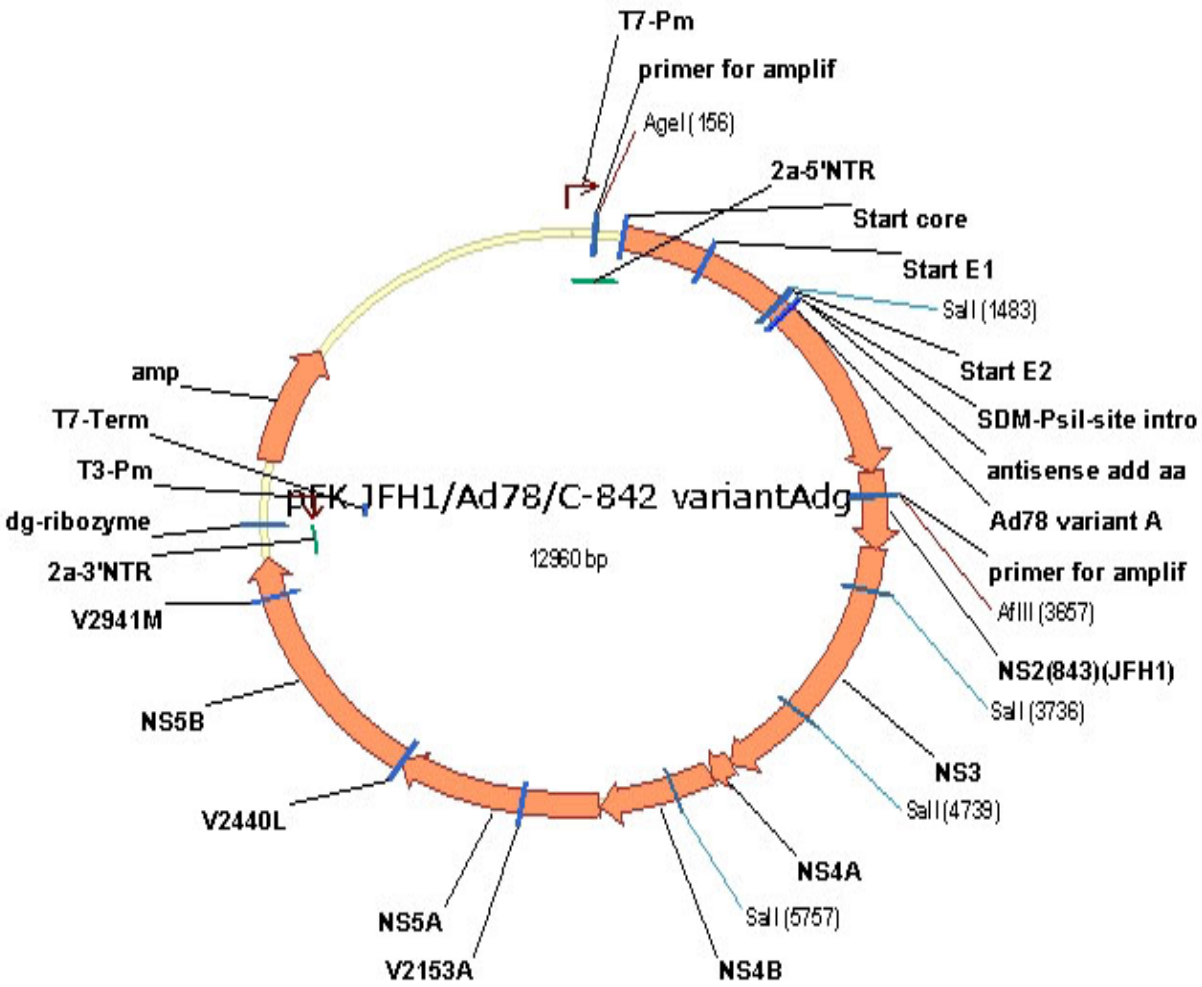

Fig. 2.3 Plasmids for production of HCVcc (AD78/JFH1)

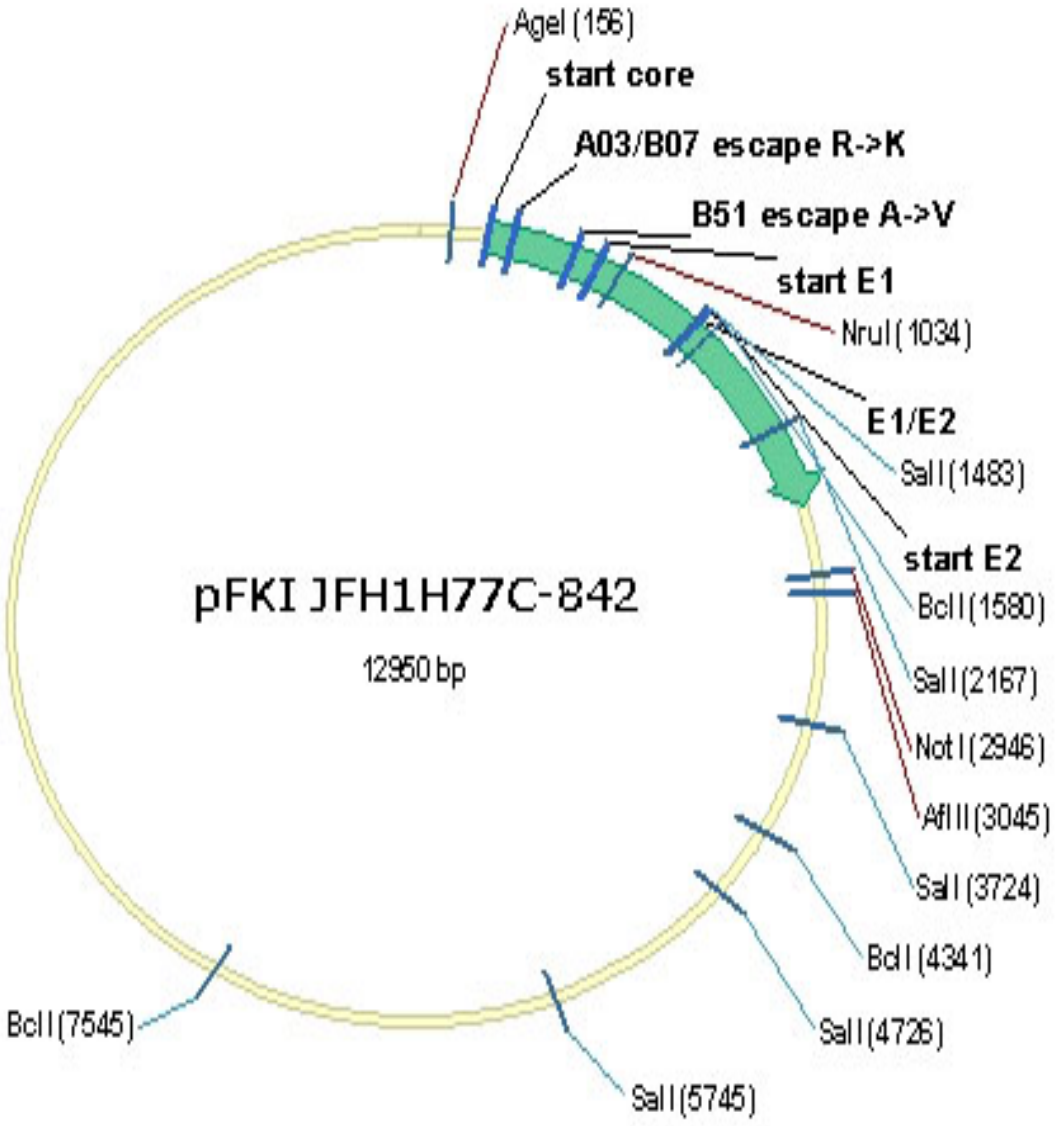

Fig. 2.4 Plasmids for production of HCVcc (H77/JFH1)

2.1.10 Buffers and Solutions

DNA-loading buffer	5.5 mM Orange G
	1.2 M Saccharose

Lysis buffer (SDS-PAGE)	0.5 M Tris, pH 6.6 1 % Glycerol 10% SDS 5 % 2-Mercaptoethanol 0.05 % Bromphenole-blue
Cytomix	120 mM KCl 0.15 mM CaCl2 10 mM K2HPO4/KH2PO4 (pH 7.6) 25 mM HEPES (1 M stock solution, Gibco cell culture grade) 2 mM EGTA 5 mM MgCl2 pH 7.6 (with KOH)
cell lysis buffer	20 mM Tris HCL, pH 8.0 137 mM NaCl 10% Glycerol 1 % Triton-X 100 2 mM EDTA add H_2O dest. to 100 %
PBS	155 mM NaCl 3 mM Na_2HPO_4 1 mM KH_2PO_4 pH 7.4
4x SDS loading buffer	10 % Glycerine 4 % SDS 125 mM Tris, pH 6.8 10 % 2-Mercaptoethanol 0.02 % Bromphenole-blue

5x DNA loading buffer	50 % Glycerine 20 mM Tris, pH 8.0 50 mM EDTA, pH 8.0 0.025 % Bromphenole-blue 0.025 % Xylenecyanole
10x TBE buffer	108 g Tris base 55 g Boric acid 0.5 M EDTA, pH 8.0
Blocking buffer (immunostaining)	1 % BSA 0.2 % skimmed milk powder 0.2 % sodium-acetate PBS
non-denaturing lysis buffer	4 mL 0.5 M Tris-HCL, pH 8.0 0.8 g NaCl 10 mL Glycerol 1 mL Triton-X 100 0.4 mL EDTA solution (0.5 %) H_2O
40 % PEG solution (precipitation of HCVcc)	40 g PEG 8000 100 mL sterile PBS

2.1.11 Antibodies

2.1.11.1 Primary Antibodies

Mouse mAb CET-3 was kindly provided by Prof. Chang-Yuli Kong (South Korea)

Mouse mAb 9E10 was kindly provided by Prof. Charles Rice (USA)

APC mouse anti-human CD8, Becton Dickinson

FITC conjugated anti human IFNγ, eBioscience

PE mouse anti-human CD4, Becton Dickinson

α-CD28/CD49d, Becton Dickinson

2.1.11.2 Secondary Antibodies

Goat-α-mouse IgG, peroxidase conjugated (A4416), Sigma

Rabbit-α-mouse IgG and IgM, peroxidase conjugated, Dianova

2.1.12 Medium for culture of E.coli

LB Agar	25 g LB-Agar powder 1 L dH_2O
LB Medium	25 g LB-Medium powder 1L dH_2O

Both media were completed to 1000 mL with bi-distilled water and sterilized by autoclaving for 15 minutes at 121 °C. Ampicillin was added to each medium at a final concentration of 100 µg/mL.

2.1.13 Bacteria strains

Strain	Genotype	Supplier
TOP10	F- mcrA Δ(mrr -hsdRMSmcrBC) φ80lacZΔM15ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(Str^R) endA1 λ^-	Invitrogen
XL10-Gold	endA1 glnV44 recA1 thi-1 gyrA96 relA1 lac Hte(mcrA)183 Δ(mcrCBhsdSMR-mrr)173	Stratagene
DH5α	supE44 ΔlacU169 (φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96thi-1 relA1	Invitrogen
Dam-	ara-14 leuB6 fhuA31 lacY1 tsx78 glnV44 galK2 galT22 mcrA dcm-6 hisG4 rfbD1 R(zgb210::Tn10) Tet^SendA1 rspL136 (Str^R) dam13::Tn9 (Cam^R) xylA -5 mtl-1 thi-1 mcrB1 hsdR2	New England Biolabs

2.1.14 Eucaryotic cell lines

2.1.14.1 Human hepatoma cell line Huh7.5

Huh7.5 cells are a sub-line derived from Huh7 hepatoma cells (Blight et al., 2002). This sub-line was established by "curing" a cell clone containing a Con1 subgenomic replicon by prolonged treatment with alpha-interferon. The receptors, important for the initiation of virus entry, are expressed on the viral surface and, therefore, these cells can be used for studying virus entry.

2.1.14.2 Human Embryonic Kidney 293T cells

293T cells are an important variant of the HEK 293 cells. This cell line bears the SV40 large T-antigen that allows episomal replication of transfected plasmids containing the SV40 origin of replication. In the current study 293T cells were used for generation of HCVpp.

2.1.14.3 Human cryo-conserved peripheral-blood monocytes (PBMCs)

Patient (in most cases drug users) derived PBMCs, patients were HLA-typed and PBMCs were cryo-conserved. These cells were used for in vitro stimulation of CD8+ T-cells with short peptides (see section 2.2.3.5).

2.1.15 Culture medium for Huh7.5 cells

DMEM	500 mL
FCS	10 %
Non-essential amino acids	1 %
HEPES	1 %
Penicillin/Streptomycin	1 %

2.1.16 Culture medium for HEK 293T cells

DMEM	500 mL
FCS	10 %
Penicillin/Streptomycin	1 %

2.1.17 Culture medium for human PBMCs

RPMI 1640	500 mL
FCS	10 %
HEPES	1 %
Penicillin/Streptomycin	1 %

2.1.18 Peptides

Peptide designation	Sequence	HLA-specificity
ADP01	APLGGAARALAHGVR	core B51, wt 1
ADP02	GAARALAHGVRVLED	core B51, wt 2
ADP03	GPKLGVRAT	core B07, mutant 1
ADP04	GPSLGVRAT	core B07, mutant 2
ADP05	GPTLGVRAT	core B07, mutant 3
ADP06	KLGVRATRK	core A03, mutant
ADP07	EVRNVSGVY	E1 A26, wt
ADP08	PASAYEVRNVSGVYH	E1 B38, wt 1
ADP09	YEVRNVSGVYHVTND	E1 B38, wt 2
ADP10	RMASCRPIDKF	E2 B57, wt
ADP11	NTRPPQGNW	E2 B57, wt
ADP12	NTRPPAGNW	E2 B57, mutant 1
ADP13	NTRPPRGNW	E2 B57, mutant 2
ADP14	NTRPPGGNW	E2 B57, mutant 3
ADP15	LLLSTTEWQI	E2 A2402, wt
ADP16	LLLSTTEWQV	E2 A2402, mutant 1
ADP17	VLPCSFTTL	E2 A2402, mutant 2
ADP18	TPLRDWAHAGLRDLA	NS2 A01/B08, wt
ADP19	RDWAHAGL	wt NS2 B08, motif
ADP20	TPLRDWAHAGL	wt NS2 B08, truncated 1
ADP21	RDWAHAGLRDL	wt NS2 B08, truncated 2
ADP22	DWAHAGLRDLA	wt NS2 B08, truncated 3
ADP23	RALAHGVRV	core B51 motif
ADP24	GAARALAHGVRV	core B51 motif, shift left
ADP25	RALAHGVRVLED	core B51 motif, shift right

2.1.19 Used Software

FigTree v1.2.2	http://tree.bio.ed.ac.uk/software/figtree/
FlowJo 7.6	Tree Star, Inc. 1997-2008
GraphPad Prism	GraphPad Software, Inc.
HCV Sequence Database	http://hcv.lanl.gov/
Mac OS X	Macintosh
iWork	Macintosh
MHC-I-binding-prediction	http://tools.immuneepitope.org/analyze/html/
NCBI Homepage	http://www.ncbi.nlm.nih.gov/
NEBcutter V2.0	http://tools.neb.com/NEBcutter2/index.php
R-Programm	http://www.R-project.org
Se-Al v2.0a11 Carbon	Sequence Alignment Editor v2.0a11 © 1996-2002 Andrew Rambaut
SISA The Fisher exact test	http://www.quantitativeskills.com/sisa/index.html
SNAP-Programm	synonymous-nonsynonymous analysis program (http://hcv.lanl.gov/)
VectorNTI	Sequence Analysis Software

2.2 Methods

2.2.1 Procaryotic Cells

2.2.1.1 Transformation of E.coli

Transformation of bacterial cells was performed according to the manufacturer's instructions. In brief, bacterial cells (Top10 cells, DH5α, dam- or XL10-Gold cells) frozen at -80 °C were thawed on ice. Two micro-liters of 2-Mercaptoethanol were added to 45 µL of XL10-Gold cells aliquot and incubated for 10 min on ice. Top10 cells were used without addition of mercaptoethanol. No more than 10 % by volume of the plasmid DNA was added to the aliquot

of bacterial cells. This mixture was incubated on ice for 30 minutes. To improve DNA absorption by bacteria, a heat shock for 30 sec. at 42 °C followed by a subsequent incubation on ice for 5 min was performed. After addition of 250 µL LB-medium a mixture was incubated on a shaker for 2 hours at 37 °C. Using a sterile spatula the complete mixture was spread over an LB-agar plate containing a selective antibiotic. The plates were incubated overnight at 37 °C.

2.2.1.2 Plasmid DNA preparation using commercial kits

One bacterial colony was picked up from the LB agar plate, using a sterile pipette tip and transferred into the flask with LB-medium containing the ampicillin (5 - 10 mL of medium for minipreps, 100 - 250 mL- for maxipreps). The bacteria culture was incubated overnight on a shaker at 37 °C. The culture was centrifuged at 5000 rpm for 15 min and cells were washed once with PBS. The DNA was extracted using the Qiaprep kits (Qiagen), according to the manufacturer's protocol. DNA was quantified by spectrophotometry at 260 nm and the size of the plasmid was checked using the electrophoresis in agarose gel.

2.2.2 Molecular Biological Methods

2.2.2.1 RNA-Extraction from human sera

HCV RNA was extracted from patients' sera using the High Pure Viral RNA Kit (Roche) according to the manufacturer's instructions. In brief, 200 µL serum was mixed with the Binding Buffer supplemented with Poly (A), transferred into a filter mini-column, and centrifuged for 30 sec at 8000 x g. Afterwards, 500 µL Inhibitor Removal Buffer was added to the upper reservoir followed by centrifugation for 1 min at 8000 x g. The column was washed 2 times with 450 µL Wash Buffer. Viral RNA was eluted in 50 µL of Elution Buffer and directly used in the reverse transcription reaction or kept at -80 °C.

2.2.2.2 Reverse-Transcription

HCV RNA isolated from patient's sera was reverse transcribed into cDNA using the Thermoscript kit (Invitrogen) according to the following procedure:

RT reaction mix I	
Primer (50 pmol/µL)	1 µL
dNTPs (5 mM)	2 µL
RNA	5 µL
RNAse free water	5 µL
Final volume	13 µL

The RNA in Mix I was denatured for 5 min at 65 °C and directly cooled down on ice.

RT reaction mix II	
5 x cDNA synthesis buffer	4 µL
0.1 M DTT	1 µL
RNAse Out (20 U)	1 µL
RNAse free water	1 µL
Final volume	7 µL

Mix II was added to Mix I to a final volume of 20 µL and incubated for 1 h at 50 °C, followed by additional incubation step (5 min at 85 °C). Five µL of generated cDNA was used for PCR 1.

2.2.2.3 Side-directed mutagenesis (SDM)

Quick-Change Lightning site-directed mutagenesis kit (Stratagene) was used to introduce single point mutations into the plasmid sequences. In this technique two complementary mutagenic primers, containing the desired nucleotide are used to amplify the entire plasmid. The non-strand-displacing action of the Pfu Ultra HF DNA Polymerase extends and incorporates the mutagenic primers, which results in a formation of nicked circular strands. After touchdown PCR, the methylated parental plasmid DNA is removed by digestion with DpnI.

Standard SDM mixture	
10x reaction buffer	5 µL
50 - 100 ng plasmid	5 µL
10 pmol/µL sense primer (5‘ - 3‘)	1.5 µL
10 pmol/µL anti-sense primer (3‘ - 5‘)	1.5 µL
dNTP-mix	1 µL
QuikSolution reagent	3 µL
QuikChange Lightning Enzyme	1 µL
H_2O	32 µL
Final volume	50 µL

PCR program for SDM			
Segment	Cycles	Temperature	Time
1	1	95 °C	2 min
2	18	95 °C	20 sec
		60 °C	10 sec
		68 °C	30 sec/kb of plasmid length
3	1	68 °C	5 min

After PCR, 1 µL DpnI was added to the PCR-product and the mixture was incubated 1h at 37 °C. Afterwards, 2 µL of the mixture were used for transformation of XL-10 Gold cells (2.2.1.1.).

2.2.2.4 DNA Dephosphorylation

In some cases, e.g. when only one enzyme was used for restriction of the plasmid, in order to prevent a re-ligation of a linearized plasmid, the restricted DNA was incubated with 3 µL Shrimp alkaline phosphatase (1 U/µL) for 1 h at 37 °C.

2.2.2.5 Amplification of DNA inserts using Polymerase Chain Reaction (PCR)

All DNA was amplified with either the GoTaq system or in a nested touchdown PCR using the Expand High Fidelity PCR System.

The PCR reaction using GoTaq:

Master-mix GoTaq PCR

5x colorless/green GoTaq reaction buffer with $MgCl_2$	10 µL
dNTPs (10 mM)	1 µL
10 pmol/µL sense primer (5‘ - 3‘)	5 µL
10 pmol/µL anti-sense primer (3‘ - 5‘)	5 µL
DNA	5 µL
GoTaq DNA polymerase	0.25 µL
H_2O	23.75 µL
Final volume	50 µL

Two separate master mixes were prepared and mixed using the Expand High Fidelity PCR System:

Master-mix PCRI

5x reaction buffer with $MgCl_2$	10 µL
dNTPs (10 mM)	1 µL
10 pmol/µL sense primer (5‘ - 3‘)	5 µL
10 pmol/µL anti-sense primer (3‘ - 5‘)	5 µL
cDNA	5 µL
High Fidelity Polymerase	0.5 µL
H_2O	23.5 µL
Final volume	50 µL

Master-mix PCRII

5x reaction buffer with $MgCl_2$	10 µL
dNTPs (10 mM)	1 µL
10 pmol/µL sense primer (5‘ - 3‘)	5 µL
10 pmol/µL anti-sense primer (3‘ - 5‘)	5 µL
DNA	2 µL
High Fidelity Polymerase	0.5 µL
H_2O	26.5 µL
Final volume	50 µL

Touchdown PCR program for both reactions			
Segment	Cycles	Temperature	Time
1	1	95 °C	2 min
2	2	95 °C	1 min
		66 - 57 °C	1 min
3	1	72 °C	3 min
4	15	95 °C	1 min
		57 °C	1 min
		72 °C	3 min
5	1	72 °C	20 min
6	1	4 °C	∞

The size of amplified DNA fragments was assessed using ethidium bromide agarose gel electrophoresis.

2.2.2.6 DNA restriction digestion

Plasmids and PCR-products were restricted either with a single endonuclease or with a combination of two enzymes. The used enzymes were provided with a single buffer for all kinds

of restriction enzymes and all possessed a temperature optimum of 37 °C for reaction. The PCR products used for restriction were previously purified by extraction from the agarose gel. For preparative purposes up to 100 µL of restriction reaction was prepared and incubated for 30 min or in case of linearization overnight at 37 °C. The control digestion of successful cloning was carried out in 20 µL reaction for 10 min.

Restriction of plasmids/PCR products

10x green FD buffer	2 µL
Plasmid DNA (µg)/PCR Product (µg)	x µL
FD Enzyme	1 µL
H_2O	x µL
Final volume	20 µL

2.2.2.7 Agarose gel electrophoresis

DNA fragments were separated in a horizontal 1-2% agarose gel containing 0.5 µg/mL of ethidium bromide. The electrophoresis was carried out in 1× TBE buffer at 120 V for approximately one hour. The visualization of the separated DNA fragments was performed using a UV-Bioimaging System (Syngene).

2.2.2.8 DNA extraction from agarose gel

Restricted plasmids or amplified DNA fragments were separated by agarose gel electrophoresis. The fragments of interest were cut out of the gel and purified with the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's protocol. The elution of purified fragments was performed in 30 µL of H_2O.

2.2.2.9 Cloning of PCR-products in intermediate plasmids

In most cases the amplified DNA fragments were cloned in pCR4-Topo plasmid (Invitrogen) without additional gel purification, according to the manufacturer's protocol.

pTopo cloning mixture	
PCR product (treated with Taq)	2 µL
Salt solution	1 µL
pCR4-Topo-vector	1 µL
H_2O	2 µL
Final volume	6 µL

The mixture was incubated for 45 min at room temperature and 2 µL were used for transformation of 25 µL supplied TOP 10 cells according to the standard protocol (2.2.1.1.).

2.2.2.10 Phenol – chloroform purification

For phenol–chloroform precipitation of the linearized plasmids two 100 µl restriction reactions were performed. After the digestion, the reactions were mixed by vortexing with 400 µl of phenol and centrifuged (16.000 xg, 10 min, 4 °C). The upper phase containing DNA was transferred to a fresh tube, mixed by vortexing with 250 µL of phenol and 250 µL of chloroform, and centrifuged (16.000 xg, 10 min, 4 °C). Again the upper phase containing DNA was transferred to a fresh tube and mixed with 500 µL of chloroform by vortexing and centrifuged (16.000 xg, 10 min, 4 °C). The DNA containing upper phase was mixed with 50 µL of 3 M sodium acetate (pH 7) and 500 µL of isopropanol. Then 1.5 µL of GlycoBlue were added and the mixture was incubated for 20 min at -20 °C. The tube was centrifuged (16.000 xg, 30 min, 4°C), the supernatant was discarded and pellet was washed with 1 mL of 70 % ethanol (16.000 xg, 10 min, 4 °C). The pellet was left to dry and afterwards suspended in 20 µL of nuclease-free H2O.

2.2.2.11 Ligation of DNA fragments

Purified, linearized plasmid and insert DNA were ligated using T4 DNA ligase. For the reaction a linearized plasmid/insert ratio of 1:3 - 1:6 was used, 1 µL of T4 ligase and 1 µL of 10x optimal ligation buffer (Rapid Ligation Kit, Roche) and the proper amount of ddH_2O were added. The mixture was incubated 15-20 min at room temperature and used for transformation of competent E.coli cells.

2.2.2.12 Plasmid DNA purification using commercial kits

One bacterial colony was picked up from the LB agar plate, using a sterile pipette tip and transferred into the flask with LB-medium containing selective antibiotic. The volume of 5 - 10 mL of the culture was used for Mini, 100 mL for Midi, and 250 ml for Maxi preparations. After overnight incubation on the shaker at 37 °C the plasmid DNA was extracted using QIAprep Kits (Qiagen) according to manufacturer's instructions. The DNA concentration was quantified by spectrophotometric OD260nm measurement as follows:

Concentration [μg/mL] = OD260nm × dilution factor × 50

Purified plasmid DNA was checked by control restriction digestion (section 4.1.4).

2.2.2.13 DNA sequencing

Sequencing was performed at the DNA-Sequencing Service of LGC Genomics. Non-standard primers designed for sequencing are listed in the Materials section (Tab. 3.5).

2.2.2.14 In vitro transcription/production of HCV-plasmid-RNA

After phenol/chloroform purification the linearized plasmid DNA was used as template for in vitro RNA transcription using the T7 Mega Script High Yield Transcription Kit (Ambion).

Modified from manufacture's instructions

Template DNA	10 μg
ATP	2 μL
CTP	2 μL
GTP	2 μL
UTP	2 μL
10x reaction buffer	3 μL
RNAse free water	x μL
T7 polymerase	2 μL
Final volume	30 μL

After incubation of the reaction for 4 h at 37 °C 1 μL of T7 polymerase was added and subsequently incubation for additional 2 h at 37 °C was performed. To remove the plasmid DNA

2 µL Turbo DNAse was added and the reaction was incubated for 30 min at 37 °C. After proteinase K treatment the transcribed HCV RNA was purified using the Mega Clear Kit (Ambion) according to manufacture‘s instructions. For protection of HCV RNA 1 µL of RNAse inhibitor was added per preparation. Concentration of RNA was determined by NanoDrop® from small aliquots and HCV RNA was stored at - 80 °C.

2.2.2.15 HCVpp purification by ultracentrifugation

The supernatant of transfected 293T cells containing the HCVpp was layered on 5 mL of 20 % (w/v) sucrose and centrifuged in rotor SW40 for 1.5 h at 40.000 rpm at 4 °C. The supernatant was discarded, the pellet was dissolved in 30 µL 2x Laemmli buffer and used for SDS-PAGE (2.3.4.2) followed by immonodetection (2.3.4.3).

2.2.2.16 HCVcc precipitation

HCVcc containing Huh7.5 cell culture supernatant was harvested and filtrated through a 45 µm membrane. The proper amount of a sterile 40 % (w/v) PEG 8000/PBS solution was added to the supernatant to final concentration of 8% PEG 8000, precipitation was performed over night at 4 °C.

2.2.2.17 HCVcc purification

After PEG precipitation, the HCVcc containing cell culture supernatant was centrifuged for 1.5 h at 8000 xg and 4 °C in 50 mL Falcon tubes. Afterwards, supernatant was removed and the virus pellets were resuspended in 70 µL DMEM (10% FCS, 1% penicillin/streptomycin, 1% non-essential amino acids, 1% HEPES) and used for serial dilutions (10^{-1} - 10^{-6}) and TCID50.

2.2.2.18 LightSNiP Assay

Genomic DNA isolated from EDTA-blood of 92 AD78-infected patients was used for detection of single nucleotide polymorphisms of HCV cell receptor genes LDLR (low-density lipoprotein receptor), SCARB1 (scavenger receptor B1) and OCLN (occludin) by LightSNiP Assay (Tib-molbiol). This test is based on melting curve analysis and is able to detect any mutation that is located within the sequence corresponding to the hybridization probe (TibMolbiol). In most cases these tests allow the differentiation between alleles (e.g. C/C, C/G or G/G):

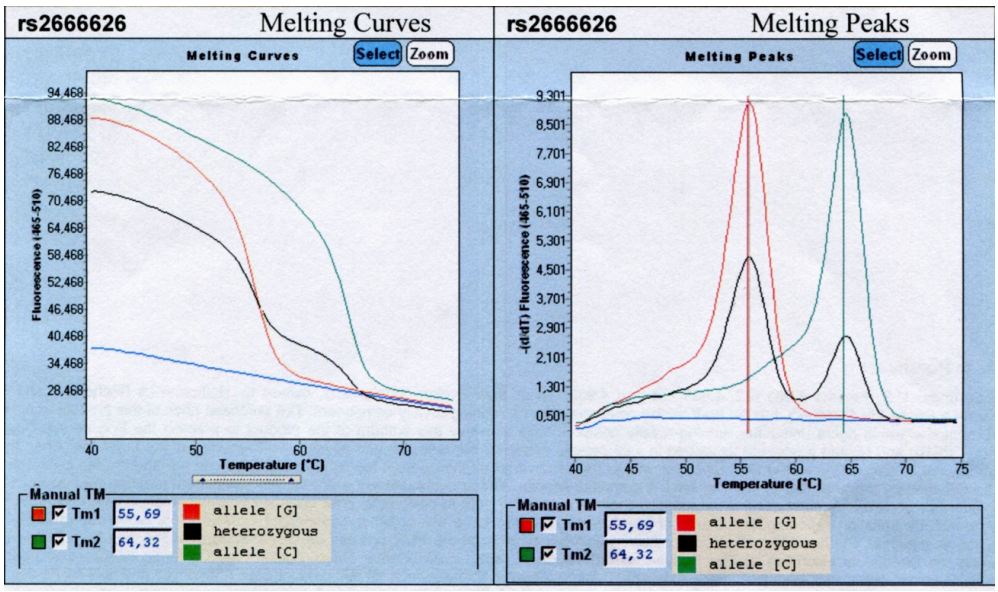

Fig. 2.5 All reactions were performed according to manufacture's instructions.

2.2.3 Cell culture

2.2.3.1 Thawing and cryo-conservation of cells

Cryo-tubes containing cells were taken out from the liquid nitrogen and thawed quickly in a warm water-bath. Cells were washed twice in 10 mL of the culture medium. Afterwards, cells were suspended in 10 mL of fresh medium, placed in 75 cm^2 flask, and cultured at 37 °C in humidified atmosphere containing 5% CO_2.

For cryo-conservation, the cell suspension was centrifuged in 50 mL tubes (300× g, 5 min) and washed once with sterile PBS. The pellet was suspended in 1 mL of culture medium supplemented with 25% FCS and 10% DMSO. Cells were frozen slowly overnight in -80 °C and then transferred to a liquid nitrogen tank.

2.2.3.2 Passaging of cells

The medium was removed and cells were washed once with 15 mL of sterile PBS. Then 1.5 mL of Trypsin-EDTA was added to cover the bottom of the 75 cm^2 flask. After 2 - 3 minutes when the cells started to detach from the bottom 8.5 mL of fresh medium was added. Cells were placed in fresh flasks in 20 mL of culture medium in the given concentration.

2.2.3.3 Culture of Huh7.5 and Huh7.5.1 cells

Adherent Huh7.5 or Huh7.5.1 cells were grown in monolayers in Dulbecco's modified minimal essential high glucose medium (4,5 g/L) with L-glutamine supplemented with 10 % FBS, 1 %

penicillin/streptomycin, 1% HEPES and 1 % non-essential amino acids. Cells were passaged two times a week at a dilution of 1:3 or 1:5 depending on the confluence.

2.2.3.4 Culture of HEK 293T cells

Adherent HEK 293T cells were grown in monolayers in Dulbecco's Modified Eagle Medium 1x (Gibco) containing 4.5 g/L glucose, L-glutamine, 25 mM HEPES, 10 % FCS, and 1 % penicillin/streptomycin. Cells were passaged 2 times a week at a dilution of 1:10 or 1:15 depending on the confluence.

2.2.3.5 Culture of human PBMCs/preparation of cryo-conserved PBMCs for peptide stimulation

Human cryo-conserved PBMCs were gently thawed, suspended in 1 mL FCS and transferred to a 15 mL Falcon tube containing 20 µL of DNAse. Cells were washed 3x with 10 mL RPMI 1640 (w/o supplements) and centrifuged for 7 min at 1800 rpm and room temperature. After the last centrifugation step cells were suspended in the appropriate amount (1 mL per stimulation) of RPMI (10 % FCS, 1 % penicillin/streptomycin, 1 % HEPES, IL-2 2.5 µL/mL, α-CD28 antibody 1 µL/mL). For subsequent peptide stimulation of PBMCs 1 mL per well of cells was transferred to a 12 well plate and 1 µg/mL of the peptide of interest was added. Cells were incubated for ten days at 37 °C and 5 % CO2. Dependent on cell growth in culture cells were supplemented with additional 500 µL of RPMI (10 % FCS, 1 % penicillin/streptomycin, 1 % HEPES) containing 2.5 µL of IL-2.

2.2.3.6 Counting of viable cells using Trypan blue exclusion microscopy

Trypan blue is a negatively charged dye that only interacts with the cell when the membrane is damaged. Therefore, all the cells, which exclude the dye, are viable. Aliquots of cell suspension were diluted with 0.4% Trypan blue stain and 10 µL of the diluted aliquot solution was transferred onto a Neubauer counting chamber. The viable cells were counted and the number of cells per mL was calculated as follows:

Number of cells/mL = number of cells in the large square × dilution factor × 10^4

2.2.3.7 Transfection of HEK 293T cells using $CaCl_2$ method/production of HCVpp

293T cells were harvested and seeded in a 10 cm^2 dish at a final concentration of 2.5 x 10^6 cells in 8 mL medium (20-30 % confluence). The next day, the 293T cells were transfected with the

expression plasmid phCMV IRES-E1/E2 in combination with 2 retroviral plasmids encoding for HIV-Gag, Pol or Luc (pCMV delta R8.2; plasmid HPPT-EF1α- Luc) using the Calcium Phosphate Transfection Kit (Clontech). Briefly, 500 µL HBS 2x were put into a facs tube. Next, using an Eppendorf tube 1.5 ml, the mix was prepared as follows:

pTopo cloning mixture	
H_2O	x µL
phCMV-E1/E2	2.7 µg
Plasmid encoding for Gag, Pol	8.1 µg
Plasmid encoding for Luc	8.1 µg
$CaCl_2$ solution (added drop-wise)	62 µL
Final volume	500 µL

This mix was added drop-wise to the tube with 500 µL of g HBS 2x and incubated for 20 to 30 min. at room temperature until the mixture becomes cloudy. After addition of this cloudy solution drop-wise to the cell culture, the dishes were incubated up to 16 h at 37 °C. The medium was changed and the cell culture was additionally incubated for 48 h at 37 °C. Finally the supernatant containing the generated HCVpp was harvested and centrifuged for 5 min at 1200 rpm. The HCVpp preparation was aliquoted, frozen at -80 °C, and used for infectivity assay.

2.2.3.8 P24 assay

For quantification of HCVpp produced in 293T cells, cell culture supernatants were analyzed for p24 protein expression in the diagnostic department at University Hospital Essen.

2.2.3.9 Infectivity assay

Huh 7.5 cells were seeded in 96 well plates. For each plate, a master mix with 1.4 x 10^6 cells in 20 mL medium was prepared. The next day, the medium was removed and HCVpp in 100 µL of Huh7.5 medium were added. After incubation for 4h at 37 °C, 200 µL of fresh pre-warmed Huh7.5 medium was added and plates were incubated for additional 72 h at 37 °C. For the infectivity evaluation, the medium was removed and 50 µL of Bright Glo lysis buffer (Promega) was added to each well and incubated for 2 h at - 20 °C. After thawing, 45 µL of prepared cell

lysates were transferred into a luminometer 96 well plate. Fifty µL of the Bright Glo Luciferase Assay buffer (Promega) were added and the luciferase-activity was immediately measured in the luminescence counter (Promega). All cell lysates were measured in triplicates and a mean value was calculated.

2.2.3.10 Electroporation of Huh7.5 cells/production of HCVcc

Huh7.5 cells were harvested from 75 cm^2 flasks at a confluence of 40 - 70 % with 1.5 mL of Trypsin after washing with sterile PBS. All steps were performed at S3** level at room temperature. In Huh7.5 medium harvested cells were centrifuged for 5 min at 700 rpm in a 50 mL Falcon tube. Supernatant was discarded and the cell pellet was suspended in 50 mL sterile PBS. A small aliquot was used for counting. Cells were centrifuged for 5 min at 700 rpm and the pellet was suspended in the needed volume of „Cytomix“ with freshly added 2 mM ATP and 5 mM glutathione (1.5 x 10^7 cells/mL). For electroporation 400 µL of suspended cells (6 x 106 cells/electroporation) were gently mixed by pipetting up and down 5 - 6 times with 50 µg of HCVcc RNA and immediately transferred into the electroporation cuvette. The cuvette was immediately transferred into the electroporation chamber and pulsed once as follows:

975 µF; 270 V; expected time constant: ~ 24 ms

Cells were directly transferred into 15 mL Huh7.5 medium in a 10 cm dish, the cuvette was rinsed several times. Cells were incubated at 37 °C and 5 % CO_2 and next the medium was exchanged for 12.5 mL fresh Huh7.5 medium after washing once with PBS. After 72 h of incubation following electroporation supernatant was harvested and filtrated through a sterile 45-µm filter. Subsequently, PEG-precipitation (2.2.2.17), HCV histochemistry (2.2.3.12) including TCID50 (2.2.3.11) were performed.

2.2.3.11 Determination of the infectious HCVcc particle titer

The titer of infectious HCVcc particles was determined using TCID50 (tissue culture infectious dose 50) assay. For that purpose, Huh7.5 cells were plated in a flat-bottom 96-well plate (6×10^3 cells per well) and infected in every row (6 wells) with 100 µL of serial logarithmic dilutions (10^{-1} - 10^{-6}) of the purified virus stock. After 72 h the infected cells were monitored. The ratio of NS5A-positive wells for every dilution was determined and the TCID50 per mL of the virus stock was calculated as follows:

$$TCID50/mL = 101+d\ (S-0.5) \times 10$$

Where: d = Log 10 of the dilution; S = the sum of infection ratios for every dilution

2.2.3.12 HCV histochemistry (modified from Lindenbach et al., 2005)

Huh7.5 cells were seeded at 6×10^3 per well on a poly-L-lysine coated flat-bottom 96-well plate (16 - 24h before infection) in a total volume of 200 µL DMEM per well. The serially diluted virus inoculum (10^{-1} - 10^{-6}) was used for infection of 6 wells in a total infection volume of 100 µL, respectively. Afterwards 100 µL Huh7.5 medium per well were added. After 72 h of incubation at 37 °C and 5 % CO_2 cells were fixed with 50 µL of ice cold methanol (- 20 °C) and incubated at least for 15 min at - 20 °C (optional over night). Methanol was removed and cells washed once with PBS. By adding 50 µL of 0.5 % (v/v) Triton X-100 in PBS for 5 min at room temperature cells were permeabilized and afterwards washed once with PBS. Blocking of the plates was performed with 100 - 200 µL of blocking buffer for 1 h at room temperature on a shaker and afterwards washed once with PBS. The expression of NS5A by infected Huh7.5 cells was detected with the 9E10 primary antibody in a dilution of 1:2000 in PBS (50 µL/well). After incubation for 1 h at room temperature on a shaker plates were washed 3 x with PBS. Bound primary antibody was detected using anti-mouse HRP (Sigma, A4416) conjugated secondary antibody in a dilution of 1:200 and incubation for 1 h at room temperature on a shaker. Plates were washed 3 x with PBS and HRP activity was detected by adding DAB+ substrate (DAKO) and incubation for 30 min. Reaction was stopped by discarding the substrate and addition of H_2O. Plates can be stored at 4 °C before microscopical analysis.

2.2.4 Protein-biochemical and immunological methods

2.2.4.1 Protein purification

The medium of the Petri dishes (10 cm^2) containing the transfected 293T cells was removed and dishes were immediately put on ice. In the next step, the cell dish was transferred onto ice and 5 mL of ice cold PBS was added to each dish. The cells were scrapped and transferred into a 15 mL Falcon tubes. A centrifugation step for 5 min at 1200 rpm at 4 °C was applied. The cell pellets were resuspended in 0.5 mL of non-denaturing lyses buffer and incubated on ice for 30 min. The mixtures were clarified by centrifugation for 20 min at 4 °C at 12.000 rpm. Finally, the supernatant was transferred into a fresh tube and either stored at -20 °C or directly used for SDS-PAGE.

2.2.4.2 SDS-PAGE

Proteins were separated using the standard discontinuous SDS-PAGE (sodiumdodecylsulfate-polyacrylamide-gel electrophoresis) in the Mini-Proteom chamber (BioRad).

	stacking gel	**separation gel 12 %**
30 % Acrylamid solution (29:1)	0.75 mL	4.15 mL
Tris-HCL (1 M, pH 6.8)	0.62 mL	
Tris-HCL (1.5 M, pH 8.8)		2.5 mL
10 % SDS	0.05 mL	0.1 mL
H2O	3.55 mL	3.25 mL

For polymerization of the gels saturated APS solution (1:500) and TEMED (1:1000) were added.

2.2.4.3 Immunoblot analysis (Western Blot)

After the SDS-PAGE, the proteins were transferred from the gel to the nitrocellulose membrane. In this process membrane and gel were soaked in western-blot-transfer-buffer and placed between three layers of Whatman paper without air bubbles. The gel was turned towards the cathode and the membrane was turned towards the anode. Transfer took place for 15 minutes at 7.5 V using the SemiDry-transfer chamber (Trans-Blot SD, BioRad). After the transfer, binding sites on the membrane were blocked with blocking buffer over night at 4 °C upon a gentle shaking. The Anti-HCV E2 antibody was diluted due to its optimal binding capacity in blocking buffer before incubation with the membrane for 1 h or, alternatively, overnight at 4 °C. Following 3 cycles of washing with T-PBS for 10 minutes, the membrane was incubated for 1 h with conjugate (anti-mouse or anti-goat peroxidase labeled IgG) diluted 1:1000 in the blocking buffer. Additional 3 steps of washing with PBS followed. Binding of the conjugate to anti-HCV antibodies on the membrane was detected on a radiographic film (BioMax Film, Kodak) by using ECL Western blotting detection reagents according to the manufacturer's instruction.

2.2.5 Flow cytometry

Flow cytometry is a technology that simultaneously measures multiple physical characteristics of cells such as relative size, relative granularity and relative fluorescence intensity. The cells are transported in the fluid stream to the measuring cell where they are examined by the laser beam

one after another. Using monoclonal antibodies conjugated with a fluorescent dye, flow cytometry enables to identify a particular cell type within complex cell populations based on their individual antigenic markers.

2.2.5.1 Staining of cells for flow cytometry analysis

About $2x10^5$ PBMCs per well and preparation were seeded in a round-bottom 96-well plate and centrifuged for 5 min at 1500 rpm, supernatant was discarded and the cell pellet resolved in 100 µL RPMI 1640 (10 % FCS, 1% penicillin/streptomycin, 1 % HEPES). The relevant peptide (final amount 1 µg) was added to each well and cells were incubated for 5 h at 37 °C and 5 % CO_2. Afterwards the plate was centrifuged for 5 min at 1500 rpm and supernatant was discarded. Cells were washed with 200 µL cold PBS/FCS (1% FCS in PBS) per well and centrifuged for 5 min at 1500 rpm. Supernatant was discarded and cells stained for CD4 and CD8 molecules on their surface using anti-CD4-PE and anti-CD8-APC labeled antibodies (eBioscience) in a concentration of 1:1000, respectively, for 15 min at 4 °C. The plate was centrifuged for 5 min at 1500 rpm and cells were washed with 200 µL cold PBS/FCS per well. Cells were fixed with 100 µL IC Fixation Buffer (eBioscience) per well and incubated for 20 min at 4 °C. After centrifugation for 5 min at 1500 rpm supernatant was discarded and cells were washed with 200 µL cold Perm Buffer (eBioscience). Intracellular IFN-γ staining was performed using IFN-γ-specific FITC-labeled antibody (eBioscience, 1:1000) in 100 µL Perm Buffer per reaction and incubation for 30 min at 4 °C. After centrifugation for 5 min at 1500 rpm cells were washed with 200 µL cold Perm Buffer per well. After the last centrifugation step (5 min, 1500 rpm) cells were resuspended in 200 µL PBS per preparation and transferred to FACS tubes for subsequent FACS analysis.

2.2.6 Phylogenetic analysis

Phylogenetic analysis was performed according to the "Maximum-Likelihood" (ML) method using the phylip-3.69 software package. Up to 93 patient derived core-NS2 sequences were analyzed parallel with a set of reference sequences from the official HCV database http://hcv.lanl.gov/. Bootstrapping analysis assessed integrity of phylogenetic trees: 100 randomized replicates of the dataset were analyzed. On the basis of the different newly grouped set of sequences it can be estimated to which extend a number of sequences from the same gene and the same species would influence the validation of the phylogenetic tree.

3 Results

3.1 Generation of the HCV AD78 sequence database

The first aim of the project was to establish a sequence database of the HCV strain AD78 that caused a single-source outbreak of hepatitis C in 1978 in East Germany. The AD78 cohort consisted of women infected by administration of an HCV contaminated anti-D globulin. This unique cohort provides the possibility to address many different questions dealing with multiple aspects of HCV research. During the study a bank of HCV AD78-related sequences corresponding to the genomic fragments core-NS2 and NS4-NS5A obtained from both the contaminated anti-D globulin batches and infected patients (n=93) was generated.

3.1.1 Analysis of HCV AD78 core-NS2 sequences from the contaminated anti-D immunoglobulin

To establish this database, the viral RNA was extracted from the contaminated anti-D immunoglobulin batches 8, 9, 12 and 15 and subjected to reverse transcription. Due to degeneration of the RNA molecules during the long-term storage of the globulins the core-NS2 regions was amplified in four overlapping fragments. The PCR products were ligated into the TOPO-TA vector with sequencing of multiple clones. The phylogenetic analysis of the amplified sequences revealed that all HCV sequences derived from the contaminated globulin bathes were very closely related and were clearly separated from other HCV genotype 1b sequences available at the Los Alamos HCV database. Figure 3.1 illustrates these results and demonstrates that all sequences corresponding to the E2 genomic region (nt) from four batches of the anti-D globulin grouped together and that this cluster was separated from the bulk of other 1b sequences with a high statistical support.

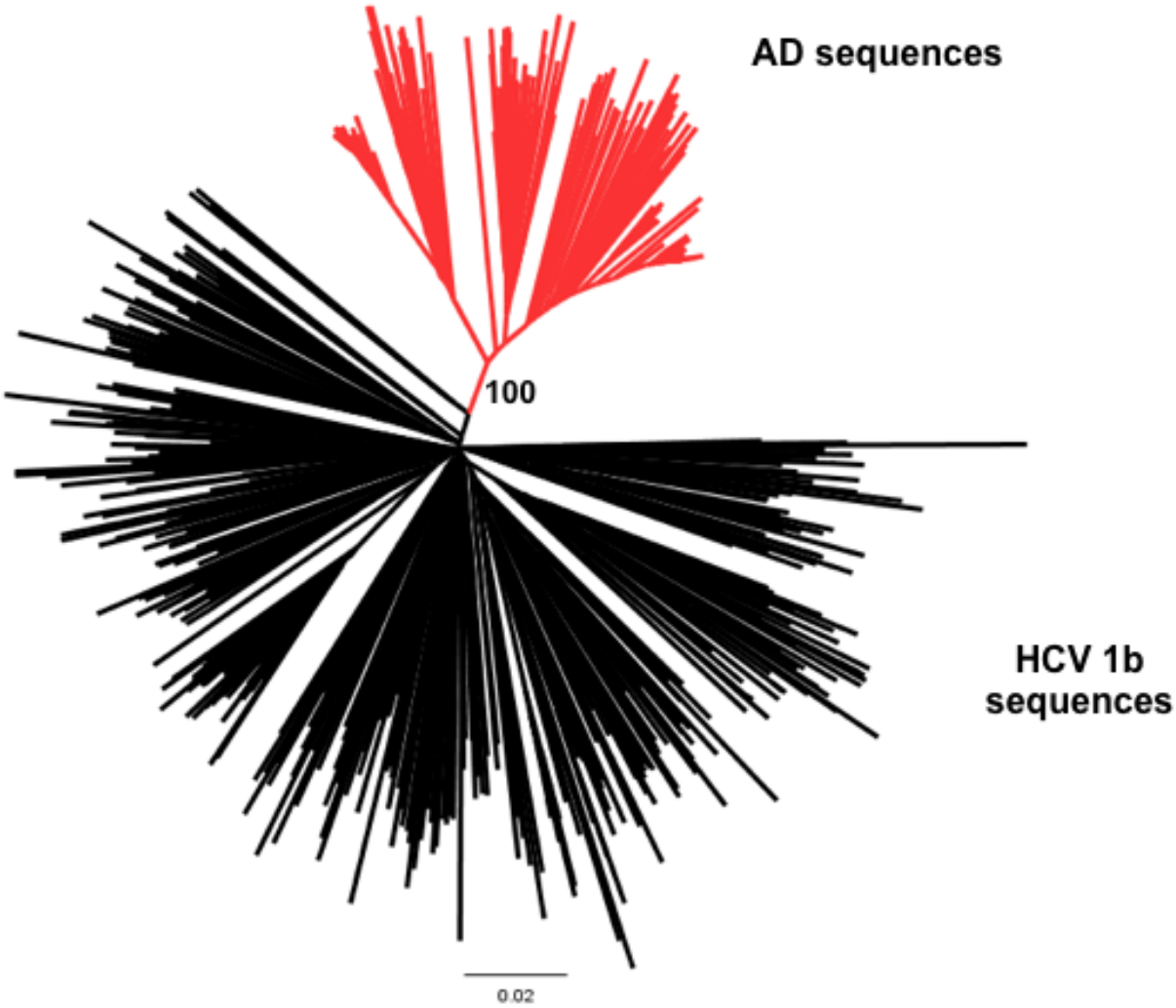

Fig. 3.1 Unrooted neighbor-joining tree of the E2 nucleotide sequences, obtained from HCV AD78-contaminated globulin batches 8, 9, 12, 15, and HCV 1b strains available from the Los Alamos HCV database. The bootstrapping value for AD78 sequences is indicated.

3.1.1.1 Identification of three variants of the HCV AD78 strain present in the contaminated anti-D immunoglobulin

Subsequent analysis of the heterogeneity of HCV clonal E2 sequences in the contaminated immunoglobulin (batches 8, 9, 12 and 15) revealed the presence of three closely related but genetically distinct variants of the AD78 strain in the infectious source (Fig.3.2). Of special note was the fact of unequal distribution of these three HCV AD78 variants (arbitrary designated as variants A, B, and C) in the different globulin batches. Figure 3.2 demonstrates that only globulin batch 12 contained sequences (labeled in blue) belonging to all three HCV A78 variants. The other three batches contained sequences belonging to two (batch 8 sequences

labeled in blue) or only to one (batch 9 sequences labeled in green and batch 15 sequences labeled in light blue) variants of the HCV AD78 strain. Such heterogeneity might be a result of different concentration of the variants A, B and C in these batches and/or inability of the used PCR protocol to provide for amplification of these three variants with an equal sensitivity. In support of this hypothesis were the previous results of our laboratory [252]. In this work the phylogenetic analysis of clonal NS3 sequences obtained from anti-D globulin batch 8 revealed the presence not of two, like in the current study, but of all three variants of the HCV AD78 strain. To address this issue, amplification of the E2 region from the batch 8 was performed using variant C-specific primers. With this approach variant 3-specific sequences could be obtained from the globulin 8 (Fig.3.3 and Fig.3.4). These results have suggested that all batches of the anti-D globulin most probably contained all three variants of the HCV AD78 strain.

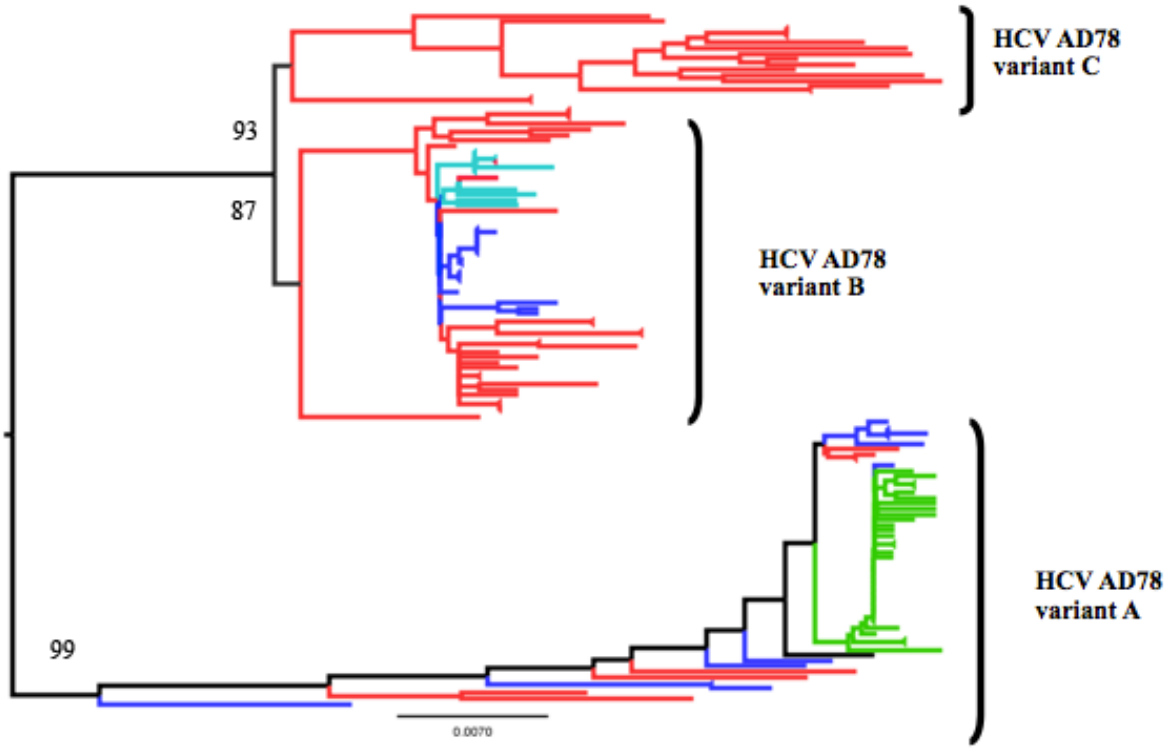

Fig. 3.2 Rooted neighbor-joining tree of the E2 sequences, obtained from anti-D globulin 8, 9, 12, and 15; separation of the AD78 strain into three closely related but genetically distinct variants (A, B, and C). The bootstrapping values are indicated.

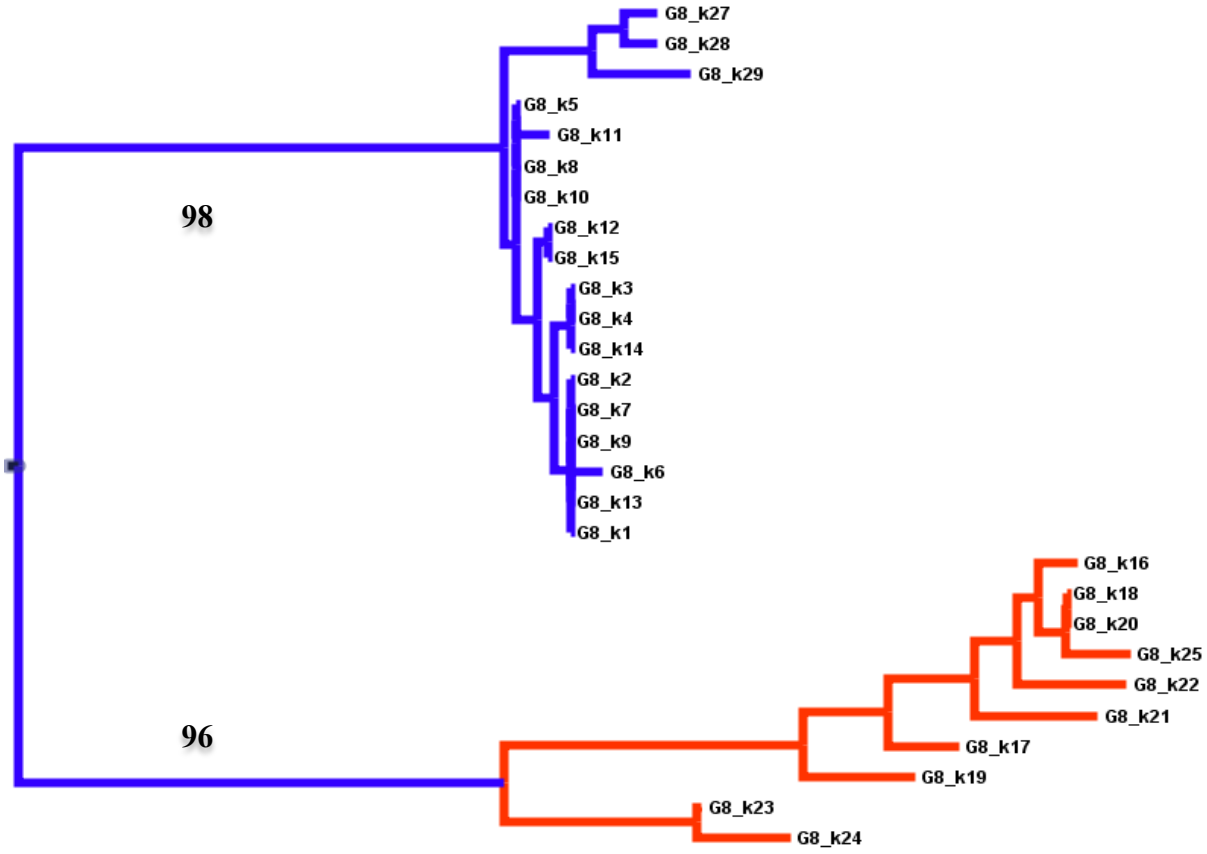

Fig. 3.3 Rooted neighbor-joining tree of E2 nucleotide sequences, (without HVRI) obtained from the anti-D globulin batch 8. The bootstrapping values are indicated.

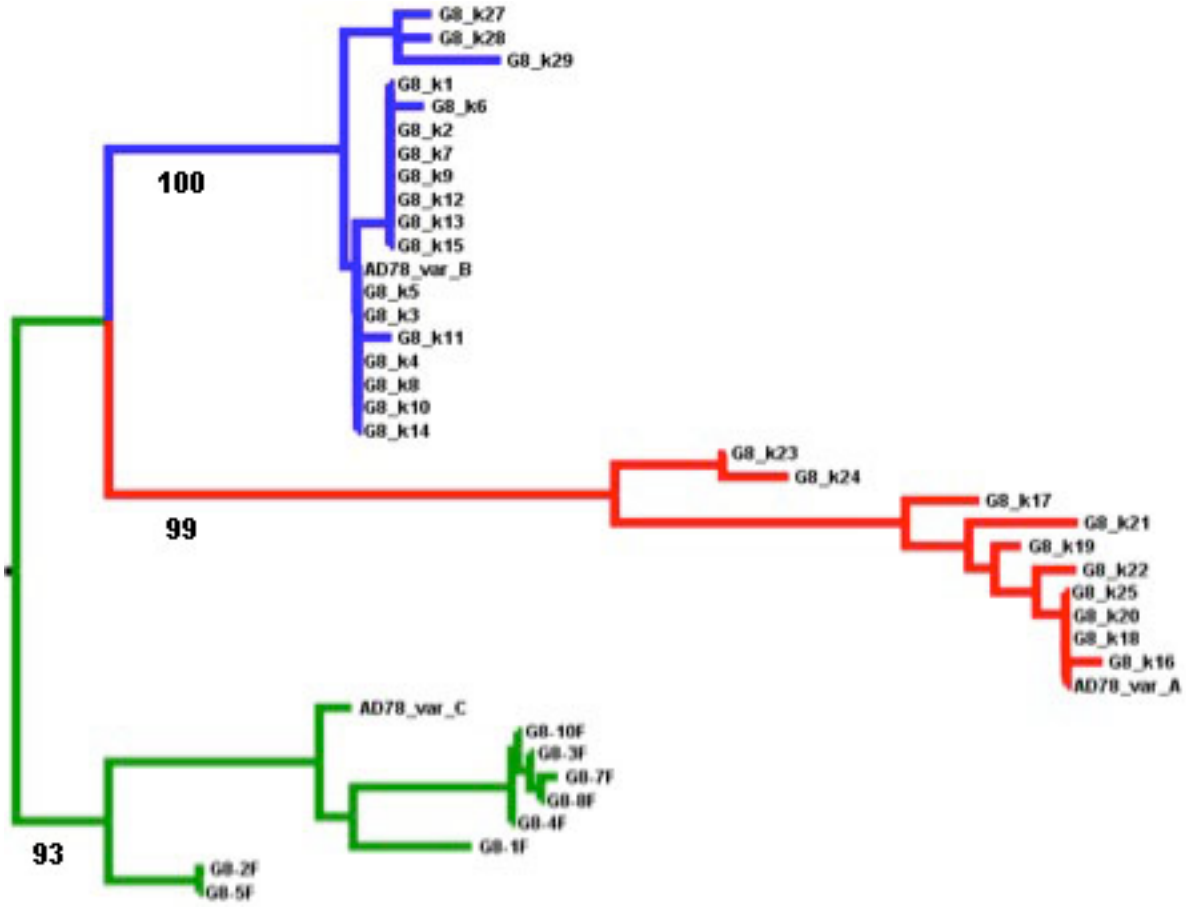

Fig. 3.4 Rooted neighbor-joining tree of E2 nucleotide sequences, obtained from anti-D globulin batch 8, sequences for variant C amplified with variant-specific primers. The bootstrapping values are indicated.

3.1.1.2 Generation of the consensus sequences for the three variants of the HCV AD78 strain

The whole set of clonal core-NS2 sequences obtained from different batches of the contaminated anti-D globulin were used for construction of the consensus sequences for HCV AD78 variant A, B, and C, respectively. As expected upon phylogenetic analysis these three consensus sequences were clearly separated from the bulk of HCV 1b sequences from the Los Alamos HCV database (Fig.3.5)

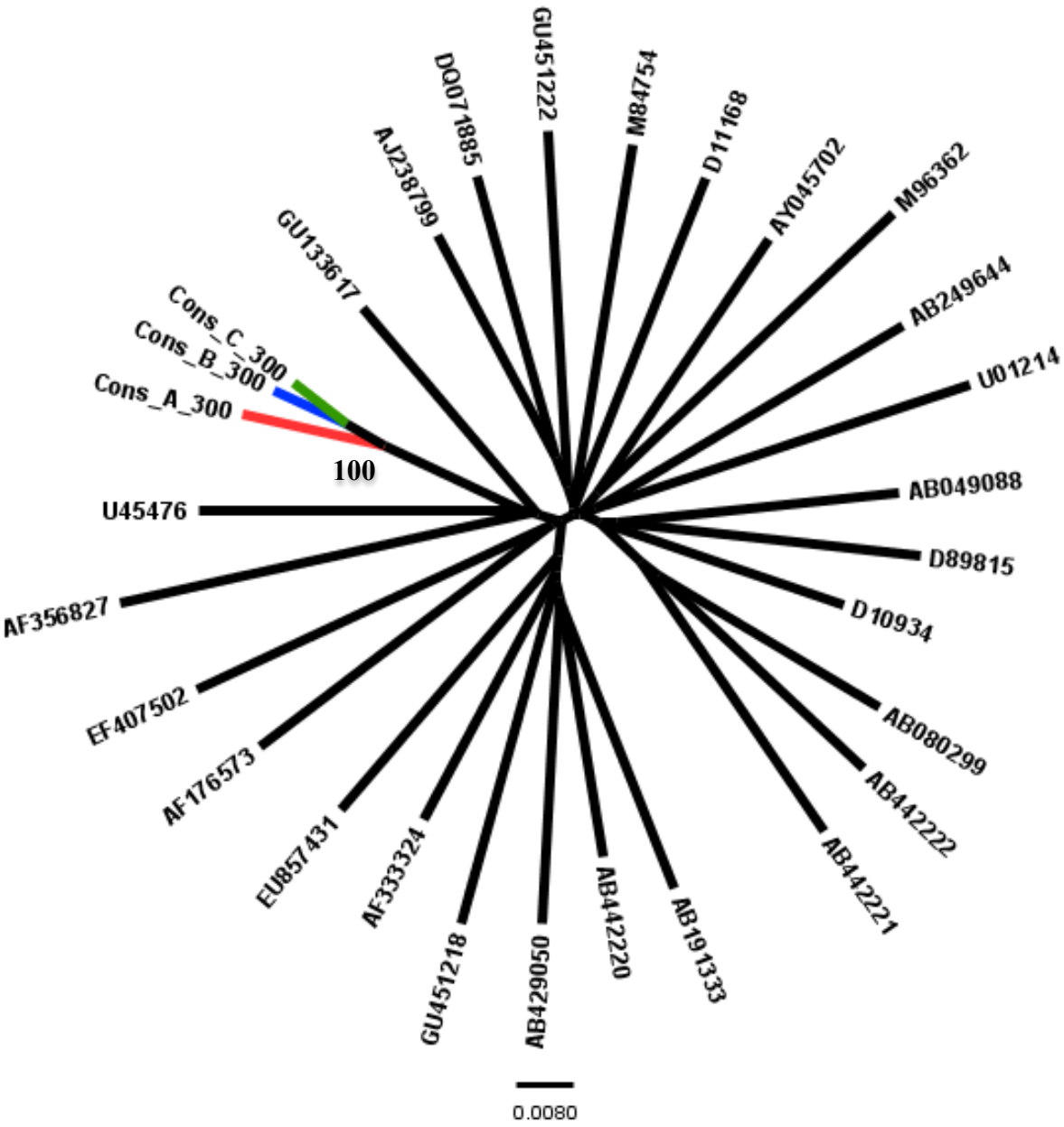

Fig. 3.5 Unrooted neighbor-joining tree of the three generated AD78,

consensus nucleotide sequences and randomly chosen HCV 1b nucleotide sequences from the Los Alamos HCV database. The bootstrapping value for HCV AD78 consensus sequences A, B, and C is indicated.

3.1.2 Analysis of HCV AD78 core-NS2 sequences from anti-D patients

At the next step of the project the core-NS2 and NS4A-NS5A genomic regions were amplified from blood samples of 93 patients obtained 30 years after infection with the HCV AD78 strain through the administration of HCV contaminated anti-D immunoglobulin. The amplified DNA fragments were subjected to direct sequencing and subsequent phylogenetic analysis together with the consensus sequences of HCV AD78 variants A, B and C obtained from the contaminated globulin batches. All these sequences demonstrated close relatedness, formed a unique cluster and were clearly separated from the HCV 1b sequences collected in the Los Alamos HCV database (Fig.3.5), supporting a previous observation of our group that the HCV isolates, which caused the infection in the anti-D cohort, came from the same origin [252]. Additional analysis of these sequences (Fig.3.6 and Fig.3.7) revealed that the cluster of anti-D sequences formed three different clades with a high bootstrapping support. This grouping corresponds to the three variants of the HCV AD78 strain identified in the contaminated globulin batches (Fig.3.2). Thus, the obtained data suggest that the infectious source (contaminated anti-D immunoglobulin) contained three closely related but genetically distinct variants of the same HCV AD78 virus strain and that all these virus variants were capable to initiate infection in the anti-D cohort.

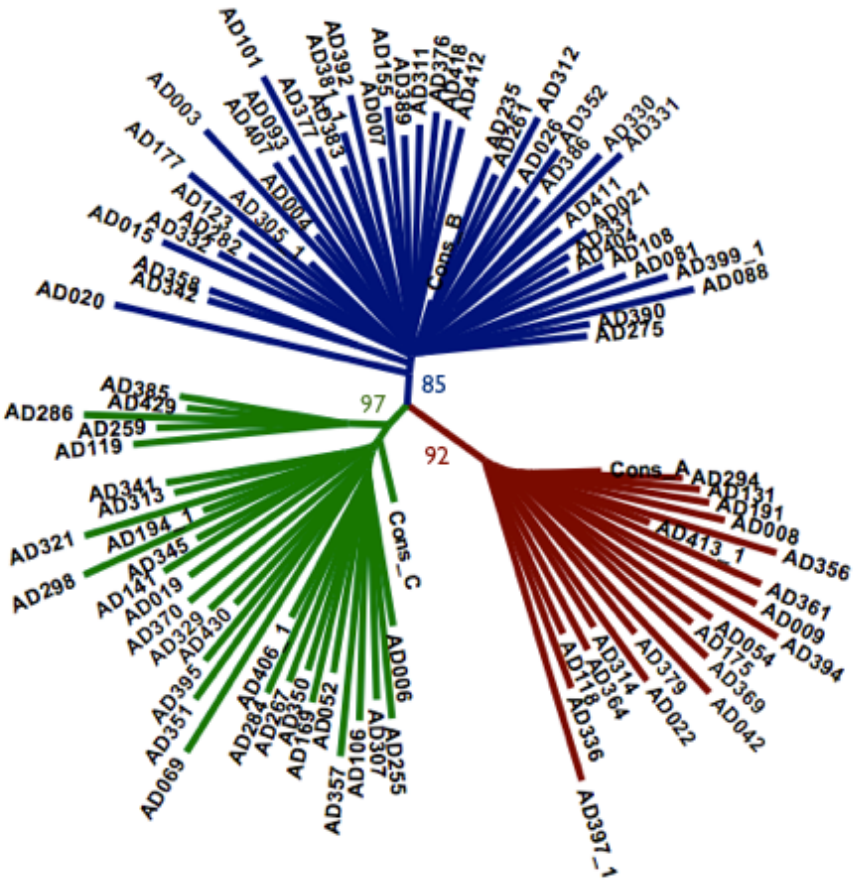

Fig. 3.6 Unrooted neighbor-joining tree of 93 patient-derived HCV AD78 core-NS2 nucleotide sequences and the three consensus sequences for HCV AD78 variants A, B and C. Bootstrapping values are indicated.

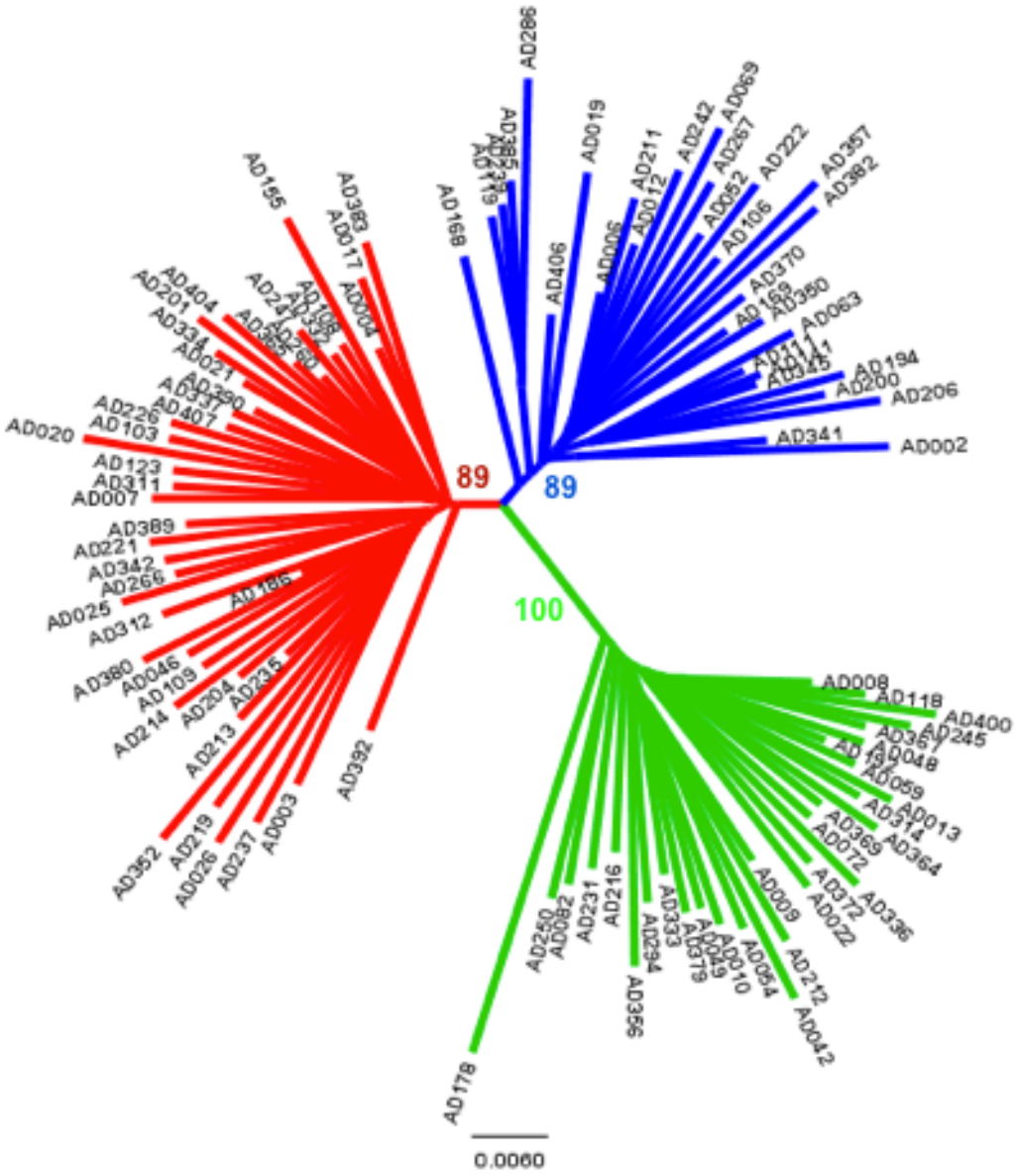

Fig. 3.7 Unrooted neighbor-joining tree of 103 patient derived HCV AD78 NS4-NS5 nucleotide sequences. Bootstrapping values are indicated.

3.1.3 Analysis of E2 sequences of the HCV AD78 strain obtained from globulin donors

Our data revealed heterogeneity of HCV AD78 sequences in the contaminated anti-D globulin batches. It was of interest to see if the three variants of the HCV AD78 strain were already

present in those donors, whose plasma samples were used for preparation of the anti-D globulin batches. Plasma samples from these 5 individuals, collected approximately 12 years later were available for analysis. Four of these samples were positive in RT-PCR and the obtained E2 sequences were subjected to phylogenetic analysis together with E2 sequences amplified from anti-D patients collected 30 years after infection. Figure 3.8 indicates that the E2 sequences from 3 of the secondary plasma donors grouped together with sequences belonging to variant C of the HCV AD78 strain. The sequence from the fourth donor localized in the clade formed by sequences belonging to variant B of this strain.

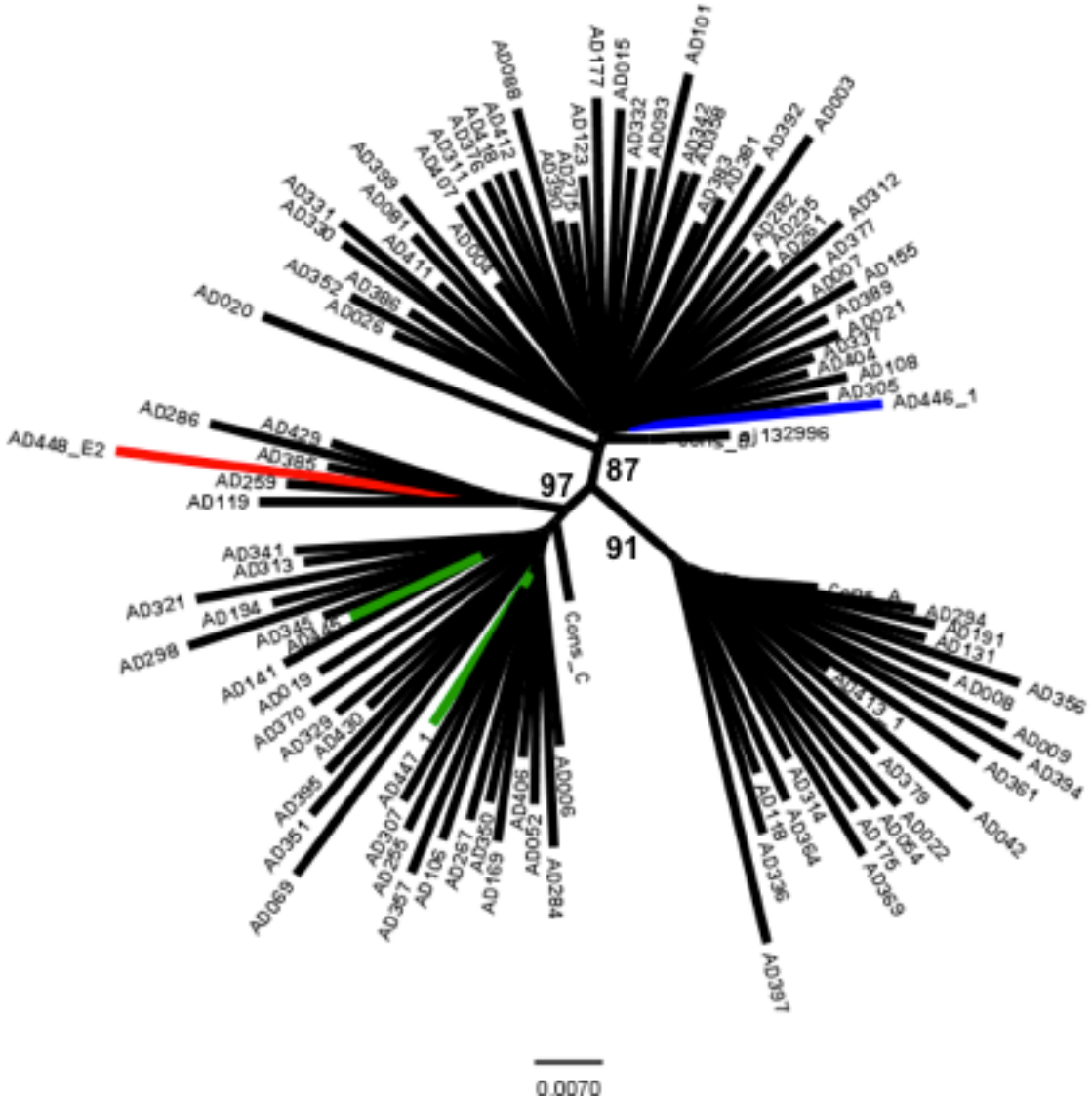

Fig. 3.8 HCV AD78 variant attribution of the HCV E2 sequences amplified from donors, whose plasma was used for preparation of the anti-D globulin batches. Three sequences labeled in green and red grouped together with sequences belonging to the viral variant C. The fourth sequence labeled in blue was found in a cluster formed by sequences belonging to virus variant B. Sequences

from the anti-D patients and consensus sequences for HCV AD78 A, B and C variants are labeled in black. Bootstrapping values are indicated.

3.1.4 Evolution of the HCV AD78 strain

Generation of the HCV AD78 sequence database allowed initiating the analysis of HCV evolution in the anti-D cohort. The core-NS2 sequences obtained from contaminated globulin and anti-D patients were compared with the HCV subtype 1b general consensus sequence constructed from sequences available from the Los Alamos database. The consensus sequences for three variants of the HCV AD78 strain present in the contaminated globulin batches 8, 9, 12, and 15 were used as the anti-D-outbreak founder sequences. Table 3.1 lists the positions in the core-NS2 fragment, where at least one of the virus variant-specific consensus sequences from the infection source differed from the HCV 1b consensus sequence. Overall, 47 such positions were found – 2 in the core, 11 in the E1, 20 in the E2 (HVR1, HVR2 and HVR3 were excluded from this analysis), 3 in the P7, and 11 in the NS2 protein sequences. Upon normalization of these results by the size of these proteins the frequencies of sites of mutations were established. As expected, the lowest value (1%) was obtained for the core protein, which reflected a high conservation of this protein. For other proteins the frequencies varied within very narrow range, between 4.8 % for P7 to 5.7 % for E1, suggesting similar "rates of hot spot of mutation". One should note, however, that for this analysis the HVRs 1, 2, and 3 were excluded from the E2 sequences and real "rate of hot spot mutation" for this particular protein, therefore, is much higher.

Table 3.1 Comparison of the HCV subtype 1b consensus sequence (aa) with HCV AD78 core-NS2 amino acid sequences from the contaminated globulin (consensus sequences for HCV AD78 variants A, B and C) and anti-D patients. Bolt lines indicate the end/beginning of the next protein, starting with core till NS2.

aa position	Consensus 1b	AD78 variant A		AD78 variant B		AD78 variant C	
		Source	Patients (n=20)	Source	Patients (n=44)	Source	Patients (n=30)
70	R	**Q**	**R (16)**	R	R (37)	R	R (23)
91	M	L	L (21)	L	L (21)	L	L (26)
202	H	Q	Q (17)	H	H (44)	H	H (29)
216	A	T	T (20)	T	T (44)	T	T (30)
233	N	D	D (12)	D	D (17)	D	D (19)

aa position	Consensus 1b	AD78 variant A		AD78 variant B		AD78 variant C	
		Source	Patients (n=20)	Source	Patients (n=44)	Source	Patients (n=30)
251	S	S	S (19)	G	G (31)	G	G (21)
257	T	T	T (19)	A	A (32)	A	A (23)
287	V	V	V (18)	V	V (37)	I	I (28)
293	F	F	F (19)	**L**	**F (30)**	**L**	**F (19)**
303	D	E	E (18)	E	E (32)	E	E (27)
314	S	T	T (19)	T	T (43)	T	T (29)
330	T	A	A (12)	T	T (43)	T	T (29)
345	V	V	V (19)	V	V (41)	M	M (18)
414	I	V	V (11)	I	I (33)	V	V (16)
492	R	Q	Q (10)	Q	Q (23)	**Q**	**R (18)**
501	Q	Q	Q (18)	E	E (40)	E	E (23)
521	R	R	R (17)	R	R (41)	**H**	**R (17)**
522	F	F	F (11)	**S**	**F (29)**	**S**	**F (22)**
528	S	S	S (15)	R	R (24)	S	S (15)
531	E	**A**	**E (17)**	E	E (39)	E	E (15)
557	S	T	T (12)	**T**	**A (22)**	T	T (15)
570	N	K	K (16)	N	N (39)	K	K (17)
574	V	L	L (12)	L	L (27)	L	L (23)
580	T	I	I (10)	I	I (25)	**I**	**T (14)**
596	K	R	R (15)	K	K (42)	K	K (29)
609	M	I	I (13)	I	I (35)	I	I (20)
636	V	V	V (19)	I	I (41)	I	I (29)
641	N	S	S (10)	S	S (20)	S	S (15)
653	D	**G**	**D (9)**	N	N (29)	N	N (26)
705	I	V	V (20)	**I**	**V (25)**	**I**	**V (22)**

aa position	Consensus 1b	AD78 variant A		AD78 variant B		AD78 variant C	
		Source	Patients (n=20)	Source	Patients (n=44)	Source	Patients (n=30)
708	A	V	V (20)	V	V (44)	V	V (29)
709	V	L	L (18)	L	L (35)	L	L (26)
719	V	I	I (20)	I	I (45)	I	I (30)
759	V	L	L (20)	L	L (41)	L	L (29)
790	F	L	L (17)	L	L (37)	L	L (14)
792	G	S	S (17)	G	G (40)	S	S (24)
824	V	V	I (10)	I	I (41)	V	V (14)
827	A	V	V (13)	V	V (28)	V	V (24)
834	H	Y	Y (18)	Y	Y (39)	Y	Y (27)
841	R	K	K (20)	K	K (44)	K	K (30)
861	I	V	V (19)	I	I (35)	I	I (23)
864	V	I	V (19)	V	V (43)	V	V (30)
885	I	V	V (19)	I	I (35)	I	I (23)
891	I	L	L (20)	L	L (44)	L	L (30)
906	I	I	I (19)	I	I (41)	M	M (29)
938	F	F	F (12)	L	L (43)	F	F (28)
1008	R	K	K (14)	K	K (29)	K	K (23)

Amino acid positions as aligned to the hepatitis C virus genotype 1 reference (NC_004102)

Most of the amino acid residues at the identified 47 sites were rather stable and were observed in sequences from most of the anti-D patients even after 30 years of infection. In 10 cases, however, reverse mutations toward the 1b consensus sequence were observed (positions 70, 293, 492, 521, 522, 531, 580, 653, and 705). Interestingly, most of these reversions (7 of 10) occurred in the E2 protein. At one position (557) mutation away from the consensus 1b sequence (from threonine to alanine) was detected in 50 % of the sequences of the patients. In 3 cases, at positions 705 and 824, where the amino acid residues in 1b consensus and infectious source sequences were similar, forward mutations were identified the majority of sequences obtained

from patients. These data suggest that in the anti-D cohort reverse mutations toward the consensus 1b sequences at the "hot spot of mutations" sites were relatively rare.

Evolution toward consensus sequence is considered as a relatively accurate assessment of a pressure toward optimal viral fitness. Therefore, at the next step, all mutations observed in the core-NS2 region in sequences obtained from all 93 anti-D patients were classified according to their direction – forward (away from the consensus sequences of AD78 variants A, B or C) or reverse (toward the 1b consensus sequence). The HVR1, HVR2 and HVR3 were excluded from this analysis. The obtained results are presented in figure 3.9 and complement those described above for the "hot spot of mutations" sites and indicate that in persistently infected anti-D patients virus genome evolution toward the general HCV 1b consensus sequence is relatively rare and observed in about 25-30 % of cases.

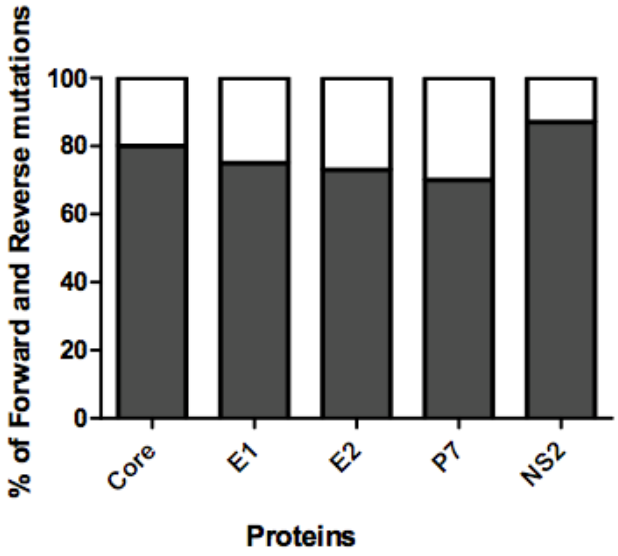

Fig. 3.9 Frequencies of forward and reverse mutations, accumulated during 30 years of infection in the core, E1, E2, p7, and NS2 sequences of HCV AD78 isolates from 93 patients. Total number of mutations is equal to 100 %.

3.2 Generation of the new AD78/JFH-1 chimeric viruses

The second aim was to generate chimeric viruses on the basis of the established consensus sequences (see section 3.1) for the three identified variants of the AD78 strain, that are capable of complete replication and infection in cell culture.

The created core-NS2 consensus sequences of all three variants of the HCV AD78 strain were chemically synthesized and used for the generation of the AD78/JFH1 chimeric viruses (fig.3.10) (collaboration with Prof. R. Bartenschlager). The first experiments demonstrated viability of all three types of the chimeric viruses with a slightly better outcome for the HCVcc bearing the core-NS2 sequence from the consensus sequence of the variant A of the HCV AD78 strain.

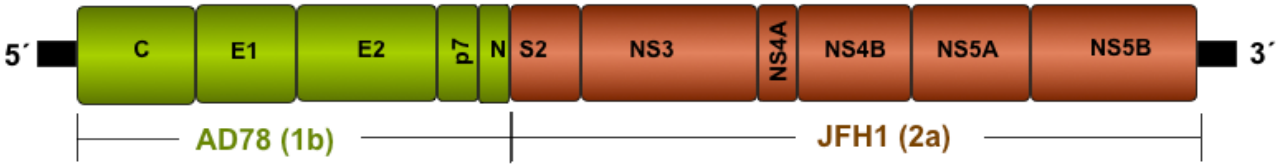

Fig. 3.10 Scheme of the AD78/JFH1 chimeric virus

At the next step the replication characteristic of this newly constructed AD78/JFH1 chimeric virus was assessed in four independent experiments. Results of the limited dilution assays presented in Figure 3.11 and 3.12 clearly demonstrate reproducibility of high levels of replication of the AD78/JFH1 virus comparable to that of the control H77/JFH1 chimeric virus. These results have indicated that the tested variant of the AD78/JFH1 chimeric viruses could be used in the subsequent experiments on the assessment of the influence of different mutations, including escape mutations, on HCVcc replication and infectivity.

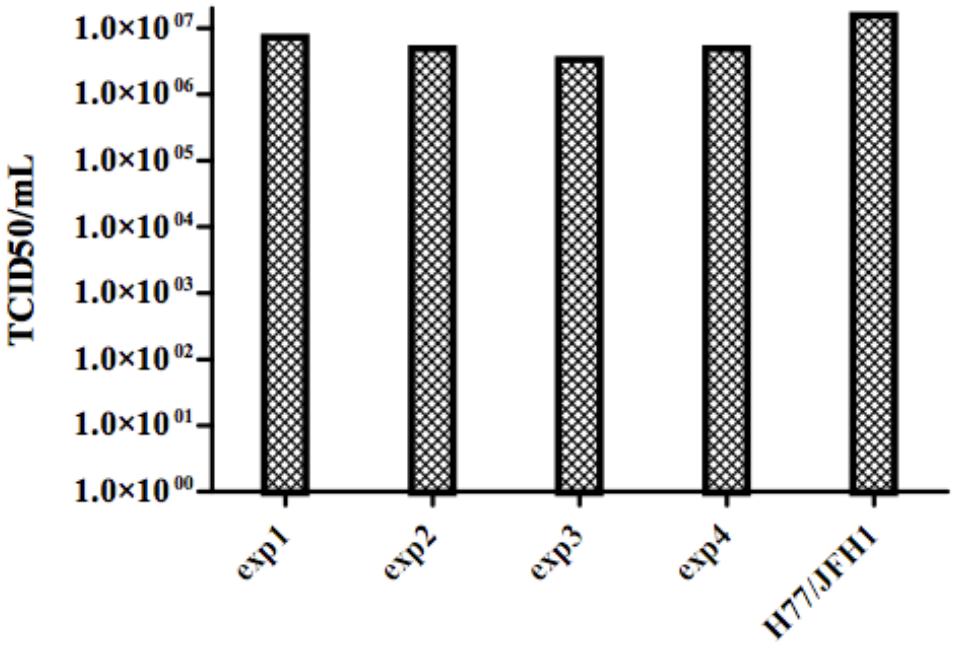

Fig. 3.11 Determination of the infectivity of the newly AD78/JFH1, chimeric virus in four independent experiments. The Huh7.5 cells were transfected with RNA transcribed from the AD78/JFH1 linearized plasmid. Supernatant collected 72 hours after transfection was used for infection of fresh Huh7.5 cells. After incubation for 3 days the infected cells were stained with monoclonal antibody directed against the NS5A protein. Virus titers were determined by TCID50 assay. The H77/JFH1 chimeric virus was used as a positive control.

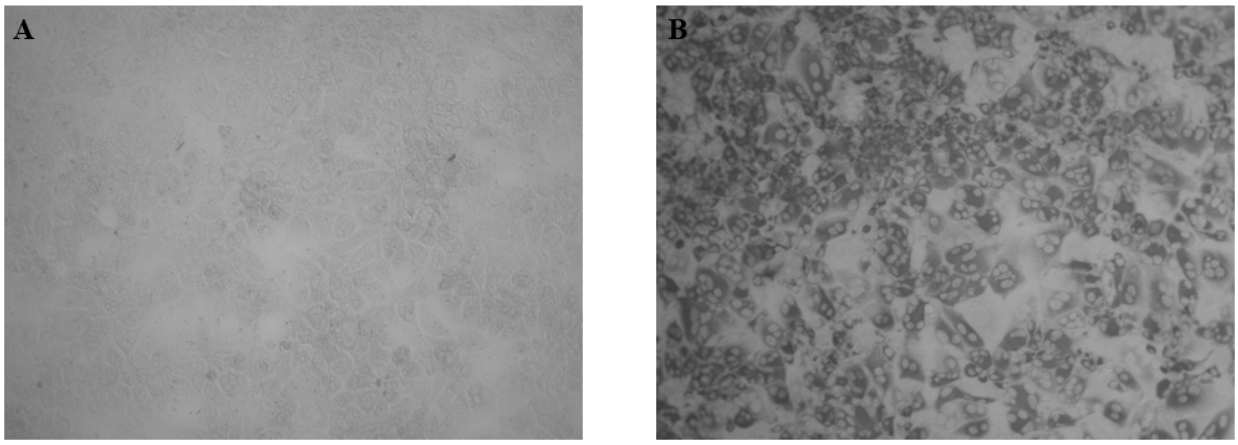

Fig. 3.12 Immunostaining of the infected Huh7.5 cells, with monoclonal antibodies directed against the NS5A protein. (A) Uninfected control, (B) Huh7.5 cells infected with the AD78/JFH1 virus.

3.3 Study of the HLA class I-associated polymorphisms in sequences from the anti-D cohort and analysis of escape mutations

The third aim of the current study was to assess the contribution of immune escape from the cellular immune response to evolution of the HCV E2 protein. A number of CD8 epitopes in HCV proteins has already been reported to the Immune Epitope Database (www.immuneepitope.org). One should note, however, that the available list of CD8 epitopes is far from being complete and novel epitopes are kept being identified during the last years [252]. Thus, for the purpose of our project it would have been imperative to check for the presence of putative novel CD8 epitopes in the sequences from the AD78 cohort. Therefore, we have applied the bioinformatic approaches, developed by Prof. D. Hoffmann in collaboration with Prof. J. Timm, for analysis of the sequences from the generated HCV AD78 sequence database [252]. With this approach a statistically significant association between expression of a particular HLA class I allele in patients and presence of amino acid substitutions at each site of the viral protein sequences in HCV isolates from patients was determined. An example for such an analysis for the HLA-B51 allele is presented in figure 3.13. Red dots located above the arbitrary cut-off value of $p < 0.01$ indicate 10 potential sites under HLA-B51 selective pressure. First, the sequences flanking the positions 147 and 153 in the core protein with the highest statistical significances were analyzed. Application of the binding prediction algorithms ANN and SMM (available at http://tools.immuneepitope.org) allowed identifying an HLA-B51-specific motif x[APG]xxxxxx[IV] around position 153 but not surrounding position 147. Sequences of anti-D patients, for whom data on HLA class I types were available (n=83), were aligned and stratified according to the expression of HLA-B51. A fragment of this alignment around position 153 is presented in figure 3.14. It is shown that 2 of 10 sequences from HLA-B51-positive patients bear the mutation H153Q. In contrast, such a substitution was absent in 73 sequences from HLA-B51-negative subjects. A similar analysis of other potential sites (Fig. 3.13) did not reveal any evidences for HLA-B51 selective pressure. Overall, these data suggested the existence of a novel CD8 epitope restricted by HLA-B51 in the core protein sequences of the HCV AD78 strain.

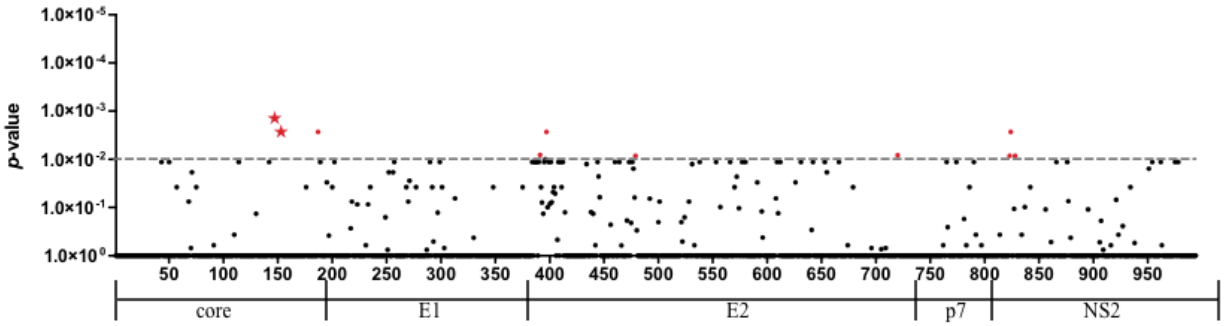

Fig. 3.13 Identification of amino acid residues under selective pressure in the presence of the HLA-B51 allele. The figure demonstrates the p-values for each amino acid position in the core-NS2 region. An arbitrary cut-off value of $p < 0.01$ is marked with a dashed line. The positions above this threshold are marked in red and the big red asterisks indicate the sequence positions highlighted in fig. 3.14.

	consensus	GAPLGGAARA LAHGVRVLED
HLA-B51 +	6	....
	1	.....V... .....
	2	.....V... ..Q.....
	1	.G..... .....
HLA-B51 -	72	....
	1	.....V... .....

Fig. 3.14 Polymorphism at positions 147 and 153 of the core sequences, obtained from anti-D patients. Aligned sequences were stratified according to the expression of the HLA-B51 allele. Position of the predicted epitope is marked with a green frame. Two of 10 sequences from HLA-B51-positive (HLA-B51 +) patients showed a mutation at position 153, whereas the same substitution was absent in all 73 sequences from HLA-B51 negative (HLA-B51 -) subjects.

The same analysis was performed for other HLA class I alleles expressed by anti-D patients included into our study. Table 3.2 lists all 10 CD8+ T-cell epitopes with a statistical support for immune selection of the putative escape mutation identified with this approach. Three of them, in the core, E1 and NS2 proteins, were putative novel epitopes.

Table 3.2 CD8+ T cell epitopes/sequence regions with statistical evidence for selection by HLA-mediated immune pressure.

Allele	Position	Protein	Sequence	*p*-value*	CD8+ T cell response	CD8+ T cell escape
A03	43 - 51	core	RLGVRATRK	0.014	+	+
A26	93 - 101	E1	EVRNVSGVY	0.0024	n.d.	n.d.
A2402	669 - 679	E2	LLSTTEWQI	0.008	+	+
A2404	679 - 687	E2	ILPCSFTTL	0.008	+	n.d.
B07	41 - 49	core	GPRLGVRAT	0.0008	+	+
B51	142 - 160	core	APLGGAARALAHGVRVLED**	0.0014	+	n.t.
B38	188 - 206	E1	PASAYEVRNVSGVYHVTND**	0.0005	n.d.	n.t.
B57	459 - 469	E2	RMASCRPIDKF	0.00003	n.d.	n.d.
B57	545 - 553	E2	NTRPPQGNW	0.0007	+	+
B08	958 - 972	NS2	TPLRDWAHAGLRDLA**	0.0002	n.d.	n.t.

* Fisher‘s exact test **sequence regions containing potential novel epitopes identified in this study n.d. - not detected n.t. - not tested

The statistical method used for the detection of epitopes and mutations that emerged under specific HLA class I selective pressure sometimes may provide false-positive results. Therefore, for identification “real” so far unknown epitopes inside viral proteins it can be used (in combination with the CD8 epitope binding prediction algorithm) only as a basic approach and needs an experimental verification. To provide an additional proof for the presence of novel epitopes or escape mutation in the already reported epitopes the peptides covering the regions of interest were designed. These peptides were used for the antigen-specific in vitro proliferation of PBMCs from the anti-HCV-positive, RNA-negative patient expressing relevant HLA class I allele. After 10 days of culture, PBMCs were re-stimulated with the same peptide, respectively. Subsequently, intracellular cytokine staining (ICS) for IFN-γ and FACS analysis were performed. Figure 3.15 exemplifies such an analysis for a putative novel HLA-B51 epitope identified in the core protein (Fig. 3.13 and Table 3.2). Stimulation of PBMCs with the peptide covering the region of interest led to increase in a number of cells (0.51%) producing IFN-γ. In control cell cultures stimulation with an HCV unrelated peptide did not increase the number of

IFN-γ producing cells (0.05 %) significantly. These results confirmed the existence of a novel HLA-B51 epitope within the peptide GAARALAHGVRVLED consisting of 15 residues, but the exact location of this epitope remained unknown

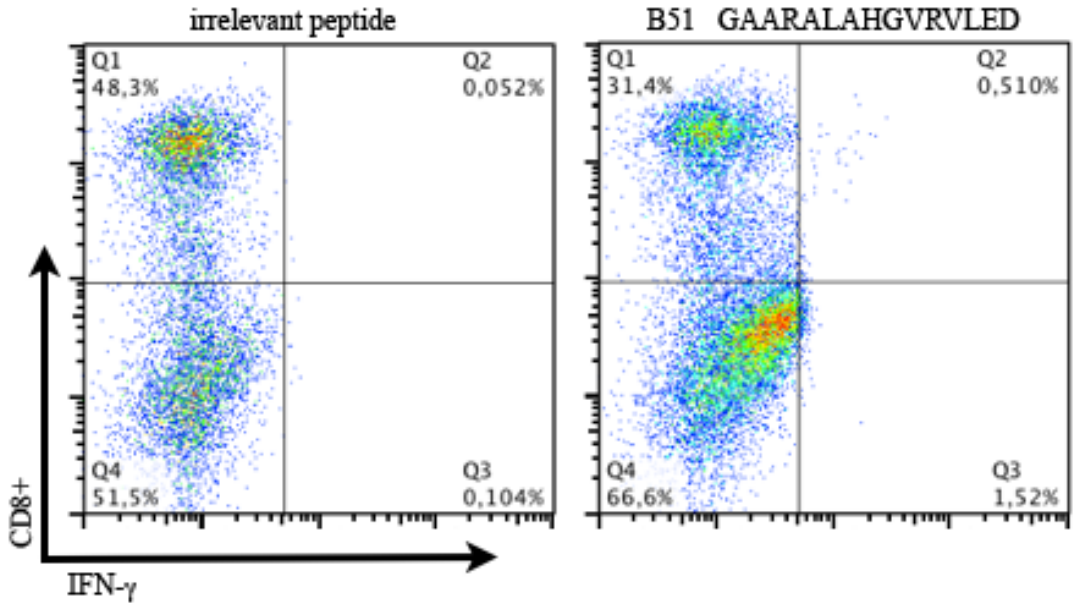

Fig. 3.15 Intracellular cytokine staining (ICS) for a putative novel HLA-B51, epitope inside the GAARALAHGVRVLED sequence region. PBMCs from an anti-HCV positive RNA-negative patient expressing HLA-B51 were expanded with the peptide GAARALAHGVRVLED and subsequently re-stimulated with the same peptide. Production of IFN-γ was detected by ICS and subsequent FACS analysis. As a negative control, stimulation of CD8+ T cells with an irrelevant peptide was performed.

As a next step, a fine mapping of this novel HLA-B51 epitope was performed with the help of a set of truncated versions of the GAARALAHGVRVLED peptide. Stimulation of HLA-B51-specific PBMCs with these truncated peptides (Fig 3.16) revealed that only the peptide RALAHGVRV corresponding to the HLA-B51 binding motif x[APG]xxxxxx[IV] was recognized by CD8 T cells in the intracellular cytokine staining (ICS) assay, confirming that this peptide truly represents a novel HLA-B51- specific CD8 epitope.

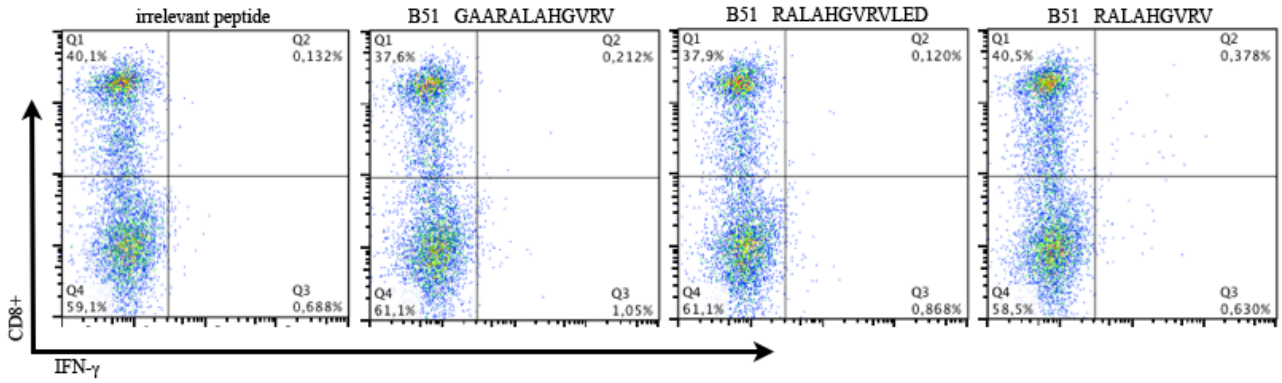

Fig. 3.16 Fine mapping of a novel HLA-B51 epitope from the core protein.
PBMCs from an anti-HCV positive, RNA-negative patient expressing HLA-B51 were expanded with 3 truncated variants of the peptide GAARALAHGVRVLED and subsequently re-stimulated with the same peptides. Production of IFN-γ was detected by ICS and subsequent FACS analysis. As a negative control, stimulation of CD8+ T cells with an unspecific peptide was performed.

Two other putative new epitopes inside the E1 and NS2 proteins (table 3.2) were additionally tested by a functional assay in vitro. Peptides corresponding to these epitopes were not recognized by CD8+ T cells in an intracellular cytokine staining assay. Thus, these preliminary data performed with PBMC from only two subjects did not provide conformation of the existence of these two putative epitopes.

Additional example of identification of the putative escape mutations in HCV AD78 core to NS2 proteins driven by CD8+ T cell responses is presented in the next two figures. First, application of the statistical approach described above allowed for identification of several positions pointing to potential sites under HLA-B07-associated selection pressure (Fig. 3.17). One of these sites (position 43 in the core protein) was located in already described CD8 epitope 41-GPRLGVRAT-49 in the core protein. Anti-D patient's sequences corresponding to the region around this epitope were aligned (Fig. 3.18) and the frequency of amino acid exchanges was assessed. Eight of 17 sequences from HLA-B07-positive individuals contained substitutions R43K, R43T, and R43S in this epitope, whereas in the sequences from 66 HLA-B07-negative patients, mutation at this site (R43K) occurred only in one case.

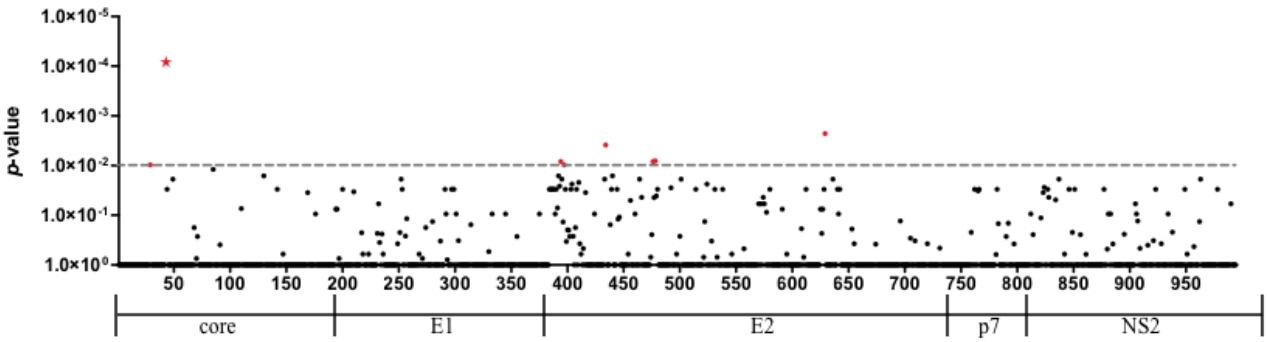

Fig. 3.17 Identification of amino acid residues under selective pressure in presence of the HLA-B07 allele. The figure demonstrates the p-values for each amino acid position in the core-NS2 region. An arbitrary cut-off value of $p < 0.01$ is marked with a dashed line. The positions above this threshold are marked in red and the big red asterisk indicates the sequence position highlighted in fig. 3.18.

		GPRLGVRATR
HLA-B07 +	9	....
	4	..K.......
	1	..KM......
	1	..S.......
	1	..T.......
	1	.........P.
HLA-B07 -	64	....
	1	..K.......
	1	.........S

Fig. 3.18 Polymorphism at position 43 of the core sequences obtained from anti-D patients. Aligned sequences were stratified according to the expression of the HLA-B07 allele. Position of the known epitope is marked with a green frame. Eight of 17 sequences from HLA-B07-positive (HLA-B07 +) patients showed amino acid substitutions at position 43, whereas the residue exchange in this position occurred only in one of 66 sequences from HLA-B07-negative (HLA-B07 -) subjects.

To get an additional proof for the presence of HLA-B07 driven escape mutations inside the 41-GPRLGVRAT-49 epitope the peptides corresponding to the wild-type variant and the potential escape variants of the peptide were designed. The wild-type and mutant peptides were tested by an intracellular cytokine-staining assay. Figure 3.19 demonstrates the result of the FACS

analysis of peptide stimulated PBMCs. Appearance of the IFN-γ-producing cells was observed only in cultures stimulated with the original wild-type peptide GPRLGVRAT but not with the mutant peptides bearing the substitutions R43K, R43S, and R43T. These data confirm that the mutations occurring at position 43 inside the HLA-B07 epitope in the core protein are truly escape mutations.

The same methodical approach was applied for analysis of other putative escape mutations identified in the current study (Table 3.2). Confirmation of the existence of HLA class I-associated escape mutations was obtained for 4 of 10 CD8 epitopes.

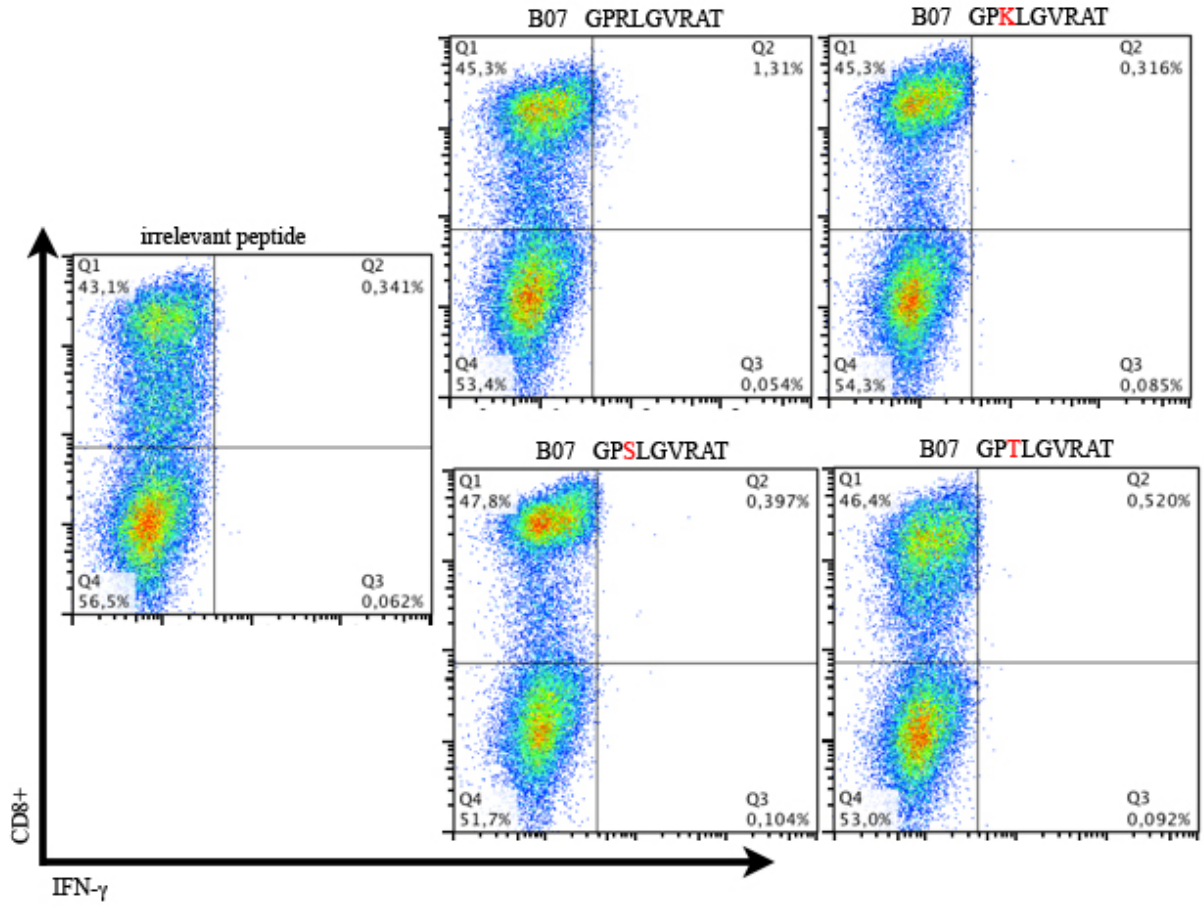

Fig. 3.19 Identification of escape mutations in the HLA-B07 epitope inside the core protein. PBMCs from an anti-HCV-positive, RNA-negative patient expressing HLA-B07 were expanded with the wild-type peptide GPRLGVRAT or with the mutated peptides bearing the substitutions R43K, R43S, or R43T and subsequently re-stimulated with the same peptides. Production of IFN-γ was detected by ICS and subsequent FACS analysis. As a negative control, stimulation of CD8+ T cells with an irrelevant peptide was performed.

3.3.1 Influence of CD8+ T cell escape mutations on viral replication

The detailed analysis of core-NS2 sequences obtained from the HCV AD78-contaminated globulin and anti-D patients led to the identification of CD8+ T cell escape mutations in this region (Table 3.2). Several recent studies, based on the application of the HCV subgenomic replicon system have shown that CD8+ T cell escape mutations inside the nonstructural HCV proteins can have severe fitness costs for the virus leading to a reduced replication capability [252,256-259]. We hypothesized that escape mutations identified in the structural genes of the HCV AD78 isolates in our study might also have a deleterious effect on the viral replication cycle. Generation of the AD78/JFH1 chimeric viruses with high replication potency in the framework of the current project made it possible to subject this hypothesis to experimental verification.

At the first step, two identified HLA-B07- and HLA-B51- specific escape mutations inside the core region (R43K and H153Q) were introduced by site-directed mutagenesis into the AD78/JFH1 construct. Analysis of the infectivity of the resulting modified viruses in Huh7.5 cell culture have shown that both escape mutations impaired virus replication, although to a different extent (Fig. 3.20). The escape mutation R43K led only to a relatively small reduction of the infectious titer of about one log. The mutation H153Q had a more pronounced effect on the viral infectivity, as the titer was reduced by approximately 2.5 logs (Fig.3.20).

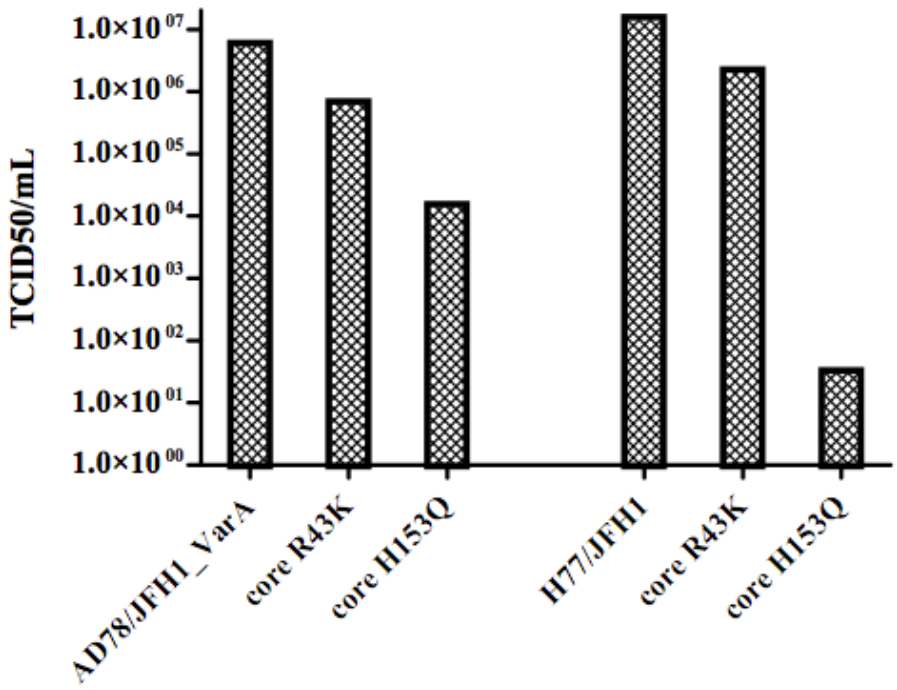

Fig. 3.20 Influence of mutations in the core gene on the infectivity of the AD78/JFH1 and H77/JFH1 viruses. Supernatant infectivity titers were determined by TCID50 assay.

The core regions of HCV genotype 1a and genotype 1b genomes are highly homologous. As the AD78 strain belongs to the HCV genotype 1b we were interested to investigate the effect of the escape mutations found in the AD78 core region on the infectivity of HCV genotype 1a. For this purpose the escape mutations (R43K and H153Q) were introduced by site-directed mutagenesis into the chimeric H77/JFH1 virus, in which the structural HCV genes were derived from the HCV subtype 1a strain H77. As expected, the effect of the R43K substitution on viral infectivity was as mild (about one log) as that observed in the AD78/JFH1 system (Fig.3.20). In contrast, the H153Q mutation caused a dramatic loss of infectivity exceeding 6 logs. These data suggest that the impact of mutations (like H153Q), which cause relatively small impairment of the infectivity of one virus, might cause much stronger fitness cost for another virus belonging to different genotype or subtype.

At the next stage, using the AD78/JFH1 system we analyzed the biological effect of the escape mutations identified inside the HLA-A2402 (666-LLSTTEWQI-674) and the HLA-B57 (541-NTRPPQGNW-549) epitopes, both located in the E2 protein of the HCV AD78 isolates (Table 3.2). The mutations were introduced by site-directed mutagenesis into the chimeric construct and the resulting modified viruses were tested for infectivity by limited dilution assay in Huh7.5 cell cultures (Fig.3.21). No significant difference in the replication capacity of the virus bearing the HLA-A2402 I674V mutation was observed as compared to the parental wild-type AD78/JFH1 virus. This unimpaired virus replication is in agreement with the observation that V at position 674 is present in about 40% of the sequences of HCV type 1b strains included into the Los Alamos HCV sequence database.

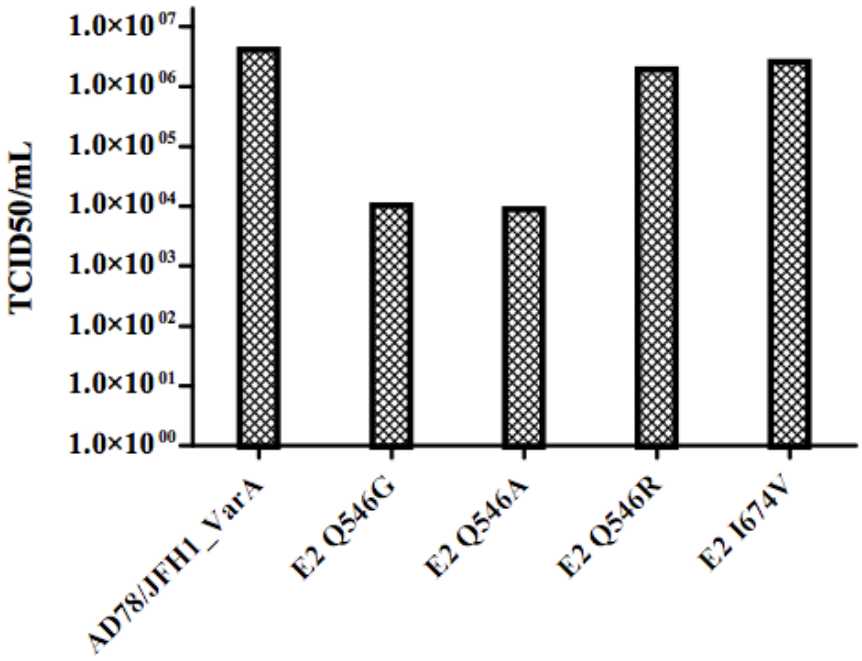

Fig. 3.21 Influence of mutations in the E2 gene on the infectivity of the AD78/JFH1 variant A virus. Supernatant infectivity titers were determined by TCID50 assay.

Of special interest are the data on the identification of putative escape mutations at position 546 in the HLA-B57 epitope 541-NTRPPLGNW-549 located in the E2 protein. Recently, conflicting data have been reported on association of this HLA-B57 epitope with spontaneous HCV clearance [197,201]. It was suggested that these disparate results might be attributed to the polymorphism at position 546 [201]. Therefore, we decided to study the influence of the identified escape mutations at position 546 on viral fitness. The mutations were introduced by site-directed mutagenesis into the AD78/JFH1 construct and the infectivity of these modified viruses was

tested in Huh7 cell cultures (Fig.3.21). The virus bearing the mutation Q546R replicated as efficiently as the virus with the "wild-type" sequence, which contained Q at position 546. One should note that at position 546 the consensus HCV 1a sequence and consensus 1b sequence contain L and Q, correspondingly, and that many isolates belonging to genotype 1b have R at this positions. Therefore, it looks like the presence of Q or R at position 546 simply reflects a natural polymorphism and not the emergence of escape mutations. In contrast, introduction of the mutations Q546G and Q546A into the AD78/JFH1 construct led to a significant loss of replicative efficiency (drop by more than 2 logs) (Fig.3.21). These two substitutions are extremely rare in the subtype 1b sequences from the Los Alamos HCV sequence database. In sum, these results suggest that the amino acid substitutions Q546G and Q546A are real escape mutations that substantially impair viral replication and that the virus variants bearing these mutations are being eventually eliminated from the virus population in the majority of infected subjects.

3.4 Influence of the specific antiviral cellular and humoral immune responses on envelope protein E2 evolution during a long-term virus persistence in anti-D patients

There is a growing body of evidence for the fact that both cellular and humoral immune responses contribute to evolution of HCV proteins [26]. At the same time, it is still not clear how big is the contribution of each type of the immune response to evolution of the envelope proteins, first of all, of the E2 protein. One possible approach to address this issue might be the assessment of selective pressures on HCV genome exerted by both the CD8+ T-cells and antiviral antibodies.

The availability of the HCV AD78 sequence database generated in the current study provides an excellent opportunity to analyze some characteristics of the evolution of the E2 gene during 30 years of viral persistence. First, we tried to assess how evident a contribution of the CD8+ T-cells to evolution of the E2 gene is. The number of synonymous and nonsynonymous substitutions inside and outside of known CD8 epitopes was determined for each individual anti-D patient according to his HLA class I genotype and a list of HLA type relevant CD8+ T cell epitopes. The aggregated data on the frequency of synonymous and nonsynonymous mutations inside and outside of all known CD8+ T-cell epitopes for all patients have not revealed significant difference between these parameters (Fig. 3.22), although, the statistical trend for a higher level of nonsynonymous mutations inside the CD8+ T cell epitopes was noted (p =

0.0684). These data suggest that the cellular responses promote the evolution of the E2 protein during the long-term viral persistence in the AD78 cohort; however, its influence probably is not profound.

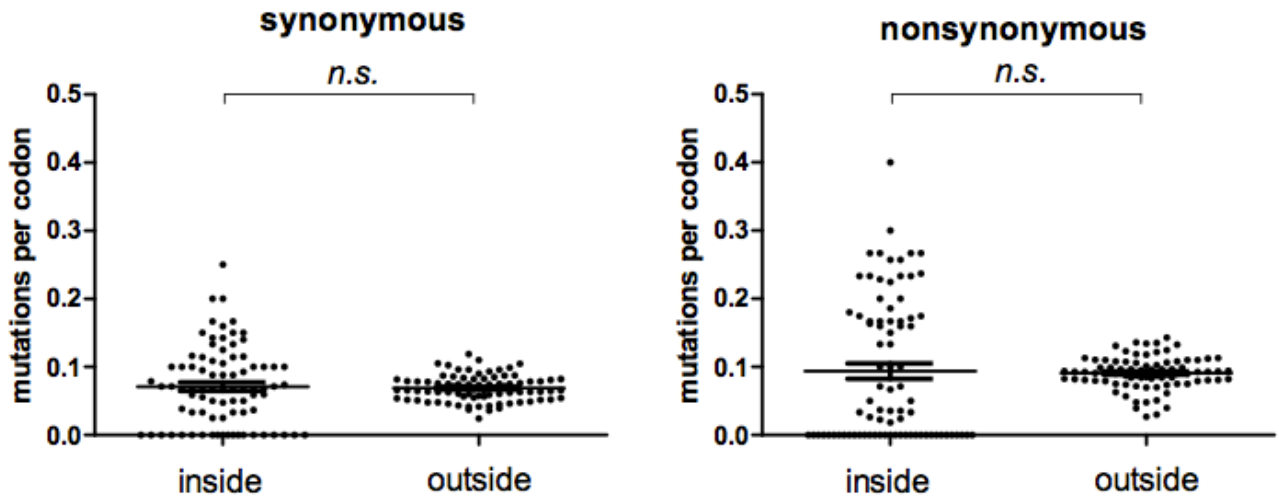

Fig. 3.22 Analysis of the frequency of synonymous and non-synonymous mutations inside and outside of known CD8+ T-cell epitopes within the E2 gene.

Next, we determined the extent to which a selective pressure of antiviral antibodies contributes to the rate of evolution of the E2 gene. To this end, the number of synonymous and nonsynonymous mutations inside and outside of previously described sites and regions [163,260-262] involved into the reactivity with human monoclonal antibodies to E2 was calculated for all subjects from the anti-D cohort. The HVRI and HVRII fragments were excluded from this analysis. Data presented on figure 3.23 demonstrate that the difference in the frequency of mutations inside and outside of all known human monoclonal antibody-binding sites was statistically significant only for the nonsynonymous, but not for the synonymous substitutions. This suggests that the humoral immune response had a distinct impact on the viral envelope evolution in the AD78 cohort.

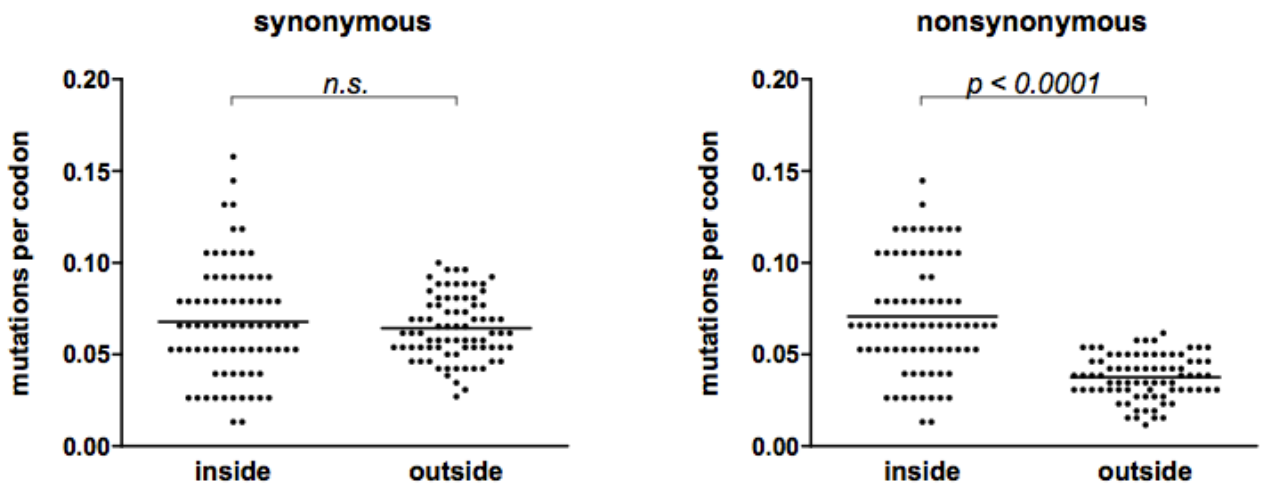

Fig. 3.23 Analysis of the frequency of synonymous and non-synonymous mutations inside and outside of known human monoclonal antibody-binding sites within the E2 gene.

Overall, these data have shown that both cellular and humoral antiviral immune responses influence the HCV E2 protein evolution during a long-term viral persistence. Different frequencies of amino acid substitution inside and outside of B- and T-cell epitopes suggest that the specific humoral immune responses probably play a leading role in HCV E2 evolution in chronically infected patients.

3.5 Special structural feature of the HCV AD78 strain – presence of an additional amino acid track at the N-terminus of HVRI

(Part of this work has been done in collaboration with Dr. M. Klein)

Comparison of the clonal HCV AD78 sequences from different batches of the contaminated anti-D globulin performed in the current study revealed the presence of additional amino acid residues at the junction between E1 and E2 proteins: RGGG, GG and SALT in sequences of the HCV AD78 variants A, B and C, correspondingly (Fig. 3.24). Subsequent analysis of the HCV AD78 sequence database generated in the current study has shown that this peculiar structural feature (presence of an additional amino acid track) was observed in the majority (83 %) of the sequences from anti-D patients. At that, the length of this insert varied from 1 to 4 residues. Twelve percent of sequences contained one, 22 % - two, 36 % - three, and 30 % - four additional residues.

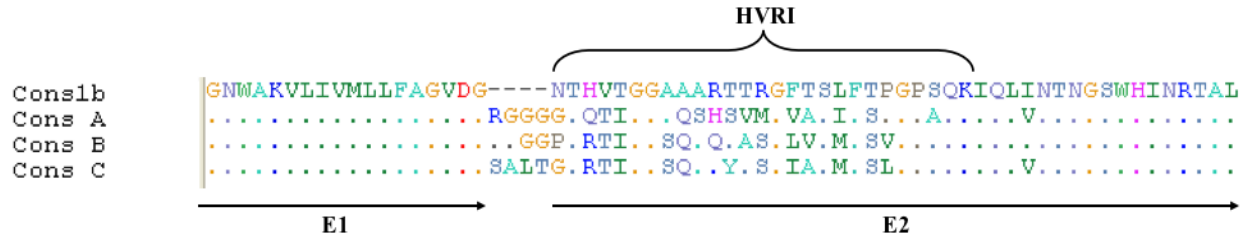

Fig. 3.24 Additional amino acid residues at the E1/E2 junction in the sequences of variants A, B and C of the HCV AD78 strain present in contaminated anti-D globulin.

Table 3.3 Changes of the additional amino acid track at the E1/E2 junction in sequences of anti-D patients obtained 30 years after infection.

Sequences from:	HCV AD78 strain		
	Variant A	**Variant B**	**Variant C**
Infectious source (anti-D globulin)	RGGG	--GG	SALT
Anti-D patients	RGGA	SRPR	AAPS
	HTYD	-VYA	TASS
	-VQA	-ASA	VTST
	-SAK	-RFS	-TAL
	--SG	--LA	-AVS
	--EM	--RG	-RAS
	--NT	---GE	--YS
	---Q	---S	--SP
	---T	---E	--VR
	----	----	----

3.5.1 Frequency of additional amino acid stretches in different HCV genotypes and characteristics of the inserted residues

Next, we searched for the presence of additional amino acid stretches in the sequences of different HCV genotypes and subtypes (Table 3.4). Overall, the frequency of such inserts in HCV sequences is low. At that, significant variations of the frequencies of those inserts in sequences belonging to different genotypes were observed. The additional amino acid residues occurred much more often in genotype 1b and 2 sequences than in sequences belonging to genotypes 1a and 3 ($p < 0.0001$). Of note is the fact of variable number of additional codons in sequences of different HCV types. Thus, for example, among HCV 1b strains we observed from 1 to 4 additional codons, while among genotype 2 strains this number varied from 2 to 5 codons. It is tempting to speculate that some sequences belonging to genotype 1b and 2 may have some specific structural features, which facilitate the emergence of these inserts at this particular position of the polyprotein. Application of several programs, which allow predicting the RNA

folding, did not, however, reveal any differences between sequences with and without additional codons at the E1/E2 gene junction.

Table 3.4 Frequency of additional codons at the 5‘ end of HVR1 sequences of HCV isolates of different types from the Los Alamos hepatitis C sequence database (related sequences are excluded).

	gt 1a (n=7146)	**gt 1b (n=3489)**	**gt 2 (n=496)**	**gt 3 (n=1711)**	**gt 4 (n=137)**	**gt 5 (n=64)**	**gt 6 (n=96)**
1 codon	0	20	0	0	1	0	0
2 codons	4	7	2	0	0	0	0
3 codons	0	2	2	0	0	0	0
4 codons	2	1	3	1	0	0	0
5 codons	1	0	3	0	0	0	0
At least one additional codon	7 (0.1 %)	30 (0.9 %)	5 (1 %)	1 (0.06 %)	1 (0.7 %)	0	0

Next, the analysis of the physico-chemical characteristics of the additional amino acid residues was performed. Its results have shown a restricted amino acid usage with predominance of small and flexible residues, especially at the very N-terminus, as well as the low frequency of Pro and large hydrophobic residues. Another important feature was the predominance of neutral and hydrophobic residues; three residues – Cys, Trp, and Asp were absent. Thus, the additional amino acid set was characterized by low frequencies or absence of residues known to restrict changes in a polyprotein conformation. Overall, these results suggested a selection for particular residues in the additional amino acid tracks located at the E1/E2 junction.

Presence of the additional amino acid residues at the E1/E2 junction raise the question to what protein these residues remain fused after the proteolytic processing of the viral polyprotein. Comparative analysis of the E1 C-terminal sequences (including the signal peptide segment) of HCV strains from the Los Alamos HCV database demonstrated absence of any special amino acid substitutions in this area in strains bearing the additional amino acid residues. Such a conservation of the E1 sequences provides for a proper processing signal and strongly suggests the invariable processing pattern. Thus, despite the absence of direct experimental proof, one

might reasonably assume that the additional amino acid residues observed at the E1/E2 junction just behind the cleavage site upon a processing became a part of the N-terminal sequence of the E2 protein.

The identified restriction of physico-chemical characteristics of residues forming the additional amino acid stretch at the N-terminus of HVRI suggest that these stretches should have a particular conformation and, most probably, are involved in interaction with the rest of HVRI and/or the E1/E2 heterodimer. This might lead in turn to changes of infectivity of HCV particles. To address this issue, two experimental approaches for measuring the HCV infectivity – the HCVpp and HCVcc systems –were used.

3.5.2 Impact of amino acid insertions on infectivity

First, a set of HCV AD78 E1/E2 expressing plasmids was generated to assess the influence of the additional amino acids at the N-terminus of the HVRI on infectivity of HCVpp. Two phCMV-IRES plasmids expressing the E1/E2 gene sequences corresponding to the consensus sequences of HCV AD78 variants A and B were used as a backbone for all subsequent manipulations. Four codons (encoding RGGG) in case of the virus variant A and two codons (encoding GG) for variant B at the 5'-end of HVRI present in these E1/E2 sequences were deleted or modified by replacing them with several natural or artificial amino acid stretches (Table 3.5). All these modified plasmids were used for transfection of HEK 293T cells. The levels of expression of the HCV envelope proteins were tested by Western blot (Figure 3.25). The supernatants of these cultures were harvested and virus pseudo-particle concentration was determined by p24-test (quantification of Gag). HCVpp-containing supernatants were normalized according to p24 concentrations. The prepared sets of HCVpps were used for infectivity assays in Huh7.5.1 cells following a standard protocol.

Table 3.5 Modifications of the additional amino acid track at the N-terminus of HVRI in the E1/E2 consensus sequences of HCV AD78 variants A and B used for preparation of the expressing plasmids.

Additional amino acid stretch in HCV AD78 variant A sequence	Source	Additional amino acid stretch in HCV AD78 variant B sequence	Source
RGGG (RG_3)	HCV AD78 variant A (wt)	--GG	HCV AD78 variant B (wt)
w/o additional stretch (Δ 4 aa)	HCV AD78 variant A without 4 aa	w/o additional stretch (Δ 2 aa)----	HCV AD78 variant B without 2 aa
-HGG	sequence from anti-D patient T98	RGGG (RG_3)	HCV AD78 variant A (wt)
SASP	sequence from anti-D patient B98	---S	sequence from anti-D patient T90
-ASA	sequence from anti-D patient H98	-TSW	sequence from anti-D patient T94
-MSV	sequence from anti-D patient H98	-MSG	sequence from anti-D patient T98
SGQL	consensus HCV type 1b sequence	-ASA	sequence from anti-D patient T06
TSSHV	consensus HCV type 2 sequence	DNDN	absent or rare amino acids
DNDN	absent or rare amino acids	-	-
RG_6	artificial stretch	-	-
RG_9	artificial stretch	-	-

(w/o = "without")

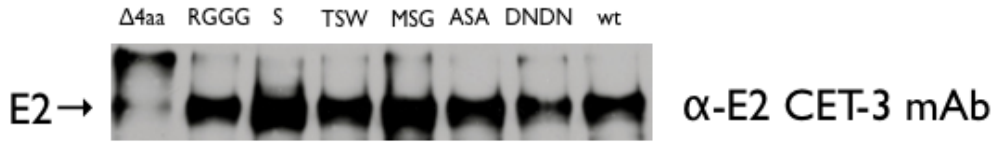

Fig. 3.25 Immunodetection of HCV AD78 E2 protein expressed in 293T cells. Cell lysates of the 293T cells transfected with the expression vector phCMV-IRES encoding the E1/E2 sequences of different HCV AD78 variants were subjected to SDS-PAGE. Consensus HCV AD78 variant B sequence was used as a backbone. The description of all modifications is listed in table 3.5. Proteins were transferred to the nitrocellulose membrane and immuno-stained with mouse monoclonal antibody to HCV 1b E2 (α-E2 CET-3 mAb).

Comparison of the infectivity of the generated set of pseudo-particles bearing either the original E2 consensus sequence of the HCV AD78 variant A or its modifications listed in table 3.5 have demonstrated that the presence (or absence) of an additional amino acid track in all cases but one (insertion of the DNDN) did not result in a significant change of infectivity (Fig. 3.26). A significant drop of infectivity was observed only after insertion of the DNDN sequence that consisted of residues, which are never (D) or exceptionally rarely (N) present in the context of additional amino acid stretches in HCV sequences. These data suggest that the presence of additional amino acid residues at the N-terminus of HVRI probably has no significant influence on virus infectivity. Principally, the same results were obtained upon assessment of infectivity of HCVpps bearing either the original E2 consensus sequence of the HCV AD78 variant B or its modifications listed in table 3.5. Despite more distinct variations of the infectivity levels in most of these cases no significant reduction of infectivity was registered (Fig. 3.26). The only exception was observed with particles bearing the E2 sequence with a deletion of the additional amino acid residues. Sequence analysis revealed the presence of proline at the first position of HVR1 in the consensus sequence of HCV AD78 variant B. Site-directed mutagenesis of the residue at this position was performed. Substitution of the Pro to Thr led to a partial restoration of the infectivity of pseudo-particles with a deleted additional amino acid residues (Fig. 3.28). The Western blot analysis has shown a reduced level of the E2 in HCVpp bearing E2 sequence without additional amino acid residues (Fig. 3.25 and 3.27) and the appearance of the E2 polypeptide band in HCVpp preparation bearing the HVRI with the exchange of Pro by Thr (Fig. 3.27). These results suggest that presence of Pro at first position of the HVRI probably causes a disruption of the polyprotein processing between E1 and E2 proteins. This interesting observation warrants a further more detailed investigation.

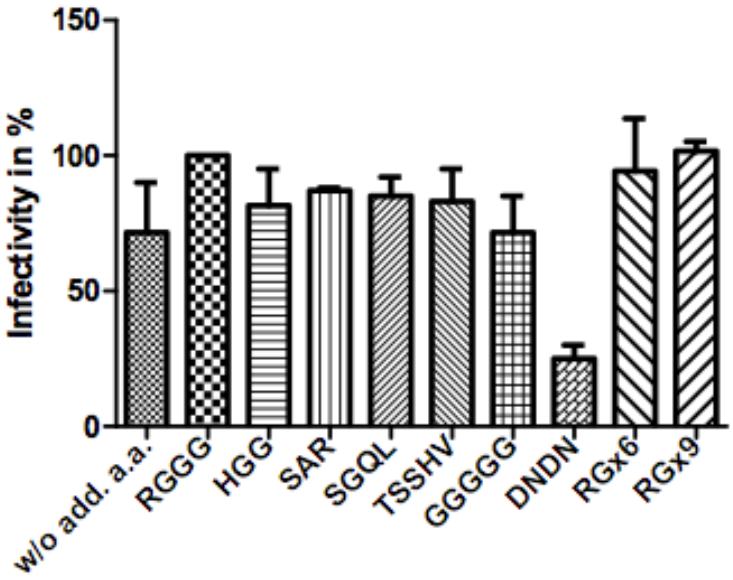

Fig. 3.26 HCVpp infectivity assay with pseudo-particles bearing wild type and modified E2 sequences. RGGG - construct bearing the wild type sequence of the HCV AD78 variant A. w/o add. aa - the same construct but with deleted RGGG insert (all other constructs see table 3.5). (w/o = "without")

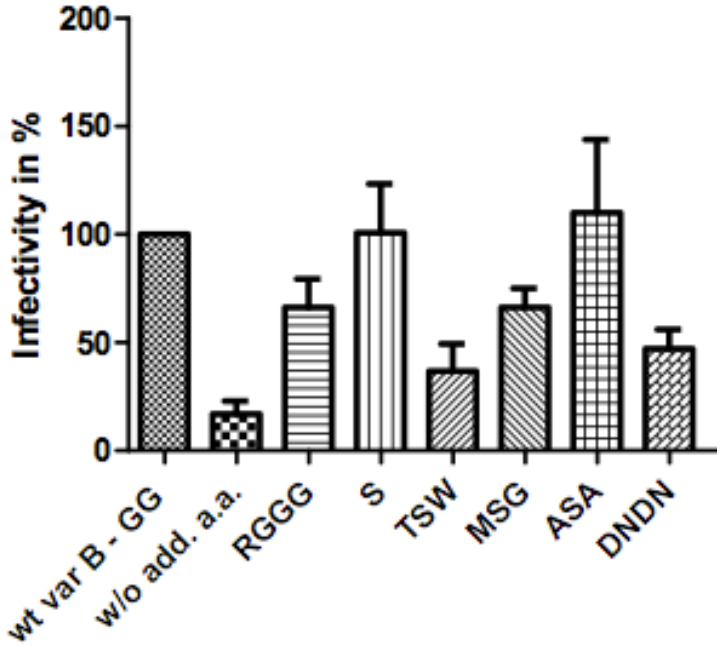

Fig. 3.27 HCVpp infectivity assay with pseudo-particles bearing wild type and modified E2 sequences. Wt var B-GG – construct bearing the wild type sequence of the HCV AD78 variant B prior to HVRI. w/o add. aa - the same construct but with deleted GG insert. RGGG - construct bearing the wild type sequence of the HCV AD78 variant B with the RGGG insert of AD78 variant B prior to HVRI. (all other constructs see table 3.5). (w/o = "without")

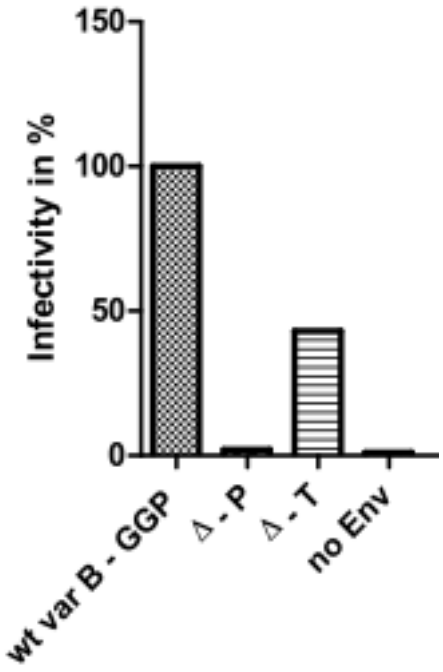

Fig. 3.28 HCVpp infectivity assay with pseudo-particles
bearing wild type and modified E2 sequences. Wt var B-GGP – construct bearing the wild type sequence of the HCV AD78 variant B with the GG insert and P at first position of the HVRI. Δ-P – the same construct but with deleted GG insert. Δ-T - construct bearing the wild type sequence of the HCV AD78 variant B with the GG insert and T instead of P at first position of the HVRI. No Env – negative control.

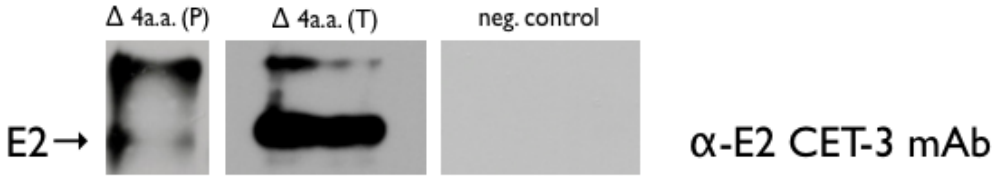

Fig. 3.29 Immunodetection of HCV AD78 E2 proteins expressed in 293T cells.
Cell lysates of the 293T cells transfected with the expression vector phCMV-IRES encoding the E1/E2 sequences of different HCV AD78 variants were subjected to SDS-PAGE. Consensus HCVAD78 variant B sequence was used as a backbone. Pro at first position of the HVRI was substituted by Thr using a site-directed mutagenesis. The proteins were transferred to the nitrocellulose membrane and immunostained with mouse monoclonal antibodies to HCV 1b E2 protein (α-E2 CET-3 mAb).

As has been documented above, the frequencies of additional amino acid stretches at the N-terminus of HVRI are much higher ($p < 0.0001$) in sequences from HCV 1b strains that from 1a strains (Table 3.4). Thus, it looks like the absence of such additional stretches is an intrinsic characteristic of HCV 1a viruses. The HCV AD78 strain belongs to subtype 1b and the genome of the majority of its isolates contains these inserts. It was of interest to see if introduction of the additional amino acid residues into the genome of 1a virus would influence its replication potentials. We decided to apply the HCVcc system to measure the influence of the additional amino acid tracks at the N-terminus of HCV AD78 isolates on virus infectivity. Three constructs were used for this investigation: AD78/JFH1 based on the consensus HCV AD78 variant A sequence containing the additional amino acid stretch RGGG at the N-terminus of HVRI; H77/JFH1 wild type (wt), and modified H77/JFH1 bearing the additional RGGG track (Fig. 3.27). Introduction of the RGGG sequence at the N-terminus of the E2 protein encoded by H77 sequence caused a dramatic impairment of infectivity of H77/JFH1 virus while the AD78/JFH1 chimeric virus bearing the same additional RGGG track was highly infectious. These results complement data on a low frequency of additional amino acid residues at the E1/E2 junction in HCV type 1a strains (Table 3.4) and suggest that for some unclear reasons most of the genotype 1a viruses (including the H77 strain) are unable to tolerate the extension of length of HVRI at its N-terminus. Further intensive studies are necessary to clarify this intriguing issue.

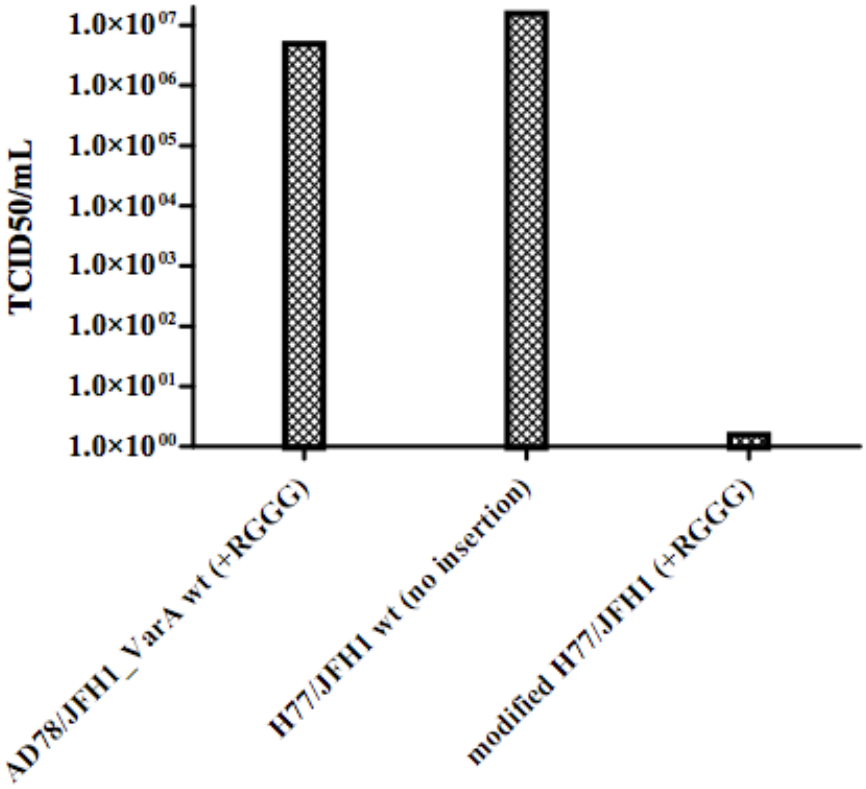

Fig. 3.30 Influence of mutations in the E2 gene on the infectivity of the AD78/JFH1 and H77/JFH1 viruses. Supernatant infectivity titers determined by TCID50 assay.

3.6 Genetic heterogeneity of HCV cell entry receptors in the AD78 cohort

Analysis of clonal core-NS2 sequences of the HCV AD78 in globulin batches 8, 9, 12, and 15 revealed the presence of three closely related but distinct virus variants of the same strain (section 3.1). In the phylogenetic analysis of viral sequences from the anti-D patients a relatively even distribution of these three virus variants was observed. Important to note, that according to the data of our group (Viazov, unpublished, [252]) none of the patients was infected with more than one virus variant, suggesting a selection mechanism for a particular virus variant in each patient. To prove the hypothesis that heterogeneity of HCV cell receptors might have influence on HCV variant selection, single-nucleotide polymorphisms (SNPs) in major HCV cell receptor genes from anti-D patients were investigated. Selection of the SNPs for analysis was performed on the basis of data kept at dbSNP-polymorphism repository (http://www.ncbi.nlm.nih.gov/SNP/ and ExPASy Molecular Biology Server http://www.expasy.ch/). We searched for SNPs that caused non-synonymous substitutions and, which occurred with relatively high or unknown frequencies. According to these criteria, seven SNPs, 2 for the LDLR (low-density lipoprotein receptor) gene, 3 for the SCARB1 (scavenger receptor B1) gene, and 2 for the OCLN (occludin) gene were chosen for subsequent analysis (Table 3.6). Two of the receptors molecules, CD81 and Claudin-1 were found to be quite conserved.

Genomic DNA isolated from EDTA-blood of AD78-infected patients was used for detection of SNP by LightSNiP Assay (Tib-molbiol). This test is based on melting curve analysis and is able to detect any mutation that is located within the sequence corresponding to the hybridization probe.

Table 3.6 Summary of selected SNPs for analysis.

Receptor gene	Polymorphism
LDLR	2x SNPs (rs11669576, rs45508991)
SCARB1	3x SNPs (rs5890, rs74830677, rs77554031)
CD81	conserved
OCLN	2x SNPs (rs2666626, rs79095497)
claudin-1	conserved

The melting curve analysis for the LDLR SNP rs45508991 showed that 32 investigated sequences bear the C/C allele and none contained the C/T or T/T allele. The results for the LDLR SNP rs116697576 demonstrated that 61 of 64 DNA sequences were positive for the G/G

allele and 3 sequences positive for the G/A allele. Thus, both investigated LDLR SNPs were predominantly present in a monoallelic manner. These results suggest that these SNPs did not have influence on the HCV AD78 variant selection in the AD78 cohort (Fig.3.31).

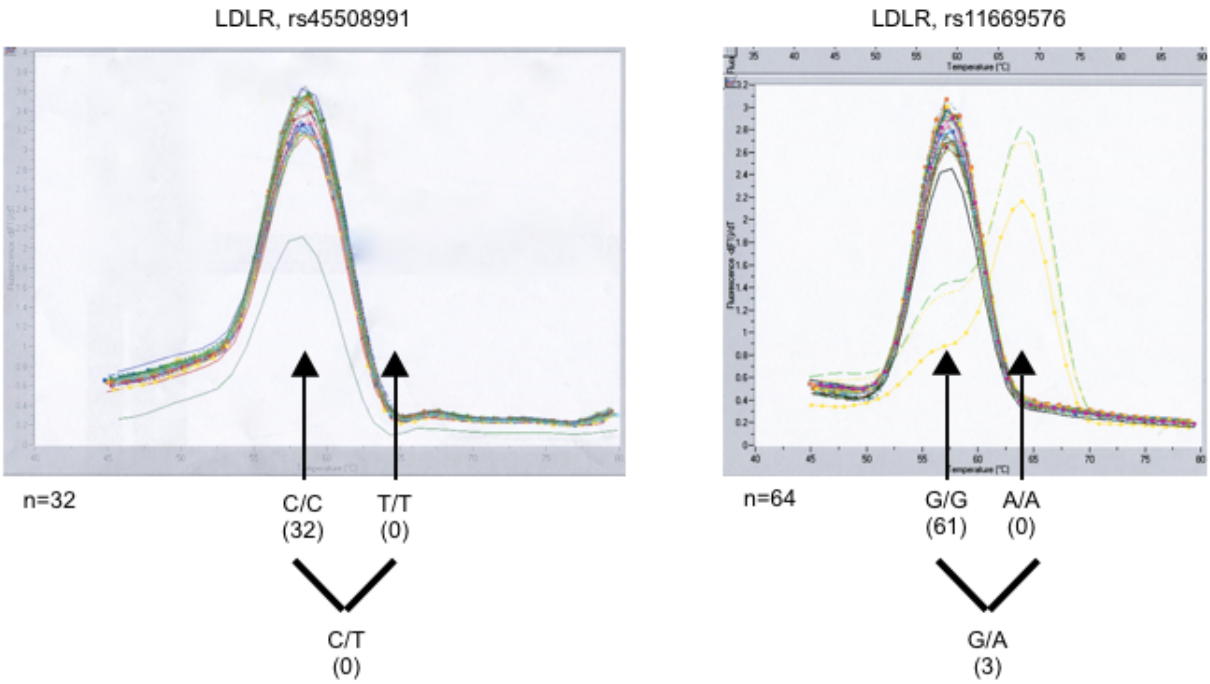

Fig. 3.31 The LightSNiP assay for the LDLR gene.
All investigated sequences (n= 32) were found to be homozygous (C/C allele) with respect to the SNP rs45508991. The SNP rs11669576 was present as G/G allele in 61 of 64 investigated DNA sequences; three individuals were positive for the G/A allele. On the graph representative results for 32 DNA samples are shown.

The melting curve analysis for the SCARB1 SNPs rs77554031, rs74830677 and rs5890 showed that all 32 investigated DNA sequences were homozygous for the tested polymorphisms. Thus these SNPs seemed not to have any influence on the HCV AD78 variant selection in the anti-D cohort (Fig.3.32).

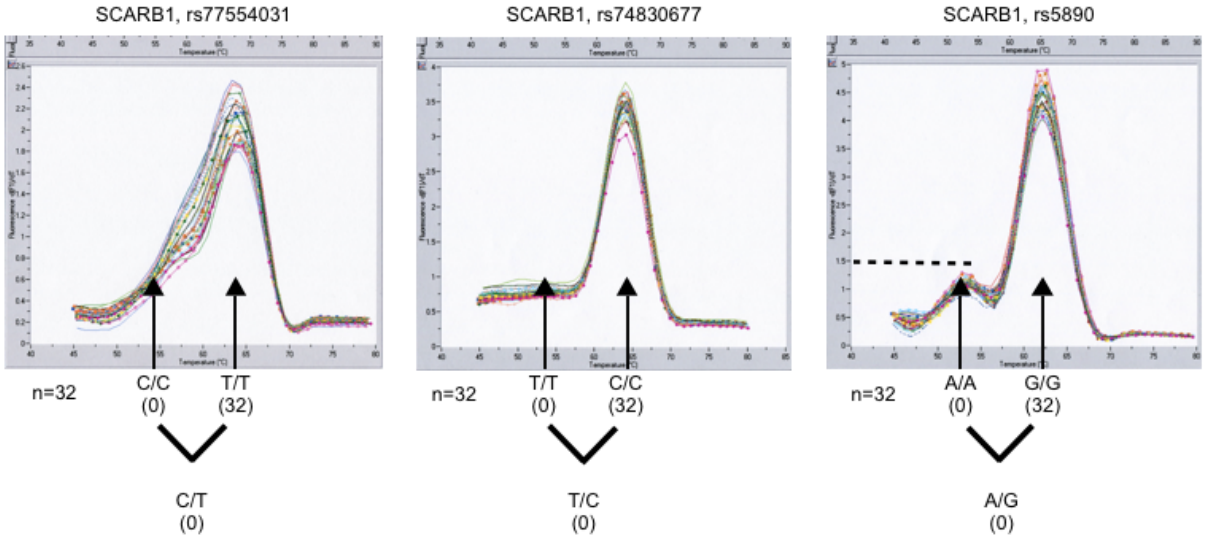

Fig. 3.32 The LightSNiP assay for the SCARB1 gene.
All investigated sequences (n= 32) were found to be homozygous (T/T allele, C/C allele, and G/G allele, respectively) with respect to the SNPs rs77554031, rs74830677, and rs5890.

The melting curve analysis for the OCLN SNP rs790995497 showed that 32 investigated sequences did bear the T/T allele and none contained the T/C or C/C alleles suggesting that these SNPs seemed also not to have influence on the HCV AD78 variant selection in the AD78 cohort (fig3.33). The LightSNiP assay results for the OCLN SNP rs2666626 showed that DNA sequences from 28 of 92 anti-D subjects were positive for the G/G allele; all other 64 sequences were positive for the G/C allele (Fig.3.33). Subsequently, the frequencies of the G/G and G/C alleles of the OCLN SNP rs2666626 in patients infected with different HCV AD78 variants was checked (Fig.3.34). Upon this analysis no association between distribution of the OCLN SNP rs2666626 and presence of a particular HCV AD78 strain variant was found.

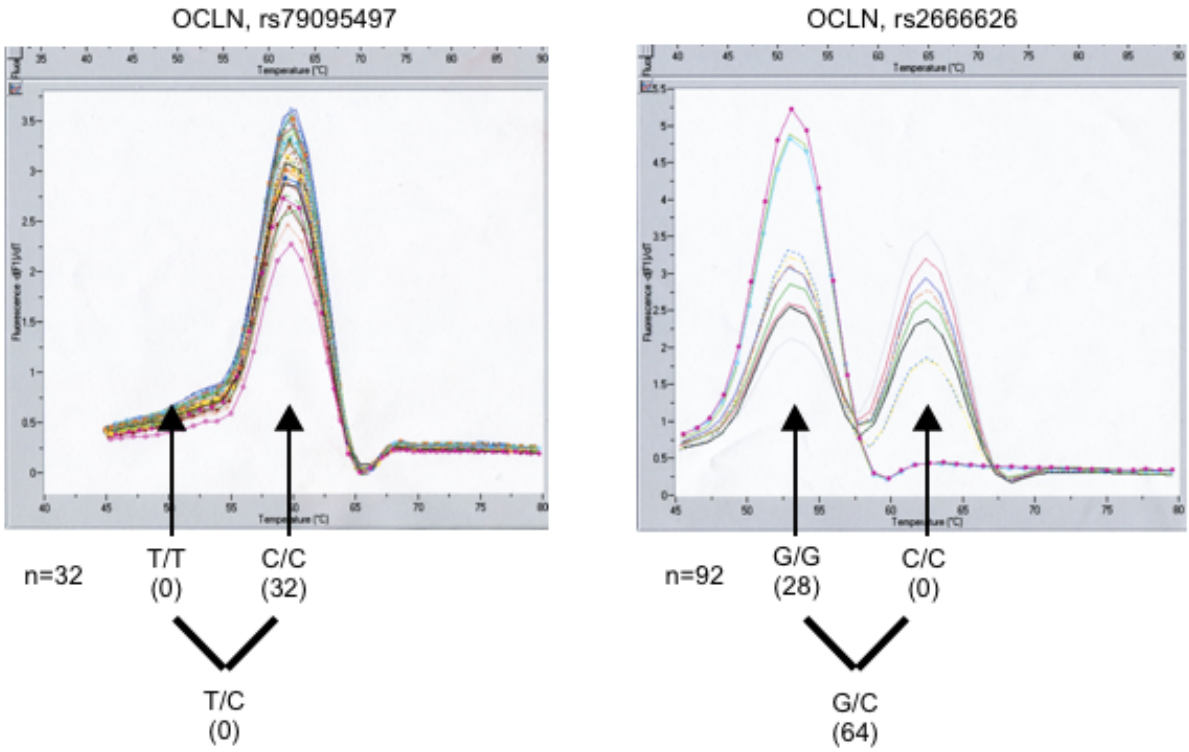

Fig. 3.33 The LightSNiP assay for the OCLN gene.
All investigated sequences (n= 32) were found to be homozygous (C/C allele) with respect to the SNP rs79095497. The SNP rs2666626 was present as G/G allele in 28 of 92 investigated DNA sequences; 64 individuals were positive for the G/C allele. On the graph representative results for 32 DNA samples are shown.

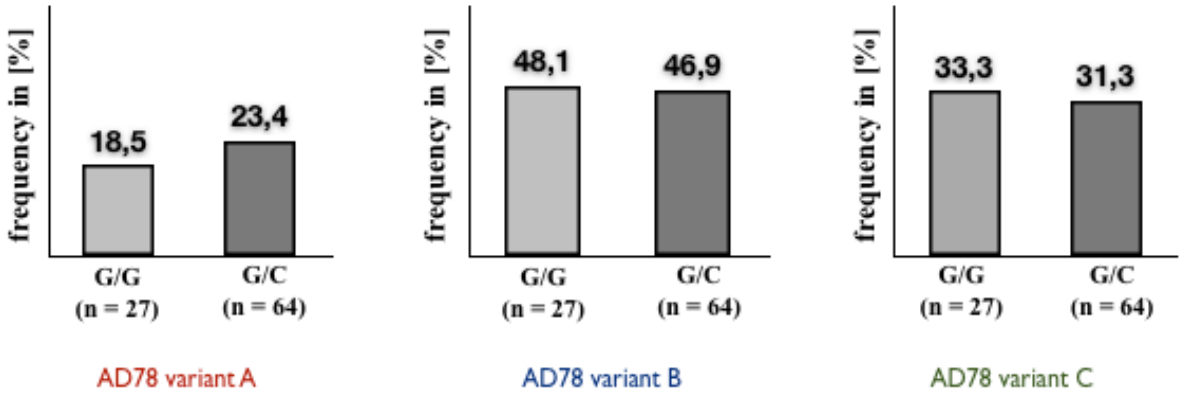

Fig. 3.34 Frequencies of the G/G and G/C alleles,
of the OCLN SNP rs2666626 in patients infected with different HCV AD78 variants.

In summary, no evident correlation between HCV cell receptor polymorphism and presence of a particular virus variant was noted. Heterogeneity of the receptors LDLR, SCARB1 and OCLN seems not to play an important role in the mechanism of virus selection at the stage of viral entry in case of natural HCV infection with multiple virus variants.

4 Discussion

The accumulating data show that the specific anti-viral immune response of the host is a major factor of HCV genome evolution [26,155,159,254]. According to the concept of immune escape mechanism, the quasispecies nature of HCV population in the infected host provides a basis for a selection of resistant variants of the virus under both humoral and cellular immune pressure and, as a result, cells bearing the viral genomes with the escape mutations are not eliminated, and HCV persistence is established. There is a growing number of publications, which demonstrate adaptation of HCV to humoral or cellular immune pressure by selection of escape mutations in targeted B- and CD8 T-cell epitopes in individual patients [26,169,245-247]. At the same time, it is not completely clear if the frequencies and characteristics of the immune escape observed in individual patients are reproducible at the population level. Several studies addressed this issue and have shown a reproducible selection pressure in a number of CD8 T-cell epitope in subjects with the same HLA class I type [26,250,252]. One should note that all of these studies dealt predominantly with the analysis of the epitopes located in nonstructural proteins of HCV and the degree, to which both cellular and humoral immune selection pressure contribute to evolution of the envelope proteins, remains unclear. One of the aims of the current project was to find answer to this important question and to get an insight into the role of humoral and cellular immune responses in evolution of the envelope protein E2 of HCV. Studies in this field, however, usually are hampered due to the fact that investigators have to deal with patients infected with different HCV strains and no information on the ancestral virus sequences is available. The essential feature of the project was the use of a special group of patients – women infected with the same HCV strain (AD78) by administration of the contaminated anti-D globulin in a single-source outbreak. This unique cohort of patients infected with the same virus strain allowed for minimization of factors, including route of transmission, size of inoculum, duration of infection, age and sex of infected individuals, playing a significant role in evolution of the virus during the chronic infection.

The first step of the current work was the generation of the HCV AD78 sequence database. The core-NS2 and NS4A-NS5a genome regions of the HCV AD78 strain from contaminated globulin batches and anti-D patients serum samples collected 30 years post infection were amplified and the genetic heterogeneity of HCV AD78 isolates was investigated. Previous data from our group ([252]; Viazov et al, unpublished), based on the study of the heterogeneity of the NS3 and NS5B genes, demonstrated that the HCV AD78 outbreak was in fact caused not by one but by three closely related but slightly different variants of the AD78 virus strain. The results

obtained in the current study upon an analysis of the created HCV AD78 sequence database confirmed the previous data from our group and suggested the presence of these three virus variants in each of the anti-D globulin batches. Interestingly, the genetic heterogeneity of the infectious source of the HCV AD78 outbreak distinguishes it from the well-known HCV outbreak, which occurred in 1978 in Ireland and was caused by a contaminated anti-D globulin that contained only one variant of the founder virus [248].

Of special interest is the question, in which individual(s) the three variants of the HCV AD78 have arisen from the original variant of this strain. The available history of the anti-D outbreak, which took place in East Germany in 1978, indicates that the "source" of the original virus (index HCV AD78-infected donor) provided blood for preparation of the erythrocytes concentrate. This concentrate was used for immunization of 5 individuals (secondary plasma donors). Three to five weeks later the plasma of these 5 subjects was mixed in different proportions and used for preparation of eight immunoglobulin lots (batches 8 to 15). Two of these donors later on developed jaundice. A phylogenetic analysis of the E2 sequences from 4 of these secondary plasma donors, collected approximately 12 years after blood donation, was performed in the current study. It revealed that one subject has been chronically infected with the HCV AD78 variant B, while three others were infected with the variant C of this virus strain. The presence of HCV AD78 variants in different secondary plasma donors and persistence of the same virus variant in three of them allow us to rebut a hypothesis that splitting of the original HCV AD78 strain into the three lineages occurred during the acute infection of these secondary plasma donors [252]. It is likely that the separation of the original HCV AD78 strain into the three variants might have taken place during the chronic infection of the index donor. An alternative explanation could be the existence of more than one index donors already infected by closely related variants of the same virus strain. These subjects probably were the remunerated donors engaged by the same blood collection center and were persistently infected with the HCV AD78 strain. Cross-contamination of these donors in the blood center and circulation of the same virus among them for years could have eventually led to a separation of the HCV AD78 strain into the three variants. In our opinion, to clarify the issue and to find the answers to most of the questions will be possible only if the samples from the index HCV AD78-infected donor and/or other donors from the same blood center become available for a detailed analysis.

Establishment in the framework of the current study of the HCV AD78 sequence database provided a basis for a series of experiments. First of all, availability of the clonal core-NS2 sequences from different batches of anti-D globulin led to the generation of the consensus

founder sequences for HCV AD78 variants A, B and C. These three consensus sequences were used in collaboration with the group of Prof. R. Bartenschlager for the development of the HCV AD78/JFH1 chimeric viruses. These new constructs were viable and capable of initiating relatively high levels of virus replication in Huh7.5 cells. Availability of such robust cell culture system represents a significant step in the extension of the currently available JFH1-based systems of genotype 1. Especially promising these new chimeric viruses are for the studies based on the materials from the HCV-AD78 outbreak. In particular, this system is essential for elucidation of the biological significance of the mutations occurring in the viral genome during both acute and persistent phases of infection.

Analysis of the generated HCV AD78 sequence database allowed identifying 47 positions in the core-NS2 fragment, where at least one of the virus variant-specific consensus sequences from the infection source is differed from the HCV 1b consensus sequence. Remarkably, most of these substitutions were very stable and have not been changed in most of the anti-D patients even after 30 years of viral persistence. Interestingly, about 40% of these stable substitutions were located inside the CD8 epitopes, suggesting that these mutations did not compromise substantially a viral fitness in a spectrum of hosts. An alternative explanation might be a presence in most of these sequences of additional compensatory mutations, which reconstitute, at least to some extent, the replicative capacity of the mutated viruses. Availability of the AD78-based HCVcc system makes it possible now to subject this intriguing topic to experimental verification.

It is important to note that only 10 of 47 substitutions (21%) reverted toward HCV 1b consensus sequence over 30 years. These data are complemented by our analysis of all mutations that were observed in the sequences from all the patients from the anti-D cohort, where selection of the general consensus 1b residue occurred only in a minority of cases. In sum, the obtained data indicate that in persistently infected anti-D patients evolution of the virus genome toward the HCV 1b consensus sequence is relatively rare and observed in about 25-30% cases.

For a further analysis of the generated HCV AD78 sequence database a statistical sequence analysis algorithm was applied [252]. This approach is directed at the detection of a statistically significant association between expression of a particular HLA class I allele and presence of amino acid substitution at each site of viral protein sequence and allowed for identification of sites under selective CD8 T-cell immune pressure at a population level. Implementation of this type of analysis led to identification of HLA class I specific polymorphisms inside 7 already

described CD8 epitopes in the core, E1, E2, and NS2 proteins. Introduction of two identified HLA-B07 and HLA-B51-specific escape mutations into the genome of the HCVcc constructs have impaired virus replication. However, for the HLA-B51-specific mutation (H153Q) a reduction of the infectious titers depended very much on the origin of the sequence included into the HCVcc genome. This mutation caused relatively mild impairment of the infectivity of the AD78/JFH1 virus, while in the H77/JFH1 virus it led to a dramatic loss of infectivity exceeding 6 logs. These data indicate that the impact of the escape mutations on viral fitness might to a significant extent depend on the sequence context. Moreover, this conclusion might apply not only to viruses belonging to different subtypes of the same genotype but, probably, even to different strains of the same subtype. From this point of view, availability of the AD78/JFH1 system, as well as of materials from anti-D outbreak, provide us with a unique possibility to assess the biological significance of different mutations in the context of a homologous genomic sequence.

Of special interest are the results of the testing of mutations in the HLA-B57 epitope in the E2 protein. A protective character of HLA-B57 allele has been described for a group of patients predominantly infected with HCV subtype 1a [201]. At the same time this allele was not protective in the Irish anti-D outbreak caused by 1b virus [197] and seems not to be protective in German anti-D cohort. The consensus 1a sequence contains L at position 546, while the Irish 1b strain contained R546. The HCV AD78 strain as well as the consensus 1b sequence has Q546. We have introduced the putative escape mutations in the HLA-B57 epitope (Q546G and Q546A), as well as substitution Q546R into the AD78/JFH1 construct and measured the infectivity of the resulting mutant viruses. Substitutions Q546G and Q546A caused a drastic drop of infectivity (more than 2 logs) and, probably, represent real escape mutations. In contrast, both viruses bearing Q or R at position 546 replicated equally well. It is likely that the Q/R polymorphism is a general characteristic of the HCV 1b strains and has nothing to do with the escape mechanism. Most probably, the disparate results on the protective value of the HLA-B57 E2 epitope are due to the fact that the Q/R variants of this epitope are not recognized by HLA-B57-restricted responses. These data highlight the fact that minor differences between sequences of HCV subtypes (and probably even between some strains of the same subtype) might have important consequences for the CD8 T-cell responses and ability to contain HCV infection.

Important aspect of immunological research of HCV infection deals with identification of novel CD8 epitopes. In most of the publications on epitope mapping the series of overlapping peptides derived from the HCV 1a sequences were used. This approach is rather cumbersome and not

always provides the unambiguous results. We decided to use an alternative approach, which revealed its value in the recent study of nonstructural NS3 and NS5 proteins [252]. At the first step, a statistical approach for detection of the HLA class I-associated polymorphisms in sequences from the anti-D cohort was used. The identified polymorphic sites were further evaluated with the epitope prediction programs for the relevant HLA allele, and the peptides covering the region under interest were investigated by antigen-specific expansion of PBMC obtained from HLA-relevant patients and subsequent intracellular cytokine staining after re-stimulation with the same peptide. This combined approach allowed us to get a confirmation of existence and to perform a fine mapping of one novel epitope in the core protein. We were not able to get an experimental conformation by a functional assay for other two potential epitope candidates despite the fact that high statistical support for a strong HLA-associated selective pressure was obtained. One should note, however, that for confirmation by a functional assay the samples from intravenous drug users were used. It is unclear to which genotype/subtype and exact sequence these subjects had been exposed to. Therefore, one should consider the negative results of the functional assay with a caution. This issue warrants farther, more detailed investigation.

The data demonstrating the HLA-associated polymorphism in the core, E1, E2, and NS2 protein sequences from anti-D patients provided evidence that positive selection pressure by CD8 T-cells plays a role in evolution of these proteins, including E2. In order to assess a contribution of both the cellular and humoral immune responses to evolution of the E2 protein gene in sequences from anti-D cohort the frequencies of synonymous and nonsynonymous substitutions inside and outside of known B- and CD8 T-cell epitopes was determined. As expected, no differences were observed in the frequency of synonymous mutations both inside and outside of these B- and T-cell epitopes. In contrast, a statistical trend was noted for a higher mutation rate inside CD8 epitopes compared to regions outside, indicating that selection pressure by CD8 T-cells evidently has some influence on evolution of the E2 protein in this group of patients. One should note, however, that novel CD8 epitopes are being continuously identified ([225,252]; current study) and that the real rate of evolution at nonsynonymous sites inside the CD8 epitopes might be somewhat higher. Importantly, the elevated mutation frequency of nonsynonymous mutations within the binding sites and regions described for human monoclonal anti-HCV antibodies rather than outside of these areas was registered and the observed difference was highly significant. In sum, these data suggest that both specific antiviral humoral and CD8 T-cell immune responses contribute to the E2 evolution during the long-term HCV persistence. At that, the humoral response probably plays a leading role, while the contribution of the CD8 T-cell response seems to be less profound.

Development of the HCV AD78 database also allowed us to identify a peculiar structural feature of the viral genome – the presence of additional codons at the E1/E2 gene junction. Previously, insertions at this position have been reported both for the AD78 strain [263] and other HCV isolates [177,264,265]. Our data have extended these original findings and indicated that such insertions may not only be found in sequences of a small number of HCV isolates but, in fact, may be present in the majority of isolates of a particular HCV strain. The functional meaning of naturally occurring amino acid stretches at the N-terminus of HVRI has not been investigated yet. Using the HCVpp system we have shown that presence of these stretches in the context of AD78 sequence had no significant influence on infectivity of pseudovirus particles. Application of a more sensitive and reproducible HCVcc system has demonstrated that the chimeric AD78/JFH1 viruses bearing the sequences of 3 variants of the HCV AD78 strain, which contained the insertions RGGG, GG, or SALT were highly infectious. These results mean that the RNA molecules with additional codons at the E1/E2 junction, which are found in HCV particles from patients' sera, are not artifacts or by-products of RNA replication, but genomes of viable virions. Overall, these data indicate that at least some of the HCV strains can tolerate the insertion of additional amino acid stretches at the N-terminus of HVRI and that these insertions have no evident influences on replicative potentials of these viruses. It is important to note, however, that the presence of additional amino acid residues at the E1/E2 junction may have important impact on structure and function of envelope proteins of HCV. In the current study the physico-chemical characteristics of the stretches of amino acid residues encoded by additional codons were analyzed. The predominance of neutral and hydrophobic residues in most of the observed positions of additional amino acid tracks suggested that these stretches might be oriented toward the hydrophobic E2 protein core. A high prevalence of small and flexible residues in these stretches, especially at the very N-terminus, might facilitate the maintaining of the varied conformational states of the HVR I. Absence of Trp and Cys, as well as the very low frequency of Pro evidence for this hypothesis. Finally, an enlargement of the HVR1 could affect some functional characteristics of the E2 protein, including its antigenic properties and ability to recognize and bind to HCV cell receptors.

Another interesting question concerns the limit of the length of the fragment that can be inserted at the E1/E2 junction. Our data have demonstrated preservation of the infectivity of HCVpp after insertion of the artificial RG_{30} track into the E1/E2 sequence derived from the AD78 strain (Viazov et al., in preparation). Recently, a report on the insertion of the flag tag at the E1/E2 junction of the chimeric J6/JFH1 virus was presented [213]. The modified virus with insertion of 13

additional amino acid residues was fully viable and retained its physico-chemical properties. Thus, at least for the genotypes 1b and 2 the presence of relatively long amino acid tracks at the N-terminus of E2 does not lead to a significant impairment of replicative potentials of the virus. In contrast to these findings, however, are the data indicating that the maximal length of insertions at this site in naturally occurring HCV isolates is five residues. These data suggest an existence in vivo of a selective mechanism that restricts the size of insertions at the N-terminus of the E2 protein.

The results of a longitudinal study performed by our group have shown that for most HCV AD78 isolates from the anti-D cohort no conservation of the length or composition of the additional amino acid stretches at the N-terminus of the E2 was observed (Viazov et al., in preparation). These findings may be explained by the naturally occurring quasispecies dynamics of HCV populations and raise the question on specificity of the mechanism of generation of such insertions. Considering very low frequency of amino acid insertions at this site in genotype 1a and 3 sequences, one might suggest that genomic sequences of some HCV isolates possess certain structural features, which facilitate the appearance of these insertions. Casino et al. suggested a mechanism of generation of extra codons at the extreme 5'-end of the E2 gene [264]. They have observed an imperfect direct repeat in the clonal sequence of one HVRI variant and suggested that a COOH-terminal region of the HVRI was duplicated and replaced the NH2-terminal region resulting in appearance of one extra amino acid residue. Our analysis of the E2 sequences retrieved from the Los Alamos HCV database as well as from the generated AD78 dataset did not reveal the presence of any repeat in any sequence. Moreover, our attempts to identify any differences in secondary structure of viral RNA molecules of HCV isolates with and without extra codon insertions by application of several RNA folding prediction programs were unsuccessful. Thus, the insertion of additional amino acid stretches at the N-terminus of the E2 protein represents a very interesting structural feature of many HCV isolates and functional significance of such inserts warrant further, more detailed studies.

One of the interesting observations in the current study as well as of our previous investigations (Viazov et al, unpublished; [252]) is that none of the anti-D patients harbored more than one variant of the HCV AD78 despite the fact that the inoculum, the anti-D globulin batches contained all three variants of the virus. These data suggest the existence of mechanism(s) that restrict either the coinfection of hepatocytes with two or several virus variants or superinfection or both. In favor of this postulate are the data on a very low frequency of mixed infection in HCV infected subjects, including multiple exposed individuals [266]. Recently, a phenomenon of HCV

superinfection exclusion by already infected Huh7 hepatoma cells due to interference at the level of HCV RNA translation and/or replication has been described [267,268]. Such a restriction may also function at other stages of the HCV replicative cycle, including the entry step. Thus, the Huh7 cells infected with one HCV strain demonstrate downregulation of expression of two key factors for HCV entry – claudine-1 and occludin [269]. Our data showing an absence of mixed infection of anti-D patients with two or three variants of HCV AD78 allowed us to propose another explanation of the restriction phenomenon. It is possible that one or several of the HCV cell receptors in cells from anti-D patients are heterogeneous and that polymorphism of these receptors might have influence on HCV variants selection in case of an inoculum containing a mixture of viruses. The anti-D cohort represented a unique group to verify this hypothesis. A single-nucleotide polymorphism (SNP) in major HCV cell receptors LDLR, SCARB1, and OCLN genes from anti-D patients was analyzed. No evident correlation between HCV cell receptor polymorphism and presence of a particular virus variant was found. These data indicate that heterogeneity of the tested major HCV cell receptors is very low and has no influence on the selection of virus variants at the stage of virus entry.

5 Summary

The specific anti-viral immune response of the host is considered as one of the major factors of HCV genome evolution. Adaptation of the virus to both cellular and humoral immune pressures by selection of the escape mutations in the targeted B- and T- epitopes is the basic mechanism of the evolution. Many details of HCV adaptation to selective forces, both in individual patients and, especially at the population level, are not completely clear. The current study address several aspects of this issue and its major aim was to get an insight into the evolution of the HCV genome at a population level and, in particular, into the contribution of humoral and cellular immune responses to evolution of the HCV envelope protein E2.

Many investigations of HCV evolution were hampered by the absence of information on the viral ancestral genomic sequence. The essential characteristic of the current project was the use of a special group of patients – women infected with the same HCV AD78 strain via contaminated anti-D globulin in a single-source outbreak. This allowed performing the accurate analysis of viral evolution at a population level using the HCV AD78 sequences present in the anti-D globulin as the index founder sequences.

At the first stage of the project a sequence database (core-P7 and NS4-NS5 genomic fragments) for multiple isolates of the HCV AD78 strain obtained from 93 anti-D patients persistently infected for 30 years and from contaminated immunoglobulin was generated.

Availability of this database allowed to analyze in detail the heterogeneity of the infectious source of the anti-D outbreak and to establish a potential time point of separation of the original HCV AD78 strain into the three closely related variants. This led to the establishment of the consensus sequences for 3 variants of the HCV AD78 strain. These sequences have been used in collaboration with the group of R.Bartenschlager for generation of the novel chimeric AD78/JFH1 viruses, which were capable to replicate in Huh7.5 hepatoma cells to high levels. Availability of such robust cell culture system represents an important step in the extension of the currently available JFH1-based systems of genotype 1. Especially promising are these new chimeric viruses for studies based on the materials from the HCV AD78 outbreak. In particular, this system is essential for elucidation of the biological significance of the mutations occurring in viral genome during both acute and persistent phases of infection.

Analysis of the heterogeneity of HCV AD78 sequences from anti-D patients has demonstrated a number of mutations both inside and outside of the CD8 T-cell epitopes and antiviral antibody binding sites and regions. Both reverse (toward the consensus 1b sequence) and forward (away from the infecting inoculum sequence) mutations were present with a predominance of the latter. A large number of mutations inside the CD8 epitopes were remarkably stable over 30 years of persistence. A least some of them were real escape mutations that, most probably, were preserved due to appearance of the compensatory mutations. The analysis of the frequency of mutations inside and outside of known and putative B- and T-cell epitopes within the E2 protein from AD78 isolates has shown higher rates of nonsynonymous amino acid substitutions inside the B-cell epitopes and, to a lesser extent inside the CD8 epitopes, than outside of these epitopes, suggesting that both humoral and CD8 T-cell immune responses contribute to the E2 evolution during the long-term HCV persistence. At that, the humoral response probably plays a leading role, while the contribution of the CD8 T-cell response seems to be less profound.

Application of a statistical approach allowed for identification of three putative novel CD8 T-cell epitopes in the core, E2 and NS2 proteins; one of them was confirmed by a functional assay in vitro. In several known CD8 epitopes an association between expression of different HLA class I alleles and presence of particular polymorphic sites was reproducibly found, suggesting immune pressure at the population level. Introduction of several escape mutations, identified in the core and E2 proteins, into the genome of the HCVcc constructs has impaired virus replication confirming that these were truly escape mutations. For the HLA-B51-specific mutation H153Q a reduction of the infectious titers depended very much on the origin of the sequence included into the HCVcc genome. This mutation caused relatively mild impairment of the infectivity of the AD78/JFH1 virus, while in the H77/JFH1 virus it led to a dramatic loss of infectivity exceeding 6 logs. These data indicate that the impact of the escape mutations on viral fitness to a significant extent might depend on the sequence context.

Generation of the HCV AD78 sequence database also allowed identifying a peculiar structural feature of some HCV strains - presence of additional 1 to 5 amino acid residues at the N-terminus of the E2 protein. Most of the AD78 isolates demonstrate the presence of such inserts. Both the HCVcc system and the HCVpp–based approach were used for analysis of the influence of the additional amino acid tracks on virus infectivity and replication. Presence of such inserts in the HCVpp bearing the AD78E1/E2 sequence or in the AD78/JFH1 virus has not significantly changed the infectivity of virus particles. In contrast, the insertion of the same additional stretches into the H77/JFH1 virus, which bears the structural genes of the H77 subtype 1a strain,

caused a complete abolishment of infectivity. Analysis of the composition of these additional amino acid stretches has shown a high prevalence of small and flexible residues, which might facilitate the maintenance of the varied conformational states of the HVRI. That, in turn, could affect some functional characteristics of the E2 protein, including its antigenic properties and ability to recognize and bind to HCV cell receptors.

6 Zusammenfassung

Die spezifische, antivirale Immunantwort des Wirtes stellt einen der Hauptfaktoren für die Evolution des HCV Genoms dar. Die Anpassung des Virus an den zellulären und humoralen Selektionsdruck erfolgt über die positive Selektion von „escape"-Mutationen in B- und T-Zell Epitopen und stellt den Basismechanismus der HCV Evolution dar. Viele Aspekte, die besonders auf Populationsebene zur Adaption von HCV an selektive Einflüsse führen, sind noch nicht ausreichend untersucht. Die vorliegende Studie befaßt sich mit einigen dieser Aspekte, wobei das Hauptziel in der Untersuchung der Evolution des HCV Genomes auf Populationsebene lag. Besonders der Beitrag der humoralen und zellulären Immunantwort zur Evolution des HCV Hüllproteins E2 wurde untersucht.

Viele Ansätze, die sich bisher mit der Evolution von HCV beschäftigten, waren relativ eingeschränkt, da keine Informationen über die ursprüngliche Sequenz des Inokulums vorlagen. Eine große Besonderheit der vorliegenden Studie ist die Untersuchung einer einzigartigen Patientengruppe - Frauen, die mit demselben HCV AD78 Stamm durch Verabreichung eines HCV kontaminierten anti-D Immunglobulins in einem „single-source"-Ausbruch infiziert wurden. Durch den Zugang zu verschiedenen Materialien dieser Kohorte war eine detaillierte Analyse der viralen Evolution auf Populationsebene möglich, wobei die AD78 Sequenzen im kontaminierten Immunglobulin als Indexsequenzen dienten.

Im ersten Schritt des Projektes wurde eine Sequenzdatenbank (core-NS2 und NS4-NS5 Genomregion) für multiple Isolate des HCV AD78 Stammes von 93 Patienten erstellt, welche persistierend über einen Zeitraum von 30 Jahren mit HCV AD78 infiziert waren, sowie von Sequenzen aus dem kontaminierten Immunglobulin.

Mit Hilfe der erstellten Sequenzdatenbank konnte die Heterogenität der Infektionsquelle des anti-D Ausbruchs detailiert analysiert werden und ein potenzieller Zeitpunkt der Separation des original AD78 Stammes in die drei eng verwandten Varianten konnte festgelegt werden. Außerdem konnten die Konsensusequenzen für alle drei AD78 Varianten erstellt werden, auf deren Grundlage in Kollaboration mit Prof. R. Bartenschlager die neuen AD78/JFH1 Viruschimären generiert wurden. Diese neuen chimären Konstrukte waren in der Lage, erfolgreich in Huh7.5 Zellen zu replizieren. Die Verfügbarkeit dieses neuen und robusten Zellkultursystems stellt einen bedeutenden Schritt für die Erweiterung der bisher kreierten JFH1-basierten Genotyp 1b Systeme dar. Besonders geeignet sind diese neuen Viruschimären für

Studien, die sich mit Proben des HCV AD78 Ausbruchs befassen. Dieses neue System stellt ein essentielles Hilfsmittel für die Aufklärung der biologischen Signifikanz von Mutationen, die im viralen Genom während der akuten und persistierenden Phase der Infektion auftreten, dar.

In der Analyse der Heterogenität von HCV AD78 Sequenzen aus anti-D Patienten wurden verschiedene Mutationen innerhalb und außerhalb von CD8 T-Zell-Epitopen und Bindungsstellen für neutralisierende Antikörper identifiziert. Mutationen weg von der Inokulumsequenz (forward mutations) wurden dabei häufiger detektiert als Mutationen in Richtung der allgemeinen 1b Konsensusequenz (reverse mutations). Eine große Anzahl von Mutationen innerhalb von CD8 T-Zell-Epitopen zeigte sich als bemerkenswert stabil in einem Zeitraum von 30 Jahren Persistenz. Einige von diesen Mutationen waren echte „escape“ Mutationen und wurden durch das Auftreten von kompensatorischen Mutationen in der Population fixiert. Die Analyse der Frequenz von Mutationen innerhalb und außerhalb von bekannten und putativen B- und T-Zell-Epitopen im E2 Protein aus AD78 Isolaten zeigte eine höhere Rate nonsynonymer Aminosäuresubstitutionen innerhalb von B-Zell-Epitopen und weniger ausgeprägte nonsynonyme Mutationen innerhalb von CD8 T-Zell-Epitopen. Diese Mutationen konnten vermehrt innerhalb bekannter Epitope beobachtet werden, als außerhalb dieser Epitope, was daraufhin deutet, dass sowohl die humorale als auch die zelluläre Immunantwort zur Evolution des E2 Hüllproteins in der langzeit-persistierenden HCV Infektion beitragen. Desweiteren scheint die humorale Immunantwort eine leitende Rolle in der Evolution des HCV E2 Proteins einzunehmen, wogegen die CD8 T-Zell Antwort einen geringeren Einfluß auf die Entwicklung des HCV E2 Proteins zu haben scheint.

Die Anwendung eines statistischen Ansatzes ermöglichte die Identifikation von drei putativen neuen CD8 T-Zell-Epitopen im core, E2 und NS2 Protein. Eines der potenziellen neuen Epitope konnte mittels funktionellem in vitro Zellkultur-Assay verifiziert werden. Innerhalb einiger bekannter CD8 T-Zell-Epitope konnte reproduzierbar eine Assoziation zwischen Expression eines bestimmten HLA Klasse 1 Allels und dem Auftreten bestimmter Polymorphismen an definierten Positionen hergestellt werden, was auf eine Immunselektion auf Populationsebene hindeutet. Die Einführung verschiedener „escape“ Mutationen, die in der core- und E2-Region identifiziert wurden, in das Genom von HCVcc Konstrukten führte zu einer Beeinträchtigung der Virusreplikation und Infektion. Diese Daten bestätigen, dass die beobachteten Polymorphismen tatsächlich „escape“ Mutationen waren. Im Falle der HLA-B51 spezifischen Mutation H153Q zeigte sich eine Reduktion des infektiösen Virustiters in Abhängigkeit der Herkunft der Sequenz, die in das HCVcc Genom integriert wurde. Während diese Mutation im

AD78/JFH1 System zu einer sehr milden Beeinträchtigung der Infektiosität führte, wurde durch Einführung dieser Mutation in das H77/JFH11 System die Infektiosität dramatisch reduziert (> 6 logs). Diese Daten lassen darauf schließen, dass der Einfluß von „escape“ Mutationen auf die virale Fitness zu einem großen Teil vom Sequenzkontext abhängig sein kann.

Durch die Erstellung der HCV AD78 Sequenzdatenbank war es möglich eine strukturelle Besonderheit einiger HCV Stämme zu identifizieren, nämlich das Vorhandensein von zusätzlichen 1 - 5 Aminosäuren am N-Terminus des E2 Proteins. Die meisten der AD78 Isolate wiesen solche Insertionen auf. Mit Hilfe des HCVpp und HCVcc Systems wude der Einfluß dieser zusätzlichen Aminosäuren auf die virale Replikation und Infektion untersucht. Insertion dieser zusätzlichen Aminosäuren in das HCVpp System, basierend auf AD78 E1/E2 Sequenzen, und das AD78/JFH1 HCVcc System hatte keinen signifikanten Einfluß auf die Infektiosität dieser Partikel. Im Gegensatz dazu führte die Insertion der gleichen zusätzlichen Aminosäuren in das H77/JFH1 HCVcc System zu einem vollständigem Verlust der Infektiosität. Die genaue Analyse der zusätzlichen Aminosären zeigte auf, daß bevorzugt kleine, flexible Aminosären vorkommen, welche zum Erhalt der konformationellen Variabilität der HVR1 beitragen könnten. Dies wiederum könnte einige funktionelle Charakteristika des E2 Proteins, einschließlich seiner Antigeneigenschaften und Fähigkeit HCV Zellrezeptoren zu erkennen und zu binden, beeinflussen.

7 References

1. Choo, Q.L.*, et al.* Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* **244**, 359-362 (1989).
2. Feinstone, S.M., Kapikian, A.Z., Purcell, R.H., Alter, H.J. & Holland, P.V. Transfusion-associated hepatitis not due to viral hepatitis type A or B. *N Engl J Med* **292**, 767-770 (1975).
3. Santantonio, T., Wiegand, J. & Gerlach, J.T. Acute hepatitis C: current status and remaining challenges. *J Hepatol* **49**, 625-633 (2008).
4. Seeff, L.B. Natural history of chronic hepatitis C. *Hepatology* **36**, S35-46 (2002).
5. Kwo, P.Y.*, et al.* Efficacy of boceprevir, an NS3 protease inhibitor, in combination with peginterferon alfa-2b and ribavirin in treatment-naive patients with genotype 1 hepatitis C infection (SPRINT-1): an open-label, randomised, multicentre phase 2 trial. *Lancet* **376**, 705-716 (2010).
6. Lawitz, E.*, et al.* A phase 2a trial of 12-week interferon-free therapy with two direct-acting antivirals (ABT-450/r, ABT-072) and ribavirin in IL28B C/C patients with chronic hepatitis C genotype 1. *J Hepatol* **59**, 18-23 (2013).
7. Chevaliez, S. & Pawlotsky, J.M. HCV Genome and Life Cycle. in *Hepatitis C Viruses: Genomes and Molecular Biology* (ed. Tan, S.L.) (Norfolk (UK), 2006).
8. Lindenbach, B.D. & Rice, C.M. *Flaviviridae: the viruses and their replication.*, (Lippincott-Williams & Wilkins, Philadelphia, 2001).
9. Kaito, M.*, et al.* Hepatitis C virus particle detected by immunoelectron microscopic study. *J Gen Virol* **75 (Pt 7)**, 1755-1760 (1994).
10. Thurner, C., Witwer, C., Hofacker, I.L. & Stadler, P.F. Conserved RNA secondary structures in Flaviviridae genomes. *J Gen Virol* **85**, 1113-1124 (2004).
11. Brown, R.J.*, et al.* Evolutionary dynamics of hepatitis C virus envelope genes during chronic infection. *J Gen Virol* **86**, 1931-1942 (2005).
12. Wang, C., Le, S.Y., Ali, N. & Siddiqui, A. An RNA pseudoknot is an essential structural element of the internal ribosome entry site located within the hepatitis C virus 5' noncoding region. *RNA* **1**, 526-537 (1995).
13. Honda, M.*, et al.* Structural requirements for initiation of translation by internal ribosome entry within genome-length hepatitis C virus RNA. *Virology* **222**, 31-42 (1996).
14. Kolykhalov, A.A., Feinstone, S.M. & Rice, C.M. Identification of a highly conserved sequence element at the 3' terminus of hepatitis C virus genome RNA. *J Virol* **70**, 3363-3371 (1996).
15. Tanaka, T., Kato, N., Cho, M.J. & Shimotohno, K. A novel sequence found at the 3' terminus of hepatitis C virus genome. *Biochem Biophys Res Commun* **215**, 744-749 (1995).
16. Tanaka, S., Takenaka, K., Matsumata, T., Mori, R. & Sugimachi, K. Hepatitis C virus replication is associated with expression of transforming growth factor-alpha and insulin-like growth factor-II in cirrhotic livers. *Dig Dis Sci* **41**, 208-215 (1996).
17. Friebe, P. & Bartenschlager, R. Genetic analysis of sequences in the 3' nontranslated region of hepatitis C virus that are important for RNA replication. *J Virol* **76**, 5326-5338 (2002).
18. Ito, T. & Lai, M.M. Determination of the secondary structure of and cellular protein binding to the 3'-untranslated region of the hepatitis C virus RNA genome. *J Virol* **71**, 8698-8706 (1997).

19. Yi, M. & Lemon, S.M. 3' nontranslated RNA signals required for replication of hepatitis C virus RNA. *J Virol* **77**, 3557-3568 (2003).
20. Yi, M. & Lemon, S.M. Structure-function analysis of the 3' stem-loop of hepatitis C virus genomic RNA and its role in viral RNA replication. *RNA* **9**, 331-345 (2003).
21. Suzuki, T. Morphogenesis of infectious hepatitis C virus particles. *Front Microbiol* **3**, 38 (2012).
22. Simmonds, P. & Midgley, S. Recombination in the genesis and evolution of hepatitis B virus genotypes. *J Virol* **79**, 15467-15476 (2005).
23. Drake, J.W., Charlesworth, B., Charlesworth, D. & Crow, J.F. Rates of spontaneous mutation. *Genetics* **148**, 1667-1686 (1998).
24. Neumann, A.U.*, et al.* Hepatitis C viral dynamics in vivo and the antiviral efficacy of interferon-alpha therapy. *Science* **282**, 103-107 (1998).
25. Simmonds, P. Genetic diversity and evolution of hepatitis C virus--15 years on. *J Gen Virol* **85**, 3173-3188 (2004).
26. Timm, J. & Roggendorf, M. Sequence diversity of hepatitis C virus: implications for immune control and therapy. *World J Gastroenterol* **13**, 4808-4817 (2007).
27. Yasui, K.*, et al.* The native form and maturation process of hepatitis C virus core protein. *J Virol* **72**, 6048-6055 (1998).
28. Chang, S.C., Yen, J.H., Kang, H.Y., Jang, M.H. & Chang, M.F. Nuclear localization signals in the core protein of hepatitis C virus. *Biochem Biophys Res Commun* **205**, 1284-1290 (1994).
29. Suzuki, R.*, et al.* Nuclear localization of the truncated hepatitis C virus core protein with its hydrophobic C terminus deleted. *J Gen Virol* **76 (Pt 1)**, 53-61 (1995).
30. Suzuki, R.*, et al.* Molecular determinants for subcellular localization of hepatitis C virus core protein. *J Virol* **79**, 1271-1281 (2005).
31. Schwer, B.*, et al.* Targeting of hepatitis C virus core protein to mitochondria through a novel C-terminal localization motif. *J Virol* **78**, 7958-7968 (2004).
32. Grakoui, A., Wychowski, C., Lin, C., Feinstone, S.M. & Rice, C.M. Expression and identification of hepatitis C virus polyprotein cleavage products. *J Virol* **67**, 1385-1395 (1993).
33. Harada, S.*, et al.* Expression of processed core protein of hepatitis C virus in mammalian cells. *J Virol* **65**, 3015-3021 (1991).
34. Santolini, E., Migliaccio, G. & La Monica, N. Biosynthesis and biochemical properties of the hepatitis C virus core protein. *J Virol* **68**, 3631-3641 (1994).
35. McLauchlan, J. Properties of the hepatitis C virus core protein: a structural protein that modulates cellular processes. *J Viral Hepat* **7**, 2-14 (2000).
36. Fukutomi, T.*, et al.* Hepatitis C virus core protein stimulates hepatocyte growth: correlation with upregulation of wnt-1 expression. *Hepatology* **41**, 1096-1105 (2005).
37. Chou, A.H.*, et al.* Hepatitis C virus core protein modulates TRAIL-mediated apoptosis by enhancing Bid cleavage and activation of mitochondria apoptosis signaling pathway. *J Immunol* **174**, 2160-2166 (2005).
38. Kountouras, J., Zavos, C. & Chatzopoulos, D. Apoptosis in hepatitis C. *J Viral Hepat* **10**, 335-342 (2003).
39. Meyer, K., Basu, A., Saito, K., Ray, R.B. & Ray, R. Inhibition of hepatitis C virus core protein expression in immortalized human hepatocytes induces cytochrome c-independent increase in Apaf-1 and caspase-9 activation for cell death. *Virology* **336**, 198-207 (2005).

40. Nunez, O.*, et al.* Increased intrahepatic cyclooxygenase 2, matrix metalloproteinase 2, and matrix metalloproteinase 9 expression is associated with progressive liver disease in chronic hepatitis C virus infection: role of viral core and NS5A proteins. *Gut* **53**, 1665-1672 (2004).
41. Ray, R.B., Lagging, L.M., Meyer, K., Steele, R. & Ray, R. Transcriptional regulation of cellular and viral promoters by the hepatitis C virus core protein. *Virus Res* **37**, 209-220 (1995).
42. Shih, C.M., Lo, S.J., Miyamura, T., Chen, S.Y. & Lee, Y.H. Suppression of hepatitis B virus expression and replication by hepatitis C virus core protein in HuH-7 cells. *J Virol* **67**, 5823-5832 (1993).
43. Barba, G.*, et al.* Hepatitis C virus core protein shows a cytoplasmic localization and associates to cellular lipid storage droplets. *Proc Natl Acad Sci U S A* **94**, 1200-1205 (1997).
44. Moriya, K.*, et al.* The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. *Nat Med* **4**, 1065-1067 (1998).
45. Moriya, K.*, et al.* Hepatitis C virus core protein induces hepatic steatosis in transgenic mice. *J Gen Virol* **78 (Pt 7)**, 1527-1531 (1997).
46. Walewski, J.L., Keller, T.R., Stump, D.D. & Branch, A.D. Evidence for a new hepatitis C virus antigen encoded in an overlapping reading frame. *RNA* **7**, 710-721 (2001).
47. Baril, M. & Brakier-Gingras, L. Translation of the F protein of hepatitis C virus is initiated at a non-AUG codon in a +1 reading frame relative to the polyprotein. *Nucleic Acids Res* **33**, 1474-1486 (2005).
48. Deleersnyder, V.*, et al.* Formation of native hepatitis C virus glycoprotein complexes. *J Virol* **71**, 697-704 (1997).
49. Bartosch, B., Dubuisson, J. & Cosset, F.L. Infectious hepatitis C virus pseudo-particles containing functional E1-E2 envelope protein complexes. *J Exp Med* **197**, 633-642 (2003).
50. Nielsen, S.U., Bassendine, M.F., Burt, A.D., Bevitt, D.J. & Toms, G.L. Characterization of the genome and structural proteins of hepatitis C virus resolved from infected human liver. *J Gen Virol* **85**, 1497-1507 (2004).
51. Cocquerel, L., Meunier, J.C., Pillez, A., Wychowski, C. & Dubuisson, J. A retention signal necessary and sufficient for endoplasmic reticulum localization maps to the transmembrane domain of hepatitis C virus glycoprotein E2. *J Virol* **72**, 2183-2191 (1998).
52. Cocquerel, L., Wychowski, C., Minner, F., Penin, F. & Dubuisson, J. Charged residues in the transmembrane domains of hepatitis C virus glycoproteins play a major role in the processing, subcellular localization, and assembly of these envelope proteins. *J Virol* **74**, 3623-3633 (2000).
53. Weiner, A.J.*, et al.* Sequence variation in hepatitis C viral isolates. *J Hepatol* **13 Suppl 4**, S6-14 (1991).
54. Farci, P.*, et al.* Prevention of hepatitis C virus infection in chimpanzees by hyperimmune serum against the hypervariable region 1 of the envelope 2 protein. *Proc Natl Acad Sci U S A* **93**, 15394-15399 (1996).
55. Zibert, A., Kraas, W., Meisel, H., Jung, G. & Roggendorf, M. Epitope mapping of antibodies directed against hypervariable region 1 in acute self-limiting and chronic infections due to hepatitis C virus. *Journal of virology* **71**, 4123-4127 (1997).

56. Penin, F.*, et al.* Conservation of the conformation and positive charges of hepatitis C virus E2 envelope glycoprotein hypervariable region 1 points to a role in cell attachment. *J Virol* **75**, 5703-5710 (2001).
57. Farci, P.*, et al.* The outcome of acute hepatitis C predicted by the evolution of the viral quasispecies. *Science* **288**, 339-344 (2000).
58. Vieyres, G., Dubuisson, J. & Patel, A.H. Characterization of antibody-mediated neutralization directed against the hypervariable region 1 of hepatitis C virus E2 glycoprotein. *J Gen Virol* **92**, 494-506 (2011).
59. Barth, H.*, et al.* Cellular binding of hepatitis C virus envelope glycoprotein E2 requires cell surface heparan sulfate. *J Biol Chem* **278**, 41003-41012 (2003).
60. Bartosch, B.*, et al.* An interplay between hypervariable region 1 of the hepatitis C virus E2 glycoprotein, the scavenger receptor BI, and high-density lipoprotein promotes both enhancement of infection and protection against neutralizing antibodies. *J Virol* **79**, 8217-8229 (2005).
61. Voisset, C.*, et al.* High density lipoproteins facilitate hepatitis C virus entry through the scavenger receptor class B type I. *J Biol Chem* **280**, 7793-7799 (2005).
62. Roccasecca, R.*, et al.* Binding of the hepatitis C virus E2 glycoprotein to CD81 is strain specific and is modulated by a complex interplay between hypervariable regions 1 and 2. *J Virol* **77**, 1856-1867 (2003).
63. Sklan, E.H., Charuworn, P., Pang, P.S. & Glenn, J.S. Mechanisms of HCV survival in the host. *Nature reviews. Gastroenterology & hepatology* **6**, 217-227 (2009).
64. Zeisel, M.B. & Baumert, T.F. HCV entry and neutralizing antibodies: lessons from viral variants. *Future Microbiol* **4**, 511-517 (2009).
65. Albecka, A.*, et al.* Identification of new functional regions in hepatitis C virus envelope glycoprotein E2. *J Virol* **85**, 1777-1792 (2011).
66. McCaffrey, K., Gouklani, H., Boo, I., Poumbourios, P. & Drummer, H.E. The variable regions of hepatitis C virus glycoprotein E2 have an essential structural role in glycoprotein assembly and virion infectivity. *J Gen Virol* **92**, 112-121 (2011).
67. Brazzoli, M.*, et al.* Folding and dimerization of hepatitis C virus E1 and E2 glycoproteins in stably transfected CHO cells. *Virology* **332**, 438-453 (2005).
68. Michalak, J.P.*, et al.* Characterization of truncated forms of hepatitis C virus glycoproteins. *J Gen Virol* **78 (Pt 9)**, 2299-2306 (1997).
69. Flint, M. & McKeating, J.A. The role of the hepatitis C virus glycoproteins in infection. *Rev Med Virol* **10**, 101-117 (2000).
70. Rosa, D.*, et al.* A quantitative test to estimate neutralizing antibodies to the hepatitis C virus: cytofluorimetric assessment of envelope glycoprotein 2 binding to target cells. *Proc Natl Acad Sci U S A* **93**, 1759-1763 (1996).
71. Carrere-Kremer, S.*, et al.* Subcellular localization and topology of the p7 polypeptide of hepatitis C virus. *J Virol* **76**, 3720-3730 (2002).
72. Gonzalez, M.E. & Carrasco, L. Viroporins. *FEBS Lett* **552**, 28-34 (2003).
73. Jones, C.T., Murray, C.L., Eastman, D.K., Tassello, J. & Rice, C.M. Hepatitis C virus p7 and NS2 proteins are essential for production of infectious virus. *J Virol* **81**, 8374-8383 (2007).
74. Sakai, A.*, et al.* The p7 polypeptide of hepatitis C virus is critical for infectivity and contains functionally important genotype-specific sequences. *Proc Natl Acad Sci U S A* **100**, 11646-11651 (2003).

75. Santolini, E., Pacini, L., Fipaldini, C., Migliaccio, G. & Monica, N. The NS2 protein of hepatitis C virus is a transmembrane polypeptide. *J Virol* **69**, 7461-7471 (1995).
76. Yamaga, A.K. & Ou, J.H. Membrane topology of the hepatitis C virus NS2 protein. *J Biol Chem* **277**, 33228-33234 (2002).
77. Grakoui, A., McCourt, D.W., Wychowski, C., Feinstone, S.M. & Rice, C.M. Characterization of the hepatitis C virus-encoded serine proteinase: determination of proteinase-dependent polyprotein cleavage sites. *J Virol* **67**, 2832-2843 (1993).
78. Hijikata, M.*, et al.* Two distinct proteinase activities required for the processing of a putative nonstructural precursor protein of hepatitis C virus. *J Virol* **67**, 4665-4675 (1993).
79. Jirasko, V.*, et al.* Structural and functional characterization of nonstructural protein 2 for its role in hepatitis C virus assembly. *J Biol Chem* **283**, 28546-28562 (2008).
80. Bartenschlager, R., Lohmann, V., Wilkinson, T. & Koch, J.O. Complex formation between the NS3 serine-type proteinase of the hepatitis C virus and NS4A and its importance for polyprotein maturation. *J Virol* **69**, 7519-7528 (1995).
81. Lin, C. & Rice, C.M. The hepatitis C virus NS3 serine proteinase and NS4A cofactor: establishment of a cell-free trans-processing assay. *Proc Natl Acad Sci U S A* **92**, 7622-7626 (1995).
82. Satoh, S., Tanji, Y., Hijikata, M., Kimura, K. & Shimotohno, K. The N-terminal region of hepatitis C virus nonstructural protein 3 (NS3) is essential for stable complex formation with NS4A. *J Virol* **69**, 4255-4260 (1995).
83. Foy, E.*, et al.* Regulation of interferon regulatory factor-3 by the hepatitis C virus serine protease. *Science* **300**, 1145-1148 (2003).
84. Li, K.*, et al.* Immune evasion by hepatitis C virus NS3/4A protease-mediated cleavage of the Toll-like receptor 3 adaptor protein TRIF. *Proc Natl Acad Sci U S A* **102**, 2992-2997 (2005).
85. Gwack, Y., Kim, D.W., Han, J.H. & Choe, J. DNA helicase activity of the hepatitis C virus nonstructural protein 3. *Eur J Biochem* **250**, 47-54 (1997).
86. Tai, C.L., Chi, W.K., Chen, D.S. & Hwang, L.H. The helicase activity associated with hepatitis C virus nonstructural protein 3 (NS3). *J Virol* **70**, 8477-8484 (1996).
87. Borowski, P.*, et al.* Non-structural protein 3 of hepatitis C virus inhibits phosphorylation mediated by cAMP-dependent protein kinase. *Eur J Biochem* **237**, 611-618 (1996).
88. Hassan, M., Ghozlan, H. & Abdel-Kader, O. Activation of c-Jun NH2-terminal kinase (JNK) signaling pathway is essential for the stimulation of hepatitis C virus (HCV) non-structural protein 3 (NS3)-mediated cell growth. *Virology* **333**, 324-336 (2005).
89. Hugle, T.*, et al.* The hepatitis C virus nonstructural protein 4B is an integral endoplasmic reticulum membrane protein. *Virology* **284**, 70-81 (2001).
90. Lundin, M., Monne, M., Widell, A., Von Heijne, G. & Persson, M.A. Topology of the membrane-associated hepatitis C virus protein NS4B. *J Virol* **77**, 5428-5438 (2003).
91. Egger, D.*, et al.* Expression of hepatitis C virus proteins induces distinct membrane alterations including a candidate viral replication complex. *J Virol* **76**, 5974-5984 (2002).
92. Elazar, M., Liu, P., Rice, C.M. & Glenn, J.S. An N-terminal amphipathic helix in hepatitis C virus (HCV) NS4B mediates membrane association, correct

localization of replication complex proteins, and HCV RNA replication. *J Virol* **78**, 11393-11400 (2004).
93. Gretton, S.N., Taylor, A.I. & McLauchlan, J. Mobility of the hepatitis C virus NS4B protein on the endoplasmic reticulum membrane and membrane-associated foci. *J Gen Virol* **86**, 1415-1421 (2005).
94. Jones, D.M., Patel, A.H., Targett-Adams, P. & McLauchlan, J. The hepatitis C virus NS4B protein can trans-complement viral RNA replication and modulates production of infectious virus. *J Virol* **83**, 2163-2177 (2009).
95. Evans, M.J., Rice, C.M. & Goff, S.P. Phosphorylation of hepatitis C virus nonstructural protein 5A modulates its protein interactions and viral RNA replication. *Proc Natl Acad Sci U S A* **101**, 13038-13043 (2004).
96. Shimakami, T.*, et al.* Effect of interaction between hepatitis C virus NS5A and NS5B on hepatitis C virus RNA replication with the hepatitis C virus replicon. *J Virol* **78**, 2738-2748 (2004).
97. Bartenschlager, R., Frese, M. & Pietschmann, T. Novel insights into hepatitis C virus replication and persistence. *Adv Virus Res* **63**, 71-180 (2004).
98. Behrens, S.E., Tomei, L. & De Francesco, R. Identification and properties of the RNA-dependent RNA polymerase of hepatitis C virus. *EMBO J* **15**, 12-22 (1996).
99. Ivashkina, N.*, et al.* The hepatitis C virus RNA-dependent RNA polymerase membrane insertion sequence is a transmembrane segment. *J Virol* **76**, 13088-13093 (2002).
100. Schmidt-Mende, J.*, et al.* Determinants for membrane association of the hepatitis C virus RNA-dependent RNA polymerase. *J Biol Chem* **276**, 44052-44063 (2001).
101. Watashi, K.*, et al.* Cyclophilin B is a functional regulator of hepatitis C virus RNA polymerase. *Mol Cell* **19**, 111-122 (2005).
102. Di Marco, S.*, et al.* Interdomain communication in hepatitis C virus polymerase abolished by small molecule inhibitors bound to a novel allosteric site. *J Biol Chem* **280**, 29765-29770 (2005).
103. Ma, H.*, et al.* Inhibition of native hepatitis C virus replicase by nucleotide and non-nucleoside inhibitors. *Virology* **332**, 8-15 (2005).
104. Pawlotsky, J.M. & McHutchison, J.G. Hepatitis C. Development of new drugs and clinical trials: promises and pitfalls. Summary of an AASLD hepatitis single topic conference, Chicago, IL, February 27-March 1, 2003. *Hepatology* **39**, 554-567 (2004).
105. Pawlotsky, J.M. Therapy of hepatitis C: from empiricism to eradication. *Hepatology* **43**, S207-220 (2006).
106. Blanchard, E.*, et al.* Hepatitis C virus entry depends on clathrin-mediated endocytosis. *J Virol* **80**, 6964-6972 (2006).
107. Ploss, A. & Dubuisson, J. New advances in the molecular biology of hepatitis C virus infection: towards the identification of new treatment targets. *Gut* **61 Suppl 1**, i25-35 (2012).
108. Sabahi, A. Hepatitis C Virus entry: the early steps in the viral replication cycle. *Virol J* **6**, 117 (2009).
109. Tang, H. & Grise, H. Cellular and molecular biology of HCV infection and hepatitis. *Clin Sci (Lond)* **117**, 49-65 (2009).
110. Levy, S., Todd, S.C. & Maecker, H.T. CD81 (TAPA-1): a molecule involved in signal transduction and cell adhesion in the immune system. *Annu Rev Immunol* **16**, 89-109 (1998).

111. Masciopinto, F., Campagnoli, S., Abrignani, S., Uematsu, Y. & Pileri, P. The small extracellular loop of CD81 is necessary for optimal surface expression of the large loop, a putative HCV receptor. *Virus Res* **80**, 1-10 (2001).
112. Pileri, P.*, et al.* Binding of hepatitis C virus to CD81. *Science* **282**, 938-941 (1998).
113. Major, M.E.*, et al.* Hepatitis C virus kinetics and host responses associated with disease and outcome of infection in chimpanzees. *Hepatology* **39**, 1709-1720 (2004).
114. Walker, C.M. Comparative features of hepatitis C virus infection in humans and chimpanzees. *Springer Semin Immunopathol* **19**, 85-98 (1997).
115. Flint, M.*, et al.* Diverse CD81 proteins support hepatitis C virus infection. *J Virol* **80**, 11331-11342 (2006).
116. Lindenbach, B.D.*, et al.* Complete replication of hepatitis C virus in cell culture. *Science* **309**, 623-626 (2005).
117. Cormier, E.G.*, et al.* CD81 is an entry coreceptor for hepatitis C virus. *Proc Natl Acad Sci U S A* **101**, 7270-7274 (2004).
118. Masciopinto, F.*, et al.* Expression of human CD81 in transgenic mice does not confer susceptibility to hepatitis C virus infection. *Virology* **304**, 187-196 (2002).
119. Koutsoudakis, G.*, et al.* Characterization of the early steps of hepatitis C virus infection by using luciferase reporter viruses. *J Virol* **80**, 5308-5320 (2006).
120. Krieger, M. Scavenger receptor class B type I is a multiligand HDL receptor that influences diverse physiologic systems. *J Clin Invest* **108**, 793-797 (2001).
121. Scarselli, E.*, et al.* The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. *EMBO J* **21**, 5017-5025 (2002).
122. Babitt, J.*, et al.* Murine SR-BI, a high density lipoprotein receptor that mediates selective lipid uptake, is N-glycosylated and fatty acylated and colocalizes with plasma membrane caveolae. *J Biol Chem* **272**, 13242-13249 (1997).
123. Yamada, E.*, et al.* Analysis of the binding of hepatitis C virus genotype 1a and 1b E2 glycoproteins to peripheral blood mononuclear cell subsets. *J Gen Virol* **86**, 2507-2512 (2005).
124. Silver, D.L., Wang, N., Xiao, X. & Tall, A.R. High density lipoprotein (HDL) particle uptake mediated by scavenger receptor class B type 1 results in selective sorting of HDL cholesterol from protein and polarized cholesterol secretion. *J Biol Chem* **276**, 25287-25293 (2001).
125. Bartosch, B.*, et al.* Cell entry of hepatitis C virus requires a set of co-receptors that include the CD81 tetraspanin and the SR-B1 scavenger receptor. *J Biol Chem* **278**, 41624-41630 (2003).
126. Forns, X.*, et al.* Hepatitis C virus lacking the hypervariable region 1 of the second envelope protein is infectious and causes acute resolving or persistent infection in chimpanzees. *Proc Natl Acad Sci U S A* **97**, 13318-13323 (2000).
127. Barth, H.*, et al.* Scavenger receptor class B type I and hepatitis C virus infection of primary tupaia hepatocytes. *J Virol* **79**, 5774-5785 (2005).
128. Harris, H.J.*, et al.* Claudin association with CD81 defines hepatitis C virus entry. *J Biol Chem* **285**, 21092-21102 (2010).
129. Evans, M.J.*, et al.* Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry. *Nature* **446**, 801-805 (2007).
130. Krieger, S.E.*, et al.* Inhibition of hepatitis C virus infection by anti-claudin-1 antibodies is mediated by neutralization of E2-CD81-claudin-1 associations. *Hepatology* **51**, 1144-1157 (2010).

131. Zheng, A.*, et al.* Claudin-6 and claudin-9 function as additional coreceptors for hepatitis C virus. *J Virol* **81**, 12465-12471 (2007).
132. Lupberger, J.*, et al.* EGFR and EphA2 are host factors for hepatitis C virus entry and possible targets for antiviral therapy. *Nat Med* **17**, 589-595 (2011).
133. Gardner, J.P.*, et al.* L-SIGN (CD 209L) is a liver-specific capture receptor for hepatitis C virus. *Proc Natl Acad Sci U S A* **100**, 4498-4503 (2003).
134. Lozach, P.Y.*, et al.* DC-SIGN and L-SIGN are high affinity binding receptors for hepatitis C virus glycoprotein E2. *J Biol Chem* **278**, 20358-20366 (2003).
135. Lozach, P.Y.*, et al.* C-type lectins L-SIGN and DC-SIGN capture and transmit infectious hepatitis C virus pseudotype particles. *J Biol Chem* **279**, 32035-32045 (2004).
136. Pohlmann, S.*, et al.* Hepatitis C virus glycoproteins interact with DC-SIGN and DC-SIGNR. *J Virol* **77**, 4070-4080 (2003).
137. Geijtenbeek, T.B.*, et al.* Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses. *Cell* **100**, 575-585 (2000).
138. Bashirova, A.A.*, et al.* A dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN)-related protein is highly expressed on human liver sinusoidal endothelial cells and promotes HIV-1 infection. *J Exp Med* **193**, 671-678 (2001).
139. Ludwig, I.S.*, et al.* Hepatitis C virus targets DC-SIGN and L-SIGN to escape lysosomal degradation. *J Virol* **78**, 8322-8332 (2004).
140. Chung, N.S. & Wasan, K.M. Potential role of the low-density lipoprotein receptor family as mediators of cellular drug uptake. *Adv Drug Deliv Rev* **56**, 1315-1334 (2004).
141. Agnello, V., Abel, G., Elfahal, M., Knight, G.B. & Zhang, Q.X. Hepatitis C virus and other flaviviridae viruses enter cells via low density lipoprotein receptor. *Proc Natl Acad Sci U S A* **96**, 12766-12771 (1999).
142. Monazahian, M.*, et al.* Low density lipoprotein receptor as a candidate receptor for hepatitis C virus. *J Med Virol* **57**, 223-229 (1999).
143. Molina, S.*, et al.* The low-density lipoprotein receptor plays a role in the infection of primary human hepatocytes by hepatitis C virus. *J Hepatol* **46**, 411-419 (2007).
144. Hsu, M.*, et al.* Hepatitis C virus glycoproteins mediate pH-dependent cell entry of pseudotyped retroviral particles. *Proc Natl Acad Sci U S A* **100**, 7271-7276 (2003).
145. Moradpour, D., Penin, F. & Rice, C.M. Replication of hepatitis C virus. *Nat Rev Microbiol* **5**, 453-463 (2007).
146. Ji, H., Fraser, C.S., Yu, Y., Leary, J. & Doudna, J.A. Coordinated assembly of human translation initiation complexes by the hepatitis C virus internal ribosome entry site RNA. *Proc Natl Acad Sci U S A* **101**, 16990-16995 (2004).
147. Lukavsky, P.J., Otto, G.A., Lancaster, A.M., Sarnow, P. & Puglisi, J.D. Structures of two RNA domains essential for hepatitis C virus internal ribosome entry site function. *Nat Struct Biol* **7**, 1105-1110 (2000).
148. Otto, G.A. & Puglisi, J.D. The pathway of HCV IRES-mediated translation initiation. *Cell* **119**, 369-380 (2004).
149. Moradpour, D.*, et al.* Membrane association of the RNA-dependent RNA polymerase is essential for hepatitis C virus RNA replication. *J Virol* **78**, 13278-13284 (2004).

150. Miyanari, Y.*, et al.* The lipid droplet is an important organelle for hepatitis C virus production. *Nat Cell Biol* **9**, 1089-1097 (2007).
151. Serafino, A.*, et al.* Suggested role of the Golgi apparatus and endoplasmic reticulum for crucial sites of hepatitis C virus replication in human lymphoblastoid cells infected in vitro. *J Med Virol* **70**, 31-41 (2003).
152. Strahotin, C.S. & Babich, M. Hepatitis C variability, patterns of resistance, and impact on therapy. *Adv Virol* **2012**, 267483 (2012).
153. Pereira, A.A. & Jacobson, I.M. New and experimental therapies for HCV. *Nature reviews. Gastroenterology & hepatology* **6**, 403-411 (2009).
154. Brenndorfer, E.D. & Sallberg, M. Hepatitis C virus-mediated modulation of cellular immunity. *Arch Immunol Ther Exp (Warsz)* **60**, 315-329 (2012).
155. Thimme, R., Binder, M. & Bartenschlager, R. Failure of innate and adaptive immune responses in controlling hepatitis C virus infection. *FEMS Microbiol Rev* **36**, 663-683 (2012).
156. Carney, D.S. & Gale, M. HCV Regulation of Host Defense. in *Hepatitis C Viruses: Genomes and Molecular Biology* (ed. Tan, S.L.) (Norfolk (UK), 2006).
157. Li, K. & Lemon, S.M. Innate immune responses in hepatitis C virus infection. *Semin Immunopathol* **35**, 53-72 (2013).
158. Schoggins, J.W. & Rice, C.M. Innate immune responses to hepatitis C virus. *Curr Top Microbiol Immunol* **369**, 219-242 (2013).
159. Walker, C.M. Adaptive immunity to the hepatitis C virus. *Adv Virus Res* **78**, 43-86 (2010).
160. Codran, A.*, et al.* Entry of hepatitis C virus pseudotypes into primary human hepatocytes by clathrin-dependent endocytosis. *J Gen Virol* **87**, 2583-2593 (2006).
161. Lagging, L.M., Meyer, K., Owens, R.J. & Ray, R. Functional role of hepatitis C virus chimeric glycoproteins in the infectivity of pseudotyped virus. *J Virol* **72**, 3539-3546 (1998).
162. Di Lorenzo, C., Angus, A.G. & Patel, A.H. Hepatitis C virus evasion mechanisms from neutralizing antibodies. *Viruses* **3**, 2280-2300 (2011).
163. Keck, Z.Y.*, et al.* Therapeutic control of hepatitis C virus: the role of neutralizing monoclonal antibodies. *Curr Top Microbiol Immunol* **317**, 1-38 (2008).
164. Johansson, D.X.*, et al.* Human combinatorial libraries yield rare antibodies that broadly neutralize hepatitis C virus. *Proc Natl Acad Sci U S A* **104**, 16269-16274 (2007).
165. Kato, N.*, et al.* Humoral immune response to hypervariable region 1 of the putative envelope glycoprotein (gp70) of hepatitis C virus. *J Virol* **67**, 3923-3930 (1993).
166. Meunier, J.C.*, et al.* Isolation and characterization of broadly neutralizing human monoclonal antibodies to the e1 glycoprotein of hepatitis C virus. *J Virol* **82**, 966-973 (2008).
167. Owsianka, A.*, et al.* Monoclonal antibody AP33 defines a broadly neutralizing epitope on the hepatitis C virus E2 envelope glycoprotein. *J Virol* **79**, 11095-11104 (2005).
168. Perotti, M.*, et al.* Identification of a broadly cross-reacting and neutralizing human monoclonal antibody directed against the hepatitis C virus E2 protein. *J Virol* **82**, 1047-1052 (2008).
169. von Hahn, T.*, et al.* Hepatitis C virus continuously escapes from neutralizing antibody and T-cell responses during chronic infection in vivo. *Gastroenterology* **132**, 667-678 (2007).

170. Krawczynski, K., *et al.* Effect of immune globulin on the prevention of experimental hepatitis C virus infection. *J Infect Dis* **173**, 822-828 (1996).
171. Yu, M.Y., *et al.* Neutralizing antibodies to hepatitis C virus (HCV) in immune globulins derived from anti-HCV-positive plasma. *Proc Natl Acad Sci U S A* **101**, 7705-7710 (2004).
172. Bjøro, K., Frøland, S.S., Yun, Z., Samdal, H.H. & Haaland, T. Hepatitis C infection in patients with primary hypogammaglobulinemia after treatment with contaminated immune globulin. *N Engl J Med* **331**, 1607-1611 (1994).
173. Razvi, S., Schneider, L., Jonas, M.M. & Cunningham-Rundles, C. Outcome of intravenous immunoglobulin-transmitted hepatitis C virus infection in primary immunodeficiency. *Clin Immunol* **101**, 284-288 (2001).
174. Dowd, K.A., Netski, D.M., Wang, X.H., Cox, A.L. & Ray, S.C. Selection pressure from neutralizing antibodies drives sequence evolution during acute infection with hepatitis C virus. *Gastroenterology* **136**, 2377-2386 (2009).
175. Pestka, J.M., *et al.* Rapid induction of virus-neutralizing antibodies and viral clearance in a single-source outbreak of hepatitis C. *Proc Natl Acad Sci U S A* **104**, 6025-6030 (2007).
176. Zeisel, M.B., Cosset, F.L. & Baumert, T.F. Host neutralizing responses and pathogenesis of hepatitis C virus infection. *Hepatology* **48**, 299-307 (2008).
177. Kato, N., *et al.* Genetic drift in hypervariable region 1 of the viral genome in persistent hepatitis C virus infection. *J Virol* **68**, 4776-4784 (1994).
178. Shimizu, Y.K., *et al.* A hyperimmune serum against a synthetic peptide corresponding to the hypervariable region 1 of hepatitis C virus can prevent viral infection in cell cultures. *Virology* **223**, 409-412 (1996).
179. Weiner, A.J., *et al.* Evidence for immune selection of hepatitis C virus (HCV) putative envelope glycoprotein variants: potential role in chronic HCV infections. *Proc Natl Acad Sci U S A* **89**, 3468-3472 (1992).
180. Ray, S.C., *et al.* Acute hepatitis C virus structural gene sequences as predictors of persistent viremia: hypervariable region 1 as a decoy. *J Virol* **73**, 2938-2946 (1999).
181. Brimacombe, C.L., *et al.* Neutralizing antibody-resistant hepatitis C virus cell-to-cell transmission. *J Virol* **85**, 596-605 (2011).
182. Cooper, S., *et al.* Analysis of a successful immune response against hepatitis C virus. *Immunity* **10**, 439-449 (1999).
183. Cox, A.L., *et al.* Comprehensive analyses of CD8+ T cell responses during longitudinal study of acute human hepatitis C. *Hepatology* **42**, 104-112 (2005).
184. Day, C.L., *et al.* Broad specificity of virus-specific CD4+ T-helper-cell responses in resolved hepatitis C virus infection. *J Virol* **76**, 12584-12595 (2002).
185. Diepolder, H.M., *et al.* Immunodominant CD4+ T-cell epitope within nonstructural protein 3 in acute hepatitis C virus infection. *J Virol* **71**, 6011-6019 (1997).
186. Diepolder, H.M., *et al.* Possible mechanism involving T-lymphocyte response to non-structural protein 3 in viral clearance in acute hepatitis C virus infection. *Lancet* **346**, 1006-1007 (1995).
187. Lechner, F., *et al.* Analysis of successful immune responses in persons infected with hepatitis C virus. *J Exp Med* **191**, 1499-1512 (2000).
188. Thimme, R., *et al.* Viral and immunological determinants of hepatitis C virus clearance, persistence, and disease. *Proc Natl Acad Sci U S A* **99**, 15661-15668 (2002).
189. Thimme, R., *et al.* Determinants of viral clearance and persistence during acute hepatitis C virus infection. *J Exp Med* **194**, 1395-1406 (2001).

190. Shin, E.C., *et al.* Delayed induction, not impaired recruitment, of specific CD8(+) T cells causes the late onset of acute hepatitis C. *Gastroenterology* **141**, 686-695, 695 e681 (2011).
191. Takaki, A., *et al.* Cellular immune responses persist and humoral responses decrease two decades after recovery from a single-source outbreak of hepatitis C. *Nat Med* **6**, 578-582 (2000).
192. Gerlach, J.T., *et al.* Recurrence of hepatitis C virus after loss of virus-specific CD4(+) T-cell response in acute hepatitis C. *Gastroenterology* **117**, 933-941 (1999).
193. Missale, G., *et al.* Different clinical behaviors of acute hepatitis C virus infection are associated with different vigor of the anti-viral cell-mediated immune response. *J Clin Invest* **98**, 706-714 (1996).
194. Schulze zur Wiesch, J., *et al.* Broad repertoire of the CD4+ Th cell response in spontaneously controlled hepatitis C virus infection includes dominant and highly promiscuous epitopes. *J Immunol* **175**, 3603-3613 (2005).
195. Grakoui, A., *et al.* HCV persistence and immune evasion in the absence of memory T cell help. *Science* **302**, 659-662 (2003).
196. Hong, X., *et al.* Human leukocyte antigen class II DQB1*0301, DRB1*1101 alleles and spontaneous clearance of hepatitis C virus infection: a meta-analysis. *World J Gastroenterol* **11**, 7302-7307 (2005).
197. McKiernan, S.M., *et al.* Distinct MHC class I and II alleles are associated with hepatitis C viral clearance, originating from a single source. *Hepatology* **40**, 108-114 (2004).
198. Rowan, A.G., *et al.* Hepatitis C virus-specific Th17 cells are suppressed by virus-induced TGF-beta. *J Immunol* **181**, 4485-4494 (2008).
199. Gruner, N.H., *et al.* Association of hepatitis C virus-specific CD8+ T cells with viral clearance in acute hepatitis C. *J Infect Dis* **181**, 1528-1536 (2000).
200. Fitzmaurice, K., *et al.* Molecular footprints reveal the impact of the protective HLA-A*03 allele in hepatitis C virus infection. *Gut* **60**, 1563-1571 (2011).
201. Kim, A.Y., *et al.* Spontaneous control of HCV is associated with expression of HLA-B 57 and preservation of targeted epitopes. *Gastroenterology* **140**, 686-696 e681 (2011).
202. Kuniholm, M.H., *et al.* Specific human leukocyte antigen class I and II alleles associated with hepatitis C virus viremia. *Hepatology* **51**, 1514-1522 (2010).
203. Neumann-Haefelin, C., *et al.* Dominant influence of an HLA-B27 restricted CD8+ T cell response in mediating HCV clearance and evolution. *Hepatology* **43**, 563-572 (2006).
204. Thio, C.L., *et al.* HLA-Cw*04 and hepatitis C virus persistence. *J Virol* **76**, 4792-4797 (2002).
205. Jo, J., *et al.* Analysis of CD8+ T-cell-mediated inhibition of hepatitis C virus replication using a novel immunological model. *Gastroenterology* **136**, 1391-1401 (2009).
206. Shoukry, N.H., *et al.* Memory CD8+ T cells are required for protection from persistent hepatitis C virus infection. *J Exp Med* **197**, 1645-1655 (2003).
207. Dolganiuc, A., *et al.* Hepatitis C virus core and nonstructural protein 3 proteins induce pro- and anti-inflammatory cytokines and inhibit dendritic cell differentiation. *J Immunol* **170**, 5615-5624 (2003).
208. Lohmann, V., *et al.* Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* **285**, 110-113 (1999).

209. Boettler, T.*, et al.* T cells with a CD4+CD25+ regulatory phenotype suppress in vitro proliferation of virus-specific CD8+ T cells during chronic hepatitis C virus infection. *J Virol* **79**, 7860-7867 (2005).
210. Radziewicz, H.*, et al.* Impaired hepatitis C virus (HCV)-specific effector CD8+ T cells undergo massive apoptosis in the peripheral blood during acute HCV infection and in the liver during the chronic phase of infection. *J Virol* **82**, 9808-9822 (2008).
211. Bukh, J., Christensen, E. & Krogsgaard, K. [Treatment and prevention of hepatitis C--progress and challenges. The Danish Society of Hepatology]. *Ugeskr Laeger* **165**, 1233 (2003).
212. Fernandez, J.*, et al.* Long-term persistence of infection in chimpanzees inoculated with an infectious hepatitis C virus clone is associated with a decrease in the viral amino acid substitution rate and low levels of heterogeneity. *J Virol* **78**, 9782-9789 (2004).
213. Prentoe, J.*, et al.* Hypervariable region 1 differentially impacts viability of hepatitis C virus strains of genotypes 1 to 6 and impairs virus neutralization. *J Virol* **85**, 2224-2234 (2011).
214. Bankwitz, D.*, et al.* Hepatitis C virus hypervariable region 1 modulates receptor interactions, conceals the CD81 binding site, and protects conserved neutralizing epitopes. *J Virol* **84**, 5751-5763 (2010).
215. Keck, Z.Y.*, et al.* Mapping a region of hepatitis C virus E2 that is responsible for escape from neutralizing antibodies and a core CD81-binding region that does not tolerate neutralization escape mutations. *J Virol* **85**, 10451-10463 (2011).
216. Gal-Tanamy, M.*, et al.* In vitro selection of a neutralization-resistant hepatitis C virus escape mutant. *Proc Natl Acad Sci U S A* **105**, 19450-19455 (2008).
217. Keck, Z.Y.*, et al.* Definition of a conserved immunodominant domain on hepatitis C virus E2 glycoprotein by neutralizing human monoclonal antibodies. *J Virol* **82**, 6061-6066 (2008).
218. Dreux, M.*, et al.* High density lipoprotein inhibits hepatitis C virus-neutralizing antibodies by stimulating cell entry via activation of the scavenger receptor BI. *J Biol Chem* **281**, 18285-18295 (2006).
219. Zhang, P.*, et al.* Hepatitis C virus epitope-specific neutralizing antibodies in Igs prepared from human plasma. *Proc Natl Acad Sci U S A* **104**, 8449-8454 (2007).
220. Zhang, P.*, et al.* Depletion of interfering antibodies in chronic hepatitis C patients and vaccinated chimpanzees reveals broad cross-genotype neutralizing activity. *Proc Natl Acad Sci U S A* **106**, 7537-7541 (2009).
221. Tarr, A.W.*, et al.* Hepatitis C patient-derived glycoproteins exhibit marked differences in susceptibility to serum neutralizing antibodies: genetic subtype defines antigenic but not neutralization serotype. *J Virol* **85**, 4246-4257 (2011).
222. Timpe, J.M.*, et al.* Hepatitis C virus cell-cell transmission in hepatoma cells in the presence of neutralizing antibodies. *Hepatology* **47**, 17-24 (2008).
223. Witteveldt, J.*, et al.* CD81 is dispensable for hepatitis C virus cell-to-cell transmission in hepatoma cells. *J Gen Virol* **90**, 48-58 (2009).
224. Lauer, G.M. & Walker, B.D. Hepatitis C virus infection. *The New England journal of medicine* **345**, 41-52 (2001).
225. Neumann-Haefelin, C.*, et al.* Virological and immunological determinants of intrahepatic virus-specific CD8+ T-cell failure in chronic hepatitis C virus infection. *Hepatology* **47**, 1824-1836 (2008).

226. Nakamoto, N.*, et al.* Synergistic reversal of intrahepatic HCV-specific CD8 T cell exhaustion by combined PD-1/CTLA-4 blockade. *PLoS Pathog* **5**, e1000313 (2009).
227. Spangenberg, H.C.*, et al.* Intrahepatic CD8+ T-cell failure during chronic hepatitis C virus infection. *Hepatology* **42**, 828-837 (2005).
228. Wedemeyer, H.*, et al.* Impaired effector function of hepatitis C virus-specific CD8+ T cells in chronic hepatitis C virus infection. *J Immunol* **169**, 3447-3458 (2002).
229. Golden-Mason, L.*, et al.* Upregulation of PD-1 expression on circulating and intrahepatic hepatitis C virus-specific CD8+ T cells associated with reversible immune dysfunction. *J Virol* **81**, 9249-9258 (2007).
230. Nakamoto, N.*, et al.* Functional restoration of HCV-specific CD8 T cells by PD-1 blockade is defined by PD-1 expression and compartmentalization. *Gastroenterology* **134**, 1927-1937, 1937 e1921-1922 (2008).
231. Penna, A.*, et al.* Dysfunction and functional restoration of HCV-specific CD8 responses in chronic hepatitis C virus infection. *Hepatology* **45**, 588-601 (2007).
232. Radziewicz, H.*, et al.* Liver-infiltrating lymphocytes in chronic human hepatitis C virus infection display an exhausted phenotype with high levels of PD-1 and low levels of CD127 expression. *J Virol* **81**, 2545-2553 (2007).
233. Golden-Mason, L.*, et al.* Negative immune regulator Tim-3 is overexpressed on T cells in hepatitis C virus infection and its blockade rescues dysfunctional CD4+ and CD8+ T cells. *J Virol* **83**, 9122-9130 (2009).
234. Callendret, B. & Walker, C. A siege of hepatitis: immune boost for viral hepatitis. *Nat Med* **17**, 252-253 (2011).
235. McMahan, R.H.*, et al.* Tim-3 expression on PD-1+ HCV-specific human CTLs is associated with viral persistence, and its blockade restores hepatocyte-directed in vitro cytotoxicity. *J Clin Invest* **120**, 4546-4557 (2010).
236. Bengsch, B.*, et al.* Coexpression of PD-1, 2B4, CD160 and KLRG1 on exhausted HCV-specific CD8+ T cells is linked to antigen recognition and T cell differentiation. *PLoS Pathog* **6**, e1000947 (2010).
237. Schlaphoff, V.*, et al.* Dual function of the NK cell receptor 2B4 (CD244) in the regulation of HCV-specific CD8+ T cells. *PLoS Pathog* **7**, e1002045 (2011).
238. Semmo, N.*, et al.* T-cell responses and previous exposure to hepatitis C virus in indeterminate blood donors. *Lancet* **365**, 327-329 (2005).
239. Cabrera, R.*, et al.* An immunomodulatory role for CD4(+)CD25(+) regulatory T lymphocytes in hepatitis C virus infection. *Hepatology* **40**, 1062-1071 (2004).
240. Sugimoto, K.*, et al.* Suppression of HCV-specific T cells without differential hierarchy demonstrated ex vivo in persistent HCV infection. *Hepatology* **38**, 1437-1448 (2003).
241. Abel, M.*, et al.* Intrahepatic virus-specific IL-10-producing CD8 T cells prevent liver damage during chronic hepatitis C virus infection. *Hepatology* **44**, 1607-1616 (2006).
242. Accapezzato, D.*, et al.* Hepatic expansion of a virus-specific regulatory CD8(+) T cell population in chronic hepatitis C virus infection. *J Clin Invest* **113**, 963-972 (2004).
243. Tacke, R.S.*, et al.* Myeloid suppressor cells induced by hepatitis C virus suppress T-cell responses through the production of reactive oxygen species. *Hepatology* **55**, 343-353 (2012).
244. Crispe, I.N. Liver antigen-presenting cells. *J Hepatol* **54**, 357-365 (2011).

245. Cox, A.L., *et al.* Cellular immune selection with hepatitis C virus persistence in humans. *J Exp Med* **201**, 1741-1752 (2005).
246. Tester, I., *et al.* Immune evasion versus recovery after acute hepatitis C virus infection from a shared source. *J Exp Med* **201**, 1725-1731 (2005).
247. Timm, J., *et al.* CD8 epitope escape and reversion in acute HCV infection. *J Exp Med* **200**, 1593-1604 (2004).
248. Ray, S.C., *et al.* Divergent and convergent evolution after a common-source outbreak of hepatitis C virus. *J Exp Med* **201**, 1753-1759 (2005).
249. Bowen, D.G. & Walker, C.M. Mutational escape from CD8+ T cell immunity: HCV evolution, from chimpanzees to man. *J Exp Med* **201**, 1709-1714 (2005).
250. Gaudieri, S., *et al.* Evidence of viral adaptation to HLA class I-restricted immune pressure in chronic hepatitis C virus infection. *J Virol* **80**, 11094-11104 (2006).
251. Rauch, A., *et al.* Divergent adaptation of hepatitis C virus genotypes 1 and 3 to human leukocyte antigen-restricted immune pressure. *Hepatology* **50**, 1017-1029 (2009).
252. Ruhl, M., *et al.* CD8+ T-cell response promotes evolution of hepatitis C virus nonstructural proteins. *Gastroenterology* **140**, 2064-2073 (2011).
253. Timm, J., *et al.* Human leukocyte antigen-associated sequence polymorphisms in hepatitis C virus reveal reproducible immune responses and constraints on viral evolution. *Hepatology* **46**, 339-349 (2007).
254. Petrovic, D., Dempsey, E., Doherty, D.G., Kelleher, D. & Long, A. Hepatitis C virus--T-cell responses and viral escape mutations. *Eur J Immunol* **42**, 17-26 (2012).
255. Wiese, M., *et al.* Outcome in a hepatitis C (genotype 1b) single source outbreak in Germany--a 25-year multicenter study. *J Hepatol* **43**, 590-598 (2005).
256. Neumann-Haefelin, C., *et al.* Human leukocyte antigen B27 selects for rare escape mutations that significantly impair hepatitis C virus replication and require compensatory mutations. *Hepatology* **54**, 1157-1166 (2011).
257. Oniangue-Ndza, C., *et al.* Compensatory mutations restore the replication defects caused by cytotoxic T lymphocyte escape mutations in hepatitis C virus polymerase. *J Virol* **85**, 11883-11890 (2011).
258. Salloum, S., *et al.* Escape from HLA-B*08-restricted CD8 T cells by hepatitis C virus is associated with fitness costs. *J Virol* **82**, 11803-11812 (2008).
259. Uebelhoer, L., *et al.* Stable cytotoxic T cell escape mutation in hepatitis C virus is linked to maintenance of viral fitness. *PLoS Pathog* **4**, e1000143 (2008).
260. Edwards, V.C., Tarr, A.W., Urbanowicz, R.A. & Ball, J.K. The role of neutralizing antibodies in hepatitis C virus infection. *J Gen Virol* **93**, 1-19 (2012).
261. Keck, Z.Y., *et al.* Human monoclonal antibodies to a novel cluster of conformational epitopes on HCV E2 with resistance to neutralization escape in a genotype 2a isolate. *PLoS Pathog* **8**, e1002653 (2012).
262. Wang, Y., Keck, Z.Y. & Foung, S.K. Neutralizing antibody response to hepatitis C virus. *Viruses* **3**, 2127-2145 (2011).
263. Hohne, M., Schreier, E. & Roggendorf, M. Sequence variability in the env-coding region of hepatitis C virus isolated from patients infected during a single source outbreak. *Arch Virol* **137**, 25-34 (1994).
264. Casino, C., *et al.* Variation of hepatitis C virus following serial transmission: multiple mechanisms of diversification of the hypervariable region and evidence for convergent genome evolution. *J Gen Virol* **80 (Pt 3)**, 717-725 (1999).
265. Torres-Puente, M., *et al.* Contribution of insertions and deletions to the variability of hepatitis C virus populations. *J Gen Virol* **88**, 2198-2203 (2007).

266. Viazov, S.*, et al.* Hepatitis C virus recombinants are rare even among intravenous drug users. *J Med Virol* **82**, 232-238 (2010).
267. Schaller, T.*, et al.* Analysis of hepatitis C virus superinfection exclusion by using novel fluorochrome gene-tagged viral genomes. *J Virol* **81**, 4591-4603 (2007).
268. Tscherne, D.M.*, et al.* Superinfection exclusion in cells infected with hepatitis C virus. *J Virol* **81**, 3693-3703 (2007).
269. Liu, S.*, et al.* Tight junction proteins claudin-1 and occludin control hepatitis C virus entry and are downregulated during infection to prevent superinfection. *J Virol* **83**, 2011-2014 (2009).

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9 Curriculum vitae with publication list

Professionelle Erfahrungen

Seit 10/2010	Doktorandin - „Genetic heterogeneity of virus isolates in chronic hepatitis C patients infected in a single-source HCV outbreak - special structural features and evidences that both humoral and cellular immune responses contribute to evolution of the envelope 2 viral protein“ Institut für Virologie, Universitätsklinikum Essen Betreuer: Prof. Dr. Michael Roggendorf Assoziiert an das Graduiertenkolleg GRK1045 “Modulation of host cell functions to treat viral and bacterial infections”, Universität Duisburg-Essen
04/2009 - 10/2009	Diplomandin - „Charakterisierung der antiretroviralen Aktivität humaner Apobec3 Proteine gegen das humane Gammaretrovirus XMRV“ Institut für Medizinische Mikrobiologie, Virologie und Hygiene, Universitätsklinikum Hamburg-Eppendorf Betreuerin: Dr. Nicole Fischer
10/2002 - 09/2010	Kranken- und Gesundheitspflegerin Innere Medizin/Gastroenterologie/Infektiologie, Universitätsklinikum Hamburg-Eppendorf (zunächst ein Jahr vollzeitbeschäftigt, danach auf Reduktion der Arbeitszeit auf 25 % wegen Studienbeginn)

Schul- und Hochschulbildung

10/2003 - 10/2009	Diplomstudium Biochemie/Molekularbiologie an der Universität Hamburg, (Schwerpunkte im Hauptstudium: Molekulare Medizin, Wahlfach: Immunologie) Abschlussnote: 1.56 (gut)
10/1999 - 09/2002	Ausbildung zur staatlich examinierten Krankenschwester am Nordwest-Krankenhaus Sanderbusch, Sande
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Publikationsliste

Lipskoch M., Luppus S., Wiese M., Schreier E., Timm J., Viazov S., Roggendorf M. „Genetic heterogeneity of HCV cell entry receptors presumably has no influence on virus selection in patients infected with inoculum containing different virus variants.“ (Manuskript in Vorbereitung)

Lipskoch M., Luppus S., Wiese M., Hoffmann D., Budeus B., Roggendorf M., Timm J., Viazov S. „Evidence for HCV escape from specific CD8+ T-cell immune response in a group of patients infected with the AD78 viral strain in a single source outbreak.“ (Manuskript in Vorbereitung)

Poster-Präsentationen

03/2013	23. Jahrestagung der Deutschen Gesellschaft für Virologie in Kiel
10/2012	19th International Symposium on Hepatitis C Virus and Related Viruses in Venedig, Italien
03/2012	22. Jahrestagung der Deutschen Gesellschaft für Virologie in Essen
11/2011	Forschungstag der medizinischen Fakultät Duisburg-Essen am Universitätsklinikum Essen

Preise

11/2011	Poster-Preis für die Präsentation der Arbeit mit dem Titel „Single nucleotide polymorphisms (SNPs) of HCV cell entry receptors presumably have no influence on virus selection in patients infected with the inoculum containing different virus variants.“ Forschungstag der medizinischen Fakultät Duisburg-Essen am Universitätsklinikum Essen

Sprachen

Deutsch	Muttersprache
Englisch	fließend in Wort und Schrift
Französisch	Grundlagen

10 List of tables

11 List of figures

12 Abbreviations

aa	amino acid
ARFP	alternate reading frame protein
α-	anti
C	core protein
cons	consensus
DC	dendritic cell
DC-SIGN	dendritic cell-specific intercellular adhesion molecule-3 grabbing nonintegrin
DNA	desoxyribonucleic acid
ds	double-stranded
E1	envelope protein 1
E2	envelope protein 2
EGFR	endothelial growth factor receptor
eIF2	eukaryotic initiation factor 2
ER	endoplasmic reticulum
g	gramm
HCC	hepato-cellular carcinoma
HCV	hepatitis C virus
HCVcc	HCV cell culture infectious
HCVpp	HCV pseudoparticle
HDL	high-density lipoprotein
HLA	human leukocyte antigen
HVR1	hypervariable region 1
HVR2	hypervariable region 2
kDa	kilo Dalton
kb	kilo base
IFN-α	interferon-α
IRF	interferon regulatory factor

ISG	interferon stimulated gene
L	liter
LD	lipid droplet
LDL	low-density lipoprotein
LDLR	LDL receptor
LEL	large extracellular loop
L-SIGN	liver/lymph node-specific intercellular adhesion molecule-3 (ICAM) grabbing integrin
Mda5	melanoma differentiation associated protein 5
min	minute
ms	milli-second
mL	milli-liter
µg	micro-gramm
µL	micro-liter
nAb	neutralizing antibody
NLS	nucleus localization signal
ng	nano-gramm
nm	nano-meter
NS	nonstructural
nt	nucleotide
n.d.	not deteced
n.t.	not tested
NTP	nucleotide tri-phosphate
OAS	2‘,5‘-oligoadenylate synthetase
OCLN	occludin
ORF	open reading frame
PKR	protein kinase R
RdRp	RNA-dependent RNA-polymerase
RIG-I	retinoic acid inducible gene-I

RNA	ribonucleic acid
RLR	RIG-I like receptor
sec	second
SEL	small extracellular loop
SNP	single-nucleotide polymorphism
SR-BI	scavenger
ss	single-stranded
SVR	sustained virological response
TLR	Toll-like receptor
TM	transmembrane
UTR	untranslated region
V	Volt
w/o	without

13 Statement

Erklärung:
Hiermit erkläre ich, gem. § 6 Abs. 2, Nr. 7 der Promotionsordnung der Math.-Nat.- Fachbereiche zur Erlangung der Dr. rer. nat., dass ich das Arbeitsgebiet, dem das Thema „Genetic heterogeneity of virus isolates in chronic hepatitis C patients infected in a single-source HCV outbreak - special structural features and evidences that both humoral and cellular immune responses contribute to evolution of the envelope 2 viral protein “ zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von Maren Lipskoch befürworte.

Essen, den _____ _____
Michael Roggendorf

Erklärung:
Hiermit erkläre ich, gem. § 6 Abs. 2, Nr. 6 der Promotionsordnung der Math.-Nat.- Fachbereiche 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 _____ _____
Maren Lipskoch

Erklärung:
Hiermit erkläre ich, gem. § 6 Abs. 2, Nr. 8 der Promotionsordnung der Math.-Nat.- Fachbereiche 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 _____ _____
Maren Lipskoch