A new vaccine strategy for HCV: Presentation of Hepatitis C Virus hypervariable region 1 on HBV capsid-like particles

Inaugural-Dissertation

zur

Erlangung des Doktorgrades

Dr. rer. nat.

der Fakultät für

Biologie

an der

Universität Duisburg-Essen

vorgelegt von

Milena Lange

aus Bergisch Gladbach

April 2012

Für meine Familie!

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

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

1. Gutachter: Prof. Dr. Michael Roggendorf

2. Gutachter: Prof. Dr. Astrid Westendorf

3. Gutachter:

Vorsitzender des Prüfungsausschusses: Prof. Dr. Daniel Hoffmann

Tag der mündlichen Prüfung: 30.08.2012

Contents

1 Introduction 1

1.1 The Hepatitis C Virus 1
1.1.1 Morphology of the virion 2
1.1.2 The genome organization 3
1.1.3 The replication cycle 6
1.1.4 The role of envelope proteins during entry 8
1.2 Model systems of HCV infection 14
1.2.1 in vitro models 14
1.3 The immune response during an HCV infection 17
1.3.1 The innate immune response 17
1.3.2 The cellular immune response 18
1.3.3 Envelope proteins and the humoral immune response 18
1.4 Vaccination trials in HCV infection 23
1.5 The Hepatitis B Virus 25
1.5.1 The HBc Protein 25
1.6 Aim of the study 28

2 Materials 30

2.1 Laboratory animals 30
2.1.1 Wild-type mice 30
2.1.2 Guinea pigs 30
2.2 Anesthetics 30
2.3 Bacteria strains 30
2.4 Eukaryotic cell lines 31
2.4.1 Human Hepatoma cell line Huh7.5 31
2.4.2 Human Embryonic Kidney 293T cells 31
2.5 Chemicals and reagents 31
2.6 Antibiotics 32
2.7 Cell culture media 33
2.8 Buffers and solutions 33
2.9 Enzymes and commercial Kits 36
2.10 Standards 36
2.11 Plasmids 36
2.12 Antibodies 37
2.13 Peptides 38
2.14 Membranes and films 38
2.15 Oligonucleotides 39
2.16 Materials and equipment 41

3 Methods 43

3.1 Working with prokaryotic cells 43
3.1.1 Transformation of chemically competent E. coli strains 43
3.1.2 Plasmid DNA preparation using commercial kits 43
3.1.3 Cultivation of E.coli for protein expression 43
3.1.4 Cell Disruption under non-denaturing conditions 44
3.2 Cell Culture 44
3.2.1 Thawing of cells 44
3.2.2 Cryoconservation of cells 45
3.2.3 Passaging of cells 45
3.2.4 Counting of viable cells using Trypan blue exclusion microscopy 45
3.2.5 Transfection of HEK-293T cells 46
3.2.6 Infectivity assay 47
3.2.7 Neutralization assay with HCVpp's 47
3.3 Molecular biology methods 48

3.3.1 Polymerase Chain Reaction ... 48
3.3.2 DNA restriction digest ... 49
3.3.3 Agarose gel electrophoresis ... 50
3.3.4 DNA extraction from agarose gels ... 50
3.3.5 Cloning of PCR-products in intermediate plasmids ... 50
3.3.6 Ligation of DNA fragments ... 51
3.3.7 DNA-Sequencing ... 52
3.4 Protein-biochemical methods ... 52
3.4.1 Determination of the protein concentration ... 52
3.4.2 SDS-PAGE ... 52
3.4.3 Native Gel electrophoresis ... 53
3.4.4 Coomassie-Brilliant-Blue Staining ... 53
3.4.5 Immunoblot Analysis (Western Blot) ... 54
3.4.6 Native Capillary Transfer ... 54
3.4.7 Analytical Sucrose Density Gradient Centrifugation ... 55
3.4.8 Preparative Dialysis ... 55
3.4.9 Triton X-114 phase separation ... 55
3.4.10 Endotoxin determination ... 56
3.5 Animal experiments ... 58
3.5.1 Anesthetization ... 58
3.5.2 Blood withdrawal ... 58
3.5.3 Immunization trials ... 58
3.6 Enzyme-linked Immunosorbent Assay (ELISA) ... 60
3.7 Electron microscopy ... 61
4 Results ... 62
4.1 Generation and characterization of SplitCore-HVRI-CLPs ... 62
4.1.1 HVRI-CLPs displaying different HVRI-variants are assembly competent ... 63
4.2 Immunization with HVRI-CLPs elicits an HVRI-specific immune response in mice ... 66
4.2.1 Single HVRI-CLPs are highly immunogenic in mice ... 66
4.2.2 Single HVRI-CLPs induce partially cross-reactive antibodies ... 68
4.3 Characterization of the neutralizing capacity of mouse sera after immunization with single HVRI-CLPs ... 69
4.3.1 Evaluation of the neutralizing capacity to homologous HVRI-variants after single immunization ... 69
4.3.2 Evaluation of the neutralizing capacity to heterologous HVRI-variants after single immunization ... 71
4.4 A mixture of HVRI-CLPs induces high titers of cross-reactive antibodies ... 73
4.5 Characterization of the neutralizing capacity of mouse sera after mixture immunization with HVRI-CLPs ... 76
4.5.1 Evaluation of the neutralizing capacity to homologous HVRI-variants after mixture immunization ... 76
4.5.2 Evaluation of the neutralizing capacity to heterologous HVRI-variants after mixture immunization ... 77
4.6 A modified mixture immunization with HVRI-CLPs increases cross-reactive and cross-neutralizing antibody titers ... 78
4.6.1 Evaluation of the neutralizing capacity to homologous HVRI-variants after improved mixture immunization ... 81
4.6.2 Evaluation of the neutralizing capacity to heterologous HVRI-variants after improved mixture immunization ... 82
4.7 The true cross-neutralizing potential after mixture immunization in mice ... 83
4.8 Immunoglobulin isotype switch in mice immunized with HVRI-CLPs ... 86
4.9 Immunization with HVRI-CLPs elicits an HVRI-specific immune response in guinea pigs ... 89
4.9.1 A mixture of HVRI-CLPs is less immunogenic in guinea pigs compared to mice ... 89
4.10 Characterization of the neutralizing capacity of guinea pig sera after immunization with HVRI-CLPs ... 91

4.10.1 Evaluation of the neutralizing capacity to homologous HVRI-variants after mixture immunization ... 91
4.10.2 Evaluation of the neutralizing capacity to heterologous HVRI-variants after mixture immunization ... 92
4.11 WHc-CLPs displaying HVRI-R9 are assembly competent ... 93

5 Discussion ... 96

5.1 Characterization of SplitCore-HVRI-CLPs ... 98
5.2 Immune response in mice after immunization with HVRI-CLPs ... 99
5.2.1 Characterization of the HVRI-specific immune response after single HVRI-CLP immunization in mice ... 99
5.2.2 Characterization of the HVRI-specific immune response after mixture HVRI-CLP immunization in mice ... 100
5.2.3 Characterization of the neutralizing capacity in mice after single HVRI-CLP immunization ... 101
5.2.4 Characterization of the neutralizing capacity in mice after mixture HVRI-CLP immunization ... 102
5.2.5 Immunization with an improved HVRI-CLP mixture induces a more vigorous neutralizing immune response in mice ... 103
5.2.6 Optimization of the mixture immunization leads to the induction of a potent cross-neutralizing response in mice ... 105
5.2.7 Characterization of the immunoglobulin isotype switch in mice immunized with the improved HVRI-CLP mixture ... 107
5.2.8 Characterization of the immune response in mice against the vaccine carrier HBc ... 108
5.3 Immune response in guinea pigs after immunization with HVRI-CLPs ... 110

6 Summary ... 111

7 Zusammenfassung ... 113

8 References ... 115

9 Appendix ... 127

9.1 Vector-cards ... 127
9.2 Supplementary tables ... 130

10 Abbreviations ... 131

11 List of figures ... 136

12 List of tables ... 138

13 Publications ... 139

13.1 Presentations ... 139

14 Acknowledgements ... 140

15 Curriculum vitae ... 141

16 Declaration (Erklärung) ... 143

1 Introduction

1.1 The Hepatitis C Virus

The Hepatitis C Virus (HCV) is a major cause of acute and chronic hepatitis in humans and is considered as a serious public-health problem. According to estimates of the World Health Organization (WHO) about 130–170 million people are chronically infected and 3-4 million people are newly infected with the blood-borne virus each year (WHO, 2011b). While 20% to 40% of acute hepatitis patients eliminate the virus and recover from the infection, 60% to 80% develop chronic hepatitis. About 10% to 20% of chronically infected individuals progress to cirrhosis and 1% to 5% of hepatic cirrhosis patients develop hepatocellular carcinoma (HCC) (WHO, 2011b). In the western industrial countries, HCV is the major cause of liver transplantations (Brown, 2005). Lasting beneficial treatment of chronic HCV infections can only be achieved by treating patients with pegylated interferon alpha (IFN-α) in combination with Ribavirin. However, usually only in 50% to 80% of patients a sustained virological response (SVR) is achieved, depending on the viral genotype (Zeuzem, 2004). The current improved understanding of the reproductive machinery of HCV has led to the discovery of numerous potential targets for antiviral therapy including processing and replication of the HCV polyprotein, viral entry and fusion, RNA translation, virus assembly and release, and several host cell factors. Inhibitors of the non-structural proteins 3/4A (NS3/4A) protease seem to be the best initial target and are currently the most advanced drugs in clinical development. The first two successful compounds, VX950 (Telaprevir) and SCH503034 (Boceprevir) were approved by the Food and Drug Administration (FDA) in May 2011, each given in combination with standard care. The results achieved with this treatment led to SVR rates to as high as 75% in genotype 1 (Jensen, 2011). Although constantly studies of alternative targets are under way, improved formulations of current HCV therapies are also being developed. However, in future a combination of antiviral agents with different mechanisms of action may lead to the eventual possibility of interferon-free regimens (Vermehren and Sarrazin, 2011). This situation clearly shows the need for new prophylactic and therapeutic approaches that prevent the spread of HCV and would provide more efficient antiviral therapy for individuals suffering from HCV.

Despite the permanent progress which is been made in the development of treatment, a prophylactic vaccine is the most important aim to significantly reduce the number of HCV infections in the future.

1.1.1 Morphology of the virion

HCV virions have a diameter of 55 to 65 nm (Fig. 1.1 A). The virus has been classified as the only member of the *Hepacivirus* genus and was assigned to the family of *Flaviviridae* due to its similarity to other viruses in this family. HCV particles adopt to a classical icosahedral scaffold by anchoring to the host cell-derived double-layer lipid membrane containing the two envelope proteins 1 (E1) and 2 (E2). The genomic RNA of the virus is encapsidated by the nucleocapsid which lies underneath the membrane and is formed by multiple copies of the core protein (Fig. 1.1 B). In the serum of an infected host HCV circulates in various forms: virions bound to very-low-density lipoproteins and low-density lipoproteins (representing the infectious fraction), virions bound to immunoglobulins and free virions. Also viral particles exhibiting physicochemical, morphologic, and antigenic properties of non-enveloped HCV nucleocapsids have been detected in plasma (Penin *et al.*, 2004).

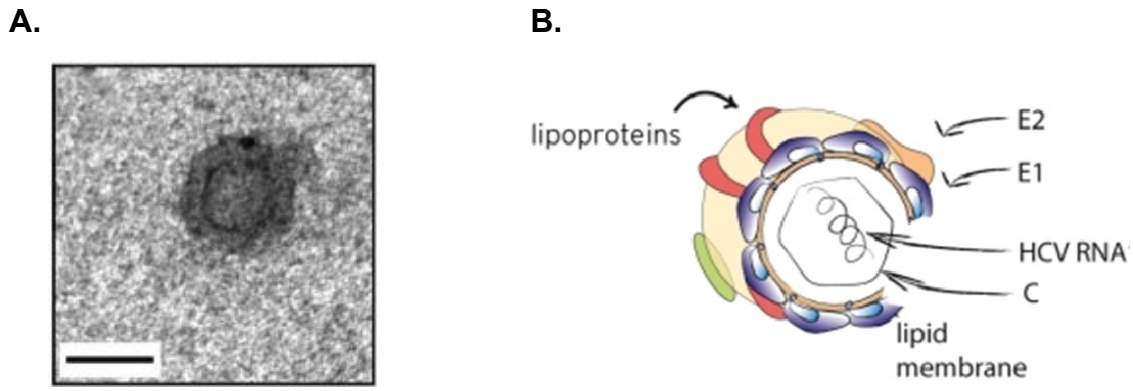

Fig. 1.1: The Hepatitis C Virus

A. Electron microscopic picture of a recombinant virus particle. The diameter corresponds to 50 nm (Wakita *et al.*, 2005). **B.** Schematic drawing of a HCV particle. The c stands for the viral core protein (nucleocapsid) [modified from:(Steinmann and Pietschmann, 2010)].

1.1.2 The genome organization

HCV contains a single-stranded, uncapped, positive-sense RNA molecule of 9.6 kilo bases (kb) in length. Translation of one single open reading frame (Pestka *et al.*, 2007) produces one large polyprotein of about 3000 amino acids (Bartenschlager *et al.*, 1993) which is proteolytically cleaved, by host and viral proteases, to yield ten viral proteins: core, E1, E2, p7, NS2, NS3, NS4A and NS4B and NS5A and NS5B (Fig. 1.2). The open reading frame is flanked by the most conserved regions of the genome; the 5' and 3' untranslated regions (UTRs) (Pestka *et al.*). The 5'-UTR is a well-conserved, 341 nucleotide sequence element that precedes the internal ribosome entry site (IRES) mediating translation of the polyprotein (Tsukiyama-Kohara *et al.*, 1992). Downstream of the coding region the approximately 200 nucleotides long 3'-UTR is located. It can be divided in 3 parts (Hsu *et al.*, 2003): The stop codon of the ORF is followed by a variable region (approx. 40 nucleotides (nt)), a poly-uracil track of 20 to 90 nucleotides and a highly conserved sequence of 98 nucleotides which is essential for replication (Kolykhalov *et al.*, 1996), (Yanagi *et al.*, 1999).

On the basis of the dissimilarity of nucleotide sequences, HCV can be classified into 7 major genotypes as well as into numerous subtypes. These 7 genotypes differ from each other by more than 30% - 35% at the nucleotide level leading to differences in disease progression, mode of transmission and worldwide distribution. The most common one is HCV genotype 1 which is also the most difficult to treat (Hnatyszyn, 2005). HCV is characterized by a very high sequence variability which arises from the error-prone RNA polymerase in combination with high selective immune pressure. Therefore, in an infected host HCV virions circulate in a large population of closely related but still distinct genetic variants or so called quasispecies (Simmonds, 2004).

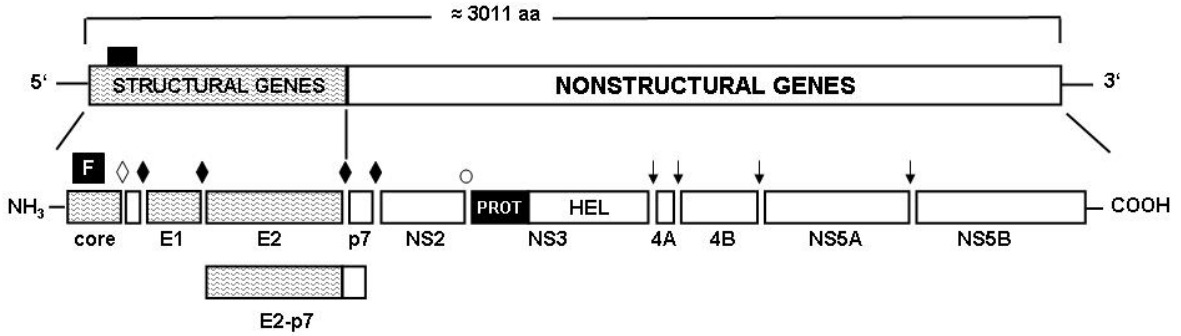

Fig. 1.2: Genomic organization of the HCV genome: polyprotein processing and cleavage products [modified from: (Knipe, 2007)]

Precursor and mature proteins generated by the proteolytic processing cascade are indicated by boxes below the genome. Nonstructural proteins are white, whereas structural proteins are colored shaded. The cleavage sites for host signalase ♦ and the viral serine protease (downward arrow) are indicated. The NS2/3 cleavage is mediated by the NS2 cystein autoprotease (open bullet). The frame shift protein is indicated by an "F" in a black box.

While the structural proteins of HCV are located at the amino-terminus (N-terminus) of the genome, the non-structural proteins are located at its carboxy-terminus (C-terminus). The first structural protein encoded by HCV is the core (c) protein which encapsidates and probably interacts with the genomic RNA (Baumert *et al.*, 1998).

HCV glycoproteins E1 and E2 are targeted to the endoplasmatic reticulum (ER) where they are released from the polyprotein by a host signal peptidase cleavage (Dubuisson *et al.*, 2002). E1 and E2 are type-I transmembrane glycoproteins of about 35 kilo Dalton (kD) and 70 kD (746 amino acids (aa)) respectively, with a large N-terminal extracellular domain and a C-terminal transmembrane domain. They assemble as noncovalent heterodimers (Deleersnyder *et al.*, 1997). The transmembrane domain of E1 and E2 contains two short (< 20 aa) hydrophobic stretches separated by fully conserved charged residues. The second hydrophobic stretch acts as an internal signal peptide for the downstream protein (Cocquerel *et al.*, 2002). The transmembrane domains are not conical but dynamic changes have been shown to occur in these domains after cleavage by the signal peptidase. Before cleavage the transmembrane domains adopt a hairpin structure while after cleavage, the signal-like sequence is reoriented towards the cytosol which in turn leads to a transmembrane passage (Cocquerel *et al.*, 2001).

The E1/E2 heterodimer is mainly retained in the ER, although when over-expressed the HCV glycoproteins can be detected at the plasma membrane (Rouille *et al.*, 2006), (Duvet *et al.*, 1998). In addition to a membrane-proximal heptad repeat sequence in E2, the determinants for ER retention have been mapped in the

transmembrane domains of E1 and E2 (Cocquerel *et al.*, 2001). Furthermore, it was shown that these domains are essential for E1/E2 heterodimerization (Op De Beeck *et al.*, 2000).
The ectodomains of E1 and E2 are heavily modified by N-linked glycans (up to 6 and 11 glycosylation sites, respectively) and contain multiple disulfide-linked cysteins (Bartenschlager *et al.*, 2004). Some glycans have been shown to be strongly conserved among HCV genotypes and are important for proper folding, secretion, assembly, and release of HCV particles (Goffard *et al.*, 2005).

The E2 glycoprotein contains a hypervariable region I (HVRI) which is encoded by the first N-terminal 27 residues (aa 384 to 410). In this region most likely the major neutralizing epitope of HCV is located (Kato *et al.*, 1992). Antibodies against this region are in principle neutralizing and until now are the only ones assumed to be protective (Farci *et al.*, 1996). The HVRI does not only show a high level of sequence variability (even within a single patient), which is largely driven by antibody selection of immune escape-variants, but also appears to play an important role in E2 function (Dubuisson, 2007). Thus, in the whole viral genome the highest sequence variability can be found in the HVRI. However, some functional constrains narrow this variability, which is evident since this region was recently shown to play an important role during virus entry (Penin *et al.*, 2001). The involvement of the HVRI in entry and the immune response during an HCV infection will be discussed in more detail in section 1.1.4 and 1.3.3.

A second hypervariable region, HVRII, has been described in the E2 protein which includes a stretch of 9 aa (position 474 to 482) (Weiner *et al.*, 1991) and has been proposed to modulate E2 host receptor CD81 binding (Roccasecca *et al.*, 2003).
Recently a third hypervariable region, HVRIII (aa 431 to 466), positioned in between HVRI and HVRII was integrated in the canonical model of E2 (Torres-Puente *et al.*, 2008), (Law *et al.*, 2008).

Following the structural region of the HCV polyprotein is a small integral membrane protein, p7 (Dubuisson, 2007). p7 is an ion-channel, that is required for HCV particle release (Stgelais *et al.*, 2009).

The remainder of the polyprotein consists of the nonstructural proteins NS2, NS3, NS4A, NS4B, NS5A and NS5B. NS2 is acting as a membrane protein which is essential for HCV RNA replication. (Blight *et al.*, 2000). The HCV NS3 is a multifunctional protein, serving as both a proteolytic enzyme and a helicase. Together with NS2 and NS4A, NS3 process the presumed NS region of the polyprotein. The C-terminal part of the NS3 protein contains the RNA helicase/nucleoside triphosphate hydrolase (NTPase) domain that is essential for translation and RNA replication (Bartenschlager *et al.*, 1993). The NS4A protein is a membrane protein, forming the membrane structure and supporting RNA replication (Egger *et al.*, 2002). NS4B is an integral membrane protein, that reorganizes the cellular membrane during replication (Liefhebber *et al.*, 2009). The NS5A phosphoprotein is important for RNA replication, and a potential involvement in suppression of PKR (protein kinase regulated) mediated antiviral cellular response is still discussed (Gale, Blakely et al. 1998). NS5B is the RNA-dependent RNA polymerase needed for viral replication (Dubuisson, 2007).

In addition to the large ORF encoding the polyprotein, the HCV genome contains an overlapping +1 reading frame which overlaps the sequence of the core protein (Branch *et al.*, 2005). This alternative reading frame protein (ARF) or frame shift protein (F protein) lacks an in-frame AUG stop codon with a frameshift efficiency of 1% to 2%. The role of the F protein in the HCV life cycle and/or pathogenesis remains unclear. However, the F protein is not required for HCV RNA replication. (Dubuisson, 2007)

1.1.3 The replication cycle

An infection with HCV starts with the binding of the viral particle to the cell surface (Fig. 1.3) which is belived to be facilitated by the interaction of E1 and E2 proteins with glycosaminoclycanes (GAGs), dendritic cell-specific intracellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), liver/lymph node-specific intracellular adhesion molecules-3 grabbing non-integrin (L-SIGN) as well as the binding of the virus to the (very)-low-density lipoprotein receptor ((V)LDL) (Sabahi, 2009). Binding of the latter receptor is presumably mediated by the apolipo-protein E which is an integral component of an infectious viral particle (Jiang and Luo, 2009). While this interaction at the surface of the hepatocyte only “fixes” the virus, 4 different

molecules are responsible for the ultimate infection: tetraspanin CD81, SR-B1 as well as both tight junction components Claudin-1 and Occludin (Popescu and Dubuisson, 2009). Due to the receptor-mediated endocytosis the virus reaches the endosomes. It is assumed that acidification of the endosomes alters the conformation of the viral envelope which interferes with the fusion of the viral lipid-envelope with the endosomal membrane. Up to now, it is only known that uncoating of the viral genome takes place at the cytoplasm of the rough ER where the viral genome is transcribed and the polyprotein is cleaved. Following the polyprotein processing, the proteins retain either directly or indirectly associated at the membrane of the ER (Welsch *et al.*, 2009). In an event not yet exactly understood, a so called “membraneous web” is formed. RNA replication takes place in invaginations of the membrane in which the viral genome is transcribed into minus-strand-copies. These copies are used as a template for new copies of the genome ((+) RNA) which in turn are utilized for the translation of new viral polyproteins, repeated synthesis of minus-strand-copies or for assembly of newly formed virus particles. Assembly of infectious particles occurs directly at or close to the lipid droplets which are integral components of the membraneous web (Moradpour *et al.*, 1996), (Barba *et al.*, 1997).

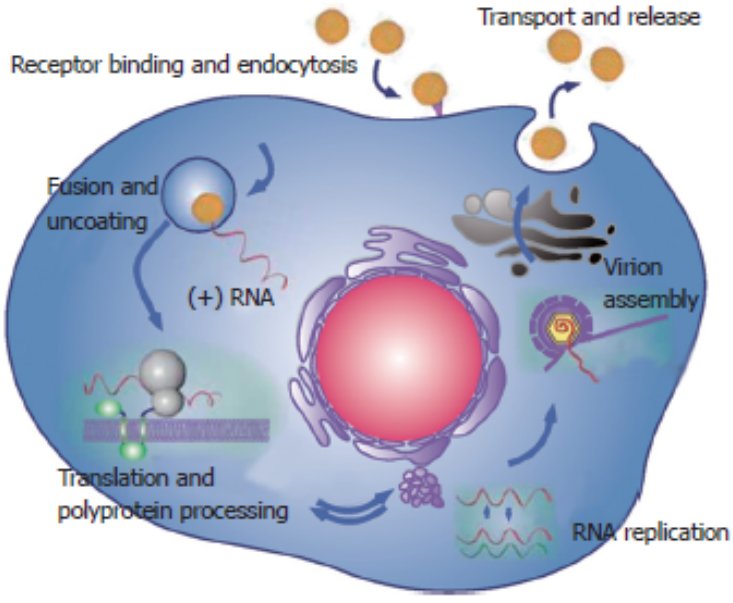

Fig. 1.3: Schematic representation of the major steps of the HCV replication cycle (Dubuisson, 2007)

Post infection of the cell, the viral RNA is delivered to the cytoplasm and translated. After processing of the proteins, the RNA is replicated in the replication-complex bound to the ER and packed into newly formed virions. The viral particles are then secreted.

1.1.4 The role of envelope proteins during entry

Virus entry is defined as the steps from particle binding to the host cell up to the delivery of the viral genome to its replication site within the target cell, which, in case of HCV, is the human hepatocyte. Viral entry is a complex multistep process based on specific interactions between virus components, mainly envelope proteins and multiple cellular factors (Burlone and Budkowska, 2009).

Virus receptors actively promote entry, whereas attachment factors serve to bind the particles and thus help to concentrate viruses on the cell surface (Dubuisson *et al.*, 2008). One of these attachment factors was identified as GAGs. The GAG heparan sulphate (HS), a glycosaminoglycan chain on cell surface proteoglycans, serves usually as attachment site for the binding of a number of viruses and other microorganisms (Barth *et al.*, 2003). Attachment of the virus to GAGs, which serve as 'primary', low affinity, but abundant receptors, may play an essential role in the early steps of HCV infection (Fig. 1.4). The pre-treatment of HCVpp (HCV pseudoparticle system) and HCVcc (HCV cell culture derived system) with heparinase (an enzyme degrading HS) inhibited the infectivity of Huh7 cells (Koutsoudakis *et al.*, 2006), (Basu *et al.*, 2007). However, the exact role of GAGs in HCV entry is still not completely revealed (Callens *et al.*, 2005).

CD81 was discovered early as an entry receptor (Pileri *et al.*, 1998) followed by the discovery of SR-B1 (Fig. 1.4) (Scarselli *et al.*, 2002). CD81 is ubiquitously expressed as a 25 kDa unglycosylated membrane protein belonging to the tetraspanin family (Levy and Shoham, 2005). Studies in non-permissive human hepatoma cell lines such as HepG2 and HH29, both not expressing CD81, confirmed the role of CD81 in HCV infections as these cells become susceptible to HCVcc and HCVpp infection upon ectopic expression of CD81 after transduction (Bartosch *et al.*, 2003a), (Zhang *et al.*, 2004), (Cormier *et al.*, 2004), (Lavillette *et al.*, 2005). Antibodies against CD81 and human recombinant CD81 LEL (large extracellular loop of CD81) inhibited HCV infection only after virus attachment. This and other studies suggest that CD81 acts as a post-binding entry molecule (Cormier *et al.*, 2004), (Flint *et al.*, 2006), (Koutsoudakis *et al.*, 2006). The cellular pathways triggered by HCV binding to CD81 were also elucidated. Engagement of CD81 plays a fundamental role in HCV infectivity through the activation of Rho guanosine triphosphate hydolase (GTP-ases)

and the actin-dependent relocalization of the E2/CD81 complex to cell-cell contact areas. Here CD81 comes in contact with the tight junction proteins Occludin and CLDN-1, two other HCV co-receptors (explained in more detail below). Finally, post-entry steps of the virus life cycle are affected by CD81 engagement activating the Raf/upstream mitogen-activated protein kinase (upstream MAPK, also called MEK) / extracellular signal-regulated kinase (also called MAPK) (Raf/MEK/ERK) signalling cascade (Brazzoli *et al.*, 2008).

SR-B1 is a 82 kDa glycoprotein with 2 cytoplasmic domains, 2 transmembrane domains and a large extracellular loop with 9 potential N-glycosylation sites. It is expressed in various mammalian cells, but is mostly expressed in the liver and steroidogenic tissues (Acton *et al.*, 1994), (Calvo and Vega, 1993), (Rhainds and Brissette, 2004). SR-B1 binds to various classes of lipoproteins, high-, low- and very-low-density lipoproteins (HDL, LDL and VLDL, respectively), as well as to chemically modified lipoproteins like oxidized and acetylated LDL and thus was referred to as being a 'multi-ligand' receptor (Fig. 1.4). Mouse SR-B1 does not bind sE2 which shows that SR-B1 appears to be species-specific. It was also shown that the deletion of the HVRI region of E2 impairs the interaction between SR-B1 and sE2 and reduces HCVpp infectivity (Scarselli *et al.*, 2002), (Bartosch *et al.*, 2003a). Furthermore, antibodies against SR-B1 also significantly reduce HCVpp infectivity (Bartosch *et al.*, 2003b). Similar to CD81, SR-B1 acts as 'post-binding' receptor which was demonstrated by antibodies inhibiting infection against both receptors when added until 60 min after virus binding (Cormier *et al.*, 2004), (Zeisel *et al.*, 2007). It was shown that HDL enhances the HCVcc infectivity only when CD81 is expressed suggesting that SR-B1 co-operatively interacts with CD81 in HCV cell entry (Fig. 1.4) (Zeisel *et al.*, 2007). In contrast to the enhancing effect, other natural ligands of SR-B1 like VLDL and oxidized LDL had significant inhibitory effects on serum HCV and HCVpp cell entry (Fig. 1.4) (Maillard *et al.*, 2006), (von Hahn *et al.*, 2006). The crucial role of SR-B1 in cell infection by HCV can be underlined by the finding that the antiviral action of interferon is linked to a decrease in the expression levels of SR-B1 on the cell surface, thereby restricting virus attachment and entry into hepatocytes (Murao *et al.*, 2008).

Tight junctions are major components of cell-cell adhesion complexes that separate apical from basolateral membrane domains and maintain cell polarity by forming an intramembrane (Shin *et al.*, 2006). The tight junction protein CLDN1, a member of the Claudin family, has also been identified as being involved in HCV entry (Fig. 1.4) (Evans *et al.*, 2007). It is composed of 211 aa with two extracellular loops, four transmembrane segments and three intracellular domains (Van Itallie and Anderson, 2006). Although CLDN1 is expressed in all epithelial tissues, it is predominantly expressed in the liver where it forms networks at tight junctions (Furuse *et al.*, 1998). HCV entry was shown to be likely mediated by the highly conserved domain in the first extracellular loop (EL1), as non-hepatic cell lines such as 293T and SW13 become susceptible to HCVpp infection when expressing CLDN1 (Evans *et al.*, 2007).

After showing that human cell lines such as HeLa and HepH (CD81- and SR-B1-positive) remained HCV-resistant when over-expressing CLDN1 it was suggested that additional factors are needed for a successful HCV entry (Evans *et al.*, 2007). Thereby another transmembrane component of the tight junctions, Occludin, was identified as a co-receptor for HCV (Fig. 1.4) (Liu *et al.*, 2009). Occludin is a 60 kDa protein with four transmembrane regions, two extracellular loops and N- and C-terminal cytoplasmic regions (Furuse *et al.*, 1993). After targeting CLDN1 and Occludin by siRNA and shRNA interference it was demonstrated that the reduction of the expression of both of these molecules inhibited HCVpp and HCVcc cell entry (Liu *et al.*, 2009). Moreover, it was shown that human Occludin renders murine cells infectable with HCVpp (Ploss *et al.*, 2009).

As already mentioned above, in the serum of an infected host HCV particles are mostly found to be associated with lipoproteins and most circulating particles are of low density due to their association with ß-lipoproteins (Thomssen *et al.*, 1992), (Prince *et al.*, 1996), (Agnello *et al.*, 1999). It was already shown that apolipoprotein B (ApoB) is associated with virus particles and plays a key role in the initial interaction of the receptor by serum-derived authentic HCV, as these virions did not recognize SR-B1 directly via viral envelope glycoproteins. In this context the lipoprotein receptor LDL-R was also shown to be involved in the HCV infection process (Fig. 1.4). The usual role of LDL-R is a sequestering of cholesterol in the form of LDL and VLDL from the circulation. The correlation of cell surface expression

of LDL-R in patients with chronic HCV infection and with a high viral load has demonstrated the involvement of LDL-R in the viral replication cycle (Petit *et al.*, 2007). Moreover, treatment of hepatocytes with monoclonal antibodies against LDL-R of LDL also inhibited HCV infection (Molina *et al.*, 2007).

The main lipoprotein component in HCV patient sera is the triglyceride-rich VLDL, produced and secreted by hepatocytes (Shelness and Sellers, 2001). Studies already demonstrated that HCV-associated lipoproteins are not simply absorbed at the surface of virions circulating in patient sera, but that VLDLs are an integral part of HCV particles (Nielsen *et al.*, 2006). This underlines the importance of VLDL in the assembly and secretion of infectious HCV particles (Fig. 1.4).

With regard to the current knowledge it is possible that HCV infection is initiated by the interaction between the lipoprotein-associated virus particle and lipoprotein receptors SR-B1 and/or LDL-R. In addition, cell surface proteoglycans facilitate infection, probably in a lipoprotein-dependent manner (Burlone and Budkowska, 2009).

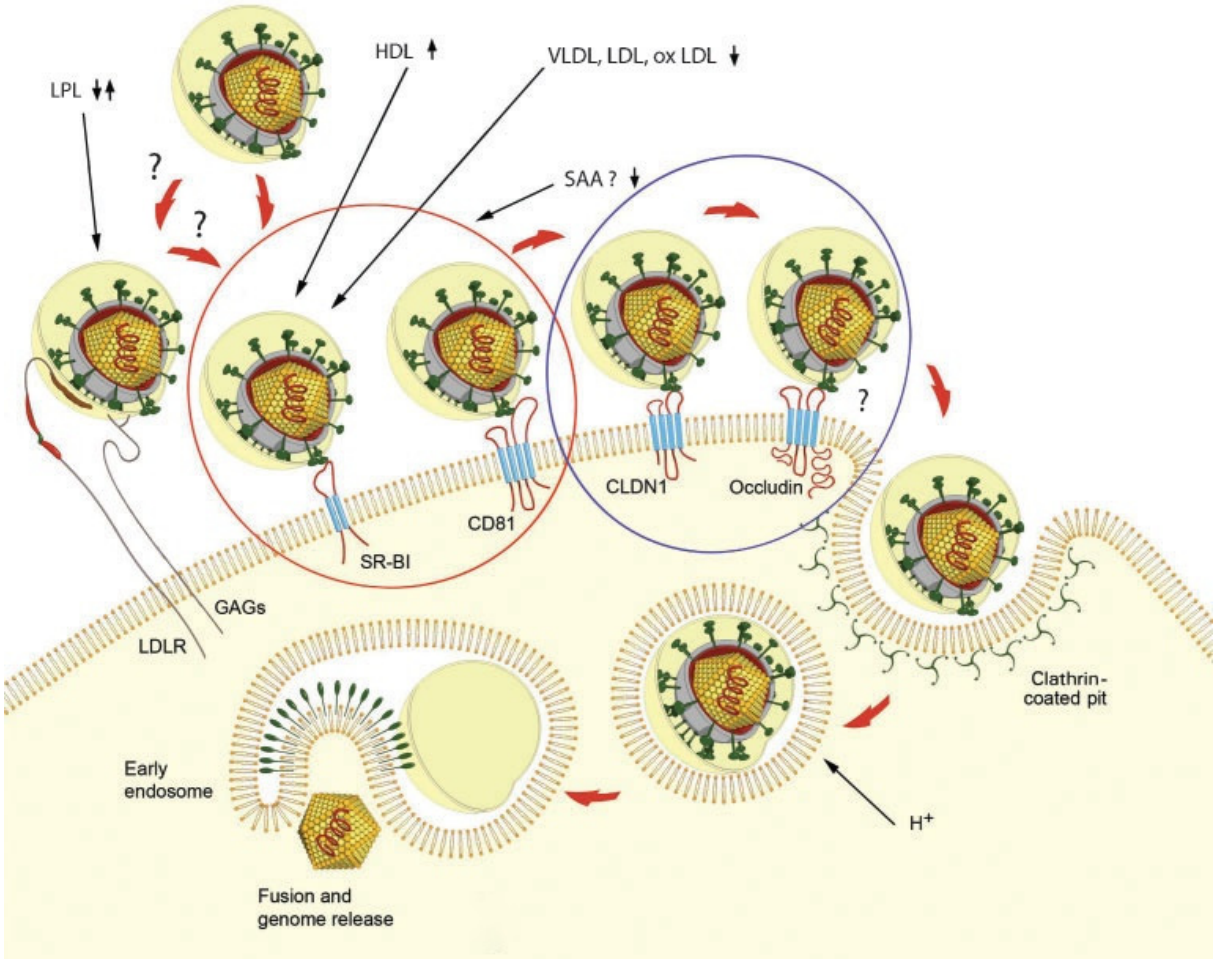

Fig. 1.4: Model of natural ApoB-associated HCV entry into to the hepatocytes [modified from: (Burlone and Budkowska, 2009)]

Virus binding and internalization is initiated by the interaction between HCV-associated lipoproteins (mainly VLDL) with lipoprotein receptors SR-B1 and/or LDL-R and/or GAGs. HCV cooperates with the SR-B1–CD81 complex and the virus is subsequently transferred by CD81 to tight junction proteins CLDN-1 and occludin. Virus enters the cell from the tight junction via endocytosis and fusion is mediated by envelope glycoproteins; this event permits the virus to escape the lipoprotein degradation pathway. Lipoprotein-mediated HCV cell entry is inhibited by natural ligands of lipoprotein receptors such as VLDL, LDL and oxidized LDL. Cell entry can also be inhibited by serum amyloid A (SAA, an acute phase protein produced primarily by hepatocytes during infection), enhanced by HDL and regulated by LPL. This model corresponds to the cell entry of HCVcc and natural HCV from patient sera, which are associated to various extents with ApoB-containing lipoproteins (Burlone and Budkowska, 2009).

Fewer is known about the interaction of the envelope proteins E1/E2 with the cellular receptors during entry. However, studies using HCVpp provided evidence for the involvement of the E1/E2 heterodimer in virus entry, as HCVpp expressing E1 or E2 separately are non-infectious (Drummer *et al.*, 2003), (Bartosch *et al.*, 2003a). In addition, E2 has been shown to interact with CD81 (Pileri *et al.*, 1998), SR-B1 (Scarselli *et al.*, 2002), DC-SIGN and L-SIGN (Gardner *et al.*, 2003), (Pohlmann *et al.*, 2003), and glycosaminoglycans (Barth *et al.*, 2003). Experiments indicated that at least CD81 and SR-B1 play a role in HCVpp entry (Bartosch *et al.*, 2003a), (Bartosch

et al., 2003b), (Hsu *et al.*, 2003), (Lavillette *et al.*, 2005), (Zhang *et al.*, 2004). Furthermore, the CD81 binding region of E2 is required to be correctly folded for the interaction between the virus and CD81 and SR-B1 (Flint *et al.*, 1999), as several regions in E2 have been identified to be engaged in CD81 binding (Flint *et al.*, 1999), (Owsianka *et al.*, 2001), (Clayton *et al.*, 2002). Moreover, specific amino acid residues have been identified (W420, Y527, W529, G530 and D535) which are critical for CD81 binding and thus are conserved across all genotypes (Owsianka *et al.*, 2006).

Beside these binding regions more and more is reported about the modulation of the accessibility of HVRI to either SR-B1 or CD81, since deletion of HVRI increased binding to CD81 but abrogated binding to SR-B1 (Scarselli *et al.*, 2002), (Roccasecca *et al.*, 2003). The conformation and structural properties of HVRI are highly conserved and HVRI is a globally basic stretch, with basic residues located at specific sequence positions, suggesting that these residues within HVRI might affect the interaction of E2 with molecules involved in HCV entry (Penin *et al.*, 2001), (Callens *et al.*, 2005). Indeed it was shown that in the absence of basic residues, infectivity was reduced to the same level as that of a mutant deleted of HVRI. In addition, HCVpp infectivity increased with the number of basic amino acids in HVRI, and the presence or absence of basic residues at specific positions modulated HCVpp infectivity (Callens *et al.*, 2005).

Up to now it is still unclear how HCV actually associates with lipoproteins and which viral determinants modulate this interaction. However, the recent observation of Bankwitz *et al.* showed that the deletion of HVRI reduces the number of VLDL-HCV particles which suggests that HVRI may influence the interplay between HCV lipids or lipoproteins. Furthermore, it was observed that soluble CD81 (hCD81-LEL) is able to neutralize and precipitate ΔHVRI particles much more readily than wild-type particles. These data indicate that HVRI masks the viral CD81 binding site. It is possible that until the interaction with a host factor which elicits a conformational change, fully exposing the CD81 binding site, the conserved CD81 binding site in wild-type particles is mostly hidden, thus preparing the virus for contact with this crucial receptor. This kind of strategy would be in line with the interplay of HIV-1 with CD4 and the chemokine receptors (Bankwitz *et al.*, 2010). HVRI has been reported as a target for neutralizing antibodies (nAbs) (Farci *et al.*, 1996) which would be reminiscent with the above mentioned strategy which may facilitate successful

evasion from neutralizing immune response through protection of conserved viral epitopes necessary for essential receptor interactions (Bankwitz *et al.*, 2010).
Because the HVRI seems to be important for virus replication and persistence in several ways; it is involved in assembly and release of virus particles with optimal composition, it influences the HCV membrane fusion process, and this domain physically protects a conserved neutralizing epitope (the viral CD81 binding site). This knowledge should be used for devising vaccine formulations that induce potent neutralizing antibody responses (Bankwitz *et al.*, 2010).
The role of E1 in HCV infection remains poorly understood, but it appears to be involved in the fusion process (Garry and Dash, 2003), (Lavillette *et al.*, 2007).

1.2 Model systems of HCV infection

The only species in which both, early antiviral immune responses and pathogenesis of HCV can be studied is the chimpanzee. Because of the extremely narrow species tropism of HCV, all rodent models require xenografting of human cells and constitutive lack of immune rejection toward these engrafted cells (Boonstra *et al.*, 2009).

1.2.1 in vitro models

To study the complexity of the HCV life cycle, a series of *in vitro* models were developed over the last decades. The two most important systems frequently used, are described in the following:

The HCV pseudoparticle-system (HCVpp):
This very successful cell-based model system is based on pseudotyping of particles produced by other viruses, including lentiviral or retroviral core particles, with HCV E1 and E2 glycoproteins (Lagging *et al.*, 1998), (Drummer *et al.*, 2003), (Bartosch *et al.*, 2003a), (Lavie *et al.*, 2007). For most assembly platforms of HCVpp, Murine Leukemia Virus (MLV) or Human Immunodeficiency Virus (HIV) vectors were used because their cores can incorporate a variety of different cellular and viral glycoproteins (Ott, 1997), (Sandrin *et al.*, 2002). Furthermore, they can easily package and integrate genetic markers into DNA of infected cells (Negre *et al.*, 2002). Monitoring of viral infection is possible due to the pseudoparticles expressing

E1/E2 at their surface and packaging of a reporter gene. HCVpp are produced by transfection of human embryonic kidney 293T cells with expression vectors encoding the E1/E2 polyprotein, the retroviral Gag-Pol core proteins, and a packaging-competent retroviral derived genome encoding a reporter gene like the green fluorescent protein (GFP) or the firefly luciferase gene (Fig. 1.5). The produced HCVpp are infectious for cell lines of hepatocyte origin, principally for Huh7.5 cells and their derivatives as well as for human primary hepatocytes (Bartosch *et al.*, 2003a), (Hsu *et al.*, 2003).

HCVpp can mimic the early steps of HCV infection, because they exhibit a preferential tropism for hepatic cells and they are specifically neutralized by anti-E2 monoclonal antibodies as well as sera from HCV-infected patients (Bartosch *et al.*, 2003a), (Hsu *et al.*, 2003), (Op De Beeck *et al.*, 2004). Furthermore, HCVpp led to the identification of multiple viral entry receptors, such as glycosaminoglycan, low-density lipoprotein receptor, DC-SIGN, L-SIGN, Claudin-1, -6 and -9, and Occludin (von Hahn and Rice, 2008), (Ploss *et al.*, 2009). Therefore, HCVpp represent the best tool available to study functional HCV envelope glycoproteins (Flint *et al.*, 2004).

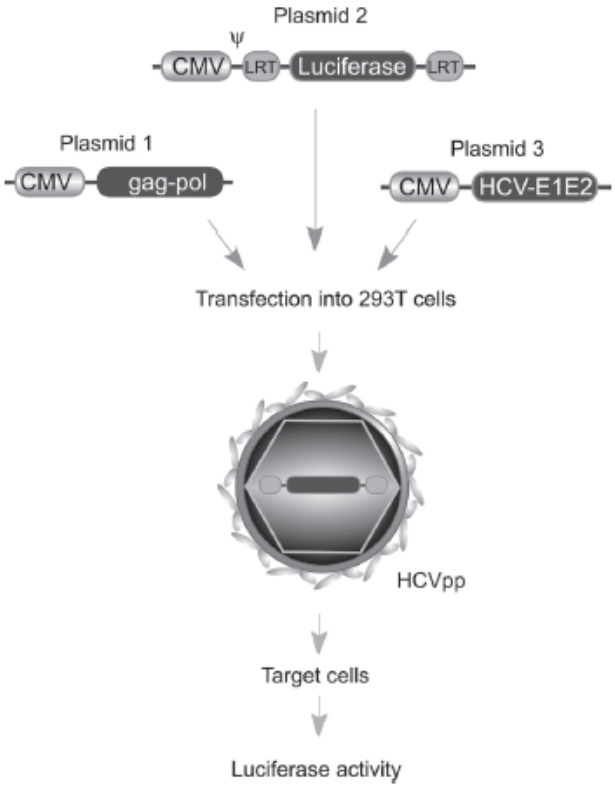

Fig. 1.5: Production of HCV pseudoparticles (HCVpp) (Lavie *et al.*, 2007)

293T cells are transfected with three expression vectors. The first vector encodes retroviral Gag andPol protein. Gag proteins are responsible for particle budding at the plasma membrane and RNA encapsidation via recognition of the specific retroviral encapsidation sequence (Ψ). The second vector harbors a Ψ sequence for encapsidation and encodes a reporter protein (Luciferase). This vector also contains retroviral sequences that are necessary for the reverse transcription of genomic RNA into proviral DNA by retroviral protein Pol encoded by the first vector. The third vector encodes HCV envelope glycoproteins, which are responsible for the cell tropism and fusion of HCVpp with the target cell membrane (Lavie *et al.*, 2007).

The HCV cell-culture derived virus-system (HCVcc)

The finding of an HCV subgenomic and, a few years later, a full genomic replicon system led to completely new opportunities to study different aspects of HCV replication. HCV replicons became extremely valuable in many laboratories worldwide (Lohmann *et al.*, 1999), (Blight *et al.*, 2002), (Lohmann *et al.*, 2003), (Blight *et al.*, 2003). However, the disadvantage of these replicons was despite their relatively high rates of HCV RNA replication their inability to produce infectious virions. A different situation emerged when the first genotype 2a consensus genome was established (Kato *et al.*, 2001), (Kato *et al.*, 2003). A subgenomic replicon that did not require adaptive mutations for replication *in vitro* and replicated up to 20-fold higher in Huh7 cells was constructed from a clone called JFH-1, isolated from a Japanese patient with fulminant Hepatitis C (Wakita *et al.*, 2005). This system was

based on the transfection of Huh7.5.1 cells with the *in vitro*-transcribed full length JFH-1 genome or a recombinant chimeric genome with another genotype 2a isolate, J6. Transfection resulted in the secretion of viral particles that were infectious in cultured cells, chimeric mice, and in chimpanzees, thus referred to as a cell-culture derived virus or HCVcc (Wakita *et al.*, 2005), (Zhong *et al.*, 2005), (Lindenbach *et al.*, 2005), (Lindenbach *et al.*, 2006). Neutralization of infectivity of cells could be achieved by antibodies against the HCV receptor CD81, antibodies against E2, or immunoglobulins from chronically infected patients. Furthermore, in this system IFN-α as well as several HCV-specific antiviral compounds were able to inhibit replication of cell-cultured HCV (Lindenbach *et al.*, 2005). Up to now, chimeric JHF-1-based genomes have been constructed of all seven known HCV genotypes (Pietschmann *et al.*, 2006), (Gottwein *et al.*, 2009). Thus, the HCVcc system provides an excellent opportunity to study the complete HCV life cycle.

1.3 The immune response during an HCV infection

1.3.1 The innate immune response

In the early phase of an HCV infection the innate immune response is induced. The first response is thought to be type I interferon (IFN-α/ß) production by infected hepatocytes and plasmacytoid dendritic cells (pDC) which is initiated by the pattern recognition receptors Toll-like receptors (TLR3) and retinoic-acid-inducible gene I (RIG-I) (Saito *et al.*, 2008). Natural killer (NK) cells which are frequently found in the liver get also activated by type I IFN and are able to rapidly exert cytotoxicity and release cytokines. This process initiates hepatitis (Ahlenstiel *et al.*, 2008). In turn the destruction of hepatocytes stimulates myeloid DCs (mDC). These DCs subsequently promote the secretion of IFN-γ through the activation of NK cells and natural killer T-cells (NKT). IFN-γ then activates hepatic macrophages to enhance local inflammation (Racanelli and Rehermann, 2006). However, the virus has evolved several mechanisms to attenuate IFN response and is thus able to persist within the liver. One of the key players is the HCV NS3/4A protein (Foy *et al.*, 2003), (Li *et al.*, 2005).

1.3.2 The cellular immune response

Although the innate immune response can be observed relatively early in HCV infection, the adaptive cellular response seems to be essential for viral clearance. The acute phase of an HCV infection endures approximately 6 month post infection. While the viral load in the serum increases exponentially during the first weeks, the first T-cells can only be detected in the periphery after four to eight weeks (Thimme *et al.*, 2001). $CD8^+$ T-cells are detectable in the blood of acutely infected patients regardless of virological outcome (Kaplan *et al.*, 2007). Spontaneous clearance of the virus in the acute phase of infection is associated with a long lasting vigorous T-cell response which is accompanied by the intrahepatic production of cytokines (Cooper *et al.*, 1999), (Lechner *et al.*, 2000). Subsequently to antigen contact, $CD8^+$ T-cells produce IFN-γ and TNF-α in the blood and in the liver and develop cytotoxic activity which by means of perforin and granzyme induces Fas-mediated apoptosis (Grakoui *et al.*, 2003). In parallel to a vigorous $CD8^+$ T-cell response a strong $CD4^+$ T-cell response also seems to be essential for viral clearance. These cells are required for the induction and maintenance of virus-specific $CD8^+$ T-cells (Grakoui *et al.*, 2003).
Chronic infection is associated with weak or no detectable $CD4^+$ and $CD8^+$ T-cell responses (Wedemeyer *et al.*, 2002), (Cox *et al.*, 2005). Detectable cells show only decreased cytotoxicity, cytokine secretion and proliferation (Ulsenheimer *et al.*, 2003). Reasons for the impaired T-cell response seem to be dysfunction or an increasing exhaustion during presence of high viremia. Mechanisms causing the T-cell failure include HCV escape mutations (Timm *et al.*, 2004), induction of regulatory T-cells (Sugimoto *et al.*, 2003), or the increased expression of the co-receptor programmed death-1 (PD-1) on T-cells (Rutebemberwa *et al.*, 2008).

1.3.3 Envelope proteins and the humoral immune response

The immune system of the host recognizes viral proteins as non-self and induces the production of antibodies (Abs). During the natural course of infection, Abs targeting both structural and non-structural proteins are produced. Only a small fraction of Abs demonstrates antiviral activity and is able to bind viral particles. These Abs are directed against epitopes that play an essential role in virus entry and are referred to as “neutralizing Abs” (nAbs) (Keck *et al.*, 2008a). Antibody binding can lead to virus neutralization which is mediated by aggregation of virus particles, preventing virus

entry through steric hindrance (Burton *et al.*, 2001). Virus neutralization will be higher with high affinity antibodies, whereas non-neutralizing Abs do not bind to the virion surface or bind with low affinity only (Houghton, 1996).

The role of the humoral immune response in controlling HCV infection remains controversial, as patients with persistent infection develop high titers of neutralizing antibodies that do not appear to clear the infection. Moreover, once chronic HCV infection is established nAbs increase in titer and breadth, typically exhibiting cross-reactivity against multiple HCV genotypes (Logvinoff *et al.*, 2004). At this stage nAbs continue to exert selection pressure on viral variants and contribute to the evolution of HCV envelope sequences throughout the course of infection but they fail to clear the virus (Farci *et al.*, 2000), (von Hahn *et al.*, 2007). In chronic HCV the overall concentration of Immunoglobulin G (IgG) and the frequency of IgG-secreting B-cells is also increased. Most of these Igs, and the B-cells that secrete them, are not HCV specific. Therefore it was assumed that HCV stimulates B-cells in a B-cell receptor-independent manner (Racanelli *et al.*, 2006).

On the other hand, HCV can be cleared without humoral immune response in immunocompromised (e.g., hypogammaglobulinemic) patients (Semmo *et al.*, 2006). In immunocompetent patients, nAbs appear late and are isolate-specific (Pestka *et al.*, 2007), (Dowd *et al.*, 2009). However, in a single-source outbreak of HCV involving multiple subjects, HCV clearance was associated with rapid induction of neutralizing antibodies in the early phase of infection. In contrast, in the same cohort of patients it was shown that chronic HCV infection was characterized by absence or low-titer of nAbs in the early phase of infection (Pestka *et al.*, 2007).

About 20 to 25% of patients in the acute phase of the disease are able to clear viremia. A study of intravenous drug users, in which a comparison of patients without evidence of previous HCV infection (HCV Ab negative and RNA negative) with those of previously infected (HCV Ab positive and RNA negative) was performed, showed that individuals with evidence of a previous infection were 12 times less likely than those with first time exposure to develop HCV persistence, suggesting that humoral immunity can prevent disease progression (Mehta *et al.*, 2002).

These findings suggest that a strong broad nAb-response may contribute to control HCV in the acute phase of infection and moreover points to the use of nAbs for therapeutical purpose.

The involvement of the complete E2 protein:

The majority of nAbs generated during acute infection have been mapped to the envelope glycoproteins E1 and E2 (Kato *et al.*, 1993), (Owsianka *et al.*, 2005; Keck *et al.*, 2008b; Meunier *et al.*, 2008). As described in section 1.1.4 it was already demonstrated that E2 is critical for host cell entry and represents an important target for virus neutralization. Several discontinuous regions of E2 contain highly conserved residues involved in CD81 binding and are targeted by nAbs (Owsianka *et al.*, 2006). The mouse monoclonal antibody (mAb) AP33 and the rat mAb 3/11 have broad neutralizing activity that can be attributed to the importance of the targeted region in CD81 binding and the extreme conservation of their epitopes (Owsianka *et al.*, 2005), (Tarr *et al.*, 2006) (Sabo *et al.*, 2011).

E1/E2 N-glycosylation sites are highly conserved across all genotypes (>97%), suggesting that the glycans associated with these proteins play an essential role in the HCV life cycle (Helle *et al.*, 2007). Recently experiments in the HCVcc system demonstrated that at least five glycans on E2 (E2N1, E2N2, E2N4, E2N6 and E2N11) reduce HCVcc sensitivity to neutralization. This indicated that glycans limit the recognition of neutralizing epitopes at the surface of E2 (Helle *et al.*, 2010). Indeed, the absence of one of these glycans leads to a higher sensitivity to neutralization by Abs purified from HCV seropositive patients, as well as mAbs (Helle *et al.*, 2007), (Falkowska *et al.*, 2007).

The HVRI and its involvement in the humoral immune response:

Early studies in chimpanzees showed that the onset of acute infection could be delayed with IgG therapy (Krawczynski *et al.*, 1996) and HVRI-specific antibodies are able to protect them partially from infection with HCV that encodes the immunizing HVRI (Lee, Suh et al. 1997), (Farci *et al.*, 1996), (Esumi *et al.*, 1999). Also in humans, early induction of HVRI-specific antibodies correlates with viral clearance and Abs from chronically infected patients are directed against the HVRI (Rosa *et al.*, 1996), (Owsianka *et al.*, 2001). In chimpanzees, a deletion of HVRI within an HCV genome was infectious, although the resulting virus was highly attenuated (Forns *et al.*, 2000). Thus, the HVRI region of E2 was identified as being the major target for nAbs. HVRI possesses multiple linear epitopes between aa 384 and 410. *In vivo* antibodies targeting HVRI have been identified (Weiner *et al.*, 1992), (Kato *et al.*, 1993), (Kato *et al.*, 1994), however these Abs tend to be highly strain-specific (Shimizu *et al.*, 1996; Vieyres *et al.*, 2011). A study on the structural conformation of

HVRI showed either a broad amino acid repertoire at each position despite a remarkable residue conformation in specific sites or replacements with amino acids with similar physicochemical properties, usually positively charged basic residues, in the most variable parts. A substantial conformational conservation was revealed by the very similar hydropathy and antigenicity profiles of HVRI variants. These data provide a plausible explanation for the extensive cross-reactivity demonstrated by Abs and confirming the existence of an active selection process (Penin *et al.*, 2001). The epitopes recognized by HVRI-specific mAbs that also show neutralizing capacity have been mapped within the C-terminal portion of HVRI (aa 396-407) (Hsu *et al.*, 2003), (Vieyres *et al.*, 2011). In contrast, the non-neutralizing mAbs bind to the N-terminal portion of HVRI (aa 384-395) (Hsu *et al.*, 2003). Thus, it appears that there are two immunogenic regions within the HVRI, with the C-terminal portion containing the neutralizing determinants.

As already mentioned earlier it was shown that the HVRI is also able to mask nAb epitopes within E2. A panel of human mAbs and patient sera, targeting the CD81-binding site within the E2 protein, made HVRI deletion mutants much more susceptible to neutralization (Bankwitz *et al.*, 2010), (Prentoe *et al.*, 2011). This is likely due to masking of the CD81-binding site, as HVRI may function to protect viral entry determinants within E2 against neutralization during early stages of entry (Bankwitz *et al.*, 2010). Thus initial contact with SR-B1 may be needed to unmask the CD81- binding region on E2 and enable the particle to interact with CD81. It has been suggested that the lipoproteins associated with the viral particle interact with this entry factor, although also a direct interaction between HVRI and SR-B1 could take place (Maillard *et al.*, 2006). Accordingly, HVRI may act as an immunological decoy that shields conserved neutralizing epitopes and stimulates a strong Ab response towards HVRI that does not result in viral clearance, but instead drives the selection of antibody-escape mutants (Ray *et al.*, 1999).

Other mechanisms used by HCV to escape from humoral immune response:

An additional strategy used by HCV may represent lipid shielding to evade antibody response. As already mentioned earlier, in patient sera low density particles of HCV are the most infectious, and recent data suggest that key neutralizing epitopes are less accessible on LVPs which are associated with VLDL such as apoB and apoE. Furthermore, several *in vitro* studies have demonstrated that HDL components of human serum (such as apoCI) can enhance the infectivity of HCVpp and HCVcc via

an HVRI-dependent mechanism (Meunier *et al.*, 2005), (Bartosch *et al.*, 2005), (Dreux *et al.*, 2007). HDL can also reduce the sensitivity of HCVpp to Ab neutralization which probably takes place by accelerating the entry of HCV via SR-B1-mediated lipid uptake (Dreux *et al.*, 2006). And ApoCl is able to further enhance infectivity by promoting fusion between viral and cellular membranes (Dreux *et al.*, 2007). Thus, lipids clearly play a crucial role in the infectivity and entry of viral particles as well as the neutralization sensitivity.

More recently, HCV has been found to be capable of direct cell-to-cell transmission, which is largely resistant to Ab neutralization (Timpe *et al.*, 2008), (Witteveldt *et al.*, 2009). Nevertheless, some antibodies could be detected that are able to partially inhibit cell-to-cell transmission. Cell-to-cell transmission requires all four entry receptors: CD81, SR-B1, Claudin1 and Occludin. However, SR-B1 appears to be particularly important (Brimacombe *et al.*, 2011). The exact mechanism of HCV cell-to-cell transmission is still unknown, nevertheless this route of virus transmission represents an ideal method of immune evasion and may explain why nAbs do not always clear the virus. Moreover, many other enveloped viruses, including Herpes Simplex Virus 1, Human T-cell Lymphotropic Virus and Measles Virus, also utilize this way to evade the host immune response (Mothes *et al.*, 2010). However, as mentioned before it was already demonstrated that HCV cell-to-cell transmission can be limited by antibodies, notably those targeting HVRI (Brimacombe *et al.*, 2011).

Lastly, the presence of non-nAbs in patient sera has been postulated to bind distinct epitopes within E2 and block or inhibit the binding of nAb to neutralizing epitopes (Zhang *et al.*, 2007), (Zhang *et al.*, 2009).

Involvement of the E1 protein:

Antibodies targeting epitopes within the E1 protein are generally rare, as they can be detected in only a few patient sera (Pestka *et al.*, 2007). This might be due to technical difficulties in detecting E1 responses which appear because of protein misfolding (unless co-translated with E2) (Dubuisson *et al.*, 1994). Alternatively, the presence of E2 might mask E1 epitopes or be immunologically dominant (Garrone *et al.*, 2011).

As a result, the failure of nAbs in controlling HCV infection could be caused by several different factors. In an infected individual HCV can rapidly evolve into many quasispecies outpacing the nAb response; surrounding cells can be infected by HCV

through cell-to-cell contact which avoids the exposition to nAbs; virion-associated lipoproteins and glycans may protect the envelope glycoproteins from nAbs; and due to the possible enhancement of virus entry by HDL the time window during which Abs can bind to and neutralize the virus is reduce (Zeisel *et al.*, 2008), (Angus and Patel, 2011). However, all the present findings about the involvement of E2 in the humoral immune response are required to be fully elucidated in the future. Nevertheless, the importance of HVRI in this context was already clearly demonstrated. To overcome the natural diversity of a virus such as HCV, the generation of broadly reactive antibodies may represent a useful approach, suggesting that mimotope-based vaccines can be used as potentially effective HCV immunogens.

1.4 Vaccination trials in HCV infection

Developing a prophylactic vaccine against HCV is a complex task concerning all the above mentioned mechanisms of HCV to evade from immune response. However, since HCV infection can be cleared by an appropriate immune response, numerous HCV vaccine approaches have been carried out with varying degrees of success over the last decade. Four main vaccine strategies have been investigated in human clinical studies: recombinant protein vaccines, peptide vaccines, DNA vaccines and vector vaccines. The details of this studies are reviewed in (Halliday *et al.*, 2011). However, the most promising vaccine approaches for HCV include virus-like particle (VLP) or rather capsid-like particle (CLP)-based vaccines that have been successfully applied for viral infections such as hepatitis B (Hilleman, 2001), (Kao and Chen, 2002). CLPs are multimeric structures that lack the viral genome but mimic the organization and conformation of authentic native viruses. In case of HCV it was already assumed that induction of a vigorous, long-lasting, and cross-reactive antiviral antibodies as well as a multispecific cellular immune response that includes both helper and cytotoxic T-lymphocytes are necessary for an effective HCV vaccine (rev. by (Lechmann and Liang, 2000), (Shiina and Rehermann, 2006), (Inchauspe and Michel, 2007), (Mikkelsen and Bukh, 2007), (Lauer and Chung, 2007), (Strickland *et al.*, 2008), (Thimme *et al.*, 2008)). Thus, CLPs bearing the HCV epitopes would be attractive candidates for the role of such a potent protein-containing immunogen, which should be able to induce vigorous humoral and cellular immune responses. The most promising variant of such CLP is the Hepatitis B Virus core antigen (HBcAg) (Pumpens and Grens, 2001), which will be described in more

detail in the next section. However, a major intrinsic advantage of recombinant HBc particles is their improved immunogenicity due to formation of a covalent link between B- and helper T-cell epitopes (Th-epitopes), and the ability of HBcAg to act as both T-cell dependent and independent antigen. As a result, the HBc particles induce high titres of antibodies and vigorous T-cell proliferative responses. Furthermore, HBc-CLPs show a high epitope density on a well organised particle which means that foreign epitopes can be exposed to the surface and the correct folding of the monomeric proteins results in preservation of conformational antigenic determinants. Moreover, it is feasible to simultaneously insert different epitopes at two and, probably, three different positions. Finally, it is relatively easy to generate and purify recombinant HBc-CLPs to vaccine quality (Pumpens and Grens, 2001). A CLP approach could create new and very promising possibilities for the development of a prophylactic vaccine to HCV.

In most cases vaccines are administered mixed with adjuvants, which is also the case in pre-clinical studies using animals. Adjuvants are substances that enhance the immune response to an antigen with which it is mixed (Murphy *et al.*, 2008). By promoting rapid, long-lasting and broad immunity this leads to an enhanced protection provided by the vaccine (Pulendran and Ahmed, 2006). A lot of different adjuvants have been developed, each stimulating an immune response by its specific action. Due to their actions adjuvants can be classified into: depot/carrier-, immunostimulation and carrier-, and immunostimulation-adjuvants (Guy, 2007). Two prominent examples belonging to the class of immunistimulating and carrier-adjuvants are, Incomplete Freunds Adjuvant (IFA) and the adjuvant AS03 which are water-in-oil and oil-in water-emulsions, respectively. IFA is often used in immunization trials in mice while AS03 is an approved adjuvant for human use, applied for instance in the human influenza vaccine (H5N1 A/Vietnam/1194/04 split virus + $AS03_A$) (Leroux-Roels *et al.*, 2007). While water-in-oil emulsions (like IFA) are believed to stimulate a T_H1-response, oil-in-water emulsions (like AS03) are known to induce T_H2-responses (Guy, 2007). These two adjuvants were also used in the immunization trials in the current study.

1.5 The Hepatitis B Virus

The Hepatits B virus (HBV) is the causative agent of acute and chronic hepatitis in humans. According to the WHO, about 2 billion people worldwide have been infected of which approximately 350 million chronically (WHO, 2011a). However, in contrast to HCV, there is already a prophylactic vaccine for HBV available, consisting of viral surface proteins produced in recombinant yeast. HBV is the main representative of *Hepadnaviridea*. The name of this family implies hepatotrophic DNA viruses which show tropism of the liver and posses a circular, partially double stranded DNA as genetic material. The molecular biology of HBV is reviewed in detail in (*World J Gastroenterol* 2007 January 7;13(1): 22-38), (Beck and Nassal, 2007), (Nassal and Schaller, 1996). In short, HBV consists of 7 proteins: 3 envelope proteins (L-, M-and S-protein), the polymerase (P-protein), the pre-core protein, the core protein (HBc), and the x-protein. The HBc protein is described in more detail in the next section.

1.5.1 The HBc Protein

The HBc protein of HBV can be divided into two parts (Fig. 1.6). Firstly, an assembly domain consisting of the first 140 amino acids which form the protein shell of the nucleocapsid (Birnbaum and Nassal, 1990). This region also includes the immunodominant c/e1 epitope which is targeted by the most antibodies. Secondly, the C-terminus of the core protein (starting from amino acid 150) which covers the nucleic acid binding domain that binds the viral RNA.

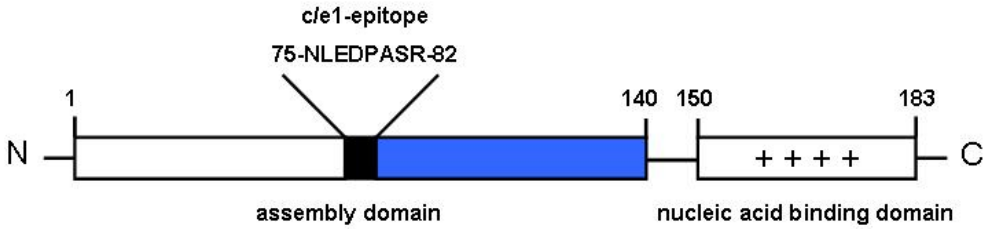

Fig. 1.6: Primary sequence of the HBV core protein

The assembly domain contains amino acids 1-140. The immunodominant c/e1 epitope spans amino acids 75-82. A short linker sequence (aa 140-150) links the assembly domain with the nucleic acid binding domain. The nucleic acid binding domain is characterized by a large amount of basic amino acids (++++).

The core protein is largely helical with two large central helices (Fig. 1.7 A & B.). The central helices are connected by a flexible loop that contains the c/e1-epitope. When

two core proteins form a dimer the central helices of each core protein form a four-helix bundle that protrudes as spikes from the capsid surface (Wynne *et al.*, 1999). HBV capsids consist of 240 core protein monomers arranged in icosahedral symmetry (Fig. 1.7 C.).

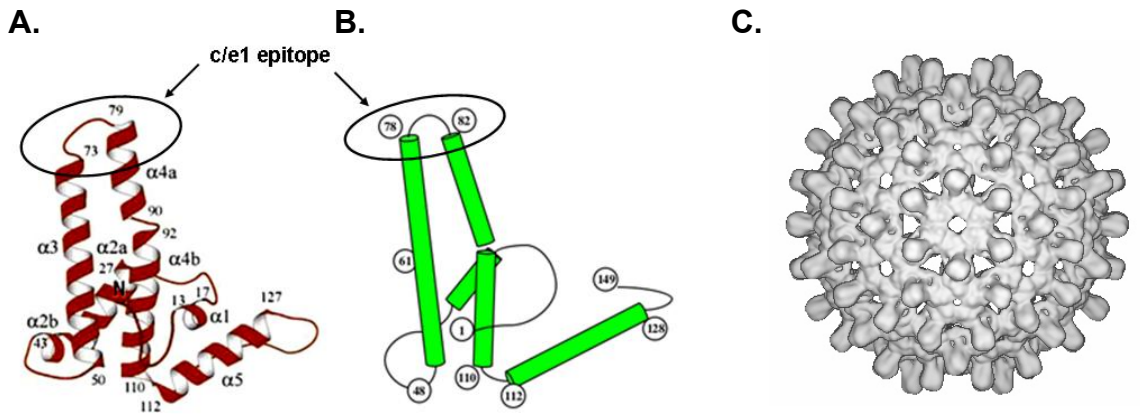

Fig. 1.7: Structure of the HBV-core protein and the HBV-capsid

A. Tertiary structure of the core monomer obtained from x-ray cristallography (Wynne *et al.*, 1999). **B.** Structure of the core monomer obtained from cryo-electonmicroscopy (Wynne *et al.*, 1999). The c/e1 epitope is located at the tips of the spikes of the core protein **C.** 3-dimensional reconstruction of the HBV-capsid obtained from cryo-electronmicroscopy. The demonstrated particle with a triangulation number T=4 consists of 120 core-protein dimers arranged in icosahedral symmetry (Wynne *et al.*, 1999).

As already stated above, the repetitive and symmetric arrangement of the HBV capsid has unique immunological features (Pumpens and Grens, 2001). HBcAg is able to trigger T-cell dependent as well as T-cell independent immune responses (Milich and McLachlan, 1986) abd the spikes of the capsid are able to unspecifically cross-link B-cell receptors (BCRs) (Bottcher *et al.*, 1997). 25 ng of particulate core protein are able to induce immune response without any adjuvant, whereas denatured core protein is not (Milich and McLachlan, 1986). After investigation of the T-cell dependent immune response it was shown that HBV capsids which contain the nucleic acid binding domain and consequently also RNA (Fig. 1.6), induced predominantly a T_H1 immune response. In contrast, HBV capsids without this domain rather induced a T_H2-response (Riedl *et al.*, 2002). At the same time it was shown that only packed RNA serves as T_H1-adjuvant. The reason for this is that only packed RNA is able to activate intravesicular TLR-7 and 8 (Takeda and Akira, 2007).

The core protein can be recombinantly expressed in *E. coli* which spontaneously assembles to icosahedral shaped capsid-like particles (CLPs) of extreme immunogenicty (Pumpens and Grens, 2001). This immunogenicity can be assigned

to foreign proteins especially if they are inserted into the surface exposed but centrally located c/e1-epitope of the core protein. The presentation of short peptides and selected full-length proteins like the GFP and the outer surface protein C (OspC) of *Borrelia burgdorferi*, has already been established (Skamel *et al.*, 2006), (Kratz *et al.*, 1999). However, many other foreign sequences impair the formation of CLPs. This originates from sterical constrains induced by the unavoidable two-sided linkage of the insert with the carrier protein (Walker *et al.*, 2008). To solve this problem Walker *et al.* designed a system where the core protein is split within the c/e1-epitope into two fragments, CoreN and CoreC. CoreN consists of the first 80 aa, CoreC of the rest of the core protein either with or without the nucleic acid binding domain. When co-expressed in *E. coli* the two fragments can self-complement each other and are able to assemble to CLPs that are undistinguishable from wild type CLPs in electronmicroscopy. These so called SplitCore CLPs have now two artificial surface exposed termini that can be used for further protein fusions (Walker *et al.*, 2011) (Fig. 1.8).

A. Native sequence of the HBc protein

B. Sequence of the SplitCore-System

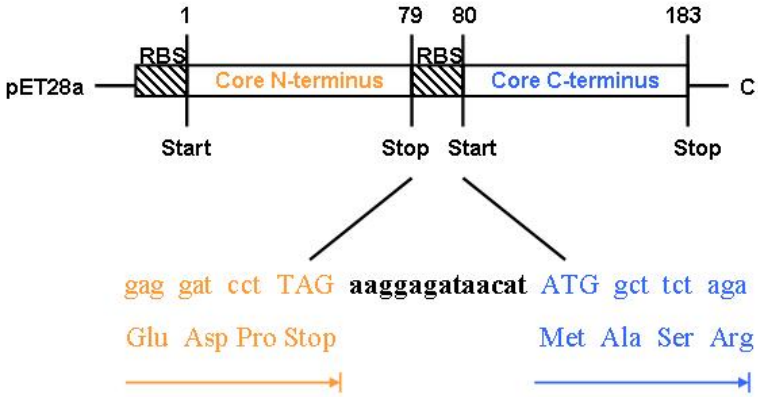

Fig. 1.8: Schematic demonstration of the SplitCore construct

A. In the used plasmids, translation of the core protein is initiated by a ribosome-binding-site (RBS) in front of the core protein. **B.** To split the core protein in two fragments, an artificial stop-codon is inserted behind proline79. Translation-initiation of the second fragment is mediated by a directly joined second RBS that is identical to the first RBS.

All the described features of SplitCore CLPs offer entirely new possibilities to use the concept of recombinant HBc particles as a carrier of foreign B and T-cell epitopes and thus for the creation of a new type of HCV immunogen.

1.6 Aim of the study

The standard antiviral therapy against HCV is successful only in 50% to 80% of patients and still difficult to tolerate. This makes therapy against HCV in most cases not satisfactory. However, due to the different genotypes and the rapid turnover rate of HCV which results in many distinct but closely related HCV variants found in each infected individual, the development of a prophylactic vaccine is a significant challenge. In order to circumvent the high genetic variability of HCV a new vaccination strategy is necessary. The most promising approach includes the application of capsid-like particle (CLP)-based vaccines. The CLPs formed by the HBV core protein (HBc), are able to induce cellular as well as humoral immune responses which can be used to present HCV envelope proteins. It was shown that the HVRI of HCV envelope protein 2 is involved in the first contact between the cellular receptor SR-B1 and the virus. For this reason, antibodies against this region are neutralizing and until now the only ones assumed to be protective. Moreover, it was demonstrated that the HVRI is involved in the direct spread of HCV from cell to cell. Despite the high variability of the HVRI, sequence data revealed that the HVRI is not a truly hypervariable region. It contains highly conserved residues surrounded by variable amino acids. The HVRI can adopt only a few related conformations which still allow binding its cellular receptor SR-B1. It is widely accepted that antibodies are able to neutralize the virus. To circumvent the problem of the high variability of the amino acids, artificially selected cross-reactive HVRI-variants (mimotopes), as well as the naturally occurring HVRI-variants, cross-reacting with a large panel of sera from chronically infected HCV patients, can be used. To enhance the immunogenicity of these short peptides and to induce vigorous humoral immune response, CLPs bearing these HCV epitopes should be applied. Thus, the aim of this study is to evaluate the innovative prophylactic vaccination strategy based on HBc-CLPs, expressing different variants of the HVRI in mice and guinea pigs.

In order to achieve this goal, the following steps are performed:

- Generation of plasmids bearing different variants of the HVRI.

- Testing the generated recombinant particles for their expression, the ability to self-assemble and maintain the antigenicity of the inserted foreign epitopes.

- Evaluation of the ability of the HVRI-CLPs to induce specific and broad cross-reacting antibodies against the HVRI in mice and guinea pigs.

- Characterization of the cross-neutralizing HVRI-antibody response using the HCV pseudoparticle-system.

As a result of the project we expect to generate a set of recombinant HVRI-CLPs which induce antibodies with a broad neutralizing activity and can be used as a prototype HCV vaccine.

2 Materials

2.1 Laboratory animals

2.1.1 Wild-type mice

Ten weeks old female C57BL/6 mice (genotype H-$2_{b/b}$) were kept under specific pathogen-free (SPF) conditions in Essen. The mice had free access to drinking water and standard food. They were purchased from Harlan Winkelmann Laboratories (Borchen, Germany).

2.1.2 Guinea pigs

Six weeks old female guinea pigs (*Cavia procellus*) were kept in Cairo, Egypt with cooperation partners.

2.2 Anesthetics

Isofluran	Delta Select, Germany

2.3 Bacteria strains

Eschrichia coli BL21*CP (Novagen, USA)	F– ompT gal [dcm] [lon] hsdSB (rB–mB–;an *E.coli* B strain) with E3, a λ prophage carrying the T7 RNA polymerase gene
Eschrichia coli Top10 (Invitrogen, Germany*)*	F-mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(Str^R)endA1 $λ^-$

Eschrichia coli XL-10 Gold (Stratagene, USA)	endA1 glnV44 recA1 thi-1 gyrA96 relA1 lac Hte (mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 tet^RF'[proAB $lacI^qZ\Delta M15$ Tn10(Tet^R Amy Cm^R)]

2.4 Eukaryotic cell lines

2.4.1 Human Hepatoma cell line Huh7.5

Huh7.5 cells are a subline derived from Huh7 hepatoma cells (Blight *et al.*, 2002). Huh7 cells were established from hepatocellular carcinoma which was found to replicate continuously in a chemically defined medium (Nakabayashi *et al.*, 1982). The subline Huh7.5 was established by "curing" a cell clone containing a Con1 subgenomic replicon by prolonged treatment with α-interferon. The receptors, important for the initiation of virus entry, are expressed on the viral surface and can therefore be used for studying virus entry.

2.4.2 Human Embryonic Kidney 293T cells

293T cells are modified 293 cells that constitutively express the Simian Virus 40 (SV-40) large T-antigen (Patel and Tikoo, 2006). The presence of this antigen allows episomal replication of transfected plasmids containing the SV40 origin of replication. In the current study 293T cells were used for generation of HCVpp.

2.5 Chemicals and reagents

Acetic acid, Cesium chloride, EDTA solution pH 8.0, Saccharose, SDS (sodium dodecyl sulphate), Tween 20	AppliChem, Germany
Acrylamide solution, Ethidium bromide, Hydrogen peroxide, LB-Agar (Luria/Miller), LB Medium (Luria/Miller), Milk powder	Roth, Germany

Boric acid	JT Baker, Netherlands
Bradford staining solution	BioRad, Germany
BSA (bovine serum albumin) fraction IV	Serva, Germany
D-PBS, TBE ultrapure 10x, Trypan Blue, Trypsin	Invitrogen, Germany
Glycine, Tris-Base	MP Biomedicals, Germany
HEPES, Non essential amino acids	PAA Laboratories, Austria
Plasmocin 25mg	Invivogen, Germany
Potassium chloride, Potassium dihydrogenphosphate, Sodium acetate, Sodium azide, Sodium carbonate, Sodium chloride, Sodium dihydrogenphosphate, Sodium hydrogencarbonate, Sodium oxide	Merck, Germany
β-mercaptoethanol, Agarose, APS (ammonium persulfate), Bromophenol blue, DMSO (Dimethyl sulfoxide), Ethanol, Freund's Adjuvant Incomplete, Glycerol, Hydrochloric acid, Isopropanol, Methanol, OPD tablettes (o-Phenylenediamine), Orange G, TEMED, Triton X-100, Triton X-114	Sigma, Germany

2.6 Antibiotics

Ampicillin	AppliChem, Germany
Carbenicillin	Serva, Germany
Chloramphenicol	AppliChem, Germany
Kanamycin	AppliChem, Germany
Penicillin/Streptomycin	PAA Laboratories, Austria

2.7 Cell culture media

Huh7.5 cell line	DMEM medium 10% FCS 1% Penicillin/Streptomycin 1% HEPES 1% Non essential amino acids
HEK-293T cell line	DMEM medium 10% FCS 1% Penicillin/Streptomycin

2.8 Buffers and solutions

Coomassie Brillant-Blue Staining Solution	0.06% (w/v) Coomassie brillant-blue R250 50% (v/v) Methanol 10 % (v/v) Acetic acid
Carbonate buffer (pH 9.6) (ELISA)	3.18 g Na_2CO_3 5.88 g $NaHCO_3$ 0.2 g NaN_3 1 L H_2O
Destaining Solution	5 % (v/v) Methanol 7.5 % (v/v) Acetic acid
Blocking solution (ELISA)	PBS 5% (v/v) FCS
T-PBS washing buffer (ELISA / Western blot)	PBS Tween 20
OPD-substrate solution (ELISA)	o-Phenylenediamine (1 pill) 10 ml PBS 10 µl H_2O_2

STOP solution (ELISA)	0.5 N H_2SO_4
5x DNA loading buffer	50% Glycerine 20 mM Tris, pH 7.5 50 mM EDTA, pH 8.0 0.025% Bromphenoleblue 0.025% Xylenecyanole
1x TBE buffer (pH 8.4)	100 mM Tris Base 90 mM boric acid 1 M EDTA
TTBS buffer	10 mM Tris-HCl pH 8.8 50 mM NaCl 0.2% Tween
Buffer P1, pH 8.0 (plasmid preparation)	50 mM Tris Base 12 mM $Na_2EDTA\ H_2O$ 100 mg RNAse 1L H_2O
Lysis buffer P2 (plasmid preparation)	200 mM NaOH 35 mM SDS
Neutralisation buffer P3 (plasmid preparation)	3 M potassium acetate 150 ml acetic acid 1 L H_2O
1x TAE buffer	40 mM Tris 5.7 % Acetic acid 50 mM EDTA pH 8.0
1x TE buffer (pH 8.0)	1 % (v/v) 1 M Tris-HCl 1 mM EDTA

4x SDS loading buffer	Glycerine 10 % SDS 4 % Tris, pH 6.8, 125 mM ß-Mercaptoethanol 10 % Bromphenol-blue 0.02 %
Blocking solution (Western blot)	PBS 5% (m/v) milk powder
10x SDM reaction buffer	100 mM KCl 100 mM $(NH_4)_2SO_4$ 200 mM Tris-HCl (pH 8.8) 20 mM $MgSO_4$ 1% Triton X-100 1 mg/ml BSA
Transfer buffer (Western blot)	72 g glycine 15 g Tris Base 25 ml 20% SDS 1L H_2O
TN_{150} buffer (Protein expression preparation)	20 mM Tris-HCl, pH 7.5 150 mM NaCl
TN_{300} buffer (Protein expression preparation)	20 mM Tris-HCl, pH 7.5 300 mM NaCl
TNE buffer	10 mM Tris-HCl pH 7.5 150 mM NaCl 1 mM EDTA
Coating buffer (ELISA)	0.1 M Carbonate buffer H_2O

2.9 Enzymes and commercial Kits

Benzonase (250 U/µl)	Novagen, USA
Bright-Glo Luciferase Assay System	Promega, Germany
CalPhos mammalian transfection kit	Clontech, Germany
ELC Western Blotting Detection Kit	GE Healthcare, UK
Gen Elute Plasmid Miniprep Kit	Qiagen, Germany
Glo Lysis Buffer	Promega, Germany
Go *Taq*-Polymerase (1 U/µl)	Promega, Germany
Limulus Amebocyte Lysate Pyrotell	Cape COD, USA
KOD-Polymerase (2.5 U/µl)	Novagen, USA
Lysozyme (46 U/µl)	Sigma, Germany
pcDNA3.1/V5-His TOPO TA Expression Kit	Invitrogen, Germany
Protease inhibitor cocktail complete	Roche, Germany
QIAprep Mini-, Maxiprep Kit	Qiagen, Germany
QIAprep Gel Extraction Kit (250)	Qiagen, Germany
QIAquick PCR purification Kit (50)	Qiagen, Germany
Restriction endonucleases (10 U/µl)	NEB, Germany
T4-DNA-Ligase (20 U/µl)	NEB, Germany

2.10. Standards

PeqGold Protein-Marker I	PeqLab, Germany
PeqGold Prestained Protein-Marker IV	PeqLab, Germany
Protein-MW-Marker (Low Range Rainbow)	GE Healthcare, UK
GeneRuler 1kb Ladder Plus	Fermentas, Germany

2.11. Plasmids

The plasmids used in this project are shown and described in the appendix section 9.1.

2.12. Antibodies

Monoclonal mouse anti-HBV-Core antibodies 10E11, 11E2, 13C9 and CET4 used for Western blotting analysis, were kindly provided by Prof. Paul Pumpems (Latvian Biomedical Research and Study Centre, Riga, Latvia), Prof. Yuri Khudyakov (Centers For Disease Control and Prevention, Atlanta, USA) and C. Yuli Kong (South Korea). Manufacturer information of the other antibodies are also listed in table 2.1.

Tab. 2.1 Antibodies and conjugates

Antibody	Usage	Manufacturer
monoclonal mouse anti-HBV-Core (10E11)	Western blot	P. Pumpens, Riga, Latvia
monoclonal mouse anti-HBV-Core (11E2)	Western blot	P. Pumpens, Riga, Latvia
monoclonal mouse anti-HBV-Core (13C9)	Western blot	P. Pumpens, Riga, Latvia
monoclonal mouse anti-HBV-Core (10E11)	Western blot	Dianova
monoclonal mouse anti-HBV-Core (13A9)	Western blot	Dianova
monoclonal mouse anti-rabbit, HRP-coupled	Western blot ELISA	Dianova
monoclonal guinea pig anti-rabbit, HRP-coupled	ELISA	Dianova
monoclonal human anti-HBV-Core (3120)	Western blot	Dianova
monoclonal goat anti-mouse IgG1, HRP-coupled	ELISA	SouthernBiotech
monoclonal goat anti-mouse IgG2a, HRP-coupled	ELISA	SouthernBiotech
monoclonal goat anti-mouse IgG2b, HRP-coupled	ELISA	SouthernBiotech
monoclonal goat anti-mouse IgG3, HRP-coupled	ELISA	SouthernBiotech

2.13. Peptides

Peptides used for coating in ELISA assays (500 ng/well) were synthetized by EMC microcollections (Germany). Other peptides were kindly provided by Prof. Yuri Khudyakov (Centers For Disease Control and Prevention, Atlanta, USA). The list of peptides is shown in table 2.2.

Lyophilisates of peptides were diluted in PBS containing 10% DMSO to the concentration 1mg/ml, aliquoted and stored at -20 °C.

ELISA plates were also coated with protein (50 ng/well) pET28a2-HBc_c1-79_80-149H6 (149-St1), the vector-card is shown in the appendix.

Tab. 2.2 Peptides

Name	Sequence
R9 (H16)	QTTVVGGSQSHTVRGLTSLFSPGASQN
YK5807 (H19)	VTYTTGGSQARHTQGVASFFTPGPAQK
YK5829 (H20)	TTTVSGGHASQITRGVTSFFSPGSAQK
G31 (H21)	TTHTVGGSVARQVHSLTGLFSPGPQQK
preS2pep1	MKNQTFRLQGFVDGL

2.14. Membranes and films

PVDF membrane	Millipore, Germany
High performance chemiluminescence film	GE Healthcare, UK

2.15. Oligonucleotides

All oligonucleotides used in the study were synthesized by Biomers (Germany). The nucleotide sequence, annealing temperature (T_{ann}) employed for PCR and usage of the oligonucleotides are presented in table 2.3.

Tab. 2.3 Oligonucleotides

Primer	Orientation	Sequence (5' -> 3')	T_{ann} [°C]	Usage
Primer-(-)-HVR-AP33-CoreN	anti-sense	gtt tta gga tcc tca ttg ctg ttt agg gca gtt ctg	-	cloning
Primer-(-)-HVR-AP33-CoreC	anti-sense	ccc acc tct aga gtc att gct gtt tag ggc agt tct g	-	cloning
Primer-(-)-HVR-R9	anti-sense	ggt gtg ggt agg atc ctc gtt ctg aga agc acc ccg	60	PCR
Primer-(-)-HVR-G31	anti-sense	gcc taa gga tcc tct ttc tgc tgc gga ccc gga gag aac aga ccg gtc aga gaa tga acc tgg cga gc aac aga acc acc cac aag tat ggg tct gtc cgg	60	PCR
Primer-(-)-HVR-YK5807	anti-sense	gcc taa gga tcc tct ttc tag ccg gac ccg ggt gaa gaa aga agc aac acc ctg ggt gtg acg agc ctg aga acc acc ggt ggt gta agt aac ctg tcc gga gcc acc	60	PCR
Primer-(-)-HVR-YK5829	anti-sense	gcc taa gga tcc tct ttc tga gca gaa ccc gga gag aag aaa gag gta aca cca cgg gtg atc tga gaa gcg tgt cca cca gag acg gta gtg gtc tgt ccg gag cc	60	PCR
Primer-(+)-HCVpp-R9	sense	gct ggc gtc gac ggc cag acc act gtt gtg ggt ggt tct cag tct cac acc gtt cgt ggt ctg acc tct ctg ttc tct ccc ggg gct tct cag aac atc cag ctt ata aac acc	60	PCR

Primer-(+)-HCVpp-G31	sense	gct ggc gtc gac ggc acc acc cat act gtg ggt ggt tct gtt gct cgc cag gtt cat tct ctg acc ggt ctg ttc tct ccg ggt ccg cag cag aaa atc cag ctt ata aac acc	60	PCR
Primer-(+)-HCVpp-YK5807	sense	gct ggc gtc gac ggc gtt act tac acc acc ggt ggt tct cag gct cgt cac acc cag ggt gtt gct tct ttc ttc acc ccg ggt ccg gct cag aaa atc cag ctt ata aac acc	60	PCR
Primer-(+)-HCVpp-YK5829	sense	gct ggc gtc gac ggc acc act acc gtc tct ggt gga cac gct tct cag atc acc cgt ggt gtt acc tct ttc ttc tct ccg ggt tct gct cag aaa atc cag ctt ata aac acc	60	PCR
Primer-(+)-HCVpp-J6-M22P	sense	cca ccg ggc gcc tga ccg cct gtt cga ccc ggg tcc ccg gca g	60	PCR
Primer-(+)-HCVpp-J6	sense	gct ggc gtc gac ggc cgc cca cac cgt ggg cgg cag cgc cgc cca gac cac cgg gcg cct gac cag cct gtt cga cat ggg tcc ccg gca gaa aat cca gct tat aaa cac c	60	PCR
Primer-(-)-pHCMV-Minus-BglII	anti-sense	ggc aga ggg aaa aag atc tca gtg g	52	PCR
Primer-(-)-phCMV-IRES-E1+E2	anti-sense	gcc acc acc ttc tga tag gc	-	sequencing
T7-promotor	sense	att aat acg act cac tat agg g	-	sequencing PCR
T7-terminator	anti-sense	gct agt tat tgc tca gcg g	-	sequencing
pcDNA3.1	anti-sense	tag aag gca cag tcg agg ct	-	sequencing
SV711	anti-sense	tat gta cgt ggg gga tct ttg	-	sequencing

2.16. Materials and equipment

Balance Vibra AJ-2200CE	Shinko Denshi, Japan
Beakers	Schott, Germany
Capillaries (heparinised)	Hirschmann Laborgeräte, Germany
Cell culture flasks (250 ml)	Greiner bio-one, Germany
Cell culture plates (96-well)	Greiner bio-one, Germany
Centrifuge Rotina 420R	Hettich, Germany
Centrifuge Avanti J-26XPi	Beckman Coulter, Germany
Centrifuge: Ultracentrifuge Optima L-70K	Beckman Coulter, Germany
Centrifuge tubes (14x95 mm, 25x89 mm)	Beckman Coulter, Germany
Centrifuge 5415D	Eppendorf, Germany
Combitips (2.5 ml; 5 ml, 10 ml)	Eppendorf, Germany
Concentration tubes (Centricon; 30K, 100K)	Millipore, Germany
CO_2 incubator Cytoperm2	Heraeus, Deutschland
Cryo-tubes (2 ml)	Greiner bio-one, Germany
Dialysis tubes	Spectrapore, USA
Dialysis cassettes	Pierce, USA
Dishes; 10 cm^2 also for cell culture	Greiner bio-one, Germany
Disposable scalpels	HMD Healthcare, UK
Disposable syringes (2 ml; 5 ml; 10 ml)	B. Braun, Germany
Erlenmeyer flasks	Schott, Germany
Electronmicroscope EM 902A	ZEISS, Germany
Electrophoresis chambers	BioRad, Germany
Elisa Reader Asys Expert Plus	Asys Hiteck
Fridge / Freezer (-20 °C)	Liebherr, Germany
Freezer (-80 °C)	Thermo Forma, Germany
Flat-bottom 96-well microplates	Falcon BD, Germany
Forceps (pointed and curved)	Oehmen, Germany
Gel running chamber	peqLab, Germany
Glomax Multi Detection System	Promega, Germany
Heating block (Thermo mixer comfort)	Eppendorf, Germany
Laminar flow (Hera Safe)	Heraeus, Germany
Microscope (inverted) Primo Vert	ZEISS, Germany
Micro screw-cap tubes (1.5 ml)	Sarstedt, Germany

Needles (0.4x19 mm; 0.9x40 mm)	Becton Dickinson, Germany
NUNC Immunoplates (96-well)	NUNC, Denmark
Parafilm	American National Can, USA
Pipette tips (10 µl; 200 µl; 1000 µl)	STARLAB, Germany
Plastic sterile pipettes (5 ml; 10 ml; 25 ml)	Greiner bio-one, Germany
Reaction tubes (1.5 ml; 2 ml)	Eppendorf, Germany
Rotary Mixer 526	Oehmen, Germany
Scissors	Oehmen, Germany
Screw-cap tubes (15 ml; 50 ml)	Falcon BD, Germany
Single-, multichannel pipettes, multipettes	Eppendorf, Germany
Shaker (GFL 3020)	GFL, Germany
Shaker (Duomax 1030)	Heidolph, Germany
Sonicator (Labsonic P)	Sartorius, Germany
Spectrophotometer (Gene Quant pro)	Amersham Bioscience, USA
Spectrophotometer Ultrospec III	Pharmacia LKB
Thermocycler 3000	Biometra, Germany
Thermocycler MJ Mini	BioRad, Germany
Thermoshaker	Gerhardt, Germany
Neubauer cell counting chamber	Marienfeld, Germany
Transblot SemiDry transfer cell	BioRad, Germany
UV transiluminator FLX-20M MWG	BioTech, Germany
UV- Bioimaging System	Syngene
Vacuum pump Integra Vacusafe	Integra, Germany
Vortex Genie 2	Bender & Hobein AG, Switzerland
Vortexer PV-1	Grant-bio, Germany
Waterbath Julabo ED	Julabo, Germany
Whatman Paper	Whatman, Germany

3 Methods

3.1 Working with prokaryotic cells

3.1.1 Transformation of chemically competent *E. coli* strains

Bacterial cells (Top10 cells, XL10-Gold cells or BL21*CP cells (for strain specific information see 2.3.) frozen at -80 °C were thawed on ice. No more than 10% by volume of the DNA to be transformed was added to 50 µl bacterial cells. This mixture was incubated on ice for 10 minutes. To improve DNA absorption by bacteria, a heat shock was performed for 1 minute at 37 °C followed by a subsequent incubation on ice for 5 minutes. To express the resistance encoded by the transformed plasmid, the mixture was incubated in a shaker for 30 minutes at 37 °C after the addition of 120 µl LB-medium. Using a sterile spatula the complete mixture was spread over an LB-agar plate containing the selective antibiotic. The plate was incubated over night at 37 °C.

3.1.2 Plasmid DNA preparation using commercial kits

One bacterial colony obtained after transformation (3.1.1.) was picked and transferred to a flask containing LB-medium and the appropriate antibiotic (5 ml of medium for minipreps, 100 ml for midipreps, and 250 ml for maxipreps). The bacterial culture was incubated shaking at 37 °C over night. Plasmid-DNA was isolated using the Qiaprep kits (Qiagen) according to the manufacturer's protocol. Purified DNA was resuspended in TE buffer and adjusted to a concentration of 1 µg/µl by spectrophotometric OD_{260nm} measurement.

3.1.3 Cultivation of *E.coli* for protein expression

Competent *E.coli* BL21*CP cells were used for production of protein. After transformation (3.1.1.) and incubation over night, 5 ml of LB-medium (containing ampicillin and chloramphenicol) was inoculated with one colony and incubated on a shaker over night at 37 °C. Two and a half ml of this overnight culture was added to 250 ml LB-medium (containing ampicillin and chloramphenicol) and cultured shaking

at 37 °C until reaching OD_{600} of 0.6-0.8. Afterwards, protein production was induced with 1 mM IPTG and the culture was incubated at romm temperature (RT). After 3 to 5 hours (alternatively over night) the culture was centrifuged 15 minutes at 5,000 rpm and 4°C. The supernatant was discarded, the precipitate was resuspended in 25 ml TN_{300} and centrifuged again for 15 minutes at 5,000 rpm and 4 °C. The supernatant was discarded and the precipitate was stored at -20 °C until further analysis.

3.1.4 Cell Disruption under non-denaturing conditions

To extract protein from *E.coli* cells, the frozen cell pellet of the induced culture (3.1.5.) was thawed on ice and resuspended in 5 ml TN_{150} buffer. One mg/ml lysozyme, Triton X-114 (1.5 %) and protease inhibitor was added to the mixture. Lysis of the cells was performed by incubation on ice for 15 minutes. Nucleic acids were fragmented by addition of 10 U/ml benzonase and incubation for 10 min at RT while shaking. For complete cell lysis, cells were sonicated. Cell lysate was transferred to a 15 ml Falcon-Tube and for 2 minutes exposed to sonication at 50% and 1 minute cooled on ice. This procedure was repeated once. Insoluble proteins and cell debris were sedimented by centrifugation at 4 °C for 15 min and 14,000 rpm. To separate membrane vesicles the supernatant was transferred into a new Falcon-Tube and incubated at 30 °C for 5 min which leads to phase separation of TX-114. Afterwards, this mixture was centrifuged at 25 °C for 15 minutes at 4000 rpm. The supernatant was layered over a SW40 sucrose-gradient which was then centrifuged for 2 hours at 20 °C and 40,000 rpm.

3.2 Cell Culture

3.2.1 Thawing of cells

Cryo tubes containing cells were taken out of liquid nitrogen and thawed quickly. Afterwards, cells were suspended in 18 ml of fresh medium, placed in a 75 cm^2 flask, and cultured at 37 °C in humidified atmosphere containing 5% CO_2.

3.2.2 Cryoconservation of cells

For cryoconservation, the cell suspension was centrifuged in 50 ml tubes (1200 rpm, 5 min) and washed once with 5 ml sterile PBS. The pellet was suspended in 900 µl FCS and 100 µl DMSO. Cells were frozen slowly overnight in -80 °C and then transferred to a liquid nitrogen tank.

3.2.3 Passaging of cells

Medium was removed from adherent Huh7.5 cells and cells were washed with 5 ml sterile PBS. Then 3 ml of Trypsin-EDTA was added to cover the bottom of the 75 cm^2 flask. After approximately 2 minutes when the cells started to detach from the bottom 10 ml of fresh DMEM medium was added. Cells were placed in fresh flasks in 18 ml of culture medium in the designated concentration.

HEK-293T cells are less adherent compared to Huh7.5 cells, therefore medium was removed and cells were resuspended in 10 ml fresh DMEM medium. Afterwards, cells were placed in fresh flasks in 18 ml of culture medium in the designated concentration.

3.2.4 Counting of viable cells using Trypan blue exclusion microscopy

Trypan blue is a negatively charged dye that only interacts with a cell when the membrane is damaged. Therefore, all cells which exclude the dye are viable. Aliquots of the cell suspension were diluted 1:10 with Trypan blue stain and 10 µl of the diluted aliquot solution was transferred onto a Neubauer hemocytometer. The viable cells were counted and the number of cells per ml was calculated as follows:

$$number\ of\ cells\ /\ ml = number\ of\ cells\ in\ the\ large\ square \times dilution\ factor \times 10^4$$

3.2.5 Transfection of HEK-293T cells

HEK-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). Prior to that, dishes were coated with 2 ml Poly-L-Lysine hydrobromide and washed with PBS. After dishes got dry, cells were added. The next day, cells were transfected with the expression plasmid phCMV IRES-E1/E2 in combination with a retroviral plasmid encoding for HIV-Gag,Pol and Luc (pHCMV-IRES-AD78-E1E2) using the Calcium Phosphate Transfection Kit (Clontech). One to 2 h before transfection of the cells, the medium was exchanged. Briefly, 500 µl HBS were put in a FACS tube. Next, using an Eppendorf tube 1.5 ml, the mix was prepared as follows:

Tab. 3.1 Mix for the generation of HCVpp

Reagent	Concentration	Volume [µl]
H_2O	-	432.6
Plasmid encoding Gag, Pol, Luc	1 µg/µl	2.7
phCMV IRES-E1/E2	1 µg/µl	2.7
$CaCl_2$ solution (added dropwise)	2M	62
		500

This mix was added dropwise and under constant slight mixing to the tube containing 500 µl HBS and incubated for 20 to 30 min at room temperature. Afterwards the solution was added dropwise to the cell culture. The dishes were incubated up to 16 h at 37 °C. Medium was changed and the cell culture was additionally incubated for 48 h at 37 °C. Every 24 h the supernatant containing the generated HCVpp's was harvested and centrifuged for 5 min at 1200 rpm. The HCVpp preparation was kept at 4 °C (not longer than 4 days) and used for infectivity assay or neutralization assay.

3.2.6 Infectivity assay

Huh7.5 cells were seeded in 96 flat bottom well plates. (5 x 10^3 cells in 100 ml). The next day, the medium was removed and HCVpp in 100 µl of medium was added. One hundred µl of fresh pre-warmed DMEM medium (containing 6% FCS) was added and plates were incubated for additional 72 h at 37 °C. For infectivity evaluation, medium was removed and 50 µl of lysis buffer (Promega) was added to each well and incubated for 2 h at -20 °C. Afterwards, 50 µl of prepared cell lysat were transferred into a luminometer 96-well plate. Fifty µl of the Bright Glo Luciferase Assay buffer (Promega) was added and the luciferase-activity was measured after 10 minutes incubation time at RT in the luminescence counter (Glomax Multi Detection System, Promega). All cell lysates were measured in triplicates and the mean value was calculated.

3.2.7 Neutralization assay with HCVpp's

Huh7.5 cells were seeded one day before preparation of neutralization assay as described in 3.2.6. Dilutions of mouse or guinea pig sera were prepared in DMEM and stored at 4 °C until the next day. The following day, HCVpp's (harvested 4 days before) were mixed with the diluted sera, and incubated for 2 h at 37 °C. Afterwards, the mixture of HCVpp with sera in the volume of 120 µl plus 100 µl DMEM medium (containing 6% FCS) was added to Huh7.5 cells. The plates were incubated for 72 h at 37 °C. For infectivity evaluation was done as described in 3.2.6. The luciferase activity of all cell lysates was measured in triplicates and a mean value was calculated. Inhibition of infectivity by more than 50 % was considered as a positive neutralization.

3.3 Molecular biology methods

3.3.1 Polymerase Chain Reaction

Polymerase chain reaction is used for the amplification of DNA sequences. Therefore the double-stranded template DNA is denatured by heating to 95 °C. After cooling specific single stranded DNA-primers are able to bind to the template DNA due to their specific character which determines temperature (annealing temperature; TM). This temperature can be calculated using the following formula:

$$TM \text{ in } °C = \Sigma(2(A+T) + 4(G+C))$$

For elongation of the primer, and amplification of the DNA sequence, the PCR mixture is kept at a temperature at which the used polymerase is able to work best. This cycle; denaturation, binding of the primer and elongation, is repeated several times. Subsequently, the mixture is heated to 72 °C which aids the polymerase to fill up incomplete strands.
The reaction mixture components and the PCR conditions used in this project are presented in tables 3.2 and 3.3.

Tab. 3.2 Standard PCR mastermix

Reagent	Concentration	Volume [µl]
Template DNA	1 µg/µl	0.5
Primer 5'- 3'	100 pmol/µl	1.0
Primer 3'- 5'	100 pmol/µl	1.0
$MgCl_2$	25 mM	6.0
dNTPs	per 10 mM	2.5
KOD - buffer	10x	10.0
KOD - Polymerase	2.5 U/µl	0.5
H_2O	-	78.5
		50

Tab. 3.3 Standard PCR Programme

PCR step	Temperature [°C]	Time [min]	Number of cycles
Denaturation	95	5	1
Denaturation	95	0:30	
Annealing	52-60*	0:30	20
Elongation	72	0:30	
Final elongation	72	5	1
Storing	4	for ever	-

***** The annealing temperature depends on the primer which was used in the PCR. Primer specific annealing temperatures are listed in table 2.3.

3.3.2 DNA restriction digest

To specifically cut plasmids and PCR-products, they were subjected to restriction digest either with a single endonuclease or with a combination of two enzymes. In the latter case, the set of restriction enzymes that work optimally in the same reaction buffer and temperature (usually 37°C) were chosen. The PCR products used for restriction were previously purified by gel extraction (3.3.4.). For preparative purposes 50 µl of restriction reaction was prepared and incubated 1-3 h (alternatively over night) at the optimal temperature given by the provider. The control digestion of successful cloning was carried out in 20 µl reaction for 1 h (Tab. 3.4).

Tab. 3.4 Restriction of plasmids and PCR-products

Reagent	Preparative digestion	Control digestion
DNA	5 µg	10 µl
Enzyme I	0.5 – 1 µl	0.25 µl
Enzyme II	0.5 – 1 µl	0.25 µl
10x buffer	5 µl	2 µl
10x BSA (if required)	5 µl	2 µl
H_2O	add 50 µl	add 20 µl

The products of the restriction reaction were separated by agarose gel electrophoresis and purified using the QIAquick Gel Extraction Kit (Qiagen) (3.3.4.).

3.3.3 Agarose gel electrophoresis

Gel electrophoresis is used for the separation of molecules according to their mass. In agarose gels, agarose forms a netlike matrix in which DNA fragments can be separated. After applying voltage (mostly 80 - 120 V) the smaller fragments move faster through the gel than shorter ones. For the separation of DNA fragments of approximately 300 to 2,500 bp, a 1.0 % agarose gel was used (10 ml 1x TAE-buffer per 0.1 g agarose). To visualize the DNA after separation, ethidium bromide was added. The visualization of the separated DNA fragments was performed using a UV- Bioimaging System (Syngene).

3.3.4 DNA extraction from agarose gels

After separation of DNA (from restriction digest or PCR) on an agarose gel, DNA can be extracted from the agarose and used for further analysis. Therefore, the desired DNA fragment was cut out of the gel (visualized by UV light) and stored in an Eppendorf tube. DNA extraction was done using the QIAprep Gel Extraction Kit (250) according to the manufacturer's protocol.

3.3.5 Cloning of PCR-products in intermediate plasmids

An easy step for rescue of PCR products is the usage of the invitrogen pTOPO-vector. The pTOPO-vector is a linearized plasmid with single overhanging 3' deoxythymidine (T) residues that are kept together by the topoisomerase. *Taq* polymerase shows a terminal transferase activity which is nontemplate-dependent and adds a single deoxyadenosine (A) to the 3' ends of PCR products, which then are inserted by the topoisomerase into the pTOPO-vector. As already mentioned, the plasmid vector (pcDNA3.1/V5-His-TOPO; Invitrogen) has single overhanging 3' deoxythymidine (T) residues which allow PCR inserts to ligate efficiently with the vector (Invitrogen, 2004). For insertion, the PCR product was stored on ice and 1 µl Go *Taq*-Polymerase was added. This mixture was incubated at 72 °C for 10 minutes and cooled on ice afterwards. The pTOPO cloning mixture was prepared as follows after manufacturer's protocol:

Tab. 3.5 pTOPO cloning mixture

Reagent	Volume [µl]
PCR product (treated with *Taq*)	2.0
Salt solution	1.0
pTOPO3.1-vector	1.0
H_2O	2.0
	6.0

After mixing and centrifugation of this mixture, the sample was incubated for 10 minutes at RT. Two µl mixture was added to 25 µl TOP 10 cells following standard transformation procedure (3.1.1.). Alternatively, 1 µl solution was added to 25 µl XL 10 Gold cells.

3.3.6 Ligation of DNA fragments

For the ligation of DNA fragments T4-DNA-ligase was used.

Tab. 3.6 Ligation mixture

Reagent	Volume / Concentration
Vector	50-200 ng
Fragment	100-400 ng
10x t4-buffer	2.0 µl
H_2O	add 20 µl

0.5 µl T4-DNA-ligase was added to this mixture and incubated for at least 1 h at RT. After transformation (3.1.1.), single clone picking and Mini-DNA preparation (3.1.2.) of TOP 10 cells (alternatively XL 10 Gold cells) with 2.5 µl ligation solution, the isolated plasmid-DNA was tested for correctness using restriction digest (3.3.2.) or sequencing methods (3.3.7.).

3.3.7 DNA-Sequencing

Sequencing was outsourced to the company AGOWA in Berlin. Primers used for sequencing are indicated in table 3.7. A standard sequencing mixture is shown in the table below.

Tab. 3.7 Sequencing mixture

Reagent	Volume / Concentration
Plasmid DNA	0.5-1 ng
Primer	20 ng*
H_2O	add 14 µl

* was added either by self or by AGOWA

3.4 Protein-biochemical methods

3.4.1 Determination of the protein concentration

To determine protein concentration the Bradford method was used. For that, 10 µl standard BSA concentrations (50 ng, 75 ng, 100 ng, 200 ng, 300 ng, 500 ng) as well as 10 µl of a 1:2, 1:5 and 1:10 dilution of the protein-solution to be measured were added to a 96-well plate. Subsequently, 200 µl of a 1:5 diluted Bradford solution were added to each well and incubated for 5 minutes. Finally, the absorption at 595 nm was measured and the protein concentration was calculated in consideration of the dilution factor.

3.4.2 SDS-PAGE

In discontinuous SDS-PAGE (sodiumdodecylsulfate-polyacrylamide-gel electrophoresis) proteins are separated due to their denaturation with SDS and hence consistent negative charge. Separation occurs predominantly according to the mass of the proteins. During electrophoresis samples are focused in the stacking gel and subsequently separated within the separation gel. Prior to application of the samples to the gel they were denatured for 5 min at 100 °C. Electrophoresis was done at 220 V until bromophenole blue was leaking out of the gel. The ingredients

needed for preparation of 15% separation gel and 5% stacking gel are presented in Tab. 3.8.

Tab. 3.8 Reagents used for preparation of SDS gels

Reagent	Stacking Gel (5%)	Separation Gel (15%)
30% Acrylamid solution (29:1)	0.75 ml	5.0 ml
Tris-HCl (1 M, pH 6.8)	0.62 ml	-
Tris-HCl (1.5 M, pH 8.8)	-	2.5 ml
SDS 10%	0.05 ml	0.1 ml
H_2O	3.55 ml	2.4 ml

For polymerisation 1:500 saturated APS solution and 1:1000 TEMED was added per gel.

3.4.3 Native Gel electrophoresis

In native agarose gel electrophoresis, proteins are separated in their native form according to their isoelectric point. For that purpose 5x DNA loading dye was added to the protein samples which were then separated on a 1% agarose gel (3.3.3.). When HBV core protein with nucleic binding domain is expressed in *E.coli*, the CLPs contain unspecific packed mRNA (see section 3.3.). In case of HBV-CLPs with nucleic acid binding domain and ethidium bromide within the gel, nonspecifically packed RNA can be detected under UV-light.

3.4.4 Coomassie-Brilliant-Blue Staining

Staining of the proteins after separation was done using Coomassie brilliant-blue staining solution while shaking for approximately 30 minutes at RT. Subsequent to staining, the gel was put in destaining solution. After complete destaining, the gel was put into water until drying.

3.4.5 Immunoblot Analysis (Western Blot)

After separation with SDS-PAGE, proteins were transferred from the gel to a PVDF-membrane by means of electrophoretic transfer. In this process membrane and gel were both put between three layers of Whatman paper, free from air bubbles. The gel was turned towards the cathode and the membrane was turned towards the anode. Beforehand, gel and membrane were soaked in western-blot-transfer-buffer for 5 minutes. Additionally the PVDF-membrane was soaked in methanol for 1 minute prior to the buffer. Transfer took place per gel for 15 minutes at 7.5 V using a SemiDry-transfer chamber. In this procedure, proteins were immobilized to the membrane. To block free protein binding sites the membrane was incubated in blocking solution (5% milk powder + 0.05% Tween in PBS) for 1 h at RT. Incubation with the first antibody (concentration antibody-dependent) was done over night at 4 °C while shaking in TTBS with 5% milk powder. Unbound antibody was removed by washing the membrane three times with TTBS for 10 minutes. Incubation with the second isotype specific antibody (1:5000) was done for 1 h at RT in TTBS. This antibody was coupled to horseradish-peroxidase (HRP). Subsequently the membrane was again washed three times with TTBS and incubated 5 minutes with ECL-solution for HRP detection. Thereby a luminiferous reaction starts so that proteins marked with antibodies can be visualized using a radiographic film.

3.4.6 Native Capillary Transfer

After separation by native gel electrophoresis, proteins were transferred from the gel to a PVDF-membrane using capillary transfer. The agarose gel was washed two times for 5 minutes in TNE-buffer. An inverted gel tray was put into a large bowl which was filled with 500 ml TNE-buffer. Two Whatman papers were put along and across onto the gel tray facilitating buffer uptake. After putting the gel with the top downwards onto the Whatman papers, the membrane was placed onto the setup without air bubbles. Three more Whatman papers were used to cover the membrane. Whatman paper and the membrane were previously soaked in TNE-buffer (5 minutes) and methanol (60 seconds) respectively. Due to the capillary flow, proteins were transferred, in native form, from the gel to the membrane. To maintain a constant flow 30 layers of thick paper were put above the Whatman paper. Evaporation of the buffer was prevented by covering the buffer reservoir with a foil.

Transfer was done over night. Afterwards the membrane was treated as in immunoblot analysis (3.4.4.).

3.4.7 Analytical Sucrose Density Gradient Centrifugation

To investigate the formation of CLPs from the core fusion-proteins, an analytical sucrose gradient was used. The density is highest at the bottom of the tubes and gets lower towards the top of the tubes. This prevents faster sedimentation of the proteins when moving farther away from the driveshaft. For analysis a sedimentation gradient was used. A gradient with a total volume of 9 ml was used. To do so, a discontinuous sucrose-gradient was prepared in a polycarbonate-tube (Beckmann). First, 60% sucrose (w/v) in TN_{300}-buffer was added to the tube. In steps of 10% the concentration was reduced to 10% sucrose. Afterwards the top sucrose concentration was layered with the sample. Exactly balanced centrifuge tubes were centrifuged for 120 minutes at 40000 rpm at 20 °C. In 14 equal fractions the gradient was then picked up from the top to the bottom. Fractions were analysed for protein by SDS-PAGE (3.4.1.)

3.4.8 Preparative Dialysis

For further usage, samples of the sucrose gradient had to be dialysed. Briefly, peak fractions of the preparative sucrose gradient were pooled and were put into a dialysis tube (Cellulose-ester, 100,000 Da elimination-volumes) and dialysed against buffer TN_{150}. Per 1 ml pooled sucrose fraction, 500-1000 ml TN_{150}-buffer were used. For concentration, the sample was added to a concentration tube (Centricon 100,000 Da elimination-volume) and concentrated by centrifugation.

3.4.9 Triton X-114 phase separation

To reduce the endotoxin content, a method which uses the hydrophobic character of endotoxins (especially the lipopolysaccharides) was applied. In a solution which contains sufficient detergent to reach the critical micelle concentration (CMC), the critical micelle temperature (CMT) is that temperature at which the detergent is mainly present in its micellular form. At a certain temperature above the CMT non-ionic detergents become cloudy and a separation in a detergent-rich and a detergent-

poor phases takes place. This temperature is called “cloudy point” and can be explained by the decrease of the hydrate shell at the polar end.

Triton X-114 is a non-ionic detergent with a CMC of 0.35 mM and a cloudy point of 22 °C. To reach the CMC, the sample was mixed with 1% Triton X-114 (~20 mM) and gently mixed for 20 min at 4 °C. Subsequently, this mixture was incubated for 5 min and heated above the CMC at 25 °C leading to phase separation (turbidity). The two phases were separated by centrifugation for 10 min at 13,000 rpm and 25 °C. After collection of the aqueous phase in a new collection tube, the phase separation was repeated two times. The detergent-rich phase was stored at -20 °C for future analysis. The aqueous phase contains 0.35 mM Triton X-114, however the endotoxin-concentration of samples purified with this method lies between 5 to 500 endotoxin units (EU)/mg protein (Liu *et al.*, 1997).

3.4.9.1 Elimination of excessive Triton X-114

For the elimination of excessive Triton X-114 in the detergent-poor phase, samples were dialysed against PBS. To prevent endotoxin contamination of purified samples sterile buffers and equipment was used. In brief, the sample was added in a sterile dialysis cassette (Pierce, Slide-A-Lyzer, MWCO 10 kDa) using a syringe. Excessive Triton X-114 was removed via dialysis, which means per 1 ml of the sample 1000 ml PBS was used for dialysis. Dialysis was carried out at 4 °C for 12 hours and repeated twice with fresh PBS. Microbial growth in the sample was prevented by sterile filtration and subsequent storage at 4 °C.

3.4.10 Endotoxin determination

To determine endotoxin or lipopolysaccharide (LPS) content of a sample, the *Limulus* amoebocyte-lysate-test was used. *Limulus* amoebocyte-lysate is an aqueous extract of blood cells (amoebocytes) of the horseshoe crab *Limulus polyphemus*. During contact with gram negative bacteria or cell wall extracts, coagulation takes place. This enzymatic reaction is not completely understood, yet; it is known that the enzyme cascade is activated by lipopolysaccharides and in the last a clotting factor is cleaved by an activated clotting enzyme, this leads to coagulation (Levin and Bang, 1964b), (Levin and Bang, 1964a), (Levin and Bang, 1968).

3.4.10.1 Quantification of endotoxin using Pyrotell®

The Pyrotell® endotoxin quantification test is a coagulation-test with a sensitivity of 0.03 EU/ml (threshold value). This means, after reaching the threshold value a tightly formed gel matrix can be observed. The LPS concentration of the samples to be measured can be determined by the preparation of different dilutions. For analysis, endotoxin-free micro tubes and sterile injection water or limulus reagent water (LRW) was used. In brief, the freeze-dried lyophilisate was incubated with reconstitution buffer (Pyrosol) for 5-10 min on ice and mixed gently. To reach the threshold value samples were diluted with LRW in a ratio of 1:100, 1:500, 1:1000, 1:5000 and 1:10000. A standard-row of 10 - 10000 pg/ml LPS was used as positive control. As negative control LRW was used. Afterwards, 100 µl of the sample was added to a sterile micro screw-cap tube and the coagulation was started after addition of 100 µl reconstituted Pyrotell. This mixture was incubated free from vibration for 60 min at 37 °C. To analyse the results of this test the reaction tubes were gently taken out of the thermo-block one by one, and inverted 180 °C. If, after turning of the reaction tube a tight, not collapsing, gel could be observed the test was assessed as positive. The endotoxin-concentration can be calculated with the following formula:

Endotoxin Units /ml (EU/ml) = S x V

S= sensitivity of the test (0.3 EU/ml)

V= lowest positive dilution

The maximum dose of endotoxin approved for intravenous products in humans is 5 EU/kg/h (European-Pharmacopoeia, 2008). As suggested by Malyala and Singh (Malyala and Singh, 2008), this safety value should also be followed in animal experiments as well. The maximum allowed endotoxin concentration of the sample can be calculated with the following formula:

$$Max.EU = 5\ EU \times h / (d/g)$$

h: hour

d: administered dose in mg

g: weight of the animal in kg

In case of immunizations the maximum dose per day is applied (h =24)

$$Max.EU_{day} = 5\ EU \times 24 / (d/g)$$

The results of several LAL-measurements are shown in the appendix in table 9.1.

3.5 Animal experiments

All animal experiments were carried out in accordance with the "*Guide for the Care and Use of Laboratory Animals*" and were approved by the local Animal Care and Use Committee (Animal Care Center, University of Duisburg-Essen, Essen, Germany and the district government of Düsseldorf, Germany).

3.5.1 Anesthetization

For blood withdrawal and immunization mice were anesthetized for several seconds with isofluran vapor. Mice were put in a glass jar filled with Isofluran-soaked cloth until the animals got numb.

3.5.2 Blood withdrawal

Blood withdrawal was carried out from anesthetized mice by retroorbital punction using 3 mm heparin-coated glass capillaries. The blood was collected in 1.5 ml Eppendorf tubes.
Blood withdrawal from guinea pigs was done by punctuation of the *Vena saphena lateralis* using 0.4×19 mm needles. The blood was collected in 1.5 ml Eppendorf tubes.

3.5.3 Immunization trials

Intramuscular (i.m.) injections of mice were performed into the *Tibialis anterior* muscle of the hind limbs using 0.4×19 mm needles. Subcutaneous (s.c.) injections were administered into the loose skin fold of the neck skin. Peptides were diluted to the given concentration in 1 ml of sterile PBS and equal volume of 100 µl was injected into each muscle (50 µl per leg), 100 µl was injected into the neck skin fold.

Each vaccine preparation was equally diluted 1:2 in incomplete Freund's adjuvant (IFA) or AS03.

Subcutaneous injections of guinea pigs were also administered into the loose skin fold of the neck skin. Peptides were diluted to the given concentration in 1 ml of sterile PBS and equal volume of 200 µl was injected into the loose skin fold of the neck skin. Each vaccine preparation was equally diluted 1:2 in incomplete Freund's adjuvant.

3.5.3.1. Immunization of C57BL/6 mice with four different HBc-HVRI plasmids

Five different groups (each group contained 6 mice, 4 mice formed the control group) of ten week old female C57BL/6 mice were vaccinated subcutaneously with 20 µg peptide in IFA at day zero. Mice were boosted at day 14, 28 and 56 with µg peptide in IFA. Serum obtained after 1st (day 28), 2nd (day 56) and 3rd (day 84) immunization was pooled for further analysis. Serum taken at day zero served as zero value. Mice which were immunized with wild type HBc-protein served as controls. Mice were sacrificed one week after the last immunization.

3.5.3.2. Immunization of C57BL/6 mice with a pool of four different HBc-HVRI plasmids

A group of six ten weeks old female C57BL/6 mice were injected (s.c.) with a pool of four different HBc-HVRI plasmids (5 µg/variant) in IFA at day zero. Mice (four animals) which were immunized with wild type HBc-protein (20 µg) served as controls. The immunization scheme was done as explained in 3.5.3.1.

3.5.3.3. Immunization of C57BL/6 mice with a pool of four different HBc-HVRI plasmids; second trial

Three different groups of ten week old female C57BL/6 mice were vaccinated with a pool of four different HBc-HVRI plasmids (5 µg/variant). The first group which comprised 10 mice was immunized i.m. with in AS03. The second group comprised 5 mice which were immunized s.c. with 5 µg/variant in IFA. The last group of 2 mice served as control group. In this group mice were immunized i.m. with 100µl PBS (50 µl per leg) in AS03. In all groups mice were immunized three times every two weeks with the indicated formulation. Blood was taken at day 0, 14, 28 and 42 and serum was used for further analysis.

3.5.3.4. Immunization of guinea pigs (*Cavia procellus*) with a pool of four different HBc-HVRI plasmids

A group of five six weeks old female guinea pigs (*Cavia procellus*) were injected s.c. with 5 µg/variant vaccine preparation in IFA at days 0, 30 and 90. Blood withdrawal was done at days 0, 30, 90 and 120 and serum was used for further analysis. Guinea pigs were sacrificed one week after the third immunization. The control group (5 animals) was treated as explained above. These animals were immunized with 20 µg wild type HBc-protein.

3.6 Enzyme-linked Immunosorbent Assay (ELISA)

The technique of ELISA was used to detect the presence of HCV HVRI-specific antibodies in mouse and guinea pig serum after immunization (3.5.3) and to characterize the IgG-subclasses.

Nunc immunoplates were coated with 50 ng/well protein or 500 ng/well peptide in coating-buffer and kept over night at 4 °C. Excess protein/peptide was removed by washing the plates with ELISA-wash buffer (200 µl/well). After blocking unsaturated binding sites with blocking buffer (200 µl/well; 1h at 4 °C), nunc plates were washed again three times using ELISA-wash buffer. Preparing serial dilutions (dilution row depends on the sample), 200 µl/well of serum sample diluted in PBS/0.1% BSA was added to the plates and incubated slightly shaking 2 h at RT. Unbound antibody was removed by washing the plates three times with ELISA-wash buffer. Detection of the bound antibodies was carried out by adding 100 µl/well of HRP-coupled isotype specific secondary antibody in PBS/0.1% BSA for 1 h slightly shaking at RT. Unbound antibodies were again removed by washing the plates three times with ELISA-wash buffer. 100 µl of OPD substrate solution was added to each well in order to detect peroxide activity, and the plates were incubated in the dark for 10-30 min. The reaction was stopped by adding 25 µl/well of stop solution. Finally, the OD_{492nm} was measured using the Elisa Reader Asys Expert Plus.

3.7 Electron microscopy

Fractions from the sucrose gradient were examined by electron microscopy. Particles were visualized after being negatively stained with 2 % uranyl acetate. Micrographs were taken at a magnification of 140,000. Electron microscopy was done by the department of Pathology at the University Hospital in Essen.

4 Results

4.1 Generation and characterization of SplitCore-HVRI-CLPs

The best way for the induction of a broad immune response to HCV would probably comprise the presentation of the total range of epitopes or highly conserved epitopes (e.g. AP33) of the E2 protein on CLPs. However, previous results showed that the complete E2 (E2-655) is insoluble and due to this cannot be purified (master thesis M. Lange, 2009). Furthermore, the highly conserved epitope AP33 is not able to form particles and thus is unsuitable for a candidate vaccine (master thesis M. Lange, 2009). Nevertheless, during the last decades it was demonstrated that HCV uses the HVRI to escape from immune response (von Hahn *et al.*, 2007). On the other hand, this region is essential for virus entry into the cell and contains a neutralizing epitope (Scarselli *et al.*, 2002), (Kato *et al.*, 1992). Moreover, the use of conserved HVRI mimotopes has already been proposed to overcome problems of restricted specificity which means that Abs targeting the conserved residues may still have therapeutic promise (Cerino *et al.*, 2001), (Roccasecca *et al.*, 2001), (Zucchelli *et al.*, 2001). Therefore, the HVRI might be an important target to inhibit HCV from entering the cell and prevent infection.

The scope of this work was to find an improved and optimized vaccine strategy to induce a potent B-cell response against the HVRI in mice. For this purpose, particles expressing four different HVRI-variants on HBV-CLPs were used and HVRI mimotopes were compared to naturally occurring HVRI-variants (Fig. 4.1.A). The immunogenicity of small peptides, like the HVRI, can be considerably enhanced when presented on HBV-CLPs. The most promising insertion site was shown to be the c/e1-epitope located at the tips of the spikes of the core protein (Wynne *et al.*, 1999). To avoid miss-folding of core particles due to sterical constrains after fusion of the peptides to the core protein, the so-called SplitCore-system was used (Fig. 4.1.B). Four different variants of the HVRI, called R9, G31, YK5829 and YK5807, were generated by PCR (see 3.3.1). R9 and G31 are artificial mimotopes, generated in the laboratory of Alfredo Nicosia (Puntoriero *et al.*, 1998). YK5829 and YK5807 are naturally occurring variants which were isolated from patients (kindly provided by Yury Khudyakov). The amino acid sequence of the different HVRI-variants is shown

in Fig. 4.1.A. In order to expose the conserved C-terminal domain on the surface of the CLPs, the HVRI-variants were fused to CoreN (for cloning strategy and vector-cards see appendix 9.1).

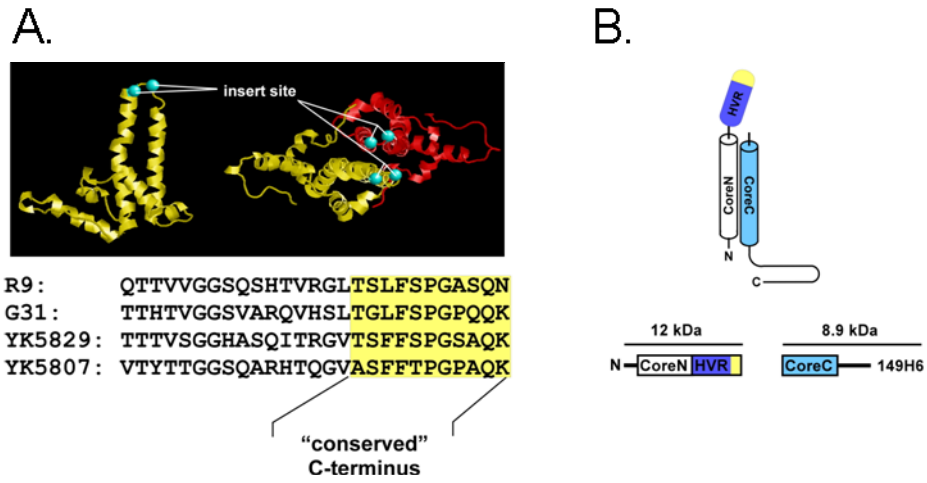

Fig. 4.1: Generation of SplitCore-CLPs displaying 4 different variants of the HVRI

A. 4 different variants were fused to the N-terminal part (CoreN) of the HBc-protein. The insertion site (blue circle) is located at the tips of the spikes of the core protein (the core protein with its insertion site is shown from the side- and from the top- view). R9 and G31 are artificial mimotopes, generated in the laboratory of Alfredo Nicosia; Dipartimento di Biologia Cellulare e dello Sviluppo, Università di Palermo, Italy. YK5829 and YK5807 are naturally occurring variants which were isolated from patients in the laboratory of Yury Khudyakov; Centers For Disease Control and Prevention, Atlanta, USA. The 4 variants show highly variable parts as well as a more conserved region located at the C-terminus (shown in yellow). **B.** By usage of the SplitCore-system, the translation of the core protein is initiated by the ribosome-binding-site at CoreN followed by the fused HVRI-variant. Translation of CoreC is mediated by the directly joined second ribosome binding site (RBS). The nucleic acid binding domain at CoreC was removed and is now followed by 6 histidines indicated by "149H6". The conserved part (yellow) of the HVRI-variants is exposed to the outside at the tips of the spikes of HBc.

4.1.1 HVRI-CLPs displaying different HVRI-variants are assembly competent

After successful generation of the four different constructs, namely SplitCore-R9, SplitCore-G31, SplitCore-YK5807, SplitCore-YK5829, their ability to form CLPs was analyzed (Fig. 4.2). SplitCore protein without any foreign protein fusion (SplitCore183-St1) was used as positive control (K). After protein expression of SplitCore-HVRI-CLPs in BL21*CP cells (see 3.1.3), cells were lysed under non-denaturing conditions (see 3.1.4) and the cell lysate was added to a preparative sucrose gradient (see 3.4.7). After centrifugation 14 fractions were collected from the top and were separated by SDS-PAGE (15%) (see 3.4.2). SplitCore-HVRI-CLPs showed co-sedimentation of CoreN and CoreC fragments into the particle typical fractions 7 to 9 (Vogel *et al.*, 2005). Some material was also detected in fractions 10

to 13 (Fig. 4.2 A, lane 7-13). The 12 kDa band corresponds to the CoreN-HVRI-fragment (white arrow), the band below 9 kDa to the CoreC fragment (green arrow). These results indicate that all 4 SplitCore-HVRI-constructs are expressed in nearly equimolar amounts, complement each other and assemble to CLPs. Identity of CoreN and CoreC fragments was analyzed by western blotting using mAb 10E11 (CoreN) and mAb 13A9 (CoreC). A band which corresponds to the right size (9 kDa) could be detected with 10E11 in fractions 6 to 11 in all SplitCore-HVRI-constructs, indicating that the CoreN-fragment was intact (Fig. 4.2 B). Also a band which corresponds to the right size (12.2 kDa) could be detected in all SplitCore-HVRI-constructs in fractions 6 to14 (Fig. 4.2 C). Further confirmation of CLP formation was done by native agarose gel electrophoresis (NAGE) (see 3.4.3 and 3.4.6). The different fractions of the gradient were applied to a native agarose gel (1 %) and the proteins in their native form were separated according to their surface charge. In principle, protein monomers diffuse strongly and appear as smear, while particulate structures move through the gel as distinct bands. Aggregates are too large to enter the gel and typically remain in the slot. Upon staining with Coomassie, distinct bands could be detected in fractions 6 to 8 indicating formation of particulate structures (Fig. 4.2 C). The blue smears, which appeared specifically in the first lanes, indicated protein monomers. Particle formation could be observed in fractions 6 to 14 in all SplitCore-HVRI-constructs, using either the particle-specific antibody 3120 or the CoreN-specific antibody 10E11 (Fig. 4.2 C). Further confirmation of CLP-formation was shown by electron microscopy (Fig. 4.2 D). Material from peak fraction number 8 was negatively stained with 2% uranyl acetate and investigated at a magnification of 140,000 showing regular shaped CLPs. Thus, the possibility of chimeric HBc-protein bearing the HVRI to form particles was demonstrated.

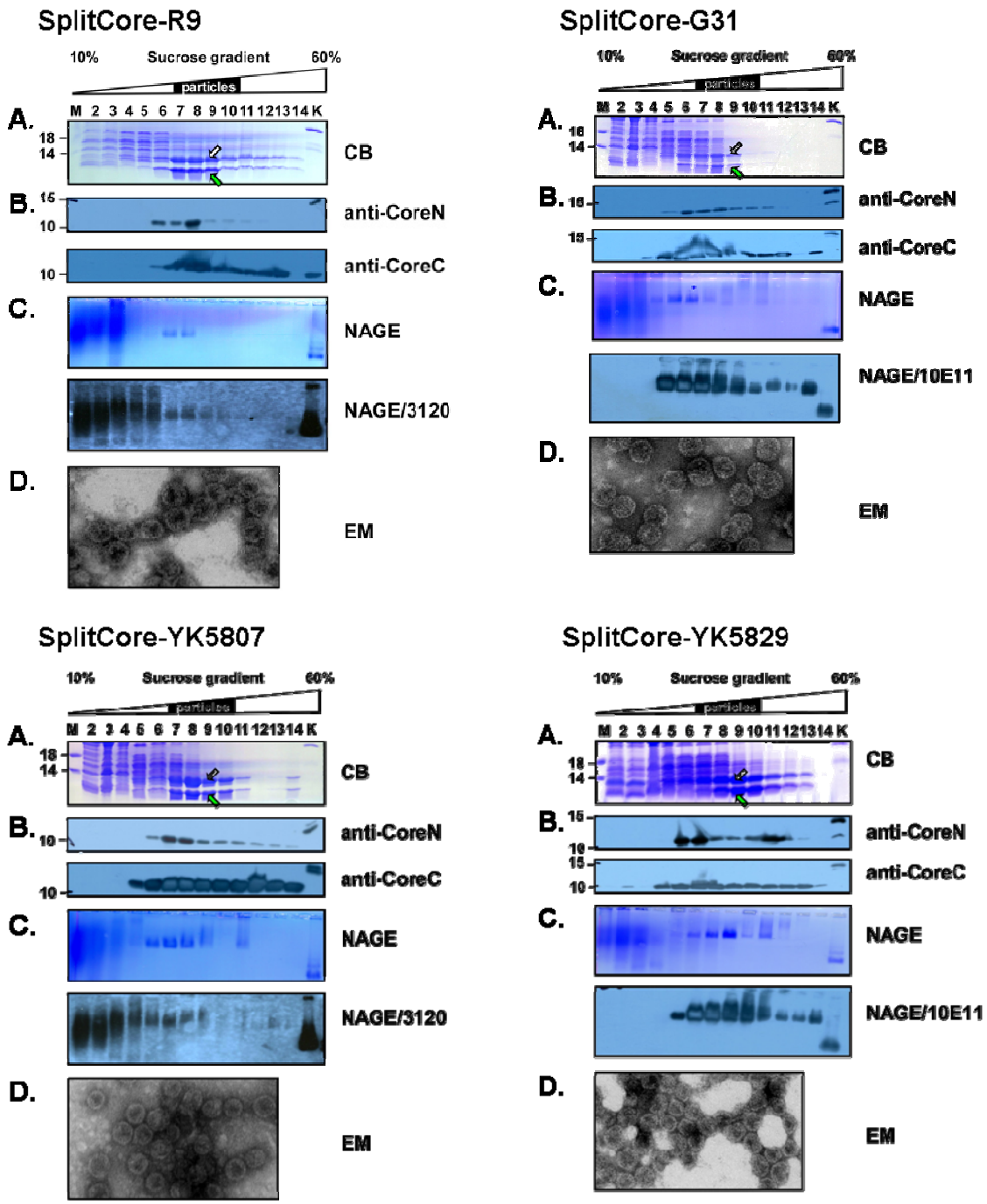

Fig. 4.2: HVRI-CLPs displaying different HVRI-variants are assembly competent

A. Coomassie SDS-PAGE After sedimentation of non-denatured cell lysate on a sucrose gradient, 14 fractions were separated on a 15% SDS-gel and proteins were visualized with Coomassie. The arrows indicate the position of bands of the corresponding HVRI-variant in the gel. Marker: PeqGold protein marker I; PeqLab. Wild-type core protein served as positive control (K). **B. CoreN/CoreC-specific WB** Fractions were separated by SDS-PAGE and transferred to a PVDF-membrane. Detection was done with the CoreN specific Ab, 10E11, or the CoreC specific Ab 13A9 and a second antibody coupled to peroxidase. Marker: PeqGold protein marker IV; PeqLab. Wild-type core protein served as positive control. **C. CLP-specific native agarose gel (NAGE)** 10 µl aliquots of gradient fractions were loaded on a 1% agarose gel visualized by Coomassie. Particulate structures appear as distinct bands in particle-typical fractions. Alternatively, proteins on an agarose gel were transferred to a PVDF-membrane. Detection was done with the particle-specific Ab 3120 or 10E11. Wild-type core protein served as positive control. **D. EM-picture** HVRI-CLPs were stained with 2% uranyl acetate and investigated by electron microscopy (Pathology, UK Essen).

4.2 Immunization with HVRI-CLPs elicits an HVRI-specific immune response in mice

After demonstrating that HVRI-SplitCore fusion proteins are able to assemble to regular particles, they were purified, concentrated and Endotoxin extracted. Subsequently, the immunogenicity of these HVRI-CLPs was analyzed in mice.

4.2.1 Single HVRI-CLPs are highly immunogenic in mice

In order to test the immunogenicity of the HVRI-CLPs, 4 groups of C57BL/6 mice were immunized with 20 µg of HVRI-CLPs s.c. in IFA on days 0, 14, 28 and 56 (see 3.5.3.1). Serum titers were determined after bleeding at days 28, 56 and 84 by ELISA specific for the HVRI-variants (Fig. 4.3).

Twenty-eight days after immunization, antibody titers reached values of 12,000 to 15,000 in all variants. Mice immunized with R9 showed antibody titers of up to 24,000 (Fig. 4.3, day 28). After the last booster immunization at day 56, antibody titers of 24,000 could be detected for R9 and G31. Serum titers of mice immunized with YK5807 and YK5829 increased to 30,000 (Fig. 4.3, day 56). Twenty-six days after the last immunization (day 84) serum titers remained constant for G31, antibody titers of mice immunized with R9 and YK5829 raised to 30,000 in contrast to serum titers of YK5807 which declined again from 30,000 to 24,000 (Fig. 4.3, day 84).

As a result, except for G31 which showed the highest antibody titers at 24,000, the remaining construct yielded antibody titers of up to 30,000 after the last bleeding (day 84). This shows a very high and early antibody response in mice against the presented HVRI-variant.

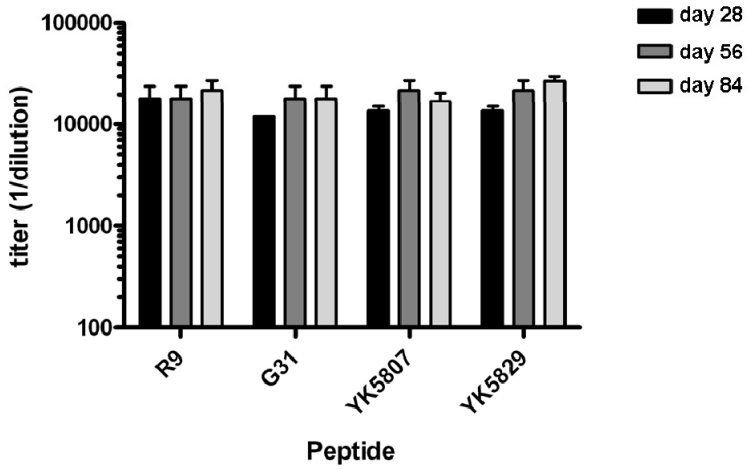

Fig. 4.3: Antibody response titers of sera from mice immunized with single HVRI-CLPs

Groups of 6 mice (C57BL/6-mice) were immunized s.c. with 4 different HVRI-variants (20 µg/variant) in IFA. Mice were immunized at days 0, 14, 28 and 56. Bleeding was done 4 times at days 0, 28, 56 and 84. Serum obtained after each bleeding was tested for specific antibodies in ELISA. Serial dilutions were made and dilutions yielding at least 2 times the optical density at λ=495 nm obtained with pre-immune serum (day 0) were scored positive. Bars represent the mean of 3 replicate values and the error bars represent standard error of the mean (S.E.M.) -values.

As core particles were shown to increase the immunogenicity of the presented peptides and they are expected to show a very high immunogenicity by themselves, in addition, the antibody response to the core protein was analyzed. Again serum titers were determined after bleeding at days 28, 56 and 84 by core-specific ELISA. As assumed, antibody titers were up to 80 times higher compared to titers against the HVRI-variants (Fig. 4.4). After 28 days each construct showed antibody titers of 60,000. Mice immunized with G31 generated titers of up to 2,400,000 (Fig. 4.4, day 28). At day 56 antibody titers increased to 2,400,000. Only the R9 titers remained constant at 60,000 throughout the immunization (Fig. 4.4, day 56). Twenty-six days after the last immunization (day 84), all serum titers remained constant (Fig. 4.4, day 84).

Analysis of the antibody response of serum from mice immunized with single HVRI-variants confirmed the high immunogenic potential of the core protein.

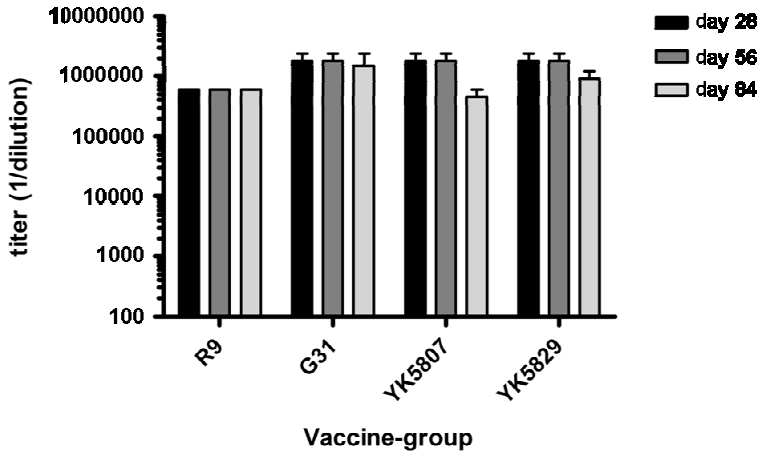

Fig. 4.4: Antibody response to the HBc-protein of sera from mice immunized with single HVRI-CLPs

Serum obtained from mice after each bleeding was tested for antibodies against the HBc-protein in ELISA. For more details see Fig. 4.3.

4.2.2 Single HVRI-CLPs induce partially cross-reactive antibodies

In order to test the cross-reactive potential of serum from mice immunized with single HVRI-variants, anti-sera (after day 84) of each group was tested against homologous and heterologous HVRI-variants in ELISA (Fig. 4.5). Monoclonal Ab against R9 was used as control. As expected, heterologous variants showed high antibody titers of 24,000 in each case (shaded in grey). Serum of mice immunized with the R9 variant (anti-R9) showed antibody titers against the heterologous variants YK5807 and YK5829 of 3000. A relatively low titer of 750 was measured against the heterologous variant G31 (Fig. 4.5). Anti-G31 serum reacted weakly (titer of 750) with the R9 variant. However, very high titers of 12,000 could be detected against the heterologous variants YK5807 and YK5829 (Fig. 4.5). Except for the very low antibody titer of <750 to R9, anti-YK5807 serum showed titers of 3,000 and 6,000 to the heterologous variants G31 and YK5829 respectively (Fig. 4.5). Anti-YK5829 serum yielded a titer of 6,000 to the R9 variant but only an antibody titer of 750 or less could be detected against G31 and YK5807 (Fig. 4.5). The mAb R9 reacted only with the R9 peptide (titer of 3,000 at a concentration of 1.2 µg/µl Ab) (Fig. 4.5).

As shown, analysis of the cross-reactive potential of serum from mice immunized with single HVRI-variants, revealed variable reactivity across the different groups. In general, serum of all groups showed potential for the induction of cross-reactive antibodies which could certainly be increased.

Cross-reactivity (peptide level)

Sera \ Peptide	R9	G31	YK5807	YK5829
anti-R9	24000	750	3000	3000
anti-G31	750	24000	12000	12000
anti-YK5807	<750	3000	24000	6000
anti-YK5829	6000	<750	750	24000
mAb R9	3000	-	-	-

Fig. 4.5: Single HVRI-CLPs induce partially cross-reactive antibodies

Cross-reactive potential in ELISA (day 56). Serum of mice immunized with single HVRI-variants was tested for its cross-reactivity to the 4 different peptides used for immunization. Titers of homologous variants are shaded in grey. Titers of heterologous variants are white. Serial dilutions were made and dilutions yielding at least 2 times the optical density at λ=495 nm obtained with pre-immune serum were scored positive. Monoclonal Ab R9 (kindly rovided by P. Pumpens; Riga, Latvia) only reacted with the R9 peptide.

4.3 Characterization of the neutralizing capacity of mouse sera after immunization with single HVRI-CLPs

As already described previously, increasing evidence indicates that neutralizing antibodies play an important role in controlling HCV infection (Shimizu *et al.*, 1994), (Shimizu *et al.*, 1996). It was shown, that despite the induction of cross-neutralizing Abs in the late phase of infection, lack of neutralizing Abs in the early phase of infection was associated with the development of chronic HCV. Instead viral clearance was associated with rapid induction of nAbs in the early phase of infection (Pestka *et al.*, 2007). As the HVRI of HCV contains a major neutralizing epitope and is involved in viral entry, the induction of nAbs against the HVRI in the early phase could help to control HCV infection. Therefore, the neutralizing capacity of the anti-sera to the HVRI was investigated.

4.3.1 Evaluation of the neutralizing capacity to homologous HVRI-variants after single immunization

To assess whether the HVRI-peptide specific response measured by ELISA could neutralize autologous HCVpp infectivity, sera (day 84) of mice immunized with single HVRI-variants were screened at different dilutions (1:25, 1:50, 1:100 and 1:200) for their ability to neutralize HCVpp infectivity bearing the four HVRI-variants included in

the vaccine. HCVpp's encoding a luciferase reporter were pre-incubated with pre- or post-immune sera for 2h and allowed to infect Huh-7.5 hepatoma cells. After 72h post-infection luciferase activity was measured and infectivity in the presence of pre-immune serum was compared to infectivity of post-immune serum. This was used to determine the percentage neutralization which is defined to start at 50% (Fig. 4.6; black continuous line) (see 3.2.7). Sera from all four groups of single immunized mice showed high neutralizing capacity against the homologous HCVpp (Fig. 4.6). R9 serum inhibited homologous HCVpp infectivity still by 70% at a dilution of 1:200 (Fig. 4.6 A, black dots). In comparison, G31 serum was able to show 70% neutralization of G31 HCVpp at a dilution of 1:100. Neutralization with 1:200 diluted serum revealed borderline neutralization (Fig. 4.6 B, black squares). At a 1:200 dilution, serum of HVRI-variants YK5807 and YK5829 still inhibited homologous HCVpp infectivity by 67% and 60% respectively (Fig. 4.6 C & D, black open dots & triangles). Sera from all groups of mice were also able to neutralize HCVpp of the other groups to a different extend. G31, YK5807 and YK5829 serum showed cross-neutralization of R9 HCVpp of 60 to 80% at a dilution of 1:50 (Fig. 4.6 A), whereas R9, YK5807 and YK5829 serum was hardly or not at all able to neutralize G31 HCVpp (Fig. 4.6 B). In contrast, YK5807 HCVpp could be neutralized relatively efficiently at a 1:50 dilution and declined to borderline neutralization at a 1:100 dilution (Fig. 4.6 C). YK5829 HCVpp were still neutralized efficiently at a dilution of 1:100 and started to become inefficient at a 1:200 dilution (Fig. 4.6 D).

Analysis of the neutralizing capacity of sera from single immunized mice demonstrated high neutralization of homologous variants and even cross-neutralizing capacity. However, none of them was able to neutralize all four variants efficiently.

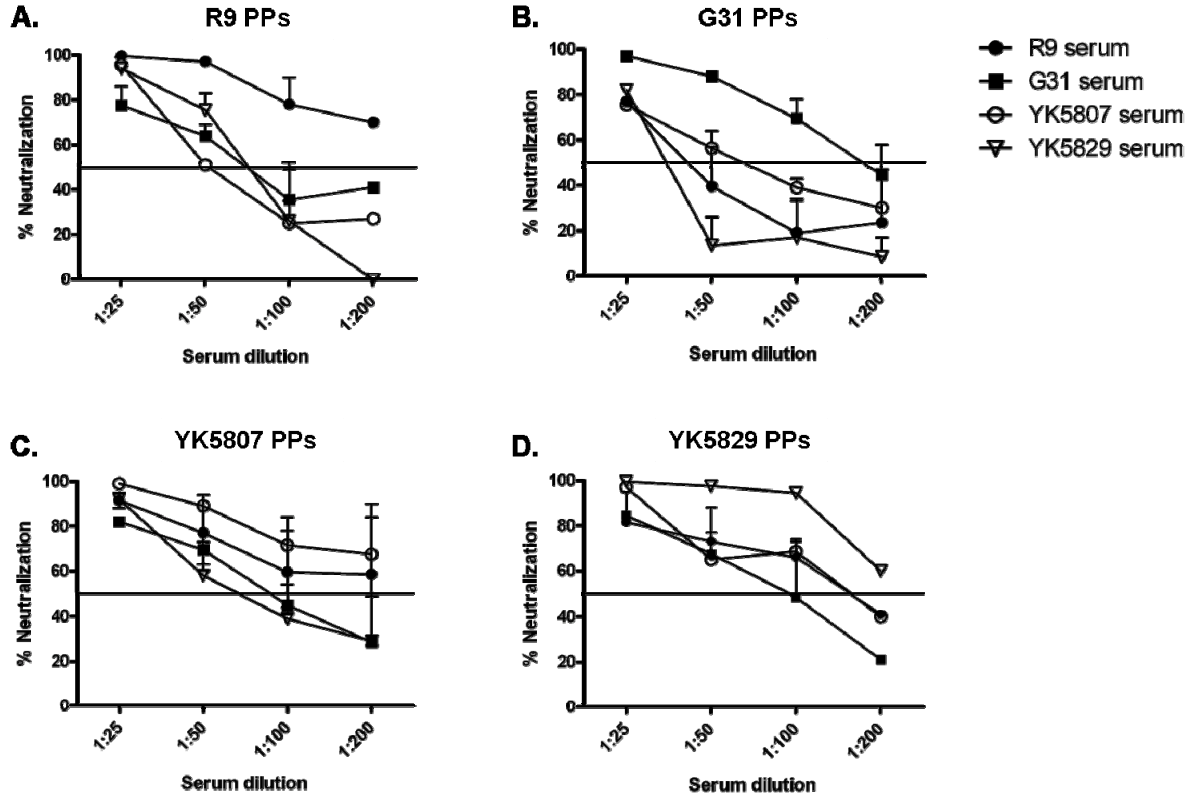

Fig. 4.6: Neutralizing capacity to homologous HVRI-variants after single immunization in mice

Day 84-serum of mice immunized with single HVRI-variants was tested in different dilutions (x-axis) for neutralization of HCVpp expressing the 4 HVRI-variants included in the vaccine. Percent neutralization was determined by comparing infectivity (luciferase relative light units – RLU) of HCVpp not incubated with serum to the infectivity in the presence of test immune sera. Symbols represent the mean of 3 replicate values (pooled serum per group) and the error bars represent S.E.M. values.

4.3.2 Evaluation of the neutralizing capacity to heterologous HVRI-variants after single immunization

To further study the breadth of the immunizing response in mice, sera were also tested for their ability to neutralize HCVpp expressing diverse HVRI-variants. Fig. 4.7 shows the amino acid sequences of the HVRI-peptides included in the vaccine (labelled in black), compared to the ones used to analyze the cross-neutralizing capacity of sera (labelled in grey). These three different HCVpp comprise HCV genotypes 1a, 1b and 2a.

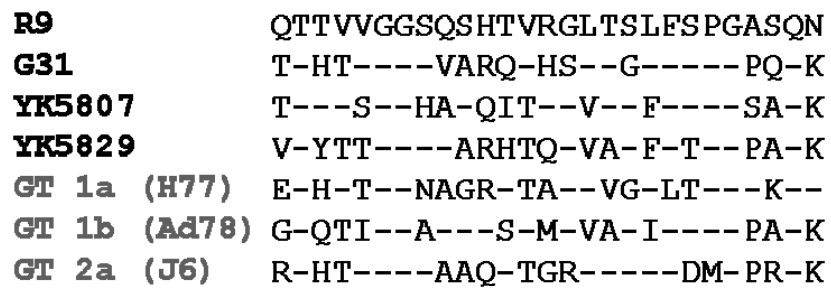

Fig. 4.7: HVRI-sequences used for the construction of HCVpp

To test cross-neutralizing capacity of the serum, beside the generation of HCV pseudoparticles expressing HVRI-variants included in the vaccine (indicated in black), also HCVpp bearing HVRI-variants of other genotypes were generated (indicated in grey). H77 HCVpp are GT1a, Ad78 HCVpp are derived from GT1b (these plasmids were kindly provided by S. Viazov from our Institute) and J6 HCVpp are GT2a (kindly constructed by A. Walker from our Institute).

Antibody responses generated following immunization with four different HVRI-variants were able to neutralize infection of HCVpp bearing HVRI-variants of genotype 1a (H77) and genotype 2a (J6) but were less efficient against genotype 1b (Ad78) (Fig. 4.8). In brief, all four sera were able to efficiently neutralize H77 HCVpp with 50% to 80% at a dilution of 1:100. At a dilution of 1:200 sera started to become inefficient (Fig. 4.8 A). In contrast to that, only at a dilution of 1:25 R9, G31 and YK5807 serum was able to neutralize Ad78 HCVpp. The only exception was shown by YK5829 serum which inhibited infection up to a dilution of 1:100 (Fig. 4.8 B). J6 HCVpp could be neutralized more efficiently. Sera of mice showed neutralization of approximately 70% at a 1:50 dilution and then started to decline drastically. Only G31 serum was not able to neutralize infection at all (Fig. 4.8 C).

Overall this shows that sera of mice immunized with single HVRI-variants were to some extend able to neutralize naturally HCV strains which bear quite different HVRI-sequences from the ones used for immunization.

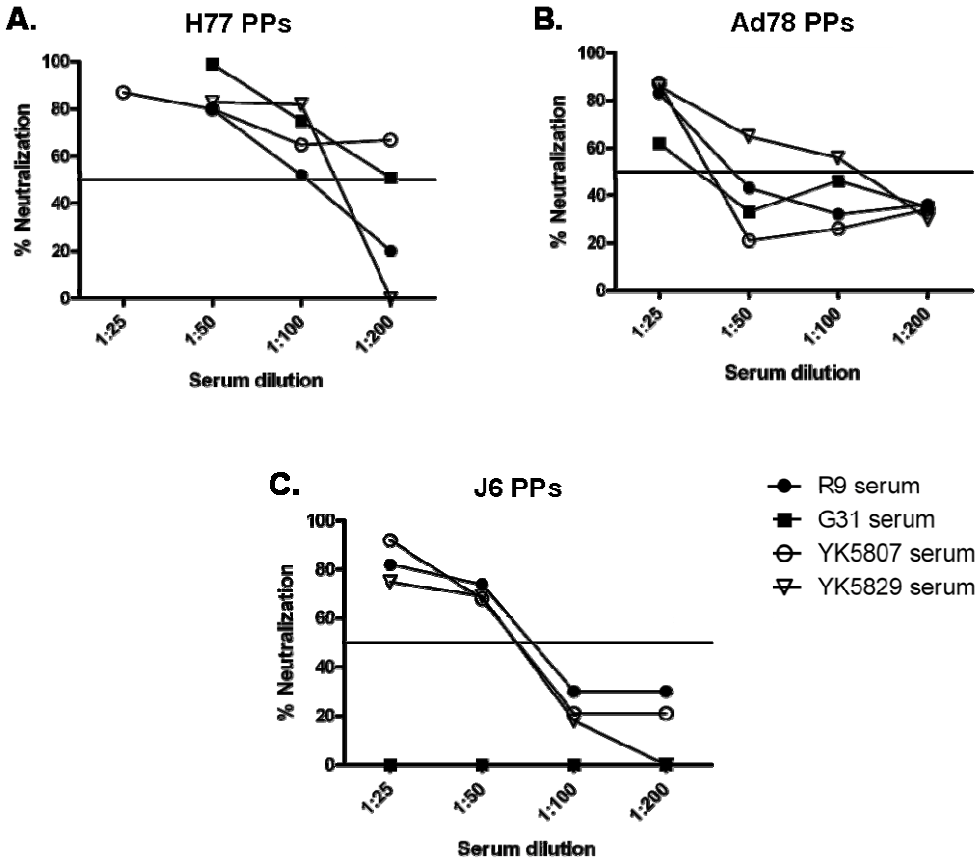

Fig. 4.8: Neutralizing capacity to heterologous HVRI-variants after single immunization in mice

Serum of mice immunized with single HVRI-variants, was obtained 84 days after immunization and tested in different dilutions for neutralization of HCVpp expressing HVRI-variants not included in the vaccine. Percent neutralization was determined by comparing infectivity RLU of HCVpp not incubated with serum to the infectivity in the presence of test immune sera. Symbols represent the mean of 3 replicate values (pooled serum per group) and the error bars represent S.E.M. values.

4.4 A mixture of HVRI-CLPs induces high titers of cross-reactive antibodies

Based on the previous results and in order to increase the cross-reactivity of the anti-serum, a second immunization scheme was applied (see 3.5.3.2). A group of C57BL/6 mice was immunized s.c. with a pool of the four different HVRI-variants. The mice were immunized with 5 µg/variant (total of 20 µg) in IFA at days 0, 28, 56 and 84. Antibody titers were determined by ELISA specific for the HVRI-variants. Two weeks after the first immunization anti-serum of variants G31, YK5807 and YK5829 showed titers of up to 15,000. The R9 group reached an antibody titer of 30,000 (Fig. 4.9, day 28). At day 56 antibody titers of groups R9, G31 and YK5829 remained constant. In contrast to that, the titer of group YK5807 increased to 30,000 (Fig. 4.9, day 56). Serum titers of groups R9, YK5807 and YK5829 remained

constant after the last booster immunization. An increase of the titer from 15,000 to 60,000 could be detected for the anti-serum of group G31 (Fig. 4.9, day 84).
In summary, immunization of mice with a pool of the four HVRI-variants induced a higher antibody titer compared to mice immunized with single variants.

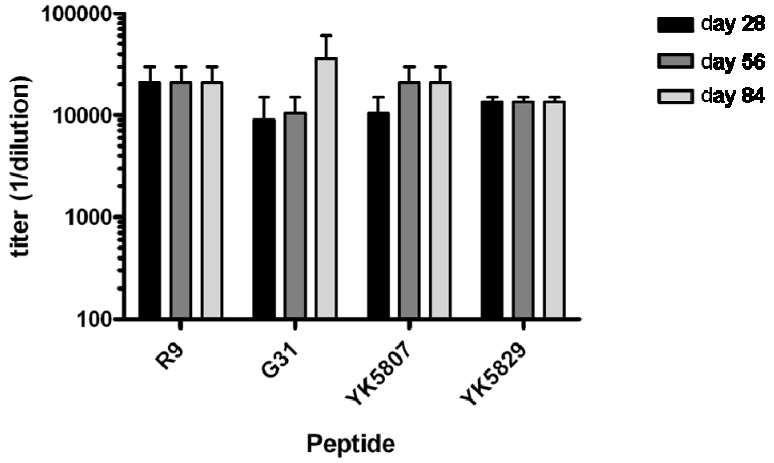

Fig. 4.9: Antibody response titers of sera from mice immunized with a mixture of HVRI-CLPs
A group of 6 mice (C57BL/6-mice) was immunized s.c. with a mixture of 4 HVRI-variants (5 µg/variant) in IFA. For more details see Fig. 4.3.

Further, the anti-core response was analyzed in these mice. After the second immunization at day 28, an antibody titer of 600,000 could be detected which increased to 1,200,000 at day 56 (Fig. 4.10). Twenty-eight days after the last immunization the antibody titer increased again to 2,400,000 (Fig. 4.10, day 84).
In conclusion the antibody response to the core protein yielded very high titers which are, like in the first immunization scheme, around 80 times higher compared to the anti-HVRI response. This confirms again the high immunogenic potential of the vaccine carrier.

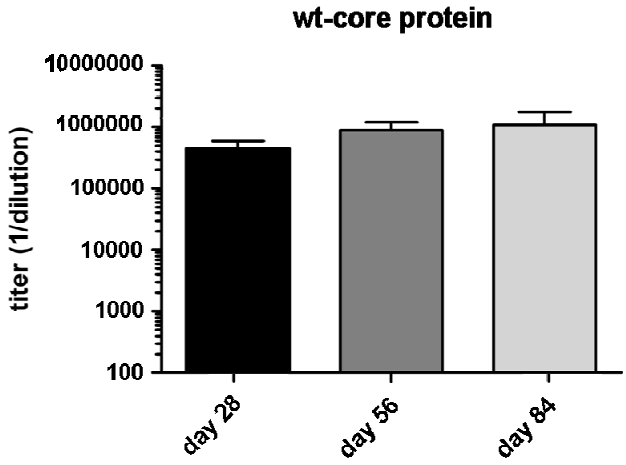

Fig. 4.10: Antibody response to the HBc-protein of sera from mice immunized with a mixture of HVRI-CLPs

Serum obtained from mice after each bleeding was tested for antibodies against the HBc-protein in ELISA. Serial dilutions were made and dilutions yielding at least 2 times the optical density at λ=495 nm obtained with pre-immune serum (day 0) were scored positive. Bars represent the mean of 3 replicate values (pooled serum per group) and the error bars represent S.E.M. values.

Next, the true cross-reactive potential of the anti-sera was tested. Therefore, the binding capacity of sera to 326 randomly chosen HVRI-sequences from patients was analyzed by ELISA. To analyze whether immunization with the mixture of CLPs induced more cross-reactive Abs compared to the immunization with single HVRI-CLPs, these two sera were compared. As shown in Fig. 4.11 A, the mixture (mixture immunization) reacted with about 25% of heterologous HVRI peptides compared to the pool of single sera (single immunization) which reacted with 19% of heterologous HVRI peptides. Also the strength of the response was much better when the mice were immunized with all four HVR-CLPs simultaneously compared to serum from mice immunized with single HVRI-variants (Fig. 4.11. B). Furthermore, it should be pointed out, that the anti-sera were tested in a 1:800 dilution which means that most likely an even higher antibody response could be detected with more concentrated serum. The experiments were carried out in the laboratory of Yury Khudyakov at the Centers For Disease Control and Prevention, Atlanta, USA.

As a result, a mixture of HVRI-CLPs induced high titres of anti-HVRI antibodies, which were able to cross-react with 25% of naturally occurring HVRI, demonstrating that HVRI-CLPs are indeed able to induce cross-reactive HCV antibodies.

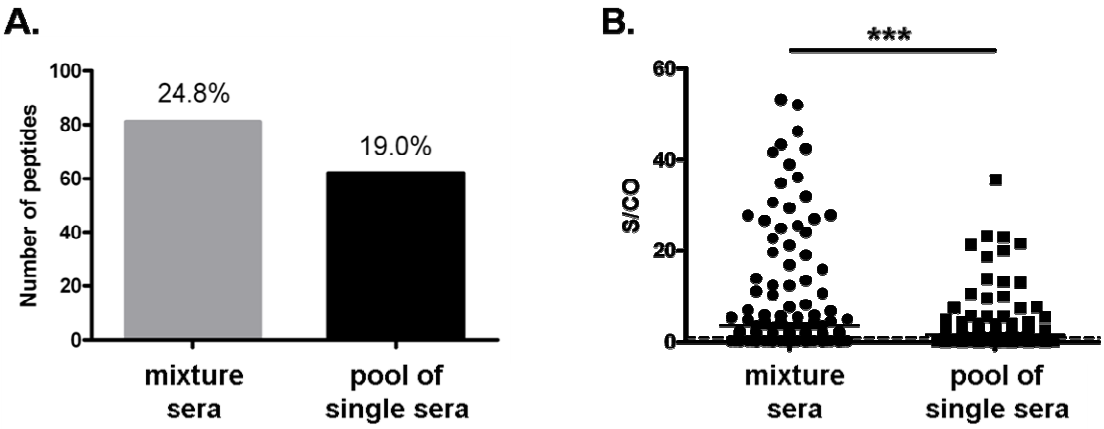

Fig. 4.11: Reactivity of mouse serum with patient derived HVRI-variants

A. The cross-reactive potential of mouse serum with 326 randomly chosen HVRI-variants was analyzed by ELISA. Mixture sera (mice immunized with the mixture of HVRI-CLPs) reacted with 24.8% of peptides tested. Pool of single sera (mixture of sera from mice immunized with the single HVR-CLPs) reacted with 19% of peptides tested. **B.** The strength of the response of mouse serum with patient derived peptides was tested by ELISA. Peptides giving a signal to cutoff (S/CO) > 1 were scored positive (p-value = 0.0001). Serum was tested in a dilution of 1:800. Experiments were carried out in the laboratory of Yury Khudyakov; Centers For Disease Control and Prevention, Atlanta, USA.

4.5 Characterization of the neutralizing capacity of mouse sera after mixture immunization with HVRI-CLPs

After showing an improved antibody response in mice immunized with a mixture of HVRI-variants, this hypothesis was also proven in neutralization experiments.

4.5.1 Evaluation of the neutralizing capacity to homologous HVRI-variants after mixture immunization

In the first series of experiments, the ability of sera (day 84) from mice immunized with the mixture of HVRI-variants to neutralize HCVpp expressing the four variants included in the vaccine was tested (Fig. 4.12). HCVpp bearing HVRI-variants R9, YK5807 and YK5829 were neutralized very efficiently. At a dilution of 1:200 the serum was still able to neutralize R9 and YK5829 HCVpp with approximately 90% (Fig. 4.12, black dots and open triangles). At the same dilution YK5807 HCVpp neutralization showed borderline values, but a 1:100 dilution revealed neutralization of 80% (Fig. 4.12, open dots). Only HCVpp bearing G31 could not be neutralized as efficiently as the other variants. Serum did not show further neutralization at higher dilutions after a neutralization of 60% at a 1:50 dilution (Fig. 4.12, black squares).

As predicted from ELISA data, the sera of mice immunized with the mixture showed significantly higher neutralization ability (with the only exception to neutralize G31 HCVpp) than sera from mice immunized with single HVRI-variants.

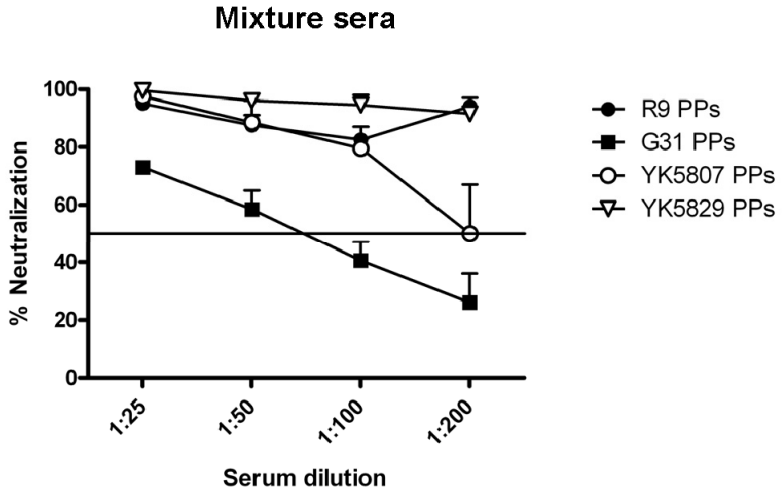

Fig. 4.12: Neutralizing capacity to homologous HVRI-variants after mixture immunization in mice

Day 84-serum of mice immunized with a mixture of 4 HVRI-variants was tested in different dilutions for neutralization of HCVpp expressing the 4 HVRI-variants included in the vaccine. Percent neutralization was determined by comparing infectivity RLU of HCVpp not incubated with serum to the infectivity in the presence of test immune sera. Symbols represent the mean of 3 replicate values (pooled serum per group) and the error bars represent S.E.M. values.

4.5.2 Evaluation of the neutralizing capacity to heterologous HVRI-variants after mixture immunization

The next series of experiments was directed to the ability of sera obtained from mice immunized with the mixture of HVRI-variants to neutralize the series of HCVpp which bear HVRI-sequences quite different form the ones included in the vaccine preparation (sequences shown in Fig. 4.7).

As already demonstrated in mice immunized with single HVRI-variants, serum showed very high activity to neutralize infection with HCVpp bearing genotype 1a (H77, black dots), however values were low against infection with HCVpp bearing genotype 1b (Ad78, black squares) and 2a (J6, black triangles) (Fig. 4.13). Serum inhibited heterologous HCVpp H77 infectivity by 80% at a 1:25 dilution and started to show borderline values at a dilution of 1:200. In contrast to that, neutralization of HCVpp Ad78 and J6 was relatively ineffective. Already at a dilution of 1:50,

neutralization of HCVpp Ad78 was ineffective while at the same dilution the serum was unable to inhibit HCVpp J6 infectivity at all.

Results showed that serum of mice was able to inhibit HCVpp infectivity bearing heterologous HVRI-variants, but demonstrated lower neutralization activity compared to inhibition of HCVpp bearing homologous sequences.

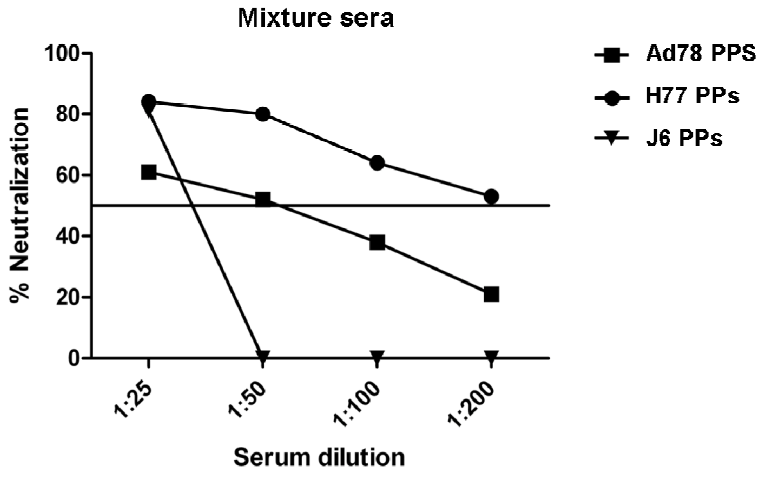

Fig. 4.13: Neutralizing capacity to heterologous HVRI-variants after mixture immunization in mice

Serum of mice immunized with a mixture of 4 HVRI-variants, was obtained 84 days after immunization and tested in different dilutions for neutralization of HCVpp expressing HVRI-variants not included in the vaccine. Percent neutralization was determined by comparing infectivity RLU of HCVpp not incubated with serum to the infectivity in the presence of test immune sera. Symbols represent the mean of 3 replicate values (pooled serum per group) and the error bars represent S.E.M. values.

4.6 A modified mixture immunization with HVRI-CLPs increases cross-reactive and cross-neutralizing antibody titers

In order to analyze the immunogenicity of the mixture immunization with HVRI-CLPs with an approved adjuvant and to shorten the vaccination protocol, the adjuvant AS03 was used in a 14-day immunization scheme (see 3.5.3.3). Therefore, the course of immunization and bleeding as well as the administration routes has been modified. In brief, C57BL/6 mice were immunized with a pool of the 4 different HVRI-variants (5 µg/variant) at days 0, 14 and 28. One group of mice was immunized i.m. applying AS03 (Fig. 4.14 A) while the second group was immunized s.c. applying IFA (Fig. 4.14 B). Evaluation of the immune response was performed as before. As shown in Fig. 4.14 A, 14 days after the first immunization, AS03 vaccinated mice showed antibody titers of around 3,000 to all peptides, except for G31 which elicited

a titer of only 200. In contrast to AS03 vaccinated mice, IFA vaccinated mice showed decreased titer levels of around 500 (Fig. 4.14 B, day 14). After the next bleeding, antibody titers were increased significantly in both groups. AS03 vaccinated mice demonstrated antibody titers of up to 32,000 to peptides R9, YK5807 and YK5829 whereas G31 elicited titers of 2,500 (Fig. 4.14 A, day 28). In comparison, the induction of antibodies was lower in IFA vaccinated mice. Titers of up to 4,000 were detected for G31 and YK5807, while R9 and YK2829 elicited only titers of 500 at most (Fig. 4.14 B, day 28). Two weeks after the last booster immunization AS03 vaccinated mice showed a repeated increase of antibody titers. Titers of 20,000 were detected for R9 and G31, whereas YK5807 and YK5829 elicited titers of 80,000 and 40,000 respectively (Fig. 4.14 A, day 42). Also an increase in antibody titers could be detected in IFA vaccinated mice however, the overall values were lower. While R9 revealed a titer of 2,000, a titer of up to 10,000 was detected for G31 and a value of 20,000 could be measured for YK5807. YK5829 induced a titer of 5,000 at most (Fig. 4.14 B, day 42). Comparison of the antibody response to HVRI-variants included in the vaccine, measured for the group immunized i.m., were significantly higher than those detected in mice immunized s.c..

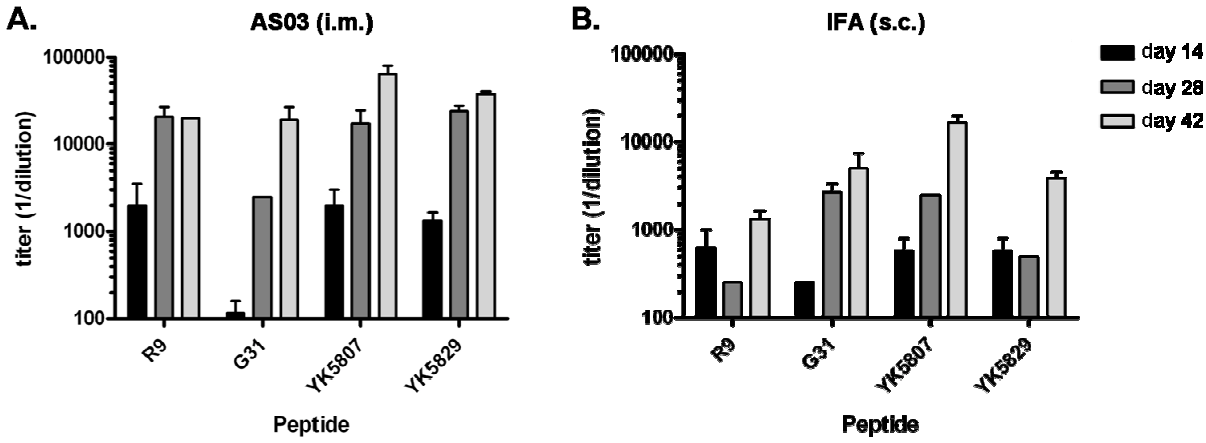

Fig. 4.14: Antibody response titers of sera from mice immunized with a mixture of HVRI-CLPs (i.m. and s.c.)

A. A group of 10 mice (C57BL/6-mice) was immunized i.m. with a mixture of the 4 HVRI-variants (5 µg/variant) in AS03. Mice were boosted 3 times and bled 4 times every 2 weeks. Serum obtained after each bleeding was tested for specific antibodies in ELISA. Serial dilutions were made and dilutions yielding at least 2 times the optical density at λ=495 nm obtained with pre-immune serum (day 0) were scored positive. **B.** A group of 5 mice was immunized s.c. with a mixture of the 4 HVRI-variants (5 µg/ variant) in IFA. The bleeding scheme and analysis of the obtained serum was done as indicated in A. Bars represent the mean of 3 replicate values (pooled serum per group) and the error bars represent S.E.M. values.

In addition, the immune response of both groups to the core protein was measured. AS03 vaccinated mice showed a titer of 20,000 after 14 days and a titer of around 40,000 at day 28. Two weeks later, the titer increased to 100,000 (Fig. 4.15 A). In comparison, a titer of around 10,000 was measured for IFA vaccinated mice at day 14. At day 28 the titer increased to 40,000 and a further cumulation to 100,000 could be measured two weeks after the last immunization (Fig. 4.15 B). In general, both groups showed the same titer after the last bleeding.

Analysis of the immune response to the core protein showed considerably lower titers compared to the values of the first mixture immunization. However, the antibody response to core was still higher in comparison to the HVRI-peptides.

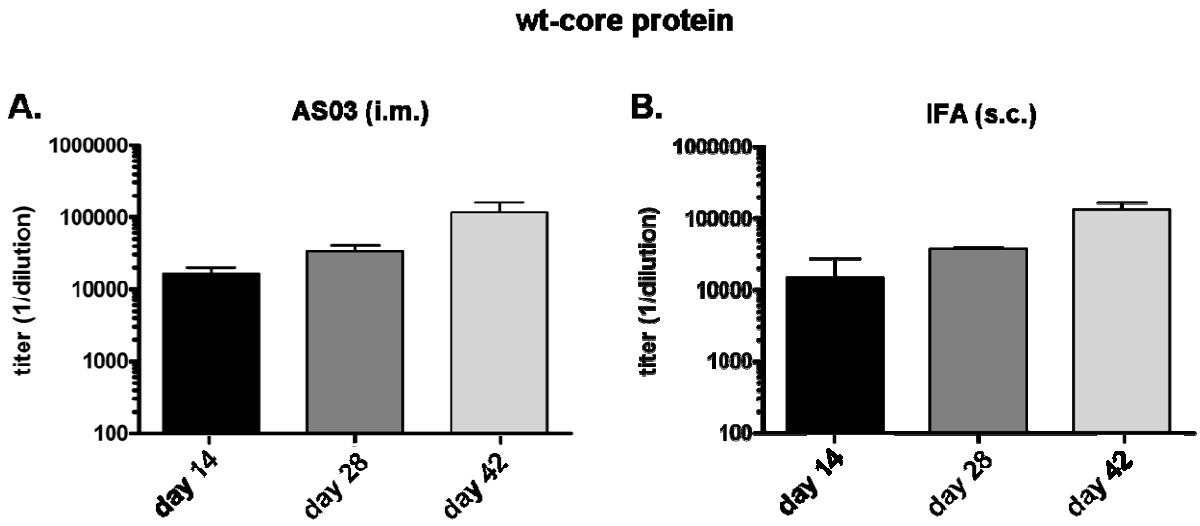

Fig. 4.15: Antibody response to the HBc-protein of sera from mice immunized with a mixture of HVRI-CLPs (i.m. and s.c.)

A. AS03 vaccinated mice were immunized i.m. with a mixture of 4 HVRI-variants. **B.** IFA vaccinated mice were immunized s.c. with a mixture of 4 HVRI-variants. Serum obtained from mice after each bleeding was tested for antibodies against the HBc-protein by ELISA. Serial dilutions were made and dilutions yielding at least 2 times the optical density at λ=495 nm obtained with pre-immune serum (day 0) were scored positive. Bars represent the mean of 3 replicate values (pooled serum per group) and the error bars represent S.E.M. values.

4.6.1 Evaluation of the neutralizing capacity to homologous HVRI-variants after improved mixture immunization

Compared to previous neutralization results with mixture immunization (Fig. 4.12) a similar and even better pattern of neutralization activity was observed in sera (day 42) of mice which obtained the shortened vaccination strategy of s.c. and i.m. administration (Fig. 4.16). In this case, AS03 vaccinated mice showed extremely effective neutralization. At a 1:50 dilution, neutralization activity to all four HCVpp started very slowly to decrease from up to 100% showing only borderline values at a 1:3200 dilution with around 40% to 60% neutralization (Fig. 4.16 A). In comparison, serum of IFA vaccinated mice reacted less effectively with HCVpp bearing the HVRI-variants included in the vaccine preparation. Infection with HCVpp R9 and YK5807 was inhibited the best by the serum, which means that borderline values were detected at a dilution 1:400 (Fig. 4.16 B, black dots and open dots). Neutralization to HCVpp YK5829 and G31 became already ineffective at a dilution of 1:200 and 1:100, (Fig. 4.16 B, open triangles and black squares).

In general, these data correspond to the ELISA data (Fig. 4.14) showing that mice immunized with AS03 i.m. had high antibody titers and these Abs are able to better neutralize the Abs from mice immunized with IFA as adjuvant (Fig. 4.9). Furthermore, G31 HCVpp were regularly the most difficult to neutralize.

In conclusion, serum of AS03 immunized mice showed a very high capacity to neutralize HCVpp bearing the HVRI-variants included in the vaccine.

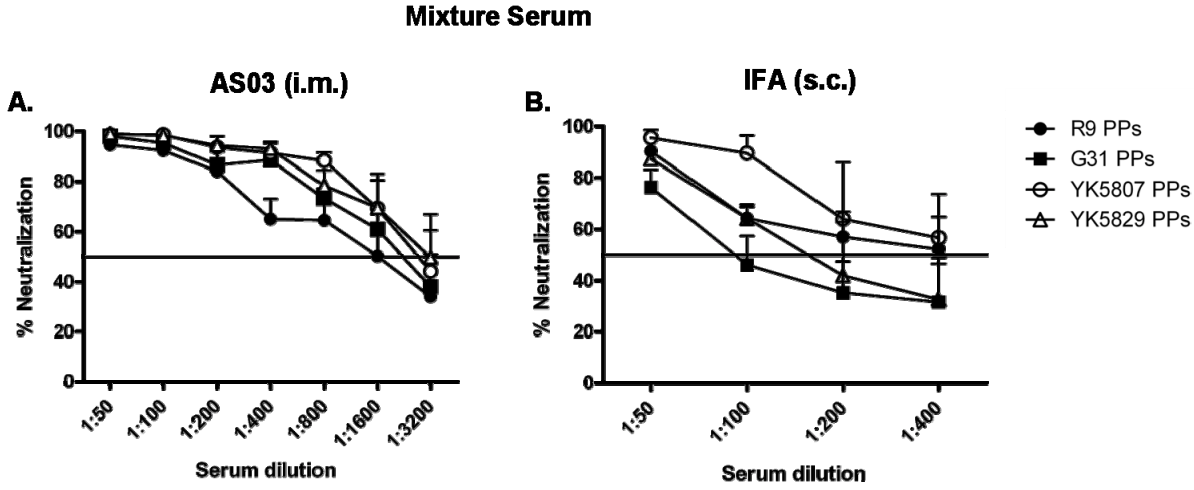

Fig. 4.16: Neutralizing capacity to homologous HVRI-variants after mixture immunization i.m. and s.c. in mice

A. AS03 vaccinated mice were immunized i.m. with a mixture of 4 HVRI-variants. **B.** IFA vaccinated mice were immunized s.c. with a mixture of 4 HVRI-variants. Serum of day 42 post immunization, of both groups was tested in different dilutions for neutralization of HCVpp expressing the 4 HVRI-variants included in the vaccine. Percent neutralization was determined by comparing infectivity RLU of HCVpp not incubated with serum to the infectivity in the presence of test immune sera. Symbols represent the mean of 3 replicate values (pooled serum per group) and the error bars represent S.E.M. values.

4.6.2 Evaluation of the neutralizing capacity to heterologous HVRI-variants after improved mixture immunization

To assess whether serum of mice immunized with the modified vaccine strategy induced more potent neutralization activity to HCVpp bearing heterologous HVRI-variants, different dilutions of serum (day 42) of AS03 vaccinated mice and IFA vaccinated mice were also analyzed in the HCVpp assay (Fig. 4.17). As expected, serum of AS03 vaccinated mice appeared to induce more potent neutralization titers, especially for HCVpp H77 and Ad78 (Fig. 4.17 A, black dots and black squares). Infection with HCVpp J6 could not be inhibited by the serum at all (Fig. 4.17 A, black triangles). Serum of IFA vaccinated mice showed neutralization of HCVpp H77 and Ad78 at a dilution of 1:50 with 65% and 71%. Thereafter, neutralization efficiency decreased rapidly. As shown for AS03 vaccinated mice serum of IFA vaccinated mice was neither able to block infection with HCVpp J6 at all (Fig. 4.17 B).

In general, these data again showed a high cross-neutralizing capacity of the serum. Furthermore, these data are consistent with values of homologous neutralization assays as well as ELISA data, which showed that serum of mice immunized with the modulated vaccine strategy revealed continuously better neutralization activity or

antibody responses (see Fig. 4.14 and Fig. 4.16). In addition, in homologous as well as in heterologous neutralization assays, HCVpp J6 were hardly neutralized.

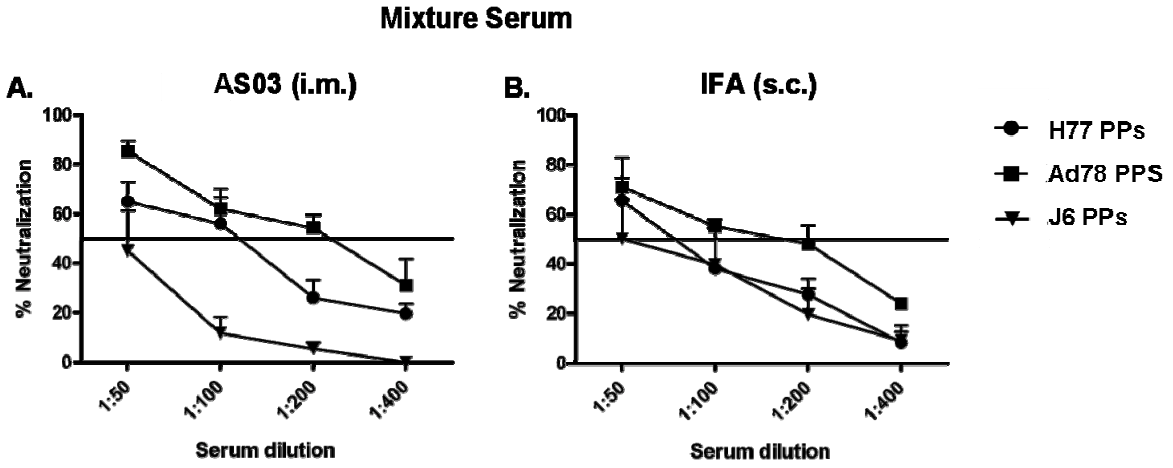

Fig. 4.17: Neutralizing capacity to heterologous HVRI-variants after mixture immunization i.m. and s.c. in mice

A. AS03 vaccinated mice were immunized i.m. with a mixture of 4 HVRI-variants. **B.** IFA vaccinated mice were immunized s.c. with a mixture of 4 HVRI-variants. Day 42-serum of both groups was tested in different dilutions for neutralization of HCVpp expressing HVRI-variants not included in the vaccine. Percent neutralization was determined by comparing infectivity RLU of HCVpp not incubated with serum to the infectivity in the presence of test immune sera. Symbols represent the mean of 3 replicate values (pooled serum per group) and the error bars represent S.E.M. values.

4.7 The true cross-neutralizing potential after mixture immunization in mice

The next goal was to determine the true cross-neutralizing potential of the serum to randomly selected HVRI-variants isolated from patients. As serum of AS03 vaccinated mice always showed the best antibody responses as well as neutralization activity, this serum, obtained at day 42 after immunization, was used for further characterization. Experiments were carried out in the laboratory of William O. Osburn at the Johns Hopkins School of Medicine, Baltimore, USA. The serum was screened at a single dilution of 1:50 for its ability to neutralize HCVpp bearing different patient derived envelope sequences (HVRI-variants). The amino acid sequences as well as the corresponding genotype of the different variants in comparison to the sequence of R9 are shown in Fig. 4.18. Serum showed weak neutralization of 4 variants, namely HCVpp 1a31, 1a38, 1a46 and 1b20 by approximately 47% indicated in orange. Four other variants 1a129, 1b44, 1b52, and 1b58 could be highly neutralized by the serum. HCVpp 1a129 was neutralized by

55%, while HCVpp 1b44 was neutralized by 59%, HCVpp 1b52 by 63.7% and infection with HCVpp 1b58 was inhibited by 73.3%. These values are indicated in green in Fig. 4.18. Neutralization of the remaining 11 HCVpp bearing other HVRI-variants showed values of less than 50%. Pseudotype viral particles expressing the Murine Leukemia Virus (MLV) glycoprotein were used as negative control. The same results are depicted as a histogram in Fig. 4.19. The dashed line indicates the starting point for positive neutralization at 50%, while the spotted line indicates weak neutralization (approximately 47%) of the corresponding bars.

This study showed that the serum was indeed able to neutralize 21% of randomly selected HVRI-variants, demonstrating a high cross-neutralizing potential. When also including the variants which were neutralized weakly, the cross-neutralizing capacity of the serum accounts for even 42%.

GT	HVRI-sequence	% Neutralization
R9	QTTVVGGSQSHTVRGLTSLFSPGASQN	
1a09	E-Y-T---AG-AAS--A---TT--K--	<50%
1a129	E-H-S---VAR-TSR--N------M--	55.0%
1a142	R-R-T--TAGRETA-FA-I--R--K--	<50%
1a154	E-H-T--NAGR-TA--VG-LT---K--	<50%
1a157	E-H-T--NAG---AR-AG------K--	<50%
1a31	E-YTS--VVAYGT-A--GF--Q-SN--	47.0%
1a38	G-H-T---AGR-TA-IAG-LTQ--K-S	47.6%
1a46	G-YIS---AARATS--V--LT---K--	47.0%
1a53	N-VLI--QAAY-ASSF-ALLT---KQ-	<50%
1a72	E-H-T--TAGRAAA--AG--TQ--R-S	<50%
1a80	N-VLI--QAAY-ASSF-A-LT---K--	<50%
1b09	S-H-T--TA---T-HFA----S----R	<50%
1b14	D-HTM--AAGRDTHKF-----F-----	<50%
1b20	E-RTI-SEVGSATHRF--V----SR-K	46.0%
1b35	A-HTI--T-RGNTY---T-----S--R	<50%
1b38	G-H-T---AGR-TA-IAG-LTQ--K-S	<50%
1b44	S-H-S---AA-AAS--T-F----P--K	59.0%
1b52	T-Y-T--ATAR-TS-FA---T-----K	63.7%
1b58	T-NIM----ARD-SRV--F-------K	73.3%
MLV	negative control	N/A

Fig. 4.18: Immunization of mice with a mixture of HVRI-CLPs induces cross-neutralizing antibodies

Mice were immunized i.m. with a mixture of 4 HVRI-variants in AS03. Day 42-serum was tested in a 1:50 dilution for neutralization of HCVpp expressing diverse HVRI-variants of GT1. As these HVRI-variants were not included in the vaccine, sequences are aligned to the R9-variant included in the vaccine. Percent neutralization was determined by comparing infectivity (luciferase relative light units – RLU) of HCVpp in the presence of pre-immune sera to the infectivity in the presence of test immune sera. Pseudotype viral particles expressing MLV gp were used to confirm serum neutralization specificity. Sera showed neutralization of <50% to most HVRI-sequences. 4 HVRI-variants (GT1a129, 1b44. 1b52 and GT1b58), indicated in green, were highly neutralized. 4 other HVRI-variants indicated in orange, were weakly neutralized (GT1a31, 1a38, 1a46 and GT1b20). Experiments were carried out in the laboratory of William O. Osburn; Johns Hopkins School of Medicine, Baltimore, MD, USA.

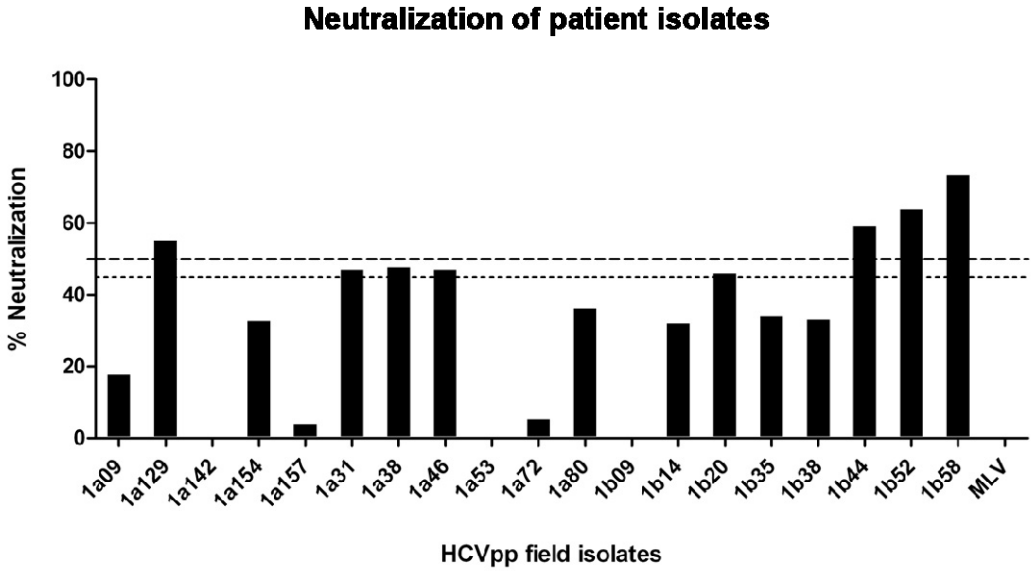

Fig. 4.19: Mixture immunization of mice (i.m.) elicits cross-neutralizing antibodies to naturally occurring HVRI-variants

Serum of AS03 vaccinated mice was tested in a 1:50 dilution for neutralization of HCVpp expressing diverse HVRI-variants of GT1 not included in the vaccine preparation. For more details see Fig. 4.18.

4.8 Immunoglobulin isotype switch in mice immunized with HVRI-CLPs

In order to stimulate B-lymphocytes to produce antibodies, antigens presented on MHC-II require the specific recognition by helper T-cells. Stimulation of antibody responses towards certain antigens may result in a selective increase in IgG antibodies of certain subclasses (Soderstrom *et al.*, 1985), (Burton and Woof, 1992). In contrast to CD8 T-cells, CD4 T-cells can differentiate into a number of different effector T-cell subsets like T_H1, T_H2 and T_H17 cells. T_H1 and T_H2 cells can contribute to humoral immunity by inducing the production of antibodies. Besides the activation of macrophages, T_H1 cells activate B-cells to produce strongly opsonizing antibodies belonging to certain IgG subclasses (IgG1 ang IgG3 in humans, and their homologs IgG2a and IgG2b in the mouse). T_H2 cells, in contrast, can subsequently stimulate B-cells to differentiate and produce immunoglobulins of other isotypes as well as neutralizing and/or weakly opsonising subclasses of IgG (Murphy *et al.*, 2008).

Comparative to the previous results and in order to show a possible link to the neutralization efficiency of the sera to IgG subclass generation, the ratio of IgG subclasses IgG2a, IgG2b and IgG3 in comparison to IgG1 in the serum of mice was determined by ELISA (see 3.6). The following tables represent the levels of IgG-

subclasses present in the serum of mice immunized with the mixture of HVRI-variants either delivered in AS03 (Tab. 4.1) or in IFA (Tab 4.2).

Coating of plates with the peptide R9 revealed a slight increase in IgG2a and IgG2b compared to IgG1 levels during vaccination of AS03 mice. IgG3 levels also increased but remained below the amount of IgG1 (Tab. 4.1). ELISA to the other peptides G31, YK5807 and YK5829, demonstrated besides IgG1, also induction of IgG2a and IgG2b levels. Slightly lower amounts of IgG3 were measured in most tests (Tab. 4.1). In general, the ratio of IgG subclasses determined in the serum of AS03 vaccinated mice points towards a mixed T_H1/ T_H2-response with a T_H1-trend. However, also the fact that mice were immunized with constructs not bearing the nucleic acid binding domain of the HBc protein which normally induces a T_H2-response, should be taken into account.

Tab. 4.1 Ratio of IgG-subclasses in AS03 vaccinated mice

coating	IgG Subclass	day 14 serum	day 28 serum	day 42 serum
R9	**IgG1**	1	1	1
	IgG2a	0.76	1.18	1.08
	IgG2b	0.61	1.13	1.09
	IgG3	0.48	0.80	0.90
G31	**IgG1**	1	1	1
	IgG2a	1.09	0.75	0.97
	IgG2b	1.0	0.7	0.8
	IgG3	0.7	0.5	0.6
YK5807	**IgG1**	1	1	1
	IgG2a	0.77	0.91	0.98
	IgG2b	0.96	0.99	0.97
	IgG3	0.77	0.85	0.91
YK5829	**IgG1**	1	1	1
	IgG2a	0.97	0.75	0.92
	IgG2b	1.06	0.98	0.99
	IgG3	0.65	0.70	0.96

In comparison to AS03 vaccinated mice (i.m.), the serum of IFA vaccinated mice (s.c.) showed a significantly increased IgG2a/b level against G31. These levels started to decrease during the remaining course of vaccination (Tab. 4.2). The other peptides induced slightly higher amounts of IgG2b compared to values measured for IgG2a during the vaccination trial, but remained lower compared to values in AS03 vaccinated mice (Tab. 4.2). The IgG3 levels to all four peptides were slightly lower compared to the results of AS03 vaccinated mice

In the early phase, the IgG ratio of subclasses determined in the serum of IFA vaccinated mice also points more towards a T_H1-response. However, in the later phase the IgG ratio shifted to a T_H1/T_H2-response. Also in this group it should be considered that mice were immunized with HVRI-CLPs not bearing the nucleic acid binding domain anymore, which normally leads to the induction of a T_H2-response.

Tab. 4.2 Ratio of IgG-subclasses in IFA vaccinated mice

coating	IgG Subclass	day 14 serum	day 28 serum	day 42 serum
R9	**IgG1**	1	1	1
	IgG2a	0.85	0.29	0.29
	IgG2b	1.86	0.75	0.74
	IgG3	0.59	0.35	0.63
G31	**IgG1**	1	1	1
	IgG2a	1.58	0.56	0.77
	IgG2b	1.80	1.02	0.94
	IgG3	0.83	0.19	0.22
YK5807	**IgG1**	1	1	1
	IgG2a	0.79	0.88	0.93
	IgG2b	1.02	0.93	0.98
	IgG3	0.63	0.76	0.89
YK5829	**IgG1**	1	1	1
	IgG2a	0.85	0.25	0.31
	IgG2b	1.03	0.51	0.54
	IgG3	0.88	0.20	0.28

4.9 Immunization with HVRI-CLPs elicits an HVRI-specific immune response in guinea pigs

In 2007 Stamataki *et al.* (Stamataki *et al.*, 2007) demonstrated an enhanced anti-glycoprotein response in guinea pigs after immunization with different variants of HCV GT1a glycoproteins E1 and/or E2, compared to mice. In order to proof this outcome, also guinea pigs were immunized with a mixture of the four different HVRI-variants used before (3.5.3.4). Therefore, groups of five female guinea pigs (*Cavia procellus*) were immunized s.c. with 5 µg/variant at 0, 30 and 90 days. The adjuvant IFA was used with the mixture of antigens described. In addition to the anti-HVRI response, the serum was also analyzed for its neutralizing capacity. The experiments were carried out in collaboration with Abdel-Rahman Zekri in Cairo; Virology and Immnunology Unit, National Cancer Institute, Cairo, Egypt.

4.9.1 A mixture of HVRI-CLPs is less immunogenic in guinea pigs compared to mice

The HCV HVRI-specific antibody response in guinea pigs was measured at days 30, 90 and 120. Thirty days after the first immunization an antibody titer of 1,600 was detected to R9 and a titer of up to 400 to the variant YK5829. Due to volume limitations of serum at this time point, the reactivity with G31 and YK5807 could not be tested (Fig. 4.20, day 30). After 90 days, the antibody titer increased to levels of 8,000 to 16,000 against R9, and levels of 2,000 to 4,000 could be detected to YK5807 and YK5829, respectively. G31 reacted with a titer of 100 (Fig. 4.20, day 90). After the third immunization, antibody titers increased further to approximately 32,000 to R9 and YK5807. At this time a titer of 2,000 was detected to G31 and a titer of up to 16,000 was detected to YK5829 (Fig. 4.20, day 120).
Analysis showed an antibody response in guinea pigs against the presented HVRI-variant which was lower compared to antibody responses in mice.

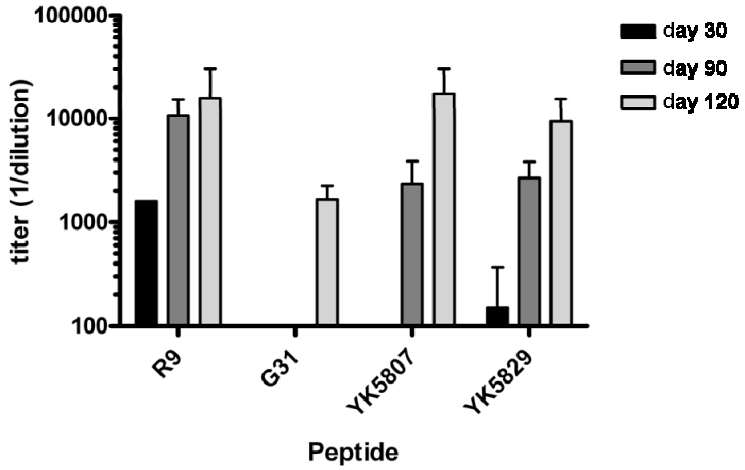

Fig. 4.20: Antibody response titers of sera from guinea pigs immunized with a mixture of 4 HVRI-CLPs

A group of 5 guinea pigs (*Cavia procellus*) was immunized with a mixture of 4 HVRI-variants (5 µg/variant) in IFA. Guinea pigs were immunized at days 0, 30, 90. Bleeding was done 4 times at days 0, 30, 90 and 120. The obtained serum was tested for specific antibodies by ELISA. Serial dilutions were made and dilutions yielding at least 2 times the optical density at λ=495 nm obtained with pre-immune serum (day 0) were scored positive. Bars represent the mean of 3 replicate values (pooled serum per group) and the error bars represent S.E.M. values.

In addition, the antibody response to the carrier of the vaccine, the HBc-protein, was analyzed. At day 30, antibody titers of 320,000 to 800,000 to the core protein could be detected in the serum of guinea pigs (Fig. 4.21, day 30). After the second booster immunization (day 30), increased antibody titers of 3,200,000 at day 90 could be detected (Fig. 4.21, day 90). Antibody titers stayed constant at 3,200,000 30 days after the last booster immunization (Fig. 4.21, day 120).

In conclusion, the guinea pigs showed a very high anti-core response compared to a relatively low anti-HVRI response. This is in contrast to mice which showed a high antibody response to the HVRI. The core response was much higher in guinea pigs.

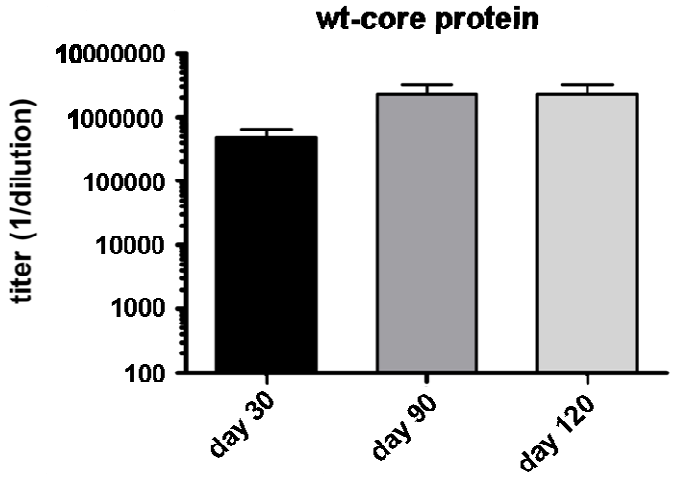

Fig. 4.21: Antibody response to the HBc-protein of sera from guinea pigs immunized with a mixture of HVRI-CLPs

Serum obtained from guninea pigs after each immunization was tested for antibodies against the HBc-protein by ELISA. Serial dilutions were made and dilutions yielding at least 2 times the optical density at λ=495 nm obtained with pre-immune serum (day 0) were scored positive. Bars represent the mean of 3 replicate values (pooled serum per group) and the error bars represent S.E.M. values.

4.10 Characterization of the neutralizing capacity of guinea pig sera after immunization with HVRI-CLPs

The importance of nAbs in HCV infections was already described. In order to analyze whether the anti-HVRI Abs measured by ELISA could neutralize HCVpp infectivity and compare the neutralizing capacity in guinea pigs to mice, the serum of guinea pigs immunized with the mixture of HVRI-variants was also tested for its neutralizing activity.

4.10.1 Evaluation of the neutralizing capacity to homologous HVRI-variants after mixture immunization

Sera obtained at day 120 after immunization of guinea pigs were tested in the HCVpp neutralization assay as described (3.2.7). The serum showed very high neutralizing capacity against all the homologous HCVpp (Fig. 4.22). Only neutralization of infection with HCVpp G31 (Fig. 4.22, black squares) showed a rapid decrease in neutralization activity, becoming inefficient at a 1:400 dilution (45%), compared to the efficiency of the serum to inhibit infection with the other three HCVpp. The neutralization efficiency started to decline and drop below 50% at a dilution of 1:1600 for YK5829 and 1:3200 for YK5807 (Fig. 4.22, open triangles and

open dots). Interestingly, at a dilution of 1:3200, serum was still able to neutralize HCVpp R9 with 65% (Fig. 4.22, black dots).

In comparison to the neutralizing capacity in mice (immunized with the mixture) which already showed very good results, neutralization activity in guinea pigs was even more potent. However, serum of both animals showed the most potent neutralization activity to HCVpp R9 but again relatively inefficient neutralization of HCVpp G31.

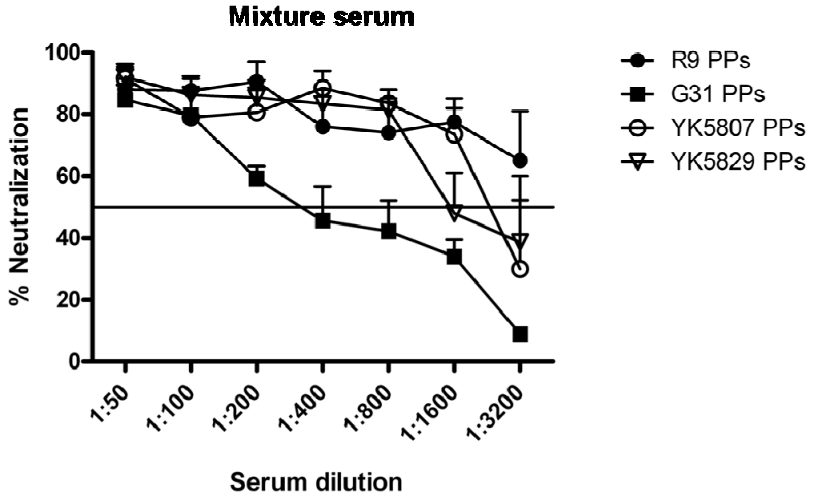

Fig. 4.22: Neutralizing capacity in guinea pigs to homologous HVRI-variants after mixture immunization

Day 120-serum of guinea pigs immunized with a mixture of 4 HVRI-variants was tested in different dilutions for neutralization of HCVpp expressing the 4 HVRI-variants included in the vaccine. Percent neutralization was determined by comparing infectivity RLU of HCVpp not incubated with serum to the infectivity in the presence of test immune sera. Symbols represent the mean of 3 replicate values (pooled serum per group) and the error bars represent S.E.M. values.

4.10.2 Evaluation of the neutralizing capacity to heterologous HVRI-variants after mixture immunization

The serum of guinea pigs (day 120) was further tested at different dilutions (1:50, 1:100, 1:200 and 1:400) for its ability to neutralize HCVpp bearing heterologous variants of the HVRI (Fig. 4.23). Serum showed relatively high neutralization of HCVpp J6 and HCVpp Ad78 of approximately 80% at a dilution of 1:50. Thereafter, neutralization activity started to decrease slowly and showed a final value of approximately 40% at a dilution of 1:400 (Fig. 4.23, black triangles). In comparison, the serum was only able to inhibit HCVpp H77 infection at a dilution of 1:100 (Fig. 4.23, black squares).

Surprisingly, neutralization of HCVpp H77 showed relatively low values compared to serum of mice which showed the least neutralization activity to HCVpp J6. In general, also a high cross-neutralizing potential could be demonstrated in guinea pigs.

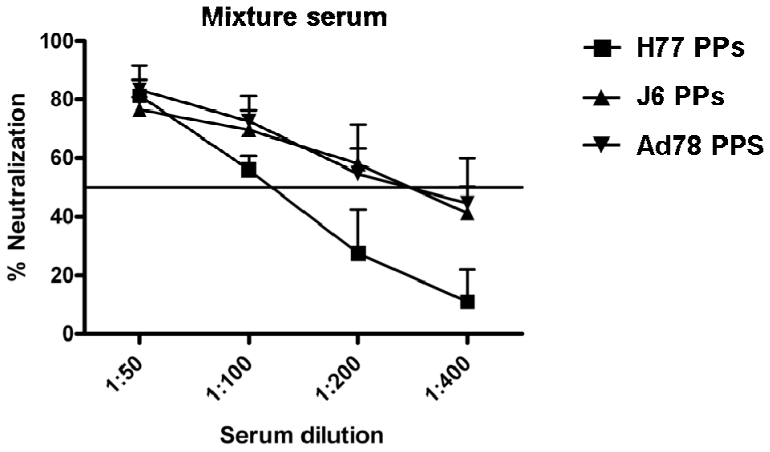

Fig. 4.23: Neutralizing capacity to heterologous HVRI-variants after mixture immunization in guinea pigs

Serum of guinea pigs immunized with a mixture of 4 HVRI-variants, was obtained 120 days after immunization and tested in different dilutions for neutralization of HCVpp expressing HVRI-variants not included in the vaccine. Percent neutralization was determined by comparing infectivity RLU of HCVpp not incubated with serum to the infectivity in the presence of test immune sera. Symbols represent the mean of 3 replicate values (pooled serum per group) and the error bars represent S.E.M. values.

4.11 WHc-CLPs displaying HVRI-R9 are assembly competent

The application of HBc-CLPs as vaccine carrier is particularly difficult because anti-HBc Abs are used as diagnostic markers for HBV. During vaccination against HBV only antibodies to the HBs-protein are induced, while antibodies against the HBc-protein and the related HBeAg are routinely used as diagnostic marker for acute or chronic HBV infections (Trepo *et al.*, 1993). One possibility to circumvent the problem of cross-reactivity in diagnostic tests is the usage of different hepadnaviral core-proteins as vaccine carriers. The possibility to use the Woodchuck Hepatitis C Virus core protein (WHc) as vaccine carrier was already demonstrated resulting in a reduced cross-reactivity of anti-WHc antibodies with the diagnostic anti-HBc test-system (Billaud *et al.*, 2005a), (Billaud *et al.*, 2005b). Another fact that also supports the usage of the WHc protein as vaccine carrier is that patients chronically infected with HBV show T-cell tolerance to the HBV core-protein (Whitacre *et al.*, 2009). For this reason it was analyzed whether WHc CLPs bearing HVRI-variants would still be able to form particles. As a representative for the 4 HVRI-variants used before, the

R9-variant was fused to the N-terminal part of the SplitCore-WHc-protein as described in section 4.1. The vector-card of this construct is shown in the appendix in Fig. 9.4

The ability of the WHc-protein bearing the HVRI-variant R9 to form CLPs was analyzed by SDS-PAGE (see method section 3.4.2) after protein expression in BL21*CP cells (see 3.1.3), cell lysis (see 3.1.4) and sucrose gradient centrifugation (see 3.4.7). Co-sedimentation of fragments of the corresponding size (12 kDa and 8.9 kDa) into fractions 8 to 14 (peak in fraction 13) was observed suggesting that the fusion protein at least partially was present as intact CLPs (Fig. 4.24 A, lane 8-14). The band at 12 kDa corresponds to the CoreN fragment (white arrow), and the band at the bottom of the gel corresponds to the small CoreC fragment (8.9 kDa, white arrow). In order to confirm the previous results, native gel electrophoresis was done (see 3.4.3). Upon staining with Coomassie, distinct bands, corresponding to particulate structures of the putative fusion protein, were detected in fractions 8-10 (Fig. 4.24 A, lane 8-10). The blue smears which appear specifically in the first lanes indicate protein monomers. The same results were shown by transfer of a 1% NAGE onto a PVDF-membrane and staining with Ab 10E11. Distinct bands indicating particle formation of WHc-R9 were observed in fractions 6 to 11 (Fig. 4.24 B lane 6-11). Protein monomers which stayed in the slot were detected in the remaining fractions 13 to 14 (Fig. 4.24 B lane 12-14). To further prove the presence of WHc-R9 CLPs, fraction 9 of the gradient was investigated by electron microscopy at a magnification of 140,000 showing regular formed particles (Fig. 4.24 C). This confirms that SplitCore-WHc-R9 is able to form CLPs.

In summary, these observations demonstrate in proof of principle that WHc-CLPs bearing HVRI-variants are potential vaccine candidates for HCV.

The generation and successful assembly of chimeric SplitCore CLPs consisting of different hepadnaviral core proteins would also be a desirable aim for the future.

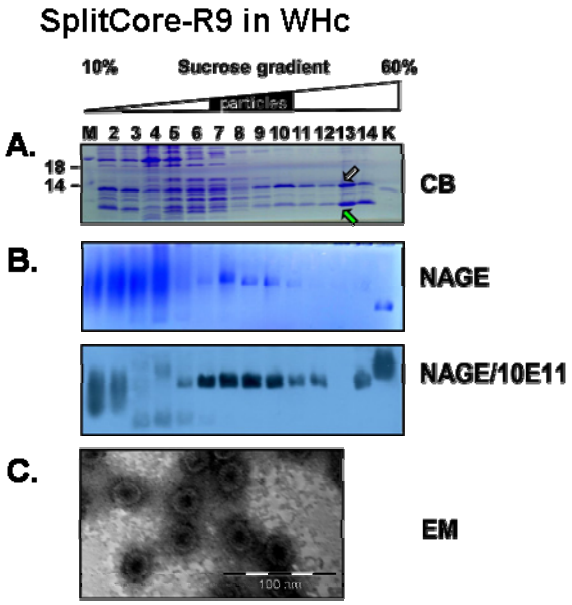

Fig. 4.24: WHV-CLPs displaying R9 are assembly competent

A. Coomassie SDS-PAGE After separation of non-denatured cell lysate on a sucrose gradient, 14 fractions were separated on a 15% SDS-gel and proteins were visualized with Coomassie. The arrows indicate the position of bands of HVRI-R9 in the gel. Marker: PeqGold protein marker I; PeqLab. Wild-type core protein served as positive control (K). **B. CLP-specific native agarose gel (NAGE)** 10 µl aliquots of gradient fractions were loaded on a 1% agarose gel visualized by Coomassie. Particulate structures appear as distinct bands in particle-typical fractions. Alternatively, proteins on a agarose gel were transferred to a PVDF-membrane. Detection was done with the particle-specifi Ab 10E11. Wild-type core protein served as positive control. **C. EM-picture** HVRI-CLPs were stained with 2% uranyl acetate and investigated by electron microscopy (Pathology, UK Essen).

5 Discussion

Since there is still no protective or therapeutic vaccine available against HCV, the number of new infections did not decrease during the last decades. The WHO estimates that over 170 million people are persistently infected with HCV, of whom 350,000 people die each year from hepatitis C-related liver diseases (WHO, 2011b). Despite ongoing research, therapeutic options are still limited. Current standard therapy of direct-acting antivirals (DAAs) with pegylated interferon and Ribavirin (PEG-IFN/RBV) is often difficult to tolerate and results in a sustained virological response in only 50% of patients which makes therapy against HCV in most cases not satisfactory (Zeuzem, 2004). However, enormous costs limit the therapy for most patients. Even though the treatment of hepatitis C with specific targeted antiviral therapies (STAT-C) directed against protease, polymerase or other nonstructural proteins continues to evolve striving for constant improvement, new approaches for a prophylactic vaccine against HCV are needed.

It is well documented that not only a broad and vigorous HCV specific T-cell response but also the induction of virus-neutralizing antibodies is needed to efficiently control HCV infection (Lechner *et al.*, 2000), (Pestka *et al.*, 2007). Indeed, studies indicate that patients who induce nAbs in the early phase of infection, subsequently clear the viral infection (Pestka *et al.*, 2007). In contrast, patients who do not completely eliminate the virus develop high titers of neutralizing antibodies during the chronic phase which are not able to control HCV infection. HCV has therefore developed various mechanisms to evade immune elimination, allowing it to persist in the majority of infected individuals (Di Lorenzo *et al.*, 2011). Despite the continuous generation of HCV escape mutants predominantly in the HVRI, studies have shown that positive selection is not evenly distributed across HVRI but instead is focused on a few discontinuous residues (McAllister *et al.*, 1998), (Penin *et al.*, 2001), (Brown *et al.*, 2007). Therefore, it was assumed that a number of HVRI-variants would be able to induce antibodies that could cross-react with different HCV strains occurring in nature (Puntoriero *et al.*, 1998). Moreover, recent data have demonstrated that antibodies to HVRI are able to block both cell-free as well as cell-to-cell transmission of HCV (Brimacombe *et al.*, 2011). Hence, the presentation of HVRI-variants on HBc-CLPs which leads to the induction of cross-neutralizing anti-HVRI antibodies, would demonstrate a new type of immunogen for the protective

immunization against HCV. Numerous clinical trials of prophylactic immunization exploited peptide, recombinant protein, DNA and vector-based vaccines; however none of them has reached approval yet (Halliday *et al.*, 2011). A novel approach to overcome viral persistence includes virus-like particles (VLPs) or CLPs-based vaccines that have been successfully employed for viral infections such as hepatitis B (Hilleman, 2001), (Kao and Chen, 2002). A HCV CLP vaccine approach could facilitate the induction of nAbs and specific T-cell epitopes in a single construct resembling mature HCV virions which could enhance immunity (Halliday *et al.*, 2011). By the presentation of foreign peptides or proteins on CLPs, general properties of the viral capsid, like the repetitive arrangement of the surface, and activation of the innate immune system can be transferred to foreign sequences. HBV-CLPs are the most promising carrier systems as they are able to trigger vigorous immune responses in humans without eliciting serious side effects. The immune boosting effect was already demonstrated for several different foreign peptides and protein fragments (Koletzki *et al.*, 1999), (Ulrich *et al.*, 1999) (Pumpens and Grens, 2001). The most adequate insertion site of the HBc protein is the surface exposed but centrally located c/e1 epitope (Pumpens and Grens, 2001). Due to the application of the so-called SplitCore-system, splitting the HBc protein in two fragments the structure of the inserted antigen as well as the potential B- and T-cell epitopes are still conserved. After co-expression in *E. coli* the HBc protein is again able to spontaneously assemble into CLPs.

In the current study it was shown that SplitCore-CLPs displaying different HVRI-variants are assembly competent and after immunization of mice show exceptional immunogenicity. Furthermore, immunization with single HVRI-CLPs induced partially cross-reactive antibodies. However, a mixture of HVRI-CLPs induced significantly high titers of cross-reactive and cross-neutralizing antibodies. Similar results could be observed in guinea pigs. Also the possibility of the WHc protein, bearing the R9 variant and to assemble to CLP demonstrates the possibility of a new strategy for a prototype vaccine against HCV.

5.1 Characterization of SplitCore-HVRI-CLPs

The first aim of this study was to prove whether SplitCore-CLPs carrying four different variants of the HVRI are assembly-competent. These variants were fused via a short linker to CoreN (N-terminal part) of the HBc protein lacking the nucleic acid binding domain. The results of this study showed that SplitCore-CLPs are able to present all four different variants of the HVRI which can be expressed in high amounts and assemble to properly formed spherical particles as demonstrated by co-sedimentation in gradients (see Fig. 4.2 A), migration as distinct bands in NAGE (see Fig. 4.2 C) and EM pictures (see Fig. 4.2 D).

In the generated constructs, particles sedimented also in the bottom fractions (9-14) and not only in the middle fractions as it was already shown (Birnbaum and Nassal, 1990). However, due to the collection of the gradient from the top fractions, most likely material is displaced in deeper fractions. In order to prevent this displacement, the gradient could be collected from the bottom. So far, no attempts in this direction were undertaken since this gradient is used only for enrichment of particles.

As mentioned in section 4.1, it was already shown in former experiments that the complete E2 ectodomain (E2-655) presented on HBc-CLPs successfully forms particles but is insoluble when purified (master thesis M. Lange, 2009). Due to this reason, this construct exhibits no properties for a possible immunogen preparation. Furthermore, the highly conserved neutralizing epitope AP33 of HCV which would demonstrate another promising construct (Owsianka *et al.*, 2005), (Tarr *et al.*, 2006), (Sabo *et al.*, 2011) was also shown to be unable to form particles (master thesis M. Lange, 2009). The reason for its insolubility is not completely clear. One explanation could be that the HVRI located directly in front of AP33 is needed for proper protein functionality. Although HVRI-sequences vary between HCV strains, the overall charge properties remain conserved making this region very hydrophilic. It was already shown that HCV uses this conservation of positively charged residues most likely to interact with negatively charged molecules such as lipids, proteins or glycosaminoglycans (Basu *et al.*, 2004). This could lead to the fact that AP33 alone is not able to form particles. Furthermore, sterical constrains may also lead to aggregation of this construct as the absence of the flexible HVRI may reduce the flexibility of the chimeric protein.

In general, the current study showed successful generation and particle formation of HBc-CLPs carrying different variants of the HVRI. Moreover, the formed particles

could easily be purified and used for immunization studies in mice. Most likely every other sequence of the HVRI could be presented by HBc-CLPs.

5.2 Immune response in mice after immunization with HVRI-CLPs

Vaccines based on VLPs have gained a great interest because of their ability to stimulate robust humoral and cellular immune responses. As this system should lead to an optimal presentation of the antigen, and the induction of a robust immune response *in vivo,* the immunization of mice with single HVRI-CLPs was performed.

5.2.1 Characterization of the HVRI-specific immune response after single HVRI-CLP immunization in mice

The results of the presented study clearly showed that expression of HVRI-variants on HBc-CLPs resulted in a very strong humoral immune response in mice as a high anti-HVRI antibody response could be detected (see Fig. 4.3). Serum of these mice was also partially cross-reactive to the other HVRI-variants (see Fig. 4.5). These results confirmed the high antigen expression provided by the HVRI-CLPs. A pattern of HVRI-sequence similarity of the used constructs with respect to the ability of the corresponding sera to induce cross-reactive antibodies preferably to one construct compared to another one could not be detected. Furthermore, it is not evident whether immunization with mimotopes induces some difference in the immune response compared to the outcome after immunization with naturally occurring variants. Nevertheless, most likely the induced antibodies seem to be predominantly directed at the C-terminus of the HVRI. The observed results are consistent with previous findings, which also demonstrated the simultaneous presence of variant-specific and cross-reactive Ab responses in the serum of mice immunized with HVRI-mimotopes. The induced antibodies were also directed to the C-terminus of the HVRI (Cerino *et al.*, 2001). These results demonstrate the possibility to induce a cross-reactive B-cell response to HVRI isolates which is not immediately evident as the HVRI has been traditionally considered structurally flexible and antigenically variable (Taniguchi *et al.*, 1993). Moreover, it was already shown, that anti-HVRI antibodies effectively captured HCV *in vitro*. Furthermore, it was found that anti-HVRI antibodies could immunoprecipitate an isolate of HCV unrelated to the original antigenic HCV isolate. These findings suggest that anti-HVRI antibodies induced in mice have the

ability to bind HCV particles in an isolate cross-reactive manner. Therefore, the combination of several HVRI-sequences should generate a broadly reactive anti-HVRI response (Esumi *et al.*, 1998).

In general, these results showed the possibility of the induction of partially cross-reactive antibodies after immunization of mice with single HVRI-CLPs.

5.2.2 Characterization of the HVRI-specific immune response after mixture HVRI-CLP immunization in mice

Due to the results obtained after single immunization of mice and the suggestions of previous reports in order to enhance cross-reactivity (Puntoriero *et al.*, 1998), (Esumi *et al.*, 1998), mice were also immunized with a mixture of the generated HVRI-variants (immunization with all four HVRI-variants in one group). In contrast to the anti-HVRI response induced in mice immunized with single HVRI-variants, the induced anti-HVRI response was much higher (see Fig. 4.9). Those results support the findings of Roccasecca *et al*., who demonstrated the possibility to achieve a broadly cross-reactive immune response in rabbits by immunization with a mixture of HVRI derived mimotopes (Roccasecca *et al.*, 2001).

To further analyze the improved antibody response observed in mice immunized with a mixture of HVRI compared to the one of mice immunized with single HVRI-variants (see section above), both sera were tested for their true cross-reactive potential by binding to 326 randomly selected HVRI-sequences isolated from patients (see Fig. 4.11) (Experiments were carried in the laboratory of Yury Khudyakov; Centers for Disease Control and Prevention, Atlanta, USA). Results showed 19% and 25% cross-reactivity of single immunized sera and mixed immunized sera to the panel of naturally occurring HVRI-variants. Also the strength of the response was much higher in mixed immunized mouse serum compared to single immunized mouse serum. Due to the fact that this analysis was done with serum diluted 1:800, most likely the cross-reactive activity could be significantly increased with the use of more concentrated serum. One reason for a better immune response in serum of mice immunized with the mixture, could be that a broader antibody repertoire can be induced resulting from the fact that 4 different HVRI-variants provide more alternatives for the induction and combination of various antibodies. Concordant to the presented cross-reactivity demonstrated by the induced Abs, Cerino *et al.* already provided a plausible

explanation saying that the very similar hydropathy and antigenicity profiles of HVRI-variants reveal a substantial conformational conservation. This confirms also the existence of an active selection process (Cerino *et al.*, 2001). On the other hand, Zucchelli *et al.* stated that titers *per se* are not responsible for the observed increase in cross-reactivity after mixture immunization, but rather that some immunogenic cooperation occurs between the different chimeric proteins when they are simultaneously presented to the immune system (Zucchelli *et al.*, 2001).

Shang *et al.* already suggested that with an appropriate HVRI peptide immunization scheme, high titer, broadly cross-reactive, blocking antibodies to HCV can be produced (Shang *et al.*, 1999). In order to achieve a protection against most HCV isolates, Campo *et al.* already showed that 19 cross-reactive HVRI-variants would in principal be sufficient to achieve this protective rate (Campo *et al.*, 2012). Also the human pneumococcal polysaccharide vaccine (PPSV23) showed that it is indeed possible to induce antibodies to 23 antigens (Manoff *et al.*, 2010), (Musher *et al.*, 2010). According to these data, an immunizatin sheme applying a higher number of HVRI-variants would be a desirable experiment for the future.

In summary results showed that immunization of mice with a mixture of HVRI-CLPs induces high titers of anti-HVRI antibodies, which are able to cross-react with 25% of naturally occurring HVRI-sequences, showing the high potential of this vaccine strategy.

5.2.3 Characterization of the neutralizing capacity in mice after single HVRI-CLP immunization

The development of neutralizing antibodies directed against the HVRI is crucial for the resolution of the HCV infection in humans. Therefore, one of the most important tasks of this project was to analyze whether the induced anti-HVRI antibodies were, besides their cross-reactivity, also able to neutralize virus infection. After immunization of mice with the single HVRI-variants, serum of each group was highly able to neutralize HCVpp bearing the homologous variants (see Fig. 4.6). Serum was also able to partially cross-neutralize HCVpp bearing the HVRI-variants present in the vaccine of the other groups, but none of the sera was able to neutralize all of the variants used for immunization of the different groups (see Fig. 4.6). When sera were tested for their neutralizing ability to heterologous variants not included in the vaccine

(H77, Ad78 and J6) a similar pattern was observed. Infection with HCVpp bearing H77 could be neutralized effectively while infection with HCVpp, Ad78 and J6 demonstrated reduced efficiency of neutralization (see Fig. 4.8). Nevertheless, results showed cross-neutralizing ability of the sera. These data are in accordance with other studies which evaluated the neutralization effects of sera in BALB/c mice immunized with mimotope proteins. These data also showed that the mimotope proteins could react to the HCV positive sera (Yang *et al.*, 2008).

The obtained results indicate clearly that immunization with single HVRI-CLPs induces partially cross-neutralizing antibody responses in mice.

5.2.4 Characterization of the neutralizing capacity in mice after mixture HVRI-CLP immunization

After showing an improved antibody response in mice immunized with a mixture of HVRI-variants, this hypothesis was also proven in neutralization results. As expected, compared to the neutralization results demonstrated after single immunization of mice, the immunization of mice with mixed HVRI-variants revealed even better neutralization results (see Fig. 4.6 and Fig. 4.12). Outstanding in both immunization schemes (single and mixture) is that HCVpp bearing the G31 variant was mostly the least neutralized (compare Fig. 4.6 and Fig. 4.12). These data are not in accordance with ELISA data which did not show differences in the immune response of this sequence to the other variants (compare Fig. 4.3 and Fig. 4.9). One explanation could be that antibodies against G31 are able to bind but they are not able to neutralize infection. Moreover, it was already argued by others that Abs raised against HVRI peptides are unable to recognize the same sequence when expressed in the context of a correctly folded complete E2 glycoprotein that includes HVRI (Cerino *et al.*, 2001).

The neutralizing capacity to heterologous variants, not included in the vaccine (HCVpp bearing H77, Ad78 or J6), showed similar results after mixture (see Fig. 4.13) and single immunization (see Fig. 4.8). In general the cross-neutralizing capacity of the sera from mice immunized with the mixture was shown very clearly. The neutralization results generated to heterologous variants showed in most cases a reduced capacity of the sera to neutralize HCVpp bearing J6 (GT2a). However, this was not an unexpected result as most likely this is due to the fact that this variant is

GT2a and could just not be recognized by the antibodies. This is also in accordance with previous experiments, which tested the true cross-reactivity of the used HVRI-variants (Fig. 4.11), and already showed that a cross-reactivity of 25% was achieved, providing a plausible explanation for the fact that specific HVRI-variants are just not recognized. However, for all used HVRI-variants it applies that specific aa residues of the HVRI may determine (cross)-reactivity levels in general, which was already demonstrated by several gropus. Consistently, Callens *et al.* showed that HCVpp infectivity increased with the number of basic residues in HVRI and the presence or absence of basic residues at specific positions modulated HCVpp infectivity (and were found to modulate virus entry) (Callens *et al.*, 2005). Similarly, differential incorporation of E1E2 (resulting from a different aa composition of the HVRI) into HCVpp was shown to result in altered neutralization sensitivities in other studies (Tarr *et al.*, 2006). On the other hand, it was already shown by several groups that the inter-genomic swab in HVRI had little effect on virus infectivity, consistent with the flexibility of this segment, its relative independence from the rest of E2 (Forns *et al.*, 2000), (McCaffrey *et al.*, 2007) and it shows tolerance to certain mutations (Vieyres *et al.*, 2010). Moreover, it was already believed that cross-neutralizing antibodies recognize overall shapes rather than primary sequences (Puntoriero *et al.*, 1998) as shown for the V3 loop of HIV gp120 (Nara *et al.*, 1991).

In general, it should be considered that only three different variants of the HVRI (representing 2 out of seven genotypes) were used to analyze the cross-neutralizing capacity. To achieve more significant results, the sera were tested with a higher amount of variants (see section 5.2.6).

In conclusion, these results demonstrate that sera from mice immunized with the mixture of HVRI-CLPs are highly able to cross-neutralize naturally HCV strains which bear a quite different HVR-sequence compared to the ones used for immunization.

5.2.5 Immunization with an improved HVRI-CLP mixture induces a more vigorous neutralizing immune response in mice

In order to analyze the immunogenicity in mice with an approved adjuvant (AS03), immunization with the mixture of the HVRI-variants in AS03 (i.m.) was compared to the immunization results with the mixture applied in IFA (s.c.) (improved vaccine strategy). ELISA data revealed a much better immune response in mice when the

vaccine was applied in AS03 compared to IFA (see Fig. 4.14). Also in accordance with ELISA, the mixture immunization of mice applied i.m. revealed an even better neutralizing capacity compared to s.c. immunization (see Fig. 4.16). Serum of these mice demonstrated a significantly high ability to neutralize infection with homologous HCVpp (see Fig. 4.16 A) and similar values to heterologous variants compared to s.c. immunization (see Fig. 4.17). The reason for the demonstrated improvement of the immune response by the i.m. route is not completely clear, yet. However, when also taking into account the core-specific immune response, a reduced response in comparison to the first single and mixture immunization (see Fig. 4.4. and Fig. 4.10) can be oserved in both vaccine preparations (see Fig. 4.15). A reason for the different core response in mice immunized with the improved vaccine strategy, AS03 i.m, could be that during the immunization procedure with IFA, HVRI-CLPs degrade, meaning that IFA has some unfavourable influence on the stability of CLPs while AS03 has not. Moreover, it is likely that during immunization with AS03 HVRI-CLPs stay intact which leads to a high, correctly folded, epitope presentation on the particles accompanied by a high HVRI-specific immune response and a lower core response as the core protein is more hidden by the presented epitopes. However, in this case a difference in the titer to the core protein in mice immunized s.c. with the improved vaccine strategy as well as with the “first mixture” in comparison to mice immunized with the improved vaccine strategy i.m. would be expected, but this was not the case, both titers showed the same values (see Fig. 4.15). Only in the first mixture immunization trial (s.c.), a higher core response could be detected (see Fig. 4.10). A difference in titer with respect to the administration route was detected only against the HVRI but not against core (compare Fig. 4.14 and Fig. 4.15), why this was not the case also in the improved vaccine strategy administered s.c. remains still unclear.

Another explanation for the demonstrated improvement of the immune response by the i.m. route could be, besides the adjuvant used, the applied injection modes. This explanation would be in accordance with the arguments stated by Nakano *et al*., who argued that at least in the HCV E2 model, a different spectrum of immunogenic sequences appears to be exploited by the host and/or at different degrees, in part depend on the injection mode. It remains determined whether differences in the amount of expressed antigens or in the processing of the expressed antigen on the presentation of the determinant(s) to the immune system account for the observed differences. Expression of the injected plasmid probably takes place in different types

of cells, and processing of the expressed antigens could consequently be different. In addition, depending on the immunization mode, different antigen-presenting cells (APCs) may be recruited. Unfortunately, to date only very few studies have addressed this question (Nakano *et al.*, 1997).

Nevertheless, in our opinion the enhanced results demonstrated by the improved vaccine preparation may most likely be linked to the adjuvant used. As already suggested from the ELISA results, these data show again that most likely, i.m. application of the vaccine is the most favourable administration route for HVRI-CLPs. This result is not only favourable when considering the use of this prototype vaccine in humans as the use of IFA in humans in forbidden. Further this application could be confirmed by a study of Frey *et al.* in which three different dosages of HCV E1E2 adjuvanted in AS03 to healthy HCV-negative adults were applied, who showed a well tolerability of the vaccine administered i.m. (Frey *et al.*, 2010).

Taken together, these findings led to the conclusion that improvement of the vaccine strategy results in significantly higher induction of neutralizing antibodies in mice. These results confirm the effective strategy to improve HCV HVRI-specific immune responses making our strategy very promising.

5.2.6 Optimization of the mixture immunization leads to the induction of a potent cross-neutralizing response in mice

Further, the true cross-neutralizing potential of the serum to randomly selected HVRI-variants isolated from patients was determined (see Fig. 4.18 and Fig. 4.19). The results showed the ability of the serum to weakly neutralize 4 out of 19 patient derived HVRI-sequences, while 4 other variants could be highly neutralized (see Fig. 4.18 and Fig. 4.19). When taking into account both, the variants which were weakly neutralized as well as the ones which could be highly neutralized, the cross-neutralizing capacity of the serum accounts for 42%. When evaluating these data one should consider that examination of the data was done in a stricter way compared to analysis of the residual neutralization data (experiments were carried out in the laboratory of William O Osbourn; John Hopkins School of Medicine, Baltimore, USA). After taking into account all the limitations used for the analysis of these data, the significantly high capacity of the serum (AS03, i.m.) to neutralize

HVRI-variants bearing quite different sequences compared to the once included in the vaccine becomes clear one more time.

When comparing the sequences of HVRI-variants which could be highly neutralized to the ones which were only weakly or not neutralized by the serum, no specific pattern in amino acid composition becomes obvious (compare Fig. 4.7 and Fig. 4.18). In the beginning of our experiments we thought that the PG-motif (Proline, and Glycine) at position 22/23 may influence the binding of nAbs, however the variants tested by W. Osburn which were highly neutralized by the serum do only exhibit the G residue at position 23. Possibly the Glutamine (Q) at position 26 also regulates some binding. This would be in accordance with data obtained in rabbits immunized with the HVRI, which suggested the presence of a conserved, partially conformational, epitope located at the C-terminus of HVRI, including the conserved G and Q amino acids in positions 23 and 26 (Shang *et al.*, 1999). This was further confirmed by Li *et al.* who showed that anti-HVRI cross-reactivity of a rabbit polyclonal antibody and one mouse mAb was highly dependent on the presence of these two conserved amino acids in their respective positions. The replacement of either or both by Leucine (L) or Valine (V) amino acids resulted in a significant loss or a complete disappearance of antigenic recognition by cross-reactive HVRI antibodies (Li *et al.*, 2001). Nevertheless, it is still not completely clear whether some other residues regulate binding of nAbs or whether this binding occurs arbitrarily.

It has often been argued that vaccines using a nAb approach to HVRI will fail, as the HVRI has been traditionally considered structurally flexible and antigenically variable. However, not only the results of this project but also other groups already suggested that utilizing virus neutralization Abs that target highly conserved, functionally constrained epitopes will limit the likelihood of viral escape, and therefore maximize virus neutralizing Abs therapeutical potential. Even if escape occurs within these conserved epitopes, it is likely that this will come at a replicative cost to the virus, such as decreased receptor binding (Beaumont *et al.*, 2004), (Pinter *et al.*, 2004), (Pugach *et al.*, 2004). Moreover, even an immune response of 40% to HCV (as demonstrated by the neutralization data of W. Osburn; see Fig. 4.18, 4.19) would probably result in a lower viral load. In this case one could speculate about a stronger and earlier stimulation of the T-cell response leading to an acute or even resolving HCV infection and no progression to the chronic phase which makes the vaccine strategy generated in this project again very promising.

In general, the results showed that anti-HVRI antibodies are able to neutralize 40% of patient derived HCVpp, demonstrating that HVRI-CLPs are indeed able to induce a high level of cross-neutralizing HCV antibodies.

5.2.7 Characterization of the immunoglobulin isotype switch in mice immunized with the improved HVRI-CLP mixture

In order to determine the balance of T_H1 and T_H2-responses among AS03 and IFA immunized mice, the IgG-subclass ratio in this serum was analysed. Analysis of IgG-subclasses in mice immunized with the HVRI-mixture in AS03 revealed the induction of a mixed T_H1/ T_H2-response with a T_H1-trend (see Tab. 4.1). In mice immunized with the HVRI-mixture in IFA the induction of a T_H1-response was not as strong as in AS03 vaccinated mice but rather a mixed T_H1/T_H2-response was detectable (see Tab.4.2).

The haplotype of C57BL/6-mice is $H2^b$ which preferentially drives the immune response into T_H1 direction in these animals. Vaccination of these mice with CLPs bearing the nucleic acid binding domain would even potentiate the T_H1-response however, vaccination trials with HBc-CLPs not harbouring the nucleic acid binding domain, as done in this project (see Fig. 4.1), are able to shift the immune response to activate T_H2-cells. This should result in the production of higher amounts of specific antibodies to the presented peptide (Riedl *et al.*, 2002). Furthermore, application of immunogens in adjuvants composed of oil-in-water emulsions, like AS03 are known to induce T_H2-responses, contrary, the use of water-in-oil emulsions, like IFA induce a T_H1-response (Guy, 2007). Moreover, the lack of co-stimulatory TLR-ligands (e.g. RNA) and the repetitive arrangement of the used CLPs normally leads to the activation of T_H2-cells to produce cytokines that drive B-cells to differentiate and produce IgGs of other types and to proliferate and secrete IgM to the detected virus-like structure. Subsequently T_H2-cells can initiate the production of neutralizing antibodies (Murphy *et al.*, 2008). It was also shown that adjuvantation of vaccines leads to better persistence of neutralizing antibodies (Moris *et al.*, 2011). Taking into account all these influencing factors, one could most likely expect the induction of a T_H2-respsonse in the conducted experiments. However, in comparison to the data obtained in these experients, the induction of T_H1-responses in both groups of mice is uncommon and the expected T_H2-response was not confirmed.

Also with respect to the obtained neutralization data in which the serum of both groups showed high neutralizing capacity (pointing to a T_H2-response), the expected results were not cofirmed by this experiment (see Fig. 4.16). The reason for this difference is not completely clear, yet. One reason might be the ration of IgG-subclasses and their ability to bind but not being able to neutralize. The ratio of the different IgG-subclasses could also influence the capability of the antibodies to neutralize infection. Even though, we expected a T_H2-response in our experiments, some studies in which the same adjuvant (AS03) was used in humans, confirmed our results. Patients who obtained the influenza vaccine to strain H1N1 delivered in AS03, showed an overall bias to a H5N1-specific T_H1-response. Moreover, it was shown that all responses, i.e. antibodies, B-cell memory, and CD4 T-cells, were increased when the vaccine was formulated with AS03 adjuvant (Moris *et al.*, 2011). Also exploratory analyses in children immunized against influenza indicated that the induction of CD4 T-cell responses polarized in favour of a T_H1-profile (Diez-Domingo *et al.*, 2010). On the other hand, one could also argue that the vaccine carrier used (not bearing the nucleic acid binding domain) had no or minor effects on a shift of the immune response to a T_H2-induction. However, most likely the reason for the detected difference in IgG-subclass ratio and neutralization response was caused by both, the amount and the isotype ratio of the antibodies. Because it was already demonstrated that vaccine adjuvants change the clonal composition of antigen-specific CD4 T-cell populations responding to vaccines and favouring the selection of higher-affinity T-cells (Malherbe *et al.*, 2008). In general these findings suggest that most likely the adjuvant changes the T-cell response both, quantitatively and qualitatively.

5.2.8 Characterization of the immune response in mice against the vaccine carrier HBc

Finally, the expected effect of a high immune response to the carrier protein, HBc, was analyzed and could be confirmed in all immunization trials. The immunization with single HVRI-variants as well as the mixture immunization revealed high titers to the core protein (see Fig. 4.4 and Fig. 4.10). Nevertheless, due to several reasons (see section 1.5.1) the induction of a preferably little amount of antibodies to HBV core protein based vaccine-carriers would be desirable. As shown in the performed

experiments (see sections 4.2.1, 4.4 and 4.6) it would not be possible to completely avoid the induction of core-specific antibodies as in all immunization trials also antibodies against the core protein were detected. However, in principle anti-carrier antibodies do not provide an additional advantage as they do not present any protection. Furthermore, in case of a higher induction of antibodies to the carrier compared the induction of antibodies to the antigen, the intense self-immunity of the vaccine-carrier could also have negative influence on the effectiveness of a vaccine, as a too high core response could also increase the risk of a refreshment of the anti-carrier immune response, while disregarding the presented peptide after each booster immunization. This leads to a problem in HBV-core protein based vaccine carriers, as over 2 billion people have already been infected with HBV and therefore already show a core protein-specific B-cell response. Nevertheless, the possibility to use the woodchuck Hepatitis B Virus core protein as vaccine carrier was already demonstrated and resulted in a reduced cross-reactivity of anti-WHc antibodies with the diagnostic test-system for anti-HBc. The reason for this reduced cross-reactivity is probably the sequence difference between the human and woodchuck core protein in the area of the tip of the spikes (Billaud *et al.*, 2005a), (Billaud *et al.*, 2005b). In order to test this hypothesis and to further improve our vaccine, it was analyzed whether WHc-CLPs bearing HVRI-variants would still be able to form particles. As shown in Fig. 4.24, the generation of SplitCore-CLPs carrying the HVRI-variant R9 was successfully demonstrated. The insertion of HVRI-variants in the SplitCore-WHc was as possible as the insertion in the SplitCore-HBc system. After successful generation of WHc-CLPs carrying the other HVRI-variants or even more variants, it would be interesting to analyze their immunogenic potential in mice and compare these data to the values obtained with HBc-CLPs.

In conclusion, HBc SplitCore-CLPs as well as WHc SplitCore-CLPs carrying HVRI-variants are assembly competent. For this reason WHc-CLPs carrying HVRI-variants could be used as new type of immunogen which would be absolutely effective and show no cross-reactivity with diagnostic HBc-tests.

5.3 Immune response in guinea pigs after immunization with HVRI-CLPs

In order to compare the current data demonstrated in mice to the publication of Stamataki *et al.* who showed an increased immune response and neutralizing capacity to HCV glycoproteins in guinea pigs compared to mice, the generated HVRI-CLPs were also tested in guinea pigs. After the last booster, high antibody titers specific to the HVRI could be detected in guinea pigs (see Fig. 4.20), while an even higher antibody-response to the core-protein was shown (see Fig. 4.21). Also the neutralizing capacity of the serum was comparable to mice (compare Fig. 4.22 and Fig. 4.16 A). The ability to inhibit infection with heterologous variants (H77, Ad78 and J6) was slightly higher in guinea pigs. Surprisingly, in comparison to heterologous neutralization in mice which always showed least neutralization of HCVpp J6, serum of guinea pigs was able to neutralize HCVpp J6, but HCVpp bearing H77 was neutralized less efficient (see Fig. 4.23) Nevertheless, interpretation of the results should be done with caution as the vaccination experiments were carried out in Egypt under non pathogen free conditions. It may be argued that during the vaccination approach, animals were already infected with other e.g. flaviviruses like the Yellow Fewer Virus which could lead to a cross-reactive immune response. Furthermore, simultaneous bacterial infections could activate the complement system which in turn interfered with the neutralization results. In order to prevent this intervention in the sera of guinea pigs, in the current project sera were heat inactivated at 56°C. However we did not see any variation in the results compared to sera not heat inactivated. This may be due to other heat-stable compounds of the innate immune system. Consistently, also other groups already showed that neutralization titers of E2 significantly increased mAbs to HVRI mimotopes upon addition of guinea complement (Meyer *et al.*, 2002). Due to this reason, Stamataki *et al.* purified their antibodies before using them in neutralization experiments which specified their results.

As already mentioned, in general our data are similar to the data demonstrated by Stamataki *et al*.. Nevertheless, these experiments should be repeated under specific pathogen free conditions.

6 Summary

More than 170 million people worldwide are chronically infected with the Hepatitis C Virus (HCV). The recommended treatment with interferon-α in combination with Ribavirin does not lead to satisfactory results. A prophylactic vaccine against HCV is not available yet, and its development is a challenging task. It is well documented that the viral persistence is caused by effective evasion of the virus from adaptive immune response. On the other hand, HCV clearance is associated with rapid induction of neutralizing antibodies in the early phase of infection, as well as a cooperation between $CD8^+$ cytotoxic and $CD4^+$ helper T-cell responses. Recent data have demonstrated that antibodies to the hypervariable region I (HVRI) of HCV E2 are able to block both, cell-free as well as cell-to-cell, transmission of HCV. Therefore, the induction of HVRI-specific B-cells by a prophylactic vaccine is a very promising strategy for future vaccines. Previous vaccination trials with peptide, recombinant protein, DNA and vector-based vaccines in patients did not result in an induction of an effective immune response, which would effectively prevent the HCV infection. Due to this, a new strategy is required for the generation of a prophylactic vaccine. A particularly encouraging approach is the usage of cross-reactive HVRI variants presented on capsid-like particles (CLPs) to boost their immunogenicity

For this purpose, an improved HBc-CLP vaccine displaying four different highly cross-reactive HVRI-variants was constructed. The efficiency of the vaccine was tested in mice and guinea pigs. Upon immunization of mice with single HVRI-variants, high titers of cross-reactive anti-HVRI antibodies were detected. A significantly stronger immune response was detected after immunization with a mixture of these CLP-HVRI-variants. These particles induced high titers of anti-HVRI antibodies, which were able to cross-react with 25% of naturally occurring HVRI sequences. In a neutralization assay with HCV pseudoparticles (HCVpp) the anti-HVRI antibodies were able to neutralize homologous as well as heterologous HCVpp. Moreover, the analysis of the neutralizing capacity of sera from these mice to HCVpp bearing envelope proteins derived from 19 patients showed that anti-HVRI antibodies were able to neutralize 40% of patient-derived HCVpp, demonstrating that HVRI-CLPs are indeed able to induce cross-neutralizing HCV antibodies.

Furthermore, the induction of cross-reactive and cross-neutralizing antibodies could be verified in guinea pigs. Here guinea pig serum showed similar neutralizing capacity compared to mice serum.

Additionally, it was demonstrated that CLPs formed by the woodchuck core protein (WHc) that presents HVRI-variants, are similarly competent for assembly.

The presented results indicate that this new vaccine approach induces a potent anti-HVRI cross-reactive immune response, as well as cross-neutralizing HVRI-specific antibody responses. They consequently constitute a new strategy for a prototype vaccine against HCV infection in humans.

7 Zusammenfassung

Weltweit sind derzeit mehr als 170 Millionen Menschen chronisch mit dem Hepatitis C Virus (HCV) infiziert. Die empfohlene Behandlungsstrategie, eine Kombinationstherapie mit Interferon-α und Ribavirin, führt jedoch zu keinen zufriedenstellenden Ergebnissen. Die Entwicklung einer prophylaktischen Impfung stellt eine anspruchsvolle Aufgabe dar, welche bis heute nicht gelöst werden konnte. Forschungsergebnisse haben gezeigt, dass die Viruspersistenz durch die Evasion des Virus vor der adaptiven Immunantwort verursacht wird. Die Eliminierung des Virus wird mit einer raschen Induktion von neutralisierenden Antikörpern, sowie einer Kooperation zwischen $CD8^+$ zytotoxischen T-Zellen und $CD4^+$ T-Helferzellen assoziiert. Aktuelle Studien beweisen, dass Antikörper gegen die Hypervariable Region I (HVRI) des E2-Proteins in der Lage sind, zellfreie, aber auch eine Übertragung des Virus von Zelle-zu-Zelle zu verhindern. Daher wurde bereits angenommen, dass die Induktion von HVRI-spezifischen B-Zellen eine der vielversprechendsten Ansatzmöglichkeiten für eine neuartige Vakzinierungsstrategie ist. Vorangegangene klinische Studien zur Entwicklung neuer Impfstoffe gegen HCV unter Verwendung von Peptiden, rekombinantem Protein, DNA und vektorbasierenden Vakzinierungsstrategien in Patienten waren ohne Erfolg und führten zu keiner ausreichenden Immunantwort. Infolgedessen werden neue wirksame Vakzinierungsstrategien dringend benötigt. Einen besonders vielverpsrechenden Ansatz stellt die Präsentation von kreuz-reaktiven HVRI-Varianten auf partikulären Trägern wie CLPs (*capsid like particles*) dar, welche deren Immunogenität um ein Vielfaches steigern können.

Zu diesem Zweck wurde eine Vakzine bestehend aus vier verschiedenen stark kreuz-reaktiven HVRI-Varianten, die auf HBc-CLPs präsentiert werden, konstruiert. Die Wirksamkeit wurde zunächst in Mäusen und anschließend in Meerschweinchen getestet. Nach der Immunisierung von Mäusen mit Einzelvarianten der HVRI konnten hohe Titer kreuz-reaktiver HVRI-Antikörper detektiert werden. Eine signifikant stärkere Immunantwort wurde beobachtet, nachdem die Mäuse mit einer Mischung der CLP-HVRI-Varianten immunisiert wurden. Diese Partikel induzierten starke Titer von HVRI-Antikörpern, welche außerdem in der Lage waren mit 25% natürlich vorkommender HVRI-Sequenzen kreuz zu reagieren. Im so genannten HCV-Pseudopartikel (HCVpp) Neutralisationssystem, konnte gezeigt werden, dass anti-

HVRI Antikörper sowohl homologen als auch heterologen HCVpp neutralisieren können. Des Weiteren konnte gezeigt werden, dass die HVRI-Antikörper im Serum dieser Mäuse in der Lage waren, HCVpp mit 19 verschiedenen, von Patienten isolierten HCV Hüllproteinen, zu 40% zu neutralisieren. Diese Ergebnisse zeigen, dass HVRI-CLPs tatsächlich kreuz-neutralisierende HCV Antikörper induzieren können.

Darüber hinaus konnte die Induktion von kreuz-reaktiven und kreuz-neutralisierenden Antikörpern im Meerschweinchen bestätigt werden, denn das Serum der Meerschweinchen wies vergleichbare Ergebnisse der Neutralisationseffizienz zu denen der Mäuse auf.

Abschließend konnte gezeigt werden, dass HVRI-präsentierende CLPs auch durch das Core-Protein des Waldmurmeltiers (WHc) produziert werden können.

Diese Ergebnisse verdeutlichen, dass der gewählte Ansatz zur Generierung einer neuen Prototypvakzine eine wirksame anti-HVRI kreuz-reaktive Immunantwort hervorruft, sowie zu einer Induktion von kreuz-neutralisierenden HVRI-spezifischen Antikörpern in der Lage ist. Die in dieser Arbeit beschriebenen Ergebnisse stellen somit eine neue Impfstrategie einer Prototypvakzie gegen Hepatitis C Infektionen im Menschen dar.

8 References

Acton, S. L., *et al.* (1994): Expression cloning of SR-BI, a CD36-related class B scavenger receptor. *J Biol Chem* 269(33): 21003-9.

Agnello, V., *et al.* (1999): Hepatitis C virus and other flaviviridae viruses enter cells via low density lipoprotein receptor. *Proc Natl Acad Sci U S A* 96(22): 12766-71.

Ahlenstiel, G., *et al.* (2008): Distinct KIR/HLA compound genotypes affect the kinetics of human antiviral natural killer cell responses. *J Clin Invest* 118(3): 1017-26.

Angus, A. G. and A. H. Patel (2011): Immunotherapeutic potential of neutralizing antibodies targeting conserved regions of the HCV envelope glycoprotein E2. *Future Microbiol* 6(3): 279-94.

Bankwitz, D., *et al.* (2010): Hepatitis C virus hypervariable region 1 modulates receptor interactions, conceals the CD81 binding site, and protects conserved neutralizing epitopes. *J Virol* 84(11): 5751-63.

Barba, G., *et al.* (1997): Hepatitis C virus core protein shows a cytoplasmic localization and associates to cellular lipid storage droplets. *Proc Natl Acad Sci U S A* 94(4): 1200-5.

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

Bartenschlager, R., *et al.* (1993): Nonstructural protein 3 of the hepatitis C virus encodes a serine-type proteinase required for cleavage at the NS3/4 and NS4/5 junctions. *J Virol* 67(7): 3835-44.

Barth, H., *et al.* (2003): Cellular binding of hepatitis C virus envelope glycoprotein E2 requires cell surface heparan sulfate. *J Biol Chem* 278(42): 41003-12.

Bartosch, B., *et al.* (2003a): Infectious hepatitis C virus pseudo-particles containing functional E1-E2 envelope protein complexes. *J Exp Med* 197(5): 633-42.

Bartosch, B., *et al.* (2005): 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(13): 8217-29.

Bartosch, B., *et al.* (2003b): 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(43): 41624-30.

Basu, A., *et al.* (2004): The hypervariable region 1 of the E2 glycoprotein of hepatitis C virus binds to glycosaminoglycans, but this binding does not lead to infection in a pseudotype system. *J Virol* 78(9): 4478-86.

Basu, A., *et al.* (2007): Sulfated homologues of heparin inhibit hepatitis C virus entry into mammalian cells. *J Virol* 81(8): 3933-41.

Baumert, T. F., *et al.* (1998): Hepatitis C virus structural proteins assemble into viruslike particles in insect cells. *J Virol* 72(5): 3827-36.

Beaumont, T., *et al.* (2004): Increased sensitivity to CD4 binding site-directed neutralization following in vitro propagation on primary lymphocytes of a neutralization-resistant human immunodeficiency virus IIIB strain isolated from an accidentally infected laboratory worker. *J Virol* 78(11): 5651-7.

Beck, J. and M. Nassal (2007): Hepatitis B virus replication. *World J Gastroenterol* 13(1): 48-64.

Billaud, J. N., *et al.* (2005a): Combinatorial approach to hepadnavirus-like particle vaccine design. *J Virol* 79(21): 13656-66.

Billaud, J. N., *et al.* (2005b): Comparative antigenicity and immunogenicity of hepadnavirus core proteins. *J Virol* 79(21): 13641-55.

Birnbaum, F. and M. Nassal (1990): Hepatitis B virus nucleocapsid assembly: primary structure requirements in the core protein. *J Virol* 64(7): 3319-30.

Blight, K. J., *et al.* (2000): Efficient initiation of HCV RNA replication in cell culture. *Science* 290(5498): 1972-4.

Blight, K. J., *et al.* (2002): Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. *J Virol* 76(24): 13001-14.

Blight, K. J., *et al.* (2003): Efficient replication of hepatitis C virus genotype 1a RNAs in cell culture. *J Virol* 77(5): 3181-90.

Boonstra, A., *et al.* (2009): Experimental models for hepatitis C viral infection. *Hepatology* 50(5): 1646-55.

Bottcher, B., *et al.* (1997): Determination of the fold of the core protein of hepatitis B virus by electron cryomicroscopy. *Nature* 386(6620): 88-91.

Branch, A. D., *et al.* (2005): The hepatitis C virus alternate reading frame (ARF) and its family of novel products: the alternate reading frame protein/F-protein, the double-frameshift protein, and others. *Semin Liver Dis* 25(1): 105-17.

Brazzoli, M., *et al.* (2008): CD81 is a central regulator of cellular events required for hepatitis C virus infection of human hepatocytes. *J Virol* 82(17): 8316-29.

Brimacombe, C. L., *et al.* (2011): Neutralizing antibody-resistant hepatitis C virus cell-to-cell transmission. *J Virol* 85(1): 596-605.

Brown, R. J., *et al.* (2007): Cross-genotype characterization of genetic diversity and molecular adaptation in hepatitis C virus envelope glycoprotein genes. *J Gen Virol* 88(Pt 2): 458-69.

Brown, R. S. (2005): Hepatitis C and liver transplantation. *Nature* 436(7053): 973-8.

Burlone, M. E. and A. Budkowska (2009): Hepatitis C virus cell entry: role of lipoproteins and cellular receptors. *J Gen Virol* 90(Pt 5): 1055-70.

Burton, D. R. and J. M. Woof (1992): Human antibody effector function. *Adv Immunol* 51: 1-84.

Burton, D. R., *et al.* (2001): A model for neutralization of viruses based on antibody coating of the virion surface. *Curr Top Microbiol Immunol* 260: 109-43.

Callens, N., *et al.* (2005): Basic residues in hypervariable region 1 of hepatitis C virus envelope glycoprotein e2 contribute to virus entry. *J Virol* 79(24): 15331-41.

Calvo, D. and M. A. Vega (1993): Identification, primary structure, and distribution of CLA-1, a novel member of the CD36/LIMPII gene family. *J Biol Chem* 268(25): 18929-35.

Campo, D. S., *et al.* (2012): Hepatitis C virus antigenic convergence. *Sci Rep* 2: 267.

Cerino, A., *et al.* (2001): Monoclonal antibodies with broad specificity for hepatitis C virus hypervariable region 1 variants can recognize viral particles. *J Immunol* 167(7): 3878-86.

Clayton, R. F., *et al.* (2002): Analysis of antigenicity and topology of E2 glycoprotein present on recombinant hepatitis C virus-like particles. *J Virol* 76(15): 7672-82.

Cocquerel, L., *et al.* (2001): Coexpression of hepatitis C virus envelope proteins E1 and E2 in cis improves the stability of membrane insertion of E2. *J Gen Virol* 82(Pt 7): 1629-35.

Cocquerel, L., *et al.* (2002): Topological changes in the transmembrane domains of hepatitis C virus envelope glycoproteins. *EMBO J* 21(12): 2893-902.

Cooper, S., *et al.* (1999): Analysis of a successful immune response against hepatitis C virus. *Immunity* 10(4): 439-49.

Cormier, E. G., *et al.* (2004): CD81 is an entry coreceptor for hepatitis C virus. *Proc Natl Acad Sci U S A* 101(19): 7270-4.

Cox, A. L., *et al.* (2005): Cellular immune selection with hepatitis C virus persistence in humans. *J Exp Med* 201(11): 1741-52.

Deleersnyder, V., *et al.* (1997): Formation of native hepatitis C virus glycoprotein complexes. *J Virol* 71(1): 697-704.

Di Lorenzo, C., *et al.* (2011): Hepatitis C virus evasion mechanisms from neutralizing antibodies. *Viruses* 3(11): 2280-300.

Diez-Domingo, J., *et al.* (2010): Immunogenicity and Safety of H5N1 A/Vietnam/1194/2004 (Clade 1) AS03-adjuvanted prepandemic candidate influenza vaccines in children aged 3 to 9 years: a phase ii, randomized, open, controlled study. *Pediatr Infect Dis J* 29(6): e35-46.

Dowd, K. A., *et al.* (2009): Selection pressure from neutralizing antibodies drives sequence evolution during acute infection with hepatitis C virus. *Gastroenterology* 136(7): 2377-86.

Dreux, M., *et al.* (2007): The exchangeable apolipoprotein ApoC-I promotes membrane fusion of hepatitis C virus. *J Biol Chem* 282(44): 32357-69.

Dreux, M., *et al.* (2006): High density lipoprotein inhibits hepatitis C virus-neutralizing antibodies by stimulating cell entry via activation of the scavenger receptor BI. *J Biol Chem* 281(27): 18285-95.

Drummer, H. E., *et al.* (2003): Cell surface expression of functional hepatitis C virus E1 and E2 glycoproteins. *FEBS Lett* 546(2-3): 385-90.

Dubuisson, J. (2007): Hepatitis C virus proteins. *World J Gastroenterol* 13(17): 2406-15.

Dubuisson, J., *et al.* (2002): Interaction of hepatitis C virus proteins with host cell membranes and lipids. *Trends Cell Biol* 12(11): 517-23.

Dubuisson, J., *et al.* (2008): Early steps of the hepatitis C virus life cycle. *Cell Microbiol* 10(4): 821-7.

Dubuisson, J., *et al.* (1994): Formation and intracellular localization of hepatitis C virus envelope glycoprotein complexes expressed by recombinant vaccinia and Sindbis viruses. *J Virol* 68(10): 6147-60.

Duvet, S., *et al.* (1998): Hepatitis C virus glycoprotein complex localization in the endoplasmic reticulum involves a determinant for retention and not retrieval. *J Biol Chem* 273(48): 32088-95.

Egger, D., *et al.* (2002): Expression of hepatitis C virus proteins induces distinct membrane alterations including a candidate viral replication complex. *J Virol* 76(12): 5974-84.

Esumi, M., *et al.* (1998): Murine antibodies against E2 and hypervariable region 1 cross-reactively capture hepatitis C virus. *Virology* 251(1): 158-64.

Esumi, M., *et al.* (1999): Experimental vaccine activities of recombinant E1 and E2 glycoproteins and hypervariable region 1 peptides of hepatitis C virus in chimpanzees. *Arch Virol* 144(5): 973-80.

European-Pharmacopoeia (2008): European-Pharmacopoeia. Strasbourg, France.

Evans, M. J., *et al.* (2007): Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry. *Nature* 446(7137): 801-5.

Falkowska, E., *et al.* (2007): Hepatitis C virus envelope glycoprotein E2 glycans modulate entry, CD81 binding, and neutralization. *J Virol* 81(15): 8072-9.

Farci, P., *et al.* (1996): 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(26): 15394-9.

Farci, P., *et al.* (2000): The outcome of acute hepatitis C predicted by the evolution of the viral quasispecies. *Science* 288(5464): 339-44.

Flint, M., *et al.* (2004): Characterization of infectious retroviral pseudotype particles bearing hepatitis C virus glycoproteins. *J Virol* 78(13): 6875-82.

Flint, M., *et al.* (2006): Diverse CD81 proteins support hepatitis C virus infection. *J Virol* 80(22): 11331-42.

Flint, M., *et al.* (1999): Characterization of hepatitis C virus E2 glycoprotein interaction with a putative cellular receptor, CD81. *J Virol* 73(8): 6235-44.

Forns, X., *et al.* (2000): Characterization of modified hepatitis C virus E2 proteins expressed on the cell surface. *Virology* 274(1): 75-85.

Foy, E., *et al.* (2003): Regulation of interferon regulatory factor-3 by the hepatitis C virus serine protease. *Science* 300(5622): 1145-8.

Frey, S. E., *et al.* (2010): Safety and immunogenicity of HCV E1E2 vaccine adjuvanted with MF59 administered to healthy adults. *Vaccine* 28(38): 6367-73.

Furuse, M., *et al.* (1998): Claudin-1 and -2: novel integral membrane proteins localizing at tight junctions with no sequence similarity to occludin. *J Cell Biol* 141(7): 1539-50.

Furuse, M., *et al.* (1993): Occludin: a novel integral membrane protein localizing at tight junctions. *J Cell Biol* 123(6 Pt 2): 1777-88.

Gardner, J. P., *et al.* (2003): L-SIGN (CD 209L) is a liver-specific capture receptor for hepatitis C virus. *Proc Natl Acad Sci U S A* 100(8): 4498-503.

Garrone, P., *et al.* (2011): A prime-boost strategy using virus-like particles pseudotyped for HCV proteins triggers broadly neutralizing antibodies in macaques. *Sci Transl Med* 3(94): 94ra71.

Garry, R. F. and S. Dash (2003): Proteomics computational analyses suggest that hepatitis C virus E1 and pestivirus E2 envelope glycoproteins are truncated class II fusion proteins. *Virology* 307(2): 255-65.

Goffard, A., *et al.* (2005): Role of N-linked glycans in the functions of hepatitis C virus envelope glycoproteins. *J Virol* 79(13): 8400-9.

Gottwein, J. M., *et al.* (2009): Development and characterization of hepatitis C virus genotype 1-7 cell culture systems: role of CD81 and scavenger receptor class B type I and effect of antiviral drugs. *Hepatology* 49(2): 364-77.

Grakoui, A., *et al.* (2003): HCV persistence and immune evasion in the absence of memory T cell help. *Science* 302(5645): 659-62.

Guy, B. (2007): The perfect mix: recent progress in adjuvant research. *Nat Rev Microbiol* 5(7): 505-17.

Halliday, J., *et al.* (2011): Vaccination for hepatitis C virus: closing in on an evasive target. *Expert Rev Vaccines* 10(5): 659-72.

Helle, F., *et al.* (2007): The neutralizing activity of anti-hepatitis C virus antibodies is modulated by specific glycans on the E2 envelope protein. *J Virol* 81(15): 8101-11.

Helle, F., *et al.* (2010): Role of N-linked glycans in the functions of hepatitis C virus envelope proteins incorporated into infectious virions. *J Virol* 84(22): 11905-15.

Hilleman, M. R. (2001): Overview of the pathogenesis, prophylaxis and therapeusis of viral hepatitis B, with focus on reduction to practical applications. *Vaccine* 19(15-16): 1837-48.

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

Houghton, M. (1996): The hepatitis C virus. In: Knight DM, howley PM (eds) Fields virology. Lippincott-Raven, Philadelphia. pp 1035-1058.

Hsu, M., *et al.* (2003): Hepatitis C virus glycoproteins mediate pH-dependent cell entry of pseudotyped retroviral particles. *Proc Natl Acad Sci U S A* 100(12): 7271-6.

Inchauspe, G. and M. L. Michel (2007): Vaccines and immunotherapies against hepatitis B and hepatitis C viruses. *J Viral Hepat* 14 Suppl 1: 97-103.

Invitrogen (2004). pcDNA3.1/V5-His TOPO TA Expression Kit, Invitrogen.

Jensen, D. M. (2011): A new era of hepatitis C therapy begins. *N Engl J Med* 364(13): 1272-4.

Jiang, J. and G. Luo (2009): Apolipoprotein E but not B is required for the formation of infectious hepatitis C virus particles. *J Virol* 83(24): 12680-91.

Kao, J. H. and D. S. Chen (2002): Global control of hepatitis B virus infection. *Lancet Infect Dis* 2(7): 395-403.

Kaplan, D. E.*, et al.* (2007): Discordant role of CD4 T-cell response relative to neutralizing antibody and CD8 T-cell responses in acute hepatitis C. *Gastroenterology* 132(2): 654-66.

Kato, N.*, et al.* (1993): Humoral immune response to hypervariable region 1 of the putative envelope glycoprotein (gp70) of hepatitis C virus. *J Virol* 67(7): 3923-30.

Kato, N.*, et al.* (1994): Genetic drift in hypervariable region 1 of the viral genome in persistent hepatitis C virus infection. *J Virol* 68(8): 4776-84.

Kato, N.*, et al.* (1992): Marked sequence diversity in the putative envelope proteins of hepatitis C viruses. *Virus Res* 22(2): 107-23.

Kato, T.*, et al.* (2003): Efficient replication of the genotype 2a hepatitis C virus subgenomic replicon. *Gastroenterology* 125(6): 1808-17.

Kato, T.*, et al.* (2001): Sequence analysis of hepatitis C virus isolated from a fulminant hepatitis patient. *J Med Virol* 64(3): 334-9.

Keck, Z. Y.*, et al.* (2008a): Therapeutic control of hepatitis C virus: the role of neutralizing monoclonal antibodies. *Curr Top Microbiol Immunol* 317: 1-38.

Keck, Z. Y.*, et al.* (2008b): Definition of a conserved immunodominant domain on hepatitis C virus E2 glycoprotein by neutralizing human monoclonal antibodies. *J Virol* 82(12): 6061-6.

Knipe, D. M., Howley, P.M., Griffin, D. E. (2007): Field's Virology, Lippincott Williams & Wilkins, Philadelphia.

Koletzki, D.*, et al.* (1999): HBV core particles allow the insertion and surface exposure of the entire potentially protective region of Puumala hantavirus nucleocapsid protein. *Biol Chem* 380(3): 325-33.

Kolykhalov, A. A.*, et al.* (1996): Identification of a highly conserved sequence element at the 3' terminus of hepatitis C virus genome RNA. *J Virol* 70(6): 3363-71.

Koutsoudakis, G.*, et al.* (2006): Characterization of the early steps of hepatitis C virus infection by using luciferase reporter viruses. *J Virol* 80(11): 5308-20.

Kratz, P. A.*, et al.* (1999): Native display of complete foreign protein domains on the surface of hepatitis B virus capsids. *Proc Natl Acad Sci U S A* 96(5): 1915-20.

Krawczynski, K.*, et al.* (1996): Effect of immune globulin on the prevention of experimental hepatitis C virus infection. *J Infect Dis* 173(4): 822-8.

Lagging, L. M.*, et al.* (1998): Functional role of hepatitis C virus chimeric glycoproteins in the infectivity of pseudotyped virus. *J Virol* 72(5): 3539-46.

Lauer, G. M. and R. T. Chung (2007): Vaccine-induced T-cell responses against HCV: one step taken, more to follow. *Gastroenterology* 132(4): 1626-8.

Lavie, M.*, et al.* (2007): Assembly of a functional HCV glycoprotein heterodimer. *Curr Issues Mol Biol* 9(2): 71-86.

Lavillette, D.*, et al.* (2007): Characterization of fusion determinants points to the involvement of three discrete regions of both E1 and E2 glycoproteins in the membrane fusion process of hepatitis C virus. *J Virol* 81(16): 8752-65.

Lavillette, D., *et al.* (2005): Characterization of host-range and cell entry properties of the major genotypes and subtypes of hepatitis C virus. *Hepatology* 41(2): 265-74.

Law, M., *et al.* (2008): Broadly neutralizing antibodies protect against hepatitis C virus quasispecies challenge. *Nat Med* 14(1): 25-7.

Lechmann, M. and T. J. Liang (2000): Vaccine development for hepatitis C. *Semin Liver Dis* 20(2): 211-26.

Lechner, F., *et al.* (2000): Analysis of successful immune responses in persons infected with hepatitis C virus. *J Exp Med* 191(9): 1499-512.

Leroux-Roels, I., *et al.* (2007): Antigen sparing and cross-reactive immunity with an adjuvanted rH5N1 prototype pandemic influenza vaccine: a randomised controlled trial. *Lancet* 370(9587): 580-9.

Levin, J. and F. B. Bang (1964a): A Description of Cellular Coagulation in the Limulus. *Bull Johns Hopkins Hosp* 115: 337-45.

Levin, J. and F. B. Bang (1964b): The Role of Endotoxin in the Extracellular Coagulation of Limulus Blood. *Bull Johns Hopkins Hosp* 115: 265-74.

Levin, J. and F. B. Bang (1968): Clottable protein in Limulus; its localization and kinetics of its coagulation by endotoxin. *Thromb Diath Haemorrh* 19(1): 186-97.

Levy, S. and T. Shoham (2005): The tetraspanin web modulates immune-signalling complexes. *Nat Rev Immunol* 5(2): 136-48.

Li, C., *et al.* (2001): Production and characterization of monoclonal antibodies specific for a conserved epitope within hepatitis C virus hypervariable region 1. *J Virol* 75(24): 12412-20.

Li, K., *et al.* (2005): 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(8): 2992-7.

Liefhebber, J. M., *et al.* (2009): Hepatitis C virus NS4B carboxy terminal domain is a membrane binding domain. *Virol J* 6: 62.

Lindenbach, B. D., *et al.* (2005): Complete replication of hepatitis C virus in cell culture. *Science* 309(5734): 623-6.

Lindenbach, B. D., *et al.* (2006): Cell culture-grown hepatitis C virus is infectious in vivo and can be recultured in vitro. *Proc Natl Acad Sci U S A* 103(10): 3805-9.

Liu, S., *et al.* (1997): Removal of endotoxin from recombinant protein preparations. *Clin Biochem* 30(6): 455-63.

Liu, S., *et al.* (2009): Tight junction proteins claudin-1 and occludin control hepatitis C virus entry and are downregulated during infection to prevent superinfection. *J Virol* 83(4): 2011-4.

Logvinoff, C., *et al.* (2004): Neutralizing antibody response during acute and chronic hepatitis C virus infection. *Proc Natl Acad Sci U S A* 101(27): 10149-54.

Lohmann, V., *et al.* (2003): Viral and cellular determinants of hepatitis C virus RNA replication in cell culture. *J Virol* 77(5): 3007-19.

Lohmann, V., *et al.* (1999): Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* 285(5424): 110-3.

Maillard, P., *et al.* (2006): The interaction of natural hepatitis C virus with human scavenger receptor SR-BI/Cla1 is mediated by ApoB-containing lipoproteins. *FASEB J* 20(6): 735-7.

Malherbe, L., *et al.* (2008): Vaccine adjuvants alter TCR-based selection thresholds. *Immunity* 28(5): 698-709.

Malyala, P. and M. Singh (2008): Endotoxin limits in formulations for preclinical research. *J Pharm Sci* 97(6): 2041-4.

Manoff, S. B., *et al.* (2010): Revaccination with a 23-valent pneumococcal polysaccharide vaccine induces elevated and persistent functional antibody responses in adults aged 65 > or = years. *J Infect Dis* 201(4): 525-33.

McAllister, J., *et al.* (1998): Long-term evolution of the hypervariable region of hepatitis C virus in a common-source-infected cohort. *J Virol* 72(6): 4893-905.

McCaffrey, K., *et al.* (2007): Expression and characterization of a minimal hepatitis C virus glycoprotein E2 core domain that retains CD81 binding. *J Virol* 81(17): 9584-90.

Mehta, S. H., *et al.* (2002): Protection against persistence of hepatitis C. *Lancet* 359(9316): 1478-83.

Meunier, J. C., *et al.* (2005): Evidence for cross-genotype neutralization of hepatitis C virus pseudo-particles and enhancement of infectivity by apolipoprotein C1. *Proc Natl Acad Sci U S A* 102(12): 4560-5.

Meunier, J. C., *et al.* (2008): Isolation and characterization of broadly neutralizing human monoclonal antibodies to the e1 glycoprotein of hepatitis C virus. *J Virol* 82(2): 966-73.

Meyer, K., *et al.* (2002): Complement-mediated enhancement of antibody function for neutralization of pseudotype virus containing hepatitis C virus E2 chimeric glycoprotein. *J Virol* 76(5): 2150-8.

Mikkelsen, M. and J. Bukh (2007): Current status of a hepatitis C vaccine: encouraging results but significant challenges ahead. *Curr Infect Dis Rep* 9(2): 94-101.

Milich, D. R. and A. McLachlan (1986): The nucleocapsid of hepatitis B virus is both a T-cell-independent and a T-cell-dependent antigen. *Science* 234(4782): 1398-401.

Molina, S., *et al.* (2007): The low-density lipoprotein receptor plays a role in the infection of primary human hepatocytes by hepatitis C virus. *J Hepatol* 46(3): 411-9.

Moradpour, D., *et al.* (1996): Characterization of cell lines allowing tightly regulated expression of hepatitis C virus core protein. *Virology* 222(1): 51-63.

Moris, P., *et al.* (2011): H5N1 influenza vaccine formulated with AS03 A induces strong cross-reactive and polyfunctional CD4 T-cell responses. *J Clin Immunol* 31(3): 443-54.

Mothes, W., *et al.* (2010): Virus cell-to-cell transmission. *J Virol* 84(17): 8360-8.

Murao, K., *et al.* (2008): Interferon alpha decreases expression of human scavenger receptor class BI, a possible HCV receptor in hepatocytes. *Gut* 57(5): 664-71.

Murphy, K., *et al.* (2008): Janeway's Immunobiology. Abington, UK, Garland Science.

Musher, D. M., *et al.* (2010): Safety and antibody response, including antibody persistence for 5 years, after primary vaccination or revaccination with pneumococcal polysaccharide vaccine in middle-aged and older adults. *J Infect Dis* 201(4): 516-24.

Nakabayashi, H., *et al.* (1982): Growth of human hepatoma cells lines with differentiated functions in chemically defined medium. *Cancer Res* 42(9): 3858-63.

Nakano, I., *et al.* (1997): Immunization with plasmid DNA encoding hepatitis C virus envelope E2 antigenic domains induces antibodies whose immune reactivity is linked to the injection mode. *J Virol* 71(9): 7101-9.

Nara, P. L., *et al.* (1991): Neutralization of HIV-1: a paradox of humoral proportions. *FASEB J* 5(10): 2437-55.

Nassal, M. and H. Schaller (1996): Hepatitis B virus replication--an update. *J Viral Hepat* 3(5): 217-26.

Negre, D., *et al.* (2002): Lentiviral vectors derived from simian immunodeficiency virus. *Curr Top Microbiol Immunol* 261: 53-74.

Nielsen, S. U., *et al.* (2006): Association between hepatitis C virus and very-low-density lipoprotein (VLDL)/LDL analyzed in iodixanol density gradients. *J Virol* 80(5): 2418-28.

Op De Beeck, A., *et al.* (2000): The transmembrane domains of hepatitis C virus envelope glycoproteins E1 and E2 play a major role in heterodimerization. *J Biol Chem* 275(40): 31428-37.

Op De Beeck, A., *et al.* (2004): Characterization of functional hepatitis C virus envelope glycoproteins. *J Virol* 78(6): 2994-3002.

Ott, D. E. (1997): Cellular proteins in HIV virions. *Rev Med Virol* 7(3): 167-180.

Owsianka, A., *et al.* (2001): Functional analysis of hepatitis C virus E2 glycoproteins and virus-like particles reveals structural dissimilarities between different forms of E2. *J Gen Virol* 82(Pt 8): 1877-83.

Owsianka, A., *et al.* (2005): Monoclonal antibody AP33 defines a broadly neutralizing epitope on the hepatitis C virus E2 envelope glycoprotein. *J Virol* 79(17): 11095-104.

Owsianka, A. M., *et al.* (2006): Identification of conserved residues in the E2 envelope glycoprotein of the hepatitis C virus that are critical for CD81 binding. *J Virol* 80(17): 8695-704.

Patel, A. K. and S. K. Tikoo (2006): 293T cells expressing simian virus 40 T antigen are semi-permissive to bovine adenovirus type 3 infection. *J Gen Virol* 87(Pt 4): 817-21.

Penin, F., *et al.* (2004): Structural biology of hepatitis C virus. *Hepatology* 39(1): 5-19.

Penin, F., *et al.* (2001): 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(12): 5703-10.

Pestka, J. M., *et al.* (2007): Rapid induction of virus-neutralizing antibodies and viral clearance in a single-source outbreak of hepatitis C. *Proc Natl Acad Sci U S A* 104(14): 6025-30.

Petit, J. M., *et al.* (2007): Cell surface expression of LDL receptor in chronic hepatitis C: correlation with viral load. *Am J Physiol Endocrinol Metab* 293(1): E416-20.

Pietschmann, T., *et al.* (2006): Construction and characterization of infectious intragenotypic and intergenotypic hepatitis C virus chimeras. *Proc Natl Acad Sci U S A* 103(19): 7408-13.

Pileri, P., *et al.* (1998): Binding of hepatitis C virus to CD81. *Science* 282(5390): 938-41.

Pinter, A., *et al.* (2004): The V1/V2 domain of gp120 is a global regulator of the sensitivity of primary human immunodeficiency virus type 1 isolates to neutralization by antibodies commonly induced upon infection. *J Virol* 78(10): 5205-15.

Ploss, A., *et al.* (2009): Human occludin is a hepatitis C virus entry factor required for infection of mouse cells. *Nature* 457(7231): 882-6.

Pohlmann, S., *et al.* (2003): Hepatitis C virus glycoproteins interact with DC-SIGN and DC-SIGNR. *J Virol* 77(7): 4070-80.

Popescu, C. I. and J. Dubuisson (2009): Role of lipid metabolism in hepatitis C virus assembly and entry. *Biol Cell* 102(1): 63-74.

Prentoe, J., *et al.* (2011): Hypervariable region 1 differentially impacts viability of hepatitis C virus strains of genotypes 1 to 6 and impairs virus neutralization. *J Virol* 85(5): 2224-34.

Prince, A. M., *et al.* (1996): Visualization of hepatitis C virions and putative defective interfering particles isolated from low-density lipoproteins. *J Viral Hepat* 3(1): 11-7.

Pugach, P., *et al.* (2004): The prolonged culture of human immunodeficiency virus type 1 in primary lymphocytes increases its sensitivity to neutralization by soluble CD4. *Virology* 321(1): 8-22.

Pulendran, B. and R. Ahmed (2006): Translating innate immunity into immunological memory: implications for vaccine development. *Cell* 124(4): 849-63.

Pumpens, P. and E. Grens (2001): HBV core particles as a carrier for B cell/T cell epitopes. *Intervirology* 44(2-3): 98-114.

Puntoriero, G., *et al.* (1998): Towards a solution for hepatitis C virus hypervariability: mimotopes of the hypervariable region 1 can induce antibodies cross-reacting with a large number of viral variants. *EMBO J* 17(13): 3521-33.

Racanelli, V. and B. Rehermann (2006): The liver as an immunological organ. *Hepatology* 43(2 Suppl 1): S54-62.

Racanelli, V., *et al.* (2006): Antibody production and in vitro behavior of CD27-defined B-cell subsets: persistent hepatitis C virus infection changes the rules. *J Virol* 80(8): 3923-34.

Ray, S. C., *et al.* (1999): Acute hepatitis C virus structural gene sequences as predictors of persistent viremia: hypervariable region 1 as a decoy. *J Virol* 73(4): 2938-46.

Rhainds, D. and L. Brissette (2004): The role of scavenger receptor class B type I (SR-BI) in lipid trafficking. defining the rules for lipid traders. *Int J Biochem Cell Biol* 36(1): 39-77.

Riedl, P., *et al.* (2002): Priming Th1 immunity to viral core particles is facilitated by trace amounts of RNA bound to its arginine-rich domain. *J Immunol* 168(10): 4951-9.

Roccasecca, R., *et al.* (2003): Binding of the hepatitis C virus E2 glycoprotein to CD81 is strain specific and is modulated by a complex interplay between hypervariable regions 1 and 2. *J Virol* 77(3): 1856-67.

Roccasecca, R., *et al.* (2001): Mimotopes of the hyper variable region 1 of the hepatitis C virus induce cross-reactive antibodies directed against discontinuous epitopes. *Mol Immunol* 38(6): 485-92.

Rosa, D., *et al.* (1996): 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(5): 1759-63.

Rouille, Y., *et al.* (2006): Subcellular localization of hepatitis C virus structural proteins in a cell culture system that efficiently replicates the virus. *J Virol* 80(6): 2832-41.

Rutebemberwa, A., *et al.* (2008): High-programmed death-1 levels on hepatitis C virus-specific T cells during acute infection are associated with viral persistence and require preservation of cognate antigen during chronic infection. *J Immunol* 181(12): 8215-25.

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

Sabo, M. C., *et al.* (2011): Neutralizing monoclonal antibodies against hepatitis C virus E2 protein bind discontinuous epitopes and inhibit infection at a postattachment step. *J Virol* 85(14): 7005-19.

Saito, T., *et al.* (2008): Innate immunity induced by composition-dependent RIG-I recognition of hepatitis C virus RNA. *Nature* 454(7203): 523-7.

Sandrin, V., *et al.* (2002): Lentiviral vectors pseudotyped with a modified RD114 envelope glycoprotein show increased stability in sera and augmented transduction of primary lymphocytes and CD34+ cells derived from human and nonhuman primates. *Blood* 100(3): 823-32.

Scarselli, E., *et al.* (2002): The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. *EMBO J* 21(19): 5017-25.

Semmo, N., *et al.* (2006): Maintenance of HCV-specific T-cell responses in antibody-deficient patients a decade after early therapy. *Blood* 107(11): 4570-1.

Shang, D., *et al.* (1999): Broadly cross-reactive, high-affinity antibody to hypervariable region 1 of the hepatitis C virus in rabbits. *Virology* 258(2): 396-405.

Shelness, G. S. and J. A. Sellers (2001): Very-low-density lipoprotein assembly and secretion. *Curr Opin Lipidol* 12(2): 151-7.

Shiina, M. and B. Rehermann (2006): Hepatitis C vaccines: Inducing and challenging memory T cells. *Hepatology* 43(6): 1395-8.

Shimizu, Y. K., *et al.* (1994): Neutralizing antibodies against hepatitis C virus and the emergence of neutralization escape mutant viruses. *J Virol* 68(3): 1494-500.

Shimizu, Y. K., *et al.* (1996): 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(2): 409-12.

Shin, K., *et al.* (2006): Tight junctions and cell polarity. *Annu Rev Cell Dev Biol* 22: 207-35.

Simmonds, P. (2004): Genetic diversity and evolution of hepatitis C virus--15 years on. *J Gen Virol* 85(Pt 11): 3173-88.

Skamel, C., *et al.* (2006): Hepatitis B virus capsid-like particles can display the complete, dimeric outer surface protein C and stimulate production of protective antibody responses against Borrelia burgdorferi infection. *J Biol Chem* 281(25): 17474-81.

Soderstrom, T., *et al.* (1985): Immunoglobulin subclass (IgG3) restriction of anti-P and anti-Pk antibodies in patients of the rare p blood group. *J Immunol* 134(1): 1-3.

Stamataki, Z., *et al.* (2007): Hepatitis C virus envelope glycoprotein immunization of rodents elicits cross-reactive neutralizing antibodies. *Vaccine* 25(45): 7773-84.

Steinmann, E. and T. Pietschmann (2010): Hepatitis C virus p7-a viroporin crucial for virus assembly and an emerging target for antiviral therapy. *Viruses* 2(9): 2078-95.

Stgelais, C., *et al.* (2009): Determinants of hepatitis C virus p7 ion channel function and drug sensitivity identified in vitro. *J Virol*.

Strickland, G. T., *et al.* (2008): Hepatitis C vaccine: supply and demand. *Lancet Infect Dis* 8(6): 379-86.

Sugimoto, K., *et al.* (2003): Suppression of HCV-specific T cells without differential hierarchy demonstrated ex vivo in persistent HCV infection. *Hepatology* 38(6): 1437-48.

Takeda, K. and S. Akira (2007): Toll-like receptors. *Curr Protoc Immunol* Chapter 14: Unit 14 12.

Taniguchi, S., *et al.* (1993): A structurally flexible and antigenically variable N-terminal domain of the hepatitis C virus E2/NS1 protein: implication for an escape from antibody. *Virology* 195(1): 297-301.

Tarr, A. W., *et al.* (2006): Characterization of the hepatitis C virus E2 epitope defined by the broadly neutralizing monoclonal antibody AP33. *Hepatology* 43(3): 592-601.

Thimme, R., *et al.* (2008): Adaptive immune responses to hepatitis C virus: from viral immunobiology to a vaccine. *Biol Chem* 389(5): 457-67.

Thimme, R., *et al.* (2001): Determinants of viral clearance and persistence during acute hepatitis C virus infection. *J Exp Med* 194(10): 1395-406.

Thomssen, R., *et al.* (1992): Association of hepatitis C virus in human sera with beta-lipoprotein. *Med Microbiol Immunol* 181(5): 293-300.

Timm, J., *et al.* (2004): CD8 epitope escape and reversion in acute HCV infection. *J Exp Med* 200(12): 1593-604.

Timpe, J. M., *et al.* (2008): Hepatitis C virus cell-cell transmission in hepatoma cells in the presence of neutralizing antibodies. *Hepatology* 47(1): 17-24.

Torres-Puente, M., *et al.* (2008): Using evolutionary tools to refine the new hypervariable region 3 within the envelope 2 protein of hepatitis C virus. *Infect Genet Evol* 8(1): 74-82.

Trepo, C., *et al.* (1993): Diagnostic markers of viral hepatitis B and C. *Gut* 34(2 Suppl): S20-5.

Tsukiyama-Kohara, K., *et al.* (1992): Internal ribosome entry site within hepatitis C virus RNA. *J Virol* 66(3): 1476-83.

Ulrich, R., *et al.* (1999): New chimaeric hepatitis B virus core particles carrying hantavirus (serotype Puumala) epitopes: immunogenicity and protection against virus challenge. *J Biotechnol* 73(2-3): 141-53.

Ulsenheimer, A., *et al.* (2003): Detection of functionally altered hepatitis C virus-specific CD4 T cells in acute and chronic hepatitis C. *Hepatology* 37(5): 1189-98.

Van Itallie, C. M. and J. M. Anderson (2006): Claudins and epithelial paracellular transport. *Annu Rev Physiol* 68: 403-29.

Vermehren, J. and C. Sarrazin (2011): New hepatitis C therapies in clinical development. *Eur J Med Res* 16(7): 303-14.

Vieyres, G., *et al.* (2010): Characterization of antibody-mediated neutralization directed against the hypervariable region 1 of hepatitis C virus E2 glycoprotein. *J Gen Virol* 92(Pt 3): 494-506.

Vieyres, G., *et al.* (2011): Characterization of antibody-mediated neutralization directed against the hypervariable region 1 of hepatitis C virus E2 glycoprotein. *J Gen Virol* 92(Pt 3): 494-506.

Vogel, M., *et al.* (2005): Quaternary structure is critical for protein display on capsid-like particles (CLPs): efficient generation of hepatitis B virus CLPs presenting monomeric but not dimeric and tetrameric fluorescent proteins. *Proteins* 58(2): 478-88.

von Hahn, T. and C. M. Rice (2008): Hepatitis C virus entry. *J Biol Chem* 283(7): 3689-93.

von Hahn, T., *et al.* (2006): Oxidized low-density lipoprotein inhibits hepatitis C virus cell entry in human hepatoma cells. *Hepatology* 43(5): 932-42.

von Hahn, T., *et al.* (2007): Hepatitis C virus continuously escapes from neutralizing antibody and T-cell responses during chronic infection in vivo. *Gastroenterology* 132(2): 667-78.

Wakita, T., *et al.* (2005): Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat Med* 11(7): 791-6.

Walker, A., *et al.* (2011): SplitCore: An exceptionally versatile viral nanoparticle for native whole protein display regardless of 3D structure. *Nature Scientific Reports* 1(5).

Walker, A., *et al.* (2008): Internal core protein cleavage leaves the hepatitis B virus capsid intact and enhances its capacity for surface display of heterologous whole chain proteins. *J Biol Chem* 283(48): 33508-15.

Wedemeyer, H., *et al.* (2002): Impaired effector function of hepatitis C virus-specific CD8+ T cells in chronic hepatitis C virus infection. *J Immunol* 169(6): 3447-58.

Weiner, A. J., *et al.* (1991): Variable and hypervariable domains are found in the regions of HCV corresponding to the flavivirus envelope and NS1 proteins and the pestivirus envelope glycoproteins. *Virology* 180(2): 842-8.

Weiner, A. J., *et al.* (1992): 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(8): 3468-72.

Welsch, S., *et al.* (2009): Composition and three-dimensional architecture of the dengue virus replication and assembly sites. *Cell Host Microbe* 5(4): 365-75.

Whitacre, D. C., *et al.* (2009): Use of hepadnavirus core proteins as vaccine platforms. *Expert Rev Vaccines* 8(11): 1565-73.

WHO. (2011a). Hepatitis B. Retrieved 04.11.2011, 2011, from: http://www.who.int/mediacentre/factsheets/fs204/en/.

WHO. (2011b). Hepatitis C. Retrieved 02.11.2011, 2011, from: http://www.who.int/mediacentre/factsheets/fs164/en/.

Witteveldt, J., *et al.* (2009): CD81 is dispensable for hepatitis C virus cell-to-cell transmission in hepatoma cells. *J Gen Virol* 90(Pt 1): 48-58.

Wynne, S. A., *et al.* (1999): The crystal structure of the human hepatitis B virus capsid. *Mol Cell* 3(6): 771-80.

Yanagi, M., *et al.* (1999): In vivo analysis of the 3' untranslated region of the hepatitis C virus after in vitro mutagenesis of an infectious cDNA clone. *Proc Natl Acad Sci U S A* 96(5): 2291-5.

Yang, M., *et al.* (2008): [Cross reactivity of mimotopes of hepatitis C virus hypervariable region 1]. *Bing Du Xue Bao* 24(4): 249-54.

Zeisel, M. B., *et al.* (2008): Host neutralizing responses and pathogenesis of hepatitis C virus infection. *Hepatology* 48(1): 299-307.

Zeisel, M. B., *et al.* (2007): Scavenger receptor class B type I is a key host factor for hepatitis C virus infection required for an entry step closely linked to CD81. *Hepatology* 46(6): 1722-31.

Zeuzem, S. (2004): [Standard treatment of acute and chronic hepatitis C]. *Z Gastroenterol* 42(8): 714-9.

Zhang, J., *et al.* (2004): CD81 is required for hepatitis C virus glycoprotein-mediated viral infection. *J Virol* 78(3): 1448-55.

Zhang, P., *et al.* (2007): Hepatitis C virus epitope-specific neutralizing antibodies in Igs prepared from human plasma. *Proc Natl Acad Sci U S A* 104(20): 8449-54.

Zhang, P., *et al.* (2009): 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(18): 7537-41.

Zhong, J., *et al.* (2005): Robust hepatitis C virus infection in vitro. *Proc Natl Acad Sci U S A* 102(26): 9294-9.

Zucchelli, S., *et al.* (2001): Mimotopes of the hepatitis C virus hypervariable region 1, but not the natural sequences, induce cross-reactive antibody response by genetic immunization. *Hepatology* 33(3): 692-703.

9 Appendix

9.1 Vector-cards

The maps of all plasmids that were generated and used during the experiments are shown in the following figures (Fig. 9.1 - Fig. 9.6).

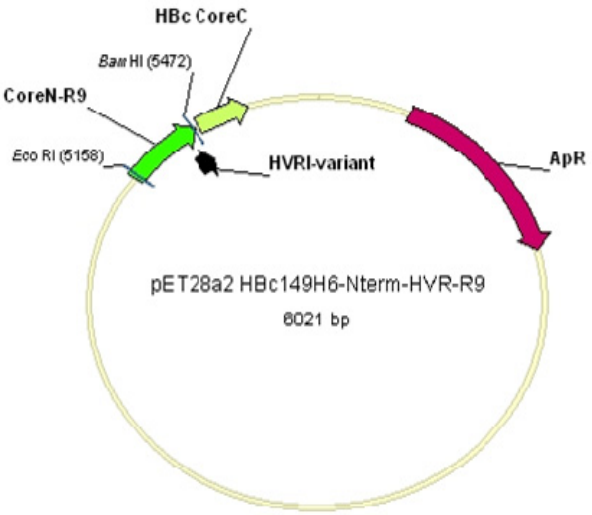

Fig. 9.1: pET28a2-HBc149-Nterm-HVR-R9

PCR was done on the template pET28a2_HBc-Nterm-HVRI-149H6-St with the primers T7-promotor and Primer-(-)-HVR-R9. The PCR product as well as the template vector were digested with *NcoI* nd *BamHI* and ligated to pET28a2_HBc149H6-Ntermn-HVR-R9 (H16). For the remaining 3 constructs PCR was done on the template pET28a2_HBc149H6-Ntermn-HVR-R9 (H16) with the primers T7-promotor and Primer-(-)-HVR-G31, indicated by the black arrow. Therefore also the primers Primer-(-)-HVR-YK5807 and Primer-(-)-HVR-YK5829 respectively, were used. The PCR product was digested with *EcoRI* and *BamHI* and inserted to CoreN in the vecotor pET28a2_HBc149H6-Ntermn-HVR-R9 (H16), digested with the same enzymes. This vector-card is a representative for all 4 generated HVRI-vriants. It contains the Ampicillin resistant gene (ApR) and was used as *E. coli* expression plasmid. The core potein does not contain the nucleic acid binding domain anymore, indicated by “149” in the name of this construct.

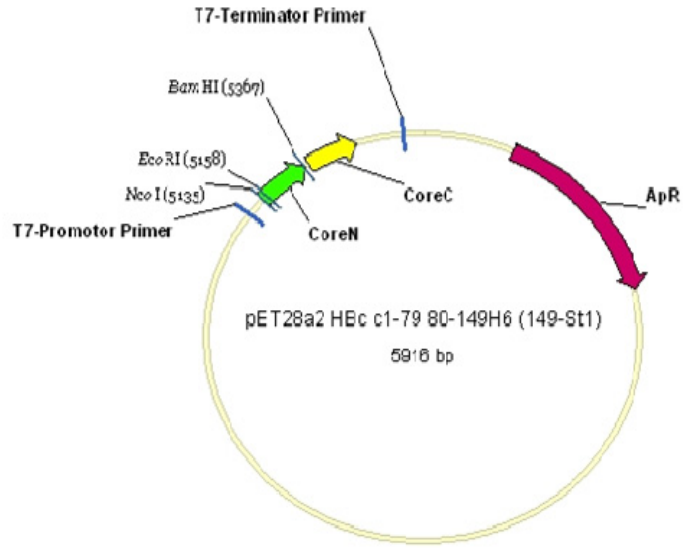

Fig. 9.2: pET28a2-HBc_c1-79_80-149H6 (149-St1)

Kindly provided by M. Nassal (Freiburg). Wild type HBc protein lacking the ribososome binding site was used as positive control, backbone for cloning, ELISA coating and protein expression. This plasmid contains an Ampicillin resistant gene.

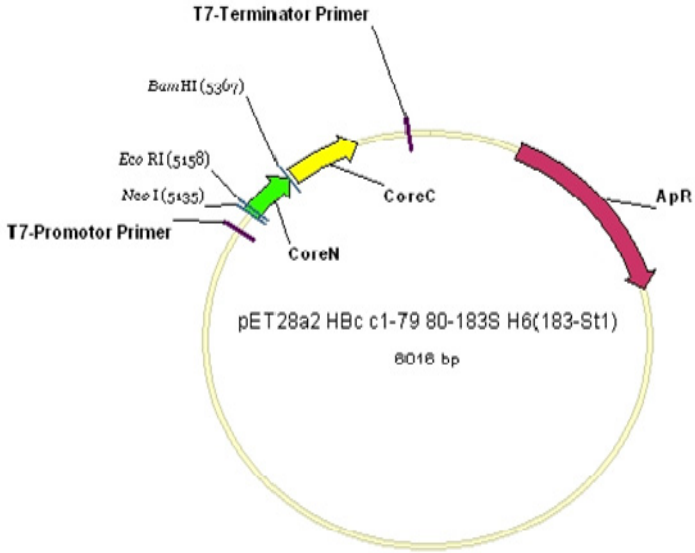

Fig. 9.3: pET28a2-HBc_c1-79_80-183S_H6 (183-St1)

Kindly provided by M. Nassal (Freiburg). The wild type HBc protein was used as positive control, backbone for cloning and protein expression. This plasmid contains an ApR.

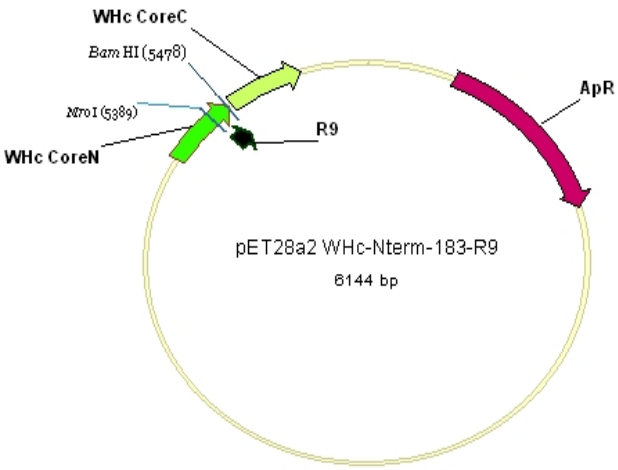

Fig. 9.4: pET28a2_WHc-Nterm-183-R9

The 89 base pair fragment of pET28a2_HBc149H6-Ntermn-HVR-R9 (H16) (digested with MroI and BamHI) was ligated with the 1.08 kb MroI/MluI fragment of pET28a2_WHc-Nterm-HVRI-149 and the 4.7 kb BamHI/MluI fragment of pET28a2_WHc-185H6-St1 W14real to pET28a2_WHc-Nterm-183-R9. The pET28a2_WHc-Nterm-183-R9 vector was used for protein expression. This plasmid contains an ApR.

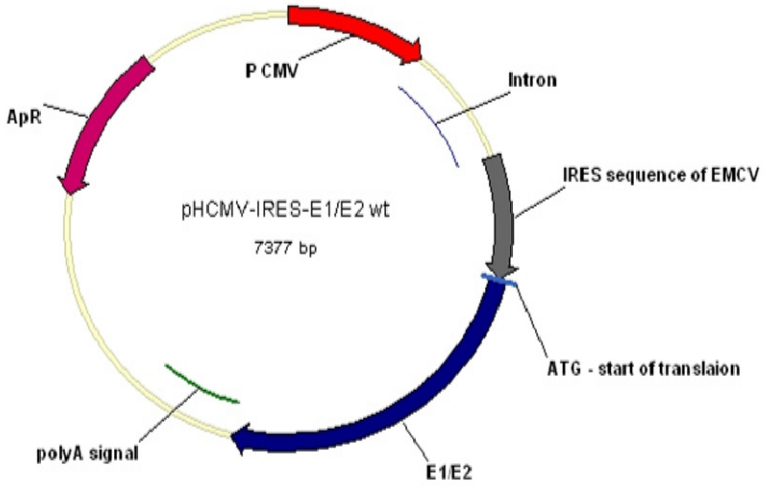

Fig. 9.5: pHCMV-IRES-AD78-E1E2

This expression plasmid contains the ApR gene, the CMV promoter (P CMV), the ß-globulin intron, the IRES sequence of EMCV and the envelope proteins of the east German anti-D cohort (AD78). E1 starts at bp 2306-2880 and E2 starts at bp 2881-3979. The polyA signal matures the process of mRNA translation. This expression vector was used as template for the construction of HCVpp's with different HVRI-sequences (kindly provided by S. Viazov from our Institute).

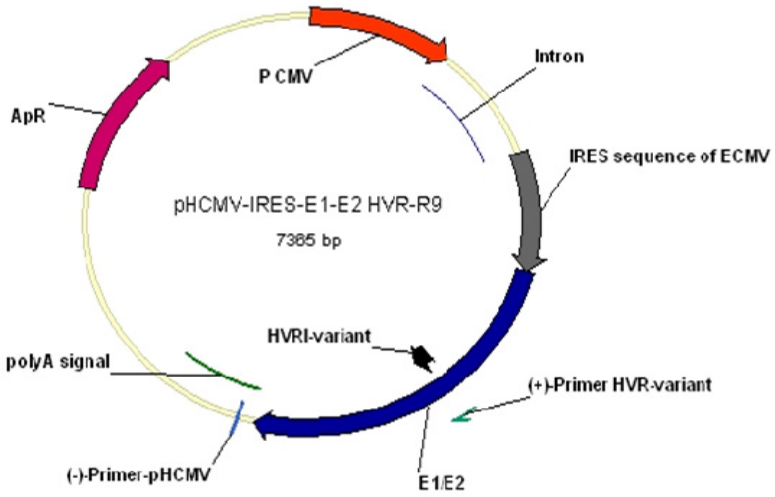

Fig. 9.6: pHCMV-IRES-E1-E2_HVR-R9

PCR was done on the template pHCMV-IRES-E1/E2 with the primers (-)-pHCMV-Minus-BglI and Primer-(+)-HCVpp-R9. The PCR product as well as the template vector were digested with *SalI* and *BglI* and ligated to pHCMV-IRES-E1-E2_HVR-R9. The same was done for all 4 HVRI-variants, indicated by the black arrow. Therefore also the primers Primer-(+)-HCVpp-YK5807, Primer-(+)-HCVpp-YK5829 and Primer-(+)-HCVpp-G31 respectively, were used. This vector-card is a representative for all 4 generated HVRI-variants. The same cloning strategy was used for the generation of heterologous HCVpp.

9.2 Supplementary tables

Tab. 9.1 Endotoxin determination using the LAL-test

Name	Protein concentration	Endotoxin concentration	EU/mg protein
1st immunization trial in mice			
R9 (H16)	2.5 mg/ml	600 EU/ml	240 EU/mg
YK5807 (H19)	1.5 mg/ml	90 EU/ml	60 EU/mg
YK5829 (H20)	0.5 mg/ml	125 EU/ml	250 EU/mg
G31 (H21)	0.6 mg/ml	60 EU/ml	100 EU/mg
wt-Core	5 mg/ml	1250 EU/ml	250 EU/mg
2nd immunization trial in mice			
R9 (H16)	1.4 mg/ml	1250 EU/ml	893 EU/mg
YK5807 (H19)	0.6 mg/ml	125 EU/ml	208 EU/mg
YK5829 (H20)	0.6 mg/ml	125 EU/ml	208 EU/mg
YK5829 (H20).1	0.4 mg/ml	60 EU/ml	150 EU/mg
G31 (H21)	0.3 mg/ml	600 EU/ml	2000 EU/mg
G31 (H21)	0.5 mg/ml	60 EU/ml	120 EU/mg

10 Abbreviations

α	anti
aa	amino acid
Ab	antibody
approx	approximately
APS	Ammonium persulfate
bp	base pair
BCR	B-cell receptor
BSA	Bovine serum albumine
°C	degree Celsius
CD	Cluster of differentiation
CLDN1	Claudin-1
CLP(s)	Capsid-like particle(s)
CMC	critical micelle concentration
CMT	critical micelle temperature
C-terminus	Carboxy-terminus
DAAs	direct-acting antivirals
DC	dendritic cell
DMEM	Dulbecco’s Modified Eagles’s Medium
DMSO	Dimethyl sulfoxide
DNA	Desoxyribonucleic acid
dNTP	Desoxynucleotidetriphosphate
dsRNA	double-stranded RNA
E1	envelope 1
E2	envelope 2

EDTA	Ethylenediaminetetraacetic acid
E.coli	*Escherichia coli*
EU	Endotoxin units
e.g.	for example
ELISA	Enzyme-linked immunosorbent assay
EM	electron microscope
ER	Endoplasmatic reticulum
et al.	and others (*lat.* et alii)
FCS	fetal calf serum
FDA	Food and Drug Administration
Fig.	figure
g	Gram
GAG(s)	Glycosaminoclycan(s)
GFP	green fluorescent protein
GT	genotype
H	hour
HBc	Hepatitis B virus core protein
HBcAg	Hepatitis B virus core antigen
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C Virus
HCVcc	Hepatitis C Virus cell culture
HCVpp	Hepatitis C Virus pseudo particle
HDL	high-density lipoproteins
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human Immunodeficiency Virus

HLA	human leukocyte antigen
HRP	Horseradish-Peroxidase
HS	heparan sulphate
HVRI	Hypervariable region 1
IFN	Interferon
Ig	Immunoglobulin
IPTG	Isopropylthiogalactoside
IRES	Internal ribosome entry site
kb	kilo base pair
kDa	kilo dalton
LB	Lurian broth
LPS	lipopolysaccharide
LRW	limulus reagent water
M	Molar
µ	micro
m	milli
mAb	monoclonal antibody
mDC	myeloid dendritic cell
MHC	Major Histocompatibility Complex
min	minute
MLV	Murine Leukemia Virus
mRNA	messenger RNA
MVA	modified vaccinia ankara
MW	Molecular Weight
n	nano
nAb	neutralizing antibody

NAGE	Native Agarose Gelelectrophoresis
NK cell	natural killer cell
NKT cell	natural killer T cell
NS	non-structural protein
Nt	nucleotide(s)
N-terminus	Amino-terminus
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD-1	programmed death-1
pDC	plasmacytoid dendritic cell
PEG	pegylated
pH	-log [H^+] (*lat.* potential Hydrogenii)
p.i.	post infection
PKR	protein kinase regulated
PVDF	Polyvinylidene fluoride
RBS	Ribosome binding site
RIG-I	retinoic-acid-inducible gene I
RLU	Relative-Light-Units
RNA	Ribonucleic acid
rpm	rounds per minute
RT	room temperature
s	second
SAA	serum amyloid A

SDS	Sodiumdodecylsulfate
SR-B1	Scavenger receptor class B member 1
STAT-C	specific targeted antiviral therapies
SVR	sustained virological response
Tab.	table
TEMED	Tertramethylethylenediamine
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TTBS	Tris tween buffered saline
Treg cell	regulatory T-cell
Tris	Tris-(hydroxymethyl)-aminomethane
U	Unit
UTR	Untranslating Region
UV-light	Ultra violet light
V	Volt
VLDL	very low density lipids
VLP	virus like particle
VSV	Vesicular stomatitis virus
WB	Western Blot
WHcAg	Woodchuck hepatitis virus core antigen
WHO	The World Health Organization

11 List of figures

Fig. 1.1: The Hepatitis C Virus 2
Fig. 1.2: Genomic organization of the HCV genome: polyprotein processing and cleavage products [modified from: (Knipe, 2007)] 4
Fig. 1.3: Schematic representation of the major steps of the HCV replication cycle (Dubuisson, 2007) 7
Fig. 1.4: Model of natural ApoB-associated HCV entry into to the hepatocytes [modified from: (Burlone and Budkowska, 2009)] 12
Fig. 1.5: Production of HCV pseudoparticles (HCVpp) (Lavie *et al.*, 2007) 16
Fig. 1.6: Primary sequence of the HBV core protein 25
Fig. 1.7: Structure of the HBV-core protein and the HBV-capsid 26
Fig. 1.8: Schematic demonstration of the SplitCore construct 27
Fig. 4.1: Generation of SplitCore-CLPs displaying 4 different variants of the HVRI . 63
Fig. 4.2: HVRI-CLPs displaying different HVRI-variants are assembly competent ... 65
Fig. 4.3: Antibody response titers of sera from mice immunized with single HVRI-CLPs 67
Fig. 4.4: Antibody response to the HBc-protein of sera from mice immunized with single HVRI-CLPs 68
Fig. 4.5: Single HVRI-CLPs induce partially cross-reactive antibodies 69
Fig. 4.6: Neutralizing capacity to homologous HVRI-variants after single immunization in mice 71
Fig. 4.7: HVRI-sequences used for the construction of HCVpp 72
Fig. 4.8: Neutralizing capacity to heterologous HVRI-variants after single immunization in mice 73
Fig. 4.9: Antibody response titers of sera from mice immunized with a mixture of HVRI-CLPs 74
Fig. 4.10: Antibody response to the HBc-protein of sera from mice immunized with a mixture of HVRI-CLPs 75
Fig. 4.11: Reactivity of mouse serum with patient derived HVRI-variants 76
Fig. 4.12: Neutralizing capacity to homologous HVRI-variants after mixture immunization in mice 77
Fig. 4.13: Neutralizing capacity to heterologous HVRI-variants after mixture immunization in mice 78

Fig. 4.14: Antibody response titers of sera from mice immunized with a mixture of HVRI-CLPs (i.m. and s.c.) 79
Fig. 4.15: Antibody response to the HBc-protein of sera from mice immunized with a mixture of HVRI-CLPs (i.m. and s.c.)..... 80
Fig. 4.16: Neutralizing capacity to homologous HVRI-variants after mixture immunization i.m. and s.c. in mice..... 82
Fig. 4.17: Neutralizing capacity to heterologous HVRI-variants after mixture immunization i.m. and s.c. in mice..... 83
Fig. 4.18: Immunization of mice with a mixture of HVRI-CLPs induces cross-neutralizing antibodies..... 85
Fig. 4.19: Mixture immunization of mice (i.m.) elicits cross-neutralizing antibodies to naturally occurring HVRI-variants..... 86
Fig. 4.20: Antibody response titers of sera from guinea pigs immunized with a mixture of 4 HVRI-CLPs 90
Fig. 4.21: Antibody response to the HBc-protein of sera from guinea pigs immunized with a mixture of HVRI-CLPs..... 91
Fig. 4.22: Neutralizing capacity in guinea pigs to homologous HVRI-variants after mixture immunization..... 92
Fig. 4.23: Neutralizing capacity to heterologous HVRI-variants after mixture immunization in guinea pigs 93
Fig. 4.24: WHV-CLPs displaying R9 are assembly competent 95
Fig. 9.1: pET28a2-HBc149-Nterm-HVR-R9 127
Fig. 9.2: pET28a2-HBc_c1-79_80-149H6 (149-St1)..... 128
Fig. 9.3: pET28a2-HBc_c1-79_80-183S_H6 (183-St1)..... 128
Fig. 9.4: pET28a2_WHc-Nterm-183-R9..... 129
Fig. 9.5: pHCMV-IRES-AD78-E1E2..... 129
Fig. 9.6: pHCMV-IRES-E1-E2_HVR-R9..... 130

12 List of tables

Tab. 2.1 Antibodies and conjugates 37
Tab. 2.2 Peptides 38
Tab. 2.3 Oligonucleotides 39
Tab. 3.1 Mix for the generation of HCVpp 46
Tab. 3.2 Standard PCR mastermix 48
Tab. 3.3 Standard PCR Programme 49
Tab. 3.4 Restriction of plasmids and PCR-products 49
Tab. 3.5 pTOPO cloning mixture 51
Tab. 3.6 Ligation mixture 51
Tab. 3.7 Sequencing mixture 52
Tab. 3.8 Reagents used for preparation of SDS gels 53
Tab. 4.1 Ratio of IgG-subclasses in AS03 vaccinated mice 87
Tab. 4.2 Ratio of IgG-subclasses in IFA vaccinated mice 88
Tab. 9.1 Endotoxin determination using the LAL-test 130

13 Publications

Manuscript in preparation:

Presentation of Hepatitis C Virus hypervariable region 1 mimotopes on HBV capsid-like particles induces cross-neutralizing antibodies. M. Lange, S. Viazov, O. Brovko, Y. Khudyakov, M. Nassal, P. Pumpens, W. Osborne, M. Roggendorf, A. Zekri, A. Walker

13.1 Presentations

- "Symposium on Immune Recognition of Pathogens and Tumors", Mülheim an der Ruhr; poster presentation
- "18th International Symposium on Hepatitis C Virus and Related Viruses", Seattle, USA; poster presentation
- "21st Annual Meeting of the Society for Virology ", Freiburg; oral presentation
- "Spring School 2011", Ettal; poster presentation
- "Bio Struct Master Class", Heinrich-Heine-Universität Düsseldorf; poste presentation
- Graduate school BIOME, regular research progress reports
- Institute of Virology, regular research progress reports and participation in the "Journal Club"

14 Acknowledgements

I am grateful to Prof. Dr. Michael Roggendorf for providing me with a very interesting scientific subject for my studies in the Institute of Virology, Essen and for his guidance during my work on the dissertation during the last years.

I am deeply thankful to Dr. Andreas Walker for his kind guidance and support throughout my dissertation, for numerous discussions and advices. He always helped me looking forward with optimism.

I am also thankful to Dr. Sergei Viazov for his helpful discussions and his pleasant support during this project.

Additionally, I would like to thank Olena Brovko for always supporting me in the lab and in scientific questions. But also I would like to thank her for the great atmosphere in our working-team and everything else besides work.
I would like to thank people who became really good friends to me: Marianne Ruhl, Marina Klein, Susanne Ziegler, Kathrin Gibbert, Simone Abel and Katrin Schöneweis for discussions of scientific topics but especially for those besides science. I thank Sina Luppus who also supported me in the lab especially during the last phase of my work. And I thank Ilsy Akhmetzyanova for joining me in boxing off our aggressions and everything else besides sport.

Furthermore, I would like to thank the HCV-lab: Maren Lipskoch, Lejla Glavinic, Christine Thöns and Kathrin Skibbe for the lively atmosphere.

Especially, I am extremely grateful to my parents Monika and Helmut and my sister Vera for supporting me in all conditions of life without having any doubts.

Finally, I am ineffable grateful to Lars for giving me his implicit trust, never giving me up even in my deepest depressive phases and for always sharing the same point of views.

15 Curriculum vitae

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

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

16 Declaration (Erklärung)

Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. 2, f der Promotionsordnung der Math.-Nat. Fakultäten zur Erlangung der Dr. rer. nat., dass ich das Arbeitsgebiet, dem das Thema „A new vaccine strategy for HCV: Presentation of Hepatitis C Virus hypervariable region 1 on HBV capsid-like particles“ zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von Milena Lange befürworte.

Essen, den _____ _____

Prof. Dr. med. Michael Roggendorf

Erklärung:

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

Essen, den _____ _____

Milena Lange

Erklärung:

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

Essen, den _____ _____

Milena Lange