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(54) Title: NEW NEUTRALIZING ANTIBODIES DIRECTED AGAINST HEPATITIS C VIRUS

(57) Abstract: The present invention relates to antibodies or fragments thereof, such as variable domains of camelid heavy-chain antibodies, directed against Hepatitis C virus glycoprotein E2, and uses thereof for preparing therapeutic or diagnostic agents.



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NEW NEUTRALIZING ANTIBODIES DIRECTED AGAINST HEPATITIS C VIRUS

The present invention relates to neutralizing antibodies and fragments thereof directed against the major Hepatitis C virus glycoprotein E2, and their use for treating or diagnosing Hepatitis C virus infection. Particularly, these antibody fragments are variable domains from camelid heavy-chain antibodies (VHH).

5 An estimated 180 million people worldwide are infected with Hepatitis C virus (HCV). Immune control of infection is uncommon and chronic infection occurs in approximately 80% of cases. The resulting liver disease has become the leading indication for liver transplantation. The engrafted liver is rapidly re-infected, with poor survival rates post-transplantation (Verna and Brown, 2008). HCV replication is rapid and error-prone; together
10 these properties result in the potential for great genetic diversity. As a consequence, strains are classified into six major genotypes each differing by up to 30% of their nucleotide sequence (Simmonds *et al.*, 2005). Within an infected host the virus exists as a population of circulating variants, providing the capacity to adapt to anti-viral selection pressures such as direct acting therapies and host immunity. This has significant implications for the design of
15 therapeutics and vaccines.

Since 1999 a combination of pegylated alpha interferon (IFN- α) and ribavirin has been the 'gold standard' therapy for chronic HCV infection. However, the limitations of this combination therapy are the associated severe side effects and, dependent on the viral genotype, variable sustained virological response (SVR) rates. While the recently introduced
20 HCV NS3 protease inhibitors Boceprevir and Telaprevir show improved efficiency in combination with IFN- α and ribavirin for genotype 1 infections (Kwo *et al.*, 2010; McHutchison *et al.*, 2010), the side effects of these new drugs still present a major limitation to therapy (Sarrazin *et al.*, 2012). The natural emergence of viruses resistant to both drugs (Welsch *et al.*, 2012) suggests that HCV will remain a major global health burden despite the
25 introduction of these direct-acting antivirals.

Several studies have investigated the role of neutralizing antibodies in the course of HCV infection *in vivo*. Screening of HCV-infected patients receiving Hepatitis B polyclonal immunoglobulins containing anti-HCV antibodies suggested a protective role for anti-HCV antibodies (Féray *et al.*, 1998). Antibodies targeting the major envelope glycoprotein E2 were
30 shown to prevent non-homologous virus infection after vaccination in chimpanzees (Youn *et al.*, 2005). Broadly neutralizing human monoclonal antibodies protected against heterologous virus challenge in a human liver-chimeric mouse model (Law *et al.*, 2007). Recent studies provided evidence that viral clearance during acute HCV infection is associated with the

presence of high titers of neutralizing antibodies (Dowd *et al.*, 2009; Pestka *et al.*, 2007), directed to specific epitopes (Ndongo *et al.*, 2010). Immunotherapy with neutralizing conventional antibodies is an attractive component of a combination therapy, and may have specific application in preventing infection of naïve liver transplants. However, production of human monoclonal antibodies is expensive (Farid *et al.*, 2007), and recent reports have demonstrated that they are unable to prevent transmission of HCV directly from cell to cell (Brimacombe *et al.*, 2011; Timpe *et al.*, 2008).

It appears from the foregoing that the need for accurate antibodies for diagnosing or treating HCV infection is important.

Conventional immunoglobulins are heterotetramers composed of two heavy and two light chains with a combined molecular weight of about 150 KD. In members of the family *Camelidae* a significant proportion of serum antibodies are homodimeric IgGs with a molecular weight of about 80 kD (Hamers-Casterman *et al.*, 1993). These heavy chain immunoglobulins (Ig) contain three domains and their variable region is referred to as VHH. Recombinant VHHs (~12-14 kD in size) constitute intact antigen-binding domains and exhibit a broad antigen-binding repertoire. Their hypervariable regions are expanded and exhibit unique characteristics, such as the substitution of three to four hydrophobic framework residues (which interact with the V_L in conventional antibodies) by more hydrophilic amino acids. To stabilize the enlarged CDRs, VHHs often possess an additional disulfide bond between CDR1 and CDR3 in dromedaries and CDR2 and CDR3 in llamas (Harmsen and De Haard, 2007; Muyldermans, 2001). The extended CDR3 loop can adopt a convex conformation, whereas conventional paratopes are limited to concave or flat structures (Muyldermans, 2001). These features allow VHHs to recognize unique epitopes that are poorly immunogenic for conventional antibodies (Lafaye *et al.*, 2009; Wernery, 2001).

VHHs have been raised to target numerous viruses (reviewed in Vanlandschoot *et al.*, 2011), among those HIV (McCoy *et al.*, 2012; Forsman *et al.*, 2008), Influenza A (Hultberg *et al.*, 2011), Poliovirus (Thys *et al.*, 2010), Foot and Mouth Disease Virus (Harmsen *et al.*, 2007) and Rotavirus (van der Vaart *et al.*, 2006). To the knowledge of the inventors, there is no VHH directed against HCV.

Although VHHs are by definition monovalent antibodies, which by default exclude any avidity effect, their biological activity measured as IC₅₀ *in vitro* can be similar to conventional, bivalent antibody molecules (Thys *et al.*, 2010). Notably, for numerous virus systems an improvement of the biological activity by up to 500-fold has been observed upon di- or trimerization of a neutralizing VHH, either by fusing two identical VHHs

(monospecific) or fusing two different neutralizing VHs (bispecific) (reviewed in Vanlandschoot *et al.*, 2011).

Within the framework of research that has led to the present invention, the inventors have prepared camelid heavy-chain antibodies raised by immunization with a monomeric form of the major hepatitis C virus glycoprotein E2. The initial challenge for generating broadly neutralizing VHH was the choice of a suitable immunogen. Guinea pig serum resulting from immunization with a soluble E2 ectodomain was previously demonstrated to induce antibodies that neutralize cell-cultured HCV and HCV pseudoparticles (Stamatakis *et al.*, 2007). Antibodies that have been induced by a glycoprotein-based vaccine (HCV genotype 1a E1/E2/p7; Choo *et al.*, 1994) neutralize isolates from genotypes 1, 4, 5 and 6, however, those antibodies failed to potently cross-neutralize isolates representing all 6 HCV genotypes (Meunier *et al.*, 2011). The inventors have used a glycoprotein construct secreting the soluble E2 ectodomain (E2e) truncated at K₇₁₅ from a genotype 2b-HCV infected patient (isolate UKN2b_2.8) (amino acid numbering is in reference to the amino acid sequence of the polyprotein of Hepatitis C virus strain H77 genotype 1a, accession number GI:130461 in the GENBANK database, and referred herein to as SEQ ID NO: 7) into *Drosophila* S2 cell supernatant. The inventors have immunized an alpaca with the E2e construct lacking the hypervariable region 1 (HVR1), in order to further increase exposure of the CD81 binding site. Correct conformation of this immunogen was demonstrated by binding to CD81 and multiple conformation-sensitive antibodies, providing evidence that this protein was similarly folded as the glycoprotein E2 in the virus particle. The inventors have thus identified and characterized four alpaca VHs directed to different epitopes on the major Hepatitis C virus glycoprotein E2, namely VHH D03, VHH C09, VHH B11 and VHH D04. The inventors have shown that two of those, namely VHH D03 and VHH C09, have neutralizing activity. The amino acid sequence alignment between VHH D03 (represented as SEQ ID NO: 2) and VHH C09 (represented as SEQ ID NO: 3) is shown in Figure 1. The consensus sequence is represented as SEQ ID NO: 1. VHH B11 and VHH D04 do not have said consensus sequence.

An expression vector encoding the VHH D03 is contained in a bacterial strain deposited at the CNCM, 25 rue du Docteur Roux, 75724 Paris Cedex 15 on October 2, 2012, under the number I-4678. An expression vector encoding the VHH C09 is contained in a bacterial strain deposited at the CNCM, 25 rue du Docteur Roux, 75724 Paris Cedex 15 on October 2, 2012, under the number I-4679.

In particular, the monovalent VHH D03 potently neutralized a broad range of HCV primary isolates. The inventors have further demonstrated that VHH D03 neutralizes the

majority of tested genotypes with an overall efficiency similar to that of the referenced bivalent, potently and broadly neutralizing human antibody 1:7 (Allander *et al.*, 2000; Johansson *et al.*, 2007). The inventors have also shown that VHH D03 inhibits cell-to-cell transmission, a route of infection that is resistant to most broadly neutralizing antibodies (Brimacombe *et al.*, 2011) and was proposed to represent a crucial *in vivo* transmission route for HCV. Furthermore, the inventors have demonstrated that the epitope recognized by this VHH D03 is distinct from any previously described for any monoclonal antibody. This epitope comprises the contacting amino acid residues G523 and T526, but does not comprise the contacting amino acid residues P525, W529, G530, D535 and N540 of a Hepatitis C virus glycoprotein E2 and is capable of binding the VHH D03. Indeed well-known mAbs 1:7 and A8 (Johansson *et al.*, 2007) bind to residues at positions G523, W529, G530 and D535, while mAb AR3A (Law *et al.*, 2008) has important contacts at S424, G523, P525, G530, D535 and N540.

Accordingly, the present invention provides an isolated neutralizing antibody or fragment thereof directed against the ectodomain of a Hepatitis C virus glycoprotein E2, characterized in that said antibody and fragment thereof bind the contacting amino acid residues G523 and T526 of said Hepatitis C virus glycoprotein E2, in reference to the amino acid sequence of the polyprotein of the Hepatitis C virus strain H77 genotype 1a of SEQ ID NO: 7.

Advantageously, said neutralizing antibody or neutralizing fragment thereof according to the present invention is a broadly neutralizing antibody or neutralizing fragment thereof that inhibits cell-to-cell transmission of HCV. A method for determining whether a neutralizing antibody or neutralizing fragment thereof is a broadly neutralizing VHH that inhibits cell-to-cell transmission of HCV is described in the Examples below.

In an embodiment, said neutralizing antibody and neutralizing fragment thereof further bind the contacting amino acid residues N415 of said Hepatitis C virus glycoprotein E2, in reference to the amino acid sequence of the polyprotein of the Hepatitis C virus strain H77 genotype 1a of SEQ ID NO: 7.

Advantageously, said neutralizing antibody and neutralizing fragment thereof do not bind amino acid residues P525, W529, G530, D535 and N540 of said Hepatitis C virus glycoprotein E2, in reference to the amino acid sequence of the polyprotein of the Hepatitis C virus strain H77 genotype 1a of SEQ ID NO: 7.

The Hepatitis C virus glycoproteins E2 and the ectodomain thereof are well known in the art. By way of example, they are described by Krey *et al.*, 2010; Lavillette *et al.*, 2005; Kato *et al.*, 2001; Kolykhalov *et al.*, 1997 and Inchauspe *et al.*, 1991.

By way of example, the amino acid sequence of the ectodomain of the Hepatitis C virus glycoprotein E2 of HCV strain H77 genotype 1a and HCV strain genotype 2b isolate UKN2b_2.8 are respectively referred herein to as SEQ ID NO: 8 and 9.

The characterization of the binding of an antibody or antibody fragment according to the present invention to contacting amino acid residues of Hepatitis C virus glycoprotein E2 can be performed by substituting an amino acid residue in the ectodomain of a Hepatitis C virus glycoprotein E2 (*e.g.*, with an alanine if said amino acid residue is not an alanine) and then carrying out a binding assay (see the Examples below).

The invention encompasses natural, recombinant or synthetic polyclonal or monoclonal antibodies, chimeric antibodies such as humanized antibodies, and also fragments thereof (for example: Fab, Fv, scFv) which have retained their ability to bind the ectodomain of a Hepatitis C virus glycoprotein E2 as defined above.

As used herein, the term “recombinant” refers to the use of genetic engineering methods (cloning, amplification) to produce said antibody or antibody fragment.

As used herein, the term “synthetic” refers to the production of said antibody or antibody fragment by *in vitro* chemical or enzymatic synthesis.

In a preferred embodiment, said antibody fragment is a variable antigen-binding domain of a camelid heavy-chain antibody (VHH).

As used herein, the term “VHH of camelid” (camel, dromedary, llama, alpaca, etc.) refers to the variable antigen-binding domain from a camelid heavy-chain antibody (See Nguyen *et al.*, 2000; Muyldermans, 2001 and for review Vanlandschoot *et al.*, 2011). A VHH can also be named Nanobody (Nb).

In another preferred embodiment, the VHH according to the present invention is from an alpaca (*Llama pacos*) heavy-chain antibody.

In another preferred embodiment, the antibody or antibody fragment, preferably the VHH, according to the present invention comprises or consists of the amino acid sequence SEQ ID NO: 1.

Advantageously, the VHH according to the present invention contains two disulfide bonds, one connecting CDR1 and CDR3 region of the VHH and the other connecting the framework upstream of CDR2 and the CDR3 region of the VHH.

In another preferred embodiment, the VHH according to the present invention consists of the amino acid sequence SEQ ID NO: 2 (VHH D03).

In another preferred embodiment, the antibody or fragment thereof, preferably the VHH, according to the present invention comprises one CDR (Complementarity Determining Region) from VHH D03 (SEQ ID NO: 2), selected from the group consisting of SEQ ID NO: 31 (CDR1 from VHH D03), SEQ ID NO: 32 (CDR2 from VHH D03) and SEQ ID NO: 33 (CDR3 from VHH D03), preferably two CDRs from VHH D03, *i.e.*, CDR1 (SEQ ID NO: 31) and CDR2 (SEQ ID NO: 32), or CDR1 (SEQ ID NO: 31) and CDR3 (SEQ ID NO: 33), or CDR2 (SEQ ID NO: 32) and CDR3 (SEQ ID NO: 33) from VHH D03, more preferably the three CDRs from VHH D03, *i.e.*, CDR1 (SEQ ID NO: 31), CDR2 (SEQ ID NO: 32), and CDR3 (SEQ ID NO: 33).

The antibody fragment, preferably the VHH, according to the present invention can be in the form of a monomer or a homomultimer, such as a homodimer or a homotrimer. An advantageous homodimer is a dimeric VHH D03 (*i.e.*, a homodimer VHH D03).

Antibody fragments, preferably VHHs, according to the present invention can also be included in a chimeric antibody in the form of a heteromultimer (*e.g.*, heterodimer or heterotrimer), comprising one antibody fragment or two different or identical antibody fragments, preferably one VHH or two different or identical VHHs, according to the present invention and a fragment Fc of a human antibody. An advantageous construct is the VHH D03 fused to a dimerizing Fc fragment derived from a human IgG1 molecule.

A VHH according to the present invention is obtainable by the method comprising the steps of:

(a) immunizing a camelid, preferably a *Llama pacos*, with a Hepatitis C virus glycoprotein E2 (HCV E2) or the ectodomain of a Hepatitis C virus glycoprotein E2 (HCV E2e) or with the ectodomain of a Hepatitis C virus glycoprotein E2 lacking the hypervariable region 1 (HVR1) (HCV E2eΔHVR1; Krey *et al.*, 2010), preferably E2eΔHVR1; optionally boosting once or twice said camelid with HCV E2e or HCV E2eΔHVR1, preferably HCV E2e,

(b) isolating peripheral lymphocytes of the immunized camelid, obtaining the total RNA and synthesizing the corresponding cDNAs (methods are known in the art; for instance see Lafaye *et al.*, 1995),

(c) constructing a library of cDNA fragments encoding VHH,

(d) transcribing the VHH-encoding cDNAs obtained in step (c) to mRNA, and

(e) expressing the VHH in an expression vector and, optionally purifying the expressed VHH.

In a preferred embodiment of said method, in step (a), the camelid is immunized at days 0, 21 and 35 with 250 µg of said HCV glycoprotein E2e or E2eΔHVR1.

5 In another preferred embodiment of said method, in step (a), the camelid is immunized with the E2eΔHVR1 of the HCV genotype 2b isolate UKN2B2.8 of SEQ ID NO: 30.

10 In a preferred embodiment of said method, in step (c), said library can be constructed by amplifying by PCR the DNA fragments encoding the VHH, and ligating the PCR products obtained into a phage vector.

The antibody and fragment directed against the ectodomain of a Hepatitis C virus glycoprotein E2 according to the present invention can be in a form of a chimeric antibody, comprising:

15 - one CDR, preferably at least two different CDRs, more preferably the three CDRs from VHH D03 (SEQ ID NO: 2), selected from the group consisting of SEQ ID NO: 31 (CDR1), SEQ ID NO: 32 (CDR2), SEQ ID NO: 33 (CDR3), and

- one or two different or the three CDRs from VHH C09 (SEQ ID NO: 3), selected from the group consisting of SEQ ID NO: 34 (CDR1), SEQ ID NO: 35 (CDR2) and SEQ ID NO: 36 (CDR3).

20 In an embodiment, said chimeric antibody comprises or consists of the VHH D03 of SEQ ID NO: 2 covalently linked to the VHH C09 of SEQ ID NO: 3.

25 The present invention also provides an isolated neutralizing antibody or fragment thereof directed against the ectodomain of a Hepatitis C virus glycoprotein E2, characterized in that said antibody and fragment thereof comprises one, preferably two different, more preferably the three CDRs from VHH C09 (SEQ ID NO: 3), selected from the group consisting of SEQ ID NO: 34 (CDR1), SEQ ID NO: 35 (CDR2) and SEQ ID NO: 36 (CDR3).

Advantageously, said fragment thereof is a VHH of camelid, preferably a VHH from an alpaca (*Llama pacos*) heavy-chain antibody, more preferably the VHH C09 of SEQ ID NO: 3.

30 The present invention also provides an isolated antigen (polypeptide) consisting of a HCV E2 ectodomain lacking the hypervariable region 1 (HVR1), corresponding to amino acids 412-715 of the polyprotein of said HCV in reference to HCV strain H77c of SEQ ID NO: 7.

In a preferred embodiment, said antigen consists of the sequence SEQ ID NO: 30 (E2eΔHVR1).

The present invention also provides a HCV vaccine composition comprising a therapeutically effective amount of an antigen according to the present invention.

5 The present invention also provides the use of an antigen according to the present invention for immunizing an animal, preferably a mammal, more preferably a camelid, such as a *Llama pacos*.

10 The present invention also provides an isolated polypeptide comprising an antibody fragment according to the present invention, preferably a VHH or a fragment thereof, preferably at least one CRD thereof, provided that said antibody fragment comprised in said polypeptide is able to bind the ectodomain of a Hepatitis C virus glycoprotein E2 as defined above.

15 The present invention also provides an isolated camelid serum, preferably an alpaca serum, comprising an antibody fragment, preferably a VHH or a fragment thereof, or a polypeptide according to the present invention.

The present invention also provides an isolated polynucleotide encoding an antibody fragment, preferably a VHH or a fragment thereof, preferably at least one CRD thereof, or a polypeptide according to the present invention.

20 Polynucleotides according to the present invention may be obtained by well-known methods of recombinant DNA technology and/or of chemical DNA synthesis.

25 The present invention also provides recombinant expression cassettes comprising a polynucleotide according to the present invention under the control of a transcriptional promoter allowing the regulation of the transcription of said polynucleotide in a host cell. Said polynucleotide can also be linked to appropriate control sequences allowing the regulation of its translation in a host cell.

30 The present invention also provides a recombinant vector comprising a polynucleotide according to the present invention. Particularly, the recombinant vector is a recombinant expression vector encoding an antibody fragment, preferably a VHH, or a polypeptide comprising an antibody fragment according to the present invention. Advantageously, said recombinant expression vector comprises an expression cassette according to the present invention.

The present invention also provides a host cell containing a recombinant expression cassette or a recombinant vector according to the present invention. The host cell is either a prokaryotic or eukaryotic host cell.

The present invention also provides a pharmaceutical composition comprising an antibody, an antibody fragment, a polypeptide comprising an antibody fragment or a polynucleotide (such as a recombinant expression vector encoding a VHH), preferably a VHH, according to the present invention, and a pharmaceutically acceptable carrier.

5 When the antibody or fragment thereof, preferably the VHH, according to the present invention is administered to a human subject, then it can be humanized in order to reduce immunogenicity in human. Methods for producing humanized antibodies or fragments thereof are known in the art (Vinckle *et al.*, 2009).

10 Further, the bioavailability of the antibody or antibody fragment according to the present invention can be improved by conjugating the neutralizing molecule(s) to inert carriers like albumin (Coppieters *et al.*, 2006) or immunoglobulins (Harmsen *et al.*, 2005).

Said vector can be delivered to a subject by gene therapy.

15 As used herein, "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. Suitable carriers are described in the most recent edition of Remington's Pharmaceutical Sciences, a standard reference text in the field. Preferred examples of such carriers or diluents include, but are not limited to, water, saline, Ringer's solutions, dextrose solution, and 5% human serum albumin. Liposomes, cationic lipids and non-aqueous vehicles such as fixed oils
20 may also be used. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with a therapeutic agent as defined hereabove, use thereof in the composition of the present invention is contemplated.

25 Advantageously, said pharmaceutical composition is intended for preventing or treating a Hepatitis C virus infection.

The present invention also provides an antibody, an antibody fragment, a polypeptide comprising an antibody fragment or a polynucleotide (such as a recombinant expression vector encoding a VHH), preferably a VHH, according to the present invention, for use as a medicament, preferably for preventing or treating a Hepatitis C virus infection.

30 The present invention also provides a method for preventing or treating a Hepatitis C virus infection, comprising administering to a subject in need thereof an antibody, an antibody fragment, a polypeptide comprising an antibody fragment or a polynucleotide encoding a VHH (such as a recombinant expression vector encoding a VHH), preferably a VHH,

according to the present invention, in an amount effective to inhibit HCV infection of susceptible cells so as to thereby prevent or treat the infection.

The antibody, antibody fragment (preferably a VHH), polypeptide comprising an antibody fragment and polynucleotide according to the present invention can be orally administered to a subject (a mammal, and preferably a human). They can also be administered to said subject by injection, such as intravenous, intraperitoneal, intramuscular or subcutaneous injection.

The term "treating" includes the administration of an antibody, antibody fragment (preferably a VHH), a polypeptide comprising an antibody fragment or a polynucleotide according to the present invention to a patient who has a Hepatitis C virus infection or a symptom of Hepatitis C virus infection, with the purpose to cure, heal, alleviate, relieve, alter, remedy, ameliorate, improve, or affect the Hepatitis C virus infection and/or the symptoms of the Hepatitis C virus infection.

The term "preventing" means that the progression of a Hepatitis C virus infection is reduced and/or eliminated, or that the onset of a Hepatitis C virus infection is delayed or eliminated.

The present invention also provides a diagnostic agent comprising an antibody, antibody fragment, or a polypeptide comprising an antibody fragment, preferably a VHH, according to the present invention.

In an embodiment of said diagnostic agent, the antibody, antibody fragment or polypeptide comprising an antibody fragment, preferably a VHH, according to the present invention is linked, directly or indirectly, covalently or non-covalently to a detectable marker.

The detectable marker can be directly and covalently linked to the antibody, antibody fragment or polypeptide comprising an antibody fragment, preferably a VHH, according to the present invention, either to one of the terminal ends (N or C terminus) of said antibody, antibody fragment or polypeptide, or to the side chain of one of the amino acids of said antibody, antibody fragment or polypeptide. The detectable marker can also be indirectly and covalently linked to said antibody, antibody fragment or polypeptide comprising an antibody fragment through a connecting arm (*i.e.*, a cross-linking reagent) either to one of the terminal ends of said antibody, antibody fragment or polypeptide, or to a side chain of one of the amino acids of said antibody, antibody fragment or polypeptide. Linking methods of a compound of interest to a peptide or antibody are well-known in the art.

In a preferred embodiment of said diagnostic agent, said detectable marker is selected from the group consisting of:

- enzymes such as horseradish peroxidase, alkaline phosphatase, glucose-6-phosphatase or beta-galactosidase;

- fluorophores such as green fluorescent protein (GFP), blue fluorescent dyes excited at wavelengths in the ultraviolet (UV) part of the spectrum (*e.g.* AMCA (7-amino-4-methylcoumarin-3-acetic acid); Alexa Fluor 350), green fluorescent dyes excited by blue light (*e.g.* FITC, Cy2, Alexa Fluor 488), red fluorescent dyes excited by green light (*e.g.* rhodamines, Texas Red, Cy3, Alexa Fluor dyes 546, 564 and 594), or dyes excited with far-red light (*e.g.* Cy5) to be visualized with electronic detectors (CCD cameras, photomultipliers);

- heavy metal chelates such as europium, lanthanum or yttrium;

- radioisotopes such as [^{18}F]fluorodeoxyglucose, ^{11}C -, ^{125}I -, ^{131}I -, ^3H -, ^{14}C -, ^{35}S , or ^{99}Tc -labelled compounds.

The present invention also provides a kit for diagnosing or monitoring, in a subject, a Hepatitis C virus infection, comprising an antibody, antibody fragment, preferably a VHH, a polypeptide comprising an antibody fragment or a diagnostic agent according to the present invention and an appropriate diagnostic reagent.

The appropriate diagnostic reagent is necessary for performing an assay for diagnosing or monitoring, in a subject, a Hepatitis C virus infection. The appropriate diagnostic reagent can be a solvent, a buffer, a dye, an anticoagulant, a Hepatitis C virus glycoprotein E2, the ectodomain of a Hepatitis C virus glycoprotein E2 (HCV E2e) or the ectodomain of a Hepatitis C virus glycoprotein E2 lacking the HVR1 domain.

The present invention also provides the use of an antibody, antibody fragment, preferably a VHH, a polypeptide comprising an antibody fragment or a diagnostic agent according to the present invention for diagnosing or monitoring a Hepatitis C virus infection in a subject.

The present invention also provides an *in vitro* method for diagnosing a Hepatitis C virus infection in a subject, comprising the steps of:

a) contacting *in vitro* an appropriate biological sample from said subject with an antibody, antibody fragment, preferably a VHH, a polypeptide comprising an antibody fragment or a diagnostic agent according to the present invention, and

b) determining the presence or the absence of a HCV envelope glycoprotein E2 in said biological sample,

the presence of said HCV envelope glycoprotein E2 indicating that said subject has Hepatitis C virus infection.

Step b) can be carried out by determining the presence or the absence of the antibody-antigen complex (*i.e.*, antibody directed to the HCV envelope glycoprotein E2 - HCV envelope glycoprotein E2 complex).

The present invention also provides an *in vitro* method for monitoring the progression or regression of a Hepatitis C virus infection in a subject, comprising the steps of:

a) contacting *in vitro* an appropriate biological sample from said subject with an antibody, antibody fragment, preferably a VHH, a polypeptide comprising an antibody fragment or a diagnostic agent according to the present invention,

b) determining the amount of HCV envelope glycoprotein E2 in said biological sample, and

c) comparing the amount determined in step (b) with the amount of HCV envelope glycoprotein E2 previously obtained for said subject,

a significant increase in amount of HCV envelope glycoprotein E2 constituting a marker of the progression of said HCV infection and a significant decrease of HCV envelope glycoprotein E2 constituting a marker of the regression of said HCV infection.

As used herein the terms “significant increase” and “significant decrease” refer to a higher amount or lower amount respectively of HCV envelope glycoprotein E2 in an appropriate biological sample with respect to the amount of HCV envelope glycoprotein E2 in an appropriate biological sample from said subject, that was previously determined and used as a reference amount.

Step b) can be carried out by determining the presence or the absence of the antibody-antigen complex (*i.e.*, antibody directed to the HCV envelope glycoprotein E2 - HCV envelope glycoprotein E2 complex).

Said appropriate biological sample can be blood, serum, urine or a liver biopsy.

In addition to the preceding features, the invention further comprises other features which will emerge from the following description, which refers to examples illustrating the present invention, as well as to the appended figures.

Figure 1 represents the sequence alignment between the amino acid sequences of VHH D03 (SEQ IDNO: 2) and VHH C09 (SEQ ID NO: 3).

Figure 2 represents the generation of alpaca heavy chain antibodies directed against HCV E2. (A) One alpaca was immunized and boosted with HCV E2e lacking the HVR1 domain (amino acids 412-715 of the HCV polyprotein; E2eΔHVR1) from patient isolate UKN2B2_8 (arrows). Serum samples (ellipses) were taken at day 50 to screen the humoral immune response by ELISA and at day 100 to generate a phage display library expressing

VHH on the surface. In between serum sampling the animal was boosted twice with full-length ectodomain E2e (polygons) (Krey *et al.*, 2010) to increase the induced immune response. (B) The native conformation of the immunogen was evaluated by ELISA assay using human mAbs recognising discrete conformation-sensitive epitopes (CBH2, CBH5, CBH7, A8, AR3A, AR3B, AR3C, 1:7, CBH4B, CBH4G and CBH8), and two well-

characterised murine mAbs recognizing linear epitopes (AP33 and ALP98) (Allander *et al.*, 2000; Hadlock *et al.*, 2000; Johansson *et al.*, 2007; Law *et al.*, 2007; Owsianka *et al.*, 2005). The immunogen was recognized by many of the mAbs that recognize the CD81 binding region (CBH5, A8, AR3A, AR3B, AR3C, 1:7, AP33), as well others that bind to epitopes outside this region (CBH4B, CBH4G, ALP98). (C) Amino acid alignment of the four isolated VHHs. Cysteines are boxed, the disulfide connectivity is indicated below the alignment, bars show the positions of the CDRs, which are shaded, according to the IMGT nomenclature (Lefranc *et al.*, 2009). Positions of somatic mutations during maturation of VHH D03 as revealed by an alignment with the closest homologous germline sequence (IGHV1S1*01) suggested by IMGT V-QUEST and junction analysis (Lefranc *et al.*, 2009) are marked by an asterisk below the sequence.

Figure 3 represents the functional characterization of VHH. (A) E2e Δ HVR1 was bound to a StrepTactin Superflow mini column and subsequently used to pull down the IMAC purified (B11, right panel) or SEC purified (D03, left panel) VHH directed against HCV E2. Eluted complexes were analyzed by SDS-PAGE under non-reducing conditions followed by Coomassie Blue staining. (B) Biotinylated VHH were bound to E1E2 proteins of strain H77 expressed on the surface of HEK293T cells transfected with an E1E2 expression construct. All four VHH were found to bind to these proteins as determined by immunofluorescent microscopy using Streptavidin-Alexafluor488 detection. Background fluorescence observed when binding each VHH to mock transfected cells was subtracted from these images. (C) CD81 binding inhibition was assayed by binding each VHH to immobilized E2e Δ HVR1 in the presence of a soluble CD81-LEL-His construct (Kitadokoro *et al.*, 2001).

Figure 4 represents the analysis of the native conformation of the immunogen. The overall conformation of HCV E2e lacking the HVR1 (amino acids 412-715 of the HCV polyprotein; E2e Δ HVR1) used as immunogen was validated by dose-response curves for the three well-characterized monoclonal antibodies AR3A, 1:7 and AP33.

Figure 5 represents the neutralizing activity of the alpaca serum. (A) Immune alpaca sera neutralized entry of HCVpp. Dilutions of immune sera sampled at day 50 (closed diamonds) and day 100 (open circles) were incubated with HCV pseudoparticles possessing

the envelope glycoproteins of strain H77. Sera from both timepoints neutralized entry equally, while sera from an animal immunized with tetanus toxin have no neutralizing effect (open triangles). (B) Performing neutralization experiments with wild type JFH1 in cell culture revealed greater neutralization potency for the sample taken at day 100 compared to day 50.

5 Serum at a dilution of 1/100 was incubated with 100 FFU of JFH1 before infecting Huh7.5 cells. Immune sera (closed diamonds) neutralized entry, while sera from an individual immunized with tetanus toxin (open triangles) had no neutralizing effect. (C) The breadth of neutralization was assessed utilizing a panel of HCV pseudoparticles representing genotypes 1-6. Differences in the neutralizing potency were observed between samples when incubated
10 with serum from day 50 (grey bars) or day 100 (white bars). The sample representing genotype 1a was resistant to neutralization by serum from both timepoints but for all other patient isolates, neutralization was observed for the later sample. No inhibition of entry was observed with control serum (black bars).

Figure 6 represents the neutralization activity of VHH. (A) Autologous
15 neutralization potency of each VHH was assessed using the vaccine strain UKN2B2.8. Each VHH was incubated at the indicated concentration with HCVpp bearing the UKN2B2.8 E1E2 proteins, and used to infect Huh7 cells. Dose dependent neutralization was observed for VHH D03 (closed squares) and C09, (closed triangles), while B11 (closed circles) and D04 (open triangles) had no observed neutralizing effect. D03 was by far the most potent VHH, exceeding the neutralization observed for the potently neutralizing anti-E2 mAb 1:7 (closed
20 diamonds) serving as a control. No neutralization was observed with the control anti-tetanus toxin mAb (open circles). (B) The neutralizing potency of each isolated VHH was determined using cell-cultured JFH1 particles. Each VHH was incubated at a concentration of 10µg/mL-1 with 100FFU of JFH1 particles before addition to target Huh7.5 cells. Neutralization was compared with the potently neutralizing mAb 1:7, and an irrelevant anti-tetanus toxin VHH. While VHH B11 and VHH D04 had little effect on JFH1 entry, VHH C09 showed limited inhibition and VHH D03 had much greater neutralizing potency, comparable to neutralization
25 by mAb 1:7. (C) A neutralization screen utilizing heterologous HCV pseudoparticles representing HCV genotypes 1-3 revealed that VHH D03 possessed the greatest neutralizing activity, with VHH C09 having neutralization activity limited to genotype 2 pseudoparticles. VHHs B11 and D04 had no effect on entry of HCVpp of any genotype. Neutralizing potency of D03 was greatest for samples representing genotypes 1b (UKN 1B12.16), 2a (UKN1A2.1) and 2b (UKN2B1.1), while 1a (H77, UKN1A20.8) and 3a (UKN3A1.28) were more resistant to neutralization. VHH samples were incubated at a concentration of 10µg/mL⁻¹ with each
30

HCVpp preparation before adding to target Huh7 cells. (D) To assess the cross-genotype efficacy of VHH D03, dose-dependent neutralization of HCV primary isolates was assessed for VHH D03 (closed circles), using VHH B11 (closed squares) and anti-tetanus toxin VHH (open triangles) as controls. Neutralization was compared to that of mAb 1:7 (closed triangles), which had previously been demonstrated to neutralize these isolates. Neutralization potency depended on the isolate used; many isolates were neutralized comparably by both VHH D03 and mAb 1:7, but UKN2A1.2 and UKN2B1.1 were more easily neutralized by VHH D03 than mAb 1:7.

Figure 7 represents the inhibition of cell to cell transmission by the VHH D03. Transmission between infected and naïve cells was achieved using co-cultures of CMFDA-labelled JFH-1 infected Huh7.5 cells and uninfected cells in the presence of antibodies that completely neutralize cell-free virus. Following culture, analysis by flow cytometry of CFMDA-/HCV NS5A+ cells revealed the number of direct transmission events. The presence of JFH-1 virions in cell supernatants was determined by titration; the point at which no cell free virus was detected, is highlighted by the bold line. The VHH was compared to fragments of the broadly neutralizing anti-E2 mAb A8.

Figure 8 represents the mapping of the epitope recognized by the VHH D03. (A) A panel of well-characterized mAbs with epitopes covering much of the surface of the E2 protein was used for competition assays with both VHH B11 and D03. Binding of a half-maximal concentration of each mAb was performed alone, or in the presence of each VHH at $10\mu\text{g mL}^{-1}$. B11 competed only with CBH-7. In contrast, VHH D03 competed with mAbs 1:7, AR2A, and AR3A, suggesting that the epitope for this VHH overlaps the CD81 binding site. (B) Binding of VHH D03 and B11 to a panel of alanine replacement mutants was assessed by immunofluorescence on cells expressing each individual mutant. Substitution of N415, G523 and T526 of the HCV polyprotein resulted in greater than 50% reduction in binding of VHH D03, while N540A of the HCV polyprotein was the only substitution that reduced binding of B11 by greater than 50%. (C) Alignment of HCV isolates used in neutralization and binding assays. Residues identified as being important to binding of VHH D03 are boxed. Asterisks highlight residues involved in interaction with the cellular receptor CD81, and the contact residues for mAbs 1:7 and A8 are labeled by triangles.

Figure 9 represents the inhibition of JFH-1 cell-cell transmission by dimeric constructs of the anti-E2 VHH D03. Transmission between HCV JFH-1 infected Huh-7.5 cells and naïve Huh-7.5 cells in the presence of VHH D03, dimeric VHH D03 and VHH D03-Fc dimer was assessed by flow cytometry. Extracellular virus was quantified by end-point

titration after 24h incubation, and a horizontal black line indicates the number of infected target cells in presence of an antibody dose that eliminated >95% of cell-free virus. Inhibition of cell-cell transmission was defined as reduced infection below this threshold. The results from two independent experiments are shown. An asterisk indicates approximately equimolar concentrations of the VHH and the dimer constructs in each experiment.

Figure 10 represents the neutralization of retroviral pseudoparticles engineered with hepatitis C virus or vesicular stomatitis glycoproteins by dimeric constructs of the anti-E2 VHH D03. Cell supernatants containing HCV H77, UKN2B2.8 or UKN3A13.6, or VSV pseudotyped particles were mixed with the indicated concentrations of the VHH D03 (15kDa), dimeric VHH D03 (30kDa), or the VHH D03-Fc dimer (80kDa). Mixtures of VHH constructs and pseudotyped particles were added to Huh7 human hepatoma cells. Infection was assayed after 72 hours incubation at 37°C. Data is presented as proportion of luciferase signal (relative light units) achieved in the absence of VHH, and indicates the mean +/- SD of three replicate experiments. An asterisk indicates approximately equimolar concentrations of the antibodies.

EXAMPLE: BROADLY NEUTRALIZING ALPACA VARIABLE HEAVY DOMAIN OF HEAVY CHAIN IgG (VHH) BLOCKS CELL-FREE AND CELL-CELL ENTRY OF HEPATITIS C VIRUS

1) Materials and Methods

Antigen

As main antigen it was used the HCV E2e of the genotype 2b isolate UKN2B2.8 (Lavillette *et al.*, 2005) lacking the hypervariable region 1 (HVR1) (SEQ ID NO: 30; E2eΔHVR1) produced in *Drosophila* S2 cells as previously described (Krey *et al.*, 2010). E2eΔHVR1 (SEQ ID NO: 30) corresponds to the fragment 412-715 of the HCV genotype 2b isolate UKN2B2.8 polyprotein (amino acid numbering is in reference to the amino acid sequence of the polyprotein of Hepatitis C virus strain H77 genotype 1a, accession number GI:130461 in the GENBANK database, and referred herein to as SEQ ID NO: 7). To verify the overall conformation 25μg of E2eΔHVR1 and 30μg of either conformation-dependent Fab-fragments e137 (Perotti *et al.*, 2008) or CBH-4D (Hadlock *et al.*, 2000), respectively, were incubated as isolated proteins as well as in complex for 1h at 10°C followed by analysis on a Superdex200 Mini column (column volume 3 ml, Amersham). Size exclusion chromatography (SEC) profiles were overlaid and used to determine the elution volumes.

Induction of a humoral immune response in an alpaca

250 μ L of HCV E2e Δ HVR1 (1mg/mL) produced in *Drosophila* S2 cells as previously described (Krey *et al.*, 2010) was mixed with 250 μ L of Freund's complete adjuvant for the first immunization, and with 250 μ L of Freund incomplete adjuvant for the following immunizations. One young adult male alpaca (*Llama pacos*) was immunized at days 0, 21 and 35 with 250 μ g of the immunogen. At day 50 a serum sample was taken and the immune response was monitored by the titration of serum samples by ELISA using HCV E2e full-length ectodomain as antigen. Subsequently the alpaca was boosted twice with the homologous HCV E2e full-length ectodomain (amino acids 384-715 of the HCV polyprotein) at 15 day intervals.

Library construction

The blood of the immunized animal was collected at day 100 and the peripheral blood lymphocytes were isolated by centrifugation on a Ficoll (Pharmacia) discontinuous gradient and stored at -80°C until further use. Total RNA and cDNA was obtained as previously described (Lafaye *et al.*, 1995). DNA fragments encoding VHH domains were amplified by PCR using CH2FORTA4 and VHBACKA6 primers, which anneal to the 3' and 5' flanking region of the V_H genes, respectively. The amplified product was used as template in a second round of PCR using either the primers VHBACKA4 and VHFOR36 or the primers VHBACKA4 and L_{HH} (5' GGACTAGTTGCGGCCGCTGGTTGTGGTTTTGGTGT CTTGGG-3'; SEQ ID NO: 6) specific for the long hinge homodimeric antibody (Lafaye *et al.*, 2009). The primers were complementary to the 5' and 3' ends of the amplified product and incorporated *SfiI* and *NotI* restriction sites at the ends of the VHH genes. The PCR products were digested and ligated into phage expression vector pHEN1. The resulting library was composed of two sub-libraries, one derived from VHH DNA-encoding genes with no hinge and the other from long hinge antibody genes. Phages were produced and isolated using both sub-libraries, and subsequently pooled.

The library was panned against HCV E2e first with the antigen coated on immunotubes as previously described (Cardoso *et al.*, 2000). Nunc Immuntubes (Maxisorp) tubes were coated overnight at 4°C with the antigen (10 $\mu\text{g/ml}$) in PBS. Phages (10^{13} transducing units) were biopanned by incubation with the coated tubes for 1h at 37°C under gentle agitation.

HCV E2e contained a strep-tag at the C terminus end and in a second panning the protein was bound on Strep-Tactin coated magnetic beads (MagStrep « type 2HC » beads,

IBA). The library was panned by incubation with the coated beads for 1 hour at 37°C. The blocking agent was changed at every round: 2% skimmed milk, Licor diluted 1:4, and 4% BSA were respectively used. The concentration of HCV E2e bound on beads decreased at every round of panning with respectively 100 nM, 50nM and 10 nM of protein. Following panning, phage clones were screened by standard ELISA procedures using a HRP/anti-M13 monoclonal antibody conjugate (GE Healthcare) for detection.

Expression of VHH

The coding sequence of the selected VHHs in vector pHEN1 was sub-cloned into a modified bacterial expression vector pET28a containing an 8-Histidine tag using the *NcoI* and *NotI* restriction sites. Transformed *E. coli* Rosetta-Gami2(DE3) cells expressed VHHs in the cytoplasm after induction with IPTG 0.2mM overnight at 16°C. Purified VHHs were obtained by IMAC from cytoplasmic extracts using a HiTrap crude column charged with Ni²⁺ (GE Healthcare) according to the manufacturer's instructions followed by size exclusion chromatography using a Superdex 75 column (GE Healthcare).

Pull-down of VHHs by E2e

50µg of E2eΔHVR1 was bound to a StrepTactin Superflow mini column (column volume 0.2ml, IBA) and washed with 10 column volumes of washing buffer. Subsequently, 30µg of IMAC purified (B11) or SEC purified (D03) VHH directed against HCV E2 were added, followed by washing with 3x5 column volumes. Complexes were eluted in 3x1 column volumes elution buffer and concentrated 20-fold by ultrafiltration. The concentrated fractions were analyzed by SDS-PAGE followed by Coomassie Blue staining.

Crystallization, data collection and structure determination

Crystals of the VHH D03 were grown at 293 K using the hanging-drop vapor-diffusion method in drops containing 1.2 µL protein (~20 mg/ml in 10 mM TRIS pH 8.0, 150 mM NaCl) mixed with 1.2 µL reservoir solution containing 2005mM LiSO₄. Diffraction quality rod-like crystals appeared after two to three weeks and were flash-frozen in mother liquor containing 18% (v/v) Glycerol. Spacegroups and cell dimensions of the crystals, resolution limits, data collection details and refinement statistics are summarized in Table 1 below.

Table 1: Crystallographic data for VHH D03

	VHH D03
<u>Data collection</u>	
Space group	P65
Cell dimensions	
a, b, c (Å)	52.81 52.81 182.47
α, β, γ (°)	90 90 120
Resolution (Å)	50.00-1.76 (1.86-1.76)
Rmerge	0.103 (0.266)
I/ σ I	7.7 (1.6)
Completeness (%)	94.7 (69.5)
Redundancy	5.0 (1.7)
<u>Refinement</u>	
Resolution (Å)	44.36-1.76
No. reflections	26863
Rwork/ Rfree	0.182 / 0.204
<u>No. atoms</u>	
Protein	1997
Ligand	-
Water	133
<u>B-factors</u>	
Protein	35.55
<u>R.m.s. deviations</u>	
Bond length (Å)	0.01
Bond angles (°)	1.14

* Values in parentheses correspond to the highest resolution shell. R.m.s. deviation - Root-mean-square deviation.

5 Data were collected at the Swiss Light Source (PX I). Data were processed with XDS (Kabsch, 1988) and scaling and reduction was performed using Pointless (Evans, 2005) and programs from the CCP4 suite (Collaborative Computational Project, 1994). The crystal structure of the VHH was determined by the molecular replacement method using Phaser (McCoy *et al.*, 2007) and a VHH (PDB 3STB) as search model. Model building was

performed using Coot (Emsley *et al.*, 2010) and refinement was done using AutoBuster (Bricogne *et al.*, 2010).

Neutralization of entry of HCV pseudoparticles by immune sera and VHH

HCV pseudoparticles representing genetically diverse primary isolates were generated as previously described (Lavillette *et al.*, 2005; Tarr *et al.*, 2007; Tarr *et al.*, 2011). Briefly, HEK293T cells were co-transfected with plasmids encoding i) amino acids 170-746 of the HCV polyprotein (Genbank accession number GI:130461), ii) a murine leukemia virus (MLV) Δenv backbone, and iii) a luciferase reporter gene flanked by the MLV long terminal repeats (LTRs). 72 hours after transfection HCV pseudoparticles were recovered from cell supernatants, and filtered through a 0.45 μm membrane. Pseudoparticles were mixed with dilutions of immune sera, defined concentrations of VHH, or monoclonal antibodies for one hour, then added to 1.5×10^4 Huh7 cells. After incubation for 48 hours cells were lysed with cell lysis buffer (Promega) and luciferase activity measured using luciferase substrate (Promega). Light output was measured with a BMG Fluostar Optima.

Neutralization of HCVcc by immune sera and VHH

RNA transcripts of the JFH1 strain of HCV were produced with a MEGAscript® T7 high yield transcription kit (Ambion) from linearized plasmid pJFH1. Genomic transcripts were cleaned up using an RNeasy® minikit (Qiagen). For each sample, 10 μg HCV RNA was electroporated into 7×10^6 Huh7.5 cells (Blight *et al.*, 2002) cells using a GenePulser Xcell™ electroporator (Bio-Rad). Media harvested after 48 hours was filtered through a 0.45 μm membrane and virus particles quantified by serially diluting cell supernatants in replicates and infecting Huh7.5 cells. Fixed cells were permeabilized with 0.5% Triton X-100 and stained with anti-NS5A mAb 9E10 (Lindenbach *et al.*, 2005) followed by HRP-conjugated Polyclonal Rabbit Anti-Mouse IgG (DakoCytomation) HRP activity was detected using the Vector® NovaRED™ (Vector) substrate kit. TCID₅₀ values were calculated using the Reed & Muench method (Reed and Muench, 1938). Neutralization assays were performed by mixing 100 focus-forming units (FFU) of virus with serum, VHH, or monoclonal antibody for one hour before adding to Huh7.5 cells. Infection was detected as described above, and percentage infection determined by comparison to the number of cells infected in the absence of inhibitors.

Binding of VHH to a panel of Alanine-substitution E1E2 point mutants.

HEK293T cells were transfected with pcDNA3.1-based vectors possessing the E1E2 genes of the H77c molecular clone of HCV bearing individual Alanine substitution point

mutants (Owsianka *et al.*, 2006). After 48 hours, transfected cells were trypsinized, harvested and seeded onto Teflon-coated microscope slides. Cells were air dried and then fixed with acetone. VHH were biotinylated using a N-hydroxysuccinamide-biotin ester (Pierce) and dialyzed into PBS using Millipore Microcon buffer exchange columns. The biotinylated VHH were then incubated with the E1E2-expressing cells. Following washing, binding was detected using a streptavidin Alexafluor488 conjugate (Invitrogen), and binding visualized using an immunofluorescence microscope. Binding was measured by integrating the total light output per field of view using a 400X magnification, subtracting the values obtained using untransfected cells as a control.

Inhibition of cell to cell transmission of HCV

HCV-infected producer Huh-7.5 cells were 5-Chloromethylfluorescein Diacetate (CMFDA)-labeled and co-cultured with unlabeled Huh-7.5 cells in a 1:1 ratio for 2 hours to allow cell-cell contacts to form. Neutralizing antibodies were then added and the culture continued for 24h. The extracellular media was collected and tested for the presence of infectious virus by infecting naïve Huh-7.5 cells. This represented the total level of infectious extracellular virus produced during the 24h period of co-culture. Purified polyclonal anti-HCV Ig (pooled from 600 donors) neutralized >95% of infectious virus.

Co-cultures of cells were fixed and permeabilized and stained for NS5A expression. Cells were analyzed by flow cytometry to identify the number of newly infected target cells (NS5A+/CMFDA-) along with the number of infected/uninfected producers in the assay. For neutralization assays, VHH or mAbs were incubated with the co-cultured cells and the residual inhibition of cell to cell transmission compared to that achieved in the presence of pooled polyclonal anti-HCV Ig.

Competition assay between VHH and defined monoclonal antibodies

Competition ELISAs were performed as described previously (Tarr *et al.*, 2012). Essentially, *Galanthus nivalis* agglutinin (GNA)-captured E1E2 proteins were probed with human or mouse monoclonal antibodies in the presence of increasing concentrations of the anti-E2 VHH. Bound mAb was detected with an anti-species IgG antibody conjugated to alkaline phosphatase. Binding was revealed with p-nitrophenol-phosphate substrate for 30 minutes. Competition was reported as inhibition compared to an uninhibited control.

Construction of dimeric VHH D03 and a VHH D03-Fc dimer

For construction of the dimeric VHH the coding sequence of VHH D03 was amplified and joined to the existing VHH expression plasmid in pET28a together with a flexible linker of 17 residues in between the two VHH's by PCR techniques. This dimeric

VHH construct was further modified by insertion of an additional proline residue upstream of the Poly-His Tag allowing for efficient removal of the tag by Carboxypeptidase A and transferred into the yeast expression vector pPIC9. Similarly, the monomeric VHH was also cloned into the same vector.

For construction of the dimeric Fc-VHH fusion construct the coding sequence of VHH D03 was amplified and joined to the Fc fragment derived from a human IgG1 antibody by PCR techniques. This fusion construct was subsequently cloned into a mammalian expression vector (pHLsec) and a Stop codon was added downstream of the coding sequence to facilitate efficient purification using Protein A.

Expression of monomeric and dimeric VHH constructs

Both the monomeric and dimeric VHHs were produced in the *P. pastoris* expression system (Invitrogen). Briefly, competent yeasts were transformed with the pPIC9 vector containing the respective VHH construct. Transformed yeast were identified by small scale expression according to the manufacturers instructions and large scale expressions of positive clones were performed in 4L fermenters according to the manufacturers instructions. Secreted VHH or VHH-Dimer was purified from the supernatant using a His^{excel} affinity column followed by Ion exchange and size exclusion chromatography on a Superdex 75 column.

Given that the N-linked glycosylation of the human Fc fragment is known to be important for serum stability, the dimeric VHH-Fc fusion construct was expressed in HEK 293F cells. Cells were grown in roller bottles and transfected with the pHLsec plasmid containing the VHH-Fc fusion construct using PEI as described before (Ariescu *et al.*, 2006). One week later secreted VHH-Fc fusion protein (VHH-Fc dimer) was purified from the supernatant using Protein A affinity chromatography followed by size exclusion chromatography on a Superdex 200 column.

Inhibition of cell to cell transmission of HCV and neutralization of entry of HCV pseudoparticles by immune sera and were carried out as described above.

2) Results

Alpaca immunization and assessment of anti-E2 serum response

One alpaca was immunized according to the protocol shown in Figure 2a with a genotype 2b HCV (isolate UKN2B2.8) E2eΔHVR1 (SEQ ID NO: 30) produced in *Drosophila* S2 cells as described previously (Krey *et al.*, 2010). This protein lacked the hypervariable region 1 (HVR1), to allow access to more conserved neutralization epitopes that have been postulated to be shielded by the HVR1 (Prentoe *et al.*, 2011; Bankwitz *et al.*, 2010). The correct conformation of E2eΔHVR1 was confirmed by testing the binding of a

panel of non-overlapping well-characterized, mostly conformation-sensitive monoclonal antibodies (Figure 2b and Figure 4).

The animal was re-boosted with HCV E2e full-length ectodomain, corresponding to amino acids 384-715 of the HCV polyprotein. Immune sera were tested for their ability to inhibit entry of HCV pseudoparticles and authentic HCV cell cultured particles. Serum sampled at day 50 and day 100 had a dose-responsive inhibitory effect on HCV entry (Figure 5). Both serum samples neutralized entry of the H77c pseudoparticles equally, but the sample taken at day 100 possessed greater neutralizing potency in the JFH1cc entry model (Figure 5b). To assess the breadth of neutralization by the immune sera, inhibition of entry of a panel of patient E1E2 isolates were assessed in the HCV pseudoparticle assay. The sample taken at day 100 consistently had greater neutralizing potency against these isolates (Figure 5c).

Identification of specific α -HCV E2 VHHs

A VHH library was generated by PCR from lymphocytes of the immunized animal into the pHEN1 vector to allow for efficient screening by phage display, yielding a library of 4×10^7 independent clones. After the panning selection of the VHH library on E2e-coated immunotubes, 96 clones were chosen and ELISA screening revealed that all of these clones bound to HCV E2e. 48 clones were sequenced and two independent VHHs - D03 and B11 - were identified in 60.5% and 39.5% of the clones, respectively. A second panning selection of the VHH library was performed, this time on HCV E2e bound on magnetic Streptactin beads, yielding 80.8% and 11.5% represented VHH D03 (SEQ ID NO: 2) and B11 (SEQ ID NO: 4), respectively. In addition, 2 new VHH - C09 (SEQ ID NO: 3) and D04 (SEQ ID NO: 5) - were identified. A full alignment of the amino acid sequences of all four VHHs is shown in Figure 2c. The limited number of identified VHHs suggests that the specific immune response against HCV E2e is remarkably oligoclonal, with VHH D03 binding to an immunodominant epitope.

Pull-down assays were performed to verify the interaction of the recombinant VHHs with the HCV E2 ectodomain. For this purpose the Strep-tagged ectodomain of HCV E2 produced in *Drosophila* S2 cells (Krey *et al.*, 2010) was bound to a streptactin column and His-tagged VHH purified either by IMAC (VHH B11, Figure 3a, left panel) or by SEC (VHH D03, Figure 3a, right panel) was added. Co-elution of the complex followed by SDS-PAGE and Coomassie staining revealed that E2e efficiently bound the VHH B11 and VHH D03 (Figure 3a). Immunofluorescence was also used to confirm binding of the four VHH to E1E2 proteins expressed on HEK 293T cells. Transiently-transfected cells were probed with

biotinylated VHH, and all four VHH clones were observed to bind specifically to these cells (Figure 3b).

Recombinant VHHS neutralize HCV entry

The four independent VHH clones were all assessed for the ability to inhibit HCV entry into cells. Firstly, HCV pseudoparticles bearing the UKN2B2.8 E1 and E2 glycoproteins were used to investigate autologous neutralization (Figure 6a). Potent, dose responsive neutralization was exhibited by VHH clone D03 (>95% at 20 µg mL⁻¹). VHH C09 also possessed neutralizing activity, reaching greater than 40% inhibition at the highest concentration tested. The two other VHH, B11 and VHH D04, possessed no inhibitory effect on HCV entry. These results were reproduced using the HCVcc model of entry (Figure 6b). Wild-type JFH1 virus was incubated with each VHH, and again, clone D03 had the most potent neutralizing effect. VHH C09 only had minor inhibitory effect on JFH1 entry. Combined, these data demonstrated that the VHH D03 was able to neutralize entry of HCV strains of an autologous genotype using *in vitro* models of entry.

To assess the breadth of neutralization by these VHHS, neutralization of a panel of patient-isolated E1E2 sequences was investigated using the HCVpp entry model (Bartosch *et al.*, 2003). Initially, all four VHHS were screened at a single concentration for neutralizing activity against a panel of pseudoparticles that had previously been demonstrated to represent a spectrum of neutralization resistance (Tarr *et al.*, 2011) (Figure 6c). Only sample VHH D03 possessed significant cross-neutralizing activity, sample VHH C09 inhibiting only HCVpp representing genotype 2. VHHS B11 and D04 did not possess neutralizing activity. While the most potent activity for VHH D03 was observed with the genotype 2 isolates, entry of pseudoparticles representing all three genotypes was inhibited by this VHH. To investigate this property of VHH D03 further, dose dependent neutralization of VHH D03 was investigated using a panel of pseudoparticles representing all six HCV genotypes. VHH B11 was also included as a control, as was the positive control mAb 1:7 (directed to a conserved neutralization epitope in the CD81 binding region of E2) and a negative control mAb directed to tetanus toxin. VHH D03 neutralized entry of isolates representing all six genotypes of HCV, while VHH B11 had no inhibitory effect (Figure 6d). The neutralization potency of VHH D03 was dependent on the HCV isolate investigated. Some clones, such as UKN2A1.2 and UKN2B1.1, were more easily neutralized by VHH D03 than mAb 1:7. However some, such as UKN3A13.6 and UKN5.15.7 were more refractive to neutralization by VHH D03.

Crystal structure of VHH D03

Sequence analysis of the four identified VHHS revealed two groups - the first one resembling the standard VHH containing only one single disulfide bond and the second one containing an additional disulfide bond connecting the CDR3 with the upstream residue of CDR2. Notably, the CD81 binding inhibition assay demonstrated that both VHHS containing a second disulfide bond interfere with CD81 binding, while the two VHHS with only one disulfide bond did not neutralize HCV infection, suggesting a possible role of this disulfide additional disulfide bond in antigen binding.

No crystal structure of a llama antibody with an additional disulfide bond between CDR3 and the framework upstream of CDR2 has been reported. The VHH D03 was crystallized to gain insights into the conformation of its potential antigen binding determinants and thus into its putative binding mode to the antigen. Diffraction quality crystals of the unliganded VHH, belonging to spacegroup P6₅, could be grown within four weeks. These crystals diffracted to 1.76Å on a synchrotron source. The crystal structure of the VHH was determined using the molecular replacement method; details and statistics of the data collection, processing and refinement are given in Table 1 above. The VHH displays the standard compact fold of an immunoglobulin domain containing nine β -strands tightly packed in two β -sheets. The three complementarity determining regions (CDR1, CDR2 and CDR3) are 8, 8 and 20 residues long (according to the IMGT nomenclature, Figure 2d), respectively, the CDR3 thus being longer than the average CDR3 from both VHH and human V_H regions (17 and 12 residues, respectively (Vu *et al.*, 1997)). The CDR3 folds over the part of the framework region that, in conventional antibodies, forms the V_H-V_L interface and is restrained in this conformation by a disulfide bond between Cys₅₂ directly upstream of the CDR2 and Cys₁₀₅ in the CDR3. Such inter-loop disulfide bonds have been identified in both camel and llama VHH (Vu *et al.*, 1997).

Antibody maturation in VHHS frequently includes somatic mutations that improve shape or charge complementarity of the paratope (Nguyen *et al.*, 2000). Amino acid alignment of VHH D03 with its closest homologous germline gene IGHV1S1*01 and mapping of the somatic mutations on the molecular surface of the α -HCV E2 VHH D03 revealed one, four and one somatic mutations in the CDR1, CDR2 and CDR3 (Figure 2d, labeled as asterisks), respectively.

VHH D03 inhibits cell-cell transmission

A significant limitation to therapeutic administration of antibodies is the ability of HCV to infect naïve cells directly, without being exposed to the extracellular environment. Having demonstrated a potent neutralizing effect on entry of cell-free HCV particles into susceptible cells, the possibility of VHH inhibition of cell-cell transmission was investigated. In addition to inhibiting the cell-free virions in this assay, VHH D03 also possessed the ability to inhibit transmission of HCV from infected producer cells to naïve huh7 cells (Figure 7).

The observed neutralizing activity of VHH D03 could be attributable to either its size, or its specificity. To discriminate between these possibilities recombinant antibody fragments of the broadly neutralizing antibody A8 (Allander *et al.*, 2000; Johansson *et al.*, 2007) was expressed in *Drosophila* S2 cells as described before (Backovic *et al.*, 2010; Gilmartin *et al.*, 2012), which possesses a similar biological activity to mAb 1:7 and binds to similar residues in E2 (Johansson *et al.*, 2007). Cell-cell transmission of JFH-1 HCVcc was neither affected by the IgG molecule of A8 (MW of ~150kD), nor by any of the recombinant fragments (Fab MW of ~50kD, single chain variable fragment (scFv), MW of ~30kD), suggesting that size is not the pivotal antibody feature that determines efficient neutralization in cell-cell transmission. Instead, this indicates that the specificity of the VHH is crucial for its efficiency to inhibit cell-cell transmission.

These antibody fragments were also tested for neutralization of cell-free HCVpp entry and directly compared to VHH D03 (Figure 8). While VHH D03 more potently neutralized clone UKN2B1.1 than mAb 1:7, when using clone H77 mAb 1:7 and its fragments were much more potently neutralizing than the VHH. In agreement with the neutralization activity on cell-cell transmission this suggests that the specificity of the VHH provides its potent neutralizing properties, rather than the small size of the protein alone.

Binding of VHH D03 to the E2 glycoproteins identifies a novel epitope overlapping the CD81 binding site

To localize the epitope recognized by VHH D03 on the surface of E2, a panel of well-characterized monoclonal antibodies raised in mice, rats or humans was assessed for direct competition for binding of VHH D03 and VHH B11 to HCV E1E2 proteins (Figure 8a). VHH D03 competed with mAbs AR3A, 1:7 and AR2A for binding to the E1E2 proteins, providing evidence that this VHH is likely to recognize an epitope overlapping the CD81 binding site in the proposed Domain 1 of E2. For comparison, competition assays were also performed with VHH B11. In contrast, binding of mAbs AR3A, 1:7 and AR2A (Law *et al.*, 2007, Johansson *et al.*, 2007) was not competed by VHH B11, but competition was observed

with mAb CBH7, which recognizes a distinct epitope. To further define the epitopes of these VHH, binding of VHH D03 and VHH B11 to a panel of alanine-substitution mutants was also performed (Figure 8b). Given that mAbs recognizing the CD81 binding site on E2 were competed by VHH D03, individual conserved residues observed in the functional patient isolates between amino acids 412 and 550 were mutated to alanine. Distinct patterns of reactivity were observed for the two VHHs. This provides further evidence that the neutralizing epitope recognized by D03 is distinct to the epitope recognized by VHH B11. Binding of VHH D03 was reduced by substitutions at amino acids T416, I422, G523 and T526 of the HCV polyprotein, while binding of VHH B11 was reduced by mutations at I422, N540 and R614. The substitutions that resulted in greater than 50% reduction in binding of D03 were N415A, G523A and T526A for D03 and only N540A of the HCV polyprotein for B11. This mapped the epitope of VHH D03 to residues overlapping the previously defined CD81 binding region consistent with the inhibition of CD81 binding by VHH D03.

Inhibition of cell-cell transmission of JFH-1 by dimeric constructs of the anti-E2 VHH D03.

Transmission between HCV JFH-1 infected Huh-7.5 cells and naïve Huh-7.5 cells in the presence of VHH D03, dimeric VHH D03 and VHH D03-Fc construct was assessed by flow cytométrie. VHH D03 demonstrated almost no inhibitory effect at 10 µg/ml (see Figure 9a), but a dose-dependent inhibition of direct infection was shown previously above. Both the dimeric VHH D03 and the VHH D03 Fc fusion protein efficiently inhibit cell-cell transmission of HCV JFH-1 in Huh-7.5 cells (see Figure 9b).

Neutralization of HCVpp by dimeric constructs of the anti-E2 VHH D03.

Cell supernatants containing HCV or VSV pseudotyped particles were mixed with the VHH D03 (15kDa), dimeric VHH D03 (30kDa), or a VHH D03-Fc construct (80kDa) at different concentrations. After incubation for one hour, mixtures of VHH constructs and pseudotyped particles were added to 15000 Huh7 human hepatoma cells. After 4 hours media was replaced and infection assayed after 72 hours incubation at 37°C using the Promega Luciferase Assay System.

The results are shown in Figure 10. While entry of strain UKN2B2.8 was neutralized equivalently by all three constructs, neutralization potency against strain H77 and UKN3A13.6 was improved for both dimeric constructs of the VHH. This demonstrates that increased neutralization breadth is achieved by the presence of higher molecular weight dimer/Fc constructs of the VHH.

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CLAIMS

1) An isolated neutralizing antibody or fragment thereof directed against the ectodomain of a Hepatitis C virus glycoprotein E2, characterized in that said antibody and fragment thereof bind the contacting amino acid residues G523 and T526, and optionally N415, of said Hepatitis C virus glycoprotein E2, in reference to the amino acid sequence of the polyprotein of the Hepatitis C virus strain H77 genotype 1a of SEQ ID NO: 7.

2) The isolated neutralizing antibody or fragment thereof according to claim 1, characterized in that they do not bind amino acid residues P525, W529, G530, D535 and N540 of said Hepatitis C virus glycoprotein E2, in reference to the amino acid sequence of the polyprotein of the Hepatitis C virus strain H77 genotype 1a of SEQ ID NO: 7.

3) The isolated neutralizing antibody or fragment thereof according to claim 1 or claim 2, characterized in that they comprise at least one Complementarity Determining Region (CDR) from VHH D03 of SEQ ID NO: 2, selected from the group consisting of SEQ ID NO: 31 (CDR1 from VHH D03), SEQ ID NO: 32 (CDR2 from VHH D03) and SEQ ID NO: 33 (CDR3 from VHH D03).

4) The isolated antibody fragment according to any one of claims 1 to 3, characterized in that it is a variable antigen-binding domain from a camelid heavy-chain antibody (VHH).

5) The isolated antibody fragment according to claim 3 or claim 4, characterized in that it comprises or consists of the amino acid sequence SEQ ID NO: 1.

6) The isolated antibody fragment according to claim 4 or claim 5, characterized in that it consists of the amino acid sequence SEQ ID NO: 2 (VHH D03).

7) The isolated antibody fragment according to any one of claims 4 to 6, characterised in that said VHH is obtainable by the method comprising the steps of:

(a) immunizing a camelid with a Hepatitis C virus glycoprotein E2 (HCV E2) or the ectodomain of a Hepatitis C virus glycoprotein E2 or with the ectodomain of a Hepatitis C virus glycoprotein E2 lacking the HVR1 domain,

(b) isolating peripheral lymphocytes of the immunized camelid, obtaining the total RNA and synthesizing the corresponding cDNAs,

(c) constructing a library of cDNA fragments encoding VHH,

(d) transcribing the VHH-encoding cDNAs obtained in step (c) to mRNA, and

(e) expressing the VHH in an expression vector.

8) The isolated neutralizing antibody or fragment thereof according to any one of claims 1 to 7, characterized in that it is in the form of a monomer, a homomultimer or a heteromultimer.

9) The isolated neutralizing antibody or fragment thereof according to any one of claims 1 to 3, characterized in that it is in a form of a chimeric antibody, comprising:

- at least one CDR from VHH D03 (SEQ ID NO: 2), selected from the group consisting of SEQ ID NO: 31 (CDR1), SEQ ID NO: 32 (CDR2), SEQ ID NO: 33 (CDR3), and

- at least one CDR from VHH C09 (SEQ ID NO: 3), selected from the group consisting of SEQ ID NO: 34 (CDR1), SEQ ID NO: 35 (CDR2) and SEQ ID NO: 36 (CDR3).

10) An isolated polypeptide, characterized in that it comprises an antibody fragment as defined in any one of claims 1 to 9.

11) An isolated polynucleotide, characterized in that it encodes an antibody fragment or a polypeptide as defined in any one of claims 1 to 10.

12) A recombinant expression cassette, characterized in that it comprises a polynucleotide according to claim 11, under control of a transcriptional promoter.

13) A recombinant vector, characterized in that it comprises a polynucleotide according to claim 11 or a recombinant expression cassette according to claim 12.

14) A host cell, characterized in that it contains a recombinant expression cassette of claim 12 or a recombinant vector of claim 13.

15) A pharmaceutical composition, characterized in that it comprises a neutralizing antibody or fragment thereof as defined in any one of claims 1 to 9 or a polypeptide as defined in claim 10 or a recombinant vector according to claim 13, and a pharmaceutically acceptable carrier.

16) A diagnostic agent, characterized in that it comprises a neutralizing antibody or fragment thereof as defined in any one of claims 1 to 9 or a polypeptide as defined in claim 10, linked, directly or indirectly, covalently or non-covalently to a detectable marker.

17) The diagnostic agent according to claim 16, characterized in that the detectable marker is selected from the group consisting of enzymes, fluorophores, heavy metal chelates and radioisotopes.

18) A kit for diagnosing or monitoring, in a subject, a Hepatitis C virus infection, characterized in that it comprises a neutralizing antibody or fragment thereof as defined in any one of claims 1 to 9 or a polypeptide as defined in claim 10 or a diagnostic agent as defined in claims 16 or 17, and an appropriate diagnostic reagent.

19) An *in vitro* method for diagnosing a Hepatitis C virus infection in a subject, characterized in that it comprises the steps of:

a) contacting *in vitro* an appropriate biological sample from said subject with a neutralizing antibody or fragment thereof as defined in any one of claims 1 to 9 or a polypeptide as defined in claim 10 or a diagnostic agent as defined in claims 16 or 17, and

b) determining the presence or the absence of a HCV envelope glycoprotein E2 in said biological sample,

the presence of said HCV envelope glycoprotein E2 indicating that said subject has Hepatitis C virus infection.

20) An *in vitro* method for monitoring the progression or regression of a Hepatitis C virus infection in a subject, characterized in that it comprises the steps of:

a) contacting *in vitro* an appropriate biological sample from said subject with a neutralizing antibody or fragment thereof as defined in any one of claims 1 to 9 or a polypeptide as defined in claim 10 or a diagnostic agent as defined in claims 16 or 17,

b) determining the amount of HCV envelope glycoprotein E2 in said biological sample, and

c) comparing the amount determined in step (b) with the amount of HCV envelope glycoprotein E2 previously obtained for said subject,

a significant increase in amount of HCV envelope glycoprotein E2 constituting a marker of the progression of said HCV infection and a significant decrease of HCV envelope glycoprotein E2 constituting a marker of the regression of said HCV infection.

21) An isolated antigen consisting of a Hepatitis C virus E2 ectodomain lacking the hypervariable region 1 (HVR1), corresponding to amino acids 412-715 of the polyprotein of said HCV in reference to HCV strain H77c of SEQ ID NO: 7.

V _H H D03 (SEQ ID NO: 2):	EVQLQASGGGLVQPGGSLRLSCTASGFT-DDYYAIGWFRQAPGKERE	49
V _H H C09 (SEQ ID NO: 3):	EVQLQA-GGGLAQPGGSLRLSCEASGFSLVNLDITIGWFRQAPGKERE	49
SEQ ID NO: 1	EVQLQA.GGGL.QPGGSLRLSC.ASGF.....IGWFRQAPGKERE	50
V _H H D03 (SEQ ID NO: 2):	CITNFDGGTYADSVKSRFTMSRDNAKNTVYLQMNSLKPEDTAVYYCAAD	99
V _H H C09 (SEQ ID NO: 3):	CVSESDGKEYYTDVSKGRFTISRDNKNTMYLQMDSLNAEDTAVYYCVAD	99
SEQ ID NO: 1:	C.....DG..YY.DSVK.RFT.SRDNAKNT.YLQM.SL..EDTAVYYC.AD	100
V _H H D03 (SEQ ID NO: 2):	K-GLCSWLRAG-GKVTFGSWGQGTQVTVSS	127
V _H H C09 (SEQ ID NO: 3):	SFAQCQPPYSGWNEDEYDWGQGTQVTVSS	129
SEQ ID NO: 1:	C.....G.....WGQGTQVTVSS	130

Figure 1

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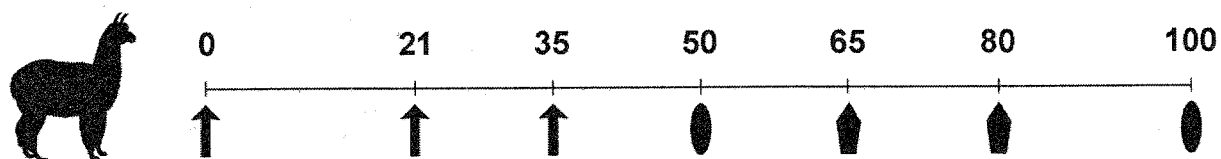


Figure 2A

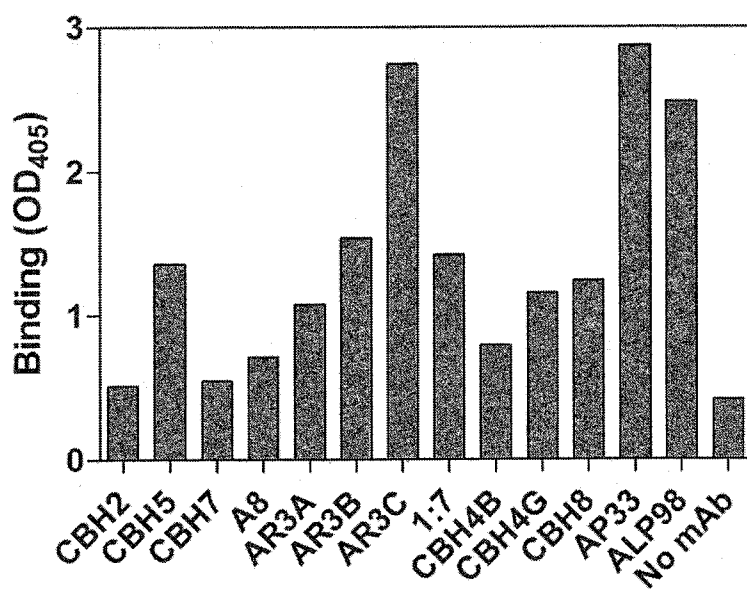
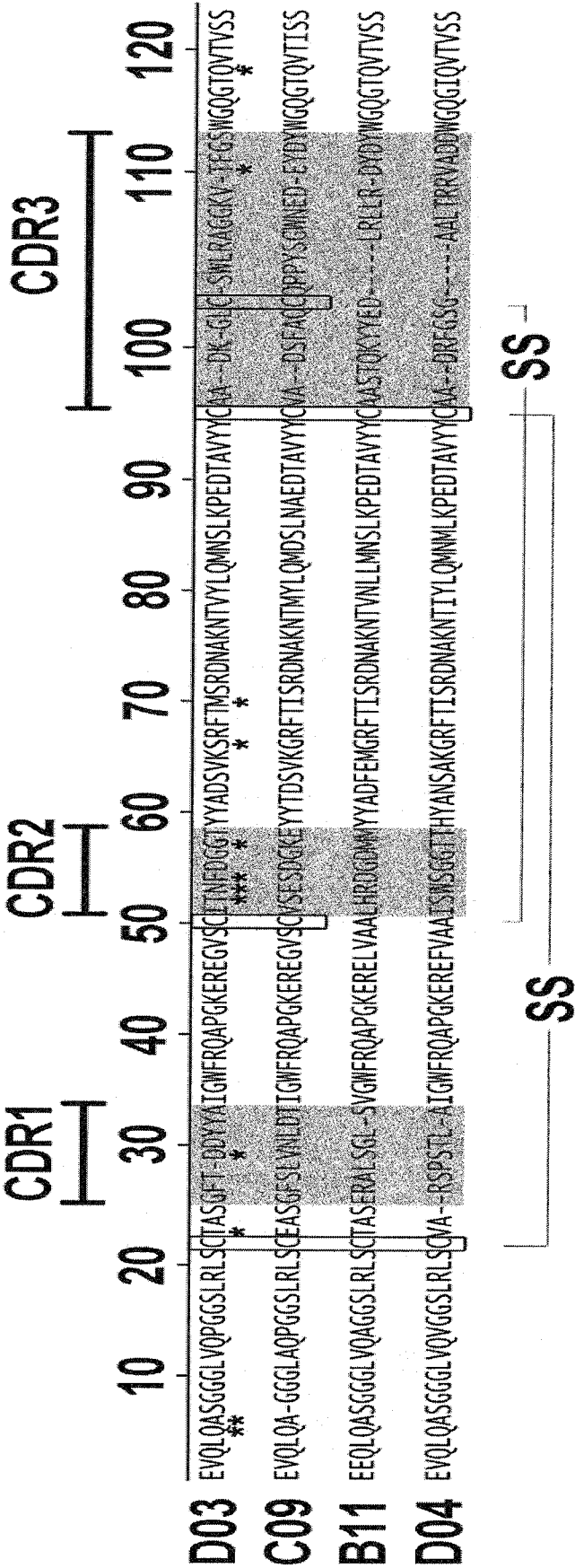


Figure 2B



D03 = SEQ ID NO: 2
C09 = SEQ ID NO: 3
B11 = SEQ ID NO: 4
D04 = SEQ ID NO: 5

Figure 2C

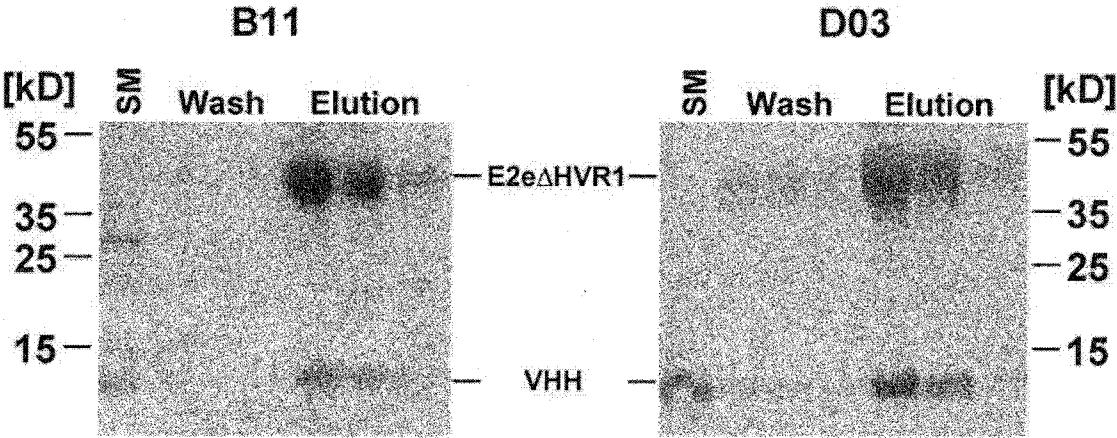


Figure 3A

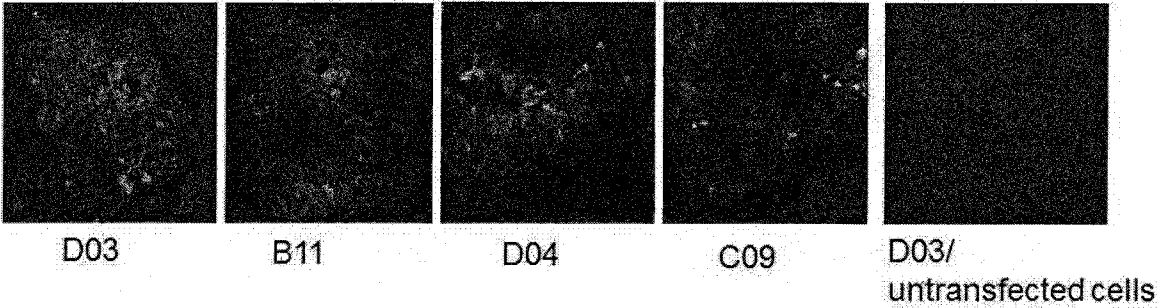


Figure 3B

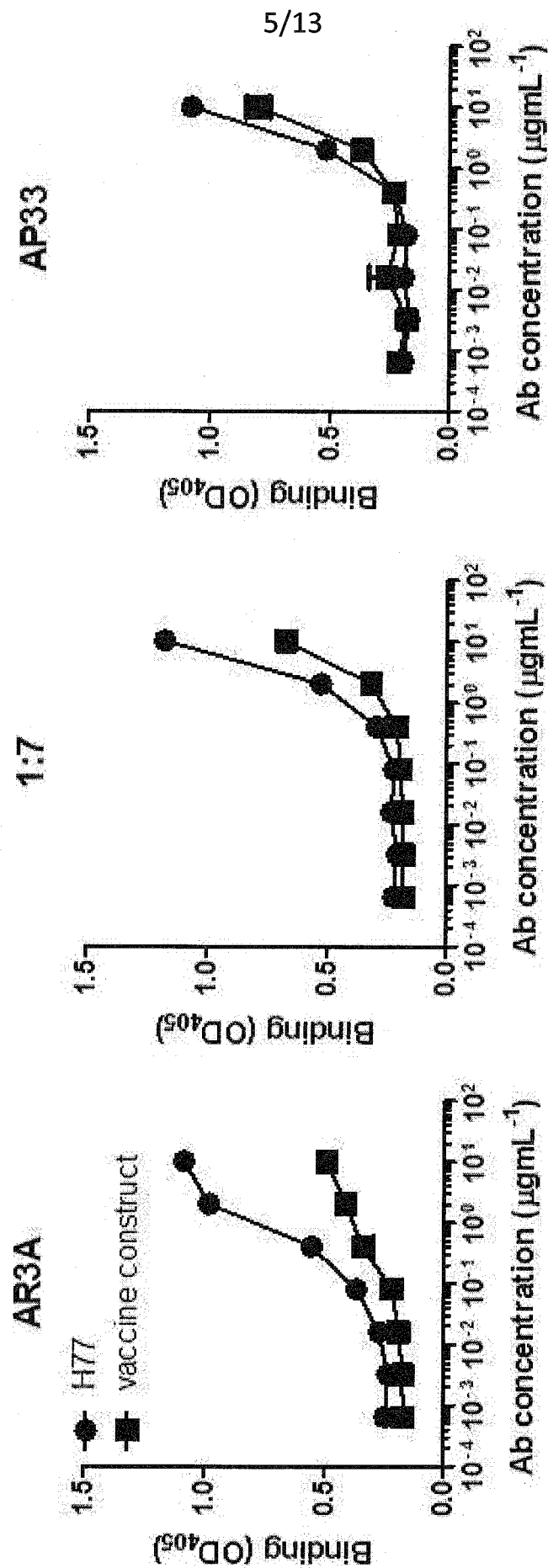


Figure 4

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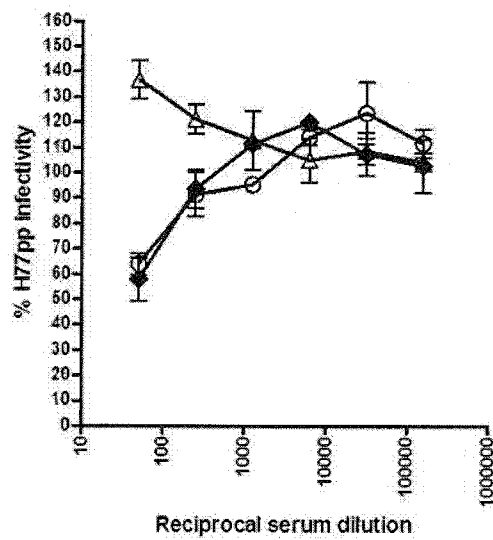


Figure 5A

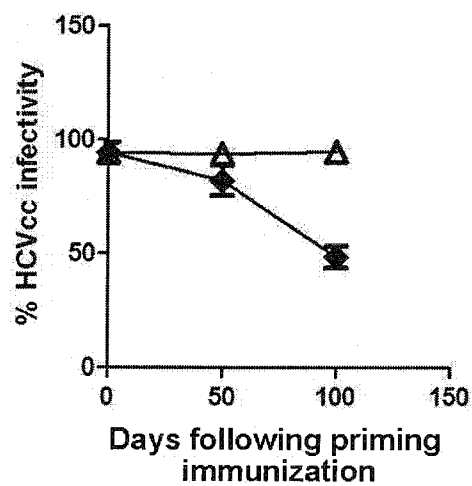


Figure 5B

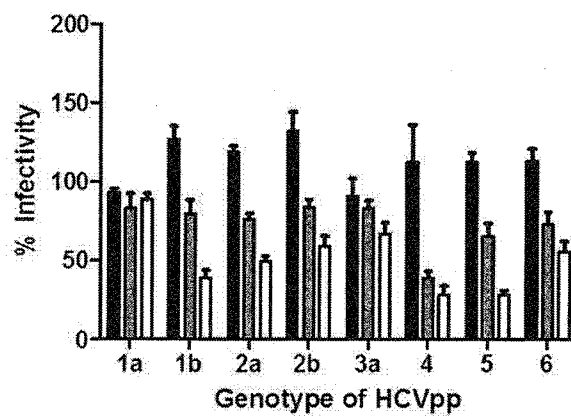


Figure 5C

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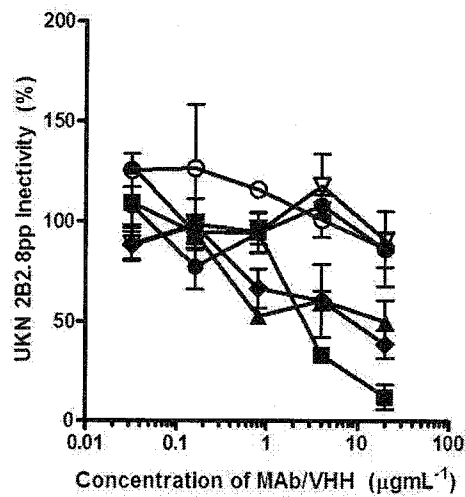


Figure 6A

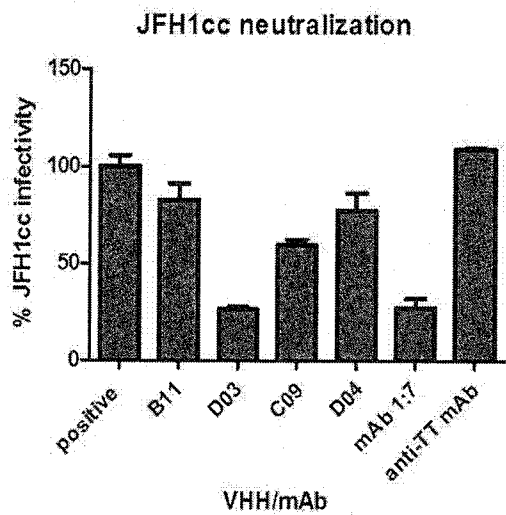


Figure 6B

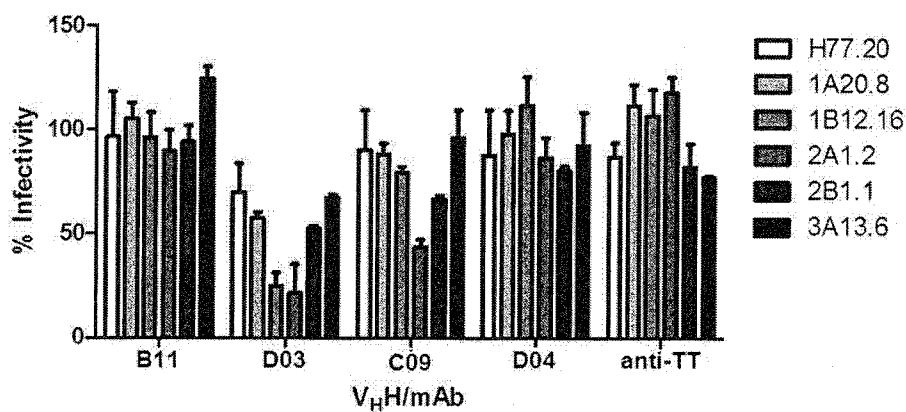


Figure 6C

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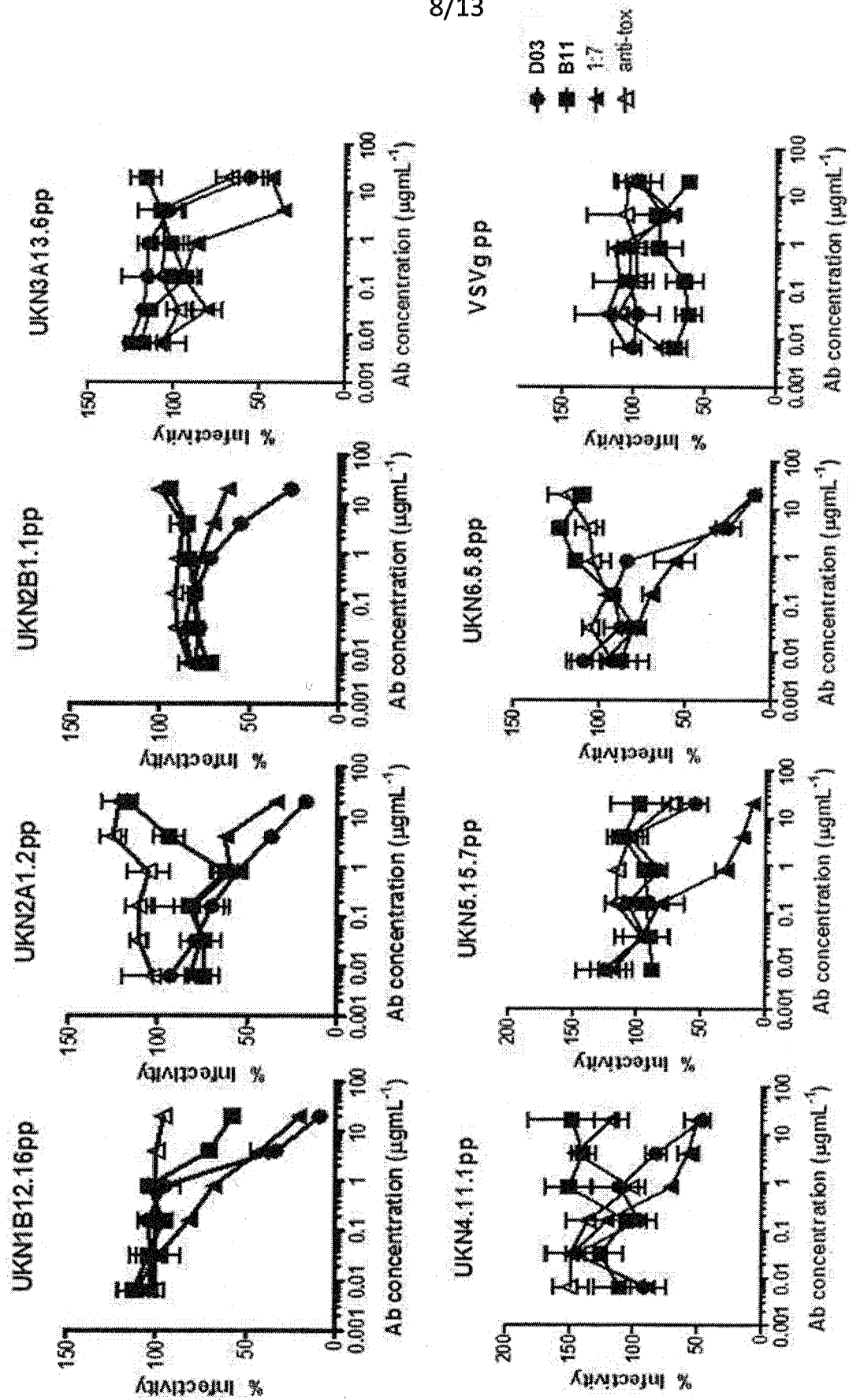


Figure 6D

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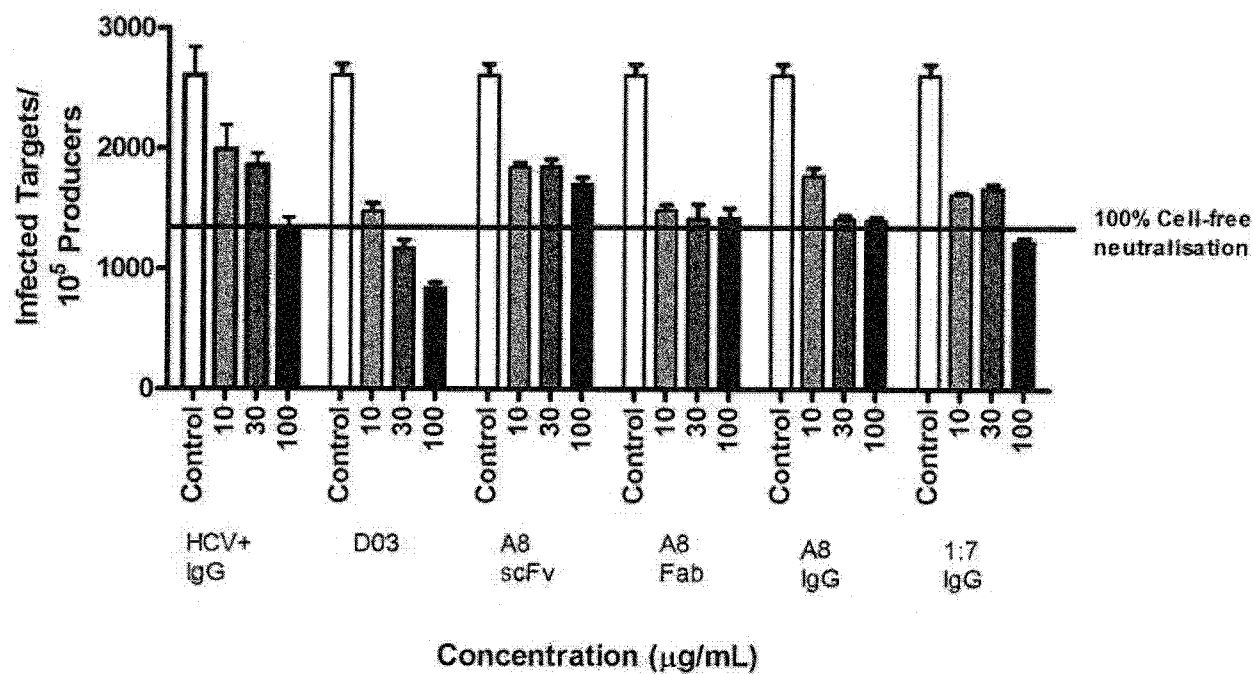


Figure 7

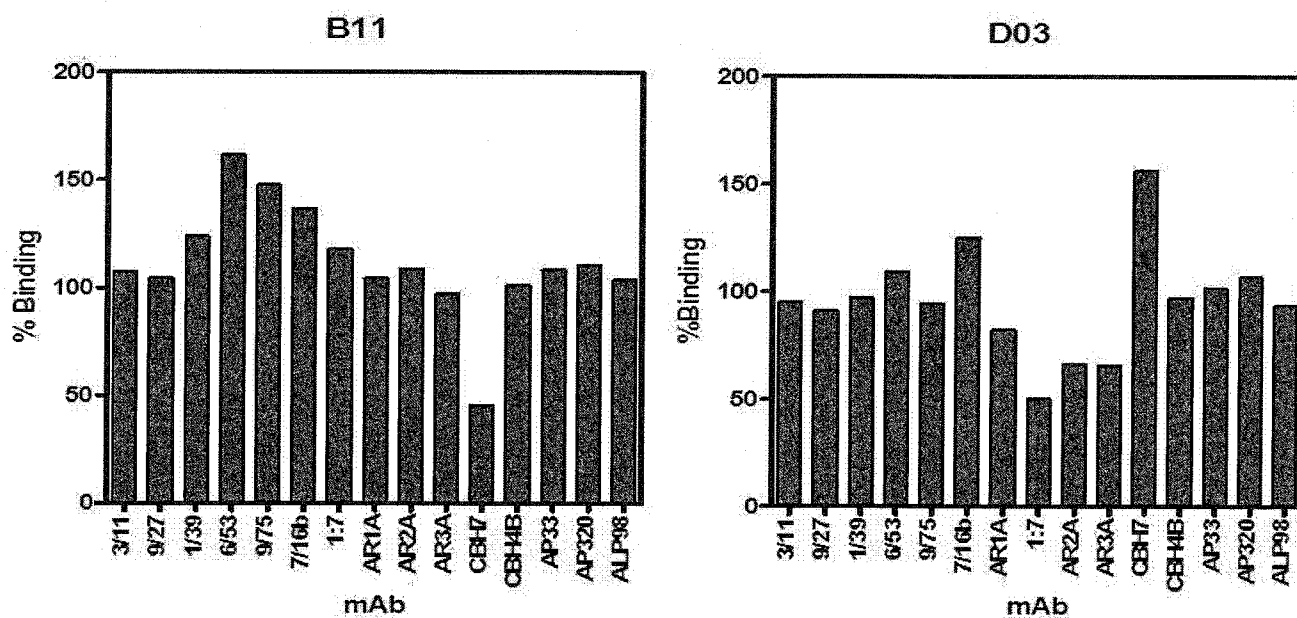


Figure 8A

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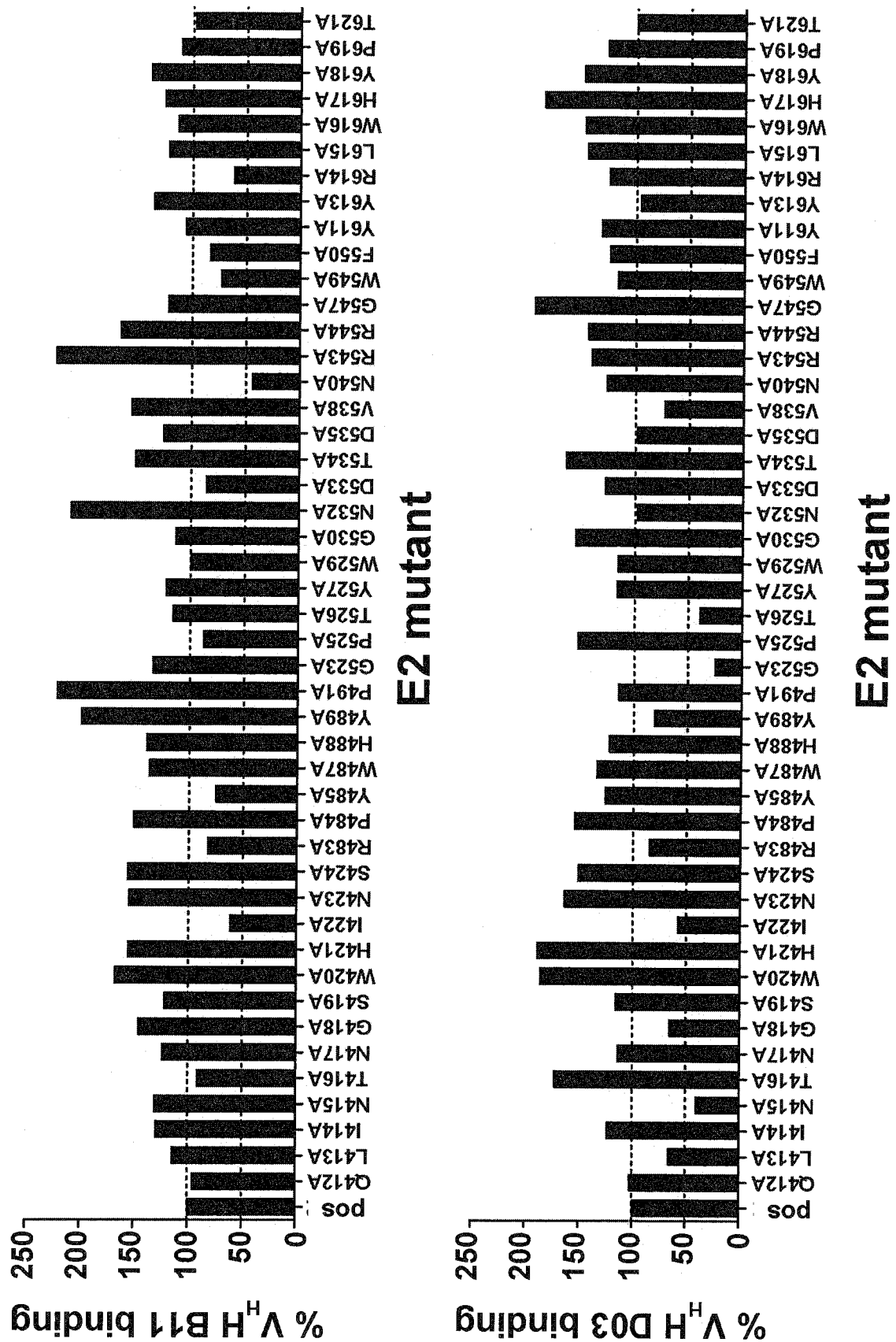


Figure 8B

412	H77.20	NIQLINTGS	*	WHINSTALNC	NESLNTGWL	SEQ ID NO:10
	UKN1A20.8	.S...		.R....	.D..T...I.	SEQ ID NO:11
	UKN1B12.16	K...V...		.R....	.D....F..	SEQ ID NO:12
	UKN1B5.23	K...V...		.R....	.D....F..	SEQ ID NO:13
	UKN2A1.2			.R....	.D..Q..FI.	SEQ ID NO:14
	UKN2B1.1	K.S...		.R....	.D..Q..F..	SEQ ID NO:15
	UKN3A13.6	RLE...		.R....	.I....F..	SEQ ID NO:16
	UKN4.11.1	.V....		.R....	.D....F..T	SEQ ID NO:17
	UKN5.15.7	.L..V...		.R....	QD..Q..FI.	SEQ ID NO:18
	UKN6.5.8	KL.....		.R....	.D..Q..FI.	SEQ ID NO:19
518	*	TTDRSGAPTY	*	SWGANDTDVF	VLNNTRPPLG	SEQ ID NO:20
	...K...		*	R..E.E....		SEQ ID NO:21
	...F...		*	T..E.E...L	I.....Q.	SEQ ID NO:22
	...V...		*	...E.E...L	L.....Q.	SEQ ID NO:23
	..NKL.V..		*	T..E.E....	L..S....R.	SEQ ID NO:24
	...EQ.L..		*	...E.E....	L..S....Q.	SEQ ID NO:25
	...PK....		*	N..E.E....	L.KSL...S.	SEQ ID NO:26
	...F.V...		*	T..D.ES...	L..A....R.	SEQ ID NO:27
	...R.Y...		*	N..S.E...I	L..S....A.	SEQ ID NO:28
	...R.N...		*	T..E.E....	M.ESL...T.	SEQ ID NO:29

Figure 8C

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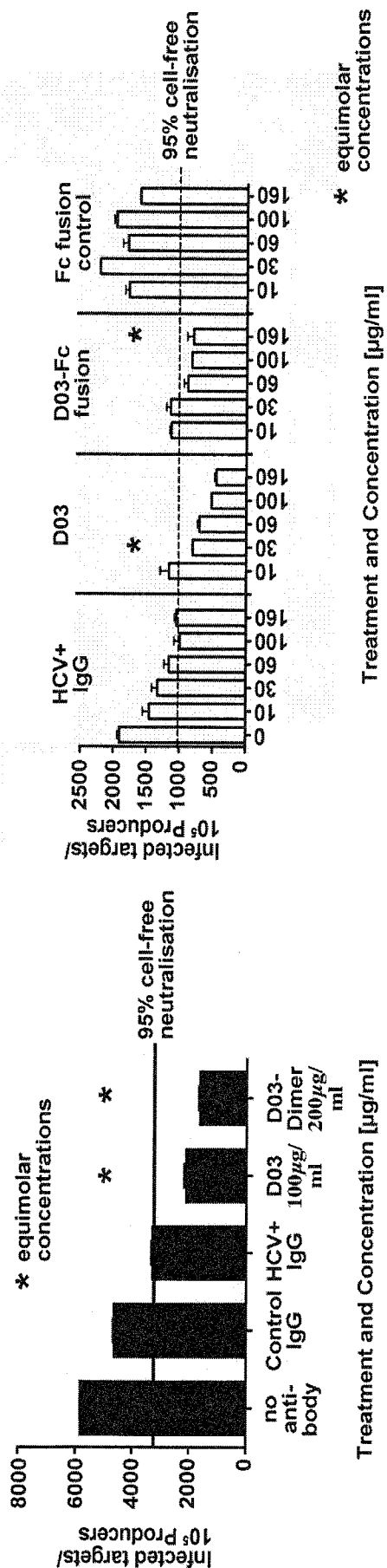


Figure 9

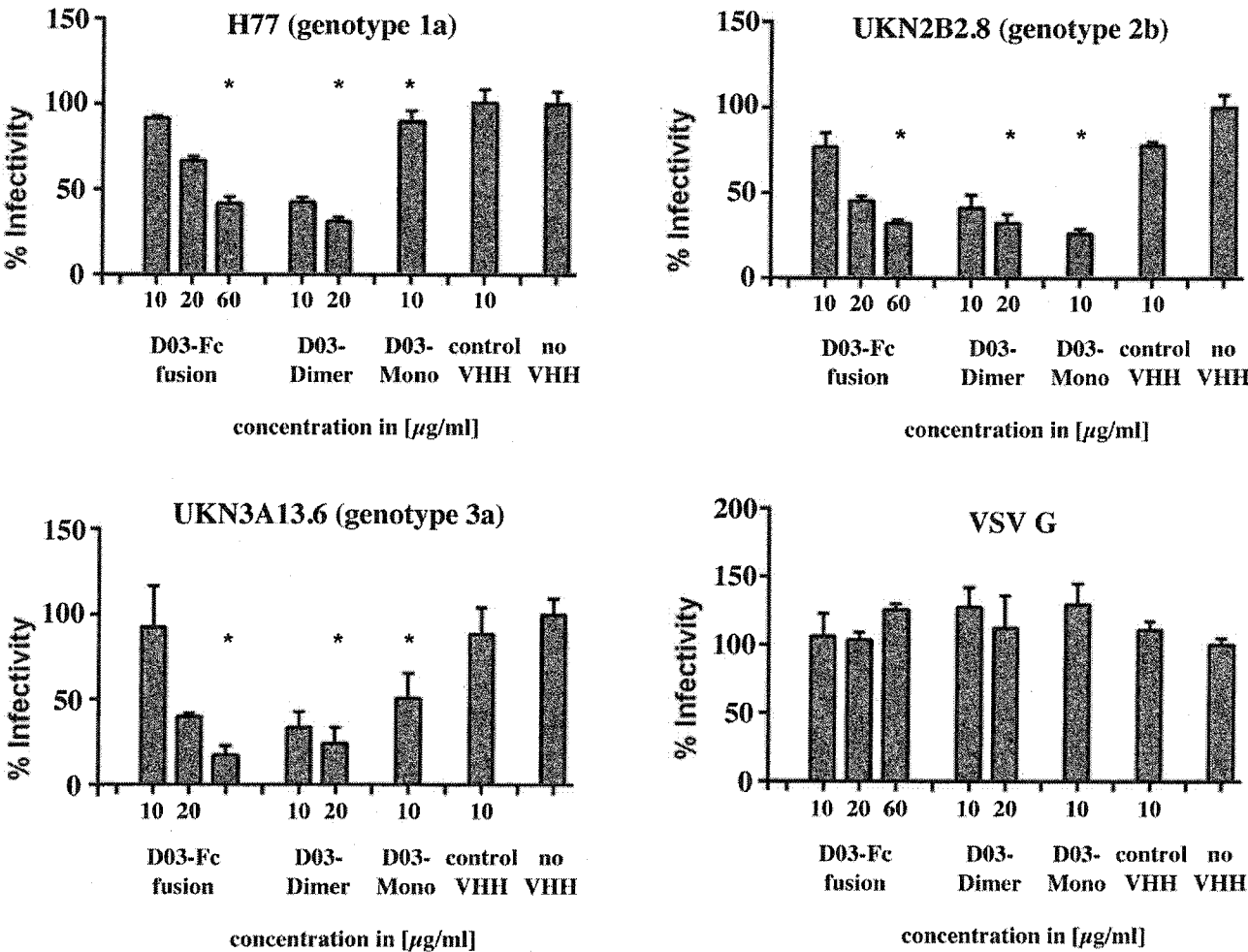


Figure 10

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2013/070698

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K16/10 G01N33/58
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOHANSSON DANIEL X ET AL: "Human combinatorial libraries yield rare antibodies that broadly neutralize hepatitis C virus", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, NATIONAL ACADEMY OF SCIENCES, US, vol. 104, no. 41, 9 October 2007 (2007-10-09), pages 16269-16274, XP002490279, ISSN: 0027-8424, DOI: 10.1073/PNAS.0705522104	1
A	cited in the application the whole document ----- -/--	2-21



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

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Date of the actual completion of the international search

13 November 2013

Date of mailing of the international search report

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INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2013/070698

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A	----- LAW MANSUN ET AL: "Broadly neutralizing antibodies protect against hepatitis C virus quasispecies challenge.", NATURE MEDICINE JAN 2008, vol. 14, no. 1, January 2008 (2008-01), pages 25-27, XP002690712, ISSN: 1546-170X cited in the application the whole document	1-21
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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2013/070698

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A	LIU JING ET AL: "Expression of hepatitis C virus E2 ectodomain in E. coli and its application in the detection of anti-E2 antibodies in human sera.", ACTA BIOCHIMICA ET BIOPHYSICA SINICA JAN 2004, vol. 36, no. 1, January 2004 (2004-01), pages 57-63, XP008159461, ISSN: 1672-9145 the whole document -----	1-21
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International application No
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International application No

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