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**Supplementary data**

**Alpaca immunization and identification of nanobodies recognizing HCV E2.**

We immunized an alpaca with HCV genotype 2b E2eΔHVR1 lacking the first hypervariable region (HVR1) (corresponding to amino acids 412-715 of HCV strain H77c polyprotein) according to the schedule illustrated in Figure S1a. We used an E2 protein lacking the hypervariable region 1 (HVR1) to increase the chances of inducing cross-reactive antibody responses, particularly those targeting the CD81 binding site, because HVR1 is likely to shield the CD81 binding site (1, 2). We demonstrated binding of a panel of conformation-sensitive monoclonal antibodies to the immunogen, suggesting a similar conformation to the native virus-associated glycoprotein (Figure S1b). The animal was subsequently boosted with homologous HCV E2e full-length ectodomain and post-immune sera inhibited HCVpp and HCVcc infection in a dose-dependent manner (Figure S3). To assess the cross-reactivity of these sera we screened these sera against a panel of pseudoparticles expressing diverse HCV glycoproteins. The serum sample taken at day 100 consistently showed greater neutralizing activity against these pseudoparticles (Figure S3c) and we therefore used the corresponding lymphocytes to generate a nanobody phage display library with the goal to identify E2 glycoprotein-specific nanobodies. Panning the library for affinity to the immunogen identified 96 clones with reactivity for HCV E2. Forty-eight of these were sequenced and two independent nanobody sequences identified. The sequence of nanobody D03 was found in 60.5% of clones, whilst the remaining 39.5% of clones corresponded to the B11 sequence. A second panning of the library on HCV E2e-Streptactin beads yielded 80.8% and 11.5% of sequences corresponding to nanobodies D03 and B11, respectively. Two additional sequences, corresponding to nanobodies D04 and C09, were identified. A full alignment of the amino acid sequences of all four nanobodies is shown in Figure S1c.

Anti-viral nanobodies have been raised by immunization of camelids with glycoproteins from many viruses, including HIV, hepatitis B virus, and influenza virus (reviewed in ([3])). However, recent reports show limited success in generating nanobodies that neutralize genetically diverse HIV-1 isolates ([4]). Nanobody libraries to HIV gp120 or FMDV particles show a greater diversity ([5], [6]), compared to the small number of discrete nanobodies isolated in this study. Our results suggest that D03 recognizes an immunodominant epitope, but further studies using a similar immunization strategy and antigen are required to understand if the narrow immune response is determined by the immunized animal, the antigen or unknown external factors.

**Cross-competition analysis of D03**

To identify the epitope recognized by nanobody D03, we first compared the ability of a well-characterized panel of mAbs to compete with binding of D03 to HCV E2. Competing mAbs were selected to cover a large area of the E2 protein (Figure S4a), including linear epitopes spanning HVR1 (mAb 9/27) and conserved regions across the E2 ectodomain (mAbs 3/11, AP33, 1/39, 2/64a, 9/75, 6/53 and ALP98). We also included antibodies recognizing well-defined conformational epitopes (mAbs AR1A, AR2A, AR3A, 1:7, CBH-4G and CBH-7).

**Supplementary Experimental Procedures**

*Analysis of immunogen E2eΔHVR1 conformation*

HCV E2 ectodomain (E2e) from strain UKN2B2.8 ([7]) lacking the hypervariable region 1 (HVR1) (amino acids 412-715 of the HCV polyprotein; E2eΔHVR1) was expressed in Drosophila S2 cells as previously described ([8]). Its native conformation was evaluated by coating onto Maxisorp assay wells (Nunc) overnight at 4°C, before blocking with PBS containing 5% non-fat milk. Following washing, captured protein was probed with monoclonal antibodies (mAbs) previously demonstrated to bind conformation-sensitive

epitopes (CBH2, CBH5, CBH7, A8, AR3A, AR3B, AR3C, 1:7, CBH4B, CBH4G and CBH8) (9-11), a partially conformation sensitive epitope (mAb AP33)(12), or linear epitope (mAb ALP98)(12, 13) of E2. Antibody interactions were revealed using anti-human/mouse antibodies conjugated to alkaline phosphatase (Sigma), followed by development with p-Nitrophenol phosphate. Absorbance was measured at 405nm using a BMG Optima spectrophotometer.

### Immunization and monitoring of polyclonal antibody response.

Purified HCV E2eΔHVR1 (1mg/mL, 250 µL) was mixed with 250 µL of Freund's complete adjuvant for the first immunization, and with 250µL of Freund's incomplete adjuvant for the following immunizations. In France, an ethical approval is not required for immunization of animals with Freund (in-)complete adjuvant. One young adult male alpaca (*Lama pacos*) was immunized at days 0, 21 and 35 with 250 µg immunogen. At day 50 a serum sample was taken and the immune response monitored by ELISA using HCV E2e full-length ectodomain as antigen. The alpaca was subsequently 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 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 (14) and DNA fragments encoding nanobody domains 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 $L_{HH}$ (14). The primers incorporated SfiI and NotI restriction sites at the ends of the nanobody genes allowing digestion of the PCR products followed by ligation into phage expression vector

pHEN1. The resulting library was composed of two sub-libraries, one derived from nanobody 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 for reactivity with HCV E2e antigen coated-immunotubes, as previously described ([15]). Phages ($10^{13}$ transducing units) were panned by incubating with the coated tubes for 1h at 37°C under gentle agitation. In a second panning HCV E2e was bound via its Strep-Tag to Strep-Tactin coated magnetic beads (MagStrep « type 2HC » beads, IBA). The library was incubated with coated beads for 1h at 37°C, with the blocking agent changing every round: 2% skimmed milk, Licor diluted 1:4, and 4% BSA, respectively. The HCV E2e concentration coated on beads decreased every round of panning (100 nM, 50nM and 10 nM, respectively). Phage clones were screened by standard ELISA procedures using a HRP/anti-M13 monoclonal antibody conjugate (GE Healthcare) for detection.

*Expression of nanobodies*

The coding sequence of the selected nanobodies was sub-cloned from plasmid pHEN1 into vector pET28a containing an 8-Histidine tag using *Nco*I and *Not*I restriction sites. Transformed *E. coli* Rosetta-Gami2 (DE3) cells expressed nanobodies in the cytoplasm after overnight induction with IPTG (0.2mM) at 16°C. Nanobodies were purified by IMAC from cytoplasmic extracts using a HiTrap crude column charged with $Ni^{2+}$ followed by size exclusion chromatography with a Superdex 75 column (GE Healthcare).

*HCVpp and HCVcc neutralization assays.*

HCVpp representing genetically diverse strains were generated as previously described ([7], [16]). Pseudoparticles were mixed with immune sera, defined concentrations of nanobodies, or mAbs for 1h and incubated with $1.5x10^4$ Huh-7 cells seeded in 96-well plates. 72h post-infection the cells were lyzed with cell lysis buffer (Promega) and luciferase activity measured with a BMG Fluostar Optima.

JFH-1 was produced as previously described ([17]). Neutralization assays were performed by mixing 100 focus-forming units (FFU) of virus with serum, nanobody, or monoclonal antibody for one hour before adding to Huh7.5 cells. Percentage infection was determined by comparison to the number of cells infected in the absence of inhibitors. Statistical significance was determined performing a one-way ANOVA with Bonferroni correction for multiple comparisons.

### Nanobody HCV E2e interactions

E2eΔHVR1 (50μg) 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) nanobody 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.

### Nanobody inhibition of HCV E2e-CD81 interaction

30μg of soluble E2 ectodomain of UKN2b_2.8 ([8]) was incubated with 30μg of CD81-LEL produced as described previously ([18]) and 60μg nanobody overnight at room temperature. Samples were separated on a Superdex 200™ PC 3.2/30 at 0.05ml/min for analytical size exclusion chromatography (SEC) or on a Superdex 200™ 10/300 GL at 0.5ml/min for preparative SEC. Peaks 1 and 2 were collected, concentrated separately by tangential flow (cutoff 5kD) and analysed by SDS-PAGE on a 4-20% gradient gel followed by Coomassie staining.

### Data collection, processing and structure solution

D03 rod-like crystals belonging to spacegroup $P6_5$ were flash-frozen in mother liquor containing 18% (v/v) Glycerol and diffracted to 1.76Å on a synchrotron source. The structure

was determined using the molecular replacement method. Spacegroups and cell dimensions, resolution limits, data collection details and refinement statistics are summarized in Table S1. Data were collected at the Swiss Light Source (PX I) and processed with XDS ([19](#)); scaling and reduction was performed using Pointless ([20](#)) and programs from the CCP4 suite ([21](#)). The crystal structure was determined by the molecular replacement method using Phaser ([22](#)) and a nanobody (PDB 3STB) as search model. Model building was performed using Coot ([23](#)) and refinement was done using AutoBuster ([24](#)). The atomic coordinates and structure factors for the crystal structure has been deposited in the Protein Data Bank, [www.pdb.org](#), under the accession number 4JVP.

### *Competition assay between nanobodies and anti-HCV E2 monoclonal antibodies*

Competition ELISAs were performed as described previously ([25](#)). Essentially, *Galanthus nivalis* agglutinin (GNA)-captured E1E2 proteins were probed with human or mouse monoclonal antibodies in the presence of D03. 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 percentage inhibition compared to an uninhibited control.

### *Nanobody reactivity to HCV E1E2 mutants.*

HEK293T cells were transfected with pcDNA3.1-based vectors expressing the E1E2 genes of the H77c molecular clone of HCV bearing individual alanine substitution point mutants ([26](#)). After 48h, transfected cells were trypsinized, harvested, seeded onto Teflon-coated microscope slides, air dried and fixed with acetone. Nanobodies were biotinylated using a N-hydroxysuccinimide-biotin ester (Pierce) and dialyzed into PBS. The biotinylated D03 was incubated with the E1E2-expressing cells, washed and binding was detected using a streptavidin Alexafluor 488 conjugate (Invitrogen), and analyzed by immunofluorescence microscopy. Binding was measured by integrating the total light output per field of view at

400X magnification, and subtracting the values obtained using untransfected cells as a control.

**Supplementary References**

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**Supplementary Figure Legends**

**Figure S1: Generation of alpaca heavy chain antibodies directed against HCV E2.**

(A) One alpaca was immunized and boosted with E2e lacking the HVR1 from patient isolate UKN2B2.8 (arrows). Serum samples (ellipses) were collected at day 50 to screen the humoral immune response by ELISA and at day 100 to generate a phage display library expressing nanobodies at the surface. The animal was boosted twice with homologous full-length ectodomain E2e (polygons) ([8]).

(B) The native conformation of the immunogen was evaluated by ELISA assay using human mAbs recognizing discrete conformation-sensitive epitopes (CBH2, CBH5, CBH7, A8, AR3A, AR3B, AR3C, 1:7, CBH4B, CBH4G and CBH8), and two well-characterized murine mAbs recognizing linear epitopes (AP33 and ALP98). 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 nanobodies. Cysteines are boxed, the disulfide connectivity is indicated below the alignment, bars show the positions of the CDRs, which are shaded in light grey, according to the IMGT nomenclature ([27]). Positions of somatic mutations as revealed by an alignment with the closest homologous germline sequence (IGHV1S1*01) suggested by IMGT V-QUEST and junction analysis ([27]) are marked by an asterisk below the sequence.

**Figure S2: Functional characterization of nanobodies.**

(A) E2eΔHVR1 was bound to a StrepTactin Superflow mini column and incubated with nanobody B11 (left panel) or D03 (right panel). Starting material (SM) of the nanobody, wash and elution fractions were analyzed by SDS-PAGE under non-reducing conditions followed by Coomassie Blue staining.

(B) Confocal images of biotinylated nanobody reactivity with HEK293T cells transfected to express HCV strain H77 E1E2 glycoproteins. Background fluorescence observed when binding each nanobody to mock transfected cells was subtracted from these images.

**Figure S3: Neutralization of HCV entry by immune sera and recombinant nanobodies.**

A) Dose-dependent neutralization of HCV H77 pseudoparticles was observed for sera obtained from day 50 following initial immunization (open circles) or day 100 (closed diamonds). No neutralizing activity was observed with a serum sample obtained from an alpaca immunized with the tetanus toxin (open diamonds). B) Neutralization of JFH-1 infectious particles in cell culture. At a dilution of 1/100, the neutralizing potency of serum from day 100 was greater than that of day 50 (Closed diamonds), and no neutralization was observed with the serum from a control animal immunized with tetanus toxin (open triangles). C) Neutralization of genetically diverse pseudoparticles with immune sera. Pre-immune serum (black bars), serum from day 50 (grey bars) and serum from day 100 (white bars) of the immunization schedule were assessed for the breadth of neutralization using patient-isolates in an HCV pseudoparticle entry assay. 1a: UKN1A20.8; 1b: UKN1B12.16; 2a: UKN2A1.2; 2b: UKN2B1.1; 3a: UKN3A13.6; 4: UKN4.11.1; 5: UKN5.15.7; 6: UKN6.5.8. The serum from day 100 consistently neutralized entry to the greatest degree, although some isolates, such as UKN1A20.8, remained resistant to neutralization by the immune sera. (D) Autologous neutralization of recombinant nanobodies was assessed using HCVpp of the immunogen strain UKN2B2.8. Each nanobody was incubated at the indicated concentration with HCVpp expressing UKN2B2.8 E1E2 and used to infect Huh7 cells. Dose-dependent neutralization was observed for D03 (closed squares) and C09, (closed triangles), while B11 (closed circles) and D04 (open triangles) had no effect. D03 was by far the most efficiently neutralizing nanobody, exceeding the neutralization observed for the human anti-E2 mAb 1:7 (closed diamonds) serving as a control. No neutralization was observed with the control anti-tetanus toxin nanobody (open circles).

**Figure S4: Inhibition of E2-CD81 interaction by nanobodies.**

(A) Linear representation of the epitopes on H77c E2 protein recognized by antibodies used in Fig. 4A. The regions involved in interaction with CD81, as well as the hypervariable regions and transmembrane region are highlighted. Linear epitopes recognized by antibodies used in competition assays are highlighted by sequences of amino acids. The contact residues involved in conformational epitopes are highlighted by colored text: red highlights the overlapping epitopes of mAbs AR3A and 1:7, while orange highlights the epitopes of mAbs AR1A and CBH7. The remaining conformation-sensitive epitopes (CBH4G, AR2A) have not been mapped yet.

 (B) A panel of well-characterized mAbs with epitopes covering several non-overlapping antigenic regions on HCV E2 were tested in competition assays with nanobody D03. Binding of a half-maximal concentration of each mAb was performed alone (100% binding), or in the presence of D03 at 10µgmL$^{-1}$. Statistical comparisons were performed using a one-way ANOVA with Bonferroni correction. * denotes $p<0.05$; ** $p<0.01$; *** $p<0.001$. Competition was observed with mAbs 1:7, AR1A, and AR3A, suggesting that the epitope recognized by D03 overlaps the CD81 binding site.

(C) Binary complexes (black line) of soluble UKN2b_2.8 E2 ectodomain (8) and nanobodies D03 (upper panel) or B11 (lower panel), respectively, and ternary complexes (light grey line) including CD81-LEL (18) were analyzed by size exclusion chromatography (SEC) in separate runs and the absorption at 280nm is shown. Both ligands were present in molar excess with respect to the HCV E2 ectodomain as indicated by the presence of peak 2. A shift in elution volume of peak 1 suggesting an increase in molecular weight of the complex upon addition of CD81-LEL was observed only for B11 (black arrows), but not for D03.

(D) Peaks 1 and 2 from SEC analysis of D03 and B11 samples containing E2 ectodomain, nanobody and CD81-LEL (both ligands in molar excess) were separated by SDS-PAGE followed by Coomassie staining. E2* represents a partially degraded E2 ectodomain. While B11 formed a ternary complex with both E2 and CD81-LEL, no CD81-LEL was found in the D03 complex, demonstrating cross-competition between D03 and CD81-LEL for E2 binding.