An alpaca nanobody inhibits hepatitis C virus entry and cell-to-cell transmission

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List of Abbreviations

HCV	- Hepatitis C virus
mAbs	- monoclonal antibodies
CDR	- complementarity determining region
JFH-1	- Japanese fulminant hepatitis isolate 1
HCVcc	- cell culture grown infectious particles
HCVpp	- HCV pseudoparticles
LT	- liver transplantation

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Abstract

Severe liver disease caused by chronic hepatitis C virus is the major indication for liver transplantation. Despite recent advances in antiviral therapy, drug toxicity and unwanted side-effects render effective treatment in liver-transplanted patients a challenging task. Virus-specific therapeutic antibodies are generally safe and well-tolerated, but their potential in preventing and treating HCV infection has not yet been realized due to a variety of issues, not least high production costs and virus variability. Heavy-chain antibodies (HCAbs) or nanobodies, produced by camelids, represent an exciting antiviral approach – they can target novel highly conserved epitopes that are inaccessible to normal antibodies and they are also easy to manipulate and produce. We isolated four distinct nanobodies from a phage-display library generated from an alpaca immunized with Hepatitis C virus E2 glycoprotein. One of them, nanobody D03, recognized a novel epitope overlapping with the epitopes of several broadly neutralizing human monoclonal antibodies. Its crystal structure revealed a long CDR3 folding over part of the framework that – in conventional antibodies - forms the interface between heavy and light chain. D03 neutralized a panel of retroviral particles pseudotyped with HCV glycoproteins from six genotypes and authentic cell-culture derived particles by interfering with E2-CD81 interaction. In contrast to some of the most broadly neutralizing human anti-E2 mAbs, D03 efficiently inhibited HCV cell-to-cell transmission. ***Conclusion:*** This is the first description of a potent and broadly neutralizing HCV-specific nanobody representing a significant advance that will lead to future development of novel entry inhibitors for the treatment and prevention of HCV infection and help our understanding of HCV cell-to-cell transmission.

An estimated 180 million people worldwide are infected with the hepatitis C virus (HCV). Chronic infection is frequent and often leads to progressive liver disease with chronic HCV infection being the leading indication for liver transplantation (1). HCV exhibits significant genetic diversity, and at least six major genotypes, which differ by up to 30% in their nucleotide sequence, have been identified (2). Within an infected individual the virus exists as a population of genetically related variants or quasispecies. This contributes to viral persistence by facilitating escape from anti-viral selection pressures (3), with significant implications for the design of anti-viral therapeutics and vaccines.

The standard treatment for chronic HCV infection is a combination of pegylated alpha interferon (IFN-α) and ribavirin. The recently-introduced protease inhibitors Boceprevir and Telaprevir show improved treatment outcomes for genotype 1 infections in combination with IFN-α and ribavirin for genotype 1 infections (4). However, therapy is limited by severe side effects and, dependent on the viral genotype, variable efficacy (5) and drug resistance(6).

Therapeutic administration of anti-HCV neutralizing antibodies may contribute to future combination therapies with protease and/or polymerase inhibitors. The HCV glycoproteins E1 and E2 are the major target for neutralizing antibodies and immunization studies have generated broadly reactive antibody responses (7). Neutralizing human monoclonal antibodies specific for HCV E2 have been shown to protect against heterologous virus challenge in a human liver-chimeric mouse model (8) and in chimpanzees (9). More recently a neutralizing human monoclonal antibody specific for HCV E2 was reported to delay viral rebound in patients following liver transplantation (10). Viral clearance during acute infection is associated with the presence of high titer neutralizing antibodies (11-13). However, reports that HCV can evade neutralizing antibodies by transmitting via cell-to-

cell contacts raised concerns on the efficacy of antibodies targeting the viral glycoproteins to limit viral transmission (14-16).

In members of the family *Camelidae* a large proportion of the humoral immune response is comprised of homodimeric IgG devoid of light chains (~80 kD) (17). These "heavy chain" antibodies also lack the C_H1 region and their variable region is referred to as VHH or nanobody. Recombinant nanobodies (~14 kD) are intact antigen-binding domains and exhibit a broad antigen-binding repertoire. They have unique characteristics, including an extended CDR3 loop that can adopt a protruding conformation allowing interaction with concave epitopes that are occluded for conventional antibodies (18). To stabilize the enlarged CDRs, nanobodies often possess an additional disulfide bond between CDR1 and CDR3 in dromedaries, and CDR2 and CDR3 in llamas (19). Nanobodies have been raised to numerous viruses (reviewed in (20)) and in spite of being monovalent they frequently exhibit biological activities comparable to conventional bivalent antibody molecules (21). As such, nanobodies are a promising tool for the targeted immunotherapy of viral infections.

Here we report the isolation and characterization of four anti-HCV alpaca nanobodies raised by immunizing an alpaca with recombinant hepatitis C virus E2 glycoprotein. One of these nanobodies neutralized HCVpp representing diverse genotypes, authentic HCVcc virions, and uniquely inhibited HCV cell-to-cell transmission. This provides the first evidence of nanobodies as potential candidates for immunotherapeutic administration in chronic hepatitis C.

Experimental procedures

HCVpp and HCVcc neutralization assays

HCVpp and 100 focus-forming units of JFH-1, respectively, were mixed with serum, nanobody, or monoclonal antibody for one hour before adding to Huh7.5 cells. Further experimental details are provided in SI Materials and Methods.

Nanobody crystallization, data collection and structure determination

Crystals of the nanobody D03 were grown at 293 K using the sitting-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 2005 mM LiSO4. Diffraction quality rod-like crystals belonging to spacegroup $P6_5$ appeared after weeks. Data collection, processing and structure solution are described in more detail in SI Materials and Methods.

Inhibition of HCV cell-to-cell transmission

Cell-to cell transmission was analysed as described before (22). HCV-infected producer Huh-7.5 cells were labeled with 5-Chloromethylfluorescein Diacetate (CMFDA) and co-cultured with naïve cells in a 1:1 ratio for 2 hours. Antibodies, antibody fragments or nanobodies were added, extracellular media was collected after 24h and infectious virus quantified by infecting naïve Huh-7.5 cells. Cell co-cultures were fixed, permeabilized, stained for HCV NS5A expression and analyzed by flow cytometry to quantify the number of newly infected target cells (NS5A+/CMFDA-). The frequency of target cells infected via the cell-to-cell transmission route in presence of antibody was compared to the polyclonal anti-HCV Ig control. Statistical significance of neutralization was determined by a one-way ANOVA with Bonferroni correction.

Results

Nanobodies neutralize HCV infectivity.

We used a phage-display library isolated from an alpaca immunized with HCV E2 to identify four nanobodies specifically recognizing E2 (Figure S1). The nanobodies were expressed in *E.coli* and antigen specificity demonstrated by pull-down and immunofluorescence assay (Figure S2). All four nanobodies were assessed for their ability to inhibit HCVpp and HCVcc infection. Determination of autologous neutralization of HCVpp bearing glycoproteins of the immunogen HCV isolate UKN2B2.8 revealed that D03 neutralized virus infection in a dose-dependent manner (>95% at 20µg mL^{-1}), while C09 possessed some neutralizing activity, and B11 and D04 had no effect on HCVpp infectivity (Figure S3d). Subsequent analysis using JFH-1 HCVcc revealed that D03 had the strongest neutralizing effect, while C09 had a minor inhibitory effect (Figure 1a). B11 and D04 did not show any neutralizing activity. Together these data demonstrated that D03 neutralizes the infectivity of HCVpp and HCVcc expressing glycoproteins of HCV genotype 2.

To assess the breadth of neutralizing activity, all four nanobodies were screened at a single concentration for their inhibitory effect on entry of pseudoparticles bearing a well-characterised and diverse panel of HCV glycoproteins that exhibited different sensitivities to serum neutralizing antibodies (23). Only D03 possessed significant cross-neutralizing activity; C09 only neutralized HCVpp pseudotyped with genotype 2 glycoproteins (Figure 1b). A more detailed analysis of the cross-reactive neutralization profile of D03 using a panel of HCVpp representing all six major HCV genotypes, showed that D03 neutralised across all genotypes, frequently exhibiting 50% inhibitory concentrations that ranged between 1-10 µgmL^{-1} for most isolates. Some isolates, such as UKN2A1.2 (genotype 2a) and UKN2B1.1 (genotype 2b), were more easily neutralized by D03 than by mAb 1:7 (used as positive control (24)). However, other strains such as UKN3A13.6 (genotype 3a) and UKN5.15.7 (genotype 5) were more refractory to neutralization by D03 and required significantly more nanobody to achieve 50% inhibition. These results indicated that the

epitope recognized by D03 is conserved across genetically diverse isolates, but presentation of the epitope at the virion surface may differ between strains.

Crystal structure of D03.

To gain insight into the conformation of its potential antigen binding determinants we crystallized D03 and determined its crystal structure to 1.8Å resolution; details and statistics of the data collection, processing and refinement are given in Table S1. As expected, the nanobody displayed an immunoglobulin fold (Figure 2a). The three complementarity determining regions (CDR1, CDR2 and CDR3) were 8, 8 and 20 residues long, respectively (according to the IMGT nomenclature, Figure S1c), the CDR3 thus being longer than the average CDR3 (25). It folds over part of the framework region, which - in conventional antibodies - forms the V_H-V_L interface. The flexibility of the extended CDR3 in D03 is restricted by a disulfide bridge (shown as yellow spheres in Figure 2a) between Cys_{50} directly upstream of the CDR2 (green, Figure 2a) and Cys_{103} in the CDR3 (red, Figure 2a).

Antibody maturation in nanobodies frequently includes somatic mutations that improve shape or charge complementarity of the paratope with the antigen (26). These mutations occur mainly in residues that are not involved in antigen contacts leading to reorganization of hydrogen bonding networks, electrostatic and van der Waals interactions, often resulting in increased affinity for antigen binding (27). Amino acid alignment of D03 with its closest homologous germline gene IGHV1S1*01 and mapping of the somatic mutations on the molecular surface revealed one, four and one somatic mutations in the CDR1, CDR2 and CDR3, respectively (Figure S1c, labeled as asterisks and Figure 2b, colored in pale yellow).

Epitope mapping of nanobody D03.

The majority of previously reported anti-HCV E2 broadly neutralizing antibodies inhibit E2 binding to the receptor CD81, their epitopes overlapping the CD81 binding site (reviewed

in (28)), which comprises mainly three discontinuous regions, aa412–425, aa428-446 and aa523-540. Remarkably, all human conformation-sensitive antibodies recognizing the latter region bind to four main contact residues (namely G523, W529, G530 or D535), and different combinations of at least two of these have been reported for individual antibodies.

The antigenic region binding D03 was identified by competition analysis of D03 with binding of a well-characterized panel of mAbs to HCV E2 (Figure S4b). D03 competed for binding of mAbs 1:7, AR3A and AR1A to HCV E2. These mAbs bind to epitopes localized in the CD81 binding region, suggesting that D03 also neutralizes HCV by interfering with E2-CD81 binding. This was further supported by the fact that no simultaneous binding of D03 and CD81-LEL to a soluble E2 ectodomain was detected, while the non-neutralizing nanobody B11 formed a ternary complex with E2 and CD81-LEL (Figure S4c-d).

We defined D03 contact residues in E2 by binding analysis of D03 to a panel of HCV E2 mutants carrying individual alanine substitutions of conserved residues between aa 412 and 621 (Figure 3a). D03 binding was reduced by more than 50% by substitutions at residues N415, G523 and T526, in line with an epitope overlapping with the CD81 binding site (Figure 3).

D03 inhibits HCV cell-to-cell transmission.

We and others have reported that cell-to-cell spread of HCV is resistant to several broadly neutralizing anti-E2 antibodies targeting the CD81 binding site, limiting their potential therapeutic capacity (14-16). The mechanism utilized by HCV for cell-to-cell spread is unknown, and as such antibody resistance is not fully understood. We investigated the ability of D03 to limit cell-to-cell virus transmission using a recently described single-cycle infectious co-culture assay (22). D03 neutralized more than 95% of the extracellular virus and inhibited cell-to-cell transmitted events (Figure 4).

HCV cell-to-cell transmission may be susceptible to the relatively small nanobody (MW ~ 15kD), but not to full IgG molecules (MW ~ 150kD). This would be in line with reports that the small molecules epigallocatechin-3-gallate (29) and El-1 (30) inhibit cell-to-cell

transmission. Alternatively, neutralizing activity of D03 on cell-to-cell spread could be attributed to a specific binding mode of the nanobody to the novel epitope described above. We expressed recombinant antibody fragments of the broadly neutralizing human antibody A8 (24) in Drosophila S2 cells (31, 32). A8 binds to an epitope defined by contact residues G523, W529, G530 and D535 (24) overlapping the novel epitope recognized by D03. Despite neutralizing >95% of extracellular JFH-1 virus, neither A8 IgG nor any of the recombinant fragments (Fab or scFv; MW ~50kD and ~30kD, respectively; Figure 4) reduced cell-to-cell transmission. Whilst these results suggest that epitope specificity rather than size *per se* underpins the inhibition of HCV cell-to-cell transmission, it is worth noting that the nanobody (MW ~15kD) is still only half the size of the scFv.

Discussion

End-stage liver disease because of HCV infection is the leading indication of liver transplantation (LT), and reinfection of the graft occurs universally (33). It is unlikely that the new direct-acting antiviral (DAA) agents, such as telaprevir and boceprevir, will prove effective in the liver transplant setting as these drugs have been shown to interfere with commonly used immunosuppressive drugs (34). Moreover combination therapy targeting multiple steps of the virus cycle is likely to be needed to limit the emergence of drug resistance. With their relatively safe profile, nanobodies have therapeutic potential in the particularly sensitive group of LT patients and as part of a combination therapy for the wider treatment of chronic HCV infection. Here we undertook the novel approach to generate HCV-specific nanobodies from the heavy chain antibody (HcAb) repertoire of an alpaca. Nanobodies combine the advantages of high affinity binding, protein stability and an ability to bind epitopes that are not recognized by conventional IgG molecules (20). Characterisation of the selected nanobodies revealed that D03 recognises a novel conserved E2 epitope. This nanobody can inhibit cell-free virus and cell-to-cell transmission, that was

previously reported to be resistant to broadly neutralizing antibodies and patient polyclonal Ig (14).

To maximise the likelihood of inducing cross-reactive nanobodies we immunized with a truncated form of E2 (35) lacking HVR1 – the main target for strain-specific neutralizing epitopes (reviewed in (28)). Crucially we assessed neutralisation breadth using HCVcc and also HCVpp supplemented with E1E2 that are resistant to antibody neutralisation (23).

Epitope mapping showed possible contact residues recognised by D03 included N415, G523 and T526. N415 forms part of the epitope by broadly neutralizing mAbs AP33 and HCV1 (36, 37). G523 has been identified as contact residue for conformation sensitive antibodies AR3A, 1:7, A8, CBH-5 and to a lesser extent also HC-1 and HC-11 ((38), reviewed in (28)), all of which interact with at least one additional residue among W529, G530 and D535. While D03 also interacts with G523, it does not depend on W529, G530 or D535 as contact residues. Instead, T526 acts as additional contact, a conserved residue that had not been implicated in a neutralizing epitope before. These data suggest that the epitope recognized by D03 overlaps with epitopes utilized by CD81 binding site-specific broadly neutralizing human antibodies, but clearly possesses a novel pattern of contact residues.

Structural analysis of D03 revealed clustering of somatic mutations at the tip of D03 suggesting that the interaction between nanobody and HCV E2 occurs likely in close proximity to CDR1 and CDR2. This indicates that the additional disulfide bridge between the framework upstream of CDR2 and the CDR3 serves to restrict the latter in a conformation that allows maximal accessibility of the tip region of the nanobody. Both neutralizing nanobodies (D03 and C09) possessed this additional disulfide bridge (Figure S1c), whereas both non-neutralizing nanobodies lacked this linkage. It is therefore tempting to speculate that the spatial restriction of the CDR3 contributes to a specific binding mode of the neutralizing nanobodies. Of note, it has been shown that binding of E2 to CD81 is

modulated by N-linked sugars forming a glycan shield (39), which is likely in close proximity of the epitope recognized by D03. This glycan shield could interfere with binding of conventional antibodies, but allow interaction with the tip of heavy-chain antibodies, the diameter of which is smaller due to the absence of light chain. However, structural analysis of D03 in complex with its antigen is required to understand the particularities of this binding mode.

This study is the first description of a nanobody that neutralizes both cell-free virus and cell-to-cell transmission of HCV, which was previously reported to be resistant to patient polyclonal Ig and broadly neutralizing antibodies targeting the CD81 binding site (14). As such, this entry inhibitor has a potentially unique application to limit HCV spread in the chronically infected liver and represents a significant advance towards therapeutic administration of antibodies to prevent 'neutralization resistant' infection of hepatocytes. The inhibitory effect of D03 on HCV cell-to-cell transmission demonstrates that the E2 glycoprotein at the surface of transmitted virions is exposed to extracellular antibodies. This suggests that the virus may be transmitting in protected pockets between cells, rather than directly forming synaptic or cellular contacts as observed for HIV-1 (40), as the latter are likely inaccessible to all extracellular antibodies.

While several nanobodies have entered clinical trials in the last few years (20), to our knowledge this study provides the first evidence that nanobodies can prevent both cell-free and direct cell-to-cell transmission of a virus, highlighting their potential to be clinically useful entry inhibitors.

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Figure legends:

Figure 1: Neutralization activity of nanobodies

(A-C) Neutralization assays were performed in triplicate and mean values calculated with ± SEM. Statistical comparisons were performed at 20 μg/ml (C) or 10 μg/ml (A+B), performing a one-way ANOVA with Bonferroni correction. n.s.=non significant; * denotes $p<0.05$; ** $p<0.01$; *** $p<0.001$ and **** $p<0.0001$. (A) The neutralizing ability of each nanobody ($10\mu gmL^{-1}$) was determined using 100 focus-forming units of JFH-1 particles before inoculating Huh7.5 target cells. Neutralization was compared to control neutralizing mAb 1:7, and an irrelevant anti-tetanus toxin nanobody. B11 and D04 had little effect on JFH-1 infectivity. C09 showed limited inhibition, while D03 possessed greatest neutralizing activity. (B) Neutralization of heterologous HCVpp representing genotypes 1-3. Nanobodies were incubated at 10μgmL-1 with each HCVpp preparation before adding to Huh7 cells. D03 possessed the greatest cross-neutralizing activity. Neutralization by C09 was limited to genotype 2-derived pseudoparticles. B11 and D04 had no effect on entry of HCVpp of any genotype. Neutralizing activity of D03 was greatest for samples representing genotypes 1b (UKN 1B12.16), 2a (UKN2A1.2) and 2b (UKN2B1.1), while 1a (H77, UKN1A20.8) and 3a (UKN3A13.6) were more resistant to neutralization. (C) Dose-dependent neutralization of HCV primary isolates was assessed for D03 (closed circles), using an anti-tetanus toxin nanobody (open triangles) and mAb 1:7 (closed triangles) as controls. Neutralization potency depended on the isolate; many isolates were neutralized comparably by both D03 and mAb 1:7. UKN2A1.2 and UKN2B1.1 were more easily neutralized by D03, while UKN5.15.7 was more efficiently neutralized by mAb 1:7.

Figure 2: Crystal structure of nanobody D03

Crystal structure of D03 viewed from the side (A). Framework regions are colored in blue and the complementarity determining regions (CDRs) are colored in brown (CDR1), green (CDR2) and red (CDR3). The sidechains of the residues in the CDRs are shown as sticks and yellow spheres represent the disulfide bridges. (B) Mapping of the somatic mutations

(pale yellow) on the molecular surface of the nanobody D03, colored as in (A), viewed from the side.

Figure 3: Mapping of the epitope recognized by D03

(A) Binding of nanobody D03 to HCV E2 (wt E2) and a panel of alanine substitution mutants was assessed by immunofluorescence on HEK293T cells expressing each individual mutant. Substitution of N415, G523 and T526 resulted in greater than 50% reduction in binding of D03. (B) Alignment of the amino acid sequences of primary HCV isolates used in neutralization and binding assays revealed that residues in E2 important to binding of D03 (boxed) were broadly conserved. Asterisks highlight residues involved in interaction with the cellular receptor CD81, and the contact residues for mAbs 1:7 and A8 are labelled with triangles.

Figure 4: D03 inhibits cell-to-cell transmission of HCV

Transmission between HCV JFH-1 infected Huh-7.5 cells and naïve Huh-7.5 cells in the presence of inhibitors was assessed by flow cytometry. Extracellular virus after 24h incubation was quantified by end-point titration, 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. D03 demonstrated dose-dependent inhibition of direct infection. Data represent the mean values of four independent experiments performed in triplicate, ± SEM. Significant neutralization by D03 was observed at 100 μg/ml, as determined by a one-way ANOVA with Bonferroni correction. **** $p<0.0001$.

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