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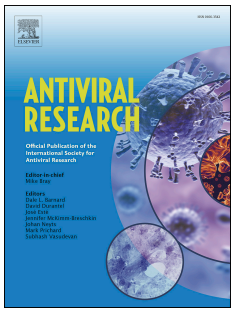

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Immunization with a synthetic consensus Hepatitis C Virus E2 glycoprotein ectodomain elicits virus-neutralizing antibodies

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Abstract

Global eradication of hepatitis C virus (HCV) infection will require an efficacious vaccine capable of eliciting protective immunity against genetically diverse HCV strains. Natural

spontaneous resolution of HCV infection is associated with production of broadly-neutralizing antibodies targeting the HCV glycoproteins E1 and E2. As such, production of cross-neutralizing antibodies is an important endpoint for experimental vaccine trials. Varying success generating cross-neutralizing antibodies has been achieved with immunogens derived from naturally-occurring HCV strains. In this study the challenge of minimising the genetic diversity between the vaccine strain and circulating HCV isolates was addressed. Two novel synthetic E2 glycoprotein immunogens (NotC1 and NotC2) were derived from consensus nucleotide sequences deduced from samples of circulating genotype 1 HCV strains. These two synthetic sequences differed in their relative positions in the overall genotype 1a/1b phylogeny. Expression of these constructs in *Drosophila melanogaster* S2 cells resulted in high yields of correctly-folded, monomeric E2 protein, which were recognised by broadly neutralizing monoclonal antibodies. Immunization of guinea pigs with either of these consensus immunogens, or a comparable protein representing a circulating genotype 1a strain resulted in high titres of cross-reactive anti-E2 antibodies. All immunogens generated antibodies capable of neutralizing the H77 strain, but NotC1 elicited antibodies that more potently neutralized virus entry. These vaccine-induced antibodies neutralized some viruses representing genotype 1, but not strains representing genotype 2 or genotype 3. Thus, while this approach to vaccine design resulted in correctly folded, immunogenic protein, cross-neutralizing epitopes were not preferentially targeted by the host immune response generated by this immunogen. Greater immunofocussing by vaccines to common epitopes is necessary to successfully elicit broadly neutralizing antibodies.

1. *Introduction*

The hepatitis C virus (HCV) infects approximately 3 million individuals each year (Gower et al., 2014). Many will go on to develop chronic liver disease, cirrhosis or liver cancer (Gerlach et al., 2003; Saito et al., 1990). HCV therapies have rapidly advanced and the newest combinations of directly acting antivirals (DAAs) achieve sustained clearance in more than 90% of recipients. However, it is unlikely that HCV will be eradicated through the use of anti-viral therapy alone. Some DAA regimens are associated with severe side-effects some patient groups cannot tolerate these therapies. The development of an effective vaccine to prevent HCV infection remains a priority.

The potential for spontaneous clearance of HCV infection in humans and experimentally infected chimpanzees highlights that vaccine-induced protective immunity is a realistic goal. (Bassett et al., 2001; Mehta et al., 2002; Nattermann et al., 2005; Weiner et al., 2001). Glycoprotein-specific antibodies are identified in individuals who spontaneously resolve infection, and infection in animal models is inhibited by acute-phase plasma, indicating a protective role for neutralizing antibodies (NAbs) (Dowd et al., 2009; Lavillette et al., 2005a; Lawitz et al., 2013; Osburn et al., 2014; Pestka et al., 2007; Saito et al., 1990; Walker and Grakoui, 2015).

Producing vaccines that elicit HCV-neutralizing antibodies is challenging. Immunisation of rodents and humans with full length E1E2 or soluble forms of E2 elicits NAbs, but these have limited cross-reactivity (Nattermann et al., 2005; Osburn et al., 2014; Thimme et al., 2001).

HCV exhibits a high degree of genetic plasticity within the E1 and E2 genes (Lavillette et al., 2005b), facilitating evasion of antibody responses. Major neutralizing epitopes are located in regions that can rapidly adapt to host immunity, leading to escape (reviewed in (Sautto et al., 2013)) or in regions that exhibit structural variability (Kong et al., 2016; Li et al., 2015; Meola et al., 2015). Individual isolates vary in their neutralization sensitivity (Bailey et al., 2015; Tarr et al., 2011) and it will be necessary for a vaccine to elicit antibodies able to neutralise the vast majority of circulating strains. Crucially, the early antibody response in individuals who resolve acute infection develop antibodies that target more conserved and therefore broadly neutralizing epitopes (Dowd et al., 2009)Pestka et al., 2007).

Previous studies have demonstrated that immunization with subunits of glycoprotein E2 induce neutralizing antibodies. Immunization with the soluble E2 ectodomain in guinea pigs elicited a potent autologous neutralizing response, although the resulting sera only poorly neutralized heterologous genotypes (Stamataki et al., 2007). Immunization with the E2 ectodomain (strain Con1; aa384-661) expressed in *Drosophila melanogaster* S2 cells is immunogenic and elicits broadly-neutralizing antibodies (Li et al., 2016). A significant challenge is to develop immunogens that steer this response to conserved broadly neutralizing epitopes. One approach to achieve this is to use protein subunits that have been manipulated to silence or remove variable regions whilst maintaining conserved conformation-dependent epitopes (Tarr et al., 2013; Vietheer et al., 2016). A soluble E2 construct lacking three hypervariable regions has been previously generated (McCaffrey et al., 2007). The monomeric form of this protein does not elicit a potent neutralizing antibody response, but high-molecular weight aggregates were recently found to be highly immunogenic and elicit neutralizing antibodies in guinea pigs (Vietheer et al., 2016). Removal of the HVR1 region

exposes broadly-conserved neutralization epitopes overlapping the CD81 binding site (Bankwitz et al., 2010). The present study aimed to improve cross-reactivity of the antibody response generated by immunization with monomeric E2, using immunogens representing consensus sequences of circulating genotype 1 HCV strains. This approach generated synthetic E1/E2 envelope glycoprotein constructs that have equal genetic distance from circulating genotype 1a viruses, mimicking possible ancestral HCV sequences.

2 Materials and methods

2.1 Consensus E1E2 sequences for generating immunogens

Synthetic consensus constructs of the HCV E1/E2 genes (nt 849-2580 referenced to Genbank AF009606) were created from 720 HCV genotype 1 strains (Los Alamos HCV database). See Supplementary Information for detailed methods.

2.2 Soluble E2 ectodomain immunogen constructs

A truncated E2 glycoprotein construct was generated with deletions of the HVR1 (aa384-409) and the C-terminus (aa645-746) of UKNP1.4.1 (UKNP1.4.1$_{410\text{-}644}$) (Genbank **KU285161**). This construct was amplified from an existing E1E2 clone (Urbanowicz et al., 2015). Similarly, the truncated constructs NotC1$_{410\text{-}644}$ and NotC2$_{410\text{-}644}$ were generated using the synthetic constructs NotC1 or NotC2 as template. The N-terminus of these constructs were truncated to Asn$_{410}$ to remove the HVR1 region, exposing epitopes overlapping the CD81 binding site. The constructs were truncated at amino acid Cys$_{644}$ to remove an unpaired cysteine at amino acid 652 that forms intermolecular disulphide bonds (Whidby et al., 2009) . The 3’ enterokinase/GlySer linker/one-STrEP-tag (IBA) was introduced by polymerase

cycling assembly (PCA). The final construct was generated by fusion PCR using the core structure and enterokinase/glyser linker/one-STrEP-tag as template in equimolar concentrations.The truncated sE2 constructs were cloned into the pMT vector [derived from plasmid pMT/BiP/V5-His].

2.3 Stable transfection of S2 cells and sE2 expression. *D. melanogaster* S2 cells were stably co-transfected with pMT plasmids containing the sE2 genes and pCoBlast (Invitrogen), using FuGene HD transfection reagent (Johansson et al., 2007a). Strep-tagged soluble E2 constructs expressed in S2 cells were purified from cell culture supernatants using a 5ml Strep-Tactin Superflow column (IBA). The flow-through, wash and eluate were collected in 1 mL fractions and analysed by western blot analysis and ELISA. Size exclusion chromatography was performed for the peak fractions using a HiLoad 16/600 Superdex 200 PG column (GE Healthcare Life Sciences) and phosphate buffered saline pH 7.2. One millilitre fractions containing monomeric protein were collected and analysed.

2.4 Detection of E2 protein.

Reactivity of different monoclonal antibodies to the purified E2 constructs was determined by GNA-capture ELISA as previously described using anti-E2 mAbs AP33 (Owsianka et al., 2005), 1:7 (Johansson et al., 2007b) or AR1A (Law et al., 2008)). GNA was coated on Nunc Maxisorp assay plates at 5μg.mL^{-1}. E2 proteins were used at a concentration of 5μg.mL^{-1}, and detecting antibodies were used at 1μg.mL^{-1}.

2.5 Guinea pig (GP) immunization. Three protein constructs were used to immunize guinea pigs (five per immunogen; Covalab, France) (Figure 3). Proteins NotC1$_{410\text{-}644}$, NotC2$_{410\text{-}644}$

and UKNP1.4.1$_{410-644}$ were prepared in PBS (pH 7.2). Animals received 500μg protein four times at 21 day intervals (Figure 3B). See Supplementary Information for further details.

2.6 Neutralization of HCV pseudoparticles (HCVpp) by GP sera.

The full-length NotC1 sequence, including the E1 signal peptide (NotC1$_{170-746}$) was cloned into pcDNA3.1 V5-His D-TOPO (Invitrogen) and used for production of HCV pseudoparticles (HCVpp). HCVpp were prepared by co-transfection of plasmids expressing packaging constructs, a luciferase reporter and the full-length E1E2 (either NotC1, JFH-1 (**AB047639**), UKNP1.4.1, UKNP2.1.1 (**KU285209**) , UKNP2.4.1 (**KU285213**), UKNP3.2.1 (**KU285218**) or H77c (**AF009606**)), as previously described (Tarr et al., 2007). Pseudoparticles were used to infect Huh7 cells. Neutralization of HCVpp entry by GP sera was assessed at a dilution of 1:100, comparing inhibition achieved with post-immunisation sera (D74) to a matched pre-immune sera for each animal (D0). Normal human serum (Sigma-Aldrich) was used as an additional negative control. Serial dilutions of monoclonal antibodies (AP33, 1:7, L1, AR1A, or AR2A) were also used to neutralize HCVpp entry as previously described (Urbanowicz et al., 2015).

3 Results

3.1 Generation of synthetic consensus E2 constructs Nucleotide sequences representing two alternative HCV E1E2 synthetic consensuses were generated by comparison of 720 genotype 1 circulating HCV strains. Both NotC1 and NotC2 were generated using the LANL Consensus Maker tool (hcv.lanl.gov; (Kuiken et al., 2008)). The computationally-derived nucleotide sequences were then added to selected members of original sequence set and a

new maximum likelihood tree was reconstructed, including the E1E2 genes of a previously-described genotype 1a synthetic sequence Bole1a (Munshaw et al., 2012). The clustering patterns demonstrated that these new samples are accurate representations of possible ancestral sequences of genotype 1a, and present a more basal sequence than Bole1a (Figure 1A). NotC1 was generated by making consensus sequences from groups of consensuses (supplementary methods). This approach resulted in a sequence that was more representative of genotype 1a than genotype 1b. NotC2 aligned as a more common ancestor of both genotype 1a and genotype 1b. Comparison of the amino acid sequences of these constructs revealed differences spread across the E2 ectodomain (Figure 1B). Importantly, all cysteine residues were conserved in these samples, as were the conserved N-linked glycosylation sites.

3.2 Expression of monomeric E2 protein representing a genotype 1 consensus sequence

$E2_{410\text{-}644}$ constructs were expressed using a Drosophila expression system (DES) and purified by Strep-tag purification and size exclusion chromatography. Soluble E2 $NotC1_{410\text{-}644}$, $NotC2_{410\text{-}644}$ and $UKNP1.4.1_{410\text{-}644}$ were resolved by western blotting (Figure 2A) and Coomassie Brilliant Blue staining (Figure 2B). The consensus constructs NotC1 and NotC2 were found to produce a mainly monomeric protein. although multiple bands for each sE2 were observed, possibly representing differently-glycosylated forms of these proteins, as previously described (Cocquerel et al., 2001). The conformation of the purified sE2 proteins was interrogated by binding of two monoclonal antibodies (Figure 2C). mAb 1:7 is a broadly reactive antibody that targets a conformational epitope on E2 overlapping the CD81 binding site (Johansson et al., 2007b), while mAb AR1A recognises a discrete conformation-sensitive epitope (Law et al., 2008). All constructs bound both 1:7 and AR1A, indicating that these sE2 constructs were correctly folded. Their ability to bind to cell surfaces and inhibit entry of

HCV pseudoviruses was also assessed (Figure 2D). NotC1 inhibited entry of all three HCVpp strains tested in a dose-dependent manner. The IC_{50} for NotC1was 0.16µg/mL (HCVpp UKNP1.4.1), 1.7µg/mL (HCVpp UKNP2.1.1) and 1.1µg/mL (HCVpp JFH1). However, minimal neutralization was observed when proteins representing either NotC2 or UKNP1.4.1 proteins were used, with approximately 50% inhibition of entry at the highest concentration tested. Unexpectedly, the presence of the UKNP1.4.1 at low concentrations appeared to have a marginal enhancing effect on entry of the three HCVpp preparations. Overall, this demonstrated that, despite having similar overall conformations, interaction of the NotC1 protein with host cells blocked virus binding the most.

3.3 Immunization with purified E2 ectodomain proteins elicits cross-reactive anti-E2 antibodies

Following immunization of guinea pigs with NotC1, NotC2 or UKNP1.4.1 $E2_{410-644}$ proteins (Figure 3A and B), autologous antibody reactivity in serum samples were measured by ELISA (Figure 3C). Pre-vaccination samples showed minimal reactivity to the immunogen. Sera taken at Day 53 or Day 74, following three/four immunizations, displayed high titres of antibodies with reactivity detectable at a 1:156,250 dilution. No increase in antibody titres occurred beyond day 53. The antibody titres achieved in each of the five animals in each group were broadly consistent. Maximal reactivity in animals immunized with NotC1 was less than that achieved with E2 representing NotC2 or UKNP1.4.1. To determine the proportion of the antibody reactivity directed to linear or conformational epitopes, binding was performed with either native or denatured target E2 proteins. Sera from each of the three guinea pig groups were pooled and diluted 1:30,000. Reactivity to each of the three

immunogens was assessed using time point D0 and D74. Sera were tested for reactivity against autologous and heterologous immunogens to assess cross reactivity (Figure 3D). Denaturation resulted in only minimal decrease in binding from native to denatured, indicating that the majority of the antibody response was directed at linear epitopes, and that these epitopes were conserved between the different proteins. Greatest reactivity was observed for the autologous immunogen for all vaccinations, although these differences were small. Pooled sera taken at D0 and D74 from guinea pigs immunised with NotC1 were also used to probe cell-expressed E1E2 by immunofluorescent microscopy. Antibodies cross-reacted to patient-derived E1E2 clones UKNP1.4.1, UKNP2.1.1, UKNP2.4.1 and UKNP3.2.1 (Figure 3E). However, this signal was not as strong as that observed with control monoclonal antibody AP33.

3.4 Consensus E1E2 NotC1 is functional in the HCV pseudovirus entry model

To assess autologous neutralization potency of antibodies generated following immunization, a full-length E1E2 (aa192-746) synthetic construct was created using the same approach as for the consensus sequence NotC1. This clone possessed an identical E2 ectodomain to the immunogen. HCVpp corresponding to NotC1 were infectious (Figure 4A). A similar construct corresponding to NotC2 was not infectious in this model (data not shown). Further characterisation of the NotC1 HCVpp in a neutralization assay was performed using broadly neutralizing monoclonal antibodies (AP33, 1:7 and AR3A) and mAbs with restricted neutralization profiles (AR2A and L1) (Law et al., 2008; Urbanowicz et al., 2015). The NotC1 construct was susceptible to neutralization by AP33 (Figure 4B), 1:7 (Figure 4C) AR2A (Figure 4D) and AR3A (Figure 4E), in a dose-dependent manner. The AR2A neutralization curve for the NotC1 sequence was similar to H77 suggesting it possesses an

intermediate epitope capable of eliciting an increased breadth of neutralization. Neutralization of the constructs in the presence of L1 was less potent (Figure 4F) and no neutralization was observed with immunoglobulins obtained from healthy HCV-negative donors (not shown).

3.5 Breadth of neutralization of vaccine-induced sera. The neutralizing potency of antibodies generated by immunization was determined by testing heat-inactivated guinea pig sera in an HCVpp entry assay. The reference strain H77c, the infectious NotC1 sequence and a panel of six HCVpp possessing patient-derived E1/E2 were tested. These clones represented three of the major HCV genotypes (Figure 5A), and displayed a range of neutralization resistance phenotypes (Urbanowicz et al., 2015). Sera sampled at day 0 (D0; before immunization) and day 74 (D74) from animals immunized with NotC1, NotC2 or UKNP1.4.1 were used at a dilution of 1:100 in neutralization assays (Figure 5B). Interestingly, some D0 sera demonstrated potent neutralization of HCVpp entry. The level of inhibition was dependent on the strain of HCV glycoprotein incorporated into particles, with clones UKNP2.1.1 and UKNP2.4.1 inhibited ≥50% compared to uninhibited controls. To determine if background neutralization could be eliminated by titrating sera, neutralization was performed with HCVpp possessing the glycoproteins of UKNP2.1.1 and UKN2.4.1 at serum dilutions of 1:50, 1:100, 1:200 and 1:400. In each case, specific vaccine-induced neutralization could not be resolved. This neutralization effect with pre-immune sera was also observed when using HCVcc representing UKN2.4.1 and reference strain J6 (Figure 5D). Despite this non-specific neutralizing effect, comparison of the neutralizing effect of D74 sera with matched D0 sample revealed that serum from guinea pigs that received the immunogen NotC1 significantly inhibited entry of the H77 and NotC1 pseudoviruses ($p<0.001$). However, this was not observed with the other guinea pig groups. The animals

that received UKNP1.4.1 vaccine generated autologous neutralizing antibodies, but only cross-neutralized the H77c strain. Consistent with this, animals that received the NotC2 immunogen produced antibodies that only neutralized H77c. Thus, despite cross-reactive antibodies being elicited by these vaccine constructs, broadly cross-neutralizing antibodies were not generated by immunization with the consensus immunogens.

4 Discussion

HCV genetic diversity poses a major challenge to the development of an effective vaccine. Selection of HCV immunogens has focused on existing, well-characterized strains, such as the genotype 1a strain H77 (Reyes-del Valle et al., 2012) or HCV1 (Stamataki et al., 2007). However, the genetic diversity between strains within a genotype can be 20–25% of their amino acid sequence (Simmonds et al., 2005). By comparison, as little as 2% amino acid difference can cause a failure in cross-reactivity of the polyclonal response to influenza vaccine (Gaschen et al., 2002). An effective way to minimize the degree of sequence dissimilarity between a vaccine strain and contemporary circulating viruses is to create artificial sequences that share key neutralization epitopes, using a consensus sequence based on the most common amino acid in each position in an alignment (Gao et al., 2005; Gupte and Arankalle, 2012; Leng et al., 2009; Liao et al., 2006). This study generated synthetic functional E2 constructs that have increased sequence similarity to improve the generation of broadly neutralizing antibodies than an E2 from a single HCV strain. Analysis of computationally derived nucleotide sequences (NotC1 and NotC2) revealed approximately 5-13% difference on an amino acid level between these consensus sequences and circulating strains of genotype 1a, and approximately 17-20% difference from genotype 1b. While this represents a reduction in distance between circulating genotype 1a and 1b strains, the amino

acid difference between other genotypes is still greater than 30% in the E1E2 genes, and as such still presents a significant challenge to generating cross-neutralizing antibodies. While further averaging of amino acid diversity using different genotype E2-coding regions might further broaden the antibodies generated by immunization, the length polymorphisms between genotypes presents a barrier to effective prediction of a consensus of these genotypes.

The immunization strategy employed in this study is developed from previous studies (Stamataki et al., 2007), using truncated, minimally diverse E2 constructs ($NotC1_{410-644}$ and $NotC2_{410-644}$), in order elicit a broadly neutralizing antibody response. Immunization was performed using purified monomeric forms of the E2 ectodomain, as these were believed to be the correctly folded version of the protein. Aggregated forms of the E2 protein do not interact with CD81 (Roccasecca et al., 2003; Tarr et al., 2011) and may elicit antibodies with limited neutralizing potency and breadth. However, when the immunogenicity of aggregate forms was directly compared to that of monomeric E2 expressed in mammalian cells, the aggregate form resulted in much broader neutralizing serum antibody responses in immunized animals (Vietheer et al., 2016). It is plausible that conserved neutralization epitopes are created on the surface of these aggregate forms of the E2 ectodomain, similar to that identified for stabilised trimers of the HIV-1 envelope glycoproteins (de Taeye et al., 2015; Julien et al., 2015).

The E2 glycoprotein requires significant post-translational modification to ensure a conformationally-correct structure including glycosylation and the formation of disulphide bridges (Dubuisson et al., 2002; Goffard and Dubuisson, 2003; Lavie et al., 2007; Patel et al.,

2001). The correctly-folded, truncated, reduced diversity constructs ($NotC1_{410-644}$ and $NotC2_{410-644}$) were expressed as monomeric protein in *D. melanogaster* S2 cells. Both of the consensus constructs interacted with conformation-sensitive antibodies. However, the antibodies produced following guinea pig immunization were almost exclusively directed to linear epitopes. The E2 protein is known to possess regions of structural instability, particularly in the regions aa412-423 (reviewed in (Tarr et al., 2015)) and aa532-540 (Vasiliauskaite et al., 2017), and as such the flexible nature of the recombinant protein (outside the context of a virion) might result in generation of antibodies directed to linear epitopes rather than those in correct conformation. It is also possible that the adjuvant used to enhance immune response to E2 promotes the generation of such antibodies (Kenney et al., 1989). Previous studies of HCV glycoprotein immunogens have used ISOMATRIX (Vietheer et al., 2016), FCA (Reyes-del Valle et al., 2012) and MF59-0 citrate (Stamataki et al., 2007) in animal studies, and MF59-1 in humans (Frey et al., 2010). Direct comparison between Addavax and FCA adjuvants in immunized goats found that FCA enhanced the production of antibodies targeted to epitopes overlapping the CD81 binding site (Wong et al., 2014), increasing the neutralizing potency of the immune sera. As a successful vaccine for HCV is likely to need to elicit conformation-sensitive antibodies, a systematic comparison of different clinically approved adjuvants is required to determine their effect on the ability to induce these antibodies. The antibodies generated were mainly directed to linear epitopes conserved between different HCV strains, as demonstrated by reactivity to diverse E1E2 glycoproteins. Despite this, the antibodies did not neutralize genetically diverse viruses. Cross neutralization by antibodies generated by NotC1 was limited to the autologous HCVpp, UKNP1.4.1 and the H77 strain and did not extend to the genotype 1a strain UKNP1.4.1. This is consistent with our previous observation that HCV cannot be categorised into clear

neutralization serotypes based on genetic similarity (Tarr et al., 2011). Subtle differences in protein sequence and fold might account for the differential ability to induce neutralizing antibodies to key epitopes. In contrast, the consensus sequence NotC2 inhibited entry of pseudoviruses to a much lower level than NotC1 and was non-infectious in the HCVpp model. This difference might be due to amino acid substitutions occurring in the region aa436-a447, which has previously been shown to affect receptor interactions. The W437L substitution in NotC2 has previously been demonstrated to reduce E2 interaction with CD81 by 80-90% and reduce infectivity of HCVpp, and mutation F442L observed in UKNP1.4.1 reduced CD81 interaction and infectivity by around 50% (Drummer et al., 2006). Interestingly, the addition of lower concentrations of the sE2 representing UKNP1.4.1 resulted in marginal enhancement of infectivity. While this enhancement may be within the experimental error of the assays used, it is plausible that binding of sub-neutralizing concentrations of soluble E2 protein could enhance colocalization of receptor complexes, which has previously been demonstrated enhance infectivity of HCV (Harris et al., 2010; Harris et al., 2008).

The observed neutralization of virus entry in both HCVpp and HCVcc models by pre-immune sera in the absence of specific antibodies was unexpected. Previous immunization studies using HCV glycoproteins (Stamataki et al., 2007; Vietheer et al., 2016) did not investigate the neutralizing potential of pre-immune serum in experimental animals. The evidence presented in Figure 5 indicates that other factors present in serum may contribute to inhibition of virus entry. Complement components can neutralize virus entry (reviewed in (Tarr et al., 2012)), but it is likely that in this case other host factors contributed to

neutralization. It will be important to determine which components of serum are acting to inhibit entry of HCV in these animals.

The HCVpp isolates used in this study were selected based on their similarity to the immunogen (NotC1 and UKNP1.4.1), one known to be highly sensitive to antibody-mediated neutralization (H77), and three genetically heterologous viruses known to display different neutralization resistance profiles (UKNP2.1.1, UKNP2.4.1 and UKNP3.2.1) (Urbanowicz et al., 2015). The consensus vaccine NotC1 elicited antibodies that neutralized the autologous virus and the H77 strain, but failed to neutralise genetically distinct viruses. The immunogens used by Stamataki and colleagues elicited sera that potently neutralized some genotype 1 isolates, but not representatives of genotype 2 (Stamataki et al., 2007). Likewise, immunization of mice with H77-recombinant measles virus resulted in antibodies that neutralized H77, but was less potent against Con1 (gt1) and J6 (gt2a). More recently Li and colleagues immunised rhesus macaques with a sE2 construct in a variety of adjuvants. The immunised animals produced cross-neutralising antibodies, although the potency of these against many isolates tested was modest (Li et al., 2017). Standardization of the strains used for future immunization studies is essential, as we have shown conclusively that differences in neutralization sensitivity will impact on the apparent effectiveness of vaccine-induced sera (Tarr et al., 2011; Urbanowicz et al., 2015).

We have demonstrated that an *in silico*-derived consensus E2 ectodomain sequence can be expressed to high yield and purity from drosophila S2 cells. This protein was immunogenic and elicited antibodies that neutralize entry of some genotype 1a isolates. However, the broader objective of eliciting antibodies that neutralize patient strains representing multiple

genotypes will require further refinement of immunization protocols. A vaccine construct comprising the consensus of a minimal CD81 binding domain might be able to focus the antibody response to conserved epitopes on E2. Additionally, boosting immunized animals with glycoproteins representing different strains might be required to focus the antibody response on to conserved conformational epitopes.

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Figure 1: Generation of ancestral consensus E2 sequences NotC1 and NotC2. A) Nucleotide sequences representing the consensus sequences NotC1 and NotC2 were generated using the consensus maker (hiv.lanl.gov) from 720 non-redundant nucleotide sequences from circulating HCV strains. Maximum likelihood reconstruction of the phylogeny using the General Time Reversible model was performed using 100 representatives of this population with these consensus sequences, reference strain H77c (AF096006) the UKNP1.4.1 strain (all highlighted with ●) (Urbanowicz et al., 2015) and the Bole1a/Con1 synthetic HCV consensus sequences (both highlighted with ▲) (Munshaw et al., 2012). This revealed that NotC1 and NotC2 are accurate representations of ancestral HCV sequences. The tree with the highest log likelihood (-21508.23) is shown. B) An alignment of the amino acid sequences of the three immunogens and Bole1a highlighted amino acid substitutions between the sequences across the entire aa410-644 region. Three regions are

highlighted: 'I' 'II' and 'III'. These linear regions are components of the discontinuous CD81 binding site and possess conserved neutralization epitopes.

Figure 2: Expression of recombinant E2. E2 constructs were expressed in S2 cells and purified by Strep-tag affinity purification and size-exclusion chromatography. These proteins were analysed by western blot (A) and Coomassie staining (B), revealing a relatively homogeneous preparation of protein at a size of approximately 45 kDa. In the lane corresponding to NotC1, a larger band of ~70 kDa was also evident. C) The conformation of these expressed E2 proteins was assessed by binding of conformation-sensitive mAbs 1:7 (White bars) and AR1A (Black bars), and the partially conformation sensitive mAb AP33 (Grey bars). Data are presented as OD_{405} after subtraction of background signal from a negative control from a mock expression experiment . All three samples reacted similarly to all three mAbs. D) Binding of recombinant E2 proteins NotC1 (○), NotC2 (▲) or UKNP1.4.1 (●) to HuH7 cells was used to inhibit entry of HCVpp bearing the glycoproteins of strains UKNP1.4.1, UKNP2.1.1 and JFH-1. A protein-free preparation following protein purification was used as a negative control (◇).

Figure 3: Immunization of guinea pigs with recombinant $E2_{410\text{-}644}$. A) Three sets of five guinea pigs were immunized with one of three proteins. Animals 1-5 received NotC1, animals 6-10 received NotC2 and animals 11-15 received UKNP1.4.1. B) The immunization schedule administered four doses of each protein, at day 1, 21, 42 and 63. A pre-bleed sample was taken at day 0, followed by sampling at day 53 and day 74. C) Each of the animals' antibody responses to the matched protein immunogen was assessed by ELISA. Serum taken at D0 (●), D53 (▲) and D74 (□) were assessed for binding. D) Pooled serum recovered at D74 from five animals that received the same immunogen were assessed for binding to native

(light grey bars) and denatured protein (dark grey bars) from each of the three vaccine constructs. E) Pooled immune sera from all NotC1-immunized animals were tested for binding to E1E2 proteins from different HCV genotypes using cell-expressed full-length E1E2.

Figure 4: A NotC1 HCV pseudovirus is infectious in vitro. A) An infectivity assay was performed using HCVpp possessing the glycoproteins of strain H77, or the consensus sequence of a full-length E1/E2 representing the NotC1 protein. Entry of NotC1 (■) and H77 (▼) HCVpp were neutralized with five different neutralizing antibodies: AP33 (B); 1:7 (C); AR2A (D); AR3A (E); or L1 (F).

Figure 5: Neutralization of diverse HCVpp by sera recovered from immunized guinea pigs. A) Pseudoviruses possessing the E1/E2 proteins of six patient-derived strains were used to represent genotype 1, 2 and 3, in addition to the reference strain H77 and the NotC1 functional clone. These were used for neutralization with the sera obtained at D74 after four immunizations with different proteins. Sequences used to generate pseudoviruses are highlighted by circles. B) Neutralization of HCVpp strains by immune sera. Sera isolated at D0 and D74 was used at a dilution of 1:100 to neutralize entry of the panel of eight HCV pseudoviruses and a VSV-G pseudovirus as control. Each panel represents a single type of pseudovirus. Serum from guinea pigs that were immunized with E2 representing NotC1, NotC2 or UKNP1.4.1 immunogens was tested against each pseudovirus. Neutralization potency of vaccine-induced antibodies was compared by One-Way ANOVA, followed by Sidak's multiple comparisons test. Significance values are indicated (*$p<0.05$, ***$p<0.001$, ****$p<0.0001$). C) Dose-dependent neutralization of HCVpp by both pre-immune sera and immune sera. Entry of HCVpp bearing the glycoproteins of clones UKNP2.1.1 and

UKN2.4.1 were neutralized with dilutions of sera between 1:50 and 1:400. D) Neutralization of HCVcc by guinea pig serum diluted 1:100. Entry of replicating HCV virions possessing the E1/E2 glycoproteins of clones J6 and UKN2.4.1 were neutralized by serum.

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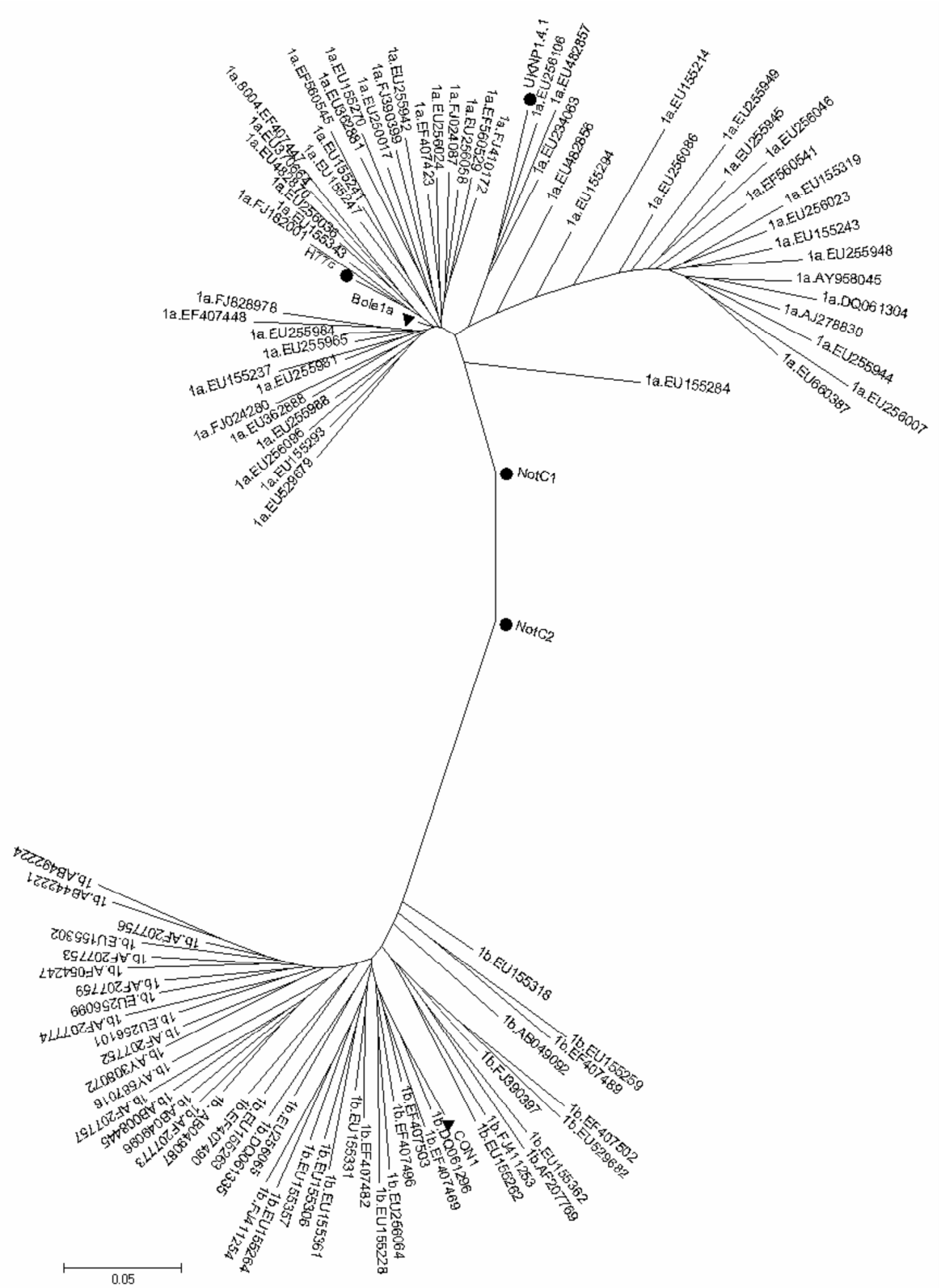
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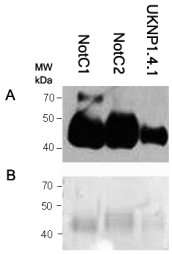
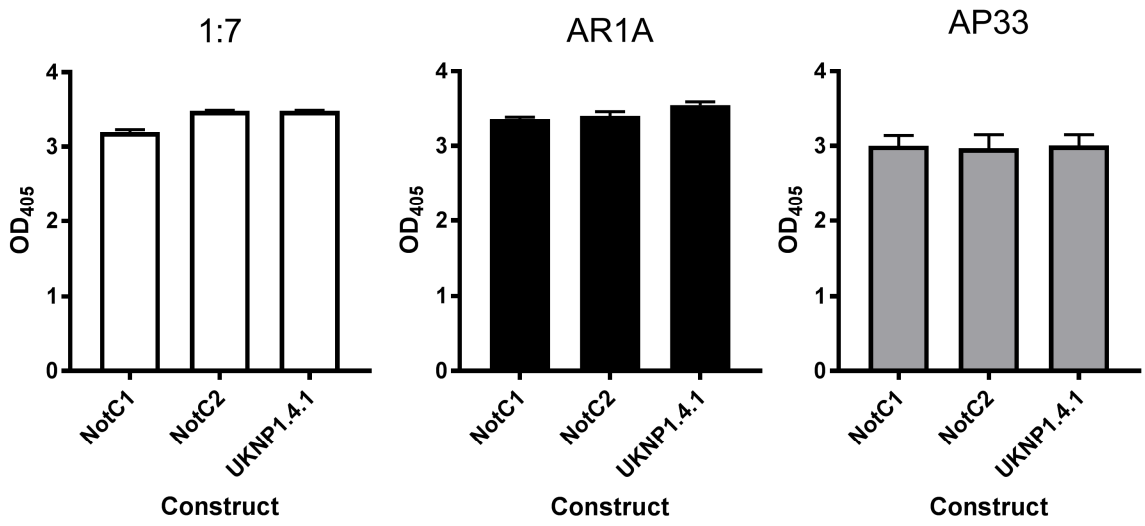
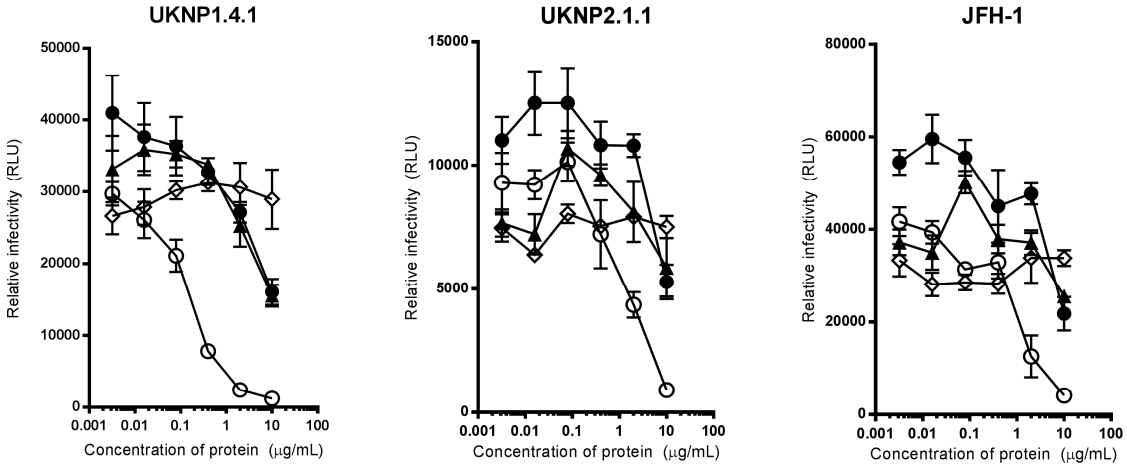
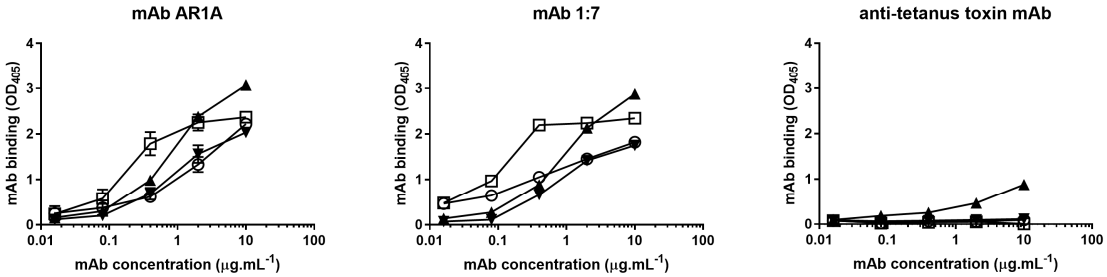
I
II
III
NotC1 NIQLINTNGSWHINRTALNCNDSLNTGWLAGLFYYHKFNSSGCPERMASCRPLADFAQGWGPISYANGSGPDQRPYCWHYP
NotC2 ..........T....L......N..........E........T..K.............A
Bole1a ..........HN..........L......T.....
UKNP1.4.1 .....S.....T..I...L.H..........T..D......G.T..G...H.....
NotC1 PKPCGIVPASKVCGPVYCFTPSPVVVGTTDRSGAPTYNWGENETDVLVLNNTRPPLGNWFGCTWMNSTGFTKVCGAPPCDI
NotC2 .....T......N.
Bole1a .....KS.....D...F.....N.
UKNP1.4.1 .....LS.....K.....R.....F.....A..Y.....N.
NotC1 GGVGNNTLHCPTDCFRKHPEATYSRCGSGPWMTPRCLVDYPYRLWHYPCTVNYTIFKVRMYVGGVEHRLEAAC
NotC2 .....N.....F.....
Bole1a .....I.....I.....
UKNP1.4.1 .....D.....I.....D...



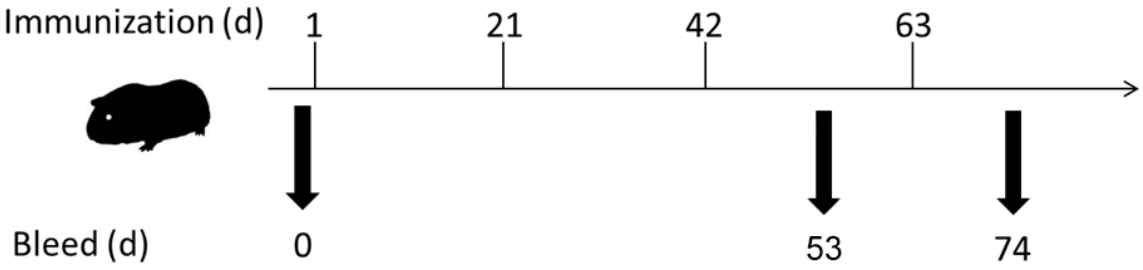


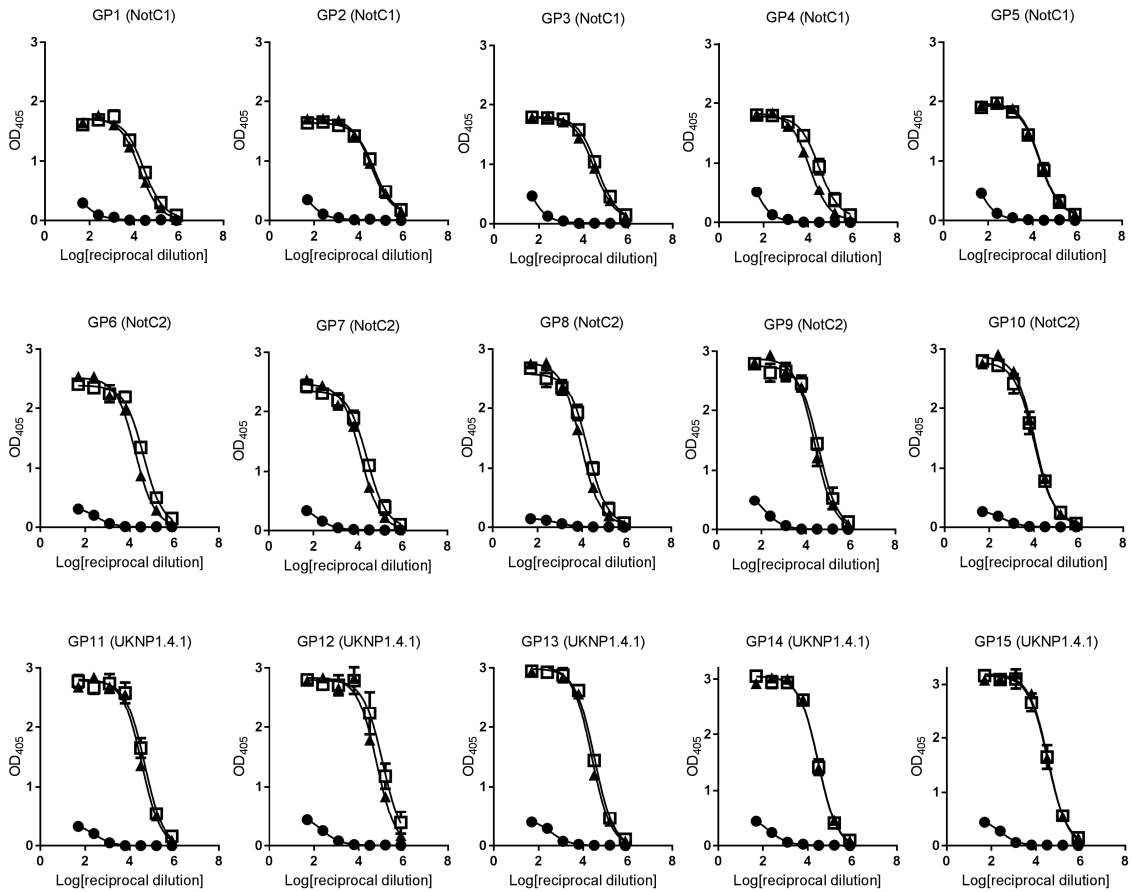


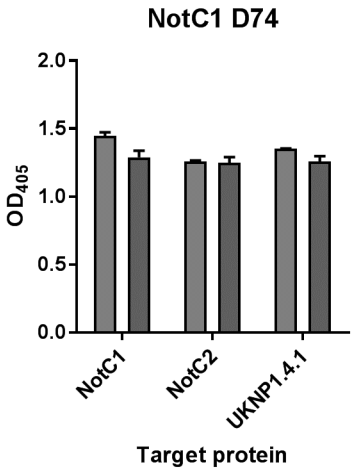
E

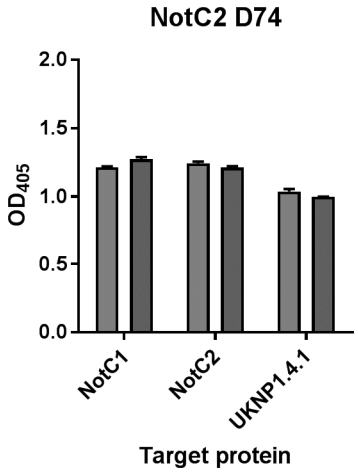


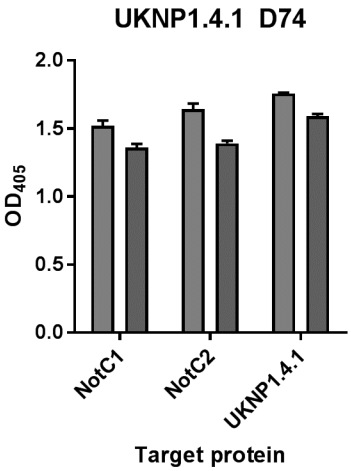
Covalab No. Guinea pig No.	Immunisation Constructs 1-4 immunizations
CUK-1260 1-5	$NotC1_{410-644}$
CUK-1262 6-10	$NotC2_{410-644}$
CUK-1276 11-15	$UKNP1.4.1_{410-644}$

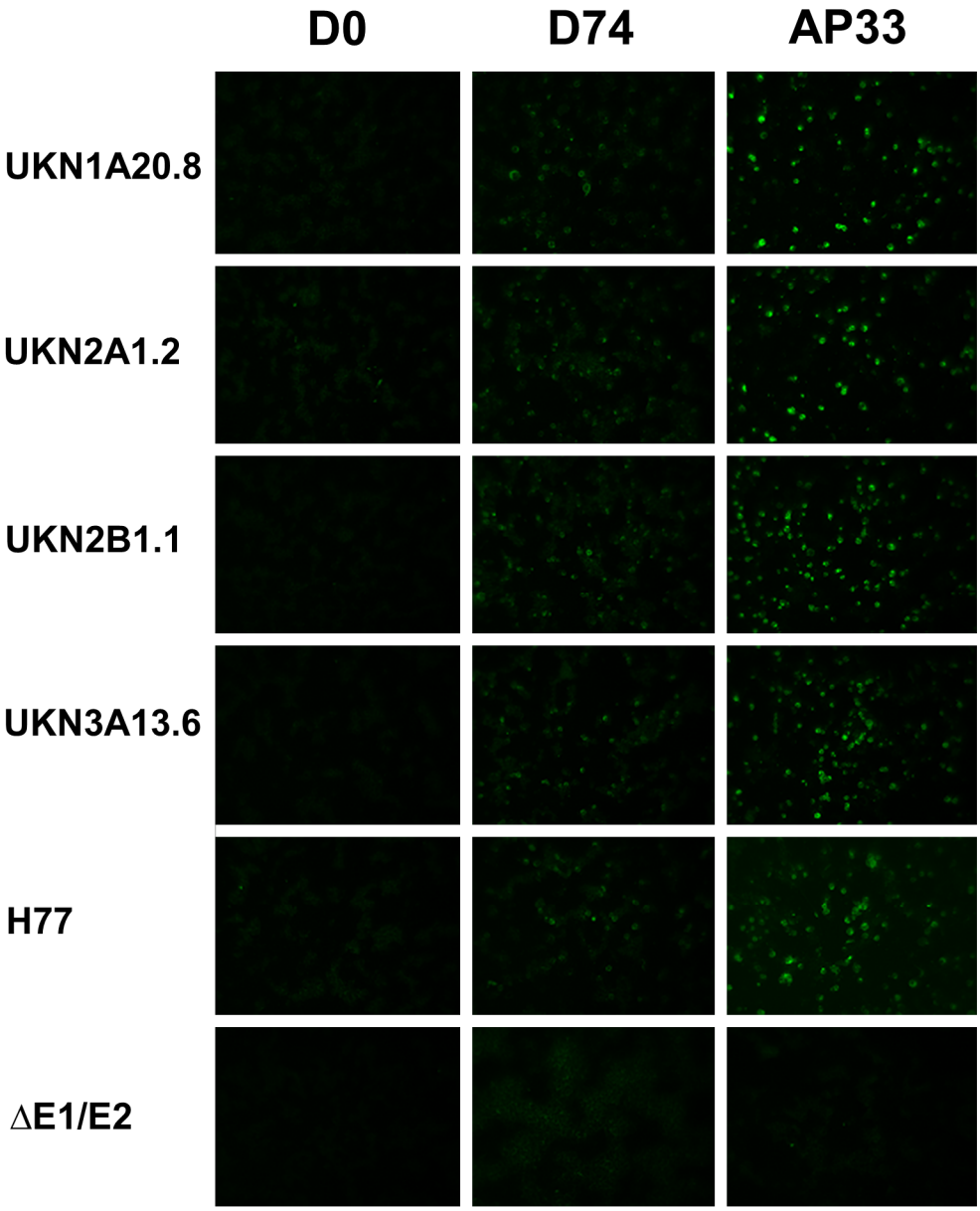


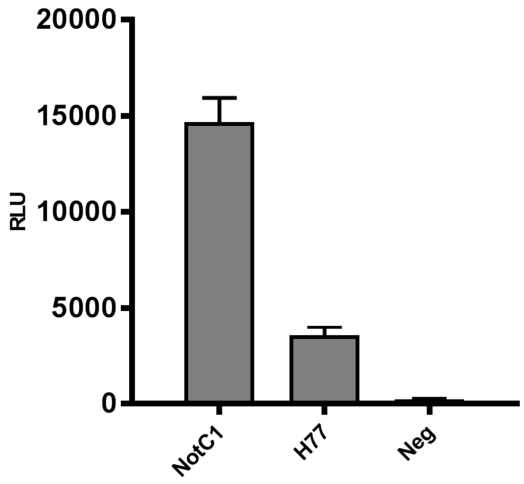



NotC1 D74
2.0
1.5
1.0
0.5
0.0
OD405
NotC1
NotC2
UKNP1.4.1
Target protein


NotC2 D74
2.0
1.5
1.0
0.5
0.0
OD405
NotC1
NotC2
UKNP1.4.1
Target protein


UKNP1.4.1 D74
2.0
1.5
1.0
0.5
0.0
OD405
NotC1
NotC2
UKNP1.4.1
Target protein





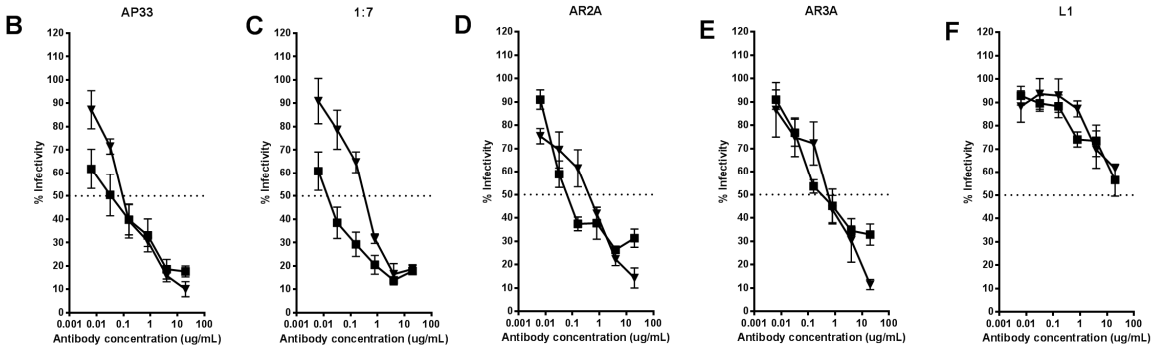
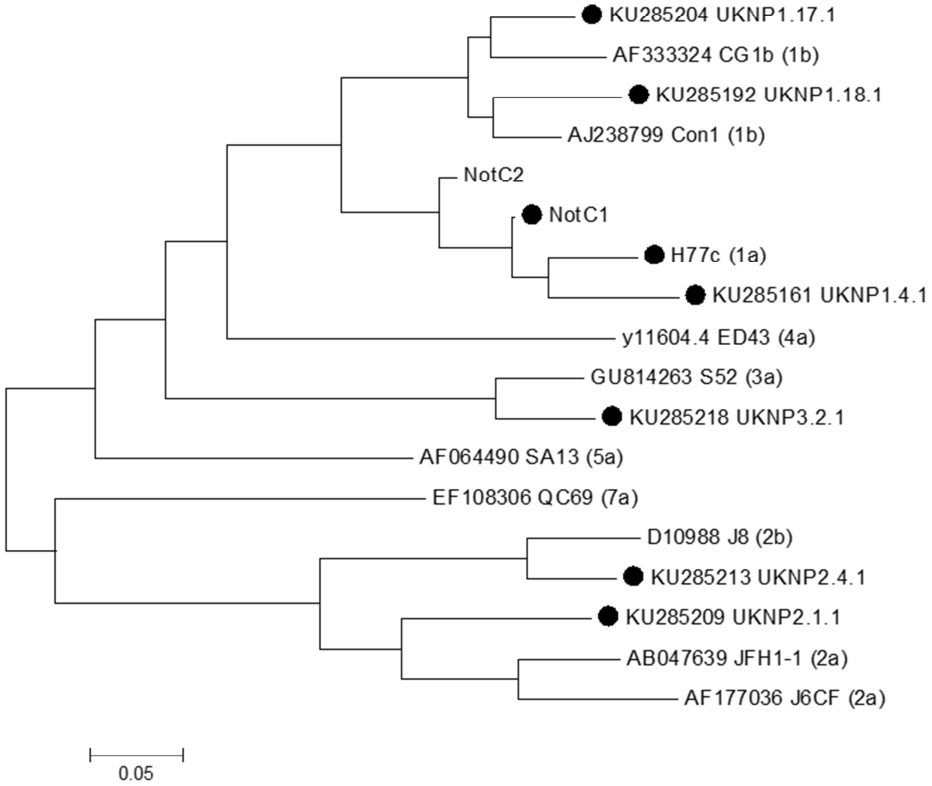
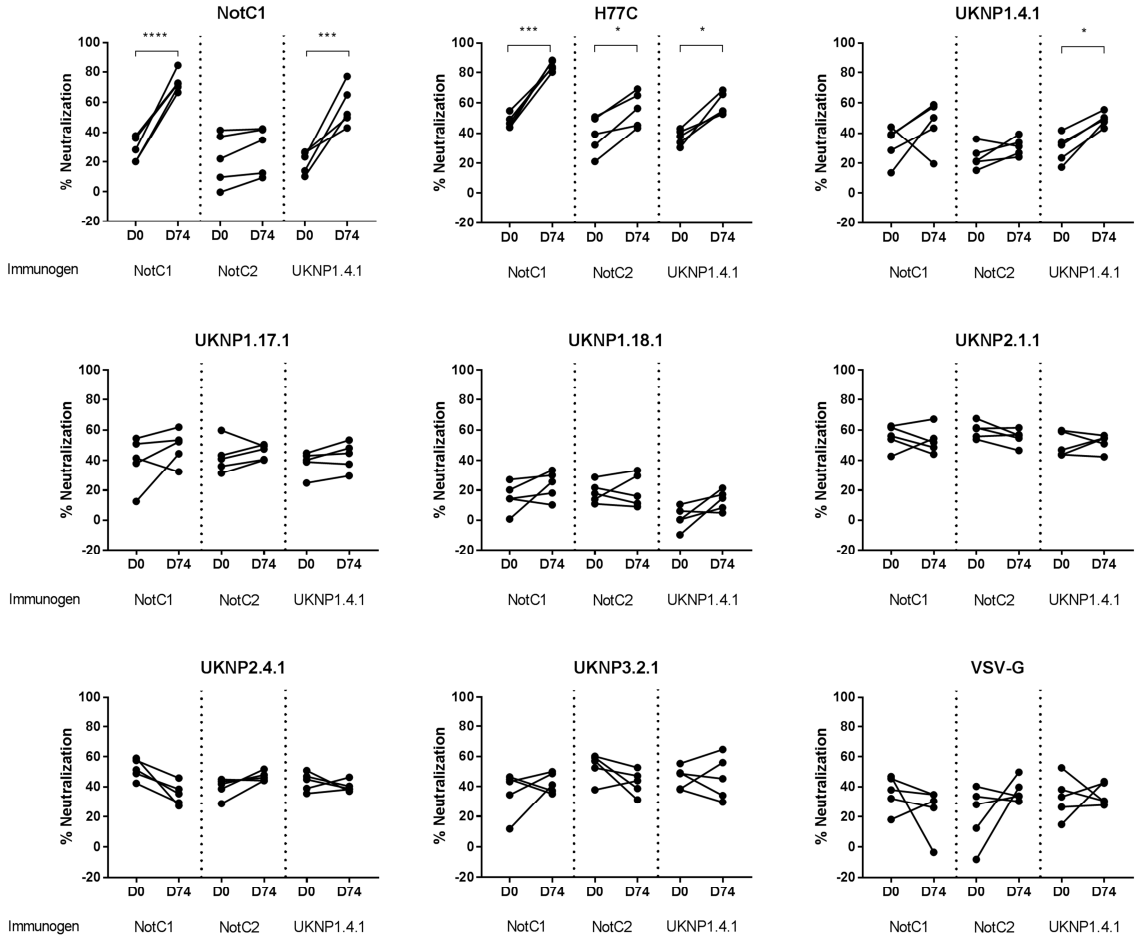
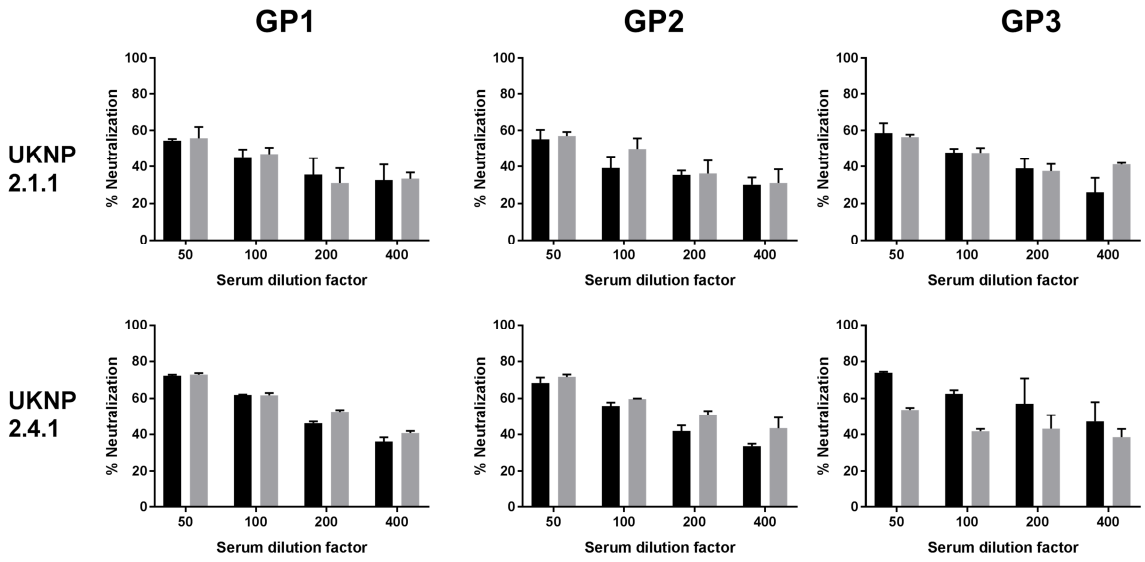
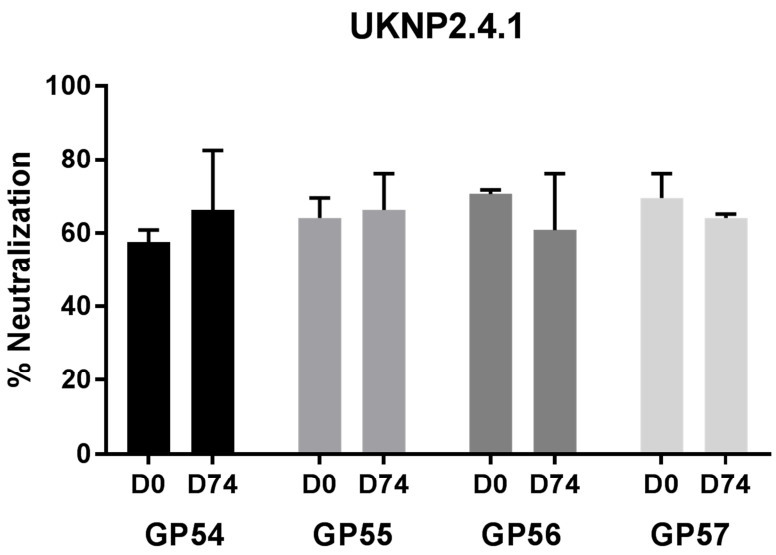


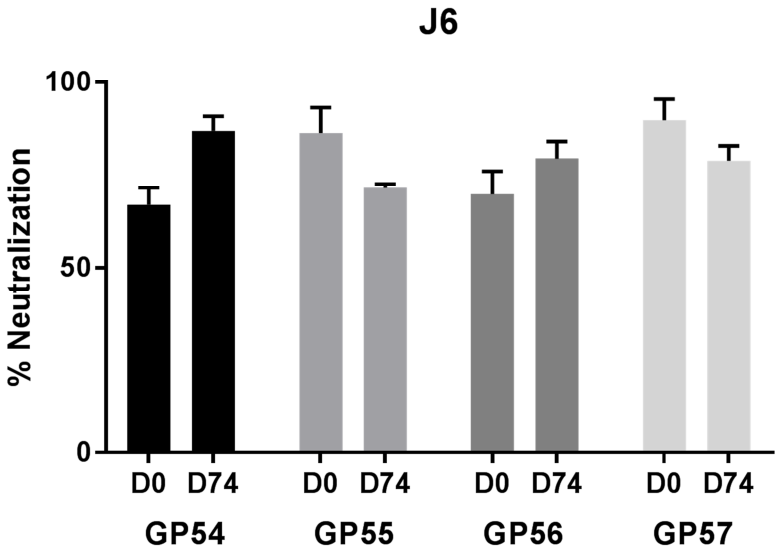
Fig 5A











- We describe a method for designing genetically conserved consensus vaccines
- We applied this approach to create synthetic hepatitis C virus E2 protein immunogens
- These cloned genes produced correctly-folded protein that bound to conformation-sensitive anti-E2 antibodies
- These synthetic consensus vaccines induced high titers of anti-E2 antibodies in immunized animals
- These immunogens were able elicit antibodies that could block infection of HCV