Characterization of a hepatitis C virus-like particle vaccine produced in a human hepatocyte-derived cell line

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An effective immune response against hepatitis C virus (HCV) requires the early development of multi-specific class I CD8⁺ and class II CD4⁺ T-cells together with broad neutralizing antibody responses. We have produced mammalian-cell-derived HCV virus-like particles (VLPs) incorporating core, E1 and E2 of HCV genotype 1a to produce such immune responses. Here we describe the biochemical and morphological characterization of the HCV VLPs and study HCV core-specific T-cell responses to the particles. The E1 and E2 glycoproteins in HCV VLPs formed non-covalent heterodimers and together with core protein assembled into VLPs with a buoyant density of 1.22 to 1.28 g cm⁻³. The HCV VLPs could be immunoprecipitated with anti-ApoE and anti-ApoC. On electron microscopy, the VLPs had a heterogeneous morphology and ranged in size from 40 to 80 nm. The HCV VLPs demonstrated dose-dependent binding to murine-derived dendritic cells and the entry of HCV VLPs into Huh7 cells was blocked by anti-CD81 antibody. Vaccination of BALB/c mice with HCV VLPs purified from iodoxanol gradients resulted in the production of neutralizing antibody responses while vaccination of humanized MHC class I transgenic mice resulted in the production of HCV core-specific CD8⁺ T-cell responses. Furthermore, IgG purified from the sera of patients chronically infected with HCV genotypes 1a and 3a blocked the binding and entry of the HCV VLPs into Huh7 cells. These results show that our mammalian-cell-derived HCV VLPs induce humoral and HCV-specific CD8⁺ T-cell responses and will have important implications for the development of a preventative vaccine for HCV.
INTRODUCTION
The development of a vaccine against hepatitis C virus (HCV) has been challenging, although advances in our understanding of the immune response against HCV makes the prospect of vaccine an achievable goal. Individuals who clear HCV spontaneously and become reinfected are far more likely to clear infection compared with individuals with primary infection (Sacks-Davis et al., 2013). Although previous infection and clearance of HCV does not necessarily result in sterilizing immunity against HCV, the development of immunity that is protective against the development of chronic infection is possible (Grebely et al., 2012). These findings indicate that producing a vaccine to prevent the development of persistent infection is possible.

An effective immune response against HCV includes the early development of broad CD8+ and CD4+ T-cell and neutralizing antibody (NAb) responses against several HCV structural and non-structural proteins (Lauer et al., 2004; Pestka et al., 2007; Schulze zur Wiesch et al., 2005; Smyk-Pearson et al., 2006). Neutralizing antibodies to epitopes in the E1 and E2 glycoproteins also have an important role in providing protection against several genotypes of HCV (Broering et al., 2009; Dowd et al., 2009; Mancini et al., 2009; Owsianka et al., 2008; Puig et al., 2006; Raghuraman et al., 2012). A successful vaccine for HCV will need to reproduce these immune responses.

Several studies have highlighted the importance of NAb in providing protection against HCV. In humanized uPA-SCID liver chimeric mice, human IgG is protective against homologous (Vanwolleghem et al., 2008) and heterologous HCV challenge (Law et al., 2008; Meuleman et al., 2011). Broadly reactive NAb is able to cure these mice of HCV infection (de Jong et al., 2014), and vaccination with a recombinant vaccinia virus encoding the HCV structural proteins is protective against HCV challenge (Dorner et al., 2011). Vaccination of chimpanzees with mammalian cell-derived recombinant E1 and E2 administered intramuscularly has also been shown to prevent the development of persistent infection after homologous and heterologous intravenous virus challenge (Choo et al., 1994). Finally, a recombinant HCV gpE1E2 vaccine has been shown to result in the production of broad cross-neutralizing antibody responses against several genotypes of HCV in humans (Frey et al., 2010; Law et al., 2013; Wong et al., 2014).

HCV virus-like particles (VLPs) could serve as a viable vaccine candidate for HCV as they are able to produce both NAb and cellular immune responses (Beaumont et al., 2013; Chua et al., 2012; Patient et al., 2009). Furthermore, as HCV-specific NAb recognizes tertiary or quaternary structures (Giang et al., 2012), the repetitive and ordered particulate structure of HCV VLPs makes VLPs an attractive vaccine candidate (Beaumont et al., 2013; Chua et al., 2012; Elnowalid et al., 2007; Garrone et al., 2011).

We have previously shown that mammalian-cell-derived HCV VLPs adjuvanted with Toll-like receptor 2 (TLR2) agonists are strongly immunogenic (Chua et al., 2012). In this study we have focussed on describing the biochemical and morphological characterization of our genotype 1a HCV VLP vaccine. We have not previously reported this; however, this characterization is important for the further development of our vaccine. Our HCV VLPs resemble HCV morphologically and biochemically and like HCV have a heterogeneous size distribution. We have also extended on previous work by studying the immunogenicity of HCV VLPs purified through iodixanol density gradients, and show that HCV VLPs are rapidly taken up by bone marrow derived dendritic cells (BMDC), elicit humoral and HCV core protein specific CD8+ T-cell responses in vaccinated mice, and are recognized by anti-HCV IgG from infected humans.

RESULTS
Characterisation of HCV VLPs
Infection of Huh7 cells with rAdHCVcoreE1E2 resulted in the production of HCV structural proteins as shown by SDS-PAGE and immunoblotting with mAbs against the core, E1 and E2 proteins (Fig. 1). The molecular mass of the core protein was 22 kDa, while the E1 glycoprotein (gpE1) had a molecular mass of 35 kDa and the E2 glycoprotein (gpE2) of 72 kDa. Both gpE1 and gpE2 were deglycosylated with endoglycosidase H, which cleaves high-mannose glycans yielding a mixture of glycoforms (Fig. 1a, b), a finding consistent with maturation in the trans-Golgi complex (Vieyres et al., 2010). Both the gpE1 and gpE2 bands migrated to the expected monomer sizes in a non-reducing SDS-PAGE (Fig. 1c).

Purified HCV VLPs were immunoprecipitated with both anti-ApoE and anti-ApoC and the HCV structural proteins detected by anti-E1 mAb A4, anti-E2 mAb H52 and anti-core (Fig. 1d), indicating that both apoproteins E and C had become incorporated into HCV VLPs. ApoE and ApoC were also detected in HCV VLPs by Western blot analysis (Fig. 1e).

HCV VLPs purified from lysates of rAdHCVcoreE1E2-infected Huh7 cells by CsCl equilibrium gradient ultracentrifugation (immunoreactive fractions 7–10) (Fig. 2a) were found to have buoyant densities of 1.22 to 1.28 g cm−3 (Fig. 2b). Core, E1 and E2 proteins co-localized to the same gradient fractions in both CsCl (fractions 7–10) and iodixanol (fractions 8–11) (Fig. 2c) gradients, indicating the formation of VLPs.

To confirm that the HCV structural proteins had assembled into VLPs, gradient fractions 7–10 showing immunoreactivity for HCV envelope proteins were examined by electron microscopy. Transmission electron microscopy revealed abundant VLPs (Fig. 3a). The VLPs appeared to consist of double- and single-membrane particles (Fig. 3b) and ranged in size from 30 to 80 nm (Fig. 3c). Immunogold localization with anti-E1 (A4) mAb confirmed that the VLPs contained HCV E1 envelope protein (Fig. 3d).

Cryo-electron microscopy of the HCV VLPs and reconstruction of the tomographic volume from the tilt series acquired showed that the HCV VLPs consisted of a heterogeneous...
mixture of particles (Fig. 3e). Isosurface three-dimensional (3D) reconstructions of HCV VLPs demonstrated that the HCV VLPs consisted of a mixed population of single- or double-membrane particles (Fig. 3f).

**Dose-dependent binding of HCV VLPs to mouse BMDC**

We have previously shown that HCV VLPs are taken up by immortalized D1 cells, and this was particularly effective if HCV VLPs were mixed with a TLR2 agonist (Chua et al., 2012). In this study, we determined the uptake of HCV VLPs by native murine BMDC (CD11c+). BMDC are considered a more representative model following vaccination in vivo. FITC-labelled HCV VLPs were detected in the cytosol of BMDC by confocal microscopy (Fig. 4a) and by flow cytometry following quenching of extracellular fluorescence with trypan blue (Fig. 4b). After a 4 h incubation with 7.5 µg FITC-labelled HCV VLPs, 99.2% of BMDC had already bound VLPs. Following quenching of extracellular fluorescence with trypan blue, 56.8% of BMDC demonstrated entry of FITC-labelled HCV VLPs. Similar results were obtained after 24 h of incubation, demonstrating that the uptake of HCV VLPs by BMDC is a rapid process (Fig. 4b). In addition, saturation of binding of FITC-labelled HCV VLPs occurred at a concentration of 500 ng ml⁻¹ (Fig. 4c).

**Induction of HCV VLP- and E2-specific humoral immune responses in mice**

We have previously reported the immunogenicity of CsCl-purified HCV VLPs. To determine the antigenicity of iodixanol-purified HCV VLPs in vivo, Balb/C mice were vaccinated with three doses of HCV VLPs in alum...
(Alhydrogel) or complete Freund’s adjuvant (CFA), and antibody titres determined using iodixanol-purified HCV VLPs as the coating antigen on ELISA plates. Mice developed strong anti-HCV VLP (Fig. 5a) and anti-E2 (Fig. 5b) specific antibody responses. The geometric mean titre for anti-HCV VLP antibody was highest in mice receiving HCV VLPs in alum \(3.58 \log_{10}\); 95% confidence interval (CI) 3.05–4.19) compared with HCV VLPs in CFA (3.16 \log_{10}; 95% CI 2.32–4.31; \(P=\text{ns}\)) and HCV VLPs in PBS (2.78 \log_{10}; 95% CI 2.2–3.5; \(P<0.05\)). The difference in geometric mean titre in mice inoculated with HCV VLPs mixed with either alum or CFA was not significant.

Neutralizing antibody response to HCV VLPs

We then determined whether the genotype 1a HCV VLP vaccine could produce neutralizing antibody responses using two methods. In the first assay, which we have previously described (Chua et al., 2012), we determined the inhibition of binding and entry of FITC-labelled HCV VLPs into Huh7 cells by immune sera from HCV VLP-vaccinated mice and compared this against a titration of non-immune sera. The sera from the mice immunized with HCV VLPs in alum inhibited binding by 55–90%, compared with 25–35% with non-immune sera \((P=0.0002)\) (Fig. 5c). This compared well to the inhibition of HCV VLP binding (85%) by anti-CD81 antibody (Fig. 5d). In the second assay, a H77 HCVpp neutralization assay (Drummer et al., 2003), we compared the neutralization of a genotype 1a HCVpp by immune mouse sera with a positive neutralizing mAb (mAb 24) and a pooled negative non-immune mouse serum (Fig. 5e). The HCV VLP immune sera potently neutralized HCVpp entry, with inhibition of entry varying from 89% \((SD\pm1.4\% ); P=0.05)\) to 45% \((SD\pm5\% ; P=0.05)\).

Inhibition of HCV VLP cell entry by human HCV-specific IgG

We next determined whether IgG from individuals infected with HCV genotypes 1a and 3a could inhibit the entry of FITC-labelled HCV VLPs into Huh7. This was important to establish given the reports of the role of human mAbs in identifying the most potent cross-neutralizing HCV E2 epitopes recognized in humans with chronic hepatitis C (Keck et al., 2004, 2005, 2008). Genotype 1a anti-HCV IgG produced a 50% inhibition of binding \((IC_{50})\) ranging from 50 to

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**Fig. 2.** (a) Western blot of CsCl fractions of purified HCV VLPs. E1, E2 and core proteins in CsCl fractions 1 to 12 were detected using Western blot analysis probed with A4, H52 and core mAb, respectively. (b) Buoyant density plot. Relative quantity of E1 protein in fractions 1–12 was determined by densitometry of Western immunoblot. Refractive index of fractions was converted to buoyant density using tables of the CRC Handbook of Chemistry and Physics (http://www.hbcpnetbase.com/). (c) Western blot of iodixanol fractions of purified HCV VLPs. Detection with anti-E1 (mAb A4) and anti-core mAb. Molecular masses (in kD) of protein molecular mass markers are indicated on the left; HCV core, E1 and E2 proteins are indicated on the right.
750 ng ml\(^{-1}\) (Fig. 5f). Similarly, genotype 3a anti-IgG inhibited HCV VLP entry with an IC\(_{50}\) ranging from 262 to 740 ng ml\(^{-1}\) (Fig. 5g). In contrast, IgG from the sera of HCV-negative controls inhibited HCV VLP entry poorly, with an IC\(_{50}\) ranging from 1500 to greater than 2000 ng ml\(^{-1}\). The difference in the means of the inhibition of binding between HCV-positive and -negative IgG for both HCV genotype 1a and 3a sera was significant (P<0.0001) (Fig. 5h, i).

**Fig. 3.** (a, b) Transmission electron microscopy (TEM) and negative staining with uranyl acetate of purified HCV VLPs, confirming the presence of heterogenous single- and double-membrane particles. (c) TEM showing the presence of 30–80 nm particles. (d) Immunogold labelling of particles performed with a mAb directed against the viral envelope protein E1 (A4 mAb). (e) Cryo-electron microscopy of HCV VLPs. A slice through the reconstructed tomographic volume from the tilt series of HCV VLPs acquired showed VLPs containing single or double membranes. (f) Isosurface three-dimensional reconstructions of HCV VLPs containing single or double membranes. Bars, 50 nm (c) and 100 nm (e). Magnification, \(\times 45\,000\) (a); \(\times 190\,000\) (b, d).

**HCV-specific T-cell responses in HCV VLP-vaccinated MHC class I transgenic mice**

We have previously shown that our HCV VLPs produce T-cell responses in MHC class I transgenic mice. We have now extended this by determining the ability of HCV VLPs to stimulate the production of HLA A2-specific T-cell responses to HCV core in MHC class I transgenic mice. In HCV VLP-vaccinated mice, the mean number of IFN-\(\gamma\)-
secretion of IFN-γ-secreting cells per million splenocytes was 269 (±81) in splenocytes stimulated with HCV VLPs compared with HCV core peptides DLMGYIPLV (184±24) and YLLPRRGPRL (97±19) (P<0.05 vs NS5B2594–2602) (Fig. 6a). The mean number of IFN-γ-secreting cells for mice vaccinated with HCV VLP in CFA was 186 (±33) per 10⁶ splenocytes stimulated with HCV VLPs, 138 (±6) per 10⁶ splenocytes stimulated with DLMGYIPLV and 90 (±25) per 10⁶ splenocytes stimulated with YLLPRRGPRL (P<0.05 vs NS5B2594–2602) (Fig. 6b).

**DISCUSSION**

We have developed mammalian liver cell-derived HCV VLPs that contain E1 and E2 glycoproteins and core protein that have a morphology resembling HCV. Our findings are consistent with those of others who have demonstrated that HCV virions are spherical, heterogeneous single- and double-membrane particles that vary in size from 40 to 80 nm (Catanese et al., 2013; Gastaminza et al., 2010). Like HCV virions our HCV VLPs also incorporate apolipoproteins C (Meunier et al., 2008) and E (Catanese et al., 2013; Gastaminza et al., 2010). We have also shown that our genotype 1a HCV VLP vaccine produces neutralizing antibody responses and that IgG purified from the sera of patients chronically infected with HCV blocks the entry of the HCV VLPs into Huh7 cells, demonstrating that antibodies developed in the course of natural infection in humans are able to recognize potential neutralizing epitopes on the surface of the HCV VLPs. Our vaccine also induced HCV core-specific T-cell responses in MHC class I transgenic mice. The significance of this finding is highlighted by the fact that next to NS3-specific CD8 T-cell responses, HCV core-specific class I epitopes are the most important in producing HCV specific clearing and protective CD8 T-cell responses.

A robust HCV NAb response is increasingly recognized to play a pivotal role in preventing HCV infection (Broering et al., 2009; Dowd et al., 2009; Pestka et al., 2007; Raghuraman et al., 2012; Yu et al., 2004). In addition, the early appearance of broad NAb is an important determinant of clearance of HCV and it is the breadth rather than simply the presence of heterologous NAb alone that provides the strongest immunological correlate of protection against persistent infection with HCV (Osburn et al., 2014).

Several HCV vaccines have been developed but these predominantly result in the production of cellular immune responses against the non-structural proteins like NS3 (Barnes et al., 2012; Swadling et al., 2014) or result in the production of antibody alone in both mice and humans (Frey et al., 2010; Law et al., 2013; Wong et al., 2014). The importance of including structural proteins in a vaccine was reinforced in a meta-analysis of the efficacy of vaccine approaches in chimpanzees, which demonstrated that immune responses to the structural rather than non-structural proteins correlated more closely with protection against and clearance of HCV (Dahari et al., 2010).

HCV VLPs offer an approach to elicit both NAb and cellular immune responses (Beaumont et al., 2013; Chua et al., 2012). Insect-cell-derived HCV VLPs have been shown to produce protective immune responses in both murine (Murata et al., 2003) and chimpanzee (Elmowalid et al., 2007) models of HCV infection. However, this vaccine was only partially protective in chimpanzees (Elmowalid et al., 2007), an outcome that may have been the result of the relatively poor ability of insect-cell-derived E2 protein to stimulate a protective antibody response in primates (Rosa et al., 1996). Producing
HCV VLPs in a human-liver-derived cell line may therefore result in a more authentic HCV VLP vaccine.

Other HCV VLP-based approaches have provided encouraging results. Garrone et al. (2011) reported an HCV genotype 1a vaccine based on retroviral Gag and HCV E2 glycoprotein pseudotypic particles produced in 293T cells. The vaccine induced high-titre NAb in both mouse and macaque and broad cross-neutralizing antibodies against five other genotypes (Garrone et al., 2011). Chimeric HBV-HCV VLPs containing E1-E2 heterodimers of genotype 1a HCV produced in CHO (Chinese hamster ovary) cells have also been shown to result in the production of cross-

**Fig. 5.** Anti-HCV VLP antibody responses in immunized BALB/c mice vaccinated with PBS, 15 µg of HCV VLPs in Alhydrogel (alum) or CFA on days 0, 21 and 42, anti-HCV VLP antibody titres as determined by ELISA using (a) HCV VLPs and (b) recombinant E2 as capture antigens. (c) Antibody-mediated neutralization of binding (NOB) of FITC-labelled HCV VLP entry into Huh7 cells determined by serial dilutions of murine non-immune or immune sera (mice inoculated with HCV VLPs, with Alhydrogel). The mean NOB of sera from immune mice was compared with the mean NOB of sera from negative control mice at each serum dilution and found to be significantly different \( P=0.0002 \). (d) NOB of FITC-labelled HCV VLPs with anti-CD81 antibody. (e) Neutralization of H77 HCVpp by mouse immune sera. Sera from four mice immunized with HCV VLPs in alum were tested in triplicate in an HCVpp neutralization assay. Neutralizing antibody activity was compared with a potent HCV E2 neutralizing mAb, mAb 24 and a negative control pooled non-immune mouse serum. Huh7 monolayers were infected with HCVpp and at 72 h post-infection, Huh7 cells were lysed and measured for luciferase activity using the Promega luciferase substrate system and a Fluostar. (f, g) Neutralization of binding of FITC-labelled HCV VLP entry in Huh7 cells was determined using IgG purified from sera of hepatitis C positive individuals (Pt) infected with genotypes 1a (f) and 3 (g) HCV. Neutralization of binding was determined as the percentage reduction in FITC-positive Huh7 cells after incubation with sera or IgG. (h, i) Mean NOB for HCV genotype 1a (h) and genotype 3a (i) IgG. *P value for the comparison of the mean NOB between HCV-positive and -negative IgG.
neutralizing antibody responses against heterologous HCV genotypes 1b, 2a and 3 (Beaumont et al., 2013; Patient et al., 2009).

The production of strong NAb responses is dependent on the correct presentation of key neutralizing epitopes on the viral envelope. The E2 protein is known to contain the major conformational neutralizing antigenic regions AR3 and AR4 (Giang et al., 2012; Law et al., 2008). Antibodies against these epitopes are broadly cross-neutralizing and protect human liver chimeric Alb-uPA/SCID mice against challenge with human-serum-derived HCV (Giang et al., 2012; Tarr et al., 2006). Broad NAbs generally block the binding of HCV to hepatocytes by interfering with the binding of HCV E2 to CD81 on the surface of hepatocytes. However, a second subset of broad NAbs act by recognizing native E1E2 heterodimers and have the strongest neutralizing activity (Giang et al., 2012). The significance of these regions on the viral envelope is further highlighted by the ability of passive immunization with mAbs directed against these regions to protect human liver chimeric uPA/SCID mice against challenge with human-serum-derived HCV (Law et al., 2008; Meuleman et al., 2011; Vanwolleghem et al., 2008).

Distinct neutralizing antibody epitopes have also been identified in and downstream of hypervariable region 1 (HVR1) of the E2 protein (Giang et al., 2012; Grollo et al., 2006; Hsu et al., 2003; Law et al., 2008). Krey et al. (2010) have reported a 3D model of the structure of E2. Three domains were identified in the model, with critical contact residues residing within domain I and that are recognized by broad NAbs (Keck et al., 2011; Krey et al., 2010). It has also become apparent that mouse neutralizing mAbs predominantly target linear epitopes within these domains and that antibodies in humans with hepatitis C that also target these epitopes only make up a small proportion of HCV NAbs in human serum. In contrast, human mAbs derived from B-cells of patients with chronic hepatitis C are the most potently neutralizing antibodies and have been shown to target conformational epitopes that are predominantly located in domain II of the E2 protein (Keck et al., 2004, 2005, 2008). The complex nature of how neutralizing epitopes are presented on the surface of HCV would suggest that these important antigenic regions need to be closely reproduced in a vaccine.

The challenge of producing authentic HCV VLPs must also take into account the fact that in the assembly of HCV the
virus becomes associated with lipoproteins. Mature virions contain ApoC and E lipoproteins and also circulate as triglyceride-rich lipoparticles (Bartenschlager et al., 2011; Catanese et al., 2013; Gastaminza et al., 2010; Tao et al., 2009). Producing HCV VLPs with a lipoprotein profile that is representative of mature virions may be an important consideration for vaccine design. We have shown that our HCV VLPs have incorporated ApoE and C, adding to their authenticity as a vaccine for HCV. The presence of ApoE in our HCV VLPs is important as ApoE plays an important role in HCV particle assembly (Lee et al., 2014).

In conclusion, our mammalian-cell-derived HCV VLPs show promise for the development of a preventive vaccine aimed at producing both NAB and cellular immune responses against HCV. We are now extending our initial work by developing a quadrivalent HCV VLP and characterizing the breadth of NAB responses to this vaccine using the HCVcc system, an essential requirement for an HCV vaccine.

METHODS

Production and purification of rAdHCVcoreE1E2 and HCV VLPs. The construction of a recombinant adenovirus encoding the HCV structural proteins (core, E1 and E2) of HCV 77H, genotype 1a, was carried out using pCDNA3.tpaE2-661myc plasmid (provided by Dr Charles Rice, Rockefeller University, NY, USA) as described previously (Torresi et al., 2007).

Western immunoblot analysis of HCV VLP proteins. Typically, 200 µl of purified HCV VLPs from each of the 12 gradient fractions were concentrated using methanol/chloroform precipitation, then resolved by reducing and non-reducing SDS-PAGE followed by Western immunoblotting. The HCV E1 and E2 proteins were detected using the anti-E1 mAb A4, and the anti-E2 mAb H52 (gifts from Professor Jean Dubuisson, Institut Pasteur de Lille, France). HCV core protein was detected using the anti-core mAb ab2740 (Abcam). Glycosylated forms of the E1 and E2 proteins were analysed by digesting 30 µl of concentrated protein with Endo H (New England Biolabs) for 2 h at 37 °C followed by SDS-PAGE and Western immunoblotting.

Immunoprecipitation of purified HCV VLPs was performed with anti-ApoE and anti-ApoC (AbCam). Purified HCV VLPs were incubated overnight at 4 °C with Protein A sepharose (Sigma) prebound with anti-ApoE or anti-ApoC antibodies. Samples were then centrifuged at 17,000 g for 5 minutes at 4 °C and pellets washed with 0.5 ml of wash buffer A [0.05 % Triton X-100, 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA], followed by 0.5 ml of wash buffer B [0.05 % Triton X-100, 10 mM Tris-HCl (pH 7.5), 0.5 mM NaCl, 2 mM EDTA] and, finally, 0.5 ml of wash buffer C (10 mM Tris-HCl, pH 7.5) followed by centrifugation. The pellet was resuspended in 20 µl Laemmli buffer, heated to 100 °C for 2 min and separated by 10 % SDS-PAGE followed by transfer onto PVDF and immunoblotting with mAb A4 (anti-E1) or anti-HCV core antibody.

Electron microscopy of HCV VLPs. Transmission electron microscopy was performed by negatively staining 1 µl of purified HCV VLPs with 2 % aqueous uranyl acetate. Immunostaining of HCV VLPs was performed on glow-discharged 300 mesh copper grids by incubating 10 µl samples on the grids for 2 min at room temperature. After washing three times with PBS for 5 min at room temperature, the grids were incubated face-down on a 20 µl drop containing purified mouse mAb A43 (anti-E1) diluted 1:10 in PBS for 30 min at room temperature followed by a further wash with PBS. The specimens were then incubated on a 20 µl drop containing gold-coupled secondary antibodies diluted 1:10 in PBS for 20 min at room temperature. After a further PBS wash, the specimens were negatively stained with 2 % aqueous uranyl acetate and examined by electron microscopy. Electron micrographs were taken with a Zeiss EM 10 electron microscope at 80 kV (Steinbach et al., 1997).

Cryo-electron tomography of HCV VLPs. Purified HCV VLPs were diluted 1:10 in 1× PBS. A 3 µl aliquot of the suspension was applied onto a glow-discharged lacey carbon grid (ProSciTech). The droplet was blotted with filter paper to remove excess liquid resulting in a thin layer which was rapidly plunge-frozen in liquid ethane. Grids were mounted in a Gatan cryoholder under liquid nitrogen. To determine the 3D structure by cryo-electron tomography, tilt series were collected from −60° to +60° in 2° increments under low dose conditions at 200 kV using a

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**Fig. 6. T-cell ELISPOT – IFN-γ response in HCV VLP vaccinated mice.** C57/B16 MHC class I transgenic mice (n=3 per group) were vaccinated with either 30 µg of HCV VLPs alone (a) or HCV VLPs premixed with 30 µg of CFA (b) on days 0 and 9. Splenocytes (1.5×10⁶) harvested from vaccinated mice (1 week after final inoculation) were stimulated with 10 µg ml⁻¹ of either HCV VLPs, or HCV core peptides DLM or YLL for 16 h. Splenocytes were also stimulated with NS5B2594–2602 as an irrelevant peptide to determine background activation and this was subtracted from the HCV VLP-specific responses. The number of IFN-γ-secreting cells was detected by T-cell ELISPOT and reported per million splenocytes. *p<0.05 vs NS5B2594–2602.
To study binding and entry in murine-derived dendritic cells, 1.5 or 4 µg ml\(^{-1}\) FITC-labelled HCV VLPs were then dialysed against PBS overnight at 4 °C. FITC-labelled HCV VLPs were then dialysed against PBS overnight at 4 °C and stored in aliquots at –80 °C.

To study binding and entry in murine-derived dendritic cells, 1.5 or 7.5 µg ml\(^{-1}\) FITC-labelled HCV VLPs were incubated with 4×10\(^5\) CD11c\(^+\) MHC class II\(^+\) murine dendritic cells (Chua et al., 2008). For entry studies, the cells were incubated for a further 1 h at 37 °C in 5 % CO\(_2\). Cells were washed three times with 0.5 % BSA-PBS to remove unbound HCV-VLPs. The cells were fixed in Cytofix (BD Biosciences) for 30 min at 4 °C, then stained with mAb A4 (anti-E1) followed by goat anti-mouse Ig Alexa 594 (Life Technologies). The cells were washed three times with 0.5 % BSA-PBS and resuspended in Prolong Gold Anti-fade Reagent with DAPI (Life Technologies) and viewed by confocal microscopy. To quench extracellular fluorescence, an equal volume of 0.1 M citrate buffer (pH4) containing 250 µg trypan blue ml\(^{-1}\) (Merck) was added to each sample and incubated for 1 min on ice prior to flow cytometry (Chua et al., 2008).

**NAb response to HCV VLPs.** The inhibition of entry of HCV VLPs into Huh7 cells by immune mouse sera was determined using FITC-labelled HCV VLP as described previously (Chua et al., 2012). Inhibition of entry of FITC-labelled HCV VLP was determined by flow cytometry (FACS Canto II, BD Bioscience) and analysed using the WEASEL 2.0 software package (Walter and Eliza Hall Institute, Melbourne, Australia). Sera that demonstrated a decrease in specific cellular binding of 50 % or more were considered neutralizing. As a positive control, inhibition of entry of FITC-HCV VLPs into Huh7 cells was also determined using anti-CD81 antibody (Abcam).

We also determined neutralizing responses using an HCV E1E2-pseudotyped HIV-1 particle (HCVpp) entry assay. Pseudotyped particle entry assays were performed as previously described (Drummer et al., 2003). HEK 293T cells (Centre for Biomedical Research, Burnet Institute, Melbourne, Australia) were cotransfected with 1 µg each of pNL4.3.LUC. R.E’ plus either pE1E2 or empty pcDNA4HisMax vector. At 72 h post-transfection, culture supernatants were filter sterilized (0.45 µm) and applied in triplicate to Huh7 monolayers seeded at 3×10\(^5\) cells per well in 48-well tissue culture dishes (Nunc). At 72 h post-infection, Huh7 cells were lysed and analysed for luciferase activity using the Promega luciferase system and a Fluostar (BMG Labtechnologies) fitted with luminescence optics.

**Inhibition of HCV VLP entry into Huh7 cells by human anti-HCV IgG.** Total IgG was purified from HCV-positive and -negative human sera using Protein A sepharose (Sigma) as described previously (Chua et al., 2012).

For inhibition of entry by anti-HCV antibodies, 5×10\(^5\) Huh7 cells were incubated with 10 % FCS in PBS for 15 min at 4 °C to reduce non-specific binding. The cells were then washed twice and re suspended in 0.1 % FCS-PBS and incubated with 200 ng FITC-labelled HCV VLP together with purified IgG (100 ng to 4 µg) for a further 1 h at 37 °C. The cells were washed three times with 0.1 % FCS-PBS, then fixed with Cytofix (BD Bioscience) according to the manufacturer’s instructions. Inhibition of entry of FITC-labelled HCV VLP was determined by flow cytometry (FACS Canto II, BD Bioscience) and analysed using the WEASEL 2.0 software package (Walter and Eliza Hall Institute, Melbourne, Australia). IgG that demonstrated a decrease in specific cellular binding of 50 % or more were considered neutralizing. The inhibition of entry of HCV VLPs by immune mouse sera was also determined as described previously (Chua et al., 2012). As a positive control, inhibition of entry of FITC-HCV VLPs into Huh7 cells was also determined using anti-CD81 antibody (Abcam).

**HCV-specific T-cell responses in MHC class I transgenic mice.** Groups of C57/Bl6 MHC class I transgenic mice (HHD mice) were inoculated with two doses of either HCV VLPs alone or HCV VLPs adjuvanted with CFA, and splenocytes were harvested 1 week after the final dose. Splenocytes from HCV VLP-vaccinated mice were stimulated with either HCV VLPs, or HCV core-specific peptides HCV core213-219 DLMGYIPLV (DLM) or HCV core252-258 YLPRRGPR (YLL) (Langhans et al., 2004), and HCV-specific T-cell responses were determined by IFN-γ ELISPOT assay as described previously (Chua et al., 2012). In addition, non-specific background was determined by stimulating splenocytes from HCV VLP-vaccinated mice with an irrelevant HCV peptide, NSSB2594–2602, that was not a component of the HCV VLP construct and then subtracting this from HCV VLP-specific responses.

**Statistical analysis.** Statistical analysis was performed using the Prism 5.0 software (GraphPad). In all cases the means±SEM is shown unless otherwise stated. P values for statistical analysis were calculated using a non-parametric Mann–Whitney rank sum test. Differences were considered statistically significant when P values were less than 0.05 (P<0.05) with a 95 % confidence level. Comparison of the neutralization of binding of HCV-positive human IgG against HCV-negative human IgG was performed using two way analysis of variance with a Bonferroni correction for multiple comparisons.

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