Analysis of the binding of hepatitis C virus genotype 1a and 1b E2 glycoproteins to peripheral blood mononuclear cell subsets

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Hepatitis C virus (HCV) binding to hepatocytes is thought to be mediated via interaction of the E2 glycoprotein with (co-)receptors including CD81 and scavenger receptor class B type I (SR-BI). Here, the expression of CD81 and SR-BI was analysed on peripheral blood mononuclear cell (PBMC) subsets, and the binding of genotype 1 soluble truncated E2 (sE2) proteins to these cells was investigated. All PBMC subsets expressed CD81, although at varying levels. In contrast, SR-BI was only detected on monocytes and dendritic cells (DCs). The genotype 1a H77c sE2 protein showed higher PBMC binding than other genotype 1a/b sE2s. H77c sE2 binding to different PBMC subsets largely paralleled their level of CD81 expression, and could be inhibited by blocking E2–CD81 interaction. However, those PBMC subsets reported to be infected by HCV in vivo (monocytes, DCs and B cells) also exhibited residual, CD81-independent binding, indicating roles for SR-BI/other receptor(s) in mediating haematopoietic cell infection.

Approximately 3% of the world’s population is infected with hepatitis C virus (HCV), a member of the family Flaviviridae. Chronic HCV infection is an important cause of liver disease, and is also associated with extrahaematopoietic manifestations including mixed cryoglobulinemia and B cell neoplasias (Ferri & Zignego, 2000). The liver represents the primary site of HCV replication, but the existence of extrahaematopoietic sites of infection, particularly within haematopoietic cells, is supported by evidence including the detection of HCV RNA and replicative intermediates and HCV proteins in certain peripheral blood mononuclear cell (PBMC) subsets (Supplementary Table S1).

HCV attachment/entry into host cells is thought to be mediated by the envelope glycoproteins, E1 and E2, which form non-covalent heterodimers. Cell-surface molecules reported to bind E2 include CD81 (Pileri et al., 1998), scavenger receptor class B type I (SR-BI) (Scarselli et al., 2002), dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) and liver/lymph node (L)-SIGN (Gardner et al., 2003; Lozach et al., 2003; Pohlmann et al., 2003), and glycosaminoglycans (GAGs) (Takikawa et al., 2000; Yagnik et al., 2000; Basu et al., 2004). Of these, DC-SIGN, L-SIGN and GAGs have been suggested to function as capture receptors that facilitate HCV interaction with specific entry receptors (Gardner et al., 2003; Pohlmann et al., 2003; Basu et al., 2004; Lozach et al., 2004), whereas CD81 and SR-BI are thought to act as (co-)receptors that mediate HCV binding and subsequent cell entry and infection (Bartosch et al., 2003b; Zhang et al., 2004). HCV particles associated with low-density lipoprotein (LDL) may also enter cells using the LDL receptor (Agnello et al., 1999; Wunschmann et al., 2000).

E2–receptor interactions may not only be involved in virion attachment/entry, but may also block or mimic receptor functions, contributing to viral persistence/pathogenesis. CD81, a member of the tetraspanin superfamily, has a variety of biological functions, which include the regulation of...
of lymphocyte activation [CD81 cross-linking enhances T/B-cell responses to stimulation through their antigen-specific receptors and inhibits natural killer (NK) cell activation] (Levy et al., 1998; Crotta et al., 2002; Tseng & Klimpel, 2002). SR-BI, a member of the CD36 superfamily, acts as a high-density lipoprotein receptor, mediating cholesterol uptake (Krieger, 2001), but also functions as a pattern-recognition receptor on monocytes and macrophages (Pearson, 1996; Imachi et al., 2000).

To gain insight into HCV interaction with haematopoietic cells and the potential for HCV to modulate the responses of PBMC subsets via receptor interaction, we analysed the expression of HCV (co-)receptors CD81 and SR-BI on PBMC subsets and characterized the binding of genotype 1 soluble truncated recombinant E2 (sE2) proteins to these cells.

PBMC subsets were identified using monoclonal antibodies (mAbs) against distinguishing surface markers (Supplementary Table S1). CD81 and SR-BI expression were analysed by co-staining with a FITC-conjugated anti-CD81 mAb (clone JS-81; PharMingen BD) or anti-SR-BI mAb [clone 3D5 (A. Vitelli and others, unpublished data)] followed by FITC-conjugated anti-mouse IgG/IgM F(ab’), (Jackson Immuno Research). As expected, we observed CD81 expression on the hepatocyte cell line Hep3B and on PBMCs (Fig. 1a). Although all PBMC subsets expressed CD81, there was marked variation in the level of expression detected on different cell types (Fig. 1b). NK, natural T (NT) and T cells expressed high levels of CD81. B cells, monocytes and myeloid DCs expressed intermediate levels of CD81, whilst only low levels of CD81 were detected on plasmacytoid DCs. High level SR-BI expression was observed on hepatocyte cell lines (including Hep3B; Fig. 1c). It has previously been shown that SR-BI is expressed on monocytes and macrophages (Buechler et al., 1999); here, we also documented its expression on plasmacytoid and myeloid DCs (Fig. 1c). SR-BI was not detected on any other PBMC subsets.

To address E2 binding to PBMC subsets, we produced sE2 from the genotype 1a HCV clone H77c by transfecting 293T cells with a plasmid encoding H77c E2 truncated at aa 660 (E2660), carrying a C-terminal histidine tag (Patel et al., 2000). sE2660 was purified from culture supernatants using
Ni²⁺ columns (Amersham Biosciences). sE2 was incubated with PBMCs for 2 h. After washing, E2 binding was detected using anti-E2 mAb H53, which recognizes E2 bound to cell-surface receptors (Flint et al., 1999), followed by FITC-conjugated anti-mouse IgG/IgM F(ab')₂. PBMC subsets were identified by co-staining with mAbs against distinguishing surface markers. H77c E2660 binding to total PBMCs (Fig. 2a) and individual cell subsets (Fig. 2b) was found to be dose-dependent and saturable, indicative of binding to specific cell-surface receptor(s). Notably, differences were observed in the level of E2 binding to individual PBMC subsets (Fig. 2b and c). The E2-binding capacity of different PBMC populations largely paralleled their level of CD81 expression (Supplementary Table S1).

The role played by CD81 in mediating H77c E2660 binding to PBMCs was examined by testing the ability of H33, a conformation-dependent anti-E2 mAb known to block H77c E2660 binding to CD81 (Patel et al., 2000), to inhibit E2 binding to PBMC subsets. Pre-incubation of H77c E2660 with saturating quantities of H33 reduced E2 binding to all PBMC subsets (Fig. 2c), suggesting that CD81 plays a dominant role in mediating E2 binding to PBMCs. Interestingly, the binding of H77c E2660 to some PBMC subsets (NK, NT and T cells) was almost entirely dependent on CD81; but E2 binding to other subsets (monocytes, DCs and B cells) was found to have a CD81-independent component, indicative of E2 binding to additional receptor(s). Similar results were also obtained when PBMCs were pre-incubated with saturating amounts of anti-CD81 mAb before addition of sE2 (Supplementary Fig. S1). Isotype matched control mAbs did not block E2 binding to PBMCs (not shown). H77c E2660 binding to monocyte-derived DCs, which expressed both CD81 and SR-BI, also involved CD81-dependent and CD81-independent components (Supplementary Fig. S2).

Notably, the PBMC subsets found to bind H77c E2660 via both CD81-dependent and CD81-independent mechanisms are those reported to be infected by HCV in vivo (Supplementary Table S1). Thus, although CD81 plays a prominent role in HCV binding to PBMC subsets, this interaction does not necessarily correlate with HCV infection status.

Fig. 2. Binding of H77c E2660 to PBMC subsets. (a) Dose-dependent, saturable binding of H77c E2660 to total PBMCs. The grey shaded histogram represents the binding of the detection mAbs (mAb H53 plus secondary mAb) to PBMCs in the absence of E2, and the line graphs represent staining of PBMCs incubated with the indicated concentrations (µg) of H77c E2660. (b) Dose-dependent, saturable binding of H77c E2660 to NK, NT and T cells. The results shown are the MFI of staining of each subset after incubation of PBMCs with the indicated concentrations of E2, and are representative of three independent experiments. (c) H77c E2660 binding to different PBMC subsets and its blocking by anti-E2 mAb H33. E2660 binding to PBMC subsets is expressed as the MFI of E2-specific staining, i.e. the MFI of cells incubated with E2 and then H53/secondary mAb minus the MFI of cells incubated with detection mAbs only. sE2 was pre-incubated without (black bars) or with (white bars) mAb H33. The results shown are representative of three independent experiments using PBMCs from different donors. (d) Blocking of H77c E2660 binding to monocytes by anti-CD81 and anti-SR-BI mAbs. The MFI of E2-specific staining (with or without antibody pre-treatment) was calculated as described above, and E2 binding to antibody pre-treated cells then expressed as a percentage of E2 binding to monocytes in the absence of blocking mAbs (100%). The data shown are the mean of results obtained in three independent experiments; error bars indicate 1 standard error above the mean.
not appear to be sufficient to allow infection of haematopoietic cells. This observation is in agreement with other studies suggesting that CD81 is required but not sufficient for the HCV attachment/entry process (Bartosch et al., 2003a, b; Hsu et al., 2003; Cormier et al., 2004; McKeating et al., 2004).

Most PBMC subsets exhibiting CD81-independent E2 binding expressed SR-BI; we thus investigated the role of SR-BI in CD81-independent E2 binding to monocytes (Fig. 2d). PBMCs were incubated with saturating quantities of anti-CD81 mAb (clone JS-81), anti-SR-BI mAb (clone 3D5), both mAbs or an isotype control mAb prior to the addition of sE2, and E2 binding to monocytes was analysed using Alexa Fluor 488-labelled mAb H53. The anti-CD81 mAb inhibited > 50% of E2 binding to monocytes. E2 binding was also partially inhibited by the anti-SR-BI mAb, showing that SR-BI played a role in E2 binding to monocytes. When used together, anti-CD81 and anti-SR-BI mAbs blocked H77c E2 binding to monocytes in an additive fashion, but a component of binding remained that could not be eliminated by saturating amounts of both mAbs. This residual binding may be mediated via DC-SIGN and/or GAGs, because a small proportion of CD14+ cells in peripheral blood express DC-SIGN (Turville et al., 2001; Engering et al., 2002) and GAGs are ubiquitously expressed (Kjellen & Lindahl, 1991).

Whilst CD81 is proposed to have a critical role as an attachment receptor, SR-BI is a strong candidate to mediate HCV internalization into cells, as SR-BI internalizes its natural ligands to endosomal compartments (Silver & Tall, 2001; Bocharov et al., 2004). Although SR-BI expression was detected on the majority of PBMC subsets reported to be infected by HCV in vivo, B cells constituted an exception to this; there may thus be an alternative pathway for virion internalization in these cells.

Although HCV infects haematopoietic cells, the liver constitutes the major site of in vivo virus replication. Further, the observation that retroviral particles pseudotyped with the HCV glycoproteins efficiently transduce hepatocytes but not PBMCs (Bartosch et al., 2003a; Cormier et al., 2004) suggests that viral attachment/entry into PBMCs may be suboptimal. Our results show that at least some PBMC subsets are capable of binding E2 via CD81, SR-BI and additional receptor(s); but it is plausible that the level of expression of SR-BI on PBMCs may limit their susceptibility to infection, and/or that PBMCs lack expression of additional factor(s) required for efficient infection of hepatocytes.

Further experiments compared the binding of sE2s derived from H77c and other genotype 1 HCVs to PBMCs. sE2 proteins truncated at aa 661 (E2661) were produced from a second genotype 1a clone, UKN1A14, and two genotype 1b clones, BK and UKN1B12. BK is a well-characterized HCV clone (Scarselli et al., 2002). Plasmids encoding UKN1A14 and UKN1B12 E2661 were generated by subcloning aa 364–661 (corresponding to the HCV-H strain) from E1E2 sequences amplified and cloned from patient serum (as described previously Lavillette et al., 2005) into mammalian expression vector pcDNA3.1 (Invitrogen). Retroviral pseudoparticles expressing E1E2 proteins from these clones were shown to infect hepatocyte cell lines in vitro (Lavillette et al., 2005), indicating the ‘functionality’ of these E2 sequences. Supplementary Fig. S3 shows the sequences of the E2660/661 proteins used in this study. Quantities of each E2 protein normalized to contain equivalent amounts of monomeric E2 (Supplementary Fig. S4) were used in binding assays.

The binding of UKN1A14 and H77c E2660/661 to PBMCs was analysed using anti-E2 mAb H53 [which recognized both genotype 1a proteins equally well in a GNA lectin-capture enzyme immunoassay (EIA) (not shown)], followed by a FITC-conjugated secondary antibody (Fig. 3a). As H53 did not recognize genotype 1b BK and UKN1B12 E2s, an Alexa Fluor 488-conjugated anti-Penta-His mAb (Qiagen) was used for E2 detection in experiments addressing their binding to CD81- and SR-BI-negative subsets.

**Fig. 3.** Comparison of the binding of H77c and other genotype 1 E2 proteins to PBMC subsets. Binding of UKN1A14 E2661 [white bars in (a)], BK E2661 [white bars in (b)] and H77c E2661 (black bars) to PBMC subsets. The results are expressed as the MFI of E2-specific staining, calculated as described in the legend to Fig. 2(c). The results shown are representative of four independent experiments using PBMCs from different donors.
binding to PBMCs (BK E2<sub>661</sub> data are shown in Fig. 3b; similar results were obtained with UKN1B12 E2<sub>661</sub>). UKN1A14, BK and UKN1B12 E2s were all found to exhibit a much lower level of binding to PBMCs than H77c E2<sub>660</sub>. This was likely a reflection of the relative abilities of these E2 proteins to bind to CD81: BK E2<sub>661</sub> was previously reported to have a much lower CD81-binding affinity than H77 E2 (Scarselli et al., 2002), and we also found that UKN1A14, BK and UKN1B12 E2<sub>661</sub> proteins bound much less well than H77c E2<sub>660</sub> to the CD81 large extracellular loop in EIAs (not shown).

Previous studies have indicated that genotype 1 E2 proteins exhibit heterogeneity in their interaction with putative cellular receptors, with several genotype 1b E2s having been shown to bind CD81 much less well than genotype 1a H/H77 E2s (Yagnik et al., 2000; Scarselli et al., 2002; Triyatni et al., 2002; Roccasceca et al., 2003). However, these studies employed genotype 1a E2s with limited sequence diversity, e.g. E2s from clones H77c and H, the genetic distance between which is only 0.7% (McKeating et al., 2004). Our results show that high affinity binding to CD81 is not a common feature of all genotype 1a E2s. Studies with larger panels of diverse genotype 1a E2s are required to determine whether there is any overall difference in the affinity of binding of genotype 1a and 1b E2s to CD81, or whether the extremely high CD81-binding affinity of H/H77c genotype 1a E2s may be anomalous.

The variation in CD81-binding affinity of sE2 proteins from different viruses may not affect their infectivity, as retroviral particles pseudotyped with HCV glycoproteins from clones whose E2 proteins have both low and high CD81-binding abilities have been shown to be infectious in vitro (Bartosch et al., 2003b; McKeating et al., 2004). However, the interaction of E2 with CD81 and other cell-surface proteins may not only be involved in viral entry but also in modulation of host-cell responses. Notably, it has been reported that E2 cross-linking of CD81 on lymphocyte subsets in vitro can modulate their response to activating stimuli in a manner analogous to CD81 cross-linking via anti-CD81 antibodies (Wack et al., 2001; Crotta et al., 2002; Tseng & Klimpel, 2002), suggesting that E2–CD81 interactions may modulate the host-immune response in vivo. If this is the case, it might be predicted that viruses bearing E2 proteins that bind with differential affinity to CD81 may have correspondingly different immunomodulatory capacities, potentially resulting in differences in persistence/pathogenesis.

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