Patient-derived hepatitis C virus and JFH-1 clones differ in their ability to infect human hepatoma cells and lymphocytes

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Hepatitis C virus (HCV) is a hepatotropic virus that also infects cells of the immune system. HCV clones cultivated in human hepatoma Huh-7.5 cells have significantly advanced our understanding of HCV replication and candidate hepatocyte receptors. However, naturally occurring patient-derived HCV, in contrast to the HCV JFH-1 clone, is unable to infect Huh-7.5 cells, while it can replicate in human primary T-cells and selected T-cell lines. To better understand this incongruity, we examined the susceptibility of primary T-cells, PBMCs and T-cell lines to infection with patient-derived HCV, the classical HCV JFH-1 and a cell culture-adapted JFH1T known to be highly infectious to Huh-7.5 cells. We also tested whether Huh-7.5 cells are prone to virus readily infecting T-lymphocytes. The results revealed that while primary T-cells and Molt4 and Jurkat T-cell lines were susceptible to patient-derived HCV, they were resistant to infection with either JFH1T or JFH-1. However, the JFH1T clone interacted more firmly, although non-productively, with the cells than JFH-1. Further, Huh-7.5 cells robustly supported replication of JFH1T but not patient-derived, wild-type virus, despite using highly sensitive detection assays. In conclusion, JFH-1 and JFH1T clones were unable to establish productive infection in human primary T-cells, PBMCs and T-cell lines known to be prone to infection by patient-derived HCV, while Huh-7.5 cells were resistant to infection with naturally occurring virus infecting immune cells. The data showed that the ability to infect lymphocytes is a characteristic of native virus but not laboratory HCV clones.

INTRODUCTION

Hepatitis C virus (HCV) is a small, enveloped RNA virus belonging to the family Flaviviridae that causes a clinically identifiable chronic infection in over 170 million people worldwide (Anonymous, 1999). This form of infection frequently progresses to cirrhosis and hepatocellular carcinoma (Tong et al., 1995). In addition to this clinically apparent chronic infection, HCV can persist as a silent, essentially asymptomatic infection in the liver and the immune system, which is usually detectable by thorough testing for HCV genome expression using nucleic acid amplification assays of enhanced sensitivity (Castillo et al., 2006; Pham et al., 2004, 2008, 2012; Radkowski et al., 2005). Overall, the evidence accumulated in the last decade indicates that HCV not only infects hepatocytes, but also enters and replicates in immune cells, including T-lymphocytes (Blackard et al., 2006; Di Liberto et al., 2006; Durand et al., 2010; Gisbert et al., 2003; Lerat et al., 1996; MacParland et al., 2006; Pal et al., 2006; Pham et al., 2004, 2005, 2008, 2010; Sarhan et al., 2012). Since the discovery of HCV (Choo et al., 1989), there have been significant efforts to establish a robust in vitro cell-culture system and a small-animal model supporting the entire replication cycle of native, naturally occurring virus (Meuleman & Leroux-Roels, 2009; Wakita et al., 2005). With the isolation and cloning of an HCV strain from a Japanese patient with fulminant hepatitis (JFH-1) (Wakita et al., 2005), this goal was partially achieved and a clone propagating to high levels in the human hepatoma Huh-7.5 cell line and producing virus particles infectious to these cells has been established. This robust system and its subsequent modifications provided an important tool for molecular studies of the HCV life cycle and preclinical testing of novel antiviral strategies. However, the degree to which this system mimics the actual events occurring during in vivo infection with wild-type HCV (wHCV) of human hepatocytes remains unsettled. In this regard, Kato et al. (2001) demonstrated a significant genetic distance between JFH-1 and the wHCV derived from patients with...
chronic hepatitis C (CHC). The phylogenetic analysis showed that although the JFH-1 clone clusters with genotype 2a viruses from patients with CHC, there are numerous amino acid deviations from the wild-type virus sequences that are most prominent in the core, NS3 and NS5A genomic regions, with a mean genetic distance of 0.1136 ± 0.0073 (Kato et al. 2001). These differences, in addition to the unique immune-deficient milieu existing within Huh-7.5 cells and abnormalities in their signalling and endocytic pathways and function of mitochondria, may account for the clone’s ability to replicate in Huh-7.5 cells (Buck & Chojkier, 1996; Damm et al., 2005; Jones et al., 1998; Korenaga et al., 2005; Kroemer & Jäättelä, 2005). Although the JFH-1 clone is highly infectious to Huh-7.5 cells, it displays a very low replication capacity and liver pathogenic potency with a short course of infection in experimentally infected chimpanzees, which represents the closest immunopathogenic model of human HCV infection and hepatitis C (Kato et al., 2008; Lindenbach et al., 2006). Further, it has been shown that administration of plasma-derived HCV to chimpanzees induces infection of PBMCs (Shimizu et al., 1997, 1998), whereas a similar situation has not yet been reported for JFH-1. In HCV-infected patients, HCV of different genotypes, unique variants distinct from these occurring in the liver or plasma, as well as virus RNA-negative (replicative) strand and intracellularly expressed viral proteins, have been identified in different subsets of circulating immune cells (Forion et al., 2004; Lerat et al., 1996; Pham et al., 2008, 2009; Roque-Afonso et al., 2005). The ability of patient-derived wHCV to infect in vitro PBMCs, T- and B-lymphocytes, and monocytes/macrophages has also been shown (Blackard et al., 2006; Di Liberto et al., 2006; Durand et al., 2010; Gisbert et al., 2003; Lerat et al., 1996; MacParland et al., 2006; Pal et al., 2006; Pham et al., 2008). In contrast, JFH-1 was found to be non-infectious to human lymphoid cells (Marukian et al., 2008; Murakami et al., 2008).

In the current study, we utilized an adapted JFH-1 virus, designated the JFH1 T clone, which has been found to be highly infectious to Huh-7.5 cells with 100–1000-fold greater production of virus particles than the classical JFH-1 strain (Jones et al., 2011; Russell et al., 2008). We aimed to identify whether such a highly infectious, readily available virus can infect human primary T-cells and/or T-cell lines previously identified to be prone to infection with wHCV (MacParland et al., 2006, 2009; Sarhan et al., 2012) and, if so, whether the clone can be utilized in studies on HCV lymphotropism. For comparison, the same target cells exposed to the classical JFH-1 clone or patient-derived HCV were analysed. Further, to determine whether Huh-7.5 cells are resistant to infection with wHCV, we investigated cells exposed to infectious HCV derived from different patients with progressing CHC for the expression of HCV genome and evidence of virus replication using highly sensitive HCV RNA positive- and negative-strand detection assays.

RESULTS

Susceptibility of primary T-lymphocytes to infection with JFH-1 and JFH1 T

Primary human T-cells from healthy donors were exposed to either classical JFH-1 strain or JFH1 T and, after 7 or 10 day culture, evaluated for the expression of HCV RNA. As shown in Fig. 1(a), the HCV RNA positive strand was detected in T-cells exposed to JFH1 T virus, but not to

![Fig. 1. Determination of the presence of HCV genome-positive and -negative (replicative) strands in human primary T-cells exposed to HCV JFH-1 or JFH1 T. Primary total T-cells affinity-purified from a healthy donor were exposed to the same copy numbers of JFH-1 or JFH1 T clones in two parallel experiments (Exp 1 and Exp 2), as described in Methods. (a) Identification of HCV RNA positive strand. Synthetic HCV RNA positive strand (sHCV RNA) at 10^5 copies per reaction was used as a positive and specificity control. (b) Testing for HCV RNA negative strand. Synthetic HCV RNA-positive (pos) and -negative (neg) strands at 10^4 copies per reaction confirmed the assay specificity for detection of the virus RNA negative strand. Water amplified in direct (DW) and nested (NW) reactions and a mock extraction served as contamination controls. The positive signals showed the expected 244 bp 5′-UTR sequence-specific fragments.](image-url)
JFH-1 virus. Comparable results were obtained from two experiments in which T-cells obtained from a healthy donor were tested (Fig. 1a). To assess whether the detection of HCV RNA positive strand in the cells exposed to JFH1_T reflected active virus replication or virus potentially adhering to the cells, the expression of HCV RNA negative (replicative) strand was evaluated. The results showed that the detection of the HCV RNA positive strand did not coincide with the presence of the virus replicative intermediate in primary T-cells exposed to JFH1_T (Fig. 1b). Therefore, the data demonstrated that neither JFH-1 nor JFH1_T was able to establish replication in primary T-lymphocytes, although JFH1_T tended to adhere to these cells more firmly than JFH-1.

**Fig. 2.** Infectivity of patient-derived HCV and JFH1_T clone towards different human T-cell lines and Huh-7.5 cells. Molt4 and Jurkat T-cell lines susceptible to patient-derived HCV (wHCV), PM1 and CEM T-cells resistant to infection with that virus, and hepatoma Huh-7.5 cells were exposed to comparable copy numbers of wHCV or JFH1_T, as outlined as in Methods. (a) Quantification of the HCV RNA positive strand by real-time RT-PCR. (b) Molt4 and Jurkat cells exposed to wHCV or JFH1_T were evaluated for the presence of the HCV RNA positive strand by RT-PCR/nucleic acid hybridization (NAH). The relative density of the RT-PCR/NAH signals was quantified by densitometry, as described in Methods. The data represent means ± SD from four experiments. (c) Identification of the HCV RNA negative strand in T-cell lines and Huh-7.5 cells exposed to wHCV or JFH1_T clone. Specificity and contamination controls for (b) and (c) were as those described in the legend to Fig. 1. (d) Identification of HCV NS5a protein by confocal microscopy in Molt4 and Jurkat T-cells exposed to JFH1_T clone or wHCV. Green fluorescence represents HCV NS5a protein in HCV-infected cells. The cells were counterstained with DAPI to identify nuclei and examined under transmitted light (TL) to visualize the cytoplasm. Magnification, ×40.
Infectivity of patient-derived HCV versus JFH1T towards T-cell lines and Huh-7.5 cells

Molt4, Jurkat, PM1 and CEM T-cells, as well as Huh-7.5 cells, were exposed to wHCV or JFH1T virus and, after culture, the cells were evaluated for expression of HCV by real-time RT-PCR. As shown for wHCV in Fig. 2(a), HCV RNA positive strand was detected in Molt4 and Jurkat T-cell lines but not in PM1 or CEM cells, while Huh-7.5 cells showed trace signals not exceeding 10 virus genome equivalents (vge) (µg total RNA)^−1. On the other hand, T-cell lines exposed to JFH1T showed variable but overall very low levels of the HCV RNA positive strand ranging between 10 and 10^3 vge (µg total RNA)^−1 (Fig. 2a), which did not further increase following exposure to 10- and 100-fold greater vge of JFH1T (data not shown), while Huh-7.5 cells, as expected, displayed very high levels exceeding 10^7 vge (µg total RNA)^−1 (Fig. 2a). Further, Molt4 and Jurkat T-cells, which have been previously found to be prone to infection with patient-derived HCV (Sarhan et al., 2012), after exposure to wHCV showed 10^5–10^7-fold greater levels (P<0.001) of HCV RNA positive strand expression than those exposed to the same copy numbers of JFH1T (Fig. 2a, b). In contrast, PM1 and CEM T-cells, which were identified as being resistant to infection with wHCV (Sarhan et al., 2012), were HCV RNA non-reactive as expected. In order to determine which of the cells were in fact infected, the expression of HCV RNA negative strand was examined. In agreement with the previous findings (Sarhan et al., 2012), only Molt4 and Jurkat T-cells lines exposed to patient-derived HCV expressed HCV genome replicative intermediate (Fig. 2c). In contrast, PM1 and CEM T-cells exposed to wHCV, as well as all cell types exposed to JFH1T virus, except Huh-7.5 cells (Fig. 2c), were negative for HCV RNA negative strand. Thus, these results confirmed that, on the one hand, wHCV recognizes and infects cultured T-cells, which have been previously identified to be susceptible to this type of virus (Sarhan et al., 2012), but it does not infect Huh-7.5 cells. On the other hand, they showed that the JFH1T done, similar to JFH-1, does not infect patient-derived virus-susceptible T-cell lines. However, in contrast to JFH-1, JFH1T showed somewhat greater ability to interact with the cells, probably with their surface plasma membranes, as suggested by the results on HCV RNA positive-strand detection (Fig. 1a). This may be due to the presence of the N417S adaptive mutation in the JFH1T, which is proposed to increase E2–CD81 binding efficiency (Russell et al., 2008).

The susceptibility of Molt4 and Jurkat T-cells to wHCV was also confirmed by identification of cytoplasmic distribution of the virus NS5a protein (Fig. 2d), which was consistent with the data reported previously (Sarhan et al., 2012). The percentages of NS5a-positive cells enumerated under a confocal microscope were 2.5–4.5 % for Molt4 cells and 1.5–4 % for JurkaT-cells. These numbers were comparable to those found before for wHCV-infected Molt4 cells using the same enumeration method, i.e. between 2 and 5 % (Sarhan et al., 2012), and they did not change meaningfully when the ratio of wHCV to T-cells was increased by 10-fold, i.e. from 1:1 to 10:1. No NS5A-positive cells were detected when Molt4 or Jurkat cells were exposed to JFH-1 or JFH-1T.

Patient-derived HCV but not JFH1T virus infects circulating lymphoid cells

To further test whether human PBMCs are susceptible to laboratory-adapted JFH1T, phytohaemagglutinin (PHA)-stimulated PBMCs (MacParland et al., 2006) obtained from a healthy donor were exposed to JFH1T or to naturally circulating HCV from three different patients with progressing CHC. After culture, cells were evaluated for the expression of HCV RNA positive and negative strands. The results showed that circulating lymphoid cells exposed to patient-derived HCV became HCV RNA both positive strand- and negative strand-reactive, while those exposed to JFH1T displayed weak positive-strand signals and were negative strand non-reactive (Fig. 3). Thus, the data implied that PBMCs were prone to infection with wHCV, which is consistent with previous reports (MacParland et al., 2006; Sarhan et al., 2012), but not to infection with JFH1T.

![Fig. 3. Patient-derived HCV but not JFH1T virus infects human lymphomononuclear cells. PHA-stimulated PBMCs isolated from a healthy donor were exposed to 1×10^5 copies of JFH1T virus or wHCV from three different patients with progressing untreated CHC. (a) Identification of the HCV RNA positive strand. (b) Detection of the HCV RNA negative (replicative) strand. Specificity and contamination controls were as those described in the legend to Fig. 1.](image-url)
Fig. 4. Huh-7.5 cells are susceptible to infection with JFH1<sub>T</sub> but not with patient-derived HCV. Equal numbers of Huh-7.5 cells were exposed to comparable copy numbers of either JFH1<sub>T</sub> virus or wHCV. Cells exposed to normal human plasma (NHP) or Dulbecco’s modified Eagle’s medium (DMEM) served as negative controls. Green fluorescence represents HCV core protein in HCV-infected cells. Nuclei were counterstained with DAPI. Magnification, ×40.
Huh-7.5 cells are not susceptible to infection with patient-derived HCV

Finally, to further our understanding of the differences in infectivity between wHCV and the JFH1T virus, Huh-7.5 cells were exposed in parallel to either JFH1 or wHCV (10^4 vge ml^-1) and cultured. The cells were examined for the expression of HCV core protein 3 days post-infection (p.i.). The data showed that while Huh-7.5 cells robustly supported replication of JFH1T virus, they were not susceptible to infection with the wHCV, as illustrated in Fig. 4. In a supplementary experiment, exposure of Huh7.5 cells to wHCV inocula at a 10-fold greater virus:cell ratio than that above also did not produce detectable signals either on the protein level or when expression of HCV RNA negative strand was evaluated (data not shown).

DISCUSSION

Although HCV is a highly hepatotropic virus, the data accumulated in recent years have documented that the virus also invades and replicates in cells of the immune system (Blackard et al., 2006; Di Liberto et al., 2006; Durand et al., 2010; Gisbert et al., 2003; Lerat et al., 1996; MacParland et al., 2006, 2009; Pal et al., 2006; Pham et al., 2004, 2005, 2008, 2012; Sarhan et al., 2012). The lack of small-animal models and a robust tissue-culture system capable of supporting infection with patient-derived HCV hindered the studies on the virus–host cell interactions occurring in vivo, particularly those mediating the initial host cell’s target recognition and associations with functionally important intracellular factors or pathways operating within naturally infected cells. Since hepatoma HuH7 cells differ significantly from normal human hepatocytes due to their deficient innate immune response, perturbed physiological functions and functionally altered mitochondria (Buck & Chojkier, 1996; Damm et al., 2005; Jones et al., 1998; Korenaga et al., 2005; Kroemer & Jäätelä, 2005) and since the JFH-1 and related clones are highly adapted to propagate in these cells and they are molecularly distinct from wild-type virus, the virus–cell interactions observed in this system should be interpreted with caution. The finding that molecularly intact wHCV can productively infect normal human primary T-cells (MacParland et al., 2006; Sarhan et al., 2012; S. G. Skardasi & T. I. Michalak, unpublished results) provides the means to investigate naturally occurring events, although in the context of lymphoid cells only, and to assess, for example, the effectiveness and the mode of action of agents interfering with replication of fully infectious and pathogenic HCV. The application of the wHCV-T-cell infection model has already led to the following findings: (i) that unique HCV variants emerge during replication in primary T-cells (MacParland et al., 2006); (ii) that different HCV genotypes, including genotypes 1, 2a and 3, can infect cultured T-cells (MacParland et al., 2006, 2009; S. G. Skardasi & T. I. Michalak, unpublished results); (iii) that the lymphocyte-specific CD5 molecule plays an important role in HCV infection of T-lymphocytes (Sarhan et al., 2012); (iv) that infection of T-cells by wHCV can be inhibited by HCV-specific protease inhibitor telaprevir and alpha interferon, and blocked by both anti-CD5 and anti-CD81 antibodies (MacParland et al., 2006; Sarhan et al., 2012); and (v) that HCV persisting after clinically apparent sustained virological response to antiviral therapy retains infectivity in vitro (MacParland et al., 2009). However, in an attempt to simplify investigations on HCV lymphotropism, a search for a readily available and well-characterized virus was undertaken. The cell-culture-derived JFH1T clone, showing greatly enhanced infectivity and robust replication in Huh7.5 cells compared with the classical JFH-1 virus (Jones et al., 2011; Russell et al., 2008), appeared to be the best candidate. Therefore, in the current work, we wanted to delineate, via comparative analysis of the infectivity towards different T-lymphocyte targets, whether JFH1T virus would be a suitable analogue of wHCV in studies on HCV lymphotropism.

Analysis of the HCV RNA positive strand revealed a relatively strong signal in primary T-cells exposed to JFH1T that was not detectable in cells exposed to JFH-1, suggesting that the JFH1T clone tended to bind to the T-cells more tightly than JFH-1, despite routinely performed extensive washing of the cells after inoculation with virus (Fig. 1). However, examination for the HCV RNA negative (replicative) strand revealed a lack of detectable signal, indicating that JFH1T did not replicate in primary T-cells or that its replication progressed at a very low level not identifiable by otherwise highly sensitive RT-PCR/NAH assay (sensitivity approx. 100 copies per reaction). Detection of the HCV RNA positive strand in culture supernatants of T-cells exposed to JFH1T could be interpreted in support of this possibility. The JFH1T clone also interacted to a variable degree with all other types of T-cells examined, including T-cell lines either susceptible or not to infection with wHCV, as shown by analysis of the HCV RNA positive strand (Figs 2a, b, 3a). However, the HCV RNA replicative strand was not detected in any of the cells except Huh-7.5 cells, as expected (Fig. 2c). In the future, it would be interesting to explore whether this non-productive interaction between JFH1T and T-cells reflects naturally occurring HCV–cell binding or is a phenomenon unique to this clone, since JFH-1 virus did not display such binding reactivity.

In contrast to cells exposed to JFH1T virus, PBMCs as well as Molt4 and Jurkat T-cell lines exposed to patient-derived wHCV demonstrated readily detectable HCV RNA replicative strand (Figs 2c, 3b), while PM1 and CEM cells, known to be resistant to infection with wHCV (Sarhan et al., 2012), were negative strand non-reactive (see Fig. 2c). Taken together, a clear picture emerged, indicating that while molecularly intact, patient-derived HCV was infectious and capable of replication in primary lymphoid cells and T-cell lines previously identified to be prone to this type of virus (MacParland et al., 2006, 2009; Sarhan et al., 2012), the JFH1T clone which is highly infectious to Huh-7.5 cells was unable to establish replication in either primary lymphocytes or wHCV-prone T-cell lines. Similarly, it has been shown
that JFH-1 did not infect T- or B-lymphocytes, monocytes, macrophages or dendritic cells from healthy donors (Marukian et al., 2008). Also, T-cells and PBMCs were not recognized by HCV pseudoparticles; however, they were found to enter hepatoma Huh7 and HepG2 cells transfected with CD81 (Cormier et al., 2004).

On the other hand, Huh-7.5 cells were highly susceptible to infection with the JFH1T clone, as documented before (Jones et al., 2011; Russell et al., 2008), while being entirely resistant to infection with wHCV despite the fact that highly sensitive assays for detection of HCV RNA positive and negative strands were used throughout the study (Pham et al., 2004; Sarhan et al., 2012). This was consistent with the results on HCV core protein staining in Huh-7.5 cells exposed to JFH1T or wHCV. Thus, cells exposed to JFH1T, but not those incubated with patient-derived HCV infectious to T-lymphocytes, were reactive for this protein.

The results from this study documented a contrasting difference between patient-derived HCV and JFH1T or JFH-1 clones in infectivity of human primary and cultured lymphocytes, and they showed that the mutations introduced into JFH1T, while greatly enhancing its infectivity of Huh-7.5 cells, were without effect on infection of lymphocytes. This divergence could be attributed, as already mentioned, to the significant genetic differences between the naturally occurring HCV and the cloned and cell culture-adapted JFH-1 viruses. However, at this stage, it cannot be completely excluded that the plasma-derived HCV may contain a minor subpopulation of lymphotropic variants or carry host factors predisposing virus to attach, enter and/or replicate preferentially in lymphoid cells, which are absent in the preparations of the cell culture-derived JFH-1-related viruses.

In conclusion, our current study clearly shows that primary lymphocytes and selected T-cell lines can support replication of patient-derived wHCV, as has been documented previously (MacParland et al., 2006, 2009; Sarhan et al., 2012), but not the otherwise highly infectious JFH-1-related strain. Since JFH-1-derived viruses are unlikely to be suitable for investigations on HCV lymphotropism, there is a need for generation of HCV clones displaying properties more closely resembling those of the wild-type virus circulating in patients which can be applied for the type of investigations mentioned. Nonetheless, this and our previous studies (MacParland et al., 2006, 2009; Sarhan et al., 2012) showed that, although using virus directly derived from HCV-infected patients for in vitro experiments is highly challenging, it is also fully feasible and can generate valuable data, which at this stage cannot be obtained otherwise.

**METHODS**

**Cell lines, primary cells and culture conditions.** Molt4 (CRL-1582) and Jurkat (TIB-152) T-cell lines were provided by the ATCC. These cells were found to be susceptible to patient-derived wHCV and to be able to support its replication, as documented in our previous studies (Sarhan et al., 2012). PM1 cells were acquired from the NIH AIDS Research and Reference Program (Rockville, MD, USA) and CCRF-CEM cells (CEM, ACC-240) from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). These two T-cell lines were not permissive to infection with naturally occurring HCV; however, PM1 cells could be infected with wHCV after stimulation with phorbol myristate acetate (PMA) and inomycin (Sigma-Aldrich), as reported previously (Sarhan et al., 2012). The cells were cultured at 1 × 10^6 cells per well in 5 ml medium containing RPMI 1640 (Invitrogen) supplemented with 10% heat-inactivated FCS, 2 mM glutamine and 0.1 mM non-essential amino acids (Invitrogen). Total PBMCs were isolated from healthy donors by gradient centrifugation in Ficoll-HyPaque (Pharmacia), as described elsewhere (Pham et al., 2004). Primary T-lymphocytes were affinity-purified from monocyte-depleted PBMCs of a healthy human donor by negative selection using MACS magnetic microbeads (Miltenyi Biotec), as reported previously (Pham et al., 2005, 2008; Sarhan et al., 2012). T-cells were 98% pure by flow cytometry evaluations. PBMCs and affinity-purified primary T-cells were stimulated with 5 μg ml⁻¹ PHA (Sigma-Aldrich) for 24 h before exposure to virus (MacParland et al., 2006). The human hepatoma-derived Huh-7.5 cells were propagated in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) supplemented with 10% FCS and 1% penicillin/streptomycin.

**HCV JFH-1 and JFH1T infectious strains.** The classical HCV JFH-1 strain and its derivative JFH1T, provided by Dr Rodney Russell, Memorial University, St John’s, Newfoundland and Labrador, Canada, were used for a comparative infection study of T-cells and Huh-7.5 cells. The JFH1T strain carries a non-synonymous point mutation in each of E2, p7 and NS2 coding sequences and has demonstrated 100- to 1000-fold greater ability to infect Huh-7.5 cells than the classical JFH-1 virus (Russell et al., 2008).

JFH-1 or its triple mutant JFH1T was propagated in Huh-7.5 cells following a protocol reported elsewhere (Jones et al., 2011; Russell et al., 2008). Briefly, cells were transfected with RNA transcribed from linearized DNA plasmids using DMRIE-C transfection reagent (Invitrogen). Then, the cells were washed and cultured in DMEM medium for 72 h. The resulting culture supernatants were assayed for the virus infectious titre by limiting dilutions in a focus-formation assay and titres were expressed as the number of f.f.u. (ml culture supernatant)⁻¹, as reported elsewhere (Jones et al., 2011; Russell et al., 2008). In addition, JFH-1 and JFH1T genome copy numbers (virus genome equivalent, vge) were quantified by real-time RT-PCR, as described below and reported in detail previously (Pham et al., 2004).

**Patient-derived HCV.** Plasma from three male patients aged 44–52 years with progressing antiviral therapy-naïve CHC, carrying HCV amplification 2.0 kit (LIPA; Siemens Healthcare Diagnostics). HCV genotype was determined using a Versant HCV amplification 2.0 kit (LIPA; Siemens Healthcare Diagnostics). PBMCs of these individuals contained the HCV RNA positive strand at between 1.6 × 10^6 and 2 × 10^7 vge (μg total RNA)⁻¹ and were HCV RNA replicative strand-reactive. In preliminary experiments, the inocula demonstrated comparable infectivity towards HCV-naïve Molt4 and Jurkat cell lines, as well as total PBMCs and affinity-purified primary T-cells, when tested at virus:cell ratios of 0.1, 1.0 and 10 vge per cell.

**Infection assays with patient-derived HCV and JFH strains.** T-cell lines, primary T-cells and PBMCs were cultured at 1 × 10^6 cells per well in six-well plates in 2 ml culture medium. For primary T-cells and PBMCs, the cells were pretreated with 5 μg PHA ml⁻¹ for 24 h before adding inocula. Then, cells were exposed to 1 × 10^5 vge
wHCV or $1 \times 10^5$ vge JFH-1 or JFH1-T in the presence of 100 µl of normal human plasma (NHP). In addition, cells treated under identical conditions and exposed to an equivalent volume of NHP served as negative controls. After exposure to either HCV preparations or NHP, the cells were extensively washed and cultured for 7–10 days p.i. in the presence of either PHA alone or PHA and human recombinant interleukin-2 (20 IU ml$^{-1}$; Roche), as reported previously (MacParland et al., 2006). For T-cell lines, the cells were extensively washed after exposure to inoculum, supplemented with fresh medium and cultured for 4–7 days p.i., as reported in detail previously (Sarhan et al., 2012). In some experiments, $1 \times 10^5$ cells were also incubated with $1 \times 10^5$ or $1 \times 10^4$ vge of JFH1-T clone in the presence of NHP (as above).

For infection of Huh-7.5 cells, the cells were seeded at $1 \times 10^4$ in six-well plates or at $1 \times 10^5$ cells per well in eight-well chamber slides and, after 24 h incubation, exposed to $1 \times 10^5$ or $1 \times 10^4$ vge ml$^{-1}$, respectively, at an m.o.i. of 1.0 for JFH-1 or JFH1-T–containing filtered culture supernatant or patient-derived HCV. In the supplementary experiment, $1 \times 10^4$ Huh-7.5 cells per well were incubated as above with patient-derived inocula at virus:cell ratios of 1.0 or 10. Cells exposed to NHP or culture supernatant from non-infected Huh-7.5 were used as controls. After 4 h exposure, the inocula were removed, and cells were washed and cultured for 3 days.

**RNA extraction and cDNA transcription.** Total RNA was extracted from $1 \times 10^6$ to $1 \times 10^5$ cells using TRIzol or from 250 µl patient plasma or 300 µl cell-culture supernatant with TRIzol S reagent (both Invitrogen). Mock extractions were performed in parallel as contamination controls (Pham et al., 2004). cDNA was transcribed with Moloney murine leukemia virus reverse transcriptase (RT) (Invitrogen), as described previously (Pham et al., 2004; Sarhan et al., 2012).

**Detection of HCV by RT-PCR/nucleic acid hybridization (NAH) assays.** HCV RNA positive and negative (replicative) strands were detected using cDNA derived from 2 and 4 µg total RNA, respectively, using the strand-specific amplification conditions reported in detail previously (Pham et al., 2004; Sarhan et al., 2012). The specificity of the signal detection and validity of controls were routinely confirmed by NAH with $1^{38}$-labelled recombinant HCV S'-UTR-E2 fragment (rHCV UTR-E2) as a probe (Pham et al., 2004). The sensitivity of RT-PCR/NAH assay for HCV RNA-positive strand identification was $<10$ vge ml$^{-1}$ (or $<2$ IU ml$^{-1}$) or $<5$ vge (µg total RNA)$^{-1}$, while that for HCV RNA-negative strand was 25–50 vge (µg total RNA)$^{-1}$, as reported before (Pham et al., 2004; Sarhan et al., 2012). The relative density of the RT-PCR/NAH signals was quantified by densitometry computer-assisted analysis using Image* software from NIH (Bethesda, MD, USA) and presented in relative density units.

**Quantification of HCV by real-time RT-PCR.** Enumeration of HCV RNA copy numbers was done by real-time RT-PCR during 45 cycles using the LightCycler 480 (Roche) and conditions previously reported (Sarhan et al., 2012). Briefly, reactions were performed in 10 µl volumes, each containing 2 µl cDNA derived from 50 ng total RNA using primers described before (Pham et al., 2004; Sarhan et al., 2012). Tenfold serial dilutions of rHCV UTR-E2 were used as standards to determine the HCV genome copy numbers. The sensitivity of the assay was between 10 and 10$^3$ vge per reaction.

**Detection of HCV proteins by confocal microscopy.** Huh-7.5 cells exposed to wHCV or infected with JFH1-T as a positive control were grown on chamber slides and then fixed in acetone for 2 min, washed with PBS, pH 7.4 (PBS), and incubated with anti-HCV core mouse mAb (B2; Anogen-YES Biotech Laboratories) at 1:1000 for 20 min at ambient temperature. Subsequently, cells were washed with PBS and incubated with Alexa Fluor 488-conjugated anti-mouse antibody for 20 min. Finally, cells were washed again and mounted using Vectashield hard-set mounting medium with DAPI (Vector Laboratories). To detect HCV NS5A protein in Molt4 and Jurkat cells infected with wHCV, cells were fixed with 2 % paraformaldehyde and permeabilized with 0.25 % saponin (Sigma-Aldrich). Staining with mouse anti-HCV NS5A mAb (Chemicon) or appropriate isotype antibody control (BD Biosciences Pharmingen) was done as reported previously (Sarhan et al., 2012). Cells were examined under a confocal microscope and images were recorded at magnification × 40. Approximately 1000 cells per preparation were examined, positive cells were counted, and the percentage of positive cells versus the total cell number was calculated, as reported before (MacParland et al., 2009; Sarhan et al., 2012).

**Statistical analyses.** Results were analysed by a one-way analysis of variance or unpaired Student’s t-test with Welch’s correction using GraphPad Prism software (GraphPad Software). Differences between experimental conditions were considered to be significant when two-sided P values were ≤ 0.05.

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