tional, Concord, MA) and pulsed with ^{3}H-thymidine (GE Healthcare BioSciences, Piscataway, NJ). T-cell stimulation was expressed as a stimulation index that was calculated as the ratio of average counts per minute (CPM) of antigen-stimulated proliferation over average CPM of the medium background. A sample was considered positive when the average stimulation index was greater than 5. The numbers of antigen-specific interferon gamma (IFN-γ)–producing cells were analyzed by enzyme-linked immunosorbent spot (ELISpot) assay. PBMCs were stimulated with recombinant protein antigens (HCV core and NS5a proteins) and HCV overlapping peptide pools (15mers overlapped by 10 amino acids) from core (38 peptides, amino acids 1-195) and NS3 (56 peptides, amino acids 1031-315)(Mimotopes, Raleigh, NC). The NS3 overlapping peptide pools were divided into two sets. The number of spots was counted by using a computer-assisted AID ELISpot Reader System and AID software version 3.5 (Autoimmune Diagnostika GmbH, Strassberg, Germany). Antigen-specific spot-forming unit (SFU) was calculated by subtracting the average of background values (four wells without antigen, typically fewer than 10 spots) from that of the antigen-stimulated sample. The sample was considered positive when the background-corrected SFU was greater than 10 and twice or more the mean SFU of the preinfection samples in the same animal.

To specifically evaluate the T-cell response against the NS2 region containing the G838R mutation, two peptides of 18 amino acids (NS2-G: ITLFTLTPGYKTLLGQCL and NS2-R: ITLFTLTPRYKTLLGQCL) were synthesized (Sigma-Genosys, The Woodlands, TX). PBMCs from both chimpanzees were stimulated with the wild-type (WT) and mutant peptides (2 μg/mL) and analyzed for IFN-γ production by IFN-γ ELISpot assays as described.

Production of JFH-1 G838R Mutant Virus. The full genome JFH-1 construct with G838R mutation in the NS2 region was generated by site-directed mutagenesis. The replication-deficient clone of JFH1 generated by introducing a point mutation into the GDD motif of the NS5B to abolish the RNA-dependent RNA polymerase activity was used as a negative control (JFH-1 GND).[8]

Quantification of HCV RNA and HCV Core Antigen. To determine the amount of HCV, total RNA was extracted with QIAamp Viral RNA Kit from 140 μL culture medium, or with RNeasy mini kit (QIAGEN, Valencia, CA) from cell pellet. Copy numbers of HCV RNA were determined by real-time quantitative RT-PCR as described. HCV core antigen (Ag) in culture supernatant was quantified by highly sensitive enzyme immunoassay (Ortho HCV core antigen ELISA Kit, Ortho Clinical Diagnostics, Tokyo, Japan).[15] To determine intracellular HCV core Ag, the cell pellet was resuspended with 100 μL radioimmune precipitation assay buffer containing 1% sodium dodecyl sulfate, 0.5% NP40, 10 mM Tris-HCl (pH 7.4), 1 mM ethylenediaminetetra-acetic acid, 150 mM NaCl, and Complete Mini protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN), then sonicated 10 minutes and subjected to the Ortho HCV core antigen enzyme-linked immunosorbent assay after centrifugation.

Titration of HCV Infectivity. To assess the intracellular infectivity, cells were harvested by treatment with trypsin-ethylenediaminetetra-acetic acid and pelleted by centrifugation. Cell pellets were resuspended with 500 μL Dulbecco's modified Eagle's medium with 10% fetal bovine serum and lysed by four freeze–thaw cycles. The supernatant was collected after centrifugation and passage through a 0.45-μm filter. These cell lysates and culture supernatants were serially diluted fivefold and inoculated into naïve Huh7.5.1 cells seeded at 1×10^4 cells/well in 96-well flat-bottom plates and assayed for focus-forming unit (FFU) by anti-core immunofluorescence as described previously.[16]

Statistical Analysis. Data from repeated experiments were averaged and expressed as mean ± standard deviation. Statistical analysis was performed using the Mann-Whitney test. *P* values of less than 0.05 were considered statistically significant.

Results

Clinical, Virological, and Immunological Profiles of JFH-1–Infected Chimpanzees. Chimpanzee 10273 (CH10273) was inoculated with patient serum containing 9.6×10^6 copies of HCV RNA. Chimpanzee 10274 (CH10274) was inoculated with 1.4×10^7 copies of JFH-1cc in culture medium. In both chimpanzees, HCV RNA became detectable in serum by RT-PCR 3 days after inoculation. Viremia was low, with titers of approximately 10^3 copies/mL. Serum ALT levels were within normal limits, and histological observation of liver biopsy showed no evidence of hepatitis (Fig. 1). In CH10273, HCV RNA in serum fluctuated but persisted for 34 weeks after inoculation, and anti-HCV was detected from 20 weeks after inoculation (Fig. 1A). In CH10274, serum HCV RNA disappeared at 9 weeks after inoculation, and no anti-HCV seroconversion was observed (Fig. 1B).

Immunological analysis for T-cell proliferation and IFN-γ production showed that HCV-specific immune responses were induced in both animals (Fig. 1). Their responses corresponded to the profiles of viremia and remained at low levels after disappearance of viremia. The

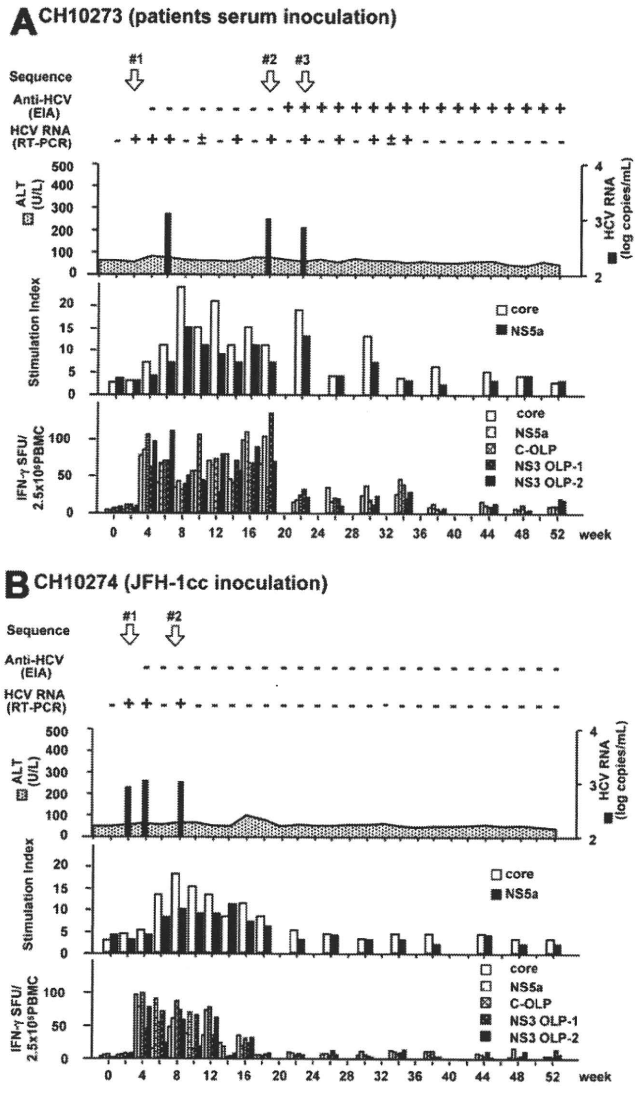


Fig. 1. Infection profiles and T cell immune responses in patient serum-inoculated and JFH-1cc-inoculated chimpanzees. (A) Chimpanzee CH10273 was inoculated with patient serum containing 9.6×10^6 copies of HCV. (B) Chimpanzee CH10274 was inoculated with JFH-1cc containing 1.4×10^7 copies of HCV. White arrows indicate the time points at which HCV sequences were determined. T cell proliferation assay results against HCV core and NS5a are shown as stimulation index (middle panel). IFN-γ responses against HCV core and NS5a proteins or overlapping peptide pools of core and NS3 are shown as SFU per 2.5×10^5 cells (bottom panel).

T-cell proliferative responses against the HCV core and NS5a proteins became positive 4 weeks after inoculation and continued up to 30 and 18 weeks in CH10273 and CH10274, respectively. Likewise, the IFN-γ responses against HCV structural and nonstructural antigens were detected 4 weeks after inoculation and maintained 34 weeks and 16 weeks in CH10273 and CH10274, respectively (Fig. 1).

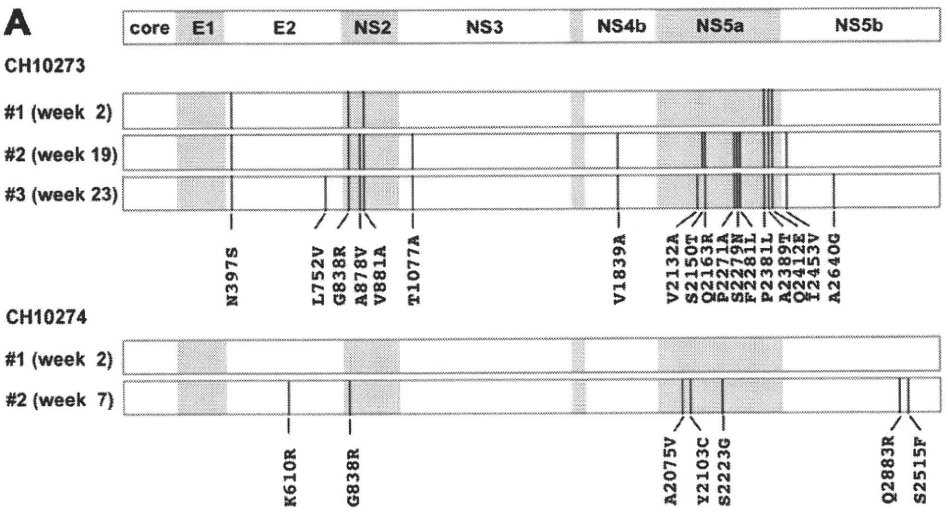
HCV Sequence Analysis. To investigate the difference and evolution of infected viruses, HCV sequences in both chimpanzees were determined directly at multiple time points as indicated in Fig. 1. In CH10273, HCV sequences were determined with sera collected at weeks 2, 19, and 23. Nineteen synonymous and six nonsynonymous mutations were already observed at week 2, and the number of mutations increased gradually with time (Table 1). Conversely, CH10274 showed no mutation at the earliest time point of infection (week 2) but subsequently developed four synonymous and seven nonsynonymous mutations at week 7 (Table 1). The mutated amino acids in the JFH-1 genome were distributed in E2, NS2, NS5a, and NS5b regions (Fig. 2A). Among these mutations, only one mutation, G838R in NS2, was identified as a common mutation between the two chimpanzees. To assess the complexity of the quasispecies, the amplified fragment encompassing HVR-1 was cloned and 10 clones in each time point were sequenced. In both animals, HVR populations of isolated HCV indicated similarly low complexity of heterogeneity (Fig. 2B). HCV clones isolated from CH10273 contained one HVR-1 mutation N397S at the earliest time point of infection, and this mutation could not be found in clones of the inoculum (Fig. 2B). To exclude the possibility of PCR artifact, sequences were confirmed by independent analyses. To ensure that the common NS2 mutant was not present as a minor species at the earliest time point of CH10274 (week 2), cloning (15 clones) and sequencing was performed and showed the WT sequence.

Effect of the NS2 Mutation on HCV Life Cycle. To assess whether this NS2 mutation could be a result of cytotoxic T-lymphocyte escape, which has been described in acutely HCV-infected chimpanzees,[17] we tested the T cell response of PBMCs from various time points during the infection against 18-mer peptides encompassing this region (both the WT and mutant sequences were tested). No T cell response could be detected against either the WT or mutant peptides throughout the infection, therefore making cytotoxic T-lymphocyte escape mutation highly unlikely. To assess the phenotype of the observed common mutation, G838R in the NS2 region, JFH-1 construct with this mutation was generated (JFH-1

Table 1. Sequence Evolution of JFH-1 in Chimpanzees

	Synonymous Mutations*	Non-synonymous Mutations*	Total
CH10273			
#1 (week 2)	19	6	25
#2 (week 19)	33	15	48
#3 (week 23)	35	17	52
CH10274			
#1 (week 2)	0	0	0
#2 (week 7)	4	7	11

*Compared with the consensus JFH-1 sequence.



B

		HVR Sequence	No./Clones
Patient Serum:		GTTTVGGAVARSTNVIAGVFSHGPQQN	17/20
(Inoculum)	:	.....P.....	1/20
	:	.....I.....	1/20
	:	.....S.....	1/20
CH10273;			
Direct #1	:	.....S.....	
Cloning #1	:	.....S.....	9/10
	:	S.....S.....	1/10
Direct #2	:	.....S.....	
Cloning #2	:	.....S.....	9/10
	:	.....S.....R..	1/10
Direct #3	:	.....S.....	
Cloning #3	:	.....S.....	10/10
CH10274;			
Direct #1	:	.....	
Cloning #1	:	.....	9/10
	:	.....A.....	1/10
Direct #2	:	.....	
Cloning #2	:	.....	10/10

Fig. 2. HCV sequence analyses. (A) Distribution of amino acid substitutions in patient serum-inoculated (CH10273) and JFH-1cc-inoculated (CH10274) chimpanzees. Positions of amino acid substitutions are indicated as vertical bars, and the mutated amino acids are shown at the bottom of each panel. The amino acid numbers correspond to the JFH-1 sequence. (B) HVR-1 populations in patient serum (inoculum) and chimpanzees. HVR-1 sequence in patient serum has been reported previously.[6] HVR-1 sequences determined by direct sequencing (Direct) or cloning and sequencing (Cloning) (10 clones at each time) in each animal are shown. Investigated time points (#1, 2, and 3) are indicated in Fig. 1. Identical amino acids are indicated as dots.

G838R). Viral replication and production of the JFH-1 G838R mutant was compared with that of the WT JFH-1 (JFH-1 WT) by transfecting the *in vitro* transcribed full-length genome RNA into Huh7.5.1 cells. HCV RNA levels in culture media of JFH-1 WT and JFH-1 G838R transfected cells were $2.96 \times 10^6 \pm 1.63 \times 10^5$ and $1.69 \times 10^7 \pm 3.61 \times 10^5$ copies/mL on day 3, and $2.67 \times 10^6 \pm 3.69 \times 10^5$ and $1.14 \times 10^7 \pm 2.23 \times 10^5$ copies/mL on day 5, respectively ($P < 0.05$)(Fig. 3A). In JFH-1 WT and JFH-1 G838R transfected cells, intracellular HCV RNA levels were $1.14 \times 10^8 \pm 1.36 \times 10^7$ and $3.66 \times 10^8 \pm 1.20 \times 10^7$ copies/well on day 3, and $1.67 \times 10^8 \pm 3.94 \times 10^7$ and $2.23 \times 10^8 \pm 1.90 \times 10^7$ copies/well on day 5, respectively ($P < 0.05$) (Fig. 3A). Thus, JFH-1 G838R could produce HCV RNA approximately fivefold higher than the JFH-1 WT in culture media and transfected cells (days 3 and 5, $P < 0.05$).

To confirm this observation, an infection study was also conducted with cell culture–generated viruses. After transfection of JFH1 WT and JFH-1 G838R genome RNA, viruses in culture media were harvested, and FFU of these viruses were titrated. The same titer of JFH1 WT or JFH-1 G838R viruses was inoculated into naïve Huh7.5.1 cells (9×10^2 FFU, multiplicity of infection = 0.003). After infection, HCV RNA titer in culture medium and infected cells was determined. Consistent with the transfection study, HCV RNA levels in culture media of JFH-1 G838R virus–infected cells were threefold to sixfold higher than those of JFH-1 WT virus (days 3 and 5, $P < 0.05$; Fig. 3B). Intracellular HCV RNA level on

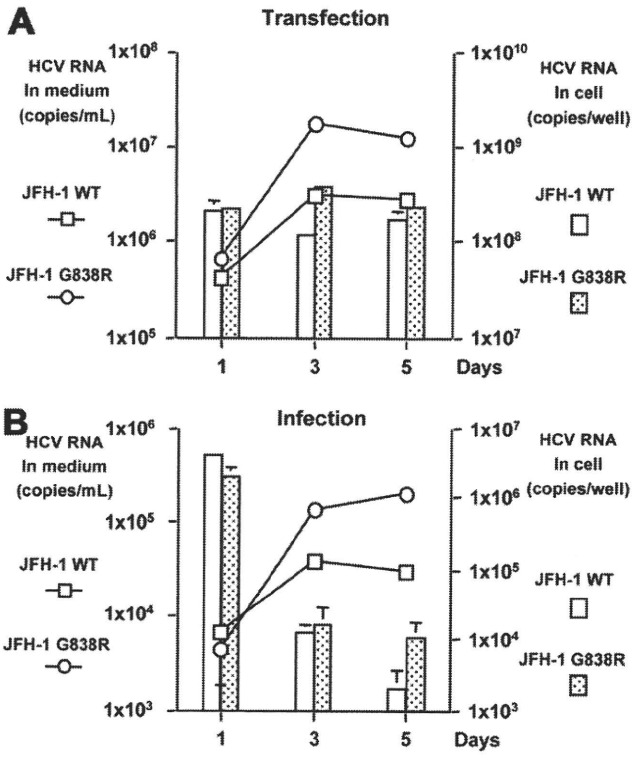


Fig. 3. Comparison of viral replication between JFH-1 WT and JFH-1 G838R in Huh 7.5.1 cells. At various times, HCV RNA was measured in culture media and cells by transfecting the same amount of *in vitro* transcribed full genome RNA (A) and by infecting the same FFU of JFH-1cc at a multiplicity of infection of 0.003 (B). Means of triplicate samples ± standard deviations are shown.

day 5 also appeared to be higher (fivefold) in JFH-1 G838R–infected cells ($P < 0.05$). Based on these data, JFH-1 G838R replicates more efficiently than the WT.

To further investigate the mechanism of this enhanced replication, we reasoned that this mutation could affect any of the viral RNA synthesis, assembly, or secretion steps. To distinguish among these possibilities, we used Huh7-25 cells, a Huh7 cells–derived cell line lacking CD81 expression.[11] This cell line cannot be reinfected by HCV but can support and produce infectious HCV on transfection with the HCV genome, therefore allowing us to address this question without the confounding effect of reinfection. HCV RNA levels of JFH-1 G838R–transfected cells in culture media were eightfold higher on day 1 and threefold higher on day 3 compared with those of JFH-1 WT transfected cells (Fig. 4A, $P < 0.05$). On day 5, the HCV RNA level was still higher in JFH-1 G838R–transfected cells, but the difference was less. The HCV RNA levels of the replication-deficient clone, JFH-1 GND, transfected cells were substantially lower than both NS2 mutant-transfected and WT-transfected cells (Fig. 4A). Similarly, HCV core Ag in culture media showed a significant difference between JFH-1 WT–transfected and JFH-1 G838R–transfected cells (days 1, 3, and 5, $P < 0.05$)(Fig. 4B). HCV core Ag of JFH-1 GND–transfected cells was under the detection limit. In contrast to culture media data, intracellular HCV RNA and core Ag levels in JFH-1 G838R–transfected cells were similar to or slightly lower than those of JFH-1 WT–transfected cells. Therefore, the G838R mutation does not appear to affect RNA replication and probably enhances either the assembly or secretion step.

To distinguish between these two possible effects, we determined the infectivity titer of intracellular viral particles in transfected cells as reported previously.[18] On day 3 after transfection, the intracellular infectivity titer in JFH-1 G838R–transfected cells was approximately four-

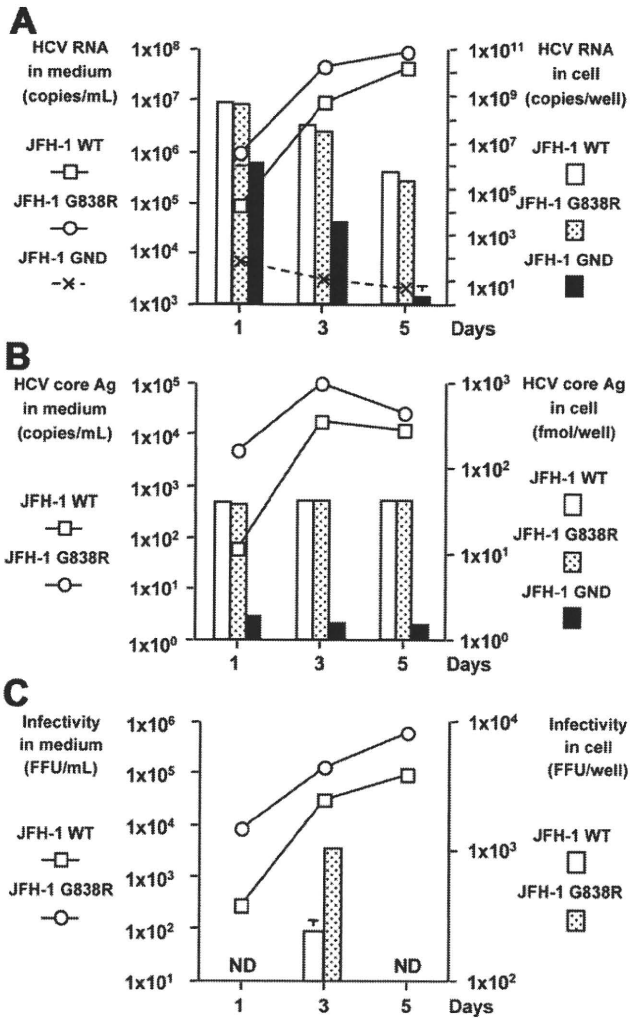


Fig. 4. Comparison of viral replication among JFH-1 WT, JFH-1 G838R, and JFH-1 GND in Huh 7-25 cells. At various times, HCV production was assessed in culture media and cells by transfecting the same amount of *in vitro* transcribed full genome RNA. HCV RNA titer (A), HCV core Ag level (B), and infectivity titers (C) are shown. The data are expressed as means of triplicate samples ± standard deviations. ND, not done.

Table 2. Specific Intectivity and Virus Secretion in Huh7-25 Cells

Clone	Intracellular HCV RNA (copies/well)	Intracellular Intectivity* (FFU/well)	Specific Intracellular Infectivity* (FFU/copies)	Extracellular HCV RNA* (copies/well)	Extracellular Infectivity* (FFU/well)	Specific Exracellular Infectivity (FFU/copies)	Infectious Virus Secretion (extra/intra)
JFH-1 WT	$4.40 \times 10^7 \pm 1.58 \times 10^7$	$2.27 \times 10^2 \pm 5.17 \times 10^1$	$1.09 \times 10^{-5} \pm 2.58 \times 10^{-6}$	$1.83 \times 10^7 \pm 1.95 \times 10^6$	$6.17 \times 10^3 \pm 9.61 \times 10^2$	$3.37 \times 10^{-4} \pm 1.38 \times 10^{-3}$	7.20 ± 2.83
JFH-1 G838R	$2.19 \times 10^7 \pm 1.11 \times 10^6$	$9.89 \times 10^2 \pm 5.02 \times 10^1$	$9.05 \times 10^{-5} \pm 2.76 \times 10^{-6}$	$5.14 \times 10^7 \pm 3.48 \times 10^6$	$2.69 \times 10^4 \pm 6.96 \times 10^3$	$5.33 \times 10^{-4} \pm 1.83 \times 10^{-4}$	6.87 ± 2.07

The data are from day 3 after HCV RNA transfection of the Huh7-25 cells.
*$P < 0.05$ comparing JFH-1 WT and G838R.

fold higher than that in JFH-1 WT–transfected cells ($P < 0.05$, Fig. 4C and Table 2). Moreover, specific intracellular infectivity of JFH-1 G838R–transfected cells was approximately eightfold higher than that in JFH-1 WT–transfected cells ($P < 0.05$, Table 2). Specific infectivity in culture medium was determined as the ratio of infectious virus (FFU) over HCV RNA copies. Specific infectivity of the JFH-1 G838R viruses was not significantly different from that of JFH-1 WT (Table 2). Finally, the rate of secretion was determined by the ratio of extracellular FFU over the intracellular FFU (Table 2), and no difference was observed between JFH-1 WT and G838R-transfected cells. Based on these data, the G838R mutation in JFH-1 enhances the assembly step of HCV.

Discussion

Although HCV-associated fulminant hepatitis is rare, several cases have been reported.[6,19-25] The HCV JFH-1 strain was isolated from one of these cases, and its unique characteristic of robust replication in cell culture might be related to the cause of fulminant hepatitis. Previously, HCV from a patient with fulminant liver failure has been shown to cause severe acute hepatitis with high viremia in a chimpanzee, although its molecular clone could not replicate in culture cells and did not induce severe hepatitis in the chimpanzee.[26,27] In our previous study, JFH-1cc induced a transient and attenuated infection in a chimpanzee.[8] The infection profile was different from the typical course of HCV infection either with patient sera or infectious RNA molecules in chimpanzees.[28-32] Because this observation was unexpected, we reasoned that the lower virulence of this strain *in vivo* might be related to the age of the chimpanzee. The chimpanzee used in the previous study was older (>25 years of age), and older chimpanzees typically do not develop significant disease on HCV infection. Another possible cause was the characteristics of the viral inoculum. JFH-1cc inoculated in the chimpanzee was monotypic because it was generated in culture cells. The original JFH-1 virus replicating in the fulminant hepatitis patient existed as a mixture of various viral species and might induce a different outcome *in vivo*. Thus, to elucidate the pathogenesis and replication capacity of the original JFH-1 strain *in vivo*, the patient serum and the JFH-1cc were inoculated into juvenile chimpanzees (5 years old). However, both chimpanzees showed attenuated infection with low-titer viremia, no ALT elevation, and absence of histological hepatitis during the acute phase of infection. Therefore, the manifestation of fulminant hepatitis of the original patient was likely a result of host factors, with the caveat that humans and chimpanzees might respond differently to HCV infection.

Similar to our previous study, the chimpanzee inoculated with monotypic JFH-1cc showed a short duration of infection and absence of seroconversion. Conversely, the chimpanzee inoculated with the patient serum showed a longer course of infection and developed anti-HCV antibodies. Immunological analysis with T-cell proliferation and IFN-γ ELISpot assays showed that HCV-specific immune responses were similarly induced in both animals and abated with the disappearance of viremia. Consistent with the longer viremia, the chimpanzee inoculated with the patient serum had a longer duration of detectable HCV immune response (Fig. 1). These differences could be explained by the sequence variations of the infecting HCV. In the chimpanzee inoculated with the patient serum, the infecting HCV showed a low sequence complexity but exhibited some sequence diversity already at week 2. The infecting HCV had a sequence alteration in the HVR-1 (N397S), but this sequence alteration could not be found in any of the 20 clones of the inoculum (Fig. 2B).[6] In addition, the NS2 G838R mutation was also not detected by cloning (six clones) and sequencing of the inoculum. Thus, this infecting HCV was probably selected from a minor species in the patient serum. It has been reported that minor clones in human serum were

selected during HCV infection in chimpanzees.[33] The selected clones were in the lighter fraction of the sucrose density gradient of the inoculum, which is devoid of immunoglobulins. Similar selection might have occurred in our study. The dominant clones in the inoculum might not be infectious because of binding to neutralizing antibodies. As a result, the infection-competent minor clone, selected during the infection, became the dominant species. Furthermore, this infecting minor clone could persist longer, although the characteristics of this clone and mechanisms for persistence are still unknown. HCV clones in CH10273 showed several other mutations at 2 weeks postinfection and accumulated additional mutations in E2, NS2, NS3, NS4b, NS5a, and NS5b regions over time (Fig. 2). Some of these regions contain known T-cell epitopes, although the major histocompatibility complex haplotype of this animal is unknown. In this chimpanzee, heterogeneity of the inoculating viruses might have contributed to the emergence of escape mutants from the host immune system, resulting in a prolonged infection. Similar observations have been reported in acute HCV infection in chimpanzees and humans.[34-36]

In HCV strains isolated from these two chimpanzees, one common mutation G838R in the NS2 region was identified. This mutation has not been reported among the adaptive mutations emerged in the JFH-1 virus passaged in cell culture.[37-39] This mutation likely arose *de novo* because one of the chimpanzees was inoculated with a molecular clone, and the week 2 sample did not harbor this mutation. NS2 is a membrane-associated cysteine protease, composed of three transmembrane domains and a protease domain.[40] Although the NS2 region is dispensable for RNA replication, it is essential for production of infectious virus in cultured cells.[41-43] Furthermore, the significance of this region has been shown in the establishment of replication-competent and infection-competent intergenotypical chimeric viruses.[44,45] The identified common mutation G838R was at the end of the first transmembrane domain,[46] and mutations in the transmembrane domains have been shown to improve the yield of infectious virus production in several studies.[45,47] Thus, some advantage of this mutation in HCV replication and production could be expected. This mutation was shown to enhance HCV production in Huh7.5.1 cells. Detailed analysis with CD81-negative Huh7-25 cell demonstrated that viral assembly was affected by this mutation. Production of infectious virus in JFH-1 G838R–transfected cells was eightfold higher than that in the JFH-1 WT–transfected cells. Thus, this mutation enhances the assembly of infectious virus particle in cultured cells, and as a result, increases infectious virus production in the culture medium. This mutation represents the first identified *in vivo* adapted mutation that is not immunologically mediated and probably confers a replication advantage to the virus *in vivo*. This adaptive mutation, unlike the other adaptive mutations reported *in vitro* with poor infectivity *in vivo*, likely results from a highly biologically relevant event in the dynamic interaction between HCV and host. Finally, it is possible that compensatory mutations in other regions of the virus may contribute to the overall biological adaptive response of the virus *in vivo*.

This study demonstrates that the HCV JFH-1 strain either generated in cell culture as a monotypic virus or obtained from patient serum is associated with attenuated infection in chimpanzees; however, the virus can rapidly evolve with adaptive mutations to facilitate propagation of the virus in a susceptible host.

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References

1. Liang TJ, Rehermann B, Seeff LB, Hoofnagle JH. Pathogenesis, natural history, treatment, and prevention of hepatitis C. Ann Intern Med 2000; 132:296-305.
2. Feld JJ, Liang TJ. Hepatitis C: identifying patients with progressive liver injury. HEPATOLOGY 2006;43(Suppl):S194-S206.
3. Shiina M, Rehermann B. Hepatitis C vaccines: Inducing and challenging memory T cells. HEPATOLOGY 2006;43:1395-1398.
4. De Francesco R, Migliaccio G. Challenges and successes in developing new therapies for hepatitis C. Nature 2005;436:953-960.
5. Di Bisceglie AM, Hoofnagle JH. Optimal therapy of hepatitis C. HEPATOLOGY 2002;36(Suppl):S121-S127.
6. Kato T, Furusaka A, Miyamoto M, Date T, Yasui K, Hiramoto J, et al. Sequence analysis of hepatitis C virus isolated from a fulminant hepatitis patient. J Med Virol 2001;64:334-339.
7. Zhong J, Gastaminza P, Cheng G, Kapadia S, Kato T, Burton DR, et al. Robust hepatitis C virus infection in vitro. Proc Natl Acad Sci U S A 2005;102:9294-9299.
8. Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, et al. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat Med 2005;11:791-796.
9. Lindenbach BD, Evans MJ, Syder AJ, Wolk B, Tellinghuisen TL, Liu CC, et al. Complete replication of hepatitis C virus in cell culture. Science 2005;309:623-626.
10. Bukh J, Pietschmann T, Lohmann V, Krieger N, Faulk K, Engle RE, et al. Mutations that permit efficient replication of hepatitis C virus RNA in Huh-7 cells prevent productive replication in chimpanzees. Proc Natl Acad Sci U S A 2002;99:14416-14421.
11. Akazawa D, Date T, Morikawa K, Murayama A, Miyamoto M, Kaga M, et al. CD81 expression is important for the permissiveness of Huh7 cell clones for heterogeneous hepatitis C virus infection. J Virol 2007;81:5036-5045.
12. Kato T, Date T, Murayama A, Morikawa K, Akazawa D, Wakita T. Cell culture and infection system for hepatitis C virus. Nat Protoc 2006;1: 2334-2339.
13. Takeuchi T, Katsume A, Tanaka T, Abe A, Inoue K, Tsukiyama-Kohara K, et al. Real-time detection system for quantification of hepatitis C virus genome. Gastroenterology 1999;116:636-642.

14. Elmowalid GA, Qiao M, Jeong SH, Borg BB, Baumert TF, Sapp RK, et al. Immunization with hepatitis C virus-like particles results in control of hepatitis C virus infection in chimpanzees. Proc Natl Acad Sci U S A 2007;104:8427-8432.
15. Aoyagi K, Ohue C, Iida K, Kimura T, Tanaka E, Kiyosawa K, et al. Development of a simple and highly sensitive enzyme immunoassay for hepatitis C virus core antigen. J Clin Microbiol 1999;37:1802-1808.
16. Kato T, Matsumura T, Heller T, Saito S, Sapp RK, Murthy K, et al. Production of infectious hepatitis C virus of various genotypes in cell cultures. J Virol 2007;81:4405-4411.
17. Erickson AL, Kimura Y, Igarashi S, Eichelberger J, Houghton M, Sidney J, et al. The outcome of hepatitis C virus infection is predicted by escape mutations in epitopes targeted by cytotoxic T lymphocytes. Immunity 2001;15:883-895.
18. Gastaminza P, Kapadia SB, Chisari FV. Differential biophysical properties of infectious intracellular and secreted hepatitis C virus particles. J Virol 2006;80:11074-11081.
19. Wright TL, Hsu H, Donegan E, Feinstone S, Greenberg H, Read A, et al. Hepatitis C virus not found in fulminant non-A, non-B hepatitis. Ann Intern Med 1991;115:111-112.
20. Liang TJ, Jeffers L, Reddy RK, Silva MO, Cheinquer H, Findor A, et al. Fulminant or subfulminant non-A, non-B viral hepatitis: the role of hepatitis C and E viruses. Gastroenterology 1993;104:556-562.
21. Yoshiba M, Dehara K, Inoue K, Okamoto H, Mayumi M. Contribution of hepatitis C virus to non-A, non-B fulminant hepatitis in Japan. HEPATOLOGY 1994;19:829-835.
22. Villamil FG, Hu KQ, Yu CH, Lee CH, Rojter SE, Podesta LG, et al. Detection of hepatitis C virus with RNA polymerase chain reaction in fulminant hepatic failure. HEPATOLOGY 1995;22:1379-1386.
23. Vento S, Cainelli F, Mirandola F, Cosco L, Di Perri G, Solbiati M, et al. Fulminant hepatitis on withdrawal of chemotherapy in carriers of hepatitis C virus. Lancet 1996;347:92-93.
24. Farci P, Alter HJ, Shimoda A, Govindarajan S, Cheung LC, Melpolder JC, et al. Hepatitis C virus-associated fulminant hepatic failure. N Engl J Med 1996;335:631-634.
25. Fukai K, Yokosuka O, Fujiwara K, Tagawa M, Imazeki F, Saisho H, et al. Etiologic considerations of fulminant non-A, non-B viral hepatitis in Japan: analyses by nucleic acid amplification method. J Infect Dis 1998;178: 325-333.
26. Farci P, Munoz SJ, Shimoda A, Govindarajan S, Wong DC, Coiana A, et al. Experimental transmission of hepatitis C virus-associated fulminant hepatitis to a chimpanzee. J Infect Dis 1999;179:1007-1011.
27. Sakai A, Takikawa S, Thimme R, Meunier JC, Spangenberg HC, Govindarajan S, et al. In vivo study of the HC-TN strain of hepatitis C virus recovered from a patient with fulminant hepatitis: RNA transcripts of a molecular clone (pHC-TN) are infectious in chimpanzees but not in Huh7.5 cells. J Virol 2007;81:7208-7219.
28. Kolykhalov AA, Agapov EV, Blight KJ, Mihalik K, Feinstone SM, Rice CM. Transmission of hepatitis C by intrahepatic inoculation with transcribed RNA. Science 1997;277:570-574.
29. Hong Z, Beaudet-Miller M, Lanford RE, Guerra B, Wright-Minogue J, Skelton A, et al. Generation of transmissible hepatitis C virions from a molecular clone in chimpanzees. Virology 1999;256:36-44.
30. Beard MR, Abell G, Honda M, Carroll A, Gartland M, Clarke B, et al. An infectious molecular clone of a Japanese genotype 1b hepatitis C virus. HEPATOLOGY 1999;30:316-324.
31. Thomson M, Nascimbeni M, Gonzales S, Murthy KK, Rehermann B, Liang TJ. Emergence of a distinct pattern of viral mutations in chimpanzees infected with a homogeneous inoculum of hepatitis C virus. Gastroenterology 2001;121:1226-1233.
32. Major ME, Mihalik K, Puig M, Rehermann B, Nascimbeni M, Rice CM, et al. Previously infected and recovered chimpanzees exhibit rapid responses that control hepatitis C virus replication upon rechallenge. J Virol 2002;76:6586-6595.
33. Kojima M, Osuga T, Tsuda F, Tanaka T, Okamoto H. Influence of antibodies to the hypervariable region of E2/NS1 glycoprotein on the selective replication of hepatitis C virus in chimpanzees. Virology 1994; 204:665-672.
34. Farci P, Shimoda A, Coiana A, Diaz G, Peddis G, Melpolder JC, et al. The outcome of acute hepatitis C predicted by the evolution of the viral quasispecies. Science 2000;288:339-344.
35. Erickson AL, Kimura Y, Igarashi S, Eichelberger J, Houghton M, Sidney J, et al. The outcome of hepatitis C virus infection is predicted by escape mutations in epitopes targeted by cytotoxic T lymphocytes. Immunity 2001;15:883-895.
36. Timm J, Lauer GM, Kavanagh DG, Sheridan I, Kim AY, Lucas M, et al. CD8 epitope escape and reversion in acute HCV infection. J Exp Med 2004;200:1593-1604.
37. Delgrange D, Pillez A, Castelain S, Cocquerel L, Rouille Y, Dubuisson J, et al. Robust production of infectious viral particles in Huh-7 cells by introducing mutations in hepatitis C virus structural proteins. J Gen Virol 2007;88:2495-2503.
38. Kaul A, Woerz I, Meuleman P, Leroux-Roels G, Bartenschlager R. Cell culture adaptation of hepatitis C virus and in vivo viability of an adapted variant. J Virol 2007;81:13168-13179.
39. Russell RS, Meunier JC, Takikawa S, Faulk K, Engle RE, Bukh J, et al. Advantages of a single-cycle production assay to study cell culture-adaptive mutations of hepatitis C virus. Proc Natl Acad Sci U S A 2008;105:4370-4375.
40. Lorenz IC, Marcotrigiano J, Dentzer TG, Rice CM. Structure of the catalytic domain of the hepatitis C virus NS2–3 protease. Nature 2006;442: 831-835.
41. Lohmann V, Korner F, Koch J, Herian U, Theilmann L, Bartenschlager R. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science 1999;285:110-113.
42. Kato T, Date T, Miyamoto M, Furusaka A, Tokushige K, Mizokami M, et al. Efficient replication of the genotype 2a hepatitis C virus subgenomic replicon. Gastroenterology 2003;125:1808-1817.
43. Jones CT, Murray CL, Eastman DK, Tassello J, Rice CM. Hepatitis C virus p7 and NS2 proteins are essential for production of infectious virus. J Virol 2007;81:8374-8383.
44. Pietschmann T, Kaul A, Koutsoudakis G, Shavinskaya A, Kallis S, Steinmann E, et al. Construction and characterization of infectious intragenotypic and intergenotypic hepatitis C virus chimeras. Proc Natl Acad Sci U S A 2006;103:7408-7413.
45. Yi M, Ma Y, Yates J, Lemon SM. Compensatory mutations in E1, p7, NS2, and NS3 enhance yields of cell culture-infectious intergenotypic chimeric hepatitis C virus. J Virol 2007;81:629-638.
46. Yamaga AK, Ou JH. Membrane topology of the hepatitis C virus NS2 protein. J Biol Chem 2002;277:33228-33234.
47. Murray CL, Jones CT, Tassello J, Rice CM. Alanine scanning of the hepatitis C virus core protein reveals numerous residues essential for production of infectious virus. J Virol 2007;81:10220-10231.

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Short Communication

Virological characterization of the hepatitis C virus JFH-1 strain in lymphocytic cell lines

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While hepatocytes are the major site of hepatitis C virus (HCV) infection, a number of studies have suggested that HCV can replicate in lymphocytes. However, *in vitro* culture systems to investigate replication of HCV in lymphocytic cells are severely limited. Robust HCV culture systems have been established using the HCV JFH-1 strain and Huh-7 cells. To gain more insights into the tissue tropism of HCV, we investigated the infection, replication, internal ribosome entry site (IRES)-dependent translation and polyprotein processing of the HCV JFH-1 strain in nine lymphocytic cell lines. HCV JFH-1 failed to infect lymphocytes and replicate, but exhibited efficient polyprotein processing and IRES-dependent translation in lymphocytes as well as in Huh-7 cells. Our results suggest that lymphocytic cells can support HCV JFH-1 translation and polyprotein processing, but may lack some host factors essential for HCV JFH-1 infection and replication.

Hepatitis C virus (HCV) is a major cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (Choo *et al.*, 1989; Saito *et al.*, 1990). Infection with HCV is frequently associated with B-cell-related diseases, such as mixed cryoglobulinaemia and non-Hodgkin's lymphoma (Hausfater *et al.*, 2000). A number of studies have suggested that HCV can replicate not only in hepatocytes, but also in lymphocytes (Ducoulombier *et al.*, 2004; Karavattathayyil *et al.*, 2000, Lerat *et al.*, 1998), whereas the determinants of HCV tropism are still unknown. The development of HCV strain JFH-1, which generates infectious HCV in culture, has made an important contribution to the study of the HCV life cycle (Lindenbach *et al.*, 2005; Wakita *et al.*, 2005; Zhong *et al.*, 2005). The HCV life cycle is divided into several steps. After entry into the cell and uncoating, the HCV life cycle leads to translation, polyprotein processing, RNA replication, virion assembly, transport and release. The JFH-1 subgenomic replicon can replicate in non-hepatic cell lines, such as HeLa cells and 293 cells, suggesting that the host factors required for HCV replication are not hepatocyte-specific (Kato *et al.*, 2005b). The SB strain of HCV (genotype 2b strain) was isolated from an HCV-infected non-Hodgkin's B-cell lymphoma and has been reported to infect B and T cells (Kondo *et al.*, 2007; Sung *et al.*, 2003). The virus titres of the SB strain in lymphocytes were, however, lower than those of JFH-1 in Huh-7 cells and the expression of HCV proteins was not confirmed (Kondo *et al.*, 2007). It is unknown whether HCV JFH-1 can infect and replicate in lymphocytes. To gain more insight into the tissue tropism of HCV infection, we investigated the infection, replication, IRES-dependent translation and polyprotein processing of the JFH-1 strain in nine lymphocytic cell lines.

We first sought to determine whether HCV JFH-1 can infect lymphocytic cell lines. We chose nine lymphocytic cell lines derived from Burkitt's lymphoma, the EBV-immortalized human B cell line, lymphoblasts and acute T-cell leukaemia. C1R, IB4, Namalwa, P3HR1 and Raji cells were Epstein–Barr virus (EBV)-positive (Table 1). Infectious HCV was generated from HCV JFH-1 RNA in Huh-7 cells (Shirakura *et al.*, 2007; Wakita *et al.*, 2005) and the calculation of the 50 % tissue culture infectious dose ($TCID_{50}$) was based on methods described previously (Lindenbach *et al.*, 2005). These cell lines (1×10^5 cells per well of a six-well plate) were incubated with 2 ml inoculum (5×10^3 or 5×10^4 $TCID_{50}$ ml^{-1}) for 3 h, washed three times with PBS, and cultured in fresh medium. The culture medium was changed every 2 days. Cells were harvested at 0 (3 h post-infection [p.i.]), 4 and 8 day p.i. HCV core antigen within cells was quantified by immunoassay (Ortho HCV-core ELISA kit; Ortho-Clinical Diagnostics). As shown in Fig. 1(a), increasing the HCV titre of the inoculum resulted in a 7.2-fold increase in the levels of HCV core protein in Huh-7 cells at 3 h p.i. Increasing the HCV titre of the inoculum resulted in a 1.5- to 3.2-fold increase in the levels of the core protein in C1R, BL41,

Table 1. Summary of the virological characterization of HCV JFH-1 in lymphocytes

Name	Source	EBV	Transfection			Concentration of G418 for selection (μg ml^{-1})	HCVcc infection	HCV-RNA replication	Translation*		Polyprotein processing†
			Buffer	Program	Efficiency				HCV-IRES	EMCV-IRES	
Bjab	Burkitt's lymphoma	−	T	T−16	>90 %	600–800	−	−	+	+ +	+
BL41	Burkitt's lymphoma	−	V	I−10	60–70 %	1000	−	−	+	+ +	ND
C1R	B lymphoblast	+	V	T−20	70–80 %	100	−	−	+ + + +	+ + + +	+
IB4	Lymphoblastoid	+	V	T−20	80–90 %	1000	−	−	+ + +	+ + + +	+
Jurkat	Acute T cell leukaemia	−	V	I−10	60–70 %	600	−	−	+ +	+	ND
Namalwa	Burkitt's lymphoma	+	V	M−13	60–70 %	600–800	−	−	+ + +	+ + + +	+
P3HR1	Burkitt's lymphoma	+	V	A−23	60–70 %	800	−	−	+ + +	+ + + +	ND
Raji	Burkitt's lymphoma	+	V	T−27	70–80 %	800	−	−	+ +	+ + + +	+
Ramos	Burkitt's lymphoma	−	V	M−13	40–60 %	400	−	−	+	+	ND
Huh7	Hepatoma	−	T	T−14	70–80 %	500	+	+	+ + +	+ + +	+

*+, <0.25 fold IRES activity of Huh-7; + +, 0.25–0.75 fold; + + +, 0.75–1.5-fold; + + + +, >1.5-fold.
†ND, Not determined.

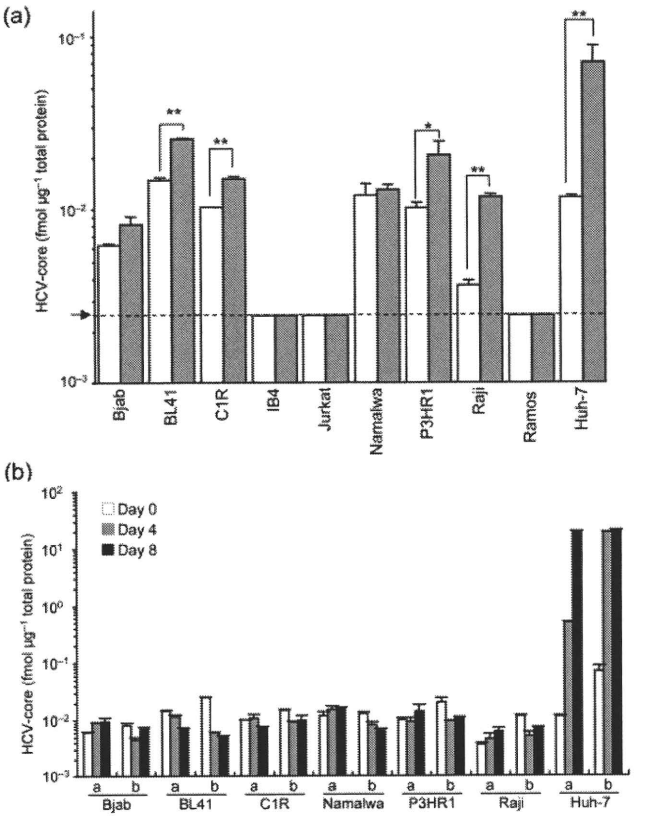

Fig. 1. HCV infection assay. (a) HCV core protein levels 3 h after infection. A total of 1×10^5 cells were infected with 2 ml of the inoculum (5×10^3 [white bars] or 5×10^4 [grey bars] $TCID_{50}$ ml^{-1}) for 3 h at 37 °C and harvested at 3 h p.i. HCV core protein in cell lysate was quantified by ELISA. The average values with standard deviations from triplicate samples are shown. The cut-off value of the immunoassay is indicated by an arrow and a dotted line. The difference between low m.o.i. (white bars) and high m.o.i. (grey bars) was significant (*, $P<0.05$; **, $P<0.01$, Student's *t*-test). (b) Time-course of HCV core protein levels after infection. In total, 1×10^5 cells were infected with 2 ml of the inoculum (5×10^3 [a] or 5×10^4 [b] $TCID_{50}$ ml^{-1}) for 3 h and harvested at 0, 4 and 8 days p.i. HCV core protein in cell lysate was quantified by ELISA. Average values ± SD from triplicate samples are shown.

P3HR1 and Raji cells, suggesting that HCV can bind to these cell lines (Fig. 1a). In contrast, the levels of HCV core protein in IB4, Jurkat and Ramos cells at 3 h p.i. were below the detection limits and there were no significant differences in the levels of the core protein in Bjab cells and Namalwa cells, suggesting that HCV binding to these cells was very inefficient (Fig. 1a). Moreover, the levels of HCV core protein increased in Huh-7 cells but, in the case of all lymphocytic cell lines, including Raji cells, the core titre did not increase at day 4 and 8 p.i., suggesting that HCV JFH-1 does not infect and/or replicate efficiently in these lymphocytic cell lines (Fig. 1b).

To assess the replication of JFH-1 in our lymphocytic cell lines, we utilized the HCV replicon system. To visualize the

replicating cells, a reporter replicon plasmid was constructed as follows. The gene encoding green fluorescence protein (GFP) was fused to the neomycin resistance gene using an overlap PCR amplification technique and the fusion product was inserted into pSGR-JFH1. The resultant plasmid was pSGR-GFPneo-JFH1. This plasmid was linearized with *Xba*I and used as a template for *in vitro* transcription using an AmpliScribe T7 High Yield Transcription kit (Epicentre Biotechnologies). RNA was transfected with high transfection efficiency and low cytotoxicity using the Nucleofector system (Amaxa Biosystems) (Coughlin *et al.*, 2004; Miyahara *et al.*, 2005; Van De Parre *et al.*, 2005). The transfection efficiencies ranged from 60 to 80 % after optimization of transfection conditions (Table 1). GFP expression was monitored periodically during the selection of HCV-replicon cells by G418 (Table 1). The GFP-expressing cells were detected at day 3 post-transfection (p.t.) in Huh-7, P3HR1, Raji, C1R and Namalwa cells. The rate of GFP expression in Huh-7 cells was more than 50 %. The rate of GFP-expression in lymphocytic cell lines was less than 1 %, despite the high transfection efficiencies. After 3 weeks of G418 selection, SGR-GFPneo-JFH1 replicon cells were established in Huh-7 cells, but not in lymphocytic cells. These data suggest that JFH-1 subgenomic replicon RNA cannot replicate in the lymphocytic cell lines.

To facilitate quantification of replication, we performed luciferase assays using subgenomic replicon RNA (SGR-JFH1/Luc) carrying firefly luciferase as a reporter. SGR-JFH1/Luc RNA was *in vitro*-transcribed using the linearized pSGR-JFH1/Luc (Kato *et al.*, 2005a) as template DNA. Cells were harvested at 4, 24, 48 and 72 h p.t. and luciferase activities were assayed with luciferase assay reagent (Promega). Assays were performed at least in triplicate. There were significant differences in luciferase activities at 4 h p.t. among the cell lines, probably because there were differences in transfection efficiencies and the doubling time of the cell lines. Thus, the replication activity was expressed relative to the reporter activity determined 4 h p.t. for each cell line, which was set to 1 (Fig. 2a). HCV subgenomic replicon RNA efficiently replicated in Huh-7 cells (Fig. 2a). Replication-deficient subgenomic replicon RNA encoding a GDD to GND mutation in NS5B served as a negative control in Huh-7 cells. The luciferase activities of replication-deficient subgenomic replicon RNA in lymphocytic cell lines also decreased rapidly (data not shown). As shown in Fig. 2(a), the luciferase activities of HCV subgenomic replicon RNA in lymphocytic cell lines decreased rapidly, suggesting that HCV subgenomic replicon RNA did not replicate efficiently in lymphocytic cell lines. Thus, these two different replicon assays demonstrated that the HCV JFH-1 subgenomic replicon failed to replicate in our lymphocytic cell lines.

To determine which steps of the HCV life cycle are impaired, we further examined translation and polyprotein processing. At first, we assessed HCV IRES-dependent translational efficiencies in the lymphocytic cell lines. Cells were co-transfected with the subgenomic replicon RNA (SGR-JFH1/Luc) and a capped RNA encoding *Renilla* luciferase (cap-luc). Cap-luc RNA was *in vitro*-transcribed using a T7 mMessage mMachine kit (Ambion). The HCV IRES activities in IB4, Namalwa and P3HR1 cells were as high as in Huh-7 cells. The HCV IRES activities in Jurkat and Raji cells were about 50 % of those in Huh-7 cells, and the HCV IRES activities in Bjab, BL41 and Ramos cells were less than 25 % of those in Huh-7 cells. On the other hand, the HCV IRES activity in C1R cells was about twofold higher than in Huh-7 cells (Fig. 2b). Replication-deficient subgenomic replicon RNA encoding a GDD to GND mutation in NS5B showed a luciferase activity level similar to that of the wild-type, suggesting that the luciferase activity at 4 h after transfection reflected translational levels but not replication levels (data not shown). Our data indicate high HCV IRES activities in all cell lines, except in Bjab, BL41 and Ramos.

The HCV polyprotein is translated in subgenomic replicon cells in an encephalomyocarditis virus (EMCV) IRES-dependent manner. To rule out the possibility that the EMCV IRES-dependent translation is impaired in lymphocytic cell lines, we assessed the EMCV IRES-dependent translational efficiencies. We assayed EMCV IRES activity using EMCV IRES-driven luciferase RNA (EMC-luc) and Cap-luc RNA. The EMCV IRES activity was five- to tenfold higher in C1R, Namalwa, IB4 and P3HR1 than in Huh-7 cells (Fig. 2c). From these results, HCV IRES and EMCV IRES exhibited sufficient translational activity in C1R, Namalwa, P3HR1 and Raji cells, suggesting that IRES-dependent translation was not impaired in these lymphocytic cell lines.

To determine whether HCV polyprotein is properly processed in lymphocytes, we examined the processing of HCV non-structural (NS) proteins. The construct pSGR-JFH1/Luc expresses the polyprotein NS3-NS4A-NS4B-NS5A-NS5B. The HCV NS3/4A protease is responsible for proteolytic processing at each cleavage site. We used the eukaryotic transient-expression system based on a recombinant vaccinia virus carrying bacteriophage T7 RNA polymerase (T7vac) (Fuerst *et al.*, 1989). To express the SGR-JFH1/Luc encoding HCV NS proteins, 5×10^6 cells were transfected with 5 μg pSGR-JFH1/Luc and infected with 2.5×10^9 p.f.u. T7vac, harvested at 24 h p.i., and analysed by Western blotting. Completely processed NS3, NS5A and NS5B proteins were detected in Bjab, Raji, IB4 and Namalwa cells as well as in pSGR-JFH1/Luc-transfected Huh-7 cells and HCV-JFH1-infected Huh-7 cells (Fig. 2c). The unprocessed polyprotein was not detected by immunoblotting in these lymphocytic cell lines (data not shown). These results suggest that the HCV polyprotein is efficiently processed in these lymphocytic cells.

In this study, we demonstrated that HCV JFH-1 failed to infect and replicate in nine lymphocytic cell lines. In contrast, HCV IRES-dependent translation and polyprotein processing by NS3/NS4A protease functioned properly

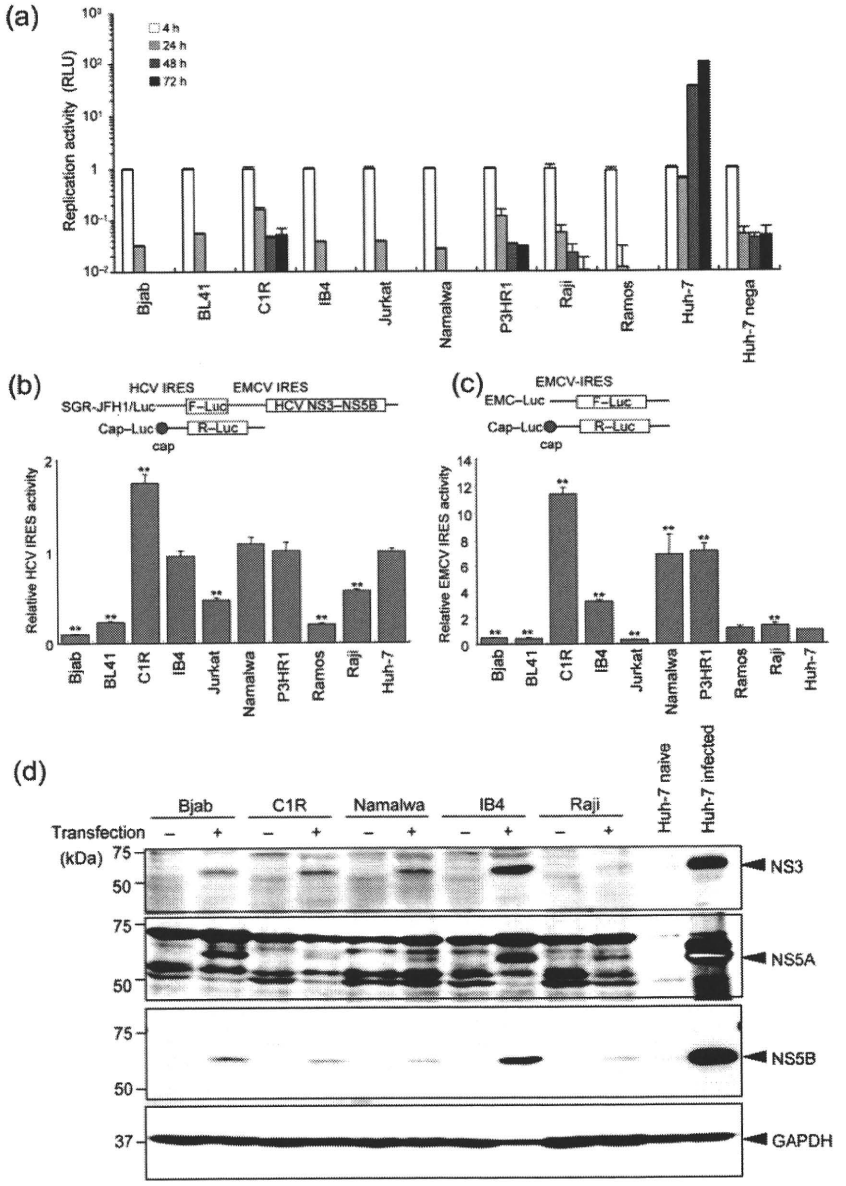

Fig. 2. Replication, HCV IRES-dependent translational efficiencies and polyprotein processing. (a) Subgenomic replicon assay. JFH-1 subgenomic replicon RNA was transfected into several cell lines and harvested at 4, 24, 48 and 72 h p.t. The replication activity was expressed relative to the reporter activity determined 4 h p.t. for each cell line, which was set to 1. RLU, Relative luciferase units; Huh-7 nega, Huh-7 cells transfected with SGR-JFH1/Luc GND, served as a negative control. (b) HCV IRES-dependent translational efficiency. To determine the HCV IRES activities, we co-transfected cells with SGR-JFH1/Luc RNA and Cap-Renilla luciferase RNA. The IRES activity of each cell line is expressed in relation to Huh-7 IRES activity, that is, as the ratio of HCV IRES-driven firefly luciferase activity to cap-driven *Renilla* luciferase activity. The difference in HCV IRES activity between Huh-7 cells and the lymphocytic cell line was significant (**, $P<0.01$, Student's *t*-test). (c) EMCV IRES-dependent translational efficiency. To determine the EMCV IRES activities, we co-transfected cells with EMCV–firefly luciferase RNA and Cap-Renilla luciferase RNA. The IRES activity of each cell line is expressed in relation to Huh-7 IRES activity, that is, as the ratio of EMCV IRES-driven firefly luciferase activity to cap-driven *Renilla* luciferase activity. The difference in EMCV IRES activity between Huh-7 cell and the lymphocytic cell line was significant (**, $P<0.01$, Student's *t*-test). (d) Polyprotein processing by NS3/4A protease in lymphocytic cell lines. pSGR-JFH1/Luc-transfected cells were infected with T7vac and harvested at 24 h p.i. HCV NS proteins, NS3, NS5A and NS5B were detected by using anti-NS3 rabbit polyclonal antibody (PAb), anti-NS5A rabbit PAb and anti-NS5B rabbit PAb. Arrowheads indicate the processed NS3, NS5A and NS5B proteins, respectively.

in these cells. Moreover, subgenomic replicon RNA failed to replicate in these cell lines. Our data suggest that lymphocytic cell lines may lack some host factors required for infection and replication of HCV-JFH1.

Viral entry often requires sequential interactions between viral proteins and several cellular factors. Several molecules (CD81, Claudin-1, Scavenger receptor class B member IR, LDL-receptor and glycosaminoglycans) have been reported to be involved in HCV binding and entry (Barth *et al.*, 2003; Evans *et al.*, 2007; Pileri *et al.*, 1998; Scarselli *et al.*, 2002). Further investigation will be required to clarify HCV binding and entry into lymphocytic cell lines.

HCV IRES and EMCV IRES exhibited sufficient translational activities in C1R, IB4, P3HR1, Namalwa and Raji cells. All these cell lines are EBV-positive. EBV-encoded nuclear antigen (EBNA1) has been reported to support HCV replication (Sugawara *et al.*, 1999). Two small EBV-encoded RNA species (EBERs) bind to the HCV IRES region (Wood *et al.*, 2001). These findings raise the possibility that HCV IRES activities may be modified by the EBV genome.

HCV JFH-1 subgenomic replicon RNA could not replicate in all lymphocytes tested in this study. The HCV SB strain, however, has been reported to infect Raji, Daudi, Molt-4

and Jurkat cells (Kondo *et al.*, 2007; Sung *et al.*, 2003). Still unknown is how hepatotropism and lymphotropism of HCV are determined. The GB virus B (GBV-B) is most closely related to HCV and the GBV-B infection of tamarins has been proposed as a good surrogate model for chronic hepatitis C (Bukh *et al.*, 2001; Jacob *et al.*, 2004; Lanford *et al.*, 2003; Martin *et al.*, 2003). A recent report has shown that GBV can disseminate to not only liver but also a variety of extrahepatic tissues such as haematolymphoid and genital tissues in tamarins (Ishii *et al.*, 2007). Viral RNA cloned from plasma and liver from the tamarins showed no sequence heterogeneity, suggesting that host factors determine the pleiotropism (Ishii *et al.*, 2007). It remains unclear how host factors and/or viral factors determine the tissue tropism of HCV. Further studies will be required to clarify the molecular mechanisms of HCV tissue tropism.

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References

Barth, H., Schafer, C., Adah, M. I., Zhang, F., Linhardt, R. J., Toyoda, H., Kinoshita-Toyoda, A., Toida, T., Van Kuppevelt, T. H. & other authors (2003). Cellular binding of hepatitis C virus envelope glycoprotein E2 requires cell surface heparan sulfate. *J Biol Chem* **278**, 41003–41012.

Bukh, J., Apgar, C. L., Govindarajan, S. & Purcell, R. H. (2001). Host range studies of GB virus-B hepatitis agent, the closest relative of hepatitis C virus, in New World monkeys and chimpanzees. *J Med Virol* **65**, 694–697.

Choo, Q. L., Kuo, G., Weiner, A. J., Overby, L. R., Bradley, D. W. & Houghton, M. (1989). Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* **244**, 359–362.

Coughlin, C. M., Vance, B. A., Grupp, S. A. & Vonderheide, R. H. (2004). RNA-transfected CD40-activated B cells induce functional T-cell responses against viral and tumor antigen targets: implications for pediatric immunotherapy. *Blood* **103**, 2046–2054.

Ducoulombier, D., Roque-Afonso, A. M., Di Liberto, G., Penin, F., Kara, R., Richard, Y., Dussaix, E. & Feray, C. (2004). Frequent compartmentalization of hepatitis C virus variants in circulating B cells and monocytes. *Hepatology* **39**, 817–825.

Evans, M. J., von Hahn, T., Tscherne, D. M., Syder, A. J., Panis, M., Wolk, B., Hatziioannou, T., McKeating, J. A., Bieniasz, P. D. & Rice, C. M. (2007). Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry. *Nature* **446**, 801–805.

Fuerst, T. R., Fernandez, M. P. & Moss, B. (1989). Transfer of the inducible *lac* repressor/operator system from *Escherichia coli* to a vaccinia virus expression vector. *Proc Natl Acad Sci U S A* **86**, 2549–2553.

Hausfater, P., Rosenthal, E. & Cacoub, P. (2000). Lymphoproliferative diseases and hepatitis C virus infection. *Ann Med Interne (Paris)* **151**, 53–57.

Ishii, K., Iijima, S., Kimura, N., Lee, Y. J., Ageyama, N., Yagi, S., Yamaguchi, K., Maki, N., Mori, K. & other authors (2007). GBV-B as a pleiotropic virus: distribution of GBV-B in extrahepatic tissues *in vivo*. *Microbes Infect* **9**, 515–521.

Jacob, J. R., Lin, K. C., Tennant, B. C. & Mansfield, K. G. (2004). GB virus B infection of the common marmoset (*Callithrix jacchus*) and associated liver pathology. *J Gen Virol* **85**, 2525–2533.

Karavattathayyil, S. J., Kalkeri, G., Liu, H. J., Gaglio, P., Garry, R. F., Krause, J. R. & Dash, S. (2000). Detection of hepatitis C virus RNA sequences in B-cell non-Hodgkin lymphoma. *Am J Clin Pathol* **113**, 391–398.

Kato, T., Date, T., Miyamoto, M., Sugiyama, M., Tanaka, Y., Orito, E., Ohno, T., Sugihara, K., Hasegawa, I. & other authors (2005a). Detection of anti-hepatitis C virus effects of interferon and ribavirin by a sensitive replicon system. *J Clin Microbiol* **43**, 5679–5684.

Kato, T., Date, T., Miyamoto, M., Zhao, Z., Mizokami, M. & Wakita, T. (2005b). Nonhepatic cell lines HeLa and 293 support efficient replication of the hepatitis C virus genotype 2a subgenomic replicon. *J Virol* **79**, 592–596.

Kondo, Y., Sung, V. M., Machida, K., Liu, M. & Lai, M. M. (2007). Hepatitis C virus infects T cells and affects interferon-gamma signaling in T cell lines. *Virology* **361**, 161–173.

Lanford, R. E., Chavez, D., Notvall, L. & Brasky, K. M. (2003). Comparison of tamarins and marmosets as hosts for GBV-B infections and the effect of immunosuppression on duration of viremia. *Virology* **311**, 72–80.

Lerat, H., Rumin, S., Habersetzer, F., Berby, F., Trabaud, M. A., Trepo, C. & Inchauspe, G. (1998). In vivo tropism of hepatitis C virus genomic sequences in hematopoietic cells: influence of viral load, viral genotype, and cell phenotype. *Blood* **91**, 3841–3849.

Lindenbach, B. D., Evans, M. J., Syder, A. J., Wolk, B., Tellinghuisen, T. L., Liu, C. C., Maruyama, T., Hynes, R. O., Burton, D. R. & other authors (2005). Complete replication of hepatitis C virus in cell culture. *Science* **309**, 623–626.

Martin, A., Bodola, F., Sangar, D. V., Goettge, K., Popov, V., Rijnbrand, R., Lanford, R. E. & Lemon, S. M. (2003). Chronic hepatitis associated with GB virus B persistence in a tamarin after intrahepatic inoculation of synthetic viral RNA. *Proc Natl Acad Sci U S A* **100**, 9962–9967.

Miyahara, Y., Naota, H., Wang, L., Hiasa, A., Goto, M., Watanabe, M., Kitano, S., Okumura, S., Takemitsu, T. & other authors (2005). Determination of cellularly processed HLA-A2402-restricted novel CTL epitopes derived from two cancer germ line genes, MAGE-A4 and SAGE. *Clin Cancer Res* **11**, 5581–5589.

Pileri, P., Uematsu, Y., Campagnoli, S., Galli, G., Falugi, F., Petracca, R., Weiner, A. J., Houghton, M., Rosa, D. & other authors (1998). Binding of hepatitis C virus to CD81. *Science* **282**, 938–941.

Saito, I., Miyamura, T., Ohbayashi, A., Harada, H., Katayama, T., Kikuchi, S., Watanabe, Y., Koi, S., Onji, M. & other authors (1990). Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. *Proc Natl Acad Sci U S A* **87**, 6547–6549.

Scarselli, E., Ansuini, H., Cerino, R., Roccasecca, R. M., Acali, S., Filocamo, G., Traboni, C., Nicosia, A., Cortese, R. & Vitelli, A. (2002). The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. *EMBO J* **21**, 5017–5025.

Shirakura, M., Murakami, K., Ichimura, T., Suzuki, R., Shimoji, T., Fukuda, K., Abe, K., Sato, S., Fukasawa, M. & other authors (2007).

E6AP ubiquitin ligase mediates ubiquitylation and degradation of hepatitis C virus core protein. *J Virol* **81**, 1174–1185.

Sugawara, Y., Makuuchi, M., Kato, N., Shimotohno, K. & Takada, K. (1999). Enhancement of hepatitis C virus replication by Epstein–Barr virus-encoded nuclear antigen 1. *EMBO J* **18**, 5755–5760.

Sung, V. M., Shimodaira, S., Doughty, A. L., Picchio, G. R., Can, H., Yen, T. S., Lindsay, K. L., Levine, A. M. & Lai, M. M. (2003). Establishment of B-cell lymphoma cell lines persistently infected with hepatitis C virus in vivo and in vitro: the apoptotic effects of virus infection. *J Virol* **77**, 2134–2146.

Van De Parre, T. J., Martinet, W., Schrijvers, D. M., Herman, A. G. & De Meyer, G. R. (2005). mRNA but not plasmid DNA is efficiently transfected in murine J774A.1 macrophages. *Biochem Biophys Res Commun* **327**, 356–360.

Wakita, T., Pietschmann, T., Kato, T., Date, T., Miyamoto, M., Zhao, Z., Murthy, K., Habermann, A., Krausslich, H. G. & other authors (2005). Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat Med* **11**, 791–796.

Wood, J., Frederickson, R. M., Fields, S. & Patel, A. H. (2001). Hepatitis C virus 3′X region interacts with human ribosomal proteins. *J Virol* **75**, 1348–1358.

Zhong, J., Gastaminza, P., Cheng, G., Kapadia, S., Kato, T., Burton, D. R., Wieland, S. F., Uprichard, S. L., Wakita, T. & Chisari, F. V. (2005). Robust hepatitis C virus infection *in vitro*. *Proc Natl Acad Sci U S A* **102**, 9294–9299.

Hepatitis C Virus–Infected Hepatocytes Extrinsically Modulate Dendritic Cell Maturation To Activate T Cells and Natural Killer Cells

Takashi Ebihara,[1] Masashi Shingai,[1] Misako Matsumoto,[1] Takaji Wakita,[2] and Tsukasa Seya[1]

Dendritic cell maturation critically modulates antiviral immune responses, and facilitates viral clearance. Hepatitis C virus (HCV) is characterized by its high predisposition to persistent infection. Here, we examined the immune response of human monocyte-derived dendritic cells (MoDCs) to the JFH1 strain of HCV, which can efficiently replicate in cell culture. However, neither HCV RNA replication nor antigen production was detected in MoDCs inoculated with JFH1. None of the indicators of HCV interacting with MoDCs we evaluated were affected, including expression of maturation markers (CD80, 83, 86), cytokines (interleukin-6 and interferon-beta), the mixed lymphocyte reaction, and natural killer (NK) cell cytotoxicity. Strikingly, MoDCs matured by phagocytosing extrinsically-infected vesicles containing HCV-derived double-stranded RNA (dsRNA). When MoDCs were cocultured with HCV-infected apoptotic Huh7.5.1 hepatic cells, there was increased CD86 expression and interleukin-6 and interferon-beta production in MoDCs, which were characterized by the potential to activate NK cells and induce $CD4^+$ T cells into the T helper 1 type. Lipid raft-dependent phagocytosis of HCV-infected apoptotic vesicles containing dsRNA was indispensable to MoDC maturation. Colocalization of dsRNA with Toll-like receptor 3 (TLR3) in phagosomes suggested the importance of TLR3 signaling in the MoDC response against HCV. *Conclusion:* The JFH1 strain does not directly stimulate MoDCs to activate T cells and NK cells, but phagocytosing HCV-infected apoptotic cells and their interaction with the TLR3 pathway in MoDCs plays a critical role in MoDC maturation and reciprocal activation of T and NK cells. (HEPATOLOGY 2008;48:48-58.)

Abbreviations: CPZ, chlorpromazine; CTL, cytotoxic T lymphocyte; DC, dendritic cell; DC-SIGN, dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin; dsRNA, double-stranded RNA; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorting; HCV, hepatitis C virus; IFN, interferon; IFNAR, type I IFN-alpha receptor; IL, interleukin; IRF, IFN regulatory factor; MβCD, methyl-beta-cyclodextrin; MDA5, melanoma differentiation associated gene 5; mAb, monoclonal antibody; MoDC, monocyte-derived dendritic cell; MOI, multiplicity of infection; MV, measles virus; NK, natural killer; NKG2D, natural killer group 2, member D; PAMP, pathogen associated molecular pattern; PBMC, peripheral blood mononuclear cells; pDC, plasmacytoid DC; poly I:C, polyinosinic:polycytidylic acid; RIG-I, retinoic acid inducible gene I; RSV, respiratory syncytial virus; RT-PCR, reverse-transcription polymerase chain reaction; siRNA, small interfering RNA; SNARF1, far red immunofluorescence dye; Th1, T helper 1; TLR, Toll-like receptor; TNF, tumor necrosis factor.

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Hepatitis C virus (HCV) is a single-strand, positive-sense RNA virus belonging to the flaviviridae family. HCV develops persistent infection in <70% of infected patients, and eventually causes chronic hepatitis, cirrhosis, and hepatocellular carcinoma in some patients.[1] Once chronic infection is established in patients with HCV, spontaneous viral clearance fails,[1] although how HCV remains persistently infecting the liver is unknown. It has been accepted that successful viral clearance by the host is largely attributed to robust induction of type I interferon (IFN) and antiviral cellular effectors, cytotoxic T lymphocyte (CTL) and natural killer (NK) cells.[2-5] In HCV-infected patients and chimpanzees, type I IFN induction and activation of HCV-specific $CD4^+$ T/$CD8^+$ T cells and NK cells are indeed detected during acute infection.[4-6] However, why these antiviral factors cannot eradicate HCV from most patients is not addressed. Facilities for inducing the antiviral effectors and their role against HCV persistence have not been well determined. A main cause for the deficiency of knowledge on the host response to HCV is the lack of an appropriate model for experimental HCV infection.

Two breakthroughs have now made it possible to investigate the immune response against HCV. First, Toll-like receptors (TLRs) and other innate immune receptors of dendritic cells (DCs) were found to be involved in the host antiviral IFN response, followed by CTL and NK cell activation.[2,7-9] Some reports revealed that HCV proteins participate in the regulation of IFN-inducing innate responses.[10-12] Second, an *in vitro* amplifiable 2a type HCV strain, JFH1, was established by Wakita et al.[13] and Zhong et al.[14] Infection studies for testing HCV replication and the immune response are therefore now feasible *in vitro*.

There are two major subsets of DCs in humans: plasmacytoid DCs (pDCs) expressing TLR7 and TLR9 and myeloid DCs expressing Toll-like receptor 3 (TLR3) for viral RNA/DNA recognition. Cytoplasmic RNA sensors, retinoic acid inducible gene I (RIG-I)-like receptors, also participate in viral RNA recognition and IFN induction.[7] RNA virus infection allows pDCs to induce type I IFN via TLR7.[15] On the contrary, myeloid DCs recognize virus-derived double-stranded RNA (dsRNA) to activate pathways for IFN-beta production and NK/CTL induction.[7-9,16] What happens in the pathway of myeloid DC maturation during HCV infection can now be experimentally followed up in infected cells as the JFH1 strain can be used for *in vitro* infection studies. Hence, we inoculated monocyte-derived (Mo)DCs with JFH1 of HCV.

Here, we show evidence that the JFH1 strain has no direct route for MoDC infection and MoDCs phagocytosing HCV-infected apoptotic vesicles participate in MoDC maturation and reciprocal activation of T cells and NK cells.

Materials and Methods

Cell Lines, Antibodies, and Reagents. Huh7.5.1 cells were kindly provided by Dr. Francis V. Chisari (The Scripps Research Institute, La Jolla, CA), and maintained in Dulbecco's modified Eagle's medium–based medium.[14] Following materials were obtained as indicated: anti-HCV-core monoclonal antibody (mAb; C7-50) from Affinity BioReagents (Golden, CO), mAbs against CD80, CD83, and CD86 from Immunotech (Fullerton, CA), anti-dsRNA mAb (K1) from English & Scientific Consulting Bt (Szirak, Hungary), biotin-conjugated anti-TLR3 mAb from eBioscience (San Diego, CA), fluorescein isothiocyanate–labeled goat anti-mouse immunoglobulin G from American Qualex (San Clemente, CA), Streptavidin Alexa Fluor 594 conjugate and SNARF1 from Molecular Probe (Carlsbad, CA), Methyl-beta-cyclodextrin (MβCD), chlorpromazine (CPZ), and bafilomycin (BAF) from Sigma-Aldrich (St. Louis, MO).

Preparation of Immature MoDCs, NK Cells, and T Cells. $CD14^+$ monocytes and autologous NK cells were isolated from human peripheral blood mononuclear cells (PBMCs) using a MACS system (Miltenyi Biotec, Bergisch Gladbach, Germany).[17] Cells purified by this technique had an average purity of 95%, as assessed by flow cytometry. Immature MoDCs were generated from monocytes using human granulocyte-macrophage colony-stimulating factor (GM-CSF; PeproTech, Rocky Hill, NJ) and interleukin (IL)-4 (PeproTech).[17] Autologous NK cells were stocked in Cell Banker (Diaton, Tokyo, Japan) at −80°C. Allogeneic $CD4^+$ and $CD8^+$ T cells were also negatively isolated by a MACS system (Miltenyi Biotec).

Stimulation of Immature MoDC, Cytokine Assay, Immunofluorescent Staining, and Flow Cytometry. The immature MoDCs (2×10^5) were inoculated with HCV and respiratory syncytial virus (RSV) at a multiplicity of infection (MOI) of one or treated with polyinosinic: polycytidylic acid (poly I:C; 10 μg/mL), and cultured in a 24-well plate. The cells and culture supernatant were harvested at indicated time points for reverse transcription polymerase chain reaction (RT-PCR), fluorescence-activated cell sorting (FACS), and enzyme-linked immunosorbent assay (ELISA; IFN-beta, IFN-gamma; Fujirebio, Inc., Tokyo, Japan; IL-6; BD Biosciences, Franklin Lakes, NJ). In some experiments, immature MoDCs (2×10^5) were cocultured with HCV- or non-infected apoptotic cells (4×10^5). MoDCs were treated with MβCD (1 mM), CPZ (5 μg/mL), and BAF (100

nM) for 1 hour before coculture. The viability of these MoDCs was examined by proidium iodide staining. After 2 days of coculture, the MoDCs were isolated from the apoptotic cells by Ficoll-Paque Plus (GE-Healthcare, Waukesha, WI) using the manufacturer's methods, and used for further analysis to assess MoDC functions. The cell lysates were produced from the apoptotic cells by three freeze/thaw cycles, followed by centrifugation at 15,000 rpm for 5 minutes or by sonication three times for 20 seconds on ice. Total RNA was extracted by Trizol (Invitrogen, Carlsbad, CA) by the manufacturer's methods. MoDCs (5×10^5 cells) were transfected with 0.625 μg total RNA by N-[1-(2,3-Dioleoyloxy)propyl]-N, N, N-trimethylammonium methyl-sulfate (DOTAP; Roche, Mannheim, Germany) and cultured in 24-well plates for 1 day. Huh7.5.1 cells were transfected with poly I:C using Lipofectamine 2000 (Invitrogen) by the manufacturer's methods. ELISA for determination of cytokine levels, flow cytometry, and immunofluorescent staining were performed as reported.[17,18]

Virus Propagation. The method to generate infectious HCV particles was referred to an *in vitro* system using the plasmid pJFH-1.[16] Noninfected cell supernatant was used as noninfected control. The concentrated virus had a titer of 1 to 2×10^6 ffu/mL. A RSV field-isolate strain (RSV2177) was propagated with Hep-2 cells as described.[17] The titer of RSV2177 was determined by 50% tissue culture infective dose (TCID50) with Hep-2 cells.

Real-time PCR Quantification of Positive-Strand and Negative-Strand HCV RNA. Total Trizol-extracted RNA was analyzed by reverse transcription-PCR (RT-PCR) with a modification of the previously described strand-specific rTth RT-PCR method.[19] RT primers for complementary DNA synthesis of positive and negative strand HCV RNA were GTGCACGGTCTACGAGACCT and GAGTGTCGTACAGCCTCCAG, respectively. Positive-strand and negative-strand HCV PCR amplifications were performed using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) with 200 nM of paired primers, forward CGGGAGAGCCATAGTGG and reverse AGTACCACAAGGCCTTTCG. The PCR conditions were 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. This PCR method could detect 10 copies of positive-strand or negative-strand HCV.

Induction and Certification of Apoptosis. A total of 1×10^5 Huh7.5.1 cells were plated in a 24-well plate and infected with the JFH1 strain at an MOI of 1. At indicated timed intervals, the infected cells and poly I:C-transfected cells were pretreated with cycloheximide

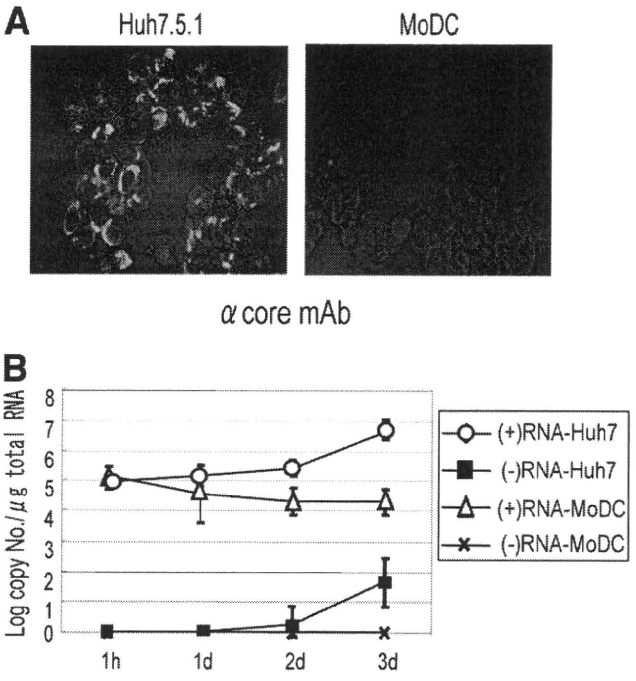

Fig. 1. MoDCs are not permissive for HCV replication. (A) Huh7.5.1 cells and MoDCs were inoculated with HCV at an MOI of 1 and cultured for 3 days. The presence of HCV core antigens was assessed by immunofluorescent staining. (B) Real-time RT-PCR to detect positive-strand and negative-strand HCV RNA. Data show means ± SD from three independent experiments using three different donors.

(20 μg/mL; Sigma) for 30 minutes, followed by tumor necrosis factor (TNF)-alpha (10 ng/mL; Pepro-Tech). The HCV-infected and noninfected apoptotic cells were harvested after another 24-hour culture. Using the HCV-infected apoptotic cells, we examined the presence of HCV core antigens and dsRNA by FACS using anti-HCV core mAb and anti-dsRNA mAb, respectively. Apoptosis was assessed by 4′,6-diamidino-2-phenylindole-staining, DNA fragmentation, and FACS by using fluorescein isothiocyanate–labeled annexin-V and proidium iodide (Roche).[20]

Assay for Lymphocyte Proliferation by MoDC. After 2 days culture of MoDCs with HCV, poly I:C (10 μg/mL), or the apoptotic cells, MoDCs were harvested and treated with mitomycin C (20 μg/mL) in phosphate buffered saline for 45 minutes. For the proliferation assay, the stimulated-MoDCs (1×10^4) were cultured with 1×10^5 allogeneic PBMCs, $CD4^+$ T cells, or $CD8^+$ T cells in U-bottom 96-well plates for 6 days. During the last 24 hours of culturing, [^{3}H]thymidine (1 mCi/well) was added to the culture medium. Then the cells and medium were harvested separately by a cell-harvester, and the radioactivity was measured by a liquid scintillation counter (Aloca, Tokyo, Japan). For the analysis of $CD4^+$ T cell polarization, the stimulated-MoDCs (1×10^4) were

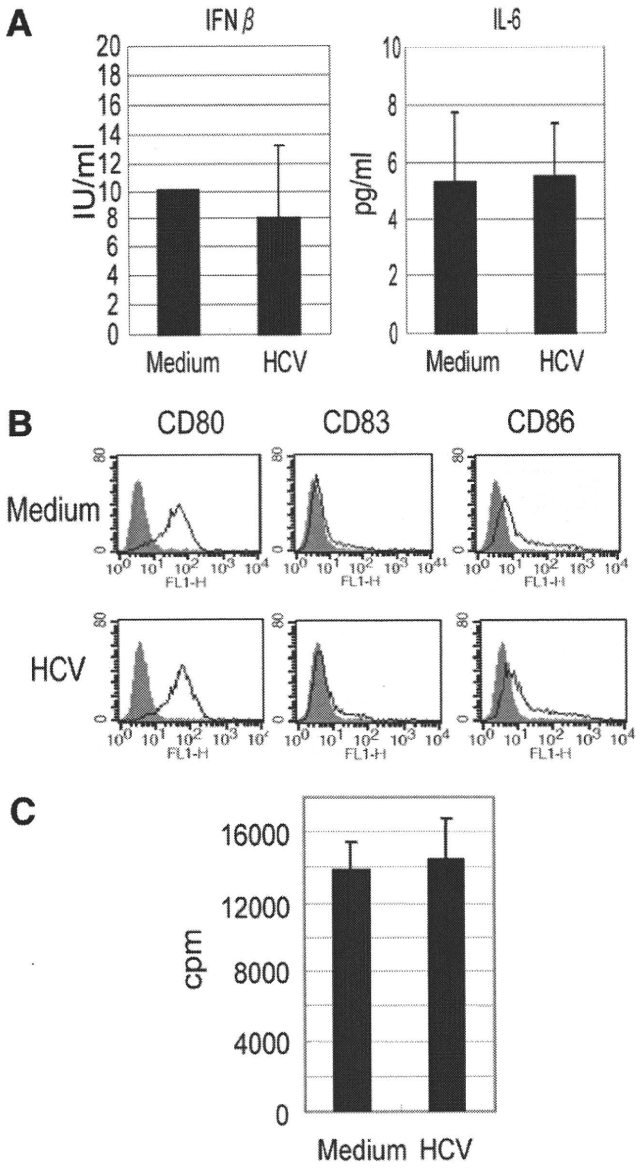

Fig. 2. HCV fails to induce MoDC maturation and cytokine response. MoDCs were inoculated with HCV at an MOI of 1 and cultured for 48 hours. (A) The supernatant was assayed for production of IFN-beta and IL-6. (B) The cells were harvested for FACS and (C) mixed lymphocyte reaction (MIR). Allogeneic PBMC were cultured with the inoculated-MoDCs for 6 days. Proliferation was determined by [^{3}H]thymidine uptake. Data show means ± SD of duplicate or triplicate samples from one experiment representative of three donors.

treated with mitomycin C and cultured with allogeneic $CD4^+$ T cells (1×10^5) for 6 days. Then the cells were washed and transferred to new round-bottom 96-well plates. Phorbol 12-myristate 13-acetate (10 ng/mL; Sigma-Aldrich) and ionomycin (1 μg/mL; Sigma-Aldrich) were added and plates were incubated for a further 24 hours. Supernatants were harvested for cytokine production (IL-4, IFN-gamma; GE-Healthcare).

MoDC-NK Coculture and ^{51}Cr Release Assay. The stimulated-MoDCs were harvested for MoDC-NK coculture at indicated time points. Autologous NK cells (5×10^5) were cocultured with the MoDCs (1×10^5) in 24-well plates for 24 hours. Transwell (Corning) was inserted to block the MoDC-NK cell contact. The supernatants and NK cells were collected from the MoDC-NK coculture and assayed for IFN-gamma production (GE Healthcare) and cytotoxicity against K562. Cytotoxicity was determined by standard ^{51}Cr release assay as described.[17]

Gene Silencing of TLR3 in MoDC. Small interfering RNA (siRNA)-based gene knockdown was preformed with MoDCs by electroporation as described.[21] siRNA duplexes (small interfering TLR3: cat #107056, negative control: cat #AM4635) were obtained from Ambion (Tokyo, Japan). Expression of TLR3 was examined by SYBR green real-time PCR using forward primer, AAGACCCATTATG-CAAAAGATTCAA and reverse primer, TCCAGATTTT-GTTCAATAGCTTGTTG. MoDCs (1×10^6 cells) were electroporated with these siRNA and cultured for 4 hours. Then, HCV-infected or noninfected apoptotic Huh7.5.1

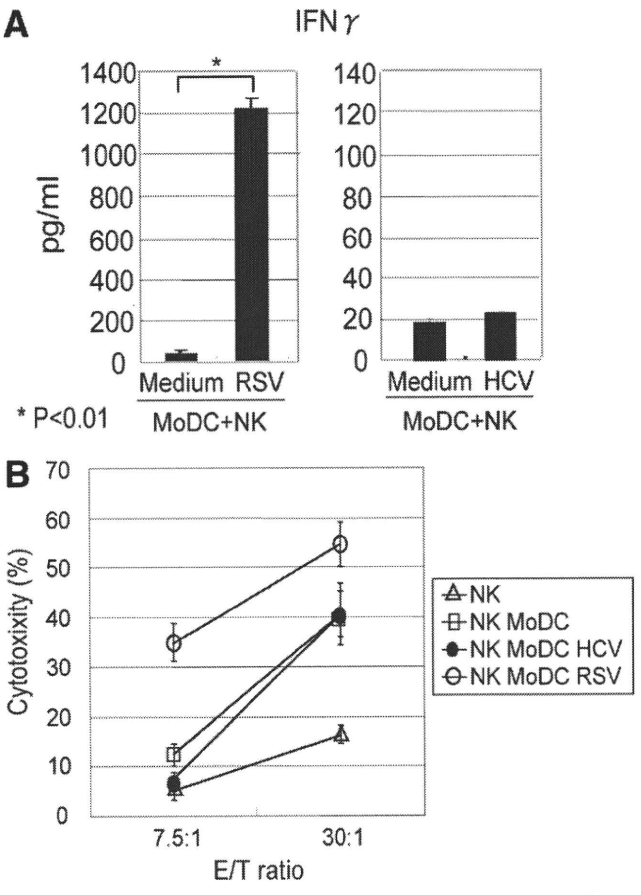

Fig. 3. MoDCs inoculated with HCV barely activate NK cells. MoDCs were harvested at 24 hours after inoculation of RSV and HCV. Autologous NK cells were cocultured with the MoDCs for 24 hours. (A) The supernatant were assayed for NK IFN-gamma production. (B) NK cells were harvested for ^{51}Cr release assay to examine NK cytotoxic activity against K562. A representative of the three similar experiments with individual donors is shown.

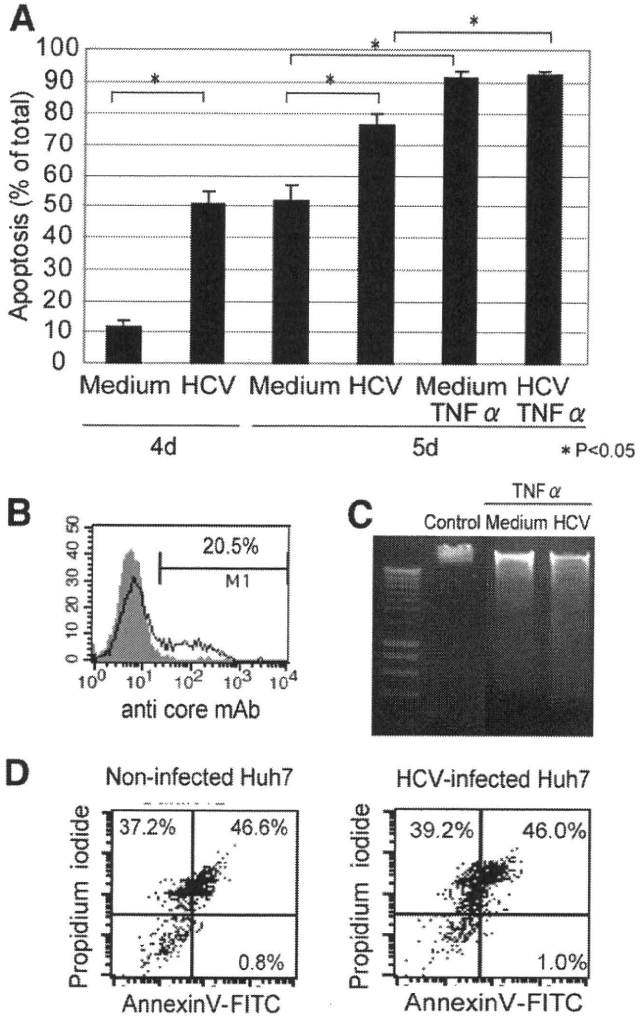

Fig. 4. Preparation of HCV-infected apoptotic Huh7.5.1 cells. Huh7.5.1 cells were infected with HCV at an MOI of 1 and culture for 4 or 5 days. Apoptosis was induced by cycloheximide and TNF-alpha after 4 days culture. At 5 days after infection, the apoptotic cells were harvested for counting 4′,6-diamidino-2-phenylindole stained apoptotic nuclei, FACS and examination of DNA fragmentation. (A) Typical apoptotic cells stained with 4′,6-diamidino-2-phenylindole were counted among <1000 cells and percent cell apoptosis was determined. Data are means ± SD from three independent experiments, each performed in triplicate. (B) HCV-core antigens were detected in the HCV-infected apoptotic cells by FACS. (C) DNA was extracted from HCV- or noninfected apoptotic Huh7.5.1 cells and electrophoresed on agarose gels to evaluate DNA fragmentation. (D) HCV- and noninfected Huh7.5.1 cells were examined for stages of apoptosis by FACS using annexin V-fluorescein isothiocyanate (FITC) and proidium iodide. Data shown are representative of three independent experiments.

cells (2×10^6 cells) were added to the wells. After 48 hours of culture, the supernatants were harvested and examined for cytokine concentrations by ELISA.

Results

MoDCs Are Not Permissive for HCV Replication. MoDCs and Huh7.5.1 cells were inoculated with the JFH1 strain at an MOI of 1, then the cells were harvested for immunofluorescent staining and sequence-specific real-time RT-PCR at indicated time points after inoculation. HCV genome RNA (negative sense of HCV RNA) was replicated in Huh7.5.1 cells but not to a detectable level in MoDCs at 2 to 3 days after inoculation (Fig. 1B). Accordingly, HCV core antigens were detected in Huh7.5.1 cells, but not in MoDCs, by immunofluorescent staining until 3 days after HCV inoculation (Fig. 1A). Similar results were obtained with monocyte-derived macrophages and BDCA4$^+$ pDCs (data not shown).

MoDC Maturation and Cytokine Response Against the JFH1 Strain. DCs work as key producers of innate inflammatory cytokines in response to pathogen-associated molecular patterns (PAMPs). However, MoDCs inoculated with JFH1 (MOI = 1) did not produce IFN-gamma or IL-6 over the noninfected control (Fig. 2A). MoDCs stimulated with PAMPs mature to up-regulate CD80/CD86 expression and activate T cells. Some reports showed that the MoDC maturation was induced following incorporation of HCV pseudotype particles into the MoDCs.[22] However, expression of costimulatory molecules (CD80, CD86) and a maturation marker (CD83) were not up-regulated by inoculation with the JFH1 strain (MOI = 1; Fig. 2B). MoDCs cocultured with JFH1 strain did not enhance the proliferation of allogeneic PBMC compared with noninoculated MoDCs (Fig. 2C).

MoDCs Exposed to the JFH1 Strain Do Not Activate NK Cells. MoDCs are known to recognize PAMPs and promote NK cell activation via MoDC/NK reciprocal interaction.[9] We have reported that NK cells are activated by MoDCs infected with RNA viruses, such as RSV, influenza virus, and measles virus.[17] We inoculated MoDCs with RSV or the JFH1 strain at an MOI of 1 and cocultured the MoDCs with autologous NK cells. After 1-day of coculture, NK cell IFN-gamma and cytotoxicity were markedly induced by RSV-treated MoDCs but not HCV-treated MoDCs (Fig. 3A,B).

HCV-Infected Apoptotic Cells Induce MoDC Maturation and Cytokine Responses. Then, we moved on to whether HCV-infected cells affect MoDC maturation. We first cocultured MoDCs with HCV-infected or noninfected Huh7.5.1 cells and examined IL-6 production by MoDCs. MoDCs cocultured with HCV-infected Huh7.5.1 cells secreted more IL-6 than those with noninfected Huh7 cells (Fig. 5A). However, since HCV infection induced apoptosis in Huh7.5.1 cells, HCV-infected and noninfected Huh7 cells were not in the same apoptotic stages (Fig. 4A). We had to exclude the possibility that apoptotic events themselves affect MoDC maturation. Therefore, we forced HCV-infected and

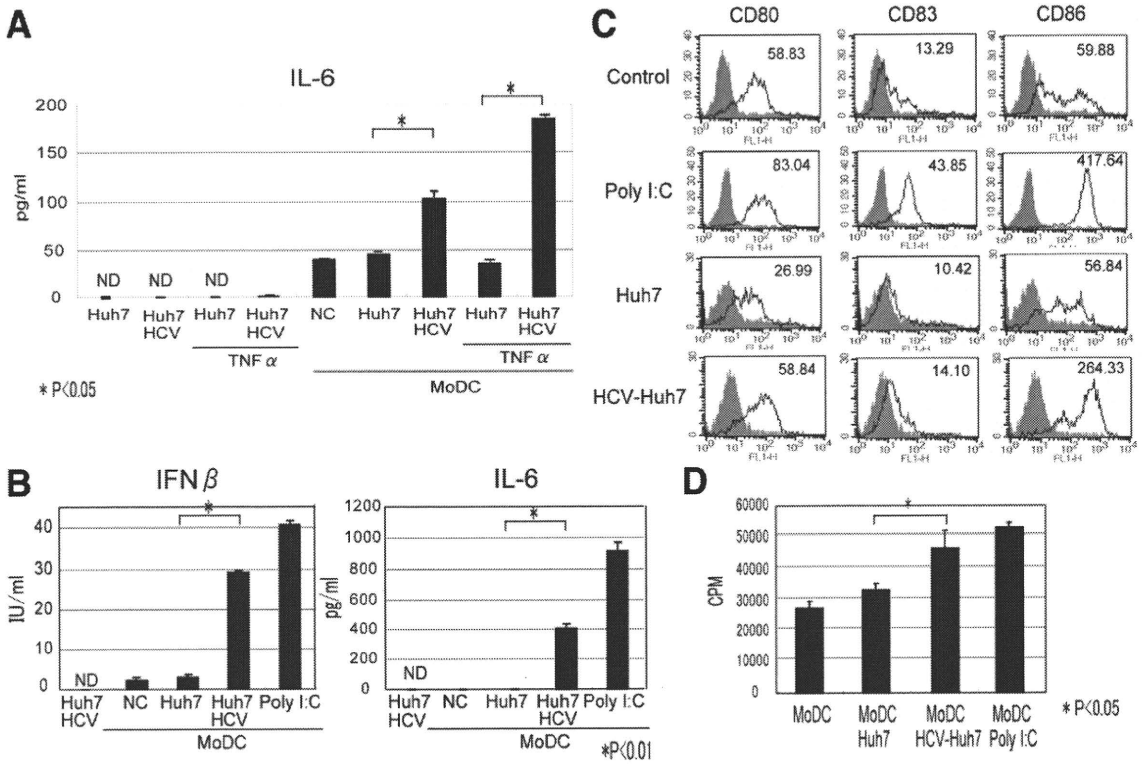

Fig. 5. HCV-infected apoptotic cells induce MoDC maturation and cytokine response. MoDCs were cocultured with HCV-infected/noninfected apoptotic or nonapoptotic cells for 2 days. Polyl:C stimulation was used as a positive control. (A,B) The culture supernatants were assayed for determination of IFN-beta and IL-6. The MoDCs were isolated from the apoptotic cells and used for (C) FACS and (D) mixed lymphocyte reaction (MLR). MoDC maturation was examined by the expression of CD80, CD83, and CD86 (C, a representative of three donor experiments). Allogeneic PBMCs were cultured with the MoDCs for 6 days. Proliferation was determined by [^{3}H]thymidine uptake (D, means ± SD of triplicate samples from one representative of three donors).

noninfected cells to induce full apoptosis by cycloheximide and TNF-alpha to the same level of apoptotic stages (Fig. 4A). HCV core antigens were detected in 20.5% of the HCV-infected apoptotic cells (Fig. 4B). Apoptotic nuclei were observed in almost all of HCV-infected and noninfected cells (Fig. 4A). DNA ladder formation, a hallmark of apoptosis, was detected in HCV-infected and noninfected apoptotic Huh7.5.1 cells to similar levels (Fig. 4C). Apoptotic cells, either infected or noninfected, gave similar profiles by flow cytometry using annexin-V for early apoptosis and propidium iodide for late apoptosis (Fig. 4D).

We applied these HCV-infected and noninfected apoptotic cells to MoDCs and determined the concentration of IFN-beta and IL-6 in the culture supernatants. HCV-infected apoptotic cells facilitated the production of IFN-beta and IL-6 by MoDCs compared with noninfected apoptotic cells (Fig. 5B). In this context, HCV products, rather than undergoing apoptosis, in infected cells are an essential factor for induction of MoDC maturation (Fig. 5A).

We next examined whether MoDC maturation was induced by HCV-infected apoptotic cells. After coculture of MoDCs with the apoptotic cells, MoDCs were isolated from the apoptotic cells using Ficol Paque. Purity of these isolated MoDCs reached over 98%, judged by 5(6)-Carboxyfluorescein diacetate N-succinimidyl ester labeled MoDCs (data not shown). CD86 of the maturation markers on MoDCs (Fig. 5C) was especially more expressed on these cells by HCV-infected apoptotic cells than by noninfected apoptotic cells. HCV-infected apoptotic cells slightly enhanced the expression levels of major histocompatibility complex class I, class II, and human leukocyte antigen-E on MoDCs (data not shown). MoDCs also acquired the increased ability to stimulate allogeneic PBMCs, $CD4^+$ T cells, and $CD8^+$ T cells in response to HCV-infected apoptotic cells (Figs. 5D and 6A).

We determined the ability of $CD4^+$ T cells to produce IFN-gamma (a Th1 cytokine) and IL-4 (a T helper 2 cytokine) after coculture of allogeneic $CD4^+$ T cells and MoDCs exposed to HCV-infected apoptotic cells. These $CD4^+$ T cells produced higher levels of IFN-gamma and lower levels of IL-4 (Fig. 6B) compared to the noninfected control, suggesting that HCV-infected apoptotic cells modulate MoDC func-

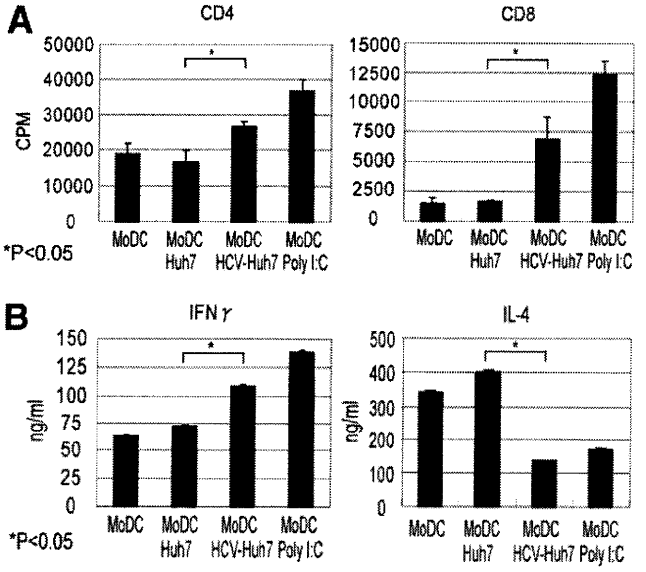

Fig. 6. HCV-infected apoptotic cells modulate MoDC function to polarize the Th1 shift. (A) After 2 days culture with HCV-infected and noninfected apoptotic cells, the isolated MoDCs (1×10^4) were cultured with allogeneic $CD4^+$T cells and $CD8^+$ T cells (1×10^5) for 6 days. Proliferation was determined by [^{3}H]thymidine uptake. (B) Allogeneic $CD4^+$ T cells were harvested after 6 days culture with the MoDCs and stimulated with phorbol 12-myristate 13-acetate and ionomycin for 24 hours. The supernatants were collected to assess the levels of IFN-gamma and IL-4 by ELISA. Poly I:C stimulation was used as a positive control. Data shown are means ± SD of duplicate or triplicate samples from one experiment representative of three donors.

tion to promote Th1-dominant immunity in the Th1/T helper 2 balance.

HCV-Infected Apoptotic Cells Stimulate MoDCs To Activate NK Cells. We next evaluated whether these mature MoDCs could enhance NK activity via MoDC-NK interaction. After exposure of MoDCs to HCV-infected or noninfected apoptotic cells, MoDCs were isolated as described above. HCV-infected apoptotic cells promoted MoDC function to augment NK cell cytotoxicity but not IFN-gamma production compared to noninfected cells (Fig. 7A,B). This up-regulation of NK cell cytotoxicity through MoDC-NK interaction was canceled by separating MoDCs from NK cells with a transwell insertion (Fig. 7C). This suggested that cell-cell contact was the key factor for MoDC-mediated NK cell cytotoxicity induced by coculture with HCV-infected apoptotic cells.

MoDC Maturation Relied on TLR3 Signal Evoked by dsRNA in Apoptotic Vesicles. We surveyed the mechanism of MoDC maturation by HCV-infected apoptotic cells. Since HCV is a positive single-strand RNA virus, dsRNA was detected in HCV-containing apoptotic vesicles by mAb against dsRNA (Fig. 8A). To investigate whether MoDCs were taking up these apoptotic vesicles, we labeled HCV-infected apoptotic cells with the far red fluorescent dye, SNARF-1. MoDCs phagocytosed the SNARF-1-labeled vesicles containing dsRNA, which partially colocalized with TLR3 (Fig. 8B,C). N-[1-(2,3-Dioleoyloxy)propyl]-N, N, N-trimethylammonium methylsulfate (DOTAP)-based transfection was employed for the targeting of RNA to the TLR3-containing endosome.[23] HCV-derived RNA allowed MoDCs to induce IL-6 production as in control poly I:C (Fig. 8D). IL-6 of

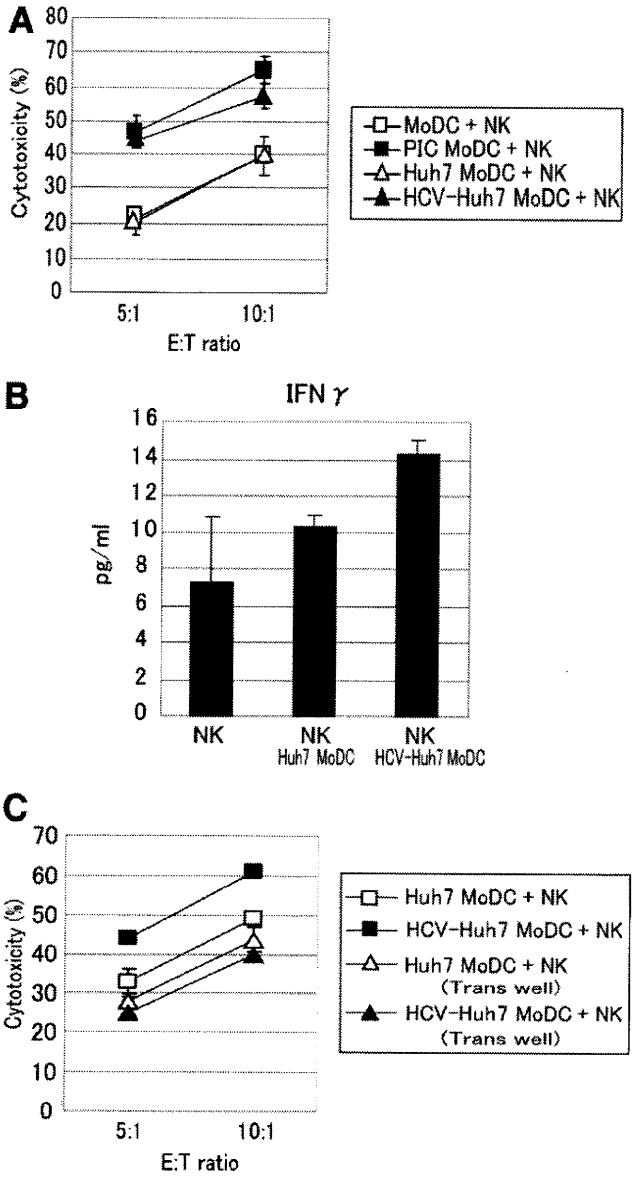

Fig. 7. NK cell activation by MoDCs exposed to HCV-infected apoptotic cells. After 2 days culture with HCV-infected and noninfected apoptotic cells, the isolated MoDCs were cultured with autologous NK cells for 24 hours. (A) NK cytotoxic activity against K562 was determined by ^{51}Cr release assay. Poly I:C stimulation was used as positive control. (B) Supernatant of the MoDC-NK coculture was assayed for NK cell IFN-gamma production. In some experiments, transwell was used to block MoDC-NK cell contact. (C) Using these NK cells, cytotoxic activity was measured by ^{51}Cr release assay. Data shown are means ± SD of duplicate or triplicate samples from one experiment representative of three donors.