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ORIGINAL ARTICLE

Genomic polymorphisms in 3β-hydroxysterol Δ24-reductase promoter sequences

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ABSTRACT

It was recently reported by the present team that 3β-hydroxysterol Δ24-reductase (DHCR24) is induced by hepatitis C virus (HCV) infection. In addition, upregulation of DHCR24 impairs p53 activity. In human hepatoma HuH-7 cells, the degree of DHCR24 expression is higher than in normal hepatic cell lines (WRL68) at the transcriptional level. The genomic promoter sequence of DHCR24 was characterized and nucleotide substitutions were observed in HuH-7 cells at nucleotide numbers −1453 (G to A), −1420 (G to T), −488 (A to C) and −200 (G to C). The mutations of these sequences from HuH-7 cell types to WRL68 cell types suppressed DHCR24 gene promoter activity. The sequences were further characterized in hepatocytes from patient tissues. Four tissues from HCV-positive patients with cirrhosis or hepatocellular carcinoma (#1, 2, 3, 5) possessed HuH-7 cell type sequences. Interestingly, one patient with liver cirrhosis (#4) possessed WRL68 cell-type sequences; this patient had been infected with HCV and was HCV negative for 17 years after interferon therapy. Next, the effect of HCV infection on these polymorphisms was examined in humanized chimeric mouse liver and HuH-7 cells. The human hepatocytes possess WRL68 cell type and did not show the nucleotide substitution after HCV infection. The HCV-replicon was removed by interferon treatment and established the cured K4 cells. These cells possess HuH-7 cell type sequences. Thus, this study showed the genomic polymorphism in DHCR24 promoter is not directly influenced by HCV infection.

Key words 3β-hydroxysterol Δ24-reductase, hepatitis C virus, promoter.

Liver cancer is one of the most prevalent forms of cancer (1). More than 80% of cases occur in developing countries; however, Japan also has a remarkably high incidence (2). Among the primary liver cancers, HCC is the most common (3). Its incidence is increasing: between 1975 and 2005, age-adjusted HCC rates tripled (4).

One crucial cause of HCC is HCV infection (5). DHCR24, which functions as an oxidoreductase during cholesterol biosynthesis (6, 7), is linked to HCV-associated hepatocarcinogenesis and development of HCC (8–10). Infection of hepatocytes with HCV results in over-expression of DHCR24. This enzyme protects cells from oxidative stress and inhibits p53 activity (8), thus

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List of Abbreviations: DHCR24, 3β-hydroxysterol Δ24-reductase; DMEM, Dulbecco's modified Eagle's medium; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IFN, interferon; SVR, sustained viral response.

contributing to the development of HCC (5). These facts prompted us to investigate whether the molecular features of *DHCR24* are linked to HCC development. To this end, we characterized the promoter region of *DHCR24* in HCC cell lines and clinical samples.

MATERIALS AND METHODS

Cell lines and growth conditions

HuH-7 and HepG2 cells were cultured in (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FCS (Sigma-Aldrich). WRL68 cells were cultured in DMEM supplemented with 1 mM sodium pyruvate (Invitrogen, Carlsbad, CA, USA), 0.1 mM non-essential amino acids (Invitrogen) and 10% FCS. HuH-7 cell-based HCV replicon harboring cell lines (R6FLR-N) (11) were cured off HCV by interferon treatment (12) and designated as K4 cells.

Northern and western blotting

Northern and western blotting were performed as previously described (8).

Sequencing of genomic DNA and reporter plasmid construction

Genomic DNA was extracted from HuH-7 and WRL68 cells using standard methods. DNA from the promoter region of *DHCR24* (~5 kb) was amplified using PCR (sense primer: 5′-CACTCCTGCTCACCACTGAT-3′; antisense primer: 5′-GTAGTAGATATCGAAGATAAGCGAGAGCGG-3′). These fragments were individually cloned into the upstream region of the firefly luciferase gene in the pGL3-Basic vector (Promega, Madison, WI, USA) at the *Xho*I and *Nco*I sites (as we had done previously for the HepG2 cell line) (6). DNA sequences were determined using standard methods. Reporter plasmids that possessed chimeric promoters were constructed using restriction enzyme sites for *Tth111*I (position −2160) and *Bss*HII (position −1030).

Dual luciferase reporter assay

Using Lipofectamine LTX (Invitrogen), HepG2 cells (1×10^4 cells/well in a 96-well plate) were transfected with a reporter plasmid (0.25 μg/well) together with an internal control plasmid (phRL-TK; 0.025 μg/well) encoding *Renilla* luciferase (Promega). Forty-eight hours after transfection, the cells were assayed with the Dual-Glo Luciferase Assay System (Promega). Luminescence was measured using a TriStar LB941 microplate reader (Berthold Technologies GmbH, Bad Wildbad, Germany).

Liver tissue samples from chimeric mice or patients infected with hepatitis C virus

Severely combined immunodeficient mice carrying human primary hepatocytes were purchased from BD BioSciences (Franklin Lakes, NJ, USA) and African American, male, 5-year-old, HCV negative mice from PhoenixBio (Hiroshima, Japan) (13). These "human liver chimeric" mice were inoculated or mock-inoculated with plasma collected from an HCV-positive (HCR6 strain (14), GenBank accession #AY045702) patient in accordance with the requirements of the Declaration of Helsinki. HCV infection in the mice thus infected was confirmed by using quantitative PCR for HCV mRNA as previously described (9). The protocols for the animal experiments were pre-approved by the local Ethics Committee, and the animals were maintained in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Informed consent for this clinical study was obtained from five patients with HCV (Table 1) at the Kumamoto University Hospital (Kumamoto, Japan), in accordance with the Helsinki Declaration prior to 2003, and the protocol was approved by the Regional Ethics Committee. LiverPool 20-donor pooled cryopreserved human hepatocytes (Celsis IVT, Baltimore, MD, USA) were purchased and used as the normal human liver tissue control. HCV RNA was detected by the COBAS TaqMan HCV test (Hoffman–La Roche, Basel, Switzerland). Liver

Table 1. Summary of patients with HCC

Patient ID	Sex	Age (years)	Diagnosis	ALT (IU/mL)	Outcome of IFN treatment	HCV RNA detection[†]
#1	F	60	LC	22	NR	+
#2	M	65	LC	31	NT	+
#3	F	57	LC	24	NR	+
#4	M	61	LC, HCC	12	SVR	−[‡]
#5	M	51	LC, HCC	91	NR	+

[†]Serum was tested for HCV RNA using quantitative PCR; [‡]In 1995, #4 was diagnosed with HCV-associated LC and HCC and HCV RNA was detected in his serum. As a result, #4 was treated with IFN. Since then, no HCV RNA has been detected in this patient's serum (>17 years). ALT, alanine aminotransferase; F, female; LC, liver cirrhosis; M, male; NR, no response; NT, not treated.

tissue was obtained from either mice or patients and processed for DNA sequencing. Two DNA fragments (corresponding to positions −1600 to −1292 and −631 to −86) were amplified using PCR with Tth-Bss forward and reverse primers (5′-ATTTCAACATGTCATTAACA-3′ and 5′-TTCTAGCACGGTGCTTTGTG-3′) and Bss-Nco forward and reverse primers (5′-CCAGCCA-TAGCCTTCCATG-3′ and 5′-AATGGCGAGCCGCGC CGG-3′), respectively. The amplified fragments were directly sequenced using the same set of primers.

Statistical analysis

Student's *t*-test was used to test the statistical significance of the results. P values of < 0.05 were considered statistically significant.

RESULTS

First, we measured DHCR24 expression in cell lines of noncancerous hepatocytes (WRL68) and hepatoma cells (HuH-7 and HepG2). Compared with noncancerous hepatocytes, DHCR24 expression in the two hepatoma cell lines was considerably increased with respect to both mRNA and protein (Fig. 1a, b). In addition, the different culture media used for the WRL68 and HuH-7 cells did not significantly influence the degree of expression of DHCR24 protein (Fig. 1c).

To identify the genetic characteristic(s) that govern DHCR24 upregulation, we isolated genomic DNA from these three cell lines and sequenced the *DHCR24* promoter region (nucleotide positions −4976 to +113, where +1 indicates the transcription start site). For this analysis, we sequenced three molecular clones from each cell line. Alignments of WRL68 and HuH-7 sequences showed different nucleotides at four positions: (i) an A to G switch at −1453 (i.e., A in WRL68 and G in HuH-7); (ii) a T to G switch at −1420; (iii) a C to A switch at −488; and (iv) a C to G switch at −200 (Fig. 2). The two hepatoma cell lines (HuH-7 and HepG2) had no nucleotide differences within these regions.

Next, we investigated whether these small changes in the promoter sequence affect gene expression in a heterologous context. We constructed reporter plasmids that placed the firefly luciferase gene under the control of *DHCR24* promoter sequences (either from HuH-7 or WRL68 cells) (Fig. 3a). We measured the promoter activity of each construct in HepG2 cells with dual-luciferase assays. The *DHCR24* promoter derived from HuH-7 cells showed significantly greater activity (i.e., induced greater expression) than the WRL68 promoter (Fig. 3b). We also constructed two reporter plasmids that contained chimeric promoters. In each of these chimeras,

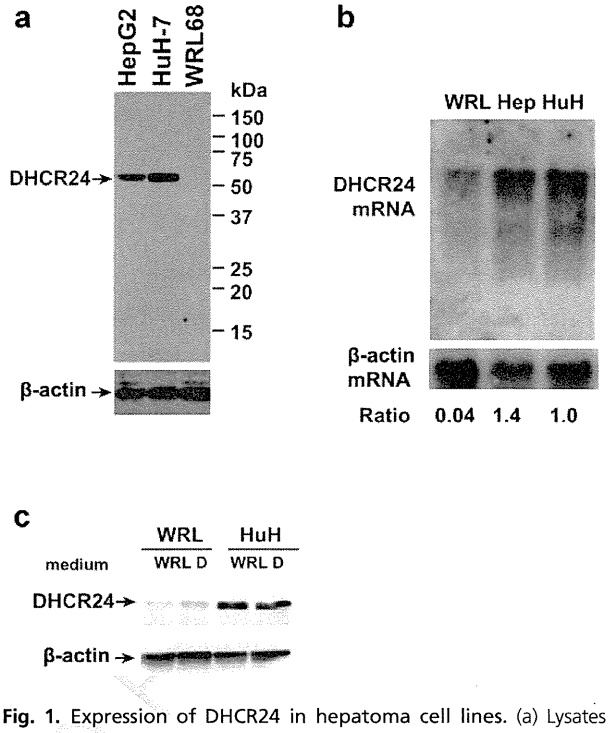

Fig. 1. Expression of DHCR24 in hepatoma cell lines. (a) Lysates from WRL68, HuH-7 and HepG2 cells were subjected to western blot analysis using antibodies directed against DHCR24 (upper panel) and β-actin (lower panel). (b) RNA was extracted from WRL68, HepG2 and HuH-7 cells and subjected to northern blot analysis using probes specific for *DHCR24* (upper panel) and β-actin (lower panel). Band intensities were quantified with a densitometer. Relative band intensity ratios (*DHCR24*/β-actin) are indicated below the gel images (the ratio for HuH-7 cells was set at 1). (c) DHCR24 protein (upper panel) and β-actin (lower panel) were detected in WRL 68 or HuH-7 cells with culture media for WRL68 cells (DMEM, 1 mM sodium pyruvate and 1 mM nonessential amino acids) or HuH-7 cells (DMEM alone).

we replaced HuH-7 fragments containing two polymorphisms with wild-type WRL68 sequences (Fig. 3a). These chimeric promoters had less activity than did intact promoters from both HuH-7 and WRL68 cells (Fig. 3b). These results indicate that the *DHCR24* promoter from HuH-7 cells contributes to the strong degree of *DHCR24* expression. In addition, all four nucleotide sequences of HuH-7 cell type in promoter fragments might be important for strong promoter activity.

Thereafter, we examined whether polymorphisms within the *DHCR24* promoter could be detected in clinical samples. We collected samples of liver tissue from five patients infected with HCV (Table 1) and sequenced the *DHCR24* promoter region (Table 2). Of the five samples tested, four (#1–3 and #5) showed all four of the polymorphisms associated with strong promoter activity (i.e., G, G, A and G nucleotides at positions −1453, −1420, −488, and −200). In contrast, promoter

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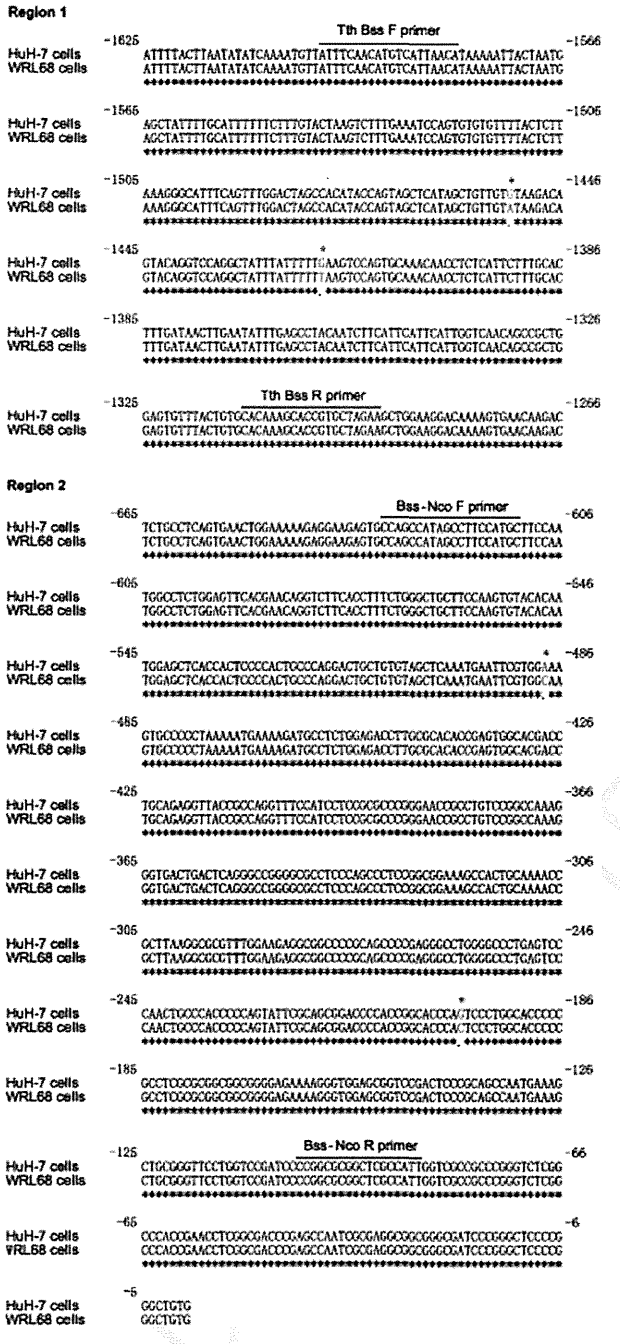

Fig. 2. Alignment of *DHCR24* promoter sequences. Nucleotide sequences from *DHCR24* promoter regions obtained from HuH-7 and WRL68 cells are shown. Cell-type-specific differences between these sequences (at positions −1453, −1420, −488, and −200) are indicated by asterisks and colors (red and blue represent nucleotides in HuH-7 and WRL68 cells, respectively). Positions of primer sequences are indicated.

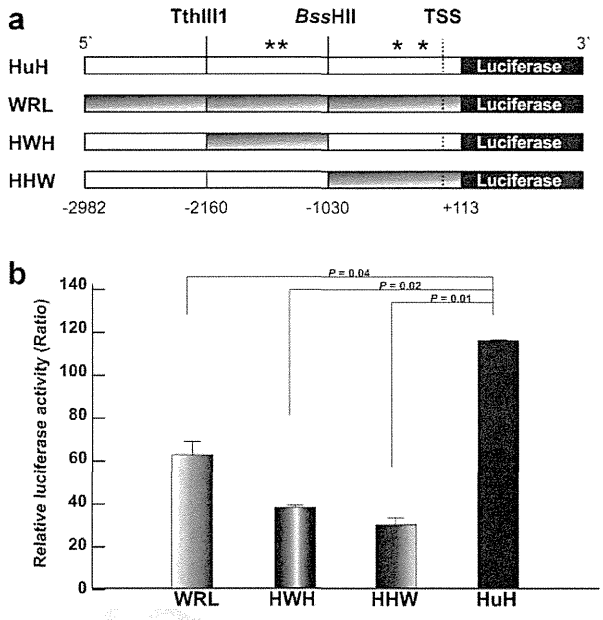

Fig. 3. Effect of nucleotide changes on *DHCR24* promoter activity. (a) Schematic diagrams of reporter constructs. Intact or chimeric *DHCR24* promoter sequences were used to drive the expression of firefly luciferase. Fragments of DNA derived from HuH-7 and WRL68 cells are colored white and grey, respectively. The asterisks indicate the position of each nucleotide polymorphism. Restriction enzyme sites (*Tth111*I and *Bss*HII) and transcription start sites are indicated. (b) Promoter activity of reporter constructs. HepG2 cells were transfected with the indicated reporter construct together with a control plasmid encoding *Renilla* luciferase. The relative ratio of firefly/*Renilla* luciferase activity is shown. Error bars indicate the standard deviation of two independent experiments. Each experiment was performed in triplicate. TSS, transcription start sites.

Table 2. Summary of nucleotide substitutions within the *DHCR24* promoter region

	Nucleotide position			
Origin of DNA sample	−1453	−1420	−488	−200
HuH-7 cells (high)†	G	G	A	G
WRL68 cells (low)‡	A	T	C	C
Patient #1	G	G	A	G
Patient #2	G	G	A	G
Patient #3	G	G	A	G
Patient #4	A	T	C	C
Patient #5 (NC)	G	G	A	G
Patient #5 (C)	G	G	A	G
20-donor pool§	A	T	C	C

†DHCR24 was expressed strongly in HuH-7 cells (Fig. 1), ‡DHCR24 was expressed weakly in WRL68 cells (Fig. 1), §Pooled normal human hepatocytes from a 20-donor pool. C, cancerous region; NC, non-cancerous region.

Table 3. Summary of nucleotide substitutions within the *DHCR24* promoter region with or without HCV infection

		Nucleotide position			
Origin of DNA sample	HCV	−1453	−1420	−488	−200
HuH-7 cells (high)	−	G	G	A	G
WRL68 cells (low)	−	A	T	C	C
Chimeric mouse liver	−	A	T	C	C
HCV infected chimeric mouse liver	+[†]	A	T	C	C
HCV replicon cells (R6FLR-N)	+	G	G	A	G
Cured K4 cells	+	G	G	A	G

[†]7.5×10^6 copies/mL of HCV in patient plasma was inoculated.

sequences from patient 4 (#4) had nucleotides associated with weak activity at these positions (i.e., A, T, C, and C). Intriguingly, only #4 exhibited an SVR, which is characterized by the absence of detectable HCV RNA in serum for >24 weeks following IFN treatment. The SVR status of #4 has persisted since 1995. In #5, promoter sequences were the same in cancerous and non-cancerous regions of the liver. These results suggest that the four polymorphisms within the *DHCR24* promoter region may influence the susceptibility to malignancy and IFN responsiveness of hepatoma cells and thus influence the fate of patients with HCC.

To assess the impact of HCV infection on genomic polymorphism in DHCR24 promoter sequences, we determined the sequences in human hepatocytes that had been transplanted into severely combined immunodeficient mice that we infected or mock-infected with HCV We detected markedly high titers of HCV only in the infected mice (Table 3). Sequencing revealed that all four polymorphic nucleotide positions were of the weak activity type. Notably, we detected no nucleotide differences between HCV- and mock-infected mice in the targeted regions (Table 3). We also established cured K4 cells by treating HCV replicon cells R6FLR-N with IFN. Analysis of the genomic sequence of these cell lines showed no nucleotide differences in R6-FLR-N and K4 cells (Table 3). These results suggest that the differences in the DHCR24 promoter sequence are ingenerate rather than induced by HCV infection.

DISCUSSION

In this study, we analyzed the promoter sequences associated with *DHCR24* in hepatocytes and identified polymorphisms that regulate the degree of expression of downstream genes (Figs. 1–3). #4 had an SVR in response to IFN treatment; thus these *DHCR24* promoter sequence polymorphisms are potential biomarkers for predicting patients' responsiveness to IFN treatment.

Genomic polymorphisms within the *DHCR24* promoter region may influence binding of transcription factors (Supplementary Fig. S1). In fact, a T-to-G nucleotide substitution at position −1420 generates a potential binding site for the protein encoded by the caudal homeobox gene (*CdxA*), a homeobox transcription factor responsible for gastrointestinal tract development and epithelial differentiation (15). A C-to-A substitution at position −488 generates potential binding sites for nuclear factor kappa-light-chain enhancer of activated B cells and STATx (16), as well as a low-affinity binding site for Nkx-2 (17). Finally, a C-to-G substitution at position −200 potentially abolishes a p300 binding site (18). These changes in transcription factor binding affinities could upregulate *DHCR24* expression, thereby promote carcinogenesis.

Previously, we discovered that DHCR24 is a host factor involved in HCV-associated development of HCC (8, 9). This protein is upregulated by HCV infection (8), and reduced degrees of expression (via siRNA knockdown) inhibit HCV replication (9). These findings are consistent with the role of DHCR24 in cholesterol biosynthesis (6, 7), which is important for HCV replication (19). Also, because the efficiency of HCV replication might have been lower in #4 than in other patients with strongly active DHCR24 promoter, the weak DHCR24 expression in this patient (Supplementary Fig. S2) might have contributed to the efficacy of IFN treatment.

In conclusion, we have discovered polymorphisms in the promoter region of DHCR24 gene that have not been induced by HCV infection. Future study will clarify their biological significance.

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DISCLOSURE

The authors have no financial relationships to disclosure.

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PLOS ONE

Immunization with a Recombinant Vaccinia Virus That Encodes Nonstructural Proteins of the Hepatitis C Virus Suppresses Viral Protein Levels in Mouse Liver

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Abstract

Chronic hepatitis C, which is caused by infection with the hepatitis C virus (HCV), is a global health problem. Using a mouse model of hepatitis C, we examined the therapeutic effects of a recombinant vaccinia virus (rVV) that encodes an HCV protein. We generated immunocompetent mice that each expressed multiple HCV proteins via a Cre/*loxP* switching system and established several distinct attenuated rVV strains. The HCV core protein was expressed consistently in the liver after polyinosinic acid–polycytidylic acid injection, and these mice showed chronic hepatitis C-related pathological findings (hepatocyte abnormalities, accumulation of glycogen, steatosis), liver fibrosis, and hepatocellular carcinoma. Immunization with one rVV strain (rVV-N25), which encoded nonstructural HCV proteins, suppressed serum inflammatory cytokine levels and alleviated the symptoms of pathological chronic hepatitis C within 7 days after injection. Furthermore, HCV protein levels in liver tissue also decreased in a CD4 and CD8 T-cell-dependent manner. Consistent with these results, we showed that rVV-N25 immunization induced a robust CD8 T-cell immune response that was specific to the HCV nonstructural protein 2. We also demonstrated that the onset of chronic hepatitis in CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice was mainly attributable to inflammatory cytokines, (tumor necrosis factor) TNF-α and (interleukin) IL-6. Thus, our generated mice model should be useful for further investigation of the immunological processes associated with persistent expression of HCV proteins because these mice had not developed immune tolerance to the HCV antigen. In addition, we propose that rVV-N25 could be developed as an effective therapeutic vaccine.

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Introduction

Hepatitis C virus (HCV) is a major public health problem; approximately 170 million people are infected with HCV worldwide [1]. HCV causes persistent infections that can lead to chronic liver diseases such as chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) [2]. Antiviral drugs are not highly effective in individuals with a chronic infection; furthermore, an effective vaccine against HVC has not been developed. A convenient animal model of HCV infection will greatly facilitate the development of an effective HCV vaccine.

Transgenic mice that express HCV proteins have been generated to study HCV expression [3,4]; however, in each of these cases, the relevant transgenes is expressed during embryonic development; therefore, the transgenic mice become immunotolerant to the transgenic products, and consequently, the adult mice are not useful for investigations of the pathogenesis of chronic hepatitis C. To address this problem, we developed a system that can drive conditional expression of an HCV transgene; our system involves the Cre/*loxP* system and a recombinant adenovirus capable of expressing Cre recombinase [5,6]. Concerns have been expressed that an adenovirus and transient expression of HCV proteins could induce immune responses [5] and, therefore, obscure any evidence of the effect of the host immune responses on chronic liver pathology. Therefore, here, we used a Cre/*loxP* switching system to generate an immunocompetent mouse model

of HCV protein expression; with this system, we could study the host immune responses against HCV proteins.

Folgori et al. (2006) reported effective vaccination of chimpanzees with an adenoviral vector and plasmid DNA encoding the HCV nonstructural region. This technique protected the liver tissues from acute hepatitis, which results when whole animals are challenged with virus [7]. However, this vaccine has not yet been shown to be effective against chronic HCV infection.

Here, we aimed to address how HCV expression causes chronic liver diseases and to provide new options for HCV vaccine development. Using LC16m8, a highly attenuated strain of vaccinia virus (VV), we generated three recombinant vaccinia viruses (rVVs) that each encoded one of three different HCV proteins and found that one recombinant virus (rVV-N25), which encoded nonstructural HCV proteins, resolved pathological chronic hepatitis C symptoms in the liver. We also found that immunization with rVV-N25 suppressed HCV core protein levels in the livers of transgenic mice; moreover, this suppression was mediated by CD4 and CD8 T cells, as has been previously reported [8].

Results

Generation of a Model of Persistent HCV Protein Expression

To produce adult mice that express an HCV transgene, we bred CN2-29 transgenic mice, which carry an HCV transgene, [5,6,9] with Mx1-Cre transgenic mice [10], which express Cre recombinase in response to interferon (IFN)-α or a chemical inducer of IFN-α, poly(I:C) (Figure 1A). Following poly(I:C) injection, the HCV transgene was rearranged, and HCV sequences were expressed in the livers of F1 progeny (CN2-$29^{(+/-)}$/$MxCre^{(+/-)}$ mice) within 7 days after poly(I:C) injection (Figure 1B).

To evaluate the characteristic features of these CN2-$29^{(+/-)}$/$MxCre^{(+/-)}$ mice, we analyzed serum alanine aminotransferase (ALT) and liver HCV core protein levels after poly(I:C) injection. As illustrated in Figure 1C, serum ALT levels increased and reached a peak at 24 h after the first poly(I:C) injection; this elevation appeared to be a direct result of the poly(I:C) treatment, which causes liver injury [11]. After this peak, serum ALT levels dropped continuously until day 4, and then ALT levels began to increase, as did HCV core protein levels. Thereafter, the HCV core protein was expressed consistently for at least 600 days.

Histological analysis showed HCV core protein expression in most hepatocytes of the transgenic mice; these mice showed evidence of lymphocytic infiltration that was caused by the HCV core proteins (Figure 1D and E). These observations, in addition to the modified histology activity index (HAI) scores, indicated that expression of HCV proteins caused chronic hepatitis in the CN2-$29^{(+/-)}$/$MxCre^{(+/-)}$ mice because a weak, though persistent, immune response followed an initial bout of acute hepatitis (Figure S1). Moreover, we observed a number of other pathological changes in these mice – including swelling of hepatocytes, abnormal architecture of liver-cell cords, abnormal accumulation of glycogen, steatosis, fibrosis, and HCC (Figures 1E and F, Table S1). Steatosis was mild in the younger mice (day 21) and became increasingly severe over time (days 120 and 180; Figure S2). Importantly, none of the pathological changes were observed in the CN2-$29^{(+/-)}$/$MxCre^{(-/-)}$ mice after poly(I:C) injection (Figure 1F).

Recombinant Vaccinia Virus Immunization in HCV Transgenic Mice

To determine whether activation of the host immune response caused the reduction with HCV protein levels in the livers of CN2-$29^{(+/-)}$/$MxCre^{(+/-)}$ mice, we used a highly attenuated VV strain, LC16m8, to generate three rVVs [12]. Each rVV encoded a different HCV protein; rVV-CN2 encoded mainly structural proteins, rVV-N25 encoded nonstructural proteins, and rVV-CN5 encoded the entire HCV protein region (Figure 2A). Because rVVs can express a variety of proteins and induce strong and long-term immunity, they have been evaluated as potential prophylactic vaccines [13].

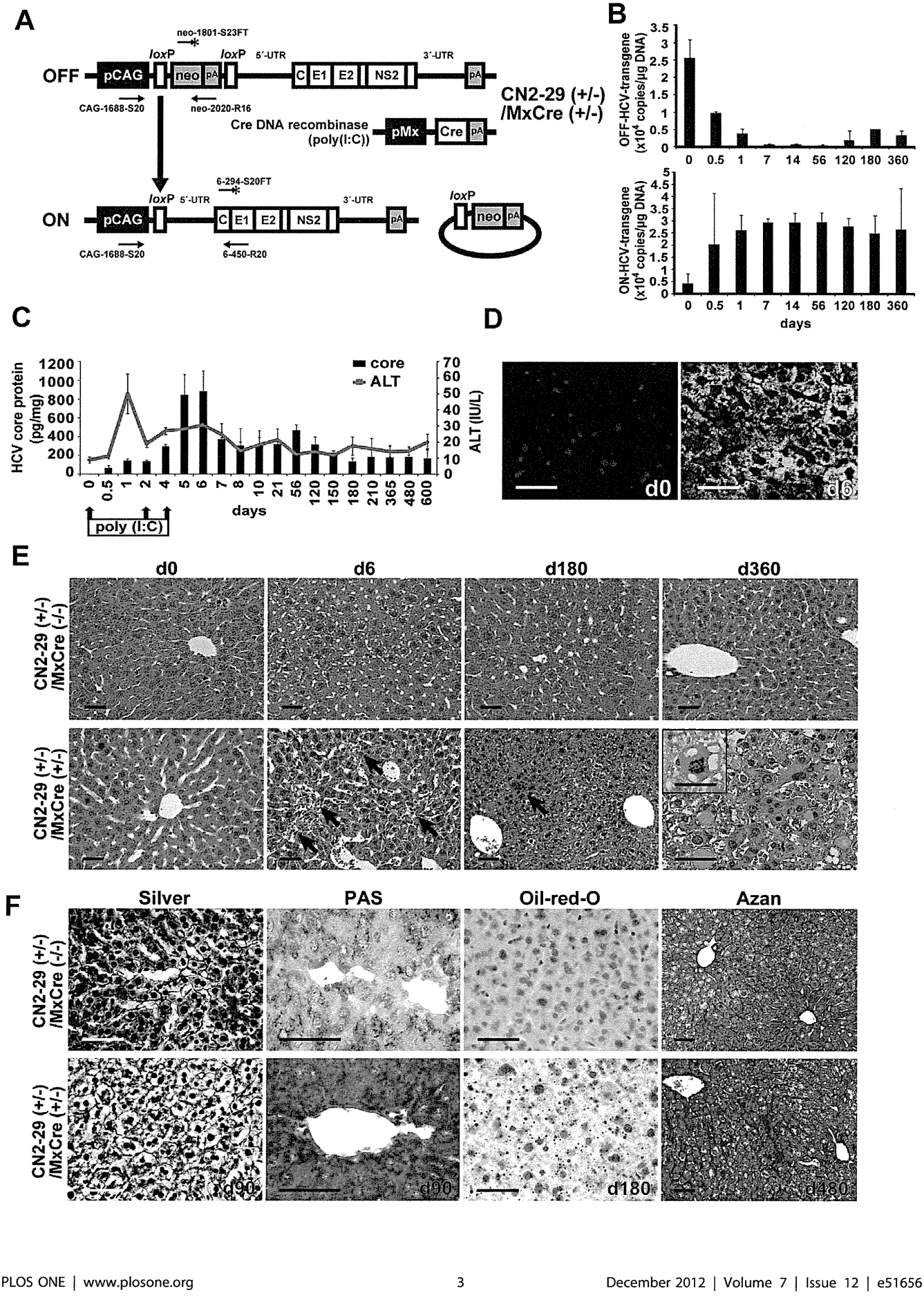
We used western blots to confirm that each HCV protein was expressed in cell lines. Each of seven proteins – the core, E1, E2, NS3-4A, NS4B, NS5A, and NS5B – was recognized and labeled by a separate cognate antibody directed (Figure S3). To induce effective immune responses against HCV proteins in transgenic mice, we injected an rVV-HCV (rVV-CN2, rVV-CN5, or rVV-N25) or LC16m8 (as the control) intradermally into CN2-$29^{(+/-)}$/$MxCre^{(+/-)}$ mice 90 days after poly(I:C) injection (Figure 2B). Analysis of liver sections 7 days after immunization with rVV-N25 revealed dramatic improvement in a variety of pathological findings associated with chronic hepatitis – including piecemeal necrosis, hepatocyte swelling, abnormal architecture of liver-cell cords, abnormal accumulation of glycogen, and steatosis (Figures 2C–E). Collectively, these results demonstrated that only the rVV-N25 treatment resulted in histological changes indicative of improvement in the chronic hepatitis suffered by the transgenic mice.

To determine whether rVV-N25 treatment induced the same effect in other strains of HCV transgenic mice, we analyzed RzCN5-$15^{(+/-)}$/$MxCre^{(+/-)}$ mice, which express all HCV proteins; in these mice, chronic hepatitis was resolved within 28 days of immunization with rVV-N25. Taken together, these findings indicated that rVV-N25 had a dramatic therapeutic effect on both types of HCV transgenic mice (Figure S4).

Treatment with rVV-N25 Reduced the HCV Core Protein Levels in the Livers

To assess in detail the effects of rVV-HCV immunization on HCV protein clearance from the livers of CN2-$29^{(+/-)}$/$MxCre^{(+/-)}$ mice, we monitored the levels of HCV core protein in liver samples via ELISA. We found that within 28 days after immunization the HCV core protein levels were significantly lower in livers of rVV-N25-treated mice than in those of control mice (Figure 3A). Immunohistochemical analysis indicated that, within 28 days after immunization, levels of HCV core protein were substantially lower in the livers of CN2-$29^{(+/-)}$/$MxCre^{(+/-)}$ mice than in those of control mice (Figure 3B). Importantly, neither resolution of chronic hepatitis nor reduction in the HCV protein levels was observed in the mice treated with LC16m8, rVV-CN2, or rVV-CN5. These results indicated that HCV non-structural proteins might be important for effects of therapeutic vaccines. In contrast, rVV-CN5 which encoded HCV structural and non-structural proteins did not show any significant effects. These results indicated that HCV structural proteins might have inhibited the therapeutic effects of the non-structural proteins. Therefore, it may be important to exclude the HCV structural proteins (aa 1–541) as antigenic proteins when developing therapeutic vaccines against chronic hepatitis C.

In addition, we measured serum ALT levels in CN2-$29^{(+/-)}$/$MxCre^{(+/-)}$ mice from all four treatment groups 28 days after rVV-HCV immunization. Serum ALT levels were not significant-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CN2-29 (+/-)
/MxCre (+/-)
Cre DNA recombinase
(poly(I:C))
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Cre
pA
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NS2
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loxP
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days
C
HCV core protein
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core
ALT
ALT (IU/L)
0 0.5 1 2 4 5 6 7 8 10 21 56 120 150 180 210 365 480 600
days
poly (I:C)
D
d0
d6
E
d0
d6
d180
d360
CN2-29 (+/-)
/MxCre (-/-)
CN2-29 (+/-)
/MxCre (+/-)
F
Silver
PAS
Oil-red-O
Azan
CN2-29 (+/-)
/MxCre (-/-)
CN2-29 (+/-)
/MxCre (+/-)
d90
d90
d180
d480

Figure 1. Pathogenesis in immunocompetent mice with persistent HCV expression. (**A**) Structure of CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ and the Cre-mediated activation of the transgene unit. R6CN2 HCV cDNA was cloned downstream of the CAG promoter, neomycin-resistant gene (*neo*), and poly A (pA) signal flanked by two *loxP* sequences. This cDNA contains the core, E1, E2, and NS2 regions. (**B**) Cre-mediated genomic DNA recombination. After poly(I:C) injection, genomic DNA was extracted from liver tissues and analyzed by quantitative RTD-PCR for Cre-mediated transgenic recombination. The transgene was almost fully recombined in transgenic mouse livers 7 days after the injection. In all cases, n = 3 mice per group. (**C**) HCV core protein expression was sustained for at least 600 days after poly(I:C) injection. (**D**) Immunohistochemical analysis revealed that most hepatocytes expressed the HCV core protein within 6 days after injection. (**E**) Liver sections from CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice after the poly(I:C) injection. Infiltrating lymphocytes (arrows) were observed on days 6 and 180; Hepatocellular carcinoma (HCC) was observed on day 360. In contrast, these pathological changes were not observed in CN2-29$^{(+/-)}$/MxCre$^{(-/-)}$ mice after the injection. The inset image shows abnormal mitosis in a tumor cell. (**F**) Hepatocyte swelling and abnormal architecture of liver-cell cords (silver staining), as well as abnormal glycogen accumulation (PAS staining) were observed on day 90 in CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice. We observed steatosis (oil-red-O staining) on day 180 and, subsequently, fibrosis (Azan staining) on day 480. The scale bars indicate 50 μm.
doi:10.1371/journal.pone.0051656.g001

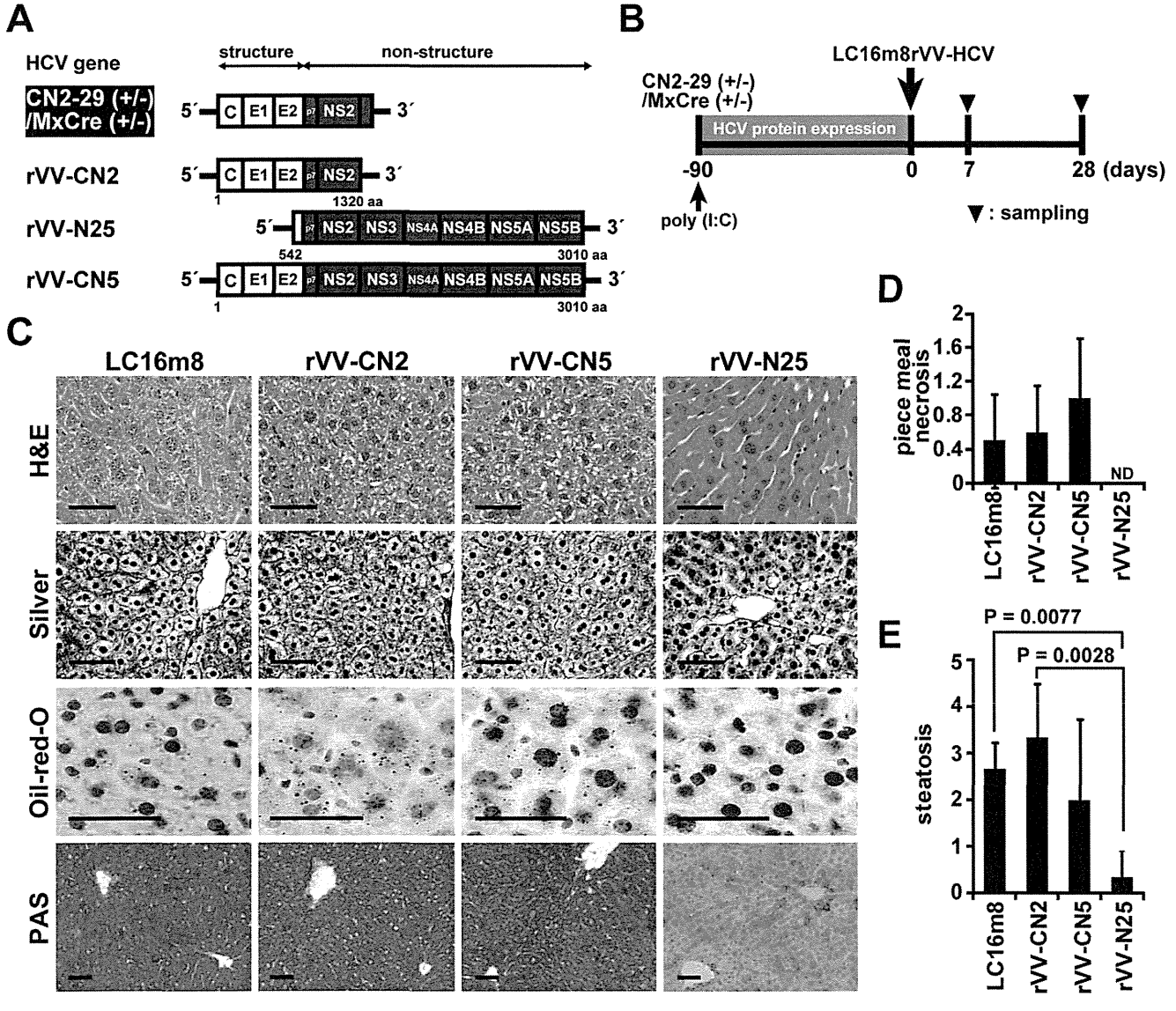

Figure 2. Effects of rVV-HCV treatment on the CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice. (**A**) HCV gene structure in the CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice and recombinant vaccinia viruses (rVV-HCV). MxCre/CN2-29 cDNA contains the core, E1, E2, and NS2 regions. The rVV-CN2 cDNA contains the core, E1, E2, and NS2 regions. The rVV-N25 cDNA contains the NS2, NS3, NS4A, NS4B, NS5A, and NS5B regions. The rVV-CN5 cDNA contains the entire HCV region. (**B**) Four groups of CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice were inoculated intradermally with rVV-CN2, rVV-N25, rVV-CN5, or LC16m8 90 days after the poly(I:C) injection. Blood, liver, and spleen tissue samples were collected 7 and 28 days after the inoculation. (**C**) Liver sections from the four groups of CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice 7 days after the inoculation. The sections were stained with H&E, silver, oil-red-O, or PAS. The scale bars indicate 50 μm. (**D**) Histological evaluation of piecemeal necrosis in the four groups of CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice 7 days after inoculation. (**E**) Histological evaluation of steatosis in the four groups of CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice 7 days after inoculation. Significant relationships are indicated by a P-value.
doi:10.1371/journal.pone.0051656.g002

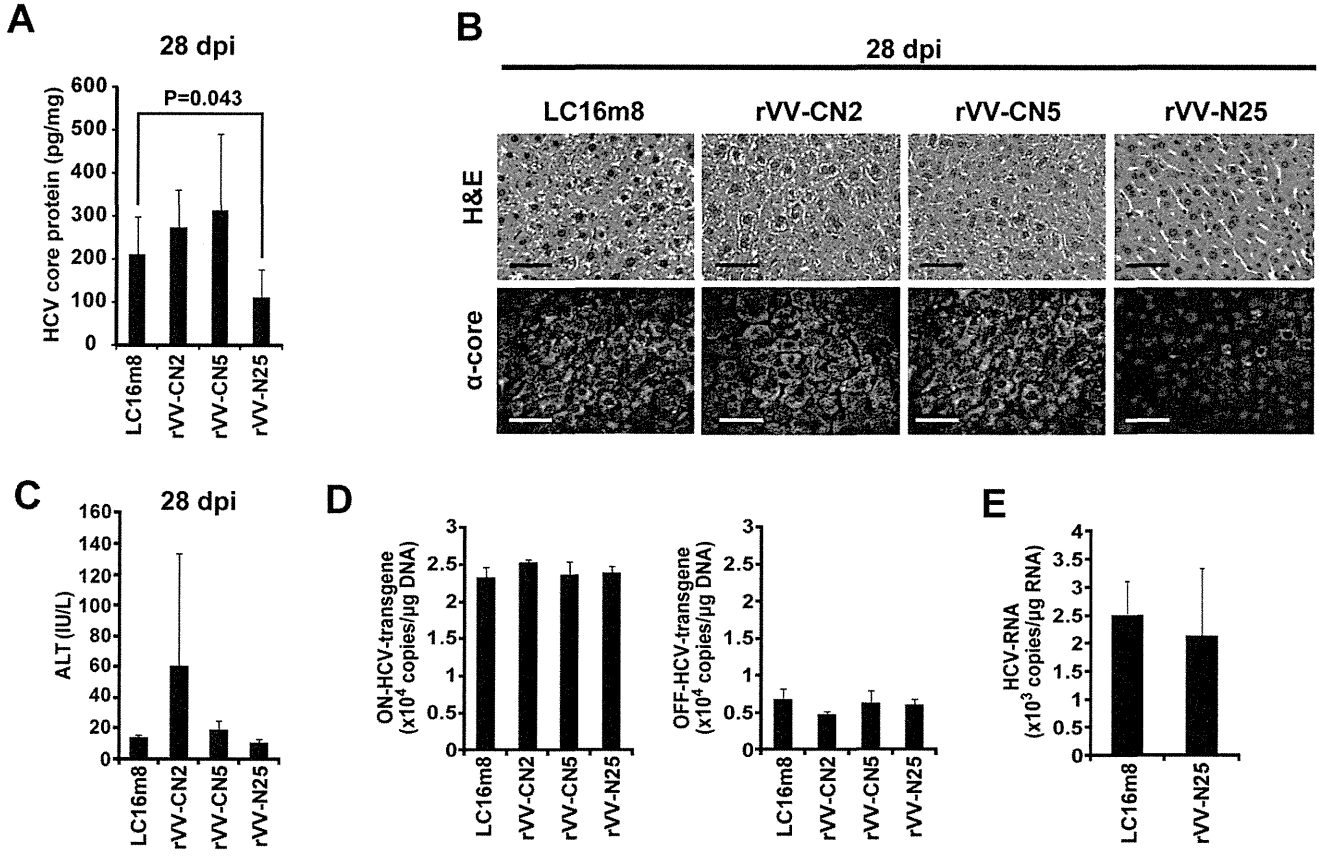

Figure 3. Effects of HCV core protein expression on the livers of CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice inoculated with rVV-HCV. (**A**) Expression of the HCV core protein in the four treatment groups of CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice 28 days after the inoculation. Significant relationships are indicated by a P-value. (**B**) H&E staining and immunohistochemical analysis for HCV core protein in the LC16m8-, rVV-CN2-, rVV-CN5-, or rVV-N25-treated CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice 28 days after the inoculation. Liver sections were stained with the anti-core monoclonal antibody. The scale bars indicate 50 μm. (**C**) Effects of HCV core protein expression on serum ALT levels in the four treatment groups of CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice 28 days after immunization. (**D**) Cre-mediated genomic DNA recombination in the four treatment groups 28 days after immunization. (**E**) Expression of HCV mRNA in the LC16m8- or rVV-N25-treated CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice 28 days after immunization. In all cases, n = 6 mice per group.
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ly different in the rVV-N25-treated mice and control mice (Figure 3C); this finding indicated that rVV-N25 treatment did not cause liver injury and that the antiviral effect was independent of hepatocyte destruction.

We hypothesized that the reduction in the levels of HCV core protein in rVV-HCV-treated mice was not caused by cytolytic elimination of hepatocytes that expressed HCV proteins. To investigate this hypothesis, we conducted an RTD-PCR analysis of genomic DNA from liver samples of CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice. The recombined transgene was similar in rVV-N25-treated and control mice 28 days after immunization (Figure 3D). We also measured the expression of HCV mRNA in LC16m8-treated CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice with that in rVV-N25-treated CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice 28 days after immunization; the HCV mRNA levels did not differ between rVV-N25-treated CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ and control mice (Figure 3E). These results indicated that rVV-N25-induced suppression of HCV core protein expression could be controlled at a posttranscriptional level.

Role of CD4 and CD8 T cells in rVV-N25-treated Mice

Viral clearance is usually associated with CD4 and CD8 T-cell activity that is regulated by cytolytic or noncytolytic antiviral mechanism [14]. To determine whether CD4 or CD8 T-cell activity was required for the reduction in HCV core protein levels in the livers of transgenic mice, we analyzed the core protein levels in CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice immunized with rVV-N25 in the absence of CD4 or CD8 T cells (Figure 4A). As expected, the mice lacking CD4 or CD8 T cells failed to show a reduction in HCV core protein levels (Figure 4B).

However, in mice lacking either CD4 or CD8 T-cells, the pathological changes associated with chronic hepatitis were resolved following rVV-N25 immunization, and the steatosis score of rVV-N25-treated mice was significantly lower than that of control mice (Figures 4C–E). These results indicated that CD4 and CD8 T cells were not responsible for the rVV-N25-induced amelioration of histological findings and that other inflammatory cell types may play an as-yet-unidentified role in the resolution of the pathological changes in these mice.

rVV-N25 Immunization Induced an NS2-specific Activated CD8 T cells Response

Because we found that HCV protein reduction in the liver required CD8 T cells, we tested whether HCV-specific CD8 T cells were present in splenocytes 28 days after immunization. To determine the functional reactivity of HCV-specific CD8$^+$ T cells, we performed a CD107a mobilization assay and intracellular IFN-γ staining. CN2-29 transgenic mice expressed the HCV structural protein and the NS2 region. However, rVV-N25 comprised only

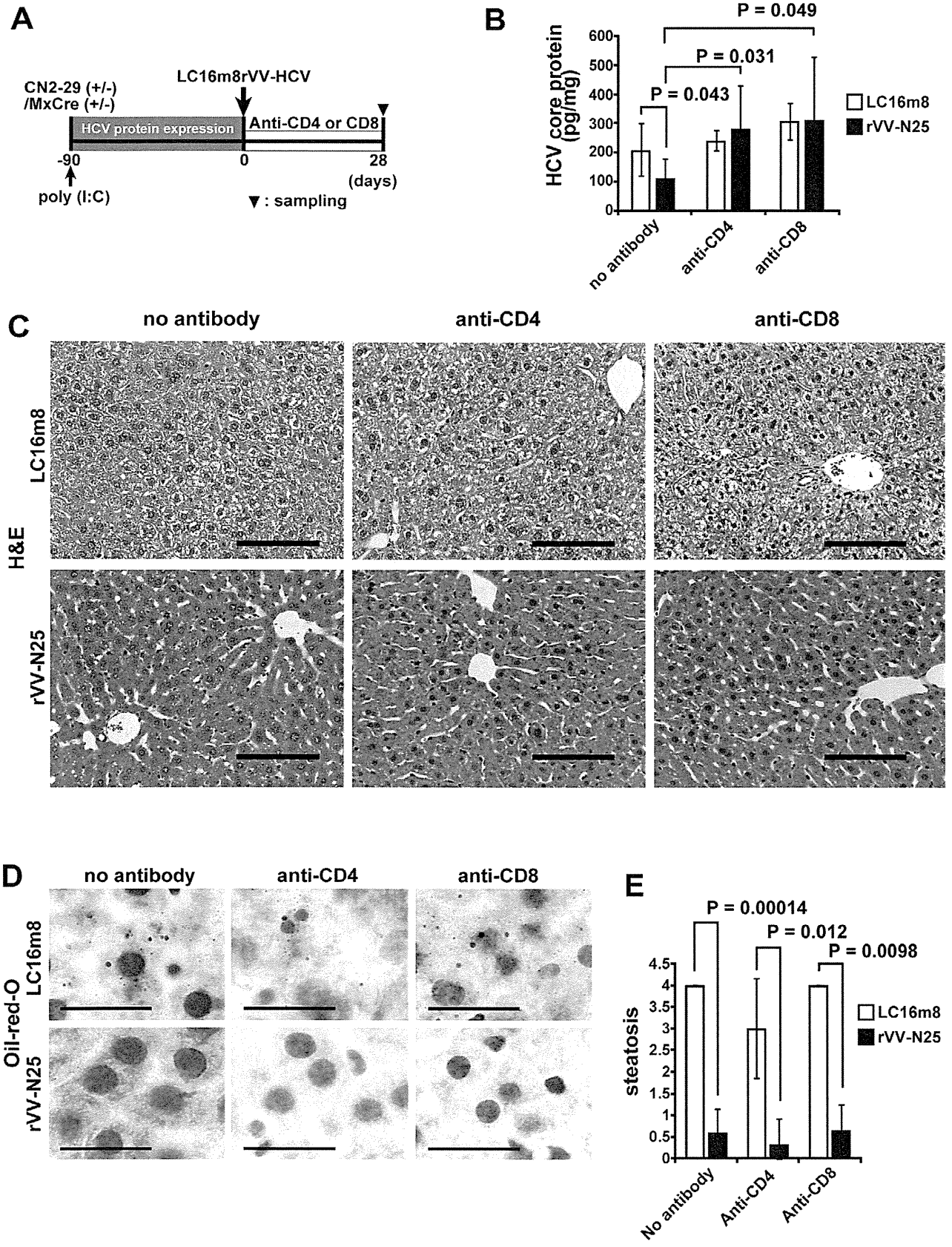

Figure 4. Role of CD4 and CD8 T cells in rVV-N25-treated mice. (**A**) Schematic diagram depicts depletion of CD4 and CD8 T cells via treatment with monoclonal antibodies. (**B**) Comparison of HCV core protein expression in control, CD4-depleted, and CD8-depleted mice 28 days after immunization with LC16m8 or rVV-N25. (**C, D**) Histological analysis of liver samples from CD4-depleted or CD8-depleted CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice

28 days after immunization with LC16m8 or rVV-N25. The scale bars indicate 100 μm (**C**) and 50 μm (**D**). (**E**) Histological evaluation of steatosis in liver samples from CD4-depleted or CD8-depleted CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice 28 days after immunization with LC16m8 or rVV-N25. Significant relationships are indicated by a P-value.
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a HCV nonstructural protein. Thus, we focused on the role of the NS2 region as the target for CD8 T cells and generated EL-4 cell lines that expressed the NS2 antigen or the CN2 antigen.

Isolated splenocytes from immunized mice were co-cultured with EL-4CN2 or EL-4NS2 cell lines for 2 weeks and analyzed.

Cytolytic cell activation can be measured using CD107a, a marker of degranulation [15]. The ratio of CD8$^+$CD107a$^+$ cells to all CD8 T cells significantly increased in rVV-N25-treated splenocytes after co-culture with EL-4CN2 or EL-4NS2 ($P<0.05$), whereas splenocytes that had been treated with any other rVV were not detected (Figure 5A, B and C). These results indicated that rVV-N25 treatment increased the frequency of HCV NS2-specific activated CD8 T cells. Consistent with these results, the ratio of CD8$^+$IFN-γ$^+$ cells to all CD8 T cells for rVV-N25-treated mice was also significantly higher than that for mice treated with any other rVV ($P<0.05$). Taken together, these findings indicated that rVV-N25 induced an effective CD8 T-cell immune response and that NS2 is an important epitope for CD8 T cells.

rVV-N25 Immunization Suppressed Inflammatory Cytokines Production

To determine whether rVV-N25 treatment affected inflammatory cytokine production, we measured serum levels of inflammatory cytokines after rVV immunization. The serum levels of these inflammatory cytokines increased in the CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice (Figure 6A, Figure S5). Immunization with rVV-N25 affected serum levels of inflammatory cytokines in CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice and caused a return to the cytokine levels observed in wild-type untreated mice (Figure 6A). In wild-type mice, the cytokine levels remained unchanged after immunization (Figure 6A). These results indicated that inflammatory cytokines were responsible for liver pathogenesis in the transgenic mice.

To test the hypothesis that inflammatory cytokines were responsible for liver pathogenesis in CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice, we administered transgenic mouse serum intravenously into nontransgenic mice. We observed the development of chronic hepatitis in the nontransgenic mice within 7 days after the serum transfer (Figures 6B and C). This finding was consistent with the

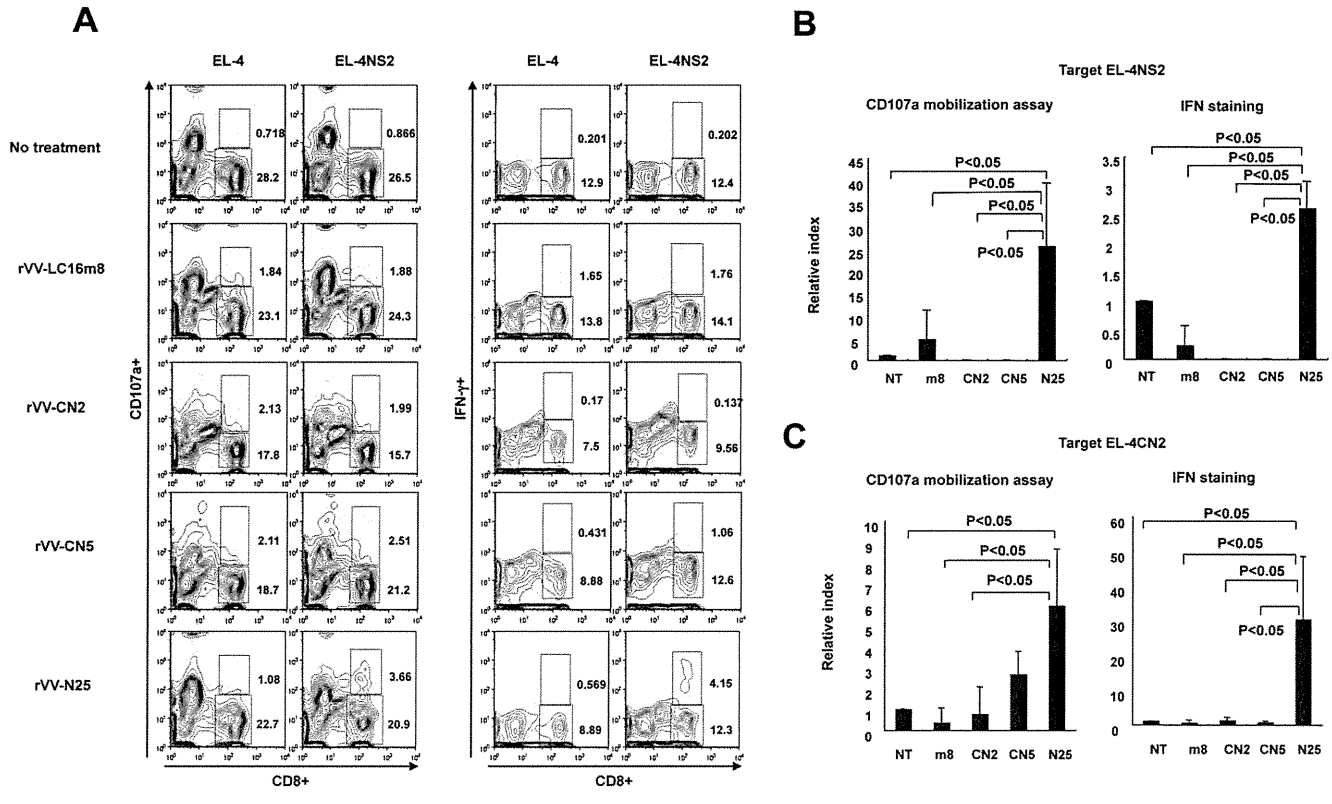

Figure 5. Immunization with rVV-N25 induced CD8 T-cell degranulation, a marker for cytotoxicity, and IFN-γ production. (**A**) The numbers represent the percentage of CD107a positive cells and negative cells (left two columns) and IFN-γ-positive cells and negative cells (right two columns). (**B, C**) The ratio of CD8$^+$IFN-γ$^+$ cells to all CD8 T cells for rVV-N25-treated mice was significantly higher than that for mice treated with any other rVV. Splenocytes (4×10^6 per well) were cultured with EL-4CN2 or EL-4NS2 cell lines in RPMI 1640 complete medium including 3% T-STIM^{TM} with ConA for 2 weeks. Harvested cells were incubated for 4 h with EL-4, EL-4CN2, or EL-4NS2 in combination with PE-labeled anti-CD107a mAb and monensin in RPMI 1640 complete medium with 50 IU/mL IL-2, according to the manufacturer's instruction. After incubation, cell suspensions were washed with PBS, and the cells were further stained with APC-labeled anti-IFN-γ mAb and Pacific blue-labeled anti-CD8 mAb. Harvested cells were stained with anti-CD107a-PE, anti-IFN-γ-APC, or anti-CD8-Pacific blue. Results that are representative of three independent experiments are shown. Significant relationships are indicated by P-value.
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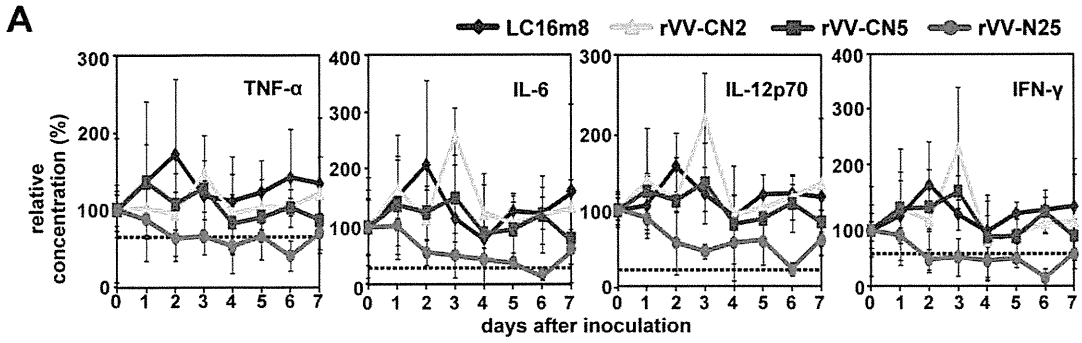
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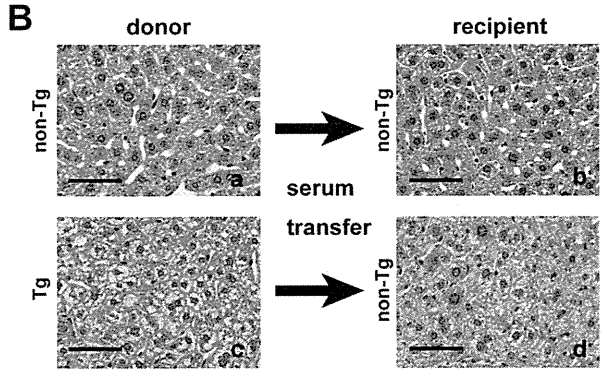
days after inoculation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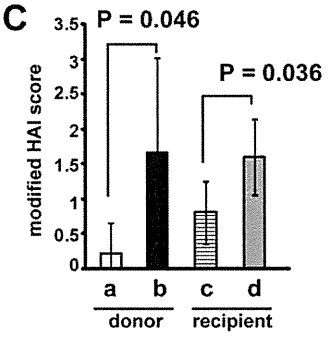
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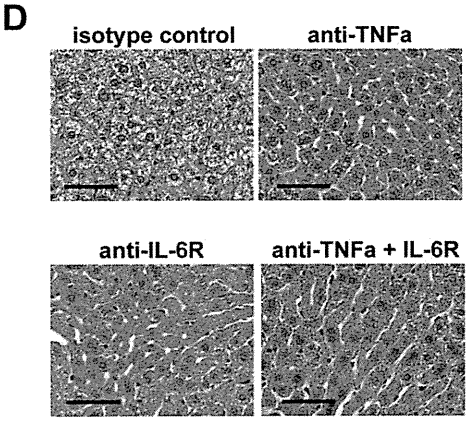
recipient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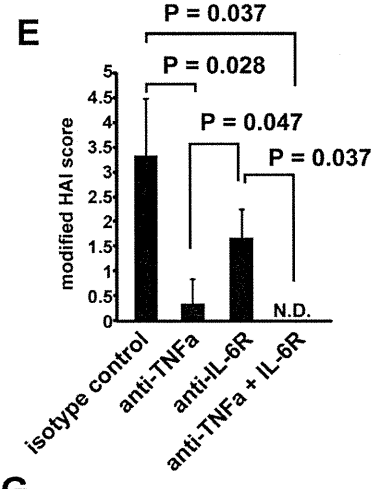
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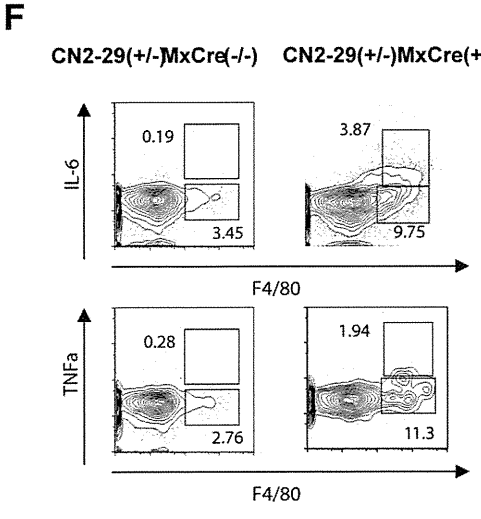
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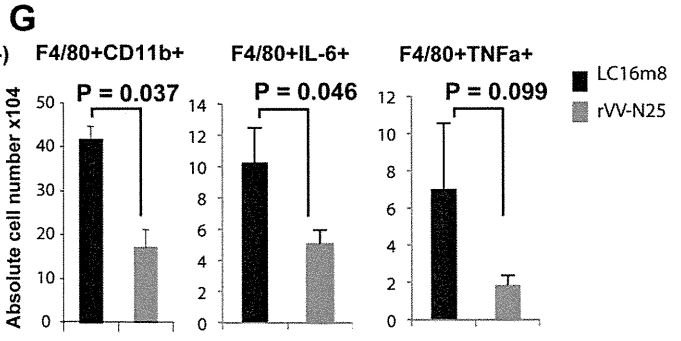
1.94
11.3


G
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F4/80+IL-6+
F4/80+TNFa+
P = 0.037
P = 0.046
P = 0.099
Absolute cell number x104
LC16m8
rVV-N25

Figure 6. Immunization with rVV-N25 suppresses serum inflammatory cytokine levels. (**A**) Daily cytokine levels in the serum of CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice during the week following immunization with LC16m8, rVV-CN2, rVV-N25, or rVV-CN5. Values represent means ± SD (n = 3) and reflect the concentrations relative to those measured on day 0. The broken lines indicate the baseline data from wild-type mice. In all cases, n = 6 mice per group. (**B**) Liver sections from CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ and CN2-29$^{(+/-)}$/MxCre$^{(-/-)}$ mice. (**C**) Histology activity index (HAI) scores of liver samples taken from CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$, or CN2-29$^{(+/-)}$/MxCre$^{(-/-)}$ mice. (**D**) Liver sections from CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice in which TNF-α was neutralized and the IL-6 receptor was blocked. The scale bars indicate 50 μm. (**E**) HAI scores of liver samples taken from CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ in which TNF-α was neutralized and the IL-6 receptor was blocked. Tg and non-Tg indicate CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ and CN2-29$^{(+/-)}$/MxCre$^{(-/-)}$, respectively. (F) Macrophages were the main producers of TNF-α and IL-6 in CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice following poly(I:C) injection. (G) Immunization with rVV-N25 reduced the number of macrophages in liver samples from CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice and suppressed TNF-α and IL-6 production from macrophages (Figure 6G). Significant relationships are indicated by a P-value.
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hypothesis that inflammatory mediators played a key role in inducing hepatitis. Furthermore, to investigate whether TNF-α and IL-6 played particularly critical roles in the pathogenesis of chronic hepatitis in the transgenic mice, we neutralized TNF-α and blocked the IL-6 receptor in the livers of these mice. As expected, chronic hepatitis did not develop in these mice. (Figure 6D and E).

Next, to determine which cell population(s) produced TNF-α, IL-6, or both during continuous HCV expression in CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice, we isolated intrahepatic lymphocytes (IHLs) and labeled the macrophages (the F4/80^{+} cells) with anti-TNF-α and anti-IL-6 antibodies using an intracellular cytokine detection method. Macrophages in CN2-29$^{(+/-)}$/MxCre$^{(-/-)}$ mice produced small amounts of TNF-α and IL-6, while those in CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice produced much larger amounts of these cytokines (Figure 6F).

Finally, we evaluated whether rVV-N25 treatment affected the number of macrophages, cytokine production by macrophages, or both; specifically, we isolated IHLs from CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice 7 days after immunization with rVV-N25 or with LC16m8. The percentage of macrophages (CD11b^{+}F4/80^{+}) among IHLs and IL-6 production from these macrophages were significantly lower in rVV-N25-treated mice than in control mice (Figure 6G). Though the percentage of TNF-α-producing macrophages was not significantly different in rVV-N25-treated and control mice (P = 0.099), rVV-N25 treatment appeared to suppress these macrophages. These results demonstrated that rVV-N25 had a suppressive effect on activated macrophages, and they indicated that this suppression ameliorated the histological indicators of chronic hepatitis.

Discussion

Various HCV transgenic mouse models have been developed and used to examine immune response to HCV expression and the effects of pathogenic HCV protein on hepatocytes [4,16,17]. However, these transgenic mice develop tolerance to the HCV protein; therefore, examining immune response to HCV protein has been difficult.

To overcome the problem of immune tolerance in mouse models of HCV expression, we developed an HCV model in mice that relies on conditional expression of the core, E1, E2, and NS2 proteins and the Cre/loxP switching system [5,6]; we showed that the injection of an Ad-Cre vector enhanced the frequency of HCV-specific activated CD8 T cells in the liver of these mice and caused liver injury. However, the Ad-Cre adenovirus vector alone causes acute hepatitis in wild-type mice. Nevertheless, the transgenic model was useful for evaluating interactions between the host immune system and viral protein (serum ALT level over 2,000 IU/L) [5]; HCV core protein levels were reduced and expression of this protein was transient (about 2 weeks). Therefore, this Ad-Cre-dependent model cannot be used to effectively investigate immune responses to chronic HCV hepatitis.

Here, we used poly (I:C)-induced expression of Cre recombinase to generate HCV transgenic mice in order to study the effect of HCV protein and confirmed that these mice developed chronic active hepatitis–including steatosis, lipid deposition, and hepatocellular carcinoma. These pathological findings in the transgenic mice were very similar to those in humans with chronic hepatitis C; therefore, this mouse model of HCV may be useful for analyzing the immune response to chronic hepatitis. However, experimental results obtained with this mouse model may not directly translate to clinical findings from patients with HCV infection because the expression of HCV proteins was not liver specific in these mice. Furthermore, poly(I:C) injection can activate innate immune responses and, consequently, might induce temporary liver injury [18]. Additionally, poly(I:C) injection has an adjuvant effect; specifically, it stimulates TLR3 signaling [19].

To evaluate whether poly(I:C) injection caused hepatitis in CN2-29$^{(+/-)}$/MxCre$^{(-/-)}$ mice, we examined serum ALT levels and liver histology following poly(I:C) injection. We found that, following poly(I:C) injection, serum ALT levels in CN2-29$^{(+/-)}$/MxCre$^{(-/-)}$ mice increased, reached a peak one day after injection, declined from day 1 to day 6, and were not elevated thereafter; this time-course indicated that poly(I:C) injection alone did not induced continuous liver injury (figure S6). Based on these findings, we believe that the effects of poly(I:C) injection in these mice did not confound our analysis of chronic hepatitis.

Immunization with rVV-N25 suppressed HCV protein levels in the liver, and this suppression was associated with ameliorated pathological chronic hepatitis findings (see Figure 3). Importantly, rVV-N25 treatment did not cause liver injury based on the serum ALT levels; therefore, this treatment was unlikely to have cytopathic effects on infected hepatocytes. These findings provided strong evidence that rVV-N25 treatment effectively halted the progression of chronic hepatitis. Immunization with plasmid DNA or with recombinant vaccinia virus can effectively induce cellular and humoral immune responses and exert a protective effect against challenge with HCV infection [20,21]. However, findings from these previous studies revealed HCV immunization of both uninfected, naïve animals and immune-tolerant animals induced a HCV-specific immune response. In the model describe here; the animals were immune competent for HCV; therefore, our findings provided further important evidence that rVV-N25 was effective in the treatment of chronic hepatitis.

In addition, we demonstrated that rVV-N25 treatment in the absence of CD4 and CD8 T cells had no effect on HCV clearance. This important observation indicated that rVV-N25-induced HCV clearance was mediated by CD4 and CD8 T cells. Many studies have shown that spontaneous viral clearance during acute HCV infection is characterized by a vigorous, broadly reactive CD4 and CD8 T-cell response. [8,22] HCV clearance and hepatocellular cytotoxicity are both mediated by CD8 antigen-specific (cytotoxic T lymphocyte) CTLs [23]. Consistent with these observations, rVV-N25 treatment effectively induced the accumulation of NS2-specific CD8 T cells, which express high levels of

CD107a and IFN-γin the spleen. Notably, even with rVV-N25 immunization, the frequency of activated CD8 T cells was very low, and a minimum of 2-weeks incubation was required to distinguish the difference between rVV treatments. Even if a small population of specific CD8+ T cells played a relevant role in the reduction of core protein, it is difficult to assert that the only NS2-specific CD8+ T cells were important to this reduction. However, based on the results presented in Figure 4B, we are able to conclude that at least CD8+ and/or CD4+ T cells were important to the reduction in HCV core protein. Therefore, to elucidate the mechanism of HCV protein clearance, further investigation of not only the other T cell epitopes but also other immunocompetent cells is required.

Interestingly, rVV-N25 treatment–but not the rVV-CN2 or rVV-CN5 treatment–efficiently induced a HCV-specific activated CD8 T cells response; this difference in efficacy could have one or more possible causes. The HCV structural proteins (core, E1, and E2 proteins) in the rVV-CN construct may cause the difference; Saito et al. reported that injection with plasmid constructs encoding the core protein induced a specific CTL response in BALB/c mice [24]. Reportedly, CTL activity against core or envelope protein is completely absent from transgenic mice immunized with a plasmid encoding the HCV structural proteins, but core-specific CTL activity is present in transgenic mice that were immunized with a plasmid encoding the HCV core [21]. In contrast, when recombinant vaccinia virus expressing different regions of the HCV polyprotein were injected into BALB/c mice, only the HCV core protein markedly suppressed vaccinia-specific CTL responses [25]. Thus, the HCV core protein may have an immunomodulatory function [26]. Based on these reports and our results, we hypothesize that the causes underlying the effectiveness of rVV-N25 treatment were as follows: 1) this rVV construct included the core and envelope proteins and 2) the core protein had an immune-suppressive effect on CTL induction. Therefore, we suggest that exclusion of the core and envelope antigen as immunogen is one important factor in HCV vaccine design.

Interestingly, immunization with rVV-N25 rapidly suppressed the inflammatory response; however, immunization with either of the other rVVs did not (see Figure 6A). This result indicated that rVV-N25 may modulate inflammation via innate immunity, as well as via acquired immunity. Reportedly, Toll-like receptor (TLR)-dependent recognition pathways play a role in the recognition of poxviruses [27]. TLR2 and TLR9 have also been implicated in the recognition of the vaccinia virus [28,29]. These findings indicate that TLR on dendritic cells may modulate the immunosuppressive effect of rVV-N25 in our model of HCV infection; however, further examination of this hypothesis is required. The finding that pathological symptoms in the HCV transgenic mice were completely blocked by intravenous injection of TNF-α and IL-6 neutralizing antibodies indicated that the progression of chronic hepatitis depended on inflammatory cytokines in serum, rather than the HCV protein levels in hepatocytes. Lymphocytes, macrophages, hepatocytes, and adipocytes each produce TNF-α and IL-6 [30,31], and HCV-infected patients have elevated levels of TNF-α and IL-6 [32,33]. Both cytokines also contribute to the maintenance of hepatosteatosis in mice fed a high-fat diet [34], and production of TNF-α and IL-6 is elevated in obese mice due to the low grade inflammatory response that is caused by lipid accumulation [35]. These findings indicate that both cytokines are responsible for HCV-triggered hepatosteatosis, and anti-cytokine neutralization is a potential treatment for chronic hepatitis if antiviral therapy is not successful.

The reduction of macrophages in number might be due to the induction of apoptosis by vaccinia virus *in vitro* infection as previous reported [36]. To understand the mechanisms responsible for the reduction of the number of macrophage, we performed another experiment to confirm whether the macrophages were infected with vaccinia virus inoculation. However, based on PCR analyses; vaccinia virus DNA was not present in liver tissue that contained macrophages (Figure S7). Furthermore, apoptosis of macrophages was not detected in liver samples (Data not shown). Based on these results, it is unlikely that the reduction in the number of macrophages was due to apoptosis induced by vaccinia virus infection. Although rVV-N25 reduced the number of macrophage, precise mechanism is still unknown. Further examination to elucidate the mechanism is required.

In conclusion, our findings demonstrated that rVV-N25 is a promising candidate for an HCV vaccine therapy. Additionally, the findings of this study indicate that rVV-N25 immunization can be used for prevention of HCV infection and as an antiviral therapy against ongoing HCV infection.

Materials and Methods

Ethics Statement

All animal care and experimental procedures were performed according to the guidelines established by the Tokyo Metropolitan Institute of Medical Science Subcommittee on Laboratory Animal Care; these guidelines conform to the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology, Japan, 2006. All protocols were approved by the Committee on the Ethics of Animal Experiments of the Tokyo Metropolitan Institute of Medical Science (Permit Number: 11–078). All efforts were made to minimize the suffering of the animals.

Animals

R6CN2 HCV cDNA (nt 294–3435) [37] and full genomic HCV cDNA (nt 1–9611) [38,39] were cloned from a blood sample taken from a patient (#R6) with chronic active hepatitis (Text S1). The infectious titer of this blood sample has been previously reported [40]. R6CN2HCV and R6CN5HCV transgenic mice were bred with Mx1-Cre transgenic mice (purchased from Jackson Laboratory) to produce R6CN2HCV-MxCre and R6CN5HCV-MxCre transgenic mice, which were designated CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ and RzCN5-15$^{(+/-)}$/MxCre$^{(+/-)}$ mice, respectively. Cre expression in the livers of these mice was induced by intraperitoneal injection of polyinosinic acid–polycytidylic acid [poly(I:C)] (GE Healthcare UK Ltd., Buckinghamshire, England); 300 μL of a poly(I:C) solution (1 mg/mL in phosphate-buffered saline [PBS]) was injected three times at 48-h intervals. All animal care and experimental procedures were performed according to the guidelines established by the Tokyo Metropolitan Institute of Medical Science Subcommittee on Laboratory Animal Care.

Histology and Immunohistochemical Staining

Tissue samples were fixed in 4% paraformaldehyde in PBS, embedded in paraffin, sectioned (4-μm thickness), and stained with hematoxylin and eosin (H&E). Staining with periodic acid–Schiff stain, Azan stain, silver, or Oil-red-O was also performed to visualize glycogen degeneration, fibrillization, reticular fiber degeneration, or lipid degeneration, respectively.

For immunohistochemical staining, unfixed frozen liver sections were fixed in 4% paraformaldehyde for 10 min and then incubated with blocking buffer (1% bovine serum albumin in PBS) for 30 min at room temperature. Subsequently, the sections were incubated with biotinylated mouse anti-HCV core mono-

clonal antibody (5E3) for 2 h at room temperature. After being washed with PBS, the sections were incubated with streptavidin–Alexa Fluor 488 (Invitrogen). The nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Fluorescence was observed using a confocal laser microscope (Laser scanning microscope 510, Carl Zeiss).

Generation of rVVs

The pBR322-based plasmid vector pBMSF7C contained the ATI/p7.5 hybrid promoter within the hemagglutinin gene region of the vaccinia virus, which was reconstructed from the pSFJ1-10 plasmid and pBM vector [41,42]. Separate full-length cDNAs encoding either the HCV structural protein, nonstructural protein, or all HCV proteins were cloned from HCV R6 strain (genotype 1b) RNA by RT-PCR. Each cDNA was inserted into a separate pBMSF7C vector downstream of the pBMSF7C ATI/p7.5 hybrid promoter; the final designation of each recombinant plasmid was pBMSF7C-CN2, pBMSF7C-N25, or pBMSF-CN5 (Figure 2). They were then transfected into primary rabbit kidney cells infected with LC16m8 (multiplicity of infection = 10). The virus–cell mixture was harvested 24 h after the initial transfection by scrapping; the mixture was then frozen at −80°C until use. The hemagglutinin-negative recombinant viruses were cloned as previously described [42] and named rVV-CN2, rVV-N25, or rVV-CN5. Insertion of the HCV protein genes into the LC16m8 genome was confirmed by direct PCR, and expression of each protein from the recombinant viruses was confirmed by western blot analysis. The titers of rVV-CN2, rVV-N25, and rVV-CN5 were determined using a standard plaque assay and RK13 cells.

Statistical Analysis

Data are shown as mean ± SD. Data were analyzed using the nonparametric Mann–Whitney or Kruskal–Wallis tests or ANOVA as appropriate; GraphPad Prism 5 for Macintosh (GraphPad) was used for all analyses. P values <0.05 were considered statistically significant.

Supporting Information

Figure S1 HAI score of liver samples taken from CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice.
(EPS)

Figure S2 Lipid degeneration in samples of liver taken from CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice.
(EPS)

Figure S3 HCV protein expression after infection of LC16m8, rVV-CN2, rVV-N25, or rVV-CN5 into HepG2 cells.
(EPS)

Figure S4 Effects of treatment with rVV-N25 in RzCN5-15$^{(+/-)}$/MxCre$^{(+/-)}$ mice.
(EPS)

Figure S5 Daily cytokine profiles of the serum from CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice during the week following inoculation with LC16m8, rVV-CN2, rVV-N25, or rVV-CN5.
(EPS)

Figure S6 The immune response following poly(I:C) injection in the acute phase.
(EPS)

Figure S7 Detection of vaccinia virus DNA in the skin, liver, and spleen after inoculation with attenuated vaccinia virus (Lister strain) or highly attenuated vaccinia virus (LC16m8 strain).
(EPS)

Table S1 Incidence of hepatocellular carcinoma in male and female transgenic mice at 360, 480, and 600 days after poly(I:C) injection.
(EPS)

Text S1 Supporting information including material and methods, and references.
(DOCX)

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Author Contributions

Performed the experiments: SS KK TC Y. Tobita TO FY Y. Tokunaga. Analyzed the data: SS KK TC MK. Contributed reagents/materials/analysis tools: KT-K TW TT MM K. Mizuno YH TH K. Matsushima. Wrote the paper: SS KK MK. Study concept and design: MK.

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Review

Role of Oxidative Stress in Hepatocarcinogenesis Induced by Hepatitis C Virus

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Abstract: Hepatitis C virus (HCV) easily establishes chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC). During the progression of HCV infections, reactive oxygen species (ROS) are generated, and these ROS then induce significant DNA damage. The role of ROS in the pathogenesis of HCV infection is still not fully understood. Recently, we found that HCV induced the expression of 3β-hydroxysterol Δ24-reductase (DHCR24). We also found that a HCV responsive region is present in the 5'-flanking genomic promoter region of DHCR24 and the HCV responsive region was characterized as (−167/−140). Moreover, the transcription factor Sp1 was found to bind to this region in response to oxidative stress under the regulation of ataxia telangiectasia mutated (ATM) kinase. Overexpression of DHCR24 impaired p53 activity by suppression of acetylation and increased interaction with MDM2. This impairment of p53 suppressed the hydrogen peroxide-induced apoptotic response in hepatocytes. Thus, a target of oxidative stress in HCV infection is DHCR24 through Sp1, which suppresses apoptotic responses and increases tumorigenicity.

Keywords: hepatitis C virus; reactive oxygen species; 3β-hydroxysterol Δ24-reductase

1. Introduction

Hepatitis C virus (HCV) is a member of the *Flaviviridae* family of RNA viruses, and possesses a positive-strand RNA genome [1]. HCV mainly replicates in the cytoplasm, but frequently establishes chronic infections, leading to the development of chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC) [2,3]. The estimated worldwide prevalence of HCV infections is 2.2%–3.0% [4], and

chronic HCV infection is a major global public health concern. HCV does not possess canonical oncogenes and is unable to integrate into the host genome, but easily establishes chronic infections, resulting in HCC with high frequency. The exact mechanism by which this occurs is not fully understood; however, possible mediators of HCV pathogenesis are reactive oxygen species (ROS). During chronic hepatitis, the immune response induces the production of ROS [5] and nitric oxide (NO) [6]. Furthermore, HCV viral nucleocapsid protein, an HCV core protein, was shown to increase oxidative stress in the liver [7,8]. Moreover, HCV affects the steady-state levels of a mitochondrial protein chaperone known as prohibitin, leading to impaired function of the mitochondrial respiratory chain with the overproduction of ROS [9]. On the other hand, HCV compromises some of the antioxidant systems, including haeme oxygenase-1 [10] and NADH dehydrogenase quinone 1 [9], resulting in the provocation of oxidative stress in the liver during HCV infections. Thus, HCV infections not only induce ROS overproduction, but also hamper the antioxidant system in the liver. The induction of oxidative stress also results in the generation of deletions in mitochondrial and nuclear DNA, which are indicators of genetic damage. NO has been shown to induce oxidative DNA damage and inhibit DNA repair [11–13]. These nucleotide abnormalities may contribute to the development of HCC [14].

2. Survey of HCV-Positive HCC-Related Host Factors

To define the host factors involved in hepatocarcinogenesis during HCV persistent infections, we established a human hepatoblastoma-derived cell line (HepG2), which expresses the full-length HCV genome under the control of a Cre/*lox*P system (RzM6 cells [15]). Using colony-formation assays and nude mice tumor-formation assays, we found that passaging of HCV-expressing cells (RzM6-LC cells) increased their tumorigenicity. To identify which pathway was responsible for the increase in tumorigenicity in RzM6-LC cells, we raised monoclonal antibodies against the RzM6-LC cells and characterized them [16]. We found that one of these clones (2-152a) recognizes 3β-hydroxysterol Δ24-reductase (or dehydrocholesterol reductase 24; DHCR24). DHCR24 functions as an enzyme that catalyzes the conversion of desmosterol to cholesterol in the post-squalene cholesterol biosynthesis pathway [17,18]. The absence of DHCR24 leads to desmosterosis [19]. Furthermore, expression of DHCR24 is down-regulated in areas of the brain affected by Alzheimer's disease [20]. DHCR24 is a multifunctional enzyme, which exerts resistance against oxidative stress and prevents apoptotic cell death when it is expressed at high levels [20–24]. Endogenous DHCR24/seladin-1 levels are up-regulated in response to acute oxidative stress [21,25,26], but the expression levels decline upon chronic exposure to oxidative stress [21,22]. DHCR24 is also reported to function as a hydrogen peroxide scavenger [24]. Thus, DHCR24 plays a crucial role in maintaining cellular physiology by regulating both cholesterol synthesis and cellular defence against oxidative stress, although the biological relevance of the hydrogen peroxide concentration (0.5–2 mM) used in some experiments requires future study.

3. HCV Induces DHCR24 Expression through Oxidative Stress

Since we observed up-regulation of DHCR24 expression in RzM6-LC cells, we decided to characterize the effects of HCV on DHCR24 expression [16,27]. Silencing of HCV by siRNA in RzM6-LC cells down-regulated the expression of DHCR24. By using chimeric mice with humanized