of a solution containing monophosphoryl lipid A and trehalose-6,6-dimycolate using the Sigma Adjuvant System (Sigma). The conjugate (0.2 mL) was injected intraperitoneally into 5-week-old female BALB/c mice, and blood samples were collected. The same volume of saline was injected as a control. Mice were injected at weeks 0, 2, 4, and 6, and blood was collected at weeks 1, 3, 5, and 7 after injection. Blood samples were collected in a BD Vacutainer SST II (Becton Dickinson, Franklin Lakes, NJ) and centrifuged at 1200 × *g* for 10 minutes at room temperature. The supernatants were collected as sera and were heat-inactivated at 56°C for 1 hour. The heat-inactivated sera was used in viral inhibition assay experiments and for IgG purification.

HCV Inhibition Assay

Inhibition of HCV infection of cultured cells was assayed using HCVpp and HCVcc for infection. Naive Huh7.5.1 cells (2×10^4) were seeded into a 48-well plate. HCVpp or HCVcc was mixed with serum that was collected from vaccine-immunized mice and the mixtures then were incubated for 30 minutes at room temperature. Naive Huh7.5.1 cells were inoculated with the virus-antibody mixtures, and after 3 hours the mixtures were removed and the cells were washed once with phosphate-buffered saline. DMEM-10 was added to each well, and the cells were cultured for 72 hours. For the HCVpp assay, cells were washed once with phosphate-buffered saline and lysed with 40 μL/well of Cell Culture Lysis Reagent (Promega, Madison, WI), and luciferase activity was quantified using a Luciferase Assay System (Promega) as described previously.[5] For the HCVcc assay, cells were washed once with phosphate-buffered saline and lysed with 100 μL/well of Passive Lysis Buffer (Promega), and HCV core protein content was quantified using the HCV core enzyme-linked immunosorbent assay kit (Ortho Clinical Diagnostics). Assays were performed in triplicate and infectivity was calculated from the value of the luciferase activity or the HCV core content.

Enzyme Immunoassay

Recombinant J6E1/FLAG or J6E2/FLAG protein (50 ng) was coated onto a 96-well plate (Nunc, Roskilde, Denmark) at 4°C overnight. Each well was blocked with Blocking One (Nacalai Tesque, Kyoto, Japan) for 1 hour at room temperature, and then was washed with phosphate-buffered saline. Sera from immunized mice were diluted 1000-fold in PBS, added to each well, and the plate was incubated for 1–2 hours at room temperature. The sera was discarded and each well was washed twice with phosphate-buffered saline. Horseradish-peroxidase–conjugated anti-mouse IgG antibody (GE Healthcare) was diluted 3000-fold with Blocking One, added into each well, and the plate then was incubated for 1 hour at room temperature. Nonbound antibody was discarded, and peroxidase activity of the horseradish-peroxidase–bound antibody was detected using a peroxidase detection kit (Sumitomo Bakelite, Tokyo, Japan).

HCV Inhibition Assay

Inhibition of HCV infection of cultured cells was assayed using HCVpp and HCVcc for infection. HCVpp or HCVcc was mixed with serum or immunoglobulin that was collected from vaccine-immunized mice and the mixtures then were incubated for 30 minutes at room temperature. Naive Huh7.5.1 cells were inoculated with the virus-antibody mixtures, and after 3 hours the mixtures were removed and the cells were washed once with phosphate-buffered saline. DMEM-10 was added to each well, and the cells were cultured for 72 hours. The cells were lysed and the lysates were assayed as described later.

Western Blot Analysis

The naive Huh7 cells that were used as a negative control and the J6/JFH-1–infected Huh7 cells that were used as a positive control were lysed using Passive Lysis Buffer (Promega), and then centrifuged to remove debris. Protein concentration was quantified using the BCA Protein Assay Kit (Pierce, Rockford, IL). The HCV core of purified HCV particles (0.5 pmol each of Frac 6 and Frac 8) and cell lysate samples (5 μg protein) then were denatured by boiling, resolved on 12% sodium dodecyl sulfate–polyacrylamide gels, and transferred to an Immobilon-P membrane (Millipore, Billerica, MA). Recombinant FLAG-tagged E1 (J6E1/FLAG) and FLAG-tagged E2 (J6E2/FLAG) proteins also were analyzed using 1, 3, 10, and 30 ng protein/lane. The membrane was blocked with BlockAce (Dainippon Sumitomo Pharma, Osaka, Japan), and probed with primary antibodies against HCV core (2H9, 3 μg/mL), E1 (B7567[6], 10 μg/mL), and E2 (AP33, 3 μg/mL; a generous gift from Genentech, Inc, South San Francisco, CA) proteins in Tris-buffered saline containing Tween-20 (20 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 0.1% [vol/vol] Tween-20 [polyoxyethylene sorbitan monolaurate]) containing 10% (vol/vol) BlockAce. After several washes with Tris-buffered saline containing Tween-20, the membrane was probed with horseradish-peroxidase–conjugated secondary antibody (GE Healthcare, 1:5000 dilution), washed repeatedly, and bound antibodies were detected using ECL-plus (GE Healthcare) and visualized using LAS3000 (Fujifilm, Tokyo, Japan). After antigen detection, the antibody on the membrane was stripped using WB stripping solution (Nacalai Tesque) and the membrane was re-probed with another antibody.

IgG Purification

Mouse IgG was purified using the KAPTIV-GY resin (Technogen). Briefly, approximately 0.5 mL of the KAPTIV-GY resin was loaded onto an empty column (Bio-Rad) and was washed with loading buffer (50 mmol/L bis-Tris, pH 6.8). Sera from mice at 7 weeks after immunization were applied onto the column and the column was washed with loading buffer. IgG then was eluted with glycine-HCl (pH 3.0) and the eluates were neutralized with Tris-HCl (pH 9.0). The eluted IgG (iHCV-IgG) was concentrated using an Amicon Ultra-15 (molecular weight cut-off 30,000) and IgG purity was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and subsequent Coomassie brilliant blue staining. Control mouse IgG was prepared similarly from saline-injected mice (Cont-IgG).

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Supplementary Table 1. Composition of Purified HCV Particles After Ultracentrifugation

	Volume, mL	Core, pmol	RNA, copies	Protein, mg	Infectivity, FFU/mL
Culture supernatant	5720	164.0 (100%)	5.0×10^{11} (100%)	4.2×10^{3} (100%)	3.0×10^{4} (100%)
Sucrose cushion purification	1.00	106.5 (65.0%)	2.5×10^{11} (49.5%)	1.4 (0.03%)	7.2×10^{7} (41.2%)
Culture supernatant	10,500	301.7 (100%)	9.1×10^{11} (100%)	7.8×10^{3} (100%)	3.0×10^{4} (100%)
Sucrose gradient purification (Frac 6)	1.05	103.0 (34.1%)	1.8×10^{11} (19.8%)	0.7 (0.01%)	3.6×10^{7} (11.9%)
Sucrose gradient purification (Frac 8)	1.05	56.6 (18.8%)	9.0×10^{10} (9.9%)	4.3 (0.06%)	5.7×10^{7} (18.8%)

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

Satoshi Sekiguchi[1], Kiminori Kimura[2], Tomoko Chiyo[1], Takahiro Ohtsuki[1], Yoshimi Tobita[1], Yuko Tokunaga[1], Fumihiko Yasui[1], Kyoko Tsukiyama-Kohara[3], Takaji Wakita[4], Toshiyuki Tanaka[5], Masayuki Miyasaka[6], Kyosuke Mizuno[7], Yukiko Hayashi[8], Tsunekazu Hishima[8], Kouji Matsushima[9], Michinori Kohara[1]*

1 Department of Microbiology and Cell Biology, Tokyo Metropolitan Institute of Medical Science, Setagaya-ku, Tokyo, Japan, **2** Division of Hepatology, Tokyo Metropolitan Komagome Hospital, Bunkyo-ku, Tokyo, Japan, **3** Transboundary Animal Diseases Center, Joint Faculty of Veterinary Medicine, Kagoshima University, Korimoto, Kagoshima, Japan, **4** Department of Virology II, National Institute of Infectious Diseases, Shinjuku-ku, Tokyo, Japan, **5** Laboratory of Immunobiology, Department of Pharmacy, School of Pharmacy, Hyogo University of Health Sciences, Chuo-ku, Kobe, Japan, **6** Laboratory of Immunodynamics, Department of Microbiology and Immunology, Osaka University Graduate School of Medicine, Suita, Osaka, Japan, **7** Chemo-Sero-Therapeutic Research Institute, Okubo, Kumamoto, Japan, **8** Department of Pathology, Tokyo Metropolitan Komagome Hospital, Bunkyo-ku, Tokyo, Japan, **9** Department of Molecular Preventive Medicine, School of Medicine, University of Tokyo, Bunkyo-ku, Tokyo, Japan

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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* E-mail: kohara-mc@igakuken.or.jp

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-

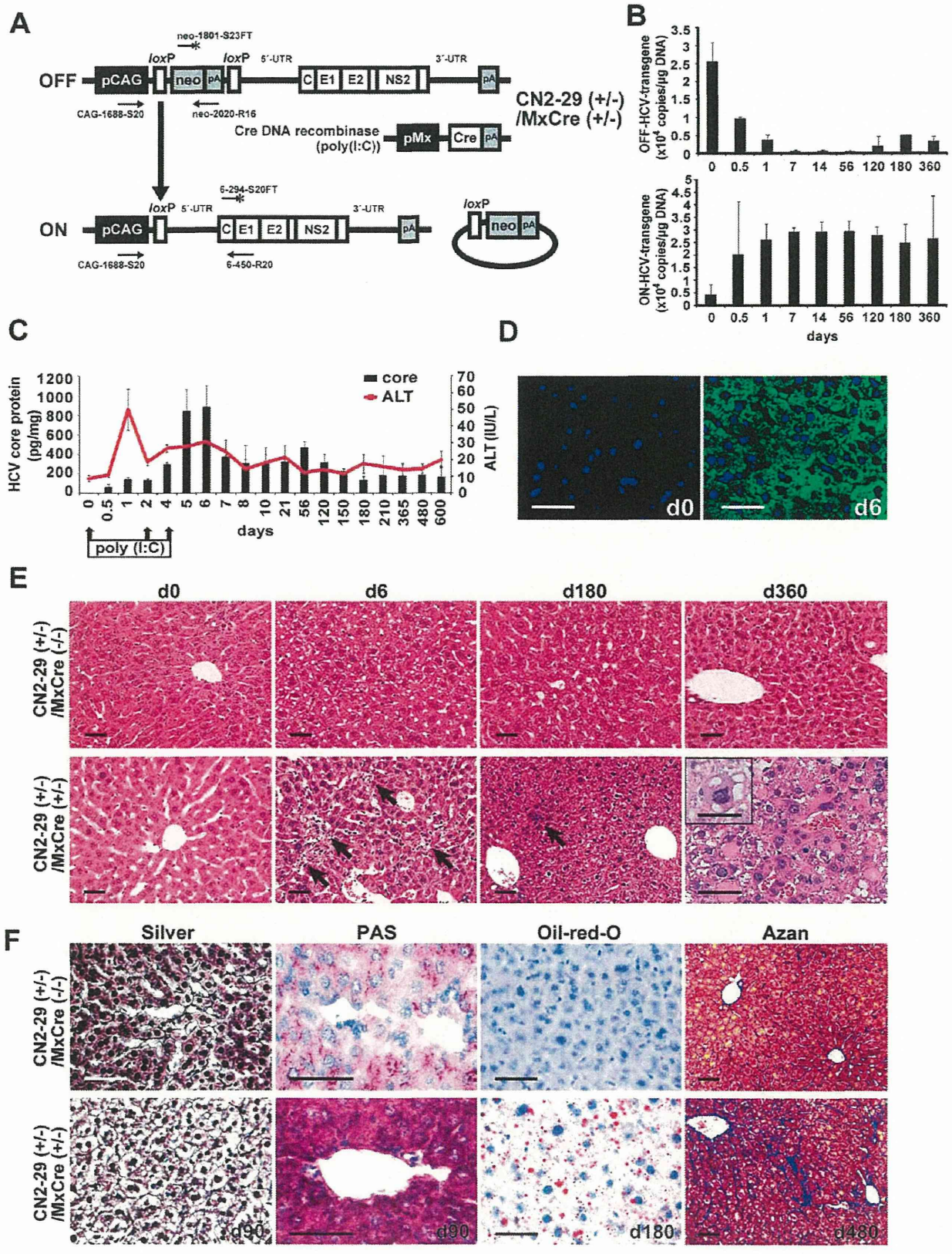
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Figure 1. Pathogenesis in immunocompetent mice with persistent HCV expression. (**A**) Structure of CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ and the Cre-mediated activation of the transgene unit. R6CN2 HCV cDNA was cloned downstream of the CAG promoter, neomycin-resistant 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

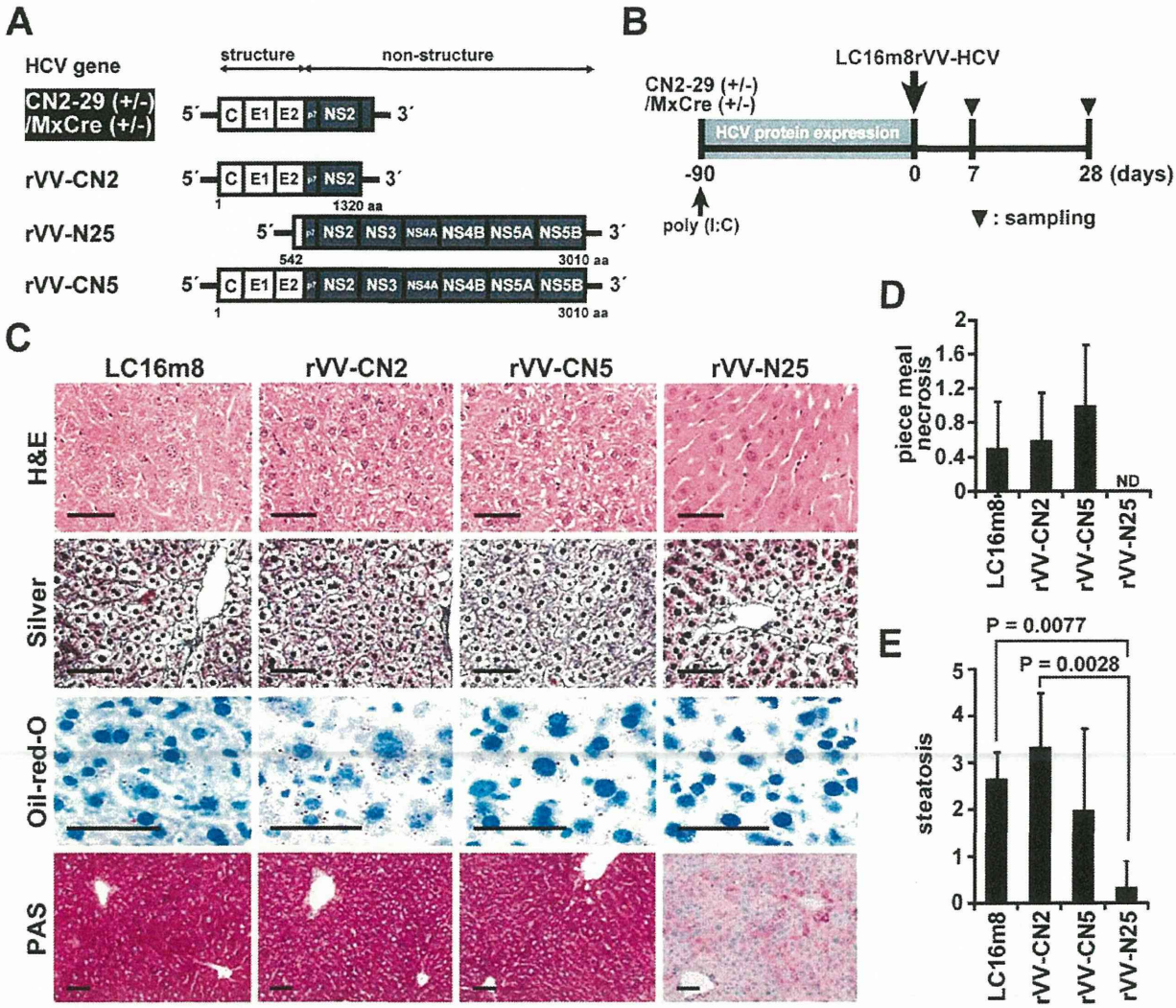

Figure 2. Effects of rVV-HCV treatment on the CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice. (**A**) HCV gene structure in the CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice and recombinant vaccinia viruses (rVV-HCV). MxCre/CN2-29 cDNA contains the core, E1, E2, and NS2 regions. The rVV-CN2 cDNA contains the core, E1, 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

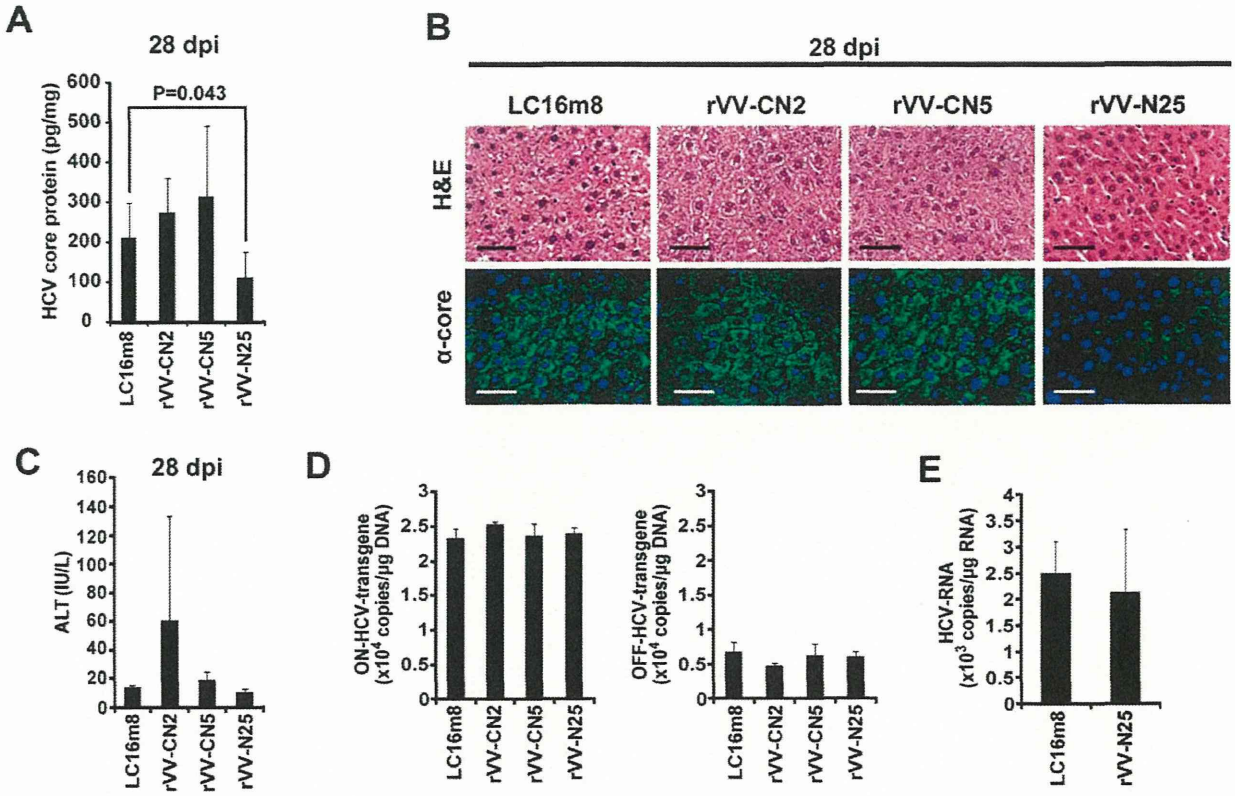

Figure 3. Effects of HCV core protein expression on the livers of CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice inoculated with rVV-HCV. (**A**) Expression of the HCV core protein in the four treatment groups of CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice 28 days after the inoculation. Significant relationships are 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

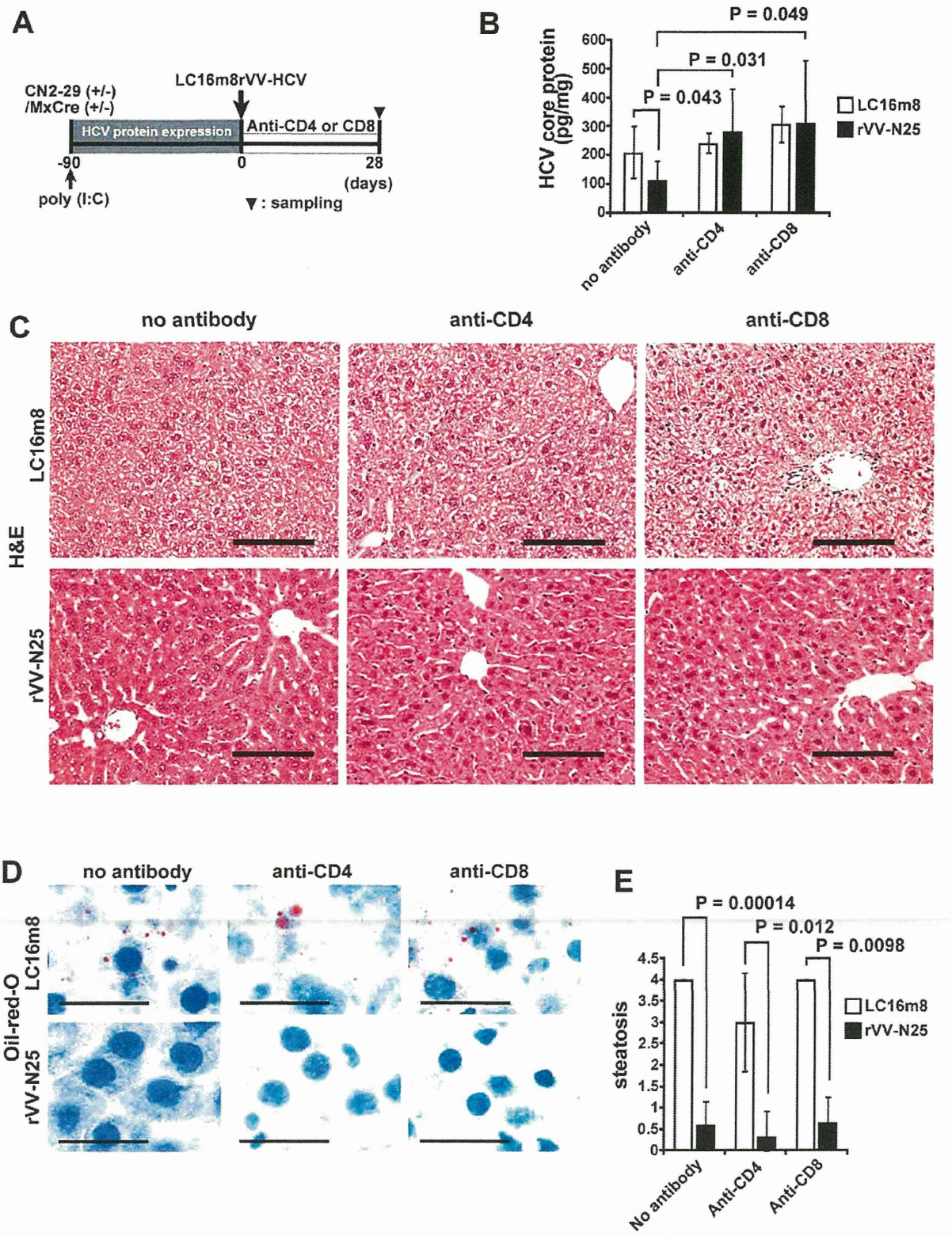

Figure 4. Role of CD4 and CD8 T cells in rVV-N25-treated mice. (**A**) Schematic diagram depicts depletion of CD4 and CD8 T cells via treatment with monoclonal antibodies. (**B**) Comparison of HCV core protein expression in control, CD4-depleted, and CD8-depleted mice 28 days after immunization with 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

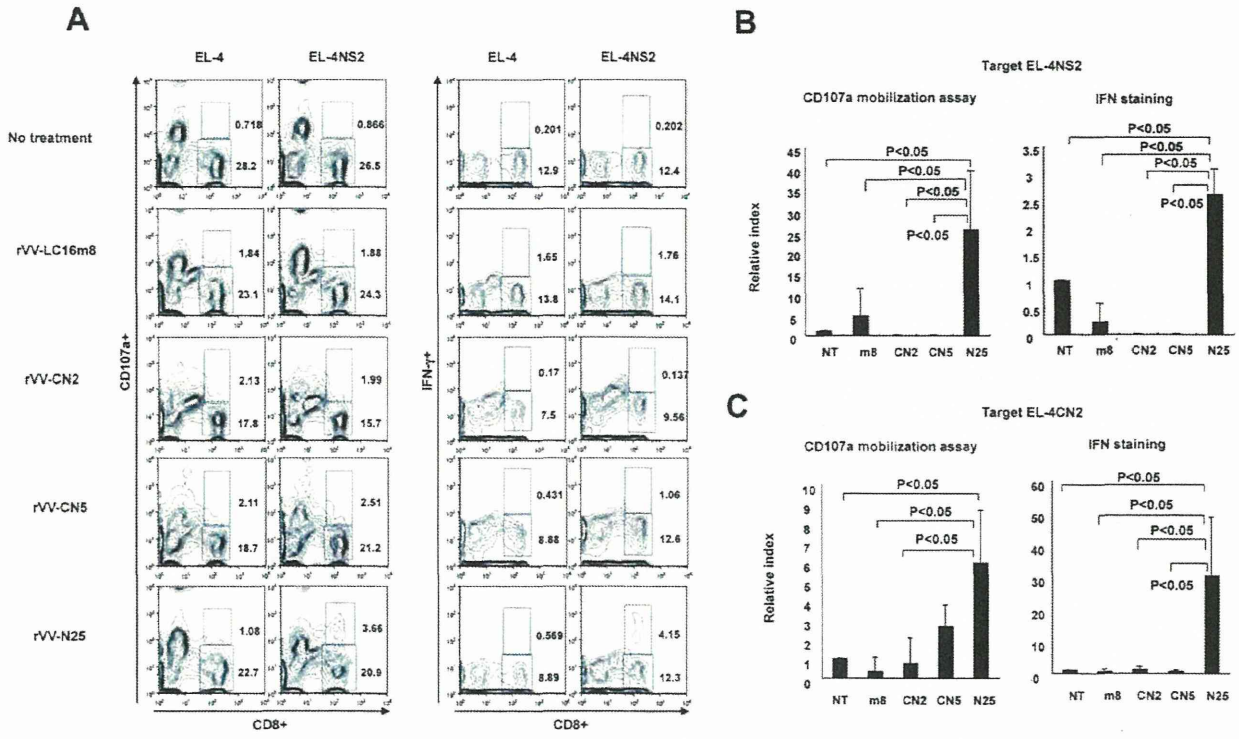

Figure 5. Immunization with rVV-N25 induced CD8 T-cell degranulation, a marker for cytotoxicity, and IFN-γ production. (**A**) The numbers represent the percentage of CD107a positive cells and negative cells (left two columns) and IFN-γ-positive cells and negative cells (right two columns). (**B, C**) 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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