Table 2. **Survival of patients undergoing radiofrequency ablation, based on tumor number, tumor size, and Child-Pugh class**

Grading	*n*	Survival (%)					Median (years)	*P* value
		1-Year	3-Year	5-Year	7-Year	10-Year		
Overall survival	1,170	96.6	80.5	60.2	45.1	27.3	6.4	—
Tumor number								
Solitary	685	97.2	82.6	64.6	50.5	32.0	7.0	0.0003
2–3	395	95.7	77.9	54.4	39.4	19.9	5.6	
≥4	90	96.5	76.4	53.6	30.1	17.6	5.3	
Tumor size								
≤3 cm	889	97.2	83.8	65.1	47.3	30.7	6.7	<0.0001
>3 cm	281	94.8	71.0	46.5	38.0	18.6	4.6	
Child-Pugh class								
A	868	98.0	86.0	65.9	50.2	30.1	7.0	<0.0001
B	291	93.2	66.4	46.5	32.4	20.6	4.6	
C	11	81.8	58.4	23.4	23.4	—	3.1	
Combination of tumor number, tumor size, and Child-Pugh class								
Solitary, ≤3 cm	534	97.6	84.7	68.0	51.4	34.3	7.1	—
Solitary, ≤3 cm, Child-Pugh A	401	98.7	90.1	74.0	57.4	41.3	8.2	—
1–3 Tumors, ≤3 cm	822	97.1	83.7	65.2	48.8	32.5	6.9	—
Solitary, ≤5 cm, or 1–3 tumors, ≤3 cm	947	97.2	82.8	63.8	48.8	30.6	6.9	—
Child-Pugh A/B								
Satisfied the indication criteria of surgical resection proposed in the BCLC protocol[a]	237	98.6	90.5	75.9	61.1	38.1	8.7	—

BCLC, Barcelona Clinic Liver Cancer; HCC, hepatocellular carcinoma.
[a]Child-Pugh class A with a normal level of bilirubin, no significant portal hypertension, and a single HCC.

The 1-, 3-, 5-, 7-, and 10-year rates of local tumor progression with or without distant recurrence were 1.4% (95% CI: 0.7–2.1%), 3.2% (95% CI: 2.1–4.3%), 3.2% (95% CI: 2.1–4.3%), 3.2% (95% CI: 2.1–4.3%), and 3.2% (95% CI: 2.1–4.3%), respectively (**Figure 3**). Univariate analysis demonstrated that prothrombin time and serum AFP, DCP, and AFP-L3 levels were correlated to local tumor progression, whereas multivariate analysis showed that serum DCP level alone was significantly correlated. Tumor size was not correlated to local tumor progression.

The 1-, 3-, 5-, 7-, and 10-year rates of distant recurrence without local tumor progression were 25.6% (95% CI: 23.0–28.2%), 63.3% (95% CI: 60.2–66.4%), 74.8% (95% CI: 71.8–77.8%), 78.1% (95% CI: 75.1–81.2%), and 80.8% (95% CI: 77.4–84.3%), respectively. Univariate analysis demonstrated 14 variables relevant to distant recurrence, whereas multivariate analysis showed that anti-HCV, Child-Pugh class, platelet count, tumor size, tumor number, serum AFP level, and serum DCP level were significantly related to distant recurrence (**Table 3**).

Complications

A total of 67 complications were encountered (**Table 4**). The incidence rates of complications per treatment and per procedure were 2.2% (67/2,982) and 1.5% (67/4,514), respectively. One patient died of hepatic failure on account of massive hepatic infarction 7 days after the last RFA procedure. He had undergone 12 RFA treatments in 8 years. The treatment mortality rate was 0.03%.

DISCUSSION

This study describes our 10-year clinical experience with RFA at a high-volume center. We performed the 2,982 RFA treatments on a total of the 1,170 primary HCC patients, showing that RFA has a high antitumor effect. Tumors were judged to have been completely ablated by final CT imaging in 99.4% of the treatments. Complete response was achieved not only in the first RFA but also in iterative RFA for recurrence. Although complete response rate differed with tumor size, there was not a sharp drop-off in effectiveness. The complete response rate may be higher in this study than others probably because we generally repeated the procedure until CT imaging demonstrated complete tumor necrosis, whereas many other studies limited the procedure number of RFA to 2–3 (11,13,15). Complete ablation of tumors has been reported to be related to improved survival (25). There were the 18 treatments in which we did not perform additional RFA for residual cancer tissue. In those treatments, usefulness of RFA had been unclear at the initial session because of liver dysfunction or tumor burden.

Table 3. Multivariate analysis of variables relevant to survival, local tumor progression, and distant recurrence

Variable	Multivariate analysis Hazard ratio (95% CI)	*P* value
Survival		
Age (per year)	1.03 (1.02–1.04)	<0.0001
Anti-HCV-positive	1.34 (1.03–1.76)	0.03
Child-Pugh class		
A	1	
B or C	2.08 (1.69–2.56)	<0.0001
Tumor size (cm)		
≤2.0	1	
2.1–3.0	1.40 (1.10–1.80)	0.007
3.1–5.0	1.80 (1.37–2.38)	<0.0001
>5.0	1.50 (0.90–2.49)	0.12
Tumor number		
Solitary	1	
2–3	1.28 (1.04–1.59)	0.02
≥4	1.58 (1.13–2.21)	0.008
Serum DCP (mAU/ml)		
≤100	1	
101–400	1.22 (0.88–1.69)	0.24
>400	1.66 (1.14–2.42)	0.008
Serum AFP-L3 (%)		
≤15	1	
>15	1.45 (1.11–1.91)	0.008
Local tumor progression		
Serum DCP (mAU/ml)		
≤100	1	
101–400	2.51 (1.02–6.20)	0.05
>400	6.52 (2.63–16.1)	<0.0001
Distant recurrence		
Anti-HCV-positive	1.44 (1.19–1.75)	0.0002
Child-Pugh class		
A	1	
B or C	1.23 (1.03–1.45)	0.02
Platelet count (/l)		
$>10^{11}$	1	
$\leq 10^{11}$	1.36 (1.12–1.64)	0.002
Tumor size (cm)		
≤2.0	1	
2.1–3.0	1.30 (1.10–1.55)	0.003
3.1–5.0	1.29 (1.05–1.60)	0.02
>5.0	1.25 (0.75–2.08)	0.4
Tumor number		
Solitary	1	
2–3	1.36 (1.16–1.59)	0.0002
≥4	2.02 (1.53–2.66)	<0.0001
Serum AFP (ng/dl)		
≤100	1	
101–400	1.15 (0.92–1.44)	0.22
>400	1.36 (1.03–1.81)	0.03
Serum DCP (mAU/ml)		
≤100	1	
101–400	1.19 (0.92–1.54)	0.19
>400	1.72 (1.22–2.42)	0.002

AFP, α-fetoprotein; CI, confidence interval; DCP, des-γ-carboxy-prothrombin; HCV, hepatitis C virus.

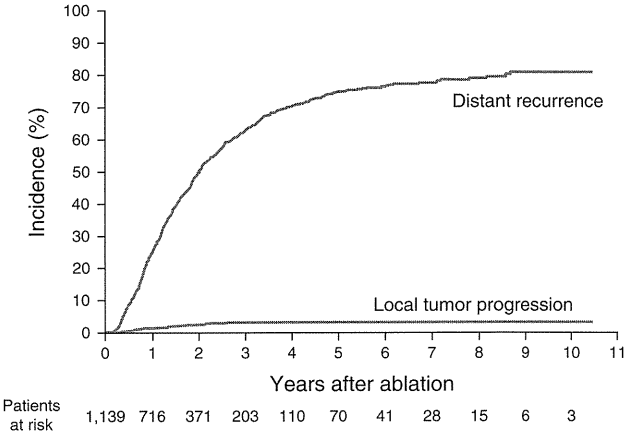

Figure 3. Local tumor progression and distant recurrence in patients who underwent radiofrequency ablation.

This study shows that RFA could achieve long-term survival for as long as 10 years. Sixteen patients treated by RFA survived for >10 years. The variables relevant to survival were similar to those found in previous studies on ethanol injection (26,27), RFA, hepatic resection (28), and transarterial chemoembolization (29). Both liver function and tumor-related factors were associated with survival. In addition, age and anti-HCV were relevant to survival in this study. Age was among the prognostic factors, probably because 23.0% of the patients were >75 years old, which resulted in a higher percentage (18.5%) of liver-unrelated deaths in this study compared with others. Anti-HCV was among the prognostic factors, probably because anti-HCV-positive patients developed distant recurrence more frequently.

HCC frequently recurred after RFA; most recurrences were, however, not local tumor progression but distant recurrence. Frequent recurrence is not specific to RFA. After hepatic resection, the

Table 4. **Complications in 2,982 treatments of radiofrequency ablation for hepatocellular carcinoma**

Complication	No. of complications
Neoplastic seeding	24
Liver abscess	6
Hemoperitoneum	12
Hemothorax	5
Symptomatic pleural effusion	1
Massive hepatic infarction	6
Gastrointestinal perforation or penetration	5
Hemobilia	2
Skin burn	1
Pneumothorax	3
Gallbladder injury	1
Cerebral infarction	1

tumor recurrence rate exceeds 70% at 5 years (30,31). In this study, periodic follow-up detected most recurrences at limited stage. RFA was performed again for first recurrence in almost 90% of cases, although multimodal treatments were used in a long-term follow-up. On the other hand, repeat resection rate for first recurrence has been reported to range from 10.4 to 30.6% (31,32). Because RFA is less invasive than hepatic resection, iterative RFA can be performed for recurrence more easily.

Local tumor progression was found less frequently in this study than in other studies, having been reported to be around 10% at 3 years following RFA (13,14). Furthermore, different from the findings in previous reports (33,34), tumor size was not related to local tumor progression in this study. These differences are probably because we repeated RFA until we considered we had ablated not only the tumor itself but also some of the liver tissue surrounding it. Furthermore, to avoid local tumor progression, we were more cautious in the treatment of larger tumors when deciding whether sufficient ablation had been performed. Only serum DCP level was significantly related to local tumor progression in this study. Elevated serum DCP level may be related to the malignant potential of HCC such as the development of portal venous invasion (35).

The frequency of distant recurrence in this study was similar to that reported in other studies (13). Among the variables significantly related to distant recurrence, tumor size, tumor number, serum AFP level, and serum DCP level were probably related to micrometastasis, which had not been detected by imaging modalities before the treatment, while anti-HCV, Child-Pugh class, and platelet count were related to metachronous multicentric carcinogenesis, which developed based on underlying chronic liver disease.

From the viewpoint of survival and distant recurrence, patients with 2.1–5.0 cm tumors had significantly worse outcomes than those with ≤2.0 cm tumors while those with tumors >5.0 cm did not have worse rates than those with tumors ≤2 cm. This is probably because the number of patients with tumors >5.0 cm ($n=35$) were not large enough for the difference to be statistically significant. Another possibility is selection bias. It is possible that patient with tumors >5.0 cm who underwent RFA had more favorable conditions for survival and distant recurrence except tumor size than those with 2.1–5.0 cm tumors.

In this study, 324 of the 1,170 patients were treated with combination of TACE and RFA at the initial treatment. Thus, we evaluated the combination as a possible variable that influences survival or recurrence. Univariate analysis demonstrated that the combined therapy was significantly correlated to overall survival, whereas multivariate analysis did not show the relationship. TACE was generally combined with RFA in patients with either ≥4 tumors or those with even one tumor >3.0 cm in diameter. This is why the correlation was significant in univariate analysis, while it was not in multivariable model in which the effect of other risk factors, such as tumor number and tumor size were adjusted. The combination of TACE and RFA was not significantly related to either local tumor progression or distant recurrence.

RFA was a safe procedure. Although many patients treated by RFA in this study were at high risk for surgical treatment because of advanced cirrhosis or other comorbidities, complications occurred in only 2.2% of the treatments. Other investigators have also reported low complication rates of 0–6.1% (11,13–16). For hepatic resection, morbidity rates of 38–47% have been reported even in recent studies (36–38).

To date, percutaneous ethanol injection has been considered the standard in ablation (5). However, randomized controlled trials have demonstrated the superiority of RFA (6–9), with RFA now largely replacing ethanol injection. We have also shifted from ethanol injection to RFA (10). At our department, RFA is currently the first option and ethanol injection is performed only on patients on whom RFA cannot be performed safely because of either enterobiliary reflux, adhesion between the tumor and the gastrointestinal tract, or other reasons.

Surgical resection has been considered the treatment of choice for HCC. Our first option for resectable HCC was also surgery. However, most patients who came to our department visited us because they did not want surgical resection. Thus, many patients in this study underwent RFA not because of unresectable tumor but because of refusal of surgery. Those who preferred surgery would have directly gone to the surgical department that has extensive experience in hepatic resection (38).

It is not easy to compare outcomes between RFA and surgical resection; the indications are different between the two treatments. Furthermore, indications for each treatment are different from institution to institution. Thus, a case adjudged to be treatable by RFA or surgical resection at an institution may not be given the same treatment at another. The best known indication criteria for surgical resection may be those proposed in the Barcelona Clinic Liver Cancer (BCLC) protocol (5), which states that surgical resection should be restricted to patients with performance status 0, Child-Pugh class A, single HCC, normal portal pressure, and normal serum bilirubin level. In patients satisfying those criteria, the 5-year survival rate is expected to be >70% (30). In this study, 237

(20.3%) of 1,170 patients satisfied those criteria and were thus considered good candidates for surgical resection; their 5-year survival rate was 75.9%, which appears satisfactory when compared with outcomes following surgical resection. Furthermore, in all 1,170 primary HCC patients treated by RFA, 5- and 10-year survival rates were 60.2% and 27.3%, respectively. In patients treated by surgical resection, 5- and 10-year survival rates were 34.4–70.0% and 10.5–52.0%, respectively (32,39–45). Although this is an observational study with no control, survivals following RFA appear comparable to those reported following surgical resection.

Two recent randomized controlled trials showed no significant difference in survival between RFA and surgical resection (46,47). Several nonrandomized controlled trials reported that RFA had similar overall survival rates to resection (48–50), while others found resection to be associated with higher survival rates (51–53). Further studies are necessary to resolve comparison of RFA with resection.

We have made strenuous efforts to standardize the RFA procedure. Although many physicians have participated in RFA at our institution, the procedure was invariably performed according to the institutional protocol and in the presence of experienced physicians. Video recording was also used to monitor the procedure. Additionally, preoperative planning and postoperative evaluation of technique effectiveness were also carried out by at least three physicians. We also believe that not only proficient practice of RFA but also detailed preoperative planning, cautious postoperative evaluation of therapeutic effect, and careful follow-up are vital to achieve satisfactory outcomes.

Source population in this study may represent selection bias, as we performed RFA on most patients who were hospitalized at our department; however, many patients with unfavorable tumor conditions for RFA might not have been referred to us. Therefore, caution is required when extrapolating our findings to the general population of HCC patients.

A second limitation is that study population cannot be clearly defined. This study was based on daily clinical practice over a 10-year period. Indication criteria of RFA have changed over time, mainly because another percutaneous ablation, that is, ethanol injection has also been performed. Furthermore, various treatments besides percutaneous ablation were available for HCC, such as surgical resection and transarterial chemoembolization, with frequently overlapping indications.

One further limitation is the fact that this was a single-center study; these results might not be reproducible consistently in other settings. To extrapolate the findings in this study to patients at other institutions, careful consideration should be given to differences in the indications, methods, expertise, performance of available ultrasound and CT equipment, and others. Treatment outcome may be influenced by the physicians' expertise and the institution's volume of care. We started ethanol injection in 1985 and microwave ablation in 1995, that is, before the introduction of RFA. Recently, we have performed over 900 RFA treatments per year, which may represent a far greater number of treatments than those in most other institutions. We would not recommend any change in daily clinical practice solely on the strength of our study findings.

In conclusion, our 10-year clinical experience shows that RFA could be locally curative, resulting in survival for as long as 10 years, and was a safe procedure. RFA might be a first-line treatment for selected patients with early-stage HCC.

CONFLICT OF INTEREST

Guarantor of the article: Shuichiro Shiina, MD, PhD.

Specific author contributions: Study concept and design, analysis and interpretation of data, and drafting of the manuscript: Shuichiro Shiina; analysis and interpretation of data and statistical analysis: Ryosuke Tateishi; study execution and data acquisition: Toru Arano, Koji Uchino, Kenichiro Enooku, Hayato Nakagawa, Yoshinari Asaoka, Takahisa Sato, Ryota Masuzaki, Yuji Kondo, and Tadashi Goto; revised the article critically for important intellectual content: Haruhiko Yoshida; Masao Omata, and Kazuhiko Koike. All authors have read and approved the submitted manuscript.

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Potential competing interests: None.

Study Highlights

WHAT IS CURRENT KNOWLEDGE

- ✓ Radiofrequency ablation (RFA) has been widely performed for hepatocellular carcinoma (HCC).
- ✓ RFA has a more reliable local antitumor effect and higher survival than ethanol injection.
- ✓ There has been no report on 10-year outcome of RFA.

WHAT IS NEW HERE

- ✓ Five- and 10-year survival rates in 1,170 patients with primary hepatocellular carcinoma (HCC) were 60.2 and 27.3%, respectively.
- ✓ Age, antibody to hepatitis C virus, Child-Pugh class, tumor size, tumor number, serum des-γ-carboxy-prothrombin level, and serum lectin-reactive α-fetoprotein level were significantly related to survival.
- ✓ Five- and 10-year local tumor progression rates were both 3.2%. Five- and 10-year distant recurrence rates were 74.8 and 80.8%, respectively.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Evaluation of molecular targeted cancer drug by changes in tumor marker doubling times

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Abstract
Background We evaluated the usefulness of tumor marker doubling time (DT) as an efficacy indicator of a molecular targeted anticancer agent.
Methods Twenty-five patients with advanced hepatocellular carcinoma (HCC) received TSU-68, a multiple tyrosine kinase inhibitor. Exponential increase in HCC-specific tumor marker levels (alpha-fetoprotein or des-gamma-carboxyprothrombin) was seen in 15 of them prior to TSU-68 administration. The relationship between tumor marker DT and tumor volume DT was evaluated. Next, tumor marker DT in the first 8 weeks of TSU-68 administration was compared with tumor marker DT before treatment. Efficacy evaluation based on changes in tumor marker DT was compared with Response Evaluation Criteria In Solid Tumors (RECIST).
Results Tumor marker DT and tumor volume DT were almost identical ($r^2 = 0.94$, $P < 0.001$) in each patient before TSU-68 administration. Efficacy evaluation based on changes in tumor marker DT on TSU-68 administration was in accordance with RECIST in 12/15 cases. Discordance was observed in three cases, for which RECIST indicated disease progression in spite of elongated tumor marker DT. Those cases showed substantial tumor necrosis without volume shrinkage or appearance of new lesions in spite of apparent effects on target lesions.
Conclusions Serum tumor marker DT can be used to evaluate viable tumor burden irrespective of the presence of tumor necrosis which can compromise radiographic evaluation. This approach may be applicable to the evaluation of responses to chemotherapy, particularly to cytostatic agents (ClinicalTrials.gov number, NCT00784290).

Keywords Doubling time · RECIST · AFP · PIVKA-II · HCC · TSU-68

Abbreviations

AFP	Alpha-fetoprotein
CEA	Carcinoembryonic antigen
CR	Complete response
CT	Computed tomography
DCP	Des-gamma-carboxyprothrombin
DT	Doubling time
FGFR	Fibroblast growth factor receptor
HCC	Hepatocellular carcinoma
PD	Progressive disease
PDGFR	Platelet-derived growth factor receptor
PR	Partial response
PSA	Prostate-specific antigen
RECIST	Response Evaluation Criteria In Solid Tumors
SD	Stable disease
TACE	Transcatheter arterial chemoembolization
VEGFR-2	Vascular endothelial growth factor receptor-2

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Introduction

Phase II trials of chemotherapeutic agents for solid tumors usually adopt an objective tumor response as the primary endpoint, the rationale being that the tumor response will be a surrogate for the effects of a particular agent on survival outcomes [1–4]. In evaluating a tumor response to a cancer drug, the Response Evaluation Criteria In Solid Tumors (RECIST) guidelines are usually adopted. However, the total tumor volume thus determined is not necessarily proportional to the number of viable tumor cells, e.g., in cases of massive tumor necrosis without tumor shrinkage [5–9].

With the progress in molecular targeted cancer drugs, concerns about the appropriate design of clinical trials of such agents have emerged [10, 11]. In contrast to conventional cytotoxic agents, molecular targeted agents often show cytostatic effects, i.e., a slowing of tumor growth. The effects of such agents upon the tumor growth rate may be better evaluated not by the changes in tumor burden but by the rate of changes for which RECIST may not be particularly suitable.

Most solid malignant tumors show an exponentially increasing volume in the natural course of their growth. The tumor volume doubling time (DT) is the parameter that defines the speed of the increase. Serum levels of several tumor markers, including prostate-specific antigen (PSA), carcinoembryonic antigen (CEA), and alpha-fetoprotein (AFP), have been reported to correlate with tumor volume in an individual patient [12–15]. The rate of changes in tumor volume may be calculated on the basis of repeated measurements of the serum tumor marker levels. The DT of serum PSA levels has also been proposed as a biological parameter that can be used to predict the prognosis of prostate cancer, and PSA determination has now become an integral part of the disease management [16–18].

The aim of the present study was to elucidate the usefulness of tumor marker DT to evaluate the efficacy of TSU-68 against hepatocellular carcinoma (HCC). TSU-68 is an orally administered, small-molecule inhibitor of multiple receptor tyrosine kinases, vascular endothelial growth factor receptor-2 (VEGFR-2), platelet-derived growth factor receptor (PDGFR), and fibroblast growth factor receptor (FGFR) [19]. As a potent antiangiogenic agent, TSU-68 is expected to be effective against HCC [20], and a phase I/II study has been recently conducted in Japan [21]. In that clinical trial, the serum levels of AFP and des-gamma-carboxyprothrombin (DCP) were also scheduled to be periodically determined. Although the effect was assessed by radiologic examinations, the effect of TSU-68 may be more accurately evaluated by changes in tumor growth speed based on specific tumor marker levels [22–26].

Methods

Clinical trial

This study was conducted according to the ethical guidelines for epidemiologic research designed by the Ministry of Education, Culture, Sports, Science and Technology and Ministry of Health, Labour and Welfare of Japan. The study design was approved by the institutional review board of the University of Tokyo Hospital.

An open-label phase I/II trial of TSU-68 for the treatment of HCC was performed between September 2003 and February 2007 at three institutions in Japan [21]. Twenty-five of the participating patients were enrolled from the University of Tokyo Hospital. In the present study, clinical data for these 25 patients, including analyses conducted before and after the trial, were further evaluated. Briefly, histologically confirmed HCC patients without indication or response to resection, ablation, or transcatheter arterial chemoembolization (TACE) were deemed eligible. The eligibility criteria also included a World Health Organization (WHO) performance status of 2 or better, a life expectancy of not less than 90 days, and a liver function of Child–Pugh class A or B. Patients were not eligible if they had received ablation, TACE, chemotherapy, or irradiation within 4 weeks, or surgery within 6 weeks, of the commencement of the trial (washout phase).

The phase I study began with a 400 mg bid oral dose of TSU-68. Because of dose-limiting toxicities, however, this was reduced to 200 mg bid in the subsequent phase II study. At the end of each 4-week cycle, dynamic contrast-enhanced computed tomography (CT) consisting of early and late arterial, and portal venous phases was performed, and contiguous transverse sections with a thickness of 5 mm were obtained. Responses were assessed on the basis on the RECIST evaluations in predetermined target lesions. The serum levels of HCC-specific tumor markers, AFP and DCP, were scheduled to be determined every 2 weeks. TSU-68 administration was discontinued when progressive disease (PD) was observed by RECIST.

Patients

Among the patients who had participated in the aforementioned trial, those who met the following criteria were included in the present study: (1) tumor growth prior to TSU-68 administration could be evaluated with two CT examinations performed 1–3 months before the trial and upon enrollment; (2) serum tumor marker levels could be determined at least three times during the washout phase, and a linear regression of the logarithmic transformation of marker levels over time showed an r^2 greater than

0.80; and (3) TSU-68 had been administered for at least 4 weeks.

Radiological evaluation of tumor volume

Radiological evaluations were performed according to RECIST guidelines version 1.0 [27]. Not more than 10 lesions, including intrahepatic tumors and extrahepatic metastases, were selected as target lesions prior to TSU-68 administration. In addition to RECIST, we also in our present analyses estimated the volume of each target lesion as a sphere taking the average of its major and minor axes as the diameter [28], and thereby calculating the radiological tumor volume DT as

$$\mathrm{DT} = \log(2) \times \frac{t_2 - t_1}{\log(V_2) - \log(V_1)}$$

where V_1 and V_2 are the volumes at times t_1 and t_2 [29].

Tumor markers

The HCC-specific tumor markers, AFP and DCP, were measured every 2 weeks for each patient registered in the trial. The serum AFP levels were measured via an enzyme immunoassay (ST AIA-PACK AFP, Tosoh, Tokyo, Japan) and DCP was measured using a chemiluminescent enzyme immunoassay (LUMIPULSE PIVKA-II, Eisai, Tokyo, Japan). These markers were also assayed after the termination of TSU-68 treatment, usually with a longer interval.

Tumor marker doubling time

In the present analyses, we assumed that the serum levels of tumor marker are proportional to the viable tumor volume with a fixed coefficient intrinsic to an individual case, when the tumor was producing the marker in question. Independently of the coefficient, the DT values can be calculated from two data points as

$$\mathrm{DT} = \log(2) \times \frac{t_2 - t_1}{\log(C_2) - \log(C_1)}$$

where C_1 and C_2 are the serum concentrations of tumor marker at times t_1 and t_2.

When data were available at more than two points, we first performed linear regression analysis of log-transformed tumor marker levels over time to determine the slope, and the DT was then calculated as

$$\mathrm{DT} = \frac{\log(2)}{\mathrm{slope}}.$$

Note in this case that the DT becomes negative when the tumor marker levels decrease following treatment.

Tumor volume and tumor marker levels during the washout phase

The total volume of target lesions was measured via two CT examinations during the washout phase: one at 4–12 weeks before and another immediately prior to the commencement of TSU-68 treatment. The tumor volume DT was then calculated as described above. The tumor marker DT was also calculated, and the relationship between the two sets of DT values was analyzed.

Changes in the DTs during TSU-68 treatment

The serum tumor marker DT during the first 8 weeks of TSU-68 administration was similarly calculated and compared with the DT measured before the drug therapy. If TSU-68 administration had been effective, the DT should be elongated, or yield a negative value. The evaluation of drug responses based on tumor marker DT was then compared with that by the RECIST method.

Tumor marker DT after the cessation of TSU-68 treatment

When a patient was observed without any anticancer treatment for more than 4 weeks after the cessation of TSU-68 treatment and tumor marker levels were determined more than once during this period, tumor marker DTs after the cessation of TSU-68 were similarly calculated and compared with those measured at 4 weeks and immediately before the cessation of treatment. The study design we used for estimating tumor marker DT before, during, and after TSU-68 administration is summarized in Fig. 1.

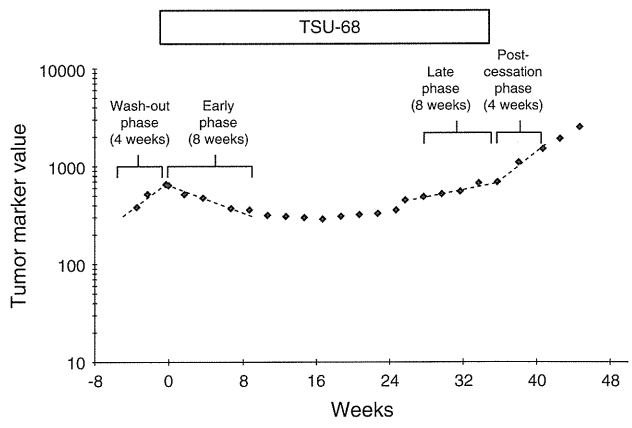

Fig. 1 Linear regression representation of the log tumor marker levels over time where DT = log(2)/slope. The slope can be calculated using a least-squares regression or two log-transformed tumor marker values: DT = log(2) × (t_2 − t_1)/[log(TM2) − log(TM1)], where t = time and TM = tumor marker level

Results

Patient characteristics

Among the 25 patients enrolled at the University of Tokyo Hospital, 15 met all of the inclusion criteria. The reasons for exclusion included insufficient tumor marker determination prior to TSU-68 administration (seven patients), results of CT prior to enrollment unavailable (one patient), no tumor marker elevation (one patient), and termination of TSU-68 administration at week 2 as a result of gastrointestinal bleeding (one patient). The baseline characteristics of the 15 patients included in the current study cohort are summarized in Table 1.

Table 1 Patient characteristics

Variable	$n = 15$
Age (years)	66.7 ± 6.3
Sex [no. (%)]	
Male	12 (80)
Female	3 (20)
Viral markers [no. (%)]	
HBs Ag+, HCV Ab−	2 (13)
HBs Ag−, HCV Ab+	13 (87)
Prior treatments[a] [no. (%)]	
TACE	13 (87)
Ablation	12 (80)
Surgery	5 (33)
Radiation	2 (13)
Systemic chemotherapy	1 (7)
Tumor stage [no. (%)[b]]	
I	0 (0)
II	0 (0)
III	7 (47)
IVa	4 (27)
IVb	4 (27)
Extrahepatic metastasis [no. (%)]	8 (53)
Portal vein thrombosis [no. (%)]	1 (7)

Plus-minus values represent the mean and standard deviation

HBs Ag hepatitis B surface antigen, *HCV Ab* hepatitis C antibody

[a] Number of pretreatments by surgery, radiofrequency ablation, transcatheter arterial chemoembolization, chemotherapy, or radiotherapy

[b] Based on the International Union Against Cancer (UICC) *TNM Classification of Malignant Tumors*, 6th edition, 2002

Tumor volume and tumor marker prior to TSU-68 administration

The relationship between the tumor volume and tumor marker DTs is shown in Fig. 2, where each point represents data from one patient. With the least-square method, the relationship was regressed to

$$y = 1.063x - 2.941$$

where x is the tumor volume DT and y is the tumor marker DT in days for both. The slope of regression was close to 1.0 with an r^2 value of 0.948, indicating that these two DTs were almost identical in each patient.

Changes in tumor marker DT during TSU-68 treatment

TSU-68 treatment was discontinued at week 4 in four patients because of the appearance of new lesions or a substantial increase in the volume of non-target lesions. The remaining 11 patients received this drug for at least 8 weeks and the response of the target lesions was evaluated in these patients as a stable disease (SD) by RECIST at week 4. Changes in the tumor marker DT before and after the commencement of TSU-68 administration are summarized in Table 2, together with the corresponding RECIST evaluation. When the tumor marker DT was increased following TSU-68 therapy, or became negative, this was considered to be an indication of at least partial drug efficacy. On the other hand, no beneficial effects were assigned to TSU-68 when the tumor marker DT was shortened following treatment. Such tumor marker-based evaluations were found to be compatible with RECIST, as a complete or partial response (CR, PR), or SD versus PD, in 12 of 15 patients. In the remaining three patients, a RECIST-based evaluation of PD was obtained in spite of an elongated tumor marker DT. In case 9, the RECIST-based evaluation became PD after cycle 2 because of the appearance of a new lesion, although the tumor marker DT was still elongated in this patient. Lymph node necrosis was observed by contrast-enhanced CT in this patient (Fig. 3), suggesting that TSU-68 remained effective. In case 10, a RECIST-based evaluation of PD was obtained because of an increase in the size of adrenal metastasis (a target lesion) although the hepatic lesions were decreased in size and the tumor marker DT was elongated. After the cessation of TSU-68 in patient 10, the left adrenal gland was excised and found to contain multiple necrotic lesions. Case 12 showed SD for the target lesion and an elongated tumor marker DT but was deemed to be a PD because of the appearance of new lesions.

In two of the other cases (nos. 1 and 2), a RECIST-based evaluation of SD was found but a negative tumor marker DT was also obtained. In case 1, the tumor marker DT became −21.1 days upon TSU-86 administration, which indicated an 84% decrease in tumor volume and 46% reduction in diameter by 8 weeks. Using RECIST parameters, a greater than 30% decrease in diameter typically corresponds to PR but case 1 was nevertheless evaluated as

SD using this system. In case 2, a tumor marker DT of −29.5 days corresponded to a decrease in diameter to 64% of baseline but the RECIST-based evaluation was SD. Importantly, necrotic lesions were found in the target tumors in both cases, possibly leading to an underestimation of anticancer effect.

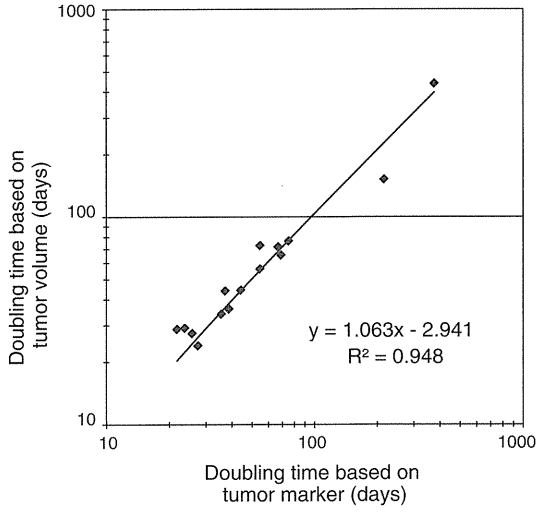

Fig. 2 Linear regression representation of the log tumor volume doubling time over the log tumor marker doubling time. *Each point* represents a data set from a single patient

Changes in tumor marker DT after the cessation of TSU-68 treatment

Changes in tumor marker DTs following the cessation of TSU-68 could be evaluated in four patients (Table 3). In each of these cases, the tumor marker DTs were elongated during TSU-68 administration compared with the baseline value and became shorter after the cessation of the treatment. Tumor marker DT after the cessation of TSU-68 was comparable with that before treatment in three patients and shorter in the remaining case.

Discussion

The production rate of a tumor marker per unit volume of the tumor mass can vary greatly among cancer patients who are positive for this marker. Hence, the serum levels of tumor marker are not directly proportional to the tumor volume. However, provided that the production rate per unit of tumor volume remains constant in each case, the changes in the serum tumor marker levels will directly correspond to the changes in tumor volume. Indeed, as we have shown in the present study, the DT of a tumor marker level and that of the corresponding tumor volume were almost identical in each patient in this study, at least during

Table 2 Tumor marker doubling time and treatment response evaluated by RECIST

Case no.	Marker	Tumor marker levels[a]		Doubling time (days)		Treatment response by RECIST
		At enrollment	At evaluation	During washout phase	During TSU-68 administration	
1	DCP	213	29	136.8	−21.1[b]	SD
2	AFP	60836	15312	38.6	−29.5[b]	SD
3	DCP	5993	5007	26.9	−231.6[b]	SD
4	AFP	144045	134030	75.3	−602.1[b]	SD
5	AFP	12004	12010	18.8	43004	SD
6	AFP	33859	33983	24.1	3010	SD[c]
7	AFP	61649	88056	71.7	115.8	SD
8	AFP	198	395	51.9	60.2	SD
9	AFP	45	92	28.0	60.2	PD
10	DCP	657	997	38.1	51.0	PD[c]
11	DCP	3188	8275	54.7	43.6	PD
12	AFP	3404	6430	24.7	32.4	PD[c]
13	AFP	53	203	88.5	15.5	PD[c]
14	AFP	19	544	376.3	12.4	PD
15	AFP	30	169	25.7	12.0	PD[c]

AFP alpha-fetoprotein, *DCP* des-gamma carboxyprothrombin, *SD* stable disease, *PD* progressive disease

[a] Unit of AFP is ng/ml and unit of DCP is mAu/ml

[b] Became negative when the tumor marker levels decreased following treatment; negative DT values correspond to the half-life

[c] Calculated using the values obtained at week 4 as treatment was discontinued at this time point. The treatment response evaluations using RECIST were also performed at week 4

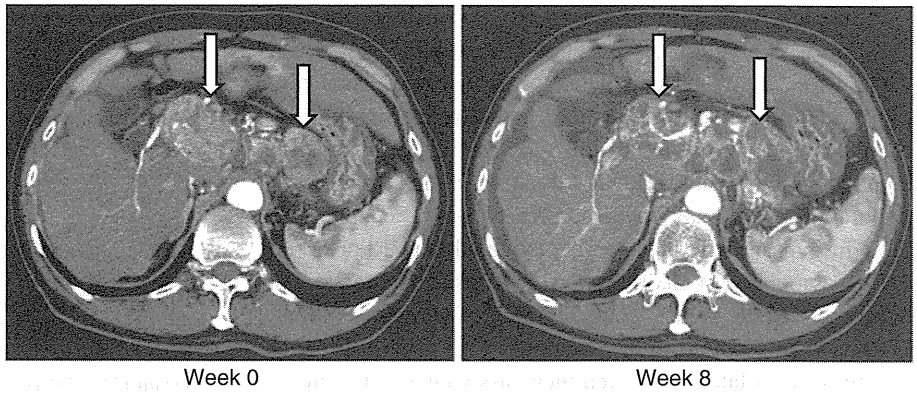

Fig. 3 A case in which a RECIST evaluation of PD was obtained even though the tumor marker levels had decreased. The lymph nodes around the hepatic arteries (target lesions, *arrows*) in this patient were enlarged and had become internally necrotic

Table 3 Tumor marker doubling time (days) before, during, and after TSU-68 treatment

Case no.	During washout phase	During the first 4 weeks of administration	During the last 4 weeks of administration	After cessation of administration
3	26.9	−97.1[b]	91.2	41.2
4	75.3	−79.2[b]	188.1	17.8
6[a]	24.1	3010	–	46.3
9	28.0	60.2	60.2	35.4

[a] Treatment was discontinued at week 4

[b] Became negative when the tumor marker levels decreased following treatment

washout phase prior to TSU-68 therapy. This indicates the possibility that tumor growth rates and any changes in them can be evaluated using tumor marker DT.

To validate the usefulness of tumor marker DTs for evaluating treatment responses, we compared this approach with the RECIST guidelines during TSU-68 administration. These two methods showed comparable results in most cases (12/15) and discrepancies were due to substantial tumor necrosis without volume shrinkage or to the appearance of new lesions in spite of the sustained effects of the drug on the target lesions. Tumor marker levels can be considered to represent viable tumor burden irrespective of the presence of necrosis or fibrosis. Evaluations based on tumor marker DTs may thus provide a better assessment of the efficacy of chemotherapeutic agents. Modified RECIST was proposed after the protocol of this study was completed. In modified RECIST, only areas with hyperattenuation were measured, excluding necrotic tissues. Modified RECIST was reported to be more useful than conventional RECIST in the evaluation of antiangiogenic agents. Although we did not directly compare tumor DT with modified RECIST in the present study, assessment based on tumor DT may be closer to modified RECIST than to conventional RECIST.

In several previous papers, early changes in AFP levels were used to assess responses to HCC treatments [30–32]. However, they evaluated only initial responses to therapy. In contrast, by evaluating tumor DT based on tumor marker levels, the effectiveness of a therapeutic agent can be monitored during its administration even when it changes over time.

In previous phase III trials of sorafenib, the response rate was not high but the overall survival was significantly improved [33, 34]. Slowing down the progression of a tumor, even if there is no reduction in the tumor volume, can therefore lead to prolonged survival. In the present study, the tumor marker DT was shortened after the cessation of TSU-68 treatment in four patients, i.e., tumor growth was accelerated, indicating that TSU-68 still inhibited tumor growth. Using RECIST evaluation, however, the treatment response in such cases will be judged as a PD, because this method does not consider time. Hence, in evaluating the response to cytostatic agents in particular, such as sorafenib and TSU-68, determination of the changes in the tumor growth rate may be substantially more adequate. Tumor marker levels can be easily measured repeatedly and, as shown in the current analysis, the corresponding DTs can thus be reliably calculated. Theoretically, the serum half-life of a tumor marker may affect the calculation of tumor DT. However, the half-life of AFP is 5 days and that of DCP is 40 h, which are much shorter than the tumor halving time even when TSU-68 is effective, and are negligible in calculations.

Another application of tumor marker DTs is the estimation of tumor growth rates when the lesions are untreated. DTs may correlate with the malignant potential of the tumor. We have shown that tumor marker DTs remained similar before the administration and after the cessation of TSU-68, which may be a characteristic of cytostatic agents in contrast to cytotoxic agents [35–37]. The decision to continue cytotoxic agents that only slow down tumor growth could be partially based on the tumor marker DT prior to treatment.

There are several limitations to the use of tumor marker DTs in the evaluation of cancer drug treatment responses. First, DTs cannot be calculated when a tumor does not produce tumor markers. Second, a tumor marker profile may change during treatment, possibly as a result of somatic mutation and clonal selection in the tumor cell population. This may make interpretation of changes in DTs difficult. Lastly, whether an elongated but still positive DT is associated with improved prognosis has yet to be confirmed. In the natural course of HCC, the tumor volume DT has been reported to be associated with prognosis [38]. We thus speculate that a treatment associated with elongation of tumor marker DT can be continued if there are no alternative treatments and the side effects are tolerable.

In conclusion, we have shown that serum tumor marker levels can be used to evaluate viable tumor burden irrespective of the presence of tumor necrosis that can compromise radiographic evaluations. This may be particularly useful in the evaluation of cytostatic agents.

Conflict of interest The authors declare that they have no conflict of interest.

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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

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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].

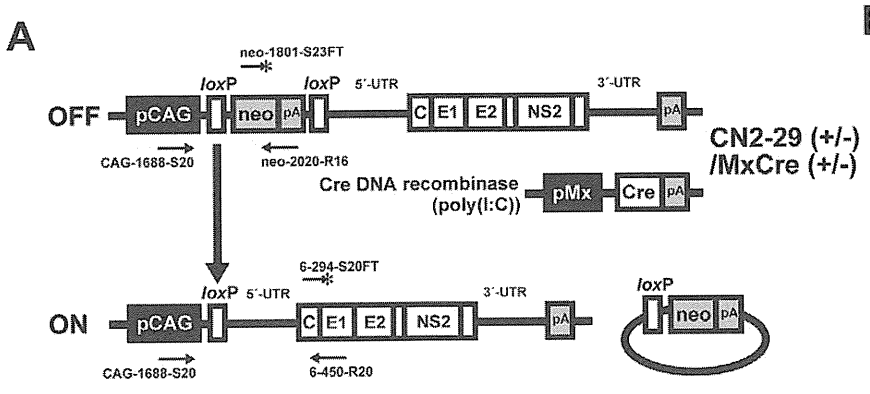
We used western blots to confirm that each HCV protein was expressed in cell lines. Each of seven proteins – the core, E1, E2, NS3-4A, NS4B, NS5A, and NS5B – was recognized and labeled by a separate cognate antibody directed (Figure S3). To induce effective immune responses against HCV proteins in transgenic 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 (+/-)
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6-294-S20FT
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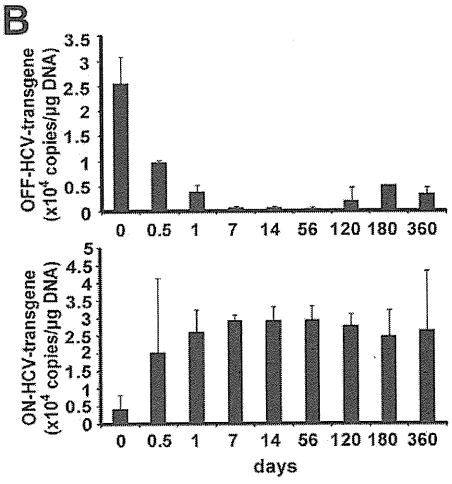
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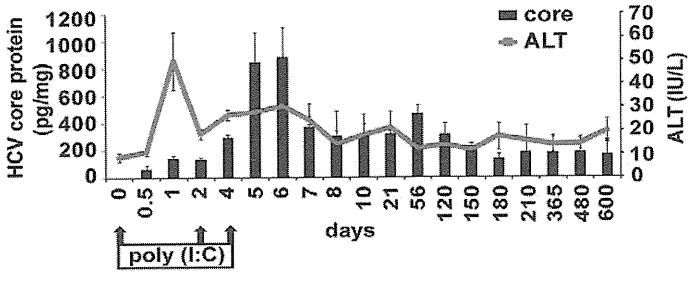
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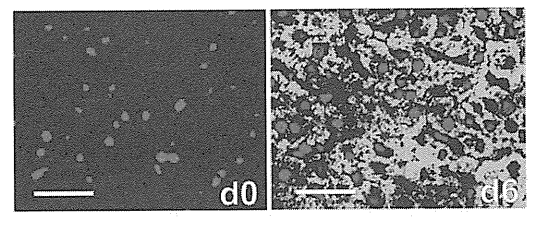
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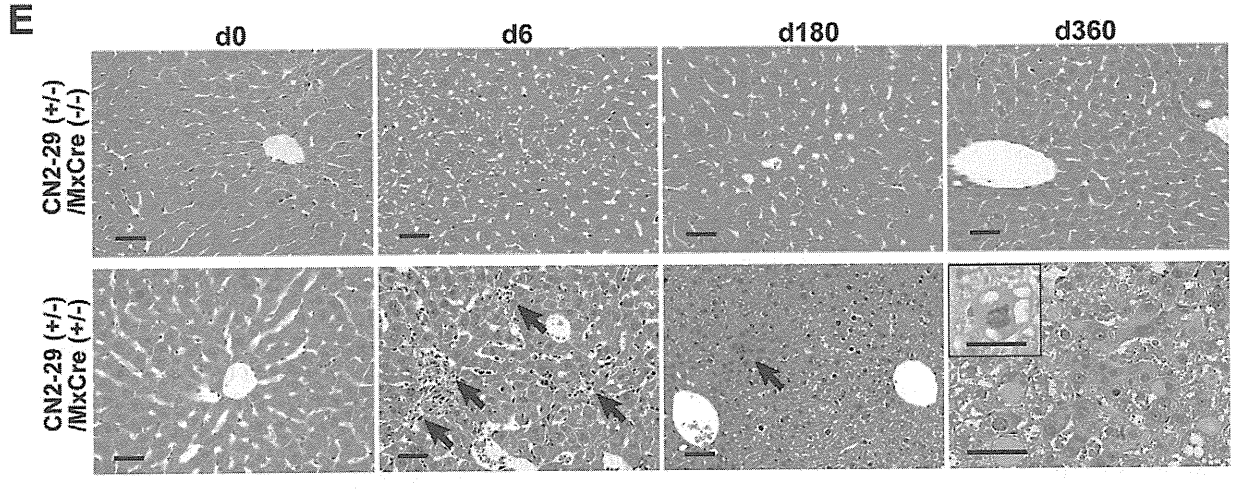
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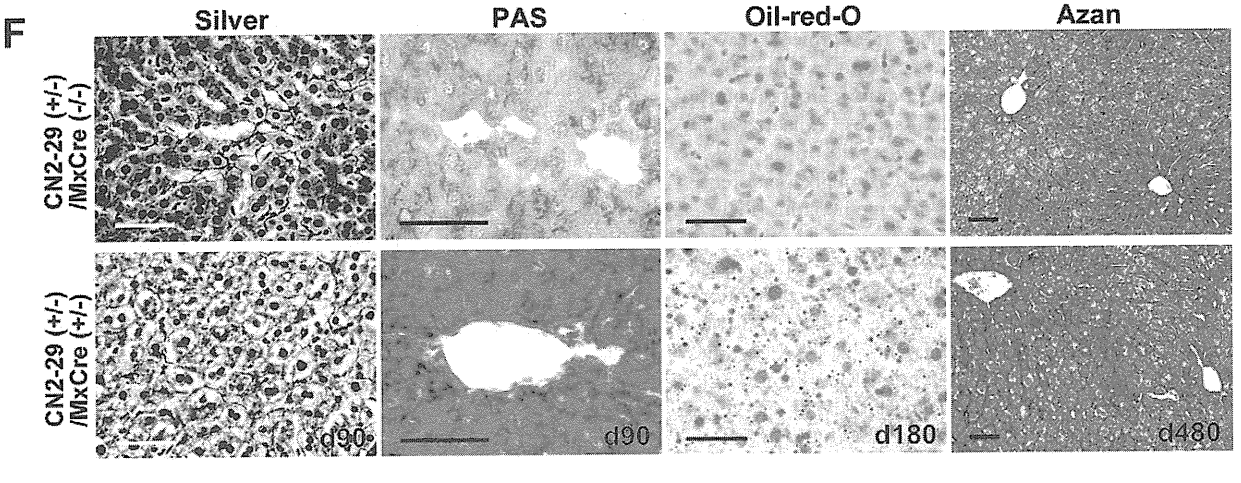
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CN2-29 (+/-)
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Figure 1. Pathogenesis in immunocompetent mice with persistent HCV expression. (**A**) Structure of CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ and the Cre-mediated activation of the transgene 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.
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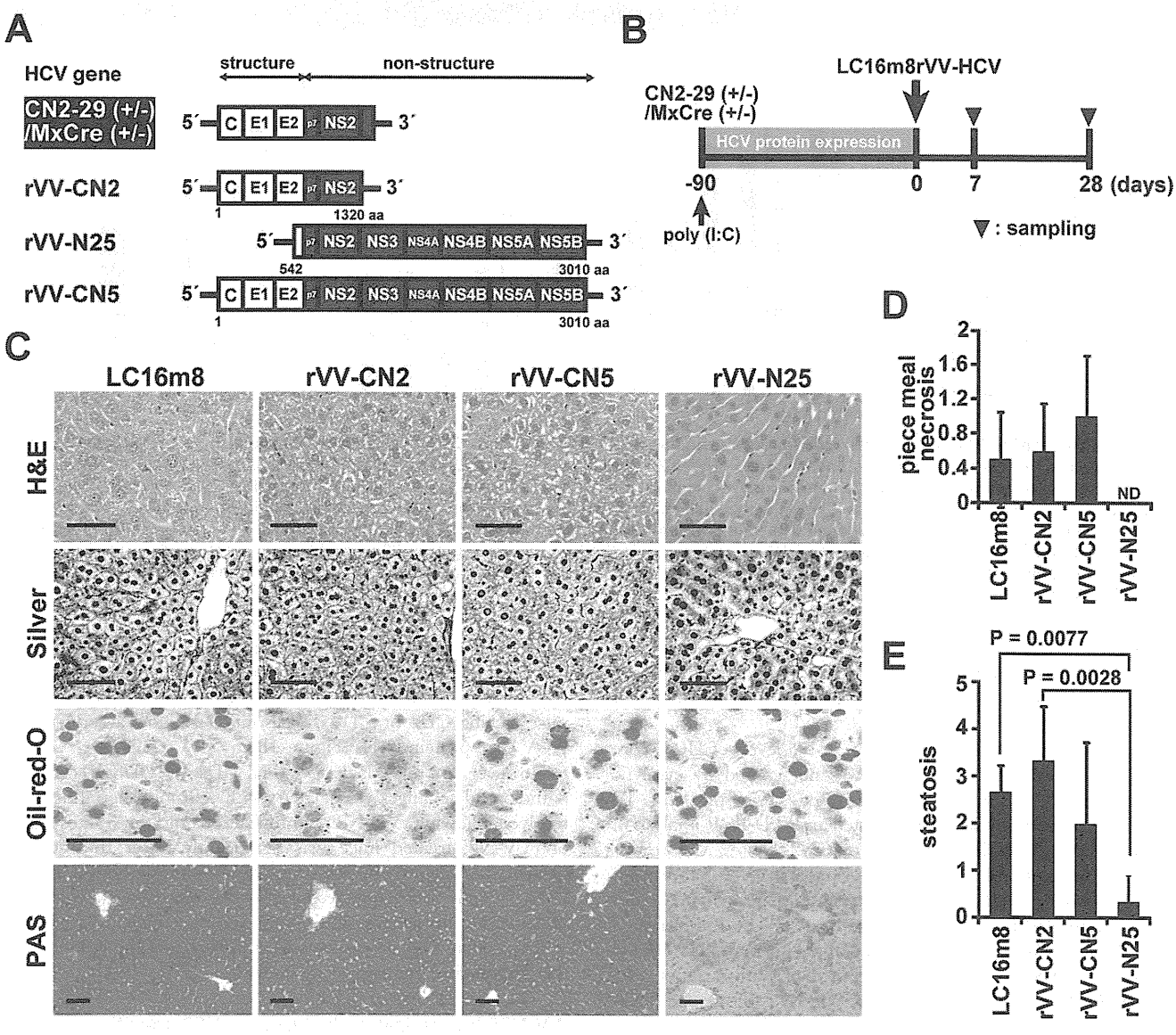

Figure 2. Effects of rVV-HCV treatment on the CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice. (**A**) HCV gene structure in the CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice and recombinant vaccinia viruses (rVV-HCV). MxCre/CN2-29 cDNA contains the core, E1, E2, and NS2 regions. The rVV-CN2 cDNA contains the core, E1,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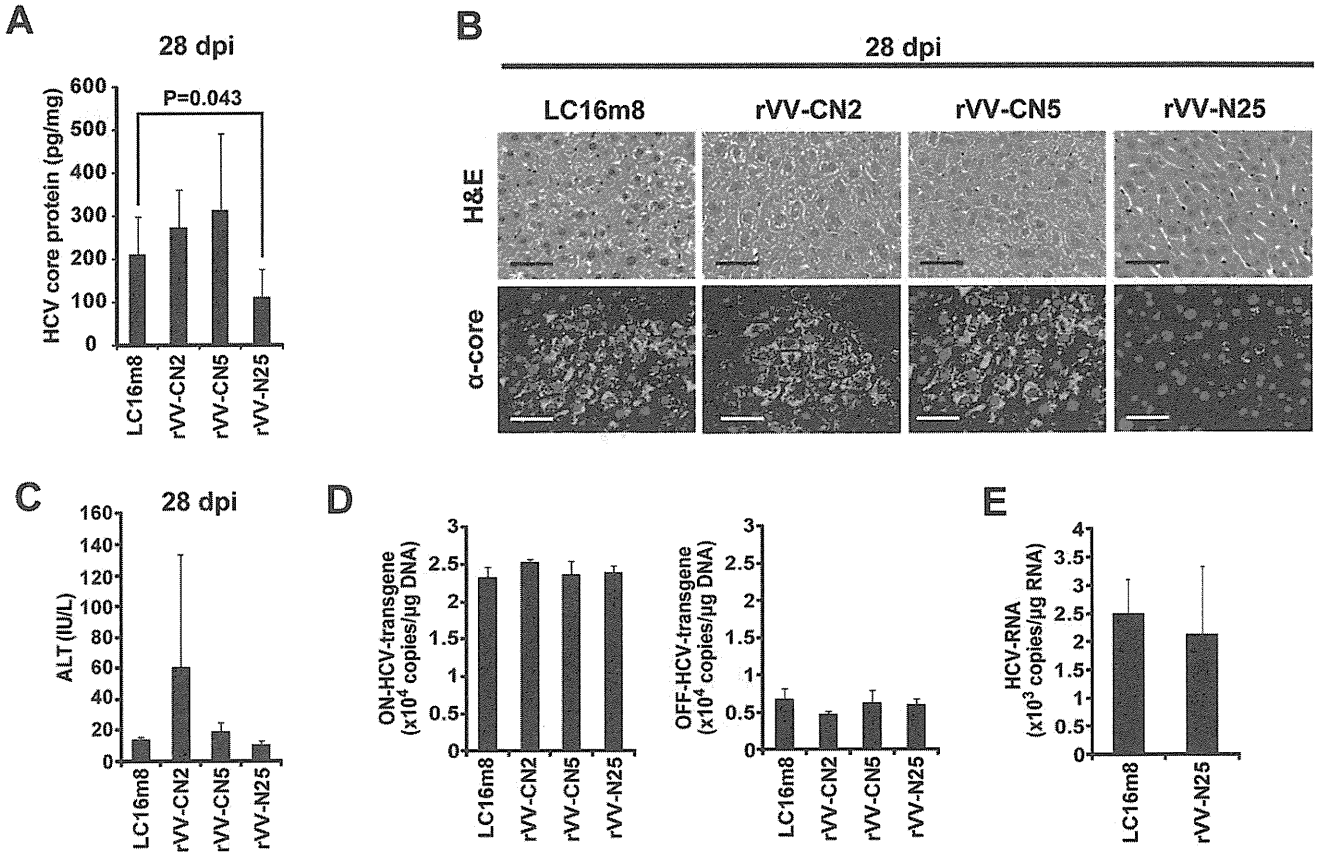

Figure 3. Effects of HCV core protein expression on the livers of CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice inoculated with rVV-HCV. (**A**) Expression of the HCV core protein in the four treatment groups of CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice 28 days after the inoculation. Significant relationships are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

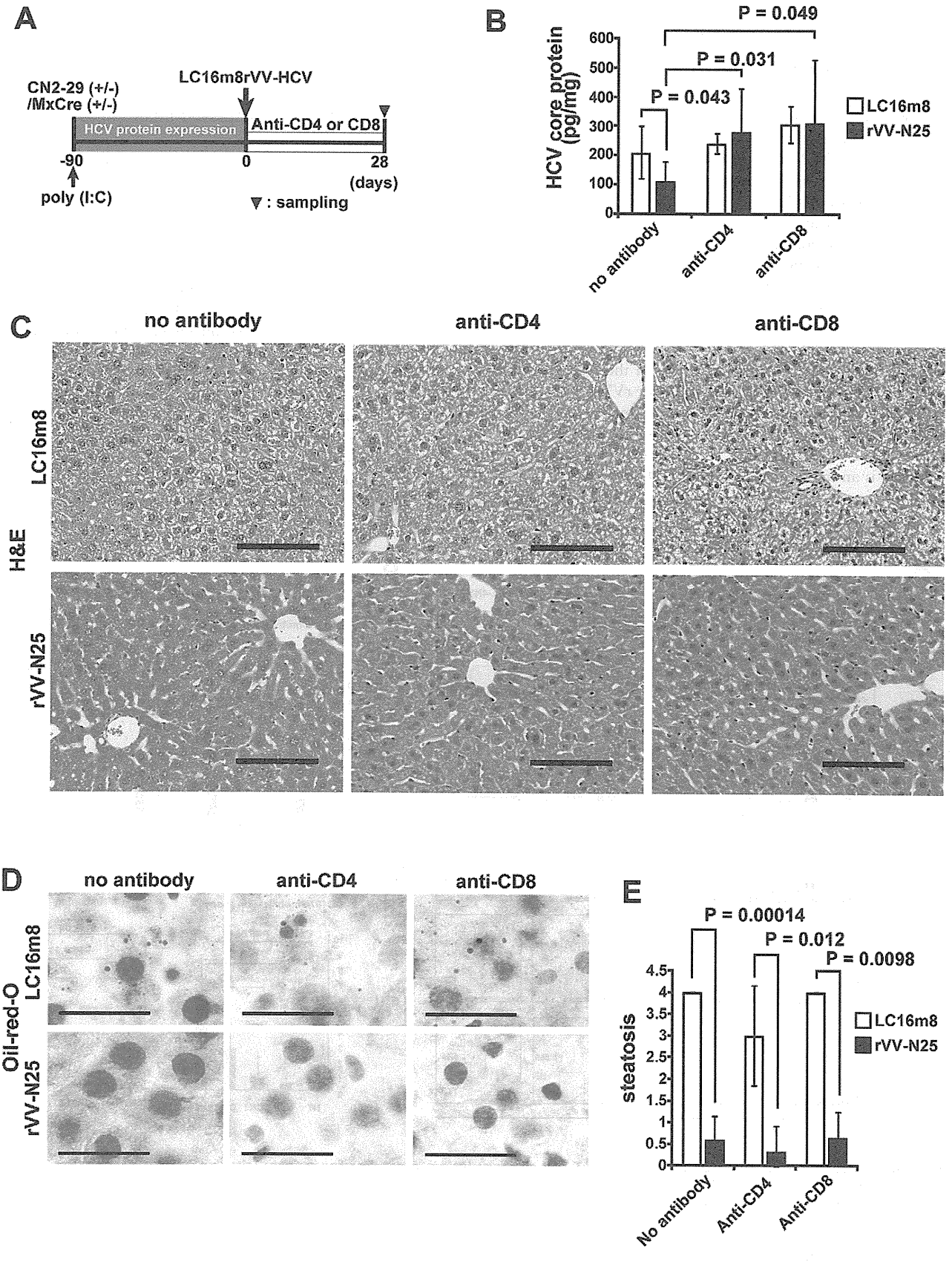

Figure 4. Role of CD4 and CD8 T cells in rVV-N25-treated mice. (**A**) Schematic diagram depicts depletion of CD4 and CD8 T cells via treatment with monoclonal antibodies. (**B**) Comparison of HCV core protein expression in control, CD4-depleted, and CD8-depleted mice 28 days after immunization with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

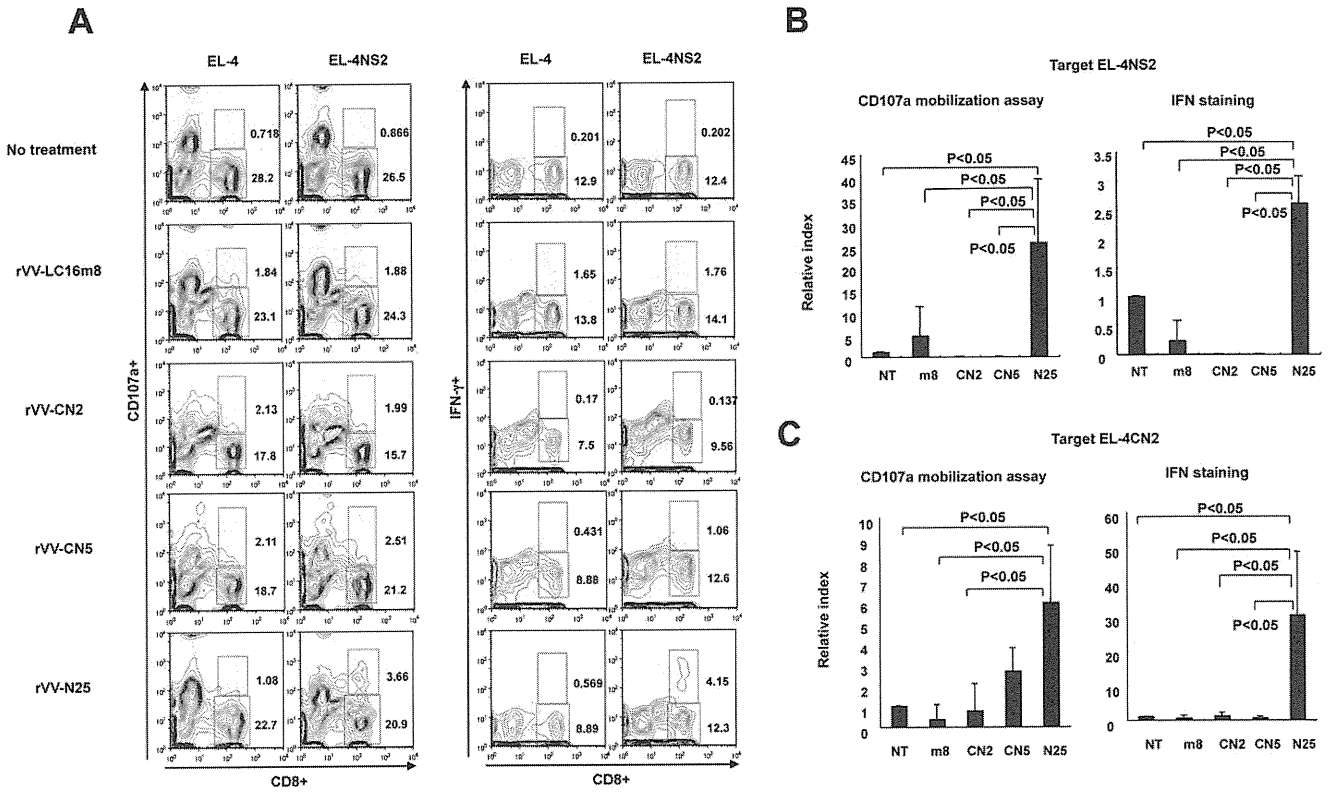

Figure 5. Immunization with rVV-N25 induced CD8 T-cell degranulation, a marker for cytotoxicity, and IFN-γ production. (**A**) The numbers represent the percentage of CD107a positive cells and negative cells (left two columns) and IFN-γ-positive cells and negative cells (right two columns). (**B, C**)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