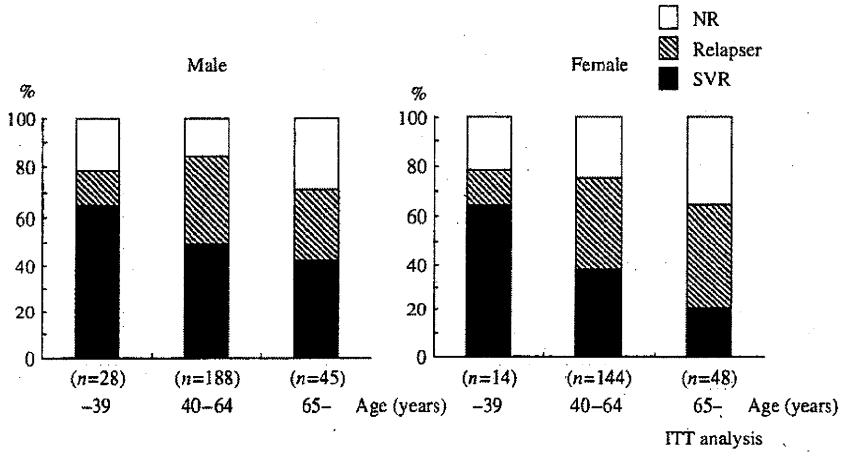

Fig. 2. A virological response to combination therapy according to the age and gender of patients with genotype 1. ITT, intention-to-treat; NR, nonresponder; SVR, sustained virological response.

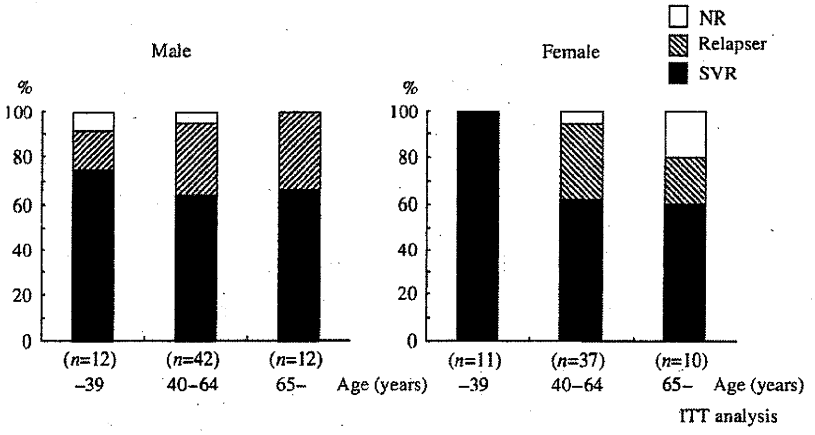

Fig. 3. A virological response to combination therapy according to the age and gender of patients with genotype 2. ITT, intention-to-treat; NR, nonresponder; SVR, sustained virological response.

Table 6. Univariate analysis of factors associated with sustained virological response in patients aged ≥65 years treated with combination therapy

	Total patients ($n=115$)	Patients who achieved a SVR ($n=43$)	Patients who did not achieved a SVR ($n=72$)	*P* value
Sex ratio (male/female)	57/58	27/16	30/42	0.0284
Age (years)	67.9 ± 2.2	67.9 ± 2.3	67.8 ± 2.1	0.7666
Body weight (kg)	56.7 ± 10.1	56.9 ± 7.1	56.5 ± 11.4	0.8417
Body mass index	22.9 ± 3.2	22.8 ± 1.9	23.0 ± 3.7	0.6980
Baseline serum ALT (IU/L)	57.7 ± 40.4	57.2 ± 41.3	58.0 ± 40.2	0.9178
GGT (IU/L)	53.3 ± 67.3	61.2 ± 98.3	48.8 ± 40.4	0.3471
Haemoglobin (g/dl)	13.7 ± 1.2	13.8 ± 1.2	13.6 ± 1.3	0.3341
Platelets ($\times 10^4/\mu l$)	16.1 ± 4.3	16.3 ± 4.7	16.0 ± 4.1	0.7412
Genotype (1/2)	93/22	29/14	64/8	0.0047
HCV RNA (kIU/ml)	1726.2 ± 1460.5	1383.6 ± 1247.0	1930.9 ± 1546.4	0.0514
Activity (A0/A1/A2/A3)	3/53/26/6	1/17/10/4	2/36/16/2	0.4132
Fibrosis (F0/F1/F2/F3/F4)	6/37/24/19/2	0/16/9/7/0	6/21/15/12/2	0.2538

ALT, alanine aminotransferase; GGT, γ-glutamyl transpeptidase; HCV RNA, hepatitis C virus RNA; kIU, kilo international units; SVR, sustained virological response.

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achieve an SVR ($P=0.0047$). The HCV load tended to be lower in patients who achieved an SVR than that in patients who did not achieve a SVR ($P=0.0514$).

In patients aged ≥65 years, the factors associated with an SVR with combination therapy were determined using multivariate analysis (Table 7). Viral load [$P=0.015$, odds ratio 1.000 (0.999–1.000)] and genotype [$P=0.022$, odds ratio 0.268 (0.087–0.830)] were significantly associated with an SVR. Gender [$P=0.052$, odds ratio 0.410 (0.166–1.009)] tended to be associated with an SVR.

To identify patients aged ≥65 years with genotype 1 (hard-to-treat population) who may benefit from combination therapy, we examined the efficacy of combination therapy according to viral load and gender (Fig. 4). Even among older male patients with high viral loads, patients with viral loads < 2 000 000 IU/ml had a significantly higher SVR than patients with viral loads over 2 000 000 IU/ml [56.7% (17/30) vs. 13.3% (2/15)] ($P=0.0094$). In contrast, there was no significant difference in the SVR rate between older female patients with viral loads < 2 000 000 IU/ml and those with viral loads over 2 000 000 IU/ml.

To evaluate the ribavirin dose during the first quarter (12 weeks) in each group of genotype 1 patients at two institutions, we calculated the percent intake of the expected dose during the first quarter. The percentage of patients who achieved a drug intake rate over 80% during the first quarter was significantly lower in elderly patients than in younger patients (75.0 vs. 88.4%; $P=0.0442$). Similarly, the patients who achieved an SVR were more likely to have a drug intake rate over 80% than patients who did not achieve an SVR (91.7 vs. 80.7%; $P=0.0464$).

Table 7. Multivariate analysis of factors associated with a sustained virological response in patients aged ≥65 years treated with combination therapy

Variable		Odds ratio (95% CI)	*P* value
HCV RNA (kIU/ml)		1.000 (0.999–1.000)	0.015
Genotype	1 vs. 2	0.268 (0.087–0.830)	0.022
Gender	Female vs. male	0.410 (0.166–1.009)	0.052

HCV RNA, hepatitis C virus RNA; kIU, kilo international units; SVR, sustained virological response.

Adverse events

The combination therapy discontinuation rate of patients aged ≥65 years was significantly higher than that of patients aged < 65 years ($P=0.0003$) (Table 2). Even when excluding genotype 1 cases in which therapy was discontinued because the virus could not be eradicated after 24 weeks, the combination therapy discontinuation rate of patients aged ≥65 years was significantly higher than that of patients aged < 65 years ($P<0.0001$). Ribavirin discontinuation was higher in older patients ($P=0.0013$). The reasons for discontinuing combination therapy and the times when therapy was discontinued are shown in Table 8. One case with a serious adverse effect occurred in each group: insulin-dependent diabetes mellitus in the younger group and bleeding from duodenal varices in the older group. The discontinuation rate because of general fatigue or anaemia was higher in older patients than that in younger patients [5.22% (6/115) vs. 1.90% (9/476) ($P=0.0418$) and 5.22% (6/115) vs. 0.63% (3/476) ($P=0.0024$) respectively].

Discussion

It is important to eradicate HCV by IFN to reduce the risk of HCC (4, 5). In addition, IFN reportedly reduces liver-related mortality in chronic hepatitis C patients over age 60 years old (11, 21, 22). However, these findings are based on studies of IFN monotherapy. The present study examined the effect of a combination of ribavirin and peginterferon. Ribavirin has been used in combination with IFN or peginterferon to treat chronic hepatitis C, and this combination therapy has been reported to be more effective than IFN monotherapy in eradicating

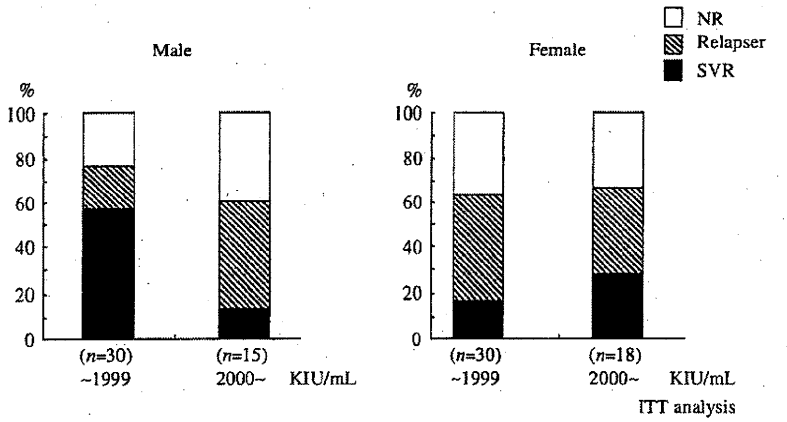

Fig. 4. A virological response to combination therapy according to virus load and gender of older patients with genotype 1. ITT, intention-to-treat; kIU, kilo international units; NR, nonresponder; SVR, sustained virological response.

Table 8. Reasons for discontinuing combination therapy

Reason	Number	Weeks after starting treatment	Reason	Number	Weeks after starting treatment
Patients aged < 65 years (n = 476)			*Patients aged ≥ 65 years (n = 115)*		
Fatigue	9	4, 8, 8, 10, 13, 20, 25, 33	Fatigue*	6	1, 4, 6, 8, 19, 32
Depression	7	1, 2, 4, 8, 13, 15, 18	Anaemia*	6	3, 8, 12, 12, 13, 15
Self-discontinuation	6	8, 16, 23, 24, 25, 28	Rash	3	1, 4, 9
Headache	3	2, 36, 37	Depression	2	2, 9
Anaemia	3	4, 11, 24	Jaundice	1	1
Rash	2	18, 25	Fiver	1	7
Hepatocellular carcinoma	2	19, 43	**Bleeding from duodenal varies‡**	**1**	**8**
Bronchitis	1	2	Anorexia	1	10
Alopecia	1	13	Hyperthyroidism	1	15
Progression of diabetes	1	14	Cholecystitis	1	16
Peritonitis due to appendicitis	1	16	Symptoms of Parkinson's disease	1	16
Fundal hemorrhage	1	17	Suspicion of interstitial pneumonia	1	20
Pneumonia	1	18	Gastric cancer	1	21
Body weight loss	1	22	Hepatocellular carcinoma	1	21
Vertigo	1	25			
Elevation of TSH	1	25			
Unknown	1	25			
Lack of funds	1	27			
Hypothyroidism	1	28			
Gastric cancer	1	38			
Insulin-dependent diabetes mellitus‡	**1**	**44**			
Reappearance of pancreatitis	1	46			
Noneradication of HCV†	34		Noneradication of HCV†	10	

*The ratio of discontinuation was significantly different between the two groups $P < 0.05$.
†Discontinued because the virus could not be eradicated after 24 weeks.
‡Serious adverse effects are shown in bold.

HCV (7–10). However, ribavirin and IFN or peginterferon in combination produce a common adverse effect, i.e. Hb levels decrease in 20–36% of treated patients with chronic hepatitis C, necessitating dose reduction or discontinuation (8, 10, 23, 24). Among elderly patients treated with combination therapy, ribavirin dose reduction is often required, resulting in a reduced SVR in older patients (15, 16). In this study, ribavirin dose reduction was higher in elderly patients than that in younger patients.

Previous studies have reported that there is no significant difference in the efficacy of IFN monotherapy between older and younger patients after normalizing for difference in background clinical characteristics, suggesting that age does not influence the outcome of IFN monotherapy (13, 14).

Adding ribavirin to IFN improves the treatment efficacy. However, ribavirin reduces Hb levels, causing greater dose reductions. Elderly patients with genotype 1 and high HCV loads have a lower SVR rate than younger patients because of a higher ribavirin dose reduction rate and discontinuation rate because of ribavirin-related anaemia (15, 16). We examined chronic hepatitis C patients with a similar background, except for age, and found that combination therapy was comparably effective between patients aged ≥60 years and those aged < 60 years, although the ribavirin discontinuation rate was higher among older patients (17). Similar results were obtained in the chronic hepatitis C patients treated with peginterferon and ribavirin, and positive responses to combination treatment were decreased for genotype 1- or 4-infected patients older than 40 years, but comparable between patients older than 65 and patients aged 40–64 years (18).

However, the background, efficacy and tolerability of peginterferon and ribavirin combination therapy in elderly patients according to gender have not been fully elucidated. Moreover, there are no data identifying which patients will achieve an SVR among older patients. Our previous report examined a 24-week regimen of ribavirin plus interferon therapy and defined advanced age as over 60 years (17). However, currently, the most common treatment protocol is prolonged ribavirin plus peginterferon-α treatment. Moreover, the patient age distribution has shifted to a more advanced age. Therefore, we need to re-evaluate an additional protocol including peginterferon and define advanced age as 65 years. We conducted a multi-institution study to evaluate the efficacy and tolerability of ribavirin plus peginterferon-α in older patients with chronic hepatitis C.

An ITT analysis indicated that the SVR rate in elderly patients was lower than that in younger patients, while a PP analysis showed that the SVR rate in elderly patients was not statistically different from that of younger

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patients. These results indicated that when treatment is not discontinued, the SVR rate of elderly patients will be high.

Multivariate analysis showed that baseline age and genotype are factors significantly associated with an SVR. Many studies have shown that baseline viral load and genotype are factors significantly associated with an SVR (8, 24). Age was associated with an SVR and the SVR rate of patients aged ≥65 years was lower than that of patients aged < 65 years (37.4 vs. 51.5%; $P=0.0067$).

Because the SVR differs according to genotype, we classified patients by genotype and compared the SVR rate for both male and female patients. In both male and female patients with genotype 1, the SVR rate decreased with age, and the SVR rate of both patients < 40 years was over 60%. These results were similar to previous studies where the SVR rate of patients < 40 years old was higher than that of another generation (17, 18, 24, 25). In patients ≥65 years old, the SVR rate of female patients was significantly lower than that of male patients [in patients with both genotype 1 and 2, 27.6% (16/58) vs. 47.4% (27/57); $P=0.0284$; in patients with genotype 1, 20.8% (10/48) vs. 42.2% (19/45); $P=0.0261$]. The result that female patients are less likely to achieve an SVR than male patients differs from that of a previous report (25). However, our results are consistent with Sezaki and colleagues, who reported that females have a poorer response to peginterferon and ribavirin combination therapy than males among patients with hepatitis C aged ≥50 years. In the older population, the gender associated with an SVR changes from male to female (26). In both male and female patients with genotype 2, the SVR of all generations was over 60%. A study by Antonucci *et al.* (18)and our previous report suggest that genotype 2 patients have a higher SVR rate, which is age-independent.

We cannot exclude the bias that better candidates were more likely to be selected among older patients than younger patients in the outpatient department. However, regardless of potential bias, elderly patients had low body weight, low Hb levels and an advanced fibrosis stage. Regarding fibrosis, elderly patients are more likely to have a long disease duration as it was reported previously that fibrosis progression was mainly dependent on age and the duration of infection (27). In this study, ITT and PP analyses indicated that the SVR rate did not differ between younger and older patients with F2–F4 fibrosis who needed treatment to prevent liver-related deaths (data not shown).

Several reports suggested that the efficacy of peginterferon and ribavirin combination therapy is lower in elderly patients with genotype 1 than in younger patients, but there are no reports establishing which elderly patients will benefit from this combination therapy. To identify genotype 1 patients ≥65 years old (hard-to-treat population) who will particularly benefit from combination therapy, we examined the efficacy of combination therapy according to viral load and gender. In older male patients with genotype 1 and HCV RNA concentrations < 2 000 000 IU/ml, the SVR rate was over 50%. Based on these results, combination therapy should be considered for male patients with genotype 1 and with HCV RNA concentrations < 2 000 000 IU/ml. Even if the treatment schedules differ between western countries and Japan, age will have to be considered, and viral load will be an important issue when treating elderly patients.

In conclusion, elderly patients in Japan who received combination therapy with peginterferon and ribavirin had a low body weight, low Hb levels and advanced fibrosis. Elderly patients had higher treatment discontinuation rates and lower SVR rates than younger patients. However, an SVR was achieved in over 50% of elderly patients with genotype 2 and in male patients with genotype 1 and HCV RNA concentrations < 2 000 000 IU/ml.

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Appendix 1

The following institutions participated in this study:

- Aihoku Hospital
- Aichi Cancer Center
- Aichi Cancer Center Aichi Hospital
- Aichi Saiseikai Hospital
- Aichi Sannomaru Hospital
- Atsumi Hospital
- Anjo Kosei Hospital
- Ichinomiya Municipal Hospital
- Ichinomiya Municipal Hospital Imaise Branch
- Inazawa City Hospital
- Ogaki Municipal Hospital
- Okazaki City Hospital
- Kainan Hospital
- Kakegawa City General Hospital
- Kamo Hospital
- Kariya Toyota General Hospital
- Gifu Social Insurance Hospital
- KumiaiKosei Hospital
- Aichi Cardiovascular and Respiratory Center
- Showa Hospital
- Tosei General Hospital
- Komaki City Hospital
- Komaki Daiichi Hospital
- Sakashita Hospital
- Saishukan Hospital
- Shizuoka Kosei Hospital
- Shizuoka Saiseikai General Hospital
- Yokkaichi Municipal Hospital
- Holy Spirit Hospital
- Kamiiida daiichi General Hospital
- Daido Hospital
- Chita City Hospital
- Chubu Rosai Hospital
- National Center for Geriatrics and Gerontology
- Tsushima City Hospital
- Tokai Memorial Hospital
- Tokai Sangyo Central Hospital
- Tokai Municipal Hospital
- Tokai Central Hospital
- Tokai Hospital
- Tohno Kousei Hospital

- Toki General Hospital
- Tokoname Municipal Hospital
- Toyota Memorial Hospital
- Toyohashi Medical Center
- Toyohashi Municipal Hospital
- Nakatsugawa Municipal General Hospital
- Nagoya Medical Center
- Nagoya Ekisaikai Hospital
- Nagoya Memorial Hospital
- Nagoya Kyouritu Hospital
- Japanese Red Cross Nagoya First Hospital
- Nishio Municipal Hospital
- Handa City Hospital
- Fukuroi Municipal Hospital
- Fujita Health University Hospital
- Brother Hospital
- Hekinan Municipal Hospital
- Mitsubishi Nagoya Hospital
- Miyoshi Municipal Hospital
- Meijo Hospital
- Meitetsu Hospital
- Yachiyo Hospital
- Yamashita Hospital

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Strong CD8$^+$ T-cell responses against tumor-associated antigens prolong the recurrence-free interval after tumor treatment in patients with hepatocellular carcinoma

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Abstract
Aim We investigated whether tumor-specific CD8$^+$ T-cell responses affect tumor-free survival as well as the relationship between CD8$^+$ T-cell responses against tumor-associated antigens (TAAs) and the clinical course after tumor treatment in patients with hepatocellular carcinoma (HCC).
Methods Twenty patients with HCC that were treated by radiofrequency ablation or trans-catheter chemo-embolization (TACE) and in whom HCC was undetectable by ultrasonography, CT, and/or MRI 1 month after treatment were enrolled in the study. Before and after treatment for HCC, analyses of TAA (glypican-3, NY-ESO-1, and MAGE-1)-specific CD8$^+$ T-cell responses were evaluated with an interferon-γ enzyme-linked immunospot (ELISpot) assay using peripheral CD8$^+$ T-cells, monocytes, and 104 types of 20-mer synthetic peptide overlapping by 10 residues and spanning the entirety of the 3 TAAs.
Results Sixteen out of 20 patients (80%) showed a positive response ($\geq$10 TAA-specific cells/10^5 CD8$^+$ T-cells) before or after treatment. When we performed univariate analysis of prognostic factors for the tumor-free period in the 20 patients, platelet count, prothrombin time, and the number of TAA-specific CD8$^+$ T-cells after treatment were significant factors ($P = 0.027$, 0.030, and 0.004, respectively). In multivariate analysis, the magnitude of the TAA-specific CD8$^+$ T-cell response ($\geq$40 TAA-specific cells/10^5 CD8$^+$ T-cells) was the only significant prognostic factor for a prolonged tumor-free interval (hazard ratio 0.342, $P = 0.022$).
Conclusions Our results suggest that strong TAA-specific CD8$^+$ T-cell responses suppress the recurrence of HCC. Immunotherapy to induce TAA-specific cytotoxic T lymphocytes by means such as the use of peptide vaccines should be considered for clinical application in patients with HCC after local therapy.

Keywords Hepatocellular carcinoma · CD8$^+$ T-cell response · Cytotoxic T lymphocyte · ELISpot assay · Immunotherapy

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Introduction

There are about 500,000 new patients with hepatocellular carcinoma (HCC) per year worldwide. Although vaccination against hepatitis B virus (HBV) and interferon (IFN)-based therapy against hepatitis C virus (HCV) will presumably reduce the number of HCC patients in the future, the incidence of HCC is still increasing in Asia and Africa because of the previous prevalence of infection with the virus. Progress in treatments for HCC has improved the prognosis of patients with HCC. However, HCC is usually associated with cirrhosis and often recurs even after complete treatment of the tumors in the remaining part of the cirrhotic liver. Thus, there is a strong need for the development of a new intervention therapy that suppresses the occurrence or recurrence of HCC effectively and that has fewer side effects. Immunotherapy may be such a treatment and may be applicable to the clinical treatment of HCC. In fact, some clinical trials have been performed [1–3].

Cytotoxic T lymphocytes (CTLs) are thought to be potent effector cells against cancers. CTLs recognize specific antigens, and the induction of CTLs specific for tumor-associated antigen (TAA) is an attractive procedure

for tumor therapy. The MAGE-1 gene was first identified as encoding a tumor-specific antigen on MZ-2-MEL cells, a melanoma cell line, in 1991 [4]. MAGE-1 gene and protein can be detected in many cancer tissues, and three articles reported the expression of MAGE-1 in HCC as 30, 68, or 78%, respectively, in a Japanese population [5–7]. In gastrointestinal tumors, immunotherapy using both dendritic cells and MAGE peptides has been performed for patients with primary malignant melanoma of the esophagus, and this therapy was able to induce peptide-specific immune responses [8].

NY-ESO-1 antigen, a member of the cancer-testis antigen family, was initially identified by a serological analysis of recombinant cDNA expression cloning in an esophageal cancer patient [9]. NY-ESO-1 mRNA was detected in 24–37% of HCCs by reverse transcription-polymerase chain reaction [10, 11].

Glypican-3 (GPC3) consists of 580 amino acids and is a heparan sulfate proteoglycan with a potential role in the control of cell division. GPC3 mRNA was detected in 74.8% of HCC tissues, but only in 3.2% of normal liver tissues [12], and GPC3 protein was detected in 72% of HCCs, but not in normal tissue using GPC-specific antibody [13]. The GPC3 protein can also be detected in sera of 40–53% of patients with HCC [14, 15].

These three antigens are thought to be attractive targets for cancer immunotherapy because they are expressed only in tumor tissues and testis, but not in normal tissues other than testis. On the basis of previous reports, it is assumed that most HCCs would express at least one of the three TAAs. Therefore, monitoring immune responses against these TAAs might help in the development of HCC immunotherapy, such as TAA-based vaccination. In this study, we investigated how the magnitude of $CD8^+$ T-cell responses against these TAAs determined by an IFN-γ enzyme-linked immunospot (ELISpot) assay is related to other clinical data and the tumor-free interval in patients with HCC, in order to explore the clinical application of such a TAA-based immunotherapy.

Methods

Patients

Twenty patients who were diagnosed with HCC at Showa University Hospital between 2006 and 2008 were enrolled in the study. They met the following study criteria: (1) pathologically confirmed as having HCC or a lesion with characteristic imaging features of HCC based on ultrasonography, CT, and/or MRI; (2) liver function classed as Child-Pugh A or B; (3) no extrahepatic metastasis or vascular invasion; (4) no previous or simultaneous cancers other than HCC; and (5) an indication for treatment such as radiofrequency ablation (RFA) or trans-catheter chemo-embolization (TACE). RFA was performed by well-trained hepatologists using usual methods according to previous reports [16]. A 16-gauge cooled-tip ablation electrode (Covidien, Boulder, CO) was used in the procedure. TACE was performed by well-experienced hepatologists and radiologists. A microcatheter was inserted from the femoral artery to the artery feeding the HCC superselectively after conventional hepatic angiography, and then a segmental or subsegmental TACE procedure was performed using gelatin, lipiodol, and either epirubicin hydrochloride or cisplatin. All patients were followed every 1–3 months by ultrasonography, CT, and/or MRI to examine the appearance of new lesions in the liver or other organs. The recurrence-free interval was defined as the period from the month of HCC treatment to the month when a recurrent and/or metastatic HCC was first detected after treatment. Clinical data (platelet count, prothrombin time, serum AST, ALT, albumin, total bilirubin level, and AFP level) were collected 1–7 days before HCC treatment. Chronic hepatitis C was diagnosed on the basis of detectable HCV RNA in serum using the Amplicor assay (Roche Diagnostics, Tokyo, Japan). Informed consent was obtained from each patient included in this study. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the Ethical Committee of Showa University.

Synthetic peptides of TAA

Twenty-mer peptides overlapping by 10 residues and spanning the entire MAGE-1, NY-ESO-1, and GPC3 proteins were synthesized based on the amino acid sequences reported previously as PepSets™ and purchased from Mimotopes (Clayton South, Victoria, Australia). These peptides were >80% pure. A total of 30 MAGE-1, 17 NY-ESO-1, and 57 GPC3 peptides were synthesized, as shown in Table 1. A total of 10–11 TTA peptides were pooled in a mixture (total 10 mixtures) at a concentration of 10 μg/ml each.

Preparation of $CD8^+$ T cells and monocytes from patients with HCC

PBMCs were isolated from heparinized peripheral blood by gradient centrifugation using Ficoll-Paque (Pharmacia-LKB Biotechnology, Uppsala, Sweden). As reported previously, peripheral $CD8^+$ T-cells and monocytes were separated from PBMCs using CD8 microbeads (MACS system; Miltenyi Biotec, Bergisch Gladbach, Germany) and a Monocyte Isolation Kit II (Miltenyi Biotec), respectively [17]. These cells were isolated using an autoMACS™ Pro Separator (Miltenyi Biotec). The purity of the cells was >95% on flow cytometry (data not shown).

Table 1 Synthetic peptides and peptide mixtures used in this study

Tumor-associated antigen	Peptide	Amino acid sequence
Glypican-3	GL1	1–20
	GL2	11–30
	GL3	21–40
	⋮	⋮
	GL57	561–580
MAGE-1	MG-1	1–20
	⋮	⋮
	MG-30	290–309
NY-ESO-1	NY-1	1–20
	⋮	⋮
	NY-17	161–180

Mix 1	Mix 2	Mix 3	Mix 4	Mix 5	Mix 6	Mix 7	Mix 8	Mix 9	Mix 10
GL1	GL2	GL3	GL4	GL5	GL6	GL7	GL8	GL9	GL10
GL11	GL12	GL13	GL14	GL15	GL16	GL17	GL18	GL19	GL20
GL21	GL22	GL23	GL24	GL25	GL26	GL27	GL28	GL29	GL30
GL31	GL32	GL33	GL34	GL35	GL36	GL37	GL38	GL39	GL40
GL41	GL42	GL43	GL44	GL45	GL46	GL47	GL48	GL49	GL50
GL51	GL52	GL53	GL54	GL55	GL56	GL57	MG-1	MG-2	MG-3
MG-4	MG-5	MG-6	MG-7	MG-8	MG-9	MG-10	MG-11	MG-12	MG-13
MG-14	MG-15	MG-16	MG-17	MG-18	MG-19	MG-20	MG-21	MG-22	MG-23
MG-24	MG-25	MG-26	MG-27	MG-28	MG-29	MG-30	NY-1	NY-2	NY-3
NY-4	NY-5	NY-6	NY-7	NY-8	NY-9	NY-10	NY-11	NY-12	NY-13
NY-14	NY-15	NY-16	NY-17	–	–	–	–	–	–

IFN-γ ELISpot assay

The ELISpot assay was performed using an IFN-γ ELISpot assay kit (Mabtech AB, Stockholm, Sweden) as previously described [17]. Briefly, a 96-well microtiter plate with a nitrocellulose membrane bottom (Millititer; Millipore, Bedford, MA) was coated with 100 μl anti-IFN-γ monoclonal antibody at a concentration of 15 μg/ml in phosphate-buffered saline (PBS) overnight at 4°C. Unbound antibody was removed by washing 6 times in Hanks' balanced saline solution. After blocking with AIM-V medium (Invitrogen Japan, Tokyo, Japan) containing 10% fetal bovine serum, 1×10^5 $CD8^+$ T-cells, 1×10^4 autologous monocytes, and a TAA peptide mixture at 10 μg/ml of each peptide were placed and incubated in duplicate in 100 μl AIM-V medium at 37°C in a humid atmosphere with 5% CO_2. After incubation for 18 h, the cells were removed by washing the plate 8 times with PBS. Next, 100 μl of biotin-conjugated monoclonal antibody was added to each well, and the plates were incubated further for 2 h at room temperature. Wells were washed 5 times with PBS and incubated with 100 μl streptavidin–alkaline phosphatase for 2 h. Unbound antibodies were removed by washing 6 times with PBS. Then, 100 μl of alkaline phosphatase substrate (Bio-Rad Laboratories, Richmond, CA) was added to each well and incubated until dark spots emerged. Color development was stopped by washing 3 times with water, and the plates were allowed to dry. Using an ELISpot reader (KS ELISPOT compact; Carl Zeiss, Oberkochen, Germany), the number of spot-forming cells (SFCs) per well was counted. Numbers of TAA-specific SFCs for each peptide mixture were calculated by subtracting the mean number of SFCs of 2 control wells (without stimulus) from the mean number of SFCs of 2 wells stimulated by TAA antigens. An SFC number was calculated for each patient as the sum of SFCs in each peptide mixture. ELISpot assays were performed before and 3–7 days after treatment. When TAA-specific $CD8^+$ T-cell responses were analyzed in 10 normal subjects, we were unable to detect any responses against TAA peptides in the ELISpot assay (data not shown).

Statistical analyses

The relationship between the number of TAA-specific $CD8^+$ T-cells and the recurrence-free period was analyzed

using a parametric survival model. The log-rank test was used to compare recurrence-free data for 2 groups. The effects of multiple explanatory variables on recurrence-free interval were analyzed using a Cox proportional hazards model. Statistical analyses were performed using the statistical software JMP version 5 (SAS Institute Inc., Cary, NC). Differences were considered as significant when the P value was less than 0.05.

Results

TAA-specific CD8$^+$ T-cells were detected by ELISpot assay before and after HCC treatment in most HCC patients

The characteristics of the 20 patients enrolled in this study are shown in Table 2. The 20 patients had no HCC detected by ultrasonography, enhanced CT, and/or MRI 1 month after treatment for HCC. In those patients with HCCs who had up to 3 HCCs and in whom the diameter of each lesion was 3 cm or less, the treatment was usually RFA; the remaining patients were treated by TACE. However, in a few patients (patients 2 and 5) in whom the diameter of each lesion was less than 3 cm, the physician in charge of the patient selected TACE because they could not deny the existence of more lesions that were undetectable by conventional enhanced CT. The clinical courses of the patients were followed for 3–29 months after therapy for HCC. The ELISpot assay was performed to detect CD8$^+$ T-cell responses to TAAs before and 3–7 days after treatment. The data are shown in Table 3 as SFCs (total count of TAA-specific CD8$^+$ T-cells/1 $\times$ 10^5 CD8$^+$ T-cells). Sixteen out of 20 patients (80%) showed a positive response (10 or more SFCs) for TAA peptides either before and/or after treatment. The numbers of SFCs (mean $\pm$ SD) before and after therapy were 33.8 $\pm$ 51.4 (0–161, median 16.5) and 32.9 $\pm$ 34.7 (0–130, median 23.0), respectively. Of the 20 patients, 5 (25%) and 7 (35%) showed a high TAA-specific immune response (40 or more SFCs) before and after treatment, respectively.

When we analyzed the TAA peptides recognized by CD8$^+$ T-cells, we occasionally observed that different peptide mixtures were identified as positive before and after HCC treatment (data not shown).

Change in TAA-specific CD8$^+$ T-cell response induced by HCC treatment does not correlate with recurrence-free period

The number of SFCs increased in 11 of 20 (55%) patients after treatment. In these patients, TAA-specific CTLs might have been induced by the treatment. There were no significant differences between the increase in TAA-specific CD8$^+$ T-cell response induced by the treatment and either therapeutic procedure, laboratory data, or background of the patients (data not shown). The increase in TAA-specific CTLs after treatment did not predict a better prognosis of HCC.

Table 2 Characteristics of HCC patients before HCC treatment

	$n = 20$	Median
Age (years)[a]	68.8 ± 9.4	73.0
Gender		
M	11	
F	9	
AST (IU/l)[a]	70 ± 49	52
ALT (IU/l)[a]	63 ± 43	54
PLT ($\times 10^4/\mu l$)[a]	9.8 ± 5.3	8.5
PT (%)[a]	81 ± 11	78
Alb (g/dl)[a]	3.5 ± 0.4	3.4
T-Bil (mg/dl)[a]	0.9 ± 0.4	0.9
AFP (ng/ml)[a]	86 ± 157	16
Virus		
HCV	17	
NBNC	3	
Child-Pugh class		
A	12	
B	8	
HCC size (mm)[a]	23 ± 8	23
No. HCCs		
1	9	
2	4	
3	7	
>3	0	
Treatment		
RFA	13	
TACE	5	
RFA + TACE	2	

NBNC Negative for neither HBV nor HCV infection, *RFA* radiofrequency ablation, *TACE* trans-catheter chemo-embolization

[a] Results are shown as mean ± SD

Platelet count, prothrombin time, and the magnitude of TAA-specific immune response after treatment correlate with the recurrence-free period by univariate analysis

When we analyzed the relationship between TAA-specific SFCs detected by the ELISpot assay or other clinical variates and the HCC-free interval using a parametric survival model, we found that platelet count, prothrombin time, and the TAA-specific CD8$^+$ T-cell response after treatment significantly correlated with the HCC-free interval

Table 3 Results of IFN-γ ELISpot assay in patients in whom HCCs were not detected after therapy

Patient no.	SFC before treatment (/10^5 $CD8^+$ T-cells)	SFC after treatment (/10^5 $CD8^+$ T-cells)	Recurrence-free interval (month)
1	0	0	5
2	15	31	10
3	12	15	5
4	159	130	26
5	58	4	12
6	5	99	29[a]
7	15	17	7
8	20	41	7
9	135	9	12
10	1	6	3
11	8	9	6
12	10	57	15
13	34	42	13[a]
14	6	4	12[a]
15	23	8	9
16	59	37	12
17	12	29	23
18	161	72	24
19	18	4	15
20	25	44	23[a]

SFC Spot-forming cells

[a] These patients had no recurrence detected by ultrasonography, enhanced CT, and/or MRI after treatment

($P = 0.005$, 0.007, and 0.001, respectively). When univariate analysis of prognostic factors for the HCC-free interval was performed, only platelet count ($P = 0.027$; Fig. 1a), prothrombin time ($P = 0.030$; Fig. 1b), and the number of SFCs after treatment ($P = 0.004$; Fig. 1c) were found to be significant. Child-Pugh class A tended to prolong the HCC-free interval, although this was not significant ($P = 0.066$). The other factors, including the number of SFCs before treatment ($P = 0.407$), ALT level ($P = 0.644$), albumin level ($P = 0.488$), total bilirubin level ($P = 0.340$), HCC size ($P = 0.756$), HCC number ($P = 0.486$), and the procedure used for HCC treatment (RFA or TACE, $P = 0.481$), did not affect HCC-free survival, as confirmed by the log-rank test.

Multivariate analysis shows that the magnitude of TAA-specific $CD8^+$ T-cell responses correlates with the HCC-free interval after treatment in patients who have no detectable HCC after therapy

In a further analysis of the 20 patients with HCC who were treated by RFA or TACE and in whom no HCC was detectable 1 month after treatment, we performed multivariate analysis using a Cox proportional hazards model. On multivariate analysis, only the magnitude of TAA-specific $CD8^+$ T-cell responses (≥40 TAA-specific cells/10^5 $CD8^+$ T-cells) was the only significant prognostic factor for a prolonged tumor-free period after treatment

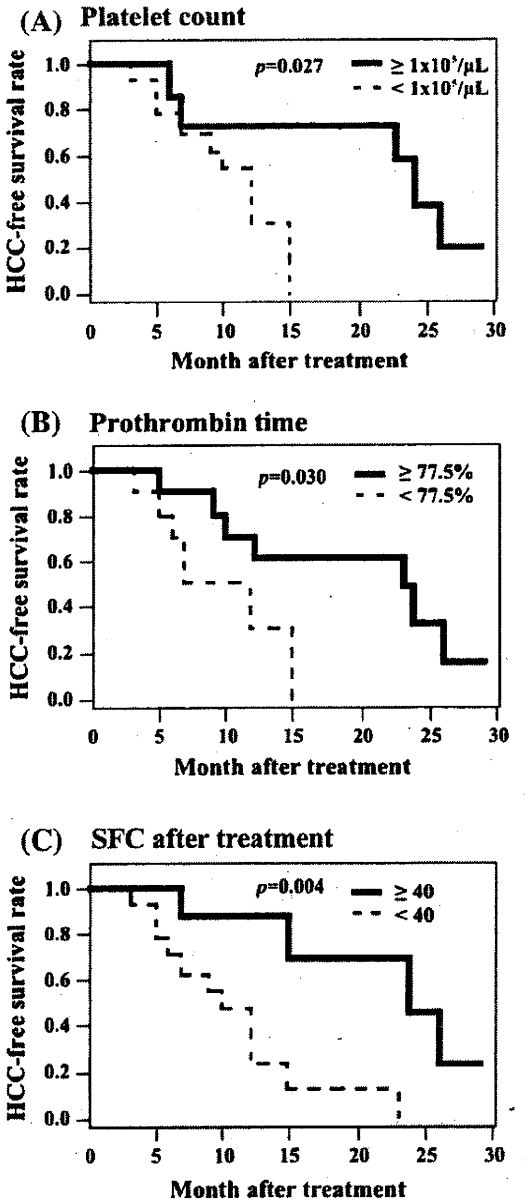

Fig. 1 Kaplan–Meier curves of HCC-free survival rate. In univariate analysis, platelet count, prothrombin time, and the tumor-associated antigen-specific $CD8^+$ T-cell response were found to be prognostic factors for the HCC-free period after treatment. Kaplan–Meier curves representing the relationship between month after treatment (HCC-free interval) and HCC-free survival rate were grouped by **a** platelet count, **b** prothrombin time, and **c** spot-forming cells (*SFCs*) specific for tumor-associated antigens after treatment

Table 4 Multivariate analyses of prognostic factors for tumor-free interval

Variable	Hazard ratio	95% Confidence limit	*P* value
Platelet count			
$\geq 1 \times 10^5/\mu L$	0.916	0.326–2.020	0.843
$<1 \times 10^5/\mu L$	1.000		
Prothrombin time			
≥77.5%	0.455	0.094–1.390	0.177
<77.5%	1.000		
Child-Pugh class			
A	1.464	0.539–6.813	0.493
B	1.000		
Spot-forming cells after treatment			
≥40	0.342	0.079–0.866	0.022
<40	1.000		

(hazard ratio 0.342, $P = 0.022$), as shown in Table 4. Therefore, the results suggest that TAA-specific CTLs detected after treatment are able to suppress the occurrence or recurrence of HCC in patients with no detectable HCCs after treatment.

Discussion

To determine whether TAA-specific CTLs suppress the occurrence or recurrence of HCC, we investigated the relationship between the magnitude of TAA-specific $CD8^+$ T-cell responses and the HCC-free interval in patients who had no detectable viable HCC 1 month after treatment for HCC. We found that potent TAA-specific $CD8^+$ T-cell responses, as observed 1 month after treatment for HCC, led to a prolonged HCC-free interval.

An HLA-A24-restricted MAGE-1 peptide-specific CTL line was established in a patient with metastatic melanoma [18], and an NY-ESO-1 DNA vaccine induced both antigen-specific effector $CD4^+$ and/or $CD8^+$ T-cell responses in most patients who did not show detectable pre-vaccination immune responses [19]. In addition, HLA-A2- and HLA-A24-restricted GPC3-derived peptide vaccine induced specific CTLs in mice [20]. In this study, we selected GPC3, MAGE-1, and NY-ESO-1 to monitor antigen-specific $CD8^+$ T-cell responses against HCC because they had been reported to be expressed commonly and frequently in HCC tissues [7, 11–13], and thus the combination of these TAAs would cover most HCCs. Among the 20 patients enrolled in the present study, 16 (80%) showed positive $CD8^+$ T-cell responses (10 or more SFCs) against the TAAs before and/or after the treatment. Although we did not examine the expression of TAAs in the HCC tissues, it would be expected that at least one of these three TAAs will be expressed in HCCs in patients who have a positive $CD8^+$ T-cell response against TAAs.

In patient 10, HCC recurrence was detected 3 months after treatment. Insufficient treatment or the pre-existence of intrahepatic metastases might be considered in a patient in whom HCCs are undetectable 1 month after treatment, but are detected within a few months after treatment. We expected that TAA-specific CTLs induced by treatment would suppress the development of a small HCC, which is not easily detected by conventional methods of examination. Thus, we enrolled and analyzed all patients in whom no HCC was detectable by ultrasonography, CT, and/or MRI 1 month after treatment, even if a recurrent or metastatic HCC was detected within a few months after treatment.

It is of interest whether tumor destruction by local HCC treatment would induce immune responses against HCCs. Apoptotic tumor cells are capable of inducing tumor-specific immune responses [21]. Dendritic cells, representing antigen-presenting cells, around damaged tumor cells take up tumor antigen released from the tumor cells and then migrate into draining lymph nodes [22]. There, they mature and stimulate tumor-specific helper T-cells and CTLs. In turn, the effector cells migrate into the tumor tissue and attack the tumor cells [23]. Tumor-specific immune responses were induced by a combination of direct dendritic cell injections into the HCC and radiation therapy that might induce tumor destruction [3]. When we compared TAA-specific $CD8^+$ T-cell responses before HCC treatment and those after treatment, about half of the patients (55%) showed an increased frequency of TAA-specific $CD8^+$ T-cells, which might have been induced by the treatment. However, the increase in TAA-specific CTLs did not affect the recurrence-free interval. Rather, it was the magnitude of TAA-specific $CD8^+$ T-cell responses after the treatment itself that affected the recurrence-free interval. Even if the frequency of these CTLs seemed to be decreased after treatment, they might infiltrate the liver. Furthermore, new CTLs other than pre-existing CTLs might be induced by the treatment because many TAA peptides recognized by CTLs were different between before and after the treatment. Although some patients showed a potent TAA-specific $CD8^+$ T-cell response before treatment, SFC before treatment did not correlate with the recurrence-free interval. We believe that TAA-specific CTLs are not able to control a large tumor burden by itself. As HCCs enlarge, they may secret immune suppressive factors such as TGF-β [24] and/or IL-10 [25] and modify gene expression of TAAs [26]. We speculate that TAA-specific CTLs detected after the treatment, but not detected before the treatment may be able to control HCCs. Otherwise, TAA-specific CTLs detected before the

treatment may be able to destroy a small HCC that was not detected by conventional examinations.

The ELISpot assay is a convenient means of detecting antigen-specific $CD8^+$ T-cells in a variety of diseases. We have detected HCV-specific $CD8^+$ T-cell responses in patients with acute HCV infection using this method and identified 6 new epitopes within the HCV protein [17]. In fact, we identified a novel GPC3-specific CTL epitope using this method (unpublished observation). At present, we are trying to identify more CTL epitopes among these TAAs that will be used as cancer vaccines.

In this study, we used peptide mixtures to stimulate $CD8^+$ T-cells. This procedure may mask responses to individual peptides because a peptide that interacts only weakly with HLA molecules is unable to attach to the molecule if the mixture contains 1 peptide with a high affinity for the same molecule. However, such a weak peptide would not contribute to tumor immune responses because of its weak interaction with the HLA molecules. Thus, we ignored this issue in this study.

Recurrence and multicentric carcinogenesis are major factors in determining the prognosis of HCC, and several treatments have been tried for the prevention of recurrence. IFN therapy [27, 28], treatment with acyclic retinoid therapy [29, 30], and adoptive immunotherapy [31] have been reported as effective in suppressing HCC recurrence. Preoperative hepatic function influenced early HCC recurrence in patients in whom small HCCs were resected [32]. This is consistent with our result that prothrombin time, reflecting hepatic function, affected the recurrence-free interval in the univariate analysis. In our study, higher platelet counts also contributed to a longer recurrence-free interval in the univariate analysis. In the multivariate analysis, however, only the magnitude of TAA-specific $CD8^+$ T-cell responses remained as an independent factor contributing to a longer recurrence-free interval.

Although the size and number of HCCs were reported to affect the period of HCC-free survival (recurrence) in patients with HCC treated by hepatic resection [33], they are not significant factors affecting the recurrence-free interval. Further investigation, such as the accumulation of analyses of HCC patients, is needed to clarify this issue. Sixteen out of 20 patients without detectable HCC 1 month after treatment had recurrent or metastatic HCCs during the observation period in this study. Our results suggest that the maintenance of strong TAA-specific $CD8^+$ T-cell responses for a long period may lead to a longer recurrence-free state. A long-term observation of TAA-specific immune responses should also be performed in any future study.

The results of our study suggest that strong TAA-specific $CD8^+$ T-cell responses would suppress HCC recurrence in patients with HCC who are treated by RFA or TACE and in whom any HCC is undetectable by ultrasonography, CT, and/or MRI 1 month after treatment. Since recurrence and intrahepatic metastasis are major risk factors influencing the prognosis of patients with HCC, immunotherapy to induce TAA-specific $CD8^+$ T-cells, such as a peptide vaccine, should be considered for clinical application in patients with HCC after local therapy.

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Review Article

Immune Response of Cytotoxic T Lymphocytes and Possibility of Vaccine Development for Hepatitis C Virus Infection

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Immune responses of cytotoxic T lymphocytes (CTLs) are implicated in viral eradication and the pathogenesis of hepatitis C. Weak CTL response against hepatitis C virus (HCV) may lead to a persistent infection. HCV infection impairs the function of HCV-specific CTLs; HCV proteins are thought to actively suppress host immune responses, including CTLs. Induction of a strong HCV-specific CTL response in HCV-infected patients can facilitate complete HCV clearance. Thus, the development of a vaccine that can induce potent CTL response against HCV is strongly expected. We investigated HCV-specific CTL responses by enzyme-linked immuno-spot assay and/or synthetic peptides and identified over 40 novel CTL epitopes in the HCV protein. Our findings may contribute to the development of the HCV vaccine. In this paper, we describe the CTL responses in HCV infection and the attempts at vaccine development based on recent scientific articles.

1. Introduction

Hepatitis C virus (HCV) was first identified in 1989 [1]. The HCV is a member of the flavivirus family and is a type of positive-strand RNA virus. The discovery of HCV contributed to the diagnosis of hepatitis C; further, HCV has been implicated in many chronic non-A and non-B hepatitis infections. This virus spreads through needles used for vaccination or drug administration, and about 180 million people in the world are presumed to be infected with HCV. It has been clarified that HCV infection often persists, causing chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC).

Cytotoxic T lymphocyte (CTL) plays a part in viral eradication [2]. These cells have been also implicated in the immunopathogenesis of viral infection [3], because HCV, by itself, does not produce cytopathic effects in hepatocytes directly. It has been thought that hepatitis is caused by the destruction of HCV-infected hepatocytes by immune cells such as natural killer (NK) cells and CTLs. Thus, the investigation of the roles of CTL in immunopathogenesis of HCV would contribute to the development of a new treatment strategy for HCV-induced hepatitis.

Interferon (IFN) therapy alone or with ribavirin and polymerase/protease inhibitor combination therapy has shown positive outcomes in more than 80% of patients with acute HCV infection and 50% of patients with chronic HCV infection. However, IFN causes severe adverse effects including flu-like symptoms, pancytopenia, hyperglycemia, depression, lung fibrosis, and cerebral bleeding. Therefore, there is an urgent need to establish an alternative therapy, which can afford a high rate of sustained virological response and performed with few adverse effects. Immunotherapy with HCV vaccine is one of the candidates of such therapies.

In this review, we have summarized the findings of recent investigations on CTL responses against HCV and the trials for the development of HCV vaccine.

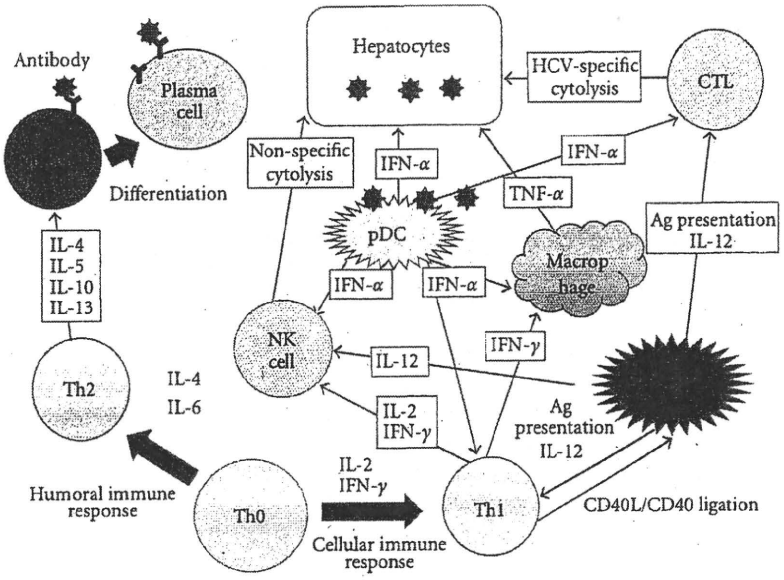

FIGURE 1: Cellular and humoral immune responses in HCV infection. Plasmacytoid dendritic cells (pDC) recognize HCV infection and produce IFN-α, which activates natural killer (NK) cells, helper T (Th) cells, macrophages, and cytotoxic T lymphocytes (CTLs). Activated NK cells destroy the HCV-infected hepatocytes in a nonspecific manner, whereas CTLs destroy the infected hepatocytes in an antigen-specific manner. Myeloid dendritic cells (mDC), which recognize dead hepatocytes, secrete IL-12, promoting the activation of NK cells, Th1 cells, and CTLs. Activated Th1 cells, in turn, promote DC maturation by interacting with the CD40/CD40 ligand. Macrophages stimulated by type 1 helper T (Th1) cells produce TNF-α, which accelerates local inflammation. In humoral immune responses, Th2 cells activate B cells. Plasma cells differentiated from B-cells secrete immunoglobulins to neutralize the circulating HCV. Abbreviated terms: CTL, cytotoxic T lymphocyte; pDC, plasmacytoid dendritic cells; mDC, myeloid dendritic cells; Th1 cell, type 1 helper T cell; Th2 cell, type 2 helper T cell; NK cell, natural killer cell; IFN, interferon; IL, interleukin; TNF, tumor necrosis factor.

2. CTL Responses in HCV Infection

2.1. Innate Immune Responses in HCV Infection. HCV infection induces cellular and humoral immune responses (Figure 1). Similar to other viral infections, nonspecific immune responses are induced in the early stages of HCV infection for the eradication of HCV. Type I IFNs produced by HCV-infected hepatocytes and plasmacytoid dendritic cells (DCs) suppress viral replication by inducing enzymes such as 2′–5′ oligoadenylate synthetase (OAS) and RNA-dependent protein kinase (PKR) in hepatocytes [4]. The plasmacytoid DC recognizes HCV infection through toll-like receptor (TLR)-7, which interacts with single-stranded RNA [5]. The TLR-signaling upregulates PDC-TREM molecules on the cell surface, and PDC-TREM-dependent signal induces further production of IFN-α [6]. Activated OAS destroys viral RNAs, whereas PKR inhibits forming polysome of viral mRNA [4]. Moreover, type I IFNs activate innate immunity components such as natural killer (NK) cells [7]. The local inflammation further activates natural killer T-cells (NKT cells) and macrophages (Kupffer cells), thereby inducing the production of cytokines such as IFN-γ and tumor necrosis factor (TNF)-α. Hepatitis is thought to be initiated in this manner, and specific immune responses are generated if innate immune responses fail to eradicate HCV.

2.2. HCV-Specific Immune Responses and Immunopathogenesis of HCV-Specific CTLs. The process of HCV-specific CTL induction and the destruction of HCV-infected hepatocytes by CTLs are shown in Figure 2. The destruction of HCV-infected hepatocytes releases HCV fragments; these fragments are taken up by myeloid DCs, consequently activating the DCs. These DCs migrate to the draining lymph nodes and express HCV antigens on human leukocyte antigen (HLA) class II molecules. Then, they enhance expression of costimulatory molecules (CD80, CD86) that interact with and activate antigen-specific helper T (Th) cells [8]. In turn, the activated Th cells promote the maturation of DCs by the expression of CD40 ligand and TNF-α. Subsequently, mature DCs stimulate specific CTLs by antigen presentation on HLA class I molecule and enhance the expression of costimulatory molecules [8]. Cytokines such as IL-2 and IL-12 produced by Th1 cells and DCs further promote CTL activation. These CTLs infiltrate the liver and recognize HCV antigens presented on the surface of HCV-infected hepatocytes together with HLA class I molecules. Then, the effector CTLs release perforin, granzyme, and TNF-α, or express Fas ligand, and initiate a direct attack on HCV-infected hepatocytes [9, 10]. In the previous study, we demonstrated that Fas ligand and TNF-α can also destroy noninfected hepatocytes in the vicinity of the HCV-infected cells [11].

When appropriate CTL responses are induced in hosts, HCV eradication is achieved. However, HCV-specific CTL responses are usually not strong enough to eradicate the virus, hence contributing to persistent infection. On the

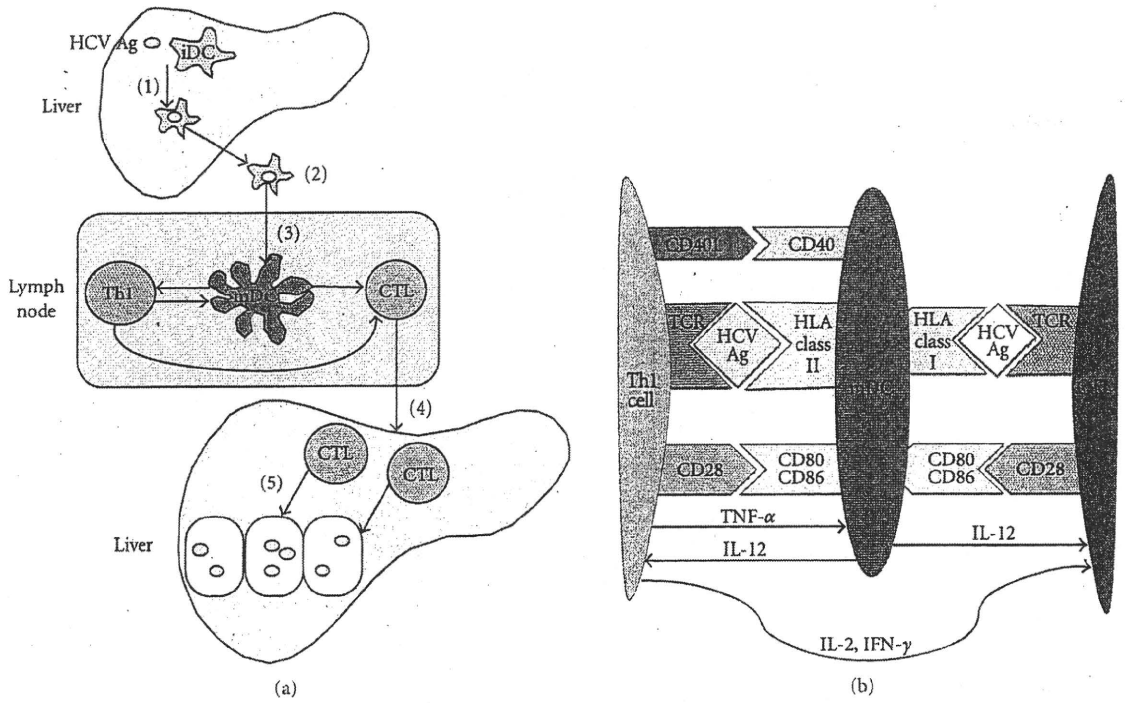

FIGURE 2: Destruction of HCV-infected hepatocytes by CTLs. (1) Immature myeloid dendritic cells (iDC) take up hepatitis C virus antigens (HCV Ag) in the liver. (2) The DCs move to a draining lymph node. (3) Matured DCs activate naïve helper T (Th) cells efficiently through stimulation with HLA class II, costimulatory molecules (CD80 and CD86), and cytokines such as IL-12. The stimulated Th cells, in turn, activate DCs by expressing CD40 ligand and secreting TNF-α. IL-12 produced by myeloid DCs differentiates these stimulated Th cells towards Th1 cells. Naïve cytotoxic T lymphocytes (CTLs) recognize the HCV Ag presented on the DCs. IL-2 and IFN-γ secreted by activated Th1 cells induce the activation and proliferation of the HCV-specific CTLs. (4) The stimulated HCV-specific CTLs leave the lymph nodes and move toward the liver. (5) They recognize HCV antigens together with HLA class I on the surface of HCV-infected hepatocytes, and try to eradicate HCV by killing the infected hepatocytes. Abbreviated terms: Th1 cell, type 1 helper T cell; mDC, myeloid dendritic cells; CTL, Cytotoxic T lymphocyte; CD, cluster of differentiation; CD40L, CD40 ligand; TCR, T-cell receptor; HLA, human leukocyte antigen; TNF, tumor necrosis factor; IL, interleukin; IFN, interferon.

other hand, markedly potent immune responses would lead to severe hepatitis and fulminant hepatitis as proven in a hepatitis B virus (HBV) model [12], although this is a rare event in HCV infection.

We evaluated the relation between HCV-specific CTL responses and the clinical course of acute HCV infection and found that HCV eradication cannot be predicted on the basis of a strong $CD8^+$ T-cell response [13]. However, Lauer et al. reported that potent and broad CTL responses against HCV peptides were observed in patients with resolved infection but not in those with persistent infection [14]. Another report indicated that patients with complete resolution of HCV infection exhibited broader CTL responses with higher functional avidity and wider cross-recognition ability than patients with persistent HCV infection [15]. The opposite observations can be attributed to the differences in the monitoring methods of the CTL responses. Race and HCV genotype might also affect the contradiction of the results. Further investigation is needed to clarify this issue.

We analyzed the immune response of chronic HCV patients by studying their HLA-B44-restricted CTLs that recognized the HCV core amino acid residues 88–96; the CTL response and viral load were found to be inversely correlated [16]. The findings of this study suggested that HCV-specific CTLs may inhibit HCV replication. Otherwise, as many reports have suggested that HCV protein impairs the CTL responses by several mechanisms (see Section 3), HCV infection with a high titer of HCV RNA may suppress the HCV-specific CTLs by an excess of HCV antigens. No relation between other CTL responses recognizing other HCV epitopes and the HCV status was found in the study. From the data, it was supposed that the HLA-B44-restricted CTLs recognizing HCV core amino acid residues 88–96 were immunodominant.

Hence, there is a need to investigate HCV-specific CTL responses and clarify some issues. First, HCV exists as quasispecies in hosts and it has a high replicative ability and low fidelity RNA polymerase [17]. Thus, many HCVs with mutations in different amino acid sequences in the epitopes may be present in the host. Other issue is that most HCV-specific CTLs may infiltrate and compartmentalize in the host liver where inflammation occurs, and thus, only a few circulating HCV-specific CTLs can be detected. Although it is very crucial to investigate liver-infiltrating CTLs, the difficulty associated with obtaining liver specimen limits such study.

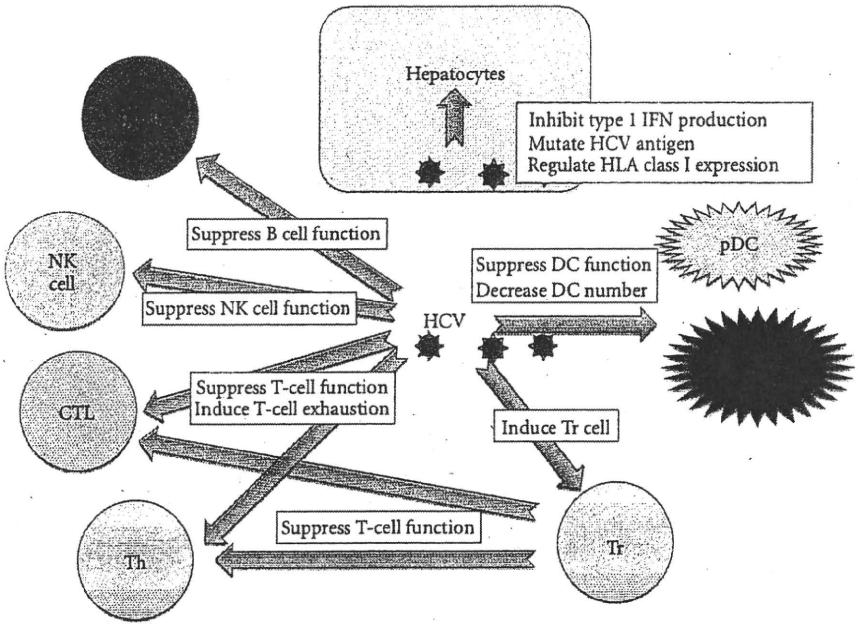

FIGURE 3: Immune suppressive mechanisms in HCV infection. HCV mutates its amino acid sequence to escape from immune surveillance, inhibits type 1 IFN production, and suppress NK cell function, T-cell function, and DC function. In addition, HCV induces Tr cells, which suppress T-cell function. Abbreviated terms: CTL, Cytotoxic T lymphocyte; pDC, plasmacytoid dendritic cells; mDC, myeloid dendritic cells; Th cell, helper T cell; NK cell, natural killer cell; IFN, interferon.

3. Immunosuppression in HCV Infection

3.1. Escape from Immune Surveillance of Cellular Immune Responses. It was reported that amino acid mutations have been detected in the immunodominant regions of HCV in all patients with acute HCV infection, and mutations by which HCV escapes from CTL surveillance have been observed only in patients with viral persistence [18]. Hughes et al. investigated the variable intensity of purifying selection on CTL epitopes, and reported that the purifying selection of CTL epitopes on nonenvelop proteins was strong, particularly when the epitope was matched [19]. Since a variety of CTLs are induced in the early stage of HCV infection, a single amino acid mutation within a CTL epitope does not appear to contribute to persistent infection. It is supposed that escape mutation is a result rather than a cause of persistent HCV infection.

3.2. Impaired Function of CTL in HCV Infection. HCV inhibits cellular immune responses in the host by several ways; immune suppressive mechanisms in HCV infection are summarized in Figure 3.

In our study, the stimulation of peripheral blood lymphocytes of HCV-infected patients with synthetic peptides corresponding to CTL epitopes revealed that patients who were infected with HCV within the past 3 years exhibited CTL responses, while those infected with HCV more than 10 years ago did not exhibit this response. There are some reasons why HCV persistence is so common although a variety of HCV-specific $CD8^+$ T-cells can be detected in the liver and peripheral blood. The impaired function of HCV-specific CTLs as effector cells is due to the reduced expression of CD3 ζ chain [20], defective IFN-γ production, low perforin content, and decreased capacity for proliferation and cytotoxicity [21]. Incomplete differentiation of the memory CTLs to effector cells in patients with acute HCV infection may be due to IL-2 deficiency during T-cell activation [22]. Programmed cell death 1 (PD-1) receptor, the ligation of which inhibits the function of effector T-cells, is upregulated on exhausted $CD8^+$ cells in patients with acute and chronic hepatitis C [23–25]. Another inhibitory receptor, namely, cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), has also been reported to be upregulated on $PD\text{-}1^+$ T-cells in the liver of HCV patients. The blockade of both these molecules is critical for the restoration of the function of HCV-specific effector cells [26].

Accumulated data have suggested that HCV itself actively suppresses host immune responses. Although spontaneous liver disease did not occur in mice expressing liver-targeted HCV NS5A transgene, both innate and adaptive immune responses were impaired [27]. HCV core protein inhibits IL-2 and IL-2 receptor α gene transcription [28], T-cell activation and proliferation, and IFN-γ production by T cells [29, 30]. HCV NS4A/B protein blocks the expression of HLA class I molecules [31].

Impaired function of DCs, which play the crucial role of antigen-presenting cells in inducing immunity, may be responsible for the impaired immune responses. It has been reported that the HCV core, E1, and NS3 proteins inhibit DC maturation [32, 33]. HCV is thought to infect DCs through the binding of HCV E2 protein and thereby suppress

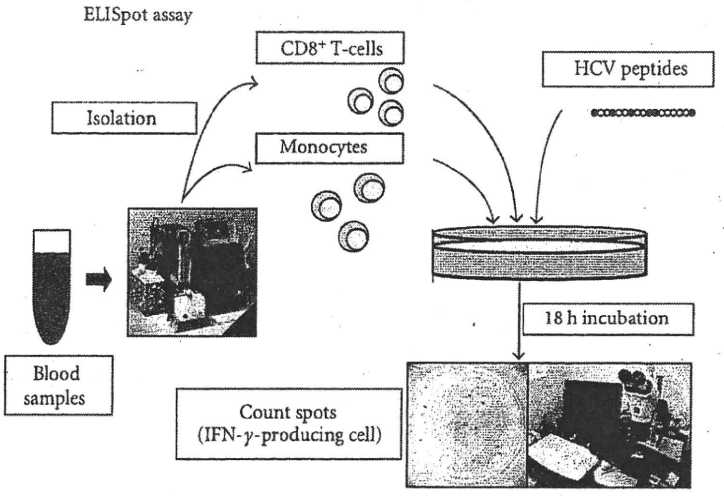

FIGURE 4: The procedure of enzyme-linked immuno-spot (ELISpot) assay. To detect CTL responses against HCV, we performed IFN-γ-based ELISpot assay. $CD8^+$ cells and monocytes were separated from peripheral blood samples by magnetic beads (MACS system; Miltenyi Biotec, Bergisch Gladbach, Germany) and used as effector cells and antigen-presenting cells, respectively. These cells with synthetic HCV peptides were incubated for 18 h at 37°C in 5% CO_2 atmosphere. Using an ELISpot reader (KS ELISPOT compact; Carl Zeiss, Oberkochen, Germany), the number of spot-forming cells (SFCs) per well was counted.

DC function [34, 35]. In addition, long-term ethanol consumption impairs CTL responses to HCV protein and subsequently alters DC function [36].

Regulatory T- (Tr) cells are also involved in HCV persistence. It has been shown that Tr cells ($CD4^+$ $CD25^+$ T cells) directly suppress T-cell function in chronic hepatitis C patients [37]. Forkhead box P3 (FOXP3)-positive Tr cells and IL-10 producing HCV-specific Tr cells infiltrate the liver of chronic HCV patients, and IL-10 mediates immune suppression in these patients [38, 39]. HCV core-specific Tr cells can be induced from the peripheral blood of patients with chronic hepatitis C [40].

4. Immunotherapy for Hepatitis C

4.1. IFN Therapy and Immune Response. Currently, chronic HCV infection can be resolved only with IFN-α-based therapy. IFN-α has been reported to have biologic effects on the immune system [41]. IFN-α upregulates HLA class I molecules on the cell surface. This cytokine appears to favor the proliferation of type 1 Th cells and activate CTLs. Ribavirin, which is used in combination with IFN-α, exerts an antiviral effect that drives the Th2 response towards a Th1 response [42]. During the primary immune response, IFN-α promotes both clonal expansion and survival of antigen-specific CTLs in vivo [43]. We also demonstrated that IFN-α prevents activation-induced cell death of CTLs [44]. A low dose of IFN-α augments cellular immune response, whereas a high dose suppresses CTL response [45]. Recently, it has been reported that although IFN-α upregulates MHC class I expression on hepatocytes, it reduces their sensitivity to CTL cytotoxicity, which may be due to the enhancement of granzyme-B inhibitor-proteinase inhibitor 9 (PI-9) expression [46]. Although it has been reported that intrahepatic and peripheral HCV-specific CTL activity was detected more often in patients with a sustained response to IFN therapy than in patients who relapsed or did not respond to the treatment [47], further study is needed to clarify the effect of IFN therapy on host immune responses in vivo.

4.2. Identification of Novel Epitopes Recognized by HCV-Specific CTLs. As described above, we first identified an HLA B44-restricted CTL epitope [48, 49]. Then, we tried to identify more novel CTL epitopes in the HCV polyprotein, and performed IFN-γ-based enzyme-linked immuno-spot (ELISpot) assay [50, 51]. The procedure of this assay is presented in Figure 4. We synthesized 297 20-mer peptides overlapping by 10 residues and spanning the entire HCV sequence based on the amino acid sequence of HCV [13]. After separation with magnetic beads, we used $CD8^+$ T-cells as effector cells and monocytes as antigen-presenting cells. After the $CD8^+$ T-cells were incubated with the monocytes and the synthetic HCV peptides for 18 hours, IFN-γ-producing cells were counted. This procedure enabled to minimize the IFN-γ production for nonspecific response. Then, we identified more than 20 CTL epitopes in the HCV protein by using the synthetic peptides (Table 1). Furthermore, our group has identified several epitopes of HCV-specific CTLs using synthetic peptides and recombinant vaccinia viruses [52].

The HLA-24 allele of HLA class I is more common among the Japanese population. Thus, CTL induction by synthetic peptides based on HLA-A24 binding motifs has been investigated mainly in Japan [53]. HCV NS5A 2132–2142 peptide corresponding to the HLA-A24 binding motif has been reported to be able to induce both cellular and humoral immune responses in most HCV-positive patients

Table 1: CTL epitopes identified by using different procedures.

(a) CTL epitopes identified by peptides overlapping by 10 residues and spanning the entire HCV sequence of genotype 1b

	HLA class I alleles	Region	Amino acid residues	Sequence	HLA restriction
Pt1	A*0207,2601 B*3501,4601 CW*0102,0303	NS3	1527–1546	WYELTPAETTVRLRAYLNTP	B*3501? A*2601?
		NS5B	2591–2605	KMALYDVVSTLPQAV	A*0207?
Pt2	A*2402,3303 B*4403,5401 CW*0803,1403	E1	332–351	LVVSQLLRIPQAVVDMVAGA	B*5401?
		NS3	1638–1656	THPITKFVMACMSADLEVV	B*5401?
		NS5B	2591–2605	KMALYDVVSTLPQAV	n.d.
Pt3	A*2602,3101 B*5101,5102 Cw1402,1502	NS3	1373–1380	IPFYGKAI	B*5101? B*5102?
Pt4	A*2402 B*0702,5201 Cw*0702,1202	E2	611–618	YPYRLWHY	n.d.
Pt5	A*1101,3101 B*6701,5101 Cw*0702,1401	NS5A	2290–2298	RPDYNPPLL	B*6701? B*5101?
Pt6	A*2402,2601 B*4002 Cw*0304	NS2	957–964	RDWAHAGL	B37
		NS5A	2122–2130	FTELDGVRL	n.d.
Pt7	A*2402,3303 B*0702,3501 Cw*0303,0702	Core	91–110	LGWAGWLLSPRGSRPSWGPT	A*3303? B*3501?
Pt8	A*2402 B*4801,5201 Cw*0803,1202	NS3	1643–1656	KFVMACMSADLEVV	n.d.
Pt9	A*2402 B*5201 Cw*1202	NS4	1760–1768	FWAKHMWNF	A*2402
		NS5B	2556–2564	TIMAKNEVF	n.d.
		NS5B	2803–2811	LTRDPTTPL	n.d.
Pt10	A*0201,0301 B*4402,4601 Cw*0102,0501	NS4	1958–1977	KRLHQWINEDCSTPCSGSWL	n.d.
Pt11	A*1101,2601 B*1501,5201 Cw*0401,1202	NS4	1858–1867	GVAGALVAFK	A*1101?
Pt12	A*2402 B*3501,4002 Cw*0303,0304	NS3	1618–1626	LHGPTPLLY	A*2402?

(b) CTL epitopes identified by HCV-derived synthetic peptides with binding motif of HLA-A24 [51]

	HLA class I alleles	Region	Amino acid residues	Sequence	HLA restriction
Pt13	A*2402,1101 B*3902,5201 Cw*0702,1202	NS3	1375–1385	FYGKAIPIEAI	n.d.
Pt14	A*2402,2601 B*4006,5401 Cw*0801,0803	E1	284–293	VFLVSQLFTF	n.d.
		E2	790–798	LYGVWPLLL	Cw*0801
		NS4	1759–1768	AFWAKHMWNF	n.d.
		NS5A	1990–1999	DFKTWLQSKL	n.d.
		NS5A	2280–2288	KFPPALPIW	A*2402
Pt15	A*2402,2601 B*3501,4002 Cw*0303,0304	NS2	910–919	PYFVRAQGLI	Cw*0303, 0304
		NS2	947–956	TYVYDHLTPL	B*4002
		NS3	1243–1252	AYAAQGYKVL	Cw*0303, 0304
Pt16	A*0206,2402 B*5201,5901 Cw*0102,1202	NS3	1443–1451	GFTGDFDSV	A*0206
Pt17	A*2402,3101 B*4801,5101 Cw*0304,0801	E2	790–798	LYGVWPLLL	Cw*0801
Pt18	A*2601,3101 B*3501,5101 Cw*0303,1402	NS5B	2456–2466	VYSTTSRSASL	n.d.

(c) CTL epitopes identified by peptides overlapping by 10 residues and spanning the entire HCV sequence [13]

	HLA class I alleles	Region	Amino acid residues	Sequence	HLA restriction
Pt19	A*2602,3101 B*5101,5102 C*1402,1502	NS3	1373–1380	IPFYGKAI	n.d.
Pt20	A*0402 B*0702,5201 C*0702,1202	E2	611–618	YPYRLWHY	n.d.
Pt21	A*1101,3101 B*6701,5101 C*0702,1402	NS5A	2290–2298	RPDYNPPLL	n.d.
Pt22	A*2402 B*5201 C*1202	NS4	1759–1768	AFWAKHMWNF	n.d.
		NS5B	2556–2564	TIMAKNEVF	n.d.
		NS5B	2803–2811	LTRDPTTPL	n.d.
Pt23	A*0201,0301 B*4402,4601 C*0102,0501	NS4	1958–1977	KRLHQWINEDCSTPCSGSWL	n.d.
Pt24	A*2402,4801 B*5201 C*0803,1202	NS3	1637–1656	LTHPITKFVMACMSADLEVV	n.d.