

Original Article

Hepatitis C Virus-specific T-cell Response Correlates with Hepatitis Activity and Donor IL28B Genotype Early after Liver Transplantation

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It is not known how the immune system targets hepatitis C virus (HCV)-infected HLA-mismatched hepatocytes under immune-suppressed conditions after orthotopic liver transplantation (OLT). In addition, the relationship between the HCV-specific immune response and IL28B variants as predictors of HCV clearance has not been well-characterized. We determined the IL28B polymorphisms for 57 post-OLT HCV carriers, and we assessed the HCV-specific immune responses by measuring the peripheral blood mononuclear cell-derived HCV-specific interferon-gamma (IFN- γ) response using an enzyme-linked immunospot assay. At 1-3 years after OLT, patients with no active hepatitis showed higher total spots on the immunospot assay. At > 3 years after OLT, patients with resolved HCV showed higher levels of core, NS3, NS5A, and total spots compared to the chronic hepatitis patients. The IL28B major genotype in the donors correlated with higher spot counts for NS5A and NS5B proteins at 1-3 years after OLT. In the post-OLT setting, the HCV-specific immune response could be strongly induced in patients with no active hepatitis with an IL28B major donor or sustained virological response. Strong immune responses in the patients with no active hepatitis could only be maintained for 3 years and diminished later. It may be beneficial to administer IFN treatment starting 3 years after OLT, to induce the maximum immunological effect.

Key words: interferon gamma, ELISPOT assay, single nucleotide polymorphisms, dendritic cell, CD4 T cell

Hepatitis C virus (HCV)-related liver cirrhosis is a common indication for orthotopic liver transplantation (OLT). Unfortunately, almost all patients become HCV-RNA-positive after OLT, and approx.

25% develop liver cirrhosis within a few years [1, 2]. The pathogenesis of and the therapeutic approaches for recurrent hepatitis C are some of the most important issues in liver transplantation research.

The main pathogenesis of HCV-related disease is

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known to involve the immune reaction to virus-infected hepatocytes [2]. A lack of strong Th1-type helper T-cell responses and cytotoxic T-cell responses against HCV leads to chronic infection by this virus [3]. A strong Th1-type helper T-cell response and the cytotoxic T-cell response correlates with spontaneous recovery and interferon (IFN)-induced sustained virological response (SVR) [4]. The importance of the immune reaction has also been noted in post-OLT chronic hepatitis C (CHC) patients.

Several studies found that HCV-specific immune responses correlate with post-OLT hepatitis C progression [5, 6]. High frequencies of regulatory T cells (Tregs) under various clinical conditions of HCV carriers have been confirmed in several studies. In patients with severe recurrent hepatitis C, regulatory-type Tr1 cells [7] or interleukin (IL)-17 might induce HCV-targeted hepatitis [6]. Despite the immune-suppressive adaptation in OLT patients, several immune functions could be exaggerated. A high frequency of Tregs inhibiting the HCV-specific CD4+ Th1 response, but not the Th17 response, was found in post-OLT patients with advanced fibrosis [6]. Such dysregulation of the immune response balance must be included in the pathogenesis of post-OLT CHC.

A human genome-wide association study uncovered many disease-susceptible genes and drug sensitivity-related genes [8]. In CHC patients, an IL28B gene single nucleotide polymorphism (SNP) was found to be related to spontaneous clearance and susceptibility to treatment with pegylated IFN (Peg-IFN) plus ribavirin [9–11]. The combination of recipient and donor IL28B genetic polymorphisms has been revealed to be important in post-OLT HCV treatment outcomes [12].

However, this genetic polymorphism and the virus-specific immune responses have not been well-characterized. An enzyme-linked immunospot (ELISPOT) assay is an efficient technique for the enumeration of cells secreting antibodies or cytokines. We hypothesized that HCV-specific immune responses evaluated by ELISPOT assay might differ between post-OLT HCV-positive patients and non-OLT CHC patients in various clinical courses. Here we examined whether correlations exist among HCV-specific CD4 T-cell responses, IL28B genetic polymorphism, and the clinical course among post-OLT hepatitis C patients.

Materials and Methods

Patients. From October 1996 to January 2012, an OLT was performed in 280 adults at Okayama University Hospital. Of the 64 patients who received OLT due to HCV-related end-stage liver cirrhosis, 57 patients underwent an IL28B SNP analysis. The characteristics of the study subjects are summarized in Table 1, 2, and 3. No patients showed co-infection with hepatitis B virus or human immunodeficiency virus. The serotype distribution of HCV was serotype 1 in 85% and serotype 2 in 15% of the patients. IFN treatment was adopted for 29 patients with histologically confirmed chronic hepatitis (CH) and high levels of alanine aminotransferase (ALT). Nine patients received non-pegylated IFN due to pancytopenia or poor general condition. Of the 29 patients receiving IFN, 12 (41.4%) showed SVR.

Of the 57 patients, 46 donors consented to undergo an SNP analysis. Recurrent hepatitis C was diagnosed by histological analysis with no rejection and by findings of parenchymal or portal inflammation. IFN treatment was adopted for the 29 patients with histologically confirmed CH and high levels of ALT. Twenty-five of the 29 IFN-adopted patients' donors consented to undergo an SNP analysis.

Informed consent was obtained from each patient included in the study, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and the ethical guidelines for analytical research on the human genome/genes in 2001 Japan as reflected in approval by the Ethics Committee at Okayama University Hospital.

Table 1 Characteristics of the post-OLT HCV patients in the SNP analysis

	Post-OLT HCV
N	57
Age at OLT (years)	58 (52–60)
Donor age (years)	36 (30–46)
Sex (M : F)	36 : 21
Interferon treatment (%)	29 (50%)
Pre-OLT MELD	13 (11–16)
CAI (FK/CyA)	23/34

OLT, orthotopic liver transplantation; HCV, hepatitis C virus; SNP, single nucleotide polymorphism; MELD, model for end-stage liver disease; CAI, calcineurin inhibitors; FK, FK506; CyA, cyclosporine A.

Table 2 Characteristics of post-OLT HCV patients for ELISPOT assay

	Post-OLT HCV		
	≤1-year post-OLT	1-to 3-year post-OLT	>3-year post-OLT
n	11	18	15
Age at assay (years)	55 (52–59)	61 (54–63)	62 (57–65)
Sex (M : F)	6 : 5	10 : 8	3 : 12
Clinical course of HCV (CH : SD : SVR)	0 : 11 : 0	4 : 12 : 2	7 : 5 : 3
HCV RNA (+)	11	16	12
Interferon (%)	0 (0)	3 (16)	8 (53)
Total bilirubin (mg/dL)	0.86 (0.74–2.15)	0.98 (0.70–1.22)	0.97 (0.79–1.41)
Albumin (g/dL)	3.5 (3.1–4.3)	4.2 (3.5–4.4)	4.0 (4.0–4.4)
ALT (IU/l)	89 (48–207)	31 (17–46)	44 (22–59)
Pre-OLT MELD	12 (8–13)	13 (11–16)	12 (10–15)
CAI (FK : CyA)	4 : 7	9 : 9	5 : 10

ELISPOT, enzyme-linked immunospot; MELD, model for end-stage liver disease; CH, patients with ALT > upper limit of normal; HCV-RNA(+); SD, patients with no active hepatitis without IFN; HCV-RNA(-); SVR, patients with sustained viral response to interferon; HCV-RNA(-); ALT, alanine aminotransferase; MELD, model for end-stage liver disease; CAI, calcineurin inhibitors; FK, FK506; CyA, cyclosporine A.

Table 3 The ELISPOT assay results of the non-OLT HCV patients

	HCV total			
		HCV-RNA (+) CH	HCV-RNA (+) PNALT	HCV-RNA (-)
n	38	20	7	11
Age at assay (years)	60 (54–71)	58 (52–63)	76 (40–78)	61 (57–74)
Sex (M : F)	22 : 16	11 : 9	2 : 5	9 : 2
HCV RNA (log copies/mL)	6.0 (0–6.5)	6.4 (6.0–6.7)	6.2 (4.7–6.7)	–
Total bilirubin (mg/dL)	0.72 (0.54–0.87)	0.71 (0.54–0.82)	0.68 (0.42–1.05)	0.81 (0.53–1.24)
Albumin (g/dL)	4.2 (3.7–4.4)	4.2 (3.6–4.4)	4.1 (4.0–4.3)	4.2 (3.3–4.5)
ALT (IU/L)	30 (18–58)	53 (31–74)*#	18 (18–19)	19 (13–55)

* $p < 0.05$ vs. HCV-RNA (+) PNALT; # $p < 0.05$ vs. HCV-RNA (-)

ALT, alanine aminotransferase; CH, patients with ALT > upper limit of normal; PNALT, patients with persistent normal ALT without IFN.

Characteristics of immunological assay subjects. We conducted immunological assays for 27 consecutive post-OLT HCV-positive recipients at 44 experimental time points. All patients showed re-infection by HCV, resulting in various degrees of CH. Patients were divided into the following groups according to the interval between the transplantation date and the experiment date and the clinical condition: 3 groups by interval: Time 1 (≤1 year after OLT, n = 11), Time 2 (>1 to ≤3 years after OLT, n = 18), and Time 3 (>3 years after OLT, n = 15); and 3 groups by clinical condition: the OLT-CH group (patients with ALT > the upper limit of normal; HCV-RNA-positive; Time 1, n = 0; Time 2, n = 4;

Time 3, n = 7), the OLT-SD group (patients with no active hepatitis without IFN [*i.e.*, stable disease]; HCV-RNA-positive; Time 1, n = 11; Time 2, n = 12; Time 3, n = 5), and the OLT-SVR group (patients with SVR following treatment with IFN; HCV-RNA-negative; Time 1, n = 0; Time 2, n = 2; Time 3, n = 3). All patients were also analyzed for IL-28B SNPs. All patients were Japanese. The general characteristics of the groups are summarized in Table 2. Patients were treated using a standard immunosuppressive regimen (tacrolimus or cyclosporine A and/or mycophenolate mofetil with steroids).

As controls, non-OLT CHC patients were included. These individuals were divided into 3 groups: the CH

group (n = 20) with active CHC (ALT > the upper limit of normal with HCV-RNA-positive status); the PNALT group (n = 7) with persistently normal ALT without IFN (ALT < the upper limit of normal with HCV-RNA-positive status) and the RNA(-) group (n = 11) with HCV-RNA-negative with or without IFN.

Laboratory tests. HCV antibody was routinely measured in serum using a commercially available chemiluminescent enzyme immunoassay (Lumipulse System; Fujirebio, Tokyo, Japan). HCV RNA was measured in serum using the qualitative COBAS Amplificor assay (Roche, Pleasanton, CA, USA).

Determination of the IL28B SNP mutation (rs8099917). To determine the genotype of IL28B SNP rs8099917, genomic DNA was extracted from peripheral blood using a silica-membrane-based DNA extraction kit (Qiagen, Tokyo, Japan) according to the protocols provided by the manufacturer. Eluted DNA was stored at -80°C until analysis. The rs8099917 SNP was determined using TaqMan predesigned SNP genotyping assays in a LightCycler 480 system (Roche Diagnostics, Tokyo, Japan), as recommended by the manufacturer. The genotype was determined to be the major homozygous allele (TT) as a beneficial genotype for pegylated IFN plus ribavirin treatment, and the minor heterozygous/homozygous allele (TG/GG). We were able to obtain the SNP genotypes of all samples with this system.

HCV recombinant proteins for ELISPOT assay. The HCV recombinant proteins (genotype 1b sequence) HCV-core, -NS3, -NS4, -NS5A and -NS5B were purchased from Mikrogen (Martinsried, Germany). These proteins were used as stimulating antigens at $1\mu\text{g}/\text{mL}$ for ELISPOT assay.

IFN- γ ELISPOT assay with myeloid dendritic cells (DCs) and CD4-positive T cells. Mononuclear cells were separated from peripheral blood by centrifugation on a Ficoll-Hypaque density gradient (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), as described [13]. CD14-positive monocytes were purified positively using microbeads (Miltenyi Biotec, Belgisch Gladbach, Germany) in accord with the manufacturer's protocol. CD4-positive T cells (T4) were subsequently positively sorted in the same manner. The positively selected fraction proved to be > 95% positive for CD14 or CD4 by flow cytometric analysis staining with fluorescein isothiocyanate (FITC)-conjugated anti-CD14 or CD4 antibody

(BD Pharmingen, San Jose, CA, USA). The T4 cells were immediately frozen.

CD14-positive cells were cultured at $1 \times 10^6/\text{mL}$ in RPMI-5% heat-inactivated human AB serum (ICN Biomedicals, Aurora, OH, USA; or MP Biomedicals, Santa Ana, CA, USA) supplemented with $100\text{ng}/\text{mL}$ of granulocyte macrophage colony-stimulating factor (GM-CSF) (kindly provided by Kirin Pharma, Tokyo, Japan) and $50\text{ng}/\text{mL}$ of IL-4 (kindly provided by Ono Pharmaceuticals, Osaka, Japan) at 37°C in 5% CO_2 for 5 days. Cells were confirmed to be CD11c-positive myeloid immature DCs (iDCs).

Each HCV protein ($1\mu\text{g}/\text{mL}$) was pulsed to the iDC culture and kept still for 1 day. An HCV protein unloaded buffer that was used for HCV protein purification was added to the iDC culture as a negative control. As a positive control, tuberculin purified protein derivative (PPD) (Japan BCG Laboratory, Tokyo) was added to the iDC culture as a recall antigen at a concentration of $0.1\mu\text{g}/\text{mL}$. To mature DCs, $1\text{ng}/\text{mL}$ of lipopolysaccharide (Sigma, St. Louis, MO, USA) was added to the culture 1 day after HCV protein addition. The same day, mouse anti-human IFN- γ antibody (Mabtech, Nacka Strand, Sweden) was diluted to $5\mu\text{g}/\text{mL}$ with ELISPOT buffer (0.159% Na_2CO_3 , 0.293% NaHCO_3) and coated overnight at 4°C on a 96-well filtration plate (Millipore, Billerica, MA, USA) with $100\mu\text{L}$ per well.

The coated plate was washed with phosphate-buffered saline (PBS) and blocked with 10% fetal calf serum RPMI1640 medium for 1-2h. Myeloid DCs were counted and seeded at 6×10^3 cells/well. Cryopreserved T4 cells were thawed, counted and seeded at 2×10^5 /well. The next day, the plate was washed 6 times with PBS. Rabbit anti-IFN- γ serum was diluted to 1/800 with PBS and used to coat the wells following incubation at 37°C for 2h. The plate was washed 6 times with PBS and coated with goat anti-rabbit IgG-AP (Southern Biotech, Birmingham, AL, USA) diluted to 1/2000 with PBS. After culturing for 1h at 37°C , the plate was washed 6 times with water, and spots were developed with 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt and nitroblue tetrazolium chloride as a substrate. Spot development was stopped after 10 min by washing with distilled water. Spot counts were calculated using a microscope.

If the positive control well with PPD showed no

spots, we did not use the results, in order to avoid assay failure. A single assay was performed for post-OLT patients, and we used a duplicate assay for some of the control non-OLT patients because we were able to collect enough PBMCs from controls.

The spots were counted by 2 specialists (authors K.K. and Y.M.) blinded to the identities of the groups and then averaged. The final spot counts results were derived as the target spot count minus the spot count for the negative control well. If the spot count for the negative control well was more than that for the target, a value of zero spots was used. Healthy controls showed less than 5 spots, indicating that the non-specific responses were minimal. Representative assay results are shown in Fig. 1.

Statistical analysis. To compare the mean values of relative indices, we used the Kruskal-Wallis test with the Dunnett post-test or Student's *t*-test on JMP version 9 software (SAS Institute, Cary, NC, USA). Differences were considered significant for values of $p < 0.05$.

Results

IL28B SNP analysis of post-OLT patients. Of the 57 recipients and 46 donors enrolled in this study, 78% and 76% carried the IL28B major genotype, respectively (Fig. 2A). The combination of recipient and donor genotypes was 68% major and major (MM), 15% mixture of major and minor (mix),

and 15% minor and minor (mm). The natural course of patients reflecting the IFN requisite was unaffected by SNPs (Fig. 2B).

Twenty-two patients finished IFN treatment and were followed for longer than 6 months, with 10 patients (45%) achieving SVR. Of the 22 patients who finished IFN treatment, 17 patients had serotype 1 HCV with 11 having no viral response (NVR) and six achieving SVR (35%). The IL28B SNP was analyzed for all of the 17 serotype 1 patients, showing 72% NVR and 83% SVR with major genotype (Fig. 2C). Seventy percent of the NVR donors carried the major genotype, whereas 100% of the SVR donors carried the major genotype. Although the recipient/donor genotype combination showed no significant association with viral response, no patients with the mm combination showed SVR.

HCV-specific IFN- γ ELISPOT assay for non-OLT CHC patients. To examine the correlation between HCV-specific IFN- γ ELISPOT results and clinical conditions, we first evaluated the difference in spot counts between the HCV antibody-positive patients with positive HCV-RNA and those with negative HCV-RNA who had achieved eradication of HCV (Fig. 3A). The numbers of HCV NS3- and NS4-specific spots were higher in the HCV-RNA-negative patients, resulting in higher spot counts. A further analysis was conducted to determine whether hepatitis activity affected the spot counts in the HCV-RNA-positive patients, by dividing the HCV-RNA-positive

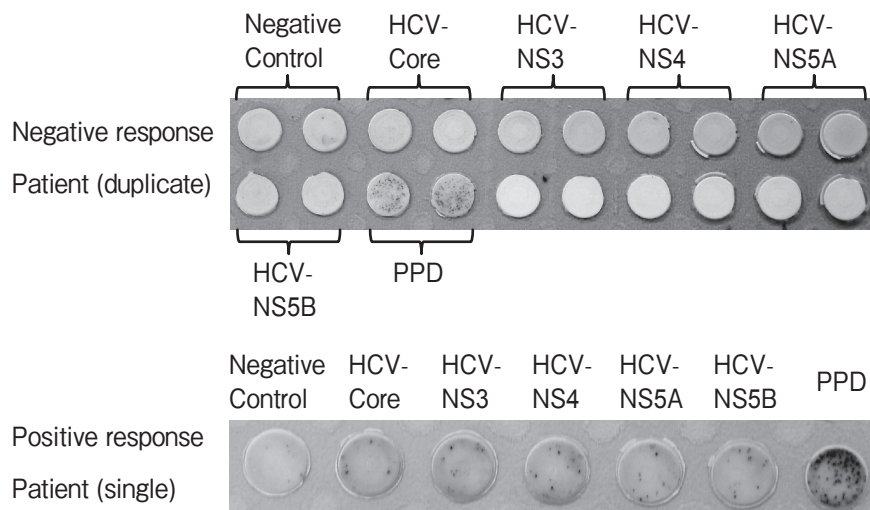


Fig. 1 Representative result of an ELISPOT assay.

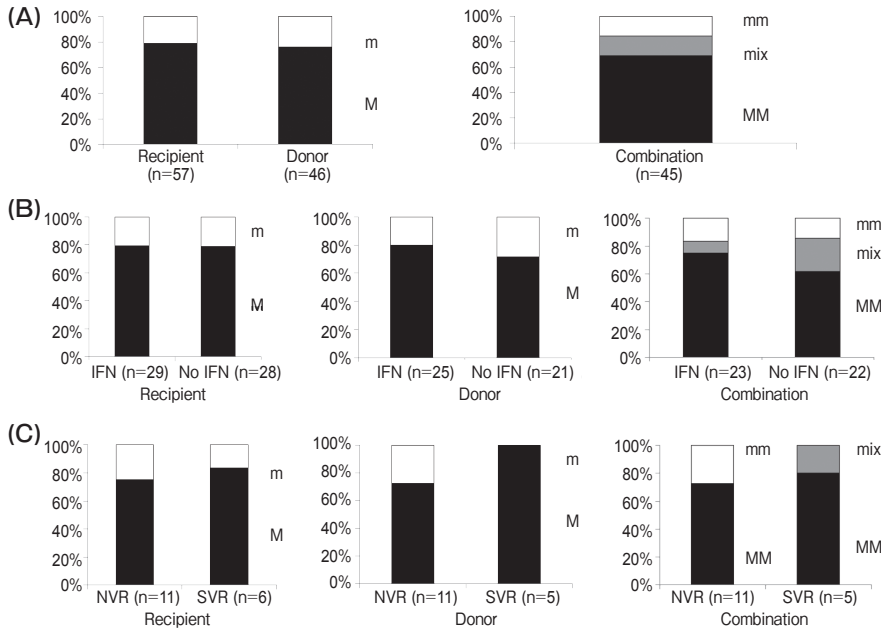


Fig. 2 IL28B SNP (rs8099917) analysis of post-orthotopic liver transplantation (OLT) patients. Results are shown as the major homozygous allele M (TT) and the minor heterozygous/homozygous allele m (TG/GG). To reveal the combination effects of the recipient and donor genotypes, the recipient M and donor M combination is shown as “MM”, the recipient m and donor m combination as “mm”, and the mixture of M and m as “mix”. **(A)** The distribution of recipient and donor genotypes. **(B)** The distribution of recipient and donor genotypes according to IFN treatment requirement. Patients who required IFN treatment because of chronic hepatitis C recurrence were regarded as the IFN group. Patients who showed no active hepatitis were regarded as the No IFN group. **(C)** The distribution of recipient and donor genotypes in HCV genotype 1-infected patients who finished more than 6 months of IFN treatment and were followed for an additional 6 months to determine the viral response. NVR, patients who showed HCV-RNA-positive results after 6 months from end of treatment. SVR, sustained viral responders who cleared the virus.

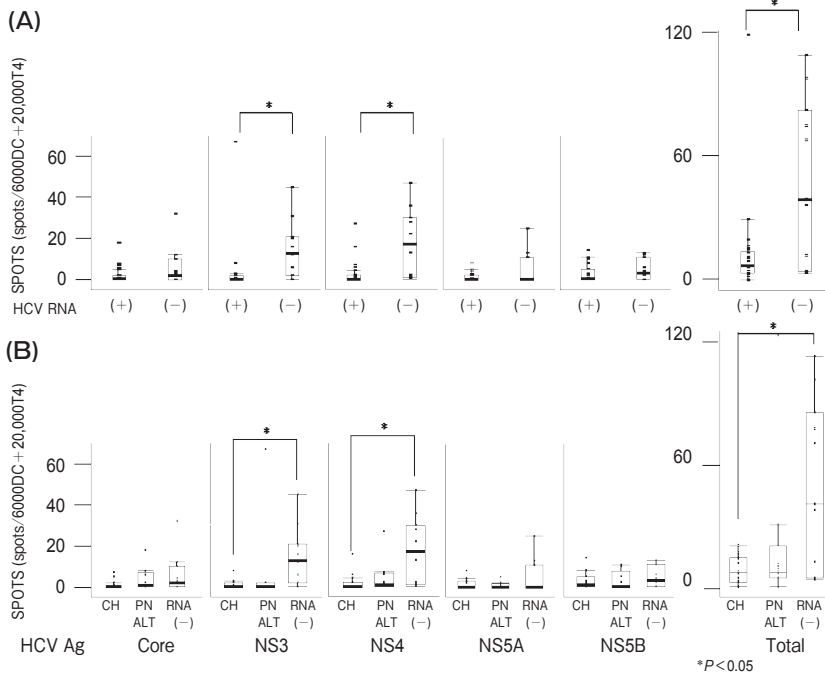


Fig. 3 HCV-specific IFN- γ ELISPOT assay for non-OLT CHC patients. The correlation between HCV-specific IFN- γ ELISPOT results and clinical conditions in the non-OLT patients was evaluated. **(A)** The difference in spot counts between the HCV antibody-positive patients with positive HCV-RNA and those with negative HCV-RNA who had achieved eradication of HCV. **(B)** The HCV-RNA-positive patients were divided into persistent normal ALT (PNALT) and chronic hepatitis (CH) groups.

patients into PNALT and CH groups (Fig. 3B). The PNALT group showed a slight increase in spot counts and no significant difference from the CH and HCV-RNA-negative groups.

HCV-specific IFN- γ ELISPOT assay for post-OLT CHC patients. Next, we analyzed the ELISPOT data of the post-OLT CHC patients. Since the effects of immune-suppressive treatment are strong in the early period after OLT, and because anti-HCV treatment would be administered as time passed, we divided the post-OLT CHC patients into 3 groups

according to time after OLT: Time 1 (T1), ≤ 1 year after OLT; Time 2 (T2), > 1 year to ≤ 3 years after OLT; and Time 3 (T3), > 3 years after OLT (Fig. 4A). The spot counts increased with time, but no significant differences were identified.

We divided the data into 3 groups according to the clinical conditions, as (1) stable disease (SD), reflecting patients with no active chronic hepatitis recurrence although ALT or bilirubin levels might be higher due to rejection or biliary complications; (2) CH, reflecting patients with chronic hepatitis; and (3)

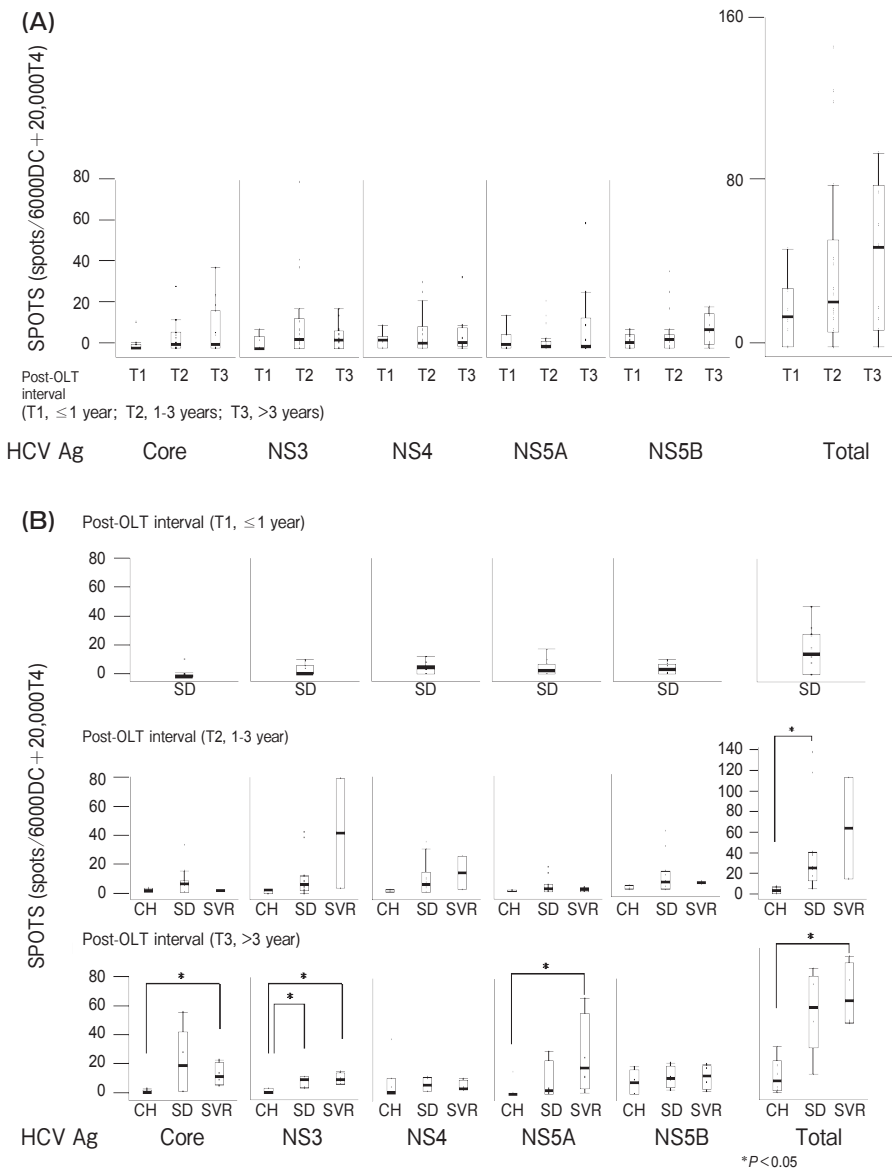


Fig. 4 HCV-specific IFN- γ ELISPOT assay for post-OLT CHC patients. The correlation between HCV-specific IFN- γ ELISPOT assay results and post-OLT clinical conditions was evaluated. **(A)** Spot counts at different time points after OLT. **(B)** Spot counts under different clinical conditions and time points after OLT. We divided the spot counts into 3 groups according to the interval between the operation and the measurement date and by the clinical condition: 3 groups by interval, Time 1 (< 1 year after OLT, $n = 11$), Time 2 (≥ 1 to < 3 years after OLT, $n = 18$), and Time 3 (≥ 3 years after OLT, $n = 15$) and 3 groups by clinical condition: the OLT-CH group (patients with ALT $>$ the upper limit of normal; HCV-RNA-positive) (Time 1, $n = 0$; Time 2, $n = 4$; Time 3, $n = 7$), the OLT-SD group (patients with no active hepatitis without IFN; HCV-RNA-positive) (Time 1, $n = 11$; Time 2, $n = 12$; Time 3, $n = 5$), and the OLT-SVR group (patients with SVR following treatment with IFN; HCV-RNA-negative) (Time 1, $n = 0$; Time 2, $n = 2$; Time 3, $n = 4$).

SVR, reflecting patients who received IFN treatment and eradicated their HCV (Fig. 4B). In the T2 group, the sum of spots was significantly higher in the SD patients compared to the CH patients, suggesting the significance of an HCV-specific immune response to control hepatitis activity in HCV-RNA carriers. In the T3 group, the HCV core-, NS3-, and NS5A-specific immune responses were higher in the SVR patients compared to the CH patients. The HCV NS3-specific immune response was higher even in the SD patients than in the CH patients.

HCV-specific IFN- γ ELISPOT results and the IL28B genotype combination after OLT. Next, we analyzed the correlation between the IL28B genotype combination and the ELISPOT data in the post-OLT patients. Since the SVR condition induces a strong response, post-OLT T2 patients other than the SVR patients were used for analysis (Fig. 5). The IL28B major (M) donors showed stronger HCV NS5A- and NS5B-specific IFN- γ ELISPOT responses compared to the minor (m) donors.

Clinical course and ELISPOT results after OLT. The temporal courses of representative post-OLT patients are shown in Fig. 6, and Fig. 6A shows patients who received IFN treatment and achieved SVR. The sum of spots increased over time

and achieved high levels after viral eradication. Patients 5 and 6 received IFN, but could not eradicate the virus (Fig. 6B). Their spot counts were very low. The patients who showed no active hepatitis without IFN (Patients 7 and 8) achieved high spot numbers in 2 years, but showed diminished levels at 5 years post-OLT.

Discussion

We investigated the impacts of IL28B genotype and HCV-specific immune responses on the clinical course of post-living donor OLT hepatitis C patients. Strong HCV-specific IFN- γ responses were seen after viral eradication and remained for several years. HCV carriers who had no active hepatitis also showed strong IFN- γ response at 1-3 years, but not after 3 years. The donor IL28B major genotype patients showed stronger HCV specific immune responses at 1-3 years post-OLT. Maintaining the HCV-specific immune response is crucial to controlling hepatitis activity.

The pathogenesis of CHC has been thought to involve immune responses, since the virus itself has no cytotoxic effects on hepatocytes. Previous studies revealed that HCV itself induces oxidative stress in

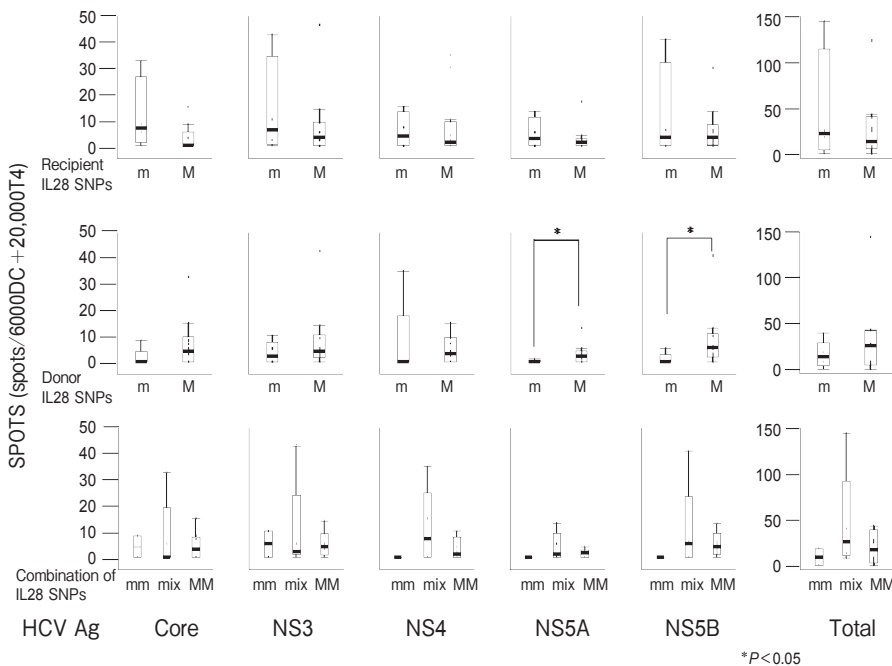


Fig. 5 HCV-specific IFN- γ ELISPOT results and IL28B genotype combination in T2 patients after OLT. The correlation between the IL28B genotype combination and the ELISPOT data in the post-OLT patients was evaluated. Post-OLT T2 patients other than the SVR patients were used for this analysis. The IL28B major (M) donors showed greater HCV NS5A- and NS5B-specific spot counts compared to the minor (m) donors.

*P<0.05

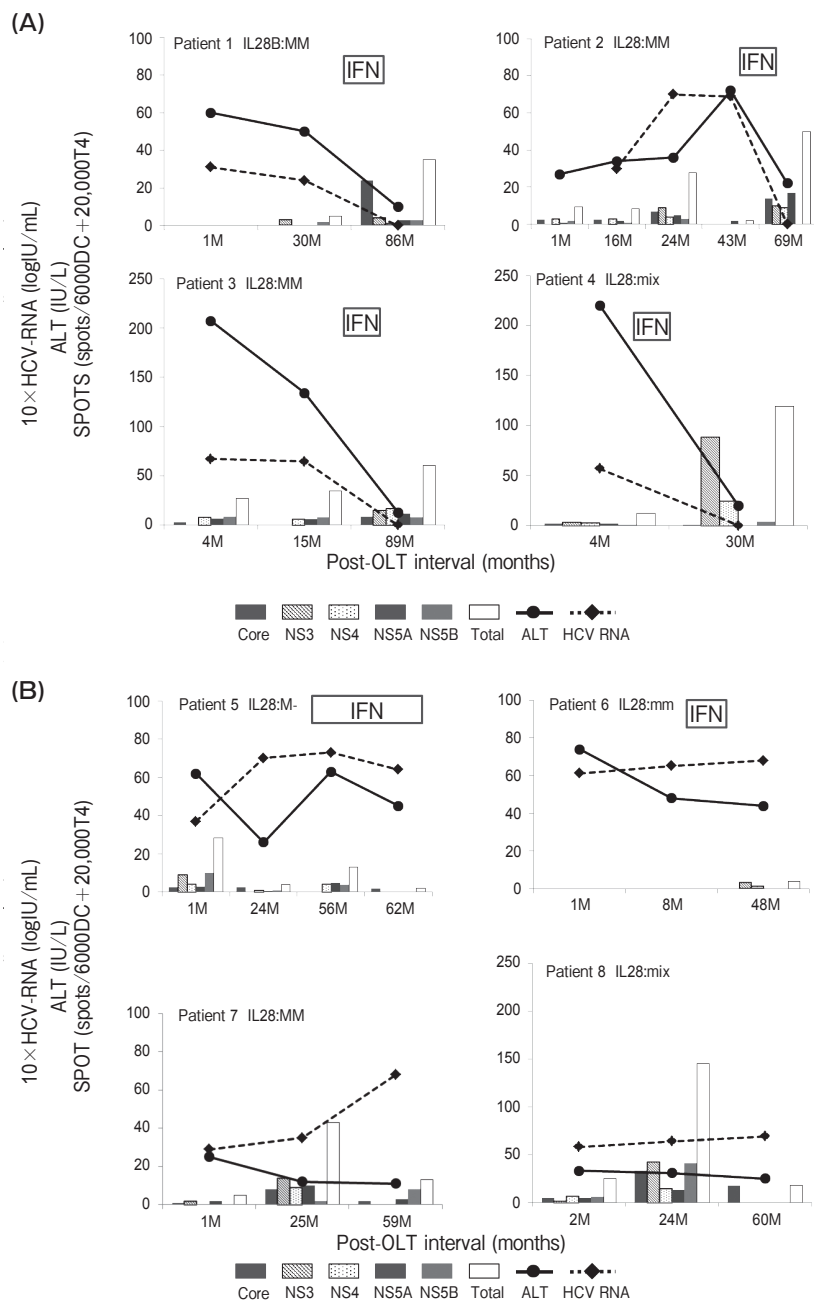


Fig. 6 Clinical course and ELISPOT data after OLT. The clinical courses of representative post-OLT patients. (A) Patients who received IFN treatment and achieved SVR. Patients 1, 2, and 3 had IL28B MM, and Patient 4 had the mix pattern of IL28B genotype. Patients 5 and 6 received IFN, but could not eradicate the virus. Patients who showed no active hepatitis without IFN (OLT-SD; Patients 7 and 8) achieved high spot numbers at 2 years, but showed diminished levels at 5 years post-OLT.

hepatocytes [14–16], resulting in worse outcomes [17]. However, immune responses including IFN treatment are still the only way to achieve virus eradication. In particular, a strong HCV-specific T-cell response correlates with viral eradication [3].

In the present study, a strong HCV-specific T-cell response was exerted in post-SVR patients even several years after OLT. This result is consistent with

previous reports on non-OLT patients and post-OLT patients [3, 18]. HCV carriers showed weak HCV-specific immune responses in both non-OLT and post-OLT settings. HCV itself has several unfavorable effects on the human immune system. HCV core protein blocks IFN signaling by interacting with signal transducer and activator of transcription protein-1 (STAT1) [19]. HCV NS3/4A serine protease cleaves

Cardif, resulting in the dislocation of Cardif from mitochondria and the reduction of downstream IFN- β production [20].

Concerning T-cell responses, high levels of persisting viral antigen result in chronic T-cell activation with a sequential loss of T-cell function including IFN- γ production [2]. In our experiments, from 1–3 years after OLT, the HCV-specific immune response in the SD patients was stronger than that in the CH patients, but was diminished later. This phenomenon suggests that a strongly maintained immune response in the early period could control hepatitis activity, but diminishes later, possibly due to sustained unfavorable effects of HCV on immune function. Introducing IFN treatment in this period with a strong HCV immune response seems likely to offer an effective protocol, whereas rejection should be addressed using IFN [21].

The present results revealed only the CD4 T-cell response, which reflects only part of the HCV-specific immune response. Elucidation of the HCV-specific CD8 T-cell response by tetramer assay is important. However, as previously reported, the HCV-derived epitopes for a tetramer assay are mainly HLA-A2- or -A24-restricted and can be adapted only to patients possessing these HLA loci. The number of such patients in the present study was too low to include them. In addition, the number of collected lymphocytes was too small to use them for a CD8 T-cell analysis.

To assess immune responses after OLT, HLA mismatches between recipient and donor represent an important issue. We used antigen-presenting cells and CD4 T cells from recipients to perform this assessment. However, in the body, HCV peptide must be expressed on donor hepatocytes, and the immune response should attack them. To identify these reactions, donor antigen-presenting cells must be used, but the allo-reactive mixed lymphocyte response in that situation might be too strong to reveal the relatively weak anti-HCV response. We therefore used the auto-response system. With this system, we cannot reveal recipient T-cell and donor hepatocyte reactions, but we can investigate immune reactions between recipient CD4 T cells and recipient-derived liver-resident antigen-presenting cells such as macrophages or monocytes. Such immune reactions play significant roles in the post-OLT hepatitis C situation.

To reveal the significant impacts of IL-28B regarding the clinical course of post-OLT HCV recurrence, analyses of many more patients are needed. Even a previous IL28B genotyping study including 327 recipients with 108 IFN receivers and 70% of the corresponding donors could not find a significant difference between SVR and non-SVR groups [22]. Another study regarding the natural course of HCV-related OLT revealed that the “favorable” IL28B SNP could be unfavorable when present in the donor [23]. We assessed only 57 recipients with 46 donors and 29 recipients with 25 donors who received IFN treatment. The natural course showed a tendency to be relatively less favorable with the donor SNP M genotype, although no significant differences were identified. The donor IL28B genotype M might be a marker to discriminate SVR patients. Here, the recipient genotypes showed the same tendency as donor genotypes.

IL28B, also called type III or lambda IFN (IFN- λ 3), is induced by viral infections and upregulated by HCV infection [9]. IL28B interacts with a heterodynamic class II cytokine receptor, IL10Rb or IL28Ra, resulting in the induction of IFN-stimulated genes through the JAK-STAT pathway [24]. IFN- λ 3 is the product of the IL28B gene, which has been found to regulate pathogens such as herpes simplex virus, cytomegalovirus, hepatitis B virus, and HCV [25–27]. In our analysis, the IL28B genotype correlated with the HCV-specific IFN- γ response in the HCV-RNA-positive post-OLT HCV patients at 1–3 years post-OLT. Although we must perform further evaluations in a greater number of patients, HCV-specific immune responses appear to be affected by IL28B genotype.

Non-OLT HCV carriers with PNALT have been shown to display higher levels of regulatory T function-related FOXP3 and CTLA4 transcripts [28]. In the present study, these PNALT patients showed no significant difference in non-OLT CH regarding the HCV-specific Th1-type cytokine IFN- γ response. This might be because our system showed very low responses in the CH and PNALT groups. In the post-OLT patients in the T2 group, the patients with SD showed a higher sum of ELISPOTs than those with CH. In the T3 group, several HCV proteins (core, NS3, NS5A) showed differences between the CH and SVR patients, resulting in higher sums of spots in the

SVR patients.

Only NS3 showed a significant difference between the CH and SD patients. Follow-up data for the same patients also indicated that the sum of spots might be high at 3 years, diminishing later. A strong HCV-specific IFN- γ response at 3 years might suggest the utility of providing treatment at that time. We could not discern any reason why the post-OLT patients exhibited strong HCV-specific responses in SD, while the non-OLT PNALT showed no such difference. In the early period after living-donor liver transplantation, regulatory T cells have been demonstrated to be decreased, probably because of the use of a calcineurin inhibitor [29]. The use of calcineurin inhibitors might be one reason for the conflicting results concerning T-cell responses.

In conclusion, strong HCV-specific immune responses could be achieved at 1–3 years post-OLT in donor IL28B-major patients. This interval might represent the best timing for IFN treatment administration. Maintaining a strong HCV-specific immune response may prove beneficial for the control of post-OLT hepatitis C activity.

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