Title: Frequency of regulatory T-cell and hepatitis C viral antigen-specific immune response in recurrent hepatitis C after liver transplantation

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Running title: Tregs in recurrent HCV after liver transplant

Table and Figure numbers: Tables: 2, Color figures: 0, Total figures: 5

Declaration: The authors declare no conflict of interest

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**Abbreviations:**

ALT, alanine aminotransferase  
CHC, chronic hepatitis C  
DC, dendritic cell  
ELISPOT, enzyme-linked immunosorbent spot  
FITC, fluorescein isothiocyanate  
FOXP3, forkhead box P3  
HCV, hepatitis C virus  
IL, interleukin  
IFN, interferon  
LPS, lipopolysaccharide  
OLT, orthotopic liver transplantation  
PBMC, peripheral blood mononuclear cell  
PBS, phosphate-buffered saline  
PNALT, persistently normal alanine aminotransferase  
RPMI, Roswell Park Memorial Institute  
SVR, sustained viral response  
T4, CD4+ T cells  
Th1, T helper cell (Th) 1  
Tr1, type 1 regulatory T cell  
TGF, transforming growth factor  
Treg, regulatory T cell
ABSTRACT

**Introduction:** Regulatory T (Treg) and type 1 regulatory T (Tr1) cells facilitate hepatitis C virus (HCV) recurrence after orthotopic liver transplantation (OLT). However, their frequencies and effects on HCV-specific immune responses have not been well investigated.

**Methods:** We determined Treg and Tr1 frequencies in OLT patients with hepatitis C and assessed their associations with HCV-specific T cell responses. These patients comprised the following groups: an early post-transplantation group (n = 14); an OLT-chronic active hepatitis C group (n = 14) with active hepatitis C (alanine aminotransferase of > upper limit of normal/positive for HCV-RNA); an OLT-persistently normal alanine aminotransferase group (n = 12) without active hepatitis C (not interferon/positive for HCV-RNA); and an OLT-sustained viral response group (n = 6) with sustained viral responses using interferon treatment (negative for HCV-RNA). The frequencies of HCV-specific CD4+ T cells that secreted interferon-γ were determined by enzyme-linked immunosorbent spot assay (except for the OLT early group).

**Results:** Treg and Tr1 frequencies were low during the early post-transplantation period. OLT patients with sustained viral responses had lower Treg frequencies than those with chronic hepatitis C, whereas Tr1 frequencies were significantly reduced in OLT patients with persistently normal alanine aminotransferase levels compared to those with chronic hepatitis C (p < 0.05). Treg frequencies positively correlated with HCV NS3 antigen-specific interferon-γ responses, which corresponded to HCV clearance.

**Conclusions:** Increased Treg frequencies and reduced HCV-NS3 antigen-specific responses recovered after viral eradication in post-OLT chronic hepatitis C patients. Reduced Tr1 frequencies were associated with hepatitis activity control, which may facilitate controlling chronic hepatitis C in patients after OLT.

**Keywords:** chronic hepatitis C, living donor liver transplantation, persistently normal alanine aminotransferase, regulatory T cell, sustained viral response, type 1 regulatory T cells
1. INTRODUCTION

Chronic hepatitis C virus (HCV) infection is prevalent worldwide and causes cirrhosis in 20% of infected patients. HCV-related liver cirrhosis is a common indication for orthotopic liver transplantation (OLT) [1]. However, HCV persists in almost all post-OLT patients and graft reinfection is universal after liver transplantation [2], leading to high-titer HCV viremia with cirrhosis within 5 years of transplantation in approximately 20% of patients and within 10 years in 50% [3]. Two-thirds of post-OLT patients do not have early hepatitis, even without therapy. Thus, HCV infection after OLT differs completely from chronic hepatitis C (CHC) without transplantation. However, the mechanisms underlying accelerated HCV-induced liver damage after OLT are poorly understood.

Several factors appear to be involved in the risk of hepatitis recurrence, particularly those related to viral and immune responses: Immunosuppressive therapy is a likely cause for the severe, accelerated course of HCV-related hepatitis after OLT [3, 4]. In particular, high-dose steroids, immunosuppressive drug combinations, powerful induction treatments, and acute rejection can worsen patient outcomes [5]. The pathology of HCV-related disease reflects immune reactions to virus-infected hepatocytes. Strong T helper cell (Th) 1 and cytotoxic T cell responses are correlated with spontaneous recovery and interferon (IFN)-induced sustained virological responses; however, diminished Th1 cell and cytotoxic T cell responses to HCV result in chronic infection [6].

Recent attention has focused on regulatory T cells (Tregs) and their contribution to CHC. Tregs are characterized by simultaneous expression of both CD4 and CD25 [interleukin (IL)-2 receptor α] surface markers [7, 8] and the absence of CD127 (IL-7 receptor) expression [9]. Their mechanisms of immunosuppression depend on both cell–cell contact and immunosuppressive cytokine secretion [10]. A subpopulation of Tregs that express CD18 and CD49b-expressing type 1 regulatory T (Tr1) cells have also attracted attention [11], because they produce large amounts of immunosuppressive cytokines, such as IL-10 and transforming growth factor-β (TGF-β), with which they inhibit type 1 and 2 helper responses [12]. Their mechanism of immunosuppression is cytokine-dependent rather than cell contact-dependent [13].
Tregs and Tr1 cells may contribute to HCV persistence by suppressing HCV-specific T cell responses [14-16]. Treg frequencies and activities are apparently higher in CHC patients than in those who have achieved viral clearance [17]. Post-OLT, Treg activities are affected by immunosuppressive therapy [18]. Tregs induce allograft tolerance [19, 20]. Moreover, Tregs and Tr1 cells are overexpressed in patients with severe hepatitis C recurrence as compared to those patients with no or minor recurrence [12, 21]. These results suggest that Tregs and Tr1 cells are involved in HCV recurrence after OLT.

Although many factors affect the severity of HCV recurrence after OLT, the exact roles of Tregs and Tr1 cells remain to be determined. Few studies have evaluated the numbers and activities of Tregs and Tr1 cells or their involvement in the accelerated progression of recurrent hepatitis C after OLT. Thus, in this study, we determined the frequencies and activities of Treg and Tr1 cells in OLT patients with post-OLT hepatitis C and assessed their associations with HCV-specific CD4+ T cell responses.

2. METHODS

2.1 Patients

Between October 1996 and January 2012, we performed OLT for 280 adults at Okayama University Hospital, Okayama City, Japan. All patients received liver transplants from living donors. Of the 64 consecutive liver transplant recipients who underwent OLT for HCV-related end-stage liver disease, all patients except one were re-infected by HCV. Thirty four HCV re-infected patients (OLT-HCV) were included in following investigations. To investigate serial changes in Tregs and Tr1 cells during the early post-OLT period (OLT-early group), 14 of these 34 patients were examined at 7 days before OLT (pre-transplant), 1–10 days post-OLT, 11–20 days post-OLT, 21–30 days post-OLT, and 31–40 days post-OLT.

Of these 34 OLT-HCV patients, 32 patients who followed for more than 6 months were divided into three groups: an OLT-CHC group (n = 14) with active hepatitis C recurrence [alanine aminotransferase (ALT) > the upper limit of normal/positive for HCV RNA/with or without histological evaluation]; an OLT-persistently normal ALT (PNALT) group (n = 12) without active
hepatitis (not IFN/positive for HCV RNA); and an OLT-sustained viral response (SVR) group (n = 6) with sustained viral responses using treatment with IFN (negative for HCV RNA). The follow-up times after OLT for these three OLT-HCV groups are shown in Table 1B. Blood samples were obtained at each of these follow-up times after OLT as well as at 3 months before and after liver biopsy was performed. Liver histology results were available for 9/14 patients in the OLT-CHC group, 10/12 in the OLT-PNALT group, and 3/6 in the OLT-SVR group. Liver tissues that had been fixed with 10% formalin were stained using hematoxylin and eosin (HE) and Azan. All liver specimens were assessed by two hepatologists (T.Y. and A.T.) who were blinded to study group allocation. The grade and stage of liver histology were assessed as activity (A0–A3) and fibrosis (F0–F4), according to the META VIR scoring system [22].

As controls, 12 healthy subjects and 37 non-OLT HCV carrier patients (non-OLT-HCV) were included. Healthy subjects were screened for HCV and hepatitis B virus infection. The non-OLT-HCV patients were divided into three groups: a CHC group (n = 25) with active CHC (ALT > the upper limit of normal/positive for HCV RNA); a CHC-PNALT group (n = 6) without active hepatitis (not IFN/positive for HCV RNA); and a CHC-SVR group (n = 6) with sustained viral responses using treatment with IFN (negative for HCV RNA). We excluded any patients with hepatocellular carcinoma.

Informed consent was obtained from each participant. Our study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ethics Committee of Okayama University Hospital.

2.2 Immunosuppression

OLT patients were treated using a standard immunosuppressive regimen (tacrolimus or cyclosporine A with steroids and/or mycophenolate mofetil).

2.3 Fluorescence-activated cell sorting analysis

A three-laser FACSaria flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) was used for fluorescence-activated cell sorting analysis. Expression levels of cell surface molecules on lymphocytes were determined by eight-color surface staining. The labeled antibodies used were as follows: AmCyan-conjugated anti-CD4; PerCP-Cy5.5-conjugated anti-CD8; APC-conjugated anti-
CD18; fluorescein isothiocyanate (FITC)-conjugated anti-CD49b or FITC-conjugated anti-CD279; PE-conjugated anti-CD127-IL7R; PE-Cy7-conjugated anti-CD25; biotin-conjugated anti-CD45RA; and brilliant violet™-conjugated anti-CCR7. Propidium iodide was used to gate for viable cells. Appropriate isotype control antibodies were used for marker settings. Peripheral blood mononuclear cells (PBMCs) were obtained from heparinized whole blood samples by density gradient centrifugation using Ficoll-Paque® PLUS (GE Healthcare, Little Chalfont, Buckinghamshire, UK). PBMCs collected from the interface were washed twice in phosphate-buffered saline (PBS) and stained with labeled monoclonal antibodies at room temperature for 30 min in the dark.

CD4+CD25+CD127 low Tregs and CD4+CD25+CD18+CD49b+ Tr1 cells were analyzed using the FACSARia flow cytometer (Figure 1). FlowJo 7.6 software for Windows (Tree Star Inc., Ashland, OR, USA) was used for data analysis.

2.4 Interferon-γ enzyme-linked immunosorbent spot (ELISPOT) assay for myeloid dendritic cells and CD4+ T cells

PBMCs were isolated from peripheral blood samples, as described above. CD14+ monocytes were positively selected using microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instructions. Subsequently, CD4+ T cells (T4) were positively sorted in the same manner. Positively selected fractions were >95% positive for CD14 or CD4 by flow cytometry analysis after staining with FITC-conjugated anti-CD14 or -CD4 antibodies (BD Pharmingen Inc., San Diego, CA, USA). T4 cells were frozen immediately. CD14+ cells were cultured at a density of 10⁶ cells/ml in Roswell Park Memorial Institute (RPMI) medium supplemented with 5% heat-inactivated human blood-type AB serum (ICN Biomedicals Inc., Orangeburg, NY, USA), 100 ng/ml of granulocyte/macrophage colony-stimulating factor (Kirin Pharma, Tokyo, Japan), and 50 ng/ml of IL-4 (Ono Pharmaceutical Co., Ltd., Osaka, Japan) at 37°C in 5% CO₂ for 5 days. These cells were CD11c+ immature myeloid dendritic cells (DCs).

HCV protein (1 μg/ml) was used to pulse immature DC cultures, which were subsequently maintained for 1 day. To mature DCs, 1 ng/ml of lipopolysaccharide (LPS; Sigma-Aldrich Corp., St. Louis, MO, USA) was added to the culture at 1 day after adding the HCV protein. On the same day,
mouse anti-human IFN-γ antibody (Mabtech AB, Stockholm, Sweden) was diluted to 5 µg/ml with enzyme-linked immunosorbent spot (ELISPOT) buffer (0.159% Na₂CO₃, 0.293% NaHCO₃), and 100 µl was coated on each well of a 96-well filtration plate (Millipore, Billerica, MA, USA) at 4°C overnight. The plate was then washed with PBS and blocked with 10% fetal bovine serum in RPMI 1640 medium for 1–2 h. Myeloid DCs were counted and seeded at a density of 6 × 10³ cells/well. Cryopreserved T4 cells were thawed, counted, and seeded at a density of 2 × 10⁵ cells/well. The next day, the plate was washed six times with PBS. Rabbit anti-IFN-γ serum was diluted to 1:800 with PBS, coated onto the wells, and incubated at 37°C for 2 h. The plate was then washed six times with PBS and coated with a goat, anti-rabbit immunoglobulin G-alkaline phosphatase antibody (Southern Biotech Associates, Birmingham, AL, USA) diluted to 1:2000 with PBS. After culture at 37°C for 1 h, the plate was washed six times with water, and spots were developed with 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt, using nitroblue tetrazolium chloride as the substrate. Spot development was stopped after 10 min by washing with distilled water. Spots were evaluated by light microscopy.

2.5 Statistical analysis

Continuous variables are presented as median with interquartile range and categorical variables as numbers (percentage). Wilcoxon rank sum test was used to compare continuous variables and the chi-square test was used to compare categorical variables. The Steel–Dwass test was used to make all pairwise comparisons for multiple groups. A p-value of <0.05 was considered significant. Statistical analysis was performed using JMP version 9 (SAS Institute, Inc., Cary, NC, USA). The error bars in the figures indicate standard errors of the means.

3. RESULTS

3.1 Baseline patient characteristics

The demographic and clinical characteristics of the patients in the non-OLT-HCV and OLT-HCV groups are summarized in Table 1. The three non-OLT-HCV groups were similar with regard to age, gender, body mass index, platelet counts, prothrombin time-international normalized ratios, total bilirubin, albumin, ALT, and viral loads. Patients in the CHC-SVR group were significantly younger
than those in the other groups (p = 0.029; Table 1A).

The characteristics of the post-OLT groups are summarized in Table 1B. The three OLT-HCV groups were also similar with regard to age, gender, model for end-stage liver disease scores, graft-to-recipient weight ratios, acute cellular rejection, immunosuppressive protocols used, and mycophenolate mofetil use. The median follow-up times after OLT were also similar between the three OLT-HCV groups (Table 1B). ALT levels determined on the same day of follow-up after OLT were significantly higher in the OLT-CHC group (p = 0.029; Table 1B). Activity and fibrosis scale scores were nonsignificantly higher in the OLT-CHC group. No chronic rejection was observed in any post-OLT patient.

3.2 Correlations between regulatory T and type 1 regulatory T cell frequencies and clinical results

We investigated possible correlations between Treg and Tr1 frequencies among CD4+ T cells and clinical results (Table 2). White blood cell counts negatively correlated with Tr1 percentages in the non-OLT-HCV group (p = 0.008; Table 2A). Platelet counts negatively correlated with Treg percentages in the OLT-HCV group (p = 0.012; Table 2B). ALT levels (p = 0.052) and fibrosis scores (p = 0.50) tended to be positively correlated with Treg percentages (Table 2B).

3.3 Regulatory T and type 1 regulatory T cell frequencies in control patients and orthotopic liver transplantation patients with hepatitis C

We determined Treg and Tr1 frequencies for 12 healthy subjects, 37 non-OLT-HCV patients, and 32 OLT-HCV patients (except for the OLT-early group). The frequencies of Tregs and Tr1 cells among CD4+ T cells were not significantly different between these three groups (Figure 2).

3.4 Regulatory T cell frequencies with HCV infection and different clinical conditions

The percentages of Tregs among CD4+ T cells were not significantly different between the three non-OLT-HCV groups (Figure 3A). Serial changes in the percentages of Tregs pre-OLT and during the early period after OLT are shown in Figure 3B. These patients were divided into two groups: OLT-early patients with later persistently normal alanine aminotransferase levels (OLT-early, later PNALT group, n = 5) and OLT-early patients with later chronic active hepatitis C (OLT-early, later CHC group, n = 5). Prior to OLT, the OLT-early, later PNALT group had a significantly higher Treg
frequency than did the OLT-early, later CHC group (p = 0.038; Figure 3B). There were no significant differences in the percentages of Tregs during the early period post-OLT. Treg frequency was relatively low during the early period post-OLT; however, it recovered after 8 weeks.

Treg frequencies were also determined in the three stable OLT-HCV groups: OLT-CHC group (n = 14); OLT-PNALT group (n = 12); and OLT-SVR group (n = 6). The percentage of Tregs among CD4+ T cells tended to be lower in the OLT-SVR group than in the OLT-CHC group, although this difference was not significant (p = 0.068; Figure 3C).

3.5 Type 1 regulatory T cell frequencies with HCV infection and different clinical conditions

Tr1 frequencies were determined for the three non-OLT-HCV, two OLT-early, and three OLT-HCV groups. The percentages of Tr1 cells were not significantly different among the three non-OLT-HCV groups (Figure 4A). After OLT, Tr1 frequency in the OLT-early, later PNALT group continuously decreased over 40 days, although these changes were not statistically significant (Figure 4B). With the stable conditions, Tr1 frequency was significantly lower in the OLT-PNALT group than in the OLT-CHC group (p = 0.001; Figure 4C).

3.6 Hepatitis C virus antigen-specific interferon-γ responses in orthotopic liver transplantation patients with hepatitis C

HCV antigen-specific immune responses were assessed for post-OLT patients. By ELISPOT assay, HCV NS3 protein-specific IFN-γ responses were stronger in the OLT-SVR group than in the OLT-CHC group (Figure 5A). HCV core, NS4, and NS5 responses were not different with different clinical conditions. We also investigated possible associations between HCV-specific IFN-γ responses and Treg and Tr1 frequencies for post-OLT patients (Figure 5B). Patients with high-HCV NS3 responses had lower Treg frequencies, and reduced numbers of Tregs were associated with HCV eradication in post-OLT patients.

DISCUSSION

In this study, we found low frequencies of Tregs and strong HCV NS3-specific IFN-γ responses among HCV-eradicated patients after OLT. Conversely, low frequencies of Tr1 cells were found among HCV RNA-positive PNALT patients even at 40 days post-OLT. The opposite manners in
which these two types of regulatory T cells affected the clinical conditions of these patients suggest that immune cell type-specific control is necessary to control post-OLT hepatitis C.

There are two major subsets of Tregs: natural Tregs, which develop in the thymus, and adaptive Tregs, which differentiate from mature classical T cells or naturally occurring Tregs under the appropriate conditions, such as exposure to TGF-β and continuous low-dose antigen [23]. Adaptive Tregs are variously classified as CD4+CD25+forkhead box P3 (FOXP3) + Tregs, IL-10-producing Tregs (Tr1), TGF-β-producing Tregs (Th3), and double-negative T cells (CD3+CD4−CD8−). The intracellular transcription factor FOXP3 is the optimal marker for discriminating between Treg subsets, whereas the extracellular markers CD4 and CD25 are insufficient for discrimination. Recently, low CD127 expression has been accepted as an optimal surface marker for Tregs, as it was definitely correlated with FOXP3 positivity [9, 24]. Thus, we considered that CD4+CD25+CD127− cells were Tregs.

Adaptive Tregs and Tr1 have been assessed in CHC in several studies. In HCV-positive patients, the broad immune regulation observed was specific to not only HCV but also to other viruses, such as influenza [25]. Treg and Tr1 frequencies are apparently higher in patients with severe HCV recurrence after OLT than in those with no or minor recurrence [12, 21, 26, 27]. However, in our study, Treg and Tr1 frequencies showed different patterns, depending on the clinical condition after OLT. Treg frequencies were higher in patients with low platelet counts or in those with relatively high ALT levels, whereas Tr1 frequencies did not show any associations with clinical test results. Low platelet counts are suggestive of splenomegaly, and high ALT levels are suggestive of active hepatitis, which explains why patients with high Treg frequencies have severe disease. OLT patients with graft fibrosis due to HCV recurrence reportedly exhibit increased Treg frequencies [28].

In our study, OLT-HCV patients with liver fibrosis had relatively higher Treg frequencies as compared to OLT-HCV patients without fibrosis, although these differences were not significant probably because of the small number of patients. Treg frequencies could easily be affected by HCV infection, especially in severe disease, and recover after HCV eradication. Low Tr1 frequencies were evident only in OLT-PNALT patients, which suggested that they were not involved in HCV-positive conditions but in HCV-positive mild hepatitis activity. This tendency was evident even at 40 days
post-OLT, which might be a marker for predicting a later clinical course.

An HCV-specific immune response, particularly an IFN-γ Th1-producing response, is reportedly related to viral clearance. The target region of HCV, which is correlated with self-limited and IFN-induced viral clearance, was reported to be the NS3 protein [6]. In our results, a strong HCV-specific response could be achieved after several months of treatment with IFNs and several months of continuous HCV RNA negativity. Patients with HCV recurrence after OLT have high Treg frequencies, which suppress Th1 responses [29]. In our results, HCV NS3-specific Th1 immune responses were positively associated with Treg frequencies, and both of these resolved after HCV eradication following OLT. These phenomena might have resulted from HCV eradication. However, eradicating this virus by controlling the Th1 immune response is not pragmatic, as such immune induction could also induce the expansion of several immune regulatory cells [30].

IFN-based treatment for post-OLT patients with hepatitis C is known to induce chronic rejection [31, 32]. For non-OLT patients with CHC, those with PNALT have a fair outcome [33]. For post-OLT patients with hepatitis C, these phenomena recommend close monitoring of patients who do not receive IFN therapy. Treg suppressor activity was demonstrated to be stronger in CHC-PNALT patients than in active hepatitis patients [34, 35]. The results of those studies provide direct evidence to link PNALT and Treg function. In our results, Treg frequency was higher in CHC-PNALT patients, as reported previously [34, 35]; however, they were nonsignificantly lower in OLT-PNALT and OLT-SVR patients. Moreover, Tr1 frequency was higher in CHC-PNALT patients and significantly lower in OLT-PNALT patients.

During the early period following living donor liver transplantation, Treg and Tr1 frequencies were decreased comparable to the results in previous reports, probably as a result of using calcineurin inhibitors [36, 37]. Although Treg frequency at several years after OLT is poorly understood, immunosuppressor use must be involved. Our results suggest that relative reinforcement of immunosuppressants might result in a better clinical course for post-OLT patients with hepatitis C when virus eradication is difficult (i.e., those patients for which viral clearance has failed after IFN treatment). Frequent steroid pulse therapy has been shown to result in an unfavorable outcome [38]. However, the effects of mild immunosuppressant reinforcement have not been well characterized for
post-OLT patients with hepatitis C. To resolve these questions, analysis of Tr1 cells in a larger number of patients with different clinical conditions over a range of time intervals after transplantation will be necessary.

In conclusion, increased Treg frequencies and reduced HCV NS3-specific immune responses could be resolved by viral eradication in CHC patients after OLT. Reductions in Tr1 frequencies were associated with normalization of ALT levels and might prove to be an important factor for controlling CHC after OLT.
ACKNOWLEDGMENTS

This study was supported by Grants-in-Aid for Scientific Research (C) MEXT KAKENHI Grant number 22590735 and Japanese Ministry of Education, Culture, Sports, Science and Technology Grant number 22790526. We thank Taiko Kameyama, Asuka Maeda, and Chizuru Mori for doing the ELISPOT assay experiments in the Department of Gastroenterology and Hepatology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences as well as Toshie Ishii for assisting with collecting clinical data and assembling the data files.

FINANCIAL DISCLOSURE

The authors have no financial relationships relevant to this article to disclose.
REFERENCES


**FIGURE LEGENDS**

**Figure 1** Gating strategies used for isolating CD4+CD25+CD127-low Treg and CD4+CD18+CD49b+ Tr1 cells.

Upper left panel shows different peripheral blood mononuclear cell populations and gating used for the lymphocyte population. Lower left panel shows gating used for the CD4+ population. (A) In the middle right panel, CD4+ cells were separated based on anti-CD25 (ordinate) and anti-CD127 (abscissa) staining with gating for the population of CD4+CD25+CD127-low Tregs. (B) In the middle center panel, CD4+ cells were separated based on anti-CD49b (ordinate) and anti-CD18 (abscissa) staining with gating for the population of CD4+CD18+CD49b+ Tr1 cells. Lower middle and left panels show the respective isotype controls.

Abbreviations: Treg, regulatory T cells; Tr1, type 1 regulatory T cells; PI, propidium iodide

**Figure 2** Regulatory T cell and type 1 regulatory T cell frequencies among CD4+ T cells.

(A) Regulatory T cell frequencies for healthy subjects (n = 12), non-orthotopic liver transplantation (OLT) patients with chronic hepatitis C (CHC) infection (non-OLT-HCV group, n = 37), and post-OLT patients with hepatitis C (OLT-HCV group, n = 32). (B) Type 1 regulatory T cell frequencies for healthy subjects, non-OLT CHC patients, and OLT-HCV patients.

Abbreviations: Treg, regulatory T cells; Tr1, type 1 regulatory T cells; OLT, orthotopic liver transplantation; CHC, chronic hepatitis C; HCV, hepatitis C virus.

**Figure 3** Regulatory T cell frequencies in non- and post-orthotopic liver transplantation patients with chronic hepatitis C.

(A) Regulatory T cell (Treg) frequencies for non-post-orthotopic liver transplantation (OLT) patients with hepatitis C. There were no significant differences between the three groups of patients, including those with active chronic hepatitis (CHC group, n = 25), those with persistently normal alanine aminotransferase levels (CHC-PNALT group, n = 6), and those with sustained viral responses (CHC-SVR group, n = 6). (B) Serial changes in Treg frequencies pre-OLT and during the early period after OLT. Patients were divided into two groups: OLT-early patients with later persistently normal
alanine aminotransferase levels (OLT-early, later PNALT group, n = 5) and OLT-early patients with later chronic active hepatitis C (OLT-early, later CHC group, n = 5). The OLT-early, later PNALT group had a significantly higher Treg frequency than the OLT-early later CHC group before OLT (p = 0.038). (C) Treg frequencies in post-OLT patients with hepatitis C. Treg frequency was determined for three stable OLT-HCV groups: post-OLT patients with active chronic hepatitis (OLT-CHC group, n = 14), post-OLT patients with persistently normal alanine aminotransferase levels (OLT-PNALT group, n = 12), and post-OLT patients with sustained viral responses (OLT-SVR group, n = 6). Treg percentages among CD4+ T cells tended to be lower in the OLT-SVR group than in the OLT-CHC group, although not significantly different (p = 0.068).

Abbreviations: Treg, regulatory T cells; OLT, orthotopic liver transplantation; CHC, chronic hepatitis C; PNALT, persistently normal alanine aminotransferase; SVR, sustained viral response; POD, Post-operative days; POD1-10, 1 to 10 days after transplantation; POD11-20, 11 to 20 days after transplantation; POD21-30, 21 to 30 days after transplantation; POD31-40, 31 to 40 days after transplantation.

**Figure 4** Type 1 regulatory T cell frequencies in non- and post-orthotopic liver transplantation patients with chronic hepatitis C.

(A) Type 1 regulatory T cell (Tr1) frequencies in non-orthotopic liver transplantation (OLT) patients with hepatitis C. There were no significant differences between the three groups, including the CHC group (n = 25), the CHC-PNALT group (n = 6), and the CHC-SVR group (n = 6). (B) Tr1 frequencies in post-OLT early patients with hepatitis C during the early post-transplantation period. Tr1 percentages in the OLT-early patients who later had persistently normal alanine aminotransferase levels (OLT-early, later PNALT group, n = 5) decreased after OLT and Tr1 percentages in the OLT-early patients who later had chronic active hepatitis C (OLT-early, later CHC group, n = 5) increased after OLT, although these were not significantly different. (C) Tr1 frequencies in post-OLT patients with hepatitis C. Tr1 frequencies were determined for three stable OLT-HCV groups: post-OLT patients with active chronic hepatitis (OLT-CHC group, n = 14), post-OLT patients with persistently normal alanine aminotransferase levels (OLT-PNALT group, n = 12), and post-OLT patients with
sustained viral responses (OLT-SVR group, n = 6). The OLT-PNALT group had a significant decrease in Tr1 frequency as compared to the OLT-CHC group.

Abbreviations: Tr1, type 1 regulatory T cells; OLT, orthotopic liver transplantation; CHC, chronic hepatitis C; PNALT, persistently normal alanine aminotransferase; SVR, sustained viral response; POD, Post-operative days; POD1-10, 1 to 10 days after transplantation; POD11-20, 11 to 20 days after transplantation; POD21-30, 21 to 30 days after transplantation; POD31-40, 31 to 40 days after transplantation

**Figure 5** Hepatitis C virus-specific interferon-γ responses.

(A) Interferon (IFN)-γ enzyme-linked immunospot (ELISPOT) assay results, using recombinant hepatitis C virus (HCV) antigens, for post-orthotopic liver transplantation (OLT) patients with hepatitis C. HCV-NS3 specific IFN-γ responses were higher in OLT patients with sustained viral responses than in those with chronic hepatitis C. (B) Associations between regulatory T cell (Treg) and type 1 regulatory T cell frequencies and HCV-specific immune responses. A high NS3-specific immune response was associated with a lower Treg frequency. No other associations were found.

Abbreviations: OLT, orthotopic liver transplantation; CHC, chronic hepatitis C; PNALT, persistently normal alanine aminotransferase; SVR, sustained viral response; Treg, regulatory T cells; Tr1, type 1 regulatory T cells.