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

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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.

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 re-infection 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

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.

☆ Declaration: the authors declare no conflict of interest.

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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 types 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 the 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 METAVIR 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. The 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 (Fig. 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 10^6 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% CO_2 for 5 days. These cells were CD11c+ immature myeloid dendritic cells (DCs).

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