9 months after LDLT. There was neither in-hospital mortality nor graft loss. None of these patients experienced any septic complications after ABO-I LDLT. High-dose IVIG was used in six patients with suspected anti-blood type isoagglutinin-mediated rejection (cases 2, 3, 4, 5, 14, and 15). Biopsy-proven acute cellular rejection occurred in four patients (case 1 at 1044 posttransplant days [postoperative day (POD)], case 2 at 650 POD, case 3 at 104 POD, and case 8 at 117 POD), all of which responded to steroid pulse therapy.

Kinetics of Anti-Blood Type Isoagglutinin Titers Before and After ABO-I LDLT

Fig. 1 and **SDC 1** (see **Figure,** http://links.lww.com/TP/A515) show that anti-blood type isoagglutinin titers ranged between 2^3 and 2^{12} before PEs, and these titers rapidly decreased after PEs. Anti-blood type isoagglutinin titers were successfully decreased to 2^6 or less in all but cases 14 and 15 at the time of LDLT. Immediate posttransplant rebound of these titers was observed in the cases 1 and 2 (rebound is defined as titer increasing up to or beyond the pretreatment titer). Rebound titers were observed for anti-donor blood type A isoagglutinin in the case 2 and for non–anti-donor blood type B isoagglutinin. Anti-blood type isoagglutinin titers after ABO-I LDLT were generally lower than each respective pretreatment titer. Both anti-blood types A and B isoagglutinin titers were measured in the cases 2, 13, 14, and 15 with blood type O. This revealed both anti-A and -B isoagglutinin titers generally lower than each pretreatment titer irrespective of the donor's blood type, although the anti-donor blood type isoagglutinin titers never reached 2^0. Anti-donor blood type isoagglutinin titers in the cases 2, 13, and 14 were constantly lower than each respective non–anti-donor blood type isoagglutinin titer. All but the hepatitis C patients maintained good liver function tests despite the sustained presence of anti-donor blood type isoagglutinin titers during the mid- and long-term posttransplant periods (hepatitis C patients had fluctuations in their liver function tests caused by recurrent hepatitis C).

Catheter-related sepsis occurred after PEs in the cases 4 and 14, and their scheduled LDLTs were postponed. The anti-blood type isoagglutinin titers rapidly returned to each respective pretreatment titer during the treatment of sepsis, and further PEs were needed to lower the anti-blood type isoagglutinin titers just before the rescheduled LDLTs.

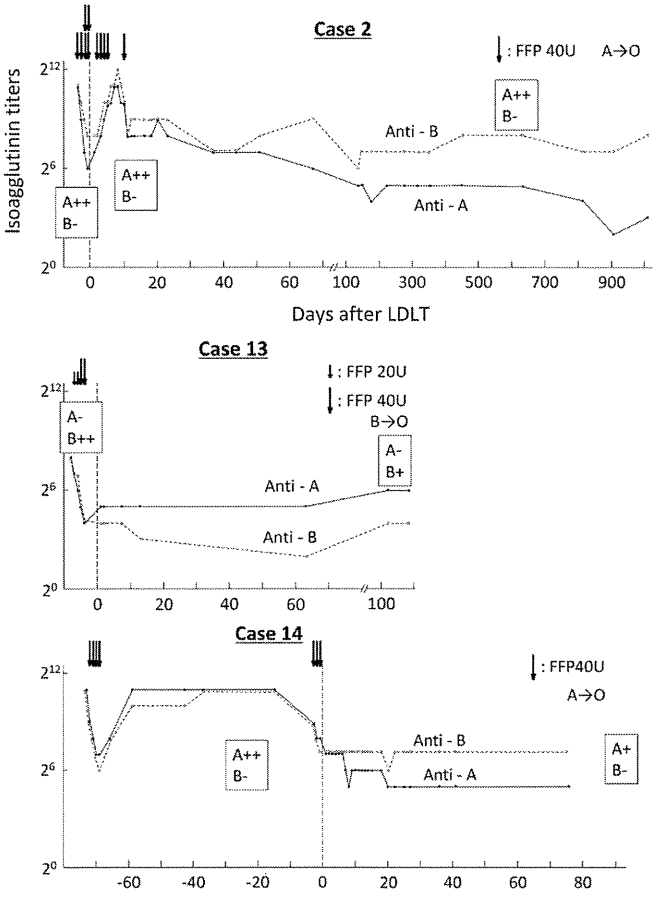

FIGURE 1. Representative kinetics of anti-blood type isoagglutinin titers and expression of blood A/B antigens in biopsy specimens before and after ABO-incompatible living donor liver transplantation. *Solid lines* and *dotted lines* indicate anti-A and -B titers, respectively. The *arrows* indicate plasma exchange. Symbols (e.g., A++ and B−) in a rectangle indicate the expression of blood A or B antigens on graft livers.

Expression of Blood A/B Antigens on Pretransplant and Posttransplant Liver Biopsies

Time-zero biopsies revealed the respective blood type antigens for each donor (Fig. 1; see **Figure, SDC 1,** http://links.lww.com/TP/A515 and Fig. 2). Positive immunostaining was mainly observed in the endothelial cells and sinusoidal cells. Posttransplant liver biopsies continued to express the blood type antigens of the donor (Fig. 2).

Restoration of B Lymphocytes After Rituximab Treatment

Seventy samples were available for flow cytometry of the peripheral blood. CD19-positive lymphocytes rapidly disappeared in the peripheral blood after rituximab treatment (Fig. 3). Restoration of CD19-positive lymphocytes started approximately 6 months after the rituximab treatment.

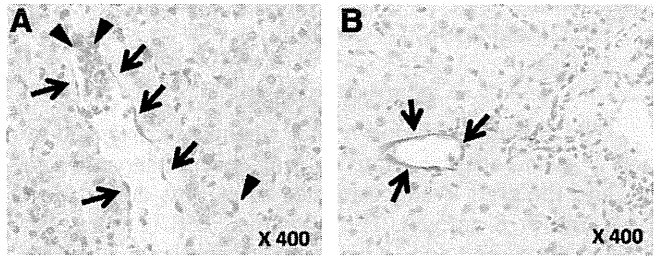

FIGURE 2. Expression of blood A antigens on posttransplant biopsy specimen. These are representative immunostaining for blood type antigens. (A) The zero-time biopsy (taken from the case 2 donor liver just before transplantation) exhibited positive for blood A antigen immunostaining for almost all endothelial cells (*arrows*) and donor red blood cells (*arrow heads*). (B) The posttransplant biopsy (taken from the case 2 recipient at 650 days after living donor liver transplantation) was also positive for blood A antigen immunostaining for almost all endothelial cells (*arrows*) but not for red blood cells.

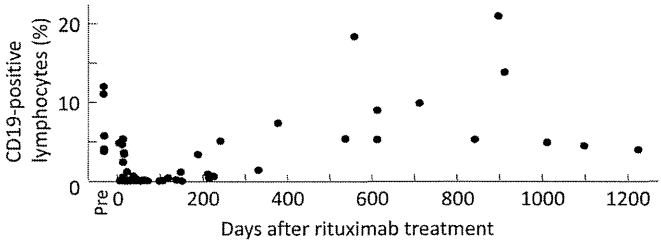

FIGURE 3. Kinetics of the percentages of CD19-positive lymphocytes in the blood after ABO-incompatible living donor liver transplantation. A total of 70 samples obtained from 15 ABO-incompatible living donor liver transplant patients were analyzed and plotted. The percentages of CD19-positive lymphocytes rapidly decreased to zero after rituximab administration and started to recover approximately 6 months later.

High Incidence of Cytomegalovirus Antigenemia in ABO-I Living Donor Liver Transplant Patients

The incidence of cytomegalovirus (CMV) antigenemia (defined as antigen detection of 10 or more in 150,000 white cells) was higher in the ABO-I group (57.1%) than the control group (11.1%; $P=0.02$); however, none of these patients became symptomatic and were easily treated by ganciclovir.

Accelerated Hepatitis C Viremia in ABO-I Living Donor Liver Transplant Hepatitis C-Positive Patients

All seven hepatitis C virus (HCV)-RNA-positive patients developed biopsy-proven recurrence of hepatitis C (case 4 at 55 POD, case 5 at 95 POD, case 6 at 184 POD, case 12 at 36 POD, case 13 at 133 POD, case 14 at 90 POD, and case 15 at 39 POD) and underwent pegylated interferon and ribavirin therapy. Only case 4 patient achieved sustained virological response by the final follow-up. Both ABO-I and control patients showed significantly increased 1-month posttransplant HCV-RNA loads in comparison to the pretransplant viral loads. However, Fig. 4 shows that the increases of HCV-RNA (HCV-RNA load at 1 month after transplantation minus HCV-RNA load at pretransplantation) in ABO-I patients (median 2.85 [range 1.8–3.8] logIU/mL) were significantly higher than those in the control patients (median 0.6 [range −0.7 to 2.2] logIU/mL) ($P<0.0001$).

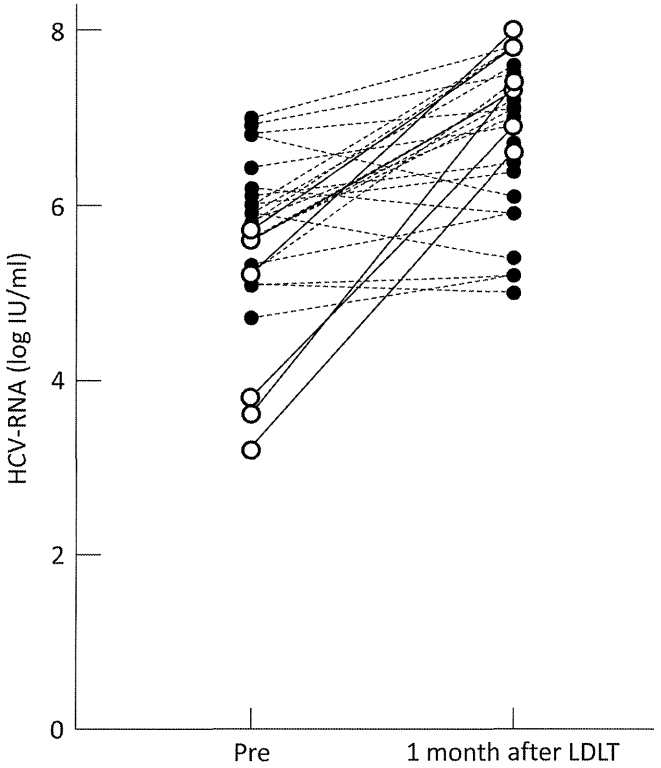

FIGURE 4. Kinetics of HCV-RNA titers before and 1 month after living donor liver transplantation. The pretransplantation HCV-RNA titers and those 1 month after transplantation are plotted. The *black dots* represent the data obtained from the control patients, whereas the *white dots* indicate the data obtained from the ABO-incompatible living donor liver transplant hepatitis C-positive patients. HCV, hepatitis C virus.

DISCUSSION

The anti-donor blood type isoagglutinin titers did not reach 2^0 even with the relatively long-term observation period, and the graft livers continued to express donor blood type A and/or B antigen. The scheduled LDLTs were postponed in the cases 4 and 14 because those patients developed catheter-related sepsis, and the interruption of PEs resulted in the rapid increase of anti-blood type isoagglutinin titers to the pretreatment levels. Liver grafting itself may cause the removal of circulating anti-blood type isoagglutinins. In other words, graft livers may absorb these antibodies. Both anti-blood type A and B isoagglutinins were analyzed in the cases 2, 13, 14, and 15 with blood type O. The cases 2, 13, and 14 showed anti-donor blood type isoagglutinin titers lower than non–anti-donor blood type isoagglutinin titers, which may indicate the graft livers were absorbing circulating anti-donor blood type isoagglutinins (*10*). Fig. 1, **SDC 1** (http://links.lww.com/TP/A515), and Figure 2 show that there was persistent expression of donor antigen on hepatic grafts with normal liver function irrespective of the persistent presence of anti-donor blood type isoagglutinins, even several months after LDLT. Do these circulating isoagglutinins attack ABO-I hepatic grafts? Or do ABO-I hepatic grafts become resistant to these isoagglutinins (*11*)? Tanaka et al. (*12*) reported intragraft expression of recipient-type ABO blood group antigens. Tanabe et al. (*13*) recently reported the decrease of antigenicity of graft endothelia after ABO-I kidney transplantation. These intragraft changes may partially contribute to this phenomenon.

The timing of rituximab administration before ABO-I LDLT is crucial because it takes at least 3 weeks after administration to achieve the maximum effect of rituximab (*14*). Rituximab was administered only 3 days before transplantation in the two fulminant cases (cases 1 and 2), which may have resulted in incomplete elimination of peripheral B lymphocytes and caused the rebound of anti-blood type isoagglutinin titers immediately after transplantation. These observations are similar to those of Egawa et al. (*15*). Rituximab was administered approximately 3 weeks before scheduled ABO-I LDLT in the remaining 13 cases, and they did not experience any rebound of anti-blood type isoagglutinin titers. These results may simply represent good fortune because the rapid increase of anti-donor blood type isoagglutinin ti-

ters may lead to a graft loss caused by accelerated anti-blood type isoagglutinin-mediated rejection, and there were only two successful cases with fulminant hepatic failure. More cases are needed to standardize the use of rituximab administration for ABO-I LDLT in patients with fulminant hepatic failure.

The most serious concern in ABO-I LDLT is that infectious complications may occur more frequently in ABO-I LDLT cases than ABO-identical or -compatible cases because of the intense immunosuppression protocol (*16*). None of the 15 patients experienced septic complications after the application of the current protocol for ABO-I LDLT. The differences between the immunosuppression protocol for ABO-I LDLT and ABO-identical or -compatible LDLT are pretransplant rituximab administration, pretransplant mycophenolate mofetil (MMF) use, and PEs just before LDLT. Local graft infusion was used early in the series (case 1 and the other two patients without rituximab use) and was often associated with catheter-related complications. Local graft infusion therapy is no longer used in ABO-I LDLT because it was associated with serious catheter-related complications or sepsis. The major cause of death in ABO-I LDLT patients in the past was septic complications secondary to intense immunosuppression and catheter-related complications (*17*, *18*). We believe that severe anti-blood type isoagglutinin-mediated rejection is mainly caused by accelerating the anti-donor blood type isoagglutinin production. Antigen-stimulated B lymphocytes differentiate into antibody-producing plasma cells (*19*). Rituximab inhibits the production of accelerating isoagglutinin by eliminating peripheral mature B lymphocytes. The use of rituximab with ABO-I LDLT may eliminate the need for graft local infusion therapy. The simplicity of the current protocol may allow patients with ABO-I LDLT to avoid septic complications.

There is also concern whether a splenectomy should be performed during ABO-I LDLT. Egawa et al. (*4*) described that prophylaxis with rituximab might take the place of splenectomy in ABO-I LDLT. Unlike cadaveric whole liver transplantation, adult-to-adult LDLT patients receive only a small partial hepatic graft, and there have been several reports regarding either persistent portal hypertension or pancytopenia caused by hypersplenism and the beneficial effects of splenectomy in adult-to-adult LDLT patients (*20–23*). We performed splenectomy in our LDLT cases to control portal venous pressure after reperfusion and to treat pancytopenia (*24*), not to induce the immunosuppressive effects associated with a splenectomy. However, all 15 patients eventually underwent simultaneous splenectomy during LDLT. The role of splenectomy in ABO-I LDLT remains to be elucidated.

Peripheral B lymphocytes in the blood disappeared for 6 months after rituximab administration and began to recover approximately 6 months later (Fig. 3). The recipients may be vulnerable to various pathogens during this period because of the lack of B-cell immunity. Although no symptomatic CMV infection occurred in the current series, there was a high incidence of CMV antigenemia in ABO-I LDLT cases. At the present time, there is no known relationship between the number of PEs or the amount of immunosuppression and the incidence of CMV antigenemia. Furthermore, a rapid increase of HCV-RNA viral loads was observed in the ABO-I LDLT patients in comparison to the control patients. All seven HCV-RNA positive ABO-I LDLT patients experienced recurrence of hepatitis C early after LDLT and were treated by pegylated interferon and ribavirin therapy. Only one patient had achieved a sustained virological response by the final follow-up. The cases 13 and 15 patients whose hepatitis C viral load steeply increased early after LDLT experienced fluctuating liver function test results with a rapid increase in the total bilirubin after LDLT. We believed that fibrosing cholestatic hepatitis might have occurred in these patients and obtained several biopsy specimens, which did not show any signs of fibrosing cholestatic hepatitis but did show many acidophil bodies. We therefore speculated that severe viremia itself caused the graft liver injury and initiated interferon therapy for these patients, which effectively stabilized their liver function tests. No conclusions can be drawn concerning the safety of this protocol for HCV-positive patients, because of the small number of HCV-positive patients and the short follow-up period.

Further cumulative case studies are required to determine the safe anti-donor blood type isoagglutinin titer levels at ABO-I LDLT. The initial assumption was that pretransplant anti-donor blood type isoagglutinin titers should be decreased to less than 2^5 by repeat PEs. However, two patients (cases 14 and 15) underwent ABO-I LDLT who had high anti-donor blood type isoagglutinin titers of 2^6 and 2^7, and they survived the early posttransplant period, although both patients received IVIG therapy under a suspicion of anti-blood type isoagglutinin-mediated rejection. The higher the anti-blood type isoagglutinin titers are at ABO-I LDLT, the higher the incidence of anti-blood type isoagglutinin-mediated rejection is likely to be. The pretreatment anti-donor blood type isoagglutinin titer was 2^3 in the case 9, and the patient did not require PEs. The results in the cases 14 and 15 suggest that pretransplant PEs may not be required if the pretransplant anti-donor blood type isoagglutinin titer is below 2^7.

Haga et al. (*25*, *26*) suggested that C4d-positive staining or periportal edema and necrosis can be a hallmark of acute humoral rejection in ABO-I LDLT. The protocol using rituximab for ABO-I LDLT was only recently developed, and this treatment may affect histological features of anti-blood type isoagglutinin-mediated rejection by depleting mature B cells. Further biopsy data are needed to establish histological diagnostic criteria for anti-blood type isoagglutinin-mediated rejection after rituximab treatment. The only effective treatment for ongoing anti-blood type isoagglutinin-mediated rejection is high-dose IVIG. This treatment has no serious adverse effects in contrast to steroid pulse therapy. Therefore, patients were treated with high-dose IVIG when the clinical data suggested anti-blood type isoagglutinin-meditated rejection. The assessment of clinical data is currently more important than biopsy evaluations for diagnosing anti-blood type isoagglutinin-mediated rejection.

In conclusion, ABO-I LDLT with our protocol is considered to be a safe option when an ABO-identical or -compatible donor is not available. Further study must be carried out to determine the safe pretransplant anti-donor blood type isoagglutinin titers and whether the application of this protocol to HCV-positive patients is justified.

MATERIALS AND METHODS

Patients

Fifteen patients underwent ABO-I LDLT in our hospital between November 2005 and December 2010. The patients' characteristics are summarized in Supplemental Table 1 (see **SDC 3,** http://links.lww.com/TP/A517). The case 2 was presented in a previous case report (*5*). Informed consent was obtained from each recipient and each donor after gaining approval from the Institutional Ethics and Indications Committees for each ABO-I LDLT. The patients were treated with previously described surgical techniques (*27, 28*). All 15 patients had a simultaneous splenectomy. The surgical and patients' medical records were retrospectively reviewed. The data of 27 HCV-positive patients who underwent ABO-identical or -compatible LDLT between July 2008 and December 2010 and survived more than 100 days after LDLT were reviewed as a control group. All HCV-positive patients underwent splenectomy for later interferon treatment. Control patients were treated with a triple immunosuppression regimen of oral calcineurin inhibitor (tacrolimus [initial target trough level at 10 ng/mL] or cyclosporine A [initial target trough level at 200 ng/mL]), MMF (initial dose of 1000 mg/day), and steroid. All patients were prophylactically administered 600 mg/day of oral acyclovir. No prophylaxis was used for CMV infection in any of the patients.

Current Protocol for ABO-I LDLT

Elective cases are treated with rituximab (375 mg/m^2) 21days before LDLT (see **Figure, SDC 2,** http://links.lww.com/TP/A516). MMF (1000 mg/body/day) is initiated 7 days before LDLT and continues several months after LDLT. Several sessions of PE using blood type AB fresh frozen plasma are performed to lower the anti-blood type isoagglutinin titers less than 2^4-fold just before LDLT. Simultaneous splenectomy is performed. One thousand milligram of methylprednisolone is administered just after graft reperfusion, and the daily doses of methylprednisolone are tapered to 20 mg/day over 7 days after LDLT. The administration of oral calcineurin inhibitor (tacrolimus [initial target trough level at 10 ng/mL] or cyclosporine A [initial target trough level at 200 ng/mL]) is initiated 1 day after LDLT. High-dose IVIG (600 mg/kg/day) is administered for 5 days when anti-blood type isoagglutinin-mediated rejection is suspected (e.g., rapid increase of total bilirubin and rapid decrease of platelet count) (*9, 29*).

The cases 1 and 2 patients could not receive rituximab 21 days before LDLT because of fulminant hepatic failure and it was administered 3 days before LDLT. The case 14 patient had sepsis caused by a catheter-related infection. The catheter was inserted for PE. Because of the severe infection, the planned LDLT had to be postponed, which resulted in an elevation of the anti-blood type isoagglutinin titers up to the pretreatment levels. Approximately 2 months later, the patient had to undergo several sessions of PE again. We were afraid that the retention of the catheter for PE for more than 3 days would cause bacterial sepsis again. We therefore planned three sessions of PE, and the catheter was removed within 3 days, although the isoagglutinin titer at LDLT was more than 2^4. As a result, the anti-blood type isoagglutinin titer at LDLT in case 14 was 2^7.

The case 15 patient had high pretreatment anti-blood type isoagglutinin titers, and we tried to lower the titers less than 2^4 by PE. However, the patient had a severe allergic reaction to fresh frozen plasma, and he could no longer tolerate any PE. We had to perform his LDLT while he still had the high anti-blood type isoagglutinin titers.

Histological Evaluation of Blood Type A/B Antigen Expression on Graft Livers

Time zero-biopsies were available in 12 cases of ABO-I LDLT, and a total of 19 posttransplant biopsies (obtained on the events of hepatitis C recurrence or acute cellular rejection) could be evaluated for the expression of blood type A/B antigen on graft livers. Monoclonal antibody 7LE for Lewis^a blood group antigen and 2-25LE for Lewis^b blood group antigen (Exbio Praha, Vestec, Czech Republic) were used for immunostaining according to the manufacturer's instructions. The expression of blood type was graded as follows: −, no positive immunostaining; +, positive immunostaining restricted to part of endothelial cells; ++, positive immunostaining for almost all endothelial cells; and +++, positive immunostaining for sinusoidal cells and endothelial cells.

Measurement of Anti-Blood Type Isoagglutinin Titers, the Number of B Lymphocytes, CMV Antigen, and HCV-RNA in the Blood

The anti-blood type isoagglutinin titers for IgG were serially measured, and those values were expressed as 2^0, 2^1, 2^2, 2^3, so forth (tests for IgM were not available in our hospital). The numbers of B lymphocytes in the blood were calculated as %CD19-positive lymphocytes using flow cytometry (FACSCanto; Becton Dickinson, Franklin Lakes, NJ). We used CD19 to follow the number of B lymphocytes because the administration of rituximab can interfere with the detection of CD20 by flow cytometry, especially when examined early after rituximab administration (*14*). CMV antigenemia (detection of pp65 antigen on white cells) was examined approximately every 2 weeks or when necessary. Previral loads of HCV before any treatment and postviral loads in the posttransplant periods were determined by real time-PCR.

Statistics

Continuous variables were expressed as the median and range and compared between two groups by the Wilcoxon rank sum test. Fisher's exact test was used to compare frequencies. Statistical significance was defined as having a P value less than 0.05. All statistical analyses were performed using the NCSS 2007 software package (Hintze JL, Kaysville, UT).

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SURGERY TODAY

Case Report

De Novo Autoimmune Hepatitis Subsequent to Switching from Type 2b to Type 2a Alpha-Pegylated Interferon Treatment for Recurrent Hepatitis C After Liver Transplantation: Report of a Case

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Abstract
Interferon (IFN), which is the only possible agent for recurrent hepatitis C after liver transplantation, may cause serious immune-related disorders. We report a case of de novo autoimmune hepatitis (AIH), which developed subsequent to switching from 2b pegylated interferon-α (peg-IFN) to 2a peg-IFN after living donor liver transplantation (LDLT). A 51-year-old man with hepatitis C-associated liver cirrhosis underwent LDLT. About 13 months after the initiation of antiviral therapy, in the form of type 2b peg-IFN with ribavirin, a negative serum hepatitis C virus (HCV)-RNA titer was confirmed. Thereafter, the 2b peg-IFN was switched to 2a peg-IFN, 3 months after which severe liver dysfunction developed, despite a constantly negative HCV-RNA. Liver biopsy showed portal and periportal inflammatory infiltrates including numerous plasma cells, indicating AIH. He was treated with steroid pulse treatment, followed by high-level immunosuppression maintenance, but eventually died of *Pneumocystis* pneumonitis 4 months after the diagnosis of de novo AIH.

Key words Liver transplantation · Autoimmune hepatitis · Pegylated interferon-α

Introduction

Although liver transplantation (LT) is often indicated for hepatitis C-associated hepatic disorders, the difficulties associated with controlling recurrent hepatitis C after LT remain a major problem.[1–4] At present, pegylated interferon-α (peg-IFN) with ribavirin is the only hopeful regimen for treating recurrent hepatitis C after LT, and it has been widely used clinically with sustained viral response rates of 43%–55%.[1–3] The major obstacles to successful interferon (IFN) treatment are its frequent serious side effects, including fever, malaise, pancytopenia, depression, and pulmonary, hepatic or renal dysfunction.[5,6] Interferon may also cause autoimmune or immune-related disorders, including autoimmune thyroiditis or rheumatoid arthritis;[5] however, chronic rejection and autoimmune hepatitis (AIH) have rarely been described as side effects of IFN after LT.[1,5–7] However, little is known about the impact of subgroups of peg-IFN, such types 2a and 2b. We report a case of de novo AIH, which may have been triggered by switching from type 2b peg-IFN to type 2a peg-IFN during treatment for recurrent hepatitis C after living donor liver transplantation (LDLT).

Case Report

The patient was a 51-year-old man with end-stage liver disease secondary to liver cirrhosis and hepatocellular carcinoma caused by hepatitis C virus (HCV) infection. His HCV genotype was 1b. He underwent LDLT using a right lobe graft donated by his wife. The patient's human leukocyte antigen (HLA) mismatch number was 5/6. Immunosuppression was induced with basiliximab and mycophenolate mofetil (MMF) with cyclosporine without steroids, followed by maintenance with cyclosporine monotherapy. An increase in the serum alanine aminotransferase level and the HCV-RNA titer (>5000 kIU/ml) was noted 5 months after the LDLT. After confirming pathological recurrent hepatitis C, he was commenced on antiviral therapy comprising peg-IFN 2b (1.5 μg/kg/week) and ribavirin (400 mg/day).

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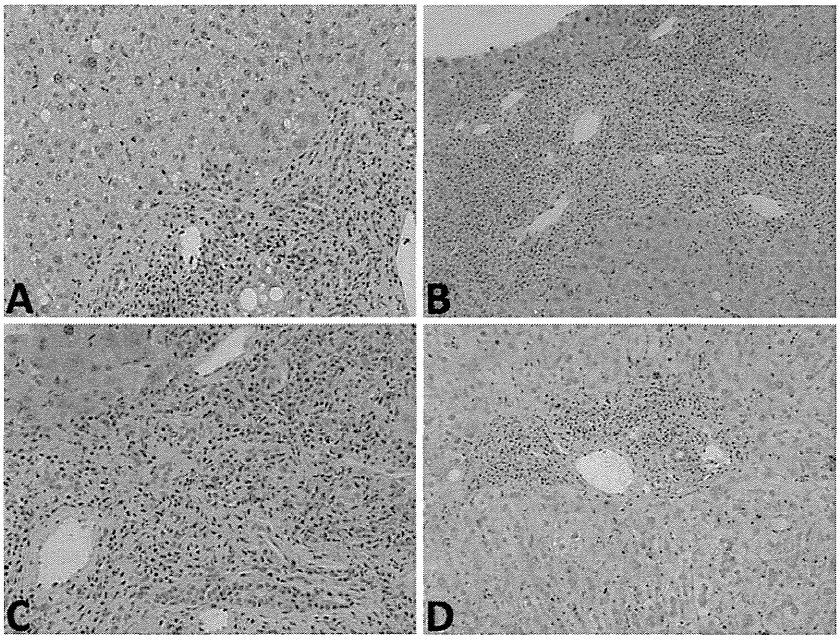

Fig. 1. A Liver biopsy 12 months after living donor liver transplantation showed only mild lymphocytic inflammatory cells in the portal and periportal zones (hematoxylin–eosin, ×200). **B** Liver biopsy at the time of the de novo autoimmune hepatitis (AIH) development showed no bile duct damage (hematoxylin–eosin, ×200). **C** Magnified image of the biopsy section in **B** showing inflammatory infiltrates. Portal and periportal inflammatory infiltrates with numerous plasma cells were also observed (hematoxylin–eosin, ×400). **D** Liver biopsy after normalization of the liver function tests. The inflammatory infiltrates remain but the plasma cells in the infiltrates have disappeared (hematoxylin–eosin, ×200)

This continued for 2 months, but was suspended for 5 months owing to side effects including anemia. The therapy was then restarted and his serum HCV-RNA titer became negative after 7 months. After 12 months of therapy, a protocol liver biopsy showed only mild lymphocytic inflammatory cells in the portal and periportal zones (Fig. 1A). After 17 months, peg-IFN 2b was switched to peg-IFN 2a (180 μg/body/week) and the ribavirin was stopped, because the general health insurance system in Japan limited the use of the same peg-IFN and ribavirin to a maximum of 48 weeks. About 3 months after this change severe hepatic dysfunction developed, with negative serum HCV-RNA titers, although the antinuclear antibodies remained positive (Fig. 2). A liver biopsy was performed, and the histology revealed portal and periportal inflammatory infiltrates including numerous plasma cells, but no eosinophils or lymphocytes, or bile duct damage. These findings were indicative of AIH and not acute or chronic rejection (Fig. 1B,C). Interferon treatment was ceased immediately and steroid pulse therapy (methylprednisolone 1000 mg/day for 3 days) was started. The immunosuppression regimen was converted from cyclosporine monotherapy to tacrolimus with daily MMF and steroids. A liver biopsy 1 month later still showed inflammatory infiltrates, although the plasma cells had disappeared (Fig. 1D). Since the serum alanine aminotransferase level was still above 50 IU/l and the serum alanine aminotransaminase level was decreasing very gradually, the maintenance immunosuppression level remained relatively high. The tacrolimus level was maintained at between 12 and 14 ng/ml, and the prednisolone dosage was tapered from 20 mg daily to 10 mg daily over 2 months. During these periods of immunosuppression, the HCV-RNA titer became positive. About 3 months after the onset of the de novo AIH the patient was readmitted in acute respiratory distress, and severe *Pneumocystis carinii* pneumonitis was diagnosed. Despite intensive treatment with sulfamethoxazole and trimetoprim under mechanical ventilation support and continuous hemodialysis, the patient died of multiorgan failure.

Discussion

Hepatitis C virus reinfection almost always occurs after LT for HCV-related liver cirrhosis, and the requirements for appropriate treatments are obvious.[1–3] Interferon is currently the only agent that can achieve HCV clearance, but this is dependent on whether this therapy can be completed without any adverse events.[1,4,5] The more common side effects of IFN, such as malaise, fever, or pancytopenia, may necessitate a reduction in the dose of IFN, while other side effects can require that the therapy is discontinued, often followed by troublesome treatment for serious events, such as depression, pancytopenia, and pulmonary, hepatic or renal dysfunction.[5,6] This case of de novo AIH, which finally caused graft loss, must be regarded as a serious complication. Interestingly, it may have been triggered by the switch from peg-IFN 2b to peg-IFN 2a.

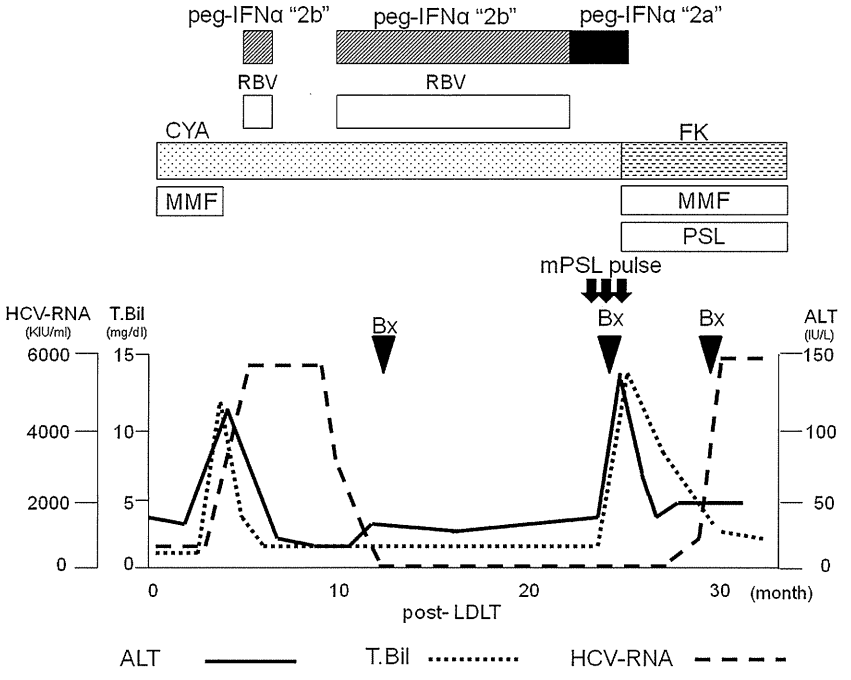

Fig. 2. Clinical course of the patient after living donor liver transplantation (*LDLT*). *peg-IFNα 2b*, pegylated interferon-α type 2b; *peg-IFNα 2a*, pegylated interferon-α type 2a; *RBV*, ribavirin; *CYA*, cyclosporine; *FK*, tacrolimus; *MMF*, mycophenolate mofetil; *PSL*, prednisolone; *mPSL*, methylprednisolone; *HCV-RNA*, hepatitis C virus RNA; *T.Bil*, total bilirubin; *ALT*, alanine aminotransferase; *Bx*, liver biopsy

Table 1. Clinical features of the 15 reported patients with de novo autoimmune hepatitis induced by pegylated interferon therapy

Age of patient (years), sex	HCV genotype	Pegylated IFN-α	Months of IFN therapy	HCV-RNA at the onset of de novo AIH	First author[Ref.]	Year
52, M	NA	2b	10	Positive	Chologitas[8]	2006
55, M	1	2a	9	Negative	Kontorinis[9]	2006
55, M	1	2b	11	Negative	Berardi[7]	2007
56, F	1	2a	9	Negative	Merli[10]	2009
58, M	1	2b	6	Negative	Berardi[7]	2007
59, M	1	2b	12	Negative	Berardi[7]	2007
59, M	1	2a	13	Negative	Merli[10]	2009
60, M	1	2b	1	Negative	Berardi[7]	2007
60, M	2	2b	12	Negative	Berardi[7]	2007
61, F	1	2b	6	Negative	Berardi[7]	2007
62, F	4	2b	10	Negative	Berardi[7]	2007
63, M	1	2a	7	Negative	Merli[10]	2009
65, M	1	2b	6	Negative	Berardi[7]	2007
66, M	1	2b	13	Negative	Berardi[7]	2007
51, M	1	2b→2a	20 (3 months after switching)	Negative	Present case	

IFN, interferon; HCV, hepatitis C virus; AIH, autoimmune hepatitis; HCV-RNA, hepatitis C virus RNA; NA, not available; 2b, pegylated interferon-α 2b; 2a, pegylated interferon-α 2a

Our search of the literature found 15 cases of de novo AIH related to IFN treatment after LT, including the present case (Table 1).[7–10] In all of the previous reports, the de novo AIH was speculated to have been caused by immune responses stimulated by IFN. Berardi et al.[7] reported that the incidence of de novo AIH in LT patients treated with IFN was 17%, which was higher than that in non-IFN-treated LT patients. Interferon induces natural killer cells and cytotoxic T lymphocytes, which polarize the adaptive immune responses to Th1, thereby causing antiviral responses.[11] Conversely, it has also been proposed that the imbalance toward Th1-mediated responses induced by IFN may be an important factor in IFN-induced autoimmunity. Interferon itself may become a major risk factor for de novo AIH.

The pathogenesis of autoimmune diseases induced by peg-IFN was reported to be associated with the dose of peg-IFN.[12] Peg-IFN, with higher activity and a longer half-life than native IFN-α, was developed for maintenance of the activity of IFN by conjugation with poly-

ethylene glycol. Since the duration of peg-IFN therapy is consequently longer, the accumulation of IFN becomes higher and the activity becomes stronger. In all but one of the previously reported cases of de novo AIH, the duration from the start of the antiviral therapy to the onset of de novo AIH was more than 6 months (Table 1). The de novo AIH in our patient, which occurred after 20 months of IFN treatment, may have been caused by an excess dose of IFN.

Hepatitis C virus itself is associated with autoimmune disorders that are independent of IFN, since antigen mimicry exists between HCV proteins and autoantigens.[13] Interestingly, in all but two of the reported cases, the HCV-RNA had disappeared before the onset of the de novo AIH (Table 1). Therefore, it has been hypothesized that loss of the viral target of the immune responses activated by IFN to promote virus clearance may lead to costimulatory signals that break the tolerance for the transplanted graft. In the present case, HCV recurred after LDLT and the HCV-RNA disappeared before the onset of the de novo AIH; thus, HCV may have triggered the de novo AIH.

Although de novo AIH may be caused by both peg-IFN 2b and peg-IFN 2a (Table 1), there have been no reports of de novo AIH after switching the IFN regimen, even among non-LT cases. Two kinds of peg-IFN are used for treating chronic hepatitis C: peg-IFN 2a (Pegasys; Roche, Basel, Switzerland) and peg-IFN 2b (Peg-Intron; Schering-Plough, Kenilworth, NJ, USA). The main difference between these products is their molecular weight, since peg-IFN 2a is 40 kDa while peg-IFN 2b is 12 kDa. The larger molecular weight of IFN accounts for a longer effective duration but less activity.[14,15] Consequently, peg-IFN 2a has a longer effective duration but less activity than peg-IFN 2b. The change in type of peg-IFN may have triggered changes to the immune system of our patient, considering that the biopsy taken before switching the IFN therapy did not reveal any signs of AIH (Fig. 1A). Moreover, no drugs other than peg-IFN were altered and the blood concentration of the immunosuppressive remained unchanged before AIH developed. Finally, the time course from the switch in the regimen to the development of the de novo AIH was short (Fig. 2). Considering these facts, the de novo AIH in the present case may have been induced by the change in the biological activity derived from switching the IFN regime.

In summary, we reported a case of de novo AIH, which may have been triggered by switching from peg-IFN 2b to peg-IFN 2a during treatment for recurrent hepatitis C after LDLT. The IFN therapy regimen is often altered in patients with chronic hepatitis C who have failed to respond to a previous IFN therapy, and such conversion has been reported to be effective.[16] Thus, it is likely that switching the IFN regimen will become more common in the treatment of nonresponders or relapsers after LT. Since a change in the IFN regimen may be a risk factor for de novo AIH, clinicians should monitor their LT patients carefully when this is done.

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Case Report

The Impact of IL28B Genetic Variants on Recurrent Hepatitis C in Liver Transplantation: Significant Lessons from a Dual Graft Case

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IL28B genetic polymorphism is related to interferon-sensitivity in chronic hepatitis C, but the significance of grafts carrying different genotypes from recipients is still unclear in liver transplantation. A 51-year-old Japanese male carrying a minor genotype underwent dual liver transplantation for liver cirrhosis due to hepatitis C virus (HCV). The left lobe graft carried a major genotype, and the right a minor genotype. He achieved virological response during the course of pegylated-interferon and ribavirin therapy against recurrent hepatitis C for 2 years, but HCV relapsed immediately at the end of the therapy. Two years after antiviral therapy, liver biopsy was performed from each graft. The specimens showed A1F0 in the left lobe graft and A2F2 in the right. Moreover, quantitative polymerase chain reaction was performed using RNA extracted from each specimen to see there was no HCV RNA in the left lobe whereas there was in the right. This case provides clear evidence that IL28B genetic variants determine interferon sensitivity in recurrent hepatitis C following liver transplantation, which could result in new strategies for donor selection or for posttransplant antiviral therapy to HCV positive recipients.

Key words: Dual grafts, hepatitis C, IL28B, liver transplantation

Abbreviations: HCV, hepatitis C virus; LT, liver transplantation; PEG-IFN, pegylated interferon; RBV, ribavirin; LDLT, living donor liver transplantation; SVR, sustained virological response; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; SLV, standard liver volume; VR, virological response; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ISG, interferon stimulated gene.

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Introduction

Hepatitis C virus (HCV) and its related diseases are the leading cause of liver transplantation (LT) worldwide (1). Despite LT, graft reinfection by HCV occurs in almost all cases following transplantation and the outcome of post-transplant antiviral therapy is very poor (2). Although the combination of pegylated interferon and ribavirin (PEG-IFN/RBV) is the standard antiviral therapy for HCV, it is expensive and has some side effects such as inducing a flu-like syndrome, pancytopenia and autoimmune hepatitis, which can be extremely detrimental to an individual after LT. Precise prediction for IFN sensitivity in recurrent hepatitis C after LT is urgently required.

The IL28B genetic polymorphism has been reported to be significantly related with the outcome of PEG-IFN/RBV therapy for chronic hepatitis C (3). A low virological response rate has been demonstrated in patients who carry the minor genotype at rs8099917 (TG or GG), located approximately 8000 bp downstream from the IL28B gene, compared with those who carry the major genotype (TT). Recently, we demonstrated that this genetic variant was also related to the outcome of posttransplant PEG-IFN/RBV therapy for recurrent hepatitis C (4). The sustained virological response (SVR) rate is relatively high in recipients carrying the major genotype with grafts from donors carrying the major genotype. No recipients carrying the minor genotype achieved a SVR with a graft from a donor carrying the minor genotype. However, accumulation of a greater number or deceased-donor cases is necessary because that study included less than a 100 living-donor cases. Many factors other than IL28 genetic variants could affect the outcome of antiviral therapy, such as age, sex, fibrosis and viral mutations (5–7). The significance of the graft from donors carrying the major genotype in IL28B for the recipients carrying the minor genotype is not yet fully understood.

We previously reported the case of a living-donor liver transplantation (LDLT) using dual grafts from two donors (8). The recipient carrying the minor genotype (T/G)

recently had liver biopsies performed from each graft after PEG-IFN/RBV therapy for recurrent hepatitis C. The donor of the right lobe carried the minor genotype (TG) at rs8099917, but the donor of the left lobe carried the major genotype (TT).

In this paper, we describe the outcomes of two liver grafts with different IL28B genetic variants in one individual.

Materials and Methods

DNA extraction and direct sequencing

The recipient's and each donor's DNA was extracted from their exenterate liver tissues at transplantation and direct sequencing was performed using a BigDye Terminator v1.1 Cycle Sequence Kit (Applied Biosystems Inc., Tokyo, Japan). The PCR primers used to detect rs8099917 were 5′-CTT CTG CAA CAA ATC GTC CC-3′ (sense) and 5′-AGG AGC TTG CAC TAG CTC TT-3′ (antisense).

RNA extraction and quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from a piece of each donor's liver biopsy specimens using ISOGEN (Nippon Gene, Tokyo, Japan) and qRT-PCR was performed using TaqMan EZ RT-PCR Core Reagents (Applied Biosystems Inc.). A TaqMan probe (5′-CTG CGG AAC CGG TGA GTA CAC-3′) and specific primers (sense, 5′-CTG CGG AAC CGG TGA GTA CAC-3′; antisense, 5′-CAC TGG GAA GCA CCC TAT CA-3′) were used for quantification of HCV RNA.

Definition of viral response and liver histology

The SVR was defined as the absence of HCV RNA by qRT-PCR (PCR Cobas TaqMan system; Roche Diagnostics) at the end of the treatment and 24 weeks after the completion of therapy. Virological response (VR) was defined as the absence of HCV RNA as well as whether or not relapse occurred. The grade of inflammation and the stage of fibrosis were defined according to the Metavir grading score (9).

A Case Report

A 51-year-old Japanese male was referred to our department as an LDLT candidate. He suffered chronic hepatitis C (genotype 1b) and had failed two previous course of PEG-IFNα2b and ribavirin therapy. LDLT was performed using dual grafts from two donors. The left lobe was from his 21-year-old son and the right lobe from his 42-year-old wife, as previously described (8). Each HLA type is as follows: recipient; A11 A24 B35 B35 DR4 DR12, his son; A24 A31 B35 B59 DR4 DR12 and his wife; A24 A26 B35 B59 DR4 DR9. Both grafts were implanted by anastomosis of hepatic veins, portal veins and arteries from each side. The bile duct reconstruction was carried out by a duct–duct method for the right lobe graft and by a Roux-en Y hepatojejunostomy for the left lobe graft. The clinical course after transplantation went on without major surgical complications. Immunosuppression was induced by mycophenolate mofetil (Cellcept®; Chugai Pharmaceutical Co. Ltd., Tokyo, Japan) with basiliximab (Simulect®; Novartis Pharma, Basel, Switzerland) and maintained with tacrolimus (Prograf®; Astellas, Tokyo, Japan), the dosages of which were adjusted to trough concentrations of 5–10 ng/mL.

His clinical course is summarized in Figure 1. HCV reinfection was detected 4 weeks post-LDLT and the viral load sharply increased up to 6.7 log IU/mL at 9 weeks post-LDLT. PEG-IFN/RBV therapy commenced 15 weeks post-LDLT with a weekly dose of 60 μg PEG-IFNα2b (Pegintron®; Schering-Plough Inc., Kenilworth, NJ, USA) and 600 mg RBV (Rebetol®; Schering-Plough Inc.) daily. The viral load decreased steadily and he achieved VR 60 weeks after the start of therapy. But 24 weeks later, HCV RNA was detected again and PEG-IFNα2b was switched to PEG-IFNα2a (Pegasys®; Hoffman-La Roche Inc, Switzerland) according to the report from Sherman et al. (10). However, his liver function gradually worsened and PEG-IFN/RBV therapy was terminated 96 weeks after commencement. Although the viral load increased sharply up to 6.0 log IU/mL, his liver function did not worsen with only liver supporting therapy. Seventy-two weeks after PEG-IFN/RBV therapy was completed, which was 4 years post-LT, liver biopsies were performed on each graft.

Prior to biopsy, the recipient's DNA was extracted from his exenterate liver tissues at transplantation and direct sequencing was performed to reveal that he carried the minor IL28B allele (rs8099917). Each donor's DNA was extracted from the liver biopsy at transplantation, and his son was identified as carrying the major genotype (TT), whereas his wife was identified as carrying the minor genotype (TG) (Figure 2). The participants of these studies were fully informed, and this work was approved by the ethical committee of Kyushu University.

The histological findings of each specimen were completely different (Figure 3). The left lobe from the donor with the major genotype in IL28B displayed only mild hepatitis and no fibrosis with a Metavir grading score of A1F0. The right lobe from the donor with the minor genotype demonstrated moderate inflammation and bridging fibrosis (A2F2). Preserved bile ducts and a lack of endotheliitis were observed in both specimens, therefore the possibility of chronic rejection was denied. Moreover, qRT-PCR using total RNA extracted from each specimen revealed that no HCV RNA could be detected in the left lobe, whereas 15.3 copies/μg of HCV RNA were detected in the right lobe. Virological outcomes were also different in each graft, and these findings can possibly be explained by the different genotypes of IL28B.

Discussion

There is no longer any doubt regarding the significant association of IL28B genetic variations with the outcome of IFN therapy for HCV (3). We previously demonstrated that both IL28B genetic variants in recipients and donors are important in posttransplant IFN therapy, suggesting that

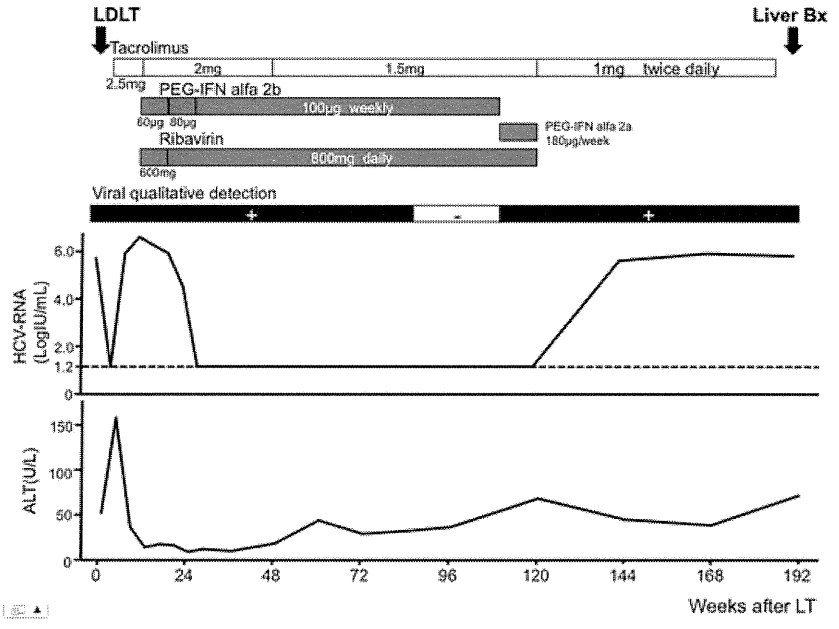

Figure 1: The clinical course after LT: the current case achieved transient VR, but did not achieve SVR with posttransplant PEG-IFN/RBV therapy for recurrent HCV.

both hepatocytes and host immune cells were associated with the relationship between IL28B genetic variants and IFN sensitivity (4). However, in cases of LDLT, many donors carry the same genetic traits as recipients. In addition, several factors other than IL28B genetic variants could influence IFN sensitivity. The significance of the grafts from donors who carry different genotypes in IL28B from the recipients is not fully understood.

In the current case, dual liver grafts from two donors carrying different IL28B genetic variants were transplanted, and specimens of liver biopsies from each graft at 2 years posttransplantation and post-PEG-IFN/RBV therapy were completely different. The left lobe graft showed displayed A1F0, which was from the recipient's 21-year-old son carrying the major genotype in IL28B, whereas the right lobe graft exhibited A2F2, which was from the recipient's 42-year-old wife carrying the minor genotype. The qRT-PCR assays revealed HCV RNA only in the graft carrying the minor genotype. It could never be concluded that left lobe graft really achieved 'SVR' because the amount of RNA extracted from each specimen was as low as about 500μg. In addition, the amount of HCV-RNA isolated in the right lobe is very low considering the high viral load in the serum, though that is inconsistent to Ramirez et al. (11). This is also due to the low amount of total RNA extracted from limited biopsy sample. We have priority over the pathological diagnosis, not over the research. We never try to perform liver biopsy again for just sampling considering patients' safety. With limited samples, we believe that, at least, there was the difference in viral load between two grafts in addition to the histological changes 4 years after transplantation. Recently, the association of IL28B genetic variations with not only treatment-induced but spontaneous clearance of HCV was reported (12), suggesting IL28B major genotype possibly had some protective function against HCV and led to prevent the graft from the progression of fibrosis even after PEG-IFN/RBV treatment for 2 years.

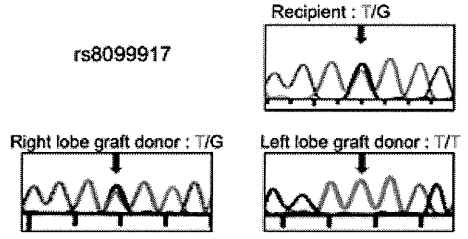

Figure 2: Direct sequencing demonstrated that the recipient and the right lobe graft donor carried the heterozygous (T/G) allele at rs8099917 (indicated by red arrow), and the left lobe graft donor carried the major homozygous (T/T) allele.

The correlation of the molecular mechanism between IL28B genetic variants and IFN sensitivity has never been clarified in detail. The key role of hepatocytes or infiltrating host immune cells, is of great interest for posttransplant IFN therapy. Honda et al. (13) reported correlation between IL28B genetic variants and pre-treatment hepatic interferon stimulated gene (ISG) expression. Several reports have demonstrated that responders to IFN therapy have pretreatment low ISG expression in hepatocytes and high ISG expression in Kupffer cells (14,15). In transplanted liver grafts, Kupffer cells, although they are also called residential macrophages, are from recipients because their duration of life is a few months at longest, whereas hepatocytes are from donors. IL28B genotypes of both donors

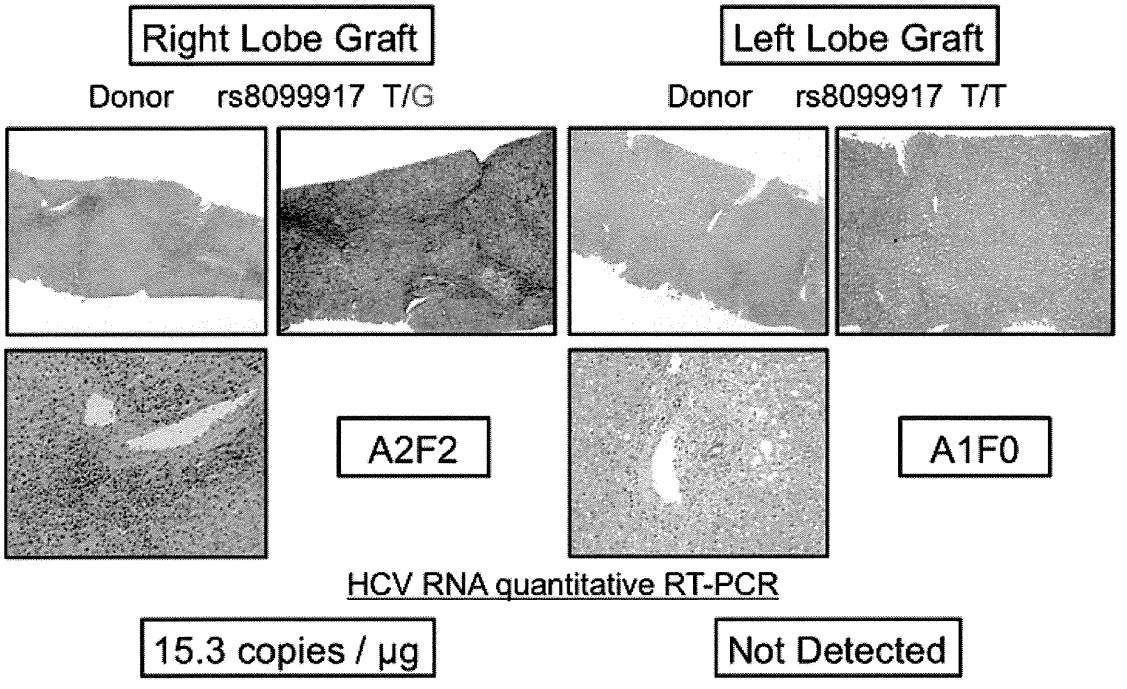

Figure 3: The liver biopsies from each graft at 4 years post-LT: (1) the left lobe graft from the recipient's son carrying the major genotype had nine portal areas and demonstrated mild chronic inflammatory infiltrates in the portal areas and lobules, accompanied by mild lobular hepatitis without fibrosis. Moderate macrovesicular and microvesicular steatosis was seen. The number of bile ducts was preserved, and endotheliitis was not observed. These features indicated chronic hepatitis, A1F0, RAI P1B0V0. (2) The right lobe graft from the recipient's wife carrying the minor genotype had three portal areas and displayed moderate chronic inflammatory infiltrates in the portal areas and lobules, accompanied by interface hepatitis and bridging fibrosis. Endotheliitis was not observed. These features were indicative of chronic hepatitis with A2F2 and RAI P1B0V0.

and recipients are still thought to correlate with hepatic ISG expression interacting each other. Sarasin-Filipowicz et al. (14) also demonstrated that hepatic ISG could not be induced above the pretreatment level by IFN therapy in patients with pretreatment high hepatic ISG expression, resulted in the lack of a VR. This suggests that the IL28B minor genotype may have some form of desensitization effect in IFN therapy in the liver by inducing pretreatment high hepatic ISG expression. On the other hand, ISG15, one of the most strongly induced ISGs, had been recently demonstrated to have a proviral function for HCV (16). Still, little is known about the relationship between IL28B genetic variants and cell-specific ISG expression in the liver. The reason why these alterations in ISG expression cause varying outcomes in IFN therapy requires further investigation. The current case, at least, strongly suggested that hepatocytes play a key role in the correlation between IL28B genetic variants and IFN-sensitivity.

All host and viral factors, other than grafts, that may influence IFN sensitivity are exactly the same in the current case. The different histological findings and viral replication of each graft suggests that IL28B genetic variant is a significantly important factor for IFN sensitivity. It is unknown whether only IL28B genetic variants affected both the histological and virological outcomes seen in the current case. Inflammation and fibrosis of the right lobe graft may have been caused by chronic rejection because of an unrelated blood donation. However, the preserved bile ducts and lack of endotheliitis seen in both grafts denies the possibility of chronic rejection. Cholestasis was never seen in both grafts. Biliary strictures following LT have been reported not to be associated with the type of biliary reconstruction; duct-to-duct or hepatojejunostomy (17). No report has demonstrated the association of the type of reconstruction with liver fibrosis. Females are widely shown to be a good prognostic factor for IFN sensitivity in chronic hepatitis C patients (5). However, Cescon et al. (18) reported that there was no relationship between gender of the donor and the SVR rate of posttransplant IFN therapy against recurrent hepatitis C. Wali et al. (19) described advancing donor age as a powerful determinant of rapid fibrosis after LT for hepatitis C, and several reports also described donor age as a predictor for the outcome of posttransplant anti-HCV therapy (18). However, these reports described that donor age of just more than 50 or 60 was unfavorable predictor for IFN outcome and progression of fibrosis. Moreover, in a recent report, Jimenez-Perez et al. (20) described that donor

age was not the predictor for SVR after LT. It could be never concluded in the current case that the difference of ages between 21-year and 42-year resulted in different graft histological changes.

In conclusion, this is the first report of two livers with different IL28B genotypes demonstrating different outcomes of posttransplant IFN therapy for recurrent HCV in one individual. Although the mechanism of cell-specific antiviral potential associated with IL28B genetic variation is yet to be determined, the current case suggests the significance of hepatocytes carrying the major genotype in IL28B for IFN therapy against HCV. This could result in new protocols for recipients with HCV-related liver diseases. In recent future, grafts from donor carrying IL28B major genotype might be transplanted to HCV positive recipients, if they have several donor candidates and have enough time to check the genotype. In cases of unfavorable genotype donors, new strategies such as HCV protease inhibitor would be proposed.

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Disclosure

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

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Modulation of CD4+ T cell responses following splenectomy in hepatitis C virus-related liver cirrhosis

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Summary

Dysfunction of T cells is a common feature in chronic persistent viral infections, including hepatitis C virus (HCV), and although hepatic and peripheral T cells have been studied extensively in chronic HCV hepatitis, the role of splenic T cell responses in such patients is poorly defined. This is an important issue, as thrombocytopenia is a complication of HCV-related liver cirrhosis (LC), due to splenic platelet sequestration and bone marrow suppression; splenectomy has been proposed to treat such patients. Herein, we studied peripheral blood mononuclear cells (PBMC) and splenic lymphoid subpopulations from a total of 22 patients, including 15 with HCV-related LC with marked thrombocytopenia treated with splenectomy, and seven controls. CD4+ T cells from peripheral blood and spleen were isolated and phenotype and function evaluated. Splenic CD4+ T cells in patients with LC expressed molecules associated with inhibitory signalling, including increased frequency of negative markers such as cytotoxic T lymphocyte associated antigen-4 (CTLA-4) and programmed death 1 (PD-1) and decreased production of cytokines. Patients with LC manifest higher levels of splenic CD4+ regulatory T cells and PD-L1- and PD-L2-expressing cells than controls. Blocking of PD-1/PD-1 ligand interaction reconstituted proliferative and cytokine responses of splenic mononuclear cells (SMC) from patients with LC. Splenectomy was followed by an increase in the ratio of interferon (IFN)-γ to interleukin (IL)-10 and a reduction of PD-1-expressing CD4+ T cells in peripheral blood. Our data suggest that peripheral tolerance is promoted by the spleen in LC via the up-regulated expression of PD-1 ligands.

Keywords: HCV, liver cirrhosis, PD-1, PD-1 ligand, splenectomy

Introduction

Hepatitis C virus (HCV) is a major public health problem, involving approximately 170 million people worldwide [1]. HCV is highly efficient at establishing persistent infection, and 70–80% of subjects are unable to clear the virus, resulting in the development of chronic liver disease and liver cancer [2]. It has long been accepted that the host immune system plays a unique role in HCV infection because of its potential to contribute to liver injury, and that HCV-specific CD4+ T cells are essential in the generation of a successful HCV-specific immune response [3]. Indeed, impaired CD4+ T cell responses have been associated with a higher rate of chronic disease and persistent viraemia [4,5]. The finding that hepatocytes are the primary site of HCV viral replication has led to the study of the role of liver HCV-specific T cell responses [4,6]. However, there are relatively few studies on the immune profile of human splenocytes and, even though the spleen has an important role in the induction and regulation of immune responses, the phenotypic and functional aspects of splenic CD4+ T cells in patients with HCV are, to a large extent, unknown.

Splenomegaly occurs occasionally in patients with liver cirrhosis (LC), including HCV infection, and is associated with the development of thrombocytopenia, due to both splenic platelet sequestration [7] and bone marrow suppression [8]. Interferon (IFN)-α/ribavirin combination therapy is the treatment of choice for HCV infection and is known to reduce the stage of disease and prevent the occurrence of hepatocellular carcinoma [9–11]. However, patients with low levels of platelets tolerate treatment with IFN-α poorly, which itself can cause thrombocytopenia [12,13];

splenectomy has been proposed as an efficient therapeutic option in patients with HCV-related LC [14] prior to antiviral therapy. Nevertheless, this practice is not accepted widely and is even considered inappropriate [15]. The purpose of this report is to evaluate splenic immune subpopulations and compare them to peripheral blood mononuclear cells in HCV-related LC and to assess the role of splenectomy in altering T cell responses in such patients, to focus and clarify whether splenectomy would be appropriate supportive therapy.

Materials and methods

Subjects

Splenic tissues from a total of 22 patients were included in this study; 15 patients were diagnosed as HCV-related LC and seven were controls who either underwent post-traumatic splenectomy or gastric cancer-associated splenectomy and lymphadenectomy (Table 1). The splenectomy in patients with HCV-related LC was performed to improve pancytopenia prior to the institution of IFN-α therapy. All control patients had normal spleen size and were considered to be immunologically intact hosts. Splenic mononuclear cells (SMC) and peripheral blood mononuclear cells (PBMC) were isolated from each of the 22 samples. In addition, in a nested substudy, PBMC were harvested from 11 of the 15 patients with HCV-related LC 2 months after splenectomy and before the administration of IFN therapy. Splenic tissue samples were subjected to phenotypic analysis utilizing immunohistochemical techniques. All samples were studied after obtaining appropriate institutional informed consent and all experimental protocols were conducted under the Guidelines of Research Ethics Committee of Kyushu University.

Preparation of cells

Freshly obtained splenic tissue was subjected to mechanical digestion and dissociated cells filtered through a 150-μm nylon mesh. The splenic cells and heparinized peripheral blood were then subjected to Ficoll-Hypaque gradient centrifugation and enriched populations of SMC and PBMC harvested from the interface, respectively. All mononuclear cells were washed and cryopreserved in media containing 7·5% dimethylsulphoxide (DMSO) and stored in liquid nitrogen until use. $CD4^+$ T cells were negatively isolated using magnetic beads (CD4 isolation kit II; Miltenyi Biotec, Aubum, CA, USA).

Isolated T cells exhibited > 95% viability confirmed by trypan blue dye exclusion and > 90% purity by flow cytometry. Myeloid-derived dendritic cells (mDC) were separated with an mDC isolation kit (Miltenyi Biotec) performed by two magnetic separation steps. The frequency of the isolated $CD1c^+CD19^-$ mDC was determined to be > 80% by flow cytometry. These procedures are standardized in our laboratory and have been described previously in detail [16].

Table 1. Characteristics of the subjects enrolled in the study

	Liver cirrhosis	Controls
Age (years)	55·7 ± 9·6	54·0 ± 14·1
Gender (male/female)	6/9	4/3
ALT (IU/l)	53·1 ± 12·7	15·4 ± 2·5
Total bilirubin (mg/dl)	1·3 ± 0·9	0·6 ± 0·2
Albumin (g/dl)	3·3 ± 0·5	3·9 ± 0·2
PT (%)	67·3 ± 10·6	92·0 ± 5·0
Platelets ($\times 10^3/mm^3$)	50·6 ± 22·4	297 ± 53·5
Child–Pugh classification (A/B/C)	7/8/0	n.a.
Disease controls (post-traumatic splenectomy/stomach cancer-associated splenectomy)	n.a.	2/5

Continuous variables are expressed as median ± standard error of the mean. ALT: alanine amino transferase; PT: prothrombin time; n.a.: not applicable.

Proliferation assay and cytokine production

Isolated $CD4^+$ T cells were cultured (1 × 105/well) in 96-well plates precoated with 10 μg/ml of monoclonal antibody to CD3 (OKT3; R&D Systems, Minneapolis, MN, USA) in the presence of human interleukin (IL)-2 (10 U/ml) for 5 days and then pulsed with 1·0 μCi of [^{3}H]-thymidine per well during the last 12 h of culture and subsequently harvested, and counted in a scintillation counter (Betaplate; Wallac, Inc., Waltham, MA, USA). Supernatant fluids were harvested before a thymidine pulse and analysed for levels of IFN-γ, IL-4 or IL-10 production by sandwich enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems), using a combination of unlabelled and biotinor enzyme-coupled monoclonal antibody to IFN-γ, IL-4 or IL-10. In all instances, known positive and negative controls were used throughout and all assays were performed in triplicate.

Additionally, a programmed death 1 (PD-1)/PD-1 ligand blocking assay was performed. Briefly, PBMC and SMC were incubated for 45 min at 37°C with a mixture of anti-PD-L1 and PD-L2 antibody (10 μg/ml each) or isotype control antibody (e-Biosciences, San Diego, CA, USA). Proliferation and IFN-γ production was performed as described above. Appropriate positive and negative controls were used throughout.

Flow cytometric analysis of the cell surface and intracellular antigens

Two- or three-colour multi-parameter flow cytometry was performed using a fluorescence activated cell sorter (FACS)Caliber Flow Cytometer (BD Biosciences, San Diego, CA, USA). Cell surface monoclonal antibodies utilized included CD4, CD25, CD28, CD154, PD-1, PD-L1 and PD-L2, and intracellular monoclonal antibodies (mAb) against cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) and forkhead box P3 (FoxP3) (BD Biosciences). mAbs specific for CD25, CD28, CD154, CTLA-4, PD-1 and

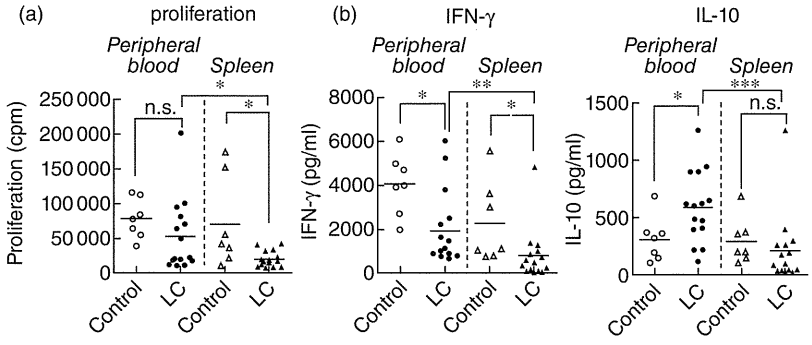

Fig. 1. Proliferation and cytokine production of $CD4^+$ T cells upon CD3 and interleukin (IL)-2 stimulation. (a) CD4 T cell proliferation, determined by [^{3}H]-thymidine incorporation, was significantly lower in spleen from liver cirrhosis (LC) patients compared to uninfected controls. (b) Cytokine production was quantified using enzyme-linked immunosorbent assays. Interferon (IFN)-γ secretion after 12 h of $CD4^+$ T cell culture was reduced significantly in LC compared to controls in both peripheral blood and spleen. Lower levels of both IFN-γ and IL-10 from spleen were observed when compared to peripheral blood in LC, but not in controls. IL-4 secretion was not detected in each group (data not shown) ($*P < 0{\cdot}05$; $**P < 0{\cdot}01$; $***P < 0{\cdot}001$).

FoxP3 were utilized for the analysis of $CD4^+$ T cells and the mAbs specific for CD40, CD80, CD83 and CD86, PD-L1 and PD-L2 (BD Biosciences) were utilized for the analysis of mDCs.

Cryopreserved mononuclear cells ($2{\cdot}5 - 5{\cdot}0 \times 105$) were stained for cell surface antigen expression at 4°C in the dark for 30 min, washed twice in 2 ml phosphate-buffered saline containing 1% bovine serum albumin and 0·01% sodium azide and fixed in 200 μl of 1% paraformaldehyde. Intracellular staining for CTLA-4 was performed following membrane permeabilization of fixed cells with 0·02% saponin (BD Biosciences). FoxP3 staining was performed according to the manufacturer's instructions. Isotype-matched control antibodies were used to determine the background levels of staining. We chose intracellular staining for CTLA-4 because CTLA-4 contains an extracellular V domain, transmembrane domain and a cytoplasmic tail; the frequencies of intracellular CTLA-4 was similar to the frequency of surface CTLA-4^+ T cells.

Immunohistochemical staining of spleen specimens

Spleen specimens were formalin-fixed, paraffin-embedded, and used for immunostaining. Deparaffinized and rehydrated sections were used for immunostaining for the cell surface markers PD-1, PD-L1, and PD-L2. Endogenous peroxidases were first blocked by incubation in normal goat serum (Vector Laboratories, Burlingame, CA, USA) for 20 min; monoclonal antibodies were diluted 1:100 (Dako, Kyoto, Japan), and immunostaining was performed on coded sections and were scored 0 as negative, 1 as positive and 2 as strongly positive by a 'blinded' and qualified pathologist.

Statistical evaluation

The data obtained were analysed using Prism software (version 5.0; GraphPad Software Inc., La Jolla, CA, USA) and then compared using the Mann–Whitney *U*-test, a non-parametric test that does not assume Gaussian variation. ELISA and proliferation data represent the average of triplicate wells. *P* values less than 0·05 were considered to be statistically significant.

Results

Splenic $CD4^+$ T cells from patients with HCV-related LC demonstrate lower levels of activation

Purified splenic $CD4^+$ T cells from patients with HCV-related LC and controls were stimulated *in vitro* with anti-CD3 mAb in the presence of IL-2. As illustrated in Fig. 1a, while splenic $CD4^+$ T cell proliferation was significantly lower in patients with HCV-related LC compared to uninfected controls ($P < 0{\cdot}05$), there was no statistical difference in peripheral $CD4^+$ T cell proliferation between patients and controls. In addition, significantly lower levels of proliferation in splenic $CD4^+$ T cells, compared with their corresponding peripheral $CD4^+$ T cell counterparts, were detected in patients with HCV-related LC ($P < 0{\cdot}05$) but not in controls, which is consistent with previous reports [3,17].

Moreover, IFN-γ secretion after 12 h of $CD4^+$ T cell culture was reduced significantly in cultures from both peripheral blood and spleen from patients with HCV-related LC compared to controls ($P < 0{\cdot}05$) (Fig. 1b). Conversely, peripheral $CD4^+$ cell cultures from patients with HCV-related LC synthesized higher relative levels of IL-10 compared to both splenic $CD4^+$ cells ($P < 0{\cdot}001$) and healthy controls ($P < 0{\cdot}05$), whereas there was no statistical difference in the levels of IL-10 synthesized by the splenic $CD4^+$ T cells (Fig. 1c). There were undetectable levels of IL-4 synthesized by either peripheral or splenic $CD4^+$ T cells (data not shown).

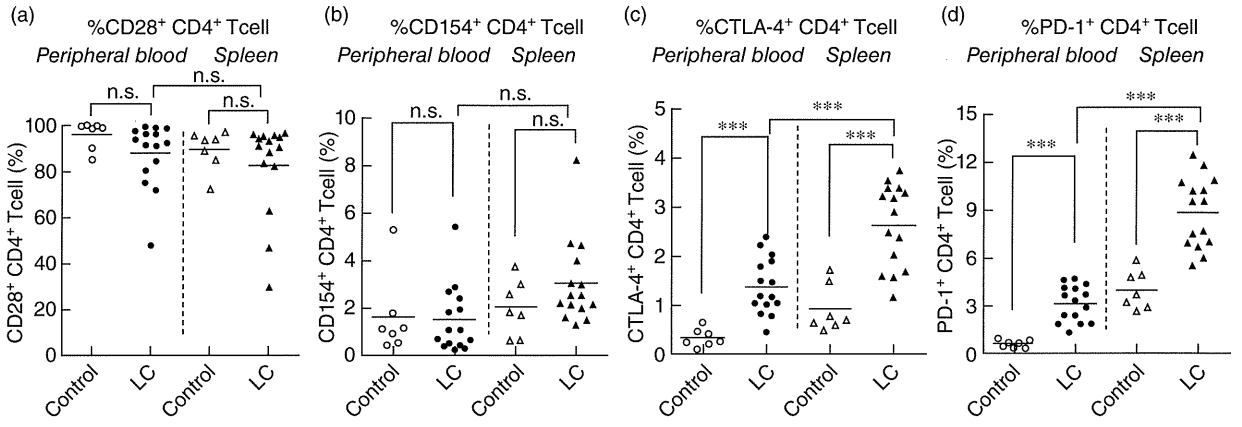

Fig. 2. CD4+ T cells phenotype. Cell surface markers were determined as the percentages of CD4+ T cells by flow cytometry. No differences in the frequencies of CD28 (a) and CD154 (b), which positively regulate CD4+ T cells, were observed. Negative markers such as cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) (c) and programmed death-1 (PD-1) (d) were significantly more expressed in liver cirrhosis (LC) than controls in both peripheral blood and spleen (***$P < 0{\cdot}001$).

Expression of inhibitory signalling molecules by CD4+ T cells from LC patients

It was reasoned that reduced levels of CD4+ T cell activation could be secondary to immune exhaustion and/or the expression of molecules associated with negative signalling. Therefore, expression of CD28, CD154, CTLA-4 and PD-1 [18–20] was analysed in peripheral and splenic CD4+ T cells from patients with HCV-related LC and controls using standard flow cytometry.

As seen in Fig. 2, there was no difference in the relative levels of CD28 or CD154 expression by CD4+ T cells from patients with HCV-related LC compared to controls either in peripheral blood or spleen (Fig. 2a,b). In contrast, the frequencies of CTLA-4- and PD-1-expressing CD4+ T cells were increased significantly in peripheral blood and spleen in patients with HCV-related LC compared to controls ($P < 0{\cdot}001$) (Fig. 2c,d). Moreover the frequency of both CTLA-4- and PD-1-expressing CD4+ T cells was higher in spleen compared to peripheral blood from patients with HCV-related LC ($P < 0{\cdot}001$).

These results indicate that impaired proliferative responses and reduced synthesis of IFN-γ in patients with HCV-related LC are associated with increased levels of expression of the inhibitory cell surface markers known to be associated with decreased immune function and exhaustion, such as CTLA-4 and PD-1.

Blocking of PD-1/PD-1 ligand interaction reconstitutes proliferative and cytokine responses of SMC from patients with HCV-related LC

To explore the hypothesis that decreased proliferation and IFN-γ production from splenic CD4+ T cells in patients with HCV-related LC were indeed associated with PD-1 increased expression, blocking experiments were performed using mAbs to both PD-L1 and PD-L2. As seen in Fig. 3, blocking of PD-1 ligands resulted in a two- to fourfold enhancement of the proliferative response ratio of PBMC and SMC, respectively (Fig. 3a) and a fourfold enhancement of IFN-γ production by SMC from LC patients ($P < 0{\cdot}05$) (Fig. 3b). Similar blocking experiments failed to show any significant increase in proliferative response in cultures of PBMC and SMC from controls.

The increased ratio of proliferation seen in cultures of SMC was significantly higher than that seen with PBMC with the use of mAbs against the PD-1 ligands (PD-L1 and PD-L2). These results in concert suggest that the decreased

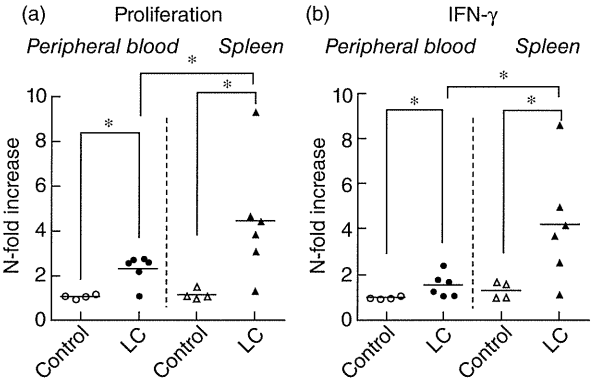

Fig. 3. Blockage of programmed death-1(PD-1)/PD-1 ligands antagonizes T cell inhibition of splenic mononuclear cells (SMC) from hepatitis C virus (HCV)-related liver cirrhosis. Comparison of proliferation (a) and interferon (IFN)-γ production (b) after treatment with a mixture of monoclonal antibodies against PD-L1 and PD-L2; the blocking effect is expressed as the ratio of the mean value after blockage relative to the basal condition (*n*-fold-increase) (*$P < 0{\cdot}05$).

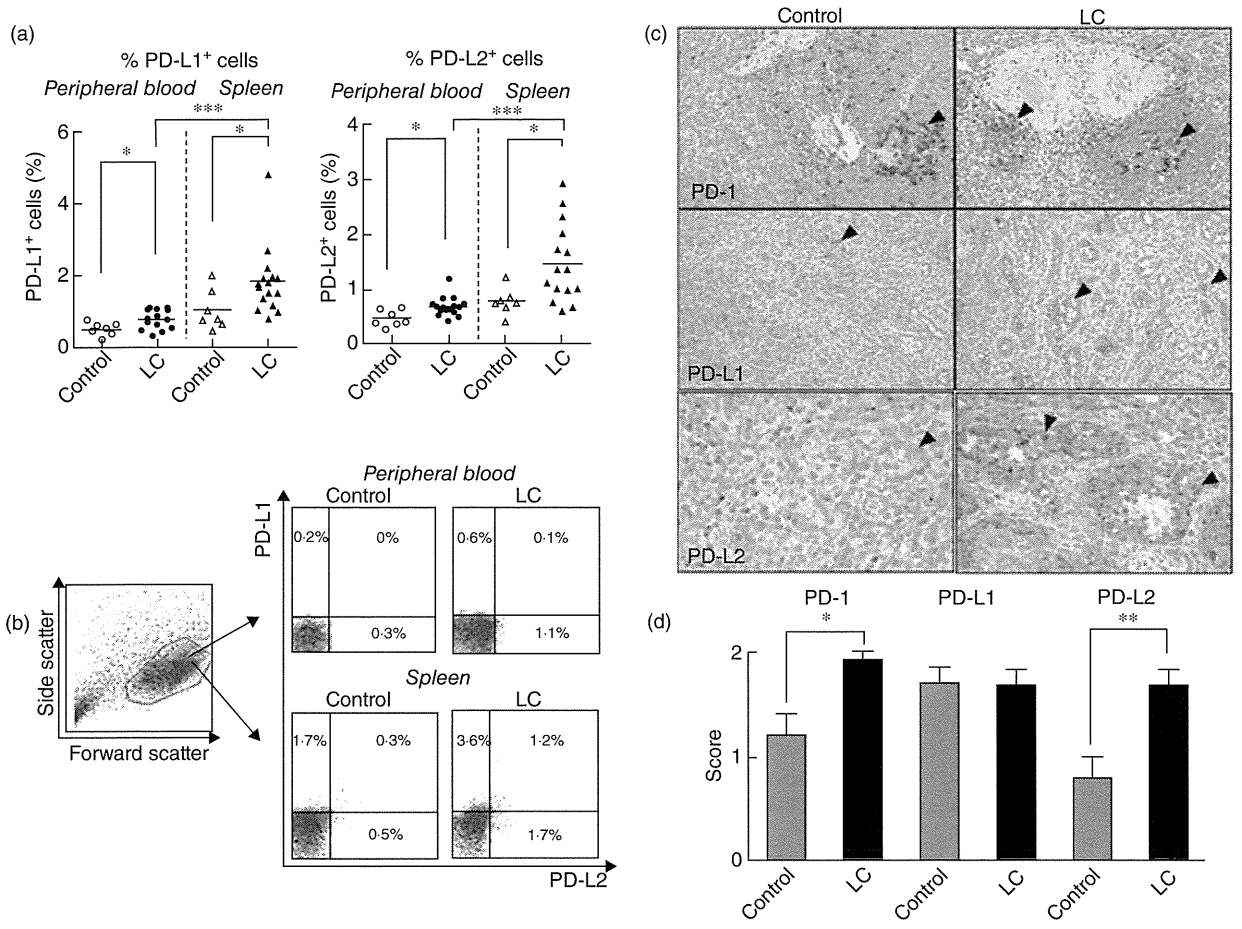

Fig. 4. Expression of negative co-stimulation molecules programmed ligand death-1 (PD-L1) and PD-L2. (a) The expression of negative co-stimulatory molecules PD-L1 and PD-L2 on peripheral blood mononuclear cells (PBMC) and splenic mononuclear cells (SMC) were analysed using flow cytometry. The expression of PD-L1 and PD-L2 was significantly higher in liver cirrhosis (LC) compared to controls (*$P < 0{\cdot}05$). (b) Representative gated plots of both PBMC and SMC are shown. (c) Spleen immunostaining from LC and controls confirmed the distribution of PD-1 and PD-1 ligands. Arrowheads indicate positive cells. (d) Their expressions were scored 0 as negative, 1 as positive and 2 as strongly positive to differentiate LC from controls. Spleens from hepatitis C virus (HCV)-related LC showed higher expression of PD-1 and PD-L2 than controls (*$P < 0{\cdot}05$; **$P < 0{\cdot}01$).

T cell responsiveness of the spleen in patients with HCV-related LC is associated at least partially with the up-regulation of PD-1/PD-1 ligands.

PD-1 ligand expression is higher in patients with HCV-related LC

Binding of the co-stimulatory molecules PD-L1 and PD-L2 on the surface of antigen-presenting cells (APC) by PD-1 expressing T cells induces down-regulation of immune responses [19,21,22]. As outlined above, $CD4^+$ T cells from patients with HCV-related LC demonstrated a marked increase of PD-1 expression compared to controls. We therefore examined PD-L1 and PD-L2 expression on unfractionated PBMC and SMC. As seen in Fig. 4a, a higher frequency of PD-L1- and PD-L2-expressing cells in SMC samples compared to PBMC was observed in both patients with HCV-related LC and control donors. The frequency of PD-L1 and PD-L2 expressing cells was higher in the PBMC and SMC from patients with HCV-related LC compared with the normal donors ($P < 0{\cdot}05$), assessed with FACS (Fig. 4b). These results were confirmed by immunohistochemical analyses of splenic tissues from patients with HCV-related LC and normal control donors (Fig. 4c,d).

$CD4^+$ $CD25^+$ $FoxP3^+$ regulatory T cells (T_{reg}) are increased in patients with HCV-related LC

To assess the role of T_{reg} and molecules involved in co-stimulation expressed by myeloid dendritic cells, we examined the frequencies of $CD4^+$ $CD25^+$ $FoxP3^+$ T_{reg} in peripheral blood and spleen from patients with HCV-related

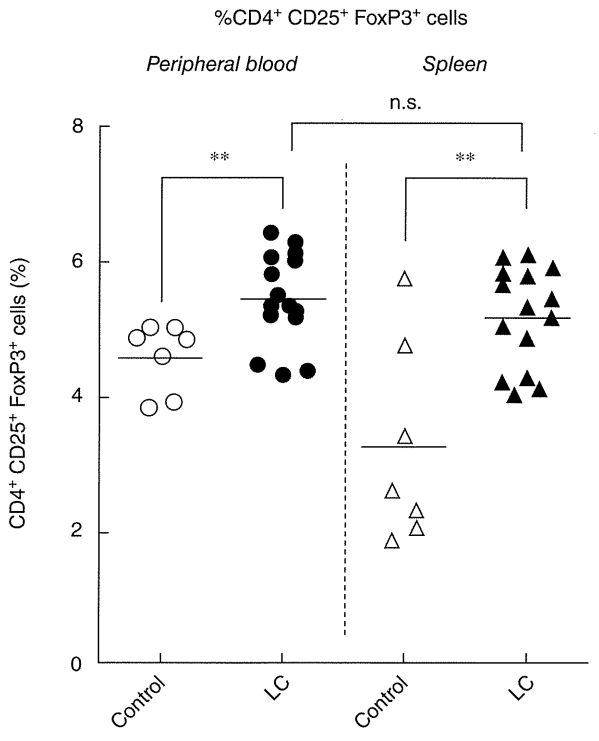

Fig. 5. $CD4^+$ $CD25^+$ forkhead box P3 (FoxP3)$^+$ regulatory T cell (T_{reg}) frequency. $FoxP3^+$ T_{reg} frequency was analysed using flow cytometry in peripheral blood and spleen and expressed as percentage. Higher frequency of T_{reg} was observed in both peripheral blood and spleen from liver cirrhosis (LC) patients (**$P < 0{\cdot}01$), whereas no differences between peripheral blood and spleen were observed in hepatitis C virus (HCV) patients.

LC and controls. As noted in Fig. 5, higher levels of $CD4^+$ $CD25^+$ $FoxP3^+$ T cells were observed in patients with HCV-related LC compared to controls in both peripheral blood and spleen. However, no differences were determined in patients with HCV-related LC between peripheral blood and spleen. Secondly, we analysed the frequency and mean density of CD40, CD80, CD83 and CD86 expressing mDC in peripheral blood and spleen from patients with HCV-related LC and control donors. There was no statistical difference in the frequency of $CD1c^+$ $CD19^-$ in the peripheral blood compared to spleen (data not shown).

Effects of splenectomy on the response of $CD4^+$ T cells in patients with HCV-related LC

To study the consequences of splenectomy on T cell function, an enriched population of $CD4^+$ T cells from peripheral blood was examined before and after the removal of spleen. As seen in Fig. 6a, while there was some increase in the rate of anti-CD3-induced proliferation by the $CD4^+$ T cells post-splenectomy, the data were found to be statistically non-significant. Conversely, the ratio of the levels of IFN-γ synthesized by $CD4^+$ T cells post-splenectomy were markedly higher ($P < 0{\cdot}05$) (Fig. 6b). Of interest to note was the finding that, whereas there was no statistical difference in the levels of IL-10 synthesized pre- *versus* post-splenectomy, there was clearly an increase in the ratio of IFN-γ to IL-10 post-splenectomy ($P < 0{\cdot}01$) (Fig. 6c).

Interestingly, when the frequency of CTLA-4- and PD-1-expressing $CD4^+$ T cells were examined, while there were no differences in the frequencies of CTLA-4-expressing $CD4^+$ T cells, there was a marked decrease in the levels of PD-1-expressing $CD4^+$ T cells post-splenectomy ($P < 0{\cdot}001$) (Fig. 7).

Discussion

It has been long been suggested that liver injury and disease progression in HCV infection are due in part to immune-mediated events [3]. Impaired adaptive immune responses have been documented in patients with chronic HCV infection and reasoned to be secondary to persistent antigenic

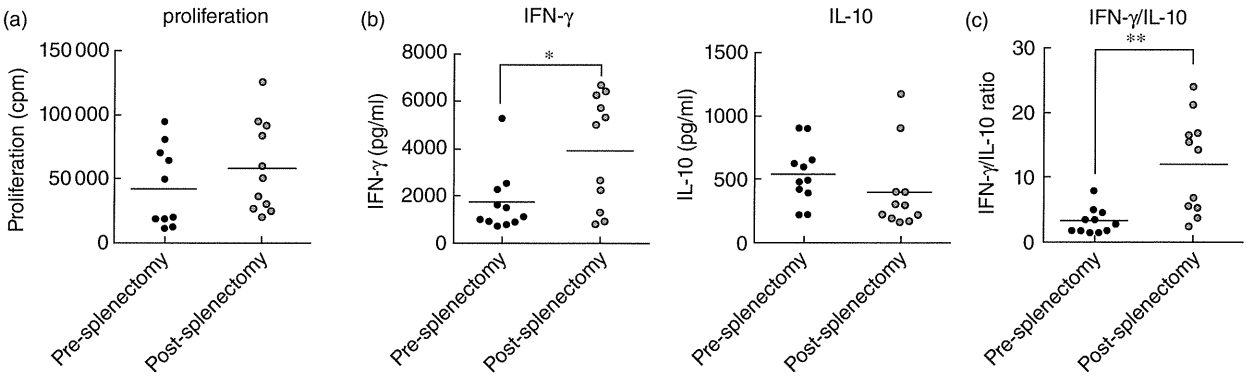

Fig. 6. Proliferation and cytokine production of $CD4^+$ T cells before and after splenectomy. Proliferation of $CD4^+$ T cells in peripheral blood was studied before and after splenectomy in patients with hepatitis C virus (HCV)-related cirrhosis. Interferon (IFN)-γ secretion (b) increased significantly after splenectomy, similar to IFN-γ/interleukin (IL)-10 ratio (d) (*$P < 0{\cdot}05$; **$P < 0{\cdot}01$).