**Table 2** Univariate and multivariate analysis of variables associated with waiting list mortality

| Variables | Univariate | | | Multivariate | | |
|---|---|---|---|---|---|---|
| | HR | 95 % CI | *P* value | HR | 95 % CI | *P* value |
| Age (per year of age) | 1.04 | 1.03–1.05 | <0.001 | 1.04 | 1.03–1.05 | <0.001 |
| Male gender | 0.93 | 0.77–1.13 | 0.48 | | | |
| Blood type | | | | | | |
| A | 1.00 | Reference | | | | |
| B | 1.07 | 0.83–1.43 | 0.61 | | | |
| O | 1.13 | 0.90–1.43 | 0.29 | | | |
| AB | 1.26 | 0.90–1.77 | 0.17 | | | |
| Etiology | | | | | | |
| HCV | 1.00 | Reference | | | | |
| BA | 0.40 | 0.22–0.72 | 0.002 | | | |
| PBC | 1.62 | 1.21–2.16 | 0.001 | 1.79 | 1.34–2.39 | <0.001 |
| PSC | 0.79 | 0.54–1.17 | 0.24 | | | |
| HBV | 0.77 | 0.56–1.05 | 0.10 | | | |
| Alcohol | 0.95 | 0.59–1.53 | 0.83 | | | |
| AIH | 0.77 | 0.34–1.74 | 0.52 | | | |
| NASH | 1.11 | 0.76–1.63 | 0.59 | | | |
| HCC | 1.46 | 1.05–2.05 | 0.003 | | | |
| Metabolic disease | 0.40 | 0.22–0.75 | 0.004 | | | |
| Polycystic disease | 0.26 | 0.10–0.70 | 0.008 | 0.27 | 0.10–0.73 | 0.01 |
| Vascular disease | 0.009 | 0.01–0.67 | 0.002 | | | |
| Others | 0.70 | 0.34–1.43 | 0.33 | | | |

*AIH* autoimmune hepatitis, *BA* biliary atresia, *HBV* hepatitis B virus, *HCC* hepatocellular carcinoma, *HCV* hepatitis C virus, *HR* hazard ratio, *NASH* non-alcoholic steatohepatitis, *PBC* primary biliary cirrhosis, *PSC* primary sclerosing cholangitis

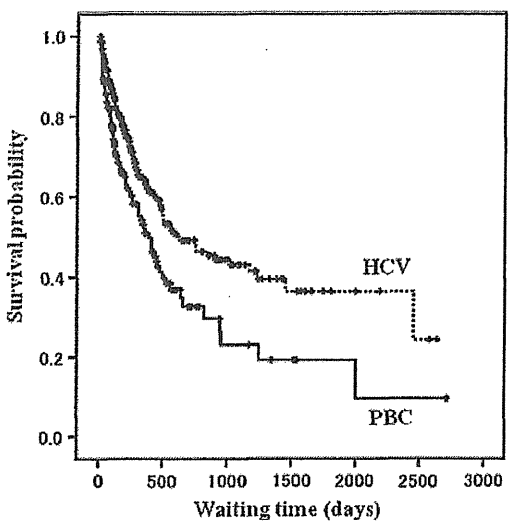


**Fig. 2** Kaplan–Meier curves comparing the cumulative waiting list survival probability of patients with chronic hepatitis C (HCV, $n = 254$) and primary biliary cirrhosis (PBC, $n = 156$)

**Table 3** Comparison of patient characteristics between HCV and PBC

| Variable | HCV ($n = 189$) | PBC ($n = 81$) | *P* value |
|---|---|---|---|
| Age (years) | 55 (29–69) | 52 (27–69) | 0.02[a] |
| Gender (male/female) | 143/46 | 15/66 | <0.001[b] |
| Platelet count ($\times 10^4/\mu L$) | 6.0 (1.7–49.0) | 10.2 (2.2–42.3) | <0.001[a] |
| Albumin (g/dL) | 2.8 (1.8–4.4) | 2.8 (1.4–4.2) | 0.96[a] |
| Total bilirubin (mg/dL) | 2.7 (0.4–39.8) | 7.2 (0.7–41.2) | <0.001[a] |
| Creatinine (mg/dL) | 0.78 (0.4–7.4) | 0.67 (0.37–2.83) | <0.001[a] |
| Prothrombin time (%) | 54.7 (11.0–103.0) | 62.2 (16.0–120.0) | 0.001[a] |
| INR | 1.51 (0.98–6.24) | 1.32 (0.91–4.31) | 0.001[a] |
| MELD score | 15 (7–52) | 17.5 (8–39) | 0.002[a] |
| CTP score | 10 (6–15) | 10 (5–15) | 0.27[a] |
| Medical point (1, 3/6, 9) | 54/135 | 22/59 | 0.81[b] |

Data are shown as median (range). Data were available for patients who were listed after June 22, 2006

*CTP* Child–Turcotte–Pugh, *HCV* hepatitis C virus, *INR* international normalized ratio, *MELD* model of end-stage liver disease, *PBC* primary biliary cirrhosis

[a] Mann–Whitney *U* test

[b] Chi-square test

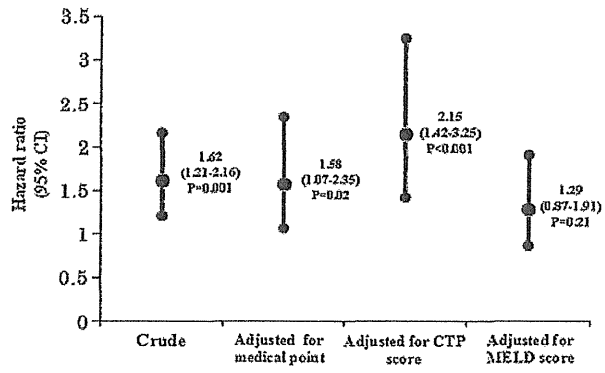


**Fig. 3** Adjusted risk of waiting list mortality for patients with primary biliary cirrhosis compared with patients with chronic hepatitis C

To examine which disease severity index was able to assess the risk of PBC patients accurately, we estimated their relative hazards with adjustment for each index. We did not estimate age-adjusted relative hazard because age was not included in the allocation measures. Figure 3 indicates the crude and disease severity index-adjusted HR for waiting list mortality of PBC patients with reference to HCV patients. In univariate analysis, PBC patients were at 62 % (HR 1.62; 95 % CI 1.21–2.16, $P = 0.001$) increased risk of waiting list mortality

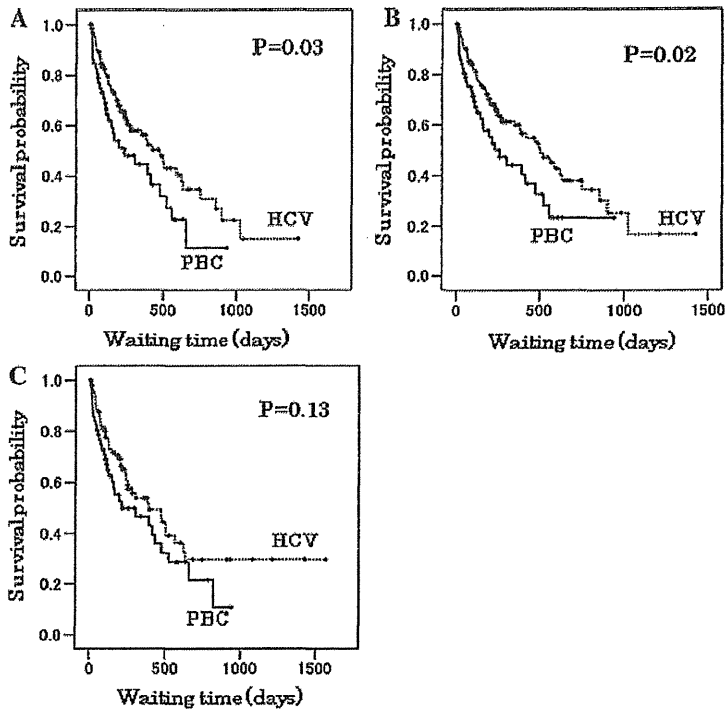


**Fig. 4** Kaplan–Meier curves comparing the cumulative waiting list survival probability of patients with chronic hepatitis C (HCV) and primary biliary cirrhosis (PBC). Patients stratified medical point = 6 (**a**), and Child–Turcotte–Pugh score ≥10 (**b**), and Model of End-Stage Liver Disease (MELD) score ≥15 (**c**)

compared with HCV patients. In bivariate analysis, the medical point-adjusted HR of waiting list mortality of PBC patients was significantly higher than that of HCV patients (HR 1.58; 95 % CI 1.07–2.35, $P = 0.02$). The CTP score-adjusted HR also showed a significantly increased risk of waiting -list mortality in PBC patients (HR 2.15; 95 % CI 1.42–3.25, $P < 0.001$). However, the MELD score-adjusted HR did not show a statistically significant risk of waiting list mortality in PBC patients (HR 1.29; 95 % CI 0.87–1.91, $P = 0.21$).

Waiting list survival of patients with HCV and PBC was compared with stratification by each of the disease severity indices (Fig. 4). Patients with medical point 6, for which most PBC and HCV patients were registered, showed a significantly shorter waiting list survival for PBC patients than of HCV patients (median 261 vs. 503 days, $P = 0.02$). In patients with CTP score ≥10, the score classified as C, the shorter waiting list survival of PBC patients was also significant (median 235 vs. 475 days, $P = 0.03$). On the other hand, when they were selected by MELD ≥15, the score indicating patients who can be expected to achieve improved survival with liver transplantation [12], there was no significant difference in the waiting list survival rate between them ($P = 0.13$).

## Discussion

The result of this study clearly indicated that the most common reason for removal from the waiting list in Japan was "waiting list death", which was a combination of death and becoming too sick for transplantation. The waiting list death included 58.1 % of all the patients removed from the list. In the United States, a recent report indicated that waiting list death was the reason for removal from the list in 25.9 % of adult patients [1]. Although this report included patients with acute liver failure and re-transplantation, high waiting list mortality in Japan was evident. Thus, the high mortality rate on the liver transplant waiting list is a major challenge in Japan. Moreover, severe donor organ shortage in Japan should contribute to the high waiting list mortality [13]; an improved organ allocation policy will be necessary to cause a decrease in waiting list death.

In this study, we found that PBC patients had a significantly higher risk of waiting list mortality compared with patients with other etiologies in the JOT registry. Since PBC is currently the third most common diagnosis in the JOT registry for liver transplantation, poor waiting list survival of PBC patients would contribute to the high waiting list mortality in Japan. PBC is a cholestatic liver disease that causes bile duct deterioration and progresses slowly to a terminal phase characterized by hyperbilirubinemia, signs of decompensated cirrhosis, ascites, and variceal bleeding. Only one type of medical therapy, involving the use of ursodeoxycholic acid (UDCA), is now widely recognized to improve the prognosis of PBC patients. Many studies have shown that UDCA therapy not only improves biochemical indices, but also delays histologic progression and improves survival without transplantation [14–16]. However, evidence has also accumulated that the

favorable effect of UDCA therapy is limited to patients with early-stage disease. In histologically advanced patients or biochemical non-responders, the transplant-free survival rate of UDCA-treated patients was not different from spontaneous survival [16, 17]. This means that PBC patients have no effective medical therapeutic option to prolong their survival when they have progressed to end-stage liver disease, and liver transplantation remains the only hope of a cure [18, 19]. PBC patients in our cohort also showed a consistently poor survival of a median period of 392 days.

The reason why PBC patients have a higher risk for waiting list mortality compared with patients with other etiologies of chronic liver disease is not clearly understood. Interestingly, PBC patients were younger, and their INR and serum creatinine levels were lower than for HCV patients at registration. This indicated that neither age nor liver and renal function at registration alone caused poor waiting list survival of PBC patients; the registration of PBC patients was not later than that for HCV patients. The rate of disease progression and lethal complications might be involved in their short waiting list survival rate. Moreover, the actual waiting list survival rate in PBC patients was not greater than the updated Mayo score-predicted spontaneous survival rate. This observation indicated that the PBC patients on the waiting list were refractory to the medical therapy and their waiting list survival suddenly deteriorated. Further analyses, particularly on the cause of death, are required to clarify the pathophysiology of PBC patients who have progressed to end-stage liver disease.

In general, deceased donor livers are allocated for transplantation on the basis of "sickest first", i.e., those who are more likely to die without a liver transplantation are assigned the highest priority. Therefore, the disease severity index used in the liver allocation system should consider the urgency of PBC patients for liver transplantation. However, our results have clarified the inability of the currently used Japanese allocation system to identify the risk of PBC patients. The medical point-adjusted HR of PBC patients revealed that they were at 58 % increased risk of waiting list mortality compared with HCV patients. In addition, the CTP score-adjusted HR showed that PBC patients were at 115 % increased risk for waiting list mortality. Thus, it is not only the current allocation system but also the CTP score-based allocation that cannot capture the risk for waiting list mortality in PBC patients. On the other hand, we found that the MELD score-adjusted HR of PBC patients lost statistical significance, and stratification by MELD score revealed comparable survival curves between patients with PBC and HCV. These results indicated that PBC patients had a similar risk of waiting list mortality compared with patients with other etiologies when they were stratified by MELD score. At the time of registration, the patients with HCV and PBC had different characteristics; however, only the MELD score accurately evaluated their disease severity, and therefore, MELD-based allocation would adequately assign priority to the patients according to their risk of waiting list mortality. Thus, our results demonstrated that the MELD score was superior to both the current Japanese allocation and CTP score-based allocation for ranking patients in the JOT registry by their risk of waiting list mortality.

In addition, patients should be re-evaluated according to their chronological change of hepatic failure to improve allocation. However, most patients with chronic liver disease were waiting at medical point 6 as an upper limit, because the highest priority at medical point 9 was generally awarded to the patients with acute liver failure or early graft failure in the current Japanese allocation system. Therefore, the current allocation system did not completely reflect the chronological change in the degree of liver failure. Thus, the MELD score, which was expressed numerically as a continuous variable with a wide dynamic range in the evaluation of hepatic decompensation, would have an advantage over the medical point system for assessing the chronological change in patients' risk of death.

In conclusion, this study demonstrated that patients with PBC, the third most common indication for liver transplantation in Japan, have a high risk for waiting list mortality in the current Japanese allocation system. The allocation system should be changed to accurately prioritize the patients with a higher mortality risk; MELD-based allocation would be suitable for this purpose and could reduce the waiting list mortality of PBC patients.

**Acknowledgments** This study was supported by a Health Labor Sciences Research Grant, Research on Measures for Intractable Diseases, from the Ministry of Health, Labor and Welfare of Japan.

**Conflict of interest** The authors declare that they have no conflict of interest.

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ELSEVIER

# A National Survey of Patients With Intestinal Motility Disorders Who Are Potential Candidates for Intestinal Transplantation in Japan

T. Ueno, M. Wada, K. Hoshino, S. Sakamoto, H. Furukawa, and M. Fukuzawa

ABSTRACT

Intestinal motility disorders are a major cause of intestinal failure. Severe cases such as idiopathic pseudo-obstruction represent life-threatening illnessed. Intestinal transplantation is a treatment for severe motility disorders with irreversible intestinal failure. However, the prevalence of severe motility disorders is unknown. We performed a national survey to identify patients with intestinal motility disorders who require an intestinal transplant. The national survey of 302 institutions treating intestinal motility disorders identified 147 patients treated from 2006 to 2011 at 46 institutions. The mean patient age was 12.1 years (range, 0.3–77.5). The mean age of onset was 3.0 years (range, 0.0–68.8). Diagnoses included chronic idiopathic intestinal pseudo-obstruction ($n$ = 96), Hirschsprung disease ($n$ = 29), megacystis microcolon intestinal hypoperistalsis syndrome ($n$ = 18), and other ($n$ = 6). There were 126 survivors and 21 patients who died during the last 5 years. The mortality rate was 14.3%. Eighty-five percent of patients required parenteral nutrition for more than 6 months, which was defined as irreversible intestinal failure. Among surviving patients with irreversible intestinal failure, 8 (9.4 %) developed hepatic failure with jaundice and 27 (31.8%) 2 or more central vein thromboses. In all, at least 35 patients (41%) with irreversible failure due to intestinal motility disorders may be candidates for transplantation. The prevalence of severe intestinal motility disorders was elucidated in Japan. Severe cases should be referred to transplant centers.

INTESTINAL MOTILITY DISORDERS are a major cause of intestinal failure. Severe cases such as idiopathic pseudo-obstruction are life-threatening. Causes of intestinal motility disorders seem to be multifactorial, and only a few have been elucidated. The prognosis is poor for patients with severe illness. The outcome for intestinal failure has improved dramatically due to the development of parenteral nutrition (PN). However PN-related complications, such as central venous catheter infection, thrombosis of venous access points, and PN-associated cholestasis of the liver, are still major problems for patients with intestinal failure. Intestinal transplantation is a treatment for irreversible intestinal failure due to severe disorders of intestinal motility that can significantly improve the prognosis and quality of life for patients. Progress in intestinal transplantation has improved survival. However, the prevalence of severe intestinal motility disorder is unknown. The Therapeutic Guidelines for Intestinal Failure Study Group performed a national survey to identify patients with intestinal motility disorders requiring an intestinal transplant.

## METHODS

This national survey was designed as a 5-year retrospective observation study involving 302 institutions that treat intestinal motility disorders. These institutions were members of the Japanese Society of Pediatric Surgeons, the Japanese Society for Small Bowel Transplantation, and the Japanese Study Group for Home Parenteral and Enteral Nutrition. After an initial survey, a questionnaire about each patient was sent to responding institutions from the data center based at Osaka University. Patients with intestinal

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failure treated at each institution from 2006 to 2011 were included. Exclusion criteria were: (1) final diagnosis other than intestinal failure, (2) intestinal failure ultimately resolved, (3) intestinal failure resulting from malignancy, and (4) intestinal failure secondary to diseases in other organs. There were 354 patients reported by 69 institutions. Irreversible intestinal failure was defined as dependence on PN for more than 6 months. Out of these 354 patients, patients with intestinal failure due to motility disorders were identified. The following factors were assessed for possible associations with indications for intestinal transplantation: diagnosis, patient age, age of onset, sex, patient outcome, PN status, liver function tests (LFTs), and central line access. This study was approved by the Osaka University Hospital institutional review board and was supported by Health Science Research Grants from the Ministry of Health, Labor and Welfare of Japan.

## RESULTS

There were 147 patients with intestinal motility disorders identified from 46 institutions. The prevalence was approximately one in one million. There were 55 male and 92 female patients. The female-to-male ratio was about 2:1. The mean patient age was 12.1 years (range, 0.3–77.5 years). The mean age of onset was 3.0 years (range, 0.0–68.8 years). Causes of intestinal failure are shown in Fig 1. During the observation period, 126 patients survived and 21 patients died. The mortality rate was 14.3%.

Detailed analysis was added for survivors to determine indications for intestinal transplantation. Of the surviving patients, 91 (62.0%) needed PN at least once a week, and 85 (57.8%) required PN for more than 6 months. Those 85 patients were defined as having irreversible intestinal failure. The following analyses were carried out for patients with irreversible intestinal failure. Catheter-related complications were assessed. The site of central vascular access (internal jugular vein, subclavian vein, and femoral vein) was reported. The number of venous access failures is shown in Fig 2. Twenty-seven patients (31.9%) had 2 or more instances of central vascular access loss.

There were 61 patients (71.8%) who developed abnormal LFTs suggestive of liver injury from PN, including 8 patients (13%) with jaundice. They were considered to have severe liver injury resulting from PN.

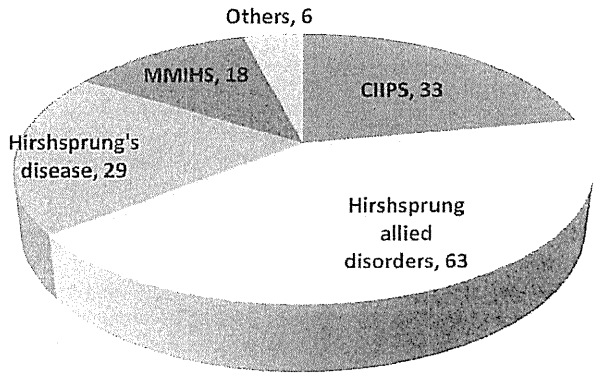


**Fig 1.** Causes of intestinal failure ($n$ = 147). CIIPS, chronic idiopathic intestinal pseudo-obstruction; MMIHS: megacystis microcolon intestinal hypoperistalsis syndrome.

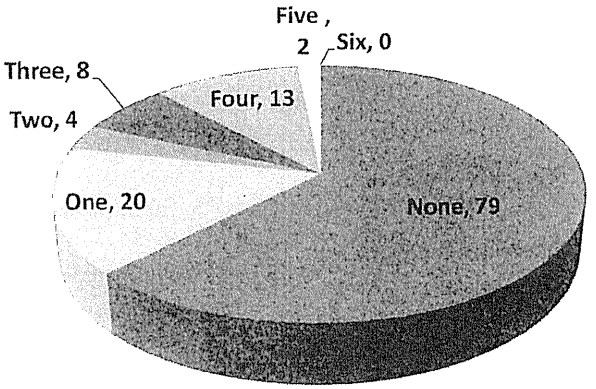


**Fig 2.** Number of central vascular access losses ($n$ = 126). The number on the left indicates the number of vascular access losses.

Fifty-eight patients required at least 1 hospitalization in the previous year. Nineteen patients (22.4%) required hospitalization for more than 6 months over the previous year. Their quality of life was severely impaired.

A flowchart for identifying possible candidates for intestinal transplantation is shown in Fig 3. Patients dependent on PN for more than 6 months were defined as having irreversible intestinal failure. Those with more than 2 central vascular access losses, and abnormal LFTs with jaundice were considered for candidates for intestinal transplantation. Patients who died from liver failure or infection might be saved by intestinal transplant. They might be candidates for intestinal transplant too. In total, 45 patients were potential candidates for intestinal transplantation. Additionally, the 19 patients who were hospitalized for more than 6 months can be potential candidates given their poor quality of life.

## DISCUSSION

Intestinal motility disorders include a wide range of diseases. Chronic intestinal pseudo-obstruction, the most common type of intestinal mobility disorder, is caused by ineffective intestinal contraction. It is characterized by symptoms and signs of intestinal obstruction.[1] Intestinal transplantation can significantly improve the prognosis and quality of life of patients with intestinal motility disorders in Japan.[1] Survival rates in Japan are comparable with rates from the international intestinal transplant registry.[2]

Previously, the prevalence of intestinal motility disorders in Japan was unknown. It was estimated that there were 100 severe cases nationwide. This study supports this figure because surveillance was of a large enough scale to cover the entire nation.

There were over 40 patients who may need intestinal transplantation. However, only 3–4 a year intestinal transplants are performed in Japan, even if 10 times as many patients may be cured by intestinal transplantation.

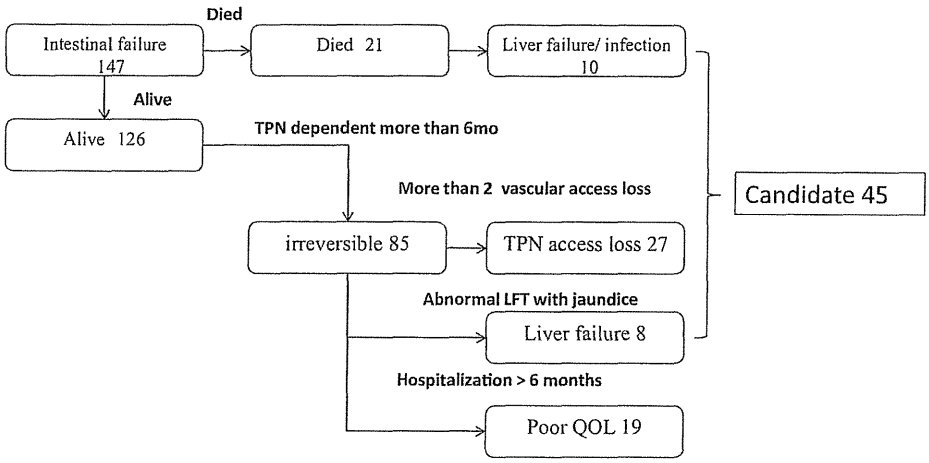


**Fig 3.** Candidates for intestinal transplantation. TPN, total parental nutrition; QoL, quality of life.

There were 2 major reasons for the relative paucity of intestinal transplants in Japan. One reason is the lack of available organs. For a long time, very few organs from deceased donors were obtainable in Japan. As with other solid organs, most intestinal transplants in Japan are performed with living donors. The shortage of organs has been alleviated due to a new act on organ transplantation that went into effect in 2010. However, the number of intestinal transplant has remained steady.

The financial barrier is the other, more profound reason preventing greater use of intestinal transplantation in Japan. Since the procedure is not covered by health insurance, either the patient or the transplant institution must pay the considerable costs out of pocket.

Some patients develop liver failure with intestinal motility disorders. These patients need simultaneous liver-intestine transplants. A combined liver-intestine transplant has less risk of acute rejection than an isolated intestinal transplant because the liver may have protective effects on the intestine. Current organ allocation guidelines do not allow for simultaneous combined liver-intestine organ retrieval; thus, a simultaneous liver-intestine transplant is impossible from deceased donor sources.

Previously, the laws on organ transplantation banned donors below 15 years of age. Intestinal transplants were not previously possible in infants because of organ size mismatch. Such patients will benefit from intestinal transplants in the future. Moreover, younger patients sometimes develop liver failure.[3] Multiorgan transplantation is a good option for such patients.[4]

It is difficult to determine the optimal timing for intestinal transplants to treat intestinal failure associated with intestinal motility disorders. Severe cases of intestinal motility disorders should be referred to institutions with expertise in transplantation.

In conclusion, the prevalence of severe motility disorders in Japan was elucidated. Patients with irreversible intestinal failure from intestinal motility disorders may be candidates for intestinal transplantation. Severe cases of motility disorder should be referred to transplant centers. Further investigation for patient details is required.

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# Experience Using Extended Criteria Donors in First 100 Cases of Deceased Donor Liver Transplantation in Japan

H. Furukawa, M. Taniguchi, M. Fujiyoshi, M. Oota, and the Japanese Study Group of Liver Transplantation

ABSTRACT

Because of the serious organ shortage in Japan, the use of extended criteria (EC) donors is inevitable to increase the number of deceased donors. However, the influence of this practice on recipient outcomes has not been clarified yet. We analyzed donor and recipient factors to determine whether those factors, especially from EC donors impacted early recipient outcomes. From February 1999 to January 2011, 100 deceased liver transplantations were performed in Japan, including 85 consecutive adult cases (age ≥18 years) who were studied to evaluate whether 6 recipient and 16 donor factors affected 3-month (90-day) recipient survival. Upon univariate analysis, Model for End-stage Liver Disease (MELD) score ≥ 25 ($P$ = .018), donor age ≥ 55 years ($P$ = .040), and cold ischemia time (CIT) ≥ 10 hours ($P$ = .00013) significantly reduced 3-month survival. Multivariate analysis confirmed the independent contributions of, three adverse factors including MELD score ≥ 25 ($P$ = .0133, odds ratio [OR] = 12.3, 95% confidence interval [CI] = 1.7–90.3), donor age ≥ 55 years ($P$ = .013, OR = 14.0, 95% CI = 1.6–119.5), and CIT ≥ 10 hours ($P$ = .0024, OR = 67.6, 95% CI = 4.5–1024.9). Three-month recipient survivals with 0, 1, 2, and 3 positive factors were 100% (n = 34), 94.4% (n = 36), 53.8% (n = 13), and 0% (n = 2), respectively ($P$ < .0001). In conclusion, to improve recipient short-term survivals, minimizing CIT is the first priority. In the long-term, we must promote deceased donation to reduce recipient MELD scores by shortening the waiting time, and revise the allocation system to minimize CIT by giving priority to the local area.

Because of the limited number of available deceased donors in Japan, organ shortage has become a major limitation. Although the revision of the Organ Transplantation Law in July 2010 allowing organ procurement with family consent has increased the number of deceased donors, it remains insufficient. In this situation of a serious organ shortage, the use of extended criteria (EC) donors is inevitable. However, the influence of those EC donors on recipient outcomes has not been clarified yet. We analyzed EC donor and recipient factors to determine which ones impacted early recipient outcomes.

## MATERIAL AND METHODS

From February 1999 to January 2011, 100 deceased liver transplantations were performed in Japan, including 85 consecutive adult cases studied herein except one subject who experienced an immediate death after surgery. Six recipient and 16 donor factors were analyzed for their impact on 3-month recipient survivals. The survey was performed at 21 deceased donor transplant centers in Japan. Recipient factors included age, sex, Model for End-stage Liver Disease (MELD) score, retransplantation, and type of liver disease (Table 1). Donor factors (extended donor criteria) included age (≥ 55 years), sex, cause of death, duration of cardiopulmonary resuscitation (≥10 minutes), hypotension (systolic pressure ≤ 60 mm Hg for ≥2 hours), intensive care unit stay (≥ 10 days), dopamine dosage (≥15 mcg/kg/min), use of pressers (≥2 pressers),

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**Table 1. Univariate Analysis of Recipient Risk Factors for 3-Month Patient Survival**

| Risk Factor | n | 3-Month Survival (%) | *P* Value |
|---|---|---|---|
| Recipient age | | | |
| <50 y | 54 | 85.2 | .3142 |
| ≥ 50 y | 31 | 93.5 | |
| Recipient sex | | | |
| Male | 45 | 86.7 | .7431 |
| Female | 40 | 90.0 | |
| MELD score | | | |
| < 25 | 59 | 93.2 | .01752 |
| ≥ 25 | 21 | 71.4 | |
| Diagnosis | | | |
| BA | 8 | 75.0 | .822 |
| CMD | 6 | 100.0 | |
| FHF | 11 | 90.9 | |
| LC (Other) | 12 | 91.7 | |
| LC (Viral) | 24 | 87.5 | |
| Other | 2 | 100.0 | |
| PBC | 7 | 100.0 | |
| PSC | 2 | 100.0 | |
| Re-LTx | 13 | 76.9 | |

Abbreviations: MELD, Model for End-stage Liver Disease; BA, biliary atresia; CMD, congenital metabolic disorders; FHF, fulminant hepatic failure; LC, liver cirrhosis; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis; Re-LTx, retransplantation.

sodium level (≥ 160 mEq/L), total bilirubin (≥ 2.0 mg/dL), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) (≥ 200 IU/l), body mass index (BMI; ≥ 28 kg/m$^2$), macrosteatosis (≥ 20%), cold ischemia time (CIT; ≥10 hours), warm ischemia time (≥50 minutes), and use of a split liver (Table 2). Subgroup analysis affecting for 3-month survival was performed dividing the recipients based upon the number of significant risk factors (RFs).

Statistical analyses were performed with software "R version 2.13.0"; univariate analysis with the Fisher exact test and multivariate analysis with logistic regression analysis. *P* values less than .05 were considered statistically significant.

## RESULTS

Patient survival was 91.8%, 89.4%, and 88.2%, at 1, 2 and 3 months respectively. Upon univariate analysis significantly worse 3-month survival rates. Center were associated with MELD score ≥ 25 ($P$ = .018), donor age ≥ 55 years ($P$ = .040), and CIT ≥10 hours ($P$ = .00013), (Tables 1 and 2). Upon multivariate analysis, three factors independently worsened 3-month survival rates: MELD score ≥ 25 ($P$ = .0133, OR = 12.3, 95% confidence interval (CI) 1.7–90.3), donor age ≥ 55 years ($P$ = .013, OR = 14.0, 95% CI = 1.6–119.5), and CIT ≥ 10 hours ($P$ = .0024, OR = 67.6, 95% CI = 4.5–1024.9 (Table 3).

**Table 2. Univariate Analysis of Donor Risk Factors for 3-Month Patient Survival**

| Risk Factor | n | 3-Month Survival | *P* Value | Risk Factor | n | 3-Month Survival | *P* Value |
|---|---|---|---|---|---|---|---|
| Donor age | | | | Na | | | |
| < 55 | 66 | 92.4 | .04025 | < 160 | 77 | 88.3 | 1 |
| ≥ 55 | 19 | 73.7 | | ≥ 160 | 8 | 87.5 | |
| Donor sex | | | | T-Bil | | | |
| Male | 47 | 91.5 | .3311 | < 2.0 | 71 | 90.1 | .3574 |
| Female | 38 | 84.2 | | ≥ 2.0 | 14 | 78.6 | |
| Cause of death | | | | AST | | | |
| CVA | 47 | 89.4 | .6808 | < 200 | 81 | 88.9 | .3997 |
| MVA | 19 | 78.9 | | ≥ 200 | 4 | 75.0 | |
| | | | | ALT | | | |
| Other | 6 | 100.0 | | < 200 | 82 | 87.8 | 1 |
| Suicide | 11 | 90.9 | | ≥ 200 | 3 | 100.0 | |
| | | | | BMI | | | |
| Tumor | 2 | 100.0 | | < 25 | 63 | 88.9 | .7141 |
| CPR | | | | ≥ 25 | 22 | 86.4 | |
| < 10 min | 53 | 84.9 | .3108 | Steatosis | | | |
| ≥ 10 min | 31 | 93.5 | | < 20% | 58 | 93.1 | .1217 |
| BP < 60 | | | | ≥ 20% | 7 | 71.4 | |
| < 2H | 78 | 89.7 | .1899 | Graft | | | |
| ≥ 2H | 7 | 71.4 | | Whole | 78 | 87.2 | .592 |
| ICU stay | | | | Split | 7 | 100.0 | |
| < 7 days | 38 | 92.1 | .5007 | CIT | | | |
| ≥ 7 days | 47 | 85.1 | | < 600 min | 57 | 98.2 | .00013 |
| Dopamine | | | | ≥ 600 min | 28 | 67.9 | |
| < 15 gamma | 57 | 87.7 | 1 | WIT | | | |
| ≥ 15 gamma | 28 | 89.3 | | < 50 min | 58 | 89.7 | .6932 |
| Vasopressor | | | | ≥ 50 min | 21 | 85.7 | |
| 0–1 drug | 42 | 88.1 | 1 | | | | |
| ≥ 2 drug | 43 | 88.4 | | | | | |

Abbreviations: Na, sodium; T-bil, total bilirubin; CVA, cerebrovascular accident; AST, aspartate aminotransferase; MVA, motor vehicle accident; ACT, alanine aminotransferase; BP, blood pressure; ICU, intensive care unit; BMI, body mass index; CPR, cardiopulmonary resucitation, CIT, cold ischemia time; WIT, warm ischemia time.

**Table 3. Multivariate Analysis For 3-Month Patient Survival**

| | Coefficient (SE) | Odds Ratio [95% CI] | *P* Value |
|---|---|---|---|
| MELD score ≥ 25 | 4.213 (1.387) | 12.3 [1.7–90.3] | .0133 |
| Donor age ≥ 55y | 2.514 (1.015) | 14.0 [1.6–119.5] | .0159 |
| CIT ≥ 600 min | 2.639 (1.094) | 67.6 [4.5–1024.9] | .0024 |

Abbreviations: SE, standard error; CI, confidence interval; MELD, Model for End-stage Liver Disease; CIT, cold ischemia time.

When the recipients were divided into four subgroups based on the number of those three RFs, 3-month survival rates of the recipients with 0, 1, 2, and 3 positive RFs were 100% (n = 34), 94.4% (n = 36), 53.8% (n = 13), and 0% (n = 2), respectively ($P < .0001$). When recipients with a CIT < 10 hours had another 1 or 2 RFs, the 3-month survival rates were 100% and 66.7%, whereas among those with CIT ≥ 10 hours, another 1 and 2 RFs yielded 3-month survival rates of 50% and 0% respectively.

## DISCUSSION

A number of studies over the past 2 decades have shown that both prolonged CIT and older age are major deleterious factors worsening early recipient outcomes.[1–5] Several recent studies showed recipient factors, especially MELD score in association with extended donor criteria, also adversely impact recipient outcomes.[6–8] Thus, the importance of analyzing both donor and recipient factors simultaneously has been emphasized to match donors and recipients to compensate for their risks.

From this study, MELD score, CIT, and donor age were observed to independently impact 3-month recipient survival rates. Subgroup analysis showed recipients with more RFs to have inferior 3-month survival; however, it was more than 66.7% when CIT was maintained within 10 hours. To minimize CIT, further efforts to reduce transportation time and to adjust donor and recipient operative times are mandatory. The allocation system must be revised to give priority to the local area in the future when more deceased donors become available.

In conclusion, because it is not realistic to eliminate patients with high MELD scores or older donors when there is a chance for a recipient, minimizing CIT is the first priority to improve recipient outcomes in Japan. In the long-term, we must promote an increased number of deceased donors to reduce the MELD score of the recipients by shortening the waiting time, and revise the allocation system to minimize CIT by giving priority to the local area.

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# Long-Term Hepatic Allograft Acceptance Based on CD40 Blockade by ASKP1240 in Nonhuman Primates

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**Blockade of the CD40–CD154 costimulatory signal is an attractive strategy for immunosuppression and tolerance induction in organ transplantation. Treatment with anti-CD154 monoclonal antibodies (mAbs) results in potent immunosuppression in nonhuman primates (NHPs). Despite plans for future clinical use, further development of these treatments was halted by complications. As an alternative approach, we have been focusing on the inhibition of the counter receptor, CD40 and have shown that a novel human anti-CD40 mAb, ASKP1240, markedly prolongs renal allograft survival in NHPs, although allografts eventually underwent chronic allograft nephropathy. On the basis of our previous findings that a CD40–CD154 costimulation blockade induces tolerance to hepatic, but not cardiac, allografts in rodents, we tested here our hypothesis that a blockade of CD40 by ASKP1240 allows acceptance of hepatic allografts in NHPs. A 2-week ASKP1240 induction treatment prolonged liver allograft survival in NHPs; however, the graft function deteriorated due to chronic rejection. In contrast, a 6-month ASKP1240 maintenance monotherapy efficiently suppressed both cellular and humoral alloimmune responses and prevented rejection on the hepatic allograft. No serious side effects, including thromboembolic complications, were noted in the ASKP1240-treated monkeys. We conclude that CD40 blockade by ASKP1240 would be a desirable immunosuppressant for clinical liver transplantation.**

**Key words: ASKP1240, CD40–CD154 costimulation blockade, liver transplantation, monoclonal antibody, nonhuman primates**

**Abbreviations: mAbs, monoclonal antibodies; LTx, liver transplantation; NHPs, nonhuman primates; DSA, donor-specific antibody; $T_{EM}$, effector memory T cell; $T_{reg}$, regulatory T cell.**

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## Introduction

Costimulation blockade is an effective strategy for preventing allograft rejection in experimental transplantations (1, 2). Among other examples, the inhibition of CD40–CD154 pathway induced tolerance in rodents (3), and the administration of anti-CD154 monoclonal antibodies (mAbs), such as hu5C8, IDEC-131 or ABI793, markedly prolonged kidney allograft survival in nonhuman primates (NHPs) (4–6). Although clinical applications were anticipated, further research was discontinued because these mAbs stimulated platelet (PLT) activation and caused thromboemboli (7,8). As an alternative, we and other researchers have considered the partner molecule, CD40. The anti-CD40 chimeric mAbs ch5D12 and chi220 were shown to prolong kidney allograft survival in NHPs (9,10). As a single agent, however, they were less effective than anti-CD154 mAbs for prolonging graft survival.

ASKP1240 is a newly developed fully human anti-CD40 mAb, which interrupts the CD40–CD154 axis by masking, does not cause antibody-dependent cell-mediated cytotoxicity or complement-dependent cytotoxicity, and consists of type 4 immunoglobulin G (11). In previous studies, we showed that a single use of ASKP1240 in both the induction (6-week) and maintenance (6-month) therapy markedly prolonged renal allograft survival in cynomolgus monkeys without causing apparent side effects (11,12). In particular, ASKP1240 maintenance treatment at 10 mg/kg completely suppressed both donor-specific antibody (DSA) and anti-drug antibody (ADA) formation (12). Although ASKP1240 ameliorated alloimmune responses and allowed prolongation of kidney allograft survival in NHPs, donor-specific tolerance was not achieved by the treatment, and these allografts underwent chronic allograft nephropathy (11,12).

In our previous studies, we showed that inhibition of the CD40–CD154 signaling pathway by the CD40Ig-encoded adenovirus vector allowed long-term acceptance of both liver and heart allografts in rats (13,14). Histopathology revealed that these cardiac allografts presented signs of cardiac allograft vasculopathy, whereas the hepatic allografts were normal. Furthermore, a skin-challenging test at more than 100 days after transplantation confirmed that donor-specific tolerance was induced to the liver allografts, but not to the cardiac allografts (13,14). A similar result concerning the tolerogenic effect of the CD40–CD154 signaling blockade on hepatic (15)—but not cardiac (16,17)—allografts has been shown by other researchers in rat transplantation models.

Here, we tested our hypothesis that the blockade of CD40–CD154 pathway by ASKP1240 leads to acceptance of hepatic allografts in NHPs.

## Materials and Methods

### *Animals*

Forty-nine purpose-bred male cynomolgus monkeys (*Macaca fascicularis*) including one blood donor animal, 49–75 months old (median, 56 months) with body weights ranging from 3.8 to 6.7 kg (median, 0.9 kg), were used. Animals were maintained and operated upon at the Shin Nippon Biomedical Laboratories (SNBL, Kagoshima, Japan). The experiment protocol was approved by the Animal Care and Use Committee of the SNBL, and all procedures were performed in accordance with the standards described in the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health.

### *Liver transplantation*

One donor animal was used per recipient. Twenty-four donor/recipient pairs were selected by ABO blood-type compatibility and by one-way mixed lymphocyte reaction (MLR) assays revealing a stimulation index greater than 5. In addition, the selection of monkey pairs was based on their genetic nonidentity according to major histocompatibility complex (MHC) class II DRβ, as confirmed by a direct sequencing of the second exon of DRβ as previously described (11,12). In the donor surgery, the portal vein (PV), suprahepatic vena cava (SHVC) and infrahepatic vena cava (IHVC) were dissected out. The hepatic artery was kept in continuity with the celiac trunk and abdominal aorta up to the iliac bifurcation. Before perfusion, donor blood was collected. The liver was perfused *in situ* through the aorta using cold histidine-tryptophan-ketoglutarate solution (Odyssey Pharmaceuticals, East Hanover, NJ, USA) and collected. Recipient surgery was conducted in parallel. Before removing the native liver, the superior mesenteric artery (SMA) and, if necessary, the middle colic artery, was clamped to prevent bowel congestion. Following hepatectomy, the graft liver was placed into the right hepatic fossa, and the SHVC and PV were anastomosed to those of the graft. The SMA was declamped, and, subsequently, the graft was reperfused. After completion of the IHVC anastomosis, the graft aorta conduit was finally anastomosed to the recipient aorta in an end-to-side fashion. For biliary reconstruction, cholecystoduodenostomy (18) was performed. In 2 cases (animals #23, #24), a side-to-side choledochocholedochostomy (19) was used. Animals were administered cefazolin (10 mg/kg; Astellas Pharma, Tokyo, Japan) before and after liver transplantation (LTx) for 3 days. Intravenous fluid was given for 3 days, until oral intake became adequate. Vital signs, appetite and attitude of LTx animals were monitored daily. Animals were euthanized when exhibiting severe weakness, weight loss or abnormal behavior, as determined by veterinary staff and investigators.

### *Experimental groups and treatment protocols*

The animals were randomly divided into three groups. Three animals receiving no treatment served as a control (n = 3). For the induction treatment group (n = 4), ASKP1240 (10 mg/kg) was given intravenously on days 0, 4, 7, 11 and 14. For the maintenance treatment group (n = 7), a weekly ASKP1240 (5 mg/kg) administration was subsequently continued for up to 6 months. No additional therapy was given to prevent rejection. Transplant recipients that died within 48 h after transplantation, nine due to a severe ischemia reperfusion injury (IRI) and one due to bleeding, were excluded.

### *Biochemical and immunological analyses*

***Hematology and blood chemistry:*** Laboratory assessments were performed three times per week for the first two postoperative weeks and weekly thereafter. Peripheral blood hematology—including red blood cell (RBC) and white blood cell (WBC) counts, hemoglobin, hematocrit and PLT counts—was performed with the ADVIA120 (Bayer Diagnostics, Sudbury, UK). The serum levels of total protein, albumin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (T-Bil), gamma-glutamyl transpeptidase (γ-GTP), total cholesterol, high-density lipoprotein cholesterol, creatinine, blood urea nitrogen and electrolytes were measured using the JCA-BM8 (Nippon Denshi, Tokyo, Japan).

***Serum ASKP1240 trough level:*** For the pharmacokinetic monitoring of ASKP1240, peripheral blood samples were obtained immediately before drug administration. Serum ASKP1240 trough levels were measured using an enzyme-linked immunosorbent assay as described previously (11,12).

***Donor-specific antibody (DSA):*** DSA was assessed by incubating the donor splenocytes with serum obtained from transplanted recipients as described previously (11,12).

***Anti-ASKP1240 antibody:*** Anti-drug antibody (ADA) was examined by applying surface plasmon resonance technology as described previously (11,12).

***Mixed lymphocyte reaction (MLR):*** One-way mixed lymphocyte reaction was performed before transplantation and periodically after grafting. Gamma-irradiated (30 Gy) peripheral blood mononuclear cells (PBMCs) of donor animals were used as stimulator cells, and responder PBMCs were obtained from recipient animals as described previously (11,12).

***ImmuKnow:*** Anticoagulated whole blood was diluted with sample agent and incubated for 15–18 h with phytohemagglutinin (PHA). The following day, $CD4^+$ T cells were positively selected with magnetic particles coated with antihuman CD4 monoclonal antibodies (Dynabeads, Oslo, Norway), washed to remove residual cells, and lysed to release intracellular adenosine triphosphate (ATP). Released ATP was measured using luciferin/luciferase and a luminometer (Turner Biosystems, Madison, WI, USA). The procedure was performed according to the manufacturer's instructions.

***ELIspot:*** The frequencies of donor-reactive interferon (IFN)-γ-secreting T cells in the periphery were determined by the ELIspot$^{PLUS}$ Monkey IFN-γ kit (Mabtech, Cleveland, OH, USA). PHA or gamma-irradiated (30 Gy) splenocytes of donor and third-party animals were used as stimulators. For the direct-response assay, the recipient's PBMCs ($5 \times 10^5$/well) were cocultured with stimulators ($1 \times 10^6$/well) for 24 h. For the indirect-response assay, the recipient's PBMCs ($5 \times 10^5$/well) were cocultured with lysed

stimulator splenocytes (1 × $10^6$/well) for 40 h. ELIspot plates were precoated with IFN-γ capture antibody (GZ-4) for 16 h and blocked with FBS-supplemented RPMI 1640 medium for 30 min. After incubation, the plates were washed, and biotin-conjugated anti–IFN-γ Ab (7-B6-1) was added. After 2 h, the plates were incubated with streptavidin-HRP for 1 h. Spots were developed using TMB solution and were counted by computer-assisted KS ELISPOT 4.1 Software (Carl Zeiss, Thornwood, NY, USA).

***Leukocyte phenotype:*** PBMCs were labeled with the following mAbs: CD4 (L200), CD8 (RPA-T8), CD25 (M-A251), CD28 (CD28.2), CD95 (DX-5), CD154 (TRAP1) (all from BD Biosciences Pharmingen, Mountain View, CA, USA) and FoxP3 (PCH101; eBioscience, San Diego, CA, USA) and were assessed by flow cytometry. Phenotypic surface markers for effector memory ($CD28^-CD95^+$), central memory ($CD28^+CD95^+$) and naive ($CD95^-$) T cells were assessed according to the methods reported by Pitcher et al. (20) and Koyama et al. (21). Staining was performed according to the manufacturer's instructions.

***Gene expression:*** Total RNAs were extracted from biopsy-obtained graft tissues by the RNeasy kit (QIAGEN, CA, USA) and from PBMCs by the TRIzol reagent and PureLink RNA Mini Kit (Invitrogen, Carlsbad, CA, USA). Complementary DNA was then synthesized. Oligonucleotide primers and TaqMan probes for measurement of mRNA expression of FoxP3, IL-10, TGF-β and GAPDH were designed with the Primer express software (Applied Biosystems, Foster City, CA, USA). A ready-made primer and probe kit, TaqMan Gene Expression Assays for Rhesus Macaque (Applied Biosystems), was used for measurement of IL-15 (Assay ID; Rh02621777 m1), IFN-γ (Rh02788577 m1), Perforin (Rh02621761 m1) and Granzyme B (Rh02621701 m1). PCR analysis was performed with the ABI PRISM 7900HT Fast Real-Time PCR System (Applied Biosystems). The relative mRNA expression levels in 2 μg of total RNA were calculated using the standard curve and were normalized by the GAPDH levels.

### *Histopathological determinations*

***Histological study:*** Liver biopsies were performed monthly after transplantation, using a 16-gauge Biopsy-Cut needle (C.R. Bard, Covington, GA, USA). Allograft rejection was evaluated histopathologically according to the Banff classification method (22). Necropsy was performed immediately after euthanization to evaluate the graft and extrahepatic organs, including the brain, lung, heart, thymus, gallbladder, spleen, pancreas, stomach, duodenum, jejunum, ileum, colon, rectum, kidney, ureter, bladder and lymph nodes. Tissue samples were fixed in 10% neutral buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin. In addition, graft tissues were stained with cytokeratin 7, α-smooth muscle actin and Masson trichrome. Histopathological examination was performed by two pathologists who were not aware of the nature of the treatment group or the clinical findings.

***Immunohistochemistry:*** Frozen tissue sections (4 μm) were fixed with cold acetone and stained by an indirect immunofluorescence technique using antihuman C4d mAb (Quidel, San Diego, CA, USA) and fluorescein isothiocyanate-conjugated rabbit antimouse IgG polyclonal antibody (Zymed, South San Francisco, CA, USA). For detection of regulatory T cells in grafts, double staining with Texas Red-conjugated rabbit polyclonal anti-FoxP3 antibody (Abcam, Cambridge, UK) and fluorescein isothiocyanate-conjugated anti-CD4 mAb (BD Biosciences, San Jose, CA, USA) was performed in the frozen sections.

### *Statistical analysis*

All values are represented as mean (SD). An intergroup statistical analysis was performed using the Mann–Whitney *U*-test. The differences were considered statistically significant when a p-value was less than 0.05.

## Results

### *Animal survival and clinical course*

Allograft survival, development of DSA and ADA and final causes of death are summarized in Figure 1A. Under the standard LTx technique without using veno-veno bypass, transplant recipients died during or just after operation mainly due to the circulatory and respiratory failure without signs of rejection because this monkey was very susceptible to IRI. To overcome this, we adopted the SMA clamping technique (manuscript in preparation). Without immunosuppression, the control allografts were promptly rejected by acute cellular rejection within a week (Figure 1A). Serum T-Bil (Figure 1B) and ALT (Figure 1C) levels were exacerbated soon after LTx. The ASKP1240 induction treatment prolonged hepatic allograft survival to 90, 188 and 209 days, except for one animal that died of a biliary complication; however, these allografts were rejected by chronic rejection (Figure 1A). Serum ASKP1240 trough levels declined slowly after cessation of ASKP1240 and became undetectable at 2 months after LTx (Figure 1D). From this point, serum T-Bil levels began to increase (Figure 1F), whereas ALT levels were unstable (Figure 1H). In contrast, maintenance ASKP1240 treatment markedly prolonged liver allograft survival to 272, 278 and 1035 days (Figure 1A). The animal who survived for more than 1000 days was euthanized at day 1035 without signs of rejection. The other two of three long-term survivors were lost due to repeated cholangitis (Figure 1A) as determined by clinical course, data and histopathology. In these animals, WBC elevated along with increased differential leucocyte count during cholangitis, and by fasting and administration of antibiotics, laboratory data recovered. Another two animals were lost because of abscess formation (Figure 1A) despite treatment of cholangitis, and the other two grafts failed shortly after LTx because of a severe IRI (Figure 1A). These four grafts failed without apparent sign of acute cellular rejection. During the ASKP1240 treatment course, serum ASKP1240 trough levels were maintained for up to 200 days after LTx (Figure 1E). Within this period, serum T-Bil (Figure 1G) and ALT (Figure 1I) levels did not rise, except in some cases that experienced transient cholangitis. No serious side-effects, including thromboemboli, were observed in the animals receiving ASKP1240 treatment.

### *Hematology*

No significant changes in RBC, WBC and PLT counts were noted in the animals, except for two cases in the maintenance treatment group that experienced spur cell anemia mainly due to liver dysfunction after LTx. This anemia, however, gradually normalized along with recovery of liver function during the ASKP1240 treatment period. Gross peripheral lymphopenia was not significant (Figures 2A and B), although absolute numbers of $CD4^+$ (Figures 2C and D) and $CD8^+$ (Figures 2E and F) T cells and the non–T-cell population (Figures 2G and H) in the periphery decreased

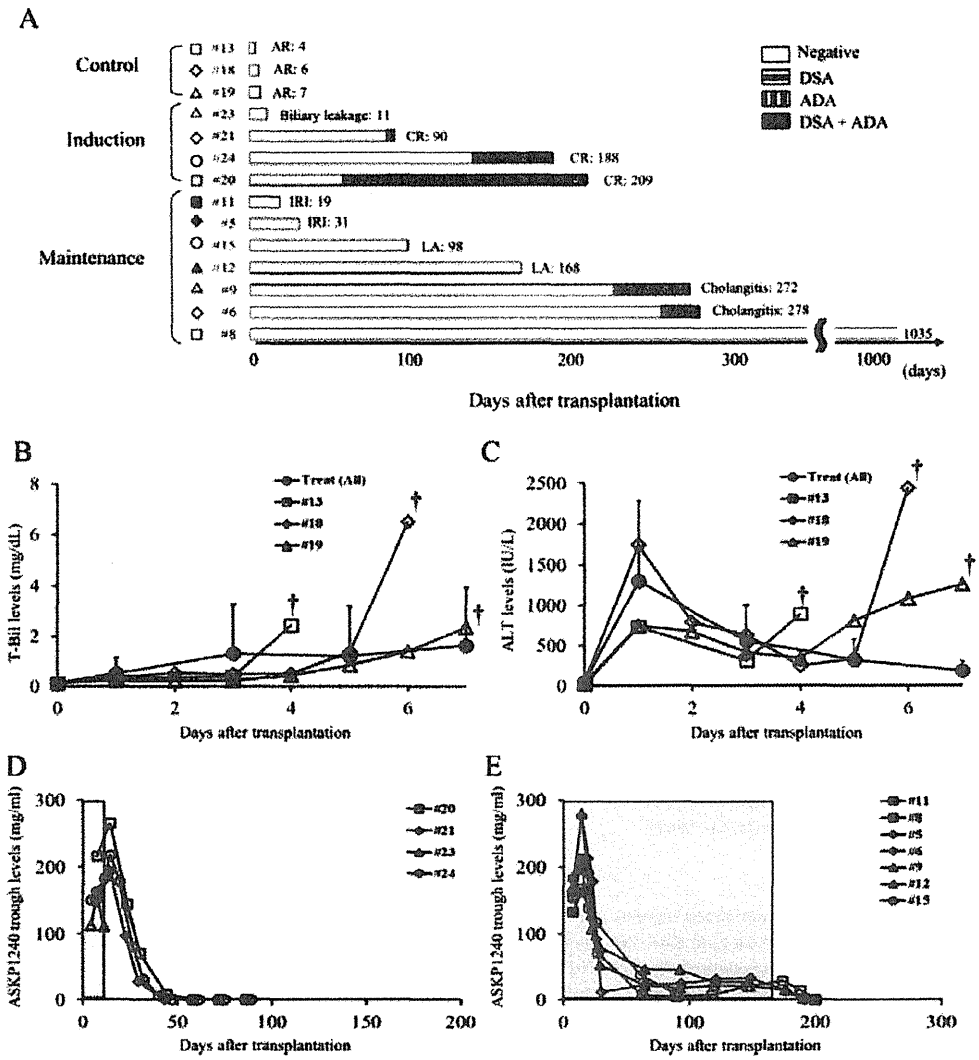


**Figure 1: Clinical courses and histopathological findings in grafts of individual transplanted monkeys.** (A) Results of histopathological examination showing allograft survival, treatment and cause of death. The horizontal bars represent the duration of survival of each animal after liver transplantation (LTx). The graft survival time and final diagnosis are shown at the end of the survival bar. The course of death was determined by periodical biopsy, necropsy, and clinical findings (AR, acute cellular rejection; CR, chronic rejection; IRI, ischemia reperfusion injury; LA, liver abscess). The bars also show the presence of serum donor-specific antibody (DSA) (vertical line), antidrug antibody (ADA) (cross line) or both (black fill). In the figure, #13 (open square), #18 (open diamond) and #19 (open triangle) represent the nontreated animals; #20 (open square), #21 (open diamond), #23 (open triangle) and #24 (open circle) represent the induction-treated animals and #11 (closed square), #8 (open square), #5 (closed diamond), #6 (open diamond), #12 (closed triangle), #9 (open triangle), and #15 (open circle) represent the maintenance-treated animals. The control animals (#13, #18, and #19) died shortly after LTx because of AR. The ASKP1240 induction treatment prolonged allograft survival to 90 (#21), 188 (#24) and 209 (#20) days; 1 animal died on day 11 because of biliary leakage (#23). In the ASKP1240 maintenance treatment group, 2 grafts failed at 19 (#11) and 31 (#5) days because of severe IRI, and another 2 animals died at 98 (#15) and 168 (#12) days because of LA, without signs of rejection. The other 3 animals survived for 272 (#9), 278 (#6) and 1035 (#8) days without apparent rejection. The serum ASKP1240 trough levels and both serum total bilirubin (T-Bil) and aspartate aminotransferase (AST) levels were periodically assessed before and after LTx. The shaded areas are the periods of ASKP1240 administration. Within a week after LTx, the control animals showed exacerbated serum T-Bil (B) and alanine aminotransferase (ALT) (C) levels compared with the average levels in the ASKP1240-treated animals (closed circle with error bars). The changes in ASKP1240 trough (D and E), T-Bil (F and G) and ALT (H and I) levels in the induction (D, F and H) and maintenance (E, G and I) treatment groups are shown.

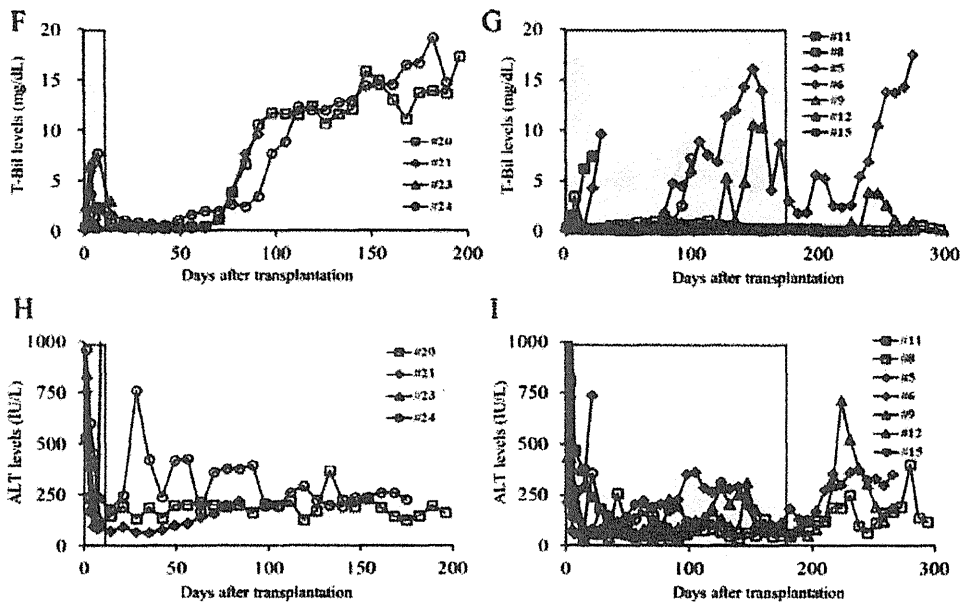


**Figure 1: Continued**

during ASKP1240 administration. The reduction of these lymphocytes was approximately proportional to the given dose of ASKP1240: a robust decrease within the first 2 weeks and a trend to recover slowly thereafter. However, we do not deny other possibilities for the change of lymphocyte counts such as surgery or cholangitis. The numbers of these cells recovered when ASKP1240 treatment was discontinued.

### *Histopathology*

The failed grafts in the control group exhibited typical histopathological features of acute cellular rejection. Severe periportal lymphocyte infiltration (Figure 3A) and bile duct inflammation were observed, along with severe central venous endotheliitis (Figure 3B) and perivenular hepatocyte necrosis. In all transplanted animals treated with ASKP1240, there were, to a varying degree, pathological hallmarks of IRI and cholangitis including bile duct injury and portal fibrosis in their liver graft. In the induction treatment group, all the allografts were rejected by chronic rejection, except for one animal that had biliary leakage (Figure 1A). Histopathology revealed that periportal cellular infiltration (Figure 3C) and bile duct atrophy were observed without ductular reaction. Later, the central veins showed veno-occlusive changes (Figure 3D). In the maintenance treatment group, the allografts suffered from a severe IRI and repeated cholangitis; however, they manifested no signs of either acute or chronic rejection. Some grafts showed a slight fibrotic change in the portal tract, without persistent cellular infiltration, even after discontinuation of the maintenance ASKP1240 therapy. Although ductular reaction was observed, bile duct loss did not occur (Figure 3E). Two animals died due to IRI. Histopathology of grafts, obtained from these animals at the time of necropsy, revealed a severe dropout of hepatocytes in the perivenular area along with only a minimal cell infiltrate in the portal tract but there was no sign of acute cellular rejection. Hepatic allografts of animals euthanized at PODs 272 and 278 revealed that there were bile duct damages including ductular reaction and bridging fibrosis in the portal area, but again, without a sign of either acute or chronic rejection. A long-surviving allograft recovered at 1035 days after LTx did not show any signs of rejection; fibrotic change in the portal tract was minimal (Figures 3F–H). Microscopic abnormalities were not found in the extrahepatic organs, including the spleen, which was intact (data not shown).

### *Immunological studies*

***IFN-γ-secreting alloreactive T cells (ELIspot Assay):*** During ASKP1240 treatment, both direct and indirect antidonor cellular responses, as assessed by the frequency of IFN-γ-secreting alloreactive T cells in the periphery, were completely abolished. However, after ASKP1240 cessation, these alloreactive T cells increased in the induction therapy group (Figure 4A), whereas this was not the case following maintained ASKP1240 treatment even after ASKP1240 cessation (Figure 4B). In the longest surviving animal (#8), direct and indirect responses against donor antigens were suppressed, whereas those against third party antigens or mitogens were intact (Figure 4C).

***Memory cells:*** The number of peripheral $CD4^+$ and $CD8^+$ effector memory T cells ($T_{EM}$: $CD28^-CD95^+$) had declined at 1 month after LTx (Figures 4D and E). The $T_{EM}$,

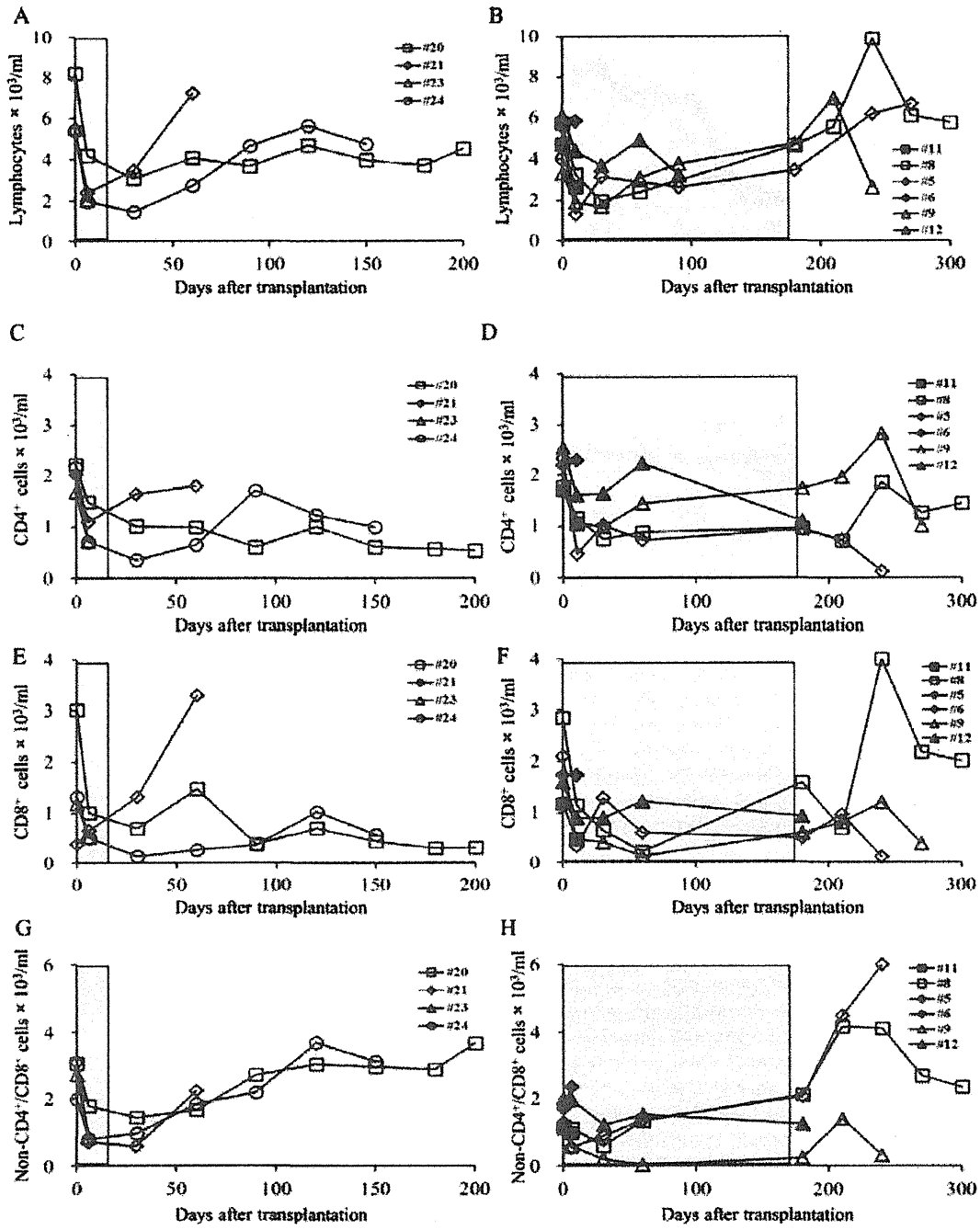


**Figure 2: Changes in peripheral lymphocyte counts following induction and maintenance treatments.** The counts of peripheral total (A and B), $CD4^+$ (C and D), $CD8^+$ (E and F) and non-$CD4^+/CD8^+$ (G and H) lymphocytes following induction (A, C, E and G) and maintenance (B, D, F and H) ASKP1240 treatment are presented. In the figure, #20 (open square), #21 (open diamond), #23 (open triangle) and #24 (open circle) have been used to represent the induction-treated animals, and #11 (closed square), #8 (open square), #5 (closed diamond), #6 (open diamond), #12 (closed triangle) and #9 (open triangle) have been used to represent the maintenance-treated animals. The shaded areas are periods of ASKP1240 administration. The peripheral lymphocyte counts decreased to values ranging from one-third to two-thirds of the preoperative value within 50 days after LTx. In the ASKP1240 induction treatment group, peripheral lymphocyte counts recovered gradually after cessation of treatment. In the maintenance treatment group, although the same tendency was observed shortly after LTx, lymphocyte count recovery was noted during the ASKP1240 treatment course.

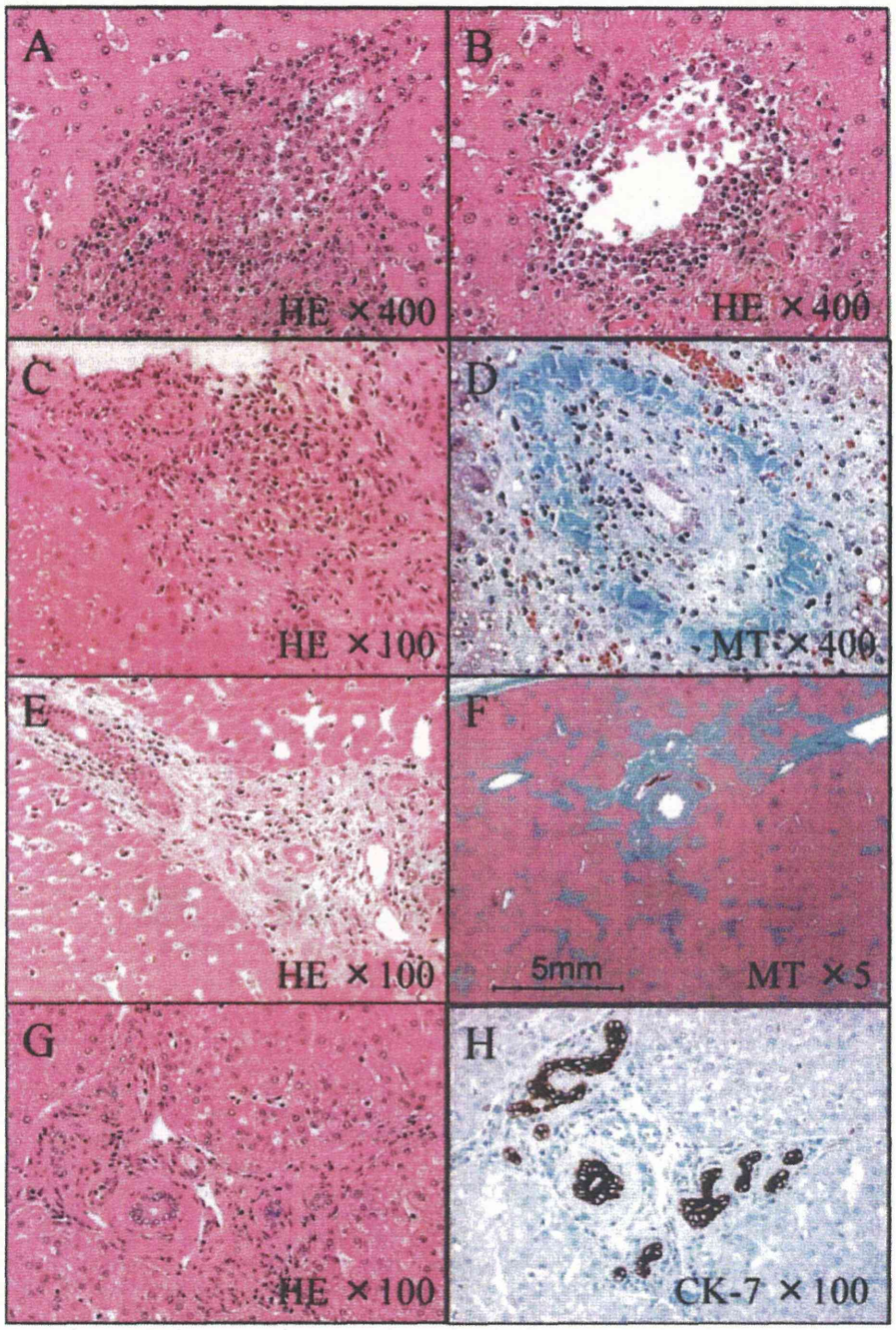


**Figure 3: Histopathology of hepatic allografts.** Microscopic appearance of liver allografts from the control (A, B), induction treatment (C, D) and maintenance treatment (E–H) groups. The control allograft at postoperative day (POD) 4 (#13) manifested acute cellular rejection with severe leukocyte infiltration in the periportal area (A), bile duct and central vein (B). Hepatic allografts, obtained from the induction treatment group at POD 88 (#24: C) and POD 209 (#20: D), manifested chronic rejection with mild periportal cell infiltration (C), bile duct atrophy and loss and veno-occlusive changes (D). Graft liver excised at POD 174 (#9: E) and POD 1035 (#8: F, G and H) from the ASKP1240 maintenance treatment group manifested no signs of acute cellular or chronic rejection. The portal tract showed mild fibrotic change and edema with minimal inflammation and eosinophilic infiltration (E). A ductular reaction was observed without bile duct loss. Minimal portal fibrosis and ductular reaction in the central vein was noted without apparent fibrosis. A long-term surviving liver graft showed occasional bridging portal fibrosis (F) and portal fibrosis with minimal inflammatory cell infiltration with good interlobular bile duct preservation (G), and with ductular proliferation (H). (HE, hematoxylin–eosin stain; CK-7, cytokeratin-7 stain; MT, Masson trichrome stain.)

particularly $CD4^+$ cells, significantly increased after the termination of ASKP1240-induction therapy (Figure 4D), whereas they did not rise at all when ASKP1240 was maintained (Figure 4E). A similar trend was observed in changes of $CD8^+$ $T_{EM}$ (Figures 4D and E).

***Antigen-specific $CD4^+CD154^+$ cells:*** Recently, it has been shown that *de novo* CD154 expression on $CD4^+$ T cells after stimulation identifies antigen-specific (23, 24) and also alloantigen-specific (25) phenotypes. Thus, we examined this population in the periphery. In the untreated control animals, the number of $CD4^+CD154^+$ T cells responding to the donor antigens increased, whereas the ASKP1240 treatment abrogated the elevation of this $CD4^+CD154^+$ T-cell population even after drug cessation in both the induction and maintenance treatment groups (Figure 4F).

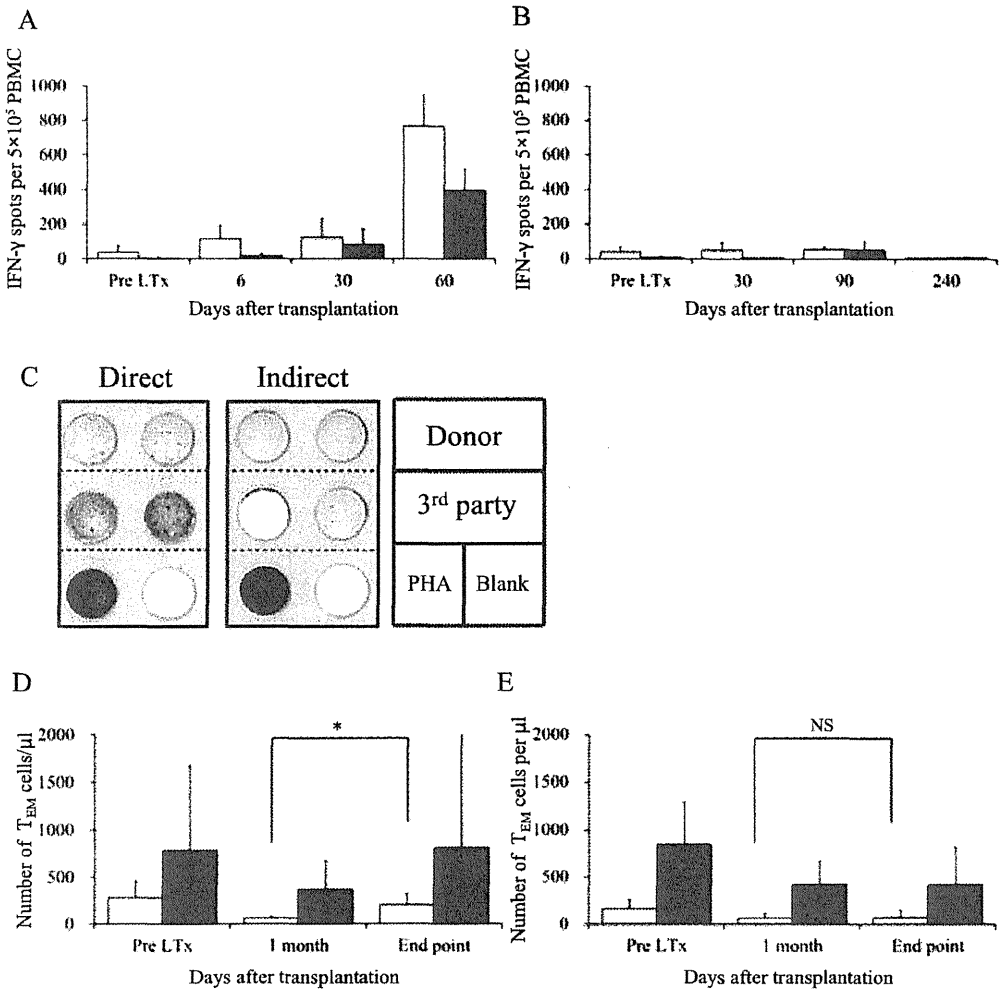


**Figure 4: Cellular alloimmune responses to hepatic allografts.** The frequencies of directly and indirectly stimulated, donor-reactive IFN-γ–secreting T cells in the induction (A) and maintenance (B) treatment groups were evaluated with ELIspot assay. White and black vertical bars represent the directly and indirectly stimulated IFN-γ spots, respectively. In the ASKP1240 induction treatment group, the frequencies of donor-reactive T cells in the periphery increased after drug cessation (A), whereas alloreactive T cells responding through direct and indirect pathways did not increase even after drug cessation in the ASKP1240 maintenance treatment group (B). In the longest surviving animal (#8), the frequencies of donor-reactive IFN-γ–producing cells were still minimal at day 389, although responses against third-party antigens and phytohemagglutinin (PHA) were maintained (C). The number of peripheral $T_{EM}$ ($CD28^{-}CD95^{+}$) cells following induction (D) and maintenance (E) treatment was examined. The white and black vertical bars indicate cell counts of $CD4^{+}$ and $CD8^{+}$ $T_{EM}$, respectively. The terminal end point was defined at 12 months after LTx or just before death if the animal did not survive for >12 months. In the ASKP1240 induction treatment group (D), the numbers of circulating $CD4^{+}$ $T_{EM}$ cells significantly increased after LTx. A similar trend was observed for the generation of $CD8^{+}$ $T_{EM}$ cells. In the ASKP1240 maintenance treatment group (E), neither $CD4^{+}$ nor $CD8^{+}$ $T_{EM}$ cells increased, even after drug cessation. Dot plots show that the proportion of donor-specific $CD4^{+}CD154^{+}$ T cells in the control allografts was higher than those of the naive and ASKP1240-treated allografts (F). The individual ATP values are presented in the induction (G) and maintenance (H) treatments. In the figure, #20 (open square), #21 (open diamond), #23 (open triangle) and #24 (open circle) represent the induction-treated animals, and #11 (closed square), #8 (open square), #5 (closed diamond), #6 (open diamond), #12 (closed triangle) and #9 (open triangle) represent the maintenance-treated animals. † and * indicate chronic rejection (G) and cholangitis (H), respectively. ImmuKnow ATP values were not affected by either rejection or infection episodes (G and H). The shaded areas are periods of ASKP1240 administration (*$p < 0.05$, Mann–Whitney *U*-test; NS, not significant).

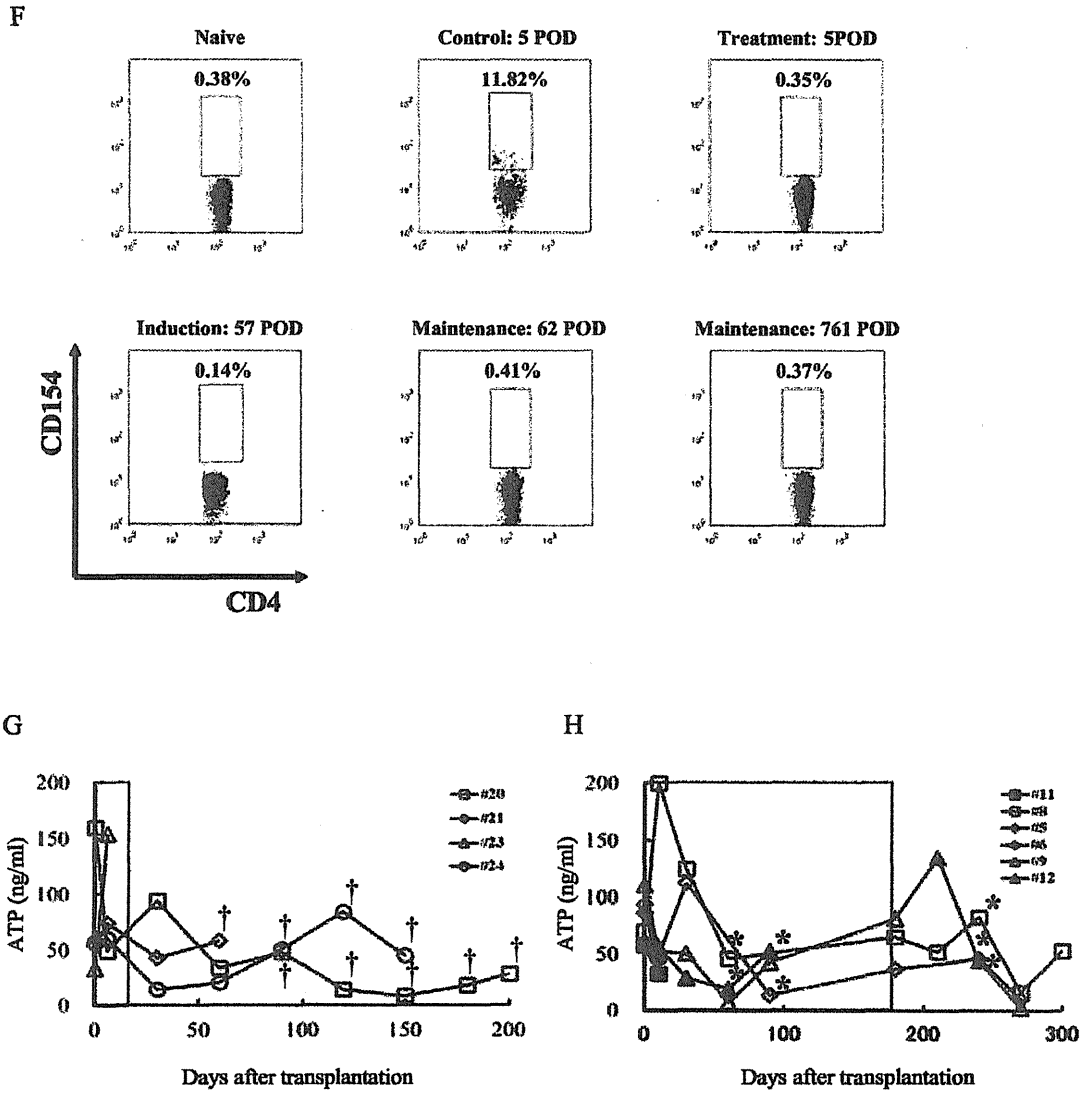


**Figure 4:** Continued

***ImmuKnow ATP values:*** In all groups, the ImmuKnow ATP values (ng/mL) were not affected by either rejection or infection throughout the course of the study (Figures 4G and H).

***DSA and ADA formation:*** Formation of antidonor IgM and IgG antibodies in sera was suppressed during the treatment course, whereas they tended to rise after ASKP1240 cessation in both the induction and maintenance treatment groups (Figures 1A and 5A–D). Both the induction and maintenance ASKP1240 treatments completely suppressed anti-ASKP1240 antibody formation (Figure 1A), except for 1 animal (#20) that experienced a transient increase after drug cessation.

***Immunohistochemistry:*** Neither C4d nor IgG deposition was observed in the allografts that underwent chronic rejection or survived without a sign of rejection (Figures 5E and F). The proportion of $Foxp3^+$ cells in the long-term surviving allografts did not rise at all as compared with those of the rejected allografts (data not shown).

***Regulatory T cells ($T_{reg}$):*** The peripheral $CD4^+$ $CD25^+Foxp3^+$ regulatory T-cell subset did not increase, even in the long-term surviving animals without rejection. Rather, a higher proportion of this subset was observed in animals that rejected allografts by acute cellular rejection (Figure 6A). At early a time point after LTx, $T_{EM}/T_{reg}$ ratio of the ASKP1240-treated animals was low. After the

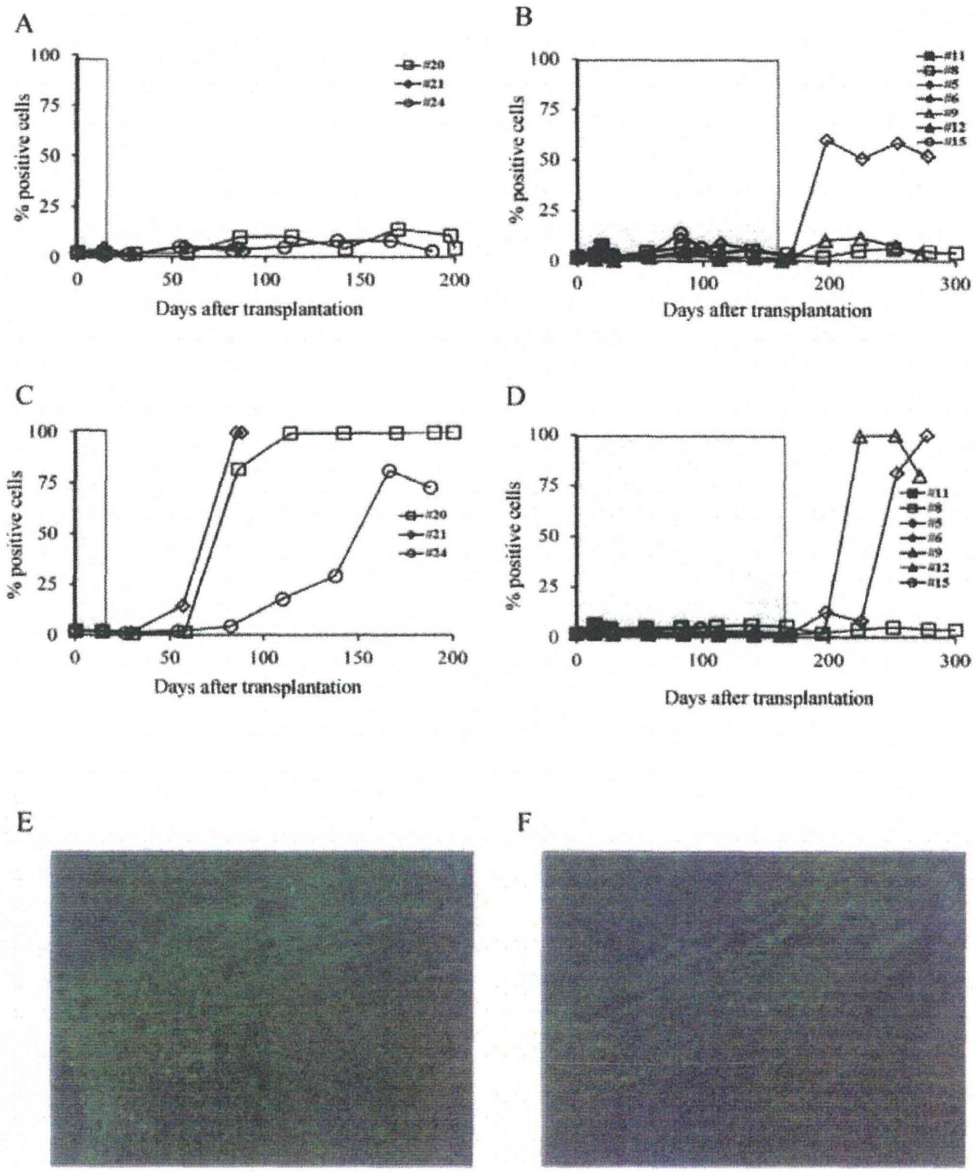


**Figure 5: Humoral immune responses to hepatic allografts.** The formation of antidonor IgM (A and B) and IgG (C and D) antibodies in sera following ASKP1240 induction (A and C) and maintenance (B and D) treatment was monitored by flow cytometric analysis. In the figure, #20 (open square), #21 (open diamond) and #24 (open circle) represent the induction-treated animals, and #11 (closed square), #8 (open square), #5 (closed diamond), #6 (open diamond), #12 (closed triangle), #9 (open triangle) and #15 (open circle) represent the maintenance-treated animals. The shaded areas are periods of ASKP1240 administration. During the ASKP1240 treatment period, antidonor antibodies were not detected. After cessation of ASKP1240 treatment, the serum antidonor IgG level increased. No allografts showed deposition of C4d (E) or IgG (F) (#8; POD 445).

drug cessation, this ratio significantly increased in both the induction and maintenance treatment groups; however, elevation of $T_{EM}/T_{reg}$ ratio was significantly higher in the induction-treated animals as compared to that of maintenance-treated ones (Figure 6B). Intragraft Foxp3 expression, as detected by immunohistochemistry (data not shown) and PCR (Figure 6C), did not show any significant change by the ASKP1240 treatment in the transplanted animals.

***PCR:*** The intragraft Granzyme B mRNA expression level (Figure 6D) was significantly down-regulated in the animals