Table 2. The demographics of the non-ACR, ACR, and SRR groups of pediatric ALF patients after LT

	non-ACR	ACR	SRR	p
n	16	17	7	
Age at LT (median)	17 days–12 yr 4 months (1 yr 3 months)	1 months–4 yr 4 months (9 months)	6 months–12 months (10 months)	0.427
BW at LT (kg [median])	2.6–32 (8.1)	3.5–17 (8.6)	7.6–9.6 (8)	0.669
Gender male/female	8/8	12/5	5/2	0.411
Living donor (n [%])	13 (76.5)	17 (100)	7 (100)	0.036
ABO-incompatible (n [%])	4 (25)	5 (29.4)	1 (14.3)	0.739
Etiologies (n [%])				
Unknown	11 (68.8)	14 (82.4)	7 (100)	0.215
Others	5 (31.3)	3 (17.7)	0 (0)	
EBV	2 (12.5)	1 (5.9)		
CMV	1 (6.3)			
HSV 1		1 (5.9)		
Echovirus 3		1 (5.9)		
MDS	1 (6.3)			
Hemochromatosis	1 (6.3)			

HSV, herpes simplex virus; MDS, mitochondria DNA depletion syndrome.

Table 3. The outcomes of basiliximab therapy in the SRR patients

Case	The interval between treatment and discharge (days)	ACR after basiliximab	CMV	EBV	Other infection	Outcome	Follow-up (months)	Present IS
1	18	+	+	–		Alive	27	TAC
2	90	+	+	–	Pneumocystis pneumonia	Alive	24	PSL, SRL
3	40	–	+	–		Alive	47	TAC
4	23	–	+	–		Alive	51	TAC
5	223	+	+	+		Graft loss, retransplant (POM 9), Alive	18	TAC, PSL, MMF, SRL
6	41	–	+	+		Alive	15	TAC, PSL
7	23	–	+	+		Alive	2	TAC, PSL, MMF

POM, postoperative months.

All patients survived, although one patient (Case 5) required retransplantation due to graft failure. The histopathological findings of his explanted graft revealed severe centrilobular rejection, including severe necrosis of zone 3, accompanied by suspected veno-occlusive disease. Veno-occlusive disease was not present in the other patients. Three patients (Cases 1, 3, and 4) could discontinue steroids after basiliximab therapy. Two patients (Cases 2 and 6) remained on prednisolone for more than two yr and one yr after basiliximab therapy, respectively. Two patients required low doses of prednisolone due to a slight increase of the values of liver function tests after basiliximab therapy at the end of the follow-up.

The changes in the results of liver function tests, such as the levels of total bilirubin, AST, and ALT after basiliximab therapy in all but one patient (Case 5) are shown in Fig. 1, which revealed a dramatic improvement of the liver function test after basiliximab therapy. The patients were discharged from the hospital with a median period from the date of basiliximab therapy to the resolution of ACR of 31.5 days (range: 18–90 days). In spite of improvement of the liver function, the duration of hospitalization was extended because the administration of ganciclovir was required in all but one patient (Case 5). Three patients (Cases 1, 2, and 5) experienced ACR at seven, 10, and 1.5 months after basiliximab therapy, respectively. All patients improved following treatment with single steroid pulse therapy. There was the same sentence in this paper.

Infectious complications after basiliximab therapy

Opportunistic infections were common, as evidenced by the CMV and EBV viremia and pneumocystis pneumonia. None of the patients developed CMV disease, although CMV-pp65 antigenemia was detected in all patients after basiliximab therapy. CMV immunoglobulin-G was

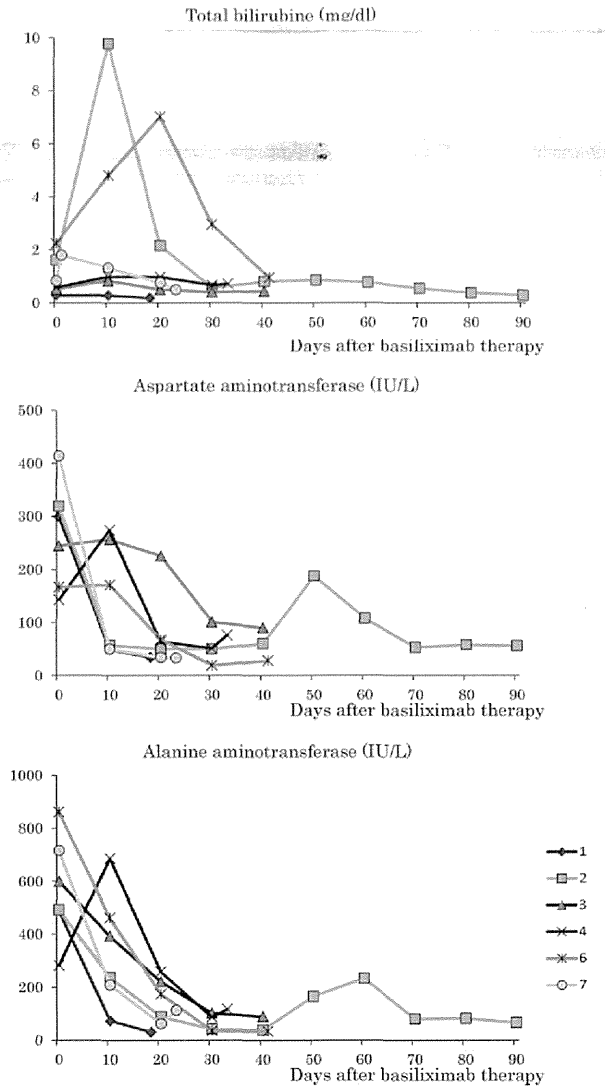

Fig. 1. Changes in liver function test parameters in six patients who exhibited improvements from the administration of basiliximab therapy to hospital discharge.

positive in all recipients before LT, and all but one (Case 5) had received livers from CMV-positive donors. The CMV-pp65 antigenemia in Cases 1 and 6 became positive within four days after basiliximab treatment; thus, the second dose of basiliximab was not administered. The CMV-pp65 antigenemia in Cases 2, 3, 4, and 7 became positive within two wk after basiliximab therapy, and in Case 5, it became positive after retransplantation. Although EBV-DNA was positive in three patients (Cases 5, 6, and 7) during the follow-up period, none of the patients developed post-transplant lymphoproliferative disease. One patient (Case 2) suffered from PCP nine months after basiliximab therapy and was successfully treated with the intravenous administration of trimethoprim–sulfamethoxazole.

The histopathological findings of the SRR patients

The histopathological findings are shown in Table 4. The histopathological grade of rejection on the Banff criteria was classified as moderate to severe rejection in all patients, and the venous endothelial inflammation scores for the central vein region were high. Centrilobular fibrosis was observed in six patients at the first rejection episode, with an interval from 11 to 44 days after LDLT. Centrilobular necrosis and hemorrhage were detected in the majority of patients. The RAI, including the presence of centrilobular perivenulitis, improved in three patients who received follow-up biopsies. No patient showed an anastomotic stricture of the hepatic vein by Doppler ultrasonography.

The changes in the proportions of $CD4^+CD25^+$ T cells and regulatory T cells before and after basiliximab therapy

The changes in the proportions of $CD4^+CD25^+$ T cells and Treg cells in the peripheral blood before and after basiliximab therapy are shown in Fig. 2. The proportions of $CD4^+CD25^+$ T cells in the patients before basiliximab therapy (mean ± standard deviation: 16.56 ± 6.49%) were significantly higher than those observed in the healthy control children (9.67 ± 2.17%, $p = 0.034$). The proportions of $CD4^+CD25^+$ T cells were significantly suppressed after basiliximab therapy compared to those measured in the patients before basiliximab therapy (16.56 ± 6.49% vs. 9.07 ± 3.96%, $p = 0.026$). There were no significant differences in the proportion of Treg cells in the peripheral blood measured before basiliximab therapy in the patients and those observed in the healthy control children (6.18 ± 3.01% vs. 6.91 ± 2.04%, $p = 0.853$). The proportions of Treg cells were also suppressed in the patients after basiliximab therapy compared to those observed in the patients before basiliximab therapy (2.71 ± 2.21% vs. 6.18 ± 3.01%, $p = 0.062$). The proportions of $CD4^+CD25^+$ T cells and Treg cells in the patients who developed graft loss after basiliximab therapy were 3.13% and 1.52%, respectively, which were the same as those observed in the other patients.

Discussion

Previous studies of LDLT for ALF in children, especially infants, have documented poor outcomes. Possible reasons for these results include

Table 4. The histopathological findings of the SRR patients

Case	Centrilobular injury				RAI	
	POD of the 1st rejection	Venulitis*	Fibrosis	Necrosis	Hemorrhage	Before the therapy	After the therapy
1	9	2	F0	–	–	6	–
2	23	3	F2	+	–	7	2
3	16	2	F2	+	+	5	0
4	14	2	F2	+	+	7	1
5	44	2	F2	+	+	6	–
6	11	2	F1	+	+	4	–
7	7	3	F2	+	+	7	–

*Venous Endothelial Inflammation Score according to the Banff schema.

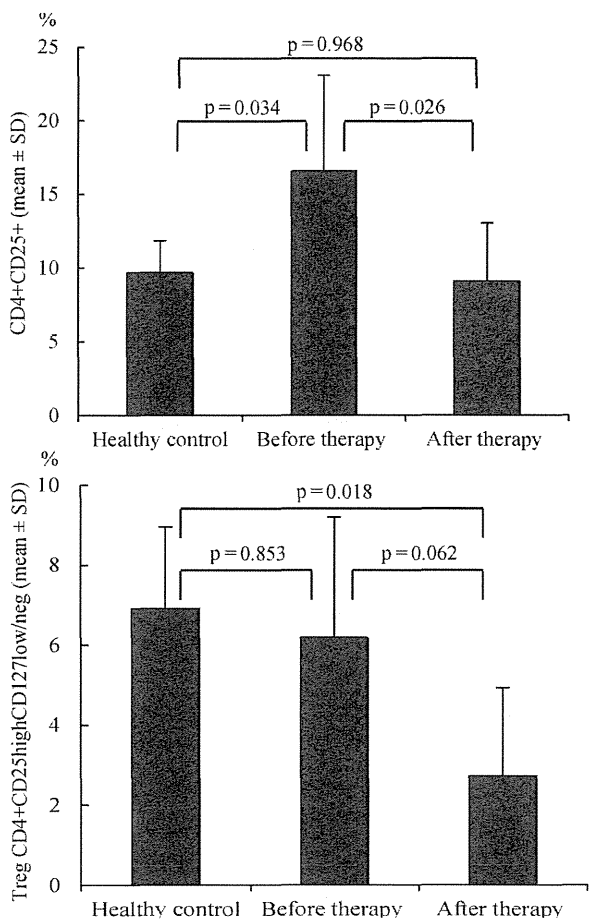

Fig. 2. Comparison of the levels of peripheral $CD4^+$ $CD25^+$ T cells and $CD4^+$ $CD25^{high}$ $CD127^{low/neg}$ regulatory T cells.

the age of the recipients, the cause of ALF, postoperative complications, for example, vascular complications, and the incidence or pattern of rejection (19–21). Among these reasons, technical difficulties in performing LDLT in infants are associated with problems of "large-for-size" grafts (33). The unknown etiology of ALF despite precise investigations before LDLT remains a big issue in this field (34). Patients classified with "cryptogenic hepatitis" may have possible causes of ALF that contraindicate the use of LT, such as mitochondrial respiratory chain disorders and familial hemophagocytic lymphohistiocytosis, although the amount of time for investigating the etiology may be limited owing to disease progression before LT (34).

With respect to the incidence or pattern of rejection after LDLT for ALF in children, previous reports have demonstrated a high incidence of ACR that is often refractory to steroids, as well as common pathological features of centrilobular changes, such as central perivenulitis (20, 21, 35). Centrilobular changes, including perivenulitis and necrosis, are associated with late acute rejection during the long-term follow-up after pediatric LT (36). The presence of central perivenulitis is a significant risk factor for the development of centrilobular fibrosis and indicates a trend toward future chronic ductopenic rejection (37). In the current study, central fibrosis was detected in six patients at the first rejection episode, although the significance of centrilobular fibrosis in the long-term follow-up of pediatric LT was uncertain (38). Therefore, managing ACR with centrilobular changes, synonymously defined as SRR in the current study, is a key issue for improving the outcomes of LDLT for cryptogenic ALF in infants.

We demonstrated the effectiveness of basiliximab as rescue therapy for SRR in pediatric LT patients with ALF. The physiological mechanisms of basiliximab therapy acting on SRR are currently unexplained. Several possible pathways related to the effects of basiliximab on SRR have been described to date (39, 40). Cellular IL-2 production contributes to the development of steroid resistance. The multidrug resistance-1 gene product is P-glycoprotein 170, which

actively transports steroids and has been reported to be upregulated by IL-2 (39). Basiliximab has been also used to treat steroid-resistant ulcerative colitis. Creed et al. reported the effectiveness of basiliximab in cases of moderate to severe steroid-resistant ulcerative colitis and described the induction of apoptosis in large numbers of steroid-resistant T-cell clones in the bowel following basiliximab therapy (40). Basiliximab appears to restore the sensitivity of these cells to steroids, resulting in rapid cell depletion in the presence of steroids. The authors also demonstrated a marked synergistic effect of basiliximab in combination with steroids. We therefore consider that treatment with basiliximab as rescue therapy decreases the rate of steroid resistance.

We experienced a high incidence of infections, such as CMV and *Pneumocystis* pneumonia. The patients suffering from SRR had already become severely immunocompromised due to multiple cycles of steroid pulse therapy and the use of additional ISs before the introduction of basiliximab therapy. Aw et al. reported that five of seven pediatric recipients with SRR were successfully treated with basiliximab, and CMV-DNA became positive in two of their patients, but none of the patients developed CMV disease (16). In our series, CMV-pp65 antigenemia was positive in all patients after basiliximab therapy. Because ALF is considered to be a risk factor for CMV infection after LT, the correlation between basiliximab therapy and CMV infection is still unclear (30). One patient suffered from PCP nine months after basiliximab therapy. The patient did not receive PCP prophylaxis after basiliximab therapy. The correlation between basiliximab therapy and *Pneumocystis* Pneumonia is also uncertain. After the experience of PCP following basiliximab, we started PCP prophylaxis at least one yr for the patient with basiliximab therapy and/or with multiple ISs.

In this study, the levels of peripheral $CD4^+$ $CD25^+$ T cells were suppressed following the administration of basiliximab therapy. Treg cells, which play an important role in preventing graft rejection, were also suppressed after basiliximab therapy. Three patients experienced ACR after receiving basiliximab. However, there were no significant differences in the proportion of Treg cells between the patients with ACR (Cases 1, 2 and 7) and the other patients (Cases 4–6) (data not shown). The usefulness of monitoring the levels of peripheral $CD4^+$ $CD25^+$ T cells and Treg cells in the patients with SRR after LT was unclear, because we had no data for pediatric LT patients without SRR. Briem-Richter et al. reported that patients who undergo LDLT have significantly higher numbers of Treg cells and associated cytokine IL-4 serum concentrations than patients who undergo deceased donor LT (41). The rate of prior ACR episodes of 16% observed in the LDLT patients is lower than the 25% noted in the deceased donor LT patients, with no significant differences ($p = 0.75$). Further investigations should be conducted to determine the significance of Treg cells as an indicator of transplantation tolerance.

In conclusion, we herein demonstrated the effectiveness and safety of rescue therapy consisting of basiliximab for SRR. Basiliximab is a possible first-line rescue therapy for SRR after LT, and its use is recommended as soon as possible when the histopathological findings demonstrate centrilobular injuries at the first ACR episode to prevent SRR of pediatric LT patients with ALF.

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Authors' contributions

T.S.: Participated in data acquisition, data analysis and interpretation, and drafting of the article; S.S. and M.K.: Participated in critical revision of the article for important intellectual content, approval of the article; H.U., K.S., I.H., H.K., A.F., T.K., M.O., and A.N.: Participated in data acquisition and analysis.

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Two-step transplantation for primary hyperoxaluria: A winning strategy to prevent progression of systemic oxalosis in early onset renal insufficiency cases

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Abstract: Several transplant strategies for PH1 have been proposed, and LT is performed to correct the metabolic defects. The patients with PH1 often suffer from ESRD and require simultaneous LKT, which leads to a long wait due to the shortage of suitable organ donors. Five patients with PH1 underwent LDLT at our institute. Three of the five patients were under dialysis before LDLT, while the other two patients were categorized as CKD stage 3. An isolated LDLT was successfully performed in all but our first case, who had complicated postoperative courses and consequently died due to sepsis after retransplantation. The renal function of the patients with CKD stage 3 was preserved after LDLT. On the other hand, our second case with ESRD underwent successful LDKT six months after LDLT, and our infant case is waiting for the subsequent KT without any post-LDLT complications after the early establishment of PD. In conclusion, a two-step transplant strategy may be needed as a life-saving option for patients with PH1 and may be possible even in small infants with systemic oxalosis. While waiting for a subsequent KT, an early resumption of PD should be considered from the perspective of the long-term requirement of RRT.

Kengo Sasaki[1], Seisuke Sakamoto[1], Hajime Uchida[1], Takanobu Shigeta[1], Masatoshi Matsunami[1], Hiroyuki Kanazawa[1], Akinari Fukuda[1], Atsuko Nakazawa[2], Mai Sato[3], Shuichi Ito[3], Reiko Horikawa[4], Tadashi Yokoi[5], Noriyuki Azuma[5] and Mureo Kasahara[1]

[1]Transplantation Center, National Center for Child Health and Development, Tokyo, Japan, [2]Division of Clinical Pathology, National Center for Child Health and Development, Tokyo, Japan, [3]Division of Nephrology and Rheumatology, National Center for Child Health and Development, Tokyo, Japan, [4]Division of Endocrinology and Metabolism, National Center for Child Health and Development, Tokyo, Japan, [5]Division of Ophthalmology, National Center for Child Health and Development, Tokyo, Japan

Key words: infantile oxalosis – living donor liver transplantation – primary hyperoxaluria type 1

Seisuke Sakamoto, MD, PhD, Transplantation Center, National Center for Child Health and Development, 2-10-1 Okura, Setagaya, Tokyo 157-8535, Japan
Tel.: +81 3 3416 0181
Fax: +81 3 3416 2222
E-mail: sakamoto-si@ncchd.go.jp

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PH1 is an autosomal recessive inherited disorder of glyoxylate metabolism, caused by a deficiency of the liver-specific peroxisomal AGT. Overproduction and urinary excretion of oxalate leads to urolithiasis and nephrocalcinosis, which may consequently result in renal failure. With advancing renal failure, the extrarenal deposition of oxalate in tissues such as the bones, retinas, and/or cardiovascular system occurs (1). Although the prognosis of PH1 has improved because of an increased awareness and earlier diagnosis, leading to earlier treatment, some patients have a rapidly progressing course, and other patients are diagnosed at advanced stages of renal failure (2). Among the various clinical phenotypes of PH1, the infantile form, which often exhibits a rapidly progressing course to ESRD and

Abbreviations: AGT, alanine-glyoxylate aminotransferase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CKD, chronic kidney disease; ESRD, end-stage renal disease; GFR, glomerular filtration rate; GRWR, graft-to-recipient body weight ratio; HD, hemodialysis; KT, kidney transplantation; LDKT, living donor kidney transplantation; LDLT, living donor liver transplantation; LKT, liver and kidney transplantation; LL, left lobe; LLS, left lateral segment; LT, liver transplantation; MMF, mycophenolate mofetil; PD, peritoneal dialysis; PH1, primary hyperoxaluria type 1; RRT, renal replacement therapy.

systemic oxalosis (infantile oxalosis) generally before three yr of age, may be difficult to manage and shows poor outcomes (3, 4).

Transplantation strategies for PH1 have been proposed based on concomitant renal insufficiency (5). Simultaneous or sequential LKT is an appropriate therapeutic option for patients with ESRD, although most patients, especially children, have to wait to obtain two organs due to the shortage of suitable organ donors (6). While waiting for transplantation, the continuation of RRT is a crucial element of survival; however, there are some dilemmas that in which all forms of dialysis are inadequate to keep up with the high production of oxalate (7).

We herein review the previous cases of PH1 treated using LDLT at our institute, and present the detailed clinical courses of three cases with ESRD, especially the infantile case in which we performed early LT to avoid further deposition of oxalate and succeeded in early resumption of PD after LT.

A review of LDLT in our PH1 cases

Since November 2010, five children with PH1 underwent LDLT at our institute. These cases are summarized in Table 1. In our institute, we recommend preemptive LT for pyridoxine-resistant PH1 patients with CKD stage 3, isolated LT (if necessary, sequential KT) for CKD stage 4, and sequential or simultaneous LKT for CKD stage 5. Three patients were under dialysis at the time of LDLT, and we planned sequential LKT. The GFR of the other two patients were 58.9 and 47.8 mL/min/1.73 m^2, categorized as CKD stage 3. Because pyridoxine therapy could not prevent disease progression, we planned preemptive LT.

Case 1

A 17-yr-old male was suspected to have PH1 from his clinical history of recurrent nephrolithiasis when he was four yr old and started to take pyridoxine and citrate. He was diagnosed based on the low AGT catalytic activity of his liver. His renal function gradually declined, and PD had been introduced at the age of 13 yr. He had a past history of bilateral femoral neck fractures due to bone lesions related to PH1, and he was confined to a wheelchair. He received an LL graft from his mother. After LDLT, he suffered from portal vein thrombosis, which led to graft failure. He underwent retransplantation and received a whole liver graft from a deceased donor. However, he died due to sepsis, which might have

Table 1. The characteristics of donors and recipients

Case No.	Age at LDLT	BW at LDLT (kg)	Age at Dx/Sex	CKD Stage at Pre-Tx.	Genetic mutation	Mode of dialysis/Period	Pre-Tx eGFR (mL/min/1.73 m^2)	Donor/Age (yr)	Graft Type/ GRWR (%)	AGT Activity of Graft Liver (%)	I/S	Complication	Current GFR (mL/min/ 1.73 m^2)	Outcome (F/U period)
1	17 yr	45.3	4 yr/M	Stage 5	N/A	PD/5 yr	N/A	Mother/46	LL/0.63	N/A	Tacrolimus Steroids	PVT	N/A	Died* (2 months)
2	15 yr	50.0	5 yr/F	Stage 5	N/A	HD/1 yr	N/A	Mother/39	LL/0.73	N/A	Tacrolimus Steroids	Massive ascites	N/A	Alive† (32 months)
3	8 months	7.1	4 months/F	Stage 5	p.Arg11X homozygous	PD/4 months	N/A	Mother/32	LLS/3.04	30.9	Tacrolimus Steroids	None	N/A	Alive (5 months)
4	10 yr	22.3	4 yr/F	Stage 3	p.Gln110 fs/ p.Lys12 fs	None	58.9	Mother/31	LLS/1.02	35.0	Tacrolimus Steroids MMF	None	67.8	Alive (20 months)
5	7 yr	23.5	7 yr/F	Stage 3	p.Try251Lys/ p.Ser275 fs	None	47.8	Mother/28	LLS/1.11	77.2	Tacrolimus Steroids MMF	None	54.0	Alive (12 months)

BW, body weight; CKD, chronic renal disease; Dx, diagnosis; F, female; F/U, follow-up; I/S, immunosuppressants; M, male; N/A, not assessed; Tx, transplantation.
*The patient underwent retransplantation due to graft failure eight days after LDLT.
†The patient underwent LDKT six months after LDLT.

been related to the requirement for continuous HD after retransplantation.

Case 2

A 15-yr-old female was suspected to have PH1 from her clinical history of recurrent nephrolithiasis when she was five yr old. She was diagnosed based on her urine and plasma oxalate level and her family history that her father had been diagnosed as PH1, although no conservative treatment was introduced. Her renal function deteriorated into ESRD, and HD was introduced at the age of 10 yr. She could conduct almost normal activities of daily living despite intermittent HD treatment before LDLT. She received an LL graft from her mother. She suffered from massive ascites for a few months after LDLT. She finally succeeded in undergoing LDKT by receiving a kidney graft from her grandmother six months after LDLT. Her hepatic and renal grafts are currently functioning well.

Case 3

A four-month-old female presented with ESRD, and PD was immediately introduced. Her plasma oxalate level was 167.2 μmol/L (reference range <1.8 μmol/L) and genetic testing confirmed a diagnosis of PH1. Medical management with pyridoxal phosphate was also initiated; however, it did not have much effect on her clinical course. Ophthalmoscopy revealed a rapid progression of oxalate deposition in the bilateral retinal epithelium (Fig. 1a,b). No other complications were found during the preoperative examinations, and her cardiac function was normal based on echocardiography. At the age of eight months, the patient underwent LDLT by receiving an LLS from her mother to decrease the risks associated with systemic oxalosis under PD, especially the rapid progression of retinal lesions. The previously inserted PD catheter was kept and protected during the operation. Intestinal manipulation was kept as a minimum, and anti-adhesive materials (Seprafilm; Genzyme Corporation, Cambridge, MA, USA) were used to prevent abdominal adhesions at the end of the operation. HD was performed for three days prior to LDLT to decrease the plasma oxalate level. Continuous HD had been performed based on the patient's cardiovascular condition and renal function for 12 days after LDLT, and thereafter, PD was successfully resumed. The postoperative clinical courses of the recipient and donor were uneventful, without any complications. After LDLT, the rapidly progressing oxalate deposition in retinal epithelium stopped, and the patient favorably gained weight to 8.8 kg at the age of 12 months without any neurodevelopmental delay. The patient continues to be managed under PD and renal anemia has been corrected by intermittent blood transfusion. KT will be performed when her body weight reaches 10 kg, although the plasma oxalate level has still been higher than the normal range after the LDLT (Figs. 2 and 3).

Discussion

The outcomes of organ transplantation in PH1 have improved over time, with recent LKT having been highly successful (8, 9). LKT is currently the most widely used procedure for the PH1 patients with severe renal insufficiency, and we also planned LKT for such patients (Case 1, 2, and 3). However, the number of the deceased donors is still low, and living donors remain the main source of donor organs in our country. The two-organ donation increases the burdens and the risks for living donors, and therefore, the timing of organ transplantation for PH1 patients used to be delayed, resulting in disappointing

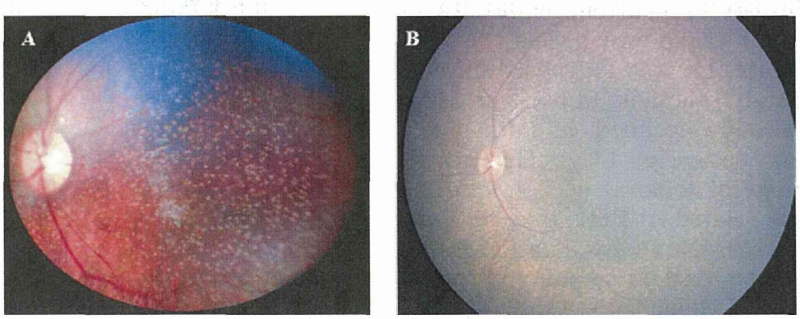

Fig. 1. The ophthalmoscopic findings of oxalate deposition in the retinal epithelium. The examination at the age of four months (no photo) showed normal findings; however, the findings at the age of five months (A) revealed multiple oxalate deposits in the retinal epithelium, and the intensity of oxalate deposition had increased at the age of seven months (B).

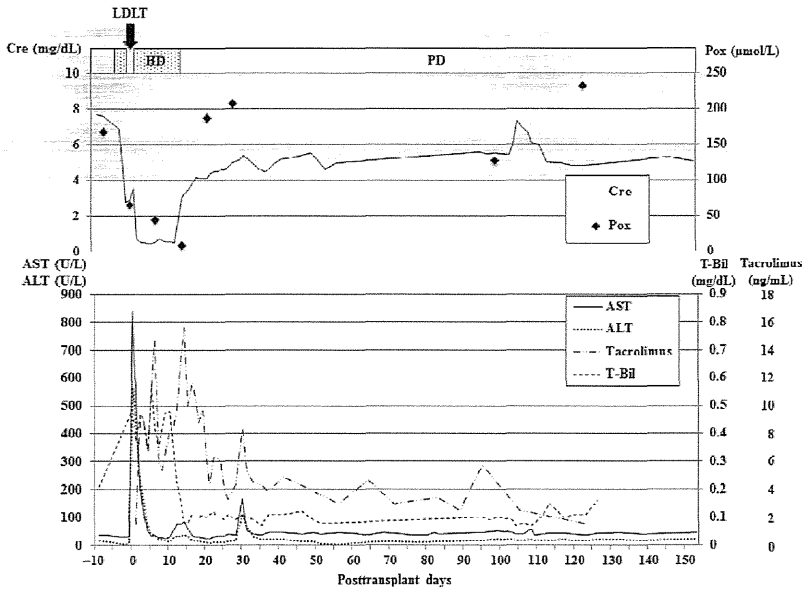

Fig. 2. The clinical course. The patient's postoperative clinical course was uneventful. The plasma oxalate level (Pox) was maintained at lower levels during the period of HD. Thereafter, it increased under PD. BW, body weight; Cre, creatinine; T-Bil, total bilirubin.

outcomes, as described in Case 1. We changed our policy to perform the organ transplantation before renal functions become severer. Preemptive LT may be an ideal transplantation strategy; however, it is difficult to decide the optimal timing because of heterogenous clinical courses of PH1 patients and the risks of the operation and the long-term immunosuppression. Previous literature reported that the threshold GFR below which degradation is unavoidable was estimated to be between 40 and 50 mL/min/1.73 m^2 (10), and therefore, we chose the pyridoxine-resistant PH1 patients with CKD stage 3 as the indication of preemptive LT (Case 4 and 5). For the PH1 patients with CKD stage 4, we recommend isolated LT and followed by sequential KT, if necessary. Even though the renal function deteriorates after LT, sequential living donor kidney donation is probably less aggressive for the donor than concurrent donation of two organs (11).

If a low body weight infant requires simultaneous LKT, it may be impossible to implant two adult organs into a very small abdominal cavity. Waiting for an infant with PH1 to be large enough for simultaneous LKT from an ideal size-matching pediatric deceased donor permits ongoing oxalate deposition throughout the waiting period, leading to the progression of complication and poor outcome, such as Case 1. The presented infant case revealed a rapid progression of retinal lesions, which could lead to vision impairment, and we decided to perform early LT (12). Sequential LKT may therefore be an appropriate therapeutic option for infants with systemic oxalosis, because an isolated LT, which is performed before the KT, can allow for earlier interruption of the oxalate accumulation (6).

When considering sequential LKT for a small patient, the use of RRT will be an important and challenging issue after LT. To the best of our knowledge, nine children with PH1 who were planned to undergo sequential LKT were previously reported (2, 6, 13–18) (Table 2). Although PD was tried after LT in two cases, both of these cases died before receiving sequential KT due to peritonitis and cytomegalovirus infection. PD is generally employed in low body weight pediatric patients, although this procedure may be difficult to perform after abdominal surgery. HD must be performed for patients with PH1 during the perioperative period for LT, although the long-term dependence on HD after LT is considered to be a

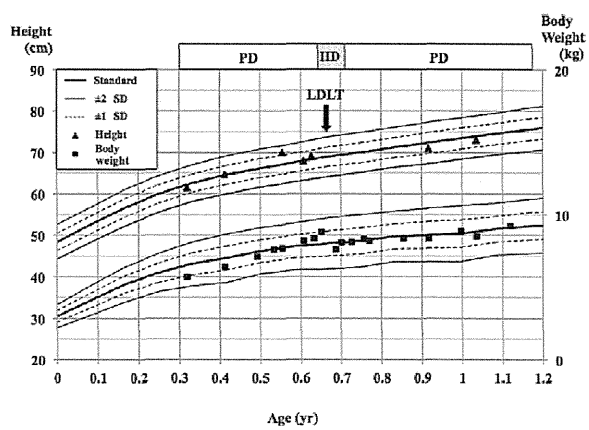

Fig. 3. The growth curve. An appropriate growth was obtained during the follow-up period after LDLT.

Table 2. A review of the previously published cases planned to undergo sequential liver-kidney transplantation for PH1

Case No.	Age (yr)/ Gender	Body Weight (kg)	Mode of dialysis Before LT	Donor Type (liver/kidney)	Interval between LT and KT	Mode of dialysis After LT	Outcome	Reference No.
1	1.3/F	6.2	PD	Father/Father	1.7 months	N/A	Alive	13
2	4.5/F	N/A	N/A	Living donor/Deceased donor	N/A	N/A	Alive	15
3	9/M	18.0	HD	Mother/Mother	4.7 months	N/A	Alive	14
4	0.9/F	N/A	N/A	N/A	9 months	HD	Alive	16
5	1.1/M	N/A	PD + HD	Deceased donor, Mother/Mother	8 months	N/A	Alive	6
6	1.4/M	9.1	PD	Father/Father	6 months	HD	Alive	17
7	3/M	N/A	HD	Deceased donor/Living donor	4 months	HD	Alive	18
8	2.3/M	N/A	PD	Living donor/Not performed	N/A	HD→PD	Died*	2
9	5/M	N/A	PD + HD	Deceased donor/Not performed	N/A	HD→PD	Died*	2
10†	17/M	45.3	PD	Mother, Deceased donor/ Not performed	N/A	HD	Died*	–
11†	15/F	50.0	HD	Mother/Grandmother	6 months	HD	Alive	–
12†	0.7/F	7.1	PD	Mother/Not performed	N/A	HD→PD	Alive	–

N/A, not assessed.
*All patients died due to infectious episodes.
†Our presented cases.

risk factor for various postoperative complications, such as catheter-related infections, especially in immunosuppressed patients, as was Case 1. Therefore, our strategy for RRT after LT prior to KT for a low body weight infant is an early resumption of PD, which can be achieved by performing meticulous surgical procedures, such as minimal intestinal manipulation and the use of anti-adhesive materials. Seprafilm has been widely used to reduce the incidence and severity of postoperative adhesion in pediatric abdominal surgery (19). In addition to these surgical techniques, postoperative management to keep the patency of the PD catheter using a heparin lock solution is important for the early resumption of PD.

When LDLT is considered for patients with PH1, the heterozygous carriers of this disorder may not be living donor candidates due to their potential for decreased enzyme function. It has been reported that the use of heterozygous donors in patients with various autosomal recessive diseases shows no negative impact on either the donors or recipients (20), although the reference articles contributing this evidence did not include LDLT cases with PH1. The previous articles related to LT or LKT from living donors did not profoundly discuss this issue (2, 6, 13, 14, 21–24). On the other hand, there are several studies reporting cases of domino LT retrieving compound heterozygous organs with PH1 (25–29). Not surprisingly, all of those reported cases developed renal insufficiency after domino LT, and Farese et al. suggested that the compound heterozygous organs with PH1 should not be used for domino LT (27). We measured the AGT catalytic activity of the graft livers. We sampled living tissue before interrupting the blood flow, and the sample was immediately frozen. The values of the native and graft livers were described as the ratio to the healthy liver value. The values of graft livers were available for three cases, including 30.9% (Case 3), 35.0% (Case 4), and 77.2% (Case 5) (30), respectively. None of the donors showed any clinical symptoms or abnormal laboratory findings related to PH1. A previous report showed that the mean AGT activity in the PH1 patients was 3.3% and that of PH1 heterozygous carrier was intermediate at 30–50% (31). The interpretation of the relatively lower AGT catalytic activity of the graft livers in our series is unclear, and further evaluation of the impact of the use of organs from heterozygous carriers of PH1 will be necessary.

In conclusion, LT via a two-step transplant strategy, which can allow for the interruption of the oxalate accumulation, may be needed as a life-saving option for the patients with PH1, and may be possible even in low body weight infants. While waiting for a subsequent KT, an early resumption of PD should be considered from the perspective of the long-term requirement for RRT.

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Authors' contributions

K.S.: Study design, writing of the paper; S.S.: Study design, critical revision of the article for clinical content; H.U., T.S., M.M., H.K., A.F.: Collection of the data; A.N.: Critical revision of the article for laboratory content; M.S., S.I.: Critical revision of the article for nephrological content; R.H.: Critical revision of the article for clinical content; T. Y., N.A.: Critical revision of the article for ophthalmological content; M.K.: Study design, critical revision of the article for clinical content.

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Evaluation of the immune function assay in pediatric living donor liver transplantation

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Abstract: The immune function (ImmuKnow) assay is a measure of cell-mediated immunity based on the peripheral CD4+ T cell ATP activity. The efficacy of ImmuKnow in pediatric LDLT is not well documented. The aim of this study was to assess the correlations between the ImmuKnow and the clinical status in pediatric LDLT recipients. A total of 716 blood samples were obtained from 60 pediatric LDLT recipients (one month to 16 yr of age). The recipient's status was classified as follows: stable, infection, or rejection. The ImmuKnow values in the pediatric LDLT recipients with a clinically stable status had a lower immune response (IQR 85–297 ATP ng/mL) than that previously reported in adults. Meanwhile, the ImmuKnow values of the stable patients were not correlated with age. Furthermore, a significant difference was found in the ImmuKnow values between the bacterial or fungal infection and stable groups, but not between the CMV or EBV infection and stable groups. The ImmuKnow levels in the pediatric LDLT were lower than those observed in the adult LDLT. The proposed reference value is between 85 and 297 ATP ng/mL in pediatric LDLT recipients. We conclude that the ImmuKnow assay could be helpful for monitoring pediatric LDLT recipients with bacterial or fungal infections.

Akinari Fukuda[1], Ken-Ichi Imadome[2], Seisuke Sakamoto[1], Takanobu Shigeta[1], Hajime Uchida[1], Masatoshi Matsunami[1], Kengo Sasaki[1], Hiroyuki Kanazawa[1], Fuyuko Kawano[2], Atsuko Nakazawa[1], Shigeyoshi Fujiwara[2] and Mureo Kasahara[1]

[1]Transplantation Center, National Center for Child Health and Development, Tokyo, Japan, [2]Department of Infectious Diseases, National Research Institute for Child Health and Development, Tokyo, Japan

Key words: pediatric liver transplantation – living donor liver transplantation – immune responses – T lymphocytes – infectious risk – rejection

Akinari Fukuda, MD, Department of Transplant Surgery, National Center for Child Health and Development, 2-10-1 Okura, Setagaya-ku, Tokyo 157-8535, Japan
Tel.: +81 3 3416 0181
Fax: +81 3 3416 2222
E-mail: fukuda-a@ncchd.go.jp

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The main goal of treatment of transplant recipients is to provide sufficient immunosuppression to prevent rejection without causing over immunosuppression that may result in opportunistic infections (1, 2). Monitoring the efficacy of immunosuppressive treatment is based on the analysis of liver enzyme measurements and liver function tests along with assessments of the blood drug levels (3–6). Not all immunosuppressive drug levels are measurable in routine clinical work-ups, and the development of a reliable and comprehensive immune function test for immune monitoring is essential, regardless of the regimen of immunosuppression.

Abbreviations: ALT, alanine transaminase; AST, aspartate transaminase; ATP, adenosine triphosphate; AUROC, area under the receiver operator characteristic curve; EBV, Epstein–Barr virus; GCV, ganciclovir; IQR, interquartile range; LDLT, living donor liver transplantation; LT, liver transplantation; PHA, phytohemagglutinin; ROC, receiver operator characteristic; s.d., standard deviation.

The ImmuKnow assay, which was approved by the Food and Drug Administration in 2002, belongs to the new generation of tests that directly measure the immune function of T cells and has been shown to reliably be used to distinguish between an immune profile of over immunosuppression and that of under immunosuppression in adults (7). The ImmuKnow assay has also been reported to be a user-friendly, non-invasive, *in vitro* assay with demonstrated effectiveness as an immune monitoring tool for use in organ transplantation recipients (8). Comprehensive reports have described the use of this test in the immune monitoring of adult transplant recipients (9), while smaller scale studies have reported its efficacy in pediatric renal transplant recipients (10). However, there have been few reports regarding the application of the ImmuKnow assay in pediatric LDLT patients, most of which relied on a small sample size. The aim of this study was therefore to clarify the reference value of the ImmuKnow assay in

pediatric LDLT recipients and to assess the usefulness of this test for distinguishing between recipients with infection and those with rejection.

Patients and methods

Patients

This study reports the results of an analysis of 60 pediatric LDLT recipients treated at the National Center for Child Health and Development, Tokyo, Japan, between December 2009 and February 2013. The characteristics of the liver transplant patients are presented in Table 1. The mean patient age at LT was 3.1 ± 4.0 yr (range: one month–16 yr). The median post-LDLT follow-up period was 2.8 yr (range: 0.9–6.7 yr). The patients included 18 males and 42 females. A total of 31 patients (51.7%) were <1 yr of age at the time of transplantation. The indications for LT included cholestatic disease (n = 35: biliary atresia, congenital hepatic fibrosis, Alagille syndrome, primary sclerosing cholangitis, and progressive familial intrahepatic cholestasis type 2), metabolic liver disease (n = 15: ornithine transcarbamylase deficiency, carbamoylphosphate synthetase 1 deficiency, glycogen storage disease type 1b, methylmalonic acidemia, propionic acidemia, and primary hyperoxaluria), acute liver failure (n = 7), vascular disease (n = 2: congenital absence of the portal vein), and cryptogenic liver cirrhosis (n = 1).

Indications for the immune function assay

There were two indications for performing an immune function assay in this study: programmed tests (pre-LDLT, every week until one month after LDLT, then two, three, six and nine months, and one yr after LDLT; n = 41) and event-driven tests. The reasons for ordering an additional immune function assay included the presence of infectious symptoms (fever, diarrhea, and coughing), signs of rejection, or significant elevation of liver function parameters (the levels of AST or ALT were elevated above 100 IU/L) in the recipient.

Immunosuppression

The immunosuppression protocol consisted of tacrolimus and low-dose steroids. The target whole-blood trough level of tacrolimus was 10–12 ng/mL during the first two wk, then approximately 10 ng/mL and 5–10 ng/mL starting the second month after LDLT. Methylprednisolone (1 mg/kg/day administered intravenously) was given on postoperative days 1–3, followed by 0.5 mg/kg/day on postoperative days 4–6. The steroid treatment was then switched to oral prednisolone (0.3 mg/kg/day) on postoperative day 7, and the dose was reduced to 0.1 mg/kg/day one month after LDLT. If the liver function was stable, the recipient was weaned off steroids at 3–6 months after LDLT. We did not use immunosuppressive agents for induction based on our immunosuppression protocol.

Table 1. Characteristics of the 60 pediatric LDLT recipients

Characteristics	Data at transplantation (n = 60)
Age [yr, mean ± s.d. (range)]	3.1 ± 4.0 (0.1–16)
0–5 months	7 (11.7%)
6–11 months	24 (40.0%)
1–4 yr	14 (23.3%)
5–9 yr	9 (15.0%)
10–17 yr	6 (10.0%)
Male/female	18/42
Blood type combination identical/compatible/incompatible	37/18/5
Follow-up period [yr, mean ± s.d. (range)]	2.8 ± 1.4 (0.9–6.7)
Primary diagnosis of recipient	
Cholestatic disease	35 (58.3%)
Metabolic disease	15 (25.0%)
Acute liver failure	7 (11.7%)
Vascular disease	2 (3.3%)
Cryptogenic liver cirrhosis	1 (1.7%)

Immune function assay

The sodium-heparinized peripheral blood samples obtained from the transplant patients were submitted for an analysis of the T cell immune function (ImmuKnow assay; Cylex, Columbia, MD, USA). All blood samples were processed on the day of sample collection. Briefly, 250 μL of anticoagulated whole blood was diluted with the provided sample diluent to a final volume of 1000 μL. The samples were added to wells and incubated for 16 h with PHA in an incubator (37 °C, 5% CO_2). Following enrichment in CD4+ T cells with the addition of magnetic particles coated with antihuman CD4 monoclonal antibodies, the blood cells were washed and lysed to release intracellular ATP. The released ATP was measured with a luciferin/luciferase assay using a luminometer. The patient's immune response was expressed as the amount of ATP (ng/mL).

Evaluation of the blood concentration and concentration/dose (C/D) ratio of tacrolimus and the ImmuKnow ATP levels

The daily dose of tacrolimus was recorded, and its weight-adjusted dose (mg/kg/day) was calculated. The blood tacrolimus concentration was normalized according to the corresponding dose per body weight blood sampling to obtain the concentration/dose (C/D) ratio. The correlation between the tacrolimus C/D ratio and the ImmuKnow ATP levels was evaluated.

Patient monitoring and clinical status

The patient's clinical status was monitored, including assessments of the complete blood cell count, liver function tests, and trough levels of immunosuppressants. The patients were divided retrospectively as having a status of stable, bacterial, fungal, or viral (cytomegalovirus and EBV) infection or rejection based on clinical information in their medical chart. Post-transplant bacterial and fungal infections were diagnosed based on clinical features, positive microbiologic tests, and imaging findings. The patients were routinely screened for antigenemia due to cytomegalovirus (CMV) in addition to blood polymerase chain reaction for EBV. CMV-pp65 antigenemia was measured weekly for the first three months postoperatively, while the recipient was hospitalized, and then monthly in the outpatient setting until six months after LT. We have used the cutoff for a positive CMV-pp65 antigenemia as 5/50 000 cells. Measurements of the EBV-DNA load were obtained every two wk while the patient remained in the hospital, then every 1–3 months thereafter until one yr after transplantation, followed by testing at the physician's discretion. Quantification of EBV-DNA was performed using a real-time quantitative PCR assay. A peripheral blood EBV-DNA

load of more than 2.4×10^3 copies/μg DNA (1.6×10^4 copies/mL) was considered to indicate significant elevation of the DNA load (11, 12). In cases of CMV-pp65 antigenemia or a positive EBV-DNA load, the dose of tacrolimus was reduced to 75% of the regular dose (13). Treatment with GCV (5 mg/kg/dose, every 12 h) was initiated in the CMV-positive patients for the first two wk, followed by the administration of a maintenance dose of IV GCV (5 mg/kg/dose, every 24 h). The treatment was continued until CMV-pp65 antigenemia became negative. Rejection was identified based on the results of liver function tests, a pathological biopsy analysis, or clinical suspicion. A stable post-transplant condition was defined as a normal liver function without any episodes of infection or rejection. The ImmuKnow ATP values obtained within three days before and after a clinical event were selected for the analysis. Throughout the study period, no clinical intervention protocol was implemented based upon the ImmuKnow ATP assay results, and the clinicians were discouraged from intervening in the immunosuppression regimen based on these results. This research was approved by the Institutional Review Board of the National Center for Child Health and Development (#410).

Statistical analysis

The Mann–Whitney *U*-test and Kruskal–Wallis test were used to compare continuous variables between two groups and three groups or more, respectively. The Pearson correlation coefficient was used to determine the relationship between the blood concentrations of tacrolimus and the ImmuKnow ATP levels. Estimates of the thresholds of the ImmuKnow ATP values for rejection and infection were determined using ROC curves. The AUROC was calculated. An AUROC of 1.0 is characteristic of an ideal test, whereas 0.5 indicates a test of no diagnostic value. The results were considered to be statistically significant for p-values of <0.05. All statistical analyses were performed using the SPSS 19.0 statistical software program (SPSS, Inc., Chicago, IL, USA).

Results

A total of 716 blood samples in 60 patients were available for the analysis. The mean patient age at the time of the ImmuKnow assay was 3.9 ± 3.8 yr. Among the samples, 157 were collected at the time of infection, 91 at the time of rejection, and 468 while in stable condition (without infection or rejection). Among the patients with infections, 52 examinations (in 22 recipients) were associated with bacterial infections, nine examinations (in six recipients) were associated with fungal infections, 14 examinations (in 11 recipients) were associated with cytomegalovirus infections, and 107 examinations (in 28 recipients) were performed in patients with EBV infection (including coinfections) (Table 2). All cases of EBV infections were classified as asymptomatic EBV viremia. There were 37 episodes of biopsy-proven acute cellular rejection in 14 recipients (23.3%). The grade of rejection included four episodes of severe rejection, 17 episodes of moderate rejection, and 16 episodes of mild rejection, according to the Banff criteria (14). There were no significant differences in the severity of rejection or values of the ImmuKnow assay (p = 0.663, Kruskal–Wallis test).

Table 2. The infections detected within three days before and after the sampling points of the immune function assay were reported. There were several patients with contained coinfections

Type of infection	Number of examinations
Bacterial	52 (in 22 recipients)
Escherichia coli	10
Enterobacter cloacae	7
Klebsiella pneumoniae	5
Pseudomonas aeruginosa	5
Enterococcus faecalis	4
Klebsiella oxytoca	3
Enterobacter aerogenes	2
Enterococcus faecium	2
Haemophilus influenzae	2
Methicillin-resistant Staphylococcus aureus	2
Staphylococcus epidermidis MRS	2
Streptococcus pneumoniae	2
Group A Streptococcus	1
Moraxella catarrhalis	1
Mycoplasma	1
Pseudomonas putida	1
Staphylococcus epidermidis	1
Streptococcus oralis	1
Fungal	9 (in 6 recipients)
Candida albicans	6
Candida spp.	2
Pneumocystis pneumonia	1
Viral	126 (in 33 recipients)
EBV (EBV-DNA loads ≥2400 copies/μg DNA)	107
Cytomegalovirus (C7-HRP ≥5/50 000 WBC counts)	14
Adenovirus	2
Influenza virus	2
Herpes simplex virus	1

Evaluation of the blood concentration and concentration/dose (C/D) ratio of tacrolimus and the ImmuKnow ATP level

The average tacrolimus trough level and C/D ratio were 5.6 ± 3.3 ng/mL and 63.5 ± 47.5 ng/mL per mg/day, respectively, in the stable group, 4.9 ± 3.7 ng/mL and 107.7 ± 170.7 ng/mL per mg/day, respectively, in the infection group, and 7.8 ± 3.7 ng/mL and 111.0 ± 109.4 ng/mL per mg/day, respectively, in the rejection group. A scatterplot was drawn to evaluate the correlation between the tacrolimus blood concentration or C/D ratio and the ImmuKnow level at each examination point (Fig. 1a,b). The tacrolimus blood C/D ratio ranged from 2.5 to 963.1 ng/mL per mg/day. There were no correlations between the tacrolimus blood concentration or C/D ratio and the ImmuKnow level ($R = 0.051$ or $R = -0.092$). There was no significant difference

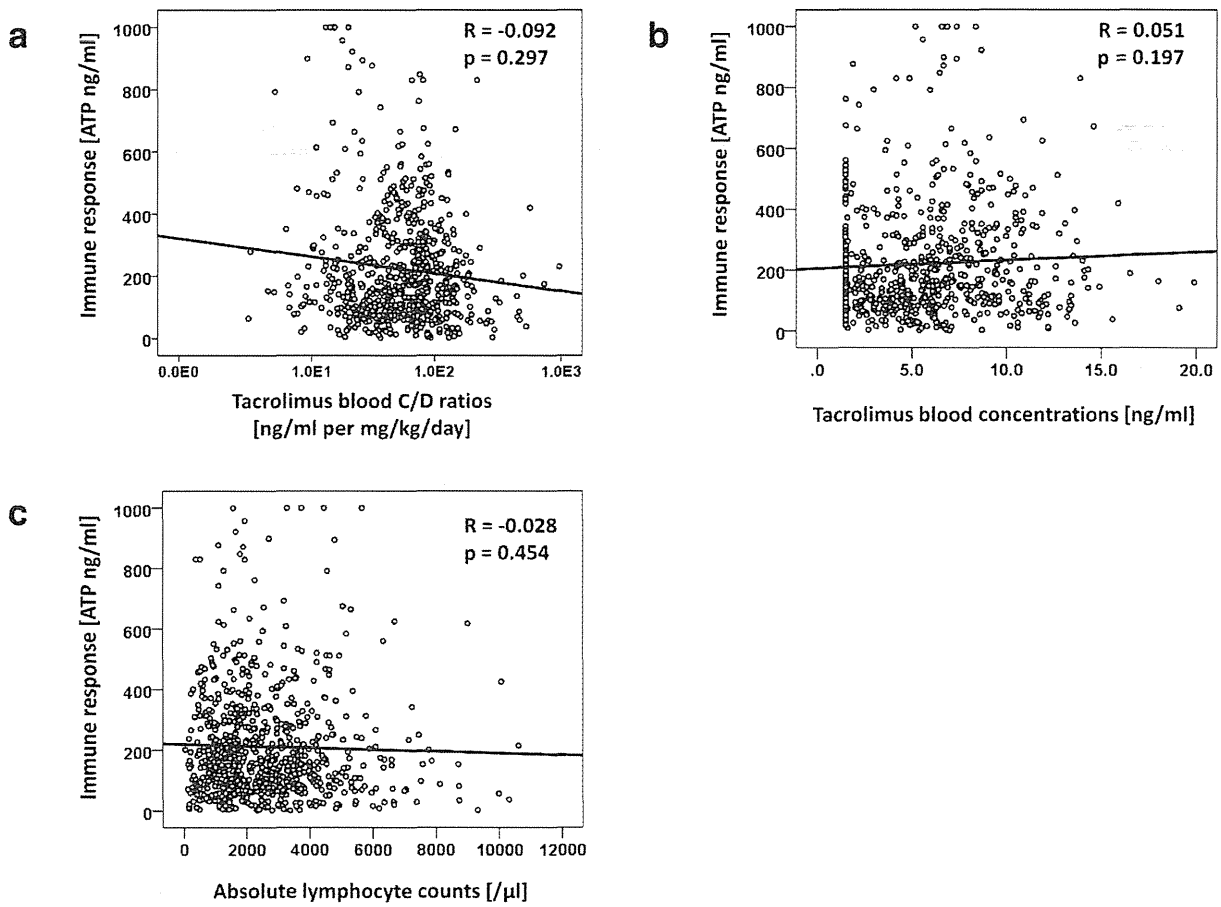

Fig. 1. The relationship between the tacrolimus blood concentration (a) or C/D ratios (b) and the ImmuKnow ATP values. The Pearson correlation coefficients were $R = 0.051$ (p = 0.297) and $R = -0.092$ (p = 0.197). (c) The correlation between the absolute lymphocyte count (ALC) and the ImmuKnow results was analyzed, but there were no significant correlations ($R = -0.028$, p = 0.454).

in the ImmuKnow values during high-dosage steroid bolus therapy compared to conventional treatment (p = 0.281, Mann–Whitney *U*-test).

Correlations between the ImmuKnow measurements and a stable immune function

A total of 468 of the 716 (65.4%) samples were collected from recipients in a clinically stable state. The median ImmuKnow level in the stable state LDLT recipients was 162 ATP ng/mL. Half of the measurements were between 85 and 297 ATP ng/mL, between the 25th and 75th percentiles (Fig. 2).

ImmuKnow values and age distribution in the pediatric LDLT recipients

The ImmuKnow values according to the age of the pediatric LDLT recipients at examination were plotted using the locally weighted regression smoother values of the stable state recipients (Fig. 3a). There were no significant differences in the distribution of the ImmuKnow values between infants and children older than one yr old at the time of the transplant (p = 0.109). The ImmuKnow values of the stable pediatric LDLT patients showed no evidence of age dependence. The median ImmuKnow value was 162 ATP ng/mL among the 468 stable state patients.

Impact of the liver transplant operation on the ImmuKnow values in pediatric LDLT recipients

The ImmuKnow values at each time point after LDLT for the pediatric LDLT recipients were plotted using the locally weighted regression smoother of the stable state recipients (Fig. 3b). There was an inflection point three months after LDLT. The median ImmuKnow level within the first three months after LDLT was

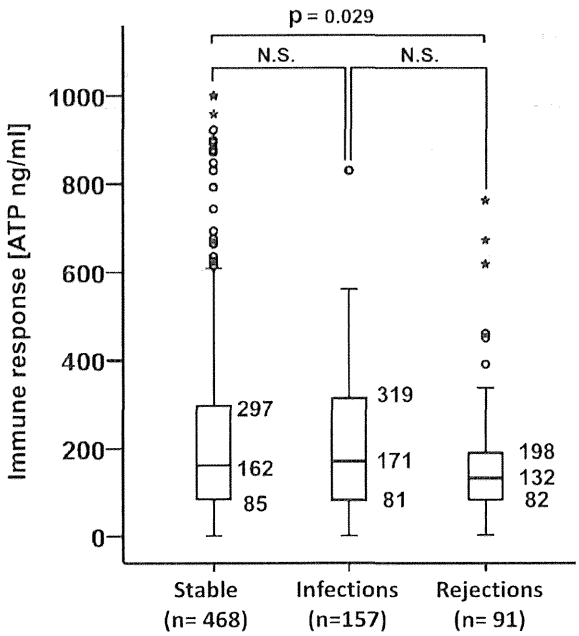

Fig. 2. ImmuKnow measurements grouped according to clinical patient status on the day of sample collection. The immune response measurements collected during events of rejection or infection did not differ between the groups. The ImmuKnow levels were low in the rejection group compared with that observed in the stable group ($p = 0.029$; Kruskal–Wallis test). The horizontal line indicates the median ImmuKnow level in each group, the vertical lines indicate the s.d., and the boxes describe the range of the central 50% of the measurements in that group.

196 ATP ng/mL. The median levels decreased significantly after three months to 139 ATP ng/mL ($p = 0.001$).

Comparison of the ImmuKnow values according to the type of infection

The ImmuKnow values in the patients with bacterial or fungal infections were significantly lower than those observed in the stable patients (Fig. 4a,b). To determine a reference value for the ImmuKnow level for diagnosing infection, a ROC curve analysis was performed. When the ImmuKnow value was set at 102.5 ATP ng/mL and 91.5 on the ROC curve for the patients diagnosed with bacterial and fungal infections, the sensitivity was 68.2% and 72.7%, the specificity was 45.5% and 66.7%, and the AUROC was 0.602 and 0.798, respectively (Fig. 5a,b).

Fourteen episodes of CMV infection and 107 episodes of EBV infection were diagnosed. There were no significant differences in the ImmuKnow values in the patients with CMV infection (Fig. 4c). There was a paradoxical significant difference in the ImmuKnow values in the patients with EBV infection in that the ImmuKnow values in the patients with EBV viremia were higher than those observed in the non-infected patients (Fig. 4d).

Discussion

The T-cell immune function assay can be used to categorize adult individuals as low, moderate, and strong immune responders, and the correlation with clinical quiescence in adults is defined as an ATP measurement within the

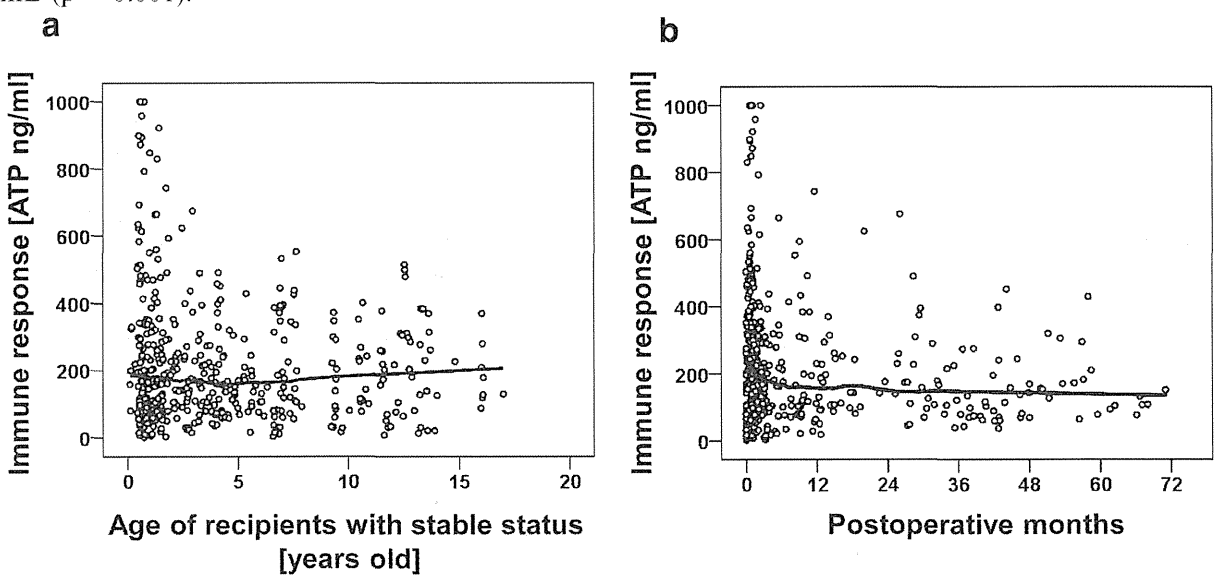

Fig. 3. A scatterplot comparing the immune responses according to the age in years (a) and the time after operation (b) among the stable liver transplant recipients. The solid line was calculated using the locally weighted regression of the ImmuKnow values. (a) There were no points of inflection in the solid line. (b) There was an inflection point three months after LDLT.

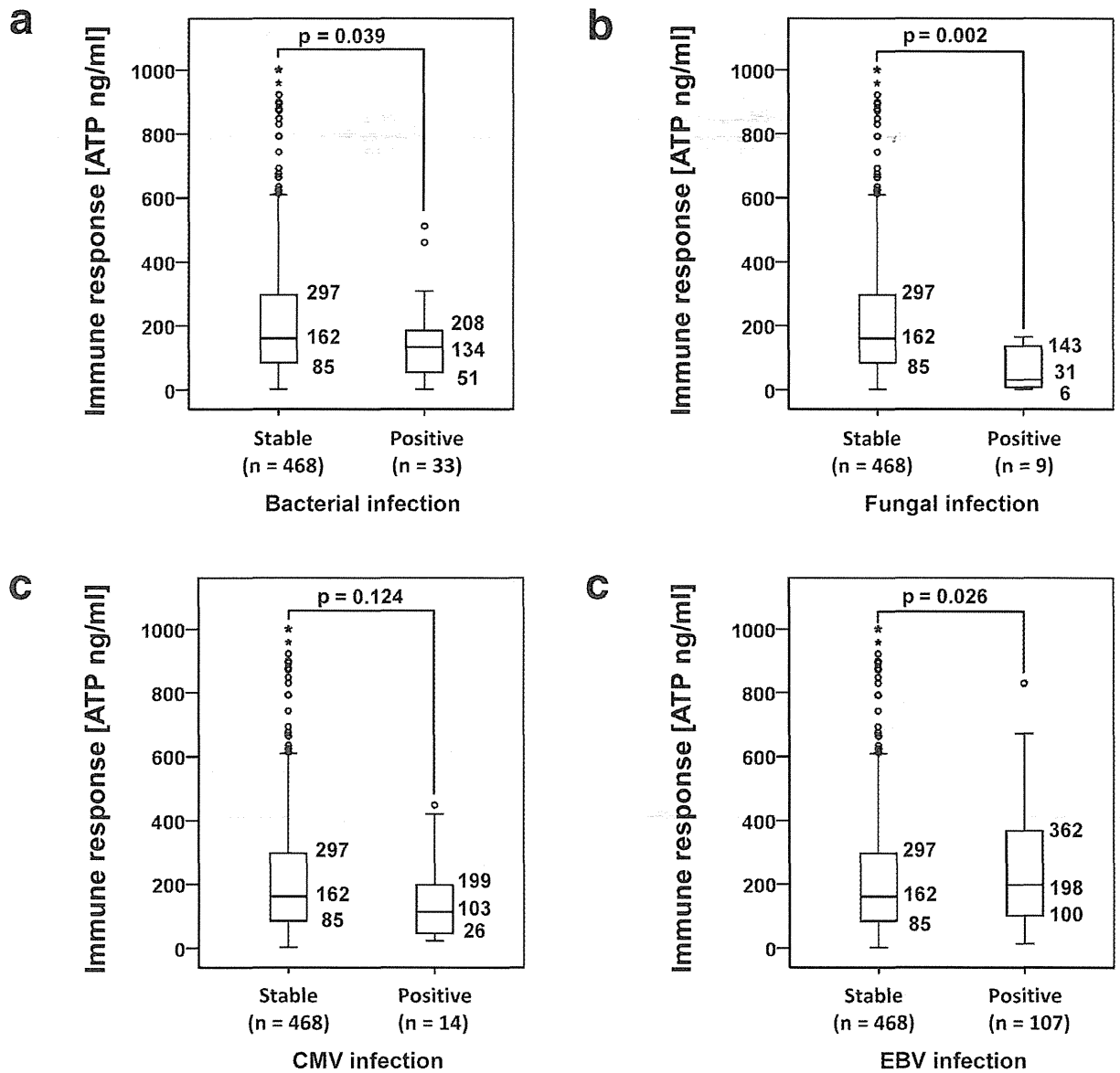

Fig. 4. ImmuKnow measurements grouped according to (a) bacterial infections, (b) fungal infections, (c) CMV infections, and (d) EBV infections, compared with the stable group.

established "moderate" range, between 225 and 525 ATP ng/mL (7). However, our study showed that the ImmuKnow ATP levels are lower in the pediatric LDLT population than in the adult LDLT population. We propose that the "moderate" range of the ImmuKnow levels in the pediatric LDLT population is between 85 and 297 ATP ng/mL based on the results of our stable state pediatric LDLT recipients analysis.

In the present study, low values on the T-cell immune function assay were associated with susceptibility to infection, although this issue is controversial (15–17). In addition, we found a significant difference in the distribution of the ImmuKnow values between the patients with a stable status and those with infection, with the exception of the patients with asymptomatic EBV viremia. Furthermore, a low ImmuKnow value was more helpful for identifying patients at higher risk of bacterial and fungal infection, and the ROC analysis showed that the sensitivity and specificity for diagnosing fungal infection episodes were 72.7% and 66.7%, respectively. Therefore, the possibility of a fungal or bacterial infection should be considered in patients in

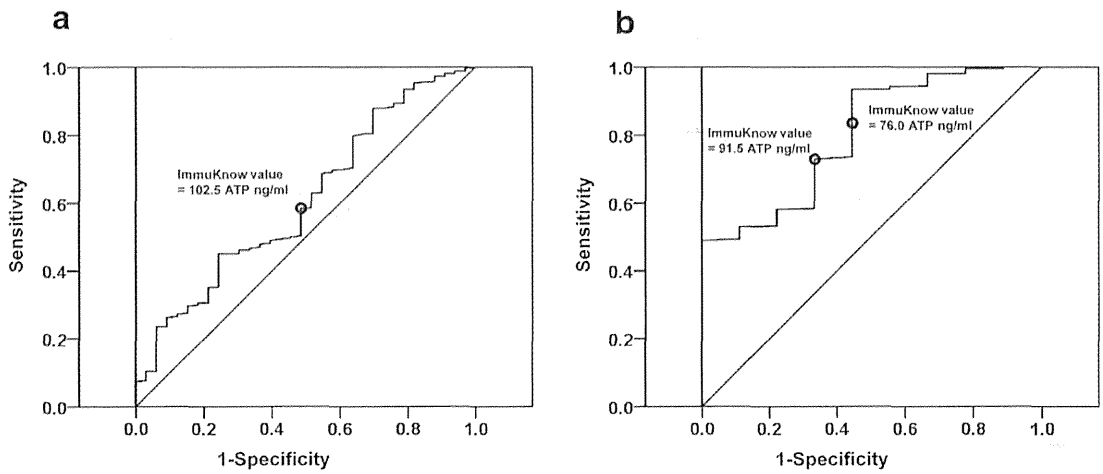

Fig. 5. Receiver operating characteristic (ROC) curve for predicting bacterial and fungal infections. (a) ROC curve: stable vs. bacterial infection. When the cutoff ImmuKnow value was set at 102.5 ATP ng/mL, the sensitivity and specificity were 68.2% and 45.5%, respectively (AUROC: 0.602). (b) ROC curve: stable vs. fungal infection. When the cutoff ImmuKnow value was set at 91.5 ATP ng/mL, the sensitivity and specificity were 72.7% and 66.7%, respectively (AUROC: 0.798). When the cutoff ImmuKnow value was set at 76.0 ATP ng/mL, there were significant differences between fungal infections and a stable status (the sensitivity was 0.803 and specificity was 0.556, p = 0.008, Mann–Whitney *U*-test).

whom the ImmuKnow value is lower than 91.5 ATP ng/mL. When the ImmuKnow value is <90 ATP ng/mL, it is better to increase the monitoring or to introduce prophylactic treatments for bacterial and/or fungal infections.

Regarding the ImmuKnow values in the patients with bacterial or fungal infection, there were no significant differences in the ImmuKnow values between the patients with CMV and EBV infections. Pediatric transplant recipients are generally dosed at higher concentrations of immunosuppressants per kilogram of body weight than adults. We investigated the relationship between the ATP levels and the pharmacokinetics of tacrolimus and found no correlations between the tacrolimus blood C/D ratios and the ImmuKnow ATP levels. Typically, measuring the peripheral blood trough levels of immunosuppressive drugs after transplantation is considered a critical step in therapeutic management to prevent toxicity and to provide effective immunosuppression. These data imply that measuring the immunosuppressive drug levels remains an important aspect of therapeutic management; however, such measurements have limited clinical value in monitoring for over immunosuppression and/or infection (18).

High values are associated with the risk of rejection in kidney (9), heart (19), liver (20–23), and pancreas (24) transplant patients, although other studies have shown no significant associations (16, 25–28). Kowalski et al. reported the results of a meta-analysis of 504 adult patients treated with a variety of transplant procedures, including LT, that recipients with an immune response of 700 ATP ng/mL were 30-fold more likely to develop rejection than those with a lower immune response value (8). The present study results differ from those of a prior study of adult solid organ transplant patients in that our pediatric recipients in the rejection group did not exhibit high ImmuKnow levels. It is important to note that the adaptive immune system is immature in children compared to adults and that a lower response to mitogen stimulation is observed in children (29). The factors which were not related with ATP levels of the T-cell immune function might be mainly associated with acute cellular rejection in pediatric liver transplant population, such as antibody-mediated rejection (30).

In the present study, we found that pediatric liver transplant recipient immune responses are age-independent. Within the pediatric patient population (age <12 yr), we found no age-related effects on the immune function. Similar findings have been reported in pediatric renal and liver transplant recipients (29, 31). In addition, Hooper et al. assessed the mean ImmuKnow values among 50 healthy children (32 children <12 yr of age and 18 children ≥12 yr of age) and 155 healthy adults and found that the mean ImmuKnow value remained constant around 327 ng/mL until 10 yr of age, after which it progressively became elevated at 10–12 yr of age and plateaued at the level observed in adults (around 433 ng/mL) after 12 yr of age (29).

We analyzed the relationship between the height/weight and the ImmuKnow value pre-transplant. There were no correlations between the clinical status of recipients' development and the ImmuKnow values (the Pearson correlation coefficients were $R = 0.131$ for height and 0.231 for weight, data not shown). A subgroup analysis based on the preoperative ImmuKnow values was performed; however, we did not find any significant differences in the incidence of developing infections.

We did not observe an adequate, absolute cutoff value for the ImmuKnow level for predicting EBV infection. A previous study reported that infectious episodes are accompanied by low of ImmuKnow values. In the present study, the ImmuKnow values were significantly higher in the patients with EBV infection than in those with a stable status, a paradoxical change. PHA activation of CD4+ T cells usually necessitates co-stimulation by other cells, such as B cells (32, 33). EBV-transformed B-cell lines are approximately 4–20 times as efficient on a per cell basis as non-T cells in stimulating the amplitude of the co-stimulation function (15, 32). Consequently, in cases of EBV infection, transformed B cells enhance the activation process, resulting in the overproduction of ATP, thus inducing an elevated ImmuKnow level (34). The unpredictably high ImmuKnow results observed in our EBV-infected recipients may be explained by this phenomenon, in contrast to the expected low values observed in the cases of viral infections (9, 10, 35).

The independent risk factors associated with the clinical status (stable vs. bacterial, fungal, CMV or EBV infection, and acute cellular rejection) were the recipients' age, time since LDLT, and ImmuKnow values, as determined by a logistic regression test. The ImmuKnow values were associated with bacterial infections ($p = 0.031$, $OR = 0.997$), fungal infections ($p = 0.014$, $OR = 0.987$), and acute cellular rejection ($p = 0.005$, $OR = 0.998$). It can be concluded that the ImmuKnow values were a useful tool for diagnosing fungal infections in our study. When the cutoff ImmuKnow value was set at 76.0 ATP ng/mL, there were significant differences between fungal infections and a stable status (the sensitivity was 0.803 and specificity was 0.556, $p = 0.008$, Mann–Whitney U-test). Ling et al. reported that the ImmuKnow value was not able to determine individuals at risk for infection or rejection in their relatively large meta-analyses in cases of adult solid organ transplantation using a ROC curve analysis (35). They concluded that it was not a useful test for diagnosing infections, because the AUROC for infection was 0.77. We intended to validate the cutoff ImmuKnow value for diagnosis rejection and infection using a ROC curve analysis in pediatric LDLT recipients. However, we also could not set an adequate cutoff value for the ImmuKnow assay for diagnosing a bacterial infection, because the AUROC was 0.602.

There were a few possible limitations associated with this study. We have used CMV-pp65 in the PCR era, because our previous study revealed the effectiveness of a universal pre-emptive therapy for CMV infection based on the cutoff for a positive CMV-pp65 antigenemia of ≤5/50 000 cells (36). However, this is not the standardized value. Further studies are necessary to determine the optimal cutoff value for CMV-pp65 antigenemia and to compare it with the results of CMV-DNA PCR. There was another limitation, that this study was retrospective study and we need to validate the cutoff values using an independent dataset in another prospective study.

Conclusions

The ImmuKnow ATP levels are lower in the pediatric LDLT population than in the adult LT population. We herein proposed a reference value between 85 and 297 ATP ng/mL in pediatric LDLT recipients, although we did not identify any age-related effects on the immune function in the pediatric LDLT recipients. The ImmuKnow assay could be helpful for monitoring pediatric LDLT recipients with infection, particularly those with fungal infections. Meanwhile, the correlations between the ImmuKnow values and the status of rejection or EBV viremia were limited.

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Disclosure

All authors declare no conflict of interests.

Authors' contributions

Akinari Fukuda, Mureo Kasahara, Ken-Ichi Imadome, and Seisuke Sakamoto participated in the design, data analysis, research, and writing of the manuscript. Takanobu Shigeta,

Hajime Uchida, Masatoshi Matsunami, Kengo Sasaki, Hiroyuki Kanazawa, Fuyuko Kawano, Atsuko Nakazawa, and Shigeyoshi Fujiwara participated in the data analysis and research.

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