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ORIGINAL ARTICLE

Factors affecting operational tolerance after pediatric living-donor liver transplantation: impact of early post-transplant events and HLA match*

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Conflicts of Interest
All authors have no potential interest to declare.

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Summary

Pediatric recipients of living-donor liver transplants (LDLT) can often discontinue immunosuppression (IS). We examined factors affecting development of operational tolerance (OT), defined as off IS for >1 year, in this population. A historic cohort analysis was conducted in 134 pediatric primary semi-allogeneic LDLT. Multivariate logistic regression analysis was used. The frequency of peripheral regulatory T cells (Tregs) was determined at >10 years post-Tx by FACS analysis. IS was successfully discontinued in 84 tolerant patients (Gr-tol), but not in 50 intolerant patients (Gr-intol). The Gr-intol consisted of 24 patients with rejection (Gr-rej) and 26 with fibrosis of grafts (Gr-fib). The absence of early rejection [odds ratio (OR) 2.79, 95% CI 1.11–7.02, $P = 0.03$], was a positive independent predictor, whereas HLA-A mismatch (0.18, 0.03–0.91, $P = 0.04$) was a negative predictor. HLA-DR mismatches did not affect OT. The Treg frequency was significantly decreased in Gr-intol (4.9%) compared with Gr-tol (7.6%) ($P = 0.003$). There were increased levels of tacrolimus in the first week in Gr-Tol ($P = 0.02$). Although HLA-B mismatch (8.73, 1.09–70.0, $P = 0.04$) was a positive independent predictor of OT, its clinical significance remains doubtful. In this large cohort of pediatric LDLT recipients, absence of early rejection, HLA-A match and the later predominance of Tregs are factors associated with OT.

Introduction

Living-donor liver transplantation (LDLT) has become a standard approach to save lives of patients suffering from end-stage liver disease [1]. There has been a rapid increase in the number of living-donor transplants (Tx) for both liver and kidney because of the shortage of deceased donors. One problem that has remained unsolved is the dependency of the patients on nonspecific life-long immunosuppression (IS) accompanied by complications [2]. Therefore, the establishment of operational tolerance (OT) in a proportion of liver Tx patients provides an opportunity to assess factors that promote tolerance [3–6]. For the following analysis, we have defined OT as continued function of a transplanted organ in the absence of IS with a follow up of >1 year.

Although immunomodulatory strategies efficiently induce tolerance in animals, tolerance after clinical organ Tx is most unusual [7–14]. However, we have developed an elective protocol that enables a substantial proportion of selected LDLT patients to be weaned off IS [15]. Some patients who stopped IS nonelectively because of infection, severe side effects or noncompliance, but did not exhibit any clinical manifestation of rejection, were included [15]. Altogether, OT occurred in 15% of all pediatric patients, regardless of whether IS was weaned electively or nonelectively, comparable with other Tx centers [16,17]. Thus, the strategy for weaning from IS has been very successful, providing a large patient cohort to investigate mechanisms of OT [18–21]. The patient cohort in this study consists of parental donors to child recipients (parent to F1). In most recipients, this led to a one haplotype difference at all HLA loci (referred to here as mismatched), except where both the mother and the father shared the same HLA allele at a particular locus. Tx in these individuals were matched at these loci (referred to here as matched).

There is considerable interest currently as to whether complete cessation of IS in the setting of pediatric LDLT is the most appropriate approach for these patients. Thus, we have investigated this population to identify factors that contribute to OT. In marked contrast to renal Tx, for which the overall survival is better when there are fewer donor–recipient HLA mismatches, HLA matching does not affect the overall survival of deceased donor liver Tx [22–28]. Nonetheless, to our knowledge, only limited data are available on the significance of HLA matches in the development of OT after liver Tx. Two reports showed that more HLA matches were associated with the development of OT after deceased donor liver Tx [29,30]. Consequently, one of the factors used in our analysis was HLA match. Previous studies from our group showed that patients who have been removed from IS can develop advanced or progressing graft fibrosis, often associated with deposition of C4d [31]. As this fibrosis often improves with re-introduction of IS [32], patients with fibrosis who recommenced IS were included in the intolerant group.

Research design and methods

Study design, statistical analysis, and population

This was a historic cohort analysis based on 670 pediatric patients who underwent LDLT at Kyoto University Hospital from June 1990 to April 2008. All operations were performed by the Kyoto method [33]. OT patients (Group-tolerance – Gr-tol) were defined as stable normal graft function for more than 1 year off IS [3–6]. On the other hand, failure to discontinue IS because of rejection (Gr-rej) or fibrosis (Gr-fib) during the process of weaning IS or after cessation of IS were referred to as intolerance (Group-intolerance – Gr-intol) [15,31]. We compared baseline characteristics of Gr-rej with Gr-fib using the *t*-test for continuous variables and the chi-square or Fisher's exact test for dichotomous variables. We also compared baseline characteristics for Gr-tol and Gr-intol by the same statistical approach. Multiple logistic regression analysis was used to determine independent predictors of OT and to obtain odds ratios (OR) that were adjusted for possible confounding factors. All the variables found to be different at $P < 0.10$, notably: HLA-A mismatch; early rejection; tacrolimus trough levels within the first week; and time after Tx; as well as those thought to be important on logical and/or biomedical grounds, notably: HLA-B/HLA-DR mismatch, and ABO compatibility, were entered into logistic regression analysis. An early episode of rejection and average tacrolimus trough levels within the first week had been reported to affect OT [29,34]. A trough level of tacrolimus administered orally was adjusted to that administered intravenously (intravenous = per oral × 1.4) [35]. Follow-up (time after Tx) was also included. The 95% confidence interval for each OR was calculated. Statistical significance was determined by 95% confidence intervals not including 1.00 for logistic analyses. Software for this analysis and all other statistical analyses reported here was Stata version 11 (Stata, College Station, TX).

IS protocol

In brief, patients are currently immunosuppressed with tacrolimus and low-dose steroids [16]. As an induction therapy, cyclosporine A was administered with steroids for the first 14 cases, and then it was switched to tacrolimus for all subsequent patients. For the next 60 cases, tacrolimus was continuously given intravenously immediately after LDLT. Subsequently, it was given orally

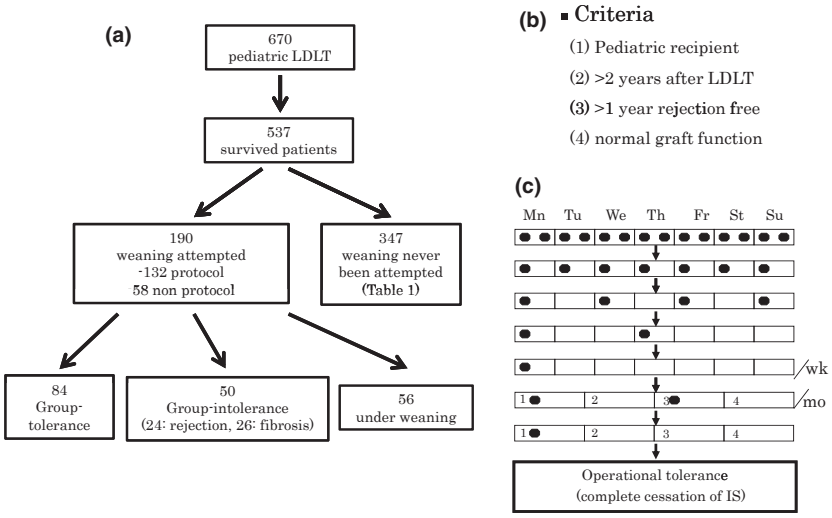

Figure 1 (a) Outcome of Weaning in 670 pediatric patients who underwent LDLT. Of the total number, 537 patients survived. A total of 190 patients attempted weaning immunosuppression (IS) either electively or nonelectively. Eighty four patients successfully discontinued IS, whereas 50 patients were unable to stop IS. Of these, 24 patients failed because of clinically overt rejection after or during the process of weaning IS, whereas 26 patients failed because of fibrosis detected by protocol biopsy. (b) Criteria for selection for the elective weaning protocol. Patients who satisfied the criteria started the Kyoto elective protocol for weaning IS. (c) Protocol for weaning off tacrolimus. The frequency of tacrolimus administration was gradually decreased with intervals of 3–6 months for each step as follows: conventional b.i.d.; q.d.; four times a week; twice a week; once a week; twice a month; once a month, and finally it was completely stopped.

from 1 day pre-Tx for all patients. In our current protocol, the target trough level of tacrolimus was 10–12 ng/ml for the first 2 weeks and 5–10 ng/ml for the following 2 months. After discharge, the dose of tacrolimus was determined individually, depending upon each patient's condition. Steroids were started during LDLT, and were then tapered gradually and stopped after 3 months.

Patients began the weaning protocol when they fulfilled the following criteria [15]: greater than 2 years post-LDLT; normal graft function and no episodes of rejection for more than 1 year (Figure 1). The frequency of tacrolimus administration was reduced in steps (Figure 1). In addition, some patients stopped IS nonelectively mainly because of complications of IS, or noncompliance [15,16]. During the weaning process or after the cessation of IS, patients were carefully monitored for rejection by serum transaminase assay. When an increase in transaminases was observed, the dosage of tacrolimus was increased or corticosteroid pulse therapy was administered with or without assessment of graft histology (deemed intolerance). Additional immunosuppressants such as Orthoclone (OKT3) were used to counteract steroid-resistant rejection [16].

Protocol biopsy

Since January 2003, protocol biopsy of liver grafts has been performed at 1, 2, 5, and 10 years post-Tx on pediatric LDLT patients, even in the presence of a normal liver test. If the biopsy showed pathologic features consistent with acute or chronic rejection, IS was increased and the patients were included in Gr-rej. If advanced fibrosis (bridging fibrosis) was observed by single biopsy, or progression of fibrosis by repeated biopsy, tapering was interrupted and IS was increased [32]. Those cases were included in Gr-fib because fibrosis is regarded as a symptom of rejection, especially as it usually improves after the re-introduction of IS [31].

HLA typing

Haplotypes were serologically defined for HLA-A, HLA-B, and HLA-DR loci in donors and recipients according to the standard methods of the NIH, except emergency LDLT for fulminant hepatitis [36].

Isolation of peripheral blood mononuclear cells

Venous blood was taken from tolerant and intolerant patients who were followed by Kyoto University hospital. Also the venous blood was taken from age-matched healthy volunteers. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by density gradient centrifugation over Ficoll-Paque PLUS^{TM} (GE Healthcare Bio-sciences, Uppsala, Sweden). PBMCs were washed twice with phosphate-buffered saline containing

0.1% sodium azide (Wako, Osaka, Japan) and 2% fetal calf serum [18,21].

Fluorescence-activated cell sorting analysis

Cells were stained for 30 min at 4 °C with the following fluorescence-conjugated mAbs: Allophycocyanin-conjugated anti-CD4, PeCy7-conjugated anti-CD25, and phycoerythrin-conjugated anti-IL7-Rα. All mAbs and their isotype controls were purchased from BD Biosciences. The cells were acquired with a FACS Calibur Becton Dickinson Pharmingen (San Diego, CA, USA) and analyzed with BD CellQuest software version 3.3. The frequency of $CD4^{+}CD25^{++}IL\text{-}7R\alpha^{low+}$ cells was analyzed by the "*t*"-test. The Human Research Ethics Committee of the Faculty of Medicine, Kyoto University approved this study. Informed consent was provided for pediatric patients according to the Declaration of Helsinki [37].

Results

Outcome of weaning IS

Five-hundred and thirty-seven pediatric patients of 670 patients survived at analysis (April 2008) (Figure 1). Of these, 347 patients had never been assessed as weaning IS, and were excluded from this analysis (Table 1). One-hundred and ninety patients were assessed as weaning IS. Among them, 132 patients were weaned by our elective protocol, and 58 patients stopped IS nonelectively (Figure 1). Eighty-four patients (15.6% of all the surviving patients) were included in Gr-tol (50 electively and 34 nonelectively weaned). Fifty patients were included in Gr-intol (31 electively and 19 nonelectively). Among them, 24 patients encountered acute cellular rejection (Gr-rej) while fibrosis was detected by liver biopsy in 26 patients (Gr-fib). Weaning IS was in progress in 56 patients at the time of this analysis (Figure 1).

Characteristics of excluded patients

Immunosuppression withdrawal had never been attempted in 347 patients, and they remained on maintenance IS (Table 1). Eighty-five of these patients (25%) exhibited unstable liver tests. Ninety-three patients (27%) had not been followed up at Kyoto University Hospital and their current clinical status and laboratory data were missing. In 18 patients (8%), although liver function was stable, weaning IS had never been attempted because of the absence of collaboration of local physicians. The other patients did not satisfy the criteria because of the presence of fibrosis or insufficient time from Tx (<2 years) (Table 1).

Table 1. Characteristics of excluded patients.

	Excluded patients	Patients not followed up
	(*n* = 347)	(*n* = 93)
Recipient age in years, mean (SD)	5.2 (5.1)	5.4 (5.1)
Recipient female, *N* (%)	214 (63%)	61 (66%)
Underlying disease, *N* (%)		
Biliary atresia	256 (76%)	68 (73%)
Blood test before Tx, mean (SD)		
WBC ($X10^3$/l)	7.9 (5.4)	7.7 (5.0)
PLT ($X10^3$/μl)	185.5 (309.2)	229.5 (549.2)
CRP (mg/dl)	1.6 (2.8)	1.8 (3.6)
AST (U/l)	191 (156)	180 (129)
ALT (U/l)	128 (141)	108 (90)
Albumin (mg/dl)	3.6 (0.6)	3.6 (0.6)
Total bilirubin (mg/dl)	11.6 (10.3)	11.6 (10.0)
Creatinine (mg/dl)	0.28 (0.23)	0.29 (0.23)
PT (s)	15.0 (5.5)	14.7 (4.9)
Condition, *N* (%)		
Hospitalized	207 (62%)	54 (58%)
Donor age in years, mean (SD)	35.3 (7.9)	34.7 (8.0)
Donor female, *N* (%)	172 (51%)	49 (53%)
ABO compatible, *N* (%)	287 (84%)	81 (87%)
Graft weight (GRBW%), mean (SD)	2.3 (1.1)	2.3 (1.2)
Follow up time after Tx in days, mean (SD)	2787 (1492)	3629 (1151)
The reason for not weaning, *N* (%)		
unstable LFT	85 (25%)	
ACR	45 (13%)	
fibrosis	19 (6%)	
AIH	16 (5%)	
CR	12 (4%)	
APOLT	3 (1%)	
re-Transplantation	16 (5%)	
not followed up	93 (27%)	
stable, but not assessed as weaning	18 (8%)	
other	5 (1%)	
<2 years after transplantation	37 (11%)	

LFT, liver function test; ACR, acute cellular rejection; AIH, autoimmune hepatitis; CR, chronic rejection; APOLT, auxiliary partial orthotopic liver transplantation. WBC, white blood cell; PLT, platelet, CRP, C-reactive protein; AST, aspartate aminotransferase; ALT, alanine aminotransferase; PT, prothrombin time; GRBW, graft recipient to body weight.

Characteristics of eligible patients

Fifty patients who were not able to fulfill the requirement for OT were included, consisting of patients who had acute or chronic rejection (Gr-rej), and those who had advanced or progressive fibrosis (Gr-fib). The characteristics of Gr-rej and Gr-fib patients are compared in Table 2. There was a significant difference between the groups ($P < 0.001$) in the reason for IS withdrawal as Gr-rej had been mainly nonelectively

Table 2. Characteristics of Gr-rej and Gr-fib patients.

	Group-rejection (n = 24)	Group-fibrosis (n = 26)	P value
Recipient age in years, mean (SD)	2.0 (3.3)	2.2 (2.6)	0.9
Recipient female, *N* (%)	18 (75%)	18 (69%)	0.7
Underlying disease, *N* (%)			
Biliary atresia	21 (88%)	22 (85%)	0.8
Blood test before transplantation, mean (SD)			
WBC ($X10^3$/l)	8.6 (4.0)	8.0 (5.7)	0.7
PLT ($X10^3$/μl)	161.6 (77.3)	153.1 (86.0)	0.7
CRP (mg/dl)	1.5 (1.4)	3.2 (4.8)	0.1
AST (U/l)	270 (157)	223 (136)	0.3
ALT (U/l)	143 (81)	145 (108)	0.9
Albumin (mg/dl)	3.5 (0.6)	3.5 (0.7)	1.0
Total bilirubin (mg/dl)	13.2 (12.4)	15.4 (10.0)	0.5
Creatinine (mg/dl)	0.18 (0.08)	0.15 (0.05)	0.2
PT (s)	14.9 (5.6)	15.8 (6.3)	0.6
Condition, *N* (%)			
Hospitalized	12 (50%)	15 (58%)	0.6
Donor age in years, mean (SD)	31.8 (8.3)	31.0 (4.3)	0.7
ABO compatible, *N* (%)	18 (75%)	25 (96%)	0.03
Graft weight (GRBW%), mean (SD)	3.2 (1.2)	3.0 (1.0)	0.9
The presence of HLA-A mismatch, *N* (%)	17 (85%)	23 (92%)	0.5
The presence of HLA-B mismatch, *N* (%)	17 (85%)	23 (92%)	0.5
The presence of HLA-DR mismatch, *N* (%)	16 (80%)	22 (88%)	0.5
The absence of early rejection, *N* (%)	13 (54%)	17 (65%)	0.4
Tacrolimus level (ng/ml), mean (SD) (average within the first week post-LDLT) all adjusted to intravenous level	14.8 (5.9)	14.9 (4.3)	1.0
Reason for IS withdrawal			<0.001
Protocol, *N* (%)	7 (29%)	24 (92%)	
Infection, *N* (%)	14 (58%)	1 (4%)	
Others, *N* (%)	3 (13%)	1 (4%)	
Follow-up time after Tx in days, mean (SD)	3889 (1236)	4418 (906)	0.08

HLA, human leukocyte antigen; LDLT, living-donor liver transplants; IS, immunosuppression.

weaned because of infection whereas Gr-fib were almost all weaned according to protocol. The only other difference was a higher incidence of ABO compatibility in Gr-fib (96%) compared with Gr-rej (75%) ($P = 0.03$). Other factors of clinical significance between the groups, such as HLA mismatch and absence of early rejection were quite similar and because of this they were pooled (Gr-intol) for univariate and multivariate analysis.

Univariate analysis of Gr-tol and Gr-intol is shown in Table 3. Patients in Gr-intol had experienced rejection within the first month post-LDLT at a significantly higher incidence (40%) than Gr-tol (17%) ($P < 0.01$). The average trough level of tacrolimus within the first week post-LDLT was significantly higher in Gr-tol (17.1 ng/ml) than in Gr-intol (11.0 ng/ml) ($P < 0.01$). In addition, the follow-up time (time after Tx) in Gr-tol (4 725 days) was significantly longer than Gr-intol (4 135 days) ($P < 0.01$). No other parameters differed between the two, although a higher rate of HLA-A mismatch in Gr-intol approached significance ($P = 0.07$).

Multivariate analysis of Gr-tol versus Gr-intol

Multivariate analysis is shown in Table 4. The absence of early rejection was associated with OT and had an OR (95% CI) of 2.79 (1.11–7.02) ($P = 0.03$). Matching at the HLA-A locus was also associated with OT with an OR of 0.18 (0.03–0.91) ($P = 0.04$). ABO compatibility or HLA-DR mismatch was not statistically significant. Higher trough levels of tacrolimus in the first week after Tx showed an association with OT ($P = 0.02$). In contrast, patients who were matched at the HLA-B locus were less likely to develop tolerance. The OR for HLA-B mismatch was 8.73 (1.09–70.0) ($P = 0.04$). Although this level is statistically significant, the large confidence interval and the very small percentage of patients in both groups who were matched at HLA-B question its clinical significance.

Table 3. Univariate analysis of Gr-tol versus Gr-intol patients.

	Group-tolerance	Group-intolerance	
	(*n* = 84)	(*n* = 50)	*P* value
Recipient age in years, mean (SD)	2.9 (3.7)	2.1 (2.9)	0.20
Recipient female, *N* (%)	49 (58%)	36 (72%)	0.11
Underlying disease, *N* (%)			
Biliary atresia	67 (80%)	43 (86%)	0.36
Blood test before transplantation, mean (SD)			
WBC ($X10^3$/l)	8.0 (4.6)	8.3 (5.0)	0.78
PLT ($X10^3$/μl)	169.7 (101.8)	157.2 (81.2)	0.46
CRP (mg/dl)	2.1 (2.4)	2.4 (3.7)	0.63
AST (U/l)	235 (157.8)	245 (147.1)	0.70
ALT (U/l)	134 (103.2)	144 (94.8)	0.58
Albumin (mg/dl)	3.5 (0.7)	3.5 (0.6)	0.59
Total bilirubin (mg/dl)	15.8 (9.5)	14.3 (11.1)	0.41
Creatinine (mg/dl)	0.18 (0.08)	0.16 (0.07)	0.28
PT (s)	14.3 (3.2)	15.4 (5.9)	0.21
Condition, *N* (%)			
Hospitalized	51 (61%)	30 (60%)	0.87
Donor age in years, mean (SD)	33.0 (6.3)	31.4 (6.5)	0.15
ABO compatible, *N* (%)	77 (92%)	43 (86%)	0.30
Graft weight (GRBW%), mean (SD)	2.9 (1.3)	3.1 (1.1)	0.32
The presence of HLA-A mismatch, *N* (%)	54 (75%)	40 (89%)	0.07
The presence of HLA-B mismatch, *N* (%)	68 (94%)	40 (89%)	0.27
The presence of HLA-DR mismatch, *N* (%)	60 (83%)	38 (84%)	0.87
The absence of early rejection, *N* (%)	70 (83%)	30 (60%)	<0.01
Tacrolimus trough level (ng/ml), mean (SD) (average within the first week post-LDLT)	17.1 (12.0)	11.0 (5.1)	<0.01
Reason for IS withdrawal			0.2
Protocol, *N* (%)	50 (60%)	31 (62%)	
Infection, *N* (%)	18 (22%)	15 (30%)	
Others, *N* (%)	15 (18%)	4 (8%)	
Follow up time after Tx in days, mean (SD)	4725 (1102.3)	4135 (1098.9)	<0.01

HLA, human leukocyte antigen; LDLT, living-donor liver transplants; IS, immunosuppression.

Table 4. Multivariate analysis of Gr-tol versus Gr-intol.

Variable	*P* value	Odds ratio	95% CI
ABO compatible	0.720	1.41	0.22–8.94
The absence of early rejection	0.030	2.79	1.11–7.02
The presence of HLA-A mismatch	0.040	0.18	0.03–0.91
The presence of HLA-B mismatch	0.040	8.73	1.09–70.0
The presence of HLA-DR mismatch	0.710	0.78	0.21–2.84
Follow up time after Tx (days)	0.22	1	1.00–1.00
Tacrolimus trough level	0.020	1.104	1.016–1.200

HLA, human leukocyte antigen; CI, confidence interval.

Frequency of $CD4^+CD25^{++}$IL-$7R\alpha^{low+}$ cells

Blood samples could be obtained from 32 of 134 patients enrolled in this study. Of them, 24 patients were Gr-tol whereas eight were in Gr-intol. The frequency of $CD4^+CD25^{++}$IL-$7R\alpha^{low+}$ cells within the peripheral $CD4^+$ cells at more than 10 years post-Tx was significantly decreased in the Gr-intol cohort (4.9 ± 1.0%) compared with the Gr-tol cohort (7.6 ± 1.7%) ($P = 0.003$) (Figure 2a). Twenty-five patients were HLA-A mismatched whereas seven were matched. The HLA-A mismatched cohort included 19 tolerant and 6 intolerant patients. The HLA-A matched cohort included five tolerant and two intolerant patients. The frequency of $CD4^+CD25^{++}$IL-$7R\alpha^{low+}$ cells was comparable between the HLA-A matched cohort and the mismatched cohort (7.2 ± 2.6 vs. 6.9 ± 1.8%, HLA-A matched vs. mismatch, $P = 0.761$) (Figure 2b). In contrast, there were fewer $CD4^+CD25^{++}$ IL-$7R\alpha^{low+}$ cells in the HLA-B matched cohort compared with the HLA-B mismatched cohort and healthy volunteers (4.7 ± 1.1 vs. 7.3 ± 1.9, 9.0 ± 2.9%, HLA-B matched vs. mismatch, $P = 0.011$, HLA-B match vs. healthy volunteers, $P = 0.013$) (Figure 2c), although the low numbers in the matched cohort question the validity of this result.

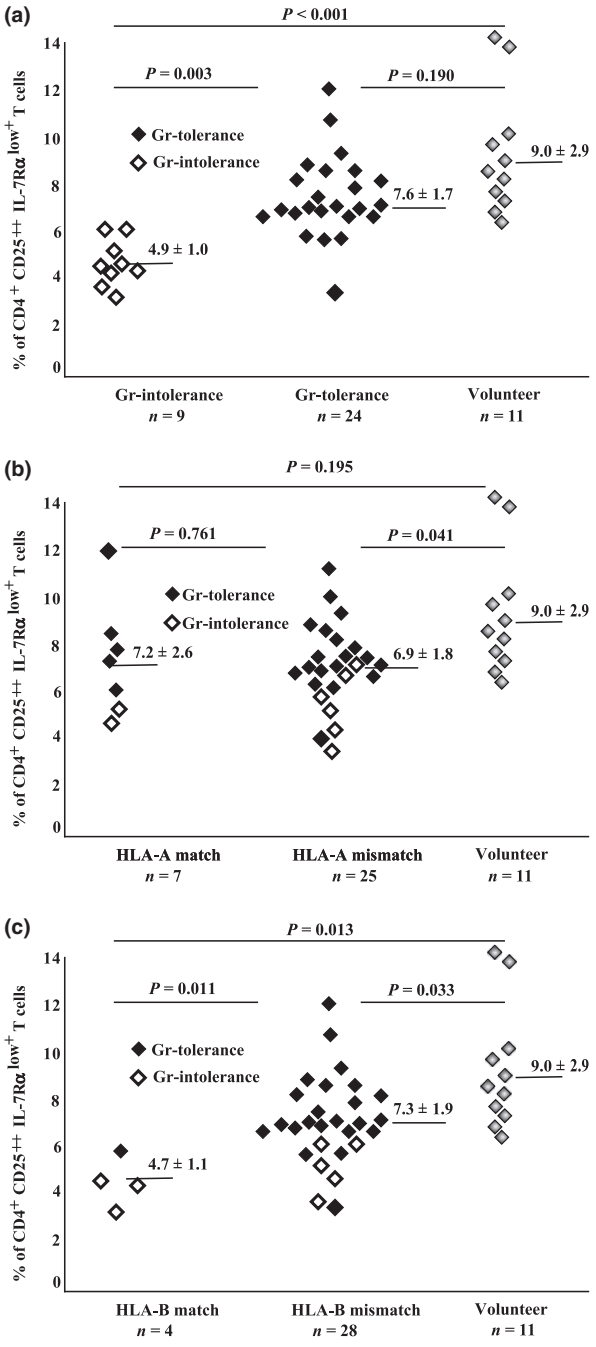

Figure 2 The frequency of $CD4^+CD25^{++}IL\text{-}7R\alpha^{low+}$ T cells with HLA match. (a) The frequency of $CD4^+CD25^{++}IL\text{-}7R\alpha^{low+}$ T cells between group-tolerance versus group-intolerance. Of 134 patients, peripheral blood was available for phenotypic analyses in 33 patients. The frequency of $CD4^+CD25^{++}IL\text{-}7R\alpha^{low+}$ T cells within $CD4^+$ cells was significantly lower in Gr-intol than that in Gr-tol (at >10 years post-Tx) (4.9 ± 1.0 vs. 7.6 ± 1.7%, Gr-intol vs. Gr-tol, $P = 0.003$). (b) Effect of HLA-A match. The frequency of $CD4^+CD25^{++}IL\text{-}7R\alpha^{low+}$ T cells within $CD4^+$ cells did not differ between the HLA-A matched and mismatched cohorts (7.2 ± 2.6 vs. 6.9 ± 1.8%, HLA-A matched vs. mismatched, $P = 0.761$). (c) Effect of HLA-B match. In the HLA-B matched cohort, the frequency of $CD4^+CD25^{++}IL\text{-}7R\alpha^{low+}$ T cells within $CD4^+$ cells was significantly decreased compared with those in the HLA-B mismatch cohort and age-matched healthy volunteers (4.7 ± 1.1 vs. 7.3 ± 1.9, 9.0 ± 2.9%, HLA-B matched vs. mismatch, $P = 0.011$, HLA-B match vs. healthy volunteers, $P = 0.013$).

Discussion

This is the first retrospective analysis of weaning of IS in a large cohort of living-related (parent to child) pediatric liver Tx patients. Although parent to child (F1) Tx are not as common as deceased donor Tx, the lack of sufficient deceased donors has led to their increasing incidence in both liver and kidney Tx. As we have previously reported that advanced fibrosis or progression of fibrosis is an indicator of the need for increased IS, we compared patients who required recommencement of IS because of rejection with those who had IS restarted for fibrosis. Characteristics for these two groups were similar; with the notable exception of the reason for IS withdrawal, where the majority of the patients in Gr-rej had been rapidly weaned nonelectively mainly due to Epstein-Barr virus infection [16] whereas Gr-fib patients were almost all electively weaned according to protocol. Perhaps, the rapid removal of the Gr-rej patients from IS during nonelective weaning predisposes these patients to frank rejection that is easily diagnosed on the basis of graft pathology on biopsy. In contrast, the slower removal of IS according to protocol of the Gr-fib patients might have led to a low-grade immune response to the graft that manifests itself as graft fibrosis in these patients. This is especially the case as long-term pediatric liver transplant recipients show a very high tendency to develop fibrosis in association with inflammation [38]. An alternative explanation is that severe viral infection could induce cross-reactive (heterologous) immunity that increases the likelihood of rejection, as shown in animal models [39]. Further statistical analysis of the two separate intolerant groups could not be performed, because of their small sample sizes. However, it will be interesting to compare other immune parameters such as extent and type of antibody deposited, composition of cellular infiltrate, and expression of cytokines and growth factors.

As both the intolerant groups were similar in other clinical features, except for the incidence of ABO compatibility, which was higher in the fibrosis group, they were combined for further analysis (Gr-intol). Comparison of Gr-tol with Gr-intol showed that the presence of rejection episodes in the first month was associated with intolerance, which accords well with published findings [29].

Increased levels of trough tacrolimus in the first week after Tx have been associated with OT of liver Tx patients, which is supported by the findings reported here. Although HLA matching is not thought to play a role in the outcome of deceased donor liver Tx, we found that matching at the HLA-A locus was associated with OT in this pediatric LDLT population, which is in accordance with the findings of improved outcomes in HLA-A matched patients in deceased donor renal Tx [22–25].

In contrast to these published results, we describe here a statistically significant association between HLA-B mismatch and OT in the multivariate, but not the univariate analysis. This finding is unexpected as in the setting of renal Tx, mismatches of HLA-B between donors and recipients negatively impact the outcomes of graft survival more strongly than those of other HLA loci [40]. This is in accordance with the *in vitro* observations that HLA-B is more immunogenic than HLA-A and HLA-DR [41]. Therefore, our findings in LDLT patients stand in contrast to those in both liver and kidney Tx patients. Consequently, it is tempting to discount this effect as a statistical aberration, because of the very small proportion of HLA-B matched patients in both groups with a consequent large confidence interval and the lack of significance in the univariate analysis. Despite this, some support for the finding comes from the completely independent area of maternal/fetal tolerance. In the Hutterites (an inbred population of European descent), significantly increased fetal loss rates were observed among couples matched for HLA-B [42]. Thus, if our findings that HLA-B mismatch favorably affects OT are correct, they appear to be consistent with maternal/fetal tolerance, suggesting that there may be common features between the two.

Recently, we have shown that donor-antigen specific regulatory T cells (Tregs) can be generated in LDLT patients and play a critical role in the induction and the maintenance of OT [18–21]. Our data are consistent with those in deceased liver Tx and kidney Tx reported by others [43,44]. The findings here in an expanded population confirm that the frequency of $CD4^{+}CD25^{++}IL\text{-}7R\alpha^{low+}$ Tregs within the peripheral $CD4^{+}$ cells derived from Gr-intol patients were significantly decreased compared with Gr-tol patients. Of interest, there was a higher percentage of Tregs in the HLA-B-mismatched patients which is in agreement with our findings of an improved outcome in HLA-B mismatched recipients. Such a correlation was not observed between HLA-A and Tregs, despite the association between HLA-A matching and tolerance. However, there are very small numbers of patients in the mismatched groups which questions the validity of these results.

Some potential limitations of the present study should be considered. Firstly, a large number of patients in the original cohort were excluded because they were not being followed up or had not been assessed as weaning. However, examination of the baseline characteristics of those patients showed that they were similar to those included in the study, suggesting that selection bias was not a major issue. Second, it is unclear whether the results reported here for HLA matching and OT in semi-allogeneic pediatric LDLT can be generalized to fully allogeneic deceased donor liver Tx. The original studies of MHC restriction by Zinkernagel and Doherty showed that F1 targets behaved similar to fully allogeneic targets [45]. Similarly, in NK cell-mediated killing of target cells lacking MHC expression, first described by Karre, there was a quantitative, but not a qualitative difference between F1 and fully allogeneic systems [46]. In animal models of liver Tx tolerance and rejection, there was also a quantitative rather than a qualitative difference between F1 and fully allogeneic grafts, with the F1 showing an outcome that was intermediate between the two parental strains [47]. On the basis of these findings, results here may be extended, to some degree, to fully allogeneic grafts.

A third factor that could influence the outcome was that this study included patients who started weaning IS electively and stopped nonelectively. It is possible that this could lead to heterogeneity of patients that could jeopardize analyses. However, regardless of weaning being elective or nonelective, the probability of successful cessation of IS was comparable between Gr-Tol or Gr-Intol (Table 3). The main difference we observed between elective and nonelective weaning was its effect on rejection compared to fibrosis as a cause of intolerance as discussed above. It is also possible that subjects enrolled in this study could have been heterogeneous in terms of IS administration as this was changed over the course of the study. Initially, IS was delivered intravenously and later orally. To adjust for this, the trough level of tacrolimus administered orally was multiplied by 1.4, according to a published report [35]. Finally, the possibility that surgical techniques and post-Tx management were modified with time was taken into account.

In conclusion, this is the first retrospective analysis from a large cohort of pediatric LDLT patients examining factors which affect the subsequent development of tolerance. This is the first demonstration that HLA-A matching may be one of the determinants for OT in semi-allogeneic pediatric liver transplantation. It accords with the previously reported association between tolerance and absence of early rejection episodes in deceased donor recipients of liver Tx. The association between higher levels of Tregs in tolerant patients is consistent with previous studies [18–21,43,44]. Last but not least, we cannot rule out the possibility that both rejection and fibrosis play a different role in the pathologic progression of intolerance. Further large scale studies are needed to

identify clinically significant effects of differences between rejection and fibrosis that lead to post-Tx intolerance.

Authorship

HO, KW, MY, HN-H, YL, XZ, TM and TK: carried out the research. HO, HN-H, YL, TK, SS and AB: wrote the manuscript. TK: participated in research design. HO, KW and HN-H: conducted the data analysis. SU: directed the transplant program.

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