Effectiveness of Perioperative Immunologic Markers Monitoring for Predicting Early Acute Cellular Rejection After Living Donor Liver Transplantation

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ABSTRACT

Background. The objective of this study was to determine whether perioperative immunologic markers monitoring could predict early acute cellular rejection (ACR) after living donor liver transplantation (LDLT).

Materials and methods. From September 2010 to June 2013, a total of 172 patients underwent LDLT at our transplant center. Of them, 26 patients were excluded because of infection. We retrospectively reviewed the remaining 146 patients. CD4 lymphocyte activity, T cell subsets test, and serum cytokine panel were checked on the day before transplantation and at 20 days after transplantation. These patients were divided into 3 groups: 1. normal liver function test (LFT) group; 2. increased LFT without rejection group; and 3. early ACR group. We excluded the increased LFT without rejection group in order to rule out multiple factors influencing immunologic factors.

Results. CD4 lymphocyte activity ($P = .004$) was significantly increased while CD4+/CD25+/FOXP3+ cells ($P < .001$) and interleukin (IL)-17 ($P = .002$) levels were significantly decreased during the perioperative period. Pretransplant IL-6 ($P = .014$) and IL-17 ($P = .029$) levels in the early ACR group were significantly lower than those in the normal LFT group. The proportion of patients with increased IL-6 during perioperative period in the early ACR group was higher than that in the normal LFT group, although the difference was not statistically significant ($P = .065$).

Conclusion. Our results suggest that IL-6 and IL-17 levels are associated with early ACR in LDLT patients. However, whether monitoring perioperative immunologic markers could predict early ACR remains unclear. Further prospective studies are needed to reach a definite conclusion.

LIVER transplantation not only saves lives but also improves quality of life of patients suffering from end-stage liver disease. Although there are many theoretical explanations for the resistance of a liver allograft to a cytotoxic antibody, acute rejection is 1 of the main causes of graft failure [1]. The incidence of acute rejection under current immunosuppressive regimens ranges from 20% to 40% [2]. However, immunosuppressive regimens may produce severe complications such as infection, hypertension, hyperlipidemia, malignancy, and chronic renal insufficiency [3]. Therefore, identification of immunologic markers for acute rejection may provide important information to adjust immunosuppression to improve long-term graft survival and minimize adverse effects of immunosuppressive agents.

Recently, experimental and clinical observations have indicated that regulatory T cells and serum cytokine play important roles in immune regulation [4–6]. However,

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there is no consensus on immunologic markers for predicting acute rejection after liver transplantation. Although immunologic responses in living donor liver transplantation (LDLT) are different from those in deceased liver transplantation, there are few reports about LDLT. Therefore, the objective of this study was to determine the effectiveness of perioperative immunologic markers monitoring for predicting early acute cellular rejection (ACR) after LDLT and examine changes of immunologic markers during perioperative period and the relationship of early ACR with perioperative immunologic markers.

MATERIALS AND METHODS

Patients

A total of 172 patients underwent LDLT from September 2010 to June 2013 at our transplant center. Immunologic markers could be affected by infection. Thus, 26 patients were excluded due to infection after LDLT. Infection was defined by fever and positive blood culture. We retrospectively reviewed the remaining 146 patients. The median follow-up period after LDLT was 25 months (range, 1–39 months). These patients were divided in 3 groups: 1. normal liver function test (LFT) group (n = 67, 45.9%), 2. Increased LFT without rejection group (n = 51, 34.9%), and 3. early ACR group (n = 28, 19.2%). We excluded those with increased value in LFT without rejection in order to rule out multiple factors influencing immunologic factors. We then compared immunologic markers between the normal LFT group and early ACR group. This study was approved by the institutional review board of our center.

Acute Cellular Rejection

ACR was diagnosed based on increased liver enzyme levels according to histologic evidence. Increased LFT was defined when total bilirubin or alanine transaminase level was more than 3 times the upper normal level. A liver biopsy was performed if acute rejection was suspected. Biopsy specimens were evaluated by an experienced transplant pathologist and scored according to the Banff scheme. In this scheme, portal inflammation, bile duct inflammation/damage, and subendothelial inflammation of portal veins or terminal hepatic venules were each graded semiquantitatively on a scale ranging from 0 (absent) to 3 (severe). These individual scores were added to produce an overall rejection activity index ranging from 0 to 9. ACR was defined as clinically suspected rejection with rejection activity index ≥ 3. Early ACR was defined as ACR within 1 year after transplantation.

Immunologic Markers

Immunologic markers were checked within 24 hours before transplantation and at 20 days after LDLT. CD4 lymphocyte activity, T cell subsets, and serum cytokine panel were evaluated. CD4 lymphocyte activity was examined by Luminex method. T cell subsets including CD4+, CD8+, CD4+/CD25+, and CD4+/CD25+/FOXP3+ cells were examined by flow cytometry method. In the serum cytokine panel, levels of interleukin (IL)–2, IL–6, IL–10, IL–12, IL–17, tumor necrosis factor α, and interferon gamma were examined by the Luminex method.

Liver Transplant and Post-Transplant Follow-up

LDLT was performed according to a standard technique using a modified right lobe with middle hepatic vein reconstruction. Immunosuppression regimen was a triple-drug regimen, including tacrolimus or cyclosporin, mycophenolate mofetil, and prednisolone. Steroids were withdrawn at 1 month after surgery. Mycophenolate mofetil was withdrawn at 6 months after surgery. An interleukin–2 receptor blocker was administered on the day of surgery and on postoperative day 4. Patients were followed monthly for the first year, every 2 months for 5 years, and every 3 months thereafter.

Statistical Analysis

Continuous variables are reported as means ± standard deviations. They were compared using Student t test. Categorical variables were analyzed using χ^2 test. Graft survival and overall survival rates were calculated using the Kaplan-Meier method. A univariate analysis was performed using the Kaplan-Meier method and evaluated using the log-rank test to identify risk factors for graft survival. To determine prognostic accuracy of immunologic markers in predicting of early ACR, receiver operating characteristic (ROC) curves are performed. All statistical analyses were performed using SPSS 18.0 for Windows (SPSS, Inc., Chicago, IL, USA). A P value $< .05$ was considered significant.

RESULTS

Clinical Characteristics of Study Populations

We evaluated immunologic markers of patients belonging to the normal LFT group (n = 67, 70.5%) and the early ACR group (n = 28, 29.5%). Among them, 68 (71.6%) were men. The mean age of these subjects was 52.2 ± 7.8 years. The most common etiology of transplantation was hepatitis B (n = 64, 67.4%), followed by excess alcohol use (n = 10, 10.5%), autoimmune disorders (n = 7, 7.4%), hepatitis C (n = 4, 4.2%), and other causes (n = 10, 10.5%). The mean model for end-stage liver disease score was 13.4 ± 9.0. Clinical characteristics of study populations were not significantly different between the normal LFT group and the early ACR group (Table 1). The median follow-up period after LDLT was 25 months (range, 1–39 months). One-year and 3-year overall survival rates in the entire group of patients were 97.9% and 87.2%, respectively. One-year and 3-year overall survival rates were 97% and 88.9%, respectively, in the normal LFT group and 100% and 82.3%, respectively, in the early ACR group. Overall survival rates were not significantly different between the 2 groups ($P = .559$).

Changes of Immunologic Markers During Perioperative Period

Immunologic markers were checked twice (within 24 hours before transplantation and at 20 days after LDLT) to examine changes of immunologic markers during the perioperative period. CD4 lymphocyte activity was significantly increased during the perioperative period ($P = .004$) (Table 2). CD4+ T cells and CD8+ T cells were not significantly different. However, regulatory T cell subsets including CD4+/CD25+ ($P < .001$) and CD4+/CD25+/FOXP3+ ($P < .001$) were

Table 1. Patient Characteristics

	Total (n = 95)	Normal LFT (n = 67)	Early ACR (n = 28)	*P* value
Donor age (y)*	34.3 ± 11.3	33.6 ± 11.0	35.8 ± 12.0	.409
Donor sex (male), n (%)	56 (58.9)	36 (53.7)	20 (71.4)	.110
Donor relationship, n (%)				.615
Relatives	73 (76.9)	52 (77.6)	21 (75.0)	
Non-relatives	22 (23.1)	15 (22.4)	7 (25.0)	
Recipient age (y)*	52.2 ± 7.8	52.9 ± 7.8	50.6 ± 7.7	.195
Recipient sex (male), n (%)	68 (71.6)	48 (71.6)	20 (71.4)	.983
Etiology, n (%)				.213
Hepatitis B	64 (67.4)	45 (67.2)	19 (67.9)	
HCC, n (%)	49 (51.6)	36 (53.7)	13 (46.4)	.516
ABO blood type, n (%)				.242
Incompatible	5 (5.3)	2 (3.0)	3 (10.7)	
MELD score*	13.4 ± 9.0	13.0 ± 8.6	14.4 ± 10.0	.492
Operative transfusion (unit) *	7.9 ± 7.1	8.3 ± 7.2	6.8 ± 7.0	.334
Ischemic time (min)*	95.6 ± 36.8	93.0 ± 37.9	101.6 ± 34.2	.314
Immunosuppressant, n (%)				.079
Cyclosporin	17 (17.9)	9 (13.4)	8(28.6)	
Prograf	78 (82.1)	58 (86.6)	20 (71.4)	

Abbreviations: ACR, acute cellular rejection; HCC, hepatocellular carcinoma; LFT, liver function test; MELD, model for end-stage liver disease.
*Values are shown as means ± standard deviation unless stated otherwise.

significantly decreased during the perioperative period. They were almost undetectable at 20 days after LDLT. For serum cytokines, IL–17 ($P = .002$) and interferon gamma ($P = .002$) levels were significantly decreased during the perioperative period. However, levels of other cytokines were not significantly changed during the perioperative period.

Relationship of Preoperative Immunologic Markers With Early ACR

We evaluated the relationship of preoperative immunologic markers with early ACR. Patients were divided into 2 groups: 1. normal LFT group (n = 67, 70.5%) and 2. early ACR group (n = 28, 29.5%). Values of immunologic markers were then compared between the 2 groups (Table 3). CD4 lymphocyte activities or T cell subsets were not significantly different between the 2 groups. For serum cytokine, preoperative IL–6 ($P = .014$) and IL–17 ($P = .029$) levels in the early ACR group were significantly lower than those in the normal LFT group. Levels of other cytokines were not significantly different between the 2 groups. However, preoperative interferon gamma level in the early ACR group was lower than that in the normal LFT group, although the difference was not statistically significant ($P = .087$).

Relationship of Postoperative Immunologic Markers With Early ACR

Postoperative immunologic markers were also compared between the 2 groups. Postoperative interferon gamma level in the early ACR group was significantly lower than that in the normal LFT group ($P = .025$) (Table 4). However, levels

Table 2. Changes of Immunologic Markers During Perioperative Period

			Correlation analysis		Paired *t* test
Factors	Preoperative	POD 20	r	*P* value	*P* value
CD4 lymphocyte activity	198.8 ± 159.2	340.6 ± 177.1	0.378	.111	.004
T4 (CD4+)	43.81 ± 10.29	41.86 ± 10.08	0.476	< .001	.127
T8 (CD8+)	22.88 ± 7.89	23.39 ± 6.07	0.573	< .001	.652
Treg (CD4/CD25)	2.596 ± 1.601	0.100 ± 0.001			< .001
Treg (CD4/CD25/FOXP3)	1.207 ± 0.805	0.100 ± 0.001			< .001
IL–2	7.03 ± 27.74	4.33 ± 16.61	0.152	.178	.424
IL–6	36.14 ± 87.57	22.70 ± 62.23	0.624	< .001	.084
IL–10	15.45 ± 49.96	21.86 ± 43.55	0.103	.366	.364
IL–12	9.37 ± 57.91	3.16 ± 26.05	−0.006	.959	.385
IL–17	34.67 ± 83.74	13.20 ± 38.23	0.743	< .001	.002
TNF-α	12.80 ± 10.94	10.97 ± 11.64	0.427	< .001	.179
IFN-γ	41.79 ± 98.86	15.44 ± 40.13	0.743	< .001	.002

Values are shown as means ± standard deviation unless stated otherwise.
Abbreviations: IFN-γ, interferon gamma; IL, interleukin; POD, postoperative day; TNF-α, tumor necrosis factor α.

Table 3. Relationship of Preoperative Immunologic Markers With Early ACR

Factors	Normal LFT	Early ACR	*P* value
CD4 lymphocyte activity	208.3 ± 145.1	131.6 ± 162.5	.273
T4 (CD4+)	42.78 ± 11.37	45.03 ± 8.21	.381
T8 (CD8+)	24.89 ± 10.05	29.17 ± 8.77	.151
Treg (CD4/CD25)	2.780 ± 1.964	2.773 ± 1.695	.992
Treg (CD4/CD25/FOXP3)	1.405 ± 1.428	1.409 ± 0.867	.993
IL–2	4.04 ± 9.79	11.69 ± 45.62	.396
IL–6	47.59 ± 99.41	13.93 ± 23.91	.014
IL–10	15.67 ± 53.87	13.21 ± 27.65	.823
IL–12	7.06 ± 31.98	22.02 ± 98.42	.446
IL–17	51.64 ± 111.62	17.00 ± 35.45	.029
TNF-α	14.42 ± 13.45	11.32 ± 11.58	.300
IFN-γ	64.52 ± 147.37	26.67 ± 60.14	.087

Values are shown as means ± standard deviation unless stated otherwise.
Abbreviations: ACR, acute cellular rejection; IFN-γ, interferon gamma; IL, interleukin; LFT, liver function test; TNF-α, tumor necrosis factor α.

of other postoperative immunologic markers were not significantly different between the 2 groups.

Relationship of Changes of Immunologic Markers During Perioperative Period With Early ACR

We evaluated the relationship of changes of immunologic markers during the perioperative period with early ACR. Changes in immunologic markers were defined as differences in levels of immunologic markers between preoperative day and 20 days after transplantation. Outcomes were classified either as an increase or a decrease in immunologic marker levels during the perioperative period. CD4 lymphocyte activity was not significantly different ($P = .530$) (Table 5). Proportions of patients with decreased CD4+ T cells ($P = .022$) and CD8+ T cells ($P = .040$) during the perioperative period in the early ACR group were significantly higher than those in the normal LFT group. Because regulatory T cell subsets were almost decreased during the perioperative period, there were no significant differences between the 2 groups. Although all serum cytokines showed no significant difference, the proportion of patients with increased IL–6 during the perioperative period in the early ACR group was higher than that in the normal LFT group, although the difference was not statistically significant ($P = .065$).

ROC Analysis of Immunologic Markers for Early ACR

To evaluate the diagnostic power of immunologic markers for early ACR, ROC curve analysis was performed (Fig 1). The analysis showed that all ROC curves failed to conclude immunologic markers (Fig 1A: preoperative markers, Fig 1B: postoperative markers) could be a statistically significant predictor for early ACR ($P > .05$ each).

DISCUSSION

The first human liver transplantation was performed in 1963 by Dr Thomas Starzl [7]. However, in the early 1980s, results of liver transplantation were poor, with 1-year survival less than 30% due to technical problems and the lack of adequate immunosuppression [8]. In 1979, a major advance came with clinical introduction of cyclosporine for immunosuppression in solid organ transplantation. Its use in liver transplantation recipients resulted in further development in this field [9]. Although survival rates after liver transplant have improved dramatically, rejection is 1 of the main

Table 4. Relationship of Postoperative Immunologic Markers With Early ACR

Factors	Normal LFT	Early ACR	*P* value
CD4 lymphocyte activity	347.3 ± 160.5	315.6 ± 213.8	.718
T4 (CD4+)	41.50 ± 11.13	40.71 ± 7.90	.762
T8 (CD8+)	22.88 ± 5.68	24.77 ± 6.74	.381
Treg (CD4/CD25)	0.264 ± 0.768	0.100 ± 0.001	.556
Treg (CD4/CD25/FOXP3)	0.109 ± 0.043	0.100 ± 0.001	.556
IL–2	3.06 ± 10.59	6.78 ± 24.99	.335
IL–6	25.14 ± 63.73	21.50 ± 62.66	.810
IL–10	20.18 ± 46.99	24.11 ± 29.36	.700
IL–12	3.98 ± 30.07	0.56 ± 2.32	.573
IL–17	16.02 ± 43.19	5.51 ± 13.56	.094
TNF-α	11.07 ± 12.36	10.14 ± 8.46	.733
IFN-γ	19.03 ± 45.39	4.75 ± 10.68	.025

Values are shown as means ± standard deviation unless stated otherwise.
ACR, acute cellular rejection; IFN-γ, interferon gamma; IL, interleukin; LFT, liver function test; TNF-α, tumor necrosis factor α.

Table 5. Relationship of Changes of Immunologic Markers During Perioperative Period With Early ACR

Factors		n	Normal LFT	Early ACR	*P* value
CD4 lymphocyte activity	D	4	4 (28.6%)	0 (0.0%)	.530
	I	15	10 (71.4%)	5 (100.0%)	
T4 (CD4+)	D	40	24 (50.0%)	16 (80.0%)	.022
	I	28	24 (50.0%)	4 (20.0%)	
T8 (CD8+)	D	15	7 (30.4%)	8 (66.7%)	.040
	I	20	16 (69.6%)	4 (33.3%)	
Treg (CD4/CD25)	D	27	19 (100.0%)	8 (100.0%)	
	I	0	0	0	
Treg (CD4/CD25/FOXP3)	D	27	19 (100.0%)	8 (100.0%)	
	I	0	0	0	
IL–2	D	54	39 (69.6%)	15 (62.5%)	.532
	I	26	17 (30.4%)	9 (37.5%)	
IL–6	D	55	42 (75.0%)	13 (54.2%)	.065
	I	25	14 (25.0%)	11 (45.8%)	
IL–10	D	25	19 (33.9%)	6 (25.0%)	.430
	I	55	37 (66.1%)	18 (75.0%)	
IL–12	D	79	55 (98.2%)	24 (100.0%)	1.000
	I	1	1 (1.8%)	0 (0.0%)	
IL–17	D	66	47 (83.9%)	19 (79.2%)	.749
	I	14	9 (16.1%)	5 (20.8%)	
TNF-α	D	44	33 (58.9%)	11 (45.8%)	.281
	I	36	23 (41.1%)	13 (54.2%)	
IFN-γ	D	62	43 (76.8%)	19(79.2%)	.815
	I	18	13 (23.2%)	5 (20.8%)	

Abbreviations: ACR, acute cellular rejection; IFN-γ, interferon gamma; IL, interleukin; LFT, liver function test; TNF-α, tumor necrosis factor α.

causes of graft failure. The incidence of acute rejection under current immunosuppressive regimens ranges from 20% to 40%. The mainstay of immunosuppression after liver transplantation is the use of a combination of calcineurin inhibitor (tacrolimus or cyclosporine), antiproliferative agent (eg, mycophenolate mofetil), and steroids. Immunosuppressive agents may produce severe complications such as nephrotoxicity, infection, hypertension, diabetes, and neurologic complication. Therefore, identification of immunologic markers for acute rejection may provide important information to adjust immunosuppression to improve long-term graft survival and minimize adverse effects of immunosuppressive agents. In this study, we evaluated changes of immunologic markers during the perioperative period and monitored the effectiveness of perioperative immunologic markers for predicting ACR.

The regulatory T cells as a subset of CD4+ T cells play a critical role in the ability of the immune system to temper its response [10]. They can downregulate the immune response by acting on effector cells or antigen presenting cells. These cells also have the ability to suppress cytokines, adhesion molecules, and costimulatory signals. CD4+ T cells that express CD25 are the most extensively studied population of regulatory T cells. In the early 1970s, Gershon et al demonstrated the existence of suppressor T cells that possessed immunoregulatory activity other than immune activation for the first time [11]. Kilshaw et al have proposed that suppressor T cells could inhibit the rejection of a murine skin allograft [12]. Subsequent work by Hall et al has shown that CD4+CD25+ T cells are responsible for the prolonged survival of cardiac grafts in an experimental model of allotransplantation [13]. A major development in the characterization of regulatory T cells was the identification of transcription factor FoxP3 as the master regulator of CD4+CD25+ T cells [14]. FoxP3 has been shown to be critical to the development and function of regulatory T cells. In the present study, regulatory T cell subsets including CD4+/CD25+ and CD4+/CD25+/FOXP3+ were significantly decreased during the perioperative period. They were almost undetectable at 20 days after LDLT. Because postoperative levels of regulatory T cells in both groups were very low, we could not conclude that the relationship between levels of regulatory T cells during the perioperative period and ACR was significant.

Cell surface receptors provide an interface through which adjacent cells can transfer signals vital to immune response. Although such cell-to-cell contact is a critical component of cellular communication, soluble mediators are also used extensively to accomplish similar tasks. These polypeptides termed cytokines are critical to the development and function of both innate and acquired immune processes. Cytokines are particularly fundamental to interactions between T cells and antigen-presenting cells. IL–2, the prototypical cytokine of T cell activation, has been particularly well studied. Akoglu et al have demonstrated that patients experiencing ACR show significantly higher intracellular percentage of IL–2 in CD8+ T cells compared to stable liver transplant recipients [15]. They also showed a good correlation between the percentage of

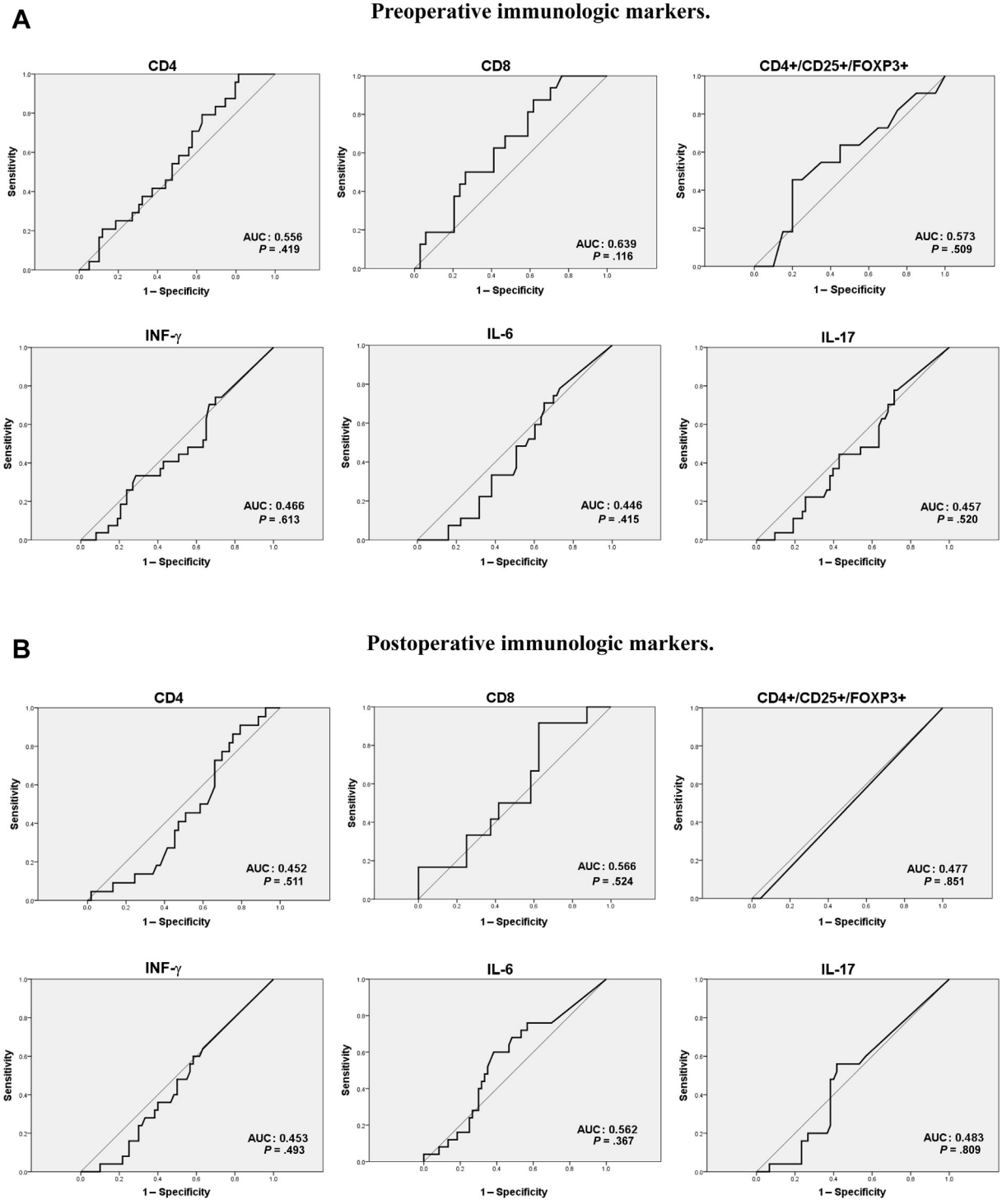

Fig 1. Receiver operator characteristic (ROC) curves for prediction of early acute cellular rejection. **(A)** Preoperative immunologic markers. **(B)** Postoperative immunologic markers. AUC, area under the curve; IL, interleukin; INF-γ, interferon gamma.

CD8+IL−2+ cells and Banff score. In a recent study, Millan et al have evaluated intracellular expression and soluble production of interferon gamma and IL−2 in 47 liver transplanted patients [16]. A pretransplant cutoff value of 55.8% for the percentage of CD8+interferon gamma+ identified patients at high risk of ACR. In the first week after transplantation,

patients with a percentage of inhibition for soluble interferon gamma, a percentage of CD8+interferon gamma+, and a percentage of CD8+IL−2+ lower than 40% developed ACR. When plasma levels of tumor necrosis factor α were measured in 50 adult patients following liver transplant, its concentration was significantly higher in patients experiencing ACR than that in those with a stable clinical course. However, in our study, we could not conclude the relationship between IL−2 and ACR.

Until recently, it was believed that antigen-activated helper T cells became terminally differentiated Th1 or Th2 cells with opposite effects. However, Th1 vs Th2 paradigm is incorrect because Th1 and Th2 can each mediate graft rejection whereas regulatory T cells are key inhibitors of cytopathic, allospecific immune responses. The current paradigm is that rejection or graft acceptance is determined by the relative balance between cytopathic Th1 and IL−17 producing T cells (Th17) vs cytoprotective regulatory T cells [17]. This balance depends on the level of inflammation in the microenvironment in which T-cell activation takes place. In the absence of proinflammatory cytokines, transforming growth factor β induces expression of Foxp3 and differentiation of CD4+ T cells into regulatory T cells [18]. In contrast, IL−6 or IL−21 prevents the development of regulatory T cells and induces differentiation of CD4+ T cells into Th17 cells that are highly cytopathic [19]. In the present study, preoperative IL−6 and IL−17 and postoperative interferon gamma levels in the early ACR group were significantly lower than those in the normal LFT group. These findings are in contrast with results of previous studies. However, the proportion of patients with increased IL−6 during perioperative period in the early ACR group was higher than that in the normal LFT group without showing statistical significance ($P = .065$). Our results suggest that changes of IL−6 levels during perioperative period could be more important than the absolute value of preoperative or postoperative IL−6 level for early ACR.

In the present study, immunologic markers were checked within 24 hours before transplantation and at 20 days after LDLT to evaluate the effectiveness of immunologic markers monitoring during the perioperative period. Although preoperative IL−6 and IL−17 levels, postoperative interferon gamma level, and changes of IL−6 during perioperative period were related to early ACR, most of the remaining immunologic markers were not related to early ACR. The limitation of the present study was that immunologic markers were not checked at episode of early ACR, although the purpose of the present study was to determine the effectiveness of immunologic markers monitoring during the perioperative period.

In conclusion, our results suggest that IL−6 and IL−17 levels are associated with early ACR of LDLT patients. However, it is difficult to predict early ACR by such simple correlation. A prospective study focusing on immunologic markers at the episode of ACR is needed to propose a model of early ACR prediction through immunologic markers.

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