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RESEARCH ARTICLE

Ischemic time of graft liver forces Th1-to-Th2 activity toward Th1 activity in patients who underwent living donor liver transplantation

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ABSTRACT. Recipient's immune responses are an important factor in allograft survival in transplantation. Cytokines are reflected with immune responses. In the present study, we aimed to evaluate potential affecting factors of liver allograft survival and their possible correlation with seroum cytokine levels in living donor liver transplantation (LDLT). One hundred and seventy-one adult patients' data were collected retrospectively. Five cytokines were collected: interferon (IFN)-γ, interleukin (IL)-2, IL-10, IL-6, and IL-17. Ischemic time of liver grafts was divided into two periods: cold and warm ischemic times (CIT and WIT, respectively). CIT had no statically significant correlation, but WIT showed a significant correlation with IFN-γ, IL-2, and IL-17 serum levels ($r = 0.0252$, 0.282, 0.178, respectively; $P < 0.05$). WIT was dichotomized as T1 (<22 min), T2 (22-70 min), and T3 (>70 min). IFN-γ was significantly increased in T2 and T3 as compared to T1. IL-6 was in T3 compared to T1 and T2. IL-17 was in T3 compared to T1. For the Th1-to-Th2 ratio, IFN-γ/IL-10, IFN-γ/IL-6, and IL-2/IL-10 were significantly different in T2 and T3 as compared to T1, and also in T3 as compared to T2. Th1 cell activities were enhanced with increased WIT. In conclusion, the longer WIT (>70 min) in LDLT is more likely to induce immunological reactions of recipients by leading to a deleterious cytokine balances in favor of an reinforced production of Th1 cytokines.

Key words: liver transplantation, intraoperative, inflammation, cytokine, donor, ischemic time

End-stage of live disease (ESLD) has a fatal and progressive course of disease. Therapeutic modality in patients with ESLD has been developed and researched continuously; however, liver transplantation (LT) is a unique and eventual treatment of ESLD till now [1-3]. The key of successful LT is accompanied by both surgical technique and medical treatment of transplantation immunology due to allograft transplantation [4]. Therefore, immunomodulation as a representative medical treatment for allogeneic transplantation is one modality for survival of the allograft like immunosuppression; furthermore, many methods have been attempted to regulate the recipient's immunity for achieving a higher survival rate of allografts during the intraoperative period [5]. For example, ischemic preconditioning as surgical intervention is well known and administering magnesium sulfate has been reported as favorable immunomodulation affected intraoperative immune status of recipients [6-8].

According to medical treatments for recipients, it is important to accurately define the recipient's immune status. The classical method of identification of immune state is to use whole blood differential counts and level of serum C-reactive protein (C-RP) as well known and commonly has been used until now. However, clinicians need more sensitive and specific immunologic indexes to identify and measure a recipient's immune status. The cell mediator substance is introduced as a biological response modifier in the 1970s, and it is called cytokines now. The cytokines are known to regulate intercellular activities, so it is some kinds of language to play a part in the immune activity of cell level [9, 10].

Abbreviations

GRWR	graft-recipient weight ratio
LDLT	living donor liver transplantation
MELD	model for end-stage liver disease
IL	interleukin
TNF	tumor necrosis factor
IFN	interferon
IT	ischemic time
LT	liver transplantation
Th	T helper cell

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There is a lack of research related to the survival of liver grafts based on serum levels of cytokines as mediators of immunity. In this opinion, we evaluate an interrelationship between pre- and post-operative serum levels of cytokines and factors of liver grafts which possibly affect survival of liver grafts.

METHODS

We reviewed data of 171 adult (age $\geq$19 years) patients who had undergone LDLT between January 2009 and December 2017 retrospectively in our hospital. Data collection was performed using an Electronic Medical Record system. The present study protocol and patient data collection were approved by the Institutional Review Board of our hospital (KC17RESI0180).

LDLT was proceeded our institute LDLT protocol using the right hepatic lobe of the living donor, and the piggyback technique for the anastomosis between donor hepatic vein and inferior vena cava of the recipient. All of LDLT was performed by two surgeons and the same LDLT protocol. A venovenous bypass was applied to limited patients in case of above 10 to 15 mmHg of the pressure gradient between portal vein and central venous pressure. A portal vein anastomosis was performed and then a hepatic artery anastomosis and bile duct reconstruction were done. The liver grafts were preserved using Histidine-tryptophan-ketoglutarate (HTK) solution. Balanced anesthesia was performed using 2.0-5.0 vol.% desflurane with remifentanil infusion at 0.1-0.2 μg/kg/min. For muscle relaxation, atracurium was infused at a rate of 7-10 μg/kg/min.

Mechanical ventilation was adjusted to maintain $PaCO_2$ at 30-35 mmHg. After anesthetic induction, a pulmonary artery and radial arterial catheter were applied to measure continuous hemodynamic monitoring and blood sampling. Packed red blood cells (PRBCs) were transfused to maintain a hematocrit between 25 and 30%. Transfusion of coagulation-related blood components was performed by the guide of thromboelastography to achieve point-of-care. Vasopressors were administered to overcome severe hemodynamic instability based on invasive hemodynamic monitors.

Patient's variables are included as follows. Pretransplant recipient variables were age, body mass index, sex, cause of LT, model for end-stage liver disease (MELD), diabetes mellitus, heart disease, hepatorenal syndrome, hepatic encephalopathy, emergency, and laboratory findings including serum cytokines and C-RP. Serum cytokines were collected as five different cytokines: interferon (IFN)-γ, interleukin (IL)-2, IL-10, IL-6, and IL-17. IFN-γ and IL-2 were collected as the cytokines for T helper (Th) 1, and IL-6, and IL-10 were collected as the cytokines for Th2. The ratio of Th1-to-Th2 was subsequently calculated. Intraoperatively, surgical time, extubation in operating room, transfused PRBCs, central venous pressure (CVP), mean blood pressure (MBP), heart rate, reperfusion syndrome, administered drugs including vasopressors, serum lactate change, and arterial pH were noted. Donor variables were age, graft fatty changes, graft-recipient weight ratio (GRWR), and ischemic time (IT) of graft.

The cytokine analysis was measured two times; 1 h before operation (pre-operative level of cytokines), and 1 h after operation (post-operative level of cytokines). The cytokines were quantified by a sandwich enzyme-linked immunoassay (ELISA) using a quantitative sandwich enzyme immunoassay test kit with specific first and second antibody pairs and recombinant proteins (Quantikine®; R&D Systems, Inc., Minneapolis, MN, USA).

In the transplant setting, graft ischemia time is used to describe two physiologically distinct periods of ischemia:

– warm ischemia time (WIT), from the removal of the organ from ice until reperfusion;

– cold ischemia time (CIT), during organ retrieval from the time of cross clamping (or of asystole in non-heart-beating donors) until cold perfusion is commenced.

WIT is a term used to describe ischemia of cells and tissues under normothermic conditions [11, 12]. WIT was dichotomized into three groups (T1, T2, T3) based on their interquartile. T1 is below 25% of WIT, T2 is between 25 and 75%, and T3 is above 75%.

Correlation analysis was performed between serum cytokines, and pre-operative, intraoperative and donor's factors. WIT groups according to serum cytokines were evaluated by the Kruskal-Wallis rank sum test. Demographic data are expressed as means $\pm$ SD or as numbers (percentages). Cytokines data are expressed as median (interquartile). All the tests were two-sided, and $P < 0.05$ was deemed statistically significant. All statistical analyses were performed using the Statistical Package for the Social Sciences version 18.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

A total of 177 LDLT patients were indicated for the present study, excluding six cases having critical missing in data-related cytokines and IT of liver grafts. The mean age of the patients was 52 ± 9 years, and men were predominant (64%). Overall MELD score was 16 ± 10 points. The most common cause of LT was hepatitis virus B infection (35.5%), and the second was hepatocellular carcinoma (28.9%). It results from regional characteristics. Asian is a more prevalence area of hepatitis virus B than C unlikely to western area. As intraoperative factors, C-RP as a classical inflammation index showed 1.17 ± 1.91, which was slightly increased than the normal range. The duration of post-reperfusion syndrome (PRS) after reperfusion of graft livers showed 3 ± 6 min. For donor factors, GWRW was 1.1 ± 0.4, and the degrees of fatty change of graft livers were 4 ± 6%. CIT and WIT were 94 ± 109, 44 ± 27 min, respectively, and the difference between CIT and WIT had a statistical significance ($P < 0.001$). Duration of WIT was relatively constant than its CIT, as WIT had a standard deviation of 27 min, whereas CIT had a larger standard deviation of 109 min. The different time characteristics between WIT and CIT are assumed to the variabilities of surgical conditions such as vascular variations and intraperitoneal adhesions of donor or recipient (*table 1*).

Table 1
Demographic data of 171 adult patients who underwent living donor liver transplantation

Variables	
Age (yr)	52 ± 9
Sex male (n, %)	106 (64)
BMI	24.6 ± 3.7
Cause of LT (n, %)	
Hepatitis B	59 (35.5)
Hepatitis C	27 (16.3)
Alcoholic	23 (13.8)
HCC	48 (28.9)
Others	9 (5.5)
MELD score	16 ± 10
C-RP (mg/dL)	1.17 ± 1.91
PRS (min)	3 ± 6
Vasopressor for rescue of PRS (μg)	12.9 ± 50.0
Total operation time (h)	9.2 ± 1.6
Donor	
GWRW (%)	1.1 ± 0.4
Fatty change (%)	4 ± 6
Ischemic time (min)	
Cold	94 ± 109
Warm	44 ± 27

Values are presented as mean ± standard deviation or *n* (%). BMI: body mass index; LT: liver transplantation; HCC: hepatocellular carcinoma; MELD: model for end-stage liver disease; C-RP: C-reactive protein; PRS: post-reperfusion system; GWRW: graft-to-recipient weight ratio.

Table 2 shows the results of correlation analysis between pre- and post-operative serum cytokines, and pre- and intraoperative factors. The MELD score and C-RP had a statistically significant positive correlation with IL-6, IL-10, and IL-17, both pre- and post-operatively. The longer duration of PRS showed more decreasing levels of IL-6. The analysis of donor factors was performed only with post-operative cytokines. GWRW and fatty change of graft liver were positively correlated with IL-2. For IT, CIT had no significant effect on the concentration of serum cytokines; however, WIT showed a significant positive correlation with IFN-γ, IL-2, and IL-17.

To investigate the changes of concentration of serum cytokines according to the duration of WIT, the duration of WIT was dichotomized with three intervals using its interquartile; less than 25% (<22 min), between 25 and 75% (22-70 min), more than 75% (>70 min). *Table 3* shows the results of comparison of three dichotomized times to concentrations of the five serum cytokines. IFN-γ, IL-6, and IL-17 had a statistical significance with the dichotomized WIT. IFN-γ was significantly increased in T2 and T3 compared to T1. IL-6 was increased at T3 compared to T1 and T2. IL-17 shows a significant difference in T3 compared to T1.

The Th1-to-Th2 ratio was calculated by calculating the IFN-γ/IL-10, IFN-γ/IL-6, and IL-2/IL-10 (*figure 1*). IFN-γ/IL-10, IFN-γ/IL-6, and IL-2/IL-10 were significantly different in T2, and T3 compared to T1, and also in T3 compared to T2. Those results showed Th1 cell activity was significantly increased as much as WIT increased.

DISCUSSION

This study clarified the effects of IT of liver grafts on the immune response of recipients, on recipient immunities in patients who underwent LDLT. Immune responses are induced by the release of inflammatory mediators such as cytokines [13]. These cytokine responses have an important effect on the function and survival of allografts in patients undergoing LT [14, 15].

IT of liver grafts is considered as two different parts into CIT and WIT. The CIT is a period of process of perfusing a preservative solution such as HTK into grafts and preparing grafts to be grafted to recipients. This period is maintained at a low temperature by ice water; therefore, this period helps to minimize ischemic injuries to grafts. The WIT refers to a period from the time of anastomosis to the reintroduction of grafts by placing the graft in the recipient's body. The grafts are rewarming by recipient's temperature and do not maintain low temperature of grafts in this period.

Table 2
Correlation coefficient between pre- and post-operative cytokines and patient factors

	Pre-operative					Post-operative				
	IFN-γ	IL-6	IL-2	IL-10	IL-17	IFN-γ	IL-6	IL-2	IL-10	IL-17
MELD score	0.026	0.291*	0.032	0.245*	0.163*	0.043	0.141*	-0.017	0.100*	0.190*
C-RP (mg/dL)	-0.017	0.352*	0.022	0.262*	0.152*	-0.041	0.113*	-0.046	0.114*	0.136*
RPS (min)	0.072	-0.062	-0.027	-0.023	-0.035	0.009	-0.118*	-0.023	0.051	-0.034
Vasopressor for rescue of RPS	-0.012	0.148*	-0.039	0.110*	0.064	0.050	0.011	-0.082	0.038	0.058
Donor										
GRWR						0.127	0.051	0.155*	-0.047	0.011
Fatty change (%)						0.101	0.097	0.160*	-0.054	0.113
Ischemic time (min)										
Cold						-0.124	0.024	-0.003	0.027	-0.075
Warm						0.252*	-0.166	0.282*	0.070	0.178*

MELD: model for end-stage liver disease; C-RP: C-reactive protein; PRS: post-reperfusion system; GWRW: graft-to-recipient weight ratio; IFN: interferon; IL: interleukin. *$P < 0.05$.

Table 3
Level of cytokines in the pre-operative and post-operative periods according to the categorized warm ischemic time.

	Pre-operative	Post-operative		
		T1	T2	T3
IFN-γ (pg/mL)	5.90 (2.72-19.55)	3.40 (1.83-12.13)	7.50 (4.10-15.35)*	21.70 (4.85-28.85)*
IL-6 (pg/mL)	11.98 (4.60-44.35)	8.80 (4.28-26.83)	17.00 (6.60-50.70)	21.70 (7.33-50.25)*†
IL-2 (pg/mL)	1.96 (0.50-6.00)	1.70 (0.65-3.20)	1.60 (0.40-7.80)	1.65 (0.18-8.10)
IL-10 (pg/mL)	5.30 (1.40-19.83)	4.25 (1.78-21.60)	3.50 (0.10-24.55)	8.25 (0.95-22.03)
IL-17 (pg/mL)	5.75 (1.15-26.73)	3.20 (0.10-16.58)	4.50 (1.03-14.13)	10.20 (3.63-66.40)*

Values are presented as median (interquartile). T1: less than 25% (<22 min) of WIT; T2: between 25 and 75% (22-70 min) of WIT; T3: more than 75% (>70 min) of WIT; IFN: interferon; IL: interleukin; WIT: warm ischemic time. *$P < 0.05$ *versus* T1; †$P < 0.05$ *versus* T2.

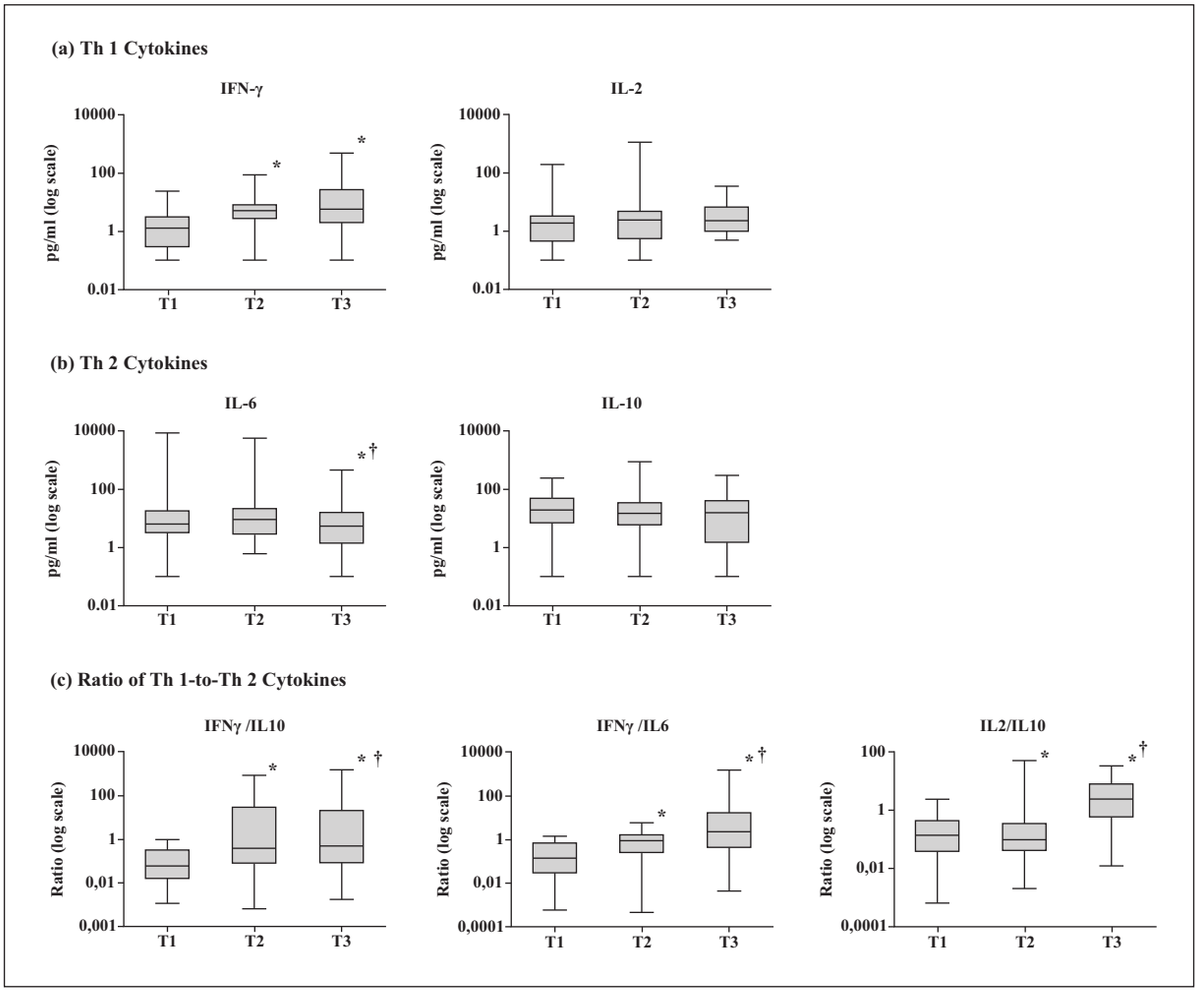

Figure 1
Level of cytokines and ratio of T helper 1 cell and T helper 2 cell cytokines post-operatively according to dichotomized warm ischemic time. Horizontal line; median, box; interquartile range; vertical bar, range. *$P < 0.05$ *versus* T1; † <0.05 *versus* T2. **a)** Th1 cytokines, **b)** Th2 cytokines, **c)** ratio of Th1-to-Th2 cytokines.

It can provoke damage of grafts by leading the ischemia as increasing the metabolism of grafts under normo-temperature than hypo-temperature.

The results of the present study-related IT showed that the duration of WIT had a significant effect on the recipient's immunologic status; however, CIT did not. The only WIT is correlated positively with three cytokines: IFN-γ, IL-2, and IL-17. In the previous literature focused on IT, there have been reports of cadaveric donor regarding the relationship between outcomes such as survival rate, and IT. The literature on cadaveric donor LT deals with CIT more important than WIT [16, 17]. It is contrary to this study's result. Another literature showed WIT is correlated with the duration of intensive care and graft survival, and only prolonged CIT (more than 18 h) is correlated with the duration of hospital stay [11]. As described above, the previous literature showed a different result compared to the results of the present study about IT. The reason is considered as the CIT as published previously is relatively longer than LDLT due to the characteristics of process nature of the cadaveric donor. For example,

one of the previous literature suggests that CIT has been gotten significance in the situation of prolonged CIT of more than 18 h. Otherwise, the CIT in our results showed within 4 h because of donor's characteristics of process in LDLT. In the cases of LDLT, the duration of CIT is not long. Therefore, WIT should be considered a more important factor that affects the immune responses than CIT as shown in our study, which is not the same as that in previous literatures. In the present study's results, the CIT, which is the preparation period of the liver grafts, was found to be significantly different among individual patients and the effect on recipient immune responses was not significant. However, in the case of WIT, IFN-γ, IL-2, and IL-17 were found to increase as the duration of WIT prolongated. This result contains that the immune responses in the CIT period of LDLT are insignificant due to decreasing metabolism in low temperature within limited time such as the situation of prolonged CIT likely to the cadaveric donor, while the immune responses in the case of WIT are activated and it affects the immune status of the recipient in the WIT period. As detailed individual cytokines, IFN-γ and IL-2 are the pro-inflammatory cytokines which are acknowledged to be the role of biomarkers in chronic liver diseases [18]. Therefore, pro-inflammatory cytokines such as IFN-γ, and IL-2 triggered by WIT are forced to increase recipient's inflammatory process, and the increasing inflammatory process would lead to reducing the function of graft livers, which results in poor outcomes of LT eventually. IL-17 was also increased by increasing the duration of WIT. IL-17 is as a pro-inflammatory cytokine and the biomarker of inflammatory responses released by Th17. Eventually, increase in IL-17 is meant to increase in inflammatory process [19, 20]. This result supports the claim that prolonged WIT leads to an increase in the inflammatory process. Therefore, we confirm that more than 70 min of WIT, as the upper quartile of WIT, triggers an inflammatory process in the recipients of LDLT, based on the results of our present study.

From the viewpoint of classical methods for estimating inflammatory status, the WBC differential counts and C-RP were used as a classical method for confirming the immunological status. C-RP showed a positive correlation with pre- and post-operative IL-6, IL-10, and IL-17. IL-6 and IL-17 are as pro-inflammatory cytokines; otherwise, IL-10 is as an anti-inflammatory cytokine [21]. IL-17 is well known as the biomarker of inflammation even it is released by Th17 and triggers a release of pro-inflammatory cytokines which led to inflammatory process eventually [22, 23].

Recently, cytokines, mediators of immune responses, have been used for evaluating the immunological responses. Cytokines are classified into pro- and anti-inflammatory cytokines depending on their action, and Th cells, one of the immunology-related cells, release their specific pattern of cytokines; they produce and evoke a specific effect. For example, Th1 cells secrete IFN-γ and activate cytotoxic T lymphocytes and macrophage *via* the action of IFN-γ. Th1 cells promote protective cell-mediated immune responses that may result in fewer post-operative infections. Alternatively, Th2 cells, which produce IL-4, IL-6, and IL-10, drive B cells to differentiate and produce immunoglobulins and are associated with humoral or antibody-mediated immunity [24, 25]. Therefore, the polarization of Th cells toward either a Th1 or a Th2 can significantly influence subsequent immune responses [26]. In this study, WIT increased Th1 cytokine activity compared to Th2 cytokine according to the ratio of Th1-to-Th2. This means that prolonged WIT led to an increase in the proinflammatory activity, and it is supported that IL-17 showed a higher concentration with prolonged WIT in this study [20]. Therefore, the increase of Th1 cytokines is highly likely to affect the function and survival of liver grafts negatively.

Our study has some limitations about the interpretation of our results. First, we measured levels of Th1 and Th2 cytokines in peripheral blood mononuclear cells using ELISA for distinguishing between a Th1 and Th2 response. Some cytokines assessed are produced by a variety of cell types including macrophages, monocytes, Th1 and Th2 cells, and endothelial cells, etc. Therefore, whether a Th1 or Th2 response is predominant should be confirmed by flow cytometry. In the previous literature, however, the method of distinguishing between Th1 and Th2 cytokines was used by the method of measurement of Th released cytokines instead of flow cytometry. Serum concentration of cytokines is more advantageous in terms of the process and cost of the method [27-30]. Second, WIT has a meaningful affecting factor to allografts under a limited situation in cases with a short CIT such as LDLT. LT with cadaveric donation or prolonged CIT (>8 h) is not appropriate to apply the present study's results.

In conclusion, the longer WIT (more than 70 mins) in LDLT is more likely to induce more immunological reactions of recipients, which shifts pro-inflammatory activity. It leads to a deleterious Th1-to-Th2 cytokines balance by reinforcing Th1 cytokines releases. Therefore, it is necessary to try to reduce WIT as much as possible in LDLT for improving the outcome of LDLT. It is definitely a concern to physicians who are proceeding LT. A long-term study of the effect of WIT-mediated immunological responses on the prognosis or outcomes of LT is needed in the future.

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