

Relationship between immune function recovery and infectious complications in patients following living donor liver transplantation (running title: Recipient immune function recovery after LDLT)

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Abstract

Background and Aim

The ImmuKnow (IK) assay enables the evaluation of peripheral blood CD4+ adenosine triphosphate activity to facilitate an objective assessment of the cellular immune function in immunosuppressed patients. However, it is unclear whether the IK assay is utilized during the acute postoperative periods following living donor liver transplantation (LDLT).

Methods

The IK values of 43 LDLT recipients were measured during the month following LDLT to evaluate the relationship between the measured IK values and infectious events.

Results

The IK values after LDLT were significantly increased compared with the IK values before LDLT ($P < 0.01$). During the month following transplantation, the rate of bacterial infection in the recipients with IK values > 225 ng/mL was significantly lower than that in the recipients with IK values ≤ 225 ng/mL (42.1% vs. 91.7%, respectively, $P < 0.01$). The rate of severe infections among the recipients who

maintained IK values > 150 ng/mL was significantly lower than that among the recipients with IK values ≤ 150 ng/mL during the month following transplantation (3.7% vs. 56.3%, respectively, $P < 0.01$).

Conclusions

The immune system of LDLT recipients dramatically improved following transplantation. The IK values of LDLT recipients were associated with the incidence of infectious events during the perioperative period after LDLT. Monitoring IK values was useful during both the acute and long-term postoperative periods.

Introduction

In patients with end-stage liver disease that is considered to be an indication for living donor liver transplantation (LDLT), immune dysfunction may be present due to long-term malnutrition or pancytopenia due to hyper-splenism.¹ LDLT recipients must also take immunosuppressants to prevent acute rejection even though infection is a major cause of death during the acute postoperative period.^{2, 3} In most institutions, the immunosuppressant dosage is determined based on therapeutic drug monitoring (TDM). However, TDM does not always reflect the effects of immunosuppression due to the lack of an objective immune function evaluation. To improve the management of LDLT recipients, a better parameter is required.

Kowalski *et al.* reported the utility of the ImmuKnow (IK) assay (Cylex Inc., Columbia, MD, USA) for postoperative management following organ transplantation.⁴ The IK assay uses phytohemagglutinin (PHA) to stimulate lymphocyte activation. Most immune cell functions depend on a cellular energy supply; thus, the assay is designed to measure the increase in intracellular adenosine triphosphate following

mitogen activation or antigenic or allogenic stimulation. The IK values were not related to the TDM of calcineurin-inhibitor drug.⁵⁻⁷ The efficacy of IK values has been reported for the late phase following liver transplantation.⁷⁻¹⁰ However, it still remains unclear how IK values change after LDLT. Furthermore, the efficacy of the IK assay during perioperative periods for LDLT recipients remains insufficient. In this study, we examined perioperative IK values in LDLT recipients and evaluated the relationship between IK values and clinical outcomes, which were primarily the occurrence of bacterial and fungal infections.

Patients and Methods

Study design

Between May 2010 and May 2013, 65 adult patients underwent LDLT at Nagasaki University Hospital. Blood samples for the IK assay were obtained from 43 of these patients prior to LDLT. The IK assay was also conducted in 21 healthy volunteers, who served as the control group (Figure 1). The IK values of the LDLT recipients

were chronologically measured at postoperative weeks 1, 2, 3, and 4. The IK values were also evaluated during infectious events.

This study protocol was approved by the institutional review board of Nagasaki University Hospital, and informed consent was obtained from each recipient and healthy volunteer.

Demographics of the living donor liver transplant recipients

The 43 LDLT recipients consisted of 25 males and 18 females with a mean age of 57.6 \pm 7.6 years. The indications for LDLT were as follows: type C cirrhosis in 26 patients; type B cirrhosis in 5 patients; non-B, non-C cirrhosis in 4 patients; primary biliary cirrhosis in 4 patients; Wilson's disease in 1 patient; alcoholic cirrhosis in 1 patient; acute liver failure in 1 patient; and multiple liver metastases of a rectal carcinoid in 1 patient. The patient demographics are summarized in Table 1. Splenectomy was performed in 33 patients with severe thrombocytopenia (platelet counts < 50,000/ μ l) or type C liver cirrhosis prior to postoperative interferon therapy. Splenectomy had been performed in 3 patients prior to LDLT.

Immunosuppressive protocols

As a basic perioperative immunosuppressive protocol, bi-therapy with tacrolimus and steroids was administered to most patients. A steroid bolus was administered intraoperatively. Tacrolimus was initiated on postoperative day (POD) 1 while tapering the steroid dosage. The target trough level of the orally administered tacrolimus was between 10 and 15 ng/mL within the first month following LDLT and 5-10 ng/mL thereafter. The corticosteroid was gradually decreased within the first three to four months. The immunosuppressant doses were adjusted after considering the clinical course and blood tacrolimus trough levels, regardless of the IK values. For patients with severe renal dysfunction, an interleukin-2 receptor inhibitor (basiliximab) was also administered on days 1 and 4 without a calcineurin inhibitor. Adjunctive mycophenolate mofetil (MMF) was also administered. When triple therapy consisting of tacrolimus, MMF, and steroids was administered, the target trough level of tacrolimus was adjusted to 5-10 ng/mL.

Postoperative bacterial and fungal infections

Post-transplant bacterial and fungal infections were defined as cases that required an additional antibacterial or antifungal agent due to a positive culture after the discontinuation of antibacterial prophylaxis. Severe infections were defined as cases requiring mechanical ventilation or surgical drainage with septic shock.

IK measurement

The perioperative immune status was evaluated using the IK assay. The ImmuKnow assay protocol has been described in detail elsewhere.^{2, 4} Briefly, 250 μ L of whole blood with 750 μ L of sample diluent was dispensed in 100- μ L aliquots to 4 wells of a 96-well microtiter plate and was incubated for 15–18 hr with PHA in a 37°C, 5% CO₂ incubator. The CD4⁺ T-cells were positively selected within the microwells using magnetic particles coated with anti-human CD4 monoclonal antibodies (Dynabeads, Dynal, Oslo, Norway) and a strong magnet (Cylex Magnet Tray 1050, Cylex Inc., Columbia, MD). The selected CD4⁺ T-cells were lysed to release intracellular ATP. Released ATP was measured using luciferin/luciferase and a luminometer (Berthold, Knoxville, TN or Turner Biosystems, Sunnyvale, CA). The

concentration of ATP was determined from the relative light units of an ATP calibration curve according to the IK data analysis calculator. The concentrations were averaged after outliers were excluded.

Evaluating the recovery of post-transplant IK values related to infections

We evaluated the relationship between the chronological changes in post-transplant IK values and infections during the month following LDLT. According to a previous report by Kowalski et al., the immune cell response can be stratified as strong (≥ 525 ng/mL), moderate (226-524 ng/mL), or low (≤ 225 ng/mL).⁴ Consequently, the 225 ng/mL IK value was defined as the cut-off value in the immunosuppressive group. The patients were divided into 2 groups according to the IK value at 2-4 weeks after LDLT to evaluate the recovery of IK values following post-LDLT bacterial and fungal infections. The low IK group demonstrated low IK values (≤ 225 ng/mL) at least once within the 2-4 weeks after LDLT, and the remaining group underwent an evaluation to determine the sensitivity of the IK values to the bacterial and fungal infections.

Evaluating the recovery of post-transplant IK values relative to severe infections

We evaluated the relationship between chronological changes in the post-transplant IK values and severe infections during the month following LDLT.

The 150 ng/mL IK value was defined as the cut-off value based on receiver operating characteristic curves. The patients were divided into 2 groups. The first group contained patients with an average IK value of 150 ng/mL in the 2-4 weeks following LDLT. This group was used to evaluate the recovery of IK values following LDLT. The second group contained patients who experienced severe post-transplant infections. Moreover, the patients were also divided into a very low IK group, who demonstrated IK values < 150 ng/mL at least once within the 2-4 weeks following LDLT, and the remaining group, who were evaluated to determine the sensitivity of the IK values to severe infections.

Statistical analyses

The ages, Child-Pugh scores, the end-stage liver disease (MELD) scores, operation time, intraoperative bleeding, and IK values of the patients are reported as means and

standard deviations. The Wilcoxon test or Student's t-test were used for non-categorical variables. Spearman's rank correlation coefficient was used to analyze ordinal qualitative variable. For the categorical analyses, Fisher's exact test or chi-squared test were used. One-way ANOVA with post-hoc Tukey HSD Test was used for comparing multiple groups. A P-value < 0.05 was considered to be significant. The SAS-JMP software program (Cary, North Carolina, USA) was used to perform all statistical analyses.

RESULTS

Comparison of the preoperative IK values

The median preoperative IK value of the 43 LDLT recipients was 48 ng/mL (range, 4-598), which was significantly lower than that of the 21 healthy volunteers (415 ng/mL) (range, 311-438) ($P = 0.01$) (Figure 1). The IK values of the male LDLT recipients prior to LDLT (79 ng/ml; range, 27-115 ng/ml) were significantly higher than those of the females (31.5 ng/ml; range, 15-54.5 ng/ml) ($P = 0.01$). There were no

significant relationships between IK values and age, DM, Child-Pugh score, or MELD score.

Post-transplant trends in the IK values

The IK value was 86.9 ± 113.9 ng/mL before LT and 204.7 ± 169.1 ng/mL at 1 week, $303.9.7 \pm 192.2$ ng/mL at 2 weeks, 302.8 ± 180.5 ng/mL at 3 weeks, and 309.8 ± 217.6 ng/mL at 4 weeks following LDLT. The IK values following LDLT significantly increased with time (Figure 2A). At 4 weeks following LDLT, the IK values of the patients who had undergone splenectomy were significantly higher than those of the patients who did not undergo splenectomy ($P < 0.01$) (Figure 2B). There were no significant relationships between IK values after LDLT and other factors such as age, gender, operation time, blood loss, DM, Child-Pugh score, MELD score, or pre-IK values.

IK values and postoperative bacterial and fungal infections

Bacterial and fungal infections within one month following LDLT were observed at a high rate (42 episodes in 30 patients, 69.8%). Positive bacterial cultures were detected in samples from blood ($n = 18$), sputum ($n = 12$), and the abdominal cavity ($n = 12$). Severe infections within one month following LDLT were observed in 10 patients (23.3%). There were no significant relationships between IK values after LDLT and other factors such as age, gender, operation time, blood loss, DM, Child-Pugh score, MELD score, or pre-IK values.

The recovery of postoperative IK values in the patients who developed bacterial and fungal infections was significantly slower than that in the remaining patients at 2 and 3 weeks ($P = 0.007$, $P = 0.014$) (Figure 3A). Based on the average postoperative IK values, the occurrence of bacterial and fungal infections significantly increased the IK values in patients with an IK value less than 225 ng/mL ($P = 0.001$) (Figure 3B). The rate of bacterial and fungal infections in the low IK group (91.67%) was significantly higher than that in the remaining group (45%) ($P < 0.001$) (Figure 3C).

The postoperative IK values in patients who developed severe infections were significantly lower than those in the remaining patients at 1 to 4 weeks ($P = 0.006$, $P =$

0.001, $P = 0.003$, $P = 0.001$) (Figure 4A). Based on the average postoperative IK values, the occurrence of severe infections was significantly increased in the group of patients with IK values less than 150 ng/mL ($P < 0.001$) (Figure 4B). The rate of bacterial and fungal infections in the very low IK group (56.3%) was significantly higher than that in the remaining group (3.6%) ($P < 0.001$) (Figure 4C). Representative cases from the very low IK group and the remaining group are presented in Figure 5.

The IK values significantly differed between the three groups (patients without infections ($n = 13$), patients with infections ($n = 20$), and patients with severe infections ($n = 10$) at 2 to 4 weeks after LDLT) (Figure 6).

Other than bacterial infections, cytomegalovirus infection (CMV) was recognized in 9 patients. Furthermore, biopsy-proven acute cellular rejection (ACR) was detected in 3 patients. However, the IK values were not significantly different between patients with CMV infections or ACR and patients without these events.

Discussion

This study demonstrated that the IK values dramatically improved in almost all LDLT recipients, and the improvements in the IK values differed due to bacterial and fungal infections during the acute perioperative periods. Specifically, the patients who developed severe infections demonstrated almost no improvement in their IK values. These results suggest that the IK assay could be useful during not only the postoperative period but also during the acute perioperative periods.

Because patients with liver cirrhosis are often immuno-compromised, various types of bacterial infections, such as spontaneous bacterial peritonitis, urinary infection, and pneumonia, can occur. Severe infectious diseases cause the high death rate during end-stage liver disease.¹ This study used the IK assay to objectively demonstrate the preoperative immunocompromised status in LDLT recipients with end-stage liver disease. Although the preoperative IK values in LDLT recipients were significantly lower than those in healthy volunteers, most recipients demonstrated recovery within a month after transplantation. Measuring the concentration of ATP in activated T cells is clinically useful for evaluating T-cell function.^{11, 12} Therefore, patients with end-stage liver disease who demonstrate significantly low ATP levels are in a state of

CD4+ T cell dysfunction. Although patients with end-stage liver disease often experience leukocytopenia, there was no relationship between the IK values and lymphocyte counts (data not shown). In patients with liver cirrhosis accompanied by hypersplenism, the balance of immune function often changes; the absolute number of T cells and the CD4/CD8 ratio decrease.^{13, 14} As helper T cells, CD4-positive T cells adjust the immune response. The risk of sepsis increases following abdominal surgery in human immunodeficiency virus (HIV) patients with a low number of CD4-positive T cells.^{14, 15} In this study, the IK values of the patients who developed severe infections were extremely low, even though the IK values of the other patients improved to the same level as that in healthy volunteers. At 4 weeks post-LDLT, the IK values in the patients who underwent splenectomy were significantly higher than those in the patients who did not undergo splenectomy, although LDLT without splenectomy was performed in only 5 patients. Splenectomy may improve portal vein hemodynamics and the function of CD4-positive T cells.^{16, 17}

In this study, only one patient experienced a postoperative decrease in IK values. This patient had multiple metachronous metastases from a rectal neuroendocrine tumor

and had normal liver function. All of the other patients with primary liver disease demonstrated a post-transplant increase in the IK values. This result demonstrated the efficacy of liver transplantation in liver transplant recipients with end-stage liver disease with respect to the recovery of their immune function. Goralzyk et al. also reported patients who had quickly recovered from preoperative pneumonia following liver transplantation.¹⁸

The postoperative increase in IK values in the present study explains the above-mentioned findings. Li et al. also reported an immunofunctional assessment based on IK values and CD4+ cell counts, and the CD4+/CD8+ ratio was useful for 2 weeks following whole liver transplantation.¹⁹ The immune function of the recipients was not sufficiently restored at 2 weeks following LDLT; however, the immune function in patients who undergo whole liver transplantation may be restored within this period. Thus, assessments of immune function after LDLT would be required for at least 4 weeks. A representative case is presented in Figure 5A.

Our study also demonstrated that IK value monitoring can be performed to effectively assess the risk of bacterial and fungal infections. The high rate of bacterial

blood infections (18/42 episodes) suggests that the immunosuppressive state caused the infectious morbidity. However, the IK values of the recipients with infections improved to nearly the same values in the no infections group for one month following LDLT. Thus, infectious morbidity without severe infections did not substantially affect IK recovery after LDLT, although low IK values may cause infectious morbidity without severe infections. Notably, severe infection and low IK values may influence each other, and there were continuous low IK values prior to and after severe infectious events. In the previous study, severe sepsis caused low IK values and the proliferation of CD4 T cells; however, non-septic patients experienced non-substantial changes in immunological functions.²⁰⁻²² In the aforementioned case (presented in Figure 5B), the post-LDLT patient developed postoperative pneumonia prior to the recovery of liver function, and the IK values remained low for 3 weeks. However, she recovered from the pneumonia, and the liver function and IK values also recovered.

It may be difficult to define a quantitative cut-off value to diagnose infection or rejection due to large inter- and intra-individual variations in the IK values, even among healthy individuals.^{5, 19} In our study, because the IK values in healthy

volunteers were similar to those found by Kowalski et al., we used the classification defined by Kowalski of low, moderate, and strong immune function according to the IK values. Because of the possible variations caused by the procedure or different environments in each facility, IK values could be an index based on semi-quantitative or qualitative evaluations rather than quantitative evaluations. It is necessary to perform constant quality control checks and assess the IK values of healthy people at each facility.

Biopsy-proven acute rejection within one month following surgery was recognized in only three cases. As in previous reports, a high IK value was identified in the patient with ACR during the long-term observation period following liver transplantation.^{8, 10, 23-25} This study evaluated a small number of patients, and further investigations are needed.

The limitation of this study is that it is difficult to intervene in the immunosuppressive drug management of bacterial infections during the initial postoperative periods. However, this prospective study demonstrated that the immune function following transplantation in LDLT recipients improved during the first month

and that the IK values of LDLT recipients were associated with bacterial infections during the early postoperative period following LDLT. The FK dose should be prospectively decided prior to infectious events according to the IK values. The IK values are another important parameter in determining the FK dose when reducing it in the treatment of severe sepsis.

In conclusion, the immune system of LDLT recipients dramatically improved following transplantation. The IK values of the LDLT recipients were associated with infectious event incidence during the perioperative period. Monitoring IK values was useful during both the acute and long-term postoperative periods. Thus, randomized controlled studies should be performed to assess the utility of IK-based intervention therapy employing individual doses of immunosuppressive drugs in an attempt to prevent bacterial infections during the perioperative period following LDLT.

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Figure legends

Figure 1. Comparison of the ImmuKnow values between healthy volunteer donors and living donor liver transplant recipients.

Small bars indicate the median IK values.

IK, ImmuKnow; LDLT, living donor liver transplantation.

*; $P = 0.01$

Figure 2. Chronological trends in the ImmuKnow values during the perioperative period in living donor liver transplant (LDLT) recipients.

A: Recovery of IK values following LDLT

B: Longitudinal changes in IK values in patients who did or did not undergo splenectomy

IK values are mean \pm standard error.

IK, ImmuKnow; Ope, operation; Pre, preoperative state; w, week.

*; $P < 0.01$ vs. Pre

** ; $P < 0.01$ vs. splenectomy (-)

Figure 3. Development of post-transplant bacterial and fungal infections according to IK values following living donor liver transplantation (LDLT).

A: The IK values following LDLT. IK value recovery was delayed in patients with bacterial and fungal infections.

Bars indicate the standard error.

B: The rate of bacterial and fungal infections among the patients with average IK values less than 225 ng/mL or not.

C: The rate of bacterial and fungal infections in patients with low IK values (≤ 225 ng/mL)

IK values are mean \pm standard error.

IK, ImmuKnow; Pre, preoperative state; w, week.

*; $P < 0.01$

Figure 4. Development of severe infections according to the IK values after living donor liver transplantation.

A: IK values following LDLT. Recovery of IK values was markedly delayed in patients with severe infections. Bars indicate the standard error.

B: The rate of severe infections among patients with average IK values less than 150 ng/mL or not.

C: The rate of severe infections among patients with and without low IK values (≤ 150 ng/mL).

IK values are mean \pm standard error.

IK, ImmuKnow; Pre, preoperative state; w, week.

**; $P < 0.05$ *; $P < 0.01$

Figure 5. IK value changes following living donor liver transplantation (LDLT) among the patients who did and did not develop severe infections.

A A 59-year-old man underwent LDLT for liver cirrhosis. In this patient, no

infectious events occurred during the first month following LDLT.

B A 62-year-old woman underwent LDLT for liver cirrhosis. In this patient,

postoperative pneumonia occurred during the 2 weeks following LDLT. IK values

quickly increased after the pneumonia was cured.

IK, ImmuKnow; Pre, preoperative state; w, week.

Figure 6. IK value changes of post-transplant infections with or without severe

infections according to IK values following living donor liver transplantation (LDLT).

IK values are the means \pm standard error.

IK, ImmuKnow; Pre, preoperative state; w, week.

**; $P < 0.05$ *; $P < 0.01$

Fig. 1

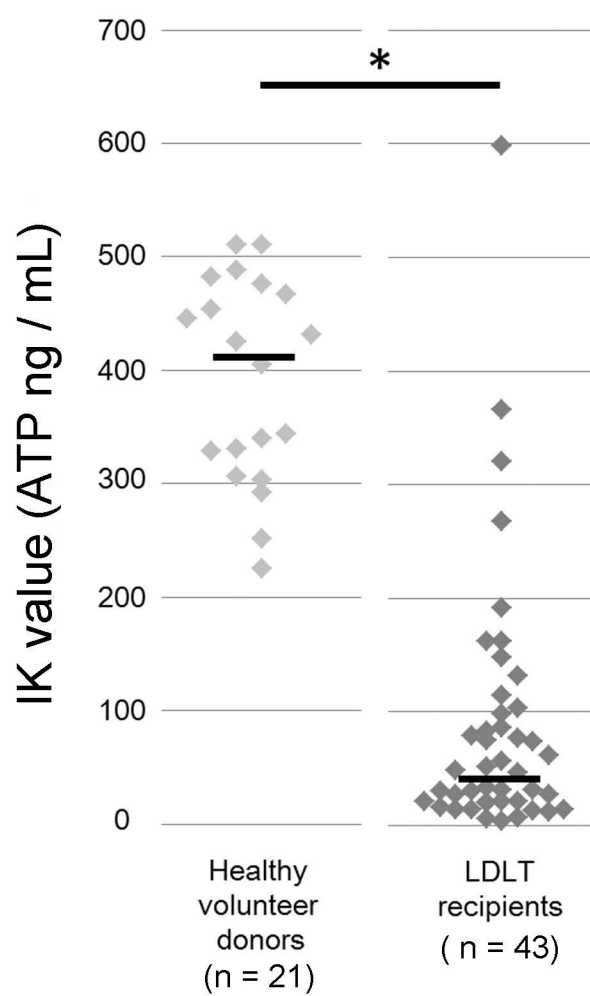
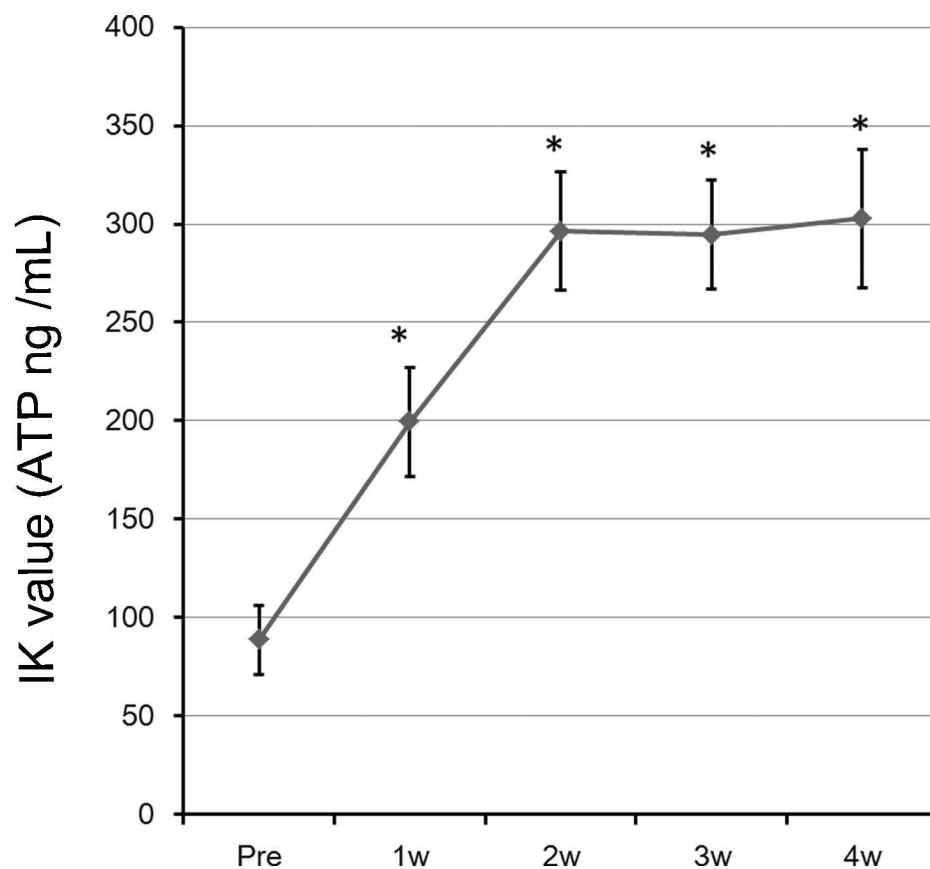
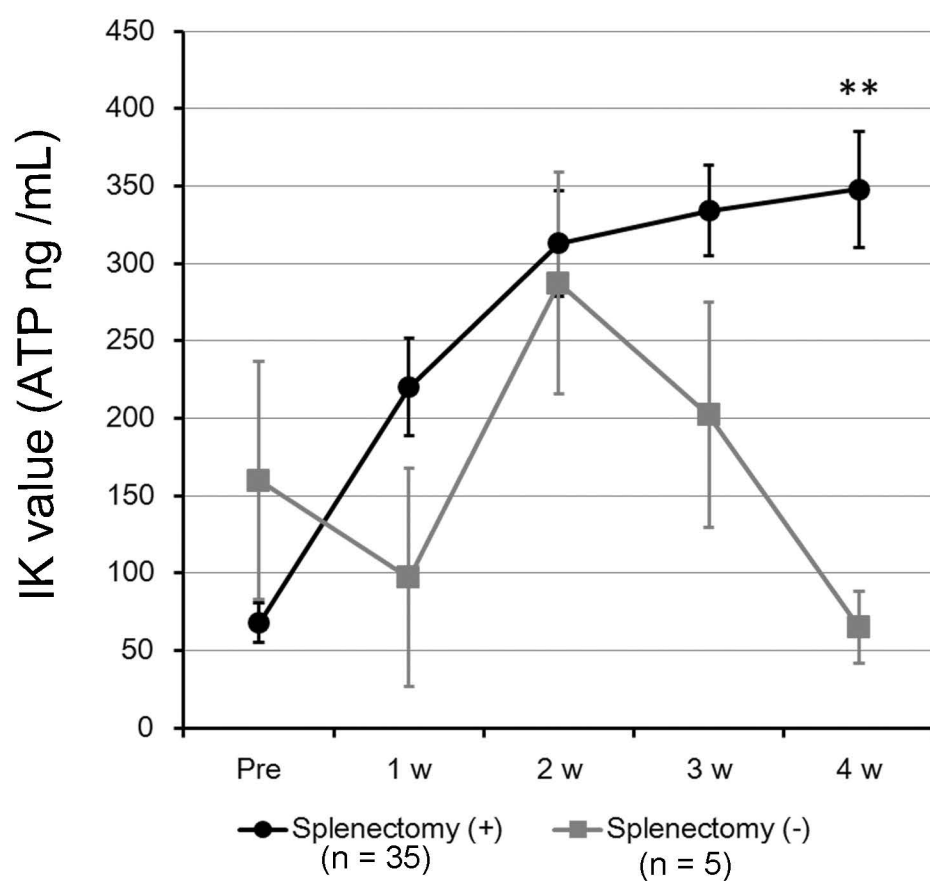


Fig. 2

A

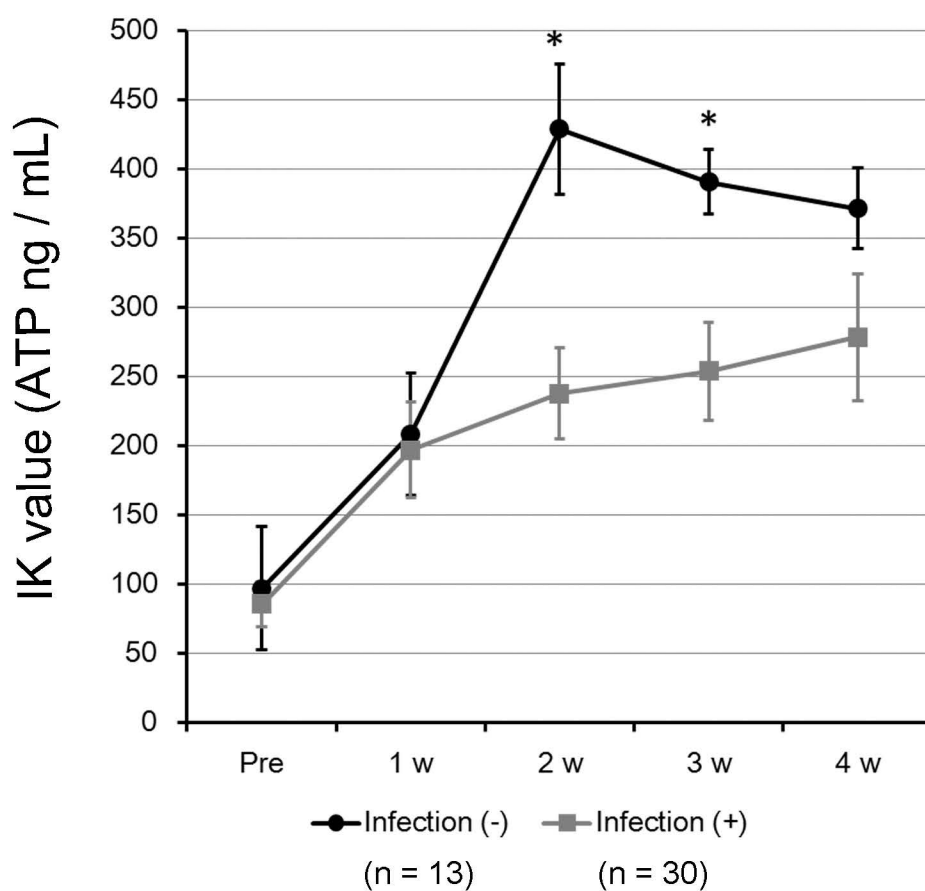
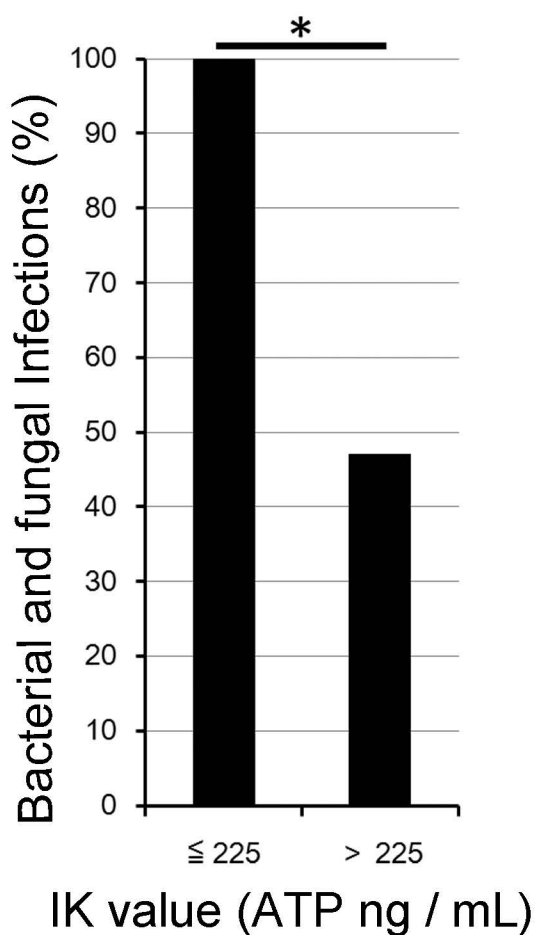
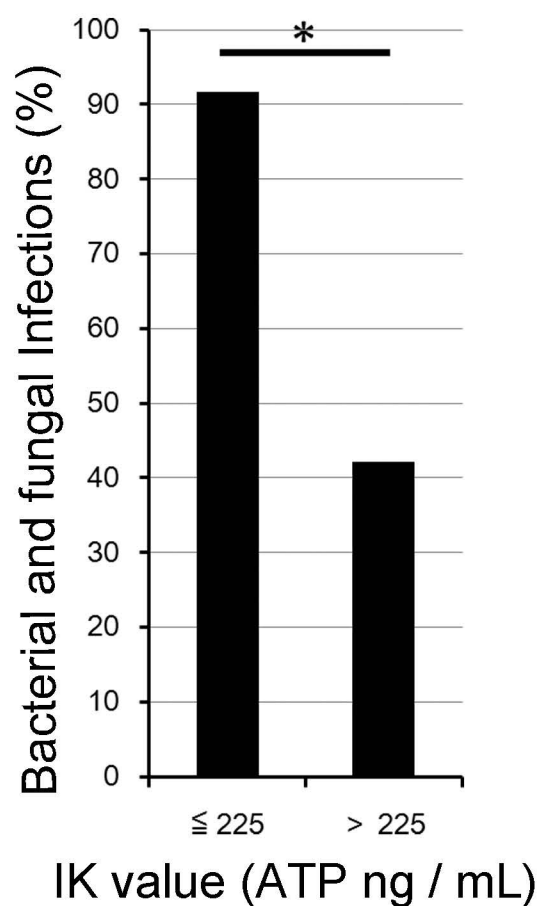


B



A

Fig. 3

**B****C**

A

Fig. 4

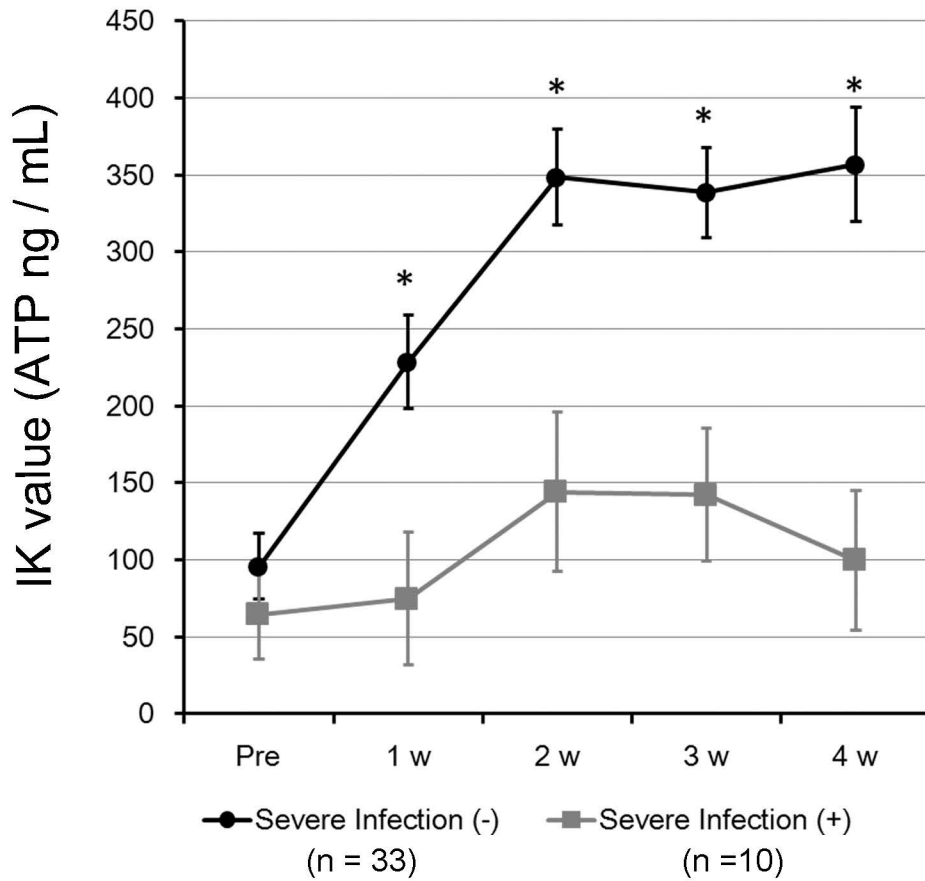
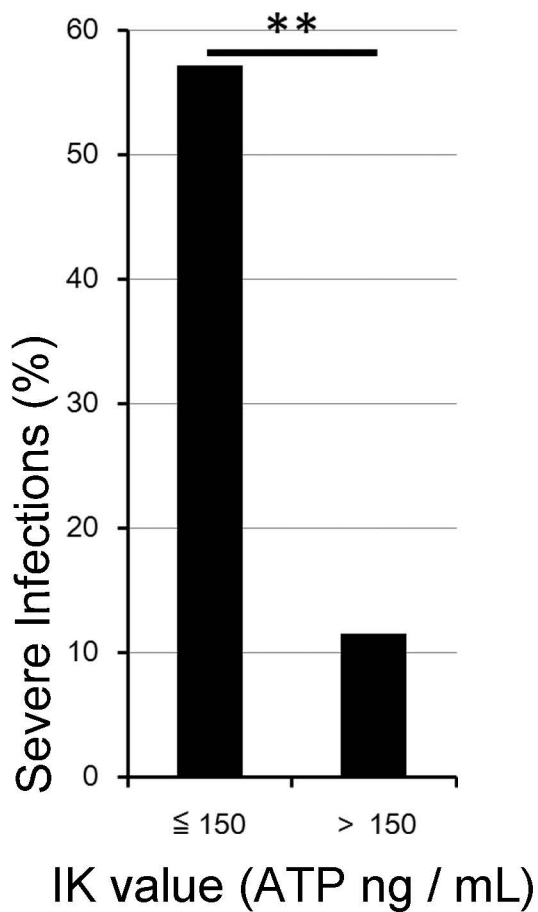
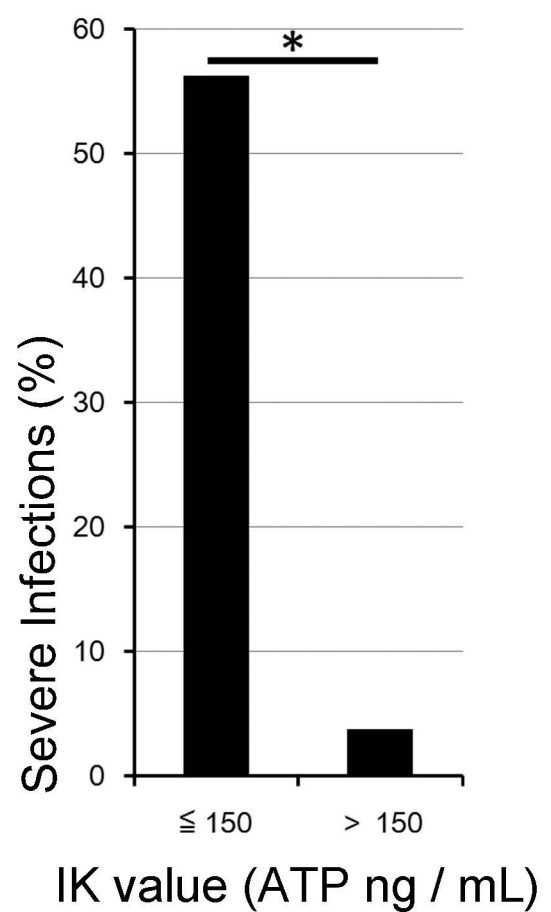
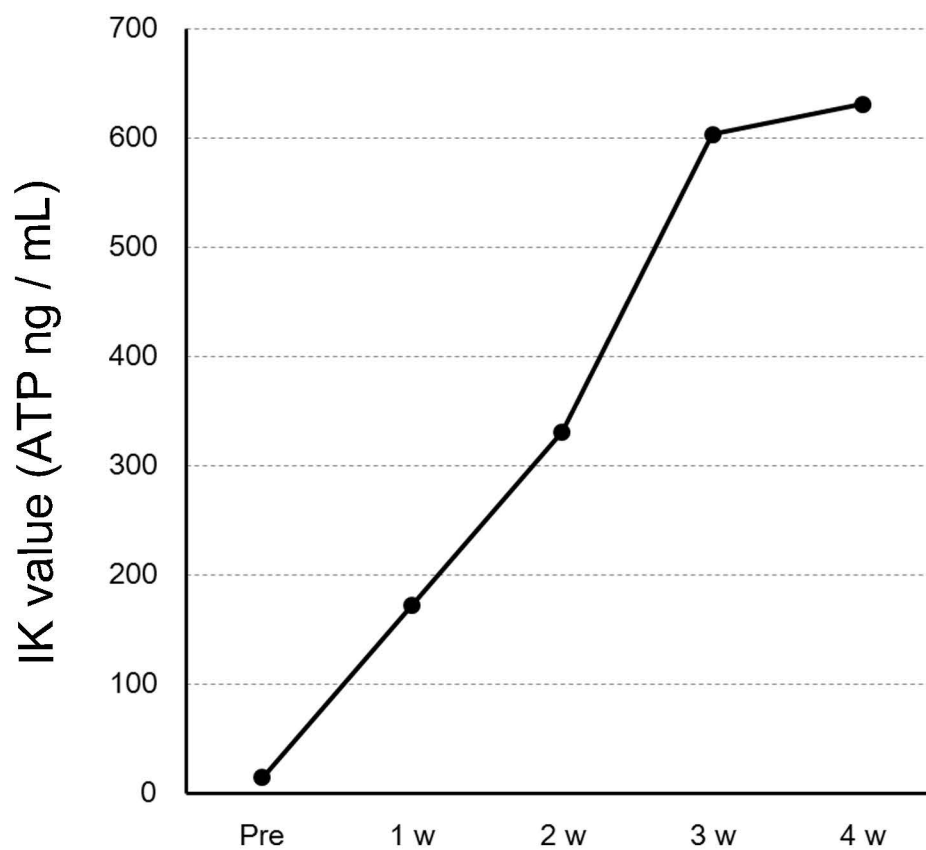
**B****C**

Fig. 5

A



B

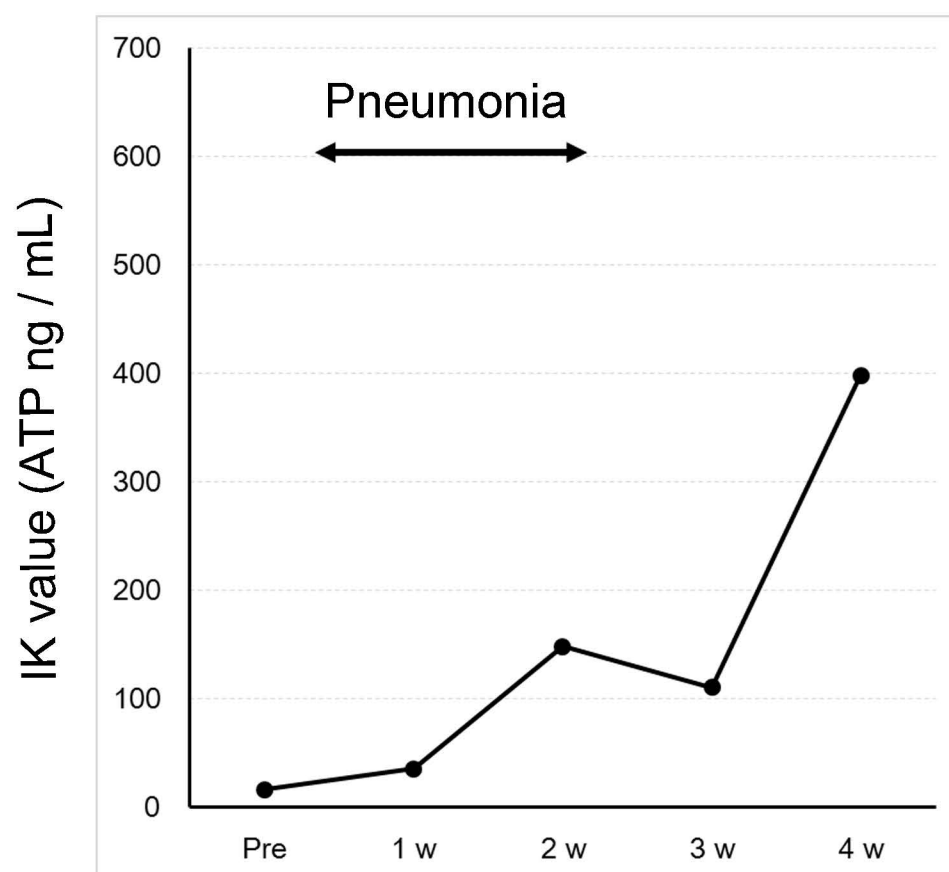


Fig. 6

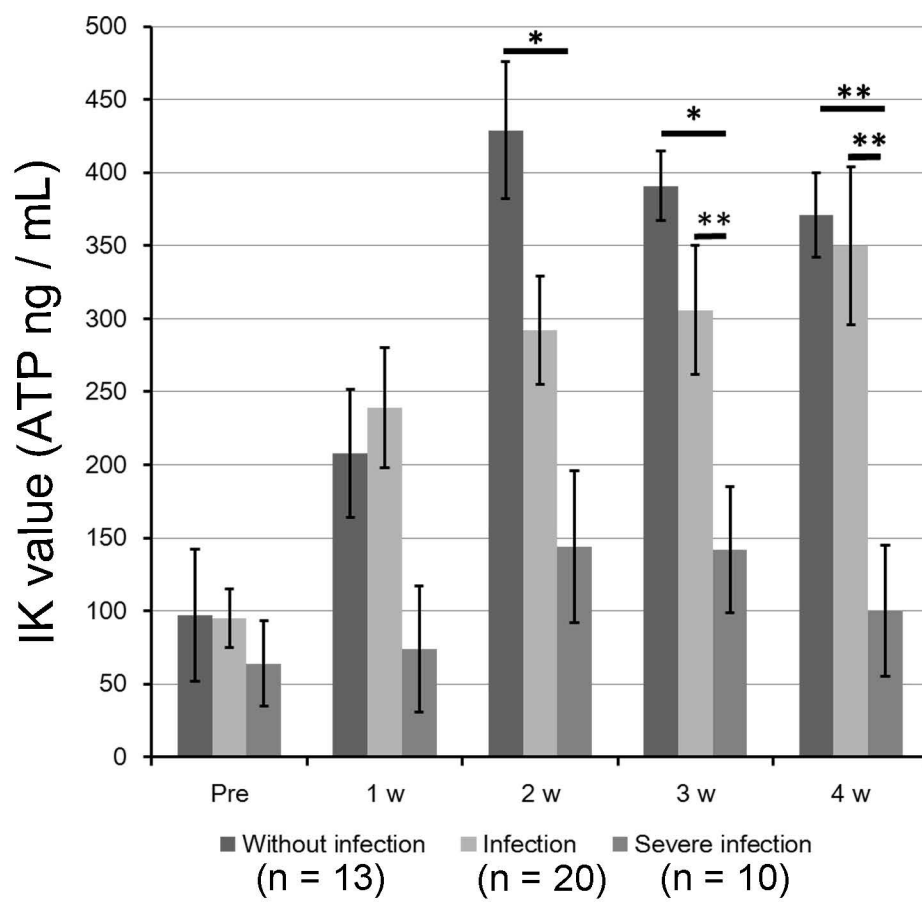


Table 1. Pre-transplant characteristics of the 43 LDLT recipients

Age (years)	57.6 \pm 7.6
Gender (male:female)	25/18
Primary disease	
Hepatitis B virus-associated liver cirrhosis	5
Hepatitis C virus-associated liver cirrhosis	26
Acute liver failure	1
Others	11
Child-Pugh score	10.0 \pm 2.3
MELD score	17.6 \pm 8.8
Incompatible blood type	13
Other viral carrier	
HIV infection	0
HTLV-1 infection	3
Splenectomy	36
ImmuKnow (ATP ng/ml)	88.6 \pm 114.7

LDLT: Living donor liver transplant

MELD : Model for End-Stage Liver Disease

HIV : Human immunodeficiency virus

HTLV-1 : Human T-cell leukemia virus type 1