Clinical relevance of a CD4+ T cell immune function assay in the diagnosis of infection in pediatric living-donor liver transplantation

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Abstract. The aim of the present study was to investigate the potential of the Immuknow immune cell function assay for the diagnosis of infection after pediatric living-donor liver transplantation (LDLT). Based on clinical data obtained following liver transplantation, 66 patients were divided into infection (n=28) and non-infection (n=38) groups. The following factors were considered in the present analysis: Primary disease, lymphocyte count, tacrolimus plasma concentration/dose (C_0/D) ratio, CD4+ T lymphocyte ATP levels, at pre-transplant stage and at weeks 1-4, and 2 and 3 months post-transplant. The CD4+ T lymphocyte ATP values were plotted in a receiver operating characteristic (ROC) curve. The CD4+ T lymphocyte ATP value of the infection group was significantly lower compared with that of the non-infection group (188.6±93.5 vs. 424.4±198.1 ng/ml, respectively; P<0.05). No correlation was observed between the ATP value and tacrolimus plasma C_0/D ratio (R^2=0.0001484); however, a correlation was reported between the ATP value and lymphocyte count (R^2=0.2149). Analysis of the ROC curve indicated that the ATP levels of CD4+ T cells were significantly associated with the diagnostic value of infection (area under the curve=0.866). These findings suggest that low CD4+ T lymphocyte ATP levels may be an independent risk factor for infection following pediatric LDLT, and that the Immuknow assay may be used as a tool to evaluate T lymphocyte function in such patients to predict the risk of infection.

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Introduction

At present, liver transplantation is one of the most effective treatments for pediatric end-stage liver diseases. With developments in surgical procedures and immune suppressive medication, the short-term and long-term survival rates of transplant recipients and grafts have markedly increased (1). However, infectious complications following pediatric liver transplantation have been noted as important causes of patient mortality, as they are major factors that affect survival (2). Therefore, early diagnosis and the treatment of infectious complications may markedly improve the survival of pediatric patients following liver transplantation.

Few studies (3,4) have monitored the immune status of pediatric patients after liver transplantation. The majority of investigations into patients' immune status after living-donor liver transplantation (LDLT) are conducted in adult patients. Patients receiving a liver transplant are administered immunosuppressants for life following the procedure; thus, it is critical to evaluate the post-transplant immune status of patients to aid clinical management. Monitoring the blood concentration of immunosuppressants is a method used to indirectly determine the immunological status of patients at post-transplantation stage, but this does not reflect their actual immune status. The Immuknow cell function assay may be used to measure CD4+ T lymphocyte ATP levels and directly monitor the immune status of patients. A number of studies revealed that bacterial and viral infections may suppress the function of CD4+ T lymphocytes in adult patients following liver transplantation (5,6). However, the clinical association between CD4+ T lymphocyte ATP levels and infection in pediatric LDLT remains unknown. The present study aimed to evaluate the clinical relevance between the value of CD4+ T lymphocyte ATP levels and post-transplant infection for pediatric patients who have undergone LDLT, based on the retrospective analysis of data from pediatric post-transplant patients with or without episodes of infection.

Materials and methods

Patients and samples. A retrospective analysis of 66 pediatric LDLT patients from the Tianjin First Central Hospital, enrolled between June and December 2017, was conducted. All patients who received liver transplantation were aged 0-3 years,

and were administered post-transplant immunosuppressive agents, including tacrolimus, mycophenolate mofetil and methylprenol. The exclusion criteria included treatment with tacrolimus for <3 months or administration of other immunosuppressive agents during the course of treatment, secondary liver transplantation or combined organ transplantation, and follow-up of <3 months (Table I).

Based on whether the patients were diagnosed with infection post-liver transplantation, the patients were divided into the infection group (28 cases; 19 male and 9 female) and the non-infection group (38 cases; 13 male and 25 female). The original diagnosis was for congenital biliary atresia. The criteria of diagnosis for infection were systemic inflammatory response syndrome-positive suspect lesions or infectious agent-positive (pathogenic microorganisms, imaging examination or biopsy), as well as effective anti-infection treatments (7).

Analysis of peripheral blood CD4+ T lymphocyte ATP levels. Assays were conducted according to the manufacturer's protocol (Cylex, Inc.). Briefly, 2-5 ml whole blood was collected from fasting patients into heparin sodium anticoagulant tubes, followed by addition of 25 μl phytohemagglutinin solution (Cylex, Inc.) and incubation for 15-18 h at 37˚C in a 5% CO_2 incubator. After incubation, 50 μl anti-CD4 monoclonal antibody coupling magnetic beads (Cylex, Inc.) were added to isolate CD4+ T lymphocytes. After washing (200 μl/time, three times; Cylex, Inc.), the cells were lysed (Cylex, Inc.) and the released intracellular ATP was measured by luminometry using the luciferin/luciferase mixture (Cylex, Inc.). The concentration of ATP was calculated from a calibration curve generated with calibrators (0, 1, 10, 100 and 1,000 ng/ml).

Blood tacrolimus monitoring. The whole blood samples of 200 μl were fully mixed with 200 μl whole blood precipitators and centrifuged (9,500 x g; 4 min; room temperature). The concentration of tacrolimus in blood was determined via a fluorescence polarization immunoassay (Abbott, Inc), and the concentration/dose (C_0/D) was calculated based on the administered dose.

Clinical data collection. A total of 462 peripheral blood samples were collected from patients with LDLT pre-transplant, as aforementioned, and at 1-4 weeks and 2 and 3 months post-transplant. The ATP values of CD4+ T cells and tacrolimus concentration were determined for each specimen. The serum C_0/D ratio was calculated. The lymphocyte counts were detected using a Sysmex hematology analyzer (XS-800i; Sysmex, Inc.).

Statistical analysis. Statistical analysis was performed using SPSS (version 20.0, IBM Corp.) and GraphPad Prism (version 5.0; GraphPad Software, Inc.) software. The normality test and homogeneity test of variance were applied to the continuous variable data, and a Student's t-test was used to analyze data with normal distribution. The results were expressed as the mean ± standard deviation. For data that did not have normal distribution, a Mann-Whitney test was conducted and data were presented as median values (interquartile range). Pearson's correlation analysis was used to determine the association between tacrolimus blood concentration and ATP levels. Receiver operating characteristic (ROC) curves were generated to analyze decreases in ATP levels to predict the sensitivity and specificity of infection. P<0.05 was considered to indicate a statistically significant difference.

Results

Comparison of cellular immunity responses between the infection and non-infection groups. The pre-transplant CD4+ T lymphocyte ATP levels of the 66 children who underwent liver transplantation were 302.5±195.7 ng/ml. The post-transplant CD4+ T lymphocyte ATP levels were 188.6±93.5 and 424.4±198.1 ng/ml for the infection and non-infection group, respectively. The ATP levels of the infection group were significantly lower compared with those of the non-infection group (P<0.05; Fig. 1). In addition, the ATP levels of the infection group significantly decreased after infection. (P<0.05; Fig. 2).

Analysis of the types of infection. A total of 28 LDLT patients displayed different degrees and sites of infection that occurred between 2 weeks and 6 months post-transplantation. Among these patients, 14 cases of pulmonary infection (50%), 4 cases of intra-abdominal infection (14.3%), 6 cases of bloodstream infection (21.4%) and other infections (14.3%) were reported. Bacteriological analysis revealed 6 cases of *Klebsiella pneumoniae*, 6 cases of *Enterococcus faecium*, 4 cases of *Acinetobacter baumannii* and 3 cases of *Staphylococcus epidermidis*. Fungal infections included 1 case of *Stenotrophomonas maltophilia*, 1 case of *Candida parapsilosis*, 1 case of *Candida tropicalis* and 1 case of *Candida albicans*. Viral infections included 4 cases of cytomegalovirus and 1 case of Epstein-Barr virus (Table II).

Immuknow analysis of ATP levels and immunosuppressant serum trough C_0/D ratio correlation analysis. A total of 396 peripheral blood samples were collected from 66 patients at weeks 1-4, and at 2 and 3 months following liver transplantation. The trough tacrolimus (FK506) C_0/D ratio and ATP levels were determined at the same time. The mean FK506 C_0/D ratio was 5.6±3.8 ng/ml and the mean ATP value was 313.9±195.1 ng/ml. No correlation was observed between the trough tacrolimus C_0/D ratio and ATP levels (P>0.05, R^2=0.0001484; Fig. 3).

Correlation between CD4+ T cell ATP levels and lymphocyte count. The ATP levels of the 66 children with LDLT were measured using 396 samples obtained at weeks 1-4, and at 2 and 3 months post-transplantation. The mean ATP levels was 313.9±195.1 ng/ml and the mean lymphocyte count was 6.05±3.6x10^9/l. Correlation analysis revealed a positive correlation between the CD4+ T cell ATP levels and the total lymphocyte count from each specimen (P<0.05, R^2=0.2149; Fig. 4).

ROC curve analysis for the sensitivity and specificity of infections. A ROC curve was generated to determine a reference ATP level for the diagnosis of infection. The results revealed that, when ATP levels were set at 200.5 ng/ml for the ROC curve analysis of patients diagnosed with infection, the sensitivity and specificity were 89.5 and 64.3%, respectively; the area under the ROC curve was 0.867 (Fig. 5).

Table I. Characteristics of pediatric living-donor liver transplantation recipients (n=66).

Characteristics	Data at transplantation
Age [mean ± SD (range)]	9.3±11.7 (5-36)
0-6 months, n (%)	28 (42.4)
7-12 months, n (%)	24 (36.4)
1-2 years, n (%)	9 (13.6)
2-3 years, n (%)	5 (7.6)
Sex, n	
Male/female	34/32
Blood type combination, n	
Identical/compatible/incompatible	41/15/10
Follow-up period, years [mean ± SD (range)]	1.6±0.9 (0.8-3.4)
Primary diagnosis of recipient	
Congenital biliary atresia and biliary cholestatic cirrhosis	63
Alagille syndrome	1
Budd-Chiari syndrome	1
Methylmalonic acidemia and liver cirrhosis	1

SD, standard deviation.

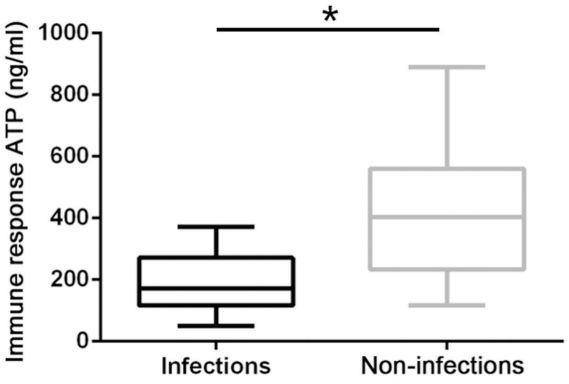

Figure 1. Immuknow assay for the comparison of ATP levels between the infection and non-infection groups. *P<0.05

Discussion

In the present study, immune cell function following transplantation was evaluated by determining the function of the transplanted organ, using detection of the lymphocyte subsets, and the blood concentration of immunosuppressants; however, the specificity and sensitivity of these two methods for the post-transplantation evaluation of cellular immune function are low (8). As a technique to evaluate cellular immune function, the clinical value of the Immuknow assay lies with permitting assessment of the risk of infection or rejection in transplant recipients.

Table II. Specific types of bacterial, fungal and viral infections following pediatric liver transplantation.

Type of infection	Number of examinations
Bacterial	19
Klebsiella pneumoniae	6
Enterococcus faecium	6
Acinetobacter baumannii	4
Staphylococcus epidermidis	3
Fungal	4
Stenotrophomonas maltophilia	1
Candida parapsilosis	1
Candida tropicalis	1
Candida albicans	1
Viral	5
Cytomegalovirus	4
Epstein-Barr virus	1

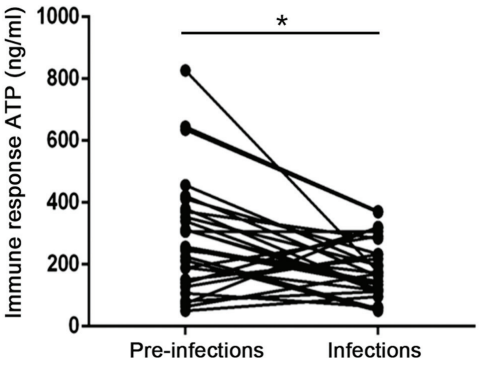

Figure 2. Immuknow assay for the ATP levels of the infection group before and after infection. *P<0.05.

The ATP levels of LDLT patients in the infection group were found to be significantly lower compared with those in the non-infection group; there was a statistically significant difference in the ATP levels pre- and post-transplant in the infection group. In the present study, low values on the T cell immune function assay were associated with the susceptibility to infection; however, controversial findings have been reported (9,10). In the present study, the ATP levels in the infection group were significantly lower compared with the non-infection group. Kobashigawa *et al* (11) and Mandras *et al* (12) analyzed the post-transplant Immuknow assay-derived data of heart transplant recipients, and revealed that the mean ATP levels of patients at the time of infection were significantly lower compared with those in patients without infection. Naderi *et al* (13) conducted an Immuknow analysis using samples from 113 kidney transplant patients and reported that the intracellular ATP concentrations were significantly lower in patients who suffered from infection vs. the renal transplant recipients with stable graft function. Of note, the iATP levels were increased in those that had experienced an episode of allograft rejection. Additionally, the Immuknow assay has

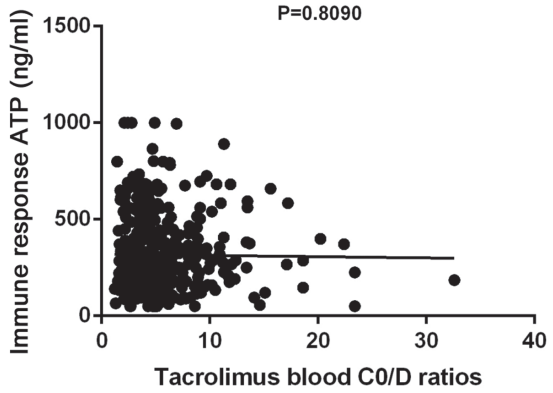

Figure 3. Correlation analysis between tacrolimus blood C_0/D ratio and Immuknow ATP levels. No correlation was observed between the two (P=0.8090).

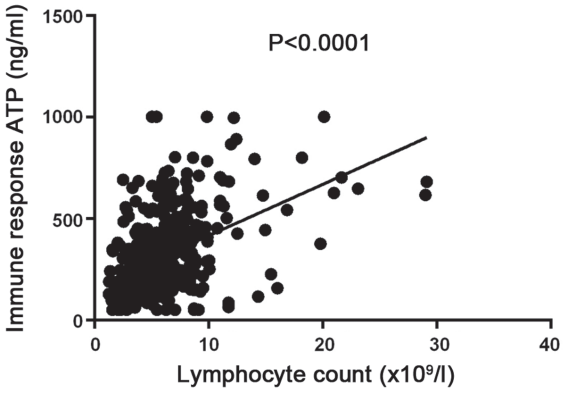

Figure 4. Correlation between lymphocyte count and Immunknow ATP levels. Positive correlation was observed between the CD4+ T cell ATP levels and the total lymphocyte count (P<0.0001).

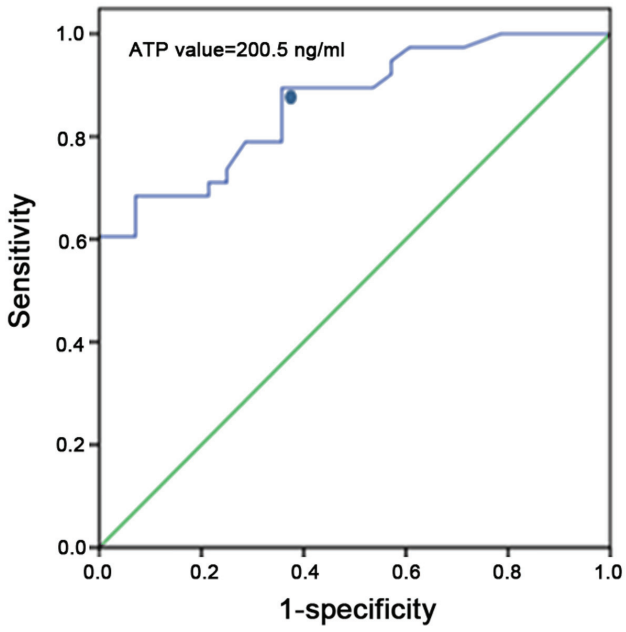

Figure 5. Receiver operating characteristic curve for predicting infections.

been proposed to predict the risk of post-transplant infection more effectively than predicting rejection (14).

In the present study, a variety of bacterial, fungal and viral infections were observed in the infection group (Table I). Regarding the diagnosis of bacterial infections, positive etiological identification may aid analysis; however, the rate of positive bacterial culture is low, time consuming, and specimens are not easily obtained, whereas other factors notably inhibit correct and timely diagnosis. Chiereghin *et al* (15) analyzed 98 symptomatic infections in 202 transplant patients, retrospectively within 1 year post-operation. The results revealed 77 (57.1%) bacterial, 45 (33.3%) viral and 13 (9.6%) fungal infections, with the bacterial infections mainly comprising *Escherichia coli* (21 strains) and *Klebsiella pneumoniae* (19 strains). In addition, bacteria were determined to be the cause of most symptomatic infections and occur more frequently in the first month after transplantation (15). Furthermore, the ATP levels of CD4+ T lymphocytes in patients with bacterial and fungal infections were significantly lower compared with those in uninfected patients, whereas the intracellular ATP levels in patients with viral infections did not differ significantly from those of uninfected patients. Furthermore, several studies have determined that alterations in the abundance of CD4+ T lymphocytes in the peripheral blood are associated with the outcome of severe lung infections following early renal transplantation. The incidence of cytomegalovirus pneumonia in CD4+ T lymphocyte-depleted patients was significantly increased compared with those possessing stable CD4+ T lymphocyte counts (16,17).

Considering the immature state of children's immune system and the administration of immunosuppressants following liver transplantation, pediatric patients who have undergone this procedure are at high risk of contracting a variety of infections. The incidence of pulmonary infections is particularly high among such patients, and is an important factor affecting the rates of patient and graft survival (18). It was observed that patients with infection had reduced lymphocyte counts; a positive correlation between CD4+ T lymphocyte ATP levels and lymphocyte counts was also reported. Thus, the Immuknow assay combined with the monitoring of lymphocyte count may have the potential to determine the risk for contracting opportunistic infections, and may reflect the patient immune status, providing a good basis for developing individualized treatment regimens.

It is widely known that administering appropriate doses of immunosuppressive agents is crucial for improving allograft outcome; however, the blood concentration of drugs is not directly correlated with the dose of drug administered due to individual pharmacokinetic differences and variations in the methods used for their detection (19). On the contrary, under conditions of hyper- and hypo-immune suppression, detrimental effects on the graft may occur, and increase the risk of infection and graft rejection (20). The present study, along with other reports, support the hypothesis that monitoring the ATP concentrations of CD4+ T cells may aid in distinguishing between hyper- and hypo immunity for the identification of LDLT patients at risk of infection or graft rejection, and may be applied to increase the efficacy of immunosuppressive therapies (21).

The present study also demonstrated that Immuknow assay-derived data were not correlated with the serum immunosuppressive drug C_0/D ratio of the patients, but were positively correlated with the lymphocyte count, indicating that modifications in administering immunosuppressive agents should be considered with respect to patient immune status. A prospective study conducted by Ravaioli *et al* (22) adjusted for the clinical benefits of immunosuppressive therapy in patients who had undergone liver transplantation based on Immuknow assays. In that study, the dose of tacrolimus was reduced by 25% when the ATP value was <130 ng/ml (weak immune cell response), but was increased by 25% when the ATP value was >450 ng/ml (strong immune cell response).

$CD4^+$ T lymphocytes play important roles in initiating immune responses, and the activity of these cells may reflect the status of the body's immune function (23,24). As the majority of immune cell functions have been directly and indirectly associated with intracellular ATP activity, measuring the ATP levels of $CD4^+$ T lymphocytes may be used to assess body immune function.

In conclusion, the Immuknow assay may be employed to evaluate the functional status of immune cells in pediatric patients following LDLT to predict the risk of infection. The application of this assay may permit individualized adjustments in the administration of immunosuppressive agents for patients to achieve an immune status that may reduce the risk of opportunistic infections and promote graft survival.

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Availability of data and materials

The datasets generated and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

DHL designed the experiments. WL performed the experiments, analyzed the data and wrote the first draft of the manuscript. KW collected the data and revised the draft. YHZ and GPS performed the measurement of $CD4^+$ T lymphocyte ATP levels and other tests. WG interpreted the patient data regarding pediatric liver transplantation and diagnosed the clinical cases. All authors discussed the results and reviewed the manuscript.

Ethics approval and consent to participate

Written informed consent was obtained from all participants or their parent/guardian in the case of children under 18 years of age, or patients otherwise considered minors under local legislation. The present study was approved by the Medical Ethics Committee of Tianjin First Central Hospital.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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