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Impaired T-Lymphocyte Proliferation Function in Biliary Atresia Patients With Chronic Cholestatic Jaundice After a Kasai Operation

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ABSTRACT: To investigate the association between chronic cholestatic jaundice, systemic immunity, and various infectious complications in patients with biliary atresia (BA), we performed a survey of the systemic immune function in 30 children with BA. Patients were divided into a jaundice group (total serum bilirubin ≥2 mg/dL for >6 mo) and control group (total serum bilirubin <2 mg/dL for >6 mo) with comparable age. Patients were tested for serum immunoglobulin and complement levels, mitogen response, interleukin (IL)-4, IL-5, and interferon-gamma production after phytohemagglutinin (PHA) stimulation, blood cell and lymphocyte subpopulation counts, phagocytic function, and leukocyte adhesion complex. They were then followed prospectively for 6 mo, and severe infectious complications requiring hospitalization were recorded. Compared with jaundice-free patients, T-lymphocyte proliferation function, determined by PHA mitogen test was significantly lower ($p = 0.02$) in BA patients with chronic cholestatic jaundice after a Kasai operation. During the study period, patients with chronic cholestatic jaundice had a higher risk of severe infectious complications than their jaundice-free counterparts (risk ratio = 5.87; $p = 0.001$). In conclusion, BA patients with chronic cholestatic jaundice are associated with impairment of T-lymphocyte proliferation and increased incidence of severe infectious complications. **(*Pediatr Res* 60: 602–606, 2006)**

BA is one of the most common cholestatic liver diseases in early infancy. Early diagnosis with a prompt Kasai operative intervention before the age of 60 d is an important prognostic factor (1). However, about one third of patients with BA remain jaundiced even after prompt intervention, and in another one third of patients, jaundice reappears after recurrent episodes of ascending cholangitis (2). Increased susceptibility to various infectious pathogens has been noted in patients with cholestatic jaundice and cholestatic animal model (3–6). A previous study showed that the incidence of sepsis in jaundiced patients ranged from 25% to 72%, and the mortality rate was around 20% to 30% (7). Infectious complications play an important role in the morbidity and mortality of jaundiced patients, although the exact mechanism of this increased susceptibility to infectious pathogens remains largely unclear. Depressed systemic and local reticuloendothelial immune functions may be responsible for the susceptibility of these patients to infectious pathogens (8,9). Impaired Kupffer cell phagocytic function and increased intestinal permeability to intestinal pathogens or endotoxins have been demonstrated in previous studies (10–13). Artificial bile duct ligation in animal models with short-term obstructive jaundice results in suppression of systemic immunity in rats and dogs (14–16). However, little is known about the association between chronic cholestatic jaundice and the systemic immune function in BA patients after a Kasai operation.

We conducted this study to evaluate the systemic immune function and infectious complications after a Kasai operation in BA patients.

METHODS

Subjects. From January 2004 to July 2005, this cross-sectional survey recruited 30 children with BA who underwent a Kasai operation (10 boys and 20 girls) at the Department of Pediatrics, National Taiwan University Hospital. The diagnosis of BA was established by intraoperative cholangiography. The ages at entry into the study ranged from 1 to 22.3 y (median, 3.5). Patients with serum total bilirubin levels >2 mg/dL for more than 6 mo were designated as the chronic cholestatic jaundice group. Twelve children (age range, 1–22.3 y; median, 1.8; three boys and nine girls) were noted to have serum total bilirubin levels >2 mg/dL for >6 mo (range, 6–114) before enrollment in our study. Another 18 jaundice-free BA children (age range, 1–16.5 y; median, 4.6; seven boys and 11 girls) with serum total bilirubin levels <2 mg/dL for >6 mo served as the control group (Table 1).

Ursodeoxycholic acid (UDCA) was prescribed to the majority of BA patients in our institute. Eleven (91.7%) of the 12 jaundice patients and 14 (77.8%) of the jaundice-free patients received UDCA therapy for >3 mo. There is no difference between these two groups in terms of UDCA use ($p = 0.32$). All patients were free of any immunosuppressant from at least 2 wk before the study began until the end of the follow-up period. The immune studies would not be done until the patients were free of infection for at least 2 wk to avoid the possible confounding on systemic immune function. Nutritional assessment and blood sampling were performed at enrollment.

Hospitalization events due to various infectious complications such as pneumonia, cholangitis, peritonitis, urinary tract infection, bacteremia, and sepsis were defined as severe infectious complications. Patients were prospectively followed for 6 mo after enrollment to record their infectious

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The authors have no potential, perceived, or real conflicts of interest. Informed consent was obtained from each child's parents or guardian.

Abbreviations: BA, biliary atresia; **PHA,** phytohemagglutinin; **PMN,** polymorphonuclear leukocyte; **PWM,** pokeweed mitogen; **SI,** stimulation index

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Table 1. *General characteristics of the study population*

Characteristics	Jaundice group (n = 12) Range	Median	Nonjaundice group (n = 18) Range	Median	p
Age (y)	1.0–22.3	1.8	1.0–16.5	4.6	NS
Hemogram					
WBC (cells/μL)	2630–22,990	7940	2510–11,990	6860	NS
Hb (g/dL)	8.1–14.5	11.6	7.5–14.4	12.4	NS
Platelet (k/μL)	54–327	156.5	28–546	183	NS
ANC (cells/μL)	1315–9288	3341	1340–4817	2283	NS
Biochemical data (mg/dL)					
Albumin	1.9–4.5	3.6	3.1–4.5	3.9	NS
Globulin	1.6–4.7	3.2	1.2–3.4	2.8	NS
AST	37–44	219	20–338	81	0.004
ALT	33–387	108	14–253	81	0.05
Total bilirubin	2.8–32	14.8	0.2–1.8	1	0.0000
Direct bilirubin	1.3–24	10.6	0.1–1.4	0.3	0.0000
Triglyceride	40–134	63	37–193	68	NS
Cholesterol	30–270	168	120–228	171	NS
PT/INR	1–2	1.5	0.9–1.4	1	0.005
Nutritional status*					
Body weight	−4.5 to 1.26	−0.8	−0.83 to 1.54	0	0.003
Body length	−4.0 to 1.78	−0.2	−1.73 to 3.0	0	NS
TSF	−3.0 to 1.5	−1.2	−1.5 to 1.33	−0.8	NS
MM	−4.51 to 2.34	−1.2	−2.21 to 1.82	−0.21	0.0009

* Profiles of nutritional status (including body weight, body length, triceps skinfold, and muscle mass) are presented as z scores.

WBC, white blood cell; Hb, hemoglobin; ANC, absolute neutrophil count; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, γ-glutamyl transpeptidase; PT/INR, prothrombin time/international normalized ratio; TSF, triceps skinfold; MM, muscle mass; NS, not significant.

complications. Case mortality, liver transplantation, or the end of the scheduled 6-mo follow-up period, whichever came first, was defined as the endpoint of this study. This study was approved by the Institutional Review Board and Research Ethics Committee of National Taiwan University Hospital.

Blood analysis. Blood samples were collected to measure serum total and direct bilirubin, albumin, cholesterol, triglyceride, calcium, alanine aminotransferase, aspartate aminotransferase, γ-glutamyl transpeptidase, prothrombin time/international normalized ratio, serum total immunoglobulin (IgG, IgA, and IgM), complement (C3 and C4), peripheral white blood cell count, hemoglobin, platelet count, absolute neutrophil count, and absolute lymphocyte count. All blood samples were analyzed immediately after blood collection.

Nutritional assessment. Body weight, body length, mid-arm muscle mass derived from upper arm anthropometry, and mid-arm triceps skinfold were measured at enrollment. Each measurement was repeated 3 times, and the average value was recorded on source documents. Data on these measurements were translated into z scores for further statistical analysis.

Mitogen assay. Lymphocyte proliferation was evaluated by mitogen assay using the mitogens PHA and pokeweed mitogen (PWM) as stimulants. PHA is a strong mitogen of T lymphocyte, and PWM is a mitogen for both T and B lymphocytes. Lymphocytes were isolated by centrifugation on Ficoll-Hypaque Density Medium (Pharmacia Ltd., Milton Keynes, UK) and diluted at a concentration of 2×10^6 cells/mL. These lymphocytes were then stimulated with PHA (4 μg/mL) and PWM (4 μg/mL). A control using RPMI 1640 was also added. All samples were run in triplicate, and the counts for the three measurements were averaged. Plates were incubated in a humidified incubator with 5% CO_2 at a temperature of 37°C for 54 h. Blast transformation was labeled by 1 μCi [^{3}H]-thymidine in culture for a further 18 h. The cells were finally removed in FILTERMATE 196 Cell Harvester (Packard Instrument Co., Meriden, CT) onto filter papers. The incorporation of [^{3}H]-thymidine was counted using the tritium window of a PACKARD Scintillation Counter Model 6400M (Packard Instrument Co.). The results were recorded in stimulation index (SI) as scintillation counts per minute of stimulant over control.

IL-4 chemiluminescent immunoassay. Supernatant of cell culture after PHA mitogen stimulant were collected at 54 h of stimulation for cytokines analysis. Human IL-4 Chemiluminescent Immunoassay (R&D Systems, Inc., Minneapolis, MN) was applied for the quantitative determination of human IL-4 concentrations in cell culture supernates after 54 h of PHA stimulation. The concentration of IL-4 was determined by a luminometer (R&D Systems, Inc.).

IL-5 and interferon-gamma (IFN-γ) immunoassay. Supernatants at 54 h of cell culture of PHA mitogen studies were collected for cytokine analysis. Human IL-5 and IFN-γ immunoassay (R&D Systems, Inc.) were applied for the quantitative determination in cell culture supernatants after PHA stimulation.

Polymorphonuclear leukocyte (PMN) superoxide release chemiluminescent assay. Chemiluminescent qualitative assay was applied to investigate the superoxide release function of PMNs. Blood samples (100 μL) were mixed with 200 μL PMA (10 μg/mL) or 200 μL normal saline, 200 μL Luminol (10^{-4} M, Sigma Chemical Co., St. Louis, MO), and 500 μL phosphate-buffered saline (PBS) containing azide (0.05%) in activated and controlled tubes, respectively. Both activated and controlled specimens were counted by Luminometer (Bio-Orbit Oy, Turku, Finland)

Phagocytic function. A Phagotest was used to investigate the phagocytic function of granulocytes and monocytes. Whole blood samples (200 μL) were incubated with fluorescein isothiocyanate (FITC)–labeled *Escherichia coli* (ORPEGEN Pharma Co., Heidelberg, Germany) in a 37°C warm water bath for 10 min. They were then washed in PBS; red blood cell lyse was later added to lyse the red blood cells. The percentage of granulocyte and monocyte *E. coli*-FITC phagocytosis was determined by a flow cytometer (BD FACSCalibur, Becton Dickinson Co.).

Neutrophil basal CD11b/CD18 adhesion molecule expression. The baseline expression of CD11b/CD18 leukocyte adhesion complex was determined by the following method: whole blood samples (0.5 mL) were incubated on ice with fluorescein-conjugated monoclonal antibody (MAb) to CD11b (Becton Dickinson Co.) and CD18 (IMMUNOTECH, Marseille, France) for 30 min. Lysing fluid was added; the tubes were incubated at room temperature for 10 min and then washed in PBS (0.05%) and resuspended in PBS-formaldehyde (1%). All MAbs directed against leukocyte surface antigens were counted by a flow cytometer (BD FACSCalibur, Becton Dickinson Co.).

Lymphocyte subpopulation. Whole blood samples (30 μL) were incubated with 5 μL fluorescein-conjugated MAb to CD3, CD4, CD8, CD16, CD19, CD45, CD45RA, CD45RO, and CD56 antigens (Becton Dickinson Co.) in test tubes for 30 min for the analysis of lymphocyte subpopulations. Lysing solution (50 μL) was added to the test tubes for another 30 min. Then 400 μL double-distilled H_2O was added for an additional 15 min. All MAbs directed against lymphocyte surface antigens were counted by a flow cytometer (BD FACSCalibur, Becton Dickinson Co.).

Statistical analysis. Statistical analyses were performed with the use of the STATA statistical software package (StataCorp LP). The z score was applied to the data management of nutritional status including body weight, body length, muscle mass derived from the mid-arm and triceps skinfold. Mann-

Whitney *U* test was applied for analysis of all continuous variables, and the Fisher exact test was used for categorical data. Backward stepwise multiple regression was applied to determine the association between different factors. A p value <0.05 was defined as statistically significant.

RESULTS

All patients in this study group were diagnosed as BA by intraoperative cholangiography. Among these BA patients with chronic cholestatic jaundice ($n = 12$), five (42%) of them had persistent jaundice after a Kasai operation, whereas the other seven (58%) became jaundiced again after recurrent episodes of cholangitis. All patients in the control group ($n = 18$) remained jaundice free after the Kasai operation.

In this study, there were eight children (66.7%) between 1 and 5 y old, three (25%) between 5 and 10 y old, and one (2.3%) older than 10 y in the jaundice study group ($n = 12$), whereas there were nine children (50%) between 1 and 5 y old, eight (44.4%) between 5 and 10 y old, and one (5.6%) older than 10 y in the control group ($n = 18$). There was no difference between these two groups in age distribution ($p > 0.05$).

Six (50%) of the 12 patients in the jaundice group received liver transplant due to chronic liver insufficiency or uncontrolled esophageal variceal bleeding 3–4 mo (median, 3.5) after enrollment in our study. One patient (8%) in the jaundice group died of *Candida* sepsis after 3.5 mo of follow-up. The follow-up period in the jaundice group ranged from 3 to 6 mo (median, 5). One child (6%) in the nonjaundice control group received a liver transplant 4 mo after enrollment due to uncontrolled esophageal variceal bleeding. The follow-up period in the control group ranged from 4 to 6 mo (median, 6). No difference in the follow-up period was identified between these two groups ($p > 0.05$).

There were 11 severe infectious events in the 57 person-months of follow-up in the jaundice group and three events in the 106 person-months of follow-up in the control group (Table 2). There were three episodes of sepsis in the jaundice group (one episode due to *Candida albican* and another two events due to *E. coli*). Sputum culture showed no bacterial pathogen in the five episodes of pneumonia. Viral isolation identified influenza A virus and adenovirus in two episodes among the four pneumonia events in the jaundice group. Blood culture in the five cholangitis events all failed to identify bacterial pathogen. Another patient in the jaundice group who suffered from an *E. coli* urinary tract infection was also identified. Patients with cholestatic jaundice had more severe infectious events than jaundice-free patients did (risk ratio: 5.87; 95% confidence interval (CI): 1.70–20.31; $p = 0.001$).

Serum levels of IgG, IgA, and IgM were not statistically different between the jaundice and nonjaundice groups (Table 3). Serum IgG was found to be lower than age-matched normal values in Taiwan (17) in three patients (25%) in the jaundice group ($n = 12$) and four patients (22%) in the control group. There was no difference between these two groups (odds ratio: 1.12; 95% CI: 0.30–4.16; $p = 0.86$). During the follow-up period, two (29%) of the seven patients whose serum IgG level below normal age-matched levels had infectious events. Compared with other 23 children with normal age-matched serum IgG levels in our study, these seven patients did not have clinical evidence of increased susceptibility to infectious complications (risk ratio: 0.73; 95% CI: 0.20–2.62; $p = 0.62$).

Significant impairment of T-lymphocyte proliferation function was observed in the jaundice group compared with the nonjaundice group, as demonstrated by a lower SI ratio in the PHA ($p = 0.02$) mitogen stimulation test (Fig. 1*A*). There was a significant correlation between serum total bilirubin levels and the PHA SI ratio ($R = -0.38$, $p < 0.05$) in our study population (Fig. 1*B*).

Patients of BA with cholestatic jaundice were associated with lower z scores in body weight ($p = 0.003$) and muscle mass ($p = 0.0009$) than jaundice-free patients in our study (Table 1). Hence, patients of BA with cholestatic jaundice were noted to have poor protein caloric status in our study. Muscle mass derived from upper arm anthropometry is considered to be a more reliable marker reflecting the nutritional status of protein as compared with body weight alone. To exclude the possible confounding effect of poor protein caloric status on T-lymphocyte proliferation, we applied backward

Table 2. *Infectious complications in the study population*

	Jaundice group	Control group	Risk difference	95% CI	p
Sepsis	3	0	0.136	−0.01 to 0.28	0.0001
Atypical pneumonia	4	1	0.056	−0.01 to 0.12	0.039
Cholangiitis	3	2	0.031	−0.03 to 0.09	NS
UTI	1	0	0.017	−0.02 to 0.05	NS
Total events	11	3	0.134	0.04 to 0.23	0.0013

There were 57 person-months of follow-up in the jaundice group and 106 person-months of follow-up in the nonjaundice group.

UTI, urinary tract infection.

Table 3. *Systemic humoral and cellular immunity of the study population*

	Jaundice group ($n = 12$)		Nonjaundice group ($n = 18$)		
	Range	Median	Range	Median	p
Immunoglobulin					
IgG (mg/dL)	529–2000	1330	362–1862	1000	NS
IgA (mg/dL)	36–518	257	25–330	146	NS
IgM (mg/dL)	59–396	137	59–221	123	NS
Complement					
C3 (mg/dL)	36–159	99	66–150	84	NS
C4 (mg/dL)	7–23	14	8.2–20.9	14	NS
Mitogen test (SI)					
PHA	1.0–64.8	17	1.7–355	144	0.02
PWM	1–143	17	1.7–158.8	18.4	NS
Phagotest					
Granulocyte (%)	48.3–91.3	85	45–92.7	83.5	NS
Monocyte (%)	26.4–85.5	52	30.1–80.5	59.6	NS
Granulocyte CD11b/18 (%)	98.3–100	99.9	99.9–100	100	NS
Cytokines (pg/mL)					
IL-4	1.6–15	4.3	1.6–84.9	3.1	NS
IL-5	7.8–591.3	44.1	7.8–477.3	46.3	NS
IFN-γ	15.6–1900	185.5	15.6–2047.6	248.3	NS

C3, complement 3; C4, complement 4.

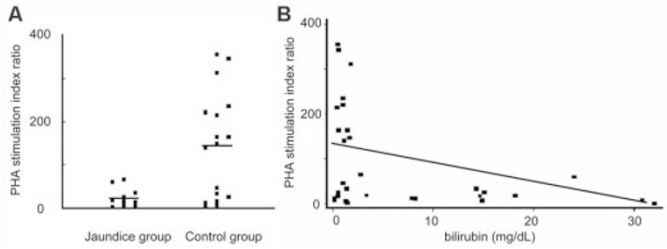

Figure 1. (*A*) PHA SI ratio is significantly lower in BA patients with chronic cholestatic jaundice ($n = 12$) as compared with the jaundice-free control group ($n = 18$;$p = 0.02$). (*B*) Scatter plot demonstrates a significant correlation between serum total bilirubin level and PHA SI ratio ($n = 30$, $R = -0.38$, adjusted $R^2 = 11.18\%$, standard error = 2.40, $p < 0.05$, Y = 126.85 − 4.96X).

stepwise multiple regression to analyze the correlation of serum total bilirubin and *z* scores of muscle mass with the SI ratio for PHA mitogen stimulation test. A significant correlation between serum total bilirubin levels and PHA SI ratio was identified (correlation coefficient = −4.96; standard error = 2.40; $p < 0.05$). Poor correlation between the *z* scores of muscle mass and PHA SI ratio was also noticed.

The IL-4, IL-5, and IFN-γ levels of cell culture supernatants after PHA mitogen stimulation showed no differences between BA patients with or without cholestatic jaundice (Table 3). Indicators of nonspecific cellular immunity, such as polymorphonuclear cell count and absolute cell count of each lymphocyte subpopulation, are summarized in Tables 3 and 4. No significant correlation of these aspects was observed in BA patients with cholestatic jaundice ($p > 0.05$).

DISCUSSION

We demonstrated significant impairment of T-lymphocyte proliferation function by PHA mitogen stimulation study in BA patients with chronic cholestatic jaundice after a Kasai operation. The detailed mechanism of suppressed T-lymphocyte proliferation is unclear (13,18). Various plasma circulating inhibitory factors such as bile acids, cytokine, bilirubin, endotoxin, and lipoproteins are possible causes of T-lymphocyte defects (19). High serum bile acids have been shown to inhibit lymphocyte proliferation in a previous study (20). To minimize the effects of circulating factors in our study, we repeated cell washing during the processing of peripheral blood mononuclear cells for *in vitro* mitogen stimulation tests, removing most unbound plasma inhibitory factors. Fraser *et al.* (21) demonstrated no difference in T-lymphocyte function after 2 wk of bile duct ligation in dogs, whereas Roughneen *et al.* (15) showed significant impairment in T-lymphocyte proliferation after 3 wk of obstructive jaundice. Such phenomena further suggest that the time course of obstructive jaundice itself plays a key role in the T-lymphocyte defect. Hence, our patients with chronic jaundice for >6 mo could demonstrate a more reliable effect of the cholestatic jaundice on T lymphocytes.

Table 4. *Absolute lymphocyte subpopulation of the study population*

	Jaundice group ($n = 12$)		Nonjaundice group ($n = 18$)		
k/μL	Range	Median	Range	Median	*p*
Total no. of lymphocytes	807–10,460	3775	590–6660	3140	NS
T cell ($CD3^+$)	179–5034	2239	370–4777	1742	NS
B cell ($CD19^+$)	158–3908	1019	82–1830	530	NS
Natural killer cell ($CD16^+56^+$)	32–1513	286	19–745	270	NS
Suppressor T cell ($CD3^+8^+$)	66–1890	630	126–1996	611	NS
Helper T cell ($CD4^+$)	123–3032	1296	214–2406	938	NS
CD $4^+/8^+$ ratio	1.1–3.5	1.8	0.8–3.7	1.6	NS
Naïve T cell ($CD3^+45RA^+$)	54–2087	862	117–1723	665	NS
Memory T cell ($CD3^+45RO^+$)	68–964	362	97–751	326	NS

Chronic protein caloric malnutrition is another factor that possibly contributes to the suppression of T lymphocyte–mediated immune function. The defect of T-lymphocyte function was demonstrated by Roughneen *et al.* (15) in the absence of nutritional depletion in animal model. In our study, patients in the jaundice group had lower *z* scores in body weight and muscle mass than patients in the control group. However, low protein-caloric nutritional status itself was not a confounding factor between T-lymphocyte defects and cholestatic jaundice in our study population by the analysis of backward stepwise multiple regression. These cholestatic BA patients received intensive nutritional support in our hospital. It is possible that the nutritional status of our patients was maintained to an extent that no significant related impact on T-lymphocyte function could be observed.

Defective PMN phagocytic function with obstructive jaundice and impaired PMN superoxide release function by bilirubin and bile acids challenge has been demonstrated in animal studies (16,22). However, no PMN dysfunction, including superoxide release, phagocytic function, absolute neutrophil cell number, and proportion of basal expression of leukocyte adhesion molecule (CD11b/CD18 complex) was identified in our study. Previous studies were limited to animal models, and the short-term jaundice was induced by artificial bile duct ligation for 2 to 3 wk. The physiologic and anatomic differences, as well as the etiology of obstructive jaundice, between human and animal studies could account for the different results. On the other hand, the number of BA patients with persistent cholestatic jaundice was not large in our study and it is possible that in such a small sample size, some borderline deterioration in PMN-mediated immunity would not be detected.

We also compared the effect of activated T lymphocyte on humoral and nonspecific cellular immunity between BA patients with or without cholestatic jaundice by cytokines assay. IL-4 produced by activated T lymphocytes is known to modulate isotype switching, IgE production of B lymphocytes, differentiating precursor helper T lymphocytes from the type II helper T lymphocytes, which regulates humoral immunity and antibody production (23). IL-5, another cytokine produced by T lymphocytes, is also known to exert pleiotropic effects on B-lymphocyte and eosinophilic lineage (24). IFN-γ influences the class of antibody produced by B lymphocytes, upregulates major histocompatibility complex antigens, and

increases the efficacy of macrophages (25). The concentrations of IL-4, IL-5, and IFN-γ of cell culture supernatants after PHA mitogen stimulation were not different between these two groups. Such a phenomenon suggests that the amounts of IL-4, IL-5, and IFN-γ released by activated T lymphocytes on humoral and nonspecific cellular immunity between cholestatic and nonjaundiced BA patients are not different.

UDCA therapy has been shown to reduce aberrant HLA class I antigens on cell surfaces in a previous study, which could be a confounding factor in our study (26). At our institute, the majority of BA patients received long-term UDCA treatment. Because the percentages of patients on UDCA therapy were not different between these two groups, the effect of UDCA on systemic immune function in this study may be mitigated when we do the comparisons.

This study demonstrates a strong association between chronic cholestatic jaundice and infectious complications in BA patients. Impaired T-lymphocyte proliferation function was evident in BA patients with chronic cholestatic jaundice, which may related to the increased infectious events in chronic cholestatic BA patients.

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