

The correlation between plasma cytokine levels in jaundice-free children with biliary atresia

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Background: T helper (Th) cell cytokines modulate inflammation and play a role in biliary atresia (BA). The aim of the study is a cross-sectional assessment of the levels of Th cytokines in the jaundice-free post Kasai procedure patients.

Methods: There were 40 jaundice-free patients with BA and 28 normal controls enrolled. Patients were divided into 3 groups, including normal liver function, impaired liver function, and portal hypertension. Plasma concentration of Th1 [interferon- γ (INF- γ), interleukin (IL)-2], Th2 (IL-4, IL-10), Th3 [transforming growth factor- β 1 (TGF- β 1)], Th17 (IL-17) cytokines, and stromal cell-derived factor-1 α (SDF-1 α) were investigated.

Results: The INF- γ level was significantly higher in the BA patients with impaired liver function and portal hypertension than controls ($P < 0.0001$ and $P < 0.0001$, respectively). There was a significantly increase of TGF- β 1 in all BA groups compared with controls ($P = 0.003$). The reduction of SDF-1 α expression was found in BA groups ($P < 0.0001$). IL-10 levels significantly correlated with aspartate aminotransferase to platelet ratio index ($r = 0.496$, $P = 0.001$). For the cytokine correlations, there were no correlations of Th1, Th2 and Th17 cytokine with the other measured cytokines, but TGF- β 1 was negatively correlated with SDF-1 α levels ($r = -0.327$, $P = 0.039$).

Conclusions: INF- γ and IL-10 are likely to be involved in the disease progression in BA. Besides, TGF- β 1 is found to be a suppression marker associated with SDF-1 α levels

and reduced production of TGF- β 1 may be associated with the disease progression.

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Key words: biliary atresia;
gastroenterology;
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Introduction

Biliary atresia (BA) is an inflammatory fibrosing condition in infancy which affects both extrahepatic and intrahepatic biliary ducts and leads to fibrous obliteration of the biliary tract and cirrhosis.^[1,2] Most BA patients become jaundice-free after the Kasai operation.^[3] However, despite initial sufficient biliary drainage, the long-term results are still not satisfactory because of ongoing liver cirrhosis. Esophageal varices caused by ongoing cirrhosis and portal hypertension (PH) make life difficult for children. Therefore, liver transplantation has been a standard treatment for end-stage liver disease for the long-term survival.^[4]

Although precise mechanisms for BA have not been elucidated, immunologic abnormalities have been proposed as a potential mechanism in the pathogenesis of BA.^[5-7] Activated T helper (Th) cells and different Th-cell cytokines are considered to play an important role in BA pathophysiology.^[8] Traditionally, clusters of differentiation (CD)4+ Th cells are divided into three main subsets, Th1, Th2, and Th3 cells, according to the types of cytokines they secrete.^[9] Interleukin (IL)-2 and interferon- γ (INF- γ) are selected as Th1 cytokines, and IL-4 and IL-10 as Th2 cytokines. Th1 cells are involved in macrophage activation and INF- γ and IL-2 productions, whereas Th2 cells are responsible for IL-4 and IL-10 secretions, humoral immunity, and inactivation of several macrophage functions.^[9] A characteristic of Th3 cells is the production of the immune modulating cytokine, transforming growth factor- β 1 (TGF- β 1) which has multiple suppressive actions on T cells, B cells, macrophages, and other cells, and correlation with protection and/or recovery from autoimmune diseases.^[10]

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An effector CD4+ T cell lineage called Th17 cells is recognized by their production of IL-17 and causes organ-specific disease by inducing pro-inflammatory cytokines and chemokines which recruit Th1 cells to the target tissue.^[11] Th17 cells play an active role in shaping the local inflammatory response in the liver.^[12] Besides, stromal cell-derived factor (SDF)-1 α plays an important role in mediating progenitor cell homing, trafficking and domiciliation to peripheral tissues^[13] and is associated with liver injury.^[14]

There were rare studies about investigations of Th1/Th2/Th3 cellular immunity in pediatric patients with BA.^[6,8,15] Moreover, data regarding the possible existence of a difference in Th17 cytokine level were not found in pediatric patients with the disorder. The aim of this study was to find the roles of Th cell cytokines and inflammatory process as expressed by levels of cytokines in jaundice-free BA patients, including Th1 (IL-2, INF- γ), Th2 (IL-4, IL-10), Th3 (TGF- β 1), Th17 (IL-17), and SDF-1 α .

Methods

Study population

This study was approved by the Institutional Ethics Committee of Taichung Veteran General Hospital. Informed consent was obtained from participating parents and/or patients before enrollment in the study. The diagnosis of BA was based on the pathological examination of resected biliary remnants.

Twenty-eight control subjects (17 boys and 11 girls; age range: 2-16 years; median: 10.5 years) were selected from children who underwent a healthy examination in our outpatient department or from hospitalized patients who had been admitted for elective surgery, such as inguinal hernia and undescended testis without infection or inflammatory conditions. A sample was obtained from all control subjects during healthy screening or before induction of anesthesia for surgery.

Forty jaundice-free pediatric patients with BA post Kasai procedure (21 boys and 19 girls; age range: 4.4 months-16 years; median: 6.5 years) were enrolled between February and December 2009. The range of follow-up after the Kasai procedure was between 3 months to 15.8 years. The jaundice-free was defined as bilirubin levels less than 2.0 mg/dL. PH was defined by the presence of ascites or esophageal varices as demonstrated by endoscopy. These BA patients were divided into 3 groups based on clinical status: group 1 consisted of 10 patients with normal liver function, aspartate aminotransferase (AST) <40 IU/L, alanine transaminase (ALT) <35 IU/L, and γ -glutamyl transpeptidase (γ -GT) <60 IU/L; group 2 consisted of

17 patients with liver function impairment, AST \geq 40 IU/L, ALT \geq 35 IU/L, or γ -GT \geq 60 IU/L without PH; and group 3 consisted of 13 patients with PH. None in this study received liver transplantation or exhibited symptoms and signs of fever, ascending cholangitis or acute inflammation at the time of blood sampling.

Even in BA patients with normal liver function, the inflammation and fibrosis are progressive in the liver. Although liver biopsy is the gold standard for evaluating liver fibrosis, it is invasive and may result in life-threatening complications. AST to platelet ratio index has been used as a simple tool for assessing liver fibrosis in patients with BA during postoperative follow-up care.^[16] All patients with BA were further divided into two groups by the median of aminotransferase to platelet ratio index (APRI) values to evaluate the differences in the levels of cytokines. APRI was calculated according to Wai et al,^[17] with the following equation:

$$\text{APRI} = \frac{\text{AST/upper normal limit (IU/L)}}{\text{Platelets } (\times 10^9/\text{L})} \times 100$$

The upper normal limit for AST was 40 IU/L according to the laboratory reference values.

Quantification of cytokines in plasma

Blood samples were collected into sterile ethylenediamine tetraacetic acid-containing tubes, and plasma was separated by centrifugation at 3000 g for 10 minutes. An additional centrifugation at 13 000 g for 10 minutes was done to avoid platelet contamination. Samples were stored at -70°C until analysis. The plasma concentrations of IL-2, IL-4, IL-10, IL-17, IFN- γ , SDF-1 α , and TGF- β 1 were measured by an enzyme linked immune sorbent assay technique (R&D Systems, Minneapolis, MN, USA). To avoid inter-assay variations, all samples were tested in the same experiment. The concentrations were calculated on a standard curve by the manufacturer's instructions. Minimal detectable levels of various cytokines were as follows: IL-2, 7 pg/mL; IL-4, 10 pg/mL; IL-10, 3.9 pg/mL; IL-17, 15 pg/mL; IFN- γ , 1.5 pg/mL; TGF- β 1, 7 pg/mL; SDF-1 α , 18 pg/mL.

Statistical analysis

All analyses were performed using the SAS ver. 9.2 software package (SAS Institute, Cary, NC, USA). Demographic data between the groups were compared by the Chi-square test. Data were expressed as median and interquartile range because of non-normal distribution or heterogeneity of variance. The Kruskal-Wallis test was applied to compare variables between multiple groups, followed by pairwise comparisons with the Mann-Whitney *U* test. Spearman's rank-order correlation coefficient was used to evaluate any association among various cytokines, with *P*<0.05 considered to be significant.

Table 1. Demographic and laboratory data in postoperative jaundice-free patients with biliary atresia

Group	Normal liver function (n=10)		Impaired liver function (n=17)		Portal hypertension (n=13)		P value
	Median	IQR	Median	IQR	Median	IQR	
Sex (M/F)	8/2		9/8		4/9		0.0660
Age (y)	5.8	3.9-10.7	6.1	1.3-11.5	6.5	2.4-8.1	0.7230
AST (IU/L)	29	25-35	70	54-93	63	40-96	0.0003*/0.0003†/0.0004‡/0.5720§
ALT (IU/L)	16	15-19	66	45-125	60	33-79	0.0002*/<0.0001†/0.0030‡/0.2760§
γ-GT (IU/L)	20	18-31	131	89-239	113	54-171	0.0001*/<0.0001†/0.0030‡/0.1940§
Platelet (10 ³ /μL)	240	123-289	216	121-290	129	110-146	0.0260*/0.9600†/0.0220‡/0.0180§
APRI	0.34	0.24-0.45	0.68	0.42-1.70	1.20	0.81-1.48	0.0009*/0.0120†/0.0002‡/0.1320§

M: male; F: female; AST: aspartate aminotransferase; ALT: alanine aminotransferase; γ-GT: γ-glutamyl transpeptidase; APRI: aminotransferase to platelet ratio index; IQR: inter-quartile range. *: Kruskal Wallis test for comparison among 3 groups, Mann-Whitney *U* test for post-hoc comparisons; †: normal liver function and impaired liver function; ‡: normal liver function and portal hypertension; §: impaired liver function and portal hypertension.

Table 2. Plasma cytokine concentrations of postoperative jaundice-free patients with biliary atresia

Group	Control (n=28)		Normal liver function (n=10)		Impaired liver function (n=17)		Portal hypertension (n=13)		P value
	Median	IQR	Median	IQR	Median	IQR	Median	IQR	
IL-2 (pg/mL)	73.4	70.7-75.0	73.9	72.2-76.1	64.8	60.0-73.2	72.3	60.0-74.9	0.2630
IL-10 (pg/mL)	11.8	8.0-14.0	10.4	9.0-12.7	12.5	9.3-14.9	13.4	9.6-14.0	0.5310
IL-17 (pg/mL)	52.0	50.5-56.6	47.1	41.3-60.4	44.6	41.3-51.6	49.3	43.5-54.0	0.5160
IFN-γ (pg/mL)	7.9	4.1-10.8	8.1	7.5-12.0	10.1	8.7-11.7	8.9	7.5-9.5	0.0410*/0.2470†/<0.0001‡/<0.0001§
TGF-β1 (ng/mL)	3.4	2.6-3.8	7.0	3.0-12.1	6.1	3.7-17.7	6.4	3.3-7.0	0.0030*/0.0270†/0.0030‡/0.0120§
SDF-1α (ng/mL)	8.0	5.8-11.8	1.9	1.9-2.1	1.8	1.7-2.2	2.5	2.1-2.9	<0.0001*/<0.0001†/<0.0001‡/<0.0001§

IL: interleukin; INF-γ: interferon-γ; TGF-β1: transforming growth factor-β1; SDF-1α: stroma cell-derived factor-1α; IQR: inter-quartile range. *: Kruskal Wallis test for comparison among 4 groups, Mann-Whitney *U* test for post-hoc comparisons; †: control and normal liver function; ‡: control and impaired liver function; §: control and portal hypertension.

Table 3. Plasma cytokine levels in relation to the median of APRI in post operative jaundice free patients with biliary atresia

Group	Control (n=28)		Low APRI (<0.7) (n=20)		High APRI (>0.7) (n=20)		P value
	Median	IQR	Median	IQR	Median	IQR	
IL-2 (pg/mL)	73.4	70.7-75.0	72.2	59.9-76.0	72.4	61.5-74.5	0.7290
IL-10 (pg/mL)	11.8	8.0-14.0	9.5	8.2-12.4	13.5	11.9-18.1	0.0100*/0.4140†/0.0250‡/0.0030§
IL-17 (pg/mL)	52.0	50.5-56.6	45.7	40.7-55.2	49.2	42.9-64.0	0.3970
IFN-γ (pg/mL)	7.9	4.1-10.8	8.8	7.5-10.9	9.1	8.3-11.6	0.0650
TGF-β1 (ng/mL)	3.4	2.6-3.8	9.4	4.0-16.0	5.1	2.8-6.8	0.0002*/<0.0001†/0.0310‡/0.0780§
SDF-1α (ng/mL)	8.0	5.8-11.8	2.0	1.8-2.2	2.2	1.8-2.6	<0.0001*/<0.0001†/<0.0001‡/0.1760§

IL: interleukin; INF-γ: interferon-γ; TGF-β1: transforming growth factor-β1; SDF-1α: stroma cell-derived factor-1α; APRI: aminotransferase to platelet ratio index; IQR: inter-quartile range. *: Kruskal Wallis test for comparison among 3 groups, Mann-Whitney *U* test for post-hoc comparisons; †: control and low APRI; ‡: control and high APRI; §: low and high APRI.

Results

Demographics, clinical observations, and laboratory data

Demographic data, liver function test, platelets and APRI in BA patients with normal liver function, impaired liver function, and PH are shown in Table 1. No significant differences of age and gender among BA patients were observed. The patients with impaired liver function and PH had higher levels of AST, ALT, γ-GT and APRI, as compared with BA patients with normal liver function. However, the BA patients with PH had significantly lower platelet counts than those without PH.

Concentration of cytokines in plasma

For Th1 cytokine expressions, the values of IL-2 were not significantly different among 4 groups ($P=0.263$) (Table 2). IFN-γ was significantly increased in BA patients with impaired liver function ($P<0.0001$) and PH ($P<0.0001$) when compared with the control group. There was no significant difference in IFN-γ among BA groups.

Regarding Th2 cytokines assay, there were no

Table 4. Correlations between measured cytokines levels and APRI in post operative jaundice free patients with biliary atresia

Cytokines	IL-2	IL-10	IL-17	IFN-γ	TGF-β1	SDF-1α	APRI
IL-2							
<i>r</i>	1.000	0.152	0.099	0.139	-0.063	0.069	0.022
<i>P</i>	0.000	0.349	0.544	0.400	0.698	0.674	0.892
IL-10							
<i>r</i>		1.000	0.126	0.177	-0.199	0.096	0.496
<i>P</i>		0.000	0.437	0.279	0.219	0.553	0.001
IL-17							
<i>r</i>			1.000	0.126	0.055	-0.130	-0.030
<i>P</i>			0.000	0.444	0.734	0.425	0.852
IFN-γ							
<i>r</i>				1.000	-0.228	0.119	0.133
<i>P</i>				0.000	0.162	0.469	0.420
TGF-β1							
<i>r</i>					1.000	-0.327	-0.222
<i>P</i>					0.000	0.039	0.169
SDF-1α							
<i>r</i>						1.000	0.244
<i>P</i>						0.000	0.129
APRI							
<i>r</i>							1.000
<i>P</i>							0.000

IL: interleukin; INF-γ: interferon-γ; TGF-β1: transforming growth factor-β1; SDF-1α: stroma cell-derived factor-1α; APRI: aminotransferase to platelet ratio index.

significant differences in IL-10 levels among these 4 groups ($P=0.531$). However, the values of IL-4 in the controls and BA patients were under the detectable limit for the assay (<10 pg/mL).

With regard to the Th3 cytokine assay, all BA patients had significantly increased TGF- β 1 levels when compared with the controls ($P=0.003$). There was no significant difference in TGF- β 1 levels among the BA patients.

For the Th17 cytokine assay, there were no significant differences in IL-17 expression among all groups ($P=0.516$).

Compared with the controls, the BA patients had significantly lower SDF-1 α levels ($P<0.0001$). However, no significant difference in the SDF-1 α levels was found among the BA patients.

Concentration of cytokines by APRI

The BA patients were further divided into 2 groups by the median of APRI (cut-off value: 0.70) (Table 3). IL-10 levels in the high APRI group were significantly higher than those in low APRI group ($P=0.003$). The level of TGF- β 1 was lower in the high APRI group than in the low APRI group, but this was statistically insignificant ($P=0.078$).

Correlations between measured cytokines in patients with BA

Correlations between measured cytokines were further analyzed (Table 4). The levels of Th1 (IL-2 and IFN- γ), Th2 (IL-10) and Th17 (IL-17) cytokines were not correlated with any of the other measured cytokines. The Th3 cytokine (TGF- β 1) was negatively correlated with SDF-1 α ($r=-0.327$, $P=0.039$). Besides, there were positive correlations of IL-10 with APRI ($r=0.496$, $P=0.001$), AST ($r=0.405$, $P=0.009$), and ALT ($r=0.469$, $P=0.002$) (ALT and AST were not shown in Table 4).

In addition, there were no gender differences in IL-2, IL-10, IL-17, IFN- γ , TGF- β 1 and SDF-1 α in the controls. We did not find the correlation of the measured cytokines with the age of both controls and BA patients (data not shown).

Discussion

Th1/Th2 balance is important to maintain the immune system under normal conditions.^[11] A number of features suggest that BA is immune-mediated, and dysregulated CD4+ Th cells are thought to play an important role in the pathogenesis of BA.^[8,18,19] The polarization towards either Th1 or Th2 immunity relies on the level of the cytokines. There have been inconsistent viewpoints about polarization of immune response to BA in the early stages of the disease.^[8,19] Narayanaswamy et al^[6] analyzed the levels of plasma Th1 (IL-2 and IFN- γ)

and Th2 (IL-4 and IL-10) cytokines in 21 BA patients at the time of Kasai procedure and 6 months after the operation. The response was non-polarized, and the levels of IFN- γ , IL-2 and IL-4 markedly increased by 6 months after portoenterostomy. Vejchapipat et al^[20] found that there was a modest increase of IFN- γ in a Th1 cytokine study with 46 patients with BA (median age of 9 years) compared with controls. In the present study, we did not find the correlations between Th1 and Th2 cytokines. But the level of INF- γ increased in patients with impaired liver function and PH. The level of IL-10 was also increased in patients with high APRI. This finding indicates that the inflammatory process is progressive and non-polarized in these patients. Besides, there were no correlations between INF- γ and IL-2, and between IL-4 and IL-10. This may be due to the small sample size or the complex pathways involved in the interaction.

For Th2 cytokine analysis, plasma IL-4 was undetectable in all participants in this study (i.e., all participants showed less than the detectable limit of 10 pg/mL). In a cross-sectional study in Taiwan, the median IL-4 level was 3.1 pg/mL (range: 1.6-84.9 pg/mL) in 18 jaundice-free BA children (age range: 1-16.5 years; median: 4.6 years).^[21] This may explain why the levels of plasma IL-4 were undetectable in our study.

IL-10 can further limit T cells activation and differentiation, leading to suppression of proinflammatory responses and reducing tissue damage from the excessive effects of inflammation.^[22] Our results showed that IL-10 level was correlated with APRI and transaminases. However, no significant difference was found in IL-10 levels between the patient and control groups. This result may be attributable to the relatively small sample size. Therefore, we can reasonably suggest that the increased levels of IL-10 in patients with BA reflect the degree of inflammation and fibrosis. This finding requires further evaluations whether the increase of IL-10 is only a consequence of persistent inflammation or whether it may play a role in favoring the progression of BA.

IL-17 induces liver fibrosis through activating inflammatory and liver resident cells.^[23] However, we did not find any correlation with the other measured cytokines. And nor any significant difference was observed in IL-17 levels among all groups.

TGF- β (Th3) was found to play an important role in immune regulation and to suppress some Th1 and Th2 cell-mediated autoimmune diseases.^[24] TGF- β 1 is an important biomarker of liver injury and correlated with the rate of fibrosis progression.^[25] However, our results did not show that TGF- β 1 level was significantly correlated with the levels of Th1, Th2, or Th17 cytokines. Vejchapipat et al^[26] analyzed the levels of

TGF- β 1 in 67 BA patients with a median age of 7 years and found that TGF- β 1 levels in BA patients were higher than those in the controls. In a study of 32 postoperative BA patients (mean age 11.2 years), Kobayashi et al^[27] found that TGF- β 1 level was significantly higher in jaundice-free patients than in the controls and patients with jaundice end-stage liver fibrosis. At the time of liver transplantation, patients with BA had been reported to have low TGF- β 1.^[15] Although it was not significant in our results, patients with a lower APRI level tended to display a higher TGF- β 1 level. These studies were consistent with our results. The lack of a correlation between patients' APRI with their TGF- β 1 levels may be due to the relatively small sample size.

The elevation of plasma SDF-1 α was found in adult patients with liver cirrhosis and correlated with the number of mobilized CD133+ hematopoietic progenitor cells.^[28] Compared with normal controls, the plasma levels of SDF-1 α were 1.2 times higher in patients with chronic liver diseases.^[29] Although there were no differences in SDF-1 α levels in adult patients with cirrhosis according to the Child-Pugh class, SDF-1 α level was 1.5 times higher than that in healthy controls.^[30] However, little is known about the association between SDF-1 α and pediatric patients with BA. In contrast with adult patients with cirrhosis, SDF-1 α was lower in BA patients than in the controls, and was negatively correlated with TGF- β 1. Although down-regulating SDF-1 α expression by TGF- β 1 had been seen in the bone marrow,^[31] the exact significance of the relationship between plasma TGF- β 1 and SDF-1 α levels in patients with BA requires further investigation.

There were several limitations in this study. First, the cross-sectional design of this study limits its ability to provide causal relationships between disease status and cytokine levels. Second, the plasma levels of cytokines did not necessarily reflect the action of their signaling pathways within the liver. Investigation of these cytokine levels in the liver tissue will elucidate possible roles in BA more precisely. Third, there still are other inflammatory cytokines or markers involved in the pathophysiology of progressive inflammatory liver injury. Fourth, we would like to emphasize that the results are not strong because of small sample size and that more data are needed to confirm our findings.

In conclusion, IFN- γ and IL-10 are likely to be involved in the disease progression in BA. In addition to non-polarization, higher TGF- β 1 levels (Th3) are associated with lower APRI levels, thus resulting in a lower degree of liver fibrosis. TGF- β 1 is a suppression marker associated with SDF-1 α levels. Serial measurements of different cytokines levels may be helpful in advancing our understanding of BA and developing new strategies for effective treatment.

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Competing interest: None declared.

Contributors: JZH participated in the design, analyzed data and contributed to the writing of the manuscript. WLC interpreted the results and helped to draft the manuscript. LCC participated in the design of the study and helped to revise the manuscript. WJD participated in the design and conducted the study, interpreted the results and helped to edit the manuscript. All the authors have read and approved the final version. WJD is the guarantor.

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