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BRIEF REPORT

Association of T-helper cell cytokine level with age in patients with biliary atresia: a preliminary study

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Abstract

Background The pathogenesis of biliary atresia (BA) is associated with an inflammatory process involving the biliary tree. This study aimed to investigate the association of T-helper cell cytokine levels with age in patients with BA.

Methods Twenty-eight patients with BA were divided into three groups according to their age (<2 months, 2–3 months, and ≥3 months). All the patients underwent Kasai portoenterostomy. Blood samples were collected from the patients preoperatively, and the liver tissue specimens were obtained during surgery. We detected serum levels of interleukin (IL)-1β, IL-12p70, interferon (IFN)-γ, IL-6, IL-10, and transforming growth factor (TGF)-β1 and liver expression of IL-1β, IL-6, and TGF-β1.

Results The serum levels of IL-1β, IL-12p70, IL-6, and IL-10 in patients aged ≥3 months were significantly higher than those in patients aged <2 months. There were no significant age-related differences in the IL-1β, IL-6 and TGF-β1 expression levels in the liver tissue of patients with BA.

Conclusions The serum levels of IL-1β, IL-6, IL-10 and IL-12p70 showed significant age-related differences in patients with BA. Interpretation of the role of cytokines in BA needs to take patient's age into consideration.

Keywords Biliary atresia · Cytokines · Pathogenesis · T-helper cell

Introduction

Biliary atresia (BA) is a frequent obliterative cholangiopathy of neonates, occurring in about 1 out of 17,000–19,000 live births in the UK and France, but with an estimated incidence up to 1:5000 in East Asian countries [1, 2]. As the most frequent identifiable cause of neonatal cholestasis, BA affects both extrahepatic and intrahepatic bile ducts, and can lead to duct obstruction and progressive liver cirrhosis [3–5]. The most effective treatment for BA is the Kasai operation [6]. However, despite surgical intervention, about 50–80% of cases still develop significant fibrosis and liver failure, and eventually require liver transplantation [7]. Early diagnosis and timely operation are vital for the treatment of BA. Better understanding of the underlying pathogenesis is important for us to develop new strategies for effective treatment.

However, the pathogenesis of BA is still poorly understood and several hypotheses have been reported, such as fibrosis, dysregulation of immunity and genetic predisposition [3, 8–10]. Bile duct injury in BA is suggested to be caused by exaggerated inflammatory or autoimmune response. The presence of $CD4^+$ T cells in portal tracts of BA patients has been reported [11]. Immunologic abnormalities especially the disorders of CD4+ T-helper (Th) cells play important roles in the pathogenesis of BA [12–15]. Some studies have suggested polarized Th-cell immunity at the early stages of BA, the Th1/Th2 paradigm, might be

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the cause of inflammation and biliary obstruction [16, 17]. However, many recent findings that examined the plasma and local cytokine levels did not find a skewed bias toward specific Th-cell immunity [18–20]. One of the reasons for the conflicting results might be that the Th-cell cytokine levels may change with age of the patients. The initiation of biliary injury in BA might have begun before birth, and the cytokine levels undergo a fast change in BA patients from birth to several months. The above studies included BA patients with large age variance (e.g., 37 days vs. 90 days) in the same group, so large differences in the cytokine levels were observed [20]. In this study, we divided the BA patients into three groups according to their age (< 2 months, 2–3 months, and ≥ 3 months), and investigated serum levels and expression of Th-cell cytokines in the liver tissue of patients with BA. By examining the Th-cell cytokines in the serum and liver of BA infants among different age groups, we want to provide new insights into the pathogenesis and clinical treatments of BA.

Methods

Patients

This study was approved by the Ethics Committee of the Children's Hospital of Zhejiang University, School of Medicine (no. 2014-IRB-006). Written informed consents were obtained from all patients' guardians. From Jan 2015 to Dec 2015, 28 consecutive patients diagnosed with BA were enrolled. Patients were divided into three groups according to their age (< 2 months, 2–3 months, and ≥ 3 months).

Quantification of serum cytokines

Peripheral blood was collected from the 28 patients with BA preoperatively in our hospital. Serum was separated immediately by centrifugation at 2000 r/min for 10 min, then was collected and stored at − 80 °C for enzyme-linked immunosorbent assay (ELISA) analysis. The serum concentrations of interleukin (IL)-1β, IL-6, IL-10, IL-12p70, interferon (IFN)-γ, and transforming growth factor (TGF)-β1 were determined using ELISA kit (R&D Systems, Wiesbaden, Germany) following the manufacturer's instructions.

Histology staining

All the 28 patients were treated with Kasai portoenterostomy and the liver tissue specimens were obtained during surgery. Liver tissue specimens were paraffin embedded for further analysis after formalin fixation. Liver sample sections (3 μm) were stained with a standard hematoxylin and eosin (H&E), or MASSON, following standard procedure. Immunohistochemistry (IHC) staining was performed according to the IHC kit instructions (EnVision™ Two-Step, DAKO, Denmark). The first antibodies were purchased from Abcam (Cambridge, MA, USA), including IL-1β (ab2105, 1:250), IL-6 (ab6672, 1:150), and TGF-β1 (ab92486, 1:250). After a secondary antibody was applied for 30 min at room temperature, the slides were stained with diaminobenzidine and hematoxylin. The appearance of yellow or brown particles in cytoplasm was considered as positive expression. The semi-quantitative scoring of the expression intensity in each sample was performed in a blinded manner and determined independently by three senior pathologists.

The Metavir scoring system was used to grade liver fibrosis based on the liver tissue specimens. There are five grades: F0 for no hepatic fibrosis, F1 for mild hepatic fibrosis, F2 for significant or obvious hepatic fibrosis, F3 for advanced hepatic fibrosis, and F4 for cirrhosis.

Statistical analysis

Data were shown as means ± SD. SPSS 22.0 software (SPSS Inc, IL, USA) was used for statistical analysis. One-way ANOVA was used to analyze the results between groups, with $P < 0.05$ considered to be significant.

Results

Demographics and laboratory data of the subjects

Demographic data are shown in Supplementary Table 1 and Fig. 1. There were no significant differences regarding gender, alanine aminotransferase, aspartate aminotransferase, direct bilirubin, and total bilirubin among the three groups ($P > 0.05$).

Serum cytokine levels

The results of ELISA showed that the serum levels of IL-1β, IL-10, IL-12p70, and IL-6 increased with age; and the levels were significantly higher in patients aged ≥ 3 months than those in patients aged < 2 months. No significant difference in IFN-γ and TGF-β1 was observed among BA patients with different ages ($P > 0.05$) (Fig. 2).

Expression of cytokines in the liver tissue of patients with BA and liver fibrosis

As shown in Fig. 3, there was no significant variation on the expression of IL-1β, IL-6 and TGF-β1 in the liver tissue of BA patients at different ages. There were no differences in

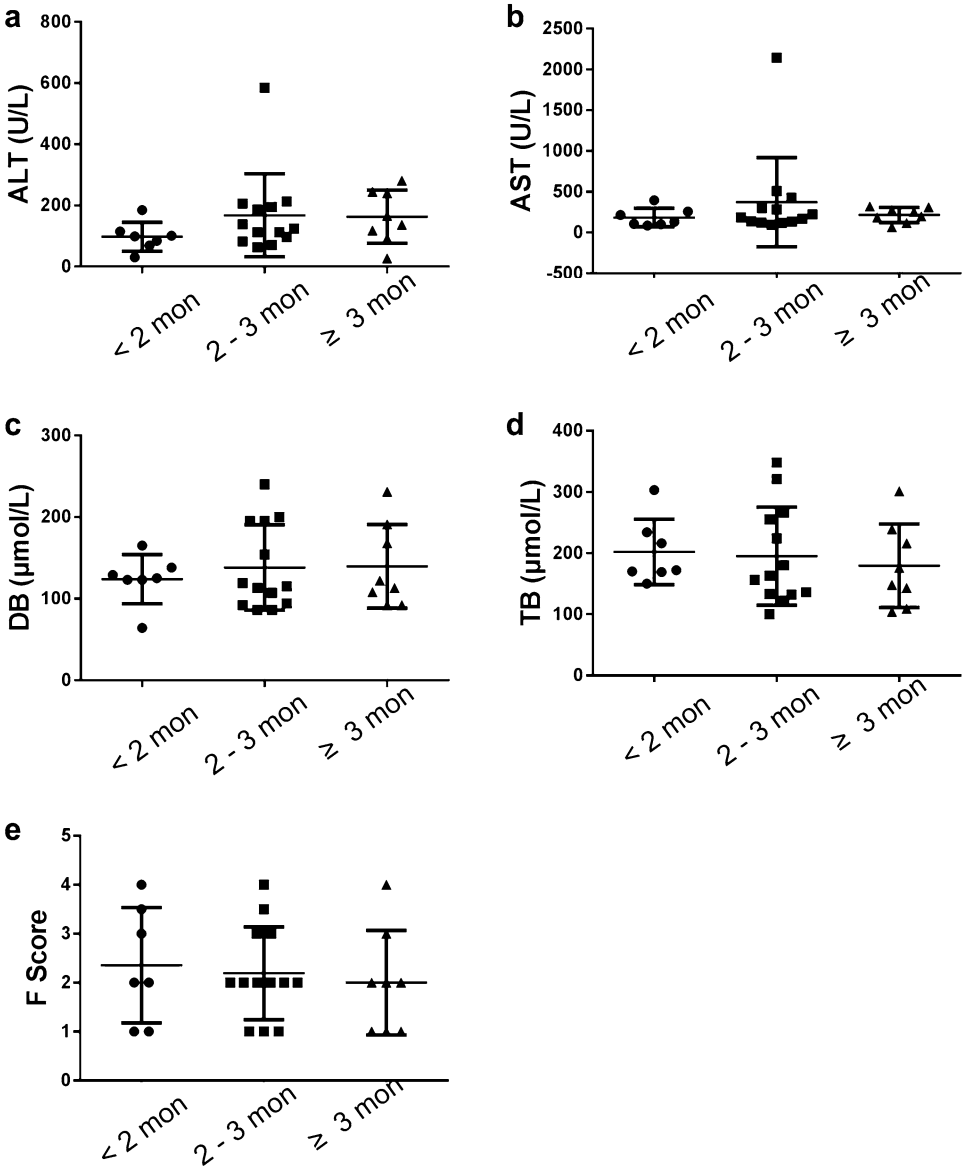

Fig. 1 ALT (**a**), AST (**b**), DB (**c**), TB (**d**) levels and liver fibrosis score (**e**) of the BA patients of different ages. *ALT* alanine aminotransferase, *AST* aspartate aminotransferase, *DB* direct bilirubin, *TB* total bilirubin, *BA* biliary atresia

levels of liver fibrosis among the three groups (Supplementary Table 1, Fig. 1).

Discussion

Th-cell cytokines, cellular adhesion molecules and macrophage markers play important roles in the pathogenesis of BA. Soluble intercellular adhesion molecule-1 and tumor necrosis factor α had significant positive correlations with post-natal age [20]. BA is characterized by progressive obstruction of the bile ducts with fibrosis and inflammation. Time point is an important variable for the fibrosis and inflammation process, so Th cytokines may change with patient's age. In this study, differences of serum levels of IL-1β, IL-12p70, IL-6 and IL-10 were observed among patients with BA of different age groups. These findings may be helpful for the understanding of the underlying pathogenesis of BA.

Many studies have explored the profiles of cytokines in patients with BA. Narayanaswamy et al. [20] examined the plasma levels of several cytokines before and after Kasai operation, and there were no significant differences in the baseline values for Th1, Th2 and macrophage cytokines between the BA group and controls. In Saito et al.'s study on preoperative blood samples, no significant differences were observed between the BA and control groups in serum levels of cytokine, including IL-1β, IL-10, IL-12p70, IFN-γ and IL-6 [19]. Although the differences in patient's age at examination may have significant influence on the levels of cytokines, no study has divided the BA patients into several sub-groups by age, which was due to

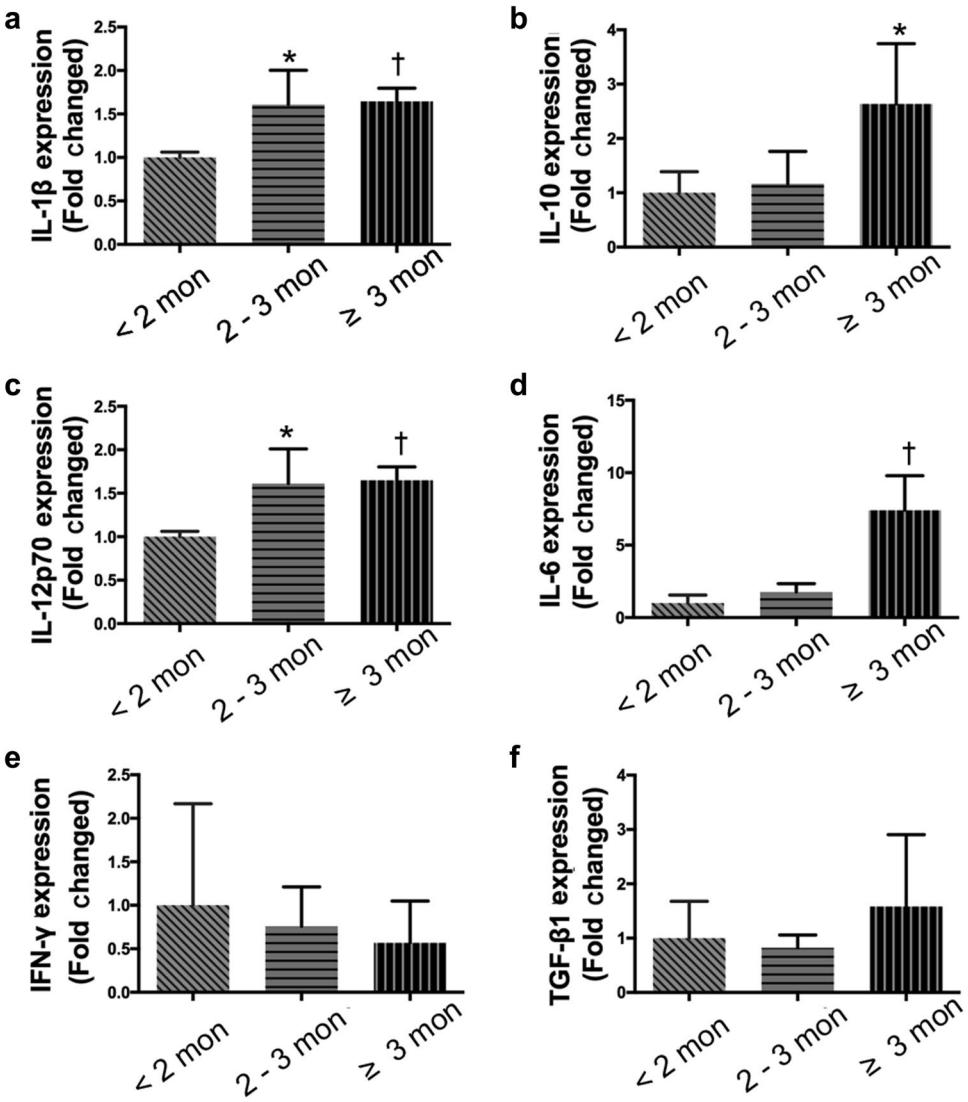

Fig. 2 Relative expression of serum cytokines from peripheral blood of patients with biliary atresia (BA) measured by enzyme-linked immuno sorbent assay (ELISA). **a** Interleukin (IL)-1β; **b** IL-10; **c** IL-12p70; **d** IL-6; **e** interferon (IFN)-γ; **f** transforming growth factor (TGF)-β1. $^{*}P < 0.05$ and † $P < 0.01$ compared with BA patients < 2 months

the challenging situations for sample collection and difficulty in including large sample size. The present study showed that the levels of some Th1 and Th2 cytokines in plasma changed with patients' age.

Activated T-helper (Th) cells are involved in BA pathophysiology [12]. The inflammatory process in liver is characterized by an initial infiltration of CD4+ T and natural killer cells. CD4+ T cells are capable of differentiating into either Th1 (driven by IL-2, IFN-γ, IL-12, and TNF-α) or Th2 effector cells (driven by IL-10 and IL-4). Before these cells infiltrate, macrophage/monocytes activate, infiltrate and proliferate to produce IL-18 and TNF-α, which initiate fibrosis.

We found that IL-1β, IL-6, IL-10 and IL-12p70 at the age of ≥ 3 months were significantly up-regulated compared with those aged < 2 months, indicating that the expressions of IL-1β, IL-6, IL-10 and IL-12p70 are different in the early and medium stages of BA. These cytokines were actively involved in the progressive inflammatory process of bile ducts in patients with BA. Interestingly, differences in IL1b and IL12p70 appeared at an earlier time (< 2 months vs. 2–3 months), while differences in IL6 and IL10 appeared later. These results indicated that during the process of BA, inflammation (IL1β) was followed by anti-inflammatory regulation (IL10). Previous study found TGF-β1 levels were increased after Kasai operation in patients with BA [18]. The relationship among IL-1β, IL-12p70, IL-10 and TGF-β1 in BA still needs further investigations.

There were several limitations to be addressed in this study. First, this was a cross-sectional study with a relatively small group of patient population, which may decrease the robustness of the conclusions. Second, there were no age-matched control groups; though comparison between BA infants in different age groups is the main aim of the current study, it is better to include three groups of healthy controls with matched age. Third, the number of

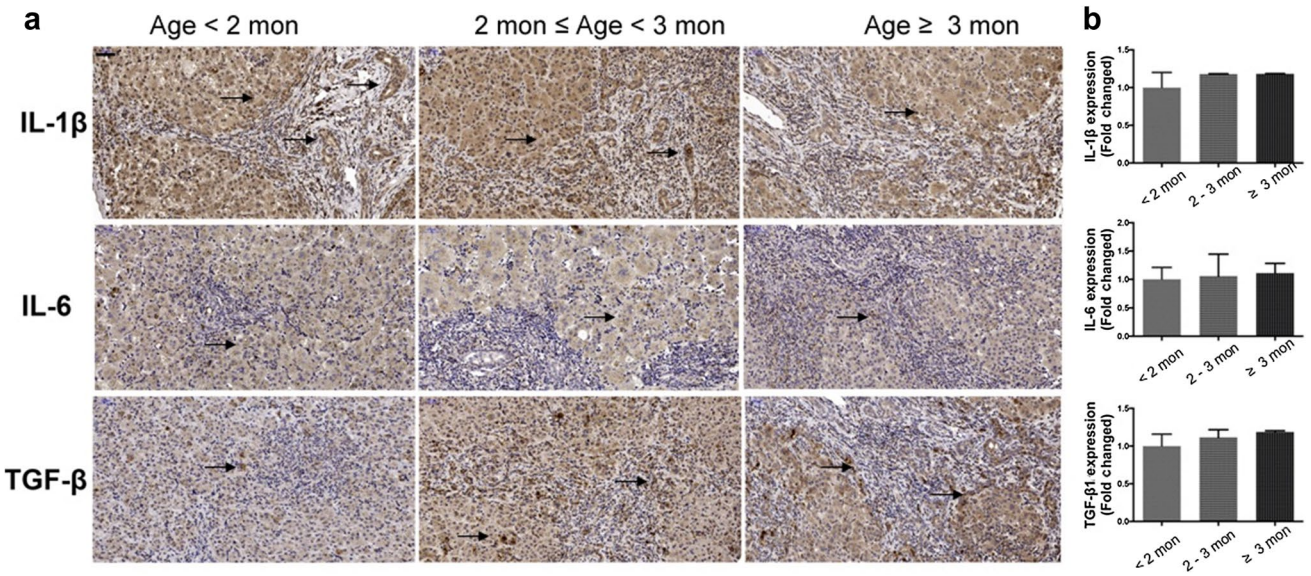

Fig. 3 Immunostaining of cytokines in the liver tissue of patients with BA at different ages. **a** Immunostaining of interleukin (IL)-1β, IL-6, and transforming growth factor (TGF)-β1 in the liver tissue of patients with biliary atresia; **b** the final immunoreactivity scores. Bar = 50 μm. Arrows indicate positive cells

Th cytokines being investigated in this study, however, is also relatively small. Thus, further studies with a larger patient cohort, more parameters and more precise testing methods are required to validate these results. Nevertheless, we compared the serum and liver Th-cell cytokine levels and further investigated the potential correlation between these cytokines and patient ages for the first time, which might provide useful insights into the pathogenesis of BA.

In conclusion, the levels of some Th1 and Th2 cytokines in plasma changed with time and disease progression. IL-1β, IL-10 and IL-12p70 were actively involved in the course of BA. However, differences in cytokine levels in liver tissues are not observed among patients of different age groups. Interpretation of the role of cytokines in BA needs to take patients' age into consideration. Further studies concerning patient's age, cytokines' levels and patient's outcome are warranted.

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Author contributions FBL participated in the design, analyzed data and contributed to the writing of the manuscript. XLS, HZ and WZG interpreted the results and helped to draft the manuscript. XXZ, KRP and SJH participated in the design of the study and helped to revise the manuscript. JFT 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. JFT is the guarantor.

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Compliance with ethical standards

Ethical approval This study was approved by the Ethics Committee of the Children's Hospital, Zhejiang University School of Medicine. Written informed consents were obtained from all patients' guardians.

Conflict of interest No financial or nonfinancial benefits have been received or will be received from any party related directly or indirectly to the subject of this article.

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