Expression of programmed death-1 and its ligands in the liver of biliary atresia

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Original article

Background: **An aberrant immune response is the predominant pathogenetic factor in biliary atresia (BA). Programmed death-1 (PD-1) and its two ligands, programmed death ligand-1 and programmed death ligand-2 (PD-L1 and PD-L2, respectively) play an important inhibitory role in immune reactions. We aimed to illustrate the expression of these molecules in BA.**

Methods: **Liver specimens were obtained from infants with BA during the Kasai procedure (early BA) and liver transplantation (late BA). Intrahepatic expression of PD-1, PD-L1, and PD-L2 were examined by immunostaining and compared with that in patients with neonatal hepatitis syndrome and normal controls. The correlation between the expression levels of these molecules in the liver and clinicopathological parameters was analyzed for each group.**

Results: **Enhanced expression of PD-1 and its ligands occurred in the livers with early BA. In the BA-affected livers, PD-1 was correlated with the degree of peri-biliary inflammation, while PD-L2 was linked more directly with portal fibrosis. None of the three molecules was correlated with the prognosis of the Kasai procedure in patients with early BA.**

Conclusion: **Only PD-1 and PD-L1 are involved in the immune reactions of early BA. Elucidation of the detailed role of PD-L2 in BA requires further research.**

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Introduction

Most patients with biliary atresia (BA) have the acquired type, which is characterized by progressive cytotoxic lymphocyte-induced inflammation and fibro-obliteration of the extrahepatic and/or intrahepatic bile ducts.[1,2] This immune response may be triggered by prenatal maternal microchimerism or perinatal viral/toxic insults.[3,4] The immunologic pathogenesis of BA includes activation of innate immunity, dysregulation of adaptive immunity, and development of other contributors to immune dysregulation such as regulatory T-cell (Treg) deficits.[5] BA is described as a CD4+ T helper 1 cell-mediated immunity with increased infiltration of CD4+, CD8+ T lymphocytes and CD68+ Kupffer cells in the portal tracts.[6] Additionally, combinatory effects of natural killer (NK) and CD8+ lymphocytes are involved in injury to the bile ducts.[7]

The two most important factors of immune regulation are the programmed death-1 (PD-1)/programmed death ligands (PD-Ls) pathway and Foxp3+ Tregs.[8] Compared with the abundance of studies on Tregs in BA,[9-11] no studies have yet addressed the role of the PD-1/PD-Ls pathway in BA. PD-1 is a member of the CD28/cytotoxic T lymphocyte antigen-4 superfamily. Engagement of PD-1 by its ligands PD-L1 (also known as B7-H1) or PD-L2 (also known as B7-DC) leads to depression of T-cell proliferation, cytokine production, and cytolytic function.[12] Crosstalk exists among the PD-1/PD-Ls pathway, T helper 17 cells, and Tregs in feto-maternal tolerance.[13] The PD-1/PD-Ls pathway also plays an important role in T-cell exhaustion in patients with virus hepatitis.[14] Expression of the PD-1 inhibitory pathway is more directly associated with the degree of inflammation than with the underlying etiology of liver damage.[15]

The aim of this study was to elucidate the expression

of PD-1 and its ligands in BA. We also evaluated the correlation between the expression of these molecules and clinicopathological parameters of BA.

Methods

Patients

Liver specimens were obtained from 28 infants with BA during Kasai procedure (early BA), another 15 infants with BA during liver transplantation after a failed Kasai operation (late BA), 15 infants with neonatal hepatitis syndrome (NHS) during operative cholangiography, and 5 normal living liver donors from July 2014 to July 2015. Fresh specimens were washed with sterile phosphate-buffered saline, fixed in formalin, and embedded in paraffin. Informed consent was obtained from each donor and the patient's parent or guardian, and the experimental protocol was approved by the Ethics Committee of Xinhua Hospital.

The diagnosis of BA relied on intraoperative cholangiography and liver histopathologic examination. All patients with early BA had type III BA and were followed up regularly after the Kasai procedure. The effect of the Kasai procedure was evaluated as "good" if the serum bilirubin level returned to <50 μmol/L within 3 months after surgery, "partial" if the bilirubin level dropped transiently after surgery but re-appeared and did not return to <50 μmol/L, and "poor" if the bilirubin level continued to rise.[16]

Histopathologic studies

Liver specimens were evaluated for peri-biliary inflammation and portal fibrosis as described by Russo et al.[17] Specifically, 4-5 μm thick sections were cut from the liver specimens. Hematoxylin-eosin staining was performed to evaluate the degree of infiltration of mononuclear inflammatory cells in the ducts. Filtration was graded as follows: A, absent; B, mild (present in occasional ducts); and C, multiple (present in many ducts). Masson's trichrome stain was used to grade portal fibrosis as follows: F0, absent or fibrous expansion of some portal tracts; F1, fibrous expansion of most portal tracts; F2, focal porto-portal bridging; F3, marked bridging; and F4, cirrhosis. Two observers (K.Z. and J.W.) independently interpreted the slides without knowledge of the clinical data, and differences were resolved by discussion.

Immunohistochemistry

Polyclonal rabbit antihuman PD-1 (Sigma, St. Louis, MO, USA; catalog number: HPA035981), polyclonal rabbit antihuman PD-L2 (Abcam, Cambridge, UK; catalog number: ab200377), and monoclonal rabbit antihuman PD-L1 (CST, Danvers, MA, USA; catalog number: 15165) were used for immunohistochemical staining according to a previously described protocol.[18] Negative controls were set using isotype-matched, concentration-matched immunoglobulin without primary antibodies; brown coloration of cells represented positive staining. The number of PD-1-, PD-L1-, and PD-L2-stained cells per high-power field (HPF; × 400) were counted using a light microscope (Eclipse; Nikon, Tokyo, Japan). One or two representative slides were taken from each specimen. Five HPFs in the portal areas were examined, and periductal areas were chosen.[19] The mean number of positive cells per HPF was calculated for each slide. Two authors (K.Z. and J.W.) evaluated the immunostained slides in a blinded fashion.

Immunofluorescence double staining

Immunofluorescence dual staining was performed to determine which cell types express PD-1, PD-L1, and PD-L2. Briefly, liver slides were incubated at 4°C overnight with diluted primary antibodies followed by diluted secondary antibodies for 45 minutes at room temperature. The following mouse-derived monoclonal primary antibodies (Abcam) were used to match PD-1 and its ligands: anti-CD4 (catalog number: ab846), anti-CD8 (catalog number: ab187279), anti-CD20 (catalog number: ab9475), anti-CD68 (catalog number: ab201340), anti-CD11b (catalog number: ab36939), anti-CD83 (catalog number: ab123494), anti-CD56 (catalog number: ab9018), anti-CK7 (catalog number: ab9021), and anti-CD31 (catalog number: ab24590). Alexa Fluor 488- and 594-conjugated polyclonal goat anti-rabbit (Jackson ImmunoResearch, West Grove, PA, USA; catalog number: 115-545-003) and polyclonal goat anti-mouse IgG antibodies (Jackson ImmunoResearch; catalog number: 115-585-003) were used as secondary antibodies. Finally, slides were incubated with 1 μg/mL 4',6-diamidino-2-phenylindole (CST; catalog number: 4083) for 10 min to stain the nuclei. Sections incubated with the appropriate isotype control primary antibodies were set as negative controls. The results were analyzed using a fluorescence microscope (Eclipse).

Statistical analysis

Descriptive results are expressed as mean±standard deviation or number (percentage). SPSS 19.0 software (IBM, Armonk, NY, USA) was used for data analyses. The Mann-Whitney *U* test or Chi-square test was used to assess comparisons between two groups as appropriate. Spearman's rank correlation was used to analyze the association between the number of stained cells and clinicopathological variables. A *P* value of <0.05 was considered statistically significant.

Results

Demographic information and clinical parameters

The infants in the late BA group were older than those in the NHS or early BA group. The sex distribution was similar among the three groups. No differences existed in the alanine aminotransferase (ALT) or aspartate aminotransferase (AST) concentration among the three groups except that the ALT concentration showed a marginal difference between the NHS and early BA groups. The early and late BA groups had similar γ-glutamyltransferase concentrations, but both were higher than those in the NHS group. The early BA and NHS groups had similar direct and total bilirubin concentrations, and both were lower than those in the late BA group (Table 1).

Peri-biliary inflammation and portal fibrosis grading in each group

We first evaluated the liver specimen length and the number of portal tracts in each group (Supplementary Table 1). Both the liver specimen length and number of portal tracts in the late BA group were higher than those in the early BA group or the NHS group; this may be because it is easier to obtain larger liver specimens during liver transplantation. However, there were no differences in the number of portal tracts per unit length (the ratio of portal tract number and length) among the three groups. We further analyzed whether differences existed among the NHS, early BA, and late BA livers according to inflammatory and fibrosis grading (Table 2). First, the distribution of specimens in the early BA group was similar to that in the late BA group with respect to the degree of peri-biliary inflammation (P=0.837). Specimens from the early and late BA groups had more severe portal fibrosis than those from the NHS group (P<0.01); in particular, more specimens from the late BA group exhibited cirrhosis than those from the early BA group.

Significantly elevated expression of PD-1 and its ligands in early BA

The expression of PD-1 and PD-Ls in liver specimens from normal controls, patients with NHS, patients with early BA, and patients with late BA were detected by immunohistochemistry. The results showed that PD-1 expression was absent or rare in the control and late

Table 2. Inflammatory and fibrosis scores in the NHS, early BA, and late BA livers

Variables	NHS (n=15)	Early BA (n=28)	Late BA (n=15)	P value		
				Early BA *vs.* NHS	Late BA *vs.* NHS	Early BA *vs.* late BA
Peri-biliary inflammation				0.046	0.078	0.837
A	8	7	3			
B	5	11	8			
C	2	10	4			
Portal fibrosis grading				<0.010	<0.010	<0.010
F0	7	2	0			
F1	5	4	0			
F2	2	7	1			
F3	1	12	3			
F4	0	3	11			

NHS: neonatal hepatitis syndrome; BA: biliary atresia. A: absent; B: mild (present in occasional ducts); C: multiple (present in many ducts); F0: absent or fibrous expansion of some portal tracts; F1: fibrous expansion of most portal tracts; F2: focal porto-portal bridging; F3: marked bridging; F4: cirrhosis.

Table 1. Patients' demographic and clinical features

Variables	Control (n=5)	NHS (n=15)	Early BA (n=28)	Late BA (n=15)	P (early BA *vs.* NHS)
Age	30.2±3.5 (y)	2.2±0.9 (mon)	2.5±1.2 (mon)	7.3±1.5 (mon)	0.139
Gender (male)	4	6	17	5	0.194
ALT (U/L)	44.4±12.7	97.4±52.0	175.5±138.4	151.1±107.8	0.041
AST (U/L)	19.4±6.3	213.4±150.4	268.4±176.2	279.7±271.3	0.198
γ-GT (U/L)	24.6±5.7	141.2±151.1	582.4±421.0	337.7±327.9	<0.010
DB (μmol/L)	2.6±1.5	88.8±33.6	109.6±46.3	187.5±98.4	0.202
TB (μmol/L)	8.8±3.3	153.2±51.1	180.3±86.8	299.1±168.7	0.111

NHS: neonatal hepatitis syndrome; BA: biliary atresia; ALT: alanine aminotransferase; AST: aspartate aminotransferase; γ-GT: γ-glutamyltransferase; DB: direct bilirubin; TB: total bilirubin.

Table 3. Number of positive cells in the normal control, NHS, early BA, and late BA livers

Variables	Control (n=5)	NHS (n=15)	Early BA (n=28)	Late BA (n=15)	P (early BA *vs.* NHS)
PD-1 (cells/HPF)	0.3±0.4	13.0±21.5	37.3±21.5	1.4±2.0	<0.010
PD-L1 (cells/HPF)	0.6±0.8	18.6±25.2	49.6±25.5	5.0±4.1	<0.010
PD-L2 (cells/HPF)	1.2±1.2	9.5±10.0	47.3±27.7	93.4±38.3	<0.010
PD-L1/PD-1	1.0±1.6	1.1±1.0	1.5±0.5	2.3±4.0	0.231
PD-L2/PD-1	1.6±2.4	0.7±0.7	1.8±1.6	24.5±32.3	<0.010
PD-L1/PD-L2	0.2±2.3	1.7±1.3	1.6±1.3	0.1±0.1	0.878

NHS: neonatal hepatitis syndrome; BA: biliary atresia; PD-1: programmed death-1; PD-L1: programmed death ligand-1; PD-L2: programmed death ligand-2; HPF: high-power field.

BA groups (Fig. A&J), while a small quantity of PD-1-positive cells was found mainly in the portal areas of 67% (10/15) of livers in the NHS group (Fig. D). However, abundant PD-1+ cells were observed within both the portal and lobule areas in the early BA group (Fig. G). Similar to the characteristic expression of PD-1, the expression of PD-L1 was absent or rare in normal control and late BA liver tissues (Fig. B&K), while its expression was found in 87% (13/15) of livers in the NHS group; the expression was restricted to the portal area (Fig. E). The expression of PD-L1 was abundant in all early BA specimens and was also mainly located in the portal area (Fig. H). The expression of PD-L2 was infrequent in normal control and NHS specimens (Fig. C&F). Increased expression of PD-L2 was found predominantly in the lobule areas of early BA liver specimens (Fig. I). However, in late BA liver tissues, PD-L2 expression was strongly expressed throughout the whole tissue section (Fig. L).

All of the PD-1+, PD-L1+, and PD-L2+ cells in the liver tissues of these detected samples were counted and compared (Table 3). The number of positive cells was significantly higher in the early BA group than that in the NHS and control groups. Interestingly, an obvious decrease in PD-1+ and PD-L1+ cells and an increase in PD-L2+ cells were found in the late BA group. We

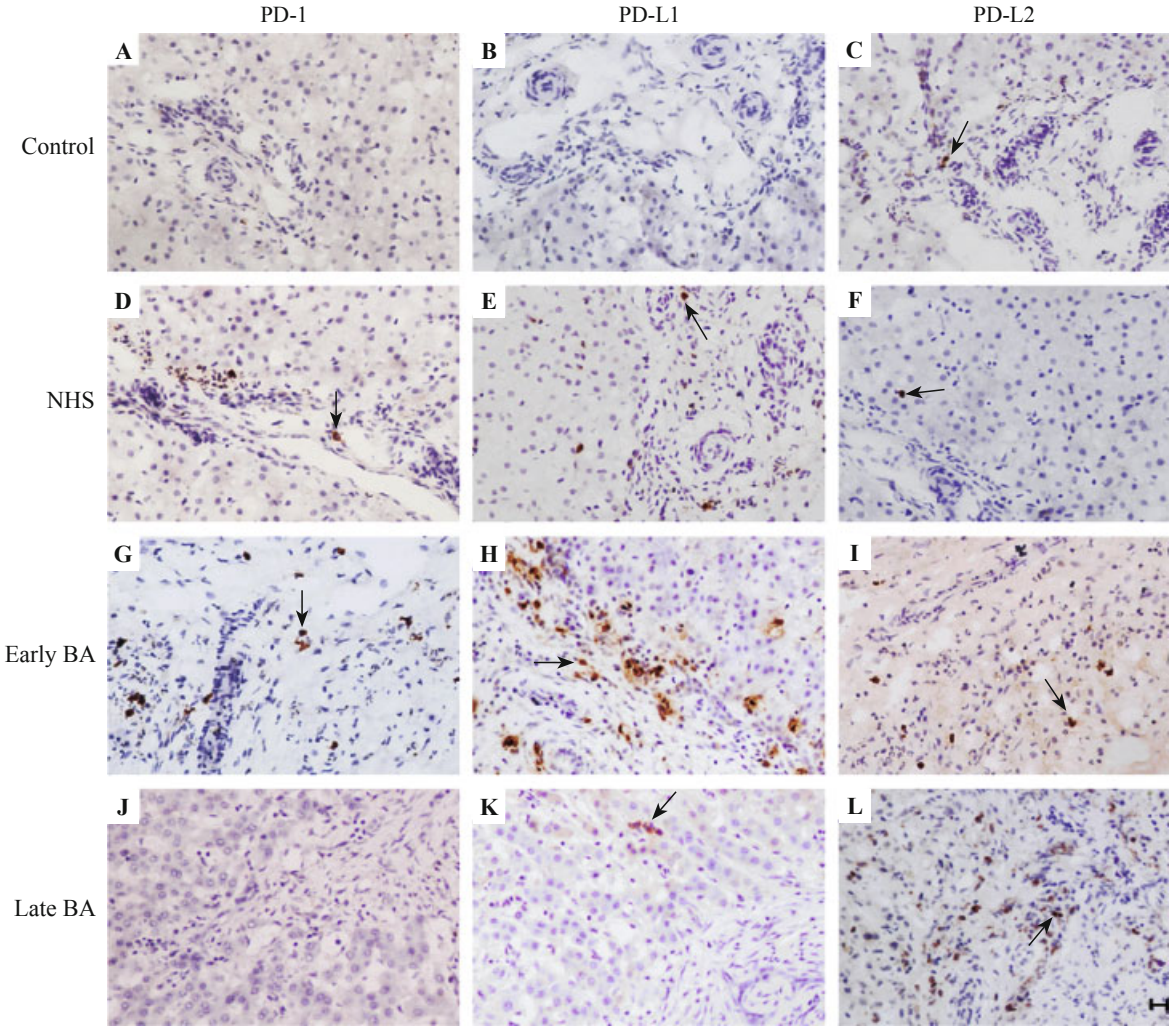

Fig. Expression of PD-1, PD-L1, and PD-L2 in the liver tissues from the normal control, NHS, early BA, and late BA groups were detected by immunohistochemistry. **A&B:** Expression of PD-1 and PD-L1 was absent in the normal liver; **C:** Small quantities of PD-L2-positive cells were found in the normal control liver tissues, and the expression was restricted to the periportal area; **D&E:** Small quantities of PD-1- and PD-L1-positive cells were found in the portal area in the NHS tissues; **F:** The expression of PD-L2 in NHS was similar to that in the normal controls; **G-I:** Elevated expression of PD-1, PD-L1, and PD-L2 was found in the early BA livers. PD-1-positive cells were found in the portal and lobular areas; the expression of PD-L1 was restricted to the portal area, while PD-L2 was found mainly in the lobular area. Expression of PD-1 and PD-L1 were absent in the livers of the late BA group (**J&K**), while significantly increased PD-L2 expression was found throughout the whole tissue section (**L**). The arrows indicate infiltrating positive cells, scale bar=20 μm. PD-1: programmed death-1; PD-L1: programmed death ligand-1; PD-L2: programmed death ligand-2; NHS: neonatal hepatitis syndrome; BA: biliary atresia.

also evaluated the PD-L1/PD-1, PD-L2/PD-1, and PD-L1/PD-L2 ratios in each group. The PD-L1/PD-1 ratio was very stable among the NHS, early BA, and late BA groups. The PD-L2/PD-1 ratio was not different between the early and late BA groups, while the PD-L1/PD-L2 ratio in late BA livers was clearly different from that of the other two groups.

Correlation of PD-1 and PD-L1 with degree of inflammation and stronger correlation of PD-L2 with fibrosis score

We analyzed the correlation between PD-1 and its two ligands as well as the correlations between these three molecules and the clinicopathological parameters in each group. In both the NHS and early BA groups, there was a significant correlation between PD-1 and PD-L1 expression; neither PD-1 nor PD-L1 was correlated with the expression of PD-L2 (Supplementary Tables 2&3). In the late BA group, we found no correlation between PD-1 and its two ligands (Supplementary Table 4). In the livers of all three groups, PD-1 was closely correlated with the degree of liver inflammation and PD-L2 was more directly correlated with the fibrosis score. Although PD-L1 was correlated with liver inflammation in the NHS and early BA groups, this correlation was not present in late BA livers. The expression of PD-1 was correlated with the serum ALT and AST concentrations in the NHS group, while both PD-1 and PD-L1 were correlated with the serum direct bilirubin and total bilirubin concentrations in the early BA group.

Phenotypes of positive cells in patients with early BA

The phenotypes of PD-1+, PD-L1+, and PD-L2+ cells in early BA livers were further examined by immunofluorescence double staining. PD-1 was expressed on CD4+ T cells, CD20+ B cells, CD68+ macrophages (Kupffer cells), CK-7+ epithelial cells, and CD31+ endothelial cells (Supplemental Fig. 1). PD-L1 expression was only observed on CD11b+ monocytes and granulocytes and CD83+ dendritic cells (Supplemental Fig. 2). Similarly, PD-L2 expression was only observed on CD4+ T cells and CD56+ natural killer (NK) cells (Supplemental Fig. 3).

Discussion

This is the first report to illustrate the PD-1/PD-Ls pathway in BA. In this study, we first found higher expression of PD-1 and its ligands in patients with early BA than that in patients with NHS and normal controls. These findings are consistent with those in adults with chronic virus hepatitis and autoimmune liver diseases.[20] However, the different distribution and function of PD-1 and its ligands may distinguish BA from other inflammatory liver diseases.

There was a significant correlation between PD-1 and PD-L1 in early BA livers; PD-1 or both PD-1 and PD-L1 were linked more directly with the degree of liver inflammation, while PD-L2 was more closely correlated with the degree of liver fibrosis in both early and late BA livers. This shows that the PD-1/PD-L1 axis plays an important role in the immunopathological damage to the biliary system in patients with BA. PD-1 was expressed in the portal and lobule areas of early BA livers, and the phenotype of positive cells included CD4+ T lymphocytes, B lymphocytes, macrophages, epithelial cells, and endothelial cells. The former three types of cells are reportedly involved in bile duct inflammation and obstruction in patients with BA.[21-23] The positive staining of these cells indicates the inhibitory role of PD-1 in immune reactions. Interestingly, however, PD-1 but not PD-Ls is expressed on epithelial and endothelial cells. Overexpression of major histocompatibility complex class II molecules and costimulatory factors (B7-1, B7-2, and CD40) have been found in the biliary epithelium and/or vessels in patients with BA.[23,24] The expression of PD-Ls should accompany the expression of these molecules. One theory regarding the pathogenesis of BA is the autoimmunity theory, in which biliary epithelia act as "autoantigens" and sustain attacks from all types of inflammatory cells.[25] The overexpression of PD-1 on epithelial and endothelial cells may occur by decreasing the "autoantigen" concentration to alleviate the immune reaction. However, the potential PD-1-induced dysfunction of epithelial and endothelial cells could aggravate the pathological damage. Determination of the detailed effects of PD-1 on the epithelium and vessels requires further study.

Compared with broader expression of PD-1 in the early BA livers, the expression of PD-L1 and PD-L2 were relatively restricted. PD-L1 was mainly expressed on monocytes, granulocytes, and mature dendritic cells in the portal area. The recruitment of myeloid monocytes and granulocytes in BA was nonspecific and associated with bile duct obstruction rather than an antigen-specific immune reaction against the biliary epithelium.[26,27] Studies have shown that the expression of PD-L1 alone may lead to monocyte dysfunction.[28] In a murine BA model, an increased population of plasmacytoid dendritic cells worked with conventional dendritic cells to stimulate proliferation of CD4+ and CD8+ T lymphocytes and activate NK cells in the early phase of the pathogenesis of biliary injury.[29] Another study showed that mature dendritic cells can also modulate liver fibrogenesis.[30] High expression of PD-L1 on dendritic cells conferred T lymphocyte and NK cell immune suppression by engagement of PD-1.[31]

Another interesting finding of this study is the discordant expression of PD-L2 in early BA livers and its correlation with the progression of fibrosis. The effect of PD-L2 on CD4+ T lymphocytes and NK cells is still unclear because aside from the inhibitory effect, PD-L mutants with abolished PD-1 binding capacity can still co-stimulate proliferation and cytokine production of T cells.[32] Studies have found that a portion of patients with BA exhibit predominance of the T helper 2 cell response[21] and that T helper 2 cytokines in the liver promote liver fibrosis.[33] NK cells are key initiators of biliary epithelial injury in experimental BA; however, NK cells can ameliorate liver fibrosis by killing activated stellate cells.[34,35]

None of the three molecules were expressed on CD8+ lymphocytes, another vital effector cell type in cholangiocyte injury. Coupled with Treg deficiency in BA,[9] the CD8+ lymphocytes were basically uninhibited. This also demonstrates that the PD-1/PD-Ls pathway is not correlated with the prognosis of the Kasai procedure. Moreover, a significant decrease in PD-1 and PD-L1 expression was shown in late BA. Related studies have found that inflammation is often less severe in late diagnosis of BA.[36]

In summary, this study showed elevated PD-1/PD-Ls pathway expression in early BA. PD-1 and/or PD-L1 expression were correlated with liver inflammation, and the PD-1 distribution on epithelial cells in early BA livers shows that these two molecules are involved in the process of immunopathological damage to the biliary tract in BA livers. The dissimilar expression of PD-L2 was more directly associated with liver fibrosis and the detailed role of PD-L2 in BA requires further research. None of the three molecules were expressed on CD8+ lymphocytes, which suggests that they are not related to the prognosis of patients who undergo the Kasai procedure.

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Ethical approval: Informed consent was obtained from each enrolled donor and the patient's parent or guardian before sample collection. The experimental protocol was approved by the Ethics Committee at Xinhua Hospital.

Competing interest: The authors of this manuscript have no conflict of interest to disclose.

Contributors: Cai W and Zhang JJ contributed to conception and design of the research, and critically revised the manuscript. Wang PL, Wang J, Zhou Y, Chen XS, Zhou KJ and Wen J contributed to acquisition, analysis, or interpretation of the data. Wang PL drafted the manuscript. Wang PL, Zhou Y, Zhou KJ, and Wen J agreed to be fully accountable for ensuring the integrity and accuracy of the work. All authors read and approved the final manuscript.

References

1 Balistreri WF, Grand R, Hoofnagle JH, Suchy FJ, Ryckman FC, Perlmutter DH, et al. Biliary atresia: current concepts and research directions. Summary of a symposium. Hepatology 1996;23:1682-1692.
2 Schreiber RA, Kleinman RE. Genetics, immunology, and biliary atresia: an opening or a diversion? J Pediatr Gastroenterol Nutr 1993;16:111-113.
3 Hayashida M, Nishimoto Y, Matsuura T, Takahashi Y, Kohashi K, Souzaki R, et al. The evidence of maternal microchimerism in biliary atresia using fluorescent in situ hybridization. J Pediatr Surg 2007;42:2097-2101.
4 Howard ER, Tan KC. Biliary atresia. Br J Hosp Med 1989;41: 123-124, 128-130.
5 Feldman AG, Mack CL. Biliary atresia: cellular dynamics and immune dysregulation. Semin Pediatr Surg 2012;21:192-200.
6 Mack CL, Tucker RM, Sokol RJ, Karrer FM, Kotzin BL, Whitington PF, et al. Biliary atresia is associated with CD4+ Th1 cell-mediated portal tract inflammation. Pediatr Res 2004;56:79-87.
7 Guo C, Zhu J, Pu CL, Deng YH, Zhang MM. Combinatory effects of hepatic CD8+ and NK lymphocytes in bile duct injury from biliary atresia. Pediatr Res 2012;71:638-644.
8 Germanidis G, Argentou N, Hytiroglou P, Vassiliadis T, Patsiaoura K, Germenis AE, et al. Liver FOXP3 and PD1/PDL1 expression is down-regulated in chronic HBV hepatitis on maintained remission related to the degree of inflammation. Front Immunol 2013;4:207.
9 Brindley SM, Lanham AM, Karrer FM, Tucker RM, Fontenot AP, Mack CL. Cytomegalovirus-specific T-cell reactivity in biliary atresia at the time of diagnosis is associated with deficits in regulatory T cells. Hepatology 2012;55:1130-1138.
10 Lages CS, Simmons J, Chougnet CA, Miethke AG. Regulatory T cells control the CD8 adaptive immune response at the time of ductal obstruction in experimental biliary atresia. Hepatology 2012;56:219-227.
11 Miethke AG, Saxena V, Shivakumar P, Sabla GE, Simmons J, Chougnet CA. Post-natal paucity of regulatory T cells and control of NK cell activation in experimental biliary atresia. J Hepatol 2010;52:718-726.
12 Riley JL. PD-1 signaling in primary T cells. Immunol Rev 2009; 229:114-125
13 Tripathi S, Guleria I. Role of PD1/PDL1 pathway, and TH17 and treg cells in maternal tolerance to the fetus. Biomed J 2015;38:25-31.
14 Xie Z, Chen Y, Zhao S, Yang Z, Yao X, Guo S, et al. Intrahepatic PD-1/PD-L1 up-regulation closely correlates with inflammation and virus replication in patients with chronic HBV infection. Immunol Invest 2009;38:624-638.
15 Kassel R, Cruise MW, Iezzoni JC, Taylor NA, Pruett TL, Hahn YS. Chronically inflamed livers up-regulate expression of inhibitory B7 family members. Hepatology 2009;50:1625-1637.
16 Ernest van Heurn LW, Saing H, Tam PK. Cholangitis after hepatic portoenterostomy for biliary atresia: a multivariate analysis of risk factors. J Pediatr 2003;142:566-571.
17 Russo P, Magee JC, Boitnott J, Bove KE, Raghunathan T, Finegold M, et al. Design and validation of the biliary atresia research consortium histologic assessment system for cholestasis in infancy. Clin Gastroenterol Hepatol 2011;9:357-362.e2.

18 Konishi J, Yamazaki K, Azuma M, Kinoshita I, Dosaka-Akita H, Nishimura M. B7-H1 expression on non-small cell lung cancer cells and its relationship with tumor-infiltrating lymphocytes and their PD-1 expression. Clin Cancer Res 2004;10:5094-5100.
19 Kotb MA, El Henawy A, Talaat S, Aziz M, El Tagy GH, El Barbary MM, et al. Immune-mediated liver injury: prognostic value of CD4+, CD8+, and CD68+ in infants with extrahepatic biliary atresia. J Pediatr Surg 2005;40:1252-1257.
20 Mataki N, Kikuchi K, Kawai T, Higashiyama M, Okada Y, Kurihara C, et al. Expression of PD-1, PD-L1, and PD-L2 in the liver in autoimmune liver diseases. Am J Gastroenterol 2007;102:302-312.
21 Li J, Bessho K, Shivakumar P, Mourya R, Mohanty SK, Dos Santos JL, et al. Th2 signals induce epithelial injury in mice and are compatible with the biliary atresia phenotype. J Clin Invest 2011;121:4244-4256.
22 Feldman AG, Tucker RM, Fenner EK, Pelanda R, Mack CL. B cell deficient mice are protected from biliary obstruction in the rotavirus-induced mouse model of biliary atresia. PLoS One 2013;8:e73644.
23 Kobayashi H, Puri P, O'Briain DS, Surana R, Miyano T. Hepatic overexpression of MHC class II antigens and macrophage-associated antigens (CD68) in patients with biliary atresia of poor prognosis. J Pediatr Surg 1997;32:590-593.
24 Kobayashi H, Li Z, Yamataka A, Lane GJ, Miyano T. Role of immunologic costimulatory factors in the pathogenesis of biliary atresia. J Pediatr Surg 2003;38:892-896.
25 Zagory JA, Nguyen MV, Wang KS. Recent advances in the pathogenesis and management of biliary atresia. Curr Opin Pediatr 2015;27:389-394.
26 Changho S, Ahmed AA. Neutrophils in biliary atresia. A study on their morphologic distribution and expression of CAP37. Pathol Res Pract 2010;206:314-317.
27 Tracy TF Jr, Dillon P, Fox ES, Minnick K, Vogler C. The inflammatory response in pediatric biliary disease: macrophage phenotype and distribution. J Pediatr Surg 1996;31:121-125; discussion 125-126.
28 Zhang Y, Zhou Y, Lou J, Li J, Bo L, Zhu K, et al. PD-L1 blockade improves survival in experimental sepsis by inhibiting lymphocyte apoptosis and reversing monocyte dysfunction. Crit Care 2010;14:R220.
29 Saxena V, Shivakumar P, Sabla G, Mourya R, Chougnet C, Bezerra JA. Dendritic cells regulate natural killer cell activation and epithelial injury in experimental biliary atresia. Sci Transl Med 2011;3:102ra94.
30 Blois SM, Piccioni F, Freitag N, Tirado-Gonzalez I, Moschansky P, Lloyd R, et al. Dendritic cells regulate angiogenesis associated with liver fibrogenesis. Angiogenesis 2014;17:119-128.
31 Ray A, Das DS, Song Y, Richardson P, Munshi NC, Chauhan D, et al. Targeting PD1-PDL1 immune checkpoint in plasmacytoid dendritic cell interactions with T cells, natural killer cells and multiple myeloma cells. Leukemia 2015;29:1441-1444.
32 Wang S, Bajorath J, Flies DB, Dong H, Honjo T, Chen L. Molecular modeling and functional mapping of B7-H1 and B7-DC uncouple costimulatory function from PD-1 interaction. J Exp Med 2003;197:1083-1091.
33 Tanabe K, Taura K, Koyama Y, Yamamoto G, Nishio T, Okuda Y, et al. Migration of splenic lymphocytes promotes liver fibrosis through modification of T helper cytokine balance in mice. J Gastroenterol 2015;50:1054-1068.
34 Radaeva S, Sun R, Jaruga B, Nguyen VT, Tian Z, Gao B. Natural killer cells ameliorate liver fibrosis by killing activated stellate cells in NKG2D-dependent and tumor necrosis factor-related apoptosis-inducing ligand-dependent manners. Gastroenterology 2006;130:435-452.
35 Shivakumar P, Sabla GE, Whitington P, Chougnet CA, Bezerra JA. Neonatal NK cells target the mouse duct epithelium via Nkg2d and drive tissue-specific injury in experimental biliary atresia. J Clin Invest 2009;119:2281-2290.
36 Muraji T, Suskind DL, Irie N. Biliary atresia: a new immunological insight into etiopathogenesis. Expert Rev Gastroenterol Hepatol 2009;3:599-606.

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