

# Comparison of CD4Foxp3and CD4IL-17

*by* Bagus Setyoboedi

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## Research



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### Comparison of CD4+/Foxp3+ and CD4+/IL-17+ cells counts on cholestatic infants with and without biliary atresia

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## Abstract

**Introduction:** biliary atresia (BA) is a progressive obstructive cholangiopathy with high mortality which is caused by immune system dysregulation and multiple hit mechanism that involves T-reg cells (represented by CD4+/FoxP3+ cells) and Th17 (CD4+/IL-17+). This study aims to compare CD4+/FoxP3+ and CD4+/IL-17+ cells counts on cholestasis infants with and without BA. **Methods:** liver biopsies of cholestasis infants which are range of age 1 -12 months old hospitalized in Pediatric Ward of Dr. Soetomo General Hospital, Surabaya for a period of 6 months. Hematoxylin-eosine (HE) staining was performed for diagnosing of BA. Immunohistochemistry (IHC) staining was done for counting of CD4+/FoxP3+ and CD4+/IL-17+ cells. **Results:** a total of 34 samples in this study, 20 infants with BA and 14 without BA. The number of CD4+/FoxP3+ cells on BA group is fewer than non-BA group (median: 6 vs. 14 cells/high power field (hpf);  $p < 0,001$ ). The number of CD4+/IL-17+ cells on BA group higher than non-BA group (median: 7 vs. 5 cell/hpf;  $p = 0,04$ ). **Conclusion:** the number of CD4+/FoxP3+ cells is fewer, while number of CD4+/IL-17+ is higher on cholestasis infants with BA compared to without BA.

## Introduction

Biliary atresia (BA) is a progressive fibro-obliterative cholangiopathy of intrahepatic and extrahepatic bile duct in varying degrees and produce obstruction to the flow of bile, cholestasis and jaundice in neonates [1-3]. Without adequate surgical therapy such as Kasai procedure (hepatoportoenterostomy) that forming a drainage of bile from the bile duct residual in the liver, liver fibrosis will take place progressively lead to cirrhosis, portal hypertension, liver failure, and death at the age of 2 years [1,2]. Biliary atresia (BA) is also the most common cause of liver transplantations in infants [2]. Biliary atresia (BA) relatively rare in the United States and Europe with the incidence rate of 1: 15000-19000 live births [3], while in Asia the incidence is higher, ranging from 1: 5400-5800 live

births in Taiwan and 1: 9000-10000 live births in Japan [4]. The etiology of BA is not known with certainty, the theory of the pathogenesis of the disease include: genetic disorders, exposure to toxins, viral infection, and autoimmune processes that cause damage to the bile ducts. The pathogenesis of BA involving the hepatobiliary infection by cholangiotropic viruses and bile duct cells damage mediated by autoreactive T cells, so that even if the virus has been eliminated, persistent inflammation and bile duct epithelial damage is still continuing [5,6]. Bile duct epithelial cell damage occurs because bile duct epithelial cells is expressed as an antigen or "self" antigens that are recognized as foreign and cause auto reactivity of T cells (bystander activation pathway) and activation of inflammatory mediators on the epithelium of the bile duct. Another mechanism is the presence of viral proteins that are structurally similar to the protein bile duct epithelium initiates the autoimmune process called molecular mimicry [5,7].

Efforts to control the immune response in order to prevent bystander damage to cells or healthy tissues yet widely known. Regulatory T cells (T-reg) are considered have a role to prevent the activation of autoreactive T cells or bystander activation [5,7]. Meanwhile Th17 cells by proinflammatory cytokines actually increase the effects of autoimmunity [8,9]. Several studies, both in animals and humans experimental indicated a decrease in the number or function of T-regs in subjects with AB, so it is not able to suppress the immune response to self-antigens, in this case were cholangiocytes, so that the inflammatory process goes on chronically [10-13]. The imbalance between the number of CD4+/FoxP3+ and CD4+/IL-17+ in this case a decrease in CD4+/FoxP3+ and an increase in CD4+/IL-17+ suspected aggravate autoimmune conditions, where the effector cells more active and progressive in attacking the body's own cells [8,9,14]. This prompted the need for research studies to determine the profile of CD4+/FoxP3+ and CD4+/IL-17+ in order to understand the pathogenesis of AB. By knowing the pathogenesis of BA mainly via the CD4 + / FoxP3 +

and CD4+/IL-17+ is able to detect and intervene early occurrence of chronic inflammatory and fibrotic process so as to prevent the incident of AB. This study was aimed to compare CD4+/FoxP3+ and CD4+/IL-17+ cells counts on cholestasis infants with and without BA.

## Methods

This research was an analytic observational within cholestasis patients 1-12 months as subjects. Liver biopsies were performed to all of the subjects. Then, subjects were divided into biliary atresia (BA) and non-biliary atresia (non BA) according to its histopathology examination. CD4+/FoxP3+ cells were defined as cells expressing CD4+/FoxP3+ that seemed to glow red in the preparation slice paraffin blocks of liver biopsies, base stained with Mayer hematoxylin and then stained with a human monoclonal antibody CD4 + followed by double staining with a human monoclonal antibody FoxP3 +, and then viewed below light microscopes (Olympus) with a magnification of 1000X (each containing approximately 1500 cells) in 20 visual fields, then counted the number of cells in the portal area and around the biliary tracts, then the numbers were averaged. CD4+/IL-17+ cells were defined as cells expressing CD4+/IL-17+ that seemed to glow red in the preparation of slice of paraffin blocks of liver biopsies base stained with Mayer hematoxylin and then stained with a human monoclonal antibody of CD+ followed by double-staining with a human monoclonal antibody IL-17, and then seen under a microscope (Olympus) with 1000X magnification (which each contain approximately 1500 cells) in 20 visual fields, then counted the number of cells in the portal areas and around the biliary tracts and then the numbers were averaged. The statistical analysis used is descriptive analysis to determine the characteristics of the sample, cholestasis profile, and the number of CD4+ /FoxP3+ cells and CD4+ /IL-17+ cells in infants with cholestasis by frequency distribution table. Different test using Chi-square / Fisher exact test for nominal data, and t-test or

Mann-Whitney U test for numerical data.  $P < 0.05$  was considered statistically significant.

## Results

Thirty-four samples of liver biopsies were obtained from 34 patients with cholestasis. Demographic data, clinical condition, as well as laboratory data on each of the study groups as shown in Table 1. From these data it was found that in the group of biliary atresia female number was larger than male, while the age of the subjects in both groups were relatively similar. Weight and height are a larger in BA group than the non-BA. Nutritional status in both groups also mostly into the category of good nutrition. Symptoms of pale stools and dark urine were also obtained in both groups in the same relative proportions. Hepatomegaly obtained in all patients in BA group, and in almost all patients in the group of non-BA. The laboratory results (direct bilirubin, total bilirubin, ALT AST, and platelets) did not show any significant difference in both groups. Based on the criteria of bile plug, ductular proliferation, and periportal inflammation / fibrosis as the basis for determining the non-atresia and biliary atresia, then obtained 20 samples in accordance with biliary atresia and 14 non biliary atresia. Figure 1 below shows histopathologic features of BA group. Figure 2 shows the immunohistochemistry staining, there were expressions of FoxP3 in CD4+/FoxP3+ cells, and interleukin 17 on CD4 + / IL-17 +. The number of CD4+/FoxP3+ cells was significantly much lower obtained in the BA group compared with the non-AB. Instead CD4+/IL-17+ cells count in the BA group is higher than the non BA group (Table 2).

## Discussion

This study is part of a study on human BA through a percutaneous liver biopsy which has never been done in Indonesia, given that most studies of BA conducted in experimental animals. In this study, the diagnosis of BA determined by histopathological examination of percutaneous liver biopsies and immunohistochemically staining

to performed determine differences in the number of CD4+/FoxP3+ and CD4+/IL-17+ cells among infants with cholestasis. Based on histopathology, in this study, 20 of 34 subjects were biliary atresia in which the majority were female. This is consistent with several previous studies that showed that female have a risk for BA 2-3 times compared to male [1,4]. In this study, in which the samples were obtained by total sampling, found the median age is relatively similar between BA groups and non-BA. Nonetheless effect on the length and weight were obtained. Average weight is higher in group BA compared with non-BA. Similarly, the median length of the body of BA group are also longer than non-BA. However, when viewing the nutritional status, it seemed there was no significant difference between the 2 study groups. Symptoms of pale stools and dark urine also appeared in both study groups. It could happen, given the level of direct bilirubin of both groups equally increased, suggesting that in the non-BA group may also occur an obstructive cholestasis. But because no diagnostic approach performed on non-BA group, so it was not known which type of obstructive cholestasis. AST, ALT, and platelets are relatively similar in both groups. This indicated that in the both groups might also occur inflammation that caused destruction of hepatocytes cells. The median value of platelets in both groups showed similar results, and still within the range of normal values. Several studies have demonstrated the value of platelets decline in the state of cholestasis [15,16]. Yet another report platelet value was within normal limits, or even the research in platelet values have increased in BA group [17].

<sup>24</sup> In this study, there are significant differences in the number of CD4 + / FoxP3 + on liver biopsy between BA and non-BA groups. Furthermore, with the different test Mann-Whitney U test did seem that the number of CD4 + / FoxP3 + on liver biopsy differ highly significant with  $p < 0.001$  between BA and non-BA groups. These results suggest that a decrease in CD4 + / FoxP3 + T-reg cell or in group BA happen dramatically. This is consistent with some previous studies both in animals and in

humans. Previous study stated that specifically inducible CD25 is a marker of T-reg (iT-reg) and not found in natural Treg (nT-reg), which nT-reg usually more common in early infancy [14]. Another study compared liver biopsies of children BA with control healthy children, found the number of T-reg cells (CD4 + / FoxP3 +) from the peripheral blood and in liver tissue decreased in children dg AB, while Th17 (CD4 + / IL17A +) increased in group AB [9]. While studies in mice almost all showed a decrease in the number of T-reg cells in the BA group than in the control group [10, 12]. Previous study used the definition CD4 + / CD25 + FoxP3 + as T-reg cells [10,11] while another study defined T-reg cells as CD4 T cells that express FoxP3 [12]. Previous study depleted T-reg cells (CD4 + / CD25 + / FoxP3 +) mice and then induct them with CMV, found that BA condition occurred with the increased CD3, CD8, TNF- $\alpha$ , IFN- $\gamma$  activated genes (STAT-1), lymphotactin, IL-12p40, MIP-1Y, MCSF in groups of mice BALB/c which T-reg were depleted [13]. The environment of existing cytokines showed elevated levels of TGF $\beta$  without an increase in the others so that the direction of CD4 naïve T cell differentiation was driven into the T-reg cells differentiation [8,14].

The CD4 + / IL-17 + or Th17 cells seemed had a role in non-BA cholestasis group, which unfortunately in this study was not conducted alot of diagnosis for each case or samples. Furthermore, in this study, with Mann-Whitney U test it appeared that the number of CD4 +/IL-17 + cells on a liver biopsy between BA and non-BA groups differ significantly with  $p = 0.04$ . This is consistent with some previous studies both in animals and in humans. The number and percentage of Th17 (CD4 + / IL17 +) from the peripheral blood and in liver tissue increased in children with BA [9]. Previous study reported that Th17 cells infiltrating the liver with AB and associated with poor output of surgery, as well as the acquisition of Th1 dominance on the AB associated with CMV. Later in the qRT-PCR analysis found that there was a 5-6 fold difference in terms of specific transcription factor ROR- $\gamma$ t Th17 and IL-17a in the AB group compared with controls [16]. High dominance of CD4 T cells that secrete IL-17

over the CD4 + T cells expressing FoxP3 pointed to evidence that the effects of pro-inflammatory interleukin 17 accompanied by dysregulation of the immune in the case of peripheral tolerance by T-reg cells (CD4 + / FoxP3 +) aggravate the injury of cholangiocyte that lead to BA conditions. The theory of subset of T-helper cells plasticity mentioned the possibility of the CD4 + / FoxP3 + cells transitions into Th17 cells, as seen in some other autoimmune diseases, such as autoimmune hepatitis and psoriasis [14]. On the other hand, there is the plasticity of Th17 into Th1 through the influence of cytokines IL-1 $\beta$ , IL-23 and IL-12 in autoimmune diseases aggravate injury to the cells themselves (self).

This study strengthens the evidence that the reduction in the number of CD4 + / FoxP3 + cells and increasing of the CD4 + / IL-17 cells + were associated with biliary tracts injuries. Biliary atresia can be avoided if we are able to maintain the number of CD4 + / FoxP3 + stays high and the levels of IL-17 is low. On the other hand differences in the number of CD4 + / FoxP3 + significantly between cholestasis infants with and without BA can be used for screening or diagnostic test in the determination of BA in infants with cholestasis. However, this needs to be studied further by comparing the number of CD4 + / FoxP3 + cells on liver biopsy with circulating cells in the peripheral blood. This study is the first study assessing the number of CD4 + / FoxP3 + and CD4 + / IL-17 + from percutaneous liver tissue biopsies in infants with cholestasis by immunohistochemistry in Indonesia, where operations Kasai is still relatively rare, especially liver transplants. Blinding was done through codification samples in paraffin blocks containing tissue biopsy of the liver that is sent to the inspector of CD4 + / FoxP3 + and CD4 + / IL-17 + by immunohistochemistry not knowing about clinical condition, laboratory, and the diagnosis which were based on histopathological examination. The limitations of this study include: 1) sampling technique that uses total sampling method; 2) diagnosis of AB instead of using the gold standard (intraoperative cholangiography).

## Conclusion

The number of CD4 + / FoxP3 + cells in BA infants with cholestasis is lower than without BA and the number of CD4 + / IL-17 + cells in BA infants with cholestasis is higher than without AB. The further research on T-reg and Th17 in infants cholestasis by considering the larger size of the sample is needed, examination markers of these cells and their associated cytokines or chemokines both in the peripheral blood and in liver biopsies, as well as the possibility to become a marker in screening or diagnostic tests.

### What is known about this topic

- Biliary atresia (BA) is a progressive obstructive cholangiopathy with high mortality;
- BA is caused by immune system dysregulation;
- There is a multiple hit mechanism that involves T-reg cells (represented by CD4+/FoxP3+ cells) and Th17 (CD4+/IL-17+) in BA.

### What this study adds

- The number of CD4 + / FoxP3 + cells in BA infants with cholestasis is lower than without BA;
- The number of CD4 + / IL-17 + cells in BA infants with cholestasis is higher than without AB;
- The reduction in the number of CD4 + / FoxP3 + cells and increasing of the CD4 + / IL-17 cells + were associated with biliary tracts injuries.

## Competing interests

The authors declare no competing interests.

## Authors' contributions

Bagus Setyoboedi: conceived and designed analysis, collected the data, contributed to data and analysis tools, performed the analysis, drafted the article. Abdul H Khoironi: collected the data and analysis tool, performed the analysis, contributed in the drafting of the article. Sjamsul Arief, Anang Endaryanto: conceived and designed analysis, contributed data and analysis tool, final approval of the version to be published. Rendi Aji Prihaningtyas: revised the article and approved the version to be published. All authors read and approved the final version of the manuscript.

## Tables and figures

**Table 1:** demographic data, clinical, laboratory, and histologic condition of BA and non-BA groups

**Table 2:** the data and results of statistical analysis of the number of CD4+/FoxP3+ cells and CD4+/IL-17+ cells

**Figure 1:** micrograph image of liver biopsy in group BA depicted yellow bile plug; (A) HE staining (magnification 200x); fibrosis surrounded periportal area (B); mason trichrome staining (magnification 200x); as well as the proliferation of the biliary duct with bile plug thrombi (C) (HE staining, magnification 100x)

**Figure 2:** in immunohistochemistry staining, there were expressions of FoxP3 in CD4+/FoxP3+ cells (brown-red, indicated by the arrows (A) 1000x magnification), and interleukin 17 on CD4 +/IL-17 + (red (B) magnification 1000x)

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**Table 1:** demographic data, clinical, laboratory, and histologic condition of BA and non-BA groups

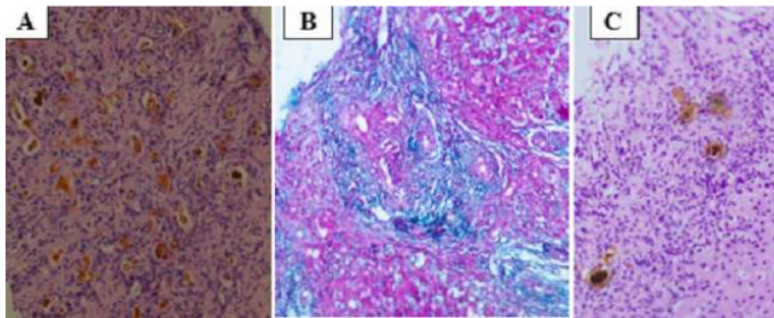
Variable	Biliary atresia (BA) (N=20)	Non-atresia bilier (non-BA) (N=14)	Significance (p)
Sex			
Male (%)	6 (30%)	9 (64.3%)	0.048 <sup>a</sup>
Female (%)	14 (70%)	5 (35.7%)	
Age (months), median (IQR)	8(5.25-9.75)	5.5(3-9)	0.186 <sup>b</sup>
Body weight (kgs), mean (±SD)	5.93(±1.19)	4.58(±1.67)	0.009 <sup>c</sup>
Body length (cms), median (IQR)	63.5(58-67.75)	56(50.75-60,.25)	0.005 <sup>b</sup>
Nutritional status			
Good N (%)	14 (70%)	8 (57.1%)	0.49 <sup>a</sup>
Less / bad N (%)	6 (30%)	6 (42.6%)	
Disease duration (months), median (IQR)	7(5-9)	5.5(3-9)	0,341 <sup>b</sup>
Pale stools N (%)	15 (75%)	8 (57.1%)	0.458 <sup>a</sup>
Dark urine N (%)	15 (75%)	10 (71.4%)	1.0 <sup>a</sup>
Hepatomegaly N (%)	20 (100%)	13 (92.9%)	0.412 <sup>a</sup>
Direct Bilirubin (mg/dl), median (IQR)	11,09 (8.53-13.17)	9.63(7.73-12.6)	0.227 <sup>b</sup>
Total Bilirubin (mg/dl), median (IQR)	14.49 (10.78-23.60)	11.32(6.72-16.32)	0.054 <sup>b</sup>
SGOT (mg/dl), median (IQR)	264 (159.8-357)	204 (126.8-275)	0.142 <sup>b</sup>
SGPT (mg/dl), median (IQR)	185 (111-334,3)	151 (103-232)	0,421 <sup>b</sup>
Platelets (x1000 cell/mm <sup>3</sup> ), mean (±SD)	335(±235)	344(±193)	0,945 <sup>c</sup>

IQR: interquartil range, SD: standar deviasi, <sup>a</sup>significance test using Chi-square/Fisher's exact test, <sup>b</sup>significance test using Mann Whitney U test, <sup>c</sup>significance test using t-test

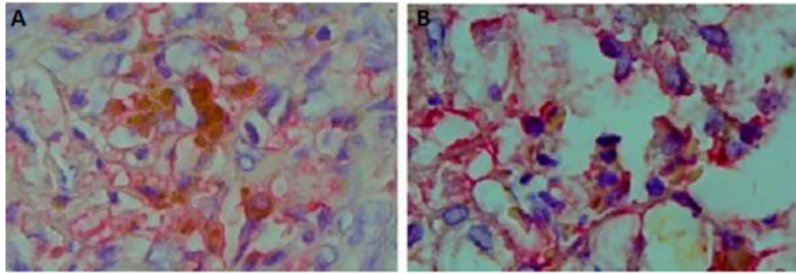
**Table 2:** the data and results of statistical analysis of the number of CD4+/FoxP3+ cells and CD4+/IL-17+ cells

Variable	BA	NonBA	Significance (p)
CD4+/FoxP3+ (cells/field), median (IQR)	6(4-8)	14(11.75-15.5)	<0.001 <sup>a</sup>
CD4+/IL-17+ (cells/lp), median (IQR)	7(6.25-8)	5(4.75-6.75)	0.04 <sup>a</sup>

IQR: interquartile range, <sup>a</sup>significance test using Mann-Whitney U test



**Figure 1:** micrograph image of liver biopsy in group BA depicted yellow bile plug; (A) HE staining (magnification 200x); fibrosis surrounded periportal area (B); mason trichrome staining (magnification 200x); as well as the proliferation of the biliary duct with bile plug thrombi (C) (HE staining, magnification 100x)



**Figure 2:** in immunohistochemistry staining, there were expressions of FoxP3 in CD4+/FoxP3+ cells (brown-red, indicated by the arrows (A) 1000x magnification), and interleukin 17 on CD4 +/IL-17 + (red (B) magnification 1000x)

# Comparison of CD4Foxp3and CD4IL-17

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