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The Expressions of $CD4^+$ and $CD8^+$ in Biliary Atresia Mice Model after Exposure with Rhesus Rotavirus (RRV)

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Abstract

Background: *Biliary atresia (BA) causesmore than 95% chronic liver disease or cirrhosis and 50-60% liver transplantation. The pathogenesis is unclear.It is proposed that viral infection of the bile duct epitheliumcausing progressive autoreactive inflammation mediated by autoimmune process, resulting fibrosis and bile duct obstruction.*

Objective: *Determine the effect of induction and duration of illness after rhesus rotavirus (RRV) exposure to expression of $CD4^+$ and $CD8^+$ in mice models BA.*

Methods:*Experimental research using 48 babiesborn from 20 mice balb/c, randomized into two groups, placebo (buffered saline) and 1.5 x 10^6pfu RRV intraperitoneally in the first 24 hours antenatally. Each group was terminated on 3^{rd}, 7^{th}, 14^{th} and 21^{st} day, then $CD4^+$ and $CD8^+$ expression was measured by flowcytometry. Statistical analysis performed using independent sample t-test, Mann-Whitney and Kruskal-Wallis.*

Results: *There were an increase of $CD4^+$ and $CD8^+$ expression in intervention group compared to control group ($p<0.05$), except $CD4^+$ in day 3^{rd} ($p=0.493$), showed the influence of the RRV induced. Influence of interaction duration of illness and RRV induction was also found statistically significant in mice models of BA ($p<0.001$). The increase of $CD4^+$ and $CD8^+$ expression was noted after 7^{th}day, peaked at 14^{th} day, and decreased at 21^{st} day.*

Conclusions:*RRV induction and duration of illness improves the adaptive cellular immune responses as reflected by the increased expression of $CD4^+$ and $CD8^+$ in mice models BA.*

Keywords:*Biliary atresia, rhesus rotavirus (RRV), $CD4^+$, $CD8^+$*

BACKGROUND

Biliary atresia is a progressive inflammatory cholangiopathy that leads to fibrosis of extrahepatic and intrahepatic bile duct.[1] The disease is estimated about 1:8.000 to 1:18.000 live births and appeared in neonatal periods accompanied by a yellowish, pale stools (acholic) and hepatomegaly.[2] It is still an issue currently, because patients may experience chronic liver disease and cirrhosis more than 95% and it is cause of liver transplantation in approximately 50-60% children.[3-5] Perinatal hepatobiliary viral infection causes apoptosis or early cholangiocyte necrosis, bile duct damage mediated by an autoimmune process, which is followed by obliteration of the bile duct.[6-9]Damaged bile duct

epithelial cells cause auto-reactive inflammation known as molecular mimicry and bystander activation followed by cholangitis, bile duct obstruction and biliary cirrhosis.[10] Th1 (T helper 1) cell response mechanism that resembles the incidence of biliary atresia in humans are often found in animal models, the induction of RRV in newborn mice.[6,11-13] In biliary atresia, infiltration of T cells surround and attack the intrahepatic bile ducts, characteristic largely by $CD4^+$ T cells, $CD8^+$ T cells or a mixture of both. Activated effector T cells produce cytokines that can directly damage epithelial cells or indirectly through stimulation of immune cells destroy the other.[11,14-18] Current research is focused on the role of cellular immunity biliary atresia bile duct damage, many researchers have shown that the portal tract infiltrates around the bile duct is composed of $CD4^+$ T cells and $CD8^+$.[19,20] The purpose of this study was to determine the effect of induction and duration of illness after rhesus rotavirus (RRV) exposure to changes in the cellular adaptive immune response, as reflected by the expression of $CD4^+$ and $CD8^+$ in the murine model of biliary atresia.

MATERIALS AND METHODS

Biliary atresia mice model

Twenty pregnant BALB/c mice were devided into two groups those were ten mice in study group and the other ten mice as control group respectively. The mice were kept separated in separate cages and a virus-free environment at the Laboratory of Molecular Biology the Faculty of MedicineUniversitas Brawijaya Malang Indonesia. They had free access to food and water. Each neonates from mice in the study group were given a single intraperitoneal (i.p.) injection of as much as 0.05 mL containing 1.5 X 106 pfu/mL of RRV strain MMU 18006 (ATCC Virginia # VR 1739), while neonate mice from control group were injected with 0.05 mL of balanced salt solution (BSS). All of the injections were performed not more than 24 hours after birth. Infected neonate mice which died within the first 2 days after birth, or were not fed by their mothers, were not included for further analysis. The neonate mice were weighted on the day of delivery and then were sacrificed by cervical dislocation on day 3, 7, 14 and 21 after birth. Liver and biliary tissues were removed by standard surgical procedure for further processes The protocol of this study had been approved by the Ethical Committe of Health Research Faculty of Medicine Universitas Brawijaya Malang Indonesia (Reg #.. 361/EC/KEPK-83/11/2012).

Histological slides of liver and biliary tissue

Isolated liver and biliary tissues were fixed with formalin, before being embedded in paraffin block. After the tissues were cut by microtome with 4-5 µm of thickness, the slices were put on the object glasses, and after deparafinized they were stained with Hematoxylin Eosin. All of histological slides were examined under light microscope with 400.x magnifications. Only the slides containing hepatic artery, portal vein and bile duct components were selected. The examination of the selected slides focused on the lumen and infiltration of inflammatory cells surrounding the bile duct. Digital photographs were obtained using the Olympus BX51 microscope with DP20 camera.

Flowcytometry analysis

Tissueswere homogenized and red cells were lysed with ACK (Ammonium-Chloride-Potassium) Lysing Buffer. Liver immune cells were enriched by Percoll gradient (40/60). Single-cell suspensions were incubated with Fc-block and ready for stained. A mouse $CD4^+$ and $CD8^+$ staining kits were used according to the manufacturer's instructions (Bioscience, San Diego, CA). Cells were visualized with BDFACSCalibur Flow-Cytometer (Becton-Dickinson, Mountain View, CA), FlowJo (Tree Star, Inc., Ashland, OR) software used for analysis. Flowcytometric analysis was done at the Laboratory ofBiomedicsBrawijayaUniversityMalang Indonesia.

Statistical analysis

The differences of histological features were compared qualitatively, while the differences of

the expression of CD4+ and CD8+ in each group from different days of sacrifices were analyzed using independent sample t-test, Mann Whitney test, One-way Annova, and Kruskal-Wallis.Data were analyzed using 95% confidence level (α=0.05).

RESULTS

The baseline characteristics of weight initially were homogeneous because the sample preparation in accordance with the inclusion criteria, but in general there are differences in mean final body weight of mice when terminated. During the study it was found a total of 9 (19%) infants dead mice so that the total sample that could be analyzed was 39. As per the study design, on 3rdday, 7th, 14th and 21st samples of each group were terminated.

After histological processing and staining of liver and biliary tissues of all samples, the results of their histological featur are shown at Figure 1, 2, 3, and4 below. There were differences of histological features especially the lumen and the thickness of biliary duct walls and the inflitration of inflammatory cells in liver tissues of study group and control group on day 3, 7, 14, and 21 respectively.

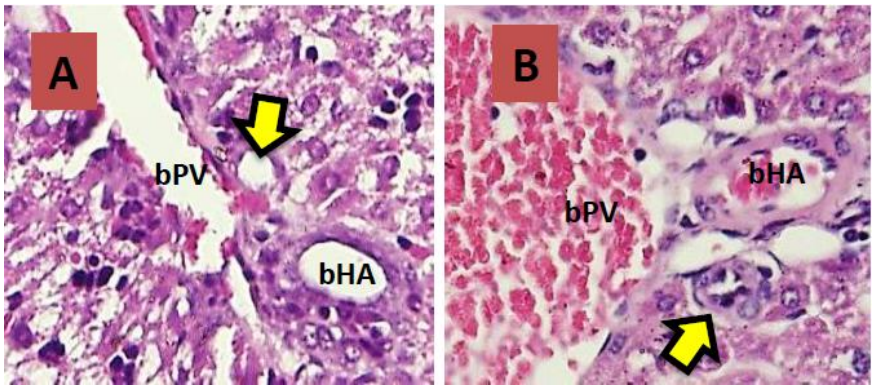

Figure1. Histologicalfeatures of liver and biliary tissues of mice on day 3.

A. Control group: no infiltration of inflammatoy cells and no narrowing of the lumen of bile duct (yellow arrow); B. RRV Group: infiltration of inflammatory cells accompanied by mucosal swelling that causes narrowing of the lumen of bile duct (yellow arrow).bHA: hepatic artery; bPV: portal vein

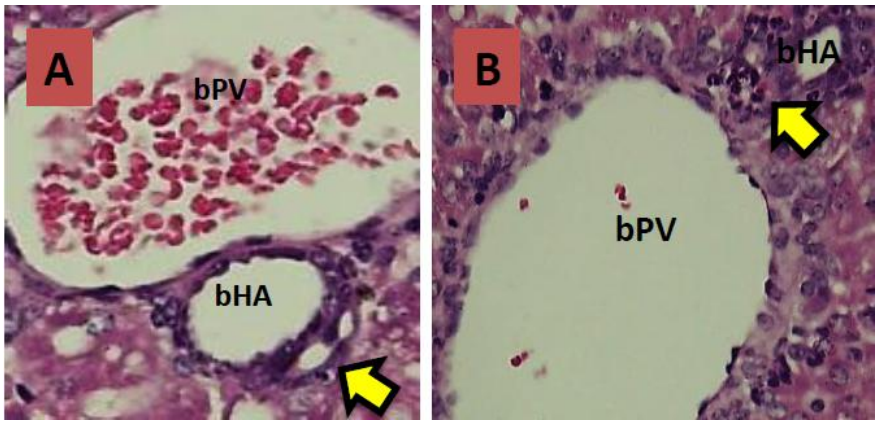

Figure 2. Histological features of liver and biliary tissues of mice on day 7.

A. Control group: no infiltration of inflammatoy cells nor narrowing of the lumen of blie duct (yellow arrow); B. RRV Group: infiltration and multiplication of inflammatory cells, bile duct mucosal swelling andlumenal narrowing become more prominent (yellow arrow). bHA: hepatic artery; bPV: portal vein.

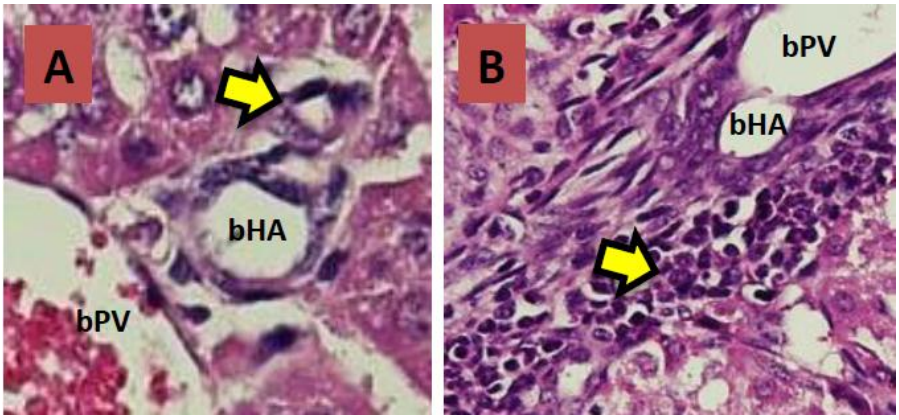

Figure 3. Histological features of liver and biliary tissues of mice on day 14.

A. Control group .No infiltration of inflammatoy cells nor narrowing of the lumen of bile duct (yellow arrow); B. RRV Group: excessive infiltration of inflammatory cells causes the narrowing of bile duct lumen become more apparent (yellow arrow).bHA: hepatic artery; bPV: portal vein.

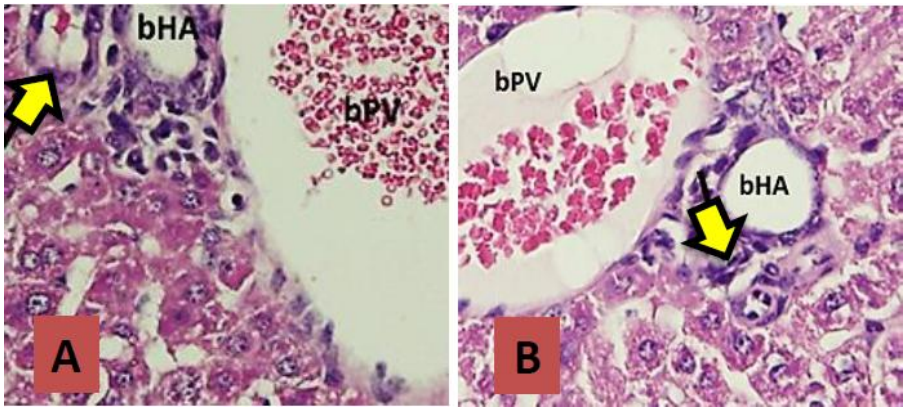

Figure 4.Histological features of liver and biliary tissues of mice on day 21.

A. Control group: no infiltration of inflammatoy cells neither narrowing of the lumen of bile duct (yellow arrow); B. Expression of inflammatory cells decrease, but bile duct lumen looked has undergone atresia (yellow arrow). bHA: hepatic artery; bPV: portal vein.

In this study, the results are generally based on statistical analysis of $p < 0.05$ indicates a significant difference in the expression of $CD4^+$ mice when compared with the interventiongroup to control between groups per variable on the 7^{th} day, 14^{th}, and 21^{st}, except on 3^{rd} day, where $p = 0.493$ (Table 1). While based on overall, control group and the interventiongroup to obtain $p < 0.001$.

Table 1.CD4$^+$ expression on the induction of RRV and control groups

Variable Day	Control groups Median (interquartile)	Interventiongroups Median (interquartile)	*p** between groups for each variable
CD4$^+$			
3	2,32 (0,39)	2,40 (0,45)	0,493
7	1,94 (0,08)	3,85 (0,10)	0,011*
14	1,23 (0,07)	8,20 (0,13)	0,006*
21	0,88 (0,22)	6,50(3,07)	0,010*
*p**	<0,001**	<0,001**	<0,001**

**Significant differences with the Mann-Whitney test on the value of α = 0.05*

***Significant differences by Kruskal-Wallis test on the value of α = 0.05*

The following chart changes in expression of CD4$^+$(median) in the control group compared to the interventiongroup from time to time:

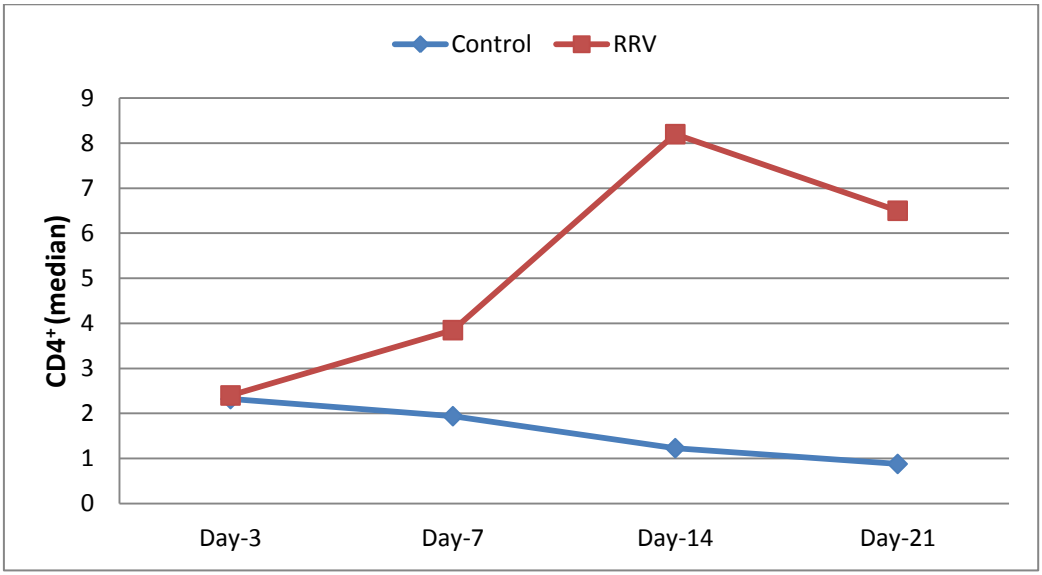

Figure 5. Effects of induction RRV and duration of illness after RRV exposure to changes in the expression of CD4$^+$ (median) control group compared to the intervention group.

Expression of CD4$^+$ began to increase since 3rd day after induction of RRV compared to controls, further increasing the value of the increase is more progressive and looks after 7th day, peaked on 14th day, then began to decline until 21st day. Whereas in the control group (blue line) obtained the expression of CD4$^+$after 3rd day decreased until 21st day.

The results generally p<0.05 indicates a significant difference in the expression of CD8$^+$ mice when compared with the interventiongroup to control between groups for each variable, where the 3rd day, 7th, 14th and 21st (Table 2).

While based on the overall results obtained control group p <0.001 and intervention group p <0.002. Induction RRV had significant effect on the expression of CD8$^+$ (p<0.001).

Table 2.$CD8^+$expression in the induction group and control RRV

Variable	Control groups Median (interquartile)	Interventiongroups Median (interquartile)	*p**between groups for each variable
Day			
$CD8^+$			
3	1,00 (0,02)	1,36 (0,27)	0,020*
7	1,83 (0,08)	2,42 (0,05)	0,011*
14	4,56 (0,05)	10,57 (0,22)	0,006*
21	5,04 (1,97)	7,51(1,13)	<0,001**
p	<0,001***	0,002***	<0,001***

**Significant differences with the Mann-Whitney test on the value of α = 0.05*
***Significant difference with the Independent Samples t-test value of α = 0.05*
****Significant differences with the Kruskal-Wallis test on the value of α = 0.05*

The following chart changes in expression of $CD8^+$ (median) in the control group compared to the interventiongroup from time to time:

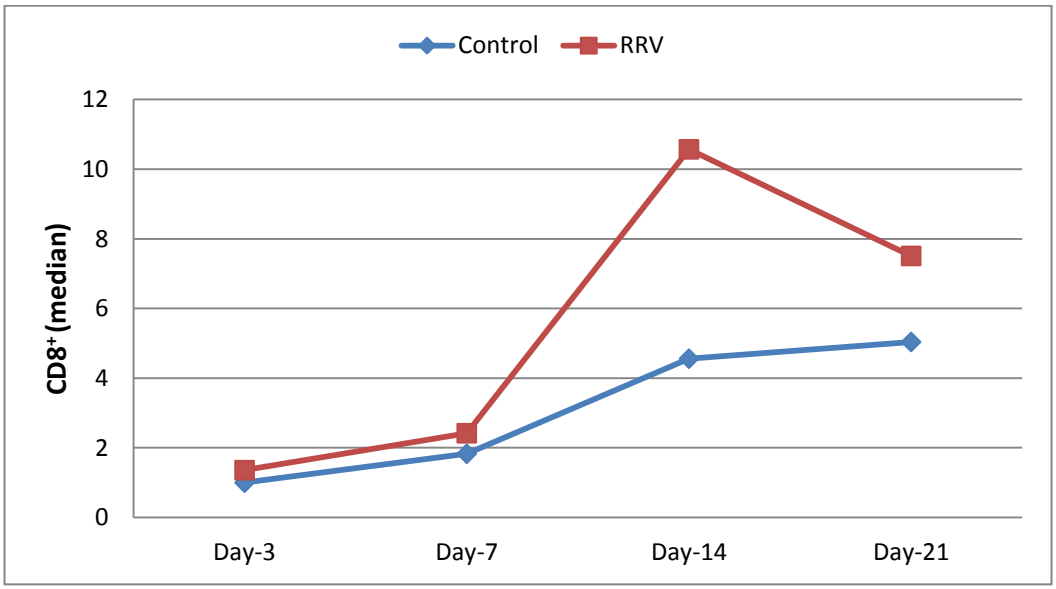

Figure 6. Effects of induction RRV and duration of illness after exposure to the expression of $CD8^+$ (median) control group compared to the intervention group.

Obtained both groups increased on 3rd day to 14thday, but the treatment group decreased until 21st day, whereas the control group remained elevated.

DISCUSSION

Histopathology examination to prove the occurrence of inflammation and biliary tract obstruction as a result of an immunological response after induction of RRV, obtained the inflammatory process 3rd day after induction of RRV were intensified on 7th day. Obstruction process or biliary tract obstruction seen on 14th day, where most of the samples have shown total obstructionand entire sample has undergone a total obstruction seen on termination 21st day. This result is also not much different from the research Carvalho (2005) and Bessho (2011) in which the occurrence of severe inflammatory process in the biliary tract on 3rd day and 7th after

induction of RRV, but they get the obstruction or complete obstruction on 14th day.[21,22] The difference can be explained here that the immune response is highly dependent on the ability of the immune system to recognize foreign molecules (antigens), the antigen recognition process performed by the main elements of the immune system are lymphocytes which are then followed by the effector phase involving various types of cells, lymphocytes must recognize all antigens on potential pathogens and at the same time he had to ignore the body's own tissue molecules, so it takes a certain time for all of it and it could be different.[1,7,23,24]

The cellular adaptive immune response that occurs is reflected by the presence of the expression of $CD4^+$ and $CD8^+$, which is in line with previous research, there is evidence to support that both $CD4^+$ and $CD8^+$ cells mediate the destruction of the bile ducts.

This study is an experimental study using animal mice balb/c which proves that the RRV induction dose of 1.5 x 10^6pfuintraperitoneal<24 hours after birth to give effect significant changes in $CD4^+$ increase in expression when compared to the overall control ($p<0.001$) and there were an increase in $CD4^+$ expression since 3rd day after RRV induction that also happened in the 7th and 14th observation. The most progressive and significant increase in $CD4^+$ expression occurred since 7th day and reached its peak in 14th day observation.(Figure 5).

In accordance with other studies, the situation is due to inflammation, $CD4^+$ T naive differentiate into effector cells Th1 (driven by IL-12 and produce IFN-γ, IL-2, TNF-β, and TNF-α) or cell effector Th2 (driven by IL-4 and produce IL-4, IL-5, and IL-10), infiltration surround and attack the bile ducts, produce cytokines that can directly damage epithelial cells or indirectly through stimulation of immune cells destroy the other.[11,14-17] On the state of the autoimmune response, this increase will occur continuously, giving rise to inflammation progressive (cholangiopathy) bile duct extrahepatic and intrahepatic, where lymphocytes Th1 primary (which is activated by $CD4^+$) and then set the immune response that damages, directly through the release of pro-inflammatory cytokines and recruitment cytotoxic T cells, causing biliary epithelial injury, cystic duct and eventually progressive occlusion of the bile duct.[1,6,8,9,14,25]

The differences were not significant expression of $CD4^+$intervention group and control on 3rd day, likely due to the response of the adaptive immune response was slow, not ready until exposed or should have previous exposure through different phases clearly, consisting of introductory phase, activation of lymphocytes and antigen elimination (effector phase), further increasing from day to day.[24] Adaptive immunity requires an immune response stimulated by repeated exposure to pathogens or antigens non-microbial, the characteristics of adaptive immunity is the specificity of the different molecules that evoke memory and the ability to respond to repeated exposure.[19,20] The proteins of bile duct apoptosis and looks likely to be seen as a foreign body, issued a mediating inflammatory autoreactive T cells that promote bile duct injury.[16]

Expression of $CD4^+$ declines after the 14th day, likely due to the small number of normal cells because it has undergone fibrosis. In the other study found fibroblast cells have been replaced with a disability in the process of molecular mimicry cholangiocytecells that act as antigens and cross-reaction or non-occurrence of bile duct epithelium protein so it does not happen again autoreactive inflammatory ductal epithelium known as bystander activation pathway.[10,21,26-28]

After a peak at 14th day, then seemed to decrease until 21st day termination, but the expression of $CD4^+$ 21st day in the interventiongroup when compared with the control was significantly higher based on statistical calculations.

This study showed that changes in the expression of $CD8^+$ in the group of mice induced RRV, both among groups for each variable or as a whole when compared with controls, with the results of statistical calculations for overall comparison obtained $p<0.001$ and there were an increase in $CD8^+$ expression since 3rd day after RRV

induction that also happened in the 7th and 14th observation. The most progressive and significant increase in $CD8^+$ expression occurred since 7th day and reached its peak in 14th day observation.(Figure 6).

In accordance with previous studies that the longer the time of illness after exposure to the virus, the higher the expression of $CD8^+$ and there is an increase that is more progressive, $CD8^+$attacks the bile ducts directly invade the epithelium and releasing molecule cytotoxic, causing damage to the epithelium of bile, cystic duct progressive and eventual obstruction of the channel bile duct.[6,11,14,25]Shivakumar (2007) found that in an effort to find the foundation of cellular relationships cytokine production and bile duct obstruction, mononuclear cells in mice newborn who examined omitted or depletion to see its contribution to the phenotype atresia, in the first study, the loss of $CD4^+$ decreased the expression of Th1 but had no effect on bile duct damage and progression to obstruction, on the contrary, the loss of $CD8^+$ greatly reduce the occurrence of injury or damage to the bile ducts, to prevent blockage or obstruction and restore the flow of bile after infection with rotavirus (RRV), but still allow for further cholangitis.

After peaked on 14th day, it seemed to decrease until 21st dayafter termination, but the expression of $CD8^+$ 21st day in theintervention group compared with controls still significantly higher based on statistical calculations, the expression of $CD8^+$ decreased after the 14th day, it is the same cause as $CD4^+$ which has been described previously in which the small number of normal cells because it has undergone fibrosis.

This study has limitations;no clinical assessment of the mice from time to time, laboratory markers, immune response, and it cannot distinguish the expression of the biliary tract or liver.

CONCLUSIONS

Induction of RRV is enhancing the adaptive immune response cellular reflected by the expression of $CD4^+$ in mice models of biliary atresia on 7th day, 14thand 21stafter induction, except on 3rd day, whereas the expression of $CD8^+$ 3rd day, 7th, 14thand 21st. Expressions of $CD4^+$ and $CD8^+$ progressively increased after 7th day of induction RRV and peaked at 14thday and then decreased.

RRV duration of exposure increases the cellular adaptive immune response, as reflected by the expression of $CD4^+$ and $CD8^+$ in mice models of biliary atresia on 3rd days, 7th, 14th and 21st after induction. Expression of $CD4^+$ and $CD8^+$ in the control group provides new information about the profile based on the sequence of observations.

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