Regulatory T Cells CD4+ CD25+ Expression in Biliary Atresia Mice Model after Exposure with Rhesus Rotavirus (RRV)

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Abstract

Biliary atresia (BA) is still a major challenge for clinicians, as without surgical intervention, more than 80% of patients will develop liver cirrhosis, or die within 2 years of life. The pathogenesis is still unknown, but a theory of primary perinatal hepatobiliary viral infection followed by auto-immune-mediated bile duct injury had been hypothesized. The objective of this study is to determine the change of regulatory T cells and inflammatory reaction in liver after rhesus rotavirus (RRV) infection in BA mice model. Two groups of newborn mice, those were RRV group consisting of 24 newborn mice injected by RRV 1.5 x 106 PFU intraperitoneally less than 24 hours after birth, and control group comprising of 24 newborn mice injected with buffered saline were used as BA model. The expression of Tregs and infiltration of inflammatory cells in the liver of the two groups of newborn mice were studied on day 3, 7, 14, and 21 after birth.

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The results show that Tregs expressions of RRV group on day 3, 7, 14 and 21 were significantly higher than control group (p<0.05). There were also significant increases of Tregs expressions on day by day, both in RRV group (p<0.001), as well as in control groups (p=0.002). The excessive infiltration of inflammatory cells and narrowing of bile duct lumens were detected on day 14. As a conclusion we can say that the Induction of Rotavirus (RRV) in newborn mice model BA increases the expressions of Tregs, and may cause damage of bile ducts within two weeks of life.

**Keywords:** mice model biliary atresia; rhesus rotavirus (RRV); regulatory T cells; CD4+ CD25+.

1. **Introduction**

Biliary atresia (BA) is a serious pediatric health problem due to its potency and progressivity in damaging both the intra- and extra-hepatic biliary ducts. Approximately 80% of patients require a liver transplantation to survive and almost all patients die by 2 years of age without surgical intervention [1;2;3]. The etiology of BA remains unclear. The pathogenesis of BA involving viral infections of the hepatobiliary tract and bile duct damage mediated by auto reactive T cells had been hypothesized. However, so far there is no infectious or toxic agents proven to be directly implicated in biliary atresia, as although the virus had been eliminated, persistent inflammation of the bile duct and epithelial cells damage still occur [4;5].

Current management of biliary atresia including surgical (Kasai porto-enterostomy / KPE) and medical approaches still has no satisfactory outcomes [6]. Some authors reported that the outcomes of treatment of the patients with BA are strongly related to the course of disease and the time of intervention. Diagnosis performed within the first two months of life, followed by performing KPE can improve the prognosis and prevent worsening of the disease [2;7;8]. On the other hand, regulatory T cells (Tregs), either natural or induced, suppress a variety of physiological and pathological immune responses. In certain diseases Treg-mediated suppression is a multi-step process and impairment or augmentation of each step can alter the ultimate effectiveness of Treg-mediated suppression [9].

In BA, the damage of bile duct occurs due to the expression of epithelial cells as an antigen, or the “self” proteins that are recognized as an “non-self”, leading to auto-reactivation of T-cells and release of inflammatory mediators. It can be explained by the evidences of bystander effect of Tregs on immune responses under particular conditions (bystander activation pathway) as well as Tregs that exert multiple effects on different cell targets [10].

Based on the facts mentioned above, it seems there is an opportunity to get a new approach in preventing the progressiveness of BA, or to stop the subsequence damage process on biliary duct by utilizing or manipulating the change of Treg expression following the onset of BA on mice model infected by rhesus rotavirus (RRV).

2. **Materials and Methods**

2.1. **Biliary atresia mice model**

Twenty pregnant BALB/c mice were divided 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 Medicine Universitas 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 \times 10^6 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).

2.2. **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.

2.3. **Flowcytometric analysis**

Tissues were 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 Fe-block and ready for stained. A mouse Treg staining kit was 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 of Biomedics Universitas Brawijaya Malang Indonesia.

2.4. **Statistical analysis**

The differences of histological features were compared qualitatively, while the difference of the expression of regulatory T cells CD4+CD25+ 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).

3. **Results**

There were totally 48 newborn miceelligible in this study, consisting of 24 neonates infected by RRV 1.5 x 106 PFU intraperitoneally less than 24 hours after birth (RRV group) and 24 neonates injected with buffered saline.
Expression of Tregs both in the RRV and control group were studied on day 3, 7, 14, and 21 after birth. Table 1 shows that the expression of Tregs in RRV group were significantly higher than those of control group, and significant differences in the expression of Tregs on day by day in RRV group were also noted (p<0.001), as well as in control groups (p=0.002).

<table>
<thead>
<tr>
<th>Day</th>
<th>RRV group</th>
<th>Control group</th>
<th>p</th>
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<tbody>
<tr>
<td></td>
<td>Median (Interquartile)</td>
<td>Median (Interquartile)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1.06 (0.05)</td>
<td>0.92 (0.09)</td>
<td>0.003 *</td>
</tr>
<tr>
<td>7</td>
<td>1.37 (0.07)</td>
<td>0.24 (0.08)</td>
<td>0.011**</td>
</tr>
<tr>
<td>14</td>
<td>3.67 (0.4)</td>
<td>1.51 (0.24)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>21</td>
<td>10.46 (0.10)</td>
<td>2.43 (0.73)</td>
<td>0.011**</td>
</tr>
<tr>
<td>p</td>
<td>0.002****</td>
<td>&lt;0.001***</td>
<td>&lt;0.001****</td>
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* Significant different with independent t-test, α=0.05
** Significant different with Mann Whitney, α=0.05
*** Significant different with one-way annova, α=0.05
**** Significant different with Kruskal Wallis, α=0.05

**Figure 1**: The comparison of regulatory T cells expressions between control group and RRV group on different days of life.

*Letter notification: differences between group

*Number notification: differences among group*
Figure 1 shows that even there is a trend of increasing expression of Tregs in control group as well as in RRV group during the 21 days of life, but the upward trend of Tregs expression in RRV group (red boxes) was more prominent compared to the control group (blue boxes) on day by day. The expression of Tregs in control group is slightly decreased on the day 7 after birth.

After histological processing and staining of liver and biliary tissues of all samples, the results of their histological features are shown at Figure 2, 3, 4, and 5 below. There were differences of histological features especially the lumen and the thickness of biliary duct walls and the infiltration of inflammatory cells in liver tissues of study group and control group on day 3, 7, 14, and 21 respectively.

**Figure 2**: Histological features of liver and biliary tissues of mice on day 3.

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

**Figure 3**: Histological features of liver and biliary tissues of mice on day 7.
A. Control group: no infiltration of inflammatory cells nor narrowing of the lumen of bile duct (black arrow); B. RRV Group: infiltration and multiplication of inflammatory cells, bile duct mucosal swelling and lumenal narrowing become more prominent (black arrow). bHA: hepatic artery; bPV: portal vein.

Figure 4: Histological features of liver and biliary tissues of mice on day 14.

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

Figure 5: Histological features of liver and biliary tissues of mice on day 21.

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

This study was conducted to confirm the profile of regulatory T cells expressions and their role in the pathogenesis of biliary atresia in newborn mice induced with rhesus rotavirus (RRV) as a model of biliary atresia.

Histological examination was conducted to prove the occurrence of inflammation and bile duct obstruction (atresia) as a result of the immunological response after induction of rhesus rotavirus. Overview of the pathogenesis of biliary atresia in newborn mice induced by RRV in this study showed that on day 3 of the inflammatory process had been going on. On day 7 it intensified the inflammatory process, while on day 14 most of the subjects had shown the condition of bile duct atresia and, finally, on day 21 all subjects had experienced a total obstruction. On the other hand, the subjects in control group did not experience inflammation, narrowing nor obstruction of the bile duct. These results are also consistent with studies conducted by Carvalho (2005) who reported that the severe inflammation of the bile ducts in the subject also occur on days 3 and 7 after induction of RRV. However, that is different from our study. In this study the entire subjects already undergo atresia on day 14 [11].

Induction RRV in less than one day after birth in Balb/c mice in our study was able to increase the number of Regulatory-T cells, while this condition did not occur in the control group. There was only a slight increase in the number of Regulatory-T cells on day 3, but the greater and sharp increase occurred on day 7 and 14. The absence of Regulatory-T cells within the first 3 days of life caused liver of the newborn susceptible to pro-inflammatory response due to viral challenge. A sharp increase in the number of newborn mice liver Regulatory-T cells 7 days after RRV infection occurred at the same time with the start of blockage of the bile ducts in the biliary atresia mice. A study in human subjects found that up regulation of Regulatory-T cells cytokines genes in the liver of newborn babies suffered from biliary atresia, whereas in the liver of normal newborns this did not happen [12]. Immunologically, definition of Regulatory-T cells activation in humans, as reflected by the activity of some existing markers, including FOXP3, is still difficult, because these markers can be transiently activated upon activation of T cells [13].

In normal conditions, which in our study were represented by the control group, the number of Regulatory-T cells was lower on day 3 and even lower on day 7 compared to the group induced by RRV. Research has shown that the slow development of Regulatory-T cells is a risk factor of developing specific autoimmunity strain [14,15]. The lower increasing number of regulatory-T cells than cytotoxic-T cells or NK-cells is associated with the pathogenesis of biliary atresia occurring in mice. Biliary atresia which is modeled with RRV infection has a lower number of regulatory-T cells compared with cytotoxic-T cells or NK-cells and repression-immunological function [16]. Further studies on the role of higher number of regulatory-T cells than cytotoxic-T cells or NK-cells compared with Treg cells associated with the development of fibrosis and autoimmunity needs to be done.

In biliary atresia condition that was modeled by RRV infected mice and enriched with Regulatory-T cells, the development of biliary atresia can be prevented. While the reduction in Regulatory-T cells in non-biliary atresia mice increased the incidence of biliary injury [17]. Previous research on autoimmune models had demonstrated
changes in both the number of Regulatory-T cells or function [18;19]. Adoptive transfer of Regulatory-T cells function has been pressing the autoimmune response and the development of disease [16]. Regulatory-T cells population intact is correlated with immunological tolerance, while the loss of Regulatory-T cells population results in inflammation due to organ-specific autoimmunity [20;21].

RRV-induced mice model of biliary atresia is characterized by the specific bile duct inflammatory cells infiltration containing autoreactive CD4+ T cells. Our study proves that inflammation and bile duct obstruction (atresia) are the result of the immunological response after RRV induction which delays the development of Regulatory-T cells implicated as the cause of autoimmunity. In the prevention of autoimmune processes, conditions Regulatory-T cells in mice are difficult to distinguish in their expression patterns (whether they are in the "activated condition", "conditions as memory cells", or "condition as effectors"), the type of molecules on their cell surfaces (including CD45RB and CD5), as well as the cytokines produced. When CD25-T cells population increases, the activation of CD25+ effector T cells increases. From this study, although we know that the population of basal and time of availability of Regulatory-T cells determine the effectiveness of the prevention of autoimmunity process, but we have not obtained information on how large the number-needed Regulatory-T cells population. What we know so far is just the information that the improvement and activation of Regulatory-T cells can lower the immune response against non-self-antigens. A complete explanation of the control mechanisms of Regulatory-T cells need to be rechecked for whether cytokines such as IL-10 or TGF-β produced by Regulatory-T cells is responsible for supporting self-reactive T cells in CD25+ dormant status [22].

5. Conclusion

Induction of rhesus rotavirus increases the expression of Regulatory-T cells in mice model of biliary atresia, which sharply increase after day 7 and peaks on day 14. It also may cause damage of bile ducts within two weeks of life.

Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this article.

References


