Aryl hydrocarbon receptor involved in Th17/Treg imbalance in chronic hepatitis B via the AhR–RORγt–FoxP3 axis

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Research Article

Keywords: Aryl hydrocarbon receptor, Th17/Treg imbalance, RORγt, FoxP3, chronic hepatitis B

Posted Date: June 30th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1748079/v1

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Abstract

**Objectives:** To investigate the involvement of aryl hydrocarbon receptor (AhR) in the Th17/Treg imbalance of chronic hepatitis B (CHB) via the AhR–RORγt–FoxP3 axis.

**Methods:** The proportions of Th17 and Treg cells in peripheral venous blood collected from 18 healthy controls (HCs) and 30 patients with CHB were determined by flow cytometry. AhR, retinoic acid-related orphan receptor gamma t (RORγt), and forkhead box protein 3 (FoxP3) mRNA levels were tested by RT-qPCR. Moreover, AhR, RORγt and FoxP3 protein levels were detected by western blot.

**Results:** The proportions of Th17 and Treg cells were significantly higher in the CHB group than those in the HC group ($p < 0.005$). The Th17/Treg ratio was remarkably higher in the CHB group than that in the HC group ($p < 0.001$). Furthermore, $AhR$, $RORγt$, and $FoxP3$ mRNA levels were higher in the CHB group than those in the HC group ($p \leq 0.001$). The expression levels of AhR, RORγt and FoxP3 protein were higher in the CHB group than those in the HC group. Moreover, there were positive correlations between $AhR$ and $RORγt$ mRNA ($p < 0.001$, $r = 0.632$) and between $AhR$ and $FoxP3$ mRNA ($p < 0.001$, $r = 0.798$).

**Conclusion:** The AhR–RORγt–FoxP3 axis may mediate Th17/Treg balance in CHB to influence disease development.

Introduction

Chronic hepatitis B (CHB), caused by the hepatitis B virus (HBV), is a major life-threatening public health concern worldwide and the most common cause of chronic hepatitis in China. It can progress to liver failure, liver cirrhosis, and hepatocellular carcinoma.

Host immunity determines the outcome of CHB during HBV infection [1]. The adaptive immune response, mediated by CD4+ and CD8+ T cells, play an essential role in HBV clearance and HBV infection control [2, 3]. The immune functions of Th17 and Treg cells belonging to CD4+ T cells are contrary. Th17 cells may be involved in immune activation and disease progression, but Treg cells inhibit the process and play a critical role in maintaining immune homeostasis. Retinoic acid-related orphan receptor gamma t (RORγt) and forkhead box protein 3 (FoxP3) are key transcription factors affecting Th17 and Treg cell differentiation and development [4, 5]. Others and we have previously shown that Th17/Treg imbalance is associated with the occurrence and progression of CHB [6, 7] [8, 9]

The aryl hydrocarbon receptor (AhR) is a ligand-dependent transcription factor activated by various endogenous and exogenous ligands [10]. Its physiological functions include cell differentiation, proliferation, apoptosis, adhesion and migration; and cytokine production [11, 12]. It mediates the development of obesity-mediated non-alcoholic fatty liver disease [13], infectious diseases[14], and autoimmune hepatitis. It develops and activates the immune system [15]. It regulates Treg and Th17 cell differentiation and plays an essentialization in Th17/Treg balance. Veldhoen et al. [16] and Quintana [17]et al. showed that AhR activated by 6-formylindolo[3,2-b]-carbazole promoted Th17 differentiation,
but AhR activated by 2,3,7,8-tetrachlorodibenzo-p-dioxin could expand Treg population in experimental autoimmune encephalomyelitis. Moreover, Liu et al. reported that baicalein could regulate the balance of Th17/Treg cells through AhR in attenuating colitis [18].

However, the role of AhR in regulating Th17/Treg balance in CHB has remained unclear. Therefore, this study detected the proportions of Th17 and Treg cells, and the expression of AhR mRNA and protein in CHB to reveal the possible involvement of AhR in the Th17/Treg imbalance of CHB. Furthermore, to investigate the signaling involved in the Th17/Treg imbalance, mRNA and protein expression of specific transcription factors (RORγt, FoxP3) were respectively detected.

**Materials And Methods**

**Patients**

The ethics committee of the First Affiliated Hospital of Kunming Medical University approved the study protocol. The healthy controls (HC) included 18 people from the First Affiliated Hospital of Kunming Medical University, Kunming, China. The CHB group included 30 patients with CHB from the Department of Infectious Diseases and Hepatology, the First Affiliated Hospital of Kunming Medical University, Kunming, China. Participants were enrolled from October 2020 to January 2021. The inclusion criterion for the CHB group was a diagnosis of CHB following the 2019 guidelines for the prevention and treatment of CHB in China [19]. Exclusion criteria were the diagnoses of other viral hepatitis, autoimmune, immunodeficiency, fatty liver, alcoholic liver diseases, current immunosuppressive or immunomodulatory drugs. Blood samples were collected from the study participants.

**Sample collection**

Peripheral blood samples (8 mL) were respectively collected from HCs and patients with CHB before treatment with nucleotide analogues and/or interferon α-2b. They were conserved in an ethylenediamine tetra acetic acid anticoagulant tube for flow cytometry, qPCR, and WB.

**Flow cytometry assay**

Fresh anticoagulant-containing peripheral venous blood (3 mL) was extracted from HCs and patients with CHB to separate the peripheral blood mononuclear cells (PBMCs) using Ficoll® as a density gradient medium. PBMCs of a density of 1×10⁶/mL were diluted with phosphate-buffered saline. The RPMI 1640 medium containing 10% fetal bovine serum was added to the 1×10⁶/mL PBMC and cultivated in 5% CO₂ at 37°C in an incubator overnight. The following day, cell-stimulating fluid (50 ng/ml of phorbol 12-myristate 13-acetate, 1 µg/ml ionomycin, Solarbio Science & Technology Co., Ltd., and 2 µmol/L of BD GolgiPlug™) was added to the cell culture medium for 5 h. Then, PE-Cy™7 Mouse Anti-Human CD4 was used for staining CD4 + T lymphocytes. BD Cytofix/Cytoperm™ was added in cell suspension for cell permeabilization. Intracellular antibodies (BD Alexa Fluor® 488 Mouse anti-Human FoxP3, PE Mouse anti-
Human IL-17A) were added separately to the aforementioned mixture. Finally, flow cytometry (Attune®, NxT) was performed to detect Th17/Treg frequency.

**Measurement of AhR, RORγt, or FoxP3 mRNA**

PBMCs were separated using Ficoll® as a density gradient medium. Trizol reagent was added into PBMCs to lyses cells. The mixture was then centrifuged at 12,000 × g for 10 minutes at 4°C, and the supernatant was collected. Next, glacial acetic acid was added to the supernatant and mixed well with the phase-separating reagent, then placed at room temperature for 3 minutes after shaking well and were centrifuged at 12,000 × g for 15 minutes at 4°C. Furthermore, the supernatant was transferred to a new centrifuge tube, an equal volume of isopropanol was added, mixed and stored at -20 °C overnight. The following day, the supernatant was discarded after centrifuging at 12,000 × g for 15 minutes at 4°C, and the pellet was resuspended with 80% ethanol. After centrifuging for 4 °C, 12000xg for 10 minutes again, the RNA pellet was dissolved with RNase-free water. Finally, RNA concentration and purity were assessed by OD 260/280 ratio. Reverse transcription and PCR were carried out in accordance with the manufacturer's instructions (BlazeTaq™ SYBR® Green qPCR Mix 2.0). Real-Time PCR primer sequences were listed in Table 1. The mRNA levels of target genes was calculated by the comparative CT (2−△△ct) method and normalized to GAPDH.

**Measurement of AhR, RORγt, or FoxP3 protein expressions**

After extracting the total protein from the PBMCs of the peripheral venous blood, the protein concentration was determined using the bicinchoninic acid protein concentration determination kit (Beyotime, Shanghai, China). Subsequently, protein bands separated from the gel were transferred to 0.45 μm of the poly (vinylidene fluoride) membrane using 90 V electrophoresis for 30 min and to the membrane for 70 min. After membrane transfer, the protein bands were sealed with 5% skimmed milk powder at room temperature for 30 min. The anti-FoxP3 antibody (1:1000, CST, USA,12632), anti-AhR antibody (1:500, affinity, USA, AF6278), and anti RORγt antibody (1:1000, Abcam, ab113434) were separately added to the membrane, with the marker as the standard, and incubated at room temperature for 60 min. They were washed with tris-buffered saline with 0.1% Tween® 20 detergent (TBST) until there was no skimmed milk powder. Subsequently, anti-rabbit IgG (whole molecule) peroxidase antibody for FoxP3(1:5000, sigma, USA, A6154), AhR (1:5000, sigma, USA, A6154) and Rabbit anti-Goat IgG horseradish peroxidase antibody (1:10000, zs, China, ZF- 0314) for RORγt were added and incubated at room temperature for 60 min. After the incubation, the secondary antibody was washed with TBST thrice. Finally, electrogenerated chemiluminescence was performed for development. Membrane-bound antibodies were detected with a horseradish peroxidase-conjugated secondary antibody and visualized by ECL advanced Western blotting detection kit. β-Actin (1:5000,Abcam,AB 6276) served as a loading control.

**Statistical analyses**
All data were analyzed using SPSS 24.0 (IBM). The data are expressed as mean ± standard deviation or median (interquartile range). Between-group differences were evaluated using the t-test (normal distribution) and Kruskal–Wallis H test (non-normal distribution). Spearman's correlation test was used to assess the correlation between two continuous variables. All the tests were two-tailed, and p-values < 0.05 were considered to be statistically significant.

Results

Proportion of Th17 and Treg cells and the Th17/Treg ratio were increased in patients with CHB

The percentages of Th17 and Treg cells were significantly higher in the CHB group than those in the HC group (p < 0.005; Fig. 1). Moreover, the Th17/Treg ratio was remarkably higher in the CHB group than that in the HC group (p < 0.001; Fig. 1.E). These results suggest that there is Th17/Treg imbalance in chronic hepatitis B patients, which is consistent with the previous studies[6, 20] [8, 9].

The mRNA expressions of AhR, RORγt and FoxP3 were increased in patients with CHB

Compared to the HC group, AhR, RORγt, and FoxP3 mRNA levels were higher in the CHB group (p ≤ 0.001, Fig. 2), indicating the possible involvement of AhR in the pathogenesis of CHB.

The protein expressions of AhR, RORγt and FoxP3 were increased in patients with CHB

WB results showed that the protein expressions of AhR, RORγt and FoxP3 were higher in the CHB group than those in the HC group (Fig. 3).

The mRNA level of AhR was positively correlated with RORγt and FoxP3 mRNA level

The correlation of AhR mRNA level with the proportions of Th17 and Treg cells was not statistically significant (p > 0.05). However, the mRNA level AhR was positively correlated with RORγt mRNA level (p < 0.001, r = 0.632) and FoxP3 mRNA level (p < 0.001, r = 0.798; Fig. 4). This suggests that AhR plays an important role in Th17 and Treg cell differentiation and function.

Discussion

In this study, the proportions of Th17 and Treg cells, the Th17/Treg ratio, and the expressions of RORγt and FoxP3 at mRNA and protein levels were increased significantly in patients with CHB. We found that there was Th17/Treg imbalance in CHB, which was consistent with the previous findings [6]. At present, there are a few reports on the Th17/Treg imbalance mechanism in CHB. Li et al demonstrated that High-mobility group box 1 (HMGB1) participated in Th17/Treg imbalance via the TLR4-IL-6 pathway[21]. Zhang et al found transcription factor C/EBPα bound in higher affinity to rs1800796C allele of IL-6 in CHB, suggested IL-6 gene allele-specific C/EBPα-binding activity regulated Th17/Treg balance to
participate HBV infection progression[22]. However, the imbalance mechanism has not been elucidated completely in CHB. The role of transcription factor RORγt and FoxP3 which have a crucial role for Th17 and Treg cells functions and differentiation is unclear in Th17/Treg imbalance in CHB.

AhR is an intracellular receptor belonging to the basic helix–loop–helix superfamily and can bind to various ligands such as 2,3,7,8-tetrachlorodibenzo-p-dioxin, 6-formylindolo[3,2-b]-carbazole, kynurenine, gut microbial products, and leukotrienes [10, 23–25]. PAS-B domain of AhR binds to ligands. The AhR complex is transferred to the nucleus. The PAS domain, mainly PAS-B, of AhR combines with the AHR nuclear translocator and activates the transcription of downstream genes, including cytochrome P450 family 1 subfamily A member 1, cytochrome P450 family 1 subfamily B member 1, and interleukin 21 [19, 26, 27]. Therefore, AhR mediates numerous cellular responses and maintains normal cell physiology. Moreover, AhR is involved in the pathogenesis of several diseases [14, 28].

AhR expresses innate and adaptive immune cells and can participate in the activation and functional development of the immune system [29]. Th17 and Treg cells, belonging to CD4 + T lymphocytes, play a role in promoting and reducing inflammation in cellular immunity. Th17/Treg imbalance is involved in the pathogenesis of many autoimmune [30], metabolic [31], and infectious diseases [32]. Therefore, AhR plays an important role in Th17 and Treg cell differentiation and function [17, 29] and is involved in Th17/Treg imbalance mechanism. However, the immunopathogenic role (especially Th17/Treg balance) of AhR in CHB remained unknown.

Moreover, we noticed that AhR expression levels were significantly elevated in CHB compared to HCs, indicating the involvement of AhR overexpression in CHB pathogenesis. This finding has not been previously reported in literature. We also found that AhR was not directly associated with the proportion of Th17 or Treg cells but was significantly correlated with the proportions of RORγt and FoxP3. Therefore, we suppose that AhR may mediate the RORγt–FoxP3 axis to participate in Th17/Treg imbalance in patients with CHB.

There are similar findings in other diseases. RORγt, the transcription factor of TH17 cells, is essential for TH17 differentiation and immune function. AhR mediates RORγt binding to the interleukin-17A promoter to regulate TH17 differentiation [33]. FoxP3 directs the differentiation of Treg cells. Quintana et al. [17] found three non-evolutionarily conserved AhR-binding sites (mainly NCABS-2) in the FoxP3 promoter to control FoxP3 expression resulting in Treg cell generation in mice. In autoimmune hepatitis, Vuerich et al showed that AhR expression increased in Treg cells, but decreased in TH17 cells. Xiong et al. found that AhR influenced the RORγt–FoxP3 axis by regulating GPR15 to control Treg intestinal homing. Palani Dinesh et al. showed that berberine (BBR) inhibited TH17 cells proliferation by downregulating RORγt and accelerated the differentiation of Treg cells through promoting FoxP3 activation via AhR to regulate the Th17/Treg imbalance[34]. Jianmin Xie also found Semaphorin 4D (Sema4D) could enhance RORγt expression and reduce FoxP3 expression in an AhR-dependent manner, resulting to Th17/Treg imbalance in ankylosing spondylitis[35]. The above results supported hypothesis of AhR– RORγt–FoxP3 axis.
However, in the study, we did not detect AhR expression level on Th17 and Treg cells, the mechanism about AhR affected expression of RORγt and FoxP3 to regulate Th17/Treg balance was not elucidated. Our conclusion should be further validated in future studies.

In conclusion, Th17/Treg imbalance was found in CHB, and AhR may influence Th17/Treg balance via the AhR–RORγt–FoxP3 axis. Thus, immunotherapy aimed at regulating the AhR–RORγt–FoxP3 axis to restore Th17/Treg balance may help in delaying CHB progression.

**Abbreviations**

CHB: Chronic hepatitis B; HBV: hepatitis B virus; RORγt: Retinoic acid-related orphan receptor gamma t; FoxP3: forkhead box protein 3; AhR: aryl hydrocarbon receptor; HC: healthy controls; PBMCs: peripheral blood mononuclear cells; HMGB1: high-mobility group box 1; NCABS-2: non-evolutionarily conserved AhR-binding sites; BBR: berberine; Sema4D: Semaphorin 4D

**Declarations**

**Acknowledgements**

The authors would like to thank all the reviewers who participated in the peer review for this manuscript.

**Authors’ contributions**

RZ collected blood samples and wrote the original draft. HL revised the first draft. JY, JG reviewed data and edited the manuscript. JL contributed to the statistical analysis. JL, JD, and YZ helped collect data and performed the statistical analysis. All authors read and agreed to the final version of the manuscript.

**Funding**

This work was supported by the National Natural Science Foundation of China (81760111;81760617) and the Yunnan Applied Basic Research Project (2017FE468(-033)). The Yunnan Health Talents Project (H-2017071) and the Yunnan Province Innovation Team of Intestinal Microecology-Related Disease Research and Technological Transformation (202005AE160010), Eminent Doctors Program of Yunnan Province (YNWR-MY-2019-072).

**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Ethical approval and consent to participate**

The experimental procedures were conducted in line with the Declaration of Helsinki. Written informed consent was obtained for all participants included in this study. The ethics committee of the First
Affiliated Hospital of Kunming Medical University approved the study protocol ([2020]47).

**Consent to publication**

Not applicable.

**Competing interest**

The authors declare no conflicts of interest with respect to the data and content of this manuscript.

**References**


Table

Table 1 Primer sequences in RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5'-3')</th>
<th>Reverse primer (5'-3')</th>
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<td>CACCACCTCCATTGCTCTG</td>
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<td>human GAPDH</td>
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<td>GCTGATGATCTTGAGGCTGTC</td>
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</tbody>
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Figures

Figure 1

Proportion of Th17 cells, Treg cells, and Th17/Treg ratio in HC and CHB groups. (A) Scatter plot of flow cytometry findings showing the percentages of Th17 cells in HC and CHB groups. (B) Scatter plot of flow cytometry findings showing the percentages of Treg cells in HC and CHB groups (C) Percentages of Th17 cells in HC and CHB groups. (D) Percentages of Treg cells in HC and CHB groups. (E) Th17/Treg ratio in HC and CHB groups. *p < 0.05; **p < 0.005; ***p ≤ 0.001.
AhR, RORγt, and FoxP3 mRNA levels in HC and CHB groups. (A) RORγt mRNA level was increased compared with the HC group ($p \leq 0.001$). (B) FoxP3 mRNA level was increased compared with the HC group ($p \leq 0.001$). (C) AhR mRNA level was increased compared with the HC group ($p \leq 0.001$). *$p < 0.05$; **$p < 0.005$; ***$p \leq 0.001$. AhR aryl hydrocarbon receptor.
Protein expression levels of AhR, RORγt, and FoxP3 in HC and CHB groups. Full length imprinting / gel is provided in supplementary figure1. HC: healthy control; CHB: chronic hepatitis B. HC1-4: representative healthy control case1,2,3,4. CHB1-4: representative chronic hepatitis B patient 1,2,3,4.
Figure 4

Correlation between AhR, RORγt mRNA, and FoxP3 mRNA. (A) Correlation between AhR and RORγt mRNA. (B) Correlation between AhR and FoxP3 mRNA.

Supplementary Files

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- supplementaryfigure1.tif