Effects of Aryl Hydrocarbon Receptor on T cells differentiation in Primary Biliary Cholangitis

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Abstract

The aim of this study was to observe the effect of Dendritic Cell (DCs) activated by AhR (Aryl hydrocarbon Receptor) on Th17 differentiation in PBC (Primary Biliary Cholangitis) patients. Ten suitable PBC patients and ten healthy people were selected as the experimental group and the control group. CD14+ mononuclear cells were isolated by Magnetic cell separation (MACS) and induced into DCs. These cells were observed and the cells surface marker were detected by flow cytometry. Two days after DCs was stimulated by BPA, the cells were cultured with the naïve CD4+ T cells in a ratio of 1:2 for three days. Then the proliferation activity of T cells was detected by CCK-8 method, and Th17 differentiation related cytokines were detected by real-time fluorescence quantitative polymerase chain reaction (qRT-PCR) and enzyme-linked immunosorbent assay (ELISA). The results showed that DCs increased in size, irregular shape and a large number of protrusions on the surface.CD11 and CD14 positive cells accounted for 96.2%.After co-culture of DCs activated by AhR and naïve CD4+ T cells, the proliferation capacity of naïve CD4+ T cells in PBC patients was significantly higher than that in the control group, and the expression levels of cytokines IL-22 and IL-17 were also significantly higher than that in the control group. These demonstrated that DCs activated by AhR can affect the differentiation of Th17 and induce its secretion of related inflammatory factors, thus promoting the occurrence of PBC.

Keywords

Aromatic hydrocarbon receptor; Dendritic cells; T cells; Primary biliary cholangitis.

1. Introduction

Women with Primary biliary cholangitis (PBC) is one of the most common chronic autoimmune disease, clinical performance for Anti-Mitochondrial Antibodies (AMAs) positive, elevated Alkaline phosphatase (ALP), pathology of immune cells infiltrating small bile ducts in the liver injury, leading to medium and small bile duct stenosis, occlusion, accompanied by portal inflammation, can develop into liver cirrhosis and liver cancer [1,2]. It is generally believed that PBC is triggered by environmental factors in genetically susceptible populations, but the specific mechanism is unknown[3].

Dioxin-like compounds (DLCs) are colorless, odorless and highly toxic environmental pollutants, mainly derived from the incineration of municipal and industrial wastes, of which Bisphenol Propane (BPA) is the most important toxic[4,5]. Existing research shows that the DLCs can produce a series of biological toxicity by activating Aryl hydrocarbon receptor (AhR), main show is the secretion of cytokines in body disorders and repression of the immune system[6].

AhR is a ligand-activated transcription factor that responds to both endogenous ligands and exogenous ligands, including DLCs[7]. A growing body of evidence supports the physiological functions of AhR in both cellular development and immune response[8]. Activation of AhR leads to up-regulation of metabolic enzymes on cytochromeP450 (CYP450), which is crucial for the biological transformation of toxic substances into low-toxic substances[9]. The role of AhR in immune regulation is multidimensional. For example, BPA can promote Dendritic Cell (DCs) maturation and activation through AhR[10-12].

DCs are the strongest antigen-presenting cells (APCs) in the immune system of the body[13]. As a key link of host immune response, DCs can directly activate the naïve $CD4^+$ T cells in the body to generate helper T cells and killer T cells, thus promoting the occurrence of immune diseases[14,15].Th17 cells can secrete IL-17A, IL-17F, IL-22 and Tumor necrosis factor-α (TNF-α), and these cytokines can mobilize, recruit and activate neutrophils collectively, thus effectively mediating tissue inflammation[16]. After binding to the receptor, IL-17 can play its biological role through the mitogen-activated protein kinase (MAPK) and nuclear factor κB (NF-κB) pathways[17]. Studies have shown that the activation of AhR can induce Th17 cell polarization and aggravate the severity of autoimmune diseases[18,19].

It is worth noting that PBC patients have significant abnormal differentiation of T cells and immune disorders, and activation and proliferation of Th17 cells can aggravate the inflammatory response of the small bile duct epithelium in PBC patients. In the PBC model mice, when the AhR gene of T cells was knocked out, the differentiation and proliferation of Th17 cells in the mice were significantly inhibited. Therefore, we intended to explore whether the activation of AhR has the same effect on the pathogenesis of PBC patients, that is, whether the DCs activated by AhR have an effect on the differentiation of Th17 in PBC patients.

2. Experimental

2.1 Materials

Lymphocyte separation medium (Ficoll-Paque™ PREMIUM, 1.077±0.001 g/mL, GE Healthcare, USA), Magnetic column sorting column and magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany), real-time fluorescence quantitative polymerase chain reaction Kit (Takara, Dalian, China), enzyme-linked immunosorbent assay Kit (Cloud-Clone corp Wuhan China), CCK-8 (Solarbio, China), GM-CSF, IL-4 (Peprotech, USA), BPA (sigma, USA), antibody (Biosciences, Sunnyvale, CA, USA).

2.2 Separation of monocytes and T cells

Peripheral blood samples were collected from 10 PBC patients and 10 healthy people with age and sex matching. After being diluted with phosphate buffer (PBS), an equal amount of lymphocyte separation solution was added to maintain a clear interface. Gradient centrifugation at room temperature (2100 rpm × 20 min) was used to extract the intermediate white cell membrane layer, which was washed twice with equal volume of PBS at the speed of 1500× rpm. CD14 and CD4 magnetic beads were added, and CD14 mononuclear cells and naïve $CD4^+$T cells were isolated according to the magnetic bead sorting instructions. The isolated $CD14^+$ mononuclear cells and naïve $CD4^+$T cells were cultured in RPMI 1640 medium containing 20% FBS and 100 U/mL dual antibody. This study was approved by the Ethics Committee of affiliated hospital of Qingdao university and obtained informed consent from patients (Approval number: QYFY WZLL 25571).

2.3 Induction of DCs maturation and activation

After $CD14^+$ mononuclear cells were separated by MACS, DCs were induced by human granulocyte-macrophage colony stimulating factor (GM-CSF,20 ng/ml) and of human interleukin-4 (IL-4, 20 ng/ml) and cultured for 7 days, followed by a half-volume fluid exchange the next day[20]. Cells were collected on the 7th day, and cell surface antibodies were detected by flow cytometry.

2.4 DCs stimulated by BPA were co-cultured with T cells

DCs from patients and healthy people were cultured separately, and 10 μM BPA were added to induce the DCs for 48 hours. After that, the DCs were cultured for 3 days with the selected T cells in a ratio of 1:2, and T cells and supernatant were collected.

2.5 Detected the proliferation ability of T cells by CCK-8

The co-cultured naïve CD4+T cells were cultured at 37°C for 24 hours, 48 hours and 72 hours respectively, and 10μl/well CCK-8 detection reagents were added to the 96-well culture plate. After incubation for 2 hours at 37°C, OD value at 450nm was measured.

2.6 Real-time fluorescence quantitative polymerase chain reaction

Unadherent naïve CD4+T cells were collected after co-culture. Total RNA was extracted from naïve CD4+T cells with Trizol RNA separation reagent, and cDNA was synthesized in strict accordance with the instructions of reverse transcription kit. The main pro-inflammatory cytokines IL-17 and IL-22 in Th17 cells were detected. Primer sequence is shown in Table 1. Relative gene expression using $2^{-\Delta\Delta CT}$ calculation method.

Table 1 Sequence of primers used for quantitative polymerase chain reaction assays.

Gene		Primer Sequence (5'-3')
IL-17	FORWARD:	CCCGGACTGTGATGGTCAAC
	REVERSE:	CCTCCCAGATCACAGAGGGATA
IL-22	FORWARD:	TCTGATGAAGCAGGTGCTGAACR
	REVERSE:	TGCAGGTCATCACCTTCAATATG

2.7 Enzyme-linked immunosorbent assay

Naïve CD4+T cells were cultured with BPA treated DC for 3 days, and supernatant was collected. Cytokines IL-22 and IL-17 were detected by ELISA.

2.8 Statistical analysis

Data analysis was performed using GraphPad Prism version 7.0 software. All experiments were repeated at least three times. The data were expressed as mean ±SEM. T test was used for comparison between groups, and ANOVA was used for comparison among groups. $P < 0.05$ was considered statistically significant.

2.9 Results

2.9.1 Induction and maturation of DCs

$CD14^{+}$ mononuclear cells were obtained from the peripheral blood of patients and healthy people by MACS. Under the induction of GM-CSF and IL-4, the cell morphology was gradually irregular, the volume increased, and the cell processes increased, which were typical DCs characteristics (Figure. 1B). Flow cytometry analysis of DCs surface markers CD11 and CD14 showed that 96.2% of CD11 and CD14 cells were double positive (Figure. 1A).

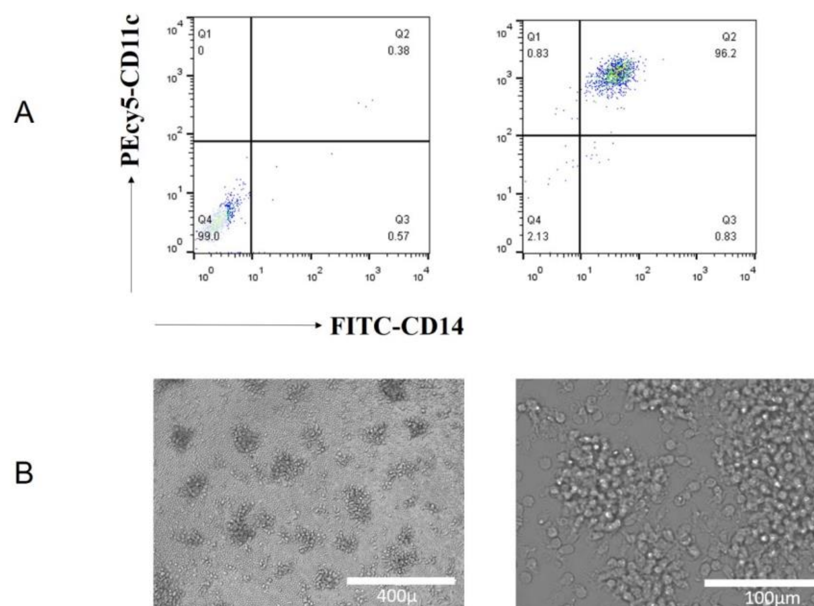

Figure. 1 Induction and maturation of DCs. A: Flow analysis of DCs surface markers CD11 and CD14 showed that 96.2% of the cells were double positive. B: Microscopic view of DCs induced by GM-CSF and IL-4.

2.9.2 After DCs co-culture, naïve CD4+ T cells proliferation was enhanced

After the success of the DCs induced, add 10 μM BPA after 48 hours respectively with the same sample of sorting out the naïve $CD4^+$ T cells co-culture in accordance with the proportion of 1:2. Naïve $CD4^+$T cells were collected at 24 h, 48 h and 72 h, respectively, and measured the sample OD value respectively. The results show that the PBC patient group, 48 h group is 24 h OD value of OD value higher, statistically significant difference (0.82 ± 0.04 versus 0.69 ± 0.04, $P < 0.05$). In the PBC group, the OD value of the 72h group was higher than that of the 48h group, and the difference was statistically significant (1.02 ± 0.04 versus 0.82 ± 0.04, $P < 0.01$). (Figure. 2)

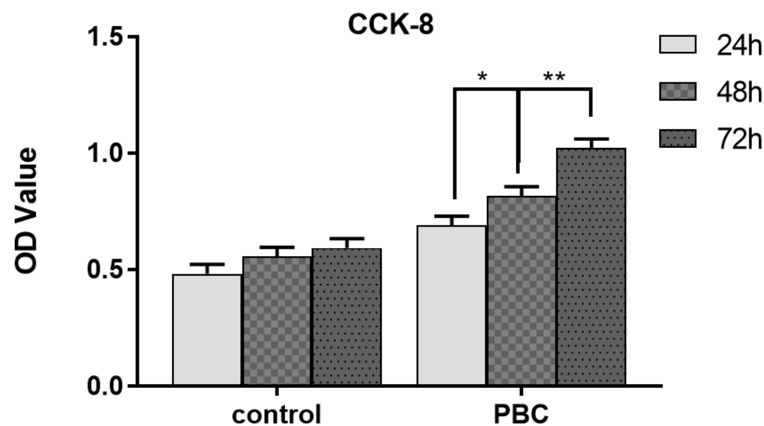

Figure. 2 CCK-8 for naïve CD4+ T cells activity. The results showed that compared with the control group, the naïve CD4+ T cells of PBC patients were stimulated by DCs for 48h and 72h with enhanced cell activity, which was statistically significant ($P<0.05$).

2.9.3 In co-culture supernatant, IL-17 and IL-22 were increased

After DCs were co-cultured with naïve $CD4^+$T cells, the expression of IL-17 and IL-22 in supernatant were detected by ELISA. As showed in Fig. 3, the expression level of IL-22 in the PBC group was higher than that in the healthy control group, and the difference was statistically significant (77.38 ± 2.90 versus 49.63 ± 4.04, $P < 0.05$). The expression level of IL-17 was also higher than that in the healthy control group, and the difference was statistically significant (170.93 ± 2.68 versus 130.61 ± 7.10, $P < 0.01$).

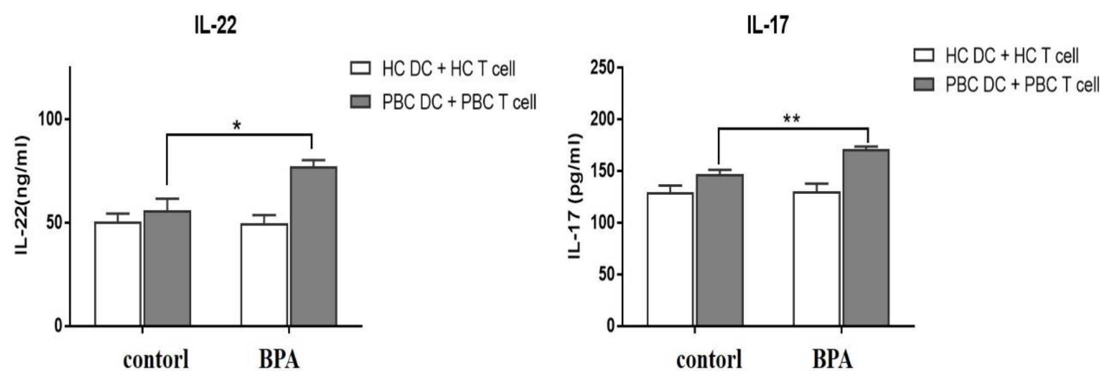

Figure. 3 Increased expression of Th17 related cytokines. After DCs were co-cultured with naïve $CD4^+$ T cells, the expression levels of IL-17 and IL-22 in supernatant were detected by ELISA. Compared with the normal control, the expression of IL-22 and IL-17 were statistically different ($P < 0.05$).

2.9.4 The expression of Th17 related cytokines increased

After DCs were co-cultured with naïve $CD4^+$ T cells, these T cells were collected and total RNA was extracted for qRT-PCR detection. The results showed that the expression of IL-17 in the PBC group (1.24 ± 0.14) was significantly higher than that in the healthy control group (0.61 ± 0.18), and the

difference was statistically significant ($P < 0.01$).The expression level of IL-22 (0.96 ± 0.20) was also significantly higher than that of the healthy control group (0.42 ± 0.15), and the difference was statistically significant ($P < 0.05$).

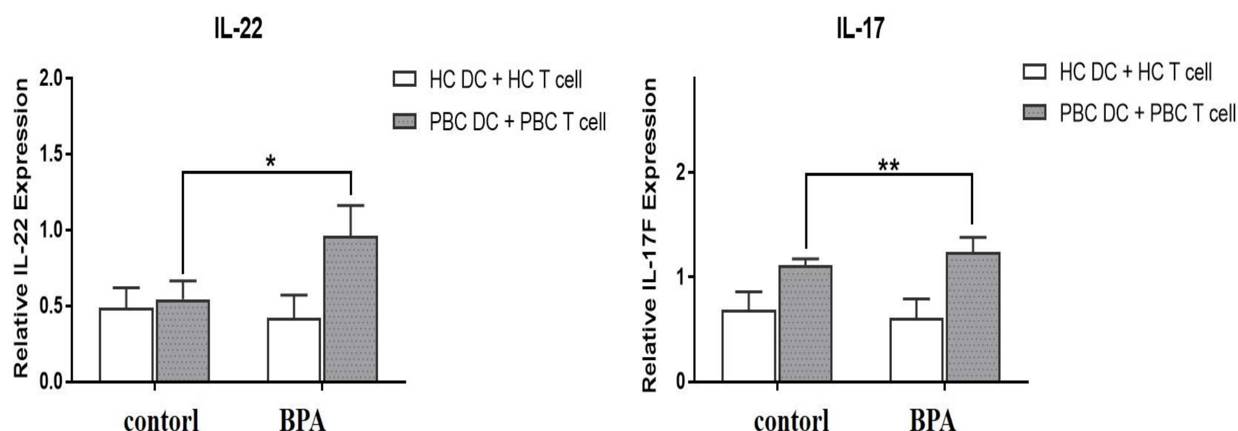

Figure. 4 Increased expression of Th17 related cytokines. Compared with the control group, the results showed that the expression of IL-22 and IL-17 in the PBC group were significantly higher than that in the healthy control group ($P < 0.05$).

2.10 Discussion

PBC is a typical autoimmune disease, characterized by the production of a large number of autoantibodies and liver function damage, abnormal activation of T cells in the body[21]. Abnormal differentiation and dysfunction of Th cells, secretion of a large number of inflammatory factors to stimulate the production of autoantibodies, thus accelerating the development of PBC disease[22]. With the aggravation of environmental problems and the improvement of medical treatment, the incidence of PBC is on the rise[23].

More and more attention has been paid to the role of environmental factors in the occurrence and development of diseases[24]. Environmental pollutants activate antigen presenting cells by AhR, and the differentiation of T cells is affected by the action of related inflammatory factors and chemokines[17]. In this study, qRT-PCR and ELISA were used to confirm that AhR-activated DCs in the PBC group had a more significant effect on the naïve $CD4^{+}$T cells than that in the normal control group, and Th17-related differentiation markers were significantly increased. It is suggested that AhR plays an important role in the occurrence and development of autoimmune diseases.

Th17 cells are a subset of T lymphocytes that play an important role in the development of inflammatory diseases[25]. The function of DCs in T cell differentiation and its immunomodulatory effect depend on the regulation and expression of cytokines and chemokines[25,26]. In this study, it was found that DCs of PBC patients stimulated by BPA could promote the differentiation of Th17, and the expression levels of related cytokines IL-22 and IL-17 were significantly higher than those of the control group. It was speculated that PBC patients had a more obvious response to xenobiotic, making individuals more prone to autoimmune diseases.

This experiment showed that AhR was more easily activated in PBC patients than normal people, and its differentiation related factors were also easily activated, suggesting that DCs activated by AhR may promote the proliferation and differentiation of Th17 cells and the secretion of its related cytokines. Th17 played an important role in the pathophysiological process of PBC, and the increase of Th17 leads to the disorder of T cells and the autoimmune response[27]. In addition, the expression of AhR in DCs of PBC patients is positively correlated with the expression of IL-17 and IL-22. Therefore, whether the AhR protein and its related downstream signaling pathway are highly expressed and how the AhR pathway plays a key role in the pathogenesis of PBC patients have become a new direction to explore the pathogenesis of PBC.

3. Conclusion

In summary, AhR-activated-DCs influenced the Th17 cells differentiation by stimulating naïve $CD4^+$ T cells to secrete IL-22 and IL-17 in PBC patients. These abnormal differentiation of T cells can cause immune dysfunction, promoting the pathogenesis of PBC. Therefore, AhR may be a potential target of PBC, which also expands a new direction for the further study of AhR on abnormal T cell differentiation in PBC.

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