Unbalanced Aryl Hydrocarbon Receptor Expression in Peripheral and Lesional T Cell Subsets of Atopic Dermatitis

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Background and Objective: The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor, which is involved in the pathogenesis of a variety of skin diseases such as atopic dermatitis (AD). In this study, we aimed to study the AhR-expressing cells in T helper 17 (Th17), T helper 22 (Th22), regulatory T cells (Treg) and B cells in peripheral blood and in AD skin lesions.

Methods: Twenty AD patients defined according to the Chinese criteria of atopic dermatitis and eighteen healthy subjects were included in our study. The AhR-expressing Th17, Th22, Treg and total B cells in peripheral blood were measured by flow cytometry. The AhR+ Th17 cells and AhR+ Th22 cells in AD skin lesions were measured by immunofluorescence. The mRNA of AhR, interleukin (IL)-22, IL-17A, IL-10, Foxp3, RORγT and TGF-β in peripheral blood mononuclear cells (PBMCs) was measured by real-time quantitative polymerase chain reaction.

Results: The expression of AhR in peripheral CD4+ T cells, Th22 cells, Treg cells and total B cells was significantly increased in AD. AhR+IL-17A+ and AhR+IL-22+ lymphocytes were also increased in AD skin lesions. The mRNA levels of AhR, IL-22 and IL-17A in PBMCs in AD patients were significantly higher. AhR mRNA levels in PBMCs positively correlated with peripheral basophil count, peripheral eosinophil count and mRNA levels of IL-22.

Conclusion: AhR was highly expressed in subpopulations of CD4+ T cells in peripheral blood and skin lesions of AD, suggesting that AhR might contribute to the pathogenesis of AD.

Keywords: aryl hydrocarbon receptor, atopic dermatitis, CD4+ T cells, Th22 cells, Th17 cells, B cells

Introduction

Atopic dermatitis (AD) is an inflammatory skin disease characterized by chronic recurrent dermatitis with profound pruritus. The pathogenesis of AD includes a multitude of factors such as disrupted epidermal barrier, exaggerated Th2 inflammation and inadequate antimicrobial peptides. AD is currently considered as a biphasic T cell-mediated disease, which exhibits Th2- and Th22-deviated immune reactions during disease chronicity. The immunologic dysregulation in AD is characterized by a type 2 helper (Th2)-dominant inflammatory signature leading to increased cytokine production such as IL-4 and IL-13. Immunoglobulin E (IgE) autoreactivity and the Th17/Th22 inflammatory axis have also been implicated.

Aryl hydrocarbon receptor (AhR) is a transcription factor which plays important roles in cell proliferation, apoptosis, differentiation, adhesion and migration. AhR signaling pathway represents an important link between environmental stimuli and immune-mediated inflammatory disorder and has become the research interest recently. The expression of AhR in a majority of immune cell types such as T cells (Th17 cells, Treg cells, Th22 cells) and B cells demonstrates the importance of this receptor in immunological processes. It has been shown that AhR plays an important role in the realm of CD4+ helper T cell mediated inflammation. The AhR expression is found to be significantly increased in PBMCs and lesional skin of patients with AD. AhR modulation can lead to potential therapeutic effects. However, little
was known about the expression of AhR in subpopulation of lymphocytes. In this study, we investigated the expression of AhR in subpopulation of T cells of peripheral blood and AD skin lesions.

**Patients and Methods**

**Patients and Healthy Controls**

Twenty adult AD patients (12 females, 8 males) were recruited. All patients satisfied the criteria of Hanifin and Rajka and Chinese criteria for atopic dermatitis. The severity of AD was determined by the eczema area and severity index (EASI) score. Peripheral eosinophils, basophils and total serum immunoglobulin E (IgE) levels were measured. Eighteen age- and gender-matched healthy subjects were also recruited. Seven normal skin biopsies were obtained from plastic surgeries. This study was approved by the Peking University People’s Hospital Ethics Committee (No.2022PHE001). The informed consent was obtained from all the participants.

**Isolation and Stimulation of Peripheral Blood Mononuclear Cells (PBMC)**

Peripheral blood samples were collected from AD patients and healthy controls, and the PBMCs were prepared by density gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare, Stockholm, Sweden). Cell suspensions were adjusted to a concentration of $5 \times 10^6$/mL and then incubated for 5 hours at 37°C in a 5% CO2 incubator, with or without phorbol myristic acid (PMA, 50 ng/mL)/ionomycin calcium (1μg/mL) mixture and brefeldin A (3μg/mL)/monensin (1.4 μg/mL) mixture (MultiSciences Biotech Co Ltd, Hangzhou, China) dissolved in RPMI 1640 medium supplemented with 10% fetal bovine serum (Gibco, Life Technologies, Grand Island, NY).

**Flow Cytometry**

For Treg staining (CD4⁺ CD25⁺ FOXP3⁺), 100μL cell suspensions ($5 \times 10^5$ cells per subject) of PBMCs were stained with surface marker fluorescein isothiocyanate (FITC)-cluster of differentiation (CD) 4 (3μL, eBiosience, USA) and peridinin chlorophyll protein complex (PerCP)-CD25 (5μL, eBiosience, USA). The mixed samples were incubated in the dark for 30 min at 4 °C. Upon adding 3 mL PBS and centrifuging at 400g for 5 min, the supernatant was removed. Upon fixation and permeabilization, nuclear markers phycoerythrin (PE)-AhR (3μL, eBiosience, USA) and allophycocyanin (APC)-FOXP3 (5μL, eBiosience, USA) were added and incubated in the dark for 30 min at 4 °C. The supernatant was removed upon centrifugation at 400g for 5 min and subjected to flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA) following dilution in 100 μL PBS.

For the staining of Th17 and Th22 cells, 100μL cell suspensions ($5 \times 10^5$ cells per subject) of PBMCs were prepared and 2 μL PMA/ionomycin alongside 2 μL brefeldin A/monensin mixture was added to the cells. And then the cells were stained with surface marker FITC-CD4 (3μL) and nuclear markers APC-IL-17A (5μL, eBiosience, USA), PerCP-IL-22 (5μL, eBiosience, USA) and PE-AhR (3μL, eBiosience, USA). Total B cells were stained with surface marker FITC-CD19 (3μL, eBiosience, USA).

Finally, the cells were resuspended and evaluated by flow cytometry.

**Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR)**

Five milliliters of venous blood was obtained from all AD patients and healthy controls in blood collection tubes containing dipotassium ethylenediamine tetra-acetic acid (K2EDTA) for PBMC separation. The PBMCs were separated from peripheral blood by Ficoll Hypaque density gradient centrifugation. The total RNA was extracted from PBMCs using RNA blood mini kit (Qiagen, Germany) according to the manufacturer’s instructions. Complementary DNA synthesis was performed using the FastKing RT Kit (Tiangen Biotech Co., Ltd., Beijing, China). RT-qPCR was performed using the SYBR Green Dye method which was carried out using cDNAs supplemented with SYBR Green Supermix (Bio-rad, Hercules, CA, USA). The PCR amplification used gene-specific primers for β-actin (forward, 5’-TGGCACACCAGCACAATGAA-3’; reverse, 5’-CTAAGTGTGTCCTACGTGAAGCA-3’), AhR (forward, 5’-TAACCCAAGACCAGATTCCCTCAGA-3’; reverse, 5’-CCCTTGGAAATTTCAATCCAGA-3’), IL-22 (forward, 5’-CCTATATCACCACCAACCCGACCTTC-3’; reverse, 5’-AGATTGGGGAACACGACTTCTTC-3’), IL-17A (forward,
5'- AGATTACTACAACCGATCCACCT-3'; reverse, 5'- GGGGACAGAGTTCATGTGGTA-3'), RORγt (forward, 5'- GTGGGGACAAGTCGTCTGG-3'; reverse, 5'- AGTGCTGGCATCGGTTTCG-3'), FOXP3 (forward, 5'- GCAGCTGAGGCCGACTA-3'; reverse, 5'- CGGAGCTCTGATGTGTTGAA-3'). The PCR protocol consisted of a cycle at 95 °C for 60 seconds followed by 40 cycles consisting of 15s at 95°C, 15s at 60°C and 15s at 72°C. The average Ct was calculated for the target genes and internal control (β-actin). ΔCt (Ct_target – Ct_β-actin) values were determined. The expression levels of target genes in AD patients were determined relative to controls as 2^{−ΔΔCt}, where ΔΔCt = ΔCt (patient or control) – ΔCt (average for controls). The results were converted into relative expression.

**Immunofluorescence**

Dual immunofluorescence was performed to colocalize CD4, CD19, IL-17A and IL-22 to AhR. Briefly, optimum cutting temperature embedded skin tissues were cut into 5-μm sections and boiled in ethylenediaminetetraacetic acid buffer (pH, 9.0) for antigen retrieval. The sections were incubated with the primary antibody overnight at 4°C. The following primary antibodies (mouse antihuman) were used: anti-CD4 (Servicebio, Wuhan, China, 1:100), anti-CD19 (Servicebio, Wuhan, China, 1:100), anti-IL-17A (Proteintech, Beijing, China, 1:200), anti-IL-22 (Bioss, Beijing, China, 1:100) and anti-AhR (Abcam, Cambridge, US). Then, the sections were incubated with corresponding fluorescence secondary antibody and counterstained using 4',6-diamidino-2-phenylindole. The sections were observed under an inverted fluorescent microscope (Nikon, Tokyo, Japan) at original magnification ×200 under appropriate absorption/emission wavelengths (Alexa Fluor, 594 nm or 488 nm). The images were captured using a Nikon Digital Still Camera and analyzed by 2 independent investigators. Immunostained cells in the dermis were counted, and results were expressed as the number of positive cells/high power field (HPF). Interobserver variations for the counts were <10%.

**Statistical Analysis**

All data were analyzed by SPSS 22.0 software. Mann–Whitney U-tests and Kruskal–Wallis test were used for non-normally distributed variables. Simple linear regression analysis and Spearman correlation index were applied to describe the correlation between measurement data. P < 0.05 was considered significant.

**Results**

**CD4+ T Cells, Th17, Th22 and Treg Cells in Peripheral Blood of AD Patients**

CD4+ T cells, Th17 cells, Th22 cells and Treg cells in PBMCs of AD patients and healthy controls were measured by flow cytometry in Figure 1. Th17 cells were significantly increased in patients with AD compared with healthy controls (P=0.007, Figure 1b). No significant difference was found in peripheral CD4+ T cells, Th22 and Treg cells between AD patients and healthy controls (P>0.05, Figure 1b and c).

**Expression of AhR in Treg, Th17, Th22 Cells and B Cells in Patients with AD**

AhR-positive cells in Treg cells, Th17 cells, Th22 cells, CD4+T cells and total B cells were measured by flow cytometry in Figure 2 and Figure 3. Comparing with healthy controls, the AhR+ CD4+T cells (P=0.018, Figure 2a), AhR+ Treg cells (P=0.009, Figure 2b), AhR+ Th22 cells (P=0.007, Figure 2d) and AhR+ CD19+ B cells (P=0.043, Figure 3) were significantly increased in AD patients. However, peripheral AhR+ Th17 cells were not increased (Figure 2c).

**AhR+ Th17 Cells and AhR+ Th22 Cells in AD Lesions**

AhR+ Th17 cells (IL-17A+AhR+) and AhR+ Th22 cells (IL-22+AhR+) in AD lesions were determined by immunofluorescence staining in Figure 4. The number of AhR+ Th17 cells (P=0.003) and AhR+ Th22 cells (P=0.008) was
significantly increased in AD lesions (Figure 4a and b). No significant difference was found in AhR$^+$ CD4$^+$ T cells ($P=0.067$, Figure 4c) and AhR$^+$ CD19$^+$ B cells ($P=0.320$, Figure 4c).

**Figure 1** T-cell subpopulations in PBMCs of AD patients and healthy controls. Representative fluorescence-activated cell sorter (FACS; BD Immunocytometry Systems, San Jose, CA) plots showed CD4$^+$ T cells (a), CD4$^+$IL-17A$^+$ Th17 cells (b), CD4$^+$IL-22$^+$ Th22 cells (b) and CD4$^+$CD25$^+$Foxp3$^+$ regulatory T (Treg) cells (c) detected in cluster of differentiation CD4$^+$ T cells by flow cytometry. The portions of Th17 cells, Th22 cells and Treg cells in CD4$^+$ T cells were analyzed. Data are displayed as mean±SEM.

Abbreviations: AD, atopic dermatitis; HC, healthy controls; IL, interleukin; Foxp3, forkhead box P3.

mRNA Expression of AhR, IL-22, IL-17A, IL-10, Foxp3, RORγT and TGF-β in PBMCs of AD Patients

The mRNA expression levels of AhR and IL-22, IL-17A, IL-10, Foxp3, RORγT and TGF-β in AD patients are shown in Figure 5. Comparing with healthy controls, the mRNA levels of AhR ($P=0.022$, Figure 5a), IL-22 ($P=0.028$, Figure 5b) and IL-17A ($P=0.032$, Figure 5c) in PBMCs of AD patients were significantly increased, whereas, the mRNA expression of the IL-10, Foxp3, RORγT and TGF-βwere not increased ($P>0.05$, Figure 5d–g).
Figure 2 The percentages of AhR$^+$ cells in CD4$^+$ T cells (a), Treg cells (b), Th17 cells (c) and Th22 cells (d) in AD patients were detected by flow cytometry. Data are displayed as mean±SEM. *$P<0.05$, **$P<0.01$.

Abbreviations: AD, atopic dermatitis; HC, healthy controls; IL, interleukin; AhR, aryl hydrocarbon receptor.
Relationship Between AhR mRNA Expression and Peripheral Basophils/Eosinophils and Th17-, Th22-, Treg-Associated Cytokines/Transcription Factors

The relationship between mRNA expression of AhR in PBMCs, peripheral basophil count, peripheral eosinophil count and cytokines is shown in Figure 5. A significant positive correlation was found between AhR mRNA level and peripheral basophil count (r = 0.645, P = 0.004, Figure 5h), peripheral eosinophil count (r = 0.717, P = 0.001, Figure 5i) and IL-22 mRNA level (r = 0.545, P = 0.019, Figure 5j).

Discussion

Aryl hydrocarbon receptor (AhR) is a protein ligand-activated transcription factor. It can be activated by small molecules, including environmental toxicants or polycyclic aromatic hydrocarbons, bacterial pigments, or physiological compounds. Canonical and non-canonical signaling pathways of AhR activation have been identified, and canonical pathways were more common. When inactivated, AhR is trapped in a cytosolic multiprotein complex. Various ligands can activate AhR to translocate into the nucleus, where it is released from the complex and dimerizes with a partner molecule (AhR nuclear translocator, ARNT) to form a transcription factor. The ligand–AhR–ARNT complex binds specific DNA sequences and initiates transcription of the target gene. AhR modulates various physiological processes such as cell cycle regulation, cell migration, hematopoiesis, vascular development and lymphocyte differentiation. It is a central player in skin integrity and skin immunity, responding to exogenous and endogenous chemicals. Various experimental systems have been used to assess the role of AhR.
AhR+ CD4+ T cells, CD19+ B cells, Th17 cells (a) and Th22 cells (b) in skin lesions of AD and healthy control were shown by Immunofluorescence staining. AhR+ Th17 cells and AhR+ Th22 cells were significantly increased in AD skin lesions (c). a,b×200. Nuclei were counterstained using DAPI. Data are displayed as mean±SEM. **P<0.01.

**Abbreviations:** AD, atopic dermatitis; HC, healthy controls; DAPI, 4',6-diamidino-2-phenylindole; AhR, aryl hydrocarbon receptor.
in skin inflammation, including in vitro assays of keratinocyte stimulation and murine models of psoriasis and atopic dermatitis.\textsuperscript{13}

Atopic dermatitis is an eczematous skin disorder characterized by Th2-deviated skin inflammation, barrier disruption and chronic pruritus.\textsuperscript{14} Increased Th2 cytokines such as IL-4, IL-5, IL-13 and IL-33 were found in AD, and the Th17/Th22 axis has also been implicated.\textsuperscript{9} In this study, we found that AhR, IL-22 and IL-17 mRNA were increased in PBMCs of AD. The AhR mRNA levels were positively correlated with IL-22 levels in PBMCs. Kim et al reported that AhR was highly expressed in acute lesions of AD and psoriasis, and the AhR pathway was also activated in chronic inflammatory skin diseases.\textsuperscript{15} Recently, AhR-targeting treatment has been developed.\textsuperscript{16} Tsuji et al reported that AhR activation could induce upregulation and nuclear translocation of OVOL1, resulting in increased FLG expression.\textsuperscript{17} AhR agonists such as soybean tar and coal tar have been shown to inhibit Th2 cytokines such as IL-4 and IL-13.\textsuperscript{18}

Th17 cells and Th22 cells, which mainly produce IL-17 and IL-22, are associated with development of various autoimmune diseases such as rheumatoid arthritis, inflammatory bowel diseases, atopic dermatitis and psoriasis. It was reported that the portion of IL-17-producing CD4+ T cells was increased in peripheral blood and skin lesions of AD.\textsuperscript{19} In acute stage of AD, more Th2, Th22, and Th17 cell infiltrations were found. In chronic stage, a mixed Th1, Th2 and Th22

Figure 5 The mRNA expression of AhR (a), IL-22 (b), IL-17A (c), IL-10 (d), Foxp3 (e), ROR\gammaT (f) and TGF-\beta (g) in PBMCs of AD patients. Correlations of AhR mRNA expression and peripheral basophil count (h), r=0.645 \(P=0.004\), eosinophil count (i), r=0.717 \(P=0.001\) and mRNA levels of IL-22 (j), r=0.545 \(P=0.019\). \*\(P<0.05\).

Abbreviations: AD, atopic dermatitis; HC, healthy controls; AhR, aryl hydrocarbon receptor.
cell infiltrations were also observed, suggesting that the increased Th17 and Th22 cells may be associated with Th2 dominance in AD.

It has been reported that the AhR expression is negligible in naïve CD4+ T cells (Th0), Th1 cells, and Th2 cells. The higher AhR level was found in Th17 cells while regulatory T (Treg) cells, Th22 cells and B lymphocytes showed intermediate expression. In this study, we found that AhR+ Th17 and AhR+ Th22 cells were increased in AD lesions. Similarly, AhR+ Th22 cells and AhR+CD4+CD25+ Treg cells were increased in PBMCs of AD. These results indicate that AhR might be a regulator of the balance between subpopulations of CD4+T cells including Th17 cells, Th22 cells and Treg cells. It has been reported that 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-induced AhR activation can promote transcription of FOXP3 gene and the expansion of the CD4+CD25+FoxP3+ Treg-cell compartment. AhR agonists tapinarof, a new treatment for AD, can inhibit T-cell expansion, Th17 differentiation and IL-17 production in a T-cell expansion assay. It also inhibited IL-17A production from CD4+ T-cell. On the other hand, Formylindolo[3,2-b]carbazole (FICZ), a high-affinity ligand for AhR, can promote IL-22 production while inhibit IL-17A production by CD4+ T cells. Marc Veldhoen et al reported that activation of AhR by FICZ can significantly enhance Th17 differentiation and promote CD4+ T cells to promote IL-22.

B cells are important for the recognition of the various antigens and are involved in the pathogenesis of allergic diseases. Tanaka et al reported that Th2 cytokines such as IL-4 and IL-13 can increase the mRNA and protein expression of AhR in B cells and keratinocytes. This suggests a mutually compensatory regulation between Th2 cells, B cells and AhR signaling. In this study, we found that AhR+ CD19+ B cells were increased in PBMCs but not significantly increased in skin lesions of AD, suggesting that AhR may be involved in biological functions of B cells in AD.

Eosinophils and basophils are both involved in the pathogenesis of AD. They are usually effector cells in IgE-dependent hypersensitivity reaction in skin inflammation. Th2 cells and Th2-derived cytokines IL-4, IL-5, and IL-13 are important regulators for eosinophil proliferation and activation. In this study, we found positive correlations between AhR mRNA expression level and peripheral blood basophil and eosinophil count, suggesting that AhR might be involved in activation and mobilization of eosinophils and basophils. Tajima et al reported that the AhR activation by benzo[a]pyrene (BaP) can increase eosinophil infiltration in a mouse model of allergic airway inflammation. Activation of AhR contributes to tissue adaptation of intestinal eosinophils in mice. Exposure to BaP significantly enhances IL-8 secretion by human eosinophilic leukemia cell line in vitro. However, the relationship between AhR activation and basophils was not established.

Conclusion
In this study, we found that AhR+ Th22 cells, Treg cells and B cells were increased in PBMCs of AD. AhR+IL-22+ and AhR+IL-17A+ T cells were also increased in AD lesions. These results suggest that the expression of AhR was diversely regulated among subpopulations of lymphocytes and might contribute to the pathogenesis of AD.

Data Sharing Statement
The original contributions presented in the study are included in the article, and further inquiries can be directed to the corresponding author.

Ethics Approval Statement
Our study complies with the Declaration of Helsinki. The study design was approved by the Institutional Ethics Committee of Peking University People’s Hospital (No.2022PHE001).

Informed Consent
Written informed consent was obtained from all patients. All data were agreed to be published also by the consent.
Author Contributions
All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure
The authors report no conflicts of interest in this work.

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