Increased aryl hydrocarbon receptor expression in patients with allergic rhinitis

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Summary

Background: A predominant Th17 population is a marker of allergic rhinitis (AR). As a ligand-activated transcription factor, the aryl hydrocarbon receptor (AhR) plays a vital role in promoting or inhibiting the development of specific Th cells. However, its role in AR remains undefined.

Objective: To analyze the potential role of AhR in the pathogenesis of AR.

Methods: In total, 30 AR patients and 13 healthy controls were recruited for this study and AR patients had clinical features, as demonstrated by rhinoconjunctivitis quality of life questionnaires, total symptom scores and visual analog scale scores. The expression of AhR, IL-17 and IL-22 and the presence of Th17 cells in peripheral blood mononuclear cells were measured before and after treatment with the nontoxic AhR ligand 2-(1'H-indole-3-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE).

Results: Pretreatment ITE studies revealed that all AR patients had a significant increase in AhR expression compared with controls and AhR expression positively correlated with clinical parameters. After ITE intervention, a severe reduction in the differentiation of Th17 cells and the production of IL-17 and IL-22 was noted in both AR patients and normal subjects. Simultaneously, a dramatic enhancement of AhR expression was also observed in all healthy controls, but not in AR patients.

Conclusion: The results suggested that the AhR may be one of the mechanisms underlying the Th17 response during the pathogenesis of AR and AhR levels were closely related to clinical severity in all AR patients. Additionally, ITE may represent a new drug candidate in the treatment of AR.

Introduction

Allergic rhinitis (AR), characterized by chronic inflammation of the nasal mucosa induced by allergen exposure, is estimated to have a high incidence rate of 10–20% worldwide, reducing patient quality of life and resulting in increased healthcare expenditures. Moreover, evidence indicates that the prevalence of AR is increasing, reaching as high as 50% in some populations. While AR has been the subject of intensive research, the details of its pathogenic mechanisms are still not clear.
Recently, researchers have begun to pay close attention to the role of Th17 in the development of allergic airway inflammation. Ciprandi et al.\(^4\) demonstrated that in AR, serum interleukin (IL)-17 levels were related to clinical severity. Additionally, Bajoriuniene et al.\(^5\) found that a predominant Th17 milieu accompanied by high levels of IL-17 was the hallmark of AR disorders. These results have revealed that Th17 may be involved in the immune response to casual allergens and could contribute to increasing the severity of inflammatory disease.

Studies have demonstrated a role for the aryl hydrocarbon receptor (AhR), a member of the basic helix–loop–helix/PER–ARNT–SIM family, in modulating immunity reactions. Currently available data suggest that AhR, after activation by its ligands, such as 2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE),\(^6\) exerts protective effects against some autoimmune and allergic diseases.\(^7\)–\(^10\) Notably, Quintana et al.\(^11\) reported that AhR could also play a crucial role in regulating Th17 differentiation and the production of excreted cytokines (i.e. IL-17 and IL-22).\(^11\) Therefore, we hypothesize that AhR may contribute to the formation of chronic inflammation patterns in AR patients.

To the best of our knowledge, no studies have investigated the role of AhR in AR. The aim of this study was to characterize the role of AhR in the Th17 response associated with AR. To this end, we examined the relationship between AhR mRNA expression and Th17 reactions in AR. Additionally, the rhinoconjunctivitis quality of life questionnaire (RQLQ), total symptoms score (TSS) and visual analog scale (VAS) were used to evaluate the severity of AR, and the association between AhR and clinical parameters was also analyzed. Finally, we assessed the effects of ITE administration on the expression of AhR, Th17, IL-17 and IL-22 in order to improve our understanding of the pathogenesis of AR.

Materials and methods

Patients

In total, 30 AR patients (17 men and 13 women) who were referred to us from May 2012 to March 2013 and 13 healthy controls (8 men and 5 women) were included in this study.

Patients with AR were defined by positive skin prick tests (SPTs) and the presence of nasal symptoms, according to standard validated criteria.\(^12\) SPTs were performed according to the guidelines of the European Academy of Allergy and Clinical Immunology.\(^13\) Patients with rhinosinusitis, asthma or systemic diseases were excluded. Clinical data were collected, including patients’ ages, sexes, TSSs, RQLQ scores and VAS scores.\(^12\) All patients discontinued using oral and topical corticosteroids, antihistamines and antileukotrienes for at least 4 weeks prior to sample collection. This study was approved by the ethical committee of Chongqing Medical University (Grant No: 201212) and adhered to the tenets of the Declaration of Helsinki. Written informed consent was obtained from each patient and healthy subject before sample collection.

Cell isolation and culture

Anticoagulated blood samples were obtained using vacuum tubes containing heparin. Peripheral blood mononuclear cells (PBMCs) were prepared by Ficoll–Hypaque density-gradient centrifugation. To study AhR expression in PBMCs and Th17 cells, PBMCs were stimulated with anti-CD3 (5 μg/ml, eBioscience, San Diego, CA, USA) and anti-CD28 antibodies (1 μg/ml, eBioscience) for 72 h at a density of 1.0 × 10\(^6\) cells/ml. The supernatants were harvested and used for enzyme-linked immunosorbent assays (ELISA) to determine IL-17 and IL-22 concentrations, while the cells were used for quantitative real-time reverse transcription polymerase chain reaction (RT–qPCR) and western blot analyses. To determine the effects of the AhR ligand ITE on AhR expression and Th17 cell differentiation, PBMCs from AR patients and healthy controls were stimulated with anti-CD3 (5 μg/ml, BD Biosciences, USA) antibodies, anti-CD28 antibodies (1 μg/ml, BD, USA), IL-6 (20 ng/ml, R&D, USA) and transforming growth factor beta 1 (TGF-β1, 2 ng/ml, R&D) in the presence of ITE (50 nM/ml; Tocris Bioscience, USA) for 3 days. The addition of phosphate-buffered saline (PBS), IL-6 and TGF-β1 served as a control for ITE-treated PBMCs.\(^11\),\(^14\) After ITE treatment, the supernatants were harvested for IL-17 and IL-22 ELISAs, and the cells were harvested for RT–qPCR, western blotting and flow cytometry analysis.

RT–qPCR

To analyze the expression of AhR mRNA in PBMCs, RT–qPCR was performed. RNA was extracted using a Total RNA Extraction Kit (Bioteke, China) and was reverse transcribed to cDNA using a SYBR Real-time PCR Premixture Kit (Biotek). The level of AhR mRNA was normalized to the expression of β-actin mRNA. RT–qPCR was performed with the following primers: AhR forward: 5’-TACTCCACTTCTAGGCCACCAC-3’, reverse: 5’-TATGGACTCGGACAATATA-3’;
β-actin forward: 5′-TGACGTGGACATCCGCAAAG-3′, reverse: 5′-CTGGAAGGTGGACAGCGAGG-3′. The expression level of AhR mRNA was determined using the 2−ΔΔCt cycle threshold method.

Western blotting

PBMCs were lysed with Radio-Immunoprecipitation Assay (RIPA) lysis buffer (containing 1% phenylmethylsulfonyl fluoride). The protein concentrations of the extracts were measured with a protein assay kit (Beyotime, China). Equal amounts of protein were loaded and separated on sodium dodecyl sulfate–polyacrylamide gels (AhR: 6% gel; β-actin: 10% gel), transferred onto polyvinylidene difluoride membranes and incubated with anti-AhR (Sigma-Aldrich, USA) or anti-β-actin (Beyotime) primary antibodies. The protein bands were detected with enhanced chemiluminescence. Quantity One software, version 4.52 (Bio-Rad) was used to quantify the relative expression levels of the proteins.

ELISA for IL-17 and IL-22

Cytokines in the cell culture supernatants were detected using a Human Duoset ELISA Development Kit (R&D) according to the manufacturer’s instructions.

Flow cytometry

For intracellular protein staining, PBMCs were stimulated with phorbolmyristate acetate (PMA, 50 ng/ml; Alexis Biochemicals, CA, USA), ionomycin (1 μg/ml; Sigma-Aldrich) and monensin (1 μg/ml; BD Biosciences) for 5 h. To detect the percentage of Th17 in PBMCs, PBMCs were washed once and then incubated with Fluorescein Isothiocyanate (FITC)-conjugated anti-human CD4 antibodies (BD Biosciences). The cells were then washed, fixed, permeabilized and stained with Phycoerythrin (PE)-conjugated anti-human IL-17 A antibodies (eBioscience) or the appropriate isotypes (eBioscience). To measure AhR expression in Th17 cells, PBMCs were washed once and incubated with Allophycocyanin (APC)-conjugated anti-human CD4 antibodies (eBioscience). The cells were then washed, fixed, permeabilized and incubated with primary antibodies (anti-human AhR antibody, Sigma-Aldrich). Next, cells were washed once, incubated with FITC-labeled secondary antibodies (4 A BioTack, China) and PE-conjugated anti-human IL-17 A antibodies. PBMCs incubated with APC-conjugated anti-human CD4 antibodies, PE-conjugated anti-human IL-17 A antibodies and the appropriate isotype served as a control. Data were analyzed using a FACScan cytometer equipped with CELLQUEST software (BD Biosciences).

Statistical analysis

The Student’s t-test and nonparametric Mann–Whitney test were applied using SPSS 20.0 software. Data are expressed as the mean ± standard deviation (SD). Differences with P-values <0.05 were considered significant.

Results

Clinical features

AR severity was evaluated using RQLQ, TSS and VAS scores; the overall clinical features are summarized in Table 1. The TSS, RQLQ and VAS scores were markedly higher in AR patients than in controls (P<0.01). The mean age and the sex ratio were not significantly different between the two groups (P>0.05).

AhR expression was significantly increased in AR patients

AhR mRNA expression was dramatically higher in PBMCs from AR patients than in those from controls (P<0.01). Furthermore, AhR protein expression was also significantly increased in PBMCs from AR patients compared to those from controls (P<0.05; Figure 1).

AhR was highly expressed in Th17 cells

The level of AhR expression in Th17 cells was expressed as a ratio of CD4+IL-17+AhR+/CD4+IL-17+ cells. Intracellular staining analysis revealed that Th17 cells highly expressed AhR in both AR patients and controls (Figure 2).

ITE upregulated the expression of AhR in healthy controls, but not in AR patients

To investigate the effects of ITE on AhR expression, PBMCs were incubated with ITE or PBS in the presence of IL-6, TGF-β1, anti-CD3 and anti-CD28 antibodies. PBMCs treated with ITE exhibited significantly higher AhR mRNA expression (P<0.05) and AhR protein expression (P<0.01) than the untreated PBMCs in the controls. However, no significant ITE-induced variation in AhR mRNA or AhR protein expression was observed in AR patients (P>0.05; Figure 3).
ITE suppressed Th17 differentiation in AR patients and controls

ITE reportedly inhibits inflammatory and allergic responses in mice by suppressing Th17 differentiation.10 Using flow cytometry, we showed that the number of Th17 cells was higher in AR patients than in controls ($P<0.01$). To examine the effects of ITE on Th17 differentiation in PBMCs from AR patients and controls, the cells were stimulated with anti-CD3 antibodies, anti-CD28 antibodies, IL-6 and TGF-β1 in the presence of ITE. The results revealed that ITE greatly inhibited the differentiation of Th17 in PBMCs from AR patients ($P<0.01$) and healthy controls ($P<0.05$; Figure 4).

ITE inhibits IL-17 and IL-22 production in AR patients and controls

We observed increased levels of IL-17 and IL-22 ($P<0.01$) in AR patients compared to controls. ITE was able to inhibit IL-17 production in PBMCs from AR patients and controls ($P<0.01$). Similar results were obtained when we examined IL-22 production in the supernatants of ITE-treated PBMCs by ELISA (Figure 5).

Correlations between AhR; the Th17 response; TSS, RQLQ and VAS scores in AR patients

Next, we evaluated the correlations between AhR and the Th17 response. Significantly positive

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Table 1  Clinical features of patients with AR

<table>
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<th>Control</th>
<th>$P$-value</th>
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<tr>
<td>Age (years)</td>
<td>35 ± 12</td>
<td>36 ± 11</td>
<td>NS</td>
</tr>
<tr>
<td>Sex (female:male)</td>
<td>17:13</td>
<td>8:5</td>
<td>NS</td>
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<tr>
<td>TSS</td>
<td>10 ± 2.78</td>
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<td>RQLQ</td>
<td>3.03 ± 1.52</td>
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<td>VAS</td>
<td>46.63 ± 18.57</td>
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NS, not significant; the significance level equals a $P$-value of 0.05.

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Figure 1. Upregulation of AhR expression in PBMCs from patients with AR. (a) RT–qPCR analysis of AhR mRNA expression in cultured PBMCs after stimulation with anti-CD3 and anti-CD28 antibodies from AR patients and healthy controls. (b) Western blot analysis of AhR protein expression in cultured PBMCs after stimulation with anti-CD3 and anti-CD28 antibodies from AR patients and healthy controls. (c) Quantification of AhR protein expression.

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Figure 2. High level of AhR expression in CD4$^{+}$IL-17$^{+}$T cells in PBMCs from AR patients and controls. (a) CD4 gating in flow cytometry was used for measurement of AhR-positive cells in CD4$^{+}$T cells from PBMCs. (b) Quantification of the percentage of AhR-positive cells in Th17 cells.
correlations were found between AhR expression and Th17, IL-22 and IL-17. In addition, a positive correlation was also found between AhR expression and TSS, RQLQ and VAS scores (Table 2).

**Discussion**

In this study, we showed that AhR expression was dramatically enhanced in AR patients compared to controls and was closely associated with the clinical severity of AR. ITE, a nontoxic, high-affinity AhR ligand, significantly inhibited the differentiation of Th17 cells and the production of IL-17 and IL-22 in PBMCs collected from AR patients and controls. These results suggest that AhR may be involved in the development of AR.

AhR and its effects on immune regulation have been linked to some autoimmune disorders and allergic diseases. This motivated us to study whether AhR was associated with the pathogenesis of AR. Importantly, we found that AhR was markedly increased in PBMCs of patients with AR, which was consistent with results of studies in allergic asthma and atopic dermatitis. Significantly positive correlations between AhR expression and Th17/clinical parameters (TSS, RQLQ and VAS scores) indicated that the upregulated expression of AhR may further worsen the disproportionate distribution of Th17 and exacerbate the clinical severity of AR.

AhR is an important factor in the immune and inflammatory response; therefore, we investigated the effects of the nontoxic AhR ligand ITE on AhR expression and the Th17 response. Our results showed that ITE dramatically increased the expression of AhR in healthy controls, but not in AR patients and significantly inhibited the differentiation of Th17 cells and the production of IL-17 in both groups. In contrast, previous studies have suggested that environmental polycyclic aromatic hydrocarbons (PAHs) could not only provoke the allergic response in allergic diseases by binding to and activating AhR, but also enhance the expression of AhR, expansion of Th17 cells and an increase in inflammatory cytokine production. These data revealed that AhR, true to its dualistic nature, exerted its effects on the immunity response in the context of the environment in which it functioned. Thus, increased AhR expression in AR patients may result from AhR activation by environmental PAHs, suggesting that as air pollution worsens, AhR may be an important factor contributing to the increased incidence of AR. Furthermore, inhibition of the Th17 response and the lack of AhR upregulation in ITE-treated cells from AR patients in our research may have resulted from competition of ITE with PAHs. Recent studies have shown that AhR ligands have protective effects against allergic asthma and that ITE as well as curcumin and quercetin, which are present in vegetables and medicinal plants, suppress allergic immune responses in mice. Our results were consistent with these reports, in which ITE was found to suppress inflammatory and allergic responses by inhibiting the Th17 response.

IL-22, a member of the IL-10 cytokine superfamily, has been implicated in the pathogenesis of auto-inflammatory and allergic diseases, such as rheumatoid arthritis (RA), Crohn’s disease and allergic asthma. In this study, we observed elevated IL-22 levels in AR patients, consistent with previous reports. However, Schnyder et al. reported that...
IL-22 is a negative regulator of the allergic response. Thus, it remains to be determined whether IL-22 plays a protective or pathogenic role in AR and resolving this will likely require further studies in AR patients and animal models.

In summary, the results obtained in this study suggested that upregulation of AhR may be associated with the inflammatory response in AR patients. The nontoxic AhR ligand ITE significantly reduced the inflammatory response by inhibiting Th17 cell differentiation and downregulating the production of IL-17. Taken together, these results suggested that upregulation of AhR expression may be one of the

![Figure 4](Image)

Figure 4. The AhR ligand ITE inhibited Th17 cell differentiation in AR patients and controls. (a) Measurement of the number of Th17 cells in cultured PBMCs after stimulation with ITE or PBS in the presence of IL-6, TGF-β1, anti-CD3 and anti-CD28 antibodies by flow cytometry. (b) Quantification of the percentage of Th17 cells in PBMCs.

![Figure 5](Image)

Figure 5. The AhR ligand ITE inhibited IL-17 and IL-22 production in AR patients and controls. (a) Detection of IL-17 in the supernatants of cultured PBMCs after stimulation with ITE or PBS in the presence of IL-6, TGF-β1, anti-CD3 and anti-CD28 antibodies from AR patients and healthy controls. (b) Detection of IL-22 in the supernatants of stimulated PBMCs from the two groups.

<table>
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<th>Table 2</th>
<th>Correlations between AhR, Th17 and TSS, RQLQ and VAS scores</th>
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<tr>
<td></td>
<td>AhR</td>
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<td></td>
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<td>RQLQ</td>
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<td>VAS</td>
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IL-22 is a negative regulator of the allergic response. Thus, it remains to be determined whether IL-22 plays a protective or pathogenic role in AR and resolving this will likely require further studies in AR patients and animal models.

In summary, the results obtained in this study suggested that upregulation of AhR may be associated with the inflammatory response in AR patients. The nontoxic AhR ligand ITE significantly reduced the inflammatory response by inhibiting Th17 cell differentiation and downregulating the production of IL-17. Taken together, these results suggested that upregulation of AhR expression may be one of the
mechanisms underlying the heightened Th17 response in AR development. Based on the findings of this study, we suggest that nontoxic ITE may be a new candidate for the treatment of AR; however, further characterization of its specific mechanism of action will be required.

Funding
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Conflict of interest: None declared.

References