Original article

Unbalanced expression of aryl hydrocarbon receptor in peripheral blood CCR6⁺CD4⁺ and CD4⁺CD25⁺T cells of rheumatoid arthritis

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A R T I C L E   I N F O

Article history:
Received 24 August 2015
Accepted 17 April 2016
Available online 22 July 2016

Keywords:
Rheumatoid arthritis
Aryl hydrocarbon receptor
CD4⁺CD25⁺T cells
CCR6⁺CD4⁺T cells

A B S T R A C T

Objective: The goal of this study was to analyze the role of aryl hydrocarbon receptor in peripheral blood CCR6⁺CD4⁺ and CD4⁺CD25⁺T cells of patients with rheumatoid arthritis. Methods: Flow cytometry was applied to determine the proportion of AhR positive cells in CCR6⁺CD4⁺T, CD4⁺CD25⁺T and peripheral blood peripheral mononuclear cells from each subject. AhR mRNA and CYP1A1 mRNA relative expression levels were tested by real-time PCR.

Results: The percentage of AhR positive cells in peripheral blood mononuclear cells was higher in RA group than that in healthy cases [(35.23 ± 10.71)% vs. (18.83 ± 7.32)%, p < 0.01]. The expression levels of AhR and CYP1A1 were both increased in patients with RA while compared to controls [(3.71 ± 1.63) vs. (2.00 ± 1.27), p = 0.002; (2.62 ± 2.08) vs. (0.62 ± 0.29), p < 0.01, respectively]. In RA patients, the percentage of AhR positive cells in CD4⁺CD25⁺T cells was significantly lower than that from controls [17.90 (6.10 ± 8.01) vs. 52.49 (19.18)%], p < 0.01]. In healthy controls, the percentage of AhR positive cells in CD4⁺CD25⁺T cells was significantly higher than that in CCR6⁺CD4⁺T cells, and was also significantly higher than that in PBMCs [(52.49 ± 19.18)% vs. (23.18 ± 5.62)%, (18.06 ± 7.80)% vs. 24.03, p < 0.01]; in RA patients, the percentage of AhR positive cells in CCR6⁺CD4⁺T cells was significantly increased than that in CD4⁺CD25⁺T cells and PBMCs [(46.02 ± 14.68)% vs. 17.90 (6.10 ± 8.01) vs. (34.22 ± 10.33)%], p < 0.01]; Nevertheless, no statistically significant relationship was found between clinical data and AhR positive cells in CCR6⁺CD4⁺T and CD4⁺CD25⁺T cells.

Conclusion: AhR may participate in the pathological progress of RA by controlling the differentiation of Th17 and Treg cells in peripheral blood.

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Expressão não equilibrada do receptor de hidrocarboneto arílico nos linfócitos T CCR6+ CD4+ e CD4+ CD25+ do sangue periférico na artrite reumatoide

R E S U M O

Objetivo: Analisar o papel do receptor de hidrocarboneto arílico (AhR) nos linfócitos T CCR6+ CD4+ e CD4+ CD25+ no sangue periférico de pacientes com artrite reumatoide (AR).

Métodos: Foi aplicada citometria de fluxo para determinar a proporção de células AhR positivas em linfócitos CCR6+ CD4+ e CD4+ CD25+ do sangue periférico e células mononucleares periféricas de cada indivíduo. Os níveis de expressão relativa de ácido ribonucleico mensageiro (do inglês ribonucleic acid, RNAm,) de AhR e RNAm de enzima de primeiro estágio essencial para o AhR (CYP1A1) foram testados por reação em cadeia de polimerase (do inglês polymerase chain reaction, PCR,) em tempo real.

Resultados: A percentagem de células AhR positivas nas células mononucleares do sangue periférico foi maior no grupo AR do que nos indivíduos saudáveis [(35,23 ± 10,71)% vs. (18,83 ± 7,32)%], (p<0,01)]. Os níveis de expressão de AhR e CYP1A1 estavam aumentados em pacientes com AR quando comparados com os controles [(3,71 ± 1,63) vs. (2,00 ± 1,27), p = 0,002; (2,62 ± 2,08) vs. (0,62 ± 0,29), p < 0,01, respectivamente]. Em pacientes com AR, a percentagem de células AhR positivas nos linfócitos T CD4+ CD25+ foi significativamente inferior à dos controles [17,90 (6,10 ± 8,10)% vs. (52,49 ± 19,18)%], (p < 0,01); em controles saudáveis, a percentagem de células AhR positivas nos linfócitos T CD4+ CD25+ foi significativamente mais elevada do que nos linfócitos T CCR6+ CD4+ e também foi significativamente maior do que nas células mononucleares do sangue periférico (do inglês peripheral blood mononuclear cells, PBMC), [(52,49 ± 19,18)% vs. (23,18 ± 5,62)% vs. (18,06 ± 7,80)%], X² = 24,03, p < 0,01; em pacientes com AR, a percentagem de células AhR positivas nos linfócitos T CCR6+ CD4+ era significativamente maior em comparação com os linfócitos T CD4+ CD25+ e PBMC (46,02 ± 14,68)% vs. [17,90 (6,10 ± 8,10)% vs. (34,22 ± 10,33)%, X² = 38,29, p < 0,01]; no entanto, não foi encontrada correlação estatisticamente significativa entre os dados clínicos e células AhR positivas em linfócitos T CCR6+ CD4+ e CD4+ CD25+.

Conclusão: O AhR pode participar do progresso patológico da AR ao controlar a diferenciação de linfócitos Th17 e Treg no sangue periférico.

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Palavras-chave: Artrite reumatoide Receptores de hidrocarboneto arílico Linfócitos T CD4+ CD25+ Linfócitos T CCR6+ CD4+

Introduction

Rheumatoid arthritis (RA) is a systemic chronic inflammatory disease that affects about 1% of the population. The aryl hydrocarbon receptor (AhR), consisted of 806 amino acids, is a transcription factor that belongs to the bHLH (basic Helix-Loop-Helix)-PAS (Per-ARNT-Sim) family. AhR combines with chaperone proteins in the cytoplasm and maintains an inactive form in the absence of its ligands such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and 6-formylindolo[3,2-b]carbazole (FICZ). Once activated, AhR dissociates from the proteins and transfers to the nucleus to induce the expression of downstream genes.

The helper (Th) 17 cells and regulatory T cells (Treg) are groups of the CD4+ T cells family. Interleukin (IL) 17 – producing Th17 cells predominantly express CC chemokine receptor (CCR) 6 which is a crucial marker for Th17 cells in autoimmune diseases; previous study has suggested that rheumatoid arthritis in animal models could be improved when treated with anti-CCR6 mono clonal antibody, which may indicate CCR6 expression plays a primary role in autoimmune diseases. CCR6+ CD4+T cell is the main component of Th17 cells, and meanwhile CCR6 is regarded as a biomarker of human Th17 cells. The CD4+ CD25+ lineage of Treg is an immunosuppressive cell which plays a vital character in immune and autoimmune responses and the transcription factor forkhead box protein P3 (Foxp3) has been thought to determine the Treg cells lineage. Recently, studies have found AhR was highly expressed in Th17 and Treg cells, which could activate its signaling pathways, control the differentiation of the cells, and further involve in the pathogenesis of autoimmune diseases in a ligand-specific manner.

Although AhR seen as a major participant in autoimmune diseases has been studied and identified in prior researches, how exactly AhR linked to rheumatoid arthritis in human beings is poorly understood. In the present study, we investigate the mRNA expression levels of AhR and CYP1A1 in peripheral blood mono-nuclear cells (PBMCs) and the proportion of AhR positive cells in CCR6+ CD4+ and CD4+CD25+ T cells to study the role of AhR in CCR6+ CD4+ and CD4+CD25+ T cells from RA patients.
Patients and methods

Study subjects

Thirty-five patients with RA (mean age 49.22 ± 8.94, 8 males and 27 females) according to the 1987 revised criteria of American Rheumatism Association were recruited from the Department of Rheumatology, The Second Affiliated Hospital of Anhui Medical University. The inclusion criteria of the study (a) a definite diagnosis, (b) free of other autoimmune diseases, (c) undergoing no infectious diseases, (d) treatment with same drugs for RA patients. Meanwhile, fourteen age and sex matched healthy controls were randomly selected from health center (mean age 46.5 ± 7.5, 3 males and 11 females). The clinical parameters and the disease activity score in 28 joints (DAS28) were calculated in detail. The Ethics Committee of the Hospital approved the study. Informed consent was obtained from the patients or from their relatives if the patients were incapable of consent.

Cell isolation

5-mL venous peripheral blood was stored in anticoagulation tube containing EDTA as the anticoagulant. PBMCs were separated using density-gradient centrifugation (Beijing Solarbio Science and Technology Company, Beijing, China). Total PBMCs number of each sample was not less than 1 × 10^8.

Flow cytometry

Cell surface markers

5 μL CD25-PE and 2 μL CD4-PerCP-Cy5.5 were both added to the marked flow tubes (a, b), at the same time 5 μL CCR6-PE and 2 μL CD4-PerCP-Cy5.5 were added to the other two tubes (c, d). After the liquid in four tubes was mixed, we placed them in a refrigerator at 4 °C for 20 min. Then 2-mL buffer was prepared to wash them for once. The above-mentioned antibodies were purchased from Becton, Dickinson and Company (BD) of American.

Fixation and perforation

100-μL PBMCs was mixed with 500 μL fixatives (Becton, Dickinson and Company, American) in four tubes to give incubation avoiding light at 4 °C temperature and after that, 1-mL buffer was to wash them for two times.

The indirect method of marking AhR

Each tube was added the mixture of 2 μL first antibody (the antibody of anti-aryl hydrocarbon receptor, Abcam Company, England) and 2 μL phosphate buffered saline (PBS). 1-mL buffer was applied to wash them after put the mixture at room temperature to avoid light for 1 h and then 2 μL second antibody (mouse monoclonal (ZA9) secondary antibody to rabbit IgG heavy chain (FITC), Abcam Company, England) was added to the four tubes and placed them in chamber at room temperature to avoid light for half an hour. 2 μL buffer was used to wash them for two times and at last 200 μL PBS was added to the tubes.

Table 1 – Primers used for the quantitative real-time RT-PCR.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>AhR Forward</td>
<td>5’-ATACCGAGACGGCTGAAT-3’</td>
</tr>
<tr>
<td>AhR Reverse</td>
<td>5’-CCAGCAGACCTTAGAGCAG-3’</td>
</tr>
<tr>
<td>β-Action Forward</td>
<td>5’-GCCGCAGAGCTCATCATT-3’</td>
</tr>
<tr>
<td>β-Action Reverse</td>
<td>5’-GGGCAGCAAGCTCATCATT-3’</td>
</tr>
<tr>
<td>CYP1A1 Forward</td>
<td>5’-CATCCCCACAGACAAAGAGA-3’</td>
</tr>
<tr>
<td>CYP1A1 Reverse</td>
<td>5’-GCAGCAGATAGCAGGAAGAA-3’</td>
</tr>
</tbody>
</table>

Flow-cytometric analysis

The percentage of CD4+CD25+ and CCR6+CD4+ T cells in PBMCs and the proportion of AhR positive cells in PBMCs, CD4+CD25+T and CCR6+CD4+T cells were all detected by CellQuest software (FACSaclibur, BD Company). The number of cells was tested at least 100,000 at a time and FlowJo7.6.1 was applied to analyze the data.

Isolation and reverse transcription of total RNA

Total RNA from RA peripheral blood mononuclear cells was separated using Trizol reagent (Invitrogen, California, USA). Primers were designed and synthesized by Takara Biotechnology (Tokyo, Japan). The specific primer sequences were shown in Table 1. The PrimeScript RT reagent Kit (Takara Biotechnology, Japan) was used to perform the synthesis of cDNA according to the manufacturer’s instructions.

Real-time quantitative PCR

The PCR was performed in a 20 μL reaction system containing 1.6 μL primers (0.8 μL forward primer and reverse primer, respectively), 2 μL cDNA, 0.4 μL ROX Reference dye II, 6 μL dH2O, and 10 μL SYBR Premix Ex Taq™ II. All the samples were amplified at the same site on ABI 7500 real-time polymerase chain reaction system (Applied Biosystems, Foster City, CA). 2^−ΔΔCT was used to compute the expression value of AhR and CYP1A1.

Laboratory measurement

For all the RA patients, erythrocyte sedimentation rate (ESR), C reactive protein (CRP) and anti-cyclic citrullinated peptide antibody (A-CCP) were measured.

Statistical analysis

Statistical analysis was carried out by the Statistical Package for Social Sciences 10.0 software e (SPSS Inc.; 2000). Quantitative variables were recorded by mean ± SD, and the differences were analyzed using t test if the data meet the normal distribution and homogeneity of variance. However, nonparametric distribution data were described by median value and interquartile range (IQR). ANOVA was used to compare the
data in three groups, and the comparison between two groups in the three groups was analyzed by LSD-t or Mann–Whitney U test. Spearman correlation analysis was applied in our study. p < 0.05 was thought to be statistically significant.

Results

The percentages of AhR in PBMCs from patients with RA and controls

As shown in Fig. 1, the percentage of AhR positive cells was decreased in healthy controls (18.83 ± 7.32)% than that in patients with RA (35.23 ± 10.71)% (p < 0.01).

The increased mRNA levels of AhR and CYP1A1 in PBMCs from RA patients

As shown in Fig. 2, AhR and CYP1A1 mRNA expression levels were both increased in patients with RA when compared with controls [(3.71 ± 1.63) vs. (2.00 ± 1.27), p = 0.002; (2.62 ± 2.08) vs. (0.62 ± 0.29), p < 0.01, respectively].

The percentage of AhR in CCR6+CD4+T and CD4+CD25+T cells from RA cases and healthy subjects

As shown in Fig. 3, the percentage of AhR positive cells in CCR6+CD4+T cells was clearly higher in patients (46.02 ± 14.68)% than that in healthy subjects (23.18 ± 5.62)% (p < 0.01) (Fig. 3A). In contrast, the percentage of AhR positive cells in CD4+CD25+T cells was decreased in RA patients [17.90 (6.10, 80.10)%] than healthy group (52.49 ± 19.18)% (Fig. 3B). Significant differences were existed between two groups (p < 0.01).

The comparison about the percentage of AhR positive cells in peripheral blood CD4+CD25+T, CCR6+CD4+T cells and PBMCs from Normal control and RA group

In the healthy group, the percentage of AhR positive cells in CD4+CD25+T cells was significantly higher than that in CCR6+CD4+T cells, and was also statistically higher than in PBMCs [(52.49 ± 19.18)% vs. (23.18 ± 5.62)% vs. (18.06 ± 7.80)%, X² = 24.03, p < 0.01] (Fig. 4A). Nevertheless, the percentage of AhR positive cells in CCR6+CD4+T cells was significantly increased than that in CD4+CD25+T cells, and was also significantly higher than that in PBMCs [(46.02 ± 14.68)% vs. 17.90 (6.10, 80.10)% vs. (34.22 ± 10.33)%, X² = 38.29, p < 0.01] (Fig. 4B).

Correlation between the AhR positive cells in peripheral blood CCR6+CD4+T, CD4+CD25+T cells and major clinical parameters from RA patients

We conducted Spearman’s correlation to assess the association between AhR/CYP1A1 mRNA expression levels, AhR positive cells in peripheral blood CCR6+CD4+T, CD4+CD25+T cells and major clinical parameters. However, no significant correlation was found between them, and the details were summarized in Table 2.

Discussion

RA is a systemic autoimmune disease primarily manifest as polyarthritis, especially the small joints of hands and feet, that is characterized by joint destruction and chronic disability.13 However, the mechanisms about the immune pathways in RA have not been identified yet. For the past few years, a wealthy of data has showed self-tolerance plays a dominant role in autoimmunity including RA as it erroneously was activated in response to its self-antigen. The self-tolerance in RA patients may be regulated by various factors such as cytokine, environmental risks and lifestyle factors.14 Currently, it was believed that aberrant T-cell homeostasis plays a great part in the pathogenesis. Moreover, Treg cells dysfunction and/or Th17 cells dysregulation are thought to contribute to the
Fig. 3 – The percentages of AHR positive cells in CCR6+CD4+T (A) and CD4+CD25+T (B) cells from healthy controls and RA patients.

Fig. 4 – The percentages of AHR positive cells in PBMCs, CCR6+CD4+T, CD4+CD25+T cells from normal control (A) and RA group (B).

development of autoimmune disorders. Similarly, accumulated evidence indicates AHR plays a critical place in regulating the differentiation of Th17 and Treg cells, affecting the development of a number of autoimmune diseases including RA.11,15–20

AHR is located in the cytoplasm and maintains an inactive form in the absence of its ligands such as TCDD and FICZ. Once activated, AHR complex combines with AHR nuclear translocator protein (Arnt) to be heterodimer in the nucleus where they bind to specific DNA sequences known as xenobiotic-response elements (XRE), and this binding will produce some reaction. During this activated process, a vital phase I enzyme of AHR (CYP1A1) that is a most widely studied responsive gene had a higher expression.21 Several groups previously have shown a

<table>
<thead>
<tr>
<th>Clinical parameters</th>
<th>ESR</th>
<th>CRP</th>
<th>Anti-CCP</th>
<th>DAS28 scores</th>
<th>Course</th>
</tr>
</thead>
<tbody>
<tr>
<td>AhR mRNA r-Value</td>
<td>0.115</td>
<td>0.014</td>
<td>0.003</td>
<td>0.068</td>
<td>−0.107</td>
</tr>
<tr>
<td>AhR mRNA p-Value</td>
<td>0.511</td>
<td>0.936</td>
<td>0.987</td>
<td>0.698</td>
<td>0.541</td>
</tr>
<tr>
<td>CYP1A1 mRNA r-Value</td>
<td>0.007</td>
<td>0.149</td>
<td>−0.035</td>
<td>0.143</td>
<td>0.031</td>
</tr>
<tr>
<td>CYP1A1 mRNA p-Value</td>
<td>0.967</td>
<td>0.392</td>
<td>0.842</td>
<td>0.411</td>
<td>0.860</td>
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<tr>
<td>CCR6+CD4+T r-Value</td>
<td>−0.262</td>
<td>−0.273</td>
<td>0.055</td>
<td>−0.187</td>
<td>−0.309</td>
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<tr>
<td>CCR6+CD4+T p-Value</td>
<td>0.14</td>
<td>0.124</td>
<td>0.761</td>
<td>0.296</td>
<td>0.08</td>
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<tr>
<td>CD4+CD25+T r-Value</td>
<td>−0.129</td>
<td>0.013</td>
<td>0.140</td>
<td>−0.045</td>
<td>−0.081</td>
</tr>
<tr>
<td>CD4+CD25+T p-Value</td>
<td>0.475</td>
<td>0.943</td>
<td>0.436</td>
<td>0.804</td>
<td>0.653</td>
</tr>
</tbody>
</table>

AHR, aryl hydrocarbon receptor; anti-CCP, anti-cyclic citrullinated peptide antibody; CRP; CYP1A1, vital phase I enzyme of AHR; DAS28, disease activity score in 28 joints; ESR, erythrocyte sedimentation rate; RA, rheumatoid arthritis.
state AhR was highly expressed in ubiquitous cells including Th17, Treg, dendritic cells (DCs). 3,5,22

Interestingly, the essential factor of the pathogenesis on traditional view in autoimmune diseases is mainly due to Th1 and Th2 cells ratio imbalance. However, recent researchers had a tendency to focus more on the balance between Th17 and Treg cells belong to the family of CD4+T cells. Quintana et al. 17 showed that AhR activation by its ligand 2,3,7,8-tetrachlorodibenzo-p-dioxin induced functional Treg cells that suppressed experimental autoimmune encephalomyelitis (EAE), but boosted Th17 cells differentiation and furthermore increased the risk of EAE if the ligand was FICZ in animal models. This study further illuminated the importance of the balance on Th17 and Treg cells. A study by de Kleer et al. 23 showed a significantly higher number of CD4+CD25+Treg cells was found in juvenile idiopathic arthritis (JIA) than that in extended oligoarticular JIA (ext-OA JIA), and suggested the higher frequencies of CD4+CD25+Treg cells in synovial tissue of JIA, the lower severity of the disease. It has been reported CD4+CD25+Treg-deficiency might exacerbate symptoms in patients with RA. 24,25 In the meantime, Nguyen et al. 26 showed the disease progression could not be prevented by CD4+CD25+Treg cells, but the severity would be lowered for its immunosuppressive function.

To date, the study about AhR in peripheral blood as well as in Th17 and Treg of RA patients is much less. Based on the mentioned studies and the notion, we conducted the experiment. Consistent with previous findings, 8,10,11,17 firstly, we found a statistically higher frequency of AhR positive cells in RA patients when compared with healthy controls. In addition, we also got AhR and CYP1A1 mRNA were both overexpressed in patients with RA. Given the result, we might speculate AhR was activated and played a significant role in RA patients. Secondly, our study showed not only the percentage of AhR in CCR6+CD4+T cells was enriched but also the percentage in CD4+CD25+T cells was reduced in RA patients compared to healthy subjects. Moreover, in present study, the highest frequency of AhR positive cells in CD4+CD25+T was tested of CCR6+CD4+T and PBMCs in healthy persons, however its proportion in CCR6+CD4+T cells was increased than PBMCs and both of them were higher than in CD4+CD25+T cells. All these findings collectively supported the view that AhR plays an essential role in differentiation and function of CD4+CD25+Treg and CCR6+CD4+Th17, controlling the balance of Th17 and Treg cells, and what could provide more therapeutic approaches in RA. However, no significant correlations between AhR/ CYP1A1 mRNA expression levels, the AhR positive cells in CD4+CD25+T cells as well as CCR6+CD4+T cells and CRP, ESR, A-CCP, cDA52, course of disease were found. It potentially told us they could not reflect the disease activity or insufficient sample size might be one of the reasons.

In conclusion, our findings first demonstrate an association between AhR and CD4+CD25+Treg, CCR6+CD4+Th17 cells in peripheral blood from RA patients, which may broaden our horizons in the etiology of RA and provide new insights into the pathogenesis of RA. Our results suggest that AhR may play a central role in controlling the balance of Th17 and Treg for its unbalanced expression in peripheral blood and further participate in the pathogenesis of RA. Of course, these results may be needed to elucidate in a further research.

Conflicts of interest
The authors declare no conflicts of interest.

Acknowledgments
This work was supported by all the donors for friendly providing the blood samples.

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