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Macrophages from Behcet's Disease Patients Express Decreased Level of Aryl Hydrocarbon Receptor (AHR) mRNA

Mohammad Taghi Palizgir[1], Maryam Akhtari[1,2], Mahdi Mahmoudi[1], Shayan Mostafaei[1], Alireza Rezaeimanesh[3], Massoomeh Akhlaghi[1], and Farhad Shahram[1]

[1] Rheumatology Research Center, Tehran University of Medical Sciences, Tehran, Iran
[2] Department of Cell and Molecular Biology, University of Tehran, Tehran, Iran
[3] Department of Immunology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran

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ABSTRACT

Aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor, connecting environmental stimulators with the immune system. M1 macrophages are a part of immune system that contribute to the inflammatory events in the pathogenesis of Behcet's disease (BD). The effect of AHR on the macrophages in BD patients is still unclear. In this study, we investigated the mRNA expression of AHR in the monocyte-derived and M1 macrophages in active BD patients in comparison to healthy controls.

Isolated monocytes from 10 healthy controls and 10 active BD patients were differentiated to macrophages by macrophage-colony stimulating factor (M-CSF) for 7 days. Cells were then polarized to M1 macrophages by lipopolysaccharide (LPS) and interferon-γ (IFNγ) for 24h. Monocyte purity and macrophage markers expression were analyzed by flow cytometry. Analysis of AHR mRNA expression was performed by SYBR Green real-time PCR.

Our results showed that AHR expression is significantly down-regulated in M1 macrophages compare to monocyte-derived macrophages. It was shown that both monocyte-derived macrophages and M1 macrophages from BD patients significantly express lower level of AHR mRNA compared to healthy individuals.

Our results demonstrate an anti-inflammatory role for AHR in macrophages, which suggest that decreased AHR expression is associated with pro-inflammatory M1 macrophage and BD susceptibility.

Keywords: Aryl hydrocarbon receptor; Behcet's disease; Gene expression

Corresponding Authors: Mahdi Mahmoudi, PhD, and Farhad Shahram, MD;
Rheumatology Research Center, Shariati Hospital, Tehran University of Medical Sciences, Tehran, Iran. PO.Box: 1411713137, Tel/Fax: (+98 21) 8822 0067, E-mail: mahmoudim@tums.ac.ir and shahramf@tums.ac.ir

INTRODUCTION

Behcet's disease (BD) is a chronic auto-inflammatory multisystemic vasculitis of unknown etiology. It is characterized by genital and oral

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aphthous, skin-mucosa lesions, joint and vessel involvements, uveitis and epididymitis.[1] The disease has a higher prevalence in regions along the ancient Silk Road, extending from the Far East to the Middle East and the Mediterranean. BD is commonly observed in Turkey, Iran, China, North Korea, and Japan.[2] The prevalence of the disease in Iran is about 80-230 patients per 100,000 population.[3,4]

Macrophages are a subset of the innate immunity cells involved in the auto-inflammatory responses in the rheumatic diseases such as BD. The inflammatory M1 macrophage is predominantly seen in BD and release large amounts of pro-inflammatory cytokines, including TNF-α, IL-6, and IL-1β that contribute to tissue damage.[5]

Although the pathogenesis of BD remains still not well known, it is believed that a complex interaction between hereditary and environmental factors is involved in the induction of inflammatory and autoimmune responses.[6]

Aryl hydrocarbon receptor (AHR), a basic helix-loop-helix (bHLH)/Per-Arnt-Sim (PAS) protein, is a ligand-activated transcription factor which connects environmental stimulators such as tobacco smoke with the immunity.[7] Upon ligand binding, AHR activates dioxin response element (DRE) in the promoter of its target genes such as cytochrome P450 (*CYP1A1*, *CYP1A2*, *CYP1B1*) and a variety of genes involved in immune responses.[8] AHR regulates a wide variety of inflammatory responses, plays a significant modulatory role in the immune system and highly expressed within immune cells.[9] Previous researches have demonstrated AHR activation can induce anti-inflammatory responses in macrophages.[10, 11] AHR activation suppresses graft-versus-host responses and prevents diabetes in mice.[12,13] In addition, AHR can induce mice CD4+CD25+Foxp3 Treg cell differentiation and suppress experimental autoimmune encephalomyelitis (EAE).[14]

The effect of AHR on the macrophages from BD patients is still unclear. In this study, we investigated mRNA expression of AHR in the monocyte-derived macrophages and M1 macrophages from BD patients in comparison to healthy controls.

MATERIALS AND METHODS

Patients and Controls

The study included, 10 active BD patients (38±11.2 years) and 10 age- and sex-matched healthy controls with a negative family history of BD and other autoimmune disorders (34±6.9 years). All patients fulfilled the International Criteria for Behcet's Disease (ICBD).[15] The patients were enrolled from the outpatient clinic of the Rheumatology Research Center (RRC), Shariati Hospital, Tehran University of Medical Sciences (TUMS). Patients' clinical manifestations were extracted from the medical records at the time patients fulfilled the criteria of Behcet's Disease. Written informed consent was obtained from all patients and controls. The Ethics committee of Tehran University of Medical Sciences approved this study (No. IR.TUMS.REC.1394.1941).

Monocyte Isolation and Macrophage Differentiation

Peripheral blood mononuclear cells (PBMCs) were isolated from venous blood by density gradient centrifugation. Whole blood was obtained by venipuncture from each subject and collected into four 9-mL tubes containing EDTA. Samples were processed within 10 hours of collection. Blood was diluted 1:2 with phosphate-buffered saline (PBS; GIBCO Invitrogen, Carlsbad, CA). PBMC buffy coats isolated using lymphocyte separation medium (Lymphodex, Inno-Train, Kronberg, Germany). Cells were then washed twice with PBS. PBMCs were incubated with MACS CD14 MicroBeades and monocytes were isolated by positive selection using magnetic-activated cell sorter columns (all from Miltenyi Biotec, San Diego, CA). Purity was 90-94% CD14 positive cells by flow cytometry. Monocytes were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal bovine serum (FBS; GIBCO Invitrogen, Carlsbad, CA), 100 U/mL penicillin, 100 µg/mL streptomycin (Sigma-Aldrich, Germany), and L-glutamine (Biosera, Kansas City, MO) and differentiated to macrophages by 50 ng/mL of recombinant human macrophage-colony stimulating factor (M-CSF; eBioscience, San Diego, CA) for 7 days.

Flow Cytometry Analysis

Monocyte and macrophage were stained with allophycocyanin (APC) conjugated anti-human CD163, fluorescein isothiocyanate (FITC) conjugated anti-human CD206 (Miltenyi Biotec, San Diego, CA), and phycoerythrin-cyanin 5 (PE-Cy5) conjugated anti-human HLA-DR (Biolegend, San Diego, CA).

Appropriate isotype control staining was performed in parallel. The cells (2×10^5) were incubated with the above antibodies for 30 min 4°C in the dark. Cells were then washed and analyzed on a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ) flow cytometry system using the FlowJo software (Tree Star, Ashland, OR, USA). Flow cytometry showed that the cells after 7-day stimulation by M-CSF were >91% HLA-DR+ and expressed macrophage markers CD163 and CD206 (95% and 91%, respectively).[16]

Macrophage Polarization

M1 macrophage polarization was obtained by culturing monocyte-derived macrophages for 24h in RPMI 1640 supplemented with 10% FBS and 100 ng/mL lipopolysaccharide (LPS; Sigma-Aldrich, Germany) plus 1,000 units/mL of interferon gamma (IFNγ; R&D systems, Minnesota, United States). The M1 macrophage transcriptional profile was evaluated by real-time PCR. The expression of M0 (Monocyte-derived) macrophage specific genes (*CD36* and *MRC1*) was significantly down regulated and the expression of M1 macrophage specific markers (*CCR7* and *IDO*) was significantly increased in the macrophages after 1day stimulation by IFNγ and LPS (Figure.1).

Quantitative Real-time PCR Analysis

Total RNA was isolated from macrophages using the High Pure RNA Isolation Kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. From each sample, equal amount of total RNA was reverse transcribed to cDNA using the Transcriptor First Strand synthesis kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. The relative expression levels of AHR mRNA were measured using the StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA) and SYBR Green (Ampliqon, Odense, Denmark) master mix.

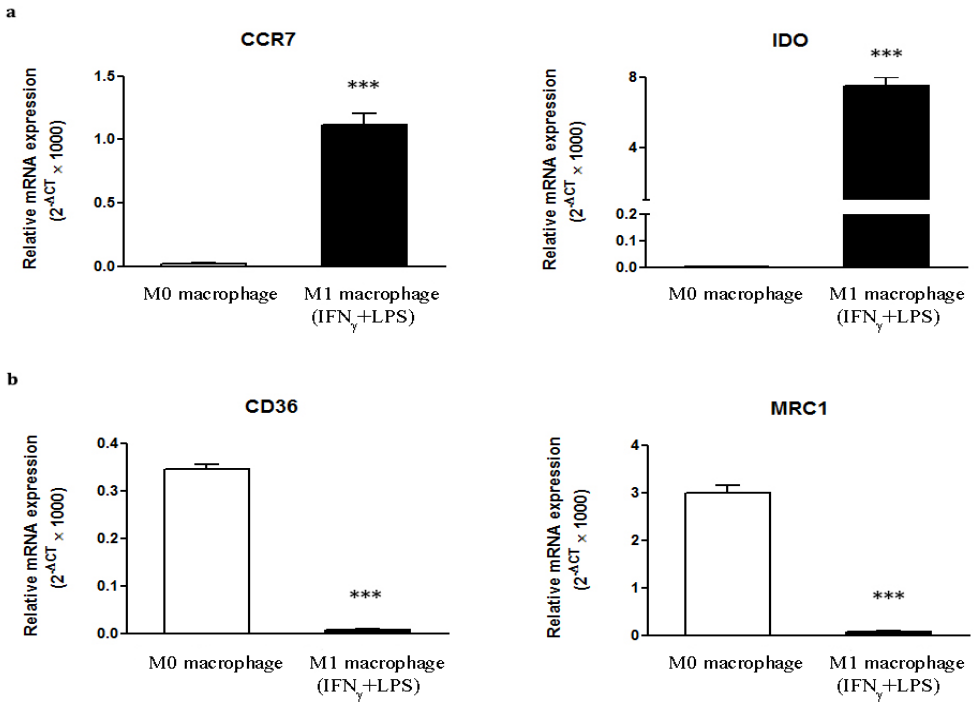

Figure 1. M0 macrophages were polarized to M1 macrophages by culturing for 1 day in Roswell Park Memorial Institute (RPMI) 1640 containing 10% fetal bovine serum (FBS) and 100 ng/mL lipopolysaccharide (LPS) plus 1000 units/mL of IFNγ. To confirm M1 macrophages polarization *CCR7*, *IDO*, *CD36*, and *MRC1* mRNA expression was evaluated in M0 macrophages and M1 macrophages from control group by real-time PCR. The expression of M1 macrophage specific genes (*CCR7* and *IDO*) was significantly increased and the expression of M0 macrophage specific markers (*CD36* and *MRC1*) was significantly down regulated, in the macrophages after 1 day stimulation by LPS and IFNγ. Data are expressed as the means±SD (* p<0.001).**

The values were normalized based on the expression of endogenous reference gene Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The following primers were used: AHR forward: 5' AGCAAGTTCACATGGAGGCA 3' and reverse: CGTGGCAGCACCCTTTCTAT, and *GAPDH* forward: 5' GAGTCAACGGATTTGGTCGT 3' and reverse: 5' GACAAGCTTCCCGTTCTCAG 3'. The relative changes in gene expression differences between healthy controls and patients were determined using the comparative CT method ($2^{-\Delta CT}$)× 1000.[17]

Statistical Methods

Quantitative variables were tested for normality by Kolmogorov-Smirnov normality test, and are expressed as mean ± SD, (normally distributed variables), or as median ± interquartile range (IQR) (not normally distributed variables). Data did have a normal distribution, one-way ANOVA and Tukey's test (as a post hoc test in ANOVA) was performed for comparing mean between groups. Correlation analysis was performed by Spearman's Rank-Order or Pearson correlation. The results were considered statistically significant at p-value<0.05. All of the statistical analysis were done by using SPSS version 23 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 6 (GraphPad Software, San Diego, CA).

RESULTS

M1 Macrophages Express Decreased Level of AHR mRNA

Monocyte-derived macrophages and M1 macrophages from normal controls were used to assay the expression of AHR mRNA. AHR is expressed in both monocyte-induced macrophages and M1 macrophages, whereas its expression is significantly down regulated by IFNγ and LPS in M1 macrophages (p<0.05; Figure 2).

Behcet's Disease Macrophages Show Reduced mRNA Expression of AHR

Our results indicated that expression of AHR mRNA was significantly reduced in monocyte-derived macrophages from BD patients as compared to healthy donors (p<0.05; Figure 3). Moreover, The AHR mRNA expression was significantly diminished in M1 macrophages from BD patients as compared to normal controls (p<0.05; Figure 3).

We next assessed whether expression of AHR mRNA was associated with specific clinical manifestations of BD. There was no significant correlation between the clinical events and the AHR mRNA expression. The prevalence of clinical criteria in patients is shown in Table 1.

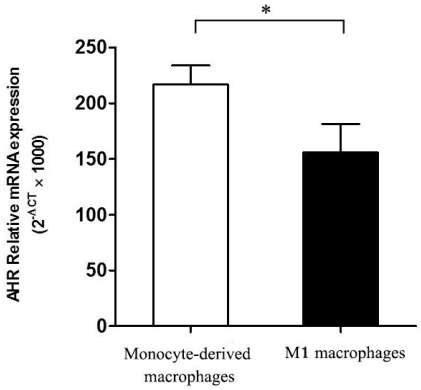

Figure 2. Aryl hydrocarbon receptor (AHR*)* gene expression is down regulated in M1 macrophages as compared to monocyte-derived macrophages. Monocytes from 10 healthy controls were differentiated to macrophages with M-CSF for 7 days. Cells were polarized to M1 macrophages by lipopolysaccharide (LPS) and IFNγ for 24 h. AHR mRNA was evaluated in monocyte-induced macrophages and M1 macrophages from healthy controls by real-time PCR and normalized to Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH)*. Data are expressed as the means±SD (*p<0.05).

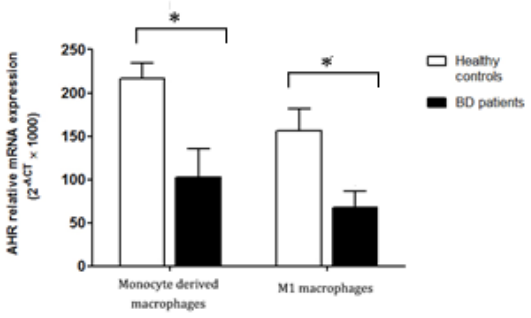

Figure 3. Monocyte-derived and M1 macrophages from Behcet's disease (BD) patients expressed significantly decreased level of AHR mRNA compared to normal controls. AHR mRNA expression was evaluated in monocyte-derived macrophages and M1 macrophages from normal controls (n=10) and active BD patients (n=10) via real-time PCR. Data are expressed as the means±SD (*p<0.05).

Table 1. Demography and clinical characteristics of the Behcet's disease (BD) patients

Characteristic	Value
Male/Female	5 (50%)/5 (50%)
HLA-B5	5 (50%)
HLA-B51	2 (20%)
HLA-B27	0 (0%)
Oral aphthosis	10 (100%)
Genital aphthosis	7 (70%)
Uveitis	7 (70%)
Retinal vasculitis	0 (0%)
Joint manifestation	1 (10%)
Pseudo folliculitis	1 (10%)
Erythema nodosum	3 (30%)
Anterior uveitis	3 (30%)
Posterior uveitis	4 (40%)
Arthralgia	0 (0%)
Monoarthritis (Sacroiliitis)	1 (10%)
Oligoarthritis	0 (0%)
Spondyloarthropathy	0 (0%)
Gastrointestinal aphthous ulcers	0 (0%)
Abdominal vasculitis	0 (0%)
Diarrhea	0 (0%)
Rectorrhagia	0 (0%)
Colitis	0 (0%)
Epididymitis	0 (0%)
Neurological manifestations	0 (0%)
Pathergy	0 (0%)
Pulmonary manifestations	0 (0%)
Cardiac manifestations	0 (0%)
Large vessel involvements	0 (0%)
Familial history of BD	0 (0%)

DISCUSSION

The innate immune system is over-activated in Behcet's disease, and macrophage as a crucial cellular component of the innate immunity has a central role in the disease pathogenesis and severity. Macrophages are the main cells of mononuclear phagocytes and play multiple roles in inflammation. Their numbers increase in auto-inflammatory diseases such as rheumatic disorders.[5,18] Macrophages have two distinct functional phenotypes in different tissue related to different stimuli: pro-inflammatory M1 and anti-inflammatory M2.[19] Classically activated M1 macrophages produce high level of pro-inflammatory cytokines and contribute to the inflammatory events in the pathogenesis of BD.[20] Levels of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α are elevated in the serums of BD patients.[21,22] It has been suggested, serum factors from BD patients are able to induce pro-inflammatory M1 macrophages and imbalance of inflammatory responses.[23]

AHR is a ligand-activated transcription factor modulates the inflammation and immune cells function. AHR signaling connects environmental xenobiotic stimulators with the immune responses.[24] It has been investigated AHR has an anti-inflammatory effect on the macrophages[11] AHR-deficient macrophages overproduce several pro-inflammatory cytokines, such as IL-6 and TNF-α, following M1 macrophage polarization.[25] However, the role of AHR on the macrophages from BD patients remains still unclear.

In this study, we first investigated the expression of AHR mRNA in the monocyte-derived and M1 macrophages in healthy controls. It was found that M1 macrophages polarization by LPS and IFNγ decreased AHR mRNA expression. AHR mRNA expression was significantly down regulated in pro-inflammatory M1 macrophages compared to monocyte-derived macrophages. Our results demonstrate an anti-inflammatory role for AHR in macrophages and support the previous studies showing that the production of several pro-inflammatory cytokines by LPS was increased in AHR-null macrophages.[25-27]

We also analyzed the AHR mRNA expression in monocyte-derived and M1 macrophages from BD patients in comparison with healthy controls. It was shown that both monocyte-derived macrophages and M1 macrophages from BD patients significantly express lower level of AHR mRNA compared to healthy individuals. This finding is in line with a study by Wang et al. who reported that peripheral blood mononuclear cells (PBMCs) from active BD patients express diminished level of AHR mRNA.[28]

Cigarette smoking as an environmental factor has been shown to affect the development of many rheumatic diseases through several systems.[29-32] It seems that cellular responses to tobacco smoke are mediated by AHR.[33,34] According to decreased AHR expression in macrophages from BD patients, it is suggested that AHR has an anti-inflammatory property and its activation can improve BD patients' symptoms. Our results are consistent with the previous studies demonstrated that oral lesions in patients with Behcet's disease can be relapsed after the cigarette smoking

cessation.[35]

This study suggests an important role of AHR in the susceptibility to BD and shows its anti-inflammatory effect on the immune system and macrophage polarization. According to our result decreased AHR expression is associated with pro-inflammatory M1 macrophage and reduced expression of AHR in macrophages is associated with Behcet's disease susceptibility. To the best of our knowledge, this study was the first to analyzed AHR mRNA expression in macrophages from BD patients. Further studies on the expression, and function of AHR in the immune system and effect of this receptor activation on the inflammatory cytokines expression and release should be done to fully determine the role of AHR in the pathogenesis of rheumatic disease.

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