Clinical Rheumatology (2018) 37:1589–1595
https://doi.org/10.1007/s10067-018-4055-9

ORIGINAL ARTICLE

# Ankylosing spondylitis monocyte-derived macrophages express increased level of $A_{2A}$ adenosine receptor and decreased level of ectonucleoside triphosphate diphosphohydrolase-1 (CD39), $A_1$ and $A_{2B}$ adenosine receptors

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Received: 18 September 2017 / Revised: 5 February 2018 / Accepted: 27 February 2018 / Published online: 9 March 2018


## Abstract

Macrophages play an important role in the ankylosing spondylitis (AS) auto-inflammatory responses and fibrocartilage destruction. Adenosine is a key modulator of inflammatory conditions. The various effects of adenosine are mediated by its interaction with adenosine receptors (AR). In this study, we investigated the mRNA expression of $A_1$, $A_{2A}$, $A_{2B}$, and $A_3$ adenosine receptors, ectonucleoside triphosphate diphosphohydrolase-1 (CD39), and ecto-5′-nucleotidase (CD73) in the monocyte-derived macrophages from AS patients in comparison to healthy controls. We also explored the correlation between analyzed gene expression and patients' clinical manifestations. Whole blood-separated monocytes from 23 healthy controls and 23 active AS patients were stimulated by macrophage colony-stimulating factor (M-CSF) for 7 days and differentiated to macrophages. Monocyte and macrophage markers were analyzed by flow cytometry. Analysis of adenosine receptors (*ADORA1 ADORA2A ADORA2B ADORA3*), *CD39* and *CD73* gene expression was performed by SYBR green real-time PCR. Our results demonstrated monocyte-derived macrophages from AS patients expressed increased level of $A_{2A}$AR and reduced level of $A_1$, $A_{2B}$AR, and CD39 mRNA compared to healthy controls. We found an inverse correlation between $A_{2A}$AR mRNA expression and Bath Ankylosing Spondylitis Functional Index (BASFI) score in AS patients. According to our results, altered expression level of adenosine-relying system would be involved in AS macrophage dysfunction and inflammation and correlated with functional status in AS patients.

**Keywords** Adenosine receptors · Ankylosing spondylitis · Ectonucleoside triphosphate diphosphohydrolase-1 (CD39) · Macrophages

**Electronic supplementary material** The online version of this article (https://doi.org/10.1007/s10067-018-4055-9) contains supplementary material, which is available to authorized users.

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## Introduction

Ankylosing spondylitis (AS) is the prototypic and most common member of a group of inflammatory rheumatic diseases named spondyloarthritis (SpA) [1]. The disease is characterized by inflammatory type of low back pain and progressive spinal stiffness [2]. Inflammation of the enthesis, erosion, and new bone (syndesmophyte) formation are the major clinicoradiographic features of AS [3, 4]. The pro-inflammatory cytokine, tumor necrosis factor alpha (TNF-α), contributed to the inflammatory process in AS [5]. Several TNF-α inhibitors have been developed to block spinal pain and inflammation in the disease [6]. Activated macrophages play a crucial role in inflammatory and immune responses through the secretion of some pro-inflammatory

cytokines such as TNF-α [7, 8]. Previous studies demonstrated that macrophages are the predominant cells in the inflammatory lesions, enthesis, and overlaying synovium of SpA and involved in the enthesis and joint inflammation and fibrocartilage destruction [9–12].

The endogenous purine nucleoside adenosine has a range of anti-inflammatory and immunomodulatory properties and involved in the pathogenesis of inflammatory rheumatic diseases [13, 14]. Adenosine is a major innate regulator of function and activation of macrophage and other phagocytic mononuclear cells [15]. The various effects of adenosine are mediated by its interaction with four specific cell surface G-protein-coupled receptors: $A_1$, $A_{2A}$, $A_{2B}$, and $A_3$ adenosine receptors that are expressed on macrophages and nearly all cell types [16]. Activation of these receptors suppresses the initiation of NF-κB pathways and the production of pro-inflammatory molecules [17–19]. According to pervious reports, $A_{2A}$, $A_{2B}$, and $A_3$ adenosine receptor activation can inhibit the secretion of pro-inflammatory TNF-α cytokine via macrophages [20–24].

Adenosine accumulates in the extracellular space under conditions of stress, cell damage, or inflammation [25, 26]. It is generated by degradation of adenosine triphosphate (ATP) by soluble and membrane-bound ectonucleotidases [27]. Following the release into the extracellular space in response to cellular inflammation, ATP breaks down to ADP and AMP by ectonucleoside triphosphate diphosphohydrolase-1 (E-NTPDase1, CD39) and then dephosphorylates into adenosine by ecto-5′-nucleotidase (CD73) [28, 29]. There are other nucleotide-metabolizing enzymes, however, CD39 and CD73 are the major ectonucleotidases that hydrolase pro-inflammatory ATP into anti-inflammatory adenosine and regulate inflammatory responses and immunity [30, 31].

Previous results indicate that lymphocytes from SpA patients express higher level of $A_{2A}$ and $A_3$ adenosine receptors [32]. However, the expression and function of adenosine receptors and ectonucleotidases in macrophages from patients with ankylosing spondylitis is still unclear. In this study, we investigated the messenger RNA (mRNA) expression of all adenosine receptors and CD39 and CD73 ectonucleotidases in the monocyte-derived macrophages from AS patients in comparison to healthy controls. We also explored the correlation between adenosine receptors and ectonucleotidases mRNA expression with patient's clinical manifestations.

## Materials and methods

### Patients and controls

The study population consisted of 23 active AS patients (4 females and 19 males with a mean age of 32 ± 10 years), who fulfilled the modified New York classification criteria for AS [33], and 23 age- and sex-matched healthy volunteers (4 females and 19 males with a mean age of 35 ± 11 years) without family history of any rheumatic disease. Patients were consecutively recruited from the outpatient clinic of the Rheumatology Research Center (RRC), Shariati Hospital, Tehran University of Medical Sciences (TUMS). Patients with Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) ≥ 4 were selected. Prior to participation, written informed consent was obtained from all participants. This study was approved by the ethics committee of Tehran University of Medical Sciences.

### Monocyte separation and macrophage derivation

Fifty milliliters of peripheral blood were collected into Ethylenediaminetetraacetic acid (EDTA) contained tubes from each subject and processed no longer than 5 h after had been taken. Samples were then diluted 1:2 with phosphate buffered saline (PBS; GIBCO Invitrogen) at pH 7.2. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll (Lymphodex, Inno-Train) density gradient centrifugation. Mononuclear cells were washed with PBS and then incubated with magnetic beads. Monocytes separated using positive selection for CD14 using MACS CD14 microbeads and magnetic-activated cell sorter columns (all from Miltenyi Biotec). Immunofluorescence staining of isolated cells was performed by phycoerythrin (PE)-conjugated anti-CD14 antibody (BD bioscience), and flow cytometry analysis showed that the purity was 92–95% (Supplementary Fig. 1). Monocytes were cultured in 24-well plates at 500,000 cells per well in complete Roswell Park Memorial Institute (RPMI) media containing 2 mM L glutamine (Biosera), 10% fetal bovine serum (FBS; Gibco BRL), 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma), with 50 ng/ml recombinant human macrophage-colony stimulating factor (M-CSF; eBioscience) for 7 days.

### Flow cytometry analysis of macrophage surface markers

Monocyte-derived macrophages ($2 \times 10^5$) were immunostained with fluorochrome-labeled antibodies after 7 days of stimulation by M-CSF. Cells were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-human CD163, PE-conjugated anti-human CD206 (BD bioscience) and appropriate isotype-matched antibodies for 30 min avoiding exposure to the light.

macrophages were analyzed on a CyFlow ML flow cytometer (Partec, GmbH, Munster, Germany) and FASC data analysis was performed using the FlowJo software (Tree Star, Ashland, OR, USA). The cells expressed macrophage

markers CD163 and CD206 (97 and 95%, respectively) (Supplementary Fig. 2a and b).

### Analysis of gene expression using real-time quantitative PCR

After 7 days of stimulation by M-CSF, total RNA from monocyte-derived macrophages were extracted using the High Pure RNA Isolation Kit (Roche). The Transcriptor First Strand synthesis kit (Roche) was used for complementary DNA (cDNA) synthesis from the equal amount of total RNA. The relative expression levels of *ADORA1*, *ADORA2A*, *ADORA2B*, *ADORA3*, *ENTPD1* (*CD39*), and *NT5E* (*CD73*) genes were performed using StepOnePlus™ real-time polymerase chain reaction (PCR) system (Applied Biosystems) and SYBR green master mix (Ampliqon). Reference gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as the endogenous control. The specific primer sequences are shown in Table 1. The relative differences in expression of selected mRNAs between healthy controls, and patients were analyzed using the comparative CT method ($2^{-\Delta CT}$).

### Statistical data analysis

Quantitative variables were assessed for normality assumption by Kolmogorov-Smirnov normality test. Statistical analysis of the relative gene expression between the patients and control groups with normal distribution was performed by one-way analysis of variance (ANOVA) and independent *t* tests and for those with non-normal distribution was performed by Kruskal-Wallis *H* and Mann-Whitney *U* tests. A simple liner regression model was used to analyze the dependence of clinical variables on gene expression levels. The differences were considered statistically significant with a *P* value less than 0.05. All of the statistical analysis was done by using SPSS version 22 and GraphPad Prism 6. All data are presented as mean ± SD.

## Results

### Demographic and clinical features

Clinical characteristics were extracted at the time patients fulfilled the criteria of AS. Recorded demographic and clinical characteristics of healthy donors and AS patients are described in Table 2.

### AS macrophages express altered mRNA level of adenosine receptors

To determine the role of adenosine receptors in AS macrophages, the mRNA expression level of $A_1$, $A_{2A}$, $A_{2B}$, and $A_3$ adenosine receptors was assessed in human monocyte-generated macrophages from healthy volunteers and AS patients by qPCR. Our results indicated that monocyte-derived macrophages preferentially expressed $A_3$, $A_{2B}$, and $A_{2A}$ adenosine receptors, and only weakly expressed $A_1$ adenosine receptor. The ratios of expression of $A_3$:$A_{2B}$:$A_{2A}$:$A_1$ receptors by macrophages were approximately 4:1:1:0.03 (Fig. 1). The $A_{2A}$AR mRNA expression in macrophages from AS patients was significantly increased by 2.03-fold in comparison to healthy subjects (Fig. 2; $P < 0.05$). Interestingly, $A_1$ and $A_{2B}$AR mRNA expressions were significantly down regulated (−2.5-fold and −1.4-fold, respectively) in macrophages from AS patients compared to normal macrophages (Fig. 3a, b; $P < 0.05$ for all). Our results showing that the mRNA expression level of $A_3$AR was not significantly different in patient's macrophages compared to healthy subjects (Supplementary Fig. 3a).

**Table 1** Primer sequences and product size of the selected genes

| Accession number | Gene name | Sequence | Size (bp) |
|---|---|---|---|
| NM_000674.2 | *ADORA1* | F: 5′ CCACAGACCTACTTCCACACC 3′<br>R: 5′ TACCGGAGAGGGATCTTGACC 3′ | 128 |
| NM_001278497.1 | *ADORA2A* | F: 5′ CATGCTAGGTTGGAACAACTGC 3′<br>R: 5′ AGATCCGCAAATAGACACCCA 3′ | 185 |
| NM_000676.2 | *ADORA2B* | F: 5′ CTGTCACATGCCAATTCAGTTG 3′<br>R: 5′ GCCTGACCATTCCCACTCTTG 3′ | 134 |
| NM_000677.3 | *ADORA3* | F: 5′ TACCCACGCCTCCATCATGT 3′<br>R: 5′ GGGGTCAATCCCACCAGGA 3′ | 220 |
| NM_001776.5 | *ENTPD1 (CD39)* | F: 5′ ACTATCGAGTCCCCAGATAATGC 3′<br>R: 5′ GGGGTCAATCCCACCAGGA 3′ | 97 |
| NM_002526.3 | *NT5E (CD73)* | F: 5′ GCCTGGGAGCTTACGATTTTG 3′<br>R: 5′ TAGTGCCCTGGTACTGGTCG 3′ | 196 |
| NM_002046.5 | *GAPDH* | F: 5′ GAGTCAACGGATTTGGTCGT 3′<br>R: 5′ GACAAGCTTCCCGTTCTCAG 3′ | 185 |

**Table 2** Clinical characteristics of AS patients and healthy controls

| Characteristic | AS patients ($n = 23$) | Healthy controls ($n = 23$) |
|---|---|---|
| Male/female | 19 (82%)/4 (18%) | 19 (82%)/4 (18%) |
| Age (years) | 32 ± 10 | 35 ± 11 |
| ESR, mm/h | 35 ± 27 | NA |
| HLA-B27 positivity, no. (%) | 17 (74%) | NA |
| Disease duration (years) | 9 ± 8 | NA |
| BASMI score | 3.5 ± 1.7 | NA |
| BASFI score | 5.2 ± 1.5 | NA |
| BASDAI score | 5.7 ± 2.0 | NA |
| BAS-G score | 7.3 ± 1.6 | NA |
| PDGA score | 6.8 ± 2.6 | NA |
| Biological agents | 0(0%) | NA |
| Methotrexate | 0(0%) | NA |

*AS*, ankylosing spondylitis; *ESR*, erythrocyte sedimentation rate; *HLA-B27*, human leukocyte antigen (subtypes B*2701-2759); *BASMI*, Bath Ankylosing Spondylitis Metrology Index; *BASFI*, Bath Ankylosing Spondylitis Functional Index; *BASDAI*, Bath Ankylosing Spondylitis Disease Activity Index; *BAS-G*, Bath Ankylosing Spondylitis Global Score; *PDGA*, patient's disease global assessment; *NA*, not applicable

## CD39 mRNA expression was decreased in AS macrophages

To study the enzymes contributed to adenosine production, we also analyzed the mRNA expression of CD39 and CD73 ectonucleotidases in human macrophages from AS patients and healthy controls. CD39 and CD73 are both expressed in monocyte-induced macrophages. Nevertheless, CD73 enzyme is weakly expressed on the cells. The results showed CD39 mRNA expression was significantly (− 1.3-fold) diminished in AS patient's macrophages compared to normal controls (Fig. 4; $P < 0.05$). However, the expression of CD73 enzyme was not significantly different between normal and patients group (Supplementary Fig. 3b).

## A2A adenosine receptor expression inversely correlated with BASFI score in AS patients

We next investigated the association of the clinical manifestation of AS patients with analyzed mRNA expressions. Clinical data included Bath Ankylosing Spondylitis Metrology Index (BASMI), Bath Ankylosing Spondylitis Functional Index (BASFI), Bath Ankylosing Spondylitis Disease Activity Index (BASDI), Bath Ankylosing Spondylitis Global Score (BAS-G), and patient's disease global assessment (PDGA). We found an inverse correlation between $A_{2A}AR$ mRNA expression and BASFI score in AS patients (Fig. 5; $P < 0.01$). There was no other significant correlation between clinical manifestations and selected gene mRNA expression.

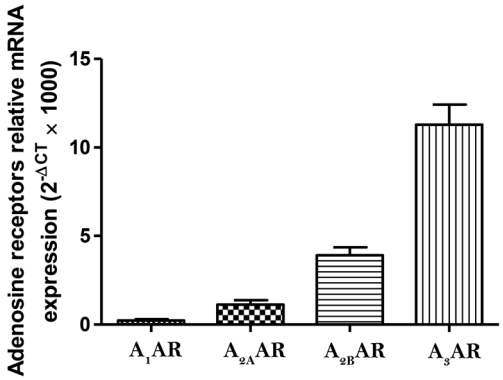


**Fig. 1** Adenosine receptor mRNA expression in monocyte-derived macrophages. The ratios of expression of A3: A2B: A2A: A1 receptors by macrophages were approximately 4:1:1:0.03. Monocytes from 23 healthy controls were differentiated to macrophages with M-CSF for 7 days. The receptor mRNA was determined by real-time PCR and normalized to *GAPDH*. Data are expressed as the means ± SD

## Discussion

To study the involvement of adenosine-relying system in ankylosing spondylitis, we primarily investigated the expression of adenosine receptors in AS macrophages compared to healthy controls. This study is the first to analyze the expression of adenosine receptors in macrophages from AS patients. All four adenosine receptors are expressed by monocyte-derived macrophages with $A_3AR$ mRNA predominance on the cells. The results of the study revealed an increase at mRNA level of $A_{2A}AR$ in monocyte-generated macrophages from AS patients. Our results are in line with the previous works demonstrated an upregulation of $A_{2A}AR$ in lymphocytes from systemic lupus erythematosus (SLE) [34], rheumatoid arthritis (RA), psoriatic arthritis (PsA), and AS patients [32]. Previous reports showed that lymphocytes from patients

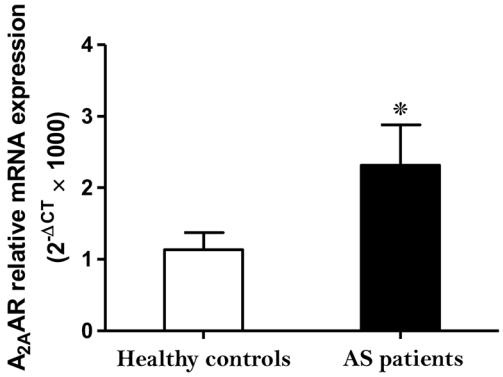


**Fig. 2** $A_{2A}AR$ mRNA expression in monocyte-derived macrophages from AS patients was significantly increased by 2.03-fold as compared to healthy controls. Isolated monocytes from 23 AS patients and 23 healthy donors were differentiated to macrophages with M-CSF. $A_{2A}AR$ mRNA was determined in monocyte-generated macrophages by real-time PCR and normalized to *GAPDH*. Data are expressed as the means ± SD (*$P \leq 0.05$)

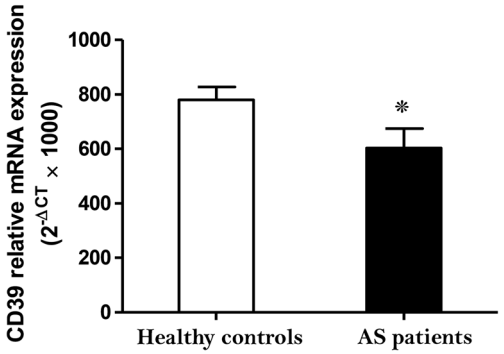


**Fig. 4** The mRNA expression of CD39 enzyme in monocyte-generated macrophages. Expression was significantly down regulated in macrophage from patients ($n = 23$) by − 1.3-fold as compared to normal individuals ($n = 23$). The expression was detected by qPCR and normalized to *GAPDH*. Data are presented as the means ± SD (*$P \leq 0.05$)

with RA, AS, and PsA inflammatory diseases [32] and PBMCs from patients with psoriasis, RA, and Crohn's disease [35] express higher level of $A_3$ adenosine receptor. However, we did not find a significant difference between $A_3AR$ mRNA level in macrophages from AS patients compared to healthy control group. We also observed a decrease at a transcriptional level of $A_1$ and $A_{2B}AR$ in AS macrophages. Our finding is different from the previous reports, showing no significant differences between mRNA levels of $A_1$ and $A_{2B}$ adenosine receptors in lymphocytes from AS and other arthritic patients comparing to normal lymphocytes [32, 34, 36]. The present study showed, for the first time, the involvement of $A_1$ and $A_{2B}AR$ mRNA alternations in the most common form of SpA. According to our results, altered expression level of adenosine receptors would be involved in macrophage dysfunction and inflammation in AS.

We next verified whether the expression of adenosine receptors in macrophages was associated with patient's clinical characteristics. Our findings, for the first time, demonstrated an inverse correlation between functional status of AS patients (BASFI score) and $A_{2A}AR$ mRNA expression in monocyte-derived macrophages. The lower level of BASFI scores were correlated with higher level of $A_{2A}AR$ mRNA expression in macrophages, showing a significant role for these receptors in AS pathogenesis. Former studies demonstrated that $A_{2A}$ adenosine receptor activation prevents differentiation of osteoclasts [37], suggesting a mechanism by which $A_{2A}AR$ could target bone erosion, formation, and functional status in AS. This finding supports the previous studies showing an inverse correlation between $A_{2A}AR$ density and Disease Activity Score (DAS) in RA patients [36], and clinical parameters in SLE patients [34].

Adenosine serves as a signaling molecule to limit inflammation. CD39 and CD73 membrane-bound ectonucleotidases degrade extracellular nucleotides to the adenosine [30]. Alterations in CD39 and CD73 expression correlate with disturbed adenosine signaling [30, 38]. Therefore, in the present work, we also analyzed the mRNA expression of CD39 and CD73 ecto-enzymes in AS macrophages in comparison to normal macrophages. Current study is the first to analyze the mRNA expression of CD73 and CD39 enzymes in macrophages from AS patients. Our results revealed that monocyte-derived macrophages from AS patients express diminished mRNA level of CD39 compared to controls. We did not find a significant difference in the mRNA level of CD73 between macrophages from AS patients and healthy

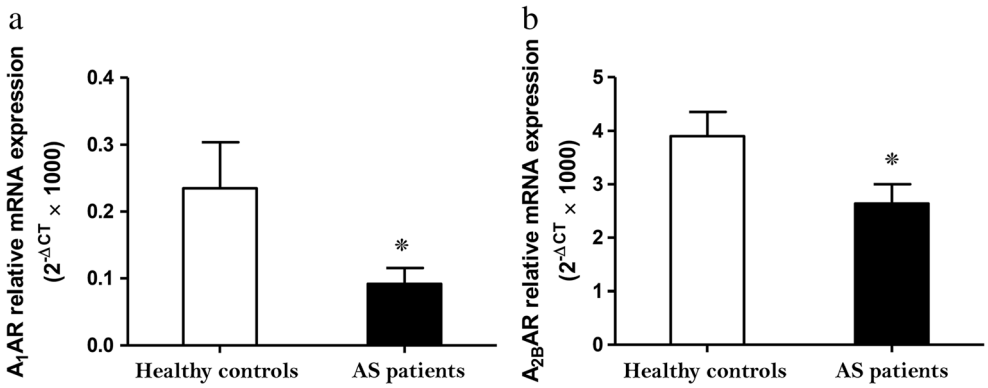


**Fig. 3** (**a**) $A_1AR$ and (**b**) $A_{2B}AR$ mRNA expression in M-CSF-generated macrophages from AS patients ($n = 23$) was significantly decreased by − 2.5-fold and − 1.4-fold, respectively, comparing to normal macrophages ($n = 23$). mRNA expression was assessed by real-time PCR and normalized to *GAPDH*. Data are expressed as the means ± SD (*$P \leq 0.05$)

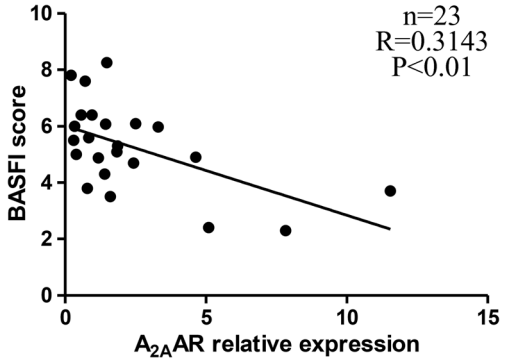


**Fig. 5** Correlation between BASFI score and $A_{2A}$AR relative mRNA expression. Simple linear regression analysis between BASFI score and the $A_{2A}$AR relative mRNA expression in monocyte-derived macrophages from 23 AS patients

individuals. We also did not observe a significant correlation between these enzymes expression and patient's clinical manifestation.

In consistent with our study, Matthew et al. have reported T cell expression of the CD39 enzyme is defective in a subset of active lupus patients [39]. Nevertheless, our data is different from the former studies on regulatory T cells from other arthritic patients. It has been reported that $CD4^+$ T cells from the synovial fluid of patients with RA [40], SpA [41], and juvenile arthritis (JIA) [42] express increased CD39 and reduced CD73 levels. There is no previous report about CD39 and CD73 expression in macrophages from AS and other rheumatic disorders. According to the anti-inflammatory role of adenosine, and the macrophage involvement in AS inflammation, it is suggested that diminished adenosine-generating enzyme CD39 in AS macrophages would be involved in the disease pathogenesis and joint inflammation.

The current study highlights, for the first time, the altered expression of ecto-enzyme CD39, $A_{2A}$, $A_{2B}$, and $A_1$ adenosine receptors in monocyte-derived macrophages from AS patients. These data indicate the emerging role of adenosine signaling molecules in AS disease as therapeutic targets. Adenosine-relying system would be involved in AS macrophage dysfunction and inflammation and correlated with functional status in AS patients. Further studies on the function of adenosine signaling molecules in the macrophages, and inflammatory pathways should be done to fully distinguish the role of adenosine in the pathogenesis of AS.

**Acknowledgements** We would like to thank all participants for their collaboration.

**Funding information** This work has been supported by a research grant from Tehran University of Medical Sciences (TUMS); grant no. 95-04-41-33869.

## Compliance with ethical standards

**Disclosures** None.

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