LETTER TO THE EDITOR
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Gene Expression Profiling of Toll-Like Receptor 4 and 5 in Peripheral Blood Mononuclear Cells in Rheumatic Disorders: Ankylosing Spondylitis and Rheumatoid Arthritis

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Spondyloarthropathies (SpA), defined by inflammatory situations, are a category of rheumatic diseases with mainly involvement of the spine, enthuses, and peripheral joints.¹ Ankylosing spondylitis (AS), as the prototype of this category, is an autoimmune disease with a chronic inflammatory arthritis which is characterized by axial skeletal ankylosis, inflammation at the enthesis, and arthritis of the peripheral limbs.² AS shows a strong association with Human Leucocyte Antigen (HLA)-B27 hereditary, and the misfolded HLA-B27 heavy chain homodimer in an animal model has supported the important role of this molecule in the pathogenesis of AS.³

Toll-like receptors (TLRs) are categorized as type I integral membrane glycoproteins which play important roles in the innate immune system. In human, 11 members of TLR family have been identified.⁴ There is an evidence demonstrating the role of TLR4 in the perforin production by helper T cells in ankylosing spondylitis.⁵ Nevertheless, it has been demonstrated that the level of TLR4 protein on peripheral-blood leucocytes of AS patients and healthy individuals is different.

However, the study almost failed to show the different level of TLR4 mRNA in AS patients.⁶ TLR4 has been also found to be an important molecule in the pathogenesis of rheumatoid arthritis.⁷ Global gene expression analysis in AS patients has revealed that TLR4 and TLR5 were the only dysregulated subtypes of TLRs in AS.⁸

Considering the existing evidences, TLR pathways and their overactivity, especially about TLR4, have some relationships with several autoimmune diseases except for AS.⁹,¹⁰ Hence in this survey, we decided to investigate the TLR4 and TLR5 expression profiles in peripheral blood mononuclear cells (PBMCs) of AS and RA patients as a confirmatory investigation of the overexpression of these molecules. RA patients were also included as disease control, in order to obtain a gene expression profile unique to AS patients. Moreover, to appraise the disease activity according to TLR4 and TLR5 mRNA levels, clinical manifestations of AS patients were evaluated in correlation with the transcript level of these two molecules.

The patients were recruited from Firoozgar Hospital, Tehran University of Medical Sciences, Tehran, Iran, and diagnosed with AS according to the modified New York criteria.¹⁰ The disease severity and functional disabilities were deliberated through Bath Ankylosing Spondylitis Disease Activity Index (BASDAI),¹¹ Bath Ankylosing Spondylitis Functional Index (BASFI),¹² and Bath Ankylosing Spondylitis Metrology Index

Keywords: Ankylosing spondylitis; Gene expression; HLA-B27 antigen; Rheumatoid arthritis; Toll-Like Receptor 4; Toll-Like Receptor 5

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(BASMI).\textsuperscript{13} Forty AS patients, 20 age and sex matched RA patients as disease control, and 50 healthy age and sex matched volunteers as healthy control group were included in the study. None of the healthy controls had a personal or family history of autoimmune diseases. Patients, except for 3 AS patients who had undergone anti-TNF immunotherapy, had received no immunomodulatory therapy for at least 3 months before they were included in the study. The Human Research Ethics Committees of Tehran University of Medical Sciences approved this study. Written informed consent was taken from all participants. About 10 ml of venous blood were obtained from each study subject. PBMCs were isolated using Ficoll-Hypaque density gradient centrifugation and total cellular RNA was extracted using the High Pure RNA Isolation Kit (Roche, Germany) according to the manufacturer’s instructions. The yield and purity of RNA were determined by a NanoDrop spectrophotometer at 260/280 nm (NanoDrop ND-2000C Spectrophotometer, Thermo Fisher Scientific, USA). Complementary DNA (cDNA) was synthesized from the RNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Germany) according to the manufacturer’s instructions. Afterwards, quantitative analysis was carried out by Real Time PCR using the TaqMan Gene Expression Assays containing the FAM dye-labeled probes (TaqMan Pre-designed Gene Expression Products, Applied Biosystems, Foster City, CA, USA) and StepOne Plus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). A widely used approach to represent relative gene expression, the comparative \( C_T \) method, was used to evaluate expression as previously described by Schmittgen and Livak.\textsuperscript{14} Relative amounts of target mRNAs in the test sample were calculated and normalized to the corresponding ACTB mRNA transcript level as a housekeeping gene. Then, the relative expression for each sample was calculated using the following equation: relative mRNA expression=$\left(2^{-\Delta C_T}\right)\times10^7$. Data analysis was carried out via SPSS software version 18 (SPSS, Chicago, IL, USA). Scale variables were evaluated for normality using the Kolmogorov–Smirnov test. Through the independent sample \( t \)-test, group comparisons were carried out. If the variable was not normally distributed, the Mann-Whitney nonparametric test was conducted. The Pearson correlation was applied for assessing the relationship between variables. All results are expressed as mean±standard deviation (SD) with statistical significance set at 5%. AS and RA patients with the mean ages of 40.7±6.9 and 43±4.7 years, respectively, were found to be age (and also sex) matched with healthy control group with a mean age of 38.9±5.3 years (\( p>0.05 \)). The ESR value was significantly higher in RA patients in comparison to healthy controls (16.1±8.4 vs. 4.4±3.82; \( p<0.001 \)). HLA-B27-positive patients showed a significantly increased ESR when compared to the HLA-B27-negative AS patients (26.21±21.44 vs. 16.5±12.3; \( p<0.001 \)), which were both significantly higher in comparison to healthy controls. ESR was significantly higher in HLA-B27-positive AS patients than RA patients (26.21±21.44 vs. 16.1±8.4; \( p=0.006 \)). The expression level of TLR4 mRNA in AS patients was approximately similar to that of healthy controls (fold change= 1.02; \( p=0.77 \)). Also, HLA-B27-positive AS patients expressed TLR4 mRNA slightly higher than the healthy controls (1.03 times upregulated; \( p=0.75 \)). On the other side, the expression of TLR4 mRNA in HLA-B27-negative AS patients was insignificantly less (0.73 times downregulated; \( p=0.09 \)) than healthy individuals (Figure 1). AS patients expressed TLR4 mRNA more than RA patients, but no significant difference was seen (1.20 times upregulated; \( p=0.11 \)). The expression level of TLR4 in RA patients was lower (fold change=0.83; \( p=0.07 \)) versus healthy individuals (Figure 1). The expression level of TLR5 in AS patients was slightly higher than that of healthy individuals (1.45 times upregulated; \( p=0.074 \)). Moreover, TLR5 mRNA expression level was rather increased in HLA-B27-positive AS patients when compared with healthy controls (1.40 times upregulated; \( p=0.13 \)). Similarly, HLA-B27-negative AS patients expressed TLR5 mRNA insignificantly more (1.80 times upregulated; \( p=0.15 \)) than healthy individuals (Figure 1). AS patients expressed TLR5 mRNA higher than RA patients, but insignificantly (1.17 times upregulated; \( p=0.18 \)). In comparison with healthy subjects, RA patients represented trivially increased expression levels of TLR5 (fold change=1.30; \( p=0.40 \)) but no significant difference was observed (Figure 1). No significant correlation was observed between the most important parameters in ankylosing spondylitis, ESR (\( p=0.33, r=-0.159 \)), BASFI (\( p=0.46, r=-0.121 \)), or BASMI scores (\( p=0.11, r=0.193 \)), with the relative expression of TLR4 mRNA in AS patients. However, the relative expression of
TLR4 had a weak positive correlation with BASDAI (p=0.02, r=0.367). Age and the duration of the disease did not correlate with the relative expression level of TLR4. None of the parameters were correlated with the relative expression level of TLR5 (Table 1).

AS patients that received anti-TNF immunotherapy demonstrated no different expression levels of both TLR4 (0.75 times downregulated; p=0.11) and TLR5 (0.93 times downregulated p=0.74) when compared to healthy individuals. On the other side, no difference was observed in mRNA expressions of TLR4 (0.67 times downregulated; p=0.09) and TLR5 (0.81 times downregulated; p=0.17) between AS patients who received and did not receive anti-TNF therapy. The expression levels of TLR4 and 5 were correlated with none of the disease activity parameters (data not shown).

TLRs, through the recognition of conserved pathogen-associated molecular patterns (PAMPs), are basically involved in innate immune responses to microbial pathogens. In human, more than 11 TLR subtypes have been identified. TLR4 has especially been linked to the pathogenesis of several immune-mediated diseases in addition to AS, like rheumatoid arthritis, multiple sclerosis and inflammatory bowel disease.

Assassi et al. reported for the first time that TLR5 expression level was high in AS patients. De Rycke et al. reported an increased expression level of TLR4, but not TLR2, in PBMCs of the patients with spondyloarthropathies (SpA) in comparison to controls. In order to re-prove the higher expression level of most concentrated Toll-like receptors in AS, we evaluated the expression level of TLR4 and compared it with RA patients. Moreover, as a recently-interested TLR in AS, the expression level of TLR5 was also analyzed. Nonetheless, both TLR4 and 5 were not differently expressed between AS patients in relation to healthy individuals.

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Table 1. The correlation of the expression of TLR4 and 5 mRNAs in PBMCs from Ankylosing Spondylitis (AS) patients with age, disease duration, BASDI, BASFI, BASMI, and ESR.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>TLR4</th>
<th>TLR5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pearson’s Correlation Coefficient</td>
<td>p value</td>
</tr>
<tr>
<td>Age</td>
<td>−0.176</td>
<td>0.23</td>
</tr>
<tr>
<td>Disease Duration</td>
<td>−0.21</td>
<td>0.22</td>
</tr>
<tr>
<td>ESR</td>
<td>−0.159</td>
<td>0.33</td>
</tr>
<tr>
<td>BASDI</td>
<td>−0.367</td>
<td>0.02*</td>
</tr>
<tr>
<td>BASFI</td>
<td>−0.121</td>
<td>0.46</td>
</tr>
<tr>
<td>BASMI</td>
<td>0.255</td>
<td>0.11</td>
</tr>
</tbody>
</table>

*Correlation significance at the level of 0.05 (2-tailed). ESR: Erythrocyte Sedimentation Rate, BASDAI: Bath Ankylosing Spondylitis Disease Activity Index, BASFI: Bath Ankylosing Spondylitis Functional Index, BASMI: Bath Ankylosing Spondylitis Metrology Index.

It has been stated that an aberrant response against pathogens may be related to the pathogenesis of AS. Moreover, Taurog et al. demonstrated that HLA-B27 transgenic rats did not develop the disease if they were raised in a germ-free environment, whereas introduction of intestinal flora caused the development of intestinal and joint inflammation. As a result, HLA-B27-transgenic rats did not develop inflammatory
intestinal or peripheral joint disease in a germ-free environment. This survey on HLA-B27-transgenic rats suggests the role of bacteria in the pathogenesis of HLA-B27-associated intestinal and joint manifestations. Through several studies in various ethnic populations of AS, a strong association has been repetitively demonstrated between AS and HLA-B27. It is estimated that the presence of the HLA-B27 allele is responsible for approximately one-third of the entire genetic contribution of AS, on top of a consensus for this association.

Our findings indicated that the expression levels of TLR4 and 5, whose importance was implied in the pathogenesis of AS, were not different between AS and both RA patients and healthy subjects. On the other side, HLA-B27-positive AS patients were not expressing TLR4 and TLR5 significantly different in comparison to RA patients and even to healthy subjects (Figure 1). The current survey may imply that the expression levels of TLR4 and 5 are not affected by the status of HLA-B27. Besides, to conclude prematurely, aberrant expression levels of TLR4 and TLR5 in PBMCs of AS patients, whether HLA-B27-positive or negative, may not indicate the AS development.

Engagement of TLR4 and TLR5 can activate downstream pathways to induce proinflammatory cytokines such as TNF-α. De Rycke et al. demonstrated that an inflammation in SpA was described through increased expression levels of TLR2 and 4. When the PBMCs of these patients were treated by infliximab (humanized antibody against TNF-α), the expression levels of both TLR2 and 4 dramatically declined. After treating with LPS, PBMCs manifested a dysfunction in production of TNF-α. On the other hand, Yang et al. reported that the expression level of TLR4 was positively correlated with the serum level of TNF-α. According to our experiments, expression levels of TLR4 and TLR5 did not indicate any significant differences between AS patients who received anti-TNF and healthy individuals. Furthermore, it was observed that the expression levels of both TLR4 and TLR5 in AS patients with anti-TNF treatment remained unaltered in comparison to AS patients without anti-TNF treatment. Besides, the expression levels of both TLR4 and TLR5 were not correlated with ESR, BASDAI, BASMI, and BASFI of AS patients who had received infliximab.

The bottom line is that our findings did not show any differences in the expression of TLR4 and TLR5 mRNAs in the PBMCs of patients with AS compared to that of RA patients and healthy individuals. Among the parameters, the BASDAI score of AS patients was negatively correlated with TLR4 mRNA expression. It should be noted that the current study was based upon

![Figure 1](http://ijaai.tums.ac.ir)
Gene Expression Profiling of TLR4 and TLR5 in Rheumatic Disorders

AS and RA patients only, and other spondyloarthritis-related disorders were not included, which could have been beneficial to gain a better understanding of the effect of TLR4 and TLR5 mRNA levels on the pathogenesis of such diseases. Various studies are not compatible to clearly demonstrate that TLRs are genetic risk factor for AS, but taking that the TLRs are the intersection point between the immune system and the environment, there is still remarkable interest with regard to the role for TLRs in the inflammatory state of AS.

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