LETTER TO THE EDITOR
Iran J Allergy Asthma Immunol
February 2016; 15(1):87-92.

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

Simin Almasi[1], Saeed Aslani[2], Hadi Poormoghim[1], Ahmadreza Jamshidi[2], Shiva Poursani[2], and Mahdi Mahmoudi[2]

[1] Firouzgar Hospital, Iran University of Medical Sciences (IUMS), Tehran, Iran
[2] Rheumatology Research Center, Tehran University of Medical Sciences, Tehran, Iran

Received: 6 April 2015; Received in revised form: 24 May 2015; Accepted: 24 May 2015

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 entheses, 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.[7,9] 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

Corresponding Author: Mahdi Mahmoudi, PhD;
Rheumatology Research Center, Shariati Hospital, Tehran, Iran
PO-BOX: 1411713137, Tel/Fax: (+98 21) 8822 0067, E-mail: mahmoudim@tums.ac.ir

Published by Tehran University of Medical Sciences (http://ijaai.tums.ac.ir)

(BASMI).[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.[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=$(2^{-\Delta Ct})\times 10^3$. 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; $p<0{\cdot}001$), 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.[15] TLR4 has especially been linked to the pathogenesis of several immune-mediated diseases in addition to AS, like rheumatoid arthritis,[16] multiple sclerosis[17] and inflammatory bowel disease.[9]

Assassi *et al*. reported for the first time that TLR5 expression level was high in AS patients.[8] 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.[6] 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.

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.[15] TLR4 has especially been linked to the pathogenesis of several immune-mediated diseases in addition to AS, like rheumatoid arthritis,[16] multiple sclerosis[17] and inflammatory bowel disease.[9]

Assassi *et al*. reported for the first time that TLR5 expression level was high in AS patients.[8] 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.[6] 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.

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

Table1. 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.

Parameter	TLR4		TLR5	
	Pearson's Correlation Coefficient	*p* value	Pearson's Correlation Coefficient	*p* value
Age	−0.176	0.23	−0.280	0.08
Disease Duration	−0.21	0.22	−0.24	0.15
ESR	−0.159	0.33	−0.259	0.10
BASDI	−0.367	0.02*	−0.239	0.14
BASFI	−0.121	0.46	−0.255	0.11
BASMI	0.255	0.11	0.033	0.84

*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.

intestinal or peripheral joint disease in a germ-free environment.[18] 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.[19]

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.[20] 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.[21] On the other hand, Yang *et al.* reported that the expression level of TLR4 was positively correlated with the serum level of TNF-α.[22] 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

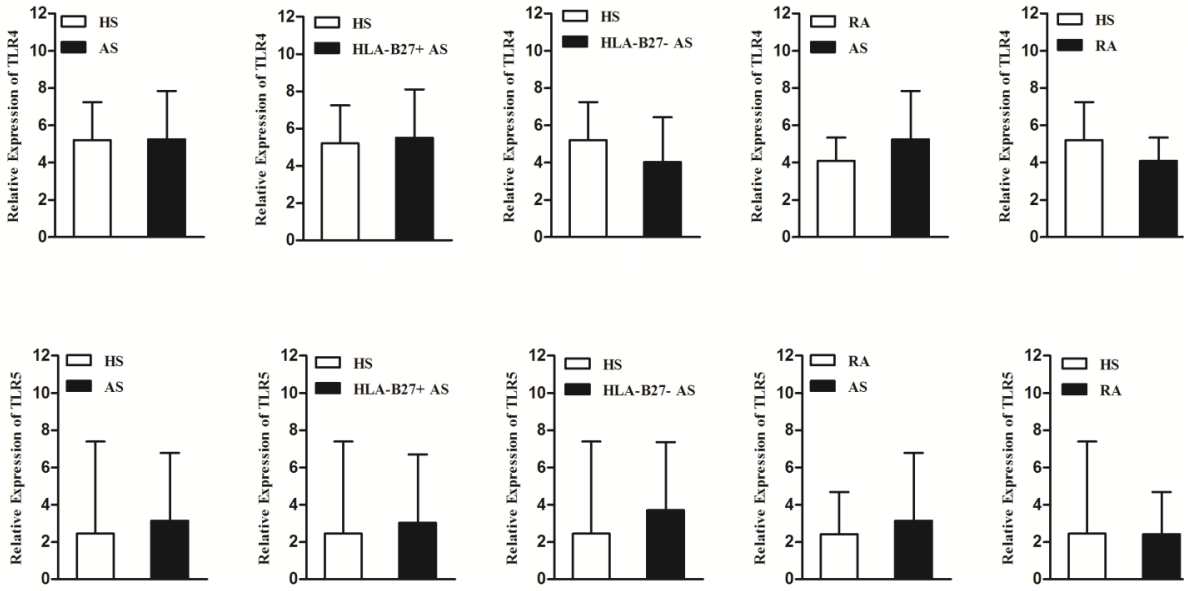

Figure1. Bar graphs illustrate relative expression of TLR4 (Upper Row) and TLR5 (Lower Row) mRNAs in PBMCs from three categories of AS patients, RA patients, and healthy subjects counter-posed with each other (AS: Ankylosing Spondylitis, RA: Rheumatoid Arthritis, HS: Healthy Subjects, HLA: Human Leukocyte Antigen).

AS and RA patients only, and other spondyloarthropathy-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.

ACKNOWLEDGEMENTS

The authors would like to express their gratitude to all participants who made the completion of this study possible. This work was partially supported by a research grant from Deputy of Research, Iran University of Medical Sciences.

REFERENCES

1. Botos I, Segal DM, Davies DR. The structural biology of Toll-like receptors. Structure 2011; 19(4):447-59.
2. Braun J, Sieper J. Ankylosing spondylitis. Lancet 2007; 369(9570):1379-90.
3. Colbert RA, DeLay ML, Klenk EI, Layh-Schmitt G. From HLA-B27 to spondyloarthritis: a journey through the ER. Immunol Rev 2010; 233(1):181-202.
4. Lories RJ, Schett G. Pathophysiology of new bone formation and ankylosis in spondyloarthritis. Rheum Dis Clin North Am 2012; 38(3):555-67.
5. Raffeiner B, Dejaco C, Duftner C, Kullich W, Goldberger C, Vega SC, et al. Between adaptive and innate immunity: TLR4-mediated perforin production by CD28null T-helper cells in ankylosing spondylitis. Arthritis Res Ther 2005; 7(6):R1412-20.
6. De Rycke L, Vandooren B, Kruithof E, De Keyser F, Veys EM, Baeten D. Tumor necrosis factor α blockade treatment down-modulates the increased systemic and local expression of toll-like receptor 2 and toll-like receptor 4 in spondylarthropathy. Arthritis Rheum 2005; 52(7):2146-58.
7. Roelofs MF, Boelens WC, Joosten LA, Abdollahi-Roodsaz S, Geurts J, Wunderink LU, et al. Identification of small heat shock protein B8 (HSP22) as a novel TLR4 ligand and potential involvement in the pathogenesis of rheumatoid arthritis. Arthritis Rheum 2006;176(11):7021-7.
8. Assassi S, Reveille JD, Arnett FC, Weisman MH, Ward MM, Agarwal SK, et al. Whole-blood gene expression profiling in ankylosing spondylitis shows upregulation of toll-like receptor 4 and 5. J Rheumatol 2011; 38(1):87-98.
9. Shi D, Das J, Das G. Inflammatory bowel disease requires the interplay between innate and adaptive immune signals. Cell Res 2006; 16(1):70-4.
10. Moll J, Wright V. New York clinical criteria for ankylosing spondylitis. A statistical evaluation. Ann Rheum Dis 1973; 32(4):354-63.
11. Garrett S, Jenkinson T, Kennedy LG, Whitelock H, Gaisford P, Calin A. A new approach to defining disease status in ankylosing spondylitis: the Bath Ankylosing Spondylitis Disease Activity Index. J Rheumatol 1994; 21(12):2286-91.
12. Calin A, Garrett S, Whitelock H, Kennedy L, O'hea J, Mallorie P, et al. A new approach to defining functional ability in ankylosing spondylitis: the development of the Bath Ankylosing Spondylitis Functional Index. J Rheumato 1994; 21(12):2281-5.
13. Jenkinson TR, Mallorie PA, Whitelock H, Kennedy LG, Garrett S, Calin A. Defining spinal mobility in ankylosing spondylitis (AS). The Bath AS Metrology Index. J Rheumatol 1994; 21(9):1694-8.
14. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative CT method. Nat Protoc 2008; 3(6):1101-8.
15. Takeda K, Akira S. Toll-like receptors in innate immunity. Inter Immunol 2005;17(1):1-14.
16. Radstake TR, Roelofs MF, Jenniskens YM, Oppers-Walgreen B, van Riel PL, Barrera P, et al. Expression of Toll-like receptors 2 and 4 in rheumatoid synovial tissue and regulation by proinflammatory cytokines interleukin-12 and interleukin-18 via interferon-γ. Arthritis Rheum 2004; 50(12):3856-65.
17. Kerfoot SM, Long EM, Hickey MJ, Andonegui G, Lapointe BM, Zanardo RC, et al. TLR4 contributes to disease-inducing mechanisms resulting in central nervous system autoimmune disease. J Immunol 2004; 173(11):7070-7.
18. Taurog JD, Richardson JA, Croft J, Simmons WA, Zhou M, Fernández-Sueiro JL, et al. The germfree state prevents development of gut and joint

inflammatory disease in HLA-B27 transgenic rats. J Exp Med 1994; 180(6):2359-64.
19. Brown M, Laval S, Brophy S, Calin A. Recurrence risk modelling of the genetic susceptibility to ankylosing spondylitis. J Exp Med 2000; 59(11):883-6.
20. Rhee SH, Im E, Riegler M, Kokkotou E, O'Brien M, Pothoulakis C. Pathophysiological role of Toll-like receptor 5 engagement by bacterial flagellin in colonic inflammation. Proc Natl Acad Sci U S A 2005; 102(38):13610-5.
21. Lai NS, Yu HC, Chen HC, Yu CL, Huang HB, Lu MC. Aberrant expression of microRNAs in T cells from patients with ankylosing spondylitis contributes to the immunopathogenesis. Clin Exp Immunol 2013; 173(1):47-57.
22. Yang ZX, Liang Y, Zhu Y, Li C, Zhang LZ, Zeng XM, et al. Increased expression of Toll-like receptor 4 in peripheral blood leucocytes and serum levels of some cytokines in patients with ankylosing spondylitis. Clin Exp Immunol 2007; 149(1):48-55.