Whole-blood Gene Expression Profiling in Ankylosing Spondylitis Shows Upregulation of Toll-like Receptor 4 and 5

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Abstract

Objective—To identify differentially expressed genes in peripheral blood cells (PBC) of patients with ankylosing spondylitis (AS) relative to healthy controls and controls with systemic inflammation.

Methods—We investigated PBC samples of 16 patients with AS and 14 matched controls, in addition to systemic lupus erythematosus (SLE) and systemic sclerosis (SSc) samples utilizing Illumina Human Ref-8 BeadChips. Candidate genes were confirmed using quantitative PCR. Subsequently, these genes were also validated in a separate sample of 27 patients with AS [before and after antitumor necrosis factor (anti-TNF) treatment] and 27 matched controls.

Results—We identified 83 differentially expressed transcripts between AS patients and controls. This gene list was filtered through the lists of differentially expressed transcripts in SLE and SSc, which resulted in identification of 52 uniquely dysregulated transcripts in AS. Many of the differentially expressed genes belonged to Toll-like receptor (TLR) and related pathways. *TLR4* and *TLR5* were the only dysregulated TLR subtypes among AS patients. We confirmed the overexpression of *TLR4* and *TLR5* in AS patients in comparison to controls (p = 0.012 and p = 0.006, respectively) and SLE (p = 0.002, p = 0.008) using quantitative PCR in the same sample. Similarly, *TLR4* (p = 0.007) and *TLR5* (p = 0.012) were significantly upregulated among the AS patients before anti-TNF treatment in the confirmatory sample. *TLR4* (p = 0.002) and *TLR5* (p = 0.025) decreased significantly after anti-TNF treatment.

Conclusion—PBC gene expression profiling in AS shows an upregulation of *TLR4* and *TLR5*. This supports the importance of TLR subtypes in the pathogenesis of AS that are responsible for the immune response to Gram-negative bacteria.

Keywords

ANKYLOSING SPONDYLITIS; TOLL-LIKE RECEPTORS; IMMUNE SYSTEM; AUTOIMMUNITY; BACTERIA; GENE EXPRESSION PROFILING

Ankylosing spondylitis (AS) is a chronic inflammatory arthritis with a predilection for the spine and sacroiliac joints that can lead to new bone formation and ultimately ankylosis. AS is the prototype of spondyloarthropathies (SpA), a related family of disorders with common clinical features and with a strong association with HLA-B27. However, HLA-B27 accounts only for ~45% of the genetic risk in AS. Genome-wide association studies have identified several other non-HLA susceptibility genes such as IL23R and ERAP1 in AS[1,2]. Other diseases belonging to the spectrum of SpA are reactive arthritis, psoriatic arthritis, and arthritis in patients with inflammatory bowel disease[3].

Functional studies also have been undertaken to identify candidate genes and pathways that play a role in the pathogenesis of SpA. Microarray data from synovium suggest a proinflammatory profile. Gu, *et al* demonstrated increased RNA expression of monocyte chemotractant protein 1 (MCP-1), interleukin 8 (IL-8), IL-1ß, endothelial-monocyte activating polypeptide II, interferon-γ, tumor necrosis factor-α (TNF-α), and BiP in SpA synovial fluid mononuclear cells[4]. Rihl, *et al* found elevated levels of IL-7 transcript and protein in sacroiliac joint cells[5]. On the other hand, transcriptional profiling of isolated peripheral blood mononuclear cells (PBMC) by several groups showed that transcripts involved in the inflammatory response are differentially expressed in SpA patients, but reports on the nature of these changes seem to vary. The earliest PBMC study indicated increased expression of proinflammatory proteins such as CXCR4/SDF-1[6]. However, recent reports suggest decreased immune responsiveness of the PBMC. Smith, *et al* found a "reverse interferon (IFN) signature" characterized by decreased expression of IFN-γ and IFN-γ-inducible genes in AS macrophages[7], and Duan, *et al* found that AS PBMC display an immunosuppressive phenotype as shown by underexpression of NR4A2, TNFAIP3, and CD69[8].

We investigated the whole-blood gene transcript profile of AS patients in comparison to controls in order to elucidate the gene expression patterns involved in this disease. Unlike previous investigators, we employed a commercially available method to stabilize RNA in the blood immediately upon phlebotomy to minimize artifacts that occur with handling and purification of living PBMC[9]. We compared their transcript profiles to healthy controls; we also compared transcript profiles to those of patients with systemic sclerosis (SSc) and systemic lupus erythematosus (SLE) in order to identify transcripts that were specific to AS and were not related only to the presence of systemic inflammation. We identified 51 genes that were differentially expressed only in AS patients. Many of the differentially expressed genes belonged to Toll-like receptor (TLR) and related pathways. We observed an overexpression of *TLR4* and *TLR5* that was confirmed by quantitative polymerase chain reaction (PCR) in 2 different cohorts of AS patients. We further demonstrated that *TLR4* and *TLR5* transcripts in whole blood decreased significantly after TNF-α inhibitor (anti-TNF) therapy. Our findings provide support for the importance of a pathogen-associated molecular pattern in the pathogenesis of AS.

MATERIALS AND METHODS

The patients with AS were recruited from the Prospective Study of Outcomes in Ankylosing Spondylitis (PSOAS) study. The PSOAS is a longitudinal study of AS patients from 3 sites in the USA: the University of Texas Health Science Center at Houston, Houston, Texas (UTHSC-H); Cedars-Sinai Medical Center, Los Angeles, California; and the National institutes of Health, Bethesda, Maryland. All AS patients met the modified New York criteria for the definitive diagnosis of AS[10]. From patients enrolled in PSOAS, we investigated 2 separate groups of patients with AS. In the initial group, no patient was receiving anti-TNF or other immunosuppressive agents. All AS patients in the initial sample set were recruited from the UTHSC-H site. In the second confirmatory cohort, 2 samples

were investigated from each patient: the first before initiation of an anti-TNF treatment; the second was obtained after the patient was treated with an anti-TNF agent for ≥ 6 months. AS patients in the confirmatory cohort were recruited from all 3 participating sites. All AS patients enrolled in the discovery group had active disease, defined as Bath AS Disease Activity Index (BASDAI) score ≥ 3.5. Similarly, all AS patients in the confirmatory cohort had a BASDAI ≥ 3.5 before the initiation of anti-TNF therapy. In addition to demographic information and BASDAI, we measured C-reactive protein (CRP) at the time of blood draw in AS patients.

The healthy controls had no history of autoimmune diseases or spondyloarthritis-related manifestations and were matched for sex, age, and ethnicity to AS patients. Patients with SLE and SSc were also investigated as disease controls in order to identify gene expression patterns that are specific to AS and are not related only to presence of systemic inflammation. Patients with SSc or SLE were recruited from the continuing longitudinal studies or clinical practice of the investigators at UTHSC-H. All SSc patients met the 1980 American College of Rheumatology (ACR) preliminary criteria for the classification of SSc[11]. Similarly, all SLE patients fulfilled the ACR classification criteria for SLE[12] and had signs of active disease in at least 2 categories of the Systemic Lupus Activity Measure-Revised[13]. Patients with SSc or SLE receiving immunosuppressive agents other than low-dose steroids (prednisone ≤ 5 mg) and hydroxychloroquine were excluded from the study. The comparison group for the patients with SSc or SLE were healthy controls matched for sex, age, and ethnicity to patients with SSc.

All study subjects provided written informed consent and the study was approved by the institutional review boards of all participating centers.

Sample processing and microarray experiments

Whole-blood samples for gene expression studies were drawn directly into PAXgene™ tubes (PreAnalytix, Hombrechtikon, Switzerland). All blood samples were processed in the laboratories of the Division of Rheumatology and Clinical Immunogenetics, UTHCS-H. Total RNA was isolated and purified according to the manufacturer's protocol using PAXgene RNA Kit. The RNA quality and yield was assessed by NanoDrop ND-10000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). A globin reduction was not done because this procedure did not increase percentage of present calls in a preliminary experiment with 9 healthy control samples. This finding might be explained by the longer transcript probes printed on the Illumina arrays (50 mer probes) in comparison to Affymetrix arrays.

Two hundred nanograms of total RNA were amplified and purified utilizing Illumina TotalPrep RNA Amplification Kit (Applied Biosystems/Ambion, Austin, TX, USA) according to the manufacturer's instructions. We hybridized the amplified cRNA on Illumina Human Ref-8v2 arrays and extracted the data utilizing the Illumina Beadstudio software (Illumina, San Diego, CA, USA).

Microarray data analysis

The raw data were exported into BRB-ArrayTools v. 3.7 (R. Simon and A. Pen Lam, National Cancer Institute, Bethesda, MD, USA).

Probes whose signal detection p values indicated no significant difference from those of the negative controls (p < 0.01) were removed from the analysis. In addition, we excluded genes whose expression values were missing or were filtered out in more than 50% of experiments. Expression data were normalized using the median over the entire array. We

used less stringent criteria for detection of differentially expressed genes in order to increase detection of any transcripts with altered gene expression. A gene was defined as differentially expressed for all comparisons when the significance level for comparison was $p < 0.01$, utilizing a random variance t test[14]. The set of differentially expressed genes was modeled in Ingenuity Pathway Analysis (Ingenuity Systems, Redwood City, CA, USA) to detect pathways or biological processes involving these genes.

Real-time quantitative PCR

Quatitative PCR (qPCR) assays for *TLR4* and *TLR5* were designed to confirm the microarray results. The assay details including the primer sequence, lowest limit of detection, and PCR efficiency are shown in Table 1. Each sample was assayed in triplicate plus a control without reverse transcriptase to access DNA contamination. Samples were reverse transcribed into cDNA using superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) for 30 min at 50°C in 384-well plates. PCR master mix containing JumpStart Taq Polymerase (Sigma, St. Louis, MO, USA) was added to the samples. Each assembled plate was run in a 7900 real-time instrument using the following PCR conditions: 95°C for 1 min, followed by 40 cycles of 95°C for 12 s and 60°C for 30 s. Results were analyzed utilizing SDS 2.3 (7900) software (Applied Biosystems, Foster City, CA, USA) with FAM reporter and ROX as the reference dye. The final data were normalized to 18s rRNA levels. The final data were presented as the molecules of the transcript divided by the molecules of 18sRNA transcript $\times 100$.

Statistical analysis

Continuous variables were analyzed by t test if the dependent variable had a normal distribution. The Mann-Whitney nonparametric test was used if the outcome variable did not have a normal distribution. We compared the gene expression values, CRP, and BASDAI scores between pre- and post-anti-TNF treatment samples utilizing a paired T test if model assumptions were met, otherwise Wilcoxon signed-rank test was applied. Linear regression was used to investigate the relationship among 2 continuous variables. Two-sided p values < 0.05 were considered significant. Analyses were performed utilizing the NCSS 2007 statistical program (NCSS, Kaysville, UT, USA).

RESULTS

Characteristics of study groups

A total of 16 patients with AS and 14 healthy controls were examined in the first group. There were no significant differences in age, sex, and ethnicity between patients and controls. Table 2 shows the demographic characteristics, presence of spondyloarthritis (SpA) related manifestations, and other clinical features in participants. No control subjects had a SpA-related manifestation. The majority of AS patients (75%) were male. Among AS patients, the mean BASDAI score was 5.31 (± 2.01 SD) and the median CRP was 0.59 mg/dl. All AS patients in this group were HLA-B27-positive. We also investigated the gene expression profile of 74 patients with SSc (female 79.7%, mean age 49.16 yrs), 21 matched controls (female 80.95%, mean age 53.53 yrs), and 17 patients with SLE (female 94.12%, mean age 38.5 yrs).

Gene expression microarrays show upregulation of nuclear factor-κB and TLR pathways in AS

A total of 8230 transcripts passed our filtering criteria across all the peripheral blood samples. Clustering analyses according to date of hybridization or chip number indicated no technical artifact.

A comparison of 16 AS patients with their matched controls revealed 83 differentially expressed transcripts. An unsupervised hierarchical clustering of these genes in AS patients and their controls is shown in Figure 1. A list of these genes is provided in Appendix 1. In a similar analysis, comparison of SLE and SSc samples to their matched controls resulted in 936 and 530 differentially expressed transcripts, respectively. The most prominent gene expression pattern among SLE and SSc patients consisted of upregulation of genes belonging IFN-related pathways[15], whereas we did not observe an overrepresentation of up- or down-regulated genes belonging to IFN pathways in comparisons of AS patients to controls in the Ingenuity Pathway Analysis.

Then we filtered the differentially expressed genes in AS through the lists of differentially expressed transcripts in SLE and SSc, which resulted in 52 transcripts corresponding to 51 genes that were uniquely differentially expressed among AS patients (Appendix 2). Ingenuity Pathway Analysis of these 52 transcripts demonstrated overrepresentation of transcripts belonging to pathways involved in TLR signaling. *TLR4* (p = 0.008) and *TLR5* (p = 0.006) were overexpressed in AS patients but not in patients with SLE or SSc. No other subtype of *TLR* was differentially expressed in AS patients.

The significantly dysregulated pathways were nuclear factor-κB signaling, dendritic cell maturation, TLR, TREM1 signaling, and BRCA1 in DNA damage (Table 3). It is notable that TLR play a major role in the activation of the first 4 pathways. Specifically, *TLR4* and *TLR5* were the only dysregulated genes that were present in all these 4 pathways.

We next assessed *TLR4* and *TLR5* levels in patients with AS, their matched controls, and patients with SLE using qPCR. In agreement with the microarray results, AS patients had higher *TLR4* (p = 0.012) and *TLR5* (p = 0.006) levels than controls. Similarly, in comparison to SLE, AS patients showed overexpression of *TLR4* (p = 0.002) and *TLR5* (p = 0.008), whereas *TLR4* (p = 0.203) and *TLR5* (p = 0.383) levels did not differ significantly between SLE patients and controls. Figure 2 shows the *TLR4* and *TLR5* levels among these 3 study groups. Further, *TLR4* and *TLR5* levels correlated highly with each other (p < 0.001, r^2 = 0.62).

Overexpression of TLR4 and TLR5 was confirmed in a separate group of AS patients

We next examined the *TLR4* and *TLR5* levels in a second larger sample of 27 patients with AS and 27 matched healthy controls. A total of 22 patients with AS (81.5%) were HLA-B27-positive; characteristics of patients and controls in the confirmation group are shown in Table 2. One sample before and another one after anti-TNF treatment were investigated on each AS patient. The CRP and BASDAI score of AS patients before anti-TNF treatment were 0.791 mg/dl (median) and 5.93 ± 1.35 (mean ± SD), respectively. As expected, both CRP (p = 0.005) and BASDAI score (p < 0.001) decreased significantly upon anti-TNF treatment to 0.095 mg/dl (median) and 3.49 ± 2.33 (mean ± SD), respectively.

Compared to their matched controls, we again observed higher *TLR4* (p = 0.007) and *TLR5* (p = 0.012) levels in AS patients before initiation of the anti-TNF treatment. However, *TLR4* and *TLR5* levels in AS samples after anti-TNF treatment did not differ significantly from controls (p = 0.126, p = 0.173, respectively). Figure 3 shows *TLR4* and *TLR5* levels in the AS samples before and after initiation of anti-TNF treatment and in controls. Similarly to the initial sample, *TLR4* and *TLR5* levels also correlated significantly with each other (p < 0.001, r^2 = 0.44).

TLR4 and TLR5 levels decreased significantly after TNF blockade

TLR4 levels decreased significantly after initiation of anti-TNF treatment from 0.072 to 0.046 (p = 0.002) in patients with AS. Similarly, the *TLR5* levels also declined significantly

on treatment with anti-TNF from 0.005 to 0.0038 (p = 0.025; Figure 3). Further, the percentage changes in *TLR4* and *TLR5* after TNF blockade correlated highly with each other (p < 0.001, r^2 = 0.75).

TLR4 and TLR5 levels in relationship with clinical features

BASDAI scores did not correlate with *TLR4* and *TLR5* levels in either study cohort (data not shown). Although CRP levels correlated with *TLR4* and *TLR5* levels in the initial sample (p = 0.015, r^2 = 0.4 and p = 0.001, r^2 = 0.6, respectively), we could not confirm this finding among pre-TNF samples in the second study group (p = 0.833 for *TLR4*, p = 0.753 for *TLR5*). Among the patients with AS, history of uveitis, psoriasis, peripheral inflammatory arthritis, or Crohn's disease did not correlate with *TLR4* and *TLR5* levels in the 2 study cohorts (data not shown). Further, there was no significant difference in *TLR4* and *TLR5* levels between the HLA-B27-positive and HLA-B27-negative AS patients (p = 0.454, p = 0.319, respectively).

DISCUSSION

We observed that PBC global gene expression profiling of patients with AS showed a dysregulation of TLR-related pathways. We specifically identified an overexpression of *TLR4* and *TLR5*, which also was confirmed by qPCR in the initial and a separate confirmatory sample. Differential regulation of these genes appeared to be unique to AS and was not observed in SLE or SSc. Our study is the first report of increased expression of *TLR5* in AS.

An abnormal host response against pathogens has been implicated in the pathogenesis of AS and other SpA subtypes. Further, 60% of patients with SpA without evidence of clinical Crohn's disease have endoscopic or histological signs of gut inflammation[16]. Moreover, studies with B27-transgenic rats provide support for the role of commensal gut flora in the pathogensis of HLA-B27-associated gut and joint manifestations. The B27-transgenic rats do not develop inflammatory intestinal or peripheral joint disease in a germ-free environment[17].

TLR are primarily involved in innate immune responses to microbial pathogens by recognition of conserved pathogen-associated molecular patterns[18,19]. More than 10 TLR subtypes have been identified. In our study, *TLR4* and *TLR5* were the only TLR subtypes that were overexpressed among patients with AS. Lipopolysaccharide (LPS) in the outer membrane of Gram-negative bacteria is the main ligand of *TLR4*[19]. In animal models, *TLR4* plays a critical role in the early cytokine response of phagocytes upon infection with reactive arthritis-associated Gram-negative bacteria such as Yersinia, Salmonella, and Chlamydia[20,21,22]. An upregulation of *TLR4* among patients with AS has been previously reported. De Rycke, *et al* reported increased expression of TLR4 but not TLR2 on PBMC of patients with SpA in comparison to controls[23]. Yang, *et al* showed that the expression of *TLR4* on lymphocytes, monocytes, and neutrophils was all significantly increased among patients with AS[24].

The main ligand for TLR5 is flagellin, a primary component of bacterial flagella that extend from the outer membrane of Gram-negative bacteria. Flagella are known to be major antigens of Gram-negative bacteria like Salmonella, *Escherichia coli*, and Yersinia, where its antigenicity serves as the basis for H serotyping[25,26,27]. There are no published reports on the role of *TLR5* in AS or other SpA subtypes. However, both *TLR5* and flagellin have been implicated in the pathogenesis of Crohn's disease (reviewed by Gewirtz[28]). A study investigating the proteins of commensal microflora that were reactive with antisera from a colitic mouse model identified flagellins as the dominant antigen. Further, serum IgG to

these flagellins was elevated in patients with Crohn's disease but not in patients with ulcerative colitis or controls[29]. In another study, flagellin exposure to injured mouse colon *in vivo*, but not intact colon, significantly worsened colonic inflammation, whereas *TLR2*-specific agonists did not have a similar effect[30]. A *TLR5* stop-polymorphism was negatively associated with Crohn's disease in Ashkenazi Jewish patients, raising the possibility that a downregulation in *TLR5* provides protection against development of Crohn's disease[31].

AS and Crohn's disease share common clinical features and genetic background. Up to one-third of patients with Crohn's disease have sacroiliac joint involvement similar to AS on computer tomography[32], whereas roughly 60% of patients with SpA have subclinical colitis by biopsy[16]. These similarities, along with our data, suggest that the role of TLR5 in the pathogenesis of AS should be further explored in mechanistic studies. We did not observe any significant difference in *TLR5* levels between the AS patients with and those without Crohn's disease in our study (p = 0.769). This finding suggests that the overexpression of *TLR5* in AS is independent of the presence of clinically apparent Crohn's disease. However, our study might have been underpowered to detect a relationship between *TLR5* expression and presence of Crohn's disease in patients with AS.

Activation of the innate immune system such as polymorphonuclear cells and macrophages plays an important role in AS-related inflammation. Patients with SpA have significantly higher neutrophil counts and lower lymphoid aggregates than patients with rheumatoid arthritis (RA) on synovial histology[33]. Further, macrophages expressing the hemoglobin scavenger receptor CD163 are increased in the synovial lining of patients with SpA compared to RA patients. CD163+ macrophages also are increased in the colonic lamina proporia in SpA patients compared to controls, supporting the hypothesis of a recirculation of similar clones in the intestinal mucosa and synovium[34]. Of interest, both TLR4 and TLR5 can induce an acute shedding of CD163 from human monocytes. As well, these 2 TLR subtypes have a synergistic effect on upregulation of CD163, whereas exogenous recombinant IFN-γ leads to downregulation of CD163[35]. Global gene expression studies of macrophages derived from AS patients reveal a "reverse" IFN signature, with IFN-γ upregulated genes being down-regulated[7]. The observed upregulation of *TLR4* and *TLR5* in our study, along with the reported "reverse" IFN-γ in AS, are both potential mechanisms that could lead to overexpression CD163+ macrophages in this disease. However, this hypothesis needs to be verified by further mechanistic studies. We did not observe a "reverse" IFN signature in whole-blood samples as described by Smith, *et al* in macrophages of patients with AS[7].

We observed downregulation of *TLR4* and *TLR5* after initiation of anti-TNF treatment in patients with AS. Both *TLR4* and *TLR5* can induce secretion of TNF-α and other proinflammatory cytokines[30,36]. The expression of *TLR4* on PBMC decreases gradually after treatment of SpA patients with anti-TNF agents *in vivo*. These PBMC have a functional impairment in their capacity to produce TNF-α after stimulation with LPS *in vitro*[23]. Further, *TLR4* mRNA correlated closely with serum TNF-α levels among patients with AS[24]. These findings suggest that TNF blockade attenuates a self-perpetuating activation of the innate immune system via the TLR pathway.

The *TLR4* and *TLR5* levels and their percentage changes after TNF blockade correlated closely with each other, which raises the possibility that they are both triggered by the same mechanism, whereas we did not observe such a strong correlation of these 2 transcripts with general markers of inflammation such as CRP.

The data on correlation of *TLR4* and *TLR5* levels with CRP in our study were not consistent. While we observed a significant correlation in the first sample, we could not confirm this

finding in the second cohort. These results did not change even after we excluded the HLA-B27-negative patients from the analysis (data not shown). Similarly, De Rycke, *et al*[23] did not observe a correlation of *TLR4* expression with CRP in SpA, whereas Yang, *et al*[24] reported correlation of *TLR4* mRNA levels with CRP in HLA-B27-positive patients with AS. Studies with larger sample sizes are needed to resolve this issue.

Potential limitations of our study are that we used less stringent criteria for identification of differentially expressed genes in microarray data analysis in order to increase our ability to detect dysregulated genes and pathways. However, we verified the overexpression of *TLR4* and *TLR5* with qPCR in the same sample in addition to a separate confirmatory cohort. We compared the global gene expression of AS patients to transcriptosomes of patients with SLE and SSc. These comparative studies should be extended to patients with other rheumatological diseases such as RA in future investigations. Further, the observed fold-changes in *TLR4* and *TLR5* between AS patients and controls were relatively small (< 2-fold). However, whole-blood samples consist of heterogenous populations of white blood cell subtypes. It is possible the observed differential expression levels in whole blood are secondary to much higher-fold changes in a particular subset of white blood cells. We focused on dysregulation of TLR transcripts. However, the other observed differentially expressed transcripts and pathways in this study also could play an important role in the pathogenesis of AS. For example, the observed dysregulations in the dendritic cell maturation pathway may provide further support for the importance of defective functional capacity of dendritic cells in SpA[37].

In summary, our global gene expression analysis revealed the TLR-related pathways as the most prominently dysregulated biological process in AS. We identified *TLR4* and *TLR5* as the only dysregulated subtypes of TLR in AS. We confirmed the overexpression of these 2 genes among patients with AS in the same sample and in a confirmatory cohort. We showed that the expression of both receptors decreased after initiation of anti-TNF treatment. Our findings provide further support for the importance of TLR subtypes responsive to Gram-negative bacteria in the pathogenesis of AS. Mechanistic studies are needed to elucidate the role of these TLR subtypes in the development of AS.

Acknowledgments

Appendix 1

Differentially expressed genes in AS compared to controls

Parametric p value	Geom mean of intensities in class 1 (AS)	Geom mean of intensities in class 2 (control)	Fold-change	GB acc	Gene symbol	Defined Gene list
0.000478	137.0849484	191.4323799	0.7161012	NM 002340	LSS	Biosynthesis of steroids

Parametric p value	Geom mean of intensities in class 1 (AS)	Geom mean of intensities in class 2 (control)	Fold-change	GB acc	Gene symbol	Defined Gene list
0.0006957	9364.319315	4899.472307	1.9112914	NM 000570	FCGR3B	Natural killer cell mediated cytotoxicity, immunology
0.0009312	70.135071	39.2769027	1.7856569	NM 198330	INPP5F	
0.0010988	71.4184127	45,7446495	1.5612408	NM 001255	CDC20	Cell cycle, Ubiquitin mediated proteolysis
0.0011141	52.8656567	70.9770793	0.7448272	NM 001013840	C10orf61	
0.0011586	392.3825626	275.9854116	1.4217511	NM 182755	ZNF438	
0.0017355	61.4036443	44.5498863	1.378312	NM 001125	ADPRH	
0.0018922	87.5243114	47.5085126	1.8422869	NM 170606	MLL3	Lysine degradation
0.002143	53.3984454	71.3663158	0.7482304	NM 014062	NOB1P	
0.0021903	233.0709896	183.0490111	1.273271	NM 001031677	RAB24	
0.002331	78.1171224	97.3888835	0.8021154	NM 017699	SIDT1	
0.002457	55.4244192	75.0410081	0.7385884	NM 020642	C11orf17	
0.0025345	956.4176519	673.3678999	1.4203493	NM 033405	PRIC285	
0.002748	111.8853999	65.758725	1.7014533	NM 015071	ARHGAP26	
0.002797	77.7048739	43.8710092	1.7712124	NM 018984	SSH1	Regulation of actin cytoskeleton
0.0031593	96.3060504	70.9280275	1.3577996	NM 001031719	FU13639	
0.0032597	74.3860187	107.9506798	0.6890741	NM 006134	TMEM50B	
0.0034606	144.2016183	198.6110208	0.7260504	NM 144638	TMEM42	Role of Ran in mitotic spindle regulation
0.0035429	121.5756396	65.759618	1.8487887	NM 052828	TRIM10	
0.0035783	76.7861621	100.233922	0.7660696	NM 181340	WDR21A	
0.0037429	1428.384827	1074.9697	1.3287675	NM 006573	TNFSF13B	TACI and BCMA stimulation of B cell immune responses., Cytokine-cytokine receptor interaction
0.0038303	2308.950828	1401.307414	1.6477118	NM 003494	DYSF	
0.003983	287.009692	225.0477753	1.2753278	NM 001776	ENTPD1	
0.004009	136.1716197	185.1636214	0.7354124	NM 006985	NPIP	
0.0040379	89.442346	60.228624	1.4850471	NM 002463	MX2	cell_signaling
0.0041087	636.3160782	820.7957534	0.7752429	NM 024619	FN3KRP	
0.0041129	54.5849904	70.7770909	0.771224	NM 024493	ZNF306	
0.0042	129.564397	206.5903427	0.6271561	NM 024048	MGC3020	
0.0043066	77.2854192	113.9056458	0.6785039	NM 004284	CHD1L	
0.0044582	313.8851602	213.5519415	1.4698305	NM 000167	GK	Glycerolipid metabolism, PPAR signaling pathway
0.004488	106.5122966	151.1224528	0.7048079	NM 018346	RSAD1	
0.0045615	64.1665543	96.0495151	0.668057	NM 138341	TMEM116	
0.0051912	433.4919731	308.0187609	1.4073557	NM 002093	GSK3B	

Parametric p value	Geom mean of intensities in class 1 (AS)	Geom mean of intensities in class 2 (control)	Fold-change	GB acc	Gene symbol	Defined Gene list
0.0053976	83.1198159	48.5045894	1,7136485	NM 203411	TMEM88	
0.0054644	249.0470921	304.1155458	0.8189226	NM 020444	KIAA1191	
0.0057117	1224.60185	822.4718523	1.4889286	NM 022059	CXCL16	
0.0057841	110.4525818	142.0882007	0.7773522	NM 080605	B3GALT6	Chondroitin sulfate biosynthesis, Glycan structures - biosynthesis 1
0.0059993	6755.193466	4676.588102	1.4444705	NM 006931	SLC2A3	Vitamin C in the Brain, cell_signaling, misc, pharmacology
0.0060617	57.8154193	37.7067951	1.5332891	NM 020439	CAMK1G	
0.0062033	107.4822883	141.3133872	0.7605952	NM 024691	ZNF419	
0.0062233	393.164908	285.6590853	1.3763431	NM 003268	TLR5	Toll-like receptor signaling pathway, immunology
0.006241	706.5141744	366.4292943	1.9281051	NM 000717	CA4	Nitrogen metabolism
0.0062475	4308.608064	2917.50243	1.4768139	NM 021642	FCGR2A	immunology
0.0063598	1500.259142	1159.001672	1.2944409	NM 004031	IRF7	gene_regulation, transcription
0.0064295	4468.985126	3070.56217	1.455429	NM 012387	PADI4	
0.0065349	162.9657928	206.0056073	0.7910745	NM 012139	SERGEF	
0.0065475	3763.198465	2463.563116	1.5275429	NM 022083	C1orf24	
0.0065818	169.8341703	202.8024016	0.8374367	NM 002857	PEX19	
0.0068421	121.5985049	67.4619564	1.8024752	NM 130385	MRVI1	
0.0069067	4094.439362	3127.288913	1.3092616	NM 139266	STAT1	Apoptotic Signaling in Response to DNA Damage, EGF Signaling Pathway, IFN alpha signaling pathway, IFN gamma signaling pathway, IL22 Soluble Receptor Signaling Pathway, Inhibition of Cellular Proliferation by Gleevec, MAPKinase Signaling Pathway, p38 MAPK Signaling Pathway, PDGF Signaling Pathway, TPO Signaling Pathway, Jak-STAT signaling pathway, Toll-like receptor signaling pathway, angiogenesis, immunology, signal_transduction
0.0069214	79.185264	50.4842138	1.5685153	NM 004347	CASP5	MAPK signaling pathway, apoptosis, immunology
0.0069767	2792.841513	2234.522646	1.2498605	NM 033306	CASP4	Caspase Cascade in Apoptosis, MAPK signaling pathway, apoptosis, immunology
0.0069791	57.8324274	41.4198501	1.3962491	NM 024496	C14orf4	

Parametric p value	Geom mean of intensities in class 1 (AS)	Geom mean of intensities in class 2 (control)	Fold-change	GB acc	Gene symbol	Defined Gene list
0.0070871	458.6254977	334.7400414	1.3700945	NM 032536	NTNG2	
0.0072365	309.1783279	239.2651331	1.2921997	NM 080424	SP110	
0.0075236	2360.736625	1641.346746	1.4382924	NM 001995	ACSL1	Perou's- Intrinsic- Breast-Cancer-Genes, Adipocytokine signaling pathway, Fatty acid metabolism, PPAR signaling pathway
0.0075429	101.3066589	143.8378404	0.7043116	NM 016311	ATPIF1	
0.0075842	55.9221565	71.0356931	0.7872402	NM 002913	RFC1	
0 007629	284.5814239	197.0116273	1.4444905	NM 138557	TLR4	immunology
0.0078644	8192.608896	5797.077578	1.4132309	NM 020980	AQP9	
0.0079295	352.6683885	228.2252792	1.5452644	NM 002243	KCNJ15	
0.0079915	69.4960341	55.0535557	1.2623351	NM 171846	LACTB	
0.0080308	4230.418121	2883.942998	1.4668869	NM 022083	C1orf24	
0.0080335	61.6309441	80.2352561	0,768128	NM 001032999	CBFA2T2	cell_cycle, immunology
0.0080701	78.1569398	53.726485	1.454719	NM 001924	GADD45A	ATM Signaling Pathway, Cell Cycle: G2/M Checkpoint, Hypoxia and p53 in the Cardiovascular system, p53 Signaling Pathway, Cell cycle, MAPK signaling pathway
0.0083262	1358.69924	985.1936551	1.379119	NM 030769	NPL	
0.0084262	961.6377224	682.6056177	1.408775	NM 006825	CKAP4	
0.0084503	87.9484113	65.2853476	1.3471386	NM 138962	MSI2	
0.0085554	597.6472872	398.0354876	1.5014925	NM 005306	FFAR2	
0.0087672	95.5304166	51.085383	1.8700147	NM 000062	SERPING1	Intrinsic Prothrombin Activation Pathway, Complement and coagulation cascades
0.0090354	158.4264733	192.559439	0.8230003	NM 004793	PRSS15	
0.0091097	198.4911419	136.4177609	1.4550242	NM 144653	BTBD14A	
0.009238	83.129073	110.3058286	0.7536236	NM 017607	PPP1R12C	
0.0092556	126.8566834	181.5263622	0.6988334	NM 139281	WDR36	
0.0092918	70.2149582	85.965983	0.8167761	NM 014149	HSPC049	
0.0093926	117.3907998	154.3682162	0.7604597	NM 017541	CRYGS	
0.0095282	154.6194436	194.177994	0.7962769	NM 024056	TMEM106C	
0.0095422	122.7966001	150.2197121	0.8174466	NM 001002880	PGEA1	
0.0095887	892.9376363	1196.945428	0.7460137	NM 019063	EML4	
0.0096633	89.6226922	56.5656362	1.5844017	NM 017409	HOXC10	development
0.0096843	85.2460615	116.6847675	0.7305672	NM 024070	MGC2463	
0.0097158	114.2832903	153.2233268	0.7458609	NM 206930	SYTL2	

Parametric p value	Geom mean of intensities in class 1 (AS)	Geom mean of intensities in class 2 (control)	Fold-change	GB acc	Gene symbol	Defined Gene list
0.0097279	59.7622428	38.6757469	1.5452124	NM 000208	INSR	Control of skeletal myogenesis by HDAC & calcium/calmodulin-dependent kinase (CaMK), Growth Hormone Signaling Pathway, Insulin Signaling Pathway, Adherens junction, Dentatorubropallidoluysian atrophy (DRPLA), Insulin signaling pathway. Type II diabetes mellitus, immunology

Appendix 2

Genes that were only differentially expressed in AS

Parametric p value	Geom mean of intensities in class 1 (AS)	Geom mean of intensities in class 2 (AS)	Fold-change	GB acc	Gene symbol	Defined Gene list	Gene involved in dysregulated pathyways in Tab
0.0006957	9364.31931	4899.47231	1.9112914	NM 000570	FCGR3B	Natural killer cell mediated cytotoxicity, immunology	Dendritic Cell Ma
0.0009312	70.135071	39.2769027	1.7856569	NM 198330	INPP5F		
0.0011141	52.8656567	70.9770793	0.7448272	NM 001013840	C10orf61		
0.0017355	61.4036443	44.5498863	1.378312	MM 001125	ADPRH		
0.0018922	87.5243114	47.5085126	1.8422869	NM 170606	MLL3	Lysine degradation	
0.002143	53.3984454	71.3663158	0.7482304	NM 014062	NOB1P		
0.0021903	233.07099	183.049011	1.273271	NM 001031677	RAB24		
0.002331	78.1171224	97.3888835	0.8021154	NM 017699	SIDT1		
0.002457	55.4244192	75.0410081	0.7385884	NM 020642	C11orf17		
0.002748	111.8854	65.758725	1.7014533	NM 015071	ARHGAP26		
0.002797	77.7048739	43.8710092	1.7712124	NM 018984	SSH1	Regulation of actin cytoskeleton	
0.0031593	96.3060504	70.9280275	1.3577996	NM 001031719	FLJ13639		
0.0035429	121.57564	65.759618	1.8487887	NM 052828	TRIM10		
0.0035783	76.7861621	100.233922	0.7660696	NM 181340	WDR21A		
0.0038303	2308.95083	1401.30741	1.6477118	NM 003494	DYSF		
0.0041087	636.316078	820.795753	0.7752429	NM 024619	FN3KRP		
0.0041129	54.5849904	70.7770909	0.771224	NM 024493	ZNF306		
0.0043066	77.2854192	113.905646	0.6785039	NM 004284	CHD1L		
0.0044582	313.88516	213.551942	1.4698305	NM 000167	GK	Glycerolipid metabolism, PPAR signaling pathway	

Parametric p value	Geom mean of intensities in class 1 (AS)	Geom mean of intensities in class 2 (AS)	Fold-change	GB acc	Gene symbol	Defined Gene list	Gene involved in dysregulated pathyways in Tab
0.004488	106.512297	151.122453	0.7048079	NM 018346	RSAD1		
0.0045615	64.1665543	96.0495151	0.668057	NM 138341	TMEM116		
0.0051912	433.491973	308.018761	1.4073557	NM 002093	GSK3B		NF-κB Signaling
0.0053976	83.1198159	48.5045894	1.7136485	NM 203411	TMEM88		
0.0057841	110.452582	142.088201	0.7773522	NM 080605	B3GALT6	Chondroitin sulfate biosynthesis, Glycan structures - biosynthesis 1	
0.0059993	6755.19347	4676.5881	1.4444705	NM 006931	SLC2A3	vitamin C in the Brain, cell_signaling, misc, pharmacology	
0.0062233	393.164908	285.659085	1.3763431	NM 003268	TLR5	Toll-like receptor signaling pathway, immunology	NF-κB Signaling, like Receptor Signaling TREM1 Signaling
0.006241	706.514174	366.429294	1.9281051	NM 000717	CA4	Nitrogen metabolism	
0.0062475	4308.60806	2917.50243	1.4768139	NM 021642	FCGR2A	immunology	Dendritic Cell Ma
0.0064295	4468.98513	3070.56217	1.455429	NM 012387	PADI4		
0.0065349	162.965793	206.005607	0.7910745	NM 012139	SERGEF		
0.0065349	162.965793	206.005607	0.7910745	NM 012139	SERGEF		
0.0065475	3763.19846	2463.56312	1.5275429	NM 022083	C1orf24		
0.0065818	169.83417	202.802402	0.8374367	NM 002857	PEX19		
0.0068421	121.598505	67.4619564	1.8024752	NM 130385	MRVI1		
0.0069791	57.8324274	41.4198501	1.3962491	NM 024496	C14orf4		
0.0075236	2360.73663	1641.34675	1.4382924	NM 001995	ACSL1	Perou's- Intrinsic- Breast- Cancer- Genes, Adipocytokine signaling pathway, Fatty acid metabolism, PPAR signaling pathway	
0.0075429	101.306659	143.83784	0.7043116	NM 016311	ATPIF1		
0.0075842	55.9221565	71.0356931	0.7872402	NM 002913	RFC1		BRCA1 in DNA D Response
0.007629	284.581424	197.011627	1.4444905	NM 138557	TLR4	immunology	NF-κB Signaling, Dendritic Cell Maturation, Toll-li Receptor Signaling, TREM Signaling
0.0078644	8192.6089	5797.07758	1.4132309	NM 020980	AQP9		
0.0079295	352.668389	228.225279	1.5452644	NM 002243	KCNJ15		
0.0079915	69.4960341	55.0535557	1.2623351	NM 171846	LACTB		
0.0080308	4230.41812	2883.943	1.4668869	NM 022083	C1orf24		
0.0080701	78.1569398	53.726485	1.454719	NM 001924	GADD45A	ATM Signaling Pathway, Cell Cycle:	BRCA1 in DNA D Response

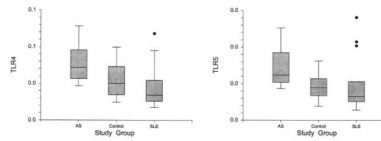| Parametric p value	Geom mean of intensities in class 1 (AS)	Geom mean of intensities in class 2 (AS)	Fold-change	GB acc	Gene symbol	Defined Gene list	Gene involved in dysregulated pathyways in Tab
						G2/M Checkpoint, Hypoxia and p53 in the Cardiovascular system, p53 Signaling Pathway, Cell cycle, MAPK signaling pathway	
0.0083262	1358.69924	985.193655	1.379119	NM 030769	NPL		
0.0090354	158.476473	192.559439	0.8230003	NM 004793	PRSS15		
0.0091097	198.491142	136.417761	1.4550242	NM 144653	BTBD14A		
0.009238	83.129073	110.305829	0.7536236	NM 017607	PPP1R12C		
0.0092556	126.856683	181.526362	0.6988334	NM 139281	WDR36		
0.0095282	154.619444	194.177994	0.7962769	NM 024056	TMEM106C		
0.0095887	892.937636	1196.94543	0.7460137	NM 019063	EML4		
0.0096633	89.6226922	56.5656362	1.5844017	NM 017409	HOXC10	development	
0.0097279	59.7622428	38.6757469	1.5452124	NM 000208	INSR	Control of skeletal myogenesis by HDAC & calcium/calmodulin-dependent kinase (CaMK), Growth Hormone Signaling Pathway, Insulin Signaling Pathway, Adherens junction, Dentatorubropallidoluysian atrophy (DRPLA), Insulin signaling pathway, Type II diabetes mellitus, immunology	NF-κB Signaling

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Figure 1.
Unsupervised hierarchical clustering of 83 genes identified as differentially expressed
between patients with AS and controls.

Figure 2.
Gene expression levels of *TLR4* and *TLR5* in AS, SLE, and controls by qPCR in the first study sample. Expression of both genes is higher in AS patients in comparison to controls and SLE patients, while there was no significant difference between SLE patients and controls.
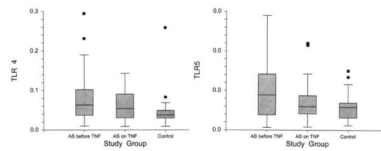
Figure 3.
Gene expression levels of *TLR4* and *TLR5* in patients with AS before and during anti-TNF treatment, as well as controls, in the second study sample. Gene expression levels of both genes decreased significantly after anti-TNF treatment among AS patients. Again, before anti-TNF treatment, samples showed higher *TLR4* and *TLR5* levels than controls. After anti-TNF treatment, there was no significant difference between AS patients and controls.

Table 1

Details of quantitative PCR

Gene	Accession No.	Primer and Probe	Amplicon Length	PCR Efficiency	Lower Limit of Detection (molecules)
Toll-like receptor 4	NM_138554	500 (+) GAG CCT TTT CTG GAC TAT CAA G *	81 bases	95%	180
		582 (−) TCC AAT GGG GAA GTT CTC TAG *			
		554 (−) FAM-AGA TTT GTC TCC ACA GCC ACC AGC-BHQ1 [†]			
Toll-like receptor 5	NM_003268	2209 (+) GCC ATC TGA CTG CAT TAA GG *	84 bases	95%	170
		2284 (−) GCA GGT AAA TCA TTG TGA GAA AG *			
		2237 (+) FAM-CCT CAA CTC CAA CAG GCT GAC AGT-BHQ1 [†]			

*
Primer sequence.

[†] Probe sequence.

Table 2

Demographic features of patients with AS and their matched controls in both samples

Feature	Discovery Group		Confirmation Group	
	AS, n = 16	Control, n = 14	AS, n = 27	Control, n = 27
Age, mean (SD) yrs	36.01 (11.94)	32.11 (7.34)	36.08 (11.4)	37.21 (10.68)
No. male (%)	12 (75)	10 (71.43)	14 (51.85)	14 (51.85)
No. Caucasian (%)	13 (81.25)	11 (78.57)	17 (62.96)	17 (62.96)
Disease duration	16.09 (12.87)	NA	11.55 (5.86)	NA
BASDAI*	5.31 (2.01)	NA	5.93 (1.35)	NA
C-reactive protein	1.4 (2.23)	NA	1.56 (2.17)	NA
Uveitis	3 (18.75)	0	14 (51.85)	0
Psoriasis	1 (6.25)	0	3 (11.11)	0
Peripheral inflammatory arthritis	10 (62.5)	0	7 (25.93)	0
Crohn's disease	0	0	3 (11.11)	0

BASDAI: Bath Ankylosing Spondylitis Disease Activity Index; NA: not applicable.

Table 3

Dysregulated pathways in comparison of patients with AS and unaffected controls according to Ingenuity Pathway Analysis

Canonical Pathways	p
Nuclear factor-κB signaling	0.0006
Dendritic cell maturation	0.008
Toll-like receptor signaling	0.008
TREM1 signaling	0.01
BRCA1 in DNA damage response	0.009