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Effects of β-D-Mannuronic Acid on TLR2 and TLR4 Expression and Associated Downstream Signaling in Monocyte-Derived Macrophages in Ankylosing Spondylitis Patients

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ABSTRACT

Objective: The β-D-mannuronic acid (M2000) is a new non-steroidal anti-inflammatory drug (NSAID) with immunomodulatory effects. We have previously shown that the gene expression level of TLR/NF-kB signaling pathway is downregulated in PBMC (Peripheral blood mononuclear cells) of treated Ankylosing Spondylitis (AS) patients with M2000 in vivo. Here, we aimed to determine the effect of M2000 on TLR2 and TLR4 expression and their downstream signaling in monocyte derived macrophages in AS patients in vitro.

Methods: The blood samples were used for isolating PBMCs and by using Magnetic Activated Cell Sorted (MACS) method, monocytes were isolated and differentiated to macrophages for evaluating protein expression of TLR2 and TLR4 by flow cytometry and gene expression of Myd88, MAPK14, NF-kB (p65 subunit) and IkB-α by Real time PCR. Cell culture supernatants were collected and the concentrations of TNF-α and IL-6 cytokines were assessed by enzyme-linked immunosorbent assay (ELISA).

Results: The gene expression of NF-kB and MAPK14 were significantly increased in the monocyte derived macrophages in AS patients compared to healthy subjects ($p < 0.05$). M2000 alone or in combination with TLR2 and TLR4 agonists (LTA/ LPS) significantly suppress the TLR2 and TLR4 expression and its downstream signaling pathway in monocyte derived macrophages. Also, the production of TNF-α and IL-6 were decreased in M2000-treated monocyte derived macrophages.

Conclusion: Since, development of inflammation through triggering TLR2 and TLR4 receptors plays a crucial role in the pathogenesis of AS, therefore, M2000 could be recommended as a therapeutic option by modulating TLR2 and TLR4 expression in AS patients.

Keywords: β-D-mannuronic Acid; TLR2, TLR4; Ankylosing spondylitis; NF-kB; MAPK14; Myd88; IkB-α

Abbreviations: NSAID: New Non-Steroidal Anti-Inflammatory Drug; PBMC: Peripheral Blood Mononuclear Cells; AS: Ankylosing Spondylitis; MACS: Magnetic Activated Cell Sorted; ELISA: Enzyme-Linked Immunosorbent Assay; MHC: Histocompatibility Complex; KIR: Killer Cell Immunoglobulin-Like Receptor; ERAP: Endoplasmic Reticulum Aminopeptidase; APCs: Antigen-Presenting Cells; DCs: Dendritic Cells; LTA: Lipoteichoic Acid; LPS: Lipopolysaccharide; TRAF: TNFα Receptor-Associated Factor; Tollip: Toll Interacting Protein; IKK: IkB Kinase; MAPK: Mitogen-Activated Protein Kinase; EAE: Experimental Autoimmune Encephalomyelitis; AIA: Adjuvant-Induced Arthritis; BASDAI: Bath Ankylosing Spondylitis Disease Activity Index; VAS: Visual Analogue Scale; BASFI: Bath Ankylosing Spondylitis Functional Index, PBMCs: Peripheral Blood Mononuclear Cells; FITC: Fluorescein Isothiocyanate; PE: Phycoerythrin; GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase; SNPs: Single Nucleotide Polymorphisms

Introduction

Ankylosing spondylitis (AS) is a progressive, systemic, and inflammatory rheumatic disease of seronegative spondyloarthropathies (SpAs) which primarily involves sacroiliac joints and spine and can manifest as inflammatory back pain and progressive spinal ankylosis and stiffness [1,2]. All these pathological changes lead to a significant loss of work productivity and decreased quality of life [3]. The prevalence of AS generally ranged 0.1% to 1.2% in different populations [4]. AS predominantly affects men in their peak of productive years [20 to 40], with a male: female ratio of approximately 2-3:1. Around 90% of patients exhibit their first symptom before age 40 [2,5,6]. The etiology and pathogenesis of AS remain unknown, but the combination of genetic, environmental, and immunological factors is thought to be important in its pathogenesis [2]. There is a strong genetic association between the major histocompatibility complex (MHC) group of molecules, specifically HLA-B27, and AS [7-9]. The population prevalence of AS is generally associated with the frequency of HLA-B27 in different societies worldwide. In the US, HLA-B27 is present in 90% of patients with AS [10,11]. A previous study showed that the frequency of AS patients with HLA-B27 is 68.9% in Iran [12]. Other susceptibility genes include IL-23 receptor (IL23R), endoplasmic reticulum aminopeptidase 1 (ERAP1) and ERAP2, killer cell immunoglobulin-like receptor (KIR) complex, and STAT3 [13-16]. Also, epidemiological and clinical studies showed the fundamental role of environmental factors, probably microbial infections, in the pathogenesis of AS [17].

The initial response to infection involves the activation of the innate immune system. Cells involved in the recognition of microbial pathogen-associated molecular patterns by Toll-like receptors (TLRs) may contribute to initiating or exacerbating inflammation in AS [18]. TLRs belong to a family of type I transmembrane glyco proteins participating in the first line of defense against invading pathogens [19,20]. These receptors recognize pathogen-associated molecular patterns or endogenous "danger" molecules and play a significant role in the regulation of innate immune responses and inflammation [21,22]. They are expressed in numerous types of antigen-presenting cells (APCs), including dendritic cells (DCs), monocytes, macrophages, and B lymphocytes [23]. Currently, 11 members of the TLR family have been identified in humans [24]. Among the TLRs, TLR2 and TLR4 are particularly important receptors, activated by various bacterial cell wall components. TLR2 is a receptor that is activated by lipoteichoic acid (LTA) from Gram-positive bacteria, whereas TLR4 is activated by lipopolysaccharide (LPS) from Gram-negative bacteria [21]. Activation of TLRs by their ligands initiates intracellular signaling pathways [25]. Upon activation of TLR2 and TLR4, myeloid differential primary response protein (MyD88) activates a family of IL-1R associated kinases (IRAKs), IRAK-1 is subsequently released and leading to the phosphorylation and activation of TNFα receptor-associated factor 6 (TRAF6).

Toll interacting protein (Tollip) is an inhibitory adaptor protein that negatively regulates the TLR-mediated signaling pathway. This protein forms a complex with IRAK in resting cells and inhibits phosphorylation and activation of IRAK [26]. Activation of TRAF6 leads to the activation of two distinct pathways: IkB kinase (IKK) complex and the mitogen-activated protein kinase (MAPK) (ERK, JNK, p38) pathways. IkB kinase (IKK) complex catalyzes the phosphorylation of inhibitory kB (IkB) protein and results in the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) [27]. Subsequent activation of the transcription factor NF-kB and the MAPK cascade leads to the synthesis and secretion of pro-inflammatory cytokines such as TNF-α, IL-6, IL-23, and IL-1β, which can induce inflammatory responses [28]. Although TLR-mediated inflammation is a significant aspect of defense against pathogens, it

may also result in the development of several inflammatory diseases [20,29]. Increased TLR expression and increased responsiveness to TLR ligands have been observed in multiple autoinflammatory diseases [30,31]. Several studies have shown the increased expression of TLR2 and TLR4 in monocytes/macrophages derived from patients with chronic inflammatory disease [23,32]. All these findings show the importance of TLRs signaling pathway in the pathogenesis of autoinflammatory diseases.

Therefore, targeting TLRs could be an important therapeutic strategy for the treatment of chronic inflammatory diseases such as AS. The TLRs antagonists are small molecules that inhibit TLRs signaling by binding to the TLRs domains and subsequently modulate the inflammatory response in the autoinflammatory disease under *in vitro* conditions. The M2000 (β-D-mannuronic Acid) is a novel nonsteroidal anti-inflammatory drug (NSAID) with low molecular weight and high tolerability and efficacy with the patent number of DE-102016113018. It has shown the potent immunosuppressive and immunomodulatory effects in various experimental models such as experimental autoimmune encephalomyelitis (EAE), adjuvant-induced arthritis (AIA), nephrotic syndrome, and acute glomerulonephritis [33-36]. Recently we demonstrated the antagonistic effects of M2000 on TLR2 and TLR4 signaling in the human embryonic kidney (HEK) 293 cell line [37]. Also, our previous *in vivo* study showed that the expression level of genes associated with TLR/NF-kB Signaling Pathway is reduced in AS patients after treatment with M2000 [38]. Based on this evidence and the significant role of TLR2 and TLR4 in the pathogenesis of chronic inflammatory disease like AS, we studied the effects of the M2000 on the TLR2 and TLR4 expression, associated downstream signal transduction pathway and cytokine production of monocyte-derived macrophages in AS patients and healthy controls under *in vitro* condition.

Methods

Extraction of β -D-Mannuronic Acid

The β-D-mannuronic Acid (M2000) was extracted from Alginic acid sodium salt (Sigma-Aldrich, St. Louis, MO). The purity of the drug was determined using Fourier Transform Infrared (FT-IR) and Carbon-13 Nuclear Magnetic Resonance (C-NMR) spectroscopy [39].

Ethics Approval

All patients signed their written informed consent. The Ethics Committee of Tehran University of Medical Sciences granted ethics approval for this study.

Patients and Samples

Ten patients with AS (8 men and 2 women; mean ± SD age of 31.5 ± 5.9 years), who fulfilled the modified New York criteria 1984, defined as a Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) score ≥4 on a 0-10 cm Visual Analogue Scale (VAS) and Bath Ankylosing Spondylitis Functional Index (BASFI) score ≥ 4 were enrolled in this study. Key exclusion criteria included any history of fever and uncontrolled concomitant diseases, malignancies, and pregnancy. The Patients were selected from the outpatient clinic of Rheumatology Research Center (Shariati Hospital, Tehran, Iran), and Iran Rheumatology center. We also recruited 10 sex- and age-matched healthy subjects as controls.

Cell Separation and Culture

Peripheral blood mononuclear cells (PBMCs) were isolated from the venous whole blood samples (40 ml per donor) by standard Ficol-paque (Biosera, France) density-gradient centrifugation. CD14+ monocytes were isolated from the PBMCs by positive immunomagnetic selection using CD14 microbeads (MACS monocyte isolation kit, Miltenyi Biotec), according to the manufacturer's instructions. Monocytes were assessed by flow cytometry; 95.7% of the cells were CD14+ (Figure 1a). The monocytes (5×105 cells/ml) were then seeded on 24-well plates and cultured for 7 days with RPMI 1640 supplemented by 10% of heat-inactivated fetal bovine serum, 2 mM of L-glutamine, 1 mM sodium pyruvate, 100 U/mL of penicillin, and 100 μg/mL of streptomycin (Gibco, Life Technologies USA). For M1-macrophage differentiation, human granulocyte macrophage colony-stimulating factor (10 ng/ml; R&D Systems, UK) was added to the dishes [40]. After 3 days, the medium was changed, and growth factors were freshly added. On day 6 cells were detached by rinsing with phosphate-buffered saline (PBS; PAA Laboratories, Germany) and cells were assessed by flow cytometry; 92.3% of the cells were CD206+ (Figure 1b).

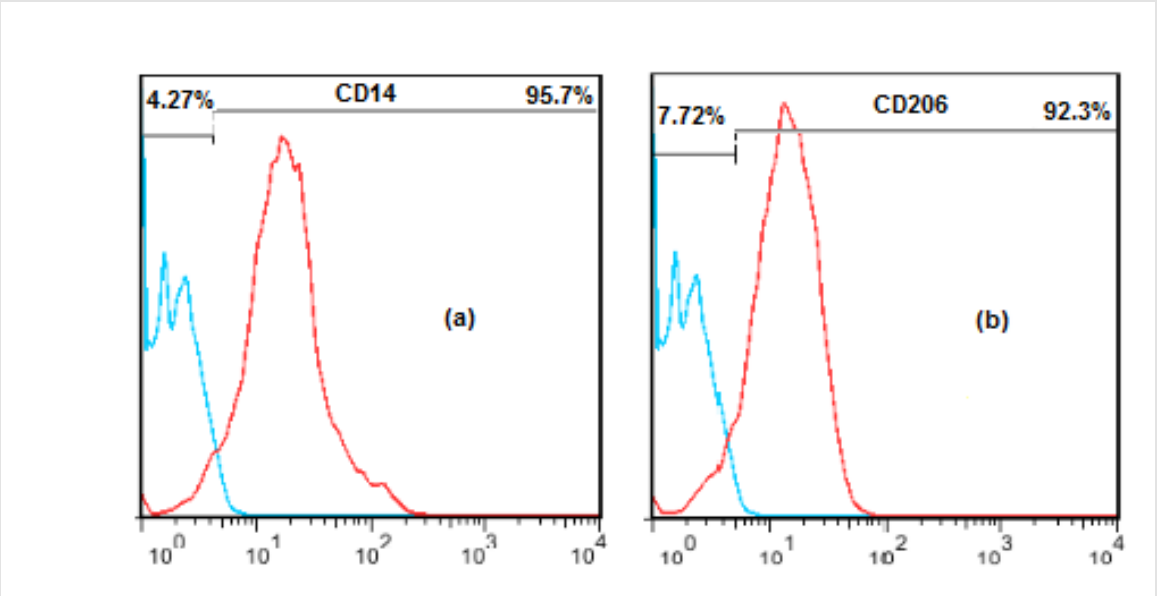

Figure 1: Representative flow cytometry histograms showing
(a) CD14 FITC+ Monocytes
(b) CD206 APC+ Monocyte derived macrophages. Red histograms show signals of specific markers; blue histogram shows signals of isotype controls.

Treatment of Cells

The monocyte-derived macrophages were pretreated with M2000 low dose (5 µg/well) and high dose (25 µg/well) for 4 hours and then incubated with and without 10 µg/ml LTA (Sigma-Aldrich, USA) as TLR2 agonist and 1 µg/ml LPS (Invivogen, USA) as a TLR4 agonist. Also, cells were stimulated with LPS and LTA without M2000 (5 and 25 µg/well) as a positive control [37,39]. Moreover, cells were treated with 30 µg/ml OxPAPC (Invivogen, USA) as TLR2 and TLR4 antagonist alone (negative control) and incubated for 24 h at 37ºC in the presence of 5% CO_2.

Flow Cytometry

For fluorescence-activated cell sorting (FACS), monocyte-derived macrophages were washed with staining buffer (PBS containing 1% BSA and 0.02% NaN3) and incubated with 5 µg fluorescein isothiocyanate (FITC) labeled anti-human CD282 (TLR2) and phycoerythrin (PE) anti-human CD284 (TLR4) monoclonal antibodies (Biolegend, USA) on ice for 15-20 minutes in the dark. The FITC Mouse IgG2a and PE Mouse IgG2a were used as isotype control antibodies. Fluorescence was measured using a BD flow cytometer (BD, USA), and data were analyzed using FlowJo software on marked cell populations on FSC-SSC dot plots.

RNA Extraction and RT-PCR

Total RNA was extracted from untreated and treated cells by a Total RNA purification kit (Hybrid RTM Gene All, Seoul, Korea) according to the manufacturer's protocol. Adequate RNA quality was determined by agarose gel electrophoresis on the GelRedTM (Biotin, USA). The purity and concentration of total RNA were assessed by UV spectrophotometer (NanoDrop ND1000) based on the A260/280 ratio, which was in the range of 1.7–2.0 for all samples. Complementary DNA (cDNA) was synthesized using oligo-dT and random 6-mer primers by using a cDNA reverse transcription kit (ABI Systems). Afterward, PCR analysis and gel agarose electrophoresis were performed to confirm the quality of synthesized cDNA and primers. The gene-specific primers were designed using the free Web-based software Primer-BLAST (National Center for Biotechnology Information). The primer sequences used in the current study are listed in (Table 1).

Table 1: Primer sequences used in Quantitative Real-time PCR.

N	Gene name	Primer sequence 5' → 3'	Product size	Accession number
1	GAPDH	5'-CCCACTCCTCCACCTTTGAC-3'	75	NM_001289746.1
		5'-CATACCAGGAAATGAGCTTGACAA-3'		
2	NF-kB (P65 subunit)	5'- GCTACACAGGACCAGGGACAGT-3'	118	NM_001145138.1
		5'- AGCTCAGCCTCATAGAAGCCATC-3'		
3	Myd88	5'-CGCCGCCTGTCTCTGTTC-3'	118	NM_001172569.1
		5'- GGTCCGCTTGTGTCTCCAGT-3'		
4	IkB-alpha	5'- CTCCACTCCATCCTGAAGGCTA-3'	167	NM_020529.2
		5'- AGGTCCACTGCGAGGTGAAG -3'		
5	MAPK14	5'- GAGGTGCCCGAGCGTTAC -3'	114	NM_139013.2
		5'- GGAGAGCTTCTTCACTGCCAC -3'		

Quantitative Real-time PCR

Real-time PCR was performed using SYBR Premix (ABI System) with a specific primer. All reactions carried out in a total volume of 20 µl included 1 µl cDNA, 10 µl SYBR Premix, 7 µl RNase Free-Water, 1 µl (100 nM) forward primer, 1 µl (100 nM) reverse primer. The PCR proceeded on the ABI StepOne Plus real-time PCR system (ABI System, USA) according to the following program: an initial step at the holding phase of 95ºC for the 30s followed by 40 cycles a cycling stage of 95ºC for 5s, 60ºC for 30s, and 60 ºC for 15s. The gene levels were normalized to the housekeeping gene Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. The relative changes in gene expression were calculated using the 2-ΔΔCt method (Ct refers to the threshold value) [41]. To reexamine the size of PCR products, amplified products were analyzed on 3% agarose gel electrophoresis.

Measurement of Cytokine Production

Cell culture supernatants were collected and the concentrations of TNF-α and IL-6 cytokines were assessed by enzyme-linked immunosorbent assay (ELISA kit eBioscience) according to the manufacturer's guidelines. The optical absorbance was read at 450 nm on a 96-well microplate ELISA reader and cytokine concentrations were determined from a curve of known concentrations of cytokine standard. The results were expressed in pg/ml.

Statistical Analysis

All statistical analyses were conducted using the SPSS 24 software. (Inc, Chicago, IL, USA). Differences between groups were then evaluated by one sample T-Test, independent samples T-Test, and one-way analysis of variance (ANOVA) using Tukey's test. The results were presented as mean ± SD and p-values less than 0.05 were considered to be significant.

Results

Gene Expression of Downstream Signaling Molecules of TLRs in AS Patients and Healthy Controls

The gene expression level of downstream signaling molecules of TLRs on unstimulated monocyte-derived macrophages in ankylosing spondylitis patients and healthy controls were determined by quantitative real-time PCR. Our results showed that the mRNA expression of NF-kB was significantly increased in patients with AS in comparison to healthy subjects ($p = 0.017$), and the expression level of IkB-α was significantly decreased in AS patients in comparison to healthy controls ($p = 0.014$) (Figure 2). To assess the effect of M2000 on the gene expression level of downstream signaling molecules of TLRs, monocyte-derived macrophages from AS patients were exposed to different concentrations of M2000, and then, we evaluated the mRNA expression of Myd88, MAPK14, NF-kB, IkB-α using quantitative real-time PCR.

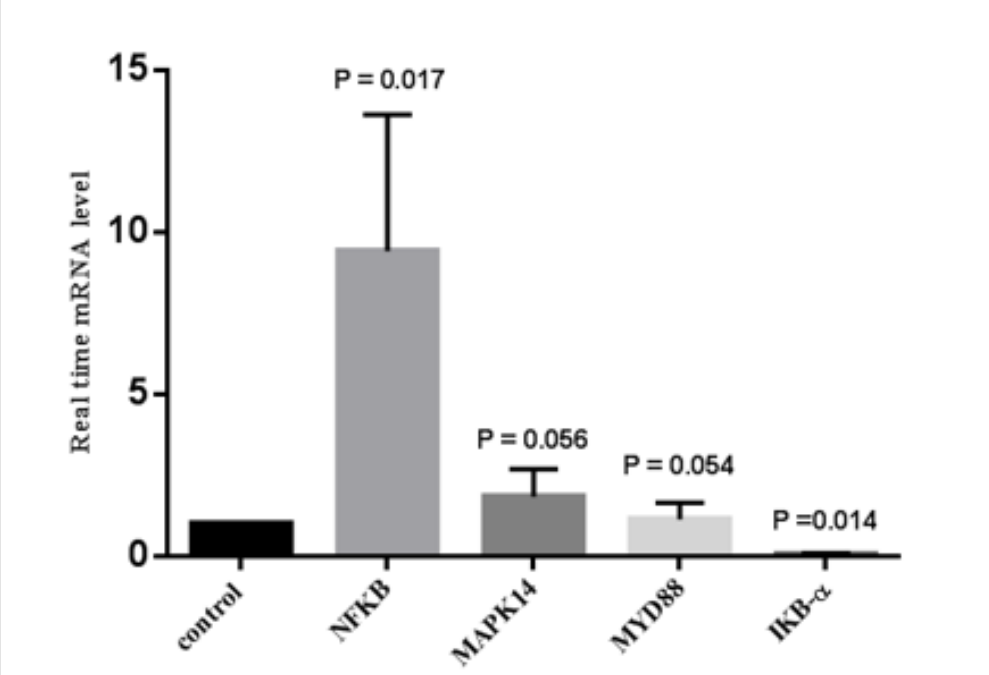

Figure 2: Relative gene expression of NF-κB, MAPK14, MyD88 and IkB-α in unstimulated monocyte-derived macrophages of 10 AS patients and 10 healthy controls. Comparison between fold changes on mRNA expression performed with the one-sample t-test. Data are presented as mean ± SD.

Effect of M2000 on NF-kB (p65 subunit)

The statistical analyses revealed significant differences between the treatment groups. Stimulation with 1 µg/ml LPS enhanced gene expression of NF-kB ($p < 0.05$) whereas, the expression level of NF-kB was significantly decreased when M2000 (25 µg/well) was added 4 h before LPS ($p = 0.03$). Similar to LPS, stimulation with 10 µg/ml LTA augmented mRNA expression of NF-kB ($p = 0.01$) and the mRNA level was reduced, when M2000 (25 µg/well) was added 4 h before LTA ($p = 0.05$) (Figure 3a).

Effect of M2000 on Myd88

The cells treated with 1 µg/ml LPS had a significantly higher level of MyD88 mRNA versus untreated cells ($p < 0.001$), while the mRNA expression was significantly reduced when M2000 (5 µg/well and 25 µg/well) was added 4 h before LPS ($p < 0.001$). Also, our results indicated that stimulation with 10 µg/ml LTA increased the mRNA expression of MyD88 and the mRNA level was reduced when M2000 (25 µg/well) was added 4 h before LTA; however, the difference was not statistically significant ($p > 0.05$) (Figure 3b).

Effect of M2000 on MAPK14

Stimulation of cells with a concentration of high (25 µg/well) dose of M2000 alone led to a decrease in gene expression of MAPK14 in comparison with the control group, but the difference was not statistically significant ($p > 0.05$). The data also revealed that stimulation with 1 µg/ml LPS enhanced the gene expression of MAPK14 ($p < 0.001$) whereas, the expression level of MAPK14 was significantly decreased when M2000 (5 µg/well and 25 µg/well) was added 4 h before LPS ($p< 0.01$). Similar to LPS, stimulation with 10 µg/ml LTA augmented mRNA expression of MAPK14 ($p = 0.07$) and the mRNA level was significantly reduced, when M2000 (25 µg/well) was added 4 h before LTA ($p = 0.03$) (Figure 3c)

Effect of M2000 on IkB-α

After stimulation with concentrations of low (5 µg/well) and high (25 µg/well) doses of M2000 alone or in combination with LPS/LTA, the expression of IkB-α was increased in comparison with the control group, but this difference was not statistically significant ($p> 0.05$). The expression of IkB-α was significantly increased when M2000 (25 µg/well) was added 4 h before LPS ($p = 0.024$). (Figure 3d). OxPAPC as an antagonist did not affect the gene expression level of downstream signaling molecules of TLRs.

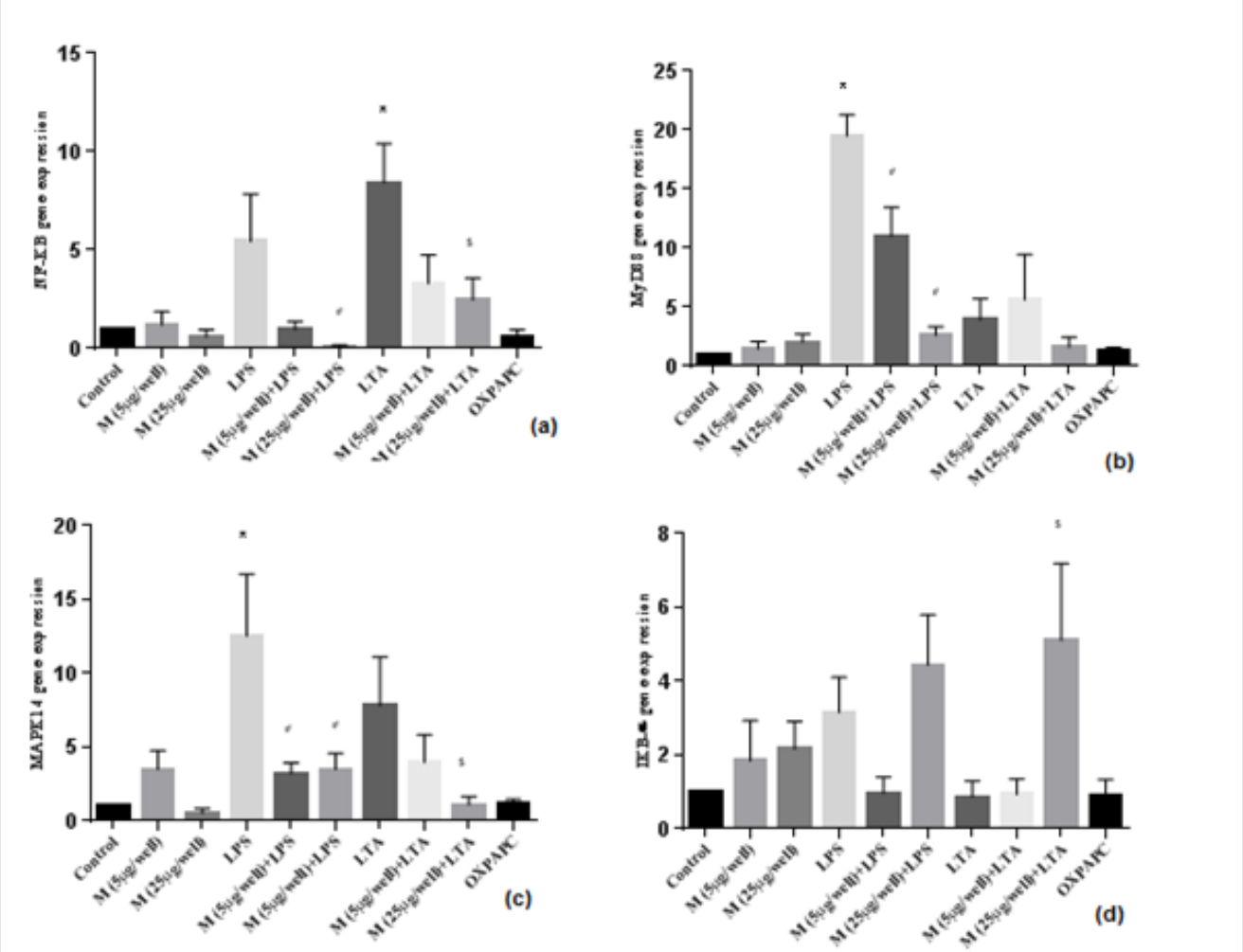

Figure 3: Real-time PCR was performed on total RNA isolated from PBMC of 5 AS patients
(a) The Real-time PCR analysis for NF-κB mRNA expression
(b) The Real-time PCR analysis for MyD88 mRNA expression
(c) The Real-time PCR analysis for MAPK14 mRNA expression
(d) The Real-time PCR analysis for IkB-α mRNA expression in AS monocyte-derived macrophages that pretreated with M2000 (M 5, 25 μg/well) in the presence or absence of LPS (1 μg/ml), LTA (10 μg/ml) and OXPAPC (30 μg/ml). Data are presented as mean ± SD. *P < 0.05, as compared with control group. #P < 0.05, as compared with LPS group. $p < 0.05, as compared with LTA group.

Effect of M2000 on the surface expressions of TLR2 and TLR4

The cell surface expressions of TLR2 and TLR4 on monocyte-derived macrophages in AS patients and healthy controls were determined by flow cytometric analysis (Figure 4). The baseline Mean Fluorescence Intensity (MFI) of TLR2 (55.97 ± 1.49) was increased in AS patients compared with healthy controls (p = 0.052). Exposure of monocyte-derived macrophages from AS patients with 25 μg/well of M2000 led to a significant decrease in the MFI of TLR2 (p < 0.01). Also, the stimulation of cells with 10 μg/ml LTA augmented the MFI of TLR2 (147.6 ± 2.24) in comparison with unstimulated control cells (p < 0. 001), while this level was significantly decreased, when M2000 at the concentration of low (5 μg/well) and high (25 μg/well) were added 4 h before LTA (p < 0. 001). (Figure 5a). The MFI of TLR4 was also increased in AS patients (43.45 ± 2.5) compared with healthy controls, but the difference was not statistically significant. Treatment of monocyte-derived macrophages from AS patients with 1 μg/ml LPS increased the MFI of TLR4 to (153 ± 0.5, p < 0. 001). On the other hand, pretreatment of cells for 4 h with 25 μg/well of M2000 before stimulation with LPS significantly decreased the MFI of TLR4 than LPS alone (p < 0. 001) (Figure 5b). Moreover, OxPAPC had no significant effect on TLR2 and 4 expressions.

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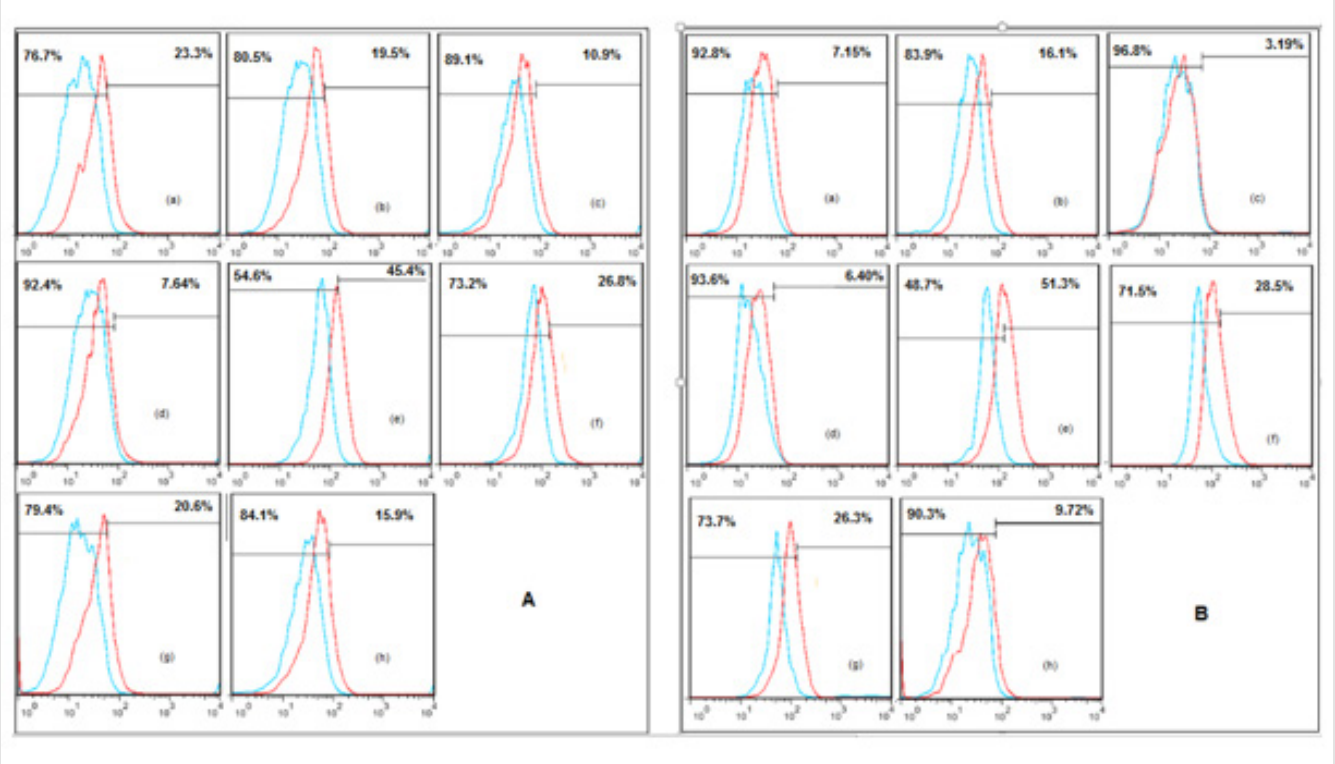

Figure 4:

A. Expression of TLR2 on monocyte derived macrophages of 5 AS patients. non-stimulated monocyte-derived macrophages of healthy subjects

a) Non-stimulated AS monocyte-derived macrophages
b) AS monocyte-derived macrophages treated with 5µg/well β-D-mannuronic acid
c) 25µg/well M2000
d) 10 µg/ml LTA
e) 5µg/well M2000+LTA
f) 25µg/well M2000+LTA
g) OxPAPC

B. Expression of TLR4 on monocyte-derived macrophages of 5 AS patients. non-stimulated monocyte-derived macrophages of healthy subjects

a) Non-stimulated AS monocyte-derived macrophages
b) AS monocyte-derived macrophages treated with 5µg/well β-D-mannuronic acid
c) 25µg/well M2000
d) 1 µg/ml LPS
e) 5µg/well M2000+LPS
f) 25µg/well M2000+LPS
g) OxPAPC

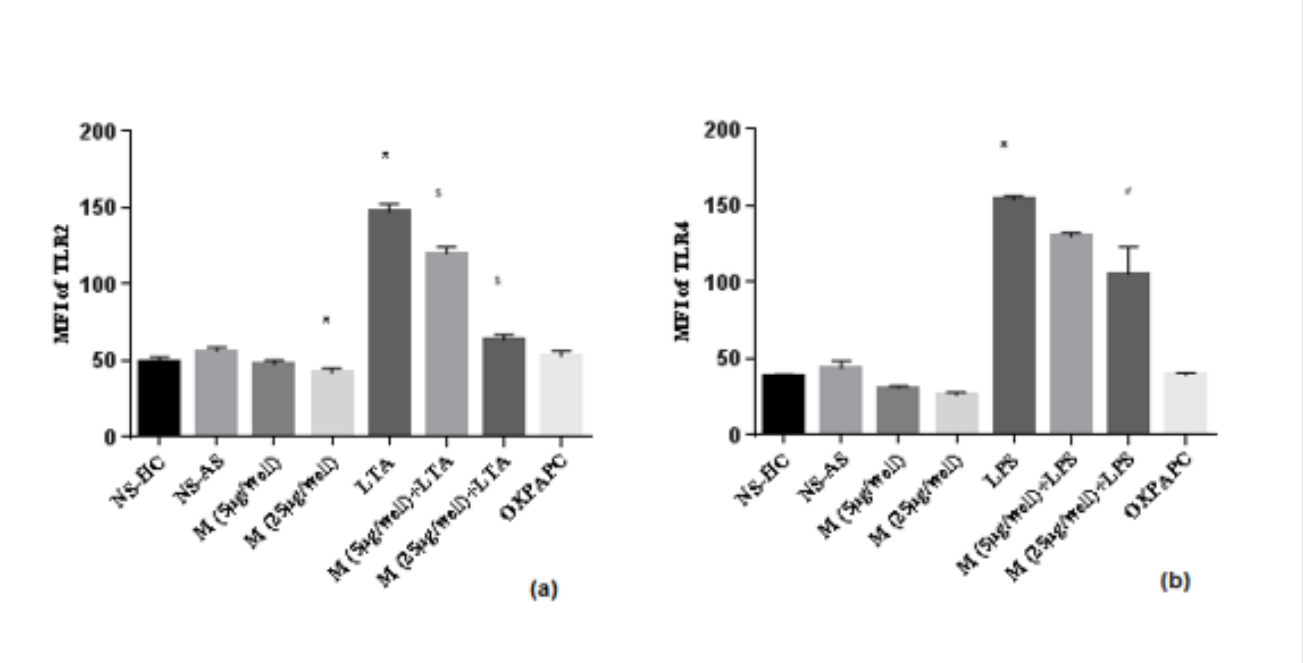

Figure 5: The effect of M2000 on the protein expression of TLR2,4 on monocyte-derived macrophages of 5 AS patients.
(a) The effect of M2000 on the protein expression of TLR2
(b) The effect of M2000 on the protein expression of TLR4 in AS monocyte-derived macrophages. Cells were pretreated with M2000 (5, 25 µg/well) in the presence or absence of LTA (10 µg/ml), LPS (1 µg/ml) and OXPAPC (30 µg/ml). Data are presented as mean ± SD. *P < 0.01, as compared with non-stimulated AS monocyte-derived macrophages. $P < 0. 001, as compared with LTA group #p < 0. 001, as compared with LPS group. NS (non-stimulated), HC (healthy control), AS (ankylosing spondylitis).

Effect of M2000 on Cytokine Production

Regarding M2000 affected the gene expression level of downstream signaling molecules of TLRs, we tested whether M2000 can decrease the production of TNF-α and IL-6 as inflammatory cytokines. Cells were treated with M2000 with or without TLR2 and TLR4 agonist and antagonist for 24 h and then cells supernatants were collected for evaluation of TNF-α and IL-6 cytokines using ELISA.

Effect of M2000 on TNF-α production

TNF-α synthesis in AS monocytes-derived macrophages is higher than that in healthy subjects; although, the difference was not statistically significant (p > 0.05). The exposure of cells to β-D-mannuronic acid (5 µg/well and 25 µg/well) led to a decrease in the production of TNF-α (p < 0.01). Our results also showed that treatment with 1 µg/ml LPS significantly increased TNF-α production (p < 0.001), whereas this level was lower when M2000 was added 4 h before LPS (p < 0.001). Moreover, challenged cells with 25 µg/well of M2000 before LTA showed a decrease in the concentration of TNF-α in comparison with stimulated cells with LTA alone (p = 0.02). OxPAPC had no effect on TNF-α production (p< 0.05) (Figure 6a).

Effect of M2000 on IL-6 production

The level of IL-6 production had no significant difference in AS monocytes-derived macrophages as compared with healthy subjects (p > 0.05). The stimulation of cells with 1 µg/ml LPS and 10 µg/ml LTA significantly increased IL-6 production in comparison with unstimulated control cells (p < 0.001), while this level was significantly decreased, when M2000 at the concentration of low (5 µg/well) and high (25 µg/well) were added 4 h before LPS and LTA (p < 0. 001). OxPAPC had no effect on IL-6 production (p < 0.05) (Figure 6b).

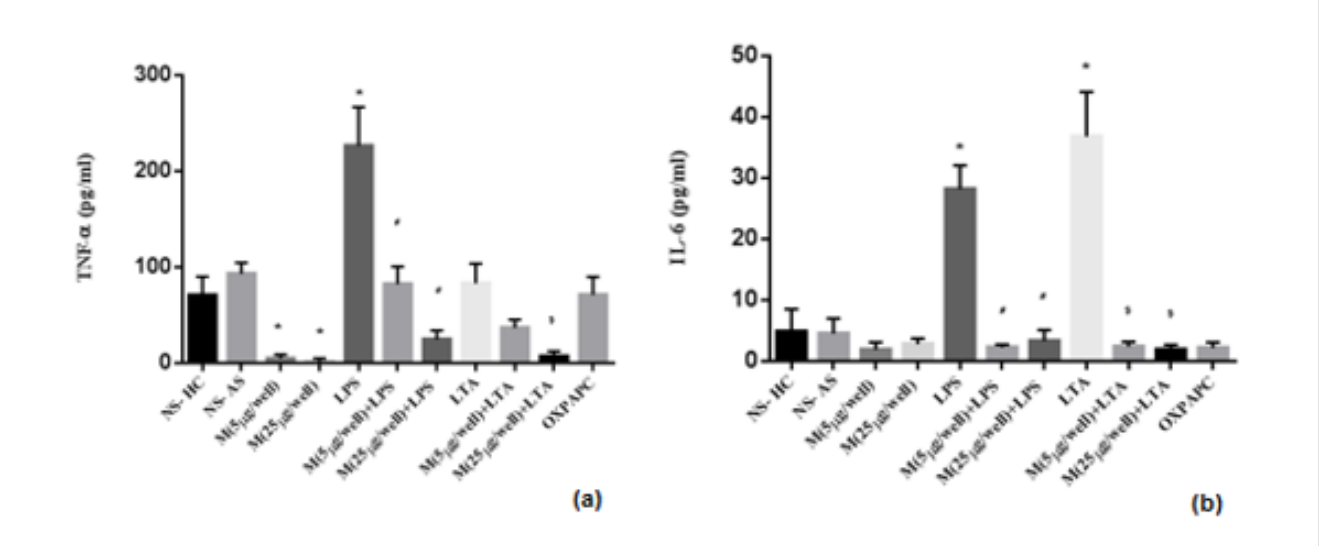

Figure 6: The effect of M2000 on the cytokine production.

(a) The effect of M2000 on TNF-α

(b) The effect of M2000 on IL-6 in supernatants of 5 AS monocytes-derived macrophages that pretreated with M2000 (M 5, 25 µg/well) in the presence or absence of LPS (1 µg/ml), LTA (10 µg/ml) and OXPAPC (30 µg/ml). Data are presented as mean ± SD. *P < 0.05, as compared with control group. #P < 0.05, as compared with LPS group. $p < 0.05, as compared with LTA group. NS (non-stimulated), HC (healthy control), AS (ankylosing spondylitis).

Discussion

The β-D-mannuronic acid (M2000) and its epimer α-L-guluronic acid (G2013) are new NSAIDs with immunomodulatory effects that have been investigated in the level of *in vitro*, *in vivo*, and clinical trials [42-46]. Although the activation of TLRs is necessary for host defense against various invading pathogens, the overactivation of TLR pathways can also lead to chronic inflammatory diseases [47]. Dysregulated TLR signaling disrupts the immune homeostasis by pro-inflammatory cytokines production and contributes to the development of many inflammatory disorders [48]. Therefore, targeting TLRs signaling may be beneficial to prevent and treat these disorders. Growing evidence shows that a dysfunctional TLR-mediated response plays a critical role in the pathogenesis of spondyloarthropathies [24,49,50]. The increased TLR2 and 4 expressions were reported in the synovium of patients with SpA, which is reduced by treatment with infliximab [51]. Yang et al. demonstrated that TLR4 expression in PBMCs in AS patients is higher than that of healthy controls [52]. Assassi et al. investigated the whole-blood gene transcript profile of AS patients and confirmed the overexpression of TLR4 and TLR5 in AS patients compared to healthy controls and also indicated the decreased expression of TLR4 and TLR5 after treatment with TNF-α inhibitor [53]. Another study showed that the TLR4 molecule and its mRNA levels were significantly increased in AS patients in comparison to healthy subjects [52].

However, it is not completely clear what kinds of mechanisms are responsible for TLRs upregulation. The ability of certain individuals to respond properly to TLR ligands may be impaired by single nucleotide polymorphisms (SNPs) within the TLR gene, resulting in altered susceptibility to infectious or inflammatory diseases [54]. Snelgrove et al. showed a link between TLR4 Asp299Gly and Thr399Ile polymorphisms and susceptibility to AS [55]. All these studies express a significant association between increased expression of TLR and the pathogenesis of AS. In the present study, we demonstrated a change in the level of TLR2 and TLR4 protein expression on the monocyte-derived macrophages in AS compared with healthy controls. Also, the statistical analysis demonstrated that NF-kB mRNA level was significantly increased in AS patients compared to healthy controls, whereas IkB-α was reduced. The M2000 was identified as a new anti-inflammatory drug with the immunosuppressive properties. Previous studies have revealed some molecular mechanisms of this novel anti-inflammatory drug [56]. Regarding the M2000 has shown inhibitory effects on TLR 2, 4 signaling in HEK293 cells and TLR/ NF-kB signaling in PBMC in AS patients [37,38], we evaluated the effects of this drug on the TLR2 and TLR4 downstream signaling transduction pathway of monocyte-derived macrophages in AS patients under *in vitro* condition. Our data indicated that TLR2 and TLR4 expression was decreased in monocyte-derived macrophages in AS patients following the exposure to M2000 (at 5 and 25 µg/well).

Moreover, treatment with TLR2 and TLR4 agonist increased surface expression of these receptors whereas pretreatment with M2000 before the agonist significantly reduced the expression level of these surface molecules, indicating that M2000 probably inhibits the attachment of agonist to TLR2 and TLR4. Also, we showed that M2000 had an inhibitory effect on gene expression of downstream signaling molecules of TLR2 and TLR4 and this was in line with the study by Aletaha, et al. [37]. The mRNA expression of NF-κB and MAPK14 was downregulated following the exposure to M2000 (at 25μg/ml). It is known that the stimulation of TLR4 by LPS activates downstream signaling pathways such as NF-κB and MAPKs [57]. In agreement with these reports, in our studies, we found that treatment with LPS/LTA augmented mRNA expression of MyD88, NF-κB, and MAPK14, and their amounts were reduced when M2000 (at 5 and 25 μg/well) was added 4 h before the agonist. IkB-α is an inhibitory protein which its function is to inhibit the NF-kB transcription factor. Stimulation of cells with various inducers, including LPS, results in the degradation of the IkB protein, releasing NF-κB to activate gene transcription [58]. Our results reveal that M2000 (at 5 and 25 μg/well) alone and in combination with TLR2 and TLR4 agonists increased the expression level of IkB-alpha compared to untreated control cells. To elucidate the anti-inflammatory effects of M2000 on TLR2 and TLR4 downstream signaling, we also determined TNF-α and IL-6 production in the monocyte-derived macrophages culture supernatant.

As seen in data analysis LPS/LTA treatment of AS macrophages strongly induce the production of TNF-α and IL-6 cytokines and M2000 alone or in combination with LPS/ LTA significantly down-regulated production of TNF-α and IL-6 cytokines in these cells. In our study, we found that M2000 treatment of stimulated macrophages from AS patients significantly decreased TLR2 and 4 surface expression and downregulated gene expression of MyD88, MAPK14, and NF-kB in TLRs downstream signaling. Thereby M2000 could reduce the TLR-mediated inflammatory responses in AS patients.

Conclusion

It is known that TLRs-triggered inflammatory response plays an important role in the pathogenesis of AS and interfering with the cytokine overproduction may improve the outcome and quality of life of the patients. In this study, we demonstrated that the M2000 could downregulate the production of pro-inflammatory mediators in AS monocyte-derived macrophages by inhibiting the TLR2 and TLR4 downstream signaling pathways. Therefore, M2000 might be a new therapeutic approach for targeting TLR-mediated cytokine production in patients with AS.

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None.

Conflict of interest

The authors declare that there is no conflict of interests.

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