Immunologic Research (2019) 67:108–115
https://doi.org/10.1007/s12026-018-9042-3

ORIGINAL ARTICLE

Assessment of immunological profile in ankylosing spondylitis patients following a clinical trial with guluronic acid (G2013), as a new NSAID with immunomodulatory properties

Seyed Shahabeddin Mortazavi-Jahromi[1] · Sepideh Nazeri[1] · Fahimeh Jafarnezhad-Ansariha[2] · Mona Oraei[1] · Abbas Mirshafiey[1]

Published online: 24 November 2018

Abstract
The present research aims to study the effects of guluronic acid (G2013) on gene expression levels of the T-bet, GATA3, RORγt, AHR, and FOXP3 transcription factors and on gene expression of their related cytokines following oral administration of this drug in ankylosing spondylitis (AS) patients. In this trial (clinical trial identifier: IRCT2016091813739N4), 14 AS patients and 12 age- and sex-matched healthy individuals were enrolled. The level of transcription factors' gene expression and expression of their related cytokines were measured by quantitative real-time PCR, before and 3 months after G2013 therapy. Our data indicated that the gene expression levels of the T-bet and IFN-γ were not significantly reduced during 12 weeks of treatment with G2013 ($p > 0.05$). The findings showed that the gene expression levels of the GATA3 and IL-4 increased significantly during 12 weeks of treatment with G2013 ($p < 0.05$). In addition, gene expression levels of the RORγt, IL-17, AHR, and IL-22 decreased significantly during the 12-week treatment with G2013 ($p < 0.05$). Moreover, the gene expression level of the FOXP3 increased significantly during 12 weeks of treatment with G2013, but the gene expression level of IL-10 did not increase significantly ($p < 0.05$, $p > 0.05$, respectively). The present study showed that oral intake of G2013 was able to modify the severity of articular and inflammatory symptoms of AS through reducing the gene expression levels of the RORγt, IL-17, AHR, and IL-22 and increasing the gene expression levels of the GATA3, IL-4, and FOXP3.

Keywords G2013 · Guluronic acid · NSAID · Immunomodulatory · Ankylosing spondylitis · Immunological profile

Introduction

Ankylosing spondylitis (AS) is a disease from the family of spondyloarthropathies (SpAs) that basically affect the sacroiliac joint and the axial skeleton and can also involve peripheral joints [1]. The inflammatory bowel disease, uveitis, and psoriasis are common symptoms of AS; therefore, it is considered a systematic disease [2, 3]. Its prevalence is approximately 0.5%, and its incidence rate in men is twice compared to that in women [3–5]. Although the exact cause of AS is still unknown, it is believed that, similar to other autoimmune diseases, a combination of genetic, immunological, and environmental factors can proceed the inflammatory reactions [6, 7]. Some of the genetic factors involved in AS include encoded MHC class I alleles, HLA-B27, endoplasmic reticulum aminopeptidase 1 (ERAP1), and IL-23R [8–10]. However, many studies have shown that the various immune cells, secreted inflammatory mediators, and different surface markers may play a major role in the pathogenesis of AS [11, 12]. In recent years, many immunological researches have shown the molecular mechanisms involved in the immunopathogenesis of this diseases. These investigations have mostly focused on various transcription factors and their related cytokines.

The T-bet transcription factor, a member of the T-box family of transcription factors, induces differentiation of naive $CD4^+$ T cells into T-helper 1 (Th1) cells [13]. T-bet causes production of Th1 cytokines, especially IFN-γ, and thus enhances the differentiation of Th1 cells by creating a positive

✉ Abbas Mirshafiey
mirshafiey@tums.ac.ir

[1] Department of Immunology, School of Public Health, Tehran University of Medical Sciences, Box 14155-6446, Tehran, Iran

[2] Department of Immunology, International Campus, Shahid Sadoughi University of Medical Sciences, Yazd, Iran

feedback loop [13]. Two separate studies have shown that T-bet gene expression and frequency of IFN-γ in peripheral blood mononuclear cells (PBMCs) increased in AS patients compared to healthy individuals [14, 15]. The GATA3 is known as a major regulator for directing the differentiation of naive $CD4^+$ T cells into Th2 cells. This transcription factor is involved in increasing emergence of Th2 cytokine genes including IL-4, IL-5, and IL-13 [16]. In two separate studies, it was shown that gene expression level and serum IL-4 level do not increase considerably in AS patients [17, 18]. The RORγt transcription factor is the main inducing factor in the differentiation of naïve $CD4^+$ T cells into Th17 cells [19]. Th17 secretes various cytokines, among them, the most important of which is IL-17 [19]. IL-17 is a pleiotropic cytokine that induces expression of proinflammatory cytokines and matrix metalloproteinases (MMPs), which play an important role in tissue inflammation and destruction [20]. Researchers have shown that the abundance of Th17 and IL-17 cells is directly related to AS activity [21]. Th22 cells are a new subset of $CD4^+$ T cells that are different from other T-helper subtypes [22]. Although the transcription factors involved in the differentiation of Th22 have not been completely identified, it seems that the aryl hydrocarbon receptor (AHR) is the main transcription factor regulating Th22 expression [23]. IL-22 is the main cytokine produced by Th22 cells [23]. Although the main role of IL-22 has not yet been clearly specified, the level of IL-22 and the frequency of Th22 increase in the PBMCs of AS patients (especially in the active phase) [24]. FOXP3 is a specific transcription factor for regulatory T cells (Tregs) that play a vital role in the development and function of these cells [25]. IL-10, TGF-β, and IL-35 are the major cytokines secreted by Tregs [25]. It has been shown that the expression level of FOXP3 and IL-10 does not significantly increase in AS patients [26].

In treatment of AS, the non-steroidal anti-inflammatory drugs (NSAIDs) are the first line of treatment protocol for reducing inflammation and pain in AS patients [27]. Studies have indicated that continuous intake of NSAIDs by patients with active AS can reduce the radiological symptoms [28]. Nevertheless, the side effects of these drugs, including gastrointestinal and cardiovascular complications, cause many problems for these patients [29]. Hence, researchers have recently tried to identify more safe and effective types of anti-inflammatory and immunomodulatory drugs. The guluronic acid (G2013) patented (DE/102016113017.6-PCT/EP2017/067920) is categorized as a novel drug of NSAID family [30, 31]. This agent with low molecular weight and anti-inflammatory and immunomodulatory properties is derived from a linear polymer of alginate, which is a major component of cell walls of brown algae and polysaccharide capsule among certain bacteria [30]. According to a study, in addition to its therapeutic effects, G2013 is less toxic than other NSAIDs for the gastrointestinal system and kidney function [30]. G2013 has shown its positive effects in experimental models of multiple sclerosis and anti-aging [32–34].

The present research aims to study the effects of G2013 on gene expression levels of the T-bet, GATA3, RORγt, AHR, and FOXP3 transcription factors and on gene expression of their related cytokines following oral administration of this drug in AS patients.

Material and methods

Ethics statement

The study was approved by the ethics committee of Tehran University of Medical Sciences (TUMS) and followed by an issued approval (clinical trial identifier: IRCT2016091813739N4) and was conducted under guidelines established by the assessment of spondyloarthritis international society (ASAS) and Helsinki manifest and its later amendments or comparable ethical standards. Written informed consent was obtained from all patients.

Preparation of G2013

The guluronic acid (G2013) with molecular formula ($C_6H_{10}O_7$) and IUPAC name [(2R/3S/4S/5S)-2/3/4/5-tetrahydroxy-6-oxohexanoic acid] was prepared from alginic acid sodium salt (Sigma-Aldrich, St. Louis, MO) as a reference sample. The purification method was carried out based on a modified procedure of the acid hydrolysis method by Nazeri et al [30]. The method was validated by characterizing the hydrolytic products using Fourier transform infrared (FT-IR) spectroscopy and carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectroscopy for confirming its molecular weight (194.139 g/mol) and exact/monoisotopic mass (194.043 g/mol).

Patients and study design

In this study, 14 patients with AS that fulfilled the modified New York criteria were selected for G2013 therapy during 12 weeks clinical trial. All patients had active disease, defined as a bath ankylosing spondylitis disease activity index (BASDAI) score ≥ 4 and bath ankylosing spondylitis functional index (BASFI) score ≥ 4 on a 0–10 numerical rating scale (NRS) which were examined. The majority of patients enrolled in this study were male (73.9%) and positive for the HLA–B27 allele (86.9%). The mean age of the patients was 34.1 ± 6.2 years (range18–45 years), and the mean disease duration was 6.4 ± 6.8 years. Other relevant eligibility criteria were the presence of axial involvement, no peripheral involvement, and the need for daily treatment with NSAIDs. Moreover, patients were excluded if they required the use of

concomitant prednisolone > 10 mg/day and methotrexate > 15 mg/week. TNF-α inhibitor treatment was not allowed before and during the study. All of the patients were recruited from the outpatient rheumatology clinic of Rheumatology Research Center (Shariati Hospital, Tehran, Iran) and Iran Rheumatology Center (Tehran, Iran). Treatment of these patients with G2013 was started on 23 October 2016 (clinical trial identifier: IRCT2016091813739N4) based on the ASAS guidelines for AS. Based on our preclinical assessment, the drug G2013 was administered with the same dose of two capsules of 500 mg per day orally after food during a period of 12 weeks to all patients. The medical history, physical examinations, and clinical outcome assessments (BASDAI score, BASFI score, ankylosing spondylitis quality of life [ASQoL], morning stiffness, physicians' global assessment, and total back pain) were performed at baseline, weeks 4 and 12. In this trial, we had 12 healthy, age- and sex-matched subjects (10 males, 2 females; mean age, 32.1 ± 6.9) without any background disease that was selected as a normal group.

Isolation of PBMCs

The blood sample was collected from AS patients (before treatment), AS patients (after treatment), and healthy subjects into EDTA containing tube (BD Vacutainer, Plymouth, UK) by venipuncture. The PBMCs were isolated from whole blood samples by standard Ficoll-Paque (Lymphosep, Biosera, France) based on density-gradient centrifugation. The viability of isolated PBMCs is measured by staining with trypan blue.

RNA extraction and cDNA synthesis

The total RNA was extracted from PBMCs of AS patients and healthy subjects using Hybrid-R™ Mini kit (GeneAll, Republic of Korea) according to the manufacturer's guidelines. The quality of isolated RNA was determined by agarose gel electrophoresis on the GelRed™ (Biotin, USA), the total RNA concentration was assessed by NanoDrop 2000 UV spectrophotometer (Thermo Scientific, UT, USA), and the samples with A260/280 ratio in the range 1.7–2.0 were selected for cDNA synthesis. Equal amounts of cDNA were synthesized using RNA (1 μg) via oligo (dT) and random hexamer primers in the presence of cDNA reverse transcriptase by the use of PrimeScript™ RT reagent kit (Takara, Shiga, Japan). The list of used primer sequences in the current study has been represented in Table 1.

Quantitative real-time PCR

PCR amplifications were performed using Syber premix Ex Taq™ II (Takara, Shiga, Japan) in ABI Step One Plus™ Real-time PCR system (Thermo Scientific, USA). Briefly, the reaction conditions included 2 μl cDNA, 12.5 μl SYBR Green Master Mix, 8.5 μl RNase-free water, 1 μl forward primer, and 1 μl reverse primer. The amplification was carried out as follows: an initial denaturation at 95 °C for 30 s, followed by 40 cycles of denaturation for 5 s at 95 °C and annealing for 30 s at 60 °C. The gene levels were normalized to the housekeeping gene (β-actin) as an internal control. The relative changes in gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method (Ct refers to the threshold value).

Statistical analysis

The statistical analysis was performed using SPSS software version 24 (IBM Corporation, Armonk, New York, USA). Paired Student *t* test and standard repeated analysis of variance (ANOVA) were used for the statistical analyses of clinical outcomes and gene expression data. The results were

Table 1 Oligonucleotide sequences of primers used in this study for qRT-PCR

Gene name	Accession number	Primer sequences	PCR product size
β-actin	NM_031144.3	F: 5′-CCTCTATGCCAACACAGTGC-3′ R: 5′-GTACTCCTGCTTGCTGATCC-3′	211
T-bet	NM_013351.1	F: 5′-TCTCCTCTCCTACCCAACCA-3′ R: 5′-CTGACTGCTCGAAACTCAGC-3′	143
IFN-γ	NM_000619.2	F: 5′-GAGTGTGGAGACCATCAAGGAA-3′ R: 5′-GGCGACAGTTCAGCCATCA-3′	162
GATA3	NM_001002295.1	F: 5′-AGGACGAGAAAGAGTGCCTC-3′ R: 5′-GAAGAGTCCGGAGCTGTACT-3′	170
IL-4	NM_000589.3	F: 5′-TTTGCTGCCTCCAAGAACAC-3′ R: 5′GTCGAGCCGTTTCAGGAATC-3′	165
RORγt	NM_005060.3	F: 5′-TTCCGAGGATGAGATTGCCC-3′ R: 5′-CAGCTTTGCCAGGATGCTTT-3′	163
IL-17	NM_002190.3	F: 5′-AGGCAGGAATCACAATCCCA-3′ R: 5′-TCCTCATTGCGGTGGAGATT-3′	178
AHR	NM_001621.4	F: 5′-AGCCACCTTCATCATCCGTCA-3′ R: 5′-TGACGTCCAACAGGTGACAGT-3′	153
IL-22	NM_020525.4	F: 5′-CCTGCATTTGACCAGAGCAA-3′ R: 5′-TAACGCAGGGGTTCATTTGG-3′	160
FOXP3	NM_014009.3	F: 5′-GGTTTCCACTGTCTTGCCTG-3′ R: 5′-TTGTGAAGGCTCTGTTTGGC-3′	176
IL-10	NM_000572.3	F: 5′-GTTCTTTGGGGAGCCAACAG-3′ R: 5′-GCTCCCTGGTTTCTCTTCCT-3′	155

F, forward; *R*, reverse

Table 2 Clinical parameter outcomes in 14 AS patients before and after G2013 therapy

Parameters	Before treatment (baseline)	After treatment (after 12 weeks)	*p* value
BASDAI score (0–10 NRS)	8.7 ± 2.1	6.1 ± 1.5	0.018
BASFI score (0–10 NRS)	7.2 ± 4.1	5.0 ± 1.0	0.015
ASQoL (range 0–18)	14.3 ± 4.8	9.0 ± 4.1	0.008
Morning stiffness (0–10 NRS)	5.8 ± 2.4	3.7 ± 2.2	0.011
Physicians' global assessment (0–10 NRS)	8.5 ± 3.6	5.1 ± 1.1	0.042
Total back pain (0–10 NRS)	7.6 ± 3.2	5.3 ± 2.4	0.021

Values are shown as mean ± SD. *BASDAI*, bath ankylosing spondylitis disease activity index; *BASFI*, bath ankylosing spondylitis functional index; *ASQoL*, ankylosing spondylitis quality of life; *NRS*, numerical rating scale

presented as mean ± standard deviations (SD). A p value < 0.05 was considered to be a statistically significant difference.

Results

Clinical outcome

The improvement in the patient status was observed after 2 weeks and also continued during the treatment course. The mean of BASDAI score, BASFI score, ASQoL, morning stiffness, physicians' global assessment, and total back pain had met a significant reduction after 12 weeks of treatment (Table 2).

Effect of G2013 on gene expression of T-bet and IFN-γ

Our findings demonstrated that the gene expression of T-bet in PBMCs of patients with AS was decreased 26% ± 5 by G2013, in comparison to the patient group (before treatment) (164% ± 7) with $p = 0.12$ (Fig. 1a). The results also showed that the gene expression of IFN-γ in patients with AS was decreased 16% ± 1 by G2013, in comparison to the patient group (before treatment) (149% ± 6) with $p = 0.18$ (Fig. 1b).

Effect of G2013 on gene expression of GATA3 and IL-4

The results illustrated that the gene expression of GATA3 in PBMCs of patients with AS was significantly increased 33% ± 1 by G2013, in comparison to the patient group (before treatment) (27% ± 7) with $p < 0.05$ (Fig. 2a). The results also revealed that the gene expression of IL-4 in PBMCs of patients with AS was significantly increased 20% ± 3 by G2013, in comparison to the patient group (before treatment) (19% ± 1) with $p < 0.05$ (Fig. 2b).

Effect of G2013 on gene expression of RORγt and IL-17

Our data revealed that the gene expression of RORγt in PBMCs of patients with AS was significantly decreased 82% ± 4 by G2013, in comparison to the patient group (before treatment) (285% ± 24) with $p < 0.05$ (Fig. 3a). The results also indicated that the gene expression of IL-17 in PBMCs of patients with AS was significantly decreased 56% ± 4 by

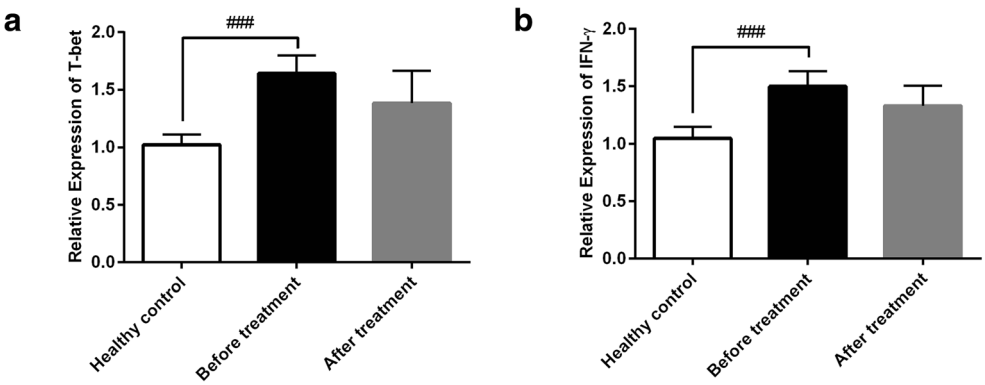

Fig. 1 The effect of G2013 on T-bet (**a**) and IFN-γ (**b**) gene expression in PBMCs AS patients. The groups were healthy control, patient group (before treatment), and patient group (after treatment). The T-bet and IFN-γ gene expression were measured by qRT-PCR. Values were normalized by β-actin. Data were expressed as mean ± SD. ###$p < 0.001$ vs. healthy control group

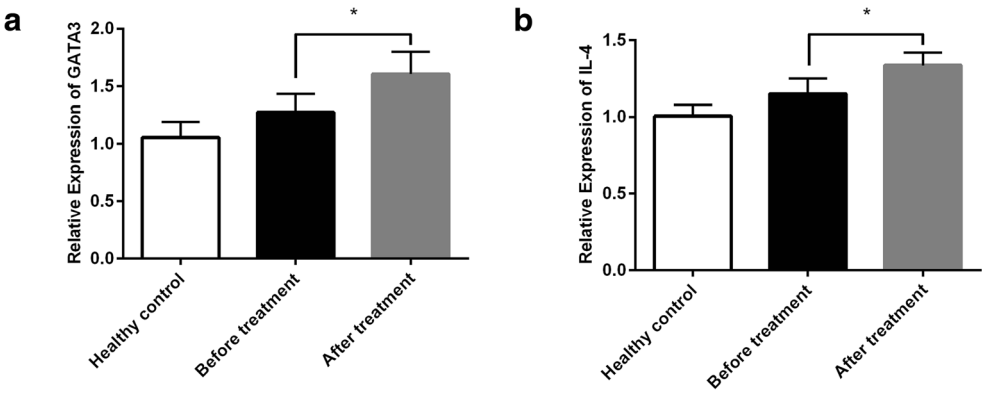

Fig. 2 The effect of G2013 on GATA3 (**a**) and IL-4 (**b**) gene expression in PBMCs AS patients. The groups were healthy control, patient group (before treatment), and patient group (after treatment). The GATA3 and IL-4 gene expression were measured by qRT-PCR. Values were normalized by β-actin. Data were expressed as mean ± SD. $^{*}p < 0.05$ vs. patient group (before treatment)

G2013, in comparison to the patient group (before treatment) (305% ± 17) with $p < 0.05$ (Fig. 3b).

Effect of G2013 on gene expression of AHR and IL-22

Our findings illustrated that the gene expression of AHR in PBMCs of patients with AS was significantly decreased 49% ± 10 by G2013, in comparison to the patient group (before treatment) (179% ± 6) with $p < 0.05$ (Fig. 4a). The results also demonstrated that the gene expression of IL-22 in PBMCs of patients with AS was significantly decreased 74% ± 6 by G2013, in comparison to the patient group (before treatment) (387% ± 17) with $p < 0.05$ (Fig. 4b).

Effect of G2013 on gene expression of FOXP3 and IL-10

Our data indicated that the gene expression of FOXP3 in PBMCs of patients with AS was significantly increased 24% ± 1 by G2013, in comparison to the patient group (before treatment) (23% ± 1) with $p < 0.05$ (Fig. 5a). The results also revealed that the gene expression of IL-10 in PBMCs of patients with AS was increased 13% ± 1 by G2013, in comparison to the patient group (before treatment) (10% ± 1) with $p = 0.43$ (Fig. 5b).

Discussion

The NSAIDs are widely used to treat patients with AS [27]. However, the use of common NSAIDs and the newly developed cyclooxygenase (COX) inhibitors is accompanied by gastrointestinal, renal, and cardiovascular side effects that limit their long-term use [29]. For decades, scientists have tried to identify more safe and effective types of NSAIDs. The G2013 for the first time was introduced in a published paper as a novel NSAID with immunomodulatory property based on its effects on cyclooxygenase (COX)-1 and COX-2 gene expression and activity [31]. The therapeutic effects of this drug with the highest tolerability, safety, and efficacy have been proven in experimental autoimmune encephalomyelitis (EAE) as well as in vitro and in vivo experiments [30–35]. The study found that G2013 can regulate TLR4 signaling pathway without changing the expression of miR-146a (as an anti-inflammatory agent) during inflammation through reducing the downstream signaling molecules, such as interleukin-1

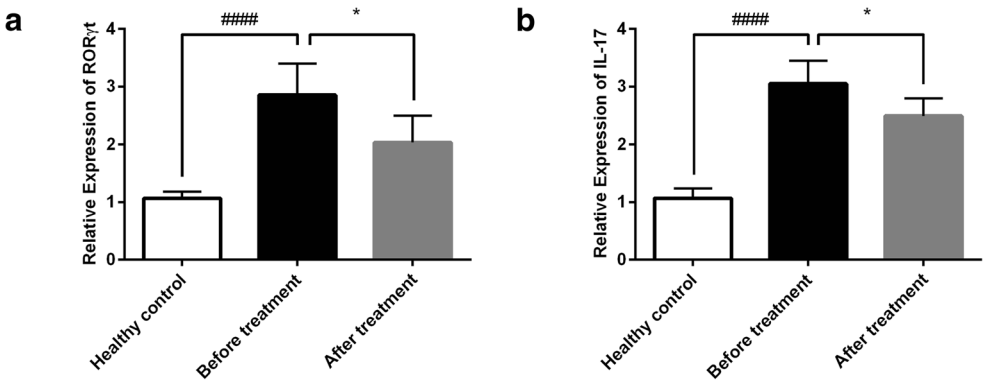

Fig. 3 The effect of G2013 on RORγt (**a**) and IL-17 (**b**) gene expression in PBMCs AS patients. The groups were healthy control, patient group (before treatment), and patient group (after treatment). The RORγt and IL-17 gene expression were measured by qRT-PCR. Values were normalized by β-actin. Data were expressed as mean ± SD. $^{*}p < 0.05$ vs. patient group (before treatment) and $^{\#\#\#\#}p < 0.0001$ vs. healthy control group

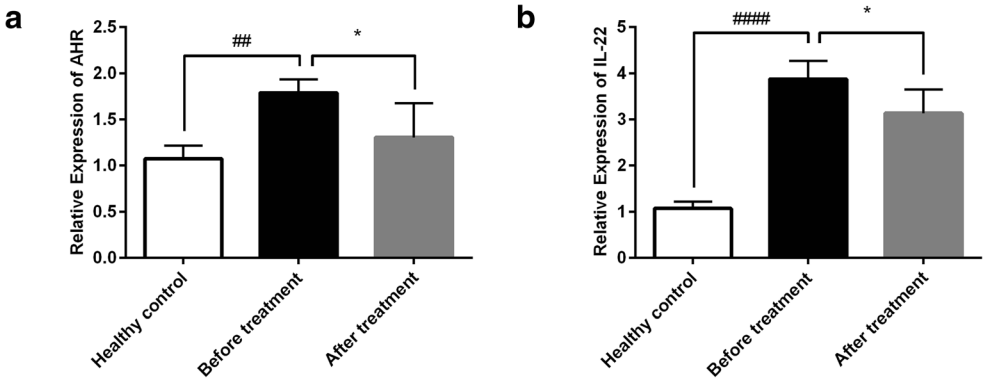

Fig. 4 The effect of G2013 on AHR (**a**) and IL-22 (**b**) gene expression in PBMCs AS patients. The groups were healthy control, patient group (before treatment), and patient group (after treatment). The AHR and IL-22 gene expression were measured by qRT-PCR. Values were normalized by β-actin. Data were expressed as mean ± SD. $^{*}p < 0.05$ vs. patient group (before treatment) and $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ vs. healthy control group

receptor-associated kinase (IRAK1) and tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) [36]. In another study, our results showed that G2013 as a novel immunomodulatory agent can significantly diminish miR-155 gene expression after stimulation with LPS [37]. The existing data revealed that G2013 is able to induce SHIP1 and SOCS1 and reduce TLR4, MyD88, and NF-κB at the level of gene expression and decrease IL-1β as a pro-inflammatory cytokine which might be recommended for reduction of inflammatory reactions [38]. Moreover, the results of another study showed that G2013 has an immunomodulatory effect on the TLR2 and TLR4 downstream transduction pathway in the healthy donors PBMCs [39]. Taeb et al. indicated that G2013 as a novel NSAID with immunomodulatory properties can modify the expression of SOD2, GPX1, CAT, GST, iNOS, and myeloperoxidase (MPO) genes up to normal gene expression and may reduce the pathologic process of aging and age-related inflammatory diseases [34].

Study of the various types of immune cells in patients with AS is of crucial importance for clarifying the pathogenesis of AS and understanding the mechanisms involved in AS development [40]. Lau et al. showed that the expression levels of the T-bet gene in the PBMCs of patients with AS increased compared to healthy subjects [14]. In a study by Mexican scientists, it was found that IFN-γ, which produces T-CD4^{+} (Th1) cells, increased in the PBMCs of AS patients compared with healthy subjects [15]. Wang et al. observed that the frequency of Th1 and the ratio of Th1/Th2 increased significantly in two groups of patients with mild and severe AS. An imbalance in T cell subsets increases IFN-γ, which may lead to persistent inflammation and further progression of AS [17]. It has been shown that the increased levels of Th2 could have a therapeutic effect in collagen-induced arthritis (CIA) (the best experimental model for studying patients with autoimmune diseases) [41]. In a study, it was observed that serum levels of IL-4 (cytokine Th2) did not increase considerably in AS patients compared to healthy subjects [18]. Researchers have proved that Th17 cells play an important role in many autoimmune diseases such as multiple sclerosis (MS), rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and AS [42]. Various studies have reported that serum levels of IL-17 and percentage of Th17 cells increase in patients with AS [43–45]. It has been stated in several studies that IL-22 levels rise in several autoimmune diseases including psoriasis, Crohn's disease, RA, SLE, and AS [24, 46, 47]. In 2012, Zhang et al. reported that the absolute number of Th22 cells in peripheral blood and plasma levels of IL-22 increased in patients with AS [24]. In a recent study by El-Zayadi et al., it

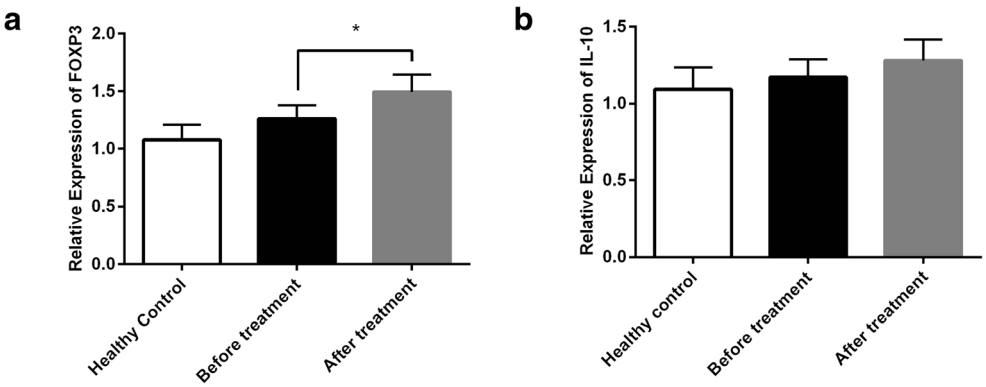

Fig. 5 The effect of G2013 on FOXP3 (**a**) and IL-10 (**b**) gene expression in PBMCs AS patients. The groups were healthy control, patient group (before treatment), and patient group (after treatment). The FOXP3 and IL-10 gene expression were measured by qRT-PCR. Values were normalized by β-actin. Data were expressed as mean ± SD. $^{*}p < 0.05$ vs. patient group (before treatment)

was shown that IL-22 played a key role in the mechanism of new bone formation in SpA [48]. Researchers have shown an increased ratio of Th17/Treg cells in AS patients [49, 50]. The findings of two separate studies indicated that FOXP3 expression levels and serum levels of IL-10 in peripheral blood of AS patients did not increase considerably [26, 51].

Collectively, a high number of studies have shown an important role for immune cells in the pathogenesis of AS disease; therefore, NSAIDs could be considered as an appropriate treatment for AS. The present study aimed to evaluate the effects of G2013 on gene expression levels of T-bet, GATA3, RORγt, AHR, and FOXP3 transcription factors and on gene expression of their related cytokines following oral administration of this drug in AS patients. Our results showed that after 12 weeks of G2013 consumption by patients, the averages of BASDI and BASFI declined, the score for ASQol, and morning stiffness, physicians' global assessment, and total back pain significantly improved compared to the patient group (before treatment with G2013) (Table 2). Our data indicated that the gene expression levels of the IFN-γ and T-bet were reduced during 12 weeks of treatment with G2013, but no significant relationship was observed in these groups (Figs. 1a and 2a). The findings showed that the gene expression levels of the GATA3 and the IL-4 increased significantly during the 12 weeks of treatment with G2013 (Fig. 2a, b). In addition, gene expression levels of the RORγt, IL-17, AHR, and IL-22 decreased significantly during the 12-week treatment with G2013 (Figs. 3a, b and 4a, b). Moreover, the gene expression level of the FOXP3 increased significantly during 12 weeks of treatment with G2013, but the gene expression level of its related cytokine (i.e., IL-10) did not increase significantly (Fig. 5a, b).

Conclusion

Many researchers are interested in controlling the progression of inflammatory and autoimmune diseases through the use of NSAIDs with the lowest toxicity and side effects. The present study showed that oral administration of G2013, as a novel NSAID with immunomodulatory properties, was able to modify the severity of articular and inflammatory symptoms of AS through reducing the gene expression levels of the RORγt, IL-17, AHR, and IL-22 and increasing the gene expression levels of the GATA3, IL-4, and FOXP3. Therefore, this drug might be recommended for the treatment of AS and other autoimmune inflammatory diseases.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval and consent to participate The study was approved by the ethics committee of Tehran University of Medical Sciences (TUMS) and followed by an issued approval (clinical trial identifier: IRCT2016091813739N4). Written informed consent was obtained from all patients.

Consent for publication Not applicable.

Reference

1. Dakwar E, Reddy J, Vale FL, Uribe JS. A review of the pathogenesis of ankylosing spondylitis. Neurosurg Focus. 2008;24:E2.
2. Thomas GP, Brown MA. Genetics and genomics of ankylosing spondylitis. Immunol Rev. 2010;233:162–80.
3. Hammer RE, Maika SD, Richardson JA, Tang JP, Taurog JD. Spontaneous inflammatory disease in transgenic rats expressing HLA-B27 and human beta 2m: an animal model of HLA-B27-associated human disorders. Cell. 1990;63:1099–112.
4. Dean LE, Jones GT, MacDonald AG, Downham C, Sturrock RD, Macfarlane GJ. Global prevalence of ankylosing spondylitis. Rheumatology (Oxford). 2014;53:650–7.
5. Gran JT, Husby G, Hordvik M. Prevalence of ankylosing spondylitis in males and females in a young middle-aged population of Tromsø, northern Norway. Ann Rheum Dis. 1985;44:359–67.
6. Tsui FW, Tsui HW, Akram A, Haroon N, Inman RD. The genetic basis of ankylosing spondylitis: new insights into disease pathogenesis. Appl Clin Genet. 2014;7:105–15.
7. Sieper J, Braun J, Rudwaleit M, Boonen A, Zink A. Ankylosing spondylitis: an overview. Ann Rheum Dis. 2002;61:iii8–18.
8. Brown MA. Non-major-histocompatibility-complex genetics of ankylosing spondylitis. Best Pract Res Clin Rheumatol. 2006;20:611–21.
9. Mahmoudi M, Jamshidi AR, Amirzargar AA, Farhadi E, Nourijelyani K, Fallahi S, et al. Association between endoplasmic reticulum aminopeptidase-1 (ERAP-1) and susceptibility to ankylosing spondylitis in Iran. Iran J Allergy Asthma Immunol. 2012;11:294–300.
10. Roberts AR, Vecellio M, Cortes A, Knight JC, Cohen CJ, Wordsworth BP. Investigation of a possible extended risk haplotype in the IL23R region associated with ankylosing spondylitis. Genes Immun. 2017;18:105–8.
11. Madej M, Nowak B, Świerkot J, Sokolik R, Chlebicki A, Korman L, et al. Cytokine profiles in axial spondyloarthritis. Reumatologia. 2015;53:9–13.
12. Sveaas SH, Berg IJ, Provan SA, Semb AG, Olsen IC, Ueland T, et al. Circulating levels of inflammatory cytokines and cytokine receptors in patients with ankylosing spondylitis: a cross-sectional comparative study. Scand J Rheumatol. 2015;44:118–24.
13. Zhu J, Jankovic D, Oler AJ, Wei G, Sharma S, Hu G, et al. The transcription factor T-bet is induced by multiple pathways and prevents an endogenous Th2 cell program during Th1 cell responses. Immunity. 2012;37:660–73.
14. Lau MC, Keith P, Costello ME, Bradbury LA, Hollis KA, Thomas R, et al. Genetic association of ankylosing spondylitis with TBX21 influences T-bet and pro-inflammatory cytokine expression in humans and SKG mice as a model of spondyloarthritis. Ann Rheum Dis. 2017;76:261–69.
15. Limón-Camacho L, Vargas-Rojas MI, Vázquez-Mellado J, Casasola-Vargas J, Moctezuma JF, Burgos-Vargas R, et al. In vivo peripheral blood proinflammatory T cells in patients with ankylosing spondylitis. J Rheumatol. 2012;39:830–5.
16. Zhang Y, Zhang Y, Gu W, He L, Sun B. Th1/Th2 cell's function in immune system. Adv Exp Med Biol. 2014;841:45–65.

17. Wang C, Liao Q, Hu Y, Zhong D. T lymphocyte subset imbalances in patients contribute to ankylosing spondylitis. Exp Ther Med. 2015;9:250–56.
18. Wang J, Zhao Q, Wang G, Yang C, Xu Y, Li Y, et al. Circulating levels of Th1 and Th2 chemokines in patients with ankylosing spondylitis. Cytokine. 2016;81:10–4.
19. Jadidi-Niaragh F, Mirshafiey A. Th17 cell, the new player of neuroinflammatory process in multiple sclerosis. Scand J Immunol. 2011;74:1–13.
20. Kuwabara T, Ishikawa F, Kondo M, Kakiuchi T. The role of IL-17 and related cytokines in inflammatory autoimmune diseases. Mediators Inflamm. 2017;2017:3908061.
21. Jethwa H, Bowness P. The interleukin (IL)-23/IL-17 axis in ankylosing spondylitis: new advances and potentials for treatment. Clin Exp Immunol. 2016;183:30–6.
22. Trifari S, Kaplan CD, Tran EH, Crellin NK, Spits H. Identification of a human helper T cell population that has abundant production of interleukin 22 and is distinct from T(H)-17, T(H)1 and T(H)2 cells. Nat Immunol. 2009;10:864–71.
23. Azizi G, Simhag A, El Rouby NM, Mirshafiey A. Th22 cells contribution in immunopathogenesis of rheumatic diseases. Iran J Allergy Asthma Immunol. 2015;14:246–54.
24. Zhang L, Li YG, Li YH, Qi L, Liu XG, Yuan CZ, et al. Increased frequencies of Th22 cells as well as Th17 cells in the peripheral blood of patients with ankylosing spondylitis and rheumatoid arthritis. PLoS One. 2012;7:e31000.
25. Campbell DJ. Control of regulatory T cell migration, function, and homeostasis. J Immunol. 2015;195:2507–13.
26. Ciccia F, Accardo-Palumbo A, Giardina A, Di Maggio P, Principato A, Bombardieri M, et al. Expansion of intestinal CD4+CD25(high) Treg cells in patients with ankylosing spondylitis: a putative role for interleukin-10 in preventing intestinal Th17 response. Arthritis Rheum. 2010;62:3625–34.
27. Maksymowych WP. Update on the treatment of ankylosing spondylitis. Ther Clin Risk Manag. 2007;3:1125–33.
28. Wanders A, Heijde Dv, Landewé R, Béhier JM, Calin A, Olivieri I, et al. Nonsteroidal antiinflammatory drugs reduce radiographic progression in patients with ankylosing spondylitis: a randomized clinical trial. Arthritis Rheum. 2005;52:1756–65.
29. Elyan M, Khan MA. The role of nonsteroidal anti-inflammatory medications and exercise in the treatment of ankylosing spondylitis. Curr Rheumatol Rep. 2006;8:255–9.
30. Nazeri S, Khadem Azarian S, Fattahi MJ, Sedaghat R, Tofighi Zavareh F, Aghazadeh Z, et al. Preclinical and pharmacotoxicology evaluation of α-l-guluronic acid (G2013) as a non-steroidal anti-inflammatory drug with immunomodulatory property. Immunopharmacol Immunotoxicol. 2017;39:59–65.
31. Mirshafiey A, Mortazavi-Jahromi SS, Taeb M, Cuzzocrea S, Esposito E. Evaluation of the effect of α-L-guluronic acid (G2013) on COX-1, COX-2 activity and gene expression for introducing this drug as a novel NSAID with immunomodulatory property. Recent Pat Inflamm Allergy Drug Discov. 2018;12:162–168.
32. Afraei S, Azizi G, Zargar SJ, Sedaghat R, Mirshafiey A. New therapeutic approach by G2013 in experimental model of multiple sclerosis. Acta Neurol Belg. 2015;115:259–66.
33. Mirshafiey A, Hosseini S, Afraei S, Rastkari N, Zavareh FT, Azizi G. Anti-aging property of G2013 molecule as a novel immunosuppressive agent on enzymatic and non-enzymatic oxidative stress determinants in rat model. Curr Drug Discov Technol. 2016;13:25–33.
34. Taeb M, Mortazavi-Jahromi SS, Jafarzadeh A, Mirzaei MR, Mirshafiey A. An in vitro evaluation of anti-aging effect of guluronic acid (G2013) based on enzymatic oxidative stress gene expression using healthy individuals PBMCs. Biomed Pharmacother. 2017;90:262–67.
35. Arjomand Fard N, Tabrizian N, Mirzaei R, Hadjati J, Tofighi Zavareh F, Salehi Nodeh AR, et al. Efficacy and safety of G2013 as a novel immunosuppressive agent on differentiation, maturation and function of human dendritic cells. Iran J Public Health. 2017;46:216–21.
36. Hajivalili M, Pourgholi F, Majidi J, Aghebati-Maleki L, Movassaghpour AA, Samadi Kafil H, et al. G2013 modulates TLR4 signaling pathway in IRAK-1 and TARF-6 dependent and miR-146a independent manner. Cell Mol Biol (Noisy-le-grand). 2016;62:1–5.
37. Mortazavi Jahromi SS, Jamshidi MM, Yousefi M, Navabi SS, Motamed N, Zavareh FT, et al. Inhibitory effect of G2013 molecule as a novel immunomodulatory agent, on miR-155 gene expression in HEK-Blue hTLR4 cell line. Eur J Inflamm. 2016;14:86–92.
38. Mortazavi-Jahromi SS, Farazmand A, Motamed N, Navabi SS, Mirshafiey A. Effects of guluronic acid (G2013) on SHIP1, SOCS1 induction and related molecules in TLR4 signaling pathway. Int Immunopharmacol. 2018;55:323–29.
39. Sharifi L, Mohsenzadegan M, Aghamohammadi A, Rezaei N, Zavareh FT, Bokaie S, et al. Immunomodulatory effect of G2013 (α-L-Guluronic Acid) on the TLR2 and TLR4 in human mononuclear cells. Curr Drug Discov Technol. 2018;15:123–131.
40. Rezaiemanesh A, Abdolmaleki M, Abdolmohammadi K, Aghaei H, Pakdel FD, Fatahi Y, et al. Immune cells involved in the pathogenesis of ankylosing spondylitis. Biomed Pharmacother. 2018;100:198–204.
41. Guo Y, Xing E, Song H, Feng G, Liang X, An G, et al. Therapeutic effect of dioscin on collagen-induced arthritis through reduction of Th1/Th2. Int Immunopharmacol. 2016;39:79–83.
42. Tabarkiewicz J, Pogoda K, Karczmarczyk A, Pozarowski P, Giannopoulos K. The role of IL-17 and Th17 lymphocytes in autoimmune diseases. Arch Immunol Ther Exp (Warsz). 2015;63:435–49.
43. Jandus C, Bioley G, Rivals JP, Dudler J, Speiser D, Romero P. Increased numbers of circulating polyfunctional Th17 memory cells in patients with seronegative spondylarthritides. Arthritis Rheum. 2008;58:2307–17.
44. Shen H, Goodall JC, Hill Gaston JS. Frequency and phenotype of peripheral blood Th17 cells in ankylosing spondylitis and rheumatoid arthritis. Arthritis Rheum. 2009;60:1647–56.
45. Xueyi L, Lina C, Zhenbiao W, Qing H, Qiang L, Zhu P. Levels of circulating Th17 cells and regulatory T cells in ankylosing spondylitis patients with an inadequate response to anti-TNF-α therapy. J Clin Immunol. 2013;33:151–61.
46. Brand S, Beigel F, Olszak T, Zitzmann K, Eichhorst ST, Otte JM, et al. IL-22 is increased in active Crohn's disease and promotes proinflammatory gene expression and intestinal epithelial cell migration. Am J Physiol Gastrointest Liver Physiol. 2006;290:G827–38.
47. Lo YH, Torii K, Saito C, Furuhashi T, Maeda A, Morita A. Serum IL-22 correlates with psoriatic severity and serum IL-6 correlates with susceptibility to phototherapy. J Dermatol Sci. 2010;58:225–7.
48. El-Zayadi AA, Jones EA, Churchman SM, Baboolal TG, Cuthbert RJ, El-Jawhari JJ, et al. Interleukin-22 drives the proliferation, migration and osteogenic differentiation of mesenchymal stem cells: a novel cytokine that could contribute to new bone formation in spondyloarthropathies. Rheumatology (Oxford). 2017;56:488–93.
49. Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, et al. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature. 2006;441:235–8.
50. Wu Y, Ren M, Yang R, Liang X, Ma Y, Tang Y, et al. Reduced immunomodulation potential of bone marrow-derived mesenchymal stem cells induced CCR4+CCR6+ Th/Treg cell subset imbalance in ankylosing spondylitis. Arthritis Res Ther. 2011;13:R29.
51. Wen JT, Zhang DH, Fang PF, Li MH, Wang RJ, Li SH. Role of Th1/Th2 cytokines in the diagnosis and prognostic evaluation of ankylosing spondylitis. Genet Mol Res. 2017;16. https://doi.org/10.4238/gmr16019322.