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In Vitro Effects of Sodium Benzoate on the Expression of T Cells-related Cytokines and Transcription Factors in Adjuvant-induced Arthritis Model

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ABSTRACT

Though the exact etiology of rheumatoid arthritis (RA) is unknown, the contribution of immune cells in the disease process is completely acknowledged. T helper (Th) 1 and Th17-related cytokines are required for the disease development and progression, while Th2 and regulatory T cells (Tregs)-derived cytokines are protective. Studies have shown that sodium benzoate (NaB) can switch the balance of Th cell subsets toward Th2 and Tregs. The present study aimed to evaluate the possible effects of NaB on the expression of $CD4^+$T cells-related cytokines and transcription factors in splenocytes derived from an animal model of RA, adjuvant-induced arthritis (AIA).

AIA was induced in rats by injection of Freund's adjuvant containing mycobacterial antigens (Mtb). Splenocytes were isolated from AIA rats and restimulated ex vivo with Mtb in the presence or absence of NaB for 24 h. To determine the effects of NaB on the expression of T cells-related cytokine and transcription factor genes, real-time PCR was performed.

NaB treatment of Mtb-stimulated splenocytes derived from arthritic rats resulted in significant increases in the gene expressions of Tregs-related cytokines (IL-10 and TGF-β) and Foxp3 transcription factor, and significant decreases in the expression of Th1-related cytokines (TNF-α and IFN-γ) and the T-bet transcription factor. The ratios of Th1/Th2 (IFN-γ/IL-4), Th1/Treg (IFN-γ/TGF-β and IFN-γ/IL-10) and Th17/Treg (IL-17/IL-10 and IL-17/IL-10+TGF-β)-related cytokines were also significantly decreased.

In conclusion, NaB can potentially be considered as a useful therapeutic agent for the treatment of RA and other Th1 and Th17-mediated diseases.

Keywords: Adjuvant-induced arthritis; $CD4^+$ T cells; Cytokines; Sodium benzoate; Transcription factors

INTRODUCTION

Rheumatoid arthritis (RA) is a common systemic autoimmune disease affecting about 1% of the population worldwide.[1] It is characterized by chronic inflammation of the synovium, particularly of small

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joints, which in the absence of treatment often leads to the destruction of articular cartilage and juxta-articular bone.[1,2] While the exact etiology of RA is unknown, it is widely accepted that RA is a multifactorial disease with genetic and environmental predisposing factors. In addition, the contribution of immune cells in the disease process is completely acknowledged.[3] In this regard, affected joints in RA patients harbor various types of infiltrating immune cells and cytokines.[2,3]

Among the immune cells infiltrating the joints, Th1 and Th17 cells have critical roles in the disease development and progression.[2] Hereof, studies have shown a direct correlation between circulating numbers of Th1 and Th17 cells as well as their cytokines with disease activity or severity in RA patients.[4] In contrast, quantitative and qualitative defects of Th2 and regulatory T cells (Tregs) have been reported in RA patients.[2] Similar to RA, studies on the pathogenesis of adjuvant-induced arthritis (AIA), a well-known animal model of RA, indicated that Th1[5] and Th17-related cytokines[6] are required for the disease induction and progression, whereas Th2-[7] and Treg-derived cytokines are protective.[8,9] Therefore, treatments aimed at enhancing Th2 and Tregs function or suppression of Th1 and Th17 induction can be useful in amelioration of RA.

Currently, treatment options for RA include the use of non-steroidal anti-inflammatory drugs (NSAIDs), traditional disease-modifying anti-rheumatic drugs (DMARDs) and biologic DMARDs which all are associated with various side effects and in some cases increased morbidity and mortality. Also, they are not cost-effective and impose a significant financial burden on patients.[10] Thus, alternative therapeutic agents with lower cost and side effects in comparison with current treatments have drawn much attention.

Sodium benzoate (NaB) is the sodium salt of benzoic acid and a metabolite of cinnamon that is widely used as a food and drink preservative due to its antimicrobial properties. It is also an FDA-approved drug against urea cycle disorders and hyperammonemia in humans.[11,12] Several studies have suggested the ability of NaB in switching the balance of Th cell subsets toward Th2 and Tregs.[13,14] *In vitro* studies have shown that NaB significantly suppressed neopterin production and tryptophan degradation by peripheral blood mononuclear cells (PBMCs) which led to the downregulation of Th1 immune responses.[15] Similarly, treatment of PBMCs of healthy individuals or patients suffering from multiple sclerosis (MS) with NaB deviated antigen-specific Th1 responses towards Th2 type by increasing the percentages of Th2 type cells.[13] Moreover, an in vivo study showed that NaB administration through drinking water could ameliorate clinical symptoms and disease progression in a mouse model of relapsing-remitting MS through various mechanisms including suppression of the expression of the proinflammatory molecules, switching the differentiation of myelin-specific T cells from Th1 to Th2 and enrichment of Tregs population.[14] In accordance with the above-mentioned studies, other studies have shown that oral, inhalation or dermal exposure to NaB promotes and exacerbates Th2-mediated allergic reactions including asthma, anaphylaxis, dermatitis, and rhinitis.[16] Hence, it is reasonable to suggest that the administration of NaB may be a simple, cost-effective and efficient treatment for Th1-related diseases such as RA. Therefore, in the present study for the first time, we investigated whether NaB might be useful in the treatment of RA by investigating its capability in diverting antigen-specific Th1 and Th17 responses towards Th2 and Tregs in splenocytes derived from AIA as an animal model of RA.

MATERIALS AND METHODS

Materials

RPMI 1640 and fetal bovine serum (FBS) were purchased from Gibco (NY, USA). L-glutamine, penicillin G, and streptomycin obtained from Shellmax (China). Trypan blue was purchased from Biosera (France). Incomplete Freund's adjuvant (IFA), sodium benzoate, dimethyl sulfoxide (DMSO) and Concanavalin A (Con A) were obtained from Sigma (St. Louis, USA) and 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl tetrazolium bromide (MTT) was purchased from GoldBiotechnlogy (USA). Heat-killed *Mycobacterium tuberculosis* (Mtb) strain H37Ra was obtained from BD Biosciences (USA). Ketamine and xylazine were obtained from Alfasan Company (Netherland). Total RNA extraction kit, High-capacity cDNA reverse transcription kit and SYBR Premix Ex Taq II were purchased from Parstous Biotechnology (Tehran, Iran), ABI (USA) and Takara Bio, Inc. (Japan), respectively. The primers were purchased from Takapouzist (Iran).

Animals

Male Sprague-Dawley rats aged 4 weeks (100±10 g) were purchased from the Center of Comparative and Experimental Medicine, Shiraz University of Medical Sciences. All the experimental protocols involving animals and their care were carried out by institutional, legal regulation as well the approval by the Ethical Committee of Shiraz University of Medical Sciences. Rats were maintained in the room at 23±1°C with alternating 12 h light-dark cycles and standard humidity to eliminate the effects of stress. Food and water were provided *ad libitum* throughout the experiments. The study was approved by the Ethics Committee of Shiraz University of Medical Sciences, Shiraz, Iran (code: IR.SUMS.REC.1396.S791).

Induction of AIA

Arthritis was induced by the inoculation of the rats with Freund's complete adjuvant (CFA). CFA was prepared by mixing heat-inactivated Mtb with IFA at a final concentration of 20 mg/mL. Briefly, rats were anesthetized with an intraperitoneal injection of a mixture of ketamine (70 mg/kg body weight) and xylazine (6 mg/kg) on day 0 and the AIA model was induced by a single intradermal (ID) injection of 0.1 mL of prepared CFA at the base of the tail of each rat. Disease development and progression were evaluated every other day from day 10 to 23 post-immunization by macroscopic inspection including walking ability, skin redness and swelling at the site of the ankle, wrist joints, and small interphalangeal joints. Lesions on all four paws of each rat were graded by two separate investigators from 0 to 4 according to the extent of both edema and erythema of the periarticular tissues.[17] From the injected animals, five rats that developed AIA were used for the study. The clinical score of selected animals was between 3.5-10.5. Schematic representation of the study design is shown in Figure 1.

Cytotoxicity Assay

Cytotoxicity of NaB on the isolated splenocytes was determined by MTT assay. Splenocytes were cultured in 96-well plate (Nunc, Denmark) at a concentration of 5×10^5 cells/well in a total volume of 200 µL in RPMI 1640 containing 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin G, and 100 µg/mL streptomycin. Cells were treated with different concentrations of NaB (500-4000 µg/mL) for 24 h. Negative control was cells without NaB (untreated) and positive control was cells treated with a toxic dose of DMSO (2%). Three hours before the termination of the experiment, MTT solution at a final concentration of 0.5 mg/ml was added to the cell culture wells. The culture plate was then centrifuged and the cell pellet was dissolved in DMSO. The absorbance was read in a microplate reader (BioTek Instruments Inc, USA) at 570 nm and 630 nm. The MTT test was performed in triplicate on three rats.

Effect of NaB on the Expression of $CD4^+$ T Cell-related Cytokines and Transcription Factors

Splenocytes Stimulation

To determine the effects of NaB on the expression of Th-related cytokines and transcription factors, splenocytes were isolated from five AIA rats on day 23 post-immunization. Cells were washed with RPMI 1640 medium and then cultured in 24-well plate (Nunc) at a concentration of 2.5×10^6 cells/well in a total volume of 0.6 ml in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin G, and 100 µg/mL streptomycin.

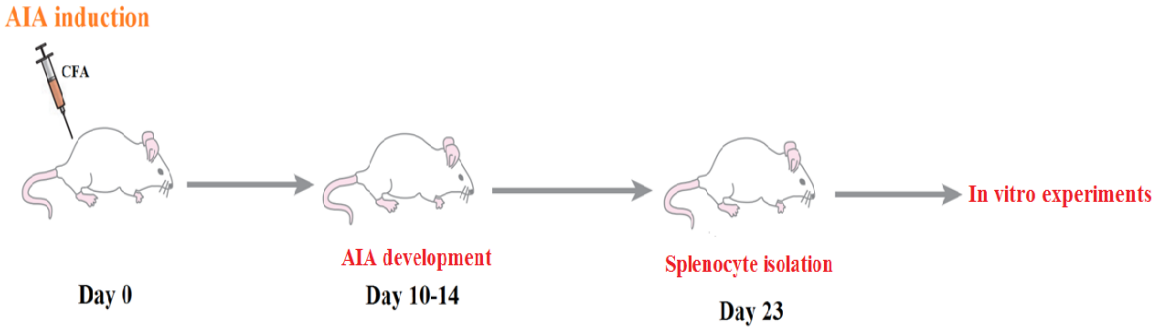

Figure 1. Schematic representation of the experimental design

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The cells were stimulated with 100 µg/mL of Mtb antigen for 24 h in the presence or absence of a nontoxic concentration of NaB (1000 µg/mL). Untreated cells (without Mtb antigen and NaB) were used as a negative control. In addition, cells treated with Con A as a T cell mitogen (at 2.5 µg/mL) was used as another control.

Total RNA Isolation and cDNA Synthesis

After treatments, splenocytes were collected and total RNA was extracted using a total RNA extraction kit. RNA purity and concentration were determined using a UV spectrophotometer (Pico 100 µL Spectrophotometer, UK). The RNA integrity was also checked by electrophoresis of samples on a 1.1% agarose gel in the presence of loading dye and visualization by a UV-transilluminator. After that, 500 ng of total RNA was reverse-transcribed to cDNA using the high capacity cDNA reverse transcription kit according to the manufacturer's protocol.

Quantitative Real-time PCR

Real-time PCR was used to determine the effect of NaB on the mRNA expression of $CD4^+$ T cells-related cytokines including interferon (IFN)-γ and tumor necrosis factor (TNF)-α (Th1), interleukin (IL)-4 (Th2), IL-17 (Th17), IL-10 and transforming growth factor (TGF)-β (Treg) as well as transcription factors including T-bet (Th1), GATA binding protein 3 (GATA3, Th2), retinoid-related orphan receptor c (RORc, Th17) and forkhead box P3 (Foxp3, Treg). Details on primer sequences and PCR amplicon size are listed in Table 1. Primers were designed using Primer-BLAST tool available at the National Center for Biotechnology Information (NCBI) website (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). In addition, the specificity of primers for the targeted mRNAs was determined by BLAST searches (at https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and AlleleID software version 7.5 (Premier Biosoft, USA) was also used to exclude primers with a tendency in forming potential hairpin, self-dimer or cross dimer.

Table 1. Rattus norvegicus target mRNA and gene-specific primer sequences

Gene	Accession number	Primer sequence	Amplicon size (bp)
β-Actin	NM_031144.3	F: 5 GCAAATGCTTCTAGGCGGAC 3′	51
		R: 5′ AAGAAAGGGTGTAAAACGCAGC 3′	
T-bet	NM_001107043.1	F: 5′TTCAACCAGCACCAGACAGAGAT 3′	127
		R: 5′CCAAGACTACATCCACAAACATCCT 3′	
IFN-γ	NM_138880.2	F: 5′ GGCCCTCTCTGGCTGTAACT 3′	149
		R: 5 TCGTGTTACCGTCCTTTTGC 3′	
TNF-α	NM_012675.3	F: 5′ TCAGCCTCTTCTCATTCCTGC 3′	203
		F: 5′ TTGGTGGTTTGCTACGACGTG 3′	
GATA3	NM_133293.1	F: 5 ′GAGGAGGAACGCTAACGGA 3′	123
		R: 5 ′GACATCTTACGGTTTCGGGTCT 3′	
IL-4	NM_201270.1	F: 5 ′TACGGCAACAAGGAACAC 3′	101
		R: 5 ′ TCTTCAAGCACGGAGGTA 3′	
RORc	XM_006232926.3	F: 5′TGAGGATGAGATTGCCCTCTAC 3′	95
		R: 5 ′TGTATTGCAGATGCTCCACTCT 3′	
IL-17	NM_001106897.1	F: 5′CAGACTACCTCAACCGTTCCAC 3′	63
		R: 5′ATCTATCAGGGTCCTCATTGCG 3′	
Foxp3	NM_001108250.1	F: 5′ GTGGCAGGGAAGGAGTGTCA 3′	95
		R: 5′TCCAAGTCTCGTGTGAAGGCA 3′	
TGFβ	NM_021578.2	F: 5′ AGAAGTCACCCGCGTGCTAAT 3′	115
		R: 5′ CACTGCTTCCCGAATGTCTGA 3′	
IL-10	NM_012854.2	F: 5 ′CCTCTGGATACAGCTGCGAC 3′	122
		R: 5 ′TCATGGCCTTGTAGACACCTT 3′	

F: forward primer, R: reverse primer

Table 2. The efficiency of real-time PCR for studied genes

Genes	Slope	Efficiency (%)
β-Actin	-3.32	99.71
RORc	-3.35	98.84
Foxp3	-3.36	98.43
IL-4	-3.58	90.25
IL-10	-3.12	109.17
IL-17	-3.29	101.35
IFN-γ	-3.26	102.65
TGF-β	-3.128	108.79
T-bet	-3.371	97.99
GATA-3	-3.38	97.63

Real-time PCR was carried out using a QuantStudio 3 quantitative PCR system (Thermo Fisher Scientific, USA) in a total reaction volume of 20 µL for 40 cycles. Briefly, 1.5 µL cDNA, 0.4 µL of forwarding and reverse primers (10 pM) and 10 µL SYBR Green master mix was used for each reaction. PCR conditions for gene amplification began with a 30 sec 95°C enzyme activation step, followed by 40 cycles of 5 sec of denaturation at 95°C, 15 sec of annealing at 59°C and 30 sec of extension at 72°C. The mRNA expressions of the respective genes were normalized to the level of β-actin mRNA in all samples and the relative expression of the target gene was calculated using the $2^{-\Delta\Delta CT}$ comparative method. In the case of a lack of similarity in the amplification efficiency between the gene of interest and housekeeping gene, the Pfaffl method was used for calculation. For each gene, the results of Mtb-stimulated cells were considered as 1. Accordingly, the relative expression of mRNA in untreated and Mtb+NaB-treated cells was calculated. The calculated efficiency and slope for the designed primers are listed in table 2.

Statistical Analysis

Data were analyzed by Statistical Package for the Social Sciences (SPSS) software version 22 (SPSS Inc, USA). Data were expressed as the mean±standard deviation (SD). Parametric data were analyzed using a one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison post hoc test. In the cases of non-parametric data, Mann–Whitney U was applied for comparison of differences among the groups. *p* values <0.05 were considered statistically significant.

RESULTS

Determination of Toxic Doses of NaB for Rat Splenocytes

Those concentrations of NaB that after 24 h did not significantly reduce the number of cultured splenocytes compared to untreated controls were considered nontoxic. MTT assay showed the toxicity of NaB at a concentration of 4000 µg/mL against splenocytes (Figure2). The half inhibitory concentration (IC50)

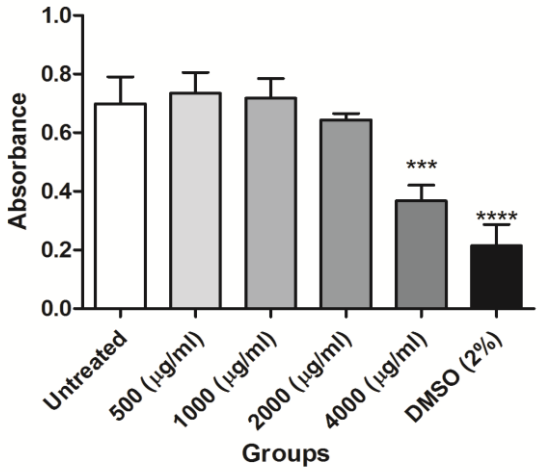

Figure 2. Cytotoxicity of NaB against rat splenocytes. Splenocytes from Sprague-Dawley rats were cultured with different concentrations of NaB for 24 h and cytotoxicity was determined using MTT assay. Absorbance is expressed as a measure of cell viability and data represent mean±SD of three experiments. DMSO at a toxic concentration (2%) was used as positive control. ******p*< 0.001, *******p*<0.0001 compared to untreated control

value of NaB was calculated to be 2254 µg/mL. In the same experiments using trypan blue staining, NaB concentration of ≥2000 µg/mL was determined as toxic doses (data are not shown).

Therefore, to make sure of using nontoxic concentrations of NaB, in the present study concentration of 1000 µg/mL was used as a suboptimal nontoxic dose of NaB for the next experiments.

Effects of NaB on the Expression of Th1-related Genes

To determine the effects of NaB on the expression of $CD4^+$ T cells-related genes, splenocytes were isolated from rats with AIA and stimulated *ex vivo* with100µg/ml of Mtb antigen in the presence (1000 µg/mL) or absence of nontoxic concentrations of NaB for 24 h. As shown in Figure 3A1, the expression level of T-bet was significantly higher in splenocytes treated with Mtb compared to those of untreated splenocytes (0.39±0.24; p=0.008). NaB significantly decreased the expression level of this transcription factor in Mtb-stimulated cells to 0.58±0.19 (p=0.008), thereby no significant differences remained in the expression of T-bet when Mtb+NaB-treated and untreated splenocytes were compared to each other (Figure 3 A1). In the same way, stimulation of splenocytes by Mtb induced significant increases in the expression levels of IFN-γ and TNF-α compared to untreated splenocytes (p<0.03). Treatment of Mtb-stimulated splenocytes with NaB significantly reduced the expression of both cytokines to 0.52±0.25 (IFN-γ, p=0.008) and 0.82±0.16 (TNF-α, p=0.03). However, in contrast to T-bet, this level of reduction did not reach to the expression levels of IFN-γ and TNF-α genes in untreated splenocytes (Figures 3 A2 and 3 A3).

Effects of NaB on the Expression of Th2 Cytokine and Transcription Factor

We found no significant difference in the expression of GATA3 between untreated (1.12±0.45) and Mtb-stimulated splenocytes. However, the expression of the IL-4 gene showed a significant increase after the treatment of splenocytes with Mtb compared to untreated splenocytes (1 *versus* 0.33±0.24, p=0.008). On the other hand, NaB treatment increased the expression levels of both GATA3 and IL-4 genes in Mtb-stimulated splenocytes, though the differences did not reach the statistical significance (Figures 3 B1 and 3 B2).

Effects of NaB on the Expression of Th17-related Cytokine and Transcription Factor

Stimulation of splenocytes with Mtb induced a significant increase in the expression level of IL-17 compared to the untreated splenocytes (0.01±0.007, p=0.008). Unexpectedly the results of the same experiment showed a significant decrease in RORc expression in Mtb-stimulated splenocytes compared to the untreated splenocytes (8.47±3.64, p=0.008). Treatment of splenocytes with NaB did not significantly affect the expression levels of RORc and IL-17 genes in Mtb-stimulated splenocytes (Figures 3 C1 and 3 C2).

Effects of NaB on the Expression of Treg-related Cytokines and Transcription Factor

No significant difference was observed in the expression of Foxp3 and TGF-β genes between untreated and Mtb-stimulated splenocytes of AIA rats (Figures 3D1 and 3D2). However, stimulation with Mtb significantly up-regulated the expression of IL-10 gene from 0.1±0.08 in untreated splenocytes to 1, p=0.008). On the other hand, as shown in Figure 3D1 to 3D3, NaB significantly increased the expression of Tregs-related genes including Foxp3 (1.64±0.14), TGF-β (1.89±1.08) and IL-10 (1.53 ±0.3) (p=0.008 for all) upon Mtb stimulation of splenocytes.

Effects of NaB on the Ratios of T Cell Subsets-related Cytokines and Transcription Factors

In addition to the evaluation of the $CD4^+$ T cell subsets-related gene expressions, in the present study, the ratios of pro-inflammatory-to-anti-inflammatory genes expressed by $CD4^+$ T cell subsets were also analyzed. The results showed that in Mtb+NaB-treated splenocytes the ratio of T-bet/GATA-3 (0.53±0.09) and IFN-γ/IL-4 (0.37±0.07) was significantly decreased compared to Mtb-treated group (p=0.008, Figure 4 A1 and 4 A2). Of interest, NaB decreased the ratio of the Th1/Th2 cell to a degree that no significant differences were observed in the ratios of T-bet/GATA-3 or IFN-γ/IL-4 between Mtb+NaB-treated and untreated groups (Figure 4A1 and 4 A2). We also examined the T-bet/Foxp3, IFN-γ/TGF-β and IFN-γ/IL-10 and (TNF-α+IFN-γ) /(TGF-β+IL-10) ratios as representatives of Th1/Tregs cells ratio. As shown in Figures 4B1, 4B2, 4B4 and 4B6, a significant reduction for all studied ratios was observed when the Mtb+NaB-treated splenocytes were compared to Mtb-stimulated

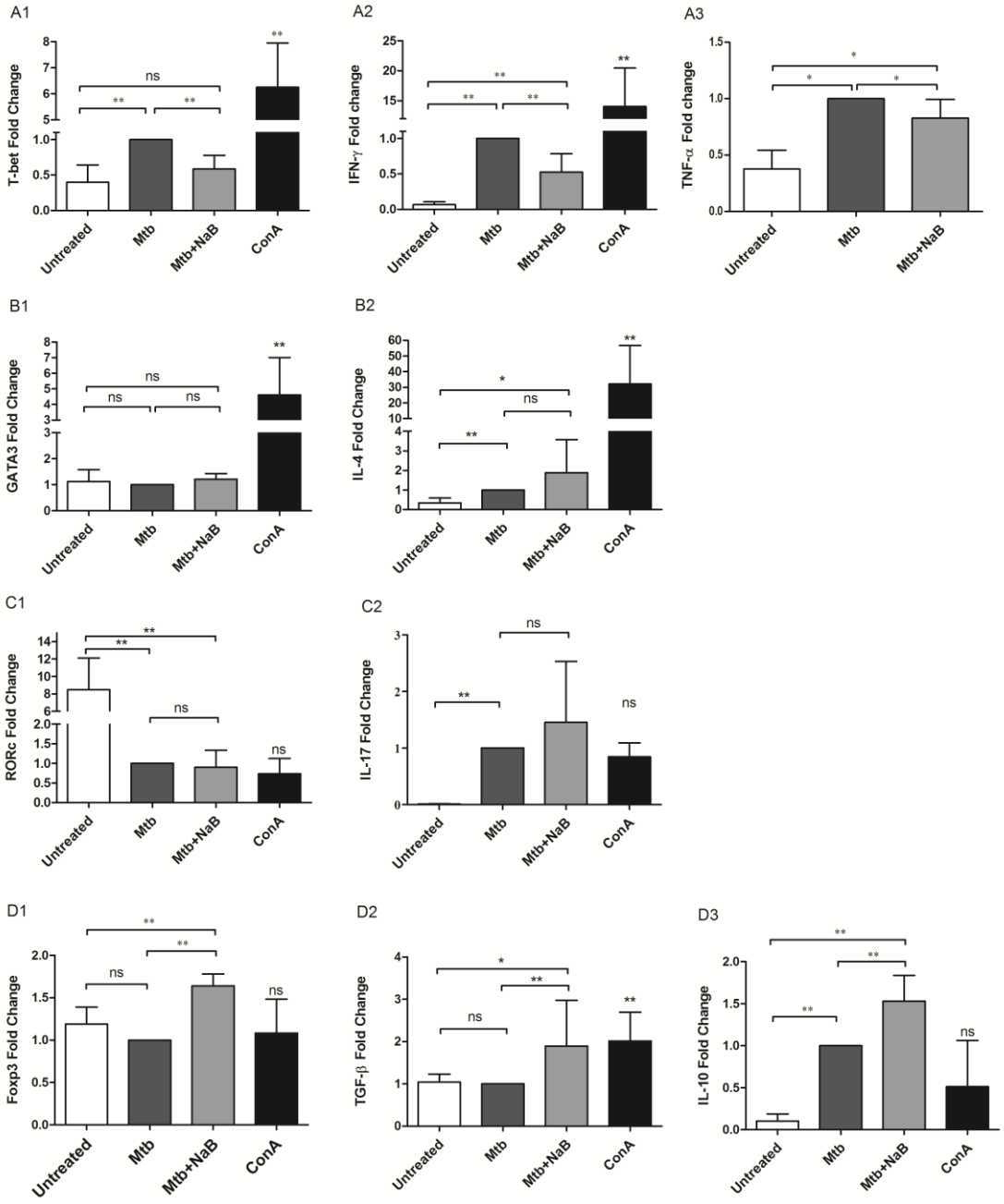

Figure 3. Effect of NaB on the CD4$^+$ T cells-related gene expressions of splenocytes derived from rats with AIA using real-time PCR. Splenocytes were stimulated *ex vivo* with whole mycobacterial antigen (Mtb,100µg/ml) in the presence or absence of NaB (1000µg/ml) for 24 h. Cells cultured alone without Mtb or NaB treatment were used as negative control (untreated). Gene expression values represent the mean of experiments from five AIA rats and error bars represent SD. Levels of mRNA in the Mtb-treated group were considered equal to 1 of gene expression and relative fold change was calculated for other groups. A1-A3: Th1-related genes (T-bet, IFN-γ, and TNF-α), B1-B2: Th2-related genes (GATA3 and IL-4), C1-C2: Th17-related genes (RORc and IL-17), D1-D3: Treg-related genes (Foxp3, TGF-β, and IL-10). *p<0.05, **p<0.01, ns: non-significant. The levels of significance for Con A versus Mtb are shown on the Con A bar.

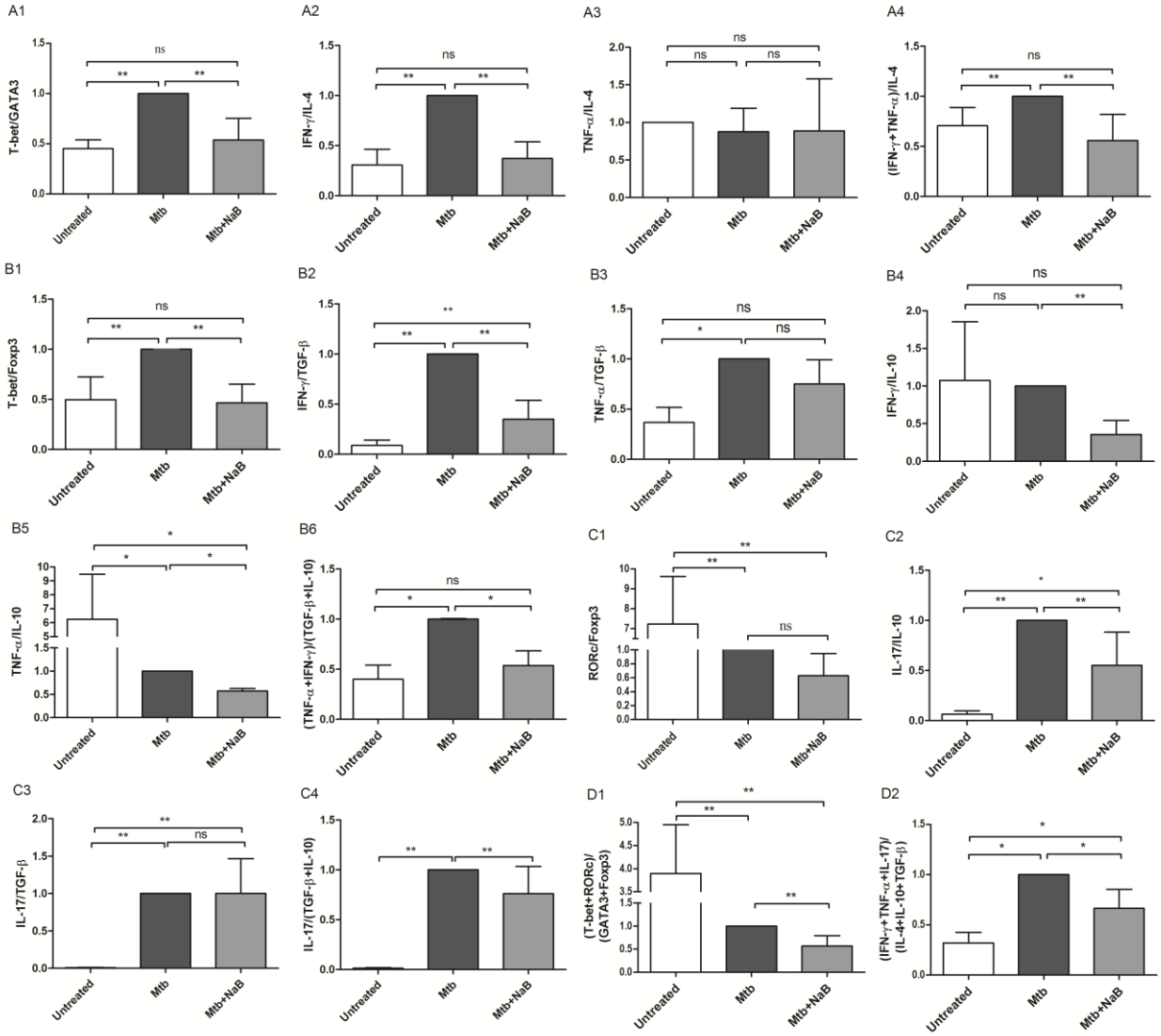

Figure 4. Effects of NaB on the ratios of CD4$^+$ T cells-related gene expressions. The effects of NaB on the balance between different T cell subsets (Th1, Th2, Th17, and Treg) were determined by calculating the ratios of related cytokines and transcription factors. A1-A4: Th1/Th2 ratios; B1-B6: Th1/Treg ratios; C1-C4: Th17/Treg ratios; D1-D2: Th1+Th17/Th2+Treg ratios

cells. The reduction in T-bet/Foxp3 ratio in the presence of NaB was quite high, as no significant statistical differences remained between Mtb+NaB-treated (0.46±0.08) and untreated splenocytes (0.49±0.1, Figure 4 B1).

In the same way, RORc/Foxp3, IL-17/IL-10, and IL-17/(IL-10+TGFβ) ratios were determined as representatives of the Th17/Tregs ratio. The result showed that although NaB did not significantly reduce the ratio of RORc/Foxp3 in treated Mtb-stimulated cells (0.62±0.14, Figure 4 C1), it caused a significant decrease in the IL-17/IL-10+TGFβ ratio (0.76±0.12, p=0.008, Figure 4 C4).

We also evaluated T-bet+RORc/GATA3+Foxp3 and IFN-γ+TNF-α+IL-17/IL-4+IL-10+TGF-β ratios as representatives of Th1+Th17/Th2+Treg-related transcription factors and cytokines, respectively. Our results showed that NaB treatment of Mtb-stimulated splenocytes significantly decreased all these ratios (Figures 4 D1 and 4 D2).

DISCUSSION

Th1 and Th17 cells are critical T cell subsets in the development and pathogenesis of the RA,[2,18] while a protective role for Th2 and Tregs cells in RA pathogenesis has been reported.[2,18-20] Given the fact that most functions of Th1 and Th17-related disease worsening cytokines are opposed by Th2- or Treg-related cytokines, the study of ratios between RA promoting and RA protecting cytokines can provide more detailed information than the study of one-to-one cytokines. Accordingly, successful therapeutic agents for RA should also effectively shift the disease exacerbating Th1 and Th17 profile towards disease attenuating Th2 and Treg profile so that a significant decrease in the ratios of Th1 or Th17-related cytokines to Th2- or Treg-related cytokines can be observed. Therefore, in the present study, for the first time, we investigated whether NaB might be useful in the treatment of RA by investigating its capability in diverting antigen-specific Th1 and Th17 responses towards Th2 and Tregs in splenocytes of rats suffering from AIA.

Our results showed that ex vivo re-stimulation of splenocytes derived from a rat model of AIA with Mtb in the presence of 1000 µg/mL NaB induced significant decreases in the expression of Th1-related genes including T-bet, IFN-γ, and TNF-α as well as significant increases in the expression of Treg-related gens (Foxp3, TGF-β, and IL-10). Given the fact that Th1 related cytokines are critical in the development and pathogenesis of RA while Treg-related cytokines are protective, it might be conceivable that NaB can be considered as a potential therapeutic agent for RA. These results were also in accordance with those of Brahmachari et al who reported that NaB diminished the mRNA expression of IFN-γ and increased the mRNA expression of Foxp3 and IL-10 in myelin basic protein (MBP)-primed splenic T cells from experimental autoimmune encephalomyelitis (EAE) mice.[14] Our study also was in good agreement with Kundu et al study demonstrated TGF-β mRNA gene up-regulation by NaB in EAE mice[21] and with Yadav et al study which showed TNF-α down-regulation by NaB in lipopolysaccharide-stimulated splenocytes from Balb/c mice.[22]

In previous studies, the higher ratios of Th1/Th2,[23,24] Th1/Treg and Th17/Treg[18,25] responses have been reported in RA patients or AIA rats compared to normal humans and animals.[26,27] These ratios were also shown to be positively associated with the degree of disease severity in RA patients.[18,28] Accordingly, new therapeutic candidates for RA should be able to normalize the above-mentioned impaired balance of T helper cells. Based on our results, the ratios of Th1-/Th2-related genes (IFN-γ/IL-4 and T-bet/GATA3), Th1-/Treg-related genes (IFN-γ/TGF-β, IFN-γ/IL-10, T-bet/Foxp3) as well as Th17/Treg-related genes (IL-17/IL-10, IL-17/IL-10+TGF-β) and Th1-+Th17-/Treg-+Th2-related cytokines (IFN-γ+TNF-α+IL-17/IL-4+IL-10+TGF-β) were significantly reduced in splenocytes derived from AIA rats after *ex vivo* re-stimulation in the presence of NaB compared to those only re-stimulated with Mtb. Among Th1-related cytokines, TNF-α and IFN-γ play fundamental roles in the pathogenesis of RA through recruitment of inflammatory cells into the joint and promotion of cartilage and bone damage by activation of chondrocytes and osteoclasts.[29,30] In contrast to Th1, Th2-related cytokines protects bone and cartilage during arthritis through inhibition of osteoclast differentiation, bone loss, and cartilage damage.[31,32] The results of our study regarding the reducing effect of NaB on the ratio of Th1/Th2 related cytokines may suggest the potential ameliorative effects of NaB on immunopathogenic mechanisms involved in RA through decreasing Th1/Th2 balance.

Th17 cells have also shown to mediate synovitis and articular damage through induction of proinflammatory cytokines and matrix metalloproteinases as well as promoting osteoclasts development.[33,34] Although, IL-17 plays an important role in RA pathogenesis, in our study NaB not only did not reduce the expression of IL-17 gene but also insignificantly increased the expression of this gene in splenocytes treated with Mtb+NaB compared to those treated with Mtb. However, given the fact that the biological effects of IL-17 might subside by overexpression of IL-10 after exposure to NaB, the effect of NaB on the insignificant increase of IL-17 expression might not be biologically important. In this regard, our results showed a significant reduction in the IL-17/IL-10+TGFβ ratio by the treatment of Mtb-activated spleen cells with NaB (Figure 4 C4). It is also worth mentioning that the expression of RORc was unexpectedly higher in un-treated splenocytes compared to other treated cells (Figure 3 C1) while at the same time the expression of IL-17 was lower in this

group compared to others (Figure 3 D1). These results point to the need for additional studies to explain the cause of this controversy.

Regulatory T cells (Treg cells) play a crucial role in $CD4^+$ T-cell tolerance by releasing anti-inflammatory cytokines including IL-10 and TGF-β. These cytokines can regulate RA by sustaining the expansion of Treg cells, suppression of inflammatory responses,[35] and suppression of osteoclastogenesis.[36,37] Interestingly, our result showed that NaB increased the expression FoxP3, TGF-β, and IL-10 (Figure 3). In addition, the results of the present study showed that NaB significantly shifted the balance of Th1 and Th17 cytokines toward Treg, thereby reduced the ratios of Th1/Treg and Th17/Treg related cytokines. Thus, it can be suggested that NaB may abrogate the detrimental effects of Th1 and Th17 cells on the progression of AIA through the induction of Tregs-dependent cytokines.

In summary, the results of the present study have demonstrated that NaB significantly decreases the expression of Th1-related genes and increases the expression of Treg-related genes after *ex vivo* antigen-specific stimulation of splenocytes derived from arthritic rats. Our study also showed that NaB was effective in decreasing the impaired balance of Th1/Th2, Th1/Treg and Th17/Treg-, as well as (Th1+Th17)/(Th2+Treg)-related cytokines in splenocytes of arthritis rats after re-stimulation with Mtb. NaB is a nontoxic FDA-approved compound for humans and is widely used as a food preservative. Moreover, NaB can be administered through drinking water or food, the easiest route for taking medicine. The low price of NaB can also be considered as another advantage for this chemical compound to be used in the treatment of RA or other Th1-mediated diseases.[11,12,38,39] Though, checking the production of different cytokines at the protein levels and determining the frequency of each T cell subset by flow cytometry as well as checking the cytokine levels at the affected joints will be necessary to reach a conclusive result, based on the results of the present study NaB can be suggested as a useful therapeutic agent for the treatment of RA and other Th1 and Th17-mediated diseases. In addition, study the effect of NaB on the treatment of AIA in a larger group of animals and at different stages of the disease is recommended for future studies.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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