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Induction of Systemic Lupus Erythematosus-like Syndrome in BALB/c Mice Leads to Disturbance in Splenic T Cell Subpopulations

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ABSTRACT

Mechanisms underlying the systemic lupus erythematosus (SLE) have not yet been elucidated. In this study, we evaluated the balance of T cell subsets in BALB/c mice model of SLE induced; using Con A and polyamines as DNA immunogenicity modifiers.

BALB/c mice were immunized subcutaneously with 50 μg extracted DNA from cells cultured in different conditions: splenocytes+ polyamines (group P), splenocytes+ Con A (group A), splenocytes+ polyamines+ Con A (group PA) and splenocytes only (control). Anti-double-stranded DNA –(ds-DNA) antibodies, proteinuria, and antinuclear autoantibodies were assessed by enzyme-linked immunosorbent assay, Bradford method, and immunofluorescence respectively. Transcription factors of different T helper subsets were examined by real-time polymerase chain reaction.

The serum level of the anti-dsDNA antibody in group PA was higher than that in the other groups ($p>0.05$). Antinuclear antibody (ANA) titer increased in groups A and PA. Proteinuria level in group PA was significantly higher than that in the control group ($p<0.001$). Expression of *Foxp3* was decreased in group A ($p=0.001$). Additionally, the ratios of *T-bet/GATA3* and *T-bet/Foxp3* were also increased in group A. ($p>0.05$).

Our results revealed an increased ratio of Th1 to Th2 and decreased expression of *Foxp3* in group A, but group PA manifested more obvious signs of the disease. These results suggest that

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other mechanisms rather than disturbance in T cells' balance may involve the development of disease symptoms.

Keywords: Animal models; Regulatory T-lymphocytes; Systemic lupus erythematosus; Th1 cells; Th2 cells; Th17 cells

INTRODUCTION

Systemic lupus erythematosus (SLE) is considered as a prototype of systemic autoimmune diseases and is characterized by the production of auto-antibodies against the nuclear components such as double-stranded DNA (dsDNA), single-stranded DNA (ssDNA), and histones.[1] Deposition of immune complexes in different organs such as kidneys, skin, and CNS can cause inflammation, damage, and SLE-related complications.[2] Although the etiology of SLE has remained unclear; genetics context, environmental factors, and hormonal milieu might contribute to the disease development and severity.[3,4] SLE was previously considered as an autoantibody-mediated disease, but nowadays there are plenty of data denoting the direct and indirect role of T cells and their secreted cytokines in the pathogenesis of SLE.[5] Delayed and /or impaired clearance of apoptotic cells and their remnants by phagocytes can also trigger SLE. SLE is accompanied by an increased rate of apoptosis in different cells; during this process, dsDNA is exposed to surface blebs of the apoptotic cells[2,6] which can activate autoreactive T cells. Polyamines (Putrescine, Spermidine, and Spermine) are involved in many biologic processes, especially in cell proliferation and apoptosis. Polyamines change the conformation of DNA from physiologic native B-DNA to more immunogenic Z-DNA and can be involved in the pathogenesis of SLE.[7-9] Animal models are valuable tools to study different aspects of complicated diseases. Variousmurine models of spontaneous and inducible SLE have been established using different protocols and most of them used modified autoantigens as the main trigger.[10-12] In this study, we applied modified DNA (by Con A and/or Polyamines) prepared by using different protocols as the main trigger to establish animal models for SLE and evaluated changes in splenic T cell populations in these models.

MATERIALS AND METHODS

Mice

Female BALB/c mice between 6 and 8 weeks of age were purchased from Pasteur Institute Center of Experimental Animals and housed in a pathogen-free animal facility. All animal experiments were performed according to the Guide for the Care and Use of Medical Laboratory Animals (Approved by Ethics committee of Tehran University of Medical Sciences, Code: 89023010777).

Preparation and Culture of Spleen Cells

Spleens of the naive BALB/c mice were aseptically removed and cut in small fragments with a blade and then smashed by syringe plunge with chilled RPMI-1640 medium (Biosera, USA) in a plastic dish. The splenocytes were isolated by density gradient centrifugation; using Lymphoflot (Bio-Rad, Germany). The isolated cells were washed twice in RPMI-1640, erythrocytes were lysed using Tris–NH_4Cl, and the remaining cells were resuspended in RPMI-1640 with the addition of 10% fetal bovine serum (Gibco, USA) and 2 mM glutamine (Sigma, USA), 100 IU/mL penicillin G and 100 mg/mL streptomycin. The splenocytes were diluted to a final concentration of $2x10^6$ cells/mL. To increase the immunogenic properties of DNA, the splenocytes were cultured in four conditions. The first condition involved stimulation with Con A (Sigma, USA) at a final concentration of 5 µgr/mL for 48 hr (group A), the second with 20 µmoL/L of each of Polyamines (Putrescine, Spermine, and Spermidine) (Sigma, USA) for 18 h (group P), the third with both Con A and Polyamines (Putrescine, Spermine, and Spermidine) at the same concentrations and time as mentioned (group PA) and the fourth group was left without stimulation as the control group. For Con A we used the concentration suggested by Li H[13] and to get the final concentration of polyamines we tried series of dilutions to check the maximum tolerable concentration in in-vitro

splenocyte culture. We chose the concentration in which the total number of live cells was more than 50%.

DNA Preparation

Splenocytes were harvested and treated with proteinase K (Sigma, USA) according to the manufacturer's instructions, and then genomic DNA was purified using the Phenol– chloroform extraction method. DNA concentration and quality were determined by absorbance (A) measurement at 260 and 280 nm. The final A260/A280 for all the DNA preparations was>1.8.

Immunization

Syngeneic BALB/c mice were divided into four groups each including 7-8 mice and were actively immunized by subcutaneous injection on the back with 0.2 mL of an emulsion containing 50 µgr of DNA in 100 λ phosphate-buffered saline (PBS) plus 100 λ complete Freund's adjuvant (CFA), (Sigma, USA) (week 0). Two booster immunizations were performed at weeks 2 and 4 consisting of DNA and incomplete Freund's adjuvant. Blood and urine samples were drawn from mice after 2 weeks of the last immunization and at 2-weeks intervals until 6 weeks after the final boost.

Detection of Anti dsDNA Antibodies by Enzyme-linked Immunosorbent Assay (ELISA)

Total levels of anti-dsDNA antibodies were assessed; using an enzyme-linked immunosorbent assay (ORGENTEC Company, Germany). Briefly, sera were diluted at 1:300 in PBS and were added to each dsDNA coated 96-well plate and incubated for 30 min at 37ºC. Following the washing, horseradish peroxidase (HRP)-conjugated goat anti-mouse Ig secondary antibody (DakoCytomation, Denmark) diluted at 1:1000 in PBS was added. After 30-minute incubation and three times of washing, substrate solution of H_2O_2 and TMB were added. Finally, after adding HCl, the absorbance was measured at 490 nm using the microtiter plate reader (Hypersion, Japan).

Detection of Antinuclear Antibody (ANA) by Indirect Immunofluorescence (IIF) Assay

Sera from each group of mice were assessed by Indirect Immunofluorescence (IIF) assay for antinuclear antibodies. Briefly, Hep-2 ANA slides (Padtan Zist Pajooh, Iran) were incubated with different dilutions of sera for 30 minutes and then washed three times in PBS. Afterward, slides were incubated with FITC-conjugated goat anti-mouse Ig (Dako, Denmark). Hep-2 slides were examined by an expert under a fluorescence microscope (BX-40, Olympus, Japan).

Detection of Proteinuria

Proteinuria was measured by Bradford protein assay kit (Bio-Rad, USA). Bovine serum albumin (Sigma, USA) was used for drawing a standard curve. Urine collected from mice was centrifuged for 20 min at 4500 rpm; supernatants were taken and diluted at 1:10 with normal saline. Bradford stock solution was diluted 1/5. The optical density of the samples was observed at 595 nm and the concentrations of protein were calculated; using a standard curve.

Quantitative Real-time Polymerase Chain Reaction for *T-bet*, *GATA3*, *FOXP3*, and *ROR γt*

To quantify the expression of *T-bet*, *GATA3*, *FOXP3*, *ROR γt*, and *β-actin* as housekeeping genes, mRNA was extracted from splenocytes; using RNX^{TM}-Plus solution (Cinna Gen, Tehran, Iran) according to the manufacturer's instruction and checked for quality (OD 260/280). cDNA was synthesized by reverse transcriptase; using random hexamer in the presence of RNase inhibitor (all provided from Vivantis, Malaysia). Each cDNA sample was amplified by ABI step one plus System (Applied Biosystems); using Precision^{TM} 2X qPCR Mastermix (Primer Design, UK). The list of primers used for amplification is mentioned in table 1.

All primers were purchased from Metabion Company, Germany. Amplification was conducted in a total volume of 20 µL for 45 cycles, 15 seconds each at 95°C and 60 seconds at 60°C (two steps real-time PCR (RT-PCR)). Data analysis was performed; using Microsoft Excel (Microsoft Corporation). Gene expression was determined after using the normalized $2^{-\Delta\Delta CT}$ method. Melt curve analysis was performed to check the specificity of amplification.

Statistical Analysis

In this study, we used one-way analysis of variance (ANOVA), checked normality assumption with one sample Kolmogorov – Smirnov test, and homogeneity of variance with Levene´s test. We consider the statistical significance (p<0.05) in all of the tests. The data were analyzed using SPSS software version 16. The results are expressed as the mean±standard deviation (mean±SD).

Table1. The list of required primers used for amplification genes of interest.

Gene name	Forward primer (5'→3')	Revers primer(5'→3')
β-actin	ACGGCCAGGTCATCACTATTG-	CAAGAAGGAAGGCTGGAAAAGA-
T-bet	GCCAGGGAACCGCTTATATG	AACTTCCTGGCGCATCCA
Foxp3	GCAGGGCAGCTAGGTACTTGTAG	TCGGAGATCCCCTTTGTCTTATC
GATA3	GGTGGACGTACTTTTTAACATCGA	CGTAGCCCTGACGGAGTTTC
ROR γt	CAGCCAACATGTGGAAAAGCT	GGGAAGGCGGCTTGGA

RESULTS

Comparison of Anti dsDNA Ab Levels between Different Groups in SLE–induced mice

Isolated splenocytes from BALB/c mice were stimulated by Con A (group A), Polyamines (group P), and polyamines plus Con A (group PA). The extracted DNA from these cells (50 µg) was injected subcutaneously into syngenic mice. The control group (C) received equal amounts of the extracted splenocytes DNA without stimulation.

Production of autoantibodies against dsDNA was assessed; using enzyme-linked immunosorbent assay (ELISA) (Figure1). Comparing to other groups, the serum level of anti dsDNA antibodies in group PA was increased; however, this difference was not statistically significant (p>0.05)

Measurement of ANA by Indirect Immunofluorescence (IIF)

Sera from different immunized groups were assessed for ANA level; using quantitative IIF. The results showed that ANA titer was increased in group A and group PA respectively compared to the control group, but the difference was not significant (p>0.05) (Figure 2).

Assessment of Proteinuria in Different Groups

The urine of different immunized groups was investigated for the existence of protein by Bradford solution. We observed that the level of proteinuria in group PA and group P was significantly higher than the level in the control group (Figure 3).

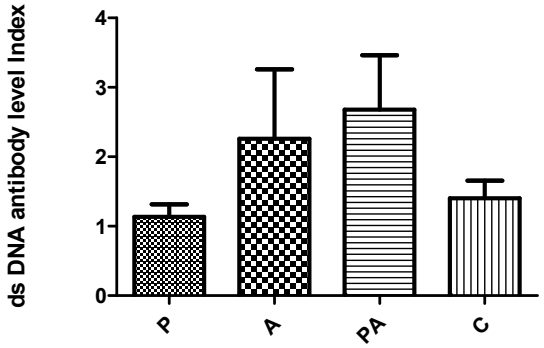

Figure 1. Comparison of serum anti-dsDNA antibody levels between different groups. Twenty-nine female BALB/c mice were immunized by subcutaneous injection with 50 µg DNA extracted from cells cultured in different conditions: splenocytes+ polyamines (P), splenocytes + Con A (A), splenocytes + polyamines + Con A (PA), and control (splenocytes only). Sera obtained 2, 4, and 6 weeks after the last injection were examined by enzyme-linked immunosorbent assay (ELISA). Data shown in the figure are the mean level of antibodies measured in three obtained samples. These differences were not statistically significant (p> 0.05).

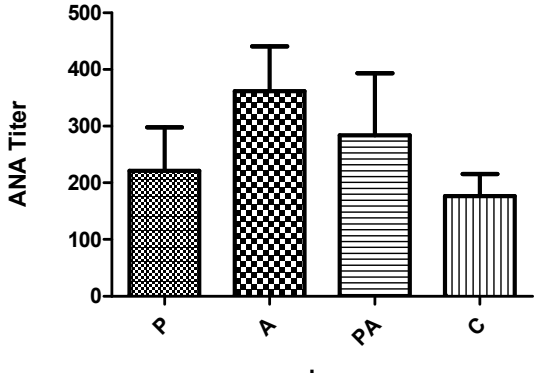

Figure 2. Comparison of serum anti-nuclear antibody levels between different groups. Twenty-nine female BALB/c mice were immunized by subcutaneous injection with 50 µg DNA extracted from cells cultured in different conditions: splenocytes+ polyamines (P), splenocytes + Con A (A), splenocytes + polyamines+ Con A (PA), and control (splenocytes only). Sera obtained 2, 4, and 6 weeks after the last injection was examined by Immunofluorescence. Data shown in the figure are the mean level of antibodies measured in three obtained samples. These differences were not statistically significant (p> 0.05).

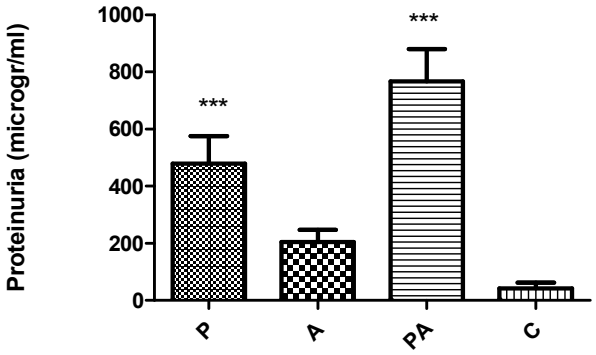

Figure 3. Comparison of urine protein levels between different groups. Twenty-nine female BALB/c mice were immunized with 50 µg DNA extracted from cells cultured in different conditions; splenocytes + polyamines (P), splenocytes + Con A (A), splenocytes + polyamines + Con A (PA), and control (splenocytes only). Urine samples were obtained 2, 4, and 6 weeks after the last injection and examined by a Bio-Rad protein assay kit. The data shown in the figure are the mean level of protein measured in three obtained samples. The result showed that the level of proteinuria in group PA and group P was significantly higher than the level in the control group ***p<0.001.

Quantitative Assay of *T-bet*, *GATA3*, *ROR γt*, and *Foxp3* mRNA Expressions

Splenocytes of different immunized groups were isolated 45 days after the last injection. *T-bet*, *GATA3*, *ROR γt*, and *foxp3* mRNA levels were analyzed; using quantitative RT-PCR. Our results showed that expression of *Foxp3* decreased in all groups in comparison with control, and this decrease in group A (immunized with DNA extracted from splenocytes+ Con A) was statistically significant (p<0.05). We calculated the ratio of *T-bet/GATA3*, *T-bet/Foxp3*, *GATA3/Foxp3*, and *ROR γt/Foxp3* expression. The ratio of *T-bet/GATA3* and *T-bet/Foxp3* in group A showed an increase compared to the control group but these differences were not significant (p>0.05) The ratios of *GATA3/Foxp3* and *ROR γt/Foxp3* were not notably different between the case and control groups (Figure 4. A-E).

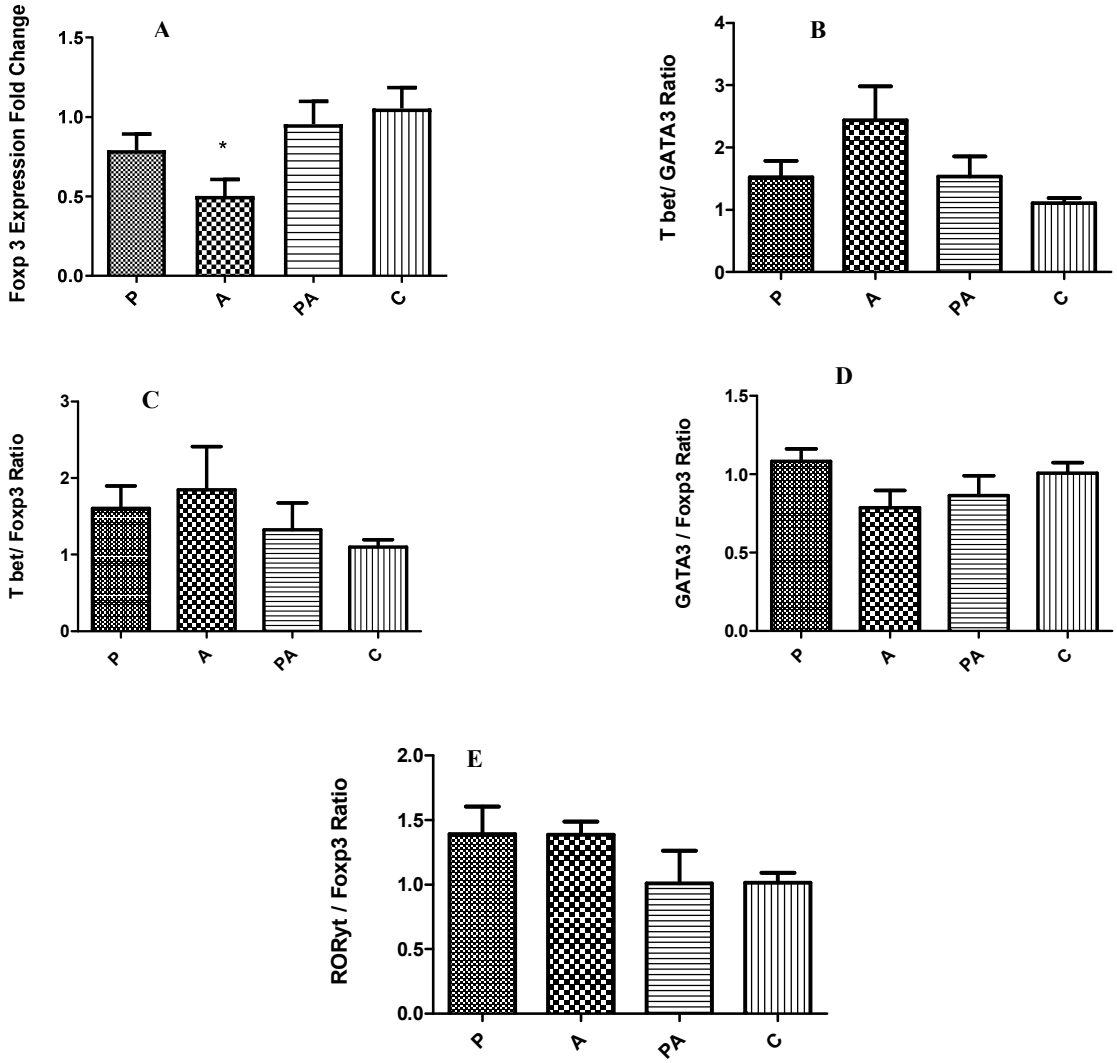

Figure 4. The ratio of gene expression in splenocytes from different groups: Twenty-nine female BALB/c mice were immunized with 50 µg DNA extracted from cells cultured in different conditions; splenocytes + polyamines (P), splenocytes + Con A (A), splenocytes + polyamines + Con A (PA), and splenocytes only (control). Splenocytes were isolated 6 weeks after the last injection and total RNA extracted and the expression of T-bet, GATA3, FOXP3, and ROR γt was evaluated. Expression of Foxp3 is demonstrated in figure 4A. Foxp3 decrease in group A was statistically significant (p=0.001). T-bet/ GATA3, T-bet/Foxp3, GATA3/Foxp3, and ROR γt/Foxp3 ratios are illustrated in figures 4B to 4E respectively. These differences were not significant (p>0.05).

DISCUSSION

SLE is a prototype of multi-system autoimmune disease with vague etiology. Autoantibodies rise against various components of nuclear antigens especially against dsDNA in SLE patients. Genetic susceptibility, environmental factors, and epigenetic effects are believed to be involved in the disease onset.[1,3,14-17]

Failure of self-tolerance mechanisms resulting from abnormal auto-antigen presentation mainly during apoptosis leads to autoantibody production against nuclear antigens and deposition of the immune complexes in different organs.[1,3,6,18]

Animal models (induced or spontaneous) are indispensable tools for the study of SLE pathogenesis. Spontaneous models that usually occur due to genetic defects are suitable to study the effects of genetic factors in the development of the disease[19-22]. Induced SLE mice models demonstrate SLE-like manifestations and help us to understand the influence of environmental and epigenetic factors in the development of SLE.[10,23,24] Although different protocols have been applied to reach the perfect SLE model, no standard procedure has yet been suggested.[10,13,23,24]

In the present study, we compared the potency of extracted DNA from splenocytes treated with Polyamines (P group), Con A (A group), or both (PA group) to induce SLE-like syndrome in BALB/c mice. Elevated levels of Polyamines in SLE patients and their role in cell proliferation and death has already been shown[7-9,12,25] and they inspired us to use them as an in-vitro modifier of DNA immunogenicity.

To evaluate the disease activity, proteinuria, anti-dsDNA, and ANA levels were measured as a standard protocol. Satoh et al reported that peritoneal injection of Pristane (2,6,10,14 tetramethylpentadecane), as a part of the monoclonal antibody production process in BALB/C mice, leads to the production of anti-su, anti-u1RNP, and anti-Sm antibodies.[26]

Li H and his colleagues induced SLE-like syndrome in BALB/C mice; using extracted DNA from Con A-stimulated splenocytes called activated lymphocyte-derived DNA (ALD-DNA).[13] They demonstrated that these mice produced autoantibody against dsDNA, ssDNA, and histone. Their induced model had proteinuria and histological defect in the kidney and they deduced that production of high-level auto-antibody was pathogenic. Our results show that the extracted DNA from Polyamines+ConA-treated splenocytes (PA) was more potent in the induction of SLE-like syndrome. The level of proteinuria in PA, P, and A groups were significantly higher than in the control group. A combination of polyamines and Con A (PA group) produced more potent immunogenic DNA as shown by the higher levels of anti-dsDNA antibody. Wen and colleagues demonstrated that DNA hypomethylation resulted from apoptosis in the ALD-DNA model can lead to augmentation of DNA immunogenicity.[27] We found out in this study, one of the proposed mechanisms of action for polyamines is, apoptosis induction and DNA hypomethylation[28,29] which can lead to more DNA immunogenicity. The group induced by extracted DNA from Con A stimulated splenocytes (group A) had the highest level of ANA. Although the results of the study by Li H et al are compatible with our findings; we extended the study using variable modification methods. Polyamines cause changes in DNA structure and Con A results in hypomethylation, two well-described phenomena that increase DNA immunogenicity. By simultaneous use of Con A and Polyamines we tried to check their synergetic pathogenic effects.

Recently more attention is attracted toward the role of T helper subpopulations mainly Th1, Th2, and Th17. The role of regulatory T cells in the maintenance of peripheral tolerance and preventing the occurrence of autoimmune diseases is also relatively well studied.[5,30-43]

In this study, we measured the T helper cells subset specific transcription factors in mRNA level by RT-PCR. We measured the expression of *T-bet*, *GATA3*, *Foxp3*, and *ROR γt* as indicators of Th1, Th2, Treg, and Th17 cells, respectively. Our results demonstrated that expression of Foxp3 in mRNA level was down-regulated in all groups and this decrease in group A was significant. This finding may explain the impaired role of regulatory T cells during the autoimmune process.[44]

We also evaluated the ratio of T helper subsets including Th1/Th2, Th1/Treg, Th2/Treg, and Th17/Treg using the specific transcription factors. Our results indicated that the ratio of T-bet/GATA3 and T-bet/foxp3 mRNA levels were increased in group A in comparison to other groups showing skewness of Th subsets towards Th1. Chan, R.W.Y et al[5] utilized urinary sedimentation in a patient with active lupus and demonstrated that mRNA and protein expressions of T-bet significantly were up-regulated in a patient with active nephritis.

The results of the study by Tshilela KAT, et al are following our findings. They measured Th subsets cytokines in glomeruli of lupus-prone MLR/lpr mice and showed that Th1 cytokines, especially tumor necrosis factor-alpha (TNF-α) have increased, while we examined Th transcription factors in splenocytes of induced models.[45]

Talaat RM et al [46] analyzed T helper subtype (Th1, Th2, Th17, and Treg) in patients by measuring plasma cytokines levels and showed altered balance in Th cells. In contrast to our results, they reported a decrease in Th1 cytokines. They also reported an increased Th17/Treg ratio in patients compared with healthy controls but we did not see any remarkable change in this proportion in the mice model. We also did not

observe a notable change in the ratio of Th2/Treg. It seems that controversy between Th1 and Th2 subsets in this disease still exists. With the emerging and elucidation of T Follicular Helper (TFH) subsets and their importance, more investigations in human and mouse models are needed.

Our results revealed an increased ratio of Th1 to Th2 and decreased expression of *Foxp3* in group A, but group PA manifested more obvious signs of the disease. These results suggest that other mechanisms rather than disturbance in T cells' balance may involve the development of disease symptoms.

CONFLICT OF INTEREST

The authors report no conflicts of interest.

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