Toll-like receptor-9 is involved in the development of B cell stimulating factor-induced systemic lupus erythematosus

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Received January 3, 2017; Accepted August 25, 2017

DOI: 10.3892/etm.2017.5411

Abstract. The objective of the present study was to investigate the role of Toll-like receptor (TLR)-9 in B lymphocyte stimulating factor (BLyS)-induced systemic lupus erythematosus (SLE) in mice. The anti-double stranded (ds)DNA antibody titer, levels of complement proteins (C3 and C4), interleukin (IL)-10 and the disease activity [assessed by the erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) level] were measured. A total of 21 transgenic female mice (aged 8-10 weeks and weighing 30-40 g) expressing the Epstein-Barr virus membrane antigen, BLLF1, were studied. Mice were randomly divided into the control, the BLyS inhibition and the TLR-9 inhibition groups, with 7 mice in each group. Mice in the blank control group received intraperitoneal injections of normal saline, mice in the BLyS inhibition group received intraperitoneal injections of anti-BR3 monoclonal antibody (5,000 ng/day) and mice in the TLR-9 inhibition group received intraperitoneal injections of anti-human TLR-9 antibody (250 ng/day). The treatment regimens continued for 10 days, followed by the collection of peripheral venous blood. The relative levels of TLR-9 mRNA were measured by reverse transcription-quantitative polymerase chain reaction. Furthermore, the BLyS protein concentration and IL-10 levels were measured by ELISA. TLR-9 mRNA, BLyS, IL-10, anti-dsDNA antibody titer, C3, C4, ESR and CRP levels of the blank control group were significantly higher than those of the other two groups ($P<0.05$). The differences in comparison of these indexes between the BLyS inhibition and TLR-9 inhibition groups were not statistically significant ($P>0.05$), with the exception of TLR-9 mRNA and BLyS. In conclusion, the TLR-9 signaling pathway may be important for BLyS-induced SLE, and regulation of the inflammatory immune level.

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Key words: toll-like receptor signal, B lymphocyte stimulating factor, systemic lupus erythematosus, signal pathway, double stranded DNA, interleukin-10

Introduction

Systemic lupus erythematosus (SLE) is a chronic, progressive and recurrent autoimmune disease, which affects multiple systems and organs of the body, including the skin, serosa, joints, kidneys and the central nervous system (1). It is characterized by the activation, proliferation and hyperfunction of B lymphocytes, as well as humoral and cellular immunity functional disorder (2). The majority of cases of SLE are diagnosed in females of childbearing age (3). SLE is a difficult disease to diagnose as it present with multiple nonspecific early symptoms, meaning it is not possible to detect SLE using a single laboratory test (4). In the majority of cases a confirmed diagnosis of SLE is only possible following organ damage (5). Children who are affected by SLE typically present with severe diseased states requiring special management (6). Older patients with SLE may have complicating co-morbid conditions, which makes treatment difficult (7). Extensive therapeutic advances have been made, over the last decade, however, treatment regimens are often long and there have been multiple previous reports of the use of ineffective drugs that do not target the desired site (8,9). There are several promising strategies that are being studied as potential novel treatments for SLE (10,11).

Animal and clinical studies of SLE pathogenesis have revealed that B lymphocyte stimulating factor (BLyS) may promote the activation and proliferation of B-lymphocytes, which leads to the production of large amounts of immunoglobulins and autoantibodies (12,13). Immunologic injury is caused by formation of immune complexes, complement-mediated cytolysis, opsonophagocytosis and antibody-mediated cell-dependent cytotoxicity (14). Toll-like receptors (TLRs) are a family of proteins that recognize an innate immunity pattern. They are widely expressed in various tissues and cells of the human body and are able to recognize and bind to conserved pathogen-associated molecules. This may trigger a series of signal transduction pathways that lead

to the release of inflammatory mediators, which may then activate acquired immunity. TLRs are regarded as a bridge between innate and acquired immunity (15). Furthermore, human B lymphocytes only express TLR-9 and TLR-10 (16). A study by Medzhitov *et al* (17) previously reported that TLRs regulate the activation of B lymphocytes and antibody production *in vivo*. Based on these observations, the aim of the present study was to investigate the TLR-9 signal transduction pathway in BLyS-induced SLE in transgenic mice.

Materials and methods

SLE transgenic mice. In total, 21 transgenic female mice (aged 8-10 weeks and weighing 30-40 g) expressing the Epstein-Barr virus membrane antigen, BLLF1, were used for experiments. Mice were purchased from the Laboratory Animal Research Center, Peking University Health Science Center (Beijing, China). Mice were fed as usual for 1 week, after which experiments were performed. Mice were kept in standard air-conditioned rooms, under a 12 h light/dark cycle, maintained at 25˚C in 40-60% humidity with food and water available *ad libitum*. The present study was approved by the Medical Ethics Committee of Hainan General Hospital (Haikou, China).

Methods. Mice were randomly divided into the blank control, BLyS inhibition and TLR-9 inhibition groups, with 7 mice in each group. The mice in the blank control group received intraperitoneal injections (0.5 ml) of normal saline (0.90% w/v NaCl in water), mice in the BLyS inhibition group received intraperitoneal injections of anti-BR3 monoclonal antibody (cat. no. D201-3; 5,000 ng/day for 10 days; Beijing Hanpu Medical Biology Research Institute, Beijing, China), and mice in the TLR-9 inhibition group received intraperitoneal injections (250 ng/day for 10 days) of anti-human TLR-9 antibody (1:50 dilution; cat. no. IMG-305a; Imgenex; Novus Biologicals, LLC, Littleton, CO, USA). Peripheral venous blood was collected prior to intervention and after maintaining the mice on normal feed for 10 days. The relative levels of TLR-9 mRNA were measured by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The BLyS protein concentration and IL-10 level were measured by ELISA (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The anti-double stranded (ds)DNA antibody titer was measured using a dot blot assay, which determines whether an antibody-based detection system would work effectively. Purified bovine serum albumin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and the test sample were spotted onto the membrane and the membrane was subsequently incubated with the appropriate primary (25˚C for 4 h) and secondary antibodies (4˚C for 24 h), to determine whether a signal could be detected. The antibodies used were obtained from the following kits: The complement C3 and C4 levels were estimated using Abcam Complement ELISA kits (cat. nos. ab157711 and ab108824; Abcam, Cambridge, UK). The erythrocyte sedimentation rates (ESRs) were measured using an ESR kit (cat. no. 21200213; Thermo Fisher Scientific, Inc.) and C-reactive protein (CRP) levels were measured using an ELISA kit (cat. no. RAB0096; Sigma-Aldrich; Merck KGaA).

RT-qPCR. The blood was centrifuged at 1,006.2 x g for 20 min at 4˚C (Beijing Liuyi Instrument Factory, Beijing, China), the serum was isolated and an equal volume of lymphocyte separation solution (Shanghai Yanjin Biology & Science Co., Ltd., Shanghai, China) was added to isolate the mononuclear cells. Total RNA was extracted with TRIzol (Sigma-Aldrich; Merck KGaA). 1.1% agarose gel electrophoresis and visualized using ethidium bromide (Thermo Fisher Scientific, Inc.) and an ultraviolet spectrophotometer (Thermo Fisher Scientific, Inc.) were used to measure the quality and concentration of the total RNA, respectively. Furthermore, RNA was treated with RNAase A (Sigma-Aldrich; Merck KGaA) and stored at -80˚C. Following this, cDNA was synthesized according to the manufacturer's protocol of the reverse transcription kit (Fermentas; Thermo Fisher Scientific, Inc.). Primers were designed by Shanghai Shengong Biology Co., Ltd., (Shanghai, China). The PCR machine used was from Shanghai Sanke Instrument Co., Ltd. (Shanghai, China). The primer sequences were as follows: TLR-9 forward, 5'-TGGATACGTTTCCTTATAAG-3' and reverse, 5'-GAAATG GAGGCACCCCTTC-3' (418 bp); and β-actin (internal control) forward, 5'-ATCATGTTTGAGACCTTCAACA-3' and reverse, 5'-CATCTCTTGCTCGAAGTCCA-3' (300 bp). For qPCR, the reaction system included 2 μl cDNA template, 0.5 μl of each primer, 9 μl 2.5X Real Master mix, 9 μl 20X SYBR solution (Thermo Fisher Scientific, Inc.) and water to a total volume of 20 μl. The thermal profile was as follows: Pre-degeneration at 95˚C for 2 min, degeneration at 95˚C for 45 sec, 60˚C for 20 sec and 75˚C for 60 sec for a total of 30 cycles, and with an extension at 72˚C for 5 min. Each sample was detected three times, and the mean values were obtained as the target gene expression as determined by the relative quantification method ($2^{-\Delta\Delta Cq}$) (18). Following the reaction, the specificity of primers was analyzed using melting curves, and 1.1% agarose gel electrophoresis was performed to identify the PCR amplification products.

Other indexes. ELISA (cat. no. A20180) and an Immunogold Labeling kit were purchased from Invitrogen (Thermo Fisher Scientific, Inc.) and used according to the manufacturer's protocols. The automatic biochemical analyzer, AU5800, was purchased from Beckman Coulter, Inc. (Brea, CA, USA).

Statistical analysis. SPSS 20.0 software (IBM Corp., Armonk, NY, USA) was used for statistical analysis. The measurement data were presented as the mean ± standard deviation. Comparisons between groups were performed by one-way analysis of variance followed by Fisher's least significant difference method. Comparisons within groups were performed using the paired t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Comparison of the relative expression of TLR-9 mRNA. The differences in the levels of TLR-9 mRNA between the three groups prior to intervention were not statistically significant. There was no significant alteration to the TLR-9 level in the control group following intervention. However, the levels of TLR-9 mRNA in the BLyS and TLR-9 inhibition groups significantly decreased following intervention compared with their levels prior to intervention ($P<0.05$), the levels of TLR-9

Table I. Relative mRNA expression of TLR-9 mRNA in the different groups.

Group	TLR-9 mRNA expression level		t-value	P-value
	Before intervention	After intervention		
Control	0.4547±0.0219	0.4562±0.0315	-0.089	0.932
BLyS inhibitor	0.4627±0.0311	0.2581±0.0270	12.845	<0.01
TLR-9 inhibitor	0.4630±0.0248	0.1513±0.0311	22.792	<0.01
F-value	0.226	187.009	-	-
P-value	0.800	<0.01	-	-

BLyS, B lymphocyte stimulating factor; TLR-9, Toll-like receptor-9.

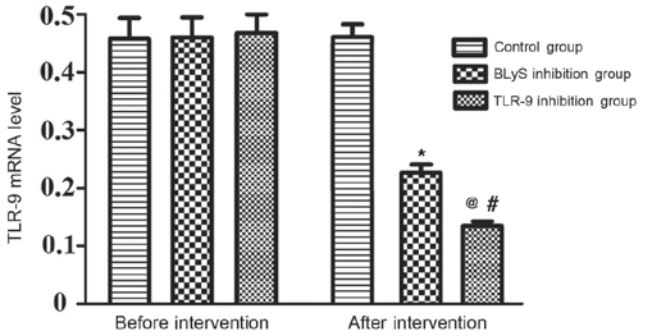

Figure 1. Comparison of the relative expression of TLR-9 mRNA. *P<0.05 vs. the BLyS inhibition group prior to intervention; #P<0.05 vs. the TLR-9 inhibition group prior to intervention; @P<0.05 vs. the BLyS inhibition group after intervention. BLyS, B lymphocyte stimulating factor; TLR-9, Toll-like receptor-9.

mRNA in the TLR-9 inhibition group were significantly lower than those in the BLyS inhibition group following intervention (P<0.05) (Table I and Fig. 1).

Comparison of the levels of BLyS and IL-10. The differences in the levels of BLyS and IL-10 in the three groups prior to intervention were not statistically significant (P>0.05). Additionally, there was no significant change in the levels in the control group following intervention. The BLyS protein concentration and IL-10 level of the BLyS and TLR-9 inhibition groups significantly decreased following intervention compared with the levels before intervention (P<0.05). The differences between the BLyS and TLR-9 inhibition groups were not statistically significant prior to intervention (Table II and Fig. 2). However, following intervention there was a significant difference between the IL-10 expression in the BLyS and TLR-9 inhibition groups (P<0.05).

Comparison of anti-dsDNA, C3, C4, ESR and CRP levels. The differences in the levels of anti-dsDNA antibody, C3, C4, ESR and CRP levels of the three groups prior to intervention were not statistically significant. Furthermore, there were no significant alterations to these levels observed in the control group following intervention. The above indexes in the BLyS and TLR-9 inhibition groups decreased significantly following intervention compared with the levels before intervention (P<0.05). Furthermore, the differences in the levels of the above indexes between the BLyS and TLR-9 inhibition groups following intervention were not statistically significant (Table III and Fig. 3).

Discussion

Mice with congenital deficiency of BLyS exhibit a reduced number of B lymphocytes and decreased levels of immunoglobulin (19). By contrast, B lymphocytes in transgenic mice with overexpression of BLyS increase in number and may lead to hyperimmunoglobulinemia (20). In patients with SLE, various high-titer autoantibodies, such as anti-dsDNA, have been detected in circulation, and the level of immune complexes has been demonstrated to increase, as well as the immunoglobulin deposited in the kidney (21). BLyS antagonists may be used to inhibit progression and improve the survival rate of SLE (22). In the present study, it was revealed that the levels of BLyS in the plasma or serum of patients with SLE were significantly higher than those of the control subjects. Additionally, the biological activity of BLyS in circulation was significantly higher compared with the control group, and was closely associated with anti-dsDNA antibody titer, disease activity, ESR, CRP levels, and serum immunoglobulin G (23). A previous study indicated that BLyS may block the expression of apoptosis-related genes in B lymphocytes downstream of signals from the B cell receptor (BCR) (24). Furthermore, TLR signaling was demonstrated to serve an indispensable role in BLyS transgenic mice by upregulating the expression of anti-apoptotic genes, including cluster of differentiation (CD)40 (25).

A total of 13 members of the TLR family have been identified (26). Lipopolysaccharide (LPS) from the wall of Gram-negative bacteria is the main ligand of TLR-9 (27). Additionally, TLR-9, CD14, myeloid differentiation protein 2 and LPS binding protein combine to form the LPS recognition receptor complex, with a high affinity and signal transduction function (28). The LPS recognition receptor may cause the translocation of nuclear factor-κB (NF-κB) from the cytoplasm to the nucleus by myeloid differentiation protein 88 (MyD88)-dependent or independent signaling pathways and bind with the NF-site in the promoter region of inflammatory response regulator genes, promoting the initiation of transcription and translation of genes encoding inflammatory cytokines, as well as the large release of cytokines (29). The immune response of the organism is thereby initiated (30).

Table II. Protein expression of BLyS and IL-10 in the different groups.

Group	BLyS, μg/l				IL-10, pg/ml			
	Before intervention	After intervention	t-value	P-value	Before intervention	After intervention	t-value	P-value
Control	49.6±3.4	47.4±2.8	1.630	0.154	273.1±24.7	292.8±32.1	-1.342	0.228
BLyS inhibitor	52.1±3.7	13.1±1.6	21.726	<0.01	290.3±35.3	171.8±16.5	8.355	<0.01
TLR-9 inhibitor	53.2±1.9	26.5±1.6	33.203	<0.01	275.0±31.3	182.3±38.7	4.983	0.002
F-value	2.451	493.896	-	-	0.661	33.730	-	-
P-value	0.114	<0.01	-	-	0.528	<0.01	-	-

BLyS, B lymphocyte stimulating factor; TLR-9, Toll-like receptor-9; IL-10, interleukin-10.

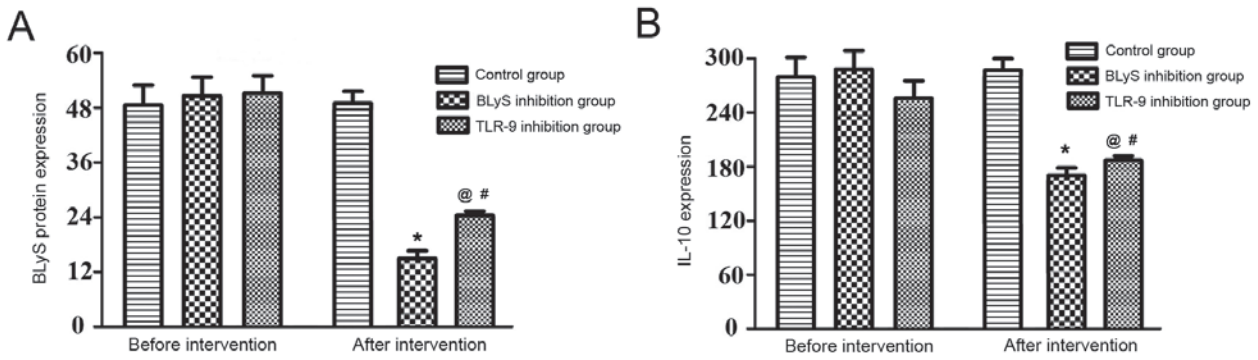

Figure 2. Comparison of the relative levels of (A) BLyS and (B) IL-10. *P<0.05 vs. the BLyS inhibition group prior to intervention; #P<0.05 vs. the TLR-9 inhibition group prior to intervention; @P<0.05 vs. the BLyS inhibition group after intervention. BLyS, B lymphocyte stimulating factor; TLR-9, Toll-like receptor-9; IL, interleukin.

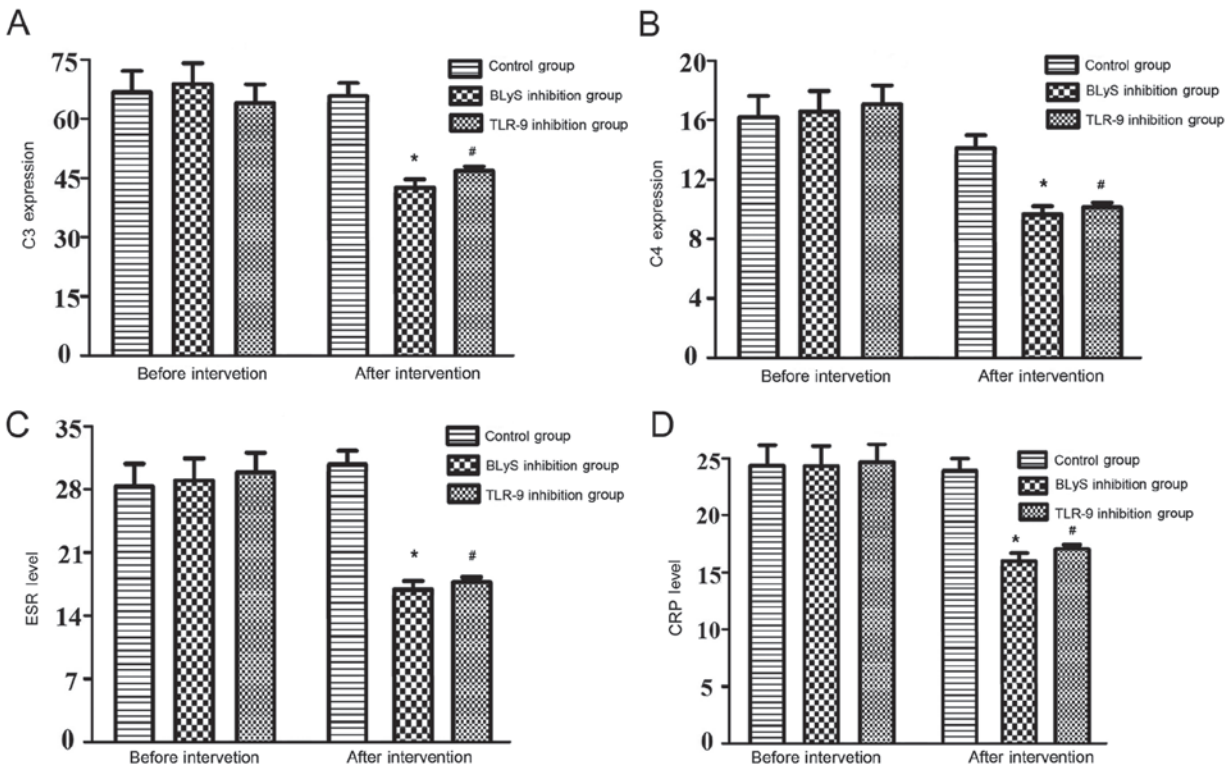

Figure 3. Comparison of anti-dsDNA, (A) C3, (B) C4, (C) ESR and (D) CRP relative levels in the different groups. *P<0.05 vs. the BLyS inhibition group prior to intervention; #P<0.05 vs. the TLR-9 inhibition group prior to intervention. BLyS, B lymphocyte stimulating factor; TLR-9, Toll-like receptor-9; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; C3, complement 3; C4, complement 4.

Table III. Levels of anti-dsDNA antibody, C3, C4, ESR and CRP in the three groups.

Group	Level of anti-ds DNA		C3, mg/ml		C4, mg/ml		ESR, mm/h		CRP, mg/l	
	Before intervention	After intervention	Before intervention	After intervention	Before intervention	After intervention	Before intervention	After intervention	Before intervention	After intervention
Control	1:16	1:14	69.3±4.7	69.6±2.7	16.1±1.7	14.7±1.2	27.7±3.2	29.1±2.5	24.1±3.6	24.1±2.3
BLyS inhibitor	1:18	1:7	70.6±3.8	45.7±4.0	16.7±2.2	8.6±0.6	30.8±2.8	16.6±3.2	23.6±2.2	16.2±2.4
TLR-9 inhibitor	1:15	1:6	67.5±1.8	48.6±1.4	17.6±1.1	8.7±0.5	29.5±2.6	18.9±2.0	24.1±2.2	17.1±2.0
F-value	0.865	56.324	1.245	143.790	1.334	129.512	2.014	45.910	0.086	25.893
P-value	0.423	<0.01	0.312	<0.01	0.288	<0.01	0.162	<0.01	0.918	<0.01

BLyS, B lymphocyte stimulating factor; TLR-9, Toll-like receptor-9; dsDNA, double stranded DNA; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; C3, complement 3; C4, complement 4.

When BCR and CD40 are combined or triggered by CpG DNA, TLR expression increases (31). Furthermore, the induced expression of TLRs on B lymphocytes may serve a role in the pathological process of autoimmune diseases (32). A study by Marshak-Rothstein *et al* (33) reported that B lymphocytes that express membrane-bound immunoglobulin M rheumatoid factor may be activated by chromosome-chromosome antibody immune complexes through the TLR9-MyD88 dependent pathway. The chromosome-chromosome antibody immune complex may then be endocytosed into the endoplasmic reticulum through BCR-mediated mechanisms, which then transmit signals by TLR9 that is expressed in the endoplast. Therefore, TLRs on B lymphocytes connect innate immunity with autoimmunity (34).

The differentiation and activation of helper T cells is not sufficient for T cell-dependent activation of B lymphocytes. In addition to the assistance of $CD4^+$ T cells, antigen-specific T cell-dependent antibody responses require the activation of TLRs on B lymphocytes (35). The function of TLRs on B lymphocytes may assist BCR to identify antigens of microbial origin, and assist with the anti-infection response (36).

Cytokines serve a critical role in regulating disease activity and organ injury in SLE. Of these cytokines, IL-10 is predominantly produced by mononuclear macrophages, fibroblasts and endothelial cells and functions to stimulate the maturation of B-lymphocytes and the secretion of immunoglobulins (37). A study in New Zealand Black and New Zealand White mice revealed that IL-10 directly caused the pathogenesis of SLE (38), indicating that the rise of exogenous IL-10 *in vitro* may lead to increased levels of immunoglobulin G and anti-dsDNA antibodies that are produced by B lymphocytes of old B/W mice, and may reduce albuminuria as well as the fatality rate. A previous study reported that IL-10 is highly and spontaneously expressed in the peripheral blood of patients with lupus, and is associated with disease activity (39). Lymphocytes isolated from patients with SLE may spontaneously increase IL-10 production *in vitro*, and anti-IL-10 may reduce the anti-ds-DNA level (40). Furthermore, multiple models of lupus have demonstrated the positive therapeutic effects of IL-10 and IL-10 receptor antagonists (41). In addition to inhibiting the ultimate IL-10 output, inhibition of the source of IL-10 production is an attractive concept.

At present, there are three types of murine model of SLE: Spontaneous, artificial induction and gene regulation types (42). The spontaneous type has a specific genetic background and good genetic stability, which is of great significance in the studies of genetic factors that affect SLE (43). The artificial induction type is suitable for short-term studies, and the majority of the mice succumb to the disease ~5 months after induction of SLE. Mice of the gene regulation type, including transgenic and knockout mice, may be used to perform genetic level analyses for studies on the mechanism of SLE (44).

In the present study, it was concluded that TLR-9 mRNA, BLyS, IL-10, anti-dsDNA antibody titer, C3, C4, ESR and CRP levels of the blank control group were significantly higher than those of the other two groups. These results are consistent with the findings of previous studies (45-48). Additionally, the difference in comparison of the above indexes between the BLyS and the TLR-9 inhibition groups were not statistically significant, with the exception of TLR-9 mRNA and BLyS.

This implied that TLR-9 represents an important signaling pathway that may regulate the inflammatory immune level for BLyS-induced SLE. Therefore, inhibiting TLR-9 or BLyS expression may inhibit the process of autoimmune injury in SLE.

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