Inhibiting Notch-1 reduces the expression of Toll-like receptor 9 in BABL/C-lpr mouse kidneys and improves glucocorticoid sensitivity

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Abstract. Systemic lupus erythematosus (SLE) is a chronic autoimmune disease, characterized by the development of a pathogenic autoantibodies. Lupus nephritis is a major cause of mortality in patients with SLE. Glucocorticoids are used for the treatment of lupus, however, corticosteroids have no effect on the expression of Toll-like receptor 9 (TLR9), which may limit response to corticosteroids in certain patients with SLR. The expression of TLR9 can be used as a predictor of glucocorticoid response in patients with active SLE. The present study analyzed urine proteins and pathological kidney sections of BABL/C-lpr mice and found that, following the inhibition of Notch1, glucocorticoid treatment improved the symptoms of lupus nephritis. Furthermore, glucocorticoid treatment reduced the expression of TLR9 in the BABL/C-lpr mouse kidneys, according to immunohistochemical and western blot analyses. These results suggested that inhibition of the expression of Notch-1 enhanced corticosteroid sensitivity in BABL/C-lpr mice.

Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease, characterized by the development of autoantibodies. SLE can damage every physiological system, including the skin, joints and kidneys. Lupus nephritis is a major cause of mortality in patients with SLE, and ~60% of patients with SLE develop clinically relevant lupus nephritis during the course of the disease (1,2).

CD4+/CD8+ T cells, antigen presenting cell, dendritic cells and macrophages are involved in the development of SLE. Previous studies have demonstrated that the Notch signaling pathway is involved in the function, proliferation and differentiation of these cells (3-9). It has been reported that miR-23b promotes the tolerogenic properties of ovalbumin-challenged dendritic cells (DCs) *in vitro* via inhibition of the Notch1/nuclear factor κB signaling pathways (10). Notch signaling also facilitates the CD8+ T cell lineage (11). Naive CD4+ T cells can differentiate into Th1, Th2, Th17 or regulatory T cells (12), and the expression of Notch ligands on antigen-presenting cells (APCs) is induced by Th1- or Th2-promoting stimuli (13). Accumulating evidence indicates that Notch and its ligands may be important mediators of T cell differentiation in SLE (9,14).

Notch signaling is a well-conserved signaling pathway in evolutionary terms. It includes four cell surface reporters (Notch1-4), which regulate the differentiation, maturation and function of a wide array of cell types (15). Whilst Notch signaling is involved in tissue and organ formation during embryonic development, evidence indicates that it continues to affect developmental processes postnatally and is implicated in the pathogenesis of human disorders, including cancer and autoimmune diseases (16).

Previous studies have demonstrated that the inhibition of Notch1 signaling is a potential therapeutic approach for SLE (17). Chemical inhibition of all four Notch receptors in response to nonspecific γ-secretase inhibitors suppresses Th1- and Th17-type differentiation and ameliorates signs of autoimmunity and renal damage in lupus-prone MRL-lpr mice (18). A previous study demonstrated significantly lower levels of Notch-1 in T cells from patients with clinically active SLE, compared with healthy patients, at the mRNA and protein levels (19).

The present study aimed to investigate the effects of the Notch signaling inhibitor, DAPT, and glucocorticoids, in combination and alone on lupus nephritis in BABL/C-lpr mice. Recognition of self nucleic acids by Toll-like receptors (TLR9) on B cells and plasmacytoid dendritic cells (PDCs) is an important step in the pathogenesis of SLE (20). Therefore, he present study used immunohistochemical and western blot analyses in order to examine changes in the expression of TLR9 in the kidneys of BABL/C-lpr mice, following treatment with glucocorticoids and DAPT.

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Materials and methods

Ethical approval. The procedures used in the present study were performed in accordance with the Regulations for the administration of affairs concerning experimental animals (1998, China). The procedures were approved by the Committee on the Ethics of Animal Experiments of Central South University (Changsha, China).

Mice. A total of 28 4 week-old female BABL/C-lpr mice (SYXK (xiang) 2011-0001) were obtained from the Department of Animal Experiments, Central South University. The mice were housed in a specific pathogen-free room under controlled temperature (22˚C) and humidity, and underwent a 12 h light/dark cycle. The experiments were performed according to the Guide for the Care and Use of Medical Laboratory Animals (Ministry of Health, People's Republic of China, 1998), with approval from the Shanghai Medical Laboratory Animal Care and Use Committee and the Ethical Committee of Central South University (Changsha, China).

Generation of the SLE murine model. The mice were randomly divided into four groups (n=7): Model 1, BABL/C-lpr mice without treatment; Model 2, BABL/C-lpr mice treated with DAPT (Selleck Chemicals, Houston, TX, USA); Model 3, BABL/C-lpr mice treated with prednisone (Xianju Company, Xianju, China); and Model 4, BABL/C-lpr mice treated with prednisone following treatment with DAPT. BABL/C-lpr mice develop nephritis, which is similar to that observed in humans with SLE (21). At 8 weeks of age, all of the mice were injected with 0.5 ml pristane (2,6,10,14-tetramethylpentadecane; Sigma-Aldrich, St. Louis, MO, USA). Following 24 weeks of pristane treatment, serum (20 μl) was obtained from the mice via retro-orbital bleeding. The serum samples were then frozen at the time of collection and were analyzed for mouse anti-double stranded DNA (anti-dsDNA) immunoglobulin (Ig)G-specific antibodies and anti-nuclear antibody IgM using a 96-well quantitative enzyme-linked immunosorbent assay (ELISA) kits from Alpha Diagnostic International (Department of Rheumatism, Xiangya Hospital, Changsha, China).

DAPT treatment. DAPT was prepared using reconstituting DAPT powder with 100% ethanol (EtOH) and stored as a stock solutions, at -20˚C. Each day, fresh stock solution was diluted in a mixture of corn oil and EtOH to a final concentration of 5% EtOH and 95% corn oil, according to previously described methods (21). The experiments were performed under an Institutional Animal Care and use Committee-approved protocol.

Following pristane treatment for 24 weeks, the Model 4 mice were treated with DAPT (5 mg/kg $day^{-1;}$ 5 days/week) for 8 weeks, Model 3 mice were treated with prednisone (9 mg/kg day^{-1}) for 4 weeks. After 4 weeks, the treatment in Model 4 was changed to prednisone for a further 4 weeks (9 mg/kg day^{-1}). All drugs were administered intragastrically.

The levels of urine protein were measured prior to and following 8 weeks of treatment. Urinary collections (24 h) were obtained at weeks 24 and 32. Albuminuria was measured in the urine collections using a Mouse Albumin ELISA Quantification kit (Lanpai Company, Shanghai, China).

Histological renal injury. Following completion of the treatment period, the mice were sacrificed by cervical dislocation and the kidneys were removed and fixed in 4% formalin. The kidney sections (1x1 cm^2) were stained using hematoxylin and eosin (Sbjbio Company, Nanjing, China) and the histopathology was assessed by a pathologist in a blinded-manner.

Glomerular TLR9 immunostaining. The levels of glomerular TLR9 were assessed in 6-μm frozen kidney sections using rabbit polyclonal antibody conjugated to TLR9 IgG (1:400; Abcam, Shanghai, China). Scores were assigned based on the intensity of the expression of IgG.

Western blot analysis. Whole, cytoplasmic and nuclear protein extractions and western blot analyses were performed, according to previously described methods. The antibodies used were Rabbit polyclonal TLR9 IgG (2 μg/ml; 1:200; cat. no. ab13928; Abcam) and goat anti-mouse IgG-horseradish peroxidase (cat. no. sc-2970; Santa Cruz Biotechnology, Inc., Dallas, TX, USA).

Statistical analysis. All data are presented as the mean ± standard deviation. Comparative analysis was performed for the protein expression levels of TLR9. A two-tailed Mann Whitney T-test was used to determine the presence of significant differences between the Models. $P<0.05$ was considered to indicate a statistically significant difference. Analysis of the western blotting was performed using GraphPad Prism (Version 5) software (GraphPad Software, Inc., La Jolla, CA, USA) and the immunostaining was analyzed using Image-Pro® Plus software (Media Cybernetics, Inc., Rockville, MD, USA) following microscopy (CKX41; Olympus Corporation, Tokyo, Japan).

Results

Notch inhibition improves nephritis in BABL/C-lpr mice. BABL/C-lpr mice develop nephritis in a similar way to humans with SLE (22). In order to determine the effect of inhibition of Notch on lupus nephritis, pristane-induced BABL/C-lpr mice were treated with DAPT for 8 weeks and urine protein levels were measured and compared between 12 treated and 12 control mice. The urine protein levels of the mice treated with DAPT decreased significantly compared with the control mice, according to ANOVA ($P<0.001$; Fig. 1).

Renal pathobiology was identified as a diagnostic factor for lupus nephritis (Fig. 2). The pathological results demonstrated a marked improvement in the renal structures of the mice treated with DAPT, indicating that DAPT treatment effectively inhibited lupus nephritis in the BABL/C-lpr mice. The response to glucocorticoids increased following Notch inhibition. Following DAPT treatment, the effect of glucocorticoid treatment increased, improving lupus nephritis in the BABL/C-lpr mice. The urine protein levels in the mice treated with DAPT and glucocorticoid were lower than those in other Model groups. The pathological results suggested that the kidneys from the DAPT and glucocorticoid-treated mice

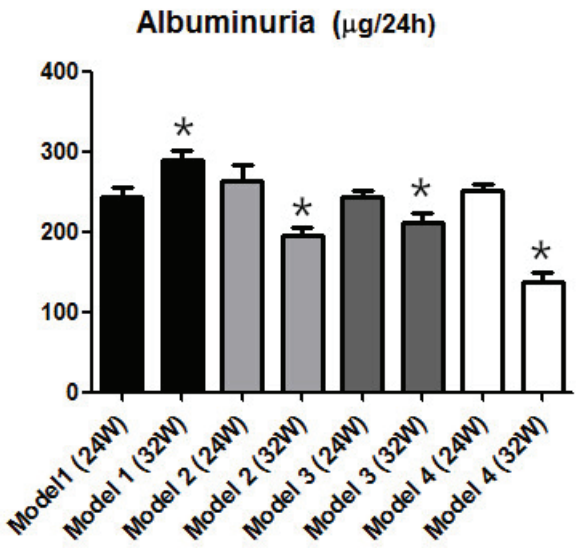

Figure 1. Following pristane treatment of lpr-BABL/C mice for 24 weeks, Model 2 mice were treated with the Notch-1 inhibitor, DAPT. Model 3 mice were treated with glucocorticosteroid and Model 4 mice were treated with glucocorticosteriod, followed by DAPT after 4 weeks. Urine protein levels in Model 4 mice decreased markedly, compared with the other groups following 8 weeks of treatment (at 32 weeks; *P<0.05).

exhibited structural improvement, compared with the other Model groups.

The expression of TLR9 can be used to predict glucocorticoid response in patients with SLE (17). Therefore, the expression of TLR9 in the kidneys of the BABL/C-lpr mice were examined. The immunohistochemical (Fig. 3) and western bolt analyses (Fig. 4) suggested that the expression of TLR9 in kidneys of the mice treated with DAPT and glucocorticoid was markedly decreased, compared with the other treatment groups. These results suggested that DAPT may improve the response to glucocorticoids in lupus nephritis by reducing the expression of TLR9. There may be an association between DAPT and TLR9.

Discussion

TLRs are pattern recognition receptors in the innate immune system, which recognize specific pathogen-associated molecular patterns, conserved among micro-organisms (23). A total of 12 TLRs have been identified in a number of autoimmune diseases, including rheumatoid arthritis and SLE, exhibit increased expression levels in patients with SLE (24,25). The expression of TLR9 is increased in patients with SLE (26), and the SLE disease activity index (SLEDAI), and expression levels of anti-dsDNA, anatoxin-a synthetase and TLR9 are significantly higher in patients with active SLE, with the expression of TLR9 significantly higher in steroid-resistant, compared with steroid-sensitive blood samples prior to treatment with corticosteroid. Positive correlations have also been observed between the expression of TLR9, the SLEDAI score and anti-dsDNA, and negative correlations have been observed between the expression of TLR9 and the expression levels of C3 and C4 (27). In the present study, the development of lupus nephritis was observed in mice treated with DAPT. Specifically, the proteinuria and renal histopathology of the mice treated

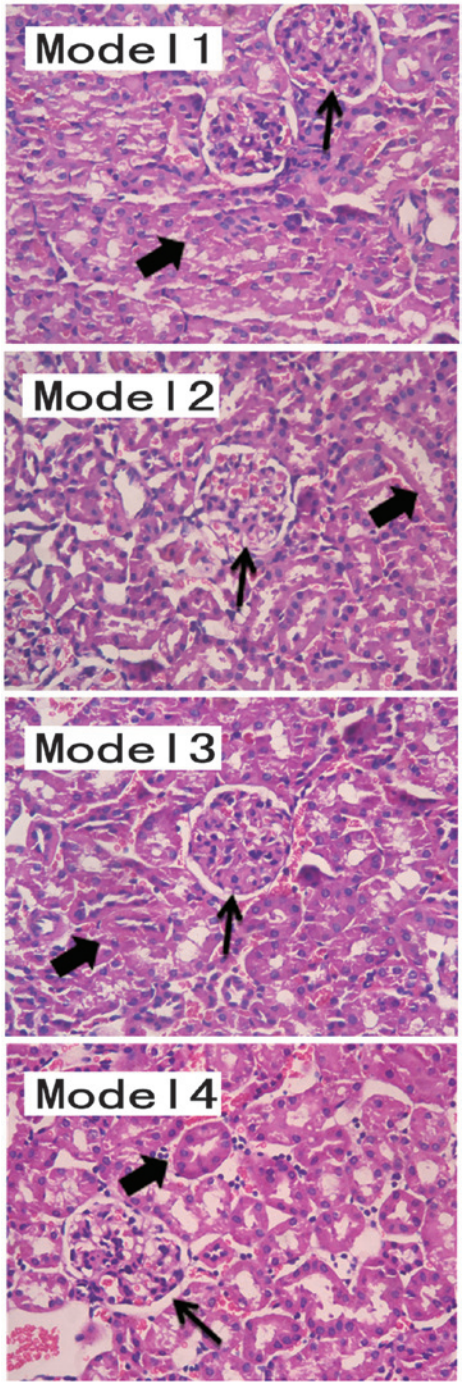

Figure 2. Model 1, diffuse thickening of the glomerular capillary (thin arrows; proliferation of mesangial cells and endothelial cells), glomerular basement membrane thickened irregular, tubular, interstitial infiltration of inflammatory cells, renal tubule degeneration and luminal stenosis (thick arrows). Model 2 and Model 3, the glomerular lesion is improved compared with that in Model 1. However, renal tubules are present. Model 4, fewer pathological histological changes were observed in the structure of the kidney sample, compared with the other models. The results were evaluated by three pathologists. Hematoxylin and eosin staining (magnification, x200).

with DAPT and glucocorticoid were improved, compared with those treated with either drug alone. Therefore, DAPT may have enhanced the anti-lupus effects of glucocorticoid treatment in the BABL/C-lpr mice. The expression of TLR9 has been observed to correlate with glomerular injury (28). TLR7 and TLR9-induced PDC stimulation can account for the

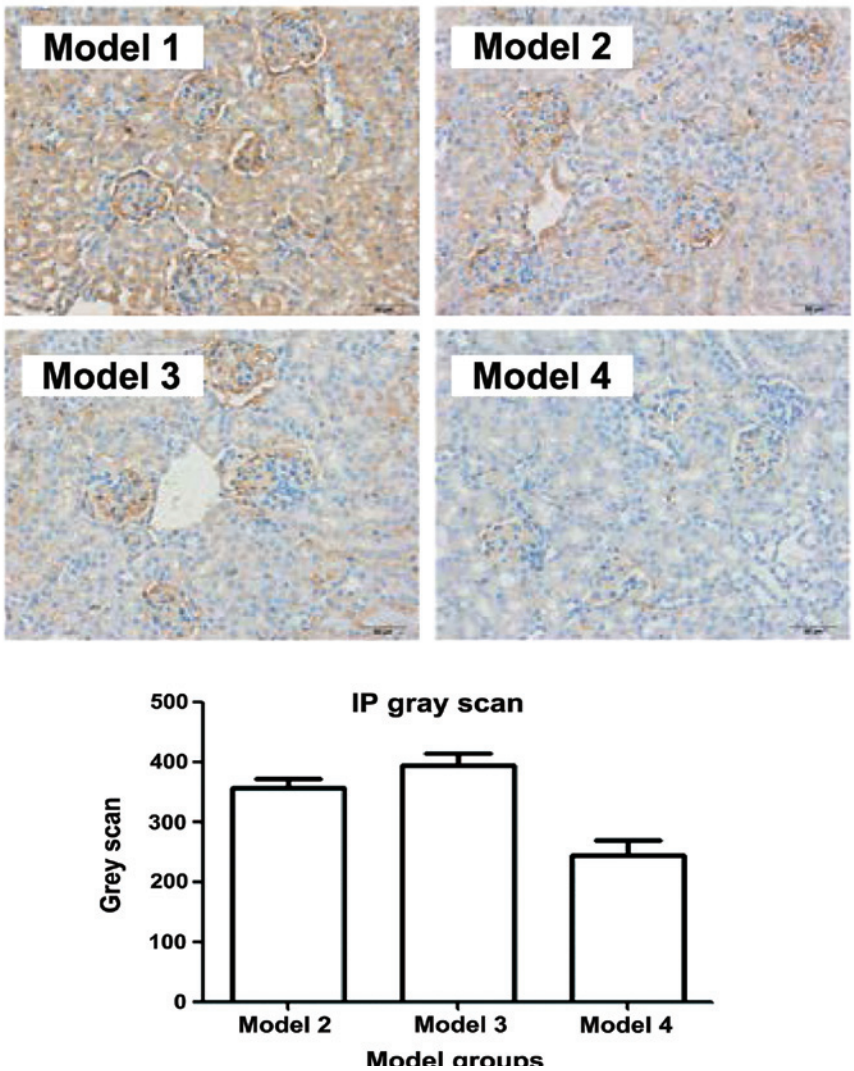

Figure 3. Following treatment of the lpr-BABL/C mice with pristane for 24 weeks, the model 2 mice were treated with the Notch-1 inhibitor, DAPT. Model 3 was treated with glucocorticosteroid and Model 4 mice were treated with DAPT following 4 weeks of glucocorticoid treatment. The expression of TLR9 was significantly reduced in Model 4, compared with the other treated groups (*P<0.05). The immunohistological staining was analyzed using Image-Pro® Plus software.(Magnification, x400).

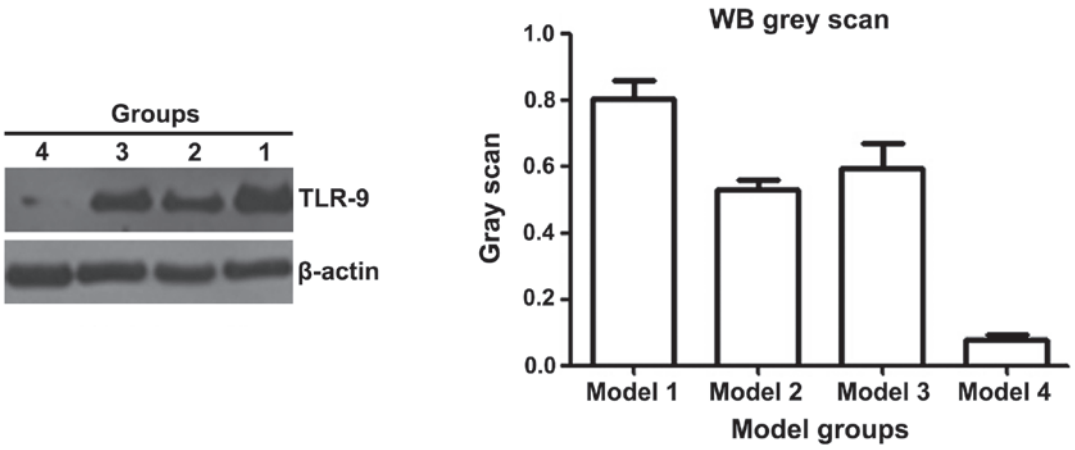

Figure 4. Following treatment of the lpr-BABL/C mice with pristane for 24 weeks, model 2 mice were treated with the Notch-1 inhibitor, DAPT, Model 3 mice were treated with glucocorticosteroid and Model 4 mice were treated with DAPT following 4 weeks of glucocorticoid treatment. The expression of TLR9 in Model 4 was significantly reduced, compared with the other Models (*P<0.05), as determined by GraphPadPrism software. WB, western blot.

reduced activity of glucocorticoids in inhibiting the interferon pathway in patients with SLE *in vivo* and in two lupus-affected mouse strains *in vitro* (29). Corticosteroids have no effect on the expression of TLR9, which explains to t lack of corticoid response in certain patients with SLE. The expression of TLR9 can be used to predict glucocorticoid response in patients with SLE (28). Therefore, the expression of TLR9 in BABL/C-lpr mouse kidneys was analyzed using immunohistochemistry. The results demonstrated that the expression of TLR9 in the mice treated with DAPT and glucocorticoid was lower than those treated with either drug alone and those, which received no treatment. Notch1 inhibition may reduce the expression of TLR9 in glomerular tissues and improve the response to corticosteroid treatment in BABL/C-lpr mice.

TLR9 is localized to the cell surface or endosomes of several types of cell, notably of APCs, including dendritic cells and B cells (30-32). TLR9 deficiency in certain lupus models may lead to reductions or alterations in anti chromatin antibodies (33). Genome-wide association investigations have revealed TLR9 genes located in susceptibility regions for SLE (34). The expression of TLR9 may contribute to renal and immunological disorders and to the presence of anti-dsDNA antibodies (35). In the present study, a positive correlation was observed between the expression of TLR9 and clinical and laboratory indices (SLEDAI, anti-dsDNA, IL10, C3 and C4) in patients with SLE, which suggested that TLR9 may represent a potential biomarker for SLE. Although the mechanisms underlying the involvement of TLR9 in SLE remains to be fully elucidated, a previous study demonstrated that the expression of TLR9 is associated with disease active indexes, including the systemic SLEDAI (36). The failure of T cells to upregulate Notch1 upon activation may be a key feature of active SLE and represents a potential therapeutic target (37). The level of Notch1 may be negatively associated with SLE activity. In the present study, DAPT was used to inhibit the Notch-1 pathway in order to investigate the effects on lupus nephritis in BABL/C-lpr mice. Lower expression levels of TLR9 were observed in the Model 4 mice compared with the mice in Models 1-3. Corticosteroids in combination with cyclophosphamide has been used previously to suppress proliferative lupus nephritis (38). The results of the present study suggested that DAPT reduced the expression of TLR9 and relieve corticosteroid resistance in BABL/C-lpr mouse kidneys. These results suggested a novel method for reducing the dosage of corticosteroids in the clinical treatment for patients with SLE.

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