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ORIGINAL ARTICLE

Silencing of STAT4 Protects Against Autoimmune Myocarditis by Regulating Th1/Th2 Immune Response *via* Inactivation of the NF-κB Pathway in Rats

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Abstract— Signal transducer and activator of transcription 4 (STAT4) has been implicated in the progression of myocarditis. The aim of the current study was to investigate the role by which STAT4 influences autoimmune myocarditis in an attempt to identify a theoretical therapeutic perspective for the condition. After successful establishment of an autoimmune myocarditis rat model, the expression patterns of STAT4, NF-κB pathway–related genes, Th1 inflammatory cytokines (IFN-γ and IL-2), and Th2 inflammatory cytokines (IL-6 and IL-10) were subsequently determined. The rats with autoimmune myocarditis were treated with oe-STAT4 or sh-STAT4 lentiviral vectors to evaluate the role of STAT4 in autoimmune myocarditis, or administrated with 1 mL 10 μmol/L of BAY11-7082 (the NF-κB pathway inhibitor) *via* tail vein to investigate the effect of the NF-κB pathway on autoimmune myocarditis. Finally, cell apoptosis was evaluated. The serum levels of IFN-γ and IL-2, extent of IκBα and P65 phosphorylation, and the expression of STAT4 were elevated, while the serum levels of IL-6 and IL-10 as well as the expression of IκBα were reduced among the rats with autoimmune myocarditis, which was accompanied by an increase in the apoptotic cells. More importantly, the silencing of STAT4 or the inhibition of the NF-κB pathway was detected to result in a decrease in the serum levels of IFN-γ and IL-2 and an elevation of the serum levels of IL-6 and IL-10, and inhibited myocardial cell apoptosis in rats with autoimmune myocarditis. Moreover, STAT4 silencing was also observed to decrease the extent of IκBα and P65 phosphorylation while acting to elevate the expression of IκBα. Taken together, silencing of STAT4 could hinder the progression of autoimmune myocarditis by balancing the expression of Th1/Th2 inflammatory cytokines *via* the NF-κB pathway, which may provide a novel target for experimental autoimmune myocarditis (EAM) treatment.

KEY WORDS: signal transducer and activator of transcription 4; NF-κB pathway; autoimmune myocarditis; Th1/2 cytokines.

Yu-Long Xue and Sheng-Xiao Zhang contributed equally to this work.

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INTRODUCTION

Autoimmune myocarditis manifests itself as consistent inflammation that inflicts severe and irreversible damage to myocardial tissues. Autoimmune myocarditis represents a chief contributor to dilated cardiomyopathy [1]. Cardiac injury arises generally as a consequence of autoimmunity between T cell and cardiac antigens in autoimmune myocarditis in the presence of relatively infective pathogens [2]. CD4+ T cells have been indicated to aid in the transition and progression of cardiomyopathy pathogenesis, while heart-specific auto-aggressive inflammatory responses are of great significance in the occurrence and development of chronic cardiomyopathy [3]. A significant variety in relation to the clinical courses of myocarditis exists in addition to a wide range of symptoms ranging from silent disease to fulminant heart failure [4]. At present, endomyocardial biopsy is regarded as the gold standard for myocardial status evaluation while its application is not habitually used in conventional clinical practice, with cardiovascular magnetic resonance deemed as the more desirable noninvasive tool for acute myocarditis diagnosis, which is susceptible to other factors due to its sensitivity [5]. At present, the therapeutic approaches available for myocarditis are limited to supportive care for heart failure and arrhythmias [4]. Therefore, it is urgent to find new diagnostic methods and biomarkers, thereby improving diagnosis and characterization of myocarditis.

Signal transduction and activation of transcription (STAT) proteins are cytoplasmic factors that are activated following the interaction of cytokines with cell surface receptors [6]. STAT4 and interferon-γ (IFN-γ) in CD4+ T cells triggered by G$\beta\gamma$ signaling could induce differentiation of T helper (Th1) cells and pro-inflammatory (M1) macrophage phenotype in a variety of autoimmune diseases [7]. STAT4 plays an essential role in the production of IFN-γ during the Th1 immune response generation [8]. As predominant members of inflammatory T cells, Th17 and Th1 have been linked to the pathogenesis of myocarditis; both of which were found to act in a respective manner to produce pathogenic cytokines such as interleukin-17 (IL-17) and IFN-γ in an animal model of experimental autoimmune myocarditis (EAM) [9]. Th cytokines such as IFN-γ are capable of mediating macrophage activation, while IL-2 has been reported to contribute to CD8+ T cell differentiation and expansion [10]. The protective effect against EAM conferred by lipoxin A4 has been reported to be achieved *via* an inflammatory response reduction (decreased levels of Th1 inflammatory cytokines including tumor necrosis factor-α (TNF-α) and IL-6 while increased levels of Th2 inflammatory cytokines including IL-4 and IL-10) as well as suppression of both the NF-κB and PI3K/Akt pathways [11]. Based on the above literature, a hypothesis was proposed that STAT4 and the NF-κB pathway are both involved in the Th1/Th2 balance in autoimmune myocarditis. Therefore, the current study was performed to identify the specific mechanism by which STAT4 influences the balance of Th1/Th2 inflammatory cytokines through the NF-κB pathway in rats with autoimmune myocarditis.

MATERIALS AND METHODS

Materials

A total of 108 male Lewis rats (aged 8 weeks and weighed 200–250 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). The STAT4 overexpression (oe)-STAT and STAT4 siRNA (sh-STAT4) lentiviral vectors were synthesized by Shanghai Genechem Co., Ltd. (Shanghai, China). Myocardial myosin was extracted and purified from a pig heart in accordance with a method described in literature [12]. Phosphate-buffered saline (PBS), tris-buffered saline (TBS), normal saline, polymerase chain reaction (PCR) buffer, deoxynucleotide triphosphates (dNTPs), complete Freund's adjuvant, proteinase K, and radio-immunoprecipitation assay (RIPA) cell lysis buffer and cocktail were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO), diaminobenzidine (DAB), paraformaldehyde, ethanol, xylene, paraffin, hematoxylin, and phenylmethylsulfonyl fluoride were all obtained from Aladdin Chemicals (Shanghai, China). Neutral resin, wax, polyacrylamide gel electrophoresis, and polyvinylidene fluoride (PVDF) membrane were all purchased from Biofuraw™ (Shanghai, China). NF-κB pathway inhibitor BAY11-7082 (ab141228) was obtained from Abcam Inc. (Cambridge, MA, USA). ELISA kit and Trizol kit (16096020) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). An RT-qPCR kit was purchased from ABI Company (Oyster Bay, NY, USA). Taq enzyme (S10118) was obtained from Yuanye Biotechnology (Shanghai, China). Optical microscope (XSP-01) was purchased from Shenzhen Aosiv Microoptics Instrument Co., Ltd. (Shenzhen, China).

Ethics Statement

The study and its experiments were conducted with the approval of the Animal Ethics Committee of Shanxi Dayi Hospital Affiliated to Shanxi Medical University. All animal experiments were performed in accordance with the principles of management and use of local laboratory animals, in addition to adhering to the National Laboratory for the Management and Use of Laboratory Animals issued by the National Institutes of Health.

Establishment of Rat Models with Autoimmune Myocarditis

Lewis rats were placed on an adaptive feeding plan for 7 days [13]. The normal rats were assigned into the normal (normal rats) and blank (normal rats injected with 0.15 mol/L PBS) groups with 12 rats placed in each group. The purified porcine cardiac myosin was extracted from a pig heart and purified based on a method prescribed in previous literature [14], after which it was dissolved in 0.15 mol/L PBS and adjusted to a final concentration of 2.0 mg/mL, followed by further mixing with an equal volume of complete Freund's adjuvant and sufficient emulsification. Next, the completely emulsified emulsion was injected into the underarm region and bilateral groin as well as the footpad regions of the rats (200 μL for each rat in each time) on the 8th and 15th day, respectively.

After the first injection of complete Freund's adjuvant into the rats, following a 4-week period of waiting, the successfully modeled rats with autoimmune myocarditis were assigned randomly into the following groups: autoimmune myocarditis (rats with autoimmune myocarditis), oe-STAT4 (rats with autoimmune myocarditis injected with 1 mL normal saline containing oe-STAT4 lentiviral vectors), oe-negative control (NC) (rats with autoimmune myocarditis injected with 1 mL normal saline containing oe-STAT4 lentiviral empty vectors), sh-STAT4 (rats with autoimmune myocarditis injected with 1 mL normal saline containing sh-STAT4 lentiviral vectors), sh-NC (rats with autoimmune myocarditis injected with 1 mL normal saline containing sh-STAT4 lentiviral empty vectors), BAY11-7082 (rats with autoimmune myocarditis injected with 1 mL 10 μmol/L NF-κB pathway inhibitor BAY11-7082) [15], and DMSO (rats with autoimmune myocarditis injected with 1 mL DMSO) groups. All the lentiviral vectors were adjusted to a concentration of 5×10^7 TU/mL with normal saline, respectively, followed by an injection into the tail vein of rats with autoimmune myocarditis on the first day of autoimmune myocarditis induction. The rats were euthanatized after the first injection of complete Freund's adjuvant [16] with the heart extracted, and the left ventricle freed, all of which were frozen in liquid nitrogen, stored at − 80 °C or fixed in 4% paraformaldehyde.

Enzyme-Linked Immunosorbent Assay

The serum levels of Th1 inflammatory cytokines (IFN-γ and IL-2) and Th2 inflammatory cytokines (IL-6 and IL-10) were detected based on the instructions of the ELISA kit. Initially, the frozen serum was thawed at the room temperature and diluted into five concentrations including 180 ng/L, 120 ng/L, 60 ng/L, 30 ng/L, and 15 ng/L with gradient standard with 50 μL samples per well. Next, the sample well was added with 40 μL sample dilution buffer followed by the addition of 10 μL sample. After being coated with the film, the ELISA plate was warmly bathed at 37 °C for 30 min, washed five times, colored with 50 μL ELISA reagent at 37 °C for 15 min under dark conditions, and then added with a termination solution. Finally, the optical density (OD) value was determined at a wavelength of 450 nm. A standard curve was subsequently constructed from the standard to calculate the sample concentration.

Hematoxylin-Eosin Staining

The specimens were fixed in 4% paraformaldehyde for 24 h, and 5-mm myocardial tissue blocks were cut from the transverse section of the heart. The obtained myocardial tissues were routinely dehydrated by gradient ethanol at concentrations of 70%, 80%, 90%, 95%, and 100%, respectively (2 h each time). Next, the myocardial tissues were cleared twice with xylene (30 min each time), immersed in wax, embedded in paraffin, and cut into 4-μm sections which were placed in an oven at 60 °C for 2 h. After the sections had cooled, they were routinely dehydrated with gradient ethanol, cleared with xylene, and rinsed. The sections were subsequently stained with hematoxylin for 4 min and washed and differentiated with hydrochloric acid-ethanol for 10 s. After that, the sections were rinsed and immersed for 5 min, stained with eosin for 2 min, and then dehydrated with gradient ethanol (1 min for each time). The sections were cleared twice with xylene (1 min each time) and mounted in neutral resin. Finally, the pathological changes of myocardial tissues were observed under an optical microscope under a hood.

TdT-Mediated dUTP-Biotin Nick End-Labeling Staining

The randomly selected myocardial tissues in each group were paraffin-embedded and cut into 5-μm sections. The sections were conventionally dewaxed, dehydrated, treated with 3% H_2O_2 for 10 min at room temperature, detached with proteinase K at 37 °C, labeled in a wet box at 37 °C for 2 h, and reacted with 1% TBS at 37 °C for

60 min. The sections were then developed with DAB for 10 min, counterstained with hematoxylin for 1 min, and sealed in neutral resin. Finally, the captured images were analyzed by Image-pro plus 6.0 image analysis system. Five sections were randomly selected from each rat, with five high magnification fields selected in a random fashion from the injured area of each section. The apoptosis index was calculated according to the following formula: apoptosis index = the number of apoptotic cells/the number of total cells × 100%.

Reverse Transcription Quantitative Polymerase Chain Reaction

Total RNA was extracted using a Trizol kit and then reversely transcribed into cDNA based on the instructions of reverse transcription quantitative polymerase chain reaction (RT-qPCR) kit. Next, a 25-μL system was applied to amplify the target genes, which consisted of 300 ng cDNA, 1 × PCR buffer, 200 μmol/L dNTPs, 80 pmol/L forward primers, 80 pmol/L reverse primers, and 0.5 U Taq enzyme. The reaction conditions were performed as follows: pre-denaturation at 94 °C for 5 min, 40 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s and extension at 72 °C for 30 s, and final extension at 72 °C for 10 min. Then, the products were preserved at 4 °C. The primer sequences are illustrated in Table 1. β-actin was regarded as the internal reference. The experiment was repeated three times.

Western Blot Analysis

A total of 100 mg of myocardial tissues was extracted from the rats in each group; after which, they were ground and lysed with 100 μL RIPA cell lysis buffer containing phenylmethylsulfonyl fluoride and cocktail for 30 min. After the tissues had been centrifuged at 4 °C, the total protein in the supernatant was collected and stored at either − 20 °C or − 80 °C. Next, the total protein was quantified *via* the Bradford method (Shanghai Yeasen Company, Shanghai, China). The total protein was separated by means of polyacrylamide gel electrophoresis with approximately 20 μg of sample placed in each lane, followed by transfer onto a PVDF membrane. After that, the membrane was blocked and incubated with primary rabbit monoclonal STAT4 (1: 1000, ab68156), rabbit monoclonal p-IκBα (1: 10000, ab133462), rabbit monoclonal IκBα (1: 1000, ab32518), rabbit polyclonal p-P65 antibodies (1: 2000, ab86299), and rabbit polyclonal β-actin antibodies (1: 5000, ab32518) overnight at 4 °C. The next day, the membrane was washed and further incubated with the horseradish peroxidase–labeled goat anti-rabbit immunoglobulin G (IgG) antibody (1: 10000, ab672) at 37 °C for 1 h. All the aforementioned antibodies were purchased from Abcam Inc. (Cambridge, MA, USA). About 1 mL electrochemiluminescence (ECL) working solution was prepared by SuperSignal® West Dura Extended Duration Substrate according to the instructions. The transfer film was incubated at room temperature for 1 min. Then the superfluous ECL reagent was removed, the film was sealed, and the X-ray film exposed for 5–10 min was placed in the cassette for development and fixation. Finally, the membrane was imaged with the gel imager, photographed with the Bio-Rad Image Analysis System (Bio-Rad Inc., Hercules, CA, USA), and analyzed by the Quantity One v4.6.2 software (Bio-Rad Inc., Hercules, CA, USA). Finally, the relative protein levels of STAT4, p-IκBα, IκBα, and p-P65 were calculated using the gray value of the target protein band/the gray value of the internal reference β-actin band. The experiment was repeated three times.

Statistical Analysis

The SPSS 19.0 statistical analysis software (IBM Corp. Armonk, NY, USA) was used for data analysis. The measurement data were expressed as the mean ± standard deviation. Comparison between two groups was analyzed using a *t* test, while an unpaired *t* test was performed if the data were in accordance with the normal distribution and homogeneity of variance. Comparison among multiple groups was processed with one-way analysis of variance. Data normality was assessed using the Kolmogorov-Smirnov method, while comparisons among multiple groups with normal distribution data were performed using a one-way analysis of variance with post-host tests for Tukey-Kramer. A value of $p < 0.05$ was considered to be indicative of statistical significance.

RESULTS

Rats with Autoimmune Myocarditis Exhibit High Expression of STAT4

After the rat autoimmune myocarditis models had been established, hematoxylin-eosin (HE) staining was performed to ascertain as to whether the model had been successfully established. Compared with the normal and blank groups, the myocardial tissues from the autoimmune myocarditis group exhibited signs of focal necrosis, fibrosis formation, interstitial hyperplasia, inflammatory cell

Table 1. Primer Sequences for RT-qPCR

Gene	Forward (5′-3′)	Reverse (5′-3′)
STAT4	GCTGCATCTTTGCCGAAATGGTG	GCTGCATCTTTGCCGAAATGGTG
β-actin	CAGCTTCTTCTAGTGCCGTTCC	GGAGTCAGGTGTTTCTGGTGGAC

RT-qPCR, reverse transcription quantitative polymerase chain reaction; *STAT4*, signal transducer and activator of transcription 4

infiltration, residual myocardial hypertrophy, and irregular arrangement. However, the myocardial tissues in the normal and blank groups did not display any signs of cardiomyocyte necrosis with regular arrangement and normal intercellular space, while no significant difference was found between the normal and blank groups (Fig. 1a). These results suggested that the autoimmune myocarditis rat model had been successfully established. Next, TdT-mediated dUTP-biotin nick end-labeling (TUNEL) staining was performed on myocardial cells to detect cell apoptosis in the normal, blank, and autoimmune myocarditis groups, and the results revealed that the number of apoptotic cells increased in the autoimmune myocarditis group when compared with the normal and blank groups ($p < 0.05$; Fig. 1b, c). There was no significant difference detected in relation to cell apoptosis between the normal and blank groups ($p > 0.05$). ELISA was employed to detect the serum levels of Th1 and Th2 inflammatory cytokines. Compared with the normal and blank groups, levels of the Th1 inflammatory cytokines (IFN-γ and IL-2) increased, while levels of the Th2 inflammatory cytokines (IL-6 and IL-10) decreased in the autoimmune myocarditis group ($p < 0.05$); the results between the normal and blank groups did not display any significant difference ($p > 0.05$; Table 2). RT-qPCR and western blot analysis methods were employed to determine the STAT4 expression in the myocardial cells; the results of which revealed there to be higher levels of STAT4 expression in the autoimmune myocarditis group than in the normal and blank groups ($p < 0.05$); STAT4 expression did not differ significantly between the normal and blank groups ($p > 0.05$; Fig. 1d, e). Taken together, rats with autoimmune myocarditis were confirmed to have elevated expression of STAT4 and Th1 inflammatory cytokines along with decreased levels of Th2 inflammatory cytokines.

STAT4 Silencing Hinders the Autoimmune Myocarditis Progression in Rats Through Balancing Th1 and Th2 Inflammatory Cytokines

The rats with autoimmune myocarditis were infected with oe-STAT4 or sh-STAT4 lentiviral vectors in order to alter STAT4 in an attempt to elucidate the effect of STAT4 on the progression of autoimmune myocarditis in addition to the serums levels of the Th1 and Th2 inflammatory cytokines. STAT4 expression was determined by RT-qPCR and western blot analysis. The results obtained revealed there to be a higher expression of STAT4 in the oe-STAT4 group when compared to the oe-NC group ($p < 0.05$), while lower STAT4 expression was observed in the sh-STAT4 group than that of the sh-NC group ($p < 0.05$). In addition, no significant difference was detected between the oe-NC and sh-NC groups ($p > 0.05$; Fig. 2a, b). Next, the serum levels of Th1 inflammatory cytokines (IFN-γ and IL-2) and Th2 inflammatory cytokines (IL-6 and IL-10) were evaluated by ELISA. In comparison with the oe-NC group, the oe-STAT4 group displayed elevated levels of IFN-γ and IL-2 but decreased levels of IL-6 and IL-10 ($p < 0.05$). The sh-STAT4 group exhibited decreased levels of IFN-γ and IL-2 and increased levels of IL-6 and IL-10 when compared with the sh-NC group ($p < 0.05$). No significant difference was detected between the oe-NC and sh-NC groups ($p > 0.05$; Table 3). The pathological changes of myocardial tissues and cells were detected following the application of HE staining, which indicated that the myocardial tissues of the oe-STAT4 group manifested focal necrosis, fibrosis formation, interstitial hyperplasia, inflammatory cell infiltration, residual myocardial hypertrophy, and irregular arrangement when compared with the oe-NC group. The myocardial tissues in the sh-STAT4 group did not display myocardial cell necrosis with regular arrangement and normal intercellular space in comparison with the sh-NC group. Then, no significant difference was found between the oe-NC and sh-NC groups ($p > 0.05$; Fig. 2c). Next, TUNEL staining was performed to detect myocardial cell apoptosis after lentiviral infection, the results of which revealed there to be an elevated number of apoptotic cells in the oe-STAT4 group compared with the oe-NC group ($p < 0.05$); the number of apoptotic cells was lower in the sh-STAT4 group than that in the sh-NC group ($p < 0.05$). The results between the oe-NC and sh-NC groups did not differ significantly ($p > 0.05$; Fig. 2d, e). The aforementioned results provided evidence

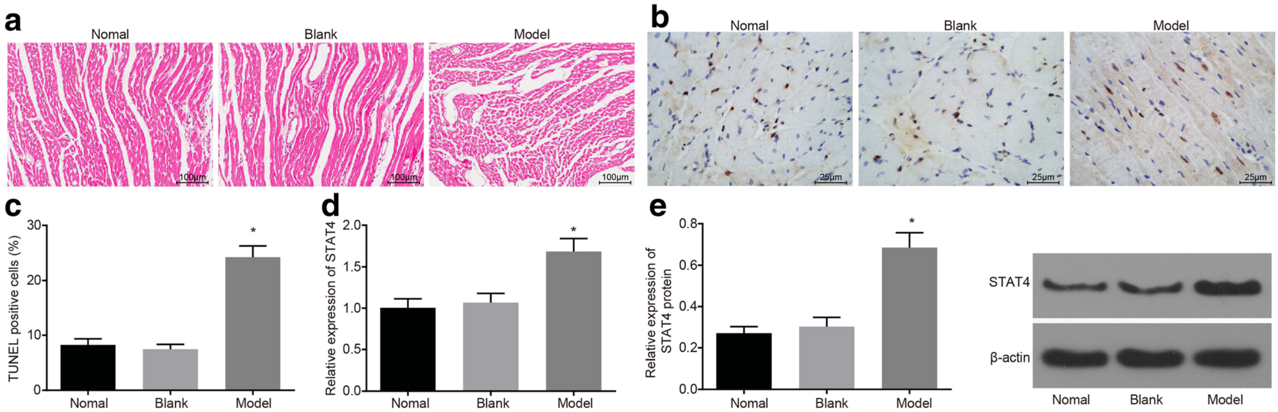

Fig. 1. High expression of STAT4 is observed in myocardial tissues of rats with autoimmune myocarditis ($n = 12$). **a** The results of HE staining (× 100). **b** The results of TUNEL staining (× 400). **c** TUNEL positive cells. **d** The mRNA expression of STAT4. **e** The protein level of STAT4. *$p < 0.05$ *vs.* the normal and blank groups; all the data were measurement data and presented as mean ± standard deviation; one-way analysis of variance was performed for data analysis; the experiment was repeated three times; STAT4, signal transducer and activator of transcription 4; HE, hematoxylin-eosin; TUNEL, TdT-mediated dUTP-biotin nick end-labeling; RT-qPCR, reverse transcription quantitative polymerase chain reaction.

suggesting that STAT4 silencing could decrease the level of Th1 inflammatory cytokines and elevate Th2 inflammatory cytokines, to balance Th1 and Type-2 cytokines, thus inhibiting the progression and development of autoimmune myocarditis in rats.

STAT4 Silencing Inhibits the NF-κB Pathway in Rats with Autoimmune Myocarditis

In order to investigate the relationship between STAT4 and the NF-κB pathway in the rats with autoimmune myocarditis, the protein levels of pathway-related proteins (p-IκBα, IκBα, and p-P65) were determined by western blot analysis. Compared with the blank group, the myocardial cells in the autoimmune myocarditis group showed increased extent of IκBα and P65 phosphorylation while decreased level of IκBα ($p < 0.05$; Fig. 3a). Moreover, the extent of P65 phosphorylation was significantly decreased in the cytoplasm while it increased in the nucleus of myocardial cells in the autoimmune myocarditis group ($p < 0.05$; Fig. 3b). No significant difference was observed between the normal and blank groups ($p > 0.05$).

Subsequently, the effect of STAT4 on the level of IκBα as well as the extent of IκBα and P65 phosphorylation in the myocardial cells was investigated by infecting myocardial cells with oe-STAT4 or sh-STAT4 lentiviral vectors, whereby the level of IκBα and the extent of IκBα and P65 phosphorylation were determined by western blot analysis. The results demonstrated that the extent of IκBα and P65 phosphorylation was significantly elevated, while the IκBα level was markedly decreased in the oe-STAT4 group when compared with the oe-NC group ($p < 0.05$). In comparison with the sh-NC group, the myocardial cells in the sh-STAT4 group exhibited significantly downregulated the extent of IκBα and P65 phosphorylation but upregulated IκBα ($p < 0.05$; Fig. 3c). Furthermore, the extent of

Table 2. Levels of Th1-Type and Th2-Type Cytokines in Autoimmune Myocarditis Rats After Modeling ($n = 12$)

Group	Normal	Blank	Autoimmune myocarditis
IFN-γ (pg/mL)	17.42 ± 2.01	18.96 ± 1.96	40.41 ± 5.23*
IL-2 (pg/mL)	11.23 ± 1.76	12.91 ± 1.44	39.41 ± 4.91*
IL-6 (pg/mL)	30.64 ± 3.74	33.11 ± 2.78	12.97 ± 1.93*
IL-10 (pg/mL)	301.32 ± 26.77	318.25 ± 22.53	183.21 ± 15.72*

Th, T helper; *IFN-γ*, interferon-γ; *IL*, interleukin; *ELISA*, enzyme-linked immunosorbent assay. *$p < 0.05$ *vs.* the normal group. All the data were measurement data and presented as mean ± standard derivation. One-way analysis of variance was performed for data analysis. The experiment was repeated three times

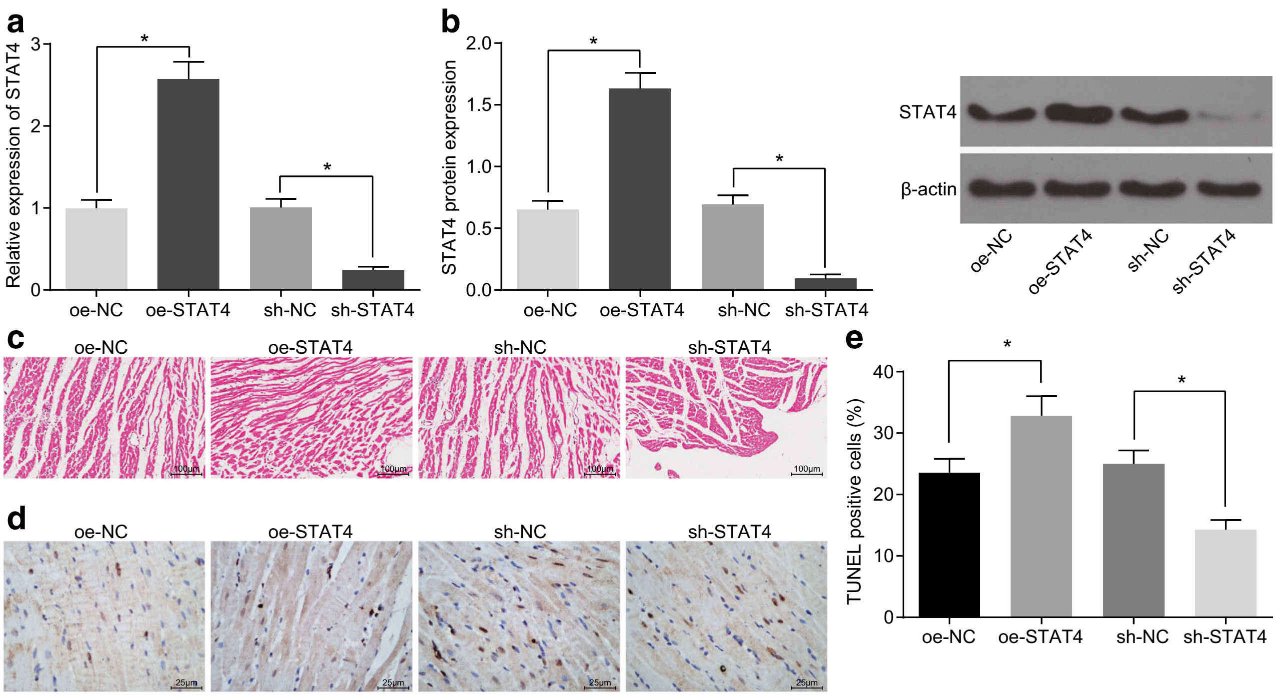

Fig. 2. The autoimmune myocarditis progression in rats is inhibited by STAT4 silencing *via* balance of Th1 and Th2 inflammatory cytokines ($n = 12$). **a** The mRNA expression of STAT4 after alteration of STAT4. **b** The protein level of STAT4 after alteration of STAT4. **c** The results of HE staining after alteration of STAT4 (× 100). **d** The results of TUNEL staining after alteration of STAT4 (× 400). **e** TUNEL positive cells after alteration of STAT4. *$p < 0.05$ *vs.* the oe-NC/sh-NC group; all the data were measurement data and presented as mean ± standard deviation; one-way analysis of variance was performed for data analysis; the experiment was repeated three times; STAT4, signal transducer and activator of transcription 4; HE, hematoxylin-eosin; TUNEL, TdT-mediated dUTP-biotin nick end-labeling; RT-qPCR, reverse transcription quantitative polymerase chain reaction; NC, negative control.

P65 phosphorylation significantly decreased in the cytoplasm while it increased in the nucleus of myocardial cells in the oe-STAT4 group ($p < 0.05$). The extent of P65 phosphorylation significantly increased in the cytoplasm while it decreased in the nucleus of myocardial cells in the sh-STAT4 group ($p < 0.05$; Fig. 3d). No significant difference was detected between the sh-NC and oe-NC groups ($p > 0.05$). These obtained results suggested that STAT4 silencing was capable of inhibiting the NF-κB pathway in rats with autoimmune myocarditis.

NF-κB Pathway Suppression Impedes Autoimmune Myocarditis Progression in Rats by Balancing Th1 and Th2 Inflammatory Cytokines

In order to determine the influence of NF-κB pathway on the serum levels of Th1 and Th2 inflammatory cytokines, rats with autoimmune myocarditis were administered with DMSO or BAY11-7082; after which, ELISA was employed to measure the levels of the Th1 inflammatory cytokines (IFN-γ and IL-2) as well as the

Table 3. Levels of Th1-type and Th2-Type Cytokines in Autoimmune Myocarditis Rats After Alteration of STAT4 ($n = 12$)

Group	oe-NC	oe-STAT4	sh-NC	sh-STAT4
IFN-γ (pg/mL)	43.44 ± 4.12	62.39 ± 7.16*	42.09 ± 5.17	28.31 ± 2.29*
IL-2 (pg/mL)	42.77 ± 4.09	58.66 ± 6.13*	41.99 ± 5.02	25.03 ± 2.98*
IL-6 (pg/mL)	11.23 ± 1.11	4.64 ± 0.69*	12.04 ± 1.49	27.09 ± 2.34*
IL-10 (pg/mL)	192.21 ± 20.24	98.54 ± 10.02*	187.22 ± 19.10	245.56 ± 19.77*

STAT4, signal transducers and activators of transcription 4; *Th*, T helper; *IFN-γ*, interferon-γ; *IL*, interleukin; *ELISA*, enzyme-linked immunosorbent assay; *NC*, negative control. *$p < 0.05$ *vs.* the oe-NC/sh-NC group. All the data were measurement data and presented as mean ± standard derivation. One-way analysis of variance was performed for data analysis. The experiment was repeated three times

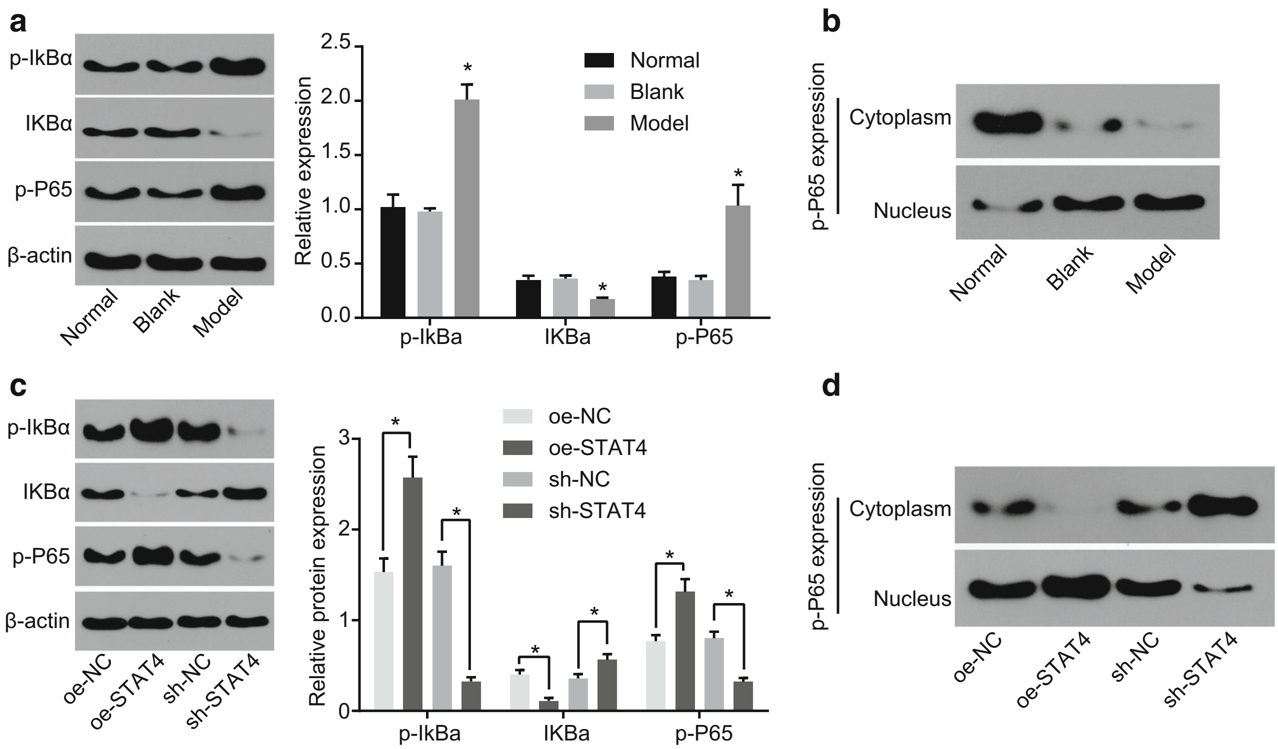

Fig. 3. The NF-κB pathway is inhibited by STAT4 silencing in rats with autoimmune myocarditis ($n = 12$). **a** The level of IκBα and the extent of IκBα and P65 phosphorylation in rats with autoimmune myocarditis. **b** The extent of P65 phosphorylation in cytoplasm and nucleus in rats with autoimmune myocarditis. **c** The level of IκBα and the extent of IκBα and P65 phosphorylation in rats with autoimmune myocarditis after alteration of STAT4. **d** The extent of P65 phosphorylation in cytoplasm and nucleus in myocardial cells of rats with autoimmune myocarditis after alteration of STAT4. All the data were measurement data and presented as mean ± standard deviation; one-way analysis of variance was performed for data analysis; the experiment was repeated three times; STAT4, signal transducer and activator of transcription 4; NF-κB, nuclear factor-κB, NC, negative control.

Th2 inflammatory cytokines (IL-6 and IL-10). The results showed that the levels of IFN-γ and IL-2 decreased while IL-6 and IL-10 levels increased in the BAY11-7082 group compared with the DMSO group ($p < 0.05$; Table 4). Next, the pathological changes of myocardial tissues were examined through HE staining; the results of which revealed that the myocardial tissues of the BAY11-7082 group had a regular arrangement of myocardial fibers and normal intercellular space without myocardial cell necrosis when compared with the DMSO group (Fig. 4a). The TUNEL staining results indicated that the number of apoptotic cells had noticeably decreased in the BAY11-7082 group when compared with the DMSO group ($p < 0.05$; Fig. 4b). Taken together, our results demonstrated that inhibition of the NF-κB pathway could restrain the levels of Th1 inflammatory cytokines (IFN-γ and IL-2) while promoting Th2 inflammatory cytokines (IL-6 and IL-10), thereby repressing the progression and development of autoimmune myocarditis in rats.

DISCUSSION

Autoimmune myocarditis is a condition triggered by an abnormal interaction between infection and immunity [2]. Leukocytes producing cytokines including T-lymphocytes and macrophages function play a critical role in the pathogenesis of myocardial damage [17]. STAT4 is expressed in T cells and has been previously reported to play a contributory role in the development of Th1 and Th2 immune responses during monophosphoryl lipid A adjuvant activity [8]. In the current study, the potential effects of STAT4 by regulating the levels of Th1/Th2 inflammatory cytokines on rat models with autoimmune myocarditis were investigated. The results obtained suggested that silencing of STAT4 balanced the levels of Th1/Th2 inflammatory cytokines by inhibiting the activation of the NF-κB pathway, thus alleviating autoimmune myocarditis.

Initially, the present study revealed that STAT4 was upregulated in rats with autoimmune myocarditis

Table 4. Levels of Th1-Type and Th2-Type Cytokines in Autoimmune Myocarditis Rats After Alteration of the NF-κB Pathway ($n = 12$)

Group	DMSO	BAY11-7082
IFN-γ (pg/mL)	41.88 ± 3.97	26.67 ± 3.02*
IL-2 (pg/mL)	43.05 ± 4.56	27.73 ± 2.99*
IL-6 (pg/mL)	11.97 ± 2.02	27.65 ± 3.07*
IL-10 (pg/mL)	177.97 ± 18.32	222.87 ± 24.76*

NF-κB, nuclear factor-κB; *Th*, T helper; *IFN-γ*, interferon-γ; *IL*, interleukin; *ELISA*, enzyme-linked immunosorbent assay; *DMSO*, dimethyl sulfoxide. *$p < 0.05$ *vs.* the DMSO group. All the data were measurement data and presented as mean ± standard derivation. One-way analysis of variance was performed for data analysis. The experiment was repeated three times

and that silencing of STAT4 could suppress the NF-κB pathway activation by increasing IκBα level and decreasing the phosphorylated content of IκBα and P65 in rats with autoimmune myocarditis. Activation of STAT was majorly regulated by suppressor of cytokine signaling 1 (SOCS), the DNA therapy of which ameliorated autoimmune myocarditis, and restored expression of SOCS by pitavastatin could hinder STAT3 and STAT4 phosphorylation [18]. The content of STAT1, STAT3, and STAT4 phosphorylation has been previously found to be increased in cases of autoimmune myocarditis, highlighting the vital role of the JAK/STAT pathway activation in myocardial cell damage [19]. It has indicated the presence of cross regulations between STAT and the NF-κB pathway in experimental autoimmune encephalomyelitis [20]. A significant increase in terms of the NF-κB level in the serum of rats with autoimmune myocarditis was discovered in a previous study [21], which was consistent with the results of the present study. Activation of the NF-κB by the inhibitor of the IκB kinase (IKK) has been implicated in the progression of various inflammatory diseases [22]. An analysis into the cloning and function of the STAT4 promoter, which is a NF-κB binding site, localized at − 969/− 959 bp upstream of the transcriptional start site, has been revealed to participate in the regulation of STAT4 in primary human dendritic cells (DC) [23], providing evidence of cooperation of STAT4 and NF-κB at gene promoters. Additionally, rheumatoid arthritis synovial fibroblasts stimulated with peptidoglycan displayed upregulated expression of NF-κB, p50, STAT1, and STAT4, while NF-κB activation was required for the function of peptidoglycan [24].

A key observation of the current study revealed that the silencing of STAT4 or inhibition of the NF-κB pathway could contribute to a balance between Th1 and Th2 inflammatory cytokines, thereby reducing inflammatory response in rats with autoimmune myocarditis. It is worth noting that autoimmune diseases are often triggered by the imbalance between Th1 and Th2, with previous studies indicating that alleviation of autoimmune myocarditis by apigenin administration is accompanied by diminished levels of Th1 inflammatory

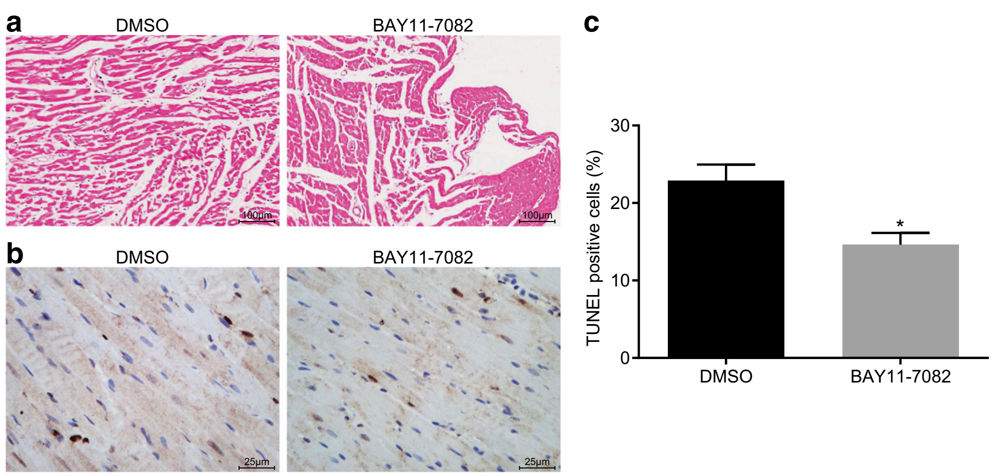

Fig. 4. The autoimmune myocarditis progression in rats is suppressed by inhibition of the NF-κB pathway *via* balance of Th1 and Th2 inflammatory cytokines ($n = 12$). **a** HE staining of myocardial tissues in rats with autoimmune myocarditis treated with DMSO or BAY11-7082 (× 100). **b** TUNEL staining of myocardial cells in rats with autoimmune myocarditis treated with DMSO or BAY11-7082 (× 400). **c** TUNEL positive cells in rats with autoimmune myocarditis treated with DMSO or BAY11-7082. *$p < 0.05$ *vs.* the DMSO group; all the data were measurement data and presented as mean ± standard deviation; one-way analysis of variance was performed for data analysis; the experiment was repeated three times; NF-κB, nuclear factor-κB; HE, hematoxylin-eosin; TUNEL, TdT-mediated dUTP-biotin nick end-labeling; DMSO, dimethyl sulfoxide.

cytokines (IFN-γ and IL-2) along with increases in Th2 inflammatory cytokines (IL-4 and IL-10) [25]. Suppression of the phosphorylated extent of STAT1, STAT3, and STAT4 achieved through berberine led to repressed Th1 and Th17 cell differentiation [19]. Interestingly, another study also concluded that the phosphorylated extent of STAT3 and STAT4 was reduced by pitavastatin treatment, which is a key factor for Th1 and Th17 lineage commitment, respectively, and restrained IFN-γ and IL-17 production from autoreactive CD4+ T cells in the heart [18]. Both cytokines and NF-κB pathways have been shown to be closely related cascades that are critical for immune response and inflammation, whereby the NF-κB regulated expression of pro-inflammatory cytokines such as IFN-c and IL-6 [20]. NF-κB signaling was suggested to trigger the downstream inflammatory cytokines including TNF-β, IL-2, IL-12, and INF-α, which contributed to immune response induction of Th1 cells [21]. Moreover, a reduction in NF-κB activation as well as IFN-γ and IL-2 mRNA expression in myocardial tissues has been shown to be triggered by IKK inhibitor administration, which ameliorated autoimmune myocarditis by attenuating inflammatory responses through inhibition of T cell activation [22].

CONCLUSIONS

In conclusion, the key findings of the current study provide evidence indicating that silencing STAT4 can inhibit the inflammatory response of myocardial cells, highlighting the potential of STAT4 as a promising biomarker for the diagnosis and treatment of myocarditis. Silencing of STAT4 could confer a suppressive effect on the activation of the NF-κB pathway, thereby downregulating Th1 inflammatory cytokines yet upregulating Th2 inflammatory cytokines, which could finally be favorable for a restrained inflammatory response (Supplementary Fig. 1). On the one hand, whether STAT4 mediates other T cells related to myocardial cell inflammatory response *via* the NF-κB pathway still remains unclear. Therefore, further studies are imperative in order to rule out influence of other T cells involving STAT4, while also clarifying the specific mechanisms of STAT4 in regulating inflammatory response in autoimmune myocarditis. On the other hand, due to lack of time and funds, whether BAY treatment could influence the expression of STAT4 was not investigated. However, we will take into consideration exploring the effect and mechanism of BAY-inhibited NF-κB pathway on STAT4 in our next research plan.

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COMPLIANCE WITH ETHICAL STANDARDS

Competing Interests. The authors declare that they have no conflict of interest.

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