Intervening in the S1PR1/STAT3 Signaling Pathway Attenuates Valvular Damage Due to Rheumatic Heart Disease

Shenglin Xian
　The First Affiliated Hospital of Guangxi Medical University

Ang Chen
　The First Affiliated Hospital of Guangxi Medical University

Yunjiao Wu
　The First Affiliated Hospital of Guangxi Medical University

Hong Wen
　The First Affiliated Hospital of Guangxi medical University

Chuanghong Lu
　The First Affiliated Hospital of Guangxi Medical University

Jianlin Wen
　The First Affiliated Hospital of Guangxi Medical University

Feng Huang
　The First Affiliated Hospital of Guangxi Medical University

Zhiyu Zeng (✉ doctorzyz@126.com)
　The First Affiliated Hospital of Guangxi Medical University　https://orcid.org/0000-0002-7383-2700

Research article

Abstract

Background: Rheumatic heart disease (RHD) affects many patients every year, but its pathogenesis is still unclear. Recent studies have found that the sphingosine 1-phosphate receptor 1 (S1PR1)/signal transducer and activator of transcription 3 (STAT3) signaling pathway is involved in valvular damage by promoting the differentiation of T helper 17 (Th17) cells during the development of valvular damage caused by RHD. In this work, we investigated whether altering the S1PR1/STAT3 signaling pathway attenuates valvular damage due to RHD.

Methods: Inactivated Group A streptococci (GAS) was used to establish the RHD rat model. Recombinant adeno-associated virus (serotype 9) vectors carrying the S1PR1 overexpression sequence was used to overexpress the expression of S1PR1. STAT3-small interfering RNA (STAT3-siRNA) was used to inhibit the expression of STAT3. Reverse transcription-quantitative PCR (RT-qPCR) was used to detect the mRNA expression of S1PR1, STAT3, collagen type III α1 chain (Col3a1) and fibroblast-specific protein 1 (FSP1). Western blotting (WB) and immunohistochemistry were used to detect the protein expression of S1PR1, STAT3, phosphorylated (p-) STAT3, retinoic acid-related orphan receptor gamma T (RORγt). Enzyme-linked immunosorbent assay (ELISA) and immunohistochemistry were used to detect the levels of interleukin (IL)-6 and IL-17. Haematoxylin and eosin (H&E) staining and Sirius red staining were used to evaluate the degree of inflammation and fibrosis in valve tissues.

Results: The expression of S1PR1 in valve tissues of RHD model rats was decreased. The levels of IL-6 and IL-17 in valves and serum of RHD model rats were increased as well as an increase of p-STAT3. The degree of valvular inflammation and fibrosis of RHD model rats was increased. Overexpression of S1PR1 and Inhibition of STAT3 overexpressed S1PR1 expression and decreased STAT3 expression respectively and reduced the total amount of p-STAT3, resulting in decreased expression of IL-6, IL-17 and RORγt and reduced the degree of valvular inflammatory and fibrosis.

Conclusions: These results suggest that the S1PR1/STAT3 signaling pathway is involved in regulating the Th17 cell-related cytokines during the vavular damage due to RHD, and altering the S1PR1/STAT3 signaling pathway could affect the expression of Th17 cell-related cytokines then attenuate the vavular damage due to RHD.

1. Background

RHD is a preventable heart disease that is caused by *Streptococcus pyogenes* or group A streptococcal (GAS) infection [1]. RHD continues to be a leading cause of deaths and disability in young patients and remains a serious public health problem worldwide [2]. At present, the number of people with RHD disability in the world exceeds one quarter of the number of people with cancer disability, and the number of deaths caused by RHD each year is as high as 250,000 [3]. However, the research progress on RHD is still exploring the pathogenesis, which is still unclear. There are no precise intervention targets for the development of RHD. Most RHD research is about the relationship between pathogenesis and signaling pathways [4–6]. There is a lack of research on intervention targets that can attenuate valvular damage due to RHD.

S1PR1 is a G protein-coupled receptor belonging to sphingosine 1-phosphate receptor (S1PR) family. S1PR1 mediates lymphocyte migration and is associated with multiple immune diseases [7] and heart diseases [8]. In these heart diseases S1PR1 participates in, it mostly plays a role in protecting the heart [9–11], and the high expression of S1PR1 can usually protect the heart during the pathogenesis of heart disease [9, 12]. And Garris CS et al. found that downregulation of S1PR1 expression increases phosphorylation levels of STAT3 [13]. A recent study discovered that the S1PR1/STAT3 signaling pathway is involved in the development of vavular damage due to RHD from the study on RHD model rats, and the expression of S1PR1 is also down-regulated as well as increased phosphorylation levels of STAT3 and increased levels of Th17-related cytokines [14].

STAT3 is a cellular signal transcription factor involved in the regulation of many cellular activities [15]. STAT3 may regulate the differentiation of CD4$^+$ T cells into Th17 cells [16]. Several studies have shown that Th17 cells and related cytokines mediate inflammatory responses and autoimmune responses [17–19]. Studies by Bas et al. showed that both the ratio of Th17/Treg cells and the level of IL-17A in patients with RHD were significantly increased than those in the control group [20]. Similar results were found in RHD animal models using Lewis rats [21]. There is a close relationship between S1PR1 and STAT3, and there have been many reports on the role of S1PR1 in regulating STAT3 in various diseases [22–25]. Therefore, we hypothesize that the STAT3 pathway is activated in the development of RHD, which induces CD4$^+$ T cells to differentiate into Th17 cells, and then Th17 cell-related inflammatory factors participate in the development of RHD. However, it is unclear whether this pathway influences or prevents RHD after intervention. What is more important is how to intervene in this pathway to influence or prevent the occurrence of RHD.

Therefore, in this study, we interfered with S1PR1/STAT3 signaling pathway by overexpress S1PR1 and inhibiting STAT3 respectively to determine whether it could attenuate the valvular damage caused by RHD.

2. Methods

In this study, we aimed to find out whether altering S1PR1/STAT3 signaling pathway can attenuate the valvular damage caused by RHD. In order to achieve this goal, RHD rat model was established following previous reports [14, 21, 26–30], overexpression sequence was used to overexpress the expression of S1PR1, the expression of STAT3 was inhibited by STAT3-siRNA, Th17 cell-related cytokines (IL-6 and IL-17) and transcription factor (RORγt) were detected. Then, the degree of inflammation and fibrosis in valve tissues was evaluated to finally verify our conjecture.

2.1 Antigen preparation

First, brain heart infusion fluid medium (Guangdong Huankai Microbial Sci. & Tech. Co., Ltd.) was used to culture the group A streptococci (GAS, ATCC19615; American Type Culture Collection), the temperature during cultivation process was kept constant at 37 °C. After 24 h, the GAS were washed with normal saline (NS), and then transferred into 10% neutral formalin for 12 h for inactivation. Second, NS was used to wash and resuspend the inactivated GAS, and the concentration was simultaneously adjusted to 4.0×10^{11} CFU/ml. Finally, after emulsifying the suspensions by sonication (Sonics & Materials, Inc.), we obtained the antigen.

2.2 In vivo gene therapy

We used the recombinant adeno-associated virus (AAV; serotype 9) vectors carrying the rat S1PR1 overexpression sequence (S1PR1-overexpress; Hanbio Biotechnology Co., Ltd.) with c-TNT promoter to interfere with the expression of S1PR1, and used an AAV-control as a negative control to test whether the AAV vectors had an effect on the experiment. The S1PR1 overexpression sequence is listed in Table 1. In order to directly inhibit the expression of STAT3, a rat STAT3 small interfering RNA (siRNA) sequence (5'-GGCTGATCATTTATATAAA-3'; STAT3-siRNA; Hanbio Biotechnology Co., Ltd.) with c-TNT promoter carried by recombinant AAV vectors was used. In addition, to determine whether the AAV vector had an effect on the rats, we also used an AAV-control as a negative control.

Table 1
Sequencing result (S1PR1 overexpression sequence)

Sequence name	Sequencing result
S1PR1 overexpression sequence	ATGGTGTCCTCCACCAGCATCCCAGTGGTTAAGGCTCTCCGCAGCCAAGTCTCCGACTATGGCAACTATGATATCATAGTCCGGCATT ACAACTACACAGGCAAGCTGAACATCGGAGTGGAGAAGGACCATGGCATTAAACTGACTTCAGTGGTGTTCATTCTCATCTGCTGCTT GATCATCCTAGAGAATATATTTGTCTTGCTAACTATTTGGAAAACCAAGAAGTTCCACCGGCCCATGTACTATTTCATAGGCAACCTAG CCCTCTCGGACCTGTTAGCAGGAGTGGCTTACACAGCTAACCTGCTTGTTGTCTGGGGCCACCACCTACAAGCTCACACCTGCCCAGT GGTTTCTGCGGGAAGGAAGTATGTTTGTGGCTCTGTCTGCCTCAGTCTTCAGCCTCCTTGCTATCGCCATTGAGCGCTACATCACCAT GCTGAAGATGAAACTACACAACGGCAGCAACAGCTCGCGCTCCTTTCTGCTGATCAGTGCCTGCTGGGTCATCTCCCTCATCCTGGG TGGGCTGCCCATCATGGGCTGGAACTGCATCAGCTCGCTGTCCAGCTGCTCCACCGTGCTCCCGCTCTACCACAAGCACTATATTCT CTTCTGCACCACCGTCTTCACCCTGCTCCTGCTTTCCATCGTCATCCTCTACTGCAGGATCTACTCCTTGGTGAGGACTCGAAGCCG CCGCCTGACCTTCCGCAAGAACATCTCCAAGGCCAGCCGCAGTTCCGAGAAGTCTCTGGCCTTGCTGAAGACAGTGATCATTGTCC TGAGTGTCTTCATTGCCTGCTGGGCCCCTCTCTTCATCCTACTACTTTTAGATGTGGGGTGCAAGGCGAAGACCTGTGACATCCTGTA CAAAGCAGAGTACTTCCTGGTTCTGGCTGTGCTGAACTCAGGTACCAACCCCATCATCTACACTCTGACCAATAAGGAGATGCGCC GGGCCTTCATCAGGATCATATCTTGTTGCAAATGCCCCAACGGAGACTCCGCTGGCAAATTCAAGAGGCCCATCATCCCGGGCAT GGAATTTAGCCGCAGCAAATCAGACAACTCCTCCCACCCCCAGAAGGATGATGGGGACAATCCAGAGACCATTATGTCTTCTGG AAACGTCAATTCTTCTTCT
S1PR1, sphingosine-1-phosphate receptor 1.	

2.3 Animals and groups

We purchased 48 Lewis rats (150−180 g) from Beijing Vital River Animal Technology Co., Ltd. All the rats were female and at 8 weeks of age, and weighing 150−180 g. 48 rats were first randomly divided into two groups: Group Ⅰ (n = 24) and Group Ⅱ (n = 24). Group Ⅰ was used to study the effect of expressing S1PR1; Group Ⅱ was used to study the effect of inhibiting STAT3. Our pathogen-free animal laboratory at the Animal Experiment Center of Guangxi Medical University provides a good environment for captive rats: the temperature is constantly set to 23˚C and the fluctuation does not exceed 2˚C; the cycle of day and night is 12 hours; the movement of rats in the cage is completely unrestricted; and we provide convenient drinking conditions and standard rat feed. We allowed all the rats to adapt to the environment for 5 days before the start of the experiment. All animal experimental procedures were conducted according to the ethical guidelines for the care and use of laboratory animals and were approved by the Medical Ethics Committee of the First Affiliated Hospital of Guangxi Medical University (grant no. 2019-KY-E-069). Rats in Group Ⅰ were divided randomly into four subgroups: control group, S1PR1-overexpress group, AAV-control group and RHD group. Each subgroup had 6 rats. The RHD group was the established RHD model. The method we used to establish the RHD rat model was following previous reports [14, 21, 26−30]. Footpad injection of CFA (Sigma-Aldrich, Merck KGaA) is essential in establishing the RHD rat model. And all rats were maintained on soft bedding and not in wire-bottomed cages. We used 9 weeks to establish the RHD rat model. In the beginning, one hind footpad of the rat was injected with a 100 µl solution of inactivated GAS (4.0×10^{11} CFU/ml) and CFA mixed at a ratio of 1:1 (v/v). Then, after one week, we performed a subcutaneous injection of 500 µl of inactivated GAS (4.0×10^{11} CFU/ml) and CFA mixed at a ratio of 1:1 (v/v) into the abdomen once a week at the same interval for 4 weeks. For the last 4 weeks, we performed subcutaneous abdominal injection once a week at the same interval with an adjustment of the injection solution to 500 µl of inactivated GAS (4.0×10^{11} CFU/ml). Rats in the S1PR1-overexpress group were injected with 2.5×10^{11} viral genome particles at one time through the tail vein (AAV-S1PR1-overexpress, diluted with 200 µl normal saline) at the beginning of the experiment. Three weeks later, the rats received exactly the same treatment as the RHD group. The rats in the AAV-control group received an injection of 2.5×10^{11} viral genome particles at one time through the tail vein (AAV-control, diluted with 200 µl normal saline) at the beginning of the experiment. Three weeks later, they were injected following the same protocol as that of the RHD group. The rats in the control group were injected using the same protocol as that of the RHD group from the beginning, but the injection solution was the same volume of NS.

Rats in Group Ⅱ were divided randomly into four subgroups: control group, STAT3-siRNA group, AAV-control group and RHD group. Each subgroup had 6 rats. The rats in the control group, AAV-control group and RHD group were injected using the same protocol as that of the control group, AAV-control group and RHD group in Group Ⅰ, respectively. The rats in the STAT3-siRNA group were injected using the same protocol as that of the S1PR1-overexpress group in Group Ⅰ, excepted that the solution for tail vein injection was changed from 2.5×10^{11} viral genome particles (AAV-S1PR1-overexpress, diluted with 200 μl normal saline) to 2.5×10^{11} viral genome particles (AAV-STAT3-siRNA, diluted with 200 μl normal saline).

2.4 Sacrifice

After the treatments, we collected a total of 1 ml blood via tail veins of rats from each group without anaesthesia, then an intraperitoneal injection of sodium pentobarbital (150 mg/kg) was used to sacrifice the rat. Animal death was determined when there was more than 5 min without breathing or heartbeat. Humane endpoint was defined as animals losing > 15% of their body weight with a decreased ability to consume food and water.

2.5 Sample preparation

We collected samples from the valves of every rat. All samples were frozen in liquid nitrogen rapidly and then stored at -80 °C for follow-up experiments. Since no animals died during the modeling process, there were 6 samples in each subgroup. The next five experimental methods (RT-qPCR, WB, immunohistochemistry, histochemistry and ELISA) were based on the previously mentioned experimental grouping and tested 6 samples in each subgroup.

2.6 RT-qPCR

First, total RNA was extracted from each sample. We used TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the protocol supplied by the manufacturer to finish this step. Second, the concentration of RNA must be measured for quantitative reverse transcription. We used a NanoDrop™ 2000 spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc.) to finish this step. Third, reverse transcription of RNA into cDNA. In this step we reverse transcribed 0.5 μg of total RNA from each sample into cDNA. The kit we used was PrimeScript RT reagent kit (Takara Bio, Inc.). And the entire reverse transcription process is strictly in accordance with the instructions supplied by the manufacturer. Finally, RT-qPCR was performed. In this step, the kit we used was TB Green Premix Ex Taq Ⅱ (Takara Bio, Inc.), the instrument we used was a StepOne system (Applied Biosystems; Thermo Fisher Scientific, Inc.), and the internal reference was set to β-actin, and the entire process was still strictly in accordance with the manufacturer's instructions. In Table 2 we list the sequences of the primer sequences in detail. The final result was expressed by the fold difference between the expression level of each mRNA and that of the internal reference using the $2^{-\Delta\Delta Ct}$ method [31]. All samples were measured 3 times.

Table 2
Sequences of primers used in reverse transcription-quantitative PCR.

Gene	Primer sequence (5'-3')
STAT3	Forward: TTTGAGACAGAGGTGTACCACCAAG
	Reverse: ACCACAGGATTGATGCCCAAG
S1PR1	Forward: GCTTCATCACTCACTACCCTAGCA
	Reverse: TTCTCCCTTCCCTCCCTCTC
Col3a1	Forward: ACTTCTGGTCCTCCTGGTCTGC
FSP1	Reverse: CGCCTGGCTCACCCTTTTCAC
	Forward: TGGGGAGAAGGACAGACGAAGC
	Reverse: TGGCAATGCAGGACAGGAAGAC
β-actin	Forward: GGAGATTACTGCCCTGGCTCCTA
	Reverse: GACTCATCGTACTCCTGCTTGCTG

In Table 2 we list the sequences of the primer sequences in detail. STAT3, signal transducer and activator of transcription 3; S1PR1, sphingosine-1-phosphate receptor 1; Col3a1, collagen type III α1 chain; FSP1, fibroblast-specific protein 1.

2.7 Western blotting

First, total protein was extracted from each sample. In this step, the kit we used was RIPA lysis buffer (Sangon Biotech Co., Ltd.), and the method used is according to the instruction supplied by the manufacturer. Second, the protein concentration was measured. In this step, the kit we used was a bicinchoninic acid (BCA) protein assay kit (Sangon Biotech Co., Ltd.). Third, the same amounts of protein (30 μg) from each sample were separated. In this step, the material we used to separate the protein was 10% SDS-PAGE. The separation conditions were: 80 V for 30 min and 120 V for 60 min, the equipment we used was a blotting system (Bio-Rad Laboratories, Inc.), and the entire process was still strictly in accordance with the

manufacturer's instructions. Third, the separated proteins were electrotransferred to 0.22 μm polyvinylidene fluoride (PVDF) membranes (EMD Millipore), the transfer conditions were: constant 80 V for 80 min. Fourth, we blocked the membranes carrying proteins for 1 h at room temperature with 3% BSA blocking solution (Sangon Biotech Co., Ltd.) and then incubated the membranes overnight at 4 °C with the following antibodies: anti-S1PR1 (1:1,000; cat. no. 55133-1-AP; ProteinTech Group, Inc.), anti-STAT3 (1:1,000; cat. no. ab68153; Abcam), anti-p-STAT3 (1:1,000; cat. no. 9145; Cell Signaling Technology, Inc.) and anti-β-tubulin (1:3,000; cat. no. 10068-1-AP; ProteinTech Group, Inc.). After that, at room temperature, we incubated the membranes in the dark for 1 h with HRP-conjugated secondary antibody (10,000; cat. no. ab6721; Abcam). Finally, the protein bands were scanned by a chemiluminescence imaging system (Alpha FluorChem FC3; Alpha, Inc.) and we used ImageJ software (1.51j, National Institute of Health) to quantify the expression of the proteins, which were normalized to β-tubulin. All samples were measured 3 times.

2.8 Immunohistochemistry

Immunohistochemistry was performed following a previous report [30] to analyse the valve tissues, which were stained for IL-6 (1:65; cat. no. ab9324; Abcam), IL-17 (1:90; cat. no. ab214588; Abcam), S1PR1 (1:80; cat. no. ab77076; Abcam), STAT3 (1:75; cat. no. ab69153; Abcam), p-STAT3 (1:70; cat. no. ab76315; Abcam). And RORγt (1:75; cat. no. 13205-1-AP; Proteintech) which is the key transcription factor that is driving the differentiation of IL-17 producing Th17 cells [32] was also detected. Briefly, the valve tissues were embedded in formalin-fixed paraffin first. Then all blocks were sectioned at 5 μm. After deparaffinization and rehydration, the 5% bovine serum albumin (BSA; Beijing Solarbio Science & Technology Co., Ltd.) solution was used to block the sections at room temperature for 1 h. After deactivation of endogenous peroxidase with hydrogen peroxide, the sections were incubated with the previously mentioned primary antibodies for 12 h at 4 °C. Then, at room temperature, the anti-rabbit horseradish peroxidase (HRP)-conjugated (1:10; cat. no. PV-6001; OriGene Technologies, Inc.) or anti-mouse HRP-conjugated (1:10; cat. no. PV-6002; OriGene Technologies, Inc.) was used as secondary antibodies to incubate the sections for 30 min. After enhancement of colour development by using diaminobenzidine (DAB), the immunostained tissues were examined under a BX43 light microscope (Olympus Corporation), and positive expression was reflected by brownish yellow staining, which was detected by microscopy. Quantitative assessment was performed using the methods provided by Friedrichs et al. in a previous report [33]. The positive cell percentage and immunohistochemical score (IHS) were also used in our experiment to describe the quantitative evaluation results.

2.9 Histochemistry

The valve tissues were fixed for 24 h at 4 °C in 4% paraformaldehyde before decalcifying and embedding in paraffin blocks. All blocks were serially sectioned at 5 μm for haematoxylin and eosin (H&E) staining and Sirius red staining. H&E staining was performed at room temperature, the sections were first stained with haematoxylin for 4–10 min and then with eosin for 0.5-2 min. Then, in order to capture the images reflecting the results of H&E staining, a BX43 light microscope (Olympus Corporation) was used. Sirius red staining was also performed at room temperature, the Sirius red solution was used to stain the sections for 1 h. Then, in order to capture the images reflecting the results of Sirius red staining, a BX43 confocal microscope (magnification, × 100; Olympus Corporation) was used.

2.10 Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) kits (cat. nos. E04640r and E07451r; Cusabio) were used to measure the levels of IL-6 and IL-17 in the serum. The entire process was strictly in accordance with the manufacturer's instructions. All samples were measured 3 times.

2.11 Statistics

Results are expressed as the mean ± standard deviation of at least three independent experiments. The software we used for statistical analysis was SPSS software 16.0 (SPSS, Inc.). The method we used to compare the differences between 2 groups is Student's t-test. The method we used when comparing the differences among 4 groups was one-way ANOVA. We used $p < 0.05$ as the criterion for identifying significant differences.

3. Results

3.1 Group Ⅰ (overexpress S1PR1)

3.1.1 In vivo gene therapy improved the expression of S1PR1, which is originally decreased during RHD-induced vavular damage

Results of RT-qPCR, WB and immunohistochemistry showed that the expression of S1PR1 in AAV-control group and RHD group was significantly lower than that of control group (P < 0.05) which is consistent with previous reports [34, 35]. The expression in the S1PR1-overexpress group was similar to that in the control group, and was significantly higher than that in the RHD group (P < 0.05; Fig. 1, 2A, 2B). These results indicated that the S1PR1 overexpression sequence we used in this experiment successfully increased the expression of S1PR1 that was originally down-regulated during the pathogenesis of RHD.

3.1.2 Elevated S1PR1 expression reduced the phosphorylation level of STAT3 that is originally elevated during RHD-induced vavular damage

The results of WB, RT-qPCR and immunohistochemistry all showed that the expression of STAT3 was not significantly different among the four groups. The expression of p-STAT3 in AAV-control group and RHD group was significantly higher than that in control group (P < 0.05). The expression of p-STAT3 in the S1PR1 group was significantly lower than that in the RHD group (P < 0.05; Fig. 2A, 2C, 2D and 3A-D). The ratio of p-STAT3/total (t-)STAT3 also showed the same trend (P < 0.05; Fig. 2E and 3E). These results suggested that the increased expression of S1PR1 in the S1PR1-overexpress group reduced the originally elevated phosphorylation level of STAT3.

3.1.3 Th17 cell-related factors expression in S1PR1-overexpress group was significantly lower than that in RHD group

This conclusion can be concluded from the two experiments of immunohistochemistry and ELISA. In the results of these two experiments, the expressions of RORγt, IL-6 and IL-17 in the AAV-control group and the RHD group were significantly higher than the control group (P < 0.05). The S1PR1-overexpress group reflected that the expressions of RORγt, IL-6 and IL-17 in this group were significantly lower than those in the RHD group (P < 0.05; Fig. 4). These results suggested that increasing the expression of S1PR1 can reduce the expression levels of Th17 cell-related factors in the valve and serum.

3.1.4 Elevated S1PR1 expression could attenuate the valvular damage due to RHD

H&E staining and Sirius red staining showed inflammation and fibrosis on the valves in the AAV-control group and RHD group, the control group was a normal valve, while on the valves of the S1PR1-overexpress group, a reduced inflammation and fibrosis can be observed than in the RHD group (Fig. 5A and 5B). Previous studies have shown that type 1 collagen (COL1) fibres are the main type of collagen in nonfibrotic valves [36]. The ratio of type 3 collagen (COL3) and COL1 can be used to reflect the degree of fibrosis of the valve tissue. In the process of fibrosis of the valve tissue, the proportion of COL3 will become larger and larger, so the larger this ratio is, the more severe the fibrosis is. Therefore, we used the COL3/COL1 (COL3/1) ratio to quantify the degree of fibrosis of the valve tissue. In the pictures of Sirius red staining, type 1 collagen fibres are closely packed yellow and red fibres, with obvious birefringence, while type 3 collagen fibres are loosely arranged green fibres, with weak birefringence. The ratio of COL3/COL1 in the S1PR1-overexpress group was significantly lower than that in the RHD group (P < 0.05; Fig. 5C). In addition, we detected the expression of Col3a1 and FSP1 by RT-qPCR to reflect the degree of valve fibrosis at the mRNA level. The results also showed that the expression of Col3a1 and FSP1 in the S1PR1-overexpress group was significantly lower than that in the RHD group (P < 0.05; Fig. 5D and 5E). The above results suggested that increasing the expression of S1PR1 could attenuate the valvular damage due to RHD.

3.2 Group Ⅱ (inhibit STAT3)

3.2.1 STAT3-siRNA pretreatment decreased STAT3 expression and reduced the total amount of p-STAT3

The result of RT-qPCR showed that compared with the control group, the expression of S1PR1 mRNA decreased in the three groups: AAV-control, RHD and STAT3-siRNA groups (P < 0.05; Fig. 6A). The results of western blotting and immunohistochemistry showed that the protein expression of S1PR1 was downregulated in the AAV-control, STAT3-siRNA and RHD groups compared with that of the control group (P < 0.05; Fig. 6A, 2B and 7A-C). These results indicate that the expression of S1PR1 is down-regulated in the RHD rat model, which is consistent with previous reports [14, 30]. Inhibition of STAT3 by STAT3-siRNA decreased STAT3 mRNA expression, this result can be reflected in the comparison between STAT3-siRNA group and the other three groups (P < 0.05; Fig. 6B). The protein expression of p-STAT3 was significantly higher in the RHD and AAV-control groups than in the control group (P < 0.05). And inhibition of STAT3 by STAT3-siRNA decreased the protein expression of STAT3, p-STAT3 in the valve tissues, this result can be reflected in the comparison between STAT3-siRNA group and the other three groups (P < 0.05; Fig. 7A, 7C, 7D, 8A-C). And the ratio of p-STAT3/total (t-)STAT3 was significantly higher in the STAT3-siRNA, AAV-control and RHD groups than in the control group (P < 0.05), while there was no significant difference among the STAT3-siRNA, AAV-control and RHD groups (P < 0.05; Fig. 7E and 8D), which indicates that STAT3-siRNA will not change the ratio of p-STAT3/t-STAT3, but will reduce the total amount of both STAT3 and p-STAT3.

3.2.2 The expression of Th17-related transcription factor and cytokines was reduced in STAT3-siRNA group than in RHD group

The results of immunohistochemistry and ELISA showed that the expression of RORγt, IL-6 and IL-17 was significantly higher in the RHD and AAV-control groups than in the control group (P < 0.05). And inhibition of STAT3 by STAT3-siRNA decreased the expression of RORγt, IL-6 and IL-17 in the serum and valve tissues, this result can be reflected in the comparison between STAT3-siRNA group and the other three groups (P < 0.05; Fig. 9).

3.2.3 STAT3-siRNA pretreatment attenuates the valvular damage due to rheumatic heart disease

H&E staining showed that there was an inflammatory response in the heart valves of the rats in the AAV-control and RHD groups. In the STAT3-siRNA group, we observed that the inflammatory response is reduced compared to the RHD group (Fig. 10A). All changes were observed under a

microscope. In the pictures of Sirius red staining, the pictures showed that the fibrosis in RHD group is significantly more severe than that in control group. Accordingly, the COL3/1 ratio was also significantly higher in RHD group than in control group. The degree of fibrosis in the STAT3-siRNA group was lower than that in the RHD group. Accordingly, the COL3/1 ratio was significantly lower in STAT3-siRNA group than that in RHD group (P < 0.05; Fig. 10B and 10C). And the results of detecting Col3a1 and FSP1 were consistent with the results of the histological examination (P < 0.05; Fig. 10D and 10E). Both results reflected that after inhibiting the expression of STAT3, the inflammatory response and fibrosis of the valve were reduced compared with the RHD group.

4. Discussion

RHD has a long history, and many patients have been killed by this disease. RHD caused 319,400 deaths in 2015 [37], 314,600 deaths in 2016 [38] and 285,500 deaths in 2017 [39], but the pathogenesis of this disease is still unknown. In recent years, research progress has mostly focused on the signaling pathways that are related to pathogenesis. Through the efforts of researchers, some signaling pathways related to this disease have been discovered. Recently, researchers have discovered that the S1PR1/STAT3 signaling pathway is involved in vavular damage due to RHD from the study on RHD model rats [14]. However, it is unknown how intervening in the S1PR1/STAT3 pathway affects valvular damage caused by RHD. More importantly, which method can be used to intervene in the S1PR1/STAT3 pathway and effectively attenuate valvular damage due to RHD?

S1PR1 has been extensively studied, and it is an important factor in heart diseases. The heart diseases it participates in include not only RHD [34, 35], but also myocardial infarction [9], Cardiac Remodelling, etc. [10]. In these heart diseases S1PR1 participates in, it mostly plays a role in protecting the heart [9–11], and the high expression of S1PR1 can usually protect the heart during the pathogenesis of heart disease [9, 12]. However, there is also a report of high expression of S1PR1 exacerbating heart damage [40], and the role of mediating the pathogenesis of disease through down-regulation of S1PR1 expression has also been reported in other diseases, such as multiple sclerosis [41]. So it can be concluded that the expression level of S1PR1 in different heart diseases is not static. Although the expression level of S1PR1 in each heart disease is variable, in most cases S1PR1 is up-regulated in heart disease and plays a cardioprotective role. In this study, after up-regulating the expression of S1PR1, the valvular damage caused by RHD is reduced, which may also be related to the cardioprotective effect of S1PR1. There is a close relationship between S1PR1 and STAT3, and there have been many reports on the role of S1PR1 in regulating STAT3 in various diseases [22–25]. In this study, the effects of S1PR1 and STAT3 are closely related to the pathogenesis of valvular damage in RHD. In previous studies on the process of valvular damage in RHD, it was reported that the down-regulation of S1PR1 and increased degree of the phosphorylation of STAT3 were involved in this process [34, 35]. This phenomenon of down-regulated S1PR1 expression and increased degree of STAT3 phosphorylation has also been reported in previous studies, such as Garris et al. found that the deficiency of S1PR1 expression would increase the phosphorylation level of STAT3 and then promote Th17 cell differentiation by studying the S1PR1 gene mutation mouse autoimmune encephalitis model [13]. The results of our current study also showed that the RHD group also had down-regulated S1PR1 expression and increased STAT3 phosphorylation level compared to the control group. However, some studies have reported that up-regulation of S1PR1 expression increases the phosphorylation of STAT3 [23, 42], but these studies are not about RHD. Combined with the uncertainty of the expression of S1PR1 in different heart diseases mentioned above, it can be concluded that the expression level of S1PR1 and its role of regulating the phosphorylation of STAT3 in different diseases and different physiological or pathological processes may not be static according to the current research progress in the world. Two studies that previously studied the role of the S1PR1/STAT3 signaling pathway in RHD and the experimental results in this paper indicate that down-regulation of S1PR1 may increase the phosphorylation of STAT3 during RHD-induced valvular damage, and then mediates the differentiation of Th17 cells [34, 35]. Therefore, the mechanism of S1PR1/STAT3 signaling pathway in the process of valvular damage caused by RHD may be very similar to the mechanism described in the study of Garris. It may be because autoimmune encephalitis and RHD are both the autoimmune diseases, however, this is just speculation. Since S1PR1 is down-regulated in this pathway, we wondered whether overexpress its expression can attenuate valvular damage due to rheumatic heart disease.

STAT3 plays a key pathogenic role in many inflammatory conditions. Researchers have found that STAT3 mediates immune myocarditis due to IL-6 production-induced liver complement component C3 and Th17 cell differentiation [43], and the differentiation of Th17 cells plays an important role in the occurrence and development of myocarditis [44]. Furthermore, tissue signaling cytokines such as IL-17 and IL-22 have been proposed to have actions on the heart that involve STAT3 [45]. Th17 cells and related inflammatory factors (such as IL17) play an important role in the process of inflammation and autoimmune response [18, 46, 47].Th17 cell-related factors are increased in peripheral blood and serum of RHD patients [48], and the level of Th17-related cytokines in the mitral valve is also significantly increased [49]. Therefore, Th17 cells are likely to promote the development of RHD disease. Previous studies have shown high expression of p-STAT3 in rheumatoid arthritis [50], and so we consider STAT3 to be the key part of this signaling pathway, we wondered whether suppressing its expression can attenuate valvular damage due to rheumatic heart disease.

Based on the research results of the relationship between the S1PR1/STAT3 signaling pathway and valvular damage caused by RHD, we speculated that the phosphorylation level of STAT3 is increased via S1PR1/STAT3 signaling pathway, thereby promoting the differentiation of CD4 + T cells into Th17 cells, and then released Th17 cells-related cytokines and participated in the process of valvular damage caused by RHD. So we designed experiments to overexpress S1PR1 and inhibit STAT3 to interfere with the S1PR1/STAT3 signaling pathway. The results of Group Ⅰ showed that after the overexpression treatment of S1PR1, the originally down-regulated expression of S1PR1 rosed, the phosphorylation level of STAT3 dropped, Th17 cell-related cytokines expressed in the valve and serum also decreased, and eventually the level of inflammation and fibrosis of the valve caused by RHD was attenuated. The results of Group Ⅱ showed that after the inhibition treatment of STAT3, the total amount of both STAT3 and p-STAT3 was reduced, the expression of Th17-related transcription factor and cytokines was then reduced, and the level of inflammation and fibrosis of the valve

caused by RHD was also attenuated. These results showed that the S1PR1/STAT3 signaling pathway is involved in regulating the Th17 cell-related cytokines during the vavular damage due to RHD, and altering the S1PR1/STAT3 signaling pathway could affect the expression of Th17 cell-related cytokines then attenuate the vavular damage due to RHD.

At present, there are few studies on the signaling pathways related to the pathogenesis of RHD, and there are only 6 signaling pathways with significant research progress: RhoA/Rho-dependent kinase (RhoA/ROCK) signaling pathway,Mitogen-activated protein kinase (MAPK) signaling pathway、Protein kinase B/S6 kinase (AKT/S6K) signaling pathway、TGF-β1/Smad signaling pathway、Wnt signaling pathway and S1PR1/STAT3 signaling pathway [34, 51–55]. And there are only three potential intervention targets found in these signaling pathway studies: Intervene in the expression of interferon (IFN)-γ and tumour necrosis factor (TNF)-α to regulate extracellular matrix remodeling to reduce heart damage caused by RHD; Intervene in AKT/S6K signaling pathway to inhibit TGF-β1-induced fibroblasts; Intervene in the S1PR1/STAT3 signaling pathway to reduce RHD-induced valvular damage. And whether these intervention targets are effective for the prevention and treatment of RHD requires the efforts of researchers to further study. This shows that there is a lack of research on the pathogenesis of RHD worldwide, so that the pathogenesis of RHD is still unclear. The threat of RHD to patients' lives is huge, the damage to patients' property and quality of life is devastating. RHD should be taken seriously, and it is worthy of researchers' efforts to explore its pathogenesis. The object of our study, S1PR1/STAT3 signaling pathway, may become a potential intervention target for RHD. We hope that this study will enrich the research on the signaling pathways related to the pathogenesis of RHD, and we hope that this research will contribute to revealing the pathogenesis of RHD, and it can provide a little help for the development of effective and inexpensive control methods for RHD in the future.

There are some limitations in our research. This was a rat study, and further research on humans is required. S1PR1 is already down-regulated during RHD-induced valvular damage, and what will happen if S1PR1 expression is completely blocked, higher technical requirements and further experiments are needed. How will up-regulating the expression of STAT3 affect RHD is unknown. Cell experiments may provide cell-level evidence for our research, but we have not conducted it. In addition, we did not detect the expression of FSP1 and Col3a1 at the protein level.

5. Conclusion

Therefore, the status of RHD is still severe, and although primary and secondary prevention strategies are clear, global implementation is not ideal [56]. Many researchers have made efforts, but its pathogenesis is still not understood. By summarizing previous research results and the inflammatory mechanism of RHD pathogenesis, we hypothesize that altering the S1PR1/STAT3 signaling pathway may affect the process of valvular damage caused by RHD. Animal experiments showed that the S1PR1/STAT3 signaling pathway is involved in regulating the Th17 cell-related cytokines during the vavular damage due to RHD, and altering the S1PR1/STAT3 signaling pathway could affect the expression of Th17 cell-related cytokines then attenuate the vavular damage due to RHD.

Abbreviations

RHD: rheumatic heart disease

S1PR1: sphingosine 1-phosphate receptor 1

STAT3: signal transducer and activator of transcription 3

Th17: T helper 17

GAS: Group A streptococci

CFA: Freund's adjuvant

STAT3-siRNA: STAT3-small interfering RNA

p-: phosphorylated

ELISA: Enzyme-linked immunosorbent assay

IL: interleukin

RT-qPCR: Reverse transcription-quantitative PCR

WB: Western blotting

RORγt: retinoic acid-related orphan receptor gamma T

NS: normal saline

AAV: adeno-associated viral

H&E: haematoxylin and eosin

IHS: immunohistochemical score

DAB: diaminobenzidine

PVDF: polyvinylidene fluoride

ELISA: Enzyme-linked immunosorbent assay

t-: total

COL1: type 1 collagen

COL3: type 3 collagen

Col3a1: type III α1 chain

FSP1: fibroblast-specific protein 1

MAPKs: mitogen-activated protein kinases

RhoA/ROCK: RhoA/Rho-dependent kinase

MAPK: Mitogen-activated protein kinase

AKT/S6K: Protein kinase B/S6 kinase

Declarations

Ethics approval and consent to participate

Protocols involving animals were approved by the Medical Ethics Committee of the First Affiliated Hospital of Guangxi Medical University (permit no. 2019-KY-E-069).

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

Funding

Authors' contributions

Z.Y.Z. and F.H. conceived and designed the study. S.L.X. and A.C. participated in the experimental design. S.L.X., A.C., Y.J.W. and C.H.L. performed the experiments. S.L.X., A.C., H.W. and J.L.W. analyzed the data. S.L.X. wrote the manuscript and all authors read and approved the final manuscript.

Acknowledgements

References

1. DA W, CO J, SM C, *et al*: Global, Regional, and National Burden of Rheumatic Heart Disease, 1990-2015. 377: 713-722, 2017.

2. Leal MTBC, Passos LSA, Guarçoni FV, *et al*: Rheumatic heart disease in the modern era: recent developments and current challenges. Rev Soc Bras Med Trop 52: e20180041-e20180041, 2019.

3. Mirabel M, Narayanan K, Jouven X and Marijon E: Cardiology patient page. Prevention of acute rheumatic fever and rheumatic heart disease. Circulation 130: e35-e37, 2014.

4. Zhao Z, He D, Ling F, *et al*: CD4(+) T cells and TGFβ1/MAPK signal pathway involved in the valvular hyperblastosis and fibrosis in patients with rheumatic heart disease. Exp Mol Pathol 114: 104402-104402, 2020.

5. Messias-Reason IJ, Schafranski MD, Kremsner PG and Kun JF: Ficolin 2 (FCN2) functional polymorphisms and the risk of rheumatic fever and rheumatic heart disease. Clin Exp Immunol 157: 395-399, 2009.

6. Li M, Xu S, Geng Y, *et al*: The protective effects of L-carnitine on myocardial ischaemia-reperfusion injury in patients with rheumatic valvular heart disease undergoing CPB surgery are associated with the suppression of NF-kappaB pathway and the activation of Nrf2 pathway. Clin Exp Pharmacol Physiol 46: 1001-1012, 2019.

7. Aoki M, Aoki H, Ramanathan R, Hait NC and Takabe K: Sphingosine-1-Phosphate Signaling in Immune Cells and Inflammation: Roles and Therapeutic Potential. Mediators Inflamm 2016: 8606878-8606878, 2016.

8. Jozefczuk E, Guzik TJ and Siedlinski M: Significance of sphingosine-1-phosphate in cardiovascular physiology and pathology. Pharmacological research 156: 104793, 2020.

9. Deng S, Zhou X, Ge Z, *et al*: Exosomes from adipose-derived mesenchymal stem cells ameliorate cardiac damage after myocardial infarction by activating S1P/SK1/S1PR1 signaling and promoting macrophage M2 polarization. The international journal of biochemistry & cell biology 114: 105564, 2019.

10. Liu X, Wu J, Zhu C, *et al*: Endothelial S1pr1 regulates pressure overload-induced cardiac remodelling through AKT-eNOS pathway. Journal of cellular and molecular medicine 24: 2013-2026, 2020.

11. Liu Y, Zhi Y, Song H, *et al*: S1PR1 promotes proliferation and inhibits apoptosis of esophageal squamous cell carcinoma through activating STAT3 pathway. Journal of experimental & clinical cancer research : CR 38: 369, 2019.

12. Chen YZ, Wang F, Wang HJ and Liu HB: Sphingosine 1 phosphate receptor-1 (S1PR1) signaling protects cardiac function by inhibiting cardiomyocyte autophagy. Journal of geriatric cardiology : JGC 15: 334-345, 2018.

13. Garris CS, Wu L, Acharya S, *et al*: Defective sphingosine 1-phosphate receptor 1 (S1P1) phosphorylation exacerbates TH17-mediated autoimmune neuroinflammation. Nature immunology 14: 1166-1172, 2013.

14. Wu XD, Zeng ZY, Gong DP, Wen JL and Huang F: Potential involvement of S1PR1/STAT3 signaling pathway in cardiac valve damage due to rheumatic heart disease. Biotech Histochem 94: 398-403, 2019.

15. Hu YS, Han X and Liu XH: STAT3: A Potential Drug Target for Tumor and Inflammation. Curr Top Med Chem 19: 1305-1317, 2019.

16. Liu X, Hu H, Fan H, *et al*: The role of STAT3 and AhR in the differentiation of CD4+ T cells into Th17 and Treg cells. Medicine (Baltimore) 96: e6615, 2017.

17. Gaffen SL, Jain R, Garg AV and Cua DJ: The IL-23-IL-17 immune axis: from mechanisms to therapeutic testing. Nat Rev Immunol 14: 585-600, 2014.

18. Whibley N, Tritto E, Traggiai E, *et al*: Antibody blockade of IL-17 family cytokines in immunity to acute murine oral mucosal candidiasis. J Leukoc Biol 99: 1153-1164, 2016.

19. Zhang Y, Shao Z, Zhang X, *et al*: TIPE2 Play a Negative Role in TLR4-Mediated Autoimmune T Helper 17 Cell Responses in Patients with Myasthenia Gravis. J Neuroimmune Pharmacol 10: 635-644, 2015.

20. Bas HD, Baser K, Yavuz E, *et al*: A shift in the balance of regulatory T and T helper 17 cells in rheumatic heart disease. J Investig Med 62: 78-83, 2014.

21. Y W, Z Z, C G, L L and Pathology LWJCptojotSfC: Changes in the expression of Th17 cell-associated cytokines in the development of rheumatic heart disease. 24: 382-387, 2015.

22. Lankadasari MB, Aparna JS, Mohammed S, *et al*: Targeting S1PR1/STAT3 loop abrogates desmoplasia and chemosensitizes pancreatic cancer to gemcitabine. Theranostics 8: 3824-3840, 2018.

23. Lin Q, Ren L, Jian M, *et al*: The mechanism of the premetastatic niche facilitating colorectal cancer liver metastasis generated from myeloid-derived suppressor cells induced by the S1PR1−STAT3 signaling pathway. Cell Death & Disease 10: 2019.

24. Lee H, Deng J, Kujawski M, *et al*: STAT3-induced S1PR1 expression is crucial for persistent STAT3 activation in tumors. Nature medicine 16: 1421-1428, 2010.

25. Deng J, Liu Y, Lee H, *et al*: S1PR1-STAT3 signaling is crucial for myeloid cell colonization at future metastatic sites. Cancer cell 21: 642-654, 2012.

26. Galvin JE, Hemric ME, Kosanke SD, Factor SM, Quinn A and Cunningham MW: Induction of myocarditis and valvulitis in lewis rats by different epitopes of cardiac myosin and its implications in rheumatic carditis. The American journal of pathology 160: 297-306, 2002.

27. Lymbury RS, Olive C, Powell KA, *et al*: Induction of autoimmune valvulitis in Lewis rats following immunization with peptides from the conserved region of group A streptococcal M protein. Journal of autoimmunity 20: 211-217, 2003.

28. Gorton D, Govan B, Olive C and Ketheesan N: B- and T-cell responses in group a streptococcus M-protein- or Peptide-induced experimental carditis. Infection and immunity 77: 2177-2183, 2009.

29. Batzloff MR, Fane A, Gorton D, *et al*: Preclinical immunogenicity and safety of a Group A streptococcal M protein-based vaccine candidate. Human vaccines & immunotherapeutics 12: 3089-3096, 2016.

30. Chen A, Wen J, Lu C, *et al*: Inhibition of miR-155-5p attenuates the valvular damage induced by rheumatic heart disease. Int J Mol Med 45: 429-440, 2020.

31. Wang X-T, Wu X-D, Lu Y-X, *et al*: Egr-1 is involved in coronary microembolization-induced myocardial injury via Bim/Beclin-1 pathway-mediated autophagy inhibition and apoptosis activation. Aging (Albany NY) 10: 3136-3147, 2018.

32. Guendisch U, Weiss J, Ecoeur F, *et al*: Pharmacological inhibition of RORγt suppresses the Th17 pathway and alleviates arthritis in vivo. PLoS One 12: e0188391-e0188391, 2017.

33. Friedrichs K, Gluba S, Eidtmann H and Jonat W: Overexpression of p53 and prognosis in breast cancer. Cancer 72: 3641-3647, 1993.

34. Wu XD, Zeng ZY, Gong DP, Wen JL and Huang F: Potential involvement of S1PR1/STAT3 signaling pathway in cardiac valve damage due to rheumatic heart disease. Biotechnic & Histochemistry 94: 398-403, 2019.

35. Chen A, Wen J, Lu C, *et al*: Inhibition of miR-155-5p attenuates the valvular damage induced by rheumatic heart disease. International journal of molecular medicine 45: 429-440, 2020.

36. Purushothaman KR, Purushothaman M, Turnbull IC, *et al*: Association of altered collagen content and lysyl oxidase expression in degenerative mitral valve disease. Cardiovasc Pathol 29: 11-18, 2017.

37. Mortality GBD and Causes of Death C: Global, regional, and national life expectancy, all-cause mortality, and cause-specific mortality for 249 causes of death, 1980-2015: a systematic analysis for the Global Burden of Disease Study 2015. Lancet 388: 1459-1544, 2016.

38. Collaborators GBDCoD: Global, regional, and national age-sex specific mortality for 264 causes of death, 1980-2016: a systematic analysis for the Global Burden of Disease Study 2016. Lancet 390: 1151-1210, 2017.

39. Collaborators GBDCoD: Global, regional, and national age-sex-specific mortality for 282 causes of death in 195 countries and territories, 1980-2017: a systematic analysis for the Global Burden of Disease Study 2017. Lancet 392: 1736-1788, 2018.

40. Zhang F, Xia Y, Yan W, *et al*: Sphingosine 1-phosphate signaling contributes to cardiac inflammation, dysfunction, and remodeling following myocardial infarction. American journal of physiology Heart and circulatory physiology 310: H250-261, 2016.

41. Edmonds Y, Milstien S and Spiegel S: Development of small-molecule inhibitors of sphingosine-1-phosphate signaling. Pharmacology & therapeutics 132: 352-360, 2011.

42. Silva VRR, Micheletti TO, Katashima CK, *et al*: Exercise activates the hypothalamic S1PR1-STAT3 axis through the central action of interleukin 6 in mice. Journal of cellular physiology 233: 9426-9436, 2018.

43. Camporeale A, Marino F, Papageorgiou A, *et al*: STAT3 activity is necessary and sufficient for the development of immune-mediated myocarditis in mice and promotes progression to dilated cardiomyopathy. EMBO Mol Med 5: 572-590, 2013.

44. Yuan J, Yu M, Lin Q-W, *et al*: Th17 cells contribute to viral replication in coxsackievirus B3-induced acute viral myocarditis. J Immunol 185: 4004-4010, 2010.

45. Kurdi M, Zgheib C and Booz GW: Recent Developments on the Crosstalk Between STAT3 and Inflammation in Heart Function and Disease. Front Immunol 9: 3029-3029, 2018.

46. Gaffen SL, Jain R, Garg AV and Cua DJ: The IL-23-IL-17 immune axis: from mechanisms to therapeutic testing. Nature reviews Immunology 14: 585-600, 2014.

47. Zhang Y, Shao Z, Zhang X, *et al*: TIPE2 Play a Negative Role in TLR4-Mediated Autoimmune T Helper 17 Cell Responses in Patients with Myasthenia Gravis. Journal of neuroimmune pharmacology : the official journal of the Society on NeuroImmune Pharmacology 10: 635-644, 2015.

48. Bas HD, Baser K, Yavuz E, *et al*: A shift in the balance of regulatory T and T helper 17 cells in rheumatic heart disease. Journal of investigative medicine : the official publication of the American Federation for Clinical Research 62: 78-83, 2014.

49. Wen Y, Zeng Z, Gui C, Li L and Li W: Changes in the expression of Th17 cell-associated cytokines in the development of rheumatic heart disease. Cardiovascular pathology : the official journal of the Society for Cardiovascular Pathology 24: 382-387, 2015.

50. Turkson J and Jove R: STAT proteins: novel molecular targets for cancer drug discovery. Oncogene 19: 6613-6626, 2000.

51. Jiang L, Wei XF, Yi DH, *et al*: Synergistic effects of cyclic strain and Th1-like cytokines on tenascin-C production by rheumatic aortic valve interstitial cells. Clinical and experimental immunology 155: 216-223, 2009.

52. Li M, Yi XIN, Ma L and Zhou Y: Hepatocyte growth factor and basic fibroblast growth factor regulate atrial fibrosis in patients with atrial fibrillation and rheumatic heart disease via the mitogen-activated protein kinase signaling pathway. Experimental and Therapeutic Medicine 6: 1121-1126, 2013.

53. Zhang P, Wang W, Wang X, *et al*: Focal adhesion kinase mediates atrial fibrosis via the AKT/S6K signaling pathway in chronic atrial fibrillation patients with rheumatic mitral valve disease. International Journal of Cardiology 168: 3200-3207, 2013.

54. Zhang L, Zhang N, Tang X, Liu F, Luo S and Xiao H: Increased alpha-Actinin-2 Expression in the Atrial Myocardium of Patients with Atrial Fibrillation Related to Rheumatic Heart Disease. Cardiology 135: 151-159, 2016.

55. Guo F, Yi X, Li M, Fu J and Li S: Snail1 is positively correlated with atrial fibrosis in patients with atrial fibrillation and rheumatic heart disease. Exp Ther Med 14: 4231-4237, 2017.

56. Remenyi B, ElGuindy A, Smith SC, Jr., Yacoub M and Holmes DR, Jr.: Valvular aspects of rheumatic heart disease. Lancet 387: 1335-1346, 2016.

Figures
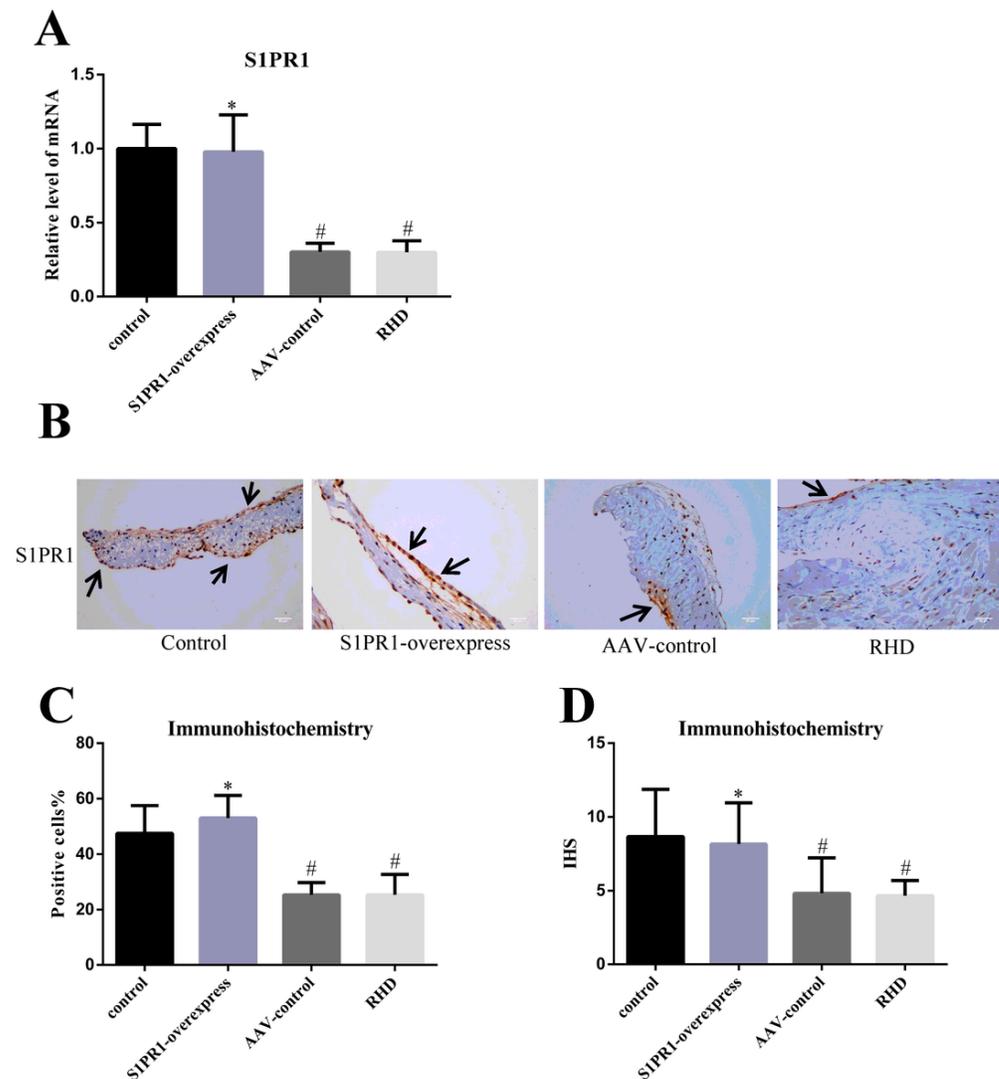
Figure 1

RT-qPCR analysis and immunohistochemistry analysis for S1PR1. (A) Relative levels of S1PR1 mRNA expression. (B) Immunohistochemical images of S1PR1; magnification, x400; scale bar: 50 μm; positive cells were represented by brownish yellow staining pointed by arrows. (C) Proportion of positive cells of S1PR1 in four groups. (D) The IHS of S1PR1 in four groups. This figure showed that the S1PR1 expression in S1PR1-overexpress group was higher than RHD group. Data was presented as the mean ± SD; #$P<0.05$ vs. the control group. *$P<0.05$ vs. the RHD group.
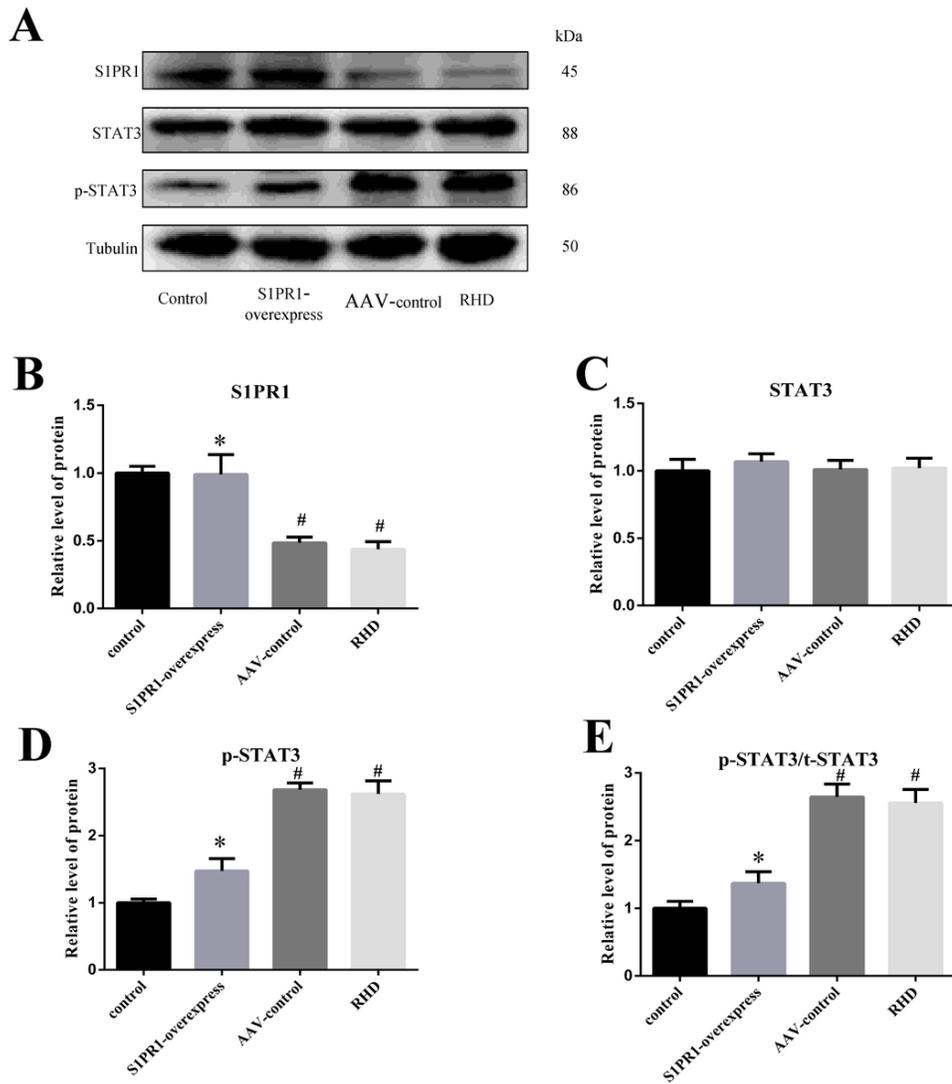
Figure 2

WB analysis. (A) Blot images of S1PR1, STAT3 and p-STAT3. (B) Relative levels of S1PR1 protein expression. (C) Relative levels of STAT3 protein expression. (D) Relative levels of p-STAT3 protein expression. (E) Fold change of the ratio of p-STAT3/t-STAT3 among 4 groups. This figure showed that the overexpression treatment of S1PR1 improved the protein expression of S1PR1, and the the protein expression of p-STAT3 in S1PR1-overexpress group was lower than that in RHD group. Data was presented as the mean ± SD; #P<0.05 vs. the control group. *P<0.05 vs. the RHD group.
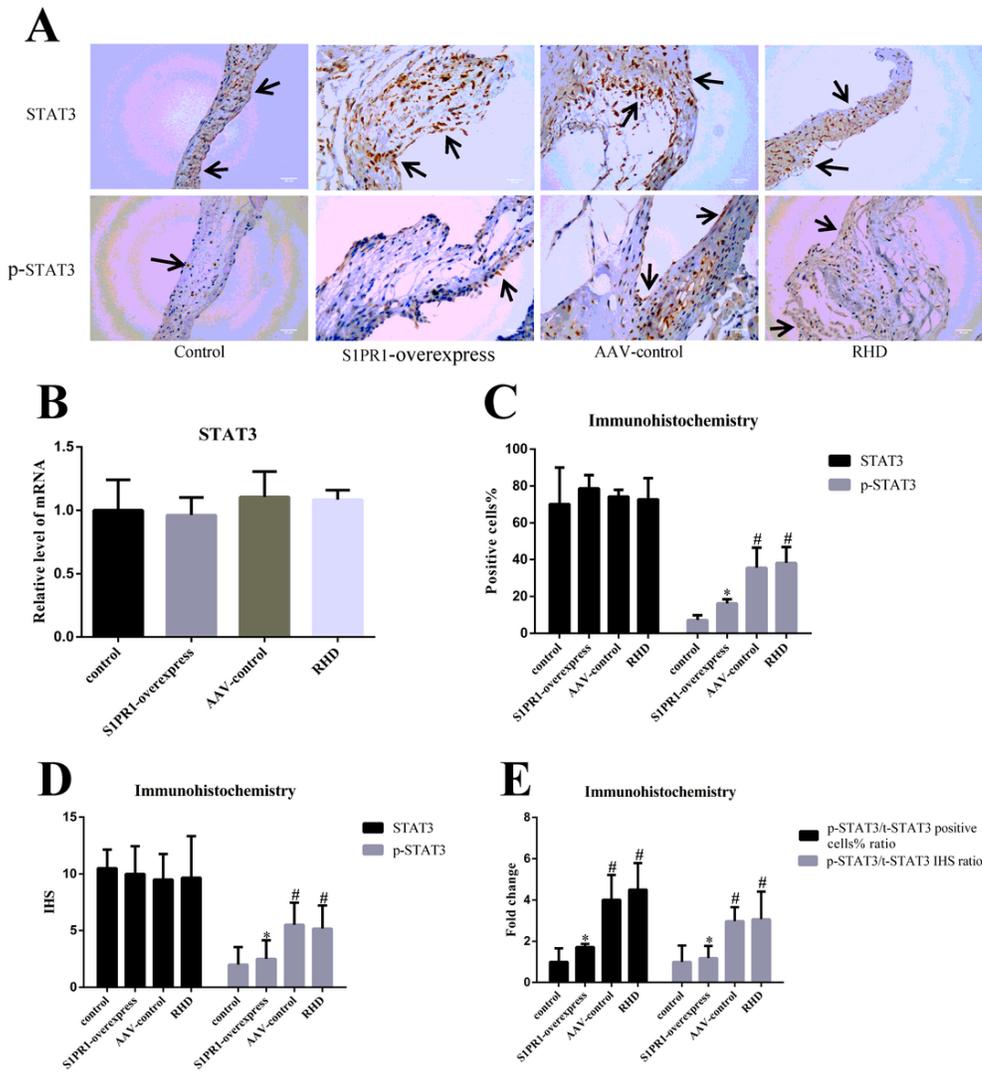
Figure 3

RT-qPCR analysis and immunohistochemistry analysis for STAT3 and p-STAT3. (A) Relative levels of STAT3 mRNA expression. (B) Immunohistochemical images of STAT3 and p-STAT3; magnification, x400; scale bar: 50 μm; positive cells were represented by brownish yellow staining pointed by arrows. (C) Proportion of positive cells of STAT3 and p-STAT3. (D) The IHS of STAT3 and p-STAT3 in four groups. (E) The ratio of p-STAT3/t-STAT3 in positive cells and IHS respectively. This figure showed that there was no significant difference in the expression of STAT3 in the 4 groups, and the the expression of p-STAT3 in S1PR1-overexpress group was lower than that in RHD group. Data was presented as the mean ± SD; #P<0.05 vs. the control group. *P<0.05 vs. the RHD group.
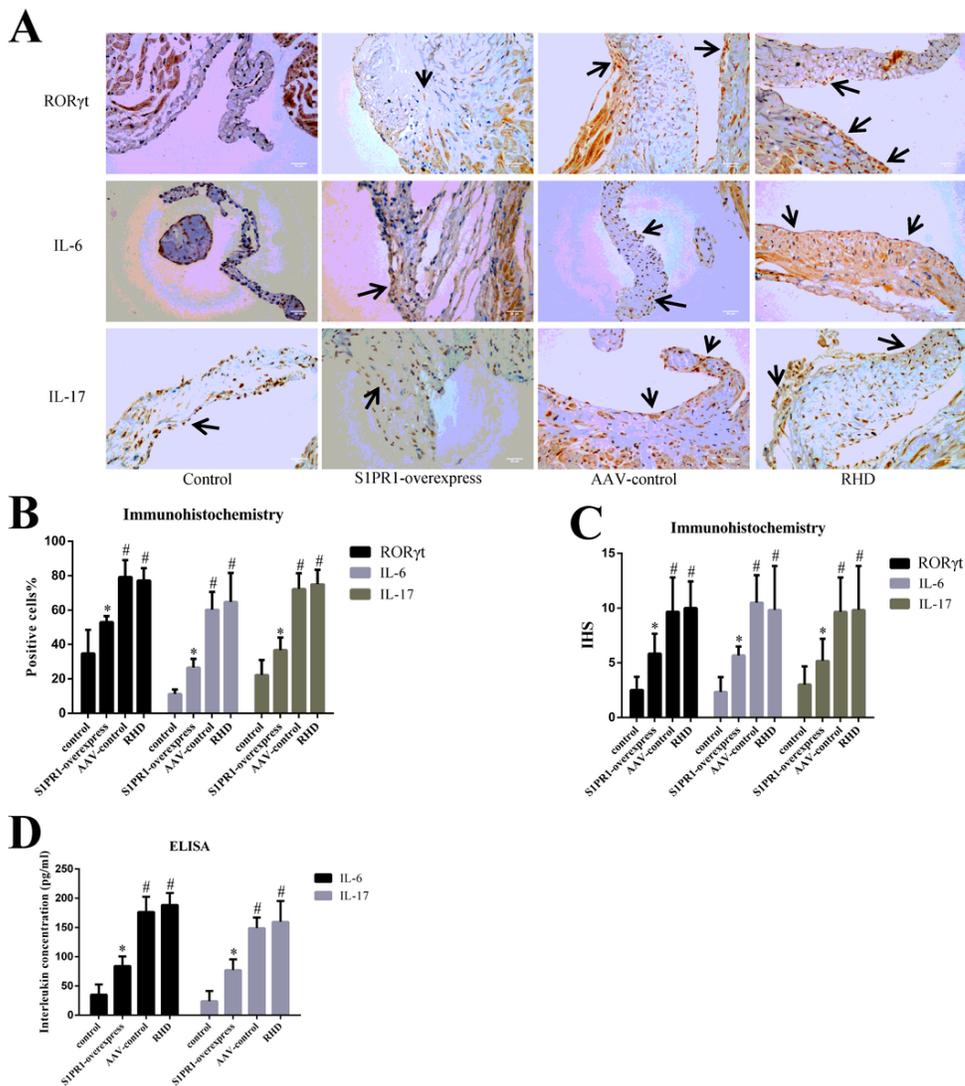
Figure 4

Immunohistochemistry and ELISA test results for Th17 cell-related factors. (A) Immunohistochemical images of RORγt, IL-6 and IL-17; magnification, x400; scale bar: 50 μm; the brownish yellow staining shown by the arrow indicates positive cells. (B) Proportion of positive cells. (C) The IHS of every group. (D) Levels of IL-6 and IL-17 in serum detected by ELISA. This figure showed that the expression of Th17 cell-related factors (RORγt, IL-6 and IL-17) was reduced in S1PR1-overexpress group than in RHD group. Data was presented as the mean ± SD; #P<0.05 vs. the control group. *P<0.05 vs. the RHD group.
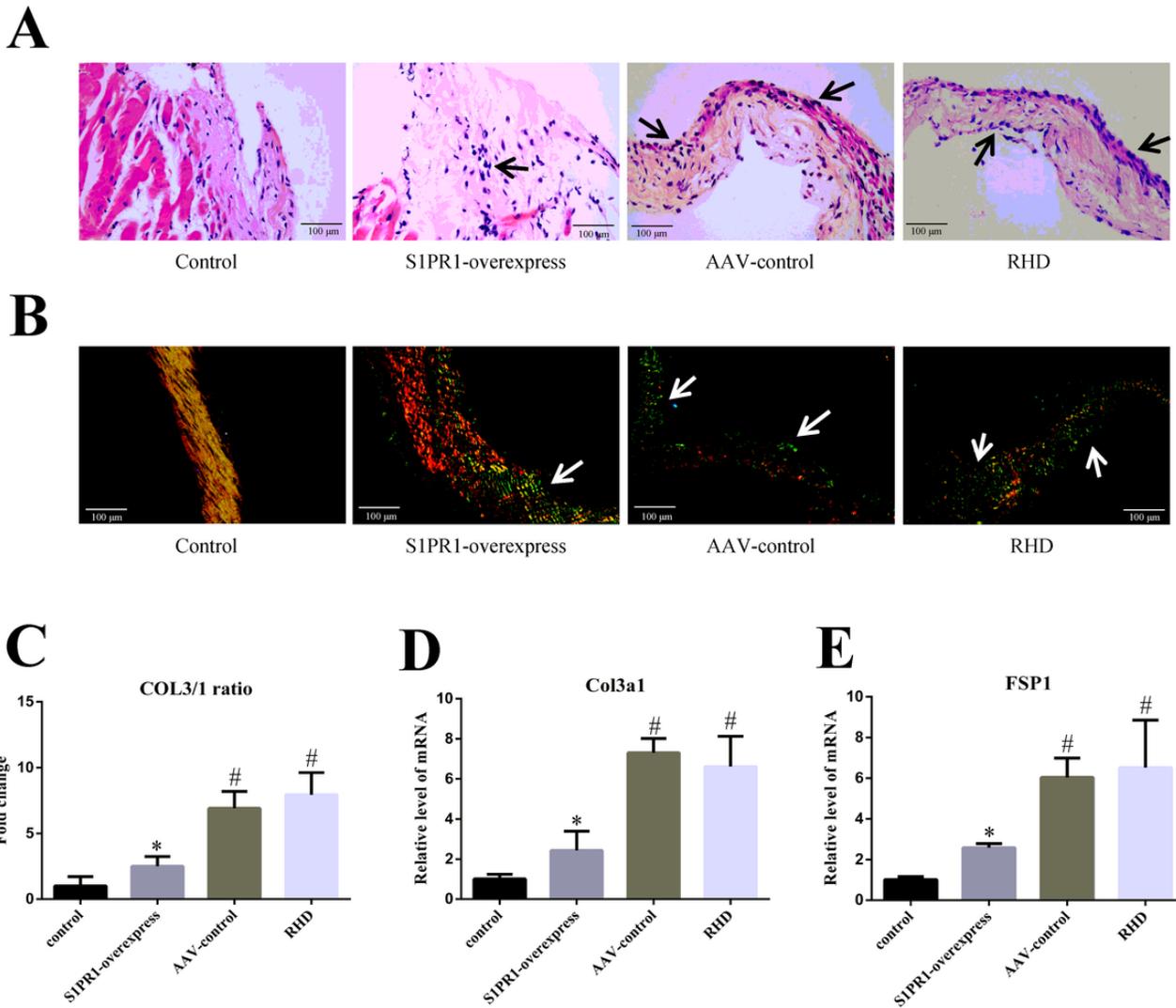
Figure 5

Valve staining results and PCR analysis of fibrosis factors. (A) H&E staining images of valves; it can be found that there was an inflammatory reaction on the valves of AAV-control group and RHD group. In S1PR1-overexpress group, the inflammatory response was significantly reduced compared with RHD group; magnification, x400; scale bar: 100 μm; inflammatory response was pointed by arrows. (B) Sirius red staining images of valves; it can be found that the valves in AAV-control group and RHD group were markedly fibrotic. In S1PR1-overexpress group, the degree of fibrosis was significantly reduced compared with RHD group; magnification, x400; scale bar: 100 μm; the loosely arranged green staining shown by the arrow indicates COL3. (C) It can be seen that COL3/1 ratio was significantly higher in the RHD group and the AAV-control group than in the control group, while the COL3/1 ratio in the S1PR1-overexpress group was significantly lower than the RHD group. (D) & (E) mRNA levels of Col3a1 and FSP1; It can be seen that the mRNA levels of Col3a1 and FSP1 were significantly higher in the RHD group and the AAV-control group than the control group, while they were significantly lower in the S1PR1-overexpress group than in the RHD group. Data was presented as the mean ± SD; #P<0.05 vs. the control group. *P<0.05 vs. the RHD group.
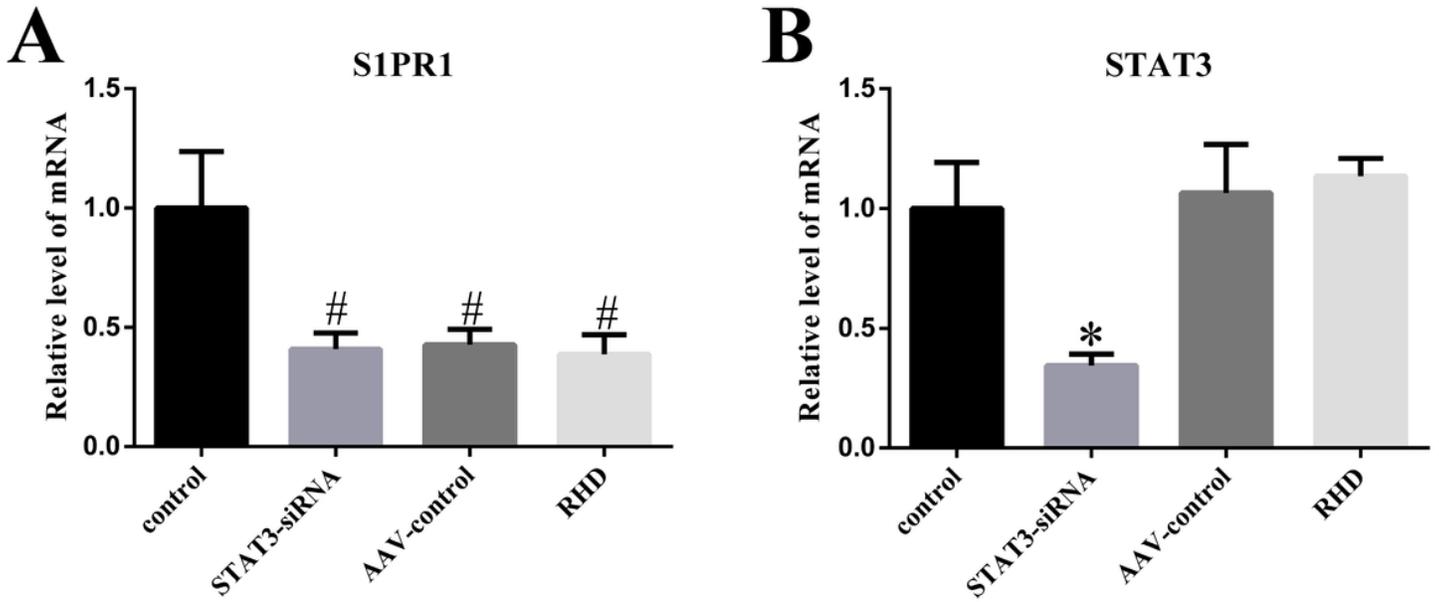
Figure 6

Reverse transcription-quantitative PCR. (A) Fold change of S1PR1 mRNA expression among 4 groups. (B) Fold change of STAT3 mRNA expression among 4 groups. This figure showed that the mRNA expression of S1PR1 in control group was higher that other three groups, and the mRNA expression of STAT3 in STAT3-siRNA group was lower than that in RHD group. Data was shown as the mean ± standard deviation; #P<0.05 compared to the control group. *P<0.05 compared to the RHD group.
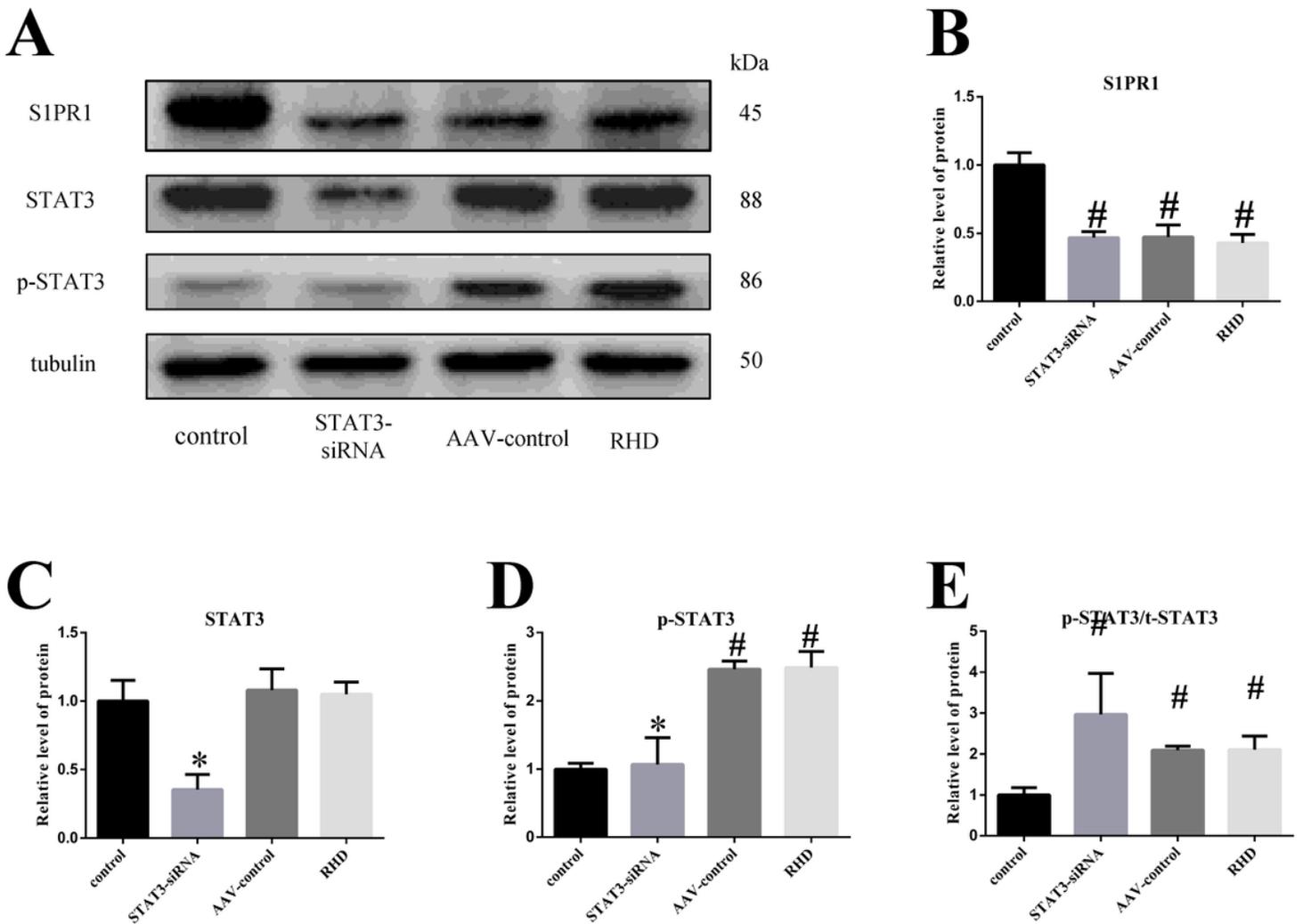
Figure 7

Western blot analysis. (A) The protein bands of S1PR1, STAT3 and p-STAT3 in the four groups. (B) Fold change of S1PR1 protein expression among 4 groups. (C) Fold change of STAT3 protein expression among 4 groups. (D) Fold change of p-STAT3 protein expression among 4 groups. (E) The relative protein level of the ratio of p-STAT3/t-STAT3. This figure showed that the protein expression of S1PR1 in control group was higher that other three groups, and the inhibition of STAT3 by STAT3-siRNA decreased the protein expression of STAT3 and the total amount of p-STAT3 in the valve tissue. Data was shown as the mean ± standard deviation; #P<0.05 compared to the control group. *P<0.05 compared to the RHD group.
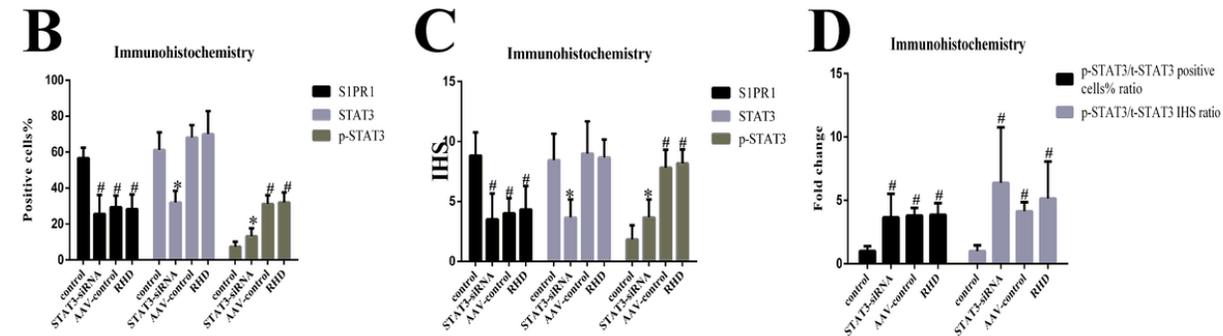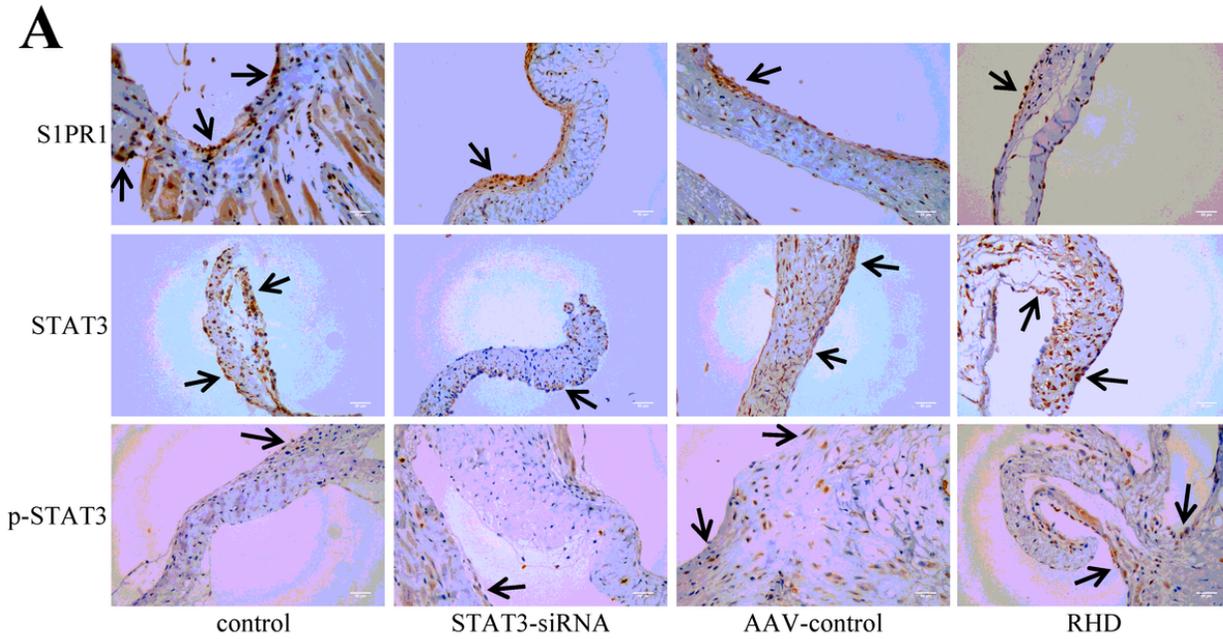

Figure 8

Immunohistochemistry analysis results of S1PR1, STAT3 and p-STAT3. (A) Immunohistochemical analysis results of S1PR1, STAT3 and p-STAT3 in valve tissues; magnification, x400; scale bar: 50 µm; the arrows marked the positive staining results. (B) The positive cells percentage. (C) The IHS. (D) The ratio of p-STAT3/t-STAT3. This figure showed that the protein expression of S1PR1 in control group was higher that other three groups, and the inhibition of STAT3 by STAT3-siRNA decreased the protein expression of STAT3 and the total amount of p-STAT3 in the valve tissue. Data was shown as the mean ± standard deviation; #P<0.05 compared to the control group. *P<0.05 compared to the RHD group.
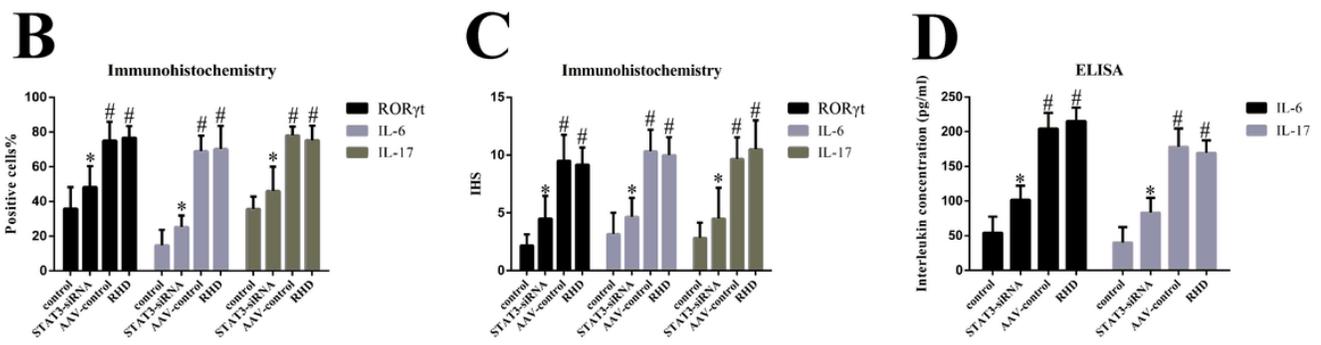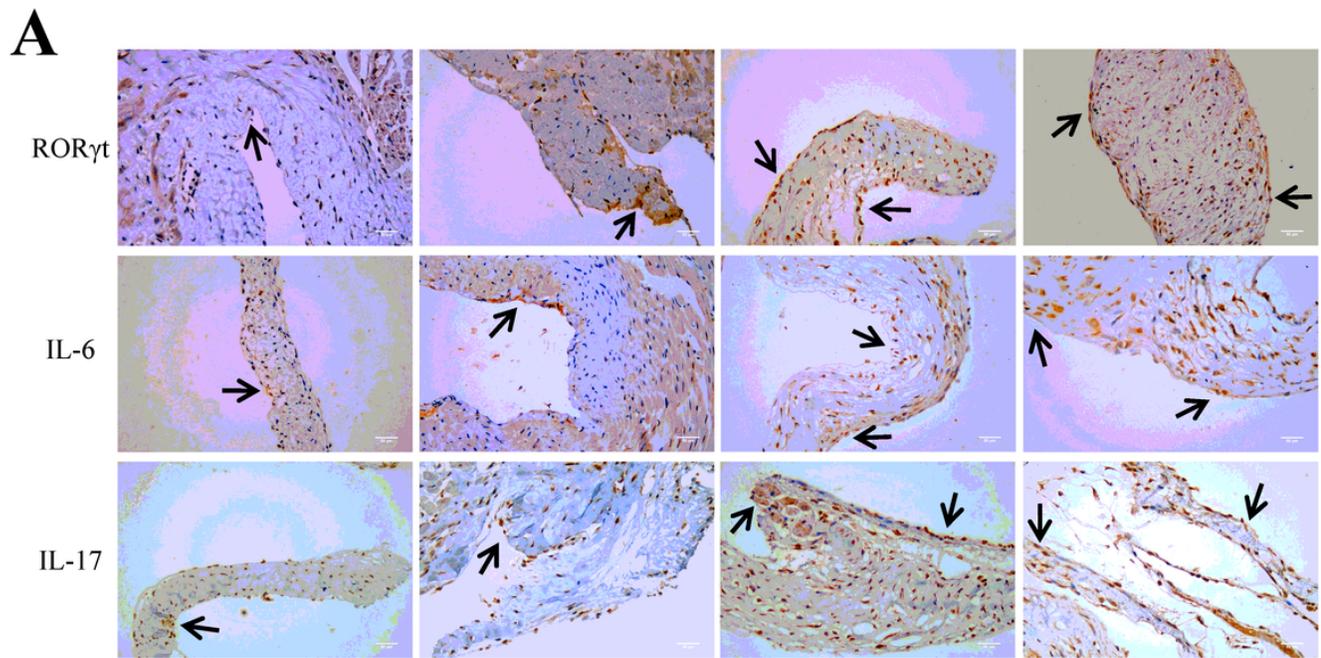
Figure 9

Th17 cell-related transcription factor and cytokines. (A) Immunohistochemical analysis results of RORγt, IL-6 and IL-17 in valve tissues; magnification, x400; scale bar: 50 μm; the arrows marked the positive staining results. (B) The positive cells percentage. (C) The IHS. (D) ELISA analysis for IL-6 and IL-17 in serum. This figure showed that the expression of RORγt, IL-6 and IL-17 was attenuated in STAT3-siRNA group. Data was shown as the mean ± standard deviation; #P<0.05 compared to the control group. *P<0.05 compared to the RHD group.
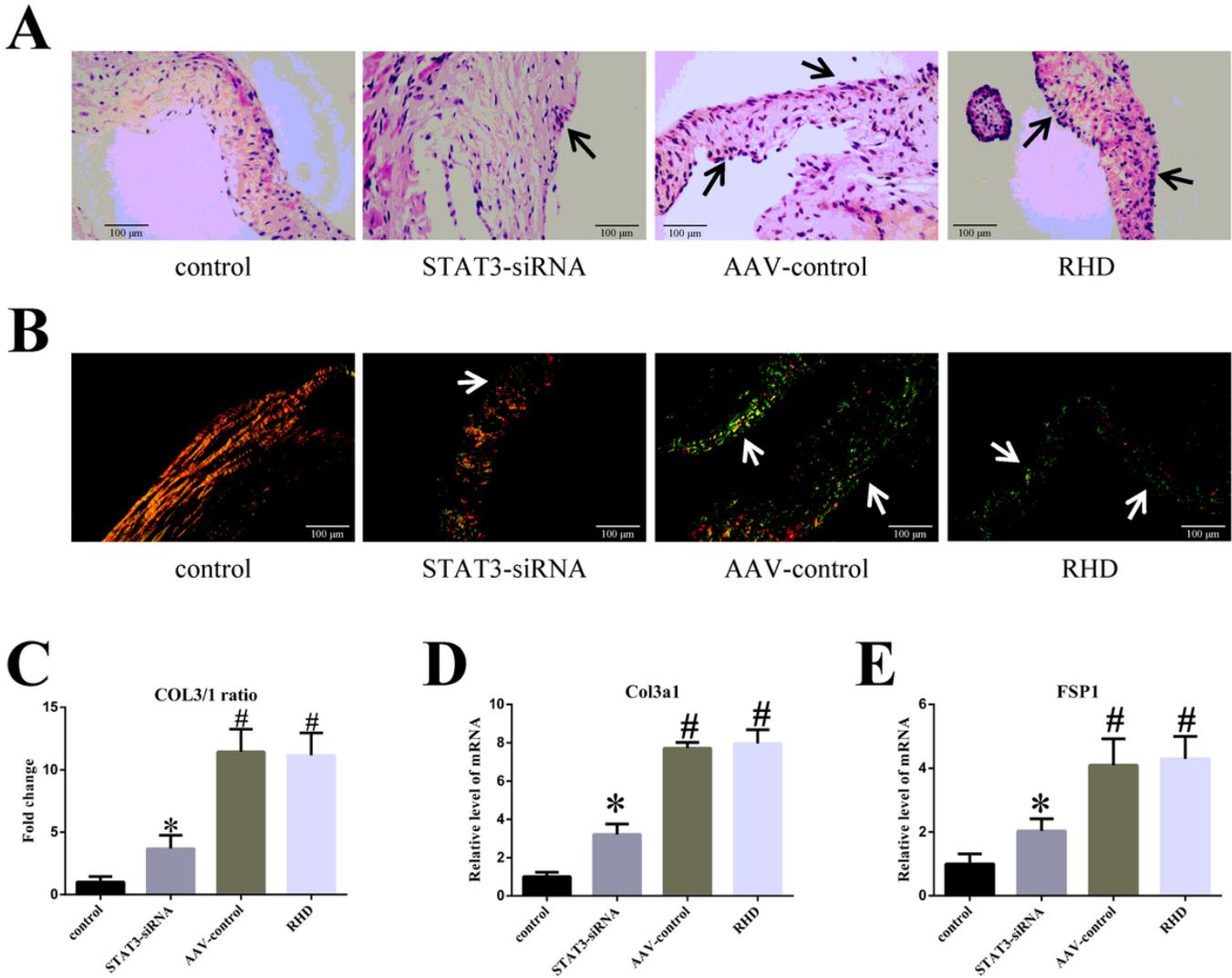
Figure 10

H&E, Sirius red staining of valve tissues and RT-qPCR analysis of fibrosis-related factors. (A) H&E staining; there was an inflammatory response in the heart valves of the rats in the AAV-control and RHD groups. In the STAT3-siRNA group, inhibition of STAT3 by STAT3-siRNA reduced inflammatory response; magnification, x400; scale bar: 100 μm; the arrows marked inflammatory response. (B) Sirius red staining, fibrosis was observed in AAV-control and RHD groups. Inhibition of STAT3 by STAT3-siRNA reduced the degree of fibrosis; magnification, x400; scale bar: 100 μm; the arrows marked COL3. (C) COL3/1 ratio was significantly higher in RHD group than in control group, inhibition of STAT3 by STAT3-siRNA reduced the COL3/1 ratio. (D) & (E) RT-qPCR analysis of Col3a1 and FSP1; both Col3a1 mRNA expression and FSP1 mRNA expression were significantly higher in RHD group than in control group, and inhibition of STAT3 by STAT3-siRNA could reduce all of them. Data was shown as the mean ± standard deviation; #P<0.05 compared to the control group. *P<0.05 compared to the RHD group.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- ARRIVEchecklist.pdf
- Originalgels.pdf
- ARRIVEchecklist.docx