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Effect of Inhibition of the JAK2/STAT3 Signaling Pathway on the Th17/IL-17 Axis in Acute Cellular Rejection After Heart Transplantation in Mice

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Abstract: Acute immune rejection is one of the most serious complications of heart transplantation, and its mechanism has always been a hot spot. Th17 cells and cytokine interleukin-17 (IL-17) have been proved to be involved in acute immune rejection, and the signaling pathway mechanism has attracted our interest. It has been confirmed that the Janus kinase 2-signal transducer and activator of transcription 3 (JAK2/STAT3) signaling pathway is involved in the differentiation of $CD4^+$ T cells, so we focus on whether the JAK2/STAT3 signaling pathway is involved in the occurrence of acute immune rejection by regulating the Th17/IL-17 axis. In this study, we used Bagg's Albino c mice and C57BL/6 mice to construct heterotopic heart transplantation models, which were divided into the acute rejection group and AG490-treated group (n = 5), and donor tissue and serum were collected in 3 experimental days from the recipient mice for H&E staining analysis of paraffin sections and ELISA, Western blot, flow cytometry, and real time-polymerase chain reaction. The results showed that the acute rejection rating of the heart decreased, and the expression of related factors decreased significantly after using the inhibitor AG490, suggesting that the JAK2/STAT3 signaling pathway regulates expression of the Th17/IL-17 axis in cardiac allograft rejection.

Key Words: cardiac transplantation, allograft rejection, JAK2/STAT3, AG490, Th17/IL-17 axis

(*J Cardiovasc Pharmacol*™ 2021;77:614–620)

INTRODUCTION

Heart transplantation has been in use for more than 50 years and is still the standard method for treatment of end-stage heart failure. Acute cellular rejection is one of the main causes of death after transplantation.[1] It is mediated mainly by T cells and accompanied by inflammatory cell infiltration and myocardial injury. The mechanisms underlying immune rejection have long been of great interest. Among the mechanisms, $CD4^+$ T cells are important effector cells in acute cardiac allograft rejection; even without $CD8^+$ T cells and B cells, $CD4^+$ T cells are sufficient to mediate rejection.[2]

Early studies have shown that Th1 and Th2 cells differentiated by $CD4^+$ T cells mainly mediate the rejection process. In recent years, studies have found that naive $CD4^+$ T cells can also differentiate into Th17 cells in the presence of interleukin-6 (IL-6) and growth transforming factor-β (TGF-β),[3] and Th17 cells are involved in the process of inflammation in the early stage of acute cellular rejection of human allogeneic heart transplantation.[4]

Interleukin-17 (IL-17) is the main inflammation-related factor secreted by the Th17 subset of $CD4^+$ T cells. IL-17 participates in the development of autoimmune diseases such as lupus nephritis and psoriasis by inducing the expression of proinflammatory factors, chemokines, and other inflammation-related factors.[5,6] In studies of transplant immune rejection, endocardial biopsies of transplanted hearts have revealed that IL-17 mRNA expression is increased and that blocking endogenous IL-17 activity reduces the Th1 immune response.[7]

The Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling pathway has been a research hotspot since its discovery in the 1990s.[8] Ligands such as interleukins bind to specific transmembrane receptors to activate JAK, which then activate downstream effectors by their corresponding STAT transcription factors.[9] The differentiation of $CD4^+$ T cells is regulated by a variety of signal pathways, among which JAK2/STAT3 signal pathway is the key signal pathway for differentiation into Th17 cells.[10]

Activated $CD4^+$ T cells activate the JAK2/STAT3 pathway through IL-6,[10] then STAT3 induces the expression of lineage-specific master regulator RORγt to participate in the differentiation of Th17 cells.[11]

AG490 is a specific blocker of the JAK2/STAT3 signaling pathway. Early studies showed that AG490 inhibits the growth and metastasis of tumor cells by inhibiting the increase in the level of STAT3 in gastric cancer cells and reducing the increase in downstream REGIα protein expression.[12] Recent studies have shown that AG490 blocks the JAK2/STAT3 signaling pathway to reduce the secretion of proinflammatory factors in autoimmune arthritis and inflammatory bowel disease and inhibit the inflammatory process.[13,14] However, whether this signaling pathway is

Received for publication October 11, 2020; accepted February 11, 2021.
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Supported by Natural Science Foundation of Hubei Province (2016CFB633).
The authors report no conflicts of interest.
Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's Web site (www.jcvp.org).
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involved in regulating the expression of related factors in acute cellular rejection of transplanted hearts has not been studied. In this article, a mouse model of abdominal heart transplantation was used to observe the regulatory effect of the JAK2/STAT3 signaling pathway in acute rejection.

MATERIALS AND METHODS

Animals and Drug Preparation

SPF male Bagg's Albino c mice and C57BL/6 mice, 8 weeks old, weighing about 20 g, were purchased from the Animal Experimental Center of Wuhan University. The JAK2 inhibitor AG490 was purchased from Abcam, Cambridge, United Kingdom. All animal experiments were approved by the Ethics Committee of the Animal Experiment Center of Wuhan University.

Construction of the Mice Abdominal Cavity Heart Transplantation Model

The heterotopic abdominal heart transplantation method of Wang et al[15] was used. Mice were anesthetized with i.p. 1% pentobarbital sodium at 50 mg/kg. Thirty Bagg's Albino c mice were used as donors, and 30 C57BL/6 mice were used as recipients. One donor mouse and one recipient mouse were selected randomly for surgery. The recipients were randomly divided into 2 groups of 15, an untreated acute rejection group and an AG490-treated group. After referring to the use of AG490 in other studies,[16,17] we decided to administer the drug in the following way: beginning on the day of transplantation and once daily for 5 days, mice were injected s.c. with 0.2 mL saline, whereas mice in the AG490-treated group were injected s.c. with 1 mg AG490 suspension in 5% dimethyl sulfoxide with saline (prepared just before use). On the first, third, and fifth day after transplantation, 5 mice from each group were anaesthetized (1% pentobarbital sodium at 50 mg/kg) and sacrificed by decapitation(n = 5). Blood samples were collected from the inferior vena cava of the recipient mice under anesthetic, and donor hearts were taken for pathological and molecular biological studies.

Recipient Mouse Observations

Daily, the recipient mice's heartbeat was palpated to detect whether the mice were alive, and the abdomens of the recipient mice were palpated to determine whether the donor heart is working normally. When the mouse was found dead or the donor heart had no heartbeat during the examination, no follow-up experiments would be performed. At the same time, the mice's limb activities were routinely checked to observe their survival status.

Donor Hearts Histology

Myocardial tissue of the donor heart in each group was fixed in 4% formaldehyde solution for 24 hours to prepare paraffin sections with a thickness of 4 μm, which were stained with hematoxylin and eosin (H&E) and observed under an optical microscope. Because there is no biopsy standard for acute cellular rejection of mice myocardial tissue, according to related research,[18] the ratings of this study were based on the heart transplant rejection standard revised by ISHT in 2004[19] (Table 1).

RNA Preparation and Real-Time PCR

Myocardial tissue was taken from the donor heart, ground in prechilled Trizol, and centrifuged at 10000 g to extract RNA. Next, according to the manufacturer's instructions, this was reversed transcribed into cDNA using the PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa, Kyoto, Japan). Then we took the transcript for polymerase chain reaction (PCR) amplification and used the SYBR Premix Ex Taq kit (TaKaRa) to perform amplification reaction on the StepOne Real-Time PCR machine. The primers were synthesized by Wuhan Tianyi-Huayu Gene Sci-Tech. The primer sequences are described in detail in the supplementary material (see **Table 2**, **Supplemental Digital Content 1**, http://links.lww.com/JCVP/A624). The reaction conditions were as follows: predenaturation at 95°C for 1 minute, denaturation 95°C for 15 seconds, annealing 58°C for 20 seconds, and elongation 72°C for 45 seconds for 40 cycles. The relative expression of mRNA was calculated using the $2-\Delta\Delta Ct$ method.

Enzyme Linked Immunosorbent Assay

Blood was collected from the inferior vena cava of the recipient under anesthetic and centrifuged to extract serum and use pure serum as samples. Then IL-17A ELISA Kit (MultiSciences, Hangzhou, China) was used according to the instructions. After adding standard and sample to the well, detection antibody was added; then the samples were incubated at 37°C for 1.5 hours, washed 6 times, 100 μL of enzyme-labeled reagent was added to each well, then 100 μL of color reagent was added to each well. After mixing, the samples were incubated in the dark at 37°C for about 15 minutes, then 100 μL of stop solution was added to each well to stop the reaction. The optical density of each well was measured at a wavelength of 450 nm after zeroing. After plotting a standard curve, the concentration of IL-17 was determined from the standard curve using the optical density values for each sample; the units are pg/mL.

Western Blot

For each group, the donor heart muscle tissue was lysed using RIPA lysate (ASPEN, Durban, South Africa), and the protein concentration of the resulting supernatant was measured with a bicinchoninic acid protein assay kit (ASPEN).

TABLE 1. Heart Biopsy Rating Standard

Grade	Standard
Grade 0 R*	No rejection
Grade 1 R, mild	Interstitial and/or perivascular infiltrate with up to 1 focus of myocyte damage
Grade 2 R, moderate	Two or more foci of infiltrate with associated myocyte damage
Grade 3 R, severe	Diffuse infiltrate with multifocal myocyte damage ± edema, ± hemorrhage, and ±vasculitis

*Revised grade to avoid confusion with 1990 scheme.

Five × loading buffer was added into protein sample and heat in a dry bath at 100°C for 5 minutes. Proteins (50 μg/well) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a poly vinylidene fluoride membrane (0.45 μm, Millipore, Penzberg, Germany), and blocked. Primary antibodies IL-17(1:1000, ab79056; Abcam) and GAPDH (1:10000, ab37168; Abcam) were added and incubated at 4°C overnight, and subsequently secondary antibodies(1:10000, AS1107; ASPEN). Protein bands were visualized using ECL chemiluminescence reagent (ASPEN) with incubating for 30 seconds. The AlphaEaseFC software processing system (http://genetictechnologiesinc.com/alpha/alpha_ease_fc.h tm) was used to analyze the optical density values of the target bands.

Flow Cytometry to Detect Proportion of Th17 Cells

After referring to the method of digesting the heart in other study,[20] type I collagenase was added to the donor myocardial tissue, which was then shaken at 200 r/min to digest at 37°C for 20 minutes to obtain a single-cell suspension. Rat antimouse fluorescent antibodies CD4-FITC, CD3-APC, CD8-PERCP, and IL-17-PE(all purchased from BD, NJ) were added and incubated at 4°C for 20 minutes, washed twice with phosphate buffer saline. Samples were fixed in the dark for 20 minutes, then added rat antimouse fluorescent antibody PE-IL-17, protected from light for 45 minutes at 4°C. After washing twice with phosphate buffer saline, Th17 cells were detected as $CD4^+$ $CD3^+$ CD8-IL17+ cells by flow cytometry (FACSVerse, BD).

Statistical Analysis

The results were presented as mean ± SEM. Data were analyzed by one-way analysis of variance which can analyze statistical differences between more than 2 groups by using SPSS 16.0 software; then one-way analysis of the variance test with post-hoc contrasted by the Student–Newman–Keuls test. $P < 0.05$ was considered statistically significant.

RESULTS

General Observation of Recipient Mice

The untreated acute rejection group had a strong heartbeat on the first day, with good limb movements; on the fifth day, the heartbeat pulsation was weakened. Observation of the transplanted hearts showed that the surrounding tissue was sticky, the heart was hard, and the color was dark red. In the AG490-treated group, the heartbeat was strong through the fifth day, and the limbs were very mobile. Observation of the transplanted heart showed no obvious peripheral adhesions, soft texture, and bright red color.

AG490 Reduced the Grading of Acute Cardiac Rejection in Donor Myocardial Tissue after Surgery

The mice were sacrificed on the first, third, and fifth days after transplantation, the donor heart tissue was taken out, and H&E staining was used to observe the changes after transplantation in both groups. The acute rejection group was mainly characterized by interstitial edema and a large number of monocyte infiltration. Rejection was grade 1R on the first day, grade 3R on the third day, and widespread cardiomyocyte damage and necrosis with interstitial hemorrhage on the fifth day which were grade 3R. The AG490-treated group showed mild mononuclear cell infiltration, mild interstitial edema, and rejection grade 1R on the first, third, and fifth days post-transplant (Fig. 1).

AG490 Reduced the Expression of IL-17 in Recipient Mice after Surgery

Because IL-17 is one of the main inflammatory factors involved in acute cellular rejection, we detected the expression levels of IL-17 in the donor myocardial tissue and the

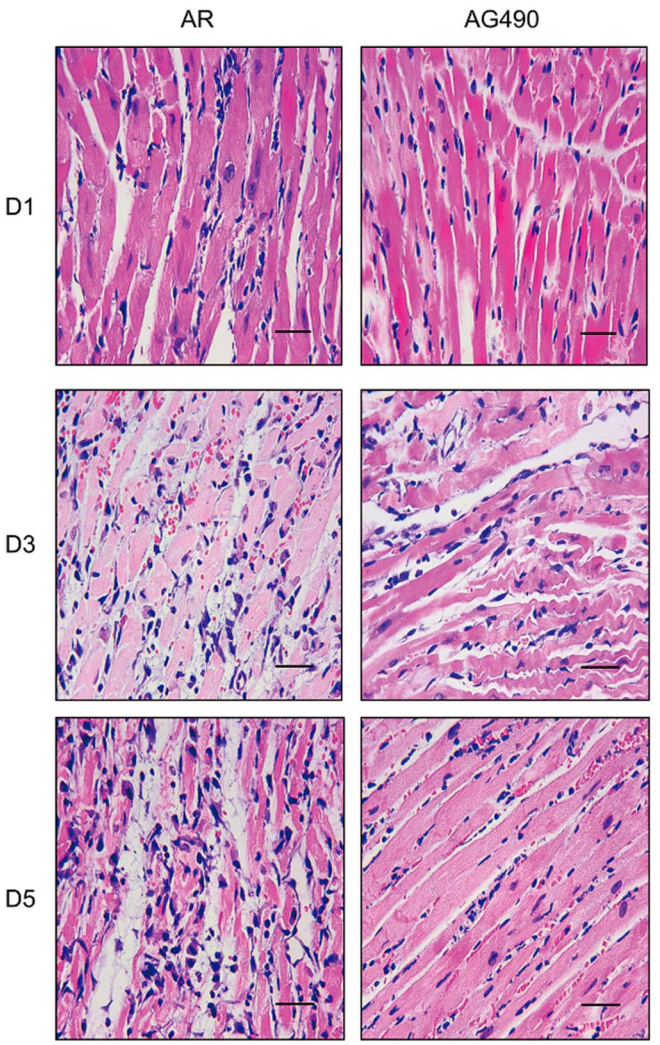


FIGURE 1. H&E staining of donor heart tissue (×400). Bar = 100 μm. D1, D3, and D5 represent the first, third, and fifth days post-transplant. AR represents the acute rejection group, and AG490 represents the AG490-treated group. Compared with the AR group, AG490-treated groups' cardiac biopsy grades are significantly reduced across 5 days.

recipient serum in the 2 groups. The experimental results suggest that the expression of IL-17 in the serum of the acute rejection group continued to increase across 5 days after the operation; comparisons between experimental days were statistically different; the value for the AG490-treated group was significantly lower than that for the acute rejection group, and the expression of IL-17 was significantly lower each day, which was statistically different between experimental days. Besides, there was a continuous downward trend in the AG490-treated group, which was statistically different from the acute rejection group (Fig. 2).

At the same time, Figure 3A shows that compared with the rejection group, the expression band of the AG490 group was significantly reduced, that is, the expression of IL-17 protein was significantly reduced. Analysis of the relative densities showed that the expression of IL-17 protein in the acute rejection group continued to increase across all 5 days after transplantation. Also comparisons between the third and first day, fifth and third day were statistically different; compared with the acute rejection group, the IL-17 protein expression in the AG490-treated group was significantly reduced, which was statistically different between experimental days and showing a continuous downward trend (Fig. 3).

AG490 Reduced Th17 Cell Infiltration in the Myocardial Tissue of Mice after Transplantation

Th17 is the main component of acute cellular rejection caused by $CD4^+$ T cells; detecting the proportion of IL-17 positive cells is helpful to detect the influence of AG490 on acute rejection. We use $CD3^+$ $CD4^+$ $CD8^-$ IL17+ to define Th17 cells. The proportion of infiltrating Th17 cells in the acute rejection group continued to increase across 5 days post-transplant; the percentages of Th17 cells were 2.37 ± 0.042%, 2.60 ± 0.056%, and 8.32 ± 0.24%; compared with the acute rejection group, the proportion of infiltrating Th17 cells in the AG490-treated group was significantly reduced, and the values showed a continuous downward trend (the percentages were 2.05 ± 0.029, 1.65 ± 0.035, and 0.81 ± 0.0097), with statistically significant differences. At the same time, for both experimental groups, comparisons between experimental days were statistically different (Fig. 4).

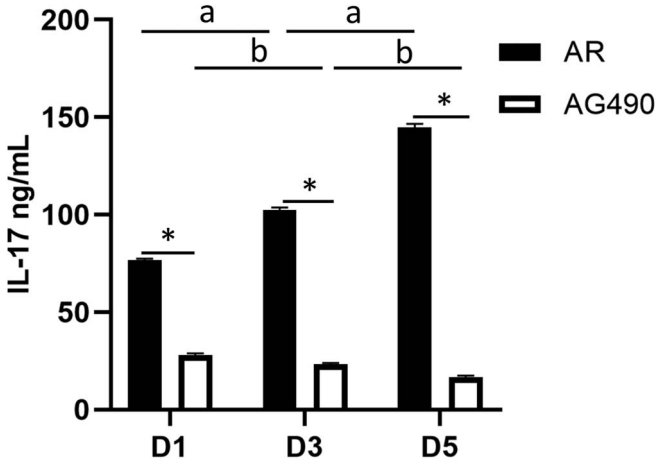
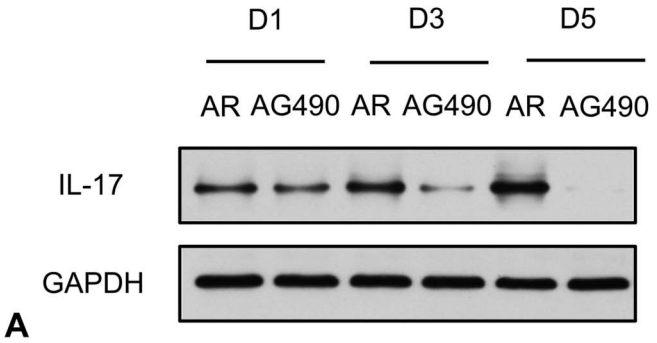


FIGURE 2. Expression levels of IL-17 in serum detected by ELISA on the first, third, and fifth days post-transplant. Compared with the AR group, the expression of IL-17 protein in serum of the AG 490-treated group was significantly reduced. Values are the mean ± SEM (n = 5). $*P < 0.05$. AR group: compared with D3, $^aP < 0.05$. AG490-treated group: compared with D3, $^bP < 0.05$.

AG490 Reduced IL-17 Expression by Reducing the Production of Factors Related to the JAK2/STAT3 Signaling Pathway

After studying the effect of AG490 on the expression of the inflammatory factor IL-17, we mainly focused on whether AG490 regulates the JAK2/STAT3 signaling pathway to reduce the infiltration of inflammatory factors in immune rejection, so we detected the mRNA levels of related factors



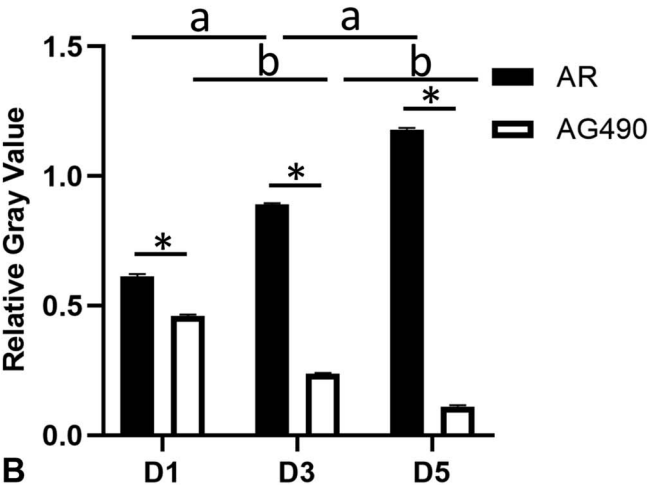


FIGURE 3. Expression of protein IL-17 in myocardial tissue in mice. A, Representative WB showing the protein detection of IL-17. AR = acute rejection group; AG490 = AG490-treated group. B, Relative intensity values from band densitometry of IL-17 in myocardial tissue. Compared with the AR group, the expression of IL-17 protein in the AG 490-treated group was significantly reduced across 5 days. Values are the mean ± SEM (n = 5). $*P < 0.05$. AR group: compared with D3, $^aP < 0.05$. AG490-treated group: compared with D3, $^bP < 0.05$.

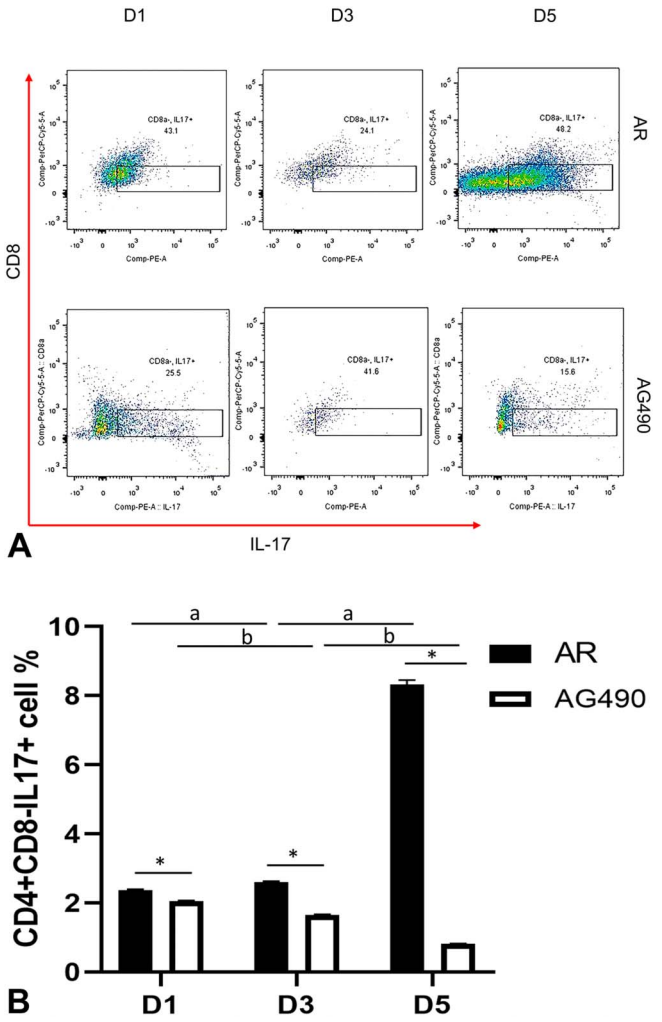


FIGURE 4. Effect of AG490 on the proportion of Th17 cells in myocardial tissue in mice. A, Flow cytometry of IL-17 cells. B, The proportion of Th17 cells. Compared with the AR group, the proportion of Th17 cells infiltrated in the donor heart tissue of the AG490-treated group was significantly reduced. Values are mean ± SEM (n = 5). $*P < 0.05$. AR group: compared with D3, $^{a}P < 0.05$. AG490-treated group: compared with D3, $^{b}P < 0.05$.

in this signaling pathway. As a result, the levels of JAK2, p-STAT3, IL-17, IL-6, TNF-β, and RORγt in myocardial tissues in the acute rejection group were continued to increase for first, third, and fifth days; The mRNA levels of JAK2, p-STAT3, IL-17, IL-6, TNF-β, and RORγt in AG490-treated group were significantly reduced compared with the acute rejection group, and continued to decline for the first, third, and fifth days post-transplant. There are statistical differences between the 2 groups, also between experimental days for the experimental groups (Fig. 5).

DISCUSSION

Acute cellular rejection is a serious complication and a significant barrier to patient and organ longevity and survival. $CD4^+$ T cells recruits a series of effector cells to mediate rejection. The Th1/Th2 pattern generated by $CD4^+$ T differentiation is generally considered to mainly mediate inflammation. However, recent studies have revealed the emerging role of other $CD4^+$ T-cell subgroups (Th17, Th22, etc.) in rejection.[21] IL-17, IL-21, and IL-22 produced by human Th17 cells have been proven to be effective inducers of tissue inflammation.[22] Experiments have shown that IL-17 participates in the acute rejection process after heart transplantation,[23] and the use of IL-17 antagonists can significantly prolong the survival time of allografts.[24]

Naive $CD4^+$ T cells are activated by the JAK2/STAT3 signaling pathway through the costimulation of TGF-β and IL-6 or TGF-β and IL-21 to differentiate into Th17 cells and then generate IL-17.[25] Inhibiting the STAT3 signaling pathway inhibits the Th17/IL-17 axis, which can reduce the proinflammatory effects of abnormally elevated IL-17. JAK2 inhibitor AG490 has been proven to regulate tumor Th17 cell/regulatory T cell (Treg) balance.[13] However, the role of the JAK2/STAT3 pathway in heart transplantation has not been studied, so the current study focused on the role of this pathway in heart transplant rejection.

In our study, a large number of inflammatory cell infiltration and myocardial cell necrosis appeared in the donor heart tissue in the acute rejection group, suggested that the acute cellular rejection model of heart transplantation was successfully constructed. At the same time, the proportion of Th17 cells and the expression of IL-17 protein in the AG490 treatment group were significantly lower than those in the acute rejection group, which further verified that the Th17/IL17 axis is involved in acute cellular rejection, and AG490 can reduce immune rejection that the Th17/IL17 axis involves.

It is worth mentioning that the expression of STAT3 and IL-17 can stimulate the production of IL-6 itself, which is the main regulator of Th17 cell differentiation and function. At the same time, RORγt is a specific transcriptional activator of the Th17 cell lineage, which can produce high levels of IL-17,[26] so it can be inferred that activation of the Th17/IL-17 axis through the JAK2/STAT3 pathway is accompanied by increased expression of IL-6, TGF-β, RORγt, and other factors.[27]

Our results showed that the expression of JAK2, p-STAT3, IL-17, IL-6, TNF-β, and RORγt mRNA in the acute rejection group gradually increased across the first, third, and fifth days post-transplant. The expression of IL-17 protein in tissues and serum also gradually increased. In the presence of the JAK2-STAT pathway inhibitor, expression of JAK2, p-STAT3, IL-17, IL-6, TNF-β, and RORγt mRNA was significantly lower than in the rejection group and showed a gradual downward trend at first, third, and fifth days. All of them were statistically significant, which is suggested that after adding the signal pathway inhibitor, the JAK2/STAT3 pathway level and its downstream protein level–related factors decreased. It further illustrates that the JAK2/STAT3 signaling pathway regulates the production of factors related to acute rejection of heart transplantation, specifically the regulation of Th17/IL-17 changes.

In histopathology, compared with the acute rejection group, the AG490-treated group had significantly reduced

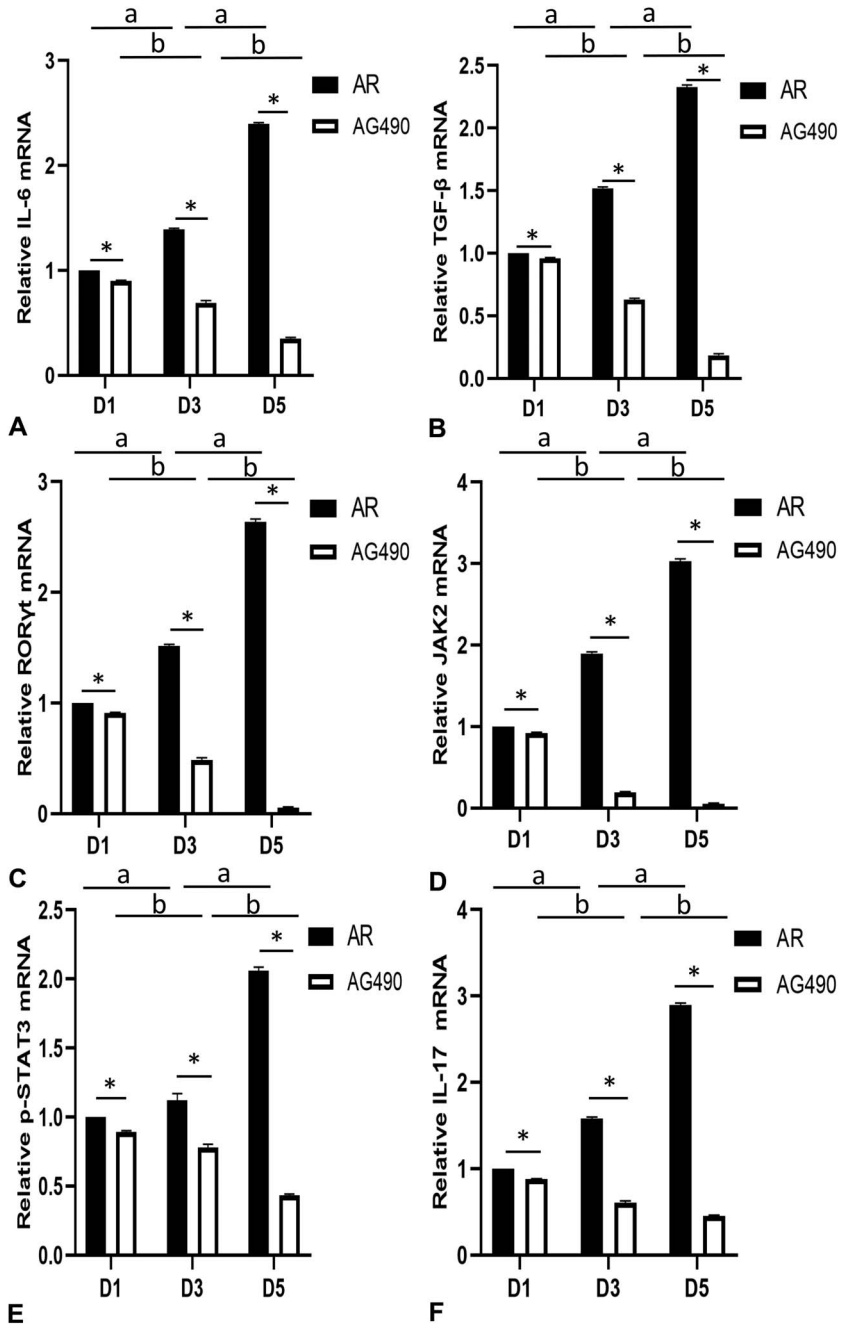


FIGURE 5. AG490 reduces mRNA expression of JAK2/STAT3 signaling pathway–related factors in donor myocardial tissue. A, IL-6 mRNA, (B) TGF-β mRNA, (C) RORγt mRNA, (D) JAK2 mRNA, (E) p-STAT3 mRNA, and (F) IL-17 mRNA. Values are mean ± SEM (n = 5). $^{*}P < 0.05$. AR group: compared with D3, $^{a}P < 0.05$. AG490-treated group: compared with D3, $^{b}P < 0.05$.

inflammatory cell infiltration and a lower grade of immune rejection of heart transplantation. It is further verified that the inhibition of signal pathways can improve acute cellular rejection.

In consideration of the molecular biological and pathological findings, we propose that the JAK2/STAT3 signaling pathway participates in the immune rejection of heart transplantation, regulates the increase of IL-17 levels, and promotes a proinflammatory response. By inhibiting the activation of the JAK2/STAT3 signaling pathway, the expression of IL-17 is reduced, thereby reducing transplantation immune rejection.

CONCLUSION

In this study, we focused on whether the JAK2/STAT3 signaling pathway is involved in acute cellular rejection of heart transplantation. To this end, we successfully constructed a mouse heterotopic heart transplantation model. According to pathological and molecular biology experiments, the signaling pathway inhibitor AG490 can reduce acute cellular rejection by reducing the expression of related factors in the JAK2/STAT3 signaling pathway. However, the specific mechanism of the JAK2/STAT3 signaling pathway involved in the regulation remains to be

studied. Whether the JAK2/STAT3 signaling pathway can be used as a new direction for regulating acute cellular rejection drugs remains to be studied in depth.

REFERENCES

1. Kim IC, Youn JC, Kobashigawa JA. The past, present and future of heart transplantation. *Korean Circ J.* 2018;48:565–590.
2. Wiseman AC, Pietra BA, Kelly BP, et al. Donor IFN-gamma receptors are critical for acute CD4(+) T cell-mediated cardiac allograft rejection. *J Immunol.* 2001;167:5457–5463.
3. Yoshimura A, Muto G. TGF-β function in immune suppression. *Curr Top Microbiol Immunol.* 2011;350:127–147.
4. van Besouw NM, Caliskan K, Peeters AM, et al. Interleukin-17-producing CD4(+) cells home to the graft early after human heart transplantation. *J Heart Lung Transpl.* 2015;34:933–940.
5. Biswas PS. IL-17 in renal immunity and autoimmunity. *J Immunol.* 2018;201:3153–3159.
6. Blauvelt A, Chiricozzi A. The immunologic role of IL-17 in psoriasis and psoriatic arthritis pathogenesis. *Clin Rev Allergy Immunol.* 2018;55:379–390.
7. Duan L, Chen J, Xia Q, et al. IL-17 promotes Type 1 T cell response through modulating dendritic cell function in acute allograft rejection. *Int Immunopharmacol*. 2014;20:290–297.
8. Sundrud MS, Trivigno C. Identity crisis of Th17 cells: many forms, many functions, many questions. *Semin Immunol.* 2013;25:263–272.
9. Bousoik E, Montazeri Aliabadi H. "Do we know jack" about JAK? A closer look at JAK/STAT signaling pathway. *Front Oncol*. 2018;8:287.
10. Zhu J, Paul WE. Peripheral CD4+ T-cell differentiation regulated by networks of cytokines and transcription factors. *Immunol Rev.* 2010;238:247–262.
11. Wu X, Shou Q, Chen C, et al. An herbal formula attenuates collagen-induced arthritis via inhibition of JAK2/STAT3 signaling and regulation of Th17 cells in mice. *Oncotarget*. 2017;8:44242–44254.
12. Sekikawa A, Fukui H, Fujii S, et al. REG Ialpha protein mediates an anti-apoptotic effect of STAT3 signaling in gastric cancer cells. *Carcinogenesis*. 2008;29:76–83.
13. Park JS, Lee J, Lim MA, et al. JAK2/STAT3 blockade by AG490 suppresses autoimmune arthritis in mice via reciprocal regulation of regulatory T Cells and Th17 cells. *J Immunol.* 2014;192:4417–4424.
14. Guo J, Wang LY, Wu J, et al. The JAK2 inhibitor AG490 regulates the Treg/Th17 balance and alleviates DSS-induced intestinal damage in IBD rats. *Clin Exp Pharmacol Physiol.* 2020;47:1374–1381.
15. Wang C, Wang Z, Allen R, et al. A modified method for heterotopic mouse heart transplantion. *J Vis Exp.* 2014;e51423.
16. Gasparitsch M, Schieber A, Schaubeck T, et al. Tyrphostin AG490 reduces inflammation and fibrosis in neonatal obstructive nephropathy. *PLoS One*. 2019;14:e0226675.
17. Mei D, Bin W, Lijian X, et al. The effects of AG490 on balance of Th17/Treg in C57BL/6 EAE mice [in Chinese]. *J Apoplexy Nervous Dis.* 2015;32:680–683.
18. Qiu S, Lv D. Triptolide inhibits CD4+ memory T cell-mediated acute rejection and prolongs cardiac allograft survival in mice. *Exp Ther Med.* 2017;14:2817–2822.
19. Billingham M, Kobashigawa JA. The revised ISHLT heart biopsy grading scale. *J Heart Lung Transpl.* 2005;24:1709.
20. Ming L, Daying W, Wenquan Z, et al. Preparation and flow cytometry of single cell suspension in adult mouse heart [in Chinese]. *Med Innovation China*. 2018;15:27–31.
21. Yamada Y, Brüstle K, Jungraithmayr W. T helper cell subsets in experimental lung allograft rejection. *J Surg Res.* 2019;233:74–81.
22. Crome SQ, Wang AY, Levings MK. Translational mini-review series on Th17 cells: function and regulation of human T helper 17 cells in health and disease. *Clin Exp Immunol.* 2010;159:109–119.
23. Yinggan D, Ming X, Shaoping Z, et al. Effect of halofuginone on cardiac allograft acute rejection in rats [in Chinese]. *Med J Wuhan Univ.* 2015;36:204–208.
24. Yuan X, Paez-Cortez J, Schmitt-Knosalla I, et al. A novel role of CD4 Th17 cells in mediating cardiac allograft rejection and vasculopathy. *J Exp Med.* 2008;205:3133–3144.
25. Ouyang W, Kolls JK, Zheng Y. The biological functions of T helper 17 cell effector cytokines in inflammation. *Immunity*. 2008;28:454–467.
26. Lin YC, Lin YC, Wu CC, et al. The immunomodulatory effects of TNF-α inhibitors on human Th17 cells via RORγt histone acetylation. *Oncotarget*. 2017;8:7559–7571.
27. Camporeale A, Poli V. IL-6, IL-17 and STAT3: a holy trinity in auto-immunity? *Front Biosci (Landmark Ed).* 2012;17:2306–2326.