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A New Concept of the Old Inhibitor NSC 74859 in Alleviating Cardiac Allograft Rejection and Extending Allograft Survival in Mice

Authors' Contribution:
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Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: STAT1/4 has been suggested to be involved in cardiac allograft rejection. However, no direct evidence regarding STAT3 has been established in cardiac allograft rejection. Here, we hypothesized that inhibition of STAT3 attenuates cardiac allograft rejection.

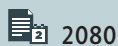
Material/Methods: To test our hypothesis, homotopic mouse heart transplantation was carried out in syngeneic C57BL/6 to C57BL/6 strain mice with or without oral gavage with NSC 74859, an inhibitor of STAT3. The immune response was investigated using real-time PCR for CD4 and CD8 surface makers of T cells and CD14 of monocytes and cytokines, including IL-2, IL-15, and IL-6 of allografts at 3, 6, and 9 days after transplantation. Prognosis was also evaluated.

Results: We found that allografts with oral gavage of NSC 74859 whose CD4, CD8 T, and CD14 monocytes were significantly lower than that of allograft without oral gavage of NSC 74859, and the same was true for the expression of IL-2, IL-15, and IL-6. Immunohistochemical analysis of grafts showed reduced infiltration of monocytes/macrophages into the graft myocardium. Survival was also markedly extended in the NSC 74859 group.

Conclusions: Inhibition of IL-6/STAT3 using NSC 74859 was shown to remarkably alleviate cardiac allograft rejection in mice, indicating that the target against IL-6/STAT3 pathway might be clinically used as an alternative therapy for cardiac allograft rejection.

MeSH Keywords: **Models, Animal • Receptors, Interleukin-6 • STAT3 Transcription Factor**

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Background

Although heart transplantation in humans has been widely used to save lives of patients with refractory end-stage heart disease or severe coronary artery disease, various scientific and clinical problems still remain [1]. Clinical problems after heart transplantation include the fact that cardiac allograft rejection leads to significant graft dysfunction, cardiac allograft vasculopathy, and eventual mortality, especially in the first year after transplantation [2]. Thus, heart allograft rejection seems to be the overarching clinical problem. Transplanted heart rejection involves interaction between the mechanisms that maintain tolerance to the graft and those that promote rejection. Although immunologic factors are important for both, the process of rejection can be very much an inflammatory one [3]. Consequently, local production of many proinflammatory cytokines, including interleukin IL-6 and IL-15, is increased in cardiac allograft rejection [4–6].

Elevated IL-6 from donor heart, kidney and liver has been reported to worsen ischemia-reperfusion injury in recipients, leading to the development of pathologic hypertrophy and fibrosis in chronic cardiac allograft rejection [7], suggesting that IL-6 is a therapeutic target to prevent chronic cardiac allograft rejection. The question of what produces high levels of IL-6 in heart allograft rejection remains largely unanswered and deserves to be investigated [8]. Nevertheless, the present study investigated how to block IL-6, a pleiotropic cytokine involved in heart disease [9], in cardiac allograft rejection. In consideration of the regulation between IL-6 and the STAT3 pathway involved in various inflammatory diseases, it stands to reason that blocking STAT3 activation would suppress production of IL-6, thereby attenuating heart allograft rejection [10,11].

On the other hand, previous sporadic studies have shown that inhibition of STAT1 [12–14] and STAT4 [15] using siRNA and knock-out technique alleviates cardiac allograft rejection in a mouse model, suggesting that STAT1/4 plays an important role in cardiac allograft rejection. Although no direct evidence has been established regarding the role of STAT3 in cardiac allograft rejection, several lines of evidence support that STAT3 is involved in cardiac allograft rejection [16,17]. Based on these previous studies, we hypothesized that inhibition of STAT3 would achieve the same effect as STAT1 and STAT4 in cardiac allograft rejection. Therefore, the aim of the present study was to investigate whether inhibition of STAT3 using NSC 74859, a specific inhibitor of IL-6/STAT3, could attenuate the response of cardiac allograft rejection in a mouse model. We found that NSC 74859 significantly attenuated the immune response of allografts in mice. We also found that it can remarkably extend allograft survival compared with controls. Our study suggests that targeting against STAT3 could be used as an alternative therapy for suppression of cardiac allograft rejection.

Material and Methods

Mice

Six-week-old C57BL/6NHsd male and female mice, weighing 18–22 grams, housed and bred under Specific Pathogen-Free (SPF) conditions, were purchased from Charles River Laboratories (Beijing, China). All experiments were carried out in strict accordance with protocols and regulations set up by the Animal Care and Well-being Committee of Xiamen University. The study was approved by the Medical Ethics Committee of Xiamen University.

Heart transplantation

Homotopic heart transplantation was performed using microsurgery on male and female C57BL/6NHsd mice, as previously described [18,19]. Cold ischemia times were controlled to be less than 25 min. Graft survival time was defined as the last day of transabdominally palpable cardiac contractions. Recipients remained untreated or were treated by oral gavage every other day for 14 days (6 treatments) with 10 mg/kg NSC 74859, based on a previous report [20]. Control group mice were subjected to heart transplantation and treated with DMSO alone. The results, expressed as mean survival time \pm standard deviation, were assessed for statistical significance by the Gehan survival test.

Real-time quantitative PCR (qPCR)

For real-time qPCR analysis, myocardium samples were snap-frozen in liquid nitrogen immediately after collection and stored at -80°C . Total RNA was extracted from frozen tissue using the RNeasy fibrotic tissue mini kit with DNase treatment (catalogue number 74704; Qiagen) and quantified by NanoDrop 2000 Microvolume Spectrophotometers. Complementary DNA was synthesized with a reverse transcriptase kit (catalogue number N8080234, Thermal, USA). Real-time qPCR was performed using standard curve method as previously described and established [21]. All the primers involved were designed and synthesized by Sangon Biotech Company (Sangon Biotech, Shanghai) and are listed in Table 1.

Immunohistochemistry

Cryostat sections were stained with polyclonal rabbit anti-murine CD14 primary antibody (Catalogue number: ab106285; working dilution at 1: 50; Abcam, Cambridge, MA, USA) for 90 min at room temperature. After washing in phosphate-buffered saline, biotinylated goat anti-rabbit secondary antibody (Catalogue number: ab7010; dilution at 1: 200, Abcam, Cambridge, MA, USA) in 2% mouse serum was added for 45 min, followed by avidin-biotin-horseradish peroxidase complex (Vectastain ABC

Table 1. All the primers involved in qRT-PCR detection of murine cytokines.

Accession num	Gene		Sequence 5'-3'	T _m (°C)	Annealing	Size(bp)
NM_008366.3	IL-2	Forward	TGGAGCAGCTGTTGATGGAC	62.41	60	120
		Reverse	TCAATTCTGTGGCCTGCTTG	62.31		
NM_031168.2	IL-6	Forward	TCCATCCAGTTGCCTTCTTG	61.18	60	136
		Reverse	AAGCCTCCGACTTGTGAAGTG	61.75		
NM_008357.2	IL-15	Forward	GCAGTCTCTCTGCAAGTCT	61.92	60	123
		Reverse	TCGTCCAACCTGCAACTGG	62.41		

β -actin primers of mouse, as the internal loading control, were readily commercial from Sangon Biotech (Sangon biotech, Shanghai, China), whose catalogue number was B661302. The detailed annealing temperature was seen on the instructions that accompanied the primers.

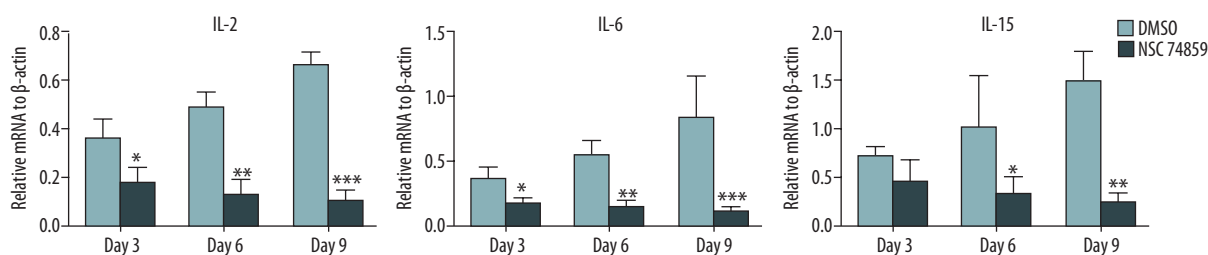


Figure 1. qRT-PCR detection of IL-2, IL-6, and IL-15 expression on mRNA level from allograft hearts of mice with or without oral gavage of NSC 74859 at 3, 6, and 9 days after transplantation. There were 2 groups – allograft hearts of murine model with DMSO as control group and with NSC 74859 as experimental group – with each group having 10 mice (n=20 mice). Three mice were euthanized at the designed time point (day 3, 6, and 9). Total RNA was extracted followed by qRT-PCR analysis using standard curve method. Relative expression of IL-2, IL-6, and IL-15 was normalized to β -actin as the internal loading control. The experiment was performed independently 3 times in triplicate samples. Two-tailed independent sample *t* test was used to analyze the differences. * Means $P < 0.05$, ** denotes $P < 0.01$, *** stands for $P < 0.001$ in comparison with its control.

kit; Vector Laboratories, Peterborough, UK). The sites of peroxidase binding were developed in phosphate-buffered saline containing diaminobenzidine tetrachloride (0.3 mg/mL) and hydrogen peroxide (0.01%) (Sigma fast; Sigma Aldrich, Poole, UK). Slides were counterstained with hematoxylin (Sigma Aldrich).

Statistical analysis

Each experiment involved was conducted at least 3 independent times with each in triplicate. All data are expressed as mean \pm standard error of the mean (SEM). All data to be analyzed were first evaluated for normal distribution using the Kolmogorov-Smirnov (KS) test. Statistical differences among groups were analyzed using one-way ANOVA or independent sample *t* test if the data were normally distributed; otherwise, we used the Mann-Whitney U test. Survival analysis was analyzed using the log-rank test with Kaplan-Meier survival curves. Significance was set at $P < 0.05$ with a two-tailed test using the SPSS17.0 software package (SPSS Inc., Chicago, IL, USA).

Results

Expression of IL-2, IL-6, and IL-15 was remarkably reduced by administration of NSC 74859

Given that IL-2, IL-6, and IL-15 are redundantly present in cardiac allograft rejection [22], we evaluated the variation of expression at the mRNA level of IL-2, IL-6, and IL-15 in grafts using real-time RT-PCR. Expression of IL-2, IL-6, and IL-15 was significantly reduced in allografts treated with NSC 74859 at 3, 6, and 9 days after transplantation in comparison with controls, compared to allografts without administration of NSC 74859 (Figure 1). Although we used only 1 method of detection, our results were entirely in agreement with a previous study by Van Hoffen et al. [23] who utilized *in situ* hybridization and immunohistochemistry to study the cytokine mRNA and protein expression inside the graft during rejection. Our finding suggests that treatment with NSC 74859 strongly reduces the production of IL-2, IL-6, and IL-15 within the graft.

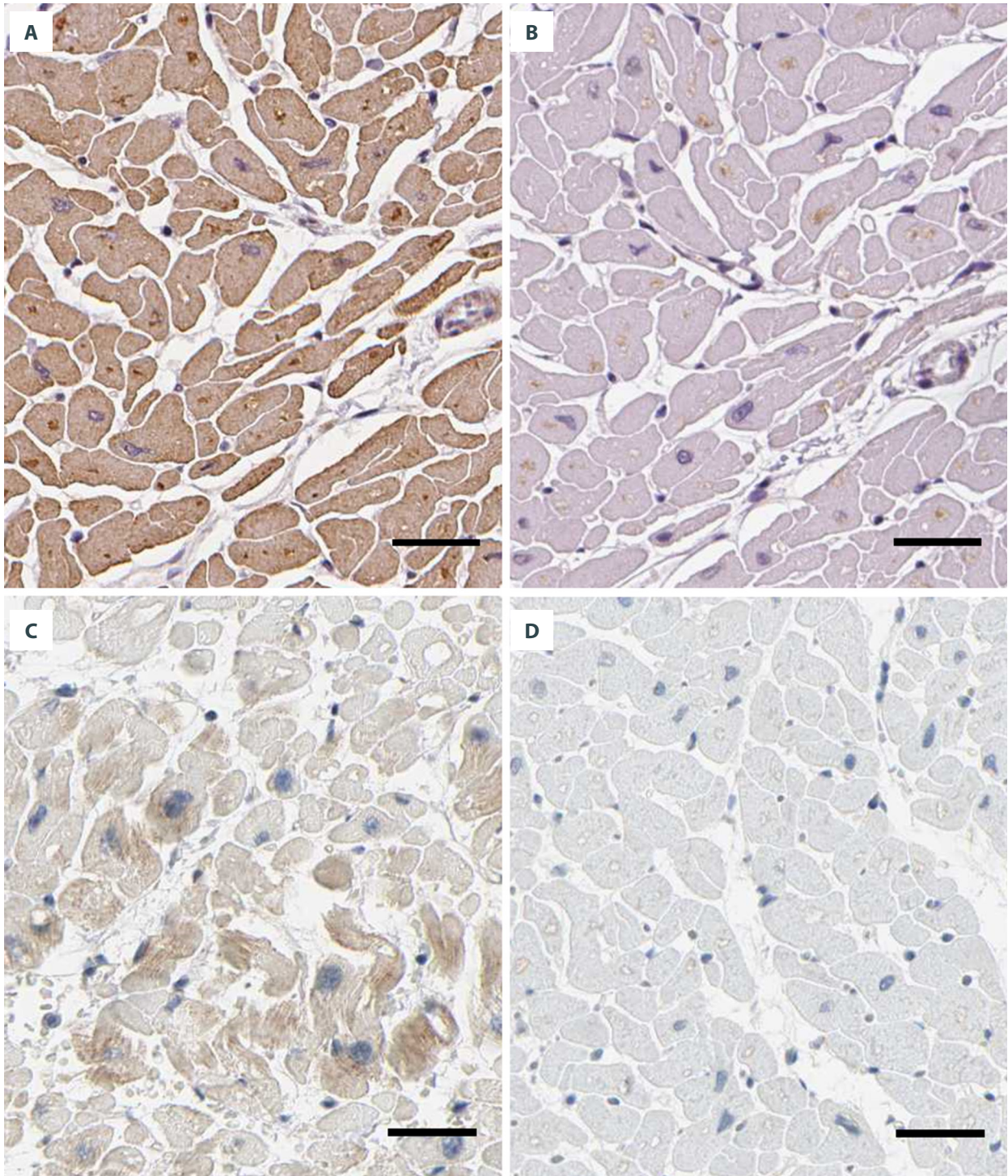


Figure 2. Immunohistochemical analysis of CD14 as well as p-STAT3 expression in allograft hearts from murine model at the ninth day after transplantation. (A) p-STAT3 expression in control group; (B) p-STAT3 expression in cardiac allograft with NSC 74859; (C) expression of CD14, a typical marker of infiltrated macrophages, in control group; (D) at 9 days after transplantation, showing CD 14 expression. Scale bar denotes 100 μ m. Magnification is 200 times. Representative figures selected from the 3 mice euthanized in each group.

The infiltrated monocytes/macrophages were markedly diminished

Because NSC 74859 is a specific inhibitor of activated or phosphorylated STAT3 and is able to prevent phosphorylation of STAT3 [24], we subsequently assessed the level of activated STAT3 and inactivated STAT3 within the grafts from the 2 groups. There was a significantly lower level of phosphorylated STAT3 in allografts treated with NSC 74859 than in the control group, but no remarkably apparent variation of the inactivated STAT3 was observed in grafts from the 2 groups (Figure 2A, 2B), indicating the effectiveness of NSC 74859 in our experiment. Therefore, we looked for infiltrated monocytes/macrophages in grafts from the 2 groups using the typical marker CD14 via immunohistochemistry. There were significantly fewer infiltrated monocytes/macrophages in allografts treated with NSC 74859 compared with controls (Figure 2C, 2D), suggesting that NSC 74859 decreased recruitment of infiltrated monocytes/macrophages in allografts.

Allograft survival was significantly prolonged relative to controls

We compared survival of allografts in the 2 groups. We found that survival of allografts treated with NSC 74859 was pronouncedly superior to that of allografts without administration of NSC 74859 (Figure 3), suggesting that administration of NSC extends allograft survival.

Discussion

The present study is the first to show direct experimental evidence that blockage of IL-6/STAT3 using the specific inhibitor NSC 74859 can markedly attenuate the immune response in cardiac allograft rejection in mice. We found that expression of classical cytokines that are higher in cardiac allograft rejection – IL-2, IL-6, and IL-15 – were significantly suppressed in allografts treated with NSC 74859 relative to controls. We also found that cardiac allografts of mice treated with NSC 74859 had far fewer infiltrated monocytes and macrophages. Moreover, survival of treated allografts was greatly extended. Importantly, our results show a linkage between cardiac allograft rejection and the IL-6/STAT3 signaling pathway, suggesting that blockage of IL-6/STAT3 could be used as an alternative clinical therapy for management of cardiac allograft rejection.

Our study was prompted by previous sporadic reports [12–14] that knock-down of STAT1 using decoy oligodeoxynucleotide approach alleviated cardiac allograft rejection in mice. Therefore, our study is an extension of these previous investigations. Based on these earlier studies, we reasoned that inhibition of STAT3 might also play a role similar to that of STAT1

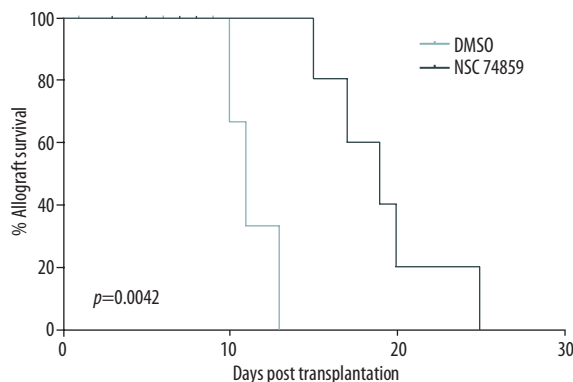


Figure 3. Prognostic analysis of the cardiac allograft murine model. There were 10 mice left in the experimental group and 9 mice in the control group after the other mice were euthanasia for analysis of cytokines or CD surface maker expression. There was an extremely significant difference in overall prognosis between the DMSO group and NSC 74859 group the using log-rank test ($p < 0.01$).

in cardiac allograft rejection. No direct experimental evidence has been established for blockage of STAT3; nonetheless, cardiac allograft rejection is considered to be an inflammatory process. On the other hand, considering that IL-6 has been reported to be heavily implicated in the allograft rejection [3] and the regulation between STAT3 pathway and IL-6 [25], we postulated that blockage of IL-6/STAT3 using the specific inhibitor NSC 74859 could alleviate the immune response in cardiac allograft rejection of mice. It was shown that cytokines that are abundantly produced in cardiac allograft rejection – IL-2, IL-6, and IL-15 – were expectedly and significantly suppressed in allografts of mice treated with NSC 74859 in comparison with controls. In addition, the infiltrated monocytes and macrophages that play the major role in rejection were also shown to be remarkably diminished relative to controls. Our findings presented here are fundamentally in line with those of previous reports on Janus kinase 3 (JAK 3) [26–29], an important member of the IL-6/STAT3 signaling pathway in cardiac allograft rejection, which leads to the suggestion that inhibition of IL-6/STAT3 signaling could alleviate cardiac allograft rejection. However, as for the mechanism by which block of IL-6/STAT3 pathway attenuates the immune response in rejection remains unknown that deserves to be further investigated. Although our current study in a sense is an extension of those earlier studies on STAT1 [12–14], our study distinctively differs from them in that the previous reports did not evaluate the variation of cytokines, including IL-2, IL-6, and IL-15, nor did they assess the marker of the infiltrated monocytes, CD14, after knock-down of STAT1. Our finding that inhibiting the IL-6/STAT3 pathway extends allograft survival is supported

by Stepkowski et al. [26], who reported that allograft survival can be prolonged by PNU156804, a selective inhibitor of JAK 3, which is another member involved in the IL-6/STAT3 signaling pathway.

The original report on NSC 74859, also named S31-201, was from the exploration of chemical inhibition with anti-tumor activity [24]. Extensive studies demonstrated the high specificity and efficiency of NSC 74859 in the inhibition of STAT3 activity, which is heavily involved in cancers. It was then extended to investigations on transition from inflammation to cancer [30]. However, the present report is the first to show the effectiveness of treatment with NSC 74859 in preventing cardiac allograft rejection. Our study is the first to evaluate NSC 74859 in cardiac allograft rejection of mice, indicating that NSC 74859 has clinical potential attenuating cardiac allograft rejection. In using NSC 74859 in a murine model, previous investigators have generally used 2 generally concentrations: 5 mg/kg [31] and 10 mg/kg [20]. In our pilot study before experimentation, we found that 5 mg/kg NSC 74859 was inadequate in suppression of the IL-6/STAT3 signaling pathway (data not shown). Thus, we selected a 10 mg/kg concentration for our experiments.

Our study is the first to present the potential novel application of NSC 74859 in the alleviation of cardiac allograft rejection

in mice. However, several limitations need to be acknowledged. First, given that our observations were made merely based on a mouse model of homotopic heart transplantation, further evaluation of NSC 74859 should be extended to a heterotopic heart transplantation mouse model. Second, from the technical perspective, a different method, for example, ELISA or flow cytometry, would be complementary to our approach used in the detection of biomarkers of interest we selected. Third, extrapolation of the direction function of NSC 74859 from our findings should be approached with caution due to the organ-specific differences in rejection [32]. Finally, another animal model of heart transplantation, for example, rats or miniature swine, with larger sample sizes are warranted to further confirm our results.

Conclusion

Inhibition of the IL-6/STAT3 pathway using inhibitor NSC 74859 can attenuate the immune response in cardiac allograft rejection of mice, underscoring the potential clinical use of NSC 74859 in the management of cardiac allograft rejection.

Conflict of interests

None.

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