BMC Cardiovascular Disorders

RESEARCH

Open Access

Neurogenic differentiation 2 promotes inflammatory activation of macrophages in doxorubicin-induced myocarditis via regulating protein kinase D

Xinyu Tan[1], Changyu Yan[1], Gang Zou[2] and Ran Jing[1*]

Abstract

Background Although it has been established that protein kinase D (PKD) plays a crucial role in various diseases, its precise role in myocarditis remains elusive.

Methods To investigate PKD's involvement in myocarditis, we established a mouse model of myocarditis using doxorubicin (DOX) to assess cardiac function, observe pathological changes, and quantify inflammatory cytokine levels in heart tissues. Additionally, macrophages were isolated from heart tissues of both control and DOX-treated groups to assess PKD expression and inflammatory cytokines in these macrophages. We explored the molecular mechanism of Neurogenic Differentiation 2 (NeuroD2) in myocarditis by utilizing NeuroD2 overexpression plasmids and NeuroD2 small interfering RNA (siRNA). Furthermore, we conducted dual-luciferase reporter and chromatin immunoprecipitation (ChIP) assays to investigate the interaction between NeuroD2 and PKD.

Results We observed significant upregulation of PKD in macrophages and heart tissues induced by DOX. The administration of a PKD inhibitor reduced inflammatory cytokine levels, improved cardiac function, and mitigated pathological changes in myocarditis-afflicted mice. Mechanistically, we found upregulated expression of NeuroD2 in both macrophages and heart tissues exposed to DOX. NeuroD2 could directly target PKD, enhancing the NLRP3/NF-κB signaling pathway and exacerbating macrophage inflammation.

Conclusions Our study demonstrates that NeuroD2 can directly bind to the PKD promoter, potentially promoting inflammatory activation of macrophages in DOX-induced myocarditis via the NLRP3/NF-κB pathway. This suggests that the NeuroD2/PKD axis may hold promise as a potential therapeutic approach for treating DOX-induced myocarditis.

Keywords Myocarditis, NeuroD2, PKD, Macrophages, Inflammatory activation, NLRP3/NF-κB pathway

*Correspondence:
Ran Jing
rjing811@gmail.com
[1]Xiangya hospital of central south university, No. 87, Xiangya road, Changsha, China
[2]Zhong Ke Zhu Ying Intelligence Technology Academy, No. 96, Tongzipo road, Changsha, China

Introduction

Myocarditis is an inflammatory disease of the cardiac muscle, typically triggered by viral infections and subsequent immune-mediated responses [1, 2]. Patients with myocarditis can manifest a wide spectrum of clinical symptoms, ranging from asymptomatic cases to cardiogenic shock and unexpected sudden death [3]. Recent studies investigating sudden cardiac death in young individuals have reported myocarditis in 2–42% of autopsied cases [4]. Besides supportive care, there are currently limited treatment options for both the acute and chronic stages of myocarditis [2], emphasizing the need to develop innovative therapeutic interventions.

The primary cause of myocarditis is localized or widespread myocardial interstitial inflammation, which leads to the degeneration and necrosis of myocardial cells and fibers, ultimately impairing cardiac function [5]. Cardiac tissue macrophages, the immune cells in the myocardium, perform a myriad of roles in normal physiological tissue maintenance and various pathological conditions [6]. In response to cardiac injury, the population of cardiac macrophages significantly increases, promoting inflammatory activation and the release of inflammatory cytokines such as IL-1β, IL-6, and TNF-α, thereby accelerating myocardial damage [7, 8]. As crucial players in the inflammatory response, macrophages also profoundly affect the development of cardiovascular diseases, including unfavorable cardiac remodeling, hypertension, myocardial infarction, and more [9]. Previous studies have reported that in human CVB3-induced myocarditis, infiltrating macrophages exhibit classical activation phenotypes (M1-type) and enhance cardiac inflammation by releasing pro-inflammatory cytokines such as TNF-α and IL-6. Importantly, these M1-type macrophages can also influence the ensuing adaptive immune response to pathological Th1 responses, thereby exacerbating myocarditis, highliting the critical pathological role of macrophages-mediated inflammation in CVB3-induced myocarditis [10]. The NLR family pyrin domain-containing protein 3 (NLRP3) inflammasome is a crucial multiprotein complex that is essential for modulating the innate immune system and inflammatory signaling pathways [11]. And nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), a pivotal activator of inflammatory responses, initiates the activation of the NLRP3-inflammasome by stimulating the expression of pro-IL-1β and NLRP3 [12]. A prior study indicated that the NF-κB/NLRP3 pathway holds a pivotal role in the pyroptosis of cardiomyocytes [13]. Therefore, targeting the inflammatory activation of macrophages, along with the NF-κB/NLRP3 axis, could be a promising therapeutic approach for myocarditis.

Protein kinase D (PKD), a family of serine/threonine protein kinases, plays a vital role in the signaling network of the second messenger diacylglycerol [14]. It has been established that PKD is activated by various extracellular stimuli and transmits crucial cell signals that impact fundamental cellular functions, including secretion, migration, proliferation, survival, angiogenesis, and immune responses [15]. Dysregulation of PKD expression and activity has been implicated in numerous human diseases, such as cancer, metabolic disorders, central nervous system disorders, as well as cardiac and inflammatory conditions [16]. For instance, Venardos et al. reported that the PKD inhibitor CID755673 enhances cardiac function in diabetic *db*/*db* mice [17]. Additionally, studies have highlighted PKD's involvement in inflammatory bowel disease and protease-induced neurogenic inflammation and pain [18, 19]. Nevertheless, the role and potential mechanisms of PKD in myocarditis remain unexplored.

Through JASPAR database mining, we found that NeuroD2 was functionally targeted by PKD. Neurogenic Differentiation 2 (NeuroD2), a member of the basic-helix-loop-helix (bHLH) transcription factor family, acts as a master regulator of cell proliferation, neuronal differentiation, and specification [20]. Additionally, NeuroD2 has been reported to be linked to inflammation, as evidenced by its upregulation in mice with spinal cord injury, where it plays a role in regulating inflammation and oxidative stress [21]. However, its role in myocarditis remains largely unknown.

Based on previous research and bioinformatic analysis, we investigated the role of PKD in a mouse model of myocarditis induced by doxorubicin (DOX) and assessed its impact on pathological conditions and cardiac function in this model. Furthermore, we explored the role of PKD in the inflammatory activation of macrophages isolated from mice in different experimental groups. We also conducted additional studies to elucidate the interaction between PKD and neurogenic differentiation 2 (NeuroD2) and the involvement of the NLRP3/NF-κB pathway in DOX-induced myocarditis.

Materials and methods

Database analysis

The JASPAR database (http://www.jaspar.genereg.net)) was used to explore the possible binding sites of the NeuroD2 and PKD promoters.

Animal model

All in vivo experiments were performed according to the guidelines for the Care and Use of Laboratory Animals of Xiangya Hospital of Central South University. This study was conducted in accordance with the ARRIVE Guidelines and approved by the Institutional Animal Care and Use Committee (IACUC) of Xiangya Hospital of Central South University (Approval No. 2022101065).

24 healthy male C57BL/6 mice (4–6 weeks old, 16–18 g) were purchased from the Laboratory Animal Center of Southern Medical University. Mice were provided with food and water ad libitum and kept under controlled conditions (Temperature 22.2 °C, air humidity 40–70%) with 12 h:12 h light/dark cycles. The mice were randomly divided into four groups ($n = 6$ per group): the control group, the DOX group, the DOX + DMSO group, and the DOX + CID755673 group. CID755673 was dissolved in DMSO. Mice were treated with intraperitoneal saline injections as a control, and mice received 1.5 mg/kg DOX once every two days for a total of three weeks by intraperitoneal injection in the DOX group. Mice in the DOX + DMSO group and DOX + CID755673 group were treated with equal volume intraperitoneal injections of DOX (once every two days for three weeks), and these mice received daily intraperitoneal injections of 10 mg/kg CID755673 (Abmole Biosciences, Houston, Texas, USA) or equal volume of DMSO solution during DOX treatment for two weeks. On day 21 of injection, echocardiography was performed to detect myocardial functions. Then, the mice were sacrificed, and heart tissues were harvested for subsequent experiments.

Echocardiography

Mice were placed on a heating pad at 37 °C and keep continuous anesthetized with 2-3% isoflurane. Pulse oximetry was used to monitor heart rate and oxygen saturation, while the depth of anesthesia was regularly controlled by evaluating the toe-withdrawal reflex and monitoring heart rate (The desired heart rate target was 400 ± 50 bpm). Echocardiography was carried out on SomnoSuite small animal anaesthesia system (Kent Scientific Corporation, USA), standard echo views (parasternal long axis view [PLAX], parasternal short axis view [PSAX] and apical four-chamber view [4-CV]) were obtained as previously reported. All measurements were obtained from M-mode images captured in the parasternal long-axis view (PLAX) at the papillary muscle level of mice. The left ventricular end-diastolic diameter (LVEDd), left ventricular end-systolic diameter (LVESd) were measured, and left ventricular fraction shortening (LVFS) and left ventricular ejection fraction (LVEF) were calculated as follows.

$$\mathrm{EF} = 100\,\mathrm{x}\frac{(\mathrm{LVEDV} - \mathrm{LVESV})}{\mathrm{LVEDV}}\,;\mathrm{FS} = 100\,\mathrm{x}\,\frac{(\mathrm{LVEDD} - \mathrm{LVESD})}{\mathrm{LVEDD}};$$

Hematoxylin and Eosin staining

Heart tissues obtained from different groups were fixed in 4% paraformaldehyde at room temperature overnight. The tissues underwent dehydration through a series of graded ethanol, followed by embedding in paraffin and slicing into sections with 5-μm-thickness. After dewaxing and dehydration, slices were stained with Hematoxylin (Sigma Aldrich) for a duration of 3–6 min, subsequently rinsed for 1–2 min, and then differentiated for 1–3 s using 1% hydrochloric acid alcohol, and encouraged the liquid to turn blue for 5–10 s, rinsed under running water for 15–30 s, and then stained with 0.5% eosin solution (Sigma Aldrich) for 2–3 min.The sections then underwent washing with distilled water for 1 to 2 s, followed by immersion in 80% ethanol for 15–30 s, then in 95% ethanol for another 15–30 s, and ultimately in anhydrous ethanol for 1 to 2 s. After being dried, they were sealed with neutral gum. The histological examination was performed using a light microscope (Olympus Corp.) with the Olympus DP70 digital camera.

Immunofluorescence staining

Mouse heart tissues were fixed in 4% paraformaldehyde and embedded in paraffin. The paraffin blocks were then sectioned into 5 μm thick slices. Following dewaxing and rehydration, sections were incubated with 5% bovine serum albumin for 1 h. After being washed with PBS, the slides were incubated with primary antibodies against CD68 (1:50, Abcam, Cambridge, MA, USA) at 4℃ overnight. Subsequently, they were incubated with the corresponding secondary antibodies for 1 h. Images were acquired using confocal microscopy (Carl Zeiss, Germany), and the average fluorescence intensity was quantified using ZEN software.

Enzyme-linked immunosorbent assay

The levels of tumor necrosis factor (TNF-α), interleukin (IL)-6, IL-18, and IL-1β in macrophages supernatant or heart homogenate were measured using respective enzyme-linked immunosorbent assay (ELISA) kits (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions.

Cell culture and flow cytometry

Macrophages were isolated from mice heart tissues from different groups. Briefly, mice were sacrificed, and hearts were collected and cut into small pieces of 1–2 mm^3 on ice. Heart tissues were digested by 0.125% trypsin at 37 °C for 5–6 times for a total of 10 min. A single-cell suspension was collected after centrifugation and filtration. Macrophages were isolated from the single-cell suspension using the Anti-F4/80 MicroBeads UltraPure kit (Novobiotec, Beijing, China), according to the manufacturer's protocol. The purity of freshly isolated macrophages was determined by a FACSAria II flow cytometer (BD Biosciences, San Jose, CA, USA).

Quantitative real-time reverse transcription polymerase chain reaction

The total RNA was extracted from macrophages or heart tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and the Reverse Transcription Kit (Takara, Dalian, China) was used to convert RNA into complementary DNA (cDNA). Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) was conducted using SYBR Select Master Mix on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Relative expression was quantified following the $2^{-\Delta\Delta Ct}$ method. The primers used in this research are listed in Table 1.

Western blotting

Mice heart tissues and macrophages were lysed in ice-cold RIPA lysis buffer containing protease inhibitors (Beyotime Biotechnology, Shanghai, China) to extract the total protein. The BCA Protein Assay Kit (Beyotime Biotechnology) was utilized to measure the total protein concentrations. The protein extracts were separated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes. After blocking 1 h with 5% skimmed milk, the membranes were incubated with the following primary antibodies at 4℃ overnight, including PKD (1:2000; Affinity Biosciences, Cincinnati, OH, USA), NLRP3 (1:2000; Affinity Biosciences), p-p65 (1:1000; Affinity Biosciences), p65 (1:1000; Affinity Biosciences), Neurod2 (1:1000; Biorbyt, Cambridge, UK.), GAPDH (1:3000; Affinity Biosciences). Followed by treatment with horseradish peroxidase (HRP)-conjugated secondary antibody (1:3000; Affinity Biosciences) at room temperature for 2 h. The protein expression was detected by an enhanced chemiluminescence (ECL) detection system.

Table 1 The sequences of primers used in this study

TNF-α-F	GCACCACCATCAAGGACTCA
TNF-α-R	CAGGGAAGAATCTGGAAAGGT
IL-6-F	GGAGTGGCTAAGGACCAAGAC
IL-6-R	GTGAGGAATGTCCACAAACTGA
IL-18-F	TCAGTCTGGTCTGGGGTTCAC
IL-18-R	TGGAGACCTGGAATCAGACAA
IL-1β-F	CTCGTGCTGTCGGACCCAT
IL-1β-R	ATTCTTTCCTTTGAGGCCCA
PKD-F	AGGACCTATCCAGGACACCAT
PKD-R	ACATTTACGCCCTCTCCTCAT
NLRP3-F	GCACTGCTCTTCACTGCTATCA
NLRP3-R	AAGTCACCAAGAGGGAACACC
Neurod2-F	CCTGGTGATTTCTGGTGGCT
Neurod2-R	TCTCCCAGCTCACTGGATGA
β-catin-F	TGCTGTCCCTGTATGCCTCT
β-catin-R	TTTGATGTCACGCACGATTT

Plasmid, oligonucleotides and transfection

The full length of the PKD cDNA sequence was amplified and cloned into the pcDNA3.1 vector to construct a PKD overexpression plasmid named pcDNA3.1- PKD. Besides, the NeuroD2 overexpression plasmid was constructed. The NeuroD2 small interfering RNA (siNeuroD2) and its negative control (NC siRNA) were obtained from Genechem (Shanghai, China). The sequences of oligonucleotides were as follows: NC siRNA: GCAAGC UGACCCUGAAGUUC; siNeuroD2: GAAUCUCUUGU CUUACGAUAU. These overexpression plasmids and oligonucleotides were transfected into macrophages using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Dual-Luciferase reporter assay

The wild (wt) and mutant (mut) types in the PKD promoter binding site were subcloned into psiCHECK-2 plasmids (Promega, Madison, WI, USA) and co-transfected with overexpression NeuroD2 plasmids (Promega) in macrophages using Lipofectamine 2000. After 48 h, the luciferase activity was measured using a dual-luciferase assay kit (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Chromatin Immunoprecipitation

A Chromatin immunoprecipitation (ChIP) experiment was conducted using the SimpleChIP Mix (Cell Signaling Technology, Beverly, MA, USA) according to the manufacturer's protocol. 6×10^6 cells were treated with 1% formaldehyde for 10 min. The cell lysate was then sonicated until the length of DNA remained between 200 and 1000 bp. An equivalent chromatin volume was immunoprecipitated at 4 °C overnight with the antibody Neurod2 (1:50, Biorbyt). Immune complexes were collected after incubating with Magnetic Beads Protein A/G. The immunoprecipitated DNA was isolated and examined by qPCR, and the primers were as follows: PKD primers: forward 5'-GCACTGCTCTTCACTGCTATCA-3'; reverse 5'- AAGTCACCAAGAGGGAACACC – 3'.

Statistical analyses

All statistical analyses were performed using SPSS21.0 (SPSS Inc., Chicago, IL, USA). Data were expressed as the mean ± standard deviation (sd). Differences between the two groups were compared using the student's *t*-test. Differences among multiple groups were analyzed using one-way analysis with Tukey's multiple comparisons test. A P-value < 0.05 was statistically significant.

Results

Protein kinase D is upregulated in heart tissues of mice induced by doxorubicin

To assess cardiac function, transthoracic echocardiography was performed, revealing enhanced LVEDd and LVESd alongside decreased LVFS and LVEF in the DOX group compared to normal mice, indicative of impaired cardiac function (Fig. 1a -1e). Histological examination via H&E staining revealed pronounced inflammatory cell infiltration and cardiomyocyte necrosis in mice with myocarditis, while normal mice exhibited minimal inflammation. The pathological score for tissue from mice with myocarditis was also significantly higher (Fig. 1f). And immunofluorescence staining assay suggested that DOX administration effectively increased the protein expression of macrophages maker CD68 in mice heart tissues compared with normal heart tissues (Fig. 1g). Furthermore, levels of inflammatory cytokines, including TNF-α, IL-6, IL-18, and IL-1β, were significantly elevated in heart tissue from the DOX group compared to the control group (Fig. 1h -1i). These findings validate the successful establishment of the DOX-induced myocarditis model. Given the known role of the NLRP3 inflammasome in inflammatory disorders as well as the effects of PKD on inflammasome formation and activation [8, 22], we assessed the tissue levels of PKD, NLRP3, phosphorylated p65 (p-p65), and p65, finding that PKD, NLRP3, p-p65, and p65 expressions were significantly upregulated in the DOX group compared to the control group (Fig. 1j). These results substantiate high

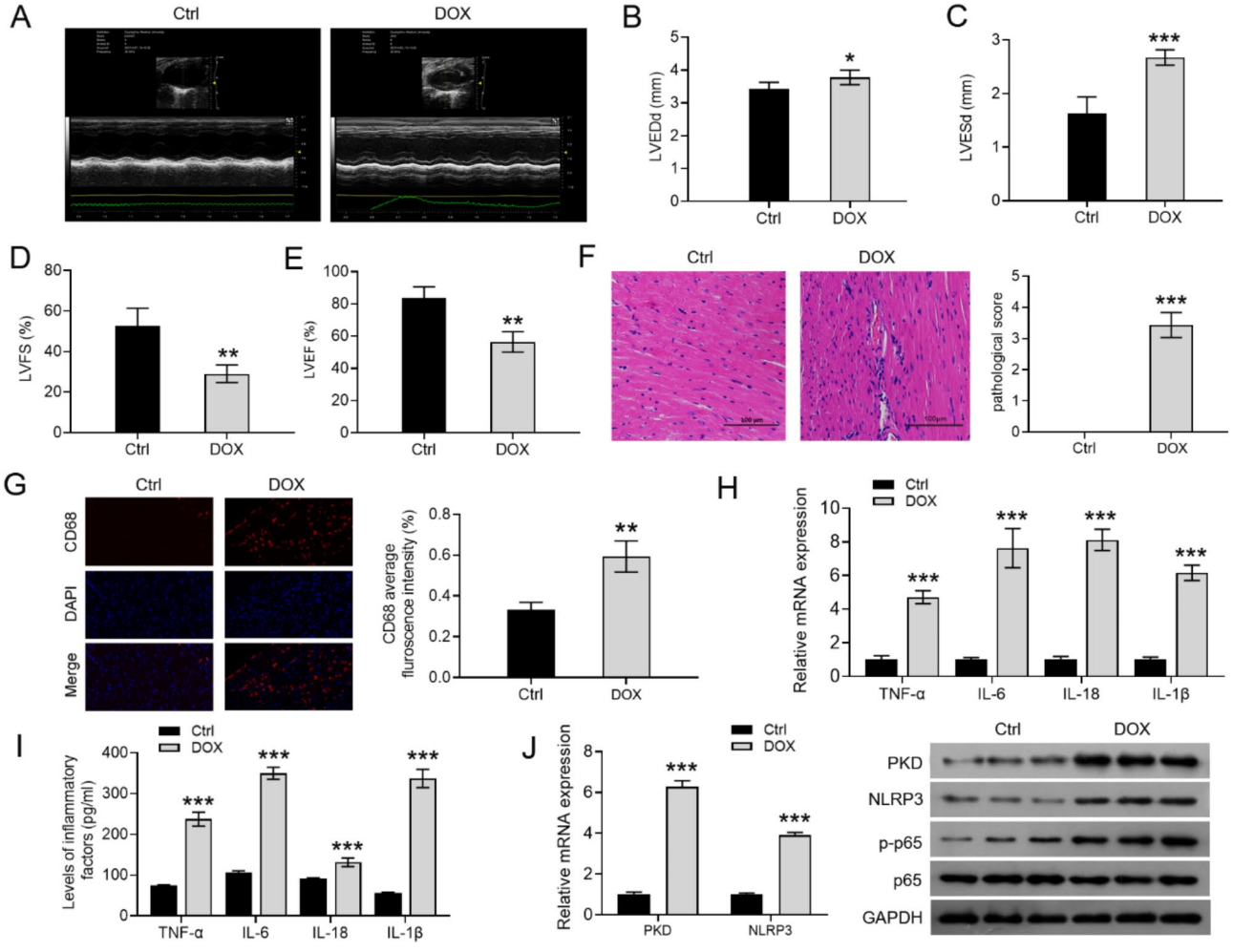

Fig. 1 Protein kinase D is highly expressed in the heart tissues of mice induced by doxorubicin. (**A**-**E**) On day 21 of doxorubicin (DOX) injection, echocardiography was conducted to detect the cardiac function of mice. LVEDd (**B**), LVESd (C), LVFS (**D**), and LVEF (**E**) were measured. (**F**) Hematoxylin and Eosin (H&E) staining of pathological changes in heart tissues in each group. (**G**) Mice heart tissue were subjected to immunofluorescence staining for macrophage marker CD68. (H-I) The mRNA expressions and cytokine levels of TNF-α, IL-6, IL-18, and IL-1β in heart tissues were measured by quantitative real-time reverse transcription polymerase chain reaction (qPCR) and Enzyme-linked immunosorbent assay (ELISA), respectively. (J) PKD, phosphorylated p65 (p-p65), p65, and NLRP3 expression in macrophages from mice heart tissues was examined using qRT-PCR analysis and western blotting, respectively. Values were presented as the mean ± SD of triplicate experiments. Compared with the control group, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

cardiac tissue expression of PKD and inflammatory factors in mice induced by DOX.

Protein kinase D inhibitor CID755673 improves the inflammation and cardiac function in mice with myocarditis

To investigate the role of PKD in myocarditis, we treated mice with myocarditis by intraperitoneal injection of CID755673 (a PKD inhibitor). As expected, CID755673 treatment significantly reduced PKD expression (Fig. 2a and 2b). Echocardiographic data demonstrated reduced LVEDd and LVESd and improved LVFS and LVEF in the inhibitor-treated group (Fig. 2c - 2g), indicating a significant amelioration of cardiac dysfunction caused by DOX injection. Additionally, CID755673 intervention significantly decreased inflammatory cell infiltration and pathological scores (Fig. 2h), along with the protein expression of the macrophage marker CD68 in heart tissues (Fig. 2i). Furthermore, CID755673 treatment reversed the increased mRNA expressions of TNF-α, IL-6, IL-18, and IL-1β in myocarditis tissue (Fig. 2j), which was further confirmed by ELISA analysis (Fig. 2k). Considering the significance of the NLRP3/p65 pathway in PKD-mediated inflammation [8], we detected the levels of NLRP3 and p-p65 in myocardial tissues treated with or without CID755673, it was observed that CID755673 effectively reduced NLRP3, p65, and p-p65 levels in myocardial tissues (Fig. 2l and 2m). These results collectively indicate that downregulating PKD can improve inflammation and cardiac function in mice with myocarditis.

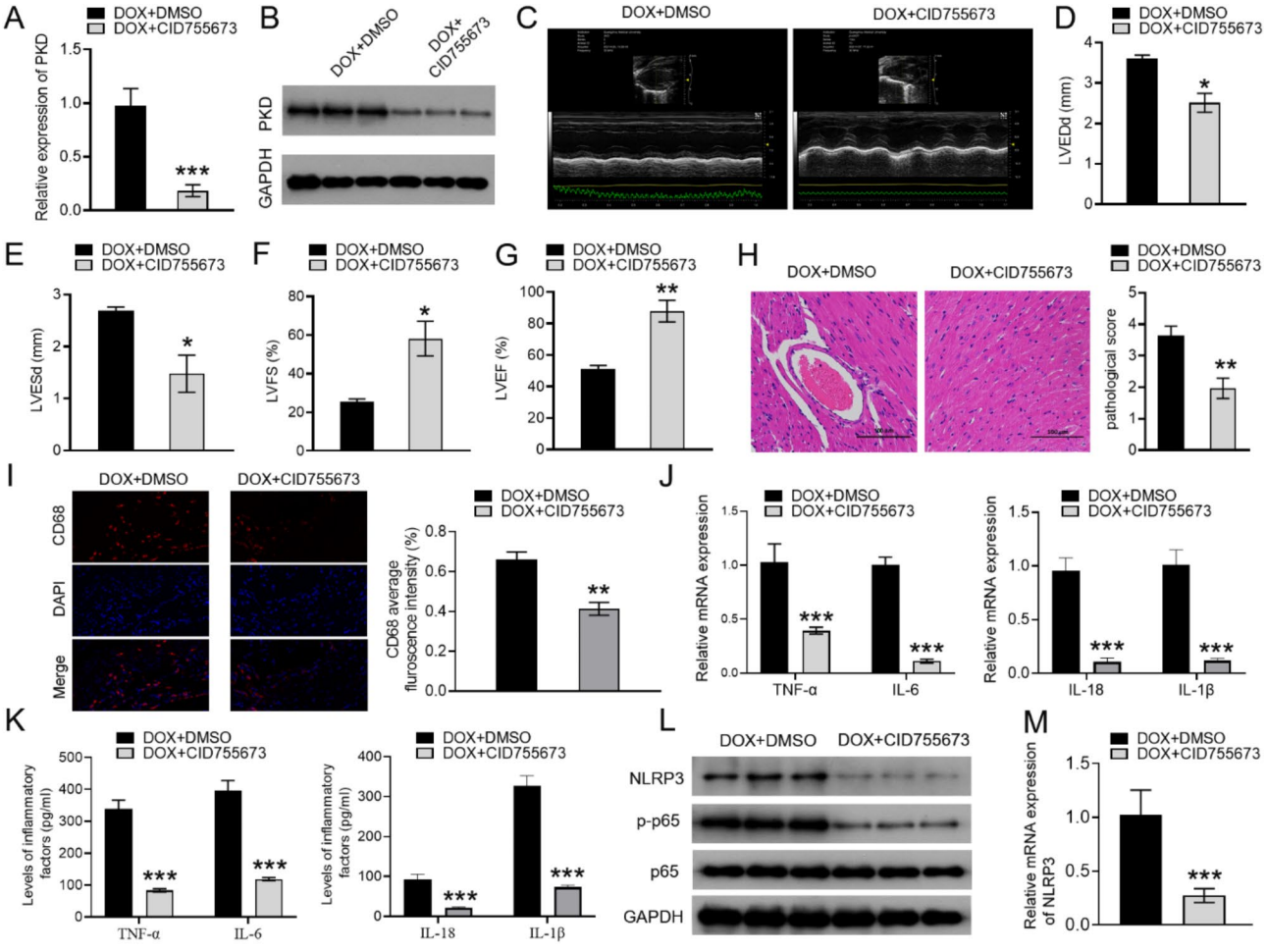

Fig. 2 Protein kinase D inhibitor CID755673 improves inflammation and cardiac function in mice with myocarditis. (**A**-**B**) Mice with myocarditis treated with or without PKD inhibitor CID755673, the expression of PKD was detected by qPCR analysis and western blotting. (**C**-**G**) The cardiac function (**C**), LVEDd (D), LVESd (**E**), LVFS (**F**), and LVEF (**G**) were explored by echocardiography. **(H)** H&E staining for inflammatory cell infiltration in each group. (**I**) Immunofluorescence staining for the macrophage marker CD68 was performed on mouse heart tissue. (**J**-**K**) The levels of TNF-α, IL-6, IL-18, and IL-1β in mice heart tissues were measured by qPCR (**J**) and ELISA (**K**). (**L**) The protein levels of NLRP3, p65, and p-p65 in different groups. (**M**) The relative expression of NLRP3 was measured by qPCR analysis. Values were presented as the mean ± SD of triplicate experiments. Compared with DOX group, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

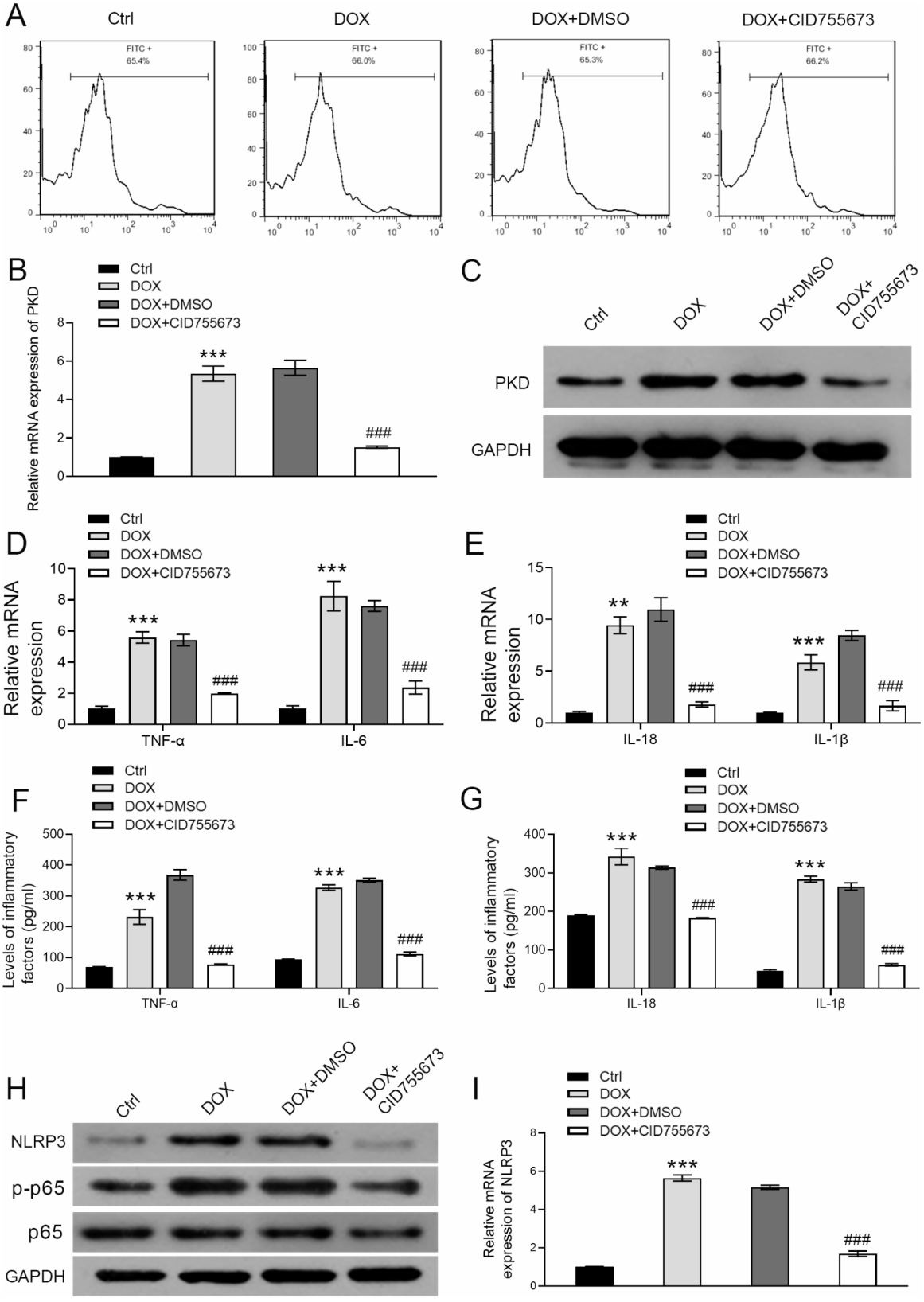

Fig. 3 (See legend on next page.)

(See figure on previous page.)

Fig. 3 Protein kinase D is involved in the DOX-induced inflammatory activation of macrophages. Macrophages were isolated from the heart tissues of mice in different groups. (**A**) The purity of macrophages was determined by a FACSAria II flow cytometer. (**B**-**C**) The mRNA expressions and protein levels of PKD were detected using qPCR analysis and western blotting. (**D**-**G**) qPCR and ELISA assay were carried out to measure the levels of TNF-α, IL-6, IL-18, and IL-1β in macrophages. (**H**) The protein levels of NLRP3, p-p65, and 65 in each group. (**I**) The expression of NLRP3 was analyzed in macrophage using qPCR analysis. Values were presented as the mean ± SD of triplicate experiments. Compared with the control group, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; Compared with DOX + DMSO group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$

Protein kinase D is involved in the doxorubicin-induced inflammatory activation of macrophages

Macrophages were isolated from the heart tissues of mice in four groups: control group, DOX group, DOX + DMSO group, and DOX + CID755673 group. Large numbers of macrophages were present in the F4/80 + stained cells from heart tissues across different groups (Fig. 3a). Notably, PKD mRNA expression was significantly elevated in the DOX group but reversed by CID755673 administration (Fig. 3b and 3c). Additionally, the TNF-α, IL-6, IL-18, and IL-1β levels were efficiently inhibited by CID755673 compared to DOX alone, as confirmed by both ELISA and qPCR analyses (Fig. 3d -3g). Furthermore, CID755673 significantly reduced the levels of NLRP3, p65, and p-p65 in macrophages compared to the DOX group (Fig. 3h -3i). Collectively, these results indicate the crucial role for PKD in macrophage inflammatory activation.

Functional interaction between neurogenic differentiation 2 and protein kinase D

In silico analysis using the JASPAR database revealed putative binding sites between NeuroD2 and PKD (Fig . 4a). Western blotting and qPCR confirmed elevated NeuroD2 expression in both macrophages and tissues induced by DOX (Fig. 4b-4c). Further experiments involved interfering with or overexpressing NeuroD2 in macrophages isolated from normal heart tissues and treated with DOX. NeuroD2 knockdown significantly downregulated NeuroD2 expression compared to the control group, while the opposite findings were observed for NeuroD2 overexpression (Fig. 4d-4e). Moreover, PKD mRNA expression and protein levles in macrophages significantly decreased with NeuroD2 knockdown but was restored with NeuroD2 overexpression (Fig.4f-4g). Subsequently, we investigated the interaction between NeuroD2 and PKD, luciferase reporter assay revealed that NeuroD2 elevated the luciferase activity of the PKD promoter WT in macrophages without affecting the MUT (Fig. 4h), suggesting the combination between NeuroD2 and PKD, which was further confirmed through a chromatin immunoprecipitation (ChIP) experiment (Fig. 4i). These results suggest that PKD is a functional target of NeuroD2.

The overexpression of protein kinase D can reverse the impact of neurogenic differentiation 2 on doxorubicin-induced inflammatory activation of macrophages

A PKD overexpression plasmid was constructed to further confirm the role of NeuroD2 in macrophage inflammatory activation. Compared to the DOX alone group, the mRNA expressions of PKD and NLRP3 decreased after NeuroD2 interference, which was reversed by PKD overexpression (Fig. 5a and 5b). Similarly, NeuroD2 inhibition significantly reduced PKD, p-p65, p65, and NLRP3 protein levels in the DOX alone group, but PKD overexpression reversed these effects (Fig. 5c). Additionally, the levels of TNF-α, IL-6, IL-18, and IL-1β were significantly reduced by NeuroD2 downregulation compared to the DOX alone group, and these trends were reversed after PKD upregulation (Fig. 5d -5g). In summary, these results indicate that the effects of NeuroD2 on DOX-induced macrophage inflammatory activation can be reversed by overexpressing PKD.

Discussion

Myocarditis poses a significant challenge due to the absence of effective treatment options in current practice, highlighting the need for innovative approaches in cardiovascular medicine [23, 24]. Previous studies have emphasized the pivotal role of excessive macrophage activation-driven inflammation in the pathogenesis of myocarditis [25]. Notably, macrophages are the predominant cardiac inflammatory cell subset during the early stages of CVB3-induced viral myocarditis, as documented by Fairweather et al. [26]. Epelman et al. have also elucidated the critical role of macrophages in initiating, amplifying, and sustaining inflammation [27]. In this context, our study utilized a DOX-induced mouse model to investigate the influence of PKD and NeuroD2 on macrophage inflammatory activation in myocarditis.

Clinically, myocarditis can be caused by a diverse range of infectious agents, such as viruses, bacteria, chlamydia, rickettsia, fungi, and protozoa, along with toxic and allergic reactions. Among these infectious agents, viruses are the most commonly reported to be associated with myocarditis [28, 29]. Following infection, changes in the quantity and function of macrophages are commonly observed in patients with both acute and chronic myocarditis [29]. Moreover, inflammatory markers such as inter leukin 6, 8, and 10 are found significantly elevated [30], and inflammatory cellular infiltrates can be observed in

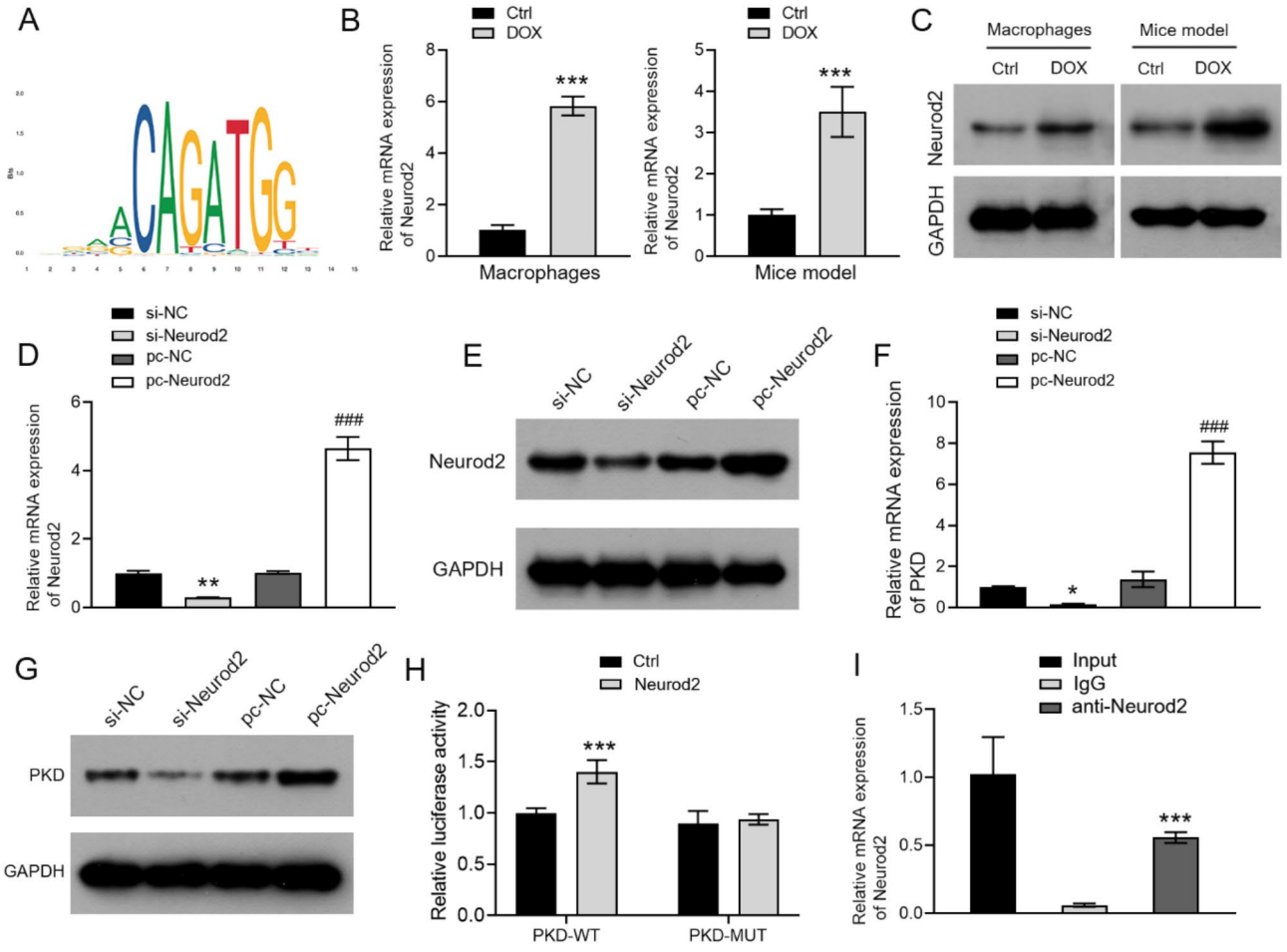

Fig. 4 Functional interaction between Neurogenic Differentiation 2 and protein kinase D. Macrophages were isolated from normal tissues and treated with 1μmol/L DOX for 12 h. (**A**) The binding site of PKD and Neurod2 was predicted by the JASPAR database. (**B**-**C**) qPCR analysis and western blotting were performed to detect the expression of Neurod2 in macrophages and tissues induced by DOX. (**D**-**E**) Macrophages isolated from the heart of normal mice were co-transfected with Neurod2 overexpression plasmid, and siNeurod2. qPCR and western blotting were performed to test the expression of Neurod2 mRNA and protein, respectively. (**F**-**G**) The levels of PKD in each group were explored by qPCR and western blotting. (H-I) The binding ability between PKD and Neurod2 was confirmed by dual-luciferase reporter assay (H) and CHIP assay (I). Values were presented as the mean ± SD of triplicate experiments. Compared with the control group or si-NC group, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; Compared with the pc-NC group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$

heart tissue section stained using conventional methods [31]. DOX is a potent and commonly utilized anticancer medication. However, the clinical utilization of DOX is constrained due to its severe cardiotoxicity and cardiac injury, typically myocarditis [32]. In this research, we constructed myocarditis mouse model by intraperitoneal injection of DOX, it was found that the introduction of DOX significantly elevated the levels of inflammatory cytokines including IL-6, IL-18 and IL-1βin mice heart tissues. And similar results were observed in macrophages isolated from mice heart tissues, suggesting that DOX treatment may trigger macrophages inflammatory activation. Moreover, H&E staining indicated an obvious inflammatory cell infiltration in heart tissues harvested from DOX group, which is in consistent with the pathological conditions in clinical virus-induced myocarditis.

PKD plays a multifaceted role in various biological processes, including angiogenesis, heart contraction, cell differentiation, apoptosis, immunomodulation, and cancer [33–35]. Studies have reported PKD's involvement in cardiomyocyte hypertrophy through regulating extracellular signal-regulated and myocyte enhancer factor 2D pathways [36]. Additionally, another study suggested that PKD has been implicated in stress signaling modulation within the heart, affecting gene expression, cell survival, excitation-contraction coupling, and metabolism [37]. Our research revealed significant upregulation of PKD in cardiac-infiltrating macrophages, and PKD inhibition was associated with enhanced cardiac function, reduced pathological conditions, and decreased levels of inflammatory cytokines in mice with myocarditis. These findings suggest a potential role for PKD in

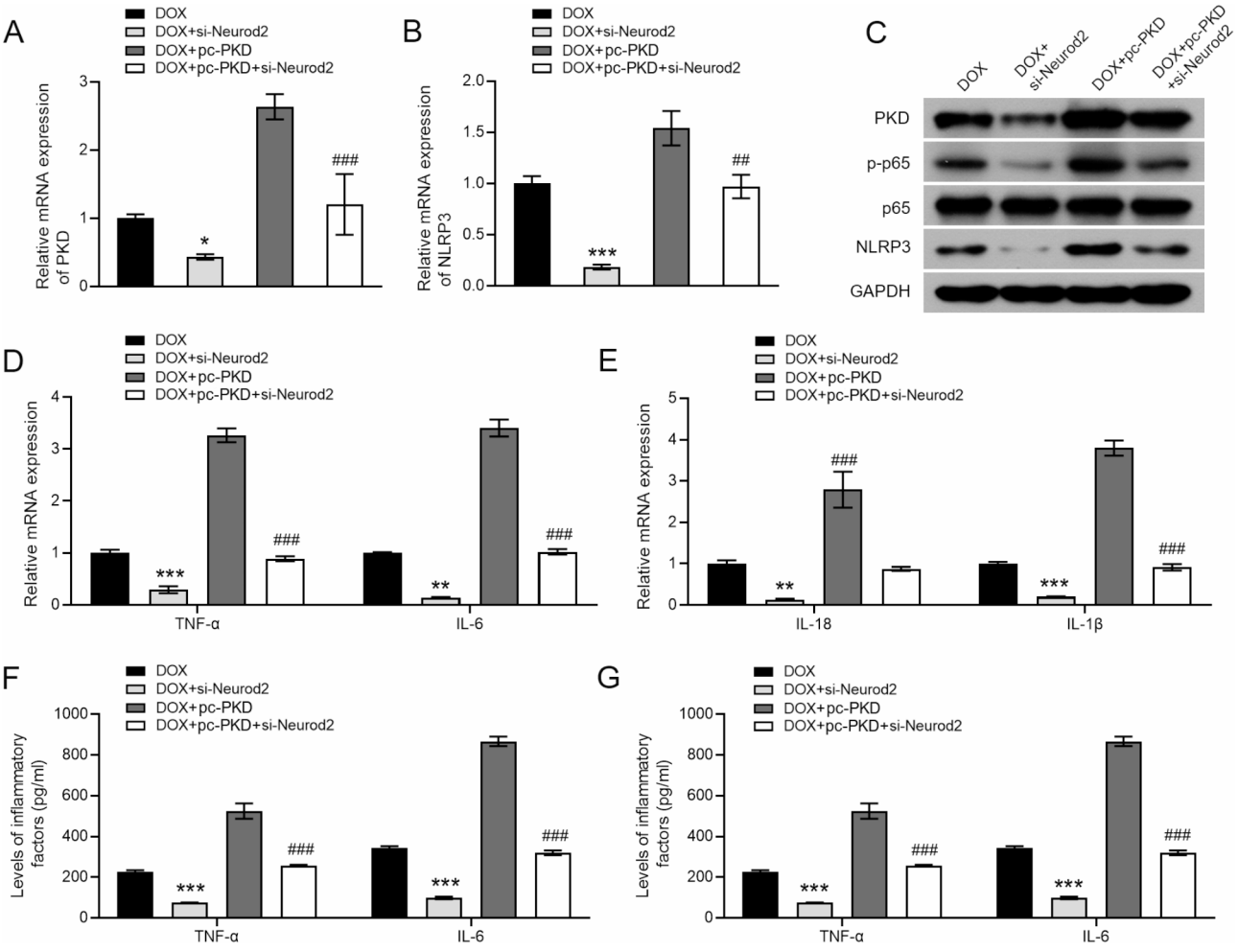

Fig. 5 The overexpression of Protein kinase D can reverse the impact of neurogenic differentiation 2 on doxorubicin-induced inflammatory activation of macrophages. Macrophages isolated from normal tissue and treated with 1μmol/L DOX for 12 h. (**A**-**B**) Macrophages were co-transfected with PKD overexpression plasmid and si-Neurod2. qPCR was used to test the mRNA expressions of PKD and NLRP3. (**C**) The protein levels of PKD, p-p65, p65, and NLRP3 were detected. (**D**-**G**) The levels of TNF-α, IL-6, IL-18, and IL-1β in each group were measured by qPCR and ELISA. Values were presented as the mean ± SD of triplicate experiments. Compared with the DOX group, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$; Compared with DOX + pc-PKD group, $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$

macrophage-mediated inflammation during the myocarditis process.

The JASPAR database predicted a potential interaction between NeuroD2 and PKD. Subsequent experiments in our study confirmed this prediction by demonstrating the direct binding of NeuroD2 to the PKD promoter, thereby regulating PKD expression. NeuroD2, a neurogenic transcription factor, have associated with multiple diseases, including its involvement in the regulation of inflammation and oxidative stress [21]. However, studies investigating the role of NeuroD2 in myocarditis are limited. Our study observed elevated NeuroD2 expression in both macrophages and myocardial tissues induced by DOX. And addition of NeuroD2 knockdown significantly reduced the PKD mRNA expression and protein levles in macrophages, while these levels were restored with NeuroD2 overexpression.We further confirmed the interaction between NeuroD2 and the PKD promoter through luciferase activity assays and ChIP experiments. Overexpression of PKD reversed the impact of NeuroD2 on inflammatory cytokine levels to some extent.

Inflammasomes serve as pattern recognition receptors that are crucial for host defense and sterile inflammatory disorders. It is now understood that the NLRP3 inflammasome can be activated by a wide range of pathogen-associated molecular patterns (PAMPs) and endogenous danger-associated molecular patterns (DAMPs) [38, 39]. Previous studies have implicated PKD in inflammasome activation and development [8], including its role in the phosphorylation and release of NLRP3 from Golgi membranes, facilitating the assembly of active inflammasome complexes. Inhibition of PKD was shown to retain NLRP3 at Golgi membranes, preventing inflammasome assembly [40]. Our study revealed a significant increase

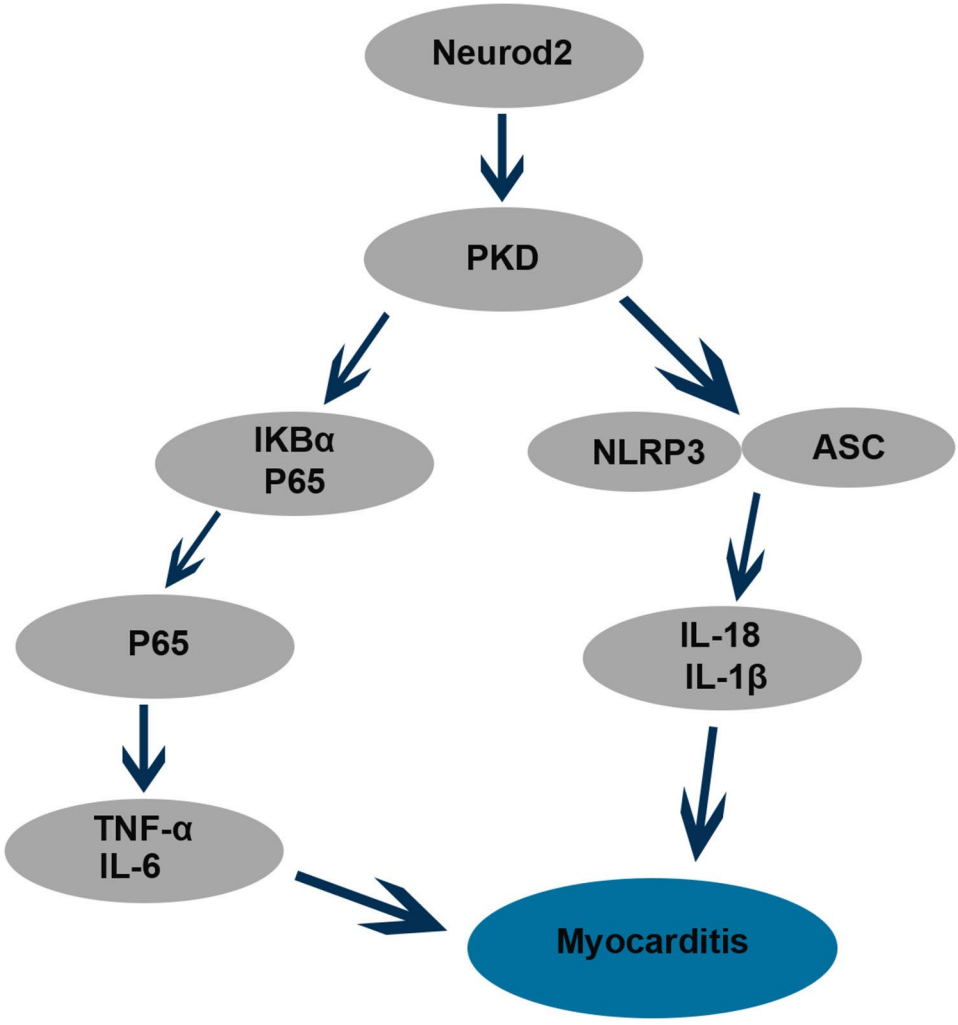

Fig. 6 PKD-mediated macrophage inflammasome activation in heart tissue. The combination of NeuroD2 and PKD promoter could enhance NLRP3/NF-κB signaling,

in NLRP3 levels in both DOX-induced mice tissues and macrophages, and PKD inhibitor CID755673 treatment effectively restrained the NLRP3 elevation, indicating the involvement of PKD in NLRP3 inflammasome activation, which was further supported by the increased release of IL-1βand IL-18. NF-κB is a nuclear transcription factor critical for various physiological reactions, particularly those related to inflammatory responses [41]. It serves as a signal for the transcriptional activation of NLRP3 inflammasome components [42], especially in response to oxidative stress [43]. And our results also revealed that DOX injections upregulated the levels of phosphorylated p65, TNF-α and IL-6, which were also reversed by CID755673 addition. These results establish PKD as a crucial regulator in triggering inflammatory responses. Moreover, the interaction between NeuroD2 and the PKD promoter amplified the NLRP3/NF-κB signaling pathway, exacerbating macrophage inflammation and finally contributing to the progression of myocarditis (Fig. 6).

However, there are still some limitations to our study. First, the limited number of mice utilized in each group may potentially impact the results, In future research, we plan to increase the number of subjects per group to enhance statistical power and ensure more robust findings. Second, another limitation of our study is the lack of validation using clinical samples. it is crucial to incorporate clinical sample analysis to validate our findings in future studies. Third, in this research, we investigated the role of the NeuroD2/PKD axis in DOX-induced myocarditis. For further studies, other types of animal models, such as those for viral myocarditis, can be developed to expand our investigation.

Conclusions

Our findings highlight the upregulation of both NeuroD2 and PKD in myocarditis. Inhibition of PKD led to improvements in pathological status and cardiac function in the DOX-induced mouse model. Furthermore, NeuroD2 functionally targeted PKD, enhancing the NLRP3/NF-κB signaling pathway and promoting macrophage inflammation, ultimately exacerbating myocarditis progression. These results suggest that targeting the NeuroD2/PKD axis may hold promise as a potential therapeutic approach against DOX-induced myocarditis.

Abbreviations

PKD	protein kinase D
DOX	Doxorubicin
NeuroD2	Neurogenic Differentiation 2
LVEDd	Left ventricular end-diastolic diameter
LVESd	Left ventricular end-systolic diameter
LVFS	Left ventricular fraction shortening
LVEF	Left ventricular ejection fraction

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12872-025-04626-7.

Supplementary Material 1

Supplementary Material 2

Acknowledgements

Not applicable.

Author contributions

TXY designed and drafted the manuscript. JR made critical revision of the manuscript. ZG and YCY collected and analyzed the data.

Funding

This research was funded by the Science and Technology Innovation Project 2021 from the Department of Transportation of Hunan Province (NO.202142).

Data availability

The datasets generated and/or analyzed during the current study are not publicly available as the data is confidential but are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

All in vivo experiments were performed according to the guidelines for the Care and Use of Laboratory Animals of Xiangya Hospital of Central South University. This study was conducted in accordance with the ARRIVE Guidelines and approved by the Institutional Animal Care and Use Committee (IACUC) of Xiangya Hospital of Central South University (Approval No. 2022101065).

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Clinical trial number

Not applicable.

Received: 10 November 2023 / Accepted: 3 March 2025

Published online: 18 March 2025

References

1. Ma WH, Zhang XG, Guo LL, Zhang JB, Wei FT, Lu QH, Du H, Kong YR, Wang X, Xu DL. Androgen receptor Inhibition alleviated inflammation in experimental autoimmune myocarditis by increasing autophagy in macrophages. Eur Rev Med Pharmacol Sci. 2021;25(10):3762–71.
2. Pollack A, Kontorovich AR, Fuster V, Dec GW. Viral myocarditis–diagnosis, treatment options, and current controversies. Nat Rev Cardiol. 2015;12(11):670–80.
3. Kawada JI, Takeuchi S, Imai H, Okumura T, Horiba K, Suzuki T, Torii Y, Yasuda K, Imanaka-Yoshida K, Ito Y. Immune cell infiltration landscapes in pediatric acute myocarditis analyzed by CIBERSORT. J Cardiol. 2021;77(2):174–78.
4. Caforio AL, Pankuweit S, Arbustini E, Basso C, Gimeno-Blanes J, Felix SB, Fu M, Helio T, Heymans S, Jahns R et al.,. *Current state of knowledge on aetiology, diagnosis, management, and therapy of myocarditis: a position statement of the European Society of Cardiology Working Group on Myocardial and Pericardial Diseases. Eur Heart J. 2013; 34(33):2636-48, 48a-48d.*
5. Li HD, Chen B, Yang F. IGF-1 affects the development of myocarditis in LDL-R knockout mice by inhibiting peritoneal infiltration of macrophages. Eur Rev Med Pharmacol Sci. 2020;24(15):8104–11.
6. Moskalik A, Niderla-Bielinska J, Ratajska A. Multiple roles of cardiac macrophages in heart homeostasis and failure. Heart Fail Rev. 2022;27(4):1413–30.
7. Zhang Z, Tang J, Cui X, Qin B, Zhang J, Zhang L, Zhang H, Liu G, Wang W, Zhang J. New insights and novel therapeutic potentials for macrophages in myocardial infarction. Inflammation. 2021;44(5):1696–712.
8. Sutton JA, Rogers LM, Dixon B, Kirk L, Doster R, Algood HM, Gaddy JA, Flaherty R, Manning SD, Aronoff DM. Protein kinase D mediates inflammatory responses of human placental macrophages to group B Streptococcus. Am J Reprod Immunol. 2019;81(3):e13075.
9. Xu X, Wang Y, Li Y, Zhang B, Song Q. The future landscape of macrophage research in cardiovascular disease: A bibliometric analysis. Curr Probl Cardiol. 2022;47(10):101311.
10. Zhang H, Yue Y, Sun T, Wu X, Xiong S. Transmissible endoplasmic reticulum stress from myocardiocytes to macrophages is pivotal for the pathogenesis of CVB3-induced viral myocarditis. Sci Rep. 2017; 7(42162.
11. Blevins HM, Xu Y, Biby S, Zhang S. The NLRP3 Inflammasome Pathway: A Review of Mechanisms and Inhibitors for the Treatment of Inflammatory Diseases. Front Aging Neurosci. 2022; 14(879021.
12. Zhong Z, Umemura A, Sanchez-Lopez E, Liang S, Shalapour S, Wong J, He F, Boassa D, Perkins G, Ali SR, et al. NF-κB restricts inflammasome activation via elimination of damaged mitochondria. Cell. 2016;164(5):896–910.
13. Zhang H, Lin J, Shen Y, Pan J, Wang C, Cheng L. Protective effect of Crocin on immune checkpoint Inhibitors-Related myocarditis through inhibiting NLRP3 mediated pyroptosis in cardiomyocytes via NF-κB pathway. J Inflamm Res. 2022;15:1653–66.
14. Aicart-Ramos C, He SD, Land M, Rubin CS. A novel conserved domain mediates dimerization of protein kinase D (PKD) isoforms: DIMERIZATION IS ESSENTIAL FOR PKD-DEPENDENT REGULATION OF SECRETION AND INNATE IMMUNITY. J Biol Chem. 2016;291(45):23516–31.
15. Steinberg SF. Decoding the cardiac actions of protein kinase D isoforms. Mol Pharmacol. 2021;100(6):558–67.
16. Zhang X, Connelly J, Chao Y, Wang QJ. Multifaceted functions of protein kinase D in pathological processes and human diseases. Biomolecules. 2021; 11(3).
17. Venardos K, De Jong KA, Elkamie M, Connor T, McGee SL. The PKD inhibitor CID755673 enhances cardiac function in diabetic Db/db mice. PLoS ONE. 2015;10(3):e0120934.
18. Chu E, Saini S, Liu T, Yoo J. Bradykinin stimulates protein kinase D-mediated colonic myofibroblast migration via cyclooxygenase-2 and heat shock protein 27. J Surg Res. 2017; 209(191–98.
19. Amadesi S, Grant AD, Cottrell GS, Vaksman N, Poole DP, Rozengurt E, Bunnett NW. Protein kinase D isoforms are expressed in rat and mouse primary sensory neurons and are activated by agonists of protease-activated receptor 2. J Comp Neurol. 2009;516(2):141–56.
20. Tutukova S, Tarabykin V, Hernandez-Miranda LR. The Role of Neurod Genes in Brain Development, Function, and Disease. Front Mol Neurosci. 2021; 14(662774.

21. Zhu Y, Xu Q, Sha WP, Zhao KP, Wang LM. MiR-219-5p promotes spinal cord injury recovery by inhibiting NEUROD2-regulated inflammation and oxidative stress. Eur Rev Med Pharmacol Sci. 2019;23(1):37–43.
22. Bao J, Sun T, Yue Y, Xiong S. Macrophage NLRP3 inflammasome activated by CVB3 capsid proteins contributes to the development of viral myocarditis. Mol Immunol. 2019;114:41–8.
23. Jahandideh A, Uotila S, Stahle M, Virta J, Li XG, Kyto V, Marjamaki P, Liljenback H, Taimen P, Oikonen V, et al. Folate receptor beta-Targeted PET imaging of macrophages in autoimmune myocarditis. J Nucl Med. 2020;61(11):1643–49.
24. Wang Y, Jia L, Shen J, Wang Y, Fu Z, Su SA, Cai Z, Wang JA, Xiang M. Cathepsin B aggravates coxsackievirus B3-induced myocarditis through activating the inflammasome and promoting pyroptosis. PLoS Pathog. 2018;14(1):e1006872.
25. Lei Y, Yang Y, Zhao J, Gao H, Chen R, Bai B, Kang X, He Y, Ding L, Wei T, Fu X, Zhao L, Li X. P-AKT2/SPK1 (P-SPK1) and P-MEK/P-ERK cell signaling pathways are involved in LPS-induced macrophage migration. Am J Transl Res. 2019;11(5):2725–41.
26. Fairweather D, Rose NR. Coxsackievirus-induced myocarditis in mice: a model of autoimmune disease for studying immunotoxicity. Methods. 2007;41(1):118–22.
27. Epelman S, Liu PP, Mann DL. Role of innate and adaptive immune mechanisms in cardiac injury and repair. Nat Rev Immunol. 2015;15(2):117–29.
28. Fairweather D, Beetler DJ, Di Florio DN, Musigk N, Heidecker B, Cooper LT. Jr. COVID-19, myocarditis and pericarditis. Circ Res. 2023;132(10):1302–19.
29. Sagar S, Liu PP, Cooper LT. Jr Myocarditis Lancet. 2012;379(9817):738–47.
30. Sellers SA, Hagan RS, Hayden FG, Fischer WA 2. The hidden burden of influenza: A review of the extra-pulmonary complications of influenza infection. Influenza Other Respir Viruses. 2017;11(5):372–93.
31. Cooper LT. Jr. Myocarditis. N Engl J Med. 2009;360(15):1526–38.
32. Wang Y, Yan S, Liu X, Deng F, Wang P, Yang L, Hu L, Huang K, He J. PRMT4 promotes ferroptosis to aggravate doxorubicin-induced cardiomyopathy via Inhibition of the Nrf2/GPX4 pathway. Cell Death Differ. 2022;29(10):1982–95.
33. Simsek Papur O, Sun A, Glatz JFC, Luiken J, Nabben M. Acute and Chronic Effects of Protein Kinase-D Signaling on Cardiac Energy Metabolism. Front Cardiovasc Med. 2018; 5(65.
34. Youssef I, Ricort JM. Deciphering the role of protein kinase D1 (PKD1) in cellular proliferation. Mol Cancer Res. 2019;17(10):1961–74.
35. Renton MC, McGee SL, Howlett KF. The role of protein kinase D (PKD) in intracellular nutrient sensing and regulation of adaptive responses to the obese environment. Obes Rev. 2021;22(3):e13145.
36. Yuan H, Xiang Q, Yang L, Geng J. Protein kinase D participates in cardiomyocyte hypertrophy by regulating extracellular signal-regulated and myocyte enhancer factor 2D. Rev Port Cardiol (Engl Ed). 2021;40(3):191–200.
37. Wood BM, Bossuyt J. Emergency Spatiotemporal shift: the response of protein kinase D to stress signals in the cardiovascular system. Front Pharmacol. 2017; 8(9.
38. Abderrazak A, Syrovets T, Couchie D, El Hadri K, Friguet B, Simmet T, Rouis M. NLRP3 inflammasome: from a danger signal sensor to a regulatory node of oxidative stress and inflammatory diseases. Redox Biol. 2015;4:296–307.
39. Tschopp J, Schroder K. NLRP3 inflammasome activation: the convergence of multiple signalling pathways on ROS production? Nat Rev Immunol. 2010;10(3):210–5.
40. Zhang Z, Meszaros G, He WT, Xu Y, de Fatima Magliarelli H, Mailly L, Mihlan M, Liu Y, Puig Gamez M, Goginashvili A, et al. Protein kinase D at the golgi controls NLRP3 inflammasome activation. J Exp Med. 2017;214(9):2671–93.
41. Hoseini Z, Sepahvand F, Rashidi B, Sahebkar A, Masoudifar A, Mirzaei H. NLRP3 inflammasome: its regulation and involvement in atherosclerosis. J Cell Physiol. 2018;233(3):2116–32.
42. Ding Y, Ding X, Zhang H, Li S, Yang P, Tan Q. Relevance of NLRP3 Inflammasome-Related pathways in the pathology of diabetic wound healing and possible therapeutic targets. Oxid Med Cell Longev. 2022; 2022(9687925.
43. Storz P, Toker A. Protein kinase D mediates a stress-induced NF-kappaB activation and survival pathway. EMBO J. 2003;22(1):109–20.

Publisher's note