Deletion of P110δ promotes the development of myocarditis in ApoE-deficient mice by increasing mononuclear cell peritoneal infiltration

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Abstract. Phosphoinositide 3-kinase catalytic subunit δ isoform (P110δ) is mainly expressed in white blood cells. It is involved in T and B lymphocyte differentiation, maturation and the neutrophil chemotaxis process. Apolipoprotein E (ApoE) is an arginine-rich alkaline protein, which is present in plasma chylomicron, low-density lipoprotein and very low-density lipoprotein. The present study aimed to determine the effects of P110δ deletion on myocarditis in $ApoE^{-/-}$ mice. A mouse model of ApoE and P110δ double deletion was initially constructed; hematoxylin and eosin (H&E) staining was performed to detect the histological alterations in the mouse myocardium. Systolic and diastolic alterations, and alterations in the left ventricular fractional shortening (LVFS) and left ventricular ejection fraction (LVEF) were examined by electrocardiogram. Blood cell of ApoE and P110δ double mice was used to detect changes in white blood cells and monocytes. Western blotting was used to detect the expression levels of apoptosis-associated proteins, whereas flow cytometry was used to detect the percentage of apoptosis. Morphological alterations in myocardial cells were observed under a microscope. The results of polymerase chain reaction demonstrated that double deletion mice were successfully constructed. H&E staining revealed that cells in the $ApoE^{-/-}$ mice were spindle-shaped; however, the nuclei were smaller in the double deletion mice. There was no change in cardiac contraction in normal mice; however, in double deletion mice, the systolic and diastolic contractions were markedly reduced. LVFS and LVEF were decreased compared with in the control group. Blood cell analysis indicated that the content of white blood cells and monocytes in the experimental group was significantly higher than that in the control group. Western blotting demonstrated that the expression levels of apoptotic proteins in double deletion mice were significantly higher compared with in the control group. Flow cytometry revealed that the apoptotic ratio was increased in double deletion mice compared with in the control group (42 vs. 21%). These findings suggested that deletion of P110δ may induce monocyte peritoneal infiltration and increase apoptosis, thus promoting the development of myocarditis.

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Introduction

Cardiac disease is currently the largest risk factor for of adult mortality and is associated with ≤17 million cases of mortality per year worldwide (1). Myocarditis is an inflammatory condition of the heart, which can be induced by viral infection, hypersensitivity to certain substances and autoimmune reactions (2). If it is not treated properly, myocarditis may lead to dilated cardiomyopathy, which may result in the development of heart failure. Although research in this area has advanced rapidly in recent years, the therapeutic strategies used to cure myocarditis remain limited. At present, physicians generally believe that myocarditis is the result of various pathogenic factors. The development of myocarditis is accompanied by lipid metabolism disorders, and inflammatory and immune responses (3,4).

Apolipoprotein E (ApoE) is an arginine-rich alkaline protein, which is present in plasma chylomicron, low-density lipoprotein and very low-density lipoprotein (5). ApoE can be synthesized in various tissues, mainly in the liver, brain and kidney. ApoE is a ligand for low-density lipoprotein receptors and for hepatocyte remnant receptors; therefore it is closely associated with lipoprotein metabolism. ApoE is polymorphic, and is dependent on individual lipid levels and is closely associated with the development of atherosclerosis. In addition, ApoE is involved in the activation of hydrolyzed fats, immunoregulation and regeneration of nerve tissue (6-8). However, to the best of our knowledge, the relationship between ApoE and myocarditis has not yet been investigated. Given the important role of ApoE in cardiac development, the present study aimed to determine whether ApoE is also involved in myocarditis.

Phosphoinositide 3-kinase (PI3K) is an intracellular phosphatidylinositol kinase, which is known to regulate

inflammatory responses in numerous diseases (9,10). The inhibition of PI3K is also able to promote infarct resorption and prevent adverse cardiac remodeling following myocardial infarction in mice (11). A study using PI3Kγ-deficient mice demonstrated a complex contribution of PI3Kγ to reparative angiogenesis in myocardial infarction (12). PI3K catalytic subunit δ isoform (P110δ) is an enzyme that in humans is encoded by the PIK3CD gene (13), which is widely involved in cell growth, differentiation, and immune regulation and other effects. P110δ is mainly expressed in white blood cells, and it is involved in T and B lymphocyte differentiation, maturation and the neutrophil chemotaxis process; therefore, P110δ is considered an important molecule that regulates the leukocyte immune response (14). In addition, it has been suggested that P110δ is an attractive pharmacological target that modulates unwanted immune responses and certain blood cancers (15). Notably, P110δ-selective inhibitors are currently being tested in clinical trials to treat autoimmunity, allergies and lymphoid malignancies (16).

In recent years, there have been many reports about the relationship between P110δ and inflammation. Previous studies have reported that B-cell lymphoma 2 (Bcl-2)-associated X protein (Bax) and BH3 interacting-domain death agonist (Bid) have an important role in transforming growth factor (TGF)-β1-induced myocarditis and apoptosis. TGF-β1 was able to promote the expression and activation of P110δ, and phosphorylate p21 through Bax and Bid (15). The lack of P110δ can effectively reverse TGF-β1-induced myocarditis (17); therefore, it may be suggested that P110δ and myocarditis are closely related. A previous study suggested that P110δ is able to inhibit monocyte infiltration in ApoE knockout (KO) mice (18). In present study, ApoE$^{-/-}$/P110δ$^{-/-}$ mice were constructed on the basis of ApoE$^{-/-}$ mice and investigated the effects of P110δ deletion on myocarditis in ApoE KO mice.

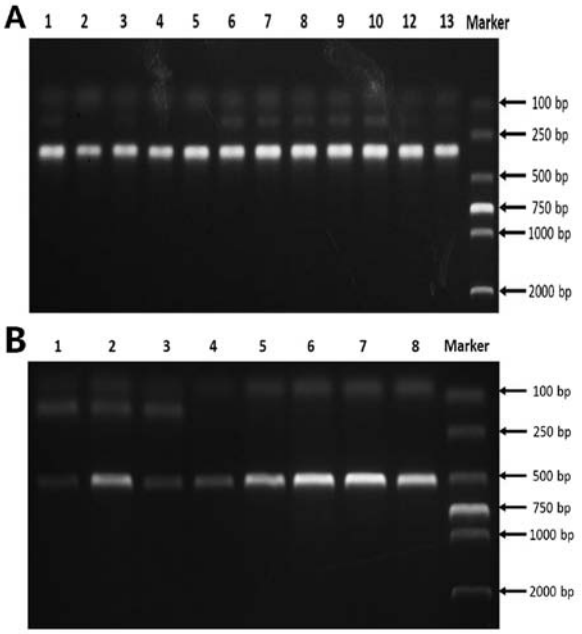

Figure 1. Construction of a mouse model. (A) Identification of ApoE$^{-/-}$ mice. ApoE$^{+/-}$ with two bands at 165 and 330 bp. Lanes 1, 6, 7, 8, 9 and 10 are ApoE$^{+/-}$ with two bands at 165 and 330 bp; Lanes 2, 3, 4, 5, 11 and 12 are ApoE$^{-/-}$with one band at 330 bp. (B) Identification of P110δ$^{-/-}$ mice. P110δ$^{-/-}$ homozygous with one band at 485 bp. Lanes 1, 2 and 3 are P110δ$^{+/-}$ heterozygous with two bands at 145 and 485 bp; Lanes 4, 5, 6, 7 and 8 are P110δ$^{-/-}$ homozygous with one band at 485 bp. ApoE, apolipoprotein E; P110δ, phosphoinositide 3-kinase catalytic subunit δ isoform.

Materials and methods

Animal model. ApoE$^{-/-}$ mice were purchased from Jackson Laboratory (Ben Harbor, ME, USA). P110δ$^{-/-}$ mice were constructed by ourselves as described previously (19). Wild-type (WT) mice were purchased from the Experimental Animal Center of Guangdong Province (Guangzhou, China). The 12 mice (6 female and 6 male, weighted 20-25 g) were weaned on the 22nd day after birth and fed a normal diet and food and water was free including 4% fat and 0.07% cholesterol). The mice were maintained under a specific pathogen-free conditions with 12-h light/dark cycles at 26-28˚C and 50-65% humidity. DNA was extracted from the tail tissues according to the MagBeads Tissues Gen DNA Extraction kit (D1700, Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). The expression levels of ApoE and P110δ were determined by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The primers used are as follows: ApoE, sense 5'-GCCTAGCCGAGGGAG AGCCG-3', antisense 5'-TGTGACTTGGGAGCTCTGCAG C-3'; and P110δ, sense 5'-CTGTCATCTCACCTTGCTCC-3' and antisense 5'-AGCGAACCGCCCTATGAC-3'. The reaction conditions were as follows: 94˚C for 3 min, followed by 35 cycles at 94˚C for 20 sec, 65˚C for 30 sec and 72˚C for 15 sec. Mice with ApoE/P110δ deletion were screened from the hybrid F2 generation and were used as experimental mice, whereas ApoE$^{-/-}$ mice were used as control mice. Mice in the experimental group (n=6) and control group (n=6) were 50% female and 50% male. The present study was approved by the Institutional Animal Care and Use Committee of The Second Hospital of Shandong University (Jinan, China).

Characterizing myocarditis by hematoxylin and eosin (H&E) staining. After obtaining mouse myocardial tissue samples (n=6), the myocardium tissues were fixed for 24 h using 10% formalin at room temperature and embedded in paraffin and sectioned to 3-5 μm; tissue sections were floated in a water bath and placed onto glass slides. The glass slides were then placed in staining racks. Paraffin was cleared from the samples in three changes of xylene (2 min per change). After hydrating the samples, the sections were stained in hematoxylin solution for 1 min and were washed under running tap water at room temperature for ≥5 min. Samples were then stained in working eosin Y solution for 10 sec, after which the samples were dehydrated and cleared in three changes of xylene (2 min per change). The slides were viewed under a light microscope. Myocarditis was characterized by inflammatory cell infiltration, myocardial cell degeneration, necrosis and a disordered myocardial arrangement (20).

Characterizing myocarditis by echocardiogram examination. Cardiac function and morphology were monitored by M- and B-mode transthoracic echocardiography, respectively (21). Left ventricular fractional shortening (LVFS) and left ventricular ejection fraction (LVEF) were assessed by cardiac magnetic resonance imaging in all mice (n=6/group).

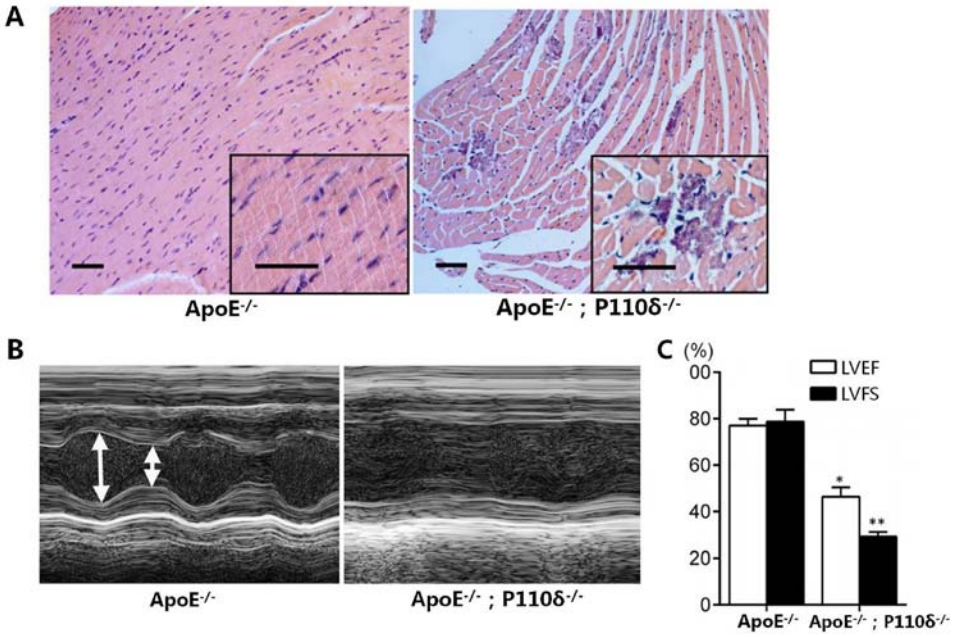

Figure 2. Deletion of P110δ promotes myocarditis in mice. (A) Hematoxylin and eosin staining of myocardial cells in ApoE$^{-/-}$ and ApoE$^{-/-}$/P110δ$^{-/-}$ mice (n=6). Scale bar=1 μl (B) Echocardiogram of the two groups of mice (n=6/group). (C) LVFS and LVEF of two groups of mice (n=6/group). *P<0.05 and **P<0.01 vs. ApoE$^{-/-}$ mice. ApoE, apolipoprotein E; H&E, hematoxylin and eosin; LVEF, left ventricular ejection fraction; LVFS, left ventricular fractional shortening; P110δ, phosphoinositide 3-kinase catalytic subunit δ isoform.

Counting of white blood cells and monocytes. Ascites were collected from the mice following sacrifice, and measurement of white blood cells was performed using an automated blood cell analyzer (Beckman Coulter, Inc., Brea, CA, USA). The whole blood was used to measure monocytes and neutrophils. The number of cells was expressed as means ± standard deviation of three independent measurements.

Western blotting. Proteins were extracted from mouse myocardial tissues using general protein kits from Beyotime Institute of Biotechnology (Haimen, China). All protein samples were adjusted to equal concentrations via a Bicinchoninic Acid protein assay, followed by the addition of bromophenol blue. Equal amounts of proteins were loaded on 10% SDS-PAGE. A total of 6 μl protein marker (EMD Millipore, Billerica, MA, USA) was added at the same time. The protein samples were separated according to a predetermined voltage. Subsequently, the protein was transferred to nitrocellulose membranes. Then the membrane was blocked in room temperature for 2 h with TBS containing 5% skim milk and 0.1% Tween-20, and incubated with primary antibodies against caspase-3 (ab13585), Bax (ab32503), Bcl-2 (ab32124) and GAPDH (ab8245) (Abcam, Cambridge, MA, USA) at a dilution of 1:1,000 at 4˚C overnight, followed by incubation with horseradish-peroxidase-conjugated goat anti-rabbit (A11008, Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and anti-mouse IgG secondary antibodies (A-11077, Invitrogen; Thermo Fisher Scientific, Inc.) for 60 min at room temperature. Detection was performed using the LI-COR Odyssey Scanning Infrared Fluorescence Imaging system (LI-COR Biosciences, Lincoln, NE, USA).

Flow cytometry. The red blood cell lysis buffer, ACK Lysis Buffer (Beyotme Institute of Biotechnology) was added to the blood samples, and centrifuged at 350 x g for 5 min at 4˚C. The precipitate was then washed three times with 0.5% bovine serum albumin (BSA, Beyotme Institute of Biotechnology) for 5 min. Anti-CD4-fluorescein isothiocyanate antibody (F1773, 1:100; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was added and incubated for 30 min at 4˚C in the dark, followed by centrifugation at 350 x g for 5 min at 4˚C. The precipitate was washed with 0.5% BSA three times and the supernatant was discarded. The final precipitate was resuspended with 200 μl 0.5% BSA for detection. The protein was detected using FACSCanto™ II (Becton Dickinson, Franklin Lakes, NJ, USA). The results are expressed as the means ± standard deviation of three independent measurements.

Statistical analysis. All experimental data are expressed as the means ± standard deviation and were analyzed by Image-Pro-Plus 6.0 (Media Cybernetics, Inc., Rockville, MD, USA) and Graph Pad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). One-way analysis of variance was used for multi-group comparisons followed by Bonferroni method as a post hoc test for multiple comparisons. Paired t-test was used to compare two groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Construction of a mouse model. Male ApoE$^{-/-}$ mice and female P110δ$^{-/-}$ mice (age, 6 weeks) were placed in one cage, and F1 mice were identified by DNA detection. Male and female ApoE$^{-/-}$ mice from the F1 generation were hybridized with F1 generation P110δ$^{-/-}$ mice to obtain the F2 generation. F2 generation mice were also identified with DNA detection. ApoE$^{-/-}$ mice were selected as the control group, whereas ApoE$^{-/-}$/P110δ$^{-/-}$ mice from the F2 generation were selected as

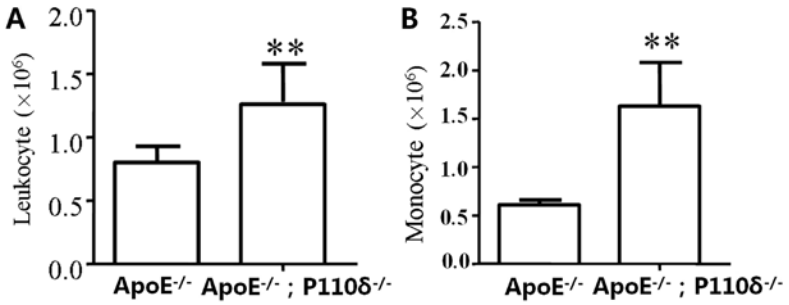

Figure 3. Deletion of P110δ promotes inflammatory cell infiltration in mice in ApoE$^{-/-}$ mice. (A) Blood cell analysis was used to detect alterations in leukocyte content in ApoE$^{-/-}$ and ApoE$^{-/-}$/P110δ$^{-/-}$ mice (n=6). (B) Cell analysis was used to detect alterations in monocyte content in ApoE$^{-/-}$ and ApoE$^{-/-}$/P110δ$^{-/-}$ mice (n=6). **P<0.01. ApoE, apolipoprotein E; P110δ, phosphoinositide 3-kinase catalytic subunit δ isoform.

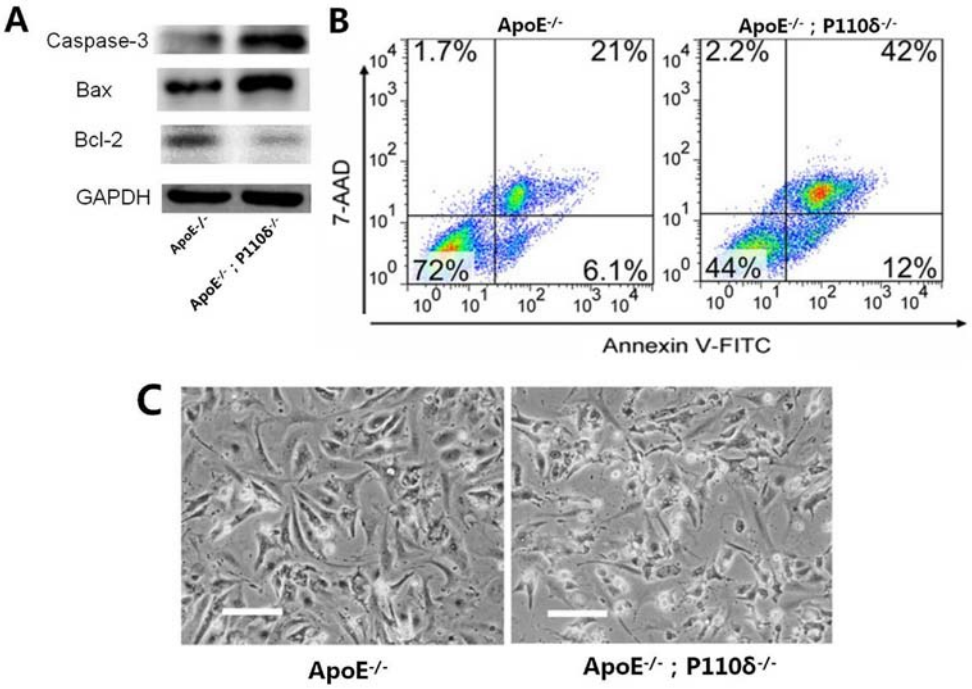

Figure 4. Deletion of P110δ promotes apoptosis of mouse cardiomyocytes. (A) Apoptotic protein expression in ApoE$^{-/-}$/P110δ$^{-/-}$ mice was detected by western blotting. (B) Percentage of apoptosis was detected by flow cytometry. (C) Morphology of mouse cardiomyocytes. Scale bar=5 μm. 7-AAD, 7-amino-actinomycin D; ApoE, apolipoprotein E; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2; FITC, fluorescein isothiocyanate; P110δ, phosphoinositide 3-kinase catalytic subunit δ isoform.

the experimental group. The results of the identification are presented in Fig. 1A; lanes 1, 6, 7, 8, 9 and 10 are ApoE$^{+/-}$ with two bands at 165 and 330 bp; lanes 2, 3, 4, 5, 11 and 12 are ApoE$^{-/-}$ with one band at 330 bp. As shown in Fig. 1B, lanes 1, 2 and 3 are P110δ$^{+/-}$ heterozygous with two bands at 145 and 485 bp; lanes 4, 5, 6, 7 and 8 are P110δ$^{-/-}$ homozygous with one band at 485 bp.

Deletion of P110δ promotes myocarditis in mice. P110δ is involved in T and B cell differentiation, lymphocyte maturation and the neutrophil chemotaxis process. Therefore, the present study aimed to investigate whether the absence of P110δ would induce myocarditis in ApoE$^{-/-}$ mice. As shown in Fig. 2A, cells were spindle-shaped and regular in ApoE$^{-/-}$ mice; however, in the ApoE$^{-/-}$/P110δ$^{-/-}$ mice the nuclei of cells were smaller and the number of inflammatory cells was markedly increased. The electrocardiogram results presented in Fig. 2B demonstrated that contractions in the control group were normal, whereas in the ApoE$^{-/-}$/P110δ$^{-/-}$ mice, the systolic and diastolic contractions were notably smaller; and further examination indicated that LVFS and LVEF were decreased by 38.5 and 61.3% compared with in the control group (Fig. 2C).

Deletion of P110δ promotes infiltration of inflammatory cells. H&E staining revealed that infiltration of inflammatory cells occurs in P110δ-deficient mice. Leukocyte adhesion and infiltration is achieved through endothelial cells; therefore, these functional alterations may lead to a series of heart diseases. In particular, monocytes serve an important role in initiating cell adhesion. Therefore, peritoneal cells were extracted and analyzed in the present study. Blood cell analysis demonstrated that the amount of white blood cells in the experimental group was significantly higher than in the control group, and there was a statistically significant difference (Fig. 3A). In addition,

the number of monocytes in ApoE$^{-/-}$/P110δ$^{-/-}$ mice was significantly increased compared with in the control group (Fig. 3B).

Deletion of P110δ promotes apoptosis of mouse cardiomyocytes. Apoptosis is a process of programmed cell death, which induced by naturally occurring cellular processes that is different from pathological cell necrosis. Apoptosis of myocardial cells in the two mouse groups was analyzed. Western blotting demonstrated that the expression levels of Bcl-2 in ApoE$^{-/-}$/P110δ$^{-/-}$ mice were markedly decreased compared with in the control group, while the expression levels of Bax and caspase-3 were notably increased within ApoE$^{-/-}$/P110δ$^{-/-}$ mice (Fig. 4A). As shown in Fig. 4B, apoptosis of myocardial cells in ApoE$^{-/-}$/P110δ$^{-/-}$ mice was increased by 2-fold compared with in the control group as measured by flow cytometry. In addition, the morphology of myocardial cells in the control group were spindle-shaped, whereas cells in the experimental group appeared shrunken and apoptotic by florescence microscopy (Fig. 4C). Therefore, it may be hypothesized that deletion of P110δ promotes apoptosis of mouse cardiomyocytes in ApoE$^{-/-}$/P110δ$^{-/-}$ mice.

Discussion

Numerous clinical and experimental animal studies have confirmed that inflammation serves an important role in the pathophysiology of myocarditis (22,23). In an experimental model of myocarditis, it was revealed that inflammatory cells, such as monocytes, macrophages and dendritic cells, may infiltrate the vascular adventitia. These inflammatory cells could improve myocarditis by increasing the infiltration of inflammatory cells into the adventitia (24). The present study indicated that cardiomyocytes were significantly infiltrated by inflammatory cells and the nuclei were clearly disorganized in ApoE$^{-/-}$/P110δ$^{-/-}$ mice (data not shown). An electrocardiogram revealed that the control group exhibited normal systolic contractions; however, in ApoE$^{-/-}$/P110δ$^{-/-}$ mice systole and diastole were markedly reduced, and LVFS and LVEF were significantly decreased. Furthermore, the results revealed that ApoE$^{-/-}$/P110δ$^{-/-}$ mice had a significant increase in leukocyte and monocyte levels. Apoptosis is a process of programmed cell death. A previous study reported that anti-apoptotic drugs could pass the blood-brain barrier to reach the drug-target site, thereby reducing intracranial excitotoxicity and release of cytochrome *c*. Blocking the release of cytochrome *c* would prevent the transcriptional translation of apoptotic genes, thereby increasing the expression of the anti-apoptotic protein, Bcl-2, through a series of cascade reactions, which have a role in the regulation of apoptosis (25). Western blotting demonstrated that the levels of apoptotic proteins, such as Bax and caspase-3, were significantly increased in ApoE$^{-/-}$/P110δ$^{-/-}$ mice compared with in the control group. Apoptosis of myocardial cells in ApoE$^{-/-}$/P110δ$^{-/-}$ mice was also higher than that in the control group (42 vs. 21%). Furthermore, the morphology of myocardial cells was spindle-shaped in the control group, whereas, the cells were shrunken and apoptotic in the experimental group. Therefore, it may be hypothesized that deletion of P110δ promotes apoptosis of mouse cardiomyocytes in ApoE$^{-/-}$/P110δ$^{-/-}$ mice.

In conclusion, it may be hypothesized that deletion of P110δ is an inducing factor in the development of myocarditis in mice. In addition, P110δ may have an important role in the absence of ApoE. The mechanism of action underlying the development of myocarditis may be associated with monocyte infiltration and apoptosis.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

QZZ, AYX, WW and FL conducted the experiments. QZZ, AYX, AMP, LNC and WW drafted the manuscript and acquired and analyzed the data, and made substantial contributions to the concept and design of the present study. QZZ, AYX, WW, AMP, LNC and FL interpreted the data and revised the manuscript.

Ethics approval and consent to participate

The present study was approved by the Institutional Animal Care and Use Committee of The Second Hospital of Shandong University (Jinan, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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