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A complete heart regeneration model with inflammation as a key component

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Abstract: The neonatal mice myocardial infarction (MI) has been established as one of the heart regeneration models. However, the role of inflammation in this model is still unclear. We sought to systematically evaluate this model and explore the role of inflammation in it. Postnatal day 1 (P1) or day 7 (P7) mice were conducted left anterior descending coronary artery (LAD) ligation. Cardiac damage, repair, and regeneration were examined by histology and echocardiography. Inflammation was detected by heart section hematoxylin and eosin (HE) staining and tissue qPCR. Dexamethasone (Dex) was used to inhibit inflammation and its effects on heart regeneration were evaluated. Two days after P1 mice MI, cardiomyocytes in ischemia area died and heart function decreased. Then surrounding cardiomyocytes proliferated to repair the injury. At day 28 after MI, hearts were almost fully regenerated with a little fibrosis existed. In contrary, P7 mice MI resulted in thinning and fibrosis of the ventricular wall. Inflammation was induced by LAD ligation after P1 mice MI and dynamic changed during the process. Inhibition of inflammation by Dex impaired heart regeneration. These demonstrated that cardiomyocytes death and heart regeneration occurred in this model and inflammation might play a crucial role in it. Modulating inflammation may provide a promising therapeutic strategy to support heart regeneration.
Key words: dexamethasone, heart regeneration, inflammation, myocardial infarction

Introduction

Since heart regeneration was proposed 150 years ago, it has been intensely investigated [1]. Lower vertebrates such as urodele amphibians and teleost fish have strong heart regenerative capacity. For example, adult zebrafish can fully regenerate the heart when subjected to 20% of the ventricle amputating [2]. However, the adult mammalian heart has long been considered an end-differentiated organ and is insufficient for regeneration in response to injury such as acute myocardial infarction (MI). Instead, MI in adult people leads to fibrotic scar formation, ventricular dilatation and contractile dysfunction[3]. Researchers have long been using zebrafish heart regeneration model to explore what blocks adult mammalian heart regeneration. However, the evolutionary distance of them limits their translational research.

An intriguing recent study suggests that resection of the left ventricular apex of neonatal mice heart can cause a regenerative response reminiscent of that in the adult zebrafish [4]. Not long after that, another heart regeneration model in neonatal mice was produced by left anterior descending coronary artery (LAD) ligation [5]. Compared with apex resection, LAD ligation induces myocardial infarction (MI) which more resembles the pathophysiologic aspects of common human MI injury.

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These models may provide more useful insights into mechanisms of mammal heart regeneration. However, recently the neonatal mice regeneration model processed by LAD ligation was challenged on whether neonatal mice hearts could completely regenerate after injury [6, 7], resulting in a controversy on the usefulness of these models for cardiac regeneration study.

As we know, myocardial infarction in adult mice or humans triggers an intense inflammatory response that is essential for the clearance of dead cells and cardiac remodeling[8]. However, the role of inflammation in neonatal mice myocardial infarction is still unknown. We sought to explore the effects of inflammation on neonatal mice heart regeneration.

Materials and Methods

Animals

Male and female C57BL/6 mice aged 8–12 weeks were maintained and bred under conventional housing conditions in our animal facility. All the animal experimental protocols were conducted under the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Neonatal mice MI model

MI surgeries were performed on neonatal mice at postnatal day 1 (P1) and postnatal day 7 (P7) as described [9]. In brief, neonates were anaesthetised by cooling on ice until the cessation of hands movement. After skin incision thoracotomy at the fourth intercostal space was performed. Visualise the LAD and pass an 11-0 nylon suture below it near the left auricle, then tied to induce infarction. After LAD ligation, neonates were removed from the ice bed and warmed at 37°C. Thoracic wall incisions and the skin wound were closed with 8-0 nylon sutures. Sham-operated mice underwent the same procedure involving hypothermic anaesthesia and thoracotomy without LAD ligation. After surgery, neonates were warmed until recovery. The total numbers of mice used for surgery were 200, the survival numbers were 183. Survival rates in our protocol were above 90% both in sham-operated and MI mice. Among the 183 survival mice, the numbers of successfully used models were about 175. In our previous research, the average left ventricular ejection fraction at P3 after P1 MI is 50.8% with 95% confidence interval of 36.3–65.3%. The average left ventricular ejection fraction at P3 after P1 Sham is 87.3% with 95% confidence interval of 82.3–92.4%. P1 MI mice or sham mice with the ejection fraction in the corresponding range could be defined as successful.

Transthoracic echocardiography

Cardiac function was evaluated by 2-dimensional echocardiography using a VEVO-1100 machine (Visual Sonics) on anaesthetized mice. M-mode tracings were used to measure left ventricular internal diameter (LVID) in either diastole or systole, which were used to calculate left ventricular fractional shortening and ejection fraction.

Dexamethasone treatment

Dexamethasone (Dex, Sigma, St. Louis, MO, USA) in sterile saline was intraperitoneally administered daily from the first day of myocardial infarction to the seventh day [10–12]. The control mice were treated with same volume sterile saline as that of Dex.

Histology and immunostaining

Mice were sacrificed at various stages following surgery. Dissected hearts were rinsed in warm PBS, absorbed excess fluid, weighed, and then fixed overnight in 4% paraformaldehyde. After washing, hearts were embedded whole in paraffin and sectioned. Hematoxylin and eosin (HE) staining and Masson's trichrome were performed on deparaffinized heart sections according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, MO, USA).

Immunofluorescence

Sections were deparaffinized in xylene and rehydrated in ethanol before antigen retrieval in sodium citrate solution. Blocking was performed in 1% BSA (Roche, Mannheim, Germany) for 30 min, and tissue sections were incubated overnight at 4°C with mouse monoclonal antibody cTNT (1:100 dilution; Abcam, Cambridge, MA, USA) or rat monoclonal antibody BrdU (1:100 dilution; Abcam). This was followed by incubation with the corresponding Alexa Fluor-conjugated secondary antibodies for 1h at room temperature. Sections were then stained with DAPI (Invitrogen, Carlsbad, CA, USA), and photographed by fluorescence microscopy or confocal microscope.

In vivo BrdU pulse-chase labelling experiments

For detection cardiomyocyte proliferation *in vivo*, 50 mg/kg of BrdU (Sigma) was injected intraperitoneally one day before collecting the hearts [13, 14]. Hearts at P7 after surgery were harvested and processed histologically as described above. Rat polyclonal antibody against BrdU (1:100 dilution; Abcam) was used for immunofluorescence.

qPCR

Total RNA was isolated using Trizol (Takara Bio, Otsu, Japan). cDNA was synthesised using RNA PCR Kit (Takara Bio). qPCR amplification was performed with an ABI PRISM 7900 Sequence Detector system (Applied Biosystem, Foster City, CA, USA). All processes were according to the manufacturer's instructions. Relative gene expression was normalised to *Gapdh*.

Statistical analysis

All data are presented as means ± SEM. All data obtained from various experiments followed a normal distribution. Means comparisons between two groups were analysed using an unpaired two-tailed *t*-test. Means comparisons between more than two groups were analysed using one-way ANOVA. *P* values of less than 0.05 were considered significant. Statistical analyses and figures were carried out using the GraphPad Prism software.

Results

Ventricular dysfunction, myocardial infarction, and cardiomyocyte proliferation were confirmed after P1 neonatal mice LAD ligation

To explore the inflammation process of neonatal mice myocardial infarction model, we firstly identified what happened at different time points of this model. P1 mice heart LAD was visualised and ligated under the surgery microscope (Fig. 1A). Then at P3, the heart showed a relatively pale area under the ligation knot (Fig. 1B), which indicated heart injury in the coronary blood supply region. Echocardiography was used to evaluate the heart function at P3 and found significantly decreased EF in P1 ligation group compared with sham group (Fig. 1C). Immunofluorescent staining of cardiomyocyte marker cardiac Troponin T (cTnT) showed great scar absent of cardiomyocytes after LAD ligation (Fig. 1D), which indicated myocardial infarction after P1 mice LAD ligation. Cardiomyocyte proliferation at P7 significantly increased after P1 LAD ligation (Figs. 1E and F). Thus, LAD ligation in P1 mice resulted in myocardial infarction, which may cause inflammation, impaired heart function, and cardiomyocyte proliferation.

Nearly complete regeneration ability of P1 mice hearts and little regeneration ability of P7 mice hearts

Myocardial infarction in neonatal may lead to inflammation, however, whether these hearts could fully regenerated is still controversial. We then try to determine the regeneration ability of postnatal mice. P1 sham-operated and P1 MI mice underwent echocardiography and heart Masson's trichrome staining at P28. Although there is still limited fibrosis both in ligation site and ischemia region after P1 mice MI (Fig. 2A), the cardiac function and weight were comparable with sham-operated mice (Figs. 2B and C). However, the P7 MI mice had a huge scar of fibrosis and reduced heart function at P28 (Fig. 2D). These results indicated that MI leads to full regeneration in P1 mice, but leads to fibrosis in P7 mice.

An acute inflammatory response was induced by neonatal mice myocardial infarction

Inflammation is often implicated in necrosis clearance and tissue repair. We next sought to explore the inflammation process after neonatal mice MI. HE staining of P3 hearts after P1 MI showed a large number of inflammatory cells in infarcted area (Fig. 3A). With the recovery of the heart, inflammatory cells and the infarcted area gradually decreased thereafter (Fig. 3A). Representative inflammatory factors expression such as *TNF-α, IL-1β, Ccl2, IL-6, Cxcl1, IL-17* and *IL-23* all changed during this process (Fig. 3B). The expression levels of all these inflammatory factors peaked at P3 except that *IL-17* level peaked at P7.

Dex inhibited inflammation and neonatal mice heart regeneration

To explore the role of inflammation in neonatal mice heart regeneration, different concentrations of Dex (0.1 mg/kg, 0.5 mg/kg, 1 mg/kg) was used to inhibit inflammation and Dex at 0.5 mg/kg or 1mg/kg could significantly inhibit the expression of inflammatory factors, especially at P3 and P7 (Fig. 4A). However, Dex at 1 mg/ml lead to mice death at a rate of about 40%. Then we used Dex at 0.5 mg/kg for heart regeneration evaluation at P28. Hearts from Dex group showed more considerable scar of fibrosis and reduced heart function compared with the control group at P28 (Figs. 4B and C). These results demonstrated that cardiac regeneration was impaired by inflammatory inhibition.

Discussion

Cardiac regeneration is a major scientific and medical objective worldwide. The neonatal mice heart LAD ligation model seems to introduced a revolutionary new prospect to study the mechanisms of endogenous mammalian cardiac regeneration. In this study, we demonstrated that inflammation might play a key role in neonatal mice heart regeneration after MI.

To explore the role of inflammation in neonatal mice LAD ligation model, we firstly confirmed myocardial

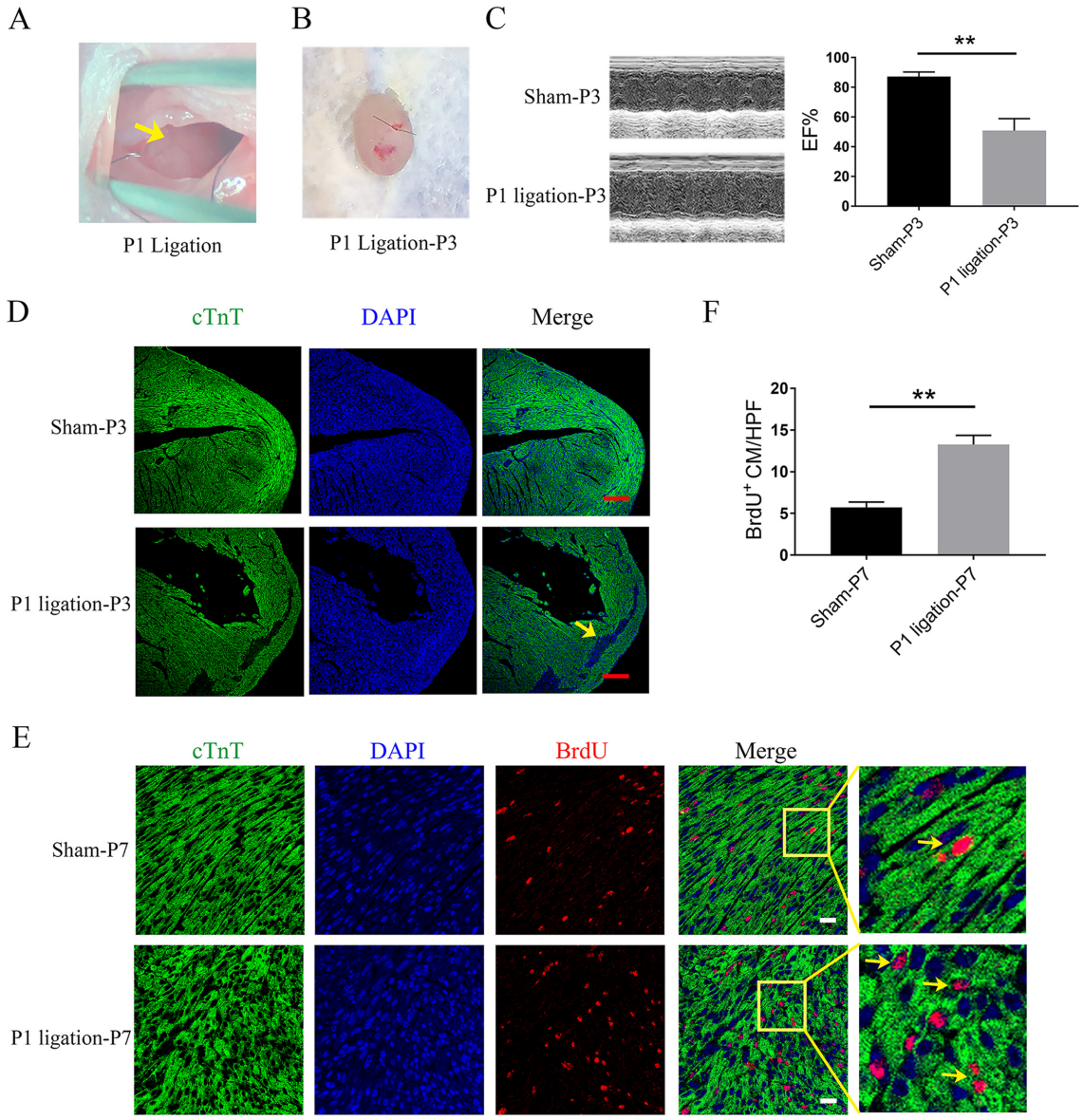

Fig. 1. Left anterior descending coronary artery (LAD) ligation in postnatal day 1 (P1) mice induces ventricular dysfunction, myocardial infarction (MI), and cardiomyocytes proliferation. (A) Arrowhead shows the ligation site of LAD of P1 mice. (B) P3 heart shows the ligated site and infarcted area. The upper red part is the ligation site. The lower red part is the site where the infarct area contacts with the chest wall. The infarct area is relatively pale. (C) Representative images of M-mode echocardiography of P3 mice after P1 sham-operation or P1 LAD ligation. Ejection fraction (EF) of the heart was calculated. $^{**}P<0.01$, n=4 for each group. (D) Representative images of cardiomyocytes death at P3 after P1 LAD ligation. Arrowhead indicates the infarcted area of the heart. Cardiomyocytes were stained for cardiac Troponin T (cTnT), and nuclei with DAPI. Scale bars, 200 μm. (E) Representative immunofluorescent staining images show cardiomyocyte proliferation at P7 after P1 LAD ligation BrdU+ cardiomyocytes were indicated (arrowheads). Cardiomyocytes were stained for cTnT, and nuclei with DAPI. Scale bars, 50 μm. (F) Quantification of BrdU+ cardiomyocytes (CM). HPF: high power field, n=4 for each group. $^{**}P<0.01$.

infarction after neonatal mice LAD ligation, which may trigger subsequent inflammation. Our results confirmed cardiomyocytes death in both sites of ligation and the below area supplied by LAD. These indicated that the damage was caused by not only mechanical injury but also ischemia injury, which is similar to adult mice LAD ligation. A recent study compared the injury of the heart between P1 and P7 mice LAD ligation using TUNEL staining [15]. The TUNEL positive area in their study showed apoptotic cells while our cTnT negative area showed dead cardiomyocyte cells. These two methods showed similar injured areas after P1 mice LAD ligation. Impaired heart function in P3 mice showed a simultaneous change in morphology and function. Then cardiomyocyte proliferation which may account for heart repair after an injury was also confirmed in our study as others

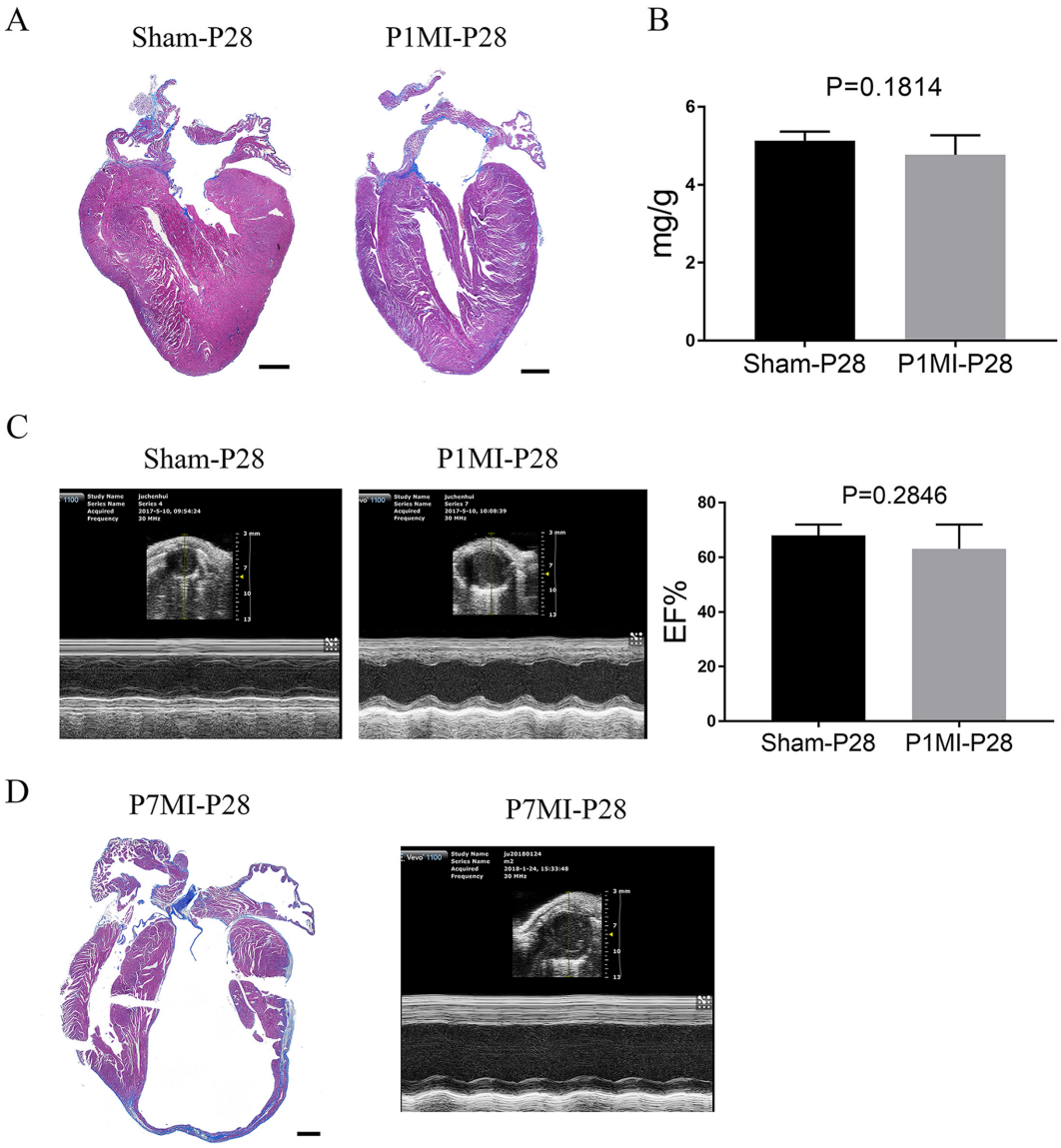

Fig. 2. P1 mice hearts show nearly full regeneration while P7 mice hearts show little regeneration after LAD ligation. (A) Representative images of Masson's trichrome staining of P28 mice heart after P1 sham-operated or P1 MI. Hearts were longitudinally cut. Scale bar: 0.5 mm. (B) Heart weight to body weight ratio at P28. (C) Representative images of M-mode echocardiography and EF values at P28, n=5 for each group. (D) Masson's trichrome staining and echocardiography of P28 mice after P7 MI. Scale bar: 0.5 mm.

[16]. It has been reported that c-kit+ precursors support postinfarction myogenesis in the neonatal [6]. However, genetic fate mapping indicated that the majority of cardiomyocytes within the regenerated tissue originated from preexisting cardiomyocytes [16].

Although this neonatal mice heart model has been widely used since it was introduced in 2012 [13, 17, 18], there is still controversy on whether the heart can fully regenerate after inflammation [6, 7]. Then we sought to determine the extent of regeneration at P28 after P1 LAD ligation. Results showed limit fibrosis both in ligation site and the ischemic area, while the cardiac function recovered to the normal level. A contrary study reported a transmural infarct in heart and considered this model as incomplete regeneration [7]. Although a little fibrosis still existed in note site and ischemia area, the neonatal heart has almost completely functional and morphological recovery after injury. It is appropriate to call it complete regeneration. Of all the operated mice, we did not observe as large fibrosis as those reported [6, 7].

We next used this model to explore the role of inflammation response in heart regeneration. Results showed immediately rise and then quickly decrease of inflammation after P1 mice LAD ligation, which is in line with adult heart repair procedure [8]. According to the inflammatory factors, we speculated that acute sudden death of cardiomyocytes in the infarcted heart firstly triggers neutrophils to clear the dead cells and extracellular ma-

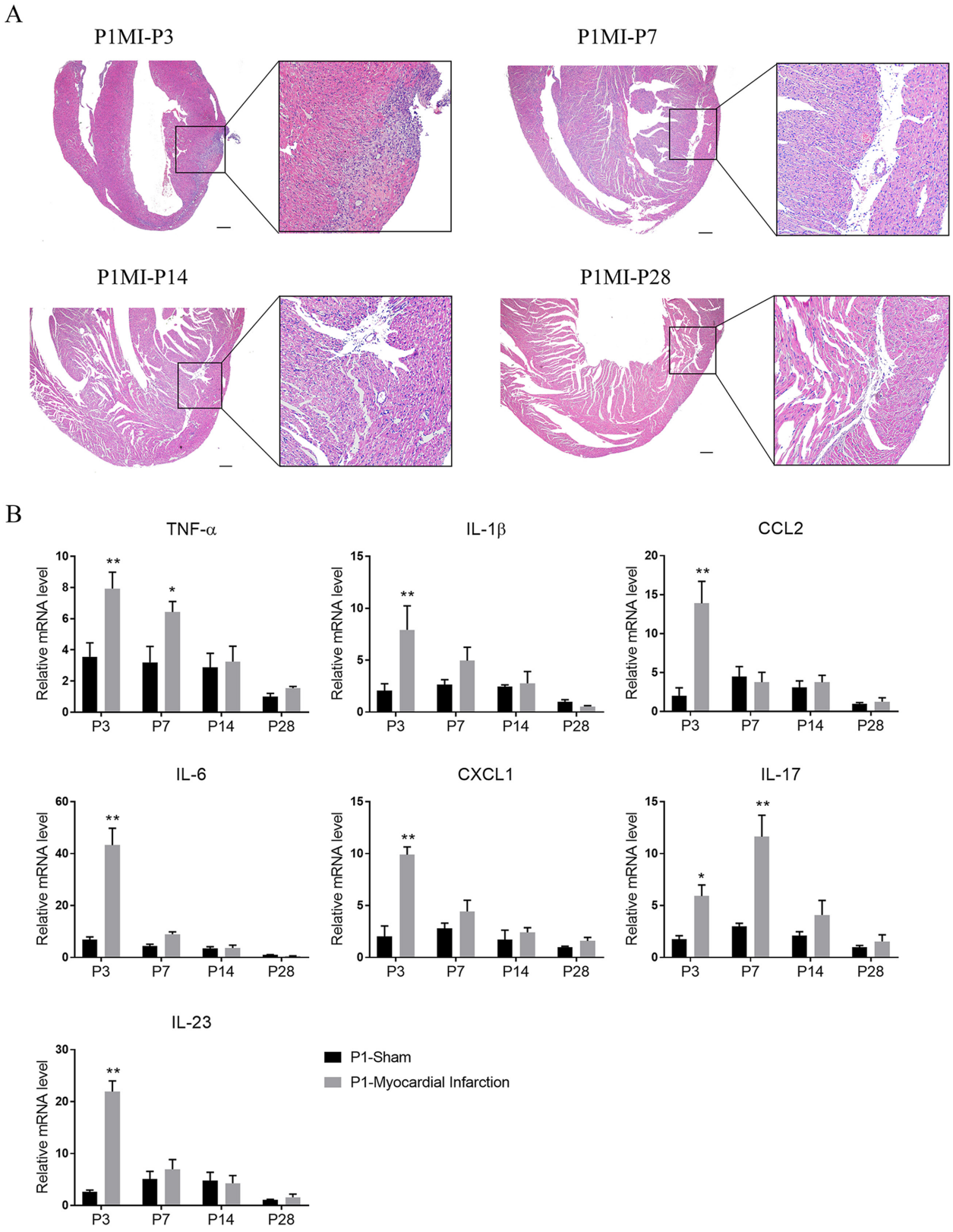

Fig. 3. Time-course of the acute inflammatory response in mouse hearts after P1 MI. (A) Hematoxylin and eosin (HE) staining of P3, P7, P14 and P28 hearts after P1 MI. Representative images of low and high magnification views are shown. Scale bars, 200 μm. (B) qPCR assays of the expression of inflammatory markers (*TNF-α, IL-1β, Ccl2, IL-6, Cxcl1, IL-17* and *IL-23*) in P3, P7, P14 and P28 heart tissues after P1 MI, n=5 per group, P1-Sham P28 group was set as control, **P*<0.05, ***P*<0.01 compared with P1-Sham group at the same time point.

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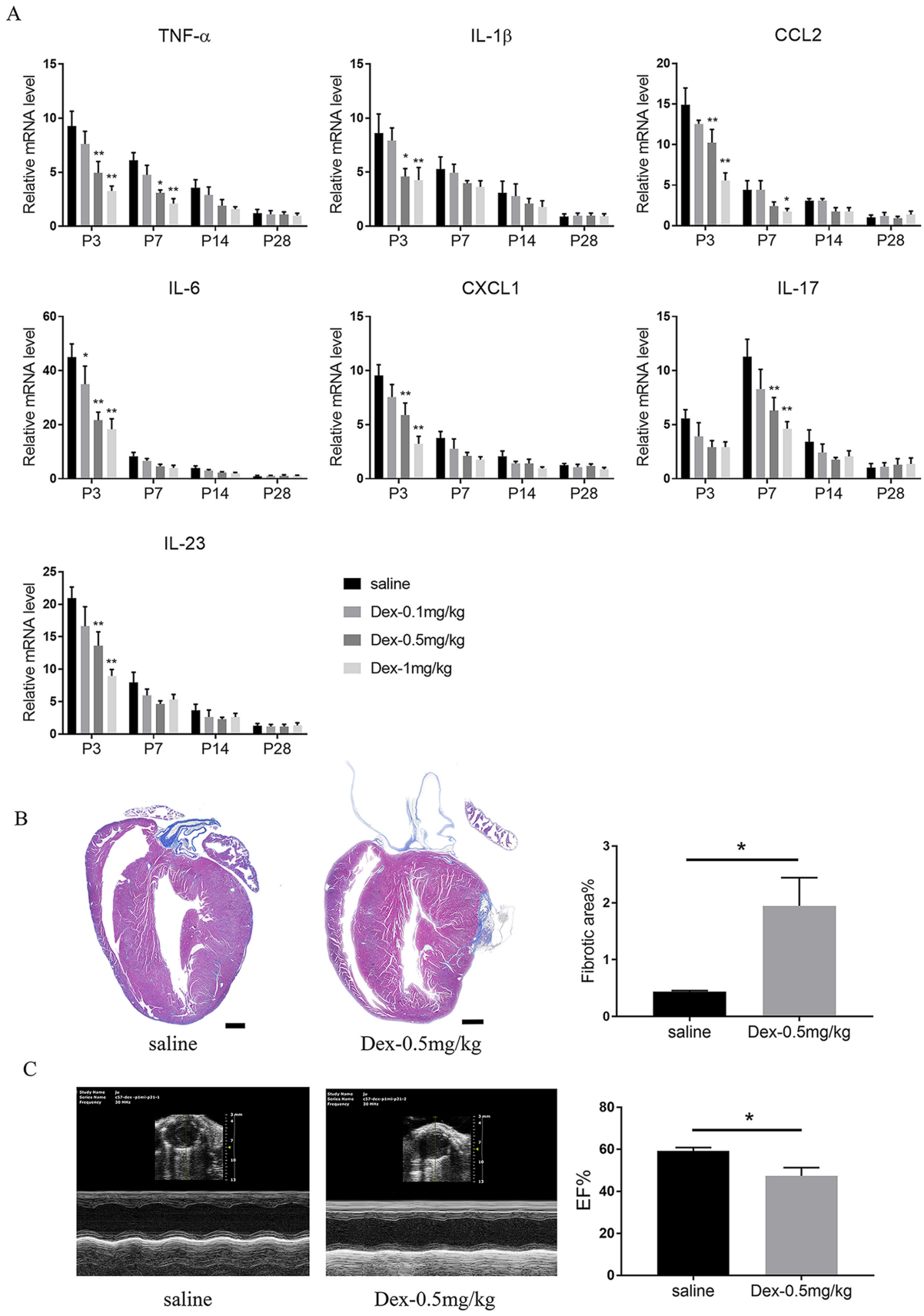

Fig. 4. Inflammation inhibition by Dex impairs hearts regeneration after P1 LAD ligation. (A) Expression of inflammatory factors at P3, P7, P14, P28 with different concentration of Dex treatment after P1 LAD ligation. n=3–5 per group, saline P28 group was set as control, **P*<0.05, ***P*<0.01 compared with each saline group. (B) Masson's trichrome staining of P28 hearts from saline and Dex group. Scale bar: 0.5 mm. Statistical graph showed the percentage of blue area in left ventricle. n=5 per group, **P*<0.05. (C) Echocardiography and ejection fraction (EF) of each group are shown, n=5 per group, **P*<0.05.

trix debris. Then mononuclear cell and macrophage subpopulations secrete growth factors that promote cardiomyocyte proliferation, angiogenesis, and inflammation reservation. T and B lymphocytes also participant in inflammatory regulation. Studies about the role of immune cells in adult mice myocardial infarction may give us some hints. B cells have been reported to trigger monocyte mobilization [19]. Treg cells have been reported to inhibit proinflammatory cytokines production by cardiomyocytes [20]. Th17 cells exhibit protective effects against cardiac fibrosis after myocardial infarction [21]. The activity of the *IL-23*/*IL-17* pathway has been reported to play an important role in remodeling after ischemia-reperfusion injury and acute myocarditis [22]. *IL-17A* is essential for the progression of dilated cardiomyopathy [23]. These factors may also play vital roles in neonatal mice MI.

Dex was reported to bind to glucocorticoid receptor and inhibit inflammation through genomic effects and nongenomic mechanisms [24]. Thus, we used different concentrations of Dex daily from P1 to P7 to inhibit inflammation and found Dex at 0.5mg/kg could significantly and appropriately inhibit the expression of inflammatory factors. According to the expression of inflammatory factors, it seems that the cell types do not change due to Dex administration. Dex at 0.5 mg/kg could also impair cardiac regeneration. These results demonstrated that inflammation promoted heart regeneration rather than inhibition. The role of inflammation in heart regeneration has been investigated in zebrafish which suggest an early *Jak1/Stat3* injury response was required for zebrafish heart regeneration [25]. The central inflammatory cells macrophages are also required for neonatal heart regeneration in neonatal mice MI model [17]. Another study reported that inflammatory was required for neonatal mice heart regeneration after apex resection [10]. However, the apex resection may not induce as strong inflammation as LAD ligation in the heart due to different pathophysiology of them. Inflammation may also play distinct roles in these two models.

In conclusion, we identified inflammation might play a key role in neonatal mice heart regeneration. This may be beneficial for further development of more specific biomarker-based therapies to enhance cardiac regeneration in the adult human heart.

Conflicts of Interest

The authors declare no conflicts of interest in association with the present study.

Acknowledgments

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