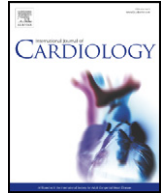




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Dual roles of calpain in facilitating Coxsackievirus B3 replication and prompting inflammation in acute myocarditis

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ABSTRACT

Background: Viral myocarditis (VMC) treatment has long been lacking of effective methods. Our former studies indicated roles of calpain in VMC pathogenesis. This study aimed at verifying the potential of calpain in Coxsackievirus B3 (CVB3)-induced myocarditis treatment.

Methods: A transgenic mouse overexpressing the endogenous calpain inhibitor, calpastatin, was introduced in the study. VMC mouse model was established via intraperitoneal injection of CVB3 in transgenic and wild mouse respectively. Myocardial injury was assayed histologically (HE staining and pathology grading) and serologically (myocardial damage markers of CK-MB and cTnI). CVB3 replication was observed in vivo and in vitro via the capsid protein VP1 detection or virus titration. Inflammation/fibrotic factors of MPO, perforin, IFN γ , IL17, Smad3 and MMP2 were evaluated using western blot or immunohistology stain. Role of calpain in regulating fibroblast migration was studied in scratch assays.

Results: Calpastatin overexpression ameliorated myocardial injury induced by CVB3 infection significantly in transgenic mouse indicated by reduced peripheral CK-MB and cTnI levels and improved histology injury. Comparing with CVB3-infected wild type mouse, the transgenic mouse heart tissue carried lower virus load. The inflammation factors of MPO, perforin, IFN γ and IL17 were down-regulated accompanied with fibrotic agents of Smad3 and MMP2 inhibition. And calpain participated in the migration of fibroblasts in vitro, which further proves its role in regulating fibrosis.

Conclusion: Calpain plays dual roles of facilitating CVB3 replication and inflammation promotion. Calpain inhibition in CVB3-induced myocarditis showed significant treatment effect. Calpain might be a novel target for VMC treatment in clinical practices.

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1. Introduction

Viral myocarditis (VMC) is a common cardiovascular disease in clinical practice [1]. While many VMC cases showed a self-limited regression, there are still approximately 30% cases progressing into dilated cardiomyopathy (DCM) and heart failure that might require mechanical circulatory support or heart transplant at the end stage despite of the optimal pharmacotherapy [1,2]. So an effective treatment of VMC in the early stage is meaningful.

VMC treatment has always been a challenge, especially with regard to the immunosuppressive treatment [2]. Many researchers considered

VMC an autoimmune disease [3], which made immunosuppression treatment reasonable. However, virus persistence in the myocardium [1,4] holds back the intended host immune suppression because this might enhance virus replication. Clinical observations of myocarditis immunosuppression therapy also returned controversial results [1]. As such, guidelines also weight the words on this issue [2].

This ambiguity might be attributed to several reasons. 1) The core concern might be that it is a risk to prescribe immunosuppressant agents considering the role of host immune responses in virus clearance. 2) Besides, we haven't completely clarified the life cycle of the intruded-viruses. So we don't know the timing to interfere the disease course. 3) The patterns that viruses interact with hosts were basically vague. So we don't know the appropriate methods to interfere. 4) Last but not least, myocarditis-causing viruses' spectrum has always been shifting temporally and regionally [5], which adds another variable for clinical specialists when facing a unique patient suffering from VMC.

Thus, logically speaking, a candidate compromising these two factors, virus replication and host inflammation response, might be an

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optimal choice. We found in our former study that calpain, a family of neutral cysteine proteases, could facilitate Coxsackievirus B3 (CVB3), a common causing virus of VMC replication *in vitro* [6]. Besides, many studies demonstrated that calpain mediates systemic inflammation response *in vivo* [1]. So, calpain might participate both in the virus life cycle and the host inflammation response simultaneously. If it is the case, calpain would be an optimal choice of myocarditis treatment.

Calpain activity is regulated by the endogenous inhibitor, calpastatin. Calpastatin is an intracellular 110-kDa protein that inhibits calpain activity specifically. Binding of calpastatin across the calpain active site would block its access to substrates *in vivo*. However, calpastatin could not penetrate across the cell membrane for the large molecular weight and cannot be used as an inhibitor *in vitro*. Thus, we introduced here a transgenic mouse overexpressing calpastatin *in vivo* to discuss this hypothesis in the mouse model of CVB3-induced viral myocarditis.

2. Materials and methods

2.1. Virus and reagents

Coxsackievirus B3 (Nancy strain) was preserved in Key Laboratory of Viral Heart Diseases, Zhongshan Hospital, Fudan University.

Calpain inhibitor I (N-acetyl-Leu-Leu-Norleucinal, ALLN), an inhibitor of neutral cysteine proteases, was purchased commercially (BIOMOL Research Laboratories Inc. USA, Catalog NO.: P-120) and dissolved in dimethyl sulfoxide (DMSO, AMRESCO LLC., USA, Product Code: N182) with a final concentration of less than 0.1% in cell culture studies. ALLN was further diluted using culture medium into target concentrations during the research.

2.2. Mice

The calpastatin transgenic mouse strain (Tg-CAST) was introduced from the laboratory of Tianqing PENG (Lawson Health Research Institute, Canada) and was bred in the Department of Laboratory Animal Science, Fudan University in a standard specific pathogen free (SPF) environment. Male inbred C57BL/6 mice aging 3–6 weeks were used for the experiment and were grouped as: wild type control group (Con. n = 10), transgenic mouse control group (Tg-CAST, n = 10), CVB3-infected group (Virus n = 15) and CVB3-infected transgenic mouse group (Virus + Tg-CAST, n = 15). All littermates were genotyped following the protocol of PENG's lab. We give assurance that all animals received humane care and that study protocols was approved by the Ethical Committee of Fudan University.

2.3. Animal model establishment

The CVB3-induced viral myocarditis model was established following the protocol as previous [7]. In short, 100,000 TCID₅₀/ml × 0.3 ml CVB3 was injected intraperitoneally with the same amount of PBS for the controls. Then, all mice were kept in SPF environment for 7 days before sacrifice. Occasional dead mouse were excluded from the study. Blood samples were collected via picking the eye balls and serum were separated via centrifuge (3000 r/min for 5 min).

2.4. Tissue histopathology and myocarditis grading

Methods and grading standards were prescribed elsewhere [7].

2.5. ELISA assay

Level of peripheral cTnI and CK-MB were measured by ELISA assays following the protocol in former study [6].

2.6. Western blot analysis

Heart tissue were grinded using homogenizer in lysis buffer (50 mM Tris-HCl, pH 7.2, 250 mM NaCl, 0.1% NP-40, 2 mM EDTA, 10% glycerol) containing protease inhibitor cocktail on ice. Tissue homogenate were centrifuged at 12,000 g for 10 min at 4 °C. The protein concentration was determined by the Bradford assay (Bio-Rad). Detailed western blot protocol was described elsewhere in our former study [6]. In brief, equal amounts of protein were loaded for SDS gel electrophoresis and then transferred to nitrocellulose membranes. After block, the blots were incubated overnight at 4 °C with the primary antibody of α -fodrin (Enzo Life Sciences, USA, Catalog NO.: FG 6090, 1:1000), enterovirus capsid protein VP1 (Leica Biosystems Newcastle Ltd., United Kingdom, Catalog NO.: NCL-ENTERO, 1:500), perforin (Cell Signaling Technology, Inc. USA, Catalog NO.: #3693, 1:1000), IFN γ (Bio Legend Inc. USA, Catalog NO.: 505811, 1:1000), IL17 (Abcam Inc. USA, Catalog NO.: ab79056, 1:1000), Smad3 (Santa Cruz Biotechnology, Inc. USA, Catalog

NO.: sc-101154, 1:1000) or MMP2 (Bioworld Technology, Inc., USA, Catalog NO.: BS1236, 1:500) respectively followed by incubation for another hour with the corresponding secondary antibody (1:4000). Bands were visualized by ECL (Super Signal West Pico kit; Pierce) and quantified by densitometry using Quantity One software (Bio-Rad). GAPDH was used as internal reference.

2.7. Viral titration

Virus titer was measured using TCID₅₀ assay in HeLa cells. Heart tissue homogenates were prepared at 4 °C using ice cold homogenizer followed by centrifugation to harvest virus-containing supernatant. Decimal serial dilutions of cell culture supernatant or tissue homogenate supernatant were added to the cultured HeLa cells and incubated for 1 h at 37 °C. Then the infected HeLa cells were cultured at 37 °C. Cytopathic effect was observed daily for 7 days. And TCID₅₀ were calculated using Reed–Muench method.

2.8. MMP activity assay

MMP activity was detected according to the instruction of the commercial assay kit (AnaSpec, Inc., CA, Catalog NO.: 71151). Tissues homogenate in assay buffer were centrifuged for 15 min at 10,000 g at 4 °C followed by incubation with APMA at a final concentration of 1 mM in the assay buffer for 1 h at 37 °C. Activate MMP immediately before the experiment. Dilute MMP-2 substrate 1:100 in assay buffer. Add 50 μ l per well of sample and MMP2 substrate solution in 96-well plate. Mix the reagents by shaking the plate gently for 30 s. Incubate the reaction at 37 °C for 50 min. Keep plate away from direct light. Add 50 μ l per well of stop solution. Mix the reagents and measure fluorescence intensity at Ex/Em = 490/520 nm. Plot data as RFU fold change comparing with the control.

2.9. Immunohistochemistry

Immunohistochemistry staining of VP1 (Leica Biosystems Newcastle Ltd., United Kingdom, Catalog NO.: NCL-ENTERO, 1:200), IFN γ (Bio Legend Inc. USA, Catalog NO.: 505811, 1:500) and IL17 (Abcam Inc. USA, Catalog NO.: ab79056, 1:500) were carried out according to the method prescribed elsewhere [7].

2.10. Myeloperoxidase (MPO) activity assay

MPO activity was assayed using commercial kit (Sigma-Aldrich Co. LLC, USA, Catalog NO.: MAK069). Standard curve was prepared according to the instruction. Then tissue samples (10 mg heart tissue + 5 volume MPO assay buffer) were prepared using MPO assay buffer followed by making master reaction mix. At room temperature, the fluorescence intensity was read and MPO activity was calculated according to the instruction.

2.11. Cells culture and cardiomyocytes infection

Primary cardiomyocytes were separated from neonatal rat heart. In short, neonatal rat (1–3 days old, supplied by Department of Laboratory Animal Science, Fudan University) were fertilized and hearts were taken out quickly, sliced into pieces and digested in 0.1% trypsin at 37 °C for 10 min and centrifuged at 3000 r/min to collect the digested cells and suspended the cells in DMEM. Repeat the digest-centrifuge cycles until the left tissue become apparent. The collected cells were suspended in DMEM containing 10% FBS and cultured in 37 °C for 2 h. The adherent cells are fibroblast and the still suspending cells are primary cardiomyocytes. Then the cardiomyocytes were counted and seeded for research. The fibroblasts were passaged as routine and the third generation of cells was used for the study.

Prepared cardiomyocytes were fasted in DMEM overnight followed by inoculation with CVB3 for 1 h in 37 °C. Then the supernatant were changed into DMEM containing 10% FBS and cultured in 37 °C for the designed time course.

2.12. Fibroblast scratch assay

Fibroblasts were seeded into 6-well plate at the density of 1×10^5 cells/well and cultured for 48 h. Then the cells were synchronized by fasting in DMEM without FBS for 24 h. Scratch injury were made using the tips of 200 μ l pipets. Then DMEM containing 10% FBS were changed with or without 5 μ g/ml of calpain inhibitor ALLN (BIOMOL Research Laboratories Inc., USA, Catalog NO.: P-12) and cultured for another 24 h. Then pictures of the scratch trace were taken and the distance were measured and analyzed.

2.13. Statistical analysis

Data were specified as mean values \pm SD and analyzed using SPSS software (version 16.0; SPSS, Inc., Chicago, IL, USA). 2-Group comparison was performed using Student t test. $P < 0.05$ was considered statistically significant.

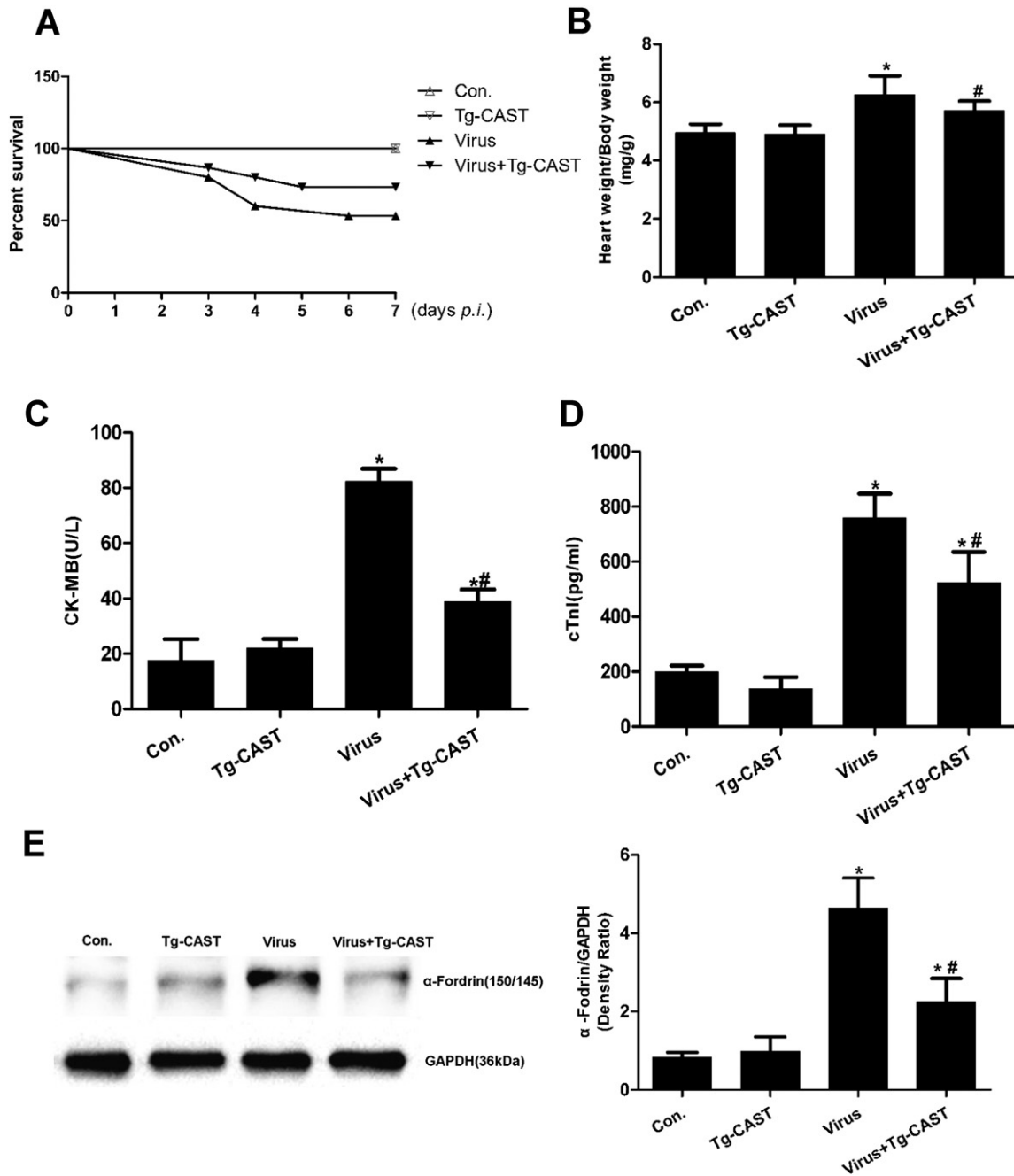


Fig. 1. Calpain inhibition ameliorated CVB3-induced myocardial injury. A: Survival curve of the mouse in different groups. B: Heart weight/body weight ratio of different groups of mouse. * $P < 0.05$ vs. Con. group; # $P < 0.05$ vs. Virus group. C, D: ELISA assays of the peripheral myocardial damage markers of CK-MB and cTnI. * $P < 0.05$ vs. Con. group; # $P < 0.05$ vs. Virus group. E: Western blot of calpain cleavage product of α -fodrin. * $P < 0.05$ vs. Con. group; # $P < 0.05$ vs. Virus group. All experiments were repeated for 3 times unless specification. Con.: the wild type control mouse group; Tg-CAST: the transgenic mouse group; Virus: the virus infection of wild type mouse group; Virus + Tg-CAST: the virus infection of transgenic mouse group.

3. Results

3.1. Calpastatin overexpression ameliorates CVB3-induced myocardium injury

Transgenic mouse had lower mortality post CVB3 challenge (Fig. 1A). Calpain inhibition decreased the HW/BW ratio significantly (Fig. 1B). And myocardial damage markers of CK-MB and cTnI were also decreased (Fig. 1C, D) in the peripheral blood indicating the protective effect of calpastatin overexpression. To make certain the calpain activity in the transgenic mouse, the degradation product of

calpain-specific substrate, α -fodrin, was blotted and as expected, the calpain activity was down-regulated in the transgenic mouse heart tissue (Fig. 1E).

3.2. Calpain activity is involved in CVB3 replication in vivo and in vitro

To observe the role of calpain activity in CVB3 replication, CVB3 capsid protein VP1 was blotted. It showed that comparing with wild type mouse CVB3-infected transgenic mouse had a lower level of VP1 expression in the myocardium (Fig. 2A). VP1 detection with immune histology method also showed that virus capsid protein decreased

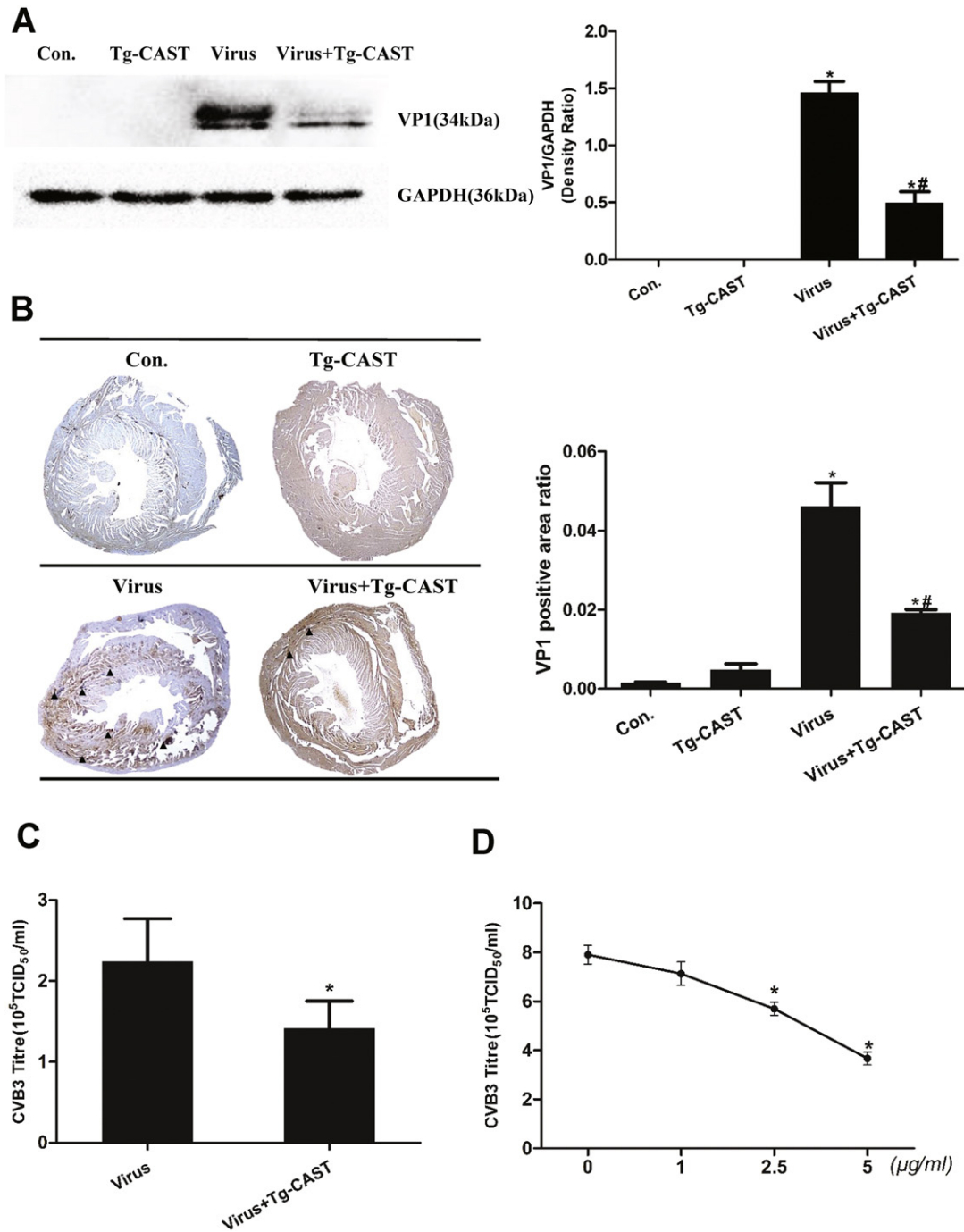


Fig. 2. Calpain activity was involved in CVB3 replication. **A:** western blot of CVB3 capsid protein VP1. * $P < 0.05$ vs. Con. group; # $P < 0.05$ vs. Virus group. **B:** immunohistology stain of CVB3 capsid protein VP1 in heart tissue. Black triangles indicated the positive staining of VP1. Histogram showed VP1 positive area/total tissue area ratio. * $P < 0.05$ vs. Con. group; # $P < 0.05$ vs. Virus group. **C:** virus load titration in heart tissue. $n = 5$ for each group. * $P < 0.05$ vs. Virus group. **D:** titration of CVB3 in vitro without or with ALLN of various dilutions (5 μ g/ml, 2.5 μ g/ml and 1 μ g/ml). * $P < 0.05$ vs. normal controls without ALLN. All experiments were repeated for 3 times unless specification.

significantly (Fig. 2B). Virus load in heart tissue were titrated and it demonstrated that virus titer were lower in the heart of transgenic mouse (Fig. 2C).

In further, we verify this finding in vitro. Neonate rat cardiomyocytes were separated accordingly. Post virus infection, the cells were inoculated with culture medium containing calpain inhibitor ALLN of various dilutions (5 μ g/ml, 2.5 μ g/ml and 1 μ g/ml) respectively. Cardiomyocytes inoculated with culture medium without ALLN were set as normal control. And 12 h later, virus load in the supernatant were titrated. We found that comparing with

the normal control 2.5 and 5 μ g/ml of ALLN hindered virus replication significantly (Fig. 2D).

3.3. Calpain inhibition ameliorates inflammation infiltration in CVB3-induced myocarditis

To observe the effect of calpastatin over-expression on CVB3-induced myocardial inflammation, tissue sections were HE stained. Comparing with wild littermates, inflammation infiltration in transgenic mouse heart tissues was ameliorated significantly (Fig. 3A). MPO, the marker

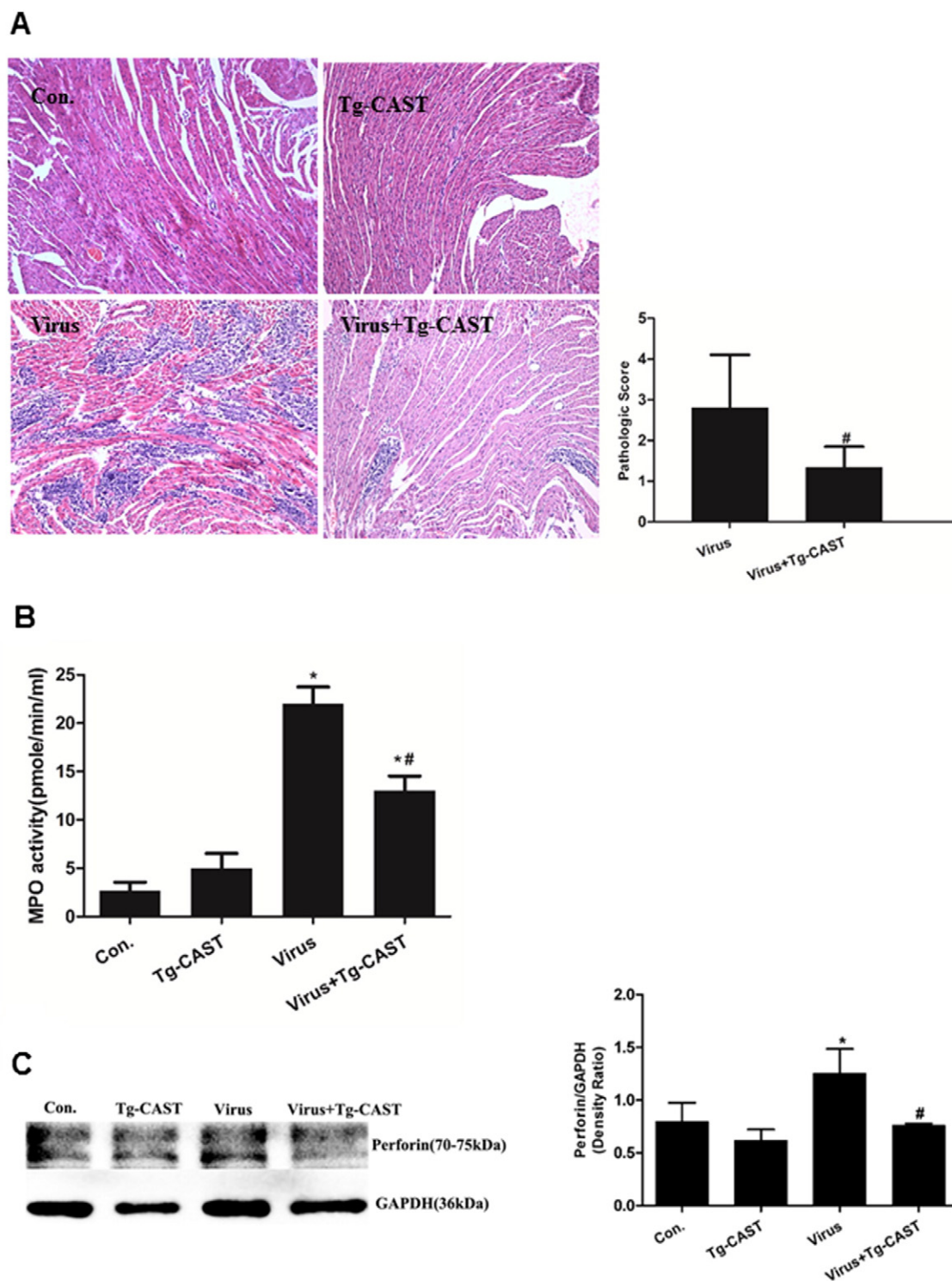


Fig. 3. Calpastatin overexpression reduced myocardial inflammation infiltration. A: Representation pictures of heart sections HE staining. Blue stained areas indicated inflammatory cell infiltration. Histogram showed the pathologic grade data. $^{\#}P < 0.05$ vs. Virus group. B: neutrophil MPO activity assay. $^*P < 0.05$ vs. Con. group; $^{\#}P < 0.05$ vs. Virus group. $n = 4$ for each group. C: Western blot of perforin. $^*P < 0.05$ vs. Con. group; $^{\#}P < 0.05$ vs. Virus group. All experiments were repeated for 3 times unless specification.

of neutrophils infiltration, was also decreased in transgenic mouse hearts (Fig. 3B). Perforin is the effective protein of cytotoxic lymphocytes and natural killers. In the transgenic mouse's heart, calpain inhibition was accompanied with significant perforin down-regulation post virus infection (Fig. 3B). This was also the case for the inflammation factors of IFN γ (Fig. 4AB) and IL17 (Fig. 4CD), which demonstrated the role of calpain in mediating inflammation infiltration in virus-induced myocarditis.

3.4. Calpain activity was involved in myocardial fibrosis in viral myocarditis

Fibrosis is the key pathophysiology course in myocarditis progression. Thus, we observed here whether calpain participated in fibrosis in CVB3-induced myocarditis. The classic fibrotic agents of smad3 (Fig. 5A) and MMP2 (Fig. 5B) were blotted and it

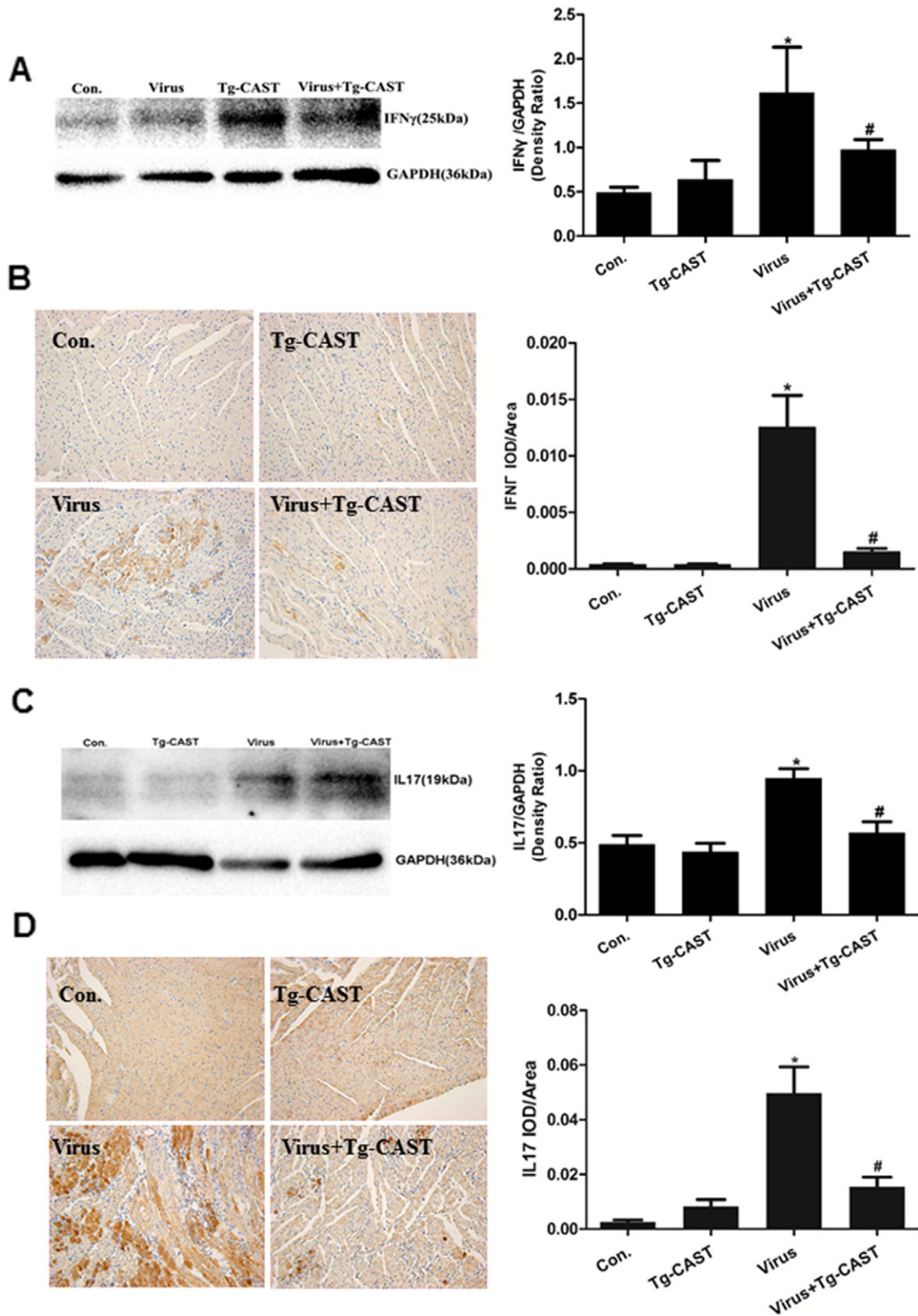


Fig. 4. Calpain participated in the regulation of IFN γ and IL17 production. A, C: western blot of IFN γ and IL17 respectively. * $P < 0.05$ vs. Con. group; # $P < 0.05$ vs. Virus group. B, D: Representation pictures of the heart sections immunohistology stain of IFN γ and IL17 respectively. Dark-brown area indicated the positive stained proteins. Histogram showed the pathologic grade data. * $P < 0.05$ vs. Con. group; # $P < 0.05$ vs. Virus group. All experiments were repeated for 3 times unless specification.

demonstrated that calpain inhibition resulted in the down-regulation of the two factors as well as the total MMPs activity (Fig. 5C).

To observe if calpain could regulate myocardial fibroblasts function, we proceed scratch assays with fibroblast. Cultured fibroblasts were incubated with the calpain inhibitor ALLN (5 $\mu\text{g/ml}$) for 24 h with a

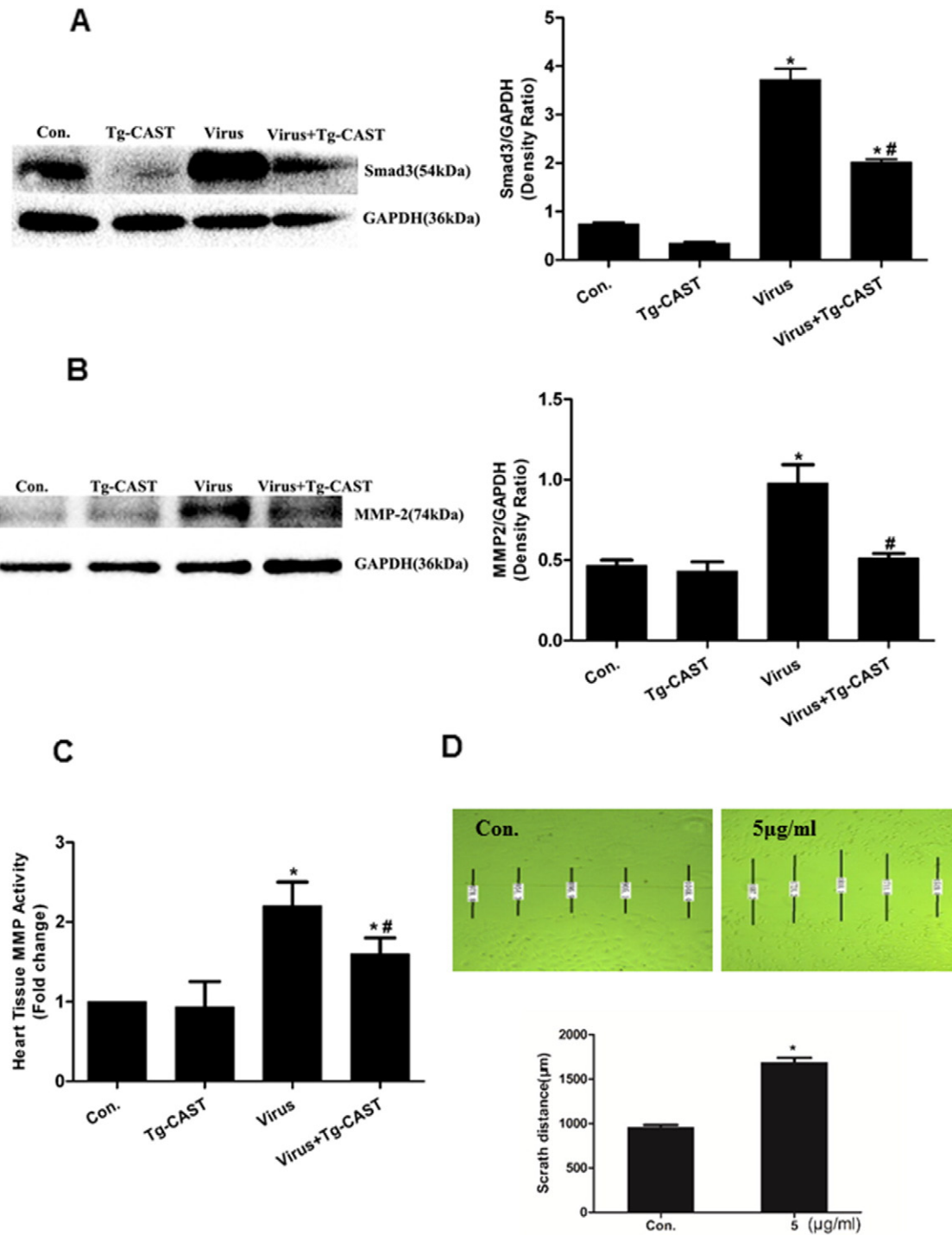


Fig. 5. Calpain was involved in myocardium fibrosis. A, B: western blot of Smad3 and MMP2 respectively. $n = 6$ for each group. * $P < 0.05$ vs. Con. group; # $P < 0.05$ vs. Virus group. C: heart tissue total MMP activity assay. Con. group MMP activity was set 1 arbitrarily. * $P < 0.05$ vs. Con. group; # $P < 0.05$ vs. Virus group. $n = 4$ for each group. D: Represented scratch assay picture of cultured fibroblasts with or without calpain inhibitor ALLN. Longer distance indicated weaker migration ability of the cells. * $P < 0.05$ vs. Con. group. The experiment was repeated for 3 times.

prepared scratch at the bottom of the culture plate. It demonstrated that ALLN intervention reduced fibroblasts migration (Fig. 5C) indicating partly roles of calpain in regulating myocardial fibroblast function.

4. Discussion

This study, utilizing a calpastatin-overexpression transgenic mouse model of viral myocarditis, demonstrated that endogenous calpain inhibition ameliorated myocardial injury significantly.

Simultaneously, calpain inhibition was associated with decreased CVB3 replication and ameliorated inflammation/fibrosis formation. These data indicated the dual involvements of calpain in both virus replication and host inflammation. This might be an answer for the ambiguous problem of immunosuppressive therapy in viral myocarditis treatment [1].

Calpain is a family of proteinases that cleave substrates into peptide segments. Its activity was regulated by the endogenous inhibitor of calpastatin [8]. As we have demonstrated, transgenic mouse

overexpressing calpastatin have a lower level of calpain activity in vivo (Fig. 1D).

Many researchers have reported the involvement of calpain in viruses' life cycles. For examples, calpain participated in the replication of influenza A virus [9], severe acute respiratory syndrome coronavirus [10], echovirus 1 [11] and human immunodeficiency virus [12]. In our former study, we found that CVB3-induced calpain activation facilitates the progeny virus replication in the early phase of infection in vitro [6]. Bozym et al. [14,13] reported the roles of calpain in mediating CVB both the entry into and the release from endothelial cells. Yoon et al. [15] demonstrated that CVB4 uses autophagy for replication after calpain activation in rat primary neurons. As we presented in this paper, calpain inhibition reduced CVB3 loads both in vivo and in vitro (Fig. 2), which further proves the calpain involvement in facilitating CVB3 replication.

Role of calpain in regulating inflammation has been observed in different animal models, such as allergic encephalomyelitis [16], sepsis [17], abdominal aortic atherosclerosis [18], ventilator-induced lung injury [19] and hypercholesterolemic nephropathy [20]. In the realm of viral myocarditis, DeBiasi et al. [21] found in a mice model of reovirus strain 8B-induced myocarditis that calpain inhibition protects against virus-induced apoptotic myocardial injury, which presented the role of calpain in treating myocarditis. But few evidence of inflammation was mentioned as we presented here in a transgenic mouse.

Mechanisms of calpain involvement in inflammation might be associated with its regulation on inflammation cell behaviors. This point is supported by evidences from several studies focusing on different inflammation cells. In neutrophil, calpain participated in cell migration, adhesion, arrest and oxidative burst [22]. Calpain1 also contributes to IgE-mediated mast cell activation [23]. Calpastatin overexpression can impair post infarct scar healing in mice by compromising reparative immune cell recruitment and activation [24]. And in leukocytes, calpain deficiency reduces angiotensin II-induced inflammation and atherosclerosis [25].

We presented here in CVB3-induced myocarditis model that myocardium inflammation infiltration was significantly ameliorated in transgenic mouse, as well as the inflammation factors of MPO activity, IL17, perforin and IFN γ . Although there might be some background levels of expression of the above factors, it is reasonable considering the wide spread of inflammatory cells even in physiology conditions without virus infection. Actually, a variety of inflammation factors whose production or secretion were regulated by calpain have been reported elsewhere including IL5 [26], IL6 [27], IL16 [28] and IL33 [29]. Combined what we have reported in this paper, we could conclude that calpain participates in the regulation of a wide spectrum of inflammation factor production. Researches from PENG's lab [30,31] in the animal models of sepsis and myocardial infarction have demonstrated respectively that calpain induces myocardial NF- κ B activation, which might explain the mechanism that calpain regulates inflammation factors production. Data from another group of our lab also found the role of calpain in regulating NF- κ B activity in CVB3-induced myocarditis mice (unpublished data).

Inflammation factors are always fibrotic agents. The most solid evidence of calpain involvement of tissue fibrosis might be from cystic fibrosis patients [32]. And Jiang et al. [33] reported that calpain1 regulates MMP2 activity in vascular smooth muscle cells to facilitate age-associated aortic wall fibrosis. So we further observed the effect of calpain on myocardium fibrosis. As we have mentioned here that Smad3/MMP2 pathway was inhibited in the transgenic mouse indicating role of calpain in regulating this classic fibrosis pathway. This was coincided with what PENG's lab have demonstrates that calpain inhibition ameliorates myocardial remodeling after myocardial infarction [31] and myocardial fibrosis in diabetic cardiomyopathy [34]. What's more, we also observed the role of calpain in regulating fibroblasts migration in vitro and found calpain inhibition hinders fibroblast migration in

scratch assays. This might explain in a novel angle the mechanisms of calpain in mediating fibrosis.

This study also has some drawbacks. 1) this is an animal study, and the results should be interpreted in caution in clinical practices. Data from VMC patients should be collected in future clinical studies. 2) The data were mainly observational. Mechanisms studies including roles of calpain in CVB3 replication and mediating inflammation in vivo should be performed in further studies respectively.

In summary, in CVB3-induced myocarditis model, we presented here that calpain plays a dual role of both facilitating CVB3 replication and mediating inflammation/fibrosis. Endogenous inhibition of calpain activity showed significant treatment effect for CVB3-induced myocarditis. This might provide a novel target for viral myocarditis treatment in the future.

Conflicts of interests

We declare no conflicts of interests.

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