Abstract. The pathogenesis of viral myocarditis (VMC) is not fully understood. This study aimed to examine the relationship between coxsackie-adenovirus receptor (CAR) and the p38 mitogen activated protein kinase (MAPK) pathway mechanisms in a mouse model. Three groups of mice were established: 5 mice in a control group injected with saline, 15 in the model group injected with coxsackie virus B$_3$ (CVB) and 15 in the intervention group injected with CVB$_3$ but treated with the p38 MAPK inhibitor SB203580. Mice were sacrificed at days 1, 5, 10, 15 and 30 and cardiac tissues were isolated to perform the tests. Quantitative PCR and western blot analysis showed CAR mRNA and protein expression levels were highest in the model group at all time-points (P<0.05). The expression levels of p38 MAPK protein by western blot analysis at days 1, 5 and 10 were obviously higher in the model group (P>0.05). H&E staining used to observe myocardial pathological changes showed the inflammatory infiltration was also higher in the model group at all the time-points (P<0.05). Our results show a direct relationship between CAR and p38 MAPK levels, and since the p38 MAPK inhibitor treatment resulted in reduced levels of CAR as well as lower inflammatory infiltration, it is possible that the signaling pathway may mediate CAR expression during the pathogenesis of VMC.

Introduction

In most individuals with viral myocarditis (VMC) the course is self-limited. However, there are a small number of patients who develop sequelae such as arrhythmias or acute heart failure that may be severe enough to result in high death rates (1). An acute infection leading to VMC can also lead to dilated cardiomyopathy in adults, and coxackie virus B (CVB) and adenovirus (AdV) are the most common etiological agents. The CVB-AdV receptor (CAR) has key functions in virus-infected myocardial cells (2). The virus is able to regulate replication by the host cell to form viral particles; moreover, the host is able to prevent viral damage through signal transduction in activated cells (3).

The signaling pathway of mitogen activated protein kinase (MAPK) may play important roles in VMC, p38 MAPK has been associated with many types of viral infections (4). p38 MAPK in newborn myocardial cells of mice can be activated by the HIV capsid protein gp120, mediating a negative inotropic action (5). It is hypothesized in the present study that the p38 MAPK signaling pathway is capable of increasing CAR expression of VMC, thereby inducing and aggravating the pathophysiological process.

Materials and methods

Animal model. Thirty-five 4-week-old BALB/c, SPF class mice weighing approximately 15 g each were provided by the Animal Experiment Center of Fudan University. The mice were fed routinely, and were kept at temperatures of 20-25˚C with a relative humidity of 50-55%. Based on random grouping, the mice were separated into 3 groups: 5 in the control group, 15 in the model group and 15 in the intervention group. The model and intervention group mice were injected intraperitoneally with 0.1 ml Dulbecco’s modified Eagle’s medium (DMEM) with 1x10$^2$ TCID$_{50}$ CVB$_3$. The intervention group mice received an additional injection of 0.1 ml of the p38 MAPK inhibitor SB203580. The control group mice were injected with 0.1 ml DMEM without any viral load. CVB$_3$ (Nancy strain) was provided by a key laboratory of viral heart diseases in Fudan University: Briefly, after a HeLa cell passage, the cells were frozen and then centrifuged 3 times, the liquid supernatant was separated, and viral loads were titrated using HeLa cell testing to obtain 50% tissue infection rate TCID$_{50}$ with 1x10$^7$, and the infectious material was kept at -70°C. SB203580 was purchased from Sigma-Aldrich (St. Louis, MO, USA). The study was approved by the Animal Ethics Committee of Fudan University Animal Center.

Experimental methods. The mice were sacrificed at 1, 5, 10, 15 and 30 days by cervical dislocation. A quantitative PCR method was used to test CAR mRNA expression levels in
Western blot analysis was used to detect CAR and p38 MAPK protein levels at the same tissue. Hematoxylin and eosin (H&E) staining allowed for observation of the myocardial pathological changes and the Kishimoto method was used to calculate integral inflammation.

**Observation index and testing method**

**Main reagents and equipment.** The following is a list of the main reagents and specialized equipment used in the study:

- TRIzol reagent and SuperScript II RT-PCR kit (both from Invitrogen Life Technologies, Carlsbad, CA, USA).
- PCR marker (Shanghai Dingguo Biological Technology Co., Ltd., Shanghai, China).
- Rabbit anti-mouse CAR, p38 MAPK monoclonal antibody and goat anti-rabbit secondary antibody marked by HRP (all from Cell Signaling Technology, Inc., Danvers, MA, USA).
- PVDF film (Bio-Rad Laboratories, Hercules, CA, USA).
- BCA protein quantification kit and enhanced chemiluminescence kit (both from Pierce Biotechnology, Inc., Rockford, IL, USA).
- Centrifugal machine (Heraeus, Hanau, Germany).
- RNA concentration determinator and PCR thermocycler (both from Eppendorf, Hamburg, Germany).
- PIP-2020 image analyzer (Chongqing Tianhai Medical Equipment Co., Ltd., Chongqing, China).
- Protein electrophoresis transfer system (Bio-Rad Laboratories) and gel quantitative software Quantity One (both from Bio-Rad Laboratories).
- Image-pro plus 5.0 color image analysis system (Media Cybernetics, Inc., Rockville, MD, USA).

**Main steps of real-time quantitative PCR method.** Cardiac muscle tissue (100 mg) from the free wall of the left ventricle were excised and total RNA was extracted based on conventional TRIzol reagent method. To verify the RNA purity and concentration the A260/A280 ratio was calculated based on ultraviolet spectrophotometry. cDNA synthesis and design of primers were done by following the instructions on the kit:

- CAR (F): 5'-GCATCACTACACCCGAACA-3', (R): 5'-ACAAGAACGGTCAGCAGGA-3';
- Internal control GAPDH (F): 5'-CTGCACCACCAACTGCTT-3', (R): 5'-GTCTGGAAATTGTGA-3'.

The reaction system included: 2.5 µl 10X buffer + 0.75 µl 50 mmol MgCl2 + 0.5 µl 10 mM NTP + 0.2 µl Taq enzyme + 2 µl cDNA + water to 25 µl. The reaction conditions included a pre-denaturation step for 3 min at 95°C, and then 35 cycles of a pre-denaturation for 30 sec at 95°C, then an annealing step for 30 sec at 55°C, and then an extension step for 30 sec at 72°C, plus a final extension for 5 min at 72°C and then 4°C to preserve the product. After 2% ethidium bromide sepharose gel electrophoresis, imaging and scanning and then calculation, the results are shown as a ratio of target gene and internal control gene, using the 2^-∆∆Ct method.

**Main steps of western blot analysis.** After conventional gel pouring, electrophoresis and membrane transfer, the membranes were locked in TBST. Then rabbit anti-mouse CAR and p38 MAPK (1:1,000) and internal control β-actin (1:1,000) diluted in 5% BSA were used to incubate the membranes at 4°C overnight. Next day the membranes were washed in TBST and then incubated with goat anti-rabbit secondary antibody (1:2,000) marked by HRP, at room temperature for 1 h. After washing the membranes again in TBST, ECL luminescence was developed. Results show the optical density of target and internal control proteins.

**Myocardial pathology observation.** The basic preparation for microscopy involved the following steps: cardiac muscle tissue was sliced, a dimethyl benzene dewaxing procedure followed by hydration gradient alcohol immersion. Staining was done using H&E, followed by dehydration-gradient alcohol immersion, xylene transparency and finally neutral balsam sealing. Myocardial pathology changes were observed under a light microscope. The Kishimoto method was used to calculate inflammatory infiltration and the necrosis score in cardiac muscle. Five high-power fields were observed in each slice to calculate the percentage of inflammatory infiltration and the necrosis area compared to the total area, a scoring system was established: 0 point for no lesion, 1 point for <25%, 2 points for 25-50%, 3 points for 50-75% and 4 points for >75%.

**Statistical analysis.** SPSS 19.0 software was used for statistical analysis; quantitative data are shown as mean ± standard deviation; comparison between groups were analyzed by single factor ANOVA; quantitative data are presented as case number or percentage (%); comparison between groups was done through χ^2 test; P<0.05 refers to differences with statistical significance.

**Results**

**Comparison of CAR mRNA expression levels.** CAR mRNA expression levels in the model group were the highest at all
time-points. The intervention group had the next highest levels and the control group the lowest; the differences were of statistical significance (P<0.05). CAR mRNA began to increase after 1 day in the model group, reached its peak at 10 days and then began to go down at 15 days, and continued to be higher than the level in the control group at day 30 (Figs. 1 and 2).

Expression of CAR and p38 MAPK protein. CAR protein expression levels in the model group were the highest at all time-points. The intervention group had the next highest levels and the control group the lowest; the differences were of statistical significance (P<0.05). CAR mRNA began to increase after 1 day in the model group, reached its peak at 10 days and then began to go down at 15 days, and continued to be higher than the level in the control group at day 30 (Figs. 1 and 2).

Myocardial pathology observation. In the model group, myocardial cells began to swell on day 1: transverse striation became blurred and there were no inflammatory cells in the stroma. At day 5, acidophilia was obvious in the stained cytoplasm and there were karyopyknosis and karyorrhexis in the nucleus, also a small amount of inflammatory cells had accumulated. At 10 days, the cytoplasm stained homogenously with clearer acidophilia. At day 15, anaplastic cells were further necrotic and disintegrated and even merged, the nuclei and cell structures disappeared and a large amount of inflammatory cells accumulated with collagenous fibers in the stroma. Finally at day 30, there were less inflammatory cells accumulated in the cardiac muscle, hyperplasia of fibrocyte groups at days 15 and 30 (P>0.05). The p38 MAPK protein in the model group began to increase at day 1 and reached its peak at day 5 and then declined at day 10 (Figs. 3 and 4).
began, and clear myocardial fibrosis ensued. Inflammatory infiltration began to increase on day 1 and reached its peak at 15 days, to then begin to decline at day 30. The differences found were all statistically significant (P<0.05) (Figs. 5 and 6).

Discussion

There are four main mechanisms for myocardial damage caused by viruses: i) direct damage of viral protein kinase on the skelemin of the myocardial cell, inhibiting protein synthesis of the host cell (6); ii) persistent virus infection causing chronic myocarditis or its development towards dilated cardiomyopathy (7); iii) cell-mediated and autoimmune mechanism disorders (8); and iv) heart function and structural changes caused by cytokines, effects of nitric oxide, neutralizing antibodies and microvascular injury (9).

As the co-receptor of CVB and AdV, CAR, plays a role of ‘middle bridge’ in the pathogenesis of VMC. Previous findings showed that cells expressing CAR can become infected with either CVB3 or AdV5 by transfecting cDNA expressing human CAR into otherwise CAR-negative, NIH 3T3 cells and then measuring infection titers (10). Additionally, using a monoclonal antibody (Rmcb) to block the CAR protein before exposure to the virus, resulted in obviously lower titers than in a control group (11). Another group showed that newborn mouse myocardial cells cultured in vitro and pre-treated with CAR antibody prevent CVB3 infection (12). These studies show CAR mediation is necessary in order for CVB and AdV to be activated inside the cells.

Human CAR is a transmembrane glycoprotein with a molecular weight of 46 kDa, which belongs to the immunoglobulin superfamily and has high homology to the primary structure of the mouse CAR protein. CAR expression is species and tissue specific. Human CAR is highly expressed in heart, pancreas, brain and small intestine. It has been indicated in studies that CAR expression is related to age, its expression levels in heart during the embryonic and neonatal periods in mice is high, and then decline with age and become undetectable in adults (13). This may be the reason why CVB infects newborns and children more easily with acute severe VMC. Furthermore, studies have shown that the cardiac muscle CAR expression level increases significantly in active stage of autoimmune myocarditis in mice and DCM patients, which points to the higher CAR expression being related to the incidence of myocarditis and cardiomyopathy (14).

The p38 MAPK signaling pathway plays an important role in the inflammatory response and its regulation. In mice suffering from burns with cardiac muscle contractile dysfunction, p38 MAPK is highly activated in cardiac muscle. A p38 MAPK inhibitor is able to alleviate the contractile dysfunction and reduce inflammatory cytokines secreted by myocardial cells such as tumor necrosis factor-α (TNF-α) (15). Another study showed that after injecting perfusion-isolated rat hearts with lipopolysaccharide, the left ventricular systolic function decreased and expression of inflammatory cytokines such as TNF-α, interleukin (IL)-1α increased (15). Subsequently, administration of the p38 MAPK inhibitor SB203580 prior to lipopolysaccharide injection, was able to improve left ventricular function and reduce TNF-α mRNA level in cardiac muscle (16). A recent study has shown an association between higher apoptosis rates and a decrease in contractile function in VMC (17) and the relationship between
p38 MAPK activation cell apoptosis induction is known (17). Finally, SB203580 inhibits p38 MAPK by reducing downstream activation of MAPKAP kinase-2 and kinase-3, which effectively reduces the signal transduction pathway induced by inflammatory factors such as IL-1β and TNF-α (18).

Throughout this study CAR mRNA and protein expression levels were the highest in the model group at all time-points. In the model group, the expression levels of p38 MAPK protein at 1, 5 and 10 days were also obviously higher than those in the other two groups. In addition, in the model group, inflammatory infiltration at all time-points was higher than in the other two groups. Since SB203580 inhibits p38 MAPK pathways and our study showed decreased CAR levels in the intervention group, it is possible that p38 MAPK signaling pathway mediates the CAR expression of VMC and is involved in its pathophysiological process.

References
1. Burns DJ and Quantz MA: Use of the impella 5.0 device as a bridge to recovery in adult fulminant viral myocarditis. Innovations Phila 10: 279-281, 2015.
2. Sharma M, Mishra B, Saikia UN, Bahl A, Ratho RK and Talwar KK: Role of coxsackie virus and adenovirus receptor (CAR) expression and viral load of adenovirus and enterovirus in patients with dilated cardiomyopathy. Arch Virol 19: 102-103, 2015.
3. Valaperti A: Drugs targeting the canonical NF-κB pathway to treat viral and autoimmune myocarditis. Curr Pharm Des 22: 440-449, 2016.
4. Xu HF, Meng L, Yao J, Gu ZY, Liu GQ, Shen YW and Zhao ZQ: Myocardial expression of Spry1 and MAPK proteins of viral myocarditis. Fa Yi Xue Za Zhi 29: 164-167, 2013 (In Chinese).
5. Ma XL, Kumar S, Gao F, Louden CS, Lopez BL, Christopher TA, Wang C, Lee JC, Feuerstein GZ and Yue TL: Inhibition of p38 mitogen-activated protein kinase decreases cardiomyocyte apoptosis and improves cardiac function after myocardial ischemia and reperfusion. Circulation 99: 1685-1691, 1999.
6. Sun S, Ma J, Zhang Q, Wang Q, Zhou L, Bai F, Hu H, Chang P, Yu J and Gao B: Argonate proteins in cardiac tissue contribute to the heart injury during viral myocarditis. Cardiovasc Pathol 25: 120-126, 2016.
7. Huber SA: Viral myocarditis and dilated cardiomyopathy: etiology and pathogenesis. Curr Pharm Des 22: 408-426, 2016.
8. Su N, Yue Y and Xiong S: Monocytic myeloid-derived suppressor cells from females, but not males, alleviate CVB3-induced myocarditis by increasing regulatory and CD4(+)CD10(+) T cells. Sci Rep 6: 22658, 2016.
9. Zhu H, Lou C and Liu P: Interleukin-27 ameliorates coxsackie virus B3-induced viral myocarditis by inhibiting Th17 cells. Virol J 12: 189, 2015.
10. Shi Y, Chen C, Lisewski U, Wrackmeyer U, Radke M, Westermann D, Sauter M, Tschöpe C, Poller W, Klingel K and Gotthardt M: Cardiac deletion of the Coxsackievirus-adenovirus receptor abolishes Coxsackievirus B3 infection and prevents myocarditis in vivo. J Am Coll Cardiol 53: 1219-1226, 2009.
11. Tomko RP, Xu R and Philpson L: HCAR and MCAR: the human and mouse cellular receptors for subgroup C adenoviruses and group B coxsackie viruses. Proc Natl Acad Sci USA 94: 3352-3356, 1997.
12. Xu HF, Chen JI, Da XP, Wu KR, Liu GQ, Zhao ZQ and Han XH: Expression of CAR in myocardial viral myocarditis and dilated cardiomyopathy. Fa Yi Xue Za Zhi 26: 328-331, 2010 (In Chinese).
13. Fischer R, Poller W, Schultheiss HP and Gotthardt M: CAR-diology - a virus receptor in the healthy and diseased heart. J Mol Med Berl 87: 879-884, 2009.
14. Shi Y, Chen C, Lisewski U, Wrackmeyer U, Radke M, Westermann D, Sauter M, Tschöpe C, Poller W, Klingel K, et al: Cardiac deletion of the coxsackie virus-adenovirus receptor abolishes coxsackievirus B3 infection and prevents myocarditis in vivo. J Am Coll Cardiol 53: 1219-1226, 2009.
15. Ballard-Croft C, White DJ, Maass DL, Hybki DP and Horton JW: Role of p38 mitogen-activated protein kinase in cardiac myocyte secretion of the inflammatory cytokine TNF-alpha. Am J Physiol Heart Circ Physiol 280: 1970-1981, 2001.
16. Wang M, Sankula R, Tsai BM, Meldrum KK, Turrentine M, March KL, Brown JW, Dinarello CA and Meldrum DR: P38 MAPK mediates myocardial proinflammatory cytokine production and endotoxin-induced contractile suppression. Shock 21: 170-174, 2004.
17. Henke A, Launhardt H, Klement K, Stelzner A, Zell R and Munder T: Apoptosis in coxsackievirus B3-caused diseases: interaction between the capsid protein VP2 and the proapoptotic protein siva. J Virol 74: 4284-4290, 2000.
18. Sreekanth GP, Chunchuranne C, Sirimontaporn A, Panaampon J, Noisakran S, Yenchitsomanus PT and Limjindaporn T: SB203580 modulates p38 MAPK signaling and dengue virus-induced liver injury by reducing MAPKAPK2, HSP27, and ATF2 phosphorylation. PLoS One 11: e0149466, 2016.