R5 HIV-1 gp120 Activates p38 MAPK to Induce Rat Cardiomyocyte Injury by the CCR5 Coreceptor

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Keywords

Human immunodeficiency virus-1 glycoprotein 120 · C-C chemokine receptor 5 · p38 mitogen-activated protein kinase · Cell injury · Cardiac troponin-I

Abstract

Background: Effective antiretroviral therapy extends the survival of patients with human immunodeficiency virus (HIV)/acquired immune deficiency syndrome, but these patients remain at higher risk for heart diseases compared with the general population. Previous studies have suggested that HIV-1 glycoprotein 120 (gp120) may be associated with heart disease. However, the underlying mechanisms by which HIV-1 gp120-mediated myocardial injury occurs remain unknown. **Objective:** The current study aimed to uncover the mechanism of C-C chemokine receptor 5 (CCR5) coreceptor (R5) HIV-1 gp120-induced myocardial injury. **Methods:** Morphology analysis, determination of the percentage of cell apoptosis, as well as lactate dehydrogenase (LDH) and creatine kinase (CK) assays were used to analyze whether R5 HIV-1 gp120 induced myocardial cell injury. We analyzed the phosphorylation of p38 mitogen-activated protein kinase (MAPK) with the CCR5 antagonist D-Ala-

peptide T-amide (DAPTA) and NMDA receptor antagonist MK801, detected LDH and CK assays with p38 MAPK antagonist SB203580 (SB), and detected the percentage of cell apoptosis and death with DAPTA to investigate the mechanism of R5 HIV-1 gp120-induced myocardial cell injury. **Results:** R5 HIV-1 gp120 damaged myocardial cells and induced p38 MAPK phosphorylation. SB blocked R5 HIV-1 gp120-induced myocardial cell injury. DAPTA blocked R5 HIV-1 gp120-mediated p38 MAPK phosphorylation, while MK801 did not. DAPTA inhibited R5 HIV-1 gp120-induced myocardial cell injury. **Conclusion:** Our data indicate that R5 HIV-1 gp120 activated p38 MAPK to trigger myocardial cell injury by the CCR5 coreceptor.

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Introduction

Acquired immune deficiency syndrome (AIDS) is a fatal infectious disease caused by human immunodeficiency virus (HIV) infection, which results in immunocompromised patients who are susceptible to opportunistic infections and malignancies. At present, highly effective antiretroviral therapy has improved the quality of life and

extended the survival of patients with HIV/AIDS, making AIDS a chronic infectious disease [1]. Patients with HIV infection have higher rates of clinical cardiovascular diseases (RR 1.61; 95% CI 1.43–1.81; $p < 0.001$), including coronary heart disease (RR 3.36; 95% CI 3.36–13.58; $p < 0.001$) [2] and cardiac diastolic dysfunction (2.4-fold greater odds; $p = 0.019$) [3] compared with individuals in the general population. In addition, patients with HIV experience increased subclinical cardiovascular damage, including coronary calcification (HIV-infected vs. uninfected people 51 vs. 32%) [4], silent myocardial ischemia (10.9% in HIV-infected people with ECG) [5], and endothelial dysfunction (flow-mediated dilation: HIV-infected vs. uninfected people $7.3 \pm 4.4\%$ vs. $11.1 \pm 6.3\%$) [6]. Thus, due to the prolonged survival of patients with HIV, heart disease may become an important cause of mortality. Therefore, it is of great theoretical value and of practical significance to elucidate the pathogenesis of HIV/AIDS-associated heart disease.

The pathogenesis of HIV/AIDS-associated heart disease is considered to be associated with a variety of factors, including opportunistic infections, Kaposi sarcoma and drug toxicity [7]. The large Data Collection on Adverse Events of Anti-HIV Drugs study found that the risk of cardiovascular disease increased by 26% per year of antiretroviral therapy exposure [8]. However, even in the current era of treatment, the high risk of cardiovascular disease still persists [9]. A study from the National Institutes of Health's Strategies has found that interruption of antiretroviral therapy was surprisingly associated with increased risk of cardiovascular disease, which indicated that HIV viral activities might be the prevailing reason of cardiovascular disease risk rather than antiretroviral therapy [10]. Further studies have indicated that HIV itself can be the cause of cardiomyopathy [11, 12]. Based on differences in HIV gene expression, HIV was divided into two types: HIV-1 and HIV-2 [13]. HIV-1 is more virulent and more infective than HIV-2 and is the cause of the majority of HIV infections globally [14]. HIV-1 invades host cells via envelope protein binding to the CD4 receptor and a coreceptor, including C-C chemokine receptor 5 (CCR5) or C-X-C chemokine receptor (CXCR4) [15]. Glycoprotein 120 (gp120) is one of the most important HIV-1 envelope proteins [16]. Based on the coreceptors used by HIV-1 gp120, HIV-1 was further divided into the R5, X4, and R5X4 subtypes [15]. The R5 subtype binds to CCR5, X4 to CXCR4, and R5X4 to CXCR4 and CCR5.

As cardiac muscle cells do not express CD4 [17], HIV cannot induce myocardial cell damage by binding to this marker. However, cardiac cells express CCR5 and CXCR4

coreceptors [11, 18]. In HIV-associated neurocognitive disorders, HIV-1 gp120 causes nerve cell injury via binding to CCR5 or CXCR4 [19]. Recently, Kelly et al. [20] used a rhesus animal model infected with simian immunodeficiency virus to demonstrate that CCR5 is a critical target in cardiac dysfunction. In addition, HIV-1 gp120 further induces myocardial diastolic dysfunction via the CXCR4-p38 mitogen-activated protein kinase (MAPK)-cardiac troponin-I (cTnI) signaling pathways [21, 22]. However, it remains unclear whether HIV-1 gp120 induces myocardial injury by binding to CCR5.

In the current study, neonatal rat cardiomyocytes were cultured and the effects of R5 HIV-1 gp120 (JRFL, viral envelope from CCR5-dependent)-mediated myocardial injury were detected. The CCR5 antagonist D-Ala-peptide T-amide (DAPTA) and the p38 MAPK antagonist SB203580 (SB) were assessed in their ability to limit R5 HIV-1 gp120-mediated myocardial injury and to elucidate the mechanism of HIV-1 gp120-mediated myocardial cell injury.

Materials and Methods

Materials

All antagonists, including SB (ab120235), DAPTA (ab120810), and MK801 (ab120027), and antibodies, including anti-p38 (ab31828), anti-p-p38 (ab4822), anti-cTnI (ab47003), anti-p-cTnI (ab58546), anti-active caspase-3 (ab49822), anti-GAPDH (ab8245), secondary antibody (ab6734), and Alexa Fluor 594 secondary antibody (ab150160) were purchased from Abcam (Cambridge, UK). R5 HIV-1 gp120 (JRFL; IT-001-0024p) soluble in PBS was purchased from Immune Technology Corp. (New York, NY, USA). The Click-iT terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) Alexa Fluor 488 Imaging Assay kit was purchased from Life Technologies (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Dulbecco's modified Eagle's medium (DMEM) was purchased from HyClone (GE Healthcare Life Sciences, Logan, UT, USA), and fetal bovine serum (FBS) was purchased from Gibco (Thermo Fisher Scientific, Inc.). Type II collagenase and trypsin were obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Sprague-Dawley neonatal rats (age <3 days) were acquired from the Fujian Medical University Animal Center (Fuzhou, China). The bicinchoninic acid kit (A045-3), the Western blotting kit (W001), the enhanced chemiluminescence kit (W028-2), the lactate dehydrogenase (LDH; A020-2) and creatine kinase (CK; A032) detection kits, and loading buffer were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Isolation and Culturing of Cardiomyocytes from Neonatal Rats

Anaesthetized neonatal Sprague-Dawley rats (total number: 330; weight: 5–8 g; sex: half male, half female) were disinfected with 75% alcohol and the skin and chest were cut with ophthalmic scissors. The ventricular tissues of exposed heart were cut off, cut into small pieces, and rinsed with PBS. Digestive enzymes (0.08% type II collagenase and 0.125% trypsin in PBS) were added to the

tissues and incubated for 5 min (37 °C). Supernatants were removed, a second portion of digestive enzymes was added, and tissues were digested for another 10 min at 37 °C. The supernatant was transferred to a centrifuge tube, an equal volume of DMEM containing 5% FBS was added, and the sample was centrifuged (1,500 g, 5 min, ordinary temperature). The tissue digestion was repeated several times until the tissue was no longer visible. Isolated cells were placed in dishes and incubated for 2 h (37 °C; air containing 5% CO₂). The supernatant was transferred to a new dish and incubated for 2 h (37 °C; air containing 5% CO₂) prior to another transfer and incubation for 24 h (37 °C; air containing 5% CO₂). Then, the medium was replaced with fresh DMEM containing 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. Subsequently, medium was replaced every 48 h, and myocardial cells cultured for 4 days were used in subsequent experiments.

Live Cell Imaging

Treated myocardial cells were cultured for 24 h (37 °C; air containing 5% CO₂). Cells were then observed with the Zeiss Axiovert 200 Inverted Fluorescence Phase Contrast Microscope (Zeiss GmbH, Jena, Germany) equipped with a Tucsen TCH-5.0 digital camera (Tucsen Photonics, Fuzhou, China) at 20× magnification. Cells were counted in 20 fields of view. The percentage of cell death was calculated as the number of rounded and detached cells divided by the total number of cells. Images were captured digitally and analyzed using ImageJ 1.8.0 (National Institutes of Health, Bethesda, MD, USA).

Immunostaining of Active Caspase-3

Myocardial cells (cell density 5×10^5) were cultured (37 °C; air containing 5% CO₂) in 12-hole plates containing coverslips for 4 days and were divided into the following four groups ($n = 5$; three repeats): control, 0.4, 0.8, and 1.0 µg/mL R5 HIV-1 gp120. Myocardial cells were cultured for 24 h (37 °C; air containing 5% CO₂). Culture supernatant was removed and coverslips were washed twice with PBS. Cells were fixed for 20 min (ordinary temperature) in 4% paraformaldehyde and washed thrice with PBS containing 0.1% bovine serum albumin (BSA; B2064, Sigma-Aldrich Corp., St. Louis, MO, USA). Cells were blocked for 45 min at ordinary temperature with PBS containing 10% FBS and 0.3% TritonX-100 and incubated overnight at 4 °C with anti-active caspase-3 antibody diluted 1:400 in PBS with 0.3% TritonX-100. Coverslips were washed twice with PBS containing 0.1% BSA and incubated with Alexa Fluor 594 secondary antibody for 1 h at room temperature. Coverslips were washed thrice with PBS containing 0.1% BSA, mounted onto a glass slide with DAPI (Thermo Fisher Scientific, Inc.), and fluorescent images were obtained using the Zeiss Axiovert microscope (20× magnification). Active caspase-3-immunopositive cells were expressed as a percentage of total cells, which were determined using DAPI-positive cells. Images were captured digitally and analyzed using ImageJ 1.8.0.

TUNEL Assay

TUNEL assays were performed to supplement the immunostaining of cleaved caspase-3. Myocardial cells were fixed for 60 min in 4% paraformaldehyde at ordinary temperature. Apoptosis levels were determined using the Click-iT TUNEL Alexa Fluor 488 Imaging Assay kit according to the manufacturer's instructions. After the TUNEL process, coverslips were washed thrice with PBS. Then, 100 µL DAPI (100 ng/mL) was added and

incubated for 5 min at ordinary temperature, avoiding light. Coverslips were washed thrice with PBS. Fluorescence decay-resistant seal tablets (Nanjing Jiancheng Bioengineering Institute) were used to seal up. Fluorescent images of TUNEL-positive cells were obtained using the Zeiss Axiovert microscope (40× magnification; 20 fields of view). TUNEL-positive cells were expressed as a percentage of total cells, determined by DAPI-positive cells. Images were captured digitally and analyzed using ImageJ 1.8.0.

Western Blotting

For the preparation of cell lysate, cells were washed three times with PBS at 37 °C, scraped off, and transferred into a precooled microfuge tube. A total of 50 µL radioimmunoprecipitation assay buffer (Nanjing Jiancheng Bioengineering Institute) containing phenylmethylsulfonyl fluoride (1 mM) was added to the tube and incubated on ice for 30 min, with mixing intervals of 30 s every 5 min. Samples were centrifuged (15 min; 18,000 g; 4 °C), placed on ice, and supernatants were transferred into fresh tubes kept on ice. Protein concentrations were determined using 1 µL supernatant and a bicinchoninic acid assay. The remaining supernatant was mixed with loading buffer and incubated for 10 min at 100 °C. Separating gels were chosen according to the target protein (p38, p-p38, cTnI, p-cTnI, and GAPDH for 12% separating gels). Samples (30 µg loaded protein per lane) were separated using SDS-PAGE and transferred onto PVDF membranes. Membranes were blocked with 5% skimmed milk powder (dissolved in 1× TBST) at ordinary temperature for 2 h and washed three times with 1× TBST. Primary antibodies were added at an appropriate dilution (1:500 for anti-p-cTnI; 1:1,000 for anti-p38, anti-p-p38, and anti-cTnI; 1:2,000 for anti-GAPDH) and incubated at 4 °C overnight. Following a wash with 1× TBST (three times), secondary antibodies were added at an appropriate dilution (1:2,000 for secondary antibody) and incubated rocking for 2 h at ordinary temperature. Membranes were washed with 1× TBST (three times), and the enhanced chemiluminescence kit was used for visualizations according to the manufacturer's instructions (1:2,000 secondary antibodies as the loading control). ImageJ 1.8.0 was used to analyze the gray values of the bands.

LDH and CK Analysis

Treated myocardial cells were cultured for 24 h (37 °C; air containing 5% CO₂). Following incubation, culture supernatants were collected and LDH and CK contents were measured by relevant kits (volume of measured samples 20 µL, wavelength for LDH 450 nm, wavelength for CK 660 nm).

Statistical Analysis

Data are presented as mean ± SD. Differences between multiple groups were assessed using one-way analysis of variance followed by Student-Newman-Keuls post hoc tests. SPSS 20.0 (IBM Corp., Armonk, NY, USA) was used for the analysis. A p value <0.05 was considered to indicate a statistically significant difference.

Results

R5 HIV-1 gp120 Mediates Myocardial Cell Injury

To determine whether R5 HIV-1 gp120 induced myocardial cell injury, HIV-1 gp120 was added to rat cardiac

Fig. 1. R5 HIV-1 gp120-mediated myocardial cell injury. **a** Phase contrast microscope images (20× magnification) of cardiomyocytes exposed to HIV-1 gp120 at varying concentrations (0.4, 0.8, and 1.0 µg/mL). Rounded and detached cells were considered dead and are presented as the percentage of total cells. **b** Cardiomyocytes exposed to HIV-1 gp120 at varying concentrations (0.4, 0.8, and 1.0 µg/mL) were stained for active caspase-3 using DAPI. Immunopositive cells (red) were considered apoptotic

and are presented as the percentage of total cell nuclei (blue). **c** Quantification of cardiomyocytes exposed to HIV-1 gp120 at varying concentrations (0.4, 0.8, and 1.0 µg/mL) revealed a dose-dependency of TUNEL-positive cells. * $p < 0.01$ versus control. HIV-1 gp120, human immunodeficiency virus-1 glycoprotein 120; R5, HIV-1 glycoprotein 120 C-C chemokine receptor 5 co-receptor; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

Fig. 2. R5 HIV-1 gp120-mediated p38 MAPK and cTnI phosphorylation. **a** Representative Western blotting images highlighting the effect of varying gp120 concentrations (0.4, 0.8, and 1.0 µg/mL) on p38 MAPK and cTnI phosphorylation. **b** Relative quantitative ratios of p-p38 MAPK/total p38 MAPK and p-cTnI/total cTnI. Ex-

periments were repeated three times. * $p < 0.05$ versus control. cTnI, cardiac troponin-I; HIV-1 gp120, human immunodeficiency virus-1 glycoprotein 120; MAPK, mitogen-activated protein kinase; p-, phosphorylated; R5, HIV-1 glycoprotein 120 C-C chemokine receptor 5 coreceptor.

muscle cells (incubated for 24 h). Microscopic analysis revealed that cells were rounded and detached in the culture dish and had died (Fig. 1a). Cell death significantly increased in a dose-dependent manner compared with untreated control ($p < 0.01$; Fig. 1a). Immunostaining of active caspase-3 indicated that R5 HIV-1 gp120 induced myocardial cell apoptosis (Fig. 1b). Staining indicating the presence of active caspase-3 increased significantly in myocardial cells exposed to 0.8 and 1.0 µg/mL R5 HIV-1 gp120 compared with control ($p < 0.01$; Fig. 1b). TUNEL staining identified significantly increased numbers of TUNEL-positive myocardial cells in cells exposed to R5 HIV-1 gp120 (0.4, 0.8, and 1.0 µg/mL) compared with control ($p < 0.01$; Fig. 1c). To detect further damage in myocardial cells, LDH and CK levels were measured. LDH and CK levels significantly increased in the 0.8 and 1.0 µg/mL gp120 groups compared with control ($p < 0.01$;

Table 1). These results indicated that R5 HIV-1 gp120 damaged myocardial cells.

R5 HIV-1 gp120 Increases p38 MAPK and cTnI Phosphorylation

In a previous report, HIV-1 gp120 induced myocardial diastolic dysfunction via the p38 MAPK signaling pathway [18]. To determine whether R5 HIV-1 gp120 was associated to p38 MAPK and cTnI phosphorylation, varying concentrations of HIV-1 gp120 (0.4, 0.8, and 1.0 µg/mL) were added to the culture medium (incubated for 4 h). Western blot analysis suggested that compared with control, p38 MAPK and cTnI phosphorylation levels were significantly elevated in the 0.8 and 1.0 µg/mL gp120 groups ($p < 0.01$; Fig. 2). The results indicated that R5 HIV-1 gp120 induced p38 MAPK and cTnI phosphorylation.

Fig. 3. The CCR5 antagonist DAPTA reverses R5 HIV-1 gp120-mediated p38 MAPK and cTnI phosphorylation. **a** Representative Western blots revealing the effect of DAPTA (10 μ M) and R5 HIV-1 gp120 (0.8 μ g/mL) treatment on p38 MAPK and cTnI phosphorylation. **b** Relative quantitative ratios of p-p38 MAPK/total p38 MAPK and p-cTnI/total cTnI. Experiments were repeated

three times. * $p < 0.05$ versus control, # $p < 0.05$ versus gp120. CCR5, C-C chemokine receptor 5; cTnI, cardiac troponin-I; DAPTA, D-Ala-peptide T-amide; HIV-1 gp120, human immunodeficiency virus-1 glycoprotein 120; MAPK, mitogen-activated protein kinase; p-, phosphorylated; R5, HIV-1 glycoprotein 120 C-C chemokine receptor 5 coreceptor.

Table 1. LDH and CK levels in the culture supernatant of CCR5 coreceptor of HIV-1 gp120-treated myocardial cells

Group	LDH, U/L	CK, U/L
Control	39 $\pm$ 4	32 $\pm$ 3
gp120		
0.2 μ g/mL	41 $\pm$ 3	30 $\pm$ 2
0.4 μ g/mL	40 $\pm$ 2	35 $\pm$ 4
0.8 μ g/mL	51 $\pm$ 3 ^a	48 $\pm$ 2 ^a
1.0 μ g/mL	69 $\pm$ 5 ^a	72 $\pm$ 3 ^a

Data are presented as mean $\pm$ SD of $n = 5$, with three repeats. CCR5, C-C chemokine receptor 5; CK, creatine kinase; HIV-1 gp120, human immunodeficiency virus-1 glycoprotein 120; LDH, lactate dehydrogenase. ^a $p < 0.05$ versus control.

Table 2. Effects of the p38 MAPK inhibitor SB on myocardial cell injury induced by HIV-1 gp120

Group	LDH, U/L	CK, U/L
Control	39 $\pm$ 4	32 $\pm$ 3
gp120	67 $\pm$ 3 ^a	74 $\pm$ 4 ^a
gp120 + SB	41 $\pm$ 3 ^b	34 $\pm$ 2 ^b
SB	37 $\pm$ 3 ^c	33 $\pm$ 2 ^c

Data are presented as mean $\pm$ SD of $n = 5$, with three repeats. Treatment was based on 1.0 μ g/mL gp120 and 10 μ M SB. CK, creatine kinase; HIV-1 gp120, human immunodeficiency virus-1 glycoprotein 120; LDH, lactate dehydrogenase; MAPK, mitogen-activated protein kinase; SB, SB203580. ^a $p < 0.05$ versus control, ^b $p < 0.05$ versus gp120, ^c $p > 0.05$ versus control.

Fig. 4. The NMDA receptor antagonist MK801 does not alleviate R5 HIV-1 gp120-mediated p38 MAPK and cTnI phosphorylation. **a** Representative Western blots revealing the effects of MK801 (2, 20, and 200 μM) and R5 HIV-1 gp120 (0.8 μg/mL) on p38 MAPK and cTnI phosphorylation. **b** Relative quantitative ratios of p-p38 MAPK/total p38 MAPK and p-cTnI/total cTnI. Experiments were

repeated three times. * $p < 0.05$ versus control. cTnI, cardiac troponin-I; HIV-1 gp120, human immunodeficiency virus-1 glycoprotein 120; MAPK, mitogen-activated protein kinase; p-, phosphorylated; R5, HIV-1 glycoprotein 120 C-C chemokine receptor 5 coreceptor.

The p38 MAPK Inhibitor SB Prevents Myocardial Cell Injury Induced by R5 HIV-1 gp120

SB (10 μM) was tested to determine whether it affected R5 HIV-1 gp120 (1.0 μg/mL)-mediated myocardial cell injury (treated cells were cultured for 24 h). Compared with control, LDH and CK levels significantly increased in the gp120 treatment group ($p < 0.01$), and no significant differences were observed for the SB treatment groups ($p = 0.177$ for LDH, $p = 0.999$ for CK; Table 2). Compared with the gp120 treatment group, LDH and CK levels significantly decreased in the gp120 + SB treatment group ($p < 0.01$; Table 2). These results suggested that R5 HIV-1 gp120 induced myocardial cell injury via the p38 MAPK signaling pathway.

The CCR5 Antagonist DAPTA Inhibited R5 HIV-1 gp120-Mediated p38 MAPK and cTnI Phosphorylation

To determine whether CCR5 is crucial in inducing p38 MAPK and cTnI phosphorylation, experiments with DAPTA (20 μM) were performed (treated cells were cultured for 4 h). Western blot analysis revealed that compared with control, p38 MAPK and cTnI phosphorylation levels were significantly elevated in the gp120 treatment group (0.8 μg/mL; $p < 0.01$), and no significant differences were observed in the DAPTA treatment groups ($p = 0.996$ for p-p38 MAPK, $p = 0.975$ for p-cTnI; Fig. 3). Compared with the gp120 treatment group, p38 MAPK and cTnI phosphorylation significantly decreased in the gp120 + DAPTA treatment group ($p < 0.05$; Fig. 3). These results indicated that R5 HIV-1 gp120 activated the

Fig. 5. The CCR5 antagonist DAPTA inhibits R5 HIV-1 gp120-mediated myocardial cell injury. **a** Phase contrast microscopy images (20× magnification) of cardiomyocytes exposed to HIV-1 gp120 (1 µg/mL) and DAPTA (20 µM) or the p38 MAPK inhibitor SB (10 µM). Rounded and detached cells were considered dead and are presented as the percentage of total cells. **b** The quantification of TUNEL-positive myocardial cells in the gp120 group significantly increased. TUNEL-positive cells revealed DAPTA and SB

alleviated HIV-1 gp120-mediated cardiomyocyte injury. * $p < 0.01$ versus control, # $p < 0.01$ versus gp120. CCR5, C-C chemokine receptor 5; DAPTA, D-Ala-peptide T-amide; HIV-1 gp120, human immunodeficiency virus-1 glycoprotein 120; MAPK, mitogen-activated protein kinase; R5, HIV-1 glycoprotein 120 C-C chemokine receptor 5 coreceptor; SB, SB203580; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

p38 MAPK signaling pathway through binding to the CCR5 coreceptor.

The NMDA Receptor Antagonist MK801 Does Not Affect R5 HIV-1 gp120-Mediated p38 MAPK and cTnI Phosphorylation

To determine whether HIV-1 gp120 induced myocardial cell autophagy by binding to the NMDA receptor [12], MK801 was applied at various concentrations (2, 20, and 200 µM) in experiments evaluating its effect on R5 HIV-1 gp120 (0.8 µg/mL)-mediated p38 MAPK and cTnI phosphorylation (treated cells were cultured for 4 h). Western blot analysis suggested that compared with control, p38 MAPK and cTnI phosphorylation levels were significantly elevated in the gp120 and the various gp120 + MK801 treatment groups ($p < 0.01$ vs. control; Fig. 4). Compared with the gp120 treatment group, p38 MAPK and cTnI phosphorylation levels were not significantly different in the various gp120 + MK801 treatment groups (2 µM, $p = 0.978$ for p-p38 MAPK, $p = 0.352$ for p-cTnI;

20 µM, $p = 0.765$ for p-p38 MAPK, $p = 0.999$ for p-cTnI; and 200 µM, $p = 1.0$ for p-p38 MAPK, $p = 0.995$ for p-cTnI; Fig. 4). These results indicated that the NMDA receptor was not a target of R5 HIV-1 gp120-mediated p38 MAPK or cTnI phosphorylation.

The CCR5 Antagonist DAPTA Inhibits R5 HIV-1 gp120-Mediated Myocardial Cell Injury

In addition to SB preventing myocardial cell injury induced by R5 HIV-1 gp120, DAPTA was used in experiments to analyze the suppression of R5 HIV-1 gp120-mediated myocardial cell injury (treated cells were cultured for 24 h). Morphological observations were performed and the percentages of dead myocardial cells in samples exposed to R5 HIV-1 gp120 (1 µg/mL) and DAPTA (20 µM) or SB (10 µM) were determined. The ratio of cell death in the gp120 group was higher than in the control group ($p < 0.01$; Fig. 5a). However, cell death was significantly reduced in cells exposed to gp120 + DAPTA or gp120 + SB compared with the gp120 treatment group

($p < 0.01$; Fig. 5a). TUNEL staining of myocardial cells further suggested that compared with the control group, TUNEL-positive myocardial cells in the gp120 group significantly increased ($p < 0.01$; Fig. 5b), while gp120 + DAPTA or gp120 + SB treatment of myocardial cells decreased the cell susceptibility to R5 HIV-1 gp120-induced apoptosis ($p < 0.01$ vs. gp120; Fig. 5b). These results indicated that the CCR5 antagonist DAPTA inhibited R5 HIV-1 gp120-mediated myocardial cell injury.

Discussion

In the current study, the mechanism of R5 HIV-1 gp120-mediated myocardial cell injury was elucidated. Rat myocardial cells were treated with R5 HIV-1 gp120, and it was observed that R5 HIV-1 gp120 induced myocardial cell injury and activated p38 MAPK. In addition, the p38 MAPK inhibitor SB reversed R5 HIV-1 gp120-induced p38 MAPK phosphorylation, indicating that R5 HIV-1 gp120 induced myocardial cell injury via p38 MAPK signaling pathway activation. It was further determined that the CCR5 inhibitor DAPTA alleviated R5 HIV-1 gp120-mediated p38 MAPK phosphorylation, but MK801, an NMDA receptor antagonist, did not, indicating that R5 HIV-1 gp120 activated p38 MAPK through the CCR5 coreceptor. Finally, the morphology and percentage cell death in DAPTA-treated myocardial cells exposed to R5 HIV-1 gp120 were determined and suggested that DAPTA inhibited R5 HIV-1 gp120-induced myocardial cell injury.

Previous studies demonstrated that HIV-1 gp120 induced myocardial dysfunction by binding to the CXCR4 coreceptor [18, 22]. To determine whether R5 HIV-1 gp120 induced myocardial cell injury, varying doses of R5 HIV-1 gp120 were added to rat myocardial cells, and it was revealed that LDH and CK levels at high doses of R5 HIV-1 gp120 (1.0 and 2.0 $\mu\text{g/mL}$) were significantly increased compared with control. The number of apoptotic cells was further observed to increase in a dose-dependent manner. The results demonstrated that R5 HIV-1 gp120 caused myocardial cell injury. Previous studies have suggested that HIV-1 gp120 is associated with HIV-associated neurocognitive disorders [23] and HIV-associated dementia [24]. Additionally, HIV-1 gp120 has been reported to activate the p38 MAPK signaling pathway triggering neurotoxic activity [25–28]. In the cardiovascular system, HIV-1 gp120 activates p38 MAPK by binding to CXCR4 inducing inducible nitric oxide synthase mRNA expression, which increased inducible nitric

oxide synthase protein and nitric oxide levels, leading to myocardial cell injury [29]. Phosphorylated p38 MAPK activated calcium-independent phospholipase A2 and induced cTnI phosphorylation, which resulted in myocardial diastolic dysfunction [21, 22]. Therefore, the p38 MAPK signaling pathway may play an important role in HIV-1 gp120-mediated myocardial cell injury and myocardial diastolic dysfunction. In the present study, it was suggested that R5 HIV-1 gp120 activated p38 MAPK in rat myocardial cells. p38 MAPK is an important member of the MAPK family and serves a role in the pathophysiology of various heart diseases, including ventricular remodeling [30], myocardial hypertrophy [31], and myocardial dysfunction [32]. However, it is unknown whether R5 HIV-1 gp120 triggers myocardial cell injury through the p38 MAPK signaling pathway. To characterize the association between the p38 MAPK signaling pathway and R5 HIV-1 gp120-mediated myocardial cell injury, cells were treated with the p38 MAPK inhibitor SB. The data suggested that SB significantly reduced R5 HIV-1 gp120-mediated myocardial cell injury, indicating that R5 HIV-1 gp120 may induce myocardial cell injury by activating p38 MAPK.

As HIV-1 gp120 binds to receptors on cardiomyocyte surfaces, R5 HIV-1 gp120-mediated p38 MAPK phosphorylation may occur via binding to specific receptors. CCR5, a member of the G-protein-coupled receptor family, is an important coreceptor of gp120 for HIV infection of target cells [15]. In addition to mediating HIV entry into host cells, CCR5 is associated with a variety of inflammatory conditions, including rheumatoid arthritis [33], multiple sclerosis [34], and asthma [35]. CCR5 is further associated with HIV-1 gp120-mediated central nervous system injury [36–38] and in the cardiovascular system and promotes monocyte aggregation to accelerate the development of atherosclerosis [39]. To determine whether R5 HIV-1 gp120 activates p38 MAPK by binding to CCR5, R5 HIV-1 gp120-treated myocardial cells were incubated with the CCR5 inhibitor DAPTA. The data suggested that DAPTA specifically blocked R5 HIV-1 gp120-mediated p38 MAPK phosphorylation and reversed R5 HIV-1 gp120-mediated myocardial cell injury. The results indicated that R5 HIV-1 gp120 induced p38 MAPK phosphorylation via the CCR5 coreceptor. A recent study reported that HIV-1 gp120 increased the expression of the autophagy-associated proteins ATG7, Beclin 1, lysosomal-associated membrane protein 1, and light chain 3 in myocardial cells, whereas the NMDA receptor inhibitor MK801 (10 $\mu\text{g/mL}$) inhibited the expression of HIV-1 gp120-induced autophagic proteins [12].

These results demonstrated that HIV-1 gp120 induced myocardial cell injury by binding to the NMDA receptor. To determine whether R5 HIV-1 gp120-mediated p38 MAPK phosphorylation was associated to the NMDA receptor, the NMDA receptor inhibitor MK801 was used in conjunction with R5 HIV-1 gp120 to treat myocardial cells. The results indicated that MK801 (200 μ M; 67 μ g/mL) could not inhibit R5 HIV-1 gp120-mediated p38 MAPK phosphorylation, while a previous study reported that MK801 (10 μ g/mL) blocked HIV-1 gp120-mediated autophagy in cardiomyocytes [12], indicating that R5 HIV-1 gp120 may not activate p38 MAPK via the NMDA receptor. However, it required further study whether R5 HIV-1 gp120 induced myocardial cell injury by other receptors or by other signal pathways. A previous report demonstrated that cTnI was associated with cardiac negative inotropic activity [21]. In the current study, it was further discovered that R5 HIV-1 gp120 induced cTnI phosphorylation and that the CCR5 inhibitor DAPTA reversed R5 HIV-1 gp120-mediated cTnI phosphorylation. These results suggest that R5 HIV-1 gp120 triggered cTnI phosphorylation via the CCR5 coreceptor. A previous report demonstrated that CCR5 inhibition prevents cardiac dysfunction in a simian immunodeficiency virus/macaque model of HIV [20]. Thus, R5 HIV-1 gp120/CCR5-mediated cTnI phosphorylation may be associated with R5 HIV-1 gp120-associated cardiac negative inotropic activity. Due to primary cardiomyocytes cultured through isolated hearts, it cannot completely eliminate other cells such as fibroblasts. Further study is needed whether R5 HIV-1 gp120 indirectly induced myocardial cell injury by other cells.

In the current study, cells were derived from neonatal rat cardiomyocytes in primary culture rather than studying the H9c2 rat cardiomyocyte cell line. The biological activity of cardiac muscle cells obtained by this approach is more comparable to that of adult rats [40]. Differential adherence was used to improve the purity of the cardiac muscle cells. Thus, there is no guarantee for the purity of the cells, and other types of cells could be interspersed among the studied cardiomyocytes.

In summary, the current study used the p38 MAPK inhibitor SB, the CCR5 inhibitor DAPTA, and the NMDA receptor inhibitor MK801 to demonstrate that R5 HIV-1 gp120 activated the p38 MAPK signaling pathway to induce myocardial cell injury via the CCR5 coreceptor. Although SB reversed R5 HIV-1 gp120-mediated cardiomyocyte injury, it was hypothesized that the CCR5 inhibitor has an advantage for the treatment of HIV/AIDS-associated heart disease as the CCR5 inhibitor not

only hinders HIV entry, but also potentially provides myocardial protection. The findings further clarified the underlying mechanism of R5 HIV-1 gp120-mediated myocardial cell injury. Furthermore, this study may provide a novel theoretical and experimental basis for the treatment of HIV-associated heart diseases.

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Statement of Ethics

The animal experiments performed in the current study were approved by the Laboratory Animal Welfare and Ethics Committee of Fujian Medical University (Fuzhou, China) (No. FJMU IACUC 2015-042).

Disclosure Statement

The authors declare no conflicts of interest, financial or otherwise.

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Author Contributions

Q. Fang was the major in experimental design, guidance, and paper writing. R. Gao was the main participant in experimental design, implementation, and article writing. Q. Xu, J. He, W. Guo, and X. Zhang participated in literature retrieval and experimental implementation. H. Ye and Y. Chen participated in experimental design and clinical guidance. R. Wang, Z. Wu, and J. Yu participated in experimental implementation guidance.

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