Effect of N-terminal region of human parvovirus B19-VP1 unique region on cardiac injury in naïve mice

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Abstract. A unique region of human parvovirus B19 virus-VP1 (B19V-VP1u) has been linked to a variety of cardiac disorders. However, the precise role of B19V-VP1u in inducing cardiac injury remains unknown. The present study investigated the effects of B19V-VP1u and different regions of B19V-VP1u, including B19V-VP1uA (residues 1-60), B19V-VP1uB (residues 61-129), B19V-VP1uC (residues 130-195) and B19V-VP1uD (residues 196-227), on inducing cardiac injury in naïve mice by zymography, immunoblotting, H&E staining and cytokine immunoassay. A significantly higher MMP-9/MMP-2 ratio and increased levels of inflammatory cytokines, including IL-6 and IL-1β, were detected in the left ventricles of the mice injected with B19V-non-structural protein 1 (B19V-NS1) and B19V-VP1u, accompanied by increased expression levels of phosphorylated (p-)ERK and p-P38. Significantly upregulated expression levels of atrial natriuretic peptide (ANP), heart-type fatty acid-binding protein (H-FABP) and creatine kinase isoenzyme-MB (CK-MB), which are well-known cardiac injury markers, as well as increased infiltration of lymphocytes, were detected in the left ventricles of the mice injected with B19V-VP1, B19V-NS1 and B19V-VP1u. Moreover, a significantly higher MMP-9/MMP-2 ratio and increased levels of IL-6 and IL-1β were observed in the left ventricles of the mice injected with B19V-VP1u, B19V-VP1u-A, B19V-VP1u-B and B19V-VP1u-C, accompanied by upregulated p-ERK and p-P38 expression. Notably, significantly lower levels of IL-6 and IL-1β were observed in the left ventricles of the mice injected with B19V-VP1uD. Furthermore, significantly increased ANP, H-FABP and CK-MB expression levels were detected in the left ventricles of the mice injected with B19V-VP1u, B19V-VP1u-A and B19V-VP1u-B, along with enhanced infiltration of lymphocytes. Significantly higher serum IL-1β, IL-6, TNF-α and IFN-γ levels were also detected in the mice injected with B19V-VP1u, B19V-VP1u-A and B19V-VP1u-B. To the best of our knowledge, the findings of the present study were the first to demonstrate that the N-terminal region (residues 1-129) of B19V-VP1u induces an increase in the levels of cardiac injury markers, thus providing evidence for understanding the possible functional regions within B19V-VP1u.

Introduction

B19 virus (B19V), known as a human parvovirus, is a widespread DNA virus that may be found in patients across a wide age range (0-94 years); the immunity to B19V was reported to be ~78% in individuals >50 years old in various countries between 1992 and 1998 (1). The B19V genome encodes a non-structural protein (NS1), two capsid proteins (VP1 and VP2) and two smaller proteins, 7.5 and 11 kDa in weight (1). Of the B19V capsid proteins, ~95% are VP2, and the remaining 5% are VP1. The 227 amino acids at the N-terminal region of B19V-VP1u are known as the B19V-VP1-unique region (VP1u), which shares the same amino acid sequence with B19V-VP1 (2). B19V-NS1 is known to possess enzymatic activities, including helicase and ATPase activities, and DNA-binding activities that regulate viral transcription and replication (3,4). B19V-NS1 also increases the expression of IL-6 and TNF-α in host cells and induces cell apoptosis (5,6). The major capsid protein of B19V, VP2, can bind P antigen (globoside) on the cell surface for viral attachment, and the minor capsid protein VP1 is associated with the induction of immune responses during infection (7-9).

B19V infection has been linked to a range of clinical manifestations, such as myocarditis (10,11), hepatitis (12), nephrotic syndrome (13), thyroid disease (14), nerve damage (15), several types of cancer (16) and autoimmune diseases (17). To

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determine the association between B19V infection and cardiac disorders, the study of the presence of the B19V genome in cardiac tissues has attracted increased attention. Indeed, various studies have reported the presence of the B19V genome in endomyocardial tissues (18,19). Similar results were also obtained from the myocardium of patients with dilated cardiomyopathy (DCM) (20,21), fulminant myocarditis, sudden heart failure or chronic inflammatory cardiomyopathy (22,23).

B19V-VP1u has been reported to significantly increase TNF-α expression in H9c2 cells (24). Accumulating evidence has indicated that immunization of BALB/c mice with B19V-VP1u proteins can cause DCM (11). Moreover, the administration of B19V-VP1u in naïve mice can elicit ultrastructural changes of the heart and significantly increase the levels of various markers of cardiac injury, such as lactate dehydrogenase, creatine kinase isoenzyme and α-hydroxybutyric acid dehydrogenase (25). Significantly aggravated inflammation, including elevated MMP-9 activity and levels of inflammatory cytokines (IL-1β, IL-6 and TNF-α), has been detected in the left ventricles of the hearts of NZB/W F1 mice receiving B19V-VP1u IgG (26). Similar findings were also observed in patients with systemic lupus erythematosus with DCM, who possessed antibodies against B19V-VP1u, indicating an association between B19V-VP1u and DCM (27). These studies strongly suggest a pathological role of B19V-V1u in cardiomyopathy. Notably, our previous studies revealed the differential effects of truncated B19V-VP1u fragments on autoimmunity, particularly anti-phospholipid syndrome (28,29). However, the antigenicity of B19V-VP1u in causing cardiac damage remains unknown. Therefore, the aim of the present study was to further elucidate the roles of different B19V-VP1u fragments in inducing cardiac injury.

Materials and methods

Human parvovirus B19V proteins. Human B19V recombinant proteins were prepared as described in our previous publications (28,29). The B19V-NS1 protein was purified using the Profinia protein purification system (Bio-Rad Laboratories, Inc.) (28). The DNA sequences encoding B19-VP1, VP1u and truncated B19V-VP1uA (residues 1-60), B (residues 61-129), C (residues 130-195) and D (residues 196-227) were ligated into a pET-32a vector-Novagene (MilliporeSigma). These recombinant parvovirus B19 proteins were further induced with 1 mM IPTG for 3 h at 37°C and purified with a PureProteome™ Nickel Magnetic Beads system (MilliporeSigma). The purified proteins were further analyzed using high performance liquid chromatography (Waters HPLC 600 System; Waters Corporation). Briefly, 400 μl (0.2 mg/ml) purified proteins were injected into a 13-μm column (Superdex® 75 10/300 GL; MilliporeSigma) and eluted with elution buffer (25 mM Tris-HCL, pH 8.0; 150 mM NaCl) at a flow rate of 0.5 ml/min. The purity of the purified proteins ranged between 98.1 and 99.3%, and the endotoxin levels of purified recombinant proteins were all under the limits of detection (0.25 endotoxin units/ml).

Ethics and animal. Animal experiments were performed in accordance with the principles of replacement, refinement and reduction and were approved by the Institutional Animal Care and Use Committee (IACUC) of Chung Shan Medical University, Taichung (approval no. 1676; approved on December 2015). A total of 32 BALB/c BYJNarl female mice (age, 7 weeks; weight, 19-21 g) were obtained from the National Laboratory Animal Center and administrated under the IACUC at Chung Shan Medical University (Taichung, Taiwan). All animals were maintained in an air-conditioned room with a 12-h light-dark cycle at 22°C, and the relative humidity in the room was 55%. Animals were allowed free access to water and standard laboratory chow (Lab Diet 5001; PMI Nutrition International Inc.).

After 1 week of acclimation, the 8-week-old animals were randomly divided into eight groups (n=4 per group) as follows: i) Control group (PBS); ii) B19V-VP1 group; iii) B19V-NS1 group; iv) B19V-VP1u group; v) VP1u-A group; vi) VP1u-B group; vii) VP1u-C group; and viii) VP1u-D group. As described in previous reports (30,31) and in our previous publication (29), the initial and booster injections (2-3 times) are necessary to obtain optimal antibody responses. A total of 20 μg purified recombinant proteins or PBS was mixed 1:1 (v/v) with Freund's complete adjuvant (MilliporeSigma), and subcutaneously injected into mice of each group on day 1. The mice were then boosted with 20 μg recombinant proteins or PBS mixed 1:1 (v/v) with Freund's incomplete adjuvant (MilliporeSigma) twice times each on days 14 and 28. All animals were fasted for 12 h before sacrifice with CO_2 when they reached 16-weeks of age. A displacement rate of 20% of the chamber volume with CO_2 per min was used to euthanize the mice (performed in April 2018). The blood from the heart and the left ventricle tissues of mice were harvested (at 10 a.m.) and stored at -80°C until required for subsequent analysis. To confirm the induction of immunity by B19V-V1u and truncated B19V-VP1u recombinant proteins, absorption experiments were performed using a competitive (comp) ELISA (29) to verify the specificity of the antibodies in naïve mice receiving B19V-VP1u or truncated B19V-VP1u recombinant proteins (Fig. S1). Whole blood samples were obtained from the hearts of mice immunized with B19-VP1u or truncated B19V-VP1u recombinant proteins as aforementioned and were centrifuged at 1,500 x g for 10 min at room temperature to obtain the heart sera. For the absorption experiments (comp ELISA), the sera were pre-incubated with 500 μM VP1u or truncated VP1u proteins for 1 h at 37°C. Subsequently, all anti-sera (non-comp or comp groups) against 2 μg B19-VP1u or truncated B19V-VP1u recombinant proteins were immobilized on the surface of each sample well of 96-well plates. After incubation at room temperature for 120 min, the liquid from each sample well was removed, and the wells were washed with PBS and subsequently incubated with horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG (cat. no. A9044; MilliporeSigma) at a dilution of 1:1,000. After incubation at room temperature for 60 min, the liquid was removed from each sample well and the wells were washed with PBS. The color reaction was performed using 1 mg/ml substrate 2,2'azino-di-(3-ethylbenzthiazolin-6-sulphonic acid) (MilliporeSigma) in the presence of 0.005% H_2O_2 at room temperature for 15 min. A cutoff value was determined using sera from CTL mice, and the value was regarded as positive. Notably, the non-'comp' groups had enhanced absorbance as inoculation raised an immune response, whereas the 'comp'
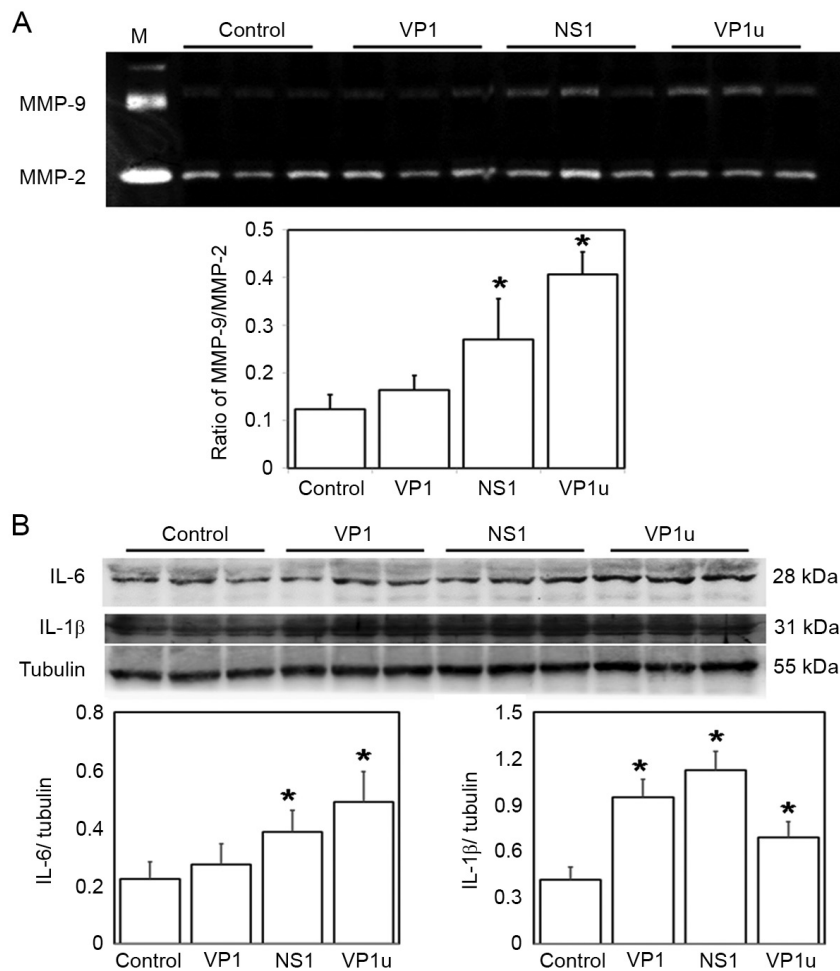
Figure 1. Effects of B19V-NS1, VP1 and VP1u on MMP-9 and MMP-2 activities and IL-6 and IL-1β expressions. (A) MMP-9 and MMP-2 activities in left ventricle tissues of the mice receiving different treatments (PBS, VP1, NS1 or VP1u). The activity ratio of MMP-9/MMP-2 is shown in the lower panel. M indicates mouse serum and was used as a positive control. (B) Protein expression levels of IL-6 and IL-1β in left ventricle tissues of the mice receiving different treatments. The lower panel shows the relative levels of IL-6 and IL-1β based on tubulin amount. Similar results were obtained in three-repeated experiments. *P<0.05 vs. Control (mice treated with PBS). n=4 for each group. B19V, B19 virus; B19V-NS1, B19V-non-structural protein 1; VP1u, VP1-unique region.

groups had reduced absorbance as antibodies were bound to the proteins from the initial absorption experiment (Fig. S1).

Gel zymography. MMP-9 and MMP-2 activities were measured as described previously (32). Protein concentrations were determined using a Bradford assay (Bio-Rad Laboratories, Inc.) and quantified using a U3000 spectrophotometer (Hitachi Ltd.). A total of 25 µg left ventricular tissue of mice from each experimental group was resolved on an 8% SDS-gel, via SDS-PAGE, that contained 0.1% gelatin. MMP-9 and MMP-2 activities were detected by staining the gel with 0.5% coomassie brilliant blue R-250 for 1 h at room temperature after soaking in reaction solution (40 mM Tris-HCl, 10 mM $CaCl_2$ and 0.02% NaN_3) for 18 h. A gel densitometry system (Alpha-Imager 2200; ProteinSimple) was used to quantify the relative MMP levels.

Protein preparation and immunoblotting. Protein concentration was determined by a modified Bradford's assay using a U3000 spectrophotometer at 595 nm with BSA (MilliporeSigma) as the standard. A total of 15 µg left ventricular tissue from each animal was resected and homogenized in 600 µl lysis buffer (PRO-PREP™; Intron Biotechnology, Inc.) with a tissue homogenizer (Polytron RT 3000; Brinkmann Instruments, Inc.). The homogenized tissues were then placed on ice for 30 min, and the supernatants of tissue lysates were harvested by centrifuging at 4˚C at 18,928 x g for 15 min. For immunoblotting, extracted proteins (25 µg/lane) were separated using 12 or 15% SDS-gels, and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Inc.). After blocking in 5% non-fat dry milk for 1 h at 25˚C, membranes were incubated with antibodies against IL-6 (1:500; cat. no. sc-1265), IL-1β (1:500; cat. no. sc-7884), ERK (1:1,000; cat. no. sc-514302), P38 (1:1,000; cat. no. sc-7972), phosphorylated (p)-P38 (1:1,000; cat. no. sc-7973), atrial natriuretic peptide (ANP; 1:500; cat. no. sc-20158) (all from Santa Cruz Biotechnology, Inc.), heart-type fatty acid-binding protein (H-FABP; 1:500; cat. no. ab16915), creatine kinase-MB (CK-MB; 1:500; cat. no. ab71722 (both from Abcam), p-ERK (1:1,000; cat. no. 05-797R; MilliporeSigma) and α-tubulin (1:500; cat. no. PA5-16891; Thermo Fisher Scientific, Inc.) for 3 h at 25˚C. The membranes were subsequently incubated with HRP-conjugated secondary antibodies (1:5,000; cat. nos. sc-2004 or sc-2005; Santa Cruz Biotechnology, Inc.)
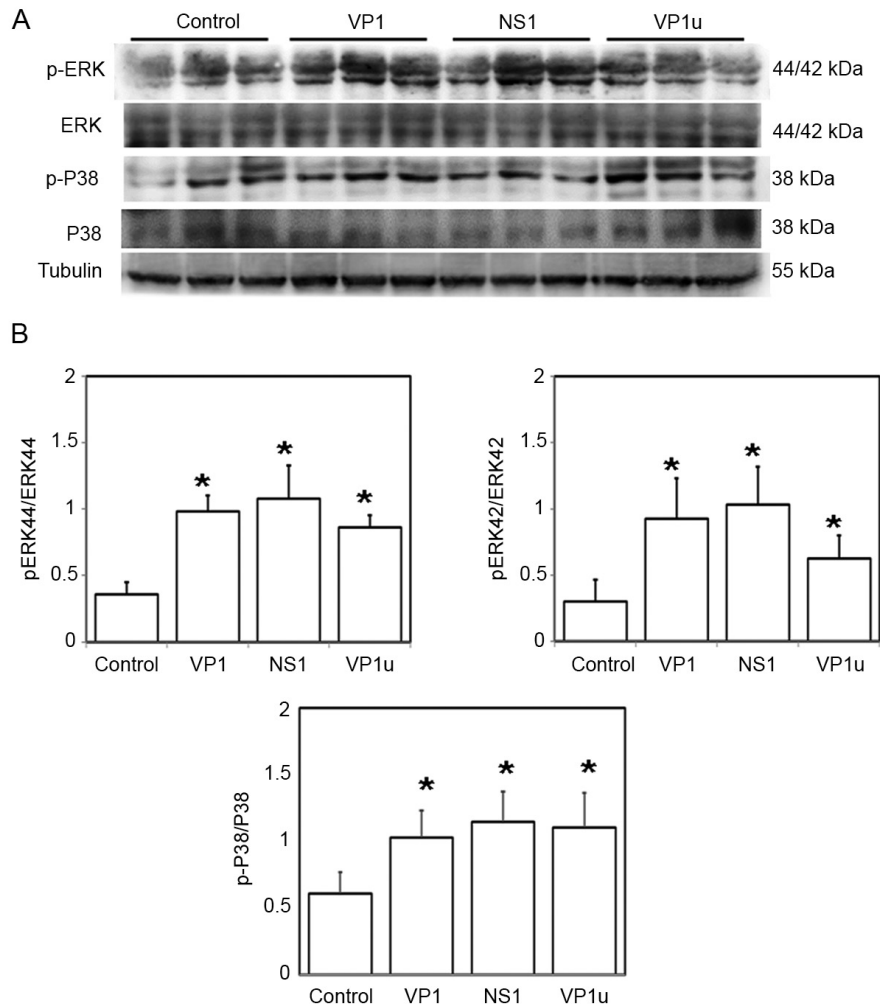
Figure 2. Effects of B19V-NS1, VP1 and VP1u on the phosphorylation of ERK and P38. (A) Protein expression levels of ERK, p-ERK, P38, and p-P38 in the left ventricle tissues of the mice receiving different treatments (PBS, VP1, NS1 or VP1u). (B) Relative levels of p-ERK and p-P38 are based on total protein expression. Similar results were obtained in three-repeated experiments. *P<0.05 vs. Control (mice treated with PBS). n=4 for each group. B19V, B19 virus; B19V-NS1, B19V-non-structural protein 1; VP1u, VP1-unique region; p-, phosphorylated.

at 25˚C for 1.5 h. Signals were visualized using Immobilon Western Chemiluminescent HRP Substrate (MilliporeSigma), and densitometry analysis was performed using an imaging analyzer (GE ImageQuant TL 8.1; GE Healthcare Life Sciences).

H&E staining. For H&E staining, the left ventricle tissues of mice were immersed in 10% formalin at 25˚C for 24 h and embedded in paraffin. The tissue blocks were sliced into 4-mm sections, deparaffinized and dehydrated. Next, the slides were stained with hematoxylin and eosin after processing at 25˚C for 5 min with 100, 95 and 75% ethanol. Each slide was subsequently immersed in 85% ethanol and 100% ethanol twice (20 min each immersion). Finally, the slides were immersed in xylene twice for 2 min. Photomicrographs were observed using a light microscope (Zeiss Axiophot microscope; Zeiss AG) at x200 and x400 magnifications.

Mouse cytokine immunoassay. The serum IL-1β, IL-6, TNF-α and IFN-γ levels of mice from each experimental group were detected with Bio-Plex mouse cytokine 8-plex assay kit, according to manufacturer's instructions (cat. no. Z6000004CF; Bio-Rad Laboratories, Inc.) and Bio-Plex

manager software version 3.0 using five parametric curve fitting (Bio-Rad Laboratories, Inc.).

Statistical analysis. GraphPad Prism 5 software (GraphPad Software, Inc.) was used to calculate the significant differences among groups using a one-way ANOVA followed by Tukey's multiple-comparisons test. All data are presented as the mean ± SEM, and were verified in ≥3 independent experiments. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of different recombinant B19V proteins on inducing markers of cardiac inflammation in naïve mice. To determine the influence of different B19V proteins on increasing cardiac inflammation, MMP-9 and MMP-2 activities were detected in the left ventricles of mice treated with PBS (Control), or recombinant B19V-VP1, B19V-NS1 or B19V-VP1u proteins. A significantly higher MMP-9/MMP-2 ratio was observed in left ventricular tissues of the mice from the B19V-NS1 and B19-VP1u groups compared with the control group (Fig. 1A).
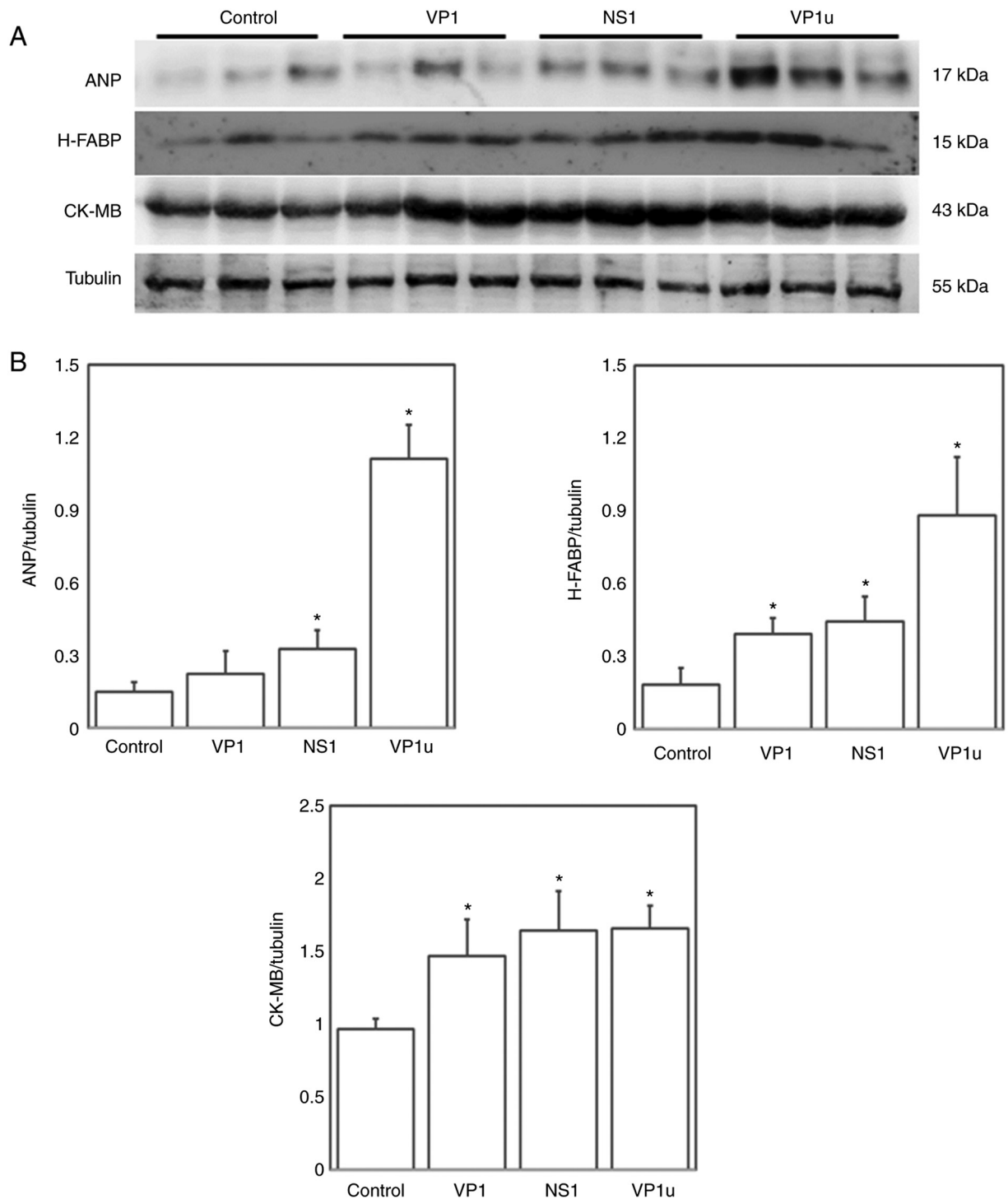
Figure 3. Effects of B19V-NS1, VP1 and VP1u on the expression levels of ANP, H-FABP and CK-MB. (A) Protein expression levels of ANP, H-FABP and CK-MB in the left ventricle tissues of the mice receiving different treatments (PBS, VP1, NS1 or VP1u). (B) Relative levels of ANP, H-FABP and CK-MB are based on tubulin expression. Similar results were obtained in three-repeated experiments. *P<0.05 vs. Control (mice treated with PBS). n=4 for each group. B19V, B19 virus; B19V-NS1, B19V-non-structural protein 1; VP1u, VP1-unique region; ANP, atrial natriuretic peptide; H-FABP, heart-type fatty acid-binding protein; CK-MB, creatine kinase isoenzyme-MB.

The levels of the inflammatory cytokines, IL-6 and IL-1β, were also detected. Significantly higher amounts of IL-6 were observed in the left ventricular tissues of the mice from the B19V-NS1 and B19-VP1u groups compared with the control group (Fig. 1B). Moreover, significantly increased IL-1β levels were detected in left ventricular tissues of mice from the B19V-VP1, B19V-NS1 and B19-VP1u groups compared with

the control group (Fig. 1B). In addition, significantly elevated p-ERK/ERK and p-P38/P38 ratios were observed in left ventricular tissues of the mice from the B19V-VP1, B19V-NS1 and B19-VP1u groups compared with the controls (Fig. 2).

Effects of different recombinant B19V proteins on inducing cardiac injury indicators in naïve mice. To understand the
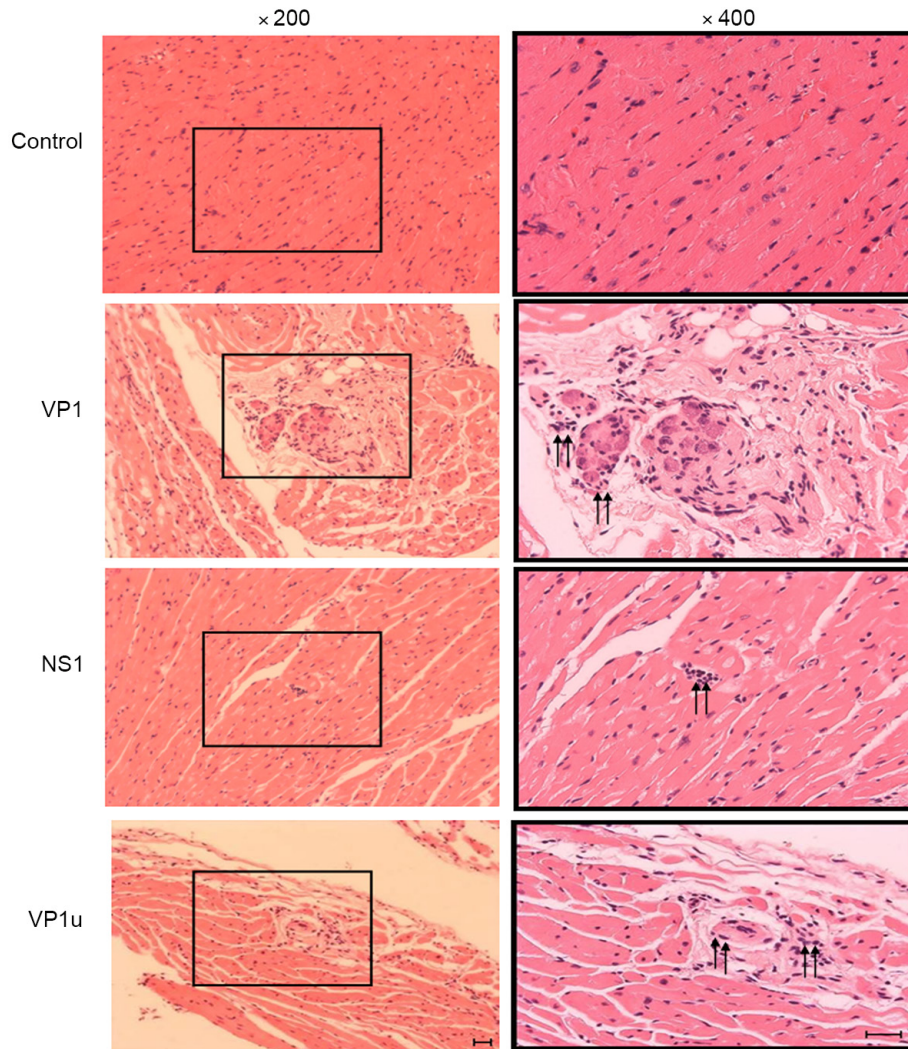
Figure 4. H&E staining of the left ventricles in naïve mice treated with B19V-NS1, VP1 or VP1u. Lymphocyte infiltration is indicated by an arrow in left ventricle tissues of naïve mice receiving different treatments (PBS, VP1, NS1 or VP1u). The images in the left panel and right panel were magnified by x200 and x400, respectively. Scale bar, 10 μm. B19V, B19 virus; B19V-NS1, B19V-non-structural protein 1; VP1u, VP1-unique region.

influence of different B19V proteins on increasing cardiac injury in naïve mice, various indicators of myocardial injury, including ANP, H-FABP and CK-MB, were measured via immunoblotting. Significantly increased ANP expression was detected in the left ventricular tissues of mice from the B19V-NS1 and B19V-VP1u groups compared with the control group (Fig. 3). Moreover, significantly elevated H-FABP and CK-MB expression was observed in left ventricular tissues of mice from the B19V-VP1, B19V-NS1 and B19V-VP1u groups compared with the control group (Fig. 3). Accordingly, infiltration of lymphocytes was observed in the left ventricles of mice injected with B19V-VP1, B19V-NS1 and B19V-VP1u proteins (Fig. 4).

Effects of different regions of recombinant B19V-VP1u proteins on inducing increases in the levels of cardiac inflammatory markers in naïve mice. To further verify the effects of different regions of B19V-VP1u on cardiac inflammation, the MMP-9 and MMP-2 activities, as well as IL-6 and IL-1β levels were detected in the left ventricles of mice treated with PBS, or recombinant B19V-VP1u, B19V-VP1u-A,

B19V-VP1u-B, B19V-VP1u-C or B19V-VP1u-D proteins. A significantly higher MMP-9/MMP-2 ratio was detected in left ventricular tissues of mice from the B19V-VP1u, B19V-VP1u-A, B19V-VP1u-B and B19V-VP1u-C groups compared with the controls (Fig. 5A). Moreover, significantly higher levels of IL-6 and IL-1β were detected in the left ventricular tissues of the mice from the B19V-VP1u, B19V-VP1u-A, B19V-VP1u-B and B19V-VP1u-C groups compared with the controls (Fig. 5B). Conversely, significantly lower IL-6 and IL-1β levels were observed in the left ventricular tissues of mice from the B19V-VP1u-D group compared with the controls (Fig. 5B). Moreover, significantly increased ratios of p-ERK/ERK and p-P38/P38 were observed in the left ventricular tissues of mice from the B19V-VP1u, B19V-VP1u-A, B19V-VP1u-B and B19V-VP1u-C groups compared with the controls (Fig. 6).

Effects of different regions of recombinant B19V-VP1u on indicators of cardiac injury in naïve mice. To investigate the effects of different regions of B19V proteins on increasing cardiac injury in naïve mice, the expression levels of ANP, H-FABP and CK-MB proteins were detected
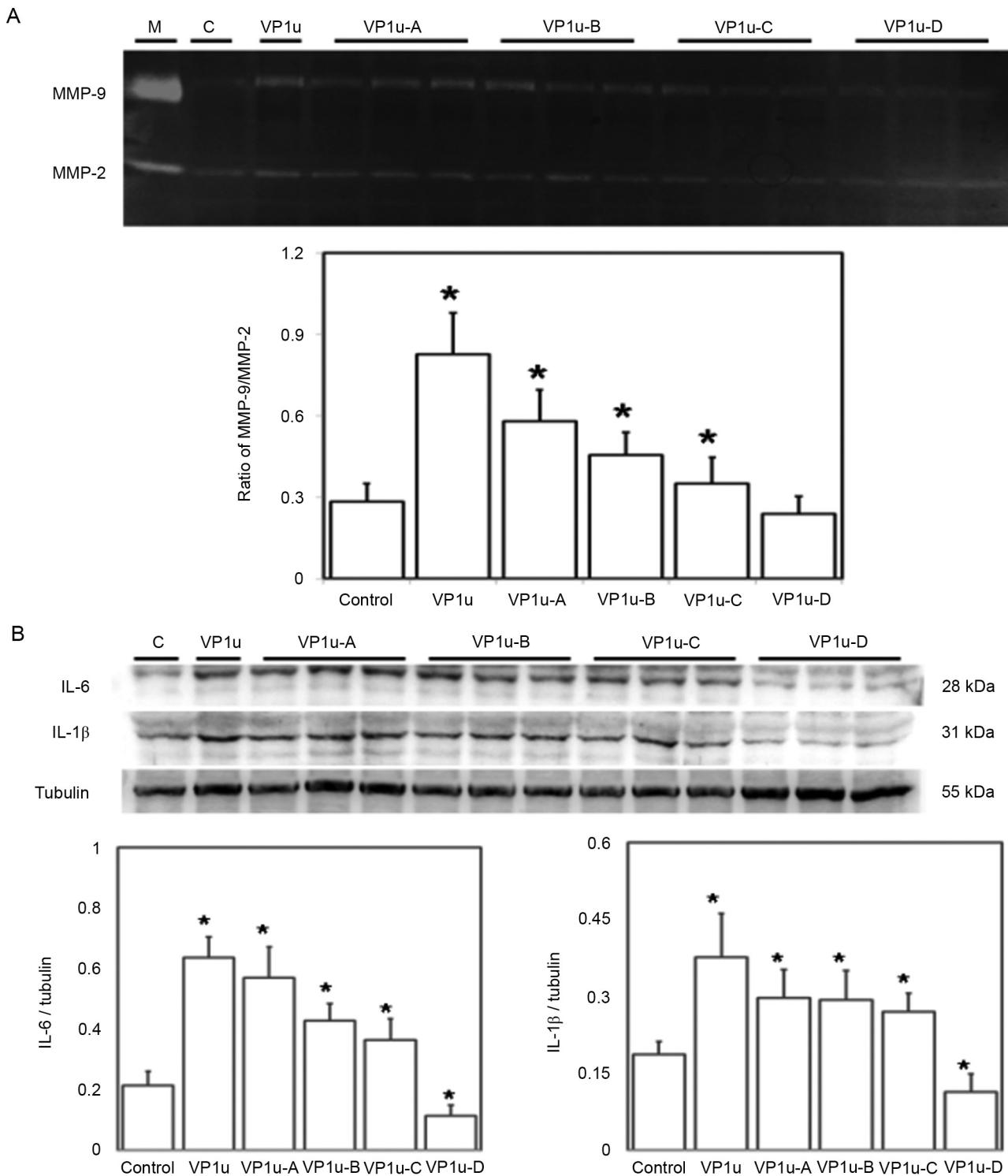
Figure 5. Effects of B19V-VP1u and its truncated fragments on MMP-9 and MMP-2 activities and IL-6 and IL-1β expression. (A) MMP-9 and MMP-2 activities in left ventricle tissues of mice receiving different treatments (PBS, VP1u, VP1u-A, VP1u-B, VP1u-C or VP1u-D). The MMP-9/MMP-2 activity ratio is shown in the lower panel. M indicates mouse serum and was used as a positive control; C indicates control mice treated with PBS. (B) Protein expression levels of IL-6 and IL-1β in the left ventricle tissues of the mice receiving different treatments. The lower panel shows the levels of IL-6 and IL-1β based on tubulin expression. Similar results were obtained in three-repeated experiments. *P<0.05 vs. Control (mice treated with PBS). n=4 for each group. B19V, B19 virus; VP1u, VP1-unique region.

via immunoblotting. Significantly higher expression levels of ANP, H-FABP and CK-MB were observed in the left ventricles of mice injected with B19V-VP1u, B19V-VP1u-A and B19V-VP1u-B proteins compared with the controls (Fig. 7). Accordingly, increased infiltration of lymphocytes

was observed in the left ventricles of mice injected with B19V-VP1u, B19V-VP1u-A, B19V-VP1u-B and B19V-VP1u-C (Fig. 8). In addition, significantly higher serum IL-1β, IL-6, TNF-α and IFN-γ levels were detected in mice injected with B19V-VP1u and B19V-VP1u-A, and only higher serum
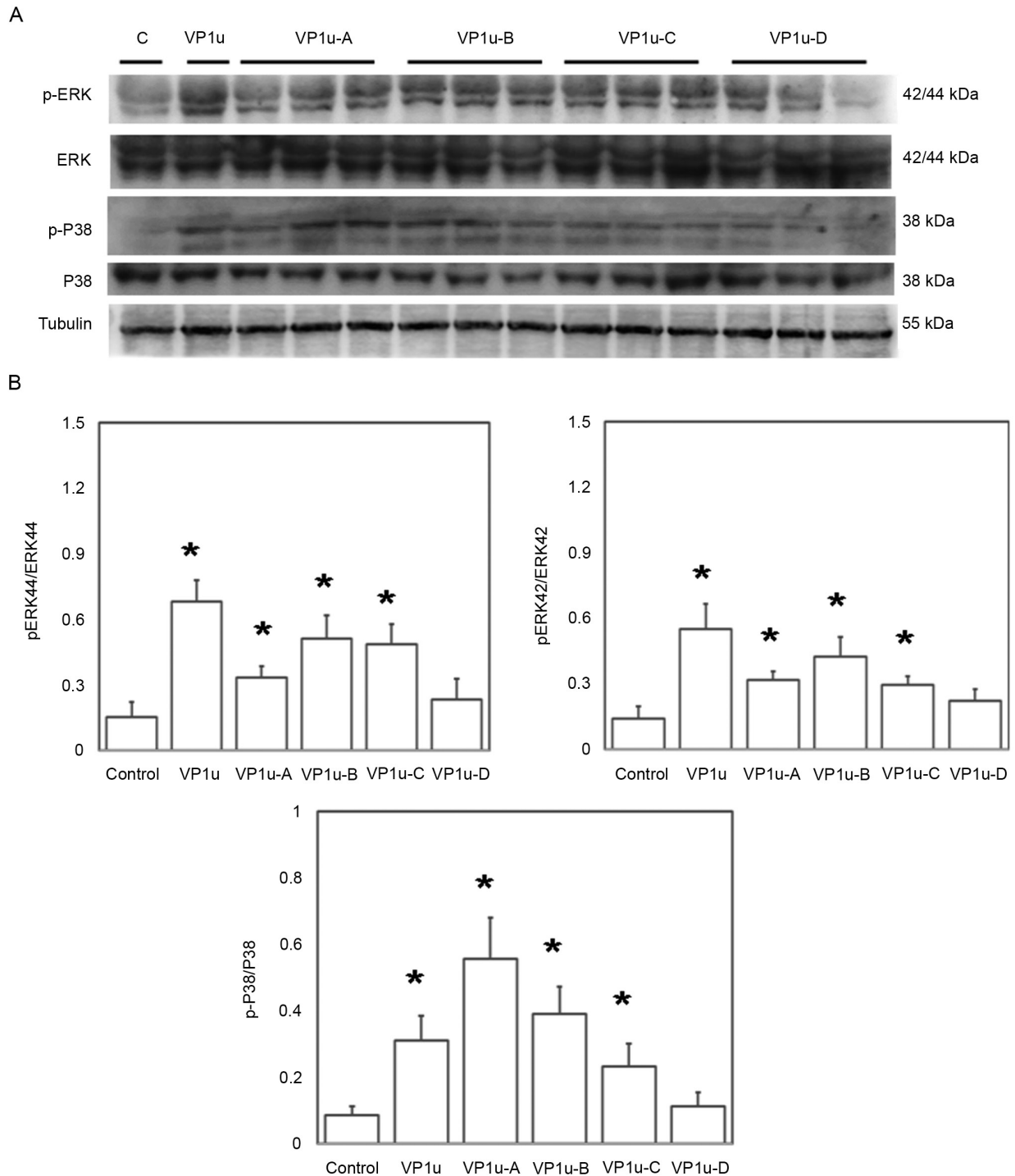
Figure 6. Effects of B19V-VP1u and its truncated fragments on the phosphorylation of ERK and P38. (A) ERK, p-ERK, P38 and p-P38 expression in left ventricle of the mice receiving different treatments (PBS, VP1u, VP1u-A, VP1u-B, VP1u-C or VP1u-D). C indicates control mice treated with PBS. (B) Relative expression levels of p-ERK and p-P38 are based on total protein expression. Similar results were obtained in three-repeated experiments. *P<0.05 vs. Control (mice treated with PBS). n=4 for each group. B19V, B19 virus; VP1u, VP1-unique region; p-, phosphorylated.

IL-1β and IL-6 levels were detected in mice injected with B19V-VP1u-B proteins compared with the controls (Fig. 9).

Discussion

Both B19V-NS1 and B19V-VP1u proteins have been linked to various heart disorders such as inflammatory cardiomyopathy and DCM (11,24-25). Notably, significantly increased cardiac inflammation was detected in NZB/W F1 mice receiving

B19V-VP1u IgG (26). In addition, among patients with systemic lupus erythematous, a potential association between B19V-VP1u and DCM was also reported (27). Although these findings revealed an association between B19V-VP1u and cardiac disorders, the roles of the antigenicity and functional motifs of B19V-VP1u in the induction of cardiac injury remain unknown. The present study identified a significantly increased MMP-9/MMP-2 ratio and elevated IL-6, IL-1β, ANP, H-FABP and CK-MB levels in the left ventricles of mice
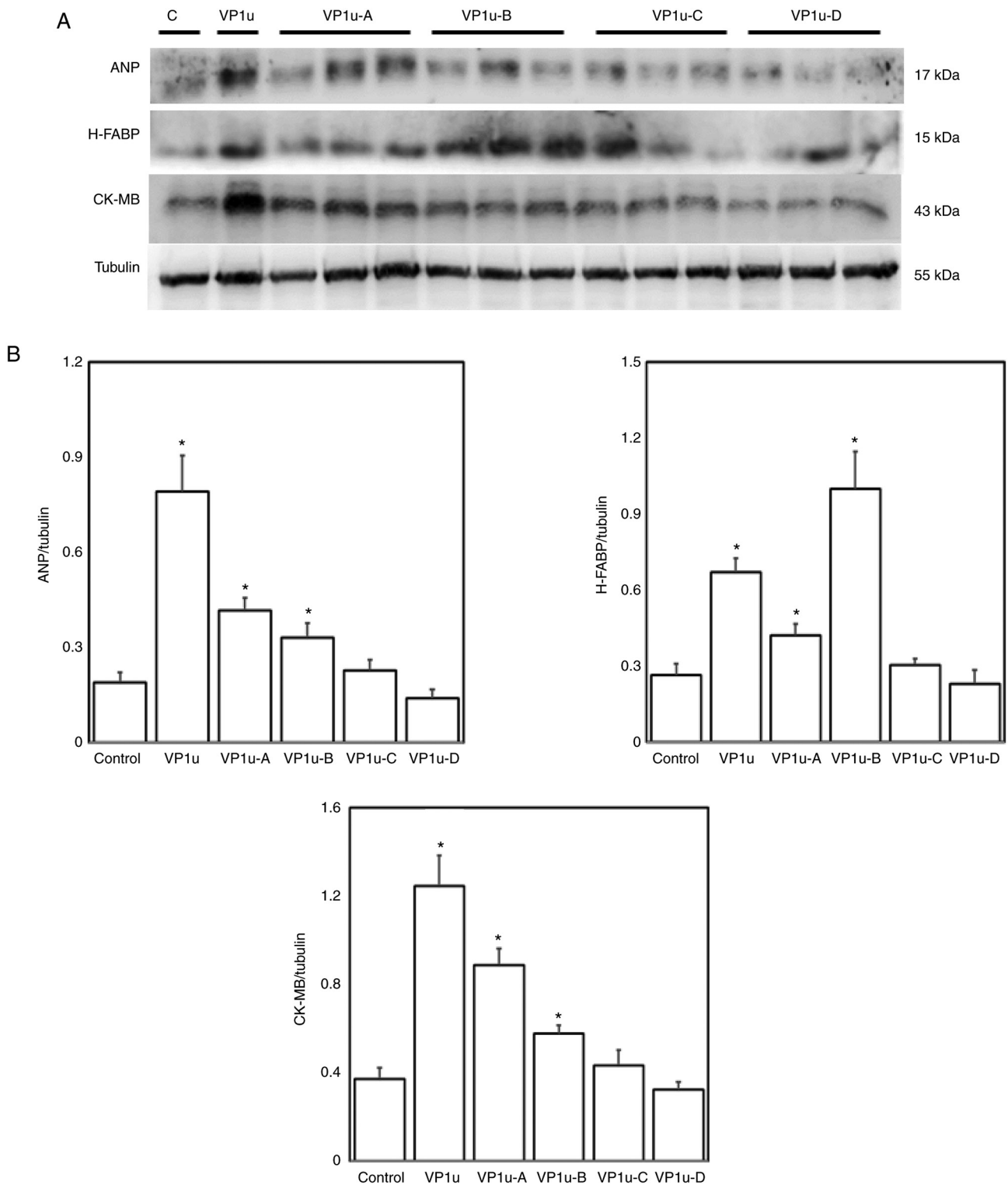
Figure 7. Effects of B19V-VP1u and its truncated fragments on expression levels of ANP, H-FABP and CK-MB. (A) Protein expression levels of ANP, H-FABP and CK-MB in the left ventricle tissues of the mice receiving different treatments (PBS, VP1u, VP1u-A, VP1u-B, VP1u-C or VP1u-D). C indicates control mice treated with PBS. (B) Relative levels of ANP, H-FABP and CK-MB are based on tubulin expression. Similar results were obtained in three-repeated experiments. *P<0.05 vs. Control (mice treated with PBS). n=4 for each group. B19V, B19 virus; VP1u, VP1-unique region; ANP, atrial natriuretic peptide; H-FABP, heart-type fatty acid-binding protein; CK-MB, creatine kinase isoenzyme-MB.

treated with B19V-NS1 and B19V-VP1u. Moreover, signifi-cantly increased MMP-9/MMP-2 ratios and IL-6 and IL-1β levels were detected in the left ventricles of mice treated with B19V-VP1u-A, B19V-VP1u-B and B19V-VP1u-C. Significantly higher levels of ANP, H-FABP and CK-MB proteins were

also observed in the left ventricles of mice treated with B19V-VP1u, B19V-VP1u-A and B19V-VP1u-B, accompanied by significantly higher serum IL-1β, IL-6, TNF-α and IFN-γ levels. Conversely, significantly lower levels of IL-6 and IL-1β were observed in the left ventricles of mice treated with
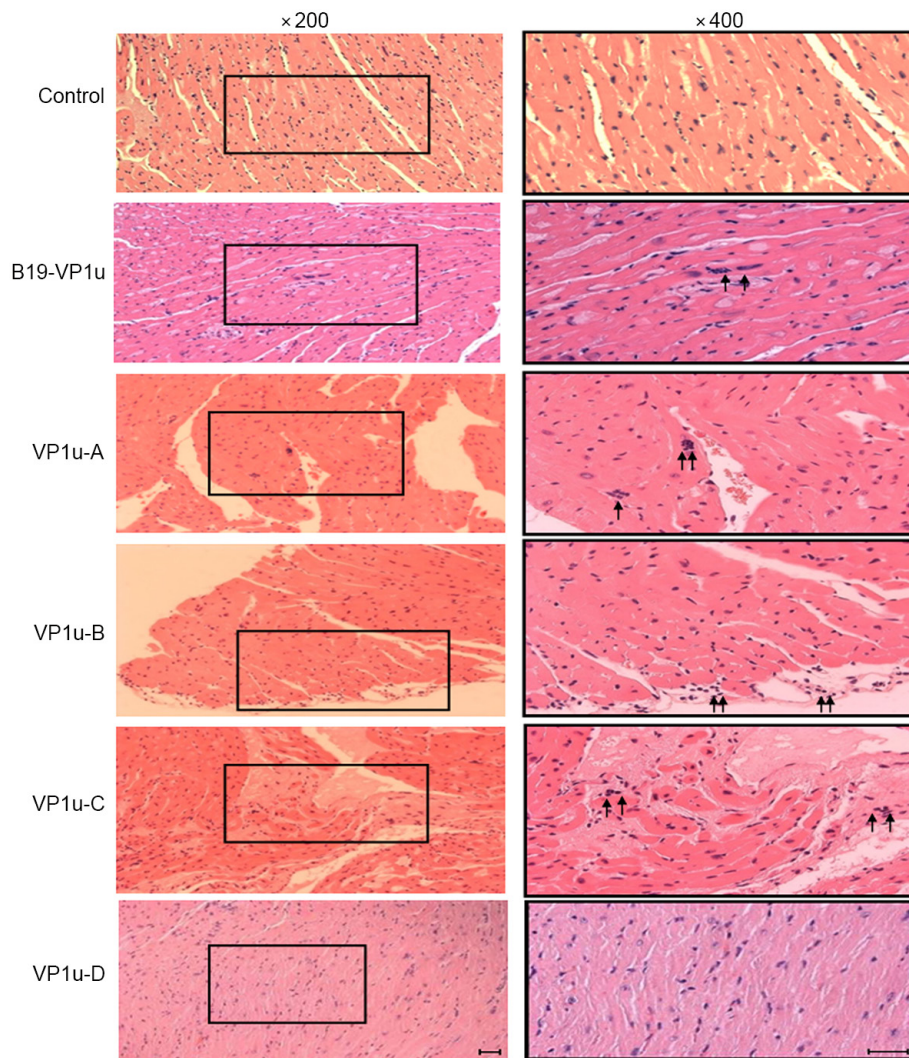
Figure 8. H&E staining of the left ventricles in naïve mice treated with PBS (control), B19-VP1u, VP1u-A, VP1u-B, VP1u-C or VP1u-D. Lymphocyte infiltration was indicated by an arrow in the left ventricle tissues of naïve mice receiving different treatments (PBS, VP1u, B19-VP1u-A, B19-VP1u-B, B19-VP1u-C or B19-VP1u-D). The images in the left panel and right panel were magnified by x200 and x400, respectively. Scale bar, 10 μm. B19V, B19 virus; VP1u, VP1-unique region.

B19V-VP1u-D. These results are similar to previous findings, which revealed that B19V-VP1u could significantly increase cardiac injury (11,25) and the first to show the differential effects of recombinant B19V-VP1u fragments on inducing the expression of cardiac injury markers.

MMPs, metal-bound enzymes that require zinc and calcium for their enzymatic activity and stability, are pivotal mediators involved in the remodeling of the extracellular matrix (33). Numerous studies have reported the essential roles of MMPs in organ development and subsequent tissue remodeling during inflammation and injury (34). MMPs, specifically MMP-9, also participate in the remodeling processes that occur following various cardiac injuries, such as myocarditis, atherosclerosis, heart failure, myocardial infarction and DCM (35). Indeed, MMP-9 has been shown to be involved in the inflammation and cardiac remodeling of Chagas disease, and the ratio of MMP-9/MMP-2 is considered as a potential biomarker in delineating the risk of cardiovascular injury (36).

ANP is expressed in the atria and ventricular myocardium, and its major physiological function is to reduce cardiac output, as well as to lower blood volume and systemic blood pressure (37). ANP is directly associated with increased stress caused by stretching, and is considered as a marker of congestive heart failure in both animal models and humans (38). ANP is known as a biochemical marker of cardiac hypertrophy, which ultimately leads to ventricular dilatation and heart failure (39). FABPs are expressed in various cells and tissues, such as cardiomyocytes, red skeletal muscle, kidney, hepatocytes, small intestine and adipocytes, and are associated with fatty acid metabolism (40). H-FABP is representative of its family in cardiac and red skeletal muscle, and is recognized as a biomarker of heart failure, chronic obstructive pulmonary disease and long-term postischemic prognosis (41,42). Although CK-MB is not heart-specific, CK-MB is still used in the diagnosis of acute myocardial infarction (43,44). To the best of our knowledge, the present study was the first to reveal that B19V-VP1u-A and B19V-VP1u-B increased ANP, H-FABP and CK-MB expression levels in the left ventricles of naïve mice compared with the controls, whereas no differences in ANP, H-FABP and CK-MB expression levels were observed in
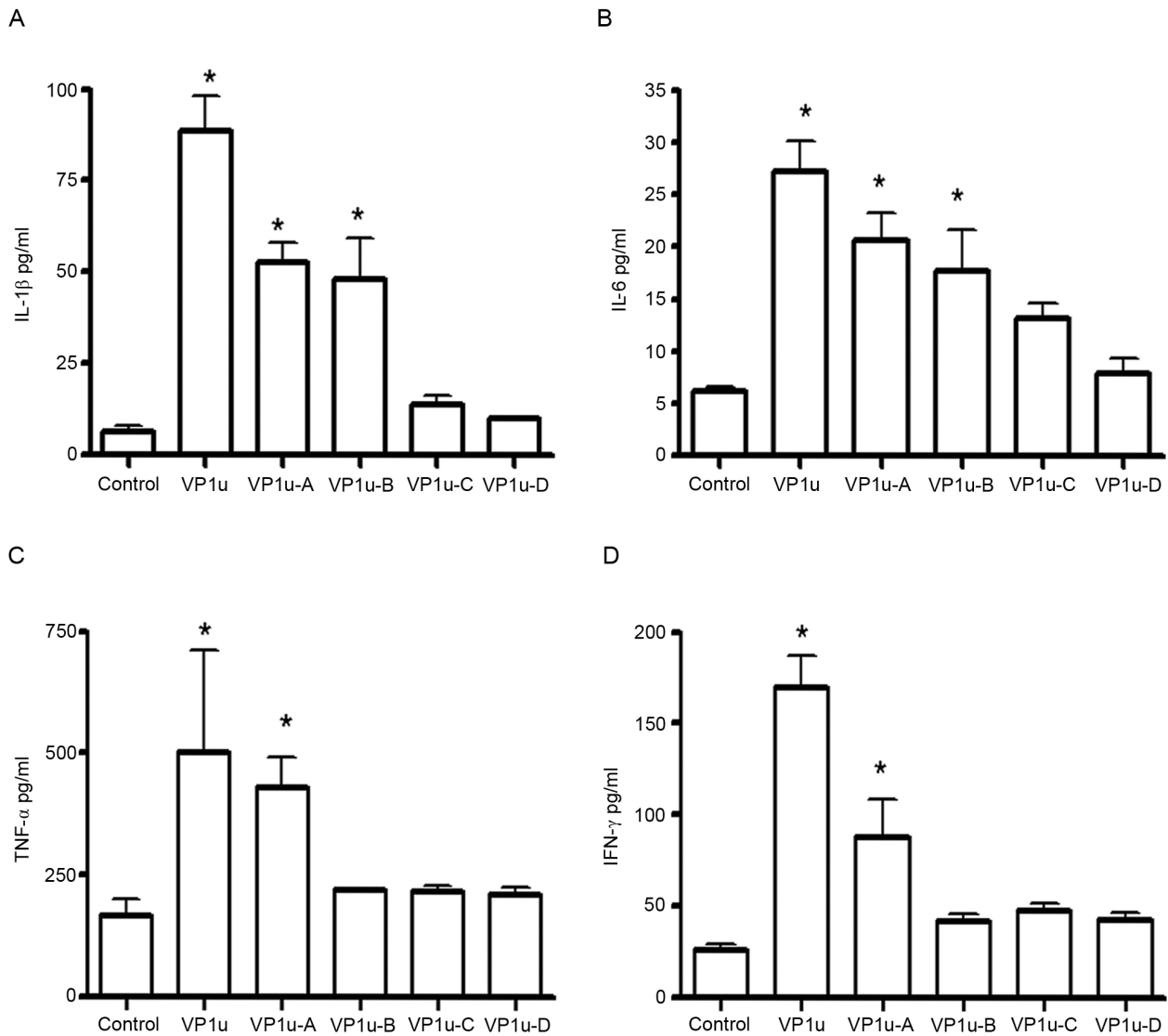
Figure 9. Levels of serum cytokines. The serum (A) IL-1β, (B) IL-6, (C) TNF-α and (D) IFN-γ levels in mice treated with B19V-VP1u, VP1u-A, VP1u-B, VP1u-C or VP1u-D were detected using Bio-Plex mouse cytokine assay kits. Data are presented as the mean ± SD. Similar results were obtained in three-repeated experiments. *P<0.05 vs. Control. n=4 for each group. B19V, B19 virus; VP1u, VP1-unique region.

the mice treated with B19V-VP1u-C and B19V-VP1u-D. These findings suggest that the N-terminal residues (residues 1-129) of B19V-VP1u exert a notable effect on the induction of the expression of various cardiac injury markers in the left ventricle.

Various cytokines serve essential roles in the pathogenesis of myocarditis and cardiomyopathy (45,46). Elevated plasma TNF-α, IL-6 and IL-1β levels have been reported in patients suffering from acute myocardial infarction (47,48). Evidence from both human and animal models has shown that proinflammatory cytokines, such as IL-1β, IL-2, IL-6 and TNF-α, are involved in myocarditis, cardiac depression and heart failure (49,50). The present study reported similar results, that B19V-VP1u, B19V-VP1u-A and B19V-VP1u-B increased the levels of IL-1β and IL-6 in the left ventricle of naïve mice. Moreover, significantly increased levels of phosphorylation of P38 and ERK were also detected, which was consistent with previous findings showing that both ERK and P38 signaling are involved in the induction of IL-1β and IL-6 (51). These results demonstrated that the 129 N-terminal amino acids of

B19V-VP1u (B19V-VP1u-A and B19V-VP1u-B) served key roles in inducing IL-1β and IL-6 levels via the ERK and P38 signaling pathways.

Previous studies have shown that the neutralizing epitopes of B19V are located within the N-terminal 100 amino acids of B19-VP1u, which contributes to B19V-induced immune responses (52,53). The N-terminal amino acids from residues 5-80 of B19-VP1u are essential for cellular binding and B19V uptake (54), which indicates an important role of the N-terminal region of B19V-VP1u in immune regulation following B19V infection. However, little is known regarding the functional motifs in the C-terminal domain of B19V-VP1u. To the best of our knowledge, the present study was the first to show that IL-1β and IL-6 levels were significantly lower in the left ventricles of mice treated with B19V-VP1u-D (amino acids 196-227) compared with mice from the control group. This finding prompts a rational assumption that a negative regulatory motif on IL-1β and IL-6 may be located within the C-terminal region of B19V-VP1u. However, further studies are required to verify the precise location of the motif and determine its function.

There were certain limitations to the present study. Firstly, evidence has revealed that engagement of B19V-VP2 and globoside (P-antigen), and the following interaction of B19V-VP1 and α5β1 integrin are essential for B19 infection of erythroid cells and their progenitors (55). However, to the best of our knowledge, no study has investigated the potential receptor for B19V-VP1u and its truncated fragments on cardiac cells. Therefore, further research is required to verify the possible receptors on cardiac cells, as well as their downstream interactions. Second, the present study lacked the assessment of cardiac function using echocardiography or electrocardiography, which may provide further understanding of the physiological role of truncated B19V-VP1u fragments on cardiac injuries. Third, the underlying mechanism and related signaling molecules involved in truncated B19V-VP1u fragment-induced cardiac injury remain unknown, as do the mechanism via which B19V DNA entering cardiomyocytes. These issues require further investigations to elucidate the differential effects and mechanisms of truncated B19V-V1u fragments on cardiac injury.

In conclusion, the present study identified the differential effects of truncated B19V-VP1u fragments and its ability to induce an increase in the levels of cardiac inflammatory and injury markers in naïve mice. The 129 N-terminal residues of B19V-VP1u, including B19V-VP1u-A and B19V-VP1u-B, predominantly induced the expression of inflammatory proteins, such as MMP-9, cardiac IL-1β and IL-6, and increased the serum levels of IL-1β, IL-6, TNF-α and IFN-γ, as well as cardiac injury markers, such as ANP, H-FABP and CK-MB. Conversely, the C-terminal residues (amino acids 196-227) attenuated the levels of proinflammatory cytokines, including IL-1β and IL-6, but did not affect MMP-9 activity or the induction of cardiac injury markers. Taken together, these findings may provide further information and improve the current understanding of the regulatory roles of B19V-VP1u in inducing cardiac injury.

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Availability of data and materials

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

Authors' contributions

KCH was involved in the study conception and design, and analysis of data. ZYH and JLY performed experiments and analysis of data. TCH was involved in the study conception and design, drafting and revising of the manuscript, performing experiments, analysis of data, and study supervision. BST was involved in the study conception and design, drafting and revising of the manuscript, and analysis of data. TCH and BST confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Animal experiments were performed in accordance with the principles of replacement, refinement and reduction and were approved by the Institutional Animal Care and Use Committee (IACUC) of Chung Shan Medical University, Taichung (approval no. 1676; approved in December 2015).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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