Human parvovirus B19 VP1u-induced osteoclastogenesis is enhanced by RANKL in RAW264.7 macrophage cells

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Abstract. Human parvovirus B19 (B19V) DNA and antigens are frequently detected in synovial tissue; however, the mechanisms linking B19V infection to bone destruction remain unclear. The present study investigated the B19V VP1 unique region (VP1u) and its influence on osteoclast differentiation. RAW264.7 macrophages were cultured with or without receptor activator of NF-κB ligand (RANKL) and B19V-VP1u for up to 7 days. Tartrate-resistant acid phosphatase staining, bone resorption assays and western blotting were used to assess osteoclastogenesis. The involvement of signaling pathways was investigated using an anti-TNF-α antibody, and inhibitors of MAPK, ERK, JNK and NF-κB signaling. B19V-VP1u alone induced osteoclastogenesis in a dose- and time-dependent manner and, in the presence of RANKL, further increased osteoclast size and number. B19V-VP1u significantly increased pit resorption, and nuclear factor of activated T cells (NFAT)2 (also known as NFATc1), cathepsin K, c-FOS, MMP9 and dendritic cell-specific transmembrane protein expression. The inhibition of ERK, JNK, NF-κB or TNF-α did not alter the effects of VP1u, whereas blockade of p38 MAPK nearly abolished VP1u-induced osteoclastogenesis. These findings indicate that B19V-VP1u acts as a strong osteoclastogenic factor through a p38-dependent but TNF-α-independent pathway *in vitro*. The present study supports a dual mechanism of B19V-induced bone pathology, whereby VP1u directly promotes osteoclastogenesis, whereas inflammation mediates bone resorption via cytokine signaling. Targeting viral protein-host signaling interactions may offer novel therapeutic approaches for B19V-associated bone erosion.

Introduction

Osteoclasts are multinucleated cells derived from the macrophage lineage that serve as the principal effectors of bone resorption. Their differentiation, which is governed primarily by receptor activator of NF-κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF), is essential for skeletal homeostasis (1,2). However, when aberrantly activated, osteoclasts drive pathological bone loss in conditions such as rheumatoid arthritis (RA), osteoporosis and chronic inflammatory diseases (3,4). In RA, osteoclast-mediated bone erosion develops rapidly and persists despite disease-modifying therapies, underscoring the need to identify alternative mechanisms of osteoclastogenesis beyond canonical cytokine-driven pathways (3).

Human parvovirus B19 (B19V) is a small, nonenveloped, single-stranded DNA virus with global distribution. Seroprevalence increases with age and transmission primarily occurs through respiratory droplets, with less frequent spread reported via blood products (5,6). B19V preferentially infects erythroid progenitor cells; additionally, it can abortively infect diverse cell types, resulting in a broad spectrum of clinical outcomes, including erythema infectiosum and arthralgia in genetically susceptible or autoimmune-affected hosts, such as those with RA (5,7).

Among environmental triggers, viral infections have gained attention. B19V is frequently detected in the synovium and blood of patients with RA, where it is associated with increased disease activity and cytokine expression (as TNFα, IL6, IL1β) (7-9). Osteoclast differentiation is governed by RANKL-RANK signaling, which activates intracellular cascades, including those involving MAPKs (p38, ERK and

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JNK) and NF-κB. These pathways converge on c-FOS and nuclear factor of activated T cells (NFAT)2 (also known as NFATc1), which are master transcription factors essential for osteoclastogenesis (1,10,11). Inflammatory cytokines, such as TNF-α and IL-1β, further amplify pathological bone resorption by engaging the same signaling pathways, often synergizing with RANKL (1,11). NF-κB activation is also indispensable, with TRAF6 and related adaptors coupling RANKL stimulation to both the MAPK and NF-κB pathways (11-13). Evidence for the role of B19V infection in osteoclastogenesis is lacking; however, prior studies have shown that proinflammatory stimuli can exploit these canonical cascades to enhance osteoclast formation (1,10,11).

B19V-VP1 unique region (VP1u) contains a secreted phospholipase A2 (PLA2) domain as a group XIII PLA2 enzyme that can induce fibroblast-like synoviocyte migration, macrophage activation, and the production of IL-6, TNF-α and IL-1β (8,14-17). These cytokines directly promote RANKL expression and osteoclast differentiation (18-20); therefore, VP1u has emerged as a plausible viral mediator of osteoclast-driven bone pathology. In addition, pathogen-mediated osteoclastogenesis has been described for several viruses, including SARS-CoV-2, dengue virus and chikungunya virus, all of which enhance osteoclast differentiation or resorptive activity (21,22). However, whether B19V-VP1u promotes osteoclast differentiation and function through proinflammatory signaling pathways remains unknown. To address this, the current study investigated the effects of the recombinant VP1u protein on osteoclastogenesis in RAW264.7 cells, a murine macrophage cell line widely used as an *in vitro* model of osteoclast precursors (23). Additionally, the underlying signaling pathways were investigated with the aim of elucidating the mechanism by which VP1u induces osteoclastogenesis.

Materials and methods

Recombinant B19V-VP1u protein. The recombinant B19V-VP1u protein was prepared as described previously (24). Briefly, the transformation of plasmid pET32a-B19V-VP1u into *Escherichia coli* BL21-DE3 competent cells (Yeastern Biotech Co., Ltd.) was induced by the addition of IPTG (1 mM) at 37˚C for 3 h and purified with Ni-NTA spin columns (Qiagen, Inc.). Endotoxin tests were conducted using the Limulus Amebocyte Lysate Endochrome Assay (Charles River Laboratories, Inc.), and endotoxin levels were below the detection limit (0.25 endotoxin unit/ml).

Cell culture and differentiation. The murine macrophage cell line RAW264.7 (BCRC no. 60001; Bioresource Collection and Research Center) was cultured in DMEM (Gibco; Thermo Fisher Scientific Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific Inc.) without antibiotics at 37˚C in 5% CO_2. For osteoclast differentiation, the $1x10^4$ cells were seeded into 8-well Millicell EZ chamber slides (cat. no. PEZGS0816; MilliporeSigma) and simultaneously cultured in the presence or absence of recombinant RANKL (100 ng/ml; Thermo Fisher Scientific, Inc.) and/or B19V-VP1u protein (1 μg/ml). M-CSF was not added to the culture media. Tartrate-resistant acid phosphatase (TRAP) staining was performed at 25˚C on days 1, 3, 5 and 7. In addition, RAW264.7 cells were also cultured with decreasing concentrations of VP1u (1, 0.1, 0.01, 0.001 μg/ml) for 5 days to determine whether B19V-VP1u-induced osteoclastogenesis is dose-dependent. To explore the signaling pathways involved in B19V-VP1u-induced osteoclastogenesis, anti-TNF-α antibody (1 μg/ml; ABclonal Biotech Co., Ltd.) or pathway inhibitors, including the p38 MAPK inhibitor SB203580 (10 μM; Cayman Chemical Company), ERK inhibitor PD98059 (10 μM; Cayman Chemical Company), JNK inhibitor SP600125 (10 μM; Cayman Chemical Company) and NF-κB inhibitor BAY11-7085 (10 μM; Cayman Chemical Company) (25,26), were added 1 h before stimulation.

TRAP staining. TRAP staining was performed using a kit (cat. no. 387A; Sigma-Aldrich; Merck KGaA) according to manufacturer's protocol. TRAP-positive cells appeared purple to dark red under light microscopy and yellow/green-colored cells are TRAP-negative, and cells containing ≥3 nuclei were defined as osteoclasts (25-27). The number and morphology of TRAP-positive multinucleated cells were recorded for each well, and cell size was quantified using ZEN 3.0 software (Zeiss AG).

Bone resorption assay. A bone resorption assay kit (cat. no. CSR-BRA-24KIT; Cosmo Bio Co., Ltd.) was used according to manufacturer's protocol. A total of $1x10^4$ RAW264.7 cells were seeded into 24-well plates, purchased precoated with calcium phosphate and chondroitin sulfate, and cultured with or without RANKL (100 ng/ml) (25-28) and B19V-VP1u (1 μg/ml) in DMEM/F-12 (Gibco; Thermo Fisher Scientific Inc.) supplemented with 20% FBS and without phenol red at 37˚C in 5% CO_2. After 7 days, the cells were removed with 5% sodium hypochlorite and resorption pits were visualized by inverted microscopy (Carl Zeiss AG) and quantified using ImageJ version 1.54 (National Institutes of Health).

Western blotting. The cells were harvested after 5 days of culture, centrifuged at 300 g for 10 min at room temperature, and then lysed in PRO-PREP protein extraction solution (Intron Biotechnology, Inc.) on ice for 2 h. The lysates were further centrifuged at 16,100 g for 30 min at 4˚C, and the supernatants were collected as protein extracts. 25 μg of Proteins loaded per lane were separated by 12% SDS-PAGE using the Mini-PROTEAN system (Bio-Rad Laboratories, Inc.) and transferred onto PVDF membranes (Immobilon-E, 0.45 μM; MilliporeSigma). After blocking with 5% non-fat dry milk for 1 h at 25˚C, antibodies against NFAT2 (1:1,000; cat. no. A1539; Abclonal Biotech Co., Ltd.), cathepsin K (CTSK; 1:500; cat. no. A5871; Abclonal Biotech Co., Ltd.), c-FOS (1:1,000; cat. no. ab190289; Abcam), MMP9 (1:1,000; cat. no. A0289; Abclonal Biotech Co., Ltd.), dendritic cell-specific transmembrane protein (DC-STAMP; 1:1,000; cat. no. A14630; Abclonal Biotech Co., Ltd.) and loading controlβ-actin (1:5,000; cat. no. MAB1501; MilliporeSigma) were incubated with the membranes at 25˚C for 3 h with gentle agitation. To detect antigen-antibody complexes, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:5,000; cat. nos. sc-2004 and sc-2005; Santa Cruz Biotechnology, Inc.) at 25˚C for 1.5 h. Immunoreactive

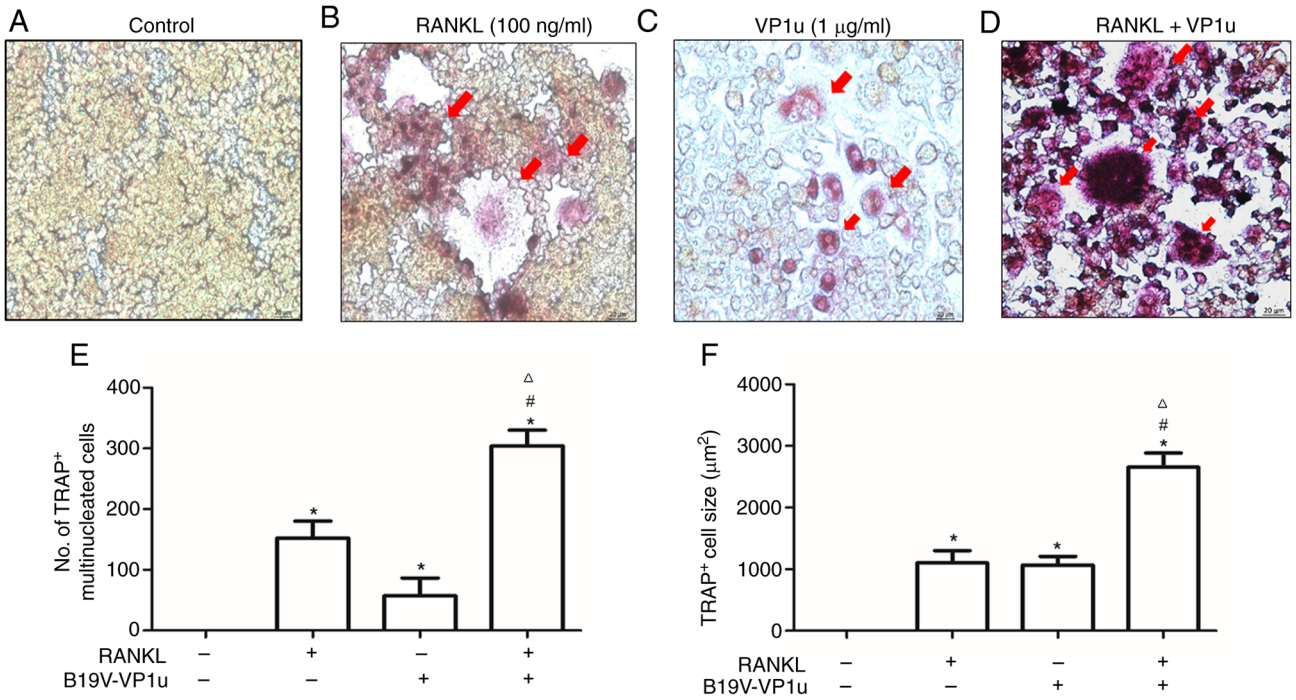

Figure 1. B19V-VP1u induces osteoclast formation. Representative images of TRAP staining in RAW264.7 cells cultured under different conditions for 7 days: (A) Control (untreated), (B) RANKL (100 ng/ml), (C) B19V-VP1u (1 μg/ml) and (D) RANKL + B19V-VP1u. Red arrows indicate TRAP-positive multinucleated osteoclasts. Scale bar=20 μm. (E) Quantification of TRAP-positive multinucleated cells. (F) Measurement of TRAP-positive cell size (μm^2). *P<0.05 vs. control; #P<0.05 vs. RANKL; ΔP<0.05 vs. VP1u. B19V-VP1u, parvovirus B19 virus VP1 unique region; RANKL, receptor activator of NF-κB ligand; TRAP, tartrate-resistant acid phosphatase.

bands were visualized with ECL reagents (MilliporeSigma) and detected using a FUJIFILM LAS-4000 imaging system (FUJIFILM Corporation). Band intensities were semi-quantified using ImageJ.

Statistical analysis. All experiments were performed in triplicate. Data are presented as the mean ± SD and were analyzed using GraphPad Prism 5.0 (Dotmatics). Statistical significance was determined using one-way ANOVA with Bonferroni's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

B19V-VP1u induces osteoclast differentiation. To assess whether B19V-VP1u promotes osteoclastogenesis, RAW264.7 cells were cultured with RANKL, VP1u or both for 7 days and stained for TRAP activity. As shown in Fig. 1A-D, control cells exhibited minimal TRAP positivity, whereas RANKL induced the formation of multinucleated osteoclasts. Stimulation with VP1u alone also induced TRAP-positive multinucleated cells, and co-stimulation with RANKL markedly increased osteoclastogenesis compared with stimulation with either stimulus alone, resulting in the production of larger, more abundant osteoclasts. Quantification confirmed that the number of TRAP-positive multinucleated cells was significantly greater in the VP1u group than in the control group, and was further increased in the RANKL + VP1u group relative to the RANKL group (Fig. 1E). Consistent with these findings, the average size of TRAP-positive multinucleated cells was significantly greater with combined stimulation than with single treatments (Fig. 1F).

Dose- and time-dependent effects of B19V-VP1u-induced osteoclastogenesis. To determine whether B19V-VP1u-induced osteoclastogenesis is dose-dependent, RAW264.7 cells were cultured with decreasing concentrations of VP1u. Robust TRAP-positive multinucleated osteoclast formation was observed at 1 μg/ml, whereas markedly fewer osteoclasts were detected at 0.1 μg/ml and virtually none were detected at ≤0.01 μg/ml (Fig. 2A-D). Time-course analysis further revealed that compared with RANKL, VP1u required prolonged stimulation to induce osteoclastogenesis. Quantification revealed minimal osteoclast formation on day 1 in all conditions; however, by days 5-7, VP1u significantly increased both the number (Fig. S1) and size (Fig. S2) of TRAP-positive multinucleated cells, particularly when combined with RANKL (Fig. 2E and F). Together, these findings suggest that VP1u promotes osteoclastogenesis in a dose- and time-dependent manner, in the presence of RANKL, further increasing osteoclast differentiation and maturation.

B19V-VP1u enhances osteoclastic bone resorption activity, and NFAT2, MMP9, CTSK, c-FOS and DC-STAMP protein expression. To assess the functional consequences of VP1u-induced osteoclastogenesis, bone resorption was evaluated on calcium phosphate-coated plates. VP1u alone modestly increased resorption, but co-stimulation with RANKL resulted in markedly increased resorption areas (Fig. 3A-D). Quantitative analysis confirmed that the resorption pit area was significantly greater in the combined treatment group (Fig. 3E). In addition

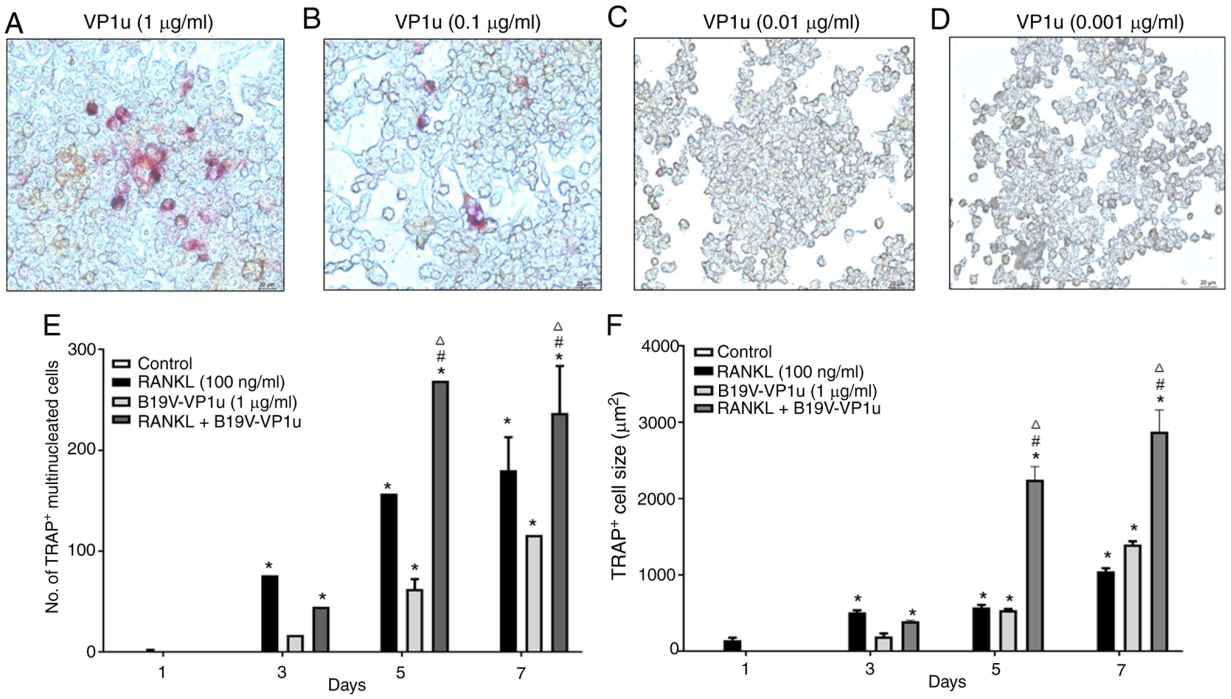

Figure 2. Dose- and time-dependent effects of B19V-VP1u on osteoclast formation. Representative TRAP staining of RAW264.7 cells cultured for 7 days with decreasing concentrations of B19V-VP1u: (A) 1 μg/ml, (B) 0.1 μg/ml, (C) 0.01 μg/ml and (D) 0.001 μg/ml. Scale bar, 20 μm. (E) Quantification of TRAP-positive multinucleated cells at days 1, 3, 5 and 7 under four conditions: Control, RANKL (100 ng/ml), B19V-VP1u (1 μg/ml) and RANKL (100 ng/ml) + B19V-VP1u (1 μg/ml). (F) Quantification of TRAP-positive cell size (μm^2) at corresponding time points. *P<0.05 vs. control; $^\#$P<0.05 vs. RANKL; $^\Delta$P<0.05 vs. VP1u. B19V-VP1u, parvovirus B19 virus VP1 unique region; RANKL, receptor activator of NF-κB ligand; TRAP, tartrate-resistant acid phosphatase.

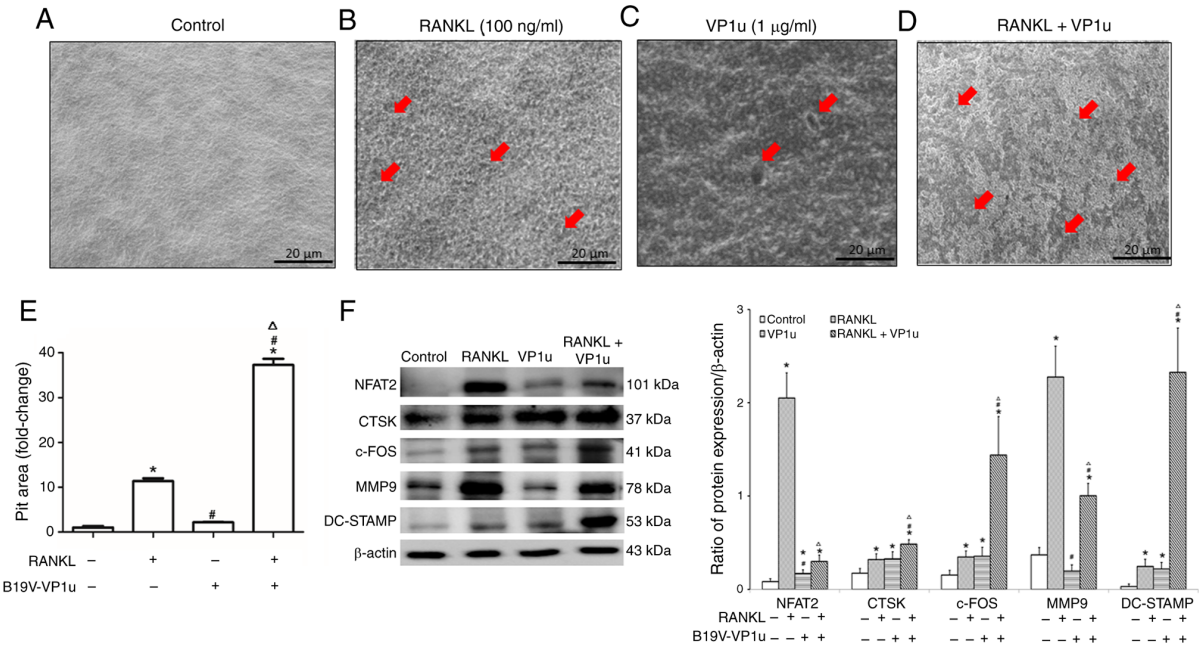

Figure 3. B19V-VP1u enhances osteoclastic bone resorption activity. Representative images of bone resorption pits on calcium phosphate-coated plates after 7 days of culture under different conditions: (A) Control, (B) RANKL (100 ng/ml), (C) B19V-VP1u (1 μg/ml) and (D) RANKL + B19V-VP1u. Red arrows indicate resorption pits. Scale bar, 20 μm. (E) Quantification of resorption pit area. (F) Western blot analysis of NFAT2, CTSK, c-FOS, MMP9 and DC-STAMP expression in osteoclasts, with densitometric semi-quantification shown. Data are presented as the mean ± SD of triplicate experiments. *P<0.05 vs. control; $^\#$P<0.05 vs. RANKL; $^\Delta$P<0.05 vs. VP1u. B19V-VP1u, parvovirus B19 virus VP1 unique region; CTSK, cathepsin K; DC-STAMP, dendritic cell-specific transmembrane protein; NFAT2, nuclear factor of activated T cells 2; RANKL, receptor activator of NF-κB ligand.

NFAT2 and MMP9 expression levels were decreased in the VP1u group compared with RANKL group, CTSK, c-FOS and DC-STAMP were upregulated in the VP1u group compared with the control group, with the strongest expression observed in the RANKL+VP1u group (Fig. 3F). These findings demonstrate that VP1u enhances osteoclast bone-resorptive activity.

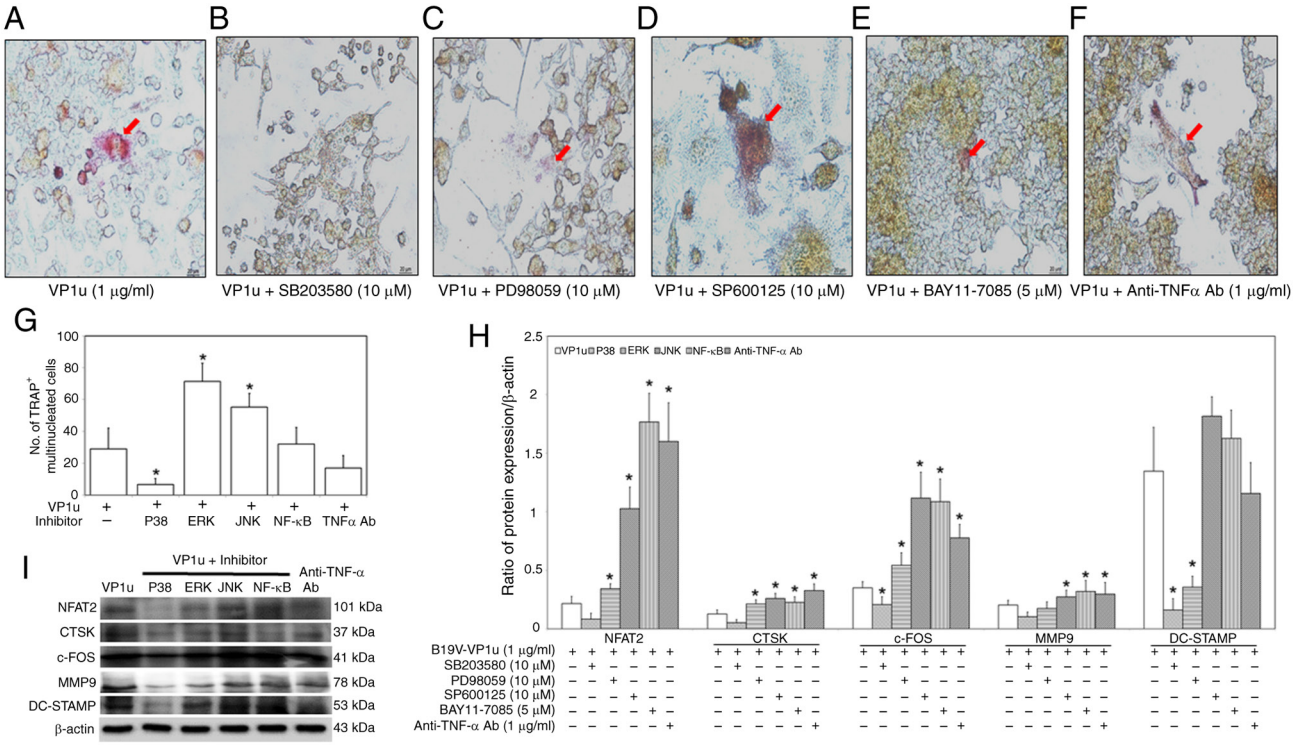

Figure 4. p38 MAPK signaling mediates B19V-VP1u-induced osteoclastogenesis. Representative TRAP staining of RAW264.7 cells cultured for 7 days with B19V-VP1u (1 μg/ml) in the presence of different inhibitors: (A) No inhibitor, (B) p38 MAPK inhibitor (SB203580, 10 μM), (C) ERK inhibitor (PD98059, 10 μM), (D) JNK inhibitor (SP600125, 10 μM), (E) NF-κB inhibitor (BAY11-7085, 5 μM), (F) anti-TNF-α antibody (1 μg/ml). Red arrows indicate TRAP-positive multinucleated osteoclasts. Scale bar=20 μm. (G) Number of TRAP+ multinucleated cells in the presence of different inhibitors. (H) Densitometric quantification of protein expression of NFAT2, CTSK, c-FOS, MMP9, and DC-STAMP. (I) Western blot analysis of NFAT2, CTSK, c-FOS, MMP9, and DC-STAMP in each group. *P<0.05 vs. VP1u. Ab, antibody; B19V-VP1u, parvovirus B19 virus VP1 unique region; CTSK, cathepsin K; DC-STAMP, dendritic cell-specific transmembrane protein; NFAT2, nuclear factor of activated T cells 2; TRAP, tartrate-resistant acid phosphatase.

p38 MAPK signaling mediates B19V-VP1u-induced osteoclastogenesis. To elucidate signaling pathways, RAW264.7 cells were stimulated with VP1u in the presence of inhibitors targeting specific signaling pathways. TRAP staining revealed that inhibition of ERK, JNK, NF-κB or TNF-α had little effect on VP1u-induced osteoclast formation, whereas the p38 MAPK inhibitor almost completely blocked osteoclastogenesis (Fig. 4A-G). Similarly, the expression levels of NFAT2, CTSK, c-FOS, MMP9 and DC-STAMP were strongly reduced only by p38 MAPK inhibition (Fig. 4H-I). B19V-VP1u-induced osteoclastogenesis was markedly suppressed by the p38 MAPK inhibitor, but not by ERK, JNK or NF-κB inhibition, or TNF-α neutralization (Fig. 4H-I). These results indicate that VP1u primarily promotes osteoclast differentiation through the p38 MAPK pathway (Fig. 5).

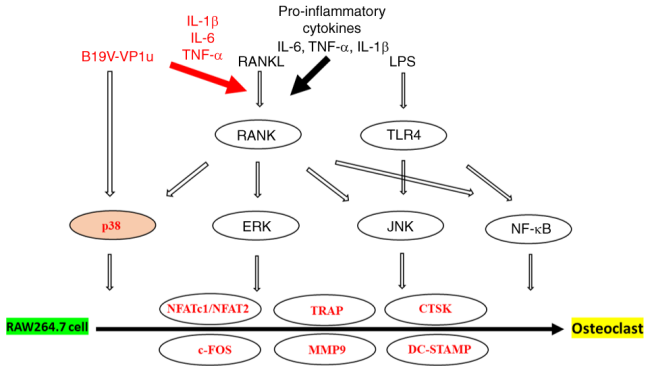

Figure 5. Proposed mechanism of B19V-VP1u-induced osteoclastogenesis. Recombinant B19V-VP1u stimulates RAW264.7 macrophages, leading to the production of proinflammatory cytokines (IL-1β, IL-6 and TNF-α) (15). VP1u directly activates the p38 MAPK pathway, which promotes the expression of osteoclast markers, including TRAP, NFAT2, CTSK, c-FOS, MMP9 and DC-STAMP, thereby driving differentiation into multinucleated osteoclasts with enhanced bone-resorptive capacity. B19V-VP1u, parvovirus B19 virus VP1 unique region; CTSK, cathepsin K; DC-STAMP, dendritic cell-specific transmembrane protein; LPS, lipopolysaccharide; NFAT2, nuclear factor of activated T cells 2; RANKL, receptor activator of NF-κB ligand; TLR4, Toll-like receptor 4; TRAP, tartrate-resistant acid phosphatase.

Discussion

The findings of the present study extend previous work showing that B19V-VP1u, through its secreted PLA2 activity, activates fibroblast-like synoviocytes and macrophages to secrete IL-6, TNF-α and IL-1β (15,29). Such cytokines are known to promote RANKL expression and osteoclast differentiation (18-20,30). However, this direct effect on osteoclast precursors suggests a mechanism by which B19V infection may induce bone destruction even when TNF-α is neutralized, potentially explaining the persistence of erosive disease in patients with RA despite cytokine-targeted immunosuppression (8,14).

B19V DNA and antigens are frequently detected in the synovial tissue from patients with RA, and their presence is associated with elevated cytokine levels and increased disease activity (8,14,31,32). The findings of the present study support

a model in which VP1u contributes to joint destruction by both stimulating cytokine release from macrophages and directly promoting osteoclastogenesis. This dual action could explain why some patients with RA continue to develop erosive disease despite immunosuppressive therapy targeting cytokines.

Previous studies have demonstrated that VP1u enhances osteoclastogenesis, promoting pathological bone resorption both *in vitro* and *in vivo*, likely through its PLA2 activity and subsequent activation of downstream inflammatory pathways (5-7). These findings suggest that compared with classical endogenous mediators, VP1u is a plausible environmental trigger for bone destruction in patients with RA. The activation of p38 MAPK by VP1u has been observed in macrophages and synoviocytes, suggesting that p38 MAPK is a critical node in VP1u-driven inflammatory and osteoclastogenic signaling (15). The p38 MAPK pathway is critically implicated in RA, where it drives chronic inflammation and joint destruction by upregulating proinflammatory cytokines and catabolic mediators, highlighting its role as a central therapeutic target in inflammatory arthritis (15,33). Targeting this pathway may offer new strategies against virus-mediated bone erosion.

The current study has several limitations, primarily due to its reliance on *in vitro* cell experiments. Rather than using bone marrow macrophages (BMMs), RAW264.7 cells were used for osteoclastic differentiation in the present study, as they are readily available, easily cultured and highly responsive to RANKL stimulation (26,27). By contrast, BMMs require additional M-CSF (often 30 ng/ml) alongside RANKL for osteoclastic differentiation (3,27). In addition, while quantitative PCR could provide complementary information, the current study focused on functional, protein-level analyses of NFATc1/NFAT2, c-Fos, MMP9 and DC-STAMP, providing additional robust evidence of B19V-VP1u involvement in osteoclastogenesis. In addition, the findings indicated that both VP1u (independent of RANKL) and VP1u + RANKL effectively induced osteogenic differentiation in RAW264.7 cells, enhancing the understanding of bone erosion during B19V viral infection. Further evidence from *in vivo* experiments is needed to elucidate the role of VP1u in osteoclast differentiation (34) and to support its contribution to erosive disease, such as in patients with RA (8-9,14). However, B19V has high host specificity (infecting only humans), and at present, there is no natural animal model that fully mimics the human infection process (7). Bone erosion is a chronic, progressive disease influenced by multiple factors (3). Because B19V cannot replicate in mice, it typically induces a human-like immunopathological response (such as skin fibrosis or thrombosis) when specific viral proteins, such as recombinant B19V-VP1u, are injected (16,17). Thus, the use of appropriate animal models, such as intra-articular or systemic administration of recombinant VP1u in mice, combined with micro-CT analysis and TRAP staining (35), may validate the pathological relevance of the present findings and VP1u-mediated bone destruction in future studies.

In conclusion, the present study identified B19V-VP1u as a novel viral mediator of osteoclast differentiation and function via the p38 MAPK pathway that acts in a dose-dependent manner and is enhanced by RANKL. These findings provide mechanistic insight into how B19V may contribute to bone erosion in RA and other inflammatory conditions, highlighting VP1u-p38 MAPK signaling as a potential therapeutic target to mitigate virus-induced osteoclastogenesis.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contribution

CYL, BST, CCT and TCH were involved in the study conception and design, drafted and revised the manuscript, and performed data analysis. YHL and TCH performed the experiments and analyzed the data. BST and TCH confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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