International Microbiology (2023) 26:757–764
https://doi.org/10.1007/s10123-023-00330-8

ORIGINAL ARTICLE

Immune escape of bovine parvovirus by VP1 inhibiting IFN-β production through the RIG-I-like receptor pathway

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Received: 27 July 2022 / Revised: 6 January 2023 / Accepted: 17 January 2023 / Published online: 27 January 2023

Abstract

Objective The present study aimed to explore if bovine parvovirus (BPV) impacts beta interferon (IFN-β) production and to reveal further molecular mechanism of BPV immune escape.

Method The pCMV-Myc-BPV-VP1 recombinant plasmid was verified with both double-enzyme digestion and sequence. HEK 293 T cells were transfected with this recombinant protein and then infected with the vesicular stomatitis virus (VSV). Expression levels of IFN-β mRNA were detected using qPCR.

Results The expression level of BPV VP1 mRNA in the pCMV-Myc-BPV-VP1 group was significantly higher than those of the untreated group (UT) and pCMV-Myc vector group. BPV virus copies in bovine turbinate (BT) cells of the BPV-VP1 group were raised ($P < 0.05$) with an increment of 5.8×10^4. Expression levels of IFN-β mRNA of the BPV VP1 group in HEK 293 T cells were decreased ($P < 0.01$). Following treatment of TBK1 and IRF3(5D), IFN-β expression levels in HEK 293 T cells were depressed. Additionally, expression levels of TBK1, IRF3(5D), MDA5, and MAVS were less than those of the flag empty vector, respectively.

Conclusion pCMV-Myc-BPV-VP1 could heighten transcription levels of VP1 protein in BT cells, promote BPV proliferation, and ascend the production of IFN-β. Overexpression of pCMV-Myc-BPV-VP decreased IFN-β mRNA expression in HEK 293 T cells and inhibited IFN-β production induced by TBK1 and IRF3(5D). Furthermore, BPV VP1 obviously declined expression levels of TBK1, IRF3(5D), MDA5, and MAVS in the RIG-I-like receptor (RLR) pathway. Our findings revealed a novel mechanism evolved by BPV VP1 to inhibit type I IFN production and provided a solid scientific basis into the immunosuppression of BPV.

Keywords Bovine parvovirus · VP1 protein · IFN-β · RIG-I-like receptor

Introduction

Bovine parvovirus (BPV) was firstly identified in the 1960s in diarrhea calves. BPV is a member of the *Bocaparvovirus* genus with a non-enveloped capsid (Dudleenamjil et al. 2010), and it can cause not only diarrhea in neonatal calves, but also respiratory and reproductive disease in adult cattle (Dudleenamjil et al. 2010). Morbidity of BPV infections is about 83 to 100% worldwide (Qinghe Zhu et al. 2022). The viral capsid plays a critical role in the infection of host cells by adhering the virus to specific receptors on target cells (Mietzsch et al. 2018). The genome comprises of single-stranded DNA (ssDNA) and possesses three open reading frames (ORFs; ORF1, ORF2, and ORF3). ORF3 encodes two capsid viral proteins (VP1 and VP2). Both VP1 (75 kDa) and VP2 (61 kDa) share a C-terminal end.

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Currently, the majority of the data available for these viruses are epidemiological. Few effective measures are available for the treatment or prevention for *Bocaparvovirus* infections. In this study, we are expected to develop a novel real-time PCR (RT-qPCR) based on the TaqMan probe, besides to provide accurate and rapid method for detecting BPV (NJ. et al. 2006).

Retinoic acid–inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5) could recognize the RNA viruses after these viruses infect host cells, which recruits mitochondrial antiviral signaling (MAVS), a downstream linker protein. MAVS combined tumor necrosis factor (TNF) receptor-associated factor (TRAF) and NF-κB to form MAVS signal corpuscle compound. This compound may activate TBK1 and IKKε to induce production of type I interferon (IFN). However, it is scarcely known if BPV exerts a similar role in infecting hosts (Rehwinkel and Gack 2020; Yang et al. 2021). The present study was designed to explore if BPV impacts IFN-β production in the host cells and to reveal further molecular mechanism of BPV immune escape. The authors also expect to find a novel mechanism of BPV inhibiting type I IFN production and provide a solid scientific basis for developing novel strategy of the diagnoses and therapy of BPV disease.

Additionally, earlier researches indicated that nucleocapsid (N) protein of SARS-CoV and peste des petits ruminants virus (PPRV) inhibited production of beta interferon (IFN-β) by targeting RIG-I signaling. The N protein of porcine delta coronavirus (PDCoV) (Chen Jun et al. 2019) and porcine epidemic diarrhea virus (PEDV) can inhibit production of beta interferon (IFN-β) via RIG-I signaling. Nowadays, scarce researches were performed on the pathogenic mechanism of BPV (Tseng et al. 2021; Workman et al. 2019). To better understand the role of BPV-VP1 protein in the suppression of the RIG-I-like receptor (RLR) pathway–mediated antiviral response, we investigated the effect of BPV-VP1 protein on type I IFN production so as to reveal further molecular mechanism of BPV VP1 pathogenesis and provide a solid scientific basis for explaining the immunosuppression of BPV VP1 (Anastasia and Saif 2021).

Materials and methods

Construction of eukaryotic expression vector of BPV VP1 gene

Specific primers of the BPV-VP1 gene were designed and synthesized referring to BPV Haden strain (GenBank no.: DQ335247) using DNAStar Primer 5.0 software (Table 1).

Total RNAs were extracted from BPV using the TIANamp Virus DNA/RNA Kit in accordance with the manufacturer's standard protocol and reversely transcribed into cDNA using the M-MLV reverse transcriptase (Invitrogen) system according to the manufacturer's protocols (Wei et al. 2014, 2017).

The full-length BPV VP1 genes were generated by PCR in a 50-μL reaction system, including 0.25 μL 10 × Ex Taq Buffer, 4.0 μL dNTP mixtures, 2.0 μL forward primer, 2.0 μL reverse primer, 30.0 μL diethyl pyrocarbonate (DEPC) water, and 3 μL cDNA, and at the PCR reaction conditions 94 °C 5 min, 94 °C 30 s, 67.75 °C 2 min, 72 °C 1 min (35 cycles), and 72 °C 10 min.

BPV VP1 gene and pCMV-Myc vector were restricted in double-enzyme systems, respectively. The reaction was carried out in a 50-μL system, including 5 μL 10 × M, 5 μL 10 × H 2.5 μL Sal I *(EcoR I)*, 2.5 μL Not I *(Kpn I)*, 4 μL pCMV-Myc-BPV-VP1, and 26 μL DEPC water at 37 °C for 4 h.

BPV VP1 was bound with double-enzyme digestion products using T4 ligase and set overnight at 16 °C. The plasmid was extracted using TransGen Biotech Plasmid Minipreparation kit (Beijing, China) according to the manufacturer's instruction. Extracted recombinant plasmids were verified with both double-enzyme digestion and sequence.

Table 1 Primer sequences

Genes	Primer	Primer sequences (5′ → 3′)
BPV-VP1	qF	ACGC**GTCGAC**CATGCCGCCAACCAATAAAGC (***Sal I)***
	qR	AAGGAAAAAA**GCGGCCGC**CTACAGGACTTTGTG GTGATTGAATCT (***Not I)***
IFN-β	qR	TTGTTGAGAACCTCCTGGCT
	qF	TGACTATGGTCCAGGCACAG
GAPDH	qF	CTTTGGCATCGTGGAGGGAC
	qR	ACAGATCGCTCTGAAGGGCT

The bold underlined part respectively indicated the enzyme digestion site

The specificity of the primers was verified and blasted in the NCBI

Transfection of bovine turbinate cells with recombinant protein pCMV-Myc-BPV-VP1 and cell viability

HEK 293 T cells and bovine turbinate (BT) cells purchased from American Type Culture Collection) were cultured in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies, Shanghai, China) supplemented with 10% FBS (Gibco) in 5% CO_2 at 37 °C for 24 h. HEK 293 T cells were transfected using fluid A (250 µL Opti-MEM media and plasmid) and fluid B (250 µL Opti-MEM media and Liposome 2000) for 6 h when the cell density was 80%, or index of fusion was over 95%. BPV VP1 was cloned into the pCMV-Myc vector with the flag tags. Plasmids were constructed by cloning the synthesized sequence into pCMV-Myc with Myc tags fused to the 3′ end. All constructed plasmids were confirmed through sequencing. In order to determine whether pCMV-Myc-BPV-VP1 could be successfully expressed in the BT cells which are susceptible to BPV, these BT cells were retrieved and transfected with pCMV-Myc-BPV-VP1 recombinant plasmid, pCMV-Myc, and blank control, respectively. Total RNA was extracted and amplified using real-time qPCR. Additionally, the cell viability was measured using Cell Counting kit-8 (CCK8, MedChemExpress LLC, Shanghai, China) referring to the manufacturer's instruction. Briefly, the cells were seeded in 96-well plates. A total of 10 µL CCK-8 reagent (APExBIO) was added into each well, and after incubation at 37 °C for 2 h, the absorbance was measured at 450 nm on a multifunction microplate reader (BioTek). The percentage at each concentration relative to the control was presented as cell viability. The tests were carried out in triplicate.

Detection of BPV copies using TaqMan qPCR

BT cells were cultured in 6-well plates, and they were infected with BPV at 0 h, 3 h, 6 h, 9 h, 12 h, and 24 h, respectively. Total RNAs were extracted from BT cells at different times. Meanwhile, BT cells without infection of BPV were set as the control group. The TaqMan qPCR method established in our laboratory was used to detect virus copies with the standard curve (regression equation) of YBPV = −3.536 XLg (BPV) + 39.773.

Expression levels of IFN-β mRNA induced by vesicular stomatitis virus (VSV)

To investigate whether BPV-VP1 protein has an inhibitory effect on IFN-β production and the interaction between BPV-VP1 protein and host IFN-β production, the human embryonic kidney (HEK) 293 T cells were infected with vesicular stomatitis virus (VSV)-EGFP for 6–8 h; then, these cells were transiently transfected with 2 µg pCMV-Myc-BPV-VP1 for 24 h. IFN-β mRNA expression levels were detected using qPCR in HEK 293 T cells.

Expression levels of VSV-induced key factors in JAK/STAT pathway

As describe above, HEK 293 T cells were transfected with pCMV-Myc-BPV-VP1 recombinant plasmid, pCMV-Myc, and blank control, respectively. Total RNA was extracted. The qPCR was performed using the PowerUp SYBR Green Master Mix on the ABI StepOnePlus system. All PCR data were analyzed using the StepOnePlus software, and the relative mRNA level of these genes was normalized based on the bovine glyceraldehyde 3-phosphate dehydrogenase (GAPDH, GenBank no.: AJ786261.1) mRNA level. The PCR amplification primers are listed in Table 1.

IFN-β levels induced by TBK1, IRF3 (5D), MDA5, and MAVS

The well-grown HEK 293 T cells were cotransfected with empty vector (pCMV-Myc), pCMV-Myc-BPV-VP1 protein, TANK-binding kinase 1 (TBK1), interferon regulatory factor 3(5D) (IRF3(5D)), melanoma differentiation-associated protein 5 (MDA5), and mitochondrial antiviral signaling (MAVS), respectively. The transcription levels of IFN-β, TBK1, IRF3 (5D), MDA5, and MAVS mRNAs in the RLR pathway were detected using RT-qPCR and western blotting, respectively.

Data statistics

Statistical analysis was performed using IBM SPSS Statistics version 21.0 (SPSS Inc., USA) and GraphPad Prism version 7.0 (GraphPad Software, Inc.; La Jolla, CA, USA). Data was presented as the means ± SE. Student's *t*-test was utilized to analyze the differences between the two groups. Inter-group differences were analyzed by one-way analysis of variance, followed by a post hoc Tukey test for multiple comparisons. *P* values less than 0.05 were considered to indicate a statistically significant difference.

Results

Construction of pCMV-Myc-BPV-VP1 recombinant plasmid and transcription levels of BPV VP1 mRNA

The recombinant plasmid of pCMV-Myc-BPV-VP1 was successfully constructed. The recombinant plasmid was digested with EcoRI/KpnI double enzymes. PCR assay displayed a distinct band of pCMV-Myc-BPV-VP1 gene (Fig. 1A). The product length was 2022 bp, which was in

agreement to the expected size. The expression level of BPV VP1 mRNA in the pCMV-Myc-BPV-VP1 treatment group was significantly higher than those of the untreated group (UT) and pCMV-Myc blank vector group (Fig. 1B), with an increase of 5.5×10^4 folds as compared to the UT group ($P < 0.001$). The finding indicated pCMV-Myc-BPV-VP1 plasmid could heighten transcription levels of BPV VP1 protein in BT cells.

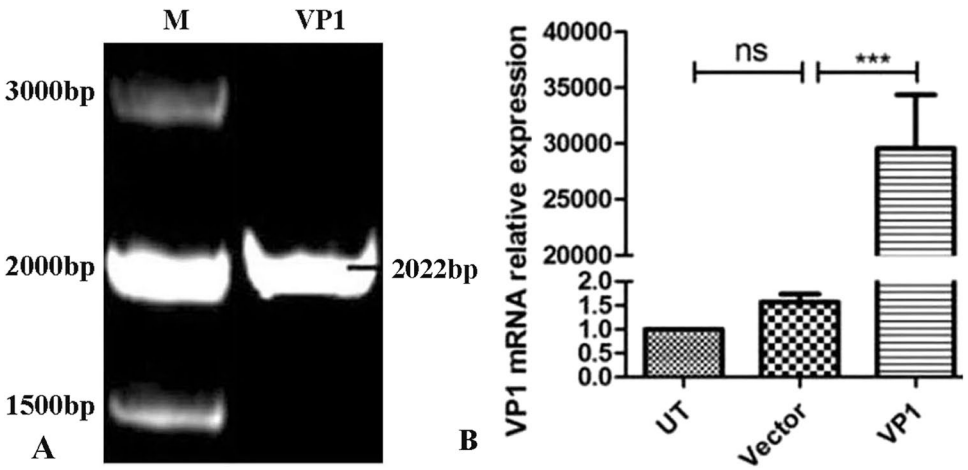

Fig. 1 The recombinant plasmid of pCMV-Myc-BPV-VP1 was verified by double enzyme digestion. M: 1000bp DNA Marker; VP1: pCMV-Myc-BPV-VP1 recombinant plasmid. The product size was 2022bp

BPV VP1 proteins promoted virus proliferation and regulated IFN-β production

In bovine turbinate (BT) cells, BPV virus copies in the pCMV-Myc-BPV-VP1 group were significantly raised when compared to the BPV group (Fig. 2A). The increment was 5.8×10^4 copies in comparison with the uninfected cells.

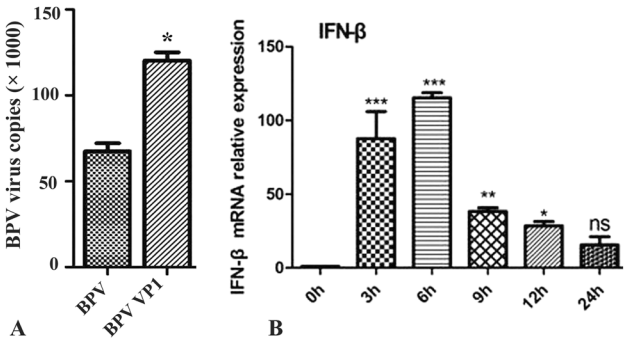

Fig. 2 BPV proliferation and IFN-β expression levels of pCMV-Myc-BPV-VP1 increased BPV proliferation (**A**). IFN-β expression levels in HEK 293 T cells were heightened at the different times following BPV infection (**B**). The maximum increment was found at 6 h after BPV infection. **A** $*P < 0.05$ as compared to BPV infection. **B** $*P < 0.05$ as compared to 0 h, $*P < 0.01$ as compared to 0 h, and $***P < 0.001$ as compared to 0 h

Total RNAs were extracted from HEK 293 T cells infected with BPV at 0 h, 3 h, 6 h, 9 h, 12 h, and 24 h, respectively. The expression levels of IFN-β mRNA were significantly increased at 3 h and 6 h ($P < 0.001$) as compared to 0 h (Fig. 2B). However, the effects were gradually descended after 9 h. The outcomes demonstrated that BPV VP1 protein increased copy numbers of BPV and promoted obvious BPV proliferation. The initial stage of BPV infection could enhance the production of IFN-β.

Overexpression of pCMV-Myc-BPV-VP1 and transcription levels of IFN-β mRNA

Human embryonic kidney (HEK) 293 T cells were transfected with pCMV-Myc-BPV-VP1 recombinant plasmid, pCMV-Myc, and blank control, respectively. Western blotting assay showed clear bands of 50 kD pCMV-Myc and 75 kD pCMV-Myc-BPV-VP1 (Fig. 3A) which was consistent with the prediction size. Expression levels of IFN-β mRNA in the HEK 293 T cells of the pCMV-Myc-BPV-VP1 group were significantly declined ($P < 0.01$) in comparison with that of the VSV infection group (Fig. 3B). The findings demonstrated that pCMV-Myc-BPV-VP1 recombinant protein was successfully overexpressed in the HEK 293 T cells. Overexpression of pCMV-Myc-BPV-VP1 could remarkably decrease IFN-β mRNA level.

BPV-VP1 proteins influenced expression levels of IFN-β, TBK1, and IRF3(5D) in the RLR pathway

The results showed both TBK1 and IRF3(5D) increased the IFN-β expression levels in the HEK 293 T cells (Fig. 4). But pCMV-Myc-BPV-VP1 protein significantly depressed IFN-β expression levels in comparison to TBK1 and IRF3(5D). The outcomes indicated that BPV VP1 protein could inhibit

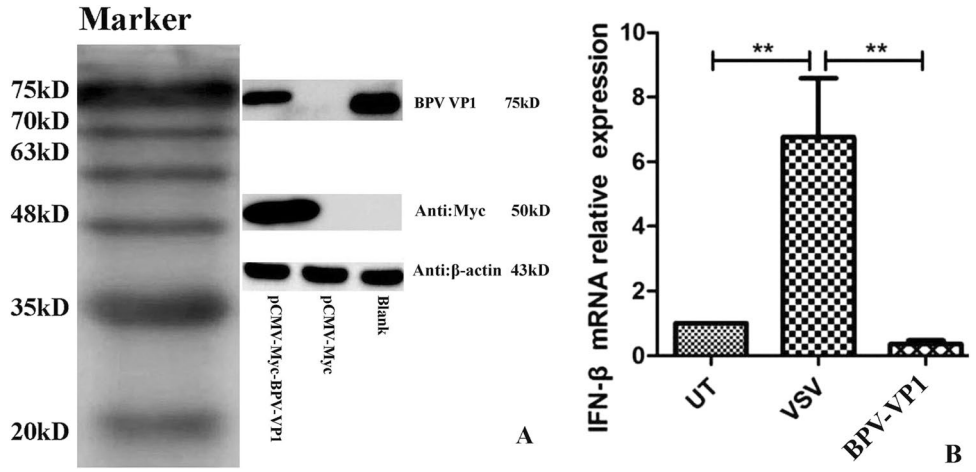

Fig. 3 Overexpression of pCMV-Myc-BPV-VP1 and IFN-β mRNA transcription level induced by VSV. These figure panels are taken from the different areas of the same gel or different gels and then joined together with a white space between the images. Western blotting assay of showed pCMV-Myc and pCMV-Myc-BPV-VP1 (Fig. 3A). Expression levels of IFN-β mRNA in the HEK 293 T cells (Fig. 3B). $P < 0.05$ as compared to UT group, ** $P < 0.01$ as compared to UT group, *** $P < 0.001$ as compared to UT group

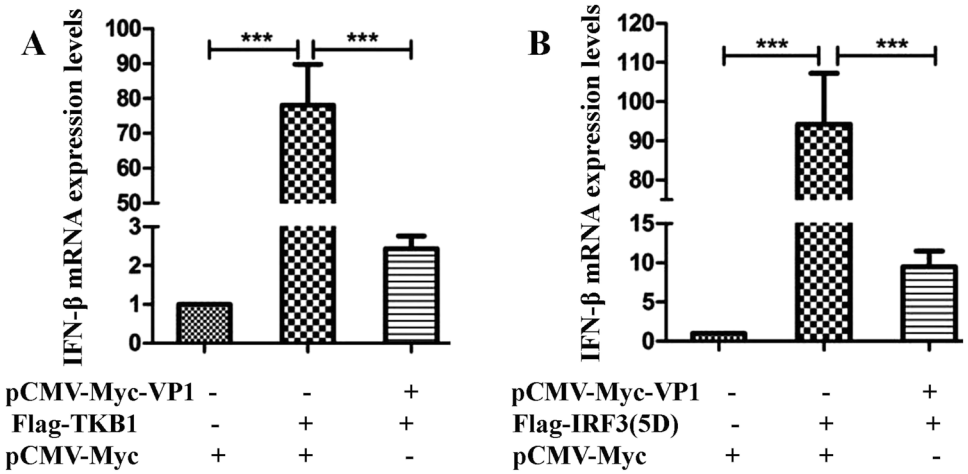

Fig. 4 Effects of BPV VP1 protein on the production of IFN-β induced by exogenous key factors. HEK-293T cells were cotransfected with empty vector (pCMV-Myc), pCMV-Myc-BPV-VP1 protein or Flag-N-key factors (TBK1 and IRF3(5D)). **A**, **B**: IFN-β expression levels induced by TBK1 and IRF3(5D), respectively. ***$P < 0.001$ as compared to pCMV-Myc group

IFN-β production induced by TBK1 and IRF3(5D). TBK1 and IRF3(5D) were potential target genes of BPV VP1 proteins.

In addition, to determine whether BPV VP1 protein effects the activation of IFN-β through the RLR pathway, HEK-293 T cells were transfected with pCMV-Myc-BPV-VP1 expression protein and single factors (TBK1, IRF3(5D), MDA5, and MAVS) as described above, respectively. Western blotting and RT-qPCR assay showed that levels of TBK1, IRF3(5D), MDA5, and MAVS were distributed and expressed in the HEK-293 T cells (Fig. 5). TBK1, IRF3(5D), MDA5, and MAVS were 84 kDa, 55 kDa, 118 kDa, and 58 kDa, respectively. Nevertheless, after the treatment of pCMV-Myc-BPV-VP1, the expression levels of TBK1, IRF3(5D), MDA5, and MAVS were lower than those of their self-flag empty vector, respectively ($P < 0.001$). The findings demonstrated that pCMV-Myc-BPV-VP1 could obviously attenuate the expression levels of these factors in the RLR pathway. Namely, pCMV-Myc-BPV-VP1 depressed pCMV-Myc efficacy on the expression levels of TBK1, IRF3(5D), MDA5, and MAVS, mainly by declining the self-expression levels of these four factors.

Discussion

The natural immune system is an important defense pattern against infection of foreign pathogens. Natural immune receptors include Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), NOD-like receptors (NLRs), and DNA receptors (Mogensen 2009). Interferon (IFN) can act on other cells and interfere with the replication of the virus (Shilin 2004). Type I interferon (IFN) response is one of the

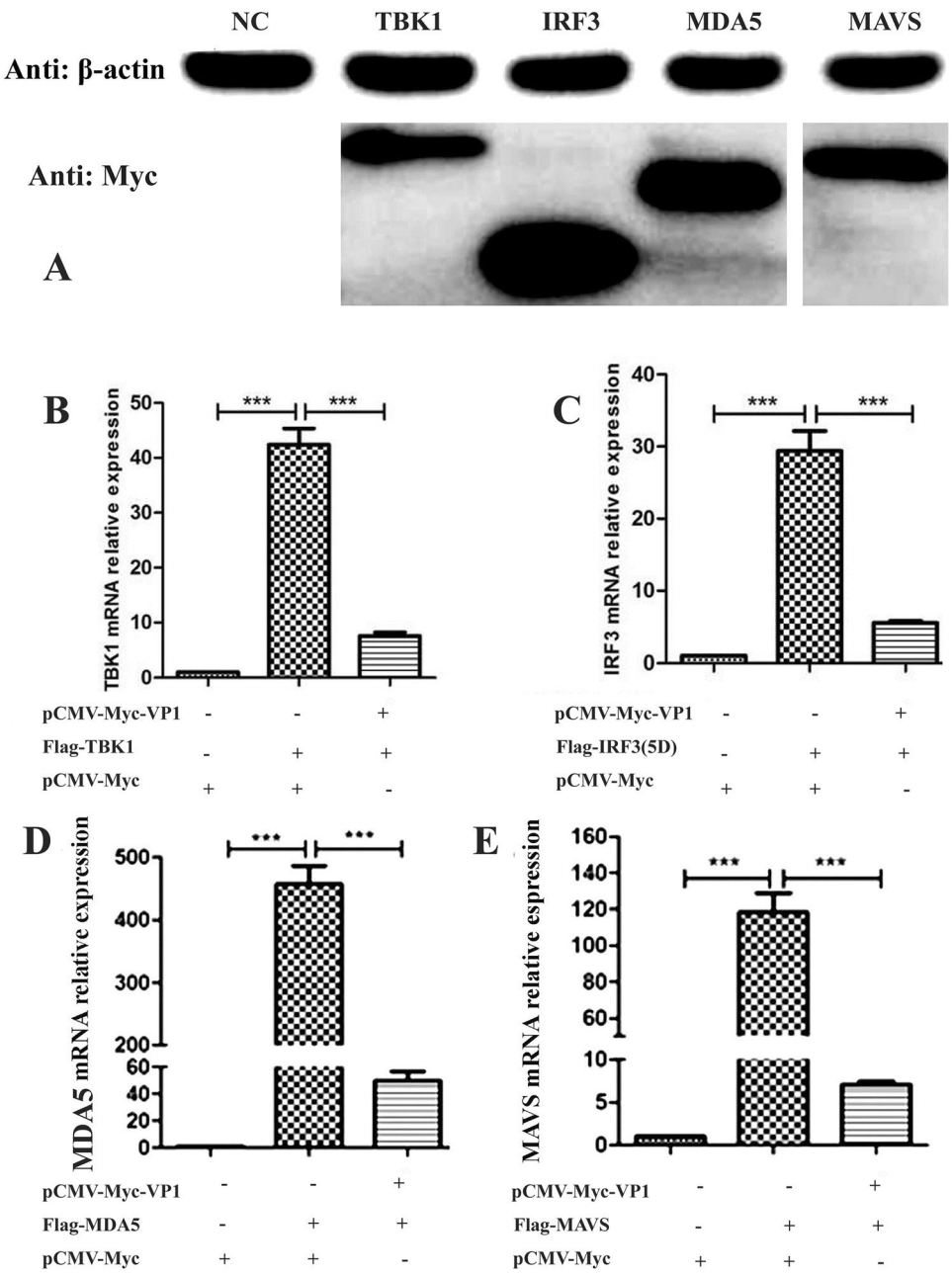

Fig. 5 Expression levels of TBK1, IRF3(5D), MDA5 and MAVS in HEK-293T cells. Western blot assay of TBK1, IRF3(5D), MDA5 and MAVS (Fig. 5A). The expression levels of TBK1, IRF3(5D), MDA5 and MAVS in HEK-293T cells, respectively (Fig. 5B, C, D and E). ***P<0.001 as compared to Flag-N-key factor treatment

most antivirus mechanisms in the natural immune system. It plays key roles in resisting virus invasion, terminating virus replication, and establishing specific immune response (Carty et al. 2021; Crosse et al. 2018a, b). However, little is known about whether BPV-VP1 suppressed IFN-β production via the RLR pathway. Furthermore, there has been scarce document about the immune escape of BPV.

Previous researches indicated that NP1 protein of porcine bocavirus (PBoV) inhibited production of type I interferon (IFN) by interferon regulatory factor 3 (IRF3) (Samad Lotfollahzadeh et al. 2020). Nonstructural protein NS1 of porcine parvovirus (PPV) activated activator of nuclear factors κB(NF-κB) through the TLR2 signaling pathway. However, there have been few researches on regulating IFN-β roles of bovine parvovirus (BPV). The molecular mechanism of BPV pathogenesis remains unclear, such that it is urgent to explain fully the BPV infection mechanism and escaping natural immunity of hosts, so that an effective vaccine may be developed timely (Carty et al. 2021).

In the present study, recombinant plasmids of pCMV-Myc-BPV-VP1 were successfully structured. TaqMan RT-qPCR assay showed that BPV VP1 protein increased significantly the BPV copy numbers and promoted proliferation of BPV. BPV infection ascended IFN-β production in early stage. Overexpression of pCMV-Myc-BPV-VP1 decreased

IFN-β mRNA expression in HEK 293 T cells and inhibited IFN-β production induced by TBK1 and IRF3(5D).

Innate immunity is a crucial defense barrier of the host cells against virus infection (Haoqin Liang et al. 2019). The interaction between pathogen-associated molecular patterns (PAMP) and pattern recognition receptors (PRR) is extremely important for the initiation of innate immune response (Liang et al. 2012). Retinoic acid–inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5) can recognize viral RNA after cells infected by RNA viruses and recruit mitochondrial antiviral signaling (MAVS) (Cao et al. 2015; Haller et al. 2006; Vazquez and Horner 2015). TBK1, IRF3, MDA5, and MAVS are activated by their respective RNA ligands to induce the downstream cascade pathway (Chen Jun et al. 2019; Wu and Hur 2015). RIG-I-like receptor (RLR), a key PRR, may recognize dsRNA produced by virus and induce the production of type I IFN through TBK1 and IRF3 to initiate the innate immune response (Liu et al. 2021). Therefore, the RLR signal pathway has a critical function for antivirus infection in the body (Zheng et al. 2021).

In order to explore if the main factors of the JAK/STAT pathway participate in the regulation process that BPV VP1 protein inhibits type I IFN and then achieves immune escape, the well-grown HEK 293 T cells were cotransfected with empty vector (pCMV-Myc), pCMV-Myc-BPV-VP1 protein, TBK1, and IRF3 (5D), respectively. TBK1 and IRF3 (5D) increased the IFN-β expression levels in HEK 293 T cells. However, BPV-VP1 proteins could depress their efficacy on enhancing IFN-β production by reducing their self-expression levels of TBK1 and IRF3 (5D) mRNAs in the RIG-I-like receptor (RLR) pathway (Crosse et al. 2018a; Liang et al. 2012). Our findings demonstrated that TBK1 and IRF3 (5D) are key target proteins of BPV-VP1 functioning in RLR pathway.

BPV-VP1 protein is an IFN-β antagonist through depression of MDA5, MAVS, TBK1, and IRF3 in the RLR pathway. However, it is unclear which factor plays a core role in this process. It is urgent to clarify this effect in the future (Anastasia and Saif 2021; Samad Lotfollahzadeh et al. 2020).

Conclusion

The pCMV-Myc-BPV-VP1 could heighten transcription levels of VP1 protein in BT cells, promote BPV proliferation, and ascend the production of IFN-β. Overexpression of pCMV-Myc-BPV-VP1 decreased IFN-β mRNA expression in HEK 293 T cells by obviously declining the expression levels of TBK1, IRF3(5D), MDA5, and MAVS in the RIG-I-like receptor (RLR) pathway. Our findings revealed a new mechanism evolved by BPV VP1 to inhibit the host innate immune activity by attenuating type I IFN production and provided a solid scientific basis into expounding the immunosuppression of BPV.

Authors' contribution Mr Yuan Zhaofang optimized TaqMan probe RT-qPCR. Professor Gong Zhuandi made the paper design and did data analyses. Dr Li Dianyu did the data statistics analyses. Mr Pei Mengyuna assessed the specificity and sensitivity. Professor Wei Suocheng was responsible for the experimental designs and writing the manuscript. All authors interpreted the data, critically revised the manuscript for important intellectual contents, and approved the final version.

Funding The innovation team for animal diseases in the plateau areas of Northwest Minzu University (2020–88-07–3). The Science and technology support program project of Gansu province of China (Grant No: 1604NKCA044).

Data availability Data availability are provided if requested.

Declarations

Ethics approval This article does not contain any studies with human or animal subjects performed by any of the authors.

Consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

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