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Original Article

The dUTPase of caprine arthritis-encephalitis virus negatively regulates interferon signaling pathway

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Abstract

Background: Deoxyuracil triphosphate nucleotide (dUTP) pyrophosphatase (dUTPase, DU) is an enzyme of caprine arthritis-encephalitis virus (CAEV) that minimizes incorporation of dUTP into the DNA. Caprine arthritis-encephalitis virus relies partly on its ability to escape from innate immunity to cause persistent infections. Interferon β (IFN-β) is an important marker for evaluating the innate immune system, and it has a broad spectrum of antiviral activity. **Aims:** This study was conducted to investigate the details of the IFN-β response to CAEV infection. **Methods:** The expression of IFN-β and the proliferation of Sendai virus (SeV) and vesicular stomatitis virus (VSV) were determined by real-time quantitative polymerase chain reaction (qPCR). The effect of DU on the IFN signaling pathway was evaluated using luciferase reporter assays. **Results:** In our study, the expression of IFN-β was significantly inhibited and the proliferation of SeV and VSV was promoted in cells overexpressing CAEV-DU. DU affected interferon stimulated response element (ISRE) and IFN-β promoter activities induced by RIG-I/MDA5/MAVS/TBK1 pathway, while did not affect them induced by interferon regulatory factor 3 (IRF3-5D). **Conclusion:** DU protein downregulated the production of IFN-β by inhibiting the activity of the signal transduction molecules upstream of IRF3, thereby, helping CAEV escape innate immunity. Findings of this work provide an evidence to understand the persistent infection and multiple system inflammation of CAEV.

Key words: Caprine arthritis-encephalitis virus, dUTPase, Innate immunity, Interferon type I

Introduction

Caprine arthritis-encephalitis virus (CAEV) is classified as a small-ruminant lentivirus (SRLV) (Adedeji *et al*., 2013), and is an enveloped, single-stranded RNA virus, belonging to the genus *Lentivirus* in the family Retroviridae (Cheevers *et al*., 1981; Hess *et al*., 1986). Caprine arthritis-encephalitis virus causes caprine arthritis-encephalitis (CAE), which is a chronic progressive infectious disease in goats and other ruminants (Huang *et al*., 2012; Lamara *et al*., 2013; Nardelli *et al*., 2020). Caprine arthritis-encephalitis virus has a tropism for the monocyte-macrophage lineage, and the maturation of monocytes to macrophages can control the expression of the viral genome (Narayan *et al*., 1982; Narayan *et al*., 1983; Blacklaws, 2012; Crespo *et al*., 2012). Infection by CAEV usually results in chronic inflammatory diseases, such as interstitial pneumonia and leukoencephalomyelitis in lambs, interstitial mastitis, and chronic arthritis in adult goats (Li *et al*., 2013). Besides, the yield and quality of milk can be reduced due to CAEV infection (Peterhans *et al*., 2004; Kaba *et al*., 2012; Juste *et al*., 2020). Caprine arthritis-encephalitis virus infections have been identified worldwide (Greenwood *et al*., 1995; Bandeira *et al*., 2009; Oem *et al*., 2012; Tageldin *et al*., 2012). Since there is no effective treatment, CAEV has caused serious economic losses for goat farmers (Leitner *et al*., 2010; Tu *et al*., 2017).

Deoxyuracil triphosphate nucleotide (dUTP) pyrophosphatase (dUTPase, DU) is a ubiquitous enzyme that exists widely in prokaryotic cells, eukaryotic cells, and some groups of viruses (el-Hajj *et al*., 1988; Gadsden *et al*., 1993; Hizi and Herzig, 2015). The *DU* gene exists in most SRLV strains and other retroviruses, such as equine infectious anemia virus (EIAV), maedi visna virus (MVV), and CAEV (Payne and Elder, 2001; Crespo *et al*., 2012; Adedeji *et al*., 2013; Michiels *et al*., 2018). In lentivirus genomes, DU is encoded by the *pol* gene (Elder *et al*., 1992). DU hydrolyzes dUTP into Deoxyuracil monophosphate nucleotide (dUMP) and pyrophosphate (PPi). During reverse transcription, viral DU prevents the misincorporation of dUTP into the synthetic cDNA (McIntosh and Haynes, 1997). It has been determined that DU plays an important role in CAEV replication, pathogenesis, and genetic stability

(Turelli *et al*., 1997). However, dUTPase is not essential for SRLV to infect or replicate (de Pablo-Maiso *et al*., 2018). Molecular clones of CAEV-Co lacking dUTPase are fully replicative (Saltarelli *et al*., 1990) and dUTPase is dispensable for MVV infection (Jonsson and Andresdottir, 2011). Even more, the whole SRLV genotype E does not encode a functional dUTPase and naturally infects goats with high efficiency (Juganaru *et al*., 2010).

Innate immune responses are prominent components of the host immune system, and inhibit viral infection through early detection of viral invasion and rapid initiation of defense mechanisms (Busca and Kumar, 2014; Sun and Lopez, 2017). In this process, Interferon β (IFN-β) is implicated in the activation of innate immune responses, and it has strong antiviral, antiproliferative, and immunomodulatory activities (Fensterl *et al*., 2015; Klotz *et al*., 2017). During RNA virus infection, cytoplasmic sensors, including retinoic acid-inducible gene 1 (RIG-I) and melanoma differentiation-associated protein 5 (MDA5), recognize viral RNAs and activate the mitochondrial antiviral signaling protein (MAVS) (Loo and Gale Jr, 2011). MAVS recruits tumor necrosis factor (TNF) receptor-associated factors (TRAFs), which subsequently activate IkB kinase (IKK) and TANK-binding kinase 1 (TBK1) complexes to phosphorylate IκBα and interferon regulatory factor 3 (IRF3), respectively. Then, nuclear factor (NF)-κB and phosphorylated IRF3 translocate into the nucleus to activate type I IFN signaling (Cui *et al*., 2014).

Recent findings have shown that sendai virus (SeV)-induced immune response can counteract SRLV infection (Pablo-Maiso *et al*., 2020). Therefore, SRLV may have strategies to escape innate immunity. Previous studies have demonstrated that the DU protein of CAEV can inhibit the production of IFN-β (Fu *et al*., 2020), but the molecular mechanism is still unclear. In this research, the relationship between DU and IFN-β was investigated.

Materials and Methods

Cells, virus, and antibody

Human embryonic kidney (HEK) 293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) added with 10% fetal bovine serum (FBS) 100 U/ml penicillin and 10 μg/ml streptomycin sulfate in a 37°C, 5% CO_2 incubator. Sendai virus and the vesicular stomatitis virus encoding green fluorescent protein (VSV-GFP) were cryopreserved in our laboratory (Shi *et al*., 2018). Internal reference antibody, anti-β-actin mouse monoclonal antibody, anti-Flag mouse monoclonal antibody, anti-GFP mouse monoclonal antibody, and goat anti-mouse IgG horseradish peroxidase (IgG HRP) conjugate were purchased from TransGen Co. (Beijing). SRE-luc/NF-κB-luc/IFN-β-luc plasmids and Flag-RIG-I/Flag-MDA5/Flag-MAVS/Flag-TBK1/IRF3-5D expression plasmids were kindly provided by Dr. Zexing Li from Tianjin Medical University. Poly (I:C) was purchased from Invitrogen.

Reverse transcription-polymerase chain reaction (RT-PCR) for complete coding sequence (CDS) amplification

Intracellular total RNAs were extracted from CAEV-infected goat joint synovial cells (GSM) cells using TRIzol reagent (Invitrogen, USA) as previously described (Huang *et al*., 2012). RNAs were reverse transcribed into cDNA using reverse transcriptase (TaKaRa, Dalian, China). A pair of oligonucleotide primers were designed based on the Shanxi strain of CAEV-SH (GU120138.1) to amplify the CDS region of DU protein and cloned into pcDNA3.1 vector (Clontech, China), as shown in Table 1.

Plasmid constructions

DU was amplified and cloned into the vector pEGFP-C1 and pFlag-CMV-2 to generate the fusion plasmid of pEGFP-DU and pFlag-CMV-DU. The primers were supplied in Table 1, *EcoR I* and *Xba I* sequences were marked by underline. The above sequences were verified by gene sequencing.

Western blot analysis

Cell lysates were heated for 10 min and isolated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Then, the separated proteins were transferred from SDS-PAGE to nitrocellulose (NC) fitter membranes (ExPro). After the transfer, the membrane was blocked with 5% skim milk in Tris buffer saline tween 20 (TBST) (0.05% Tween 20) for 1 h. The membrane was subsequently incubated with primary antibodies overnight at 4°C, and it was then washed 3 times with TBST and stained with HRP-conjugated secondary antibodies for 1 h. The membrane was detected using a chemiluminescence detection kit (Thermo Scientific, Waltham, MA, USA) and observed through Gel Imaging System (BIO-RAD, USA).

Real-time quantitative PCR (qPCR)

Total RNA was extracted using TRIzol reagent (Invitrogen, USA) and cDNA was synthesized using

Table 1: Primers used for PCR amplification

Primer name	GenBank No.	Sequence of primer (5´-3´)
pcDNA3.1-DU-F	GU120138.1	CCACACTGGACTAGTGGATCCATGGCAGGATATGATTTAATATGT
pcDNA3.1-DU-R		CTTGGTACCGAGCTCGGATCCTAATATTAATTGTGCAAACTTT
Flag-CMV2-DU-F	GU120138.1	CGGAATTCAATGGCAGGATATGATTTAATATGT
Flag-CMV2-DU-R		GCTCTAGATCATAATATTAATTGTGCAAACTTT

PCR: Polymerase chain reaction, and CMV2-DU: CMV promotor DU expression plasmid

HiScript Q RT SuperMix (Vazyme, China). The comparative quantification of gene expression was analyzed by quantitative PCR (qPCR) carried out by ABI 7500 real-time PCR system (Applied Biosystems, Foster city, CA, USA). The thermal cycler program was made up of 95°C for 10 min, 40 cycles of 15 s at 95°C, then, 55°C for 30 s and 72°C for 30 s. This experiment used DBI Bioscience-2043 Bestar SybrGreen qPCR Mastermix. Relative levels of mRNA were normalized to the β-actin RNA levels in each sample. The relative transcript levels of the target gene were determined by the $2^{-\Delta\Delta Ct}$ threshold method. The data were normalized to the expression level of the *β-actin* gene and analyzed using GraphPad Prism 6.0 software. All gene-specific primers are listed in Table 2.

Table 2: Primers used for real-time qPCR amplification

Primer name	GenBank No.	Sequence of primer (5´-3´)
SeV-F	NC_001552.1	GAAAGAGATACCGAACCCAGAG
SeV-R		GCTTGAGGGAGTGTATTGTAGG
IFN-β-F	NM_002176.3	CAACAAGTGTCTGCTCGAAAT
IFN-β-R		TCTCCTCAGGGATGTCAAAG
β-Actin-F	NM_001101.4	GGAAATCGTGCGTGACATTAA
β-Actin-R		AGGAAGGAAGGCTGGAAGAG
IRF3-F	NM_213770	AGACGCTCACCACGCTACACC
IRF3-R		GCTTGAGGGAGTGTATTGTAGG
NF-κB-F	NM_003998.3	GAACCACACCCCTGCATATAG
NF-κB-R		GCATTTTCCCAAGAGTCATCC
VSV-F	JX121109.2	ATGTGAGCACTAAAGTAGCCCT
VSV-R		GTTCTTCCAAAGCTAACCCAGT
Q-DU-F	GU 120138.1	TAAAGAAATCACAATGGGCTA
Q-DU-R		ATTATTACCTGTATTTGTCCCT

qPCR: Quantitative PCR, SeV: Sendai virus, IFN: Interferon, IRF: Interferon regulatory factor, NF: Nuclear factor, VSV: Vesicular stomatitis virus, DU: dUTPase, NC, NM, JX, and GU are marks of Genbank registered number

Luciferase reporter assay

293T cells were seeded in 24-well plates and co-transfected with 100 ng of IFNβ-luc, pFlag-DU (100 ng), and 100 ng of internal control -LacZ plasmid (Promega). Polyethylenimine (PEI) (Sigma, USA) was used for plasmid transfection, according to a previously described protocol (Yang *et al*., 2017). After 12 h of transfection, cells were inoculated with SeV or VSV at 0.5 multiplicity of infection (MOI) for another 16 h. The whole cell lysates were collected and luciferase activity was measured and normalized to the LacZ activity as previously described (Liu *et al*., 2019). HEK293T cells were co-transfected with the eukaryotic expression plasmid Flag-RIG-I, Flag-MDA5, Flag-MAVS, Flag-TBK1, or IRF3-5D (an IRF3 mutant that can activate downstream gene expression when expressed alone), and the related luciferase reporter plasmid to evaluate the activation of IFN signaling pathway as described previously (Fu *et al*., 2020).

Statistical analysis

The above experiments were done with at least three independent replicates. Statistical significance among different results was determined by GraphPad Prism software using Student's t-test. P-values less than 0.05 were considered statistically significant (* P<0.05, ** P<0.01, *** P<0.001, and NS: No significant).

Results

DU is a negative regulator of IFN-β

To investigate whether the expression of DU affects the IFN-β production, pFlag-CMV-DU expression vector was constructed and the expression was identified by Western blot (Fig. 1A). Next, 293T cells were transfected with pFlag-CMV-DU or empty vector for 24 h before SeV stimulation, and the mRNA levels of IFN-β and its transcription factors IRF3 and NF-κB were detected by RT-qPCR. Consistent with the previous study (Fu *et al*., 2020), our results suggested that overexpression of DU significantly reduced the mRNA levels of IFN-β, IRF3, and NF-κB, as compared with controls (Fig. 1B).

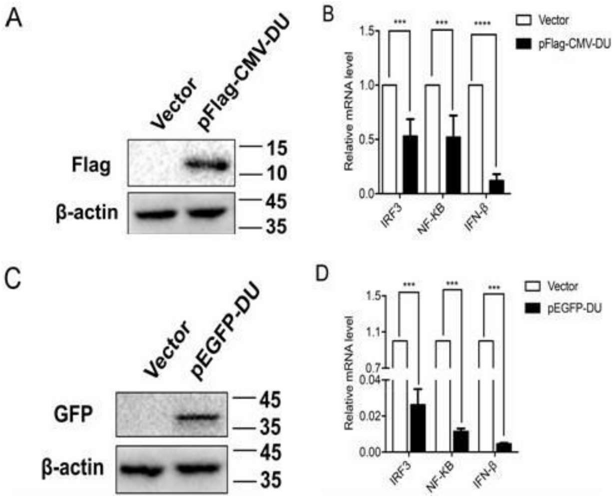

Fig. 1: Label proteins do not affect the inhibitory effect of DU on IFN-β. (**A** and **C**) 293T cells were transfected with pFlag-CMV-DU (0.5 µg) (**A**), and pEGFP-DU (0.5 µg) (**C**) and empty vector. The cells were collected at 24 h post-transfection and tested by Western blot. Molecular weight markers in kDa are shown on the right. (**B** and **D**) 293T cells were transfected with pFlag-CMV-DU (**B**), pEGFP-DU (**D**), and empty vector. Twelve hours after transfection, cells were infected with 0.5 MOI SeV. The infected cells were collected at 24 h post-infection, mRNA levels of IRF3, NF-κB, and IFN-β were tested by real time quantitative reverse transcription-polymerase chain reaction (RT-qPCR). Differences in data considered statistically significant at p-value less than 0.05 (* P<0.05, ** P<0.01, *** P<0.001, and NS: No significant). CMV: Cytomegalovirus, DU: dUTPase, IRF3: Interferon regulatory factor 3, NF: Nuclear factor, IFN-β: Interferon beta, pEGFP: Enhanced green fluorescence protein plasmid, MOI: Multiplicity of infection, and SeV: Sendai virus

Considering that the molecular weight of DU protein is about 10 kDa, which is relatively small, to exclude the effect of tag protein on DU function, we constructed a DU expression plasmid with GFP tag (pEGFP-DU).

Western blot analysis showed that the pEGFP-DU expression vector was successfully constructed (Fig. 1C). Consistently, transfection of the pEGFP-DU expression vector also dramatically decreased the mRNA levels of SeV-activated IFN-β, IRF3, and NF-κB compared to the pFlag-CMV-DU results (Fig. 1D). All over, these data demonstrate that DU is a negative regulator of IFN-β.

DU facilitates SeV and VSV proliferation

Since we have determined that DU inhibits IFN-β mRNA level, it prompted us to investigate the effect of DU on viral proliferation. 293T cells were transfected with pFlag-CMV-DU expression plasmids or empty vector and infected with SeV or VSV-GFP at 24 h post transfection. Then, total RNA was extracted from the cells at 16 h post infection and analyzed for the mRNA levels of SeV and VSV by RT-qPCR. As shown in Figs. 2A-C, the overexpression of DU upregulated the mRNA levels of SeV and VSV in a dose-dependent manner. Correspondingly, the results of immunofluorescence and Western blot analyses further confirmed that DU dose dependently promoted VSV replication (Figs. 2D and E). Together, these observations suggest that DU facilitates the proliferation of SeV and VSV in a dose-dependent manner.

DU inhibits IFN-β production in a dose-dependent manner

To further confirm the inhibitory effect of DU on the expression of IFN-β, the transcription level of the IFN-β in cells treated with SeV, VSV-GFP or poly (I:C) (1 μg/ml) were compared between transfection plasmid of DU and blank vector cells. As shown in Fig. 3A, overexpression of DU significantly reduced the mRNA level of IFN-β induced by SeV, VSV-GFP, and poly (I:C). This inhibitory effect was dose dependent (Figs. 3B-D).

Next, 293T cells were co-transfected with the luciferase reporter plasmid of IFN-β (IFN-β-luc) and different doses of pFlag-CMV-DU expression vector to verify the above RT-qPCR assay results. The results demonstrated that DU inhibited SeV, VSV, and poly (I:C)-activated IFN-β promoter activity in a dose dependent manner (Figs. 3E-G). Collectively, the DU dose dependently inhibits the production of IFN-β to promote viral proliferation.

DU has an inhibitory effect on the upstream of IRF3 in the signaling pathway

Previous studies have shown that SeV, VSV, and poly (I:C) activate downstream IFN signaling pathways through activating MAVS, RIG-I, and MDA5, respectively. To further clarify the specific mechanism by which DU inhibits the production of IFN-β, we explored the role of DU in the IFN-β signaling pathway. The luciferase reporter plasmids of interferon stimulated response element (ISRE), NF-κB and IFN-β (ISRE-luc, NF-κB-luc, and IFN-β-luc), and eukaryotic expression plasmids of RIG-I, MDA5, MAVS, and TBK1 (Flag-RIG-I, Flag-MDA5, Flag-MAVS, and Flag-TBK1), and pEGFP-DU or empty vector were co-transfected into 293T cells. The effect of DU on the signaling pathway was determined by detecting the luciferase activity of the reporters. As shown in Figs. 4A-C, overexpression of RIG-I, MDA5, MAVS, and TBK1 induced the promoter activation of ISRE, NF-κB, and IFN-β, however, co-transfection with DU significantly attenuated this activation, suggesting that DU was a negative regulator of the IFN-β signaling pathway.

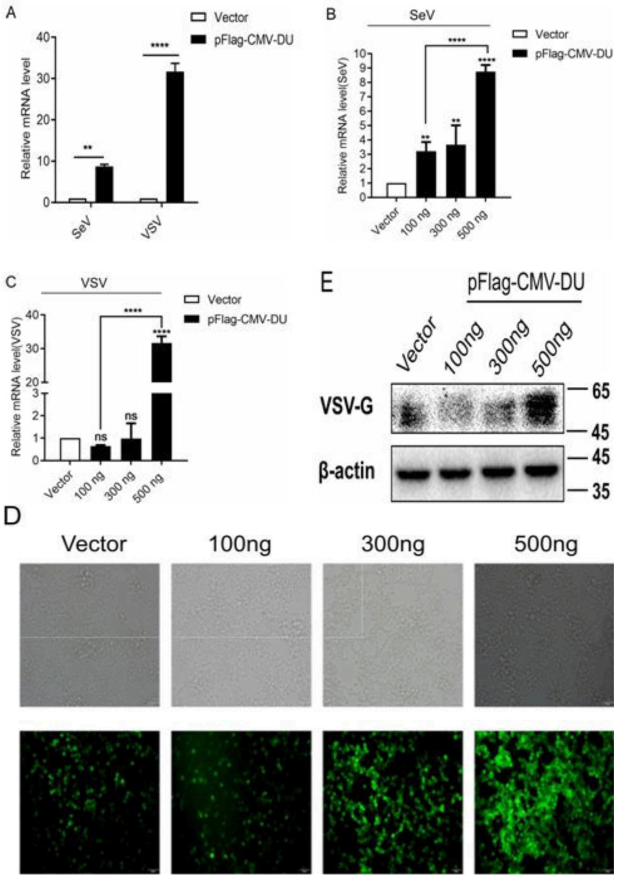

Fig. 2: Overexpression of DU promotes SeV/VSV proliferation. (**A**) 293T cells were transfected with pFlag-CMV-DU expression plasmid or empty vector, 12 h after transfection, cells were infected with 0.5 MOI SeV or VSV. The mRNA level of SeV or VSV was examined by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis at 16 h post-infection. (**B** and **C**) 293T cells were transfected with different doses of pFlag-CMV-DU expression plasmids (100 ng/300 ng/500 ng), 12 h after transfection, cells were infected with SeV or VSV and the mRNA level of SeV or VSV was tested by RT-qPCR analysis at 16 h post-infection. (**D** and **E**) 293T cells were transfected with pFlag-CMV-DU expression plasmid or control plasmid, after 12 h, cells were infected with VSV-GFP, immunofluorescence microscope imaging (**D**), and Western blot (**E**) used for observing viral proliferation at 16 h post-infection. Molecular weight markers in kDa are shown on the right. Results were normalized to those of the control gene *β-actin* and are presented relative to those of control cells. Differences in data considered statistically significant at p-value less than 0.05 (* $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$, and NS: No significant). CMV: Cytomegalovirus, DU: dUTPase, MOI: Multiplicity of infection, SeV: Sendai virus, and VSV-GFP: Vesicular stomatitis virus harbored green fluorescence protein gene

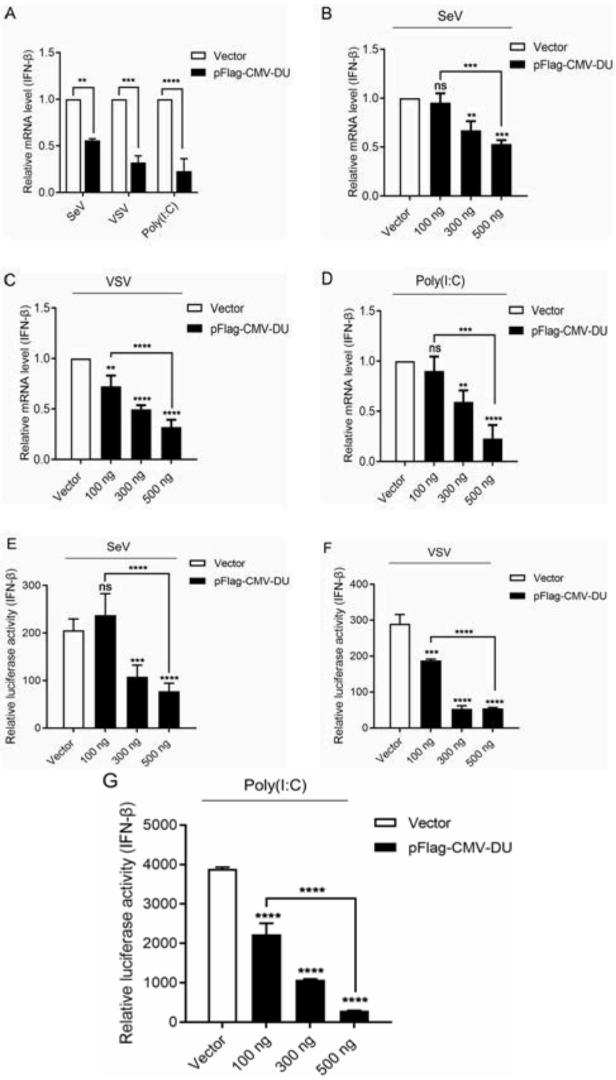

Fig. 3: DU inhibits IFN-β expression in a dose-dependent manner. (**A**-**D**) 293T cells were transfected with pFlag-CMV-DU or empty vector. At 12 h post-transfection, cells were infected with SeV/VSV-GFP/poly (I:C). The infected cells were collected at 16 h post-infection and mRNA levels of IFN-β were tested by quantitative reverse transcription-polymerase chain reaction (RT-qPCR). (**E**-**G**) 293T cells were co-transfected with IFN-β-luc and pFlag-CMV-DU and then, inoculated with SeV/VSV-GFP/poly (I:C), finally, the luciferase activity of IFN-β promotor was tested. Differences in data considered statistically significant at p-value less than 0.05 (* P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001, and NS: No significant). CMV: Cytomegalovirus, DU: dUTPase, SeV: Sendai virus, VSV-GFP: Vesicular stomatitis virus harbored green fluorescence protein gene, and IFN: Interferon

IRF3 is an important downstream transcription factor of MAVS and TBK1, and it can be stimulated by upstream signals to undergo phosphorylation and dimerization, then, it enters the nucleus and activates transcription (Liu *et al*., 2015; Bakshi *et al*., 2017; He *et al*., 2017). To verify the effect of DU on the transcription activity of IRF3 IRF3-5D plasmid, a constitutively active form of IRF3 (Sen *et al*., 2010), was co-transfected into 293T cells with DU plasmid. It was found that the ISRE and IFN-β promoter activities activated by IRF3-5D were not affected by DU (Figs. 4D and E). The results indicated that the inhibitory effect of DU on the IFN signaling pathway was located at upstream of IRF3 transcription factor.

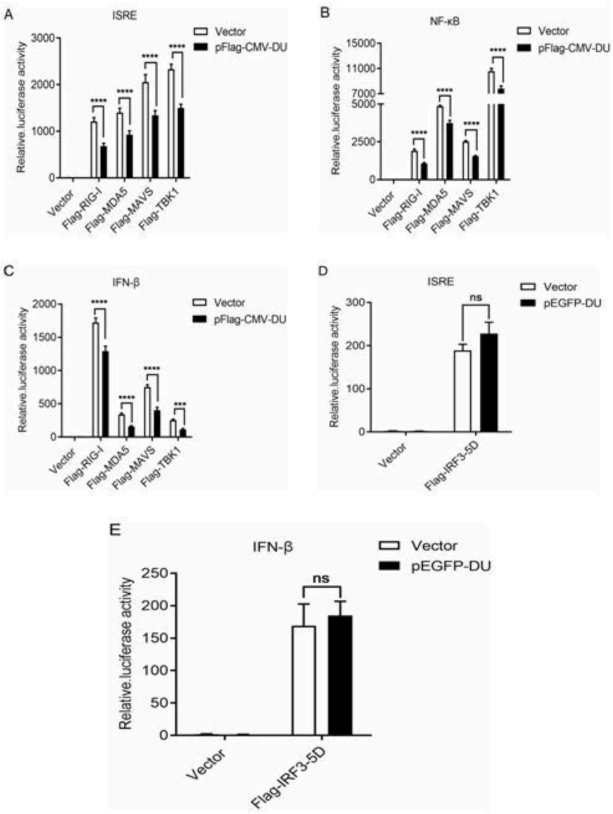

Fig. 4: DU is an inhibitory factor in IFN-β signaling pathway. (**A**-**C**) 293T cells were co-transfected with ISRE-luc/NF-κB-luc/IFN-β-luc plasmids (100 ng), Flag-RIG-I/Flag-MDA5/Flag-MAVS/Flag-TBK1, and pEGFP-DU expression plasmids or empty vector, then, the luciferase activity of ISRE, NF-κB, and IFN-β promoters was examined. (**D** and **E**) 293T cells were co-transfected with ISRE-luc/IFN-β-luc plasmids and pEGFP-DU expression plasmids or empty vector, then, the luciferase activity of ISRE and IFN-β promotors was tested. Differences in data considered statistically significant at p-value less than 0.05 (* P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001, and NS: No significant). Data are representative of three independent experiments. ISRE: Interferon stimulated response element, CMV: Cytomegalovirus, RIG: Retinoic acid-inducible gene I, MDA5: Melanoma differentiation-associated gene 5, MAVS: Mitochondrial antiviral signaling protein, TBK1: TANK-Binding kinase 1, NF: Nuclear factor, IFN: Interferon, IRF3: Interferon regulatory factor 3, and pEGFP-DU: dUTPase

Discussion

DU is a ubiquitous enzyme regulated by the cell cycle and is abundant both in differentiated and undifferentiated cells (Turelli *et al*., 1997). When the ratio of dUTP/dTTP (Deoxyuracil triphosphate nucleotide/Thymidine triphosphate deoxynucleotide) in the virus-infected cells increases, the reverse transcription of the virus can proceed normally (Hizi and Herzig, 2015), but the process of viral DNA integration into host cell DNA and the expression of most viral

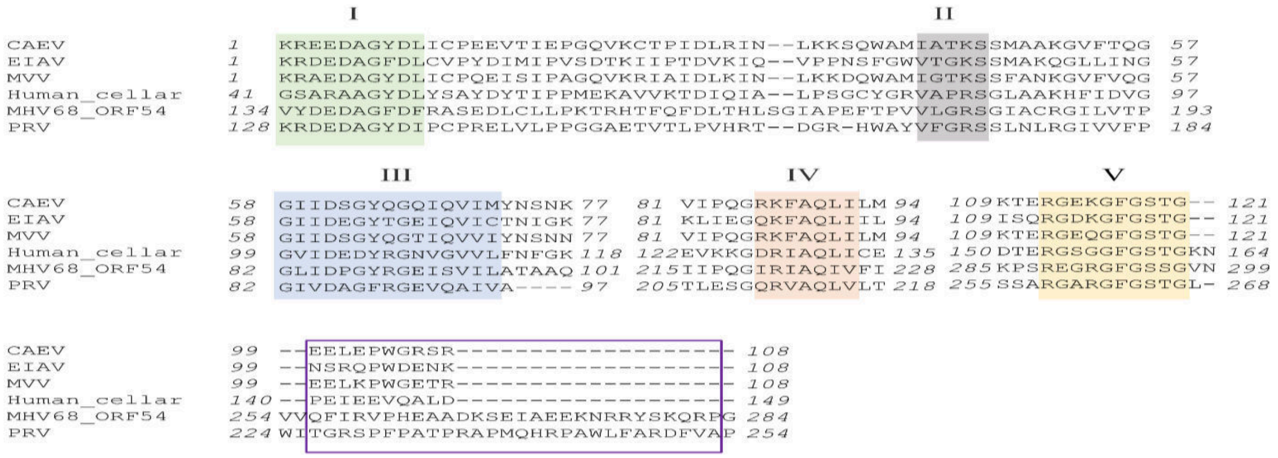

Fig. 5: Multiple sequence alignment of dUTPases between different viruses and humans. The five highly-conserved domains typical of dUTPases are indicated above the sequences. The functional region of PRV UL50 in charge of anti-IFN activity reported by Zhang (2017) is marked by a purple rectangle. The sequences of the following dUTPases are as follows: MVV (GI:9626549), CAEV (GI:266706151), EIAV (GI:323836), MHV68 (GI:114782444), PRV (GI: 51557490), and humans (GI:4503423). CAEV: Caprine arthritis-encephalitis virus, EIAV: Equine infectious anemia virus, MVV: Maedi-visna virus, MHV: Mouse hepatitis virus, ORF: Open reading frame, PRV: Pseudorabies virus, and IFN: Interferon

proteins are blocked (Weil *et al*., 2013). As a dUTPase, DU can catalyze the conversion of dUTP to dUMP and PPi, help to maintain a low ratio of dUTP/dTTP (McGeoch, 1990; Payne and Elder, 2001; Vertessy and Toth, 2009), thereby, reducing the possibility of dUTP incorporation into cDNA, providing an advantageous environment for viral replication (Chen *et al*., 2002; Kato *et al*., 2014).

IFN-β is an important antiviral cytokine in innate immunity that inhibits the proliferation of most viruses. On contrast, many viruses downregulation IFN expression by disturbing IFN signaling pathway using their coding proteins to escape from innate immunity. Small-ruminant lentiviruses weakly induce type-I IFN (Zink and Narayan, 1989). Besides, dUTPase is dispensable for MVV infection (Jonsson and Andresdottir, 2011). The inhibition effects of dUTPase in IFN-β production were also detected in our previous report (Fu *et al*., 2020) CAEV with a point mutation in the gene coding dUTPase has been shown to revert to the wild type (WT) in an infected goat (Turelli *et al*., 1997). All those results showed that dUTPase prompts clinical signs and pathogenesis of retrovirus infection. Accordingly, dUTPase is not essential for SRLV infection, SRLV *Roccaverano* strain (genotype E) lacking the entire dUTPase and Vpr-like genes replicates efficiently in non-dividing cells (Juganaru *et al*., 2010), which has been described as a low pathogenic strain in goats (Reina *et al*., 2009). Thus, we speculated that the inhibition of IFN-β by DU is one of the probable reasons for the low production of IFN-β. To further validate the inhibitory effect of DU on IFN-β production, we overexpressed different doses of plasmid encoding dUTPase (100, 300, 500 ng, respectively) in 293T cells. DU showed a dose-dependent inhibition of IFN-β expression. Correspondingly, DU could promote the replication of SeV and VSV.

SeV/VSV/Poly (I:C) can be recognized by the organism's pattern recognition receptors (PRRs), which activate the downstream signal transduction pathway and activate IFN-β. dUTPase can be recognized by RIG-I, which induces the downstream activation of IFN-β; poly (I:C) induces the activation of MDA5 (Kato *et al*., 2008); SeV infection leads to the activation and aggregation of MAVS on mitochondria, and then MAVS activates IFN-β signaling pathway. Although SeV/VSV/Poly (I:C) are recognized by different receptors, these receptors ultimately activate IFN-β through the downstream "MAVS-TBK1-IRF3" signaling pathway. In our study, we found that CAEV-DU can inhibit the RIG-I/MDA5/MVAS/TBK1-activated IFN-β signaling pathway, but it has no influence on the activity of IRF3-induced IFN-β signaling pathway, indicating that the target molecule for DU to inhibit IFN-β production is located upstream of IRF3.

Pseudorabies virus dUTPase UL50 and mouse herpesvirus 68 (MHV68) ORF54 are two kinds of herpesviral dUTPases. Recent studies have shown that MHV68 ORF54 and PRV dUTPase can antagonize type I IFN signaling independent of their dUTPase activity (Leang *et al*., 2011; Zhang *et al*., 2017). Deoxyuracil triphosphate nucleotide (dUTP) pyrophosphatase usually have five conserved amino acid (aa) motifs. Multiple sequence alignment of dUTPases between different viruses (SRLV and herpesvirus) and humans is shown in Fig. 5. The study by Zhang *et al*. (2017) showed that the region between motifs IV and V is critical for PRV UL50 inhibition of type I IFN signaling. However, this region is almost absent in several SRLV dUTPases and cellular dUTPase but appears in MHV68 ORF54, which can also inhibit type I IFN signaling. Considering that CAEV is a kind of retrovirus and herpesvirus belonging to DNA viruses. It is probably an important reason for the sequence difference between CAEV dUTPase and herpesviral dUTPases. Hence, we speculated that CAEV dUTPase may have different mechanisms compared to herpesviral dUTPases to help the virus evade host type I IFN response, and this requires further investigation.

In summary, our study preliminarily determines the mechanism by which DU negatively regulates the production of IFN-β by inhibiting the upstream of IRF3 in the IFN-β signaling pathway, thereby inhibiting the transcriptional expression of IFN-β. This work presents a theoretical basis for the CAEV to evade innate immunity. However, more details about the interactions between DU and the IFN-β signaling pathway also demand further exploration.

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Conflict of interest

The authors declare no financial and/or non-financial competing interests.

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