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Caspase-1 Interacts with Bovine Viral Diarrhea Virus NS2-3 protein to modulate the viral titer in MDBK

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Abstract

Bovine Viral Diarrhea Virus (BVDV) belongs to the Flaviviridae viral family and is widely distributed worldwide, affecting millions of animals and causing significant clinical manifestations and production losses. Its genome encodes a single polyprotein that is processed into 10–12 viral proteins. Polyprotein processing is essential for viral replication, and is mediated by viral autoproteases such as Npro and NS2-3, which activate the replication machinery. Inflammasomes are innate immune system receptors/sensors that regulate the activation of caspase-1 and promote inflammation in response to viral infections. Previous studies have shown that NLRP3 and IFI16 inflammasomes are activated in bovine macrophages infected with BVDV and that this activation is related to viral titer. In this study, we hypothesized that caspase-1 interacts with BVDV proteins to cleave them during viral replication, contributing to polyprotein processing and promoting the start of the viral replication process, which has an impact on viral progeny. In silico analysis identified four putative caspase-1 cleavage sites within the Erns, NS2-3, and NS5A proteins. These sites are structurally and phylogenetically conserved across the Pestivirus genus, suggesting a common evolutionary mechanism. We observed colocalization of NS2-3 with caspase-1 in BVDV-infected MDBK. Additionally, modulation of caspases-1 activation resulted in significant changes in viral titers. Furthermore, pharmacological modulation of caspase-1 activity significantly altered viral replication levels, indicating that caspase-1 plays a key role in efficient BVDV replication. Our findings suggest that BVDV may exploit caspase-1-mediated proteolytic processing of its polyprotein to enhance replication.

Introduction

Bovine Viral Diarrhea Virus (BVDV) is a member of the *Flaviviridae* family within the *Pestivirus* genus and shares structural similarities with Classical Swine Fever Virus (CSFV) and Border Disease Virus (BDV) (1). Although BVDV primarily infects cattle, it can also infect other ungulate species. Its global distribution and high prevalence in cattle represent a major economic burden to the livestock industry, causing infertility, abortions, and reduced milk production (2, 3).

The BVDV genome consists of a single-stranded, positive-sense RNA that encodes a single polyprotein. This polyprotein is processed by both cellular and viral proteases in order to generate structural proteins (C, Erns, E1, and E2) and nonstructural proteins (Npro, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (4, 5). Nonstructural proteins (NSPs) are responsible for viral replication (6). Among these, NS2 and NS3 play a critical role in the polyprotein processing (7). Formation of the viral replication complex depends on coordinated interactions among NS3-NS4A-NS4B-NS5A-NS5B proteins, with NS3 and NS5B being particularly important. NS5B functions as an RNA-dependent RNA polymerase (RdRp), which is indispensable for viral genome replication (8).

The C-terminal region of the NS2 protein exhibits cysteine protease activity responsible for the autoproteolytic cleavage of between NS2 and NS3. This cleavage is important for early stages of RNA replication (9) and is regulated by the activation of the DNAJC14 chaperone, also known as Jiv domain.

NS3 is a multifunctional protein with protease, helicase, and NTPase activities and is critical for viral replication. It has been observed that free NS3 is essential for proper formation of the viral replication complex (10, 9, 5). The processing of these two proteins is associated with the classification of BVDV into two biotypes: cytopathic (cp) and non-cytopathic (ncp) based on their effects in cell culture. In the cytopathic biotype, the presence and accumulation of free NS3 in late stages of infection can induce apoptosis. In contrast, in the non-cytopathic biotype, the cleavage of NS2 and NS3 occurs only in early stages of infection; thus, in late stages of infection, NS2-3 remains uncleaved and the infection continues without cytopathic effects (9, 11).

During viral infection, host cells activate innate immune response mechanisms by recognizing Damage-Associated Molecular Patterns (DAMPs) or Pathogen-Associated Molecular Patterns (PAMPs) through pattern recognition receptors (PRRs), such as NOD-like receptors (NLRs) or Toll-like receptors (TLRs). Activation of these receptors triggers signaling cascades that promote the transcription of proinflammatory cytokines such as type 1 and 3 interferons (IFN), interleukin-1 beta (IL-1β), tumor necrosis factor-alpha (TNF-α), and interleukin-6 (IL-6) (12, 4, 6).

Cytokines of the IL-1 family (IL-1β, IL-18 and IL-33) require post-translational processing for secretion. This processing is mediated by a multiprotein complex known as the inflammasome (12, 13). This inflammasome consists of an NLR family receptor, the adaptor molecule ASC, and caspase-1. The classical activation of this complex occurs through ligand-receptor interaction, followed by coupling to ASC through the pyrin domain, which in turn promotes autocatalytic activation of procaspase-1. Activated caspase-1 then cleaves proIL-1β into its mature form, enabling its secretion and amplification of the inflammatory response (12).

The most common associated inflammasome with viral infections are NLRP3, AIM2, and IFI16, which can recognize DNA, RNA, and viral proteins (13, 14). Although inflammasome activation primarily promotes cytokine release, several viruses have evolved mechanisms to modulate this pathway, by either suppressing or exploiting it to enhance replication (15). For example, Influenza, Measles, and Nipah viruses can inhibit inflammasome assembly (16), while Yellow Fever, Dengue, or Zika viruses interfere with cytokine production signaling pathways through their NS5 protein (4). Among the DNA viruses reported to activate inflammasomes complex some examples are the varicella-zoster virus, adenovirus type 5, and bovine herpesvirus type 1 (14, 17). Regarding RNA viruses, BVD, influenza, Zika, dengue, chikungunya, hepatitis C, and west Nile encephalitis virus (WNV) have been described as capable of producing an immune response through the inflammasome (14, 18).

In silico predictions provide insight into potential interactions between cellular and viral molecules, identifying signaling pathways and potential therapeutic targets (19).

In BVDV infection, the activation of the NLRP3 (20) and IFI16 (21) inflammasomes has been identified. Moreover, inhibition of caspase-1 and the NLRP3 receptor has been shown to reduce BVDV viral titer, suggesting that caspase-1 that may play a role in promoting viral replication, potentially through interaction with viral proteins (21). However, the mechanisms underlying this proviral effect remain

unclear. Therefore, the present study aims to identify the effect of inflammasome activation role on the modulation of viral titer through its effector molecule, caspase-1.

Materials and methods

Bioinformatic analysis

Protein sequences reported for the BVDV NADL strain and viruses belonging to the *Flaviviridae* family were retrieved from the National Center for Biotechnology Information (NCBI) sequence database. Sequence alignment was performed using the protein Basic Local Alignment Search Tool (BLASTp) (22). Separately, each of these sequences was submitted to the ExPASy Swiss-Model program, where protein homology modeling was conducted using crystallized proteins from viruses of the same viral family using templates within the Swiss-Model database (23). De novo structural models were also created using the AlphaFold 3 program (24). Accession numbers for all sequences and templates are provided in Supplementary Material 1. Models exhibiting the highest sequence identity and the lowest QMEANDisCo scores were selected for downstream analyses. Structural comparisons were conducted using the MatchMaker tool within Chimera. Sequence identity percentages and Root Mean Square Deviation (RMSD) were obtained with an iteration cutoff of 2 Å. Visualization and analysis of these proteins and their domains were performed using the Chimera program 1.19. (25).

Prediction of Caspase-1 Cleavages

A total of 89 sequences of viral proteins from different members of the Flaviviridae family were used. These sequences showed a similarity in BLASTp analyses, this was evaluated for potential caspase-1 cleavage sites. Using ExPASy PeptideCutter tool (Swiss-Model variant) cleavage predictions were performed. Cleavage motifs consistent with known caspase-1 recognition sequences to analyze comparatively across taxa were identified.

Phylogenetic Analysis

Phylogenetic trees were constructed using RAxML under a Maximum Likelihood evolutionary model (26). Protein sequence alignments were generated by comparing BVDV-1a strain NADL against homologous sequences of proteins from viruses within the *Flaviviridae* family obtained from the NCBI sequence database (38 viruses and 144,948 sequences) using BLASTp (22). Accession numbers for the sequences are listed in Supplementary Material 1. Sequence alignment was performed using MAFFT (27), and alignments were visualized in JalView (28). Incomplete or redundant sequences were removed. Phylogenetic trees were generated with 10,000 bootstraps for each tree, and the bipartition tree was visualized using the FigTree program. To root the trees, the most taxonomically distant viral sequences from the *Flaviviridae* family were selected as outgroups.

Viral propagation

For propagation and acquisition of the viral stock of cp-BVDV (NADL-1) we used Madin-Darby Bovine Kidney Cells (MDBK) (ATCC® CCL-22, USA.). Cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) (Gibco, NY, USA) incubated at 37°C with 5% CO_2. For the infection assays, the medium was changed to DMEM supplemented with 2% FBS, agitated for 2 hours, and then incubated for 72 hours for cp-BVDV (NADL-1). Cells were freezed in order to lysate them to obtain the viral stock needed for the experiments. For the viral titration of cp-BVDV (NADL-1), MDBK cells were harvested on 96-well plates, performing serial tenfold dilutions of the viral inoculum, and the titer was determined by the Reed-Müench Method.

Cell infection and inflammasome modulation

The MDBK cells were cultured on 24-well plates at a density of 8×10^4 cells per well to perform the infection, activation or inhibition assays. Multiplicity of infection (MOI) of 2:1 was used for cp-BVDV with 500µL of DMEM medium supplemented with 2% FBS (Gibco, New York, USA). Negative control was MDBK cells cultured with DMEM and 2% FBS, positive control of inflammasome activation was treated 24 h postinfection with 300ng/mL of LPS from *E. coli* O26:B26, strain is ATCC 12795 (Merck, Boston, USA). For the inhibition treatments, the MDBK cells were incubated with CRID3 24 h post infection (50µM) (Merck, Boston, USA). The supernatants recovered from 48 hours post-infection.

Confocal Microscopy

8×10^3 MDBK cells were harvested in a chamber slide and infected with BVDV NADL at MOI 2:1 during 24h, followed the cells were fixed with 4% PFA and washed three times with PBS 1x, after the cells were blocked during one hour (PBS 1x 5% equine sera and 0.3% de triton X-100) at room temperature, after they were washed three times with PBS and incubated 4°C overnight with primary antibodies 1/500, anti-NS2/3 (Santa Cruz, California US sc-101592) and anti-caspase-1 p10 (Santa Cruz, California US sc-515). Incubation was followed by five washes with PBS 1x and after, were incubated with secondary antibodies for one hour at room temperature (Texas Red and Alexa Fluor 488), subsequently was washed three times with PBS 1x and the Vectashield with DAPI was added (Vector laboratories). The images were captured using a confocal microscope (Nikon A1R + STORM, Tokyo, Japan).

Results

BVDV Erns, NS2-3, and NS5A proteins contain putative caspase-1 cleavage sites

To identify potential *in silico* interactions between caspase-1 and the *Pestivirus* polyprotein, cleavage site prediction analysis were performed. Seven potential cleavage sites were identified within the Erns, E2, NS2-3, and NS5A proteins. These sites were, specifically located at aspartic acid residues as follows: position 48 in Erns; position 36 in E2; positions 60, 74 and 157 in NS2-3; and position 48 in NS5A (Fig. 1a-1f and Table 1). The predicted cleavage sites for each protein are highlighted in the protein models (Fig. 1g-1l).

Table 1
Predicted cleavage sites by caspase-1 in pestivirus polyprotein

Virus	Protein	Likely cleavage by caspase-1 (AA position)
BVDV	Erns	D48
	NS2-3	D74 and D157
	NS5A	D48
CSFV	E2	D36
	NS2-3	D60
BDV	NS2-3	D157
Supplemental 1. Accession numbers for all sequences and templates used		

NS2-3 protein exhibits structural and phylogenetic similarities across the *Flaviviridae* family

Following the identification of potential *In silico* interactions, it was observed that the predicted sites in the NS2-3 protein were conserved across genus. Phylogenetic analyses were conducted to determine evolutionary relationships and assess cleavage site conservation within the *Flaviviridae* family. NS2-3 protein phylogeny was constructed with four sequences of the *Pestivirus* genus. The phylogenetic tree for BVDV NS2-3 protein revealed that this protein forms an exclusive monophyletic group together with Classical Swine Fever Virus (CSFV) and Border Disease Virus (BDV) (Fig. 2a), consisting with their classification within the *Pestivirus* genus. Structural comparison analysis demonstrated high homology, with sequence identity percentages exceeding 88% and RMSD values of 0.928 Å for CSFV and 0.896 Å for BDV (Fig. 2b). Among the proteins with predicted caspase-1 cleavage sites, NS2-3 plays a central role in viral replication. Notably, the predicted caspase-1 cleavage sites were located proximal to the natural cleavage site by other viral proteases such as NS3/4A, which cut the complete protein in NS2 and NS3 proteins. NS3 protein phylogeny was constructed with 37 sequences from the viruses of the *Flaviviridae* family. This protein is a highly conserved protein throughout the *Flaviviridae* family (Fig. 2c) and also shows a high homology in terms of sequence identity percentages, exceeding 89% and structural similarities between the pestiviruses analyzed with RMSD values of less than 0.8 Å (Fig. 2d). Since the predicted cleavage *In silico* in NS2-3, would favor the release of NS3, it was determined whether the domains of this protein were conserved after caspase-1 cleavage. It was identified that none of the domains were cut by the cleavage site predicted by caspase-1 (Fig. 2e) and most likely conserved its biological activity, therefore, it was analyzed for its potential interaction with caspase-1 *In vitro*.

Caspase-1 Colocalizes with BVDV NS2-3 Protein in Infected MDBK Cells

To experimentally validate the interaction suggested by the *In silico* predictions, MDBK cells were infected with the BVDV NADL strain. Cytopathic effects were observed to be characterized by vacuolization and monolayer lysis, which were most evident at 48 hours post-infection (hpi) (Fig. 3a). Infection was confirmed by RT-PCR (Fig. 3c). As expected, non-cytopathic effects were observed in

uninfected control cells. Additionally, viral titers were calculated using the Reed-Muench method, showing a time-dependent increase (Fig. 3b).

To assess potential *In vitro* interactions between caspase-1 and the BVDV NS2-3 protein, confocal microscopy was performed. Infected cells showed an increased presence of caspase-1 compared to the negative control (Fig. 3d). Merged images of caspase-1 and the viral NS2-3 protein revealed yellow spots corresponding to a possible colocalization. This suggests a close physical association between caspase-1 and the viral NS2-3 protein expressed only during the viral replicative cycle. Besides, it was identified as the presence of caspase-1 active at 24 hpi by Western Blot (Fig. 3e)

Modulation of Caspase-1 Activity Directly Impacts BVDV Viral Replication in MDBK Cells

To explore the functional consequences of the caspase-1/NS2-3 interaction, caspase-1 activity was modulated using LPS and CRID3 in BVDV cp-NADL-infected MDBK cells (MOI 2:1). Stimulating infected cells with LPS resulted in an increase in viral titer (Fig. 4b). Conversely, inhibition of the NLRP3 inflammasome pathway drastically reduced the viral load (Fig. 4c). Representative images for both assays are shown in Fig. 4a.

Discussion

Our results demonstrate that the modulation of caspase-1 activity has a significant impact on the viral titer of BVDV in MDBK cells. We observed that stimulation with LPS increases viral titers, whereas inhibition with CRID3 drastically decreases them. These findings are consistent with previous reports; for instance, luteolin has been shown to attenuate p65 phosphorylation, reducing the expression of inflammasome-related genes and, causes the recruitment of pro-caspase-1, which ultimately limits caspase-1 activation and viral replication (29). Likewise, Gallegos-Rodarte et al. (2023) reported that the use of CRID3 in bovine macrophages infected with the NADL strain decreased viral titers. Overall, these findings suggest that inflammasome components, in both the upstream and downstream of the inflammasome pathway, exert a direct effect on the viral replication cycle. However, the precise molecular mechanism has yet to be fully elucidated.

In this context, the NS2-3 protein is a key candidate. Since NS2-3 is essential for *Pestivirus* replication, its interaction with caspase-1 (suggested by our *In silico* and *In vitro* analyses) could explain the observed biological changes. The proteolytic processing of viral proteins by host caspases is not an isolated phenomenon; it has been documented in coronaviruses, such as Transmissible Gastroenteritis Virus (TGEV) and Porcine Epidemic Diarrhea Virus (PEDV), where the nucleocapsid protein is susceptible to cleavage by caspases 6 and 7 (30, 31). Similarly, in Feline Calicivirus, caspase-2 can process capsid proteins, and in sarcoma-associated herpesviruses, similar interactions that modulate pathogenesis have been detected (32, 33). These precedents support the possibility that BVDV may exploit host proteases to optimize replication.

The colocalization between caspase-1 and the NS2-3 protein provides physical evidence linking innate immune response to the viral replication machinery. However, biochemical validation experiments such as *In vitro* cleavage assays or targeted Western Blotting are necessary to confirm the caspase-1 mediated proteolytic processing. It is particularly relevant to determine whether this mechanism differs between the cytopathic (cp) and non-cytopathic (ncp) biotypes, considering their distinct replication kinetics and that the ncp biotype specifically activates the IFI16 inflammasome. Since both biotypes induce caspase-1-mediated IL-1β release (34, 21), the interaction observed with NS2-3 could represent a point of convergence in BVDV pathogenesis, regardless of the pattern recognition receptor (PRR) initially activated.

The strong conservation observed among BVDV-1, CSFV and BDV, together with the stability of their predicted protein structures, suggest evolutionary constraints that may preserve functional interactions across pestiviruses.. When comparing NS2-3 BVDV-1 protein to CSFV and BDV, we observed a low RMSD value 0.928 Å and 0.896 Å, respectively; besides, the identity percentage is high with both viruses (BDV: 90.76% and CSFV: 88.9%). The pattern is similar when comparing the NS3 protein among these viruses; high identity percentage (CSFV: 89.9% and BDV: 91.65%) and low RMSD which is reflected in the phylogenetic tree. This parameter is lower for BDV (0.246 Å) than for CSFV (0.782 Å) which is consistent with keeping the predicted cut site by caspase-1 at the same position for BDV. Although there are subtle differences, that suggest certain amino acid changes in BDV resulted in structural modifications not observed in CSFV, which may have had a decisive evolutionary weight in the divergence of these species within the *Pestivirus* genus.

In this study, we focused solely on the cp biotype (NADL reference strain). It has been reported that the inflammasome acts as a regulator of viral load not only through caspase-1 interaction but also through various inflammasome components (16, 14). This has been observed in viruses such as Hepatitis and Classical Swine Fever, p7 viroporin induces IL-1β secretion through caspase-1 activation as a viral regulation mechanism (12). Therefore, future studies should expand the analysis to other viral proteins, other inflammasome components, and the ncp biotype in the future. However, since Gallegos-Rodarte et al. (2023) demonstrated that caspase-1 activation in the ncp biotype is specifically linked to the IFI16 inflammasome, the stimuli used in this work (targeted at the canonical pathway) might not be suitable for that model, since the innate immune response and the heterogeneity of molecules involved in the replication of both biotypes.

In silico predictions are fundamental for inferring biological functions, especially for proteins that have not yet been crystallized. In addition to NS2-3, we identified potential caspase-1 cleavage sites in the Erns and NS5A proteins, which could enhance the observed biological effect. Nonetheless, molecular docking studies are required to precisely characterize these interactions. These tools have previously enabled the demonstration of genomic proximity between Ebola and Marburg viruses (35), the evolutionary tracking of SARS-CoV-2 (36), and the homology-based susceptibility analysis of the ACE2 protein in different hosts (37). In our case, the integration of computational models optimizes *In vitro* and

In vivo resources by directing experimentation toward molecular targets with a higher probability of functional relevance.

Conclusion

Therefore, the present study identified the effect of inflammasome activation role on the modulation of viral titer through its effector molecule, caspase-1. The cytopathic NADL strain of BVDV colocalizes with caspase-1 in infected MDBK cells, and the stimulation or inhibition of this pathway directly affects the viral titer. This results in an increase or decrease of viral load, respectively. These findings suggest that caspase-1 plays a functional role in the BVDV replication cycle and may represent a novel target for antiviral strategies.

Declarations

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Competing interests

All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

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Data Availability

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

Ethics approval

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required, as no animals or human volunteers were involved in this study.

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Figures

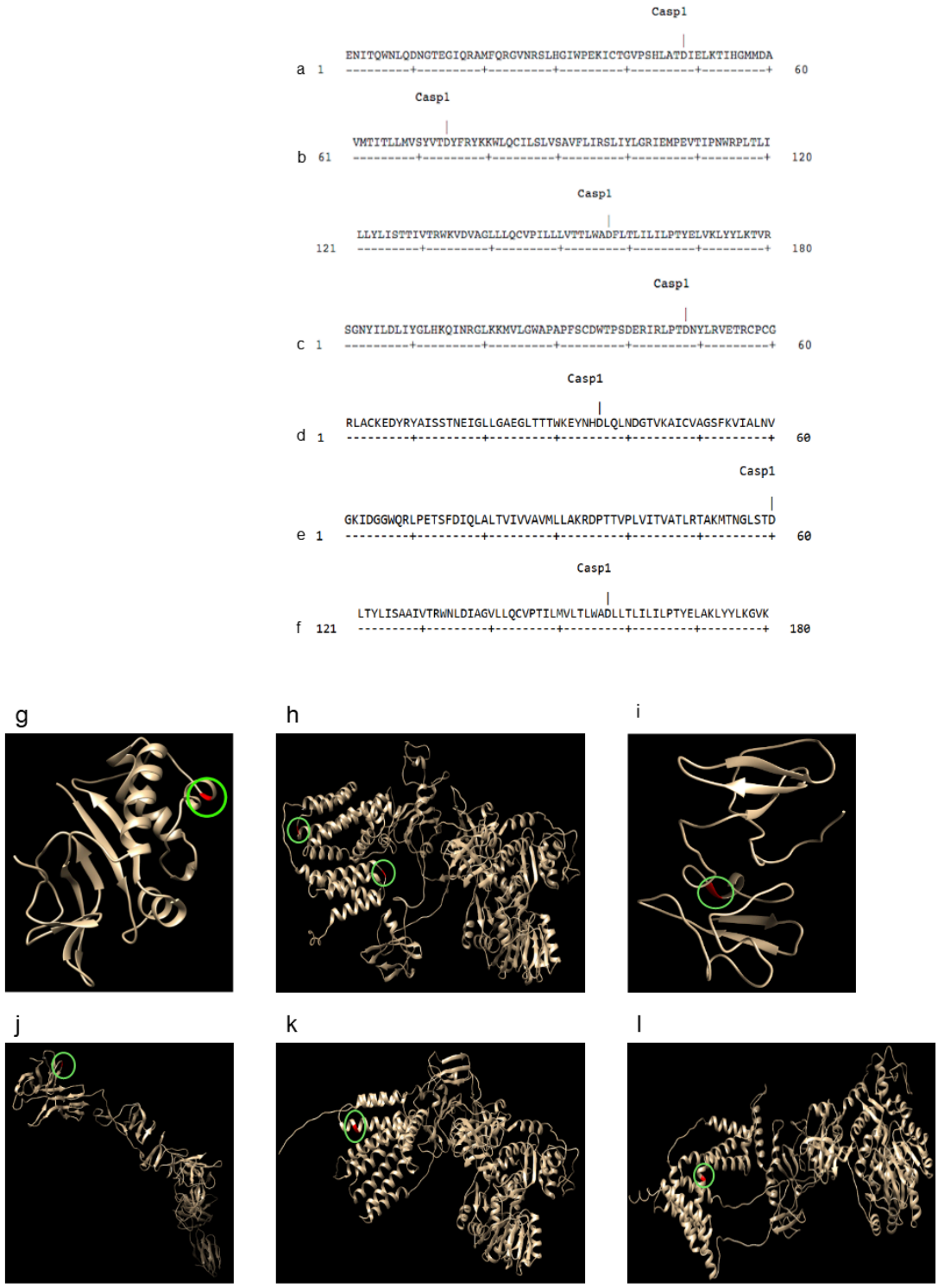


Figure 1

Structural models of BVDV, CSFV, and BDV proteins and predicted caspase-1 cleavage sites. a) Cleavage site for BVDV Erns protein. b) Cleavage sites for BVDV NS2-3 protein. c) Cleavage site for BVDV NS5A protein. d) Cleavage site for CSFV E2 protein. e) Cleavage site for CSFV NS2-3 protein. f) Cleavage site for BDV NS2-3 protein. g) BVDV Erns protein model. h) BVDV NS2-3 protein model. i) BVDV NS5A protein

model. j) CSFV E2 protein model. k) CSFV NS2-3 protein model. l) BDV NS2-3 protein model. Green circles indicate the predicted cleavage sites within the protein structures.

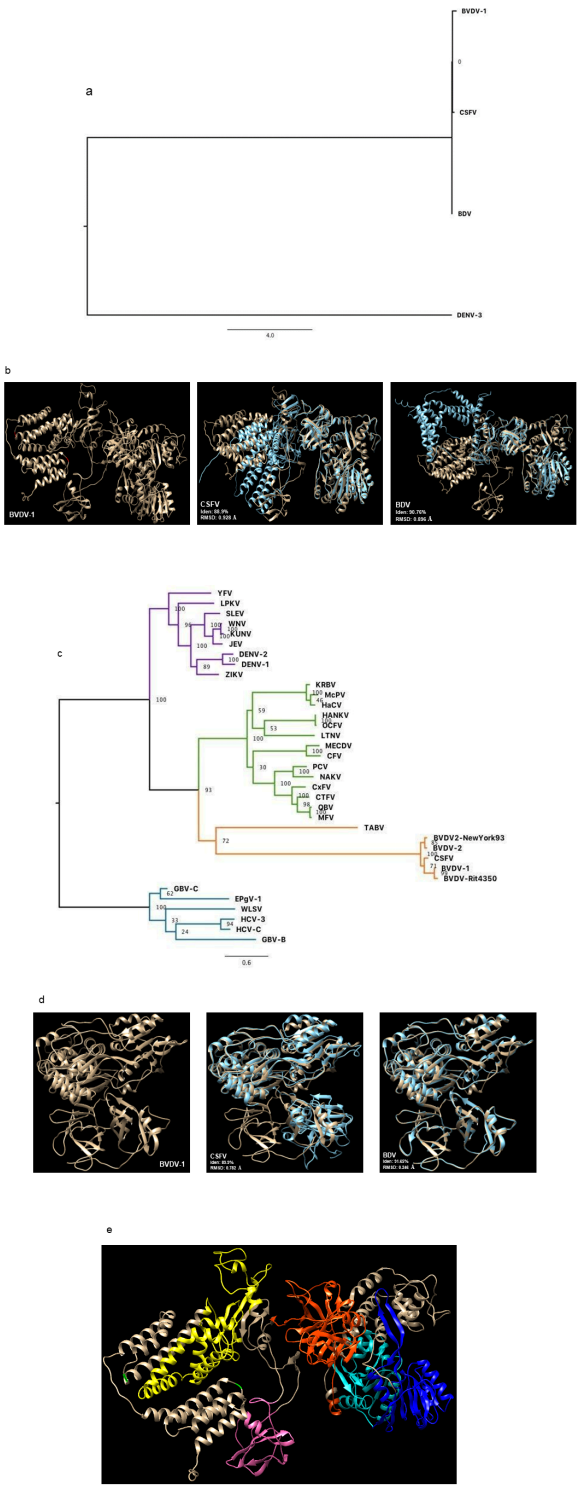


Figure 2

Phylogenetic, structural, and functional domain analysis of NS2-3 and NS3 proteins. a) Phylogenetic tree of the NS2-3 protein. b) Structural superposition of NS2-3 protein models: BVDV-1 (gold) and CSFV (blue)

or BDV (blue). c) Phylogenetic tree of the NS3 protein. d) Structural comparison of NS3 protein models: BVDV-1 (gold) and CSFV (blue) or BDV (blue). e) Functional domains of the BVDV NS2-3 protein: Peptidase C74 (Yellow), Jiv90 (Pink), Peptidase S31 (Orange), DEXH-box helicase domain of NS3 protease-helicase (Cyan), C-terminal helicase domain of the RNA helicase A (RHA) family helicases (Blue), and predicted cleavage sites (Green).

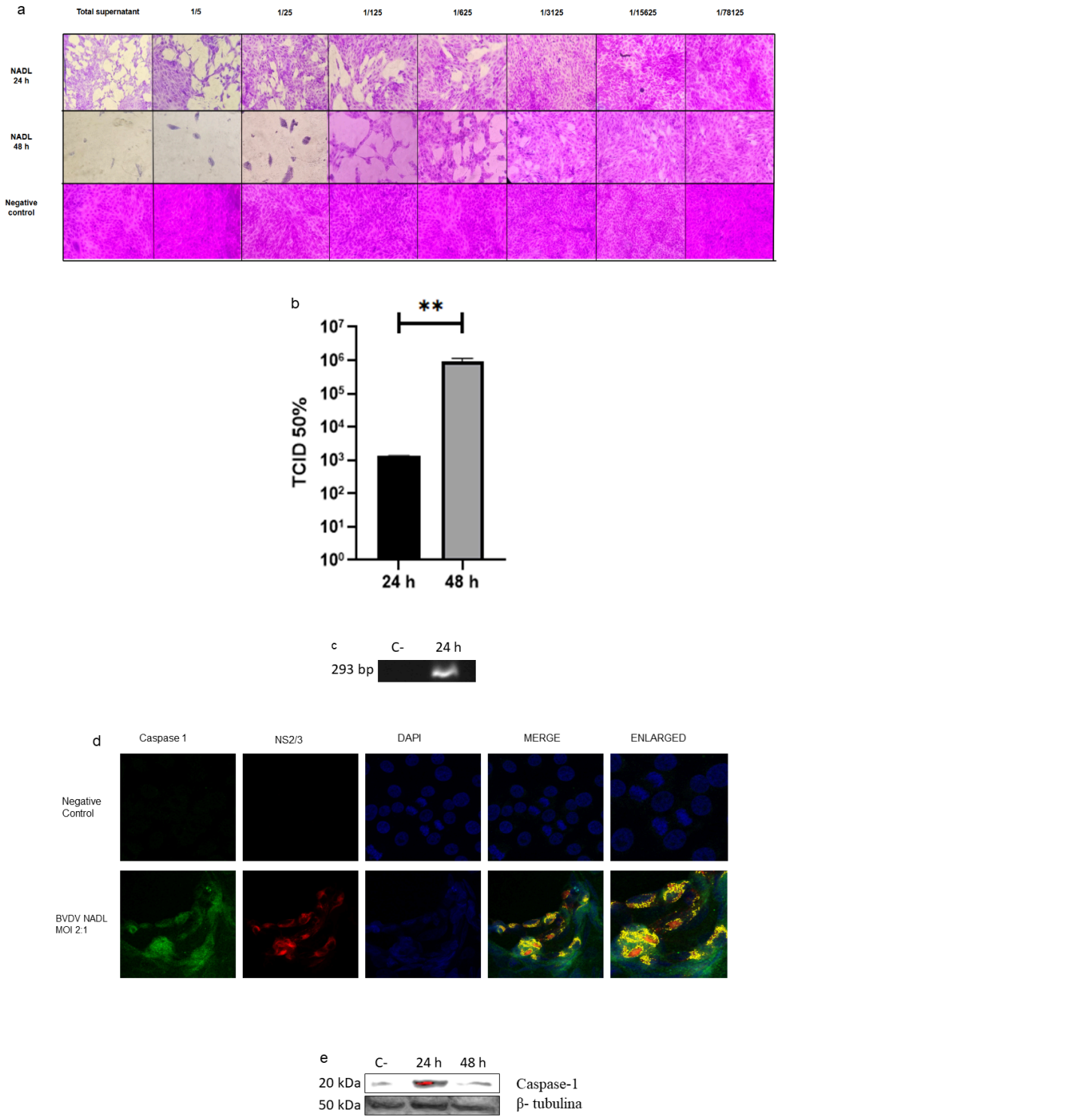


Figure 3

BVDV NS2-3 protein colocalizes with caspase-1 in MDBK cells. a) Cytopathic effect of BVDV at 24 and 48 h post-infection. b) TCID50% of BVDV infection in MDBK cells at 24 and 48 hpi. c) Identification of the BVDV 5' UTR in the infected culture. d) Colocalization of BVDV NS2-3 viral protein with caspase-1 in MDBK cells. e) Identification of active caspase-1 in MDBK cells by Western Blot (WB).

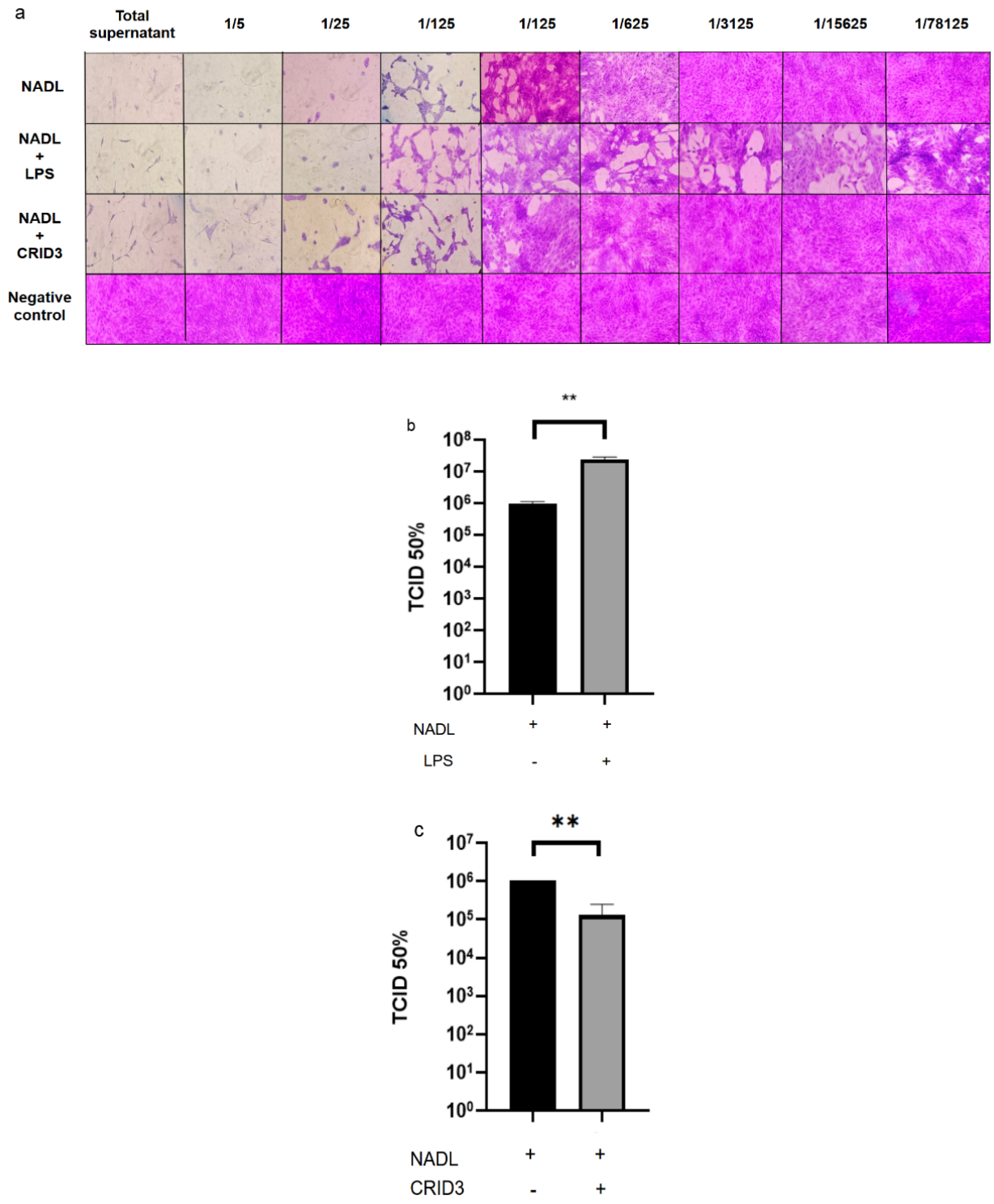


Figure 4

Effect of caspase-1 modulation on BVDV replication in MDBK cells. a) Cytopathic effect of BVDV at 48 h post-infection in MDBK cells with LPS or CRID3. b) TCID50% in BVDV-infected MDBK cells at 48 hpi with LPS stimulation. c) TCID50% in BVDV-infected MDBK cells at 48 with CRID3.

Supplementary Files

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- supplementarymaterial1.xlsx