

Mutations in Classical Swine Fever Virus NS4B Affect Virulence in Swine[∇]

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NS4B is one of the nonstructural proteins of classical swine fever virus (CSFV), the etiological agent of a severe, highly lethal disease of swine. Protein domain analysis of the predicted amino acid sequence of the NS4B protein of highly pathogenic CSFV strain Brescia (BICv) identified a putative Toll/interleukin-1 receptor (TIR)-like domain. This TIR-like motif harbors two conserved domains, box 1 and box 2, also observed in other members of the TIR superfamily, including Toll-like receptors (TLRs). Mutations within the BICv NS4B box 2 domain (V2566A, G2567A, I2568A) produced recombinant virus NS4B.VGIv, with an altered phenotype displaying enhanced transcriptional activation of TLR-7-induced genes in swine macrophages, including a significant sustained accumulation of interleukin-6 (IL-6) mRNA. Transfection of swine macrophages with the wild-type NS4B gene partially blocked the TLR-7-activating effect of imiquimod (R837), while transfection with the NS4B gene harboring mutations in either of the putative boxes displayed decreased blocking activity. NS4B.VGIv showed an attenuated phenotype in swine, displaying reduced replication in the oronasal cavity and limited spread from the inoculation site to secondary target organs. Furthermore, the level and duration of IL-6 production in the tonsils of pigs intranasally inoculated with NS4B.VGIv were significantly higher than those for animals infected with BICv. The peak of IL-6 production in infected animals paralleled the ability of animals infected with NS4B.VGIv to resist challenge with virulent BICv. Interestingly, treatment of peripheral blood mononuclear cell cultures with recombinant porcine IL-6 results in a significant decrease in BICv replication.

Classical swine fever (CSF) is a highly contagious lethal disease of swine. The etiological agent, CSF virus (CSFV), is a small enveloped virus with a positive single-stranded RNA (ssRNA) genome. Along with bovine viral diarrhea virus (BVDV) and border disease virus (BDV), CSFV is classified as a member of the genus *Pestivirus* within the family *Flaviviridae* (2). The 12.5-kb CSFV genome contains a single open reading frame that encodes a 3,898-amino-acid polyprotein and ultimately yields 11 to 12 final cleavage products (NH₂-Npro-C-E^{ms}-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH) through co- and posttranslational processing of the polyprotein by cellular and viral proteases (36, 46). NS4B is one of the nonstructural proteins of CSFV, and its function has not been clearly characterized.

Several studies of other flaviviruses, including hepatitis C virus (HCV), have revealed that NS4B is an endoplasmic reticulum (ER)-associated integral membrane protein that contains four putative transmembrane domains flanked by cytoplasmic N- and C-terminal regions (16, 25, 26, 34). The N-terminal region contains a conserved amphipathic helix that can become a transmembrane domain upon NS4B cleavage (10, 26), while the C-terminal region contains two predicted

helical domains (17). *In vitro* studies have shown that NS4B is involved in HCV RNA replication (3, 8, 24, 47). NS4B itself is sufficient to induce the formation of a membranous web, a specific membrane alteration that serves as a scaffold to facilitate HCV RNA replication (7). A recent study demonstrated that amino acids (aa) 40 to 69 in the N-terminal portion of NS4B are essential in the formation of a functional replication complex (13).

Additionally, NS4B interacts with other HCV nonstructural proteins during the viral replication cycle. Physical interaction between NS4B and nonstructural protein 3 (NS3) has been implicated in productive replication of HCV RNAs (32, 33). In studies of BVDV replication, NS3, NS4B, and NS5A have been associated as components of a multiprotein complex that serves a critical role in viral RNA synthesis (34). Further, it has been shown that NS4B not only functions in RNA replication but also plays an important role in virus assembly and release (17).

Interaction of flavivirus NS4B with molecular components of the immune system has also been reported. The double-stranded RNA-triggered, interferon regulatory factor 3 (IRF-3)-mediated antiviral interferon (IFN) expression pathway is suppressed in the presence of HCV NS4B protein (43). Expression of dengue virus NS4B strongly blocks the IFN-induced signal transduction cascade by interfering with STAT1 phosphorylation (31), an observation extended to West Nile and yellow fever viruses (30).

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In BVDV, cytopathogenicity in infected cell cultures has been linked to a single residue in NS4B. A mutation in residue 15 of NS4B (Y to C) conferred a noncytopathic phenotype in cell culture (34). Interestingly, only one study has provided *in vivo* evidence of a direct involvement of NS4B in virus virulence. Replacement of cysteine 102 with a serine in the West Nile virus NS4B protein rendered the virus temperature sensitive, with attenuated neuroinvasive and neurovirulence phenotypes in mice (50).

Investigation of the identity of CSFV NS4B with the NS4B proteins of members of the *Flaviviridae*, such as HCV, shows only a negligible resemblance (<http://www.ebi.ac.uk/clustalw/>). Despite this divergence, the topology of NS4B is similar among members of the *Flaviviridae* (26); it contains several ER and cytoplasmic domains separated by transmembrane regions (22, 26).

Using sequence analysis, we have identified a predicted Toll-interleukin-1 receptor (TIR)-like domain within CSFV NS4B. This predicted TIR domain is unique to CSFV and BVDV; it is not present in other pestiviruses or flaviviruses. TIR domains are known to participate in the signal transduction of Toll-like receptors (TLRs). TLRs constitute a large family of proteins that play essential roles in recognizing various microbial components, activating the innate immune system, and inducing cytokines needed for the adaptive immune response (1, 18, 19, 42). The signal transduction of TLRs is based on homotypic domain interactions, or TIR-to-TIR interactions, with TIR-containing adaptor proteins, such as MyD88, TIRAP/MAL, TRIF, and TRAM (1, 18). This signal transduction leads to the activation of many transcription factors, such as NF- κ Bs and IRFs, that translocate to the nucleus and regulate the transcription of genes involved in the immune response (1, 15, 19).

TIR domains contain three conserved motifs, named boxes 1, 2, and 3, which have been identified by multisequence alignments (39). Alanine substitutions in box 1 and 2 motifs within the TIR domain of the human interleukin-1 (IL-1) receptor inhibited its signaling, whereas mutations in box 3 had no effect (40). Based on those observations, we targeted box 1 and 2 motifs in CSFV NS4B for mutagenesis, replaced the wild-type sequence with alanines, and examined the phenotypic changes incurred with the mutant viruses. We report here that the substitutions in box 2 of the NS4B protein of highly virulent CSFV Brescia (BICv) produced an attenuated virus (NS4B.VGIv), with localized replication in the tonsils and limited dissemination during the infection. Regardless of its limited *in vivo* replication, animals infected with NS4B.VGIv were completely protected against virulent challenge with BICv as early as 3 days postinoculation. Cellular gene expression analyses of primary swine macrophage cultures infected with NS4B.VGIv showed significantly higher levels of activation of several genes, particularly IL-6, than those in cultures infected with the parental virus, BICv. Interestingly, most of the genes showing increased transcriptional activation upon NS4B.VGIv infection are also induced by activated TLR-7 (1, 18, 19), suggesting NS4B-mediated interference with TLR-7 activity during infection with virulent wild-type virus. The transient expression of wild-type NS4B, but not of the NS4B gene harboring mutations in either of the putative boxes, interfered with the transcriptional activation of TLR-7-induced immune-

regulatory genes (e.g., IL-6) in swine macrophages treated with a TLR-7 activator, imiquimod R837 (I-R837) (5, 11, 15, 21, 45, 49). *In vivo* analyses demonstrated significantly higher levels of IL-6 that were maintained in the tonsils of swine experimentally infected with NS4B.VGIv than in those of swine infected with BICv. Furthermore, treatment of swine peripheral blood mononuclear cell (PBMC) cultures with recombinant porcine IL-6 resulted in a significant reduction in BICv replication.

MATERIALS AND METHODS

Domain annotation of CSFV NS4B. The NS4B amino acid sequence (CSFV strain Brescia polypeptide amino acid residues 2337 to 2683) was used for domain annotations utilizing the SMART (simple modular architecture research tool) software program. This program identifies protein domains in sequence databases that share sequence homology with query sequences and outputs multiple sequence alignments of the query sequences with sequences of domain families. The porcine TIR domains of TLRs (1, 18) were also identified utilizing the SMART program software (39), along with human TLR1 and human TLR2, and were manually aligned together. The crystal structure for human TLR1 (RCSB PDB no. 11081518) was used as a template for modeling the NS4B structure, as viewed on the SWISS-MODEL program (<http://www.expasy.org/spdbv/>) (14), to compare NS4B residues to the crystallized human TLR1.

Viruses and cells. Swine kidney cells (SK6) (44), free of BVDV, were cultured in Dulbecco's minimal essential medium (DMEM) (Gibco, Grand Island, NY) with 10% fetal calf serum (FCS) (Atlas Biologicals, Fort Collins, CO). CSFV strain Brescia was propagated in SK6 cells and was used for the construction of an infectious cDNA clone (37). Growth kinetics was assessed using primary swine macrophage cell cultures prepared as described by Zsak et al. (51). Titration of CSFV from clinical samples was performed using SK6 cells in 96-well plates (Costar, Cambridge, MA). After 4 days in culture, viral infectivity was assessed using an immunoperoxidase assay utilizing the anti-CSFV monoclonal antibody (MAb) WH303 (6) and the Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Titers were calculated according to the method of Reed and Muench (35) and were expressed as 50% tissue culture infective doses (TCID₅₀) per milliliter. The sensitivity of the test, as performed, was $\geq \log_{10}$ 1.8 TCID₅₀/ml. Plaque assays were performed using SK6 cells in 6-well plates (Costar). SK6 monolayers were infected, overlaid with 0.5% agarose, and incubated at 37°C for 3 days. Plates were fixed with 50% (vol/vol) ethanol-acetone and were stained by immunohistochemistry with MAb WH303 (6).

Construction of CSFV mutants. A full-length infectious clone (IC) of the virulent strain Brescia (pBIC) (37) was used as a template in which putative TIR motifs I₂₅₃₁Y₂₅₃₂K₂₅₃₃ (box 1) and V₂₅₆₆G₂₅₆₇I₂₅₆₈ (box 2) were replaced with alanine triplets (see Fig. 2). Mutations were introduced by site-directed mutagenesis, using the QuikChange XL site-directed mutagenesis kit (Stratagene, Cedar Creek, TX), performed per manufacturer's instructions and using the following primers (only 5'→3' forward primer sequences are shown; mutations producing amino acid substitutions are italicized): for the IYK mutant, GTCATACTGAGTACCGCAGCCGCCGCAACCTACCTATCAA TCAGG; for the VGI mutant, TCACAAACCCAGTATCTGCGGCTGCA GCGGTACTGCTAGGGGTG. An IYK-VGI mutant was produced by sequential mutagenesis using the pIYK construct as a template with VGI primers.

Recombinant NS4B was expressed in swine macrophages as an N-terminal fusion of human green fluorescent protein II (hGFP II) into the Vitality phrGFP II-N mammalian expression vector (Stratagene, La Jolla, CA). Each version of NS4B (wild-type NS4B, NS4B.IYK, NS4B.VGI, or NS4B.VGI) was PCR amplified from a corresponding CSFV full-length infectious clone using primers F (5'-GAATCCGCTCAGGGGATGTGCAGAGAT-3') and R (5'-GTCGA CTTATAGCTG GCGGATCTTTCCTT-3'), followed by cloning into the vector using EcoRI and SalI restriction sites.

***In vitro* rescue of CSFV Brescia and NS4B mutants.** Full-length genomic clones were linearized with SrfI and were *in vitro* transcribed using the T7 Megascript system (Ambion, Austin, TX). RNA was precipitated with LiCl and was transfected into SK6 cells by electroporation at 500 V, 720 Ω , and 100 W with a BTX 630 electroporator (BTX, San Diego, CA). SK6 cells were seeded in 12-well plates and incubated for 4 days at 37°C under 5% CO₂. Virus was detected by immunoperoxidase staining as described above, and stocks of rescued viruses were stored at -70°C.

DNA sequencing and analysis. Full-length clones and *in vitro*-rescued virus were completely sequenced with CSFV-specific primers by the dideoxynucleotide

chain termination method (38). Viruses recovered from infected animals were sequenced in the region of the genome that contained the desired mutations. Sequencing reactions were prepared with the Dye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA). Reaction products were sequenced on a Prism 3730xl automated DNA sequencer (Applied Biosystems). Sequence data were assembled using Sequencher 4.7 software (Gene Codes Corporation, Ann Arbor, MI). The final DNA consensus sequence represented, on average, five redundancies at each base position.

Animal infections. The virulence of NS4B.VGIv relative to that of BICv was initially assessed in 10- to 12-week-old, 40-lb commercial-breed pigs inoculated intranasally (i.n.) with 10^5 TCID₅₀ of each virus. Fourteen pigs were randomly allocated into 2 groups of 8 and 6 animals and were inoculated with NS4B.VGIv or BICv, respectively. Clinical signs (anorexia, depression, purple skin discoloration, staggering gait, diarrhea, and coughing) and changes in body temperature (fever defined as a rectal temperature of $>104^\circ\text{F}$) were recorded daily throughout the experiment and were scored as previously described (28).

The effects of NS4B mutations on CSFV shedding and tissue distribution within the host were assessed in pigs i.n. inoculated with recombinant NS4B.VGIv ($n = 8$) or BICv ($n = 8$). To determine the distribution of the virus in tissues, one pig per group was sacrificed at 1, 2, 3, 4, 7, and 9 days postinfection (dpi), and tissue samples were collected. Tonsils, mandibular lymph nodes, spleens, and kidneys were removed from each pig and were either snap-frozen in liquid nitrogen for subsequent virus titration or mounted on polystyrene blocks for immunofluorescence analysis (see below). Blood, nasal swabs, and tonsil scraping samples were also obtained from each pig before necropsy and were used to assess virus shedding. The remaining 2 pigs in each group were monitored to check for the appearance of clinical signs during a 21-day observation period.

For infection-challenge studies, 12 pigs were randomly allocated into 3 groups containing 4 animals each. Pigs in groups 1 and 2 were i.n. inoculated with NS4B.VGIv, and pigs in group 3 were mock infected. At 3 dpi (group 1) or 28 dpi (group 2), animals were challenged with BICv along with animals in group 3. Clinical signs and body temperature were recorded daily throughout the experiment as described above. Blood, serum, nasal swabs, and tonsil scrapings were collected at various times postchallenge, with blood obtained from the anterior vena cava in EDTA-containing tubes (Vacutainer) for total and differential white blood cell counts. Total and differential white blood cell and platelet counts were obtained using a Beckman Coulter ACT (Beckman Coulter, CA).

Sample collection, immunofluorescence, and confocal microscopy. Triplicate samples were collected postmortem from palatine tonsils of infected-challenged animals. The tissues were mounted on polystyrene blocks using optimal cutting temperature (OCT) compound (Tissue-Tek Sakura, Torrance, CA), promptly frozen in liquid nitrogen, and stored at -70°C .

To assess the presence of VGIv and BICv, and to study the expression of IL-6, sections (thickness, 4 μm) were obtained from each of the triplicate cryopreserved tissue samples and were fixed with acetone for 10 min at -20°C . After fixation, tissue sections were incubated at room temperature (RT) for 90 min in blocking buffer containing 2% (wt/vol) bovine serum albumin (Sigma, St. Louis, MO) and 20% (vol/vol) normal bovine serum (Gibco-Invitrogen, Carlsbad, CA) in phosphate-buffered saline (PBS). Either primary MAb WH303 against CSFV E2 (6) or an anti-swine IL-6 MAb (R&D Systems, Minneapolis, MN) was diluted in blocking buffer and incubated with tissue sections overnight at 4°C in a humid chamber. After five washes with PBS at RT, tissue sections were incubated for 90 min at 37°C with the appropriate secondary antibodies, goat anti-mouse isotype-specific IgG labeled with either Alexa Fluor 488 or Alexa Fluor 594 (Molecular Probes-Invitrogen, Carlsbad, CA), diluted in blocking buffer. Following this incubation, tissue sections were washed five times with PBS at RT, counterstained with TOPRO-iodide 642/661 (Molecular Probes) for 5 min at RT, washed as before, mounted, and examined in a Leica scanning confocal microscope (TCS2; Leica Microsystems, Bannockburn, IL). Data were collected utilizing an appropriate control lacking incubation with primary antibodies in order to determine channel crossover settings and negative background levels. The captured images were adjusted for contrast and brightness using Adobe Photoshop software (Adobe, San José, CA).

Quantitative real-time PCR (qrt-PCR). Swine macrophage primary cell cultures were infected at a multiplicity of infection (MOI) of 1, and total cellular RNA was extracted at 24 and 48 h postinfection/posttreatment using a RNeasy minikit (Qiagen). Contaminant genomic DNA was removed by DNase treatment using Turbo DNA-free (Ambion). After DNase treatment, genomic DNA contamination of RNA stocks was assessed by real-time PCR amplification targeting the porcine β -actin gene. Total RNA was quantified and cDNA synthesized with random primers using a High Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA) in 100- μl reaction mixtures containing 2,500 ng of

total RNA. For gene expression quantification, first-strand cDNA was amplified by real-time PCR using Power SYBR green PCR master mix (Applied Biosystems) with primer pairs described in reference 4. A 50- μl reaction mixture contained 25 μl of Power SYBR green PCR master mix, 5 μl of cDNA, and 400 nmol/liter of each primer. Cycling conditions were as follows: activation of the AmpliTaq Gold polymerase at 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 s followed by annealing and extension at 60°C for 1 min, and a melting curve analysis performed at the end of the PCR. Real-time PCRs were run using the ABI 7500 Real-Time PCR system platform (Applied Biosystems). Gene expression data were normalized by assessing mRNA accumulation of a housekeeping gene (β -actin) in infected and mock-infected cell cultures. Relative quantities (RQ) of mRNAs were then calculated using the $2^{-\Delta\Delta\text{CT}}$ method (described in ABI Prism 7700 sequence detection system User Bulletin 2 [PN 4303859]). The normalized mRNA expression level of a cellular gene in infected cells was considered significant when it departed from its level in uninfected cells 3-fold in either direction (4).

Effect of NS4B on swine macrophages treated with imiquimod R837. Primary blood-derived swine macrophage cultures were transfected with 5 μg of phrGFP, phrGFP/NS4B, phrGFP/NS4B.IYK, phrGFP/NS4B.IYK.VGI, or phrGFP/NS4B.VGI plasmid DNA by using Lipofectamine (Invitrogen, Carlsbad, California) according to the manufacturer's protocol. Twenty-four hours after transfection, cells were treated with 20 $\mu\text{g}/\text{ml}$ imiquimod R837 (Invitrogen, San Diego, CA), and RNA was collected 24 h posttreatment using QIAshredder columns (Qiagen), followed by RNA isolation using an RNeasy kit (Qiagen). The RNA was treated to remove genomic DNA contamination, reverse transcribed, and processed by real-time PCR. Gene expression was quantified by qrt-PCR as described above.

Development of a recombinant adenovirus expressing swine IL-6. A replication-defective human adenovirus type 5 (Ad5) containing the porcine IL-6 gene was developed (29). Briefly, the IL-6 gene (GenBank accession no. DQ832259) was synthesized (Bio Basic Inc., Ontario, Canada), flanked by ClaI and XbaI restriction sites (pIL-6). The pIL-6 fragment was subsequently cloned into pAd5Blue (29), which was also digested with ClaI and XbaI (pAd5pIL6). The Ad5pIL6 plasmid was then linearized with PacI and transfected into human embryonic kidney 293 (HEK 293) cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Recombinant virus was harvested 4 days posttransfection, further amplified in HEK 293 cells, and purified using a discontinuous followed by a continuous CsCl gradient centrifugation procedure. Recombinant IL-6 was generated by infecting nonpermissive SK6 cells with Ad5pIL6v. At 24 h postinfection (hpi), the culture medium containing secreted IL-6 was harvested and used in subsequent experiments.

Effect of IL-6 treatment on swine PBMC cultures infected with CSFV. Primary swine macrophage or PBMC cultures were prepared as described by Zsak et al. (51) in 24-well plates (Primaria, Falcon; Becton Dickinson, Franklin Lakes, NY). Cultures were treated with either Ad5-derived IL-6 (at a concentration of approximately 85 ng/ml, as quantified by an enzyme-linked immunosorbent assay specific for swine IL-6 [Quantikine IL-6 immunoassay; R&D Research, MN]) or the supernatant obtained from SK6 cells infected with Ad5Blue virus, or they were mock treated. Twenty-four hours later, cultures were infected with BICv at an MOI of 0.1. Virus yield was assessed at 24 hpi. Additionally, a dose-response experiment was performed using 10-fold-decreasing concentrations of IL-6 (85 through 0.085 ng/ml) under conditions similar to those described above.

Effect of treatment with imiquimod R837 in swine macrophage cultures infected with CSFV. Primary swine macrophage cultures were prepared as described previously (51) in 24-well plates. Cultures were treated with 20 $\mu\text{g}/\text{ml}$ imiquimod R837 either 24 h before the virus infection or at 6, 18, or 24 h after virus infection. Cultures were infected with BICv or NS4B.VGIv at an MOI of 0.1. Virus yield was assessed at 24 hpi by determining the titers of samples in SK6 cells.

RESULTS

NS4B contains a putative TIR domain. Utilizing SMART program analysis software (39) for wide genome screening of CSFV strain Brescia, we observed that NS4B has a putative TIR domain located between amino acids 195 and 328 (residues 2531 to 2664 of the CSFV polyprotein). In general, TIR domains lack a specific amino acid sequence and show only 20 to 30% identity (data not shown) (<http://www.cellsignal.com/reference/domain/tir.html>). To identify TIR domains, the

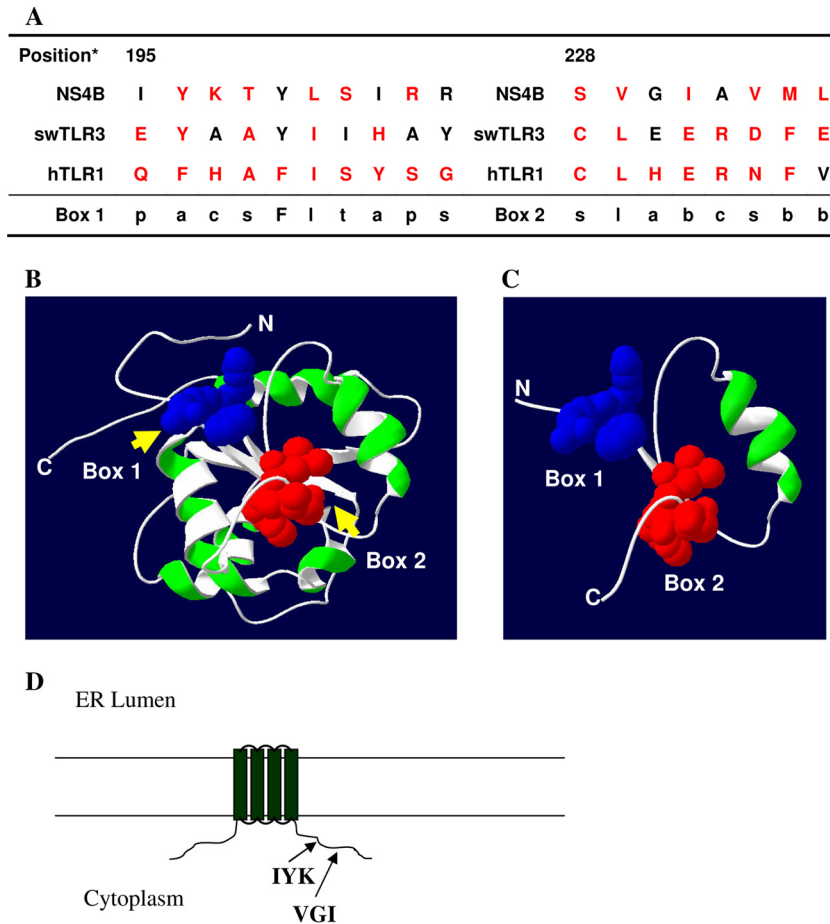


FIG. 1. (A) Alignment of the CSFV NS4B TIR domain against *Sus scrofa* Toll-like receptor TIR domains (swTLR3) and human TLR1 (hTLR1). Shown are amino acid sequences between positions 195 and 204 (box 1) and between positions 228 and 235 (box 2) of the CSFV NS4B protein. “Position *” indicates the residue position in the CSFV NS4B protein. The consensus sequence is composed of residues in 60% of TIR domains for box 1 and box 2. Amino acid consensus: a, aromatic amino acids (F, H, W, Y); b, big amino acids (E, F, H, I, K, L, M, Q, R, W, Y); c, charged amino acids (D, E, H, K, R); h, hydrophobic amino acids (A, C, F, G, H, I, L, M, T, V, W, Y); l, aliphatic amino acids (I, L, V); p, polar amino acids (C, D, E, H, K, N, Q, R, S, T); s, small amino acids (A, C, D, G, N, P, S, T, V); t, tiny amino acids (A, G, S). (B) Human TLR1 TIR domain crystal structure, with the corresponding residues of the CSFV NS4B IYK mutant highlighted in blue and those of the VGI mutant highlighted in red. The crystal structure was viewed using Swiss-PDB Viewer (<http://www.expasy.org/spdbv/>). (C) Simplified human TLR1 TIR domain crystal structure containing only residues 634 to 676, colored and modeled as in panel B. (D) Schematic representation of CSFV NS4B protein topology (based on the structure predicted from HCV [25, 26]). Predicted positions for boxes 1 and 2 are indicated.

SMART program utilizes areas of similar amino acid properties that are specific to known TIR domains. A TIR domain consists of three boxes (termed boxes 1, 2, and 3 by Slack et al. [40]); box 1 and box 2 are the most critical for function. These boxes of conserved residues are set in a core sequence ranging from 135 to 160 amino acids. In NS4B, both boxes 1 and 2 display high similarity to the consensus sequence profile of the TIR domain family. This consensus sequence, (particularly compared with swine TLR3 and human TLR1) is shown in Fig. 1A.

To avoid abolishing the putative essential function of NS4B, we determined areas in the predicted TIR domain that would be suitable for disrupting potential TIR-TIR domain interactions without completely altering the putative secondary structure of NS4B. To do this, we used the crystal structure of human Toll-like receptor 1 as a model and determined that residues IYK in box 1 and VGI in box 2 are potential protein contact sites in the TIR domain of CSFV NS4B that, if mod-

ified, would not affect the protein secondary structure (Fig. 1B and C).

Construction of CSFV NS4B mutant viruses. Mutagenesis of box 1 and 2 motifs of NS4B was conducted according to the report by Slack et al. (40). Infectious RNA was *in vitro* transcribed from a full-length IC of CSFV Brescia containing mutations at the desired amino acid positions (Fig. 1A and 2A) that was subsequently used to transfect SK6 cells. Infectious clone mutants, referred to as pNS4B.IYK, pNS4B.VGI, and double mutant pNS4B.IYK.VGI, contained putative TIR-like motifs partially replaced by alanine residues (Fig. 2A). Infectious virus was rescued from transfected cells by day 4 after transfection with the pNS4B.VGI construct. In contrast, after four independent transfection procedures, the pNS4B.IYK and pNS4B.IYK.VGI constructs did not produce infectious viruses. NS4B.IYK and NS4B.IYK.VGI RNA transcripts used in transfections were completely sequenced to verify fidelity during the *in vitro*

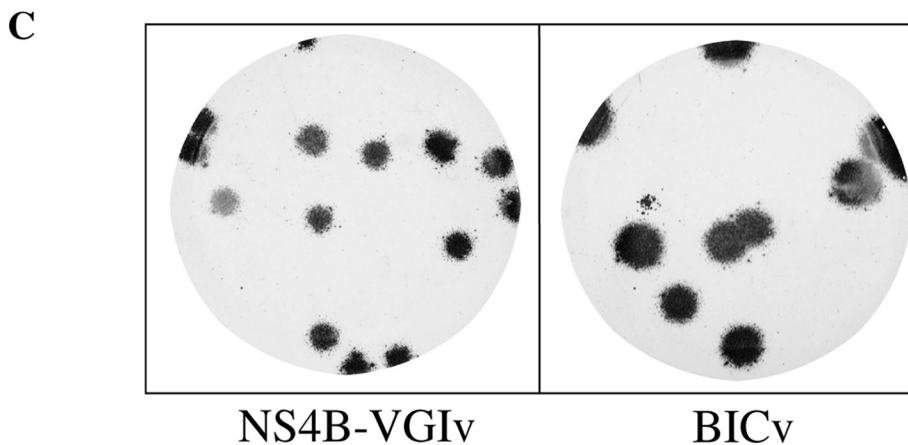
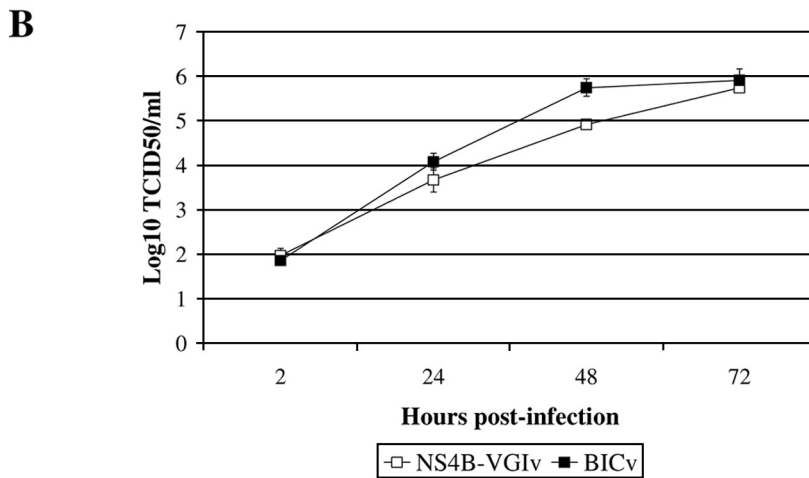
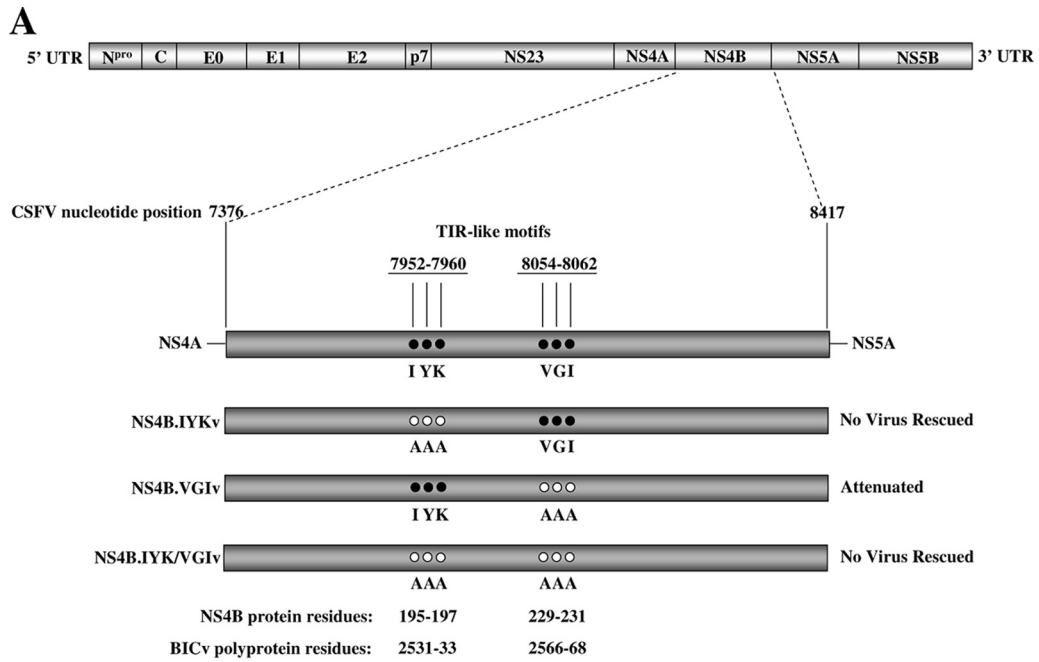


FIG. 2. (A) Schematic representation of substitutions at box 1 and box 2 of classical swine fever virus NS4B protein, generated by site-directed mutagenesis of the full-length cDNA clone pBIC. Wild-type NS4B is shown at the top; box 1 (IYK) and box 2 (VGI) are depicted, along with their positions in the CSFV nucleotide sequence, CSFV polypeptide, and NS4B amino acid sequence. Mutants are designated by the mutated residues. (B) *In vitro* growth characteristics of CSFV NS4B.VGIv and the parental virus, BICv. Primary swine macrophage cell cultures were infected

TABLE 1. Swine survival and fever response following infection with CSFV mutant NS4B.VGIv or the parental strain, BICv

Virus	Survival		Fever ^a		
	No. of survivors/ total no.	Time to death (days) ^a	No. of days to onset	Duration (no. of days)	Maximum daily temp (°F)
NS4B.VGIv	8/8	No	No	No	103.8 (0.4)
BICv	0/6	11 (0.82)	3 (0)	6.75 (1.71)	106.5 (0.6)

^a Values are means (standard deviations).

transcription reaction. The complete nucleotide sequence of the rescued NS4B.VGI virus (NS4B.VGIv) genome was identical to that of the parental DNA plasmid, confirming that only mutations at predicted motif sites were present in the rescued virus.

Replication of the CSFV NS4B.VGIv mutant *in vitro*. The *in vitro* growth characteristics of the mutant virus NS4B.VGIv relative to those of the parental virus, BICv, were evaluated in a multistep growth curve. Primary swine macrophage cell cultures were infected at an MOI of 0.01 TCID₅₀ per cell. Virus was adsorbed for 1 h (time zero), and samples were collected at various times postinfection through 72 h. NS4B.VGIv exhibited growth characteristics similar to those of BICv (Fig. 2B). Similar results were obtained when growth kinetics was assessed in SK6 cells (data not shown). Additionally, when NS4B.VGIv was tested for its plaque size in SK6 cells, it exhibited a reduction in plaque size of approximately 50% relative to the parental virus, BICv (Fig. 2C).

Virulence of CSFV NS4B.VGIv mutants *in vivo*. To examine whether the mutations in residues 2566 to 2568 of the CSFV polypeptide (residues 229, 230, and 231 of the NS4B protein) affected virulence, pigs were intranasally inoculated with 10⁵ TCID₅₀ of the NS4B.VGIv mutant (Table 1) and were monitored for clinical disease, evaluated relative to that with the parental virus BICv. While BICv exhibited a characteristic lethal phenotype, animals infected with NS4B.VGIv survived the infection and remained clinically normal throughout the observation period (21 days). All animals infected with BICv presented clinical signs of CSF starting 4 to 6 dpi, developing classical symptoms of the disease and dying around day 11 postinfection. White blood cell and platelet counts dropped by 4 to 6 dpi in animals inoculated with BICv and kept declining until death, while only a transient decrease was observed in animals inoculated with NS4B.VGIv (data not shown).

Viremia in NS4B.VGIv-inoculated animals was transient (Tables 2 and 3) and was significantly reduced, by 10⁴ to 10⁵, from that observed in BICv-infected swine. Similar patterns were observed for nasal and tonsil samples (Table 2). In all cases, nucleotide sequences of the NS4B gene from viruses recovered from infected animals were identical to those of stock viruses used for inoculation (data not shown).

TABLE 2. Titers of virus in clinical samples after intranasal inoculation with mutant NS4B.VGIv or the parental strain, BICv

Virus (dpi)	No. of animals with virus/total no. of inoculated animals per group (avg viral titer from positive infected animals [\log_{10} TCID ₅₀ /ml])		
	Blood	Nasal swabs	Tonsil scrapings
NS4B.VGIv			
4	0/4	1/4 (1.9)	0/4
6	3/4 (2.2)	1/4 (2.4)	0/4
8	1/4 (2.0)	0/4	1/4 (1.90)
12	0/4	0/4	0/4
BICv			
4	4/4 (4.5)	0/4	4/4 (2.0)
6	4/4 (7.2)	4/4 (4.5)	4/4 (4.1)
8	4/4 (7.6)	4/4 (5.0)	4/4 (4.8)
12	D ^a	D	D

^a D, all animals in this group were dead by this time point.

The ability of NS4B.VGIv to establish a systemic infection in intranasally inoculated animals was compared with that of the virulent parental virus, BICv. Randomly selected animals were euthanized at 1, 2, 3, 4, 7, and 9 dpi (one animal/time point/group), and virus titration was performed in collected tissues (tonsils, mandibular and retropharyngeal lymph nodes, kidneys, spleen, and blood). The titers measured in those tissue samples are shown in Table 3. *In vivo* replication of NS4B.VGIv was transient in tonsils, with titers 10² to 10⁵ lower than those of BICv depending on the time postinfection. Differences between NS4B.VGIv and BICv titers were also observed in mandibular and retropharyngeal lymph nodes, and no mutant virus was detected in blood, spleen, or kidneys, indicating a severely limited ability of NS4B.VGIv to spread within the host.

NS4B.VGIv protects pigs against lethal CSFV challenge. The limited *in vivo* replication kinetics of NS4B.VGIv is similar to that observed with CSICv, a CSFV vaccine strain (37). However, restricted viral *in vivo* replication could also impair protection against wild-type virus infection. Thus, the ability of NS4B.VGIv to induce protection against virulent BICv was assessed in early and late vaccination-exposure experiments. Groups of pigs ($n = 4$) were intranasally inoculated with NS4B.VGIv and were challenged at 3 or 28 dpi. Mock-vaccinated control pigs receiving BICv ($n = 4$) developed anorexia, depression, and fever by 4 days postchallenge (dpc), as well as marked reductions in circulating leukocytes and platelets by 4 dpc (data not shown), and died or were euthanized *in extremis* by 10 dpc. Notably, infection with NS4B.VGIv (under the conditions described in this report) induced protection when pigs were challenged with the virulent parental virus at 3 and 28 dpi, as evidenced by the absence of clinical signs (Table 4). All pigs survived the BICv challenge and remained clinically

(MOI, 0.01) with the mutant or BICv, and virus yields were titrated at different times postinfection in SK6 cells. Data are means and standard deviations from two independent experiments. Sensitivity of virus detection, $\geq \log_{10}$ 1.8 TCID₅₀/ml. (C) Plaque formation by the CSFV NS4B.VGIv mutant and BICv. SK6 monolayers were infected, overlaid with 0.5% agarose, and incubated at 37°C for 3 days. Plates were fixed with 50% (vol/vol) ethanol-acetone and were stained by immunohistochemistry with MAb WH303 (6).

TABLE 3. Titers of virus in tissues after intranasal inoculation with mutant NS4B.VGIv or the parental strain, BICv

Virus (dpi)	Viral titer ^a (TCID ₅₀ /ml) in:						Virus detection ^b
	Tonsils	MLN	RFLN	Spleen	Kidneys	Blood	
NS4B.VGIv							
1	Neg	Neg	Neg	Neg	Neg	Neg	Neg
2	Neg	Neg	Neg	Neg	Neg	Neg	Neg
3	Neg	1.97	2.1	Neg	Neg	Neg	Neg
4	Neg	1.97	2.2	Neg	Neg	Neg	+
7	3.2	Neg	3.2	Neg	Neg	Neg	++
9	Neg	Neg	Neg	Neg	Neg	Neg	ND
BICv							
1	Neg	Neg	Neg	Neg	Neg	Neg	Neg
2	1.97	2.3	Neg	Neg	Neg	Neg	Neg
3	3.8	3.8	2.9	2.3	Neg	2.0	+++
4	4.2	3.6	5.0	3.8	2.8	4.2	+++
7	5.0	4.5	4.8	5.6	2.6	5.8	+++
9	5	5.1	4.6	4.6	4.1	6.5	ND

^a Neg, viral titer of ≤ 1.8 TCID₅₀/ml. MLN, mandibular lymph nodes; RFLN, retropharyngeal lymph nodes.

^b Immunofluorescence was performed on tonsil tissues obtained from one animal per time point using anti-E2 MAb WH303. Neg, negative. Positive reactivity was estimated from low (+) to strong (+++). ND, not determined.

normal, without significant changes in their hematological values (data not shown). Viremia and virus shedding of vaccinated-exposed animals were examined at 4, 6, 8, 12, 14, and 21 dpc (Table 5). As expected, in mock-vaccinated control animals, viremia was observed by 4 dpc, with virus titers remaining high by 8 dpc ($10^{7.8}$ TCID₅₀/ml) in the surviving pigs. Furthermore, virus was detected in the nasal swabs and tonsil scrapings of these animals after 4 to 6 dpc. In contrast, no challenge virus was detected in any clinical sample obtained from animals previously inoculated with NS4B.VGIv. Thus, even though NS4B.VGIv showed limited *in vivo* growth, solid protection was induced shortly after vaccination.

Transcriptional activation profile of immunologically relevant genes in swine macrophages infected with NS4B.VGIv. To further understand possible mechanisms responsible for NS4B.VGIv attenuation, the pattern of activation of immunologically relevant genes in swine macrophages infected with mutant and parental viruses was analyzed using quantitative real-time PCR (4) followed by melting curve analysis. To assess changes in cellular gene expression upon infection, primary porcine macrophage cell cultures were infected at an MOI of 1 TCID₅₀ per cell with either NS4B.VGIv or BICv. Total cellular RNA was extracted from infected and mock-infected cells at 24 hpi (exponential growth) and 48 hpi (growth plateau). Prior to synthesis of cDNA, total RNA was treated with DNase and tested for genomic DNA contamination by means

of real-time PCR (see Materials and Methods). Steady-state levels of mRNA accumulation were determined for 58 swine immunomodulatory genes as described elsewhere (4). This approach identified 8 genes differentially expressed in primary porcine macrophages infected with NS4B.VGIv or BICv (Fig. 3): AMCF-1 (also known as IL-8), AMCF-2, IFN- α , IL-1 α , IL-1 β , IL-6, MCP2 (also known as CCL8), and NCP-1. Expression of AMCF-1, AMCF-2, IFN- α , IL-1 α , and NCP-1 was slightly augmented at 24 hpi. Expression of IL-1 β was significantly increased at 24 hpi, and increased levels of MCP-2 were detected at 48 hpi. Notably, levels of IL-6 were significantly increased (>10-fold) both at 24 and at 48 hpi in cultures infected with NS4B.VGIv (Fig. 3A and B).

IL-6 expression in tonsils of pigs infected with NS4B.VGIv or BICv. Since NS4B.VGIv induces a higher level of IL-6 expression than BICv in swine cells *in vitro* (Fig. 3), it was important to investigate whether these changes in expression also occur *in vivo* during CSFV infection. Two groups containing six animals each were infected intranasally with 10^5 TCID₅₀ of either NS4B.VGIv or BICv. One animal from each group was euthanized at 6 h and 1, 2, 3, 4, and 7 days postinfection, and their tonsils were removed in order to assess the presence of cells producing IL-6 by means of immunofluorescence and detection of cells harboring viral antigen (as described in Materials and Methods). The results demonstrated abundant expression of IL-6 in tonsils, which could be detected as early as 1 dpi in NS4B.VGIv-infected animals and was sustained until 3 dpi, with some cells expressing IL-6 at 4 dpi (Fig. 4). In contrast, IL-6-producing cells were detected in the tonsils of BICv-infected pigs only at days 2 and 3 postinfection, and their numbers were significantly lower than those in NS4B.VGIv-infected animals at the same time points (Fig. 4). Interestingly, viral titers and the presence of viral antigen in tonsils appear to have an inverse relationship with the extent and abundance of IL-6 expression. A significant number of infected cells were observed in the tonsils of animals infected with BICv, starting at 3 dpi and lasting until the animals died. Mean-

TABLE 4. Swine survival and fever response after challenge of NS4B.VGIv-infected animals with virulent BICv

Vaccine (challenge time)	Survival ^a		Fever ^a	
	No. of survivors/total no.	CSFV symptoms/time to death (days)	Mean time to onset (days)	Duration (days)
NS4B.VGI (3 dpi)	4/4	No	No	No
NS4B.VGI (28 dpi)	4/4	No	No	No
Mock	0/4	Yes/9.5 (1.2)	4.5 (0.7)	9.5 (0.7)

^a Values in parentheses are standard deviations.

TABLE 5. Detection of virus in nasal swabs, tonsil scrapings, and blood samples obtained after challenge of NS4B.VGIv-infected animals with virulent BICv

Challenge group and sample	No. of animals from whom virus was isolated/total no. of animals challenged (avg virus titer from positive infected animals [\log_{10} TCID ₅₀ /ml]) at the following time:						
	Day of challenge	4 dpc	6 dpc	8 dpc	12 dpc	14 dpc	21 dpc
3 dpi							
Nasal	0/4	0/4	0/4	0/4	0/4	0/4	0/4
Tonsil	0/4	0/4	0/4	0/4	0/4	0/4	0/4
Blood	0/4	0/4	0/4	0/4	0/4	0/4	0/4
28 dpi							
Nasal	0/4	0/4	0/4	0/4	0/4	0/4	0/4
Tonsil	0/4	0/4	0/4	0/4	0/4	0/4	0/4
Blood	0/4	0/4	0/4	0/4	0/4	0/4	0/4
Control							
Nasal	0/4	0/4	4/4 (4.4)	4/4 (4.9)	D ^a	D	D
Tonsil	0/4	1/4 (2.0)	4/4 (4.1)	4/4 (4.8)	D	D	D
Blood	0/4	4/4 (4.5)	4/4 (7.4)	4/4 (7.8)	D	D	D

^a D, animals died or were euthanized.

while, NS4B.VGIv was poorly and transiently detected in the tonsils of infected animals between 4 and 7 dpi only (Table 3). Thus, it appears that both *in vitro* and *in vivo*, NS4B.VGIv infection consistently induced a stronger IL-6 response than that observed during BICv infection, and the expression of IL-6 *in vivo* seems to be inversely related to the ability of the virus to replicate in the tonsils of infected animals.

Effect of IL-6 treatment of swine PBMC cultures on the replication of CSFV. The possible effect of IL-6 on the replication of CSFV was analyzed in swine PBMC and macrophage cultures. Swine primary cell cultures of PBMCs and macrophages were pretreated with approximately 85 ng/ml of adenovirus-derived swine IL-6 24 h before infection with BICv (MOI, 0.1). The presence of IL-6 had no effect on virus replication in macrophage cell cultures. Virus titers in the supernatants harvested at 24 h from IL-6 treated cultures, mock-treated cultures, or cultures containing Ad5Blue virus were practically undistinguishable (Fig. 5A). However, PBMC cultures treated with IL-6 showed a drastic decrease (by \log_{10} 3.15 TCID₅₀/ml, with a standard deviation [SD] of 0.2) in virus yield from that for mock-treated or untreated cultures (Fig. 5A). A dose-response experiment performed using different concentrations of IL-6 showed that the detrimental effect of this cytokine on BICv replication is dose dependent (Fig. 5B).

CSFV NS4B blocks the effect of imiquimod R837, a TLR-7 activator, in swine macrophage cultures. The differential pattern of immunologically relevant genes activated in macrophages during infection with NS4B.VGIv and BICv resembled that induced by a well-characterized compound that stimulates macrophage activity, imiquimod R837 (I-R837) (5, 11, 12, 21, 45, 49). I-R837, an imidazoquinoline amine analog to guanosine, is an immune response modifier with potent indirect antiviral activity (5). This low-molecular-weight synthetic drug induces the activation of TLR-7 (12, 15, 21), leading to expression of several cytokines, such as type I IFN, IL-1, IL-6, IL-8, IL-10, MCP-1, and tumor necrosis factor (TNF) (5, 11,

12, 15, 21, 45, 49). The similarity between the pattern of gene activation in macrophages stimulated with I-R837 and by infection with NS4B.VGIv suggests the possibility of interference by CSFV NS4B with TLR-7 function (Fig. 3). First, in order to analyze this possibility, primary swine macrophage cultures were transfected with either phrGFP.NS4B or phrGFP constructs, followed 24 h later by stimulation with 20 μ g/ml of I-R387. Blockage of increased transcription of the marker genes IFN- β , IL-1 β , IL-6, and TNF- α in swine macrophage cultures treated with I-R387 was assessed as an indication of suppressed TLR-7 activity. Transfection with pGFP.NS4B provokes a blockage of the transcriptional activation of the IFN- β , IL-1 β , IL-6, and TNF- α genes compared with the level of activation detected in macrophages transfected with pGFP. Thus, CSFV NS4B appears to obstruct the TLR-7 function induced by I-R837 (Fig. 6A, B, C, and D). Second, the abilities of mutant forms of NS4B protein (the IYK and VGI mutants and the IYK VGI double mutant) to block I-R837-induced TLR-7 activation were assessed by an experiment similar to that described above. Macrophage cultures were transfected with either phrGFP.NS4B, phrGFP.NS4B.IYK, phrGFP.NS4B.VGI, or phrGFP.NS4B.IYK.VGI and were stimulated with 20 μ g/ml of I-R837 at 24 h posttransfection. The transcriptional activation of the IL-6 gene (used as a marker for TLR-7 activation) was then assessed 24 h after I-R837 treatment. Transcriptional activation of the IL-6 gene was blocked in cells transfected with wild-type phrGFP.NS4B. However, cells transfected with mutant forms of NS4B, phrGFP.NS4B.IYK or phrGFP.NS4B.IYK.VGI, failed to block the transcription of the IL-6 gene. A partial block of IL-6 transcription was observed in cells transfected with mutant phrGFP.NS4B.VGI. Therefore, alterations of both the IYK and the VGI motif in NS4B appear to affect its ability to block the TLR-7 activity induced by I-R837 (Fig. 6E).

Effect of I-R837 treatment of swine macrophage cultures on the replication of CSFV. I-R837 is known to possess antiviral activity (5). Thus, the effect of I-R837 on the replication of BICv or NS4B.VGIv was analyzed using swine macrophage

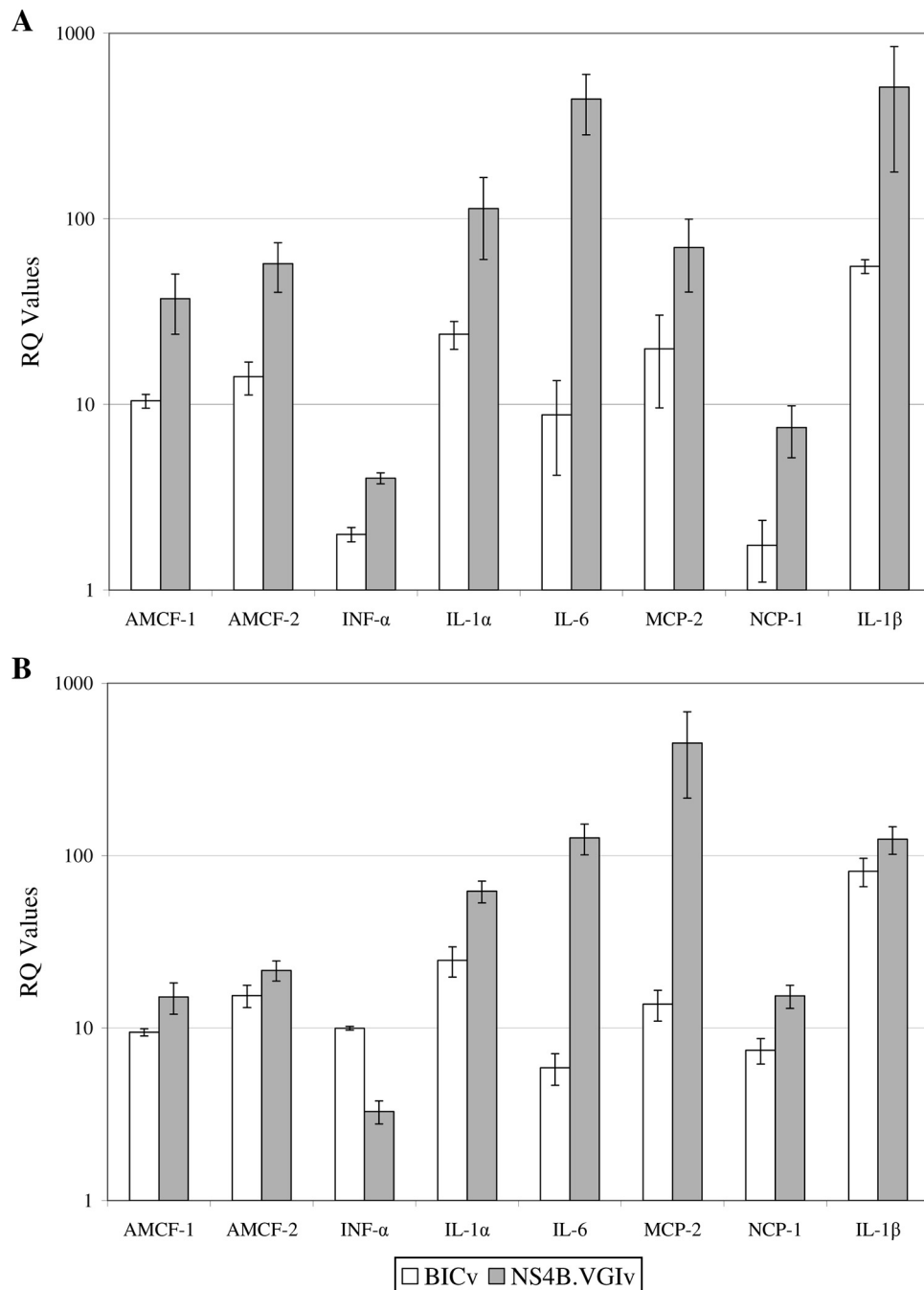


FIG. 3. Gene expression changes in peripheral blood-derived macrophages at 24 h (A) and 48 h (B) after infection with CSFV NS4B.VGIv or BICv. Gene expression was quantified by qrt-PCR. Values are expressed as RQ of mRNA accumulation (estimated by the $2^{-\Delta\Delta CT}$ method) with standard deviations.

cultures. Swine primary macrophage cell cultures were pretreated with 20 $\mu\text{g/ml}$ of I-R837 24 h before infection with either BICv or NS4B.VGIv (MOI, 0.1). The presence of I-R837 clearly reduces the yield of BICv at 24 hpi (by \log_{10} 3.17 TCID₅₀/ml [SD, 0.5]) from that for the untreated culture. Interestingly, I-R837 treatment seems to be less effective in cultures infected with NS4B.VGIv (reduction, \log_{10} 1.86 TCID₅₀/ml [SD, 0.15]) (Fig. 7A).

Additionally, the effect of I-R837 on CSFV progeny yield

was assessed in cells treated after infection. Macrophage cell cultures were infected with either the parental virus, BICv, or mutant NS4B.VGIv (MOI, 0.1) and were then treated with 20 $\mu\text{g/ml}$ of I-R837 at 6, 12, or 18 h postinfection. The presence of I-R837 clearly reduced NS4B.VGIv yields in macrophage cultures treated at 6 h postinfection (by \log_{10} 2.7 TCID₅₀/ml [SD, 0.2]). The observed effect on virus progeny yield progressively disappeared when macrophage cultures were treated at 6, 18, and 24 h postinfection. BICv

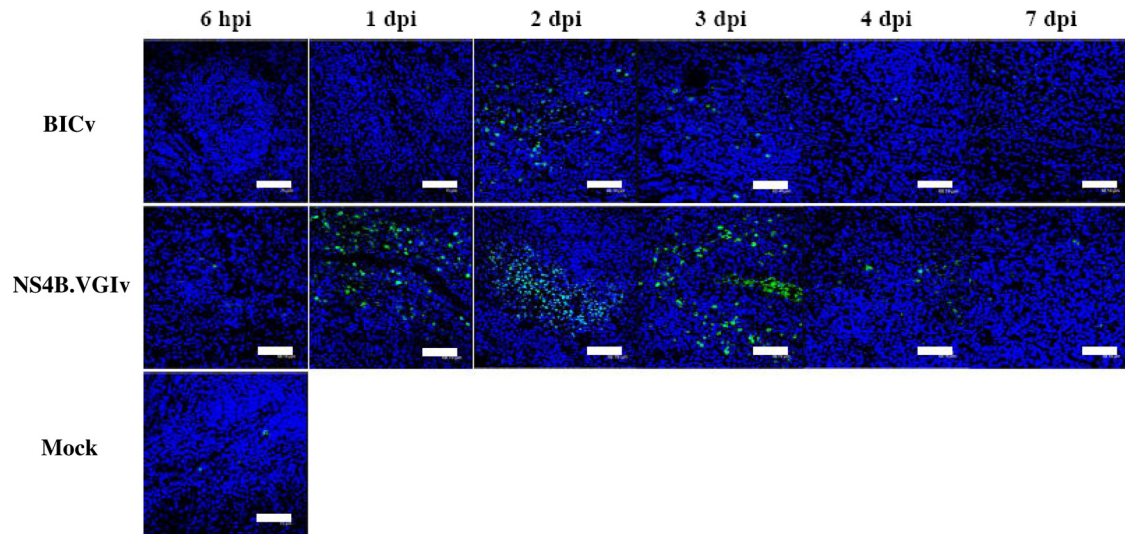


FIG. 4. Detection of IL-6-producing cells in the tonsils of animals infected i.n. with either NS4B.VGIv or BICv at 10^5 TCID₅₀/ml. The presence of IL-6 was detected by immunofluorescence.

progeny yields were not affected by treatment of macrophage cultures with I-R837 (Fig. 7B).

DISCUSSION

It has been demonstrated that the NS4B protein of flaviviruses, an endoplasmic reticulum-associated integral membrane protein (10, 16, 17, 25, 26, 34), is involved in several viral functions including RNA replication (3, 7, 8, 9, 13, 24, 47), interacting closely with other viral nonstructural proteins during the cycle of replication (32, 33, 34), and in the process of virus assembly and release (17). Although direct involvement of NS4B in flavivirus virulence has been reported infrequently (50), its role in the modulation of interferon response has been described (30, 31, 43). However, although the topology of NS4B is similar among members of the *Flaviviridae* (17, 25, 26, 27), and it contains several ER and cytoplasmic domains separated by transmembrane regions (Fig. 1D), the CSFV NS4B protein exhibits only a negligible resemblance (<http://www.ebi.ac.uk/clustalw/>) to those of other members of the *Flaviviridae*, such as HCV NS4B. This feature hampers the prediction of shared motifs or putative functional activities between the CSFV NS4B protein and those of other members of the *Flaviviridae*. For instance, in flaviviruses, motifs located toward the N-terminal cytoplasmic portion of the proteins are associated with NS4B functions, while the predicted TIR motifs found in CSFV NS4B are located toward the C-terminal cytoplasmic portion of the protein (Fig. 1D).

The data presented in this report suggest that the CSFV NS4B protein contains a region showing similarities to the TIR domain of Toll-like receptors/IL-1, including the motifs in boxes 1 and 2, shown to be critical for TIR function (40). Here we observed that alterations in NS4B box 1 are lethal for viral viability, while substitutions in box 2 (NS4B.VGIv) lead to complete virus attenuation in swine. TIR domains participate in the signal transduction of Toll-like receptors (TLRs), based on homotypic domain interaction or TIR-to-TIR interaction with TIR-containing adaptor proteins, which in turn lead to the

activation of many transcription factors, such as NF- κ Bs and IRFs (1, 18, 19, 42). These activated transcription factors translocate to the nucleus, regulating gene transcription and the cellular immune response.

TLR-7 is normally activated by ssRNA as a mechanism of host defense against ssRNA viruses (1, 15, 18, 19). TLR-7 activation subsequently leads to increased transcription levels of several immunologically relevant genes, such as type I IFN, IL-1, IL-6, IL-8, IL-10, MCP-1, and TNF (5, 11, 12, 15, 21, 45, 49). The pattern of gene activation observed in macrophages infected with BICv suggests that viral mechanisms would block the activation of TLR-7. In contrast, infection with NS4B.VGIv would allow the activation of TLR-7, suggesting that NS4B may indeed prevent the activation of TLR-7 during infection with BICv. This mechanism of inhibition in turn might be disrupted during infection with mutant NS4B.VGIv. It is possible that during BICv infection of macrophages, NS4B interferes with the activation of TLR-7, either through direct TIR-to-TIR interaction with TLR-7 or possibly by competition for MyD88. This would explain the activation of TLR-7 signaling during infection with NS4B.VGIv, which leads to cytokine production, facilitating host control of the viral infection. Indirect evidence that this may happen is provided by the observation that transfection of macrophages with wild-type NS4B reduces the responsiveness of TLR-7 to a well characterized activator, I-R837 (Fig. 6A, B, C, and D). Interestingly, transfection with an NS4B gene harboring a mutation in either the putative box 1 (IYK) or box 2 (VGI), or mutations in both box 1 and box 2 (IYK and VGI), was less efficient at mediating this reduction (Fig. 6E). Additionally, when I-R837 was added after the infection, its antiviral activity did not affect BICv replication as it affected NS4B.VGIv replication. Perhaps BICv is able to block the effect of I-R837, while NS4B.VGIv is not (Fig. 7B). To our knowledge, this mechanism of evading TLR activation has not been observed with ssRNA viruses, but it has been described for vaccinia virus (VV), a double-stranded DNA virus (41). VV expresses an early protein, A46R, which

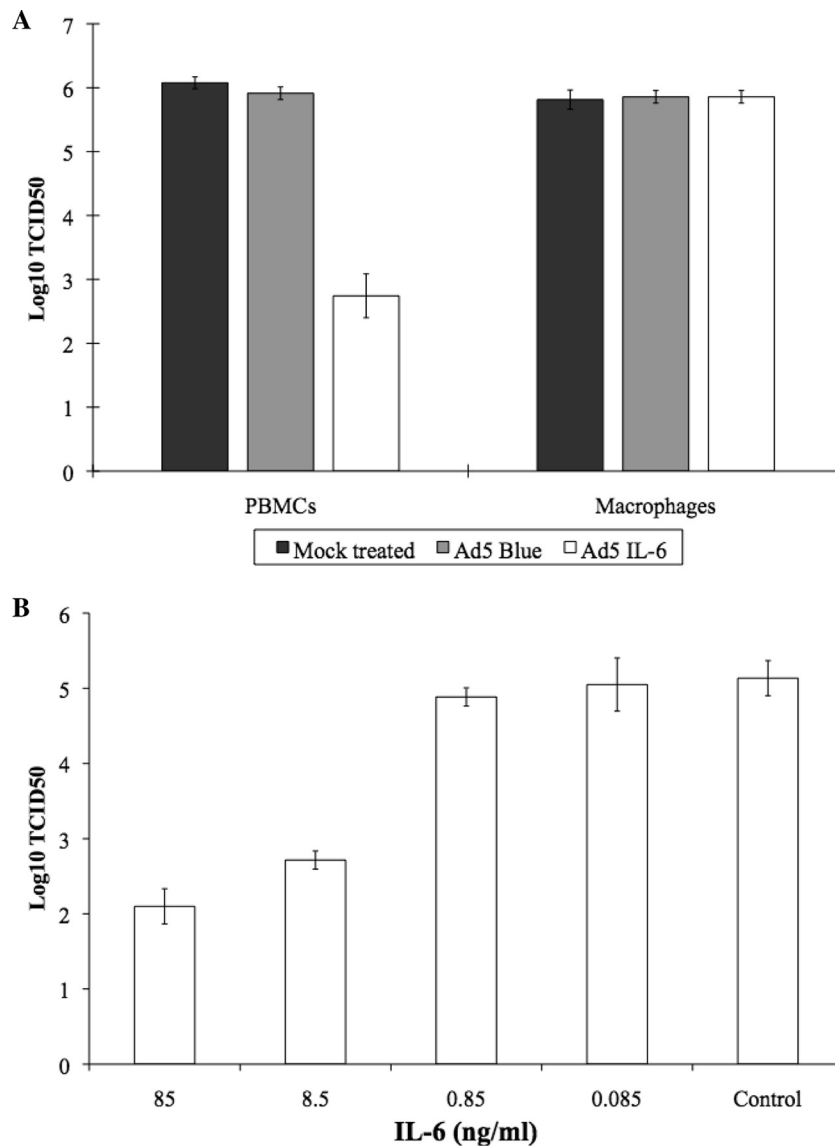


FIG. 5. Effects of IL-6 treatment on swine PBMC and macrophage cell cultures infected with CSFV. (A) Primary swine macrophage or PBMC cultures were either treated with Ad5-derived IL-6, mock treated, or treated with the Ad5-Blue culture supernatant; 24 h later, cultures were infected with BICv. (B) PBMC cultures were treated either with different IL-6 concentrations or with the Ad5Blue culture supernatant (control). Twenty-four hours later, cultures were infected with BICv. Infections were performed at an MOI of 0.1, and virus yield was assessed 24 hpi.

contains a TIR domain that interferes with host signal transduction of TLRs via interaction with the TIR domains of Toll-like receptors and TIR-containing adaptor proteins (41). Our results suggest that CSFV NS4B interferes with TLR signaling in a related manner, via its TIR-like domain.

IL-6, a cytokine produced primarily by cells of the monocyte/macrophage lineage, has been shown to be critically involved in several aspects of the acute-phase response and the development of the acquired immune response (23, 48). IL-6 plays an important role as a cofactor both in early events of cytotoxic T-lymphocyte (CTL) activation and in the process of B-cell late differentiation (48). Furthermore, it has been shown that IL-6 serves an important role during the development of an efficient immune response to viral infections. Interleukin-6-deficient mice have an impaired

T-cell-dependent antibody response to vesicular stomatitis virus infection as well as a decreased CTL response to VV (20). IL-6 significantly inhibited the replication of CSFV *in vitro*. Swine PBMCs pretreated with adenovirus-derived porcine IL-6 24 h before infection with BICv displayed a significant reduction in virus yield (Fig. 5). This ability is not restricted to BICv, since similar results were obtained with NS4B.VGIv (data not shown). Although the mechanism mediating this reduction is not known, it is clear that it requires the involvement of nonadherent PBMCs, since this effect was not seen with adherent primary swine macrophages alone. It is possible that nonadherent PBMCs pretreated with IL-6 release soluble molecules that are not released in adherent macrophages alone, and that these soluble molecules ultimately mediate the inhibition of CSFV replication

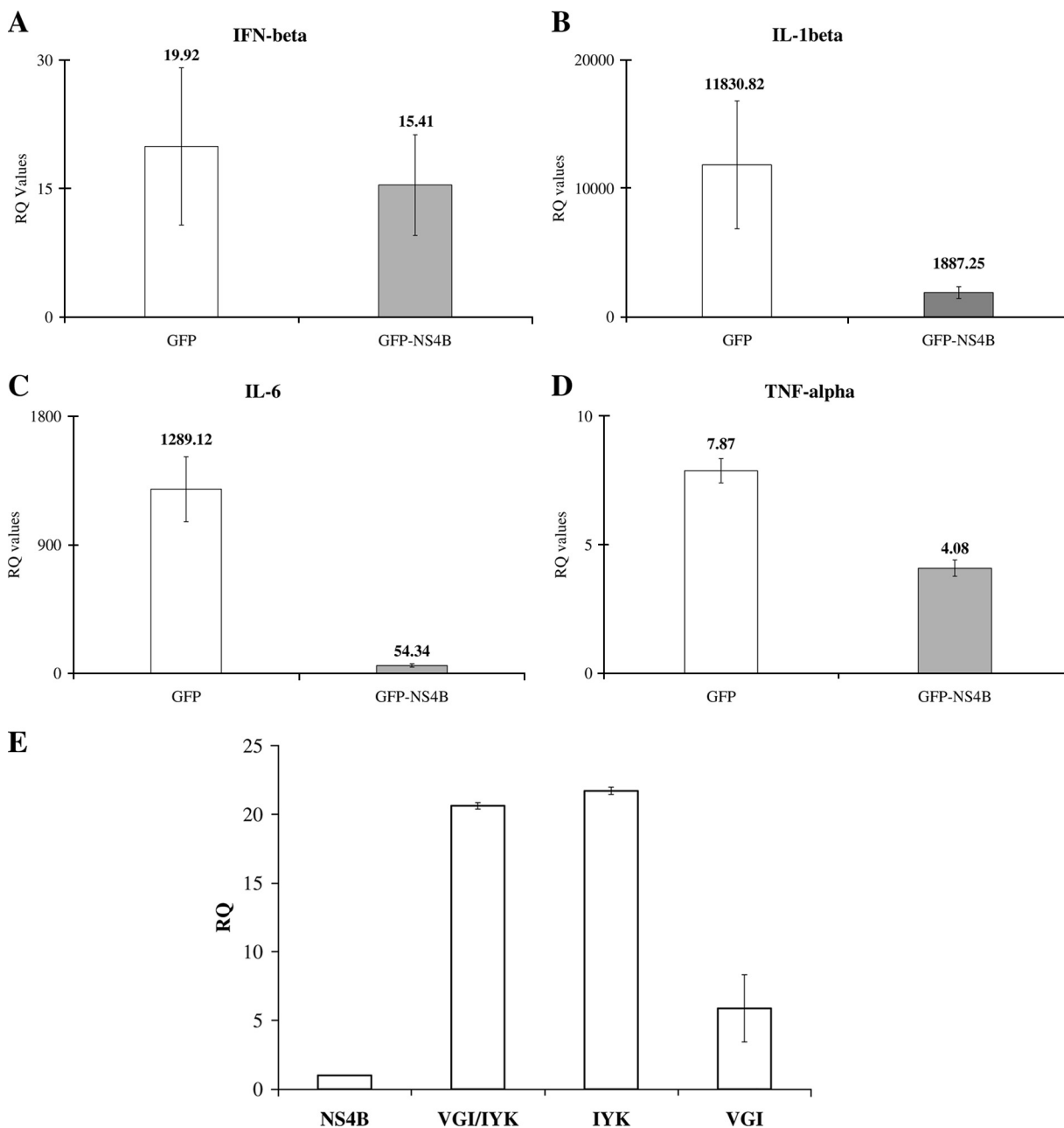


FIG. 6. Effect of CSFV NS4B protein on I-R837 activity in swine macrophages. Primary swine macrophage cultures were transfected with phrGFP or phrGFP/NS4B (A, B, C, and D) or with the phrGFP/NS4B, phrGFP/NS4B.IYK, phrGFP/NS4B.VGI, or phrGFP/NS4B.IYK.VGI construct (E), and 24 h later, the cultures were stimulated with 20 μ g/ml of I-R387. Blockage of increased transcription of the marker genes IFN- β (A), IL-1 β (B), IL-6 (C and E), and TNF (D) in swine macrophage cultures treated with I-387 was assessed as an indication of the suppression of TLR-7 activity. RNA quantification was performed as described for Fig. 3. The results in panels A, B, C, and D are expressed as RQ, while in panel E, the results are expressed as RQ relative to NS4B, which is arbitrarily assigned the value of 1 unit.

in adherent macrophages. The *in vivo* studies have provided indirect support suggesting that IL-6 plays a significant role during CSFV infection (Fig. 5). In this study, the intensity and duration of IL-6 expression suggest an inverse relationship with levels of viral antigen and titers in the tonsils of animals infected with either BICv or NS4B.VGIv. This implies that expression levels of IL-6 are inversely related to the ability of the virus to replicate in the tonsils of infected animals. Of particular note from this study, it was shown

that peak cellular IL-6 levels at 3 dpi coincided with protection when pigs were challenged with the highly virulent parental virus, suggesting that IL-6 can play an early protective role against CSFV infection.

In summary, we report here that the nonstructural NS4B protein of CSFV strain Brescia has similarities to the TLR family with regard to protein structure and motifs present in boxes 1 and 2. Alterations in box 1 are lethal for virus viability, while substitutions in box 2 (NS4B.VGIv) lead to

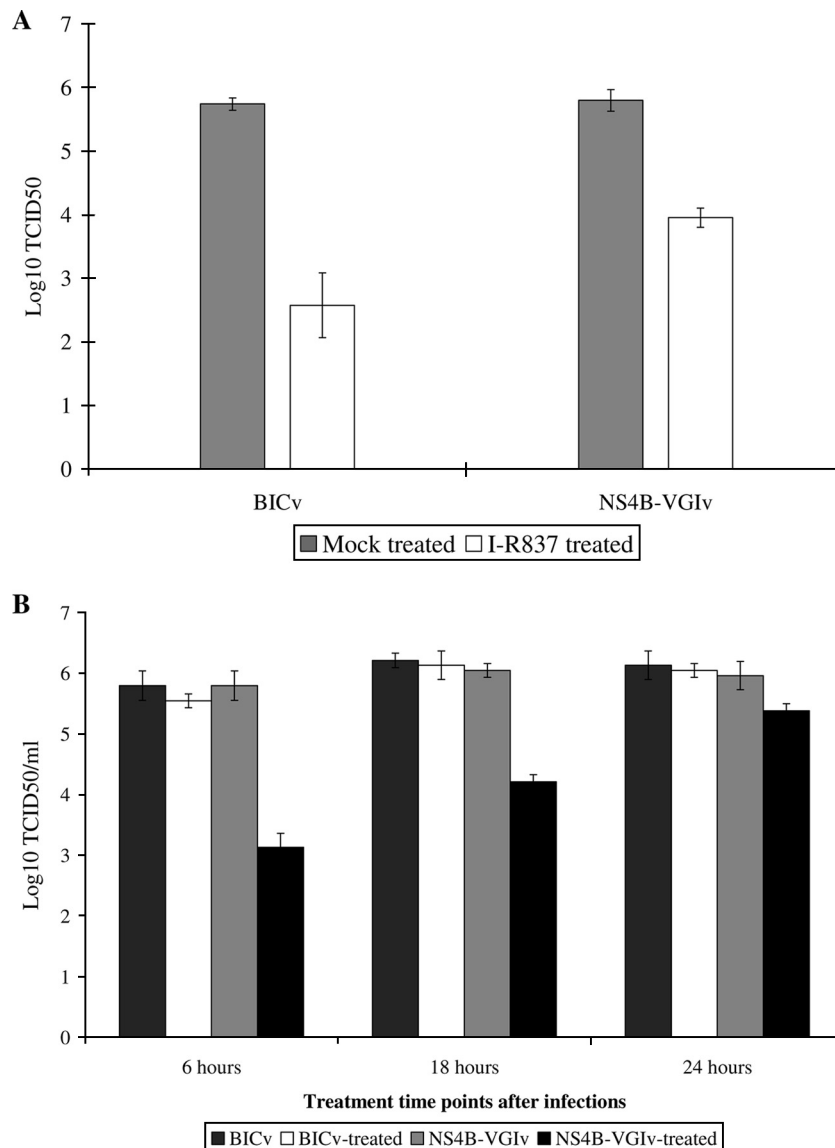


FIG. 7. Effect of imiquimod R837 (I-R837) treatment on swine primary macrophage cell cultures infected with CSFV. (A) Macrophages were either treated with I-R837 or mock treated, and 24 h later, cultures were infected with either NS4B.VGIv or BICv. (B) Macrophages were treated with I-R837 at 6, 12, or 18 h after virus infection. Infections were performed at an MOI of 0.1, and virus yields were assessed at 24 hpi.

complete virus attenuation in swine. A direct link between reduced virulence *in vivo* and alteration of the activation profile of immunologically relevant genes could not be determined. However, it is possible that disruption of the TIR domain in NS4B led to increased production of host innate immune response mediators, potentially altering the outcome of infection in the porcine host. In addition to the disruption of the TIR domain, the increased levels of IL-6 observed during *in vivo* NS4B.VGIv infection could also aid in controlling the infection, contributing to the attenuated phenotype of NS4B.VGIv. The rapid protective immunity elicited by NS4B.VGIv suggests that the VGI residues could be modified for the development of a live-attenuated CSFV vaccine that would provide rapid protection against CSF disease. An improved understanding of the underlying genetic basis of viral virulence and the host immune response

will permit future rational design of efficacious biological tools for controlling CSF disease.

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