Porcine reproductive and respiratory syndrome virus nucleocapsid protein modulates interferon-β production by inhibiting IRF3 activation in immortalized porcine alveolar macrophages

Mingeun Sagong • Changhee Lee

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Abstract Porcine reproductive and respiratory syndrome virus (PRRSV) infection appears to elicit a weak innate immune response suppressing type 1 interferon (IFN) production. Recent studies have revealed that several nonstructural proteins encoded by the PRRSV genome independently antagonize the type 1 IFN system. The present study sought to identify the structural proteins that possess the immune evasion properties in immortalized porcine alveolar macrophages (PAM). Each structural protein gene was stably expressed in a porcine monocyte-derived macrophage cell line, PAM-pCD163, and tested for its potential to inhibit IFN-β induction. We then focused on the nucleocapsid (N) protein, which has a strong inhibitory effect on dsRNA-induced IFN-β production. Upon dsRNA stimulation, IFN-β production was shown to decrease proportionally with increasing levels of N expression. Furthermore, the PRRSV N protein was found to downregulate IFN-dependent gene production by dsRNA. Taken together, these results indicate the ability of N to modulate the dsRNA-mediated IFN induction pathways. In addition, the N protein significantly interfered with dsRNA-induced phosphorylation and nuclear translocation of IRF3. Our data suggest that the PRRSV N protein is a responsible component, independent of other nonstructural elements, for evading the IFN response by antagonizing IRF3 activation.

Introduction

Porcine reproductive and respiratory syndrome (PRRS) is an important and re-emerging disease of swine that was first recognized independently in North America in 1987 and in Europe in 1990 [19, 46]. The disease is characterized by reproductive failure in pregnant sows and mild to severe respiratory distress in piglets and growing pigs [47]. Since its emergence, the PRRS situation has become endemic in most pork-producing countries, causing tremendous economic losses in the pig industry worldwide [2, 36]. The causative agent of the disease is PRRS virus (PRRSV), which belongs to the family Arteriviridae in the order Nidovirales [5, 31, 41]. The PRRSV genome is a single-stranded positive-sense RNA containing ten open reading frames (ORFs), designated ORF1a, ORF1b, and ORF2 through 7, including ORF2b and ORF5a [12, 15, 41]. Two large ORFs, 1a and 1b, occupying the 5’ two-thirds of the genome, encode the nonstructural polypeptides pp1a and pp1ab, which are proteolytically cleaved into 14 nonstructural protein (NSP) products: NSP1x, NSP1β, NSP2 through NSP12 including NSP7α and NSP7β, in order from the N-terminus [41, 44]. The remaining ORFs in the 3’-terminal region code for structural GP2, small envelope (E), GP3, GP4, 5a, GP5, membrane (M), and nucleocapsid (N) proteins [12, 15, 32, 51].

The type 1 IFN system is a central feature of the antiviral innate immune response, which is the first defense against viral infection [37, 39]. Viral replication initially generates viral double-stranded RNA (dsRNA), which is then recognized by host-cell receptors. This interaction stimulates host signaling pathways, which leads to the coordinated activation and subsequent nuclear translocation of transcription factors, including interferon regulatory factor 3 (IRF3), nuclear factor kappa B (NF-κB), and
activating transcription factor-2 (ATF-2), to induce the expression of type 1 IFN. Once IFN is secreted, it signals via IFN receptors on adjacent cell surfaces, which activates a cell-signaling cascade through the Janus kinase (JAK)-mediated signal transducer and activator of transcription (STAT) pathway. This triggers the transcription of several hundred IFN-stimulated genes (ISGs), enabling target cells to produce an antiviral response to inhibit virus replication.

PRRSV primarily infects cells of the monocyte/macrophage lineage, such as porcine alveolar macrophages (PAMs) [9, 10] and can establish persistent infection, which may last for up to six months in the natural host [1, 8, 48]. Consequently, PRRSV infection appears to suppress normal macrophage function and immune responses, and it is complicated by secondary opportunistic bacterial infections in most cases [11]. Indeed, studies have revealed that lungs of pigs infected with PRRSV are incapable of eliciting type 1 interferon (IFN) responses [4, 29, 33, 45]. The impairment of type 1 IFN production by PRRSV would result in a weak host adaptive immunity, including a delayed or slow development of humoral and cell-mediated immune responses, leading to viral persistence in infected pigs [28, 30, 34, 38].

Viruses have evolved the strategy of expressing proteins to circumvent the IFN response for their survival in the host. PRRSV has been postulated to encode viral products that possess immune evasion properties by regulating IFN activity. Thus, several recent studies have attempted to identify NSPs with innate immune evasion features encoded by the PRRSV genome. Beura et al. [3] first reported that various NSPs, such as NSP1, NSP2, NSP4, and NSP11, are able to suppress dsRNA-induced IFN-β promoter activation. In that study, NSP1α and NSP1β, auto-cleavage products of NSP1, were shown to modulate type 1 IFN induction by antagonizing IRF3 activation. Other studies have independently demonstrated that NSP1 exerts its inhibitory effect on the IFN response in different ways by interfering with other transcription factors such as CREB-binding protein (CBP), NF-κB, and STAT-1 [6, 20]. In addition, the PRRSV NSP2 has been shown to antagonize type 1 IFN production by inhibiting the NF-κB signaling pathway or IRF3 activation [27, 43]. However, little is known about structural proteins that regulate IFN activity. In the present study, therefore, we aimed to identify the structural proteins of PRRSV that are responsible for mediating IFN down-regulation in immortalized natural target cells. It was showed that several structural proteins inhibit type 1 IFN induction upon dsRNA treatment. Among those viral proteins, the N protein was chosen to investigate the inhibitory mechanism and was subsequently found to block dsRNA-induced IRF3 activation. Our data indicate that the N protein has a novel function related to the immune modulation and evasion strategy during PRRSV infection.

Materials and methods

Cells, virus, and antibodies

PAM-pCD163 cells [26] were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 1% antibiotic-antimycotic solution (100×; Invitrogen), 10 mM HEPES (Invitrogen), 1 mM sodium pyruvate (Invitrogen), and nonessential amino acids (100×; Invitrogen) in the presence of 50 μg/ml Zeocin (Invitrogen). The cells were maintained at 37°C with 5% CO₂. The PRRSV strain PL97-1 [17] was used to prepare virus stocks in PAM-pCD163 as described previously [26]. The following antibodies were used in the present study: anti-PRRSV N monoclonal antibody (MAb) SDOW17 (Rural Technologies), rabbit anti-IRF3 polyclonal antibody (Santa Cruz Biotechnology), rabbit anti-SP1 polyclonal antibody (Santa Cruz Biotechnology), anti-β-actin MAb (Santa Cruz Biotechnology), anti-α-tubulin (Sigma), anti-phosphorylated IRF3 (Ser396) rabbit MAb (Cell Signaling Technology), and anti-6× histidine tag MAb (IG Therapy).

Construction of expression plasmids

DNA manipulation and cloning were performed according to standard procedures [40]. The E. coli strain DH5α (RBC Bioscience) was used as the host for general cloning. The PRRSV strain PL97-1 was used as a template to extract viral RNA using a Viral RNA Mini Kit (QIAGEN) according to the manufacturer’s instructions. RT-PCR was performed to amplify all PRRSV structural protein genes with primer sets, and primer sequences will be provided upon request. The PCR amplicon was initially inserted into a pBudCE4.1 vector (Invitrogen) that contains six repetitive 3′ histidine codons, and each resulting plasmid was verified by nucleotide sequencing. A His-tagged cDNA fragment obtained from the corresponding plasmid vector was then subcloned into a pFB-Neo retroviral vector (Stratagene) using Sal I and EcoR I restriction sites to construct individual PRRSV structural-protein-expressing plasmids, thereby producing C-terminally His-tagged PRRSV structural proteins.

Generation of stable PAM cell lines

A retrovirus gene transfer system (Stratagene) was applied to generate cell lines constitutively expressing each recombinant PRRSV structural protein as described elsewhere [26, 35]. Antibiotic-resistant continuous cell clones were examined by RT-PCR to detect the presence of the full-length structural protein gene, and positive clones...
expressing the respective PRRSV structural proteins were amplified for subsequent analysis.

Quantitative real-time RT-PCR

PAM-pCD163 cells grown at 3 × 10^5 cells/well in a 6-well tissue culture plate were infected with PRRSV at a multiplicity of infection (MOI) of 1 for 24 h and transfected with 1 μg polyinosinic:polycytidylic acid (poly[I:C]; Sigma) using X-feet (Clontech) according to the manufacturer’s instructions. PAM cells expressing each viral protein were grown in 6-well tissue culture plates to 80% confluency for two days and treated by transfection with 1 μg poly(I:C). At 6 h post-poly(I:C) stimulation, total cellular RNA was extracted using TRizol Reagent (Invitrogen) and treated with DNase I (TaKaRa) according to the manufacturer’s manual. The RNA was used for reverse transcription using a PrimeScript 1st strand cDNA synthesis kit and then for real-time PCR for quantification of porcine IFN-β and ISG15 mRNA copy number on a Thermal Cycler Dice Real Time System using SYBR Green I (TaKaRa). Real-time PCR reactions were performed in a total volume of 20 μl of the reaction mixture containing a template cDNA, forward and reverse primers, and 2× SYBR Premix Ex Taq (TaKaRa). The following primer sets were used in the real-time PCRs: porcine IFN-β forward, 5′-TCGCTTCTCTGATGTGTTTC-3′; porcine IFN-β reverse, 5′-TTCTGACATGCCAAATCTGCT-3′; porcine ISG15 forward, 5′-GGGACCTGACGGTGAAGA-3′; porcine ISG15 reverse, 5′-GCCAGACGCTGCTGG-3′; porcine β-actin forward, 5′-GACCACCTTCACTCGATCA-3′; porcine β-actin reverse, 5′-GTGTGGCGTAGAGGTCTT-3′. The mRNA levels of the tested genes were normalized to that of porcine β-actin mRNA in all experiments. The porcine IFN-β and ISG15 mRNA levels in virus-infected cells or in viral-protein-expressing cells subjected to poly(I:C) stimulation were expressed as copy numbers relative to unstimulated cells according to a method described previously [42]. Three independent experiments were repeated, and the average of normalized values is presented.

Cell fractionation and western blot analysis

PAM cells were grown in 6-well tissue culture plates to 80% confluency for two days and transfected with 1 μg poly(I:C) for 6 h. The cells were then solubilized in a lysis buffer containing 0.5% Triton X-100, 60 mM β-glycerophosphate, 15 mM ρ-nitrophenyl phosphate, 25 mM MOPS, 15 mM, MgCl2, 80 mM NaCl, 15 mM EGTA (pH 7.4), 1 mM sodium orthovanadate, 1 μg/ml E64, 2 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM PMSF for 30 min on ice and clarified by centrifugation at 15,800 g for 30 min at 4°C. For cell fractionation, poly(I:C)-transfected PAM cells were fractionated using a Nuclear/Cytosol Fractionation Kit (BioVision) according to the manufacturer’s manual. The total protein concentrations in the supernatants were determined using a BCA protein assay (Pierce). Equal amounts of total protein were separated on a NuPAGE 4-12% gradient Bis-Tris gel (Invitrogen) under reducing conditions and electrotransferred onto Immunobilon-P (Millipore). The membranes were blocked with 3% powdered skim milk (BD Biosciences) in TBS (10 mM Tris-HCl [pH 8.0], 150 mM NaCl) with 0.05% Tween-20 (TBST) at 4°C for 2 h and incubated at 4°C overnight with appropriate primary antibodies. The blots were then incubated with horseradish peroxidase (HRP)-labeled goat anti-mouse IgG or anti-rabbit IgG (Santa Cruz Biotechnology) at a dilution of 1:5,000 for 2 h at 4°C. Protein bands were detected using enhanced chemiluminescence (ECL) reagents (Amersham Biosciences) according to the instructions of the manufacturer.

Indirect immunofluorescence

PAM cells were grown on microscope coverslips placed in 6-well tissue culture plates and induced by transfection with 1 μg poly(I:C). At 48 h post-transfection, cells were fixed with 4% paraformaldehyde for 10 min at room temperature (RT) and permeabilized with 0.2% Triton X-100 in PBS at RT for 10 min. The cells were blocked using 1% bovine serum albumin (BSA) in PBS for 30 min at RT and then co-stained with anti-IRF3 polyclonal antibody and anti-N SDOW17 MAb or anti-His tag antibody for 2 h. After washing five times in PBS, the cells were incubated for 1 h at RT with a mixture of Alexa Fluor 488–conjugated anti-rabbit and Alexa Fluor 594–conjugated anti-IRF3 (Santa Cruz Biotechnology) at a dilution of 1:5,000, 4,6-diamidino-2-phenylindole (DAPI; Sigma) and visualized using a fluorescent microscope (Leica DM IL LED).

Results

Modulation of type 1 IFN induction by PRRSV

Although several research groups have reported the ability of PRRSV to suppress type 1 IFN production, they used primary PAM cells or Marc-145 cells as target cells to determine IFN response upon PRRSV infection [3, 20, 25, 33]. In this study, therefore, we first sought to revisit whether such a feature of PRRSV is reproducible in our continuous PAM cell model [26]. For this experiment, a well-characterized PRRSV strain, PL97-1 [7, 17], corresponding to the type 2 North American genotype, was used.
to infect immortalized PAM-pCD163 cells to measure IFN-β mRNA levels by quantitative RT-PCR. As shown in Fig. 1, very little IFN-β mRNA was detected in the cells infected with PRRSV. In contrast, PAM-pCD163 cells transfected with poly(I:C), a positive control for type 1 IFN production, showed efficient induction of IFN-β mRNA. However, PRRSV infection significantly suppressed poly(I:C)-mediated IFN-β mRNA production in PAM-pCD163 cells, confirming the regulatory property of PRRSV on type I IFN induction in our cell system.

Suppression of type 1 IFN production by N

As with other viruses, PRRSV has been known to code for proteins that antagonize the IFN response by independently inhibiting its related transcription factors. To date, several NSPs of PRRSV have been identified as being responsible for the poor induction of type 1 IFN by PRRSV. The present study was further expanded to the PRRSV structural proteins in order to investigate their role in the modulation of IFN in the natural target cells. To address this issue, sublines of PAM cells were first established to stably express each recombinant structural protein of PRRSV under the control of a retroviral LTR promoter. Cell clones expressing the corresponding viral proteins were initially collected and subjected to RT-PCR and western blotting to verify expression of individual viral genes at the mRNA and protein level, respectively (Fig. 2A). Each of the PAM cell clones constitutively expressing the highest levels of the respective structural protein was chosen for subsequent studies. In addition, a stable PAM cell line expressing the PRRSV NSP1, which alone strongly suppresses type 1 IFN induction, was generated to be used as a positive control.

Each of these cell lines was treated by transfection with poly(I:C), followed by real-time RT-PCR to quantitatively measure IFN-β mRNA. As expected, NSP1 was found to dramatically down-regulate poly(I:C)-induced IFN-β mRNA production in continuous PAM cells (Fig. 2B). Furthermore, IFN-β mRNA induction by poly(I:C) was suppressed to various degrees by GP4, GP5, and N in PAM-pCD163 cells (Fig. 2B). Of these structural proteins, the N protein was found to have the strongest inhibitory effect on the IFN-β response. To confirm this observation, we used three different PAM cell lines showing low-, moderate-, or high-level expression of N for the same assay. As shown in Fig. 3A, poly(I:C)-stimulated IFN induction was gradually inhibited in proportion to increasing expression levels of N (bottom panel).

Upon virus infection, the biological activities of type 1 IFNs are initiated by binding to their receptors, and they up-regulate expression of many ISGs, which, in combination, specify the antiviral state by sensitizing cells to limit viral replication [37]. Of all the genes regulated by the IFN response, ISG15 is one of the most inducible genes [14]. Therefore, to further verify the IFN-antagonistic activity of the N protein, it was tested whether N is capable of modulating ISG15 expression induced by dsRNA by quantitative RT-PCR. While ISG15 was efficiently induced in response to poly(I:C), the PRRSV N protein significantly impaired poly(I:C)-mediated ISG15 mRNA production in PAM-pCD163 cells (Fig. 3B). Together, our results demonstrate that N is a viral component responsible for IFN down-regulation by PRRSV.

Inhibition of IRF3 phosphorylation and nuclear translocation of N

IRF3 is one of key players of the signaling cascade leading to type 1 IFN induction [18]. This transcription factor is ubiquitously present in the cytoplasm but, upon receiving appropriate extra stimuli, including virus infection, its C-terminus is phosphorylated by an upstream kinase. This event causes a conformational change leading to dimerization and the exposure of a nuclear-localization signal (NLS). Once IRF3 is translocated into the nucleus, it assembles on the IFN-β promoter with other transcription factors, leading to the recruitment of transcriptional co-activators such as CBP/p300, and ultimately up-regulation of IFN mRNA transcription [37]. Various viruses have evolved to disarm IRF3 activation to escape the host IFN defense. Furthermore, PRRSV NSPs are known to possess
the ability to interfere with the IRF3 pathway for active IFN antagonism. Thus, essential steps of the IRF3 activation process, such as phosphorylation and nuclear translocation were studied to define the mechanism by which N inhibits type 1 IFN induction. We first determined whether IRF phosphorylation can be impaired by N. As a positive control, poly(I:C) stimulation induced the phosphorylation of IRF3 in PAM cells (Fig. 4A, lane 1). In contrast, dsRNA-mediated IRF3 phosphorylation was undetectable in N-expressing PAM cells (lane 3).

Next, the nuclear translocation of IRF3 in PAM cells stably expressing N was investigated by cell fractionation from poly(I:C)-treated PAM cells in the presence or absence of N and analyzed for the quantitative detection of ISG15 mRNA by real-time RT-PCR. The level of ISG15 mRNA in the absence of N upon poly(I:C) stimulation was set at 100%, and that of the N protein was normalized accordingly. These values are representative of the average of three independent experiments, and error bars denote standard deviations.

Fig. 2 Involvement of specific PRRSV structural proteins in inhibition of IFN-β induction. (A) Established stable PAM cell lines were grown independently for 48 h, and total RNA was extracted from cells. Each viral gene was amplified by RT-PCR and visualized on a 0.8% agarose gel (top panel). Cell lysates were prepared from cells and subjected to western blot using an anti-His tag antibody to determine the expression level of each PRRSV protein in stable cell lines (middle panel). The blot was also reacted with anti-β-actin antibody to confirm equal protein loading (bottom panel). Lane 1 (M), molecular weight marker; lane 2, PAM-GP2; lane 3, PAM-E, lane 4, PAM-GP3, lane 5, PAM-GP4; lane 6, PAM-GP5; lane 7, PAM-M; lane 8, PAM-N. (B) PAM cells stably expressing each structural protein of PRRSV were stimulated with poly (I:C) as described in “Materials and methods”. Total RNA isolated from cells was subjected to real-time RT-PCR, and the porcine IFN-β mRNA levels in each stable cell line were expressed as copy numbers relative to the unstimulated control. These data are representative of the mean values from three independent experiments, and error bars represent standard deviations.

Fig. 3 Inhibitory effects on the induction of IFN-β and ISG15 by N. (A) Three different stable PAM cell lines with low-, moderate-, or high-level N expression were stimulated with poly(I:C) for 6 h and used for real-time RT-PCR for the determination of IFN-β mRNA copy number as described earlier. The bottom panels indicate increasing levels of N expression in each stable cell line, and β-actin was used as a protein loading control. (B) Total RNA was isolated from poly(I:C)-treated PAM cells in the presence or absence of N and analyzed for the quantitative detection of ISG15 mRNA by real-time RT-PCR. The level of ISG15 mRNA in the absence of N upon poly(I:C) stimulation was set at 100%, and that of the N protein was normalized accordingly. These values are representative of the average of three independent experiments, and error bars denote standard deviations.
assay. As expected, poly(I:C) treatment was able to cause translocation of IRF3 to the nucleus (Fig. 4B, top panel, lane 5). However, in the presence of N, the level of IRF3 in the nucleus was markedly reduced to the basal level (lane 7). This observation was further confirmed by visualization of subcellular localization of IRF3. As shown in Fig. 5, IRF3 is normally located in the cytoplasm with nuclear diffusion in PAM cell lines without dsRNA stimulation (top, second, third, and fourth panels). Poly(I:C) stimulation led to the translocation of IRF3 to the nucleus (top panels). In the presence of N, the level of IRF3 in the nucleus was markedly reduced to the basal level (lane 7). This observation was further confirmed by visualization of subcellular localization of IRF3. As shown in Fig. 5, IRF3 is normally located in the cytoplasm with nuclear diffusion in PAM cell lines without dsRNA stimulation (top, second, third, and fourth panels). Poly(I:C) stimulation led to the translocation of IRF3 to the nucleus (top panels). In the presence of N, the level of IRF3 in the nucleus was markedly reduced to the basal level (lane 7).
Inhibition of INF-β production by PRRSV N protein

In the present study, immortalized PAM cell lines constitutively expressing each structural protein of PRRSV were first established and used as tools for studying dsRNA-induced IFN signaling pathways. The advantages of using this continuous PAM system are that, apart from currently known PRRSV-permissive cells used in other studies, it could facilitate the unlimited supply of PAM and mimic the virus-host interactions in the natural host. GP4, GP5, and N proteins stably expressed in PAM cells are able to significantly suppress upregulation of IFN-β mRNA induced by dsRNA. To investigate the mechanism of impairment of the IFN response by the PRRSV structural protein, we focused on N, which had the strongest inhibitory effect. The N protein was also found to inhibit ISG15 induction by poly(I:C). Although all factors of the IFN system are virtually targeted by a variety of viral IFN antagonistic proteins, IRF3 is one of the ideal components of viruses at the level of the activation process for modulating the IFN response. After dsRNA stimulation in N-expressing PAM cells, our results show that N targets the IRF3 activation pathway, leading to modification of its phosphorylation and subsequent nuclear translocation. Thus, the PRRSV N protein seems to prevent the activation of IRF3, which, in turn, blocks the full assembly of an enhanceosome complex on the IFN promoter, leading to inhibition of IFN mRNA transcription.

Most of the known IFN antagonistic proteins are non-structural proteins of viruses that limit IFN production or specifically inhibit IFN signaling by infected cells by several factors of the IFN pathway [37]. There are only a few reports of viral structural proteins that function as IFN antagonists. These include the core protein of hepatitis C virus (HCV) and the N protein of severe acute respiratory syndrome coronavirus (SARS-CoV) [16, 21]. However, these viral structural proteins were found to inhibit the IFN response by blocking the NF-κB pathway, the other target element of the IFN system. To our knowledge, this is the first instance of a structural N protein directly targeting the IRF3 activation pathway to down-regulate type 1 IFN induction. The N protein of PRRSV is a multifunctional serine phosphoprotein [41, 49]. As the sole structural component of the viral capsid, N associates with itself, providing the pivotal basis for nucleocapsid assembly and virus infectivity [23, 50]. Since the entire life cycle of PRRSV occurs in the cytoplasm of infected cells, the N protein is distributed predominantly in the cytoplasmic and perinuclear regions. However, the PRRSV N protein is specifically observed in the nucleus and nucleolus of infected cells, suggesting a non-structural function(s) in these cellular compartments. Although N protein nuclear localization is dispensable for virus cytopathology and replication, it appears to play an important role in viral attenuation and pathogenesis [24]. Thus, it has been postulated that nuclear translocation of N may have effects on the nuclear function of the host cell in order to favor virus replication and modulate host antiviral responses. The present study supports this hypothesis, providing strong evidence that the N protein inhibits the host innate immune response independently of other nonstructural elements of PRRSV. It would be of further interest to evaluate whether nuclear localization of the N protein is essential for its ability to modulate the IFN response by inhibiting IRF3 activation, and this issue is currently under investigation.

Combining our findings with those from other reports, it appears that the pathogenesis of PRRSV is very complex, with multiple viral proteins contributing to evasion strategies to escape the host innate immune response by targeting different steps in IFN induction. Once the PRRSV genome is released after the uncoating process in virus-infected cells, individual NSPs are initially produced, and among these, five NSPs, including NSP1α, NSP1β, NSP2, NSP4, and NSP11, independently exert their own functions...
in counteracting the host type 1 response by various mechanisms. Later, while the newly translated N proteins are accumulating in the cytoplasm and function as the principal structural component for nucleocapsid assembly, other N protein populations being shuttled between the cytoplasm and the nucleus appear to play non-structural roles in participating in antagonizing type 1 IFN induction. Consequently, the poor type 1 IFN production mediated by non-structural and structural elements of PRRSV causes the inadequate adaptive immune response and likely leads to viral persistence and continuous virus shedding in the natural host.

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