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Intranasal treatment with CpG-B oligodeoxynucleotides protects CBA mice from lethal equine herpesvirus 1 challenge by an innate immune response

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ABSTRACT

Equine herpesvirus 1 (EHV-1) is the causative agent of a number of equine disease manifestations, including severe disease of the central nervous system, respiratory infections, and abortion storms. Our results showed that intranasal treatment with CpG-B oligodeoxynucleotides (ODN 1826) protected CBA mice from pathogenic EHV-1 RacL11 challenge. The IFN-γ gene and seven interferon-stimulated genes (ISGs) were upregulated 39.4- to 260.3-fold at 8 h postchallenge in the lungs of RacL11-challenged mice that had been treated with CpG-B ODN. Interestingly, IFN-γ gene expression was upregulated by 26-fold upon RacL11 challenge in CpG-B ODN-treated mice lungs as compared to that of CpG-A ODN (ODN 1585)-treated mice lungs; however, the seven ISGs were upregulated by 2.4–5.0-fold, suggesting that IFN-γ is a major factor in the protection of CBA mice from the lethal challenge. Pre-treatment with IFN-γ significantly reduced EHV-1 yield in murine alveolar macrophage MH-S cells, but not in mouse lung epithelial MLE12 cells. These results suggest that CpG-B ODN may be used as a prophylactic agent in horses and provide a basis for more effective treatment of EHV-1 infection.

1. Introduction

Equine herpesvirus 1 (EHV-1), a member of the *Alphaherpesvirinae*, is a major pathogen affecting equines and causes respiratory disease, abortion, and in some cases, neurological disease (Allen and Bryans, 1985; Carroll and Westbury, 1985; Jackson and Kendrick, 1971). EHV-1 infection of the horse generates a short-lived humoral response but does not confer long-term protection, as disease often occurs following natural infection (Bryans and Allen, 1986; Crandell et al., 1980). Cytotoxic T lymphocytes (CTL) with the capacity for killing EHV-1-infected target cells were detected in equine peripheral blood mononuclear cells (PBMC) as early as 1 week postinfection, reached maximal levels by 2–3 weeks, and remained detectable for a year after infection (Allen et al., 1995).

The EHV-1 nonpathogenic strain KyA is attenuated in mice and horses, whereas the wild-type pathogenic strain RacL11 induces severe inflammatory cell infiltration in the lung, such that infected mice succumb at 3–6 days post-infection (Colle III et al., 1992; Frampton et al., 2002; Lewis et al., 1997; Matsumura et al., 1996; Smith et al., 2005; Smith et al., 2000b). A murine model of EHV-1 infection that closely mimicked EHV-1 infection in the natural host was established in various strains of mice (Awan et al., 1990). Infection of CBA mice with KyA generated a vigorous $CD8^+$, class I MHC-restricted, EHV-1-specific primary cytotoxic T lymphocytes (CTL) response in the draining mediastinal lymph nodes (MLN) and a long-term memory CTL response in the spleen (Smith et al., 1998).

Synthetic oligodeoxynucleotides bearing CpG motifs (CpG ODNs) can bind to Toll-like receptor 9 (TLR9) and activate immune responses (Krieg, 2002). CpG ODNs can be classified into 4 main classes: type A (CpG-A ODNs), type B, type C, and type P (Vollmer and Krieg, 2009). CpG-A ODNs are characterized by a phosphodiester central CpG-containing palindromic motif and a phosphorothioate 3′ poly-G string and activate natural killer cells and stimulate plasmacytoid dendritic cells (pDC) and macrophages to produce IFN-α (Lenert et al., 2003; Verthelyi and Zeuner, 2003). CpG-B ODNs contain a complete phosphorothioate backbone of a linear single-stranded conformation with multiple CpG-motifs and primarily stimulate B cell proliferation and monocyte maturation. CpG-B ODNs also stimulate maturation of pDC and macrophages (Hartmann et al., 2003; Verthelyi and Zeuner, 2003). CpG-C ODNs contain a complete phosphorothioate backbone with 5′ CpG-motif and 3′ palindrome sequence and stimulate both pDC and B cells (Abel et al., 2005; Jurk et al., 2004). CpG-P ONDs form concatamers

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due to the presence of the 5' palindromic. Similar to the CpG-A ODNs, CpG-P ODNs induce the IRF7 signaling pathway (Vollmer and Krieg, 2009).

Type II Interferon gamma (IFN-γ) is critical in the regulation of the host immune response against viral and intracellular bacterial pathogens. High levels of IFN-γ are secreted by Type 1 T helper cells (Th1 cells), $CD8^+$ CTL, and NK cells in response to virus infection (Spellberg and Edwards, 2001). The production of proinflammatory cytokines and chemokines is also induced by IFN-γ in endothelial cells, epithelial cells, and fibroblasts.

Several EHV-1 vaccines are available for protection against the respiratory and abortigenic forms of EHV-1; however, they do not elicit long-term immunity or protection for the horse (Bürki et al., 1990; Burrows et al., 1984; Hannant et al., 1993). EHV-1 outbreaks occur within the horse population despite active vaccination with currently available EHV-1 vaccines. CpG ODNs can enhance innate immune responses (Ito et al., 2005) and can function as an adjuvant to improve adaptive immune responses against pathogens (Gallichan et al., 2001). CpG ODNs were able to induce type I IFN, IFN-γ, and TNF-α production in equine PBMC (Wattrang et al., 2012). To develop a safe and effective treatment for EHV-1 infections and resultant diseases, CpG ODNs were investigated for the ability to elicit protection by innate immune responses.

2. Materials and methods

2.1. Viruses, cell culture, and plaque assay

The KyA strain of EHV-1 was propagated in suspension cultures of mouse fibroblasts L-M cells as described (O'Callaghan et al., 1968; Perdue et al., 1974). Pathogenic EHV-1 RacL11 (Reczko and Mayr, 1963) was propagated in equine dermal fibroblast NBL6 cells. Murine alveolar macrophage MH-S cells were maintained at 37 °C in RPMI-1640 medium supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin, 0.05 mM 2-mercaptoethanol, and 10% fetal bovine serum. L-M and NBL6 cells were maintained at 37 °C in complete Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin, nonessential amino acids, and 5% fetal bovine serum. Mouse lung epithelial MLE12 cells were maintained at 37 °C in DMEM:Ham's F12 (50:50 mix) supplemented with 100 U/mL of penicillin, 100 μg/ml of streptomycin, 0.005 mg/ml insulin, 0.01 mg/ml transferrin, 30 nM sodium selenite, 10 nM hydrocortisone, 10 nM β-estradiol, 10 mM HEPES, 2 mM L-glutamine, and 2% fetal bovine serum. EHV-1 titers were determined on NBL6 cells as described previously (Kim et al., 2011). Serial dilutions of samples from each passage were used to inoculate NBL6 monolayers. Infected monolayers were incubated for 4 days in medium containing 1.5% methylcellulose. Plaques were quantitated after fixing with 10% formalin solution (Fisher Scientific) and staining with 0.5% crystal violet.

2.2. Mice

Female CBA mice, 3–4 weeks of age, obtained from Envigo (Indianapolis, IN), were maintained in filter-topped cages at the Animal Resource Facility of the Louisiana State University Health Sciences Center, Shreveport. The Animal Resource Facility is certified by the Association for Assessment and Accreditation of Laboratory Animal Care International. All procedures were previously approved by the University Animal Care Committee. All mice were rested for a period of 7–9 days prior to use. Experimental groups consisted of eight mice (for body weight changes and percent survival) or four mice (lung cytopathology, lung virus titers, and RT-qPCR), and all experiments were performed in duplicate. Statistical analysis was conducted with a two-tailed Student t-test.

2.3. Infection of mice and assessment of respiratory disease

CBA mice were anesthetized with isoflurane (Sigma Chemical Co., St. Louis, MO) inhalation, treated intranasally with 5 μg of CpG-A ODN (ODN 1585: 5′-ggGGTCAACGTTGAgggggg-3′, InvivoGen, San Diego, CA), CpG-B ODN (ODN 1826: 5′-tccatgacgttcctgacgtt-3′, InvivoGen), or CpG-C ODN (ODN 2395: 5′-tcgtcgttttcggcgc:gcgccg-3′, InvivoGen), or 2×10^6 plaque forming unit (PFU)/mouse of EHV-1 KyA, and challenged with 1.5×10^6 PFU/mouse of pathogenic RacL11. Mice were observed daily following infection for clinical signs of respiratory disease, including labored breathing, ruffled fur, and huddling. For weight loss experiments, mice were weighed individually immediately prior to infection and daily thereafter at the same time each day.

2.4. Western blot analysis

Preparation of total extracts of transfected cells and western blot analysis were performed as described (Kim et al., 2006). Blots were incubated for 1 h with the OC33 polyclonal antibody to the EHV-1 immediate-early protein (IEP) (Harty and O'Callaghan, 1991) or β-actin (Santa Cruz Biotechnology, Santa Cruz, CA). The blots were washed three times for 5 min each in TBST and incubated for 30 min with secondary antibody (anti-rabbit IgG [Fc]-alkaline phosphatase [AP] conjugate [Promega]). Proteins were visualized by incubating the membranes containing blotted protein in AP conjugate substrate (Bio-Rad, Hercules, CA) according to manufacturer's directions. The density of the brands on the membrane was ascertained by scanning with a HP Scanjet 8300 (Hewlett-Packard, Palo Alto, CA) and analyzed with Image Studio Lite software (LI-COR Biosciences, Lincoln, NE).

2.5. Histopathology

Lungs from CBA mice treated with control medium, 5 μg/mouse of CpG-A ODN or CpG-B ODN and challenged with 1.5×10^6 PFU/mouse of RacL11 were harvested and processed for histopathological analysis as previously described (Smith et al., 2005). On day 4 post-challenge, the mice were sacrificed and the lungs were removed. The lung tissue was mounted in tissue molds, covered in OCT, cryostat sectioned, fixed, and stained with H&E.

2.6. Real-time RT-qPCR assays

The lungs of infected CBA mice were removed at 8 hpi, and total RNA purified by using the RNeasy Mini kit (QIAGEN, CA) was analyzed by real-time RT-qPCR. Quantitative real-time RT-PCR (RT-qPCR) assays were performed as previously described (Kim et al., 2016). Primers used in RT-qPCR are shown in Table 1. Real-time RT-qPCR amplification was carried out with the CFX96™ Real-Time PCR Detection System by using the iTaq™ Universal SYBR® Green One-Step Kit according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA). Each sample was assayed in triplicate.

3. Results

3.1. Treatment with class B CpG oligodeoxynucleotides (ODN 1826) protects CBA mice from pathogenic RacL11 challenge infection

It has been shown that CpG-A, -B, and -C ODNs induced type I IFN, IFN-γ, and TNF-α production in equine peripheral blood mononuclear cells (PBMC) (Wattrang et al., 2012). Intranasal immunization of mice with formalin-inactivated bovine respiratory syncytial virus vaccine (FI-BRSV) formulated with CpG ODN resulted in both humoral and cell-mediated immunity, characterized by enhanced production of BRSV-specific serum IgG as well as increased IFN-γ (Mapletoft et al., 2008). Our previous results showed that immunization with nonpathogenic EHV-1 KyA significantly increased expression of IFN-γ and seven

Table 1
The list of primers for real-time RT-qPCR analysis.

Gene Name		Sequence	location
GAPDH-m	Forward Primer	ATCACTGCCACTCAGAAGACTGT	541–563
	Reverse Primer	ACCAGTGGATGCAGGGATGATGTT	636–613
IFI204	Forward Primer	GACAACCAAGAGCAATACACCA	112–133
	Reverse Primer	ATCAGTTTGCCCAATCCAGAAT	197–176
IFI44L	Forward Primer	GGGTCTGACGAAGCAGTATC	700–721
	Reverse Primer	CCCCATTGAAGAATCACACAGCAT	801–781
IFN-α	Forward Primer	CCTGATGGTCTTGGTGGTGATAA	21–43
	Reverse Primer	CAGTTCCTTCATCCCGACCAG	396–376
IFN-β	Forward Primer	AAGAGTTACACTGCCTTTGCCATC	225–248
	Reverse Primer	CACTGTCTGCTGGTGGAGTTCATC	359–336
IFN-γ	Forward Primer	ATGAACGCTACACACTGCATC	1–21
	Reverse Primer	CCATCCTTTTGCCAGTTCCTC	182–162
IRF7	Forward Primer	GAGACTGGCTATTGGGGGAG	38–57
	Reverse Primer	GACCGAAATGCTTCCAGGG	139–121
ISG15	Forward Primer	GGTGTCCGTGACTAACTCCAT	48–68
	Reverse Primer	CTGTACCACTAGCATCACTGTG	222–201
MX1	Forward Primer	GACCATAGGGGTCTTGACCAA	621–141
	Reverse Primer	AGACTTGCTCTTTCTGAAAAGCC	802–780
OAS1a	Forward Primer	GCCTGATCCCAGAATCTATGC	495–515
	Reverse Primer	GAGCAACTCTAGGGCGTACTG	711–691
RSAD2	Forward Primer	TGCTGGCTGAGAATAGCATTAGG	97–119
	Reverse Primer	GCTGAGTGCTGTTCCCATCT	208–189

antiviral interferon-stimulated genes (ISG) and protected CBA mice from pathogenic RacL11 challenge at 1–7 days post-immunization (Kim et al., 2016). To determine if CpG ODNs induce IFN-γ and protect mice from challenge with EHV-1 RacL11, CBA mice were treated intranasally with three types of CpG ODNs (CpG-A, CpG-B, and CpG-C) and were challenged with RacL11 at 48 h post-treatment. None of the CpG-B ODN-treated mice lost any of their preinfection body weight, and all rapidly gained weight (Fig. 1A). All of the CpG-B ODN-treated mice survived challenge with RacL11 (Fig. 1B). Treatment with CpG-C ODN 2395, which combines features of both types A and B ODNs, also protected CBA mice from RacL11 challenge (Fig. 1A). The mice treated with non-pathogenic EHV-1 KyA lost less than 5% of their preinfection body weight, and none of these mice died (Fig. 1A and B), which is consistent with our previous results (Kim et al., 2016). However, the CpG-A ODN-treated mice lost more than 20% of their preinfection body weight by day 5 post-infection (pi) (Figs. 1A), and 75% of these mice succumbed to RacL11 challenge infection (Fig. 1B). The untreated mice lost more than 20% of their preinfection body weight by day 4 post-RacL11 challenge (Fig. 1A), and all succumbed to RacL11 infection by days 4–6 post-challenge (Fig. 1B). These results demonstrated that CpG-B ODN stimulation effectively protected CBA mice from the lethal challenge.

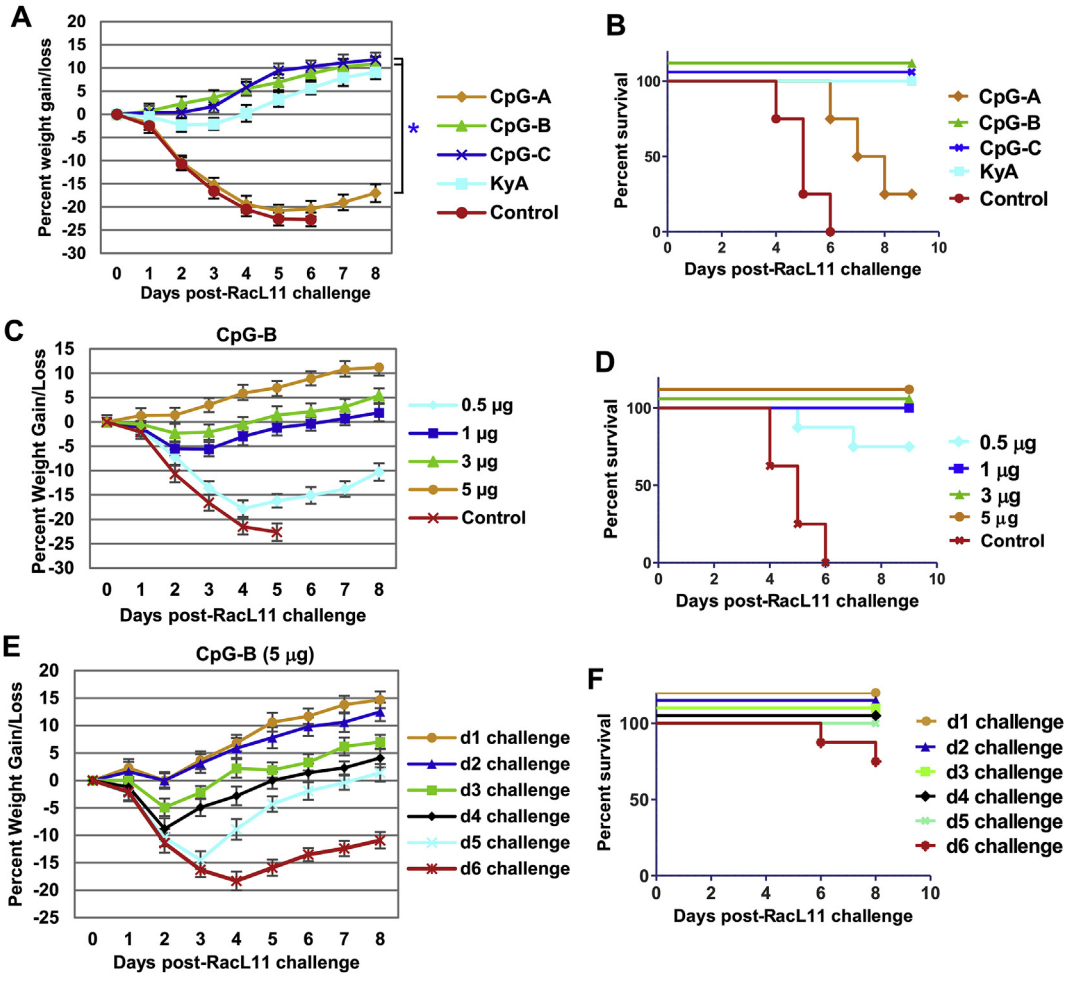

Fig. 1. Class B CpG oligonucleotides protected CBA mice from pathogenic RacL11 challenge infection. (A) Average body weights and (B) percent survivals of the CBA mice treated with CpG ODNs. CBA mice (8 mice per group) were administered intranasally with control medium, 5 μg/mouse of CpG-A ODN, CpG-B ODN, or CpG-C ODN, or were infected with 2×10^6 PFU/mouse of EHV-1 KyA, and were challenged with pathogenic EHV-1 RacL11 (1.5×10^6 PFU/mouse) at 48 h later. $P < 0.01$ for CpG-B and CpG-C ODN groups vs. CpG-A ODN group. (C) Average body weights and (D) percent survivals of the CBA mice treated with different dosages of CpG-B ODN. CBA mice (n = 8) were treated intranasally with 0.5, 1, 3, or 5 μg of CpG-B ODN per mouse and were challenged with RacL11 (1.5×10^6 PFU/mouse) at 48 h post-treatment. (E) Average body weights and (F) percent survivals of the CBA mice treated with CpG-B ODN. CBA mice (n = 8) were treated intranasally with 5 μg/mouse of CpG-B ODN and were challenged with RacL11 (1.5×10^6 PFU/mouse) at 1–6 days post-treatment.

To determine the minimal dose of the CpG-B ODN required for protection, CBA mice were treated with increasing amounts of the CpG-B ODN and challenged with RacL11 at 2 days post-treatment. The mice administered 1, 3, or 5 μg/mouse of CpG-B ODN lost less than 10% of their preinfection body weight and all rapidly regained their body weight (Fig. 1C). All mice treated with 1, 3 or 5 μg of CpG-B ODN survived challenge with pathogenic EHV-1 RacL11 (Fig. 1D). However, the mice administered 0.5 μg of the CpG-B ODN lost 18% of their pre-infection body weight by day 4 pi (Fig. 1D), and two of the eight mice succumbed to RacL11 challenge infection by day 8 pi (Fig. 1D). Repeat experiments confirmed these findings and revealed that a dose of 1 μg of CpG-B ODN elicits full protection to challenge with pathogenic EHV-1.

To determine the period of protection elicited by treatment with CpG-B ODN, CBA mice treated with 5 μg of the CpG-B ODN were challenged with 1.5×10^6 PFU/mouse of RacL11 at 1, 2, 3, 4, 5, and 6 days post-treatment. When challenged within 5 days post-treatment, all of the CpG-B ODN-treated mice lost less than 15% of their preinfection body weight and rapidly regained weight (Fig. 1E). None of these treated mice died upon challenge with RacL11 (Fig. 1F). When challenged at 6 days post-treatment, the treated mice lost 18% of their preinfection body weight and slowly regained weight (Fig. 1E). Twenty-five percent of the mice treated with CpG-B ODN died upon RacL11 challenge at 6 to 8 dpi (Fig. 1F).

Because CpG-C ODN combines features of both types A and B ODN, CpG-A and CpG-B ODNs were used in the following experiments.

3.2. Treatment with the CpG-B ODN reduces lung virus titers and severe virus-induced pathology

Our published results showed that intranasal immunization with EHV-1 KyA accelerated clearance of pathogenic EHV-1 RacL11 from the lungs of CBA mice (Kim et al., 2016). To investigate whether treatment with the CpG-B ODN inhibits EHV-1 replication in CBA mice lungs, mice were treated with medium, CpG-A ODN, or CpG-B ODN, or were immunized with EHV-1 KyA and were challenged with RacL11 at 2 days later. The amount of infectious EHV-1 in the lungs was determined at 2, 3, and 4 days post-challenge. Lung virus titers of the CpG-B-treated mice were 63-fold lower at 2 dpi, 100-fold lower at 3 dpi and 1580-fold lower at 4 dpi than those of lungs of mock-treated mice (Fig. 2A). Lung virus titers of the KyA-immunized mice were 100-fold lower at 2 dpi, 125-fold lower at 3 dpi and 2511-fold lower at 4 dpi than those of lungs of mock-treated mice (Fig. 2A). However, lung virus titers of the CpG-A-treated mice were reduced by less than 4-fold than those of lungs of mock-treated mice (Fig. 2A). These results indicated that CpG-B ODN treatment inhibits EHV-1 replication in the lungs of CBA mice.

To access whether treatment with CpG-A or CpG-B ODN can attenuate the development of severe lung cytopathogy due to infection with pathogenic EHV-1 RacL11, CBA mice treated with medium, CpG-A ODN, or CpG-B ODN were challenged with RacL11 at 2 days post-treatment, and lungs collected at 4 days post-RacL11 challenge were assessed for cytopathology. As expected, the lungs of control mice infected with RacL11 exhibited extensive peribronchial cuffing, interstitial inflammatory infiltration, and disruption of the bronchial epithelium (Fig. 2B), resulting in 100% mortality of these mice by 6 dpi. Inflammatory infiltration was not detected at 4 days post-treatment in the lungs of mice treated with CpG-A ODN or CpG-B ODN (Fig. 2B). The lungs of mice treated with CpG-B ODN exhibited an inflammatory infiltration, but no peribronchial cuffing was noted at 4 days post-challenge infection (dpc) (Fig. 2B). In contrast, the lungs of mice treated with CpG-A ODN exhibited extensive peribronchial cuffing and disruption of the bronchial epithelium at 4 dpc (Fig. 2B). Taken together, these results indicated that CpG-B ODN treatment inhibited EHV-1 replication in the lung, attenuated development of severe EHV-1-induced lung cytopathology, and protected CBA mice against lethal EHV-1 challenge.

3.3. IFN-γ and seven antiviral interferon-stimulated genes are upregulated in the lungs of CpG-ODN-treated mice

Our results showed that CpG-B ODN treatment protects CBA mice from RacL11 challenge at 1–5 days post-treatment (Fig. 1E). Our previous published results showed that EHV-1 KyA immunization effectively protected CBA mice from RacL11 challenge at 1–7 days post-immunization (Kim et al., 2016). Affymetrix microarray analysis revealed that IFN-γ and seven antiviral interferon-stimulated genes (ISGs)

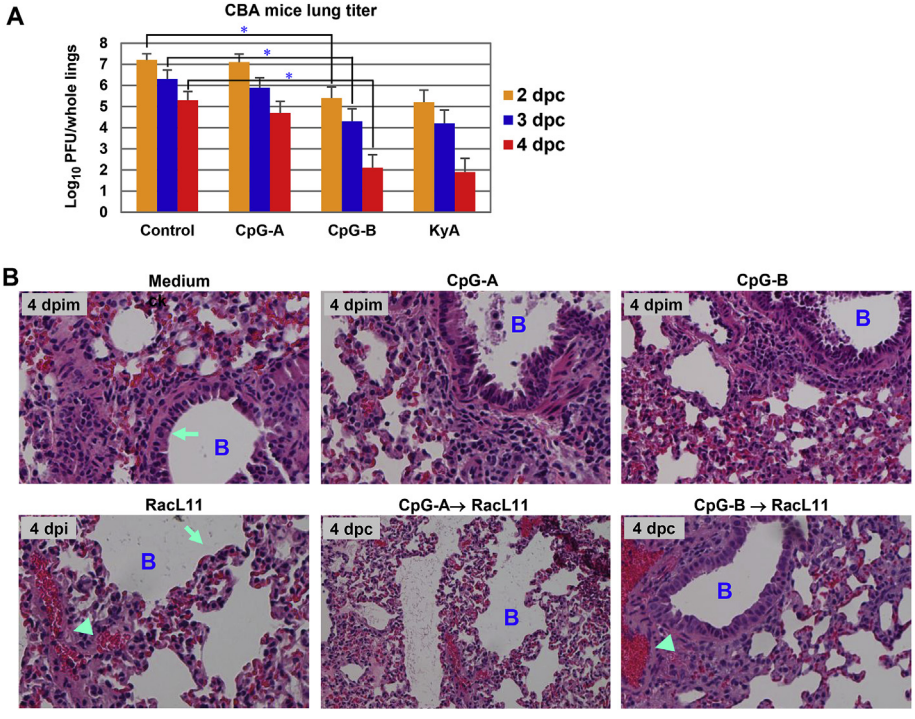

Fig. 2. Treatment with CpG-B ODN is protective against subsequent RacL11 challenge. (A) Virus titers from the lungs of CBA mice treated with CpG ODN. Mice (n = 4) were treated with 5 μg/mouse of control medium, CpG-A ODN, or CpG-B ODN and challenged with RacL11 (1.5×10^6 PFU/mouse) at 2 days post-treatment. The mice immunized with KyA (2×10^6 PFU/mouse) were challenged with RacL11 (1.5×10^6 PFU/mouse) at 2 dpi. On days 2–4 post-challenge, the lungs were removed and homogenized, and the amount of infectious EHV-1 was determined (Kim et al., 2011). *denote statistical significance ($P < 0.01$). (B) Histological sections of infected mice lungs. CBA mice (n = 4) treated with control medium, CpG-A ODN, or CpG-B ODN were challenged with RacL11 as described in Fig. 2A. On day 4 post-challenge, the lung tissue was fixed and stained with H&E as described in Materials and Methods. The resulting slides were visualized and photographed at 20× magnification with a Nikon TE300 Eclipse microscope. Dpi, days post-infection; dpim, days post-immunization; dpc, days post-RacL11 challenge; B, bronchus. Bronchial epithelial (arrows) and inflammatory infiltration (arrowheads) regions are indicated. Data are representative of those from four mice.

Table 2
Antiviral interferon-stimulated genes upregulated by CpG ODNs in CBA mice.

Gene name	Description	Targeted Viruses[a]
RSAD2	Radical S-adenosyl methionine domain containing 2	DENV, FLUAV, HCMV, HCV(r), SINV, WNV (v)
MX1	Myxovirus (influenza virus) resistance 1	CVB, FLUAV, HCV (r), HPIV3, MV, SFV, VSV
IRF7	Interferon regulatory factor 7	numerous RNA and DNA viruses
IFI44L	Interferon-induced protein 44 like	HCV
ISG15	Interferon stimulated protein (=ubiquitin-like modifier)	FLUAV, HIV-1, HSV-1, JEV, MHV-68, SINV
OAS1a	2′-5′ oligoadenylate synthetase 1a	CHIKV, DENV, EMCV, HCV(r), SINV, WNV
IFI204 (IFI16)	IFN-inducible 204	HSV-1[b], HCMV[c], KSHV[d]

[a]References are in Schoggins and Rice (2011) except for [b]HSV-1 (Diner et al., 2016; Johnson et al., 2014), [c]HCMV (Diner et al., 2016), and [d]KSHV (Kerur et al., 2011). CHIKV, chikungunya virus; CVB, coxsackie B virus; DENV, dengue virus; EMCV, encephalomyocarditis virus; FLUAV, influenza A virus; HCV, hepatitis C virus [(r), replicon]; HCMV, human cytomegalovirus; HIV-1, human immunodeficiency virus 1; HSV-1, herpes simplex virus type 1; JEV, Japanese encephalitis virus, KSHV, Kaposi's sarcoma associated herpesvirus; MHV-68, murine gammaherpervirus-68; MV, measles; SINV, Sindbis virus; VSV, vesicular stomatitis virus; WNV, West Nile virus [(v), virus-like particles]; YFV, yellow fever virus.

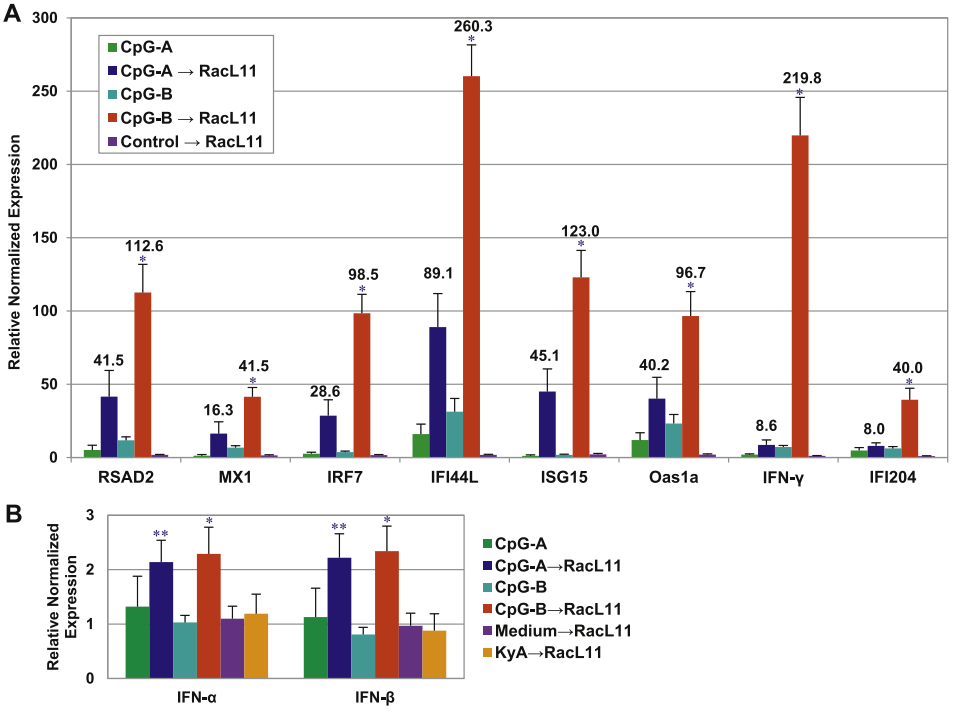

Fig. 3. Upregulation of IFN-γ gene and antiviral ISGs in the lungs of CpG-B ODN-treated CBA mice. (A) CBA mice (n = 4) were treated intranasally with 5 μg/mouse of control medium, CpG-A ODN, or CpG-B ODN and challenged with RacL11 (1.5×10^6 PFU/mouse) at 2 days post-treatment. Lungs were removed at 8 hpi and RNA was purified and used for real-time RT-qPCR. Relative mRNA expression of ISGs and IFN-γ was determined by real-time RT-qPCR by normalizing to GAPDH. Results are expressed as fold change over control mice (medium-treated). The numbers on the top of the bars represent fold changes. *, $P < 0.01$ for comparison with the lungs of RacL11-challenged mice that had been treated with the CpG-A ODN. (B) Relative mRNA expression of IFN-α and IFN-β was determined at the same time with those of panel 3A and are shown in a different graph due to the big difference in expression levels. CBA mice immunized with 2×10^6 PFU/mouse of attenuated KyA were challenged with RacL11 (1.5×10^6 PFU/mouse) at 2 dpi. *, $P < 0.01$ for comparison with CpG-B ODN-treated mice lungs. **, $P < 0.05$ for comparison with CpG-A ODN-treated mice lungs.

were significantly upregulated at 8 h post-challenge in the lungs of RacL11-challenged mice that had been immunized with KyA (Kim et al., 2016). To investigate the mechanism by which CpG-B ODN stimulation protects CBA mice from challenge with pathogenic RacL11, real-time RT-qPCR analyses were performed with RNA from infected mouse lungs. RNA from mock-treated mice lungs was used as the control. Lungs of the control mice and mice treated with CpG-A ODN or CpG-B ODN were harvested at 8 h post-RacL11 challenge and used for RT-qPCR. RT-qPCR results showed that IFN-γ and seven antiviral ISGs RSAD2, MX1, IRF7, IFI44L, ISG15, OAS1a, and IFI204 (Table 2) were significantly upregulated 39.4- to 260.3-fold at 8 h postchallenge in the lungs of mice that had been treated with the CpG-B ODN (Fig. 3A). The IFN-γ and seven antiviral ISGs were upregulated 8.0- to 89.1-fold in the lungs of RacL11-challenged mice that had been treated with CpG-A ODN (Fig. 3A). However, CpG-A- or CpG-B ODN-treatment without EHV-1 challenge upregulated the eight genes by 1.2- to 16.1-fold. Interestingly, the IFN-γ gene was significantly upregulated by 26-fold upon RacL11 challenge infection in CpG-B ODN-treated mice lungs as compared to that of CpG-A ODN-treated mice lungs (Fig. 3A). The seven ISGs were upregulated by 2.4–5.0-fold upon RacL11 challenge in CpG-B ODN-treated mice lungs as compared to that of CpG-A ODN-treated mice lungs (Fig. 3A). EHV-1 RacL11 infection was not able to induce similar levels of expression of these eight genes (less than 2.2-fold) in the lungs of control mice (medium treated) at 8 h post-infection (Fig. 3A), which is consistent with our previous results (Kim et al., 2016). Type I interferon IFN-α and IFN-β genes in the CpG-A- and CpG-B-treated CBA mice lungs were upregulated approximately 2-fold upon RacL11 challenge infection (Fig. 3B). Taken together, these results indicated that IFN-γ expression enhanced by treatment of mice with CpG-B ODN may be important for the protection of CBA mice from pathogenic EHV-1 RacL11 infection.

3.4. Pre-treatment with IFN-γ significantly inhibits EHV-1 replication in murine alveolar macrophage MH-S cells

Macrophages in the lung alveoli provide an innate defense mechanism, and IFN-γ is the prototypical macrophage-activating cytokine. The primary immune response in the lungs of mice infected with pathogenic EHV-1 was alveolar and interstitial inflammation, characterized by the sequential appearance of macrophages (Bartels et al., 1998). Lung histology of EHV-1 RacL11-infected CBA mice revealed a massive cellular consolidation of the lung, consisting primarily lymphocytes, macrophages, and neutrophils (Smith et al., 2000a). Our previous results showed that 24 h pre-treatment with 20 ng/ml of IFN-γ

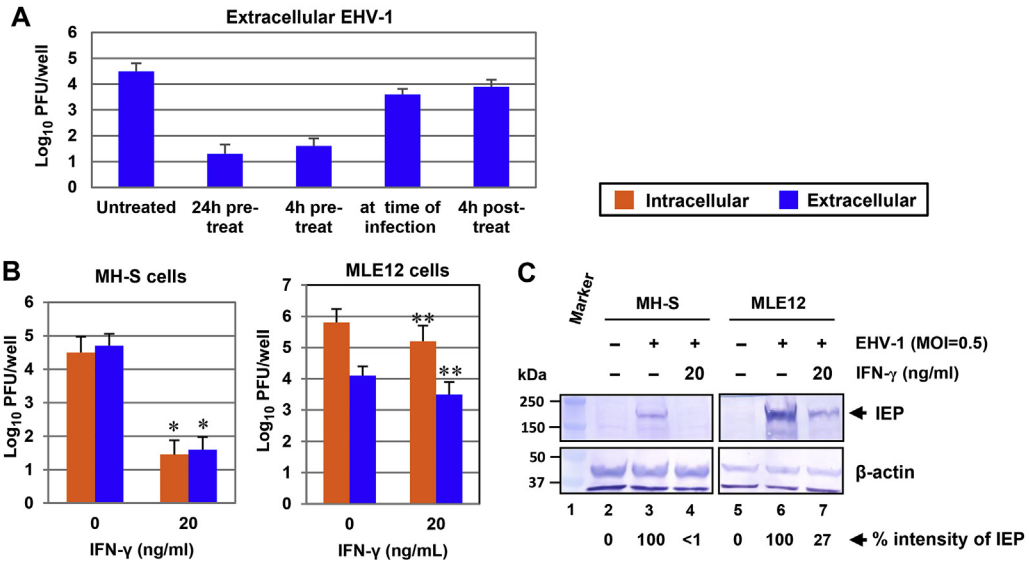

Fig. 4. IFN-γ treatment reduces EHV-1 yields in murine alveolar macrophage MH-S cells. (A) MH-S cells were plated in 12-well plates (5×10^5 cells/well) and treated with 20 ng/ml of murine IFN-γ (Cell Sciences, Canton, MA). 24 h prior to, 4 h prior to, at time of infection, or 4 h following EHV-1 KyA (MOI = 0.5) infection. At 30 h post-infection, the extracellular virus was titered by plaque assay. (B) MH-S and murine lung epithelial MLE12 cells plated in 12-well plates (3×10^5 cells/well for MLE12cells; 5×10^5 cells/well for MH-S cells) were treated with 20 ng/ml of murine IFN-γ and the cells were infected with 0.5 MOI of EHV-1 KyA at 24 h post-treatment. At 30 hpi, intracellular virus was released and titered by plaque assay on NBL6 (Kim et al., 2011). (C) The infected cells were also harvested at 30 hpi for western blot analyses using anti-IEP OC33 (Harty and O'Callaghan, 1991) and anti-actin (Santa Cruz Biotechnology, Santa Cruz, CA). Data are representative of three independent experiments. Numbers to the left represent molecular weight standards (kDa) (Bio-Rad Laboratories, Hercules, CA). Quantification was done with Image Studio Lite software (LI-COR Biosciences, Lincoln, NE). The intensities of the IEP bands were normalized to those of non-treated cells. *, $P < 0.01$ for comparison with the untreated control. **, $P < 0.05$ for comparison with the untreated control.

significantly reduced EHV-1 KyA and RacL11 yields at 24 h post-infection (hpi) in murine alveolar macrophage MH-S cells (Kim et al., 2016). To determine the length of IFN-γ treatment for EHV-1 inhibition, MH-S cells were treated with 20 ng/ml of IFN-γ 24 h prior to, 4 h prior to, at the time of infection, and 4 h after infection. Twenty-four hour and four hour pre-treatment with IFN-γ more effectively inhibited EHV-1 KyA replication than treatment at the time of infection and at 4 h post-treatment (Fig. 4A). Four-hour post-treatment of MH-S cells with IFN-γ inhibited EHV-1 replication by only 3.2-fold (Fig. 4A). Treatment with 20 ng/ml of IFN-γ reduced intracellular and extracellular EHV-1 KyA yields in MH-S cells by 1122- and 1258-fold at 30 h post-infection (hpi), respectively (Fig. 4B), which is consistent with our previous results (Kim et al., 2016). To investigate whether IFN-γ inhibits EHV-1 replication in other mouse lung cells, mouse lung epithelial MLE12 cells were employed. .IFN-γ reduced virus yields by only 4-fold in MLE12 cells (Fig. 4B). To confirm these results, the infected cells were harvested at 30 hpi for western blot analyses using antibodies to the EHV-1 immediate-early protein (IEP) and β-actin. The EHV-1 IEP acts to induce the expression of early (E) and late (L) viral genes and is essential for viral growth (Buczynski et al., 2005; Smith et al., 1992, 1995). IFN-γ reduced the expression level of the viral IEP by greater than 99% in MH-S cells (Fig. 4C lane 4) as compared to that of non-treated cells (Fig. 4C lane 3). IFN-γ reduced the expression levels of the IEP by 27% in MLE12 cells (Fig. 4B lane 7). Very similar results were obtained with EHV-1 RacL11 (data not shown). These results indicated that IFN-γ effectively inhibits EHV-1 replication in murine alveolar macrophage MH-S cells.

4. Discussion

CpG oligonucleotides (CpG ODNs) stimulate innate immune responses in mice, primates and many domestic species (Abel et al., 2005; Kamstrup et al., 2001; Kurata et al., 2004; Mena et al., 2003; Rankin et al., 2001). Addition of CpG ODN as an adjuvant to modified vaccinia Ankara (MVA) (Belyakov et al., 2006) and formalin-inactivated bovine respiratory syncytial virus vaccine (FI-BRSV) (Mapletoft et al., 2008), improved the immune responses as well as eliciting protection against lethal virus challenge. A single injection with 15 μg/mouse of CpG-B ODN (ODN, 1826) intraperitoneally prior to the 2009 H1N1 influenza virus challenge reduced mean weight loss (44.2% to 22.6%) and mortality rate (100% to 40%) in mice as compared to that of PBS-treated mice (Jiang et al., 2011). The findings of the present study showed that intranasal CpG-B ODN treatment significantly increased expression of IFN-γ and seven antiviral ISGs upon pathogenic RacL11 challenge, accelerated clearance of virus from the lungs of infected CBA mice, reduced lung cytopathology, and protected mice at 1–5 days post-treatment. When CBA mice were immunized with both EHV-1 KyA and CpG-B ODN, we saw some synergistic effect in protection from RacL11 challenge as lung virus titers were reduced (data not shown), suggesting that formulation of KyA with CpG-B ODN could result in enhanced innate immune responses as well as protection from lethal EHV-1 challenge. Analyses of total RNA in the lungs of mice challenged with RacL11 following simultaneous immunization with KyA and administration of CpG ODN would identify any changes in the ISG expression profile associated with enhanced protection due to the simultaneous administration of the ODN and the KyA vaccine virus. Taken together, these results suggest that CpG-B ODN treatment stimulates the immune response in the lungs of CBA mice upon EHV-1 challenge infection, resulting in the protection of mice. This study demonstrates for the first time that treatment of CpG ODN protects mice (100% survival rate) from lethal EHV-1 challenge at 1–5 days post-treatment.

The innate immune response is considered the first line of defense against viral pathogens. In the horse, pre-existing EHV-1-induced IFN-γ producing cells resulted in protection from developing clinical signs and viral shedding after challenge infection (Coombs et al., 2006). In the horse, equine IFN-γ increases major histocompatibility complex (MHC) Class II expression by monocytes and peripheral blood mononuclear cells (PBMC) (Wagner et al., 2005), and shows antiviral activity (Gutmann et al., 2005). *In vitro* EHV-1 infection of PBMC induced IFN-α secretion without major differences between viral strains and age groups (Wagner et al., 2011). In the horse, innate immune responses to EHV-1 such as IFN-α, IFN-β, and antiviral ISGs have not yet been investigated. In response to CpG-B ODN 2135 and CpG-C ODN 2395 stimulation *in vitro*, PBMCs of neonatal foals showed increased expression of IFN-γ, IL-6, and IL-12p35/p40 (Liu et al., 2009). Our findings showed that the IFN-γ gene was upregulated by 26-fold upon RacL11 challenge in CpG-B ODN-treated mice lungs as compared to that of CpG-A ODN-treated mice lungs. However, the seven ISGs RSAD2, MX1, IRF7, IFI44L, ISG15, OAS1a, and IFI204 in CpG-B ODN-treated mice were upregulated by only 2.4–5.0-fold in the lungs as compared to those of

CpG-A ODN-treated mice lungs. Type I interferon IFN-α and IFN-β are important inflammatory cytokines elicited by the eukaryotic host as innate immune responses against invading pathogens (Schoggins and Rice, 2011). IFN-α and IFN-β genes in the lungs of CpG-B-treated CBA mice were upregulated only 2-fold upon RacL11 challenge (Fig. 3B). Taken together, these results suggested that IFN-γ expression is a major factor for the protection of CBA mice from pathogenic EHV-1 RacL11 challenge.

The data presented in this study revealed that treatment with CpG-B ODN upregulated the IFN-inducible 204 (IFI204) gene by 40-fold at 8 h postchallenge in the lungs of RacL11-challenged mice. Since IFN-γ, IFN-α, and LPS induce IFI204 expression (Gariglio et al., 1998), it may be significant that IFN-γ upregulated IFI204 by 40-fold in the lungs of RacL11-challenged mice that were treated with CpG-B ODN two days prior to challenge. Interferon-γ-inducible protein 16 (IFI16), a human homolog of IFI204, has critical roles in antiviral immunity (Ansari et al., 2013; Kerur et al., 2011; Singh et al., 2013). IFI16 has been shown to restrict herpesvirus replication (Diner et al., 2016; Johnson et al., 2014; Kerur et al., 2011; Merkl et al., 2018; Orzalli et al., 2013).

The ubiquitin-like molecule, ISG15, plays a crucial role in the host's antiviral response against approximately 18 viruses, including herpes simplex virus type 1 (HSV-1) (Lenschow, 2010; Lenschow et al., 2007; Skaug and Chen, 2010; Zhang and Zhang, 2011). Consistent with the importance of ISG15 in innate immune responses to herpesviruses, the level of ISG15 expression was increased 123-fold in CpG-B-treated mice following RacL11 challenge (Fig. 3A). The ISG15 gene was upregulated by 45.1-fold in the lungs of CpG-A ODN-treated mice that succumbed to RacL11 challenge. Thus, upregulation of ISG15 may not be involved in the inhibition of EHV-1 replication in the lungs of CpG-B-treated CBA mice or if involved a large amount of the ISG15 gene product may be required for its anti-EHV-1 effect.

Incoming viruses are sensed by pattern recognition receptors (PRRs), leading to activation of interferon regulatory factors (IRFs) and subsequent induction of interferons. IFNs bind their cognate receptors and transcriptionally induce hundreds of interferon-stimulated genes (ISGs), many of which function to inhibit virus replication (Der et al., 1998; Schoggins and Rice, 2011; Sen and Peters, 2007). It is possible that additional ISGs are significantly upregulated in the lungs of CpG-B ODN-treated mice upon RacL11 challenge. Our data showed that pretreatment with IFN-γ significantly reduced EHV-1 yield in MH-S cells, but not in MLE12 cells (Fig. 3), suggesting that IFN-γ-stimulated protein (s) inhibit viral replication. Upcoming experiments will compare the profile of ISG genes induced by IFN-γ in MH-S cells that are protected to EHV-1 challenge to that of MLE12 cells that are comparatively resistant following treatment with IFN-γ. These comparatively analyses would help identify ISGs associated with an anti-EHV-1 phenotype so that experiments to evaluate these specific ISG gene products for the ability to suppress EHV-1 replication and pathogenicity may be carried out.

Elucidation of the mechanisms by which IFN-γ and antiviral ISGs may control EHV-1 replication will provide new strategies for the development of anti-EHV-1 agents in horses and contribute to the understanding of how ISGs gene products function as anti-herpesvirus proteins. Our approach to generate and characterize recombinant adenoviruses expressing antiviral ISGs identified in the CBA mouse model may give more insight into the importance of specific ISGs in the prevention of EHV-1 infection. Our future experiments of CpG-B ODN treatment and IFN-γ administration in the natural host horse may offer a basis for more effective treatment of this major equine pathogen.

5. Conclusions

We report here that intranasal CpG-B ODN treatment significantly increased expression of IFN-γ and seven antiviral ISGs upon pathogenic RacL11 challenge and protected mice at 1–5 days post-treatment. The IFN-γ gene was upregulated by 26-fold upon RacL11 challenge in CpG-B ODN-treated mice lungs as compared to that of CpG-A ODN-treated mice lungs. The seven ISGs were upregulated by 2.4 to 5-fold in CpG-B ODN-treated mice lungs as compared to those of CpG-A ODN-treated mice lungs, suggesting that IFN-γ is important for the protection of CBA mice from the lethal challenge. These results suggest that intranasal administration with CpG-B ODN protects CBA mice from pathogenic EHV-1 challenge by eliciting innate immune responses.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2019.104546.

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