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Viral replication and innate immunity of feline herpesvirus-1 virulence-associated genes in feline respiratory epithelial cells

Yao Lee[a,b], Roger Maes[b,c], S.-H. Sheldon Tai[d], Gisela Soboll Hussey[b,*]

[a] Graduate Program in Comparative Medicine and Integrative Biology, College of Veterinary Medicine, Michigan State University, 784 Wilson Road, East Lansing, MI 48824, USA
[b] Department of Pathobiology and Diagnostic Investigation, College of Veterinary Medicine, Michigan State University, 784 Wilson Road, East Lansing, MI 48824, USA
[c] Veterinary Diagnostic Laboratory, 4125 Beaumont Road, Building 0215, Lansing, MI 48910, USA
[d] Maryland Institute for Applied Environmental Health, School of Public Health, University of Maryland, 4200 Valley Dr, College Park, MD 20742, USA

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ABSTRACT

Feline herpesvirus-1 (FHV-1) infection occurs worldwide and is a leading cause of respiratory and ocular diseases in cats. Current vaccines reduce the severity of symptoms but do not prevent infection and, therefore, do not provide defense against an establishment of latency and reactivation. We hypothesize that immunomodulation of FHV-1 is the cause of lack in protection and that deletion of virulence/immune modulatory genes of FHV-1 will enhance safety and immunogenicity. Our objective was to use feline respiratory epithelial cell (FREC) cultures to define *in vitro* growth characteristics and immunomodulation resulting from infection of FRECs with the virulent FHV-1 strain C27 (WT) and glycoprotein C-deletion (gC-), glycoprotein E-deletion (gE-), serine/threonine protein kinase-deletion (PK-), as well as gE and thymidine kinase-double-deletion (gE-TK-) mutants generated by bacterial artificial chromosome mutagenesis.

Differentiated FRECs were mock inoculated or inoculated with WT, gC-, gE-, PK-, or gE-TK- mutants. Virus titration and real-time quantitative PCR assays were performed on samples collected at 1 hpi followed by 24 h intervals between 24 and 96 hpi to determine growth kinetics. Real-time PCR was used to quantitate IFNα, TNFα, IL-1β, IL-10, and TGFβ-specific mRNA levels. Immunoassays were performed to measure the protein levels of subsets of cytokines/chemokines secreted by FRECs.

Inoculation of FRECs with gE-TK- resulted in significantly lower end-point titers than inoculation with WT or gE-. Both PK- and gC- inoculated FRECs also produced significantly lower end-point titers at 96 hpi than WT. Overall, intracellular virus titers were higher than those of extracellular virus. PCR results for viral DNA paralleled the virus titration results. Further, in contrast to WT inoculation, an increase in IFNα and IL-10 mRNA expression was not observed following inoculation with gE-TK- and PK-, but inoculation with gE-TK- and PK- did result in increased TGFβ expression in FRECs compared to responses following infection with WT. Moreover, gE-TK- and PK- blocked the inhibition of IL-8 and neutrophil chemoattractant (KC), which was observed following inoculation with WT.

In summary, the results obtained in FRECs may be used to predict the safety and immunogenicity characteristics of these mutants *in vivo*. Our study highlights the value of the FREC system for studying replication kinetics/immune modulation factors of FHV-1 and screening prospective vaccine candidates before their use in experimental cats.

Abbreviations: ALI, Air-liquid interface; ANOVA, Analysis of variance; BAC, Bacterial artificial chromosome; BHV-1, Bovine herpesvirus-1; CRFK, Crandall Reese feline kidney; CPE, Cytopathic effect; DMEM, Dulbecco's Modified Eagle Medium; EHV-1, Equine herpesvirus-1; ELISA, Enzyme-linked immunosorbent assays; FHV-1, Feline herpesvirus-1; FREC, Feline respiratory epithelial cell; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; GM-CSF, Granulocyte-macrophage colony-stimulating factor; HSV-1, Human herpes simplex virus-1; HSV-2, Human herpes simplex virus-2; gC-, Glycoprotein C-deletion mutant; gE-, Glycoprotein E-deletion mutant; gE-TK-, Glycoprotein E & thymidine kinase-double-deletion mutant; hpi, Hours post-inoculation; IFNα, Interferon alpha; IL-1β, Interleukin 1 beta; IL-8, Interleukin 8; IL-10, Interleukin 10; IL-12p40, Interleukin 12 subunit beta; KC, Keratinocyte chemoattractant/ neutrophil chemoattractant; MOI, Multiplicity of infection; NC, Negative control; PCR, Polymerase chain reaction; PK-, Serine/threonine protein kinase-deletion mutant; PRV, Porcine pseudorabies virus; RANTES, Regulated on activation, normal T cell expressed and secreted; $TCID_{50}$, Tissue culture infectious dose 50%; TGFβ, Transforming growth factor beta; TLR3, Toll-like receptor 3; TNFα, Tumor necrosis factor alpha; WT, Feline herpesvirus-1 C27 wild type

* Corresponding author at: Department of Pathobiology and Diagnostic Investigation, College of Veterinary Medicine, Michigan State University. 784 Wilson Rd, A13, East Lansing, MI 48824, USA.
E-mail addresses: leeyao@msu.edu (Y. Lee), maes@dcpah.msu.edu (R. Maes), stai1@umd.edu (S.-H.S. Tai), husseygi@msu.edu (G. Soboll Hussey).

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1. Introduction

Feline herpesvirus-1 (FHV-1) is classified within the family *Herpesviridae*, subfamily *Alphaherpesvirinae*, genus *Varicellovirus*, species *Felid alphaherpesvirus 1*. Clinical signs commonly associated with FHV-1 include fever, nasal and ocular discharge, conjunctivitis and keratitis. Pneumonia, facial and nasal dermatitis, stomatitis, abortion and fatality in kittens are observed occasionally (Harbour et al., 1991; Hoover and Griesemer, 1971; Hoover et al., 1970; Maes, 2012; Nasisse et al., 1998). The disease is particularly severe in 6–9 week-old cats because of loss of passive immunity at this age (Dawson et al., 2001; Johnson and Povey, 1985). The virus is highly prevalent in the feline population worldwide. Transmission occurs via the oronasal route, with viral invasion and replication in conjunctival and upper respiratory epithelia (Gaskell and Povey, 1979). Similar to other herpesviruses, latency is established in neurons within the trigeminal ganglia after the acute phase of infection. Stress or immunosuppression readily lead to reactivation, resulting in renewed shedding of infectious virus, often accompanied with recrudescence of clinical signs (Gaskell et al., 2007, 1985).

Modified live (MLV) and inactivated vaccines are commercially available. Both vaccine types are typically trivalent and also include feline calicivirus, as well as feline panleukopenia virus (Lappin et al., 2006; Weigler et al., 1997). Existing vaccines only reduce clinical signs but do not prevent viral shedding or establishment of field virus latency. This incomplete protection induced by current FHV-1 vaccines is a problem, particularly in animal shelters, where it can still lead to outbreaks and disease in cats. Furthermore, vaccination with MLV is based on the temperature sensitivity of the vaccine virus that is attenuated when administered SC but remains virulent when administered IN or in case of accidental spills of the vaccine virus (Orr et al., 1978; Kruger et al., 1996). Aside from insufficient protection, the F2 strain used in the current vaccine has an almost identical cleavage pattern in restriction enzyme analysis compared to wild-type isolates (Horimoto et al., 1992), making the development of a new recombinant vaccine platform with distinguishable markers from the wild type or virulent strains desirable and a secondary objective.

Based on knowledge gained through studies of various alphaherpesviruses and the fact that many of these viral genes have homologous functions within viral families, glycoprotein E (gE), glycoprotein C (gC), thymidine kinase (TK), and serine/threonine protein kinase (PK) have been identified as potential virulence factors that are at the same time non-essential proteins for virus growth in alphaherpesviruses (Dingwell et al., 1994; Field and Wildy, 1978; Gaskell et al., 2007; Zuckermann et al., 1988). Clearly, detailed studies for these genes of FHV-1 are needed to define these functions in the feline system. Glycoprotein C of alphaherpesviruses including human herpes simplex virus-1 (HSV-1), equine herpesvirus-1 (EHV-1) and pseudorabies virus (PRV), has been shown to be associated with viral attachment and entry (Lubinski et al., 1999; Osterrieder, 1999; Rue and Ryan, 2002), whereas studies using bovine herpesvirus-1 (BHV-1) and PRV showed that gE is primarily associated with facilitating viral transmission and trans-synaptic viral spread (Otsuka and Xuan, 1996; Zuckermann et al., 1988). Further studies using HSV-1 and FHV-1 thymidine kinase showed that this alphaherpesvirus protein regulates viral replication and establishment of latency in neurons cells and is the target of anti-herpetic drugs (Field and Wildy, 1978; Hussein et al., 2008; Nunberg et al., 1989). Lastly, the Us3-encoded serine/threonine protein kinase is conserved in the alphaherpesvirus family and has been shown to function in mechanisms of anti-apoptosis, the phosphorylation process in host cells, cytoskeleton re-arrangements, and viral nuclear egress in multiple members of the alphaherpesvirus family (Baek et al., 2002; Jacob et al., 2011; Osterrieder, 1999). Another study demonstrated that the PK from PRV induces cytoskeleton rearrangement and could then facilitate cell-to-cell viral spread and invasion across the basement membrane in the respiratory tract (Lamote et al., 2016).

For other veterinary herpesviruses, vaccination with several selected deletion mutants have shown good results. Glycoprotein E-deletion mutants were a cornerstone in PRV eradication campaigns (van Oirschot et al., 1990). Administration of mutants with BHV-1 gE-deletion (gE-), TK-deletion (TK-), and gE and TK-double-deletion (gE-TK-) induced improved immunity, reduced viral shedding and protected calves from clincal disease (Kaashoek et al., 1996). Experimental intranasal or intramuscular vaccination with gE- mutants drastically reduced viral shedding and alleviated the symptoms of clinical disease after EHV-1 wild type (WT) challenge in foals (Tsujimura et al., 2009). Intranasal inoculation of pigs with gE-, PK-, and TK- pseudorabies virus mutants reduced or eliminated post-challenge virus shedding (Kimman et al., 1994). Further, gI-gE- or TK- mutants of FHV-1 have been generated previously and shown to induce protective immunity in cats; however, residual virulence remained a concern (Sussman et al., 1995; Yokoyama et al., 1996).

In addition to being important virulence factors, the genes selected for deletion likely play a role in modulating the host's immune response. Glycoprotein E of HSV-1 interferes with the antibody-depedent cytotoxicity by binding to the Fc domain of the immunoglobulin G (Johnson et al., 1988). Glycoprotein C of HSV-1 inactivates complement by interacting with component C3b (Lubinski et al., 1999). Us3-encoded PK or the orthologs in various alphaherpesviruses are versatile in immune modulation, including impeding interferon signal transduction, down-regulating the expression of major histocompatibility complex class I (MHC class I), and interfering with the apoptosis pathway to prevent cell death (Jacob et al., 2011). Recent research showed that the Us3 protein of FHV-1 is a powerful inhibitor of INFβ via inhibition of interferon regulatory transcription factor 3 (IRF3) dimerization (Tian et al., 2018).

To generate gC-, gE-, PK-, and gE-TK- mutants of FHV-1, we used recombineering of a bacterial artificial chromosome (BAC) clone containing the entire FHV-1 genome. These mutants were initially characterized in Crandall Reese feline kidney (CRFK) cells by our group (Tai et al., 2016). We also developed a primary feline respiratory epithelial cell (FREC) system grown at the air-liquid interface (ALI), and showed that differentiated FRECs resemble the natural airway of cats morphologically as well as immunologically and, as such, are a suitable *in vitro* model to study FHV-1 (Nelli et al., 2016).

The hypothesis of this study is that deletion of virulence/immune modulatory genes of FHV-1 will enhance its safety and immunogenicity. Our objective was to use the FREC system to investigate the virulence and immune modulation of the selected FHV-1 deletion mutants (gC-, gE-, PK-, gE-TK-), with the ultimate goal of identifying one or more mutants for FHV-1 vaccine development.

2. Materials and methods

2.1. FHV-1 wild type and FHV-1 mutants

FHV-1 strain C27 [ATCC, VR-636, Manassas, VA, USA] was used as the wild type (WT) in this study (Nelli et al., 2016; Tai et al., 2016). Deletion mutants of FHV-1 including a gE-deficient mutant (gE-), gE/TK-double-deficient mutant (gE-TK-), gC-deficient mutant (gC-), and PK-deficient mutant (PK-), were constructed previously via two-step Red-mediated recombination, based on a full-length C27 bacterial artificial chromosome (BAC) clone (Tai et al., 2016). The Crandall Reese feline kidney (CRFK) cell line [ATCC, CCL-94] was used for virus propagation.

2.2. Animals and sample collections

Six domestic short hair (DSH) cats were used in this study. All cats were 3–6 months old, showing no respiratory signs, and were euthanized for reasons unrelated to the collection of FRECs. Cats were anesthetized with isoflurane induction, followed by euthanasia by intravenous injection of 85.9 mg/kg pentobarbital sodium. All procedures

and protocols performed in this study were done in accordance with the animal care guidelines of the Animal Care and Use Committee at Michigan State University, East Lansing, USA. Following euthanasia, tracheas were collected and FRECs were isolated and then stored in liquid nitrogen, as previously described (Nelli et al., 2016).

2.3. FREC infection with WT and FHV-1 mutants

For infections of FRECs, 2×10^6 FRECs per well were seeded in the top wells of an air-liquid interface culture system. FRECs were cultured for up to 3 weeks to get full differentiation and confluency, as previously described (Nelli et al., 2016). For virus infection, the experiments were split into two sets. The reason for this was the fact that gC- and PK- mutants could not be propagated to high enough titers for infection experiments at an MOI of 1. Thus, infection experiments using WT, gE-, and gE-TK- were performed at an MOI of 1, and a second set of experiments was performed using WT, gC-, and PK- at an MOI of 0.1. Tissues from three cats were used for each experiment as separate biological replicates. Negative controls (NC) were FRECs with mock inoculation using DMEM/F12 [Life Technologies, Grand Island, NY, USA]. Following inoculation, the cells were incubated for 1 h at 37℃, 5% CO_2. The inoculum was then removed, and the top and plate wells were washed twice with PBS. Fresh growth medium, consisting of DMEM/F12 supplemented with 2% Ultroser-G [Pall BioSepra, Cergy St Christophe, France], 100 IU/mL of penicillin, 100 µg/mL of streptomycin and 1.25 µg/ml of amphotericin B [Life Technologies, Carlsbad, CA, USA], was then added to the plate wells. FRECs were then collected at 1 h post-inoculation (hpi), or cultured until sampling, at 24, 48, 72, 96 hpi, respectively. Prior to collection of cells, images of each well containg cells were taken in IMC (integrated modulation contrast) mode using a Leica inverted microscope [Leica, Microsystems, Buffalo Grove, IL]. For sampling, FRECs were washed and incubated with 1 ml of Accumax Cell Dissociation Solution [Life Technologies] at 37℃ 5% CO_2 for 20 min, followed by vigorous pipetting to detach the cells. Cell suspensions were collected, aliquoted, and centrifuged to collect cell pellets. Forty percent of the cells from each well were lysed in 500 µl of Trizol [Life Technologies] for cytokine/chemokine mRNA expression analysis, 30% was used for viral titration assays and the remaining 30% was for viral genome quantification assays. The sub-natants collected from plate wells were also stored for analyses of extracellular viral growth and cytokine-associated protein assays.

2.4. Determination of viral growth kinetics

The titers of WT and mutants were determined based upon the presence of typical cytopathic effect (CPE) followed by the method of Reed and Muench (1938) to calculate the values of tissue culture infectious dose 50% ($TCID_{50}$). To obtain the titers of intracellular virus, the fractions of FREC pellets suspended in PBS were serially diluted in 10-fold and were incubated with 2×10^4 CRFK cells/well for 3–5 days until CPE was noted, as previously described (Tai et al., 2016). To perform analysis for titers of extracellular virus, sub-natant with 10-fold serial dilutions were used.

2.5. Real-time PCR for viral genome quantitation

DNA from FREC pellets, as well as sub-natants, were extracted using a QIAamp DNA blood mini kit according to the manufacturer's protocol [QIAGEN, Hilden, Germany]. Total DNA concentration was measured with a Nanodrop spectrophotometer [Thermo Fisher]. For samples from FREC pellets, 4 ng/µl of DNA was used for each real time PCR assay. For samples from sub-natants, approximately 1 ng/ul of DNA was used. Real time PCR was performed using a 7500 Fast Real-Time PCR System with 7500 Software v2.0.6 [Applied Biosystems by Life Technologies Corp., Austin, TX, USA], based on a previously published protocol that amplifies an 81-bp conserved fragment from the open reading frame of glycoprotein B (gB) gene of FHV-1 (Vogtlin et al., 2002). Primers and probe used were: Forward (5′-3′): AGA GGC TAA CGG ACC ATC GA; Reverse (5′-3′): GCC CGT GGT GGC TCT AAA C; Probe (5′-3′): FAM-TAT ATG TGT CCA CCA CCT TCA GGA TCT ACT GTC GT-BHQ-1. A total volume of 25 µl PCR amplification mix was prepared, comprising 12.5 µl of TaqMan Fast Universal PCR Master Mix no AmpErase UNG [Applied Biosystems by Life Technologies Corp.], 1 µl of each primer (400 nM), 0.1 µl of probe (80 nM), 5.4 µl of sterile water, and 5 µl of the extracted DNA. The PCR conditions were: 2 min at 50℃ and 30 s at 95℃, followed by 40 cycles consisting of denaturation at 95℃ for 15 s and annealing-elongation at 60℃ for 1 min. Each sample was analyzed in duplicate.

2.6. Cytokine/chemokine mRNA gene expression assays

Total RNA from the fractions of FREC pellets suspended in 500 µl of Trizol was isolated by using a RNeasy mini kit and RNase-Free DNase [QIAGEN] treatment according to the manufacturer's protocol. The RNA concentration was measured using a Nanodrop spectrophotometer [Thermo Fisher]. Reverse transcription was performed using a qScript cDNA SuperMix [Quantabio, Beverly, MA, USA], with 100 ng of sample RNA. Sequences of primers and probes for real time PCR for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), interferon alpha (IFNα), tumor necrosis factor alpha (TNFα), interleukin 1 beta (IL-1β), interleukin 10 (IL-10), transforming growth factor beta (TGFβ), granulocyte-macrophage colony-stimulating factor (GM-CSF), and Toll-like receptor 3 (TLR3) were as previously described (Nelli et al., 2016). A total volume of 20 µl PCR cocktail was prepared, using 10 µl of TaqMan Fast Universal PCR Master Mix no AmpErase UNG [Applied Biosystems], 0.8 µl of each primer (400 nM), 0.2 µl of probe (200 nM), 3.2 µl of sterile water, and 5 µl of cDNA. GAPDH was used as an endogenous control for each gene of interest (Nelli et al., 2016). Real time PCR was performed using the 7500 Fast Real-Time PCR System with 7500 Software v2.0.6 [Applied Biosystems] The cycling conditions were as follows: 95℃ for 30 s, followed by 40 cycles consisting of 95℃ for 3 s and 60℃ for 30 s. Each sample analysis was performed in duplicate.

2.7. Multiplex immunoassays for feline inflammatory cytokines/chemokines

A Milliplex™ MAP feline cytokine/chemokine magnetic bead kit [Cat. No. FCYTOMAG-20K-PMX, MilliporeSigma, Burlington, MA, USA] was used to quantify a total number of 19 inflammation and immune-associated analytes in FREC culture sub-natants at 96 hpi. Those included sFas, Fms-related tyrosine kinase 3 (Flt-3) ligand, GM-CSF, interferon gamma (IFNγ), IL-1β, IL-2, IL-4, IL-6, IL-8, IL-12p40, IL-13, IL-18, neutrophil chemoattractant (KC, also known as keratinocyte chemoattractant), monocyte chemoattractant protein 1 (MCP-1, also known as CCL2), platelet-derived growth factor two B subunits (PDGF-BB), stem cell factor (SCF), stromal cell-derived factor 1 (SDF-1), TNF-α, as well as chemokine (C-C motif) ligand 5 (CCL5, also known as regulated on activation, normal T cell expressed and secreted (RANTES)). Twenty-five µl of assay buffer and 25 µl of neat FREC culture sub-natants from the virus/mutant infection experiments were blended and assayed according to the manufacturer's instruction [MilliporeSigma]. The results were analyzed by a Luminex 200™ machine [MilliporeSigma]. Mean fluorescence intensity (MFI) was determined for each sample, followed by absolute quantification based on standard curve. Each sample analysis was performed in duplicate.

2.8. Enzyme-linked immunosorbent assays (ELISA) for feline interferon alpha (IFNα) and feline interleukin 10 (IL-10)

A feline interferon alpha ELISA kit [Cat. No. MBS280850, MyBioSource, San Diego, CA, USA] and a feline IL-10 ELISA kit [Sigma, Saint Louis, MO, USA] were used to quantify the level of IFNα and IL-10

in FREC culture sub-natants at 96 hpi. One hundred µl of sub-natants were used for the assays according to manufacturers' instructions. The results were analyzed using a SpectraMax Plus 384 Microplate Reader with SoftMax Pro software [VWR, Radnor, PA, USA]. Each sample analysis was performed in duplicate.

2.9. Statistical analysis

For viral titrations, log transformation was performed for normalization before statistical analysis. For viral genome quantification assays, raw Ct values were used for statistical analysis. For cytokine/chemokine mRNA gene expression assays, the Ct values from each gene of interest were normalized with GAPDH values (δCt), followed by normalization using the mean values of δCt at 1 hpi (starting point) as the calibrator to calculate the fold change using the $2^{-}\delta\delta^{Ct}$ method as previously described (Livak and Schmittgen, 2001). Fold changes were then used for statistical analysis. Two-way analysis of variance (ANOVA) with post hoc Tukey's multiple comparison tests [GraphPad Prism Software v6, San Diego, CA] was conducted to compare different groups at the same time point. A p value of < 0.05 was regarded as significant difference.

3. Results

3.1. Microscopic findings following infection of FREC with WT virus and deletion mutants

After 2 weeks of culture, FRECs from all cats were confluent and fully differentiated, as previously described (Nelli et al., 2016). In the first set of experiments, in which an MOI of 1 was used, plaques indicating CPE, cell detachment and cell lysis were first noted at 24 hpi in WT and gE- infection groups (indicated by arrows in Fig. 1). The plaques in gE- infected wells at 24 hpi (Fig. 1e) were smaller than those in WT infected wells (Fig. 1a). More than 70% FRECs were lysed after 48 hpi in WT and gE- groups (Fig. 1b-d, f-h). In contrast, only a few indistinct CPE plaques were noted in gE-TK- infected FRECs at 48, 72 and 96 hpi (indicated by arrows in Fig. 1j–l). In the second set of experiments, which was performed at an MOI of 0.1 with WT, gC-, and PK- mutants (Fig. 2), small CPE plaques were first noted at 24 hpi in WT and gC- groups (indicated by arrows inFig. 2 a, e). Distinct CPE was seen at 48 hpi and at all later time points in both WT and gC- infected FRECs (Fig. 2b-d, f-h). Progression of CPE was significantly delayed in FRECs infected with PK-, where indistinct CPE was observed in FRECs from 2 out of 3 cats at 72 hpi (Fig. 2k). At 96 hpi around 20%˜30% area of FRECs showed CPE in the PK- infected FRECs from all three cats (indicated by an arrow in Fig. 2l). Uninfected control FRECs (NC) in experiments 1 and 2 maintained full confluency throughout the experimental period (Figs. 1m, 2 m).

3.2. Endpoint titers and growth kinetics of mutants compared to WT inoculation

The growth curves for WT- inoculated FREC were similar and independent of the MOI used for inoculation. Titers were maximal at 48 hpi in both experiments at $10^{6.30}$ $TCID_{50}$/ml and $10^{6.47}$ $TCID_{50}$/ml, respectively (Fig. 3a-b). WT and gE- inoculated FRECs showed similar growth kinetics with maximal infectious titers at 48 hpi of $10^{6.30}$ $TCID_{50}$/ml and $10^{5.68}$ $TCID_{50}$/ml, respectively (Fig. 3a). After 48 hpi the infectious titers tapered off to $10^{4.63}$ $TCID_{50}$/ml in FRECs inoculated with WT and $10^{4.96}$ $TCID_{50}$/ml in FRECs with gE- at 96 hpi. FRECs inoculated with gE-TK- exhibited overall significantly lower titers at 24, 48, 72, and 96 hpi ($p < 0.05$) compared to WT or gE- inoculated FRECs. Maximal titers for gE-TK- inoculated FRECs were seen at 96 hpi at $10^{3.41}$ $TCID_{50}$/ml (Fig. 3a). For inoculations at an MOI of 0.1 WT and gC- inoculated FRECs showed similar growth kinetics at 24 hpi and then titers of gC- tapered off, while WT titers reached a plateau after 48 hpi (Fig. 3b). Maximum titers for WT and gC- inoculated FRECs were seen at 48 hpi in infected FRECs, at $10^{6.47}$ $TCID_{50}$/ml and $10^{4.19}$ $TCID_{50}$/ml, respectively. PK- inoculated FRECs showed delayed growth kinetics along with significantly lower titers before 48 hpi compared to WT and gC- inoculated FRECs ($p < 0.0001$). After 48 hpi, titers in PK- inoculated FRECs were lower than those of WT-inoculated FRECs ($p < 0.0001$), but not different from gC- inoculated FRECs (Fig. 3b). All samples from mock inoculated FRECs in both experiments were negative.

Extracellular viral titers in sub-natants are shown in Fig. 3c–d. Only

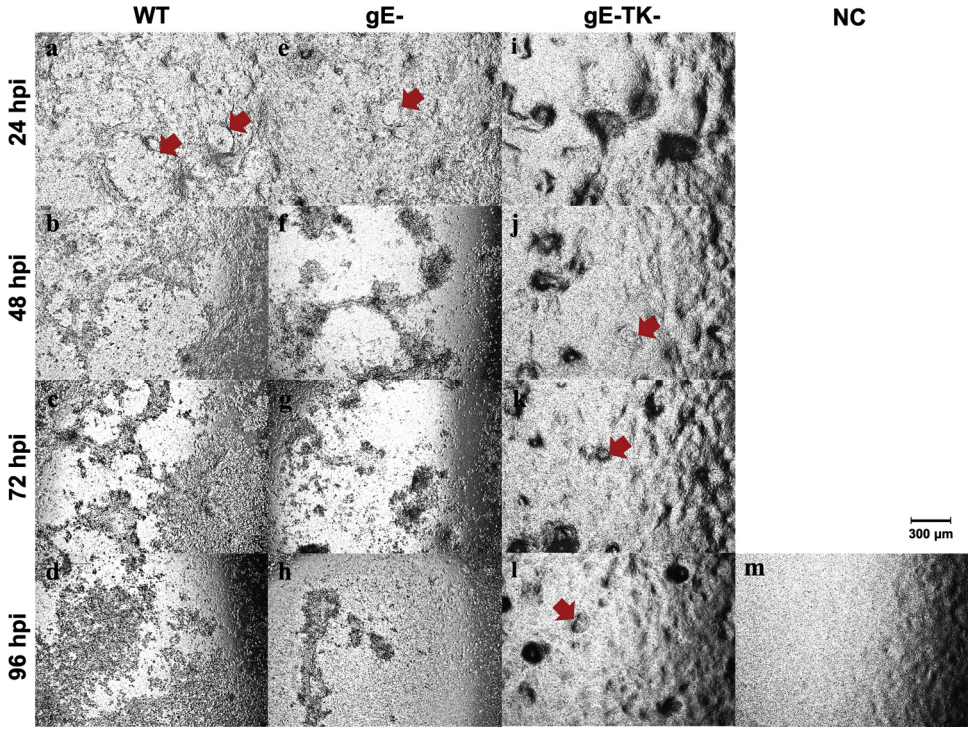


Fig. 1. Representative microscopic findings following inoculation of FRECs with FHV-1 or mutants at an MOI of 1. All images were taken at 40× magnification. A-d) FRECs infected with WT at 24, 48, 72, and 96 hpi; e-h) FRECs infected with gE- at 24, 48, 72, and 96 hpi; i-l) FRECs infected with gE-TK- at 24, 48, 72, and 96 hpi; m) mock inoculated FRECs at 96 hpi. Arrows indicate focal plaque formation due to cytopathic effects caused by viral infection. gE-: glycoprotein E-deletion mutant; gE-TK-: glycoprotein E and thymidine kinase-double-deletion mutant; hpi: hours post-inoculation; MOI: multiplicity of infection; NC: negative control (mock inoculation); WT: strain C27 wild type.

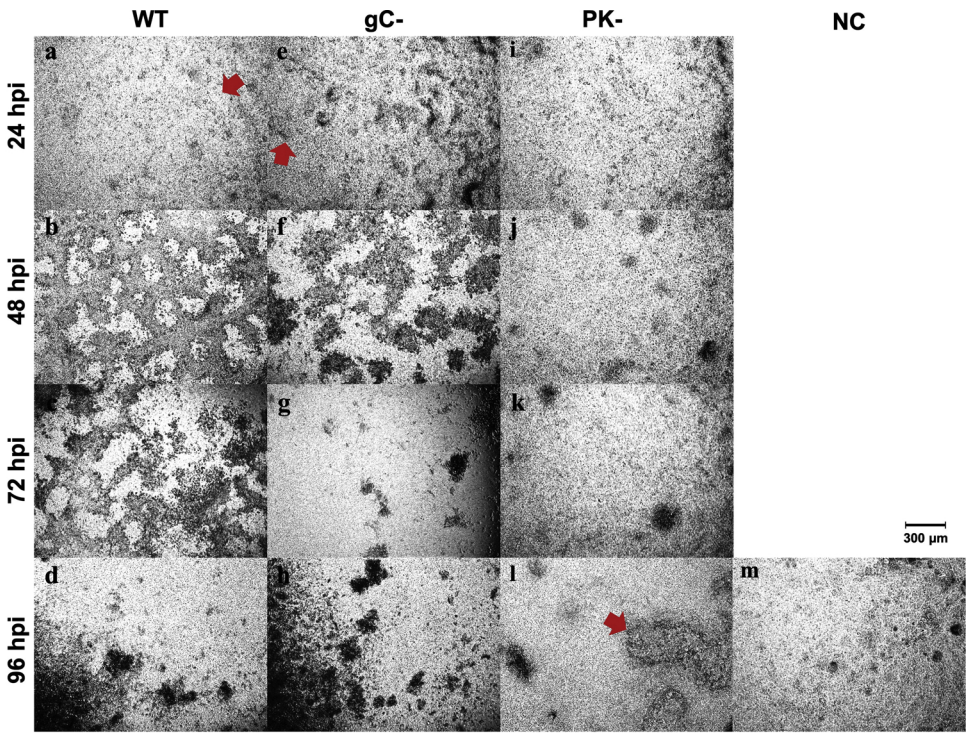


Fig. 2. Representative microscopic findings following inoculation of FRECs with FHV-1 or mutants at an MOI of 0.1. All images were taken at 40× magnification. A-d) FRECs infected with WT at 24, 48, 72, and 96 hpi; e-h) FRECs infected with gC- at 24, 48, 72, and 96 hpi; **i-l**) FRECs infected with PK- at 24, 48, 72, and 96 hpi; m) mock inoculated FRECs at 96 hpi. Arrows indicate focal plaque formation due to cytopathic effects caused by viral infection. gC-: glycoprotein C-deletion mutant; hpi: hours post-inoculation; MOI: multiplicity of infection; NC: negative control (mock inoculation); PK-: serine/threonine protein kinase-deletion mutant; WT: strain C27 wild type.

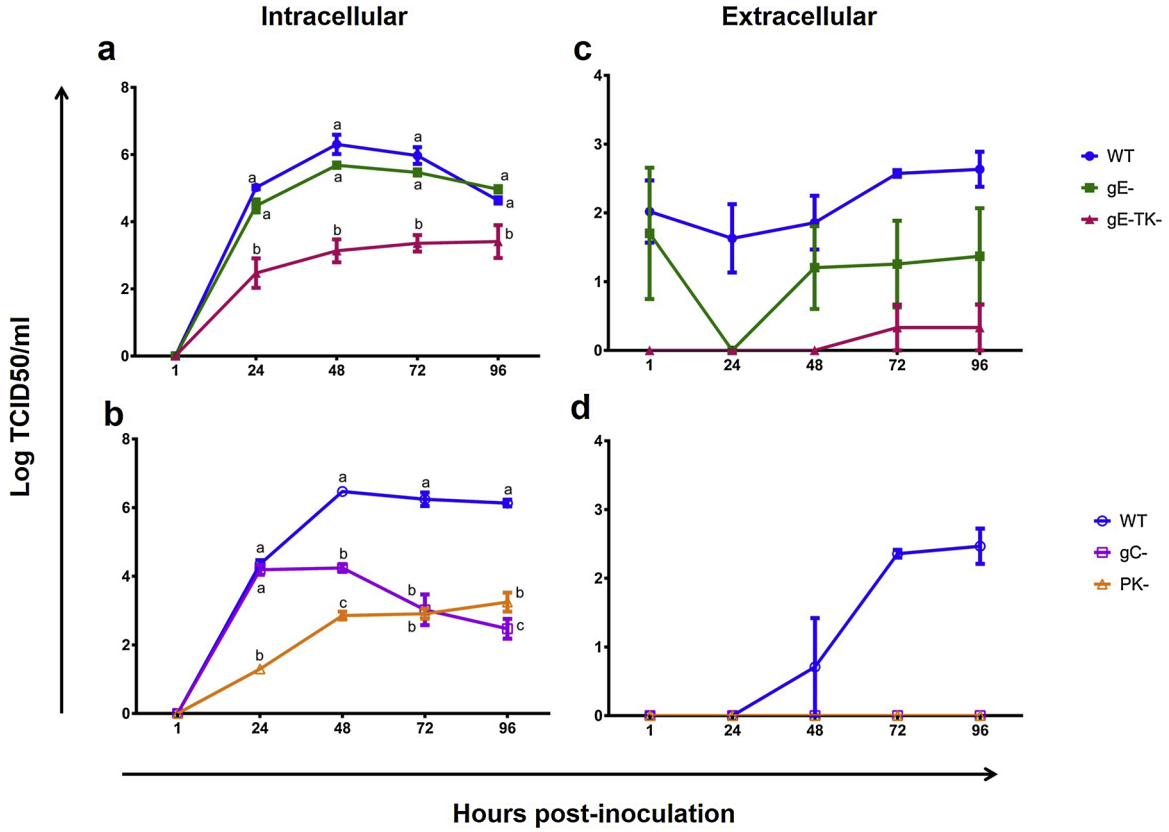


Fig. 3. Viral growth kinetics. Viral titers (log $TCID_{50}$/ml) were measured in cell pellet lysates (intracellular virus) of feline respiratory epithelial cells inoculated at an MOI of 1 (a), or an MOI of 0.1 (b), in sub-natants (extracellular virus) of feline respiratory epithelial cells inoculated at an MOI of 1 (**c**), or sub-natant infected at an MOI of 0.1 (d). Symbols at each time point represent the mean value of $TCID_{50}$/ml measured from 3 cats, and the error bar represents the standard error of the mean. [a,b,c] Different letters indicate a significant difference between infection groups at the same time point ($p < 0.05$). gC-: glycoprotein C-deletion mutant; gE-: glycoprotein E-deletion mutant; gE-TK-: glycoprotein E and thymidine kinase-double-deletion mutant; MOI: multiplicity of infection; PK-: serine/threonine protein kinase-deletion mutant; $TCID_{50}$: tissue culture infectious dose 50%; WT: strain C27 wild type.

titers in sub-natants of WT inoculated FRECs could be consistently determined at all time points post-inoculation, with maximal titers at 96 hpi ($10^{2.63}$ $TCID_{50}$/ml and $10^{2.47}$ $TCID_{50}$/ml, respectively) (Fig. 3c–d). Extracellular virus titers in sub-natants from gE- inoculated FRECs fluctuated but were always below 10^2 $TCID_{50}$/ml, whereas titers of the gE-TK- mutant in sub-natants could only be detected at 72 hpi and 96 hpi (Fig. 3c). Infectious virus was not detected in sub-natants from gC- and PK- inoculated FRECs at any of the sampling points (Fig. 3d).

3.3. Viral genome quantification mirrored viral titration results

The shape of the viral genome quantification curves and Ct values generated with samples from FRECs inoculated with WT at an MOI of 1 were similar to those inoculated at an MOI of 0.1 (Fig. 4). With an MOI of 1, viral DNA kinetic curves for WT and gE- mutant were similar at all time points post-inoculations, while the DNA kinetic curve generated from FRECs infected with gE-TK- changed more gradually and overall Ct values were significantly lower than those following WT and gE- infections ($p < 0.0001$) (Fig. 4a). For inoculation with an MOI of 0.1, the amount of FHV-1 DNA in FRECs inoculated with gC- was similar to that with WT, while the amount of viral DNA in FRECs inoculated with PK- was lower compared to WT and gC- inoculations, starting at 24 h post infection ($p < 0.0001$) (Fig. 4b). No viral genome was detected in mock inoculated FRECs.

Extracellular levels of viral DNA (Fig. 4c-d) showed similar patterns compared to intracellular FHV-1 DNA levels over time. DNA levels between FRECs inoculated with WT at an MOI of 1 were similar to levels seen following inoculation with an MOI of 0.1, with maximal amounts of FHV-1 DNA at 96 hpi in both experiments (Fig. 4c-d). Growth curves for sub-natants from WT and gE- inoculated FRECs were similar at all time points post-inoculation, while the viral DNA detected in sub-natants from FRECs infected with gE-TK- were significantly lower than those following WT and gE- infections ($p < 0.0001$) (Fig. 4c). The amount of FHV-1 DNA in sub-natants of FRECs inoculated with gC- was similar to that obtained with WT, while the viral DNA in sub-natants of FRECs infected with PK- was decreased compared to WT and FRECs inoculated with gC- starting at 48 hpi, ($p < 0.05$) (Fig. 4d). Viral DNA was not detected in sub-natants from mock inoculated FRECs.

3.4. FHV-1 virulence-associated genes modulate cytokine mRNA expression in FRECs

Cytokine mRNA expression was analyzed at 24, 48, and 72 hpi for all treatment groups. While trends were similar at all time points, differences were most significant at 72 hpi, thus only data at 72 hpi is shown in Figs. 5 and 6. Mock inoculated FRECs were regarded as the control (NC) and were applied as the baseline for interpretation of cytokine regulation. The NC was only analyzed at 1 hpi and 96 hpi in Fig. 5 due to restricted cell amounts available in the first set of experiment, whereas NC was obtained at all time points (1, 24, 48, 72, and 96 hpi) in the second set of experiment Fig. 6. IFNα gene expression was significantly up-regulated in response to WT infection compared to NC, both at an MOI of 0.1 and 1 ($p = 0.0395$ at an MOI of 1 and $p = 0.0009$ at an MOI of 0.1) (Figs. 5a and 6 a). Similar to WT infection, inoculation with gE- and gC- significantly up-regulated IFNα gene

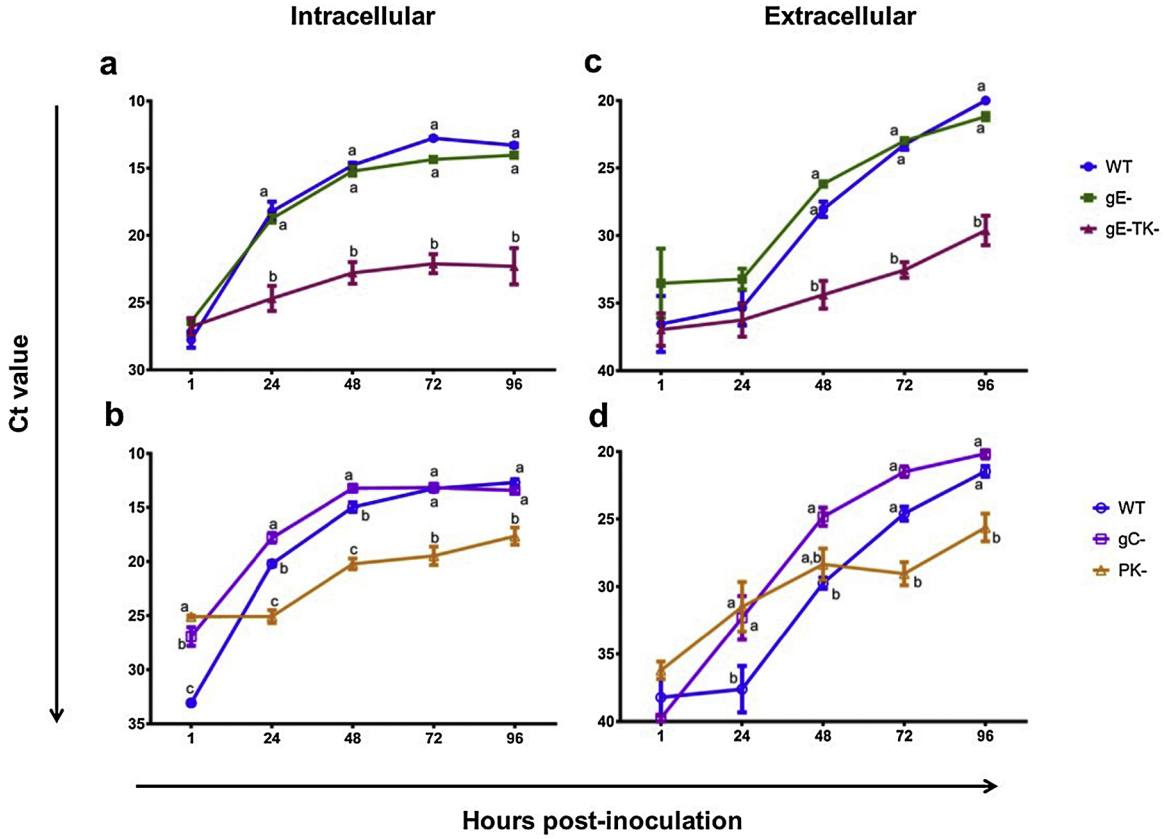


Fig. 4. Measurement of viral DNA kinetics. Ct values were determined by real-time PCR in cell pellet DNA extracts (intracellular virus) of feline respiratory epithelial cells inoculated at an MOI of 1 (a), or an MOI of 0.1 (b), and in extracts of sub-natants (extracellular virus) of feline respiratory epithelial cells inoculated at an MOI of 1 (c) or an MOI of 0.1 (d). Symbols for at each time point represent the mean Ct value of samples from 3 cats, and the error bar represented the standard error of the mean. [a,b,c] Different letters indicate significant differences between groups at the same time point ($p < 0.05$). gC-: glycoprotein C-deletion mutant; gE-: glycoprotein E-deletion mutant; gE-TK-: glycoprotein E and thymidine kinase-double-deletion mutant; MOI: multiplicity of infection; PCR: polymerase chain reaction; PK-: serine/threonine protein kinase-deletion mutant; WT: strain C27 wild type.

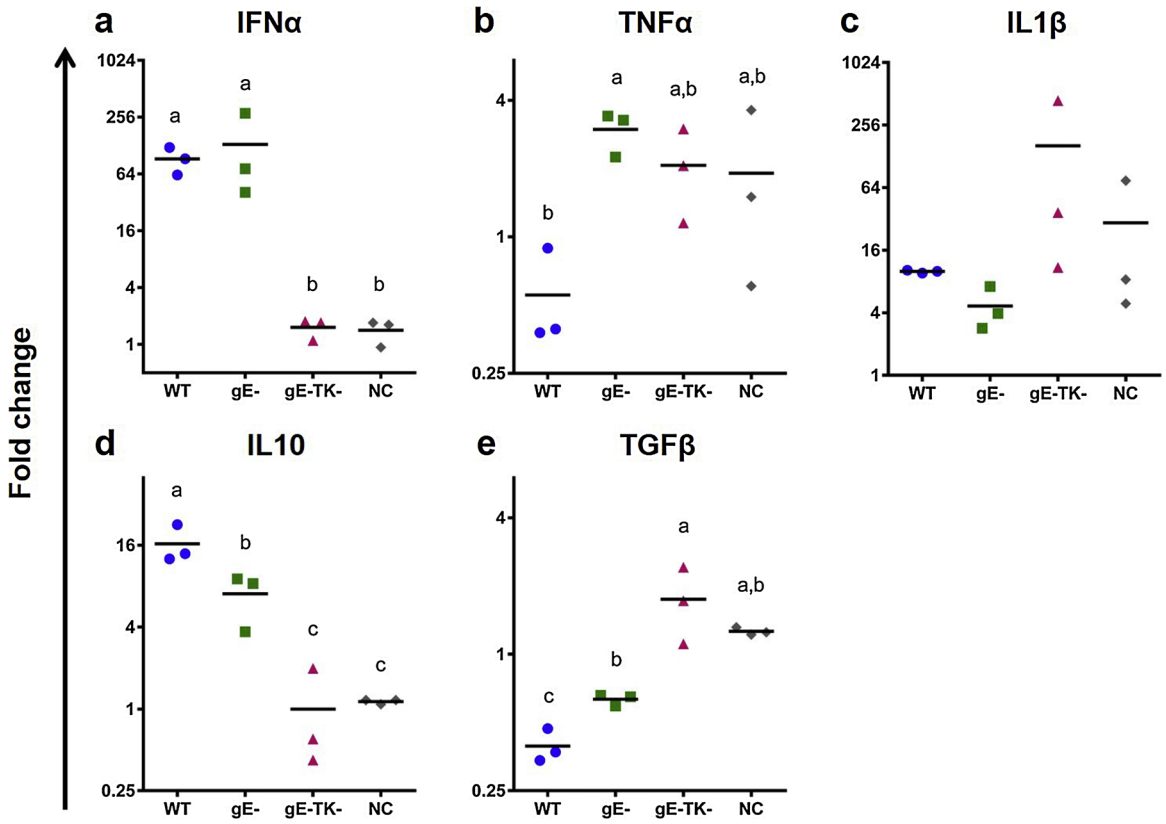


Fig. 5. Cytokine gene expression in FRECs inoculated at an MOI of 1. The levels of mRNA of IFNα (a) TNFα (b), IL-1β (c), IL-10 (d), and TGFβ (e) were determined by reverse transcription real-time PCR at 72 hpi in cell lysates from feline respiratory epithelial cells infected with WT or mutants at an MOI of 1. Fold change was calculated for each sample using the $2^{-}\delta\delta^{Ct}$ method. The horizontal dashed line in each group represents the mean value. ^{a,b,c} Different letters indicate significant differences ($p < 0.05$) in multiple comparisons between groups for each cytokine. gE-: glycoprotein E-deletion mutant; gE-TK-: glycoprotein E and thymidine kinase-double-deletion mutant; IFNα: interferon alpha; IL-1β: interleukin 1 beta; IL-10: interleukin 10; MOI: multiplicity of infection; NC: negative control at 96 hpi (mock inoculation); PCR: polymerase chain reaction; TGFβ: transforming growth factor beta; TNFα: tumor growth factor alpha; WT: strain C27 wild type.

expression in FRECs at 72 hpi compared to mock inoculated FRECs (NC) ($p = 0.0021$ and $p = 0.0001$, respectively), while in gE-TK- or PK- deletion mutants this up-regulation of IFNα gene expression was not observed. In addition to inducing INF-α, gC- mutant also enhanced TNFα and IL-1β gene expression in FRECs at an MOI of 0.1 ($p = 0.0377$ and $p = 0.0072$, respectively), although the effect of WT on TNFα and IL-1β expression were not significant ($p = 0.2770$ and $p = 0.3476$, respectively) (Fig. 6b–c). Inoculation with other mutants did not significantly influence TNFα or IL-1β gene expression ($p > 0.05$) (Fig. 5b, c, 6 b, c).

Further, WT inoculation of FRECs significantly up-regulated the expression of the regulatory cytokine IL-10 at an MOI of 1 and 0.1 ($p < 0.0001$ and $p = 0.0073$, respectively) (Figs. 5d and 6 d). Inoculation of FRECs with the gE- mutant also induced a significant up-regulation of IL-10 gene expression compared to FRECs with NC ($p = 0.0034$), though the up-regulation by gE- was significantly lower than that by WT ($p < 0.0001$). In contrast, following inoculation with gC, gE&TK or PK deletion mutants this IL-10 mRNA up-regulation was not observed ($p > 0.05$). Finally, WT inoculation of FRECs significantly down-regulated TGFβ gene expression in FRECs compared to FRECs with NC ($p = 0.05$ and $p < 0.0001$, respectively) (Figs. 5e and 6 e). This down-regulation of TGFβ gene expression was also noted in FRECs inoculated with gC- or PK- at an MOI of 0.1 compared to FRECs with NC ($p = 0.0002$ and $p = 0.0024$, respectively), whereas the down-regulation of TGFβ was noted but not significant in FRECs inoculated with gE- ($p = 0.2171$). In contrast, deletion of gE&TK did not result in a dowregulation of the TGFβ gene expression (Fig. 5e). No significant differences in GM-CSF or TLR3 gene expression was found between treatments at all time points post-inoculation (data not shown).

3.5. Deletion of FHV-1 virulence-associated genes modulates chemokines in FRECs

Cell culture sub-natants of FRECs were collected at 96 hpi and analyzed by Milliplex™ immunoassay and conventional ELISAs. Sub-natants collected from mock inoculated FRECs at 96 hpi were regarded as the control (NC) and the baseline for multiple comparison tests. Among the analytes measured, IL-8, IL-12p40, RANTES, and KC were detected and presented (Fig. 7), while the others were below detection limits and therefore the differences between groups could not be determined. Inoculation of FRECs with WT and gE- at an MOI of 1 showed a trend for IL-8 reduction in FRECs compared to NC inoculation, although differences were not significant ($p = 0.1722$ and $p = 0.5005$, respectively) (Fig. 7a), whereas WT and gC- inoculation in FRECs at an MOI of 0.1 demonstrated a significant reduction in IL-8 secretion compared to NC inoculation ($p = 0.0007$ and $p = 0.0008$, respectively) (Fig. 7e). Deletion of gE&TK or PK blocked this downregulation of the IL-8 response seen after WT inoculation to levels comparable of mock-inoculated FRECs, suggesting that TK and PK might mediate an inhibition of IL-8 secretion in FRECs. Similar trends were observed for KC and IL-12p40 secretion (Fig. 7b, d, f, h), pointing to a role of TK and PK in modulating KC and IL-12p40 secretion in FRECs, although a significant down-modulation of IL-12p40 was only observed at an MOI of 0.1 for WT inoculated FRECS (Fig. 7h). Further, inoculation of FRECs with the gE- mutant up-regulated secretion of RANTES compared to mock inoculated FRECs ($p = 0.0083$) (Fig. 7c).

Secretion of IFNα and IL-10 were determined by conventional ELISAs, and they were below the detection limit in all groups (data not shown).

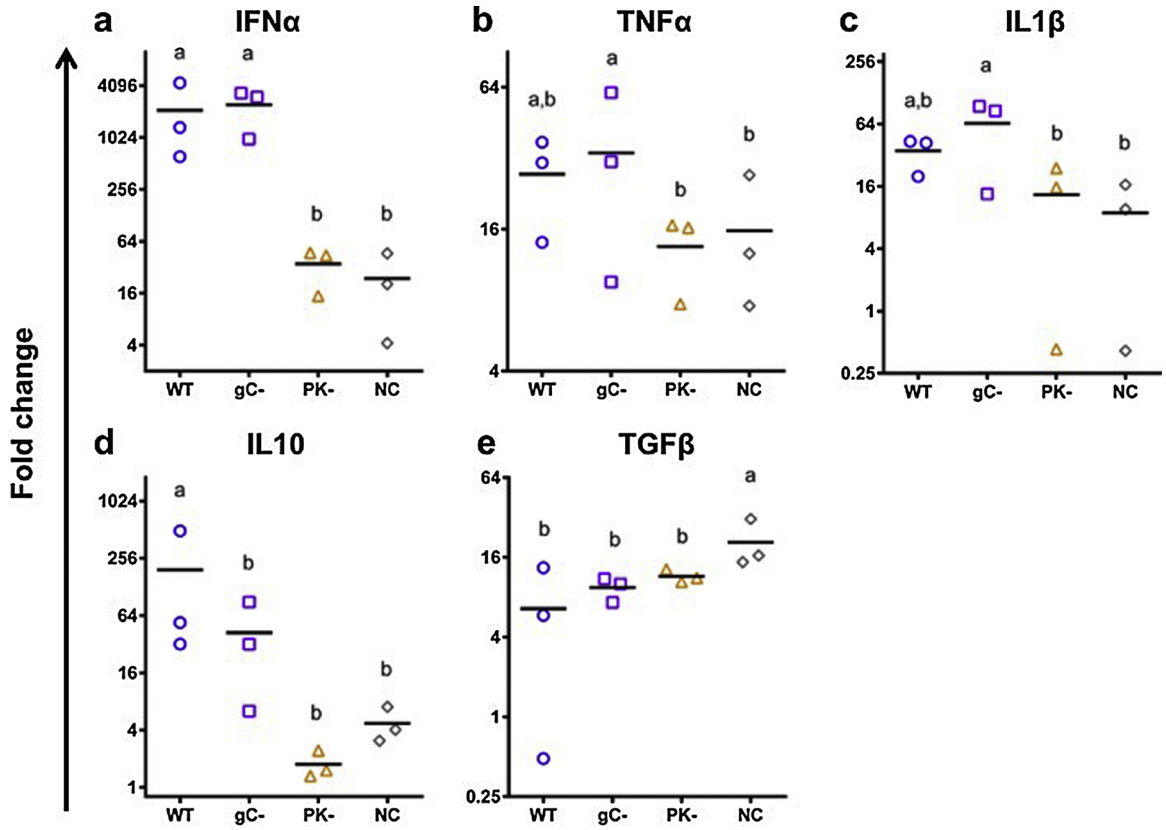
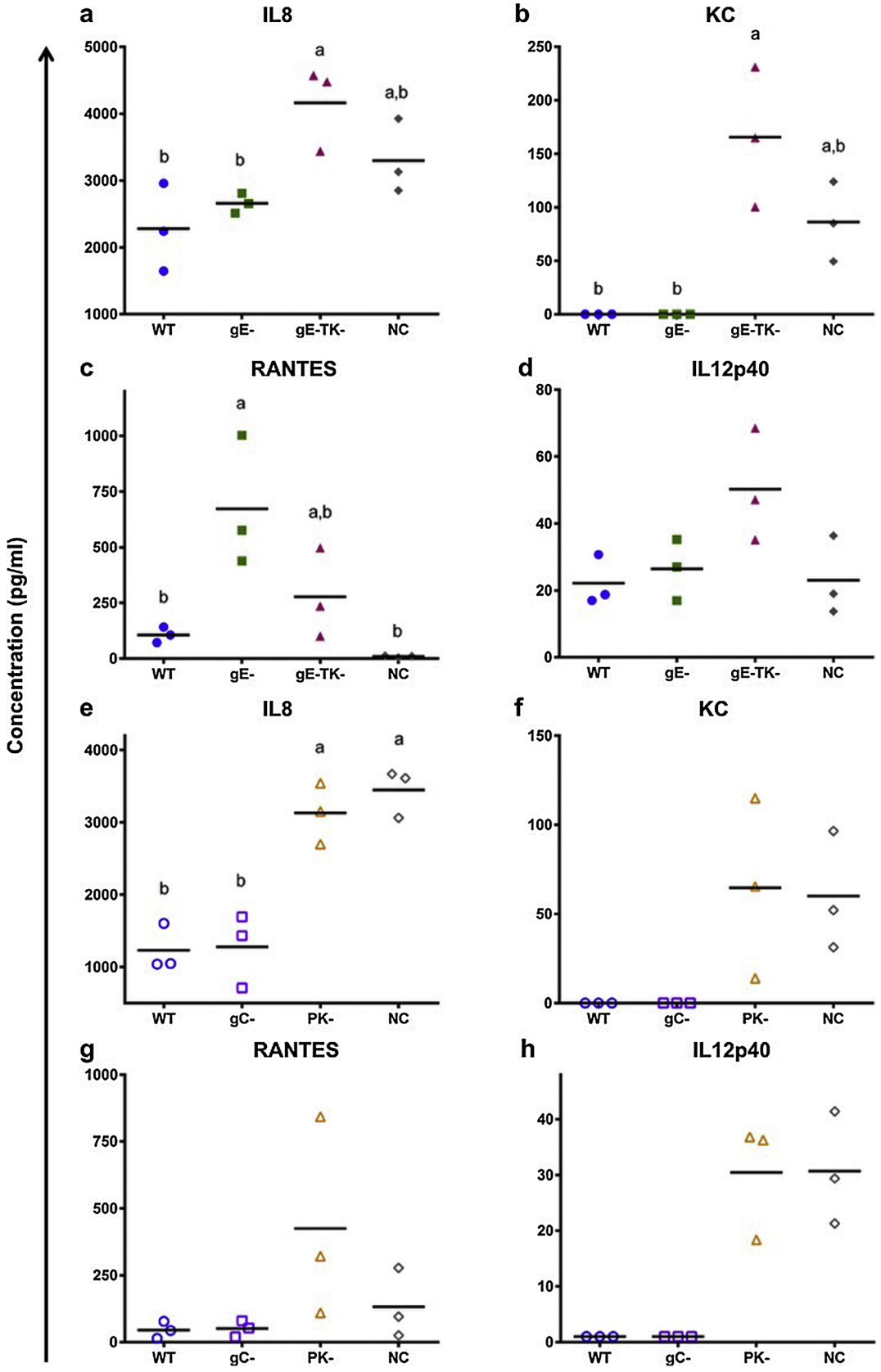


Fig. 6. Cytokine gene expression in FRECs inoculated at an MOI of 0.1. The levels of mRNA of IFNα (a) TNFα (b), IL-1β (**c**), IL-10 (d), and TGFβ (e), were determined by reverse transcription real-time PCR at 72 hpi in cell lysates from feline respiratory epithelial cells infected with WT or mutants at an MOI of 0.1. Fold change was calculated for each sample using the $2^{-}\delta\delta^{Ct}$ method. The horizontal dashed line in each group represents the mean value. [a,b] Different letters indicate significant differences ($p < 0.05$) in multiple comparisons between groups in each cytokine. gC-: glycoprotein C-deletion mutant; IFNα: interferon alpha; IL-1β: interleukin 1 beta; IL-10: interleukin 10; MOI: multiplicity of infection; NC: negative control at 72 hpi (mock inoculation); PCR: polymerase chain reaction; PK-: serine/threonine protein kinase-deletion mutant; TGFβ: transforming growth factor beta; TNFα: tumor growth factor alpha; WT: strain C27 wild type.

4. Discussion

FHV-1 mutants with deletions of gC, gE, gE and TK, or PK, were previously engineered and characterized by our group in CRFK cells (Tai et al., 2016). In addition, the development of FREC cultures and their usefulness as an *in vitro* model to study FHV-1 pathogenesis were described previously by us (Nelli et al., 2016). In the present study, growth characteristics and innate immune responses induced by the mutants were characterized in FRECs. We observed several differences between our previous study of infectivity of the mutants in CRFK cells (Tai et al., 2016) and the present study in FRECs. In the previous study performed by our group, infection of CRFK cells with the gC- at an MOI of 0.01 or 3 resulted in viral titers that were significantly lower than those obtained with WT or other mutants, suggesting that gC is important for viral infectivity by facilitating viral entry, including initial attachment and penetration similar to what has been described for HSV-1 (Herold et al., 1991). In the present study, we show that gC- infection in FRECs at an MOI of 0.1 results in similar infectious virus titers compared to WT infection at an early stage (24 hpi) and then tapers off by 48 hpi, resulting in lower total endpoint titers compared to WT infection. This implies that the impaired infectivity of gC- is more complex then suggested by experiments in monolayer cell lines, when a primary pseudo-stratified respiratory cell culture system, resembling the natural feline airway and possessing innate immune function, is used. Similar findings were previously presented in an EHV-1 study, where an EHV-1 gC-deletion mutant demonstrated faster and increased penetration into equine primary epithelial cells isolated from fresh tissues compared to results in an equine dermal cell line, though the mutant always showed lower penetration rate than WT regardless of the cell type used for infection (Osterrieder, 1999). Heparan sulfate proteoglycans expressed on the cell surface have been described to be the main receptors recognized by HSV-1 gC (Herold et al., 1991). However, other co-receptors on the surface of target cells also play a role in viral entry, including but not limited to the herpes virus entry mediator (HVEM, tumor necrosis factor receptor superfamily member 14), nectin-1 or nectin-2, and 3-O-S-site of heparan sulfate. These receptors are recognized by several glycoproteins of herpesviruses such as gB, gD, gH, and gL (Eberle et al., 1995; Montgomery et al., 1996; Shukla et al., 1999). We speculate that in FRECs, which are the target cells for FHV-1 replication *in vivo*, there may be a number of surface receptors that can compensate for the absence of gC-mediated attachment at an early stage of FHV-1 infection as has been described for other alpha herpesviruses (Herold et al., 1994; Maeda et al., 1997; Spear et al., 2000) and in contrast to CRFK cells, which are not primary target cells. Further functional assays elucidating the function of FHV-1 gC for viral entry in FRECs compared to other cell lines are clearly required to confirm this hypothesis. At 48 hpi the titer of the gC- mutant in infected FRECs was significantly lower compared to WT infection (Fig. 3b), while quantitative PCR data showed DNA amounts of the WT and gC- to be similar (Fig. 4b). Osterrieder et al. (1999) proposed that the deletion of EHV-1 gC may affect viral assembly of late gene products and thus impede viral egress. Functional assays for gC to study viral entry and egress are required to strengthen this hypothesis.

Glycoprotein E-TK- and PK- mutants showed similar growth kinetics in FRECs and were similar to those previously reported by us in CRFK cells (Tai et al., 2016). In both cell types, increases in viral production were delayed compared to WT. However, in a previous experiment performed in our laboratory, endpoint titers in CRFKs were over 10^4



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$TCID_{50}$/ml after 72 hpi (Tai et al., 2016), while infection of FRECs with either gE-TK- or PK- were significantly lower titers below 10^4 $TCID_{50}$/ml (Fig. 3a–b). Viral genome quantification data in the present study further verified the decreased viral DNA synthesis following infection with gE-TK- and PK- in FRECs compared to infection with WT. Previous studies, using either pseudorabies virus (PRV) or human herpes simplex

Fig. 7. Chemokine secretion profile. A Milliplex™ immunoassay was used to determine the protein levels of subsets of chemokines, including IL-8 (a, e), KC (b, f), RANTES (c, g), and IL-12p40 (d, h), in sub-natant samples collected at 96 hpi from feline respiratory epithelial cells infected at an MOI of 1 (a-d) or an MOI of 0.1 (e-h). The horizontal dashed line in each group represents the mean value. For KC assays, the WT and gE- mutant groups (b) as well as the WT and the gC- groups (f) were below the detection limit. In the IL-12p40 assay, the WT and gC- groups (h) were below the detection limit. [a,b] Different letters indicate significant differences ($p < 0.05$) in multiple comparisons between groups in each cytokine. gC-: glycoprotein C-deletion mutant; gE-: glycoprotein E-deletion mutant; gE-TK-: glycoprotein E and thymidine kinase-double-deletion mutant; IL-8: interleukin 8; KC: keratinocyte chemoattractant/ neutrophil chemoattractant; IL-12p40: interleukin 12 subunit beta; MOI: multiplicity of infection; NC: negative control at 96 hpi (mock inoculation); PK-: serine/threonine protein kinase-deletion mutant; RANTES: regulated on activation, normal T cell expressed and secreted; WT: strain C27 wild type.

virus-1 (HSV-1), showed that TK primarily affects deoxynucleotide metabolism and only mediates viral replication in non-dividing cells such as neurons, which may explain the differences in titers between different cell types (Kimman et al., 1994; Wilcox et al., 1992). Overall, we found that infection of FRECs with FHV-1 gC-, PK-, and gE-TK-, but not gE-, resulted in reduced viral replication, which was not observed to the same degree in CRFK cells in our previous study (Tai et al., 2016). Our data also demonstrated lower infectious titers of WT in FRECs when compared to the titers of WT in CRFK cells. Moreover, significant differences were found between intracellular and extracellular infectious virus titers, ranging from 1,000-fold to 10,000-fold at each time point post-inoculation (Fig. 3c–d) In contrast, in earlier studies by our group, intra- and extra-cellular titers were comparable to each other in CRFK cells (Tai et al., 2016). These findings highlight the importance of studying FHV-1 in its primary target cells that resemble the natural airway epithelium, are polarized and possess a functional immune defense.

FRECs inoculated with WT, gE- and gC- mutants up-regulated IFNα gene expression compared to mock inoculated or gE-TK- and PK- inoculated FRECs. This is in line with a previous study where equine respiratory epithelial cells (ERECs) inoculated with EHV-1 also showed a significant up-regulation of IFNα expression and IFNβ expression, which was further enhanced by deletion of ORF1 (pUL56) (Soboll Hussey et al., 2014). Previous studies using HSV-1 and HSV-2 showed that the Us3-encoded PK, which is conserved in various alphaherpesviruses (Jacob et al., 2011), modulates interferon signaling by trapping the expression of TLR3 and IFN receptors, or suppressing activation of regulatory transcription factor 3 gene (IRF3) (Lewandowski et al., 1994; Peri et al., 2008; Wang et al., 2013). In our study, deletion of PK blocked the upregulation of IFNα gene expression in FRECs seen in WT, gE- and gC- inoculated FRECs. This confirms a role of Us3 in the regulation of interferon responses as described previously but is different from the upregulation of the IFNβ expression observed following inoculation with an Us3 deletion mutant by Tian et al. (Tian et al., 2018). However our study looked at IFNα not IFNβ, examined cytokine mRNA expression at a much later time point than Tian et al, who found an upregulation at 4 hip that was gone by 12 hpi, and used primary FRECs in contrast to a monolayer cell line. While it is clear that modulation of the interferon response is an important component of alphaherpesvirus immune modulation, further investigation into the modulation of the interferon pathway by FHV-1 is clearly warranted. Additionally, there is evidence that immunity may be differentially regulated depending on target cell type. This has been shown in a previous EHV-1 study, where type I interferon secretion as well as other inflammatory cytokines were regulated distinctively in ERECs and in peripheral mononuclear cells (Soboll Hussey et al., 2014). The same phenomenon has been shown *in vivo* where interferon levels differed significantly between the blood, the cerebrospinal fluid (CSF) and the nasal secretion of horses infected with EHV-1 (Holz et al., 2017).

Another immunomodulatory mechanism often targeted by herpesviruses is the expression of modulatory cytokines including IL-10 and TGFβ. Our data support previous findings in humans (human simplex virus-1, human herpesvirus-4 (HHV-4, or Epstein-Barr virus), HHV-5 (human cytomegalovirus), HHV-6, and HHV-8 (Kaposi's sarcoma-associated herpesvirus)) and horses (EHV-1) showing that FHV-1 modulates the expression of IL-10 and TGFβ (Arena et al., 1999; Gupta et al., 2006; Hussey et al., 2014; Kotenko et al., 2000; Liu et al., 2012; Moore et al., 1990; Nelli et al., 2016). Deletion of gC, gE, PK, and TK all appeared to affect gene expression of IL-10 and TGFβ in FRECs to various degrees compared to WT infection (Figs. 5d–e, 6 d–e). We show a significant up-regulation of IL-10 and down-regulation of TGFβ in FRECs following WT inoculation and similar trends were noted following inoculation of FRECs with gE- and gC-. In contrast, FRECs inoculated with gE-TK- and PK- did not show up-regulation of IL-10, and FRECs inoculated with gE-TK- did not show down-regulation of TGFβ. TGFβ is a biomolecule that regulates the cell cycle and cell apoptosis. Multiple herpesviral genes have shown to target expression of TGFβ including the latency-associated transcript (LAT) from HSV-1 that has been shown to inhibit apoptosis by down-regulating TGFβ (Gupta et al., 2006). In addition, Us3-encoded PK of HSV-1, HSV-2, PRV, and Marek's disease's virus (MDV, gallid herpesvirus type 2) have been shown to have anti-apoptotic characteristics, both *in vivo* or *in vitro* (Asano et al., 1999; Geenen et al., 2005; Leopardi et al., 1997; Schumacher et al., 2008), potentially through a blockage of caspase 3 activities (Benetti and Roizman, 2007). In the present study we show that FHV-1 gC, gE, and TK modulate TGFβ expression in FRECs, but whether such modulation connects to the FHV-1 anti-apoptosis in FRECs and what pathway in the apoptosis mechanism is compromised by these genes need more investigations.

In addition to modulating antiviral and modulatory cytokines, herpesviruses are known to also target chemokines and chemoattraction of immune cells (Van de Walle et al., 2008a). We found that FHV-1 suppressed a number of chemokines including IL-8 (CXCL8) and KC (CXCL1), which are chemoattractants for T cells and neutrophils (Fig. 7a–b, e–f). Deletion of gE&TK or PK restored secretion of IL-8 and KC by FRECs, suggesting the regulation capacity of chemotaxis of FHV-1 genes. Similar results were observed for EHV-1, which is another alphaherpesvirus, revealing that ERECs inoculated with EHV-1 WT showed a lack of IL-8 and MCP-1 secretion, and such influence could be blocked when pUL56 was deleted (Soboll Hussey et al., 2014). In chickens, MDV sheds a virkine called viral IL-8 (vIL-8) to mimic and consequently modulate the chemokine networking in the host (Liu et al., 1999). In addition to the modulation of IL-8 and KC, we show that deletion of gE in FHV-1 increased secretion of RANTES in FRECs (Fig. 7c). The chemokine RANTES (CCL5) is highly associated with the migration and activation of CD8 + T cells as well as other leukocytes via IFNγ signaling, which is a critical mechanism for eliminating herpes virus infection. Last but not least, inoculation of FRECs with WT and gC- seemed to hamper the secretion of IL-12p40, which is a chemoattractant for macrophages and dendritic cells (Fig. 7h). Together, these findings suggest the FHV-1, like other alphaherpesviruses, selectively modulates secretion of chemokines at the respiratory epithelium by targeting multiple chemokines. Viral proteins including gE combined with TK and PK appear to play a role in this. The well-known viral protein that performed as a viral chemokine binding protein (vCKBP) to obstruct chemokine activites is glycoprotein G, which has been described in several herpes virus species such as EHV-1, EHV-3, bovine herpesvirus-1, MDV, infectious laryngotracheitis virus (ILTV, gallid herpesvirus-1), and canine herpesvirus-1 (Van de Walle et al., 2008a, b), and the same is likely to be true for FHV-1. Glyprotein G in FHV-1 displayed a high affinity for numerous chemokines including KC, IL-8, MIP-1α (CCL3), and RANTES, and subsequently disrupted the functions

thereof (Costes et al., 2005). Chemotaxis assays should be performed to contentiously investigate the role of gE, TK, and PK in the regulation of chemotaxis.

While FRECs are an ideal system to screen candidate viruses or mutants for viral replication kinetics and immune modulatory properties, ultimately it will be necessary to fully assess the safety and efficacy of the most promising candidates *in vivo*. Pseudorabies virus infection of pigs with double-deletion mutants (gE-PK- and gE-TK-) showed reduced viral nasal shedding. Also, pigs experimentally inoculated with a PK-mutant showed full protection, with no viral shedding following WT challenge (Kimman et al., 1994). Initial studies by our group showed that a FHV-1 mutant with a gE-deletion/partial gI-deletion reduced titers in CRFK cells (Sussman et al., 1995) and induced protective immunity *in vivo* (Kruger et al., 1996). Moreover, oronasal administration of this mutant showed decreased virulence compared to a commercial vaccine strain (Kruger et al., 1996). Another mutant, C7301dlTK, was constructed using the Japanese strain C7301 as the parent strain and contained a 450 bp deletion in the TK gene (Yokoyama et al., 1995). In clinical trials, the virulence of C7301dlTK was reduced and infection with this mutant provided partial protection against WT challenge, although challenge virus could still be recovered from eyes, nose, and the oral cavity post-challenge (Yokoyama et al., 1996). Finally, a recombinant which contained an insertion of the envelope (env) or gag protein genes of feline leukemia virus (FeLV) within the TK locus also showed protection against WT challenge (Cole et al., 1990; Wardley et al., 1992).

In summary, in the present study we performed a thorough comparison of a candidate vaccine panel using FRECs, a culture system that simulates the natural airway epithelium. Our study confirmed previous data (Nelli et al., 2016) demonstrating that FRECs are advanced tools to study FHV-1 pathogenesis and immunity compared to monolayer cell lines prior to *in vivo* studies. via this system, we found that single deletions of gC or gE were not sufficient to reduce replication of FHV-1 in FRECs. In contrast, gE and TK double deletion and PK deletion decreased replication and also impaired immune modulatory properties of the virus. The double-deletion mutant, gE-TK-, showed significantly reduced replication and CPE in FRECs, and they are logical candidates for further *in vivo* investigation to study induction of immunity and safety in the host.

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