CHARACTERIZATION OF FELINE HERPESVIRUS-1 DELETION MUTANTS AND THE INNATE IMMUNE RESPONSES

By

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ABSTRACT

CHARACTERIZATION OF FELINE HERPESVIRUS-1
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Feline herpesvirus-1 (FHV-1) is an important primary viral pathogen of cats worldwide. The clinical signs in cats include upper respiratory and ocular disease. As with all alphaherpesviruses, induction of lifelong latency with periodic reactivation is also an important part of the epidemiology of this disease. Commercial vaccines for FHV-1 only reduce clinical signs but don't prevent virus shedding and the subsequent establishment of latency. This presents an obstacle for controlling disease especially in animal shelters where infected cats, even when vaccinated, will keep spreading the virus to most of the unvaccinated population. Such circumstances lead to the recurrence of the disease and significant loss of neonatal and unvaccinated cats. Thus, there is a strong demand to develop a next-generation vaccine, which provides more efficient and effective protection, both against clinical signs and infection itself. It is hypothesized that the insufficient effectiveness of current FHV-1 vaccines is due to the fact that current vaccines contain the complete set of virulence genes and can not be safely administered mucosally, where induction of immunity is critical for protection. In fact, the genome sequences of vaccine strains are almost identical to those of clinical isolates. Moreover, several virulence factors of alphaherpesviruses, including glycoprotein C (gC), glycoprotein E (gE), and Us3-encoded serine/threonine protein kinase (PK), also have immune modulatory function that prevent induction of strong host immunity. Studies involving other alphaherpesviruses, such as pseudorabies virus, bovine herpesvirus-1, and equine herpesvirus-1, have demonstrated that experimental immunization with mutants in which virulence-associated

or immune modulatory genes were deleted could induce immune responses that protect the host from wild-type virus infection. This data led us to explore the use of deletion mutants of FHV-1 to achieve the same goal. Four FHV-1 deletion mutants, including a glycoprotein C gene-deleted mutant (gC-), a glycoprotein E gene-deleted mutant (gE-), a Us3-encoded serine/threonine protein kinase gene-deleted mutant (PK-), as well as a mutant with deletions of both the gE and thymidine kinase (TK) genes (gE-TK-), were constructed previously in our lab by bacterial artificial chromosome (BAC) mutagenesis. The research described in this thesis is composed of 1) an in vitro study with these mutants in a primary feline respiratory cell culture (FREC) model, 2) an ex vivo study performed using a feline tracheal tissue explant culture system, and 3) an in vivo study. Together, these studies provide comprehensive data regarding safety, induction of innate and adaptive immune responses and efficacy of these deletion mutants and will guide moving forward with the most promising candidate for vaccine development.

To my family

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KEY TO ABBREVIATIONS

ALI	Air-liquid interface
CPE	Cytopathic effect
FHV-1	Feline herpesvirus-1
FREC	Feline respiratory epithelial cell
gC-	Glycoprotein C-deletion mutant
gE-	Glycoprotein E-deletion mutant
gE-TK-	Glycoprotein E & thymidine kinase-double-deletion mutant
hpi	Hours post-inoculation
MOI	Multiplicity of infection
NC	Negative control
PCR	Polymerase chain reaction
PK-	Serine/threonine protein kinase-deletion mutant
$TCID_{50}$	Tissue culture infectious dose 50%
WT	Feline herpesvirus-1 C27 wild type

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION AND LITERATURE REVIEW

Feline herpesvirus-1: A ubiquitous respiratory pathogen of cats

Feline herpesvirus-1 (FHV-1) is one of the major causes of feline viral upper respiratory and ocular disease (Harbour et al., 1991; Hoover and Griesemer, 1971; Hoover et al., 1970; Nasisse et al., 1998). The prevalence rate of FHV-1 is around 30% worldwide (Binns et al., 2000; Fernandez et al., 2017; Rampazzo et al., 2003), but could be 50% or higher in a complex environment such as animal shelters (Pedersen et al., 2004). The virus is transmitted via oronasal route, followed by invasion and replication in the upper respiratory tract epithelium (Gaskell and Povey, 1979). Virus excretion starts 24 hours after infection and typically lasts for 1-2 weeks. The acute phase of the disease is characterized by fever, lethargy, sneezing, coughing, nasal and ocular discharges (Gaskell et al., 2007; Maes, 2012). Some cats may also develop chronic and recurrent lesions, such as chronic rhinosinusitis and keratoconjunctivitis (corneal scarring, also known as “dry eye”) (Maggs, 2005). Generalized infections can occur in neonatal kittens. In addition, following the acute phase of infection, FHV-1 uses retrograde axonal spread along sensory nerves to reach sensory neurons, particularly within the trigeminal ganglia, and establishes latency. During latency, the latency-associated transcript of FHV-1 is expressed but lytic gene expression is repressed (Maes, 2012). Periodic viral reactivation, potentially accompanied with recrudescence of clinical signs and viral shedding, occurs as a result of various natural stresses or administration of corticosteroids (Gaskell et al., 2007; Gaskell et al., 1985).

Incomplete protection provided by current vaccines is a major hurdle in the control of FHV-1

Currently there are two types of multivalent commercial vaccines: modified live (MLV) and inactivated vaccines. In addition to FHV-1, both types of vaccines also contain feline calicivirus (FCV) as well as feline panleukopenia virus (FPV) (Lappin et al., 2006; Weigler et al., 1997). Existing FHV-1 vaccines alleviate clinical signs but do not fully prevent infection and viral shedding (Day et al., 2016). This is a big concern particularly in animal shelters, where the majority of young unvaccinated cats are susceptible to virus spread by other FHV-1 carriers, even if these were previously vaccinated. Commercial vaccines are also not able to prevent latency establishment or reactivation. Based upon their safety profile, most MLVs can only be administered systemically. Although immunization with existing MLVs labeled for intranasal use has been shown to induce better protection than systemically administered vaccines, infection and subsequent latency are not prevented (Weigler et al., 1997). Moreover, some mild clinical signs, such as transient sneezing occur after intranasal immunization (Orr et al., 1978), pointing to concerns about residual virulence of existing MLVs.

Classification of feline herpesvirus-1 and strain characteristics

According to the Virus Taxonomy released in 2018 by the International Committee on Taxonomy of Viruses (ICTV) Feline herpesvirus-1 (FHV-1) belongs to Family *Herpesviridae*, subfamily *Alphaherpesvirinae*, genus *Varicellovirus*, *Felid herpesvirus 1*. Feline herpesvirus-1 is a double-stranded DNA virus with an icosahedral nucleocapsid surrounded by a tegument layer and a lipid bilayer envelope, from which a number of glycoproteins project. Genetically and antigenically, FHV-1 is closely related to canine herpesvirus-1 (CHV-1) and phocine

herpesvirus-1 (PhV-1), and cross-protection between FHV-1 and PhV-1 has been reported (Harder et al., 1998; Martina et al., 2001). Several strains of FHV-1 have been identified, and it has been shown that they are genetically very similar, and all belong to a single serotype (Mochizuki et al., 1977; Studdert and Martin, 1970). The C27 virulent strain, isolated from an oropharyngeal conjunctival swab of a cat with respiratory symptoms in USA (Crandell and Maurer, 1958), is the prototype frequently used in FHV-1 research. The attenuated F2 strain, discovered in Japan, became the seed strain for current commercial vaccines (Bittle and Rubic, 1975). Minor genetic differences among strains have been reported, such as variance of Mlu1 cleavage patterns (Horimoto et al., 1992), re-arrangements in the gC gene (Hamano et al., 2004), and differences in the Sal1 site in the UL17 herpes simplex virus gene homologue (Hamano et al., 2005). In general, there is no remarkable biological difference among strains that can be used to distinguish attenuated from virulent strains, whether by genetic or immunological diagnostic methods (Mochizuki et al., 1977; Studdert and Martin, 1970).

Virulence factors identified in alphaherpesviruses

Herpesviruses encode various virulence factors, which are involved in various pathogenesis mechanisms. The life cycle of an alphaherpesvirus, mostly obtained from studies of human herpes simplex virus 1 (HSV-1), represents the universal model for other alphaherpesviruses. It is composed of several steps: initial attachment, penetration (viral entry), immediate early protein synthesis, early protein synthesis along with viral DNA replication, late protein (mostly glycoproteins) synthesis, viral egress, and cell lysis. According to studies of HSV-1, pseudorabies virus (PRV) in pigs, and bovine herpesvirus-1 (BHV-1), the viral entry process is composed of two parts: viral attachment and membrane fusion (Spear, 1993). The initial

attachment is mediated by gC binding to the cellular receptor heparan sulfate (Herold et al., 1991). This is followed by fusion and penetration, facilitated by interactions between several viral glycoproteins including gB, gD, gH, and gL, and cell receptors including herpes virus entry mediator (HVEM), nectin-1 or nectin-2, 3-O-S-site of heparan sulfate, and Toll-like receptor 2 (TLR2) (Connolly et al., 2011; Eberle et al., 1995; Leoni et al., 2012; Montgomery et al., 1996; Shukla et al., 1999). Envelope glycoproteins have been extensively investigated, not only because of their role for viral entry but also because these are main targets of the host immune response, thus highlighting why they are targeted in vaccine development efforts. Three main glycoproteins, gB, gD, and gH, have been identified *in vitro* as essential glycoproteins, i.e. they are vital for virus replication. In contrast, gC, gE, gI, and gG have been identified as non-essential glycoproteins. Even though they are not required for virus replication *in vitro* (Mettenleiter et al., 1990), they still partially mediate viral egress (Ben-Porat et al., 1986; Eisenberg et al., 2012; Spear et al., 2000). In addition from glycoproteins, virulence factors involving phosphorylation and DNA replication, e.g. Us3-encoded serine/threonine protein kinase (PK) and thymidine kinase (TK), are also thought to be good candidates for constructing deletion mutants as vaccine candidates (Kimman et al., 1994; Tenser et al., 1983).

Generating and characterizing FHV-1 gene-deletion mutants as a strategy for vaccine development

The use of a pseudorabies virus (PRV) gE-deletion mutant to protect against Aujeszky's disease in pigs was an inspiration for developing new-generation vaccines in veterinary medicine (van Oirschot et al., 1990). Kimman et al. (1994) published a combined *in vitro* and *in vivo* study of PRV, which encompassed multiple deletion mutants, including a gE-deletion mutant (gE-), an

Us3-encoded serine/threonine protein kinase-deletion mutant (PK-), a thymidine kinase-deletion mutant (TK-), and gE-PK-double-deletion mutant (gE-PK-), and a gE-TK-double-deletion mutant (gE-TK-). They reported that the PK- and gE-PK- mutants reached lower titers in SK-6 cells compared to titers following inoculation with wild-type (WT) or other mutants. However, this *in vitro* finding did not correlate with findings in the *in vivo* study. Pigs inoculated intranasally with the gE-PK- or gE-TK- mutants excreted lower amounts of virus, but the levels of virus shedding in pigs inoculated with the gE-, PK-, and TK- mutants and WT raised concerns about the actual safety of the gE-, PK-, and TK- single deletion mutants. In addition, pigs immunized with the PK- mutant showed full protection with no virulent virus excretion after WT challenge, while this was not true in the other groups (Kimman et al., 1994). The study was the first full paper revealing the candidacy of several virulence-associated gene deletion mutants as vaccine candidates against an alpha herpesvirus of veterinary importance. The study also revealed that virulence-associated gene-deleted mutants did not behave the same *in vitro* using cell culture and *in vivo*, emphasizing the requirement to conduct *in vivo* experiments as an essential component of assessing ultimate safety and efficacy of a vaccine candidate. In cattle, administration of mutants with a gE-deletion (gE-), a TK-deletion (TK-), and a gE and a TK-double-deletion (gE-TK-) induced improved immunity, which protected calves from bovine herpesvirus-1 (BHV-1) infection (Kaashoek et al., 1996). Experimentally intranasal or intramuscular vaccination with gE- mutants drastically reduced viral shedding and alleviated the symptoms of clinical disease after WT equine herpesvirus-1 (EHV-1) challenge in foals (Tsujimura et al., 2009). For FHV-1, gI-gE- or TK- mutants of FHV-1 have been generated previously and shown to induce protective immunity in cats (Sussman et al., 1995; Yokoyama et al., 1996). All the above studies suggested that virulence factor-associated gene-deletion mutants

not only potentially have reduced virulence compared to their parent strain, but also retained the level of antigenicity required to protect against clinical signs and infection itself.

Several FHV-1 deletion mutants have been constructed previously by bacterial artificial chromosome cloning along with Red-mediated mutagenesis

Bacterial artificial chromosome (BAC) cloning has been used widely as vectors for holding a large foreign DNA fragments, which could be more than 300 kb (Shizuya et al., 1992). The BAC cloning vectors provides a stable and efficient way for allowing a precise insertion or recombination of DNA fragments without unitended DNA rearrangement outside of interested sequences. After combining herpesivurs genome with a BAC cloning vector, the site-specific mutations could be performed entirely in *Escherichia coli*, without a requirement of reconstitution of live virus from eukaryotic cell culture if yeast artificial chromosomes were used (Costantino and Court, 2003). There have been several successful examples of BAC-vetored herpes virus recombinants in human herpes simplex virus (Saeki et al., 1998), equine

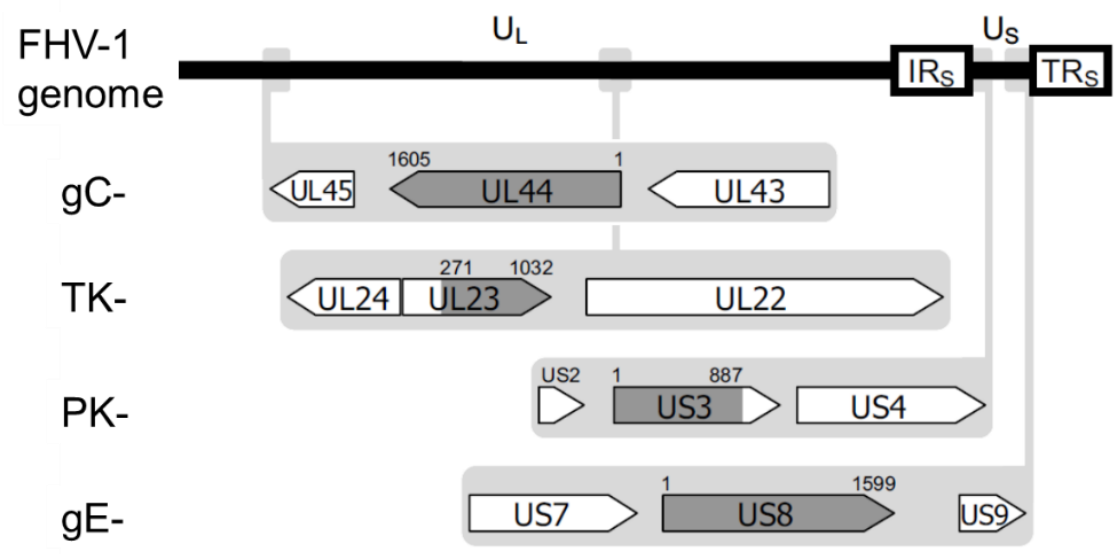

Figure 1. 1. A diagram revealing the whole genome of FHV-1 and the mutants with deleted genes accordingly, indicated by shaded region (Tai et al., 2016).

herpesvirus-1 (Rudolph et al., 2002), pseudorabies virus (Kimman et al., 1994), and Marek's disease virus or Gallid alphaherpesvirus 2 (Schumacher et al., 2000). Our lab has utilized BAC cloning followed by recombineering, or Red-mediated recombination to be specific, to generate four different FHV-1 mutants: gC-deleted mutant (gC-), gE-deleted mutant (gE-), gE/TK-double deleted mutant (gE-TK-), and PK-deleted mutant (PK-) (Tai et al., 2016). These selected virulence-associated genes of FHV-1 were described and characterized *in vitro* and *in vivo* in this dissertation.

Glycoprotein C: an anchorage protein involved in initial viral attachment, egress, cell-to cell spread and complement C3b inhibition

Glycoprotein C is the anchorage protein for initial viral attachment by recognition of the cell receptor heparan sulfate (Herold et al., 1991). Deletion of gC would interfere with viral attachment, but would not stop viral entry (Laquerre et al., 1998). Glycoprotein C also mediates viral egress and cell-to-cell spread based on a study of equine herpesvirus-1 (EHV-1) (Osterrieder, 1999). Furthermore, a study investigating a HSV-1 gC deletion mutant showed virions bound on the surface of epithelial cells for longer time when compared to wild-type virus, suggesting gC plays a role in viral penetration (Herold et al., 1994; Herold et al., 1991). In addition, gC is known to interact with complement C3b and, as such, prevent complement-mediated cell lysis of infected cells (Friedman et al., 1984; Harris et al., 1990). Glycoprotein C of Varicella zoster virus (VZV) has been identified as a viral chemokine binding protein (vCKBP) that simulates chemokine receptors, which regulate leukocyte migration (Gonzalez-Motos et al., 2017). However, for FHV-1, gG rather than gC was identified as a vCKBP that interacts with CXC, CC, and C-chemokine families (Costes et al., 2006).

Glycoprotein E: a molecule mediating viral entry and neural spread

Multiple alphaherpesviruses, including PRV, HSV-1, and FHV-1, have identified gE as important for viral cell entry, intracellular transport, and cell-to-cell spread (Mijnes et al., 1996; Polcicova et al., 2005; Tirabassi and Enquist, 1998). Glycoprotein E forms a hexodimer in conjunction with gI to initiate the fusion of the viral envelope and the cell membrane, which is followed by delivery of nucleocapsids into cytoplasmic vesicles (Brack et al., 2000). Human HSV-1 research has shown that the gE-gI complex specifically accumulates at the tight junctions, which is a key factor for lateral cell-to-cell spread of virus via tight junctions in highly polarized epithelial cells (Johnson et al., 2001). Herpes simplex virus 1 gE-deletion mutants exhibited random rather than directional virus spread within epithelial cells, and thus the infection efficiency of the mutant was drastically decreased (Wisner et al., 2000). Regarding the homology of gE among different alphaherpesviruses, two to four cysteine clusters, usually located in the N-terminal part of the protein, can be found in PRV, HSV-1, VZV, BHV-1, and EHV-1 (Davison, 1983; McGeoch et al., 1985). The C-terminal of the gE consists of five cysteine residues which are well-conserved in many alphaherpesviruses. Those conserved cysteines indicate that gE homologues possess common biological functions, e.g. viral entry, in alphaherpesviruses. Glycoprotein E is also a neurovirulence factor to facilitate trans-synaptic spread in the nervous system (Dingwell et al., 1995). It is responsible for the retrograde spread of alphaherpesvirus from epithelial cells to neurons (McGraw and Friedman, 2009), and the anterograde transition from the neuron cell body to the axon terminus (McGraw et al., 2009). In addition, gE interferes with antibody-dependent cytotoxicity by binding the Fc domain of IgG, which subsequently inhibits the activation of antibody (Awasthi et al., 2014).

Thymidine kinase: a long history of attenuating FHV-1 by deleting TK for vaccine development

The thymidine kinase (TK) gene of FHV-1 was first identified in an American strain (UC-D). The TK gene is 1029 base pairs long and encodes a 343 amino acid protein (Nunberg et al., 1989). Subsequently, a TK-deletion mutant with insertion of the genes encoding the envelope (env) or gag protein of feline leukemia virus (FeLV) was generated (Cole et al., 1990; Wardley et al., 1992). Another TK insertion mutant, C7301dlTK, was constructed based upon the Japanese C7301 parent strain. It had a deletion of a 450 bp EcoRV-SmaI fragment of TK gene (Yokoyama et al., 1995). It was demonstrated that the growth kinetics of C7301dlTK were similar to those of its parent strain, but the plaques generated by the mutant were smaller than those of the parent strain. The same group further conducted a clinical trial with this mutant and showed that the virulence of mutant was significantly reduced following ocular, intranasal, and oral inoculation and that clinical signs after challenge were reduced comared to the WT challenge-only group. Also, a significant VN antibody titer was detected in the C7301dlTK inoculation groups. However, a high titer of challenge virus was still recovered from several sites such as eye, nose, and mouth (Yokoyama et al., 1996). Thymidine kinase is not an essential protein for virus growth in regular dividing cells, but it assists viral growth in non-dividing cells, such as neurons (Field and Wildy, 1978). Studies have shown that the virulence of TK-defective mutants of HSV-1 (Field and Wildy, 1978), PRV (Tenser et al., 1983), BHV-1 (Kit et al., 1985b), and FHV-1 (Slater and York, 1976) was profoundly attenuated *in vivo* and have also shown that TK- mutants are less readily reactivated from neurons. Another study showed that TK-defective mutant of EHV-1 to be present in trigeminal ganglia *in vivo*, but that latent infection did not lead to reactivation, indicating its neurovirulent role (Coen et al., 1989). Thymidine kinase is also the target of the

anti-herpesvirus drug, acyclovir and its derivatives (Elion, 1982; Hussein et al., 2008).

US3-encoded serine/threonine protein kinase: a versatile protein mediating cytoskeleton rearrangement and mechanisms of anti-apoptosis and modulation the interferon response

Serine/threonine Us3-encoded protein kinase (PK) is a non-essential protein for virus replication. However, it functions in phosphorylation and catalyzes the process of virus envelopment during nuclear egress (Ryckman and Roller, 2004). PK-mediated phosphorylation involves lamins, the components of nuclear membranes, and viral gB, for the virus to be released from the nuclear membrane (Jacob et al., 2011; Wagenaar et al., 1995). The PK also regulates the organization of the cytoskeleton by controling Rho GTPase signaling, which is believed to facilitate virus transmission (Jacob et al., 2015; Lamote et al., 2016). In addition, PK has been recognized as an immune modulator in infection studies with HSV-1 and shown to influence interferon (IFN) signaling via multiple mechanisms, including down-regulation of the expression of Toll-like receptor 3 and type I IFN-inducible molecule MxA (Peri et al., 2008), interference with formation of interferon regulatory factor 3 (IRF3) dimerization followed by subsequent down-regulation of IFNβ expression (Tian et al., 2018; Wang et al., 2013), as well as phosphorylation of IFNγ receptors (Liang and Roizman, 2008). In addition, there is evidence of the role of PK in down-regulation of the major histocompatibility complex class I (MHC class I) to prevent antigen presentation (Eisfeld et al., 2007).

To summarize, gC, gE, and PK, all of which are virulence factors of alphaherpesviruses, serve as immune modulators to counteract host immunity. The current FHV-1 F2 vaccine strain contains all these multi-function virulencegenes, based upon the fact that this strain is genetically

almost identical to other FHV-1 virulent isolates. This is presumably one of the reasons why the current vaccine cannot provide full protection in cats. In addition, current vaccines cannot be safely administered at the mucosa, yet the route is essential for the induction of strong mucosal immunity. Therefore, it is hypothesized that **by constructing FHV-1 deletion mutants with a deficiency in one or more of these virulence factors, such as gC, gE, PK, and TK, a next--generation mucosal FHV-1 vaccine with reduced virulence and more effective immunogenicity can be developed**.

The innate immune response of the feline respiratory epithelium against viral infection

The primary infection route of FHV-1 infection is through the respiratory epithelium. Therefore, feline respiratory epithelial cells (FRECs) play a key role in immune defense against the infection. They are not only a physical barrier, but also control several pathways of the innate immune response. Respiratory epithelial cells express various pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) of herpesviruses (and other pathogens). The PRR-PAMP interactions include TLR2 to recognize glycoproteins, TLR3 to recognize dsRNA, and TLR9 to recognize DNA (Ma and He, 2014). Other PRR-PAMP interactions have been reported for HSV-1, such as RIG-I-like receptors (RLRs) to detect viral RNA in the cytoplasm (Cheng et al., 2007), and melanoma differentiation-associated gene 5 (MDA5) to detect RNA secondary structures (Pichlmair et al., 2009). Intracellular viral proteins could also be identified by DNA sensors, including the DNA-dependent activator of IFN-regulatory factors (DAI, or ZBP1), absent in melanoma 2 (AIM2), RNA polymerase III, leucine-rich repeat flightless-interacting protein (LRRFIP1), as well as IFNγ-inducible protein 16 (IFI16) (Chiu et al., 2009; Hornung et al., 2009; Takaoka et al., 2007; Unterholzner et al., 2010).

Following PRR-PAMP recognition of an invading virus, a pro-inflammatory responses will be initiated, with the activations of the transcription factors nuclear factor-κB (NF-κB), activator protein 1 (AP1), and several IFN regulatory factor (IRF) family members (Honda and Taniguchi, 2006; Takeuchi and Akira, 2010). The expression of type I interferons, comprising IFNα and IFNβ, is the main activity of pro-inflammatory responses against viral infections. Specifically, IRF3 and NF-κB initialize the expression of IFNα, while IRF7 combined with NF-κB initialize the expression of IFNβ. The production of type I IFNs further induce the expression of interferon-stimulated genes (ISGs) to expand IFN activity in order to inhibit virus replication (Randall and Goodbourn, 2008). The secretion of inflammatory cytokines such as IL4, IL6, tumor necrosis factor α (TNFα), granulocyte-macrophage colony stimulating factors (GM-CSF), as well as chemoattractants for T cells, dendritic cells, and monocytes, are also involved in stimulation of innate immune response in the respiratory tract (Kato and Schleimer, 2007).

Host--pathogen interaction during alphaherpesvirus infection

It has been shown that some viral components of alphaherpesviruses, such as serine/threonine protein kinase (PK) and thymidine kinase (TK), are able to modulate the activation of IFNα, IFNβ, and IFNγ in blood leucocytes by reducing TLR3 expression or by counteracting IRF3 (Lewandowski et al., 1994a; Peri et al., 2008; Wang et al., 2013). A recent study further showed that EHV-1, an alphaherpesvirus of horses, regulates the production of IFNα, IFNβ, and IFNγ in respiratory epithelial cells (Hussey et al., 2014). It is logical to assume that FHV-1, similar to other alphaherpesviruses, modulates TLR expression at the respiratory epithelium, leading to a dysregulation of inflammatory cytokine responses and interferons, and

resulting in interference of dendritic cells maturation and dysfunction of antigen-presentation mechanism (Nelli et al., 2016; Salio et al., 1999).

In addition to modulating IFN activity, several alphaherpesviruses including HSV-1, VZV, PRV, EHV-1, BHV-1, and FHV-1, have been shown to evade the immune defense by downregulating the expression of MHC class I on antigen presenting cells. The expression of MHC class I is crucial for activating the cytotoxicity of $CD8^+$ T cells to eradicate herpes-infected cells (Abendroth et al., 2001; Deruelle et al., 2009; Hill et al., 1994; Montagnaro et al., 2009; Rappocciolo et al., 2003; Wei et al., 2011). Glycoproteins or the Us3-encoded PK can all participate in manipulation of MHC class I expression (Imai et al., 2013; Montagnaro et al., 2009). Other cytokines or chemokines, including but not limited to IL1β, TNFα, GM-CSF, MCP-1, IL10, and TGFβ, can also be down regulated by alphaherpesviruses, (Boivin et al., 2002; Gomez-Cambronero et al., 2003; Gupta et al., 2006; Rossol-Voth et al., 1991; Sergerie et al., 2007; Smith et al., 2000).

The ultimate goal: developing a next-generation FHV-1 vaccine combined with a strategy of heterologous prime-boost immunization

The prime-boost immunization strategy has been used widely for vaccination against different diseases. In the homologous approach, the vaccine delivery routes are the same for the initial priming and subsequent boost immunizations. In the heterologous approach, the same vaccine preparation is used but the routes of administration differ between the initial administration (prime) and subsequent booster administrations. Heterologous prime-boost has been shown to induce a more effective immune response than homologous prime boost (Ramshaw and Ramsay, 2000). Specifically, both humoral and cellular immunity could be better

induced when the immunogens are delivered via different routes (Kardani et al., 2016; Lu, 2009). Intranasal immunization of mice with an inactivated bovine respiratory syncytial virus-derived vaccine was shown to trigger better antibody production, cell-mediated immunity, and mucosal immune responses compared to those resulting from subcutaneous immunization (Mapletoft et al., 2010). Intranasal priming combined with a subsequent subcutaneous boost with a *Yersinia pestis*-derived protein protected the host from aerosol challenge by inducing a marked amount of IgG (Glynn et al., 2005). Another study confirmed that an intranasal immunization induced significant production of IgA, which was not found in parenteral immunization (Fiorino et al., 2013). The same study also showed that heterologous prime-boost immunization which included a mucosal route induced a significant amount of IL17, a pro-inflammatory cytokine critical for CD4+ T cell activation, and presumably tended to drive Th1 polarization (Fiorino et al., 2013). Considering that there is no current vaccine that can fully protect the cats from FHV-1 infection, thus leading to lifelong latency and potential for reactivation (Day et al., 2016), a safe and effective next-generation vaccine is required. Moreover, an application of such vaccine utilizing a heterologous prime-boost immunization strategy is expected to stimulate both systemic and mucosal immunity, with more complete protection than existing vaccines.

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CHAPTER 2

VIRAL REPLICATION AND INNATE IMMUNITY OF FELINE HERPESVIRUS-1 VIRULENCE-ASSOCIATED GENES IN FELINE RESPIRATORY EPITHELIAL CELLS

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

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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. Our hypothesis was that immunomodulation of FHV-1 is the cause 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-hour intervals between 24 and 96 hpi to determine growth kinetics. Reverse transcription realtime 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, gE-TK- and PK- reduced IFNα and IL-10 mRNA expression, as well as 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), 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.

KEYWORDS

Feline herpesvirus-1

Feline respiratory epithelial cell

Glycoprotein C

Glycoprotein E

Serine/threonine protein kinase

Thymidine kinase

ABBREVIATIONS

ALI	Air-liquid interface
ANOVA	Analysis of variance
BAC	Bacterial artificial chromosome
BHV-1	Bovine herpesvirus-1
CRFK	Crandell 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

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; Gaskell et al., 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, MLV vaccination is based on the temperature sensitivity of the vaccine virus that is attenuated when administered sq. but remains virulent when administered i.n. or in case of accidental spills of the vaccine virus. This indicates residual virulence (Orr et al., 1978). 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 confirm 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 nervous 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 rearrangements, 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). Recent research even showed that the Us3 protein of FHV-1 is a powerful inhibitor of type I interferons via inhibition of interferon regulatory transcription factor 3 (IRF3) dimerization (Tian et al., 2018).

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; 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 Crandell 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 was 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.

MATERIALS AND METHODS

FHV-1 wild type and the 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 Crandell Reese feline kidney (CRFK) cell line [ATCC, CCL-94] was used for virus propagation.

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 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).

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 hour at 37℃ ,5% CO. 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 as 1 hour post-inoculation (hpi), or cultured until sampling, at 24, 48, 72, 96 hpi, respectively. For sampling, FRECs were washed and incubated with 1ml of Accumax Cell Dissociation Solution [Life Technologies] at 37℃ 5% CO_2 for 20 min, followed by vigorously 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.

Titrations to reveal 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.

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.

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.

Multiplex immunoassays for feline inflammatory cytokines/chemokines

A Milliplex^{TM} 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^{TM} 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.

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.

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.

RESULTS

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) (**Fig. 2. 1**). The plaques in gE- infected wells at 24 hpi (**Fig. 2. 1e**) were smaller than those in WT infected wells (**Fig. 2. 1a**). More than 70% FRECs were lysed after 48 hpi in WT and gE- groups (**Fig. 2. 1b-d, 2. 1f-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) (**Fig. 2. 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. 2**), small CPE plaques were first noted at 24 hpi in WT and gC- groups (indicated by arrows) (**Fig. 2. 2a, 2. 2e**). Clear CPE was seen at 48 hpi and at all later time points in both WT and gC- infected FRECs (**Fig. 2. 2b-d, 2. 2f-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. 2. 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) (**Fig. 2. 2l**). Uninfected control FRECs (NC) in experiments 1 and 2 maintained full confluency throughout the experimental period (**Fig. 2. 1m, 2. 2m**).

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. 2. 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. 2. 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. 2. 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. 2. 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. 2. 3b**). All samples from mock inoculated FRECs in both experiments were negative.

Extracellular viral titers in sub-natants are shown in **Fig. 2. 3c-d**. Only 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. 2. 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. 2. 3c**). Infectious virus was not detected in sub-natants from gC- and PK- inoculated FRECs at any of the sampling points (**Fig. 2. 3d**).

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. 2. 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. 2. 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 amount of viral DNA in FRECs infected with PK- was lower compared to WT and gC- inoculations starting at 24 hours post infection ($p < 0.0001$) (**Fig. 2. 4b**). No viral genome was detected in mock inoculated FRECs.

Extracellular levels of viral DNA (**Fig. 2. 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. 2. 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. 2. 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. 2. 4d**). Viral DNA was not detected in sub-natants from mock inoculated FRECs.

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 **figures 2. 5 and 2. 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. 2. 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. 2. 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) (**Fig. 2. 5a and 2. 6a**). Similar to WT infection, inoculation with gE- and gC- significantly up-regulated IFNα gene 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. 2. 6b-c**). Inoculation with other mutants did not significantly influence TNFα or IL-1β gene expression ($p> 0.05$) (**Fig. 2. 5b-c, 2. 6b-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) (**Fig. 2. 5d and 2. 6d**). 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) (**Fig. 2. 5e and 2. 6e**). 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. 2. 5e**). No significant differences in GM-CSF or TLR3 gene expression was found between treatments at all time points post-inoculation (data not shown).

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. 2. 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. 2. 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. 2. 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. 2. 7b, 2. 7d, 2. 7f, 2. 7h**), 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. 2. 7h**). Further, inoculation of FRECs with the gE- mutant up-regulated secretion of RANTES compared to mock inoculated FRECs ($p= 0.0083$) (**Fig. 2. 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).

DISCUSSION

FHV-1 mutants generated in this study, which have 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 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 previously 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. 2. 3b**), while quantitative PCR data showed DNA amounts of the WT and gC- to be similar (**Fig. 2. 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, endpoint titers in CRFKs in a previous experiment performed in our laboratory were over 10^4 $TCID_{50}$/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. 2. 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 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. 2. 3c-d**) In contrast, intra- and extra-cellular titers were comparable to each other in CRFK cell inoculations in earlier studies by our group (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 IFN□ 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 resulted in a down-regulation of IFNα gene expression in FRECs compared to WT, gE- and gC- inoculated FRECs. However, IFNα and IFN□ protein secretion was below the detection limit in our study. 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 definitely 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 (**Fig. 2. 5d-e, 2. 6d-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 in 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. 2. 7a-b, 2. 7e-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. 2. 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. 2. 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; Van de Walle et al., 2008b), 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 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, deletion of TK and PK 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 may be an ideal candidate for further *in vivo* investigation to study induction of immunity and safety in the host.

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FUNDING

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APPENDIX

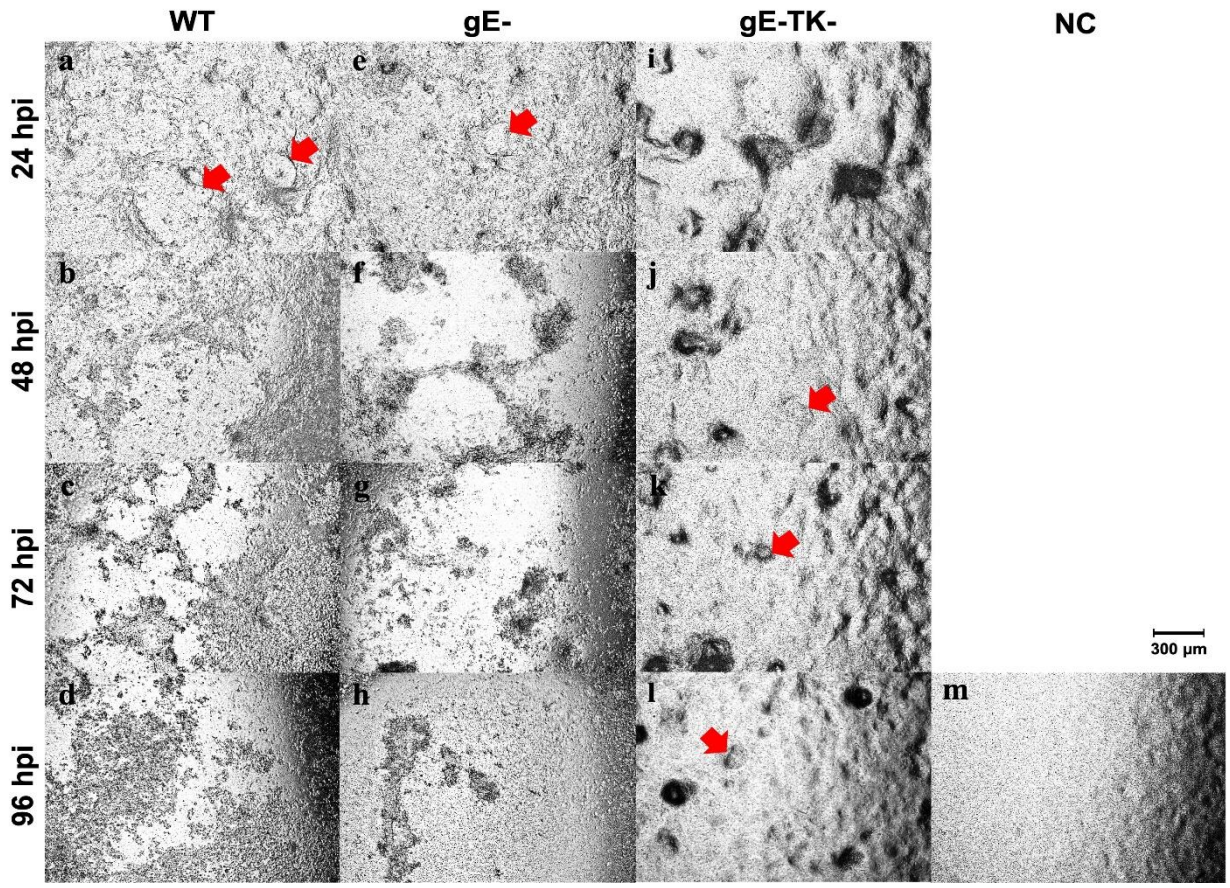

Figure 2. 1. Representative microscopic findings following inoculation of FRECs with FHV-1 or mutants at an MOI of 1. All images were taken at 40× magnification. A-d) FRECs infected with WT at 24, 48, 72, and 96 hpi; e-h) FRECs infected with gE- at 24, 48, 72, and 96 hpi; i-l) FRECs infected with gE-TK- at 24, 48, 72, and 96 hpi; m) mock inoculated FRECs at 96 hpi. Arrows indicate the 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.

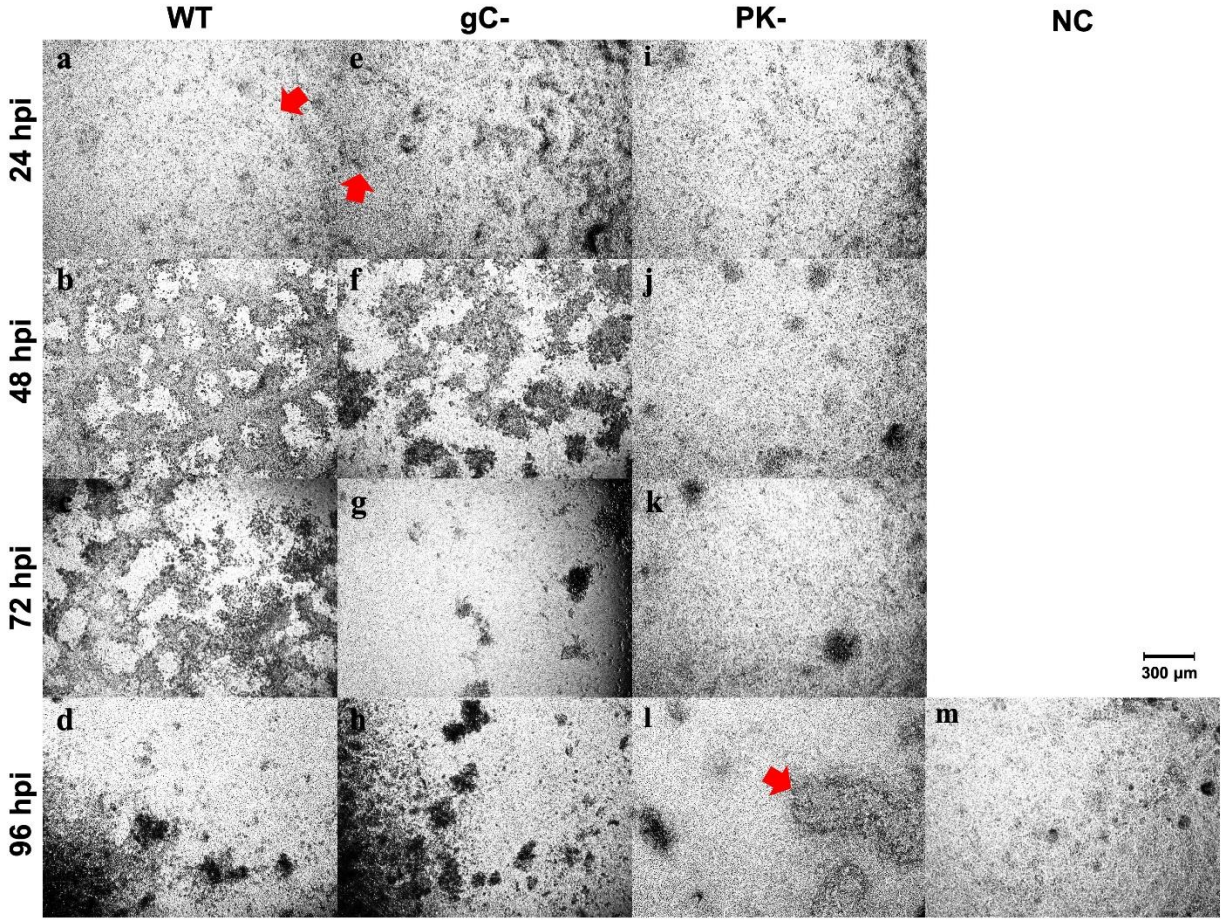

Figure 2. 2. Representative microscopic findings following inoculation of FRECs with FHV-1 or mutants at an MOI of 0.1. All images were taken at 40× magnification. A-d) FRECs infected with WT at 24, 48, 72, and 96 hpi; e-h) FRECs infected with gC- at 24, 48, 72, and 96 hpi; i-l) FRECs infected with PK- at 24, 48, 72, and 96 hpi; m) mock inoculated FRECs at 96 hpi. Arrows indicate the 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.

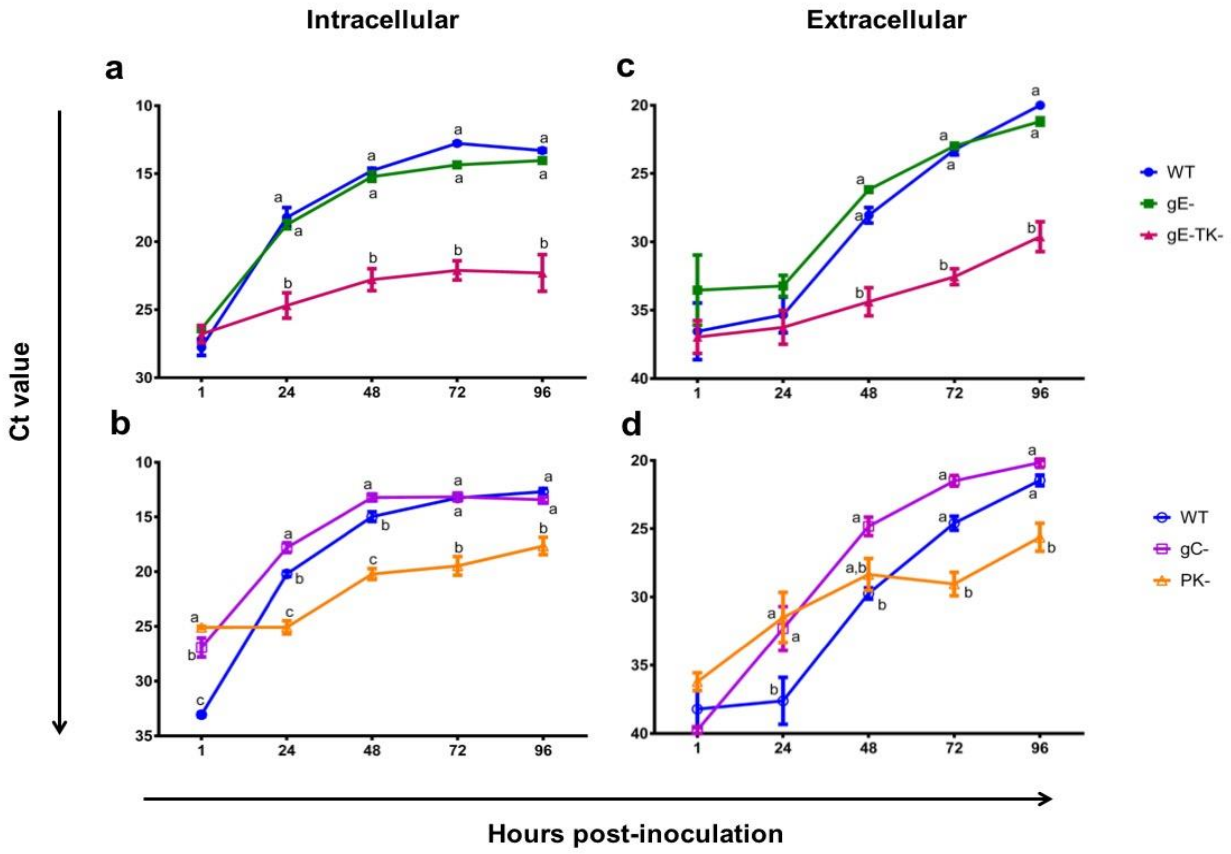

Figure 2. 3. Viral growth kinetics. Viral titers (log $TCID_{50}$/ml) were measured in cell pellet lysates (intracellular virus) of feline respiratory epithelial cells inoculated at an MOI of 1 (a), or an MOI of 0.1 (b), in sub-natants (extracellular virus) of feline respiratory epithelial cells inoculated 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.

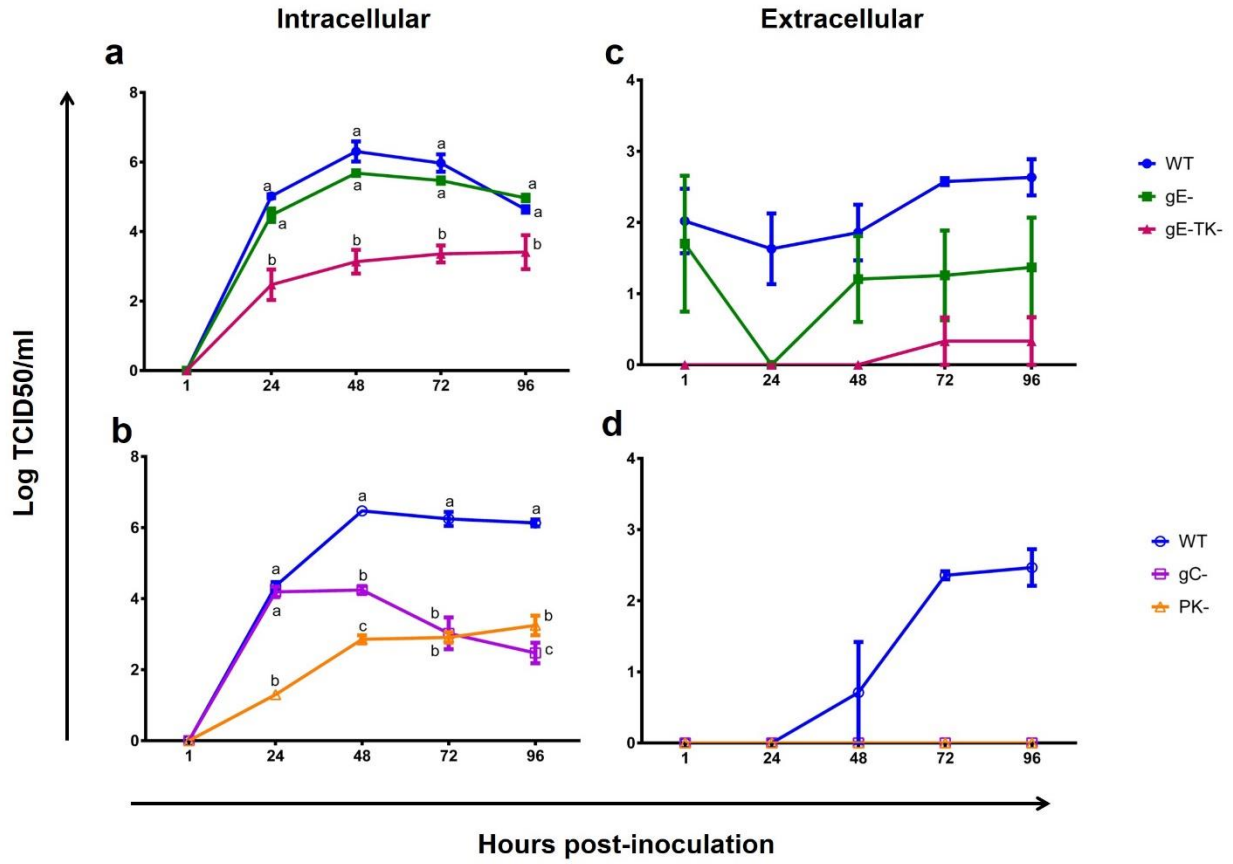

Figure 2. 4. Measurement of viral DNA kinetics. Ct values were determined by real-time PCR in cell pellet DNA extracts (intracellular virus) of feline respiratory epithelial cells inoculated at an MOI of 1 (a), or an MOI of 0.1 (b), and in extracts of sub-natants (extracellular virus) of feline respiratory 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 a significant difference 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.

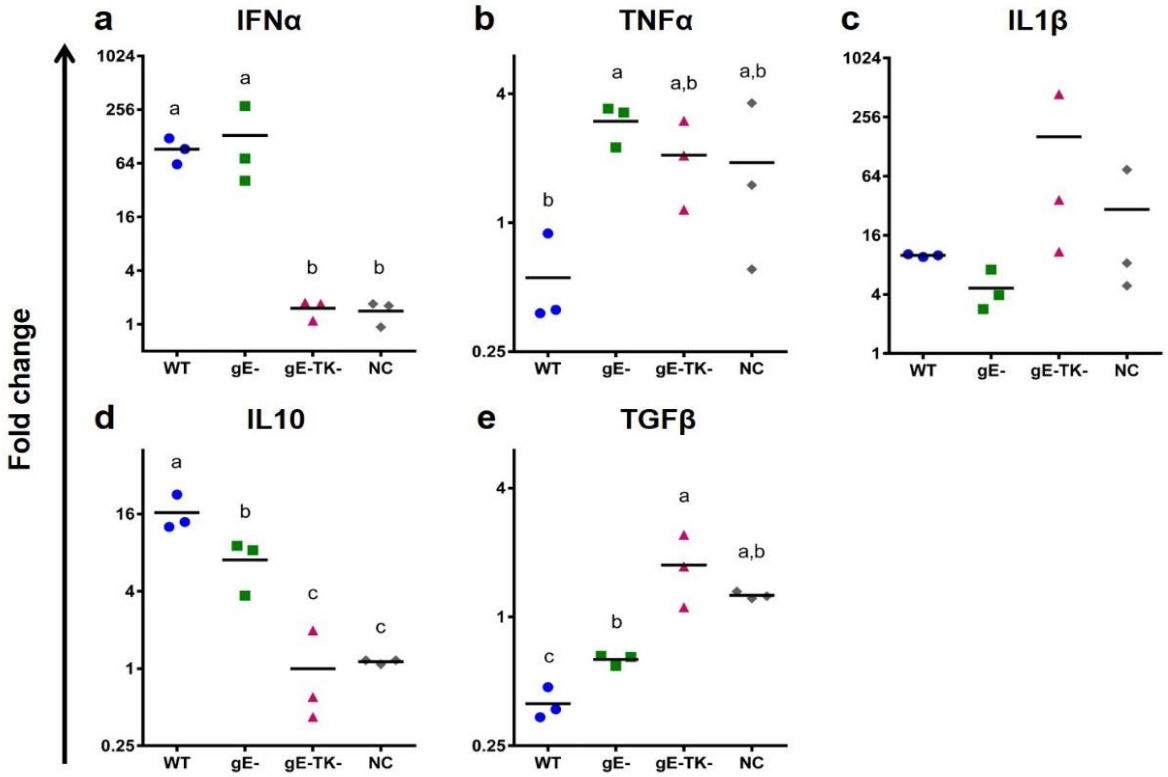

Figure 2. 5. Cytokine gene expression in FRECs inoculated at an MOI of 1. The levels of mRNA of IFNα (a) TNFα (b), IL-1β (c), IL-10 (d), and TGFβ (e) were determined by reverse transcription real-time PCR at 72 hpi in cell lysates from feline respiratory epithelial cells infected with WT or mutants at 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 a significant difference in the multiple comparisons between groups by applying post hoc Tukey's tests for each cytokine ($p < 0.05$). 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.

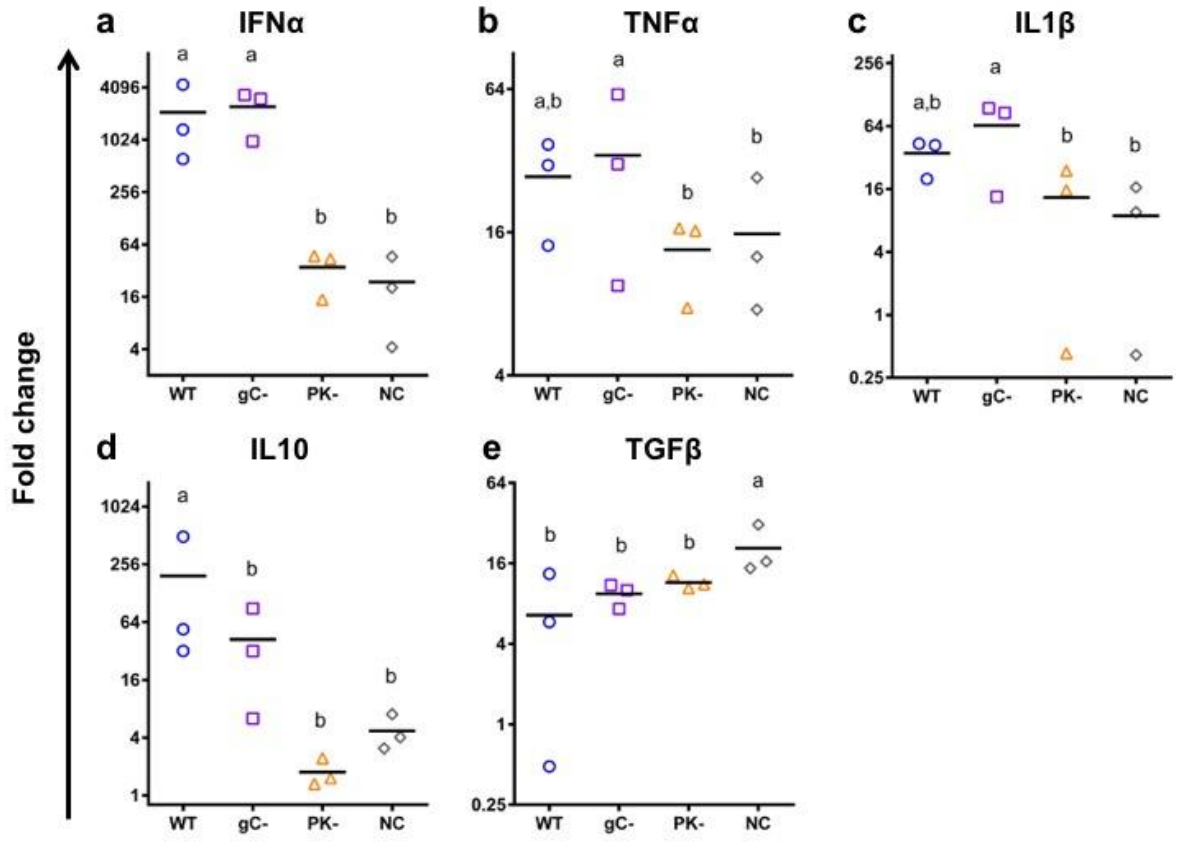

Figure 2. 6. Cytokine gene expression in FRECs inoculated at an MOI of 0.1. The levels of mRNA of IFNα (a) TNFα (b), IL-1β (c), IL-10 (d), and TGFβ (e), were determined by reverse transcription real-time PCR at 72 hpi in cell lysates from feline respiratory epithelial cells infected with WT or mutants 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 a significant difference in the multiple comparisons between groups by applying post hoc Tukey's tests in each cytokine ($p < 0.05$). 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.

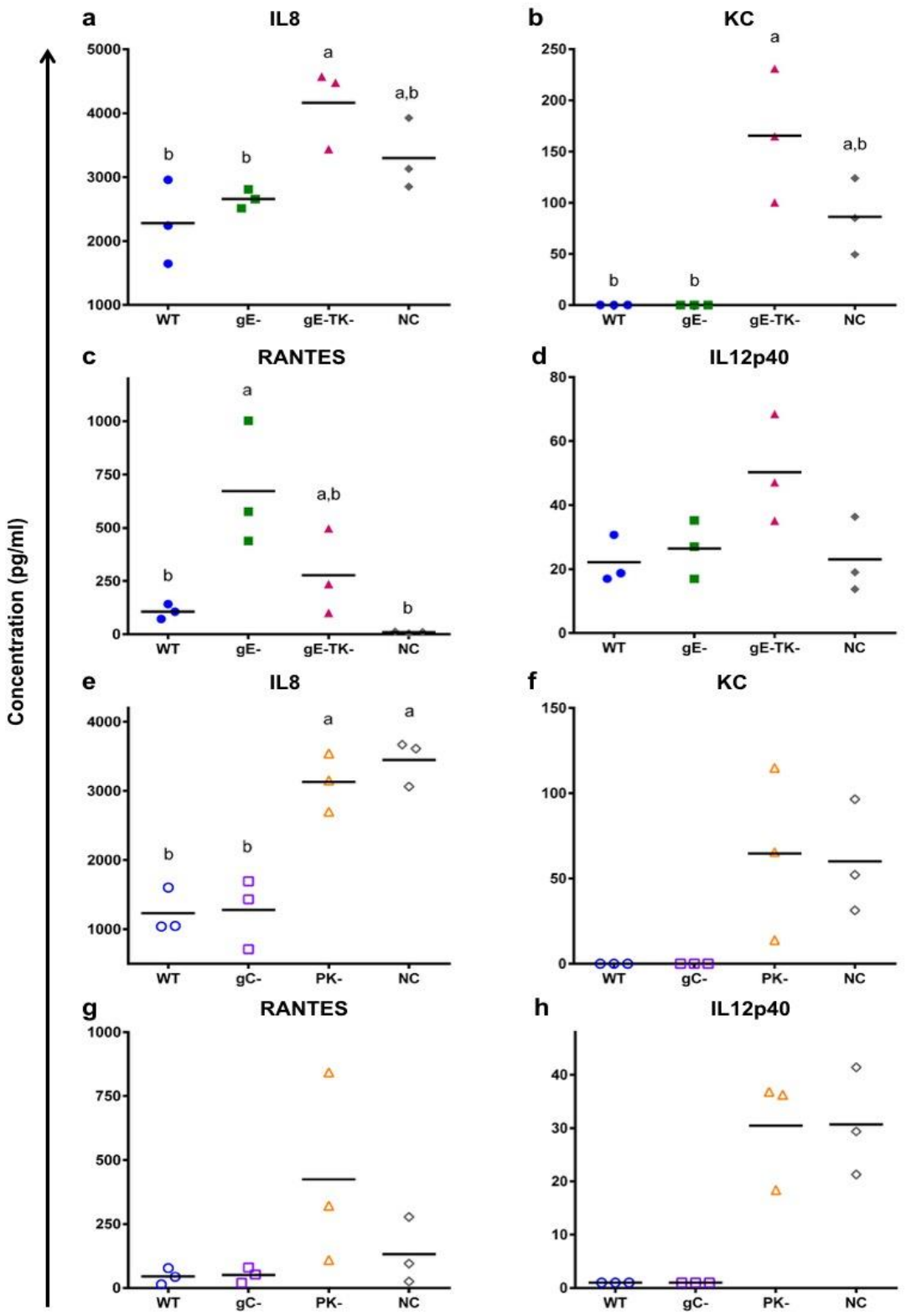

Figure 2. 7. Chemokine secretion profile. A Milliplex^{TM} immunoassay was used to determine the protein levels of subsets of chemokines, including IL-8 (a, e), KC (b, f), RANTES (c, g),

Figure 2. 7. (Cont'd) 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 in the multiple comparisons between groups by applying post hoc Tukey's tests for each chemokine, respectively ($p < 0.05$). 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.

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CHAPTER 3

CHARACTERIZATION OF FELINE HERPESVIRUS-1 DELETION MUTANTS IN TISSUE EXPLANT CULTURES

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Characterization of feline herpesvirus-1 deletion mutants in tissue explant cultures

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ABSTRACT

Feline herpesvirus-1 (FHV-1) is the primary cause of viral respiratory and ocular disease in cats. While commercial vaccines can provide clinical protection, they do not protect from infection or prevent latency. Moreover, they are not safe for intranasal administration. Our overall objective is to develop a new mucosal vaccine against FHV-1 disease to address these shortcomings. Feline herpesvirus-1 deletion mutants of glycoprotein C (gC-), gE (gE-), US3-encoded serine/threonine protein kinase (PK-), and both gE and thymidine kinase (gE-TK-) were generated by bacterial artificial chromosome (BAC) mutagenesis. Tracheal tissue explants from eight cats were used to compare the pattern of viral infection and associated tissue damage, as well as virus spread through the basement membrane following inoculation with wild-type virus (WT), and gE-, gE-TK-, PK-, and gC- mutants. Tissues were collected at 24, 48, or 72 hours post-inoculation (hpi) followed by immunohistochemistry (IHC) for FHV-1. Histological changes were graded based on the distribution of viral infected cells and the severity of tissue damage. Inoculations with the WT virus resulted in maximal scores at 72 hpi both at a multiplicity of infection (MOI) of 1 and 0.1. Inoculation with the gE- mutant produced scores similar to those inoculated with the WT virus at 24 and 48 hpi, but scores were significantly decreased at 72 hpi. Explants inoculated with the gE-TK- mutant showed significantly decreased scores at all time points. Further, the majority of explants inoculated with the PK- mutant resulted in scores of zero at all time points, regardless of MOI. Finally, inoculation with WT resulted in significant stromal invasion below the infected epithelium, while stromal invasion was observed in less than 50% of the samples following inoculation with gE-, gE-TK-, PK-, or gC- mutants and confined closely to the area surrounding the infected epithelium. In conclusion, the gE-TK- and PK- mutants exhibited significantly reduced virulence, tissue damage and spread

to the underlying stroma, suggesting that they may be good vaccine candidates for *in vivo* testing.

KEYWORDS

Feline herpesvirus-1 deletion mutant

Feline tracheal explant

Glycoprotein E

Serine/threonine protein kinase

Thymidine kinase

Stromal invasion

ABBREVIATIONS

ALI	Air-liquid interface
ANOVA	Analysis of variance
BAC	Bacterial artificial chromosome
CRFK	Crandell Reese feline kidney
CPE	Cytopathic effect
CXCL1	chemokine (C-X-C motif) ligand 1
DMEM	Dulbecco's Modified Eagle Medium
EHV-1	Equine herpesvirus-1
FHV-1	Feline herpesvirus-1
FREC	Feline respiratory epithelial cell
HSV-1	Human herpes simplex virus-1
gC-	Glycoprotein C-deletion mutant
gE-	Glycoprotein E-deletion mutant
gE-TK-	Glycoprotein E & thymidine kinase-double-deletion mutant
hpi	Hours post-inoculation
IHC	Immunochemistry
IL-10	Interleukin 10
MOI	Multiplicity of infection
NC	Negative control
PCR	Polymerase chain reaction
PK-	Serine/threonine protein kinase-deletion mutant
PRV	Pseudorabies virus

TCID50	Tissue culture infectious dose 50%
WT	Feline herpesvirus-1 C27 wild-type

INTRODUCTION

Feline herpesvirus-1 (FHV-1) belongs to the family *Herpesviridae*, subfamily *Alphaherpesvirinae*, genus *Varicellovirus*, and species *Felid alphaherpesvirus 1*. Feline herpesvirus-1 is one of the primary pathogens responsible for feline respiratory disease and an important cause of ocular disease. Common symptoms include viral rhinotracheitis, pneumonia, conjunctivitis, keratitis, stomatitis, and sometimes abortion as well as neonatal death (Harbour et al., 1991; Hoover and Griesemer, 1971; Studdert and Martin, 1970). Currently there are inactivated and modified live vaccines commercially available. However, these current vaccines only reduce clinical signs of disease but cannot prevent infection, viral shedding, and thus latency establishment or reactivation (Lappin et al., 2006; Weigler et al., 1997). Moreover, commercial vaccines show residual virulence when administered intranasally in cats (Kruger et al., 1996; Orr et al., 1978). Thus, constructing a safe second-generation vaccine that can be administered mucosally and effectively protects against clinical signs, infection, and latency is warranted.

We have previously constructed FHV-1 deletion mutants of glycoprotein C (gC-), glycoprotein E (gE-), US3-encoded serine/threonine protein kinase (PK-), and gE and thymidine kinase (gE-TK-) via bacterial artificial chromosome (BAC) mutagenesis (Tai et al., 2016). Because feline respiratory epithelial cells are the natural target cells for invasion and replication of FHV-1, and the first cells encountered by the virus during natural infection (Gaskell and Povey, 1979), we thoroughly examined the candidate deletion mutants *in vitro* in primary feline respiratory epithelial cells (FRECs) isolated from feline tracheas and cultured at the air liquid interface (Lee et al., 2019). In that study, we identified the gE-TK- and PK- mutants as the most promising vaccine candidates because they exhibited attenuated virulence and reduced viral

replication. Moreover, while infection with the virulent C27 strain resulted in down-regulation of IL8 and CXCL1 (neutrophil chemokine) and up-regulation of IL10, this modulation was not observed in FRECs inoculated with the PK- or gE-TK- deletion mutants, suggesting that the deletion of the selected virulence genes may also have allowed the host cells to maintain a better immune defense against FHV-1 infection. Furthermore, we demonstrated that the cell-to-cell spread of FHV-1 was decreased when genes for gC, PK, and both gE and TK, were deleted (Lee et al., 2019).

However, tissue damage and stromal invasion caused by viral infection are additional important indicators for evaluating the virulence of alphaherpesviruses that cannot be evaluated in FRECs. It has been shown that alphaherpesviruses specifically target and disrupt the components of extracellular matrix, such as collagen VII and integrin alpha 6, to assist viral penetration through the basement membrane barrier (Bannazadeh Baghi and Nauwynck, 2016). The disruption of extracellular matrix and tight junctions between epithelial cells likely also helps to expose the cellular N-linked glycan receptors for viral entry and spread of alphaherpesviruses (Van Cleemput et al., 2017). Tissue explants freshly obtained from feline tracheas are composed of a layer of epithelium with an intact basement membrane, thus closely simulating the *in vivo* microenvironment. Moreover, a cross section of tissue explants after viral inoculation provides a clear morphological view of tissue damage and stromal invasion, making them an ideal tool for evaluating viral respiratory infections, particularly when combined with the results obtained in FRECs. The value of tissue explant cultures has been shown in studies of human herpes simplex virus-1 (HSV-1), equine herpesivurs-1 (EHV-1), pseudorabies virus in pigs, and bovine herpesvirus-1 (BHV-1) (Glorieux et al., 2011; Glorieux et al., 2009; Negussie et al., 2016; Steukers et al., 2012). For FHV-1, one study has demonstrated viral plaques in the

epithelium of tissue explants from the feline trachea and the conjunctiva with disruption of the basement (Li et al., 2015). Other studies have used tissue explants from feline trachea to show morphological changes in the epithelium, including cell detachment and necrosis after FHV-1 infection (Leeming et al., 2006; Monne Rodriguez et al., 2017).

As a follow-up to our previous FHV-1 study in FRECs (Lee et al., 2019), our goal was to use feline tracheal explants to compare the viral infection pattern and the associated tissue damage caused by the FHV-1 wild-type (WT) strain and deletion mutants. Feline herpesvirus-1 specific immunohistological (IHC) labeling was performed and a scoring system was implemented, which took into account viral distribution and severity of associated tissue damage and semi-quantified the IHC results. Basement membrane penetration was also reviewed to evaluate stromal invasion of each virus. Our ultimate goal was to combine data from the present and our previous study (Lee et al., 2019) to use for the logical selection of vaccine candidates for future *in vivo* trials.

MATERIALS AND METHODS

Viruses

The FHV-1 wild type (WT) used in this study was strain C27 [ATCC, VR-636, Manassas, VA, USA]. Deletion mutants of the FHV-1 C27 strain, including a gE-deficient mutant (gE-), a gE/TK-double-deficient mutant (gE-TK-), a gC-deficient mutant (gC-), and a PK-deficient mutant (PK-), were previously constructed by two-step Red-mediated recombination (Tai et al., 2016) using a full-length of C27 bacterial artificial chromosome (BAC) clone. Deletions in each virus and absence of WT cross-contamination were confirmed by PCR using primers for gC, gE, TK, and PK genes, as described oreviously (Tai et al., 2016).

Animals and sample collections

Eight domestic-short-hair cats were used in this study. All cats were 3-6 months old, had no respiratory clinical signs, and were euthanized for reasons not related to this study. Cats were anesthetized with isoflurane induction, followed by euthanasia by intravenous injection of 85.9 mg/kg pentobarbital sodium. All procedures 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. Tracheas were freshly collected at necropsy using sterile instruments , followed by transportation to the lab in DMEM/F12 [Life Technologies, Grand Island, NY, USA] medium supplemented with 100 U/ml penicillin, 100 U/ml streptomycin, 1.25 μg/ml amphotericin B, and 1 μg/ml gentamicin [Life Technologies].

Tissue explant culture inoculations with WT and deletion mutants

Tracheas were trimmed into several 4 × 4 mm pieces without damaging the mucosa and cultured as previously described (Li et al., 2015). Briefly, tissue explants were placed on top of a pad with the mucosa layer on the up side and the cartilage layer on the bottom side and cultured in 6-well plates submerged in 5 ml culture media containing DMEM/F12 with 100 U/ml penicillin, 100 U/ml streptomycin, 1.25 μg/ml amphotericin B, 1 μg/ml gentamicin [Life Technologies], and 1% of GlutaMAX^{TM} [Life Technologies]. After acclimatization overnight, tissue explants were transferred from 6-well culture plates to 12-well or 24-well infection plates for inoculation with the respective viruses. According to previous estimates (Leeming et al., 2006), the tissues explants contained approximately one million epithelial cells, which was used to calculate the multiplicity of infection (MOI) for virus inoculation. Tissue explants from 4 cats (No. 1-4) were inoculated with the WT, gE-, or gE-TK- mutants, at an MOI of 1, or inoculated with the WT or PK- mutant at an MOI of 0.1, respectively. Tissue explants from 4 additional cats (No. 5-8) were inoculated with the WT or PK- mutant at an MOI of 1 or with the WT or gC- mutant at an MOI of 0.1, respectively. After 1 hr adsorption, all inoculated tissues were washed with PBS and transferred back to the culture plates, replenished with fresh culture media and cultured for 24, 48, or 72 hours post-inoculation (hpi). At each time point harvested tissue explants were preserved by fixation in formalin. Mock treatment explants consisting of inoculation with DMEM/F12 were harvested at 1 and 72 hpi.

Tissue preservation and processing

Each tissue explant was fixed for 24 hrs in 10% buffered formalin, and then dehydrated in 70% ethanol for at least 24 hrs. Subsequently, tissues were submitted to the histology lab at

Veterinary Diagnostic Laboratory (VDL) at Michigan State University (MSU) for further processing. The tissues were paraffin-embedded and stored as tissue blocks.

Immunohistochemistry for FHV-1

Serial sections of 3 μm thickness with 200 μm intervals were cut from paraffin-embedded tissue blocks and immunochemically labeled for FHV-1 using the automated IHC slide DISCOVERY ULTRA Ventana^{TM} system [Ventana Medical Systems, Tucson, AZ, USA], and processed by the histology lab in the VDL at MSU. Briefly, the sections were dewaxed by treatment with EZ Prep [Cat. No. 950-100, Roche, Indianapolis, IN, USA] followed by treatment with DISCOVERY RiboCC [Cat. No. 760-107, Roche] for antigen retrieval. Five sections were then stained with a mouse-monoclonal anti-feline herpesvirus antibody [Cat. No. FHV5, Custom Monoclonal International, W. Sacramento, CA, USA] at a 1:1000 dilution for 32 min at room temperature. This was followed by staining with a secondary antibody DISCOVERY UltraMap anti-Mouse Alk Phos [Cat. No. 760-4312, Roche] and staining with the ChromoMap AP Red Staining kit [Cat. No. 760-160, Roche]. Hematoxylin was used as the counterstain.

Microscopic observation and evaluation via a scoring system

The IHC labeling for FHV-1 was microscopically evaluated by the first author and confirmed by a board-certified pathologist. For each cat, each time point, and each treatment, a total of 5 sections representing 5 different levels of tissue depths at 200 μm intervals were examined. Patterns of viral infection of the epithelial layer were evaluated. Positive FHV-1 IHC labeling in the subepithelial stroma below the basement membrane or within cartilage was excluded from evaluation. In addition, any signal localized at the section margins was also

excluded from the evaluation. Viral distribution and severity of tissue destruction were graded per section. For viral distribution, 0 points were assigned to each section with no FHV-1 labeling being detected; 1 point was assigned to a single focal cluster of FHV-1 positive epithelial cells within the examined section; 2 points were assigned to multifocal FHV1-positive clusters of epithelial cells within the examined section. For severity, 0 points were given for an intact, uninfected epithelium; 1 point was given for a mild infection, with less than 5 epithelial cells being infected and the epithelium being structurally intact; 2 points were given for moderate infections that were characterized by slightly disrupted epithelium with at least 5 epithelial cells infected; and 3 points were given for severe infections characterized by massive necrosis or total loss of epithelium with FHV-1 positive epithelial cells sloughing off and surrounding affected areas. A total IHC score per section was calculated by multiplying the points for viral distribution with the points for severity.

Statistical analyses

The mean score of total scores of 5 sections per animal at each time point in each treatment group was calculated. A two-way-ANOVA followed by post-hoc multiple comparison tests was performed to compare the difference between treatment groups at each time point. $P < 0.05$ was considered statistically significant.

RESULTS

Deletion of the gE gene or double-deletion of the gE and TK genes reduces virulence of FHV-1 when compared to WT inoculation at an MOI of 1

Tracheal tissue explants from 4 cats (No. 1-4) were inoculated with the WT, gE-, or gE-TK- mutants at an MOI of 1. At 24 and 48 hpi, the average of total scores from WT inoculated explants were not significantly different from those of the gE- mutant inoculated explants or gE-TK- mutant inoculated explants (**Fig. 3. 1A &B**). In contrast, by 72 hpi the average of the scores for the WT inoculated group was 2.85 (**Fig. 3. 1C**), which was significantly higher than scores following gE- mutant inoculation (average score= 0.75, P= 0.0135) or gE-TK- mutant inoculation (average score= 0.3, P= 0.0022).

Inoculation with FHV-1 WT at an MOI of 1 resulted in small viral antigen clusters within the cytoplasm of one or two epithelial cells with no pathological changes by 24 hpi (**Fig. 3. 2A, arrow**). Viral antigens were clearly localized at the apical part of the epithelial cells (insert of **Fig. 3. 2A)**. By 48 hpi, multiple foci of FHV-1 infection associated with mild to moderate pathological changes such as disarrangement of the pseudostratified columnar architecture and necrosis of epithelium, with degenerated and necrotic virus-infected cells detaching from the epithelium, were noted in the WT inoculated group (**Fig. 3. 2B**). However, the majority of the epithelium was still intact. At 72 hpi, WT infection caused massive epithelial necrosis with total loss of epithelium, consequently leading to large areas of exposure of the submucosal surface (**Fig. 3. 2C, arrowhead**). Epithelial cells detaching from the basement membrane and sloughing into the medium were immunohistochemically strongly positive for FHV-1. Abundant FHV-1 labeling was also detected in the subepithelial stroma, suggesting that FHV-1 breached the integrity of the basement membrane and invaded into the stroma (**Fig. 3. 2C**). In contrast,

following inoculation with the gE- mutant at an MOI of 1, tissue explants had one or multiple confined infected cell clusters at 24 hpi, sometimes with disarrangement of the pseudostratified columnar architecture and single cell necrosis of the affected epithelium (**Fig. 3. 2D**). Infected cell clusters remained confined to the epithelium (**Fig. 3. 2D insert**). While the number of gE-mutant infected cell clusters per each section was slightly increased by 48 hpi, it was reduced by 72 hpi and the severity of the pathological changes at 48 and 72 hpi (**Fig. 3. 2E-F**) was similar to the changes observed at 24 hpi. For the gE-TK- mutant inoculated group, few small virus infected epithelial cell clusters were observed at 24 and 48 hpi, but the epithelial layer retained its integrity (**Fig. 3. 2G-H**). Most of the tissues were negative for FHV-1 at 72 hpi and the epithelium remained intact with an unremarkable brush border, identical to the negative control group at 72 hpi (**Fig. 3. 2I & 3. 2J insert**).

Deletion of the PK gene significantly reduces FHV-1 virulence at all time points regardless of MOI

Tracheal tissues from 4 cats (No. 5-8) were inoculated with FHV-1 WT or the PK- mutant at an MOI of 1. While there was no significant difference between the scores of the WT and PK-mutant inoculated explants at 24 hpi (P= 0.2565) (**Fig. 3. 3A**), by 48 hpi average scores of the WT inoculated group (score = 2.75) were significantly higher than those of the PK-mutant inoculated group (score = 0.05) (P= 0.0032) (**Fig. 3. 3B**). The differences between the scores for the WT (score = 5.6) and PK- mutant inoculated group (score= 0.1) were even higher at 72 hpi (P< 0.0001) (**Fig. 3. 3C**).

When tissues from 4 cats (No. 1-4) were inoculated with FHV-1 WT or PK- mutant at an MOI of 0.1, no significant differences were detected for the scores at 24 and 48 hpi (**Fig. 3. 3D**

& 3. 3E). However, at 72 hpi (**Fig. 3. 3F**), there was a significantly (P= 0.0014) higher average score for the WT inoculated group (score = 2.75) compared to the average score for the PK- inoculated group (score = 0.1).

Histologically, small to moderate sized foci of virus-infected cells were noted in the WT inoculated group at an MOI of 0.1 at both 24 and 48 hpi. Single or multiple foci of virus-infected cells were accompanied by some pathological changes such as disturbance of the epithelial cell architecture (**Fig. 3. 4G-H**). By 72 hpi, tissue damage characterized by epithelial cell necrosis and complete epithelial loss with exposure of the submucosal surface was observed (**Fig. 3. 4I**). However, the overall area affected at an MOI of 0.1 was not as large as the area observed in the WT inoculated group at an MOI of 1. At an MOI of 1, histological findings in the WT inoculated group were similar but slightly more widely distributed and the virus infected cell clusters were larger with individual cell necrosis of the epithelium at 48 hpi (**Fig. 3. 4A-B**). By 72 hpi there was massive necrosis and loss of epithelium with exposure of the submucosal surface (**Fig. 3. 4C**). In contrast, a change in MOI did not cause a significant difference for explants inoculated with the PK- mutant. At an MOI of 0.1, only tissues from one cat had a single small area of virus infected cells within the epithelium at 24 hpi **(Fig. 3. 4J**). In addition, a small area of virus infected cells associated with focal ulceration (**Fig. 3. 4F insert**) was observed only in a single cat in the PK- inoculated group at an MOI of 1 at 72 hpi (**Fig. 3. 4F**), while the other three cats remained negative at 72 hpi. There was no other evidence of viral infection, and samples from all other PK-mutant inoculated explants were negative at all time point regardless of the MOI (**Fig. 3. 4D-E, 3. 4K-L**).

Deletion of gC gene does not significantly reduce viral spread and tissue damage

For inoculation with the gC- mutant, only an MOI of 0.1 was used as this mutant could not be grown to sufficient titers for an inoculation with an MOI of 1. Tissues from 4 cats (No. 5-8) were inoculated with either the FHV-1 WT or the gC- mutant, at an MOI of 0.1. When comparing the average scores of the WT and gC-mutant inoculated groups, no significant differences were observed at any time point ($P > 0.9999$, $P = 0.4397$, and 0.1766 respectively at 24, 48 and 72 hpi) (**Fig. 3. 5**).

Histological findings of the WT inoculation group in this set were similar to previously observed findings in cats No. 1-4 at an MOI of 0.1. Single or multiple, small to moderate sized foci of infected cells accompanied by pathological changes such as disturbance of the epithelial cell architecture were noted at both 24 & 48 hpi (**Fig. 3. 6A, 3. 6B**). Massive necrosis was observed at 72 hpi (**Fig. 3. 6C**). Tissues inoculated with the gC- mutant had small foci of virus infected cells at 24 hpi without other remarkable histological changes (**Fig. 3. 6D**). The size of such foci of virus infected cells was increased at 48 and 72 hpi, along with histological changes such as cell swelling, disarrangement of epithelial architecture and detachment of epithelial cells from the basement membrane (**Fig. 3. 6E-F**).

Stromal invasion was reduced following inoculation with deletion mutants

Stromal invasion was reviewed separately in order to clearly demonstrate the potential of viral invasions for each virus. Breakthroughs of the basement membrane barrier from the epithelial side were observed in most explants inoculated with WT by 48 hpi, and the width and the depth of the tissue damage was increased by 72 hpi (**Table 3. 1- 3. 3**). Virus-positive cells in the mesenchymal area underneath the basement membrane were morphologically consistent with

macrophages or dendritic cells (**Fig. 3. 7A**). Breakthroughs were also observed in tissues infected with the gE- mutant, characterized by epithelial necrosis and immunohistochemically positive cells invading through the basement membrane into the underlying stroma (**Fig. 3. 7B**). However, only explants from one cat exhibited a basement membrane breach while in the other gE-mutant-infected explants virus positive cells were confined to the epithelial layer (**Table 3. 1**). Most tissues inoculated with the gE-TK- mutant had no tissue damage, but there were still multiple foci in explants from one cat that demonstrated mild epithelial cell necrosis and stromal invasion of virus positive cells (**Table 3. 1, Fig. 3. 7C**). For tissues inoculated with the PK-mutant at an MOI of 1, only one virus positive area was observed among all tissue sections (**Table 3. 2**). This area was characterized by virus positive epithelial cells as well as stromal invasion of virus positive cells (**Fig. 3. 7D**), although the breach was localized with no further detection of virus infected cells in the underlying tissues. No stromal invasion was noted in tissue sections inoculated with the PK- mutant at an MOI of 0.1 (**Table 3. 2**), as foci of virus positive cells remained confined to the epithelial layer and there was no evidence of invasion through the basement membrane. Tissues inoculated with the gC- mutant at an MOI of 0.1 did not demonstrate any stromal invasion at 24 hpi and 48 hpi, but there were areas of severe coagulative necrosis at 72 hpi (**Fig. 3. 7E**) with two cats exhibiting virus positive cells invading through the basement membrane (**Table 3. 3**).

DISCUSSION

This study corroborates and extends previous studies from our laboratory, where we used three dimensional primary feline respiratory epithelial cell (FREC) cultures to study viral kinetics and epithelial innate immunity following inoculation with FHV-1 and FHV-1 deletion mutants (Lee et al., 2019; Nelli et al., 2016). Moreover, this study provides additional information because explants allow for investigating viral invasiveness, viral spread to deeper cell layers, virus induced tissue damage and breaches of the integrity of the basement membrane. Based on the previous results (Lee et al., 2019), we used an MOI of 1 for inoculations of tracheal explants with gE-, gE-TK- and PK- mutants and the WT control. We used an MOI of 0.1 for inoculations with PK- and gC- mutants and the WT control since these mutants were difficult to propagate at higher titers. This is consistent with Leeming et al. who described FHV-1 growth kinetics in tissue explant cultures at an MOI ranging from 0.1 to 100 (Leeming et al., 2006). Leeming et al. also showed that tissue explants supported WT FHV-1 replication and that the epithelial structure could be maintained for up to 72 hpi when the MOI was equal or smaller than 1, but that at an MOI >1, it was difficult to appreciate the histological changes over time (Leeming et al., 2006). Furthermore, it has been shown that cilia movement and cell viability of tissue explants exposed to media alone were within normal range for up to 72 hpi, but started to decline thereafter (Leeming et al., 2006; Li et al., 2015). Seventy-two hpi was chosen as the end-time point in the present study accordingly. The data presented here, in combination with our previous studies, verified that the *in vitro* and *ex vivo* culture systems are emerging as valuable tools for studying FHV-1 viral characteristics and innate immune responses prior to final *in vivo* studies in cats.

Consistent with our previous study, we found that the double-deletion of gE and TK (gE-TK-) resulted in significant attenuation at an MOI of 1 and caused low pathology scores of inoculated explants, which were no significantly different from those of negative controls. In FRECS, inoculation with the gE-TK- mutant led to small and indistinct plaques, whereas FRECs inoculated with the WT exhibited significant cytopathic effect (CPE) starting at 24 hpi (Lee et al., 2019). Furthermore, tissue explants inoculated with a single gE deletion mutant (gE-) exhibited smaller sized viral plaques asscociated with mild pathological changes at 72 hpi, whereas WT inoculation led to massive necrosis at this time point. In contrast, we previously showed that FRECs inoculated with the gE- mutant exhibited identical growth curves compared to FRECs inoculated with WT (Lee et al., 2019). In multiple alphaherpesviruses including pseudorabies, HSV-1, and FHV-1, gE has been identified to be important for intracellular transport and cell-to-cell spread (Mijnes et al., 1996; Polcicova et al., 2005; Tirabassi and Enquist, 1998). The impact of the deletion of the gE gene on FHV-1 growth kinetics is more evident in the tissue explant assay than previously observed in the FREC system. This is likely due to the fact that the microstructure of the epithelium is more completely maintained in explants compared to FRECs and a complete histologic examination could be performed in the current study. However, both systems clearly identified the double-deletion of gE and TK as less virulent compared to the single deletion mutant. In the present study, only explants from one cat were positive for FHV-1 at 72 hpi following inoculation with the gE-TK- mutant. In constrast, explants from all 4 cats inoculated with the gE- mutant had positive immunohistochemical labeling for FHV-1 at 72 hpi.

Similar to double-deletion of gE and TK genes, a single-deletion of the PK gene also significantly attenuated the virulence of FHV-1 in the explant system. In our previous study using FRECs, inoculation with the PK- mutant at an MOI of 0.1 caused no CPE until 96 hpi, at

which the cells from 2 cats had multiple plaques of various sizes (Lee et al., 2019). Plaques were characterized by necrosis of individual superficial cells with some live cells with intact cell junctions at the bottom of the plaques (Lee et al., 2019). In the present study no significant pathological changes were seen in tissues inoculated with the PK- mutant, either at an MOI of 0.1 or 1. Explants from only one cat labeled immunohistochemically positive for FHV-1 following inoculation with the PK- mutant regardless of the MOI used. This cat had small sized foci of virus-infected cells located superficially in the epithelium at 72 hpi. This major reduction of virulence resulting from the deletion of PK gene was not influenced by increasing the MOI to 1. An attenuation of viral transmission in mutants with a deletion of the US3-encoded PK gene has also been shown in *ex vivo* cultures of other alphaherpesviruses such as pseudorabies virus and BHV-1 (Wagenaar et al., 1995; Zhao et al., 2017). Such attenuation may be attributed to insufficient PK-mediated phosphorylation, leading to a lack of phosphorylated proteins, including lamins from nuclear membranes and functional viral gB, all of which are required for the virus to exit through the nuclear membrane and for further viral transmission (Jacob et al., 2011; Wagenaar et al., 1995). In addition, PK has been recognized as an immune modulator in infection studies with HSV-1 and shown to influence interferon signaling via multiple mechanisms, including inhibition of expression of Toll-like receptor 3 and interferon receptors. Also, PK has been shown to disturb the activation of regulatory transcription factor 3 gene (IRF3) (Lewandowski et al., 1994; Peri et al., 2008; Wang et al., 2013). It was previously revealed that the deletion of PK gene impaired the immune modulation of FHV-1 in FRECs, reverting an up-regulation of IL10 gene expression and a down-regulation of chemoattractant secretions in host cells (Lee et al., 2019). This study suggested that the virulence of the PK gene-deficient FHV-1 mutant was significantly reduced while host innate immune defense was maintained.

The deletion of the gC gene alone did reduce end-point FHV-1 titers in FRECs *in vitro*, but the virus growth curves demonstrated a moderate reduction of virulence of gC- mutant compared to those of FRECs inoculated with the PK- mutant or the gE-TK- double-deletion mutant (Lee et al., 2019). Severe cell lysis was still observed starting at 48 hpi in FRECs inoculated with the gC- mutant at an MOI of 0.1, and CPE in gC- mutant inoculated FRECs could not be distinguished from CPE following WT inoculation (Lee et al., 2019). Similarly, in the present study, the scores of the tissue explants inoculated with the gC- mutant were lower but not statistically significantly different from the scores of the explants inoculated with the WT at 72 hpi. Nevertheless, the size of the gC- mutant-infected cell clusters seen in the tissue explants was smaller than those seen following WT inoculation. However, in contrast to the explants inoculated with the gE-TK- or PK- mutants, more than one infected cell cluster could be observerd per section in the gC- mutant group. Glycosaminoglycan heparan sulfate has been identified as the cellular receptor site used by gC for initial attachment. Therefore, it was thought that deleting gC would reduce virulence (Kaashoek et al., 1998; Osterrieder, 1999; Tal-Singer et al., 1995). However, for HSV-1 it has been shown that the initial attachment, or so-called tethering, initiated by gC binding to glycosaminoglycan heparan sulfate, is not required for viral entry (Laquerre et al., 1998). Our findings in the present study, in addition to our previous study, are consistent with those observations, since infection with the gC- mutant was not different from the WT infection at early time points. It is likely that there are gC-independent entry routes that might compensate for reduced attachment, possibly through other receptors such as herpes virus entry mediator (HVEM), nectins, and 3-O-S-site of heparan sulfate (Montgomery et al., 1996; Shukla et al., 1999). However, deletion of gC has been shown to reduce the cell-to-cell spread of alphaherpesvirus in horses in a later stage of infection (Osterrieder, 1999), which may

explain why in our present study the lesions in tissues inoculated with the gC- mutant were smaller than those following WT inoculation at 72 hpi. While functional assays to study viral entry and egress would be of interest to further investigate the role of FHV-1 gC and our assay system was limited to experiments at an MOI of 0.1 for this mutant, the goal of the present study was to study tissue damage and viral invasiveness and identify the most promising vaccine candidates for use in cats. Using our system, the gE-TK- and PK- mutants could clearly be identified as superior when compared to the gC- mutant.

Epithelial integrity is an important host defense mechanism against alphaherpesvirus infection. The ability of the virus to penetrate the basement membrane and to infect underlying tissues and cells has important implications for viral pathogenesis. Furthermore, the loss of the integrity of tight junctions between epithelial cells has been shown to play an important role in facilitating viral invasion of EHV-1 (Van Cleemput et al., 2017). Bovine herpesvirus-1, HSV-1, and pseudorabies virus have been shown to break through the basement membrane of the respiratory epithelium (Glorieux et al., 2011; Glorieux et al., 2009; Steukers et al., 2012). On the other hand, infection with EHV-1 did not disrupt the basement membrane, but instead individual virus infected mononuclear cells could be found below the basement membrane (Vandekerckhove et al., 2010). A previous study using FHV-1 strain B927 in feline tracheal rings demonstrated that viral infection was limited to the tracheal epithelium and FHV-1 did not break through the basement membrane (Monne Rodriguez et al., 2017). However, another study using FHV-1 strain C27 in feline tracheal tissue explants and corneal explants demonstrated that the virus invaded the underlying tissues by disrupting the integrity of the basement membrane (Li et al., 2015). In the present study we observed clear evidence of stromal invasion with disruption of the basement membrane following inoculation with WT. Interestingly, none of the

deletions in the present study completely prevented a breach of the basement membrane. Studies with pseudorabies have shown that PK facilitated viral invasion and breaking through the basement membrane by manipulating Rho GTPase signaling, which regulates the organization of the cytoskeleton, and a deletion of PK prevented the mutant from breaking through the basement membrane (Jacob et al., 2015; Lamote et al., 2016). Impairment of the ability to breech the basement membrane has also been shown in a US3-deleted BHV-1 following inoculation of a respiratory tissue explant (Zhao et al., 2017). In the present study, inoculation of the explants with the PK- mutant resulted in a comparatively small and very limited infection of the epithelium compared to the WT infection at all time points, but limited crossing of the basement membrane was still observed in explants from a single cat. Deletion of the gC gene has not been shown to affect the disruption of the basement membrane by BHV-1 (Zhao et al., 2017) similar to our study, where a breach of the basement membrane was still observed in tissues inoculated with the gC- mutant of FHV-1. However, the extent of the disruption was much smaller than observed with WT inoculation. We speculate that a lack of gC reduces expansion of viral invasion. The function of gE, in conjunction with gI, is to initiate fusion and to envelope the nucleocapsids for further delivery into cytoplasmic vesicles (Brack et al., 2000). Human HSV-1 research has shown that the gE-gI complex specifically accumulates at the tight junctions, which is a key factor for cell-to-cell spread of virus laterally through the tight junction in highly polarized epithelial cells (Johnson et al., 2001). HSV-1 gE- mutants exhibited random rather than directional virus spread within epithelial cells, which drastically decreased the efficiency of infection (Wisner et al., 2000). However, gE deficiency seemed insufficient to block the virus from stromal invasion based on our findings. Thymidine kinase (TK) is a key *in vivo* virulence factor for alphaherpesviruses including BHV-1, HSV-1, pseudorabies virus, and FHV-1 (Field

and Wildy, 1978; Kit et al., 1985a; Kit et al., 1985b; Yokoyama et al., 1996). Generating a double-deletion mutant (gE-TK-) has been shown to be an excellent candidate for vaccine development for pseudorabies virus (Kimman et al., 1994). Similar to what was seen following the inoculation with the PK- mutant, double-deletion of gE and TK genes significantly reduced virulence and tissue damage of FHV-1 infection, but again did not completely eliminate stromal invasion of the virus.

In the present study, FHV-1-infected cells, which were morphologically consistent with dendritic cells or macrophages, were observed in the underlying tissues below the basement membrane. It is known that dendritic cells, monocytes, lymphocytes, and fibroblasts can all be infected by herpesviruses (de Jong et al., 2008; Laval et al., 2015; Wilsterman et al., 2011), and those cells can be the carriers or so-called "trojan horses" for the virus to spread systemically or just contribute to the local immune response against infection (Laval et al., 2015; Wilsterman et al., 2011; Yang et al., 2017). Viral replication may be limited within these mononuclear cells depending on the presence of immune stimulation (van Der Meulen et al., 2000). We have previously shown that the inoculation of FRECs with WT, gE-, and gC- reduced the expressions of IL8 and CXCL1, which could consequently reduce the chemoattractions of neutrophils and T cells (Lee et al., 2019). However, the current study did not clearly show any aggregation or infiltration of the immune cells in any inoculation group, which is most likely limited in this system. The cell characterization for immune cells present amid the viral clusters, including T lymphocytes, dendritic cells, and macrophages, will be performed in the future, which chould shed some light on the viral pathogenesis at the respiratory tract.

In conclusion, this study focused on the morphological changes of respiratory tissue explants inoculated with FHV-1 WT, or gC-, gE-, gE-TK-, and PK- mutants with the goal of

comparing virulence and viral spread between WT and mutant viruses as part of the selection of FHV-1 mutants for follow-up *in vivo* experiments. Microscopic findings were evaluated via a semiquantitative scoring system and extended our previous observations in primary FREC cultures, which focused on viral growth kinetics and the modulation of innate immunity. Here we demonstrate that inoculation of tracheal tissue explants with either a PK- or a gE-TK- mutant resulted in significantly lower IHC/pathology scores compared to inoculation with WT. Tissues infected with the gC- or the gE- mutant exhibited low to moderate scores. The present study corroborates our previous findings using FREC cultures and suggest that the PK- and gE-TK- mutants are significantly attenuated. Results from our previous study using FRECs also show that the PK- and gE-TK- mutants still induce innate immune responses at the respiratory epithelium (Lee et al., 2019). Thus, the PK- and gE-TK- mutants may be ideal candidates for new approaches to immunize cats against feline viral rhinotracheitis.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

FUNDING

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APPENDIX

Table 3. 1. Number of explants from cats positive for basement membrane breach (numerator) out of the total number of cats (denominator) at each time point. MOI: multiplicity of infection; hpi: hours post-inoculation.

	Treatment	24 hpi	48 hpi	72 hpi
MOI= 1	WT	1/4	3/4	4/4
	gE-	1/4	1/4	1/4
	gE-TK-	0/4	2/4	1/4

Table 3. 2. Number of explants from cats positive for basement membrane breach (numerator) out of the total number of cats (denominator) at each time point. MOI: multiplicity of infection; hpi: hours post-inoculation.

	Treatment	24 hpi	48 hpi	72 hpi
MOI= 1	WT	3/4	4/4	4/4
	PK-	0/4	0/4	1/4
MOI= 0.1	WT	1/4	2/4	3/4
	PK-	0/4	0/4	0/4

Table 3. 3. Number of explants from cats positive for basement membrane breach (numerator) out of the total number of cats (denominator) at each time point. MOI: multiplicity of infection; hpi: hours post-inoculation.

	Treatment	24 hpi	48 hpi	72 hpi
MOI= 0.1	WT	1/4	3/4	3/4
	gC-	0/4	0/4	2/4

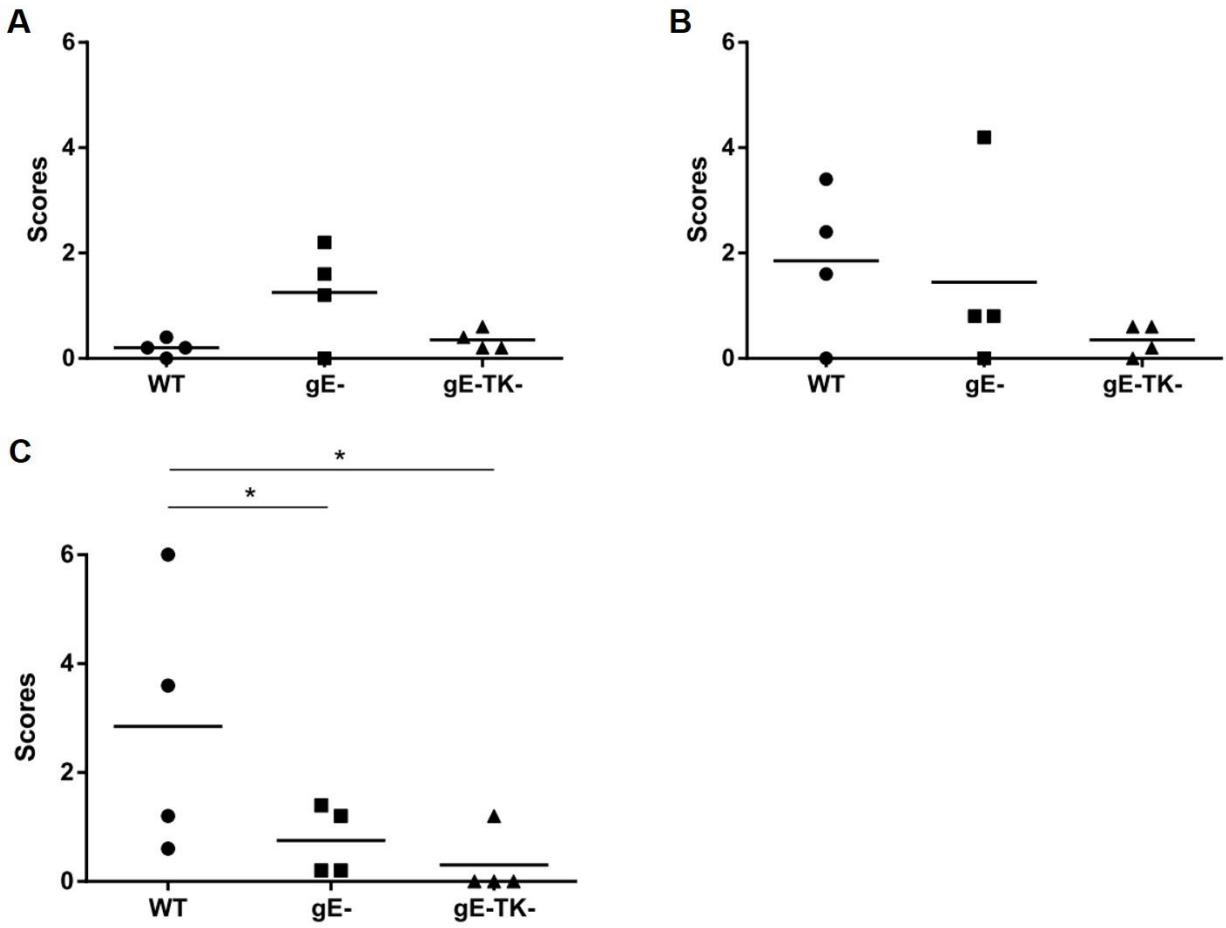

Figure 3. 1. Total IHC scores of tissue explants inoculated with WT, gE-, and gE-TK- at an MOI of 1, at 24 (A), 48 (B), and 72 hpi (C). Each data point represented the average score from 5 sections per cat, and lines represented the average score from 4 cats in each treatment group. Asterisks represented a significant difference between groups ($P < 0.05$).

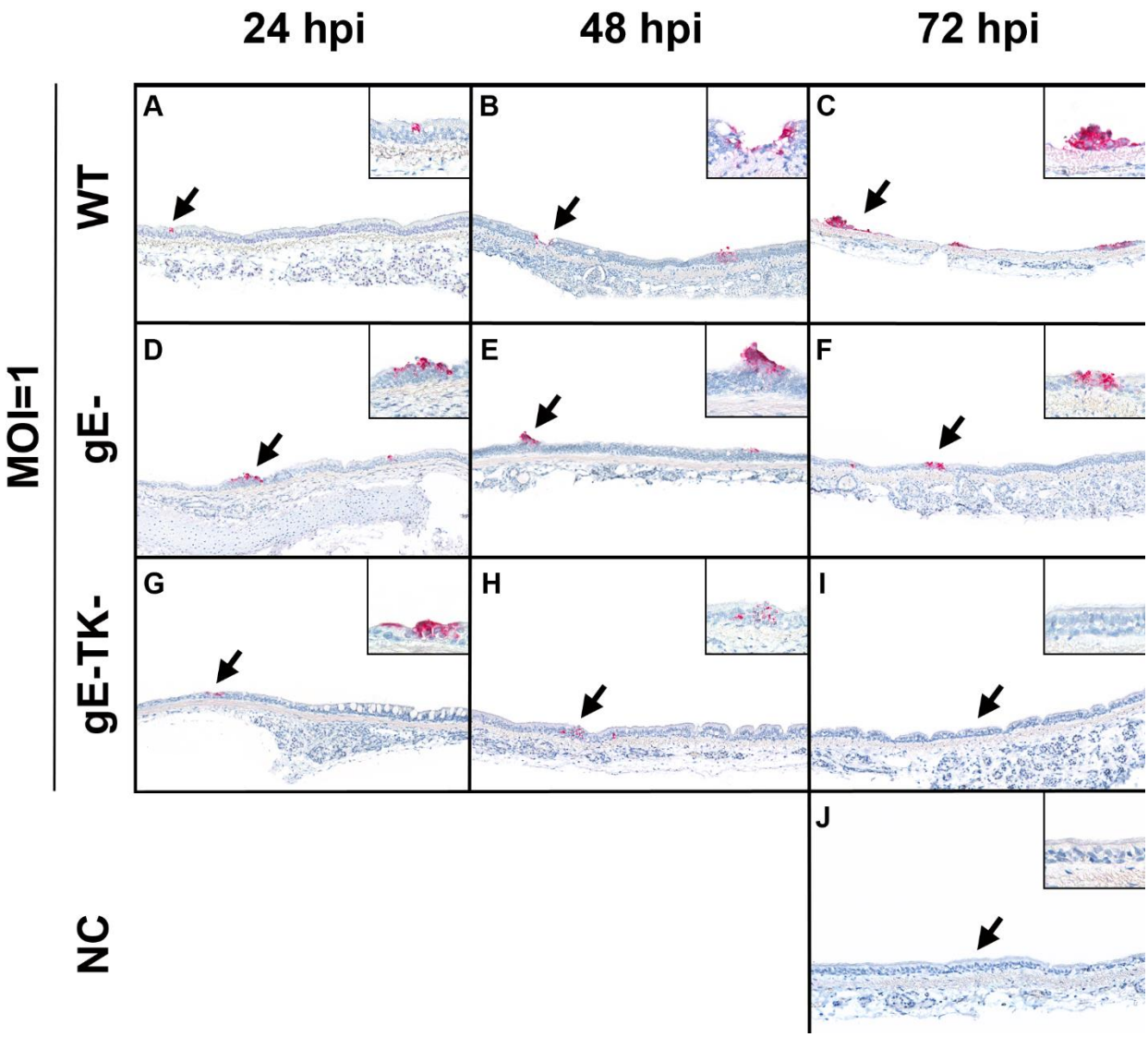

Figure 3. 2. Representative images of IHC-stained tissue sections inoculated with WT (A-C)**, gE-** (D-F)**, and gE-TK-** (G-I) at an MOI of 1, at 24, 48, and 72 hpi. A representative image of negative mock inoculated control (NC) was shown at 72 hpi (J). The FHV-1 antigen was stained in red and hematoxylin was used for counterstaining. Inserts were higher magnification of areas highlighted by the arrows.

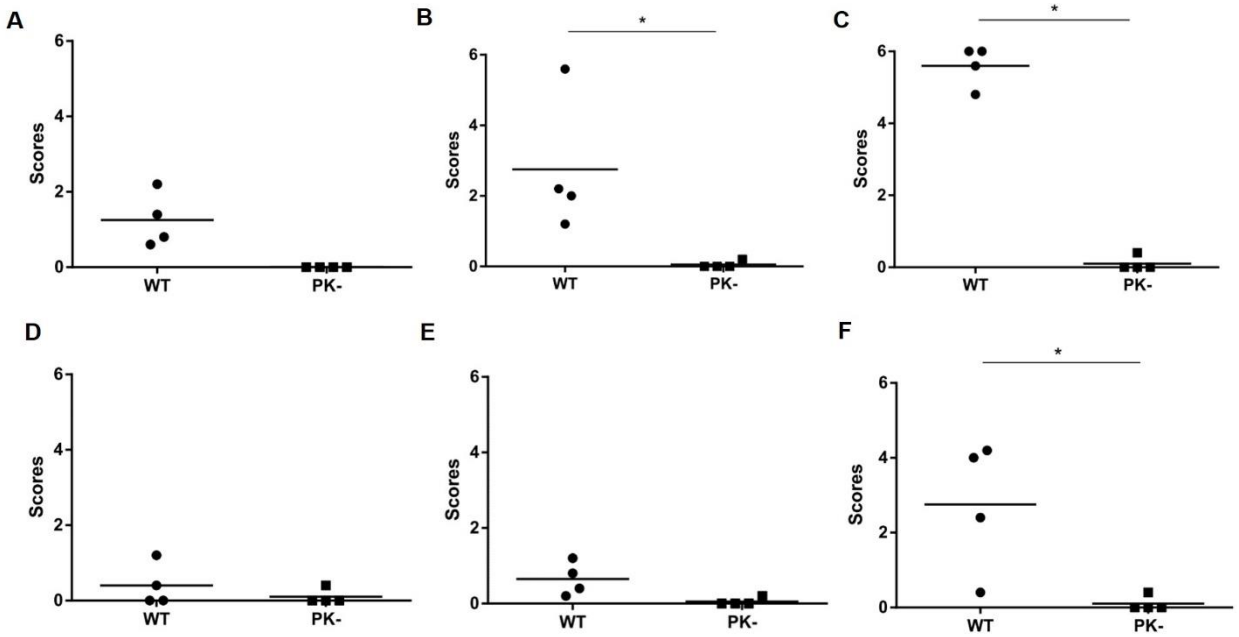

Figure 3. 3. Total IHC scores of tissue explants inoculated with WT and PK- at an MOI of 1, at 24 (A), 48 (B), and 72 hpi (C) and an MOI of 0.1, at 24 (D), 48 (E), and 72 hpi (F). Each data point represented the average score from 5 sections per cat, and the lines represented the average score from 4 cats in each treatment group. Asterisks represented a significant difference between groups ($P < 0.05$).

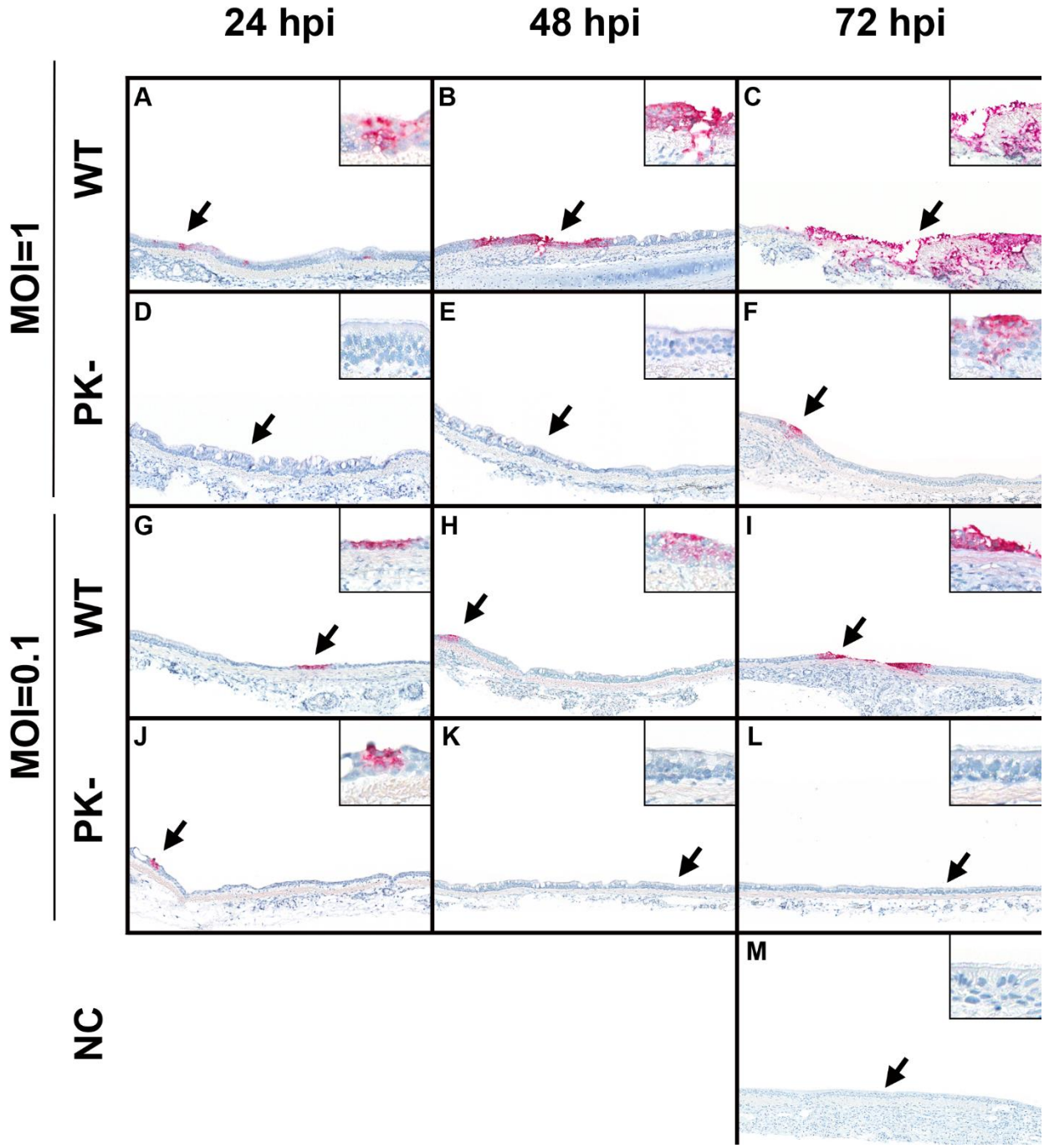

Figure 3. 4. Representative images of IHC-stained tissue sections inoculated with WT (A-C at an MOI of 1, G-I at an MOI of 0.1) **and PK-** (D-F at an MOI of 1, J-L at an MOI of 0.1) at 24, 48, and 72 hpi. A representative image of negative mock inoculated control (NC) was shown at 72 hpi (M). The FHV-1 antigen was stained in red and hematoxylin was used for counterstaining. Inserts were higher magnification of areas highlighted by the arrows.

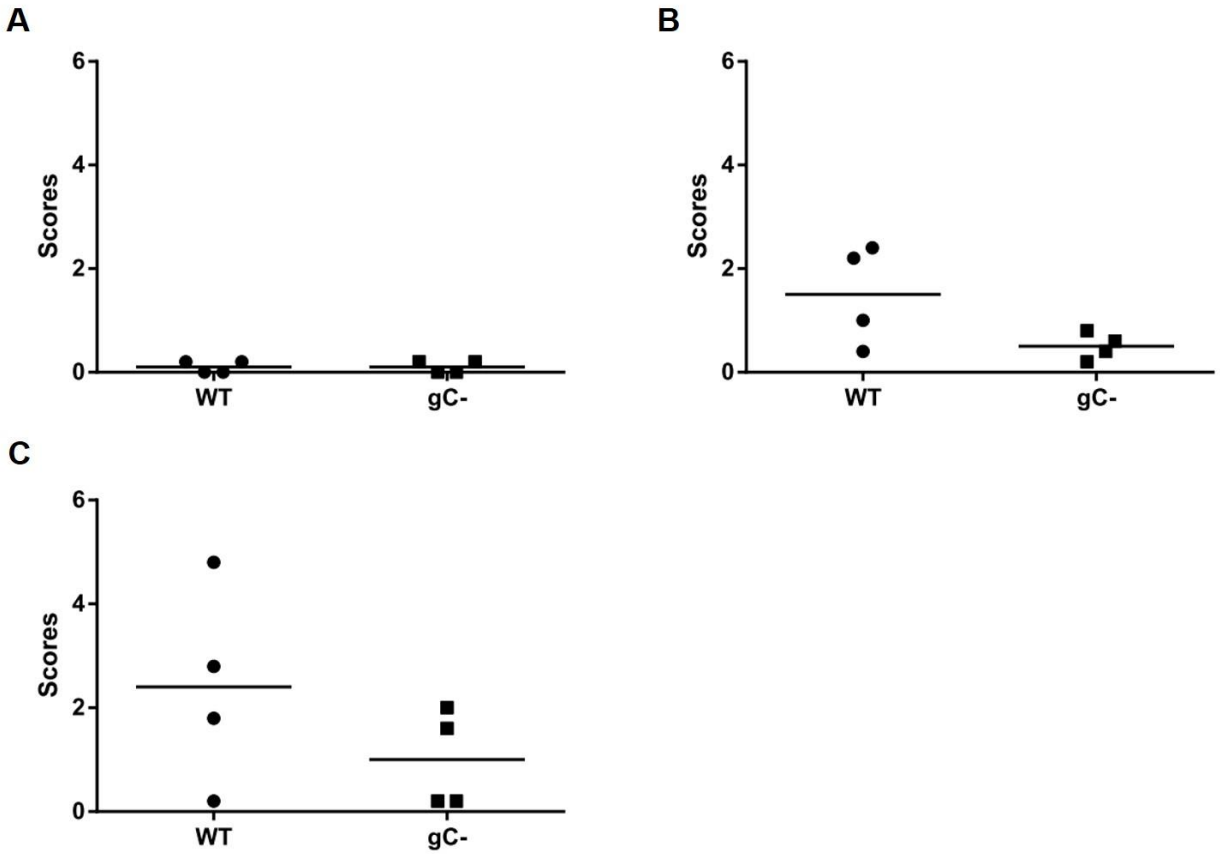

Figure 3. 5. Total IHC scores of tissue explants inoculated with WT and gC- at an MOI of 0.1, at 24 (A), 48 (B), and 72 hpi (C). Each data point represented the average score from 5 sections per cat, and the lines represented the average score from 4 cats in each treatment group.

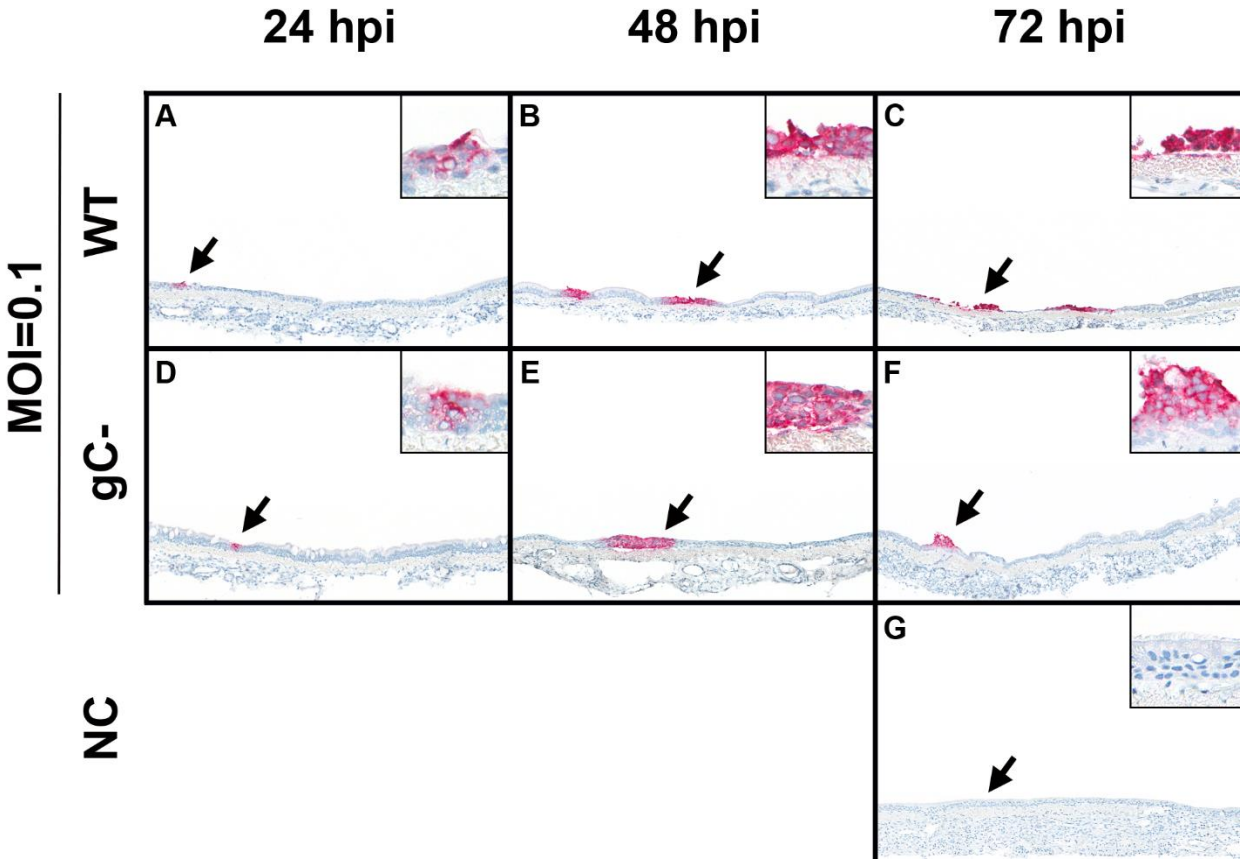

Figure 3. 6. Representative images of IHC-stained tissue sections inoculated with WT (A-C) **and gC-** (D-F) at an MOI of 0.1, at 24, 48, and 72 hpi. A representative image of negative mock inoculated control (NC) was shown at 72 hpi (G). The FHV-1 antigen was stained in red and hematoxylin was used for counterstaining. Inserts were higher magnification of areas highlighted by the arrows.

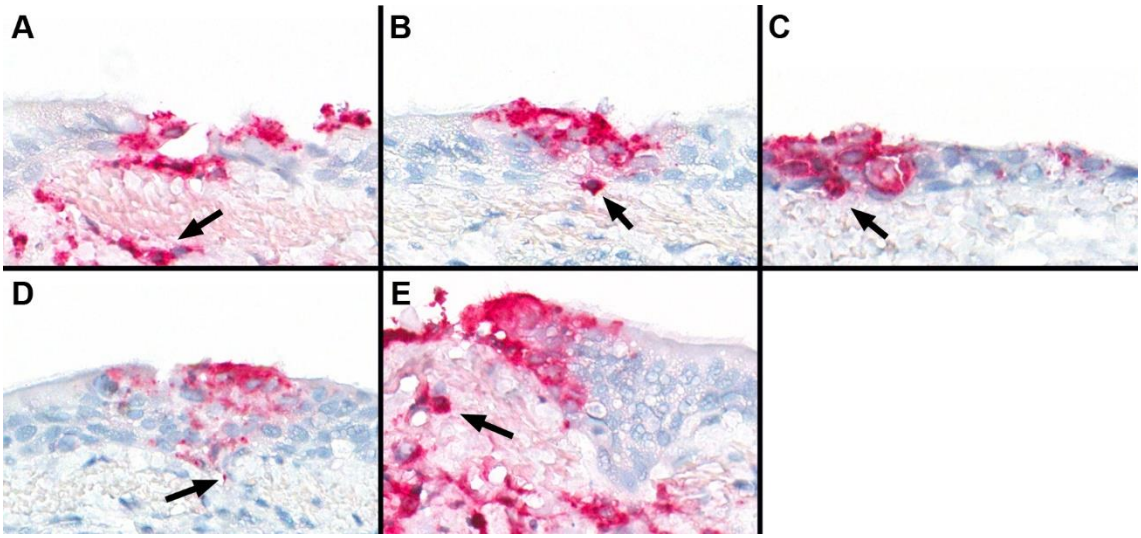

Figure 3. 7. Images of IHC-stained tissue sections exhibiting stromal invasion at 72 hpi inoculated with WT at an MOI of 1 (A), gE- at an MOI of 1 (B), gE-TK- at an MOI of 1 (C), PK- at an MOI of 1 (D), and gC- at an MOI of 0.1 (E). The FHV-1 antigen was stained in red and hematoxylin was used for counterstaining. The infected cells breaking the basement membrane were indicated by arrows.

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CHAPTER 4

SAFETY AND EFFICACY OF FELINE HERPESVIRUS-1 DELETION MUTANTS IN CATS

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Safety and Efficacy of feline herpesvirus-1 deletion mutants in cats

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ABSTRACT

Feline herpesvirus-1 (FHV-1) is an important viral respiratory and ocular pathogen of cats worldwide. FHV-1 vaccines are core vaccines in the US. However, immunity induced by current FHV-1 vaccines is limited and relatively short-lived. Specifically, current FHV-1 vaccines reduce the severity of clinical symptoms, but they do not prevent infection, viral nasal shedding and latency. To address these shortcomings, we have previously constructed FHV-1 gE-TK- and a PK- deletion mutants using bacterial artificial chromosome (BAC) and shown safety and immunogenicity in vitro. In this study we compared the safety and efficacy of a combined subcutaneous and mucosal vaccination regime using the FHV-1 gE-TK- and PK- mutants with commercial vaccination in cats.

Four groups were established and included an unvaccinated control group, a commercial vaccine group, a gE-TK- mutant group, and a PK- mutant group. Cats in gE-TK- and PK- mutant groups were vaccinated twice subcutaneously followed by an intranasal boost. Cats in the commercial vaccine group were vaccinated twice according to the manufacturers' instructions. All four groups were challenge infected, and clinical disease, nasal shedding, virus neutralization antibodies and cytokine mRNA gene expressions were evaluated.

Vaccination with gE-TK- and PK- mutants was safe and resulted in significantly reduced clinical disease and viral nasal shedding following infection. This protection was associated with induction of virus neutralization antibody and interferons after immunization and a reduction of IL-1β, an induction of TNFα and TGFβ and a prevention of IL10 reduction after challenge infection. In conclusion, the present study showed the merits of incorporating a mucosal immunization component with deletion in virulence and immune modulatory genes, for prevention of FHV-1 infection in cats.

INTRODUCTION

Feline herpesvirus-1 (FHV-1), a member of the alphaherpesvirinae, is an important viral pathogen of cats worldwide. The clinical signs associated with FHV-1 include fever, rhinotracheitis, pneumonia, conjunctivitis, keratitis, neonatal fatality, and potentially facial and nasal dermatitis and abortion (Dawson et al., 2001; Nasisse et al., 1998). It is estimated that FHV-1 accounts for approximately 50-75% of all diagnosed viral upper respiratory infections in cats (Maes, 2012) and because of this prevalence, FHV-1 vaccines are part of the feline core vaccines (Day et al., 2016). Current recommendations by the World Small Animal Veterinary Association (WSAVA) are to vaccinate kittens against infection with FHV-1 at 6-8 weeks old, followed by at least one additional administration 2-4 weeks later (Day et al., 2016). Annual revaccination should be carried out in high-risk cats, such as pregnant queens or cats living in multi-cat environments.

Both inactivated and MLV feline herpesvirus vaccines are widely used in the United States, but protection from FHV-1 is limited in efficacy and shorter in duration compared to feline calicivirus (FCV) and feline panleukopenia virus (FPV), the other two components of feline core vaccination. Currently used FHV-1 vaccines reduce the severity of clinical symptoms, but they do not prevent FHV-1 infection itself in previously vaccinated cats and, as a consequence, cannot curtail establishment of virulent FHV-1 latency (Maes, 2012; Richter et al., 2009). Environmental, physiologic and chemical stressors can all lead to reactivation from latency, and as such, are associated with renewed replication and shedding of infectious virus (Gould, 2011; Johnson and Maggs, 2005). This implies that clinically normal cats can shed virus and cause disease in unvaccinated animals, explaining the high incidence in shelters. Reactivation also plays an important role in immune-mediated dendritic keratitis.

Besides of their inability to prevent infection, mucosal administration of current vaccines labeled for SC use is not safe because the basis of their attenuation is the natural temperature sensitivity of FHV-1. Thus, current MLV vaccines contain the complete set of viral genes that are involved in replication, virulence and immunomodulation and are attenuated at body temperature. However, at the reduced temperature of the upper respiratory tract, the vaccine virus retains its virulence. Reports on virulence of the vaccine virus in cats intranasally (IN) immunized with a commercial vaccine labeled for subcutaneous (SC) use, or in cats with inadvertent mucosal exposure following subcutaneous administration, are therefore not unexpected (Day et al., 2016; Gaskell et al., 2002; Orr et al., 1978)

Despite widespread vaccination, FHV-1 continues to circulate. This accentuates the real need for a vaccine that provides more comprehensive protection than those that are currently available. It is likely that stimulation of mucosal respiratory immunity plays an important role in stimulating responses that are crucial for more comprehensive protection. With the introduction of molecular biology and recombinant DNA technology over the past years, it is possible to advance vaccine technology, for example, by generating gene-deleted viruses. This strategy has been shown to lead to mutants that could be used as vaccines for pseudorabies virus (PRV), bovine herpesvirus-1 (BHV-1), and equine herpesvirus-1 (EHV-1) (Kaashoek et al., 1996; Kimman et al., 1994; Tsujimura et al., 2009). In particular, the development and very successful use of a PRV gE-deletion mutant to protect against Aujeszky's disease in pigs was a motivator for the development of next-generation vaccines for animal use (van Oirschot et al., 1990). Furthermore, Kimman et al. reported on a combined *in vitro* and *in vivo* PRV study, which encompassed multiple deletion mutants, including a gE-deletion mutant (gE-), an Us3-encoded serine/threonine protein kinase-deletion mutant (PK-), a thymidine kinase-deletion mutant (TK-),

a gE-PK-double-deletion mutant (gE-PK-), and a gE-TK-double-deletion mutant (gE-TK-). Pigs inoculated intranasally with the gE-PK- or gE-TK- mutants excreted low amounts of virus, but the levels of virus shedding in those inoculated with the gE-, PK-, and TK- mutants and WT raised concerns about the actual safety of the gE-, PK-, and TK- mutants. In terms of protection against challenge virus replication, pigs immunized with the PK- mutant showed complete protection (Kimman et al., 1994). In cattle, immunization with mutants with deletions of gE (gE-), TK (TK-), or both gE and TK (gE-TK-) induced immunity which protected calves from BHV-1 infection. Experimentally intranasal or intramuscular vaccination with gE- mutants drastically reduced viral shedding and alleviated the symptoms of clinical disease after WT EHV-1 challenge in foals (Tsujimura et al., 2009).

Regarding FHV-1 in cats, gI-gE- or TK- mutants of FHV-1 have been generated previously and shown to induce protective immunity (Sussman et al., 1995; Yokoyama et al., 1996). All of the above studies suggest that virulence factor-associated gene-deletion mutants not only have the potential for reduced virulence compared to their parent strain, but also to retain a level of antigenicity required to protect against both clinical signs and infection itself.

We have previously described the generation of gE-, PK-, gC- and gE- TK- mutants of FHV-1 by using bacterial artificial chromosome mutagenesis (BAC) (Tai et al., 2016, 2010). Based upon their characterization in CRFK cells, feline respiratory epithelial cells (FRECs) and tracheal explants (Lee et al., 2020, 2019), the PK- mutant and the gE-TK-double deletion mutant were selected for further evaluation of safety and efficacy in cats as described in this paper.

MATERIALS AND METHODS

Animals

A total of 20 male domestic short hair specific-pathogen-free (SPF) cats were purchased from Liberty Research Laboratories for this study. All cats were 3.5 months old on arrival. Each group was housed in a separate room and cats within each group were kept separate to avoid cross-contamination. All cats were acclimatized for 7 days and cats were fed a diet of dry and moist food and had access to ad libitum water throughout the study. All protocols performed in this study were approved by the Animal Care and Use Committee at Michigan State University (MSU), East Lansing, MI, USA.

Experimental design

An experimental timeline of the study is shown in **Table 4. 1**. Cats were randomly divided into 4 groups upon arrival: 1) Unvaccinated controls, n= 4; 2) Commercial vaccinates, n= 4, immunized subcutaneously (SC) on days 21 and 42, with a commercial adjuvant-free modified-live vaccine according to the manufacturer's recommendations; 3) gE-TK- vaccinates, n= 6, vaccinated SC with the gE-TK- mutant on days 0 (V1) and 21 (V2), followed by an intranasal (IN) boost on day 42 (V3); 4) PK- vaccinates, n= 6, vaccinated SC with the PK- mutant on days 0 and 21, followed by an IN boost on day 42. All groups were challenged (CH) intranasally with 4×10^5 $TCID_{50}$ of virulent FHV-1 on day 63. Clinical signs and body temperatures were monitored daily throughout the study. Nasal swabs were collected prior to the begin of the study and on days 2, 21, 23, 42, 44, 46, 48, 50, 52, 62, 65, 67, 69, 71, 73, 75, 77, 81 of the study as well as the time of necropsy, and were placed in 1 ml of transport medium and stored at -80^{o}C until further processing. Blood for measurement of virus neutralizing antibody titers was

collected into serum separator tubes on days 0, 14, 21, 35, 42, 56, 63, and 77. In addition, 2.5 ml of whole blood for isolation of mRNA was collected in PAXgene® blood RNA tubes (IVD) [PreAnalytix GmbH, Hombrechtikon, Switzerland] on days 0, 23, 44, and 65. Necropsies with gross examinations and tissue collections were performed between days 83 and 86. On the day of necropsy, cats were initially anesthetized with isoflurane, followed by euthanasia by intravenous injection of 85.9 mg/kg pentobarbital sodium.

Viruses

Deletion mutants of FHV-1 included a double-deletion-mutant (gE-TK-) as well as a single-deletion-mutant (PK-) and were constructed using the full-length bacterial artificial chromosome (BAC) clone of the C27 strain of FHV-1 [ATCC, VR-636, Manassas, VA, USA] and two-step Red-mediated recombination (Tai et al., 2016). A low passage clinical FHV-1 isolate from the Veterinary Diagnostic Laboratory (VDL) at MSU [FHV-1 1627] was used as the challenge virus on day 63 at a dose of 4×10^5 $TCID_{50}$ per cat via intranasal instillation in anesthetized cats. For virus propagation and titration, the Crandell-Rees feline kidney (CRFK) cell line [ATCC, CCL-94] was used. For SC administrations of the gE-TK- or PK- mutants a dose of 1×10^6 $TCID_{50}$ was used per cat. For IN administration on day 42 a dose of 1×10^6 $TCID_{50}$ per cat was used for the gE-TK- mutant and a dose of 5×10^5 $TCID_{50}$ per cat was used for the PK- mutant. The total dose was divided into 2 aliquots, with 100 ul total volume of each, and administered dropwise into each nostril to cats that had been anesthetized. A lower dose was chosen for the PK- mutants because titer of the PK- mutant could not be increased in CRFK cells.

Clinical signs and body weights

The scoring method used to quantitate clinical signs of FHV-1 has been described previously (Sussman et al., 1995) and is shown in **Table 4. 2**. Briefly, clinical signs included conjunctivitis, blepharospasm, ocular discharge, nasal discharge, sneezing, nasal congestion, coughing, and fever. Cats were evaluated and quantitated daily and scores were assigned to each cat for each day. Scores for each group were then calculated for each time period after vaccination and after challenge infection and corrected for numbers of cats in each group. This resulted in a total clinical score for each group pre-vaccination, post vaccination 1 (post-V1, day 0-21), post vaccination 2 (post-V2, day 22-42), post vaccination 3 (post-V3, day 43-63) and post challenge infection (post-CH, day 64-83).

Real-time PCR on nasal swab extracts

Nasal swabs were collected into 1ml of viral transport media (EMEN [Sigma-Aldrich, St. Louis, MO, USA] supplemented with 1% penicillin-streptomycin [Life Technologies, Carlsbad, CA, USA]. Total DNA was extracted from 200 µl aliquots of nasal swab samples using the DNeasy Blood and Tissue kit [QIAGEN, Hilden, Germany]. Extracted DNA was quantitated with a Nanodrop spectrophotometer [Thermo Fisher Scientific, Waltham, MA, USA] and real-time PCR on 20 ng of extracted DNA 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] as previously described (Vögtlin et al., 2002). Briefly, 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. The PCR amplification parameters were 2 min at 50℃ and 30 s at 95℃, followed by 40

cycles of denaturation at 95℃ for 15 s and annealing-elongation at 60℃ for 1 min. Each sample was analyzed in triplicate.

Conventional PCR to differentiate between WT virus and mutant viruses

In order to differentiate WT from the mutant viruses in nasal swabs, conventional PCR assays targeting FHV-1 gE, PK, and TK genes were performed, as previously described (Tai et al., 2016). Sequences of forward and reverse primers are listed in **Table 4. 3**. A 20 µl PCR cocktail was composed of 10 µl GoTaq® G2 Green Master Mix [Promega, Madison, WI, USA], 1.2 µl of forward primer (240 nM), 1.2 µl of reverse primer (240 nM), water 3.6 µl, and template 4 µl. PCR cycling conditions were 94℃ for 2min, followed by 40 cycles of 94℃ for 1min, 55℃ for 1 min, and 68℃ for 1min, followed by final elongation at 68℃ for 10 min. Amplification products were resolved in 1% agarose gels prepared using 1X TBE [Bio-Rad, Hercules, CA, USA] and visualized using SYBR™ Safe [Life Technologies, Carlsbad, CA, USA].

Virus isolation

Nasal swab eluates were filter-sterilized using Millex syringe filters with an average pore diameter of 0.45 µm. [Cat. No. SLHA033SS, Millipore, Bedford, MA, USA]. For a standard virus isolation assay, a total number of 2.5×10^4 CRFK cells and 50 µl of filtrate were added to duplicate wells of a 96 well microtiter plate. The plates were incubated for 3 days at 37℃, 5% CO_2 and the presence or absence of characteristic cytopathic effect was evaluated for determining whether samples were positive or negative.

Virus neutralization antibody testing

Sera from all cats were analyzed for the presence of virus neutralizing (VN) antibodies as previously described (Kruger et al., 1996). Briefly, sera were heat-inactivated at 56°C for 30 min, followed by preparation of a two-fold serial dilution series. One hundred $TCID_{50}$ of FHV-1 C27 was mixed with the samples. Following a 1-hour incubation at 37C, 2×10^4 CRFK cells were added to each well and the plates were incubated for 3 days at 37°C, 5% CO_2. Each sample was analyzed in duplicate for the presence of cytopathic effect.

Cytokine mRNA gene expression analysis

Total RNA was extracted from whole blood samples preserved in PAXgene® blood RNA tubes (IVD), according to the manufacturer's protocol [PreAnalytix GmbH]. Total RNA concentration was measured with a Nanodrop spectrophotometer [Thermo Fisher Scientific] and reverse transcription was performed using 100 ng of sample RNA combined with 20 µl of a High-Capacity cDNA Reverse Transcription Kit and RNase Inhibitor [Applied Biosystems]. Sequences of primers and probes for real- time PCR for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), interferon alpha (IFNα), interferon beta (IFNβ), interferon gamma (IFNγ), tumor necrosis factor alpha (TNFα), interleukin 1 beta (IL-1β), interleukin 10 (IL-10), interleukin 12 subunit p40 (IL12p40), transforming growth factor beta (TGFβ), and chemokine C-C motif ligand 5 (CCL5, also known as RANTES) are listed in **Table 4. 4** and have been previously described (Foley et al., 2003; Ignacio et al., 2005; Kipar et al., 2001; Nguyen Van et al., 2006; Robert-Tissot et al., 2011). The cDNA was diluted 1:20 for real-time PCR analysis and a 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. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous control for each gene of interest (Lee et al., 2019; 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 was analyzed in triplicate.

Histological examination

Nasal mucosa, tonsil, trachea, lung, retropharyngeal lymph node, heart, brain, liver, kidney, spleen, adrenal glands, testes, and ocular specimens, were collected at the time of necropsy and fixed in 10% buffered formalin, paraffin embedded. The sections were examined by a pathologist.

Statistical analysis

The total clinical scores for each period after vaccination and the period after challenge were corrected by sample size per group for graphing and analyzed by Kruskal-Wallis one-way analysis of variance (ANOVA) followed by Dunn's post hoc multiple comparison test. In addition, daily total scores post-CH were analyzed by two-way ANOVA followed by Tukey's multiple comparison test [GraphPad Prism Software v6, San Diego, CA]. For viral genome quantification assays, raw Ct values were used for two way-ANOVA, followed by Tukey's post hoc multiple comparison test [GraphPad Prism Software v6]. For VN antibody titers a two-way ANOVA with Tukey's host hoc multiple comparison test was used [GraphPad Prism Software v6]. For cytokine/chemokine mRNA gene expression assays fold change compared to pre-

vaccination levels were calculated using the 2^{-ddCt} method as previously described (Livak and Schmittgen, 2001). For statistical analysis ddCt values were used for two-way ANOVA with Tukey's post hoc multiple comparison test [GraphPad Prism Software v6]. A p value of < 0.05 was regarded as significant difference for all data.

RESULTS

Vaccination with the gE-TK- or PK- mutants was safe and significantly reduced clinical scores post challenge infection

Total clinical scores per group were adjusted by the number of animals per group, and were calculated for pre-vaccination (preV), post-V1 (day 0-21), post-V2 (day 21-42), post-V3 (day 42-63), and post-CH periods (**Figure 4. 1A**). All groups had a clinical score of zero during the pre-vaccination period. After the first subcutaneous (SC) vaccination with the PK- or gE-TK- mutants (post-V1 period), the PK- mutant group had an accumulative score of 0, and the gE-TK- mutant group had a cumulative score of 1, caused by a mild fever of a single cat. During the same time period the control group had a total score of 0, and the commercial vaccine group had a score of 1 due to a mild temperature elevation in a single cat on day 21. Differences between experimental groups were not statistically significant for this period.

After the second SC inoculation with PK- or gE-TK- mutants on day 21 (post-V2 period, from day 22 to day 42), the PK- group had a total score of zero, and the gE-TK- group had a total score of 3 (adjust to 2 to get a proportion equal to the sample size of the control and commercial vaccine groups), attributed to a fever on a single day in 3/6 cats. During this same time period (which followed the first vaccination with the commercial vaccine), the total clinical scores were 3 in the commercial vaccine group due to fever for one or two days respectively in 2/4 cats. The clinical score for the controls during this period was 0. Overall, differences in clinical scores during this period were not statistically significant between groups.

Following the last vaccination, cats in PK- group had a total clinical score of 3 (adjusted to 2 to get a proportion equal to the sample size of the control and commercial vaccine groups) post-IN inoculation resulting from sneezing by a 1/6 cat on 3 different days. In the gE-TK-

mutant the clinical score was zero in the post-IN immunization period and the commercially vaccinated group had a score of 1 resulting from a slightly elevated temperature in 1/4 cats on one day. The total score of the control group prior to challenge was 0 and differences in clinical scores during this period were not statistically significant between groups.

During the post-challenge period, the control group had a total score of 143, and the commercially vaccinated group had a total score of 67. When looking at the clinical scores on individual days following challenge infection (**Figure 4. 1B**), a bi-phase nature of the clinical score was observed in both controls and commercially vaccinated cats. In the control group, the first peak was noted on day 4 post-CH. The second peak was seen on day 9 post-CH. In the commercially vaccinated group, the primary peak on day 3 post-CH was not significantly different from that in control group on the same day. On day 7, the commercial vaccine group started to have significantly lower values than those in the control group ($p= 0.0026$). The secondary peak of total clinical scores in commercial vaccine group occurred on day 11, which had a significant difference from that in the control group ($p< 0.0001$). In contrast, in the gE-TK- group a total post-CH clinical score of 7 (adjusted to 5 to get a proportion equal to the sample size of the control and commercial vaccine groups) was observed and a total score of 4 (adjusted to 3 for sample size equity) was observed in the PK- group (**Figure 4. 1A**). Both the scores of gE-TK- and PK- groups were significantly lower than the scores of the controls ($p< 0.0001$ and < 0.0001) and the commercial vaccinates ($p< 0.0109$ and 0.0032). Clinical scores in gE-TK- and PK- groups were not significantly different from each other, but they were significantly lower than clinical scores in the control group from day 3 to day 13 ($p< 0.05$) (**Figure 4. 1B**). In addition, scores in gE-TK- and PK- groups were significantly lower than those in commercial vaccine group from day 3 to 8, as well as on day 13 ($p< 0.05$).

Vaccination with the gE-TK- or PK- mutants prevented body weight loss

Following infection, 3/4 cats in the control group and 2/4 cats in the commercial vaccine group exhibited weight loss and the remaining cats in each group stopped gaining weight (**Table 4. 5**). In contrast, all cats vaccinated with the gE-TK- or PK- vaccines either maintained or continued gaining weight.

Low level vaccine virus DNA was detected in nasal secretions after intranasal administration of the PK- or gE-TK- mutants, but no infectious virus was isolated

After intranasal vaccination with the PK- mutant, viral DNA was detected in the nasal secretion for 10 days with Ct values between 39 to 22 (**Figure 4. 2A**). Shedding of viral DNA had seized by day 62 prior to challenge infection. In contrast, significant shedding of viral DNA was not detected in the gE-TK- group after intranasal vaccination and viral DNA could only be detected at Ct levels between 36 and 39 in 3 of 6 cats on individual days. Viral DNA was not recovered from any commercially vaccinated cat or control cat at any time prior to challenge infection. Further, no infectious virus was detected by VI from nasal secretions of any cat at any time prior challenge infection.

Vaccination with the gE-TK- or PK- mutants reduced viral nasal shedding compared to controls and commercially vaccinated cats following challenge infection

Cats in the control group shed viral DNA in nasal secretions for up to 3 weeks following infection and peak levels were detected on day 4 post infection (day 67 of the study) (**Figure 4. 2A**). Cats in the commercial vaccine group shed significantly decreased levels of viral DNA compared to the controls on day 4 post-CH ($p= 0.0024$), but levels were significantly higher than

levels in the gE-TK- vaccinates on days 2 (p= 0.0246) and day 4 (p= 0.0037) post-CH. The values in commercial group were also significantly higher than those in PK- group from day 4 (p= 0.0001) through day 8 (p= 0.0077). In addition, shedding of viral DNA could still be observed in 3 of 4 control cats and 3 of 4 commercially vaccinated cats at the time point of necropsy. In contrast, only 1 of 6 cats in the gE-TK- or PK- vaccinated groups showed low level shedding of viral DNA at the time of necropsy. In addition to viral DNA quantification, virus isolation (VI) on nasal swab samples was performed to test for infectious virus shedding (**Figure 4. 2B**). Concurrent with the nasal shedding of viral DNA, more cats in the control group shed infectious virus following infection than any other group. Specifically, three cats out of four in the control group shed infectious virus on day 4 and 8 post-CH, and all four cats in the control group shed infectious virus on Day 6 post-CH. In contrast, two cats in the commercial vaccine group shed infectious virus on day 6 post-CH and only one cat in gE-TK- group shed infectious virus on day 6 post-CH. None of the cats in the PK- group shed infectious virus at any time point.

Differentiation between vaccine virus and challenge virus

To differentiate between PK- vaccine virus and virulent challenge virus, a conventional PCR amplifying the FHV-1 PK gene was carried out on nasal swab samples from the PK- group, the commercial vaccine group, and control group, on the samples collected on day 69 (day 6 post-CH) and on samples from the PK- vaccinates collected on day 48 (day 6 post IN vaccination) (**Figure 4. 8**). The full length of PCR product of the PK gene is 1266 bp in size, which is the expected size of the challenge virus and all cats in the commercially vaccinated group (n= 4) and the control group (n= 4) were in addition to 3/6 cats in the PK- vaccinates were positive. The remaining 3 cats in the PK- group did not show a band of any size on day 6 post challenge

infection. In contrast, the nasal swab samples from the PK- group collected on day 48 (day 6 post intranasal vaccination), showed a product of 379 bp in size, which indicates that the viral DNA shed at this point was PK- vaccine virus.

To differentiate between gE-TK- vaccine virus and virulent challenge virus conventional PCRs amplifying the FHV-1 gE and TK genes were carried out on nasal swab samples from the gE-TK- group, commercial vaccine group, and control group, on samples collected on day 69 (day 6 post-CH) and on day 48 (day 6 post IN vaccination on the 2 cats of the gE-TK- vaccine group that were positive by real-time PCR) (**Figure 4. 9 and 4. 10**). Samples from all cats in the gE-TK- group and all cats in the commercial vaccine group, and control group exhibited a PCR products of 1867 bp on day 6 post challenge, suggesting the virus present is the challenge virus. No PCR products were detected in any of the cats in the gE-TK- group following the intranasal vaccination presumably due to no, or very low level of vaccine virus DNA in nasal secretions as a result of vaccination. The conventional PCR targeting the FHV-1 TK gene was showed a PCR product size of 1006 bp in 5 of 6 gE-TK- vaccinated cats and all cats in the commercial vaccine and control groups on day 6 post-CH, confirming the virus present in the samples was the challenge virus. Similar to the findings with the gE specific PCR, no TK-related PCR products were detected in any of the cats in the gE-TK- group following the intranasal vaccination, presumably due to no or very low-level vaccine virus DNA in nasal secretions as a result of vaccination.

VN titers increased in all vaccine groups as a result of vaccination and the increase in post-challenge VN antibody titer was highest in the commercial vaccine group

Virus neutralization antibody titers following vaccination and challenge infection are shown in **Figure 4. 3**. VN antibody titers of cats in the gE-TK- mutant group were significantly higher than in those of cats in any other group on day 21, 3 weeks post-first-SC inoculation ($p< 0.0001$ compared to control group), ($p< 0.0001$ compared to commercial vaccine group), ($p= 0.0114$ compared to PK- group). In contrast, VN antibody titers in the PK- group on day 21 were not significantly different from the control or the commercial vaccine groups. On day 35, which is 14 days after the second SC inoculation with the gE-TK- or PK- mutant, or 14 days after first SC inoculation with the commercial vaccine, antibody titers in the gE-TK- group were not significantly different from those in the commercial vaccine group. In contrast, titers in the PK- vaccinates were lower on day 35, than titers in the commercial vaccine group ($p= 0.0192$) and the gE-TK- group albeit not quite significant ($p= 0.0755$). Moreover, the titer of PK- group on day 35 were not significantly different from that of control group ($p= 0.1347$). After the IN vaccination with the gE-TK- or PK- mutants or the second SC vaccination of the commercial vaccinates on day 42, antibody titers in all three vaccination groups were significantly higher than titers in the controls. However, titers in the gE-TK- group and in the commercial vaccine were significantly higher than VN titers in the PK- vaccinates ($p= 0.0196$ and 0.0311). By day 56, titers in all vaccination groups were significantly higher than titers in controls but not significantly different from each other and this was true on day 63 right before challenge infection.

On day 77, which is 14 days after challenge infection, titers in cats from the control group were not statistically different from titers of cats in the gE-TK- group ($p= 0.5995$) or cats in the

PK- group (p= 0.7868). In contrast, the average titer of cats in the commercial vaccine group was significantly higher than those of the controls, gE-TK- vaccinates and PK- vaccinates groups (p= 0.0002, 0.0046, and 0.0016).

Cytokine mRNA gene expressions in blood following vaccination and challenge infection

Whole blood mRNA gene expression for cytokines and chemokines were evaluated at day 0 before any treatment, two days after the second SC inoculation with the PK- or gE-TK- mutant in the PK- or gE-TK- vaccinates (day 23), two days after the second SC inoculation with the commercial vaccine in the commercial vaccinates, or the IN inoculation with the PK- or gE-TK- in the PK- or gE-TK- vaccinates (day 44), and two days after challenge with the virulent FHV-1 in all groups (day 65), and results are shown in **Figures 4. 4 and 4. 5**.

On day 23, which is two days after the second SC inoculation with the PK- or gE-TK- mutant, IFNα expression was upregulated the PK- group (p= 0.0474). In addition, IFNβ mRNA expression was upregulated in both PK- and gE-TK- groups, compared to those of respective groups on day 0 (p= 0.0045 and 0.0137). In contrast, the expression of IL10 was significantly downregulated in the gE-TK- vaccinates on day 23, (p= 0.0399), and there was a significant difference of IL10 expression between PK- and gE-TK- vaccinates on day 23 (p= 0.0483). No significant difference was noted on day 23 for PK- and gE-TK- vaccinates compared to levels on day 0, for IFNγ, IL1β, CCL5, TNFα, and TGFβ mRNA expression.

On day 44, which is two days after the IN inoculation with the PK- or gE-TK- mutants, or two days after the second SC inoculation with the commercial vaccine, there was a significant upregulation of IFNβ expression in the PK- vaccinates compared to that of the same group on day 0 (p= 0.045) and an increase in IFNβ expression in the gE-TK- vaccinates and the

commercial vaccinates albeit not statistically significant. In addition, IL1β expression in the gE-TK- group was significantly lower than in commercial vaccinates ($p= 0.0283$). Finally, IL10 was significantly downregulated in all vaccine groups although only the gE-TK- vaccinates and the commercial vaccinates was statistically significant, compared to levels of the respective group on day 0 ($p= 0.0033$ and < 0.0001).

On day 65, which is two days after challenging with virulent FHV-1, upregulation of IFNα expression was noted in the commercial vaccinates and gE-TK- vaccinates, compared to their counterparts on day 0 ($p= 0.0287$ and 0.0259, respectively). Coinciding, an upregulation of IFNβ expression was noted in PK-, gE-TK-, and commercial vaccinates, comparing to day 0 ($p= 0.0228$, 0.0014, and 0.0055). Moreover, the upregulation of IFNβ in gE-TK- vaccinates on day 65 was significantly increased when compared to its counterparts on day 23 ($p= 0.0072$) and day 44 ($p= 0.0219$). The type II interferon, IFNγ was also upregulated in PK-, gE-TK-, and commercial vaccinates when compared to their counterparts on day 0 ($p= 0.0025$, 0.0041, and 0.0023), in addition to an upregulation of CCL5 in the gE-TK- vaccinates compared to that of day 0 ($p= 0.0323$). IL1β expression was significantly upregulated in all four groups when compared to day 0 ($p= 0.0271$ for PK- vaccinates, $p< 0.0001$ for gE-TK- vaccinates, $p= 0.002$ for commercial vaccinates, and $p= 0.0009$ for controls). However, the IL1β upregulation was higher in the commercial vaccinates or the controls than in the PK- or gE-TK- vaccinates ($p= 0.0084$ and 0.0119, respectively compared to commercial vaccinates) ($P= 0.0014$ and 0.0021, respectively compared to control). A significant upregulation was also noted for TNFα expression in PK- vaccinates ($p= 0.0018$) and gE-TK- vaccinates ($p= 0.0004$) and a similar trend was observed for TGFβ expression in PK- vaccinates ($p< 0.0001$), gE-TK- vaccinates ($p< 0.0001$), and controls ($p< 0.0001$). Interestingly TGFβ expression was significantly lower in the

commercial vaccinates compared to the controls (p= 0.0137). TGFβ expression was also higher in the PK- vaccinates post-challenge when compared to post-vaccination levels on day 23 (p< 0.0001) or on day 44 (p= 0.001), and in the gE-TK- vaccinates when compared to day 23 (p< 0.0001). Finally, a significant upregulation of IL10 expression was observed in the PK- vaccinates on day 65 compared to that on day 0 (p= 0.042). On the contrary, a significant downregulation of IL10 was seen in the commercial vaccinates (p= 0.0002) and the controls (p= 0.0123) compared to their levels on day 0.

Histology revealed only mild lesions in lung and nasal turbinates of gE-TK- and PK- vaccinates, while marked lesions were noted in commercial vaccinates and controls

Necropsies were performed and gross examinations were conducted 3 weeks post infection when all cats had clinically recovered. Gross lesions were not detected in any of the cats but nasal turbinate, tonsil, trachea, lung, retropharyngeal lymph node, heart, brain, liver, kidney, spleen, adrenal glands, testes, and ocular specimens from all cats were examined microscopically. Lesions in all cats were limited to the lungs and nasal turbinates and images were shown in **Figure 4. 6**. All cats in the control group (n= 4) had congested lungs with a mild interstitial pneumonia and moderate numbers of foamy macrophages in the alveoli, mild alveolar edema and few interstitial neutrophils and lymphocytes. Two cats had moderate bronchus associated lymphoid tissue (BALT) hyperplasia (**Figure 4. 6A**). All 4 cats had a moderate chronic lymphoplasmacytic rhinitis with formation of lymphoid follicles in 2 cats and ulceration of the mucosal epithelium with the necrotic surface being covered by degenerate neutrophils in one animal (**Figure 4. 6B**). In the commercial vaccine group (n= 4), all 4 cats had moderately congested lungs with few intra-alveolar macrophages and edema fluid and 2 cats had BALT

hyperplasia and one cat had a mild interstitial pneumonia with mild perivascular lymphoid infiltrates (**Fig. 6C**). In addition, three cats out of 4 in the commercial vaccine group had a mild to moderate rhinitis with erosions and suppurative inflammation in one cat (**Figure 4. 6D**). In contrast, histological findings in the gE-TK- and PK- groups were much milder, especially in the nasal turbinates. Lungs of all cats in the gE-TK- group (total n= 6) were mildly congested with some intra-alveolar edema fluid and few intra-alveolar macrophages (**Figure 4. 6E**) and 3 cats had mild BALT hyperplasia. Only one cat had a mild rhinitis, while most cats had normal nasal mucosa (**Figure 4. 6F**). In the PK- group all cats (total n= 6) had similar changes as the cats in the gE-TK- group and 3 cats had mild BALT hyperplasia in lung tissues. Two cats had a mild focal interstitial pneumonia, but overall, the lungs were unremarkable (**Figure 4. 6G**). Only one of the 6 PK- cats had a mild rhinitis while the remaining cats had an unremarkable nasal mucosa (**Figure 4. 6H**). No microscopic lesions related to this study were noted in other organs or tissues.

DISCUSSION

The current study shows safety and efficacy of a novel vaccination regime that utilizes a gE-TK- FHV-1 deletion mutant or a PK-FHV-1 deletion mutant administered twice subcutaneously (SC) followed by mucosal boosting in SPF free kittens. Our vaccination regime was chosen based on the hypothesis that adding a mucosal boost to the standard two dose regimen of SC immunization would induce protective immunity not only against clinical signs but also against virus shedding after challenge. It has previously been shown that boosting via the intranasal route following priming via the SC route has a synergistic effect on reduction of viral shedding post-challenge by stimulating robust mucosal and systemic immunity (Lappin et al., 2006; Reagan et al., 2014). Reagan et al. (2014) further showed that kittens vaccinated concurrently via the SC and IN routes were better protected against clinical signs and virus shedding of virulent virus, administered at 1-week post vaccination. Priming via the SC route, followed by an IN boost has also been recommended for cats older than 2 months old without maternal antibody protection. (Ruch-Gallie et al., 2011).

In our study we show that clinical protection was significantly improved following vaccination with either the gE-TK- mutant or the PK- mutant when compared to unvaccinated controls or cats vaccinated with a commercial MLV vaccine. Specifically, the cumulative clinical scores after challenge were extremely low in both the PK- and gE-TK- groups (score of 3 and 4 respectively), with clinical signs limited to fever for 1 day in 3/6 cats (gE-TK- group), sneezing for 1-3 days in 2/6 cats in the gE-TK- group and 1/6 cats in the PK- group, and mild nasal congestion for 1 day in 1 cat in the PK- group. In contrast, cats that were not vaccinated or cats immunized twice with a commercial vaccine prior to challenge exhibited fever, sneezing, coughing, nasal discharge, nasal congestion, blepharospasm, and ocular discharge for up to 17

days post-challenge (total scores 143 in controls and 67 in commercial vaccinates). Furthermore, 3/4 cats and 2/4 cats exhibited weight loss and the remaining cats stopped gaining weight following challenge infection in unvaccinated or commercially vaccinated cats, while all cats vaccinated with the gE-TK- or PK- vaccines maintained or continued gaining weight. The histological finding supported the clinical data after challenge infection and showed that all cats in the control group and the 2/4 commercial vaccine group had moderate to severe interstitial pneumonia and 3/4 cats showed rhinitis, while the cats in the gE-TK- and PK- groups had milder or unremarkable lesions in lungs and nasal turbinates.

In addition to the reduction of clinical disease, challenge virus DNA levels in nasal secretions were significantly lower in the groups immunized with the PK- and gE-TK- mutants than in those that were either not vaccinated or vaccinated with a commercial vaccine. Furthermore, virus isolation testing indicated that none of cats shed virulent challenge virus in the group immunized with the PK- mutant, while only one cat showed infectious viral shedding on day 6 post-challenge in the gE-TK- group. These data parallel those of a vaccination-challenge study which included a PRV PK- deletion mutant. This mutant completely protected pigs from respiratory shedding of infectious virus. (Kimman et al., 1994). However, experimental immunization with gE-TK- double deletion mutants during *in vivo* testing for porcine pseudorabies virus (PRV) and bovine herpesvirus-1 (BHV-1) did not completely prevent infectious virus shedding post challenge (Kaashoek et al., 1996; Kimman et al., 1994).

We previously reported that PK- and gE-TK- mutants of FHV-1 were attenuated and showed significantly reduced viral replication in feline respiratory epithelial cells (Lee et al., 2019), which was taken as an *in vitro* indicator for being a candidate for further *in vivo* testing. The data from the present study confirmed that the presence of gE-TK- viral DNA in nasal

secretions after IN inoculation stayed extremely low (Ct value no less than 35) in 4/6 cats or was not detectable (2/6 cats) during the whole observation period following vaccination. In contrast, all six of the cats in the PK- mutant group were positive for viral DNA detection post-intranasal vaccination, and there was an average peak (Ct= 30) on day 6 post intranasal vaccination. However, no infectious virus could be detected by virus isolation from any of the collected nasal secretions samples in either of these groups during the observation period following vaccination. This indicates that, despite 2 previous SC immunizations, the PK- mutant could still replicate at a very low level, which was detectable by PCR but not by VI because it was either below the limit of detection of VI or complexed with antibody. Interestingly, while there was low level replication detected by PCR, this replication was not associated with clinical signs except for 1 cat that showed mild sneezing on 3 occasions following the intranasal vaccination.

Our safety and efficacy data using the gE-TK- or PK- mutant as a mucosal booster following SC priming is an improvement in safety over previous studies that reported some residual virulence following mucosal administration only with a FHV-1 gE-gI deletion mutant at higher dose levels in cats. This higher dose level was however needed for induction of protection from virulent FHV-1 challenge (Kruger et al., 1996; Sussman et al., 1995). Furthermore, intranasal administration of a single TK deletion mutant of FHV-1 in cats resulted in sneezing and, following challenge, significant virus shedding was detected in oral, nasal, and ocular secretions (Yokoyama et al., 1996). It is important to recognize that, while we did show that our vaccination regime with the gE-TK- and PK- mutants was safe and efficacious, further studies are needed to directly evaluate the safety of the mutants when given intranasally without prior SC priming.

A further advantage of using deletion mutants is the ability to differentiate FHV-1 vaccine strains from virulent field strains, which can be a challenge with conventional vaccines where the genomes of vaccine virus and virulent virus are nearly identical (Maggs and Clarke, 2005). In the present study, the shedding of virulent FHV-1 post-challenge was easily differentiated from that of the deletion mutants via PCR testing targeting the PK, gE, or TK genes. The results obtained showed that the viral shedding post-challenge in PK- and gE-TK- groups completely consisted of virulent FHV-1 challenge virus.

Interestingly, fever was never observed in the group immunized with the PK- mutant, either after vaccination or after the challenge infection. In contrast, occasional fever responses lasting 1 day were noted in 3/6 cats immunized with the gE-TK- mutant following the primary or secondary SC immunization, but no clinical signs at all were noted post-challenge infection in this group. Fever can be an indicator of an ongoing systemic immune response and is often associated with the development of serum VN antibody titers following immunization (Lappin et al., 2009; Reagan et al., 2014). The lack of fever in the group immunized with the PK- mutant might correlate with the lower slope of the VN antibody titer curve following SC immunization in this group. Further, there was a sharp post-challenge spike of the VN antibody titer in the group immunized with the commercial vaccine, which was not observed in the groups immunized with either the in the PK- or gE-TK- deletion mutants or the control groups. This spike in the commercial vaccine group may reflect a boosting by significant post-challenge virulent virus shedding in this group, which was not observed in the gE-TK- or PK- vaccine groups.

We have previously shown in feline respiratory epithelial cell (FREC) cultures that inoculation with the gE-TK- or the PK- mutant did not significantly induce the INFα and IL10

responses as well as chemokine responses compared to wild type FHV-1 inoculation but instead induced a TGFβ response (Lee et al., 2019). In the present study, the gene expression of type I interferons in blood, especially IFNβ, was upregulated two days after the second SC inoculation with the PK- or gE-TK- mutants. Such upregulation was not found in the group immunized with a commercial vaccine. On the other hand, the gene expression of IFNγ, which is a type II interferon, was not altered in any vaccinated groups until the challenge occurred. Expression of type I interferons is a major antiviral immune response in the defense against viral infection. Activation of IFN-regulatory factor 3 (IRF3) and NF-κB initiate expression of IFNα, while IRF7 combined with NF-κB initiates the expression of IFNβ. However, the PK and TK genes of alphaherpesviruses have shown to modulate the activation of IFNα and IFNβ in blood leucocytes by reducing TLR3 expression or by counteracting subsequent transcriptions factors IRF3 (Lewandowski et al., 1994; Peri et al., 2008; Saira et al., 2007; Wang et al., 2013). The gene expression of cytokines including IFNs, TGFβ, and TNFα, was upregulated in all groups after challenge. However, upregulation of IL1β post-challenge in the groups immunized with the PK- or gE-TK- mutants was lower than in the group immunized with a commercial vaccine or the control group. IL1β is an inflammatory cytokine and it is likely that the increased levels observed in the groups immunized with a commercial vaccine or the unvaccinated control group reflected the increased challenge virus replication compared to the gE-TK- and PK- vaccination groups. Similarly, IL1β was one of the key cytokines indicating the severity of HSV1-associated encephalitis and neural invasion *in vivo* (Sergerie et al., 2007). The finding might also suggest that our mutants reduced viral replication and inflammation contributed to decreased neurovirulence compared to controls and commercially vaccinated groups, but viral detection in trigeminal ganglia and latency examination for all groups are still pending.

The expression of IL10 was significantly downregulated in the gE-TK- vaccinates after the second SC inoculation and IN inoculation, but such downregulation was not shown to the same degree in PK- vaccinates. It has been shown in a study of HSV-1 that alphaherpesviruses manipulate T cell activation by inhibiting the activation of the T cell signaling complex and the associated NF-κB activation, while the p38 kinase and c-Jun N-terminal kinase (JNK) is activated by the virus. Consequently, the virus-infected T cells could selectively synthesize IL10 to favor immune suppression by the virus (Sloan and Jerome, 2007). Moreover, it is believed that IL10, along with IL4, IL5, and IL6, is a Th2 cytokine which favors to regulate antibody development and B cell activation, in contrast to Th1 cytokines such as IFNγ and TNFα which induce the activation of macrophage and T cells (Mosmann et al., 1986). IL10 is known to inhibit activation of Th1 responses by suppressing the ability of antigen-presenting cells to secrete Th1-associated cytokines (Rojas et al., 2017), which is a crucial mechanism to eliminate herpesvirus infection. We speculate that the downregulation of IL10 by gE-TK- vaccination in our study might have contributed to the activation of cellular immunity by the gE-TK- mutant.

Current commercial immunization for FHV-1 consists of 2 consecutively subcutaneous (SC) inoculations (Day et al., 2016). A vaccination regime which consists of inoculating the same antigen via different administration routes for priming and subsequent boosting is not a standard procedure. However, the present study demonstrates the benefit of an additional boost via a mucosal route over standard dual SC immunization. This dual-site immunization strategy has been deployed against a variety of viral infections, such as human immunodeficiency virus (Hu et al., 1991), poliovirus (John, 2009), and measles virus (Durrheim et al., 2014). It was found to induce more effective protection by stimulating stronger humoral and cellular immunity (Kardani et al., 2016; Ramshaw and Ramsay, 2000). The addition of vaccine administration via

the IN route induces local immune responses, such as IgM and IgA as well as local cytokine and chemokine responses at the mucosal level (Cocker et al., 1986). It has been reported that in mice immunization that include a mucosal route resulted in a significant production of mucosal IgA and a significant secretion of IL17, which is a pro-inflammatory cytokine critical for CD4+ T cell activation and Th1 polarization (Fiorino et al., 2013). We specifically targeted induction of mucosal immunity with the IN administration with the PK- and gE-TK-, but further investigations of IgA or cytokine/chemokine quantification in nasal swabs samples are necessary to verify this assumption.

In conclusion, the present study consists of a comprehensive safety and efficacy evaluation of a PK- and gE-TK- deletion mutant of FHV-1, two pilot vaccine candidates. The immunization regime consisted of two SC immunizations followed by an additional dose administered IN and a virulent challenge with an FHV-1 clinical isolate and results were compared to those resulting from immunization with a commonly used commercial vaccine, following the standard vaccination protocol. Our data clearly indicates the benefit of incorporating a mucosal immunization component with deletion in virulence and immune modulatory genes, in terms of enhancing protection against clinical disease, virus shedding and latency.

APPENDIX

Table 4. 1. The timeline and the experimental design of the study. The cats in gE-TK- and PK- groups were subject to subcutaneous inoculations with gE-TK and PK- mutants, respectively, on day 0 (V1) and 21 (V2), followed by an intranasal inoculation with the respective mutant on day 42 (V3). The commercial vaccinates only got subcutaneous inoculation with the commercial vaccines on day 21 (V2) and 42 (V3). No inoculation was conducted in control group from during V1, V2, and V3 period. All four groups were subject to virulent FHV-1 challenge (CH) intranasally on day 63. The solid line represented daily physical exam for clinical scores. Dot lines represented the nasal swab sampling were taken every other day. The X indicated the conduction was performed on the specific day. Ne: the necropsy day.

	Day of the project																		
	-7	**0**	**2**	**4**	**14**	**21**	**23**	**25**	**35**	**42**	**44**	**52**	**56**	**62**	**63**	**65**	**77**	**81**	**Ne**
Treatment		**V1**				**V2**				**V3**					**CH**				
Clinical score																			
Nasal shedding	X	X	X	X	X	X	X	X	X	X		X	X	X		X	X	X	X
Seral VN test		X			X	X			X	X			X		X		X		
Blood cytokine		X					X				X					X			
Histology																			X

Table 4. 2. Clinical score criteria

Category	Signs	Score
Conjunctivitis	Normal	0
	Mild hyperemia	1
	Moderate to severe hyperemia	2
	Moderate to severe hyperemia, with chemosis	3
Blepharospasm	Normal	0
	< 25% of eye closed	1
	25-50% of eye closed	2
	50-75% of eye closed	3
	75-100% of eye closed	4
Ocular discharge	Normal	0
	Minor, serous	1
	Minor to moderate, mucoid	2
	Marked mucopurulent	3
Sneezing	Normal	0
	Sneezing observed or evident with nasal discharge with blood on walls of cage	1
Nasal discharge	Normal	0
	Minor, serous, occasional with blood	1
	Minor to moderate, mucoid or bloody	2
	Marked mucopurulent	3
Nasal congestion	Normal	0
	Mild	1
	Moderate	2
	Severe, with open mouth breathing	3
Coughing	Normal	0
	coughing noted	1
Fever	Body temperature lower than or equal to 102.56 Fahrenheit	0
	Body temperature higher than 102.56 Fahrenheit	1

Table 4. 3. Primers for differentiating FHV-1 WT and the mutants

Viral gene	Forward primer (5'-3')	Reverse primer (5'-3')	Expected product size
PK	AGGCACTCAGTGGGCCAAAGT	AGGCTGTCTTACACATGAGGCA	WT: 1266 bp PK- mutant: 379 bp
gE	GGTCATGTGTAATGTTGACG	ATACAATATACGCGTTTGACG	WT: 1867 bp gE-TK- mutant: 268 bp
TK	ACGATGGCGAGTGGAACCATCC	TACGGTGCATATCACATGCGGCTA	WT: 1006 bp gE-TK- mutant: 543 bp

Table 4. 4. Primers and probes for cytokine gene expression assays

	Forward primer	Reverse primer	Probe
GAPDH	GCCGTGGAATTTGCCGT	GCCATCAATGACCCCTTCAT	CTCAACTACATGGTCTACATGTTCCAGTATGATTCCA
IL1β	AATGACCTGTTCTTTGAGGCTGAT	CCAGAAAACTGTGGCTCAGGTT	CGAAAAGATGAAGGGCAGCCTCCAA
IL10	ACTTTAAGGGTTACCTGGGTTG	CGTGCTGTTTGATGTCTGG	TTGGAGGAGGTGATGCCCCA
IL12p40	TGGCTTCAGTTGCAGGTTCTT	TGGACGCTATTCACAAGCTCA	CGGTTTGATGATGTCCCTGATGAAGAAGCT
IFNα	CTTGACGCTCCTGGGACAAA	ACTGGTCTCCACCAGAACACG	TCCCTGCCATCTCCTGTCAGAAGG
IFNβ	TGGAATGAGACCACTGTTGAGAA	GGATCGTTTCCAGGTGTTCCT	CTCCTTGCGACACTCCACTGGCAG
IFNγ	TGCAAGTAATCCAGATGTAGCAG	GTTTTATCACTCTCCTCTTTCCAG	CAAAATGTCTACGAAAAGCGACCCACC*
TNFα	CACATGGCCTGCTGCAACTAATC	AGCTTCGGGGTTTGCTACTAC	TCTCGAACTCCGAGTGACAAGCCA
TGFβ	GGAATGGCTGTCCTTTGATG	TGCAGTGTGTTATCTTTGCTGTC	TTTCGCCTCAGTGCCCACTG
CCL5	CTACACCAGCAGCAGTGTTCC	ACACACCTGGCGCTTCCTC	TGCCCAGCAGTCGTCTTTGTCACCC

Table 4. 5. Body weight changes. Weight loss compared to the previous observation in the cat was highlighted in bold. CV: commercial vaccinates.

		Control				CV				gE-TK-						PK-					
	No	**67**	**68**	**69**	**70**	**71**	**72**	**73**	**74**	**75**	**76**	**77**	**78**	**79**	**80**	**81**	**82**	**83**	**84**	**85**	**86**
Day of the project	**-4**	2.0	2.2	2.1	2.4	3.0	2.4	1.9	2.1	2.2	2.4	2.2	2.1	2.2	2.1	2.1	2.3	2.2	2.0	2.8	2.4
	0	2.1	2.3	2.1	2.4	2.9	2.4	2.0	2.1	2.3	2.4	2.2	2.0	2.0	2.0	2.2	2.5	2.3	2.2	2.7	2.5
	6	2.4	2.6	2.3	2.8	3.2	2.6	2.2	2.5	2.5	2.7	2.4	2.3	2.4	2.4	2.5	2.9	2.4	2.2	2.8	2.8
	14	2.7	2.9	2.7	3.1	3.0	3.0	2.1	2.5	2.7	3.0	2.7	2.5	2.5	2.6	2.9	3.0	2.8	2.4	3.3	2.9
	21	3.0	3.2	2.9	3.3	3.6	3.2	2.6	2.8	2.9	3.3	2.8	2.6	2.7	2.8	2.9	3.2	2.9	2.5	3.4	3.1
	35	3.4	3.6	3.2	3.2	3.9	3.6	3.0	3.4	3.3	3.8	3.2	2.8	2.9	3.1	3.2	3.5	3.4	2.8	3.9	3.5
	42	3.6	3.8	3.5	4.1	4.2	3.8	3.3	3.7	3.5	3.9	3.3	2.9	3.1	3.3	3.5	3.8	3.6	2.9	4.1	3.7
	44	3.6	3.8	3.5	4.1	4.2	3.8	3.3	3.7	3.5	3.9	3.3	2.9	3.1	3.3	3.5	3.8	3.6	2.9	4.1	3.7
	56	4.0	4.4	3.7	4.5	4.5	4.2	3.4	3.9	3.9	4.3	3.6	3.2	3.2	3.6	3.8	4.1	3.9	3.2	4.5	4.1
	63	4.1	4.5	3.9	4.8	4.7	4.3	3.8	4.2	4.0	4.6	3.7	3.4	3.4	3.7	4.0	4.2	4.2	3.4	4.7	4.3
	72	**3.9**	**4.2**	**3.5**	4.8	4.8	**3.9**	**3.7**	4.2	4.2	4.7	3.7	3.5	3.5	3.7	4.1	4.4	4.3	3.5	4.9	4.4

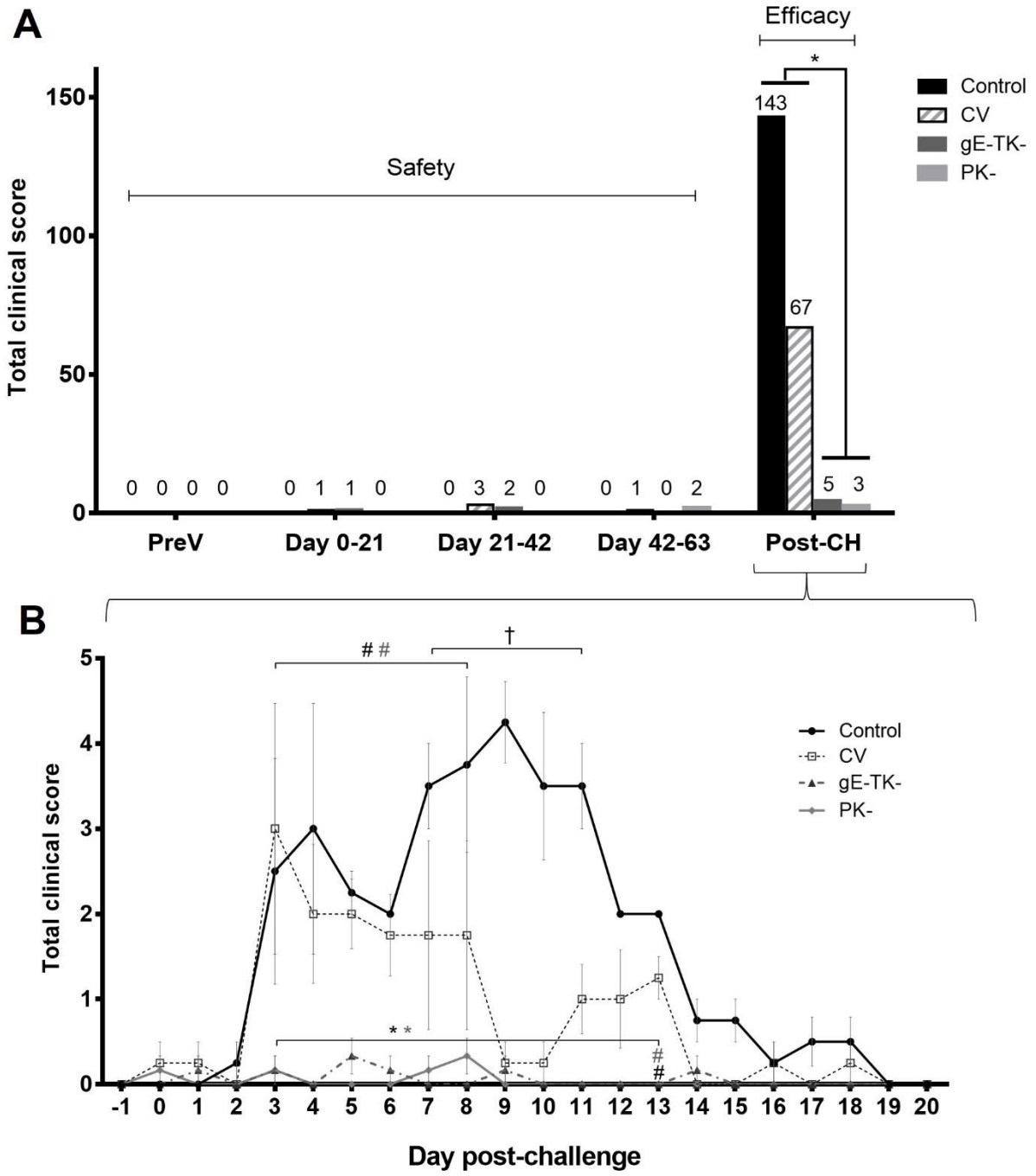

Figure 4. 1. Clinical scores. (A) Total clinical scores of controls (black bars), commercial vaccinates (CV, dashed bars), gE-TK- vaccinates (dark grey bars), and PK- vaccinates (light grey bars). Total scores pre-vaccination (pre-V), from day 0 to 21 (post-V1), from day 21 to 42 (post-V2), from day 42 to 63 (post-V3), and post-challenge (post-CH) from day 64 to day 83. Total scores in gE-TK- and PK- groups (n= 6) were adjusted to match the sample size of control and commercial vaccine groups (n= 4). An Asterisk represents a significant difference ($p< 0.05$). (B) Average daily total scores +/- SEM (standard error of the mean) of controls (black circles and solid lines), commercial vaccinates (CV, open squares and dotted lines), gE-TK- vaccinates

Figure 4. 1. (Cont'd) (triangles with dashed lines), and PK- vaccinates (diamonds with grey lines) are shown from one day before challenge to the end of the study. Black * indicate a significant difference ($p < 0.05$) between the controls and gE-TK- vaccinates. Grey * indicate a significant difference ($p < 0.05$) between the controls and PK- vaccinates. Black # indicates a significant difference ($p < 0.05$) between the commercial vaccinates and the gE-TK- vaccinates. Grey # indicates a significant difference ($p < 0.05$) between the commercial vaccinates and the PK- vaccinates. Black † indicate a significant difference ($p < 0.05$) between commercial vaccinates and controls.

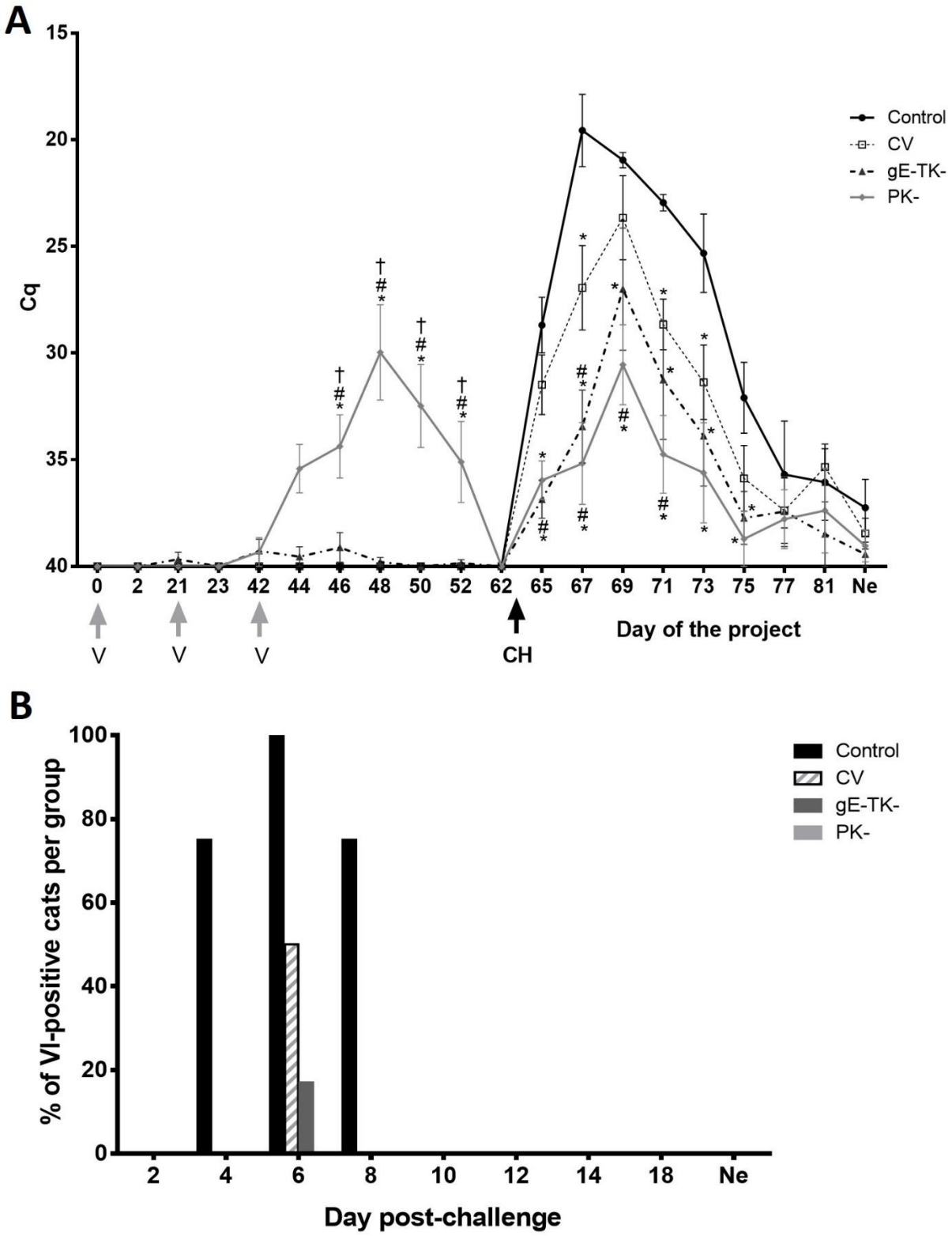

Figure 4. 2. Viral nasal shedding in nasal swab samples. (A) Presence of FHV-1 gB DNA quantitated by real-time PCR. Average viral shedding +/- SEM (standard error of the mean) of controls (black circle and solid lines), commercial vaccinates (CV, open squares and dotted

Figure 4. 2. (Cont'd) lines), gE-TK- vaccinates (triangles with dashed lines), and PK- vaccinates (diamonds with grey lines). * represents a significant difference ($p < 0.05$) from the control group. # represents a significant difference ($p < 0.05$) from the commercial vaccine group. † represents a significant difference ($p < 0.05$) between PK- and gE-TK- groups. Vaccinations on day 0, 21, and 42 are highlighted by grey arrows indicating a vaccination (V). The challenge infection on day 63 is indicated by a black arrow and CH. (B) Virus isolation following challenge infection. The percentage of cats that were positive by virus isolation are indicated by black bars (controls), dashed bars (commercial vaccinates, CV), dark grey bars (gE-TK- vaccinates), and light grey bars (PK- vaccinates) daily post-challenge.

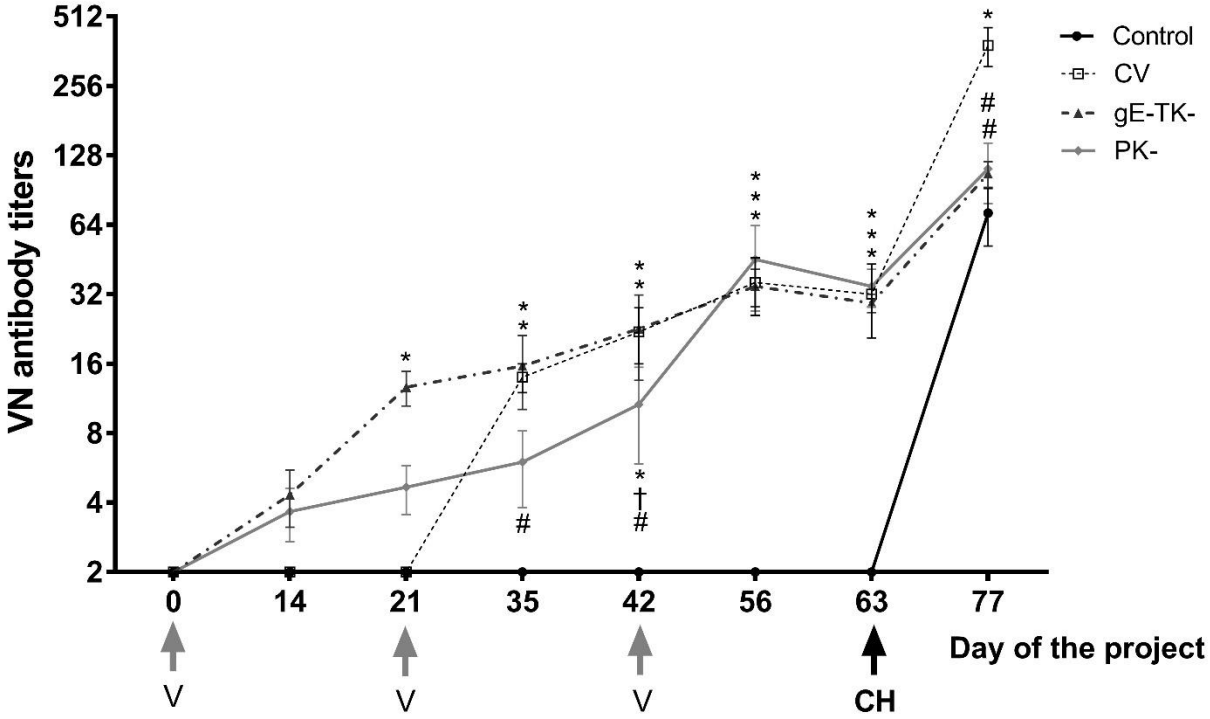

Figure 4. 3. Virus neutralization antibody titers +/- SEM (standard error of the mean) of controls (black circle and solid lines), commercial vaccinates (CV, open squares and dotted lines), gE-TK- vaccinates (triangles with dashed lines), and PK- vaccinates (diamonds with grey lines). * represents a significant difference ($p < 0.05$) from the control group. # represents a significant difference from the commercial vaccine group. † represents a significant difference ($p < 0.05$) between PK- and gE-TK- groups. Vaccinations on day 0, 21, and 42 are highlighted by grey arrows indicating a vaccination (V). The challenge infection on day 63 is indicated by a black arrow and CH.

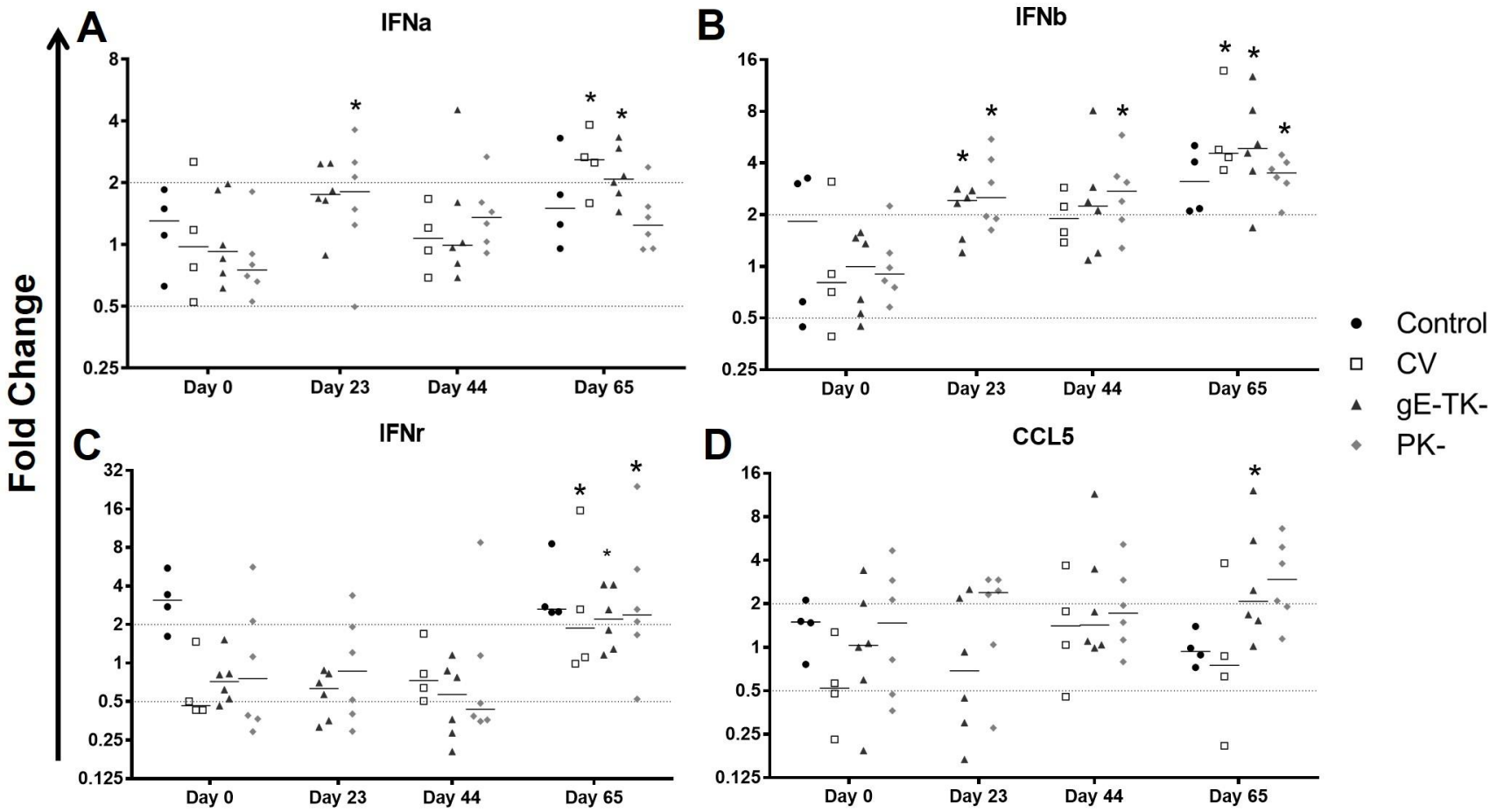

Figure 4. 4. Interferons and CCL5 mRNA expression in whole blood samples. IFNα (A), IFNβ (B), IFNγ (C), and CCL5 (D). The line is the median of fold change for each group and individual symbols represent fold changes for individual cats. * represents a significant difference ($p < 0.05$) from the same group from day 0. CV: Commercial vaccinates.

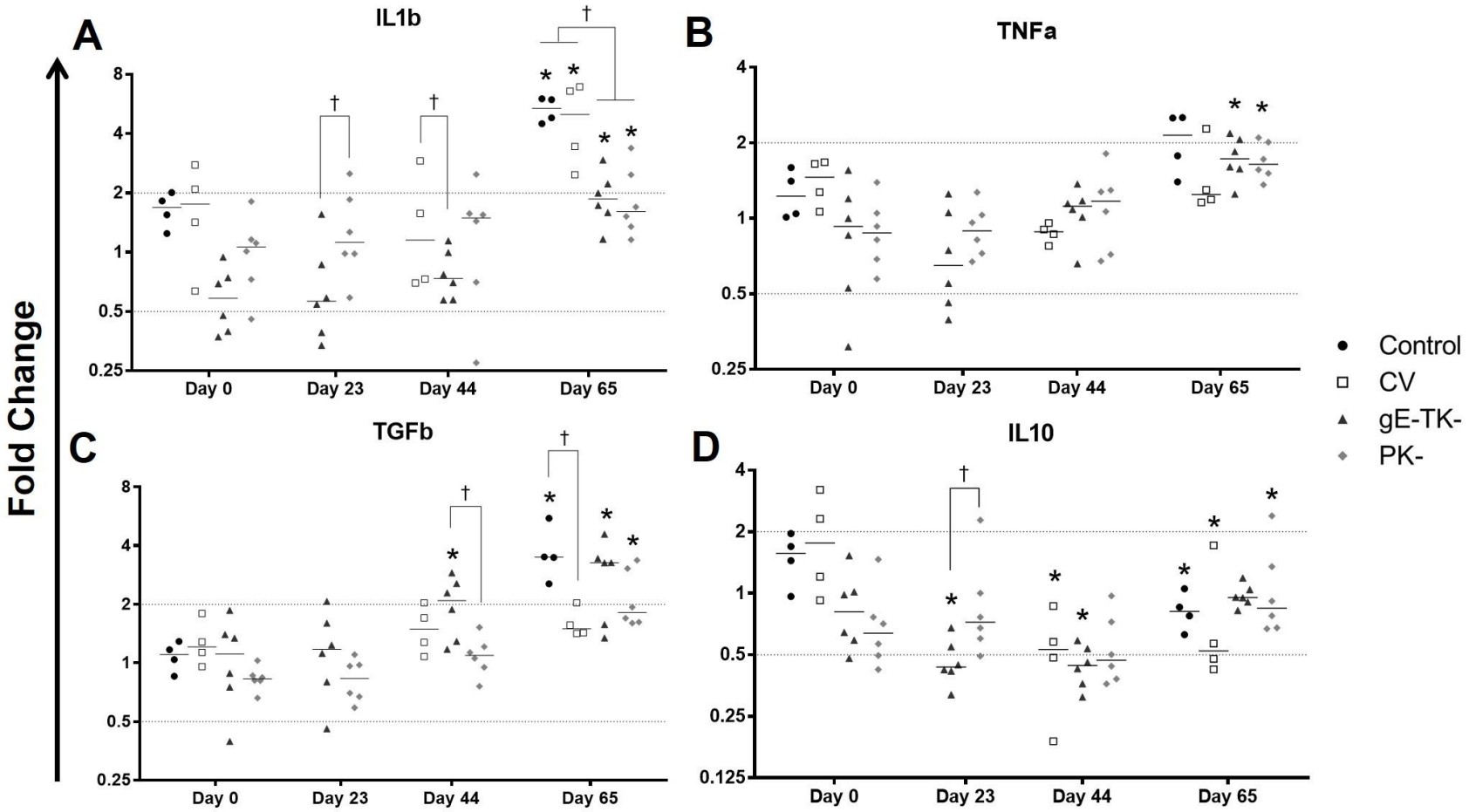

Figure 4. 5. Cytokine mRNA expression in whole blood samples. IL1b (A), TNFα (B), TGFβ (C), and IL10 (D). The line is the median of fold change for each group and individual symbols represent fold changes for individual cats. * represents a significant difference ($p < 0.05$) from the same group from day 0. † represents a significant difference ($p < 0.05$) between groups on the same sampling day. CV: Commercial vaccinates.

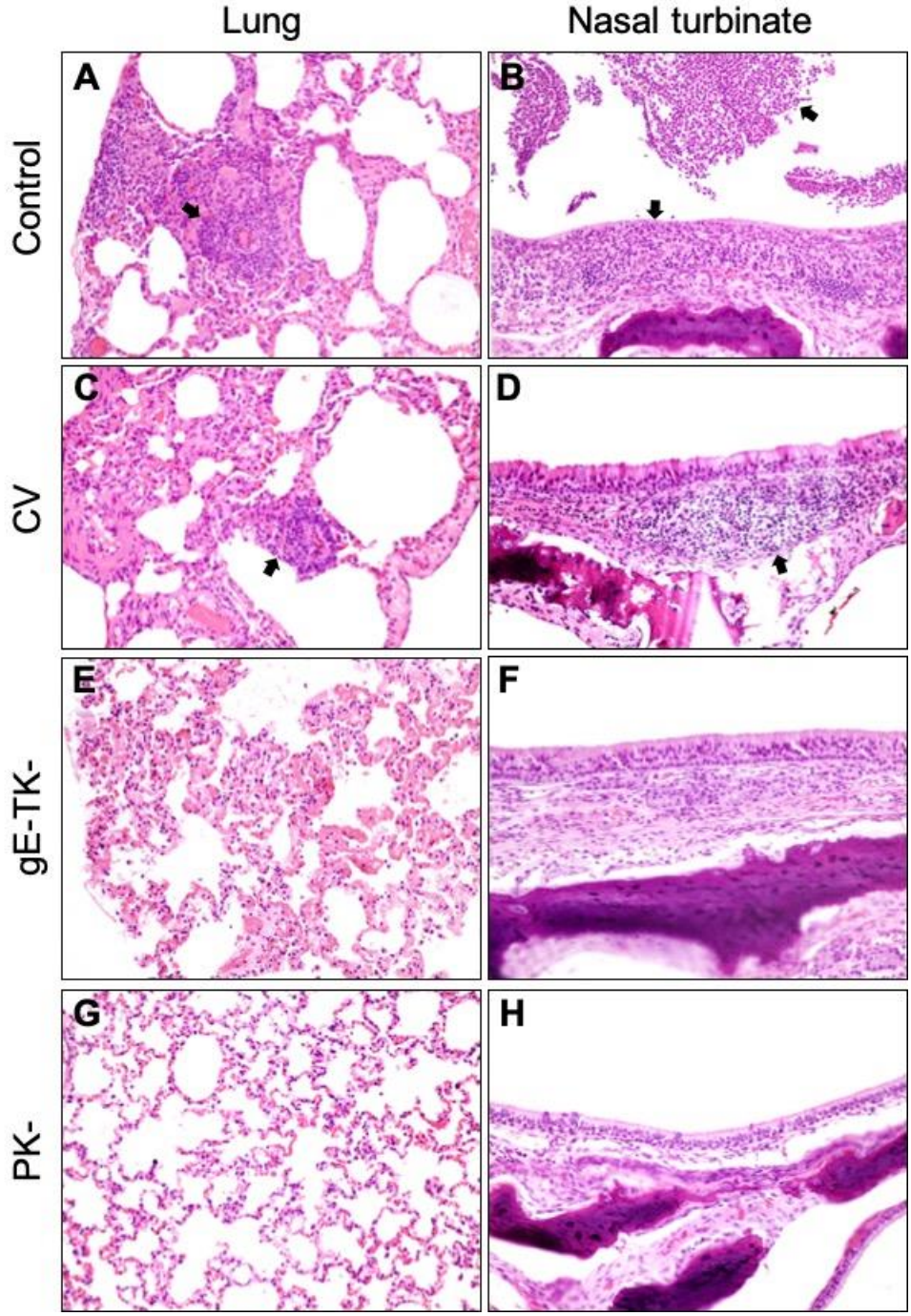

Figure 4. 6. Histological changes in lung and nasal turbinates at time of necropsy. Representative images of the lungs from controls (A), commercial vaccinates (CV) (C), gE-TK- vaccinates (E), and PK- vaccinates (G). Representative images of nasal turbinates from controls (B), commercial vaccinates (D), gE-TK- vaccinates (F), and PK- vaccinates (H). Arrows indicated the infiltration and aggregation of inflammatory cells, composed of lymphocytes and neutrophils.

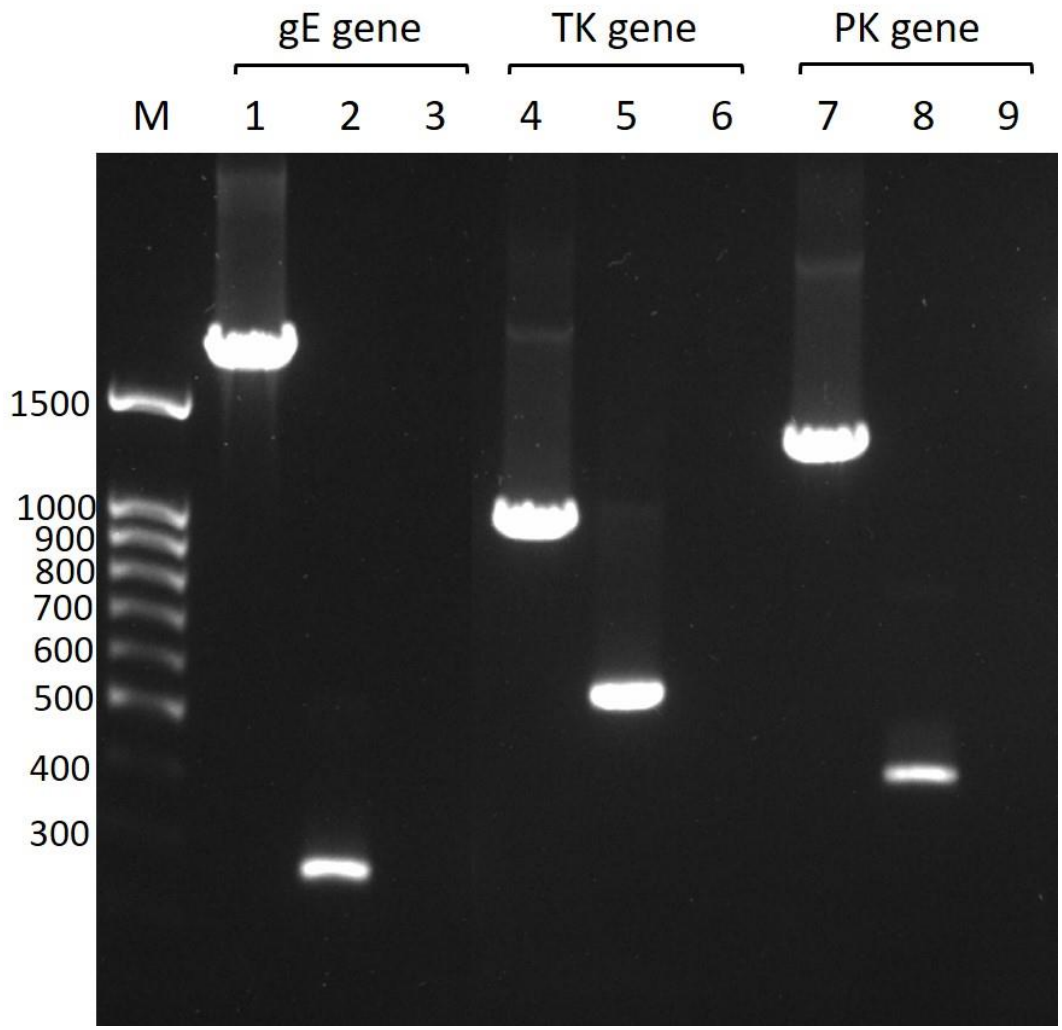

Figure 4. 7. The conventional PCR results. Conventional PCR was carried out to detect the FHV-1 genes of gE (lane 1-3), TK (lane 4-6), and PK (lane 7-9), using DNA samples extracted from the virulent FHV-1 (lane 1, 4, and 7), gE-TK- mutant (lane 2 and 5), and PK- mutant (lane 8). Lane 3, 6, and 9 were negative controls. The virulent FHV-1 contained a full length of each gene, which is 1867 base pairs (bp) for gE gene, 1006 bp for TK gene, and 1266 bp for PK gene. The gE-TK- mutant had a deficient gE gene with 268 bp in length, and a deficient TK gene with 543 bp in length. The PK- mutant had a deficient PK gene with 379 bp in length. M: DNA ladder as the marker.

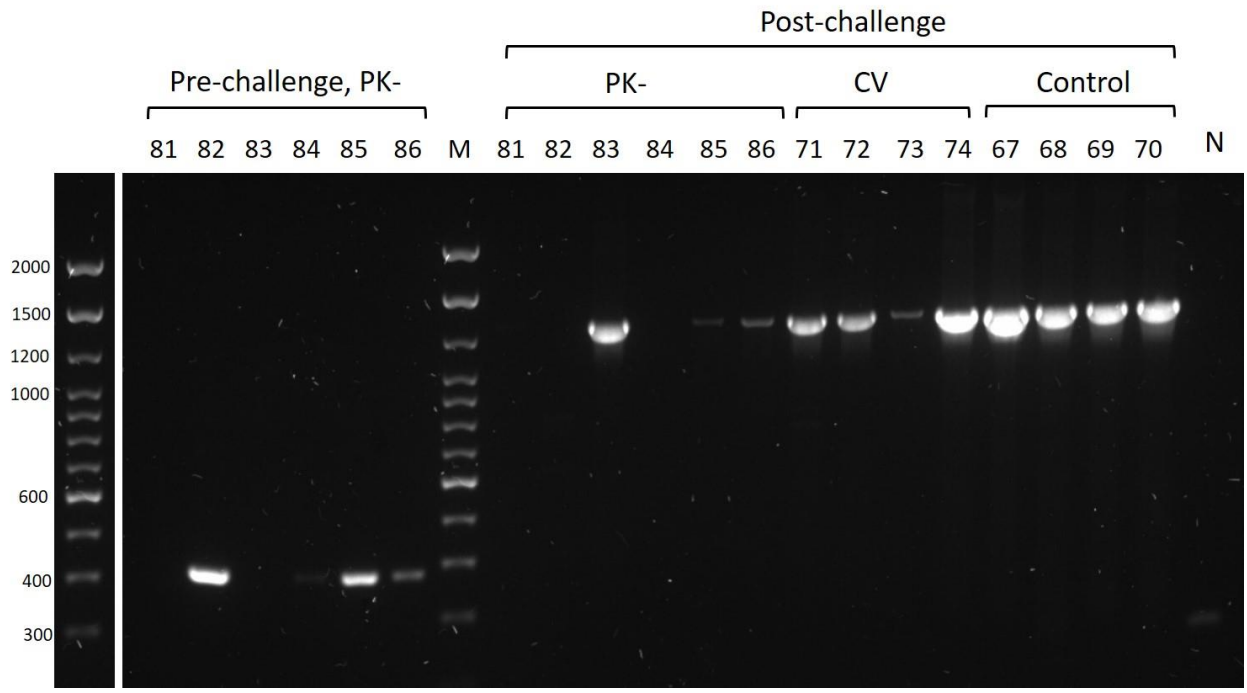

Figure 4. 8. Conventional PCR targeting FHV-1 PK gene was performed to differentiate the virus origin in nasal shedding prior to and after challenge infection. The number on the top of the gel was the case No. applied in the study. A PCR product with 379 bp in size, which matched with the size of mutated PK shown in Figure 4. 7, was found in 3 PK- vaccinates prior to challenge infection. A PCR product with a size of 1266 bp, which matched with the size of full PK gene in virulent FHV-1, was noted in 3 PK- vaccinates (CV), all 4 commercial vaccinates, and all unvaccinated controls post challenge. M: DNA ladder as the marker. N: negative control for PCR.

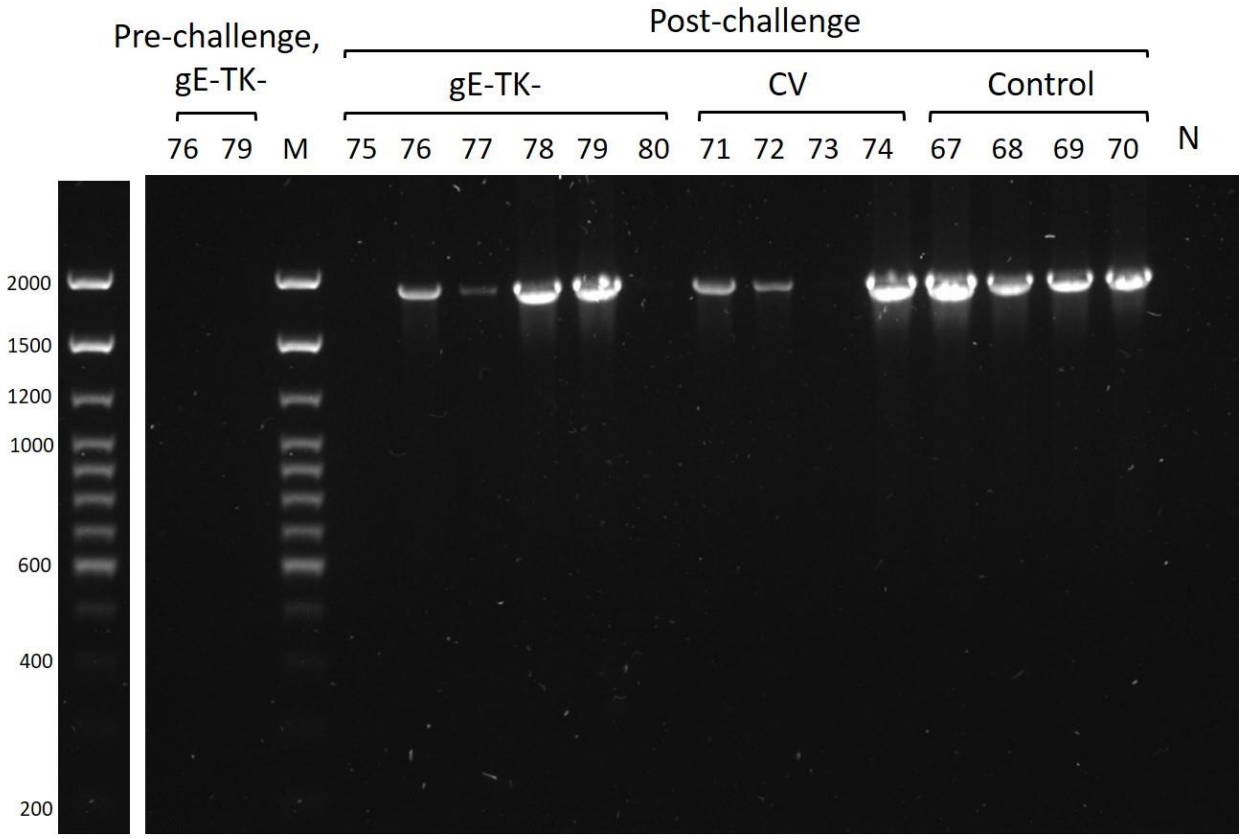

Figure 4. 9. Conventional PCR targeting FHV-1 gE gene was performed to differentiate the virus origin in nasal shedding prior to and after challenge infection. The number on the top of the gel was the case No. applied in the study. There was no PCR product revealing in 2 of the gE-TK- vaccinates prior to challenge presumably due to extremely low or no viral shedding. A PCR product with 1867 bp in size, which matched with the size of full gE gene in virulent FHV-1 shown in **Figure 4. 7**, was noted in 4 gE-TK- vaccinates, 3 commercial vaccinates (CV), and all 4 controls in the post challenge time. M: DNA ladder as the marker. N: negative control for PCR.

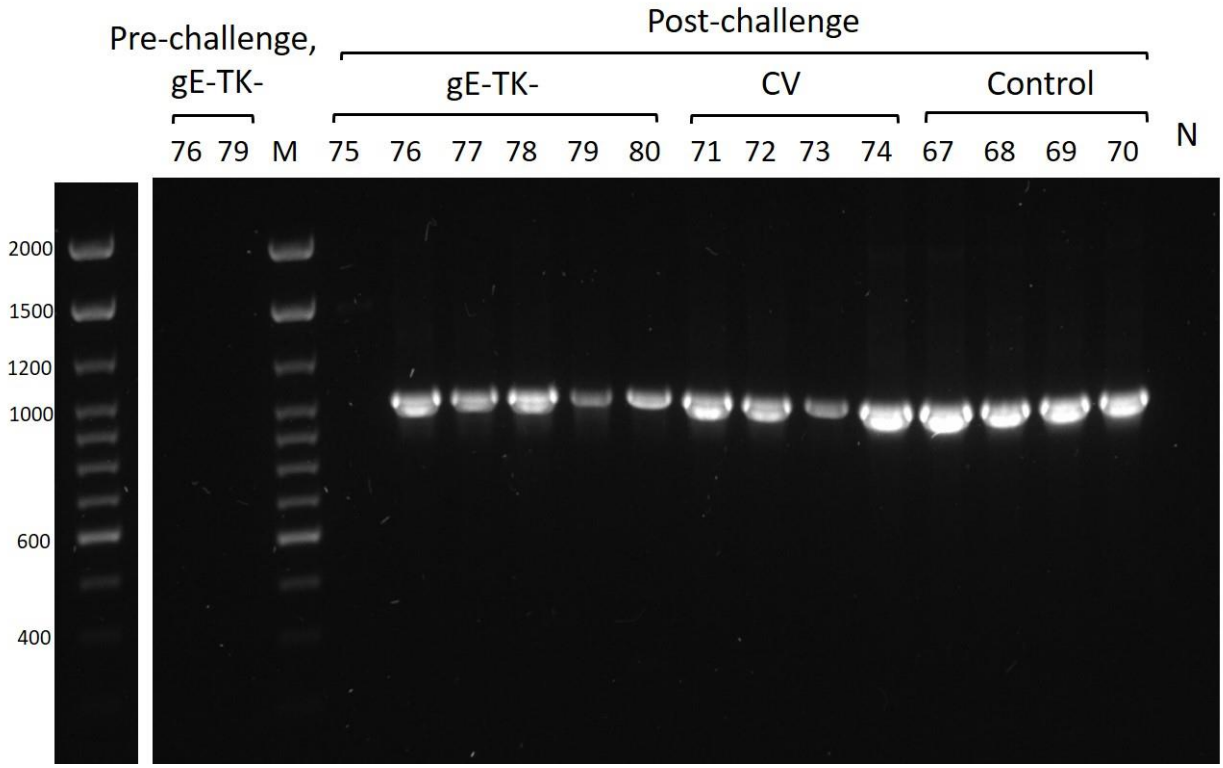

Figure 4. 10. Conventional PCR targeting FHV-1 TK gene was performed to differentiate the virus origin in nasal shedding prior to and after challenge infection. The number on the top of the gel was the case No. applied in the study. There was no PCR product revealing in 2 of the gE-TK- vaccinates prior to challenge presumably due to extremely low or no viral shedding. A PCR product with 1006 bp in size, which matched with the size of full gE gene in virulent FHV-1 shown in Figure 4. 7, was noted in 5 gE-TK- vaccinates, all 4 commercial vaccinates (CV), and all 4 controls in the post challenge time. M: DNA ladder as the marker. N: negative control for PCR.

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CHAPTER 5

CONCLUSTION AND FUTURE DIRECTIONS

CONCLUSIONS AND FUTURE DIRECTIONS

FHV-1 is an important viral respiratory and ocular pathogen of cats and the author has highlighted the shortcomings of current FHV-1 vaccination in the introduction of this thesis. Based on this information, there is significant need for novel vaccination platforms that can be safely administered at the mucosal level and strengthen respiratory immunity to FHV-1.

To address this problem, we have previously generated a bacterial artificial chromosome (BAC) containing the FHV-1 genome and constructed several FHV-1 deletion mutants. We then conducted a systematic *in vitro* characterization of these mutants, which is described in chapters 2 and 3 of this thesis. The feline primary respiratory epithelial cell (FREC) cultures utilized in chapter 2 to compare FHV-1 vaccine candidates are unique tools because they simulate the microenvironment and immune system of the natural airway epithelium (Nelli et al., 2016). We show that FREC cultures are superior to study FHV-1 pathogenesis and immunity compared to conventional *in vitro* monolayer cell lines, e.g. Crandell-Rees feline kidney (CRFK) cells (Tai et al., 2010). Using the FREC system, we showed that single deletions of gC or gE in FHV-1 did not attenuate the viral replication in respiratory epithelial cells, but that double deletion of gE and TK or single deletion of PK significantly reduced viral replication. Moreover, respiratory epithelial cells infected with gE-TK- or PK- mutants mounted better innate immune responses, suggesting that gE, TK or PK not only are virulence factors, but also contribute to immune modulation observed with wild type virus. In chapter 3, we then used an *ex vivo* tracheal tissue explant system to evaluate tissue damage and stromal invasion of FHV-1 wild-type (WT) and FHV-1 mutants, e.g. gC-, gE-, PK-, and gE-TK-. This study confirmed that the virulence of gC- and gE- mutants were not significantly different from that of WT FHV-1, but that the gE-TK-

and PK- mutants were significantly attenuated in terms of virulence, despite of the ability to penetrate the basement membrane. Based on the combined results described in chapters 2 and 3, the PK- and gE-TK- FHV-1 mutants were selected as vaccine candidates for immunizing cats against feline viral rhinotracheitis. The *in vivo* study in cats in chapter 4 examined the safety and efficacy of PK- and gE-TK- FHV-1 mutants, using a prime-boost immunization strategy (two subcutaneous inoculations followed by one intranasal inoculation). This study provided a comprehensive investigation of clinical signs, viral shedding, and immune responses following vaccination and subsequent challenge infection with virulent FHV-1. Our results verified the safety of the selected prime-boost immunization strategy using the PK- and gE-TK- mutants. Moreover, the protection resulting from vaccination with the deletion mutants was superior compared to that obtained with a commonly used commercial vaccine. Specifically, immunization with either one of the FHV-1 mutants drastically reduced clinical signs and the PK- mutant completely prevented viral shedding after exposure to the field virus.

As summarized in this thesis, we have developed a prime –boost strategy to enhance immunity at the respiratory mucosa, which is crucial because the virus primarily targets the respiratory epithelium and the conjunctiva (Lappin et al., 2006). Our current data demonstrates that the FHV-1 deletion mutants were safe for intranasal boosting. However, testing of mucosal administration with the FHV-1 deletion mutant candidates alone will be required to verify the safety for mucosal vaccination by itself, as well as to examine the mucosal immune responses without prior systemic priming. In addition, it would be worthwhile to evaluate our FHV-1 deletion mutants as an intranasal boost for the current commercial parenteral FHV-1 vaccines. The parenteral commercial vaccine claims long-term humoral responses for up to 3 years in cats (Jas et al., 2015). Therefore, the duration of immunity stimulated by our deletion mutants should

be examined as well. Furthermore, current commercial vaccines for FHV-1 are used as trivalent vaccines, aimed at protecting not only from FHV-1 infection but also from feline calicivirus (FCV) and feline panleukopenia virus (FPV) (Day et al., 2016). Another study showed that the commercial trivalent vaccine demonstrated some nonspecific protections against other common respiratory pathogens, such as *Bordetella bronchiseptica* (Bradley et al., 2012). In future experiments we will investigate the use of our FHV-1 deletion mutants for insertion of the components of other viruses, including but not limited to FCV and FPV, to create multivalent vaccines (Yokoyama et al., 1996a; Yokoyama et al., 1996b).

My work has demonstrated the merit of primary FREC cultures for studying FHV-1 pathogenesis and immunity. Currently, I am working on the immortalization of the FRECs, with the goal to expand the application of this *in vitro* system by eliminating or at least reducing the need for cats. Such *in vitro* culture systems are highly valuable tools for studying both infectious and non-infectious respiratory diseases in cats, in addition to FHV-1 infection.

For example, the current novel coronavirus pandemic disease (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a huge threat to human health and the economy worldwide. It has been shown that the C-terminal domain of spike protein (S) from SARS-CoV-2 binds to host receptor angiotensin-converting enzyme 2 (ACE2), followed by cleavage of transmembrane protease serine 2 (TMPRSS2) to prime virus entry (Hoffmann et al., 2020). By using human bronchial epithelial cell (HBEC) cultures in an air-liquid-interface system, the secretory cell subset of the bronchial epithelial cells has been quickly identified as the susceptible cell type of SARS-CoV-2, due to its high expression of both ACE2 and TMPRSS2 (Lukassen et al., 2020). Interestingly, it has also been shown that ferrets and cats are susceptible to this virus as well. Approximately 10^6 copies of viral RNA were detected in nasal

turbinates, tracheas, tonsils, and lungs in cats 3 days after a challenge with a virulent SARS-CoV-2, suggesting viral replication and the potential of viral shedding from those tissues (Shi et al., 2020). Moreover, based on sequencing, it has been shown that 16 out of 19 amino acids in the binding-domain of ACE2 from cat cells are identical to those from the human cells, suggesting that cats maybe hosts or reservoirs for this virus in addition to non-human primates (Sun et al., 2020). The primary FRECs as well as immortal feline respiratory epithelial cells (iFRECs) developed by our lab could be an excellent *in vitro* model to help investigate the pathogenesis of SARS-CoV-2 in cats. Primary and iFRECs could be stained with antibodies and analyzed by flow cytometry to examine different cell subsets and their expressions of ACE2. The sub-population of the epithelial cells which expressed the most ACE2 could be separated out, followed by next-generation sequencing and compared to the results found in human cells. Primary and iFRECs could also be inoculated with viral proteins such as a recombinant S protein of SARS-CoV-2, and then the viral protein could be traced within the cells by flow cytometry. Using this technology, the cell subset which contained the most amount of viral protein could be identified and characterized by different cell markers. The virus internalization and glycosylation within the iFRECs could be studied as well, which would strengthen our knowledge of drug or vaccine development (Kumar et al., 2020).

Primary or iFREC cultures could also be used as an *in vitro* system to study asthma, which has been widely studied for years in human medicine by using HBEC ALI culture system (Stewart et al., 2012). Feline asthma is a critical airway non-infectious disease in cats. The common treatment for feline asthma is oral administration of glucocorticoids, but such treatment is not suitable for cats with concurrent diseases including diabetes mellitus and cardiac diseases (Trzil and Reinero, 2014). Some medications for human asthma like serotonin antagonists (to

counteract serotonin's induction of bronchoconstriction) and second-generation antihistamines are ineffective for treating feline asthma (Reinero et al., 2005; Schooley et al., 2007), implying that the mechanisms of human and feline asthma might be different. Development of innovative treatments such as stem cell therapy is ongoing (Trzil et al., 2016), but most experiments were performed *in vivo* in mice or in cats primarily due to the lack of an appropriate *in vitro* respiratory cell culture systems. Our FRECs or iFRECs could provide a great platform for studies of cytokine profile induction associated with asthma, by performing co-culture of iFRECs with primary feline peripheral blood mononuclear cells (PBMCs) or FeT-J, a cell line of feline PBMCs. Moreover, the methodology of the co-culture of iFRECs with blood monocyte-derived dendritic cells or dendritic cells derived from feline lung tissues, as demonstrated previously in horses (Lee et al., 2017), could be adopted to help understanding the pathogenesis of feline asthma, considering that dendritic cells play a key role for initiating the hypersensitivity that is linked to asthma (Hammad and Lambrecht, 2008). Furthermore, the FREC or iFREC systems, the tissue explant cultures established in this study could be a valuable addition for looking at associated tissues, and thus reduce the number of *in vivo* studies.

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