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STANDARD ARTICLE

Evaluation of liposome toll-like receptor ligand complexes for non-specific mucosal immunoprotection from feline herpesvirus-1 infection

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Background: Feline herpesvirus-1 (FHV-1) infection can result in serious morbidity and mortality, especially in kittens. Immunotherapy using liposome-toll-like receptor (TLR) ligand complexes (LTC) has been shown to activate innate immune responses.

Objectives: To determine in kittens whether mucosal administration of LTC before FHV-1 inoculation would decrease severity of clinical signs and decrease quantities of FHV-1 DNA in materials collected on oropharyngeal swabs.

Animals: Nineteen, 14-week-old, purpose-bred kittens.

Methods: Pilot clinical trial with 2 groups of kittens allocated to either an LTC or control group. The LTC were administered into both nares and the oropharynx of the 12 LTC group kittens, and all 19 kittens were inoculated with FHV-1 24 hours later. Clinical scores were determined daily for 28 days, and oropharyngeal mucosal materials were collected every 7 days to assess FHV-1 DNA quantities for comparison between groups.

Results: Conjunctivitis was more common in kittens in the control group on Days 15-28 ($P = .01$) and Days 1-28 ($P = .02$). Total respiratory scores were higher in the LTC group on days 15-28 ($P = .03$). The LTC group had significantly decreased FHV-1 DNA on swabs when compared to the control group on some postinoculation days, using 2 methods of calculation.

Conclusions and Clinical Importance: Administration of LTC to kittens was shown to decrease FHV-1 DNA and some manifestations of illness in kittens when administrated 24 hours before inoculation, suggesting clinical benefit.

KEYWORDS
immunity, immunotherapy, innate, toll-like

1 | INTRODUCTION

Feline herpesvirus 1 (FHV-1) is a common cause of ocular and upper respiratory infections in cats and can be a major cause of morbidity and sometimes mortality, especially in young kittens.[1–5] Prior administration of FHV-1 vaccines may lessen illness if exposed, but vaccination against FHV-1 provides incomplete immunity.[6–9] Clinical signs of FHV-1 infection can be reactivated with repeat exposure, after induction of stress, or after administration of immunosuppressive drugs.[10–12]

One study showed that after FHV-1 challenge, a significant decrease in clinical scores was noted in kittens as soon as 4 days after administration of 1 dose of an intranasal vaccine, and this decrease occurred before development of specific FHV-1 immune responses.[7] Administration of an intranasal FHV-1 vaccine was shown to induce cross protection against *Bordetella bronchiseptica*, a primary bacterial pathogen in cats that was not contained in the vaccine.[13] These

findings suggested that intranasal administration of these 2 vaccines had induced nonspecific immune responses that imparted a positive effect against the primary pathogen. This result supports continued work to evaluate stimulation of innate immunity for protection against infections in cats.

Toll-like receptors are evolutionarily conserved receptors that activate cellular immune defenses against a variety of different pathogens.[14–16] Viruses and bacteria express specific molecular structures that are capable of activating toll-like receptors 3 (TLR3) and TLR9, respectively.[14–16] Given that TLR activation can generate nonspecific protection from infections, several different approaches to developing immunotherapeutic agents that activate TLR pathways have been developed for generating protective immunity. One widely studied immunotherapy platform is based on triggering innate immune responses using TLR9 agonists complexed to cationic liposomes, which greatly enhances the activity of the TLR9 agonist.[17,18] In a number of animal challenge studies, parenteral or inhalational administration of liposomal-TLR9 complexes generated complete or nearly complete protection against highly virulent bacterial and viral pathogens.[19–24] In addition, administration of liposome-TLR9 complexes to cats with chronic rhinitis also was shown to alleviate some clinical signs of illness.[25]

Recently, a new formulation of a liposome-TLR complex (LTC) was developed that includes a TLR9 agonist, a TLR3 agonist, and methylcellulose as a mucosal adhesive agent.[26] In a study of healthy, purpose-bred cats, cytokine and cellular immune responses to this LTC were evaluated in vitro and in vivo. Quantitative polymerase chain reaction (qPCR) assays, ELISA assays, and flow cytometry were used to evaluate nasal lavage specimens and pharyngeal swabs.[26] In that study, the in vitro experiment showed that the LTC rapidly activated cat leukocytes, including upregulation of costimulatory molecules and cytokine production. The in vivo experiment showed that topical administration of the LTC triggered rapid recruitment of monocytes to the nasal and oropharyngeal mucosa in the healthy cats. Based on the results from these in vivo and in vitro experiments in healthy cats, the objective of our pilot study was to determine whether mucosal administration of LTC before FHV-1 challenge could decrease the severity of clinical signs, hasten resolution of clinical signs, and decrease FHV-1 DNA shedding in the kittens. Our primary hypothesis was that administration of LTC before FHV-1 challenge would induce positive clinical outcomes to infection.

2 | MATERIALS AND METHODS

2.1 | Animals

Nine female and 10 male, 14-week old, purpose-bred nonvaccinated domestic shorthair kittens were included in this 28-day pilot study. Before the start of the study, all kittens were serologically negative for FHV-1, and pharyngeal swab samples obtained from each kitten were negative for DNA of FHV-1 by PCR assay (Center for Companion Animal Studies, Colorado State University, Fort Collins, Colorado). Before the start of the study, gonadectomies were performed in each of the males (19 days) and 6 randomly selected females (26 days).

2.2 | LTC formulation

Cationic liposomes (1,2-dioleoyl-3-trimethylammonium propane; Avanti, Alabaster, Alabama), poly I:C (InVivoGen, San Diego, California), and pDNA (noncoding commercial plasmid PCR2.1; Thermo-Fisher, Waltham, MA) were mixed, followed by addition of carboxymethylcellulose (Sigma-Aldrich, St. Louis, Missouri). This LTC formulation has been described in previous experiments in healthy cats.[26]

2.3 | Study design

The 19 study animals were divided into 2 separate rooms that varied in size with the smaller room housing the LTC group (n = 7; 3 intact females and 4 neutered males) and the larger room housing the untreated control group (n = 12; 6 neutered females and 6 neutered males). The dissimilar group sizes and rooms were used because of a previously planned study that included the 12 control cats.[11] The kittens were housed and cared for in accordance with a protocol approved by the Institutional Animal Care and Use Committee at the contract research facility.

Five days before the start of the study, the kittens were moved into their respective rooms. Twenty-four hours before FHV-1 inoculation, the kittens in the LTC group were given 0.2 mL of the LTC in each nare and 0.6 mL in the caudal oropharynx, and the control group was not treated. On Day 0 of the study, each of the 19 animals was sedated using the facility protocol and inoculated with $1 \times 10^{5.3}$ tissue culture infective dose (TCID-50) of a strain of FHV-1 originally obtained from the US Department of Agriculture, divided equally between the nares and oropharynx.[8,9]

2.4 | Clinical monitoring

Two trained observers (masked as to assignment of animals to the study groups) assessed the kittens for 30 minutes at approximately the same time every morning beginning on Day 0 before FHV-1 inoculation and continuing through Day 28 postinoculation. Observers used a clinical score sheet adapted from other FHV-1 vaccination or treatment studies (Table 1).[9,10] Body temperatures were determined by microchip.[27] Increased body temperature was defined as >102.5°F (39.2°C), and pyrexia therefore was classified as present or absent per kitten per day. Body weights were measured weekly. Overall health was monitored daily by 1 of the study investigators. The protocol included a rescue clause for those kittens that developed moderate to severe signs of FHV-1 and loss of appetite for 48 hours. Daily appetite during severe illness days was determined by interest in and willingness to consume a canned food formulated for critical care use (Hill's Prescription Diet a/d, Topeka, Kansas), which was offered in equal small quantities to the kittens in each room. Supportive care and treatment that could be administered included SC fluids, buprenorphine for discomfort, topical cidofovir, or PO famciclovir as needed as determined by the investigators.

2.5 | Laboratory evaluations

On Days 7, 14, 21, and 28, mucosal cells were collected from the caudal pharynx of each kitten using manual restraint without sedation.

TABLE 1 Clinical scoring rubric applied by 2 trained observers to each kitten daily for 28 days

Clinical sign	Score
Conjunctivitis	0 = None
	1 = Mild
	2 = Moderate
	3 = Severe
Blepharospasm	0 = None
	1 = Eye < 25% closed
	2 = Eye 25%-50% closed
	3 = Eye 50%-75% closed
	4 = Eye completely closed
Ocular discharge	0 = None
	1 = Mild serous (clear) discharge
	2 = Moderate mucoid (white) discharge
	3 = Severe mucopurulent (moist yellow-green) discharge
Body temperature (microchip)	0: ≤102.5
	1: >102.5
Cough	0 = None
	1 = Observed
Sneezing (yes/no)	0 = None
	1 = Observed
Nasal discharge	0 = None
	1 = Mild serous (clear) discharge
	2 = Moderate mucoid (white) discharge
	3 = Severe mucopurulent (moist yellow-green) discharge or hemorrhagic (bloody/red) discharge
Nasal congestion (if score varies during observation period, record highest score observed)	0 = None (no congestion present; able to breathe through both nares without difficulty)
	1 = Mild/minor congestion (barely audible; audible on close listening, subtle snoring sounds on inhalation ANY time during the observation period
	2 = Moderate congestion (easily audible; consistently audible throughout observation period; audible snoring sounds on inhalation or expiration that are likely to originate from the nasal cavity)
	3 = Severe congestion (audible across the room, with or without open mouth breathing; minimal nasal air flow noted from 1 or both nares after local debris is cleared away)

Swabs were stored at −80°C until assayed in batches. Total DNA was extracted from the oropharyngeal swabs and evaluated for DNA of FHV-1, DNA of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene, and feline 28S ribosomal DNA by qPCR as previously described.[28,29] Results of the FHV-1 qPCR assay were expressed as the ratio of FHV-1 DNA/GAPDH DNA in an attempt to standardize specimens and ensure sample adequacy by presence of GAPDH in the sample. To verify findings between groups of cats using the FHV-1/GAPDH ratio calculation, the delta-delta Cq method was used to compare FHV-1 to 28S results and then compared between groups in the statistical analyses.[30]

2.6 | Statistical analysis

Soon after the FHV-1 challenge and onset of clinical signs in study animals, it was determined that sneezing and nasal congestion were being underestimated in the control group because of the larger number of kittens (n = 12). Thus, the scorers were unable to reliably capture sneezing and nasal discharge occurrences in the larger study group. Sneezing and nasal congestion therefore were excluded from all subsequent data analyses.

Three time periods were used for data analysis: Days 1-14, 15-28, and 1-28. Observation occurrences were defined as the number of times a clinical sign (Table 1) was observed to be present within a time period and group. For each 14-day time period, the LTC group had 98 possible observation occurrences and the control group had 168 possible observation occurrences. For the 28-day time period, the LTC group had 196 possible observation occurrences and the control group had 336 possible observation occurrences. Total scores were calculated for each kitten each day by adding the individual clinical score variables recorded for that day, excluding sneezing and nasal congestion as described above (Table 1). Total ocular scores were calculated as the sum of conjunctivitis, blepharospasm, and ocular discharge. Total respiratory scores were calculated as the sum of nasal discharge and cough. Total clinical scores were calculated as the sum of total ocular, total respiratory, and pyrexia scores. The presence versus absence of severe ocular disease and severe respiratory disease were recorded on each day that a kitten's total ocular or total respiratory score was >2. The presence of severe total clinical disease was recorded on each day that the kitten's total clinical score was >3. Day of illness resolution was defined as the study day on which total clinical score was <2 and remained as such.

Descriptive statistics were calculated. Clinical scores were expressed as frequencies (presence or absence) of observations and total ocular, respiratory, and clinical scores as well as the FHV-1/GAPDH ratios were expressed as median, mean, and range. Continuous variables including change in body weight and day of illness resolution also were expressed as median, mean, and range. The Shapiro-Wilk test was used to evaluate outcome variables for normality. Because of nonnormal distribution of variables and clinical outcome ordinal data, the Wilcoxon rank sum test was used to compare median clinical scores between the LTC group and the control group for each of the 3 time periods; FHV-1/GAPDH ratios and FHV-1 to 28S results on Days 7, 14, 21, and 28; and body weight changes. The number of days to illness resolution also was evaluated using Kaplan-Meier curves and compared between the groups using the log-rank test for equality of survivor functions. The proportions of observations of the dichotomous (presence or absence) clinical variables of pyrexia, severe ocular, severe respiratory, and severe total clinical scores were compared between the LTC group and the control group using a 2-tailed Fisher exact test. To control for lack of independence among observations because of repeated measurements on the same kitten over time, mixed model regression analyses were used, using ranked data for continuous variables. Conjunctivitis and nasal discharge were converted to dichotomous variables of presence or absence for mixed model logistic regression analyses. Odds ratios (OR) and 95% confidence intervals (CI) were calculated for some variables. Commercially available software (Stata Statistical Software:

Release 14; StataCorp 2015, College Station, Texas, StataCorp LP) was used for all comparisons. Significance was defined as $P < .05$.

3 | RESULTS

3.1 | Clinical findings

All 19 study kittens exhibited clinical signs of FHV-1 infection after inoculation. All kittens had a minimum of 12 days of observation with a total clinical score >0 and a minimum of 3 days of observation with a total clinical score >1. Subcutaneous fluids at 15 mL/kg were needed for 1 LTC group kitten on Day 9 and 1 control group kitten on Day 10. No other rescue treatments were needed. Pyrexia, ocular discharge, and nasal discharge occurred in all 19 kittens on at least 1 day. Conjunctivitis and blepharospasm occurred on at least 1 day in all 7 LTC kittens and in 11 of 12 control group kittens (overall 95% of kittens). Coughing was rare and was only observed in 1 LTC group kitten on Day 6, 3 control group kittens on Day 6, and in an additional control group kitten on Day 10.

Analysis of a number of clinical variables including total clinical score, ocular discharge, blepharospasm, and severe total respiratory disease (results not shown) did not identify statistically significant differences when comparing the LTC group to the control group. Pyrexia occurred at 54 of 168 control group observation points (32.1%) and at 21 of 98 LTC group observation points (21.4%) during Days 1-14 ($P = .07$). On Day 28 of the study, the group median body weight had increased more for the LTC group than for the control group, but the difference was not statistically significant ($P = .08$).

Conjunctivitis scores ≥1 were detected more frequently in the control group when compared to the LTC group on Days 1-14 (30% versus 16%; $P = .01$), Days 15-28 (29% versus 10%; $P < .001$), and Days 1-28 (29.5% vs 13%; $P < .001$). After controlling for lack of independence because of repeated measurements on the same kitten over time (Figure 1), the odds of having conjunctivitis were greater among kittens in the control group as compared to kittens in the LTC group
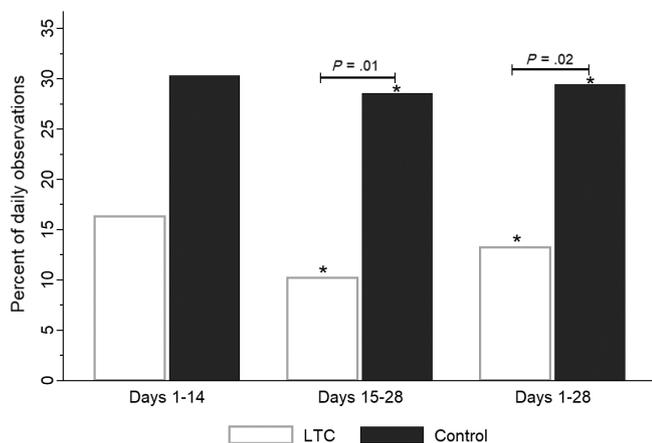
on Days 15-28 (OR, 4.4; 95% CI, 1.4-14; $P = .01$) and Days 1-28 (OR 3.1; 95% CI, 1.2-7.6; $P = .02$).

Total respiratory scores were higher in the LTC group compared to the control group on Days 15-28 ($P < .001$) and Days 1-28 ($P < .001$). After controlling for lack of independence, total respiratory scores were higher in the LTC group when compared to the control group on Days 15-28 ($P = .03$). However, all cats had total respiratory scores of ≤2 by Day 28.

For some clinical variables, statistical differences were detected between the LTC and control groups until the results were controlled for lack of independence because of repeated measurements on the same kitten over time. Total ocular score on Days 15-28 ($P = .04$), severe total clinical disease score on Days 1-14 ($P = .03$), and severe ocular disease score on Days 1-14 ($P = .02$) were higher in the control group compared to the LTC group until controlled for lack of independence. In addition, nasal discharge scores were higher in the LTC group compared to the control group on Days 15-28 ($P = .04$), until controlled for lack of independence.

In the LTC group, 4 kittens had a total clinical score of <2 by Day 28 although the other 3 kittens were clinically well but had total clinical scores of 2. In contrast, all 12 kittens in the control group had total clinical scores <2 on Day 28. When the cats with resolution of clinical disease by Day 28 were compared, the LTC group had a median of 17.5 days (range, 10 to >28) to resolution of illness compared to the control group kittens with a median of 23 days (range, 20-28). The days to resolution did not differ statistically ($P = .18$) between groups.

3.2 | FHV-1/GAPDH ratios and FHV-1 to 28S results

Detectable FHV-1/GAPDH ratios >0 were detected on at least 3 of the 4 dates of sample collection for 6 kittens in the LTC group and all 12 kittens in the control group. The largest amount of FHV-1 DNA was recovered on Day 7 for all kittens. The amount of FHV-1 DNA recovered was significantly higher in the control group on Day 21 ($P = .04$), Day 28 ($P = .002$), and when results from Days 21 and 28 were combined ($P = .001$). A total of 71 swabs (26 from the LTC group and 45 from the control group) contained quantifiable GAPDH and thus were used in this analysis. The FHV-1/GAPDH ratios did not differ between the groups on Days 7, 14, and 28 (Figure 2). On Day 21, the control group had significantly higher FHV-1/GAPDH ratios as compared to the LTC group ($P = .04$). When the results from Days 21 and 28 were combined, the control group had significantly higher FHV-1/GAPDH ratios as compared to the LTC group ($P = .01$; Figure 2). When the analyses were repeated using results of the FHV-1 to 28S calculation to verify the results from the FHV-1/GAPDH ratios (data not shown), the control group was significantly higher as compared to the LTC group on Day 28 ($P = .001$) and when the results from Days 21 and 28 were combined ($P = 0.005$).

FIGURE 1 Percentages of observations with conjunctivitis after FHV-1 inoculation of cats administered an LTC and an untreated control group. *P-values are from adjusted logistic regression model; y-axis represents percentage of daily kitten observations in which conjunctivitis was observed. FHV-1, feline herpesvirus-1; LTC, liposome-toll-like receptor complex

4 | DISCUSSION

All kittens in the study experienced acute clinical signs of FHV-1 infection, and all had measurable shedding of FHV-1 DNA documenting induction of FHV-1 infection. Our findings documenting an LTC
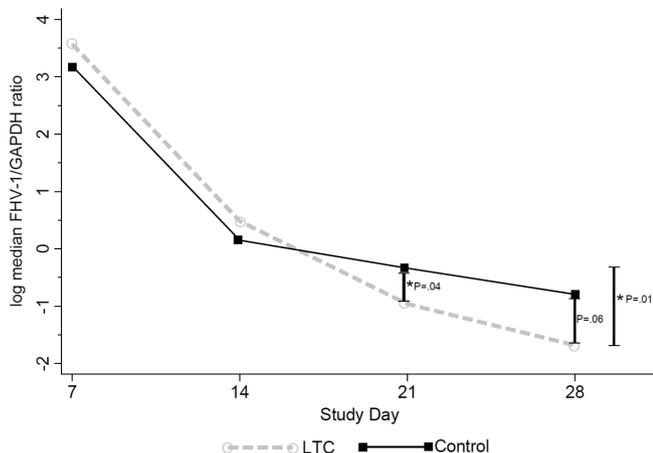
FIGURE 2 FHV-1/GAPDH ratios as detected by quantitative polymerase chain reaction from pharyngeal swabs of cats inoculated with FHV-1. Median values in logarithmic scale are depicted for the LTC group (n = 7, dotted gray line) and the control group (n = 12, solid black line with squares). *Statistical significance: P < .05. P values based on Wilcoxon rank sum test to compare median FHV-1/GAPDH ratios for the 2 groups. FHV-1, feline herpesvirus-1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LTC, liposome-toll-like receptor complex

treatment effect on the course of FHV-1 infection include significantly less conjunctivitis in the LTC-treated kittens and decreased shedding of FHV-1 DNA on some postinoculation days using 2 methods for calculation of results. Among other findings, including pyrexia, body weight, total ocular scores, severe total clinical disease scores, and severe ocular disease, the clinical scores also were consistent with a positive LTC treatment effect, but statistical significance was lost after adjusting for repeated kitten observations over time. The loss of statistical significance after this adjustment likely can be attributed to small sample sizes.[31] Additional studies are indicated to determine whether these findings would show statistical significance with larger numbers of animal.

Prior studies in cats and rodent models can help explain mechanistically why LTC treatment may lessen some clinical signs of FHV-1 and decrease shedding of viral DNA. For example, research in healthy cats demonstrated that PO and intranasal administration of LTC significantly activated immune responses locally in the mucosa of cats, as reflected by recruitment of activated monocytes into the nose and oropharynx.[26] In addition, production of several key antiviral and antibacterial cytokines was triggered by LTC treatment, including IFN-γ, IFN-α, and IL-12.[26] Previous studies in rodents and dogs also have defined the immune mechanisms of action of LTC, which include activation of monocytes and dendritic cells, production of Th1 cytokines, and stimulation of NK cell activation and proliferation.[17,19,21,22] The TLR3 pathway in particular is important in innate immune defense against viral infections.[32] For example, it also has been shown that deficiency of TLR3 in humans can be associated with susceptibility to herpes simplex virus 1 encephalitis.[33]

When normal cats were given LTC, most of the immunological effects were noted within hours to days after administration.[26] In the study described here, however, significant decreases in FHV-1 DNA shedding and some clinical abnormalities (especially ocular signs) suggest a delayed effect induced by LTC. These findings also may relate

to the large infective dose of FHV-1 administered to the kittens, because a higher infective dose is associated with a longer duration of viral excretion.[34] In future studies, a natural model of infection may show different magnitude and timing of potential treatment effects.

Detection of higher total respiratory scores in the LTC group compared to the control group on Days 15-28 was unexpected because that also was the time that significant decreases in FHV-1 DNA shedding in the LTC group occurred. The difference between groups related primarily to the finding of nasal discharge scores of 2 or 3 being reported in 21% of the LTC observations compared to only 2% for the control cats. These findings are unlikely to relate merely to direct irritation induced by the LTC because when tested on healthy cats, healthy dogs (unpublished data), and healthy cattle (unpublished data), no adverse clinical signs such as nasal discharge have been recognized.[26] Although TLRs activate the protective antiviral immune response and recruitment of cells that produce the chemokines and cytokines responsible for limiting infective load, the severity of inflammation induced by a pathogen potentially could be magnified in some animals.[16,35] Thus, a possible explanation for the transient higher nasal discharge scores on Days 15-28 in the LTC group could be related to overstimulation of immune and inflammatory responses against FHV-1.[19,35] This finding was not likely because of increased viral replication and damage because viral DNA from oropharyngeal swabs was not increased and no increases in ocular disease, pyrexia, or cough were detected concurrently. Studies to determine whether these findings can be replicated in cats with FHV-1 infection induced by contact with infected cats and in cats inoculated with LTC PO or parenterally should be performed. In addition, because PCR assay results do not prove the presence of live virus, quantitative FHV-1 culture also might provide additional information and should be considered for use in future studies.

One of the primary limitations of our study was unequal group sizes and differences in the size of the evaluation rooms. These inequalities confounded the ability of the clinical observers to record sneezing and nasal congestion variables accurately. Sneezing is 1 of the most objective and quantifiable respiratory clinical signs of FHV-1 infection and should be included in the analyses in future studies.[11] Another potential limitation was to have all 3 intact females assigned to the LTC group. Although all kittens were sexually immature at the time of the study, the effects of early gonad removal on immune function are unknown, especially in prepubescent animals. Future studies should ensure more equal distributions between groups. Lastly, although GAPDH has been used successfully as a comparison gene in other published FHV-1 treatment studies in cats[1,11,28] and the findings were similar between the FHV-1/GAPDH and FHV-1 to 28S calculations using the data described here, it is possible that the use of multiple comparison genes simultaneously could provide additional information or different results.[36]

5 | CONCLUSIONS

A single mucosal administration of LTC 24 hours before FHV-1 challenge in cats was associated with several positive clinical effects and with decreased shedding of FHV-1 DNA. Results of our pilot study support additional larger studies with LTC in client-owned cat populations, particularly in shelter settings with high risk of exposure to

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FHV-1 and other pathogens and where transmission is by more natural routes and doses of the agents.

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CONFLICT OF INTEREST DECLARATION

Drs. Lappin and Dow both hold stock options and corporate positions in Poudre Canyon Therapeutics, a Fort Collins company developing the LTC immunotherapy platform technology.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL AND CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

The in vivo work was approved by the IACUC at the research facility (High Quality Research Inc.)

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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REFERENCES

1. Low HC, Powell CC, Veir JK, Hawley JR, Lappin MR. Prevalence of feline herpesvirus 1, Chlamydophila felis, and Mycoplasma spp DNA in conjunctival cells collected from cats with and without conjunctivitis. *Am J Vet Res*. 2007;68:643-648.
2. Dinnage JD, Scarlett JM, Richards JR. Descriptive epidemiology of feline upper respiratory tract disease in an animal shelter. *J Feline Med Surg*. 2009;11:816-825.
3. Veir JK, Ruch-Gallie R, Spindel ME, Lappin MR. Prevalence of selected infectious organisms and comparison of two anatomic sampling sites in shelter cats with upper respiratory tract disease. *J Feline Med Surg*. 2008;10:551-557.
4. Gould D. Feline herpesvirus-1 ocular manifestations, diagnosis and treatment options. *J Feline Med Surg*. 2011;13:333-346.
5. Lappin MR, Blondeau J, Boothe D, et al. Antimicrobial use guidelines for treatment of respiratory tract disease in dogs and cats: Antimicrobial Guidelines Working Group of the International Society for Companion Animal Infectious Diseases. *J Vet Intern Med*. 2017;31:279-294.
6. Scherk MA, Ford RB, Gaskell RM, et al. 2013 AAFP Feline Vaccination Advisory Panel Report. *J Feline Med Surg*. 2013;15:785-808.
7. Lappin MR, Sebring RW, Porter M, et al. Effects of a single dose of an intranasal feline herpesvirus 1, calicivirus, and panleukopenia vaccine on clinical signs and virus shedding after challenge with virulent feline herpesvirus 1. *J Feline Med Surg*. 2006;8:158-163.
8. Reagan KL, Hawley JR, Lappin MR. Concurrent administration of an intranasal vaccine containing feline herpesvirus-1 (FHV-1) with a parenteral vaccine containing FHV-1 is superior to parenteral vaccination alone in an acute FHV-1 challenge model. *Vet J*. 2014;201:202-206.
9. Summers SC, Ruch-Gallie R, Hawley JR, et al. Effect of modified live or inactivated feline herpesvirus-1 parenteral vaccines on clinical and laboratory findings following viral challenge. *J Feline Med Surg*. 2016;19: 824-830. https://doi.org/10.1177/1098612X16659333.
10. Contreras ET, Hawley JR, Lappin MR. Effects of administration of Carnivora on clinical signs in cats after repeat challenge with feline herpesvirus 1. *Int J Appl Res Vet Med*. 2016;14:208-216.
11. Contreras ET, Hodgkins E, Tynes V, et al. Effect of a pheromone on stress-associated reactivation of feline herpesvirus-1 in experimentally inoculated kittens. *J Vet Intern Med*. 2017;32:406-417. https://doi.org/10.1111/jvim.14894 [Epub ahead of print].
12. Lappin MR, Roycroft LM. Effect of ciclosporin and methylprednisolone acetate on cats previously infected with feline herpesvirus 1. *J Feline Med Surg*. 2015;17:353-388.
13. Bradley A, Kinyon J, Frana T, Bolte D, Hyatt DR, Lappin MR. Efficacy of intranasal administration of a modified live feline herpesvirus 1 and feline calicivirus vaccine against disease caused by *Bordetella bronchiseptica* after experimental challenge. *J Vet Intern Med*. 2012;26:1121-1125.
14. Akira S, Takeda K. Toll-like receptor signaling. *Nat Rev Immunol*. 2004; 4:499-511.
15. Black M, Trent A, Tirrell M, Olive C. Advances in the design and delivery of peptide subunit vaccines with a focus on Toll-like receptor agonists. *Expert Rev Vaccines*. 2010;9:157-173.
16. Kanzler H, Barrat FJ, Hessel EM, Coffman RL. Therapeutic targeting of innate immunity with Toll-like receptor agonists and antagonists. *Nat Med*. 2007;13:552-559.
17. Dow S. Liposome–nucleic acid immunotherapeutics. *Expert Opin Drug Deliv*. 2008;5:11-24.
18. Shim G, Kim M-G, Park JY, Oh YK. Application of cationic liposomes for delivery of nucleic acids. *Asian J Pharm Sci*. 2013;8:72-80.
19. Dow SW, Fradkin LG, Liggitt DH, et al. Lipid-DNA complexes induce potent activation of innate immune responses and antitumor activity when administered intravenously. *J Immunol*. 1999;163:1552-1561.
20. Gowen BB, Fairman J, Dow S, et al. Prophylaxis with cationic liposome–DNA complexes protects hamsters from phleboviral disease: Importance of liposomal delivery and CpG motifs. *Antiviral Res*. 2009;81:37-46. https://doi.org/10.1016/j.antiviral.2008.09.001.
21. Logue CH, Phillips AT, Mossel EC, et al. Treatment with cationic liposome–DNA complexes (CLDCs) protects mice from lethal Western equine encephalitis virus (WEEV) challenge. *Antiviral Res*. 2010;87: 195-203.
22. Gowen BB, Fairman J, Smee DF, et al. Protective immunity against acute phleboviral infection elicited through immunostimulatory cationic liposome-DNA complexes. *Antiviral Res*. 2006;69:165-172.
23. Troyer RM, Propst KL, Fairman J, Bosio CM, Dow SW. Mucosal immunotherapy for protection from pneumonic infection with *Francisella tularensis*. *Vaccine*. 2009;27:4424-4433. https://doi.org/10.1016/j.vaccine.2009.05.041.
24. Goodyear A, Kellihan L, Bielefeldt-Ohmann H, Troyer R, Propst K, Dow S. Protection from pneumonic infection with *Burkholderia* species by inhalational immunotherapy. *Infect Immun*. 2009;77:1579-1588. https://doi.org/10.1128/IAI.01384-08.
25. Veir JK, Lappin MR, Dow SW. Evaluation of a novel immunotherapy for treatment of chronic rhinitis in cats. *J Feline Med Surg*. 2006;8:400-411.
26. Wheat W, Chow L, Coy J, et al. Activation of upper respiraty tract mucosal innate immune responses in cats by liposomal toll-like receptor ligand complexes delivered topically. *J Vet Int Med*. 2019;1-8. https://doi.org/10.1111/jvim.15426.
27. Quimby JM, Olea-Popelka F, Lappin MR. Comparison of digital rectal and microchip transponder thermometry in cats. *J Am Assoc Lab Anim Sci*. 2009;48:402-404.
28. Fontenelle JP, Powell CC, Veir JK, Radecki SV, Lappin MR. Effect of topical ophthalmic application of cidofovir on experimentally induced primary ocular feline herpesvirus-1 infection in cats. *Am J Vet Res*. 2008;69:289-293.

29. Helps C, Reeves N, Egan K, Howard P, Harbour D. Detection of *Chlamydophila felis* and feline herpesvirus by multiplex real-time PCR analysis. *J Clin Microbiol*. 2003;41:2734-2736.

30. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc*. 2008;3:1101-1108.

31. Giuffrida MA. Type II error and statistical power in reports of small animal clinical trials. *J Am Vet Med Assoc*. 2014;244:1075-1080.

32. Tabeta K, Georgel P, Janssen E, et al. Toll-like receptors 9 and 3 as essential components of innate immune defense against mouse cytomegalovirus infection. *Proc Natl Acad Sci U S A*. 2004;101:3516-3521. https://doi.org/10.1073/pnas.0400525101.

33. Zhang S-Y, Jouanguy E, Ugolini S, et al. TLR3 deficiency in patients with herpes simplex encephalitis. *Science*. 2007;317:1522-1527. https://doi.org/10.1126/science.1139522.

34. Gaskell RM, Povey RC. The dose response of cats to experimental infection with feline viral rhinotracheitis virus. *J Comp Pathol*. 1979; 89:179-191.

35. Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol*. 2010;11:373-384. https://doi.org/10.1038/ni.1863.

36. Kessler Y, Helfer-Hungerbuehler AK, Cattori V, et al. Quantitative TaqMan real-time PCR assays for gene expression normalisation in feline tissues. *BMC Mol Biol*. 2009;10:106.