THESIS

MUCOSAL AND SYSTEMIC IMMUNE CORRELATES OF PROTECTION AGAINST FELINE ENTERIC CORONAVIRUS INFECTION

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ABSTRACT

MUCOSAL AND SYSTEMIC IMMUNE CORRELATES OF PROTECTION AGAINST FELINE ENTERIC CORONAVIRUS INFECTION

Feline infectious peritonitis (FIP) is a disease with high mortality that results from a mutation in the genome of the relatively harmless and ubiquitous feline coronavirus (FCoV) (Licitra et al. 2013). FIP causes a deadly effusive and/or granulomatous disease in cats (Kipar et al. 2005). Because FIP is always fatal, our aim is to aid with the development of a vaccine against the parent virus FCoV. The goal of this study is to complete a comprehensive assessment of the mucosal immune response associated with FCoV infection and clearance. Previous research has shown that cats infected with FCoV can clear the virus, or they can become intermittent or persistent virus shedders (Marks 2016). It is thought that rapid waning of the humoral immune response predisposes cats to reinfection (Myrrha et al. 2011).

A closed cat colony with circulating FCoV infection was studied longitudinally to assess mucosal immune correlates of protection. Blood and fecal samples were collected monthly and colonic biopsies were obtained at an arbitrary time 0. Virologic assessment included PCR detection of virus in feces and colonic tissue. Immunological assessment included FECV-specific serum IgG and fecal IgA. Lamina propria lymphocytes from colon biopsies were phenotyped using flow cytometry and were assessed for FCoV-specific IgA and IFNγ expression by ELISPOT. Expression of IL17 and FoxP3 was measured by qRT-PCR. Although histopathology of colonic biopsies from cats shedding virus was unremarkable, an inflammatory state was indicated by total IgA producing cells, IFNγ production, and increased IL17:FoxP3. FCoV-

specific IgA was also associated with viral shedding. Taken together, results indicate mucosal and systemic antibody responses are responsible for limiting FECV infection while cell-mediated responses were not detected. Therefore, a vaccine strategy targeting antibody induction via a mucosal route may provide protection against FECV infection.

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CHAPTER 1 – INTRODUCTION: REVIEW OF LITERATURE

1.1 History: The recorded history of FIP began with its initial disease observation in 1963 (Scott 1999). Following this, the disease was replicated in laboratory conditions with experimental infection trials using organ homogenates of infected animals to inoculate otherwise healthy cats. The disease was replicated under these experimental conditions, and the scientists subsequently suspected a viral etiology (Wolfe and Griesemer 1966). The viral etiology was confirmed in 1968, and classified as a member of the *Coronaviridae* family in 1976 (Ward et al. 1968), (Osterhaus, Horzinek, and Ellens 1976). FCoV is now considered to be the most important cause of infectious disease-related deaths in the pet cat population, as it is the causative agent of aberrant immune-responses that lead to feline infectious peritonitis (FIP) (Hartmann 2005).

1.2 Coronavirus Classification and Background: As with all coronaviruses, FCoV is an enveloped, positive-sense, single-stranded RNA virus (Myrrha et al. 2011). Its envelope is decorated with transmembrane spike proteins, forming their characteristic "crown" appearance when viewed by electron microscopy (Myrrha et al. 2011). In addition to yielding the *Coronaviridae* family name, the spike proteins function as its main method of attachment to mediate infection (Li 2016). *Coronaviridae* contains the two subfamilies *Letovirinae* and *Orthocoronavirinae* (also known as *Coronavirinae*). Subfamily *Orthocoronavirinae (Coronavirinae)* is further divided into the 4 genera *Alphacoronavirus (*formerly CoV group 1*)*, *Betacoronavirus* (formerly group 2), *Gammacoronavirus* (formerly group 3), and *Deltacoronavirus* (Vlasova et al. 2011). FCoV belongs to species *Alphacoronavirus 1* (formerly subgroup 1a) under genus *Alphacoronavirus*, along with canine coronavirus (CCoV) and

transmissible gastroenteritis virus (TGEV), and porcine respiratory virus (PRCoV) (Kummrow et al. 2005).

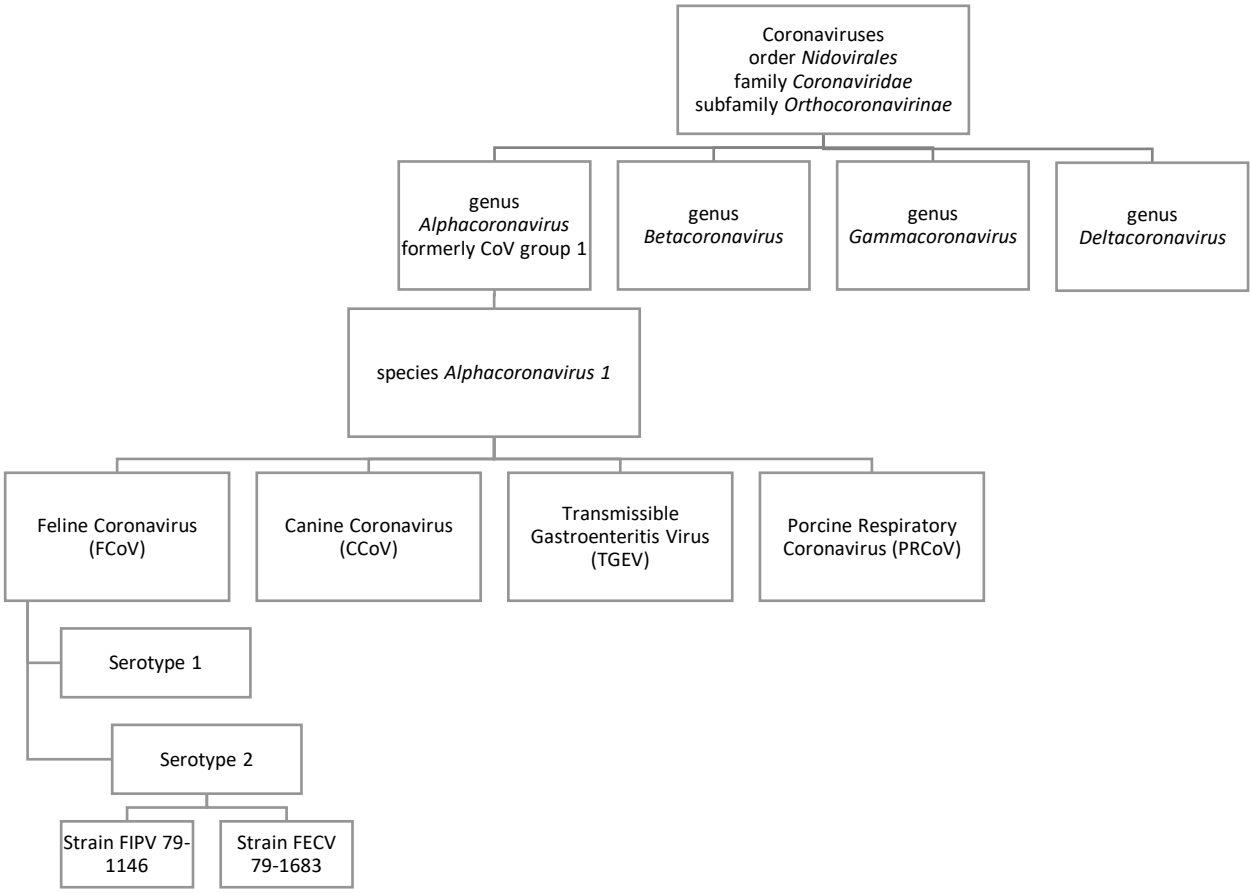

Figure 1: FCoV Phylogenetic, Serotype, and Strain Relations

Previous studies have suggested that cats are susceptible to all members of genus *Alphacoronavirus* (formerly CoV group 1) (Horzinek, Lutz, and Pedersen 1982). Two biotypes (or pathotypes) of FCoV exist: feline enteric coronavirus (FECV) and feline infectious peritonitis virus (FIPV) (An et al. 2011) . Both biotypes (FECV and FIPV) contain two serotypes (serotype 1 and serotype 2) (An et al. 2011). Conversely, this means that either serotype can result in either biotype. Serotypes 1 and 2 are differentiated by the amino acid sequence of the spike (S) protein (Motokawa et al. 1995). Serotype 1 is the most common serotype of FCoV that infects cats,

accounting for 66% to 83% of infections in most population studies (An et al. 2011) (Kummrow et al. 2005). Serotype 2 is the second most common serotype of FCoV, and is thought to have arisen from a double recombination event between FCoV type 1 and CCoV (Herrewegh et al. 1998). Two prototypical isolates (FIPV 79-1146 and FECV 79-1683) have been studied extensively (Herrewegh et al. 1998). Both serotype 2 strains encode a CCoV-like S protein and the M, N, 7a, and 7b proteins of FCoV type I (Hohdatsu et al. 1992). Serotype 2 strains have been better studied as compared to serotype 1 strains because the former can be propagated in vitro whereas the latter cannot be readily adapted to in vitro growth (Pedersen et al. 1984).

The type 2 serotype strains FIPV 79-1146 and FECV 79-1683 are the most closely related strains of the two biotypes (Rottier et al. 2005). FECV 79-1683 presents as unapparent-to-mild enteritis when inoculated in specific pathogen free (SPF) kittens, whereas FIPV 79-1146 induces effusive FIP in SPF kittens post oronasal or intraperitoneal inoculation (Pedersen et al. 1984). The difference in disease presentation is explained by the ability of FIPV 79-1146 to replicate in macrophages, an ability mediated by the C-terminal domain of the S protein (Rottier et al. 2005). Co-infection with serotype 1 and 2 is also possible and has also been documented with relative frequency (An et al. 2011). To complicate matters, biotype FECV and biotype FIP are only distinguishable based upon their disease presentations, and not by the antibodies that they induce (Hartmann et al. 2003). Similarities in antibodies between biotypes lead to complications in diagnosing ante-mortem cases of FIP, further discussed in section 1.5.

1.3 Antibody Dependent Enhancement: Cross-protection between FIPV 79-1146 and FECV 79-1183 does not occur; to the contrary, preexisting immunity to FECV 79-1683 has been shown to cause accelerated disease associated with FECV 79-1146 (Pedersen et al. 1984), likely because of antibody dependent enhancement (ADE). The reverse has also been shown, with monoclonal

antibody against the spike protein of FCoV enhancing replication of FECV 79-1183 in U937 cells (cell line derived from myeloid lineage) and primary feline monocytes (Takano et al. 2017). In the case of ADE of FIPV 79-1146, mRNA levels of inflammatory cytokines (TNF-α, IL-1β, and IL-6) are significantly increased (Takano et al. 2017). Antibody dependent enhancement is proposed to occur because the presence of antibodies allows for easier uptake into macrophages via Fc receptors. This has made the development of vaccines challenging because of the risk of inducing antibodies that will result in ADE. One study found that antibody-positive cats developed disease earlier (12 days compared with 28 days or more for controls) (FW, WV, and CW 1995). In addition, a higher proportion of antibody-positive cats died compared with antibody-negative controls (FW, WV, and CW 1995). Initially, ADE was not thought to play a major role in the field because it was not seen in cats naturally infected with FCoV (Addie and Jarrett 1990). Later it was recognized that reinfection with the same serotype induces ADE in cats infected with FIPV in experimental settings (Takano et al. 2008).

1.4 FCoV Transmission: FCoV is shed in large numbers in the feces of infected cats and spreads effectively via fomite transmission, such as through litter trays, litter scoops, shoes, hands, and clothes of handlers (Addie and Jarrett 2001). FCoV is less commonly shed in the saliva (Addie and Jarrett 2001). FIPV is reportedly present in the tonsils 24 hours after ingestion (Stoddart et al. 1988). FCoV is quite hardy in the environment, surviving up to 3-7 weeks under dry, room temperature conditions (Scott 1999). FCoV is easily destroyed through the use of household cleaners (Addie et al. 2009). Most kittens become infected with FCoV after protective levels of maternal antibodies wane, around 5-6 weeks of age (Addie and Jarrett 1992).

1.5 FCoV Prevalence: FCoV is considered to be ubiquitous in the domestic cat population and is also found in wild cat populations. Infection prevalence tends to be higher in conditions with higher population density (such as catteries and shelters) (Pedersen 2009). Approximately 25% of cats in single-cat households are serologically positive for FCoV (Rohrbach et al. 2001). Between 75% and 90% of cats in multi-cat households are serologically positive for FCoV (Rohrbach et al. 2001). Feral and wild cat populations are also susceptible to infection with FCoV, but the prevalence in these populations is currently unknown (Hartmann 2005).

1.6 Control of FCoV in Populations: To minimize the risk of FCoV infections manifesting in the FIP biotype, many catteries and shelters attempt to control for FCoV in their populations. The

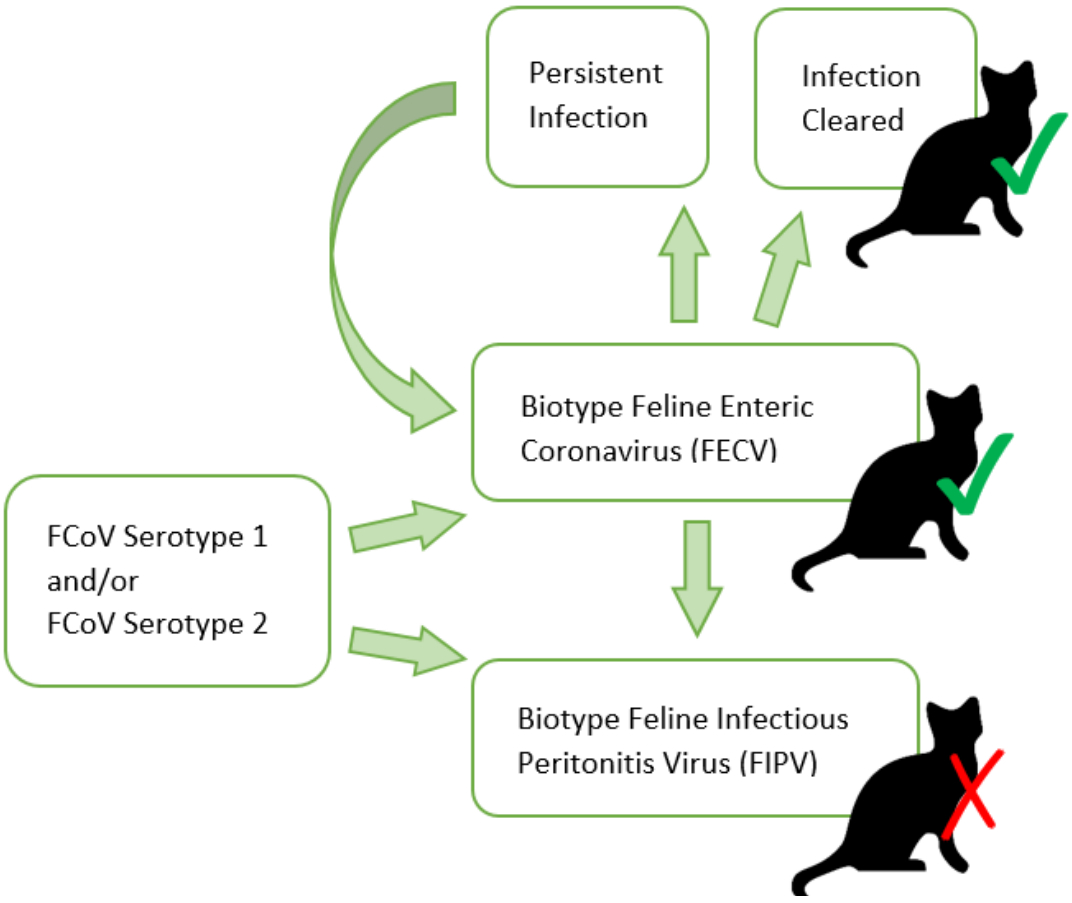

Figure 2: FCoV Biotypes and Outcomes

variability of outcomes and presence of persistent shedders is thought to perpetuate the virus within cat populations such as breeding colonies and in shelters (Marks 2016). With this in mind, the majority of recommendations tend to focus on limiting numbers of cats, regular cleaning and sanitation of litter boxes, and removing chronic shedders from the population (Drechsler et al. 2011). Although a vaccine against FCoV does exist, it is not currently recommended by the AAFP Feline Vaccination Advisory Panel due to insufficient evidence that the vaccine provides relevant protection (Scherk et al. 2013). The vaccine not licensed for kittens under the age of 16 weeks of age, which is the age when most kittens experience their first FCoV infection (Scherk et al. 2013).

1.7 Disease Manifestation of Feline Enteric Coronavirus (FECV): FECV typically causes a mild enteropathy in infected individuals. Presentation ranges from subclinical infections to mild diarrhea and enteritis associated with enterocyte destruction due to virus replication (Pedersen 1995). Previous research has shown that cats infected with FECV can clear the virus or become intermittent or persistent virus shedders (Marks 2016). It is thought that rapid waning of the humoral immune response predisposes cats to reinfection (Myrrha et al. 2011).

1.8 Disease Manifestation of Feline Infectious Peritonitis (FIP): Feline infectious peritonitis (FIP) is a deadly disease that results from a mutation in the genome of the relatively harmless and ubiquitous feline coronavirus (FCoV). It is a complicated disease pathway, that involves virus or virus antigen, antiviral antibodies, and host complement; without the development of anti-FCoV antibodies, FIP cannot occur (An et al. 2011). FIP is characterized by an intense, granulomatous, inflammatory response around blood vessels in the body where infected white blood cells circulate (Figure 3: Classic FIP Lesions with Fibrinopurulent Exudate and Granulomatous Lesions on Organs (Hsieh, 2014)). Many times, these areas of intense

inflammation are located in the abdomen, especially in the kidneys (Weiss and Scott 1981). Other organ systems potentially affected are thoracic organs, the eyes, and the CNS (Hartmann 2005).

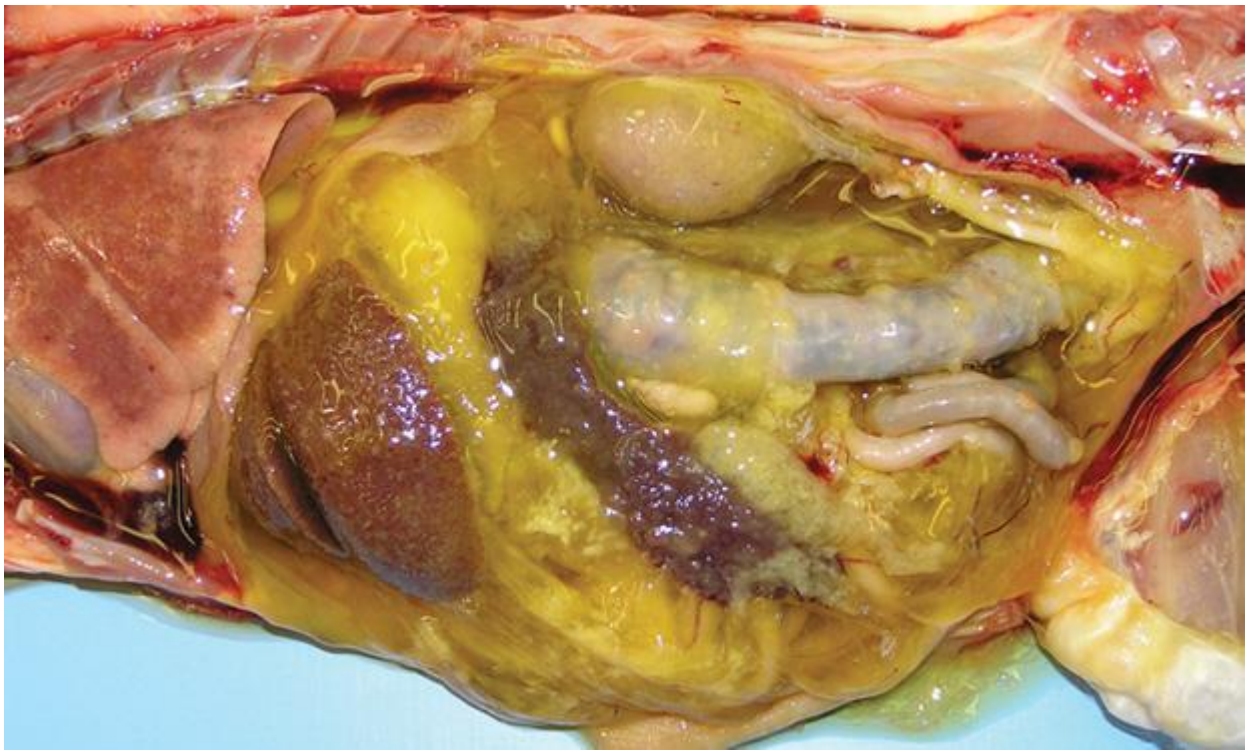

Figure 3: Classic FIP Lesions with Fibrinopurulent Exudate and Granulomatous Lesions on Organs (Hsieh, 2014)

FIP is the number one cause of death by infectious causes in young cats; literature shows that FIP develops in 7.8% to 12% of FCoV infected cats (Rohrbach et al. 2001), (Addie et al. 2009). FIP manifests as a wet form (effusive), dry form (non-effusive), or a combination of the two (Kipar and Meli 2014). The wet form of FIP is associated with the collection of fluid in the abdominal cavity (Figure 4: FIP Patient with Wet Form (Effusive) Presentation with Ascites (Hartmann 2005)), the chest cavity (pleural effusion), around the heart (pericardial effusion), or a combination thereof (Kipar and Meli 2014). In a survey of 390 cats with FIP associated effusions, 62% had ascites, 17% had thoracic effusions, and 21% had effusions in both body

Figure 4: FIP Patient with Wet Form (Effusive) Presentation with Ascites (Hartmann 2005)

cavities (pericardial effusions were not classified separately in this survey) (Hartmann, Binder, and Hischberger 2002). Individuals with ascites typically present with progressive, non-painful distension of the abdomen. The fluid is classically thick and straw-to-yellow in coloration (Figure 5: Typical Ascites Presentation of FIP (Hartmann 2005)) with more in-depth analysis revealing it to be a non-septic exudate (specific gravity 1.017 to 1.047) with a high protein content (5-12 g/dl) and moderate cellularity (Barlough and Stoddart 1988).

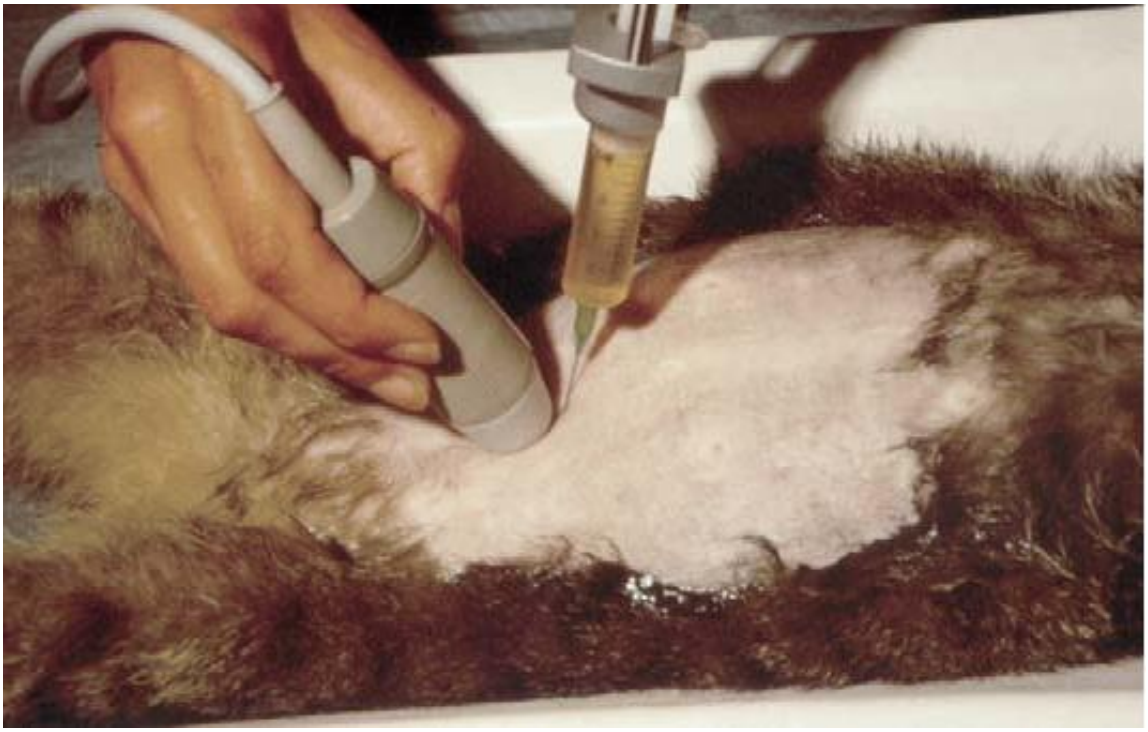

Figure 5: Typical Ascites Presentation of FIP (Hartmann 2005)

Symptoms other than effusion include the following: fever, icterus, abdominal lymphadenopathy, thickened intestinal walls, renomegaly, eye involvement (uveitis, keratic precipitates, and retinal changes), and central nervous system involvement (Hartmann 2005). Eye involvement (Figure 6: FIP Patient with Uveitis (Andrew 2000)) is reported in approximately 29% of FIP cases, with the majority (68%) of involved cases presenting with bilateral involvement (Pedersen 2009).

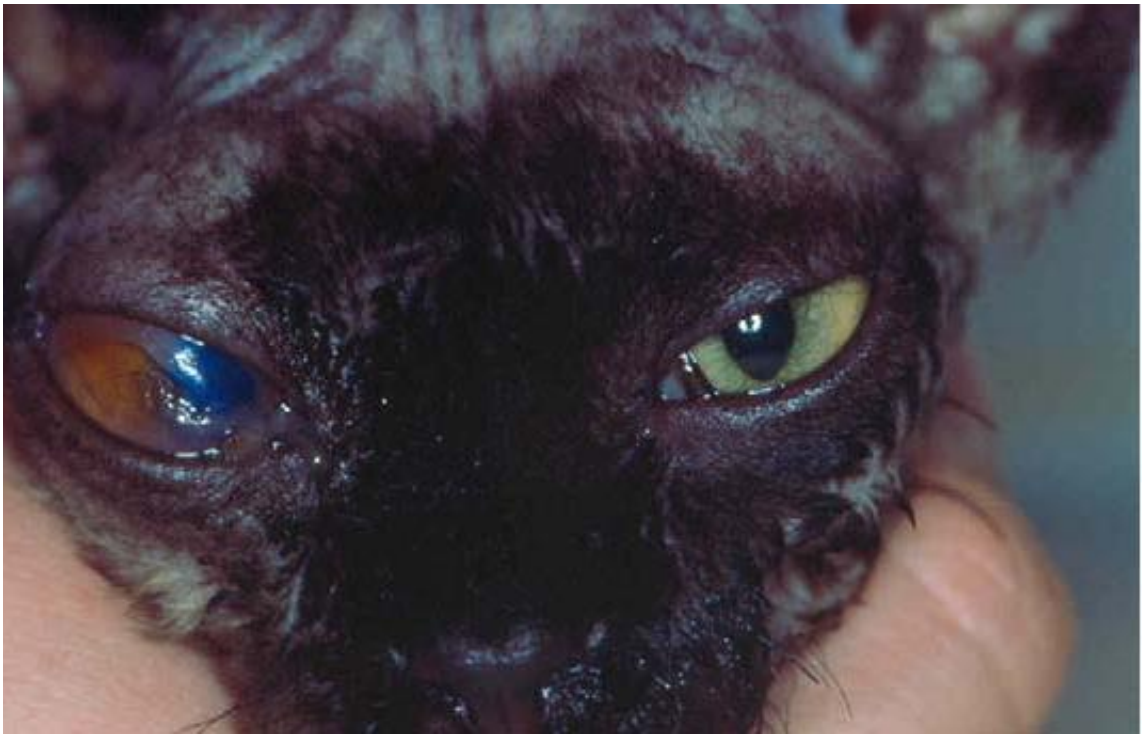

Figure 6: FIP Patient with Uveitis (Andrew 2000)

1.8.1 Hypotheses for Biotype FIP Manifestation: There is still uncertainty about what causes the presentation of the biotype FIP (Brown et al. 2009). The two main hypotheses are the internal mutation hypothesis (an avirulent strain mutates in vivo to form a virulent strain) or the hypothesis that genetically distinctive avirulent and virulent forms coexist (Brown et al. 2009). Scientific consensus is still evolving, and at this point it appears that both hypotheses hold merit.

1.8.2 Risk Factors for the Development of FIP: Important risk factors for the development of FIP following infection with FCoV include population density, stress (through management practices or [potentially] concurrent illnesses), breed, sex, neuter status, and age. Cat breeds are affected in unequal distributions, with heritability of FIP being reported >50% in some pedigree catteries (Rohrbach et al. 2001). Bengals, Abyssinians, Himalayans, Birmans, Rexes, and Ragdolls have been noted by some studies to be at higher risk. (Foley and Pedersen 1996) and (Pesteanu-Somogyi, Radzai, and Pressler 2006). Additionally, an Australian study also found an unequal

distributions of FIP between domestic cat breeds, with Burmese, Australian mist, British shorthair, and Cornish rex were overrepresented while domestic shorthair and Persian were underrepresented (Norris et al. 2005). Inheritance of FIP susceptibility is likely a polygenetic trait rather than a simple dominant or recessive mode (Foley and Pedersen 1996). Sex and neuter status also play a role in individual susceptibility to the development of FIP, with spayed females at the lowest risk for the development of FIP and intact males at the highest risk (Pedersen 2009). Most cats who develop FIP are between the ages of 6 months and 3 years (Pedersen 1989); more than half of cases are younger than 1 year (Hartmann, Binder, and Hischberger 2002).

1.8.3 Diagnosis and Medical Intervention of FIP: FIP cannot not be cured; only palliative care can be provided to affected cats (Pedersen 2019). Because FCoV is considered to be a ubiquitous virus, many cats will be seropositive for the virus as an incidental finding. This, in addition to the fact that antibodies for FCoV biotype FECV and FIP are indistinguishable, result in complications with ante-mortem diagnosis of FIP. FIP is typically listed as a differential diagnosis based on presentation, patient history, results of FCoV antibody levels, and laboratory tests. The only definitive diagnosis is through biopsy, and is rarely performed ante-mortem due because of cost and because FIP is a terminal disease. Ante-mortem diagnosis of the dry form of FIP presents an additional challenge as these cases lack effusion to test for antibodies. Treatment of FIP is typically aimed at suppressing the destructive immune response with an immunosuppressive dose of corticosteroids, although there have been no controlled studies to prove any beneficial effect (Addie et al. 2009).

1.8.4 Control of FIP in Populations: Outbreaks of FIP in populations tend to be thought as chance mutations arising simultaneously. Classically, FIPV is not considered to be contagious to

other cats. More recently, there have been reports suggesting horizontal transfer of FIP between individuals (Wang et al. 2013). Scientific consensus is still evolving.

1.9 Working with Coronaviruses: Coronaviruses are notoriously difficult to propagate in culture, which results in laboratory challenges when it comes to developing both diagnostics and treatment (Holmes 2001). As previously mentioned, most work with FCoV has been done with serotype 2 because it can be easily propagated in vitro (Pedersen et al. 1984). Coronaviruses exhibit a propensity to jump between species, a characteristic which creates serious public health concern (Menachery, Graham, and Baric 2017). Unlike many coronaviruses, FCoV is not a primary pathogen of the respiratory tract. Biotype FECV primarily affects only mature intestinal epithelial cells where it replicates (Pedersen 1989). In addition to the mature apical epithelium of the small intestine, the mesenteric lymph nodes, tonsils, thymus, and the lungs were also noted as targets of the virus under experimental infection (Pedersen et al. 1984). Biotype FIP results in disseminated infection due to its acquired tropism for macrophages (Rottier et al. 2005).

1.10 Study Background: Because of its world-wide presence, most cats come into contact with the virus at some point in their lives. Up until this point, there has not been a comprehensive assessment of the mucosal immune response associated with FCoV infection and clearance. This study aims to determine the mucosal immune correlates associated with control of virus replication and clearance.

1.11 Study Design: Colorado State University is home to a closed colony of specific pathogen free (SPF) cats with naturally circulating FCoV in the population. Thirty-three individuals from the colony were studied longitudinally to characterize the mucosal immune response associated with FCoV infection. Plasma and fecal material were collected at 7 time points (-2 month, 0 [biopsy], +1 month, +2 month, +3 month, +4 month, and +5 month), to characterize the FCoV

immunological and virological status. Plasma FCoV-specific antibody was determined by ELISA, with the microwells coated with viral antigen. Viral RNA extraction and PCR amplification from fecal samples was used to assess virus replication and shedding (Addie and Jarrett 2001). Based on results from these two assays, cats were assigned to one of the 3 following study groups: those that were PCR and serology negative, those that were serology positive and PCR negative, and those that were both PCR and serology positive. The immunological state of each group was then characterized in more detail.

Table 1: Study Groups

Group	N	Fecal FCoV	FCoV Serum IgG
1	18	Negative	Negative
2	6	Negative	Positive
3	9	Positive	Positive

Biopsies were taken from the colon of the cats to determine cell populations and their activities in relation to FCoV infection (study group designation). From the colon biopsies, mucosal lymphocytes were isolated and phenotyped by flow cytometry. The lymphocytes were assessed for production of FCoV-specific IgA and IFNγ by ELISPOT. Expression of IL-17 and FoxP3 was assessed by qRT-PCR. Functional assays were used to determine whether mucosal IgA and serum IgG represent correlates of protection. The humoral immune response was determined using FCoV-specific IgA ELISPOT and ELISA for fecal IgA. All of these assays were completed with the goal of identifying specific immune correlates associated with control of viral replication.

CHAPTER 2 – MATERIALS AND METHODS

2.1 Animals: This study used 23 intact female, and 10 neutered male, and 2 intact male domestic shorthair cats (DSH). The cats ranged in age from 1 year to 9 years of age. These animals were part of the Colorado State University cat colony managed by the Dr. Sue VandeWoude laboratory and are considered to be specific pathogen free (SPF). The colony was previously known to have circulating FCoV. The cats were group housed, with the exception of the intact males which were housed together in their own run in the same room as the other cats. Cats were maintained on a 12:12-h light:dark cycle in a room which was kept between 69-75 F with 20-70% relative humidity. The cats were fed free-choice commercial IAMs hairball care diet (IAMs 62991), any supplemental with canned food was Purina Proplan Veterinary Diets OM overweight management (Purina 64889). Cats had access to water at all times. Material supplied in litter boxes was Envigo Teklad laboratory grade aspen bedding (Envigo Teklad 7093), autoclaved prior to use to ensure sterility. Cats were group housed and cared for in accordance with Association for the Assessment of Laboratory Animal Care standards and with approval from the Colorado State University Institutional Animal Care and Use Committee.

2.2 Study Groups: Cats were assigned to one of three groups based on their plasma IgG status to FCoV (positive, negative, or "borderline") and their fecal viral status (positive or negative for the presence of virus, determined by RNA extraction and amplification through qPCR). This is shown in Table 2.

Table 2: Study Groups

Group	N	Fecal FCoV	FCoV Serum IgG
1	18	Negative	Negative
2	6	Negative	Positive
3	9	Positive	Positive

2.3 Sampling Protocols: For fecal collection, cats were separated into individual cages for sample integrity. The fecal samples were collected from the litter boxes, packaged into plastic bags, and stored at -80 °C. For plasma collection, cats were manually restrained and 2 mL of blood were collected from the cephalic vein into EDTA tubes. Plasma was isolated by centrifugation (2200 × *g* for 15 min), aliquoted into 0.5 µl tubes, and stored at -80 °C. Prior to colon biopsies, the cats were fasted overnight to prevent regurgitation under anesthesia. Colon biopsies were collected by endoscopy under general anesthesia. Cats were recovered under supervision.

2.4 FCoV Specific IgG in Plasma: The commercially available kit Feline Infectious Peritonitis Virus Antibody Test Kit for the Detection of IgG Antibody in Feline Serum of Plasma (IVD Technologies FIPV-1000) was used. All assays were performed according to manufacturer's instructions.

2.5 Mucosal Lymphocyte Isolation: Colon biopsy samples (obtained as above) were processed using modifications of a previously described protocol (Howard et al. 2005). Briefly, endoscopic biopsies were digested in 2mL of digestion medium consisting of RMPI without L-glutamine (Corning 15-040-CV), 0.125 IU/mL penicillin/streptomycin (Cellgro 30-002-CI), 50 µg/µL Liberase-DL (Sigma-Aldrich 5466202001), and 1 ng/µL DNAse I (Sigma-Aldrich 10104159001) for 30 min at 37 ºC. Tissue was then passed 10-15 times through a sterile 16

gauge needle attached to a 5 mL syringe. Disrupted cells were then passed through a 100 µm EasyStrainer (Greiner Bio-One 542000), while large pieces of undigested tissue were returned to 37 ºC for 30 min with an additional 2 mL of fresh digestion media. This process was repeated twice for a total of 3 digestion steps. After straining of the final digest through a 100 µm strainer, all digestion aliquots were combined and strained through a 70 µm EasyStrainer (Greiner Bio-One 542070) followed by a 40 µm EasyStrainer (Greiner Bio-One 542040). Cells were then washed with cell culture media, and filtered one final time through a 35 µm 5 mL Falcon Round Bottom Polystyrene Test Tube, with Cell Strainer Snap Cap (Corning 352235). Cells were counted with a Cellometer Auto 2000 Cell Viability Counter by Nexcelom Biosciences (Lawrence, MA), according to the manufacturer's directions.

2.6 Immunophenotyping: Approximately 200,000 cells obtained from the mucosal lymphocyte isolation protocol above were immunophenotyped by flow cytometry. Standard staining protocols were used, similar to those previously described (Simões, Howard, and Dean 2012). Briefly, cells were blocked with 1% bovine serum albumin (Equitech-Bio BAH66), followed by staining with the following antibodies: mouse anti-cat CD45 (clone 30.7.9), goat anti-mouse IgG2A conjugated to APC-Cy7 (SouthernBiotech, Birmingham, AL), mouse anti-cat CD4 conjugated to FITC (SouthernBiotech, Birmingham, AL), mouse anti-cat CD8 conjugated to APC (Clinical Immunology Laboratory, NCSU, Raleigh, NC), and rat anti-mouse B220 conjugated to PerCP (BioLegend, San Diego, CA). Cells were then washed, fixed, and permeabilized using Fixation/Permeabilization Solution Kit (BD Pharmingen 55471). After washing, cells were stained intracellularly with mouse anti-human Ki67 conjugated to PE (BD Pharmingen 556027). After two final wash steps, the stained cells were analyzed immediately using a Gallios flow cytometer (Beckman Coulter, Indianapolis, IN). At least 50,000 events

were typically collected. Analysis of flow cytometric data was completed using FlowJo Software, version 10 (Tree Star, Ashland, OR).

2.7 FCoV Specific IgA in Feces: For this study, a FCoV-specific IgA ELISA was developed. 100 µL Feline Infectious Peritonitis Virus Antigen (IVD Technologies FIPV-1-500) was diluted in 1X PBS (Cellgro MT 21-040-CV) to 15 mg/mL was used to coat 96 well plate (Greiner Microlon High Binding 655061). After adding antigen, the plate was covered with SealPlate film, nonsterile (Genesee 12-167) and incubated overnight at 4 °C in a plastic bag with a wet paper towel (to prevent evaporation of coating). Unbound antigen was removed from the plate and discarded after bleaching. The plate was flicked and tapped dry then 200 µL Blocking Buffer (Carnation nonfat dry milk [5% W/V] in 1X PBS (Cellgro MT 21-040-CV) with Kathon [preservative; 0.1% W/V, Sigma 5-00135]) was added , plates were covered, and incubated at room temperature for 2 hours. The buffer was discarded, and the plate was flicked and tapped dry. The plate was washed 4 times with 200 µL PBST (1X Phosphate-Buffered Saline, 0.1% Tween [Fisher BioReagents BP337-100]) with a 5 minute soak between wash 3 and wash 4. In between each wash the plate was flicked and tapped dry. Dilutions of fecal sample extracts were prepared in complete sample and secondary antibody diluent (Carnation nonfat dry milk [5% W/V] in 10X PBS and distilled water with normal goat serum [5% W/V], Kathon [preservative; 0.1% W/V Sigma 5-00135], and Tween [0.05% W/V][Fisher BioReagents BP337-100]), and 100 µL of each dilution was added to the plate. Plates were covered and incubated at 37 °C for 3 hours, then plate washed 6 times with 200 µL PBST with a 5 minute soak between wash 3 and wash 4. In between each wash the plate was flicked and tapped dry. 100 µL/well of 0.5 µg/mL goat anti-cat IgA (Bethyl A20-101P) was added for 1 hour at 37 ˚C. After 6 washes, SureBlue Reserve TMB Substrate (SeraCare 5120-0081) was added for 10 minutes at room temperature

(RT) then development was stopped with 1 N HCl and absorbance was measured (450 nm, minus background measured at 570 nm). A standard curve was generated from reference serum wells using Prism software (GraphPad, La Jolla, CA) for quantification of IgA.

2.8 Viral RNA Extraction from Fecal Samples: Fecal samples were thawed on ice, a portion was transferred into a tube and weighed, sterile saline was added (1ml per 100mg feces) and samples were homogenized for 1 min at 6.5 m/sec in a FastPrep 24 instrument (MP Biomedicals, Irvine, CA). After centrifugation (5min at 12,000xg), RNA was extracted from the supernatant with QIAamp Viral RNA mini kit (QIAGEN 52904) according to the manufacturer's protocol with 4 volumes supernatant, Buffer AVL, and 100% ethanol. RNA was eluted with 40 µL AVE, 2% murine RNase inhibitor (NEB M0314) was added, and RNA was stored at -80 ˚C. A buffer-only extraction was included in each extraction batch.

2.9 Viral RNA Extraction from Colon Tissue and PBMC: RNA was extracted from colon biopsy tissue using the RNeasy Powerlyzer Tissue & Cells kit (QIAGEN 15055-50) per manufacturer's protocol; tissues were homogenized with two 45-second cycles at 5.5 m/s in the FastPrep 24 (MP Biomedicals, Santa Ana, CA), and on-column treatment with RNase-free DNase (QIAGEN 79254) was included. RNA was extracted from PBMC using the RNeasy Mini Kit (QIAGEN 74104), including QIAshredder (QIAGEN 79654) and RNase-free DNase treatments (QIAGEN 79254) per manufacturer's protocols. RNase inhibitor was added to eluted RNA before storing at -80 ˚C. RNA was quantified with Qubit RNA BR Assay kit (Thermo Fisher Scientific Q10211) and quality was assessed by spectrophotometry and by RNA High Sensitivity ScreenTape assay (Agilent, Santa Clara, CA). A buffer-only extraction was included in each tissue or PBMC extraction batch.

2.10 FCoV real-time RT-PCR: RNA was analyzed for presence of FCoV RNA by real-time RT-PCR (rt-RT-PCR) amplification of a well-conserved region of the membrane and nucleocapsid genes using primers (400 nM each) and probe (200 nM, PrimeTime® 5' 6-FAM/ZEN/3'IB®FQ-labeled; Integrated DNA Technologies, Coralville, IA), sequences as previously described (Dye, Helps, and Siddell 2008) with Luna Universal Probe One-Step RT-qPCR reagent (NEB E3006). Samples (5 µL and 0.5 µL fecal RNA or 100 ng colon or PBMC RNA) were run in triplicate 20 µL reactions in white 96-well PCR plates (Bio-Rad HSP9631) using a Bio-Rad CFX96 (Bio-Rad, Hercules, CA) instrument and the following cycling conditions: reverse transcription (55 °C, 1 min), initial denaturation (95 °C, 10 min), 45 amplification cycles (95 °C 10 sec, 60 °C 30 sec), and final extension (72 °C, 10min). Serial dilutions of FCoV-positive RNA were included on each plate for quality control. No FCoV RNA was detected in buffer-only extraction controls or in no-template controls included on each plate.

2.11 IFNγ ELISPOT: Capture and detection antibodies from the feline IFNγ Development Module (R&D Systems) were used with MultiScreen-IP 96-well plates (MilliporeSigma MAIPSWU10) to quantify IFNγ-producing mucosal lymphocytes after stimulation with phorbol 12-myristate 13-acetate (PMA, 50 ng/mL; LC Laboratories, Woburn, MA) and ionomycin (300 ng/mL; LC Laboratories) or FIPV Antigen (60 µg/mL; IVD Technologies, Santa Ana, CA). The protocol was modified with the use of 2.5 x 10^4 cells/well (PMA/ionomycin) or 2 x 10^5 cells/well (FIPV Antigen) incubated for 40 hours at 37 ˚C, 5% CO_2. Plates were scanned with an Immunospot S5 Analyzer (Cellular Technology Limited, Cleveland, OH) and analysis was performed by CTL.

2.12 IL17 and FoxP3 real-time RT-PCR: RT-PCR reactions were carried out as for FCoV, with 100 ng colon RNA. FoxP3 primer and probe sequences were previously published (Lankford et

al. 2008). IL17 primers: forward TGGCTCCTGGGACAACTTC, reverse TCCTCGGTAGTTGGGCATCC, probe TCCCATCACTGCTGCTGCTGCTCT. All probes were 5' 6-FAM/ZEN/3'IB®FQ-labeled (Integrated DNA Technologies, Coralville, IA). Additional controls included no-RT reactions for each sample with each primer/probe set and a Real-Time Internal Control RNA reaction (Primerdesign Ltd, Plymouth Meeting, PA) for each sample for inhibitor analysis. No RT-PCR inhibition was detected.

2.13 Western Blot for Identification of Novel Antigens: Western blot was developed for the identification of novel antigens. Pierce Lane Marker Reducing Sample Buffer (Thermo Fisher Scientific 39000) was removed from -20 °C and thawed in 37 °C water bath to completely dissolve SDS. Precision Plus WesternC MWM (Bio-rad 161-0376) and Feline Infectious Peritonitis Virus Antigen (IVD Technologies FIPV-1-500) were thawed on the bench. Samples were prepared by adding the following to each PCR tube: 3 µL Loading Buffer (Pierce Lane Marker Reducing Sample Buffer [Thermo Fisher Scientific 39000]), 5 µL Virus, 7 µL 1X PBS (1X PBS w/o Ca and Mg [Cellgro MT 21-040-CV]) for a total of 15 µL. Tubes were vortexed to mix and then spun down. Tubes were heated in Bio-Rad CFX96 instrument (Bio-Rad, Hercules, CA) for 5 minutes at 95 °C. Gel was assembled (4–20% Mini-PROTEAN® TGX™ Precast Protein Gels, 15-well, 15 µL [Bio-Rad 4561096]) into the Bio-Rad Mini-Protean System (Bio-Rad, Hercules, CA). Internal chamber was filled with 1X Tris-Glycine SDS (Fisher BioReagents BP2440-4) to check for leaks. Wells were rinsed with 1X Tris-Glycine SDS prior to loading. Ladder was set up with the following: 3 µL Loading Buffer, 5 µL Ladder and 7 µL 1X PBS. Samples and ladder were loaded into the gel using a p100 with gel loading tips. Gel was run using the following mini TGX settings: 200 V, 51 mA for 31 min. The gel was then transferred to PVDF membrane via the TransBlot Turbo (Bio-Rad, Hercules, CA) following manufacturer's

instructions. Post transfer, the membrane was placed in Blocking Buffer (0.5% Carnation nonfat milk in PBST (1X Phosphate-Buffered Saline, 0.1% Tween [Fisher BioReagents BP337-100]) with Kathon [preservative; 0.1% W/V Sigma 5-00135]) and put in fridge overnight for blocking. Lanes were cut into separate strips with a scalpel (Techno Cut 6008T-10). The individual lanes were then re-blocked in Blocking Buffer on orbital shaker for 5 minutes at room temperature. Feline plasma aliquots (primary antibody) were thawed on ice, then vortexed and spun down. Plasma was diluted with Blocking Buffer (100 µL Plasma to 10 mL Blocking Buffer). The tubes were vortexed to mix. Primary antibody solution was added to the membranes and incubated at room temperature on orbital shaker for 1 hour. Membranes were washed for 5 minutes in PBST 3 times, while shaking on orbital shaker. Diluted secondary antibody of choice in Blocking Buffer to concentrations recommended by manufacture, along with Streptavidin (Bio-Rad 1610381) at 1:5,000. Goat anti-Cat IgG-Fc Fragment Antibody HRP Conjugated (Bethyl Laboratories A20-117P), Goat anti-Cat Light Chain Antibody HRP Conjugated (Bethyl Laboratories A20-121P), and Goat anti-Cat IgA Antibody HRP Conjugated (Bethyl Laboratories A20-101P) were utilized at their respective recommended concentrations. The secondary antibody solution was added to the membranes and incubated at room temperature on orbital shaker for 1 hour. During this incubation, Pierce TMB (Thermo Fisher Scientific 34021) was removed from 4 °C storage, brought to room temperature, and filtered through 0.22 µm filter (CELLTREAT 229746). Membranes were washed for 5 minutes in PBST 3 times while shaking on orbital shaker. Strips were covered with TMB and developed for 10 minutes, protected from light. Development was arrested by rinsing membranes with ultrapure water.

2.14 FCoV Serotyping by PCR: A 700 base segment of the S gene was amplified to determine whether the Colorado State University virus strain was serotype 1 or serotype 2. This was a

previously reported portion of the Colorado State University cat colony strain of FCoV S protein (Kummrow et al. 2005) using primers UCD1.3502f (5'-GCA CTT AAT GCT TAT GTG TCT CAA A-3'), FIPV79-1164.3549f (5'-AGC ACT TAA TGC ATT TGT GTC TCA-3'), and KU2.4226r (5'-CAC ACA TAC CAA GGC C-3') (Table 3).

Table 3: Primers for Serotyping Assay

Serotype	**Forward Primer Name**	**Reverse Primer Name**
1	UCD1.3500f	KU2.4226r
2	FIPV79-1664.3549f	KU2.4226r

Viral RNA was extracted from feces as described above (2.8 Viral RNA Extraction from Fecal Samples) and was used as the template for the reaction. LunaScript RT Supermix (NEB E3010) was used for reverse transcription and was run on the Bio-Rad CFX96 instrument with the following conditions: primer annealing (25 °C, 2 min), cDNA synthesis (55 °C, 10 min), heat inactivation (95 °C, 1 min). Q5 Hot Start High-Fidelity 2X Master Mix (NEB M0494S) was used for PCR Bio-Rad CFX96 instrument with the following cycling conditions: initial denaturation (94 °C, 2 min), 40 amplification cycles (94 °C 15 sec, 55 °C 30 sec, 68 °C 1 min), and final extension (68 °C, 5 min). PCR product was run on 2% agarose gel, and then purified using Monarch DNA Gel Extraction Kit Protocol (NEB #71020). The resulting purified DNA was submitted for Sanger Sequencing through Genewiz and sequences were compared to known FCoV serotypes using NCBI Blastn.

2.15 Virus Propagation: Crandell feline kidney cells (CrFK, ATCC CCL-94) (1.0×10^6) were plated into a $75cm^2$ Greiner Bio-One CELLCOAT™ Collagen Type 1-Coated cell culture flask (Greiner Bio-One 658950-005), covered with 12 mL CrFK media (DMEM [Corning 15-013-CV] with 10% FBS, 1% Pen/Strep, 1% L-glutamine 200mM (stabilized), 1.5% HEPES 1M) and

incubated 24 hours at 37 °C and 5% CO_2. Media was removed and cells were washed once with 12 mL DPBS Dulbecco's Phosphate Buffered Saline). Virus stock (1:25 dilution describe, isolate FIP 79-1149) in 12 mL DPBS, incubated 1 hour at 37 °C and 5% CO_2. Media was removed and washed 2 times with 12 mL DPBS then 12 mL CrFK media was added and cells were incubated 48 hours at 37 °C and 5% CO_2. Supernatant was removed into a 15 mL conical tube (Sarstedt Inc 15 mL SCTUBE 17X120CB GWB/CS500), vortexed, then centrifuged (10 min at 1,000 rpm) to remove cellular debris. Supernatant was decanted and aliquoted into 1.5 mL tubes (Sarstedt 72.703.600) and stored at -80 °C.

2.16 Virus Neutralization Assay: This protocol was developed determine if the plasma samples from the CSU cat colony contained neutralizing antibody against FCoV. The neutralization assay was developed in collaboration with Nexalom Biosciences with the use of the Celigo Plate Reader and Analysis software (Nexcelom Bioscience, Lawrence, MA).

2.16.1 Cell, Plasma, and Virus Protocol: CrFK cells (ATCC CCL-94) were suspended in CrFK media CrFK media (DMEM [Corning 15-013-CV] supplemented with 10% FBS, 1% Pen/Strep, 1% L-glutamine 200 mM [stabilized], and 1.5% HEPES 1M) to a concentration of 30,000 cells/ml. 200 µL of diluted cells were added to each well of a sterile, collagen type 1 coated 96 well plate (Greiner 655956). The cells were allowed to adhere with overnight incubation at 37 °C and 5% CO_2. The next day, plasma samples of interest were thawed at room temperature, vortexed, and centrifuged (10,000 rpm for 5 min). Plasma samples were heat inactivated (57 °C for 1 hour) and serially diluted in DPBS (1X Dulbecco's Phosphate Buffered Saline with calcium and magnesium, without phenol red [GE Healthcare SH30264.FS]). Plasma was diluted 1:10 for row "A" by adding 22 µL plasma into 198 µL DPBS (220 µL total). This was repeated down the

rows to create serial dilutions. This yields the dilution series as shown in Figure 7: Plasma Dilution Series for Neutralization Assay.

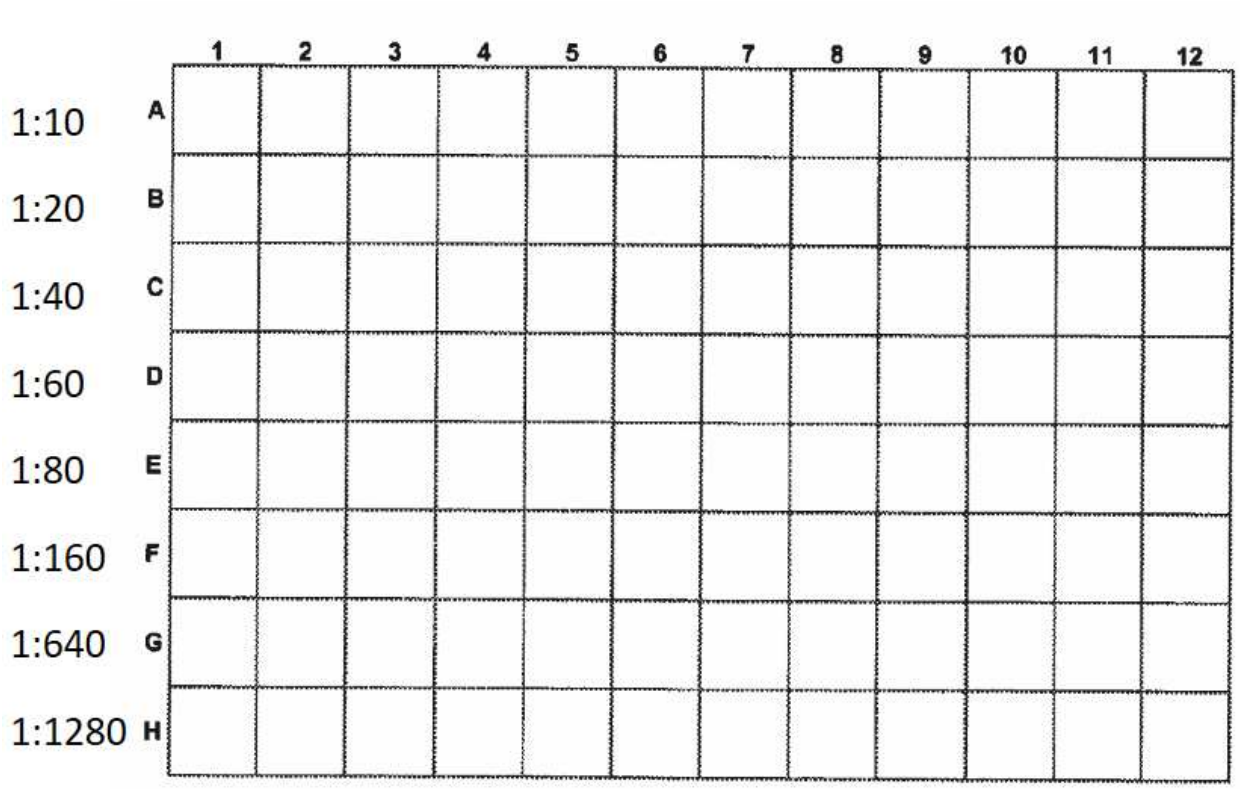

Figure 7: Plasma Dilution Series for Neutralization Assay

Diluted virus stock (1:10 in DPBS [GE Healthcare SH30264.FS]) was added (110 µL/well) and mixed by pipetting up and down 3 times, then the plate was incubated at room temperature for 1 hour. A 96 well plate (prepared as described above) was removed from the incubator. The CrFK media was discarded and the wells were washed once with 200 µL DPBS (GE Healthcare SH30264.FS) per well. Plasma dilutions with virus (100 µL) was added to each well in the CrFK 96 well plate. The plate was then incubated at 37 °C for 1 hour. Supernatant was removed and cells washed 2 times with 200 µL DPBS/well. CrFK media was added (200 µL per well) and incubated at 37 °C, 5% CO_2 for 24 hours.

2.16.2 CrFK Staining Protocol: CrFK media was removed and washed once with 200 µL DPBS. Next, the cells were fixed by adding 100 µL/well of 4% paraformaldehyde (32% Paraformaldehyde [Emsdiasum 15714-S] in 1X PBS [Cellgro MT 21-040-CV]) at room temperature for 30 minutes. The cells were then washed 2 times with 200 µL DPBS/well. The cells were permeabilized by adding 100 µL of 0.1% Triton (Sigma-Aldrich T9284-100ML) in 1X PBS to each well and incubating at room temperature for 5 minutes. The plates were washed 2 times with 200 µL DPBS/well and blocked by adding 200 µL of Blocking Buffer (10% goat serum [Equitech-Bio SG-0500] in 1X PBS [Cellgro MT 21-040-CV], filtered [EMD Millipore SE1M003M00]). The plate was blocked at room temperature for 30 minutes. The Blocking Buffer was discarded and 100 µL of primary antibody solution (Mab FIPV3-70 Custom Monoclonals International, Sacramento, CA with 3% goat serum, filtered [CELLTREAT 229748]) was added. The plate was incubated at room temperature for 1 hour, protected from light. The primary solution was removed and the plate was then washed 2 times with 200 µL DPBS and 100 µL of secondary antibody solution (Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed, Alexa Fluor Plus 488 [Thermo Fisher Scientific A32723] in 3% goat serum, filtered [CELLTREAT 229748]) was added to each well. The plate was then incubated at room temperature for 1 hour, protected from light. The secondary solution was removed and the plate was washed 2 times with 200 µL DPBS per well. 100 µL of DAPI stain dilution (1 µg/mL DAPI [Sigma-Aldrich MBD0015-1ML] in 3% goat serum) was added to each well, incubated at room temperature for 7 minutes, protected from light. The DAPI solution was then discarded and the wells were washed 2 times with 200 µL DPBS. 100 µL DPBS was added to each well for imaging and analysis with the Celigo Plate Reader and Analysis software (Nexcelom Bioscience, Lawrence, MA). Wells were also imaged using the EVOS Fluorescence Microscope (Thermo

Fisher Scientific, Waltham, MA). This further imaging allowed for the visualization of cytoplasmic viral antigens (Figure 8: Visualization of CoV Antigen via AlexaFlour488 (Green). DAPI (Blue) represents nuclear material).

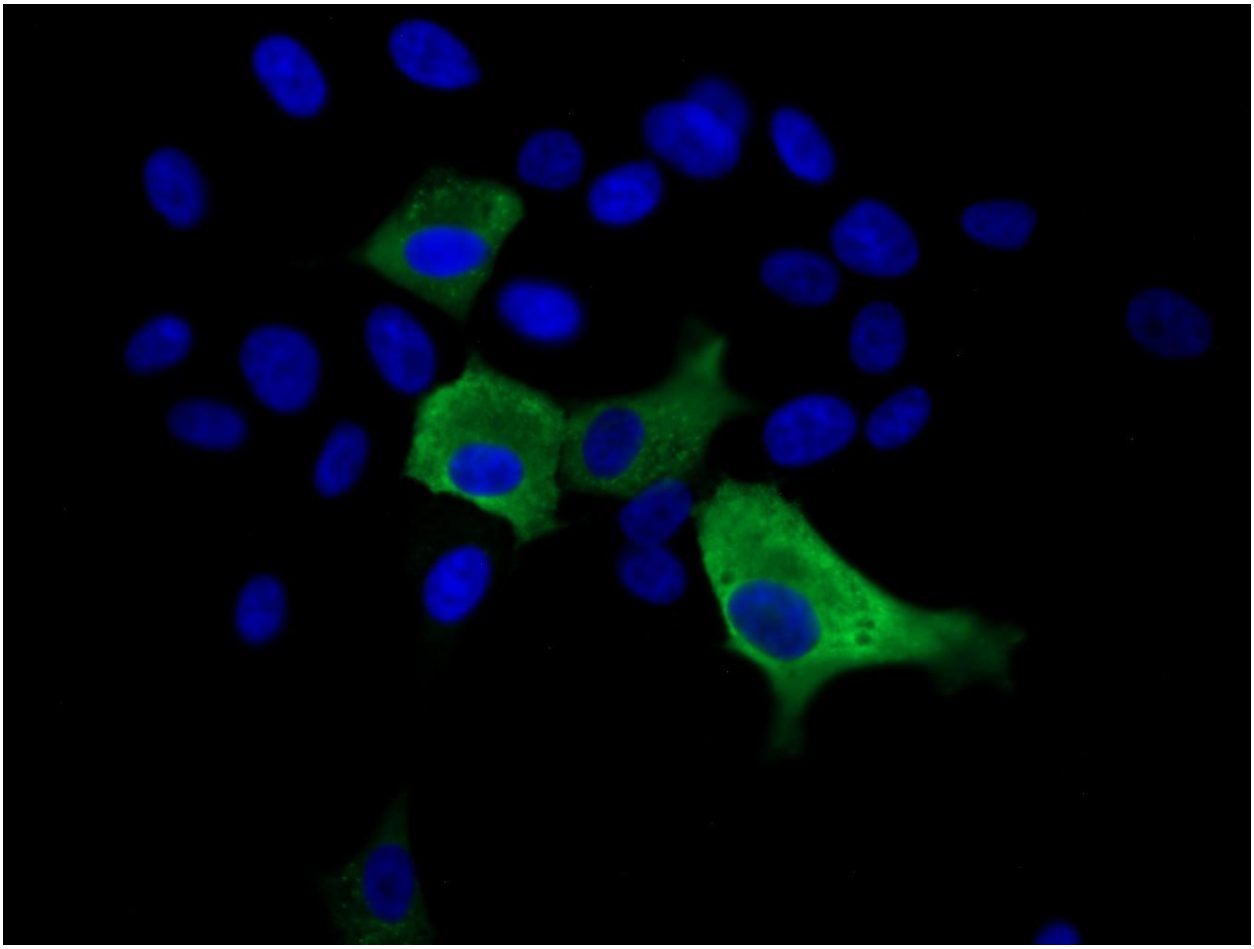

Figure 8: Visualization of CoV Antigen via AlexaFlour488 (Green). DAPI (Blue) represents nuclear material

2.17 Statistical Analysis: All analysis were performed in the open-source program R version 3.5.1. Log-transformed values of the variables were evaluated separately using linear models. Residuals for all models were tested for normality and homoskedasticity and none were found to violate those assumptions. Group means and confidence intervals were extracted from the models using the package emmeans version 1.2.3. This package was also used to create the comparison plots of model meals. Data were handled and plots created using the packages

contained in tidyverse version 1.2.1. Plot placement was facilitated with the use of packages grid version 3.5.1 and gridExtra version 2.3.

CHAPTER 3 – RESULTS

3.1 Plasma IgG and Fecal Real-Time RT-PCR: FCoV infection status of the 33 cats included in this study were typical of those previously reported as determined by ELISA for FCoV-specific plasma IgG and RT-PCR for fecal virus RNA (Addie and Jarrett 2001). These data were used to group animals for further analysis. Group 1 cats were FCoV plasma IgG negative and virus negative throughout the stud; however, because these cats are group housed, they were most likely exposed to FCoV and possibly infected at some point previously. This group represents an immune naïve or quiescent state, due to their lack of plasma IgG. Group 2 were plasma IgG positive at the time of colonoscopy, but never tested positive for virus in either feces or colon biopsy tissue. This group represents the convalescent phase of the immune response. These cats varied in the duration of plasma IgG over the study time frame, from 1 month to the duration of the study (7 months). Group 3 cats were all fecal virus positive at, or just prior, to the time of colonoscopy. These groups were anticipated to represent different immunological states that might help identify correlates of protection against FECV (Table 5: Phenotype and Proliferation of Colon Lymphocytes).

All but one individual (from Group 3) controlled virus shedding within 3 months from the start of the study. The cat with uncontrolled virus shedding remained fecal virus positive throughout the study period and the colon biopsy was also virus positive. The remaining 7 individuals were able to control the virus shedding in an average time of 2.6 months. The average time of plasma IgG after virus replication was controlled was 2.5 months. Cats were naturally infected, which presents limitations in extrapolating data of virus infection kinetics. More can be concluded

about the systemic immune response, which waned rapidly after virus was cleared. Out of the 15 cats that were seropositive at the time of colon biopsy (Groups 2 and 3), 11 were seronegative by the end of the study.

Table 4: Serological status and RT-PCR results for experimental groups.

Group	FCoV IgG+/total	Fecal FCoV+/total	Colon FCoV+/total	Blood FCoV+/total
1	0/18	0/18	0/18	0/18
2	6/6	0/6	0/6	0/6
3	9/9	9/9	3/9	0/9

3.2 CBC, Serum Chemistry, and PBMC RT-PCR: On the day that colon biopsies were taken, a complete blood count (CBC) and full serum chemistry panel was performed by the Veterinary Diagnostic Laboratory at Colorado State University. No abnormalities were identified for any individual animal and no differences were observed between groups, consistent with lack of pathogenicity of FECV. Peripheral blood mononuclear cells were analyzed for FCoV by RT-PCR and all samples were negative at the biopsy time point.

3.3 Colon Histopathology: No histopathological abnormalities were noted and no conclusive positive staining for FCoV antigen was observed for any cat. Positive and negative control tissues stained as expected.

3.4 Colon IgA ELISPOT: Lymphocytes were isolated from colonic biopsies and ELISPOT assay was used to determine the total number of IgA producing cells and the number of FCoV-specific IgA producing cells. Similar to total fecal IgA, no difference was observed in IgA producing cells (Figure 9: Colon IgA ELISPOT Data). Similar to FCoV-specific fecal IgA, the number of

FCoV-specific IgA producing cells was significantly greater (p = 0.001) in Group 3 as compared to either Group 1 or Group 2 (Figure 9: Colon IgA ELISPOT Data).

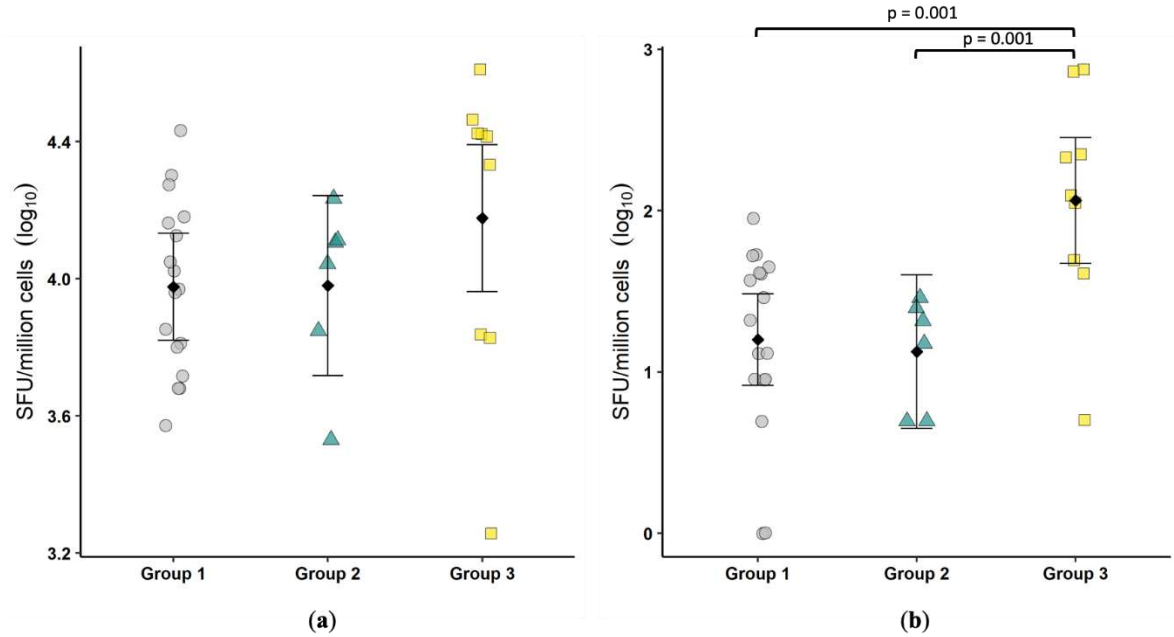

Figure 9: Colon IgA ELISPOT Data. FCoV-specific IgA secreting cells are increased in cats with replicating FECV infection. The number of IgA-secreting cells (spot forming units, SFU) (a) and the number of FCoV-specific IgA-secreting cells (b) were determined by ELISPOT. Each symbol represents a single cat. Bars show the mean and 95% confidence interval. ANOVA was used to compare group means and p values are shown when <0.05.

3.5 Flow Cytometry: Lymphocytes were isolated from colonic pinch biopsies for phenotype and proliferation analysis. The percentages of CD4+ T cells, CD8+ T cells and B cells were determined and the percentage of each population that was Ki-67+ was measured to assess proliferation. While there were no significant differences between the groups, such an analysis has not been previously reported in cats. Results are shown in Table 4 for each group as well as combined results from all groups. The lymphocytes from the biopsies represent both intraepithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL). IEL are typically CD8+, while LPL are predominantly CD4+. These data may be useful for future studies that utilize colonic biopsies to assess mucosal immune responses in cats.

Table 5: Phenotype and Proliferation of Colon Lymphocytes

Phenotype	Group 1	Group 2	Group 3	Combined
CD220+	11.0% +/- 6.5%	7.6% +/- 7.0%	9.4% +/- 5.5%	10.0% +/- 6.3%
CD4+	76.9% +/- 8.3%	81.2% +/- 10.4%	73.0% +/- 13.4%	76.7% +/- 10.2%
CD8+	1.6% +/- 1.1%	1.3% +/- 1.5%	3.8% +/- 1.9%	2.2% +/- 1.7%
CD45+Ki67+	21.7% +/- 5.2%	19.3% +/- 9.9%	19.5% +/- 9.3%	20.7% +/- 7.2%
CD220+Ki67+	62.6% +/- 13.7%	55.2% +/- 18.4%	51.8% +/- 19.7%	58.5% +/- 16.5%
CD4+Ki67+	20.5% +/- 5.3%	18.4% +/- 9.1%	19.2% +/- 7.5%	19.8% +/- 6.5%
CD8+Ki67+	17.5% +/- 14.9%	13.2% +/- 9.9%	11.0% +/- 13.2%	15.0% +/- 13.7%

3.6 Colon IFNγ ELISPOT and IL17:FoxP3 RT-PCR: The total number of colonic lymphocytes with potential to produce IFNγ was determined by stimulation with PMA/ionomycin and the total number of FCoV-specific IFNγ producing cells was determined by re-stimulation with viral antigen. There were no significant differences between groups in IFNγ-producing capacity or in antigen-specific production (Figure 10 Colon IFNγ ELISPOT Data).

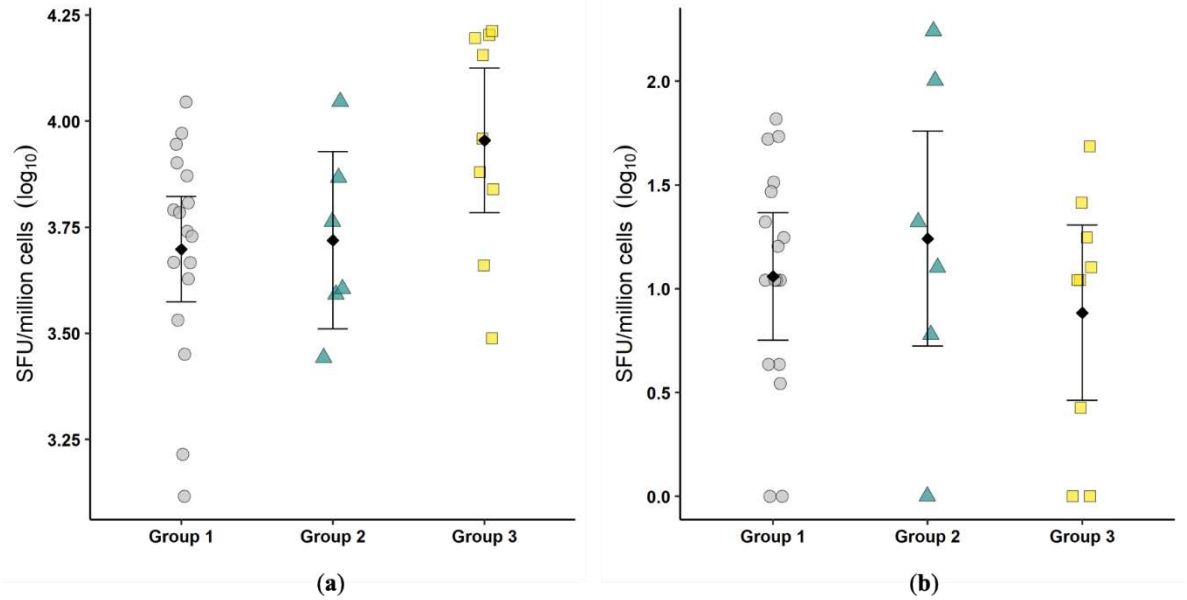

Figure 10: Colon IFNγ ELISPOT Data: Infection by FECV does not results in an increase in total IFNγ producing cells or FCoV-specific IFNγ producing cells. The total number of IFNγ-secreting cells after PMA/ionomycin stimulation (spot forming units, SFU) (a) and the number of FCoV-specific IFNγ-secreting cells (b) were determined by ELISPOT. Each symbol represents a single cat. Bars show the mean and 95% confidence interval. Linear models were used to compare group means and p values are shown when ≤0.05. One was added to the number of FCoV-specific IFNγ-secreting cells prior to log transformation.

These results suggest T cell-mediated control of FECV does not o FCoV-specific IgA was significantly elevated in cats actively shedding virus ccur in the colonic mucosa. As a means to assess the state of T cell balance between inflammation and regulation, the transcriptional ratio of IL17 and FoxP3 was determined by RT-PCR. Group 3 cats had a significantly higher ratio as compared to Group 1 indicating an overall tendency toward inflammation (Figure 11: IL17:FoxP3 RT-PCR Data).

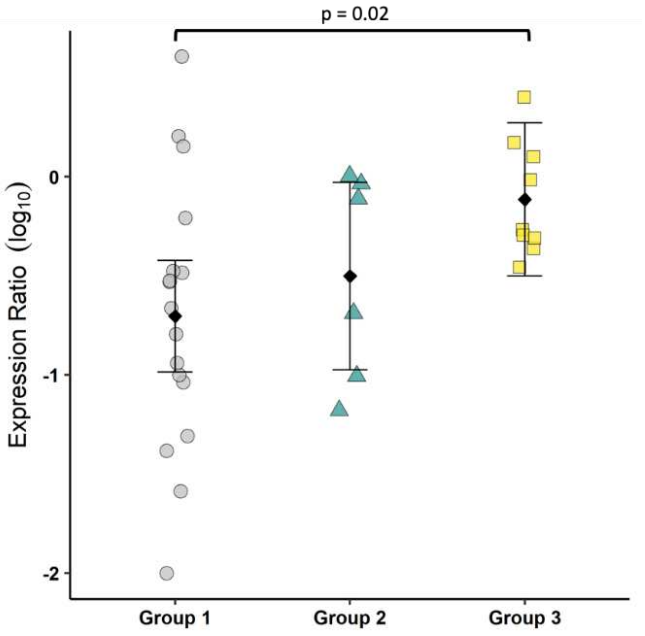

Figure 11: IL17:FoxP3 RT-PCR Data: FECV infection induces an inflammatory bias as indicated by IL17:FoxP3. The ratio of IL17 to FoxP3 transcripts was determined by real-time RT-PCR. Each symbol represents a single cat. Bars show the mean and 95% con fidence interval. ANOVA was used to compare group means and p values are shown when <0.05.

3.7 Fecal IgA Results: Total fecal IgA was measured and while there was a trend for Group 3 to have higher levels, the difference did not reach significance. FCoV-specific fecal IgA was significantly higher ($p = 0.001$) in cats actively shedding virus as compared to either Group 1 or Group 2 (Figure 12: Fecal IgA Data). This result suggests a role for mucosal IgA in the control and clearance of FECV infection.

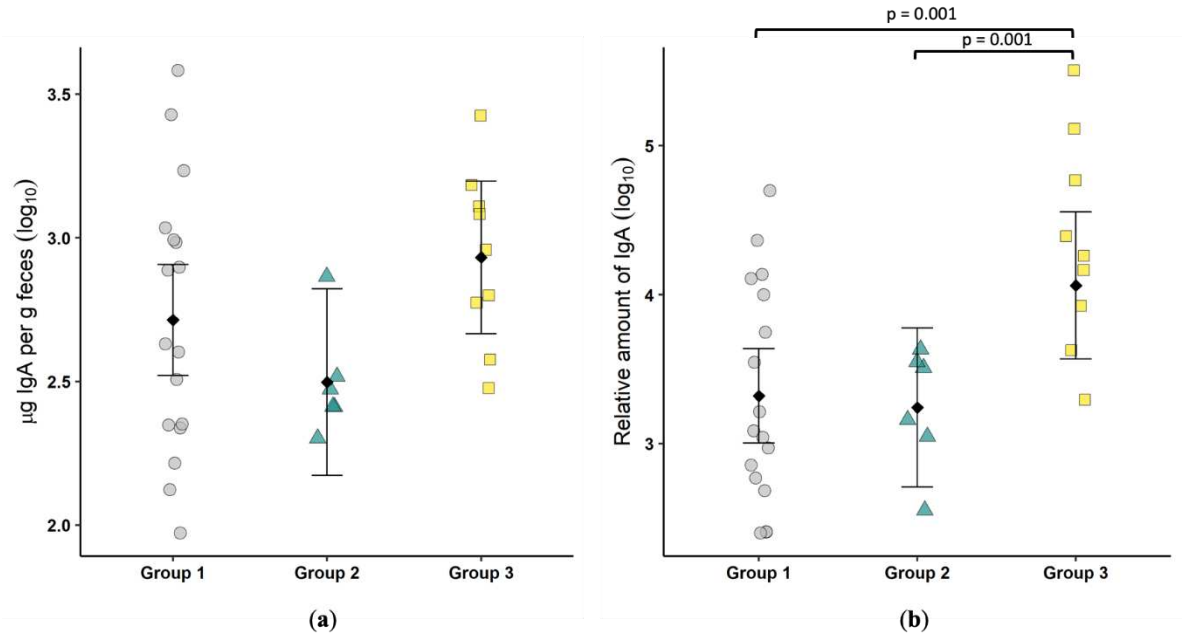

Figure 12: Fecal IgA Data: FCoV-Specific IgA is increased in cats with replicating FECV infection. Total fecal IgA, shown as µg IgA per gram of feces (a) and FCoV-specific fecal IgA, shown as relative amounts (b) were determined by ELISA. Each symbol represents Each symbol represents a single cat. Bars show the mean and 95% confidence interval. ANOVA was used to compare group means and p values are shown when <0.05.

3.8 Western Blot: Western Blot analysis was used to determine recognition of viral proteins by serum antibodies in study cats. The membrane (M) protein and the nucleocapsid (N) protein were both visualized, but the spike (S) protein was not observed most likely because it was absent or at very low concentration in the virus antigen preparation.

3.9 Serotyping CSU Laboratory Virus: Knowing the serotype of the virus was important because Serotype 1 and Serotype 2 have been noted previously to not cross-neutralize (Pedersen et al. 1984). Results from the serotyping experiment demonstrated the CSU colony virus is most similar to serotype 1.

3.10 Neutralization Assay: Initial screening of plasma from CSU cats showed no neutralizing antibodies against FIPV 79-1146 virus. This is likely due to incongruences in serotype of virus

being used in the assay (Serotype 2) and the serotype of the virus present in the CSU cat colony (Serotype 1). It has been shown in previous literature that the two serotypes are not cross-protective (Terada et al. 2014).

CHAPTER 4 – DISCUSSION AND FUTURE DIRECTIONS

Since its initial discovery over 5 decades ago, feline coronavirus has baffled both researchers and veterinarians in both its diagnosis and prevention. How can such a ubiquitous and relatively benign virus manifest into such a deadly disease? Although much research has been done about the dramatic disease manifestation of feline infectious peritonitis, very little is known about how the feline immune system responds to the presence of the biotype feline enteric coronavirus (FECV). Through the characterization of FECV control and elimination, mechanisms of protection against enteric pathology may be elucidated. During this study, special attention was given to the mucosal immune response due to FECV tropism for enterocytes and the important role of IgA at mucosal surfaces.

The utilization of the CSU cat colony (which contains endemic serotype 1 FECV) allowed for the characterization of immune responses associated with virus control. Because the colon has been demonstrated to be a potent reservoir for the virus, biopsies were taken from each of the enrolled individuals, along with blood samples and fecal samples (Addie 2003). Through these samples, the cats were categorized into the three following groups: actively infected individuals, those with evidence of prior exposure, and those in a relatively naïve state (Table 1: Study Groups). Because blood and fecal samples were taken at monthly intervals for six months, it was possible to correlate activity of the immune system and virus clearance. During the study, 8 out of the 9 cats that were identified as actively infected were able to control viral replication shortly after the time of colonic biopsy. Thus, the immune profile at the time of clearance might provide correlates of protection against FECV. Individuals who successfully cleared the virus had

significantly elevated systemic IgG and mucosal IgA against FCoV, but no measurable mucosal IFNγ T cell response. Thus, it appears that control of FECV is primarily mediated by antibody responses. Interestingly, the CSU cat colony had one persistently shedding individual. This cat had a robust FCoV antigen specific IgG response, but did not successfully eliminate the virus. Upon recheck 1 year from time of initial biopsy, it was found she had maintained a high level of FCoV antigen specific IgG. This may indicate that the presence of circulating IgG may not be solely sufficient for the clearance of FCoV in some individuals.

A rapid decline in IgG plasma antibody titer after control of FECV replication was observed with the cats in this study. The lack of durability of the presumptive protective antibody response underlies the potential for repeated reinfection, but also represents a challenge for development of an FECV vaccine. This is likely due to the complex host-pathogen balance has evolved between cats and FECV, such that the virus is rarely pathogenic and this mild infection does not elicit life-long immunity. This, in turn, allows for a perpetual endemic state in cat populations. Successful immunization against FECV will require induction of a more robust T-cell response to drive establishment of B cell memory and longer-lived plasma cells.

An orally delivered vaccine targeting protective FCoV epitopes pose an interesting possibility, particularly in higher-density and stressful environments associated with shelters and colonies. Identification of protective epitopes will require mapping of cloned feline anti-FECV antibodies and development of in vitro assays capable of testing relevant effector functions such as virus exclusion and neutralization in the context of the intestinal mucosa. Importantly, through targeting an oral route, antibody dependent enhancement (ADE) could potentially be avoided. ADE is currently associated with vigorous stimulation of systemic IgG, the devastating consequences of which has stymied the success of many previous vaccines.

To facilitate the validation of future vaccines, a virus neutralization assay was outlined. Initial testing of the plasma samples from the CSU cat colony did not reveal neutralization of FIP 79-1149. This is in accordance with previous literature that demonstrated that Serotype 1 antibodies (contained in CSU plasma) do not neutralize Serotype 2 virus (antigen utilized in the assay) (Terada et al. 2014). Development of a neutralization assay with a Serotype 1 virus will require overcoming the in vitro propagation barrier that has proven to be a persistent obstacle for researchers in the field.

Typical FECV infections result in remarkably few clinical signs and modest immune system responses. The cats in this study were a prime example of this, as all individuals demonstrated minimal to no changes in serum chemistry profile, complete blood count (CBC), or blood lymphocyte subsets. Additionally, there were no changes in mucosal lymphocyte phenotypes or proliferation, total fecal IgA, or constitutive IFNγ production. An increase the mucosal IL17:FoxP3 ratio was observed in virus-shedding individuals, but no histological abnormalities accompanied this apparent shift toward an inflammatory environment. These features confirm the overall picture of a relatively benign enteric viral infection, despite the robust viral replication demonstrated in the cats.

In conclusion, we characterized, for the first time, the mucosal humoral and cellular response to FECV and suggest the mucosal IgA and systemic IgG responses are necessary for virus control given the lack of demonstrable cell-mediated immune responses. Significant additional work is required to characterize the viral targets and the antibody effector functions needed for successful vaccination against FCoV.

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