DISSERTATION

CYTOKINES, ANTIBODIES AND PLASMA VIREMIA OF CATS INFECTED WITH FELINE IMMUNODEFICIENCY VIRUS

Submitted by

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ABSTRACT

CYTOKINES, ANTIBODIES AND PLASMA VIREMIA OF CATS INFECTED WITH FELINE IMMUNODEFICIENCY VIRUS

Feline immunodeficiency viruses (FIVs) are naturally occurring lentiviruses (family *Retroviridae*) of felid species, including domestic and wild cats. Studies on FIVs are beneficial for understanding the host immune response associated with disease progression (e.g., domestic cat FIV) or the viral kinetics and molecular ecology associated with naturally occurring infections in wildlife (e.g., bobcat and mountain lion FIVs). Here we describe the development and validation of the following microsphere immunoassays (MIAs) for evaluating the cytokine and antibody response of domestic cats: i) the quantification of cytokines (interferon gamma (IFNγ), interleukin (IL)-10, and IL-12/IL-23) in cell culture supernatant, and ii) the quantification of these cytokines in plasma; iii) the quantification of total IgG and IgA in plasma, and iv) the detection of IgG and IgA antibodies to feline CD134 (the primary cell receptor for FIV), and FIV capsid (CA) and surface (SU) proteins in plasma. These assays were used to evaluate temporal cytokine and antibody responses of domestic cats experimentally infected with various FIV strains. To analyze viral RNA loads associated with naturally occurring FIV infections in bobcats or mountain lions, we are adapting existing quantitative PCR assays for use with plasma samples. The eight assays described here are/will be beneficial for addressing questions related to lentiviral immune response and viral kinetics.

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CHAPTER 1: INTRODUCTION

Feline immunodeficiency virus. Feline immunodeficiency viruses (FIVs) are naturally occurring lentiviruses (RNA genome; family *Retroviridae*) of felids, including at least 10 species of free-ranging wild cats (19, 37) and the domestic cat (*Felis catus*). The seroprevalence of these viruses range from 0-90% (reviewed in (38)), and transmission within a species is thought to occur primarily through the exchange of infectious fluids via direct contact (i.e., transfer of infectious saliva through bites (3)). With the exception of domestic cat FIV (FIV_{Fca}), these viruses are generally thought to be apathogenic in their respective host. A number of studies suggest that African lions (6, 31, 32) and mountain lions (32) may experience pathological and/or clinical changes due to FIV infection; however, the association between infection and disease is difficult to study in free-ranging felids. FIVs have many similarities to both human and simian immunodeficiency viruses (HIV and SIV), including genomic organization (*gag*, *pol* and *env* genes), virion structure, viral replication (including the integration of the FIV DNA genome (provirus) into host cell DNA), susceptible target cells and immune response (17, 38).

Domestic cat feline immunodeficiency virus. The clinical course of domestic cat FIV mimics HIV disease progression, including the hallmark decline of peripheral $CD4^+$ T-cells (17, 27, 46). Despite a vigorous immune response, FIV and HIV infections are life-long and infected individuals eventually succumb to opportunistic infections. Given the high degree of similarity between these two viruses, the domestic cat is an appropriate animal model for evaluating the immune response associated with lentiviral infections. However, a lack of feline-specific reagents and/or assays has hampered thorough investigations of the immune response during FIV

infection. Thus, a goal of this research was to develop domestic cat microsphere immunoassays to evaluate the cytokine and antibody response of domestic cats infected with FIV.

Microsphere immunoassays. Microsphere immunoassay (MIA) technology allows for the simultaneous detection of multiple analytes within biological samples (e.g., cytokines, chemokines or antibodies; reviewed in (42)). Multiple sets of microspheres, each with a unique ratio of internal dyes and conjugated with a different capture reagent (e.g., antibodies or antigen), are combined and incubated with an aliquot of sample (Figure 1.1). The detection or quantification of each analyte is based on the fluorescence intensity of the detection antibody reporter dye. Advantages of MIAs, compared to traditional immunoassays (e.g., enzyme-linked immunosorbent assays (ELISAs)), are that less time and sample volume are required. There are commercially available MIA kits for a number of species, including humans, non-human primates, horses, pigs, rabbits, dogs, rats and mice; however, no kits are commercially available for domestic cats. Thus, a goal of this research was to develop domestic cat-specific MIAs for the quantification or detection of cytokines and antibodies.

Cytokine response of domestic cats infected with FIV. Multiple studies have evaluated the cytokine response of domestic cats infected with FIV (e.g., (2, 15, 30, 34, 35, 47)). Each of these studies examined the mRNA expression of various cytokines, such as interferon gamma (IFNγ), interleukin (IL)-2, IL-6, IL-10, IL-12 and tumor necrosis factor alpha. Although cytokine expression varies by cell type/tissue and time-point examined, the results of these studies indicate that cytokine expression during the acute stage of infection may be relevant to immunodeficiency virus pathogenesis. For example, elevated levels of IL-10 mRNA expression in $CD4^+$ and $CD8^+$ T-cells coincided with high tissue viral RNA loads (2), and increased levels of IFNγ mRNA expression in blood cells coincided with $CD4^+$ T-cell maintenance in FIV

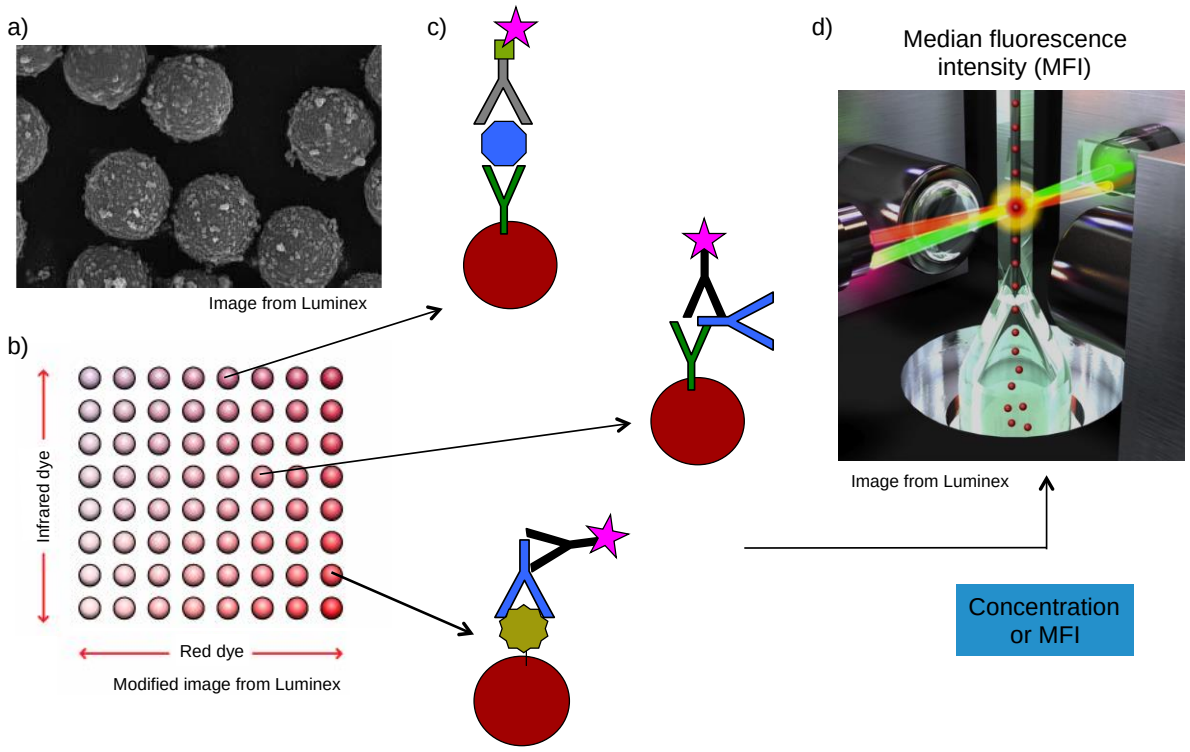

Figure 1.1. Diagram summarizing MIA technology. Magnetic microspheres (a) have two internal dyes, red and infrared (b), which come in varying ratios (regions) assigned by the manufacturer. Multiple analytes can be measured simultaneously within a biological sample by coupling different capture reagents to microspheres from spectrally unique regions (i.e., chemically activating the carboxyl groups of the surface of the microspheres and forming covalent bonds with the primary amines of the capture reagent). Capture reagents (c) can include recombinant viral proteins (yellow) or capture antibodies (dark green). Once coupled, microspheres are first incubated with the sample (e.g., plasma or cell culture supernatant). If the analyte of interest (blue; e.g., antibody or cytokine) is present in the sample, it will bind to the capture reagent. The microspheres are then incubated with detection antibodies. These antibodies can be directly conjugated with the phycoerythrin (PE) reporter molecule (black with pink star) or biotinylated (grey with light green square). If the antibody is biotinylated, the microspheres are subsequently incubated with streptavidin-PE (pink star). The microspheres are analyzed on the instrument by individually passing through two lasers (d). The red laser determines the ratio of the two internal dyes of each microsphere (i.e., the microsphere region, and therefore analyte) and the green laser determines the intensity of the reporter dye. The instrument samples a well until a designated number of microspheres per region have been analyzed (e.g., 100 microspheres). The data output is median fluorescence intensity (MFI); this output can be back calculated to concentration (ng or pg/ml) when standard curves are available/generated. Images obtained from Luminex Corporation (http://www.luminexcorp.com; Accessed October 2009 and May 2010).

co-infected cats (34). Since mRNA expression may not necessarily correlate with protein levels (i.e., the biologically active form), we sought to develop a multiplex MIA for the quantification of feline cytokines using commercially available reagents.

Cytokine MIAs specific for the domestic cat. First, we developed and validated a MIA for the quantitation of IFNγ, IL-10 and IL-12/IL-23 in cell culture supernatant (Chapter 2; (44)). These three cytokines were selected because a) mRNA expression of these cytokines during the acute stage of FIV infection have been evaluated, and differences in the temporal or relative expression may be relevant to immunodeficiency pathogenesis (2, 15, 30, 34), and b) reagents are commercially available (i.e., from ELISA kits). Cytokine concentrations of peripheral blood mononuclear cell supernatants from naïve and FIV-infected cats were compared between the MIA and ELISAs. The results obtained by the MIA correlated with the values obtained with the commercially available ELISAs, indicating that the multiplex assay is a reliable alternative to the ELISAs.

The cell culture supernatant MIA was then modified to quantify IFNγ, IL-10 and IL-12/23 in domestic cat plasma (Chapter 3; (45)). Plasma cytokine concentrations were measured in two separate *in vivo* studies, in which domestic cats were infected with apathogenic and/or pathogenic FIV ((34), Sprague et al., in preparation; Figure 1.2). Additionally, the cytokine concentrations obtained for one of the *in vivo* studies (34) were compared to the mRNA cytokine expression of blood cells. IL-12/23 was elevated ($p < 0.05$) in cats inoculated with apathogenic and/or pathogenic FIV strains relative to the sham cats during the acute stage of infection (i.e., within a month post-infection) in both of the *in vivo* studies. IL-12/23 concentrations ranged from 377 to 1,904 pg/ml in naïve cats and 552 to 3,460 pg/ml in infected cats. In contrast, the majority of plasma samples had IFNγ and IL-10 concentrations below the lowest standard tested.

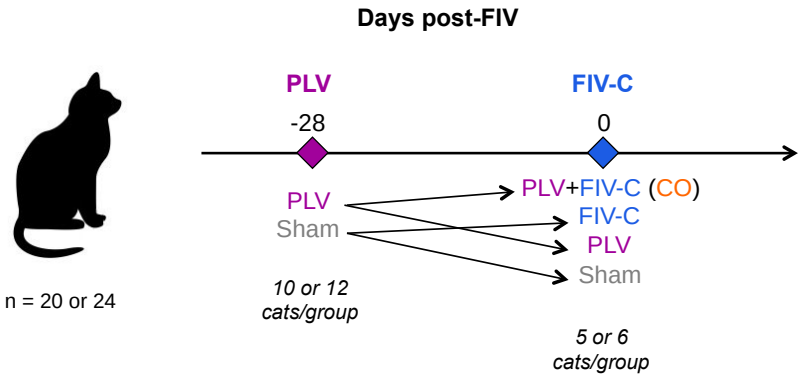

Figure 1.2. Study design for the two *in vivo* studies, in which domestic cats were infected with apathogenic and/or pathogenic FIV ((34), Sprague et al., in preparation). The two virus strains used in these studies were $FIV_{Pco}B$ or puma lentivirus-1695 (PLV), a well-characterized viral isolate obtained from a British Columbia mountain lion (41), and FIV-C36 (FIV-C), a virulent molecular clone of domestic cat FIV that has also been well-characterized (14). Inoculation of domestic cats with PLV results in a productive infection without clinical disease (i.e., apathogenic infection) (40). The 2005 (34) and 2010 (Sprague et al., in preparation) co-infection studies included 20 and 24 cats, respectively. On day -28 of the studies, half of the cats were inoculated intravenously (IV) with PLV, and the other half of the cats were sham inoculated. Twenty-eight days later (day 0), half of the PLV cats and half of the shams cats were inoculated IV with FIV-C, and the remaining cats were sham inoculated. Cats that were inoculated with PLV and FIV-C are referred to as co-infected (CO).

The inability to consistently detect levels of IFNγ and IL-10 in plasma, despite the fact that mRNA changes were detected, suggests that these cytokines may have different mRNA stability/degradation, be secreted and/or cleared in a more highly-regulated manner than IL-12/23, or perhaps exert local effects under tighter peripheral constraints and/or at a lower effective concentration.

Antibody response of domestic cats infected with FIV. Domestic cats develop a robust antibody response during the early stage of FIV-infection, which consists of both FIV and non-FIV specific antibodies. FIV-specific antibodies are detected in plasma/serum within weeks post-infection (7, 8, 16, 20, 21, 28, 29) and increase over time (8, 21, 28, 29). These antibodies

(primarily IgG, but also IgA and IgM (7, 20)) are specific to various viral proteins, including capsid (CA), matrix, surface (SU) and transmembrane proteins (16, 20, 22, 26). In addition, IgG antibodies specific to a variety of non-viral and self antigens are also detected after infection (18); these antibodies contribute to the elevated levels of total IgG antibodies (1, 20), and are thought to be generated due to polyclonal B-cell activation.

Despite the robust FIV-specific antibody response, domestic cats are unable to eliminate the viral infection; however, these antibodies may be beneficial during the later stage of disease. For example, over time antibodies to the SU protein are capable of virus neutralization (23), and as observed with HIV, the presence of antibodies to the CA/Gag protein correlates with delayed disease progression (4, 10, 11, 24, 43). Additionally, FIV-infected cats that develop autoantibodies to CD134 (the primary cell receptor for FIV (12, 33)), tend to have lower peak viral loads and less virulent disease than cats that do not develop anti-receptor antibodies (21). Grant et al. (21) demonstrated the α-CD134 antibodies bind to a cryptic epitope on the CD134 receptor, which is only exposed once the virus SU protein is bound (13), and this binding results in the displacement of SU from the cell receptor (21). By blocking viral entry into a cell, these results suggest that α-CD134 antibodies directly contributed to the observed reduction in viral loads. Although there are a number of studies that have evaluated the antibody response of FIV-infected domestic cats (1, 7, 8, 16, 18, 20-22, 26, 28, 29), there are limited data on the development of both total and FIV-specific IgG and IgA antibodies during the early stage of infection.

Antibody MIAs specific for the domestic cat. We developed and validated MIAs for a) the quantification of total IgG and IgA levels in plasma, and b) detecting IgG and IgA antibodies to feline CD134, and FIV CA and SU proteins in plasma (Chapter 4). These four assays were

then used to examine the temporal antibody response of domestic cats infected with apathogenic and pathogenic FIVs (34), and domestic cats infected with parental and chimeric FIVs of varying pathogenicity (36). The goals of these experiments were to determine whether the kinetics and/or level of the antibody response is related to virus strain/clinical outcome and/or viral load, as well as to estimate the percentage of total IgG antibodies that are CA- or SU-specific. The results from these studies demonstrated that a) total IgG antibodies increase over time after infection; b) α-CA and α-SU IgG antibodies are first detectable between 9-28 days post-infection and increase over time, and that these antibodies represent a fraction of the total IgG increase; c) α-CD134 IgG antibody production varies among individuals and over time, and was not strongly correlated with viral load during the early stage of infection; d) circulating IgA antibodies, in general, do not increase after intravenous FIV infection; and e) total IgG, and α-CA and α-SU IgG antibody kinetics and level vary with FIV viral strain/pathogenicity.

Puma lentiviruses. Although FIVs are generally species-specific, mountain lions (*Puma concolor*; also referred to as cougar, panther or puma) can be infected with two distinct FIV strains ((19), Lee et al., unpublished). Throughout North and South America, free-ranging and captive mountain lions are infected with $FIV_{Pco}B$ or puma lentivirus (PLV)-B ((9, 19), Lee et al., unpublished). In California and Florida, free-ranging mountain lions are infected with $FIV_{Pco}A$ or PLV-A, which is highly divergent from PLV-B ((9, 19), Lee et al., unpublished). Bobcats (*Lynx rufus*) in California and Florida are also infected with PLV-A, and to-date PLV-A is the only FIV sequenced from this species of cat ((19, 25), Lagana et al., in press and Lee et al., unpublished). Given that PLV-A is the only known FIV to infect bobcats and that PLV-A is less common in mountain lions than PLV-B, Franklin and Troyer et al. (19) hypothesized that PLV-A may actually be of bobcat origin and that PLV-A in the mountain lion is the result of cross-

species transmission events. Analyses of PLV-A proviral sequences from pumas and bobcats support this hypothesis, in that viral isolates from mountain lions were more likely to be undergoing positive selection than bobcat viral isolates (Lee et al., unpublished). Additional support for this hypothesis could be provided by plasma viremia data; previous cross-species infection studies with the domestic cat indicate that non-host adapted FIVs (e.g., PLV-A, PLV-B or lion lentivirus (FIV_{Ple})) typically result in lower levels of plasma viremia than host-adapted FIV strains (39, 40).

Puma lentivirus quantitative PCR. PLV-specific primers and plasmids have been developed for the quantification of proviral loads in whole blood samples from bobcats and mountain lions (Templin-Hladky et al., unpublished). We are in the process of adapting these assays for the quantification of PLV-A and PLV-B RNA in plasma (Chapter 5); these assays will be useful for the detection of active viral replication (versus a residual infection indicated by the detection provirus). Experiments have been conducted to determine the linear range of the standards and to optimize assay conditions, as well as testing plasma samples from PLV positive bobcats and mountain lions (based on provirus sequence data; Lee et al., unpublished). Of the samples tested to-date, only 4 (of 14) of the PLV-B mountain lion samples had viral loads within the linear range of the standards (10^2 to 10^7 copies/reaction); these four values are at the low end of those previously reported for PLV-B infected mountain lions (5). All of the PLV-A bobcat samples (7 of 7) tested to-date had viral loads below the linear range of the standards (10^2 to 10^7 copies/reaction). Future experiments will be conducted to concentrate viral RNA and/or cDNA, so that the sample concentrations are within the quantifiable range. Once assay development and validation are complete, plasma samples will be tested to determine whether mountain lion

PLV-A viremia is lower than bobcat PLV-A and mountain lion PLV-B viremia, which would suggest that PLV-A is of bobcat origin.

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CHAPTER 2: DEVELOPMENT AND VALIDATION OF A MULTIPLEX MICROSPHERE-BASED ASSAY FOR DETECTION OF DOMESTIC CAT (*FELIS CATUS*) CYTOKINES[1]

INTRODUCTION

Cytokines are small proteins secreted by cells to coordinate cellular communication in response to inflammation or infection. As such, these molecules have important roles in innate immune responses during an infection and contribute to the activation of the adaptive immune response (reviewed in (13)). Therefore, characterization of the cytokine response is crucial for understanding host-pathogen dynamics. Because cytokines are often secreted in small amounts locally and may have short half-lives, detection of cytokines during disease processes has not been routinely performed; however, new technologies that allow cytokine detection, either during disease states in hosts or in tissue culture systems, would provide additional tools for understanding immune modulation.

Frequently, cytokines are assessed by quantitative PCR (qPCR) or by enzyme-linked immunosorbent assay (ELISA); however, both of these methods have limitations (reviewed in (19, 20)). Although qPCR assays can be developed to detect multiple cytokines simultaneously, relative mRNA expression may not correlate with protein levels. ELISAs, on the other hand, have the capability to quantify protein, but only one cytokine can be detected in a single ELISA assay. Additionally, these assays are labor-intensive and require a significant sample volume. The latter is of particular importance with laboratory animals because of the limited sample

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volume (e.g., serum, plasma or peripheral blood mononuclear cells (PBMC)) that can be safely obtained.

Microsphere-based assays are a relatively new technology capable of detecting multiple analytes simultaneously (reviewed in (9, 20)). Microspheres with spectrally unique internal dyes act as the solid support for individual immunoassays. Using flow cytometry technology, the analyte concentration is determined by the fluorescence intensity of the reporter dye. Currently, there are 100 spectrally unique microspheres available; theoretically, 100 different analytes could be detected in a single sample. There are commercially available cytokine-multiplex kits for humans, non-human primates, mice, rats and dogs; however, no kits are currently available for domestic cats. The availability of human and mouse reagents has allowed the analysis of cytokine levels in a variety of disease states, including sickle cell disease (1), sepsis (5), viral infection in humans (8), and bacterial pneumonia in mice (6). The capacity to accurately and reproducibly evaluate cytokine profiles in domestic cats, in both naturally occurring and experimentally induced disease states, would potentially provide valuable information regarding disease pathogenesis, progression, and prognosis.

The purpose of this study was to develop a microsphere-based immunoassay for the simultaneous quantitation of the feline cytokines interferon gamma (IFNγ), interleukin-10 (IL-10) and IL-12/IL-23 p40 (subsequently referred to as IL-12/23). These cytokines were selected because their mRNA expression during the acute stage of feline immunodeficiency virus (FIV) infection has been evaluated and differences in temporal or relative expression may be relevant to immunodeficiency pathogenesis (2, 7, 15). IFNγ and IL-12 are critical for the differentiation of T-helper 1 (T_h1) cells, while IFN-γ is critical for the activation of cytotoxic T lymphocytes (CTLs). CTLs are an important component of the host defense against viral pathogens. IL-10,

on the other hand, is a suppressor of a T_h1 immune response. Our laboratory intends to apply this technology to evaluate cytokine profiles of domestic cats during infection with FIV, a naturally occurring feline virus with many parallels to human immunodeficiency virus (HIV) (14, 16). Measurements of cytokines secreted from cells in culture following experimental infections with different FIV strains could offer new insights into disease pathogenesis and progression. This assay could also be used to study other feline diseases with human applications, such as cancer and inflammatory diseases.

MATERIALS AND METHODS

Capture antibody coupling to microspheres. Carboxylated magnetic microspheres (Luminex Corporation, Austin, TX) were conjugated with feline IFNγ, IL-10, and IL-12/23 capture antibody from DuoSet® ELISA Development kits (R&D Systems, Minneapolis, MN) according to the manufacturer's recommendations for two-step carbodiimide coupling of protein to MagPlex-C magnetic carboxylated microspheres (Luminex; Table 2.1). Briefly, lyophilized capture antibody was re-suspended in 1 ml of sterile phosphate-buffered saline (PBS). Magnetic microspheres were washed with 100 µl of distilled water and re-suspended in 80 µl of activation buffer (0.1 M sodium phosphate monobasic, anhydrous, pH 6.2). Carboxyl groups on the surface of the microspheres were chemically activated using 10 µl *N*-hydroxysulfosuccinimide (50 mg/ml) and 10 µl 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (50 mg/ml) (Fisher Scientific, Pittsburgh, PA). Activated microspheres were washed twice with 250 µl of coupling buffer (0.05 M 2(*N*-morpholino)ethanesulfonic acid, pH 5.0) and then re-suspended in 100 µl of the buffer. Capture antibody was added, and the final volume was brought to 500 µl with coupling buffer. The mixture was incubated for 2 h at room temperature in the dark with gentle rotation. After incubation, coupled microspheres were washed three times (500 µl and

Table 2.1. Components used for the development of the domestic cat microsphere assay.[a]

Analyte	Microsphere region	Capture antibody	Detection antibody	Standard
Feline IFNγ	15	Goat anti-feline IFNγ	Biotinylated goat anti-feline IFNγ	Recombinant feline IFNγ
Feline IL-10	33	Mouse anti-feline IL-10	Biotinylated goat anti-feline IL-10	Recombinant feline IL-10
Feline IL-12/23	55	Goat anti-feline IL-12/23	Biotinylated goat anti-feline IL-12/23	Recombinant feline IL-12/23

[a] The analyte, capture antibody, detection antibody and standard were from the DuoSet® ELISA (R&D Systems). The microsphere region (MagPlex-C Microspheres; Luminex) was assigned by manufacturer based on ratio of two internal dyes.

two 1-ml washes) with blocking/storage buffer (PBS, 0.1% bovine serum albumin (BSA), 0.02% Tween 20, 0.05% azide, pH 7.4). The microspheres were then re-suspended in blocking/storage buffer (150 µl per 10^6 microspheres) and stored at 4°C in the dark for 2 to 4 months. Microsphere concentrations were determined using an automated counter (Invitrogen, Carlsbad, CA).

To confirm antibody coupling, microspheres (~5,000) were incubated with a phycoerythrin (PE)-labeled secondary antibody (R&D Systems) according to the manufacturer's recommendations (Bio-Plex amine coupling kit instruction manual: protein coupling validation (Bio-Rad, Hercules, CA)). A median fluorescent intensity (MFI) of >2,000 indicated successful coupling.

Microsphere assay protocol. The immunoassay protocol used in these experiments was modified from a protocol for a washed capture sandwich immunoassay using magnetic microspheres (Luminex). Aliquots of coupled microspheres were combined in assay buffer (PBS, 1% BSA, pH 7.4) for a final concentration of either 50 or 100 microspheres/µl per analyte. A 50-µl aliquot of the multiplex microsphere solution (i.e., 2,500 or 5,000 microspheres/analyte per well) was then added to each well in a 96-well, round-bottom polystyrene plate (Fisher Scientific). Wells were washed twice with 100 µl of assay buffer using a 96-well magnetic separator (Ambion, Austin, TX) and again, after each subsequent incubation. Plates were incubated for 30 min at ~800 rpm in the dark at room temperature. Microspheres were incubated in 50 µl of solution in the following order: (i) sample, standard, spike or cell culture medium control (5 to 20% heat-inactivated fetal bovine serum, 2% glutamine, 0.5% 2-mercapto ethanol, 1% penicillin-streptomycin, quantity sufficient (q.s.) RPMI 1640); (ii) detection antibody; and

(iii) streptavidin-PE (Invitrogen). After the final incubation and washes, the microspheres were re-suspended in 100 µl of assay buffer and analyzed.

A Bio-Plex™ 200 instrument (Bio-Rad) was calibrated before each analysis and validated monthly to ensure optimal performance of optics, reporters, fluidics, and classification per the manufacturer's recommendations. Each plate included an eight-point standard curve (2-fold dilution series) and four control wells, in addition to samples with known or unknown analyte concentrations. All standards and samples were run in duplicate. Median fluorescence intensity was calculated from ≥100 microspheres per analyte per well. Bio-Plex™ Manager 5.0 (Bio-Rad) was used for data collection and analysis. A five-parameter logistic standard curve was generated for each analyte from the standards by plotting concentration versus fluorescence. The computer program calculated the concentration of each sample or spike using the respective analyte standard curve. Acceptable standard recovery was 70 to130% of the nominal value (Bio-Plex cytokine assay instruction manual; Bio-Rad).

Microsphere assay development. Assay development and optimization were conducted in the following order: (i) optimization of capture antibody concentrations, (ii) optimization of detection antibody concentrations, (iii) determination of standard curve ranges, (iv) evaluation of cross-reactivity among analytes, (v) comparison of MFI between single and multiplex standard curves, (vi) optimization of streptavidin-PE concentration, and (vii) optimization of the number of microspheres per well. Capture antibody concentrations tested were 1, 2.5, 5, 7.5, 10, and 15 µg per 10^6 microspheres. Detection antibody concentrations tested were 1X, 2X,and 3X the recommended concentration for ELISA protocols. Standard curve ranges evaluated were based upon the recommendations of the ELISA protocols (7-point curves), with three additional lower concentrations (i.e., a 10-point curve was tested). To test for cross-reactivity among analytes,

each cytokine standard curve was individually tested with multiplex microspheres and detection antibody. Additionally, to determine if the MFI was affected by multiplexing analytes, single and multiplex standard curves were compared. Streptavidin-PE concentrations tested were 2, 3, and 4 μg/ml. The number of microspheres tested per well were approximately 1,000, 2,000, 2,500, and 5,000 microspheres per analyte to determine the minimum number required per well.

Multiplex assay validation. Intra- and inter-assay experiments were conducted to determine both the accuracy and precision of the multiplex assay. In these experiments, the spike concentrations tested were based upon the dilution series of the standard curve. The stock solution of each standard provided by the ELISA kit manufacturer was diluted to three concentrations and spiked into cell culture medium to test the accuracy of detecting sample concentrations based upon the standard curves generated. Spike concentrations were as follows: 31, 250, and 1,000 pg/ml for IFNγ; 63, 500, and 2,000 pg/ml for IL-10; and 39, 156, and 625 pg/ml for IL-12/23. For the intra-assay experiment, 10 wells with each spike concentration were run in a single experiment. For the inter-assay experiments, spiked samples (duplicate or quadruplicate wells) were run in nine separate experiments.

Acceptable spike recovery was 70 to130% of the nominal value (Assay qualification guidelines for Luminex assays; Panomics, Santa Clara, CA). The coefficient of variation (CV) for the mean spike recoveries at each concentration was required to be <20% for the intra-assay experiment and <30% for the inter-assay experiments (Bio-Rad, personal communication). The lower limit of quantitation (LLOQ) was the lowest concentration tested that was repeatedly recovered. The upper limit of quantitation (ULOQ) was the highest concentration tested that was repeatedly recovered.

Generation of supernatant from domestic cat PBMC. We compared cytokine values determined by commercially available, unvalidated ELISAs (DuoSet® ELISA Development kits; R&D Systems) to those determined by the multiplex assay using cell culture supernatant samples generated from domestic cat PBMC. The ELISA kits are marketed as a reagent resource for analysis of cytokines in cell culture supernatant. Cell culture supernatant samples generated in our laboratory were tested to verify that the multiplex assay was capable of detecting natural cytokines produced by domestic cat cells (i.e., other than the recombinant cytokines standards).

Specific-pathogen-free domestic cats were housed in Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC, Intl.) approved facilities and handled under protocols approved by the Colorado State University Institutional Animal Care and Use Committee (IACUC). Heparinized blood was collected from both naïve and FIV-positive cats sedated with ketamine-acepromazine. PBMC were isolated using a Histopaque gradient (Sigma-Aldrich, St. Louis, MO) and re-suspended in medium. Cells were grown in the presence of 0, 0.5, or 5 μg/ml of concanavalin A (ConA) (Sigma-Aldrich). Additionally, PBMCs from naïve cats were infected *in vitro* with one of two FIV strains, as a component of other ongoing experiments. Supernatants were harvested from 1 to 10 days post-culture and stored in aliquots of 0.5 to 1 ml at -80°C until tested with both assays.

ELISA and multiplex cytokine analysis of supernatant samples. The manufacturer's protocol was followed for each ELISA, although the standard curve range was altered to reflect the range used in the microsphere assay. IFNγ, IL-10, and IL-12/23 concentrations in all samples were tested simultaneously by both assays to minimize freeze-thaw and sample storage variability. Samples were included in the assay comparison analysis if the concentrations obtained from both assays were within the standard range tested (i.e., a sample was excluded if

one or both assays resulted in a concentration above or below the standard range). Values were compared using a Spearman's rank correlation for nonparametric data and 95% prediction intervals (GraphPad Prism 5.0; GraphPad, La Jolla, CA).

RESULTS

Microsphere assay conditions. Each component of the microsphere assay was tested individually. The findings from these experiments (summarized in Table 2.2) are as follows.

Table 2.2. Optimized concentrations of reagents.

Cytokine	Capture antibody ($\mu g/10^6$ microspheres)	Detection antibody (ng/ml)	Streptavidin-PE (μg/ml)	Microsphere input (per region per well)
IFNγ	5	2,400	3	2,500
IL-10	5	200	3	2,500
IL-12/23	5	300	3	2,500

(i) *Capture antibody concentration.* The optimal concentration was the highest point in the linear range (i.e., before saturation). Five micrograms of capture antibody per 10^6 microspheres was found to be the optimal concentration for microsphere coupling for each analyte.

(ii) *Detection antibody concentration.* Concentrations that resulted in MFIs of >2,000 for a high standard and <100 for a low standard were selected. For IL-10, the concentration of detection antibody that met these criteria was the same as the concentration recommended in the ELISA protocol (200 ng/ml). For IFNγ and IL-12/23, the concentrations that met these criteria was 3X the recommended ELISA concentrations (2.4 μg/ml and 300 ng/ml, respectively). Detection antibody saturation was not determined.

(iii) *Standard curve range.* All 10 tested concentrations were detected for each analyte (Table 2.3). The lowest concentration (8 pg/ml for IFNγ, 16 pg/ml for IL-10, and 5 pg/ml for IL-12/23) was eliminated from future standard curves because the MFI was only slightly higher than that of unspiked controls. The highest concentration (4,000 pg/ml for IFNγ, 8,000 pg/ml for IL-10, and 2,500 pg/ml for IL-12/23) was also eliminated because this was above the expected concentration for cell culture supernatant samples.

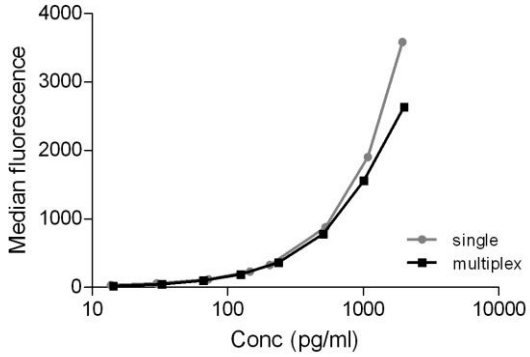
Table 2.3. Standardized range for each analyte using the microsphere assay compared to the recommended standard range for the ELISAs.

Assay and parameter	pg/ml		
	IFNγ	IL-10	IL-12/23
ELISA			
Standard curve	63-4,000	125-8,000	39-2,500
Microsphere assay			
Standard range tested	8-4,000	16-8,000	5-2,500
Standard curve	16-2,000	31-4,000	10-1,250
LLOQ	31	63	39
ULOQ	1,000	2,000	625

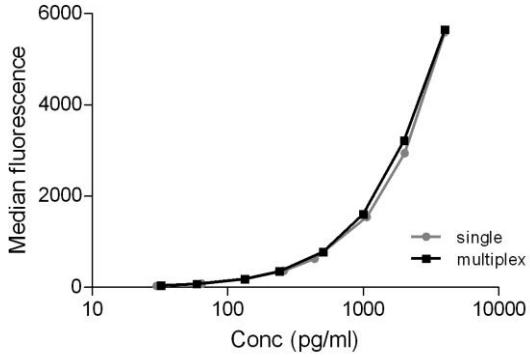
(iv) *Testing for cross-reactivity among analytes.* Minimal cross-reactivity was observed among analytes; values ranged from 3-34 MFI.

(v) *Comparison of MFI between single and multiplex standard curves.* Each analyte had only minor differences between single and multiplex standard curves (Figure 2.1), indicating that these analytes could be analyzed in multiplex format.

a)



b)



c)

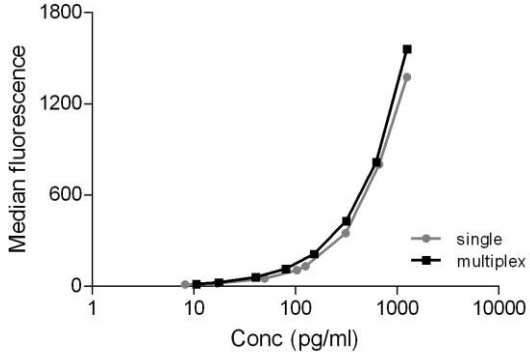


Figure 2.1. Single and multiplex standard curves are comparable. (a) IFNγ; (b) IL-10; (c) IL-12/23.

(vi) *Streptavidin-PE concentration.* The streptavidin-PE concentration used up to this stage of the optimization process (i to v) was 4 μg/ml as per the microsphere manufacturer's recommendations. All standards were detectable when the concentration of streptavidin-PE was decreased to 3 μg/ml. The lowest concentration of the IL-12/23 standard curve was not detected when the streptavidin-PE concentration was decreased to 2 μg/ml; therefore, 3 μg/ml was used for all further experiments.

(vii) *Microsphere concentration.* The microsphere input per well for each analyte used throughout the optimization process (i to vi) was 5,000 as per the microsphere manufacturer's recommendations. Standard concentrations and microsphere readings were unaffected by decreasing the microsphere input to 2,500 per analyte (i.e., the minimum of 100 microspheres were read for each analyte). Decreasing the microsphere input to 2,000 per well or below did not always result in the reading of 100 microspheres; therefore, we determined 2,500 to be the lower limit for assay reproducibility.

Multiplex assay validation. For all three analytes, the individual spike concentration recoveries (accuracy) were within the manufacturer-recommended acceptable range of 70 to 130%. The mean recoveries for each analyte at each concentration for the inter- and intra-assay experiments were within these limits (Table 2.4). Precision was measured as the percent CV of the individual recoveries for spikes at each concentration per analyte. All of the percent CVs for the inter- and intra-assay experiments were also within the validation requirements recommended by the manufacturer (<20% in the intra-assay and <30% in the inter-assay experiments; Table 2.4).

Table 2.4. Assay precision and accuracy over a range of analyte concentrations

Experiment and spike level	Mean % recovery (% CV)		
	IFNγ	IL-10	IL-12/23
Intra-assay			
High	120 (5)	106 (4)	114 (4)
Medium	120 (4)	99(13)	116 (4)
Low	109 (11)	95 (7)	110 (10)
Inter-assay			
High	105 (5)	107 (8)	108 (5)
Medium	109 (5)	107 (6)	111 (5)
Low	103 (16)	109 (8)	112 (9)

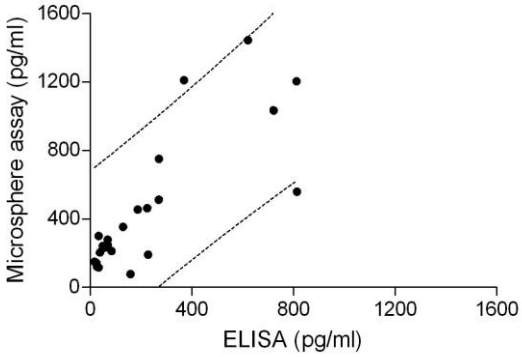
The validated limits of quantitation per analyte are summarized in Table 2.3. The lowest standard used for each analyte in the microsphere assay is one-fourth the concentration of the lowest standard recommended for each ELISA protocol.

Comparison to commercially available ELISAs. The number of supernatant samples in which cytokine concentrations were quantifiable by both ELISA and the microsphere assay varied for each analyte (Figure 2.2) (n = 24 for IFNγ, n = 38 for IL-10, and n = 27 for IL-12/23). The Spearman rank correlation (R_s) was 0.79 for IFNγ, 0.93 for IL-10, and 0.86 for IL-12/23. The values indicate a positive linear relationship between cytokine concentrations obtained by ELISA and by the multiplex microsphere assay. Most data fell within the 95% prediction interval (Figure 2.2), further demonstrating high correlation between the assays.

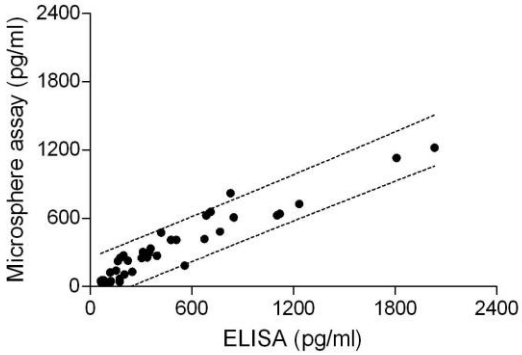
DISCUSSION

This study demonstrates the successful development and validation of a microsphere-based immunoassay for the quantification of the domestic cat cytokines IFNγ, IL-10 and

a)



b)



c)

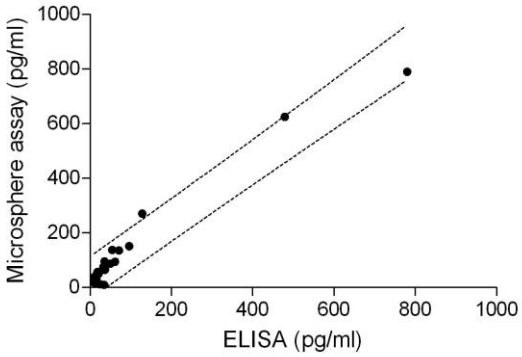


Figure 2.2. Cytokine concentrations in domestic cat PBMC samples measured by ELISA and the microsphere assay are correlated. (a) IFNγ; (b) IL-10; (c) IL-12/23. Dashed lines represent the 95% prediction intervals.

IL-12/23. To our knowledge, this is the first microsphere-based assay developed for the domestic cat.

The microsphere assay has several advantages over commercially available ELISAs. The time required for the microsphere assay is less than the time required for the three ELISAs, and the microsphere assay is much simpler to conduct. The microsphere assay detects lower concentrations of the analytes than the standard ranges recommended by the ELISA protocols (Table 2.3). Additionally, the microsphere assay requires only 50 µl of sample for analysis, compared to 300 µl of sample required for performing the three corresponding cytokine ELISAs. Finally, the microsphere assay has been optimized and validated using industry-recommended requirements, ensuring reproducible quantification of cytokine values both within and among experiments.

The true quantifiable range of each cytokine is likely broader than the range tested (i.e., the true ULOQ is likely higher and the true LLOQ is likely lower than the concentrations tested in this assay). The high spikes had the same concentration as the second-highest standard, whereas the low spikes had the same concentration as either the second- or third-lowest standard. Although the true ULOQs of the assay were not reached, the majority of the cell culture supernatant samples for each analyte were below the high spike concentration. This is demonstrated in Figure 2.2 by detectable levels of cytokines in PBMC cultures; most concentrations were detected in the lower 30 to 50% of the standard range. The highest values tended to occur in supernatants from PBMC stimulated with high concentrations of a ConA, a potent T-cell mitogen.

Cytokine concentrations of biologically relevant samples in the microsphere assay and ELISAs were compared. Since the ELISAs were not validated, we cannot state with certainty

that the absolute values obtained by both assays are equivalent; however, correlation analysis does demonstrate that values were proportionate and fell within the 95% predictive limits for the vast majority of data points. This analysis indicates that the multiplex assay has been properly established to predict cytokine concentrations in samples and that these values would be similar to those obtained with the commercially available ELISAs.

During intra- and inter-assay validation, it was noted that fluorescence decreased across the plate (i.e., MFI was higher in the first samples read and the values decreased over time), though recoveries were still within the acceptable range. Experiments conducted after assay validation revealed that re-suspension of microspheres in a 0.1% formalin solution before analysis decreased MFI drift (10, 12); see Technology tips: stop solution or fixative [Luminex]). It is possible that the accuracy and precision of the validation experiments would have improved if this fixation step had been included; however, the values reported here are similar to those reported for other assays with (10) and without (4, 11) fixation. We therefore recommend that a formalin fixation step be considered as a component of cytokine multiplex procedures to stabilize MFI and to increase assay accuracy and precision.

Microsphere-based assay development has expanded the number of analytes that can be simultaneously detected in biological samples (e.g., cytokines, antigens, and antibodies). Commercially available cytokine kits and assays developed by individual research groups (e.g., for porcine [(4)] and horse (21)] cytokines) provided valuable insights into the innate immune response of a host against infection. For example, microsphere-based assays have been used to characterize the cytokine response in the lymph nodes of chronically infected HIV patients (3) and cytokine production during acute HIV-1 infections (17). Both studies demonstrate quantifiable differences in cytokine levels between HIV-infected tissues/patients and

control/comparison groups. These differences stress the importance of characterizing the innate immune response in HIV infections in order to gain insights into future vaccine and drug development. Additionally, in a translational study for human acute lung injury (ALI), investigators characterized the cytokine response of canines with induced ALI and the effect of sphingosine 1-phosphate (S1P) treatment (18). Although there was variation in cytokine production and S1P was found to reduce lung injury, this appeared to be independent of the cytokine response. We speculate that the domestic cat cytokine assay described here may have both experimental and clinical applications for defining disease states.

In summary, this study describes an optimized and validated assay for the simultaneous quantification of three domestic cat cytokines in a convenient and reproducible platform. Commercially available reagents that could be used to expand this panel for the detection of additional cytokines include those for IL-1β, IL-2, IL-4, IL-6, and/or tumor necrosis factor alpha, adding to the utility of this assay. Further efforts will be directed toward the development of a similar assay for the detection of cytokines in domestic cat plasma/serum samples.

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CHAPTER 3: MICROSPHERE IMMUNOASSAY FOR THE DETECTION OF CYTOKINES IN DOMESTIC CAT (*FELIS CATUS*) PLASMA: ELEVATED IL-12/23 IN ACUTE FELINE IMMUNODEFICIENCY VIRUS INFECTIONS[2]

INTRODUCTION

Microsphere immunoassays (MIAs) are a relatively new technology capable of detecting multiple analytes simultaneously (reviewed in (7)). Microspheres with spectrally unique internal dyes act as the solid support for individual immunoassays. Using flow cytometry technology, the analyte concentration is determined by the fluorescence intensity of the reporter dye. MIA kits are commercially available for the quantification of various analytes, including cytokines, soluble cytokine receptors, chemokines and antibodies. These kits are available for humans, non-human primates, dogs, mice and rats; however, no kits are commercially available for the domestic cat.

Feline immunodeficiency virus is a naturally occurring lentivirus of the domestic cat (FIV_{Fca}, subsequently referred to as FIV) that is similar to human immunodeficiency virus (HIV) in terms of viral structure, transmission, target cells and disease progression (5, 10, 20). Clinical disease is characterized by flu-like symptoms and a decrease in $CD4^+$ T-cells during the acute stage of infection, followed by a long asymptomatic stage in which $CD4^+$ T-cells continually decline (reviewed in (5)). Despite a vigorous immune response, FIV and HIV infections are life-long and infected individuals succumb to opportunistic infections. Given the high degree of

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similarity between FIV and HIV, the domestic cat is an appropriate animal model for evaluating the relationship between $CD4^+$ T-cell depletion and immune activation with lentiviral infections.

Inoculation of domestic cats with FIV_{Pco} (subsequently referred to as PLV), a lentivirus native to the mountain lion and genetically related to domestic cat FIV (2), causes productive infection without clinical disease (15). PLV proviral loads decrease in peripheral blood mononuclear cells (PBMC) within three months of infection, and are higher in the gastrointestinal tract than lymphoid tissues (13). Previous studies conducted by our laboratory demonstrate that PLV infection before FIV infection (i.e., co-infection) blunts the peripheral $CD4^+$ T-cell loss observed during the acute phase of FIV single-infection (12, 14). The mechanism(s) underlying this peripheral $CD4^+$ T-cell maintenance is still under investigation, including the host cytokine response.

Here we a) describe the development and validation of a MIA to simultaneously quantify domestic cat cytokines interferon gamma (IFNγ), interleukin (IL)-10, and IL-12/IL-23 p40 (IL-12/23) in plasma (modified from (18)), b) demonstrate the use of this assay with plasma samples collected from domestic cats inoculated with FIV and/or PLV, and c) compare cytokine concentrations to mRNA expression. IFNγ, IL-10, and IL-12/23 were selected because reagents are commercially available, and their mRNA expression during the acute stage of infection may be relevant to immunodeficiency virus pathogenesis (1, 4, 11, 12).

MATERIALS AND METHODS

Antibodies and standards. Capture and detection antibodies specific for domestic cat cytokines, and recombinant cytokine standards were obtained from DuoSet® Enzyme-linked Immunosorbent Assay (ELISA) Development kits (R&D Systems, Minneapolis, MN). The reagents used in the IL-10 and IFNγ sandwich ELISAs detect both monomers and homodimers

of their respective molecules (personal communication, R&D Systems). IL-12 and IL-23 are heterodimers, which share a common p40 subunit. The sandwich ELISA detects p40 monomers and heterodimers of IL-12 and IL-23 (kit insert, R&D Systems).

Coupling capture antibody to microspheres. Methods for coupling capture antibody to microspheres and confirmation of coupling are identical to those previously described (18), with the exception that microsphere concentrations were determined using a hemocytometer. For each analyte, the concentration of capture antibody used was 5 μg per 10^6 microspheres (optimal concentration reported in (18)).

Plasma sample dilution and standard diluent. Plasma is a complex matrix, which includes endogenous and exogenous components (e.g., complement, rheumatoid factors, autoantibodies and anticoagulants) that can cause bias in immunoassays and result in inaccurate quantification of the analytes of interest (reviewed in (9, 19)). Bias can also be attributed to differences between the matrices of samples and standards (17), and can affect the measurement of individual cytokines differently (6). To reduce the effect of the sample matrix on cytokine concentrations, plasma samples were diluted 1-in-5 (as per the microsphere manufacturer's recommendations) in phosphate-buffered saline (PBS). Although this dilution reduced the effect of the matrix on cytokine detection, the limits of quantitation (see below) increased by a factor of five (i.e., the 1-in-5 dilution reduced the sensitivity of the assay).

To minimize any differences between sample and standard matrices, we initially diluted standards in PBS + 20% pooled naïve domestic cat sera (Colorado State University specific-pathogen free cat colony; equivalent to 1-in-5 sample dilution). However, background levels of IL-12/23 present in naïve cat sera affected the accuracy of the IL-12/23 standard curve at low concentrations. Alternative sera were tested, including donkey and feline (Jackson

ImmunoResearch Laboratories, West Grove, PA), fetal bovine (Atlanta Biologicals, Lawrenceville, GA), goat (MP Biomedicals, Solon, OH) and mouse (Invitrogen, Carlsbad, CA), as well as human standard diluent (Bio-Rad, Hercules, CA). Of these reagents, PBS + 20% goat sera was the only diluent in which all eight-points of the standard curve for each analyte were detectable and where there was no cross-reactivity of factors in the sera with feline antibodies.

Microsphere immunoassay protocol. The MIA protocol used was similar to the method described in Wood et al. (2011), with the following modifications: standards and spikes were prepared in PBS + 20% goat serum, diluent controls (blanks) consisted of PBS + 20% goat sera, and after the final wash microspheres were re-suspended in 100 µl of 0.5% formaldehyde (37% w/w; Fisher Scientific, Pittsburgh, PA) in assay buffer. Spikes (samples of known concentration) were prepared similar to the standards and used for assay validation. Spikes were also interspersed throughout the plate (i.e., beginning, middle and end) as additional controls during analysis of plasma samples. Approximately 2,500 microspheres per analyte were added to each well and plasma samples were diluted 1-in-5 with PBS. Each experiment included an eight-point standard curve (2-fold dilution series) and four diluent control wells.

Bio-Plex™ 200 (Bio-Rad) maintenance and data analysis (Bio-Plex™ Manager 5.0, Bio-Rad) were identical to the methods previously described (18). Briefly, median fluorescence intensity (MFI) was calculated from ≥100 microspheres per analyte per well. For each analyte, a standard curve was generated and used to calculate cytokine concentrations in spikes and plasma samples. Acceptable standard recovery was 70-130% of the nominal value (Bio-Rad).

Microsphere immunoassay validation. Inter- and intra-assay experiments were conducted as previously described (18). Spike concentrations were: 31, 250 and 1,000 pg/ml for IFNγ; 63, 500 and 2,000 pg/ml for IL-10; and 20, 156 and 625 pg/ml for IL-12/23. Acceptable

accuracy (spike recovery) was 70-130% of the nominal value (Panomics, Santa Clara, CA), and acceptable precision (coefficient of variation (CV)) for the mean spike recovery at each concentration was required to be <20% for the intra-assay and <30% for the inter-assay (personal communication, Bio-Rad) for the method to be considered validated. The lower and upper limits of quantitation (LLOQ and ULOQ, respectively) were defined as the lowest and highest spike concentrations tested that showed acceptable accuracy and precision.

Analysis of cytokines in domestic cat plasma. We quantified cytokine levels in domestic cat plasma collected from experimental co-infection studies with PLV and FIV conducted in 2005 (12) and 2010 (Sprague et al., in preparation) (Figure 1.1). Animals were housed in AAALAC-International accredited facilities and all protocols were reviewed by the Colorado State University Institutional Animal Care and Use Committee prior to initiation. The two virus strains used in these studies were PLV-1695, which is a well-characterized puma lentivirus isolate obtained from a British Columbia mountain lion (16), and FIV-C36 (subsequently referred to as FIV-C), which is a highly-virulent molecular clone of domestic cat FIV that has also been well characterized (3).

Samples selected from the 2005 study were 24 days post-PLV inoculation (or day -4 relative to FIV-C inoculation) and 24 days post-FIV-C inoculation (days 24 and 52 as per (12), respectively). Plasma samples from all cats in the study (n = 20) were tested at these two time-points. At day 24, plasma samples were collected from PLV and sham inoculated cats (n = 10 cats per group). At day 52, plasma samples were collected from PLV, FIV-C, co-infected (i.e., PLV + FIV-C; subsequently referred to as CO) and sham inoculated cats (n = 5 cats per group).

A study with similar timelines and animal groups was conducted in 2010 (Sprague et al., in preparation). Plasma samples from all cats (n = 24) were tested 28 days post-PLV inoculation

(or day 0 relative to FIV-C inoculation) and 28-32 days post-FIV-C inoculation (referred to as days 28 and 56-60 days). At day 28, plasma samples were collected from PLV and sham inoculated cats (n = 12 cats per group). At days 56-60, plasma samples were collected from PLV, FIV-C, CO and sham inoculated cats (n = 6 cats per group).

All plasma samples were stored in 0.5-1 ml aliquots at -80°C from the time of collection until cytokine quantification. The inoculation status of each cat was unknown at the time of testing.

Samples with cytokine concentrations below the lowest standard tested (i.e., extrapolated beyond the standard curve), were assigned a value that was half the concentration of the low standard multiplied by five to account for the 1-in-5 sample dilution. Median cytokine concentrations were compared between PLV and sham inoculated cats (days 24 and 28) using Mann-Whitney tests. Among the four inoculation groups (days 52 and 56-60), median cytokine concentrations were compared using Kruskal-Wallis tests, and if necessary, post-hoc comparisons were made using Dunn's multiple comparison tests (GraphPad Prism 5.0, La Jolla, CA). p-values <0.05 were considered significant. Statistical analysis was not performed if the majority of cytokine measurements fell below the established LLOQ for a given cytokine and/or time-point.

Comparison of cytokine levels in plasma to cytokine mRNA expression. We compared plasma cytokine levels and mRNA cytokine expression of blood cells at two time-points for the animals in the 2005 co-infection study (n = 20). Protein levels were detected by MIA and the mRNA data was previously published by TerWee et al. (2008), as fold-difference relative to sham.

RESULTS

Microsphere immunoassay validation. For both the inter- and intra-assay validation, the mean spike recoveries (accuracy) and percent CVs (precision) for each of the three analytes were within the acceptable ranges (Table 3.1). The validated lower limit of quantitation (LLOQ) and upper limit of quantitation (ULOQ) for each analyte were 31 and 1,000 pg/ml for IFNγ, 63 and 2,000 pg/ml for IL-10, and 20 and 625 pg/ml for IL-12/23. The LLOQ of IL-12/23 determined for plasma was lower than the value reported for the cell culture supernatant MIA (39 pg/ml) in Wood et al. (2011).

Table 3.1. Assay accuracy and precision over a range of analyte concentrations

	Mean % recovery (%CV)		
Spike level	IFNγ	IL-10	IL-12/23
Intra-assay			
High	118 (12)	93 (9)	92 (9)
Medium	104 (11)	94 (9)	101 (6)
Low	83 (16)	106 (18)	106 (10)
Inter-assay			
High	103 (9)	99 (7)	101 (8)
Medium	107 (8)	103 (4)	105 (4)
Low	106 (14)	104 (13)	100 (9)

Cytokines in domestic cat plasma. IL-12/23 concentrations were quantifiable in all plasma samples tested; all concentrations were above the LLOQ (100 pg/ml adjusting for the 1-in-5 sample dilution; Figures 3.1 and 3.2) and only two individual IL-12/23 results were above the validated ULOQ (3,460 pg/ml, Figure 3.1; 3,257 pg/ml Figure 3.2). Plasma concentrations

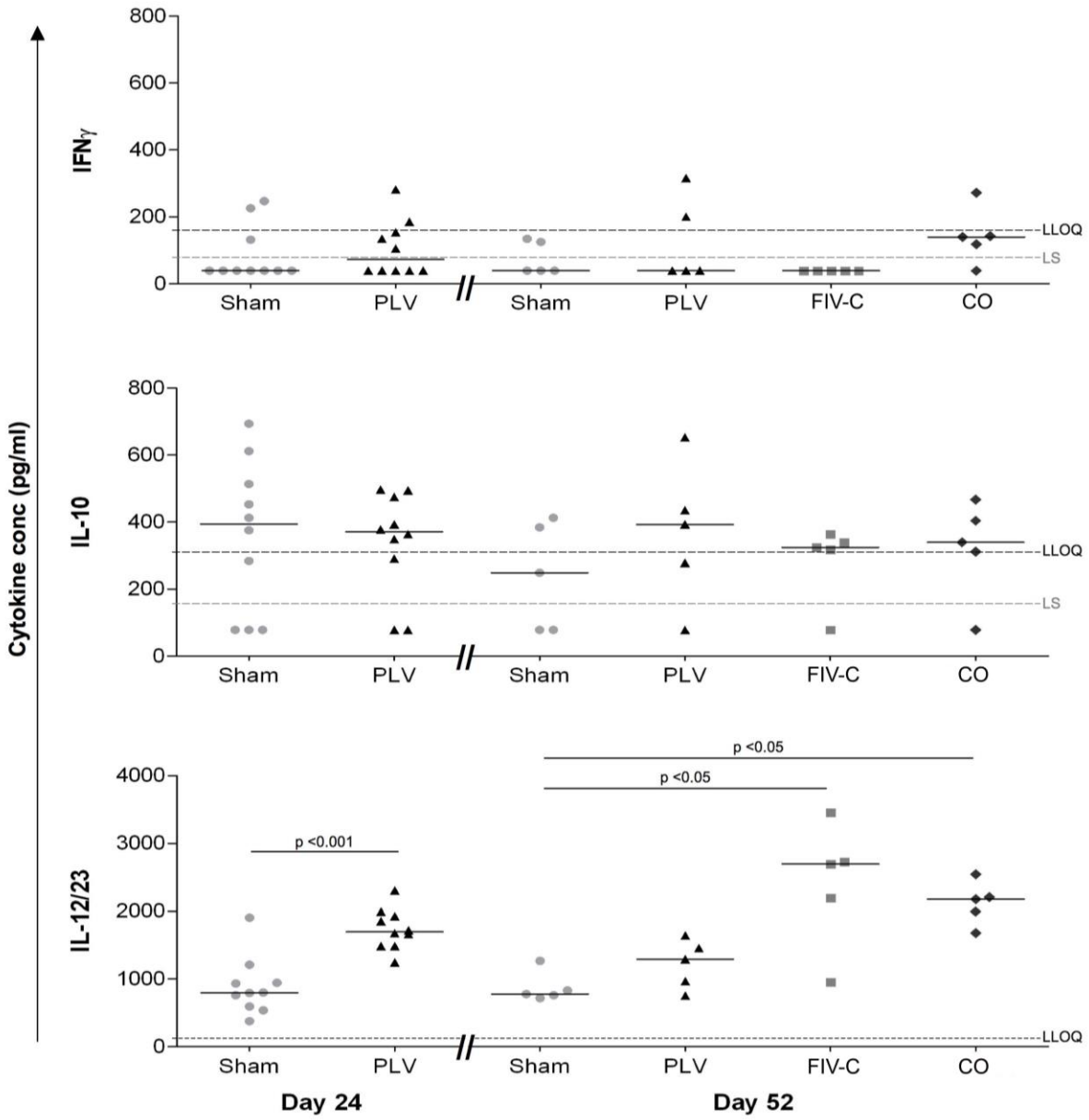

Figure 3.1. Cytokine concentrations from domestic cat plasma samples collected 24 days post-PLV inoculation and 24 days post-FIV-C inoculation (day 52) of the 2005 study. The solid lines indicate the medians for each inoculation group. The dashed lines indicate the concentrations of the lower limit of quantitation (LLOQ) and the lowest standard (LS) tested after accounting for a 1-in-5 sample dilution (i.e., the in-well LLOQ/LS concentrations were one-fifth of those indicated in the figure).

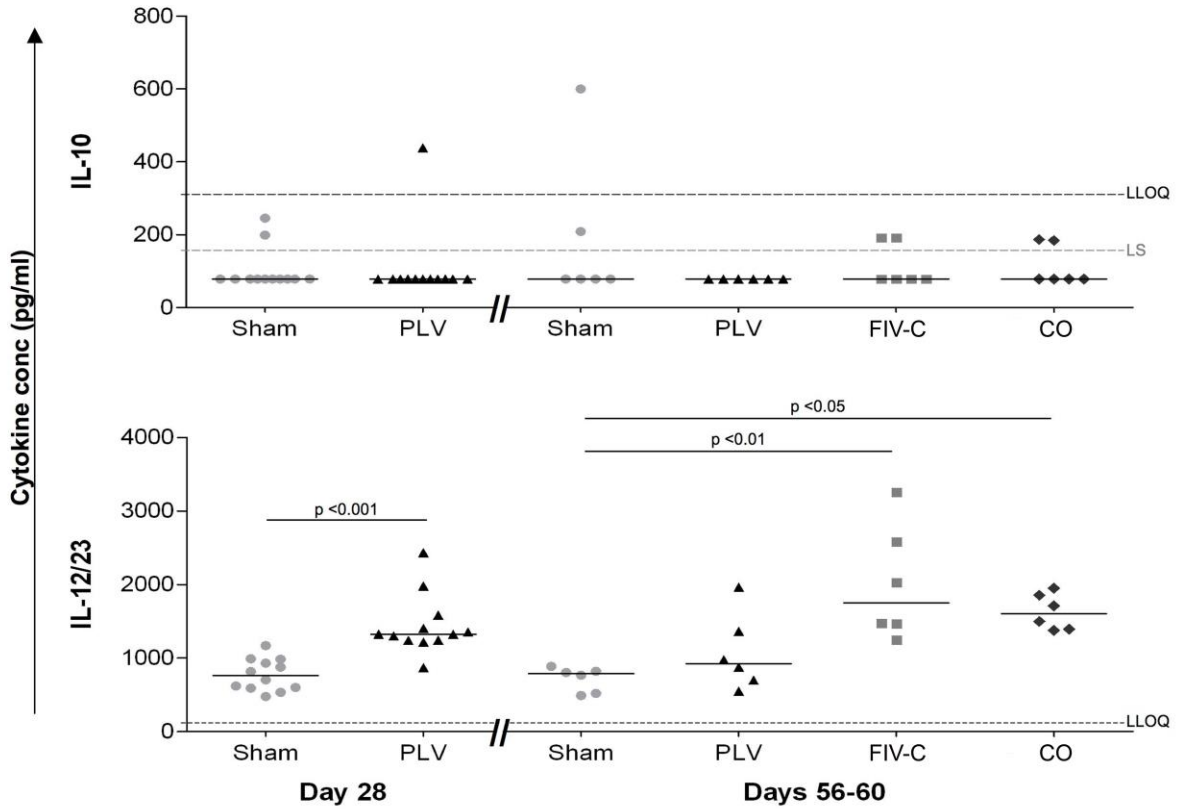

Figure 3.2. Cytokine concentrations from domestic cat plasma samples collected 28 days post-PLV inoculation and 28-32 days post-FIV-C inoculation (days 56-60) of the 2010 study. The solid lines indicate the medians for each inoculation group. The dashed lines indicate the concentrations of the lower limit of quantitation (LLOQ) and the lowest standard (LS) tested after accounting for a 1-in-5 sample dilution (i.e., the in-well LLOQ/LS concentrations were one-fifth of those indicated in the figure).

ranged from 337 to 3,460 pg/ml in the 2005 study, and from 479 to 3,257 pg/ml in the 2010 study. In both studies, the concentration of IL-12/23 was significantly higher in PLV than sham inoculated cats ($p < 0.001$). Additionally, the concentration of IL-12/23 was significantly higher in FIV-C and CO inoculated cats than sham-inoculated cats ($p < 0.05$). There were no significant differences between FIV-C or PLV groups with the CO group at day 52 (Figure 3.1) or days 56-60 (Figure 3.2).

For the majority of samples from the 2005 study, the concentrations of IFNγ, regardless of time and inoculation status, were below the lowest standard tested (Figure 3.1). Only seven of the 40 samples tested had values above the LLOQ (155 pg/ml adjusting for the 1-in-5 sample dilution); the range of these samples was 185 to 316 pg/ml. No values are reported for IFNγ concentrations from the 2010 study because the spikes bracketing the samples had recoveries lower than the acceptable range of 70-130%. Mean recoveries for these spikes ranged from 20-63%. This low recovery was cytokine specific and not an overall issue with the MIA (i.e., the recoveries of IL-10 and IL-12/23 in the same spikes were acceptable). In subsequent experiments, this problem abated when freshly thawed antibodies were used instead of antibodies that had been stored for more than 24 h at 4°C. However, the 2010 samples were not re-analyzed because of sample limitations.

The majority of samples from the 2005 study had IL-10 concentrations above the LLOQ (315 pg/ml adjusting for the 1-in-5 sample dilution; Figure 3.1); the range of these samples was 319 to 693 pg/ml. The concentration of IL-10 was not significantly different between PLV and sham inoculated cats at day 24 ($p = 0.79$) or among the four inoculation groups at day 52 ($p = 0.68$). In the 2010 study, however, the majority of samples had concentrations of IL-10 below the LLOQ (Figure 3.2). Only 2 of the 48 samples had values above the LLOQ; the concentrations of these samples were 438 and 600 pg/ml.

Comparison of cytokine levels in plasma to cytokine mRNA expression. While there were significant increases in IFNγ mRNA in the FIV-C and/or PLV infected groups at day 52 of the 2005 study (12), levels of IFNγ protein in plasma remained low (most below the LLOQ) for all groups (Figure 3.1). Similarly, IL-10 mRNA expression was increased in the FIV-C infected group at day 52 (12), but there was no detectable difference in IL-10 protein in plasma (Figure

3.1). In contrast, IL-12/23 protein levels in plasma were increased in PLV (day 24), and FIV-C and CO (day 52) infected groups (Figure 3.1), without any detectable change in mRNA expression. Comparison of the protein:mRNA ratio for each cytokine (Figure 3.3) demonstrates that IL-12/23 had higher protein abundance relative to the level of mRNA expression than IL-10 and IFNγ.

DISCUSSION

These results demonstrate the successful development and validation of a MIA for the quantification of domestic cat cytokines IFNγ, IL-10 and IL-12/23 in plasma. To our knowledge, this is the first assay developed and validated for quantifying feline cytokines in plasma; the reagents used in the MIA are from commercially available ELISA kits validated for cell culture supernatants. The advantages of developing a MIA for the detection of feline cytokines, e.g., compared to developing ELISAs, is that multiple analytes can be quantified simultaneously, and thus less time and sample volume are required to conduct comparable experiments.

In both the 2005 and 2010 co-infection study, we detected a significant increase in circulating IL-12/23 during acute viral infection. The concentration of IL-12/23 was significantly higher in PLV than sham inoculated cats at 24 and 28 days post-inoculation, but by day 52 and days 56-60 IL-12/23 concentrations were not statistically different (Figures 3.1 and 3.2, respectively). This transient elevation of IL-12/23 likely coincides with a decrease in viral load; in the 2005 study, there was a decrease in PLV proviral load in PBMCs between days 24 and 52 (12). Plasma concentrations of IL-12/23 were significantly higher in cats acutely infected with FIV-C; the concentrations of IL-12/23 were higher in FIV-C and CO inoculated cats compared to sham cats at 24 and 28-32 days post-inoculation (Figures 3.1 and 3.2, respectively). The elevation of IL-12/23 with FIV-C and CO inoculated cats is likely because of similar FIV-C

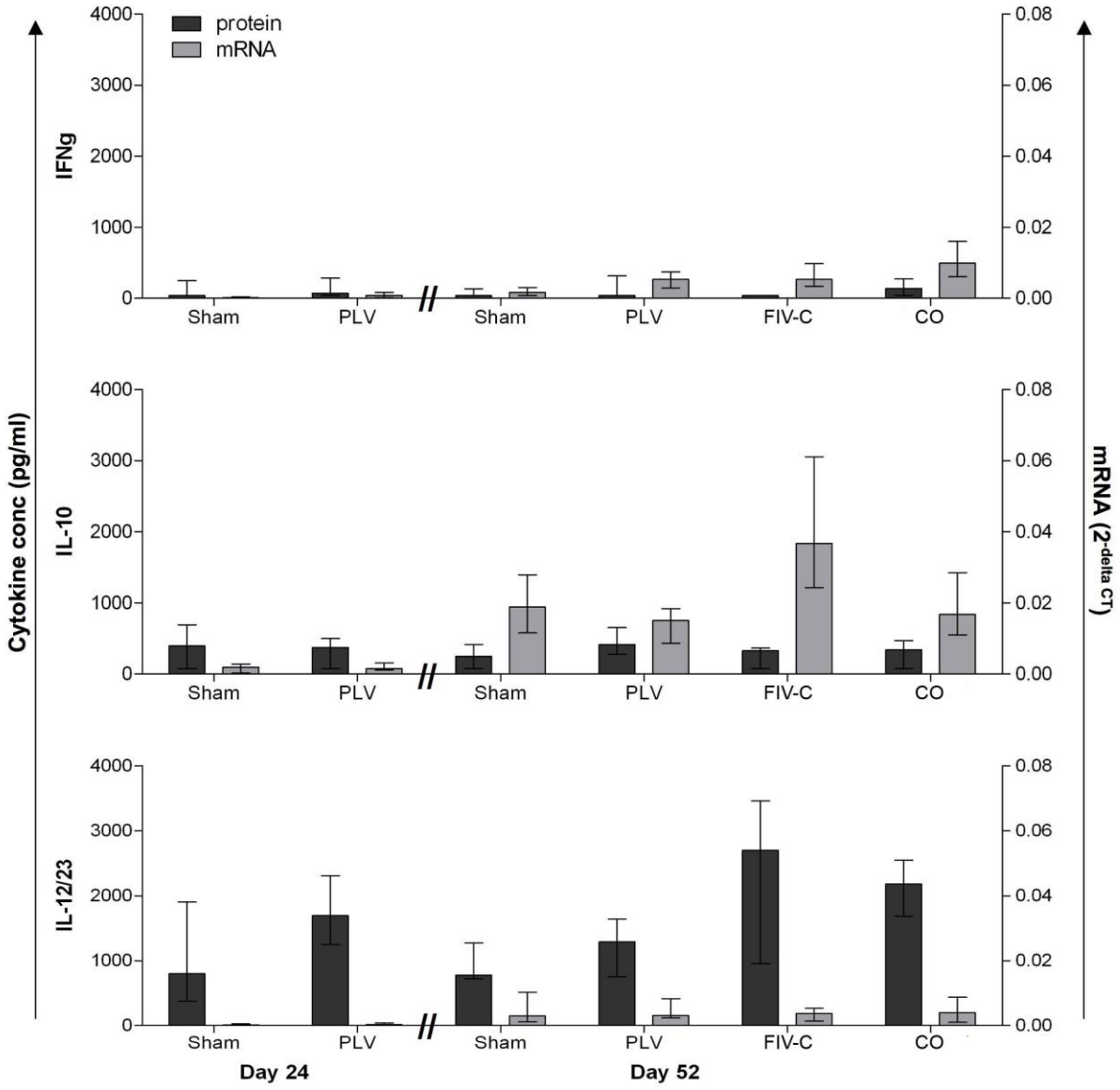

Figure 3.3. Plasma cytokine concentrations and PBMC cytokine mRNA expression from domestic cat samples collected 24 days post-PLV inoculation and 24 days post-FIV-C inoculation (day 52) of the 2005 study. The solid bars indicate the median (and range) for each inoculation group.

viral loads. The observed increase in IL-12 and/or IL-23 during early infection is likely produced by macrophages and dendritic cells for T-helper 1 (Th1) and Th17 $CD4^+$ T cell

development, respectively (8). The consistency of these results between two independently conducted studies, separated by several years, is a significant finding in an outbred species.

In contrast to IL-12/23, the majority of IFNγ and IL-10 results were below the LLOQ. It is possible that there are differences in these cytokines during PLV and/or FIV-C infection; however, at the selected time-points, these cytokines were too low to detect with the validated MIA. Alternatively, the lack of detection could be because these cytokines are not elevated in the periphery during the acute stage of infection and/or these cytokines were affected by the long-term storage of the samples.

To investigated whether the 1-in-5 plasma dilution may have contributed to the lack of IFNγ and IL-10 detection, we compared cytokine concentrations in 11 plasma samples collected from FIV and/or PLV infected cats diluted 1-in-2 and 1-in-5 (some samples were part of a different FIV-infection study). Despite the lower dilution, IFNγ and IL-10 were quantifiable in only a few more samples (three additional samples were quantifiable for both IFNγ and IL-10). Additionally, to investigate whether IFNγ and IL-10 were elevated at earlier time-points, we tested plasma samples collected a) 3 days post-PLV and 3 days post-FIV-C from the 2005 co-infection study using a 1-in-5 dilution, and b) 14 days post-PLV and 14 days post-FIV-C from the 2010 co-infection study using a 1-in-2 dilution. At 3 days post-infection, only 2 of the 40 samples had values above the LLOQ for both IFNγ and IL-10 (i.e., two separate cats per analyte). At 14 days post-infection, the majority of samples (35 of 48) had values below the LLOQ for both IFNγ and IL-10. No definitive conclusions could be made from these experiments because the samples tested were from two different time points and studies, and were tested at different dilutions. No additional sample dilution experiments were conducted,

but these results showed that IFNγ and IL-10 were only detected at very low concentrations in the majority of the samples tested.

The lack of agreement between the plasma cytokine data reported here and the mRNA data reported in TerWee et al. (2008) supports the premise that mRNA expression does not necessarily predict protein abundance. Comparison of circulating protein levels relative to mRNA transcript demonstrates that IL-10 and IFNγ tend to have a significantly lower protein:mRNA ratio than IL-12/23. The observed differences could be attributed to various factors associated with post-transcriptional regulation or protein detection (6), and/or the effect of long-term storage of samples.

In summary, the MIA described here could be used to evaluate domestic cat cytokine responses elicited by a variety of diseases. Additionally, the assay could be expanded to include other cytokines for which reagents are commercially available, including IL-1β, IL-2, IL-4, IL-6 and/or tumor necrosis factor α.

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CHAPTER 4: MICROSPHERE IMMUNOASSAYS FOR THE QUANTIFICATION OF DOMESTIC CAT ANTIBODIES AND THE DETECTION OF ANTIBODIES AGAINST FELINE IMMUNODEFICIENCY VIRUS IN PLASMA[3]

INTRODUCTION

Microsphere immunoassay (MIA) technology allows for the simultaneous detection of multiple analytes (e.g., cytokines, chemokines or antibodies; reviewed in (62)) within biological samples. Multiple sets of microspheres, each with a unique ratio of internal dyes and conjugated with a different capture reagent (e.g., antibodies or antigen), are combined and incubated with an aliquot of sample. The detection or quantification of each analyte is based on the fluorescence intensity of the detection antibody reporter dye. The advantages of MIAs, compared to traditional immunoassays (e.g., enzyme-linked immunosorbent assays (ELISAs)), are that less time and sample volume are required. MIAs have been developed for the detection of antibodies to various animal and human pathogens, including avian influenza (63), foot-and-mouth disease virus (14), human immunodeficiency virus (HIV; (4, 59)), *Plasmodium falciparum* (27), *Toxoplasma gondii* (38), West Nile virus (66), and a panel of five select agents (*Bacillus anthracis, Yersinia pestis, Francisella tularensis,* ricin toxin and staphylococcal enterotoxin B; (6)). Assays, such as these, can be used for screening or diagnostic purposes (6), or investigating antibody kinetics (63).

Feline immunodeficiency virus (FIV) is a naturally occurring lentivirus of the domestic cat (*Felis catus*) that is similar to HIV in terms of viral structure, transmission, target cells and disease progression, including the hallmark decline of peripheral $CD4^+$ T-cells (25, 44, 69).

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Despite a vigorous immune response, FIV and HIV infections are life-long and infected individuals succumb to opportunist infections. Given the high degree of similarity between these two viruses, the domestic cat is an appropriate animal model for evaluating the immune response associated with lentiviral infections.

Domestic cats develop antibodies to various FIV proteins, including core proteins, such as capsid (CA) and matrix, as well as the two membrane proteins, surface (SU) and transmembrane (TM) (24, 29, 34, 42). Antibodies to these viral proteins are generally first detected within weeks post-infection (9, 10, 24, 29, 31, 46, 47) and increase during the early stage of infection (10, 31, 46, 47). The level and kinetics of the antibody response varies depending on viral strain and/or inoculation route, as well as among individual cats (9, 10, 31, 47). Although the majority of these studies have focused on the IgG FIV-specific antibody response, IgM and IgA FIV-specific antibodies are also detected in plasma/sera (9, 29). In addition, FIV-infected domestic cats exhibit elevated levels of gamma-globulins (28, 68), as a result of elevated levels of IgG antibodies (1, 29). The elevated IgG levels are attributed to polyclonal B-cell activation, rather than entirely virus-specific, as antibodies generated after FIV infection are specific for a variety of non-viral and self antigens (26). Although the cause of the polyclonal activation with FIV is unknown, with HIV the SU protein (gp120) is able to induce a $CD4^+$ T-cell independent B-cell response (32); gp120 can activate a subset of B-cells and trigger antibody class-switching through an innate pathway involving the mannose C-type lectin receptor and B cell-activating factor of the tumor necrosis factor family (BAFF) (32). Polyclonal B-cell activation is not unique to lentiviruses; many viruses, bacteria and parasites can trigger a polyclonal B-cell response (reviewed in (41)). Despite the number of studies that have evaluated the antibody response of FIV-infected domestic cats (1, 9, 10, 24, 26, 29, 31, 34, 42, 46, 47),

there are limited data on the development of total IgG and IgA, and IgG and IgA FIV-specific antibodies during the early stage of infection.

Here we describe the development and validation of domestic cat-specific MIAs for a) the quantification of total IgG and IgA levels in plasma, and b) the detection IgG and IgA antibodies to feline CD134 (the primary cell receptor for FIV (16, 49)), and FIV CA and SU proteins in plasma (assays subsequently referred to as recombinant protein (rProtein)). The rProteins included in the MIAs were selected because previous FIV studies have documented an antibody response to these proteins (9, 10, 24, 29, 31, 34, 42, 46, 47). Domestic cats develop a robust α-CA antibody response (e.g., detectable at 1:3,200 sample dilution (9, 23)) within weeks post-infection (9, 42). Although α-CA antibodies are non-neutralizing, these antibodies may play a role in disease progression. For example, the presence of α-Gag/CA antibodies correlates with delayed HIV disease progression (7, 12, 13, 40, 64). Antibodies to SU protein are detectable within weeks post-infection (31), and antibodies to specific SU epitopes are capable of neutralizing the virus (e.g., V4 and V5 regions (48, 50, 51, 65)). Although neutralizing antibodies develop slowly over time (e.g., years post-FIV infection (35)), passive immunization experiments with plasma from cats 3 weeks post-infection (8) or sera from cats 9-10 months post-infection (33) were able to prevent FIV infection. Domestic cats develop α-CD134 antibodies that bind to a cryptic epitope on the CD134 receptor, which is only exposed once the virus SU protein is bound (18), and this binding results in the displacement of SU from the cell receptor (31). In a recent study, it was noted that FIV-infected cats that developed autoantibodies to CD134 tended to have lower viral loads and less virulent disease than cats that did not develop anti-receptor antibodies (31).

The utility of the four MIAs described here were demonstrated by testing plasma samples collected from domestic cats experimentally infected with various FIV strains. Plasma samples selected for testing were from a) a study evaluating impacts of co-infection with pathogenic and apathogenic FIVs (55); and, b) a study evaluating parental and chimeric FIVs of varying pathogenicity (57). These two studies were selected because significant information about immunopathology and viral load has already been determined. The goal of this study was to determine whether the kinetics and/or level of the antibody response is related to virus strain/clinical outcome, and to estimate the percent of IgG antibodies that are CA- or SU-specific.

Briefly, in the co-infection study, domestic cats were inoculated intravenously (IV) with PLV-1695 (subsequently referred to as PLV) and/or FIV-C36 (clade C virus; subsequently referred to as FIV-C) (55). PLV is a well-characterized puma lentivirus isolate obtained from a British Columbia mountain lion (60), and FIV-C is a highly-virulent molecular clone of domestic cat FIV that has been well characterized (19). In this study, peripheral $CD4^+$ T-cells in FIV-C cats decreased after inoculation and remained low compared to their baseline values for the duration of the study. In contrast, peripheral $CD4^+$ T-cells in co-infected (CO) cats (i.e., inoculated with PLV 28 days before FIV-C inoculation) decreased 9 days after FIV-C inoculation and then rebounded to levels similar to their baseline for the duration of the study. Of note, there was no difference in the plasma viral loads between FIV-C and CO cats at the time-points tested. Although data from this study suggested that the non-adaptive immune response contributed to the observed $CD4^+$ T-cell maintenance, the antibody response was only examined at a single time-point. At 31 days post-FIV-C inoculation, there was no difference in serum neutralizing antibody titers among the groups (55).

In the FIV-chimera study, domestic cats were inoculated IV with *in vivo* passaged FIV-C, FIV-PPR (clade A virus; subsequently referred to as FIV-A) or a chimera virus FIV-PCenv (subsequently referred as FIV-A/C) (57). The FIV-A/C virus has the FIV-A backbone with FIV-C accessory and envelope genes (i.e., FIV-C *vif, orfA, env* and part of the *rev* response element or 3' elements) (20). The parental viruses, FIV-C and FIV-A, differ in terms of virulence and tropism. FIV-A is neurotropic (22) and causes less severe disease in the periphery than FIV-C (e.g., lower viral loads, and less severe decreases in $CD4^+$ T-cells and neutrophils (43)). The purpose for constructing the chimera virus and testing *in vivo* was to determine whether the envelope region contributed to the increased pathogenicity of the FIV-C virus (21, 43). The results of the virus-passaged *in vivo* study indicated that, in general, the FIV-C cats had the highest viral and proviral loads, followed by the FIV-A/C and then FIV-A infected cats (57), suggesting that pathogenicity is linked to the 3' viral elements. To date, the antibody response has not been evaluated for any of the chimera *in vivo* studies (20, 57).

MATERIALS AND METHODS

Antibodies, standards and microspheres. Reagents and microspheres used for the development of the four MIAs are summarized in Table 4.1. MagPlex-C microspheres were obtained from Bio-Rad (Hercules, CA). Cat reference serum was used for total IgG and IgA standard curves; undiluted reference serum contains 5.4 mg/ml of IgG and 0.22 mg/ml of IgA (Bethyl Laboratories, Montgomery, TX). The concentration of albumin in the reference serum is not reported; the detection of albumin served as a control to confirm that the standard or sample was loaded into each well. Anti-cat IgG, IgA and albumin antibodies (Bethyl Laboratories) were coupled to the microspheres as capture reagents, as well as conjugated with phycoerythrin (PE; EasyLink R-Phycoerythrin conjugation kits, Abcam, Cambridge, MA) to use as detection

Table 4.1. Components used for the development of the four domestic cat MIAs.

Assay	Total IgG	rProtein IgG	Total IgA	rProtein IgA
Analytes detected	IgG Albumin	α-CA IgG α-SU_{FIV-A} IgG α-SU_{FIV-C} IgG α-CD134 IgG α-Fc IgG α-FeLV IgG Albumin	IgA Albumin	α-CA IgA α-SU_{FIV-A} IgA α-SU_{FIV-C} IgA α-CD134 IgA α-Fc IgA α-FeLV IgA Albumin
Microspheres				
Microspheres/analyte per well	2,500	2,500	2,500	2,500
Capture reagent (region)[a]	α-cat IgG Ab[b] (77) α-cat albumin Ab[b] (13)	CA (35) SU_{FIV-A} (65) SU_{FIV-C} (43) CD134 (54) Fc (29) FeLV (28) α-cat albumin Ab[b] (13)	α-cat IgA Ab[b] (63) α-cat albumin Ab[b] (13)	CA (35) SU_{FIV-A} (65) SU_{FIV-C} (43) CD134 (54) Fc (29) FeLV (28) α-cat albumin Ab[b] (13)
Standards and plasma samples				
Standard[b] range (ng/ml)	7.8-1,000	-	3.9-500	-
Plasma dilution	1:200,000	1:50	1:10,000	1:50
Volume per well (µl)	50	50	50	50
Incubation (min)	60	60	60	60
Primary Ab				
Antibody (ng/ml)	-	α-cat IgG mAb[d] (400)	-	-
Volume per well (µl)	-	50	-	-
Incubation (min)	-	30	-	-
Detection Ab				
Antibodies (ng/ml)	PE α-cat IgG[c] (400) PE α-cat albumin[c] (200)	PE α-mouse IgG[e] (400) PE α-cat albumin[c] (200)	PE α-cat IgA[c] (400) PE α-cat albumin[c] (200)	PE α-cat IgA[c] (400) PE α-cat albumin[c] (200)
Volume per well (µl)	100	50	100	50
Incubation (min)	30	30	30	30

[a] MagPlex-C microsphere region was assigned by the manufacturer; the region is based on the ratio of two internal dyes
[b] Reagents from Bethyl Laboratories
[c] Unconjugated antibodies (Bethyl Laboratories) were conjugated with phycoerythrin (PE) using the EasyLink R-Phycoerythrin conjugation kits (Abcam)
[d] Monoclonal antibody (GPB2-2B1) from Custom Monoclonals International
[e] Antibody from R&D Systems

antibodies. A primary antibody was used for the detection of α-rProtein IgG antibodies because the α-IgG detection antibody cross-reacts with the Fc-tag on the recombinant proteins; the primary antibody was a cat-specific α-IgG monoclonal antibody (Custom Monoclonals International, Sacramento, CA). The microsphere with Fc (Sino Biological Inc., Beijing, China) attached served as a control for the Fc-tag on the recombinant proteins, and the microsphere with feline leukemia virus (FeLV; FL74 provided by J. Elder) attached served as a non-FIV viral control.

Recombinant FIV-C CA protein. The sequence encoding the CA protein (1,038 to 1,703; 666 nucleotides) was amplified from a molecular clone of FIV-C (19) using standard PCR protocols. The primers, which included restriction sites (underlined) for directional insertion into the plasmid, as well as start (italicized) and stop codons (wave underlined), were as follows: Fwd (BamHI) 5'-CGCGGATCC*ATG*CCTATCCAAATAGTAAATGGAGCAC-3' and Rev (EcoRI) 5'-CCGGAATTCCTACTATAATTGCATTTTGTATGCCGGTG-3'. The pGEX-2T plasmid (GE Healthcare Life Sciences, Piscataway, NJ; provided by J. Rovnak) used for protein expression includes a glutathione S-transferase (GST) gene upstream of the insertion site, a thrombin cleavage site between the GST and insertion site, and a gene for ampicillin resistance. Purified plasmid and PCR product were digested with BamHI and EcoRI restriction enzymes, and ligated with T4 DNA ligase according to manufacturer recommendations (New England BioLabs, Ipswich, MA). The plasmid was propagated in One Shot® TOP10 Chemically Competent *E. coli* (Life Technologies, Grand Island, NY). Purified plasmid was sequenced (Proteomic and Metabolomics Facility, Colorado State University) to confirm capsid insertion using the following primers: Fwd 5'-GGGCTGGCAAGCCACGTTTGGTG-3' and Rev 5'-CCGGGAGCTGCATGTGTCAGAGG-3'.

Chemically competent BL21 *E. coli* (provided by J. Rovnak) were used for protein expression. The transformed *E. coli* were grown in 1 L of broth (LB broth + 50 μg/ml ampicillin; 140 rpm at 37°C); the cells were induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside when the OD_{600} reached 0.6 and incubated for an additional two hours. Pelleted bacteria (10,000 x g for 10 min at 4°C) were lysed with BugBuster® Protein Extraction Reagent according to manufacturer recommendations (EMD Chemicals, Inc., San Diego, CA). The GST-tag was removed from the CA protein using thrombin. GST and thrombin were removed from solution using glutathione sepharose 4B beads and benzamidine sepharose 4 fast flow beads, respectively, according to manufacturer recommendations (GE Healthcare Life Sciences). The presence of purified CA protein was confirmed by SDS-PAGE and by immunoblot analysis (band ~26 kDa). The protein storage buffer was exchanged to PBS by dialysis, and the protein concentration was determined using a BCA protein assay kit (Thermo Scientific, Waltham, MA) before storage at -80°C.

Recombinant surface proteins and CD134. Recombinant SU_{C36}-Fc, SU_{PPR}-Fc and CD134-Fc (subsequently referred to as $SU_{FIV\text{-}C}$, $SU_{FIV\text{-}A}$ and CD134; provided by J. Elder) were expressed and purified from CHO cells, as previously described (15, 17). The Fc tag is the Fc domain of human IgG1(15, 17); for $SU_{FIV\text{-}C}$ the Fc tag is N-terminus, whereas for $SU_{FIV\text{-}A}$ and CD134 the Fc tag is C-terminus. [Note: Experiments were conducted to express FIV-C SU and TM proteins in both baculovirus-insect cell and *E. coli* expression systems. However, as the initial expression experiments were unsuccessful and time was limited, the rProteins provided by J. Elder were used in these assays. See appendix for details.]

Coupling of capture reagents to microspheres. Magnetic microspheres were conjugated with capture antibodies, recombinant proteins or whole virus (Table 4.1) as

previously described (67), with the exception that microsphere concentrations were determined using a hemocytometer. To confirm protein coupling, microspheres (5,000 per well) were incubated with primary antibodies and/or PE-conjugated detection antibodies as follows: PE α-goat antibody (R&D Systems, Minneapolis, MN) for IgG, IgA and albumin microspheres; FIV^+ plasma and PE α-cat IgG antibody for CA microspheres; pooled α-FIV SU monoclonal antibodies (Custom Monoclonals International) and PE α-mouse IgG antibody (R&D Systems) for SU_{FIV-A} and SU_{FIV-C} microspheres; α-human IgG Fc (Sino Biological Inc.) and PE α-mouse IgG antibody for CD134 and Fc microspheres; and A2 mouse ascites (provided by C. Mathiason) and PE α-mouse IgG antibody for FeLV microspheres. A median fluorescence intensity (MFI) of >2,000 indicated successful coupling (Bio-Rad).

Assay development for total IgG and IgA. Assay development and optimization for the total IgG and IgA MIAs were conducted in the following order: (i) optimization of capture antibody concentration, (ii) optimization of detection antibody concentration, (iii) determination of standard range and sample dilution, and (iv) comparison of IgG and IgA standard curve MFI alone or multiplexed with albumin. Capture antibody concentrations tested were 1, 2.5, 5, 7.5, 10 and 15 μg per 10^6 microspheres. Detection antibody concentrations tested were 200, 400, 600 and 833 ng/ml. The standard curve range and sample dilution were optimized simultaneously; plasma samples from FIV-infected and uninfected domestic cats were tested at different dilutions with various standard curve ranges to ensure that at a specific dilution the majority of sample concentrations were within the standard range. Sample dilution and standard ranges tested were initially based on those reported for quantitative ELISAs (11). To determine whether the MFI of IgG or IgA standard curves were affected by multiplexing with albumin, single and multiplex

standard curves were compared. In each of these experiments, approximately 2,500 microspheres per analyte were added to each well.

Total IgG and IgA MIA protocols. The protocols used for total IgG and IgA MIAs were similar to the method described previously (67), with the following modifications: standards, samples and spikes were diluted in assay buffer (PBS, 1% BSA, pH 7.4; Sigma-Aldrich, St. Louis, MO); diluent controls (blanks) consisted of assay buffer; diluent controls, samples and standards were incubated for 60 min; detection antibody volumes were 100 μl; and after the final wash microspheres were re-suspended in 100 μl of 0.5% formaldehyde (37%, w/w; Fisher Scientific, Pittsburgh, PA) in assay buffer. The modifications to incubation time and volume were based on a protocol for a washed serological assay using magnetic microspheres (Luminex Corporation, Austin, TX). Each experiment included an eight-point standard curve (2-fold dilution series) and four diluent control wells. All samples and standards were run in duplicate. Reagent concentrations, volumes and incubation times are summarized in Table 4.1. Bio-Plex™ 200 (Bio-Rad) maintenance and data analysis (Bio-Plex™ Manager 5.0, Bio-Rad) were identical to the methods previously described (67). MFI was calculated from ≥100 microspheres per analyte per well. The standard curves were used to calculate antibody concentrations in plasma samples. Acceptable standard recovery was 70–130% of the nominal value (Bio-Rad).

Validation total IgG and IgA protocols. Intra- and inter-assay experiments were conducted to determine both the accuracy and precision of the total IgG and total IgA MIAs. These experiments were similar to the method previously described (67). Briefly, spiked samples (samples of known concentration) were prepared similar to the standards, and were run in five separate inter-assay experiments (2 wells at each spike concentration) and one intra-assay

experiment (10 wells at each spike concentration). Spike concentrations were 16, 125 and 500 ng/ml for IgG, and 8, 63 and 250 ng/ml for IgA. Acceptable accuracy (spike recovery) was 70-130% of the nominal value (Panomics, Santa Clara, CA), and acceptable precision (coefficient of variation (CV)) for the mean spike recovery at each concentration was required to be <20% for the intra-assay and <30% for the inter-assay (personal communication, Bio-Rad) for the method to be considered validated.

Assay development for rProtein MIAs. Assay development and optimization for the rProtein MIAs were conducted in the following order: (i) optimization of capture reagent concentration, (ii) comparison of FIV and naïve plasma sample MFI between single and multiplexed microspheres, (iii) establishment of FIV-C and naïve plasma pools, and (iv) determination of sample dilution. Concentrations of capture reagent tested were 0.5, 1, 2.5, 5, 7.5 and 10 μg per 10^6 microspheres for all analytes except the two SU proteins. Because stocks of the SU proteins were limited, the optimal concentration found for the other analytes was used for SU coupling (i.e., 5 μg per 10^6 microspheres). The detection antibody concentrations used were the optimal concentrations of the total IgG and IgA MIAs. To determine whether analyte MFIs were affected by multiplexing, FIV-C and naïve plasma samples were tested with single and multiplexed microspheres. Since standards are not available for the analytes in the rProtein MIAs, FIV-C and naïve plasma pools were established and used in each experiment as assay controls. Plasma samples from four FIV-C cats and four naïve cats were tested individually with both the IgG and IgA rProtein MIAs. Samples with high MFI for CA, $SU_{FIV\text{-}A}$, $SU_{FIV\text{-}C}$ and CD134 were included in the positive pool, and samples with low MFI for CA, $SU_{FIV\text{-}A}$, $SU_{FIV\text{-}A}$, CD134, Fc and FeLV were included in the naïve pool. None of the FIV-C plasma samples had high MFI values for IgA antibodies against CD134. Separate reference pools were established

for the IgG and IgA assays. Plasma samples used during steps (i) to (iii) were diluted 1:50 in assay buffer; this dilution was chosen as it is a dilution used to detect FIV antibodies by immunoblot assay (5). Samples dilutions tested were 1:25, 1:50, 1:100, 1:500 and 1:1,000 for detecting α-rProtein IgG antibodies, and 1:5, 1:10, 1:25 and 1:50 for detecting α-rProtein IgA antibodies. Because there was not an ideal dilution for all analytes (i.e., at low dilutions some analytes were maximized, whereas at high dilutions some analytes were undetectable), we standardized the assay by using a 1:50 dilution for both the IgG and IgA rProtein MIAs. In each of these experiments, approximately 2,500 microspheres per analyte were added to each well.

rProtein IgG and IgA MIA protocols. The protocols used for the rProtein MIAs were similar to the method described previously (67), with the following modifications: samples were diluted in assay buffer; diluent controls (blanks) consisted of assay buffer; diluent controls and samples were incubated for 60 min; and after the final wash, microspheres were re-suspended in 100 μl of 0.5% formaldehyde in assay buffer. Each experiment included FIV-C and naïve reference samples, and four diluent control wells. All samples and reference pools were run in duplicate. Reagent concentrations, volumes and incubation times are summarized in Table 4.1. Bio-Plex™ 200 maintenance was identical to the method previously described (67). The median fluorescence intensity (MFI) was calculated from ≥100 microspheres per analyte per well (Bio-Plex™ Manager 5.0), and the MFI per sample was used for data analysis.

Validation rProtein IgG and IgA protocols. Intra- and inter-assay experiments were conducted to determine the precision of the rProtein MIAs for CA, $SU_{FIV\text{-}A}$, $SU_{FIV\text{-}C}$ and CD134. These experiments were similar to the method previously described (67), except that FIV-C and naïve reference pools were used in place of standards. Reference samples were diluted 1:50 with assay buffer, and were run in five separate inter-assay experiments (2 wells per sample) and one

intra-assay experiment (10 wells per sample). Two separate FIV-C reference pools (early and late stage of infection) were used to validate the IgG assay because at early time-points α-CD134 IgG antibodies are not detectable and at later time-points the MFI for CA and SU_{FIV-C} were maximized. Acceptable precision (%CV) for the mean MFI of each reference was <20% for the intra-assay and <30% for the inter-assay (personal communication, Bio-Rad). The rProtein assays were not validated for Fc and FeLV, as these microspheres served as controls.

Co-infection study. Antibody levels were measured in domestic cat plasma collected from an experimental co-infection study with PLV and FIV-C (55) (Figure 1.1). Animals (n = 20; 5 cats per group) were housed in AAALAC-International accredited facilities, and all protocols were reviewed by the Colorado State University Institutional Animal Care and Use Committee prior to initiation. EDTA plasma samples were collected in 0.5-1 ml aliquots and stored at -80°C. Data for one sham cat is not included in the results presented here because of an underlying condition that affected the antibody response.

Plasma samples from -4, 9, 17, 24 and 66 days post-FIV-C inoculation were tested with each of the four antibody MIAs (24, 37, 45, 52 and 94 days post-PLV inoculation, as referred to in TerWee et al. 2008). These time-points were selected to a) determine whether antibodies to PLV cross-react with FIV-C viral proteins (i.e., antibodies at day -4), and b) determine whether CO cats developed a higher antibody response than FIV-C cats over time. Plasma samples for each cat were available for testing at nearly all of the time-points selected (89 of 95 samples). The SU_{FIV-A} microsphere was not included in the rProtein assays because only the FIV-C virus was used in this study. The inoculation status of each cat was unknown at the time of testing.

Chimera study. Antibody levels were measured in domestic cat plasma collected from an experimental infection study involving parental FIVs of high (FIV-C) or low (FIV-A)

pathogenicity, as well as a chimera (FIV-A/C) with intermediate pathogenicity (57). Housing and approval for animals (n = 20; 5 cats per group) were similar to those for the co-infection study. EDTA plasma samples were collected in 0.5-1 ml aliquots and stored at -80°C. Two cats (one FIV-C and one FIV-A) diagnosed with allergic dermatitis unrelated to the study protocol were euthanized before the conclusion of the study (57), and are not included in the data presented here.

Plasma samples from days 7, 14, 21, 28, 35, 67 and 326 post-FIV inoculation were tested in each of the four antibody MIAs. These time-points were selected to a) determine whether the antibody levels differed among the virus-infected cats over time, and b) determine whether antibody levels correlated with virus strains/clinical outcome. All plasma samples (n = 18) were available for testing from each of the selected time-points. The inoculation status of each cat was unknown at the time of testing.

In preliminary experiments, it was noted that antibodies generated against one virus bound to the SU protein of the other virus (e.g., antibodies of a FIV-C infected cat bound to SU_{FIV-A}). Nonetheless, the presence of these cross-reactive antibodies did not drastically affect the MFI of antibodies bound to the matching SU protein (e.g., antibodies of a FIV-C infected cat bound to SU_{FIV-C}). Thus, both microspheres were included in the assays; however, only the virus matched SU protein data are reported (i.e., SU_{FIV-C} data for FIV-C and FIV-A/C cats, and SU_{FIV-A} data for FIV-A cats). The values reported for the sham cats are the average MFI of SU_{FIV-A} and SU_{FIV-C}.

Estimating percent FIV-specific IgG antibodies. To estimate the proportion of the total IgG response that was FIV-specific, the MFI for total IgG (1:200,000 dilution) was compared to the MFIs for CA and SU of the same sample serially diluted (1:50 to

1:10,000/1:20,000). The dilution at which the CA or SU MFI was closest to the total IgG MFI was used for the percent estimation (e.g., 1:200,000 divided by 1:5,000 equals 0.025 or 2.5%). These CA and SU percentages were then expressed as a percentage of the increase in the total IgG response for each cat (i.e., relative to the average IgG response of the sham cats at the same time-point). Thus, the estimates reported are the percentages of CA- or SU-specific IgG antibodies relative to the increase in IgG antibodies due to infection. These experiments and calculations were conducted for FIV-C and CO cats on day 66 of the co-infection study, and for FIV-C, FIV-A/C and FIV-A cats on day 326 of the chimera study.

Statistical analyses. Repeated-measures ANOVAs were used to examine antibody responses over time among the treatment groups in the co-infection and chimera studies. Of particular interest were the time-by-treatment effects, as these indicate differences in antibody kinetics (the difference in responses among the groups over time). Prior to analysis, these data were examined and normalized by $\log_{10}$-transformation. When significant time-by-treatment effects were noted, pairwise repeated-measures ANOVAs were conducted to determine which treatments differed from one another over time. Differences in the average level of the antibody response (average across all time-points) were also noted between pairs. One-way ANOVAs and pairwise comparisons were used to examine the antibody response among the treatment groups at a select set of single time-points, such as when the antibody levels were only elevated at particular time-point(s). Analyses were conducted in R (http://www.r-project.org/), and p-values <0.05 were considered significant.

RESULTS

Microsphere assay conditions. Optimized assay conditions are summarized in Table 4.1. The optimal concentration for capture reagents was 5 μg per 10^6 microspheres. Each

analyte had only minor differences between single and multiplex standard curve MFI or between single and multiplex reference sample MFI, indicating that the analytes could be evaluated in the multiplexed format.

Microsphere immunoassay validations. The intra- and inter-assay mean spike recoveries (accuracy) and percent CVs (precision) were within the acceptable ranges for both the total IgG and total IgA MIAs (Table 4.2). For the rProtein MIAs, the intra- and inter-assay percent CVs were within the acceptable range for CA, $SU_{FIV\text{-}A}$, $SU_{FIV\text{-}C}$ and CD134 (Table 4.3). The values for IgG antibodies to Fc and FeLV in the two FIV reference pools ranged from 17-53 MFI and 7-49 MFI, respectively. The values for IgA antibodies to Fc and FeLV in the FIV reference pool were 23-52 MFI and 11-37 MFI, respectively. For the naïve reference pools, the values for IgG and IgA rProtein antibodies to all of the analytes combined ranged from 4-46 MFI and 2-68 MFI, respectively.

Co-infection study antibody response. *Total IgG*. The kinetics and level of the total IgG response differed among the four treatment groups (p-values <0.001; Figure 4.1a). There were significant differences in the antibody kinetics between all pairs (p-values <0.05), except for FIV-C and CO, and sham and PLV cats. Total IgG was on average higher for CO than FIV-cats, and higher for PLV than sham (p-values <0.001). The average level of the PLV total IgG response was similar to FIV-C cats, but significantly lower than the CO cats (p <0.0001). The decrease in total IgG for the PLV cats at day 17 is likely because three of the five plasma samples were not available for testing; the two cats with samples available on day 17 had the lowest total IgG values at the other time-points tested. The concentrations for total IgG ranged from 7.0 to 45.4 mg/ml.

Table 4.2. Total IgG and IgA assay accuracy and precision over a range of analyte concentrations. Acceptable spike recovery was 70-130% of the nominal value. The coefficient of variation (CV) for the mean spike recovery was required to be <20% for the intra-assay and <30% for the inter-assay.

Spike level	Mean % recovery (%CV)	
	IgG	IgA
Intra-assay		
High	89 (15)	101 (12)
Medium	93 (7)	102 (5)
Low	87 (3)	105 (5)
Inter-assay		
High	88 (12)	82 (5)
Medium	93 (6)	96 (5)
Low	98 (7)	90 (5)

Table 4.3. IgG and IgA rProtein mean MFI and precision for the FIV-C reference samples. The coefficient of variation (CV) for the mean MFI was required to be <20% for the intra-assay and <30% for the inter-assay. The values reported for IgG antibodies to CA and SU_{FIV-C} are from a different FIV-C plasma pool than values reported for IgG antibodies to SU_{FIV-A} and CD134. Plasma samples used for the reference samples were from the early (22 and 29 days post-infection) and late (298, 325 and 346 days post-infection) stage of infection. IgA antibodies to CD134 were not detectable in the FIV-C reference sample.

FIV reference sample	Mean MFI (%CV)			
	CA	SU_{FIV-A}	SU_{FIV-C}	CD134
IgG antibodies				
Stage of infection	Early	Late	Early	Late
Intra-assay	14,085 (2)	14,883 (2)	4,741 (1)	5,715 (1)
Inter-assay	13,699 (8)	13,883 (11)	4,585 (8)	5,422 (13)
IgA antibodies				
Stage of infection	Late	Late	Late	Late
Intra-assay	626 (5)	2,285 (5)	4,880 (2)	-
Inter-assay	694 (10)	2,455 (6)	5,380 (4)	-

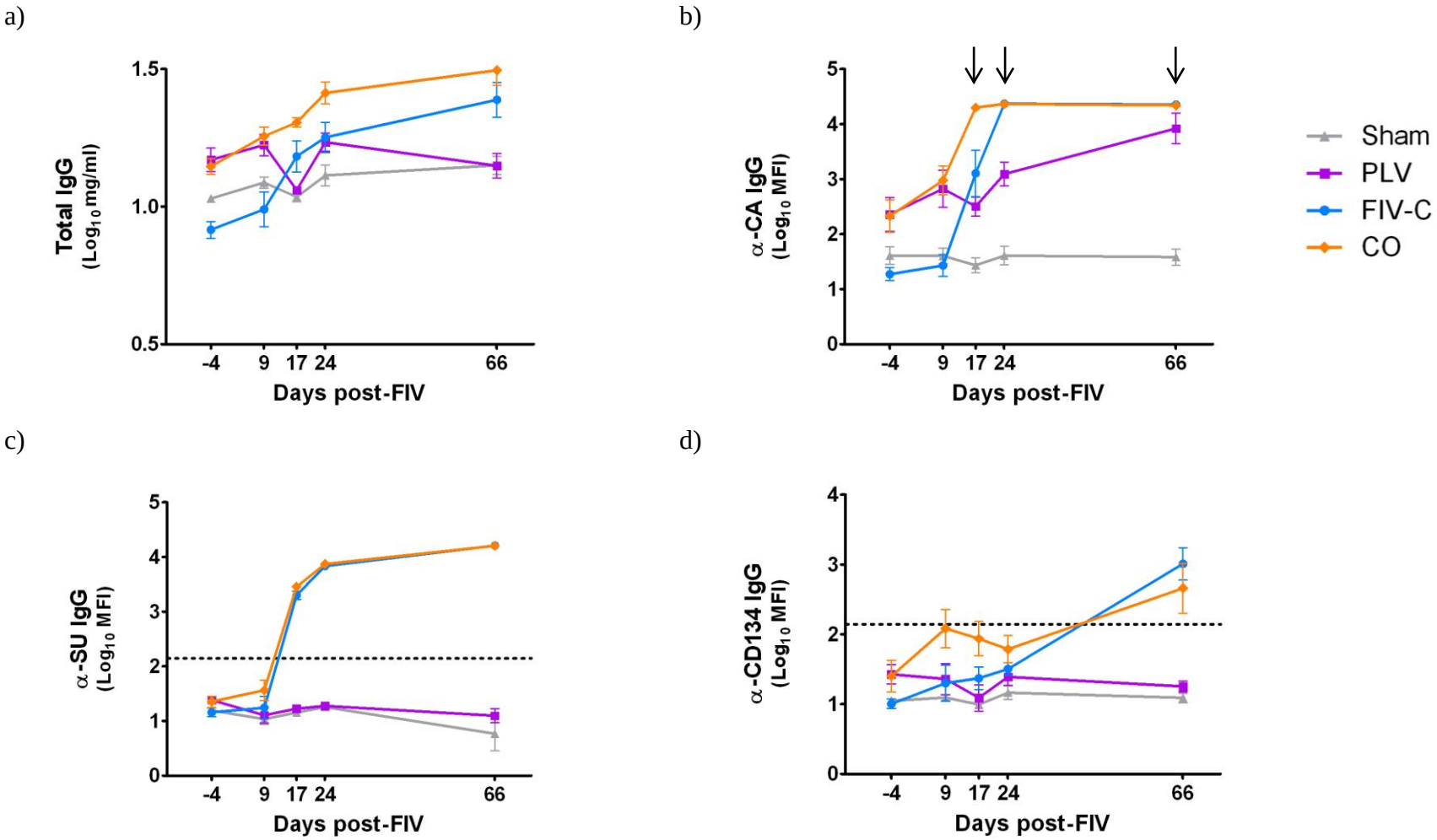

Figure 4.1. Total IgG (a), α-CA IgG (b), α-SU_{FIV-C} IgG (c) and α-CD134 IgG (d) responses for cats from the co-infection study. Cats were inoculated on day -24 with PLV (PLV and CO groups) and/or on day 0 with FIV-C (FIV-C and CO groups). Data points represent the mean MFI + SE. The dotted horizontal lines represent the level of non-specific antibody binding or the mean MFI + 2SD for α-Fc IgG antibodies (MFI 140). Arrows indicate where the MFI for individual cats/group means were maximized with current assay conditions (i.e., using a 1:50 sample dilution).

Anti-capsid IgG. The α-CA IgG MFI for individual cats/group means were maximized from day 17 through 66 with the current assay conditions (i.e., 1:50 dilution; Figure 4.1b). Days 24 and 66 were excluded from the statistical analyses because the mean MFIs reported for CO and FIV-C cats were maximized (i.e., any differences in the antibody response that might have occurred at these time-points were not detected). For day -4 through 17, the antibody response differed over time among the four treatment groups ($p < 0.001$). There were significant differences in the antibody kinetics of FIV-C cats with both sham and PLV cats, and CO cats with both sham and PLV cats (p-values <0.05), but there were no differences between FIV-C and CO or sham and PLV cats. The α-CA IgG response was on average higher for CO than FIV-C cats, and higher for PLV than sham cats (p-values <0.001). Antibodies generated during PLV infection cross-react with FIV-C CA protein, as indicated by the elevated α-CA IgG levels at day -4 (i.e., four days before FIV inoculation) in the two groups of cats inoculated with PLV (PLV and CO).

Anti-surface IgG. The α-SU_{FIV-C} IgG response differed over time among the four treatment groups ($p < 0.001$; Figure 4.1c). There were significant differences in both the kinetics and level of the antibody response of FIV-C cats with both sham and PLV cats, and CO cats with both sham and PLV cats (p-values <0.001), but there were no differences between FIV-C and CO or sham and PLV cats.

Anti-CD134 IgG. The α-CD134 IgG data were not analyzed by repeated-measures ANOVA because the majority of the values were below the non-specific antibody binding level (MFI of 140; Figure 4.1d). None of the cats had IgG antibodies attributed to CD134 on day -4, and none of the sham or PLV cats had antibodies attributed to CD134 at any of the time-points tested. At 9, 17 and 24 days post-infection, only one cat in the CO group clearly had elevated α-

CD134 IgG antibodies (MFI 732, 529 and 253, respectively); however, by day 66 this cat no longer had detectable antibodies. (Note: On day 9, two cats in the CO had MFI values of 142 and 155, which were only slightly above the non-specific antibody binding level). On day 66, three of the five cats in the CO group had detectable antibodies (MFI range 213 to 3,422), and all five of the cats in the FIV-C group had detectable antibodies (MFI range 327 to 7,452). Although there was a statistical difference among the groups at day 66 ($p < 0.001$), there was no difference between the two groups that had an IgG antibody response attributed to CD134 (i.e., FIV-C and CO cats).

Total IgA. There were no significant differences among the treatment groups over time for total IgA (range 0.2 to 7.5 mg/ml, average 1.4 mg/ml; Figure 4.2a).

Anti-capsid IgA. There were no significant differences among the treatment groups over time for α-CA IgA (MFI range 36 to 7,807; average MFI of 370; Figure 4.2b).

Anti-surface IgA. Because the majority of α-SU_{FIV-C} IgA values (87 of 89) were below the non-specific antibody binding level (MFI of 588), the IgA response observed could not be distinguished between the recombinant protein and the Fc tag (Figure 4.2c).

Anti-CD134 IgA. None of the cats had detectable IgA antibodies to CD134; all values were below the non-specific antibody binding level (MFI 588; Figure 4.2d).

Anti-FeLV antibodies. The majority of cats had low IgG and IgA MFI values for FeLV (average MFI of 90 and 77, respectively).

Estimation of percent FIV-specific IgG antibodies. Estimates of the percentage of total IgG antibodies that are CA- or SU-specific relative to the increase in total IgG antibodies due to infection are summarized in Table 4.4. The range of CA-specific IgG antibodies was 1.3 to 11.1%, and for SU-specific antibodies was 0.2 to 9.6%.

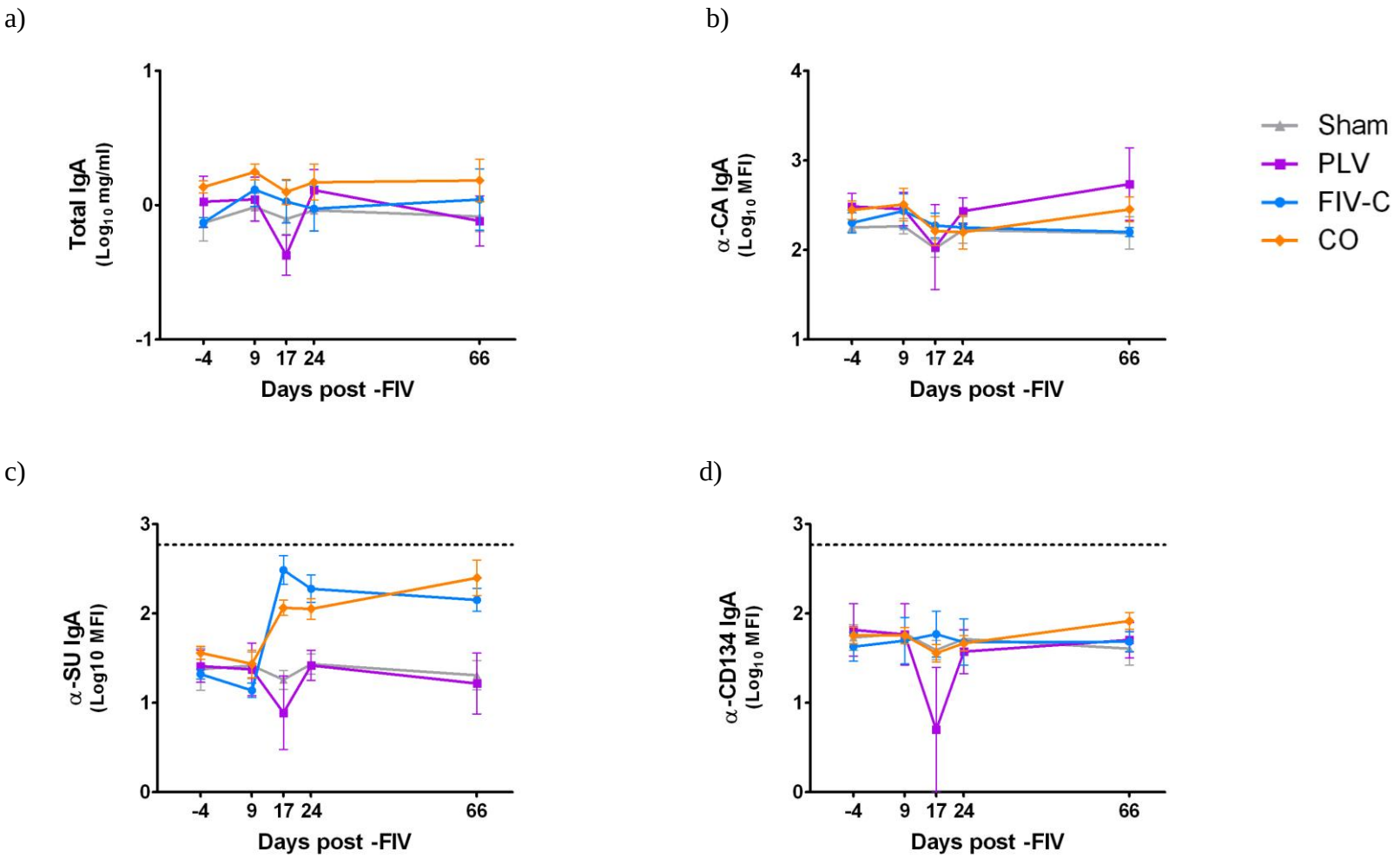

Figure 4.2. Total IgA (a), α-CA IgA (b), α-SU_{FIV-C} IgA (c) and α-CD134 IgA (d) responses for cats from the co-infection study. Cats were inoculated on day -24 with PLV (PLV and CO groups) and/or on day 0 with FIV-C (FIV-C and CO groups). Data points represent the mean MFI + SE. The dotted horizontal lines represent the level of non-specific antibody binding or the mean MFI + 2SD for α-Fc IgA antibodies (MFI 588). The decrease in IgA for PLV cats at day 17 is likely because three (of five) plasma samples were not available for testing; the two cats with samples on day 17 tended to have low IgA values at the other time-points tested.

Table 4.4. Estimates of the percentage of IgG antibodies that are CA- or SU-specific relative to the increase in total IgG antibodies due to infection. These estimates are for the final time-point examined in each of the two *in vivo* studies.

	Range of % IgG estimates (median)	
Study/group	CA-specific	SU-specific
Co-infection		
FIV-C	1.3-9.6 (3.3)	0.7-9.6 (3.2)
CO	2.1-11.1 (2.8)	0.2-1.8 (0.9)
Chimera		
*FIV-C	0.06-1.4 (1.4)	1.4-5.7 (1.4)
*FIV-A/C	0.2-1.6 (0.3)	1.5-18.5 (2.5)
FIV-A	1.6-10.1 (2.2)	6.5-19.8 (10.9)

* One cat in the group was excluded because total IgG was below the average total IgG response of the sham cats.

Chimera study antibody response. *Total IgG.* The kinetics and level of the total IgG antibodies differed among the four treatment groups ($p < 0.05$; Figure 4.3a). There were significant differences in the antibody kinetics of sham cats with both FIV-C and FIV-A/C cats, and between FIV-C and FIV-A cats (p-values <0.05). On average the level of total IgG response was higher in FIV-C than FIV-A cats ($p < 0.05$), whereas sham cats had similar IgG levels to both FIV-C and FIV-A/C cats. On average the level of the total IgG response for FIV-A cats was lower than both FIV-A/C and sham cats (p-values <0.05), and the FIV-A/C cats were lower than FIV-C cats ($p < 0.01$). The concentrations for total IgG ranged from 3.1 to 29.3 mg/ml.

Anti-capsid IgG. The α-CA IgG MFI for individual cats/group means were maximized from day 21 through 326 with the current assay conditions (i.e., 1:50 dilution; Figure 4.3b). Days 67 and 326 were excluded from statistical analyses because the mean MFIs reported for FIV-A and FIV-C cats on day 67 were maximized (i.e., any differences in the antibody response that might have occurred at these time-points were not detected). For days 7 through 35,

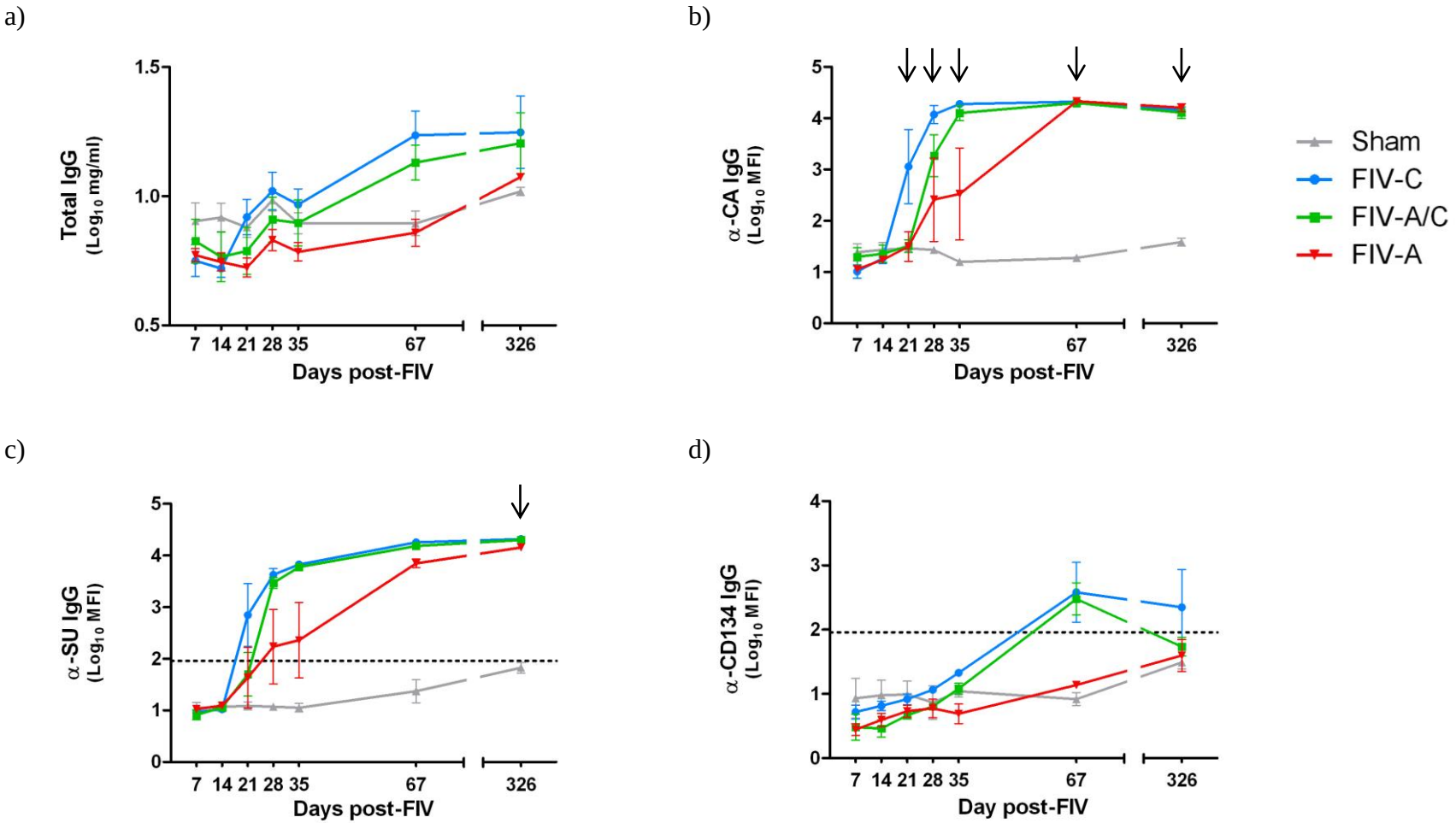

Figure 4.3. Total IgG (a), α-CA IgG (b), α-SU IgG (c) and α-CD134 (d) IgG responses for cats from the chimera study. Data points represent the mean MFI + SE. The dotted horizontal lines represent the level of non-specific antibody binding or the mean MFI + 2SD for α-Fc IgG antibodies (MFI 91). Arrows indicate where the MFI for individual cats/group means were maximized with current assay conditions (i.e., using a 1:50 sample dilution).

antibody kinetics differed among the four treatment groups ($p < 0.001$). There were significant differences in both the kinetics and level of the antibody response of sham cats with both FIV-C, FIV-A/C cats, and between FIV-C and FIV-A/C cats (p-values < 0.05).

Anti-surface IgG. The α-SU IgG MFI for group means for FIV-C and FIV-A/C cats were maximized on day 326 with current assay conditions (i.e., 1:50 dilution; Figure 4.3c). Day 326 was excluded from the statistical analyses because any differences in the level of the antibody response that might have occurred at this time-point were therefore not detected. For days 7 through 67, there was a significant difference in the antibody kinetics among the groups ($p < 0.001$). The kinetics of the three virus-challenged groups were significantly different from sham cats (p-values < 0.001); however, there were no differences in the antibody kinetics among the virus-challenged groups, despite the fact that FIV-A was generally lower over time. On average, the level of α-SU IgG responses for FIV-C and FIV-A/C cats were higher than FIV-A cats (p-values < 0.01), but FIV-C and FIV-A/C were similar to each other.

Anti-CD134 IgG. The α-CD134 IgG data were not analyzed by repeated-measures ANOVA because the majority of the values were below the non-specific antibody binding level (MFI of 91; Figure 4.3d). None of the cats, regardless of treatment group, had IgG antibodies attributed to CD134 between days 7 through 35. On day 67, seven cats (three FIV-C and four FIV-A/C) had elevated α-CD134 IgG antibodies (MFI range 154 to 3,148). Although there was a statistical difference among the groups at this time-point ($p < 0.001$), there was no difference between FIV-C and FIV-A/C cats (i.e., the two groups that had IgG antibodies attributed to CD134). By day 326, four of these seven cats no longer had detectable α-CD134 IgG antibodies, and two others had decreased levels. Only one of the FIV-C cats had an increase in IgG antibodies attributed to CD134 from day 67 to 326 (MFI 3,148 to 10,137). In addition, only one

FIV-A cat had detectable α-CD134 IgG antibodies at day 326 (MFI 213). There were no statistical differences among the groups on day 326.

Total IgA. There were no differences among the treatment groups for total IgA over time (range 0.1 to 2.8 mg/ml, average 0.9 mg/ml; Figure 4.4a).

Anti-capsid IgA. There was a significant difference among the treatment groups over time for α-CA IgA ($p < 0.05$; Figure 4.4b). There were significant differences in the antibody kinetics of FIV-A cats with both sham and FIV-A/C cats (p-values <0.01). On average the level of the α-CA IgA response was higher in FIV-A cats than either sham or FIV-C cats (p-values <0.05), and FIV-A/C cats had a higher level than FIV-C ($p < 0.01$).

Anti-surface IgA. The α-SU IgA data were not analyzed by repeated-measures ANOVA because the majority of the values were below the non-specific antibody binding level (MFI of 821; Figure 4.4c). None of the cats had IgA antibodies attributed to SU on day 7 or 14. From day 21 to 67, only 5 samples (of 72) had values above the non-specific antibody binding level; four of these were from two FIV-C cats and one from a FIV-A cat (MFI range 862 to 2,081). On day 326, three of the FIV-C cats (MFI range 1,010 to 6,578) and three of the FIV-A cats (MFI range 6,164 to 16,389) had detectable IgA antibodies to SU. Although there was a statistical difference among the groups at day 326 ($p < 0.01$), there was no difference between the two groups that had an IgA antibody response attributed to SU (i.e., FIV-A and FIV-C cats).

Anti-CD134 IgA. None of the cats detectable IgA antibodies to CD134; all values were below the non-specific antibody binding level (MFI 821; Figure 4.4d).

Anti-FeLV antibodies. The majority of cats had low IgG and IgA MFI values for FeLV (average MFI of 9 and 22, respectively).

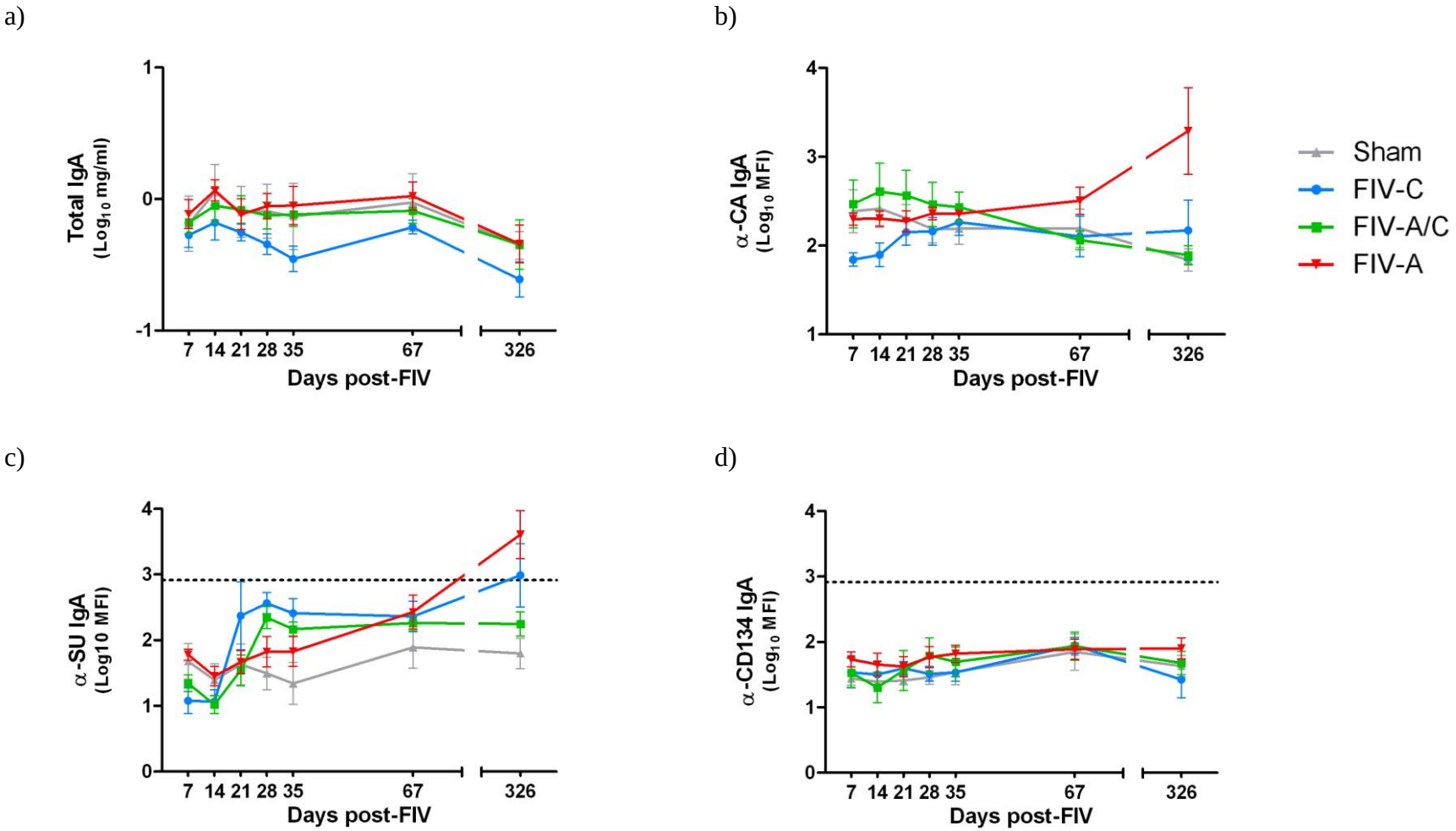

Figure 4.4. Total IgA (a), α-CA IgA (b), α-SU IgA (c) and α-CD134 IgA (d) responses for cats from the chimera study. Data points represent the mean MFI + SE. The dotted horizontal lines represent the level of non-specific antibody binding or the mean MFI + 2SD for α-Fc IgA antibodies (MFI 821).

Estimation of percent FIV-specific IgG antibodies. Estimates of the percentage of total IgG antibodies that were CA- or SU-specific relative to the increase in total IgG antibodies due to infection are summarized in Table 4.4. The range of CA-specific IgG antibodies was 0.06 to 10.1%, and for SU-specific antibodies was 1.4 to 19.8%.

DISCUSSION

This study demonstrates the successful development and validation of MIAs for the quantification of feline total IgG and IgA, and for the detection of IgG and IgA antibodies to FIV CA and SU proteins, and feline CD134 in domestic cat plasma. The utility of these assays were illustrated by examining the kinetics and level of the IgG and IgA responses in cats experimentally infected with various FIV strains (from studies where differences in immunopathology and viral kinetics had previously been described (55, 57)).

The total IgG concentrations obtained for the cats in the two *in vivo* studies are similar to values previously reported for uninfected and FIV-infected domestic cats measured by ELISA (1, 29). In the data reported here, the kinetics and level of the total IgG response varied by FIV strain. The kinetics of the total IgG response for the least virulent viruses (PLV or FIV-A) were similar to the respective sham group, whereas the kinetics for the more virulent viruses (FIV-C or FIV-C and FIV-A/C) were significantly different from the respective sham group. In the chimera study, the highest IgG antibody response was observed with the FIV-C cats (most virulent), followed by FIV-A/C cats and then FIV-A cats (least virulent). In the co-infection study, on average the level of the PLV response was similar to FIV-C cats, but lower than CO cats. These results suggest an additive effect on IgG production with co-infection, in that the initial increase in total IgG due to PLV was increased further by the subsequent FIV-C infection. In these two studies, the FIV-infected cats (specifically FIV-C and FIV-A/C cats) had increasing

levels of total IgG over time; these data support the findings of previous FIV studies, in which domestic cats have elevated levels IgG antibodies (1, 29), likely due to ongoing polyclonal B-cell activation.

In the co-infection study, cats infected with PLV developed IgG antibodies that cross-reacted with the CA protein, but not the SU protein, of FIV-C. These results are not unexpected as the CA proteins of FIV-C and PLV are 64.4% identical at amino acid sites, whereas the SU proteins of these two viruses are 19.7% identical at amino acid sites (amino acid sequence alignment in Geneious 5.6.6; Biomatters Ltd, Auckland, New Zealand). Because of this cross-reactivity, the CO cats had a higher α-CA IgG response than FIV-C cats at early time-points. It is unknown whether the increase in α-CA antibodies prior to FIV-C infection could contribute to the observed $CD4^+$ T-cell maintenance in the CO cats. However, the fact that the CO cats experienced a transient decrease in peripheral $CD4^+$ T-cells, which was similar to the decrease in the FIV-C cats at this same time-point, suggests that these antibodies may not be directly associated with the overall $CD4^+$ T-cell maintenance. Perhaps, as suggested with HIV, the presence of α-CA antibodies are an indicator of the immune system functioning efficiently (7), in that $CD4^+$ T-helper cells are able to activate B-cells for differentiation into antibody-producing plasma cells.

In the chimera study, the early temporal dynamics observed for the α-CA and α-SU IgG responses correspond with the early viral and proviral load dynamics of these cats. As noted by Thompson et al. (2011), FIV-C cats had the highest viral loads, followed by FIV-A/C and then FIV-A cats. The fact that kinetics and level of the rProtein IgG response were similar between FIV-A/C and FIV-A cats for CA, and similar between FIV-A/C and FIV-C cats for SU was consistent with the corresponding viral proteins expressed by the chimeric virus (i.e., FIV-A/C

contains FIV-A *gag* and FIV-C *env* genes). However, it was surprising that there were no significant differences between FIV-C and FIV-A cats for both α-CA and α-SU IgG antibodies, despite the fact that FIV-A cats generally have lower IgG antibody levels. The lack of significance is perhaps due to the large standard error for the FIV-A cats on days 28 and 35 for both α-CA and α-SU IgG antibodies. Similar to previous studies (9, 47), the results of the chimera study demonstrate that antibody kinetics and levels can vary by viral strain; however, the results of this study demonstrate that the antibody levels are positively associated with virulence. This would suggest that the viral antigen load contributes to the magnitude of the anti-FIV antibody response, versus a situation in which a more robust antibody response contributes to a decrease in plasma viral load.

Similar to findings reported by Grant et al. (31), the IgG antibody response to CD134 observed here was variable among individuals, and there was a trend for high CD134 antibodies associated with lower plasma viral loads among the FIV-C cats in the chimera study. The two FIV-C infected cats that had high levels of α-CD134 IgG antibodies (>1,000 MFI) at day 67, on average had lower plasma viral loads from day 173 to 326 (statistical analyses were not conducted due to the small sample size, n = 4). This trend was not observed for FIV-A/C cats, which also had α-CD134 IgG antibodies on day 67, or for the FIV-A cats, which regardless of viral load, did not have α-CD134 IgG antibodies at this time-point. It is known that FIV-A infected cats can develop α-CD134 IgG antibodies, as 21 of the 24 FIV-Petaluma (clade A virus) positive cats in the study by Grant et al. (31) had detectable antibodies at 30 months post-infection. Perhaps the period of time needed for the development of α-CD134 IgG antibodies is longer with less virulent FIV strains, such as FIV-A (i.e., > 326 days, which was the length of the chimera study). Similar antibody and viral load comparisons were not conducted for the co-

infection study due to limited plasma viremia data. Additionally, none of the PLV cats developed α-CD134 IgG antibodies. This is perhaps because of a difference in receptor usage; CD134 does not appear to be the sole, primary receptor for PLV cell entry (52). Despite the fact that PLV cats did not develop α-CD134 IgG antibodies, CO cats developed detectable IgG antibodies against CD134 earlier after FIV-C infection than the FIV-C single infection group. Although a statistical association between α-CD134 IgG antibodies and viral load/clinical outcome could not be made, we documented some trends similar to those observed by Grant et al. (31).

Given that FIV-infected domestic cats exhibit elevated levels of total IgG antibodies (1, 29) and that these antibodies are not entirely virus-specific (26), we estimated the increase in total IgG antibodies that were CA- or SU-specific. The percentage of the total IgG response that was estimated to be CA-specific ranged from 0.06 to 11.1% and SU-specific ranged from 0.2 to 19.8%. These large ranges may be artifacts of the method used for estimating FIV-specific antibodies (i.e., on average, the closest rProtein dilution was ± 936 MFI from the total IgG MFI, and the average total IgG response for the sham cats was not necessarily equivalent the total IgG baseline level for each FIV-infected cat). For individual cats from both studies, the estimated percentage of antibodies that were FIV-specific (i.e., CA plus SU) ranged from 1.8 to 21.8%. However, the total percentage of FIV-specific antibodies would be higher, as domestic cats develop antibodies to other viral proteins besides CA and SU (24, 29, 34, 42). It is unknown what the majority of IgG antibodies are directed against or how these values would compare to a 'typical' viral infection. It would be interesting to obtain similar percentages for non-viral and self antigens, as described by Flynn et al. (26), to compare with the percentages obtained here for CA and SU proteins.

The total IgA concentrations obtained for the cats in the two *in vivo* studies are similar to values previously reported for uninfected and FIV-infected domestic cats measured by ELISA (1, 29). Previous studies have documented normal levels of total IgA in FIV-infected cats during the first year of infection (data not shown (29)) and at 24-28 weeks post-infection (1); however, elevated levels of total IgA have been noted with a few healthy, FIV-infected domestics cats (29). Nonetheless, neither the co-infection nor the chimera study presented temporal differences in total IgA levels.

In general there were few differences in the IgA antibody response to the rProteins. The most notable, however, was the α-CA IgA response of FIV-A infected cats in the chimera study. The differences in antibody kinetics and level of the FIV-A cats to the other groups were due to the elevation in α-CA IgA at day 326. Interestingly, it was also the FIV-A group at day 326 that had the highest α-SU IgA response. These data suggest that either IgA antibodies to the rProteins have yet to be developed or are not present/detectable in the periphery during the early stage of infection (e.g., within two months post-infection), and that perhaps the observed differences in the IgA response among the virus-challenged groups are due to differences in viral pathogenicity. It is possible that cats in the co-infection study developed IgA antibodies to the rProteins, but similar to the chimera studies, these antibodies may only be elevated at later time-points (i.e., after two months post-infection). Burkhard et al. (9) noted variable levels of IgA antibodies to the CA protein between three to nine weeks post-infection; these levels varied based on inoculation route, viral strain and among individuals in a treatment group. Interestingly, no IgA antibodies were detected against the envelope protein (9). Grant (1995) also noted that some cats developed α-CA IgA antibodies two months after infection, and that the number of cats with detectable antibodies varied by FIV strain (data not shown (29)).

Although other studies have evaluated antibody response to FIV (1, 9, 10, 24, 26, 29, 31, 34, 42, 46, 47), the data presented here provides the most comprehensive analysis to date, particularly for early stages of infection, and for *in vivo* studies where viral kinetics and other clinical parameters have been well described. With the MIAs, we were able to detect antibodies to multiple analytes simultaneously, in addition to the detection of albumin in each sample. The detection of albumin confirmed that plasma was loaded into each well; this was particularly important when target antibodies were not detected (e.g., early time-points before FIV-specific antibodies were detectable in plasma). As demonstrated here, the MIAs are useful for characterizing the antibody response over time and studying trends related to disease outcome.

One limitation of multiplexed MIAs is that a single dilution may not be ideal for measuring each of the analytes. For example, total IgG and IgA were not multiplexed because of the difference in dilution required for quantification (i.e., 1:200,000 and 1:10,000, respectively), and the 1:50 sample dilution was not ideal for measuring the level of the IgG response for each of the analytes in the rProtein assay (i.e., at later time-points the α-CA and α-SU IgG MFI maximized). Given that the relationship between analyte concentration and signal for immunoassays are not generally linear (2), the MFI values obtained with different dilutions may not be comparable. Thus, single dilution was used in rProtein experiments in order to compare the raw MFI among the groups over time. To compare differences in the kinetics and level of each analyte across all time-points, samples would need to be tested with multiple dilutions (e.g., 1:50 for CD134, 1:500 for SU, and 1:5,000 for CA); however, this defeats the benefit of using a multiplex assay.

Another potential limitation of the rProtein MIAs is that both IgG and IgA antibodies can bind to the recombinant proteins; the only difference between these two assays is that the

detection antibodies bind to either IgG or IgA. Given that the concentrations of total IgG were much higher than the concentrations of total IgA in these two studies (IgG was ~3 to 285 times higher than the respective IgA value), it is possible that the α-rProtein IgA antibodies were out competed by the higher levels of α-IgG rProtein antibodies. This might contribute to the lack of detectable IgA antibodies to the rProteins; however, to be certain this would require the removal of IgG antibodies from each plasma sample (e.g., as described in (30)) prior to testing. Despite the above limitations, the rProtein assays would be useful to determine whether a cat has detectable α-CA or α-SU antibodies for diagnostic purposes.

It is unknown how the MIAs compare to conventional immunoassays, such as ELISA or immunoblot. For this reason, as well as others (e.g., different viral strains and time intervals examined), we were unable to make direct comparisons to previous studies (e.g., ELISA (9, 10, 31, 46, 47) or immunoblot (24, 29, 34, 42)).

Possible functions of FIV-specific antibodies, such as the ones described in this study, could include viral neutralization, viral opsonization and/or antibody-dependent cell-mediated cytotoxicity (reviewed in (3, 58)). Given that IgG subclasses have different functional properties and that the presence of human IgG subclasses can vary with HIV disease state (reviewed in (58)), it would be interesting to compare the kinetics and levels of the three (possibly four) domestic cat IgG subclasses (30) throughout FIV infection.

In summary, the results presented in this study demonstrate that a) total IgG antibodies increase over time after FIV infection; b) α-CA and α-SU IgG antibodies are first detectable between 9-28 days post-infection and increase over time, and that these antibodies are a fraction of the total IgG increase; c) α-CD134 IgG antibody production varies among individuals and over time, and was not strongly correlated to viral load during early stage of infection; d)

circulating IgA antibodies, in general, do not increase after IV FIV infection; and e) total IgG, and α-CA and α-SU IgG antibody kinetics and level vary with FIV viral strain/pathogenicity (Figure 4.5). In future studies, the total IgG and IgA MIAs described here could be used to evaluate the antibody response of elicited by a variety of diseases in domestic cats, and the rProtein MIAs could be used to evaluate the domestic cat antibody response elicited by other FIV strains or to screen samples for FIV and/or FeLV infection. These assays could be expanded to include other antibodies (e.g., IgM, IgG subtypes), additional viral proteins (e.g., TM or specific FIV protein epitopes) or adapted to test other samples types, such as saliva. Assays such as these will be useful for assessing the IgG and IgA response associated with disease pathogenesis and/or protective vaccine immunity.

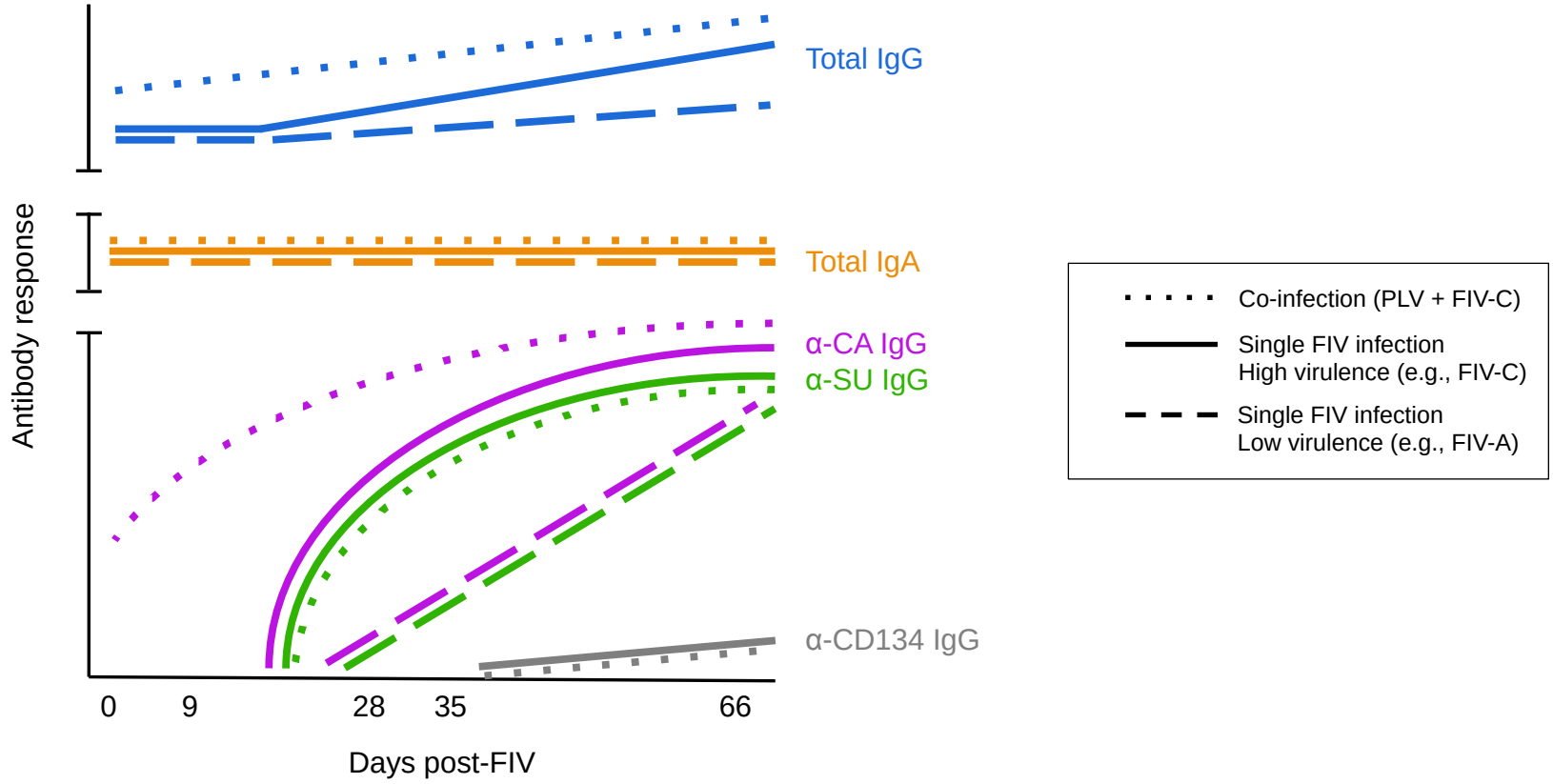

Figure 4.5. Summary of the peripheral/circulating antibody response during the early stage of FIV-single and co-infection. For FIV-single infected cats [FIV-C, FIV-A and FIV-A/C cats]: (a) the total antibody concentration ranged from 3.1 to 35.8 mg/ml for IgG, and 0.1 to 7.5 mg/ml for IgA; (b) the IgG antibody response to CD134 varied greatly among individuals/over time, but was mostly detected around 66 days post-infection; (c) the percentage of total IgG antibody increase that was estimated to be CA-specific ranged from 0.06 to 10.1%, and SU-specific ranged from 0.7 to 19.8%; and (d) the kinetics and level of the IgG response varied by FIV virulence. For co-infected cats: (a) the total antibody concentrations ranged from 12.1 to 45.4 mg/ml for IgG, and 0.6 to 3.6 mg/ml for IgA; (b) the antibody response for total IgG and α-CA IgG antibodies were higher during the early stage of FIV infection due to prior infection with PLV [the α-SU IgG and α-CD134 IgG responses were similar between single (high virulence) and co-infected cats]; and (c) the percentage of total IgG antibody increase that was estimated to be CA-specific ranged from 2.1 to 11.1%, and SU-specific ranged from 0.2 to 1.8%.

APPENDIX

Baculovirus protein expression. *Surface and transmembrane proteins.* FIV *env* encodes an envelope polyprotein (140-150 kDa), which contains an L-domain, and SU and TM proteins (54, 61). After translation, the glycosylated polyprotein undergoes two cleavage steps; the first cleavage step removes the L-domain (20 kDa), which contains the secretion signal required for protein transport outside the cell (53), and the second cleavage step separates the SU (gp95) and TM (gp40) proteins (54, 61). Since the SU and TM proteins are glycosylated and a secretion signal is required for transport outside of the cell, we attempted to express these proteins in insect cells using an insect cell secretion signal.

Honeybee melittin signal sequence. The honeybee melittin (HBM) signal sequence (63 nucleotides), was selected because it has been shown to enhance foreign protein secretion from insect cells ((56); however, protein secretion with HBM may be equivalent to the native sequence signal (36)). To generate a dsDNA HBM sequence, complementary oligonucleotides of the HBM sequence (Integrated DNA Technologies, Coralville, IA) were annealed using a touchdown PCR protocol. The sequences included additional 5' and 3' nucleotides for directional insertion into the plasmid, to eliminate the need for restriction enzyme digestion (Table 4.5).

The FastBac Dual plasmid (pFBD; Life Technologies; provided by D. Henderson) used for protein expression has ampicillin and gentamicin resistance genes for plasmid and bacmid selection, respectively. Purified plasmid was digested with BamHI and EcoRI restriction enzymes, and ligated with the dsDNA HBM sequence using T4 DNA ligase according to manufacturer recommendations (New England BioLabs). The plasmid was propagated in One Shot® TOP10 Chemically Competent *E. coli* according to manufacturer recommendations (Life

Table 4.5. Primers used for the amplification of SU and TM nucleotide sequences, and sequencing of plasmid and bacmid inserts, as well as the complementary honeybee melittin (HBM) oligonucleotide sequences. Restriction sites (underlined), His-tags (bolded), start codons (italicized) and stop codons (wave underlined) incorporated into the primers/oligonucleotides are noted.

Primer/oligonucleotide (Restriction site, tag)	Sequence (5'-to-3')
Baculovirus expression	
SU (EcoRI, 6-His) Fwd	CCGGAATTC**CATCACCATCACCATCAC**AGACTCCCACCCTTAGTAGTC
SU (PstI) Rev	AACTGCAGCTACTATCTTTTTTGTCTTGATTGATAGTTTAAAACTTCTT
TM (EcoRI, 6-His) Fwd	CCGGATTCC**CATCACCATCACCATCAC**ACCCAATATCATCAAGTCCTAGCTA
TM (PstI) Rev	AACTGCAGCTACTATTTTCCCATCCATCCCGTC
pFBD Fwd	CGTAACAGTTTTGTAATAAAAAAACCTATAAATATTCC
pFBD Rev	CCTCTACAAATGTGGTATGGCTG
Bacmid Fwd	GTTTTCCCAGTCACGAC
Bacmid Rev	CAGGAAACAGCTATGAC
HBM (BamHI[a]) Fwd	GATCCACCATGAAATTCTTAGTCAACGTTGCCCTTGTTTTTATGGTCGTATACATTTCTTACATCTATGCGGCCG
HBM (EcoRI[a]) Rev	AATTCGGCCGCATAGATGTAAGAAATGTATACGACCATAAAAACAAGGGCAACGTTGACTAAGAATTTCATGGTG
E. coli expression	
SU (BamHI) Fwd	CGCGGATCC*ATG*AGACTCCCACCCTTAGTAGTCCCA
SU (EcoRI) Rev	CCGGAATTCCTACTATCTTTTTTGTCTTGATTGATAGTTTAAAACTTCTTCTGGTACC
TM (BamHI) Fwd	CGCGGATCC*ATG*ACCCAATATCATCAAGTCCTAGCTACTCATCAA
TM (EcoRI) Rev	CCGGAATTCCTACTATTTTCCCATCCATCCCGTCCAATATTCC

[a] The restriction sites do not include the first nucleotide at the 5'-end. This was to allow for the correct 5' over-hang for insertion into the plasmid.

Technologies). Purified plasmid was sequenced (Proteomic and Metabolomics Facility, Colorado State University) to confirm HBM insertion using the pFBD primers (Table 4.5).

Surface and transmembrane nucleotide sequences. Sequences encoding SU and TM were amplified from a molecular clone of FIV-C (19) using standard PCR protocols. Based on an alignment of FIV-C with other annotated FIV strains (10, 37, 39, 45), the segment amplified for SU was 6,808 to 8,097 (1,290 nucleotides) and for TM was 8,182 to 8,601 (420 nucleotides). The coding region for TM excludes the hydrophobic region (N-terminus), and intracellular and hydrophobic transmembrane domain (C-terminus); these regions were excluded because hydrophobicity could affect protein solubility and/or secretion, and the inter-cellular region is not exposed to neutralizing antibodies. The primers, which included restriction sites for directional insertion into the plasmid and polyhistidine (6-His) tags, are listed in Table 4.5. Purified PCR products were digested with EcoRI and PstI restriction enzymes according to manufacturer recommendations (New England BioLabs), and stored at -20°C until ligation with the HBM-pFBD plasmid.

The HBM-pFBD purified plasmid was digested with EcoRI and PstI restriction enzymes, and ligated with the SU or TM sequence using T4 DNA ligase according to manufacturer recommendations (New England BioLabs). The plasmid was propagated in One Shot® TOP10 Chemically Competent *E. coli* according to manufacturer recommendations (Life Technologies). Purified plasmid was sequenced (Proteomic and Metabolomics Facility, Colorado State University) to confirm SU or TM insertion using the pFBD primers (Table 4.5).

Protein expression. DH10Bac™ *E. coli* (Bac-to-Bac® Baculovirus Expression System, Life Technologies; provided by D. Henderson) were transformed with SU-HBM-pFBD or TM-HBM-pFBD according manufacturer recommendations. DH10Bac™ *E. coli* contains a helper

plasmid and a baculovirus shuttle vector (bacmid); the helper plasmid supplies proteins required for transposition of the recombinant protein sequences into the bacmid. Recombinant bacmid DNA was isolated according to the manufacturer recommendations (Life Technologies). To confirm the insertion of SU- or TM-HBM into the bacmid, the region spanning the transposition site was amplified by PCR using bacmid primers (Table 4.5). On an agarose gel, a band ~3,000 bp suggested that TM-HBM was inserted into the bacmid, and a band ~ 4,000 bp suggested that SU-HBM was inserted into the bacmid.

The bacmids were then used to transfect Sf9 insect cells (*Spodoptera frugiperda* or fall armyworm cell line; provided by D. Henderson) according to the manufacturer recommendations (Life Technologies). Cells were incubated at 28°C in Sf-900 II media (Life Technologies) with penicillin/streptomycin and fungizone. Cells were monitored daily for cytopathic effect (CPE; e.g., increased diameter, granular appearance and lysis) and passaged according to manufacturer recommendations. Briefly, transfected cells were initially grown in 6-well tissue culture plates for four days. The supernatant from these cells were then transferred to 10 cm plates (passage 1) containing 10^7 Sf9 cells and incubated for 72 hours. The supernatant from one SU and one TM culture was then transferred into a flask (passage 2) containing $1.5x10^6$ cells/ml in 250 ml of Sf9 media, and incubated for an additional 72 hours.

Both the supernatant and the cells from passages 1 and 2 were tested by SDS-PAGE and immunoblot (α-His antibody) for the presence of SU and TM proteins. There were no visible bands in the supernatant or cells around the expected molecular weights for SU or TM expression (>51 and >18 kDa, respectively; weights expected to be above the kDa listed due to glycosylation). Multiple experiments were conducted to trouble-shoot the protein expression, such as repeating the transfection of Sf9 cells, modifying the immunoblot protocol (e.g., primary

antibody incubation time, antibody diluent and using a different α-His antibody), and using an alternative method for protein purification (i.e., fast protein liquid chromatography). Despite these efforts, SU and TM proteins were never identified/purified. Possible reasons for the lack of protein expression include bacmid DNA degradation (e.g., perhaps due to storage at 4°C from time of isolation until transfection), low transfection efficiency and/or sub-optimal growth conditions (e.g., temperature or incubation times). Additionally, it is possible that the HBM sequence did not enhance expression/secretion in the baculovirus system (36) or that there was an error with the SU- or TM-HBM insertion that prevented protein expression (e.g., frame-shift that affected translation). Due to time limitations, no additional baculovirus expression experiments were conducted.

***E. coli* protein expression.** Because the baculovirus expression of SU and TM was unsuccessful, an attempt was made to express these proteins in *E. coli*. The primers used to amplify the SU and TM regions are listed in Table 4.5. The protocols and regents used were identical to those described for the recombinant FIV-C CA protein (see above). However, unlike the CA expression, when the purified protein solution was run on a SDS-PAGE gel, there were no visible bands at the expected molecular weights for SU or TM (~51 and ~18 kDa, respectively). Upon examination of the respective cell pellets, a band was noted at the molecular weight of SU-GST (~77 kDa) or TM-GST (~44 kDa). These results suggested that perhaps the reagent used to lyse the cells was inefficient or that the recombinant proteins were in inclusion bodies. Additional protein expression experiments using a different lysing reagent, suggested that the band ~77 kDa was a bacterial protein (i.e., not SU-GST), whereas the band ~44 kDa was TM-GST. It is possible that the SU protein was expressed at low levels, but not visible by SDS-PAGE because it was masked by a bacterial protein or that culture conditions were not optimal

for expression. Due to time limitations, no additional experiments were conducted to determine why the SU protein did not appear to be expressed (e.g., immunoblot or optimizing culture conditions, such as media, incubation time or temperature) or to remove the TM protein from the inclusion bodies.

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CHAPTER 5: DEVELOPMENT OF REAL TIME PCR ASSAYS FOR THE QUANTIFICATION OF PUMA LENTIVIRUS VIREMIA[4]

INTRODUCTION

Feline immunodeficiency viruses (FIVs) are naturally occurring lentiviruses (RNA genome; family *Retroviridae*) of felids, including the domestic cat (*Felis catus*) and at least 10 species of free-ranging wild cats (4, 9). Each felid species is typically infected with a genetically-distinct FIV strain (9); however, mountain lions (*Puma concolor*; also referred to as cougar, panther or puma) can be infected with two distinct strains ((2, 4), Lee et al., unpublished). Free-ranging and captive mountain lions are infected with $FIV_{Pco}B$ or puma lentivirus (PLV)-B throughout their geographic range in North and South America ((2, 4), Lee et al., unpublished). In California and Florida, free-ranging mountain lions are infected with $FIV_{Pco}A$ or PLV-A, which is highly divergent from PLV-B ((2, 4), Lee et al., unpublished). Interestingly, bobcats (*Lynx rufus*) are also infected with PLV-A; PLV-A has been detected in bobcats from California and Florida, but not Colorado ((4, 6), Lagana et al., in press and Lee et al., unpublished). To date, PLV-A is the only FIV sequenced from bobcats ((4, 6), Lagana et al., in press and Lee et al., unpublished).

Given that a) PLV-A is the only known FIV to infect bobcats, b) PLV-A is less common in mountain lions than PLV-B, and c) PLV-A in mountain lions has only been reported in geographic regions with PLV-A infected bobcats, Franklin and Troyer et al. (4) hypothesized that PLV-A may actually be of bobcat origin, and that PLV-A in the mountain lion is the result of cross-species transmission events. Analyses of PLV-A proviral sequences support this

[4] Ryan M. Troyer and Sue VandeWoude contributed to the research presented in this chapter.

hypothesis, in that viral isolates from mountain lions were more likely to be undergoing positive selection than bobcat viral isolates (Lee et al., unpublished). Additionally, preliminary data indicates that bobcats have significantly higher PLV-A proviral loads than mountain lions ($p < 0.01$; Templin-Hladky et al., unpublished), suggesting that PLV-A replicates less efficiently in mountain lions. However, as provirus indicates a residual infection, the detection of viral RNA would be a more accurate measure of viral fitness. Thus, the aim of this project is to adapt the PLV proviral quantitative PCR (qPCR) assays (Templin-Hladky et al., unpublished) for the quantification of viral RNA in plasma. Since domestic cat cross-species infection studies indicate that non-host adapted FIVs (e.g., PLV-A, PLV-B or lion lentivirus (FIV_{Ple})) typically result in lower levels of plasma viremia than host-adapted FIV strains (11, 12), we predict that mountain lion PLV-A viremia will be lower than bobcat PLV-A and mountain lion PLV-B viremia.

MATERIALS AND METHODS

Plasma samples. Blood samples were collected from free-ranging bobcats and mountain lions in California, Colorado and Florida (2002 to 2011), as part of ongoing research projects conducted by our laboratory and collaborators. Appropriate approvals were obtained prior to the collection of samples. Plasma was separated from each blood sample as previously described (5), and stored at -80°C.

The plasma samples used thus far for assay development were from PLV-A positive bobcats (n = 7) and PLV-B positive mountain lions (n = 14) (Table 5.1); these samples are all positive based on provirus sequence data (Lee et al., unpublished). To date, no PLV-A positive mountain lion samples have been tested. Plasma samples from PLV-A positive mountain lions

Table 5.1. PLV-positive bobcat and mountain lion plasma samples tested to date. Only four PLV-B samples have viral RNA levels above the assay LLOQ (i.e., above 17,000 copies/ml of plasma). Values reported for PLV-A and PLV-B viremia below the assay LLOQ are not reliable.

Animal ID	Spp[a]	Sex	Age[b]	Location[c]	Collaborator	Anti-PLV Abs[d]	Sequenced virus[e]	PLV-A viremia (RNA copies/ml plasma)	PLV-B viremia (RNA copies/ml plasma)
599R1	Lru	F	A	CA	Boyce	+	A	8,400	ND[f]
603	Lru	M	A	CA	Boyce	+	A	4,300	ND
1133	Lru	M	A	FL	McBride	-	A	4,900	ND
1134	Lru	M	A	FL	McBride	-	A	14,000	ND
1142	Lru	M	A	FL	McBride	+	A	10,000	ND
1187	Lru	M	A	FL	McBride	-	A	2,100	ND
1206	Lru	M	A	FL	McBride	-	A	2,100	ND
85	Pco	M	A	CA	Boyce	+	B	ND	ND
88	Pco	F	A	CA	Boyce	+	B	ND	ND
92	Pco	F	A	CA	Boyce	+	B	2,200	ND
121	Pco	F	A	CA	Boyce	+	B	ND	3,300
122	Pco	M	A	CA	Boyce	+	B	ND	120,000
139R1	Pco	M	A	CO	Logan	+	B	ND	5,000
431	Pco	F	A	CO	Logan	+	B	ND	30,000
686	Pco	M	A	CO	Logan	+	B	ND	13,000
1045R1	Pco	M	A	CO	Alldredge	-	B	ND	12,000
1058	Pco	F	A	CO	Logan	+	B	ND	6,900
1077	Pco	F	Y	CO	Alldredge	+	B	ND	26,000
1346R1	Pco	F	A	CO	Alldredge	+	B	860	4,700
1377	Pco	M	-	CO	Logan	+	B	ND	130,000
1378 (296R2)	Pco	F	A	CO	Alldredge	+	B	ND	12,000

[a] Lru – bobcat (*Lynx rufus*); Pco – mountain lion (*Puma concolor*)
[b] Y – young (6 months to 2 years); A – adult (≥ 2 years)
[c] CA – California; CO – Colorado; FL – Florida
[d] Antibodies to PLV detected by western blot (VandeWoude laboratory)
[e] Provirus sequenced by J. Lee (unpublished); DNA was extracted from whole blood or peripheral blood mononuclear cells
[f] ND – Non-detectable

will only be tested once assay development is complete, as these samples are more limited in number. Plasma samples from naïve domestic cats served as negative controls for each set/batch of RNA extractions and subsequent qPCR experiments.

Ideally, each sample should remain frozen until viral RNA purification, as repeated freeze-thaws can reduce viral titers (QIAmp viral RNA mini kit handbook; Qiagen, Valencia, CA). It is unknown how many freeze-thaws the selected bobcat and mountain lion samples have undergone prior to RNA purification.

Viral RNA extraction. RNA was extracted from bobcat or mountain lion plasma using the QIAmp viral RNA mini kit (Qiagen) according to manufacturer recommendations. The volume of plasma used for each RNA extraction was 140 µl, with the exception of one sample that only had 110 µl. Purified RNA was eluted in 60 µl of AVE buffer (Qiagen) and stored at -80°C.

cDNA synthesis. Equal volumes of RNA and cDNA master mix were used for cDNA synthesis. For 10 µl of purified RNA, the 10 µl of cDNA master mix consisted of the following: 4 µl of 5x First Strand Buffer (Life Technologies, Grand Island, NY), 1 µl of 10 mM dNTP (Bio-Rad, Hercules, CA), 1 µl of 0.1 M DTT (Life Technologies), 0.25 µl of 40 U/µl RNase Out™ (Life Technologies), 0.25 µl of 200 U/µl SuperScript® II Reverse Transcriptase (Life Technologies), 2 µl of 300 ng/µl Random Primers (Life Technologies), and 1.5 µl of nuclease-free water. Samples were incubated at 42°C for 50 min, followed by 95°C for 5 min. cDNA was stored at -20°C until testing in the qPCR assays.

Primers. Primers (Templin-Hladky et al., unpublished) used in these experiments are listed in Table 5.2. PLV-A primers amplify a conserved region of *env* (103 nucleotides), while the PLV-B primers amplify a conserved region of *gag* (151 nucleotides). These regions were

selected for amplification because of conserved regions at both ends for primer binding. The conserved regions were identified by aligning 24 full-length PLV-A sequences (23 unpublished sequences from J. Lee and one sequence from GenBank: U0398) or 39 full-length PLV-B sequences (26 unpublished sequences from J. Lee, and the following 13 sequences from GenBank: DQ192583, EF455603, EF455604, EF455605, EF455606, EF455607, EF455608, EF455609, EF455610, EF455611, EF455612, EF455613, EF455615).

Table 5.2. Primers used to quantify PLV RNA loads.

Primer	Sequence (5' to 3')
PLV-A Fwd	GCAGCCCTGACGGTATCC
PLV-A Rev	GCAGTCTCCTCTGAACAATCC
PLV-B Fwd	CTGTCTGTCATGGGGAATGAGT
PLV-B Rev	GTCCTGTAGCTACCAAGGCAA

Plasmid standards. Extracted DNA from the whole blood of a PLV-A positive bobcat (cat 188) and a PLV-B positive mountain lion (cat 85), known to contain the conserved *env* and *gag* primer binding sites, respectively, were used to amplify fragments of these genes for insertion into the pCR®-2.1TOPO® vector (Life Technologies; Templin-Hladky et al., unpublished). The purified plasmids were diluted in TE buffer (10 mM Tris, 1 mM EDTA), and concentrations were determined using a NanoDrop spectrophotometer (Templin-Hladky et al., unpublished). Plasmid standards were stored at -20°C.

Quantitative PCR. The protocol used initially for the quantification of viral RNA loads was identical to the protocol used for proviral quantification (Templin-Hladky et al., unpublished). Briefly, each reaction (25 µl) contained 12.5 µl SsoFast™ EvaGreen® Supermix (Bio-Rad), 1 µl the Fwd primer (10 µM), 1 µl the Rev primer (10 µM), 5.5 µl of nuclease-free

water and 5 µl of sample cDNA. Amplification was as follows: 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec and 62°C for 10 sec. To assess amplification specificity, all reactions underwent a melt-curve analysis (65°C to 95°C in 0.5°C increments, 10 sec per increment). The iQ5 iCycler (Bio-Rad) used for these experiments was calibrated monthly, and the iQ5 Optimal System Software Version 2.0 was used for data collection and analysis. All standards, no template controls and samples (PLV-positive samples (Table 5.1) and naïve domestic cat samples) were run in triplicate. The mean starting quantity (SQ) per sample (mean copies/well) was used to calculate the viral RNA copies/ml of plasma. The equation used for these calculations was as follows:

(SQ) (1/C) (D) (E) (1/P) = RNA copies per ml of plasma

SQ: Mean starting quantity per sample

C: cDNA volume (µl) added per well of the qPCR assay (5 µl)

D: Inverse of the dilution used for cDNA synthesis (e.g., for 1-in-2 dilution D equals 2)

E: Elution volume (µl) used for viral RNA extraction (60 µl)

P: Plasma volume (ml) used for viral RNA extraction (generally, 0.14 ml)

All calculated values of RNA copies/ml of plasma were rounded to two significant digits based on the precision of the method. Samples were considered positive when at least two of the three replicates had Ct values.

Assay development. *Lower limit of quantitation.* The initial experiments conducted were to determine the lower limit of quantitation (LLOQ) of each assay; although the same standards and primers were used for proviral quantification (Templin-Hladky et al., unpublished), the LLOQ of both assays had not been investigated. To determine the LLOQ of each assay, PLV-A and PLV-B standards (diluted in TE buffer) were tested on multiple days to

determine the lowest concentration that could be reliably quantified. Standard concentrations tested were 10^2 to 10^7 plasmid copies/well (10-fold dilution), as well as values below this range, such as 10, 20, 40, 50, 60 and 80 copies/well. The lower limit that was reliably quantified in these experiments was 10^2 copies/well for both assays; the Ct values for copy numbers below 10^2 were no longer linear (i.e., the values overlapped, regardless of concentration). Using the equation noted above, an LLOQ of 10^2 copies/well is equivalent to 17,143 RNA copies/ml of plasma, which rounded to two significant digits is 17,000 RNA copies/ml plasma.

Bobcat and mountain lion viral RNA loads. To determine the range of viral RNA loads of naturally infected bobcats and mountain lions, we tested plasma samples from PLV-A or PLV-B positive cats (n = 21; Table 5.1). All samples were tested in both assays (PLV-A and PLV-B) to confirm that the primers/assays were specific (i.e., based on the sequencing data obtained by Lee et al., unpublished).

All of the PLV-A bobcat samples (7 of 7) had detectable levels of PLV-A RNA (Figure 5.1); however, these values were below the LLOQ. None of these bobcats had detectable levels of PLV-B RNA. Given that PLV-A is the only known FIV to infect bobcats ((4, 6), Lagana et al., in press and Lee et al., unpublished), these results suggest that the PLV-B primers are specific and do not cross-react with PLV-A.

Four (of 14) of the PLV-B mountain lion samples had viral loads above the LLOQ; these values ranged from 26,000 to 130,000 RNA copies/ml of plasma (Figure 5.1). These plasma viral loads are within the range previously reported for PLV-B infected mountain lions (range $2.3x10^3$ to $2.81x10^6$ RNA copies/ml; (1)). PLV-B RNA was not detected in three (of 14) mountain lion samples (Table 5.1) despite the fact the PLV-B provirus was sequenced from these cats (Lee et al., unpublished). This suggests that either the virus was not actively replicating

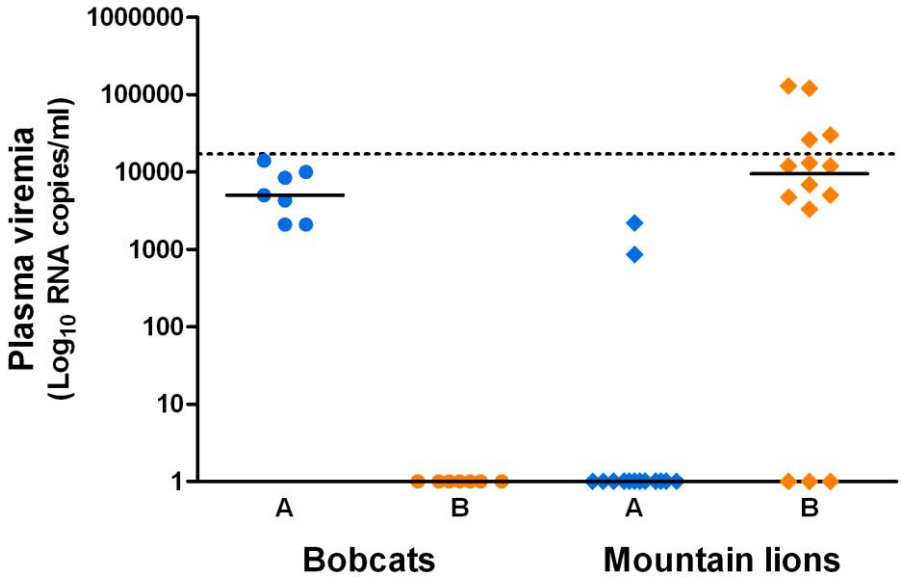

Figure 5.1. Detectable plasma viral RNA loads of PLV-A provirus positive bobcats and PLV-B provirus positive mountain lions. The solid lines indicate the medians, and the dashed line indicates the lower limit of quantitation (LLOQ; 17,000 copies) for both of the qPCR assays.

when the blood samples were collected or that the viral RNA levels were below the assay limit of detection.

In addition, two (of 14) mountain lions had detectable levels of PLV-A RNA (Table 5.1 and Figure 5.1). Given that mountain lions can be infected with PLV-A and/or PLV-B ((2, 4), Lee et al., unpublished), it is possible that these cats are co-infected with both viruses. However, as one of these cats was captured in Colorado (a region in which PLV-A has not been previously detected in either bobcats or mountain lions), it seems unlikely that this cat was infected with PLV-A. (Note: The other PLV-A positive sample was collected in California). To date, only mountain lions in California and Florida are known be infected with PLV-A ((2, 4), Lee et al., unpublished). It is possible that these two PLV-A RNA positive samples could have been contaminated (e.g., at blood collection, plasma separation, RNA extraction or qPCR) or that the

PLV-A primers cross-react with PLV-B. However, the latter seems less likely, as one of the two mountain lions with PLV-A had undetectable levels of PLV-B viremia (cat 92).

Overall, these preliminary results suggest that PLV RNA levels for both bobcats and mountain lions are within a similar range to those reported for PLV-B positive mountain lions (1). However, due to differences in methods, the majority of the samples tested here are below the LLOQ of our assays. The notable differences between our method and that used by Blake et al. (1) are the standard curve range and the plasma sample volume. The standard range used by Blake et al. (1) was $3.2x10^2$ to $3.2x10^8$ copies, with $3.2x10^2$ being set as their lower limit of detection, and the volume of plasma used ranged from 100 µl to 6.5 ml. By using larger sample volumes, and therefore presumably higher starting quantities of viral RNA, the results for the majority of samples in the study by Blake et al. (1) were above their limit of detection (i.e., 21 of 24 samples with reported values).

Assay modifications. Given that the majority of the samples tested (17 of 21) had values below the assay LLOQ, experiments were conducted to try to lower the LLOQs. Assay modifications were conducted in the following order: (i) addition of carrier nucleic acid to the standard curve, (ii) optimization of primer annealing temperature, and (iii) increase in the volume of cDNA added per well.

Yeast tRNA (Life Technologies) was added to the standard diluent (TE buffer) as a carrier. Carrier nucleic acids are added to standard curves to prevent the target nucleic acid (e.g., plasmid) from adhering to tube walls (7). The addition of a carrier can improve the reliability of standard detection, particularly at low concentrations (i.e., increasing the linear range of the standards (7)). The concentrations of yeast tRNA tested were 5, 10, 20 or 50 ng/µl; these concentrations were selected based on the range of carrier concentrations previously reported for

PCR assays (1, 3, 8). Standard concentrations tested were 10 to 10^7 (10-fold dilution), plus 20, 40, 60 and 80 plasmid copies/well. The addition of 5, 10 and 20 ng/µl yeast tRNA did not lower the LLOQ (i.e., Ct values of the standards overlapped below 100 copies/well, regardless of yeast tRNA concentration). With 50 ng/µl of yeast tRNA, the relationship between standard concentration and Ct value was no longer linear over the full-range of standards. Although the addition of yeast tRNA at 5, 10 and 20 ng/µl did not improve or hinder plasmid quantification, we selected the 10 ng/µl (middle value) as the concentration to be added to the standards for all subsequent experiments.

Next, we optimized the primer annealing temperatures to improve assay performance (i.e., improve the standard curve amplification efficiencies and R^2 values). The annealing temperatures were optimized for the proviral assays by conventional PCR (Templin-Hladky et al., unpublished); however, as the efficiency of the PLV-B standard curve tended to be low (i.e., values at or below the acceptable range of 90-105%; Real-Time PCR Application Guide, Bio-Rad), we decided to conduct an additional annealing temperature optimization experiment. PLV-A and PLV-B standards (10^3 to 10^7 copies/well) were tested at eight different temperatures (range 52 to 62°C) using the temperature gradient function on the real-time instrument. For both assays, lower annealing temperatures (52, 52.8 and 54.1°C) resulted in higher standard curve efficiencies and R^2 values. Given that the efficiency and R^2 values were similar amongst the three lowest annealing temperatures, we selected the middle temperature (52.8°C) as the annealing temperature for all subsequent experiments.

Despite the addition of yeast tRNA and lowering the annealing temperature, the LLOQs remained at 100 copies/well. Thus, we decided to increase the amount of cDNA added into each reaction (i.e., doubling the volume of cDNA added to each well by replacing 5 µl of nuclease-

free water with an additional 5 µl of cDNA). In theory, with this modification, more sample values should come above each assay LLOQ. However, the SQ values reported for these samples were not reliable, as the increase in cDNA volume resulted in non-exponential amplification curves. The samples amplified between 10-15 cycles, plateaued between 15-30 cycles and then amplified between 30-40 cycles. The reason that the amplification curves plateaued between 15-30 cycles is not known. It is possible that one of the reagents used for cDNA synthesis inhibits PCR amplification in a concentration-dependent manner.

FUTURE DIRECTIONS

The following assay modifications could be conducted in an attempt to accurately quantify PLV-A and PLV-B in plasma samples that have concentrations near/below the LLOQ of the current method: (i) purifying the cDNA to eliminate substances that may have inhibited PCR amplification (e.g., with QIAquick PCR Purification kit (Qiagen)) and then doubling the cDNA volume, (ii) increasing the starting volume of plasma used for viral RNA extraction (i.e., up to 560 µl of plasma can be used for RNA extraction), (iii) using different primers, (iv) using a different qPCR master mix, and/or (v) using a different type of carrier nucleic acid (e.g., *E. coli* tRNA). Although increasing the plasma volumes would be the easiest modification, this will only be done as a last resort because sample volumes are limited. In addition to these modifications, we need to establish PLV-A and PLV-B positive controls to use with each experiment. Ideally, these controls would be used in each RNA extraction experiment and subsequent qPCR assays; however, as plasma volumes are limited, the positive controls might only be used in the qPCR assays.

Once assay development and validation are complete, the plasma samples listed here will need to be re-tested using the updated assay conditions. Additional samples will also need to be

tested (e.g., plasma samples from PLV-A positive mountain lions, as well as additional samples of PLV-B positive mountain lions and PLV-A positive bobcats) in order to determine whether mountain lion PLV-A viremia is lower than bobcat PLV-A and mountain lion PLV-B viremia. These results could add further support to the hypothesis proposed by Franklin and Troyer et al. (4) that PLV-A is of bobcat origin, and has been transmitted to mountain lions. In addition, the assays described here could be used to (i) screen samples of unknown PLV status for evidence of infection (i.e., actively replicating virus), (ii) differentiate PLV-A and PLV-B infections in mountain lions, (iii) assess viral kinetics of individual cats (i.e., repeated samplings), and/or (iv) examine the PLV strain(s) circulating in Florida panthers before and after the introduction of mountain lions from Texas in 1995 (10).

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CHAPTER 6: CONCLUSIONS AND FUTURE DIRECTIONS

Studies on feline immunodeficiency viruses (FIVs) are beneficial for understanding viral pathogenesis and the host immune response associated with lentivirus disease progression (e.g., domestic cat FIV), and for understanding viral kinetics and molecular ecology associated with naturally occurring infections in wild felids (e.g., bobcats and mountain lion FIVs). However, the lack of feline-specific assays has hampered thorough investigations in these research areas. Thus, a goal of the research described here was to develop and validate microsphere immunoassays (MIAs) for the evaluation of the domestic cat immune response, and quantitative PCR assays for the evaluation of bobcat and mountain lion viral RNA loads.

The advantage of developing MIAs to evaluate the domestic cat immune response, compared to traditional immunoassays, is that multiple analytes can be detected/quantified simultaneously, meaning that less time and sample volume are required (reviewed in (18)). Currently, 80 different analytes could be detected within a single sample simultaneously using the magnetic microspheres and instrument described in this dissertation. These assays can be used with a variety of sample types, including cell culture supernatant (Chapter 2), tissue homogenates (1, 6), plasma (Chapters 3 and 4) and other fluids, such as saliva (5, 16) or urine (14, 17). Multiplex MIAs can only be developed as long as there is no cross-reactivity among analytes and that the analytes are present in concentrations of a similar order of magnitude. For example, total IgG and IgA could not be multiplexed because of the difference in dilution required for quantification (Chapter 4). Nonetheless, MIAs are a powerful new technology to evaluate multiple analytes within biological samples and are relatively easy to develop.

In Chapters 2 and 3, we developed, validated and demonstrated the use of cytokine MIAs for the quantification of interferon gamma (IFNγ), interleukin (IL)-10, IL-12/23 in cell culture supernatant and plasma. The results of these experiments indicated that cytokine secretion and/or detection varies both among and between sample types, and that mRNA cytokine expression may not correlate with secreted protein levels. Future use of these assays could include establishing the normal range of cytokines for domestic cats, or evaluating the cytokine response elicited by a variety of pathogens, disease states (e.g., cancer or inflammation), vaccines or therapies. To provide a more complete understanding of the domestic cat cytokine response, the MIAs could be expanded to include additional cytokines for which reagents are commercially available, such as IL-1β, IL-2, IL-4, IL-5, IL-6 and/or tumor necrosis factor alpha.

In Chapter 4, we developed, validated and demonstrated the use of antibody MIAs for the quantification of total IgG and IgA in plasma, and for the detection of IgG and IgA antibodies to feline CD134, and FIV capsid (CA) and surface (SU) proteins in plasma. The results from these experiments demonstrated that the kinetics and/or level of the antibody response can vary by FIV strain/pathogenicity. Future use of these assays could include evaluating the antibody response generated with the FIV vaccine and/or in various FIV-infection studies, as well as evaluating the total antibody response of domestic cats generated by a variety of pathogens, disease states, vaccines or therapies. To provide a more complete understanding of the antibody response to FIV, the MIAs could be expanded/modified to include different capture proteins (other viral proteins or specific epitopes), to detect different antibodies (IgM or IgG subclasses), and/or to test additional sample types (mucosal secretions, such as saliva). Additionally, a MIA could be developed as a surrogate for FIV neutralization, using methods similar to those previously described for other viruses (2, 4). For example, the level of antibodies to particular regions of

the FIV SU protein (such as the V4 and V5 regions, which are involved with neutralization (11-13, 20)) could be positively correlated with virus neutralization titers (as demonstrated with equine arteritis virus (4)). Alternatively, the presence of FIV neutralizing antibodies in a sample could be measured indirectly, as the percent inhibition of soluble CD134 binding to the SU protein attached to microspheres (as demonstrated with Nipah and Hendra viruses (2)).

With modifications and/or additional experiments, the MIAs described in this dissertation could be used for FIV diagnostic or prognostic purposes, as well as to distinguish FIV-positive from vaccinated cats. In order to use the FIV-specific IgG MIA as a diagnostic assay, experiments would need to be conducted to demonstrate that the MIA has equivalent specificity and sensitivity to the current serodiagnostic assay (SNAP FIV antibody/FeLV antigen combo test, IDEXX Laboratories). Using MIAs, the presence/absence of particular cytokines or antibodies in domestic cat plasma/serum could be used to predict disease outcome. With HIV patients, for example, low initial titers or a decline in α-CA antibodies correlate with a faster rate of disease progression (3, 7, 19); increasing levels of serum IL-10 are associated with disease progression (15); and higher plasma concentrations of IL-1α and IL-7 correlate with an increased risk of $CD4^+$ T-cell loss (10). Additionally, a MIA could be developed to differentiate infected and vaccinated animals by multiplexing the two enzyme-linked immunosorbent assays currently used for differentiation (TM peptide and formalin-treated FIV capture antigens; (8)). Alternatively, given that the FIV vaccine contains inactivated whole virus from clades A and D ((9); Fel-O-Vax FIV, Fort Dodge Animal Health), it might be possible to develop a MIA to detect antibodies specific to the formalin-treated viruses used in the vaccine. Detectable antibodies to both clade A and D formalin-treated virus/viral proteins would suggest that the cat had been vaccinated versus naturally infected.

As demonstrated in this dissertation, these newly developed feline-specific assays will be useful to evaluate the immune response of domestic cats infected with FIV (in addition to previously used assays), or to evaluate viral RNA levels of wild felids naturally infected with FIV. By validating each of the domestic cat MIAs (according to published guidelines), we demonstrated the accuracy and precision of the assays, and established a lower limit of quantitation (LLOQ). Equivalent validation experiments will also be conducted for the quantitative PCR assays once development is complete. Although data from non-validated assays are useful for determining whether an analyte of interest is detectable, the reported values lack accuracy/confidence. By validating the new assays, as described here, we demonstrated the method repeatability and LLOQ, and thus provided confidence in the values reported for samples within the quantitation range.

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