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Domestic cat microsphere immunoassays: Detection of antibodies during feline immunodeficiency virus infection

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Abstract

Microsphere immunoassays (MIAs) allow rapid and accurate evaluation of multiple analytes simultaneously within a biological sample. 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 of IgG and IgA antibodies to feline immunodeficiency virus (FIV) capsid (CA) and surface (SU) proteins, and feline CD134 in plasma. These assays were used to examine the temporal antibody response of domestic cats infected with apathogenic and pathogenic FIVs, and domestic cats infected with parental and chimeric FIVs of varying pathogenicity. The results from these studies demonstrated that a) total IgG antibodies increase over time after infection; b) -CA and -SU IgG antibodies are detectable between 9–28 days post-infection and increase over time, and these antibodies combined represent a fraction (1.8 to 21.8%) of the total IgG increase due to infection; c) measurable -CD134 IgG antibody levels vary among individuals and over time, and are not strongly correlated with viral load; d) circulating IgA antibodies, in general, do not increase during the early stage of infection; and e) total IgG, and -CA and -SU IgG antibody kinetics and levels vary with FIV viral strain/pathogenicity. The MIAs described here could be used to screen domestic cats for FIV infection, and to evaluate the FIV-specific or total antibody response elicited by various FIV strains/other diseases.

Keywords

Domestic cat; feline immunodeficiency virus; microsphere immunoassay; antibody detection; multiplexing

1. Introduction

Microsphere immunoassay (MIA) technology allows for the simultaneous detection of multiple analytes within biological samples (e.g., cytokines, chemokines or antibodies) (reviewed in (Vignali, 2000)). 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 a sample. The detection or quantification of each analyte is

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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 (Watson et al., 2009), foot-and-mouth disease virus (Clavijo et al., 2006), human immunodeficiency virus (HIV; (Bellisario et al., 2001; Tomaras et al., 2008)), *Plasmodium falciparum* (Fouda et al., 2006), *Toxoplasma gondii* (Kaul et al., 2004), West Nile virus (Wong et al., 2004), and a panel of five select agents (Biagini et al., 2005). Assays, such as these, can be used for screening or diagnostic purposes (Biagini et al., 2005), or investigating antibody kinetics (Watson et al., 2009).

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 (Yamamoto et al., 1988; Pedersen et al., 1989; Elder et al., 2010). Despite a vigorous immune response, FIV and HIV infections are life-long and infected individuals succumb to opportunistic infections. Given the high degree of similarity between these two viruses, the domestic cat is a useful 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) (O'Connor et al., 1989; Hosie and Jarrett, 1990; Egberink et al., 1992; Grant, 1995a). Antibodies to these viral proteins are generally detected within weeks post-infection and increase during the early stage of disease (Reid et al., 1991; Egberink et al., 1992; Rimmelzwaan et al., 1994; Calzolari et al., 1995; Grant, 1995a; Burkhard et al., 2001; Grant et al., 2009). The function of these virus-specific antibodies can vary in terms of disease outcome. For example, antibodies to specific FIV SU epitopes, such as the V4 and V5 regions, are capable of neutralizing the virus (Siebelink et al., 1993; Siebelink et al., 1996; Samman et al., 2010; Willett et al., 2010) and, as observed with HIV patients, the presence of -Gag/CA antibodies correlates with delayed disease progression (Lange et al., 1986; Weber et al., 1987; Cheingsong-Popov et al., 1991; Chargelegue et al., 1995; Binley et al., 1997). The level and kinetics of the FIV-specific antibody response varies depending on inoculation route and/or viral strain, as well as among individual cats (Rimmelzwaan et al., 1994; Calzolari et al., 1995; Burkhard et al., 2001; Grant et al., 2009). While most studies evaluating humoral response during FIV infection have focused on IgG FIV-specific antibodies, IgM and IgA FIV-specific antibodies are also detected in plasma/sera (Grant, 1995a; Burkhard et al., 2001).

In addition to anti-viral antibodies, FIV infected cats develop antibodies to self and non-viral antigens (Flynn et al., 1994; Grant et al., 2009). For example, some cats develop antibodies to CD134, the primary cell receptor for FIV (de Parseval et al., 2004b; Shimojima et al., 2004). These antibodies bind to a cryptic epitope on the CD134 receptor, which is only exposed once the virus SU protein is bound (de Parseval et al., 2006), and this binding results in the displacement of SU from the cell receptor (Grant et al., 2009). Of note, 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 (Grant et al., 2009). FIV-infected domestic cats also exhibit elevated levels of IgG antibodies (Ackley et al., 1990; Grant, 1995a), due to polyclonal B-cell activation (Flynn et al., 1994). Despite the number of studies that have evaluated the antibody response of FIV-infected domestic cats 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 (O'Connor et al., 1989; Ackley et al., 1990; Hosie and Jarrett, 1990; Reid et al., 1991; Egberink et al., 1992; Flynn et al., 1994;

Rimmelzwaan et al., 1994; Calzolari et al., 1995; Grant, 1995a; Burkhard et al., 2001; Grant et al., 2009).

Here we describe the development and validation of domestic cat-specific MIAs for a) the quantification of total IgG and IgA levels, and b) the detection of IgG and IgA antibodies to FIV CA and SU proteins, and feline CD134 (subsequently referred to as recombinant protein (rProtein) MIAs). The utility of these four MIAs were demonstrated by testing plasma samples collected from domestic cats experimentally infected with various FIV strains. Plasma samples selected for testing were from two studies where significant information about immunopathology and viral load have been reported (TerWee et al., 2008; Thompson et al., 2011). 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.

In a study evaluating the impact of co-infection with pathogenic and apathogenic FIV (TerWee et al., 2008), domestic cats were inoculated with a well-characterized puma lentivirus isolate (PLV-1695; subsequently referred to as PLV) obtained from a British Columbia mountain lion (VandeWoude et al., 1997) and/or a highly virulent molecular clone of domestic cat FIV (FIV-C36, subsequently referred to as FIV-C) (de Rozières et al., 2004). Results of the co-infection study indicated that, in general, peripheral CD4⁺ T-cells were maintained in co-infected (CO) cats compared to FIV-C infected cats (TerWee et al., 2008). There was no difference in serum neutralization antibody titers among the groups at the single time-point tested (TerWee et al., 2008). We hypothesized that CO cats would have a more robust and rapid antibody response than FIV infected cats because of cross-reacting antibodies generated against PLV.

In a second study, domestic cats were inoculated IV with *in vivo* passaged virulent FIV-C, avirulent FIV-PPR (clade A virus; subsequently referred to as FIV-A) or a chimera virus FIV-PCenv (subsequently referred as FIV-A/C) (de Rozières et al., 2008; Thompson et al., 2011). The chimera virus has the FIV-A backbone with FIV-C accessory and envelope genes (i.e., FIV-C *vif*, *orfA*, *env* and part of *rev*) (de Rozières et al., 2008). The purpose for constructing the chimera virus and testing *in vivo* was to determine whether the envelope and accessory genes contributed to the increased pathogenicity of the FIV-C virus (Diehl et al., 1995; Pedersen et al., 2001). Results of this study indicated that (i) FIV-A/C cats had more similar plasma viral and proviral loads to FIV-C than FIV-A infected cats, and (ii) FIV-A/C and FIV-C cats had higher virus levels than FIV-A cats (Thompson et al., 2011), suggesting that pathogenicity is linked to the 3' viral elements (de Rozières et al., 2008; Thompson et al., 2011). We hypothesized that the antibody levels would be positively correlated with plasma viremia due to higher levels of viral antigen.

2. Materials and methods

2.1 Antibodies, standards and microspheres

Reagents and microspheres used for the development of the four MIAs are summarized in Table 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 antibodies. A primary antibody was used for the detection of -rProtein IgG antibodies because the -

IgG detection antibody cross-reacts with the Fc-tag on the recombinant proteins. The primary antibody was a cat-specific -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.

2.2 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 (de Rozieres et al., 2004) 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'-CGCGGATCCATGCCTATCCAAATAGTAAATGGAGCAC-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 OD₆₀₀ 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.

2.3 Recombinant SU and CD134 proteins

Recombinant SU_{C36}-Fc, SU_{PPR}-Fc and CD134-Fc (subsequently referred to as SU_{FIV-C}, SU_{FIV-A} and CD134, respectively; provided by J. Elder) were expressed and purified from CHO cells, as previously described (de Parseval and Elder, 2001; de Parseval et al., 2004a). The Fc tag is the Fc domain of human IgG1 (de Parseval and Elder, 2001; de Parseval et al., 2004a); for SU_{FIV-C} the Fc tag is N-terminal, whereas for SU_{FIV-A} and CD134 the Fc tag is C-terminal.

2.4 Coupling of capture reagents to microspheres

Magnetic microspheres were conjugated with capture antibodies, recombinant proteins or whole virus (Table 1) as previously described (Wood et al., 2011), 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-goat antibody (R&D Systems, Minneapolis, MN) for IgG, IgA and albumin microspheres; plasma from FIV-infected cats and PE-cat IgG antibody for CA microspheres; pooled-FIV SU monoclonal antibodies (Custom Monoclonals International) and PE-mouse IgG antibody (R&D Systems) for SU_{FIV-A} and SU_{FIV-C} microspheres; -human IgG Fc (Sino Biological Inc.) and PE-mouse IgG antibody for CD134 and Fc microspheres; and A2 mouse ascites (provided by C. Mathiason) and PE-mouse IgG antibody for FeLV microspheres. A median fluorescence intensity (MFI) of >2,000 indicated successful coupling (Bio-Rad).

2.5 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 (Campbell et al., 2004). 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.

2.6 Total IgG and IgA MIA protocols

The protocols used for total IgG and IgA MIAs were similar to the method described previously (Wood et al., 2011), 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 1. Bio-Plex^{TM} 200 (Bio-Rad) maintenance and data analysis (Bio-Plex^{TM} Manager 5.0, Bio-Rad) were identical to the methods previously described (Wood et al., 2011). 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).

2.7 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 (Wood et al., 2011). 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.

2.8 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, $\text{SU}_{\text{FIV-A}}$, $\text{SU}_{\text{FIV-C}}$ and CD134 were included in the positive pool, and samples with low MFI for CA, $\text{SU}_{\text{FIV-A}}$, $\text{SU}_{\text{FIV-C}}$, 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 samples (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 (Bevins et al., 2012). Sample dilutions tested were 1:25, 1:50, 1:100, 1:500 and 1:1,000 for detecting -rProtein IgG antibodies, and 1:5, 1:10, 1:25 and 1:50 for detecting -rProtein IgA antibodies. Because there was not an ideal dilution for all analytes (i.e., at low dilutions the MFI for some analytes were limited by the binding capacity of the microspheres, whereas at high dilutions the MFI for some analytes were undetectable), we standardized the assays by using a 1:50 dilution for both of the rProtein MIAs. In each of these experiments, approximately 2,500 microspheres per analyte were added to each well.

2.9 rProtein IgG and IgA MIA protocols

The protocols used for the rProtein MIAs were similar to the method described previously (Wood et al., 2011), 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 unknown and reference samples were run in duplicate. Reagent concentrations, volumes and incubation times are summarized in Table 1. Bio-Plex™ 200 maintenance was identical to the method previously described (Wood et al., 2011). 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.

2.10 Validation rProtein IgG and IgA protocols

Intra- and inter-assay experiments were conducted to determine the precision of the rProtein MIAs for CA, $\text{SU}_{\text{FIV-A}}$, $\text{SU}_{\text{FIV-C}}$ and CD134. These experiments were similar to the method previously described (Wood et al., 2011), except that FIV-C and naïve reference samples 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 samples (early and late stage of infection) were used to validate the IgG assay because at early time-points - CD134 IgG antibodies were not detectable, and at later time-points the MFI for CA and SU_{FIV-C} were limited by the binding capacity of the microspheres. 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.

2.11 Co-infection in vivo study

Antibody levels were measured in plasma collected from an experimental co-infection study, in which domestic cats were intravenously (IV) inoculated with PLV and/or FIV-C as previously described (TerWee et al., 2008). 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 was excluded from the statistical analyses as this animal had an underlying condition that resulted in abnormal immunoglobulin profiles.

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.

2.12 Chimera in vivo study

Antibody levels were measured in plasma collected from an experimental infection study, in which domestic cats were IV inoculated with FIV-A, FIV-A/C or FIV-C as previously described (Thompson et al., 2011). 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 (Thompson et al., 2011), 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} microspheres). The presence of these cross-reactive antibodies did not significantly 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} microspheres). Both SU microspheres were included in the rProtein MIAs used for the chimera study; 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 naïve cats are the average MFI of SU_{FIV-A} and SU_{FIV-C} .

2.13 Anti-CD134 IgG antibodies and plasma viremia

For the chimera study, we compared the -CD134 IgG and plasma viremia (Thompson et al., 2011) data to determine whether cats with autoantibodies to CD134 tended to have lower viral loads than cats that did not develop anti-receptor antibodies, as observed in Grant et al. (2009). Anti-CD134 IgG levels and viral load comparisons were not conducted for the co-infection study due to limited plasma viremia data.

2.14 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.

2.15 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.

3. Results

3.1 Microsphere assay conditions

Optimized assay conditions are summarized in Table 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.

3.2 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 2). For the rProtein MIAs, the intra- and inter-assay percent CVs were within the acceptable range for CA, SU_{FIV-A} , SU_{FIV-C} and CD134 (Table 3). The MFI values for IgG antibodies to Fc and FeLV in the two FIV reference samples were 17–53 and 7–49, respectively. The MFI

values for IgA antibodies to Fc and FeLV in the FIV reference samples were 23–52 and 11–37, respectively. For the naïve reference sample, the MFI values for IgG and IgA rProtein antibodies to all of the analytes combined ranged from 4–46 and 2–68, respectively.

3.3 Co-infection study antibody response

3.3.1 IgG antibody response

Total IgG: The concentrations for total IgG ranged from 7.0 to 45.4 mg/ml. The kinetics and level of the total IgG response differed among the four treatment groups (p-values <0.001; Fig. 1a). There were significant differences in the antibody kinetics between all pairs (p-values <0.05), except for FIV-C versus CO, and sham versus 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). Three of the five plasma samples were not available for testing from PLV cats on day 17.

Anti-CA IgG: The -CA IgG MFI increased over time for FIV exposed animals. From day 17 through 66, MFI values for individual cats/group means were limited by the binding capacity of the microspheres (i.e., antibody binding was saturated using a 1:50 dilution; Fig. 1b). Therefore, statistical analyses were conducted on MFI values obtained from days -4 through 17, representing a time frame where differences among groups means could be examined within the measurable range of the assay. During this time frame, 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 compared to both sham and PLV cats, and CO cats compared to both sham and PLV cats (p-values <0.05). The -CA IgG response was on average higher for CO than FIV-C cats, and higher for PLV than sham cats (p-values <0.001). Elevated -CA IgG levels were detected at day -4 in the two groups of cats inoculated with PLV at day -28 (PLV and CO).

Anti-SU IgG: The -SU IgG MFI increased over time for FIV exposed animals. The -SU_{FIV-C} IgG response differed over time among the four treatment groups (p <0.001; Fig. 1c). There were significant differences in both the kinetics and level of the antibody response of FIV-C cats compared to both sham and PLV cats, and CO cats compared to both sham and PLV cats (p-values <0.001).

Anti-CD134 IgG: The majority of cats did not have antibodies attributed to CD134; most of the values were below the non-specific antibody binding level or the mean MFI + 2SD of antibodies binding to Fc microspheres (MFI of 140, Fig. 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. On 9, 17 and 24 days post-infection, one cat in the CO group clearly had elevated -CD134 IgG antibodies (MFI 732, 529 and 253, respectively). Two other cats in the CO groups had MFI levels slightly above the non-specific antibody level (MFI of 142 and 155) on day 9. 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). There was a statistical difference among the four groups at day 66 (p <0.001); however, there was not difference between the FIV-C and CO cats, which were the two groups with IgG antibodies attributed to CD134.

3.3.2 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. The range of CA-specific IgG antibodies was 1.3 to 11.1%, and for SU-specific antibodies was 0.2 to 9.6%. For individual

cats, the estimated percent of antibodies that were FIV-specific (i.e., CA plus SU) ranged from 2.5 to 19.2%.

3.3.3 IgA antibody response—There were no observable differences in the peripheral IgA response among the four groups. 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) or -CA IgA (MFI range 36 to 7,807; average MFI of 370). The majority -SU_{FIV-C} IgA MFI values (87 of 89) and all of the -CD134 IgA MFI values were below the non-specific antibody binding level (MFI of 588).

3.4 Chimera study antibody response

3.4.1 IgG antibody response

Total IgG: The concentrations for total IgG ranged from 3.1 to 29.3 mg/ml. The kinetics and level of the total IgG antibodies differed among the four treatment groups ($p < 0.05$; Fig. 2a). There were significant differences in the antibody kinetics of sham cats compared to 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 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 ($p < 0.01$).

Anti-CA IgG: The -CA IgG MFI increased over time for FIV exposed animals. From day 21 through 326, MFI values for individual cats/group means were limited by the binding capacity of the microspheres (i.e., antibody binding was saturated using a 1:50 dilution; Fig. 2b). Therefore, statistical analyses were performed on MFI values obtained from days 7 through 35, representing a time frame where differences among groups means could be examined within the measurable range of the assay. During this time frame, the 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 compared to both FIV-C and FIV-A/C cats, and between FIV-C and FIV-A/C cats (p -values < 0.05).

Anti-SU IgG: The -SU IgG MFI increased over time for FIV exposed animals. By day 326, MFI values for individual cats/groups means were again limited by the binding capacity of the microspheres (i.e., antibody binding was saturated using a 1:50 dilution; Fig. 2c). Therefore, statistical analyses were performed on MFI values obtained from days 7 through 67. During this time frame, 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 -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 majority of cats did not have antibodies attributed to CD134; most of the values were below the non-specific antibody binding level (MFI of 91, Fig. 2d). None of the cats 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 -CD134 IgG antibodies (MFI range 154 to 3,148). There was a statistical difference among the groups at this time-point ($p < 0.001$); however, there was no difference between FIV-C and FIV-A/C cats, which were the two groups with IgG antibodies attributed to CD134. By day 326, four of the seven cats no longer had detectable -CD134 IgG antibodies, and the MFI levels had decreased in two other cats. One of the FIV-C cats had an increase in -CD134 IgG antibodies from day 67 to 326 (MFI 3,148 to 10,137), and one FIV-A cat had detectable -CD134 IgG antibodies at day 326 (MFI 213). There were no statistical differences among the groups on day 326.

3.4.2 Anti-CD134 IgG antibodies and plasma viremia—We noted that CD134 IgG antibodies were associated with lower plasma viral loads among FIV-C cats. The two FIV-C infected cats that had high levels of α -CD134 IgG antibodies (>1,000 MFI) at day 67 had lower plasma viral loads from day 173 to 326 than the two other FIV-C infected cats in this study (data not shown). This trend was not observed for FIV-A/C cats, which also had α -CD134 IgG antibodies on day 67. Due to low sample sizes and variable α -CD134 IgG results in this study, we were unable to make statistical comparisons among the groups.

3.4.3 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. The range of CA-specific IgG antibodies was 0.06 to 10.1%, and for SU-specific antibodies was 1.4 to 19.8%. For individual cats, the estimated percent of antibodies that were FIV-specific (i.e., CA plus SU) ranged from 1.8 to 21.8%.

3.4.4 IgA antibody response—The only notable difference in peripheral IgA kinetics among the groups was for α -CA antibodies ($p < 0.05$; Fig. 3). There were significant differences in the antibody kinetics of FIV-A cats compared to 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 ($p < 0.01$).

The majority of cats did not have IgA antibodies attributed to SU. On days 7 and 14, all cats had α -SU values below the non-specific antibody binding level (MFI of 821). From day 21 to 67, five (of 72) samples were positive (MFI range 862 to 2,081; four values from two FIV-C cats and one from a FIV-A cat). 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. There was a statistical difference among the groups at day 326 ($p < 0.01$); however, there was no difference between the FIV-A and FIV-C cats, which were the two groups that had an IgA antibody response attributed to SU.

There were no differences among the treatment groups over time for total IgA (range 0.1 to 2.8 mg/ml, average 0.9 mg/ml). None of the cats detectable IgA antibodies to CD134; all values were below the non-specific antibody binding level (MFI 821).

3.5 Peripheral/circulating antibody response

The results from these analyses of temporal antibody responses after FIV exposure demonstrate that a) total IgG antibodies increase over time; b) α -CA and α -SU IgG antibodies are detectable between 9–28 days post-infection and increase over time, and that these antibodies combined represent a fraction (1.8 to 21.8%) of the total IgG increase; c) measurable α -CD134 IgG antibody levels vary among individuals and over time, and are not strongly correlated to viral load; d) circulating IgA antibodies, in general, do not increase during the early stage of FIV infection; and e) total IgG, α -CA and α -SU IgG antibody kinetics and level vary with FIV viral strain/load. In the co-infection study, total and α -CA IgG antibodies tended to be higher in the coinfection group, despite equivalent plasma FIV viral loads. In the chimera study, the α -CA and α -SU IgG levels tended to be higher in cats with higher FIV viral loads (FIV-C and A/C). A summary of peripheral/circulating antibody response is presented in Figure 4.

4. 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 (TerWee et al., 2008; Thompson et al., 2011).

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 (Ackley et al., 1990; Grant, 1995a). 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 groups. 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, the initial increase in total IgG due to PLV was increased further by the subsequent FIV-C infection. In both studies, cats with relatively higher FIV viral loads (specifically FIV-C and FIV-A/C, but not FIV-A 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 (Ackley et al., 1990; Grant, 1995a), likely due to ongoing polyclonal B-cell activation. However, here we document differences in the level of the total IgG increase relating to virulence (e.g., viral load and CD4⁺ T-cell perturbations).

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 reflect the fact that 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). This cross-reactivity or anamnestic immune response likely contributed to higher -CA IgG response in CO versus FIV-C cats at early time-points. It is unknown whether the increase in -CA antibodies prior to FIV-C infection could have contributed to the observed CD4⁺ T-cell maintenance in the CO cats (TerWee et al., 2008).

In the chimera study, the early temporal dynamics observed for the -CA and -SU IgG responses correspond with the early viral and proviral load kinetics, as FIV-C cats had the highest viral loads, followed by FIV-A/C and then FIV-A cats (Thompson et al, 2011). This finding suggests that 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. The kinetics and level of the rProtein IgG responses were similar between FIV-A/C and FIV-A cats for CA, and between FIV-A/C and FIV-C cats for SU, which are 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 -CA and -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 variability among the FIV-A cats on days 28 and 35.

Although a statistical association between -CD134 IgG antibodies and viral load/clinical outcome could not be made, we did note a trend similar to that observed by Grant et al. (2009). In the chimera study, FIV-C cats with -CD134 IgG antibodies had lower plasma viral loads. None of the cats infected with PLV developed -CD134 IgG antibodies. This is perhaps because CD134 is not the primary receptor for PLV cell entry (Smirnova et al., 2005). Despite this fact, CO cats developed detectable IgG antibodies against CD134 earlier after FIV-C infection than the FIV-C single infection group. The differences between our results and Grant et al. (2009) could be attributable to differences between the ELISA and

MIA relating to detection antibodies, reagent concentrations, sample dilution, assay sensitivity or other factors.

Given that FIV-infected domestic cats exhibit elevated levels of total IgG antibodies (Ackley et al., 1990; Grant, 1995a) and that these antibodies are not entirely virus-specific (Flynn et al., 1994), we estimated the increase in total IgG antibodies that were CA- or SU-specific. The percent 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%. The estimated percent of antibodies that were FIV-specific (i.e., CA plus SU) for all individuals ranged from 1.8 to 21.8%. The actual percent of FIV-specific antibodies would be higher as we did not measure antibodies against FIV antigens other than CA and SU. It is unknown what the majority of IgG antibodies are directed against, or how these values would compare to percentage of antibodies developed to other viral pathogens. It would be interesting to obtain similar percentages for non-viral and self antigens, as described by Flynn et al. (1994), to compare with the percentages obtained here for CA and SU proteins.

The total IgA concentrations obtained for cats in the two *in vivo* studies are similar to values previously reported for uninfected and FIV-infected domestic cats measured by ELISA (Ackley et al., 1990; Grant, 1995a). Previous studies have documented normal levels of total IgA in FIV-infected cats during the first year of infection (Grant, 1995a) and at 24–28 weeks post-infection (Ackley et al., 1990); however, elevated levels of total IgA have been noted with a few healthy, FIV-infected domestic cats (Grant, 1995a). Nonetheless, neither the co-infection nor the chimera study presented temporal differences in total IgA levels.

The most notable finding related to IgA was the elevation of -CA IgA antibodies in FIV-A infected cats at day 326. This group also had the highest -SU IgA response at this time point. These data suggest that 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 to three months post-infection), and/or that strain specific differences in the anti-viral IgA response may occur. It is possible that the elevated levels of peripheral IgA in the FIV-A infected cats are due to differences in virus virulence and/or tropism; some strains of FIV-A are neurotropic (Dow et al., 1990) and cause less severe disease in the periphery than FIV-C (e.g., lower viral loads, and less severe decreases in CD4⁺ T-cells and neutrophils (Pedersen et al., 2001)). Burkhard et al. (2001) 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. Additionally, in this study by Burkhard et al. (2001), no peripheral IgA antibodies were detected against the envelope protein after IV or vaginal inoculation. Grant (1995a) also noted that some cats developed -CA IgA antibodies two months after infection, and that the number of cats with detectable antibodies varied by FIV strain.

The results presented here provide the most comprehensive analysis to date, particularly for early stages of infection, and for *in vivo* virus challenge studies where viral kinetics and other clinical parameters have been well described. We were able to develop antibody assays that were capable of detecting multiple analytes simultaneously, including the detection of albumin, which served as a control for the presence of plasma. The detection of albumin was particularly important for samples from early time-points, as FIV-specific antibodies are not detectable in plasma. Since this study did not include comparisons of the MIAs to conventional immunoassays, such as ELISA or immunoblot, we were unable to make direct comparisons to previous studies that have evaluated the antibody response to FIV (O'Connor et al., 1989; Ackley et al., 1990; Hosie and Jarrett, 1990; Reid et al., 1991; Egberink et al., 1992; Flynn et al., 1994; Rimmelzwaan et al., 1994; Calzolari et al., 1995; Grant, 1995a; Burkhard et al., 2001; Grant et al., 2009). Nonetheless, advantages of MIAs

are that less time and samples volume are required (e.g., compared to ELISAs and immunoblots), and the data output is quantitative, meaning that more detailed information about the specific antibody response to multiple analytes can be obtained (e.g., compared to immunoblots).

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 -CA and -SU IgG MFI were limited by the binding capacity of the microspheres). Given that the relationship between analyte concentration and signal for immunoassays are not generally linear (Baud, 1993), MFI values obtained with different dilutions may not be comparable. Thus, single dilution was used in rProtein experiments in order to compare the 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 at 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 per cat), it is possible that the -rProtein IgA antibodies were out-competed by the higher levels of -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 (Grant, 1995b)) prior to testing. Despite the above limitations, the rProtein assays would be useful to determine whether a cat has detectable -CA or -SU antibodies for diagnostic purposes.

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 (Tomaras and Haynes, 2009; Baum, 2010)). Given that IgG subclasses have different functional properties and that the presence of human IgG subclasses can vary with HIV disease state (reviewed in (Tomaras and Haynes, 2009)), it would be interesting to compare the kinetics and levels of the three (possibly four) domestic cat IgG subclasses (Grant, 1995b) throughout FIV infection.

Future studies could assess total IgG and IgA response 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), additional viral proteins (e.g., TM or specific FIV protein epitopes), or adapted to test other sample types, such as saliva. Additionally, a MIA could be developed to differentiate FIV infected and vaccinated animals by either multiplexing the two enzyme-linked immunosorbent assays currently used for differentiation (TM peptide and formalin-treated FIV as capture antigens; (Levy et al., 2008)) or detecting antibodies specific to the formalin-treated FIV-A and FIV-D viruses used in the vaccine (Fel-O-Vax FIV, Fort Dodge Animal Health; (Pu et al., 2001)). Assays such as these will be useful for assessing the IgG and IgA response associated with disease pathogenesis and/or protective vaccine immunity.

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Fig. 1. Co-infected cats have elevated levels of total IgG and α -CA IgG antibodies compared to FIV-single infected cats. 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 IV 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 $\pm$ SE. For the recombinant proteins with Fc tags (SU and CD134), the dotted horizontal line represents the level of non-specific antibody binding to the Fc tag (mean MFI + 2SD for α -Fc IgG antibodies; MFI 140). Arrows indicate where the MFI values for individual cats/group means were limited by the binding capacity of the microspheres (i.e., antibody binding was saturated using a 1:50 dilution).

Fig. 2.

The trends in the temporal IgG antibody responses correspond with FIV virulence. Total IgG (a), α -CA IgG (b), α -SU IgG (c) and α -CD134 IgG (d) responses for cats from the chimera study. Cats were IV inoculated on day 0 with FIV-A, FIV-A/C or FIV-C. Data points represent the mean MFI + SE. For the recombinant proteins with Fc tags (SU and CD134), the dotted horizontal line represents the level of non-specific antibody binding to the Fc tag (mean MFI + 2SD for α -Fc IgG antibodies; MFI 91). Arrows indicate where the MFI values for individual cats/group means were limited by the binding capacity of the microspheres (i.e., antibody binding was saturated using a 1:50 dilution).

Fig. 3. FIV-A infected cats have elevated levels of α-CA IgA antibodies approximately one year after infection. Cats were IV inoculated on day 0 with FIV-A, FIV-A/C or FIV-C. Data points represent the mean MFI + SE.

Fig. 4.

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 [PLV + FIV-C]: (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; 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%.

Table 1

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

Assay	Total IgG	rProtein IgG	Total IgA	rProtein IgA
<i>Analytes detected</i>	IgG Albumin	-CA IgG -SU _{FIV-A} IgG -SU _{FIV-C} IgG -CD134 IgG -Fc IgG -FeLV IgG Albumin	IgA Albumin	-CA IgA -SU _{FIV-A} IgA -SU _{FIV-C} IgA -CD134 IgA -Fc IgA -FeLV IgA Albumin
<i>Microspheres</i>				
Microspheres/analyte per well	2,500	2,500	2,500	2,500
Capture reagent (region) ^a	-cat IgG Ab ^b (77) -cat albumin Ab ^b (13)	CA (35) SU _{FIV-A} (65) SU _{FIV-C} (43) CD134 (54) Fc (29) FeLV (28) -cat albumin Ab ^b (13)	-cat IgA Ab ^b (63) -cat albumin Ab ^b (13)	CA (35) SU _{FIV-A} (65) SU _{FIV-C} (43) CD134 (54) Fc (29) FeLV (28) -cat albumin Ab ^b (13)
<i>Standards and plasma samples</i>				
Standard ^b range (ng/ml)	7.8-1,000	-	3.9-500	-
Plasma dilution	1:200,000	1:50	1:10,000	1:50
μl/well (incubation min)	50 (60)	50 (60)	50 (60)	50 (60)
<i>Primary Ab</i>				
Antibody (ng/ml)	-	-cat IgG mAb ^d (400)	-	-
μl. well (incubation min)	-	50 (30)	-	-
<i>Detection Ab</i>				
Antibodies (ng/ml)	PE -cat IgG ^c (400) PE -cat albumin ^c (200)	PE -mouse IgG ^e (400) PE -cat albumin ^c (200)	PE -cat IgA ^c (400) PE -cat albumin ^c (200)	PE -cat IgA ^c (400) PE -cat albumin ^c (200)
μl. well (incubation min)	100 (3)	50 (30)	100 (30)	50 (30)

^aMagPlex-C microsphere region was assigned by the manufacturer; the region is based on the ratio of two internal dyes^bReagents from Bethyl Laboratories^cUnconjugated antibodies (Bethyl Laboratories) were conjugated with phycoerythrin (PE) using the EasyLink R-Phycoerythrin conjugation kits (Abcam)^dMonoclonal antibody (GPB2-2B1) from Custom Monoclonals International^eAntibody from R&D Systems

Table 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
<i>Intra-assay</i>		
High	89 (15)	101 (12)
Medium	93 (7)	102 (5)
Low	87 (3)	105 (5)
<i>Inter-assay</i>		
High	88 (12)	82 (5)
Medium	93 (6)	96 (5)
Low	98 (7)	90 (5)

Table 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
<i>IgG antibodies</i>				
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)
<i>IgA antibodies</i>				
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)	-

Table 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
<i>Co-infection</i>		
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)
<i>Chimera</i>		
^a FIV-C	0.06–1.4 (1.4)	1.4–5.7 (1.4)
^a 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)

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