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Microsphere immunoassay for the detection of cytokines in domestic cat (*Felis catus*) plasma: Elevated IL-12/23 in acute feline immunodeficiency virus infections

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Abstract

We recently described the development and validation of a highly sensitive and specific microsphere immunoassay capable of simultaneously quantifying three domestic cat cytokines in tissue culture supernatant. Here we describe the modification of this assay to measure interferon gamma (IFN γ), interleukin (IL)-10 and IL-12/IL-23 p40 (IL-12/23) in domestic cat plasma, report values obtained from plasma collected after feline immunodeficiency virus (FIV) exposure, and compare plasma concentrations to blood cell mRNA expression. The validated quantitation limits of this assay are 31 to 1000 pg/ml for IFN γ , 63 to 2000 pg/ml for IL-10, and 20 to 625 pg/ml for IL-12/23. Plasma cytokine levels from domestic cats infected with pathogenic and/or apathogenic FIV were determined at 3–4 and 7–8 weeks post-infection. IL-12/23 was elevated ($p < 0.05$) during acute infection with both FIV strains in two similar studies, conducted five years apart in different feline cohorts ($n = 44$ total animals). IL-12/23 concentrations ranged from 377 to 1904 pg/ml in naïve cats and 552 to 3460 pg/ml in infected cats. In contrast, the majority of plasma samples had IFN γ and IL-10 concentrations below the lowest standard tested. The inability to consistently detect levels of IFN γ and IL-10 in plasma, despite the fact that mRNA changes were detected, suggests that these cytokines may 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.

Keywords

domestic cat; microsphere immunoassay; cytokines; IL-12/23; IL-10; IFN γ

1. Introduction

Microsphere immunoassays (MIAs) are a relatively new technology capable of detecting multiple analytes simultaneously (reviewed in (Kellar and Iannone, 2002)). 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

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Conflict of interest statement

There are no conflicts of interest.

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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 (Yamamoto et al., 1988; Pedersen et al., 1989; Elder et al., 2010). 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 (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 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 puma and genetically related to domestic cat FIV (Carpenter et al., 1996), causes productive infection without clinical disease (VandeWoude et al., 1997a). 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 (TerWee et al., 2005). Previous studies conducted by our laboratory demonstrate that PLV infection before FIV infection (i.e., coinfection) blunts the peripheral CD4⁺ T-cell loss observed during the acute phase of FIV single-infection (VandeWoude et al., 2002; TerWee et al., 2008). 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 (IFN γ), interleukin (IL)-10, and IL-12/IL-23 p40 (IL-12/23) in plasma (modified from (Wood et al., 2011)), 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. IFN γ , 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 (Dean and Pedersen, 1998; Ritchey et al., 2001; Avery and Hoover, 2004; TerWee et al., 2008).

2. Materials and methods

2.1 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 IFN γ 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).

2.2 Coupling capture antibody to microspheres

Methods for coupling capture antibody to microspheres and confirmation of coupling are identical to those previously described (Wood et al., 2011), 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 (Wood et al., 2011)).

2.3 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 (Nickoloff, 1984; Wood, 1991)). Bias can also be attributed to differences between the matrices of samples and standards (Wild, 2001), and can affect the measurement of individual cytokines differently (Fichorova et al., 2008). 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.

2.4 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 2500 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 (Wood et al., 2011). 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).

2.5 Microsphere immunoassay validation

Inter- and intra-assay experiments were conducted as previously described (Wood et al., 2011). Spike concentrations were: 31, 250 and 1000 pg/ml for IFN γ ; 63, 500 and 2000 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.

2.6 Analysis of cytokines in domestic cat plasma

We quantified cytokine levels in domestic cat plasma collected from experimental coinfection studies with PLV-1695 and FIV-C36 conducted in 2005 (TerWee et al., 2008) and 2010 (unpublished). 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. PLV-1695 is a well-characterized puma lentivirus isolate obtained from a British Columbia puma (VandeWoude et al., 1997b). FIV-C36 is a highly-virulent molecular clone of domestic cat FIV that has also been well characterized (de Rozieres et al., 2004).

Samples selected from the 2005 study were 24 days post-PLV inoculation and 24 days post-FIV inoculation (days 24 and 52 as per (TerWee et al., 2008), 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, PLV-FIV 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 and 28–32 days post-FIV 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, PLV-FIV 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 blind 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.

2.7 Comparison of cytokine levels in plasma to cytokine mRNA expression

We compared plasma cytokine levels and mRNA cytokine expression at two time-points for the animals in the 2005 coinfection 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.

3. Results

3.1 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 1). The validated lower limit of quantitation (LLOQ) and upper limit of quantitation (ULOQ) for each analyte were 31 and 1000 pg/ml for IFN γ , 63 and 2000 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).

3.2. 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 1 and 2) and only two individual IL-12/23 results were above the validated ULOQ (3460 pg/ml, Figure 1; 3257 pg/ml Figure 2). Plasma concentrations ranged from 337 to 3460 pg/ml in the 2005 study, and from 479 to 3257 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 and PLV-FIV inoculated cats than sham-inoculated cats ($p < 0.05$). There were no significant differences between FIV or PLV groups with the PLV-FIV group at day 52 (Figure 1) or days 56–60 (Figure 2).

For the majority of samples from the 2005 study, the concentrations of IFN γ , regardless of time and inoculation status, were below the lowest standard tested (Figure 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 IFN γ 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 hours 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 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 2). Only two of the 48 samples had values above the LLOQ; the concentrations of these samples were 438 and 600 pg/ml.

3.3 Comparison of cytokine levels in plasma to cytokine mRNA expression

While there were significant increases in IFN γ mRNA in the FIV and/or PLV infected groups at day 52 of the 2005 study (TerWee et al., 2008), levels of IFN γ protein in plasma remained low (most below the LLOQ) for all groups (Figure 1). Similarly, IL-10 mRNA expression was increased in the FIV-infected group at day 52 (TerWee et al., 2008), but there was no detectable difference in IL-10 protein in plasma (Figure 1). In contrast, IL-12/23 protein levels in plasma were increased in PLV (day 24), and FIV and FIV/PLV (day 52) infected groups (Figure 1), without any detectable change in mRNA expression. Comparison of the protein:mRNA ratio for each cytokine (Figure 3) demonstrates that

IL-12/23 had higher protein abundance relative to the level of mRNA expression than IL-10 and IFN γ .

4. Discussion

These results demonstrate the successful development and validation of a MIA for the quantification of domestic cat cytokines IFN γ , 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 coinfection 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 1 and 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 (TerWee et al., 2008). Plasma concentrations of IL-12/23 were significantly higher in cats acutely infected with FIV; the concentrations of IL-12/23 were higher in FIV and PLV-FIV inoculated cats compared to sham cats at 24 and 28–32 days post-inoculation (Figures 1 and 2, respectively). The elevation of IL-12/23 with FIV and PLV-FIV inoculated cats is likely because of similar FIV 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 (Langrish et al., 2004). 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 IFN γ and IL-10 results were below the LLOQ. It is possible that there are differences in these cytokines during PLV and/or FIV 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 investigate if the 1-in-5 plasma dilution may have contributed to the lack of IFN γ and IL-10 detection, we compared cytokine concentrations from plasma samples diluted 1-in-2 and 1-in-5. Despite the lower dilution, IFN γ and IL-10 were quantifiable in only a few more samples (data not shown).

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 IFN γ 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 (Fichorova et al., 2008), 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-1 β , IL-2, IL-4, IL-6 and/or tumor necrosis factor α .

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Figure 1.

Cytokine concentrations from domestic cat plasma samples collected 24 days post-PLV inoculation and 24 days post-FIV 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 2.

Cytokine concentrations from domestic cat plasma samples collected 28 days post-PLV inoculation and 28–32 days post-FIV 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).

Figure 3. Plasma cytokine concentrations and PBMC cytokine mRNA expression from domestic cat sample collected 24 days post-PLV inoculation and 24 days post-FIV inoculation (day 52) of the 2005 study.

Table 1

Assay accuracy and precision over a range of analyte concentrations.

Mean % recovery (%CV)			
Spike level	IFN γ	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)