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Research paper

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 (IFNγ), 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 31–1000 pg/ml for IFNγ, 63–2000 pg/ml for IL-10, and 20–625 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 IFNγ and IL-10 concentrations below the lowest standard tested. 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 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.

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 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

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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 (IFNγ), 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. 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 (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 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).

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.