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Vaccination with *Vif*-deleted Feline Immunodeficiency Virus Provirus, GM-CSF, and TNF- α Plasmids Preserves Global CD4 T Lymphocyte Function after Challenge with FIV

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Abstract

Feline immunodeficiency virus (FIV) DNA vaccine approaches that included a *vif*-deleted FIV provirus (FIV-pPPR Δ vif) and feline cytokine expression plasmids were tested for immunogenicity and efficacy by immunization of specific pathogen free cats. Vaccine protocols included FIV-pPPR Δ vif plasmid alone; a combination of FIV-pPPR Δ vif DNA and feline granulocyte macrophage-colony stimulating factor (GM-CSF) and tumor necrosis factor (TNF)- α expression plasmids; or a combination of FIV-pPPR Δ vif and feline interleukin (IL)-15 plasmids. Cats immunized with FIV-pPPR Δ vif, GM-CSF and TNF- α plasmids demonstrated an increased frequency of FIV-specific T cell proliferation responses compared to other vaccine groups. Immunization with FIV-pPPR Δ vif and IL-15 plasmids was distinguished from other vaccine protocols by the induction of antiviral antibodies. Suppression of virus loads was not observed for any of the FIV-pPPR Δ vif DNA vaccine protocols after challenge with the FIV-PPR isolate. However, prior immunization with FIV-pPPR Δ vif, GM-CSF, and TNF- α plasmids resulted in preservation of CD4 T cell functions, including mitogen-induced cytokine expression and antigen-specific proliferation upon infection with FIV. These findings justify further examination of cytokine combinations as adjuvants for lentiviral DNA vaccines.

Keywords

FIV; DNA vaccine; cytokine adjuvants

1. Introduction

Vaccine development for human immunodeficiency virus (HIV) has utilized animal models to test a wide variety of vaccine approaches and to characterize correlates of protective

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immunity. Feline immunodeficiency virus (FIV) is a lentivirus that induces a slow but progressive immunodeficiency syndrome in cats similar to AIDS in HIV-1-infected humans and has provided a useful animal model for testing of candidate HIV-1 vaccine approaches [1–3]. DNA-based vaccines designed for FIV have been shown to induce potent cellular immune responses and enhanced protection in the feline animal model, particularly when co-inoculated with cytokine expression plasmids [4–7]. In general, use of cytokines as adjuvants has been reported to enhance antigen-specific immune responses and increase efficacy for prophylactic pathogen-targeted and therapeutic cancer vaccines [8–12]. A FIV DNA vaccine based on a replication-defective FIV_{RET} provirus (FIV RT) encoding an in-frame deletion in the reverse transcriptase gene (RT) [6], provided protection against homologous challenge when co-inoculated with an expression plasmid containing feline interferon- γ (IFN- γ) [6,13]. Subsequent studies tested co-inoculation of either interleukin (IL)-12 or IL-18 expression plasmids, with proviral DNA vaccines based on the pathogenic FIV-GL8 isolate and encoding deletions within either RT or the integrase gene [5]. These studies also revealed vaccine-induced protection against homologous challenge and partial protection against heterologous challenge with FIV_{RET} by demonstrating a significant reduction in viral load and suppression of disease in vaccinated cats after challenge.

Previous studies from our laboratory tested immunogenicity and efficacy of a FIV proviral DNA (FIV-pPPR Δ vif) vaccine encoding a deletion within the viral infectivity factor (*vif*) accessory gene. *Vif* deletion imposed a severe restriction on virus replication to generate a DNA vaccine more similar to a defective provirus rather than an attenuated virus vaccine [14]. One vaccine study revealed that immunization of cats with FIV-pPPR Δ vif proviral DNA alone elicited protection against challenge with homologous wild type FIV-PPR [15]. Later studies investigating this FIV-pPPR Δ vif DNA vaccine revealed improved antigen-specific cellular immune responses by co-expression of feline IFN- γ from the *vif*-deleted FIV provirus [16]. However, the latter study revealed poor efficacy for the FIV-pPPR Δ vif DNA vaccine, with or without incorporation of IFN- γ co-expression. Discrepancies in vaccine efficacy between earlier and later studies were possibly due to changes in vaccination schedule, timing of challenge, and/or route of challenge. Regardless, these findings supported further investigation of other cytokines as adjuvants for this FIV DNA vaccine.

Various other cytokines previously tested as adjuvants for DNA vaccine models, included granulocyte macrophage-colony stimulating factor (GM-CSF), tumor necrosis factor (TNF)- α , and IL-15 [8,10,11,17]. As a hematopoietic regulator, GM-CSF recruits and induces the maturation of antigen-presenting cells (APC) [18–21], and was shown to enhance CD4 T cell proliferation and antibody responses when used as an adjuvant for DNA vaccines [20,22–24]. TNF- α is a pleiotropic cytokine that plays a role in inflammation, and may also enhance antigen presentation by professional APC to T lymphocytes [25–28]. Furthermore, use of TNF- α in combination with other cytokines including GM-CSF was shown to produce a synergistic adjuvant effect resulting in improved immune responses and vaccine efficacy [17,29]. Lastly, IL-15 is a proinflammatory cytokine that shares similar properties with IL-2 to enhance humoral and cellular immune responses by inducing proliferation, activation, and cytokine release from lymphocytes [30–32], but is not associated with activation-induced apoptosis of T cells. IL-15 has been shown to promote longevity and survival of antigen-specific CD8 T cells and CD4-independent CD8 T cells, production and tissue trafficking of CD4, DC maturation, and increased antibody titers when used as either a therapeutic or a vaccine adjuvant [33–36]. Based on these observations, expression plasmids encoding either feline (fe) GM-CSF, TNF- α , or IL-15 were co-inoculated with the FIV-pPPR Δ vif DNA vaccine to investigate cytokine modulation of immunogenicity and efficacy of this proviral DNA vaccine. Cellular and humoral responses were detected after immunization of cats with different vaccine regimens. Cats co-immunized with the combination of FIV-pPPR Δ vif, and feGM-CSF and feTNF- α expression plasmids exhibited enhanced FIV-specific T cell proliferative responses.

Interestingly, vaccination with FIV-pPPRΔvif and feIL-15 plasmids proved more efficient for induction of FIV-specific antibodies. Improved vaccine-induced FIV-specific cellular or humoral immune responses did not result in suppression of virus loads after challenge with the uncloned biological FIV-PPR isolate. However, specific functional properties of peripheral blood CD4 T cells were preserved by vaccination with the FIV-pPPRΔvif DNA vaccine approach that included both feGM-CSF and feTNF-α expression plasmids.

2. Materials and Methods

2.1. Vaccine plasmids

Construction and characterization of the FIV-pPPRΔvif provirus plasmid encoding a 375 base pair deletion within the *vif* gene of FIV-pPPR molecular clone was previously described [14]. FIV-pPPR vaccine plasmids were tested for stability of virus expression by transfection of Crandell feline kidney (CrFK) cells, a feline adherent cell line (American Type Culture Collection, ATCC, Manassas, VA). Transfected-cell culture supernatants were tested for virus production by assay for FIV viral RNA using real-time TaqMan PCR [37]. Cytokine expression plasmids tested as vaccine adjuvants in these studies included previously described feIL-15 and feTNF-α expression plasmids (pND14-Lc-IL15 and pCDNA-TNF-α, respectively) [38, 39], as well as a newly constructed feGM-CSF expression plasmid. cDNA was prepared from cellular RNA extracted from mitogen-activated feline peripheral blood mononuclear cells (PBMC) using the RNeasy Mini Kit (Qiagen Inc., Valencia, CA). Primers (forward: AATGAAACGGTAGAA GTCGTCTCTG and reverse: CGTACAGCTTTAGGTGAGTCTGCA) were designed according to feGM-CSF sequence available in GeneBank to characterize 5' and 3' terminal feGM-CSF sequences. Using a commercial RACE PCR kit (GeneRacer™ Kit, Invitrogen), these primers together with the GeneRacer™ 5' Primer and GeneRacer™ 3' Primer included within the kit, were used to PCR amplify 5' and 3' terminal feGM-CSF sequences from cDNA. Nucleotide sequence was characterized for amplified 5' and 3' terminal feGM-CSF cDNA fragments after insertion into plasmid pCR4-TOPO (Invitrogen, Carlsbad, CA). Newly generated feGM-CSF terminal sequences were next used to design primers (forward: AGACCCAAGCTTAGGATGT GGCTGCAGACCTGC and reverse: ACTGGGAAGCTTTCACCTTCTAGACTGGCTTCCAGC) for PCR amplification, molecular cloning and sequencing of a full-length feGM-CSF cDNA. Feline GM-CSF cDNA was cloned into expression plasmid pCDNA 3.1 (Invitrogen) in the correct and reverse orientation to create expression plasmids designated pCDNA-feGMCSF and pCDNA-rfeGMCSF (negative control), respectively. Both plasmids were confirmed by DNA sequencing. Plasmid DNA for immunization was prepared using standard protocols for centrifugation to equilibrium twice in cesium chloride-ethidium bromide gradients [40].

2.2. Expression of recombinant feGM-CSF in mammalian cells

pCDNA-feGMCSF and pCDNA-rfeGMCSF plasmids were assayed for feGM-CSF expression by transfection of COS-7 cells, an African green monkey adherent cell line, using electroporation protocols previously described [37]. Cell culture supernatants were harvested and cell lysates prepared 48 hours after transfection. Secreted feGM-CSF was concentrated 2-fold from transfected cell supernatants with MicroCon YM-10 columns (Millipore, Billerica, MA). Cell lysates and concentrated supernatants were assayed for recombinant GM-CSF by western blot analysis as previously described [37] using goat polyclonal antibodies (R&D Systems, Inc., Minneapolis, MN) specific for feGM-CSF.

Biological activity of secreted recombinant feGM-CSF was tested using TF-1 cells (human erythroblast cell line, ATCC), for which replication depends on GM-CSF or IL-3 for long term growth [41]. Briefly, TF-1 cells were washed five times to remove recombinant human (rh)

GM-CSF from growth media prior to testing. TF-1 cells were then re-suspended in TF-1 growth medium (RPMI 1640 containing 10% fetal bovine serum and antibiotics) without rhGM-CSF and reseeded in a 96-well flat bottom plate (10^4 cells/100 μ l). rhGM-CSF standards and samples were serially diluted and added to TF-1 cells. Samples were obtained from concentrated supernatants of COS-7 cells transfected with either pCDNA-feGMCSF or pUC19 (negative control plasmid). Cells were incubated for 48 hr at 37°C in 5% CO₂. The MTT-based Cell Growth Determination Kit (Sigma Aldrich, St. Louis MO) and live cell counts by Trypan Blue were used to determine numbers of viable cells.

2.3. Vaccination of SPF cats with infectious plasmid DNA vaccines

Thirty-six specific pathogen free (SPF) juvenile cats aged 4 to 6 months were obtained from the Pet and Nutrition Cat Colony (University of California, Davis). Animals were housed and maintained according to regulations and guidelines of the Institutional Animal Care and Use committee. Experimental and control groups consisted of six cats each (Table 1). Vaccine groups included group 1 cats inoculated with FIV-pPPR Δ vif, pCDNA-feGMCSF, and pCDNA-TNF- α plasmid; group 2 inoculated with FIV-pPPR Δ vif and pND14-Lc-IL15; group 3 inoculated with FIV-pPPR Δ vif plasmid DNA; control group 4 inoculated with pCDNA-feGMCSF and pCDNA-TNF- α ; and control group 5 immunized with pND14-Lc-IL15 expression plasmid. The final control group (group 6) was immunized with sham vector plasmid, pND14. For each experimental and control group, cats were inoculated intramuscularly (IM) with 600 μ g of each plasmid re-suspended in sterile saline (1 ml). Groups 1 to 5 were boosted by IM inoculation with vaccine plasmids (600 μ g each) between 32 and 33 weeks after priming, and group 6 was boosted at 18 weeks after vaccination (Table 1). Immunization of the different vaccine groups was staggered over time with an initial entry of vaccine groups 3 and 6 that were subsequently followed by groups 2 and 5. The final groups entered into the study included groups 1 and 4.

2.4. Challenge of vaccinated cats

Unc cloned biological FIV-PPR virus stock was prepared for wild type virus challenge of immunized cats by limited passage and amplification of virus on feline PBMC cultures as previously described [15]. Vaccinated and unvaccinated control cats were challenged with biological FIV-PPR virus stock (1 ml) containing 100 50% tissue culture infectious doses by the IM route. Groups 1 to 5 were challenged between 42 to 44 weeks, and group 6 was challenged at 28 weeks after immunization. Animals were monitored for clinical signs as previously described [15].

2.5. Assay for FIV-specific CD4 T cell proliferative responses to viral antigens

Virus-specific T cell proliferative responses were tested in PBMC prepared from vaccinated and control cats as previously described [16]. Briefly, PBMC were isolated by ficoll-hypaque separation and plated at 10^6 cells per well in a 24 well plate in standard PBMC media without IL-2. PBMC were cultured overnight and stimulated with either 10 μ g of concavalin A (ConA) or 1 μ g of sucrose gradient purified FIV-PPR inactivated by treatment with aldrithiol-2 (Sigma Aldrich). After three days of incubation with antigen, cells were stained with 5-bromodeoxyuridine (BrdU) to identify proliferating cells. Cells were harvested and assayed for proliferation using a commercial BrdU Flow Kit (BD Biosciences, San Diego, CA). Virus-specific proliferation of lymphocytes was determined by double-labeling of total cellular DNA with 7-actinomycin D and BrdU incorporation. Cells were acquired using FACScan (Becton Dickinson, San Jose, CA) and analyzed by FlowJo software (Treestar, Ashland, OR). Data from 60,000 events for each sample were collected and analyzed. A significant proliferation response was defined as equal to, or greater than, 2% FIV-specific proliferating T cells, based on values previously established for uninfected control cats [16].

2.6. Assessment for antiviral antibody

Serum or plasma were assayed for antibodies specific to FIV p24Gag after immunization and challenge by an ELISA using recombinant p24Gag as the solid-phase antigen as previously described [42]. Only two serum dilutions, 10^{-2} and 10^{-3} , were assayed for reactivity to FIV p24Gag.

2.7. Detection and measurement of virus load

Genomic DNA was extracted and isolated from PBMC after immunization and challenge using a QIAamp DNA Blood Mini Kit (Qiagen Inc.). FIV proviral DNA copy number per million cells was assayed by a TaqMan® fluorogenic real-time PCR detection system based on FIV-pPPR *gag* TaqMan primer and probe sequences as previously described [37,43]. Real-time TaqMan PCR assays were performed using a 7700 ABI Prism sequence detector (Applied Biosystems, Foster City, CA).

PBMC harvested from all cats after challenge were also assayed for FIV infection by PBMC virus isolation as previously described [44]. Supernatants were collected twice weekly from PBMC cultures for 28 days and tested for virus production by a FIV p24Gag antigen-capture ELISA [44].

2.8. Stimulation and intracellular cytokine staining (ICS) of feline PBMC

Isolated feline PBMC were seeded in a 24-well plate with 10^6 cells per well, and incubated overnight at 37°C in standard PBMC medium without IL-2. The next day phorbol 12-myristate 13-acetate (PMA) and ionomycin were added to wells designated as mitogen-activated cells, for a final concentration of 0.5 µg/ml and 0.1 µg/ml respectively. Wells with untreated PBMC served as negative controls. Samples were incubated with mitogen for 1.5 hr at 37°C. Wells were next treated with 10 µg/ml golgi inhibitor brefeldin A (Sigma-Aldrich) and 1 µl of Golgi Plug (BD Biosciences), and incubated for 4.5 hr at 37°C. Cells were harvested using the protocol provided by the Fixation/Permeabilization Solution kit (BD Biosciences) with the following modifications [45,46]. Cells were incubated in 0.5 mM EDTA first and then stained with mouse anti-feline CD4-PE (clone 3–4F4, Southern Biotech, Birmingham, AL) and mouse anti-feline CD8-FITC (clone fCD8, Southern Biotech). After two washes with a commercial FACS buffer, cytofix/cytoperm (BD Biosciences) was added to all samples. Mouse anti-human TNF-α APC (clone MAb11, BD Bioscience), rat anti-human IL-2 Alexa700 (clone MQ1-17H12, Biolegend, San Diego CA), and feline CD4-PE antibody were next added to each sample. Anti-feline CD4 antibody was added during surface and intracellular staining due to down-regulation of lymphocyte CD4 receptors associated with stimulation with PMA and ionomycin. Compensation beads (CompBeads Set Anti-Mouse Ig, κ, BD Bioscience), instead of cells, were used to minimize spectral overlap. Data from 100,000 lymphocyte-gated events were collected using LSRII (Becton Dickinson) and analyzed using FlowJo software.

2.9.1. Cross-reactivity of human IL-2 antibody—Fifty million feline PBMC harvested from healthy SPF cats were stimulated with PMA and ionomycin for 10–12 hours in 2 ml of standard PBMC media without IL-2. Activated cell culture supernatants were collected and concentrated using MicroCon YM-10 columns (Millipore, Billerica, MA) for harvest of secreted feline IL-2. Feline IL-2 proteins were immunoprecipitated with 1 µg of rat anti-human IL-2 antibody (clone MQ1-17H12, BioLegend) and 30% Protein G sepharose beads (Sigma-Aldrich). Proteins were separated on an 18% polyacrylamide gel and transferred onto nitrocellulose filter paper for Western blot analysis. Blots were blocked with 5% fish gelatin for 2 hours, and then incubated with 1.5 µg of goat anti-feline IL-2 polyclonal antibody (R&D Systems, Minneapolis, MN) overnight for detection of immunoprecipitated feline IL-2. Bound antibodies were detected with donkey anti-goat conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz CA).

2.9.2 Statistics—Statistical analyses comparing values for virus loads and frequencies of lymphocytes expressing TNF- α and IL-2 was based on the Mann-Whitney *U* test (GraphPad Prism). *P* values < 0.05 were considered significant.

3. Results

3.1. Validation of the feline GM-CSF expression plasmid

Newly constructed expression plasmids pCDNA-feGMCSF and pCDNA-rfeGMCSF encoded feGM-CSF cDNAs in the forward and reverse orientation respectively. These plasmids were tested for feGM-CSF expression by transfection of COS-7 cells and assay of cell lysates and concentrated transfected-cell supernatants by western blot analysis. Analysis of both supernatants and cell lysates revealed two feGM-CSF proteins of slightly different sizes, most likely due to post-translational modification (Fig. 1). A 22–24 kD protein was the predominant feGM-CSF protein detected in cell supernatants and represented the mature glycosylated protein. A smaller 16–18 kD protein was also detected in the transfected-cell lysates and represented either a feGM-CSF unglycosylated precursor or degradation product. These experiments confirmed that vaccine plasmid pCDNA-feGMCSF was capable of expression and secretion of feGM-CSF.

The biological activity of the GM-CSF expression vector was validated *in vitro* using TF-1 cells reported to be dependent on specific hematopoietic growth factors such as GM-CSF for growth in cell culture. Addition of recombinant feGM-CSF prepared from pCDNA-feGMCSF-transfected cells resulted in cell proliferation and survival as tested by a MTT-based assay and by viable cell counts after staining with Trypan Blue. Feline GM-CSF protein produced from transiently transfected COS-7 cells increased TF-1 viability by 0.5 to 1 log (cells/ml) compared to empty vector control (data not shown).

3.2. Virus-specific cellular immune responses after immunization

Groups 1–6 containing six SPF cats each were immunized with vaccine protocols that included FIV-pPPR Δ vif plasmid alone; a combination of FIV-pPPR Δ vif DNA and pCDNA-feGMCSF and pCDNA-TNF- α ; a combination of FIV-pPPR Δ vif DNA and pND14-Lc-IL15; a combination of pCDNA-feGMCSF and pCDNA-TNF- α only; pND14-Lc-IL15 only; or sham vector plasmid only (Table 1). FIV-specific peripheral blood T-cell proliferative responses were compared between cats vaccinated with either FIV-pPPR Δ vif proviral DNA alone or combined with different cytokine adjuvants. Overall, lymphoproliferative responses were very low or undetectable for cats immunized with FIV-pPPR Δ vif proviral DNA, with or without cytokines, after priming immunization (Fig. 2). Priming immunization with FIV-pPPR Δ vif proviral DNA alone resulted in detectable lymphoproliferative responses for one cat, and a booster immunization was required for detectable responses for a second cat. Two cats demonstrated proliferative responses after priming immunization with FIV-pPPR Δ vif proviral DNA and pCDNA-feGMCSF and pCDNA-TNF- α plasmids. Furthermore, proliferative responses were detected in four of six group 1 cats following a booster immunization. FIV-specific proliferative responses were detected in PBMC from only one cat from group 2 that received FIV-pPPR Δ vif and pND14-Lc-IL15, and after priming immunization only. Lymphoproliferative responses were not detected in cats immunized with cytokine expression plasmids only, or control plasmid. These findings revealed a greater frequency of lymphocyte proliferative responses with a FIV-pPPR Δ vif proviral DNA immunization strategy that included GM-CSF and TNF- α delivered by expression plasmids, and that these responses could be enhanced by a boosting immunization.

3.3. Virus-specific humoral immune responses after immunization

Antiviral antibodies were only detected in group 2 cats immunized with FIV-pPPRΔvif and pND14-Lc-IL15 (Table 2). A low FIV p24-Gag antibody titer was observed as early as 12 weeks after priming immunization for one cat within group 2. Within 28 weeks after priming immunization, a second group 2 cat was also seropositive. Five out of six group 2 cats demonstrated low FIV p24-Gag antibody titers of 1:100 by 4 weeks after a boosting immunization with pPPRΔvif and pND14-Lc-IL15, and these low antiviral antibody titers persisted up to the day of challenge.

3.4. Vaccine efficacy

Feline PBMC samples from cats in all groups were monitored for FIV proviral DNA copy number after immunization and challenge with the biological FIV-PPR isolate. Proviral DNA was undetectable by a real-time TaqMan PCR assay in cats immunized with a FIV-pPPRΔvif proviral DNA-based vaccine or cytokine adjuvants alone (data not shown). Similarly, previous reports revealed an absence of detectable virus infection by either virus isolation or real-time PCR assays of PBMC harvested from cats inoculated with FIV-pPPRΔvif proviral DNA [15, 16]. Cats were challenged with biological FIV-PPR virus at 42 to 44 weeks after vaccination. Viral DNA was detected in PBMC from all cats in all vaccine groups when first tested at four weeks after challenge with FIV-PPR (Fig. 3A). Significant differences in virus loads between vaccine groups over time were not observed (Fig. 3B). Interestingly, three of six cats from group 1 were virus-negative by a standard PBMC virus isolation assay at four weeks after challenge, whereas all cats from groups 3–6 were positive at this same time point. Virus isolation data was not available for group 2 at four weeks after challenge. However, by 12 weeks after challenge, all cats within each vaccine group were virus-positive by PBMC virus isolation (data not shown). These findings indicated that FIV-pPPRΔvif immunization, with or without cytokine adjuvants, did not protect vaccinated cats from infection, or significantly suppress PBMC proviral DNA load after IM challenge with FIV-PPR. Importantly, cytokines including IL-15 or the combination of GM-CSF and TNF-α did not significantly improve efficacy of a FIVpPPRΔvif DNA vaccine based on the absence of suppression of virus loads.

Animals were monitored for clinical signs of acute FIV infection, viremia, and anti-FIV antibody for up to 16 weeks after challenge. All cats remained clinically healthy and did not exhibit signs of acute FIV infection. Significant changes in peripheral blood neutrophil concentrations and CD4 T cell percentages were not observed in either experimental or control groups up to 16 weeks after challenge (data not shown). These findings were consistent with previous observations where CD4 T cell depletion was not associated with early stages of infection with the FIV-PPR biological isolate [16,44]

3.5. Humoral immune responses after challenge

FIV-specific p24-Gag antibody responses were detected in all experimental and control groups after challenge with FIV-PPR (Table 3). Five of six cats immunized with FIV-pPPRΔvif and pND14-Lc-IL15 plasmids were positive for anti-FIV p24Gag antibodies prior to challenge, showing a detectable humoral response after immunization. For all other vaccine groups, seroconversion was evident by 4 or 8 weeks after challenge as measured by FIV p24Gag antibody ELISA. Furthermore, emergence of antiviral antibodies in cats immunized with FIV-pPPRΔvif either alone, or with pCDNA-feGMCSF and pCDNA-TNF-α did not precede the detection of antibodies in control groups. The absence of detectable antiviral antibody at two and four weeks after challenge for vaccine groups 1 and 3, suggested that these vaccine protocols did not significantly prime antibody responses.

3.6. Proliferative immune responses in animals after challenge

Vaccine groups designed to test for GM-CSF and TNF- α adjuvant activity (groups 1, 3, 4, and 6) were also tested for FIV-specific PBMC proliferative responses after challenge. Lymphoproliferative responses were not detected after challenge in any of the cats immunized with FIV-pPPR Δ vif alone or with sham plasmid (Fig. 4). All animals that were co-immunized with FIV-pPPR Δ vif DNA, feGM-CSF, and feTNF- α plasmids demonstrated proliferative responses for at least one time point between 2 and 16 weeks after challenge, with frequencies of proliferating cells ranging from 2 to 15%. Proliferation responses for this vaccine group were greatest at eight weeks after challenge. Control cats immunized with feGM-CSF and feTNF- α expression plasmids alone also exhibited proliferative responses. However in general, proliferative responses for group 4 were of lower magnitude and lower frequency compared to group 1. Improved FIV-specific proliferative responses observed after challenge for cats immunized with cytokine plasmids only, was surprising and not easily explained. Sustained expression of cytokine plasmid DNA after immunization may have contributed to enhanced antigen presentation and T cell responses upon challenge, as plasmid DNA has been shown to persist at the initial site of intramuscular injection [47–49]. Overall, these findings indicated that co-immunization of FIV-pPPR Δ vif DNA with feGM-CSF and feTNF- α plasmids resulted in augmented and preserved CD4 lymphocyte proliferative responses after challenge with FIV-PPR when compared to immunization with FIV-pPPR Δ vif DNA alone.

3.7. Development of a feline ICS assay for feline IL-2 and TNF- α

Previous studies demonstrated that mitogen stimulation of PBMC with PMA and ionomycin induced cytokine patterns similar to those expressed by antigen-stimulated cells and could reveal the full cytokine production potential of T lymphocytes [50,51]. Furthermore, multiple reports described defects in mitogen or antigen-induced lymphocyte expression of cytokines, particularly those sharing the common gamma chain (γ c) receptor such as IL-2, for HIV, simian immunodeficiency virus (SIV), and simian human immunodeficiency virus (SHIV)-infected hosts [52–57]. Accordingly, a multicolor ICS flow cytometric assay for detection of feline PBMC expressing different cytokines was developed as one approach to examine effects of FIV infection on peripheral blood feline CD4 T cell functions. Scatter plots representative of data generated by an ICS flow cytometric assay and the gating strategy for analysis of feline TNF- α and IL-2 expression from peripheral blood CD4 and CD8 lymphocyte subsets are shown in Fig. 5A. The four-color cytometric panels allowed differentiation between lymphocyte subsets that produced either TNF- α alone, IL-2 alone, or a combination of both cytokines (TNF- α and IL-2) in response to mitogen stimulation. Antibodies against human TNF- α and IL-2 were utilized for this protocol due to the lack of feline cytokine-specific monoclonal antibodies. Cross-reactivity of the anti-human TNF- α monoclonal antibody used for detection of feline TNF- α by flow cytometry was previously described [58]. To validate cross-reactivity of a human IL-2 monoclonal antibody (clone MQ1-17H12) for feIL-2, immunoprecipitates were generated by this human IL-2 monoclonal antibody (clone MQ1-17H12) from mitogen-stimulated feline PBMC culture supernatants. Immunoprecipitates were next assayed by Western blot using a commercial polyclonal antibody against feIL-2. This immunoblot analysis confirmed the cross-reactivity of the human IL-2 monoclonal antibody for feIL-2 (Fig. 5B) and justified the use of this antibody for a feline ICS flow cytometric assay.

3.8. Mitogen-induced PBMC expression of TNF- α and IL-2 after challenge

Assay for cytokine expression upon stimulation with mitogens PMA and ionomycin provided another approach for comparing T cell function in vaccinated and unvaccinated cats after challenge with biological FIV-PPR. Therefore, PBMC sampled from cats within groups 1 and 4 between 27 to 40 weeks after challenge were assayed for PMA and ionomycin induction of IL-2 and TNF- α expression using the newly developed feline ICS assay. This time frame was

considered representative of early chronic FIV infection and appropriate for testing the effects of vaccination on preservation of CD4 and CD8 lymphocyte function in the face of FIV infection. PBMC samples from a group of 14 SPF cats were similarly assayed and served as uninfected controls. Interestingly, ICS assays revealed a higher frequency of CD4 lymphocytes expressing TNF- α after mitogen stimulation in FIV-PPR-infected cats, compared to frequencies measured for uninfected SPF controls ($P < 0.001$) (Fig. 6A). Furthermore, a higher frequency of CD4 lymphocytes expressing TNF- α after mitogen stimulation was also observed in FIV-infected cats previously vaccinated with both FIV-pPPR Δ vif and feGM-CSF and feTNF- α plasmids (group 1), when compared to infected cats vaccinated with feGM-CSF and feTNF- α plasmids alone (group 4) ($P < 0.03$). Similar findings were observed for CD8 lymphocytes where higher frequencies of cells positive for TNF- α expression after mitogen stimulation were observed for FIV-infected cats compared to SPF cats ($P < 0.001$), and for group 1 cats compared to group 4 cats ($P < 0.001$) (Fig. 6B). These data suggested that FIV infection resulted in lymphocyte subsets capable of hyperactive TNF- α expression responses to mitogen stimulation, particularly for cats vaccinated with both FIV-pPPR Δ vif and feGM-CSF and TNF- α plasmids. It is important to note that basal TNF- α expression measured for CD4 and CD8 lymphocytes was not different between the groups. Frequencies of unstimulated lymphocytes shown to be positive for TNF- α expression were less than 1% for all FIV-infected and uninfected cats (data not shown).

Importantly, differences in frequencies of CD4 lymphocytes expressing IL-2 after mitogen activation were also observed between FIV-infected and uninfected SPF control cats (Fig. 6A). The mean frequency of CD4 lymphocytes expressing IL-2 measured for FIV-infected control group 4 cats was significantly decreased compared to that observed for SPF ($P < 0.001$) and FIV-infected group 1 ($P < 0.03$) cats. Also, significantly higher frequencies of CD4 lymphocytes expressing both IL-2 and TNF- α after mitogen stimulation were also observed for FIV-infected group 1 cats vaccinated with FIV-pPPR Δ vif, feGM-CSF, and feTNF- α plasmids, when compared to FIV-infected cats vaccinated with cytokine plasmids alone ($P < 0.001$), or to uninfected SPF cats ($P < 0.001$) (Fig. 6A). These findings suggested that vaccination with FIV-pPPR Δ vif along with feGM-CSF and feTNF- α plasmids resulted in a preservation of CD4 lymphocyte IL-2 expression for the early phase of chronic infection; this finding was not observed for control group 4 cats vaccinated with cytokine expression plasmids alone. Moreover, this vaccination strategy also resulted in a sparing of polyfunctional lymphocytes capable of expressing both IL-2 and TNF- α in cats after infection with FIV-PPR. CD8 lymphocyte frequencies expressing IL-2 in response to mitogen stimulation were not statistically significant different between groups 1, 4 and SPF cat controls, although mean frequencies for group 1 were higher (Fig. 6B). A small but significant difference ($P < 0.02$) in frequencies of mitogen-activated CD8 lymphocytes expressing both IL-2 and TNF- α was observed between group 1 and unvaccinated cats (group 4) (Fig. 6B). These observations suggested that a functional defect involving IL-2 expression was predominantly restricted to CD4 T cells in early chronic FIV infection.

4. Discussion

Previous studies testing efficacy of a *vif*-deleted FIV provirus as a DNA vaccine revealed inconsistent findings concerning vaccine efficacy with one preliminary vaccine study showing protection from challenge infection induced by immunization with the FIV-pPPR Δ vif DNA vaccine [15]. However, a subsequent study testing the FIV-pPPR Δ vif DNA vaccine administered either alone or with a feIFN- γ expression plasmid, revealed no suppression of challenge virus loads for either approach and suggested the need for testing additional candidate cytokines as adjuvants for this FIV DNA vaccine. Accordingly, current studies described herein, tested specific cytokines, including IL-15, and TNF- α and GM-CSF in combination, for adjuvant activity hypothesized to enhance FIV-pPPR Δ vif DNA vaccine immunogenicity

and efficacy. Differences in immunogenicity were observed between vaccine groups in the current study by the measurement of FIV-specific CD4 T cell proliferative responses [16]. Immunization with FIV-pPPRΔvif DNA with feTNF-α and feGM-CSF expression plasmids generated a higher frequency of FIV-specific lymphoproliferative responses when compared to the other vaccine groups. Improvement of vaccine-induced CD4 T cell responses was previously reported for combination cytokine adjuvant strategies that included GM-CSF and TNF-α [17,29] and likely accounted for enhanced T cell responses observed in the current study. However, an expected GM-CSF-induced augmentation of antiviral antibody responses elicited by the FIV-pPPRΔvif DNA was not observed for this cytokine combination strategy. Use of GM-CSF in a cytokine combination rather than as a single agent adjuvant, may have been one possible factor accounting for the absence of improved antibody responses to the FIV-pPPRΔvif DNA vaccine for this group of cats.

In contrast, FIV-specific antibodies were only detected in cats immunized with the FIV-pPPRΔvif DNA vaccine protocol that included a feIL-15 expression plasmid. The lack of detectable antiviral antibodies observed for cats immunized with FIV-pPPRΔvif DNA alone when assayed by standard serological assays was expected and previously reported in other studies testing inoculation of animals with *vif* mutants in multiple lentivirus systems [15,16, 59–61]. Therefore, detection of FIV p24Gag antibodies after FIV-pPPRΔvif DNA immunization with the IL-15 expression plasmid suggested a humoral response uniquely elicited by IL-15 adjuvant activity. These results are further supported by previous studies showing IL-15-induced B cell proliferation and secretion of immunoglobulins [36,62–64]. IL-15 was also previously shown to increase [³H]-thymidine incorporation and antibody production in B cells stimulated with heat-inactivated HIV-1 [63]. Selected reports have described IL-15 amplification of memory CD4 T cells and augmentation of antigen-specific CD4 T cell responses [35,65]. However, enhancement of FIV-specific peripheral blood CD4 responses was not observed for group 2 cats, as shown by the poor FIV-specific lymphoproliferative responses detected after both priming and boosting immunizations with FIV-pPPRΔvif and IL-15 plasmids. Alternatively, previous studies also revealed a rapid emigration of IL-15-amplified CD4 T cells from peripheral blood to tissues [35] which may account for the absence of detectable IL-15-imposed effects on CD4 responses in this current study where tissue lymphocytes were not assayed. Augmentation of antigen-specific CD8 T cell responses has been reported as a primary effect of IL-15 adjuvant activity [30–32], but was not assessed in this study.

A second objective of these studies was to test the effects of different cytokine adjuvants on the efficacy of a FIV-pPPRΔvif DNA vaccine. Although use of cytokine adjuvants improved FIV-pPPRΔvif DNA vaccine immunogenicity, protection as defined by suppression of virus loads after challenge with biological FIV-PPR was not observed for any of the vaccine protocols tested in the current study. Although viremia was delayed for three of six cats within group 1, all cats within each vaccine group demonstrated productive virus infection of PBMC by virus isolation by 12 weeks after challenge. Furthermore, PBMC proviral DNA loads were comparable between vaccine groups over the 12 week time period examined after challenge. It is important to note that data regarding viral RNA plasma loads were not available for comparisons between vaccine groups. Assessment for plasma virus loads may have revealed differences in virus replication during very early time points after infection and evidence of vaccine enhancement, as was shown by another previously reported FIV vaccine study [66]. However, based on recent FIV vaccine reports, differences in virus loads between experimental groups as reflected by viral RNA plasma concentrations and PBMC proviral DNA loads were generally comparable [66–68].

Importantly, these findings of improved immunogenicity without significant improvement of virus suppression were similar to those reported in our previous study that examined IFN-γ as

a cytokine adjuvant for this same FIV-pPPRΔvif DNA vaccine, and included a single immunization tested by challenge 13 weeks later [16]. However, the design of the current study included a booster immunization (32–33 weeks after priming vaccination) and a longer time frame (42–44 weeks) between priming immunization and challenge with FIV-PPR. This study design more closely mimicked vaccine trial conditions that revealed significant efficacy for the FIV-pPPRΔvif DNA in a earlier preliminary study [15]. This earlier trial included a booster immunization at 43 weeks after a priming vaccination followed by challenge five weeks later. Regardless of the changes in the vaccine trial design, suppression of challenge virus was not observed in cats vaccinated with FIV-pPPRΔvif and various cytokine plasmids in the current investigation.

To further test for vaccine-associated effects on viral pathogenesis after infection by a FIV-PPR challenge, a separate study was conducted to compare CD4 T cell function in cats immunized with FIV-pPPRΔvif, feGM-CSF, and feTNF-α plasmids to those control cats immunized only with cytokine plasmids. Interestingly, flow cytometric analysis revealed that vaccination with the FIV-pPPRΔvif DNA vaccine and feGM-CSF and feTNF-α plasmids resulted in a preservation of CD4 lymphocytes capable of expressing IL-2 upon mitogen stimulation in the face of FIV infection; this result was not observed in group 4 cats vaccinated with cytokine expression plasmids only. Similarly, a higher frequency of polyfunctional CD4 lymphocytes expressing both IL-2 and TNF-α after mitogen stimulation was also observed for FIV-infected cats previously vaccinated with FIV-pPPRΔvif, GM-CSF, and TNF-α plasmids when compared to cats vaccinated with cytokine plasmids alone. Previous SIV and SHIV vaccine studies also revealed sparing of CD4 T cell functions such as mitogen-induced IL-2 secretion and virus-specific CD4 T cell proliferation as a result of partially efficacious vaccines [55,57]. A reduction in the capacity for IL-2 secretion by CD4 T cells correlated with disease progression in these primate studies. A decrease in IL-2-expressing cells was also observed with the advancement of the disease in HIV-1 infection [69]. Moreover, robust peripheral blood lymphoproliferative responses were observed after challenge infection with FIV-PPR for those cats vaccinated with the combination of FIV-pPPRΔvif, GM-CSF, and TNF-α plasmids. In contrast, lymphoproliferative responses were not detected after challenge in unvaccinated cats, or cats vaccinated with FIV-pPPRΔvif DNA alone. The absence of viral-specific proliferative responses in these two latter vaccine groups was significant given that proviral DNA loads were comparable between all vaccine groups, and most likely reflected another virus-associated defect in CD4 T cell function. Similarly, an absence of virus-specific T cell proliferative responses was also reported for rhesus macaques during acute infection with SIVmac251 [70,71]. Taken together, preservation of CD4 T cell functions including mitogen-responsive cytokine expression and antigen-specific proliferation, indicated that a FIV-pPPRΔvif DNA vaccine protocol including GM-CSF and TNF-α plasmids afforded some protection against FIV-associated disease during acute and early chronic phases of infection. A comparison for production of these particular cytokines with IL-15 for induction of this vaccine-associated protection was not possible because cats vaccinated with FIV-pPPRΔvif and IL-15 plasmids or with FIV-pPPRΔvif alone, were not available for testing of these particular functions with this newly developed feline cytokine flow cytometric assay. Additional studies will be necessary to determine if these effects on preservation of CD4 lymphocyte function are specific for the combination of GM-CSF and TNF-α as cytokine adjuvants, or if the use of other cytokine adjuvants including IL-15, might yield similar outcomes.

Critical observations from these studies included findings of increased immunogenicity provided by a FIV-pPPRΔvif DNA vaccine with the use of different cytokine adjuvants delivered by expression plasmids. The failure of FIV-pPPRΔvif DNA vaccines incorporating different cytokine adjuvants to suppress virus loads after FIV-PPR challenge was disappointing. Nevertheless, findings from these studies also revealed a preservation of CD4

T lymphocyte function in the face of FIV infection by vaccination with FIV-pPPRΔvif, GM-CSF, and TNF- α plasmids. These findings illustrate the potential for enhanced DNA vaccine efficacy that may be generated by incorporation of different cytokine adjuvants. Cytokine combinations that include GM-CSF and TNF- α with other unique cytokines warrant further testing as adjuvants in future FIV DNA vaccine studies. Furthermore, incorporation of other vaccine modalities including FIV immunogens delivered by a viral vector as a boosting immunogen may be required for generation of a highly efficacious FIV vaccine capable of suppression of both FIV disease and virus loads.

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Figure 1. Expression of recombinant feGM-CSF in mammalian cells by Western blot analysis

Cell lysates (lane 1) and concentrated cell culture supernatants (lane 3) prepared from COS-7 cells transfected with pCDNA3-feGMCSF were compared to lysates (lane 2) and supernatants (lane 4) prepared from COS-7 cells transfected with control plasmid pCDNA3-rfeGMCSF by immunoblot analysis. The blot was probed with goat polyclonal antibody against feGM-CSF. Arrows indicate two GM-CSF proteins of slightly different sizes, probably due to post-translational modification. The smaller form of feGM-CSF appears as the predominant form in cell lysates and the larger form representing mature secreted glycosylated protein is the predominant form in transfected-cell culture supernatants.

Figure 2. Detection of FIV-specific lymphoproliferative responses after vaccination

PBMC from vaccinated animals were tested for FIV-specific proliferation using a flow cytometric assay based on BrdU-uptake with whole killed virus as antigen. Frequency of proliferating PBMC for different vaccine and control groups are shown for time points before and after vaccination. Arrowheads shown below the graphs for vaccine groups describe the time point of booster immunization. A significant proliferation response was defined as a value greater than, or equal to, 2% FIV-specific proliferating T cells (see dashed cut line). Data was not always available for the same time-points for each group after vaccination. Numbers next to legend symbols represent identity of the experimental animal.

Figure 3. Measurement of FIV proviral DNA concentrations in PBMC of immunized animals after challenge with FIV-PPR

PBMC preparations from cats within vaccine and control groups were assayed for FIV proviral DNA concentrations at 0, 4, 8, and 12 weeks after challenge (A). Proviral DNA concentrations were measured using TaqMan real-time PCR with FIV-PPR *gag* primer and probe sets.

Numbers next to legend symbols represent identity of the experimental animal. Median virus loads with standard error bars for each vaccine group at 0, 4, 8, and 12 weeks after challenge are also described (B).

Figure 4. Detection of FIV-specific lymphoproliferative responses after challenge with FIV-PPR PBMC from vaccinated and unvaccinated animals were tested for FIV-specific proliferation using a flow cytometric assay based on BrdU-uptake with whole killed virus as antigen. Frequency of proliferating PBMC for different vaccine and control groups are shown for time points ranging from 2 to 16 weeks after challenge. A significant proliferation response was defined as a value greater than, or equal to, 2% FIV-specific proliferating T cells (see dashed cut line). Data was not always available for the same time-points for each group after challenge. Numbers next to legend symbols represent identity of the experimental animal.

Figure 5. Development and validation of a feline intracellular staining cytokine flow cytometric assay for expression of feline IL-2 and TNF- α

A representative pseudo-color plot generated by flow cytometry illustrates intracellular TNF- α and IL-2 staining of feline PBMC after stimulation with mitogens PMA and ionomycin (A). The four-color flow cytometric panels show gating strategy and lymphocyte staining by antibodies for feline CD4, feline CD8, human TNF- α , and human IL-2 as described in Methods. Cross reactivity of a human IL-2 monoclonal antibody (clone MQ1-17H12) for feline IL-2 was confirmed by immunoprecipitation and Western blot analysis (B). Immunoprecipitates were prepared from cell lysates and concentrated culture supernatants derived from mitogen-stimulated feline PBMC and simian COS-7 cells, using the human IL-2 monoclonal antibody. Immunoprecipitates prepared from PBMC lysates (lane 3), concentrated PBMC culture supernatants (lane 4), and COS-7 cells (lane 5) were separated on a 18% polyacrylamide gel and assayed for detection of a 15 kD feline IL-2 protein using a goat anti-feline IL-2 polyclonal antibody. Molecular weight standards (lane 1) and an immunoprecipitate from recombinant human IL-2 (AIDS Research and Reference Reagent Program) (lane 2) provided controls for detection of a feline IL-2 protein of the appropriate size.

Figure 6. Cytokine expression profiles of normal, vaccinated FIV-infected, and unvaccinated FIV-infected cats

PBMC were harvested from FIV-infected cats previously vaccinated with FIV Δ vif DNA and feGM-CSF and feTNF- α plasmids (group 1) or with feGM-CSF and feTNF- α plasmids alone (control group 4), and from healthy SPF cats. PBMC were tested for cytokine expression after PMA and ionomycin stimulation using an ICS assay. Frequencies are shown as percentages of CD4 (A) and CD8 (B) lymphocytes expressing either TNF- α alone, IL-2 alone, or co-expressing both cytokines after mitogen stimulation. Mean and standard error of four repeated experiments over multiple time points are depicted in each bar graph. Asterisks above bar graphs indicate a significant difference between values, with P values < 0.05 considered significant as determined by the Mann-Whitney U test.

TABLE 1

Experimental Design for Vaccine Study

Groups	No. of cats	Plasmid Inoculum	Time of Boost (week)	Time of Challenge (week)
1	6	FIV-pPPRΔvif, pCDNA-feGMCSF, pCDNA-TNF- α	32	43
2	6	FIV-pPPRΔvif, pND14-Lc-IL15	33	44
3	6	FIV-pPPRΔvif	32	42
4	6	pCDNA-feGMCSF, pCDNA-TNF- α	32	42
5	6	pND14-Lc-IL15	32	42
6	6	pND14	18	28

TABLE 2

Detection of FIV antibody after immunization

Vaccine Plasmids	Weeks after vaccination	Animal number						
		406	407	416	418	442	444	
FIV-pPPRA vif pCDNA-feGMCSF pCDNA-TNF- α	0	- ^a	-	-	-	-	-	-
	32 ^b	-	-	-	-	-	-	-
	34	-	-	-	-	-	-	-
	42	-	-	-	-	-	-	-
		303	307	310	314	316	041	
FIV-pPPRA vif pND14-Lc-IL15	-2	-	-	-	-	-	-	-
	12	-	-	-	-	10 ²	-	-
	28	-	-	10 ²	-	10 ²	-	-
	37 ^c	10 ²	-	10 ²	10 ²	10 ²	10 ²	10 ²
	44	10 ²	-	10 ²	10 ²	10 ²	10 ²	10 ²
		102	114	119	120	123	127	
FIV-pPPRA vif	0	-	-	-	-	-	-	-
	32 ^b	-	-	-	-	-	-	-
	34	-	-	-	-	-	-	-
	42	-	-	-	-	-	-	-

^aSera with titers less than 1:100 were considered negative (-) for FIV antibody. Serum dilutions of 10⁻² and 10⁻³ were tested for reactivity to FIV p24 Gag. Serum antibody titer to FIV p24 Gag is expressed as the reciprocal of the highest dilution positive for antibody.

^bTime points where cats were given a second immunization.

^cCats were given a second immunization at 33 weeks post inoculation.

TABLE 3

Detection of FIV antibody in immunized cats after challenge

Vaccine Plasmids	Weeks after challenge	Animal number					
		406	407	416	418	442	444
FIV-pPPRA vif	0	- ^a	-	-	-	-	-
pCDNA-feGMCSE	2	-	-	-	-	-	-
pCDNA-TNF- α	4	-	-	10 ^{2b}	10 ²	-	10 ²
	8	10 ³	10 ²	10 ³	10 ³	10 ³	10 ²
	12	10 ³	10 ³	10 ³	10 ³	10 ³	10 ³
		303	307	310	314	316	041
FIV-pPPRA vif	0	10 ²	-	10 ²	10 ²	10 ²	10 ²
pND14-Lc-IL15	2	10 ²	-	10 ²	10 ²	10 ²	10 ²
	4	10 ³	10 ³	10 ³	10 ³	10 ³	10 ³
	8	10 ³	10 ³	10 ³	10 ³	10 ³	10 ³
	12	10 ³	10 ³	10 ³	10 ³	10 ³	10 ³
		102	114	119	120	123	127
FIV-pPPRA vif	0	-	-	-	-	-	-
	2	-	-	-	-	-	-
	4	10 ²	-	-	-	-	-
	8	10 ³	10 ³	10 ³	10 ³	10 ³	10 ³
	12	10 ²	10 ³	10 ³	10 ³	10 ³	10 ³
		415	417	420	433	436	443
pCDNA-feGMCSE	0	-	-	-	-	-	-
pCDNA-TNF- α	2	-	-	-	-	-	-

Vaccine Plasmids	Weeks after challenge	Animal number							
		406	407	416	418	442	444		
pND14-Lc-IL15	4	10 ²	10 ²	10 ²	10 ²	10 ²	10 ²		
	8	10 ³	10 ³	10 ³	10 ²	10 ³	10 ³		
	12	10 ³	10 ³	10 ²	10 ³	10 ³	10 ³		
		300	305	306	309	315	321		
pND14	0	-	-	-	-	-	-		
	2	-	-	-	-	-	-		
	4	10 ³	10 ²	10 ³	10 ³	10 ³	10 ³		
	8	10 ³	10 ²	10 ³	10 ³	10 ³	10 ³		
	13	10 ³	10 ³	10 ³	10 ³	10 ³	10 ³		
		229	232	233	234	235	236		
pND14	0	-	-	-	-	-	-		
	2	-	-	-	-	-	-		
	4	10 ²	10 ²	-	-	-	-		
	8	10 ²	10 ²	10 ²	10 ²	10 ³	10 ²		
	12	10 ²	10 ²	10 ²	10 ²	10 ³	10 ³		

^aSera with titers less than 1:100 were considered antibody negative.

^bSerum dilutions of 10⁻² and 10⁻³ were tested for reactivity to FIV p24 Gag. Serum antibody titer is expressed as the reciprocal of the highest serum dilution tested and found to be positive for antibody.