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Constitutive expression of types 1 and 2 cytokines by alveolar macrophages from feline immunodeficiency virus-infected cats

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Abstract

Evidence suggests that feline immunodeficiency virus (FIV), causes pulmonary immunodeficiency. The overall objective of this study was to explore FIV-induced alterations in cell counts and cytokine gene expression in the pulmonary compartment during the acute stage infection. Bronchoalveolar lavage (BAL) cells were collected from FIV-infected and control cats at 0, 4, 10, and 16 weeks post-FIV infection for phenotype and cytokine analysis. The major change in BAL cellular populations following FIV-infection was the development of a neutrophilia. Total BAL cell counts and relative numbers of alveolar macrophages (AM), eosinophils, and lymphocytes remained similar in both groups. The RT-qcPCR analyses of AM purified from BAL showed constitutive expression of TNF α , IL6 and IL10 mRNAs that peaked during the acute stage of infection then declined. The TNF α and IL6 bioactive protein secretion showed a similar response. In contrast, IFN γ expression increased progressively with time after infection and paralleled a progressive increase in FIV-gag mRNA in AM. The IL12 p40 expression also differed from the other cytokines in that there was a progressive decrease in the number of cats with AM IL12 expression following FIV infection. Infection of AM in vitro with FIV also caused an increase in

Abbreviations: AM, alveolar macrophages; FIV, feline immunodeficiency virus; BAL, bronchoalveolar lavage

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TNF α and IL6 mRNA and bioactive protein suggesting that the increased cytokine response by AM following infection of cats with FIV is an intrinsic characteristic of FIV-infected AM. In summary, pulmonary immune changes seen in FIV-infected cats are similar to those seen in HIV-infected human patients. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

The lung has been recognized as one of the most common targets of secondary infections, including *Mycobacterium tuberculosis*, *Cryptococcus neoformans*, *Histoplasma capsulatum*, and *Toxoplasma gondii*, in human immunodeficiency virus (HIV) infected patients. The primary line of defense against such pathogens is the activated alveolar macrophage (Beck and Shellito, 1989). Activation of alveolar macrophages (AM) is a biphasic process regulated by a number of cytokines generated by antigen specific T cells (IFN γ , TNF α) and the AM itself (TNF α , IL6, IL12). Early activation occurs as a result of IFN γ and TNF α production from NK cells and macrophages. A more sustained activation of AM, which is required for successful control of intracellular pathogens, is dependent upon development of a Th1 immune response (IFN γ and IL12).

Alveolar macrophage (AM) from HIV-infected patients constitutively produce cytokines, such as TNF α , IL10, IL8, IL10, and IL12, indicative of a state of activation (Agostini et al., 1991a; Denis and Ghadirian, 1994; Trentin et al., 1992; Twigg et al., 1992), yet they are not able to successfully control intracellular pathogens. As both Th1 and Th2 cytokines are produced by activated macrophages, it may be the ratio of the particular cytokines that is important in determining whether macrophages will function properly or not. For example, although low levels of IL12 are constitutively produced by the AM of HIV-infected patients, levels could not be increased following stimulation by *Staphylococcus aureus* (Denis and Ghadirian, 1994). These data suggest that although AM from HIV-infected patients constitutively secrete cytokines associated with activation, when faced with a secondary infectious agent, the AM cannot reach the level of activation necessary for successful clearance of the organism, possibly due to a failure in mounting a protective Th1 immune response.

Feline immunodeficiency virus (FIV), a lentivirus morphologically and biochemically similar to HIV, has a pathogenesis and clinical disease pattern that parallels that seen in HIV infection (Bendinelli et al., 1995; English et al., 1994). The acute infection is followed by a variably long asymptomatic period characterized by a progressive decrease of CD4⁺ lymphocytes that results in the hallmark inverted CD4⁺:CD8⁺ ratio in the peripheral blood (Bendinelli et al., 1995; English et al., 1994). The asymptomatic period is followed by the development of AIDS-associated disorders, many of which mimic AIDS in humans (Bendinelli et al., 1995; English et al., 1994). Davidson et al. (1993) and Yang et al. (1996) reported that FIV-infected cats challenged with an avirulent strain of *T. gondii* developed severe respiratory disease resulting in 60–80% mortality/euthanasia. The FIV-infected cats developed systemic toxoplasmosis characterized by necrotic/inflammatory foci in the liver, spleen, lymph nodes, and most severely in the lung. The

lesions were accompanied by abundant replication of *Toxoplasma* tachyzoites readily noticeable in the sites of inflammation. In contrast, FIV– cats developed only a transient, mild clinical disease characterized by anorexia and lethargy without clinical signs of pulmonary involvement. Levy et al. (1998) examined the peripheral and bronchial lymph nodes from normal and FIV-infected cats following challenge with *T. gondii* and reported a marked difference in the cytokine profile in the two groups of cats. As compared to control cats challenged with *T. gondii*, cells harvested from the lymph nodes of FIV-*T. gondii* co-infected cats produced high levels of IL10 and IFN γ but were deficient in IL2 and IL12 (p40) mRNA production. Dean and Pedersen (1998) also reported elevated levels of IL10 and IFN γ mRNA in lymph nodes of FIV-infected cats.

As the pulmonary macrophage microbicidal activation process and resistance to *T. gondii* is dependent upon an effective IL12-dependent Th1 immune response to *T. gondii* antigens, we were interested in determining what effect FIV-infection may have on AM in the lung. In this study, we characterized changes occurring in bronchoalveolar lavage (BAL) cell counts, differentials, and cytokine mRNA and protein expression from the AM of cats infected with FIV. To verify that any alterations in cytokine message and protein expression were associated with a productive viral infection, FIV-gag mRNA expression was quantitated in AM infected with FIV in vivo and in vitro.

2. Materials and methods

2.1. Experimental animals

Twenty-two, 6-month-old, specific pathogen free (SPF), neutered male cats were obtained from Liberty Laboratories (Liberty Corners, NJ). The SPF cats were seronegative for feline panleukopenia, feline herpesvirus, feline calicivirus, coronaviruses, feline leukemia virus, feline immunodeficiency virus, feline syncytia-forming virus, *Chlamydia*, and *Toxoplasma*.

2.2. Infection with feline immunodeficiency virus (FIV)

The NCSU₁ strain of FIV was originally isolated from a naturally infected cat at the North Carolina State University College of Veterinary Medicine and has been described in detail elsewhere (English et al., 1993, 1994; Davidson et al., 1993). Twelve cats were inoculated with FIV intravenously with 5×10^6 TCID₅₀ of cell-free virus culture supernatant as described by Davidson et al. (1993). The remaining 10 cats were sham inoculated with medium. All FIV-infected cats were antibody positive by commercial ELISA (IDEXX, Westbrook, ME) and provirus positive by PCR by 4 weeks post-FIV infection.

2.3. Collection of bronchoalveolar lavage (BAL) cells and alveolar macrophages (AM)

Bronchoalveolar lavage (BAL) was performed as previously described (Hawkins and DeNicola, 1989; Ma et al., 1995). Briefly, cats were anesthetized with ketamine and

intubated so that the end of an endotracheal tube was positioned within the distal trachea. Lavage was performed in three aliquots through the tube with the cat in left lateral recumbency using a volume of 5 ml/kg body weight of sterile phosphate-buffered saline (PBS) in each aliquot. The aliquots were pooled, total viable BAL cell counts determined by trypan blue dye exclusion, and differential cell analyses performed on Diff-Quik stained cytopins. Using the differential to determine the numbers of AM per milliliter of BAL fluid, duplicate cultures of AM (along with other contaminating BAL cells) were seeded in RPMI supplemented with 10% fetal bovine serum and antibiotics containing 1×10^6 AM/ml each. Following overnight incubation, nonadherent cells were removed by washing with HBSS. From one culture, adherent cells were dislodged and an aliquot used to verify AM purity, which was always >95% as determined by differential analyses. Remaining cells were lysed and total cellular RNA prepared for quantitation of cytokine gene expression (see below). In the duplicate culture, AM were cultured with fresh medium for an additional 24 h, when culture supernatants were harvested and frozen at -85°C until analyzed for TNF α and IL6 bioactivity.

2.4. *In vitro* infection of alveolar macrophages with FIV

Purified AM (prepared as described above) from normal SPF cats were infected *in vitro* with FIV-NCSU₁ at an MOI of 1 for 24 h. Total cytoplasmic RNA and culture supernatant were collected on days 1, 3, and 7 post-infection and frozen until analyzed for FIV-gag mRNA, cytokine mRNA, and TNF α and IL6 bioactivity.

2.5. Quantitation of bioactive IL6 and TNF α

The IL6 bioactivity was determined using an IL6-dependent murine plasmacytoma cell line, T1165, as previously described (Ma et al., 1995; Nordan and Potter, 1986). Briefly, serial two-fold dilutions of the supernatants to be assayed were made and 50 μl added to 50 μl of T1165 cells in 96-well culture plates. Controls included murine IL6 containing P388D cell supernatant (positive control) and medium alone (negative control). Each dilution was run in triplicate. After 2 days of incubation, 20 μl of medium containing 1 μCi ^3H -thymidine (ICN, Costa Mesa, CA) was added to each well and incubated overnight. Cells were then harvested and incorporation of ^3H -thymidine (in counts per minute) measured using a scintillation counter. Units of IL6 per milliliter (U/ml) were defined as the reciprocal of the dilution of the supernatant which stimulated 50% of the maximal counts per minute in each assay, multiplied by 10 since the assay volume was 0.1 ml.

The TNF α bioactivity was measured as previously described (Ma et al., 1995; Laster et al., 1988) with minor modifications. Briefly, LM cells, a mouse fibroblast cell line that is sensitive to TNF, were plated in 96 well flat-bottom plates at a concentration of 4×10^4 cells in 100 μl and cultured overnight. Fifty microliters of serial two-fold dilutions of AM cell supernatants were then added in triplicate along with 20 μl of culture medium containing 8.5 $\mu\text{g/ml}$ actinomycin D. Serial dilutions of human recombinant TNF α (UBI, Lake Placid, NY) of known bioactivity were used as a standard and positive control. Following overnight incubation, 25 μl of 3-[4,5-dimethylthiazol-2-yl]-2,5-

diphenyltetrazolium bromide at 5 mg/ml in PBS was added to each well. Following a 4 h incubation, all supernatant was removed and 100 μ l of 0.01N HCl in isopropanol was added to each well. After a 30 min incubation, 100 μ l of ddH₂O was added to each well and absorbency measured at 570/630 nm in an ELISA plate reader. Units of TNF α were determined from a standard curve derived from dilutions of the human recombinant TNF α standard. The murine fibroblasts may be either more or less sensitive to feline TNF α when compared with the human recombinant standard. Therefore, the values reported are meant for relative comparisons of feline TNF α levels between cats and timepoints and are not intended to convey an absolute quantitative measurement.

2.6. RNA isolation

Total cellular RNA was isolated from purified AM using the guanidinium thiocyanate technique as described by Rottman et al. (1995). Briefly, RNeasy-60 (Tel Test B, Friendswood, TX) was added to monolayers of purified AM (5×10^6 cells) and the cells lysed by pipetting up and down multiple times. The RNA was subsequently isolated according to kit instructions.

2.7. Reverse transcription-quantitative competitive PCR (RT-qcPCR) for cytokine mRNA

Cytokine mRNA levels were quantitated by a RT-qcPCR method shown previously to be sensitive, accurate, and reproducible (Levy et al., 1998; Rottman et al., 1996). The competitive DNA fragment consists of a linear array of upstream and downstream primer sequences for IFN γ , IL2, IL4, IL6, IL10, IL12 (p40), TNF α , and G3PDH separated by a DNA spacer (Rottman et al., 1996). Amplification of each primer pair yields a competitor product of 573 base pairs, which is different than the cDNA products. A second 489 base pair competitive DNA fragment was constructed to allow quantification of FIV-gag mRNA by RT-qcPCR. The FIV-gag primers were chosen from sequences of the NCSU₁ isolate molecular clone (Yang et al., 1996).

Following reverse transcription of an RNA sample, equal amounts of cDNA were amplified using cytokine specific primer pairs and serial five-fold dilutions of a DNA competitive fragment (500×10^{-5} to 1.02×10^{-5} amol). The PCR products were separated by electrophoresis on 2% agarose gels, stained with ethidium bromide, and photographed under 300 nm UV transillumination with a Polaroid camera. An image was acquired using Adobe Photoshop and analyzed using the NIH Image Software Program. The log of the cytokine product fluorescence was plotted against the log of the number of copies of competitor in the sample, resulting in the cytokine copy number at the x intercept. Constitutive mRNA expression for the measured cytokines is presented as a ratio of the cytokine to G3PDH to allow for quantitative comparisons between cytokines and animals.

2.8. Statistical analysis

Comparisons of results between FIV-infected and uninfected cats were performed using nonparametric analysis (Wilcoxon's ranking test). Differences between infected

and uninfected cats were considered significant at $P < 0.05$. Comparisons of FIV-p26 gag expression between cats within the FIV-infected group were made using the Student's *t*-test with an alpha level of 0.05.

3. Results

3.1. FIV infection of SPF cats

As expected, all of the SPF cats infected with FIV-NCSU₁ became PCR+ for provirus in peripheral blood mononuclear cells (PBMC) and developed antibody to the gag proteins by 4 weeks after infection (data not shown). The FIV-infected cats demonstrated a progressive decline in the peripheral blood CD4⁺:CD8⁺ ratios secondary to a decrease in CD4⁺ cells and an increase in CD8⁺ cells (Fig. 1). These findings are characteristic for infection by FIV and are similar to those previously reported for experimental infection with the NCSU₁ virus isolate (English et al., 1994; Davidson et al., 1993; Yang et al., 1996; Tompkins et al., 1991).

3.2. Total BAL cell counts and differentials following FIV infection

Examination of cell populations in BAL fluids clearly showed that the total cell counts derived from infected and control groups did not vary appreciably throughout the course of the experiment (Fig. 2A). This indicates that FIV infection of SPF cats alone does not result in measurable changes in pulmonary cell numbers. Differential counts on the recovered BAL populations from control and FIV-infected cats demonstrated that, with

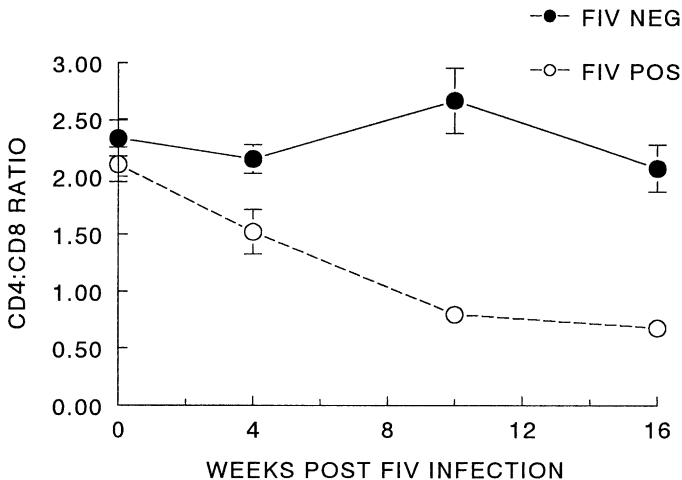


Fig. 1. Mean peripheral blood CD4⁺:CD8⁺ ratios of 12 cats infected with 5×10^6 TCID₅₀ of FIV-NCSU₁ and 10 uninfected control cats. The percent CD4⁺ and CD8⁺ cells was determined by two-color flow cytometric analysis. The plotted ratios are the means of individual ratios. Error bars represent S.E.

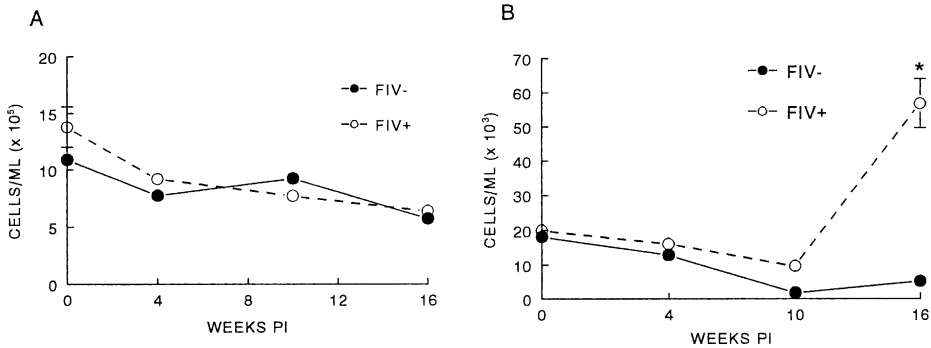


Fig. 2. Total cell counts (A) and absolute numbers of neutrophils (B) in bronchoalveolar lavages from FIV-infected and control cats. Total viable cell counts and differential analyses were determined as described in Section 2. Data represent the mean \pm S.E. of 12 FIV-infected and 10 uninfected cats. (*) Difference is significant at $P < 0.05$.

the exception of neutrophils, the absolute numbers of other cell populations (AM, eosinophils, lymphocytes) in both FIV-infected and control cats did not change significantly and were similar throughout the course of the experiment. The absolute numbers of neutrophils were significantly elevated ($P = 0.036$) in the FIV-infected cats only at 16 weeks post-FIV infection (Fig. 2B).

3.3. Constitutive cytokine production by AM

The HIV infection induces AM activation accompanied by the elaboration of a number of cytokines, such as TNF α , IL6, IL8, IL10, and IL12 (Agostini et al., 1991a; Denis and Ghadirian, 1994; Trentin et al., 1992; Twigg et al., 1992). To determine if FIV infection caused a similar activation of AM, BAL cells were collected from the FIV-infected and control cats, and constitutive cytokine production was measured. Alveolar macrophages (AM) from FIV-infected cats produced significantly greater levels of bioactive TNF α at 4 ($P = 0.001$) and 10 ($P < 0.001$) weeks post-FIV infection than did AM from control cats (Fig. 3A). Similarly, the FIV-infected cats had significant elevations in bioactive IL6 at 4 ($P = 0.002$) and 10 ($P = 0.003$) weeks post-FIV infection (Fig. 3B). The increased constitutive TNF α and IL6 secretion by AM coincided with changes in the CD4⁺ and CD8⁺ subsets in the circulation (Fig. 1). At 16 weeks post-infection, when the CD4⁺:CD8⁺ ratios had stabilized, there still remained significant levels of constitutive TNF α (Fig. 3A, $P = 0.001$) and IL6 (Fig. 3B, $P = 0.035$) production by AM.

Because of the limited availability of bioassays or ELISAs for feline cytokines, alterations in other cytokines were assessed by quantitating changes in mRNA levels using RT-qPCR. Interleukin-6, IL10, IL12 (p40), and TNF α were evaluated as monokines that are important for the initiation and regulation of innate and acquired immunity. For the most part, TNF α and IL6 mRNA expression correlated with the bioactive protein production (Fig. 4A and B). Alveolar macrophages (AM) from FIV-infected cats had elevated TNF α mRNA levels that bordered on statistical significance at 4 weeks ($P = 0.056$) and 10 weeks ($P = 0.045$) post-FIV infection. At 16 weeks post-FIV infection, the TNF α mRNA levels were similar to that of control animals. The

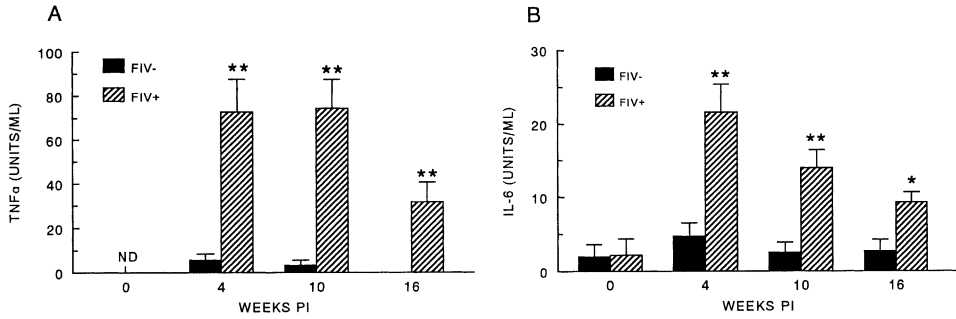


Fig. 3. Bioactive TNF α (A) and IL6 (B) in supernatants of AM collected at times post-FIV infection. Alveolar macrophages (AM) were purified by plastic adherence and cultured for 24 h. Cell supernatants were collected and assayed using the TNF α sensitive LM cell line (A) or the IL6-dependent T1165 cell line (B). Data represent the mean \pm S.E. of 12 (FIV-infected) and 10 (control) cats. (*) Difference is significant at $P < 0.05$; (**) Difference is significant at $P < 0.01$.

IL6 mRNA expression from FIV-infected cats was significantly elevated over control cats at 4 weeks post-FIV infection ($P = 0.033$), but by 16 weeks post-FIV infection had returned to levels similar to controls.

The IL10 mRNA expression in AM from FIV-infected cats peaked at 4 weeks post-FIV infection (Fig. 4C, $P = 0.007$) and declined to control levels at 16 weeks post-FIV infection. The control cats consistently expressed lower levels of IL10 mRNA than FIV-infected cats throughout the experiment (Fig. 4C).

Although generally considered a product of activated lymphocytes, IFN γ expression (mRNA and bioactive protein) from macrophages has been experimentally documented in other model systems (Munder et al., 1998; Nugent et al., 1985). Therefore, we investigated the ability of AM from FIV-infected cats to produce IFN γ . In contrast to AM from control cats, AM from FIV-infected cats produced high levels of IFN γ mRNA. Unlike the other cytokines, IFN γ mRNA levels increased progressively over the course of the infection (Fig. 4D, $P = 0.003$ at 10 weeks; $P = 0.002$ at 16 weeks). Table 1 shows that the numbers of FIV-infected cats expressing IFN γ mRNA also increased with time, whereas IFN γ mRNA was detected in only one control cat at one time point (16 weeks post-FIV infection). Thus, FIV-induced changes in IFN γ mRNA expression by AM was manifested by a time-related increase in the number of cats expressing IFN γ mRNA, as well as an increase in the level of expression for individual cats.

Table 2 shows that the AM collected from control cats consistently maintained low but measurable constitutive IL12 p40 mRNA expression at all timepoints. In contrast, the AM

Table 1
Numbers of cats with constitutive IFN γ mRNA expression in purified AM

FIV status	Weeks post-FIV infection			
	0	4	10	16
FIV+	0/12	2/12	12/12	12/12
FIV-	0/12	0/10	0/10	1/10

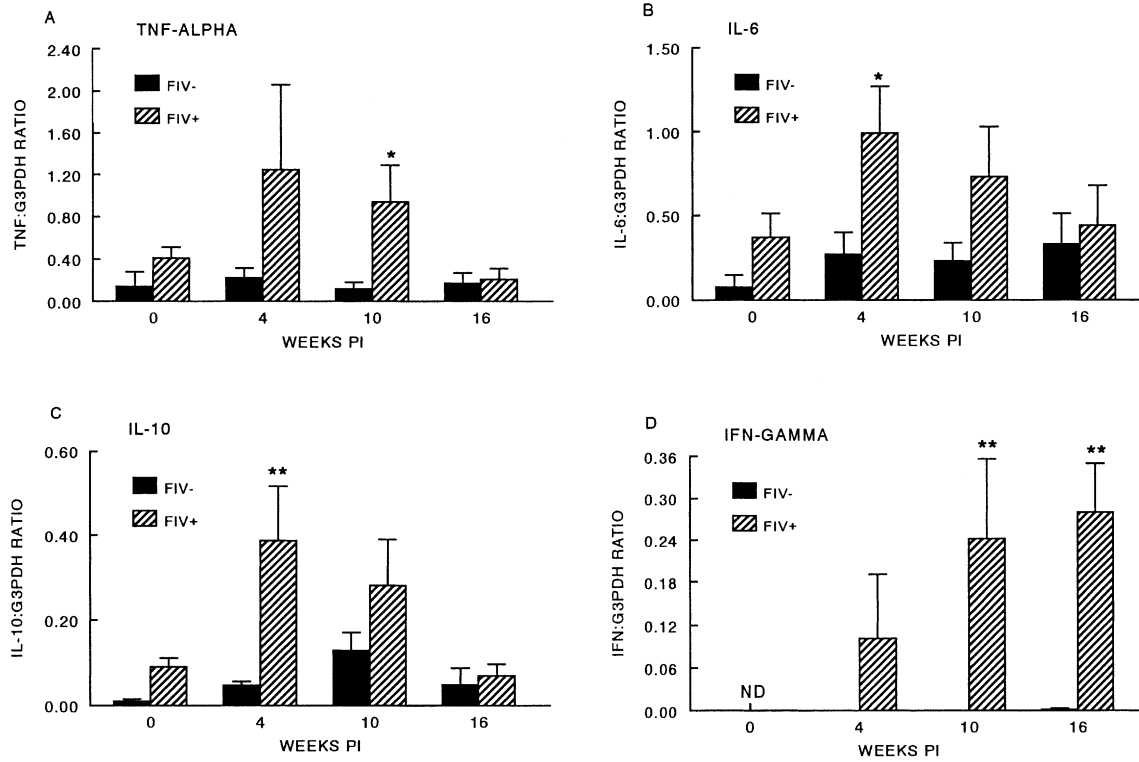


Fig. 4. Quantitation of constitutive cytokine mRNA expression in AM. Alveolar macrophages (AM) were purified by plastic adherence and total cytoplasmic RNA was extracted and analyzed by RT-qcPCR utilizing oligo-primers specific for TNF α (A); IL6 (B); IL10 (C); and IFN γ (D). Data represent the mean \pm S.E. of the cytokine:G3PDH ratio from 12 FIV-infected and 10 control cats. (*) Difference is significant $P < 0.05$; (**) difference is significant at $P < 0.01$. Expression of IFN γ mRNA was not detected (ND) from any FIV-infected or control cat at time 0 nor from control cats at any time point with the exception of one control cat at 16 weeks PI (see Table 1).

Table 2
Numbers of cats with constitutive IL12 mRNA expression in purified AM

FIV status	Weeks post-FIV infection			
	0	4	10	16
FIV+	11/12	9/12	6/12	3/12
FIV–	9/12	10/10	10/10	10/10

from the FIV+ cats demonstrated a progressive decrease in IL12 mRNA expression with time after infection, such that there was a progressive decrease in the number of cats with detectable IL12 mRNA. When compared as groups, the AM from FIV-infected cats expressed significantly less ($P = 0.032$) IL12 mRNA than control cats at week 16 (data not shown). However, the FIV-infected cats that maintained IL12 mRNA expression (3/12) had levels similar to that seen in the control cats (data not shown).

3.4. Quantitation of FIV-gag mRNA expression in AM following FIV infection

The FIV-gag mRNA was not detected in either the control or FIV-infected groups cats before FIV infection. Following FIV infection, there was a steady increase of FIV-gag expression over the 16-week course of infection; with peak gag mRNA expression occurring at 16 weeks post-FIV infection (Fig. 5). The increase in gag mRNA was due to

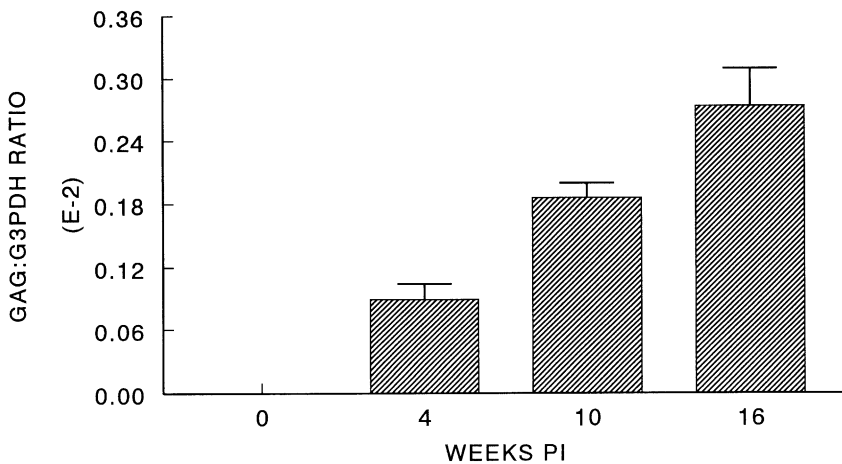


Fig. 5. Quantitation of constitutive FIV-gag mRNA expression from AM collected from FIV-infected cats. Alveolar macrophages (AM) were purified by plastic adherence and total cytoplasmic RNA was extracted and analyzed by RT-qPCR utilizing oligo-primers specific for p26 of FIV. The FIV-gag mRNA was not detected in the control cats at anytime or in FIV-infected cats before FIV infection. Data represents the mean \pm S.E. of the FIV-gag:G3PDH ratio from 12 FIV-infected cats. There was a significant increase in FIV-gag mRNA expression throughout the experiment (4 < 10 weeks, $P = 0.001$; 10 < 16 weeks, $P = 0.002$).

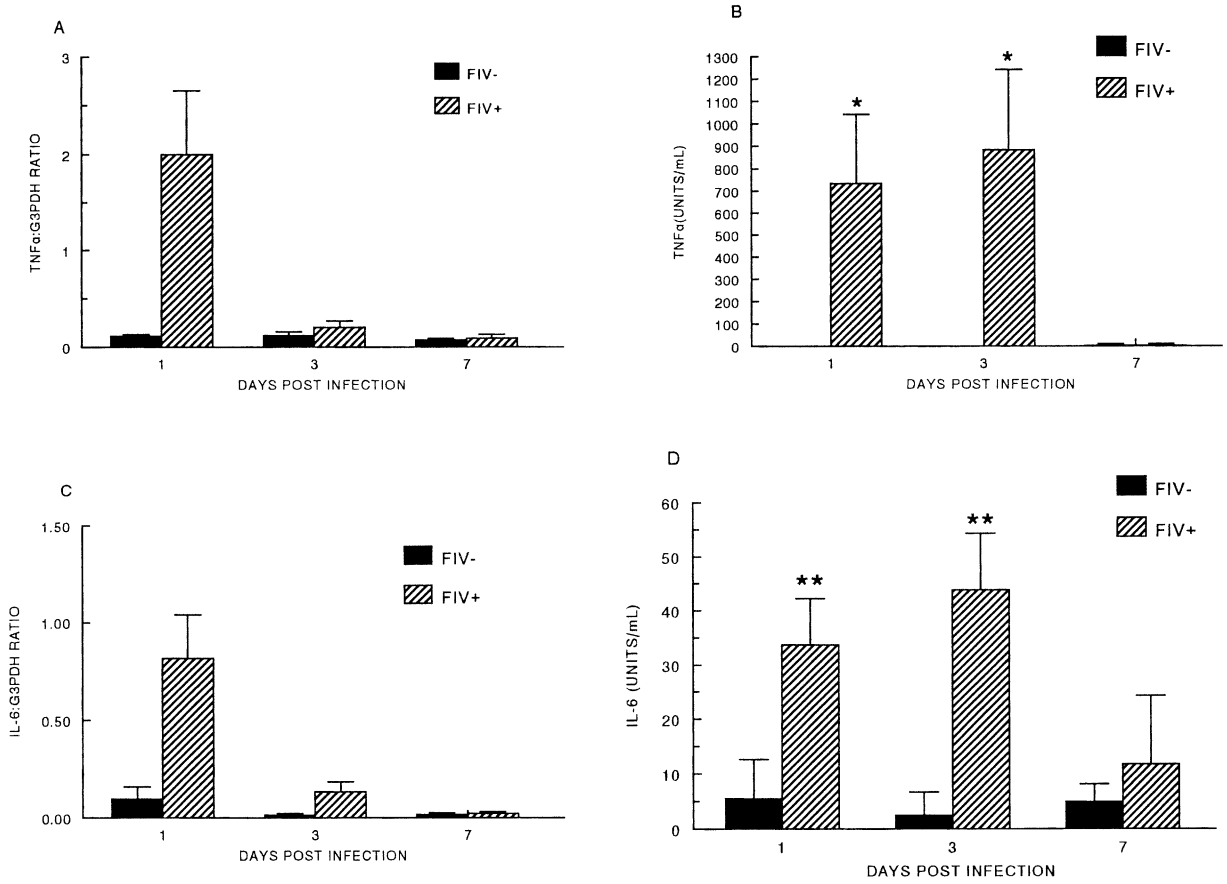


Fig. 6. TNF α (A, B) and IL6 (C, D) production by AM infected in vitro with FIV. Alveolar macrophages (AM) from normal cats were infected with FIV at an MOI of 1. The mRNA and cell supernatants were collected and assayed for cytokine message by PCR or protein by bioassay. Data represent the mean \pm S.E. of four different cat.

an absolute increase in RNA expression in each individual cat, as all of the cats expressed detectable p26 mRNA throughout the experiment.

3.5. Cytokine responses of AM infected with FIV *in vitro*

The above data suggests that the constitutive cytokine expression by AM recovered from FIV-infected cats is a direct response to FIV infection. To test this directly, AM from normal cats were infected with FIV *in vitro*, and TNF α and IL6 mRNA and protein were assayed. Fig. 6 indicates that AM cultured in the absence of virus produced little to no TNF α (Fig. 6A and B) or IL6 (Fig. 6C and D) mRNA or protein even after 7 days in culture, whereas those infected with FIV produced significant levels of TNF α and IL6 message (Fig. 6A and C) at 1 day after infection and protein (Fig. 6B and D) at 3 and 7 days after infection. Addition of irradiated FIV to AM cultures failed to induce a cytokine response (data not shown), suggesting a virus infection-dependent process. In support to this conjecture, FIV-gag mRNA was easily detectable in AM infected with live FIV but not irradiated virus at either 3 or 7 days after infection (data not shown).

4. Discussion

There is considerable evidence that cellular infiltrates and pro-inflammatory cytokines may play a role in the clinical manifestation of HIV-associated pulmonary disease. However, reports of specific cellular and cytokine changes in HIV-infected patients have been controversial. While it is clear that altered expression of IL1, IL6, IL8, IL10 and TNF α , in the pulmonary compartment is associated with HIV infection (Agostini et al., 1991a; Denis and Ghadirian, 1994; Trentin et al., 1992; Twigg et al., 1992), it is not known whether this is an intrinsic characteristic of viral infection of pulmonary cells (AM) or due to cofactors, such as secondary opportunistic infections. In this study, we addressed the question of cellular changes and cytokine expression in the pulmonary compartment of FIV-infected SPF cats, a lentivirus animal model in which interpretation of results is not confounded by potential exposure to secondary pathogens.

Total cell counts and absolute numbers of AM, lymphocytes, and eosinophils were similar in both FIV-infected and control groups. Although most studies on HIV-infected patients report no differences in total cell counts or AM numbers (Rankin et al., 1988; Meltzer et al., 1993), others have reported increases in both (Beck and Shellito, 1989; Agostini et al., 1991b). Agostini et al. (1991b) reported increased AM numbers in approximately one-third of HIV-infected patients whether or not they had concurrent opportunistic infections. Failure to demonstrate a decrease in AM numbers from HIV-infected patients and FIV-infected cats suggests that the high incidence of opportunistic pulmonary diseases in these lentiviral infections is not due to a numerical deficiency of macrophages in the lungs.

The most consistent cytologic feature of the BAL from FIV-infected cats was a neutrophilia at 16 weeks post-FIV infection. Similarly, increases in distinct cell populations including neutrophils, lymphocytes, and macrophages have also been reported in HIV-infected patients, in some cases, without altering the total cell counts

(Agostini et al., 1988; Davis et al., 1993). Because the pulmonary neutrophilia was not accompanied by either a systemic neutrophilia, clinical symptoms of illness, or bacterial contamination of the BAL fluid, the neutrophilia is related to FIV infection and not a result of a secondary bacterial pneumonia. Also, as a pulmonary neutrophilia did not develop in the control cats, the neutrophilia observed in FIV-infected cats is not a result of the sequential BAL. A neutrophilia in the BAL population has been reported in cats during the asymptomatic stage (range 8 months to 4 years) of FIV infection (Hawkins et al., 1996) that is similar to the neutrophilia reported in later stages of HIV infection (Agostini et al., 1991b). Cadore et al. (Cadore et al., 1997) also reported a neutrophilic alveolitis in cats naturally infected with FIV and suffering from a number of secondary complications. The mechanism that regulates the recruitment of neutrophils to the lung is most likely the release of macrophage-derived cytokines, perhaps in response to secondary infections. In humans, IL8 and GM-CSF have been found to be increased in patients infected with HIV-1 (Agostini et al., 1992; Denis and Ghadirian, 1994). In this study, we did not specifically evaluate chemokine expression from AM; however, it would not be unreasonable to expect that the constitutive cytokine release exhibited by AM from FIV-infected cats (TNF α , IL6, IL10, IFN γ) would also include IL8, GM-CSF, and other chemokines.

An important difference in the BAL cell populations of FIV- and HIV-infected subjects is the failure of FIV-infected cats to develop the pulmonary lymphocytosis that is characteristic of HIV infection (Agostini et al., 1988). There was no difference in the absolute numbers of lymphocytes between FIV-infected and control cats, and lymphocytes never contributed to more than 4% to the total BAL cell population. Based upon absolute lymphocyte counts, 25% of patients with early HIV infection and 50% of patients with advanced disease show increased numbers of lymphocytes, particularly CD8⁺ lymphocytes, in the lung (Agostini et al., 1991b). These CD8⁺ cells are thought to be involved in the host defense mechanism against HIV (Beck and Shellito, 1989). While this study and previous studies have shown that FIV-infected cats develop a population of activated CD8⁺ cells in the peripheral circulation (Jeng et al., 1996; Bucci et al., 1998), Hawkins et al. (1996) and the present study show that significant numbers of lymphocytes are not recoverable from the pulmonary immune compartment by BAL.

As the absence of a “lymphocytic alveolitis” in FIV-infected cats is not due to a failure in the development of activated CD8⁺ lymphocytes in the circulation, one possible explanation is that there is a difference between HIV and FIV in recruitment of these cells to the lung. This could be related to the level of virus expression in the lung of cats versus the expression of viral antigens in the lung of HIV-infected patients. Although this study quantitated FIV-gag mRNA expression in purified AM, determination of actual antigen expression and location within the lung was not performed. While Ma et al. (1995) identified FIV mRNA by *in situ* hybridization in only a small fraction (1 in 10,000) of the total BAL cell population, studies to determine the extent of FIV antigens present in interstitial lung tissue have not been reported. An alternative possibility is that the CD8⁺ pulmonary lymphocytosis characteristic of HIV-infected patients is a response to subclinical secondary pulmonary infections that would not occur in FIV-infected SPF cats maintained in isolation facilities. In support of this, Cadore et al. (1997) described an interstitial pneumonitis in cats naturally infected with FIV and suffering from a variety of

other infectious disease complications. The pneumonitis was characterized by a neutrophilic and lymphocytic alveolitis. We also have recently observed that FIV-infected cats do have large numbers of activated CD8⁺ cells in the circulation that are not observed in the lungs unless they are exposed to a secondary pulmonary pathogen. Following exposure of FIV-infected cats to *T. gondii*, there is a rapid influx (1–3 days) of activated CD8⁺ cells into the pulmonary compartment that is not seen in normal cats challenged with *T. gondii* (unpublished observations).

Constitutive elaboration of cytokines by AM from FIV-infected cats showed many similarities to their HIV-infected counterparts. Expression of IL6, IL10, and TNF α mRNA was initially upregulated during the acute stage infection and then decreased to near control levels by 16 weeks post-FIV infection. Elaboration of bioactive TNF α and IL6 proteins was still evident at 16 weeks post-infection. As this experiment was interrupted at 16 weeks post-FIV infection, it is not known if the low level of constitutive TNF α and IL6 activity would remain or eventually return to normal levels. However, Levy et al. (1998) reported elevated expression of TNF α mRNA in lymph node cells from FIV-infected cats as long as 12 months post-infection. Alveolar macrophages (AM) from clinically healthy (asymptomatic) or diseased (ARC, AIDS) HIV-infected patients produced constitutive TNF α bioactive protein throughout the course of HIV infection (Israel-Biet et al., 1991; Krishnan et al., 1990; Millar et al., 1991). In contrast to the rise and fall of TNF α , IL6 and IL10 mRNA levels, IFN γ mRNA expression by AM from FIV-infected cats continued to increase throughout the experiment.

For these experiments, plastic adherence was utilized for purification of AM from other BAL cell populations (eosinophils, neutrophils, and lymphocytes). Whereas AM purity was confirmed by differential analysis (at least 95%), there remains a possibility that a cell type other than the AM is responsible for the IFN γ expression. Contaminating lymphocytes would seem to be the leading candidate; however, lymphocytes were only rarely, if ever, demonstrated on post-plastic adherence differentials. The predominant contaminating cell type were eosinophils. Eosinophils have been demonstrated to elaborate IFN γ mRNA in some model systems (Woerly et al., 1999); however, the levels of IFN γ mRNA demonstrated in the purified AM culture were independent of the level (5% or less) of eosinophilic contamination. For this reason coupled with the precedent of macrophage derived IFN γ in the literature (Munder et al., 1998; Nugent et al., 1985), AM were considered the most-likely source of the IFN γ mRNA. The increase in IFN γ mRNA in AM is similar to that reported in the peripheral blood and lymph nodes of HIV-infected patients (Graziosi et al., 1994; Graziosi et al., 1996; Than et al., 1997) and FIV-infected cats (Levy et al., 1998; Dean and Pedersen, 1998). It has yet to be determined if AM from HIV-infected patients constitutively produce IFN γ . If subsequent studies show that the AM is in fact not the source of the IFN γ mRNA, this should not diminish the fact that significant IFN γ mRNA levels were only detected in the FIV-infected cats. It is also possible that the increased cytokine production by AM from the FIV-infected cat was due to in vitro manipulation of previously sensitized cells. However, AM from normal cats were collected and cultured in vitro in parallel to those from the FIV-infected cats, supporting our conclusions that the elevated cytokine production in the latter group is due to FIV infection and not an artifact of culture.

Alveolar macrophages (AM) from most FIV-infected cats lost their ability to produce IL12 mRNA by 16 weeks post-FIV infection. This is somewhat different from HIV infection, where AM exhibit low level constitutive production of IL12 (Denis and Ghadirian, 1994). Despite the constitutive production of IL12, HIV-infected AM stimulated with *S. aureus* do not increase IL12 production as do control AM (Denis and Ghadirian, 1994). Similarly, Levy et al. (1998) reported that FIV-infected cats co-challenged with *T. gondii* did not respond with increased IL12 mRNA expression in the lymph nodes, as did *T. gondii* challenged control cats. Therefore, quantitation of cytokine mRNA expression in the lung and lymph nodes of FIV-infected cats indicate that susceptibility to *T. gondii* may be due (at least in part) to an inability to generate an IL12-dependent Th1 immune response in the lung. Interestingly, the number of cats in this study without detectable IL12 mRNA expression (9/12 or 75%) correlates well with the expected mortality rate (60–80%) previously reported for the FIV-NCSU₁-*T. gondii* co-challenge model (Davidson et al., 1993; Yang et al., 1996).

In vitro infection of AM with FIV also induced expression of TNF α and IL6. The expression of HIV in macrophages and lymphocytes is closely related to cellular activation and cytokine signaling (Kazazi et al., 1992; Poli et al., 1990a,b; Serpente et al., 1992). In this study, FIV-gag expression that increased throughout the experiment did not correlate with TNF α or IL6 expression that peaked at 4 weeks post-FIV infection. Interestingly, IFN γ was the only cytokine that was expressed with kinetics similar to the FIV-gag expression, suggesting that mechanisms regulating expression of TNF α and IL6 in AM are different from those regulating IFN γ . Upregulation of cytokines was dependent upon virus replication in AM, as FIV infection of AM in vitro induced cytokine mRNA and protein synthesis, whereas exposure of AM to irradiated virus failed to induce either cytokine mRNA or protein. The progressive increase in virus burden in AM also differs markedly with transient acute stage (2–4 weeks) high virus burden in plasma and PBMC (Jeng et al., 1996). This observation supports the conjecture that macrophages may be a major virus reservoir during the asymptomatic stage of infection.

The IL10 mRNA synthesis was also elevated in AM during the acute stage infection. However, similar to TNF α and IL6, peak IL10 expression did not correlate with AM virus burden. The continued increase in FIV-gag and IFN γ mRNA in the presence of high levels of IL10 is somewhat unexpected, as IL10 is a potent inhibitor of HIV replication (Akridge et al., 1994; Weissman et al., 1994), as well as macrophage cytokine expression (Gazzinelli et al., 1992; Murray et al., 1997). However, when IL10 is present at levels that do not inhibit TNF α and IL6 synthesis, IL10 will actually augment TNF α and IL6 mediated HIV expression (Weissman et al., 1995). The FIV replication may be similarly regulated; however, additional studies will be needed to resolve this question.

Concurrent upregulation of IFN γ and IL10 is consistent with recent observations that both FIV and HIV causes upregulation of some Th1 (IFN γ , TNF α), as well as Th2 (IL10) cytokines and a suppression of other Th1 (IL2, IL12) cytokines (Levy et al., 1998; Dean and Pedersen, 1998; Graziosi et al., 1994, 1996; Than et al., 1997). The data reported herein indicate that AM from FIV-infected cats have a similar constitutive cytokine profile as has been reported from lymph node cells and PBMC from FIV-infected cats and HIV-infected patients.

From the standpoint of immunoprotection from intracellular pathogens, levels of both protective (TNF α , IFN γ) and nonprotective (IL10, IL6) cytokines were elevated. As both HIV patients and FIV-infected cats are susceptible to intracellular pathogens, this suggests that it is not simply the appearance of protective cytokines exclusive of nonprotective cytokines, but the relative levels of the cytokines, in both quantity and timing of appearance. In support of this, the concurrent increased IL10 and decreased IL12 mRNA expression in FIV-infected cats has been shown to be predictive of susceptibility of FIV-infected cats to *T. gondii* (Levy et al., 1998). The FIV-*T. gondii* co-infection, thus, should provide a powerful model to dissect the role of cytokines in the pulmonary immunodeficiency in FIV- and HIV-infected patients.

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