$CD8^+$ T CELL ANTIVIRAL ACTIVITY: MECHANISM OF INDUCTION AND THE SUPPRESSION OF EMERGING FELINE IMMUNODEFICIENCY VIRUS STRAINS

A Dissertation

by

ANAGHA PHADKE

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2004

Major Subject: Microbiology

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ABSTRACT

CD8$^+$ T Cell Antiviral Activity: Mechanism of Induction and the Suppression of Emerging Feline Immunodeficiency Virus Strains.

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In the present studies, the essential role of inducer cells for the induction of soluble anti-viral activity against feline immunodeficiency virus (FIV) was investigated. Induction of suppression of FIV replication was found to not strictly require autologous cells and was probably not FIV specific. Suppression was maximum when the inducer cells and the effector CD8$^+$ T cells were in contact with each other, suggesting a potential role for membrane antigen interactions and/or cytokines in the induction process. Additionally, flow cytometry analysis demonstrated a significant increase in the percentage of CD8$^+$ B7-1$^+$ T cells in the peripheral blood of chronically FIV infected cats as compared with uninfected cats.

Examination of the FIV V3-V4 envelope sequences from PBMC, lymph nodes and spleen from six cats chronically infected from three to six years with the molecular clone of FIV-PPR did not demonstrate viral variants specific for the tissues examined, emphasizing the critical role of the initial diversity and virulence of the infecting virus

inoculum. Additionally, *in vitro* $CD8^+$ T cell antiviral activity demonstrated by four of the six cats could have led to the control of virus replication *in vivo*, resulting in the uniform viral variants observed.

Infection of specific pathogen free cats with FIV-TX53, an FIV isolate that belongs to an emerging subtype more closely related to FIV clade B, demonstrated an acute stage infection characterized by lymphoadenopathy and a viral dose dependent decline of $CD4^+/CD8^+$ T cell ratios below 1 by 11 weeks post infection. Interestingly, an expansion of $CD8\beta^{low}$ population of $CD8^+$ T cells was observed in the infected cats.

The soluble antiviral activity generated from inducer T cell stimulated $CD8^+$ T cells from FIV-A-PPR infected cats also suppressed *in vitro* replication of the emerging FIV-TX53 and FIV-TX078 isolates. This is the first report demonstrating that the $CD8^+$ T cell antiviral activity is inter-clade effective among FIV strains. As the success of a FIV vaccine could be hampered by occurrence of highly divergent viral variants in the fields, the exploitation of this innate, soluble anti-FIV activity could contribute to the design of novel, safe and complementary anti-FIV therapeutic strategies.

To my family, especially my parents and husband, whose continued blessings, support, unconditional love and patience all these years, have allowed me to see this day.

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CHAPTER I

INTRODUCTION

FELINE IMMUNODEFICIENCY VIRUS

FIV genome organization

Feline immunodeficiency virus (FIV), previously called feline T–lymphotropic virus, was first isolated in 1986 from a Petaluma cat in California with an AIDS like disease (Pedersen et al., 1987). FIV is classified as a member of the lentivirus genus of the *Retroviridae* family because it resembles lentiviruses, such as human immunodeficiency virus and simian immunodeficiency virus, in virion morphology, tropism for T lymphocytes and for cells of the monocyte, macrophage lineage, Mg^{2+}-dependent reverse transcriptase activity and persistent infection in the host (Olmsted et al., 1989b; Pedersen et al., 1987; Talbott et al., 1989; Yamamoto et al., 1989). Since its discovery, FIV has been recognized as a common pathogen worldwide in both domestic and wild cats such as panthers, pumas, lions and bob cats (Olmsted et al., 1992; Yamamoto et al., 1989). The similarity of FIV infection in cats to HIV infection in humans, makes it a suitable small animal model for evaluating the potential of certain antiviral compounds, (Bisset et al., 2002; North and LaCasse, 1995), approaches for experimental vaccination (Jarrett et al., 1990; Pu et al., 2001; Yamamoto et al., 1991) and pathogenesis of lentivirus induced immunodeficiency (Yamamoto et al., 1988).

This dissertation follows the style of Virology.

Although FIV is similar to primate lentiviruses, including HIV, in its biologic activities in the host, its genetic organization and gene regulation are more similar to the lentiviruses of the ungulates (Olmsted et al., 1989a; Sparger et al., 1992; Talbott et al., 1989; Thompson et al., 1994a). The FIV genome is composed of two copies of about 9,400 bp of single stranded RNA, has three long open reading frames encoding the group associated antigen, polymerase and envelope proteins and several short ORFs encoding the regulatory proteins, such as vif, rev and ORF-A (Phillips et al., 1990; Talbott et al., 1989). The gag gene is initially translated to yield a precursor polyprotein of about 50kDa that is subsequently cleaved to yield the functionally mature matrix, capsid and nucleocapsid proteins (Bendinelli et al., 1995; Elder et al., 1993; Steinman et al., 1990). The pol gene overlaps the gag gene by 109 nucleotides, is in a -1 reading frame with respect to that of gag gene and is translated as a gag-pol fusion polyprotein by ribosomal frameshifting (Morikawa and Bishop, 1992). This polyprotein is then cleaved to yield protease, reverse transcriptase, dUTPase and integrase (Elder et al., 1992; Elder et al., 1993). The primary translation product of the FIV env gene is processed through proteolytic cleavage into the surface protein and the transmembrane protein (TM) (gp40) (Verschoor et al., 1993). Pancino et al. (1993b) have identified nine variable (V) regions throughout the FIV env protein. The first two lie in the leader region, which is also the first coding exon of the rev gene and because of proteolytic processing are not present in the mature env protein. Variable regions V3 through V6 are found in the SU, and variable regions V7 through V9 occur in the TM. The env glycoprotein plays a key role in determining viral tropism, mediates membrane fusion

involved in virus penetration and syncytium formation, and it contains linear and conformational epitopes (de Ronde et al., 1994) against which humoral immune responses are induced by the host. The V3 region of the SU, in addition to the ectodomain of the TM glycoprotein (Vahlenkamp et al., 1997) has been found to be responsible for the tropism of FIV for Crandell feline kidney (CRFK) cells (Siebelink et al., 1995b; Verschoor et al., 1995). Macrophage tropism is dictated by the V3 and V4 regions of the env gene (Vahlenkamp et al., 1999). The V3 region also contains a linear neutralization domain (de Ronde et al., 1994; Lombardi et al., 1993b) that is involved in the neutralization of CRFK cell adapted FIV strains while V4 and V5 regions were found to be necessary for FIV neutralization in feline thymocytes (Siebelink et al., 1995a). Cell culture adapted strains of FIV are able to use CXCR4 to facilitate cell fusion mediated through the V3 region (Willett et al., 1997b; Willett et al., 1997c). The 2 long terminal repeats (LTRs) of FIV, like those of maedi-visna virus, but unlike HIV, have strong basal promoter activity at least in certain cell types and are only poor transactivators (Miyazawa et al., 1992; Sparger et al., 1992). Studies on the regulatory genes of FIV have indicated similar roles for these proteins in FIV and HIV replication. The viral infectivity factor (vif) gene, essential for FIV replication in lymphocytes and macrophages, encodes a regulatory protein that is necessary for cell free virus dissemination to new target cells from the initial site of infection (Lockridge et al., 1999; Shacklett and Luciw, 1994). Rev is necessary for stabilization and transport of unspliced and singly spliced viral mRNA from the nucleus to the cytoplasm (Kiyomasu et al., 1991; Phillips et al., 1992; Tomonaga et al., 1993b). Orf-A, a tat-like gene encodes a

viral transactivator necessary for productive FIV replication in primary peripheral blood mononuclear cells (PBMC), primary feline T cells and feline T cell lines (de Parseval and Elder, 1999; Phillips et al., 1990; Sparger et al., 1994; Tomonaga et al., 1993a; Waters et al., 1996). However, unlike other lentiviral transactivators, it requires additional LTR elements, such as ATF for transactivation (Chatterji et al., 2002).

FIV env diversity

Molecular clones of FIV have been derived from the Petaluma strain designated as FIV-34TF10 and FIV-14 (Talbott et al., 1989), from the PPR strain designated as FIV-pPPR (Phillips et al., 1990), from the Japanese strain FIV-TM2 designated as pTM219 (Miyazawa et al., 1989), from the Netherlands strain (Siebelink et al., 1992), from the Glasgow strain FIV-GL_8 designated as FIV-$GL8_{414}$ (Hosie et al., 2000) and from the FIV-$NCSU_1$ strain designated as JSY3 from North Carolina (Yang et al., 1996). Under experimental conditions three FIV isolates have shown to consistently induce fatal immunodeficiency disease: Subtype A virus FIV $NCSU_1$ (English et al., 1994) which has been molecularly cloned (Yang et al., 1996), and rapidly passaged versions of FIV subtype C and B isolates (Bachmann et al., 1997; Diehl et al., 1995b).

Sequences within the env gene are least conserved among the FIV isolates (Pancino et al., 1993b; Sodora et al., 1994). Five distinct clades of FIV (A-E) have been identified with 15-30% variability in env amino acid sequence although a majority of viruses identified belong to either clade A or clade B (Bachmann et al., 1997; Elder et al., 1998; Sodora et al., 1994). Recent studies on FIV diversity in our laboratory have

provided evidence that FIV isolates from free-roaming cats in Texas, forming a distinct cluster as compared to other clades, may have evolved from FIV clade B (Weaver et al., 2004). Although these cats were present with various clinical abnormalities, such as skin lesions, lymphoid hyperplasia and presence of roundworms and tapeworms at necropsy, the acute stage of the disease caused by these FIV TX isolates has not been previously studied. For a vaccine to be effective, selection of vaccine challenge strains that are most representative of field isolates is essential. Hence studying the disease course manifested by the isolates belonging to the newly emerging subtype is essential for identifying new more virulent strains.

Different mechanisms have been put forward to explain HIV-1 env evolution *in vivo*, including recruitment of latently infected $CD4^+$ cells and T cell activation, positive selection of variants as a result of viral escape from specific host defenses, such as CTLs and also the effect of negative forces (Casado et al., 2001). Moreover non-selective mechanisms, such as random drift, founder effects, bottlenecking and Muller's ratchet and compartmentalization and selection of variants with env glycoproteins optimally adapted to locally expressed receptors (Casado et al., 2001; Wong et al., 1997), have also been found to be associated with env variation. In addition to this, the error prone reverse transcriptase with a high rate of HIV turnover also contributes to accumulation of point mutations in the HIV genome (Preston et al., 1988).

Studies regarding the evolution of FIV env gene over time have yielded contrasting results. Most of the studies carried out with naturally infected cats demonstrate very close similarity (99.3% or 98%) between the viral variants obtained

from different tissues of the same animal (Nishimura et al., 1996; Siebelink et al., 1992). Sodora et al. (1994) found that intra-cat diversity of env sequences from naturally infected cats was approximately half of that reported for sequence variation of HIV-1 from humans. A degree of sequence similarity among different proviral clones is unusual for lentiviruses, which in general show much more sequence variation, especially in the env gene. An exception to this are studies by Greene et al. (1993) that have shown that the rate of change of FIV was 3.4×10^{-3} nucleotide substitutions per site per year for the env gene and less than 10^{-4} nucleotide substitutions per site per year for the gag and pol genes, values that were similar to those found that for HIV-1. In this study though, virus isolates derived from the same cat were more highly related to each other than to isolates from other cats (10-12% env variation). Since these studies have been carried out using naturally infected cats, the length of the time that the cats had been infected, in addition to the diversity in the infecting virus inoculum, early target cells or tissues in the host, expansion of the fittest genotype (s) in each tissue and induction of host immune response would contribute to the evolution of viral variants.

In order to determine the temporal genetic variation in the env gene, it is necessary to start with a homogenous population of virus that can be studied for both, viral evolution within a host and generation of tissue specific variants by eliminating factors, such as the initial mixed inoculum of viral variants that differ in cellular tropism. An important feature of the FIV model system is the availability of molecular clones that, after transfection into cells in culture, give rise to infectious virions (Hosie et al., 2002; Miyazawa et al., 1991; Phillips et al., 1990; Siebelink et al., 1992; Talbott et al.,

1989). Hence the fate of a single FIV clone can be determined, as it replicates over time in the infected host. Relating the biological properties of such populations to the genomic structure of the molecular clone, from which they were derived, may reveal changes in amino acids required for enhanced pathogenicity and altered tropism of the virus.

Kohmoto et al. (2003) demonstrated that the molecular clone of FIV TM2 could be transmitted across the mucosal epithelium without broadening of cell tropism or lack of variation in the V3- V5 env region at 4 weeks post infection (p.i.). Infection with a molecular clone of FIV Petaluma FIV-F14 yielded viral variants from the peripheral blood of two infected cats at 135 weeks p.i. (Hosie et al., 2002), which had altered cell tropism as compared to the parental clone used for infection. Dean et al. (1999) found that experimental inoculation of cats with molecular clones of FIV-PPR and Petaluma (FIV-34TF10 and FIV-14) yielded viral variants in the bone marrow in 3 cats infected with FIV-34TF10, in the lymphoid tissues of 1 cat infected with FIV-14 and 2 cats infected with FIV-PPR at 23 weeks p.i. by heteroduplex mobility assay. However studies that screened for the presence of FIV viral variants among different tissues of chronically infected cats initially experimentally infected with a molecular clone of FIV have not been reported.

FIV receptors and tropism

Although FIV infects $CD4^+$ T cells, FIV does not use the CD4 molecule as a primary receptor or coreceptor (Hosie et al., 1993; Norimine et al., 1993; Willett and

Hosie, 1999). CD9 was identified as a putative receptor for FIV but later was found to be required for virus release from the cells rather than virus entry (de Parseval et al., 1997; Willett et al., 1997a). The primary isolates of FIV (which have been passaged 3-10 times) can be propagated only in cultures of fresh mitogen stimulated PBMC, dendritic cells, thymocytes, macrophages or IL-2 dependent T cell lines (Brown et al., 1991; Dean et al., 1996; Dow et al., 1999; English et al., 1993). FIV can also infect a range of $CD4^-$ cell types including feline monocytes and macrophages, B cells and cells of the neuronal lineage, such as primary cultures of astrocytes and microglia, but neither oligodendrocytes nor neurons (Willett and Hosie, 1999). The primary and tissue culture passaged isolates of clade A and the laboratory adapted isolates of strain B share usage of the CXCR4 receptor, the co-receptor for the entry of HIV into cells, for env-mediated fusion and productive infection of cells (Egberink et al., 1999; Frey et al., 2001; Poeschla and Looney, 1998; Richardson et al., 1999; Willett et al., 1997c). It is not yet clear if this receptor serves as a primary receptor or the co-receptor for FIV infection. A 40kDa protein (distinct from CXCR4) has also been thought to play a role in FIV-PPR infection and cell-adapted FIV-34TF10 molecular clone's SU interaction with primary feline T cells (de Parseval and Elder, 2001). FIV-PPR was also found to infect CRFK cells using CXCR4 but at very low levels, while FIV-34TF10 was shown to use cellular heparans, in addition to CXCR4, for infection of adherent cells, such as CRFK cells, G355-5 feline glial cells, human Jurkat T cells, HeLa cells and interleukin (IL)-2 independent feline lymphoma cells 3201(de Parseval and Elder, 2001; Lerner and Elder, 2000). CXCR4 was also found to mediate the entry of FIV-Petaluma into astrocytes,

maybe contributing to dementia in the later stages of FIV infection (Nakagaki et al., 2001). Detection of mRNA messages for CCR5, CXCR4, and CCR3 in various feline tissues of the reproductive tract, large intestine and PBMC also point to the possible role of multiple chemokine receptors in the entry of FIV (Caney et al., 2002; Kovacs et al., 1999). Infection of primary human PBMC and macrophages *in vitro* by two different strains of FIV, FIV Petaluma and V1-CSF was inhibited by antibodies to human CXCR4, CCR5 and CCR3 (Johnston and Power, 1999; Johnston and Power, 2002). Experimental FIV infection of cyanomolgous macaques resulting in loss of $CD4^+$ T cells and weight loss has also been shown (Johnston et al., 2001). The capacity of both primate and non-primate lentiviruses to interact with chemokine receptors suggests common determinants in the pathogenesis of these viruses that could facilitate cross-species transmission.

Mode of transmission of FIV

The major mode of transmission of FIV is thought to be via bite wounds (Yamamoto et al., 1989). FIV has been demonstrated in the saliva of naturally or experimentally infected cats (Yamamoto et al., 1989) and can also be isolated from the blood, serum, plasma, cerebrospinal fluid of naturally and experimentally infected cats by tissue culture methods (Yamamoto et al., 1988). Venereal transmission from infected males to females though never been clearly documented experimentally, is thought to be possible since replication competent FIV in cell free and cell associated forms could be detected in the semen of domestic cats (Jordan et al., 1995). At least some strains of FIV

can be transmitted vertically from mother to offspring *in utero*, post natally via milk, and potentially also during parturition, thus paralleling perinatal HIV transmission in humans (O'Neil et al., 1995; O'Neil et al., 1996). Virus strains representing three clades of FIV (A, B and C) can be experimentally transmitted across vaginal, rectal and oral mucosa by either cell associated or cell-free virus (Bishop et al., 1996; Bucci et al., 1998a; Bucci et al., 1998b; Burkhard et al., 1997; Gebhard et al., 1999; Moench et al., 1993; Obert and Hoover, 2000a; Obert and Hoover, 2000b; O'Neil et al., 1996).

Disease caused by FIV

FIV causes a disease syndrome in cats similar to that caused by HIV in humans. The clinical phases of FIV infection parallel those of HIV (Pedersen, 1992): 1) an acute phase characterized by a burst of viral replication, flu like illness, lymphadenopathy, and progressive decline in $CD4^+$ T lymphocytes with a reduction in the $CD4^+/CD8^+$ T cell ratio, 2) a prolonged asymptomatic phase with viral downregulation and a continued decline of $CD4^+$ T cells and 3) a terminal phase characterized by immunologic abnormalities, increase in the viral load, wasting, hematologic suppression and clinical immunodeficiency with opportunistic infections.

Both FIV and HIV infection have a well-defined first stage of disease. The primary phase of infection is characterized by varying degrees of fever, diarrhea, gingivitis, conjunctivitis, uveitis, jaundice, secondary bacterial sepsis, neutropenia (often associated with a mild to moderate leucopenia), and generalized lymphadenopathy. The fever and other clinical signs persist from a few days to several weeks before

disappearing (Barlough et al., 1991; Callanan et al., 1992; Yamamoto et al., 1988). The severity of the primary signs of disease varies with age (George et al., 1993). When FIV-Petaluma strain was used for infection, the most persistent lymphadenopathy developed in newborn kittens while young adult cats (8-12 months of age) developed a much less pronounced transient lymphadenopathy and lymphadenopathy was not observed in aged cats (7.5-11.5 years of age), in spite of their more rapid rate of progression to the next stages of the disease. Similarly, the severity and duration of neutropenia decreased with increasing age (George et al., 1993). During the early acute phase of FIV infection, the inversion of the $CD4^+$/ $CD8^+$ T cell ratio is compounded by a sharp increase in lymphocytes expressing the feline CD8 marker (fCD8). This expanded subset which displays reduced expression of the fCD8 marker (fCD8low) and increased levels of major histocompatibility complex (MHC) class II antigens (Willett et al., 1993). In addition to quantitative changes in lymphocyte phenotype, qualitative dysfunction of B- and T- lymphocytes has been reported for FIV infection. The onset of immunodeficiency is marked by reduced responsiveness of feline PBMC to *in vitro* T- cell and B-cell mitogens (Siebelink et al., 1990; Torten et al., 1991), decreased interleukin-2 (IL-2) production (Lawrence et al., 1995; Siebelink et al., 1990), and hypergammaglobulinemia attributable to elevated IgG levels (Ackley et al., 1990). In addition, there is a significant increase in IL-1, IL-6 and tumor necrosis factor production (Lawrence et al., 1995). Thus, as shown in HIV-infected humans, FIV produces a significant perturbation of cytokine production that may contribute to the immune dysfunction.

After the initial signs of clinical illness associated with primary FIV infection have subsided, the infection usually remains clinically inapparent for prolonged periods of varying duration during which the virus can be isolated from the blood (Yamamoto et al., 1988). The asymptomatic phase is followed by a phase characterized by persistent generalized lymphadenopathy (PGL), including vague signs of illness, such as recurrent fevers, anorexia, weight loss or nonspecific behavioral changes (Pedersen, 1992; Yamamoto et al., 1989). Chronic secondary or opportunistic infections are absent at this stage. Half of the clinically ill cats brought to the veterinarian are present in the next stage i.e. the ARC (AIDS-related complex)-like stage of the disease. Cats are presented with chronic secondary infections of the oral cavity, upper respiratory tract and other body parts, but not with opportunistic infections. The agents causing these infections include other feline viruses, bacteria, fungi, protozoa and parasites (Bendinelli et al., 1995; Pedersen, 1992). Weight loss of about 20%, chronic diarrhea, chronic stomatitis and gingivitis, chronic respiratory disease, chronic skin infections, chronic enteritis PGL, hematologic abnormalities (anemia, leukopenia, neutropenia, lymphopenia) have also been observed (Bendinelli et al., 1995; Pedersen, 1992; Yamamoto et al., 1989). Most, if not all, cases diagnosed as ARC progress to feline AIDS (FAIDS) after variable time intervals. Cats suffer from secondary infections listed above, opportunistic infections and to a lesser extent from neurologic and neoplastic disorders during the FAIDS stage (5 to 10% of clinically ill FIV infected cats) (Bendinelli et al., 1995; Hutson et al., 1991; Yamamoto et al., 1989).

While the usual natural course of FIV infection is observed over several years, strains that are associated with accelerated clinical immunodeficiency can be generated by *in vivo* passage of plasma from cats in acute phase of FIV infection (Diehl et al., 1996; Diehl et al., 1995b). This has been observed with an FIV strain belonging to the subtype C designated CABCpadyOOC (FIV-C-PGammer). This isolate rapidly killed two-thirds or more of the kittens infected intravenously at <12 weeks of age. Viremia detected in the plasma of the infected kittens was higher than that described for either acute HIV or SIV infection (Diehl et al., 1995b).

ANTIVIRAL ACTIVITY AGAINST LENTIVIRUSES

Role of antibodies

Antiviral response to FIV infection in cats includes both humoral and cell-mediated immunity. The antibodies to the capsid (p24) and env proteins appear between 4 and 6 weeks p.i. in cats infected with FIV. The timing of appearance of the antibodies to either capsid or env varies depending on the virus strain used and the route of infection (Avrameas et al., 1993; Burkhard et al., 2001; Egberink et al., 1992; Hosie and Jarrett, 1990; Rimmelzwaan et al., 1994). Major B cell epitopes have been mapped to SU glycoprotein, the TM env glycoprotein (Avrameas et al., 1993; de Ronde et al., 1994; Pancino et al., 1993a) and the gag region (Lombardi et al., 1993a). Tozzini et al. (1993) found that active production of neutralizing antibodies was evident about 5-6 weeks p.i., which reached a plateau 3 to 4 months later and persisted throughout the prolonged observation period. Peptides derived from the principal immunodominant domain (PID)

(Pancino et al., 1995) found in the FIV env TM region are recognized by 100% of the FIV- infected cat sera (Avrameas et al., 1993).

$CD8^+$ T cell mediated antiviral activity

Cellular immune responses mediated by $CD8^+$ T cells determine whether a person infected with HIV-1 develops AIDS and are often implicated in the maintenance of long-term asymptomatic state. During HIV infection, CTLs are thought to play a key role in preventing progression to the AIDS stage. The presence of CTLs has been inversely correlated with viral load in HIV infected individuals and hence thought to play a key role in maintaining the asymptomatic state in HIV infected individuals (Cohen and Fauci, 1996). Persistent $CD8^+$ T cell depletion experiments in SIV infected animals, demonstrated an increased SIV viral load, in the absence of $CD8^+$ T lymphocytes (Cohen and Fauci, 1996). This indicated that the CTLs were important in maintenance of the viral load at a steady state and thus in controlling SIV infection.

FIV specific CTLs have been detected in the peripheral blood of cats experimentally infected with FIV-GL_8, as early as 2 weeks p.i. (Beatty et al., 1996), in cats chronically infected with the FIV-Petaluma strain (Song et al., 1992; Song et al., 1995) and in cats mucosally infected with either FIV-A-PPR or FIV-B-2542 at 9 weeks p.i. (Burkhard et al., 2001). As the disease progressed following FIV-GL_8 infection of cats, the CTLs were found to reside predominantly in the lymph nodes and spleen up to one year p.i., but were not detected in the peripheral blood (Beatty et al., 1996). This is in contrast to the presence of CTLs in chronic FIV-Petaluma infection of cats (Song et

al., 1992; Song et al., 1995) and HIV infection where the CTLs persist into the asymptomatic period (Rinaldo et al., 1995; Walker et al., 1987) .

In contrast to the cytotoxic activity, $CD8^+$ T cells suppress viral replication leaving the HIV infected cells intact. The non-cytolytic function of $CD8^+$ T lymphocytes was first described about 17 years ago by Dr. Jay Levy (Walker et al., 1986). $CD8^+$ T cell antiviral response is thought to be mediated, at least in part by the so called soluble CD8 antiviral factor (CAF) which lacks identity to known cytokines and chemokines such as interleukin (IL)-2 to IL-12, IL-16, Interferon-α, β, γ, TNF-α, TNF-β, TGF-β, granulocyte-macrophage-colony stimulating factor (GM-CSF), RANTES, MIP-1 α, MIP-1 β, MCP-1, 3, GRO-α, GRO-β, leukemia inhibitory factor, lymphotactin, IP-10, granzymes A, B, granulysin, protegrins, histatins and defensins, TNF-1 receptors- I and II and sFas (Levy, 2003). This antiviral activity was not HLA restricted (Mackewicz et al., 1998; Walker et al., 1991a). The antiviral response mediated by CAF has been identified very early in primary infection, before the appearance of anti-HIV antibodies, is associated with higher $CD4^+$ T cell counts, decreased viral loads, improved clinical status and long term-nonprogression (Levy et al., 1996). Non-cytotoxic responses of $CD8^+$ T lymphocytes, a type of innate immunity, could be more important than the cytotoxic responses in controlling certain viral infections (Levy, 2001).

A similar kind of immune response involving non-cytolytic $CD8^+$ T cells which suppressed replication of FIV in target cells *in vitro*, in a non-MHC restricted manner was seen in naturally or experimentally FIV infected cats (Bucci et al., 1998b; Choi et al., 2000b; Flynn et al., 1999; Hohdatsu et al., 2000; Jeng et al., 1996). The $CD8^+$ T cell

antiviral activity against FIV-Petaluma and FIV-GL_8 could also be demonstrated in blood and lymph nodes of cats vaccinated with whole inactivated virus prepared from the FIV-Petaluma strain (Flynn et al., 1999). This $CD8^+$ T cell antiviral activity was found to be mediated through cell-cell contact between effector cells ($CD8^+$ T cells) and target cells (either PBMC or feline T cell lines, such as MYA-1 and FCD4E cells) or in part by a soluble factor (Bucci et al., 1998b; Choi et al., 2000b; Flynn et al., 1999; Hohdatsu et al., 1998). Human MIP1-α and not SDF1-α was able to suppress FIV-PPR replication in feline PBMC (Choi et al., 2000b). The $CD8^+$ T cell antiviral activity acted by inhibiting mRNA transcription during the virus life cycle and could be detected by Flynn et al. (1999) during the acute phase only or by others during the asymptomatic chronic phase and the symptomatic phase of FIV infection (Choi et al., 2000b; Hohdatsu et al., 1998; Jeng et al., 1996). Cats with high levels of FIV suppression in the thymus and blood had low virus loads in the primary and secondary lymphoid tissues (Crawford et al., 2001). As in humans and primates, virus load was inversely correlated with the strength of the FIV suppression among individual cats (Bucci et al., 1998a). In another study, the $CD8^+$ T cell antiviral activity was also found to inversely correlate with plasma viremia and $CD4^+$ T cell counts (Hohdatsu et al., 2003). This antiviral activity could be detected as early as 1-week p.i. with FIV-GL_8 strain, even before the CTL activity which was demonstrated at 4 weeks p.i (Flynn et al., 2002). Thus, both CTL and non-cytotoxic antiviral activity appeared before the humoral immune response in FIV infection indicating that this may be a part of the host's innate response.

Phenotypes of cells involved in the $CD8^+$ T cell mediated antiviral activity

Following experimental infection of cats with the $NCSU_1$ isolate of FIV, the $CD8^+$ T effector cells responsible for non-cytolytic suppression of FIV replication were found to have the activation phenotype characterized by reduced surface expression of the CD8β-chain ($CD8\alpha^+\beta^{low}$) and L-selectin (CD62L), as well as an increased expression of CD44, CD49d and CD18 (Bucci et al., 1998b; Gebhard et al., 1999). However, the correlation between the $CD8^+$ T cell anti-FIV activity and the presence of the unique $CD8\alpha^+\beta^{low}$ phenotype in cats has been controversial. In neonatal cats infected with FIV $NCSU_1$-JSY3, molecular clone, the antiviral $CD8^+$ suppressor cells emerged during the acute infection in the blood and thymus and were found to be the predominant $CD8^+$ phenotype in the blood and the lymph nodes during chronic infection (Crawford et al., 2001). Bucci et al. (1998b) have also shown that the unique $CD8\alpha^+\beta^{low}$ phenotype was responsible for suppression of FIV $NCSU_1$ in acute and long-term asymptomatic infection of cats. In contrast to these studies, both $CD8^{hi}$ and $CD8^{lo}$ T cell subpopulations prepared from PBMC of FIV-GL_8 infected cats were shown to suppress FIV replication *in vitro* (Flynn et al., 2002). Supporting the latter study, Hohdatsu et al. (2003) found no correlation between $CD8\alpha^+\beta^{low}$ or $CD8\alpha^+\beta^-$ T cells counts and anti-FIV activity in experimentally or naturally infected cats.

$CD8^+$ T cells that mediate the anti-HIV activity have been characterized by an activated phenotype expressing HLA-DR and /or CD28 markers and to correspond to $CD29^+/CD45RA^-$ memory cells (Landay et al., 1993). Copeland et al. (2002) have

shown that stronger CAF activity was observed for $CD8^+$ T cells expressing the activation markers $CD38^+$ or $CD38^+HLA\text{-}DR^+$.

Although the phenotypes characterizing the $CD8^+$ T cells involved in the antiviral activity are known, the physiological stimulus and the cells required for their stimulation and induction remain a mystery. Recent studies have shown that dendritic cells enhanced the non-cytotoxic $CD8^+$ T cell anti-HIV activity *in vitro* which was mediated primarily by production of IL-15 by the mature dendritic cells (Castelli et al., 2003). Few studies point to the role of costimulatory molecules, such as B7 in enhancing the $CD8^+$ T cell antiviral activity. Barker et al. (1999) demonstrated that while both the B7-1 and B7-2 costimulatory molecules could deliver a signal sufficient to increase $CD8^+$ T cell antiviral activity, it was the B7-2 molecule on macrophages that enhanced $CD8^+$ T cell suppression of HIV replication. Although studies by Tompkins et al. (2002) have shown significant numbers of $B7\text{-}1^+$ $CD4^+$ and $B7\text{-}1^+$ $CD8^+$ T cells in both blood and lymph nodes of FIV-infected cats with $B7\text{-}2^+$ T cells for the most part being restricted to lymph nodes, the role of B7-1 in the induction of $CD8^+$ T cells mediating anti-FIV activity has not been demonstrated.

Lack of specificity at the effector and inducer phase of control of virus replication

$CD8^+$ T cell mediated antiviral activity has been demonstrated in HIV-infected chimpanzees (Castro et al., 1991), SIV-infected monkeys (Ennen et al., 1994; Kannagi et al., 1988), HIV-infected baboons (Locher et al., 1999) and FIV-infected cats (as mentioned earlier), suggesting that this natural antiviral response is common in lentivirus

infections. $CD8^+$ T cell mediated antiviral activity in HIV and FIV infection has however, been found to be non-specific at the effector phase of control. Thus, Walker et al. (1991b) demonstrated that CAF is not virus specific and could suppress replication of wide variety of lentiviruses, such as HIV-1, HIV-2 and SIV strains. This antiviral response has also been shown to reduce LTR transcription of murine and avian retroviruses (Copeland et al., 1995). $CD8^+$ T cells of African green monkeys inhibited HIV-1 replication in human $CD4^+$ T cells (Ennen et al., 1994) while $CD8^+$ T cells of SIV-infected sooty mangabeys inhibited the replication of HIV-2, SIV-1 (SIV_{smm-7}) and HIV-1 (LAV) (Powell et al., 1990). Furthermore, $CD3^+CD8^+$ T cells of HIV-1 IIIB infected chimpanzees were shown to inhibit replication of HIV-1 strains IIIB, MN and RF *in vitro* (Husch et al., 1993). In addition, requirement for a specific HIV antigen or the virus itself for stimulation of $CD8^+$ T cells to mediate the antiviral activity has also not been found to be necessary. Supporting this, the anti-HIV activity has been demonstrated from $CD8^+$ T lymphocytes of HIV-uninfected, healthy individuals, $CD8^+$ T cell clones derived from HIV-uninfected individuals, CTLs from HIV-1 uninfected individuals and even from cord blood (supposed to contain only naïve $CD8^+$ T cells) from HIV-uninfected neonates (Bagasra and Pomerantz, 1993; Hsueh et al., 1994; Kootstra et al., 1997; Liu et al., 2003; Mackewicz et al., 1997; Rosok et al., 1997). Additionally, the $CD8^+$ T cell antiviral activity was found to be effective against heterologous FIV isolates such as FIV-Petaluma, FIV-GL_8 and FIV-PPR belonging to the FIV clade A (Choi et al., 2000b; Flynn et al., 1999). However, effective $CD8^+$ T cell

antiviral activity has not yet been demonstrated to afford cross-clade protection in FIV infection of cats.

OBJECTIVES

In our earlier studies, reproducible production of non-cytotoxic anti-FIV activity of $CD8^+$ T lymphocytes, from cats in the asymptomatic stage of FIV infection, was maximally achieved *in vitro* with effector cells stimulated by FIV-PPR strain infected inducer T cells rather than mitogen (Choi et al., 2000b). Extending on earlier work the objectives of this dissertation were to:

- Determine the cells that could be used as inducer cells for the stimulation of the $CD8^+$ T cells responsible for suppression of FIV replication *in vitro*
- Determine the phenotypes of the inducer cells and fresh PBMC with respect to B7, MHC class I or MHC class II, CD4 and CD8 expression
- Examine the kinetics of the induction process to determine the optimal days for generating the $CD8^+$ T cell generated anti-FIV activity *in vitro*
- Determine effect of the $CD8^+$ T cell antiviral activity generated from $CD8^+$ T cells of FIV-PPR infected cats, on the suppression of the newly isolated FIV TX strains
- Examine the pathogenesis of newly emerging TX isolates and compare the acute stage of the disease course with that described for other FIV isolates

- Examine the presence of viral evolution and tissue specific env variants that may have arisen in the cats after three to six years of infection with the FIV-PPR molecular clone

CHAPTER II

THE ROLE OF INDUCER CELLS IN MEDIATING SOLUBLE ANTI-VIRAL ACTIVITY AGAINST FELINE IMMUNODEFICIENCY VIRUS BELONGING TO DIFFERENT SUBTYPES*

INTRODUCTION

Feline Immunodeficiency virus (FIV) is a T-lymphotropic lentivirus in the family *Retroviridae* (Pedersen et al., 1987). Natural or experimental FIV infection of cats results in a transmissible AIDS-like disease, similar to that observed with human immunodeficiency virus (HIV) infection in humans (Pedersen et al., 1989; Yamamoto et al., 1988). FIV causes a transient acute illness characterized by fever, lymphadenopathy, neutropenia and neurologic problems that may recur following latency, resulting in clinical illness and immunosuppression (Pedersen et al., 1989; Yamamoto et al., 1988). The degree of acute clinical disease is dose-dependent on the infecting inoculum (Hokanson et al., 2000). Phylogenetic analysis have separated FIV strains into five distinct phylogenetic clades designated A to E, based on 15-30% variability in the envelope (env) amino acid sequence although the majority of viruses identified to date belong to either clade A or clade B (Bachmann et al., 1997; Elder et al., 1998; Sodora et al., 1994). However, recent phylogenetic analyses of Texas FIV isolates demonstrated

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that these isolates that were closely related to clade B isolates, formed a unique cluster distinct from the other clades (Weaver et al., 2004).

$CD8^+$ T cells are a critical component of cellular immune protection against HIV infection in humans. $CD8^+$ T lymphocytes control HIV, simian immunodeficiency virus (SIV), and FIV infection through both cytotoxic (CTL) and non-cytotoxic mechanisms (Appay et al., 2000; Barker, 1999; Barouch and Letvin, 2001; Blackbourn et al., 1994; Brinchmann et al., 1990; Charaf et al., 1993; Choi et al., 2000b; Flynn et al., 1999; Furci et al., 2002; Geiben-Lynn et al., 2001; Hohdatsu et al., 2000; Jeng et al., 1996; Kannagi et al., 1988; Le Borgne et al., 2000; Mackewicz et al., 1994; Pollack et al., 1997; Powell et al., 1990; Song et al., 1992; Walker, 1993; Walker et al., 1986; Yang et al., 1997). Non-cytotoxic responses of $CD8^+$ T lymphocytes, a type of innate immunity, may be more important than the cytotoxic responses in controlling certain viral infections (Levy, 2001). The non-cytotoxic antiviral response seen in certain HIV-infected individuals involves suppression by $CD8^+$ T cells of HIV replication in peripheral blood mononuclear cells (PBMC), monocyte-derived macrophages and acutely or naturally infected $CD4^+$ T cells (Barker et al., 1998; Brinchmann et al., 1990; Levy et al., 1996). This $CD8^+$ non-cytotoxic antiviral response is thought to be mediated, at least in part, by an undefined secreted CD8 antiviral factor (CAF) (Clerici et al., 1996; Levy, 2001; Levy et al., 1996). Such non-cytotoxic activity is not HIV-specific, not species specific, not MHC restricted and is identified immediately after acute HIV infection (Clerici et al., 1996; Levy, 2001; Mackewicz et al., 1998; Walker et al., 1991a). The antiviral activity was broadly cross-reactive, as $CD8^+$ T cells from individuals infected only with HIV-1

suppressed the replication of diverse strains of HIV-1 and HIV-2, as well as SIV (Walker et al., 1991b). The levels of $CD8^+$ T cell anti-HIV responses have been correlated with $CD4^+$ T cell counts and the absence of infection in individuals repeatedly exposed to HIV and in HIV infected, long-term survivors (Blackbourn et al., 1996; Furci et al., 2002; Gomez et al., 1994; Landay et al., 1993; Levy, 2001; Mackewicz et al., 1991; Stranford et al., 1999). In contrast, Copeland et al. (1997) did not find any correlation between the extent of $CD8^+$ T cell mediated suppression and clinical stage of infection or $CD4^+$ T cell counts in HIV-1 infected individuals.

$CD8^+$ T cell-mediated suppression of FIV replication has been described by several groups, although the mechanisms of activation and conditions for viral suppression vary with the methodologies (Bucci et al., 1998a; Choi et al., 2000b; Crawford et al., 2001; Flynn et al., 1999; Flynn et al., 2002; Hohdatsu et al., 2000; Hohdatsu et al., 1998; Hohdatsu et al., 2002; Jeng et al., 1996). Furthermore, *in vivo* reduction of FIV load has been correlated with a strong $CD8^+$ T cell mediated anti-FIV activity (Bucci et al., 1998a; Crawford et al., 2001; Unpublished data from our laboratory). Mitogen stimulated non-cytolytic T cells capable of suppressing the replication of $Glasgow_8$ isolate of FIV (FIV-GL_8) represented the first detectable anti-viral immune response in cats following FIV-GL_8 infection (Flynn et al., 2002). In our earlier studies, reproducible production of non-cytotoxic anti-FIV activity from $CD8^+$ T lymphocytes, from cats in the asymptomatic stage of FIV infection, was maximally achieved *in vitro* with effector cells stimulated by FIV-PPR strain infected inducer T cells rather than mitogen (Choi et al., 2000b).

Whereas a number of studies have attempted to characterize the $CD8^+$ T cells responsible for generating lentivirus suppression (Bucci et al., 1998b; Flynn et al., 2002; Gebhard et al., 1999; Landay et al., 1993; Toso et al., 1995; Zanussi et al., 1996) or have focused on characterizing the $CD8^+$ T cell antiviral activity (Geiben-Lynn et al., 2001; Mosoian et al., 2000), there is little known about the physiologic stimulus, that activates the effector $CD8^+$ T cells to generate suppressing activity. Previous reports suggested that HIV infection or a potential exposure to the virus was needed for the CAF activity (Levy et al., 1998; Walker et al., 1991b). However, the fact that the $CD8^+$ T cell antiviral response has been observed in asymptomatic HIV infected individuals (Blackbourn et al., 1996; Brinchmann et al., 1990; Hsueh et al., 1994; Mackewicz et al., 1991; Toso et al., 1995), HIV exposed but uninfected individuals (Furci et al., 2002; Levy et al., 1998; Stranford et al., 1999), healthy HIV-naïve individuals (Bagasra and Pomerantz, 1993; Kootstra et al., 1997; Rosok et al., 1997), as well as in FIV infected and uninfected cats (Choi et al., 2000b) suggests that mechanisms involved for the induction of this antiviral response are not virus specific.

Since the $CD8^+$ T cell antiviral activity seems to be non-specific with respect to HIV in humans or FIV in cats, the elicited immune response would seem to be of an innate nature. Both soluble and cellular components contribute to innate immunity (Levy, 2001), resulting in a general response of the immune system to an infection without specificity for a target antigen. Identifying the conditions leading to the induction of this antiviral activity is essential in exploiting this innate control of lentiviral infection. Here we report that feline skin fibroblasts (FSF) could be used as

inducer cells to mediate the soluble anti-FIV activity and the importance of inducer cell-effector cell contact in the generation of optimal suppression of FIV replication *in vitro*. Furthermore, in order to determine if this $CD8^+$ T cell antiviral activity could provide cross protection against FIV strains from a newly emerging subtype closely related to clade B, its effect on the replication of the Texas FIV isolates was also examined.

MATERIAL AND METHODS

Experimental animals

Specific, pathogen-free (SPF) cats, purchased from Harlan Sprague-Dawley, Madison, WI or Liberty Laboratories, Liberty corner, N.J., were serologically negative for feline leukemia virus. Cats were housed in a specific, pathogen-free environment at the Laboratory Animal Research and Resources Support Facility (LARR), Texas A&M University, College Station. Cats AUO2, AUO3, AWF1, AZV2 and E284 were chronically infected with FIV-PPR strain. These cats had been inoculated intravenously (i.v.), three to six years previously with 50 to 1250 $TCID_{50}$ of virus. Cats AUS3, OAE5 sham inoculated with saline solution were used as negative control cats.

Virus

FIV-PPR was propagated in feline PBMC from a SPF cat not infected with FIV. Additionally, FIV-TX53 (AY139109) and FIV-TX078 (AY139107) strains were propagated in PBMC from the feral cat from which it was isolated (Weaver et al., 2004). After 7 to 10 days of infection, virus replication was evaluated with an FIV capsid

antigen detection enzyme-linked immunosorbent assay (ELISA) (Choi et al., 2000b). Supernatants with an optical density (OD) of more than 2.0 were collected and these stocks were stored at –80^{o}C. The FIV-PPR virus stock had a titer of 0.5 x $10^{5.1}$ / ml and FIV-TX53 and FIV-TX078 had titers of 0.5 x 10^{6}/ ml, determined as described by Hokanson (Hokanson et al., 2000).

Cell culture

Feline PBMC were isolated from EDTA (K_3)-treated whole blood by Histopaque-1077 (Sigma, St. Louis, MO) density gradient centrifugation (Choi et al., 2000b). PBMC were cultured as described previously with RPMI 1640 (Gibco BRL, Grand Island, N.Y.) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Atlas Biologicals, Fort Collins, CO), 50 ug/ml of gentamicin (Gibco BRL, Grand Island, N.Y.), 5 x 10^{-5} M 2-mercaptoethanol (Gibco BRL, Grand Island, N.Y.), 2mM L-glutamine (Gibco BRL, Grand Island, N.Y.), and 100 U of human recombinant IL-2 (hr IL-2) (Gibco BRL, Grand Island, N.Y.) per ml (Choi et al., 2000b). Cells were grown at 37^{o}C in a humidified atmosphere of 5% CO_2.

Continuous culture of feline skin fibroblasts (FSF)

To develop FSF cell lines, tissues were prepared from skin plug biopsies of infected cats AUO2, AUO3, AWF1, AZV2, OLQ5 and uninfected cat OAE5 (Dr. Mary Carpino). After five to six passages in culture, the cells were transformed by transfection of SV40 T antigen-expressing plasmid DNA (Gift from David Busbee, Department of

Veterinary Anatomy, Texas A & M University). The transformed FSF were selected by adding geneticin (0.5mg/ml of the medium) (Gibco BRL, Grand Island, N.Y.). The FSF were cultured in MEM-alpha supplemented by 10% heat inactivated fetal bovine serum (FBS) (Atlas Biologicals, Fort Collins, CO), 2mM L-glutamine (Gibco BRL, Grand Island, N.Y.), 20 units/ml of epidermal growth factor (Gibco BRL, Grand Island, N.Y.) and 50 ug/ml of gentamicin (Gibco BRL, Grand Island, N.Y.).

Preparation of FIV-PPR infected inducer T cells

Feline T cells were infected with FIV-PPR strain and irradiated (11,000 rads from a ^{6o}Co source provided by the Nuclear Science Center, Texas Engineering Experiment Station, College Station) before using as inducer cells for the stimulation of effector cells, as previously described (Choi et al., 2000b; Song et al., 1992). Briefly, freshly prepared PBMC were stimulated for three days with 5 ug/ml of Concavalin A (Con A) (Sigma, St. Louis, MO). The stimulated cells were infected with FIV-PPR for 1 h at 37^{o}C. The cells were washed and cultured in complete RPMI containing 100 U hr IL-2 per ml. After 6 days of incubation, virus replication was monitored using the FIV capsid antigen ELISA. These FIV infected cells were cryo-preserved to be used later as inducer cells.

Preparation of FSF as inducer cells

The gene encoding FIV capsid antigen was amplified by PCR from FIV-PPR infected T cells using primers synthesized with Bam HI sites and ligated into Bam HI-

digested Semliki Forest virus vector (SFV) (Gibco BRL, Grand Island, N.Y.) (Phadke et al., 2004). The SFV vector RNA was prepared by transcribing *in vitro* from Spe I-linearized recombinant vectors and was transfected into FSF using DMRIE-C reagent (Gibco, BRL, Grand Island, N.Y.). After 18 hours in complete RPMI medium, the SFV-capsid expressing FSF were used as inducer cells.

Phenotype enrichment

Negative selection for enrichment of $CD8^+$ T from freshly isolated PBMC of FIV-PPR-infected cats was performed using a combination of panning and magnetic beads. Briefly, plastic adherent cells were first removed from fresh PBMC by incubating the PBMC in complete RPMI at 37^{o}C in a humidified atmosphere of 5% CO_2 for 4 hours. The non-adherent cells were washed once in phosphate-buffered saline (PBS). The washed cells were then incubated in 1 ml of PBS with 3% FBS containing 500 ul of anti-feline CD4 monoclonal antibody (CAT30A, gift from Dr. Wayne Tompkins, North Carolina State University) and 30 ul of mouse anti-human CD14 monocyte antibody (Clone TUK4, Dako, Carpinteria, CA) for 30 minutes at 4^{o}C on a mixer. The cells were washed once in PBS, resuspended in 1 ml of PBS with 3% FBS containing M450 dynabeads that had been coated with goat anti-mouse IgG (Dynal biotech, Great neck, N.Y.) and incubated for 30 minutes at 4^{o}C on a mixer. After the bead bound cells ($CD4^+$ T cells and $CD14^+$ cells) were magnetically removed, the remaining cells, mostly $CD8^+$ T cells, were resuspended in 1 ml of PBS with 3% FBS, gently agitated to release any trapped nonbound cells, and then magnetically reisolated. The purity of the $CD8^+$ T cells

was confirmed by flow cytometry with a FACscaliber flow cytometer (Becton-Dickinson, San Jose, CA) at the Veterinary Pathobiology Core Facility (Director, Dr. Roger Smith, Texas A&M University, College Station) (data not shown).

Stimulation of effector cells by SFV-capsid expressing inducer cells

FSF prepared from cat AZV2, were plated in 6 well plates (3 X 10^5 cells/well) and were transfected with SFV-capsid RNA, SFV lacZ RNA or SFV-1 RNA (vector only without any insert) using DMRIE-C (Gibco, BRL, Grand Island, N.Y.). Five ug of RNA were used for each transfection reaction. Expression of SFV-capsid on the FSF was confirmed by an immunofluorescence assay (IFA), performed as described elsewhere (Brown et al.., 1991) (Fig. 1). Expression of lacZ was confirmed by adding X-galactosidase (Promega, WI)**.** These inducer cells transfected with SFV-capsid RNA, SFV-lacZ RNA or SFV RNA were overlaid 18 hours after transfection with autologous or heterologous effector cells (either fresh PBMC or $CD8^+$ T cells) from FIV infected and uninfected cats. Every two days for six days, the non-adherent cells were harvested and centrifuged at 1200 rpm for 10 minutes (Jouan Inc., Winchester, VA), and the supernatants collected and stored at 4^oC before overlaying the cells onto freshly prepared inducer cells. Controls were non-transfected FSF cultured in the absence of effector cells, effector cells cultured without FSF and non-transfected FSF co-cultured with effector cells. The supernatants collected from these cells on days 2, 4 and 6 were stored at 4^oC.

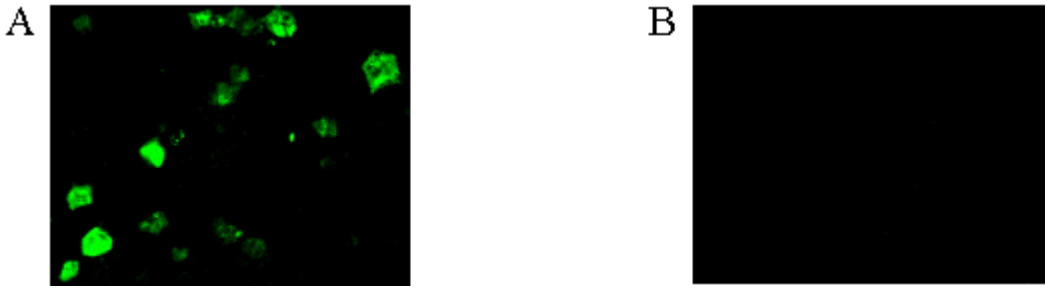


Fig. 1. Immunofluorescent staining of the FIV capsid protein expressed on FSF. Magnification 200X. FIV capsid protein expressed on FSF after transfection of FSF with SFV vector expressing FIV capsid RNA (A). Untransfected FSF cells are shown in (B) (Since these cells are untransfected, the fluorescent antibody does not recognize any antigen, as a result of which the figure 1B appears blank).

Transwell separation of cells

To assess the role of cell-cell contact in the induction of the effector cells, the irradiated inducer T cells were co-cultured with autologous effector PBMC in 6-well tissue culture plates in which the two cell populations were separated by a 0.45 um pore size polycarbonate membrane (Corning Incorporated, Acton, MA) at a ratio of 1:5 inducer to effector cells in a total volume of 3 ml of complete RPMI. Inducer cells were also co-cultured with the effector PBMC, such that the inducer cells and the effector cells were in contact with each other. PBMC cultured without any inducer cells were used as controls. Newly irradiated inducer cells were added to the effector cells on the third day. The supernatants were collected on days 3 and 6 and stored at 4^{o}C.

Cross- reactivity of the $CD8^{+}$ T cell antiviral activity from FIV-PPR infected cats

In order to determine if the $CD8^{+}$ T cell anti-viral activity from FIV-PPR infected cats was effective in suppressing replication of the Texas isolates, $CD8^{+}$ T cells were enriched by negative selection from FIV infected cats, AZV2, AUO2, AUO3, AWF1, and E284. All $CD8^{+}$ T cells with the exception of $CD8^{+}$ T cells from cat AWF1 were stimulated by irradiated autologous FIV-PPR infected T cells for 6 days. On the third day, the supernatants were harvested from the co-culture and newly irradiated inducer T cells were added to the effector $CD8^{+}$ T cells. The supernatants collected on days 3 and 6 were stored at 4^{o}C. As controls, the irradiated inducer cells were also cultured and supernatants were collected on days 3 and 6 and stored at 4^{o}C.

FIV suppression assay

Cell-free supernatants stored at 4^{o}C were ultracentrifuged at 35,000 rpm for 2 hours (Beckman L7-55 Ultracentrifuge, Palo Alto, CA) to remove the residual viral particles. The supernatants were passed through 0.22 um-pore size filters (Pall Corporation, Ann Arbor, MI) and stored at 4^{o}C. Target cells were prepared from PBMC of an FIV uninfected cat after culturing *in vitro* for six days with Con A and IL-2 (Choi et al., 2000b). They were then infected with the FIV-PPR strain and cultured with supernatants in a 96-well plate for 10 days at a medium to supernatant ratio of 1:1. Supernatant and fresh complete RPMI + hr IL-2 100 U/ml was added to the cells every 3 days. The amount of FIV in the supernatants was determined by an FIV capsid antigen detection ELISA. Suppression was considered to be positive when virus expression was ≤ 65% of virus expression in the untreated but FIV-PPR infected control cells (positive controls).

To determine the cross reactivity of the $CD8^{+}$ T cell antiviral activity, the FIV suppression assay was performed as mentioned above, with the exception that the target cells were infected with the TX078, TX53 or FIV-PPR strain.

Data analysis

The differences in FIV replication between infected control cells and cells in which suppression of FIV was seen, were analyzed using Student's t test (Ott, 1993). Statistically significant differences were set at $p < 0.05$.

RESULTS

Kinetics of the induction process using either PBMC or CD8$^+$ T lymphocytes as effector cells when FSF were used as inducer cells

In order to determine the kinetics of the non-cytolytic activity following exposure to FSF inducer cells, supernatants were collected every 2 days from the co-culture of FSF from cat AZV2 and PBMC from either autologous or heterologous effector cells. The FSF were transfected with SFV vector expressing FIV capsid or an irrelevant antigen such as lacZ or the SFV vector expressing only its polymerase. In the presence of FSF, suppression was maximum on the 2nd and the 4th day but decreased by the 6th day as observed for FIV infected cats AZV2 (Fig. 2A) and AUO2 (Fig. 2B & 2C) and also for FIV uninfected cats OAE5 (Fig. 2D) and AUS3 (Fig. 2E). Thus, induction of the anti-FIV activity did not require autologous FSF as inducer cells.

CD8$^+$ T lymphocytes were enriched by negative selection, in order to ensure that the CD8$^+$ T lymphocyte population within the PBMC contributed to the suppression of FIV replication *in vitro*. This population of CD8$^+$ T lymphocytes contained not more than 5% macrophages and not more than 10% CD4$^+$ T lymphocytes as determined by flow cytometry (data not shown). The kinetics of the soluble anti-FIV activity was determined using cat AZV2 FSF as inducer cells and enriched CD8$^+$ T lymphocytes from FIV-infected cats, AZV2 (Fig. 3A) and AUO2 (Fig. 3B) and an FIV-uninfected cat AUS3 (Fig. 3C) as effector cells. Supernatants were collected every two days from the co-culture of autologous or heterologous CD8$^+$ T lymphocytes and FSF transfected with SFV vector expressing FIV capsid or an irrelevant antigen such as lacZ or the SFV

A.

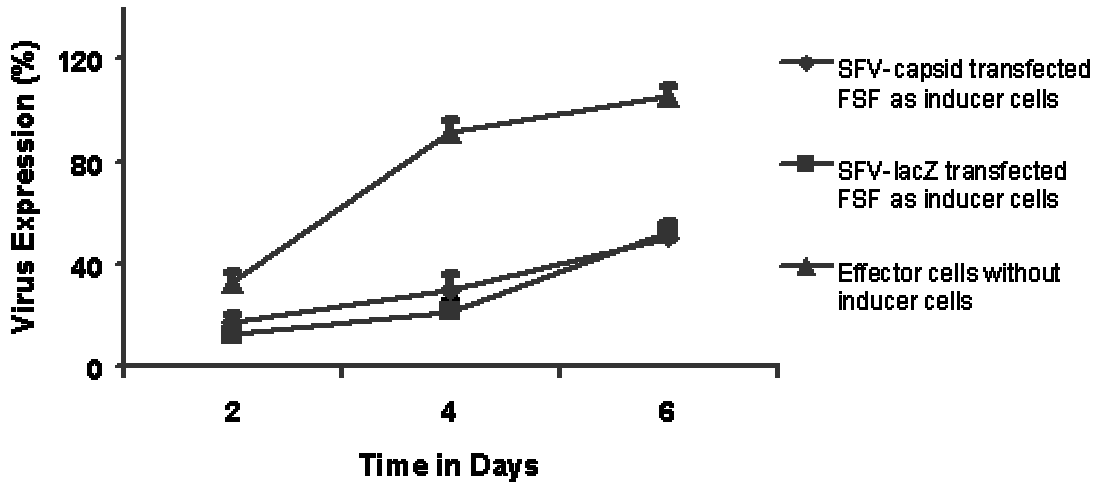
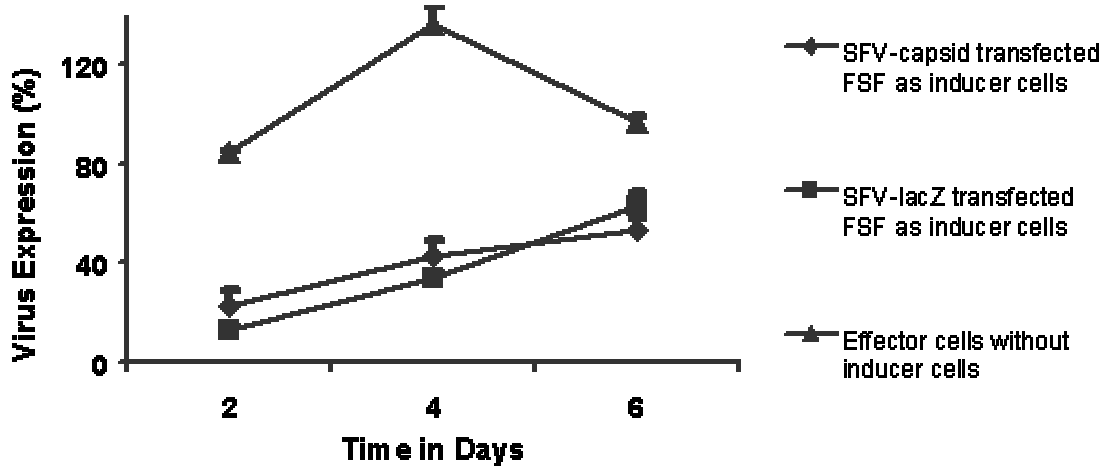


Fig. 2. Kinetics of the induction process using fresh PBMC as effector cells when FSF were used as inducer cells. FSF prepared from cat AZV2 were transfected with Semliki Forest virus vector (SFV) expressing FIV capsid, an irrelevant antigen such as lacZ or SFV vector expressing only its polymerase, or left untransfected. Fresh PBMC from FIV-infected cats AZV2 (A), AUO2 (B and C) and FIV uninfected cats OAE5 (D) and AUS3 (E) were overlaid on FSF. Every 2 days the supernatants were collected and stored at 4^{o}C and the non-adherent cells were overlaid on newly transfected FSF. As controls, supernatants were collected from untransfected FSF cultured without $CD8^{+}$ T lymphocytes and PBMC cultured in the absence of FSF. The supernatants were ultracentrifuged and added to FIV-PPR infected target cells (cat OAE5 PBMC) every 3 days at a medium to supernatant ratio of 1:1. FIV production was determined by use of an FIV-capsid antigen detection ELISA. All the values have been normalized with respect to values obtained for untransfected FSF cultured without effector PBMC.

B.



C.

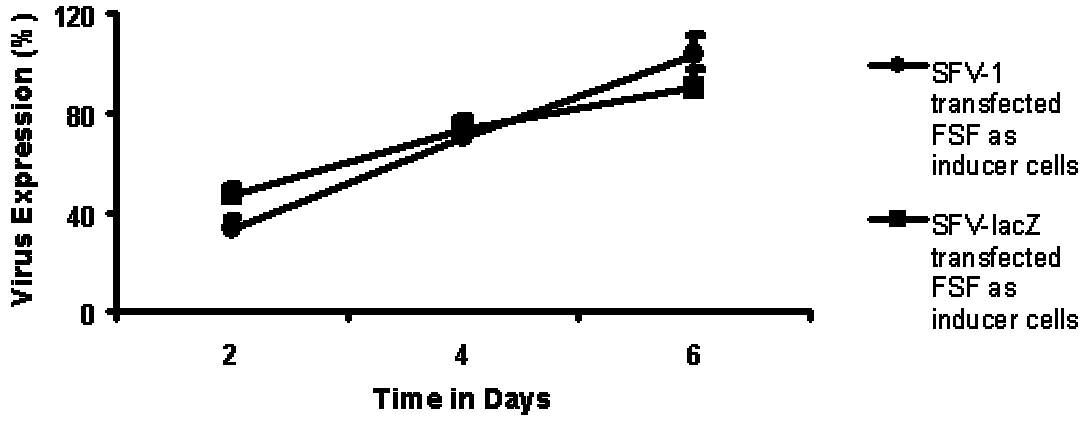
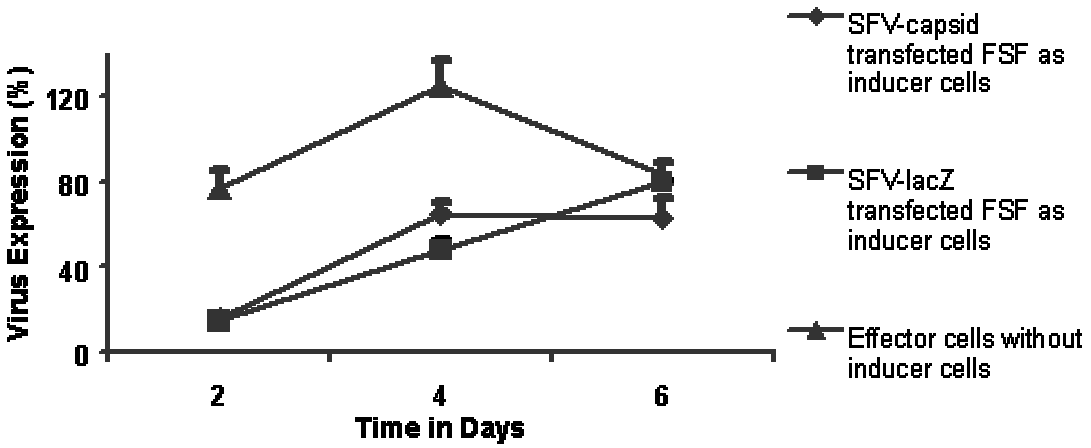


Fig. 2. Continued

D.



E.

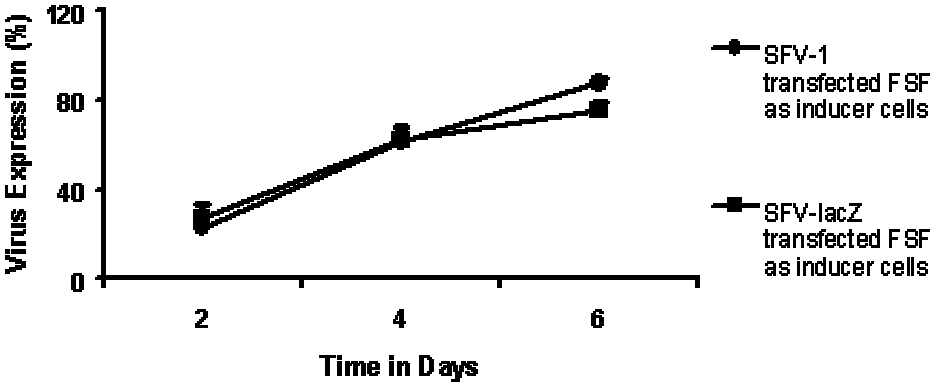


Fig. 2. Continued

vector expressing only its polymerase. In contrast to studies using PBMC as a source of effector cells, when $CD8^+$ T cells were used as effector cells, suppression of FIV replication was not only observed on day 6, but greater suppression was observed using supernatants collected on days 2 and 4 of induction of the effector $CD8^+$ T cells for the three cats that were examined (Fig. 3A-3C). The presence of macrophages and/or $CD4^+$ T cells may either dilute the suppression or actively inhibit the suppressive activity of $CD8^+$ T effector cells.

Interestingly, following induction with FSF transfected with SFV vector expressing an irrelevant antigen such as lacZ or SFV vector by itself, supernatants from effector $CD8^+$ T cells or effector PBMC of infected cats, AZV2 and AUO2 and uninfected cats OAE5 and AUS3 were also able to suppress FIV replication *in vitro* (Fig. 2A-2E, 3A- 3C). SFV vector without an insert expresses only the polymerase gene of the SFV virus during the course of transcription. It is possible that the polymerase or the viral RNA stimulates the effector cells, demonstrating that this kind of induction is not specific for FIV. Supernatants obtained from non-transfected FSF were not able to suppress FIV replication on any of the days examined (Fig. 2A-2E, 3A-3C). Supernatants from effector $CD8^+$ T cells or effector PBMC cultured without FSF did not demonstrate any suppressing activity on any of the days examined (Fig. 2A, 2B and 2D, 3A-3C). In addition, supernatants obtained from $CD8^+$ T cells co-cultured with non-transfected FSF did not show any suppressing activity (Fig. 3A-3C). Thus, non-transfected FSF cells could not induce $CD8^+$ T cells or PBMC to produce the anti-FIV activity.

A.

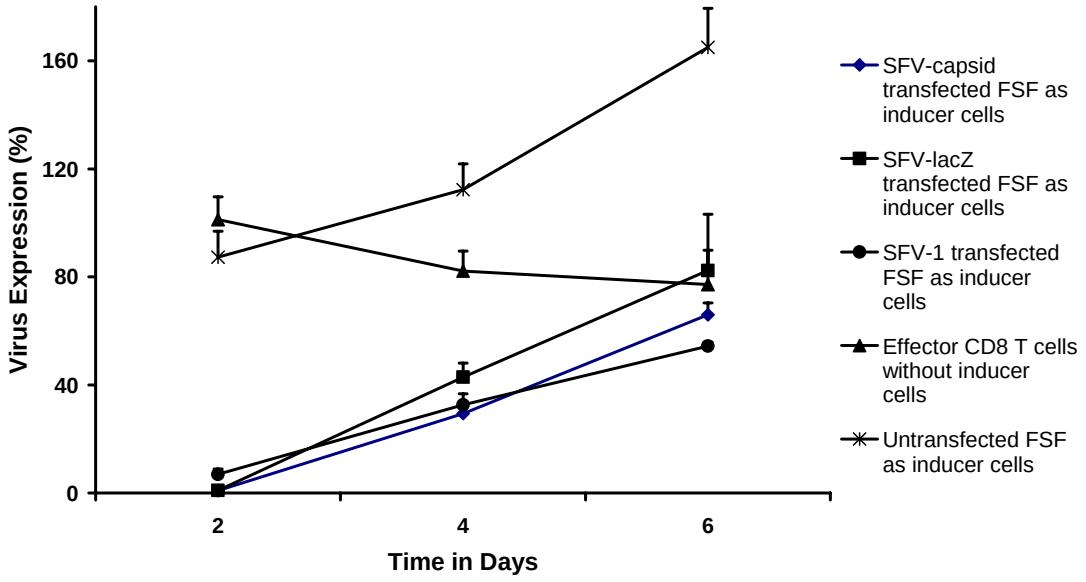
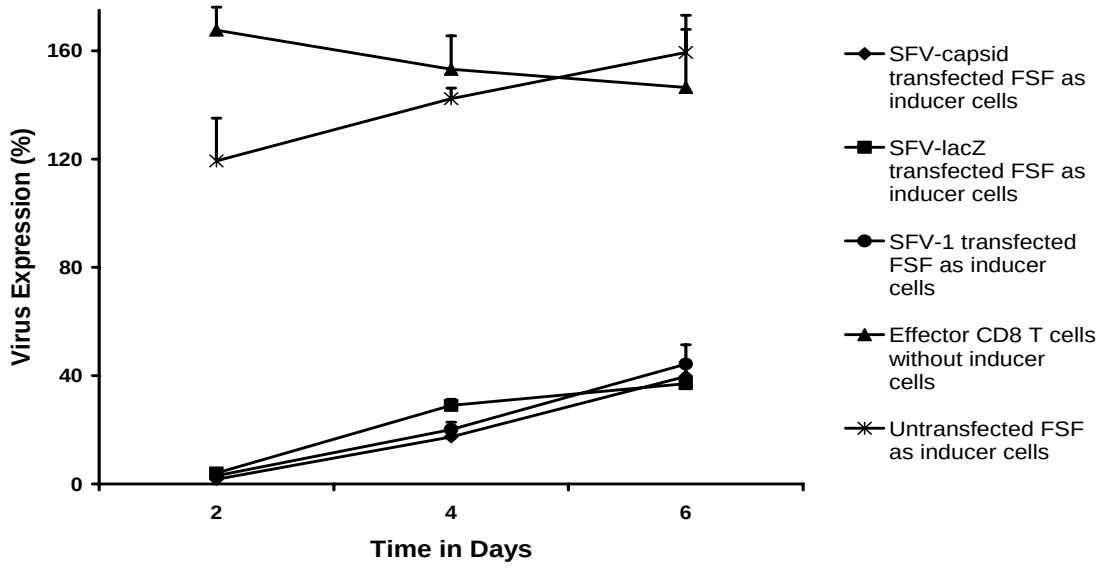


Fig. 3. Kinetics of the induction process using $CD8^+$ T lymphocytes as effector cells when FSF were used as inducer cells. FSF prepared from cat AZV2 were transfected with Semliki Forest virus vector (SFV) expressing FIV capsid, an irrelevant antigen such as lacZ or SFV vector expressing only its polymerase, or left untransfected. $CD8^+$ T lymphocytes enriched from fresh PBMC from FIV-infected cats AZV2 (A), AUO2 (B) and FIV uninfected cat AUS3 (C) were overlaid on FSF. Every 2 days the supernatants were collected and stored at 4^oC and the non-adherent cells were overlaid on newly transfected FSF. As controls, supernatants were collected from untransfected FSF cultured without $CD8^+$ T lymphocytes, $CD8^+$ T lymphocytes cultured in the absence of FSF and $CD8^+$ T lymphocytes co-cultured with untransfected FSF. The supernatants were ultracentrifuged and added to FIV-PPR infected target cells (cat OAE5 PBMC) every 3 days at a medium to supernatant ratio of 1:1. FIV production was determined by use of an FIV-capsid antigen detection ELISA. All the values have been normalized with respect to values obtained for untransfected FSF cultured without $CD8^+$ T lymphocytes.

B.



C.

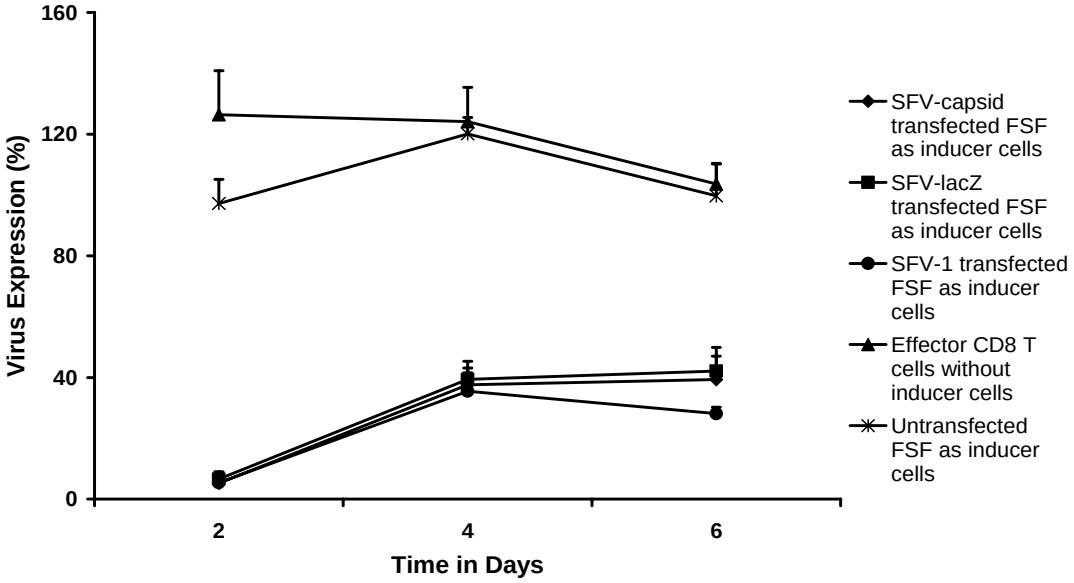


Fig. 3. Continued

Kinetics for the requirement of inducer cell-effector cell contact for the induction process

Transwell cell culture plates were used to examine whether cell contact-mediated stimulation was necessary for the production of the soluble anti-FIV activity. When supernatants collected on day 7, from effector cells without direct contact with inducer cells were added to target cells, half suppressed virus replication (FIV infected cats AUO3, AWF1 and AZV2), however supernatants from the remaining FIV infected cats E238, E284 and OLQ5 did not (Phadke et al., 2004). Overall, the amount of suppression of FIV replication observed with the supernatants collected when the inducer and effector cells were in contact with each other was reduced by approximately 50% when the inducer and effector cells were separated in a transwell plate (Phadke et al., 2004). Therefore, inducer cell contact was required for optimal stimulation of effector cells for production of the soluble anti-FIV activity.

The kinetics of the induction process using transwell culture plates indicated that the supernatants obtained from the co-culture of the autologous irradiated inducer T cells and the effector cells in contact with each other were able to suppress FIV replication on the third day (Fig. 4A) but this suppression was lost by the sixth day (Fig. 4B) for FIV infected cats AZV2, AUO2 and uninfected cat AUS3. However, when the inducer cells and the effector cells from FIV infected cats AZV2 and AUO2 were separated by a 0.45 um membrane, the amount of suppression of virus replication seen on the third day was again reduced by nearly half of that seen when the inducer and the effector cells were in contact with each other. Effector cell supernatant from FIV uninfected cat AUS3 was

able to suppress FIV replication on either day 3 or day 6, but only marginally as compared with the infected cats. The supernatants from the PBMC cultured in the absence of inducer cells did not suppress FIV replication either on day 3 or day 6 for any of the cats examined (Fig. 4A & B). Thus, under the conditions where FSF were used as inducer cells or when FIV infected inducer T cells were separated from the effector cells, the optimal days for the induction of the soluble anti-FIV activity continued until the fourth day of induction, after which a decrease in the anti-FIV activity was consistently seen.

The soluble anti-viral activity of $CD8^+$ T cells from FIV-PPR infected cats is effective against individual FIV isolates from Texas

The supernatants from $CD8^+$ T cells from five FIV-PPR infected cats suppressed replication of the FIV-PPR strain and two FIV isolates from Texas namely, TX53 and TX078 to different extents on days 3 and 6. Supernatants collected on day 3 from $CD8^+$ T cells of cat AZV2 strongly suppressed replication of FIV-PPR (virus expression 1.4%) followed by FIV-TX078 (virus expression 9.4%) and FIV-TX53 (virus expression 32.7%) (Fig. 5A). Supernatants collected on day 6 from $CD8^+$ T cells of cat AZV2 were able to weakly suppress FIV-PPR (virus expression 51.7%) and FIV-TX078 replication (virus expression 59.7%), as determined by the 65% virus expression limit, but not FIV-TX53 replication in the target cells (Fig. 5A) while. Supernatants collected either on day 3 or day 6, from irradiated inducer T cells cultured without effector cells, did not suppress replication of any of the 3 viruses; FIV-PPR, FIV-TX53 and FIV-TX078.

A.

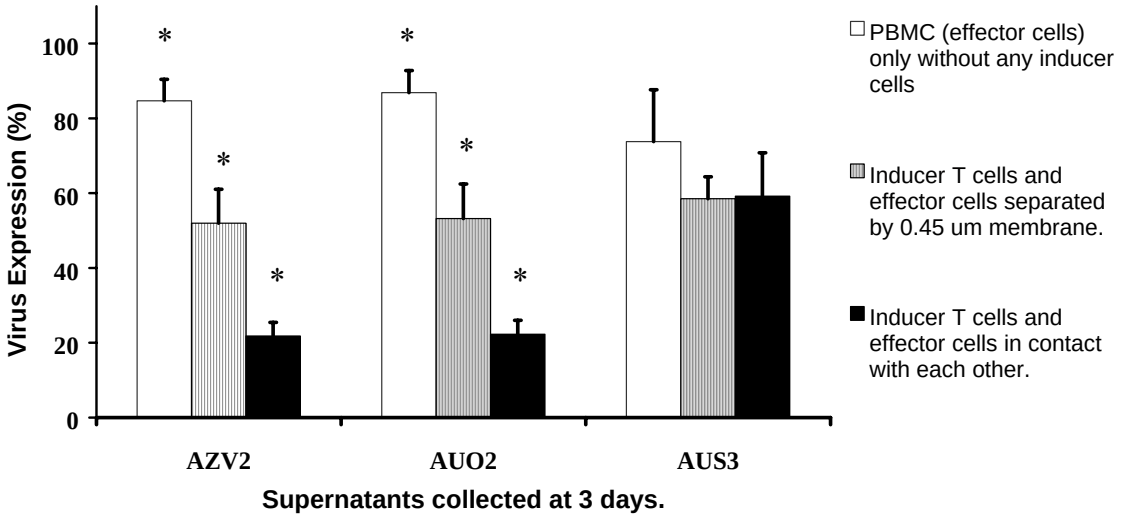


Fig. 4. Kinetics for the requirement of inducer cell-effector cell contact for the induction process. Fresh PBMC (effector cells) from FIV infected cats AZV2 and AUO2, and uninfected cat AUS3 were co-cultured in contact with autologous irradiated FIV infected inducer T cells or separated from the inducer T cells by a 0.45 um membrane. Cell culture supernatants collected on days 3 (A) and 6 (B), were ultracentrifuged and added to FIV–PPR infected target cells (cat OAE5 PBMC) every 3 days at a medium to supernatant ratio of 1:1. FIV production was determined by use of an FIV capsid antigen detection ELISA. Values indicated by asterisks are significant (as calculated by the t test) among the three parameters examined, at 3 days for FIV-infected cats AZV2 and AUO2.

B.

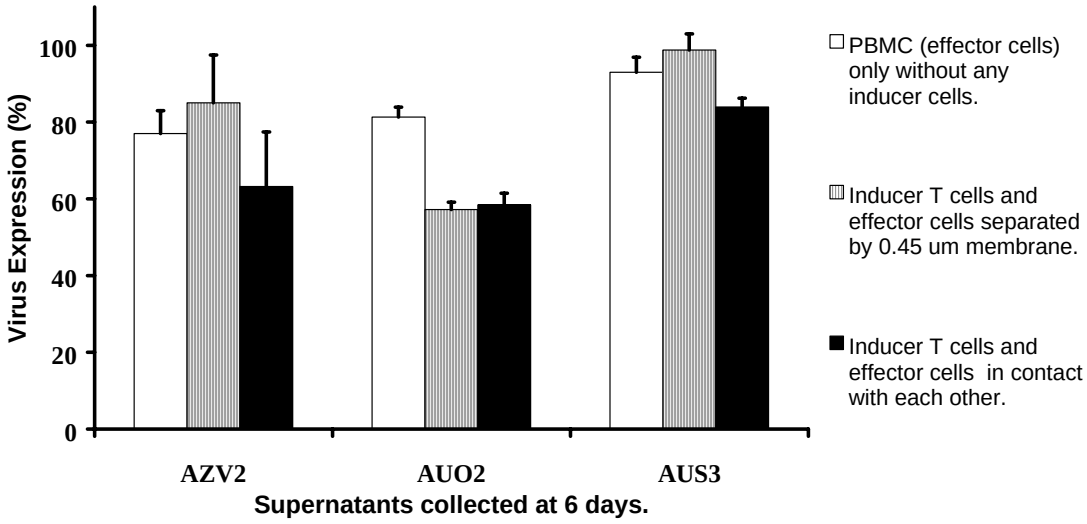


Fig. 4. Continued

A.

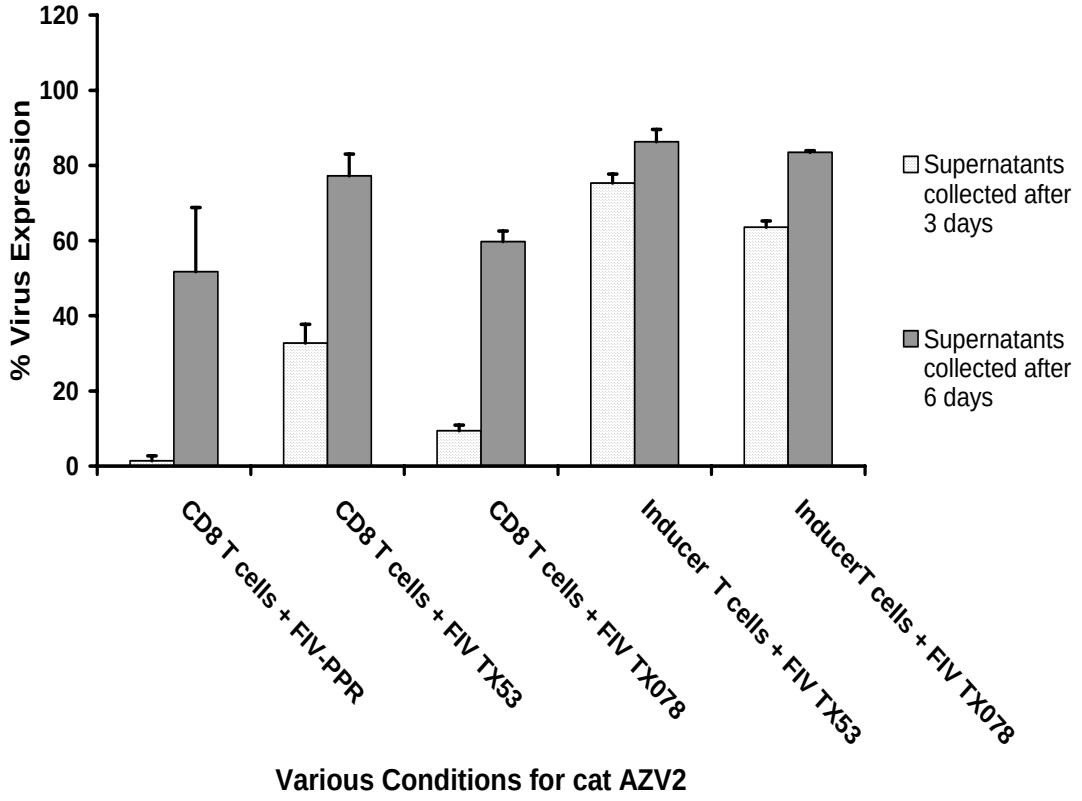


Fig. 5. Cross- reactivity of soluble anti-viral activity mediated by $CD8^+$ T cells. $CD8^+$ T lymphocytes enriched from fresh PBMC from FIV-infected cats AZV2 (A), AUO2 (B), AUO3 (C), E284 (D) and AWF1 (E), were co-cultured with autologous irradiated inducer T cells for 6 days, with the exception of $CD8^+$ T cells from cat AWF1. Irradiated inducer T cells were cultured as controls without effector cells. Cell culture supernatants collected on days 3 and 6, were ultracentrifuged and added to target cells (FIV uninfected cat AUS3 PBMC) every 3 days at a medium to supernatant ratio of 1:1. FIV production was determined by use of an FIV capsid antigen detection ELISA. The target cells were infected with FIV-PPR, FIV-TX53 or FIV-TX078 strains.

B.

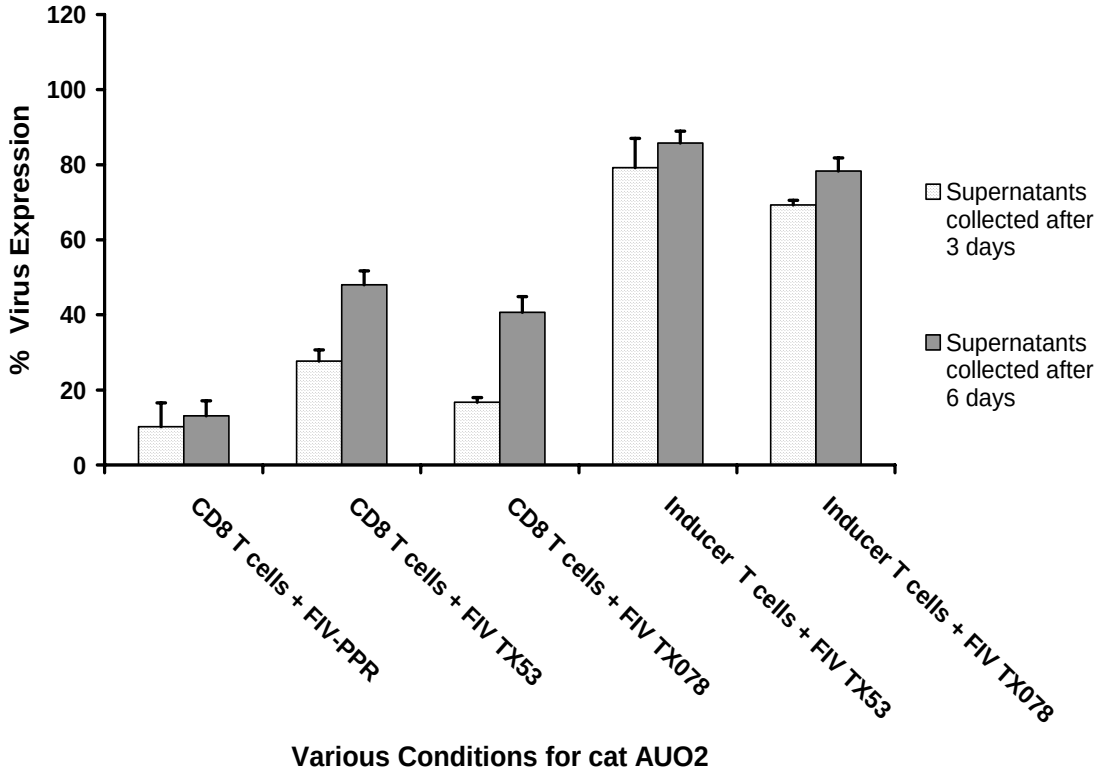


Fig. 5. Continued

C.

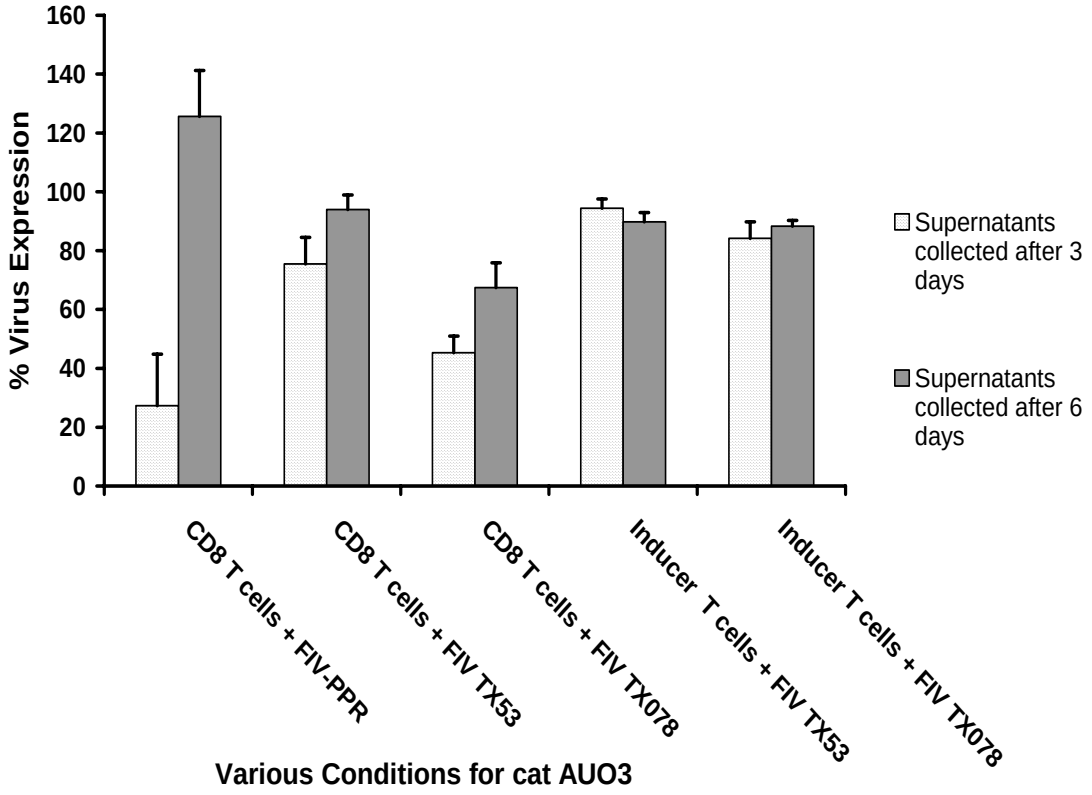


Fig. 5. Continued

D.

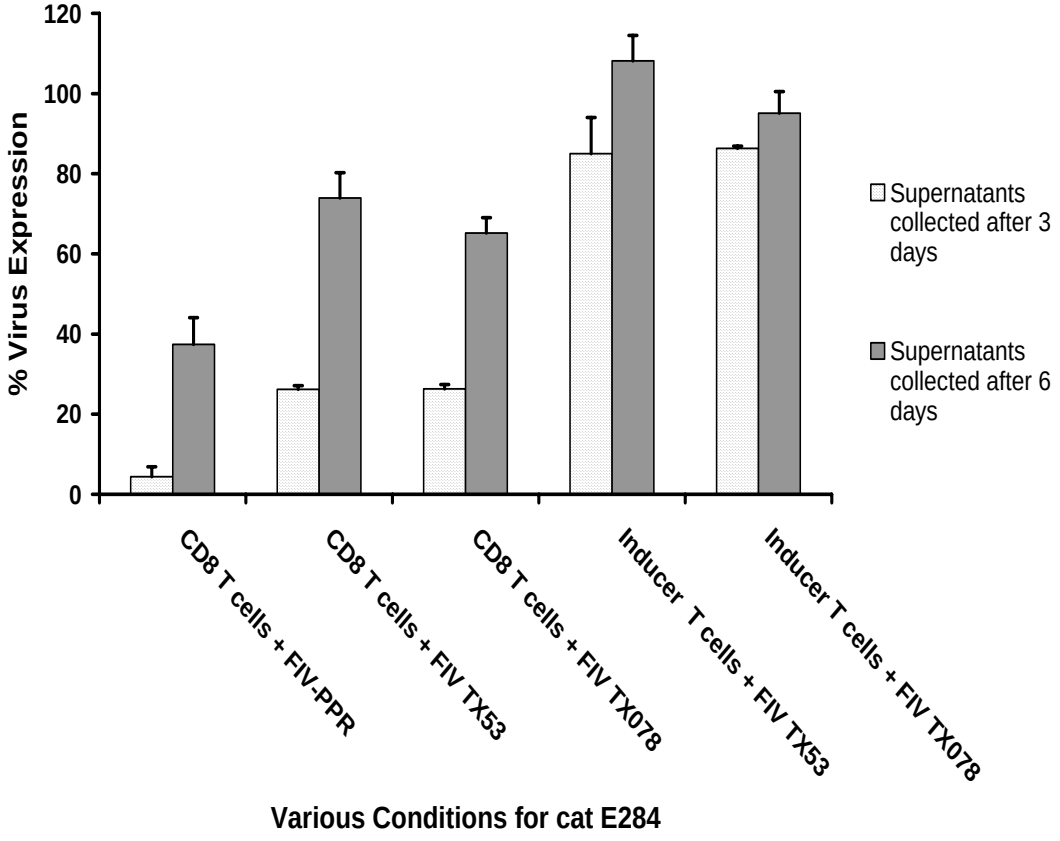


Fig. 5. Continued

E.

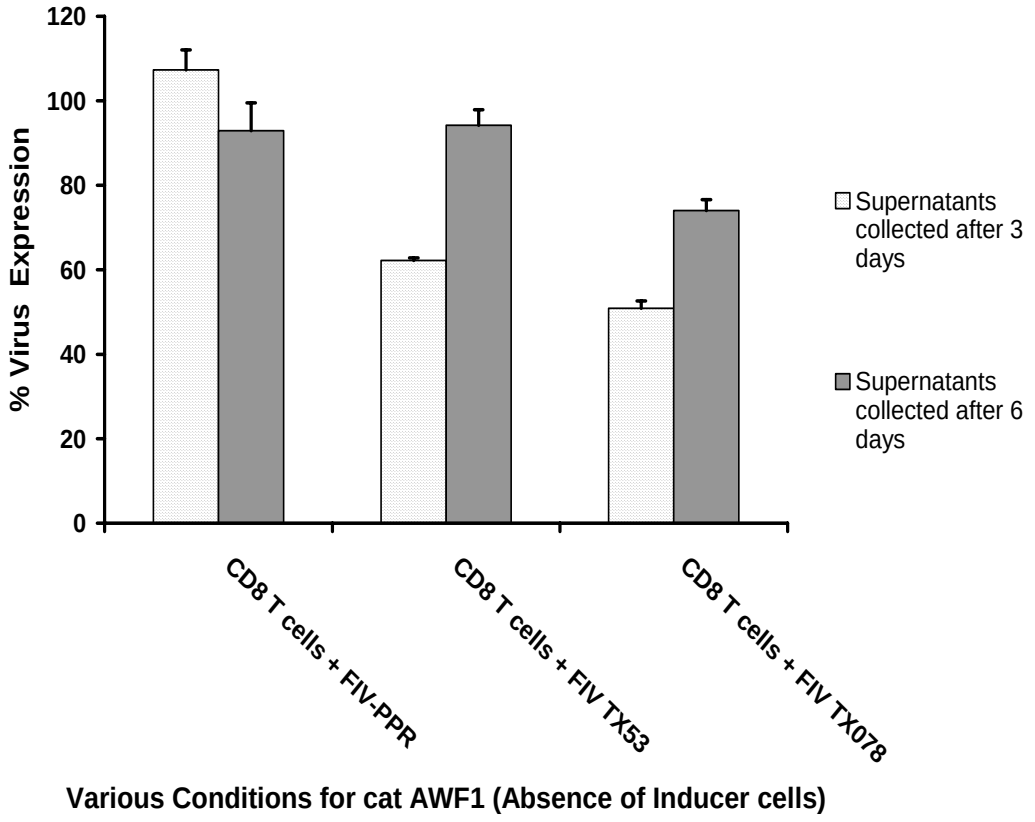


Fig. 5. Continued

Similar results were obtained for supernatants collected on day 3 from $CD8^+$ T cells of cats AUO2 with observed virus expression of only 10.2%, 16.7%, and 27.7% for FIV-PPR, FIV-TX078 and FIV-TX53, respectively (Fig. 5B). In contrast to results for cat AZV2, the supernatants collected on day 6 from $CD8^+$ T cells of cats AUO2 were able to strongly suppress replication of the three viruses in the target cells. Supernatants collected either on day 3 or day 6, from irradiated inducer T cells cultured without effector cells, did not suppress replication of any of the 3 viruses; FIV-PPR (data not shown), FIV-TX53 and FIV-TX078 (Fig. 5B).

For cat AUO3, supernatants collected on day 3 from $CD8^+$ T cells, showed stronger suppression of FIV-PPR replication (virus expression of 27.3%), as compared to FIV-TX078 (virus expression of 45.3%) with no suppression of FIV-TX53 replication (Fig. 5C). Suppression by supernatants collected on day 6 from $CD8^+$ T cells was lost for all the three viruses examined. Once again, supernatants collected either on day 3 or day 6, from irradiated inducer T cells cultured without effector cells, did not suppress replication of any of the 3 viruses; FIV-PPR (data not shown), FIV-TX53 and FIV-TX078 (Fig. 5C).

Supernatants collected on day 3 from $CD8^+$ T cells of cat E284 showed a pattern of suppression similar to that seen for $CD8^+$ T cells of cat AZV2. Thus, supernatants on day 3 strongly suppressed replication of FIV-PPR (virus expression 4.4%) followed by FIV-TX078 (virus expression 26.2%) and FIV-TX53 (virus expression 26.3%) (Fig. 5D). Supernatants collected on day 6, however, suppressed the replication of only FIV-PPR (virus expression 37.4%) but neither FIV-TX53 nor FIV-TX078 replication in the

target cells. Once again, supernatants collected either on day 3 or day 6, from irradiated inducer T cells cultured without effector cells, did not suppress replication of any of the 3 viruses; FIV-PPR (data not shown), FIV-TX53 and FIV-TX078 (Fig. 5D).

Finally, supernatants collected on day 3 from $CD8^+$ T cells of cat AWF1 did not suppress replication of FIV-PPR strain and suppressed FIV-TX53 (virus expression 62.2%) and FIV TX078 (virus expression 50.9%) replication very weakly as determined by the 65% virus expression limit (Fig. 5E). This slight suppression was also completely lost by day 6. Lack of strong suppression of virus replication of the three isolates by $CD8^+$ T cell supernatant on day 3, from cat AWF1 was not surprising, since these $CD8^+$ T cells were cultured without irradiated inducer T cells. This once again proved the necessary requirement of inducer cells for the induction of the $CD8^+$ T cells to mediate the anti-FIV activity in our system. Overall, supernatants collected on day 3 from $CD8^+$ T cells of cats AZV2, AUO2 and E284 demonstrated maximum suppression of the 3 virus isolates examined.

DISCUSSION

These studies suggest that more than one pattern of induction stimulates antiviral activity in our chronically FIV infected and uninfected control cats. Studies performed in our laboratory consistently indicate that it is the $CD8^+$ T cells within the population of PBMC that are responsible for the soluble anti-FIV activity (Choi et al., 2000b; Fig. 3A-3C). The various patterns of induction may stimulate several molecules that mediate $CD8^+$ T cell suppressive activity against FIV. It is likely that the mechanism of induction

differs quantitatively and maybe even to a greater extent, qualitatively, leading to the induction of distinct antiviral factors. Moriuchi et al. (1996) have demonstrated that the HIV-suppressor activity of $CD8^+$ T cell supernatants is multifactorial and that various factors within these supernatants including, but not limited to, the β-chemokines, may affect HIV replication at various stages of the life cycle of the virus. Due to the distinct $CD8^+$ T cell suppressor activities that have been reported from various laboratories, Copeland (2002) has also suggested that the CAF activity in humans could represent more than one factor. Thus, it is possible that the various modes of induction, lead to the secretion of several molecules mediating the $CD8^+$ T cell suppressive activity against FIV, and by inference, HIV. Suppression of FIV replication in our cats likely depends on the distinct patterns of induction resulting from the differences in the innate immune system of the cats, the course of infection and illness, and the differences in the genetics of the cats. The levels of suppressing activity generated have remained highly reproducible for each animal over several years, although variable from cat to cat. All the FIV infected cats in our colony have been infected between three to six years and are in the chronic asymptomatic stage of infection. The proviral load of these cats has been found to inversely correlate with their ability to suppress FIV replication *in vitro* (manuscript in preparation). It is possible that the suppressing activity of the $CD8^+$ T cells actually helps to maintain the asymptomatic nature of the cats, because as yet, we have been unable to demonstrate anti-FIV $CD8^+$ T cell suppressing activity in acutely infected cats.

The various types of cells that function in the induction of the effector cells and the communication required for the induction of the antiviral activity against FIV or HIV have not yet been defined. The focus of this study was to determine the requirements for induction of suppression of FIV replication, specifically whether T cells were necessary for induction of the feline $CD8^+$ T cell suppressing activity and whether FIV infection was a criterion for cells to function as inducer cells. Inducer cells used in our initial studies were irradiated FIV-infected T lymphocytes (Choi et al., 2000b). But immortalized FSF, transfected with SFV vector expressing either the FIV capsid or an irrelevant antigen, such as lacZ or FSF transfected with SFV vector expressing only its polymerase, could also mediate the induction process. Either irradiated or non-irradiated FSF could serve as inducer cells (Phadke et al., 2004). The use of FSF transfected with SFV vector expressing the capsid protein of the FIV-PPR strain, might serve as a convenient alternative to the use of infected T cells as inducer cells in our system. Thus, it appears that functional inducer cells do not need to be professional antigen presenting cells (APC) and these cells do not have to express the viable and complete virus.

The effector cells produced the soluble anti-FIV activity in the absence of MHC matched inducer cells when either FIV infected T cells or transfected FSF were used. This has been shown in our studies using autologous or heterologous inducer cells to stimulate the effector cells (Phadke et al., 2004). This is in contrast to the MHC class I restricted, FIV specific cytotoxic responses described for $CD8^+$ T cells from our FIV infected cats, where autologous FIV-infected T cells were used as APC (Song et al., 1992). In the studies with FSF, we have consistently shown that supernatants from

effector cells (either PBMC or $CD8^+$ T cells) when co-cultured with mismatched FSF transfected with SFV vector expressing either the FIV capsid or an irrelevant antigen, such as lacZ or FSF transfected with SFV vector expressing only its polymerase, suppress FIV replication. In our studies, non-transfected FSF alone do not induce effector cells and although MHC restriction is not required for the soluble anti-FIV response, it is not yet clear as to whether it might improve or otherwise contribute to the activation of the effector cells. Our studies are in accordance with studies in HIV infection, where HLA compatibility at the effector phase was not required (Mackewicz et al., 1998). Thus, heterologous $CD8^+$ T lymphocytes from one HIV infected individual were found to suppress HIV replication in naturally infected $CD4^+$ T cells from another HIV-infected individual (Brinchmann et al., 1990).

Optimal induction of the suppressing activity from the effector cells depended on cell-cell contact between inducer and effector cells, although approximately 50% of the suppressing activity could be demonstrated when the inducer and the effector cells were not in contact with each other. Therefore, membrane antigen interactions may be critical in the communication, while cytokines may play an accessory or a separate role in the induction process.

$CD8^+$ cytotoxic T cells from HIV-infected individuals have been shown to be specific for multiple epitopes within gag, env, nef, vif and pol gene products for HIV infected individuals and for env and gag gene products in FIV infected cats (Beatty et al., 1996; Flynn et al., 1994; Flynn et al., 1996; Gandhi and Walker, 2002; Li et al., 1995; Song et al., 1992; Song et al., 1995). However, earlier studies of $CD8^+$ T cell

mediated non-cytoxic antiviral activity against HIV have demonstrated lack of antigen-specificity at the induction phase. Thus, in agreement with our studies, anti-HIV activity has been demonstrated from $CD8^+$ T lymphocytes from HIV-uninfected, healthy individuals, $CD8^+$ T cell clones derived from HIV-infected and uninfected individuals, cytotoxic T lymphocytes (CTLs) from HIV-1 uninfected individuals and even from cord blood (supposed to contain only naïve $CD8^+$ T cells) from HIV-uninfected neonates (Bagasra and Pomerantz, 1993; Hsueh et al., 1994; Kootstra et al., 1997; Leith et al., 1999; Liu et al., 2003; Mackewicz et al., 1997; Rosok et al., 1997; Song et al., 1992). Thus these studies demonstrated that induction of the anti-viral activity against HIV mediated by $CD8^+$ T cells did not require prior exposure to HIV antigen and that it might not even necessarily require the presence of a virus for induction of $CD8^+$ T cells.

The $CD8^+$ T cell mediated antiviral activity against HIV has also been found to demonstrate lack of antigen-specificity at the effector phase of control. Copeland et al. (1995) showed that $CD8^+$ T cell supernatants from HIV-infected individuals (both primary and herpes-virus-samiri transformed $CD8^+$ T cell lines) inhibited activation of LTRs of human T cell leukemia virus and Rous sarcoma virus in transiently transfected Jurkat cells. Walker et al. (1991b), demonstrated cross-reactivity, by showing that $CD8^+$ T cells from an HIV-infected individual could suppress virus replication in $CD4^+$ T cells acutely infected with HIV-1, HIV-2 or even SIV_{mac}. $CD8^+$ T cells from uninfected chimpanzees and chimpanzees infected with $HIV\text{-}1_{SF2}$ could inhibit replication of $HIV\text{-}1_{SF33,}$ explaining the natural resistance of chimpanzees to HIV- induced disease (Castro et al., 1991). In addition, $CD8^+$ T cells of African green monkeys inhibited HIV-1

replication in human $CD4^+$ T cells (Ennen et al., 1994) while $CD8^+$ T cells of SIV-infected sooty mangabeys inhibited the replication of HIV-2, SIV-1 (SIV_{smm-7}) and HIV-1 (LAV) (Powell et al., 1990). Furthermore, $CD3^+CD8^+$ T cells of HIV-1 IIIB infected chimpanzees were shown to inhibit replication of HIV-1 strains IIIB, MN and RF *in vitro* (Husch et al., 1993). Moreover, this $CD8^+$ T cell antiviral activity was shown to prevent superinfection of HIV-1 infected PBMC by diverse HIV isolates such as HIV-$1_{SF33,}$ HIV-2_{UC2} and HIV-2_{UC3} (Locher et al., 1999). Prevention of superinfection due to soluble factor secreted by $CD8^+$ T cells that suppressed virus replication was also observed in HIV-2 infected baboons that were challenged with either autologous or heterologous HIV-2 strains (Locher et al., 1997).

Our results have also demonstrated that $CD8^+$ T cell antiviral activity from FIV-PPR infected cats suppressed replication of other retroviruses such as feline leukemia virus and HIV (Unpublished data from our laboratory). Hohdtasu et al., (2002) also demonstrated that the $CD8^+$ T cell mediated anti-FIV activity was effective against FIV strains of subtypes that differed from the original FIV strain used to infect the target cells. They used FIV strains belonging to clades A, B and D in their studies, which therefore implies a role for $CD8^+$ T cell antiviral activity in mediating resistance to superinfection in FIV infection. Moreover, the $CD8^+$ T cell antiviral activity was found to be effective against heterologous FIV isolates such as FIV-Petaluma, FIV-GL_8 and FIV-PPR belonging to the FIV clade A (Choi et al., 2000b; Flynn et al., 1999). In contrast to these studies, we have now demonstrated that $CD8^+$ T cells from FIV-PPR cats were able to suppress viral replication in PBMC acutely infected with either FIV-

TX53 or FIV-TX078 belonging to a newly emerging clade, which is closely related to clade B. Thus the soluble suppressing activity was interclade active in case of FIV, in agreement with the cross-reactivity and superinfection studies mentioned earlier.

The kinetics of the induction process demonstrated that antiviral activity against FIV was maximally expressed until day 4 after induction, but it was reduced appreciably or was absent by the 6^{th} day when FSF were used as inducer cells (Fig. 2A-2E, 3A-3C). However, when $CD8^+$ T cells of cat AUO2 were induced by irradiated inducer T cells and not FSF expressing FIV capsid, lacZ or transfected with SFV vector only, suppression of replication of FIV-PPR, FIV-TX53 and FIV-TX078 replication, was observed on day 6 also (Fig. 5B). This implies that interactions between the effector $CD8^+$ T cells and the different inducer cells probably involve unique membrane interactions that generate various levels of FIV suppressing activity against different virus strains. Thus, understanding the mechanisms that activate and control suppression of FIV, may lead to our understanding of the natural process for controlling chronic lentiviral infection.

FIV infected T cells and FSF transfected with SFV vector expressing the FIV capsid, an irrelevant antigen such as lacZ or FSF transfected with SFV vector only, could be used to induce the effector cells to suppress FIV replication *in vitro*. Both FSF and FIV-infected T cells would seem to require communication of some kind of crisis to induce the effector cells. Identifying alterations occurring with respect to membrane antigen expression within these two types of inducer cells will be valuable in understanding conditions needed for induction of effector cell synthesis of antiviral

activity. As the success of a FIV vaccine could be hampered by the occurrence of highly divergent viral variants in the field, the exploitation of this innate, soluble $CD8^+$ T cell anti-FIV activity would be more useful. Hence, characterization and identification of the stimuli and mechanisms involved in the induction of the antiviral activity against FIV, not addressed in other studies, could provide information for strategies of practical exploitation of this innate T cell immunity and may contribute to the design of novel, safe and complementary anti-FIV therapeutic strategies.

CHAPTER III

PHENOTYPIC CHANGES IN FRESH PERIPHERAL BLOOD MONONUCLEAR CELLS IN HEALTHY CATS AND CATS CHRONICALLY INFECTED WITH FELINE IMMUNODEFICIENCY VIRUS

INTRODUCTION

A key step towards exploiting the soluble antiviral activity mediated by $CD8^+$ T cells is to elucidate mechanisms that are responsible for stimulating the $CD8^+$ T lymphocytes. The various types of cells that could function in the induction of the effector $CD8^+$ T cells and the communication required for the induction of the antiviral activity against FIV or HIV have not as yet been defined. As described in chapter II, earlier studies in our laboratory have established that irradiated FIV-PPR infected T cells or immortalized feline skin fibroblasts (FSF) expressing either the FIV capsid, or an irrelevant antigen, such as lacZ or FSF transfected with SFV vector expressing only the polymerase, stimulate anti-FIV activity of $CD8^+$ T cells (Choi et al., 2000b; Phadke et al., 2004). These studies differ from studies in other laboratories (Bucci et al., 1998b; Flynn et al., 1999; Jeng et al., 1996) which relied on mitogen specific stimulation of $CD8^+$ T cells to induce effector cells to generate anti-FIV activity.

Production of the soluble anti-FIV activity from $CD8^+$ T cells was shown in our studies using autologous or heterologous inducer cells to stimulate the effector cells. This is in contrast to the MHC class I restricted FIV specific cytotoxic responses described for

$CD8^+$ T cells from our FIV infected cats where autologous FIV antigen expressing T cells were essential as inducer and target cells (Song et al., 1992). However, although MHC restriction is not required for the non-cytolytic anti-FIV response, it is not yet clear as to whether it might improve or contribute to the activation of the effector cells.

Optimal induction of the suppressing activity from the effector cells was shown to depend on cell-cell contact between inducer and effector cells, but lower levels of suppressing activity could be demonstrated when the inducer T cells and the effector cells were separated by a 0.45 um membrane in a transwell dish (Phadke et al., 2004). The requirement for cell contact indicates that cellular membrane antigen interactions are critical in the communication, while cytokines may play an accessory or separate role in the induction process. Cell-cell contact could depend on the interaction between costimulatory molecules and adhesion molecules, such as B7-1 (CD80), B7-2 (CD86), ICAM-1 or CD40, on the inducer cells with their respective ligands (CD28, CTLA-4, LFA-1 or CD40L) on the effector cells (Chirathaworn et al., 2002; Kochli et al., 1999; Kornbluth, 2002; Lenschow et al., 1996). It is likely that the separation of effector cells and inducer cells in the transwell prevented the various membrane antigen interactions, as a result of which the effector cells were not stimulated to generate supernatants that suppressed FIV replication in the target cells.

Determining the phenotype of feline inducer T cells, with respect to expression of the various membrane antigens would enable us to identify various membrane antigens that might be involved in the induction process. In this study, fresh peripheral blood mononuclear cells (PBMC) from FIV infected and uninfected cats were analyzed for the

expression of various membrane antigens including CD4, CD8, MHC-I, MHC-II, Pan T marker and B7-1 molecules. Two-color flow cytometric analysis demonstrated a significant elevation of B7-1 on $CD8^+$ T lymphocytes in FIV infected cats as compared to uninfected cats.

MATERIALS AND METHODS

Experimental animals and infection

Specific, pathogen-free cats, purchased from Harlan Sprague-Dawley, Madison, WI or Liberty Laboratories, Liberty corner, N.J., were serologically negative for feline leukemia virus. Cats were housed in a specific, pathogen-free environment at the Laboratory Animal Research and Resources Support Facility (LARR), Texas A&M University, College Station. Cats AUO2, AUO3, AWF1, AZV2, A306, A308, E238, E284, M165, M242 and M153 were chronically infected with FIV PPR strain. These cats had been inoculated with 50 to 1250 $TCID_{50}$ of virus i.v. three to six years prior to these studies. All the cats were in the asymptomatic stage of infection. Cats AUS3, OAE5, and OLQ4, sham inoculated with saline solution were used as uninfected control cats.

Antibodies

Fluorescein isothiocyanate (FITC) conjugated monoclonal antibodies (Mab) to feline CD4, feline CD8 (CD8β chain) (Southern Biotechnology Associates, Birmingham, AL) and unlabeled Mabs to feline MHC class I and MHC class II molecules (VMRD, Inc., Pullman, WA) were purchased commercially. Anti-feline B7-1 Mab (clone

B7.1.66) and a Mab that recognizes feline T cells (clone 1.572) were kindly provided by Dr. Wayne Tompkins (North Carolina State University, Raleigh, NC). Rabbit polyclonal antibodies to feline B7-1 and B7-2 were provided by Barbara Winslow (Schering-Plough Animal Health, San Diego, CA). Rabbit polyclonal antibody to feline CTLA-4 was developed in our laboratory (Choi et al., 2000a). The specificities of the rabbit polyclonal antibodies to feline B7-1, B7-2 and CTLA-4 respectively, have been confirmed (Tompkins et al., 2002). Secondary antibodies, including phycoerythrin (PE) conjugated goat anti-mouse (GAM) IgG1, FITC conjugated GAM IgG2a and PE conjugated GAM IgG2a, were purchased from Southern Biotechnology Associates (Birmingham, AL).

Staining of freshly prepared PBMC

Lymphocyte subsets were determined in peripheral blood collected by jugular venipuncture into EDTA (K_3)-containing tubes. Feline PBMC were isolated from EDTA (K_3)-treated whole blood by Histopaque-1077 (Sigma, St. Louis, MO) density gradient centrifugation (Choi et al., 2000b) and washed once with cold phosphate-buffered saline (PBS) containing 0.1% sodium azide. Prior to staining of the cells, the Fc receptors on the cells were blocked by incubating the cells for 10 minutes on ice with goat anti-IgG (2 mg/ml) (Sigma, St. Louis, MO). Two-color flow cytometric analysis was performed with freshly isolated PBMC to determine the expression of B7-1 on $CD4^+$ T cells, $CD8^+$ T cells, or on total T cells (using a feline pan T antibody), MHC class I on $CD4^+$ or $CD8^+$ T cells and MHC class II on $CD4^+$ or $CD8^+$ T cells. FITC conjugated Mabs to feline CD4 and feline CD8 and unlabeled Mab to feline MHC class I and feline MHC class II in cold

PBS-0.1% sodium azide were added to the cells in the respective tubes at a final dilution of 1:50. The anti-feline B7-1 Mab and anti-feline pan T Mab were used undiluted in 50 ul volume. After incubating for 30 minutes on ice, the cells were washed once with cold PBS-0.1% sodium azide and centrifuged at 1200 rpm for 10 minutes (Jouan Inc., Winchester, VA). PE conjugated GAM IgG1 (Southern Biotechnology Associates, Birmingham, AL) in cold PBS-0.1% sodium azide was added at a dilution of 1:250 to tubes containing cells stained for B7-1 and MHC class I molecules. FITC-conjugated GAM IgG2a and PE conjugated GAM IgG2a (Southern Biotechnology Associates, Birmingham, AL) in cold PBS-0.1% sodium azide were added to tubes containing cells stained for Pan T marker and MHC class II molecules, respectively, at a dilution of 1:50. After incubating for 30 minutes on ice, the cells were washed once with cold PBS-0.1% sodium azide and centrifuged at 1200 rpm for 10 minutes. Negative controls consisted of isotype-matched controls and unstained cells. After washing the cells, 300 ul of 2% formaldehyde were added to the tubes, which were wrapped in an aluminium foil and kept at 4^{o}C until they were examined the following day with a FACscaliber flow cytometer (Becton-Dickinson, San Jose, CA) at the Veterinary Pathobiology Core Facility (Director, Dr. Roger Smith, Texas A&M University, College Station).

Cell culture

Feline PBMC were isolated from EDTA (K_3)-treated whole blood by Histopaque-1077 (Sigma, St. Louis, MO) density gradient centrifugation (Choi et al., 2000b). PBMC were cultured as described previously with RPMI 1640 (Gibco BRL, Grand Island,

N.Y.), supplemented with 10% heat inactivated fetal bovine serum (FBS) (Atlas Biologicals, Fort Collins, CO), 50 ug/ml of gentamicin (Gibco BRL, Grand Island, N.Y.), 5 x 10^{-5} M 2-mercaptoethanol (Gibco BRL, Grand Island, N.Y.), 2mM L-glutamine (Gibco BRL, Grand Island, N.Y.), and 100 U of human recombinant IL-2 (hr IL-2) (Gibco BRL, Grand Island, N.Y.) per ml (Choi et al., 2000b). Cells were grown at 37^{o}C in a humidified atmosphere of 5% CO_2.

Preparation of FIV-PPR infected inducer T cells

Feline T cells were infected with FIV-PPR strain and irradiated (11,000 rads from a ^{6o}Co source provided by the Nuclear Science Center, Texas Engineering Experiment Station, College Station) before using as inducer cells for stimulation of effector cells, as previously described (Choi et al., 2000b; Song et al., 1992). Briefly, freshly prepared PBMC were stimulated for three days with 5 ug/ml of Concavalin A (Con A) (Sigma, St. Louis, MO). The stimulated cells were centrifuged at 1200 rpm for 10 minutes (Jouan Inc., Winchester, VA) and infected with FIV-PPR for 1 h at 37^{o}C. The cells were washed before culturing in complete RPMI containing 100 U hr IL-2 per ml. After 6 days of incubation, virus replication was monitored using the FIV capsid antigen ELISA. These FIV-infected cells were cryo-preserved to be used later as inducer cells.

Blocking studies

In order to determine the role, if any, of the B7 molecules in the induction process, irradiated autologous inducer T cells were incubated with the following

antibodies for 1 hour in a humidified atmosphere of 5% CO_2, Mab to feline B7-1, rabbit polyclonal antibody to feline B7-1, rabbit polyclonal antibody to feline B7-2, rabbit polyclonal antibody to feline CTLA-4, a combination of Mab to feline B7-1 and rabbit polyclonal antibody to feline B7-2 and a combination of rabbit polyclonal antibodies to feline B7-1 and feline B7-2. Negative controls consisted of irrelevant hybridoma supernatant and pre-immune rabbit serum. After 1 hour, effector cells (fresh PBMC) from FIV infected cats AU02, A306, M165 and FIV uninfected cat OLQ4 were added to the inducer cells at a ratio of 1 inducer for 10 effector cells. On the 3rd day, the cells were harvested and centrifuged at 1200 rpm for 10 minutes (Jouan Inc., Winchester, VA), and the supernatants were collected and stored at 4°C.

FIV suppression assay

Cell-free supernatants stored at 4°C were ultracentrifuged at 35,000 rpm for 2 hours (Beckman L7-55 Ultracentrifuge, Palo Alto, CA) to remove the residual viral particles. The supernatants were passed through 0.22 um-pore size filters (Pall Corporation, Ann Arbor, MI) and stored at 4°C. Target cells were prepared from PBMC of an FIV uninfected cat after culturing *in vitro* for six days with Con A and IL-2 (Choi et al., 2000b). They were then infected with the FIV-PPR strain and cultured with supernatants in a 96-well plate for 10 days at a medium to supernatant ratio of 1:1. Supernatant and fresh complete RPMI + hr IL-2 100 U/ml were added to the cells every 3 days. The amount of FIV in the supernatants was determined by an FIV capsid antigen detection ELISA. Suppression was considered to be positive when virus expression was

≤ 65% of virus expression in the untreated but FIV-PPR infected control cells (positive controls).

Data analysis

The differences in the percentages of cells between FIV infected and uninfected cats were analyzed using Student's t test (Ott, 1993). Statistically significant differences were set at $p < 0.05$.

RESULTS

Expression of various membrane molecules on freshly prepared PBMC from FIV uninfected cats and cats chronically infected with FIV-PPR strain

In order to determine the phenotype of inducer T cells used in our studies, fresh PBMC from 11 chronically FIV infected cats and from 3 uninfected cats were analyzed for surface expression of CD4, CD8, B7-1 expression on T cells, B7-1 expression on $CD4^+$ T cells or $CD8^+$ T cells, MHC class I expression on $CD4^+$ T cells or $CD8^+$ T cells and MHC class II expression on $CD4^+$ T cells or $CD8^+$ T cells. Analysis of the data indicated that there was no significant difference in the $CD4^+/CD8^+$ T cell ratio between FIV infected and uninfected cats (Table 1). In addition, there was no significant difference in the percentages of double-labeled $CD4^+$ and $CD8^+$ T cells from FIV infected and uninfected cats expressing either MHC class I or MHC class II molecules (Table 1). Analysis of two-color flow cytometry of fresh PBMC demonstrated that the percentage of B7-1^+ peripheral blood $CD8^+$ T cells from FIV-infected cats was

significantly greater ($p < 0.05$) as compared to uninfected cats (Figs. 6, 7). However, the percentage of B7-1$^+$ peripheral blood CD4$^+$ T cells and the percentage of peripheral total B7-1$^+$ T cells was not significantly different between FIV-infected and uninfected cats (Figs. 6, 7 and Table 1).

Effect of blocking costimulatory interactions mediated by B7-1 and B7-2 on the ability of the effector cells to suppress FIV replication *in vitro*

Flow cytometry studies indicated a greater percentage of B7-1$^+$ peripheral blood CD8$^+$ T cells in FIV infected cats as compared to FIV uninfected cats (Fig. 6). Costimulatory molecules B7-1, B7-2 and CTLA-4 were shown to be up regulated by stimulation of feline CD4$^+$ and CD8$^+$ T cells with concavalin A (ConA) or the combination of phorbol myristate acetate (PMA) and ionomycin (I) (Vahlenkamp et al., 2004). Based on these observations, attempts were made to block the B7 interactions in order to determine which of the B7 molecules might be involved in the ability of inducer T cells to increase the CD8$^+$ T cell antiviral activity. If the costimulatory molecules were important in the induction process, the supernatants derived from blocking B7-1 and B7-2 interactions between inducer and effector cells would not suppress FIV replication. However inconclusive results were obtained for the 4 cats studied, 3 FIV infected cats, M165, AUO2 and A306 and one uninfected cat OLQ4 (data not shown), because the supernatants derived from inducer and effector cells co-cultured with pre-immune rabbit serum (control for polyclonal antibody) suppressed FIV replication to the same extent as the supernatants generated in the presence of feline specific polyclonal antibodies,

Table 1

Analysis of phenotypes of fresh PBMC from uninfected cats and cats chronically infected with the FIV-PPR strain

Cats	FIV-PPR virus dose	% of Single-labeled cells		% of Double-labeled cells					
		T cells							
						% MHC-I $^{+}$		% MHC-II^{+}	
		$CD4^{+}$	$CD8^{+}$	CD4/ CD8 ratio	B7-1^{+}	$CD4^{+}$	$CD8^{+}$	$CD4^{+}$	$CD8^{+}$
OAE5	Uninfected	21.10	14.10	1.50	27.70	100.0	100.0	92.10	83.30
OLQ4	Uninfected	25.00	10.20	2.45	39.50	100.0	100.0	7.30	54.50
AUS3	Uninfected	14.20	10.50	1.35	32.80	100.0	100.0	96.80	98.70
AZV2	50	12.10	8.70	1.39	54.50	100.0	100.0	82.30	91.00
AUO2	50	11.70	14.30	0.82	39.20	99.9	99.8	85.30	83.50
AUO3	50	18.70	17.40	1.07	57.10	99.9	99.8	99.80	99.20
AWF1	50	16.80	8.40	2.00	49.60	99.7	100.0	85.80	91.80
E238	1250	18.20	12.60	1.44	35.80	100.0	99.9	95.50	88.40
E284	1250	29.00	19.80	1.46	33.40	100.0	99.9	83.10	85.60
A306	250	24.80	9.10	2.73	64.20	100.0	100.0	92.10	91.70
A308	250	20.80	14.10	1.48	72.40	100.0	100.0	97.60	97.50
M242	50	39.40	24.30	1.62	16.10	99.9	100.0	45.40	61.20
M153	50	19.10	33.70	0.57	33.60	100.0	99.8	11.50	22.80
M165	50	21.20	21.20	1.00	43.30	100.0	100.0	36.40	38.90

Fresh PBMC from 3 FIV-uninfected cats and 11 chronically FIV infected cats were singly or doubly stained for the expression of various membrane molecules as explained in the materials and methods.

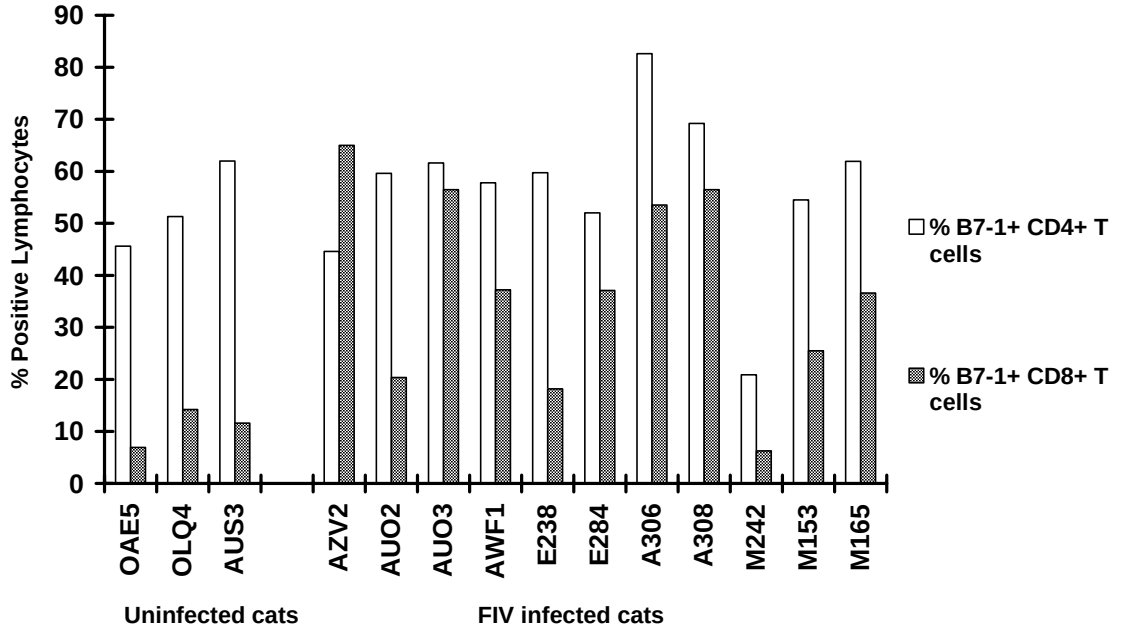


Fig. 6. Percentage of B7-1$^+$ CD4$^+$ and B7-1$^+$ CD8$^+$ T cells from freshly prepared PBMC of FIV infected and uninfected cats. Fresh PBMC from 11 FIV infected cats and 3 uninfected cats were evaluated for the expression of B7-1 on their CD4$^+$ and CD8$^+$ T cells by double labeling the cells as explained in the materials and methods.

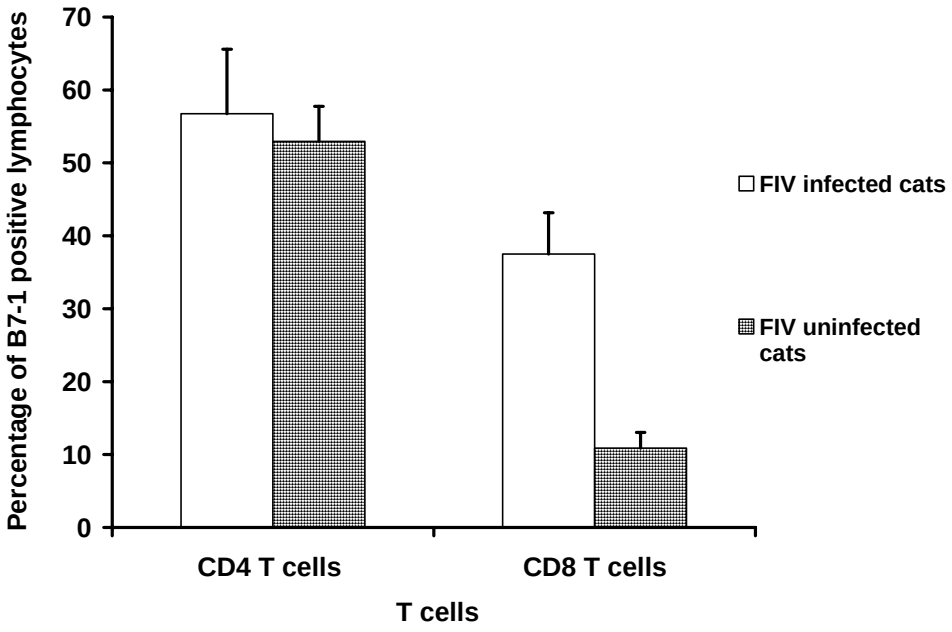


Fig. 7. Comparison of percentages of B7-1$^+$ CD4$^+$ T cells and B7-1$^+$ CD8$^+$ T cells from freshly prepared PBMC of FIV infected and uninfected cats. Freshly prepared PBMC from 11 FIV infected cats and 3 uninfected cats were double labeled as explained in the materials and methods and evaluated for the expression of B7-1 on their CD4$^+$ and CD8$^+$ T cells. The percentage of B7-1$^+$ peripheral blood CD8$^+$ T cells from FIV-infected cats was significantly greater ($p < 0.05$) as compared to uninfected cats as indicated by asterisks.

indicating that blocking was not specific with the rabbit serum. Moreover, addition of the irrelevant hybridoma supernatant to the co-culture of effector cells and inducer cells generated supernatants that were able to suppress FIV replication. This once again indicated that virus suppression observed was the result of non-specific inhibition from polyclonal or monoclonal antibodies to feline antigens.

DISCUSSION

Recently, a number of studies have attempted to elucidate the phenotype of the $CD8^+$ T cells responsible for mediating the suppression of either FIV or HIV (Bucci et al., 1998b; Gebhard et al., 1999; Landay et al., 1993; Toso et al., 1995; Zanussi et al., 1996). In cats, the $CD8^+$ T effector cells responsible for suppression of the virus bear an activation phenotype characterized by low levels of CD8β chain ($CD8\alpha^+\beta^{low}$) and L-selectin (CD62L), as well as high levels of CD44, CD49d and CD18 (Bucci et al., 1998b; Gebhard et al., 1999). Most of these studies have used mitogen stimulated $CD8^+$ T lymphocytes for generation of anti-FIV activity. However, studies carried out in our laboratory have consistently shown that $CD8^+$ T lymphocytes required stimulation by inducer cells (either T cells or FSF), and not mitogen for the generation of maximum anti-FIV activity (Choi et al., 2000b; Phadke et al., 2004). Hence, characterizing the phenotype of inducer T cells is essential for understanding the mechanisms required for stimulation of $CD8^+$ T lymphocytes.

Towards this end, fresh PBMC from 11 chronically FIV infected cats and three FIV uninfected cats were analyzed for surface expression of various membrane antigens.

There was no significant difference between the MHC class I and MHC class II expression on $CD4^+$ T cells or $CD8\beta^+$ T cells either from FIV infected or uninfected cats. This is inconsistent with other reports describing increased expression of MHC class II on $CD8\alpha^+\beta^{low}$ T cells in FIV-infected cats (Ohno et al., 1992; Rideout et al., 1992; Willett et al., 1993). Willett et al. (1993) observed an MHC class II increase in $CD8\alpha^+\beta^{low}$ PBMC of cats that were intraperitoneally infected with FIV-A-GL_8 strain between 2-18 months prior to analysis. Rideout et al. (1992) observed MHC class II upregulation on $CD8^+$ T cells from cats intravenously infected with FIV-A-Petaluma for 3 months or 5 years. In contrast, Shimojima et al. (2003) found that activated $CD8^+$ PBLs from cats that been infected vaginally by FIV-B-TM2 strain had downregulated MHC class II expression as compared to unprimed lymphocytes, which was also demonstrated by Reubel et al. (1994) on PBMC from experimentally FIV infected cats. However it is thought that expression of MHC class II molecules is not a reliable marker for feline T cell activation as it is for human T cells, because of low-level constitutive expression in naïve T cells (Rideout et al., 1990).

Surprisingly, the percentage of B7-1^+ peripheral $CD8^+$ T cells from FIV chronically infected cats was significantly greater as compared to the percentage of B7-1^+ $CD8^+$ T cells from FIV uninfected cats. There was no significant difference in the percentages of peripheral B7-1^+ $CD4^+$ T cells betwen FIV infected and uninfected cats (Fig. 6, 7). Expression of the B7-1 costimulatory molecule on T cells is unusual since these molecules under normal physiologic conditions are found on professional antigen presenting cells (APC), such as dendritic cells, B cells and macrophages (Lenschow et

al., 1996). However, a number of recent studies have shown that B7 molecules may be up regulated on T cells activated *in vitro* and on a subset of $CD4^+$ and $CD8^+$ T cells in HIV or FIV infection (Haffar et al., 1993; Kochli et al., 1999; Tompkins et al., 2002; Wyss-Coray et al., 1993b). Studies carried out by Tompkins et al. (2002), using cats infected with the North Carolina FIV isolate (NCSU) have indicated that although $B7\text{-}1^+$ $CD4^+$ and $B7\text{-}1^+$ $CD8^+$ T cells were present in both blood and lymph nodes of FIV-infected cats, $B7\text{-}2^+$ T cells were for the most part restricted to lymph nodes. Wolthers et al. (1996) reported that B7-2 and not B7-1 expression on freshly isolated $CD3^+$ T cells was significantly higher among HIV infected individuals as compared to HIV uninfected individuals. They also reported increased B7-1 and B7-2 expression on $CD4^+$ and $CD8^+$ T cells from HIV infected persons as compared to control subjects, after stimulation of these cells with either mitogen (PMA/I) or a combination of CD2 and CD28 antibodies (Wolthers et al., 1996). Kochli et al., (1999) demonstrated that B7-2 expression and not B7-1 expression on fresh peripheral blood $CD4^+$ and $CD8^+$ T cells among HIV infected individuals was significantly higher as compared to HIV uninfected individuals. According to studies by Wyss-Coray et al. (1993b), examination of a limited number of HIV-infected patients (n = 12) revealed that 0.5% - 20% of $CD3^+$ T cells in fresh PBMC expressed B7 molecules, whereas none of the $CD3^+$ T cells from PBMC of healthy patients (n = 5) expressed B7. Additionally, Jason et al. (1999) demonstrated a relatively higher proportion of HIV-capsid $(p24)^+$ $CD3^+$ T lymphocytes expressing B7-1 and B7-2 than $p24^-$ $CD3^+$ lymphocytes. In our studies, we have been unable to determine percentages of feline B7-2 for lack of a monoclonal antibody to feline B7-2. However,

increased percentage of peripheral B7-1$^+$ CD8$^+$ T cells was observed in our FIV infected cats as compared to FIV uninfected cats.

On the other hand, it is likely that the expression of costimulatory molecules is a reflection of the chronicity of T cell activation during HIV infection (Kochli et al., 1999) since the percentage of actively HIV infected CD4$^+$ T cells is < 0.02% which is far less than the percentage of T cells with an APC phenotype. Human T lymphotropic virus (HTLV- also a retrovirus) infected T lymphocytes constitutively express B7-1 and B7-2 concomitant to the down-modulation of CD28 on T cells. In HTLV infection, such B7-1$^+$/ B7-2$^+$ T cells serve as APC, leading to a sustained proliferation of T cells, and both the ligands have been found to participate in allostimulation, autologous proliferation as well as spontaneous proliferation of HTLV-II infected PBMC (Lal et al., 1996).

Activated or *in vitro* stimulated T cells also express B7-1 and B7-2 molecules in addition to MHC-II molecules (HLA-DR), suggesting that those T cells might function as APC (Azuma et al., 1993; Pichler and Wyss-Coray, 1994; Wyss-Coray et al., 1993a). Studies with HIV infected individuals have shown that, during infection, T cells themselves develop an antigen presenting phenotype by upregulating expression of HLA-DR, B7-1 and B7-2 molecules. The more the disease progresses, the less CD28 and more DR and B7-2 are found on circulating T cells (Kochli et al., 1999). Activated T cells from HIV infected individuals have also been found to present the HIV envelope protein gp120 (Lanzavecchia et al., 1988; Siliciano et al., 1988). Based on these observations and the flow cytomtery results discussed, it is possible that similar to HIV infection in humans, T cells from FIV infected cats develop an antigen presenting

phenotype and hence contribute to the stimulation of effector $CD8^+$ T cells as described earlier (Choi et al., 2000b; Phadke et al., 2004).

The B7 family of proteins, B7-1 and B7-2, provide the major costimulatory signal for augmenting and sustaining T-cell response via interaction with the CD28 costimulatory receptor. Binding to another receptor on activated T cells, CTLA-4 leads to antigen specific anergy in mice (Lenschow et al., 1996; McAdam et al., 1998). In the case of HIV infection, $CD8^+$ T cell antiviral factor production has been found to be associated with cells expressing CD8, CD38, HLA-DR and CD28 antigens (Jiang et al., 2003). Engagement of the CD28 molecule on $CD8^+$ T cells from AIDS patients during stimulation *in vitro* was found to restore and enhance the ability of these cells to suppress HIV replication (Barker et al., 1997). Moreover, blocking the interaction of CD28 and B7 molecules with a CTLA-4Ig fusion protein abrogated the ability of autologous macrophages to enhance this $CD8^+$ T cell antiviral activity (Barker et al., 1997). Barker et al. (1999) demonstrated that while both B7 molecules could deliver a costimulatory signal sufficient to increase $CD8^+$ T cell antiviral activity, B7-2 was found to be the molecule on macrophages that enhanced $CD8^+$ T cell suppression of HIV replication. Hence in order to determine the role played by B7 molecules in the induction of anti-FIV activity in our system, interactions of B7 with their receptors were blocked using anti-feline B7-1 antibody, anti-feline B7-2 antibody or anti-feline CTLA-4 antibody. However, the results obtained using antibodies to feline B7-1, B7-2 and CTLA-4 were not conclusive as the antibody controls (pre-immune rabbit serum and the irrelevant hybridoma supernatant) demonstrated non-specific suppression, making it difficult to

determine the role of B7-1 and B7-2 molecules expressed on inducer T cells in the induction of $CD8^+$ T cells to mediate the anti-viral activity.

In conclusion, the percentage of B7-1^+ peripheral $CD8^+$ T cells from FIV chronically infected cats was significantly greater as compared to the percentage of B7-1^+ $CD8^+$ T cells from FIV uninfected cats, but its relevance to the induction of suppressing activity against FIV is presently unknown.

CHAPTER IV

PATHOGENENSIS OF A TEXAS FIV ISOLATE WHICH BELONGS TO AN EMERGING SUBTYPE CLOSELY RELATED TO FIV CLADE B

INTRODUCTION

Feline immunodeficiency virus (FIV), a lentivirus, within the family *Retroviridae*, causes an AIDS-like disease in naturally and experimentally infected cats, similar to that seen in human immunodeficiency virus (HIV) infection of humans. FIV resembles HIV in terms of its morphology, genome organization and Mg^{2+} dependency of its reverse transcriptase (Olmsted et al., 1989b; Pedersen et al., 1987; Talbott et al., 1989; Yamamoto et al., 1989). After primary infection, the clinical course of FIV infection in cats also parallels that seen in HIV infection in humans. Cats develop an acute infection illness similar to that seen in HIV infection including low grade fever, transient lymphadenopathy and transient leucopenia due to an absolute neutropenia (Barlough et al., 1991; Callanan et al., 1992; Yamamoto et al., 1988). *In vivo*, FIV causes an early plasma and cell-associated viremia, followed by a strong antibody response that correlates with reduction in plasma viremia (Diehl et al., 1995a; Yamamoto et al., 1988). This is followed by a long asymptomatic period of varying duration characterized by inverted $CD4^+$/ $CD8^+$ T cell ratios due to a decrease in $CD4^+$ T cells and sometimes due to a corresponding increase in the $CD8^+$ T cells (Ackley et al., 1990; Tompkins et al., 1991; Torten et al., 1991). The increase in $CD8^+$ T cells has been

reported to include a population of $CD8^+$ T cells expressing low levels of CD8 ($CD8^{low}$) and increased levels of MHC class II antigens (Willett et al., 1993). Furthermore, the onset of immunodeficiency is also marked by a reduced responsiveness of feline peripheral blood mononuclear cells (PBMC) to *in vitro* mitogenic stimulation (Siebelink et al., 1990; Torten et al., 1991). The asymptomatic period progresses to a stage characterized by a variety of AIDS-associated disorders, such as opportunistic infections, chronic gingivitis or stomatitis, chronic upper respiratory infections, superficial and systemic fungal infections, ocular disease and chronic enteritis (Bendinelli et al., 1995; Pedersen, 1992). As with human AIDS patients, FIV infected cats usually die from a chronic wasting syndrome, neurologic disease, neoplasia or systemic opportunistic infections (Bendinelli et al., 1995; Hutson et al., 1991; Yamamoto et al., 1989). Similarities in biological characteristics and disease progression to AIDS have made FIV a relevant small animal model for studying lentiviral pathogenesis and therapeutic strategies.

FIV isolates can be subdivided into five distinct phylogenetic clades, designated A to E based on the nucleotide sequences from the envelope gene (Bachmann et al., 1997; Elder et al., 1998; Sodora et al., 1994). Those numbers can be expected to increase as further studies reveal additional diversity. Clade B viruses are significantly diverse with three phylogenetically and evolutionary distinguishable groups (Bachmann et al., 1997). In fact, recent studies from our laboratory have indicated the evolution of an emerging subtype of FIV isolates in Texas that appear to have shared a common ancestor with clade B isolates (Weaver et al., 2004). Studies by Sodora et al. (1994) have

suggested that FIV subtype B was in a more advanced state of adaptation to the host, and may be less pathogenic than subtype A. In their study, none of the cats from which subtype B viruses were obtained had evidence of disease, although some of them had low $CD4^+$ T cell numbers. Three viral isolates, namely FIV subtype B strain 2542, FIV-TM1 and FIV-TM2 belonging to clade B, have been studied for pathogenicity with contrasting results (Burkhard et al., 2001; Diehl et al., 1995a; Diehl et al., 1995b; Kohmoto et al., 1998; Yamamoto et al., 1997). Although the three isolates caused immunodeficiency with an inversion of $CD4^+$/ $CD8^+$ T cell ratios, FIV clade B strain 2542 was shown to cause fatal immunodeficiency in cats (Elder et al., 1998).

The major obstacle for acceptance of FIV as a small animal model for human AIDS has been the long and protracted disease course associated with infection with most FIV strains. Under experimental conditions 3 FIV isolates have shown to consistently induce fatal immunodeficiency disease: A molecularly cloned clade A FIV $NCSU_1$ virus (English et al., 1994; Yang et al., 1996) and rapidly passaged clade B and clade C FIV isolates (Bachmann et al., 1997; Diehl et al., 1995b; Elder et al., 1998). FIV-C-PGammer rapidly killed two-thirds or more of the kittens infected intravenously (i.v.) at less than 12 weeks of age (Diehl et al., 1996; Diehl et al., 1995b). However, the virulence appeared to be age-restricted because cats 8-12 months of age were unable to demonstrate this rapid disease course seen with kittens (Pedersen et al., 2001).

The Texas (TX) isolates were isolated from naturally infected feral cats which were either asymptomatic or showed varying clinical abnormalities, such as skin lesions, and lymphoid hyperplasia in addition to the presence of tapeworms and roundworms at

necropsy. These TX isolates formed a distinct cluster when compared with FIV strains from other known clades (Weaver et al., 2004). Disease caused by experimental infection of cats with the TX isolates has not yet been demonstrated. The following study was undertaken with the goal to determine if the TX isolates, specifically FIV-TX53 differed from other known FIV strains in its ability to cause disease in the acute stage of experimental infection.

MATERIALS AND METHODS

Animals and virus inoculations

Twelve specific, pathogen-free (SPF), 4 month old male kittens were purchased from Harlan Sprague Dawley (Indianapolis, IN). They were housed in a specific, pathogen-free environment, for the duration of the experiments at Laboratory Animal Research and Resource support facilities (LARR), Texas A&M University, College Station.

The kittens were randomly divided into four groups of 3 individuals 1 week before inoculation to re-establish dominance hierarchies. The negative control group was sham inoculated i.v. with 1 ml of sterile phosphate buffered saline (PBS) only. The three treatment groups received 1 ml of sterile PBS containing the FIV-TX53 strain of FIV at titers of 500, 2000 or 8000 $TCID_{50}$. Following inoculation, general health was monitored daily for 15 days post infection (p.i.) and then every two days until 9 weeks p.i.

Preparation of feline peripheral blood mononuclear cells (PBMC)

Whole blood was drawn by jugular venipuncture 1 week prior to infection and at 2, 4, 7, 9 and 11 weeks p.i. Feline PBMC were isolated from EDTA (K_3)-treated whole blood by Histopaque-1077 (Sigma, St. Louis, MO) density gradient centrifugation (Choi et al., 2000b). Unstimulated fresh PBMC, collected 1 week prior to infection and during weeks 2, 4, 7, 9 and 11 p.i. were used to determine $CD4^+/CD8^+$ T cell ratios, for proviral DNA PCR and complete blood counts.

Virus

FIV-TX53 (AY139109) was propagated in PBMC from the feral cat from which it was isolated. After 7 to 10 days of infection, virus replication was evaluated with an FIV capsid antigen detection enzyme-linked immunosorbent assay (ELISA) (Choi et al., 2000b). Supernatants with an optical density (OD) of more than 2 were collected and these stocks were stored at -80^o C. The FIV-TX53 virus stock had a titer of 0.5×10^6 / ml $TCID_{50}$, determined as described earlier (Hokanson et al., 2000).

Complete blood counts and $CD4^+/CD8^+$ T cell staining of freshly prepared PBMC

Complete blood counts were determined by the Texas Veterinary Medical Diagnostic laboratory (TVMDL) at Texas A&M University in order to monitor lymphocyte and neutrophil levels. PBMC from 9 FIV infected and 3 uninfected cats were isolated from EDTA (K_3)-treated whole blood by Histopaque-1077 (Sigma, St. Louis, MO) density gradient centrifugation (Choi et al., 2000b) and washed once with cold

phosphate-buffered saline (PBS) containing 0.1% sodium azide. Prior to staining of the cells, the Fc receptors on the cells were blocked by incubating the cells for 10 minutes on ice with goat anti-IgG (2 mg/ml) (Sigma, St. Louis, MO). Phycoerythrin (PE) conjugated monoclonal antibody (Mab) specific for feline CD4 (Southern Biotechnology Associates, Birmingham, AL) and fluorescein isothiocyanate (FITC) conjugated Mab specific for feline CD8β chain (Southern Biotechnology Associates, Birmingham, AL) were then added to the cells in the respective tubes at a final dilution of 1:50. After incubating for 30 minutes on ice, the cells were washed once with cold PBS-0.1% sodium azide and centrifuged at 1200 rpm for 10 minutes (Jouan Inc., Winchester, VA). Negative controls consisted of unstained cells. After washing the cells, 300 ul of 2% formaldehyde were added to the tubes, which were wrapped in an aluminium foil and kept at 4^{o}C until they were examined the next day with a FACscaliber flow cytometer (Becton-Dickinson, San Jose, CA) at the Veterinary Pathobiology Core Facility, (Director, Dr. Roger Smith, Texas A&M University, College Station).

Lymphocytes were analyzed on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA), using CellQuest (Becton Dickinson) acquisition software. Green fluorescence from conjugated fluorescein was analysed through a 530/30-nm bandpass filter, and orange fluorescence from R-phycoerythrin through a 585/42-nm bandpass filter. Electronic compensation was applied to correct for spectral overlap of fluorescein and R-phycoerythrin during data acquisition. List mode data were acquired on a minimum of 10,000 cells defined by light scatter gates. Data analysis was performed

in CellQuest / FlowJo, (Treestar, Inc., Palo Alto, CA) using forward and side light scatter to gate on the lymphocyte population.

Virus isolation

Fresh PBMC from weeks 2, 4, 7 and 9 p.i. were cultured as described previously with RPMI 1640 (Gibco BRL, Grand Island, N.Y.) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Atlas Biologicals, Fort Collins, CO), 50 ug/ml of gentamicin (Gibco BRL, Grand Island, N.Y.), 5 x 10^{-5} M 2-mercaptoethanol (Gibco BRL, Grand Island, N.Y.), 2mM L-glutamine (Gibco BRL, Grand Island, N.Y.) and with 5 ug/ml of Concavalin A (Con A) (Sigma, St. Louis, MO) for 3 days. Cells were grown at 37^{o}C in a humidified atmosphere of 5% CO_2. On the third day, the cells were washed and cultured in complete RPMI containing 100 U human recombinant interleukin -2 (hr IL-2) per ml (Gibco BRL, Grand Island, N.Y.). Thereafter, virus replication was monitored every 3 days using the FIV capsid antigen ELISA.

PCR amplification of proviral DNA

In order to amplify the proviral DNA, genomic DNA was isolated from 1 ml of blood collected at 2, 4, 7 and 9 weeks p.i. using using red blood cell lysing buffer and QIAmp DNA blood mini kit (Qiagen Inc., Valencia CA). In addition, genomic DNA was isolated using QIAmp DNA blood mini kit from PBMC, after culturing for 6 days as mentioned above. The DNA was stored at -20^{o}C. FIV capsid gene region was amplified by nested PCR using genomic DNA obtained from PBMC of the FIV infected cats and

uninfected control cats. The initial PCR used primers 628F (5'-ATGGGGAACGGACAGGGGCGA-3') and 1957R (5'-TTATAAATCCAATAGTCTCTCCTC-3') amplifying a 1329 bp gag product, while the second round nested PCR used primers 1033F (5'-CCTATTCAAACAGCAAATGGAGCA-3') and 1678R (5'-TGGCAAGAGTTGCATTTTATATCCTGG-3') amplifying a 645 bp gag product. Primers used in this study were made by IDT, Inc. (Coralville, IA). The following parameters were used for the primary amplification of the gag gene product using a GeneAmp 2400 (Perkin Elmer, Norwalk, CT): 94^{o}C for 5 min, followed by 5 cycles of 94^{o}C for 60s, 53^{o} C for 60s and 72^{o}C for 2 min., and finally 30 cycles of 94^{o}C for 15s, 53^{o} C for 45s and 72^{o} C for 2 min. The annealing step was increased by 0.1^{o}C per cycle for 30 cycles. The reaction was held at 72^{o}C for 15 min followed by 4^{o} C for infinity. The thermal cycling parameters for the secondary amplification were identical to the primary amplification except that 5 ul of the each of the primary PCR products was used as template.

Anti-FIV capsid antibody assay

Detection of antibodies against FIV-TX53 in plasma from each blood collection was evaluated using the IDEXX Petcheck anti-capsid antibody ELISA kit (IDEXX Inc., Portland, ME).

Statistical analysis

The data obtained from the experiment designed to study the pathogenesis of FIV-TX53 isolate was analyzed using a repeated measures design (Kuehl, 2000). The analysis determined if there was a significant difference in the response variables (Absolute $CD4^+$ T cells, absolute $CD8^+$ T cells, $CD4^+/CD8^+$ T cell ratio, numbers of lymphocytes and neutrophils, percentages of $CD8\beta^{low}$ and $CD8\beta^{high}$ expression) with respect to the three doses of virus inoculum administered (treatment) and over the weeks that it was examined (time). The repeated measures design was preferred since it accounted for trends in the response variables across the different time points and treatment levels. If a significant difference was observed in the main effects for treatment or time, pairwise comparisons between various doses or pairwise comparisons between different weeks were carried out using Tukey test for pairwise comparison of all treatment means (Kuehl, 2000). Statistical significance was set at $p < 0.05$. In order to compare the results obtained for the infected cats with respect to the control uninfected cats, Dunnett's method for comparison of all treatments with a control was used (Kuehl, 2000). This test determined if there was a significant difference ($p< 0.05$) between the three viral doses and the control across all the weeks examined. In order to establish significant differences between the responses for the control uninfected cats and the data values obtained for the cats infected with three doses of the virus for each week, a one way analysis of variance (ANOVA) was performed. Using ANOVA, pairwise comparisons were carried out using least significant difference test (LSD), Tukey test which is also called honestly significant difference (HSD) test, Student-Newman-Keuls

(SNK) multiple range test and Dunnett's method for comparison of all treatments with a control (Kuehl, 2000). Statistical significance was again set at $p < 0.05$. All the statistical analysis was performed using the SAS software (SAS Institute, Cary, NC).

RESULTS

Clinical illness

Four-month-old cats were infected with three different doses of the FIV-TX53 isolate. Cats in each group developed a mild to moderate generalized lymphadenopathy of their popliteal or submandibular lymph nodes starting around 10 days p.i. Surprisingly, all the cats infected with 2000 $TCID_{50}$ of the virus demonstrated lymphadenopathy while only one cat from each group of cats infected with either 500 $TCID_{50}$ (cat 3819) or 8000 $TCID_{50}$ (3825) of the virus demonstrated lymphadenopathy. There was no significant difference in the timing of lymphadenopathy among the three groups of cats infected with the varying doses of the virus. Except for the lymphadenopathy, the cats appeared to be normal.

Complete blood cell evaluations

Hematologic abnormalities were observed in the infected cats as compared to the uninfected cats. Complete blood counts indicated that the number of lymphocytes and neutrophils did decrease over time p.i., but with some exceptions did not fluctuate as compared with the counts of sham infected control cats. Lymphopenia seen by 7 weeks p.i. for cat 3821 (500 $TCID_{50}$ of the virus) persisted until 11 weeks p.i. (Fig. 8). Cat 3822

(2000 $TCID_{50}$ of the virus) showed lymphopenia between weeks 7 and 9 p.i. and the numbers returned to normal values by 11 weeks p.i. Cat 3824 from the same group also receiving 2000 $TCID_{50}$ of the virus was lymphopenic at 11 weeks p.i. (Fig.8). For all doses of virus administered, the number of lymphocytes at 4 weeks p.i. was statistically greater than at 9 weeks p.i. ($p < 0.05$) (Fig. 8). Surprisingly, the cats infected with 8000 $TCID_{50}$ of the virus were not lymphopenic at any of the times examined. The absolute lymphocytes of the sham infected control cats were within the normal range at all times examined.

The number of neutrophils after infection was statistically lower than preinfection values, at weeks 2 ($p = 0.0095$), 4 ($p = 0.0.0314$), 7 ($p = 0.0292$), 9 ($p = 0.0002$) and 11 ($p = 0.0056$) p.i. for all viral doses administered (Fig. 9). Interestingly, all three cats infected with 500 $TCID_{50}$ of the virus demonstrated neutropenia either at week 11 p.i. (Cat 3819), or at week 9 p.i. (Cat 3820) while cat 3821 continued to show a reduction in the neutrophil numbers as compared to normal values between 7 to 11 weeks p.i. (Fig. 9). In addition between 7 and 11 weeks p.i., neutrophil numbers were decreased in two cats infected with 2000 $TCID_{50}$ of the virus (cats 3822, 3824) as compared with those from sham inoculated controls (Fig. 9). The decline in neutrophil numbers of cats infected with 8000 $TCID_{50}$ was not statistically different from that of the normal neutrophil values at any time examined. Therefore, no trend was observed between the amount of virus administered and decrease in the absolute numbers of neutrophils or lymphocytes, for the uninfected and infected cats.

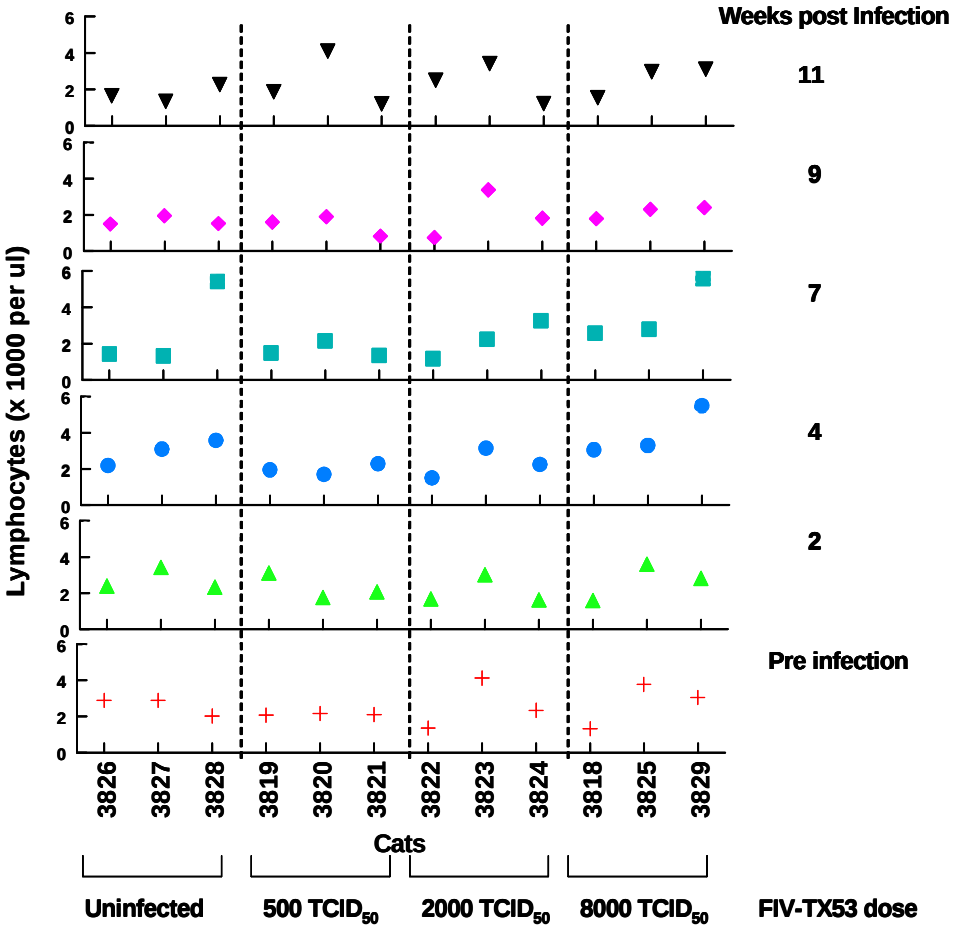


Fig. 8. Absolute blood lymphocyte counts until 11 weeks after infection of cats with varying doses of FIV-TX53 virus. Cats 3826, 3827 and 3828 were uninfected controls, cats 3819, 3920, 3821 were infected i.v. with 500 $TCID_{50}$ of the virus, cats 3822, 3823 and 3824 were infected i.v. with 2000 $TCID_{50}$ of the virus and cats 3818, 3825 and 3829 were infected i.v. with 8000 $TCID_{50}$ of the virus.

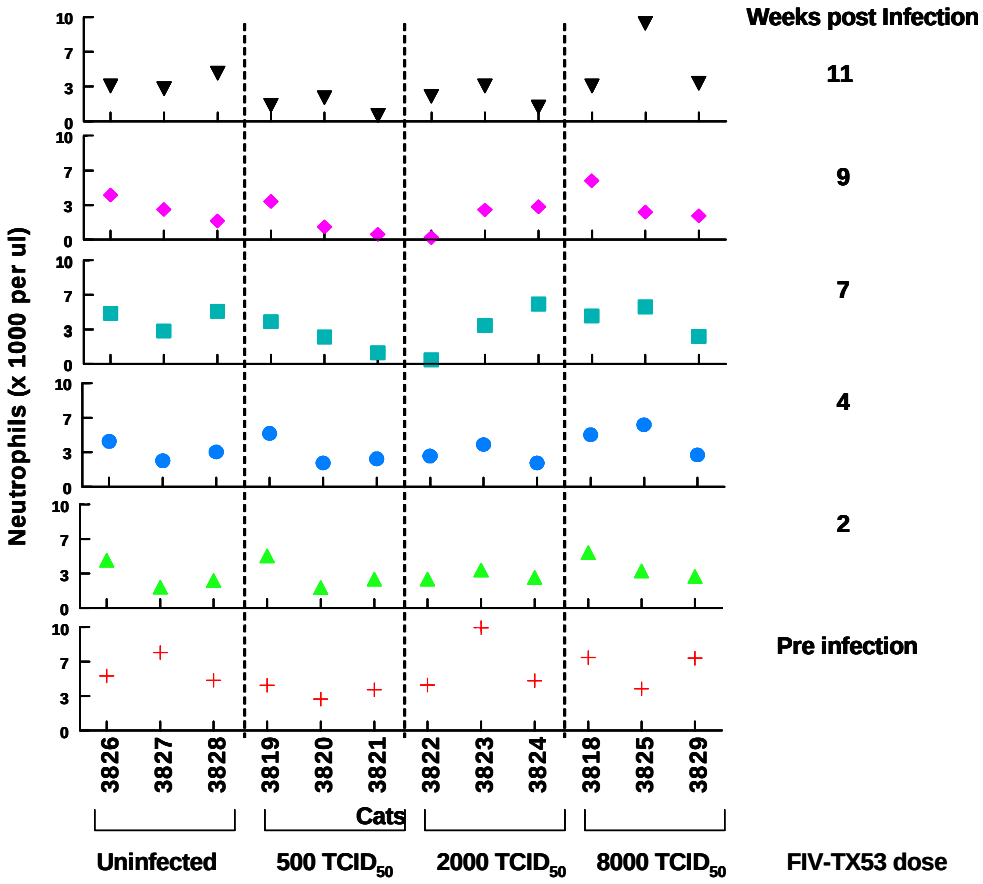


Fig. 9. Absolute blood neutrophil counts until 11 weeks after infection of cats with varying doses of FIV-TX53 virus. Cats 3826, 3827 and 3828 were uninfected controls, cats 3819, 3920, 3821 were infected i.v. with 500 $TCID_{50}$ of the virus, cats 3822, 3823 and 3824 were infected i.v. with 2000 $TCID_{50}$ of the virus and cats 3818, 3825 and 3829 were infected i.v. with 8000 $TCID_{50}$ of the virus.

$CD4^+/CD8^+$ T lymphocyte ratios

The $CD4^+$/ $CD8^+$ T cell ratio was determined for the cats before they were infected and at 2, 4, 7, 9 and 11 weeks p.i. A temporal decrease in the $CD4^+$/ $CD8^+$ T cell ratio could be observed for the infected cats depending on the viral dose administered as compared to the uninfected cats. Thus, the $CD4^+$/ $CD8^+$ T cell ratio of the cats dropped below 1 between weeks 4-7 p.i. for cats infected with the highest dose ($TCID_{50}$ = 8000) of the virus (Fig. 10). Two of the three cats (cats 3822 and cat 3823) infected with 2000 $TCID_{50}$ of virus inoculum showed a $CD4^+$/ $CD8^+$ T cell ratio below 1 at week 9 p.i., while the ratio was below 1 for the third cat from the same group (cat 3824), at week 7 p.i. The $CD4^+$/ $CD8^+$ T cell ratios for two of the cats 3819 and 3821, infected with the least amount of virus ($TCID_{50}$ = 500) dropped below 1 by only 11 weeks p.i. The $CD4^+$/ $CD8^+$ T cell ratios of the cats infected with any of the three doses of the virus were significantly lower than those of the uninfected cats at weeks 7, 9 and 11 p.i. ($p < 0.01$) (Fig. 10).

For all the times examined, the decrease in the $CD4^+/CD8^+$ T cell ratio was accompanied by a temporal and sharp decline of $CD4^+$ T cells, which remained low even at 11 weeks p.i. and did not revert to the preinfection levels except for cat 3820 at 11 week p.i. (Fig. 10 and 11). This decline in the number of $CD4^+$ T cells was not found to depend on the dose of the virus inoculum administered (Fig. 11). However, there was a significant reduction in the numbers of $CD4^+$ T cells of cats infected with either 500 or 2000 $TCID_{50}$ of the virus at all times examined, as compared to the uninfected controls ($p < 0.05$). The numbers of $CD4^+$ T cells were significantly reduced at week 11 p.i. as

compared to week 4 p.i. ($p = 0.0417$) and at week 9 p.i. as compared to the $CD4^+$ T cell numbers before infection ($p = 0.0086$), at weeks 2 ($p = 0.0128$) and 4 ($p = 0.0047$) p.i. for all the doses of virus administered.

The decline in the $CD4^+/CD8^+$ T cell ratio was also accompanied by a corresponding small increase in the number of $CD8^+$ T cells (Fig. 10 and 12). The absolute numbers of $CD8^+$ T cells did show a significant difference depending on the virus dose administered ($p = 0.029$) and over the weeks examined ($p = 0.0321$). However the small sample number of the cats prevented pairwise significant differences to be detected using the Tukey test (Kuehl, 2000). The absolute $CD8^+$ T cell numbers for cats infected with the highest dose of the virus ($TCID_{50}$= 8000) was significantly greater than $CD8^+$ T cell numbers of uninfected cats at all the times examined ($p < 0.05$) (Fig. 12). Absolute $CD8^+$ T cell numbers in cats infected with 500 $TCID_{50}$ or 2000 $TCID_{50}$ of the virus increased very slightly as compared to pre-infected values (Fig. 12). However this slight increase in the numbers of $CD8^+$ T cells from cats infected with either 500 or 2000 $TCID_{50}$ was not significantly high as compared to $CD8^+$ T cell numbers of sham inoculated control cats at all times examined.

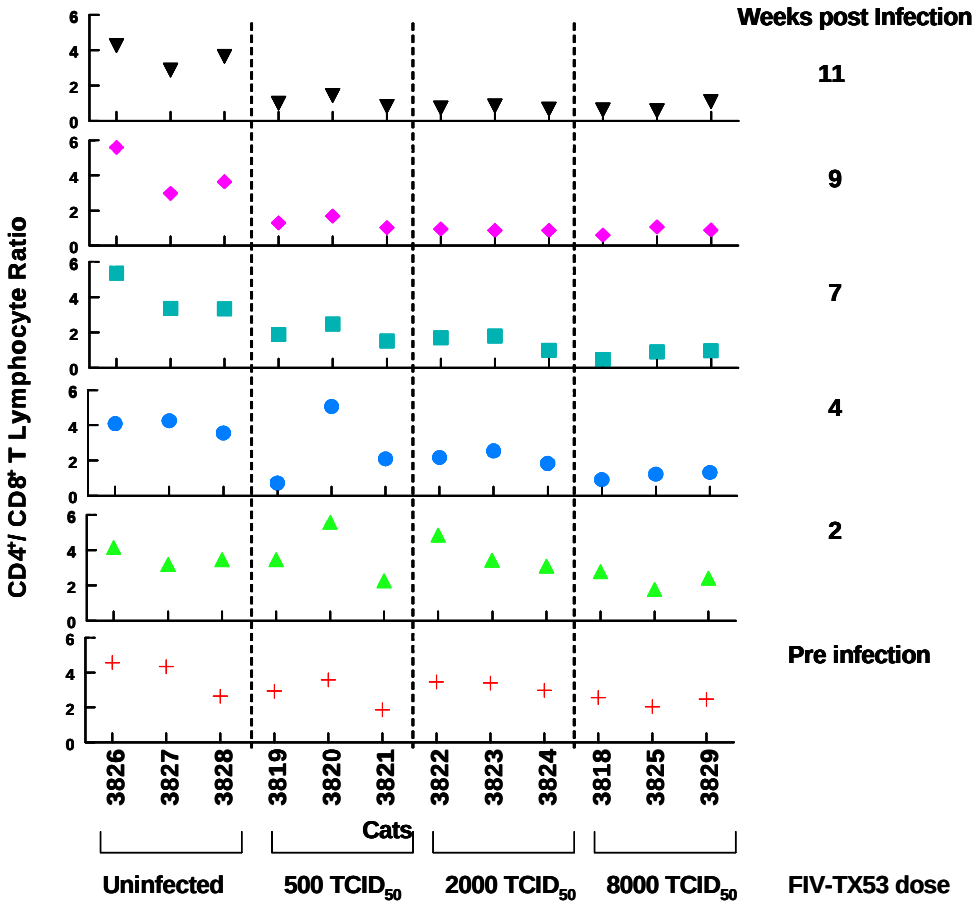


Fig. 10. $CD4^+$/ $CD8^+$ T lymphocyte ratio until 11 weeks after infection of cats with varying doses of FIV-TX53 virus. Cats 3826, 3827 and 3828 were uninfected controls, cats 3819, 3920, 3821 were infected i.v. with 500 $TCID_{50}$ of the virus, cats 3822, 3823 and 3824 were infected i.v. with 2000 $TCID_{50}$ of the virus and cats 3818, 3825 and 3829 were infected i.v. with 8000 $TCID_{50}$ of the virus.

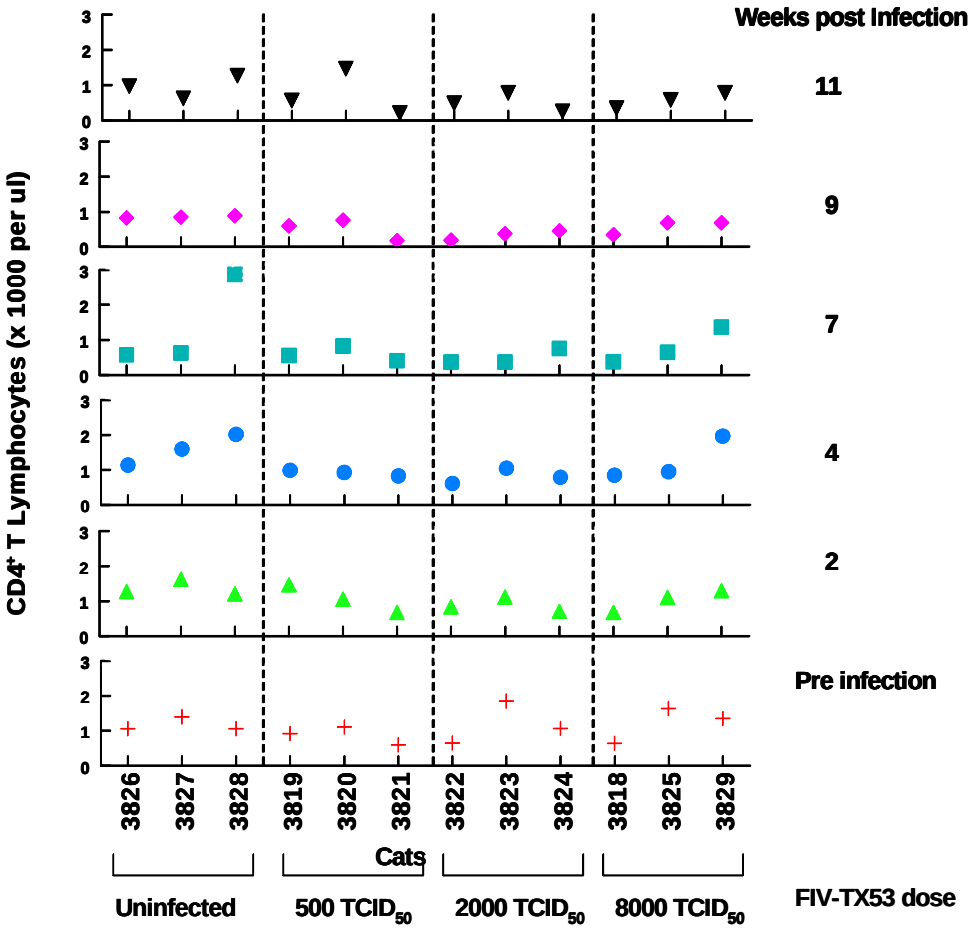


Fig. 11. Absolute $CD4^+$ T lymphocyte counts until 11 weeks after infection of cats with varying doses of FIV-TX53 virus. Cats 3826, 3827 and 3828 were uninfected controls, cats 3819, 3920, 3821 were infected i.v. with 500 $TCID_{50}$ of the virus, cats 3822, 3823 and 3824 were infected i.v. with 2000 $TCID_{50}$ of the virus and cats 3818, 3825 and 3829 were infected i.v. with 8000 $TCID_{50}$ of the virus.

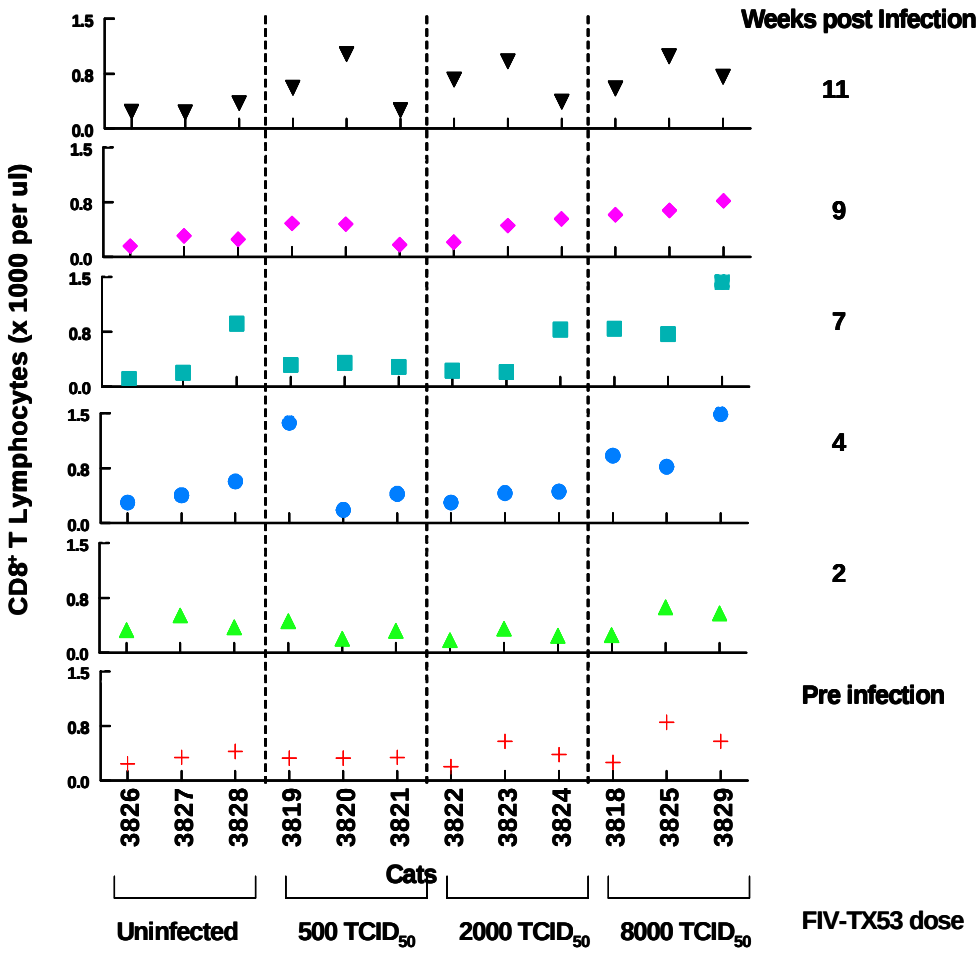


Fig. 12. Absolute $CD8^+$ T lymphocyte counts until 11 weeks after infection of cats with varying doses of FIV-TX53 virus. Cats 3826, 3827 and 3828 were uninfected controls, cats 3819, 3920, 3821 were infected i.v. with 500 $TCID_{50}$ of the virus, cats 3822, 3823 and 3824 were infected i.v. with 2000 $TCID_{50}$ of the virus and cats 3818, 3825 and 3829 were infected i.v. with 8000 $TCID_{50}$ of the virus.

Expansion of CD8β^{low} T lymphocytes in FIV-TX53 infected cats

In our studies, the increase in the numbers of $CD8^+$ T cells of the infected cats was accompanied by an expansion of a $CD8\beta^{low}$ T cell population, as indicated by flow cytometry using Mab to feline CD8β chain (Southern Biotechnology Associates, Birmingham, AL). The representative contour and dot plots in Fig. 13 demonstrate typical patterns of CD8β chain expression on fresh PBMC from uninfected cat 3828 and cat 3824 infected with 2000 $TCID_{50}$ of the virus at 7 weeks p.i. In contrast to FIV uninfected cats, CD8β chain expression on fresh PBMC from FIV infected cats was bimodal, demonstrating the presence of two distinct subpopulations of $CD8^+$ T cells, $CD8\beta^{low}$ and $CD8\beta^{high}$. The $CD8\beta^{low}$ population of $CD8^+$ T cells increased as infection progressed, reaching peak levels at 9 weeks p.i. (Fig.14). The percentages of this $CD8\beta^{low}$ population for all infected cats, started increasing by 4 week p.i. but were significantly increased by weeks 9 ($p = 0.0113$) and 11 p.i. ($p = 0.0002$) as compared to the sham infected control cats (Fig. 14). There was no trend observed between the amount of virus administered and the temporal increase of this population.

However, there was a significant difference seen in the percentages of the $CD8\beta^{high}$ T cells depending on the virus dose administered ($p = 0.0455$) (Fig. 15). However due to the small sample size, significant pairwise differences ($p = 0.0312$) could be observed only between the percentages of $CD8\beta^{high}$ T cells from cats infected with 2000 $TCID_{50}$ or 8000 $TCID_{50}$ of the virus. Thus, the percentages of $CD8\beta^{high}$ population were significantly increased ($p < 0.05$) in cats infected with 8000 $TCID_{50}$ of the virus as compared to cats infected with 2000 $TCID_{50}$ of the virus and sham infected

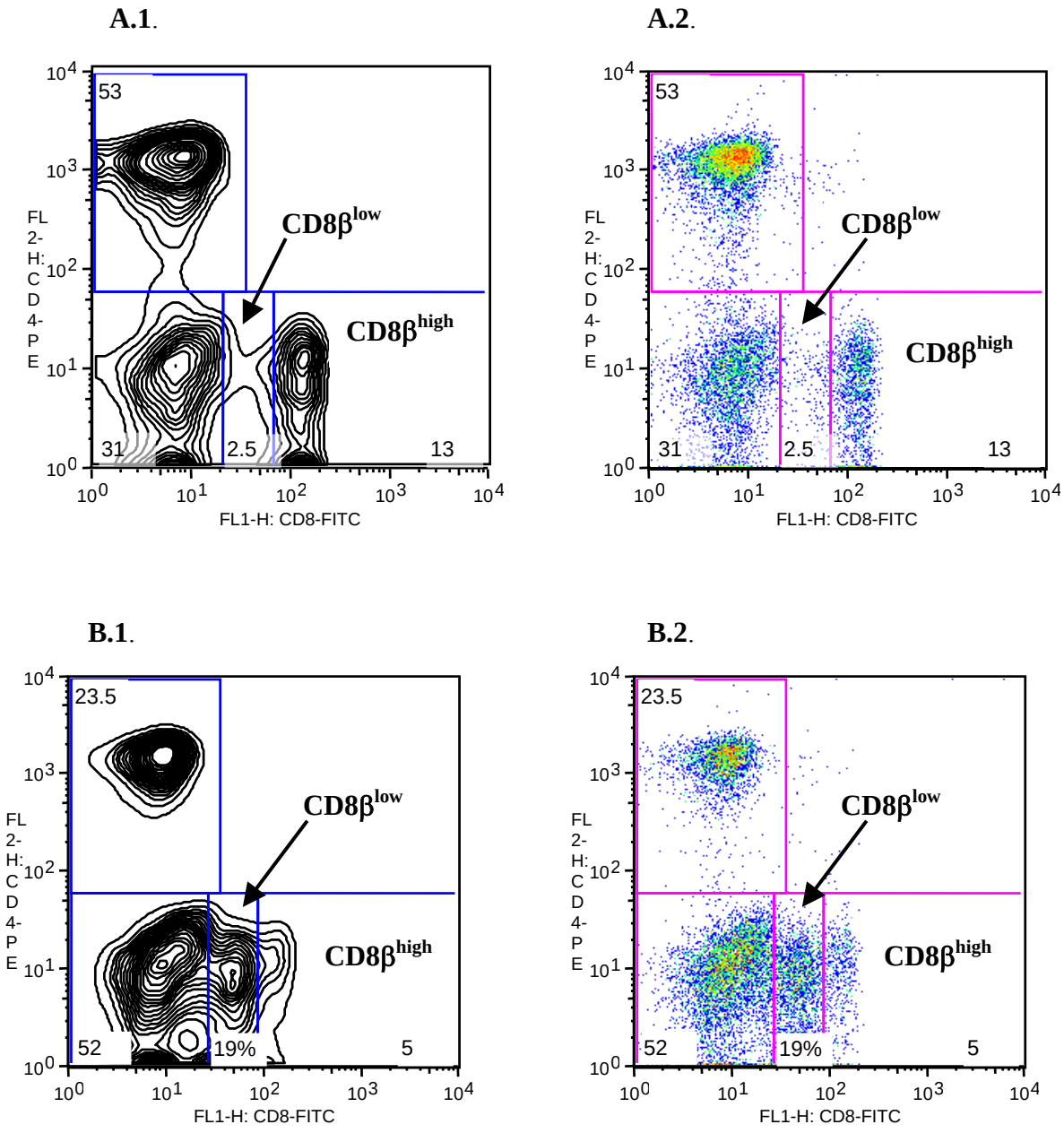


Fig. 13. Patterns of CD8β^{low} expression. Patterns of CD8β^{low} expression for FIV uninfected cat 3828 (A1 and A2) and cat 3824 (B1 and B2) infected with 2000 $TCID_{50}$ of FIV-TX53 virus at 7 weeks p.i. Contour plots (A1 and B1) or dot plots (A2 and B2) presented as fluorescence intensity are shown.

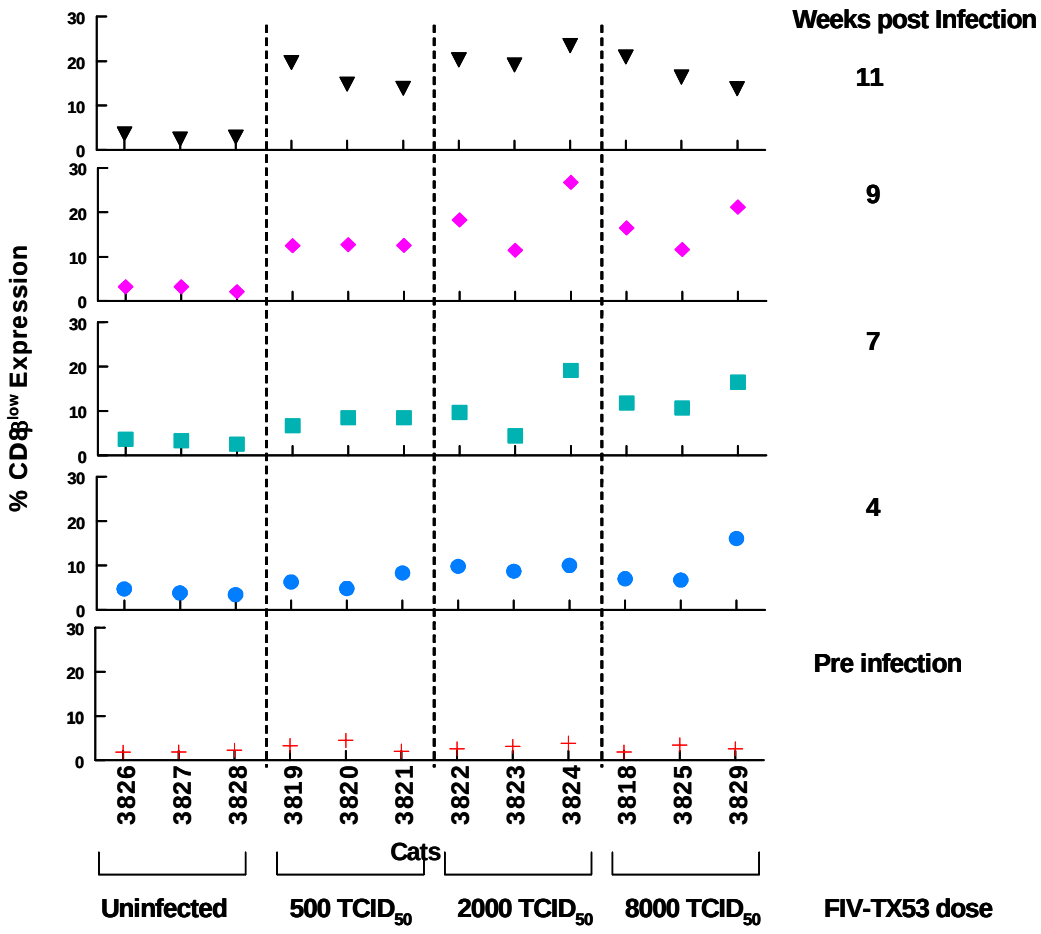


Fig. 14. Expansion of the CD8β^{low} subset of T lymphocytes until 11 weeks after infection of cats with varying doses of FIV-TX53 virus. Cats 3826, 3827 and 3828 were uninfected controls, cats 3819, 3920, 3821 were infected i.v. with 500 $TCID_{50}$ of the virus, cats 3822, 3823 and 3824 were infected i.v. with 2000 $TCID_{50}$ of the virus and cats 3818, 3825 and 3829 were infected i.v. with 8000 $TCID_{50}$ of the virus.

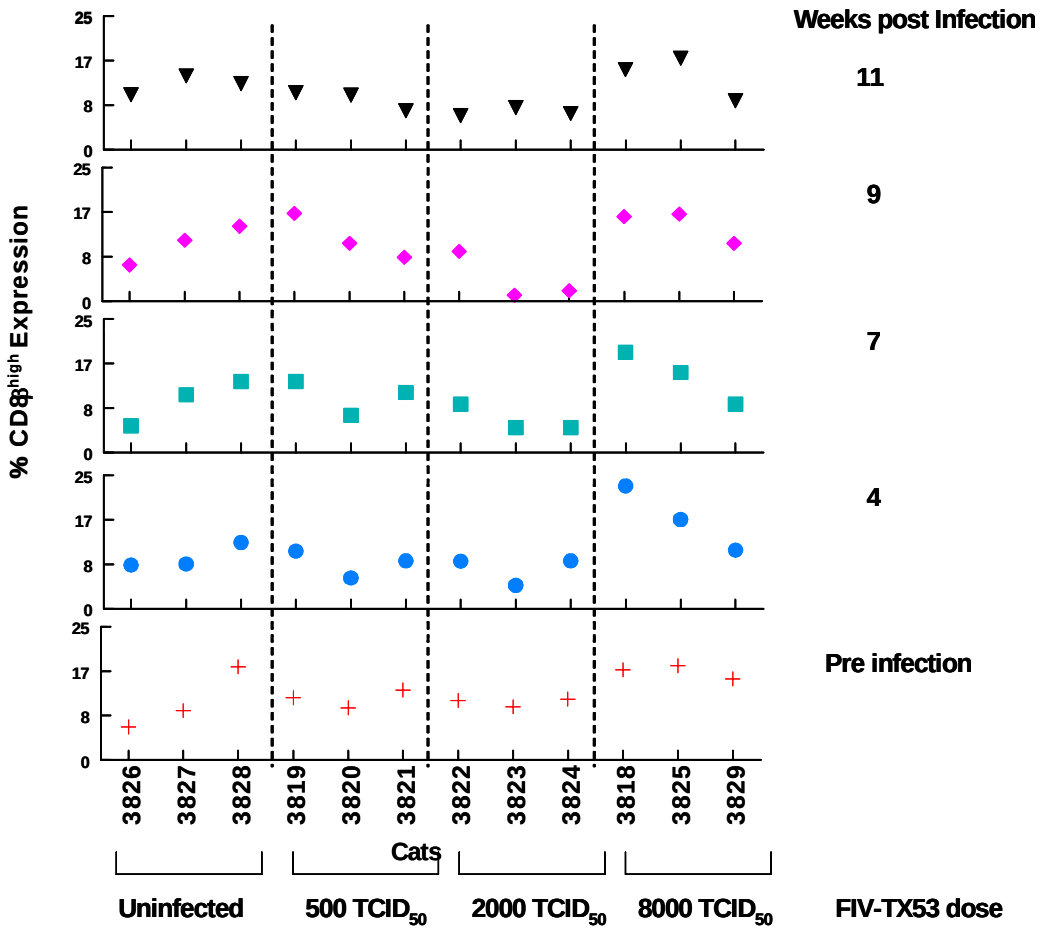


Fig. 15. The CD8β^{high} subset of T lymphocytes until 11 weeks after infection of cats with varying doses of FIV-TX53 virus. Cats 3826, 3827 and 3828 were uninfected controls, cats 3819, 3920, 3821 were infected i.v. with 500 $TCID_{50}$ of the virus, cats 3822, 3823 and 3824 were infected i.v. with 2000 $TCID_{50}$ of the virus and cats 3818, 3825 and 3829 were infected i.v. with 8000 $TCID_{50}$ of the virus.

control cats across all the weeks examined (Fig. 15). Surprisingly, the percentages of $CD8\beta^{high}$ population were significantly decreased ($p < 0.05$) for cats infected with 2000 $TCID_{50}$ of the virus as compared to the sham infected control cats across all the weeks examined. There was no significant change in the percentages of $CD8\beta^{high}$ population for cats infected with 500 $TCID_{50}$ of the virus, when compared to the sham infected control cats (Fig. 15).

Virus isolation and nested PCR for provirus detection

Virus could be detected after culturing PBMC at 9 weeks p.i. for all the infected cats, but virus isolation took longer for PBMC collected at 2, 4 and 7 weeks p.i. By day 9 of culture, PBMC supernatants collected at 2 weeks p.i. from the three cats infected with the highest dose (8000 $TCID_{50}$) were positive for the presence of FIV capsid antigen by FIV antigen capture ELISA (Table 2). By day 9 of culture, supernatants from PBMC collected at 2 weeks p.i. from two of the three cats (cat 3823 and cat 3824) infected with 2000 $TCID_{50}$ of the virus were positive while none of the PBMC supernatants from cats receiving the lowest dose of the virus (500 $TCID_{50}$) were positive (Table 2). After an additional 5 days of culture, PBMC from all the cats were positive by FIV capsid ELISA except for cat 3820 receiving only 500 $TCID_{50}$ of virus. However, PBMC supernatants from cat 3820 were positive for viral antigen following an additional 7 days of culture. For PBMC collected 4 weeks p.i., three cats in the group infected with the lowest dose of the virus and one cat each from the groups infected with either middle or highest dose of the virus were negative by FIV capsid ELISA on the 9^{th}

Table 2

Evaluation of virus isolated from peripheral blood of cats inoculated with varying doses of FIV-TX53 virus

		2 wpi					4wpi				
		PCR		Virus Isolation			PCR		Virus Isolation		
$TCID_{50}$ OF FIV-TX53 VIRUS	CATS	From fresh PBMC	From cells cultured for 6 days	After 9 days of culture	After 14 days of culture	Capsid Antibody detection	From fresh PBMC	From cells cultured for 6 days	After 9 days of culture	After 14 days of culture	Capsid Antibody detection
500	3819	–	–	–	+	–	–	+	–	+	–
	3820	–	–	–	+ **(21 days)**	–	–	–	–	+	–
	3821	–	+	–	+	–	–	+	–	+	–
2000	3822	–	+	–	+	–	–	+	–	+	+
	3823	+	+	+		–	+	+	+		+
	3824	–	–	+		–	+	+	+		–
8000	3818	–	+	+		–	–	–	–	+	+
	3825	–	+	+		–	–	–	+		+
	3829	–	–	+		–	–	+	+		+

Cats 3826, 3827 and 3828 were uninfected controls, cats 3819, 3920, 3821 were infected i.v. with 500 $TCID_{50}$ of the virus, cats 3822, 3823 and 3824 were infected i.v. with 2000 $TCID_{50}$ of the virus and cats 3818, 3825 and 3829 were infected i.v. with 8000 $TCID_{50}$ of the virus. The uninfected control cats tested negative for virus isolation by culture and PCR and were seronegative for the 9 weeks examined

Table 2 (continued)

		7 wpi					9 wpi			
		PCR		Virus isolation			PCR		Virus Isolation	
$TCID_{50}$ OF FIV-TX53 VIRUS	CATS	From fresh PBMC	From cells cultured for 6 days	After 9 days of culture	After 14 days of culture	Capsid antibody detection	From fresh PBMC	From cells cultured for 6 days	After 9 days of culture	Capsid antibody detection
500	3819	–	–	–	+	+	–	+	+	+
	3820	–	+	+		+	+	+	+	+
	3821	–	+	–	+	+	+	+	+	+
2000	3822	–	+	–	+	+	+	+	+	+
	3823	+	+	+		+	+	+	+	+
	3824	+	+	+		+	+	+	+	+
8000	3818	–	+	–	+	+	+	+	+	+
	3825	–	+	+		+	–	+	+	+
	3829	–	+	–	+	+	+	+	+	+

day of culture, but were positive by day 13 of culture (Table 2). PBMC collected at 7 weeks p.i. from all the infected cats were positive for the presence of FIV capsid by ELISA by day 14 of culture. PBMC collected at 9 weeks p.i. from all the cats regardless of viral dose administered initially, were positive by FIV capsid ELISA after only 9 days of culture. PBMC from uninfected cats were consistently negative by ELISA at all times examined (Table 2).

In agreement with results obtained for virus isolation, provirus detection for the capsid region of the virus was sporadic by nested PCR using genomic DNA (gdna) obtained from freshly collected PBMC at 2, 4 and 7 weeks p.i. At 2, 4 and 7 weeks p.i., fresh gdna from only cats 3823 or 3824 (2000 $TCID_{50}$) tested positive by PCR (Table 2). However, gdna from PBMC of infected cats 3818, 3819, 3820, 3821, 3822, 3825 and 3829 tested positive after 6 days of culture (Table 2). As shown in Table 2, at 9 weeks p.i., gdna obtained from freshly collected PBMC from all the infected cats, except cat 3819 (500 $TCID_{50}$) and cat 3825 (8000 $TCID_{50}$) tested positive by nested PCR. Thus, an increase in the virus load by 9 weeks p.i. could be deduced by the increased frequency of the virus isolation from fresh PBMC both by ELISA and by nested PCR (Table 2). The sham infected control cats were also consistently negative for FIV gag by nested PCR.

Seroconversion

The FIV uninfected control animals remained seronegative for antibodies to FIV capsid throughout the course of the experiment as indicated in Table 2. Capsid antibodies could not be detected from plasma at 2 weeks p.i. for any of the infected cats

(Table 2). Antibodies could be detected in plasma by 4 weeks p.i from all the three cats infected with the highest dose of the virus (8000 $TCID_{50}$), two of the cats infected with (2000 $TCID_{50}$) and none of the cats infected with the least dose of the virus (Table 2). By 7 week p.i. all the cats infected with the lowest dose of virus (500 $TCID_{50}$) and the third cat infected with 2000 $TCID_{50}$ had also seroconverted. The cats did not become seronegative with antibodies persisting in all the infected cats at least until 9 weeks p.i. (Table 2).

DISCUSSION

Previous studies from our laboratory indicated that FIV isolates obtained from naturally infected feral cats in Texas clustered separately from the other known clades of FIV, though it was more similar to viruses from FIV clade B (Weaver et al., 2004). The objective of the present study was to characterize the acute stage of infection by experimental infection of SPF cats with a biological clone of a Texas isolate namely; FIV-TX53 and to determine the dose response effects of virus infection.

Various strains of FIV have been examined for their pathogenicity in experimentally infected cats, though different routes of inoculation and various doses of virus used prevent absolute comparisons. Infections with FIV-A-PPR and FIV-A-Petaluma are thought to cause a milder disease as compared to FIV-A-$NCSU_1$, FIV clade B and C field isolates that have been shown to cause fatal immunodeficiency disease in cats under experimental conditions (Burkhard et al., 2002; Diehl et al., 1995b; Elder et al., 1998; Sparger et al., 1994).

The Texas isolates appear to share a common ancestor with isolates from FIV clade B (Weaver et al., 2004). Contrasting results for pathogenicity of three clade B viral isolates namely FIV subtype B strain 2542, FIV-B-TM1 and FIV-B-TM2, have been reported (Burkhard et al., 1997; Kohmoto et al., 1998; Yamamoto et al., 1997). FIV subtype B strain 2542 has been shown to be readily transmitted by prenatal and post natal mother-to-offspring transmission or by exposure of vaginal and rectal mucous membranes to cell associated or cell-free virus and by i.v. inoculation (Burkhard et al., 2001; Burkhard et al., 2002; O'Neil et al., 1996). Rapid serial passage of acute phase plasma from cats infected with FIV-B-2542 induced clinical immunodeficiency, leading to lymphadenopathy and inversion of $CD4^+$/ $CD8^+$ T cell ratios (Diehl et al., 1995a). In contrast, infection with subtype B strains TM1 and TM2 did lead to immunologic abnormalities, such as loss of T cell responses to mitogen stimulation and decreases in $CD4^+/CD8^+$ T cell ratios by 135 and 160 weeks p.i. (Yamamoto et al., 1997) but clinical disease did not appear until 8 years p.i. (Kohmoto et al., 1998).

Infection of 4 month old cats with 500, 2000, and 8000 $TCID_{50}$ of the FIV-TX53 viral strain did not lead to a fatal disease, although the cats did demonstrate transient lymphadenopathy. Complete blood counts indicated that the number of lymphocytes and neutrophils did decrease over time p.i., but with some exceptions fluctuated slightly from the normal values of sham infected control cats. Our results were similar to those obtained for experimental infection with FIV-Petaluma where the lymphocyte and neutrophil number dropped between 4 to 6 weeks p.i. (Yamamoto et al., 1988).

All the animals in our study had seroconverted by 7 weeks p.i. with 2 of the 3 cats in the 2000 $TCID_{50}$ and 3 of the 3 cats in 8000 $TCID_{50}$ group seroconverting by 4 weeks p.i. Similar to the time of seroconversion in our studies, comparisons of infection in cats by FIV-C-PGammer, FIV-A-Petaluma and FIV-$NCSU_1$ demonstrated that all the infected cats had seroconverted by 4 weeks p.i. (English et al., 1994; Pedersen et al., 2001; Yamamoto et al., 1988), while in another study, cats infected with FIV-PPR or FIV-B-2542 had seroconverted by 3 weeks p.i.(Burkhard et al., 2002). Studies by Burkhard et al. (2002) which compared provirus levels in PBMC DNA, demonstrated that PBMC proviral levels were lower in FIV-A-PPR infected cats at 3 and 6 weeks p.i., but were similar to those seen in cats infected with more pathogenic FIV-B-2542 isolate by 9 weeks p.i. Although we did not calculate viral titers, the increased frequency of virus isolations from all the infected cats, suggested an increase in the viral load in the peripheral blood by 9 weeks p.i.

In our studies, the timing of the inversion of the $CD4^+/CD8^+$ T cell ratio in the infected cats was found to depend on the amount of virus administered, with $CD4^+/CD8^+$ T cell ratio dropping below 1 by 11 weeks p.i for eight of the nine infected cats. This inversion of $CD4^+/CD8^+$ T cell ratio was accompanied in all cases by a corresponding decrease of absolute $CD4^+$ T cells for all the viral doses administered. The numbers of $CD4^+$ T lymphocytes did not revert to their pre-infection values, except for cat 3820 at 11 week p.i. A corresponding increase in the $CD8^+$ T cells could be demonstrated for all the cats though this increase was significant only for the cats infected with the highest dose of the virus as compared to the uninfected cats for all the times examined. Our

studies were similar to infection with FIV-A-GL_8 biological clone, FIV-A-$NCSU_1$ biological clone and FIV-B-2542, that led to a decline in the $CD4^+/CD8^+$ T cell ratio between 3 to 6 weeks p.i. due to decrease in $CD4^+$ and an increase in $CD8^+$ T cell numbers (Burkhard et al., 2002; English et al., 1994; Hosie et al., 2002). Although there were only three cats in each group in our study, and the timing of change in the neutrophil numbers or $CD4^+$ or $CD8^+$ T cell numbers was somewhat variable, strong trends were nonetheless evident.

The increase in the numbers of $CD8^+$ T lymphocytes was accompanied by an increase in a subset of $CD8^+$ T lymphocytes expressing low levels of the CD8β chain. Expansion of this population has also been demonstrated with experimental infection of cats with FIV-A-GL_8, FIV-$NCSU_1$ isolate, FIV-A- Petaluma, Swiss isolate of FIV (FIV Z2) and FIV-B-TM2 isolate (Bucci et al., 1998b; Lehmann et al., 1992; Shimojima et al., 1998; Willett et al., 1993). These $CD8\beta^{low}$ cells have been defined as $CD8\alpha^+\beta^{low}$, with the vpg9 and FT-2 antibodies used in the previous studies shown to recognize the CDαβ heterodimer and the β-chain of the αβ heterodimer, respectively (Shimojima et al., 1998). Although not examined in this study, Bucci et al. (1998b) and Shimojima et al. (1998) found that analysis of CD8 α chain expression using anti-CD8 α chain specific antibody did not show a reduction in the expression of the CD8 α chain similar to that seen for the β chain of CD8.

Our results are in agreement with those of Willett et al. (1993) in that development of cell associated viremia was followed by the appearance of $CD8\beta^{low}$ population of T cells. In agreement with their studies using FIV-A-GL_8 virus, the

$CD8\beta^{low}$ population of T cells in our studies expanded rapidly after infection, as early as 4 weeks p.i. In cats infected with the FIV-A-$NCSU_1$ virus, the $CD8\alpha^{+}\beta^{low}$ phenotype increased in cats before seroconversion during acute infection and became the predominant population in asymptomatic, long term infected cats (Bucci et al., 1998b). In our studies we observed a significant increase in the $CD8\beta^{low}$ population of T cells in the acute stage of infection, which increased significantly over time in all the FIV infected cats regardless of the viral dose administered. However, the correlation between the $CD8^{+}$ T cell antiviral activity against FIV and the presence of the unique $CD8\alpha^{+}\beta^{low}$ phenotype in cats has been controversial. Bucci et al. (1998b) have shown that the unique $CD8\alpha^{+}\beta^{low}$ and not the $CD8\alpha^{+}\beta^{high}$ phenotype, detected as early as 6 weeks p.i., correlated with decreased plasma and PBMC-associated viremia and was responsible for suppression of FIV-A-$NCSU_1$ in acute and long-term asymptomatic infection. In contrast, both $CD8^{high}$ and $CD8^{low}$ T cell subpopulations from PBMC of FIV-GL_8 infected cats were shown to suppress FIV replication *in vitro* (Flynn et al., 2002). Identifying whether $CD8\beta^{low}$ or the $CD8\beta^{high}$ population in our infected cats contributes to antiviral activity against FIV would be helpful in understanding the mechanism used by $CD8^{+}$ T cells from FIV-PPR cats in our system to suppress FIV-PPR replication *in vitro*. Previous studies from our laboratory have demonstrated an inverse correlation between the $CD8^{+}$ T cell mediated suppressing activity and the proviral load in cats chronically infected with the FIV-PPR strain (Unpublished data from our laboratory).

Crawford et al. (2001) demonstrated that $CD8\alpha^{+}\beta^{low}$ cells in the thymus of neonatal cats infected with FIV-A-$NCSU_1$ isolate, were linked to a proportional decline

in the $CD8\alpha^{+}\beta^{high}$ cells among a stable number of total CD8α cells, suggesting that $CD8\alpha^{+}\beta^{low}$ cells might be generated at the expense of $CD8\alpha^{+}\beta^{high}$ cells. In our studies, the percentages of $CD8\beta^{high}$ population were significantly decreased ($p < 0.05$) for cats infected with 2000 $TCID_{50}$ of the virus as compared to the control-uninfected cats across all the weeks examined. Since the decrease in the percentages of $CD8\beta^{high}$ population for cats 3823 and 3824 infected with 2000 $TCID_{50}$ of the virus coincided with their percentages of $CD8\beta^{low}$ population increasing, it is tempting to speculate that $CD8\beta^{low}$ population is increasing at the expense of the $CD8\beta^{high}$ population.

In conclusion, we have shown that infection of cats with FIV-TX53, which belongs to a newly emerging subtype, causes acute disease characterized by a decline of $CD4^{+}/CD8^{+}$ T cell ratios below 1 by 11 weeks p.i. This reduction was marked by rapid changes in the lymphocyte profiles of the cats, which included an expansion of the $CD8\beta^{low}$ population of $CD8^{+}$ T cells.

CHAPTER V

VARIATION IN THE V3-V4 ENVELOPE SEQUENCE FROM FELINE IMMUNODEFICIENCY PROVIRUS OBTAINED FROM BLOOD, SPLEEN AND LYMPH NODES OF CATS EXPERIMENTALLY INFECTED WITH THE FIV-PPR MOLECULAR CLONE

INTRODUCTION

A major obstacle in the development of a vaccine against human immunodeficiency virus (HIV) has been the high plasticity of its genome (Coffin, 1986; Temin, 1989). Lentiviruses, such as HIV and simian immunodeficiency virus (SIV), display a large degree of molecular and biological variation. This rapid evolution is generally attributed to the low fidelity of the viral enzyme reverse transcriptase (RT) in copying the viral genomic RNA to DNA (Mansky and Temin, 1995; Roberts et al., 1988). HIV-1 genome exhibits extensive sequence variation, both between patients, within different tissues of an individual patient and even within different regions of the same tissue (Ball et al., 1994; Casado et al., 2001; Chang et al., 1998; van't Wout et al., 1998; Visco-Comandini et al., 2001).

Feline immunodeficiency virus (FIV) has been reported worldwide with a prevalence rate ranging from 1-28% (Ishida et al., 1989; Yamamoto et al., 1989). FIV was first isolated from a cat with symptoms of an immunodeficiency like syndrome in

1986 (Pedersen et al., 1987). Like HIV-1, FIV can induce immunological abnormalities, including an inversion in the $CD4^+/CD8^+$ T cell ratios resulting from a decrease in $CD4^+$ T cells (Ackley et al., 1990; Hoffmann-Fezer et al., 1992; Novotney et al., 1990) and decreased lymphocyte proliferative responses to mitogens (Barlough et al., 1991; Siebelink et al., 1990; Torten et al., 1991). Such similarities in biological behavior between HIV-1 and FIV, support FIV infection of cats as a small-animal model to study the pathogenesis of lentivirus induced AIDS and as a model for the HIV vaccine development.

Similar to the envelope (env) gene of HIV, Pancino et al. (1993b) have identified nine variable regions throughout the FIV env gene. The first two are located in the leader region, which is also the first coding exon of the *rev* gene and are not present in the mature env protein because of proteolytic processing (Pancino et al., 1993b; Phillips et al., 1992; Verschoor et al., 1993). Variable regions V3 through V6 occur in the surface protein (SU), and variable regions V7 through V9 occur in the transmembrane protein (TM) (Pancino et al., 1993b). The V3 region of the SU in addition to the ectodomain of the TM glycoprotein (Vahlenkamp et al., 1997) has been found to be responsible for the tropism of FIV for Crandell feline kidney (CRFK) cells (Siebelink et al., 1995b; Verschoor et al., 1995). Macrophage tropism of FIV is dictated by the V3 and V4 regions of the env gene (Vahlenkamp et al., 1999). The V3 region also contains a linear neutralization domain (de Ronde et al., 1994; Lombardi et al., 1993b) that is involved in the neutralization of CRFK cell adapted FIV strains while V4 and V5 regions were found to be necessary for FIV neutralization in feline thymocytes (Siebelink et al.,

1995a). Cell culture adapted strains of FIV are able to use CXCR4 to facilitate cell fusion mediated through the V3 region (Willett et al., 1997b; Willett et al., 1997c).

An important feature of the FIV model is the availability of molecular clones that, after transfection into cells in culture, give rise to infectious virions (Hosie et al., 2002; Miyazawa et al., 1991; Phillips et al., 1990; Siebelink et al., 1992; Talbott et al., 1989). FIV derived in this manner can be used for experimental inoculation of cats and the fate of a single FIV clone, as it replicates over time in the infected host can be determined. The use of a molecularly cloned virus allows genetically homogenous viral populations to be obtained. This provides unique opportunities to study both viral evolution within a host and the generation of tissue specific variants by eliminating factors, such as a mixed inoculum of viral variants differing in their cellular tropism. Relating the biological properties of such populations to the genomic structure of the molecular clone, from which they were derived, could reveal the basis of biological variation and may identify determinants of viral virulence.

The level of intra-sample and intra-individual variation, of the FIV env gene during the course of infection and the effect of host factors, such as immune pressure on the extent of variation, can have a great influence on the pathogenesis of the disease caused by FIV (Dean et al., 1999; Hosie et al., 2002; Sodora et al., 1995; Sodora et al., 1994). In this chapter, results of a study involving V3-V4 env sequence variation among three tissues from six FIV infected cats have been presented.

MATERIALS AND METHODS

Experimental animals

Specific, pathogen-free (SPF) cats purchased, from Harlan Sprague-Dawley, Madison, WI or Liberty Laboratories, Liberty Corner, N.J., were serologically negative for feline leukemia virus. Cats were housed in a specific, pathogen-free environment at the Laboratory Animal Research and Resources Support Facility (LARR), Texas A&M University, College Station. Cats A306, A308, M153, M160, M165 and M242 had been experimentally infected i.v. with 50 $TCID_{50}$ (cats M153, M160, M165 and M142) or 250 $TCID_{50}$ (cats A306 and A308) of FIV-PPR molecular clone (Table 3) prepared as described elsewhere (Hokanson et al., 2000). The six cats were humanely euthanized 3 to 6 years post infection (p.i.) by intravenous (i.v.) injection of three milliliters of concentrated pentobarbital sodium solution (390 mg/ml). Prior to euthanasia, blood was collected in EDTA (K_3)-treated tubes. Complete blood counts were performed by Texas Veterinary Medical Diagnostic Laboratory, College Station, Texas. Lymph nodes and spleen tissues collected at necropsy were rapidly frozen and stored at -80^{o}C for subsequent DNA analysis.

Antibodies

Monoclonal antibodies (Mab) to feline CD4 conjugated to phycoerythrin (PE) (Southern Biotechnology Associates, Birmingham, AL) and Mab to feline CD8β chain conjugated to fluorescein isothiocyante (FITC) (Southern Biotechnology Associates, Birmingham, AL) were purchased commercially.

Table 3

History of the cats infected with the FIV-PPR molecular clone

Cats	FIV Dose $TCID_{50}$	Sex	Age at euthanasia	Year Infected
M153	50	F	4 years 5 months	2000
M160	50	M	4 years 5 months	2000
M165	50	F	4 years 5 months	2000
M242	50	M	4 years 5 months	2000
A306	250	M	6 years	1997
A308	250	M	6 years	1997

Phenotypic characterization of freshly prepared peripheral blood mononuclear cells (PBMC)

PBMC from FIV infected cats A306, A308, M160, M165 and M24 were isolated from EDTA (K_3)-treated whole blood by Histopaque-1077 (Sigma, St. Louis, MO) density gradient centrifugation (Choi et al., 2000b) and washed once with cold phosphate-buffered saline (PBS) containing 0.1% sodium azide. Prior to staining of the cells, the Fc receptors on the cells were blocked by incubating the cells for 10 minutes on ice with goat anti-IgG (2 mg/ml) (Sigma, St. Louis, MO). PE conjugated Mab to feline CD4 and FITC conjugated Mab to feline CD8 β chain were then added to the cells in the respective tubes at a final dilution of 1:50. After incubating for 30 minutes on ice, the cells were washed once with cold PBS-0.1% sodium azide and centrifuged at 1200 rpm for 10 minutes (Jouan Inc. Winchester, VA). Negative controls consisted of unstained cells. After washing the cells, 300 ul of 2% formaldehyde were added to the tubes, which were wrapped in an aluminium foil and kept at 4^oC until they were examined the next day with a FACscaliber flow cytometer (Becton-Dickinson, San Jose, CA) at the Veterinary Pathobiology Core Facility, (Director, Dr. Roger Smith, Texas A&M University, College Station).

Genomic DNA isolation

Feline PBMC were isolated from EDTA (K_3)-treated whole blood by Histopaque-1077 (Sigma, St. Louis, MO) density gradient centrifugation (Choi et al., 2000b). Genomic DNA was extracted from PBMC using QIAmp DNA blood mini kit

(Qiagen Inc., Valencia CA). Genomic DNA was also extracted from lymph nodes and spleen tissues using the mammalian genomic DNA isolation miniprep kit (Sigma, St. Louis, MO).

Cloning of the FIV env V3 through V4 regions

FIV V3-V4 env gene region was amplified by nested PCR from genomic DNA extracted from lymph node and spleen tissues and PBMC of the FIV infected cats. The first round PCR used primers 6782F (5'-GCTCAGATAGTATGGAGACTTCC-3') and 8814R (5'- ACTCCATCATTCCTCCTCTT-3') that amplified an 2051bp env product. 5 ul of the first round PCR reaction were used as a template for the second round PCR that used primers 7313F (5'-ATACCAAAATGTGGATGGTG-3') and 7844R (5'-CAAGACCA ATTCCCAGCAAT-3'), which amplified an 551 bp env product spanning the V3-V4 region. Primers used in this study were made by IDT, Inc. (Coralville, IA). The following parameters were used for the primary amplification of the env gene product using a GeneAmp 2400 (Perkin Elmer, Norwalk, CT): 94^{o}C for 5 min, followed by 5 cycles of 94^{o}C for 60s, 53^{o}C for 60s and 72^{o}C for 2 min., and finally 30 cycles of 94^{o}C for 15s, 53^{o}C for 45s and 72^{o} C for 2 min. The annealing step was increased by 0.1^{o}C for each of the 30 cycles. The reaction was held at 72^{o}C for 15 min followed by 4^{o}C for infinity. The thermal cycling parameters for the secondary amplification were identical to the primary amplification. After purification of the PCR product, using the GenElute PCR clean-up kit (Sigma, St. Louis, MO), the samples were cloned into pSTBlue-1 vector (Novagen, Madison, WI) and transformed into Novablue Singles

competent cells (Novagen, Madison, WI). Recombinant plasmids were checked for the presence of insert by colony PCR according to the instructions in the Novagen Acceptor Vector kit manual (Novagen, Madison, WI). The plasmids containing the inserts were then amplified and purified using the GenElute Plasmid miniprep kit (Sigma, St. Louis, MO.).

DNA Sequencing

Three plasmids were sequenced for each tissue examined from each cat. DNA sequencing was carried out using the dye terminator cycle sequencing method (Gene technologies Core facility, Texas A&M University). The following thermal cycling steps were used for the extension of the V3-V4 product for 45 cycles: 96^{o}C for 10 sec, 50^{o}C for 5 sec and 60^{o}C for 4 minutes. All PCR reactions were performed using a GeneAmp PCR system 2400 thermal cycler (Perkin Elmer, Norwalk, CT). After purification of the product by spin column purification protocol, the samples were sent to the Gene Technologies core facility for DNA sequencing (Department of Biology, Texas A&M University, College Station, Texas). The clones were sequenced bi-directionally using the T7 promoter sequence as the forward primer and the SP6 promoter sequence as the reverse primer.

Sequence alignment and phylogenetic analyses

Nucleotide sequences were aligned using the CLUSTAL W multiple sequence alignment program (Thompson et al., 1994b) of SDSC biology workbench. All aligned sequences were also inspected manually to correct for apparent mistakes. Positions containing gaps or ambiguously aligned positions were removed from the data set. Phylogenetic trees were established by the neighbor-joining method (Saitou and Nei, 1987), as well as the maximum likelihood method with similar branching patterns. The maximum likelihood method was implemented using PAUP* (D. L. Swofford, PAUP 4.0, version 4.0b10) and neighbor-joining method was implemented using MEGA version 2.1 (Kumar et al., 1994). The statistical value of the tree was evaluated by bootstrap analysis of 1000 replicas. Pairwise protein and nucleic acid sequence distances were calculated using the CLUSTAL W multiple sequence alignment program of SDSC biology workbench.

RESULTS

Clinical history of the cats

As indicated in Table 4, the $CD4^+$/ $CD8^+$ T cell ratios at the time of euthanasia ranged from 0.2-1.75 for the six cats, which were below the values observed normally for FIV uninfected cats. CBC analysis demonstrated few poikilocytosis of the red blood cells (presence of abnormally shaped red blood cells) for cats M153 and M160. The total number of lymphocytes from the six FIV infected cats was below average (Table 4). The neutrophil number was also below average for cats M153, M160 and A308 (Table 4).

Data from uninfected cats suggest that in general, $CD4^+$ T cell counts below 200 cells/ul are abnormally low (Sodora et al., 1994). According to this criterion, except for cat M165, all cats had abnormally low $CD4^+$ T cell levels at euthanasia (Table 4).

Table 4

Immunological and hematological characteristics of FIV-PPR infected cats at euthanasia

Cats	$CD4^+/CD8^+$ T cell ratio	Absolute number of cells per ul			
			T cells		
		Lymphocytes	$CD4^+$	$CD8^+$	Neutrophils
M153	0.41	728	139	336	1560
M160	0.2	800	26	130	2208
M165	1.24	1113	226	183	3551
M242	1.75	1450	122	70	N.D.
A306	1.3	279	72	56	2573
A308	0.38	713	91	238	1380

Normal $CD4^+/CD8^+$ T cell ratio in FIV uninfected cats > 2

Normal lymphocyte count = 1500-7000 cells/ ul

Normal $CD4^+$ T cell count > 200 cells/ ul

Normal neutrophil count = 2500-12500 cells/ ul

N.D. = not done

Records maintained at LARR indicated that cat A306 had mild gingivitis a few days before euthanasia and cat M242 had demonstrated extreme neutropenia and vomiting a year before it was euthanised. Cats A306 and A308 had also demonstrated vomiting, and diarrhea was noted for A308 two months before they both were euthanised. At the time of necropsy, cat A308 demonstrated signs of enteritis while cat M153 showed a brain lesion.

Sequence analysis

The V3-V4 env region was amplified as a 551bp product from lymph nodes, spleen and PBMC of all the six cats, except from PBMC of cat M153. Nucleotide sequences of the env V3-V4 region were obtained from the PCR amplified products. The viral variants were assigned numbers according to the number of the bacterial clone containing the env insert, followed by the letter indicating the organ from which the clones were obtained (L = lymph node, P = PBMC, S = spleen) followed by the name of the cat. Hence 3P M165 would describe a plasmid clone # 3 containing the env insert derived from PBMC of cat M165.

There were a few mutations observed among the viral variants from the three tissues, most of them being point mutations. The percentage identity (Clustal W program) of these sequences and their translated products was found to be between 97-100% for all cats except cats A308 and M160 (Table 5). V3-V4 env sequences from cats A308 and M160 demonstrated greater amino acid variation as compared to the nucleotide sequence variation in their env sequence. The percentage identity of the

Table 5

Variation in the V3-V4 envelope sequences obtained from tissues of six cats

Cats	Tissues	% identity with the original V3-V4 env sequence	
		nucleic acid	amino acid
M153	PBMC	Not done	Not done
	Lymph nodes	98- 99%	97- 98%
	Spleen	99- 100%	98- 100%
M160	PBMC	99%	98%
	Lymph nodes	97- 99%	91- 98%
	Spleen	98- 99%	96- 98%
M165	PBMC	98- 99%	97- 98%
	Lymph nodes	99%	97- 100%
	Spleen	99%	97- 99%
M242	PBMC	98- 99%	98- 99%
	Lymph nodes	98- 99%	97- 99%
	Spleen	99%	97- 98%
A306	PBMC	98- 99%	96- 99%
	Lymph nodes	99%	98- 99%
	Spleen	99%	98- 99%
A308	PBMC	96- 99%	91- 98%
	Lymph nodes	97- 98%	93- 97%
	Spleen	97- 98%	91- 97%

translated products was between 91-98% for cat A308. For cat M160 the amino acid sequence of one of the clones derived from the lymph node (1L M160) was only 91% identical to the original FIV–PPR V3- V4 env sequence (Table 5).

Determinants reported for CRFK tropism include an E → K change at position 407 and R at position 397 in the V3 region of the FIV env gene which is seen in the FIV-Petaluma molecular clone 34TF10 but not in the FIV-PPR clone or the FIV-Petaluma clone FIV-14 (Table 6) and an E → K change at position 409 which is observed in both the Petaluma clones but not in the FIV-PPR clone (Table 6) (Verschoor et al., 1995). The FIV-PPR molecular clone has an E at position 407 (Phillips et al., 1990) and this was not changed in any of the clones examined. Clone 6P from cat M165 had a K → E change at position 397 while 3P from the same cat had an E → K change at position 409 (Table 6). Clones 2S A308 and 5P A308 had a K → R change at position 397 (Table 6). Additionally, clones 4L from M160 and clone 1L from cat A306 had a replacement of E → G and E → D, at position 409, respectively (Table 6). With the exception of E (hydrophilic) → G (amphiphilic) change for clone 4L M160, all the other amino acid changes were conserved with respect to the nature of the changed amino acid. However, since these changes were observed in only one of the three clones from the specific tissue, it is difficult to conclude on their relevance to the tropism of these viral variants.

Among the nine cysteine residues present in the V3-V4 region sequenced, seven cysteines were conserved in all the clones examined. Interestingly, two clones from the lymph nodes of two cats, A306 and A308 had C→ R alteration at position 366 while a C→ Y change at position 417 was seen in clones 7S A308 and 5L A306 (Table 7).

Table 6

Mutations at sites involved in Crandell feline kindney cell tropism

V3- V4 Region of the FIV envelope gene				
Names of clones	Position 397	Position 407	Position 409	Tropism
FIV-PPR	**K**	**E**	**E**	**PBMC**
FIV- 34TF10 (FIV- Petaluma clone)	R	K	K	CRFK
FIV-14 (FIV-Petaluma clone)	R	E	K	CRFK, CXCR4 tropic
4L M160	K	E	G*	Unknown Tropism
1L A306	K	E	D	
3P M165	K	E	K	
6P M165	E	E	E	
2S A308	R	E	E	
5P A308	R	E	E	

* E (Hydrophilic) → G (Amphiphilic) change

Table 7

Change in cysteine residues

Names of clones	Mutation
3L A308	C 366 R
7S A308	C 417 Y
2P A306	C 417 Y
5L A306	C 366 R

Among the 8 potential N- linked glycosylation sites present in V3-V4 region sequenced, only 3 sites were completely conserved among all the clones examined (Table 8). Interestingly, clone #5 from the PBMC and clone # 2 from the spleen of cat A308 had point mutations at two potential N- linked glycosylation sites along with a change K→ R at position 397 (Tables 6 and 8). Clone # 2 from PBMC of cat A306 also had point mutations at two potential N- linked glycosylation sites along with a C→ Y change at position 417 (Tables 6 and 8). Both of these cats had been initially infected with virus 250 $TCID_{50}$ of the virus.

Except for the clones mentioned above, all the remaining clones from the tissues of the six cats retained the original sequence as the parental FIV-PPR clone.

Phylogenetic analysis

As demonstrated for other lentiviruses such as SIV, equine infectious anemia virus (EIAV) and caprine-arthritis encephalitis virus (CAEV) (Hotzel et al., 2002; Leroux et al., 1997; Zheng et al., 1997), the V3-V4 env region of the FIV-PPR strain in this study was assumed to have diverged from the parental FIV-PPR molecular clone used for infection, maybe resulting in tissue specific variants. Fig.16 A-F demonstrate unrooted phylogenetic trees of V3-V4 env nucleotide sequences from the tissues of six cats as compared to the V3-V4 nucleotide sequence of the parental FIV-PPR molecular clone. Contrary to our assumption, phylogenetic analysis of all the sequences for cats M153 (Fig. 16A), M160 (Fig. 16B) and M242 (Fig. 16D), indicated that for a single cat, the sequences derived from the PBMC, lymph node or spleen were similar enough to

Table 8

Change in potential N-linked glycosylation sites

Position and original sequence	Changed sequence	Variant name
418- 420 NST	NGT	7S M165
	NIT	5P A308
	NSA	2P A306
	NSP	5S M242
422- 424 NLT	**CONSERVED**	
448- 450 NKS	DKS	1S M242
	DKS	2L M165
	SKS	2P A306
	KKS	5P A308
	KKS	2L A308
	KKS	1L M153
	KKS	2S A308
469- 471 NTS	NAP	7P A306
	NTL	2L M242
481-483 NVS	DVS	4L M153
	KVS	4L M165
	KVS	4P M160
	NIS	5P M242
	NAS	2S A308
491-493 NCS	**CONSERVED**	
518-520 NMT	DMT	5L A306
531- 533 NWS	**CONSERVED**	

cluster together in the unrooted tree, without showing any tissue specific variants. For cat M165 (Fig. 16C), the sequences from the PBMC did cluster together (bootstrap value, 65%) whereas for cat A306 (Fig. 16E), the sequences from spleen clustered together (bootstrap value, 61%). However, these sequences were found to be approximately 98-99% identical with the original sequence (Table 5) when their amino acid sequences were compared. The V3-V4 env sequences from the tissues of cat A308 (Fig. 16F) demonstrated more mutations, although no tissue specific variants were observed.

FIV-PPR belongs to clade A with an an overall nucleic acid sequence identity of 91% and an env diversity of 14% as compared with the Petaluma strain from the same clade (Phillips et al., 1990). When the V3-V4 env nucleotide and amino acid sequences of representative clones for each tissue and each cat were compared to that of FIV-Petaluma and FIV-PPR env sequences, using neighbor-joining method of computing divergence, all the sequences clustered together, with FIV-Petaluma being the outlier (Fig. 17 and 18). This shows that the viral env sequences from the three tissues remained relatively unchanged as compared to FIV-PPR even after three to six years p.i.

A.

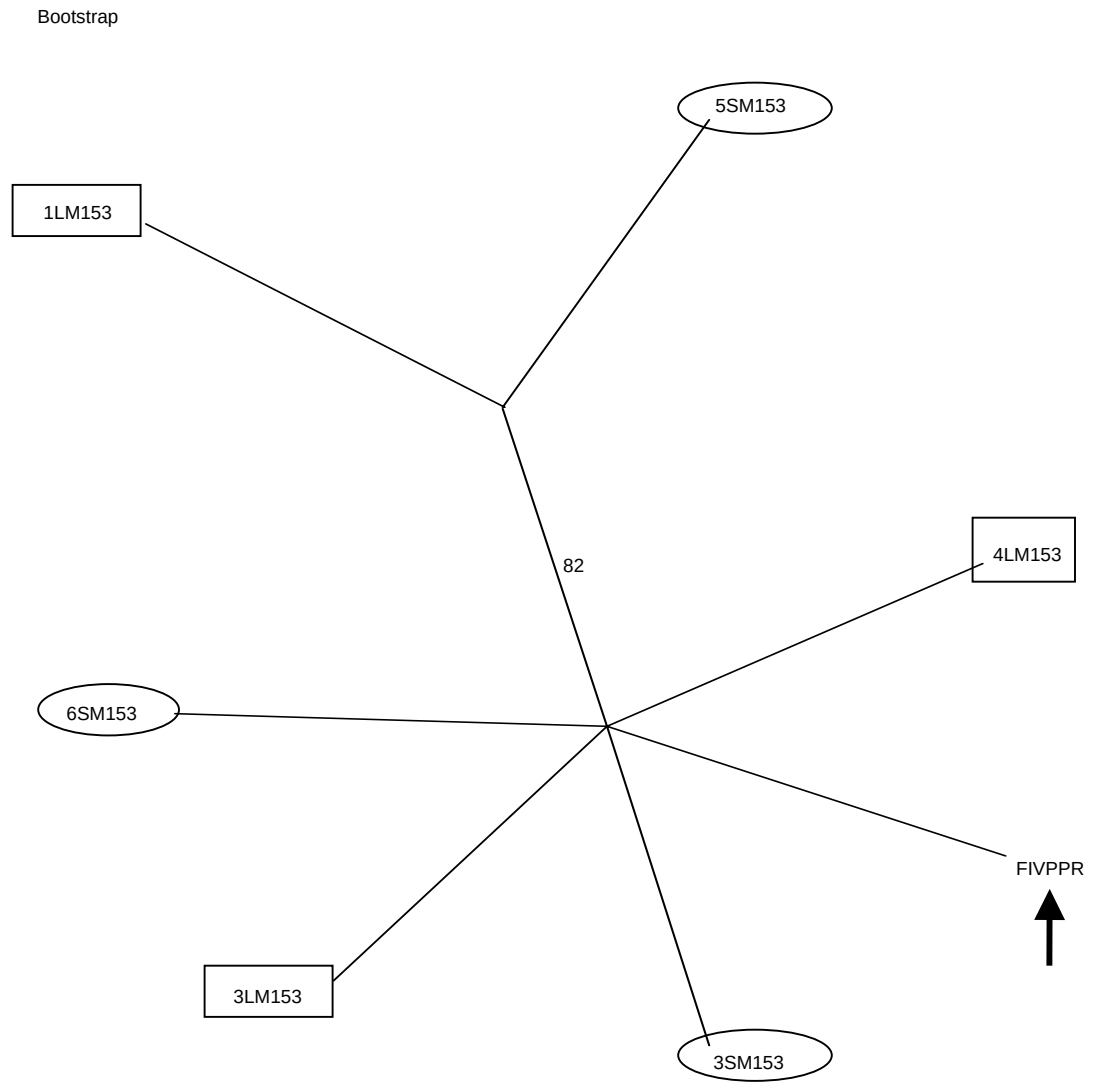


Fig. 16. Unrooted maximum-likelihood trees of V3-V4 envelope nucleotide sequences of viral variants from tissues of six FIV- PPR infected cats. Sequences from PBMC (designated as a hexagon), lymph node (designated as a rectangle) and spleen (designated as an oval) of cats M153 (Fig. 1A), M160 (Fig. 1B), M165 (Fig. 1C), M242 (Fig. 1D), A306 (Fig. 1E) and A308 (Fig. 1F) are shown. The number above each branch is the percentage of bootstrap replications supporting that branch. The single arrow points to the V3-V4 nucleotide sequence of the parental FIV-PPR clone. The viral isolates were assigned numbers composed of the number of the bacterial clone containing the env insert, followed by the letter indicating the organ from which the clones were obtained (L = lymph node, P = PBMC, S = spleen) followed by the name of the cat.

B.

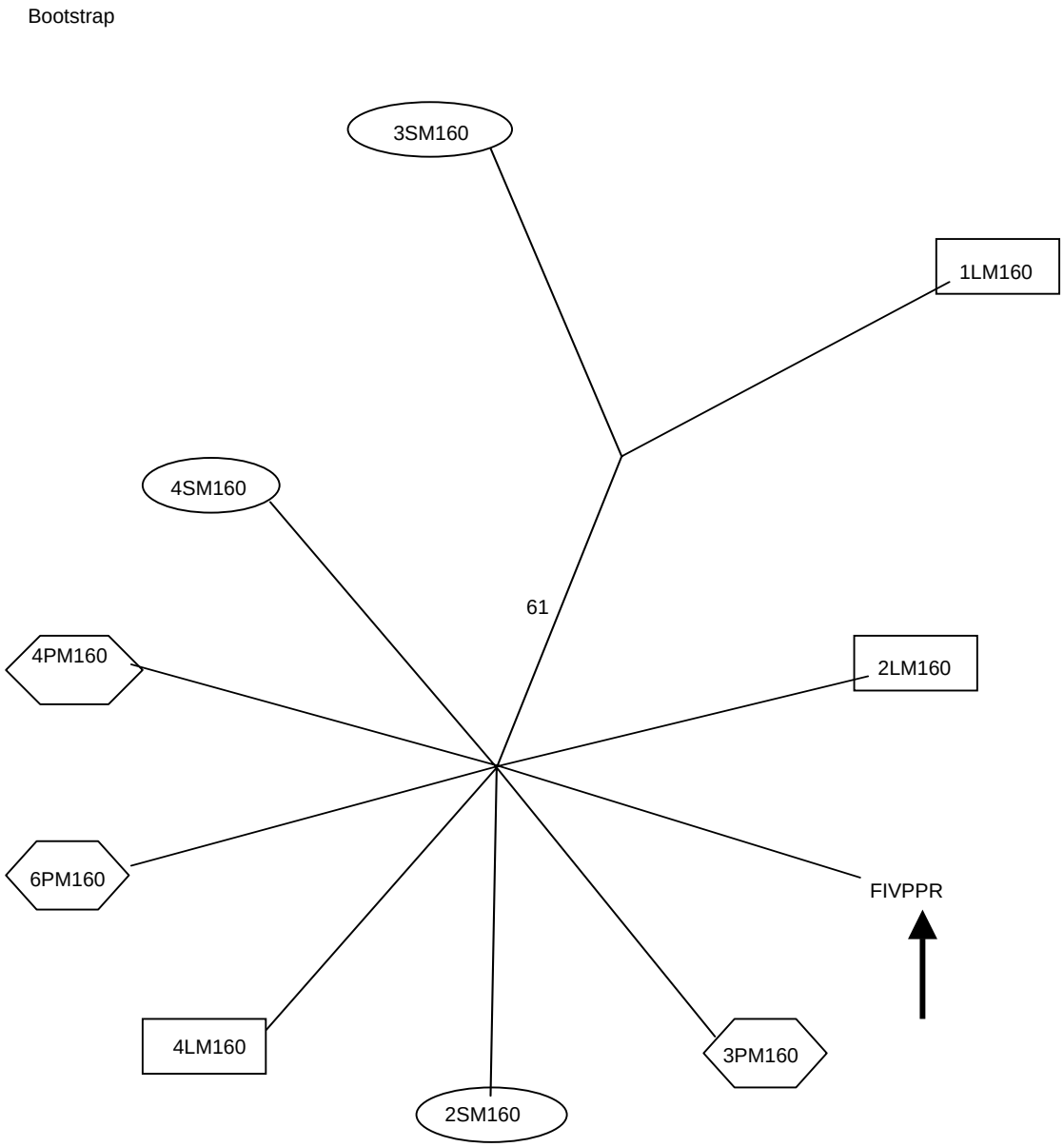


Fig. 16. Continued

C.

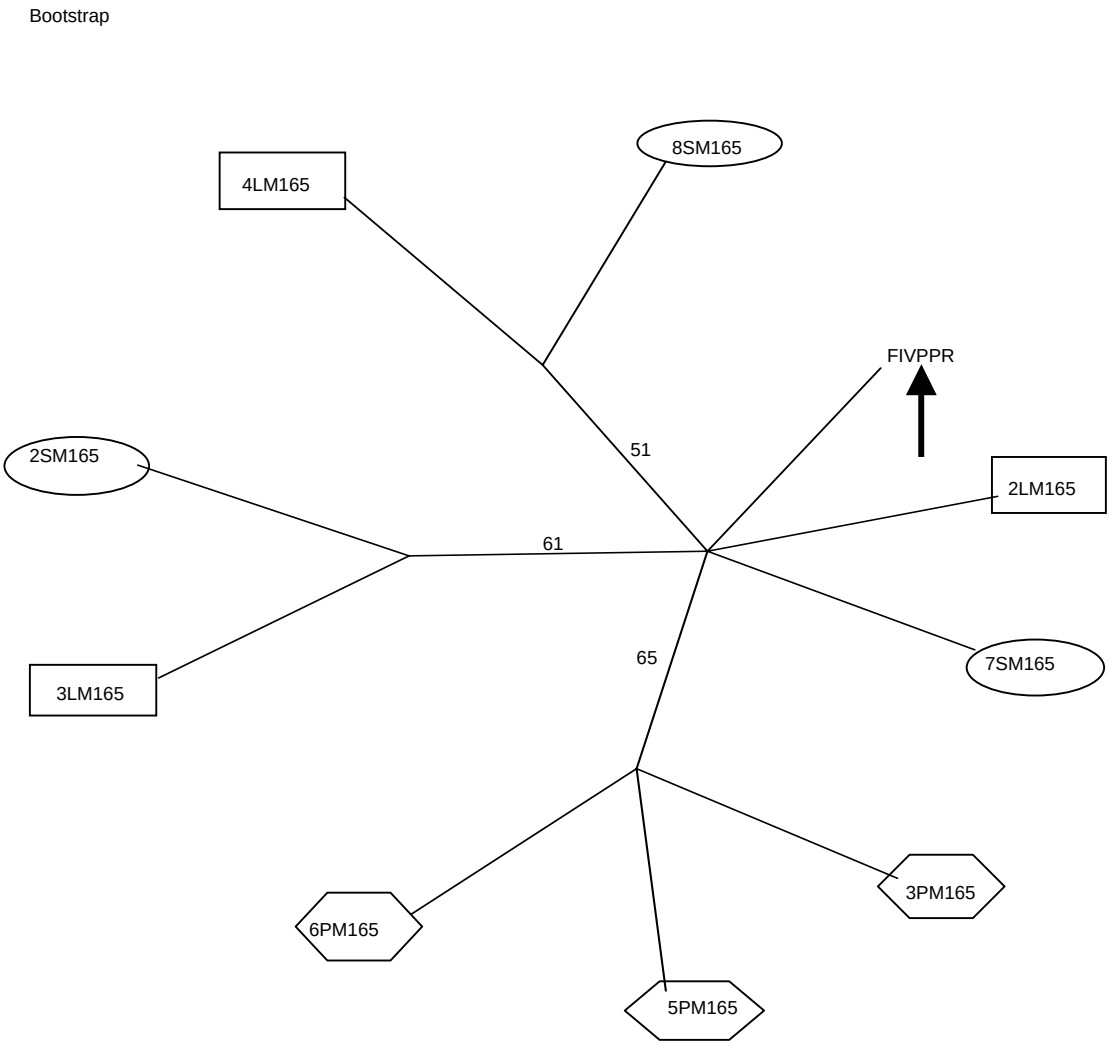


Fig. 16. Continued

D.

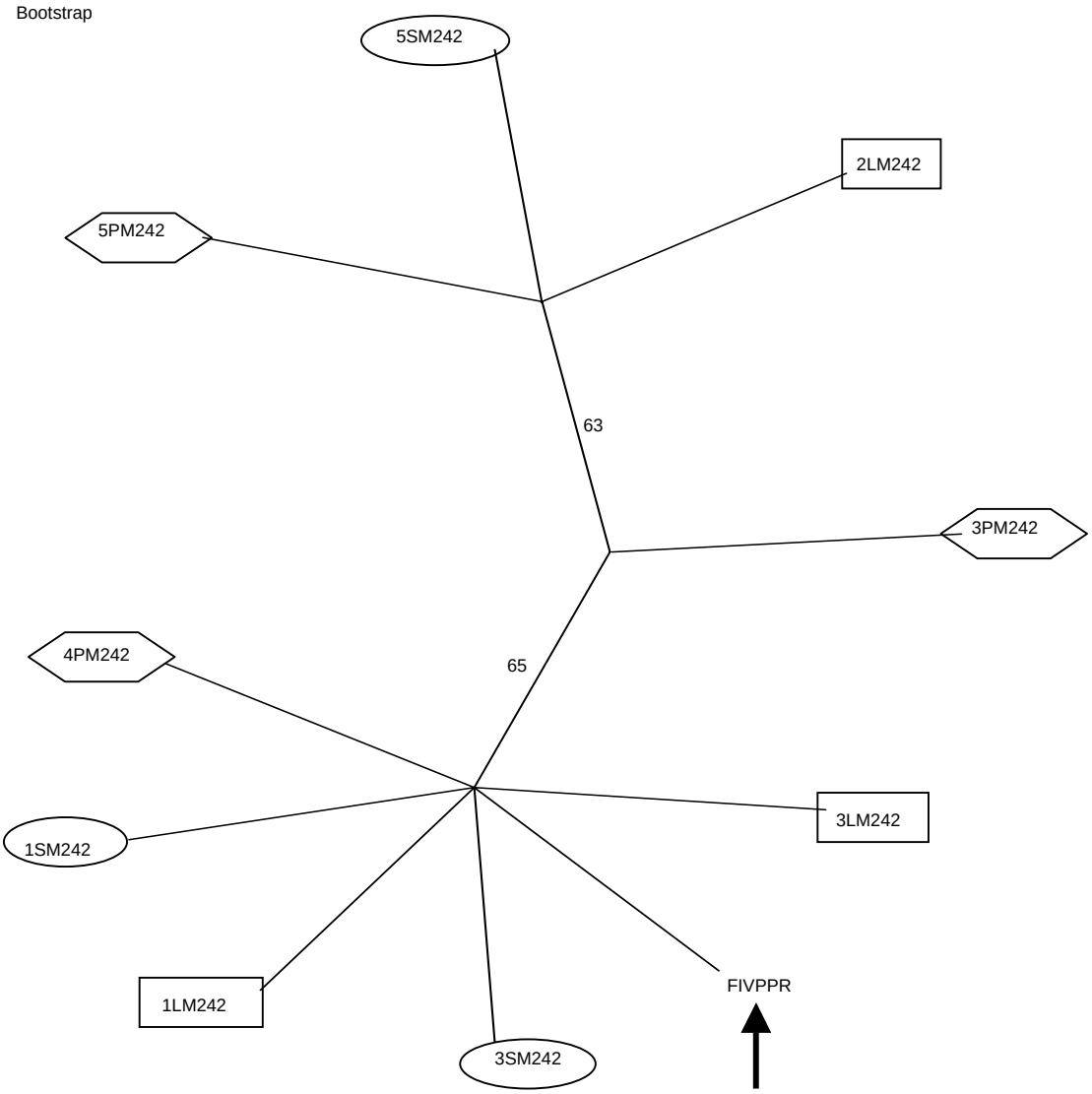


Fig. 16. Continued

E.

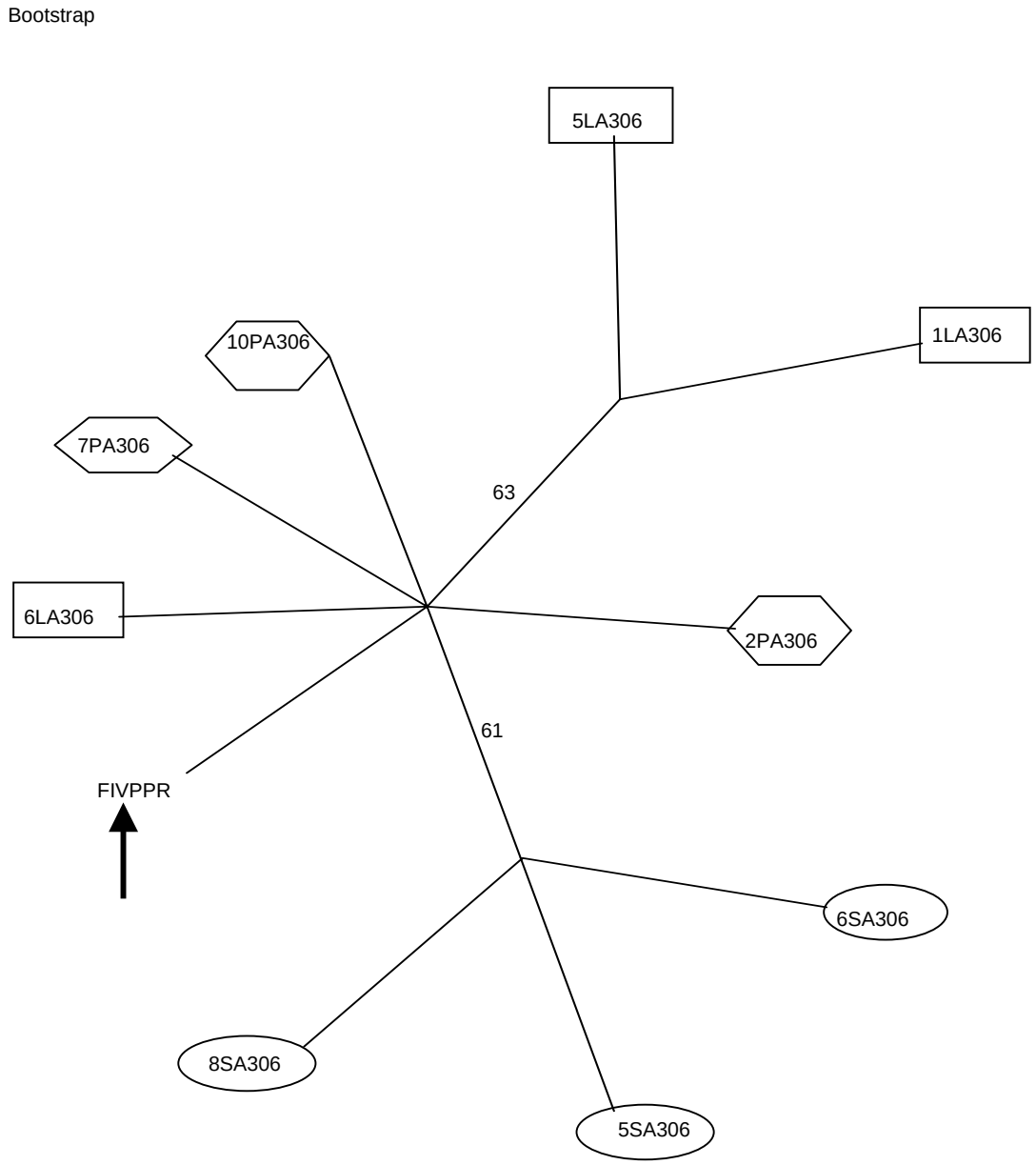


Fig. 16. Continued

F.

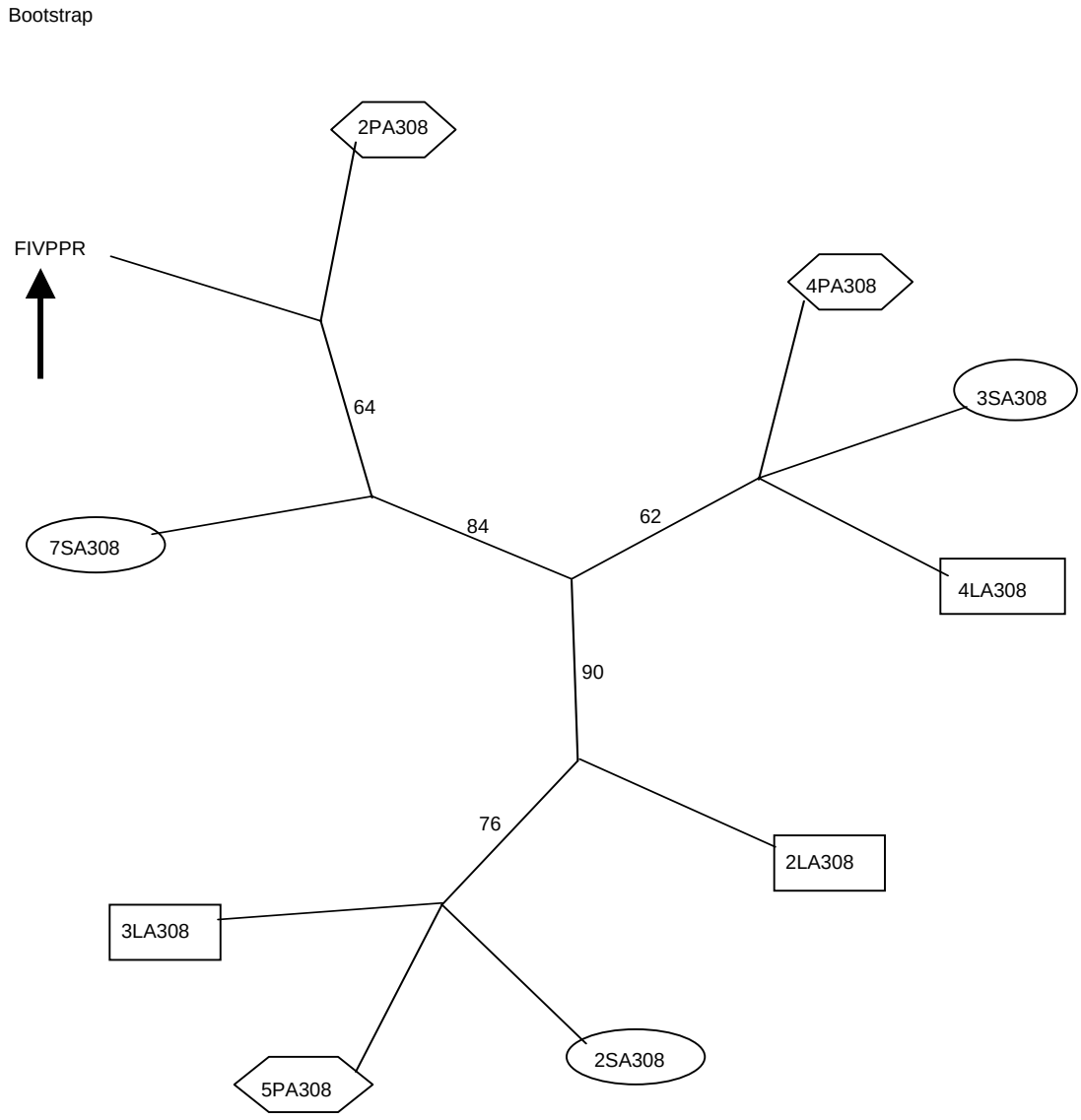


Fig. 16. Continued

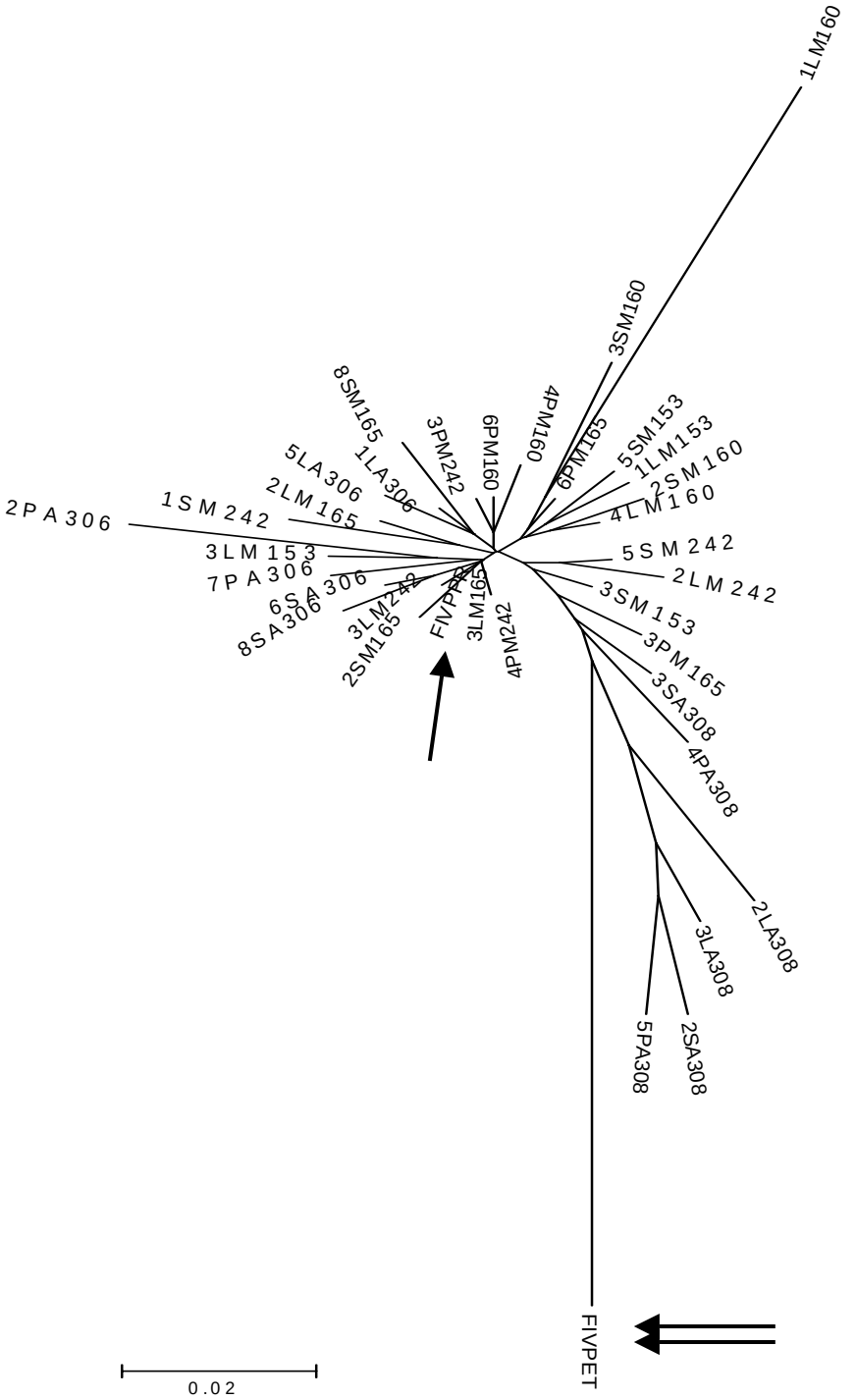


Fig. 17. Neighbor-joining phylogenetic tree of V3-V4 envelope nucleotide sequences of viral variants from lymph nodes, spleen and PBMC of six FIV-PPR infected cats. The single arrow points to the parental FIV-PPR clone used to infect the cats while the double arrow points to an outlier, FIV-Petaluma strain belonging to the same clade A as FIV-PPR.

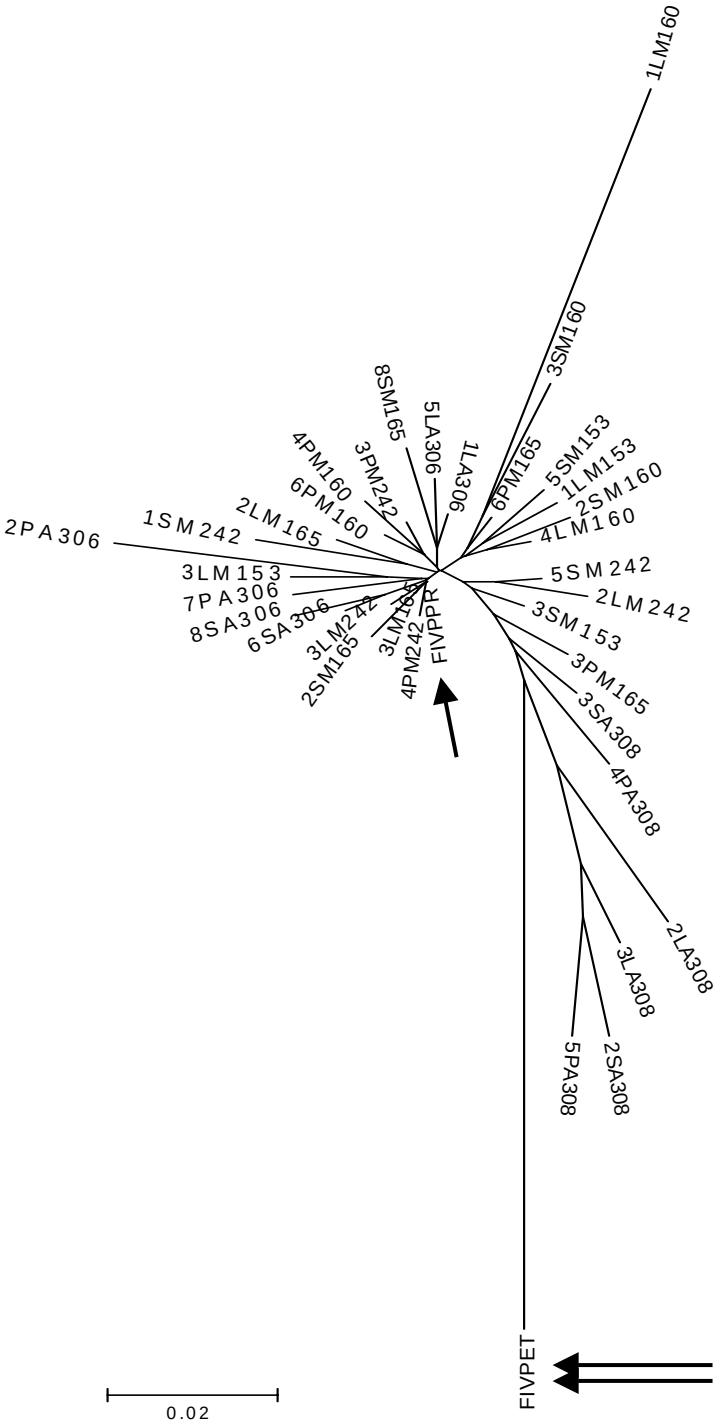


Fig. 18. Neighbor-joining phylogenetic tree of V3-V4 envelope amino acid sequences of viral variants from lymph nodes, spleen and PBMC of six FIV-PPR infected cats. The single arrow points to the parental FIV-PPR clone used to infect the cats while the double arrow points to an outlier, FIV-Petaluma strain belonging to the same clade A as FIV-PPR.

DISCUSSION

Unexpectedly, sequencing of the V3-V4 env region of provirus from PBMC, lymph nodes and spleen of cats infected with a molecular clone of FIV-PPR did not give rise to tissue specific viral variants. Surprisingly, our studies have demonstrated an almost uniform virus population in the three tissues examined from cats M153, M160, M165, M242 and A306. However, viral env sequences from the three tissues of cat A308 did demonstrate 7-9% divergence from the env sequence of the original FIV-PPR clone when the amino acid sequences were examined. In none of the groups was there any evidence of an accumulation of mutations over time leading to a change in the genotype of the quasispecies.

Genetic diversity arising as a result of evolving quasispecies or due to recombination between dually infected FIV strains has been reported (Bachmann et al., 1997; Carpenter et al., 1998). Rigby et al. (1993) have suggested that positive selection for protein sequence changes operates in the variable region of FIV env gene. Although there has been a rapid increase in available FIV sequences particularly for the env gene, there have been very few studies focusing on the rate and nature of env sequence evolution within a single animal. The few studies reported were contradictory depending on the env region sequenced and whether the animals were naturally or experimentally infected. Phylogenetic studies based on sequencing the entire FIV env region by Sibelink et al. (1992) and sequencing the V3-V6 FIV env region by Nishimura et al. (1996), using tissues of naturally infected cats demonstrated more than 98% sequence similarity between the env viral sequences. Kohmoto et al. (2003) found that, the virus was

transmitted across the mucosal epithelium without broadening of cell tropism, after sequencing a 642-bp V3-V5 env region from PBMC of FIV-B-TM2 vaginally infected cats, at an early stage of infection (4 week p.i.). In another study, three of the six cats infected with FIV- 34TF10, one of the six cats infected with FIV-14 and two of the six cats infected with FIV-PPR clone were shown to be infected with variants in the bone marrow and lymphoid tissues identified by heteroduplex mobility assays (HMA) (Dean et al., 1999). However, phylogenetic analysis was not performed on the variants. In addition, Hosie et al. (2002), demonstrated emergence of 2 variant viruses with increased virulence in two of the three cats infected intraperitoneally with FIV-14. These virulent variant strains carried a mutation at position 409 in the env region (K →Q or K →E) that reduced the net charge of the V3 loop, reducing the ability of the env proteins to induce fusion in CXCR4 expressing cells. They have therefore, suggested that the E →K mutation at position 409 contributes to attenuation of the FIV-14 strain.

In our study, clone # 3 from the PBMC of cat M165 did show an E →K mutation at position 409, which was found to be required for change of tropism to CRFK cells. However, there were no mutations associated with position 397 and 407 of the env V3 region for the same clone. Based on studies by Hosie et al. (2002), it is possible that the E →K change in 3P M165 was associated with an attenuation of the virus virulence. However, since these changes were observed in only one of the three clones from the specific tissue, it is difficult to conclude on their relevance to the tropism or virulence of these viral variants.

Our studies are in agreement with those of Kohmoto et al. (2003), in that there was no broadening of cell tropism associated with any of the FIV-PPR viral variants obtained from the three tissues, except for the clones mentioned in table 4A, whose tropism was unknown. Our studies were in contrast to those of Nishimura et al. (1996) in that the cysteines and the potential N-linked glycosylation sites in the V3-V4 region were not conserved among the viral variants obtained from the tissues of chronically infected cats. Our studies are also, in agreement with those of Siebelink et al. (1992) and Nishimura et al. (1996) in that the emerging variants in the tissues examined were very similar to the parental molecular clone used for initial infection. Overall, lack of variation in the FIV V3-V4 env region from tissues within each animal in our study was unexpected, considering that other lentivurses such as HIV, SIV, and caprine arthritis enteritis virus (CAEV) show variation in their env gene (Coffin, 1986; Hotzel et al., 2002; Johnson et al., 1991; Leroux et al., 1997; Zheng et al., 1997).

In contrast, Greene et al. (1993) examined the *in vivo* evolution of FIV isolates obtained sequentially from a naturally and persistently FIV-infected cat over a 3-year period. Their results indicated that the variation in FIV was similar to the pattern and rate of variation found in other lentiviruses including HIV-1. Sequencing subgenomic regions of the FIV gag (p15/p24), polymerase (RT) and 5' segment of the env gene coding for an N terminal region of gp120 spanning the first and second variable domains (V1-V2), they found that the major capsid protein and RT enzyme of polymerase were highly conserved as opposed to the env region which had relatively rapid and extensive variation in the order of 10^{-3} nucleotide substitutions/ site/ year, an order of magnitude

more than variation calculated for the gag and RT sequences (10^{-4} nucleotide substitutions/ site/ year). However, the V1-V2 FIV env region is located in the amino-terminal leader and signal regions and is not present in the mature env protein due to proteolytic processing (Pancino et al., 1993b; Verschoor et al., 1993). The FIV V1-V2 env region has also not been reported to be responsible for cellular tropism of FIV. Hence changes reported in that region might not be responsible for altering the pathogenicity of the variants.

Four of the six cats (cat A306, A308, M165 and M242) that were examined for the presence of variation in the V3-V4 env region, previously have demonstrated $CD8^+$ T cell mediated non-cytolytic activity against FIV *in vitro*, described in chapter II (Choi et al., 2000b; Unpublished data from our laboratory). *In vivo*, such control of virus replication would lead to fewer mutations being incorporated in the virus due to limited viral replication cycles. This would also fail to drive the host immune responses to exert selective immune pressure that would contribute to the lack evolution of viral variants observed in our study. Moreover the SPF facility used to house the cats also might have reduced the selection pressure imposed by the host immune system on the virus.

The genetic diversity of the virus in the infected host arises from competition and selection among variants and results in altered cell tropism and replication efficiency. However, the level of genetic diversity of FIV genomes in infected cats is 2-fold lower than that of HIV-1 genomes in humans (Siebelink et al., 1992; Sodora et al., 1994). Additionally, a molecular clone of FIV–PPR was used for the initial infection of the six cats in our study, which eliminated effects of the initial diverse viral inoculum,

narrowing the biological phenotypes of virus available for persistence in the host. Moreover, the FIV-PPR molecular clone used in these studies was found to be minimally pathogenic after inoculation of SPF cats, as compared to the biological clone of FIV-PPR from which it was derived (Sparger et al., 1994). However, studies using pathogenic SIV molecular clones have demonstrated the presence of tissue specific variants (Buckner et al., 2002; Johnson et al., 1991) in the infected animals. Thus, lack of env V3-V4 variation observed in our studies reemphasizes the fact that presence of diversity and virulence of the initial virus inoculum is responsible for generating viral variants within the host.

HIV viral heterogeneity is correlated with both the length of the asymptomatic period following primary infection and to a slower $CD4^+$ T cell decline (Delwart et al., 1997; Wolinsky et al., 1996) with exceptions (Markham et al., 1998), while a relative stable evolutionary stasis of viral variants is associated with both rapid loss of $CD4^+$ T cells and progression to AIDS. In our study, except for cat M165, all other cats had $CD4^+$ T cell counts lesser than 200 cells/ul. Hence, lack of variation in the V3-V4 env region, in our study is therefore surprising, considering that the cats were long–term infected, showed signs of some clinical illness and immunosuppression.

In conclusion, our results demonstrated an almost uniform population of viral variants from the three tissues of six cats chronically infected with the FIV-PPR molecular clone with no evidence of tissue specific variants.

CHAPTER VI

SUMMARY AND DISCUSSION

The critical role of $CD8^+$ cytotoxic T cells (CTLs) in the suppression of HIV has been suggested by the close relation between emergence of HIV specific CTL response and down regulation of viremia after acute infection, inverse correlation between the frequency of HIV-specific CTLS and levels of plasma load and the $CD4^+$ T cell decline, the association of vigorous HIV-specific CTL response with slow progression of disease and the decline of HIV CTL activity with disease progression (Cohen and Fauci, 1996). However, in 1986, another type of antiviral activity against HIV was described that involved suppression of HIV replication by $CD8^+$ T cells from asymptomatic, HIV infected individuals that did not involve killing the target cells (Walker et al., 1986). Subsequent studies have shown that one or more soluble molecule (s) secreted by $CD8^+$ T lymphocytes mediate this non-cytolytic anti-viral activity (Copeland, 2002; Levy, 2003; Moriuchi et al., 1996; Walker et al., 1986). Several groups also described a similar kind of antiviral activity for FIV, although the mechanisms of activation and conditions for viral suppression vary with the methodologies (Bucci et al., 1998a; Bucci et al., 1998b; Choi et al., 2000b; Flynn et al., 1999; Flynn et al., 2002; Hohdatsu et al., 2000; Jeng et al., 1996). In our earlier studies, reproducible production of non-cytotoxic anti-FIV activity from $CD8^+$ T lymphocytes, from cats in the asymptomatic stage of FIV infection, was maximally achieved *in vitro* with effector cells stimulated by FIV-PPR

infected inducer T cells rather than mitogen (Choi et al., 2000b). However, most studies describing the $CD8^+$ T cell non-cytolytic antiviral activity described, relied on mitogen (Concavalin A or phytohemagglutinin) stimulation of $CD8^+$ T lymphocytes or stimulated $CD8^+$ T lymphocytes using either anti-CD3 and anti-CD28 antibodies or a combination of the two antibodies. Thus, the use of inducer cells versus use of mitogen was the key difference between our studies and others. Although a number of reports have focused on identifying the molecules responsible for suppression of virus replication (HIV, FIV or SIV) *in vitro* (Blackbourn et al., 1994; Geiben-Lynn et al., 2001; Mosoian et al., 2000; Unpublished data from our laboratory) the physiologic stimulus required for the stimulation and induction of $CD8^+$ T cells is not yet known.

The focus of this study was to examine various aspects related to the soluble anti-FIV activity mediated by $CD8^+$ T lymphocytes. This involved determining 1) the requirements for induction of $CD8^+$ T cell mediated suppression of FIV replication, 2) the kinetics of the induction process, 3) phenotypes of the inducer T cells and 4) cross reactivity of the soluble $CD8^+$ T cell mediated anti-FIV activity. Inducer cells used in our initial studies were irradiated FIV-infected T lymphocytes (Choi et al., 2000b). But immortalized FSF, transfected with SFV vector expressing either the FIV capsid or an irrelevant antigen, such as lacZ or FSF transfected with SFV vector expressing only its polymerase, could also mediate the induction process. The effector cells produced the soluble anti-FIV activity in the absence of MHC matched inducer cells when either FIV infected T cells or transfected FSF were used. Although MHC restriction was not required for the soluble anti-FIV response, it is not yet clear as to whether it might

improve or otherwise contribute to the activation of the effector cells in our system (Phadke et al., 2004). Thus, it appeared that functional inducer cells did not need to be professional antigen presenting cells (APC) and these cells did not have to express the viable and complete virus. However, it would be worthwhile to determine if the use of professional APC such as dendritic cells or macrophages could serve to enhance the $CD8^+$ T cell antiviral activity against FIV in our system, as has been reported for HIV (Barker et al., 1999; Castelli et al., 2003).

Our studies also showed that optimal induction of the suppressing activity generated by the effector cells occurred between days 1 to 4 and depended on cell-cell contact between inducer and effector cells (Phadke et al., 2004). This implicated a critical role for membrane antigen interactions in the communication between inducer and effector cells with cytokines playing an accessory or a separate role in the induction process. Cell-cell contact could depend on the interaction between costimulatory molecules and adhesion molecules (B7-1 (CD80), B7-2 (CD86), ICAM-1 or CD40) on the inducer cells with their respective ligands (CD28, CTLA-4, LFA-1 or CD40L) on the effector cells (Chirathaworn et al., 2002; Kochli et al., 1999; Kornbluth, 2002; Lenschow et al., 1996).

One of the important membrane molecules, that has been shown to play a role in enhancing the $CD8^+$ T cell mediated suppression of HIV replication, is CD28 (Barker et al., 1997). CD28, through its interaction with the B7 family of proteins, B7-1 and B7-2, provides the major co stimulatory signal for augmenting and sustaining a T-cell response (Lenschow et al., 1996). In the case of HIV infection, $CD8^+$ T cell antiviral factor

production has been found to be associated with cells expressing CD8, CD38, HLA-DR and CD28 (Jiang et al., 2003). Engagement of the CD28 molecule on $CD8^+$ T cells from AIDS patients during stimulation *in vitro* restored and enhanced the ability of these cells to suppress HIV replication *in vitro* (Barker et al., 1997). Barker et al. (1999) also demonstrated that while both B7 molecules could deliver a costimulatory signal sufficient to increase $CD8^+$ T cell antiviral activity, B7-2 on macrophages was found to enhance $CD8^+$ T cell suppression of HIV replication *in vitro*.

In order to determine the membrane antigens that play a role in the induction process, modulations of phenotypes of freshly prepared PBMC were determined using a panel of feline antigen specific antibodies. Flow cytometry studies carried out on fresh PBMC from FIV infected and uninfected cats demonstrated a significant increase in the percentages of $CD8^+$ T cells coexpressing the B7-1 co-stimulatory molecule in FIV infected cats as compared to the uninfected cats. The percentages of $CD4^+$ T cells expressing B7-1 did not vary significantly with infection. There was no significant difference between the MHC class I and MHC class II expression on $CD4^+$ T cells or $CD8\beta^+$ T cells between FIV infected or FIV uninfected cats. Expression of B7-1 costimulatory molecule on T cells was unusual since, normally these molecules are found on professional APC (Lenschow et al., 1996). However, a number of recent studies have shown that B7 molecules may be up regulated on T cells activated *in vitro* and on a subset of $CD4^+$ and $CD8^+$ T cells in HIV or FIV infection (Haffar et al., 1993; Kochli et al., 1999; Tompkins et al., 2002; Wyss-Coray et al., 1993b). Based on these observations and the flow cytomtery results discussed, it is possible that as with HIV

infection of humans, T cells in FIV infected cats developed an antigen presenting phenotype and hence contributed to the stimulation of effector $CD8^+$ T cells in our system via B7-1 molecules. Blocking the costimulatory interactions between the inducer and effector cells would allow us to identify the exact molecules required for the induction process. However, lack of feline specific antibodies, such as feline CD28 monoclonal antibody (Mab), feline CTLA-4 Mab and feline B7-2 Mab make such a study currently difficult.

An important feature of the FIV model system is the availability of molecular clones that, after transfection into cells in culture, give rise to infectious virions (Hosie et al., 2002; Miyazawa et al., 1991; Phillips et al., 1990; Siebelink et al., 1992; Talbott et al., 1989). The use of a molecularly cloned virus provides a unique opportunity to study both viral evolution within a host and to examine the generation of tissue specific variants by eliminating factors, such as a diverse inoculum of viral variants differing in their cellular tropism. However, infection of cats with a molecular clone of FIV (FIV-PPR) did not give rise to tissue specific viral variant quasispecies in PBMC, lymph nodes and spleen tissues which were examined, based on sequencing of the V3-V4 region of the envelope (env) gene. The variable V3-V4 FIV env region was chosen, since it has been found to include determinants required for FIV tropism (Siebelink et al., 1995b; Verschoor et al., 1995), to contain neutralization domains (de Ronde et al., 1994; Lombardi et al., 1993b) and to undergo extensive mutations in other lentiviruses, such as HIV, SIV and caprine arthritis-encephalitis virus (CAEV) (Coffin, 1986; Endo et al., 2000; Hotzel et al., 2002; Johnson et al., 1991). Surprisingly, our studies have

demonstrated an almost uniform virus population in the 3 tissues examined for cats M153, M160, M165, M242 and A306. The diversity of the V3-V4 region did not increase, except in some clones from cats A308 and M160, but the variation seemed to be random.

One of the reasons for the emergence of tissue specific variants in HIV or SIV infections is the high replication rate of the virus, coupled with the rapid turnover of infected cells, lack of fidelity of the reverse transcriptase enzyme and selection of variants with envelope glycoprotein optimally adapted to locally expressed receptors on cells (Casado et al., 2001; Wong et al., 1997). However, there have been no studies supporting a high error rate associated with the FIV reverse transcriptase enzyme. Moreover, the $CD8^+$ T cells of four of the six cats (cat A306, A308, M165 and M242) that were examined for the presence of variation in the V3-V4 region of the envelope gene in our study have in the past demonstrated $CD8^+$ T cell mediated suppression of FIV replication *in vitro* [Choi et al., 2000b; Unpublished data from our laboratory]. Suppression of FIV-PPR virus in the PBMC *in vitro* would imply an *in vivo* ability of $CD8^+$ T cells to suppress virus replication in infected cells. Suppression of virus replication would lead to a reduction in the number of mutations being incorporated in the virus due to fewer viral replication cycles. Since the suppressive activity of the $CD8^+$ T cells blocked the viral replication in the cells, the cells could probably resume normal function with limited viral transmission. Since variation could also partly depend on the diversity of the initial inoculated population of virus, the use of a molecular clone of FIV-PPR could also be the reason for fewer mutations observed in the V3-V4 env region

in our studies. On the other hand, the molecular clone of FIV-PPR was prepared by transfection of a provirus plasmid into Crandell feline kidney cells (CRFK) and passage of virus stocks in PBMC cocultured with transfected CRFK cells. The molecular clone of FIV-PPR thus obtained was found to be minimally pathogenic after inoculation of SPF cats, as compared to the biological clone of FIV-PPR from which it was derived (Sparger et al., 1994). Thus, *in vitro* cultivation of FIV isolates may amplify less pathogenic variants within quasispecies that fail to drive the host immune responses of cats, to exert selective immune pressure that contributes to the evolution of viral variants. This may occur as a result of a favorable equilibrium being maintained between the infecting virus and the host, giving rise to a benign infection in the host.

Five distinct clades of FIV (A-E) have been identified with 15-30% variability in env amino acid sequence although a majority of viruses identified belong to either clade A or clade B (Bachmann et al., 1997; Elder et al., 1998; Sodora et al., 1994). Recent phylogenetic studies in our laboratory, using FIV isolates from naturally infected feral cats in Texas, have suggested that the Texas isolates seem to have evolved from FIV clade B and formed a distinct cluster as compared to other clades including clade B (Weaver et al., 2004). Experimental infection of cats with the FIV-TX53 strain which belongs to the newly emerging FIV subtype caused an acute disease characterized by viral dose dependent inversion of $CD4^+$/ $CD8^+$ T cell ratios that dropped below 1 by 11 weeks p.i. This inversion of the $CD4^+$/ $CD8^+$ T cell ratio included a progressive decline in the $CD4^+$ T cells in the infected cats accompanied by an increase in the $CD8^+$ T cells, which was significant as compared to the $CD8^+$ T cells in the uninfected cats only at the

highest dose administered. Additionally, the increase in the $CD8^+$ T cells was characterized by an expansion of the $CD8\beta^{low}$ population of $CD8^+$ T cells. $CD8\beta^{low}$ population of cells has been considered a marker for infection with more pathogenic strains of FIV, such as the molecular clone FIV-$GL8_{414}$ or the pathogenic virus variants arising after FIV-PET_{F14} infection of cats (Hosie et al., 2002). There have been controversial reports demonstrating this population to be involved in the $CD8^+$ T cell mediated *in vitro* suppression of various FIV strains, such as FIV-$NCSU_1$ and FIV-GL_8 (Bucci et al., 1998b; Flynn et al., 2002; Hosie et al., 2002). Sequencing the variable region of the envelope gene from FIV-TX53 strains obtained from the cats infected with different doses of this virus should provide us information about possible emergence of new strains that coincide with the appearance of this population. Additionally, cell-sorting studies could be carried out to determine whether the $CD8\beta^{low}$ population or the $CD8\beta^{high}$ population of $CD8^+$ T cells in the FIV-TX53 infected cats is responsible, for non-cytolytic suppression of the virus *in vitro*.

Earlier studies by Choi et al. (2000b) in our laboratory had demonstrated that supernatants collected from FIV-PPR infected cat PBMC (Clade A), which had been stimulated with irradiated FIV-PPR infected inducer T cells, suppressed viral replication of FIV-Petaluma (Clade A) infected target cells. Flynn et al. (1999) also demonstrated suppression of FIV-Petaluma strain and FIV-GL_8 strain by mitogen stimulated lymphoblasts obtained from FIV-Petaluma infected cats. Again, both these strains belonged to FIV clade A. In contrast to these studies, we have now demonstrated that $CD8^+$ T cells from FIV-PPR cats were able to suppress viral replication in PBMC

acutely infected with either FIV-TX53 or FIV-TX078 belonging to a new clade closely related to clade B. This is the first report of the soluble suppressing activity in FIV being cross-reactive among FIV clades. These studies were performed using inducer T cells to stimulate the $CD8^+$ T effector cells, although we would predict the same results using FSF as inducer cells, as demonstrated in chapter II.

Earlier studies of $CD8^+$ T cell antiviral activity against HIV have demonstrated lack of antigen-specificity both at the induction and effector phase. Thus, the fact that the $CD8^+$ T cell antiviral response has been observed in asymptomatic HIV infected individuals, HIV exposed but uninfected individuals, healthy HIV-naïve individuals, as well as in FIV infected and uninfected cats (Bagasra and Pomerantz, 1993; Blackbourn et al., 1996; Brinchmann et al., 1990; Furci et al., 2002; Hsueh et al., 1994; Kootstra et al., 1997; Levy et al., 1998; Mackewicz et al., 1991; Rosok et al., 1997; Stranford et al., 1999; Toso et al., 1995) suggests that mechanisms involved for the induction of this antiviral response are not virus specific. Lack of specificity at the effector phase has been demonstrated by Walker et al. (1991b), who showed that $CD8^+$ T cells from an HIV-infected individual could suppress virus replication in $CD4^+$ T cells acutely infected with HIV-1, HIV-2 or even SIV_{mac}, thus demonstrating cross-reactivity.

Whole inactivated FIV-Petaluma vaccine was able to elicit $CD8^+$ T cell antiviral activity in vaccinated cats, that suppressed FIV-Petaluma and FIV-GL_8 virus replication *in vitro* (Flynn et al., 1999). Also, $CD8^+$ T cells derived from macaques immunized with live, attenuated SIV have been shown to inhibit the replication of SIV via soluble factors in a non-MHC restricted manner (Gauduin et al., 1998). Moreover, studies in our

laboratory have also demonstrated suppression of feline leukemia virus (FeLV) replication *in vitro* by the supernatants of inducer T cell stimulated $CD8^+$ T cells from FIV-PPR infected cats (Unpublished data from our laboratory). Thus the nonspecific nature of the soluble $CD8^+$ T cell antiviral activity and that of induction indicates that $CD8^+$ T cells, well characterized as providing adaptive immunity, can also provide innate immunity. This seemingly innate response of the cells to inhibit either FIV or HIV replication non-cytolytically may be due to the recognition by the effector $CD8^+$ T cells of a "pathogen associated molecular pattern" (PAMP) on inducer cells or cells in close contact (Medzhitov and Janeway, 2000), making it a more general mechanism of control rather than being FIV specific. These PAMP could be highly conserved structures present on different pathogens (Medzhitov and Janeway, 2000). As the success of a FIV vaccine could be hampered by the occurrence of highly divergent viral variants in the field, the exploitation of this innate, soluble $CD8^+$ T cell anti-FIV activity would be more useful. Hence, characterization and identification of the stimuli and mechanisms involved in the induction of the $CD8^+$ T cell antiviral activity against FIV could provide information for strategies of practical exploitation of this innate T cell immunity and may contribute to the design of novel, safe and complementary anti-FIV therapeutic strategies.

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