

## ABSTRACT

**Hewitt, Tracy P.** Cellular and Molecular Mechanisms involved with feline CD8<sup>+</sup> T cell mediated anti-FIV activity (under the direction of Dr. Wayne Tompkins).

A population of activated CD8<sup>+</sup> T cells that express the B7.1 (CD80) and B7.2 (CD86) costimulatory molecules and their ligand CTLA4 exist in the blood of asymptomatic FIV-infected cats. As evidence of CD8<sup>+</sup> T cell mediated anti-FIV activity, coculture of CD8<sup>+</sup> depleted-PBMC with FCD4E cells resulted in a significant increase in FIV p24 levels as compared to total PBMC cocultures. This anti-FIV activity was not overridden by the addition of either rIL-2 or ConA. All cats were infected either intravenously or vaginally. There was no correlation between the route of infection and the presence of CD8<sup>+</sup> T cell-mediated antiviral activity. Nonsuppressor cats had a higher number of viral RNA molecules per milliliter of plasma than the suppressor cats. PBMC from suppressor cats had a higher percentage of CD8<sup>+</sup> CD25<sup>+</sup> cells as compared to nonsuppressor cats. Both CD8<sup>+</sup> CD25<sup>+</sup> and CD8<sup>+</sup> CD25<sup>-</sup> subsets inhibited FIV replication. CD8<sup>+</sup> cells from suppressor cats also had an increased in the frequency of B7.1<sup>+</sup> CD8<sup>+</sup> cells with a low expression of the CD8 $\beta$  chain, suggesting that the CD8<sup>+</sup> anti-FIV cells express the activation phenotype, CD8<sup>+</sup>  $\beta^{\text{lo}}$  B7.1<sup>+</sup>. Depletion of B7.1<sup>+</sup> cells from PBMC of suppressor cats resulted in increased viral replication similar to depletion of CD8<sup>+</sup> cells. CD8<sup>+</sup>B7.1<sup>+</sup> and CD8<sup>+</sup>B7.1<sup>-</sup> subsets cocultured with infected CD4<sup>+</sup> T cells revealed that the antiviral activity reside primarily in CD8<sup>+</sup> B7.1<sup>+</sup> subset. The presence of CD8<sup>+</sup> antiviral cells lead to a decrease in the number of FIV RNA molecules per 10<sup>6</sup> CD4<sup>+</sup> T cells in suppressor cats with no appreciable difference in the expression of IL-2 mRNA levels from either group of cats.

The results of this study showed a strong correlation between the presence of CD8<sup>+</sup> T cell anti-FIV activity, a reduction in viremia, low expression of the CD8β chain and expression of B7.1 costimulatory molecule on CD8<sup>+</sup> T cells in suppressor cats suggesting that CD8<sup>+</sup> β<sup>lo</sup> B7.1<sup>+</sup> antiviral cells may play a major role in controlling FIV replication *in vivo*.

# **Cellular and Molecular Mechanisms involved with feline CD8<sup>+</sup> T cell mediated anti-FIV activity**

by

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## **Biography**

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## LIST OF ABBREVIATIONS

**Ag**-antigen

**AIDS**-acquired immunodeficiency syndrome

**APC**-antigen presenting cells

**APC**-allophycocyanin (fluorochrome)

**B7.1**-CD80

**B7.2**-CD86

**CAF**-CD8 antiviral factor

**CBC**-complete blood count

**CD**-cluster of differentiation

**cDNA**-copy DNA

**ConA**-Concanavilin A

**CPE**-cytopathic effect

**CrFK**-Crandell feline kidney cells

**CTL**-cytotoxic T-lymphocytes

**CTLA4**-cytotoxic T-lymphocyte antigen four (CD152)

**ELISA**-enzyme linked immunoabsorbent assay

**FCD4E**-feline CD4 E cells

**FITC**-fluorescein isothiocyanate

**FIV**-feline immunodeficiency virus

**G3PDH**-glyceraldehyde 3' phosphate dehydrogenase

**HAART**-highly active antiretroviral therapy

**HIV**-human immunodeficiency virus

**IL**-interleukin

**LN**-lymph node

**LTR**-long terminal repeat

**mAb**-monoclonal

**MHC**-major histocompatibility complex

**mL**-milliliter

**mAb**-monoclonal antibody

**µg**-microgram

**mRNA**-messenger ribonucleic acid

**NCSU<sub>1</sub>** –North Carolina State University FIV isolate 1

**NFκβ**-nuclear factor kappa-β

**pAb**-polyclonal antibody

**PBMC**-peripheral blood mononuclear cells

**PCR**-polymerase chain reaction

**PE**-phycoerthyerin

**p.i.**-post infection

**rhIL-2** recombinant human interleukin-two

**RNA**-ribonucleic acid

**RT-PCR**-reverse transcription polymerase chain reaction

**SD**-standard deviation

**SIV**-simian immunodeficiency virus

**SPF**-specific pathogen free

**TCR**-T cell receptor

**TCID-** Tissue culture infectious dose

**TGF $\beta$** -tumor growth factor beta

## INTRODUCTION

Feline Immunodeficiency Virus (FIV) and Human Immunodeficiency Virus (HIV) infections cause remarkably similar diseases characterized by an early and persistent CD8<sup>+</sup> lymphocytosis and a progressive loss of CD4<sup>+</sup> T cell numbers and function leading to AIDS [Fauci et al., 1993; Giorgi et al., 1993; Elder et al., 1998]. During HIV and FIV infections, an increase in the number of CD8<sup>+</sup> T cells correlates with the emergence of a subset of CD8<sup>+</sup> T cells with potent antiviral activity [Levy et al., 1996; Mackewicz et al., 1994; Bucci, Gebhard et al., 1998; Jeng et al., 1996].

The CD8<sup>+</sup> T cells that are capable of inhibiting virus replication in infected cells are classified according to how they mediate antiviral activity. One CD8<sup>+</sup> T cell subset, cytotoxic T lymphocytes (CTL), mediates the destruction of virus infected cells by an antigen-specific, class I MHC-restricted cytotoxic mechanism whereas another population of CD8<sup>+</sup> effector cells inhibits virus replication by a non-MHC restricted, noncytotoxic mechanism [Walker et al., 1988; Walker et al., 1991; Wiviott et al., 1990; Buseyne et al. 1993].

CD8<sup>+</sup> T cells with known anti-HIV suppressor activity, when separated from the CD4<sup>+</sup> T cells by a semipermeable membrane, failed to suppress HIV replication suggesting the presence of a cell contact dependent suppressor mechanism [Levy et al., 1996; Mackewicz et al., 1998]. However, the supernatants from stimulated HIV<sup>+</sup> CD8<sup>+</sup> T cells were able to suppress virus, suggesting the presence of a soluble

inhibitory factor. [Walker et al., 1986, Walker et al., 1989]. Neutralizing antibodies against a number of cytokines and chemokines did not alleviate the antiviral effects of the soluble factor(s), demonstrating that the soluble mediator(s) lacked identity with many known cytokines and chemokines [Barker et al., 1995; Mackewicz et al., 1996; Mackewicz et al., 1996; Mackewicz et al., 1997].

In the case of FIV, CD8<sup>+</sup> T cells had strong anti-FIV activity against autologous and heterologous infected CD4<sup>+</sup> T cells, suggesting that the CD8<sup>+</sup> T cell mediated antiviral activity may occur in a non-MHC restricted manner [Bucci, English et al., 1998; Jeng et al., 1996; Flynn et al., 1999; Choi et al., 2000]. In addition, there was also a decrease in anti-FIV activity when transwell filters were used to separate the CD8<sup>+</sup> antiviral cells from the FIV infected CD4<sup>+</sup> T cells, suggesting that optimal anti-FIV activity required cell-cell contact. In another study, supernatants from ConA-stimulated FIV<sup>+</sup> CD8<sup>+</sup> antiviral T cells as well as normal CD8<sup>+</sup> T cells suppressed FIV replication, suggesting that the soluble factor may not be physiologically relevant [Flynn et al., 1999; Choi et al., 2000]. Thus, while antiviral activity of CD8<sup>+</sup> T suppressor cells may involve cell contact as well as a soluble factor, neither the soluble factor(s) nor the cell surface receptors responsible for the cell contact mechanism have been identified.

CD8<sup>+</sup> T cells that mediate immunity through cytotoxic or noncytotoxic mechanisms can be divided by differential expression of surface markers [Levy et al., 1996]. The expression of HLA-DR<sup>+</sup> and CD38<sup>+</sup> markers on CD8<sup>+</sup> lymphocytes correlates with

high anti-HIV activity [Levy et al., 1996; Landay et al., 1993; Mackewicz et al., 1992]. It has also been reported that the CD8<sup>+</sup> CD38<sup>+</sup> phenotype is associated with high viremia, high provirus burden and disease progression [Ferbass et al., 1995; Giorgi et al., 1993]. However, long-term nonprogressor HIV positive patients possess high numbers of HLA-DR<sup>+</sup> CD38<sup>-</sup> CD8<sup>+</sup> T cells [Giorgi et al., 1993]. The HLA-DR<sup>+</sup> CD38<sup>-</sup> CD8<sup>+</sup> phenotype that is predominate in these patients is noncytotoxic, which raises the possibility that these cells reduce viremia by a suppressive mechanism. It has also been reported that the predominant HIV suppressor cell in HIV-infected patients has a CD8<sup>+</sup> CD28<sup>+</sup> phenotype and that CD28 costimulation induced upregulation of CD25 expression and increased suppressor activity [Barker et al., 1997; Barker et al., 1998; Barker et al., 1999].

Previous research in our laboratory determined that the CD8<sup>+</sup> anti-FIV T cells in the blood of FIV-infected cats have an activated phenotype (CD8 $\alpha^+$   $\beta^{\text{lo}}$  CD62L<sup>neg</sup> CD44<sup>Hi</sup> CD18<sup>Hi</sup>) [Bucci, Gebhard et al., 1998; Gebhard et al., 1999]. We also recently reported that an usually high number of CD8<sup>+</sup> and CD4<sup>+</sup> T cells in the blood of FIV positive cats co-express B7.1 and B7.2 costimulatory molecules and their ligand CTLA4 [Tompkins et al., 2002; Vahlenkamp et al., 2004]. As B7 molecules expressed on activated T cells can transduce a contact dependent signal for IL-2 suppression and anergy, and as FIV replication is positively regulated by IL-2; CD8<sup>+</sup> B7<sup>+</sup> cells may function as anti-FIV cells. The overall purpose of this research project was to investigate the cellular and molecular mechanisms of CD8<sup>+</sup> T cell mediated anti-FIV activity. The specific aims of this project were:

1. To assess the presence of cell-associated and cell-free virus in asymptomatic FIV-infected cats.
2. To phenotypically characterize the cell surface markers on T lymphocytes isolated from FIV infected cats with and without CD8<sup>+</sup> T cell antiviral activity.
3. To determine the role of cell surface markers associated with anti-FIV CD8<sup>+</sup> T cells in asymptomatic FIV-infected cats.
4. To determine the effects of CD8<sup>+</sup> T cell antiviral activity on IL-2 mRNA and FIV RNA expression.

More specifically, experiments were designed to address the following questions:

1. Does CD8<sup>+</sup> T cell-mediated anti-FIV activity correlate with a reduction in cell-associated and plasma viremia?
2. What is the cell surface phenotype of CD8<sup>+</sup> and CD4<sup>+</sup> T cells in suppressor and nonsuppressor cats?
3. Does the B7 costimulatory molecule play a role in CD8<sup>+</sup> T cell mediated antiviral activity?
4. Does a specific subset of CD8<sup>+</sup> T cells mediate anti-FIV activity in long-term asymptomatic FIV infected cats?
5. Can exogenous IL-2 inhibit CD8<sup>+</sup> T cell anti-FIV activity?
6. Does the presence of CD8<sup>+</sup> anti-FIV cells have an effect on IL-2 mRNA and FIV gene transcription in infected CD4<sup>+</sup> T cells?



## **LITERATURE REVIEW**

### **A. CD8<sup>+</sup> T cell-mediated noncytotoxic anti-HIV activity**

Human Immunodeficiency Virus (HIV) is a lentivirus that causes human acquired immunodeficiency syndrome (AIDS). The acute phase of HIV infection is characterized by high viremia, lymphadenopathy, and the development of humoral and cell mediated immune responses [Fauci et al., 1993; Pantaleo et al., 1993].

The acute phase is followed by a long asymptomatic stage, which is characterized by low plasma viremia levels and inversion in the ratio of CD4<sup>+</sup> and CD8<sup>+</sup> T cells. The inversion in the ratio of CD4<sup>+</sup> to CD8<sup>+</sup> T cells is attributed to a loss of CD4<sup>+</sup> T cells and an increase in the number of CD8<sup>+</sup> T cells [Giorgi et al., 1993; Prince et al., 1991; Yagi et al., 1991]. The gradual deterioration in the function of the immune system leads to the development of AIDS related complex and AIDS. Death usually occurs within 2 years after the development of AIDS primarily as a result of opportunistic infections and the wasting syndrome [Fauci et al., 1993; Pantaleo et al., 1993].

A consistent observation in HIV infected patients is an increase in the number of CD8<sup>+</sup> T cells, which begins at seroconversion and persists throughout the asymptomatic stage of the disease [Giorgi et al., 1993; Prince et al., 1991; Yagi et al., 1991]. A subset of these CD8<sup>+</sup> T cells can suppress HIV-1 replication in CD4<sup>+</sup> T cells in a noncytotoxic, non-MHC Class I restricted

manner, when cultured in vitro [Walker et al., 1986; Mackewicz et al., 1992]. Walker and others were able to demonstrate the presence of CD8<sup>+</sup> T cell-mediated antiviral activity in PBMC isolated from HIV-infected individuals [Walker et al., 1986]. Removal of the CD8<sup>+</sup> T cells from the total PBMC population resulted in a release of virus from the CD4<sup>+</sup> T cells whereas replacement of the CD8<sup>+</sup> T cells in the culture system led to suppression of virus replication. Virus replication, which was measured by a reverse transcriptase assay, was inhibited in a dose dependent manner when autologous not allogenic CD8<sup>+</sup> T cells were added back to coculture system [Walker et al., 1986]. Virus replication increased when CD8<sup>+</sup> T cells were removed from the culture, suggesting that the mechanism of suppression did not involve lysis of the HIV infected cells [Walker et al., 1986].

The CD8<sup>+</sup> T cells suppressed HIV virus replication without affecting the proliferation or expression of activation markers and without eliminating the infected CD4<sup>+</sup> T cells [Wiviott et al., 1990; Walker et al., 1991; Mackewicz et al., 1992; Levy et al., 1996]. Phenotypic and functional characterization of the CD8<sup>+</sup> anti-HIV cells suggests that they have an activated phenotype (CD8<sup>+</sup> HLA-DR<sup>+</sup> CD28<sup>+</sup>) [Giorgi et al., 1993; Barker et al., 1997; Barker et al., 1999] and the capacity to block HIV replication in naturally and acutely infected CD4<sup>+</sup> T cells [Levy et al., 1996] and macrophages [Moriuchi et al., 1996; Barker et al., 1999].

The clinical significance of this antiviral activity is indicated by its occurrence in HIV infected individuals and the reduction in this activity as the individual advances toward AIDS [Walker et al., 1989; Gulzar et al., 2004; Landay et al., 1993; Mackewicz et al., 1994, 1996, 1997].

Many long-term nonprogressor HIV positive patients maintain high levels of CD8<sup>+</sup> T cells that are capable of controlling HIV infection of PBMC which leads to low viral loads [Cao et al., 1995]. Purified HIV-infected CD4<sup>+</sup> T cells isolated from these nonprogressors can infect PHA-stimulated PBMC, and the addition of CD8<sup>+</sup> T cells dramatically inhibits the transmission of HIV, suggesting that these patients possess HIV-infected CD4<sup>+</sup> T cells whose viral expression can be downregulated by CD8<sup>+</sup> T cells [Cao et al., 1995; Paul et al., 1995].

CD8<sup>+</sup> T cells from patients with AIDS are not as effective as CD8<sup>+</sup> T cells from nonprogressors in downregulating viral expression, suggesting that the anti-HIV activity mediated by CD8<sup>+</sup> T cells correlates with the clinical stage of disease [Brinchmann et al., 1990; Kannagi et al., 1998]. Also, disease resistant hosts such as chimpanzees have a higher percentage of circulating CD8<sup>+</sup> T cells than humans and *in vivo* treatment of HIV-1 infected chimpanzees with anti-CD8 mAb enhanced virus recovery from their PBMCs [Castro et al., 1992]. Therefore, CD8<sup>+</sup> T cells may be responsible for natural anti-HIV immune responses [Castro et al., 1991, 1992].

CD8<sup>+</sup> T cells' antiviral activity seen in asymptomatic HIV-infected patients decreases as CD4<sup>+</sup> T cell numbers decrease and as individuals progress toward the AIDS stage, suggesting that the maintenance of CD8<sup>+</sup> T cell mediated antiviral activity may be dependent upon the presence of CD4<sup>+</sup> T cells [Levy et al., 1996; Mackewicz et al., 1991]. These CD8<sup>+</sup> anti-HIV cells are categorized by the manner in which inhibition of HIV replication occurs. One subset of these CD8<sup>+</sup> T cells appears to mediate the destruction of HIV-infected cells by HIV-specific, class I major histocompatibility complex (MHC)-restricted, cytotoxic T cells (CTL), whereas the other subset of CD8<sup>+</sup> T cells suppresses HIV virus replication by a non-MHC restricted, noncytotoxic mechanism (nCTL) [Levy et al., 1998].

#### **1. Mechanism of CD8<sup>+</sup> T cell noncytotoxic anti-HIV activity**

CD8<sup>+</sup> anti-HIV activity is mediated by a CD8<sup>+</sup> T cell antiviral factor (CAF) and by cell-to-cell contact [Walker et al., 1989; Brinchmann et al., 1991]. It is not known whether cell-cell contact represents a different mechanism than CAF. The ability of peripheral blood CD8<sup>+</sup> T cells isolated from HIV-infected individuals to suppress HIV replication in a contact dependent manner and by a soluble mediator was demonstrated using a transwell coculture system [Walker et al., 1986; Mackewicz et al., 1992]. Infected CD4<sup>+</sup> T cells were separated from the CD8<sup>+</sup> T cells by a semipermeable membrane. CD8<sup>+</sup> T cells from some of the HIV-infected individuals did not produce an antiviral soluble factor but their CD8<sup>+</sup> T cells suppressed HIV replication in a contact

dependent manner. When the antiviral soluble factor was present, its activity declined over time. Optimal antiviral activity occurred when the infected CD4<sup>+</sup> and CD8<sup>+</sup> T cells were in contact with each other. The antiviral activity of CAF has also been demonstrated by adding culture supernatants collected from peripheral blood CD8<sup>+</sup> T cells of HIV-infected individuals directly to naturally and acutely infected CD4<sup>+</sup> T cells [Walker et al., 1986; Mackewicz et al., 1992]. CD8<sup>+</sup> T cells may suppress HIV replication before RNA transcription occurs. When naturally infected CD4<sup>+</sup> T cells were cocultured with CD8<sup>+</sup> T cells, there was a dramatic decrease in viral protein and RNA synthesis [Mackewicz et al., 1995]. These findings were supported by northern blot analysis of viral RNA species, which revealed a decrease in single, double and unspliced RNA species.

In a coculture system with CD8<sup>+</sup> cells, CAF blocked viral transcription [Mackewicz et al., 1995]. CAF also reduced chloramphenicol acetylase transferase (CAT) expression mediated by long terminal repeat (LTR) from Human T-lymphotropic virus and Rous Sarcoma virus [Copeland, McKay et al., 1995]. In another study, CAF was also able to suppress expression of luciferase activity in Jurkat T cells transfected with HIV-LTR linked to a luciferase reporter gene [Mackewicz et al., 1996]. This strongly suggested that the CD8<sup>+</sup> T cells might suppress virus replication by inhibiting HIV-LTR driven gene transcription [Mackewicz et al., 1996].

**a. Antiviral Activity of Cytokines**

The identity of CAF is unknown. In an effort to identify CAF, researchers examined the effects of recombinant cytokines, interferons, tumor necrosis factors, IL-4, IL-10, and TGF- $\beta_1$  on HIV replication in CD4<sup>+</sup> T cells.

Interferons, tumor necrosis factor- $\alpha$ , TGF- $\beta_1$ , and IL-8 reduced virus replication in a dose dependent manner. However neutralizing antibodies specific for these cytokines or for IL-4, and IL-6 did not reduce or abolish the antiviral effects of CAF [Barker et al., 1995; Brinchmann et al., 1991; Mackewicz, Ortega et al., 1994; Mackewicz et al., 1995; Mackewicz, Levy et al., 1996]. These studies clearly demonstrated that CAF lacked identity with these cytokines.

Progression to AIDS has been shown to correlate with a shift in the overall cytokine pattern from a T helper cell type 1 (TH<sub>1</sub>) to a T helper cell type 2 (TH<sub>2</sub>) response [Mackewicz, Ortega et al., 1994; Brinchmann et al., 1991; Barker, Mackewicz et al., 1995]. The production of cytokines such as IL-2 declined over time whereas cytokines such as IL-4 and IL-10 increased as the disease progressed toward AIDS. Also the antiviral effects of CD8<sup>+</sup> T cells declined as the disease progressed from the asymptomatic stage to AIDS [Mackewicz et al., 1994; Brinchmann et al., 1991]. Barker et al., [1995] investigated the role of IL-2, IL-4, and IL-10 in CD8<sup>+</sup> T cell mediated antiviral activity. Their results demonstrated that IL-2 enhanced the antiviral activity of CD8<sup>+</sup> T cells from HIV-infected individuals whereas IL-4 and IL-10 inhibited

the antiviral activity. However, the addition of IL-2 reversed the inhibitory effects of the T<sub>H</sub>2 cytokines [Barker et al., 1995].

#### **b. Antiviral Activity of Chemokines**

In a continued effort to identify the antiviral factor, studies showed that recombinant  $\beta$ -chemokines, RANTES, MIP-1 $\alpha$ , and MIP-1 $\beta$ , produced by CD8<sup>+</sup> T cells, inhibited HIV-1, HIV-2 and SIV in a dose-dependent manner [Cocchi et al., 1995; Barker, Bossart et al, 1998; Fransen et al., 2000; Greco et al, 1998]. Neutralizing antibodies to these chemokines blocked their antiviral activity [Cocchi et al., 1995]. All three chemokines competitively blocked HIV entry into target cells by binding to the  $\beta$ -chemokine receptor, CCR5 and preventing the binding of viral envelope protein [Cocchi et al., 1995; Cocchi et al., 1996]. However, these chemokines did not block HIV LTR-directed transcription, which is inhibited by CAF [Leith et al., 1997; Mackewicz et al., 1995]. In addition, high levels of  $\beta$ -chemokines do not inhibit acute HIV-1 infection and their presence or absence does not correlate with CD8<sup>+</sup> anti-HIV activity.

#### **c. Antiviral Activity of IL-16**

IL-16 is a CD8<sup>+</sup> T cell derived cytokine that induces chemotaxis of CD4<sup>+</sup> T cells, monocytes and eosinophils [Center et al., 1996; Cruishank et al., 1987, 1991, 1996; Rand et al., 1991]. In addition to its chemotactic activity, IL-16 induces CD4<sup>+</sup> T cell anergy by binding to and/or crossing linking the CD4

molecules, which has an inhibitory effects on IL-2 production [Cruishank et al., 1991; Center et al., 1996; Cruishank et al., 1996]. IL-16 has been cited as a potential anti-HIV molecule because of its known interaction with the CD4 molecule [Baier et al., 1995; Cruikshank et al., 1996]. The most likely mechanism for HIV inhibition by IL-16 is the production of a negative signal after cross linking the cell surface CD4 molecule and not the inhibition of viral entry [Center et al., 1996].

In one study, IL-16 repressed transcription from the HIV-1 LTR reporter gene, even in the presence of PMA and Tat activation [Maciaszek et al., 1997].

Cell lines that lacked the CD4 molecule did not respond to IL-16, which clearly indicated that the above effects were mediated by the CD4 molecule [Maciaszek et al., 1997]. It has also been reported that CD8<sup>+</sup> T cell mediated antiviral activity is not attributed to IL-16 because monoclonal antibodies against IL-16 do not inhibit the antiviral activity [Mackewicz et al., 1996].

There is not a good correlation between IL-16 levels produced by CD8<sup>+</sup> cells isolated from HIV-positive long-term nonprogressors and IL-16 levels in AIDS patients. Therefore, importance of the role of IL-16 in CD8<sup>+</sup> T cell mediated antiviral activity requires more investigation [Mackewicz et al., 1996].

#### **d. Antiviral Activity of Defensins**

Alpha-defensins are part of a family of peptides that have antimicrobial properties [Cole et al., 2003]. Recently, Zhang et al., [2002] reported that



alpha-defensins-1, 2, and 3, secreted from stimulated CD8<sup>+</sup> T cells of long-term nonprogressors, may account for the anti-HIV activity of CAF. Their determination was based on the ability of monoclonal antibodies to the alpha-defensins to eliminate CAF activity and the ability of alpha-defensins to inhibit HIV replication [Zhang et al., 2002]. However, Chang et al., [2003] found that alpha-defensins did not inhibit LTR mediated gene expression, nor did the antibodies to these alpha-defensins inhibit CAF activity in the super-natants of Herpes saimiri transformed CD8<sup>+</sup> T cells. Several studies have shown that CAF acts at the level of transcription [Copeland et al., 1996; Copeland et al., 1998; Copeland et al., 2002; Mackewicz et al., 1995]. Therefore, more research is necessary to determine which stage of HIV life cycle is affected by the alpha-defensins.

## **B. CD8<sup>+</sup> Cytotoxic T-Lymphocytes**

Major histocompatibility class I-restricted cytotoxic T lymphocytes directed against HIV have been isolated from the peripheral blood, lymph nodes, bronchioalveolar lavage, and cerebrospinal fluid of HIV-infected individuals [Plata et al., 1987; Hoffenbach et al., 1989]. The frequency of HIV-specific CTL is higher in healthy HIV-infected individuals than in AIDS patients [Nixon et al., 1988, Novitsky et al., 2002].

Most HIV-specific CTLs are predominately MHC class I restricted CD8<sup>+</sup> T cells, but there are also class II restricted CD4<sup>+</sup> CTLs in the PBMC of HIV-infected individuals [Littua et al., 1992; Kunda et al., 1992]. Cytotoxic T cell

responses are directed against HIV envelope, gag, reverse transcriptase, and the accessory protein nef [Walker et al., 1988, 1989; Culmann et al., 1990]. CTL clones recognizing the HIV nef protein persist as long as 31 months in vivo [Kalams et al., 1994] whereas other studies have determined that these CTL clones may survive longer than 5 years [Moss et al., 1995].

CD8<sup>+</sup> CTL recognize 8-11 amino acid residues presented by the class I MHC molecules and mediate the killing of infected target cells by two mechanisms [Guo et al., 1992; Berke et al., 1994]. One mechanism involves the release of perforin and granzymes that form pores in the plasma membrane of target cell, which leads to cytolysis. The CTL is believed to be protected from its cytolytic molecules by an unidentified mechanism [Stinchcombe et al., 2001; Bossi et al., 2002]. The other cytotoxic mechanism involves the induction of apoptosis of target cell, which results from the ligation of FAS on target cell with the FAS ligand on the CTL [Berke et al., 1994]. CTL effector cells have the ability to kill an infected cell, detach and kill another infected cell.

CTL also possess the ability to produce a variety of cytokines such as interferon- $\gamma$ , (IFN-  $\gamma$ ), tumor necrosis factor- $\alpha$ , TNF- $\beta$ , granulocyte macrophage colony-stimulating factor (GM-CSF), interleukin-1(IL-1), IL-2, IL-3, and IL-4 following mitogenic stimulation [Price et al., 1995]. The release of these cytokines from CTL also induces the expression of human leukocyte antigen (HLA) class I, intracellular adhesion molecule-1(ICAM-1), and secretion of

$\beta$ 2 microglobulin, suggesting that these cytokines enhance the ability of CTL to be more effective killers by upregulating the necessary cell surface proteins needed to bind and kill infected target cells [Jassey et al., 1994].

During acute HIV infection, cytotoxic responses appear to coincide with clearance of viral antigenemia. Similar observations have been made with SIV infections; Gag and nef-specific CTL were detected in the PBMC of infected macaques 2 weeks after infection [Yasutomi et al., 1993]. These studies clearly demonstrated that CD8<sup>+</sup> CTL play a role in reducing viremia during HIV infection [Clark et al., 1991]. Other observations have confirmed that virus-specific CD8<sup>+</sup> CTL are involved in controlling viremia following primary infection with HIV, even prior to seroconversion [Koup et al., 1994; Borrow et al., 1994].

It has been suggested that anti-HIV CTL responses are responsible for the clearance of virus in some perinatally-exposed infants [Levy et al., 1998]. In the absence of HIV infection, anti-HIV antibodies, infectious virus or provirus, HIV-specific CTL were observed in a 13 month old child born to a HIV<sup>+</sup> mother [Rowland-Jones et al., 1995]. In another observation, at least 25% of presumed uninfected infants born to HIV<sup>+</sup> mothers possessed anti-HIV CTL in their peripheral blood [DeMaria et al., 1994]. All of the anti-HIV CTL positive infants were seronegative and virus negative by PCR and *in vitro* PBMC cocultures.

Pollack et al., (1993) reported incidents of HIV-seroconversion in the presence of HIV-specific CTL by infants born to HIV<sup>+</sup> positive mothers. This HIV-specific CTL mediated immune response was not limited to children, as homosexual men with recent exposure to HIV mounted anti-HIV responses to HIV antigens which suggested a protective CTL response to sexually transmitted virus [Clerici et al., 1993]. HIV-specific CTL have been found in health care workers exposed to HIV by single needle sticks, even though these individuals never became viremic [Pinto et al., 1995]. HIV envelope specific CTL activity could be detected in these individuals for more than a year following exposure. The presence of HIV-specific CTL suggests that an infection occurred; it is not known whether or not a transient infection occurred [Clerici et al., 1993; Shearer et al., 1996].

The importance of CD8<sup>+</sup> anti-HIV responses suggests that CTL may be reliable prognostic indicators of disease progression [Pantelo et al., 1993]. Ferbas et al., [1995] reported that the CD8<sup>+</sup> CD38<sup>+</sup> activation phenotype was associated with strong anti-HIV activity, high viremia, provirus burden, and HIV mRNA levels in the plasma. Giorgi et al., 1993 showed that the HLA-DR<sup>+</sup> CD8<sup>+</sup> CD38<sup>+</sup> phenotype correlated with a progressive decline in CD4<sup>+</sup> cell numbers and disease progression. These studies strongly suggest that the HLA-DR<sup>+</sup> CD8<sup>+</sup> CD38<sup>+</sup> phenotype may result from chronic activation by HIV antigens and may be associated with high viremia, progressive loss of CD4<sup>+</sup> T cells and disease progression [Giorgi et al., 1993].

### **C. CD8<sup>+</sup> T cell-mediated anti-FIV activity**

Feline Immunodeficiency Virus (FIV), previously called feline T-lymphotropic lentivirus (FTLV), is a member of the lentivirus family [Pederson, et al., 1987]. The pathogenesis of FIV is very similar to HIV. As a result, FIV has become a useful animal model for studying the pathogenesis of HIV-1 infections in humans [English, et al., 1993, Tompkins, et al., 1991]. FIV infection, just as HIV, produces a long asymptomatic phase, with an inversion in the ratio of peripheral CD4<sup>+</sup>: CD8<sup>+</sup> T cells which is attributed to the loss of CD4<sup>+</sup> T cells and CD8<sup>+</sup> lymphocytosis. FIV infects feline lymphocytes, macrophages, monocytes and neuronal cells [English, et al., 1994; Dean et al., 1999]. Both antigen-specific cytotoxic T lymphocytes (CTL) and noncytotoxic CD8<sup>+</sup> T cell antiviral activity have been detected in the peripheral blood of FIV infected cats during the acute and asymptomatic stage of infection [Bucci, Gebhard et al., 1998; Hohdatsu et al., 1998; Flynn et al., 1999; Flynn et al., 2002; Choi et al., 2000].

Similar to HIV-1, the mechanism of feline CD8<sup>+</sup> T cell mediated antiviral activity is not very well characterized. Characterization of the noncytotoxic antiviral activity in mucosally infected cats showed a correlation between a strong CD8<sup>+</sup> antiviral activity and a reduction in viremia. The CD8<sup>+</sup> T cells also had strong anti-FIV activity against autologous and heterologous infected CD4<sup>+</sup> T cells. This suggested that the CD8<sup>+</sup> T cell mediated antiviral activity may occur in a non-MHC restricted manner [Bucci, Gebhard et al., 1998].

There was also a decrease in anti-FIV activity when transwell filters were used to separate the CD8<sup>+</sup> antiviral cells from the FIV infected CD4<sup>+</sup> T cells. This suggested that optimal anti-FIV activity required cell-cell contact between the CD8<sup>+</sup> antiviral cells and the infected CD4<sup>+</sup> cells.

Supernatants collected from Con A-stimulated CD8<sup>+</sup> antiviral T cells and normal CD8<sup>+</sup> T cells suppressed FIV replication, suggesting that the soluble factor (CAF) may not be physiologically relevant [Choi et al., 2000]. As with the cell-cell contact mechanism, the mechanism of feline CAF antiviral activity has not been determined. Both mechanisms involve CD8<sup>+</sup> T cells that are capable of suppressing HIV and FIV replication in CD4<sup>+</sup> T cells. Identifying the specific CD8<sup>+</sup> antiviral T cells will provide a better understanding of cellular immunity during FIV infection.

#### **D. Abnormal CD8<sup>+</sup> T cell homeostasis during FIV and HIV infections**

A marked and persistent increase in the number of CD8<sup>+</sup> T cells is a consistent feature of HIV and FIV infections. This increase in CD8<sup>+</sup> T cells may be the result of a strong CTL or non-CTL antiviral response to virus. There is also an alteration in the cell surface phenotype of the CD8<sup>+</sup> T cells in the peripheral blood, which includes an early and progressive decrease in the naïve population along with an expansion of the effector/memory CD8<sup>+</sup> T cells [Gebhard et al., 1999; Shimojima et al., 1998]. Rabin et al., [1995] reported that HIV-infected children and adults had a decrease in the number of circulating naïve CD8<sup>+</sup> T cells subset (CD8<sup>+</sup> CD62L-selectin<sup>pos</sup> CD11a<sup>low</sup>

CD45RA<sup>high</sup>) and an increase in CD8<sup>+</sup> cells with memory or activated phenotype (CD8<sup>+</sup> CD62L<sup>neg</sup> CD11a<sup>Hi</sup> CD45RA<sup>Lo</sup>). The memory cells make up more than 80% of the total CD8<sup>+</sup> T cells in the circulation of individuals with long-term HIV infections [Roederer et al, 1995]. Rabin et al., [1995] also reported an increase in the number of activated CD4<sup>+</sup> T cells. Gebhard et al., [1999] reported that FIV infection causes an increase in CD8<sup>+</sup>CD62L<sup>neg</sup>CD49<sup>Hi</sup>CD44<sup>Hi</sup>CD18<sup>Hi</sup> population in the circulation.

Tompkins et al., [2002] recently reported an unusual increase in the number of CD8<sup>+</sup> and CD4<sup>+</sup> T cells expressing B7.1, B7.2 and CTLA4 on their surface in the circulation and lymph nodes from FIV-infected asymptomatic cats. Flow cytometric analysis using antibodies specific for feline B7.1 and B7.2 costimulatory molecules along with the human CTLA4-Ig fusion protein revealed an increase in the percentage of CD8<sup>+</sup> cells expressing B7 and CTLA4 molecules in the lymph nodes and blood of FIV-infected cats as compared to uninfected cats [Tompkins et al., 2002]. The percentages of CD8<sup>+</sup> B7<sup>+</sup> cells progressively increased with time, such that the majority of the CD8<sup>+</sup> T cells in the lymph nodes of long term FIV-infected cats expressed B7 molecules [Tompkins et al., [2002]. The progressive increase in CD8<sup>+</sup> B7<sup>+</sup> cells in the blood of FIV-infected cats is also consistent with the previously described expansion of CD8<sup>+</sup> CD62L<sup>neg</sup> in the blood of infected cats [Gebhard et al., 1999]. Some of the CD8<sup>+</sup> B7<sup>+</sup> cells are CD62L<sup>neg</sup>, suggesting that they

are a subset of the larger CD8<sup>+</sup> CD62L<sup>neg</sup> anti-FIV suppressor cells previously described by Bucci et al., [1998] and Gebhard et al., [1999].

Barker, Bossart et al., [1998 ] reported that the predominant anti-HIV cells in HIV-infected individuals were CD8<sup>+</sup> CD28<sup>+</sup> T cells and the upregulation of CD25 correlated with antiviral activity. Vahlenkamp et al., [2004] discovered a subset of CD8<sup>+</sup> T cells in the blood and lymph nodes that express CD25. The number of CD8<sup>+</sup> CD25<sup>+</sup> T cells in the LN increase with time after FIV infection and these cells also express the B7 molecules on their surface as compared to CD8<sup>+</sup> CD25<sup>+</sup> T cells isolated from the LN of FIV negative cats [Vahlenkamp et al., 2004]. These data suggest that FIV infection increases both the number and activation status of CD8<sup>+</sup> CD25<sup>+</sup> T cells.

B7 costimulatory molecules are normally found on professional antigen presenting cells (APC) and sequentially interact with CD28 and CTLA4 on T cells to provide the necessary second signal for regulating the immune response [Greenfield et al., 1998; Harris et al., 1999]. B7 engagement of CD28 on T cells transduces a signal for IL-2 mRNA stabilization and transcription that is necessary for the initiation of an immune response [Harris et al., 1999]. B7 engagement of CTLA4 on activated T cells transduces a signal for transcriptional suppression of IL-2 which would terminate the immune response [Bluestone et al., 1996]. IL-2 is a growth factor that's required for the activation of T lymphocytes [Walunas et al., 1994].



As FIV and HIV replication are enhanced by IL-2 [Kinter et al., 1995; Hewitt unpublished 2004], it is possible that B7 ligation of CTLA4 on infected CD4<sup>+</sup> T cells may also indirectly terminate FIV gene transcription.

Many similarities exist between FIV and HIV antiviral responses mediated by CD8<sup>+</sup> T cells. Our laboratory previously reported that the CD8<sup>+</sup> anti-FIV activity can be attributed to a subset within the activated CD8<sup>+</sup>β<sup>lo</sup>CD62L<sup>-</sup>CD49d<sup>hi</sup>CD18<sup>hi</sup> population that is found in the blood of FIV infected cats [Bucci, Gebhard et al., 1998, Gebhard et al., 1999]. However, not all FIV positive cats with this phenotype have CD8<sup>+</sup> T cell mediated antiviral activity, suggesting that another subset of CD8<sup>+</sup> T cells may be responsible for the anti-FIV activity. Recently, we described a CD8<sup>+</sup>B7.1<sup>+</sup>B7.2<sup>+</sup>CTLA4<sup>+</sup> subset of activated CD8<sup>+</sup> T cells that are present in the lymph nodes and blood of FIV infected cats. The above observations suggest that FIV infection induces the emergence of CD8<sup>+</sup> T cells that are phenotypically and functionally distinct. In this study, experiments were designed to determine the specific CD8<sup>+</sup> cell surface phenotype(s) that mediate antiviral activity and to determine whether the subset (s) have an effect on IL-2 mRNA and FIV gag gene expression in infected CD4<sup>+</sup> T cells. The identification of the CD8<sup>+</sup> antiviral T cell subset(s) will further our understanding of their role during FIV infection.

## **MATERIALS AND METHODS**

### **Animals**

Specific pathogen-free (SPF) cats were purchased from Liberty Laboratory (Liberty Corners, NJ) or Cedar River Laboratory (Mason City, IA). Twenty-two cats were inoculated intravaginally or intravenously with cell-free or cell-associated form of NCSU<sub>1</sub> isolate of FIV as previously described [Bucci et al., 1998; Tompkins et al., 2002]. Cats were seropositive for FIV as determined by commercial ELISA (IDEXX), and proviral DNA-positive as determined by polymerase chain reaction (PCR) for gag sequences [Bucci et al., 1998; Gebhard et al., 1998]. At the time of this study, the cats were at least 3-4 years of age, were infected with FIV for at least 2 years and were clinically asymptomatic.

### **Sample Collection**

Asymptomatic FIV-NCSU<sub>1</sub> infected cats were anesthetized with ketamine (20µg/kg) given intravenously. Twenty milliliters (mL) of whole blood were collected by jugular venipuncture into EDTA anticoagulant vacutainer tubes and separated into plasma fraction by centrifugation at 1500 rpm. Peripheral blood mononuclear cells were isolated on percoll gradients as previously described by Tompkins et al., [1987]. Complete blood counts were performed on a Coulter Counter and absolute leukocyte numbers were determined from a manual differential cell count. PBMC were counted using the Trypan Blue-hemocytometer method.

## **Lymph node biopsy**

Lymph node cells were obtained from peripheral lymph node biopsies as described by [Levy et al., 1998]. Cats were anesthetized with ketamine or diazepam given intravenously and maintained with inhalant anesthesia, isoflurane, for excisional biopsy of popliteal lymph nodes. Butophanol tartrate was administered at the conclusion of each surgery to control post-operative discomfort. Single cell suspensions of lymph node cells (LNC) were prepared by gently passing the tissue through a steel mesh screen.

## **FIV-NCSU<sub>1</sub>**

The FIV-NCSU<sub>1</sub> was originally isolated from a naturally infected cat at North Carolina State University College of Veterinary Medicine [English et al., 1993]. The cat presented with acute enteritis and lymphopenia, which resolved with supportive therapy. Feline immunodeficiency virus infection was diagnosed based on the presence of serum antibodies to FIV using an ELISA method (IDEXX, Portland, ME) and western blot, and the presence of FIV provirus detected by PCR in PBMC from this cat. This cat was not infected with FeLV based on a lack of antigenemia (Pet Check FeLV Elisa, TechAmerica, Omaha, NE), a lack of Mn<sup>2+</sup> dependent RT activity in PBMC cocultures and a lack of detectable FeLV provirus in the PBMC. The cat remained clinically normal for more than 4 years, with a CD4: CD8 ratio below 0.5.

### **Generation of FIV-NCSU<sub>1</sub> virus stock**

PBMC were isolated from FIV-NCSU<sub>1</sub> infected cats and were stimulated with ConA (2 µg/mL) for 24 hours. PBMC were washed twice with culture medium and cocultured with an equal number of FCD4E cells in the presence of 200 U/mL recombinant human IL-2. Culture supernatants were harvested when the cells formed large syncytia and gag p24 antigen production was positive in the FIV p24 antigen capture ELISA assay. The virus stock was titrated in FCD4E cells and had a TCID<sub>50</sub> of 10<sup>6.5</sup>. No viral associated DNA was present in the viral stock as analyzed by PCR. The virus stock was treated with Dnase I (300 U/mL) for 30 minutes at room temperature [Joshi et al., 2004].

### **FCD4E cells**

FCD4E cells were established in our laboratory through long term culture of PBMC from SPF cats in the presence of recombinant human IL-2 (rHuIL-2). FCD4E cells are an interleukin-2-dependent feline CD4<sup>+</sup> T lymphocyte cell line [English et al., 1993]. The FCD4E cells are 100% positive for the feline pan-T cell marker 1.572, 60-65% positive for the feline CD4 marker as determined by CAT30A antibody, and negative for the CD8 marker as determined by CD8<sup>+</sup> monoclonal antibody (3.357). FCD4E cells were maintained in complete RPMI media supplemented with 100 units/mL of rHuIL-2.

### **Assessment of CD8<sup>+</sup> T cell inhibition of FIV replication in autologous PBMC**

Percoll separated PBMC ( $10^6$ ) were incubated with immunomagnetic beads precoated with goat anti-mouse IgG (M450; Dynal, Lake Success, NY) and treated with anti- CD8<sup>+</sup> monoclonal antibody (3.357 or 117) at a bead to cell ratio of 3:1. The bead:cell mixture was incubated with agitation at 4° C for 1 hour and magnetically sorted to obtain the CD8<sup>+</sup> -depleted PBMC. Total PBMC and CD8<sup>+</sup> depleted PBMC were analyzed using FACScan flow cytometry for distribution of CD4, and CD8 and B cell phenotypes by two color flow cytometry analysis.

Magnetic bead sorting routinely removed  $\geq 98\%$  of the CD8<sup>+</sup> T cells. For the modified endogenous assay, total PBMC ( $10^5$ ) or PBMC depleted of CD8<sup>+</sup> cells ( $10^5$ ) or B7.1<sup>+</sup> cells ( $10^5$ ) were cultured with uninfected feline CD4<sup>+</sup> T lymphocytes (FCD4E) ( $10^5$ ) in the presence of 100 units/mL recombinant human interleukin 2 (AIDS Research and Reference Reagent Program, NIH Bethesda, MD). Uninfected feline CD4<sup>+</sup> T lymphocytes (FCD4E) ( $10^5$ ) served as a negative control. Cell-free culture supernatants were collected at 9 days of culture and assayed for FIV p24 production by ELISA. Inhibition of FIV production was expressed as a reduction of FIV p24 optical density in the presence of CD8<sup>+</sup> T cells as compared to FIV p24 optical density of CD8<sup>+</sup>-depleted PBMC.

Supernatant collected from Crandell feline kidney cells infected with the Petaluma strain of FIV was used as the internal positive control for the p24 ELISA assay. Similar methods were used to evaluate the antiviral activity of B7<sup>+</sup> cells and CD25<sup>+</sup> cells.

### **FIV p24 antigen capture ELISA**

FIV p24 antigen was detected in culture supernatants using an antigen capture ELISA. Immunolon 2HB plates (Dyner) were coated overnight with mAb p24Cr1 (Custom Monoclonals, Sacramento, CA) at 4°C and blocked. Antigen was prepared by treating culture supernatants with 1% Triton-X-100. Samples were added to the p24Cr1-coated-plate and incubated for 1.5 hr at 37°C. At the end of the incubation, ELISA plate was washed 2X with and rinsed 3X with PBS buffer. This was followed by the addition of biotin-conjugated anti-gag antibody PAK3-2C1 (Custom Monoclonals, Sacramento, CA) and subsequently extravidin-peroxidase (Sigma), each allowed to incubate for 1hr at 37°C. The reaction was developed using TMB peroxidase substrate (KPL Labs, Maryland). Color reaction was stopped using 100µL 2M H<sub>2</sub>SO<sub>4</sub> and the optical density (O.D.) measured at 450nm (reference filter 405 nm).

### **Assessment of Plasma Viral Load**

Blood was collected into EDTA tubes and centrifuged for 5 minutes at 1200 rpm. The plasma was collected and stored at -20 or -70 °C until analyzed. The samples were slowly thawed on ice and RNA was extracted from 140 µL using Qiagen QIAmp Viral RNA kit (Qiagen, Valencia, CA) following the manufacturer's instructions. The viral RNA was reverse transcribed using the Taq Man One Step RT-PCR from Applied Biosystems (Branchburg, New Jersey). Gag specific sequence primers were designed using software from Applied Biosystems based on sequences reported by Leutenegger, et al., (1999).

The NCSU<sub>1</sub> specific probe for the real time PCR reaction was designed as specified by Leutenegger, et al., (1999).

(Forward primer sequence: FIVNC.491f 5'GATTAGGAGG TGAGGAAGT TCAGCT3', reverse primer sequence: FIVNC.617R: 5'CTTCATCCA ATATTTC TTTATCTGCA3'; Probe: FIVNC.555P 5'FAM-CATGGC CACATTAA TAATGGC CGCA-TAMRA3').

Standards consisted of the NCSU<sub>1</sub> gag plasmid and a pcDNA3.1 gag that were run in serial dilutions to generate a standard curve. Ambion's mMessage mMachine kit (Austin, TX) was used to generate the RNA gag standard. RNA standard is estimated at  $1 \times 10^{11}$  molecules/mL. Standards were used at  $10^0$ - $10^6$  molecules/sample in ten fold dilutions. Negative controls consisted of nuclease-free water, PCR master mix with primers, probe and no-RNA template. A standard curve was developed and used to calculate the number of viral particles per mL of plasma.

The real-time PCR reaction was assembled as specified by the manufacture with minor adjustments. Twenty  $\mu$ L of RNA was incubated in RT-PCR master mix with 100nM final concentration of forward and reverse primers in the presence of an RNase inhibitor (0.40U/mL) and with 250 nM final concentration of the Taq Man Probe. The real time PCR assay was performed on the BioRad i-cycler (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK) using the following thermal cycling parameters: 30 minutes at 48 °C for the Reverse Transcription, 10 minutes at 95 °C for AmpliTaq Gold Activation, 50 cycles of 15 seconds denature step at 95 °C and 1 minute anneal/extend step at 60 °C.

Fluorescence was detected at the end of each anneal/extend step and analyzed post run.

### **Flow Cytometric Analysis of lymphocyte surface antigens**

Three color flow cytometric analysis was used to determine the percentages of CD4<sup>+</sup>, CD8<sup>+</sup>, CD25<sup>+</sup> and B7<sup>+</sup> cells in whole blood samples with the use of fluorescein isothiocyanate-anti-feline CD4 (MAb CAT 30A) [Tompkin et. al 1990], allophycocyanin-anti-feline CD8 (MAb 117) or PE-anti-CD8 (MAb 3.357), allophycocyanin-anti-feline CD25 [Ohno et al., 1992]. When rabbit anti-feline B7.1 and anti-feline B7.2 antibodies were used [Tompkins et. al 2002], cells were stained first with either of these antibodies, followed by a PE-labeled donkey-anti rabbit antibody [Jackson ImmunoResearch Laboratories, West Grove, Pa.], and finally with the anti-CD4 and anti-CD8 directly conjugated antibodies. Negative controls consisted of isotype matched MAbs or preimmunization rabbit serum. CD8<sup>+</sup> cells were monitored for distinct populations as determined on the basis of fluorescence intensity of staining by antibodies specific for the CD8 $\alpha$  chain (MAb 3.357) or CD8 $\beta$  chain (MAb 117), as described by Bucci et al 1998 and Gebhard et al., 1999. The percentages of CD8 $\beta$ <sup>lo</sup> and CD8 $\beta$ <sup>hi</sup> cells were calculated on the basis of total CD8<sup>+</sup> cells as determined by staining for the CD8 $\alpha$  chain. All incubation were done at 4° C. Care was taken to perform all flow cytometry as soon after collecting samples from the cats as possible, to avoid any culture-induced changes in expression of costimulatory molecules. Data were acquired on a FACS Caliber flow cytometer (Becton Dickinson), with use of a helium-neon laser as the second excitation source for the allophycocyanin-stained samples.



For all samples, data from at least 15, 000 cells were acquired and stored list-mode fashion for subsequent analysis. Gated data were generated for fluorescent analysis of lymphocytes, as defined by forward and side scatter. Box and whisker plots were used to graphically show the distribution of the percentage of CD4<sup>+</sup> or CD8<sup>+</sup> T cells expressing B7.1, B7.2 or CTLA4 surface markers. The box and whiskers plot represents five values: the lowest value of the data set (minimum), the 25<sup>th</sup> percentile (Quartile 1), the midpoint of the data set (median), the 75<sup>th</sup> percentile (Q3), the highest value of the data set (maximum).

#### **Separation of CD8<sup>+</sup> Subsets using Cytomation MoFlo Hi-Speed Cell Sorter**

CD8<sup>+</sup>CD25<sup>+</sup> or B7.1<sup>+</sup> and CD8<sup>+</sup>CD25<sup>-</sup>, CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> subsets were sorted using Cytomation MoFlo Cell Sorter based upon the criteria of fluorescence intensity of PE conjugated to monoclonal antibody CD8 (3.357) or PE-conjugated to monoclonal antibody CD4 (30A) or FITC conjugated to monoclonal antibody CD25 or B7.1. Purity of the sorted subsets >90% was confirmed by FACS analysis. These purified CD8<sup>+</sup> subsets or CD4<sup>+</sup> subsets were washed 2X with HBSS and resuspended in complete RPMI 1640 media and used in subsequent exogenous FIV inhibition studies.

### **FIV inhibition by CD8<sup>+</sup>T cell subsets using the Acute Infection Assay**

To determine the relative antiviral activity of CD8<sup>+</sup> T cell subsets, a modified version of an acute infection assay described by Bucci, et al. [1998] was used. FCD4E cells ( $10^5$ ) were infected with FIV NCSU<sub>1</sub> at an MOI of 0.1 TCID<sub>50</sub> for 2 hours, washed and incubated for 18 hours and cultured with CD8<sup>+</sup> subsets ( $10^5$ ) in the presence of 100 units/mL of rhIL-2. CD8<sup>+</sup> subsets were obtained by incubating PBMC with immunomagnetic beads precoated with goat anti-mouse IgG (M450; Dynal, Lake Success, NY) and treated with anti-CD4 (30A) or anti-CD21 (that binds B cells) or anti-CD25 monoclonal antibodies at a bead to cell ratio of 3:1. The cell:bead mixture was incubated as previously described in the Methods section. As a positive control, FIV-NCSU<sub>1</sub> infected FCD4E cells were cultured in the absence of effector CD8<sup>+</sup> subsets. After 3, 5, 7, and 9 days of incubation, cell-free culture supernatants were assayed for FIV p24 by ELISA. To assess the antiviral activity mediated by Cytomation MoFlo purified CD8<sup>+</sup> CD25<sup>+</sup> and CD8<sup>+</sup> CD25<sup>-</sup> subsets. These subsets were cocultured with FCD4E cells infected with 0.1 MOI FIV-NCSU<sub>1</sub> RPMI 1640 at CD8:FCD4E ratios ranging from 1.0-0.1. This dose of virus yields a productive infection of FCD4E cells with syncytia formation and high levels of FIV-p24 following 9 days of coculture, with inhibition of FIV production expressed as a reduction of p24 optical density values as compared to p24 levels obtained from FCD4E cells cultured alone.

### **IL-2 Treatment of Effector Cells**

CD8<sup>+</sup>-depleted PBMC ( $10^5$ ) or total PBMC ( $10^5$ ) were cocultured with uninfected FCD4E ( $10^5$ ) cells in complete RPMI media in the presence or absence of

recombinant human IL-2 (100 U/mL or 200 U/mL or 300U/mL). After 9 days in culture, cell free supernatants (100 µL) were collected and assayed for the FIV p24 production by ELISA.

### **Assessment of IL-2 mRNA and FIV gag gene analysis**

Blood was collected from cats into EDTA tubes and centrifuged for 5 minutes at 1200 rpm and the plasma fraction was removed. Freshly isolated PBMC were collected using percoll gradients. PBMC were counted using trypan blue method. PBMC were depleted of CD8<sup>+</sup> T cells using (3.357) monoclonal antibody coated magnetic beads. CD8-depleted PBMC or total PBMC were stimulated with or without 10 µg/mL of ConA for 4-6 hours. RNA was extracted from 10<sup>6</sup> CD4<sup>+</sup> T cells using Qiagen QIAmp (Qiagen, Valencia, CA) following manufacturer's instructions. 20 µL volume of RNA was reverse transcribed using the Taq Man One Step RT-PCR from Applied Biosystems (Branchburg, New Jersey). Gag specific sequence primers were designed as previously described in methods. IL-2 and GAPDH specific probe and primers sequences were designed using software from Applied Biosystems based on sequences obtained from feline interleukin 2 sequences in PubMed GeneBank. Negative controls consisted of nuclease-free water, PCR master mix with primers, probe and no-RNA template.

Feline IL-2F 5'CTTTTGAATGGAGTTAATAATCCTGAGA 3';

Feline IL-2R 5'CCTTCTTGGGCACGTAAAATTTA3';

Probe 6famCCCCAACTCTCCAGGATCACAtamara;

Probe6famCCAGCAGCGAGATCCCGCCAtamara

Feline GADPHF5'TGGAAGCCCATCACCATCT3'

FelineGADPHR 5'CCAGCATCACCCCATTGA3'

The real-time reaction was assembled as specified by the manufacture with minor adjustments. A twenty  $\mu$ L volume containing RNA was incubated in RT-PCR master mix with 50 nM final concentration of forward and reverse primers in the presence of an RNase inhibitor (0.40U/mL) and with 250 nM final concentration of the Taq Man Probe. The real time PCR assay was performed on the BioRad i-cycler (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK) using the following thermal cycling parameters: 30 minutes at 48°C for the Reverse Transcription, 10 minutes at 95 °C for AmpliTaq Gold Activation, 45 cycles of 15 seconds denature step at 95 °C and 1 minute anneal/extend step at 60 °C. Fluorescence was detected at the end of each anneal/extend step and analyzed post run. Result were reported as cycle threshold C (t), which is defined as the amplification cycle for the signal from a particular well that exceeds the background fluorescent intensity, indicating amplified product.

### **Statistical Analysis**

FIV p24 data were analyzed using the Student's t test to determine significant differences between means. All FIV p24 data are presented as mean +/- standard deviation. The Mann-Whitney nonparametric test was used to compare the expression of surface markers on CD4, or CD8 cell populations.

A p-value <.05 was considered significant.

## Results

### **FIV-NCSU<sub>1</sub> INDUCES CD8<sup>+</sup> T CELL MEDIATED ANTI-FIV ACTIVITY THAT CORRELATES WITH A REDUCTION IN CELL-ASSOCIATED AND CELL-FREE VIRUS IN ASYMPTOMATIC FIV-INFECTED CATS**

CD8<sup>+</sup> T cell mediated antiviral activity has been demonstrated in cats infected with feline immunodeficiency virus (FIV), a lentivirus that induces feline AIDS with characteristics similar to human AIDS [Pederson et al., 1987]. We previously reported that CD8<sup>+</sup> mediated anti-FIV activity was expressed during the acute stage of infection and was sustained through the asymptomatic stage of infection [Gebhard et al., 1999; Bucci et al., 1998; Jeng et al., 1996]. We also recently reported that an usually high number of CD8<sup>+</sup> and CD4<sup>+</sup> T cells in the blood of FIV positive cats co-express B7.1 and B7.2 costimulatory molecules and their ligand CTLA4 [Tompkins et al., 2002; Vahlenkamp et al., 2004]. In this study, we investigate the phenotypic and functional correlates between the FIV-induced activation phenotype (CD8<sup>+</sup> B7.1<sup>+</sup> B7.2<sup>+</sup> CTLA4<sup>+</sup>) and CD8<sup>+</sup> T cell mediated antiviral activity in cats infected intravenously and vaginally with FIV-NCSU<sub>1</sub>.

### **Assessment of CD8<sup>+</sup> T cells inhibition of FIV replication in autologous PBMC**

To assay for the presence of feline CD8<sup>+</sup> T cell-mediated anti-FIV activity in asymptomatic FIV positive cats, CD8<sup>+</sup> T cells were depleted from the PBMC using immunomagnetic beads precoated with anti-CD8 $\alpha$  (3.357) monoclonal antibody or anti-CD8 $\beta$  (117) monoclonal antibody as previously described [Bucci et al., 1996; Jeng et al., 1996]. Anti-CD8 antibody-coated magnetic beads removed  $\geq 98\%$  of

CD8<sup>+</sup> cells from PBMC as determined by flow cytometric analysis (Figure 1). PBMC or CD8-depleted PBMC were cocultured with uninfected FCD4E cells at a ratio of 1:1. Cell-free supernatants were collected after 9 days and analyzed for FIV p24 production by ELISA. Table 1 shows the results of CD8<sup>+</sup> suppressor analysis of twenty-two FIV positive cats. Evidence of CD8<sup>+</sup> T cell mediated antiviral activity was determined using the formula developed by Bucci et al., [1998], which  $\geq 66\%$  suppression of FIV p24 production was considered strong suppressor activity. As seen in Table 1, 30% of these FIV positive cats showed significant suppressor activity and there is no correlation between the route of FIV infection and the presence of CD8<sup>+</sup> T cell-mediated antiviral activity. Figure 2 represents the results of 7 FIV positive cats.

FIV p24 was barely detectable in some cats suggesting that these cats may have cleared FIV from their circulation (Table 1). Loss of viral replication in PBMC cocultures reflects either a decrease in the number of infected cells or cellular control of viral expression as demonstrated by CD8<sup>+</sup> T cells. Cats showing evidence of CD8<sup>+</sup> antiviral activity by increased p24 expression in CD8<sup>+</sup> depleted-PBMC cocultures with uninfected FCD4E cells also showed high levels of FIV p24 when CD8-depleted PBMC were cultured alone, suggesting that there were enough FIV susceptible cells in the total PBMC population that could become infected. Cats that had little or no CD8<sup>+</sup> T cell mediated antiviral activity demonstrated very little or no reduction in cell-associated virus (Table 1). The presence of CD8<sup>+</sup> T cell

mediated antiviral activity correlates with a decrease in cell associated viremia during asymptomatic FIV infection.

### **Assessment of CD8<sup>+</sup> T cell inhibition of FIV replication in peripheral lymph node mononuclear cells**

The antiviral activity of CD8<sup>+</sup> T cells isolated from mononuclear cells collected from lymph nodes was also determined. Peripheral lymph nodes were examined in 4 suppressor cats. Figure 3 shows increased levels of FIV p24 antigen production in FCD4E cells cocultured with CD8<sup>+</sup> T cell-depleted lymph node mononuclear cells as compared to FCD4E cells cocultured with total lymph node mononuclear cells, demonstrating the presence of some CD8<sup>+</sup> T cell mediated anti-FIV activity in these cats.

### **Plasma Viral Load**

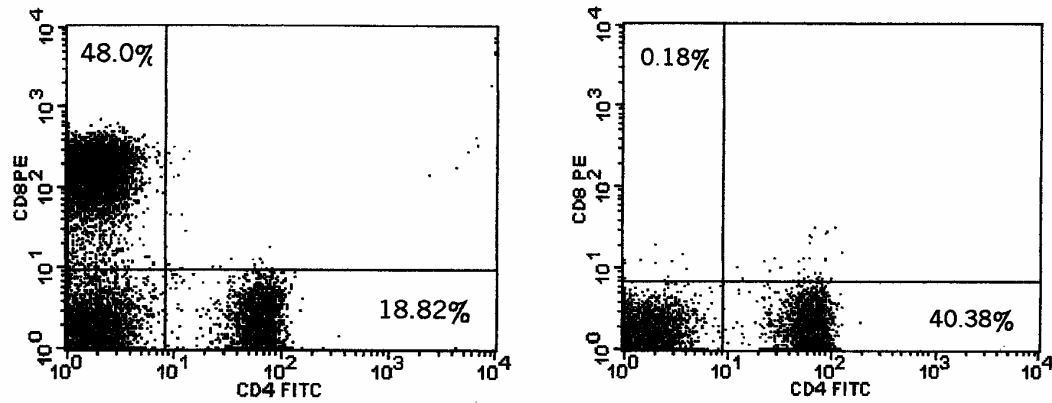
Plasma viremia represents the level of infectious virus produced *in vivo*, whereas cocultures measure the ability of stimulated PBMC to produce virus *in vitro*.

The expression of virus by PBMC in culture may not reflect the level of viral replication *in vivo*. Viral load in plasma has been shown to be a prognostic marker for disease progression in HIV-infected humans [Plaeger et al., 1998]. Several groups have reported an inverse correlation between CD8<sup>+</sup> T cells mediated antiviral activity in HIV-infected individuals and plasma viremia [Blackburn et al., 1996; Ferbas et al., 1995; Mackewicz et al., 1994]. We investigated the relationship between the presence of CD8<sup>+</sup> T cell mediated antiviral activity and plasma viremia.

Real Time-PCR analysis of plasma viremia showed that nonsuppressor cats have a significantly higher number of viral RNA molecules per milliliter of plasma than the suppressor cats, suggesting that CD8<sup>+</sup> anti-FIV cells may control FIV replication *in vivo* (Figure 4). These results also parallel the cell-associated viremia. Cell-associated and plasma virus were detected in cats infected vaginally and intravenously with FIV-NCSU<sub>1</sub>.

The results of this study show that CD8<sup>+</sup> T cell mediated anti-FIV activity in FIV-infected cats' correlates with a reduction in the plasma viral load. The cats used in this study were clinically healthy at the time these assays were performed.





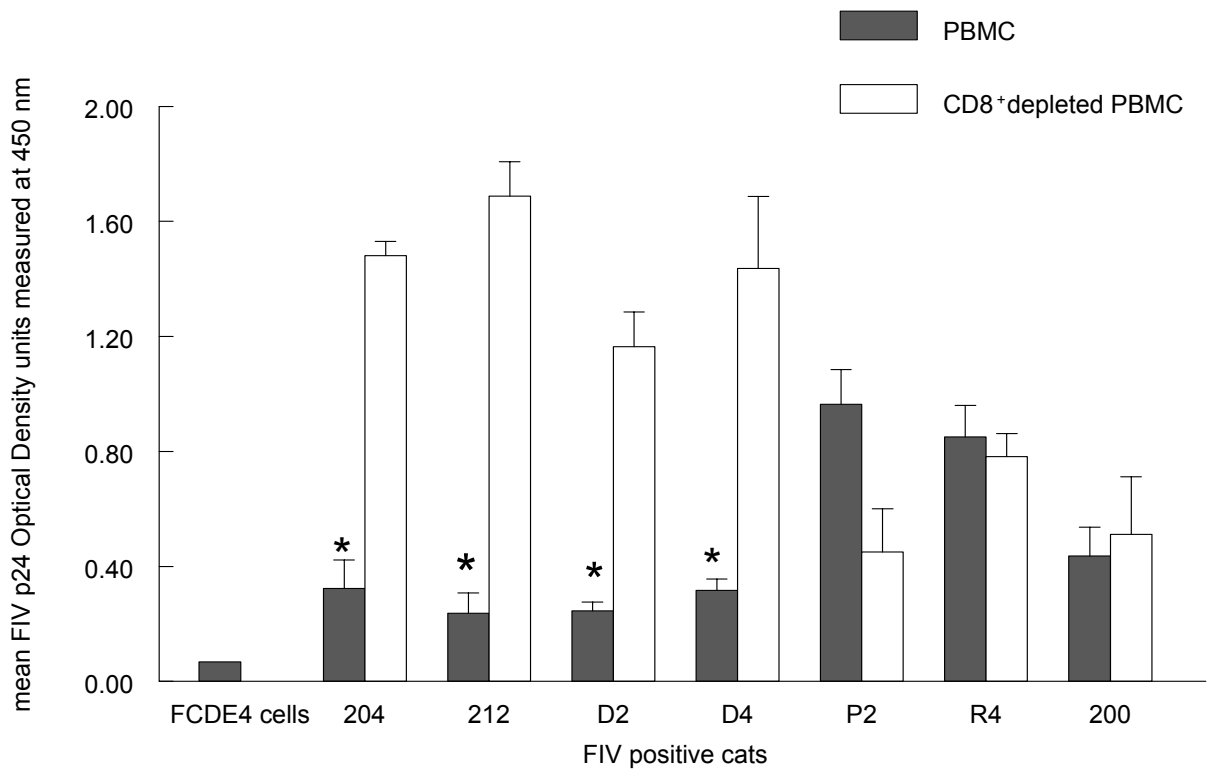
**Figure 1.** Dot plot flow cytometric analysis of CD8<sup>+</sup> T cells in PBMC isolated from FIV positive cats before and after depletion with anti-CD8 mAb coated magnetic beads. PBMC (10<sup>5</sup>) from asymptomatic FIV-NCSU<sub>1</sub> infected cats were depleted of CD8<sup>+</sup> cells using immunomagnetic beads precoated with anti-CD8 monoclonal antibody (3.357 or 117)). Total PBMC and CD8<sup>+</sup> depleted-PBMC were analyzed using two color flow cytometry for the distribution of CD8<sup>+</sup> and CD4<sup>+</sup> cells. Left panel: upper left quadrant represents the presence of CD8<sup>+</sup> T cells. Right panel: the absence of dots in the upper left quadrant represents the removal of CD8<sup>+</sup> T cells from PBMC after the depletion process.

**Table 1.**

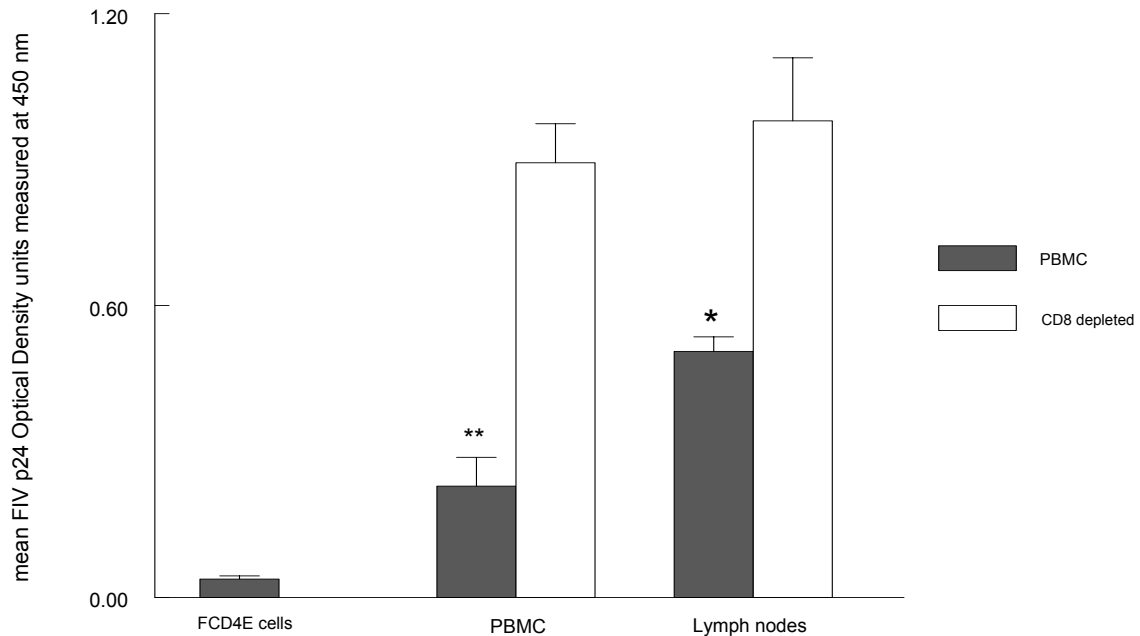
FIV p24 antigen in total PBMC or CD8<sup>+</sup> T cell-depleted PBMC from FIV-NCSU<sub>1</sub> infected asymptomatic cats.

Cats	Route of infection	Length of infection	O.D.		O.D.		% Suppression	CD4:CD8 ratio
			Total PBMC		CD8+ T cell depleted PBMC			
<b>AY</b>	<b>intravenous</b>	<b>5 years</b>	<b>0.401</b>	<b>± 0.12</b>	<b>1.109</b>	<b>± 0.18</b>	<b>66</b>	<b>0.6</b>
<b>D2</b>	<b>vagina</b>	<b>4.2 years</b>	<b>0.330</b>	<b>± 0.13</b>	<b>1.020</b>	<b>± 0.15</b>	<b>67</b>	<b>0.8</b>
<b>D4</b>	<b>vagina</b>	<b>4.2 years</b>	<b>0.421</b>	<b>± 0.26</b>	<b>1.235</b>	<b>± 0.08</b>	<b>66</b>	<b>2</b>
P2	intravenous	4.4 years	1.690	± 0.12	0.450	± 0.15	0	0.6
<b>A5</b>	<b>vagina</b>	<b>4 years</b>	<b>0.384</b>	<b>± 0.08</b>	<b>1.072</b>	<b>± 0.13</b>	<b>66</b>	<b>0.9</b>
R4	vagina	3.4 years	1.298	± 0.18	0.600	± 0.12	0	1.2
<b>204</b>	<b>intravenous</b>	<b>3.1 years</b>	<b>0.302</b>	<b>± 0.10</b>	<b>1.067</b>	<b>± 0.05</b>	<b>72</b>	<b>0.5</b>
<b>212</b>	<b>intravenous</b>	<b>3.1 years</b>	<b>0.271</b>	<b>± 0.07</b>	<b>1.673</b>	<b>± 0.12</b>	<b>84</b>	<b>0.4</b>
200	vagina	2.6 years	0.437	± 0.16	0.512	± 0.22	15	0.7
210	vagina	3 years	0.419	± 0.09	0.489	± 0.15	14	0.7
201	vagina	3.3 years	0.690	± 0.14	0.952	± 0.17	28	0.7
211	vagina	3.3 years	0.460	± 0.12	0.353	± 0.20	0	0.8
207	vagina	3 years	0.584	± 0.15	0.664	± 0.21	12	1
245	intravenous	2.2 years	0.446	± 0.15	0.610	± 0.16	27	0.8
249	intravenous	2.2 years	0.669	± 0.21	0.409	± 0.19	0	0.4
243	intravenous	2.4 years	0.690	± 0.09	0.790	± 0.23	13	0.9
245	intravenous	2.4 years	0.850	± 0.12	0.782	± 0.08	0	0.6
241	intravenous	2.1 years	0.874	± 0.21	1.182	± 0.12	26	0.9
249	intravenous	2.2 years	0.785	± 0.13	1.154	± 0.21	32	0.4
242	intravenous	2.2 years	0.635	± 0.12	1.020	± 0.16	38	0.7
<b>417</b>	<b>intravenous</b>	<b>2.2 years</b>	<b>0.314</b>	<b>± 0.11</b>	<b>0.952</b>	<b>± 0.25</b>	<b>67</b>	<b>0.6</b>
<b>421</b>	<b>intravenous</b>	<b>2 years</b>	<b>0.301</b>	<b>± 0.21</b>	<b>1.309</b>	<b>± 0.20</b>	<b>77</b>	<b>1.6</b>

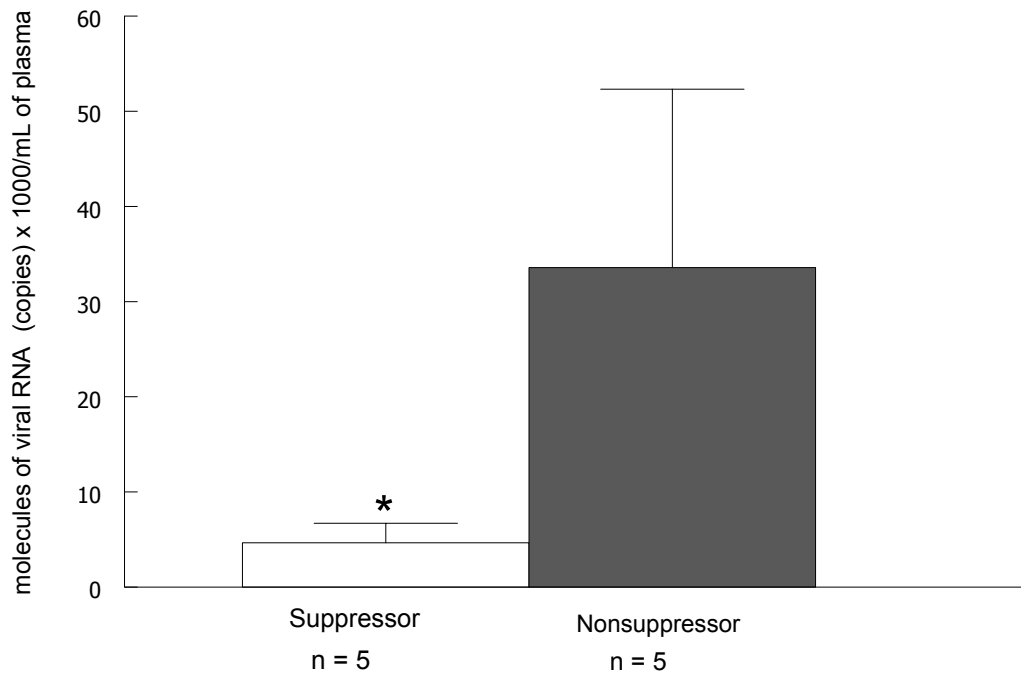
Final cell concentration was adjusted to (10<sup>5</sup> PBMC) or (10<sup>5</sup>) CD8<sup>+</sup>-depleted PBMC cocultured with (10<sup>5</sup>) uninfected FCD4E cells/200µL. After 9 days of coculture, cell-free supernatants were collected and analyzed for FIV p24 antigen using an ELISA method. Results are expressed as the mean ± standard deviation of the optical density (O.D.) at 450 nm of the anti-p24 ELISA with 3-5 replicates per cat. The average O.D. of the negative control, uninfected FCD4E cells cells, 0.09.



**Figure 2.** Assessment of CD8<sup>+</sup> T cell-mediated antiviral activity in autologous peripheral blood mononuclear cells (PBMC) using CD8<sup>+</sup> T cell depletion assay. PBMC ( $10^5$ ) from asymptomatic FIV-NCSU<sub>1</sub>-infected cats were depleted of CD8<sup>+</sup> cells using immunomagnetic beads precoated with anti-CD8 monoclonal antibody (3.357). CD8<sup>+</sup> depleted-PBMC ( $10^5$ ) and total PBMC ( $10^5$ ) were cocultured with ( $10^5$ ) uninfected CD4<sup>+</sup> T lymphocytes (FCD4E). After 9 days of culture, supernatants were assayed for FIV p24 by ELISA. Uninfected CD4<sup>+</sup> T lymphocytes (FCD4E) were used as a negative control. Striped bars, FIV p24 from total PBMC; white bars, CD8<sup>+</sup> T cell depleted-PBMC cocultures. FIV p24 values are expressed as optical densities (OD). Bars and error bars represent the mean  $\pm$  S.D. of 3-5 replicates ELISAA.\* Total PBMC and CD8<sup>+</sup> depleted-PBMC cocultures are significantly different ( $p < .005$ ) by student *t*-test.



**Figure 3.** Assessment of CD8<sup>+</sup> T cell-mediated antiviral activity in lymph nodes of suppressor cats. PBMC or lymph node mononuclear cells ( $10^5$ ) from FIV-NCSU<sub>1</sub>-infected cats were depleted of CD8<sup>+</sup> cells using immunomagnetic beads precoated with anti-CD8 monoclonal antibody (3.357). CD8<sup>+</sup> depleted-PBMC ( $10^5$ ) and total PBMC ( $10^5$ ) were cocultured with  $10^5$  uninfected CD4<sup>+</sup> T lymphocytes (FCD4E). After 9 days of culture, supernatants were assayed for FIV p24 by ELISA. Uninfected CD4<sup>+</sup> T lymphocytes (FCD4E) were used as a negative control. FIV p24 values are expressed as optical densities (OD). Bars and error bars represent the mean  $\pm$  S.D. of 5 wells/ELISA for 1 suppressor cat. Striped bars, FIV p24 from total PBMC or total LN mononuclear cells; white bars, CD8<sup>+</sup> T cell depleted cocultures. \* Denotes a significant difference between total and CD8<sup>+</sup>-depleted LN mononuclear cells (p .004). \*\* Denotes significant difference between total and CD8<sup>+</sup>-depleted PBMC (p.0003) by student t-test. Graph is representative of 4 suppressor cats used in the study.



**Figure 4.** Nonsuppressor cats have a significantly higher number of viral RNA molecules per mL of plasma. Plasma was collected from EDTA anti-coagulated whole blood by centrifugation for 5 minutes at 1200 rpm. FIV-NCSU<sub>1</sub> viral RNA was isolated from 140 µL as described by Qiagen Viral RNA Mini Kit. 20 µL of isolated plasma viral RNA was reverse transcribed in 50 µL reaction which consisted of FIV-NCSU<sub>1</sub> gag primers and PCR master mix using a Biorad *iCycler* Real Time PCR detection system. Negative control consisted of a no template mixture of 25 µL of PCR master mix, 2.0 µL of FIV-NCSU<sub>1</sub> gag primers, probe and 23 µL of dH<sub>2</sub>O. Positive controls consisted of 10 µL of serial dilution of FIV NCSU<sub>1</sub> gag standards, 2.0 µL of FIV-NCSU<sub>1</sub> gag primers, probe, 25 µL of PCR master mix and 13 µL of dH<sub>2</sub>O. \* Represents a significant difference between viral RNA molecules per mL of plasma in suppressor cats (n=5) and nonsuppressor cats (n=5) (p< .0001).

## **IMMUNOLOGICAL CHARACTERIZATION OF CATS WITH AND WITHOUT CD8<sup>+</sup> T CELL MEDIATED ANTI-FIV ACTIVITY**

Shimajima et al [1998] reported an increase in two subsets of CD8<sup>+</sup> T cells, CD8 $\alpha^+\beta^{\text{lo}}$  and CD8 $\alpha^+\beta^{\text{neg}}$  in the peripheral blood of FIV infected cats. The CD8 $\alpha^+\beta^{\text{lo}}$  subset increases as early as 3 weeks post infection and the subset is maintained throughout the asymptomatic phase [Shimajima et al 1998; Bucci et al [1998]. Bucci et al., [1998 and Flynn et al., [1997] reported that the CD8 $\alpha^+\beta^{\text{lo}}$  subset had strong antiviral activity during acute and long-term infections. Gebhard et al., [1999] reported that CD8<sup>+</sup> T cells in the majority of cats with asymptomatic FIV infection had a characteristic activation phenotype (CD8<sup>+</sup>  $\beta^{\text{lo}}$ CD62L<sup>neg</sup>CD44<sup>Hi</sup>CD49d<sup>Hi</sup>CD18<sup>Hi</sup>). However, not all FIV positive cats with this CD8<sup>+</sup> activation phenotype have CD8<sup>+</sup> T cell mediated antiviral activity, suggesting that a subset within this larger population is responsible for mediating antiviral activity [Bucci et al., 1998; Gebhard et al., 1999; Jeng et al., 1996]. We recently determined that FIV infection induces CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the blood and lymph nodes to express an unusual B7.1<sup>+</sup> B7.2<sup>+</sup> CTLA4<sup>+</sup> phenotype [Tompkins et al., 2002; VahlenKamp et al., 2004].

### **Flow Cytometric Analysis of Lymphocyte Surface Antigens**

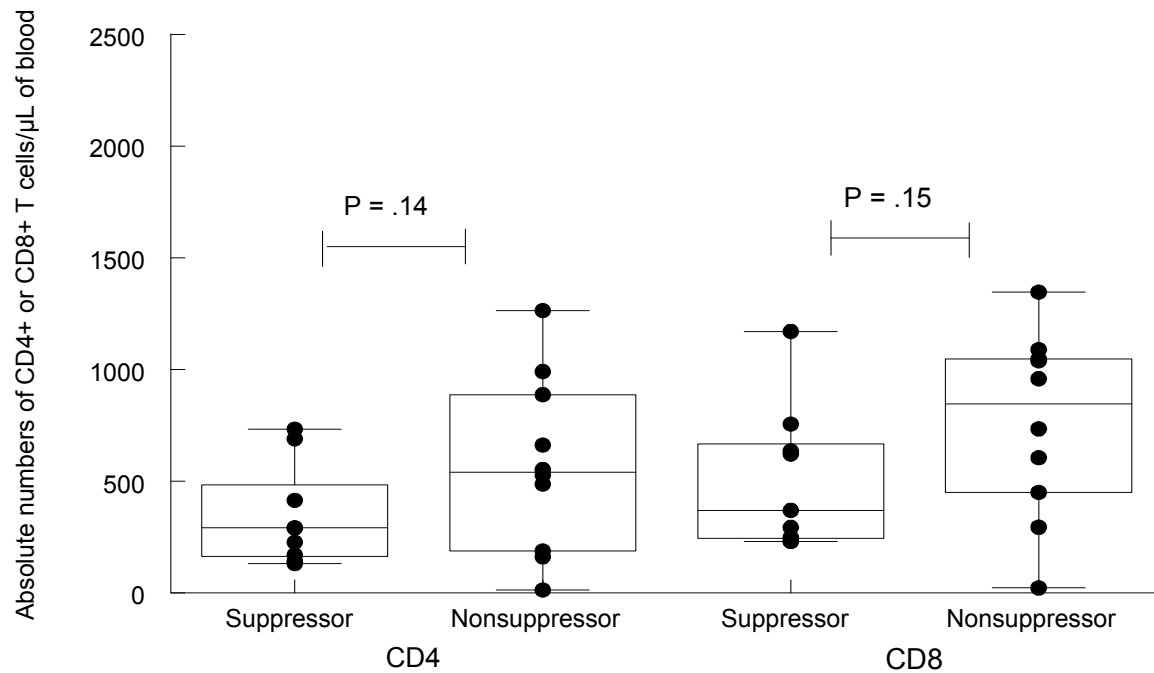
To more precisely identify the specific subset of CD8<sup>+</sup> antiviral cells, we compared the cell surface phenotype of CD8<sup>+</sup> T cells in suppressor and nonsuppressor cats using multicolor flow cytometry with antibodies specific for CD8 $\beta$  chain, B7.1, B7.2, CTLA4, and CD25. We also compared the absolute number of lymphocyte populations within both suppressor and nonsuppressor groups. There was not a

significant difference in the absolute numbers of CD4<sup>+</sup> or CD8<sup>+</sup> T cells in the peripheral blood of suppressor and nonsuppressor cats (Fig. 5). The suppressor cats were infected for more than 3 years and 45-75% of the total CD8<sup>+</sup> T cells had an activation phenotype, CD8 $\alpha^+\beta^{\text{lo}}$ . We observed the presence of CD8 $\alpha^+\beta^{\text{lo}}$  in both suppressor and nonsuppressor cats. However, suppressor cats had a significantly higher percentage of CD8 $\alpha^+\beta^{\text{lo}}$  cells, indicative of an activation phenotype, as compared to nonsuppressor cats (Fig. 6). Nonsuppressor cats had a significantly higher percentage of CD8 $\alpha^+\beta^{\text{Hi}}$  cells as compared to suppressor cats.

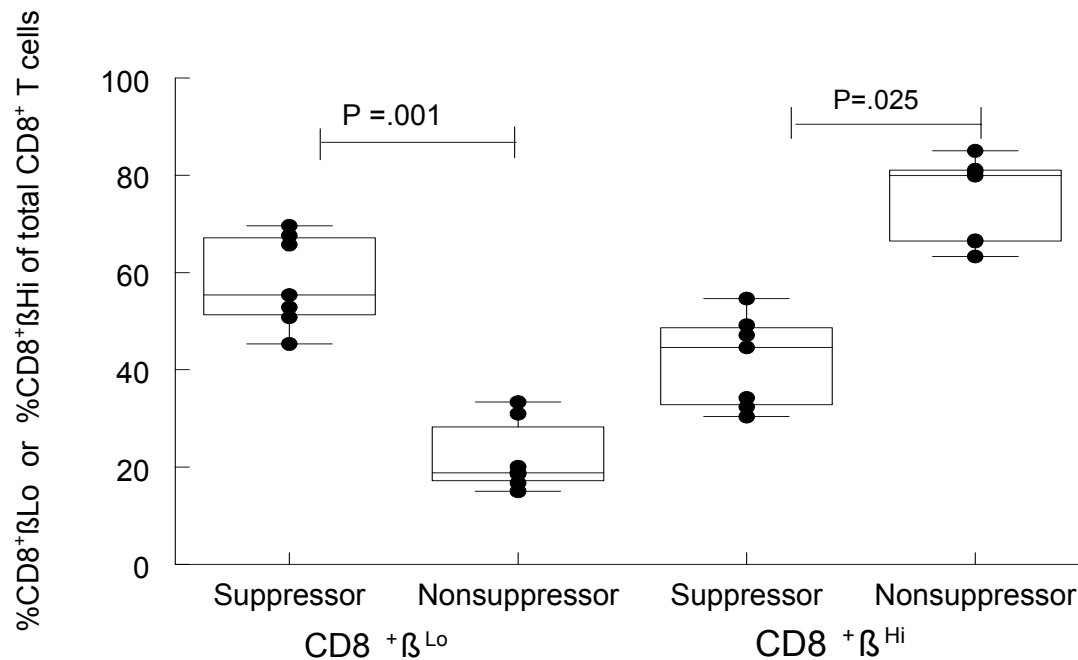
Additional phenotypic analysis of CD8<sup>+</sup> and CD4<sup>+</sup> T cells in PBMC from suppressor cats also revealed a higher percentage of the B7.1 (CD80) positive cells as compared to nonsuppressor cats (Fig. 7, 8). B7.2 or CTLA4 positive CD8<sup>+</sup> T cells do not differ between the two groups of cats. There were also a higher percentage of CD4<sup>+</sup> B7.1<sup>+</sup> T cells in suppressor cats as compared to nonsuppressor cats. However, a greater proportion of CD4<sup>+</sup> cells from nonsuppressor cats expressed CTLA4 as compared to CD4<sup>+</sup> cells from suppressor cats (Fig. 8). In addition, a greater proportion of CD8<sup>+</sup> T cells from suppressor cats revealed surface expression of CD25, another activation marker of PBMC (Fig. 9). These results suggest that FIV infection regulates the expression of several activation markers on CD8<sup>+</sup> T cells, which lead to the presence of distinct subsets of CD8<sup>+</sup> T cells. It is important to determine which CD8<sup>+</sup> T cell subsets are responsible for anti-FIV activity.

FIV infection causes a decrease in the CD4/CD8 ratio of peripheral blood lymphocytes in infected cats. CD4/CD8 ratios are often used to monitor immunological dysfunctions during FIV infection [English et al.,1994]. There was not a significant difference in the CD4/CD8 ratios of suppressor and non-suppressor cats (Table 1). There is no apparent correlation between the CD4/CD8 ratios and the increase in the percentage of CD8<sup>+</sup>B7.1<sup>+</sup> subset and the CD8<sup>+</sup> T cell mediated antiviral activity present in the suppressor cats. These observations, along with previous reports, suggest that CD8<sup>+</sup> antiviral cells may be phenotypically characterized by a reduction in the expression of CD8 $\beta$  chain on peripheral blood B7.1<sup>+</sup> CD8<sup>+</sup> T cells.

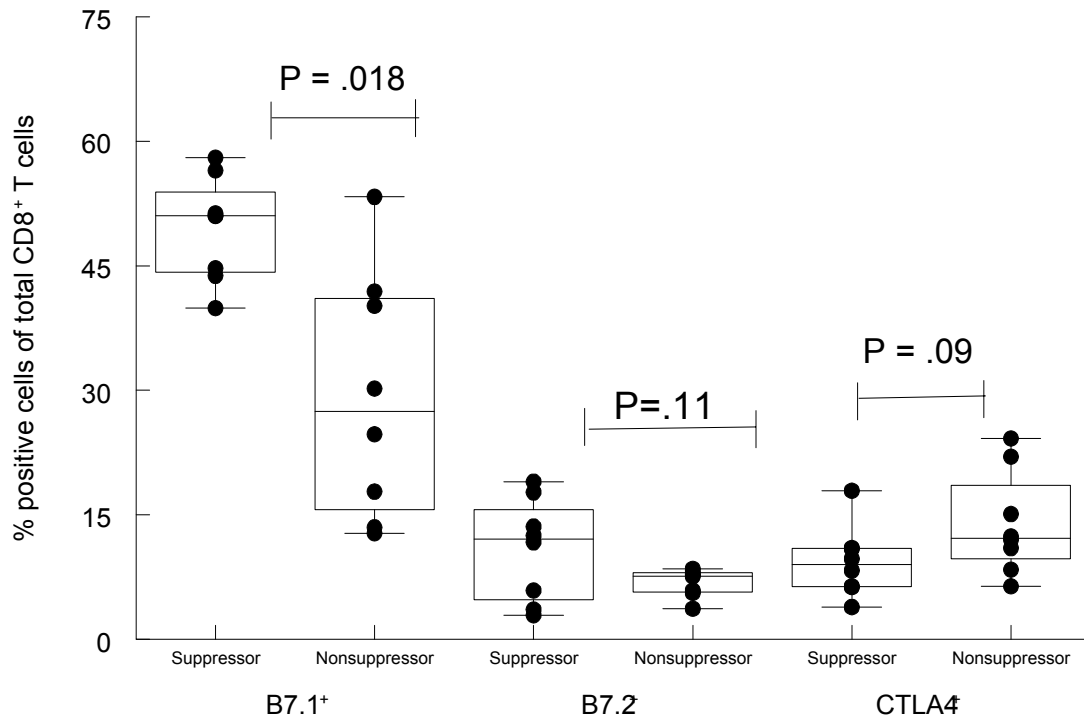




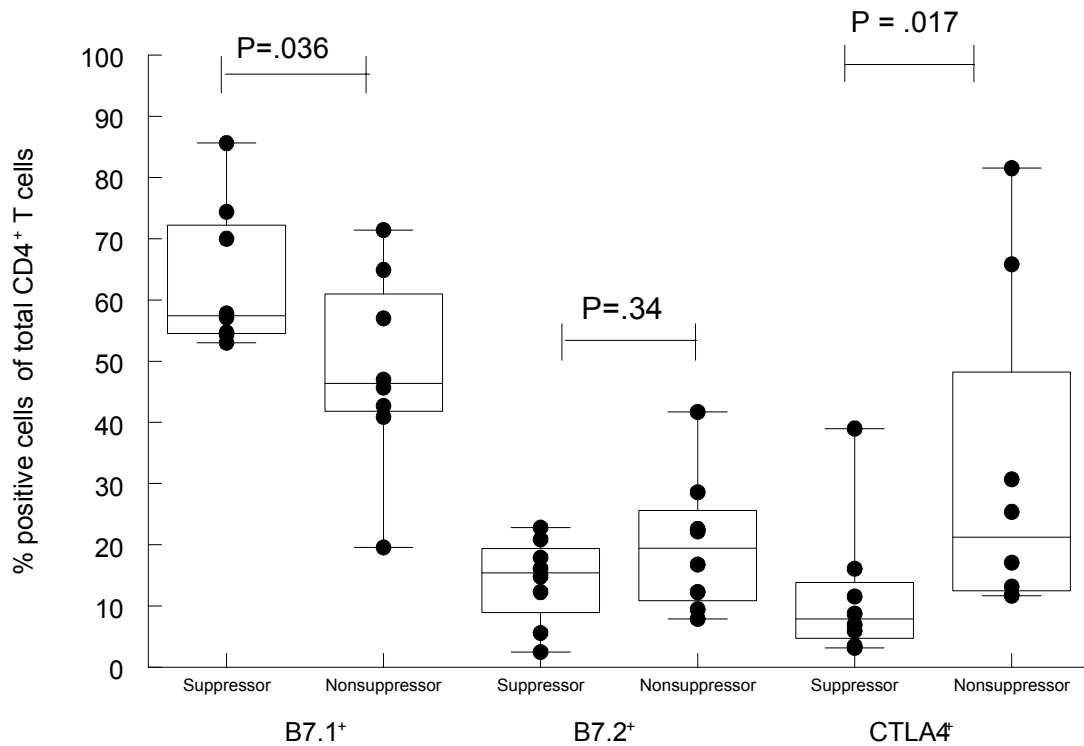
**Figure 5.** No significant differences in absolute numbers of CD8<sup>+</sup> T cells and CD4<sup>+</sup> T cells. PBMC of FIV-infected cats with (suppressor cats) and without (nonsuppressor cats) CD8<sup>+</sup> T cell mediated anti-FIV activity were analyzed for expression of CD4 or CD8 molecules by muticolor flow cytometry using anti-CD4 (CAT30A) or anti-CD8 (3.357) mAbs. The absolute number for each T cell subset was calculated by multiplying the percent of each subset times the absolute number of lymphocytes. [Each box and whiskers plot represents 5<sup>th</sup> and 95<sup>th</sup> percentiles, 25<sup>th</sup> and 75<sup>th</sup> percentiles (box), and median (middle line)]. •Represents the value for each individual cat; suppressor cats n= 6; nonsuppressor cats n=9. There is no significant difference in the absolute number of CD8<sup>+</sup> T cells (p= .14) and the absolute number of CD4<sup>+</sup> T cells (p=.15) between the two groups of cats.



**Figure 6.** Phenotypic analysis of PBMC CD8<sup>+</sup> T cells from cats with and without CD8<sup>+</sup> T cell mediated anti-FIV activity. CD8<sup>+</sup> T cells from PBMC of FIV-infected cats with (suppressor cats) and without (nonsuppressor cats) CD8<sup>+</sup> T cell mediated anti-FIV activity were analyzed for expression of CD8β-chain by multicolor flow cytometry. Box and whiskers plots are as described in Figure 5. •Represents the value for each individual cat; suppressor cats n= 7; nonsuppressor cats n=6. A significantly higher percentage of CD8<sup>+</sup> T cells from suppressor cats have a reduced expression of the CD8β-chain compared to nonsuppressor cats (p=.001).

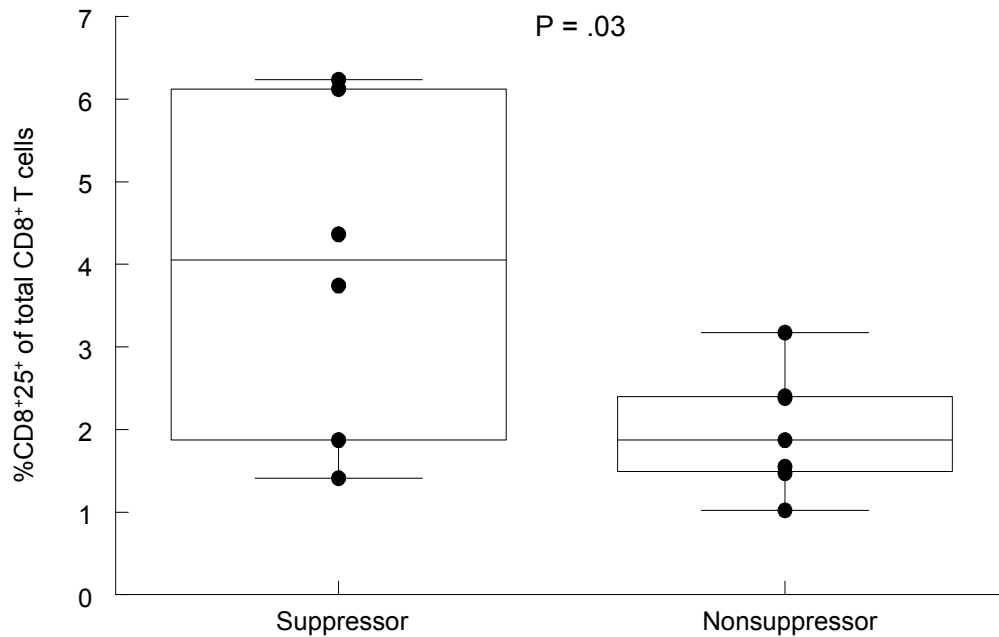


**Figure 7.** Percent of CD8<sup>+</sup> cells from PBMC of FIV-infected cats with (suppressor cats) and without (nonsuppressor cats) CD8<sup>+</sup> T cell mediated anti-FIV activity were analyzed for expression of B7.1, B7.2, and CTLA4 by multicolor flow cytometry. Box and whiskers plots are as described in Figure 5. •Represents the value for each individual cat; suppressor cats n= 6; nonsuppressor cats n=8. There is a higher percentage of B7.1<sup>+</sup> CD8<sup>+</sup> T cells in suppressor cats compared to nonsuppressor cats (p=.018). There is not a significant difference in B7.2 and CTLA4 expression on CD8<sup>+</sup> T cells between the two groups of cats (p =.11; p =.09).



**Figure 8.** Percent of CD4<sup>+</sup> cells from PBMC of FIV-infected cats with (suppressor cats) and without (nonsuppressor cats) CD8<sup>+</sup> T cell mediated anti-FIV activity were analyzed for expression of B7.1, B7.2, and CTLA4 by multicolor flow cytometry.

Box and whiskers plots are as described in Figure 5. •Represents the value for each individual cat; suppressor cats n= 6; nonsuppressor cats n=8. There is a higher percentage of B7.1<sup>+</sup> expression on CD4<sup>+</sup> T cells in suppressor cats than in nonsuppressor cats (p=.036). There is no significant difference in B7.2 expression on CD4<sup>+</sup> T cells between the two groups of cats (p=.34). There is a higher percentage of CTLA4<sup>+</sup> CD4<sup>+</sup> T cells in nonsuppressor cats ((p=.017).



**Figure 9.** Phenotypic analysis of PBMC CD8<sup>+</sup> T cells from cats with and without CD8<sup>+</sup> T cell mediated anti-FIV activity. CD8<sup>+</sup> T cells from PBMC of FIV-infected cats with (suppressor cats) and without (nonsuppressor cats) CD8<sup>+</sup> T cell mediated anti-FIV activity were analyzed for expression of CD25 (IL-2R  $\alpha$  chain), by multicolor flow cytometry. Box and whiskers plots are as described in Figure 5. •Represents the value for each individual cat. A higher percentage of CD8<sup>+</sup> cells from suppressor cats express the CD25 (IL-2R $\alpha$  chain) compared to nonsuppressor cats ( $p=.03$ ).

## **THE ROLE OF CELL SURFACE RECEPTORS ASSOCIATED WITH CD8<sup>+</sup> T CELL MEDIATED ANTIVIRAL ACTIVITY**

The role of the B7.1, B7.2, and CTLA4, cell surface markers in FIV infection or their correlation with disease progression has not been investigated. As shown in the previous section, flow cytometric analysis revealed a number of CD8<sup>+</sup> subsets expressing several activation phenotypes including CD8<sup>+</sup> T cells expressing the B7.1 molecule in the PBMC of suppressor cats (Figure 7). To better understand cellular immunity during FIV infection, we investigated the anti-FIV properties of these different CD8<sup>+</sup> subsets using a Modified Endogenous Assay and an Acute Infection Assay.

### **Assessment of B7.1<sup>+</sup> cells' inhibition of FIV replication in autologous PBMC**

To determine if there was a correlation between the expression of the B7.1 costimulatory molecule and antiviral activity mediated by CD8<sup>+</sup> T cells. B7.1<sup>+</sup> cells were depleted from PBMC isolated from suppressor cats. The B7.1<sup>+</sup> depleted subsets were cocultured with uninfected FCD4E cells in a modified endogenous assay, as previously described by Bucci, English et al [1998]. FIV p24 levels were determined by an ELISA. Depletion of B7.1<sup>+</sup> cells from PBMC significantly reduced the suppressor activity (Fig. 10). Levels of p24 are lower in B7.1 depleted PBMC cocultures compared to CD8 depleted cocultures. To assess the capacity of mitogen-activated PBMC to suppress the replication of FIV *in vitro*, PBMC and B7.1<sup>+</sup> or CD8<sup>+</sup> depleted subsets were cocultured with uninfected FCD4E cells in the presence of 10 µg/mL of ConA. ConA stimulation did not alter the CD8<sup>+</sup> T cell

mediated antiviral activity. Taken together, these results strongly suggested that CD8<sup>+</sup> B7.1<sup>+</sup> cells contribute to the antiviral activity observed in these suppressor cats.

### **Assessment of CD8<sup>+</sup> B7.1<sup>+</sup> cells inhibition of FIV replication in Acute Infection Assay**

To further explore the antiviral activity of CD8<sup>+</sup> B7.1<sup>+</sup> cells, PBMC were sorted using a MoFlo Cell Sorter into CD8<sup>+</sup> B7.1<sup>+</sup> or CD8<sup>+</sup> B7.1<sup>-</sup> subsets. Each subset was cocultured with FIV-NCSU<sub>1</sub> infected FCD4E cells. FIV p24 levels were determined by ELISA method. CD8<sup>+</sup> B7.1<sup>+</sup> cells were able to reduce FIV p24 levels in the acute infection assay (Figure 11).

### **Assessment of CD25<sup>+</sup> cells' inhibition of FIV replication in autologous PBMC**

Barker et al. [1997] reported the cell surface phenotype of anti-HIV cells in HIV-infected individuals as CD8<sup>+</sup> CD25<sup>+</sup> CD28<sup>+</sup> and the upregulation of CD25 correlated with a significant increase in suppressor activity. Phenotypic analysis of CD8<sup>+</sup> T cells in PBMC from our FIV positive cats revealed a higher percentage of CD8<sup>+</sup> CD25<sup>+</sup> cells in suppressor cats as compared to nonsuppressor cats (Figure 9). To determine whether CD25<sup>+</sup> cells played a role in CD8<sup>+</sup> T cell mediated antiviral activity, CD25<sup>+</sup> cells were depleted from the PBMC and cocultured with uninfected FCD4E cells. Depletion of CD25<sup>+</sup> cells had no effect on anti-FIV activity in the modified endogenous assay.

## **Assessment of CD25<sup>+</sup> cells inhibition of FIV replication in Acute Infection**

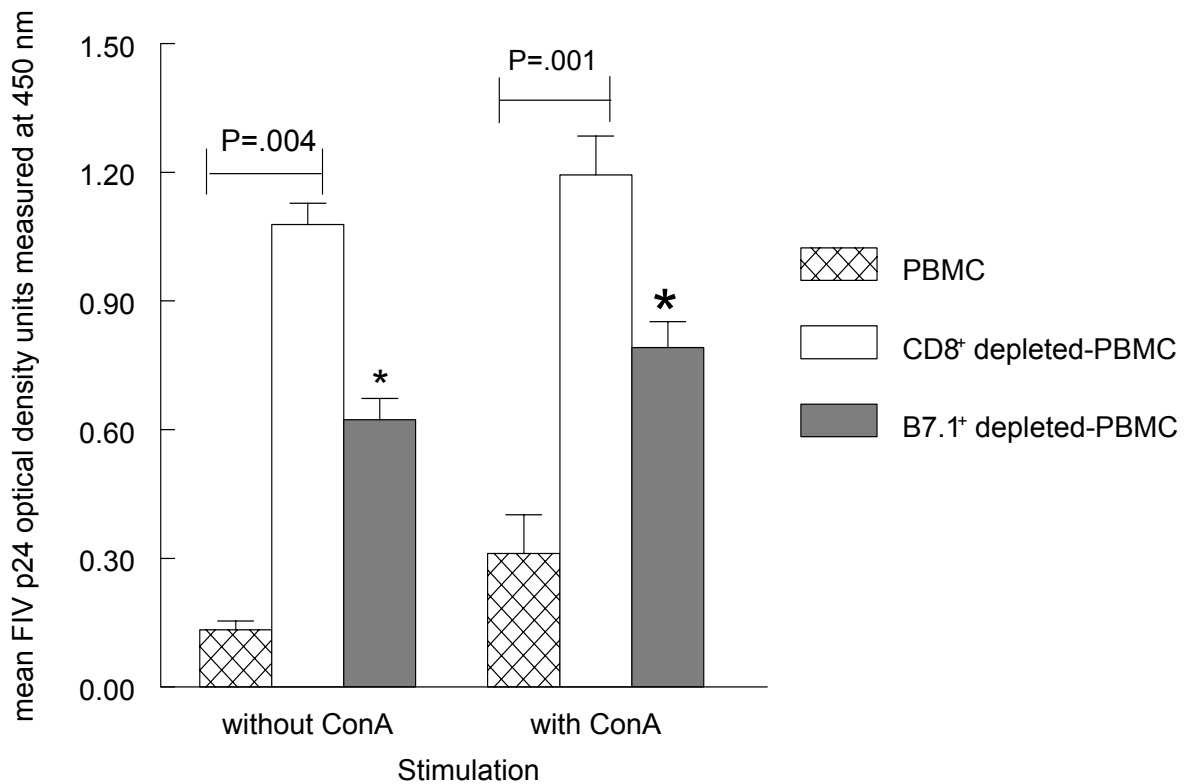
### **Assay**

To further determine if the CD25<sup>+</sup> CD8<sup>+</sup> subset could inhibit FIV replication, PBMC were either depleted of CD4<sup>+</sup>, CD21<sup>+</sup> (B cells) or CD25<sup>+</sup> cells and cocultured with infected FCD4E cells. CD4, CD21-depleted PBMC were able to significantly reduced FIV p24 production in the acute infection assay, suggesting that this population of cells does not suppress FIV replication (Figure 12). In addition, CD4, CD21, CD25 depleted PBMC were also able to reduce FIV p24 levels, suggesting that suppressor function is not a property of these cells.

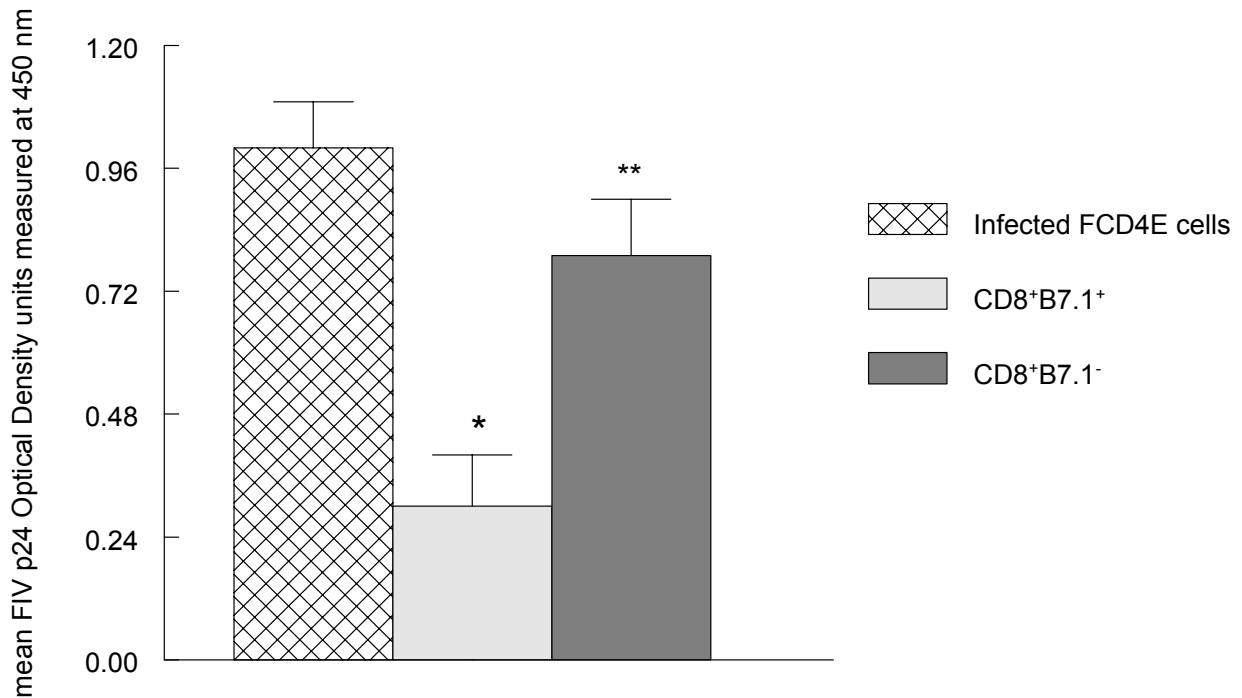
## **Assessment of sorted CD8<sup>+</sup> CD25<sup>+</sup> and CD8<sup>+</sup> CD25<sup>-</sup> subsets inhibition of FIV replication in Acute Infection Assay**

To further explore the antiviral activity of CD8<sup>+</sup> CD25<sup>+</sup>, PBMC were sorted using the Cytomation MoFlo Cell Sorter into CD8<sup>+</sup> CD25<sup>+</sup> and CD8<sup>+</sup> CD25<sup>-</sup> subsets. Each subset was cocultured with infected FCD4E cells in the Acute Infection Assay. Both subsets inhibited FIV replication in the acute infection assay (Figure 13), suggesting that one of the CD8<sup>+</sup> anti-FIV subsets have a phenotype similar to that seen in HIV-infected individuals.



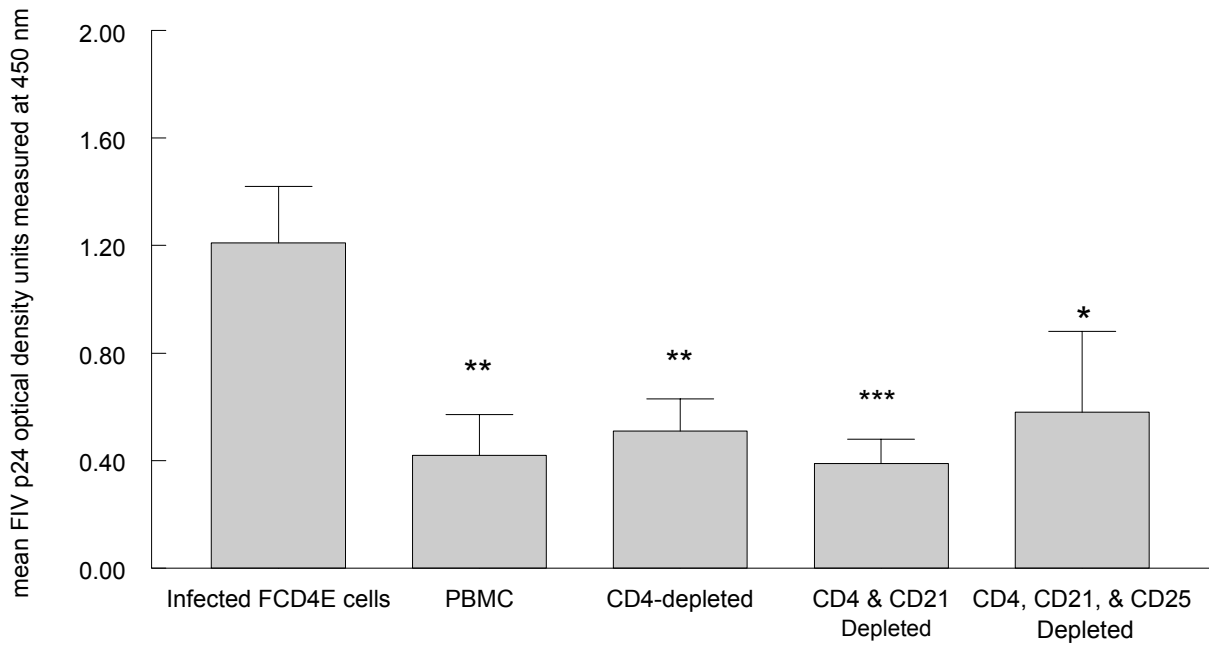


**Figure 10.** Depletion of B7.1<sup>+</sup> cells from PBMC of suppressor cats resulted in increased FIV replication. ConA did not alter the CD8<sup>+</sup> T cell mediated anti-FIV activity. PBMC of suppressor cats were depleted of CD8<sup>+</sup> or B7.1<sup>+</sup> cells using anti-CD8 mAb or anti-B7.1 antibody coated magnetic beads, respectively. Total PBMC (10<sup>5</sup>) or depleted PBMC subsets (10<sup>5</sup>) were cocultured with (10<sup>5</sup>) uninfected FCD4E cells in the absence or presence of 10 µg/mL of ConA. Supernatants were collected and assayed for FIV p24 antigen after 9 days in culture. Depletion of B7.1<sup>+</sup> cells resulted in increased viral replication similar to CD8<sup>+</sup> T cell depleted cocultures. Bars and error bars represents the mean +/- standard deviation of the optical density measurement of 5 wells/p24 ELISA for 1 suppressor cat, representative of 5 suppressor cats used in the study. \* Represents a significant difference between total PBMC and B7.1<sup>+</sup> cell-depleted PBMC cocultures. (p values <.005).

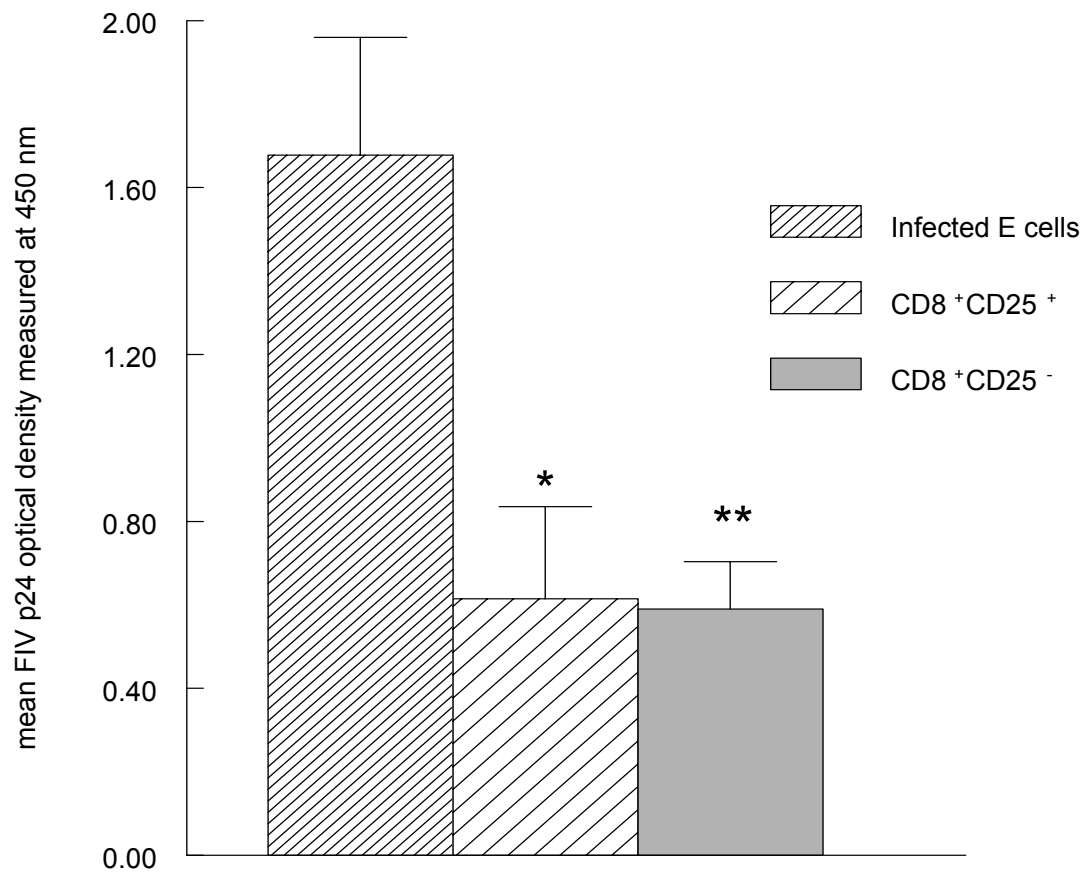


**Figure 11.** CD8<sup>+</sup>B7.1<sup>+</sup> cells suppress FIV replication in the FCD4E acute infection assay. PBMC from 4 suppressor cats were stained with FITC-conjugated anti-CD8 monoclonal antibody (3.357) and PE-conjugated anti-B7.1 antibody. Antibody stained PBMC were sorted using the Cytomation MoFlo Cell Sorter into CD8<sup>+</sup> B7.1<sup>+</sup> and CD8<sup>+</sup>B7.1<sup>-</sup> subsets. 5 x 10<sup>4</sup> cells from each subset were cocultured with FCD4E cells infected with FIV-NCSU<sub>1</sub> at MOI of 0.01 for 7 days. Data (bars and error bars) represent the mean +/- standard deviation of the O.D. measurements for 3 wells/p24 ELISA for 1 suppressor cat, representative of 4 suppressor cats used in this study.

\* Represents a significant difference between infected FCD4E cells and CD8<sup>+</sup> B7.1<sup>+</sup> cells cocultured with infected FCD4E cells (p=.001). (\*\*) indicates no significant difference between infected FCD4E cells and CD8<sup>+</sup> B7.1<sup>-</sup> cells cocultured with infected FCD4E cells (p=.06).



**Figure 12.** CD4, CD21, CD25 depleted PBMC suppress FIV replication in the FCD4E acute infection assay. PBMC ( $10^5$ ) from 4 suppressor cats were depleted of CD4<sup>+</sup> or CD21<sup>+</sup> or CD25<sup>+</sup> cells cocultured with FIV-NCSU<sub>1</sub> infected FCD4E cells ( $10^5$ ) for 7 days. FIV p24 values are expressed as optical density (O.D.) for 1 suppressor cat, representative of 4 suppressor cats used in the study. Bars and error bars represent the mean  $\pm$  S.D. of triplicate samples. (\*) indicates a significant difference between FIV-NCSU<sub>1</sub> infected FCD4E cells and CD4, CD21, CD25 depleted PBMC ( $p = .04$ ); (\*\*) indicates a significant difference between infected FCD4E cells and total PBMC or CD4-depleted PBMC ( $p = .007$ ); (\*\*\*) indicates a significant difference between infected FCD4E cells and CD4, CD21 depleted PBMC ( $p = .003$ ).



**Figure 13.** CD8<sup>+</sup> 25<sup>+</sup> and CD8<sup>+</sup> 25<sup>-</sup> cells suppress FIV replication in the FCD4E acute infection assay. PBMC from suppressor cats were stained with FITC-conjugated anti-CD8 monoclonal antibody (3.357) and PE-conjugated anti-CD25 antibody. PBMC were sorted using the Cytomation MoFlo Cell Sorter into CD8<sup>+</sup> 25<sup>+</sup> and CD8<sup>+</sup> 25<sup>-</sup> subsets. ( $5 \times 10^3$ ) cells from each subset were cocultured with FCD4E cells infected with FIV-NCSU<sub>1</sub> at MOI of 0.01 for 5 days. Data represent the mean +/- standard deviation for 3 wells/p24 ELISA for 1 suppressor cat, representative of 4 suppressor cats used in this study. (\*), (\*\*) indicate a significant difference between infected FCD4E cells and CD8<sup>+</sup> 25<sup>+</sup> or CD8<sup>+</sup> 25<sup>-</sup> cells cocultured with infected FCD4E cells ( $p=.022$ ) and ( $p=.019$ ) respectively.

## **ASSESSMENT OF MOLECULAR MECHANISMS OF CD8<sup>+</sup> T CELL MEDIATED ANTI-FIV ACTIVITY**

We recently reported that an unusually high number of CD8<sup>+</sup> and CD4<sup>+</sup> T cells in the blood of FIV positive cats coexpress B7 costimulatory molecules and their ligand, CTLA4 [Tompkins et al., 2002; Vahlenkamp et al., 2004]. It is also known that B7 ligation of CTLA4 on activated CD4<sup>+</sup> T cells transmit a negative signal for IL-2 transcription, which results in anergy and/or apoptosis [Bluestone et al., 1997; Walunas et al., 1994]. IL-2 provides a strong activation signal for naïve CD4<sup>+</sup> T cells and is also known to enhance HIV replication [Kinter et al., 1995]. In this study, we have shown that the PBMC from suppressor cats have a significantly higher percentage of CD8<sup>+</sup> B7.1<sup>+</sup> cells compared to nonsuppressor cats. In these experiments, we are testing the possibility that CD8<sup>+</sup> antiviral cells suppress IL-2 transcription and indirectly inhibit FIV replication in the infected CD4<sup>+</sup> T cells.

### **The effects of IL-2 on CD8<sup>+</sup> T cell mediated anti-FIV activity**

The addition of IL-2 combined with HAART has been shown to lead to increases in the number of CD4<sup>+</sup> T cells in peripheral blood and lymph nodes of HIV-infected individuals [Kovacs et al., 1996]. IL-2 has also been shown to enhance CD8<sup>+</sup> T cell mediated antiviral responses in HIV-infected individuals Kovacs et al., 1997; Kinter et al., 1995]. Our first approach was to investigate the effects of exogenous IL-2 on CD8<sup>+</sup> T cell mediated anti-FIV activity, *in vitro*. CD8<sup>+</sup> T cell depleted and total PBMC were cocultured with uninfected FCD4E cells in complete RPMI media in the presence or absence of recombinant human IL-2. Supernatants were collected at

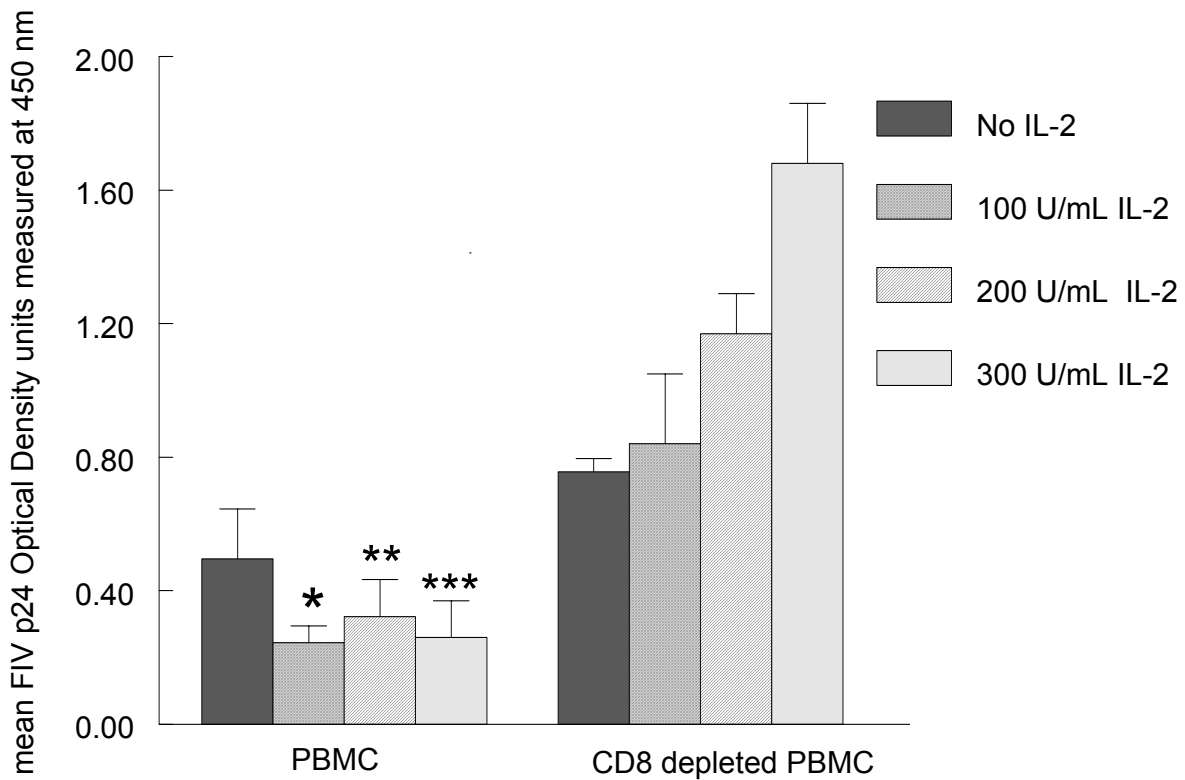
day 9 and analyzed for FIV p24 production by ELISA. The addition of exogenous IL-2 stimulation did not alter the suppressive nature of the CD8<sup>+</sup> T cells (Figure 14). As expected, IL-2 enhanced FIV replication in the absence of CD8<sup>+</sup> T cells.

### **Assessment of endogenous IL-2 mRNA and FIV gag gene expression in FIV-infected CD4<sup>+</sup> T cells**

In our next study, we investigated the effects of CD8<sup>+</sup> T cells on FIV and IL-2 mRNA expression in infected CD4<sup>+</sup> T cells. To quantify the *in vivo* viral RNA load and IL-2 mRNA expression in suppressor and nonsuppressor cats, CD4<sup>+</sup> T cells, freshly isolated from infected cats were washed and RNA extracted using the methods and reagents supplied by Qiagen. To determine the effects of CD8<sup>+</sup> T cell mediated antiviral activity on FIV replication, CD8<sup>+</sup> T cells isolated from FIV-infected cats were stimulated with or without ConA for 4-6 hours. CD4<sup>+</sup> T cells were washed and RNA was extracted. FIV and IL-2 RNA expression were determined using real time RT-PCR. Input RNA was normalized using glyceraldehydes-3-phosphate dehydrogenase (GAPDH) RNA. Reverse transcription and thermal amplification were performed using the appropriate primers, probes and parameters as described in the material and methods. There was no appreciable difference in the expression of IL-2 mRNA in the CD4<sup>+</sup> T cells isolated from suppressor and nonsuppressor cats (Figure 15). The CD8<sup>+</sup> T cells did not have a significant effect on the expression of GAPDH and IL-2mRNA in the ConA-stimulated or unstimulated CD4<sup>+</sup> T cells in either group of cats (Figure 15). There was not a significant difference in the level of GAPDH mRNA expression in CD4<sup>+</sup> T cells stimulated with or without ConA isolated

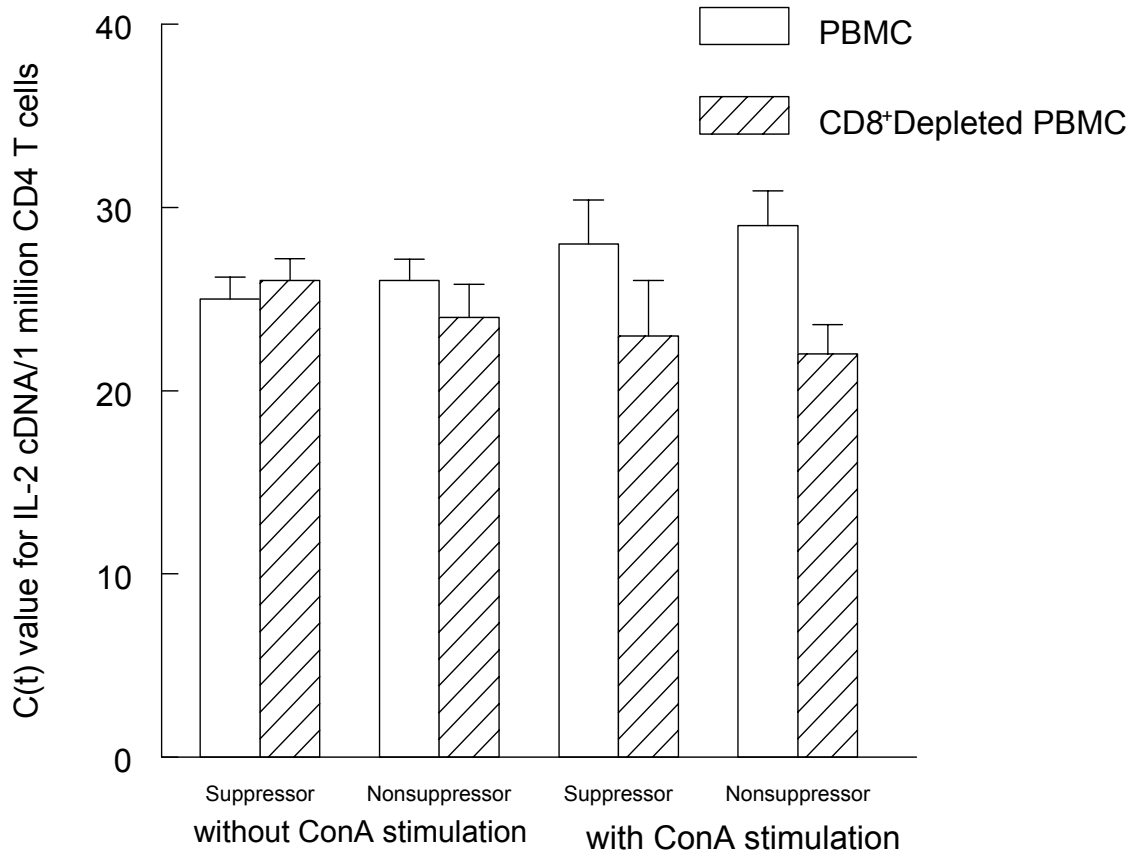
from either group of cats. There were a higher number of viral RNA molecules per  $10^6$  CD4<sup>+</sup> T cells in nonsuppressor as compared to suppressor cats (Figure 16).

Culturing the FIV-infected CD4<sup>+</sup> T cells in the presence of CD8<sup>+</sup> T cells from the suppressor cats caused a significant reduction in the number of viral RNA molecules per  $10^6$  CD4<sup>+</sup> T cells as compared to CD4<sup>+</sup> T cells cultured in the presence of CD8<sup>+</sup> T cells from nonsuppressor cats, suggesting that the antiviral activity may act at the level of FIV RNA synthesis (Figure 16).

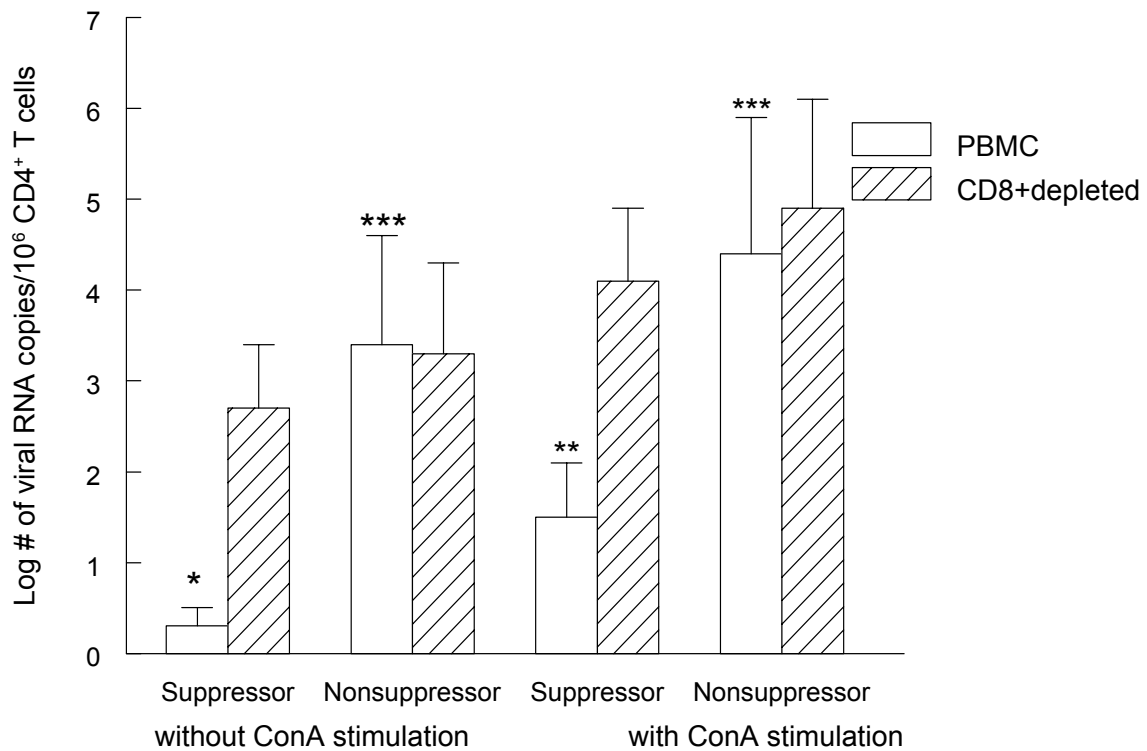


**Figure 14.** IL-2 stimulation did not alter CD8<sup>+</sup> T cell mediated anti-FIV activity. CD8<sup>+</sup> T cell-depleted ( $10^5$ ) or PBMC ( $10^5$ ) were cocultured with uninfected FCD4E cells ( $10^5$ ) in complete RPMI supplemented without rhIL-2 or with 100 U/mL or 200 U/mL or 300 U/mL of rhIL-2. After 9 days of culture, cell-free supernatants were collected and assayed for FIV p24 production by ELISA. Bars represent the mean  $\pm$  S.D. of 3- wells/p24 ELISA for 1 suppressor cat, representative of 5 suppressor cats used in this study. There was a significant difference in FIV p24 production in PBMC and CD8<sup>+</sup> T cell-depleted PBMC cocultures cultured in the presence of rhIL-2; (\*), (\*\*), (\*\*\*) represents p values =.02, .001, .007 respectively.





**Figure 15.** No significant difference in IL-2 cDNA isolated from CD4<sup>+</sup> T cells. PBMC were depleted of CD8<sup>+</sup> T cells using (3.357) antibody coated magnetic beads. CD8-depleted PBMC or total PBMC were stimulated with or without 10 µg/mL of ConA for 4-6 hours. 20 µL of isolated cellular RNA was reverse transcribed in 50 µL reaction which consisted of IL-2 primers, probe, and PCR master mix using Biorad *iCycler* Real Time PCR detection system. Negative control consisted of a no template mixture of PCR master mix, IL-2 primers, probe and dH<sub>2</sub>O. For normalization purposes and as a positive control for the assay, real time PCR was also performed using primers and probe for GAPDH. Results are reported as C(t) values. Bar and error bars represent the mean +/- S.D. of 3 PCR reactions for 1 suppressor or nonsuppressor cat. Representative of data observed in 5 cats. All p values ≥ .05.



**Figure 16.** CD4<sup>+</sup> T cells collected from nonsuppressor cats have a higher number of viral RNA molecules per 10<sup>6</sup> CD4<sup>+</sup> T cells. PBMC were collected from suppressor and nonsuppressor cats and were analyzed as described in figure 15. Bars and error bars represent the mean +/- S.D. of 3 PCR reactions for 1 suppressor or nonsuppressor cat. Representative of data observed in 5 cats. In the presence of CD8<sup>+</sup> T cells isolated from suppressor cats, there is a reduction in the number of viral RNA molecules in CD4<sup>+</sup> T cells; (\*), (\*\*) indicate p values= .014, .002 respectively. In the presence of CD8<sup>+</sup> T cells isolated from nonsuppressor cats, there is no significant reduction in the number of viral RNA molecules in CD4<sup>+</sup> T cells; (\*\*\*) indicates p values ≥ .05.

## Discussion

A population of CD8<sup>+</sup> T cells in asymptomatic HIV-infected humans and FIV-infected cats can suppress HIV and FIV replication in a noncytotoxic, non-MHC restricted manner [Bucci, English et al., 1998; Flynn et al., 1995, Flynn et al., 2002; Jeng et al., 1996; Choi et al., 2000]. The mechanism responsible for suppression of FIV and HIV replication is unknown. However, there is evidence that both cell contact and soluble factors maybe involved [Flynn et al., 1999; Choi et al., 2000; Bucci et al., 1998, Hohdatsu et al. 2000].

In this study, we investigated the cellular and molecular mechanism (s) involved with feline CD8<sup>+</sup> T cell-mediated antiviral activity in asymptomatic FIV NCSU<sub>1</sub>-infected cats. Twenty-two specific pathogen-free cats were infected intravenously or vaginally with FIV-NCSU<sub>1</sub> strain as previously described by Bucci, English et al., [1998]. At the time of this study, all cats were infected for more than 2 years and tested positive for FIV-NCSU<sub>1</sub> by ELISA and PCR. The cats did not exhibit any clinical signs of the AIDS-like disease.

To detect the presence of CD8<sup>+</sup> T cell-mediated antiviral activity, we used a modified version of the endogenous assay, an *in vitro* system of FIV replication that mimics the cellular microenvironment of peripheral blood. In this system, the antiviral activity is capable of inhibiting virus replication in a model of endogenous FIV infection in which peripheral blood cells from an infected cat are cocultured in the presence or absence of exogenous stimuli.

The spread of infection is due to viral particles from the *in vivo* infected peripheral blood cells infecting previously uninfected CD4<sup>+</sup> T cells.

In 30% of the asymptomatic FIV-NCSU<sub>1</sub>-infected cats, the culture supernatants of CD8<sup>+</sup> T cell depleted from PBMC expressed significantly higher levels of the FIV p24 antigen as compared to total PBMC, suggesting the presence of CD8<sup>+</sup> T cell mediated antiviral activity, *in vitro*. As a result, we designated those cats as suppressor cats whereas other cats in this study that showed little or no evidence of CD8<sup>+</sup> T cell mediated anti-FIV activity were designated nonsuppressor cats.

Gebhard et al., [1999] reported that antiviral CD8<sup>+</sup> T cells emerge during the acute infection phase and become the predominant population of CD8<sup>+</sup> cells in the blood and lymph nodes during the chronic infection phase. Similar to published observations [Flynn et al., 1995; Flynn et al., 2002], CD8<sup>+</sup> T cells from the lymph nodes of FIV-infected cats were able to control FIV replication *in vitro*, suggesting that these CD8<sup>+</sup> T cells may control the spread of FIV *in vivo*. The CD8<sup>+</sup> T cells isolated from the blood and lymph node of the same animals had antiviral activities.

The presence of CD8<sup>+</sup> T cell mediated-antiviral activity may be effected by time, route of infection, virus strain, cellular tropism, secondary infections, or type of tissue infected. In this study, cats were infected within 6 months of birth with FIV-NCSU<sub>1</sub>. All the FIV positive cats (suppressor and nonsuppressor) were maintained in a controlled environment with limited exposure to secondary pathogens and

continuously monitored for clinical signs of disease. The presence or absence of CD8<sup>+</sup> T cell mediated antiviral activity did not correlate with the absolute number of CD4<sup>+</sup> or CD8<sup>+</sup> T cells, the CD4:CD8 ratio, the route or length of infection. The cause of the variation of CD8<sup>+</sup> T cell mediated suppressor activity in these infected cats remains unclear and very little is known about the cellular mechanisms of controlling FIV replication.

Because the expression of virus by PBMC *in vitro* may not reflect the level of virus replication *in vivo* [English et al., 1994; Jeng et al., 1996; Plaeger et al., 1999] we compared plasma viremia levels in both groups of cats and found a significantly higher viral load in nonsuppressor cats as compared to suppressor cats, suggesting that CD8<sup>+</sup> T cell anti-FIV cells may control virus replication *in vivo*.

The findings in this study indicate that noncytotoxic FIV suppression by CD8<sup>+</sup> T cells is inversely associated with the cell-associated and plasma viral load in long-term asymptomatic FIV-infected cats. However, it is still unclear whether a lack of CD8<sup>+</sup> T cell mediated antiviral activity leads to an increase in viral loads or whether high viral loads overwhelm the action of the CD8<sup>+</sup> T cells. An understanding of the host's immune response during the asymptomatic phase of FIV infection, when virus replication is controlled, will help elucidate the host immune response during infection. This information will help provide insight that will facilitate the development of new antiviral agents, vaccines and therapies. The full nature of the immunological response during FIV infection is still unclear, but significant progress has been made

in the immunological characterization of FIV positive cats. It has been reported that the CD8<sup>+</sup> cells in cats with antiviral activity have an activation phenotype, CD8α<sup>+</sup>β<sup>Low</sup>, CD44<sup>high</sup>, CD49d<sup>high</sup>, CD62L<sup>neg</sup> [Bucci, Gebhard et al., 1998; Gebhard et al., 1999; Shimojima et al., 1998]. It was also suggested that the increase in CD8<sup>+</sup> T cells may be due to the expansion of CD8α<sup>+</sup>β<sup>low</sup> cells. This study also shows that 22 FIV-infected cats had a significantly higher percentage of CD8α<sup>+</sup>β<sup>low</sup> cells as compared to FIV negative cats [Gebhard et al., 1998; Hohdatsu et al., 2002]. In addition, suppressor cats also have a higher percentage of CD8α<sup>+</sup>β<sup>low</sup> cells as compared to nonsuppressor cats. The presence of CD8α<sup>+</sup>β<sup>low</sup> cells in long-term FIV-infected suppressor cats supports that notion that effector T cells are maintained over a long period of time.

Phenotypic analysis of the CD8<sup>+</sup> T cells from suppressor and nonsuppressor cats also revealed an increase in the percentage of B7.1<sup>+</sup> CD8<sup>+</sup> cells with low expression of the CD8β chain in the suppressor cats, suggesting that the anti-FIV cells express the activation phenotype CD8α<sup>+</sup>β<sup>lo</sup> B7.1<sup>+</sup>. In support of depletion of B7.1<sup>+</sup> cells from suppressor cats resulted in increased virus replication similar to that seen with depletion of CD8<sup>+</sup> T cells in the modified endogenous assay. However, there was not a significant difference in either the absolute number of CD8<sup>+</sup> T cells in the suppressor and nonsuppressor cats, which ensured that depletion of B7.1<sup>+</sup> cells did not remove disproportionately more CD8<sup>+</sup> cells from suppressor cats.

To determine the antiviral activity of CD8<sup>+</sup> B7.1<sup>+</sup> and CD8<sup>+</sup> B7.1<sup>-</sup> subsets, the acute infection assay was used to measure antiviral activity using target CD4<sup>+</sup> T cells infected with a known TCID<sub>50</sub> virus inoculum. The target CD4<sup>+</sup> T cells were obtained from a FIV-negative donor. One of the advantages of the acute infection assay is that the researcher can control the titer of the virus used. Secondly, this assay may also be used to compare the antiviral activity between different subsets of CD8<sup>+</sup> T cells while keeping the virus titer and target T cell number constant. The CD8<sup>+</sup> B7.1<sup>+</sup> T cells were able to significantly reduce virus replication in the acute infection system.

In HIV infected individuals the onset of opportunistic infections is associated with ineffective adaptive and innate immune responses [Levy et al., 2001; Levy et al., 2003]. The clinical relevance of the CD8<sup>+</sup> T cell mediated anti-viral activity is demonstrated by its occurrence in HIV infections and its reduction with advancement towards AIDS [Mackewicz et al., 1991; Stranford et al., 1999; Levy et al., 1996, 2001]. However, it has not been determined whether the lack of CD8<sup>+</sup> T cell mediated antiviral activity in FIV infected cats is associated with the onset of opportunistic infections [Flynn et al., 1999; Hohdatsu et al., 1998; English et al., 1994].

The decrease in the percentage of CD8 $\alpha^+\beta^{\text{lo}}$  cells and the expansion of the CD8 $\alpha^+\beta^{\text{hi}}$  subset correlates with a lack of CD8<sup>+</sup> T cell-mediated antiviral activity in some of the nonsuppressor cats. Furthermore, less than 20% of total CD8<sup>+</sup> T cells in these cats

expressed B7.1 or B7.2 costimulatory molecules as compared to cats with potent anti-FIV activity. It remains to be determined whether these findings are associated with the development of disease because the nonsuppressor cats exhibit no clinical signs of illness.

Barker et al., [1997] reported the cell surface phenotype of anti-HIV cells in HIV-infected individuals as CD8<sup>+</sup> CD28<sup>+</sup> and the upregulation of CD25 correlated with a significant increase in suppressor activity. In our study, phenotypic analysis of PBMC from suppressor cats revealed a higher percentage of CD25<sup>+</sup> CD8<sup>+</sup> cells as compared to nonsuppressor cats. However using a modified version of the endogenous assay, depletion of CD25<sup>+</sup> cells from PBMC had no effect on anti-FIV activity in the suppressor cats whereas CD8<sup>+</sup> CD25<sup>+</sup> and CD8<sup>+</sup> 25<sup>-</sup> subsets exhibited significant inhibition of FIV replication in the acute infection assay, suggesting that a subset of CD8<sup>+</sup> anti-FIV cells may have a phenotype CD8<sup>+</sup> CD25<sup>+</sup>.

IL-2 is a T cell growth factor that is required for the activation of naïve T lymphocytes and has been shown to positively regulate HIV replication in PBMC of seronegative individuals, *in vitro*. Exogenous IL-2 does not induce the replication of endogenous HIV in PBMC from HIV-infected individuals [Kinter et al., 1995]. Interestingly in our study, neither ConA nor IL-2 stimulation altered the suppressive nature of the CD8<sup>+</sup> anti-FIV cells, which is similar to findings reported for CD8<sup>+</sup> anti-HIV cells in HIV-infected patients. These data suggest that IL-2 may be necessary for potent anti-FIV activity and/or may work in synergy with some other soluble factor. These data



also suggests that CD8<sup>+</sup> T cell mediated antiviral activity may interfere with the IL-2 signal transduction pathway in infected CD4<sup>+</sup> T cells, which may also indirectly interfere with FIV replication.

The elucidation of the mechanism(s) for feline CD8<sup>+</sup> T cell mediated antiviral activity will provide critical information that can be used to help design proper therapies for both FIV and HIV infections [Levy et al., 2003; Flynn et al., 2002]. We know that FIV infection results in the upregulation of B7 costimulatory molecules and their ligand CTLA4 [Tompkins et al., 2002; Vahlenkamp et al., 2004]. As a result of this study, we know that the upregulation of B7.1 molecules correlates with the presence of CD8<sup>+</sup> T cell anti-FIV activity, in-vitro. The mechanism of CD8<sup>+</sup>B7.1<sup>+</sup> T cell suppression of FIV replication is unknown. However, it is possible that these cells may downregulate FIV gene transcription by B7 ligation of CTLA4 on activated infected CD4<sup>+</sup> T cells.

In this study, CD8<sup>+</sup> antiviral activity was tested for its effect on FIV RNA and IL-2 mRNA expression in naturally infected CD4<sup>+</sup> T cells. The presence of CD8<sup>+</sup> T cells did not affect the expression of IL-2 mRNA. This does not rule out the possibility of CD8<sup>+</sup> T cells having an effect on the translation of the IL-2 in a mature and functional protein. However, the presence of CD8<sup>+</sup> T cells resulted in a decrease in the level of FIV p24 in cell free culture supernatant and a decrease in the number of FIV mRNA molecules in CD4<sup>+</sup>T cells, suggesting that the CD8<sup>+</sup> T cell antiviral activity may act at the level of FIV mRNA synthesis. It has also been shown that CD8<sup>+</sup> anti-HIV cells

inhibit virus replication by blocking HIV mRNA transcription [Chen et al., 1993; Le Borgne et al., 2000; Leith et al., 1999].

The results of this study showed a strong correlation between the presence of CD8<sup>+</sup> T cell anti-FIV activity, a reduction in viremia, and the expression of B7.1 costimulatory molecule on the CD8<sup>+</sup> T cells in the suppressor cats, suggesting that CD8<sup>+</sup>B7.1<sup>+</sup> antiviral cells may suppress FIV replication *in vivo*. English et al., [1994] and others [Hohdatsu et al., 2000] have reported that FIV infected cats have a decrease in CD4<sup>+</sup> T cell count and an increase CD8<sup>+</sup> T cells. There is no correlation between the presence of CD8<sup>+</sup> T cell antiviral activity and the absolute number of peripheral CD8<sup>+</sup> or CD4<sup>+</sup> T cells, suggesting that the antiviral activity may not be solely dependent upon cell number.

We also showed that CD8<sup>+</sup> T cells from long-term asymptomatic FIV positive cats are capable of inhibiting virus replication without killing the infected target cell in both acute and endogenous systems. However, our data do not imply that the CD8<sup>+</sup> antiviral cells use the same mechanism of suppressing virus replication in both systems. Our observations are similar to those of others who found a correlation of the stage of the disease and the ability of CD8<sup>+</sup> T cells to inhibit FIV and HIV replication [Flynn et al., 1999; Levy et al., 1998; Levy et al., 2003].

In summary, the experimental FIV infection of domestic cats is associated with the induction of CD8<sup>+</sup> subsets which are capable of inhibiting FIV replication during the

asymptomatic stage of disease. These cells may play a major role in controlling FIV replication *in vivo*. There are compelling similarities between HIV and FIV infections. The significance of controlling virus replication in both FIV and HIV infections can not be overemphasized. Controlling virus replication may prevent mutations, which lead to more cytopathic and drug resistant strains of the virus (Levy et al., 1996). An understanding of the immune responses associated with feline CD8<sup>+</sup> antiviral activity during the asymptomatic stage of infection may be applied towards developing vaccines and a new generation of antiviral drugs against both lentiviruses.

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