ABSTRACT

JOSHI, ANJALI. **Role of $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells in feline immunodeficiency virus infection. (Under the direction of Dr. Wayne Tompkins)**

Treatment of HIV infected individuals with HAART reduces the levels of plasma viral loads to below the limit of detection by standard clinical assays. HAART however, does not result in virus eradication as a small but detectable virus reservoir persists in all individuals receiving therapy. Studies attempting to identify reservoirs of HIV-1 latency have documented that the virus persists as both a latent and productive infection in subsets of $CD4^+$ T cells. However, reports regarding establishment of a stable HIV-1 infection in quiescent T cells in vitro remain controversial. In the present study, we investigated the susceptibility of naïve ($CD4^+CD25^-$) and activated ($CD4^+CD25^+$) feline T cells to FIV infection, their ability to replicate the virus and potentially act as a reservoir for virus persistence in infected animals. While both $CD4^+CD25^+$ and $CD4^+CD25^-$ cells are susceptible to FIV infection in vitro and in vivo, only $CD4^+CD25^+$ cells produce infectious virions in the absence of a strong mitogenic stimulus like ConA. In contrast to $CD4^+CD25^-$ cells, $CD4^+CD25^+$ cells display the key characteristics of Treg cells in that they remain unresponsive to mitogenic stimulation, and are relatively resistant to apoptosis. Mechanisms regulating infection of these cells revealed that $CD4^+CD25^-$ cells are less susceptible to FIV infection, both at the level at viral entry and cellular transcriptional activity. The ability of $CD4^+CD25^+$ cells to replicate FIV efficiently in the presence of IL-2 but remain anergic and unresponsive to apoptotic signaling suggests that these cells may provide a reservoir of productive FIV infection. $CD4^+CD25^-$ cells, on the contrary, seem to establish as latent viral reservoirs capable of being reactivated after stimulation.

Role of $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells in Feline Immunodeficiency Virus Infection

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LIST OF ABBREVIATIONS

Ab - Antibody
AICD - Activation Induced Cell Death
ACAD - Activated T cell Autonomous Death
APC - Allophycocyanin
AIDS - Acquired Immune Deficiency Syndrome
CAE - Caprine Arthritis Encephalitis virus
Cdk-1 - Cyklin Dependent Kinase-1
CEBP - CAAT Enhancer Binding Protein
ConA – ConcanvalinA
CNS - Central Nervous System
CTL - Cytotoxic T Lymphocytes
DNA - Deoxyribonucleic Acid
EIA - Equine Infectious Anemia virus
ELISA - Enzyme Linked Immunosorbent Assay
EMSA - Electrophoretic Mobility Shift Assay
Env - Envelope
FACS - Fluorescence Activated Cell Sorting
FCD4E - Feline CD4E cells
FITC - Fluorescein Isothiocyanate
FIV - Feline Immunodeficiency Virus
FSC - Forward Scatter
HAART - Highly Active Antiretroviral Therapy
HIV - Human Immunodeficiency Virus
IL-2 - Interleukin-2
LN - Lymph Nodes
LTR - Long Terminal Repeat
MAb - Monoclonal Antibody
MTOR - Mammalian Target of Rapamycin
MFI - Mean Fluorescence Intensity
NFκB - Nuclear Factor associated with Kappa light chain of B cells.
NSI – Non Syncytia Inducing
ORF - Open Reading Frame
PBMC - Peripheral Blood Mononuclear Cells
PCR - Polymerase Chain Reaction
PE - Phycoerythrin
PEV - Position Effect Variegation
PI - Propidium Iodide
RNA - Ribonucleic Acid
RNAse - Ribonuclease
RT - Reverse Transcriptase
SI - Syncytia Inducing
SIV - Simian Immunodeficiency Virus
SSC - Side Scatter
TCR - T Cell Receptor

1. INTRODUCTION

Studies conducted on HIV and FIV tropism have shown that both the viruses infect a variety of cell types in their respective hosts including T cells, macrophages, and CNS glial cells (Chun and Fauci., 1999; Bendinelli et al., 1995). Although it is well established that HIV-1 displays a particular tropism for $CD4^+$ lymphocytes, which may lead to their gradual depletion, there are conflicting reports regarding HIV infection of resting T cells in vitro. Resting T cells derived from HIV infected individuals harbor replication competent integrated proviral DNA suggesting that they are infected in vivo (Chun et al., 1995). Whether resting T cells are infected de novo or are derived from activated infected cells, which subsequently revert to a resting phenotype remains to be determined. In this regard, studies have demonstrated that the HIV co-receptors CXCR4 and CCR5 are differentially expressed on activated versus naïve/resting cells, which determine the permissiveness of above T cells to HIV infection (Bluel et al., 1997). While CXCR4 is expressed on almost all of the $CD4^+$ T cells, CCR5 is not only a marker of cells infectable with R5 tropic viruses but also of activated/cycling T cells (Eckstein et al., 2001; Pierson et al., 2000). In agreement with this are the findings that while resting T cells are susceptible to infection by X4 tropic viruses, they are resistant to infection by R5 tropic viruses (Pierson et al., 2000).

With the advent of Highly Active Antiretroviral Therapy (HAART), additional information has been gained on HIV infection of $CD4^+$ T cell subsets in vivo. Treatment of asymptomatic HIV-1 infected individuals with HAART decreases the amount of plasma virus load to levels below the limit of detection by standard clinical assays (Chun at al., 2000; Perelson et al., 1996). However, HAART does not result in virus eradication as a small but

detectable reservoir of latently as well as productively infected, $CD4^+$ cells harboring replication competent HIV-1 has been shown to persist in essentially all patients receiving therapy (Dornadula et al., 1999; Finzi et al., 1997; Wong et a., 1997). Further, there is evidence for continuous ongoing viral replication on the basis of viral sequence evolution within the envelope region over time in patients receiving HAART (Gunthard et al., 1999).

It is well established that retroviral replication in T cells is governed by a series of cellular transcription factors that transactivate the viral LTR (Cullen., 1989; Bigornia et al., 2001). Since these transcription factors are predominantly upregulated in activated T cells, these cells are also the ones capable of supporting a productive retroviral infection (Ikeda et al., 1996; Kornfeld et al., 1998; Tang et al., 1999; Wu and Marsh., 2001). Earlier attempts to identify the reservoir of productive HIV infection focused on a unique subset of $CD4^+$ cells expressing the CD25 (interleukin-2 [IL-2R] alpha chain) antigen and demonstrated CD25 as a marker of cells productively infected with HIV in vitro (Borvak et al., 1995; Chou et al., 1997; Ramilo et al., 1993). These observations are of interest, as cells of the $CD4^+CD25^+$ lineage comprise an important immune regulatory population (Maloy and Powrie., 2001) that appear to be resistant to clonal deletion and apoptosis when stimulated with anti-CD3 (Taams et al., 2001) or super antigen (Banz et al., 2002), suggesting they could be long-lived in lymphoid tissues. While $CD4^+CD25^+$ cells have an activated phenotype, they remain anergic to mitogenic stimulation as detected by their lack of proliferation and once activated, suppress other T cell responses in a non-specific manner (Joshi et al., 2004; Maloy and Powrie., 2001; Vahlenkamp et al., 2004).

Keeping in mind the above mentioned facts, the current study was undertaken to elucidate the role of $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells in FIV infection and replication in

vitro and in vivo. Further, we explored the mechanisms governing the differential replication of FIV in $CD4^+CD25^+$ cells and define the role of these cells as long-lived reservoirs of productive FIV infection. With regard to the $CD4^+CD25^-$ counterparts, experiments were conducted to determine whether they were capable of harboring a replication competent latent FIV infection capable of reactivation when stimulated.

2. BACKGROUND AND SIGNIFICANCE

A. Feline Immunodeficiency virus

FIV was isolated by Pedersen et al (1987) form cats in a cattery in California showing symptoms of immunodeficiency. Due to its marked similarity with HIV-1, the virus was classified as a Lentivirus belonging to the family Retroviridae. FIV however, bears greater relatedness to the ungulate lentiviruses Caprine Arthritis Encephalitis virus (CAE) and Equine Infectious Anemia virus (EIA) than to Simian Immunodeficiency Virus (SIV) and HIV-1. FIV infection of cats results in a clinical disease that mimics HIV infection in humans. There has been a considerable effort to develop a small animal model for HIV due to the disease gaining epidemic proportions in the 20th century. In this context, FIV bears greater similarity to HIV since the two viruses cause similar disease in their respective hosts unlike SIV that replicates to high titres in monkeys without causing immunodeficiency. Moreover, there are several other reasons that make FIV an excellent small animal model for HIV induced immunodeficiency in humans and are discussed below.

B. Genomic organization of FIV

The genome of FIV, similar to that of HIV is approximately 9.8 kilo bases long and flanked at the 5' and 3' ends with LTRs comprising of U3, R and U5 regions. The genome of FIV codes for three major viral genes, namely *gag, pol* and *env*. Besides, the virus has other smaller ORFs that code for accessory proteins like *Vpr, Vif, Rev, Orf2, OrfA* etc. (Elder et al.,

1993). The *gag* gene encodes for precursors of viral structural proteins, which is cleaved by the viral protease during the process of maturation into matrix (MA), caspid (CA), and nucleocaspid (NC) proteins. The *pol* gene encodes for the viral protease (PR), reverse transcriptase (RT) and integrase (IN). In addition, the *pol* gene encodes for a protein with dUTPase activity also found in EIA and CAE virus but not in HIV-1. This protein prevents uracil misincorporations during the process of reverse transcription and hence plays an important role in FIV infection of non-dividing cells like macrophages (Bendinelli et al., 1995). The *env* gene of FIV codes for the membrane glycoprotein that comprises of the surface unit (SU) and transmembrane glycoprotein (TM) which are initially synthesized as a single precursor but later proteolytically cleaved and held together on the surface by non-covalent interactions (Inoshima et al., 1998). The *rev* gene of FIV is similar in function to *rev* gene product in HIV and transports unspliced and partially spliced viral RNA (Phillips et al., 1992) from the nucleus to the cytoplasm. *Rev* binds to the rev response element (RRE) located at the 3' end of env message and transports the mRNA outside the nucleus, bypassing the cellular splicing machinery. The *orf2* gene product in FIV, though distinct from other lentiviruses (Chatterji et al., 2002), shares functions similar to the viral transactivator *(Tat)* protein of other lentiviruses.

C. FIV receptor/co-receptor usage and viral entry

The *Env* glycoprotein of FIV is an important protein that mediates viral entry. Like other enveloped viruses, FIV enters the target cells via fusion of viral membrane with that of the target cell. While the viral envelope glycoptotein binds to the specific receptor/co-

receptor on cells, its gp41 domain mediates fusion facilitating in viral entry. It has been known for a while that FIV like HIV utilizes CXCR4 as a co-receptor for viral entry (Willet et al., 1997). However, the primary receptor for FIV has recently been identified as CD134, a $CD4^+$ T cell activation marker belonging to the TNF receptor superfamily (Shimojima et al., 2004). Similar to the CD4 independent isolates of HIV, CXCR4 alone is sufficient for mediating fusion and viral entry by lab adapted isolates of FIV. On the contrary, the primary receptor is essentially required by primary isolates of FIV. The fact that CD4 is not required as a receptor for FIV, but the two viruses still cause strikingly similar diseases in their respective hosts suggests a marked conservation between the pathogenesis of varied lentiviruses during evolution.

D. FIV as a suitable model to study HIV latency and persistence

The pathogenesis and disease pattern of FIV infection in cats parallels HIV-1 induced AIDS in humans (Bendinelli et al., 1995; English et al., 1994). Infected cats develop an acute infection syndrome including low-grade fever and transient generalized lymphadenopathy, followed by a long asymptomatic period in which the CD4:CD8 ratio declines due to an early increase in $CD8^+$ and a progressive decline in $CD4^+$ cell numbers. This asymptomatic period is followed by the development of a variety of disorders, many of which mimic HIV infection in humans (English et al., 1994; Pedersen et al., 1989). Once infection is initiated, lentiviruses usually persist for lifetime of the host and FIV is no exception. Similar to HIV, acute stage FIV infection is characterized by a high plasma viremia followed by a marked decline and persistent low level viremia during the long

asymptomatic stage of infection. Studies conducted by English et al (1994) used a PCR based assay to detect FIV proviral DNA in lymphocyte subsets sorted from peripheral blood of infected cats. They demonstrated that 2-6 weeks post infection highest level of provirus was present in $CD4^+$ cells, while after 3 months the maximum proviral load was found in the Ig^+ cells (English et al., 1994; Dean et al., 1996). In contrast, little or no provirus was detected in cells of the monocyte lineage (English et al., 1994). The mechanisms of FIV persistence in spite of the prompt and sustained immune response developed by infected cats have not been investigated. The validity of FIV as an animal model for AIDS can be emphasized by the fact that it provides an opportunity to study the disease in the absence of selection pressure of drugs. There has collected tremendous information in the recent past on the nature of HIV and AIDS in HAART treated patients especially in terms of virus persistence/reservoirs. However, it would be interesting to pursue the course of the disease and virus behavior in the absence of selection pressure. This would indeed be difficult to study in HIV-infected individuals but could easily be accomplished in FIV-infected cats.

E. Role of cell activation in HIV infection in vivo

Life cycle of retroviruses involves the generation of a DNA reverse transcript from double stranded viral RNA, which subsequently integrates into the host DNA (Farnet and Haseltine., 1990). Although, oncogenic retroviruses essentially require dividing host cells to establish a productive infection, lentiviruses are capable of establishing infection in non-dividing or terminally differentiated cells (Ostrowski et al., 1999). Indeed, human lentiviruses HIV-1 and HIV-2 possess the capability to stably infect non-dividing host cells

like macrophages and CNS glial cells (Spina et al., 1995). The ability of HIV to infect non-dividing cells is important for viral pathology in vivo (Finzi et al., 1999). HIV infection in lymphoid organs appears to be maintained in tissue macrophages and infection in the brain predominantly occurs via macrophages and microglial cells (Chun and Fauci., 1999). Moreover, the massive infection and spread of HIV within the $CD4^+$ T cell population is more readily acceptable with the observation that the virus persists in T cells in vivo even though the majority of these cells are found in a quiescent non-dividing state. Several studies have indicated that HIV found in resting $CD4^+$ T cells of the peripheral circulation and lymph nodes is maintained predominantly in a non-productive viral form either as extrachromosomal DNA or in a state of restricted transcription while retaining the potential to produce infectious virions when appropriately stimulated (Chun and Fauci., 1999; Spina et al., 1995).

F. Cell activation linked to HIV infection in vitro

The demonstration of HIV infection of primary quiescent T cells in vitro has remained controversial. Gowda et al (1989) demonstrated that unstimulated $CD4^+$ cells exposed to infectious virus for upto 48 hours were unable to synthesize any detectable integrated or unintegrated proviral DNA. However, activation of these cells with PHA or anti-CD3 antibody before viral exposure resulted in generation of unintegrated HIV DNA after 6 hours and integrated copies after 24 hours. They also demonstrated that resting T cells, in contrast to PHA stimulated PBMC were incapable of undergoing fusion and apoptosis when cultured with HIV-1 envelope expressing cells. Similarly, Chou et al (1997) showed that when unfractionated PBMC are first infected with HIV and $CD25^-$ cells then

isolated, the latter contain only partial DNA transcripts and no full-length proviral DNA or LTR circles. PHA activation of these cells resulted in generation of full-length proviral DNA and p24 antigen production. However, when $CD4^+CD25^-$ cells were first purified from PBMC and then infected, viral DNA could not be detected in these cells. Chou et al (1997) hence speculated that either $CD4^+CD25^-$ cells could not be infected de novo or that the presence of $CD25^+$ cells is required for infection of $CD25^-$ cells in vitro. In contrast to the above mentioned reports, Stevenson et al (1990) reported that T cell activation was not required for HIV-1 infection, however viral DNA was unable to integrate in resting T cells and was maintained extra-chromosomally. Subsequent T cell activation led to integration of extra-chromosomal forms and a productive viral life cycle. These extrachromosomal forms were found to persist for several weeks after infection of resting T cells (Pierson et al., 2002) and following activation these forms maintained their ability to integrate and act as a template for infectious virus. Mc Dougal et al (1985) similarly stated that activation/proliferative state of cells is not a necessary determinant for infectivity but rather, determines the amount of virus replication that will occur. Supporting the above findings, Spina et al (1995) demonstrated that HIV-1 can infect and establish a complete stable form of viral DNA in primary $CD4^+$ lymphocytes in vitro but is blocked from transcription in the absence of cell activation. In contrast to this, others have reported establishment of a complete infectious cycle of HIV in resting T cells with detectable early mRNA transcription and production of infectious virions (Gondois-Rey et al., 2001; Stevenson et al., 1990).

G. Productive infection by HIV essentially requires cell activation

Although there seem to be varied opinions regarding HIV entry in resting $CD4^+$ T cells, reports of HIV replicating productively in activated T cells are unanimous. HIV depends on both cellular and viral factors for efficient transcription of its genome and the activity of HIV promoter is highly dependent on the level of host cell activation (Zack et al., 1990 and 1992). HIV and FIV LTR regions contain binding sites for cellular transcription factors like AP-1, AP-4, ATF, CEBP, NFκB, SP-1 etc. (Bigornia et al., 2001; Cullen., 1989; Cullen and Greene., 1989; Tang et al., 1999). Binding of cellular transcription factors to the LTR region in the retroviral genome is known to be a requirement for efficient transcription via integrated proviral DNA. Although, the role of NFκB in HIV transactivation is well established, the putative NFκB binding site in FIV LTR, although variably expressed in different FIV isolates, seems to play little or no role in viral transcription (Sparger et al., 2002; Thompson et al., 2004). However, several studies have shown that the binding sites for AP-1, ATF and CEBP in the FIV LTR regulate FIV replication in infected cells. Kawaguchi et al (1995) demonstrated by site-specific mutations that the CEBP site in FIV LTR is necessary for efficient viral replication in both feline fibroblasts and a T lymphoblastoid cell line. Inoshima et al (1996) infected cats with AP-1 deleted viruses and showed that the site is essential for full viral replication and pathogencity in vivo. Ikeda et al (1998) demonstrated that mutations/deletions of AP-1 or ATF sites led to only moderate decrease in LTR dependent CAT activity while deletions of both ATF and AP-1 sites led to a severe reduction of basal promoter activity. Similarly, Bigornia et al (2001) showed that viruses encoding

deletions of both ATF and AP-1 binding sites were severely impaired in their ability to replicate both in feline lymphocytes and macrophages.

Another important factor contributing to the differential replication of HIV in activated and resting T cells is the state of cellular chromatin. Studies have shown that in latently infected cells proviral DNA integration frequently occurs into alphoid repetitive DNA, a component of centromeric heterochromatin leading to transcriptional repression by Position Effect Variegation (PEV). On the other hand, a comprehensive analysis of site of HIV integration during productive infection showed that HIV integrates preferentially in actively transcribed genes, namely the euchromatin (Bestor., 1998). It has hence been hypothesized that a repressive chromatin environment is the common underlying mechanism for transcriptional repression in latently infected cells (Jordan et al., 2003). Moreover, since heterochromatin is predominantly associated with the nuclear envelope, this is the most frequently encountered integration environment that the HIV preintegration complex sees once translocated to the nucleus. This explains the fact why most cells in retroviral infections are latently infected.

H. $CD4^+$ T cell decline in HIV is linked to apoptosis of activated cells

It is well known that T cells from HIV^+ individuals undergo spontaneous apoptosis when cultured exogenously. This emphasizes the involvement of Activated T Cell Autonomous Death (ACAD) in HIV driven T cells destruction. ACAD is associated with down-regulation of the anti-apoptotic gene Bcl-2 as detected in vivo in the blood and LN of HIV positive individuals (Gougeon., 2003). Cells that express low levels of Bcl-2 have an

activated phenotype in vivo indicating that priming for spontaneous apoptosis in T cells is mainly a result of immune activation. Activation Induced Cell Death (AICD), which is dependent on death receptors, can be induced ex vivo in T cells from HIV^+ patients following activation with mitogens, superantigens, or TCR specific antibodies (Gougeon., 2003). In vivo, increased expression of CD95 by both $CD4^+$ and $CD8^+$ T cell subsets from HIV^+ individuals leads to increased susceptibility to CD95 induced apoptosis, which correlates positively with disease progression. Increased levels of CD95 are also detected in the serum of HIV^+ individuals commonly used as a predictive marker for progression to AIDS (Gougeon., 2003). Studies conducted in vitro have demonstrated that $CD4^+$ cells can upregulate the expression of CD95L either as a result of direct infection or via viral proteins as gp120, tat or nef (Peterlin and Trono., 2003). These cells can then become possible killers of activated (not resting) CD95 expressing cells, which are found in high numbers in HIV^+ individuals (Gougeon., 2003). This is supported by the in vitro observation that activated $CD4^+$ T cells that express CD95L can kill CD95 expressing $CD8^+$ T cells independent of antigen recognition. HIV specific Cytotoxic T Lymphocytes (CTL) are also potential effectors for killing CD95 expressing activated lymphocytes induced by persistent HIV driven immune stimulation (Gougeon., 2003; Peterlin and Trono., 2003). Further emphasizing the role of immune activation in HIV induced apoptosis Gowda et al (1989) showed that cell to cell fusion characterized by syncytia formation was significantly reduced between freshly isolated resting T cells and cells constitutively expressing high levels of HIV envelope glycoprotein. Stimulation of resting T cells with PHA or OKT3 antibody restored syncytia formation between the above cell types emphasizing that T cell activation is a must for envelope dependent cell-to-cell fusion and apoptosis.

I. CD25 as a marker of cellular activation

The IL-2 receptor (IL-2R) consists of three non-covalently associated proteins called α, β and γ. The α and β chains are involved in cytokine binding and β and γ chains are involved in signal transduction (Ellery and Nicholls., 2002). The IL-2R α chain (also known as CD25) is a 55Kd glycoprotein that appears on T cell activation and was originally called Tac (for T cell activation). IL-2 binds to the α chain alone with a low affinity and this does not lead to any detectable biological response. The IL-2R β and γ chains are expressed on resting T cells and are sufficient and necessary for signal transduction (Ellery and Nicholls., 2002). However, cells that express IL-2R α together with IL-2R β and γ can bind IL-2 with greater affinity and stimulate growth and proliferation at a much lower concentration of IL-2. On antigen receptor mediated T cell activation, IL-2Rα is rapidly upregulated thereby reducing the concentration of IL-2 needed for proliferation. Binding of IL-2 to its receptor results in the activation of multiple signal transduction pathways including the JAK/STAT, PI3 kinase and the Ras signaling pathway (Ellery and Nicholls., 2002). The relative level of high to intermediate affinity receptor signaling determines whether a cell proliferates or undergoes AICD depending on the cell status.

Initial studies attempting to identify the role of CD25 in HIV infection demonstrated that the α chain of IL-2R expressed on activated T cells represents an ideal marker of cells productively infected with HIV. Ramilo et al (1993) and Borvak et al (1995) used immunotoxin labeled anti-CD25 antibody to show that normal PBMC infected with HIV-1 in vitro were markedly diminished in their ability to replicate HIV. Studies in a number of experimental models have firmly established the existence of a $CD4^+CD25^+$ T regulatory

(Treg) cell population that inhibits activation of autoreactive T cells thereby maintaining peripheral tolerance (Maloy and Powrie., 2002). There is also evidence showing that $CD4^+CD25^+$ cells can suppress immune response against infectious agents such as bacteria, fungi, viruses and intracellular parasites (Sakaguchi et al., 2003; Belkaid et al., 2002). The most useful marker to date for the identification of Treg cells is the CD25 antigen that has been demonstrated on 5-10% of the $CD4^+$ cells in the circulation of normal rodents, humans and cats (Maloy and Powrie., 2002; Vahlenkamp et al., 2004). While Treg cells have a partial activation phenotype they are anergic and their growth is dependent on exogenous IL-2 (Papiernick et al., 1998; Vahlenkamp et al., 2004). Important to the issue of virus persistence these cells appear to be resistant to clonal deletion and apoptosis when stimulated with anti-CD3 (Taams et al., 2001) or super antigen (Banz et al., 2002) suggesting that they could be long-lived in lymphoid tissues. Thus, by virtue of their anergic characteristic and resistance to clonal deletion these cells could act as ideal reservoirs of long-lived HIV infection.

J. Immunoregulatory $CD4^+CD25^+$ T cells

Sakaghuchi et al (1995) first described a subpopulation within the $CD4^+$ T cells that could prevent pathology associated with autoimmune disease model in mice. Since then, studies in a number of experiments models have firmly established the existence of a $CD4^+CD25^+$ T regulatory population with important immune regulatory properties (Anderson et al., 2004; Bluestone and Abbas., 2003; Shevach ., 2002; Walker., 2004). CD25, although a marker also expressed by activated T cells, remains to date the most useful marker for the $CD4^+CD25^+$ Treg population. These cells comprise 5-10% of the peripheral $CD4^+$ T cell

population in normal humans, rodents and cats (Maloy and Powrie., 2001; Joshi et al., 2004; Vahlenkamp et al., 2004). An important characteristics of Treg cells is that they remain anergic to stimulation via their TCR and in some instances to antibodies directed to the co-stimulatory molecule CD28 (Maloy and Powrie., 2001; Walker., 2004). However, exogenous cytokines like IL-2 and IL-4 are able to rescue Treg cells from their anergic state suggesting their proliferative potential (Walker., 2004).

In vivo, studies have demonstrated that Treg cells can proliferate in normal hosts in an antigen dependent manner especially when stimulated via antigen, mature antigen pulsed dendritic cells, or transgenic antigen expressed in various peripheral tissues (Cozzo et al., 2003; Walker., 2004). Whether maintenance of the Treg pool in the periphery is dependent on some limiting factor or whether they regulate their own homeostasis remains to be known. Although once speculated that rescue of Treg cells form their anergic state impairs their suppressive potential, studies later demonstrated that suppression is only temporarily abolished during Treg proliferation and rapidly reinstated as proliferation in these cells wanes. Indeed, stimulation of Treg cells via their TCR is required in order to induce their suppressive potential as detected by their ability to inhibit the proliferation of $CD4^+CD25^-$ T cells in co-culture assays in a dose-dependent manner (Jonuleit et al., 2001; Thornton and Shevach., 1998). However, once activated these cells can suppress $CD4^+$, $CD8^+$ and B cell responses in a non-specific manner. The suppression mediated by $CD4^+CD25^+$ Treg cells is cytokine independent but cell contact dependent (Nakamura et al., 2001; Thornton and Shevach., 2000). Some of the studies have demonstrated that cell contact mediated suppression by $CD4^+CD25^+$ T cells is due to cell surface associated TGF beta (Nakamura et al., 2001). Others have shown that Treg cells inhibit $CD4^+$ T cell responses via inhibition of

IL-2 production (Thornton and Shevach., 2001; Vahlenkamp et al., 2004). Another important property of Treg cells is that they are relatively resistant to viral superantigen induced apoptosis in vivo (Banz et al., 2002). It has also been demonstrated that $CD4^+CD25^+$ T cells activated in vitro by anti-CD3 antibody are resistant to Fas/Fas ligand induced apoptosis compared to their $CD4^+CD25^-$ counterparts (Taams et al., 2001).

Although it is well known that Treg cells comprise a unique population that originates in the thymus, it is becoming increasingly clear that cells with regulatory characteristics can also be generated in the periphery (Bach., 2003; Bluestone and Abbas., 2003). This can occur by intravenous antigen administration, co-stimulatory blockade, delivering antigens via immature dendritic cells or by delivering anti-CD4 antibodies (Bluestone and Abbas., 2003; Walker., 2004). In vitro, cells with Treg characteristics can be generated by repetitive stimulation with immature dendritic cells or by culturing cells in the presence of IL-10 and/or TGF beta (Bluestone and Abbas., 2003; Walker., 2004).

In the quest for an appropriate marker for $CD4^+CD25^+$ Treg cells, Hori et al (2003) demonstrated that Foxp3, a forkhead/winged helix family of transcription factor is specifically expressed by Treg cells and programs their development and function. Although it is known that Foxp3 is a negative modulator of IL-2 transcription (Hori et al., 2003; Garra and Vieira., 2003), it is not clear what genes are induced by Foxp3 which mediate their regulatory activity. However, it is well established that Treg cells are absent in Scrufy and Foxp3 deficient mice (Hori et al., 2003; Garra and Vieira., 2003). Furthermore, retroviral transfer of Foxp3 converts naïve T cells towards a regulatory T cell phenotype similar to that of naturally occurring $CD4^+CD25^+$ Treg cells (Richter et al., 2004).

Recent literature classifies $CD4^+CD25^+$ Treg cells into two distinct subtypes 1) natural Treg cells, that develop in the thymus and 2) adaptive Treg cells generated form naïve T cells in the periphery (Bluestone and Abbas., 2003). The natural versus adaptive Treg cells differ in terms of their development, specificity, mechanism of action and dependence on T cell receptor and co-stimulatory signaling.

K. Role of $CD4^+CD25^+$ Treg cells in infectious diseases

Although, Treg cells are believed to be specific towards self-antigens, it is unreasonable to conclude that their TCR does not cross-react to some extent with foreign epitopes as well. Data available to date suggests that $CD4^+CD25^+$ Treg cells can react to foreign proteins too. In this regard, the concept of natural versus adaptive Treg cells seems to be more plausible when comparing infectious diseases with autoimmune settings. It has been speculated that natural Treg cells would be most effective at suppressing autoreactive T cell responses locally in non-inflammatory settings where antigen specific self limiting reactions are required in order maintain homeostasis. On the other hand, adaptive Treg cells play a more important role during self-damaging inflammatory reactions or inflammatory autoimmune diseases that are more similar to infectious settings (Bluestone and Abbas., 2003).

Belkaid et al (2002) were one of the first to describe the role of $CD4^+CD25^+$ Treg cells in infectious diseases. They showed that $CD4^+CD25^+$ T cells play an important role in persistence of Leishmania major parasites in the dermis of infected mice via both IL-10 dependent and independent mechanisms. Moreover, sterilizing immunity to the parasites in

IL-10 knock out mice leads to loss of protective immunity to re-infection. Hisaeda et al (2003) showed with respect to malaria infection that depletion of $CD25^+$ cells protects mice from challenge with a lethal strain of malaria parasite. Subsequently, Aandhal et al (2004) similarly showed that in vitro depletion of $CD4^+CD25^+$ Treg cells from PBMC of HIV^+ individuals resulted in a significant increase in the number of HIV antigen specific interferon gamma producing T cells. Further, Richter et al (2004) recently demonstrated that HIV positive individuals with low $CD4^+$ T cell counts and high levels of immune activation show a reduction in the $FoxP3^+$ $CD4^+CD25^+$ high T cells. This specific depletion of Treg cells in HIV infection might be important in producing a state of hyperactivation in conventional T cells, which is an important characteristic of HIV disease progression.

Vahlenkamp et al (2004) demonstrated with regard to another retrovirus FIV, that $CD4^+CD25^+$ T cells comprise an important immunoregulatory T cell subset in cats too. These cells, like the Treg cells described in rodent and human systems comprise 5-10% of the peripheral $CD4^+$ T cell population although a higher percentage is present in the feline lymph nodes. Moreover, feline $CD4^+CD25^+$ cells also express cell surface associated CTLA4, a marker predominantly associated with Treg cells. While $CD4^+CD25^+$ T cells derived form FIV^- cats required stimulation via their TCR in order to suppress proliferation of activated $CD4^+CD25^-$ cells, Treg cells derived from FIV^+ cats bypassed the requirement of stimulation via their TCR in order to non-specifically suppress $CD4^+$ T helper responses (Vahlenkamp et al., 2004). Also, the suppression of $CD4^+$ T helper responses mediated by feline Treg cells was via inhibition of IL-2 production. The preservation of a population with important immune regulatory properties through evolution in varied species emphasizes the functional importance of $CD4^+CD25^+$ Treg cells in immune responses.

The role of $CD4^+CD25^+$ Treg cells in infectious settings is further emphasized by the fact that these cells selectively express toll like receptors and can be activated by pathogen associated molecular patterns only produced by pathogens and not by the host cells (Caramalho et al., 2003; Pasare et al., 2003). Moreover, LPS is known to markedly upregulate Treg activity as measured in suppression assays in vitro (Caramalho et al., 2003; Vahlenkamp et al., 2004). This phenomenon not only hints towards another important link between innate and adaptive immune response but also reveals a novel mechanism for control of immune responses to pathogens.

An important manifestation of a number of chronic infections like HIV, tuberculosis, malaria, toxoplasmosis is the presence of a high percentage of $CD4^+$ T cells capable of releasing large amounts of IL-10 (Sakaguchi., 2003). This might be a strategy adopted by organisms that find ways to co-exist with the host in order to favor their long-term survival. Or, on the contrary, it might be a host adaptation to prevent an exaggerated immune response during the course of a normal immune response towards the invading pathogen. This phenomenon, although to some extent detrimental to the host in terms of maintenance of chronic infections and re-infections also confers the host with an advantage of maintaining the antigen for prolonged periods of time, which facilitates an enhanced memory T cell response during re-infection.

L. Reservoirs of HIV infection

Treatment of HIV-1 infected individuals with HAART decreases the amount of virus in plasma to levels below the limit of detection by standard clinical assays (Perelson et al., 1996). HAART however, does not result in virus eradication as a small but detectable

reservoir of latently and productively infected $CD4^+$ cells harboring replication competent HIV-1 has been shown to persist in essentially all patients receiving therapy (Dornadula et al., 1999; Finzi et al., 1997; Wong et al., 1997). Moreover, rebound in plasma viremia levels after cessation of HAART in HIV infected individuals does not correlate with the frequency of latently infected cells indicating that other viral reservoirs exist. There is also evidence for continuous ongoing viral replication on the basis of viral sequence evolution within the envelope region over time in patients receiving HAART (Gunthard et al., 1999). It is unknown whether the sustained level of viremia results from the release of virus from a population of productively infected T cells or from latently infected $CD4^+$ cells that become activated or both (Hermankova et al., 2001; Perelson et al., 2002). The presence of residual infected T cells is of serious concern as they provide the basis for lifetime persistence of the virus despite potent antiviral therapy. A better understanding of the nature of the reservoir that sustains virus replication in aviremic patients on HAART may lead to the development of more effective strategies to limit virus replication.

Although there are several potential viral reservoirs including macrophages, microglial cells, seminal fluid cells, thymocytes and resting $CD4^+$ cells, long term persistence of HIV has best been documented in $CD45RO^+$ long-lived memory T cells (Van der Ende et al., 1999). A potentially stable virus reservoir is established when $CD4^+$ cells survive the cytopathic effects of the virus and revert to a resting memory phenotype carrying the provirus in the post integration phase of latency (Persaud et al., 2003). The rapidity with which the latent viral reservoir is capable of being established is emphasized by the fact that initiation of HAART as early as 10 days after the onset of symptoms of primary HIV infection did not prevent the generation of latently infected T cells (Chun et al., 1998). The half-life of

replication competent HIV in latently infected cells is so high that the natural turnover of this latent reservoir would not be sufficient to achieve eradication of HIV in infected individuals treated with HAART alone (Chun and Fauci., 1999). In this regard studies have shown that the combination of cytokines particularly IL-2, IL-6 and TNF-alpha is a potent inducer of viral replication from the pool of latently infected cells (Chun et al., 1998 and 1999). Since these cytokines are known to be naturally present in the lymphoid milieu they might explain for the commonly observed reappearance of plasma viremia after cessation of HAART in HIV-infected individuals (Chun and Fauci., 1999).

A commonly used method to identify cells productively infected with HIV/FIV is to analyze for the presence of 1-LTR, or 2-LTR circles in the nucleus of infected cells (Brown at al., 1987; Frey at al., 2002; Goedert et al., 2001). These circular forms, also referred to as circle junctions, are hallmarks of cells productively infected with HIV generally absent in resting cells (Sharkey and Stevenson., 2001; Zazzi et al., 1997). It has been demonstrated that majority of patients on HAART, showing no detectable plasma HIV RNA have LTR episomal DNA intermediates in their PBMC indicative of low level ongoing virus replication (Chun et al., 1999; Sharkey et al., 2000). Sharkey et al (2000) demonstrated that rescue of HIV-1 in vitro strongly correlates with the presence of these viral DNA intermediates in patient's PBMC. Moreover, severity of AIDS associated dementia has been linked to the presence of these circular unintegrated HIV DNA forms marking them as molecular predictors of HIV disease progression (Teo et al., 1997).

M. Role of viral proteins in inducing virus expression from latently infected cells

Resting $CD4^+$ T cells containing integrated proviral DNA constitute one of the long-lived reservoirs of HIV in vivo (Persaud et al., 2003). This cellular reservoir of HIV is quiescent based on the observation that HIV production in T cells is essentially linked to cellular activation. Based on the fact that the number of infected T cells in HIV^+ individuals is remarkably low and even a fewer proportion of these cells is capable of supporting a productive infection would restrict virus replication to a small, defined cell population (Bukrinsky et al., 1991; Chun et al., 1997). However, the virus has evolved strategies to meet these limitations. HIV encodes several proteins including the envelope glycoprotein (composed of gp120 and gp41) and *Nef* which are known to trigger a variety of signaling pathways associated with cellular activation, thereby facilitating virus production from resting/latently infected cells (Wu and Marsh., 2001).

Kornfeld et al (1988) showed that gp120, as a result of specific interaction with CD4 is capable of inducing an increase in the levels of intracellular IP3 and calcium with a simultaneous increase in IL-2R expression and cell motility. Others showed that interaction of HIV gp120 with cell surface CD4 delivers signals to the target cell resulting in activation of transcription factors NFκB and AP-1 (Bossis et al., 2002; Chirmule at al., 1995). Recent studies by Cicala et al (2002) demonstrated that HIV envelope was capable of inducing a series of signals in non-proliferating target cells that favor virus replication. Through the use of oligonucleotide microarray and high throughput western blotting they demonstrated that gp120 induced the expression of cytokines, chemokines, kinases and transcription factors associated with antigen specific T cell activation in the absence of cellular proliferation. In a

similar study, Kinter et al (2003) showed that HIV recombinant envelope glycoprotein induces virus replication in cells isolated from HIV-infected individuals. Interestingly, this induction of HIV expression continued in the presence of RT inhibitors and was not associated with markers of classical T cell activation, proliferation or apoptosis. Chun et al (2003) compared virus production in resting $CD4^+$ T cells from viremic versus aviremic HIV patients. They demonstrated that resting $CD4^+$ T cells from aviremic patients failed to produce cell free HIV spontaneously ex vivo despite the presence of HIV proviral DNA when compared to viremic patients. This apparent difference was attributed to the fact that a variety of genes involved in RNA processing, transcription regulation, protein trafficking and vesicle transport were upregulated in resting $CD4^+$ cells from viremic patients compared to aviremic patients.

N. Based on the above literature, the present study was undertaken with the following specific aims

Specific Aim 1: To address the question whether there is a difference in the inherent abilities of $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells to be infected with FIV and produce infectious virions in vitro and in vivo.

Specific Aim 2: To determine if feline $CD4^+CD25^+$ cells display the key characteristic of $CD4^+CD25^+$ Treg cells described in the rodent and human systems and whether FIV infection alters these characteristics.

Specific Aim 3: If $CD4^+CD25^+$ T cells have the potential to serve as long-term reservoirs of productive FIV infection and whether $CD4^+CD25^-$ cells comprise a latent viral reservoir capable of competent reactivation when strongly stimulated.

Specific Aim 4: To determine whether the preferential replication of FIV in $CD4^+CD25^+$ T cells is governed at the level of viral entry or at the level of cellular transcriptional activity or both.

O. LITERATURE CITED

1. **Aandahl EM, Michaelsson J, Moretto WJ, Hecht FM, Nixon DF.** 2004. Human CD4+ CD25+ regulatory T cells control T-cell responses to human immunodeficiency virus and cytomegalovirus antigens. J Virol. **78:** 2454-2459.

2. **Bach JF.** 2003. Regulatory T cells under scrutiny. Nat Rev Immunol. **3:** 189-198.

3. **Banz A, Pontoux C, Papiernik M.** 2002. Modulation of Fas-dependent apoptosis: a dynamic process controlling both the persistence and death of CD4 regulatory T cells and effector T cells. J Immunol. **169:** 750-757.

4. **Belkaid Y, Piccirillo CA, Mendez S, Shevach EM, Sacks DL.** 2002. CD4+CD25+ regulatory T cells control Leishmania major persistence and immunity. Nature. **420:** 502-507.

5. **Bendinelli M, Pistello M, Lombardi S, Poli A, Garzelli C, Matteucci D, Ceccherini-Nelli L, Malvaldi G, Tozzini F.** 1995. Feline immunodeficiency virus: an interesting model for AIDS studies and an important cat pathogen. Clin Microbiol Rev. **8:** 87-112.

6. **Bestor TH.** 1998. Gene silencing. Methylation meets acetylation. Nature. **393:** 311-312.

7. **Bigornia L, Lockridge KM, Sparger EE.** 2001. Construction and in vitro characterization of attenuated feline immunodeficiency virus long terminal repeat mutant viruses. J.Virol. **75:** 1054-1060.

8. **Bleul CC, Wu L, Hoxie JA, Springer TA, Mackay CR.** 1997. The HIV coreceptors CXCR4 and CCR5 are differentially expressed and regulated on human T lymphocytes. Proc. Natl. Acad. Sci. U S A. **94:** 1925-1930.

9. **Bluestone JA, Abbas AK.** 2003. Natural versus adaptive regulatory T cells. Nat Rev Immunol. **3:** 253-257.

10. **Borvak J, Chou CS, Bell K, Van Dyke G, Zola H, Ramilo O, Vitetta ES.** 1995. Expression of CD25 defines peripheral blood mononuclear cells with productive versus latent HIV infection. J Immunol. **155:** 3196-3204.

11. **Bossis G, Salinas S, Cartier C, Devaux C, Briant L.** 2002. NF-kappaB activation upon interaction of HIV-1 envelope glycoproteins with cell surface CD4 involves IkappaB kinases. FEBS Lett. **516:** 257-264.

12. **Brown PO, Bowerman B, Varmus HE, Bishop JM.** 1987. Correct integration of retroviral DNA in vitro. Cell. **49:** 347-356.

13. **Bukrinsky MI, Stanwick TL, Dempsey MP, Stevenson M.** 1991. Quiescent T lymphocytes as an inducible virus reservoir in HIV-1 infection. Science. **254:** 423-427.

14. **Caramalho I, Lopes-Carvalho T, Ostler D, Zelenay S, Haury M, Demengeot J.** 2003. Regulatory T cells selectively express toll-like receptors and are activated by lipopolysaccharide. J Exp Med. **197:** 403-411.

15. **Chatterji U, de Parseval A, Elder JH. 2002.** Feline immunodeficiency virus OrfA is distinct from other lentivirus transactivators. J Virol. **76:** 9624-34.

16. **Chirmule N, Goonewardena H, Pahwa S, Pasieka R, Kalyanaraman VS, Pahwa S.** 1995. HIV-1 envelope glycoproteins induce activation of activated protein-1 in CD4+ T cells. J Biol Chem. **270:**19364-19369.

17. **Chou CS, Ramilo O, Vitetta ES.** 1997. Highly purified CD25- resting T cells cannot be infected de novo with HIV-1. Proc Natl Acad Sci U S A. **94:** 1361-1365.

18. **Chun TW, Carruth L, Finzi D, Shen X, DiGiuseppe JA, Taylor H, Hermankova M, Chadwick K, Margolick J, Quinn TC, Kuo YH, Brookmeyer R, Zeiger MA, Barditch-Crovo P, Siliciano RF**. 1997. Quantification of latent tissue reservoirs and total body viral load in HIV-1 infection. Nature. **387:** 183-188.

19. **Chun TW, Davey RT Jr, Engel D, Lane HC, Fauci AS.** 1999. Re-emergence of HIV after stopping therapy. Nature. **401:** 874-875.

20. **Chun TW, Davey RT Jr, Ostrowski M, Shawn Justement J, Engel D, Mullins JI, Fauci AS.** 2000. Relationship between pre-existing viral reservoirs and the re-emergence of plasma viremia after discontinuation of highly active anti-retroviral therapy. Nat Med. **6:** 757-761.

21. **Chun TW, Engel D, Berrey MM, Shea T, Corey L, Fauci AS.** 1998. Early establishment of a pool of latently infected, resting CD4 (+) T cells during primary HIV-1 infection. Proc Natl Acad Sci U S A. **95:** 8869-8873.

22. **Chun TW, Engel D, Mizell SB, Ehler LA, Fauci AS.** 1998. Induction of HIV-1 replication in latently infected CD4+ T cells using a combination of cytokines. J Exp Med. **188:** 83-91

23. **Chun TW, Engel D, Mizell SB, Hallahan CW, Fischette M, Park S, Davey RT Jr, Dybul M, Kovacs JA, Metcalf JA, Mican JM, Berrey MM, Corey L, Lane HC, Fauci AS.** 1999. Effect of interleukin-2 on the pool of latently infected, resting CD4+ T cells in HIV-1-infected patients receiving highly active anti-retroviral therapy. Nat Med. **5:** 651-655.

24. **Chun TW, Fauci AS.** 1999. Latent reservoirs of HIV: obstacles to the eradication of virus. Proc Natl Acad Sci U S A. **96:**10958-10961.

25. **Chun TW, Finzi D, Margolick J, Chadwick K, Schwartz D, Siliciano RF.** 1995. In vivo fate of HIV-1-infected T cells: quantitative analysis of the transition to stable latency. Nat Med. **1:** 1284-1290.

26. **Chun TW, Justement JS, Lempicki RA, Yang J, Dennis G Jr, Hallahan CW, Sanford C, Pandya P, Liu S, McLaughlin M, Ehler LA, Moir S, Fauci AS.** 2003. Gene expression and viral prodution in latently infected, resting CD4+ T cells in viremic versus aviremic HIV- infected individuals. Proc Natl Acad Sci U S A.**100:** 1908-1913.

27. **Cicala C, Arthos J, Selig SM, Dennis G Jr, Hosack DA, Van Ryk D, Spangler ML, Steenbeke TD, Khazanie P, Gupta N, Yang J, Daucher M, Lempicki RA, Fauci AS.** 2002. HIV envelope induces a cascade of cell signals in non-proliferating target cells that favor virus replication. Proc Natl Acad Sci U S A. **99:**9380-9385.

28. **Cozzo C, Larkin J 3rd, Caton AJ.** 2003. Cutting edge: self-peptides drive the peripheral expansion of CD4+CD25+ regulatory T cells. J Immunol. **171:** 5678-5682.

29. **Cullen BR, Greene WC.** 1989. Regulatory pathways governing HIV-1 replication. Cell. **58:** 423-426.

30. **Dean GA, Higgins J, LaVoy A, Fan Z, Pedersen NC.** 1998. Measurement of feline cytokine gene expression by quantitative-competitive RT-PCR. Vet Immunol Immunopathol. **15:** 73-82.

31. **Dean GA, Reubel GH, Moore PF, Pedersen NC.** 1996. Proviral burden and infection kinetics of feline immunodeficiency virus in lymphocyte subsets of blood and lymph node. J Virol. **70:** 5165-5169.

32. Dornadula G, Zhang H, VanUitert B, Stern J, Livornese L Jr, Ingerman MJ, Witek J, Kedanis RJ, Natkin J, DeSimone J, Pomerantz RJ. 1999. Residual HIV-1 RNA in blood plasma of patients taking suppressive highly active antiretroviral therapy. JAMA. **282:** 1627-1632.

33. **Eckstein DA, Penn ML, Korin YD, Scripture-Adams DD, Zack JA, Kreisberg JF, Roederer M, Sherman MP, Chin PS, Goldsmith MA.** 2001. HIV-1 actively replicates in naive CD4(+) T cells residing within human lymphoid tissues. Immunity. **15:** 671-682.

34. **Elder JH, Phillips TR.** 1993. Molecular properties of feline immunodeficiency virus (FIV). Infect Agents Dis. **2:** 361-74.

35. **Ellery JM, Nicholls PJ.** 2002. Possible mechanism for the alpha subunit of the interleukin-2 receptor (CD25) to influence interleukin-2 receptor signal transduction. Immunol Cell Biol. **80:** 351-357.

36. **Ellery JM, Nicholls PJ.** 2002. Alternate signalling pathways from the interleukin-2 receptor. Cytokine Growth Factor Rev. **13:** 27-40.

37. **English RV, Johnson CM, Gebhard DH, Tompkins MB.** 1993. In vivo lymphocyte tropism of feline immunodeficiency virus. J Virol. **67:** 5175-5186.

38. **Farnet CM, Haseltine WA.** 1990. Integration of human immunodeficiency virus type 1 DNA in vitro. Proc Natl Acad Sci U S A. **87:** 4164-4168.

39. **Finzi D, Blankson J, Siliciano JD, Margolick JB, Chadwick K, Pierson T, Smith K, Lisziewicz J, Lori F, Flexner C, Quinn TC, Chaisson RE, Rosenberg E, Walker B, Gange S, Gallant J, Siliciano RF.** 1999. Latent infection of CD4+ T cells provides a mechanism for lifelong persistence of HIV-1, even in patients on effective combination therapy. Nat Med. **5:** 512-517.

40. **Finzi D, Hermankova M, Pierson T, Carruth LM, Buck C, Chaisson RE, Quinn TC, Chadwick K, Margolick J, Brookmeyer R, Gallant J, Markowitz M, Ho DD, Richman DD, Siliciano RF.** 1997. Identification of a reservoir for HIV-1 in patients on highly active antiretroviral therapy. Science. **278:** 1295-1300.

41. **Frey SC, Hoover EA, Mullins JI.** 2001. Feline immunodeficiency virus cell entry. J Virol. **75:** 5433-54340.

42. **Goedert JJ, O'Brien TR, Hatzakis A, Kostrikis LG.** 2001. T cell receptor excision circles and HIV-1 2-LTR episomal DNA to predict AIDS in patients not receiving effective therapy. AIDS. **15:** 2245-2250

43. **Gondois-Rey F, Biancotto A, Pion M, Chenine AL, Gluschankof P, Horejsi V, Tamalet C, Vigne R, Hirsch I**. 2001. Production of HIV-1 by resting memory T lymphocytes. AIDS. **15:** 1931-1940.

44. **Gougeon ML.** 2003. Apoptosis as an HIV strategy to escape immune attack. Nat Rev Immunol. **3:** 392-404.

45. **Gowda SD, Stein BS, Mohagheghpour N, Benike CJ, Engleman EG.** 1989. Evidence that T cell activation is required for HIV-1 entry in CD4+ lymphocytes. J Immunol. Feb **142:** 773-780.

46. **Gunthard HF, Frost SD, Leigh-Brown AJ, Ignacio CC, Kee K, Perelson AS, Spina CA, Havlir DV, Hezareh M, Looney DJ, Richman DD, Wong JK.** 1999. Evolution of envelope sequences of human immunodeficiency virus type 1 in cellular reservoirs in the setting of potent antiviral therapy. J Virol. **73:** 9404-9412.

47. **Hermankova M, Ray SC, Ruff C, Powell-Davis M, Ingersoll R, D'Aquila RT, Quinn TC, Siliciano JD, Siliciano RF, Persaud D.** 2001. HIV-1 drug resistance profiles in children and adults with viral load of <50 copies/ml receiving combination therapy. JAMA. **286:** 196-207.

48. **Hisaeda H, Maekawa Y, Iwakawa D, Okada H, Himeno K, Kishihara K, Tsukumo S, Yasutomo K.** 2004. Escape of malaria parasites from host immunity requires CD4+ CD25+ regulatory T cells. Nat Med. **10:** 29-30.

49. **Hori S, Nomura T, Sakaguchi S.** 2003. Control of regulatory T cell development by the transcription factor Foxp3. Science. **299:** 1057-1061.

50. **Ikeda Y, Inoshima Y, Kawaguchi Y, Maeda K, Kohmoto M, Kai C, Miyazawa T, Mikami T.** 1998. Protein-binding properties of the putative AP-1 and ATF sequences in the feline immunodeficiency virus long terminal repeat. J Gen Virol. **79:** 95-99.

51. **Ikeda Y, Kawaguchi Y, Tomonaga K, Inoshima Y, Kohmoto M, Miyazawa T, Mikami T.** 1996. Regulatory properties of the integrated long terminal repeat of the feline immunodeficiency virus. Virus Res. **41:** 201-207.

52. **Inoshima Y, Kohmoto M, Ikeda Y, Yamada H, Kawaguchi Y, Tomonaga K, Miyazawa T, Kai C, Umemura T, Mikami T.** 1996. Roles of the auxiliary genes and AP-1 binding site in the long terminal repeat of feline immunodeficiency virus in the early stage of infection in cats. J Virol. **70:** 8518-8526.

53. **Inoshima Y, Miyazawa T, Mikami T.** 1998. In vivo functions of the auxiliary genes and regulatory elements of feline immunodeficiency virus. Vet Microbiol. **60:** 141-53.

54. **Jonuleit H, Schmitt E, Stassen M, Tuettenberg A, Knop J, Enk AH.** 2001. Identification and functional characterization of human CD4(+)CD25(+) T cells with regulatory properties isolated from peripheral blood. J Exp Med.**193:**1285-1294.

55. **Jordan A, Bisgrove D, Verdin E. 2003.** HIV reproducibly establishes a latent infection after acute infection of T cells in vitro. EMBO J.**22:** 1868-1877.

56. **Joshi A, Vahlenkamp TW, Garg H, Tompkins WA, Tompkins MB.** 2004. Preferential replication of FIV in activated CD4(+)CD25(+)T cells independent of cellular proliferation. Virology. **321:** 307-322.

57. **Kawaguchi Y, Tomonaga K, Maeda K, Ono M, Miyazawa T, Kohmoto M, Tohya Y, Mikami T.** 1995. The C/EBP site in the feline immunodeficiency virus (FIV) long terminal repeat (LTR) is necessary for its efficient replication and is also involved in the inhibition of FIV LTR-directed gene expression by pseudorabies virus ICP4. Virology. **208:** 492-499.

58. **Kinter AL, Umscheid CA, Arthos J, Cicala C, Lin Y, Jackson R, Donoghue E, Ehler L, Adelsberger J, Rabin RL, Fauci AS.** 2003. HIV envelope induces virus expression from resting CD4+ T cells isolated from HIV-infected individuals in the absence of markers of cellular activation or apoptosis. J Immunol. **170:** 2449-2455.

59. **Kornfeld H, Cruikshank WW, Pyle SW, Berman JS, Center DM.** 1988. Lymphocyte activation by HIV-1 envelope glycoprotein. Nature. **335:** 445-448.

60. **Maloy KJ, Powrie F.** 2001. Regulatory T cells in the control of immune pathology. Nat Immunol. **2:** 816-822.

61. **McDougal JS, Mawle A, Cort SP, Nicholson JK, Cross GD, Scheppler-Campbell JA, Hicks D, Sligh J.** 1985. Cellular tropism of the human retrovirus HTLV-III/LAV. I. Role of T cell activation and expression of the T4 antigen. J Immunol. **135:**3151-1362.

62. **Nagler-Anderson C, Bhan AK, Podolsky DK, Terhorst C.** 2004. Control freaks: immune regulatory cells. Nat Immunol. **5:** 119-122.

63. **Nakamura K, Kitani A, Strober W.** 2001. Cell contact-dependent immunosuppression by CD4(+)CD25(+) regulatory T cells is mediated by cell surface-bound transforming growth factor beta. J Exp Med. **194:** 629-644.

64. **O'Garra A, Vieira P.** 2003. Twenty-first century Foxp3. Nat Immunol. **4:** 304-306.

65. **Ostrowski MA, Chun TW, Justement SJ, Motola I, Spinelli MA, Adelsberger J, Ehler LA, Mizell SB, Hallahan CW, Fauci AS.** 1999. Both memory and

CD45RA+/CD62L+ naive CD4(+) T cells are infected in human immunodeficiency virus type 1-infected individuals. J Virol. **73:** 6430-6435.

66. **Oswald-Richter K, Grill SM, Shariat N, Leelawong M, Sundrud MS, Haas DW, Unutmaz D.** 2004. HIV Infection of Naturally Occurring and Genetically Reprogrammed Human Regulatory T-cells. PLoS Biol. **2:** 955-966

67. **Papiernik M, de Moraes ML, Pontoux C, Vasseur F, Penit C.** 1998. Regulatory CD4 T cells: expression of IL-2R alpha chain, resistance to clonal deletion and IL-2 dependency. Int Immunol. **10:** 371-378.

68. **Pasare C, Medzhitov R.** 2003. Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells. Science. **299:** 1033-1036.

69. **Pedersen NC, Yamamoto JK, Ishida T, Hansen H.** 1989. Feline immunodeficiency virus infection. Vet Immunol Immunopathol. **21:** 111-129.

70. **Perelson AS, Neumann AU, Markowitz M, Leonard JM, Ho DD.** 1996. HIV-1 dynamics in vivo: virion clearance rate, infected cell life-span, and viral generation time. Science. **271:** 1582-1586.

71. **Persaud D, Zhou Y, Siliciano JM, Siliciano RF.** 2003. Latency in human immunodeficiency virus type 1 infection: no easy answers. J Virol. **77:**1659-1665.

72. **Peterlin BM, Trono D. 2003.** Hide, shield and strike back: how HIV-infected cells avoid immune eradication. Nat Rev Immunol. **3:** 97-107.

73. **Phillips TR, Lamont C, Konings DA, Shacklett BL, Hamson CA, Luciw PA, Elder JH.** 1992. Identification of the Rev transactivation and Rev-responsive elements of feline immunodeficiency virus. J Virol. **66:** 5464-71.

74. **Pierson T, Hoffman TL, Blankson J, Finzi D, Chadwick K, Margolick JB, Buck C, Siliciano JD, Doms RW, Siliciano RF.** 2000. Characterization of chemokine receptor utilization of viruses in the latent reservoir for human immunodeficiency virus type 1. J Virol. **74:** 7824-7833.

75. **Pierson TC, Kieffer TL, Ruff CT, Buck C, Gange SJ, Siliciano RF.** 2002. Intrinsic stability of episomal circles formed during human immunodeficiency virus type 1 replication. J Virol. **76:** 4138-4144.

76. **Ramilo O, Bell KD, Uhr JW, Vitetta ES.** 1993. Role of CD25+ and CD25-T cells in acute HIV infection in vitro. J Immunol. **150:** 5202-5208.

77. **Sakaguchi S. 2003.** Regulatory T cells: mediating compromises between host and parasite. Nat Immunol. **4:** 10-11.

78. **Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M.** 1995. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol. **155:** 1151-64.

79. **Sharkey ME, Stevenson M.** 2001. Two long terminal repeat circles and persistent HIV-1 replication. Curr Opin Infect Dis. **14:** 5-11.

80. **Sharkey ME, Teo I, Greenough T, Sharova N, Luzuriaga K, Sullivan JL, Bucy RP, Kostrikis LG, Haase A, Veryard C, Davaro RE, Cheeseman SH, Daly JS, Bova C, Ellison RT 3rd, Mady B, Lai KK, Moyle G, Nelson M, Gazzard B, Shaunak S, Stevenson M.** 2000. Persistence of episomal HIV-1 infection intermediates in patients on highly active anti-retroviral therapy. Nat Med. **6:** 76-81.

81. **Shevach EM.** 2002. CD4+ CD25+ suppressor T cells: more questions than answers. Nat Rev Immunol. **2:** 389-400.

82. **Shimojima M, Miyazawa T, Ikeda Y, McMonagle EL, Haining H, Akashi H, Takeuchi Y, Hosie MJ, Willett BJ.** 2004. Use of CD134 as a primary receptor by the feline immunodeficiency virus. Science. **303:** 1192-1195.

83. **Sparger EE, Shacklett BL, Renshaw-Gegg L, Barry PA, Pedersen NC, Elder JH, Luciw PA.** 1992. Regulation of gene expression directed by the long terminal repeat of the feline immunodeficiency virus. Virology **187:** 165-177.

84. **Spina CA, Guatelli JC, Richman DD.** 1995. Establishment of a stable, inducible form of human immunodeficiency virus type 1 DNA in quiescent CD4 lymphocytes in vitro. J Virol. **69:** 2977-2988.

85. **Stevenson M, Stanwick TL, Dempsey MP, Lamonica CA. 1990.** HIV-1 replication is controlled at the level of T cell activation and proviral integration. EMBO J. **9:**1551-1560.

86. **Taams LS, Smith J, Rustin MH, Salmon M, Poulter LW, Akbar AN.** 2001. Human anergic/suppressive CD4(+)CD25(+) T cells: a highly differentiated and apoptosis-prone population. Eur J Immunol. **31:** 1122-1131.

87. **Tang H, Kuhen KL, Wong-Staal F.** 1999. Lentivirus replication and regulation. Annu Rev Genet. **33:** 133-170.

88. **Teo I, Veryard C, Barnes H, An SF, Jones M, Lantos PL, Luthert P, Shaunak S.** 1997. Circular forms of unintegrated human immunodeficiency virus type 1 DNA and high levels of viral protein expression: association with dementia and multinucleated giant cells in the brains of patients with AIDS. J Virol. **71:** 2928-2933.

89. T**hompson FJ, Elder J, Neil JC.** 1994. Cis- and trans-regulation of feline immunodeficiency virus: identification of functional binding sites in the long terminal repeat. J Gen Virol. **75:** 545-554.

90. **Thornton AM, Shevach EM.** 1998. CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. J Exp Med. **188:** 287-296.

91. **Thornton AM, Shevach EM.** 2000. Suppressor effector function of CD4+CD25+ immunoregulatory T cells is antigen nonspecific. J Immunol. **164:** 183-190.

92. **Vahlenkamp TW, Tompkins MB, Tompkins WA.** 2004. Feline immunodeficiency virus infection phenotypically and functionally activates immunosuppressive CD4+CD25+ T regulatory cells. J Immunol. **172:** 4752-4761.

93. **Van der Ende ME, Schutten M, Raschdorff B, Grossschupff G, Racz P, Osterhaus AD, Tenner-Racz K.** 1999. CD4 T cells remain the major source of HIV-1 during end stage disease. AIDS. **13:** 1015-1019.

94. **Walker LS.** 2004. CD4+ CD25+ Treg: divide and rule? Immunology. **111:**129-137.

95. **Willett BJ, Picard L, Hosie MJ, Turner JD, Adema K, Clapham PR.** 1997. Shared usage of the chemokine receptor CXCR4 by the feline and human immunodeficiency viruses. J Virol. **71:** 6407-6415.

96. **Wong JK, Hezareh M, Gunthard HF, Havlir DV, Ignacio CC, Spina CA, Richman DD.** 1997. Recovery of replication-competent HIV despite prolonged suppression of plasma viremia. Science. **278:** 1291-1295.

97. **Wu Y, Marsh JW.** 2001. Selective transcription and modulation of resting T cell activity by preintegrated HIV DNA. Science. **293:** 1503-1506.

98. **Zack JA, Arrigo SJ, Weitsman SR, Go AS, Haislip A, Chen IS.** 1990. HIV-1 entry into quiescent primary lymphocytes: molecular analysis reveals a labile, latent viral structure. Cell. **61:** 213-222

99. **Zack JA, Haislip AM, Krogstad P, Chen IS.** 1992. Incompletely reverse-transcribed human immunodeficiency virus type 1 genomes in quiescent cells can function as intermediates in the retroviral life cycle. J Virol. **66:** 1717-1725.

100. **Zazzi M, Romano L, Catucci M, Venturi G, De Milito A, Almi P, Gonnelli A, Rubino M, Occhini U, Valensin PE.** 1997. Evaluation of the presence of 2-LTR HIV-1 unintegrated DNA as a simple molecular predictor of disease progression. J Med Virol. **52:** 20-25.

3. CHAPTER-1

Preferential Replication of FIV in Activated $CD4^+CD25^+$ T cells Independent of Cellular Proliferation

ABSTRACT

Studies attempting to identify reservoirs of HIV-1 latency have documented that the virus persists as both a latent and productive infection in subsets of $CD4^+$ cells. Reports regarding establishment of a stable HIV-1 infection in quiescent T cells in vitro, however, are controversial. In the present study we investigated the susceptibility of naïve and activated $CD4^+$ cell subsets (distinguished by differential expression of CD25) to feline immunodeficiency virus (FIV) infection, their ability to replicate the virus and potentially act as a reservoir for virus persistence in infected animals. While both $CD4^+CD25^+$ and $CD4^+CD25^-$ cells are susceptible to FIV infection in vitro and in vivo, only $CD4^+CD25^+$ cells produce infectious virions when cultured with interleukin-2 (IL-2). Latently infected $CD4^+CD25^-$ cells produce infectious virions following ConcanvalinA (ConA) stimulation, which correlates with up-regulated surface expression of CD25. In contrast to $CD4^+CD25^-$ cells, $CD4^+CD25^+$ cells remain unresponsive to mitogen stimulation, and are relatively resistant to apoptosis, whether or not infected with FIV. The ability of $CD4^+CD25^+$ cells to replicate FIV efficiently in the presence of IL-2 but remain anergic and unresponsive to apoptotic signaling suggests that these cells may provide a reservoir of productive FIV infection. On the contrary $CD4^+CD25^-$ cells seem to establish as latent viral reservoirs capable of being reactivated after stimulation.

Keywords: FIV, HIV, AIDS, latency, stimulation, anergy, apoptosis, reservoir

INTRODUCTION

Feline immunodeficiency virus (FIV) is a lentivirus of cats with a pathogenesis and disease pattern that parallels HIV-1 induced AIDS in humans (Bendenelli et al., 1995; English et al., 1994). Infected cats develop an acute infection syndrome, including low-grade fever and transient generalized lymphadenopathy, followed by a long asymptomatic period in which the CD4: CD8 ratio declines due to an early increase in $CD8^+$ and a progressive decline in $CD4^+$ cell numbers. This asymptomatic period is followed by the development of a variety of disorders, many of which mimic HIV-infection in humans (English et al., 1994).

HIV and FIV have been shown to infect a variety of cell types in their respective hosts including T cells, macrophages, and CNS glial cells (Bendinelli et al., 1995). While it is well established that HIV-1 displays a particular tropism for $CD4^+$ lymphocytes, which may lead to their gradual depletion, there are conflicting reports regarding HIV infection of resting T cells. While some studies have shown that HIV can readily enter quiescent T cells in culture and complete the infectious cycle with transcription of early mRNA (Stevenson et al., 1990), others have shown that naïve/resting T cells are resistant to de novo HIV infection (Chou et al., 1997). Whether resting T cells are infected de novo or are derived from activated infected cells, which subsequently revert to a resting phenotype remains to be elucidated. Although there is extensive documentation of FIV infecting $CD4^+$ cells in vivo and in vitro (Bendenelli et al., 1995; Dean et al., 1996; English et al., 1994), there are no studies addressing differential in vitro susceptibility of resting or activated $CD4^+$ cells.

With the advent of highly active antiretroviral therapy (HAART), additional information has been gained on HIV infection of $CD4^+$ subsets in vivo. Treatment of

asymptomatic HIV-1 infected individuals with HAART decreases the amount of virus in plasma to levels below the limit of detection by standard clinical assays (Perelson et al., 1996). However, a small but detectable reservoir of latently infected, resting $CD4^+$ cells harboring replication competent HIV-1 has been shown to persist in essentially all patients receiving therapy (Dornadula et al., 1999; Finzi et al., 1997; Wong et al., 1997). Recently Chun et al. (2003) reported that resting $CD4^+$ cells from the majority of viremic HIV-1 positive patients are capable of producing cell-free HIV-1 spontaneously ex vivo. No extracellular virus was produced by resting $CD4^+$ cells from aviremic patients despite the presence of HIV-1 mRNA. Interestingly, DNA microarray analysis revealed that a number of genes involved in mRNA and protein synthesis and processing were significantly upregulated in resting $CD4^+$ cells from HIV viremic patients compared to aviremic patients, suggesting that virus replication impacted the physiological state of resting $CD4^+$ cells or alternatively, that partial activation of $CD4^+$ cells is a prerequisite for virus replication. This latter speculation is of interest in that earlier studies have demonstrated that productive infection with HIV-1 in vitro was associated with a $CD4^+$ cell subset characterized by a partial activation phenotype as indicated by cell surface expression of the CD25 receptor (IL-2 receptor α chain).

Borvak et al (1995) and Ramilo et al (1993) used immunotoxin-labeled anti-CD25 to show that PBMC depleted of $CD25^+$ cells were markedly diminished in their ability to replicate HIV when infected in vitro. Further, they also showed that the $CD4^+CD25^+$ cells could be productively infected with HIV in vitro, whereas highly purified resting $CD4^+CD25^-$ cells were resistant to HIV infection (Chou et al., 1997). These observations are of interest, as the $CD4^+CD25^+$ cell phenotype possesses unique immunological

characteristics that could make a particularly favorable target for sustained HIV infection. Studies in a number of experimental models have firmly established the existence of a naturally occurring $CD4^+CD25^+$ T regulatory (Treg) cell population that is importanat in maintaining peripheral self-tolerance (Maloy and Powrie, 2001). These $CD4^+CD25^+$ cells also play a major role in regulating immune response to microbial infections (Sakaguchi, 2003). The most useful marker to date for identification of Treg cells is the CD25 antigen that has been demonstrated on 5-10% of $CD4^+$ cells in the circulation of normal rodents and humans (Jonuleit et al., 2001). While Treg cells have a partial activation phenotype, they are anergic in that they proliferate poorly upon TCR stimulation in vitro and their growth is dependent on exogenous IL-2 (Thornton and Shevach, 1998). Importantly, $CD4^+CD25^+$ cells appear to be resistant to clonal deletion and apoptosis when stimulated with anti-CD3 (Taams et al., 2001) or super antigen (Banz et al., 2002), suggesting they could be long-lived in lymphoid tissues. While the $CD4^+CD25^+$ cells reported to support HIV replication were not identified as Treg cells, it will be important to define some of the functional characteristics of these virus-infected cells to help ascertain their potential importance as a reservoir of productive infection.

In this study we have examined FIV entry and replication in activated and resting $CD4^+$ cell subsets distinguished by differential expression of CD25. Our studies demonstrate that although both $CD4^+CD25^+$ and $CD4^+CD25^-$ cells are susceptible to FIV infection in vitro and in vivo, only $CD4^+CD25^+$ cells replicate the virus in the absence of mitogenic stimulation. In contrast to $CD4^+CD25^-$ cells, $CD4^+CD25^+$ cells, whether or not infected with FIV, do not proliferate in response to ConcanvalinA (ConA) stimulation and are relatively resistant to activation-induced programmed cell death, suggesting that they could represent a

long-lived reservoir of productive FIV infection.

MATERIALS AND METHODS

Cats. Specific pathogen free (SPF) cats were obtained from Liberty Labs (Liberty Corners, NJ, USA) or Cedar River Laboratory (IA) and housed at the Laboratory Animal Resource Facility at the College of veterinary medicine, North Carolina State University. FIV-infected cats (32 in number) were inoculated with the $NCSU_1$ isolate of FIV as described previously (Tompkins et al., 2002). All infected cats were positive for FIV infection as confirmed by immunoblot analysis using antibody to FIV antigens and provirus positive as detected by polymerase chain reaction (PCR) using primers for the *gag* gene sequence. At the time samples were taken, cats had been infected with FIV for over five years, were asymptomatic, and had inverted $CD4^+$: $CD8^+$ ratios in the blood. Uninfected age matched control cats (16 in number) ranged in age between five to six years and were housed separately from FIV infected cats.

Blood and lymph node cell collection. Lymphocytes were obtained from either whole blood or lymph nodes. Due to a limited number of available lymph nodes for biopsy, PBMC were used whenever possible. However, if an experiment required a large number of cells, lymph node cells were collected. Preliminary studies using cells from both sources gave equivalent results. Whole blood was collected by jugular venipuncture into EDTA vacutainer tubes. PBMC were isolated by Percoll (Sigma, St. Louis, MO) density gradient centrifugation as described by Tompkins et al (1987). LN cells were obtained by peripheral LN biopsies as

described previously (Tompkins et al., 2002). Briefly, cats were anesthetized with intravenous ketamine and diazepam and maintained with inhalant isoflurane. One popliteal LN was excised and butorphanol tartrate administered to control post-operative discomfort. Single cell suspensions of LN cells were prepared by gently passing the tissue through a steel mesh screen.

Purification of T cells. To investigate single lymphocyte subsets, $CD4^+CD25^+$ and $CD4^+CD25^-$ cells were sorted with a Cytomation MoFlo fluorescence activated cell sorter (FACS) in the flow cytometry facility at University of North Carolina-Chapel Hill. The purity of FACS sorted cell populations was >99.5%. For some experiments, $CD4^+$ subsets were enriched by negative selection using goat anti-mouse IgG coated magnetic beads (Dynabeads® M-450, Dynal, Oslo, Norway). PBMC were depleted of B cells with anti-CD21 monoclonal antibody (mAb), monocytes and macrophages with anti-CD14 mAb (Dako, Carpentria, CA) followed by plastic adherence, and $CD8^+$ cells with mAb 3.357 (Tompkins et al., 1990). The CD25 expressing $CD4^+$ cells were then enriched by positive selection using anti-CD25 mAb (Ohno et al., 1992) coated beads. The purity of enriched $CD4^+CD25^+$ or $CD4^+CD25^-$ cell population was verified by flow cytometric analysis and determined to be greater than 98% for the $CD4^+$ cell population and greater than 99.5% for $CD25^+$ and $CD25^-$ cells.

Cell culture and stimulation. PBMC, LN cells or purified T cell subsets were cultured at 10^6 cells/ml in growth medium (RPMI-1640 containing 10% heat inactivated fetal bovine serum, 1% penicillin-streptomycin, 1% sodium bicarbonate, 1% sodium pyruvate, 1% L-

glutamine, and 1 mM HEPES buffer) in the presence or absence of 2 μg/ml ConA or 100U/ml recombinant human IL-2 (IL-2) kindly provided by the NIH AIDS Research and Reagent Program. FCD4E cells were established through long-term culture of PBMC from a SPF cat in the presence of IL-2 and cultured in RPMI-1640 medium (English et al., 1993). These cells are 100% positive for the feline pan T cell marker 1.572 and 60 to 65% positive for the feline CD4 homolog (English et al., 1993).

FIV-NCSU$_1$ virus stock generation and in vitro infection. PBMC were isolated from FIV-NCSU$_1$ positive cats and stimulated with ConA (2μg/ml) for 24h. Cells were washed twice with culture medium and co-cultured with an equal number of FCD4E cells in the presence of 200U/ml IL-2. Culture supernatants were harvested when the cells formed large syncytia and Gag-p24 antigen production was strongly positive in an ELISA. The virus stock was titrated in FCD4E cells and had a $TCID_{50}$ of $10^{6.5}$. In all the infection assays, total PBMC, $CD4^+CD25^+$, or $CD4^+CD25^-$ cells were infected with FIV-NCSU$_1$ using a multiplicity of infection (m.o.i.) of 0.1. No viral associated DNA was present in the viral stock as analyzed by PCR. Before the infections the virus stock was treated with DNAseI (300U/ml) for 30min at room temperature. Cells were exposed to the virus for 2h at 37^{o}C. Following virus adsorption, the cells were washed three times with culture medium and plated.

FIV p24 antigen capture ELISA. Detection of FIV Gag-p24 antigen in culture supernatants was done using an antigen capture ELISA. Immunolon 2HB plates (Dynex) were coated overnight with mAb p24Cr1 (Custom Monoclonals, Sacramento, CA) at 4^{o}C and blocked. Antigen was prepared by treating culture supernatans with 1% Triton-X-100. Samples were added to the plate and incubated for 1.5h at 37^{o}C. 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 1h at 37^{o}C. The plate was developed using TMB peroxidase substrate (KPL Labs, Maryland). Color reaction was stopped using 100µl 2M H2SO4 and the optical density (O.D.) measured at 450nm (reference filter 405nm).

Flow cytometry analysis. PBMC or purified cell populations were stained for surface expression of various markers using fluorescein isothiocyanate (FITC)-conjugated anti-CD4 (Tompkins et al., 1990), phycoerythrin (PE)-conjugated anti-CD8 (Tompkins et al., 1990) and allophycocyanin (APC)- conjugated anti-CD25 antibodies (Ohno et al., 1992). Samples were analyzed using a FACSCalibur Flow Cytometer (Becton Dickinson, Los Angeles, CA). At least 5×10^5 cells were used for staining and 15,000 cells acquired using Becton Dickinson Cell Quest software.

Polymerase chain reaction. PCR studies were conducted as described earlier (Frey et al., 2001) with minor modifications. All the primers used in the study were based on the FIV-$NCSU_1$ sequence. The episomal DNA intermediates, characteristics of cells productively infected with HIV (See Fig.1) were detected using env-sense (5'-GGC AAT GTG GCA TGT CTG AAA AAG AGG AGG AAT GAT- 3') and gag-antisense primers (5'-CGC CCC TGT CCA TTC CCC ATG TTG CTG TAG AAT CTC- 3'). In some cases, a nested PCR reaction was used for detection of circle junctions. The nested primers were env-sense-2 (5'-GAG GAG GAA TGA TGA AGT ATC TCA GAC-3') and gag-antisense-2 (5'-CCC ATG TTG CTG TAG AAT CTC TCC TAC-3'). The intermediate stages of reverse transcription were

detected either by LTR-sense (5'-GCG CTA GCA GCT GCC TAA CCG CAA AAC CAC-3') and gag-antisense primers or by FIV-gag specific nested primer pair. The early products of reverse transcription were detected using primers LTR-sense and LTR-antisense (5'- GTA TCT GTG GGA GCC TCA AGG GAG AAC TC- 3') (See Fig. 1). Sensitivity of the LTR and LTR-gag primer pairs was determined using serial 10-fold dilutions of a known copy number of the FIV-$NCSU_1$ plasmid in a PCR reaction with the primers. The resulting sensitivity was 10 copies for the LTR-gag and 100 copies for the LTR primer pairs. Due to a lack of a positive control for circle junctions, the sensitivity of the env-gag primer pair could not be determined. However, as sequences of this pair are the same as those described by Frey et al. (2001), we believe that the sensitivity should be similar and is 10 copies. DNA was isolated from equal numbers of infected cells using QIAamp® DNA Blood Mini Kit, Qiagen, Germany) and 200ng DNA was used as template. GAPDH sense (5'- CCT TCA TTG ACC TCA ACT ACA T - 3') and GAPDH antisense (5'- CCA AAG TTG TCA TGG ATG ACC - 3') primer pair was used as control to ensure the use of equal amounts of DNA template in identical experiments. PCR amplification was performed after denaturation for 3 min at 95°C followed by 40 cycles of 45 sec at 94°C, 45 sec at 55°C, 1 min at 72°C and a final incubation of 10 min at 72°C.

Cell proliferation assay. Cell proliferation was determined by measuring the uptake of ^{3}HTdR following mitogenic stimulation. Cells (10^5/well) were cultured in a round bottom 96-well plate in the presence or absence of 2 μg/ml ConA or 100U/ml IL-2. On day 6 cultures were pulsed with 1μCi of ^{3}HTdR for 18h. The cells were harvested using a Filtermake Harvester (Packard Bioscience) and ^{3}HTdR uptake determined using a Top Count NXT Microplate scintillation counter (Packard Bioscience). Lack of proliferation in $CD4^+CD25^+$

cells was also confirmed by CFSE labeling studies. Cells ($5X10^5$) were labeled with Vybrant^{TM} CFDA-SE cell tracer kit (Molecular Probes) immediately after isolation using a 1μM dye concentration following the manufacturer's protocol. Following labeling, the cells were cultured as described above. At day 6, labeled cells were analyzed using the FACSCalibur flow cytometer. Cell divisions were monitored by dilution of CFSE dye measured as a reduction in MFI in the FITC channel.

Measurement of apoptosis. Cells undergoing apoptosis were differentiated from non-apoptotic cells by staining for the presence of phosphatidyl-serine on their surface using the Annexin V Staining Kit (Roche Laboratories) following the manufacturer's instructions. Dead cells were differentiated from apoptotic cells by dual staining with PI and analyzed by two color-flow cytometric analysis using a FACSCalibur flow cytometer.

Statistical analysis. The student's t-test was used to compare differences in p24 antigen production between infected $CD4^+CD25^+$ and $CD4^+CD25^-$ cells. Differences in apoptosis, cellular proliferation and cell surface markers between $CD4^+CD25^+$ and $CD4^+CD25^-$ cells were determined using the Mann-Whitney's test (t-test-like for non-parametric data). Differences were considered significant at $p<0.05$.

RESULTS

Correlation between appearance of circle junctions and FIV p24 antigen production in acutely infected Feline CD4E (FCD4E) cells. Retroviral infection of T cells is

characterized be the formation of linear reverse transcribed double stranded DNA flanked by the 5' and 3' long terminal repeats (Fig. 1A) (Farnet and Haseltine, 1990; Tang et al., 1999). In addition to the linear products of reverse transcription, two circular forms of viral DNA have also been found in the nucleus of infected cells (Brown et al., 1987; Frey et al., 2001; Sharkey and Stevenson, 2001) and contain either two LTRs formed by circularization of linear viral DNA or one LTR formed by homologous recombination between the LTR's of linear DNA precursors (Fig. 1B) (Brown et al., 1987). These episomal DNA intermediates, also referred to as circle junctions, are characteristics of cells productively infected with HIV and are generally not present in latently infected cells (Teo et al., 1997). By selecting certain primer pairs, we detected by PCR early stages of reverse transcription (LTR-sense and LTR-antisense primers; Fig. 1C), intermediate stages (LTR-sense and Gag-antisense primers; Fig. 1C), and circle junction products (Env-sense and Gag-antisense primers; Fig 1B) of virus replication. Consistent with other studies in HIV and FIV, we were unable to detect 2-LTR circle junction products (Farnet and Haseltine, 1991; Frey et al., 2001). This is likely due to the many fold higher abundance of 1-LTR circles in the nucleus of infected cells compared to 2-LTR circles, resulting in a preferential amplification of 1-LTR circles (Farnet and Haseltine, 1991).

To validate the FIV primers and determine the time points at which different forms of reverse transcription appeared in infected cells, FCD4E cells were infected in vitro with the FIV-NCSU$_1$ isolate and analyzed for reverse transcription products using primer pairs as described in Figure 1. No amplification product was detected with any primer pair when the cells were harvested immediately after infection (Fig. 2A). The LTR band (early product) was present by 7h post infection (pi) and all time points assayed thereafter. The band

amplified with LTR-Gag primers (intermediate product) was not detected until 24h pi and persisted thereafter (Fig. 2A). The Env-Gag primer pair product (circle junctions) was detected only after 6 days pi (Fig. 2A). This band was also present at 12 days pi (data not shown). At day 6 and 8, when cells were positive for circle junctions by PCR, p24 antigen was also detected in culture supernatants (Fig. 2B). Hence, the appearance of circle junction PCR products correlates with p24 antigen production in FIV-$NCSU_1$ infected T cells.

Differential expression of circle junctions in $CD4^+CD25^+$ and $CD4^+CD25^-$ cells. Previous studies suggest that $CD4^+CD25^+$ but not $CD4^+CD25^-$ cells can be infected with HIV in vitro (Chou et al., 1997; Gowda et al., 1989; Tang et al., 1995). As shown in Figure 3, a population of $CD4^+CD25^+$ cells is present in both feline lymph nodes and peripheral blood. To investigate the role of these T cell subsets in FIV infection, lymph node (LN) cells from an FIV negative cat were sorted by flow cytometry into $CD4^+CD25^+$ and $CD4^+CD25^-$ populations, infected with FIV, and analyzed for the presence of replication DNA intermediates and p24 antigen production. FIV-infected $CD4^+CD25^-$ cells cultured in the presence of IL-2 were positive for early and intermediate DNA transcripts by 3 days pi, but showed no evidence of circle junctions (Fig. 4A) or p24 antigen in culture supernatants (Fig. 4B) at any time points pi. Absence of circular viral DNA forms in $CD4^+CD25^-$ cells was further confirmed using an Env-Gag nested primer pair to amplify the circle junction products, which also resulted in lack of any visible amplification products (data not shown). However, when stimulated with ConA immediately after infection, $CD4^+CD25^-$ cells produced circle junctions by 9 days pi (Fig. 4C), which correlated with soluble p24 antigen detection (Fig. 4D) in an ELISA. Although a low level of p24 antigen was detectable in the

supernatants of ConA treated $CD4^+CD25^-$ cells at day 6, a visible Env-Gag product was not detectable by standard PCR. However, a faint band was detectable at day 6 when the Env-Gag nested primer pairs were used (data not shown). As with the $CD4^+CD25^-$ cells, infected $CD4^+CD25^+$ cells cultured in IL-2 were positive for early and intermediate transcripts by 3 days pi (Fig. 4A). However, in contrast to the $CD4^+CD25^-$ cells, circle junctions were detected at 6 and 9 days pi (Fig. 4A), which correlated with the presence of p24 antigen in the culture supernatant (Fig. 4B). When stimulated with ConA, $CD4^+CD25^+$ cells produced all three transcripts (early, intermediate and circle junctions) (Fig. 4C) and soluble p24 (Fig. 4D) as early as 3 days pi. In the absence of IL-2 or ConA, both cell populations produced the early and intermediate transcripts, but not circle junctions or p24 (data not shown). Thus, while $CD4^+CD25^+$ cells were productively infected with FIV, activation by ConA is required for the $CD4^+CD25^-$ cells to produce virus.

$CD4^+CD25^-$ cells harbor a latent FIV infection that can be activated by ConA. Infected $CD4^+CD25^-$ cells contain specific proviral DNA without detectable p24 antigen production in the absence of cellular activation. Hence we asked if this proviral DNA form was biologically stable and whether ConA stimulation several days after infection would lead to reactivation of virus from these cells. PBMC derived $CD4^+CD25^-$ cells from an FIV negative cat were infected in vitro with FIV and cultured in the presence of IL-2 for 6 days prior to stimulation with ConA. PCR of infected cells just before addition of ConA revealed the presence of early (Fig. 5A, lane 1) and intermediate (Fig. 5A, lane 2) stages of proviral reverse transcription, but no detectable circle junctions (Fig. 5A, lane 3), or p24 antigen in culture supernatant (Fig. 5B). Following addition of ConA, p24 antigen was detectable at 12

days post stimulation and reached maximum levels by 15 days (Fig. 5B). The kinetics of virus production in cells stimulated immediately after infection was however more rapid compared to cells stimulated after 6 days of infection (Fig. 5B). These data suggest that $CD4^+CD25^-$ cells develop a latent infection, which can be reactivated upon mitogenic stimulation.

Productive infection of $CD4^+CD25^-$ cells on ConA stimulation correlates with surface upregulation of CD25. As $CD4^+CD25^-$ cells become productively infected when stimulated with ConA but not IL-2, we asked whether there is a differential activation response of $CD4^+CD25^-$ cells to these two stimuli. PBMC were depleted of $CD25^+$ cells by Ab-coated magnetic beads and shown by flow cytometry to contain less than 1% $CD4^+CD25^+$ cells (Fig. 6A). The resulting $CD25^-$ cells were cultured in the presence or absence of either ConA or IL-2 and analyzed for surface expression of CD25 by flow cytometry. By 3 days of culture, ConA stimulated $CD25^-$ cells upregulated CD25 on their surface such more than 80% of the $CD4^+$ cells expressed surface associated CD25 (Fig. 6B and 6C). In contrast, in the presence of IL-2 or culture medium alone $CD4^+CD25^-$ cells did not show an upregulation of CD25 on their surface (Fig. 6C). These data, in conjunction with the previous experiment demonstrate that ConA, but not IL-2, phenotypically activates $CD4^+CD25^-$ cells, and activated $CD4^+CD25^-$ cells are capable of supporting a productive FIV infection.

Proliferation of $CD4^+CD25^+$ cells inversely correlates with virus production. Recent literature emphasizes that naturally occurring cells bearing the $CD4^+CD25^+$ phenotype are a thymus-derived lineage distinct from $CD4^+CD25^-$ T helper cells (Maloy and Powrie, 2001).

These cells are unique in that they are anergic and relatively resistant to apoptosis (Banz et al., 2002; Taams et al ., 2001). As $CD4^+CD25^+$ cells replicated FIV in vitro, we assessed these functional characteristics of the feline $CD4^+CD25^+$ cell subset and the effect FIV infection may have on their function. To address this question, LN derived uninfected or in vitro FIV-infected $CD4^+CD25^+$ and $CD4^+CD25^-$ cells were stimulated with IL-2 or ConA and assayed for proliferative responses (tritiated thymidine [^{3}HTdR uptake]) and virus production (p24 antigen capture ELISA). In the absence of exogenous IL-2 or ConA, neither $CD4^+CD25^+$ nor $CD4^+CD25^-$ cells showed any significant proliferative response or p24 production (Fig. 7A and 7B). Stimulation of $CD4^+CD25^-$ cells with either IL-2 or ConA resulted in significant ($p<0.01$) levels of cell proliferation (Fig. 7B) compared to $CD4^+CD25^+$ cells. However, virus production by $CD4^+CD25^-$ cells was detected only in the presence of ConA (Fig. 7A). In contrast, $CD4^+CD25^+$ cells produced virus when stimulated with either ConA or IL-2 (Fig. 7A), yet did not proliferate (Fig. 7B). Thus, while FIV replicated efficiently in $CD4^+CD25^+$ cells in the presence of IL-2 and ConA, these cells themselves remained unresponsive to these mitogenic stimuli. Experiments with PBMC derived $CD4^+CD25^+$ and $CD4^+CD25^-$ cells gave similar results.

To obviate the possibility that lack of detection of proliferation in ^{3}HTdR uptake assay was because early or late proliferative events were missed in our analysis, total number of cell divisions in $CD4^+CD25^+$ and $CD4^+CD25^-$ cells were also determined by CFSE labeling. CFSE is transferred equally to daughter cells resulting in a reduction of Mean Fluorescence Intensity (MFI), an indicator of cell division. FIV-infected $CD4^+CD25^+$ and $CD4^+CD25^-$ cells were labeled with CFSE and cultured with IL-2, ConA or medium alone as in Fig. 7B. When stimulated with ConA for 6 days, $CD4^+CD25^-$ cells showed a marked

dilution in the CFSE dye content compared to $CD4^+CD25^-$ cells that were analyzed immediately after labeling (Fig. 7C). $CD4^+CD25^-$ cells in the presence of IL-2 also showed a dilution in CFSE content although not as marked as in the presence of ConA. In contrast, $CD4^+CD25^+$ cells when cultured with any of the above treatments did not show a significant shift in the mean fluorescence intensities compared to 0 day controls indicating that they did not divide (Fig. 7C). This data further confirms our results from ^{3}HTdR uptake assay suggesting that while $CD4^+CD25^-$ cells underwent cell division both in the presence of IL-2 and ConA, $CD4^+CD25^+$ cells did not proliferate under similar conditions.

$CD4^+CD25^+$ cells show a relative resistance to apoptosis. Failure of the $CD4^+CD25^+$ cells to proliferate in response to mitogenic stimulation could possibly be due to their activated phenotype, which on further stimulation might result in apoptosis in a large number of these cells. To address this possibility, uninfected or in vitro infected LN derived $CD4^+CD25^+$ and $CD4^+CD25^-$ cells were stained for surface expression of apoptotic markers under different culture conditions. When cultured in the presence or absence of IL-2, $CD4^+CD25^-$ cells showed significantly ($p<0.01$, $p<0.05$ respectively) higher percent of apoptotic cells than $CD4^+CD25^+$ cells (Fig. 8). Both infected and uninfected $CD4^+CD25^+$ and $CD4^+CD25^-$ cells showed a high percentage (approximately 40%) of apoptotic cells when stimulated with ConA. Interestingly, there was no significant difference in the percentage of apoptotic cells between the infected and uninfected groups (both $CD4^+CD25^+$ and $CD4^+CD25^-$). Thus it can be concluded that in both uninfected and infected cell populations, the $CD4^+CD25^+$ cells show similar (in the case of ConA stimulation) or less apoptosis (in the case of IL2 or no stimulation) when compared to the $CD4^+CD25^-$ cells. Thus, the lack of proliferation seen in

$CD4^+CD25^+$ cells cannot be attributed to higher percentage of apoptosis prone cells in this population. Experiments with PBMC derived $CD4^+CD25^+$ and $CD4^+CD25^-$ cells gave similar results.

Detection of circle junction products and rescue of replication competent virus from $CD4^+CD25^+$ cells from FIV-infected cats. The in vitro studies conducted thus far indicate that in vitro infected $CD4^+CD25^+$ cells efficiently replicate FIV, are unresponsive to exogenous mitogenic stimuli, and do not exhibit higher levels of apoptosis when compared to their $CD4^+CD25^-$ counterparts. To corroborate these findings in vivo, we studied $CD4^+CD25^+$ and $CD4^+CD25^-$ cells purified by flow cytometric sorting ($\geq$99.5% purity) from FIV-$NCSU_1$-infected cats. Figure 9A illustrates data from a representative asymptomatic FIV^+ cat demonstrating the presence of LTR and LTR-Gag products, but no detectable Env-gag products, in $CD4^+CD25^-$ cells indicating the absence of circle junction forms and hence a non-productive latent infection in these cells (Fig. 9A). Absence of Env-Gag products was confirmed using nested primer pairs (data not shown). In contrast, analysis of $CD4^+CD25^+$ cells from the same cat revealed circle junction products indicative of a productive infection. The circle junction products were detected in $CD4^+CD25^+$ cells but not $CD4^+CD25^-$ cells in 4 of 8 FIV-infected cats analyzed (data not shown). All the infected cats were positive when DNA from PBMC was screened using a nested primer pair for the *gag* gene (data not shown). The above results suggest that while the LTR products were always detected and LTR-Gag products were mostly detected in $CD4^+CD25^+$ cells, as well as $CD4^+CD25^-$ cells from FIV positive cats, detection of circle junction products was variable and restricted to $CD4^+CD25^+$ cells. In an attempt to rescue replication competent virus, $CD4^+CD25^+$ and

$CD4^+CD25^-$ cells from FIV positive cats were co-cultured with uninfected FCD4E cells. In the presence of IL-2, $CD4^+CD25^+$ - FCD4E co-cultures yielded a significantly strong ($p<0.01$) p24 signal, whereas $CD4^+CD25^-$ cells only gave a low p24 reading when co-cultured with FCD4E cells (Fig. 9B). However, treatment of $CD4^+CD25^-$ cells with ConA for 48h and subsequent co-culture with uninfected FCD4E cells yielded significantly higher ($p<0.01$) p24 readings than when the cells were stimulated with IL-2 alone (data not shown). Unsorted PBMC when co-cultured with FCD4E cells gave p24 readings slightly higher than $CD4^+CD25^-$ cells but significantly lower ($p<0.05$) than co-cultured $CD4^+CD25^+$ cells (Fig. 9B). Thus, $CD4^+CD25^+$ cells from FIV positive cats showed IL-2 dependant amplification of circle junctions and production of p24 in culture supernatants, which was not seen in $CD4^+CD25^-$ cells.

DISCUSSION

While it is well established that $CD4^+$ cells are prime targets for HIV infection in vivo, reports of HIV infecting naïve $CD4^+$ cells in vitro have been conflicting. Gowda et al (1989) were unable to detect the presence of HIV DNA in fresh unstimulated $CD4^+$ cells that had been exposed to the virus for prolonged periods of time. Similarly, Chou et al. (1997) reported that naïve $CD4^+$ cells were refractory to HIV infection in vitro, as evidenced by failure to detect viral DNA by PCR. In contrast, Stevenson et al (1990) reported the presence of relatively stable full-length unintegrated DNA forms in latently infected cells. Binding of HIV to resting $CD4^+$ cells followed by synthesis of partially reverse transcribed proviral DNA intermediates has also been reported (Chou et al., 1997; Zack et al., 1992). These

conflicting reports on the ability of HIV to infect naïve $CD4^+$ cells may be attributed to the presence of $CD4^+$ subsets with different degrees of susceptibility to infection (Douek et al., 2002). In this regard, there have been a number of reports indicating that $CD4^+$ cells expressing CD25, the IL-2 receptor α chain, could be productively infected with HIV-1, whereas $CD4^+CD25^-$ support neither latent nor productive infections (Borvak et al., 1995; Ramilo et al., 1993; Chou et al., 1997). Under normal physiological conditions in the blood, majority of $CD4^+$ T cells are represented by a naïve $CD4^+CD25^-$ T helper phenotype (90-95%) and a smaller, activated $CD4^+CD25^+$ phenotype (5-10%) of distinct lineage (Jonuleit et al., 2001). In this study we addressed the question of FIV infection of resting and activated feline $CD4^+$ T cells in vitro and in vivo as a relevant animal model for HIV-AIDS in humans. We focused our research on $CD4^+$ cell subsets differing in expression of the IL-2 R α chain (CD25) as potential targets of latent and productive FIV infection.

Recently, it has been demonstrated that, in addition to linear products of HIV reverse transcription, circular DNA intermediates or circle junctions are formed in the nucleus. Detection of these circular DNA intermediates is a hallmark of productively infected cells, as they are generally not found in resting $CD4^+$ cells. Frey et al. (2001) showed that production of circle junctions in cells infected in vitro or in vivo with FIV correlated with the presence of p24 antigen, indicative of a productive infection. In this study, an Env-sense and Gag-antisense primer pair was utilized to detect LTR-circular DNA intermediates in feline $CD4^+CD25^+$ and $CD4^+CD25^-$ cell subsets infected in vitro and in vivo with FIV. Circular DNA intermediates were detected as 1-LTR circles in FIV-infected $CD4^+CD25^+$ cells but not $CD4^+CD25^-$ naïve cells whether infected in vitro or in vivo. The kinetics of detection of DNA circular intermediates in FIV-infected $CD4^+CD25^+$ cells correlated with detection of

p24 antigen in the culture supernatants, and both were dependent on the presence of IL-2 in culture medium. PCR analysis of $CD4^+CD25^-$ naïve T cells did reveal evidence of early and intermediate stages of FIV reverse transcription, and ConA stimulation resulted in formation of circular DNA intermediates and production of p24, indicating that the $CD4^+CD25^-$ cells harbored full length latent replication competent FIV genome. It is unlikely that the inability to productively infect $CD4^+CD25^-$ naïve T cells is due to low expression of the FIV co-receptor CXCR4, as, in contrast to human T cells, this molecule does not appear to be expressed on feline T cells (Willett et al., 2003).

Our findings of productive FIV infection of $CD4^+CD25^+$ cells in vitro are in agreement with Borvak et al (1995) and Ramilo et al (1993), who used anti-CD25-ricin A chain immunotoxin to demonstrate that elimination of activated $CD25^+$ cells from normal PBMC before in vitro HIV infection results in a marked reduction in p24 antigen production. However, in contrast to our observation, Chou et al. (1997) reported that purified $CD4^+CD25^-$ cells were not susceptible to HIV infection in vitro as indicated by failure to detect viral DNA by PCR. It is unlikely that FIV infection of the $CD4^+CD25^-$ enriched population observed in our studies could be attributed to contaminating $CD4^+CD25^+$ cells, as flow cytometric based sorting yielded a highly purified ($\geq$99.5%) $CD4^+CD25^-$ population. Others have reported that HIV is able to establish latent, replication competent infection in naïve $CD4^+$ T cells in vitro (Zack et al., 1990; Zack et al., 1992).

It is of interest that while IL-2 is necessary and sufficient to promote FIV replication in cultured $CD4^+CD25^+$ cells, a strong mitogenic stimulus (ConA) is required to rescue infectious virus from latently infected $CD4^+CD25^-$ cells. Active virus replication in ConA stimulated $CD4^+CD25^-$ cells is preceded by surface upregulation of the CD25 molecule on

this cell population, which does not occur when $CD4^+CD25^-$ cells are incubated with IL-2 alone. It is possible that inability of FIV to replicate in IL-2-stimulated $CD4^+CD25^-$ cells is due to the absence of the IL-2-R α chain in these cells and hence failure to form the high affinity IL-2 receptor. However, it has been reported that the IL-2 receptor beta and gamma chains are responsive to IL-2 signaling in the absence of the α chain (Ellery and Nicholls, 2002). It is equally possible that $CD4^+CD25^+$ cells, but not $CD4^+CD25^-$ cells, have in place an IL-2-responsive intracellular signaling pathway necessary for FIV replication. In this regard, Chun et al. (2003) recently reported that $CD4^+$ cells harboring productive HIV-1 infection upregulated a number of genes associated with mRNA and protein synthesis and processing, suggestive of a partial activation genotype. In agreement with our observations, these authors speculated that a certain level of metabolic activity or cellular activation (e.g. IL-2 stimulation) is necessary to support a productive infection in $CD4^+$ cells. In contrast to our studies, the productively HIV-infected $CD4^+$ cells described by Chun et al. (2003) showed no evidence of phenotypic activation and did not express the CD25 antigen on their surface. Whatever the mechanisms, our data suggest that the partially activated $CD4^+CD25^+$ cells but not the naïve $CD4^+CD25^-$ cells have in place the IL-2-responsive extracellular or intracellular signaling elements necessary for active FIV replication. It will be of much interest to further define these $CD4^+$ reservoirs of infection and determine if this partial activation phenotype is the cause or effect of productive FIV infection.

A naturally occurring $CD4^+CD25^+$ phenotype has been described in rodents and humans that differs from naïve $CD4^+CD25^-$ T helper cells in that it is partially activated, yet anergic, and does not proliferate in response to antigenic or mitogenic stimulation (Maloy and Powrie, 2001). Our evidence suggests that the $CD4^+CD25^+$ phenotype harboring a

productive FIV infection may also be functionally anergic. While $CD4^+CD25^+$ cells produced significant levels of p24 antigen in the presence of IL-2 or ConA, they were unresponsive to proliferation signals (ConA stimulation) when compared to their $CD4^+CD25^-$ counterparts. Lack of proliferation in $CD4^+CD25^+$ cells was not due to higher levels of cell death in the $CD4^+CD25^+$ population, as Annexin V staining demonstrated that whether infected or uninfected, the $CD4^+CD25^+$ cells always exhibited similar or less apoptosis than $CD4^+CD25^-$ cells.

The functional characteristics of the $CD4^+CD25^+$ cell populations harboring a productive FIV infection described herein are reminiscent of $CD4^+$ Treg cells. $CD4^+CD25^+$ Treg cells perform an important function of suppressing autoreactive T cells (Maloy and Powrie, 2001) and regulating immune responses to microbial pathogens (Sakaguchi, 2003). Similar to the $CD4^+CD25^+$ cells described herein, they are partially activated and responsive to IL-2, and yet are arrested in a G0/G1 anergic state and relatively resistant to apoptosis (Banz et al., 2002; Taams et al., 2001). Phenotypically, $CD4^+CD25^+$ Treg cells are $CD45RA^-$, $CD45RO^+$, and express the co-stimulatory molecules B7.1, B7.2, and CTLA4 (Jonuleit et al., 2001, Caramalho et al., 2003). The defining feature of $CD4^+CD25^+$ Treg cells is their ability to inhibit in a contact-dependent manner proliferation of other activated T cells in vitro (Jonuleit et al., 2001). Although reagents are not available to distinguish the different isoforms of feline CD45, we have recently documented the expression of the co-stimulatory molecules on feline $CD4^+CD25^+$ cells (Vahlenkamp et al., submitted). In addition, we have been able to demonstrate that $CD4^+CD25^+$ cells from FIV-infected cats were able to suppress the proliferative response of ConA-stimulated autologous $CD4^+CD25^-$ cells (Vahlenkamp et al. submitted). The fact that $CD4^+CD25^+$ cells appear to be anergic and relatively resistant to

clonal deletion could favor a stable, long-lived reservoir of FIV infection in lymphoid tissues. This possibility was not addressed in the present study as we have not determined the frequency of FIV-infected $CD4^+CD25^+$ cells in the blood and lymph node and whether $CD4^+CD25^+$ cells support a chronic or lytic FIV infection. Our PCR analysis suggests that a relatively small fraction of $CD4^+CD25^+$ cells are productively infected with FIV in vivo in asymptomatic infected cats. However, limiting-dilution co-culture revealed that >50% of $CD4^+CD25^+$ cells can be productively infected with FIV in vitro (Joshi, unpublished) suggesting that most, if not all freshly isolated $CD4^+CD25^+$ are susceptible to ex vivo infection. Moreover, these long-term cultures show no evidence of increased cell death despite high virus burden, suggesting that the cells may be supporting a non-lytic chronic infection (Joshi, unpublished).

Studies have shown that latent infection of resting $CD4^+$ cells provides a mechanism for lifelong persistence of HIV even in patients on effective HAART therapy. More recently, a reservoir of productive HIV-infection has been reported in essentially all HIV^+ patients on HAART (Finzi et al., 1997; Sharkey et al., 2000; Wong et al., 1997). Thus, by virtue of their unique anergic characteristic $CD4^+CD25^+$ cells may provide an important sanctuary for HIV survival even in the face of potent HAART. How $CD4^+CD25^+$ cells produce infectious virions yet remain in a partially activated, non-proliferating state resisting activation-induced cell death is an important question for future investigations.

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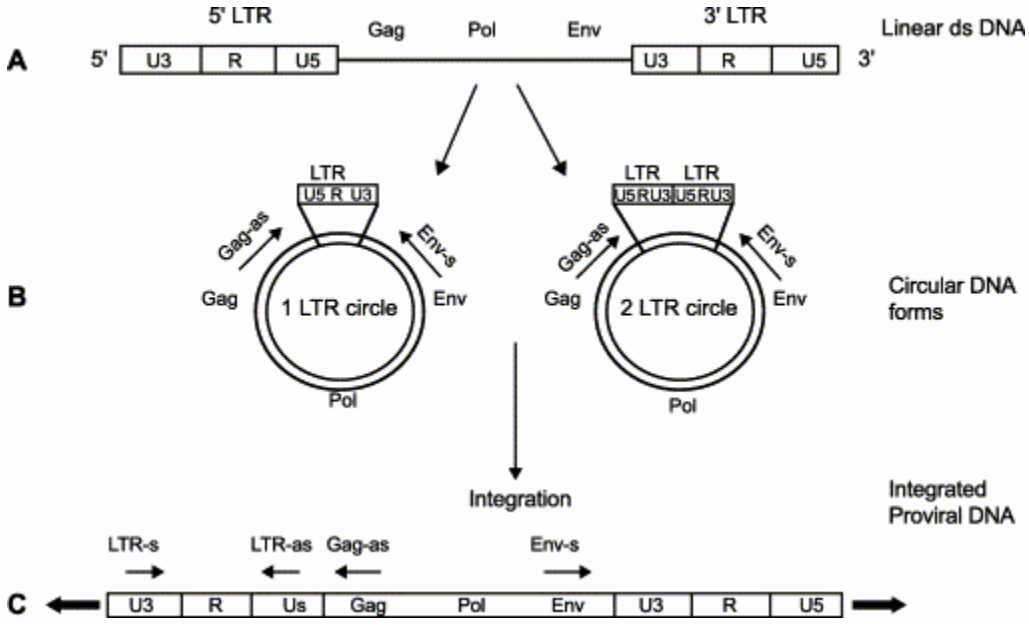


Fig. 1. Schematic representation of linear proviral DNA, 1 LTR circle junctions and 2 LTR circle junctions depicting position of primer pairs used in the study. **(A)** Double stranded linear reverse transcribed unintegrated proviral DNA showing 5' and 3' LTRs and major viral genes. **(B)** 1 LTR and 2 LTR circle junction products showing position of the En*v*-sense (Env-s) and Gag-antisense (Gag-as) primer pair used to amplify these circle junctions in productively infected cells. **(C)** Linear double stranded proviral DNA integrated into the host genome. The LTR-sense (LTR-s), LTR-antisense (LTR-as), and Gag-as primers depicted in the figure can be used to detect either linear unintegrated or linear integrated proviral DNA forms but not the circle junctions, which are specifically detected by Env-s and Gag-as primer pair.

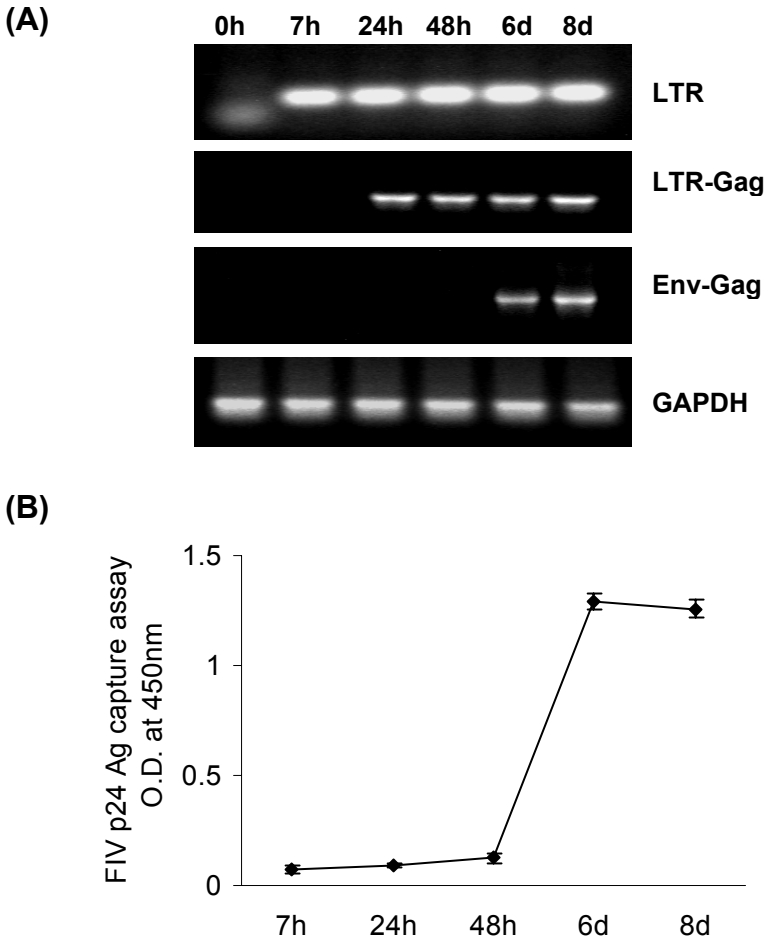


Fig. 2. Correlation between appearance of circle junctions by PCR and detectable Gag-p24 antigen in culture supernatants. FCD4E cells were infected in vitro with FIV- $NCSU_1$ at an m.o.i of 0.1 and plated in culture medium supplemented with 100U/ml IL-2. Cells and culture supernatant were harvested at 0h, 7h, 24h, 48h, 6 days and 8 days post infection for **(A)** DNA isolation and PCR using primer pairs described in Fig. 1 and **(B)** p24 antigen detection by antigen capture ELISA. The p24 values at each time point represent mean ± standard error of triplicates cultures. The experiment was repeated twice with similar results. Negative control for PCR comprised of water plus reagents and did not result in amplification of any bands (data not shown).

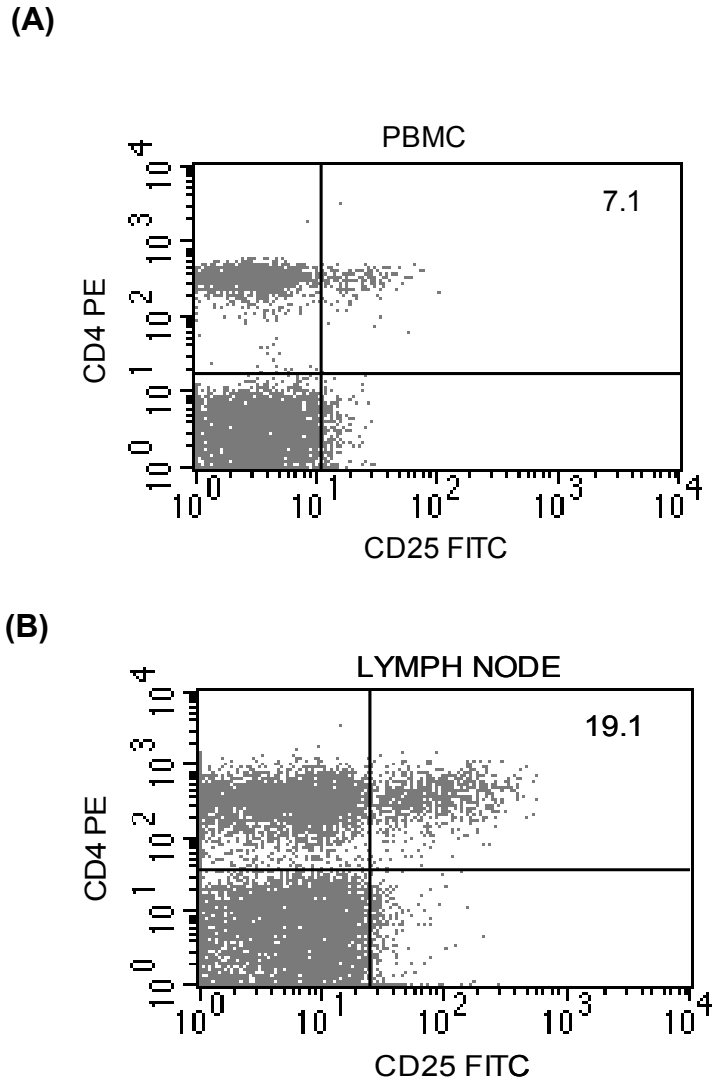
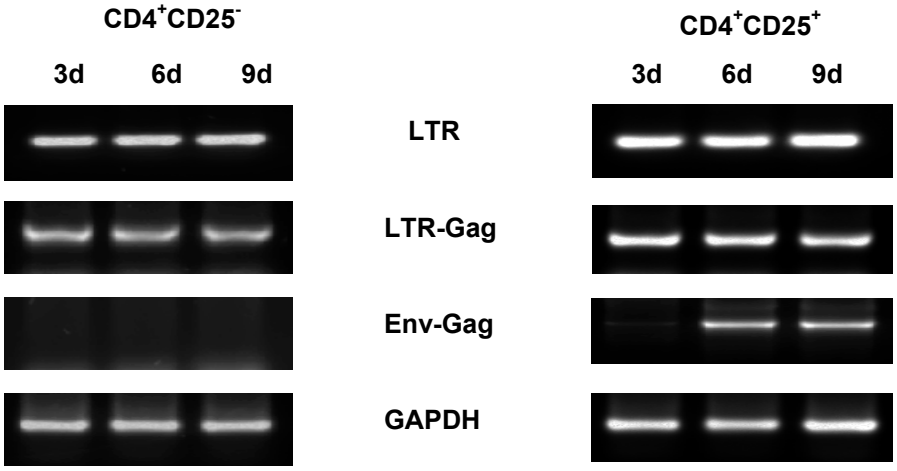
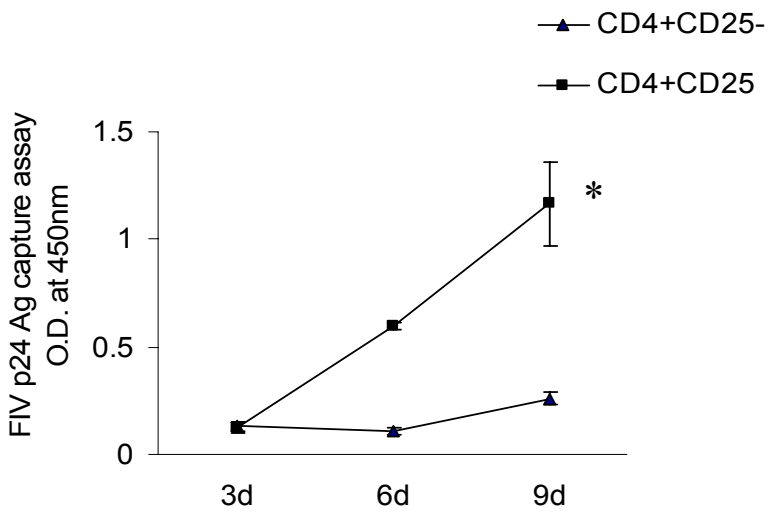


Fig. 3. Representative two-color flow cytometric dot-plot analysis of $CD4^+CD25^+$ cells in the PBMC **(A)** and LN **(B)** of normal cats. PBMC and LN cells were derived from SPF cats, stained with FITC-conjugated anti-CD25 and PE-conjugated anti-CD4 antibodies, and analyzed on a FACSCalibur flow cytometer. Numbers represent the percent $CD4^+CD25^+$ cells of the total $CD4^+$ population.

(A)


CD4+CD25-
3d
6d
9d
CD4+CD25+
3d
6d
9d
LTR
LTR-Gag
Env-Gag
GAPDH

(B)


CD4+CD25-
CD4+CD25
FIV p24 Ag capture assay
O.D. at 450nm
1.5
1
0.5
0
3d
6d
9d
*

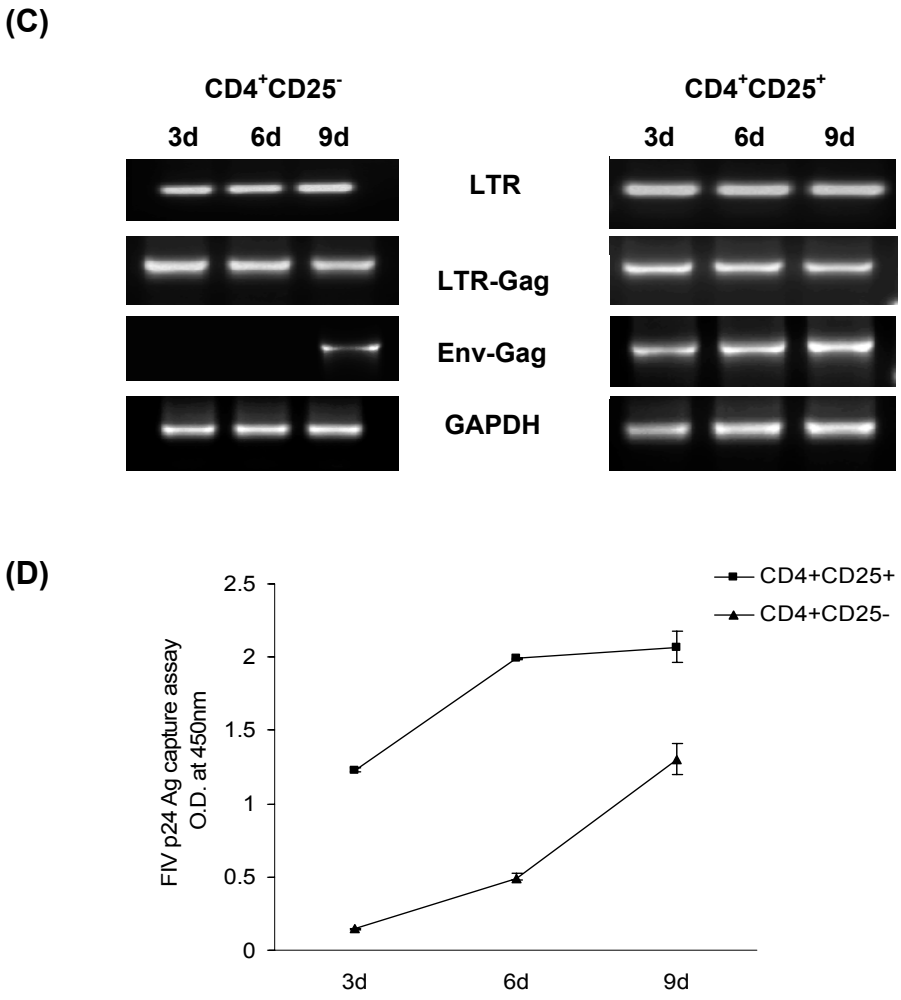


Fig. 4. PCR analysis for detection of circle junctions in $CD4^+CD25^+$ and $CD4^+CD25^-$ cells in the presence or absence of ConA stimulation. LN cells were isolated from FIV negative cats, sorted into in $CD4^+CD25^+$ or in $CD4^+CD25^-$ cell using FACS, and infected in vitro with FIV-$NCSU_1$ at an m.o.i. of 0.1. Cells (10^6/ml) were then either left unstimulated in culture medium supplemented with 100U/ml IL-2 **(A, B)** or stimulated with ConA at 2µg/ml **(C, D)**. Cells and culture supernatants were harvested at 3, 6 and 9 days post infection for PCR (A, C) and p24 antigen detection by antigen capture ELISA (B, D) respectively. The p24 values represent mean ± standard error of triplicate cultures. Negative control for PCR comprised of water plus reagents and did not result in amplification of any bands (data not shown). * significant difference ($p<0.05$) in p24 Ag production by $CD4^+CD25^+$ compared to $CD4^+CD25^-$ cells. The experiment was repeated four times. One of the representative data is shown.

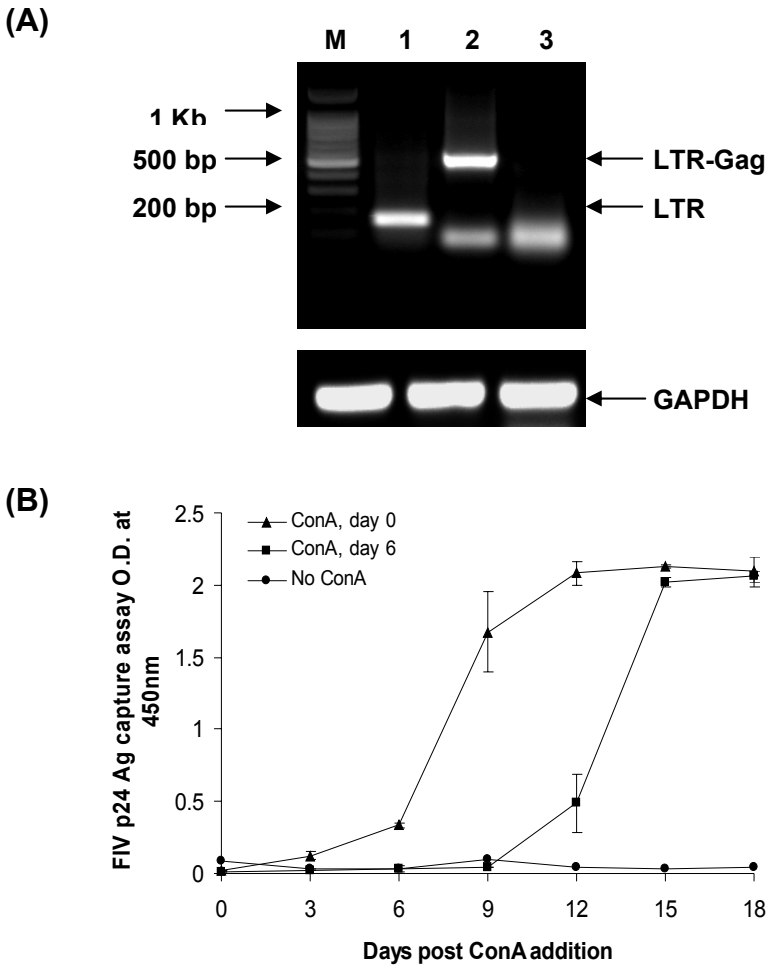


Fig. 5. Rescue of productive infection from $CD4^+CD25^-$ cells 6 days after infection. Magnetic bead purified $CD4^+CD25^-$ PBMC from a negative cat were infected in vitro with FIV (m.o.i of 0.1) and cultured in medium in the presence of 100 U/ml IL-2. After 6 days of culture, **(A)** a portion of the cells were harvested for PCR analysis using LTR-sense and LTR-antisense (LTR) primer pair (lane 1), LTR-sense and Gag-antisense (LTR-Gag) primers (lane 2), and Env-sense and Gag-antisense (Env-Gag) primers (lane 3). (M) is a 100bp molecular weight ladder. **(B)** Remaining cells were cultured in the presence of ConA (2 μg/ml) and supernatants harvested every three days for p24 antigen detection by antigen capture ELISA. Controls consisted of $CD4^+CD25^-$ cells cultured in the presence of ConA immediately after infection and in the presence of IL-2 only. The p24 values at each time point represent mean ± standard error of duplicate cultures. The experiment was repeated twice with similar results.

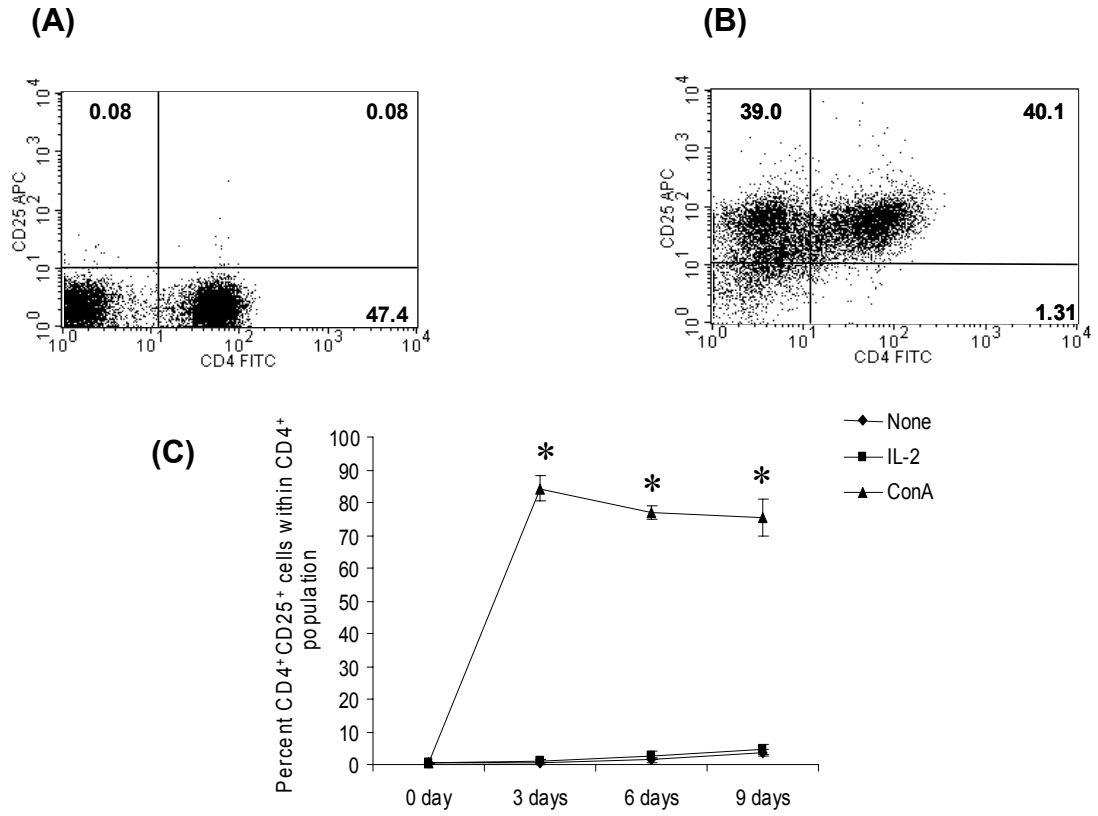


Fig. 6. Flow cytometric analysis of CD25 expression on cultured $CD4^+CD25^-$ cells in the presence or absence of stimulation. PBMC were isolated from FIV negative cats and $CD25^+$ cells depleted using anti-CD25 coated magnetic beads. The CD25 depleted cells were cultured either in 1) growth medium alone 2) growth medium supplemented with 100U/ml IL-2 or 3) growth medium containing 2µg/ml ConA. Cells were harvested, stained and analyzed by flow cytometry for the expression of CD4 and CD25 after 3, 6 and 9 days of different treatments. The dot plots demonstrate the percent of cells that **(A)** express CD25 and CD4 immediately after depletion or **(B)** after 9 days in culture with ConA. The numbers represent the percentage of cells within each quadrant. (C) The percentage of $CD25^+$ cells within the $CD4^+$ population ($CD4^+CD25^+$ cells / total $CD4^+$ cells x 100) after different treatments is plotted as a function of time. Data at each time point represent mean ± standard error of triplicate cultures. * significant increase ($p<0.01$) in CD25 expression in $CD4^+CD25^-$ cells in the presence of ConA compared to treatment with IL-2 or culture media alone. The experiment was repeated three independent times. One of the representative results is depicted.

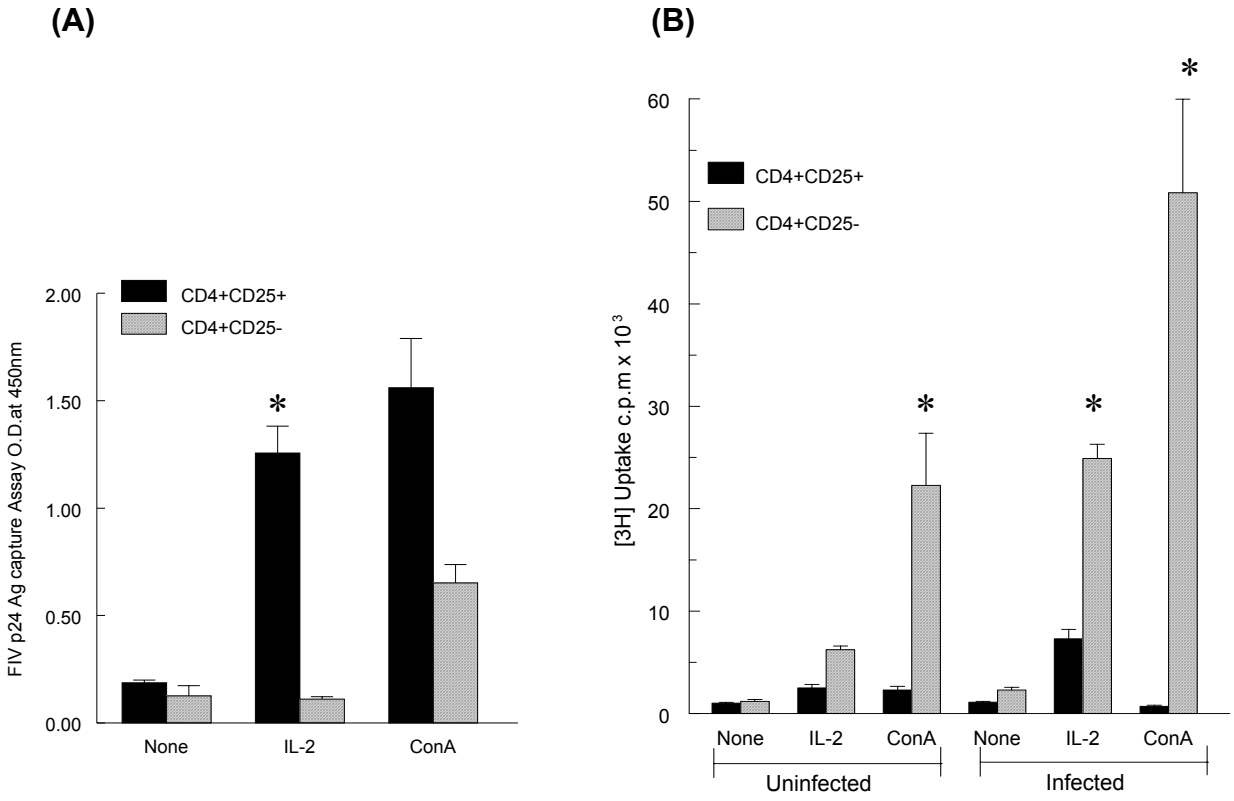


Fig. 7. FIV replication and cellular proliferation in $CD4^+CD25^+$ or in $CD4^+CD25^-$ cells under different culture conditions. Magnetic bead purified $CD4^+CD25^+$ and $CD4^+CD25^-$ cells from LN of FIV negative cats were either infected with FIV-$NCSU_1$ at an m.o.i. of 0.1 or left uninfected. Infected and uninfected $CD4^+CD25^+$ and $CD4^+CD25^-$ cells were cultured in the presence of either 100U/ml IL-2 or 2μg/ml ConA or left untreated for 6 days. **(A)** On day 6 culture, supernatants were harvested from infected cells and assayed for p24 antigen in an ELISA. * significant increase ($p<0.01$) in p24 Ag production by $CD4^+CD25^+$ cells compared to $CD4^+CD25^-$ cells cultured in the presence of IL-2. **(B)** On the same day all the cultures were pulsed with ^3H TdR at 1μCi/well at 37°C. After 18h cells were harvested and ^3H TdR uptake determined using a liquid scintillation beta counter. The p24 values and ^3H TdR readings for each treatment represent mean ± standard error of triplicates and five wells respectively. * significant increase ($p<0.01$) in uptake of 3H TdR by $CD4^+CD25^-$ cells in the presence of IL-2 or ConA compared to $CD4^+CD25^+$ cells. Results from one of the three representative experiments are depicted here.

(C)

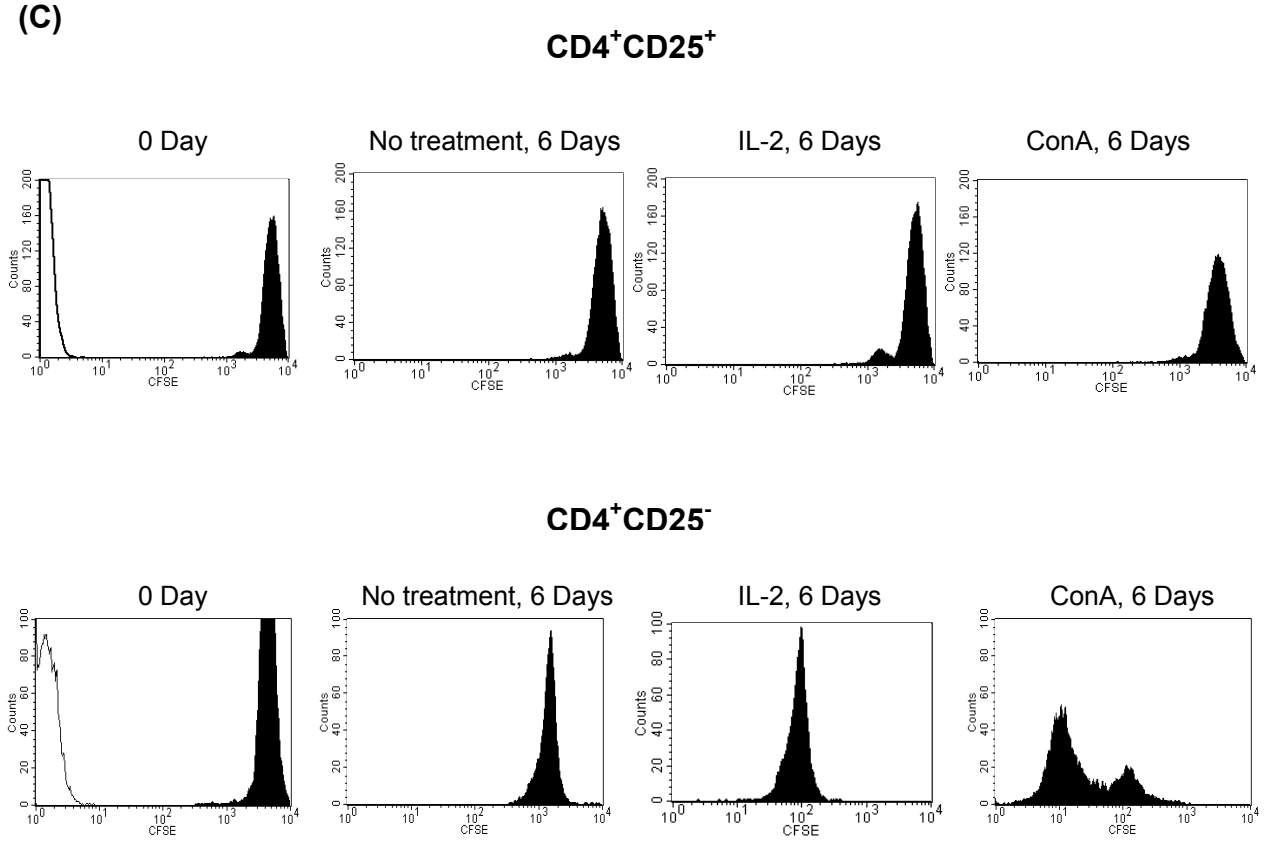


Fig. 7.C. CFSE labeling studies of infected $CD4^+CD25^+$ and $CD4^+CD25^-$ cells. Magnetic bead purified $CD4^+CD25^+$ and $CD4^+CD25^-$ cells from LN of FIV negative cats were infected with FIV-$NCSU_1$ as above and labeled with CFDA-SE. Cells were either analyzed immediately after labeling or cultured for 6 days in the presence of IL-2, ConA or medium alone. At day 6 culture supernatants were assayed as in (A) above (data not shown). Cells were also analyzed for dilution of CFDA-SE dye using flow cytometry. Analyses were performed in duplicates and the results were repeated 4 times. One of the representative data from infected cells is depicted here. Similar assays were performed with uninfected $CD4^+CD25^+$ and $CD4^+CD25^-$ cells and yielded comparable results (data not shown). 0-day data, open histograms represent unlabeled cells and filled histograms represent cells analyzed immediately after labeling.

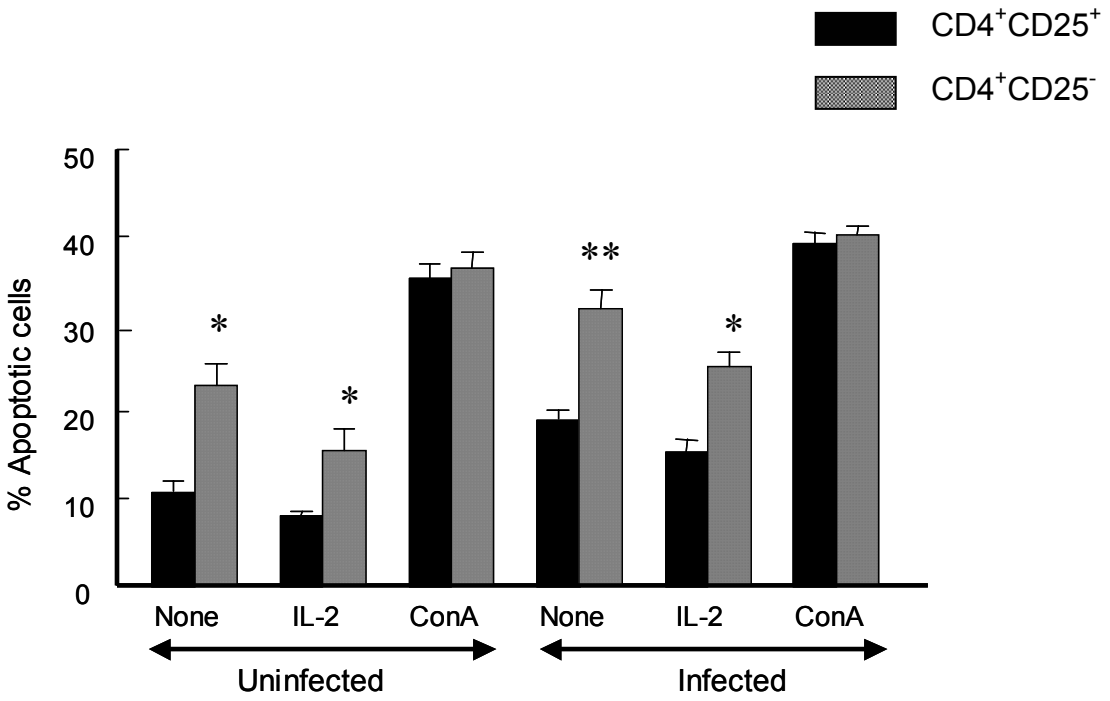
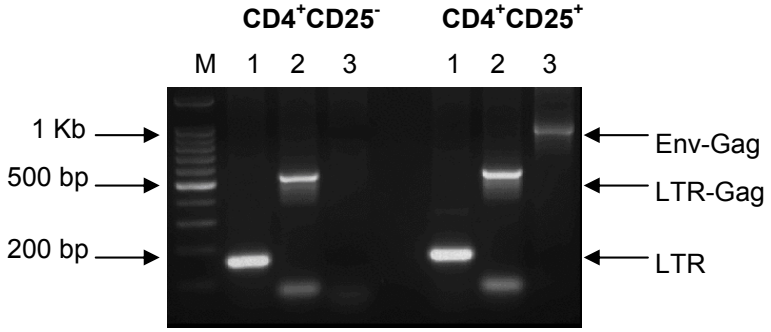


Fig. 8. Determination of percent apoptosis in infected or uninfected $CD4^+CD25^+$ and $CD4^+CD25^-$ cells. LN-derived cells were sorted into $CD4^+CD25^+$ and $CD4^+CD25^-$ populations by FACS. In vitro infected or uninfected $CD4^+CD25^+$ and $CD4^+CD25^-$ cells were either left untreated or treated with IL-2 or ConA for 6 days as in Fig. 6. On day 6 FIV p24 antigen was determined in supernatants of infected cells by ELISA. On day 7 infected, as well as uninfected $CD4^+CD25^+$ and $CD4^+CD25^-$ cells were stained with AnnexinV and analyzed by flow cytometry. Dead cells were differentiated from apoptotic cells by dual staining with PI. Percent apoptosis in $CD4^+CD25^+$ and $CD4^+CD25^-$ cells is represented as mean ± standard error of triplicates. * significant increase ($p<0.01$) in percent apoptosis in $CD4^+CD25^-$ cells compared to $CD4^+CD25^+$ cells. ** significant increase ($p<0.05$) in percent apoptosis in infected $CD4^+CD25^-$ cells compared to infected $CD4^+CD25^+$ cells in the absence of any treatment. The experiment was repeated three times with similar results.

(A)


CD4+CD25-
CD4+CD25+
M 1 2 3 1 2 3
1 Kb
500 bp
200 bp
Env-Gag
LTR-Gag
LTR

(B)

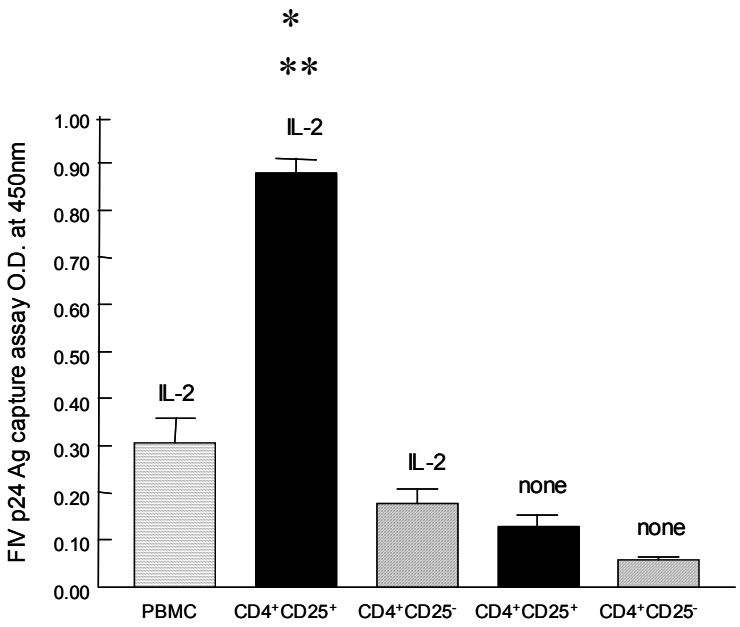

*
**
IL-2
FIV p24 Ag capture assay O.D. at 450nm
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
IL-2
IL-2
none
none
PBMC
CD4+CD25+
CD4+CD25-
CD4+CD25+
CD4+CD25-

Fig. 9. Analysis of virus replication in $CD4^+CD25^+$ and $CD4^+CD25^-$ cells from cats infected with the $NCSU_1$ isolate of FIV. **(A)** Detection of circle junction forms in $CD4^+CD25^+$ cells from a FIV positive cat. DNA was isolated from FACS purified $CD4^+CD25^+$ and $CD4^+CD25^-$ PBMC from FIV infected cats PCR reactions were set up using LTR-sense and LTR-antisense (LTR) primer pair (lane 1), LTR-sense and Gag-antisense (LTR-Gag) primers (lane 2), and Env-sense and Gag-antisense (Env-Gag) primers (lane 3). For detection of circle junctions from FIV positive cats the Env-Gag PCR product was subjected to a nested reaction. (M) 100bp molecular weight ladder. Equal amount of DNA was used for each PCR reaction as determined by intensity of GAPDH bands (data not shown). Negative control comprised of water plus reagents and did not result in amplification of any bands (data not shown). One of the PCR gels out of the 8 cats analyzed is shown. **(B)** Rescue of replication competent virus from FIV positive cats. FACS purified $CD4^+CD25^+$ and $CD4^+CD25^-$ cells from LN of FIV positive cats were either cultured alone or co-cultured with an equal number of uninfected FCD4E cells in the presence of IL-2 at 100U/ml. Culture supernatants were harvested after 9 days of co-culture and p24 antigen determined using an antigen capture ELISA. The p24 values for each cell type represent mean ± standard error triplicates. $*$ significant increase in p24 Ag production by $CD4^+CD25^+$ cells in the presence of IL-2 compared to $CD4^+CD25^+$ cells in the absence of IL-2 or to $CD4^+CD25^-$ cells in the presence or absence of IL-2. $**$ significant increase ($p<0.05$) in p24 production by $CD4^+CD25^+$ cells compared to total PBMC in the presence of IL-2. The experiment was repeated twice with similar results.

REFERENCES

1. **Banz, A., Pontoux, C., Papiernik, M.,** 2002. Modulation of Fas-dependent apoptosis: a dynamic process controlling both the persistence and death of CD4 regulatory T cells and effector T cells. J Immunol. **169,** 750-757.

2. **Bendinelli, M., Pistello, M., Lombardi, S., Poli, A., Garzelli, C., Matteucci, D., Ceccherini-Nelli, L., Malvaldi, G., Tozzini, F.,** 1995. Feline immunodeficiency virus: an interesting model for AIDS studies and an important cat pathogen. Clin Microbiol Rev. **8,** 87-112.

3. **Borvak, J., Chou, C. S., Bell, K., Van Dyke G., Zola, H., Ramilo, O., Vitetta E. S.,** 1995. Expression of CD25 defines peripheral blood mononuclear cells with productive versus latent HIV infection. J Immunol. **155,** 3196-3204.

4. **Brown, P. O., Bowerman, B., Varmus, H. E., Bishop, J. M.,** 1987. Correct integration of retroviral DNA in vitro. Cell. **49,** 347-356.

5. **Caramalho, I., Lopes-Carvalho, T., Ostler, D., Zelenay, S., Haury, M., and Demengeot, J.** (2003). Regulatory T cells selectively express toll-like receptors and are activated by lipopolysaccharide. J Exp Med **197,** 403-11.

6. **Chou, C. S., Ramilo, O., Vitetta, E. S.,** 1997. Highly purified CD25- resting T cells cannot be infected de novo with HIV-1. Proc Natl Acad Sci U S A**. 94,** 1361-1365.

7. **Chun, T. W., Justement, J. S., Lempicki, R. A., Yang, J., Dennis, G. Jr., Hallahan, C. W., Sanford, C., Pandya, P., Liu, S., McLaughlin, M., Ehler, L. A., Moir, S., Fauci, A. S.,** 2003. Gene expression and viral prodution in latently infected, resting CD4+ T cells in viremic versus aviremic HIV-infected individuals. Proc Natl Acad Sci U S A. **100,** 1908-1913.

8. **Dean, G. A.., Reubel, G. H., Moore, P. F., Pedersen, N. C.,** 1996. Proviral burden and infection kinetics of feline immunodeficiency virus in lymphocyte subsets of blood and lymph node. J Virol. **70,** 5165-5169.

9. **Dornadula, G., Zhang, H., VanUitert, B., Stern, J., Livornese, Jr L., Ingerman, M. J., Witek, J., Kedanis, R. J., Natkin, J., DeSimone, J., Pomerantz, R. J.,** 1999.

Residual HIV-1 RNA in blood plasma of patients taking suppressive highly active antiretroviral therapy. JAMA. **282,** 1627-1632.

10. **Douek, D. C., Brenchley, J. M., Betts, M. R., Ambrozak, D. R., Hill, B. J., Okamoto, Y., Casazza, J. P., Kuruppu, J., Kunstman, K., Wolinsky, S., Grossman, Z., Dybul, M., Oxenius, A., Price, D. A., Connors, M., Koup, R. A.,** 2002. HIV preferentially infects HIV-specific CD4+ T cells. Nature. **417,** 95-98

11. **Ellery, J. M., Nicholls, P. J.,** 2002. Alternate signaling pathways from the interleukin-2 receptor. Cytokine Growth Factor Rev. **13,** 27-40.

12. **English, R. V., Johnson, C. M., Gebhard, D. H., Tompkins, M. B.,** 1993. In vivo lymphocyte tropism of feline immunodeficiency virus. J Virol. **67,** 5175-5186.

13. **Farnet, C. M., Haseltine, W. A.,** 1990. Integration of human immunodeficiency virus type 1 DNA in vitro. Proc Natl Acad Sci U S A. **87,** 4164-8.

14. **Finzi, D., Hermankova, M., Pierson, T., Carruth, L. M., Buck, C., Chaisson, R. E., Quinn, T. C., Chadwick, K., Margolick, J., Brookmeyer, R., Gallant, J., Markowitz, M., Ho, D. D., Richman, D. D., Siliciano, R. F.,** 1997. Identification of a reservoir for HIV-1 in patients on highly active antiretroviral therapy. Science. **278,** 1295-300.

15. **Frey, S. C., Hoover, E. A., Mullins J. I.,** 2001. Feline immunodeficiency virus cell entry. J Virol. **75,** 5433-54340.

16. **Gowda, S. D., Stein, B. S., Mohagheghpour, N., Benike, C. J., Engleman, E. G.,** 1989. Evidence that T cell activation is required for HIV-1 entry in CD4+ lymphocytes. J Immunol. Feb **142,** 773-80.

17. **Jonuleit, H., Schmitt, E., Stassen, M., Tuettenberg, A., Knop, J., Enk, A. H.,** 2001. Identification and functional characterization of human CD4(+)CD25(+) T cells with regulatory properties isolated from peripheral blood. J Exp Med. **193,** 1285-1294

18. **Maloy, K. J., Powrie, F.,** 2001. Regulatory T cells in the control of immune pathology. Nat Immunol. **2,** 816-822.

19. **Ohno. K., Watari, T., Goitsuka, R., Tsujimoto, H., Hasegawa, A.,** 1992. Altered surface antigen expression on peripheral blood mononuclear cells in cats infected with feline immunodeficiency virus. J Vet Med Sci. **54,** 517-522.

20. **Perelson, A. S., Neumann, A. U., Markowitz, M., Leonard, J. M., and Ho D. D.,** 1996. HIV-1 dynamics in vivo: virion clearance rate, infected cell life-span, and viral generation time. Science. **271,** 1582-6.

21. **Ramilo, O., Bell, K. D., Uhr, J. W., Vitetta, E. S.,** 1993. Role of CD25+ and CD25- T cells in acute HIV infection in vitro. J Immunol. **150,** 5202-5208.

22. **Sakaguchi, M.,** 2003. Regulatory T cells: mediating compromises between host and parasite. Nat. Immunol. **4,** 10-11.

23. **Sharkey, M. E., Stevenson, M.,** 2001. Two long terminal repeat circles and persistent HIV-1 replication. Curr Opin Infect Dis. **14,** 5-11.

24. **Sharkey, M. E., Teo, I., Greenough, T., Sharova, N., Luzuriaga, K., Sullivan, J. L., Bucy, R. P., Kostrikis, L. G., Haase, A., Veryard, C., Davaro, R. E., Cheeseman, S. H., Daly, J. S., Bova, C., Ellison, R. T. 3rd., Mady, B., Lai, K. K., Moyle, G., Nelson, M., Gazzard, B., Shaunak, S., Stevenson, M.,** 2000. Persistence of episomal HIV-1 infection intermediates in patients on highly active anti-retroviral therapy. Nat Med. **6,** 76-81.

25. **Stevenson, M., Stanwick, T. L., Dempsey, M. P., Lamonica, C. A.,** 1990. HIV-1 replication is controlled at the level of T cell activation and proviral integration. EMBO J. **9,** 1551-1560.

26. **Taams, L. S., Smith, J., Rustin, M. H., Salmon, M., Poulter, L. W., Akbar, A. N.,** 2001. Human anergic/suppressive CD4(+)CD25(+) T cells: a highly differentiated and apoptosis-prone population. Eur J Immunol. **31,** 1122-1131.

27. **Tang, H., Kuhen, K. L., Wong-Staal, F.,** 1999. Lentivirus replication and regulation. Annu Rev Genet. **33,** 133-170.

28. **Tang, S., Patterson, B., Levy, J. A.,** 1995. Highly purified quiescent human peripheral blood CD4+ T cells are infectible by human immunodeficiency virus but do not release virus after activation. J Virol. **69,** 5659-5665.

29. **Teo, I., Veryard, C., Barnes, H., An, S. F., Jones, M., Lantos, P. L., Luthert, P., Shaunak, S.,** 1997. Circular forms of unintegrated human immunodeficiency virus type 1 DNA and high levels of viral protein expression: association with dementia and multinucleated giant cells in the brains of patients with AIDS. J Virol. **71,** 2928-2933.

30. **Thornton, A. M., Shevach, E. M.,** 1998. CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. J Exp Med. **188,** 287-296.

31. **Tompkins, M. B., Bull, M. E., Dow, J. L., Ball, J. M., Collisson, E. W., Winslow, B. J., Phadke, A. P., Vahlenkamp, T. W., Tompkins, W. A.,** 2002. Feline immunodeficiency virus infection is characterized by B7+CTLA4+ T cell apoptosis. J Infect Dis. **185,** 1077-1093.

32. **Tompkins, M. B., Gebhard, D. H., Bingham, H. R., Hamilton, M. J., Davis, W. C., Tompkins W. A.,** 1990. Characterization of monoclonal antibodies to feline T lymphocytes and their use in the analysis of lymphocyte tissue distribution in the cat. Vet Immunol Immunopathol. **4,** 305-317.

33. **Tompkins, M. B., Ogilvie, G. K., Franklin, R. A., Kelley, K.W., Tompkins, W.A.,** 1987. Induction of IL-2 and lymphokine activated killer cells in the cat. Vet Immunol Immunopathol. **16,** 1-10.

34. **Willett, B. J., Cannon, C. A., and Hosie, M. J.,** 2003. Expression of CXCR4 on feline peripheral blood mononuclear cells: effect of feline immunodeficiency virus infection. J Virol **77,** 709-12.

35. **Wong, J. K., Hezareh, M., Gunthard, H. F., Havlir, D. V., Ignacio, C. C., Spina, C. A., Richman, D. D.,** 1997. Recovery of replication-competent HIV despite prolonged suppression of plasma viremia. Science. **278,** 1291-1295.

36. **Zack, J. A., Arrigo, S. J., Weitsman, S. R., Go, A. S., Haislip, A., Chen, I. S.,** 1990. HIV-1 entry into quiescent primary lymphocytes: molecular analysis reveals a labile, latent viral structure. Cell. **61,** 213-222.

37. **Zack, J. A., Haislip, A. M., Krogstad, P., Chen, I. S.,** 1992. Incompletely reverse-transcribed human immunodeficiency virus type 1 genomes in quiescent cells can function as intermediates in the retroviral life cycle. J Virol. **66,** 1717-1725.

4. CHAPTER-2

Preferential FIV Infection of $CD4^+CD25^+$ T Regulatory Cells Correlates both with Surface Expression of CXCR4 and CD134 and Activation of Cellular Transcriptional Factors

ABSTRACT

Previously, we have characterized feline $CD4^+CD25^+$ T regulatory (Treg) cells with regard to their immune regulatory properties and ability to support feline immunodeficiency virus (FIV) replication in vitro and in vivo. Our studies showed that while $CD4^+CD25^+$ cells were capable of replicating FIV in the presence of IL-2 alone, $CD4^+CD25^-$ cells harbored a latent infection that required a strong mitogenic stimulus to activate virus replication. In the present study, we investigated the mechanisms governing the preferential replication of FIV in highly purified $CD4^+CD25^+$ Treg cells compared to their $CD4^+CD25^-$ counterparts. Studies aimed at elucidating mechanisms regulating infection of these cells revealed that $CD4^+CD25^-$ cells were less susceptible to FIV binding and entry compared to $CD4^+CD25^+$ cells which correlated with increased surface expression of FIV co-receptor CXCR4. In addition, a greater percentage of $CD4^+CD25^+$ cells expressed the primary receptor CD134 compared to $CD4^+CD25^-$ cells. Although increased permissiveness to FIV infection of $CD4^+CD25^-$ cells following mitogenic stimulation correlated strongly with upregulation of surface CXCR4, it did not correlate with CD134 expression. Further, study of intracellular factors regulating FIV replication, revealed that $CD4^+CD25^+$ but not $CD4^+CD25^-$ T cells showed constitutive and IL-2 responsive transactivation of ATF, CEBP and AP-1 transcription factors that are important for FIV replication. These factors were upregulated in $CD4^+CD25^-$ T cells following ConA stimulation, which correlated with FIV replication. This is the first report elucidating the mechanisms that allow for productive lentiviral infection of $CD4^+CD25^+$ Treg cells.

INTRODUCTION

Feline Immunodeficiency Virus (FIV), a lentivirus belonging to the family retroviridae, is remarkably similar to the human lentivirus HIV-1 both in terms of genomic organization and disease pathogenesis (Bendinelli et al., 1995). These similarities in pathogenesis of the two viruses makes FIV an excellent small animal model for HIV-1 induced immunodeficiency in humans. Despite the marked similarities between HIV and FIV, the two viruses also have apparent differences. While HIV-1 utilizes CD4 as the primary receptor and shows a major tropism for cells of the CD4 lineage (Maddon et al., 1986), FIV does not utilize the CD4 molecule as a receptor and its tropism is more generalized for cells of the CD4, CD8 as well as B lineage (English et al., 1993; Dean et al., 1996). In this regard, the primary receptor for FIV has only recently been identified as CD134, a $CD4^+$ T cell activation marker belonging to the TNF receptor superfamily (Shimojima et al., 2004), while the utilization of CXCR4 as the co-receptor for FIV entry is well established (Garg et al., 2004; Willett et al., 1997),

Studies have established that in HIV infected individuals, the virus persists mainly as a latent reservoir in memory $CD4^+$ T ($CD45RO^+$) cells, which possess the inherent ability to survive for a prolonged period of time (Chun and Fauci., 1999; Persaud et al., 2003). While HIV infection has also been demonstrated in naïve T cells in vivo (Ostrowski et al., 1999), establishment of a stable HIV infection in resting T cells in vitro remains controversial. Some studies suggest that HIV can efficiently bind and enter resting T cells (Spina et al., 1995; Tang et al., 1995), whereas others have shown that naïve T cells are resistant to de novo HIV infection (Gowda et al., 1989; Chou et al., 1997). In this regard, data suggests that susceptibility of different lymphocytes subsets to HIV infection is regulated, to some extent,

by differential expression of viral co-receptors. Pertaining to HIV binding and entry, studies have shown that the HIV co-receptors CXCR4 (X4) and CCR5 (R5) are differentially expressed on activated versus naïve/resting T cells. While CXCR4 is expressed on both naive and memory T cells, CCR5 is a marker of activated and replicating T cells that are infectable with R5 tropic viruses. (Eckstein et al., 2001; Pierson et al., 2000). In support of this, studies have demonstrated that while resting $CD4^+$ T cells can be infected by X4 tropic viruses they are resistant to infection by R5 tropic viruses (Pierson et al., 2000).

In addition to cell surface receptor expression, the activation/proliferative state of T cells also determines the extent of virus replication (Stevenson et al., 1990). Further, it has been demonstrated that HIV and FIV replicate efficiently in $CD4^+CD25^+$ T cells that are partially activated but not in naïve $CD4^+CD25^-$ resting T cells (Borvak et al., 1995; Chou et al., 1997; Joshi et al., 2004; Stevenson et al., 1990). To date there have been limited reports regarding FIV replication in activated versus resting T cells. We have recently reported that FIV establishes a productive infection in feline $CD4^+CD25^+$ T cells, but not in naïve $CD4^+CD25^-$ T cells (Joshi et al., 2004). We subsequently phenotypically and functionally characterized these $CD4^+CD25^+$ T cells as T regulatory (Treg) cells (Vahlenkamp et al., 2004). These cells have a partial activation phenotype, are anergic and demonstrate immunosuppressive function in the presence of IL-2 (Vahlenkamp et al., 2004). While little is known of the factors regulating anergy in Treg cells, the activation/proliferative state of lymphocytes is largely controlled by group of cellular transcription factors that regulate IL-2 gene expression, such as NFκB and AP-1 (Aringer et al., 2002) that also regulate transcription of HIV and FIV genes (Bigornia et al., 2001; Cullen., 1989).

HIV and FIV depend on both cellular and viral factors for efficient transcription of their genomes and the activity of their promoters within the LTR regions are highly dependent on the level of host cell activation (Bigornia et al., 2001, tang et al., 1999). HIV and FIV LTR's encompass binding sites for several cellular transcription factors, including AP-1, AP-4, ATF, CEBP, NFκB, and SP-1 (Cullen., 1989; Bigornia et al., 2001; Pereira et al., 2000). Although the role of NFκB in HIV mRNA transcription is well established (Cullen., 1989; Pereira et al., 2000), the role of a putative NFκB binding site in FIV LTR transactivation remains unclear. However, several studies have established that the binding sites for AP-1, ATF and CEBP in the FIV LTR are critical for FIV replication in infected cells. Kawaguchi et al (1995) demonstrated by site-specific mutations that the CEBP site in FIV LTR is necessary for efficient viral replication in both feline fibroblasts and a T lymphoblastoid cell line. A number of studies demonstrated that mutations/deletions of both ATF and AP-1 sites resulted in severely reduced basal promoter activity form FIV LTR and impaired ability of viruses to replicate both in feline lymphocytes and macrophages (Bigornia et al., 2001; Ikeda et al., 1998; Inoshima et al., 1996).

In the present study, we extended our previous observations to elucidate the mechanisms governing the differential replication of FIV in $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells. Our results demonstrate that $CD4^+CD25^-$ T cells exhibit restrictions to FIV infection both at the level of viral entry and transcriptional activation. While viral entry correlates more significantly with surface CXCR4 than CD134 expression, FIV transcriptional activation correlates significantly with AP-1, CEBP and ATF activation but not NFκB.

MATERIALS AND METHODS

Cats. Specific pathogen free (SPF) cats were obtained from Liberty Labs (Liberty Corners, NJ, USA) or Cedar River Laboratory (IA) and housed at the Laboratory Animal Resource Facility at the College of veterinary medicine, North Carolina State University. At the time of the study, cats ranged in age between six to seven years and were seronegative for FIV.

Blood and lymph node (LN) cell collection. Lymphocytes were obtained either from LN or whole blood. LN cells were obtained by peripheral LN biopsies as described previously (Tompkins et al., 2002). Peripheral blood mononuclear cells (PBMC) were isolated from whole blood by Percoll (Sigma, St. Louis, MO) density gradient centrifugation as described by Tompkins et al (1987). Preliminary studies using cells from both sources gave equivalent results.

Antibodies and flow cytometry analysis. PBMC or purified cell populations were stained for surface expression of various markers using phycoerythrin (PE), fluorescein isothiocyanate (FITC) or allophycocyanin (APC) conjugated anti-CD4 (48) and FITC conjugated anti-CD25 antibodies (Ohno et al., 1992). Cell surface CXCR4 expression was determined using the cross-reacting human anti-CXCR4 antibody (44717) followed by PE conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, PA). Surface CD134 expression was determined using cross-reacting (Shimojima et al., 2004) PE conjugated BerACT35 monoclonal antibody (Ancell Corporation, Bayport, MN) following the manufacturer's instructions. Samples were analyzed using a FACSCalibur Flow

Cytometer (Becton Dickinson, Los Angeles, CA). Generally, 3-5×10^5 cells were used for staining and 15,000 events acquired using Becton Dickinson Cell Quest software.

Purification of T cells. To investigate single lymphocyte subsets, $CD4^+CD25^+$ and $CD4^+CD25^-$ cells were stained with anti-CD4 and anti-CD25 antibodies, as described above and sorted into pure populations using Cytomation MoFlo fluorescence activated cell sorter (FACS). The purity of FACS sorted cell populations was ≥ 97.0%. For some experiments, $CD4^+$ subsets were enriched by negative selection using goat anti-mouse IgG coated magnetic beads (Dynabeads® M-450, Dynal, Oslo, Norway) as described previously (Joshi et al., 2004; Vahlenkamp et al., 2004). Briefly, PBMC were depleted of B cells, CD8 cells and monocytes using anti-CD21, anti-CD8 and anti-CD14 monoclonal antibodies respectively. The remaining cells were further treated with 5mM leucine methyl ester for 1hr at 25^{o}C (Sigma) to deplete natural killer cells. The CD25 expressing $CD4^+$ cells were then enriched by positive selection using anti-CD25 monoclonal antibody (Ohno et al., 1992) coated beads. The purity of enriched $CD4^+CD25^+$ or $CD4^+CD25^-$ cells was verified by flow cytometric analysis and varied from 90 to 97% for the $CD4^+$ cell population and > 98.0% for $CD25^+$ and $CD25^-$ cells (Fig. 1).

Cell culture and stimulation. PBMC, LN cells or purified T cell subsets were cultured at 10^6 cells/ml in growth medium (RPMI-1640 containing 10% heat inactivated fetal bovine serum, 1% penicillin-streptomycin, 1% sodium bicarbonate, 1% sodium pyruvate, 1% L-glutamine, and 1 mM HEPES buffer) in the presence or absence of 2μg/ml ConA or 100U/ml recombinant human interleukin-2 (IL-2) (NIH AIDS Research and Reagent Program). Feline

CD4E (FCD4E) cells were established through long-term culture of PBMC from a SPF cat in the presence of IL-2 and cultured in RPMI-1640 medium (English et al., 1993). These cells are 100% positive for the feline pan T cell marker 1.572 and 60 to 65% positive for the feline CD4 homolog (English et al., 1993). Viability for different cell populations was determined by Trypan Blue dye exclusion assay and was routinely greater than 90%.

FIV-NCSU$_1$ virus stock generation and in vitro infection. FIV-NCSU$_1$ virus stock was prepared from PBMC derived from FIV positive cats as described (Joshi et al., 2004). No virus associated DNA was present in the stock as analyzed by PCR using FIV specific primers. In vitro infection assays were performed using a multiplicity of infection (m.o.i.) of 0.1 or as indicated. Before infections, the virus stock was treated with DNAseI (300U/ml) for 30min at room temperature. Cells were exposed to the virus for 1-2hr at 37^{o}C. Following virus adsorption, the cells were washed three times with culture medium and plated at a concentration of 10^6 cells/ml of culture medium.

FIV p24 antigen capture ELISA. FIV Gag-p24 antigen in culture supernatants of infected cells was detected using an antigen capture ELISA as described previously (Joshi et al., 2004).

PCR and reverse transcription PCR. DNA was extracted from equal numbers of infected cells using QIAamp® DNA Blood Mini Kit (Qiagen, CA) and quantified by measuring the A_{260} using a UV-spectrophotometer (BioRad). Generally, 0.5µg of DNA was used for PCR reactions unless otherwise indicated. For some experiments, DNA was harvested by direct

cell lysis in PCR tubes to minimize loss during extraction. Briefly, 10^5 infected cells were pelleted in a PCR tube and lysed in 50µl of lysis buffer (10mM Tris-Cl [pH 8.3] 0.45% Nonidet P-40, 0.45% Tween-20, 50µg ProteinaseK/ml) and incubated for 3hr at 56^{o}C followed by denaturation for 10min at 100^{o}C. 10µl of this lysate was used for PCR. FIV specific products in infected cells were detected using primers LTR-sense and LTR-antisense and a GAPDH primer pair was used as a control to ensure the use of equal amounts of DNA template in identical experiments as described previously (Joshi et al., 2004). For RT-PCR studies, RNA was isolated using the RNeasy Micro kit (Qiagen, CA). During extraction the samples were treated with RNAse free DNAse as per the manufacturer's protocol to get rid of contaminating DNA.

Real time PCR and RT-PCR. RNA or DNA was isolated from equal numbers of infected cell populations as described above. 0.5µg of purified RNA was reverse transcribed and amplified using the TaqMan One Step RT-PCR kit (Applied Biosystems, CA) in a total reaction volume of 50µl with cycling conditions recommended by the manufacturer. In order to calculate viral copy numbers, a standard curve was generated using serial 10-fold dilutions of gag-RNA standard of known copy number. The gag-RNA was prepared by in-vitro transcription (Ambion, Austin, TX) from a plasmid containing a portion of the $NCSU_1$ *gag* gene under the control of the T7 promoter. The gag primers and probe derived from the FIV-$NCSU_1$ sequence have been previously described (Burkhard et al., 2002) and are as follows: Forward primer-5'- GAT TAG GAG GTG AGG AAG TTC AGC T -3'; Reverse primer-5'- CTT TCA TCC AAT ATT TCT TTA TCT GCA -3'; Probe-5'- 6FAM - CAT GGC CAC ATT AAT AAT GGC CGC A - TAMRA -3'. Samples and standards were amplified in 96

well plates (Axygen, CA) using the BioRad icyler (Hercules, CA). In parallel, RT-PCR reactions for GAPDH gene were used as housekeeping control. Quantitative real time PCR for cell-associated proviral DNA was also performed with the above-mentioned primer pairs in a total reaction volume of 25µl using the TaqMan Universal PCR kit (Applied Biosystems, CA). To calculate proviral copy numbers, a standard curve was generated using serial 10-fold dilutions of the $NCSU_1$ plasmid with known copy numbers. 0.5µg of DNA was used per reaction and amplified in the icycler (BioRad, Hercules, CA) under the following conditions: 95^{o}C for 10min, 45 cycles of 95^{o}C for 15sec and a final extension of 60^{o}C for 1min. PCR for GAPDH DNA was used as housekeeping gene for normalizing the DNA quantity.

Viral Binding and Entry Assays. FIV binding and entry assays were performed as described previously (Ma et al., 2003). Briefly, for viral binding, 10^6 purified $CD4^+CD25^+$ and $CD4^+CD25^-$ cells were chilled on ice for 20min followed by incubation with FIV-$NCSU_1$ for 1hr on ice. Subsequently, the cells were washed 4 times with chilled PBS and lysed immediately to isolate total RNA. For entry assays, 10^6 cells were incubated with FIV at 37^{o}C for 1hr, treated with Trypsin-EDTA (0.25%) for 7min at 37^{o}C to remove of cell surface attached virions followed by 4 washes with PBS. Cells were then lysed, total RNA isolated and used for real time RT-PCR. Efficiency of Trypsin-EDTA treatment in removing virus attached to the cell surface was determined by incubating cells with FIV on ice for 1hr followed by trypsinization as indicated above and subsequently performing RT-PCR using FIV specific primers.

Blocking studies using the CXCR4 antagonist AMD-3100. $CD4^+CD25^+$ and $CD4^+CD25^-$ cells ($2x10^5$ per treatment) were incubated for 30min with serial 10-fold dilutions of AMD-3100 (a kind gift of Dr. Edward Hoover, Colorado State University) starting with a maximum concentration of 10μg/ml. Cells were subsequently infected with FIV-$NCSU_1$ either in the presence or absence of inhibitor. Following infection, cells were washed with PBS and cultured in RPMI medium supplemented with IL-2 for $CD4^+CD25^+$ cells and ConA for $CD4^+CD25^-$ cells. Culture medium for both the cell types was replenished with the respective above-mentioned concentrations of AMD-3100 to minimize second round infections. Infected cells were cultured for 12 days and culture supernatants harvested every 3 day post infection for FIV-p24 antigen capture ELISA.

Electrophoretic Mobility Shift Assay (EMSA). Nuclear extracts were prepared from 10^6 cells as per the protocol of Dignam and co-workers (1983) either immediately after isolation or after culture for 5 days in the presence of IL-2 (100U/ml) or ConA (2μg/ml). Protein concentrations in the nuclear extracts were estimated using the colorimetric Coomassie Blue Protein Assay Reagent Kit (Pierce Biotechnology, IL) following the manufacturer's protocol. Oligonucleotides were designed using the predicted binding sites for, AP-1, ATF, CEBP and NFκB in the FIV-Petaluma and FIV-PPR genomes and incorporating the changes in the FIV-$NCSU_1$ genome. The sequences for the sense strand are as follows: AP-1, 5'- AGC ATG ACT CAT AGT TAA AG -3'; CEBP, 5'- TGC TTA ACC GCA AAA CCA CAT CCT A -3'; NFκB, 5'- GGA CTA GTG ACT GTT TAC GA -3' ATF, 5'- GCT GAT GAC GTA TAA TTT GC -3'. In addition, a consensus oligonucleotide for NFκB having the sequence 5'- AGT TGA GGG GAC TTT CCC AGG C -3' was also used in the study. The double

stranded oligonucleotides were labeled at the 5' end with radiolabeled $\gamma P^{32}ATP$ (3,000Ci/mmol at 10mCi/ml, Amersham Biosciences) using the T_4 PolyNucleotide Kinase (10units/µl, Invitrogen). Nuclear extracts (2.5µg for AP-1 and CEBP, 5µg for ATF and consensus NFκB and 10µg for LTR NFκB) were incubated with 1µl of radiolabeled oligonucleotides (0.2ng, 5-8x10^4 cpm) in binding buffer (10mM TrisHCl [pH 7.5], 50mM NaCl, 1mM $MgCl_2$, 0.5mM DTT, 0.5mM EDTA, 4% glycerol, 50µg/ml PolyI: PolyC) for 15min at room temperature. Specific competition (SC) was accomplished by using 50-fold molar excess of unlabeled probe. In addition, non-specific competitors (NSC) that did not contain the AP-1, ATF, CEBP or NFκB binding sequence were used to demonstrate specificity and have been described elsewhere (Ikeda et al., 1998; Kawaguchi et al., 1995; Mizuno et al., 2001). Nuclear extracts for specific and non-specific competition were derived from 1.5x10^6 T cells activated with 2µg/ml ConA for 5 days. Following binding, the complexes were resolved in a 6% non-denaturing acrylamide/bisacrylamide (30:1) gel in 22.5 mM Tris Borate and 0.5mM EDTA (TBE) buffer. The gels were subsequently dried and autoradiographed.

Statistical analysis. The student's t-test was used to compare differences in p24 antigen between infected $CD4^+CD25^+$ and $CD4^+CD25^-$ cells. Differences in viral copy numbers and cell surface markers between $CD4^+CD25^+$ and $CD4^+CD25^-$ cells were determined using the Mann-Whitney's test (t-test-like for non-parametric data). Differences were considered significant at $p<0.05$. Correlation analysis between variables was performed using the Pearson's correlation coefficient (R).

RESULTS

$CD4^+CD25^-$ cells show a compromised ability for FIV binding and entry compared to $CD4^+CD25^+$ cells. Previously we have shown that $CD4^+CD25^+$ cells replicated FIV in vitro in the presence of IL-2 alone, whereas $CD4^+CD25^-$ cells established a latent infection and could be activated to produce infectious virus only via a strong mitogenic stimulus such as ConA (Joshi et al., 2004). However, ConA stimulated $CD4^+CD25^-$ cells produced significantly less virus than IL-2 cultured $CD4^+CD25^+$ cells, suggesting that factors in addition to those controlling virus replication may govern permissiveness of $CD4^+CD25^-$ cells to FIV infection. To determine if these cell types differed with respect to FIV permissiveness at the level of viral entry, virus binding and entry assays were performed on highly purified $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells subsets purified by antibody coated magnetic beads or FACS sorting as demonstrated in Fig. 1. PCR amplification of proviral DNA was performed 8hr post infection on purified $CD4^+CD25^+$ and $CD4^+CD25^-$ T cell subsets infected with serial dilutions of FIV. As shown in Fig. 2A proviral DNA was detectable at 16-fold lower virus dilutions in $CD4^+CD25^+$ cells compared to $CD4^+CD25^-$ cells, suggesting that $CD4^+CD25^+$ cells are more permissive to FIV infection. Stimulation of $CD4^+CD25^-$ cells with ConA prior to infection enhanced their permissiveness to FIV, which was comparable to that seen in $CD4^+CD25^+$ cells (Fig. 2A).

Having seen a difference with respect to proviral DNA in *in vitro* infected $CD4^+CD25^+$ versus $CD4^+CD25^-$ cells, viral binding and entry assays were performed in the above cell types by determining viral RNA copy numbers by real time RT-PCR. For binding assays, purified cell populations were incubated with FIV on ice for 1hr and total RNA

isolated immediately following incubation and washing. For detecting viral entry, cells were incubated with FIV at 37^{o}C for 1hr and trypsinized to remove to cell-surface attached virions prior to RNA isolation. While $CD4^{+}CD25^{+}$ cells bound FIV twice as efficiently as $CD4^{+}CD25^{-}$ cells, the difference was approximately 50-fold in terms of viral entry (Fig. 2B). $CD4^{+}CD25^{-}$ cells, on the other hand when stimulated with a mitogen prior to virus incubation were as efficient as $CD4^{+}CD25^{+}$ cells with regard to both FIV binding and entry (Fig. 2B). Trypsinization control experiments suggested that trypsin-EDTA treatment was capable of stripping most of the virus attached to the cell surface (data not shown). Control reaction in which the RT step was omitted did not yield any visible amplification product indicating absence of contaminating DNA (data not shown). The above findings suggest that $CD4^{+}CD25^{-}$ cells are compromised with respect to FIV binding, and an additional restriction occurs at the level of viral entry into cells.

Differential expression of FIV co-receptor CXCR4 on $CD4^{+}CD25^{+}$ and $CD4^{+}CD25^{-}$ cells. Having seen a difference in $CD4^{+}CD25^{+}$ and $CD4^{+}CD25^{-}$ cells at the level of viral binding and entry, we investigated the expression of FIV co-receptor CXCR4, on the two cell types. As seen in Fig. 3A, two-color flow cytometry staining of feline LN cells with CD4 and CXCR4 antibodies revealed that >95% of the $CD4^{+}$ cells were CXCR4 positive. As $CD4^{+}CD25^{+}$ cells represent 20-30% of the total LN $CD4^{+}$ T cells (Vahlenkamp et al., 2004), these data indicate that the majority of $CD4^{+}CD25^{+}$, as well as $CD4^{+}CD25^{-}$ cells express CXCR4. However, comparison of the mean fluorescence intensity (MFI) of CXCR4 expression on $CD4^{+}CD25^{+}$ and $CD4^{+}CD25^{-}$ cells revealed that $CD4^{+}CD25^{+}$ cells inherently expressed higher levels of CXCR4 on a per cell basis (MFI 401.58 versus 116.26) when

assayed immediately post isolation (Fig. 3B). IL-2 treatment for 3 days led to only moderate increase in CXCR4 expression levels on both $CD4^+$ cell subsets (Fig.3B, 401.58 versus 425.65 for $CD4^+CD25^+$ and 116.26 versus 157.47 for $CD4^+CD25^-$ cells). Interestingly, treatment with ConA for 3 days led to a significant increase in CXCR4 expression in $CD4^+CD25^-$ cells (Fig. 3B, 116.26 versus 385.19) but down-regulated CXCR4 expression in $CD4^+CD25^+$ cells (Fig. 3B, 401.58 versus 219.48). These findings suggest that the reduced viral permissiveness exhibited by $CD4^+CD25^-$ cells when compared to $CD4^+CD25^+$ cells could be due to a lower surface CXCR4 expression in these cells.

Having determined that $CD4^+CD25^+$ and $CD4^+CD25^-$ cells differ with respect to levels of CXCR4 expression, we asked whether the specific CXCR4 antagonist AMD-3100 could block infection in a concentration-dependent manner. $CD4^+CD25^+$ and $CD4^+CD25^-$ cells were infected with FIV-$NCSU_1$ in the presence of varying concentrations of AMD-3100 and p24 antigen in culture supernatants detected by an ELISA. As seen in Fig. 4, concentrations of AMD-3100 below 0.01μg/ml had minimum effect in blocking FIV infection of both the cell types. However, concentrations of AMD-3100 in the range of 0.01 to 1μg/ml were significantly more efficacious in blocking FIV infection of $CD4^+CD25^-$ (IC_{50} 0.0295) compared to $CD4^+CD25^+$ (IC_{50} 0.2063) cells, again suggesting a higher density of CXCR4 on the Treg cells.

To further establish a correlation between CXCR4 expression levels and susceptibility to FIV infection, a time study was performed to determine kinetics of CXCR4 upregulation in parallel with viral entry into $CD4^+CD25^-$ cells. $CD4^+CD25^-$ cells were either left unstimulated or stimulated with ConA for different time periods prior to infection with FIV as indicated in Fig. 5. Following stimulation, cells were monitored for viral entry by real

time PCR and CXCR4 expression by flow cytometry. The MFI of CXCR4 expression started to increase after 4hr of stimulation and continued to increase thereafter up to 72hr (Fig. 5B). In parallel, viral entry in $CD4^+CD25^-$ cells increased in an identical pattern (Fig. 5A). These results indicate a strong correlation (R=0.9845, Fig. 5C) between increase in viral entry in $CD4^+CD25^-$ cells following ConA stimulation and an upregulation of cell surface CXCR4 expression on a per cell basis.

FIV receptor CD134 (OX-40) is differentially expressed on $CD4^+CD25^+$ and $CD4^+CD25^-$ T cell populations. Recently it has been reported that FIV uses CD134 as the primary receptor for entry into cells (Shimojima et al., 2004). Hence we investigated whether $CD4^+CD25^+$ and $CD4^+CD25^-$ cells differed with respect to the cell surface expression of CD134. Flow cytometry analysis using the human CD134 antibody clone previously described to cross-react with the feline CD134 homolog (Shimojima et al., 2004) revealed that within the PBMC or LN population the number of $CD134^+$ cells ranged from 6-14% in 8 normal cats analyzed. Fig. 6A represents a typical flow cytometry analysis of CD134 expression on feline LN cells. Similar analysis on sorted cell populations revealed that approximately 25-30% of $CD4^+CD25^+$ and 10-15% of $CD4^+CD25^-$ cells expressed CD134 (Fig. 6B). Surprisingly, stimulation with ConA at a concentration of 2μg/ml did not upregulate surface CD134 expression on either $CD4^+$ T cell subsets (Fig. 6C). On the contrary, there seemed to be a down regulation of surface CD134 expression on $CD4^+CD25^+$ and $CD4^+CD25^-$ cells following ConA stimulation (Fig. 6B versus 6C). At a similar concentration of ConA, 90-95% of $CD4^+$ cells became positive for the activation marker CD25 (Fig. 6C, top) compared to 5-10% $CD4^+CD25^+$ population present prior to stimulation

(Fig. 1A). Treatment with 100U/ml IL-2 did not have any effect on the surface expression of either CD25 or CD134 (data not shown). From these findings we can conclude that although $CD4^+CD25^+$ cells express higher levels of FIV receptor and coreceptor compared to $CD4^+CD25^-$ cells, increase in viral entry in $CD4^+CD25^-$ cells following ConA stimulation correlates with increased CXCR4 but not CD134 expression.

$CD4^+CD25^+$ cells constitutively express high levels of cellular transcription factors known to activate FIV gene expression. Previously we have shown that while $CD4^+CD25^+$ cells replicate FIV in vitro in the presence of IL-2 alone, $CD4^+CD25^-$ cells require a strong mitogenic stimulus like ConA for a productive FIV infection (Joshi et al., 2004). An important block to retroviral replication in resting/naïve T cells is at the level of cellular transcriptional activity (Tang et al., 1999). Hence, we analyzed the activation status of the transcription factors ATF, AP-1 and CEBP that are known to play an important role in FIV replication (Bigornia et al., 2001; Kawaguchi et al., 1995). Figure 7A depicts the nucleotide sequence of the FIV-$NCSU_1$ LTR from position 40 to 200, and the predicted binding sites for the transcription factors. Shift assay revealed constitutive transactivation of CEBP and ATF in $CD4^+CD25^+$ T cells when analyzed immediately after isolation (Fig. 7B, Lane 4), which were further enhanced with IL-2 treatment (Fig. 7B, lane 5). $CD4^+CD25^+$ cells also expressed low constitutive activation of transcription factor AP-1 (Fig. 7B, lane 4), which was significantly upregulated by IL-2 treatment (Fig. 7B, lane 5). In contrast, $CD4^+CD25^-$ cells expressed low or no constitutive activity of these transcription factors (Fig. 7B, lane 7) all of which were highly upregulated by ConA (Fig 7B, lane 9) but not IL-2 (Fig 7B, lane 8) treatment. Hence, while $CD4^+CD25^+$ cells demonstrated constitutive or IL-2 dependent

activation of transcription factors known to be important in FIV replication, $CD4^+CD25^-$ cells expressed these transcription factors only following treatment with ConA. This correlates with FIV replication in $CD4^+CD25^+$ cells in the presence of IL-2 alone but in $CD4^+CD25^-$ cells only when stimulated with a strong mitogenic stimulus.

NFκB binding to FIV LTR is not important for a productive infection. While a putative NFκB motif is variably expressed on isolates of FIV, specific protein binding to this sequence has not been established (Sparger et al., 1992; Thompson., 1994). We also observed that the putative NFκB oligo derived from FIV-$NCSU_1$ LTR did not bind efficiently to NFκB by EMSA (data not shown). Moreover, the binding was not enhanced in mitogen treated cells suggesting that this FIV-$NCSU_1$ sequence does not provide a good fit for NFκB. To further explore if NFκB is activated in $CD4^+CD25^+$ Treg cells, we developed consensus oligonucleotides for the NFκB binding site (Fig. 8A). With this probe $CD4^+CD25^+$ cells showed constitutive high expression of NFκB, which was further upregulated by IL-2 treatment (Fig. 8A, lane 4 versus 5). $CD4^+CD25^-$ cells revealed no constitutive NFκB activity (Fig. 8A, lane 7), but activation was observed following ConA and to a lesser extent IL-2 treatment (Fig. 8A, lane 8 versus 9). In order to elucidate the discrepancy seen with NFκB probe derived from FIV-LTR versus consensus NFκB oligo, cross-competition experiments were performed using nuclear extracts derived from FCD4E cells. Incubation of FCD4E nuclear extracts with excess of unlabeled consensus NFκB probe prior to addition of P^{32} labeled FIV-LTR NFκB probe abolished weak binding of the FIV-LTR probe (Fig. 8B, lane 3 versus lane 7). In contrast, incubation with excess of unlabeled LTR-derived NFκB oligo prior to addition of radiolabeled consensus NFκB oligo did not have any effect on NFκB

binding to consensus oligo (Fig. 8B, lane 4 versus lane 8). These results suggest that, while NFκB is upregulated in $CD4^+CD25^+$ Treg cells, the putative NFκB binding site in the FIV-LTR is not a perfect match for binding to NFκB and probably does not play an important role in FIV replication.

DISCUSSION

Previously we have characterized feline $CD4^+CD25^+$ T regulatory cells both with regard to their immune regulatory properties and ability to support FIV-$NCSU_1$ replication (Joshi et al., 2004; Vahlenkamp et al., 2004). Our studies showed that feline $CD4^+CD25^+$ cells posses the key characteristics of Treg cells with regard to their anergic state and ability to suppress proliferation of $CD4^+CD25^-$ cells via inhibition of IL-2 production. Further, we also demonstrated that these cells are arrested in the G_0/G_1 state of the cell cycle and express on their surface CTLA4, a marker associated with Treg cells (Vahlenkamp et al., 2004). Virus replication studies revealed that while $CD4^+CD25^+$ cells harbored a productive FIV infection in the presence of IL-2 alone, $CD4^+CD25^-$ cells established a latent infection and required a strong mitogenic stimulus such as ConA to produce infectious virus. While $CD4^+CD25^+$ cells efficiently replicated FIV, they themselves remained anergic to mitogenic stimulus and AICD, suggesting their potential to act as a long-term reservoir of productive FIV infection (Joshi et al., 2004). The present study was aimed at investigating the mechanisms governing infection and replication of FIV in $CD4^+CD25^+$ cells.

Ramilo et al (1993) demonstrated that PBMC depleted of $CD25^+$ cells using anti-CD25 immunotoxin were severely restricted in their ability to replicate HIV in vitro. Further,

Chou et al (1997) demonstrated that, whereas $CD4^+CD25^+$ T cells could be productively infected with HIV-1 in vitro, highly purified $CD4^+CD25^-$ cells were incapable of being infected. However, they did not elucidate the mechanism(s) restricting HIV infection of $CD4^+CD25^-$ cells. In the present study we found that $CD4^+CD25^-$ cells were less susceptible than $CD4^+CD25^+$ T cells to FIV infection in vitro which correlated with reduced surface expression of the FIV co-receptor CXCR4 on their surface. In agreement with increased density of CXCR4 on $CD4^+CD25^+$ T cells as compared to $CD4^+CD25^-$ T cells is the observation that CXCR4 antagonist AMD-3100 was able to block FIV infection of $CD4^+CD25^-$ cells at significantly lower concentrations than that required to block infection of $CD4^+CD25^+$ Treg cells. Further, $CD4^+CD25^-$ cells express significantly less CXCR4 on their surface, which is consistent with the finding that they bind two-fold less virus on their surface. However, the level of viral entry seen in the $CD4^+CD25^-$ cells was approximately 50-fold less than that seen in $CD4^+CD25^+$ cells, which is most likely explained by the fact that cell surface components other than the viral receptor and co-receptor like heparan sulphate proteoglycans (HPG) serve as facilitators in non-specific cellular attachment of HIV and FIV (Ohshiro et al., 1996; Patel et al., 1993; Parseval et al., 2001). Our findings of CXCR4 expression on feline cells corroborate the findings of others reporting that essentially all $CD4^+$ cells express CXCR4 (Eckstein et al., 2001; Pierson et al., 2000). Increased expression of CXCR4 on $CD4^+CD25^+$ Treg cells as opposed to resting $CD4^+CD25^-$ T cells may be due to the fact that the Treg cells are partially activated (Powrie., 2001; Vahkenkamp et al., 2004). It is well established, including studies herein that CXCR4 surface expression is increased following cell activation (Bluel et al., 1997). The importance of CXCR4 expression levels on a per cell basis in HIV infection has also been emphasized by others. Harouse et al

(1999) found that although comparable percentages of $CD3^+CD4^+$ $CXCR4^+$ cells are present in the gut associated lymphoid tissue (GALT) and periphery, the level of CXCR4 expression is significantly lower in $CD3^+$ lamina propria lymphocytes than the LN or peripheral blood. This could explain why X4 tropic viruses cause a more profound loss of circulating $CD4^+$ T cells than intestinal $CD4^+$ cells.

Shimojima et al (2004) recently showed that FIV utilizes CD134 (OX-40), a $CD4^+$ T cell activation marker belonging to the TNF receptor superfamily as the primary receptor for entry into cells. In the present study, we found that significantly higher percentage of $CD4^+CD25^+$ Treg cells expressed surface CD134 than did $CD4^+CD25^-$ cells, suggesting a relationship between increased susceptibility of Treg cells to FIV infection and CD134 expression. However, ConA stimulation did not upregulate surface CD134 expression on $CD4^+CD25^-$ T cells, although it markedly increased their susceptibility to FIV-$NCSU_1$ infection in vitro. Barten et al (2001) also found only a moderate increase in surface CD134 expression in rat whole blood stimulated with high concentrations of ConA. However, stimulation of feline $CD4^+CD25^-$ cells with ConA resulted in an increase in CXCR4 expression, which correlates significantly with viral entry. Moreover, the time-line of increased susceptibility of ConA-stimulated $CD4^+CD25^-$ cells showed a linear correlation with upregulation of CXCR4 on their surface. Thus, while the $NCSU_1$ isolate of FIV utilizes the CD134 receptor (B. J. Willett, personal communication), our findings suggest that CXCR4 expression levels and not CD134 may be the limiting factor for FIV entry into $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells.

To further explore potential differences in $CD4^+CD25^+$ and $CD4^+CD25^-$ cells that could account for a productive infection in Treg cells but a latent replication competent

infection in the naïve T helper cells, we analyzed expression of a number of cellular transcription factors known to regulate the FIV promoter. EMSA of several cellular transcription factors revealed a striking difference in their activation status in $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells. Consistent with other reports (Ikeda et al., 1996; Kornfeld et al., 1998; Tang et al., 1999; Wu and Marsh., 2001), our studies show that resting $CD4^+CD25^-$ cells have a low basal activity of AP-1, ATF, CEBP and NFκB that is upregulated strongly following ConA but not IL-2 stimulation. Activation of these transcription factors in $CD4^+CD25^-$ T cells by ConA stimulation but not IL-2 is consistent with the observation that ConA but not IL-2 converts a latent infection to a productive infection in these cells (Joshi et al., 2004). In contrast to $CD4^+CD25^-$ cells, $CD4^+CD25^+$ Treg cells showed constitutive high basal activity of ATF and CEBP, and a strong AP-1 transactivation in response to IL-2. As it is well established that these transcription factors are required for FIV replication, these data support the argument that the ability of $CD4^+CD25^+$ Treg cells to support FIV replication lies in the high basal and IL-2 responsive activity of these cellular transcription factors.

It is well established that NFκB is an important transcription factor in regulating HIV replication (Cullen., 1989; Tang et al., 1999). However, we found that the putative NFκB binding sequence in the $NCSU_1$ FIV LTR did not bind NFκB or compete with a consensus NFκB probe, suggesting that NFκB does not play a role in FIV replication in Treg cells. In support of this, a number of studies have reported that mutations in the putative NFκB binding site in FIV LTR does not affect FIV replication and probably does not play an important role in viral replication (Sparger et al., 1992; Thompson et al., 1994). Interestingly however, we did find using the NFκB consensus sequence that NFκB was activated in unstimulated and IL-2 treated $CD4^+CD25^+$ Treg cells but not $CD4^+CD25^-$ T cells.

Constitutive and IL-2 responsive activity of AP-1 and NFκB in $CD4^+CD25^+$ Treg cells as opposed to $CD4^+CD25^-$ cells is also of interest from the point of view that these transcription factors regulate IL-2 gene expression, and survival of anergic $CD4^+CD25^+$ Treg cells is dependent upon IL-2 (Papiernik et al., 1998; Walker., 2004). Thus it is possible that constitutive and/or IL-2 induced activation of these transcription factors not only maintains a constant basal level activation of the IL-2 promoter necessary for Treg cell survival but also provides necessary signals for FIV replication.

Overall, our results implicate an important role for $CD4^+CD25^+$ cells as a reservoir for FIV replication both in vitro and in vivo. Although IL-2 is the limiting factor for viral replication in these cells in vitro, we believe that in the lymphoid milieu or during instances of immune activation the cytokine levels in vivo would be sufficient for these cells to support a productive FIV infection. Moreover, as IL-2 is necessary for the survival of $CD4^+CD25^+$ Treg cells, activation of these transcription factors may be a component of their cell survival program and ability to resist apoptosis (Banz et al., 2002; Joshi et al., 2004; Taams et al., 2001; Vahlenkamp et al., 2004). Thus, the partial activation status of $CD4^+CD25^+$ Treg cells as manifested in part by constitutive transactivation of transcription factors capable of promoting FIV replication may be critical to maintaining a long-term stable reservoir of productive FIV infection. The $CD4^+CD25^-$ cells, on the other hand, could serve as latent viral reservoir capable of competent reactivation when stimulated.

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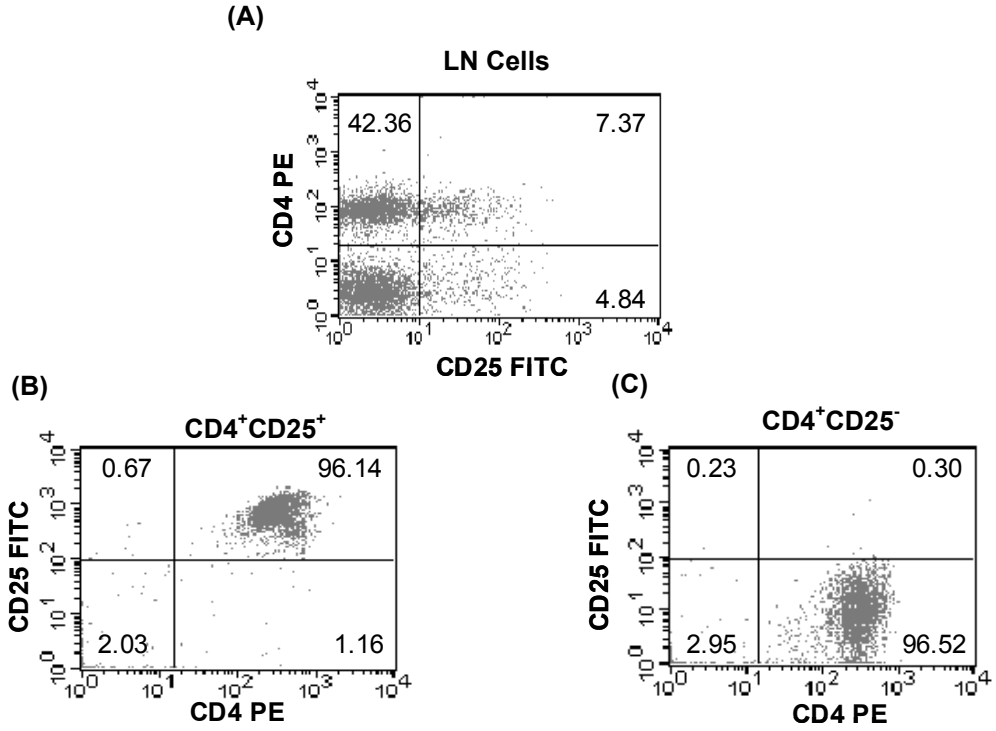
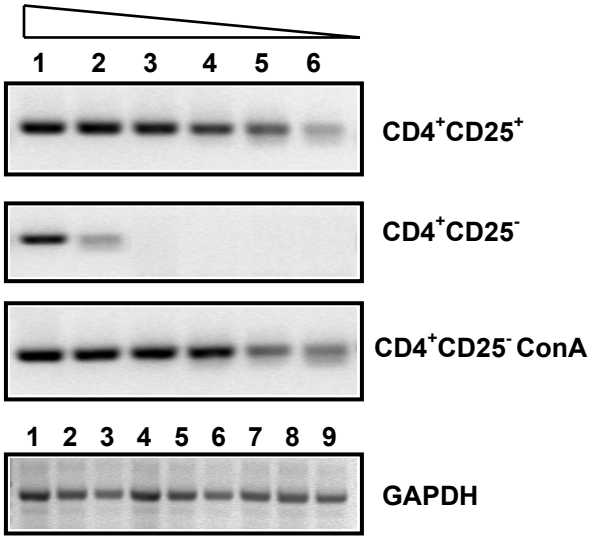
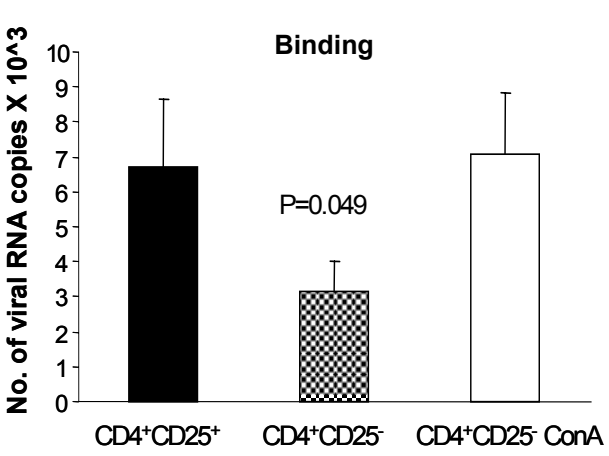


FIG. 1. Representative dot plots indicating the purity of $CD4^+CD25^+$ and $CD4^+CD25^-$ cells used in the study. LN derived cells were sorted into $CD4^+CD25^+$ and $CD4^+CD25^-$ populations using antibody coated magnetic beads as described. **(A)** Total LN cells **(B)** Purified $CD4^+CD25^+$ cells and **(C)** Purified $CD4^+CD25^-$ cells were stained with PE-conjugated anti-CD4 and FITC-conjugated anti-CD25 antibody and analyzed by flow cytometry. Numbers represent the percentage of cells within each quadrant.

(A)


1 2 3 4 5 6
CD4+CD25+
CD4+CD25-
CD4+CD25- ConA
1 2 3 4 5 6 7 8 9
GAPDH

(B)


Binding
No. of viral RNA copies X 10^3
P=0.049
CD4+CD25+
CD4+CD25-
CD4+CD25- ConA

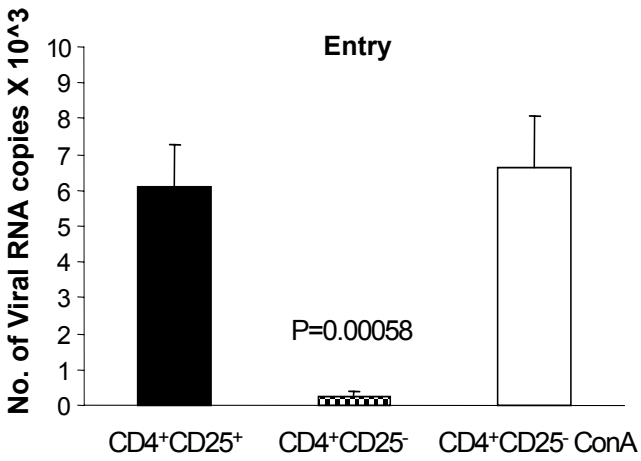
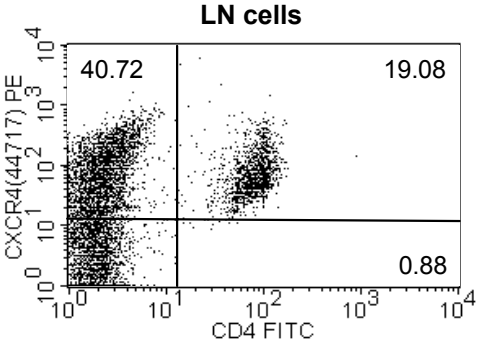
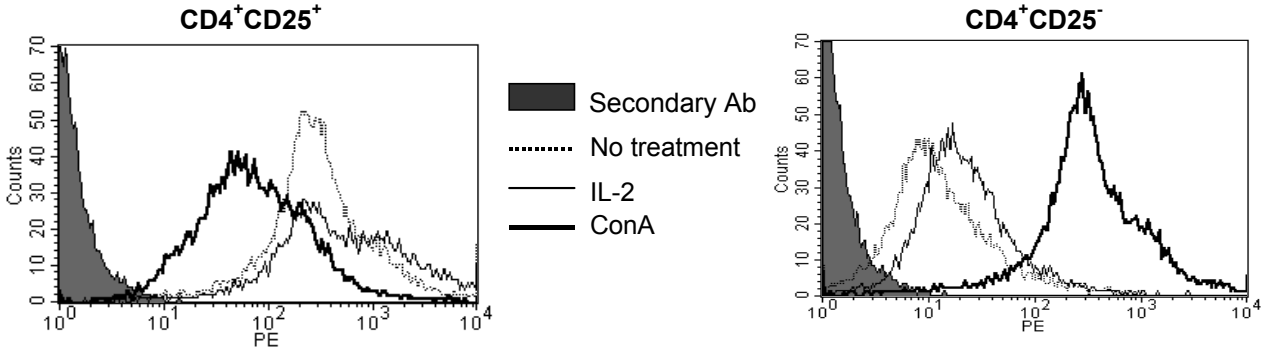

Entry
No. of Viral RNA copies X 10^3
P=0.00058
CD4+CD25+
CD4+CD25-
CD4+CD25- ConA

FIG. 2. Differential permissiveness to FIV infection in $CD4^+CD25^+$ versus $CD4^+CD25^-$ cells. **(A)** LN derived cells were sorted into highly purified $CD4^+CD25^+$ and $CD4^+CD25^-$ populations using FACS. 10^5 each of $CD4^+CD25^+$, $CD4^+CD25^-$ or $CD4^+CD25^-$ cells stimulated with ConA for 72hr were infected in-vitro with serial 2-fold dilutions of FIV-$NCSU_1$ starting with a m.o.i of 0.1 (lane 1). Cells were harvested 8hr post infection and DNA isolated by direct cell lysis for PCR of proviral DNA using FIV-LTR specific primers. Water control reaction did not yield any visible amplification product. For GAPDH controls 3 samples were picked up randomly from each $CD4^+CD25^+$ (lanes 1-3), $CD4^+CD25^-$ (lanes 4-6) and $CD4^+CD25^-$ cells stimulated with ConA (lanes 7-9). The experiment was repeated four times with similar results. **(B)** Estimation of viral copy numbers for binding and entry assays in $CD4^+CD25^+$ and $CD4^+CD25^-$ cells using Real Time RT-PCR. LN derived $CD4^+CD25^+$ and $CD4^+CD25^-$ cells of high purity (>97%) were obtained using a combination of immunomagnetic beads and flow cytometric sorting. 10^6 sorted cells were used for viral binding and viral entry experiments as described in the methods. 0.5μg of RNA from each cell type was used for Real time RT-PCR using FIV-$NCSU_1$ gag-specific primers. Viral copy numbers were determined using in-vitro transcribed gag RNA standard. To determine if $CD4^+CD25^-$ cells show an increase in viral receptiveness following mitogenic stimulation, cells were stimulated for 72hr with 2μg/ml ConA prior to binding and entry assays. The experiment was repeated thrice with similar results. Error bars represent mean ± Standard Deviation (S.D) of triplicate wells. The p-values represent significant increase in viral binding or entry in $CD4^+CD25^+$ or ConA stimulated $CD4^+CD25^-$ cells versus unstimulated $CD4^+CD25^-$ cells. RT-PCR using GAPDH specific primers was used to demonstrate equal RNA loading per reaction (data not shown). Water control reaction lacked any detectable amplification products (data not shown).

(A)



(B)



Cells	CXCR4 expression (MFI)		
	No treatment	**IL-2**	**ConA**
$CD4^+CD25^+$	401.58	425.65	219.48
$CD4^+CD25^-$	116.26	157.47	385.19

FIG. 3. Expression of CXCR4 in $CD4^+$, $CD4^+CD25^+$ and $CD4^+CD25^-$ cells. **(A)** $5x10^5$ LN derived cells were incubated with cross-reacting anti-CXCR4 antibody (44717) followed by PE-conjugated goat anti-mouse IgG. The cells were then washed, stained with FITC labeled feline anti-CD4 antibody (30A) and analyzed by flow cytometry. Numbers represent the percentage of cells within each quadrant. **(B)** LN derived cells were sorted into $CD4^+CD25^+$ and $CD4^+CD25^-$ populations. $3x10^5$ purified cells were stained for surface CXCR4 expression as described above either immediately post isolation or after stimulation for 3 days with 100U/ml IL-2 or 2μg/ml ConA and analyzed by flow cytometry. One representative of four independent experiments is shown. Thick solid line: ConA; Thin solid line: IL-2; Dotted line: No treatment; Shaded region: Secondary antibody alone.

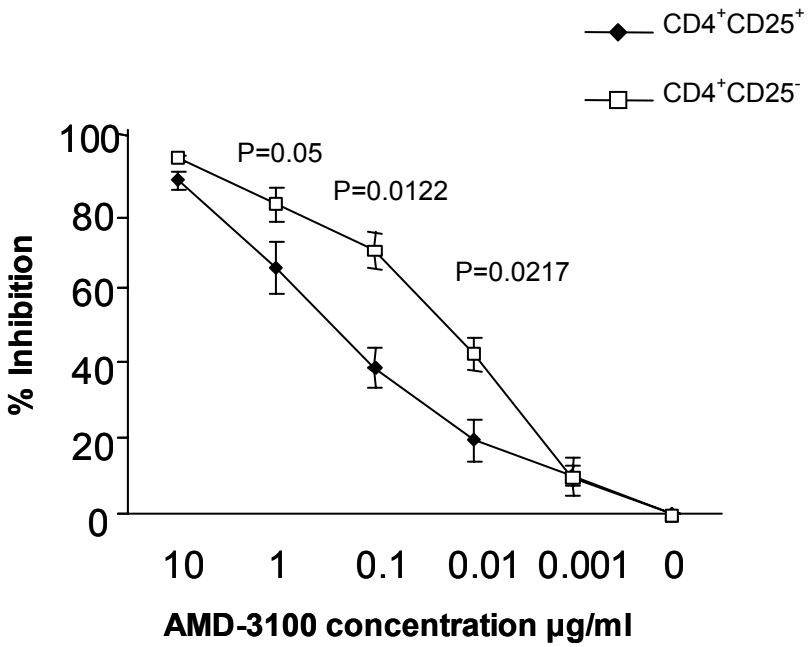
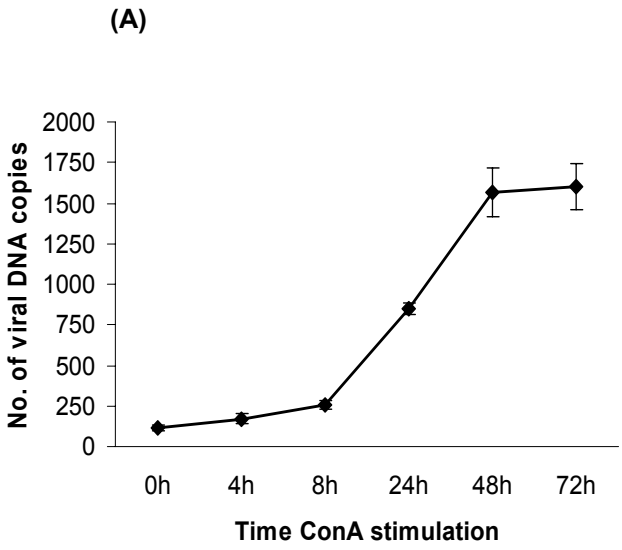
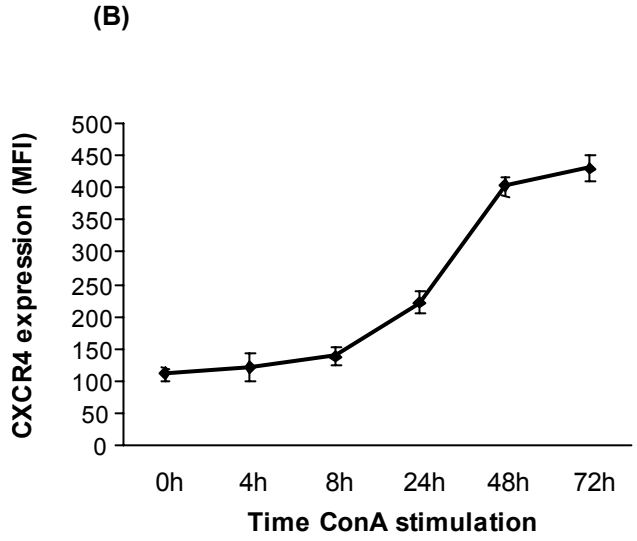


FIG. 4. Blocking FIV infection in $CD4^+CD25^+$ and $CD4^+CD25^-$ cells using the CXCR4 antagonist AMD-3100. LN derived $CD4^+CD25^+$ and $CD4^+CD25^-$ cells ($2x10^5$) were infected with FIV-$NCSU_1$ at an m.o.i of 0.1 either in the absence of AMD-3100 or in the presence of serial 10-fold dilutions of AMD-3100 starting with a maximum concentration of 10µg/ml. Following infection, $CD4^+CD25^+$ and $CD4^+CD25^-$ cells were cultured in the presence of 100U/ml IL-2 or 2µg/ml ConA respectively for 12 days. Culture media was replenished with AMD-3100 to minimize second round infections. Supernatants were harvested every 3 days for detection of p24 antigen by ELISA. Results from only 9 day harvests are shown. Data points represent mean of duplicate cultures. The experiment was repeated thrice with similar results. The p-values represent significantly higher levels of p24 antigen in culture supernatants of $CD4^+CD25^+$ versus $CD4^+CD25^-$ cells at corresponding concentrations of AMD-3100.


(A)
No. of viral DNA copies
2000
1750
1500
1250
1000
750
500
250
0
0h
4h
8h
24h
48h
72h
Time ConA stimulation


(B)
CXCR4 expression (MFI)
500
450
400
350
300
250
200
150
100
50
0
0h
4h
8h
24h
48h
72h
Time ConA stimulation

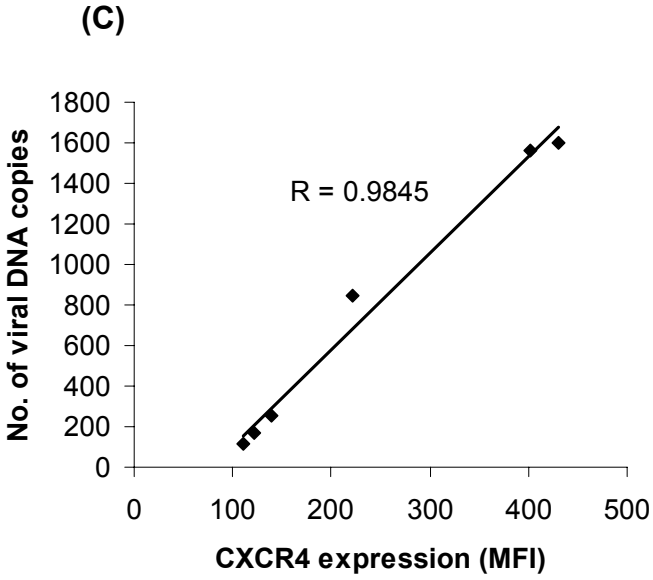

(C)
No. of viral DNA copies
1800
1600
1400
1200
1000
800
600
400
200
0
R = 0.9845
0
100
200
300
400
500
CXCR4 expression (MFI)

FIG. 5. Co-relation between increase in viral entry in $CD4^+CD25^-$ cells following ConA stimulation with increase in MFI of CXCR4 expression. Magnetic bead purified $CD4^+CD25^-$ cells were stimulated with 2µg/ml ConA for 4, 8, 24, 48, 72hr or left unstimulated. **(A)** 10^6 stimulated and unstimulated cells were then infected with FIV, trypsinised to get rid of cell surface attached virions and cultured for 8hr. Subsequently, DNA was isolated from infected cells and 0.5µg used for real time PCR using $NCSU_1$ gag specific primers. Error bars represent mean ± S.D of triplicates. The experiment was repeated twice. One representative result is depicted. **(B)** $3x10^5$ stimulated or unstimulated $CD4^+CD25^-$ cells were stained for surface CXCR4 expression as described in Fig. 3A and analyzed by flow cytometry. Data represent mean of duplicates. Real time PCR data and CXCR4 staining from the same experiment are shown. **(C)** Linear correlation between MFI of CXCR4 expression and viral copy numbers in $CD4^+CD25^-$ cells following ConA stimulation. Increase in viral entry in $CD4^+CD25^-$ cells following ConA stimulation is plotted against CXCR4 expression levels. Line represents linear correlation and the Pearson correlation coefficient (R).

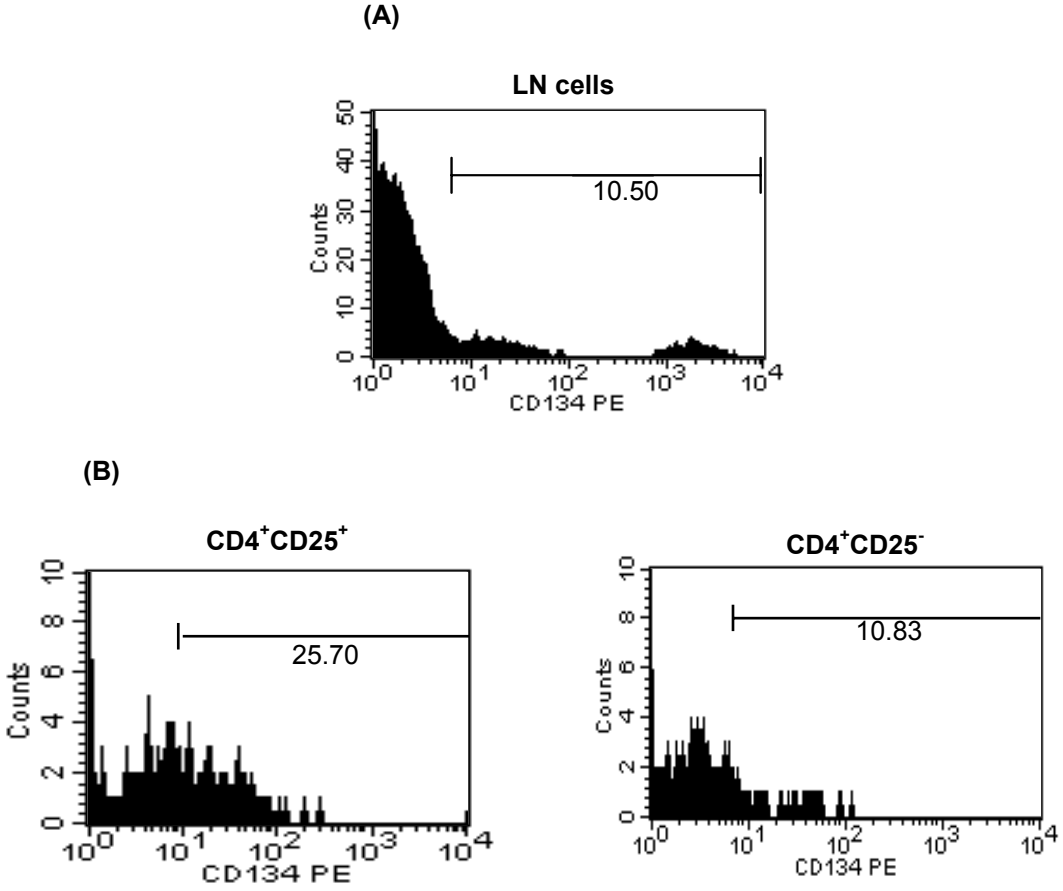


FIG. 6. Expression of FIV receptor CD134 (OX40) on total LN and $CD4^+CD25^+$ versus $CD4^+CD25^-$ cells. **(A)** $5x10^5$ LN cells were stained with PE-conjugated anti-CD134 antibody and analyzed by flow cytometry. **(B)** LN derived cells were sorted into $CD4^+CD25^+$ and $CD4^+CD25^-$ populations using FACS. Surface CD134 expression in the above cell types was determined immediately after sorting by staining with PE-conjugated anti CD134 antibody (Ber ACT35) followed by flow cytometry. Numbers represent the percentage of $CD4^+CD25^+$ or $CD4^+CD25^-$ cells expressing CD134. One representative of 4 independent experiments is depicted.

(C)

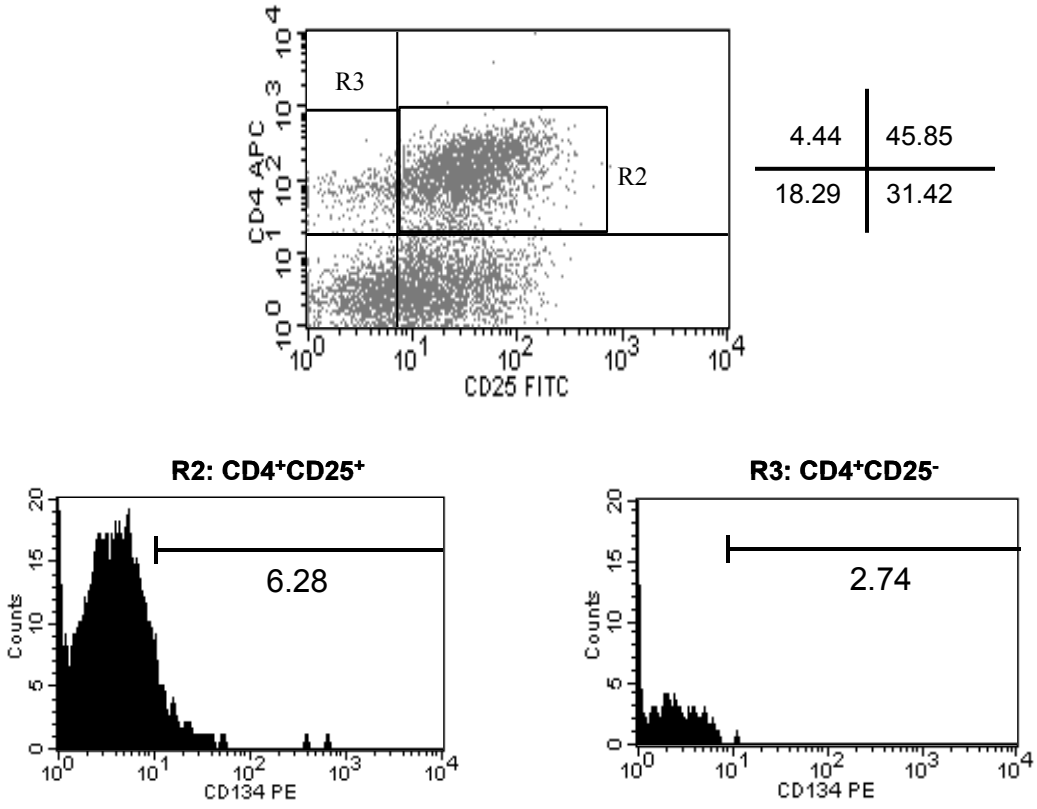


FIG. 6.C. Total unsorted LN cells were stimulated with 2µg/ml ConA for 72hr. $5x10^5$ cells were then stained with APC-conjugated anti-CD4, FITC-conjugated anti-CD25 and PE-conjugated anti-CD134 antibodies and analyzed by flow cytometry. (Top) CD25 expression on $CD4^+$ cells stimulated with ConA for 72hr. (Bottom) Gated R2: $CD4^+CD25^+$ cells and R3: $CD4^+CD25^-$ cell populations were analyzed for surface CD134 expression. Numbers represent the percentage of ConA stimulated $CD4^+CD25^+$ or $CD4^+CD25^-$ cells co-expressing CD134.

(A)

FIG. 7(A). Sequence of FIV-NCSU$_1$ LTR (sense strand only) from nucleotide positions 40 to 200. The predicted binding sites for the transcription factors NFκB, AP-1, CEBP and ATF (represented alongside) are underlined. The oligonucleotides used for the detection of NFκB, CEBP, AP-1 and ATF are indicated by bold letters.

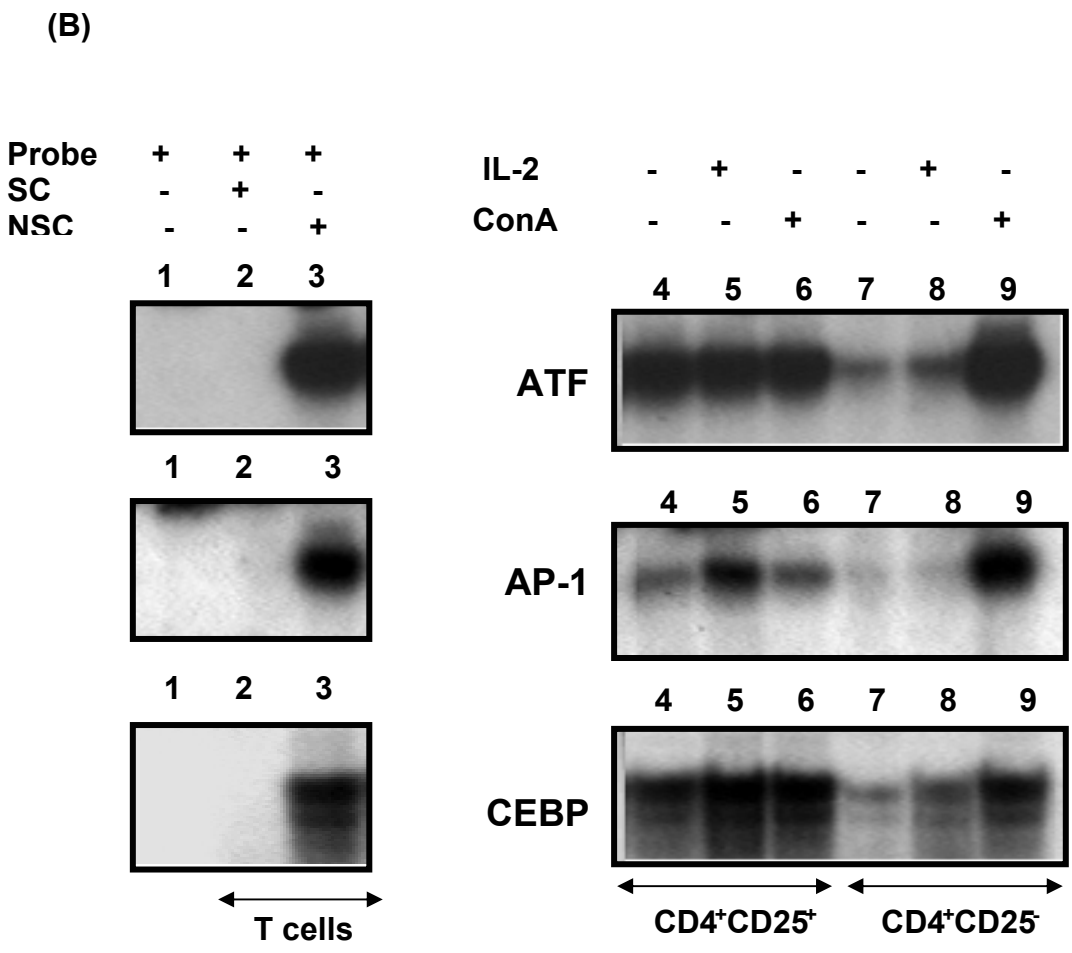
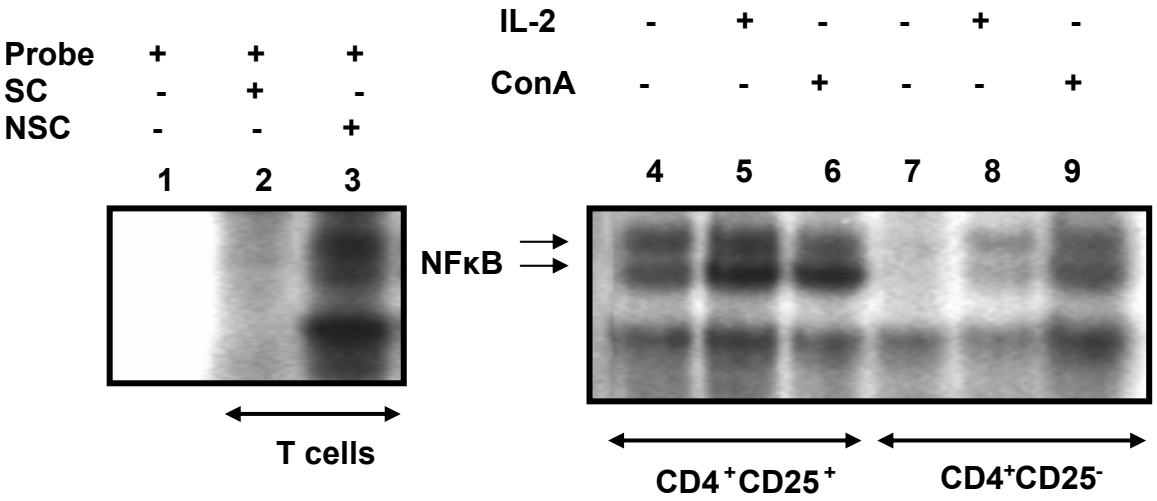


FIG. 7(B). EMSA of nuclear extracts derived from $CD4^+CD25^+$ and $CD4^+CD25^-$ cells. LN derived cells were sorted into $CD4^+CD25^+$ and $CD4^+CD25^-$ populations using immunomagnetic beads. Nuclear extracts were prepared from 10^6 cells either immediately following isolation or after stimulation for 5 days with 100U/ml IL-2 or 2μg/ml ConA as indicated (lanes 4-9). Equal amounts of extracts from different cell types or treatments were incubated with 5'γP^{32}ATP labeled ATF, AP-1 or CEBP oligonucleotides for 15min at room temperature. 50-fold molar excess of specific competitor (SC, lane 2) or non-specific competitor (NSC, lane 3) was used to demonstrate specific protein binding of each probe. Following binding, the complexes were resolved on a non-denaturing polyacrylamide gel, dried and autoradiographed. The experiments were repeated thrice with similar results.

(A)


IL-2 - + - - + -
Probe + + +
ConA - - + - - +
SC - + -
NSC - - +
1 2 3
4 5 6 7 8 9
NFκB
T cells
CD4+CD25+
CD4+CD25-

(B)

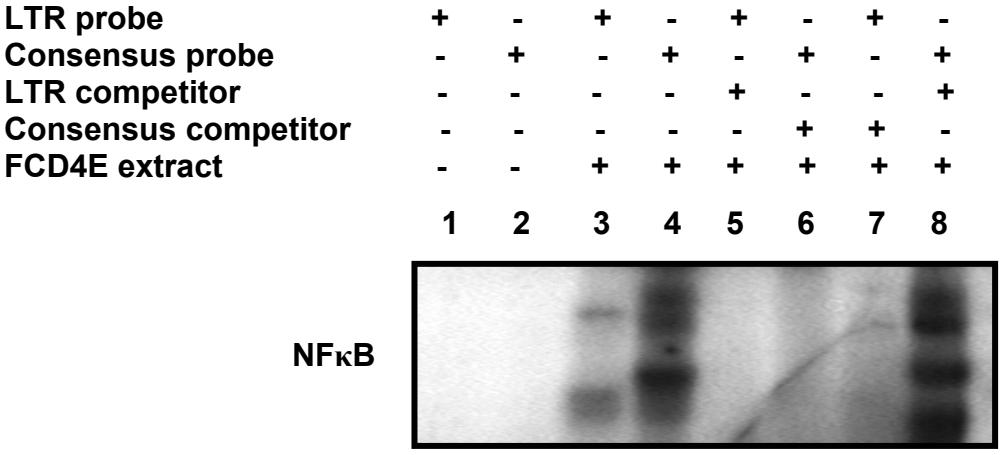

LTR probe + - + - + - + -
Consensus probe - + - + - + - +
LTR competitor - - - - + - - +
Consensus competitor - - - - - + + -
FCD4E extract - - + + + + + +
1 2 3 4 5 6 7 8
NFκB

FIG. 8. NFκB binding to FIV LTR is not required for a productive FIV infection. **(A)** EMSA of nuclear extracts from $CD4^+CD25^+$ and $CD4^+CD25^-$ cells using consensus NFκB oligonucleotide. Nuclear extracts were prepared from $CD4^+CD25^+$ and $CD4^+CD25^-$ cells after different treatments (lanes 4-9) as described in Fig. 7B. Nuclear extracts were then incubated with consensus NFκB oligo for 15min at room temperature in the presence or absence of specific competitor (SC, lane 2) or non-specific competitor (NSC, lane 3). Complexes were resolved on a polyacrylamide gel, dried and autoradiographed. One representative of three independent experiments is depicted. **(B)** Cross competition assays using radiolabeled NFκB oligonucleotide derived from $NCSU_1$ LTR sequence or consensus NFκB sequence. Nuclear extracts were prepared form $1.5x10^6$ FCD4E cells, an IL-2 dependent $CD4^+$ cell line highly permissive for FIV-$NCSU_1$ infection. 5μg of extracts were incubated either with end labeled $NCSU_1$ LTR derived (lane 3) or consensus NFκB (lane 4) oligonucleotides. Complexes were subsequently resolved on a 6% polyacrylamide gel. For competition (lanes 5-6) and cross-competition (lanes 7-8) assays, nuclear extracts were incubated with 50-fold molar excess of indicated unlabelled probes prior to incubation with the radiolabeled probe. One representative of four independent experiments is depicted.

REFERENCES

1. **Aringer, M.** 2002. T lymphocyte activation--an inside overview. Acta Med. Austriaca **29:**7-13.

2. **Banz, A., C. Pontoux, and M. Papiernik.** 2002. Modulation of Fas-dependent apoptosis: a dynamic process controlling both the persistence and death of CD4 regulatory T cells and effector T cells. J. Immunol. **169:**750-757.

3. **Barten, M. J., J. F. Gummert, T. van Gelder, R. Shorthouse, and R. E. Morris.** 2001. Flow cytometric quantitation of calcium-dependent and –independent mitogen-stimulation of T cell functions in whole blood: inhibition by immunosuppressive drugs in vitro. J. Immunol. Methods **253:**95-112.

4. **Bendinelli, M., M. Pistello, S. Lombardi, A. Poli, C. Garzelli, D. Matteucci, L. Ceccherini-Nelli, G. Malvaldi, and F. Tozzini.** 1995. Feline immunodeficiency virus: an interesting model for AIDS studies and an important cat pathogen. Clin. Microbiol. Rev. **8:**87-112.

5. **Bigornia, L., K. M. Lockridge, and E. E. Sparger.** 2001. Construction and in vitro characterization of attenuated feline immunodeficiency virus long terminal repeat mutant viruses. J.Virol. **75:**1054-1060.

6. **Bleul, C. C., L. Wu, J. A. Hoxie, T. A. Springer, and C. R. Mackay.** 1997. The HIV coreceptors CXCR4 and CCR5 are differentially expressed and regulated on human T lymphocytes. Proc. Natl. Acad. Sci. U S A. **94:**1925-1930.

7. **Borvak, J., C. S. Chou, K. Bell, G. Van Dyke, H. Zola, O. Ramilo, and E. S. Vitetta.** 1995. Expression of CD25 defines peripheral blood mononuclear cells with productive versus latent HIV infection. J. Immunol. **155:**3196-3204.

8. **Burkhard, M. J., L. Valenski, S. Leavell, G. A. Dean and W. A. Tompkins.** 2002. Evaluation of FIV protein-expressing VEE-replicon vaccine vectors in cats. Vaccine **21:**258-268.

9. **Chou, C. S., O. Ramilo, and E. S. Vitetta.** 1997. Highly purified CD25- resting T cells cannot be infected de novo with HIV-1. Proc. Natl. Acad. Sci. U S A. **94:**1361-1365.

10. **Chun, T. W., and A. S. Fauci.** 1999. Latent reservoirs of HIV: obstacles to the eradication of virus. Proc. Natl. Acad. Sci. U S A. **96:**10958-10961.

11. **Cullen, B. R., and W. C. Greene.** 1989. Regulatory pathways governing HIV-1 replication. Cell **58:**423-426.

12. **Dean, G. A., G. H. Reubel, P. F. Moore, and N. C. Pedersen.** 1996. Proviral burden and infection kinetics of feline immunodeficiency virus in lymphocyte subsets of blood and lymph node. J. Virol. **70:**5165-5169.

13. **De Parseval, A., and J. H. Elder.** 2001. Binding of recombinant feline immunodeficiency virus surface glycoprotein to feline cells: role of CXCR4, cell-surface heparans, and an unidentified non-CXCR4 receptor. J. Virol. **75:**4528-4539.

14. **Dignam, J. D., R. M. Lebovitz, and R. G. Roeder. 1983.** Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11:**1475-1489.

15. **Eckstein, D. A., M. L. Penn, Y. D. Korin, D. D. Scripture-Adams, J. A. Zack, J. F. Kreisberg, M. Roederer, M. P. Sherman, P. S. Chin, and M. A. Goldsmith.** 2001. HIV-1 actively replicates in naive CD4(+) T cells residing within human lymphoid tissues. Immunity **15:**671-682.

16. **English, R. V., C. M. Johnson, D. H. Gebhard, and M. B. Tompkins.** 1993. In vivo lymphocyte tropism of feline immunodeficiency virus. J. Virol. **67:**5175-5186.

17. **Garg, H., F. J. Fuller, and W. A. Tompkins.** 2004. Mechanism of feline immunodeficiency virus envelope glycoprotein-mediated fusion. Virology **321:**274-286.

18. **Gowda, S. D., B. S. Stein., N. Mohagheghpour, C. J. Benike, and E. G. Engleman.** 1989. Evidence that T cell activation is required for HIV-1 entry in CD4+ lymphocytes. J. Immunol. **142:**773-780.

19. **Harouse, J. M., A. Gettie., R. C. Tan, J. Blanchard, and C. Cheng-Mayer.** 1999. Distinct pathogenic sequela in rhesus macaques infected with CCR5 or CXCR4 utilizing SHIVs. Science **284:**816-819.

20. **Ikeda, Y., Y. Inoshima, Y. Kawaguchi, K. Maeda, M. Kohmoto, C. Kai, T. Miyazawa, and T. Mikami.** 1998. Protein-binding properties of the putative AP-1 and ATF sequences in the feline immunodeficiency virus long terminal repeat. J. Gen. Virol. **79:**95-99.

21. **Ikeda, Y., Y. Kawaguchi, K. Tomonaga, Y. Inoshima, M. Kohmoto, T. Miyazawa, and T. Mikami.** 1996. Regulatory properties of the integrated long terminal repeat of the feline immunodeficiency virus. Virus Res. **41:**201-207.

22. **Inoshima, Y., M. Kohmoto, Y. Ikeda, H. Yamada, Y. Kawaguchi, K. Tomonaga, T. Miyazawa, C. Kai, T. Umemura, and T. Mikami.** 1996. Roles of the auxiliary genes and AP-1 binding site in the long terminal repeat of feline immunodeficiency virus in the early stage of infection in cats. J. Virol. **70:**8518-8526.

23. **Joshi, A., T. W. Vahlenkamp, H. Garg, W. A. Tompkins, and M. B. Tompkins.** 2004. Preferential replication of FIV in activated CD4(+)CD25(+)T cells independent of cellular proliferation. Virology **321:**307-322.

24. **Kawaguchi, Y., K. Tomonaga, K. Maeda, M. Ono, T. Miyazawa, M. Kohmoto, Y. Tohya, and T. Mikami.** 1995. The C/EBP site in the feline immunodeficiency virus (FIV) long terminal repeat (LTR) is necessary for its efficient replication and is also involved in the inhibition of FIV LTR-directed gene expression by pseudorabies virus ICP4. Virology **208:**492-499.

25. **Kornfeld, H., W. W. Cruikshank, S. W. Pyle, J. S. Berman, and D. M. Center.** 1988. Lymphocyte activation by HIV-1 envelope glycoprotein. Nature **335:**445-448.

26. **Ma, J., S. Kennedy-Stoskopf, J. M. Jaynes, L. M. Thurmond, and W. A. Tompkins.** 2002. Inhibitory activity of synthetic peptide antibiotics on feline immunodeficiency virus infectivity in vitro. J. Virol. **76:**9952-9961.

27. **Maddon, P. J., A. G. Dalgleish, J. S. McDougal, P. R. Clapham, R. A. Weiss, and R. Axel.** 1986. The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. Cell **47:**333-348.

28. **Maloy, K. J., and F. Powrie.** 2001. Regulatory T cells in the control of immune pathology. Nat. Immunol. **2:**816-822.

29. **Mizuno, T., Y. Goto, K. Baba, K. Masuda, K. Ohno and H. Tsujimoto.** 2001. TNF-alpha-induced cell death in feline immunodeficiency virus-infected cells is mediated by the caspase cascade. Virology **287:**446-455.

30. **Ohno, K., R. Goitsuka, K. Kitamura, A. Hasegawa, T. Tokunaga, and M. Honda.** 1992. Production of a monoclonal antibody that defines the alpha-subunit of the feline IL-2 receptor. Hybridoma **11:**595-605.

31. **Ohshiro, Y., T. Murakami, K. Matsuda, K. Nishioka, K. Yoshida, and N. Yamamoto.** 1996. Role of cell surface glycosaminoglycans of human T cells in human immunodeficiency virus type-1 (HIV-1) infection. Microbiol. Immunol. **40:**827-835.

32. **Ostrowski, M. A., T. W. Chun, S. J. Justement, I. Motola, M. A. Spinelli, J. Adelsberger, L. A. Ehler, S. B. Mizell, C. W. Hallahan, and A. S. Fauci.** 1999. Both memory and CD45RA+/CD62L+ naive CD4(+) T cells are infected in human immunodeficiency virus type 1-infected individuals. J. Virol. **73:**6430-6435.

33. **Papiernik, M., M. L. de Moraes, C. Pontoux, F. Vasseur, and C. Penit.** 1998. Regulatory CD4 T cells: expression of IL-2R alpha chain, resistance to clonal deletion and IL-2 dependency. Int. Immunol. **10:**371-378.

34. **Patel, M., M. Yanagishita, G. Roderiquez, D. C. Bou-Habib, T. Oravecz, V. C. Hascall, and M. A. Norcross.** 1993. Cell-surface heparan sulfate proteoglycan mediates HIV-1 infection of T-cell lines. AIDS Res. Hum. Retroviruses **9:**167-174.

35. **Pereira, L. A., K. Bentley, A. Peeters, M. J. Churchill, and N. J. Deacon.** 2000. A compilation of cellular transcription factor interactions with the HIV-1 LTR promoter. Nucleic Acids Res. **28:**663-668.

36. **Persaud, D., Y. Zhou, J. M. Siliciano, and R. F. Siliciano.** 2003. Latency in human immunodeficiency virus type 1 infection: no easy answers. J. Virol. **77:**1659-1665.

37. **Pierson, T., T. L. Hoffman, J. Blankson, D. Finzi, K. Chadwick, J. B. Margolick, C. Buck, J. D. Siliciano, R. W. Doms, and R. F. Siliciano.** 2000. Characterization of chemokine receptor utilization of viruses in the latent reservoir for human immunodeficiency virus type 1. J. Virol. **74:**7824-7833.

38. **Ramilo, O., K. D. Bell, J. W. Uhr, and E. S. Vitetta.** 1993. Role of CD25+ and CD25-T cells in acute HIV infection in vitro. J. Immunol. **150:**5202-5208.

39. **Shimojima, M., T. Miyazawa, Y. Ikeda, E. L. McMonagle, H. Haining, H. Akashi, Y. Takeuchi, M. J. Hosie, and B. J. Willett.** 2004. Use of CD134 as a primary receptor by the feline immunodeficiency virus. Science **303:**1192-1195.

40. **Sparger, E. E., B. L. Shacklett, L. Renshaw-Gegg, P. A. Barry, N. C. Pedersen, J. H. Elder and P. A. Luciw.** 1992. Regulation of gene expression directed by the long terminal repeat of the feline immunodeficiency virus. Virology **187:**165-177.

41. **Spina, C. A., J. C. Guatelli, and D. D. Richman.** 1995. Establishment of a stable, inducible form of human immunodeficiency virus type 1 DNA in quiescent CD4 lymphocytes in vitro. J. Virol. **69:**2977-2988.

42. **Stevenson, M., T. L. Stanwick, M. P. Dempsey, and C. A. Lamonica.** 1990. HIV-1 replication is controlled at the level of T cell activation and proviral integration. EMBO. J. **9:**1551-1560.

43. **Taams, L. S., J. Smith., M. H. Rustin., M. Salmon., L. W. Poulter., and A. N. Akbar.** 2001. Human anergic/suppressive CD4(+)CD25(+) T cells: a highly differentiated and apoptosis-prone population. Eur. J. Immunol. **31:**1122-1131.

44. **Tang, H., K. L. Kuhen, and F. Wong-Staal.** 1999. Lentivirus replication and regulation. Annu Rev Genet. **33:**133-170.

45. **Tang, S., B. Patterson, and J. A. Levy.** 1995. Highly purified quiescent human peripheral blood CD4+ T cells are infectible by human immunodeficiency virus but do not release virus after activation. J Virol. **69:**5659-5665.

46. **Thompson, F. J., J. Elder and J. C. Neil**. 1994. Cis- and trans-regulation of feline immunodeficiency virus: identification of functional binding sites in the long terminal repeat. J. Gen. Virol. **75:**545-554.

47. **Tompkins, M. B., M. E. Bull, J. L. Dow, J. M. Ball, E. W. Collisson, B. J. Winslow, A. P. Phadke, T. W.Vahlenkamp, and W. A. Tompkins.** 2002. Feline immunodeficiency virus infection is characterized by B7+CTLA4+ T cell apoptosis. J. Infect. Dis. **185:**1077-1093.

48. **Tompkins, M. B., D. H. Gebhard, H. R. Bingham, M. J. Hamilton, W. C. Davis, and W. A. Tompkins.** 1990. Characterization of monoclonal antibodies to feline T lymphocytes and their use in the analysis of lymphocyte tissue distribution in the cat. Vet. Immunol. Immunopathol. **4:**305-317.

49. **Tompkins, M. B., G. K. Ogilvie, R. A. Franklin, K. W. Kelley, and W. A. Tompkins.** 1987. Induction of IL-2 and lymphokine activated killer cells in the cat. Vet. Immunol. Immunopathol. **16:**1-10.

50. **Vahlenkamp, T. W., M. B. Tompkins, and W. A. Tompkins.** 2004. Feline immunodeficiency virus infection phenotypically and functionally activates immunosuppressive CD4+CD25+ T regulatory cells. J. Immunol. **172:**4752-4761.

51. **Walker, L. S.** 2004. CD4+ CD25+ Treg: divide and rule? Immunology **111:**129-137.

52. **Willett, B. J., L. Picard, M. J. Hosie, J. D. Turner, K. Adema, and P. R. Clapham.** 1997. Shared usage of the chemokine receptor CXCR4 by the feline and human immunodeficiency viruses. J. Virol. **71:**6407-6415.

53. **Wu, Y., and J. W. Marsh.** 2001. Selective transcription and modulation of resting T cell activity by preintegrated HIV DNA. Science **293:**1503-1506.

5. SUMMARY

The preceding experiments have demonstrated the following regarding FIV infection of $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells:

1. **Preferential replication of FIV occurs in activated $CD4^+CD25^+$ T cells independent of cellular proliferation.**

    a) The LTR, LTR-Gag, and Env-Gag primer pairs can be successfully used to detect early, intermediate and late stages of reverse transcription respectively in *in-vitro* FIV-$NCSU_1$ infected cells.

    b) The extrachromosomal circle junction products found in the nucleus of infected cells can be amplified using the Env-Gag primer pair. These circle forms are products of reverse transcription within the cell and are hallmarks of cells productively infected with HIV/FIV, generally not found in latently infected cells.

    c) The appearance of circle junction products in FIV infected cells as detected by PCR correlates with the production of Gag-p24 antigen in the culture supernatants as detected by an antigen capture ELISA.

    d) $CD4^+CD25^+$ cells comprise 5-10% of the $CD4^+$ T cell population in the peripheral blood and upto 20% of the $CD4^+$ cells in the feline lymph nodes of normal cats.

    e) Both $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells are capable of being infected with FIV-$NCSU_1$ in vitro.

    f) Only $CD4^+CD25^+$ T cells are capable of harboring a productive FIV infection in the absence of a strong mitogen like ConA but in the presence of IL-2.

g) $CD4^+CD25^-$ cells, when stimulated with ConA immediately post-infection also replicate FIV but less efficiently than $CD4^+CD25^+$ cells.

h) $CD4^+CD25^-$ cells when infected with FIV-$NCSU_1$ in vitro and cultured in media supplemented with IL-2 alone harbor a latent replication competent provirus that can be reactivated to produce infectious virions several days post infection.

i) Productive FIV infection of $CD4^+CD25^-$ cells in the presence of ConA correlates with surface upregulation of CD25 expression.

j) Feline $CD4^+CD25^+$ T cells display the key characteristics of Treg cells described in the rodent and human systems. These cells remain anergic to mitogenic stimulation as detected by lack of proliferation and FIV infection does not alter these characteristics. On the other hand, infected as well as uninfected $CD4^+CD25^-$ T cells show high levels of cell proliferation when stimulated with ConA as determined by ^{3}HTdR uptake and CFSE dye dilution assays.

k) The lack of cell proliferation in cultures of $CD4^+CD25^+$ T cells in the presence of mitogens does not correlate with high levels of apoptosis in these cells. These cells either undergo similar or less apoptosis compared to their $CD4^+CD25^-$ counterparts under different culture conditions.

l) $CD4^+CD25^+$ cells and not $CD4^+CD25^-$ cells derived from FIV-$NCSU_1$ infected cats are productively infected. Although the LTR and LTR-Gag products were variably expressed in the $CD4^+CD25^+$ and $CD4^+CD25^-$ cells derived from FIV-infected cats, the detection of circle junction products was restricted only to $CD4^+CD25^+$ cells.

m) Replication competent virus could be rescued from $CD4^+CD25^+$ cells derived from FIV^+ cats when co-cultured with uninfected FCD4E cells in the presence of IL-2. On the other hand, the $CD4^+CD25^-$ cells required a strong mitogenic stimulus prior to co-culture with FCD4E cells in order to produce infectious virions suggesting that in vivo too they are latently infected.

2. **Preferential FIV infection of $CD4^+CD25^+$ Treg cells correlates both with surface upregulation of CXCR4 and CD134 and activation of cellular transcription factors.**

   a) $CD4^+CD25^+$ and $CD4^+CD25^-$ cells could be sorted to high purity when using the MoFlo fluorescence activated cell sorter. An equivalent level of purity could be achieved with antibody coated magnetic beads.
   b) $CD4^+CD25^+$ cells show a greater permissiveness to FIV binding and entry as detected by infection of highly purified $CD4^+CD25^+$ and $CD4^+CD25^-$ cells with serial virus dilutions followed by PCR of infected cells.
   c) Real time RT-PCR assays in order to determine the viral copy numbers for viral binding and entry assays in $CD4^+CD25^+$ versus $CD4^+CD25^-$ cells revealed that while $CD4^+CD25^+$ cells were twice more permissive to FIV binding than $CD4^+CD25^-$ cells, the difference was approximately fifty-fold in terms of viral entry.

d) Activation of $CD4^+CD25^-$ cells with ConA for three days prior to viral binding and entry assays made them as permissive as $CD4^+CD25^+$ cells both to FIV binding and entry.

e) Increased viral binding and entry in $CD4^+CD25^+$ cells as opposed to $CD4^+CD25^-$ cells correlates with higher levels of surface CXCR4 expression in these cells. Activation of $CD4^+CD25^-$ cells with ConA for 3 days resulted in an increase in the surface CXCR4 expression levels in these cells but down-regulated CXCR4 expression in $CD4^+CD25^+$ cells.

f) IL-2 activation has negligible effects on surface CXCR4 expression levels in both $CD4^+CD25^+$ as well as $CD4^+CD25^-$ cells.

g) Consistent with lower levels of surface CXCR4 expression in $CD4^+CD25^-$ cells, lower concentrations of the specific CXCR4 antagonist AMD-3100 are required to block FIV infection in $CD4^+CD25^-$ cells as opposed to $CD4^+CD25^+$ cells.

h) A time study of increase in viral entry in $CD4^+CD25^-$ cells following ConA stimulation in parallel with increase in surface CXCR4 expression revealed a perfect correlation between the two processes suggesting that increase in viral entry in $CD4^+CD25^-$ cells correlates with increase in CXCR4 expression.

i) A higher percentage of $CD4^+CD25^+$ cells express the FIV receptor CD134 as compared to $CD4^+CD25^-$ cells derived from normal cats. In unsorted lymph node cells the expression of CD134 ranged from 6-14%.

j) Stimulation of $CD4^+CD25^-$ cells with ConA did not result in upregulation of surface CD134 expression suggesting that increase in viral entry in

$CD4^+CD25^-$ cells following ConA activation does not correlate with CD134 upregulation.

k) FIV-$NCSU_1$ LTR contains binding sites for various transcription factors like ATF, AP-1, AP-4, CEBP, SP-1 which play an important role in transactivation of viral LTR.

l) $CD4^+CD25^+$ cells inherently express higher levels or show an IL-2 dependent transactivation of transcription factors described to play an important role in FIV replication namely ATF, AP-1 and CEBP.

m) $CD4^+CD25^-$ cells express low levels of ATF, AP-1, NFκB and CEBP, all of which can be strongly upregulated following ConA activation and to lower levels following IL-2 stimulaton.

n) Unlike in HIV infection, the NFκB binding site in FIV LTR does not play an important role in FIV replication. Cross-competition studies revealed that the putative NFκB binding site in FIV LTR does not bind NFκB.

o) $CD4^+CD25^+$ cells show higher levels of NFκB transactivation as detected by the consensus NFκB binding probe which is further upregulated in these cells following IL-2 and ConA treatment. $CD4^+CD25^-$ cells express lower levels of the same but show a strong ConA dependent transactivation of NFκB.

6. CONCLUSION

Studies conducted on HIV and FIV tropism have suggested that the virus mainly persists as a reservoir in latently infected cells. Memory cells with the $CD45RO^+$ phenotype act as suitable candidates for virus persistence due to their inherent ability to survive for prolonged periods of time. A potentially stable virus reservoir is said to establish when infected cells resist cytopathic effects of the virus and revert to a resting memory phenotype. Or alternatively, if activated cells are infected during reversion to a resting state when they can harbor a latent provirus but lack the necessary factors to support a productive virus infection. Although it is well known that both resting and activated T cells are infected in HIV-infected individuals, the controversy remains whether resting cells are infected de-novo or if residual virus in resting T cells is a result of viral entry in activated T cells which subsequently revert to a resting phenotype.

Studies pertaining to HIV infection of T cells in vitro have demonstrated variable results. While some have demonstrated that HIV is incapable of entering resting T cells, others have shown that they are not only readily infected but also capable of supporting viral RNA transcription. Keeping in mind the above mentioned facts we studied FIV replication in resting and activated $CD4^+$ T cells (differentiated by differential expression of CD25). CD25 comprises the alpha chain of the high affinity IL-2 receptor and is a well-characterized marker of activated T cells. CD25, to date, is also the best-characterized marker for $CD4^+CD25^+$ regulatory T cells that comprise an important immune regulatory population in humans, rodents and cats controlling the activation of autoreactive T cells thereby preventing autoimmunity.

In an attempt to differentiate productive FIV infection from a latent one, we used an inverse PCR based assay to specifically amplify circle junction products from productively infected cells. It is well established that retroviral replication in cells is characterized by the formation of double stranded DNA intermediates. Besides the linear proviral DNA forms that are precursors to proviral integration, the nuclei of HIV/FIV infected cells also harbor circular DNA intermediates. These occur as either 1-LTR or 2-LTR circle junctions found in the nucleus of productively infected cells, are surrogate markers of retroviral integration and generally absent in latently infected cells. In our studies, we were able to amplify circle junction products from productively infected cells via PCR, which correlated with the appearance of Gag-p24 antigen in culture supernatants as detected by an antigen capture ELISA. Besides, in the present study we also used LTR and LTR-Gag primer pairs to detect early and intermediate stages of reverse transcription in FIV-$NCSU_1$ infected cells.

In vitro infection studies with $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells revealed that while both the above cell types were capable of being infected with FIV in vitro, only $CD4^+CD25^+$ cells were able to replicate FIV productively in the absence of mitogenic stimulation like ConA. $CD4^+CD25^-$ cells on the other hand required a strong mitogen like ConA in order to harbor a productive FIV infection and this correlated with upregulation of CD25 on their surface. While $CD4^+CD25^+$ cells efficiently replicated FIV in the presence of both IL-2 and ConA, they themselves remained anergic to mitogenic stimulation as measured by lack of proliferation as opposed to the robust proliferation in $CD4^+CD25^-$ cells under similar culture conditions. The lack of proliferation in $CD4^+CD25^+$ cells in response to mitogens was not due to a large percentage of these cells undergoing apoptosis in culture. Both infected and uninfected $CD4^+CD25^+$ cells underwent either similar or less apoptosis

than their $CD4^+CD25^-$ counterparts under various culture conditions suggesting their ability to act as long-term reservoirs of a productive FIV infection. $CD4^+CD25^-$ cells on the other hand in the absence of mitogens did show the presence of early and intermediate sates of viral reverse transcription but lacked the circle junction products. Stimulation with ConA several days post infection was able to rescue a productive FIV infection from these cells. These results suggest that $CD4^+CD25^-$ cells hold the potential to act as long-term latent viral reservoirs capable of competent reactivation when stimulated. In support of this, studies conducted in FIV-$NCSU_1$ infected cats demonstrated that while the LTR and LTR-Gag products were variably detected in both $CD4^+CD25^+$ and $CD4^+CD25^-$ cells isolated from FIV^+ cats, the presence of Env-Gag amplified circle junction products was restricted to $CD4^+CD25^+$ cells and never detected in $CD4^+CD25^-$ cells. An attempt to rescue replication competent virus from $CD4^+CD25^+$ and $CD4^+CD25^-$ cells derived from infected cats also yielded similar results. While $CD4^+CD25^+$ cells could amplify virus in the presence of IL-2 alone, $CD4^+CD25^-$ cells again required a stronger stimulus in order to produce infectious virions when cocultured with uninfected FCD4E cells.

We further attempted to study the mechanisms governing the differential replication of FIV in $CD4^+CD25^+$ versus the $CD4^+CD25^-$ cells. We wished to determine whether the preferential replication of FIV in $CD4^+CD25^+$ cells in the absence of mitogenic stimulation was governed at the level of cell membrane or cellular transcriptional activity or both. Viral binding and entry assays were hence performed on highly purified $CD4^+CD25^+$ and $CD4^+CD25^-$ cells and viral copy numbers in infected cells determined by real time RT-PCR. Experiments demonstrated that $CD4^+CD25^-$ cells were less susceptible to both FIV binding and entry when compared to $CD4^+CD25^+$ cells and the difference was more significant at the

level of viral entry. However, stimulation of $CD4^+CD25^-$ cells with ConA prior to the binding and entry assays helped them regain their permissiveness to FIV infection comparable to that in $CD4^+CD25^+$ cells. Having seen a difference in the above cell types with respect to virus permissiveness we looked at the factors responsible for the differential permissiveness of $CD4^+CD25^+$ cells to FIV infection in vitro.

The expression levels of viral receptor and co-receptor play an important role in retroviral entry into cells. Hence we attempted to investigate the expression levels of FIV receptor (CD134) and co-receptor (CXCR4) in $CD4^+CD25^+$ cells and compared it to that in $CD4^+CD25^-$ cells. We found that $CD4^+CD25^-$ cells showed lower cell surface expression of CXCR4 as well as CD134. Stimulation of $CD4^+CD25^-$ cells with ConA resulted in surface upreulation of CXCR4 but not CD134. This suggests that increase in viral entry in $CD4^+CD25^-$ cells following mitogenic stimulation correlates with upregulation of surface CXCR4 but not CD134. In agreement with this, there was a linear correlation between increase in viral entry in $CD4^+CD25^-$ cells following ConA stimulation with increase in surface CXCR4 expression. Consistent with the above data lower concentrations of the CXCR4 antagonist AMD-3100 were required to block FIV infection in $CD4^+CD25^-$ cells as opposed to $CD4^+CD25^+$ cells.

It is well established that HIV and FIV LTRs encompass binding sites for various transcription factors, which play an important role in viral replication. In an attempt to identify differences in $CD4^+CD25^+$ and $CD4^+CD25^-$ cells with respect to their activation status, which could affect transcativation via viral LTR, gel shift assays were performed. Our studies revealed that while $CD4^+CD25^+$ cells showed constitutive or IL-2 dependent transactivation of the transcription factors ATF, AP-1 and CEBP, known to play an important

role in FIV replication, $CD4^+CD25^-$ cells lacked the same but upregulated them strongly following ConA activation. This correlates with FIV replication in $CD4^+CD25^+$ cells in the presence of IL-2 alone but in $CD4^+CD25^-$ cells only in the presence of ConA. Moreover, our shift data also suggested that unlike HIV infection, NFκB activation does not play an important role in FIV replication. Although studies using the consensus NFκB probe suggested that the factor was inherently upregulated in $CD4^+CD25^+$ Treg cells but not in $CD4^+CD25^-$ cells, cross competition assays using the LTR derived and consensus NFκB probe suggested that the putative NFκB binding site in FIV-$NCSU_1$ LTR does not bind NFκB.

To conclude, our results have important implications in the field of retroviral research pertaining to latent and productive reservoirs of HIV/FIV infection. Our studies have shown that while $CD4^+CD25^+$ cells comprise an important immune regulatory population both in humans and cats they are also highly susceptible to FIV infection both in vitro and in vivo and seem to be suitable candidates for long-term reservoirs of a productive FIV infection. Although these cells do depend on exogenous IL-2 in order to replicate FIV, we believe that the cytokine milieu in the lymph nodes will be sufficient to support a productive FIV infection by these cells. On the contrary, the $CD4^+CD25^-$ cells serve as latent viral reservoirs capable of competent reactivation when appropriately stimulated.