NOTE TO USERS

DISSERTATION

CXCR4 IN FELINE IMMUNODEFICIENCY VIRUS INFECTION AND AN EXPERIMENTAL THERAPY

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY SEAN P. TROTH ENTITLED CXCR4 IN FELINE IMMUNODEFICIENCY VIRUS INFECTION AND AN EXPERIMENTAL THERAPY BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

CXCR4 IN FELINE IMMUNODEFICIENCY VIRUS INFECTION AND AN EXPERIMENTAL THERAPY

Most strains of HIV-1 require CD4 and a chemokine receptor for attachment and entry into cells. The genetic adaptation of HIV-1 toward use of the CXCR4 chemokine receptor has been shown to play a critical role in the progression from latent infection to the development of AIDS. Whether CXCR4 receptor expression in different tissues in vivo correlates with increased susceptibility of those tissues to infection remains unclear, and is difficult to study in humans. Here we have employed the feline model for HIV to study the role of the CXCR4 chemokine receptor in lentiviral infection. In addition to mapping the distribution and infection rate of CXCR4 positive cells throughout lymphoid tissues, bone marrow and blood, we explored the effectiveness of chemokine receptor antagonists in blocking or ameliorating infection in vitro and in vivo.

The natural CXCR4 ligand SDF-1α as well as the CXCR4-specific antagonist AMD3100 (1-1*-[1,4-phenylenebis (methylene)]-bis(1,4,8,11-tetraazacyclotetradecane), were effective in inhibiting FIV infection of peripheral blood mononuclear cells (PBMC) in vitro. AMD3100 given to cats prior to intravenous virus challenge and continued

twice daily for 30 days resulted in significantly decreased proviral loads in blood for the first 12 weeks post inoculation (p.i.). Blood proviral loads rebounded to levels similar to the controls after the drug was discontinued and tissue proviral levels were equal to controls at 6 months post-inoculation. Administration of AMD3100 to cats both prior to and for 30 days following oronasal challenge was not effective in reducing blood or tissue proviral loads. We hypothesize that failure of AMD3100 to inhibit mucosal infection resulted from relatively low drug concentrations outside of the plasma compartment that resulted in inferior blockade of CXCR4 in tissues. Furthermore, the drug failed to reduce viral loads when administered for seven days during chronic infection (22 weeks p.i.). The lack of antiviral activity in chronic FIV infection may be a reflection of low viral replication or sequestration of virus in macrophages during the latent phase rendering drugs targeting viral entry less effective during this time. It is also possible that, similar to HIV-1 infections, there may be a virus phenotype change in later stages of infection allowing the use of a broader spectrum of receptors.

To study CXCR4 distribution and relationship to FIV infection, we sorted CXCR4 positive and negative fractions from lymph node, thymus, blood and bone marrow and measured proviral loads in lymphoid cell subsets by real time PCR. CXCR4 expression was significantly higher in lymph node than the other tissues examined both in proportion of cells staining and staining intensity per cell. Proviral burdens were significantly greater within the thymus than in lymph node, bone marrow or blood and higher concentrations of provirus were detected in T cells than B cells in blood and lymph node. CXCR4 was highly expressed in B and T cell subsets however proviral concentrations were similar between CXCR4 positive and negative fractions.

Tissues from these studies were additionally used to compare distribution of provirus by real time PCR over time and with route of infection. The major viral reservoir in acute infection was the thymus whereas bone marrow had the highest proviral burden in chronic infection. Route of inoculation (intravenous versus oronasal) had no effect on proviral distribution when measured at 6 months post inoculation.

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DEDICATION

To Johanna, Benjamin, Jillian and Allison

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INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1)

By the end of 2003 the number of people living with HIV/AIDS worldwide was estimated to be approximately 38 million including an estimated 4.7 million new cases occurring that year [1] . An estimated 13,700 new infections occur daily, most of which will remain asymptomatic for years before developing AIDS [2] . While therapies based upon inhibition of viral reverse transcriptase and protease have been successful in slowing the progression of AIDS, therapies targeting other phases of the viral life cycle have not been as successful. An attractive target of therapeutic intervention and an area of active research continues to be the viral attachment and entry phase of infection. A better understanding of these events particularly via prospective studies using an in vivo model would be valuable in the development of new therapies targeting viral attachment to cell surface receptors.

Viral tropism and the development of AIDS

HIV-1 requires binding of virion envelope glycoprotein gp120 to both CD4 [3-5] and to one of the seven–transmembrane, G-protein-coupled chemokine receptors [6-13] for cellular entry. Two major coreceptors, CCR5 (R5) and CXCR4 (X4), have been identified, each of which have an affinity for different HIV isolates. R5 is utilized

primarily by macrophage tropic (non syncytia-inducing) strains of HIV-1 and is the primary receptor used in the initial, asymptomatic phase of infection [14] . T-cell tropic (syncytia-inducing) isolates of HIV-1 primarily use the CXCR4 coreceptor but many can also use CCR5 [15-21] . Some strains of HIV (R5X4 HIV-1) are able to use both receptors with comparable efficiency. Infection by some isolates of HIV-1 and HIV-2 has been shown to be CD4-independent and mediated by CXCR4 alone [22-32] .

Initial infection by HIV occurs in macrophages, monocytes and CCR5-bearing T-cells [33-35]. The virus remains in these cells for several years and does not produce clinical symptoms of AIDS. Random mutations occur over time altering viral affinity for host chemokine receptors [36-38] . Eventually CXCR4 utilizing T-tropic strains are produced and it is this mutation to the T-tropic phenotype that corresponds with aggressive virus replication and rapid T-cell depletion leading to AIDS [18, 39-41] . While this adaptation of the virus to the use of CXCR4 is frequently cited as being key to the development of AIDS [37, 42] some strictly CCR5 using HIV subtypes have also been isolated from groups of individuals with AIDS [43] . Furthermore studies in HIV-infected children have provided evidence that the shift to the CXCR4 using phenotype can occur well after the onset of clinical disease [44] . Thus it remains unclear whether adaptation to the use of CXCR4 truly causes the immune dysfunction and cytopathic effects leading to AIDS or is simply a result of selective pressures following immune dysfunction after the onset of disease. Whether CXCR4 receptor expression in different tissues in vivo correlates with increased susceptibility of those tissues to infection remains unclear, and is difficult to study in humans.

FIV history and clinical course

FIV is a lentivirus that causes a disease syndrome similar to that caused by HIV with clinical phases analogous to those of HIV. Initial infection is characterized by rapid viral replication which corresponds to flu-like illness, lymphadenopathy, and an early progressive decline in CD4+ T-lymphocytes [45-48] which is followed by a prolonged asymptomatic latent phase where the virus is sequestered from the host immune system and relatively little virus replication occurs. The terminal phase of FIV infection is characterized by suppression of hemic elements (particularly CD4+ T lymphocytes) and recrudescence of plasma viremia resulting in immunological decompensation and increased susceptibility to opportunistic infection [49-52] . Other similarities between HIV and FIV infection include neurologic abnormalities, hyperplastic then depletive lymphoid tissue lesions and many hematologic disorders [49, 52-59] .

Similar to HIV, FIV is transmitted via mucosal exposure (oral, rectal and vaginal), blood transfer, and vertically via in utero, milk-borne, and intrapartum routes [60-63] . Infection via the oral mucosal route has been demonstrated in newborn kittens foster nursed by FIV infected queens [60, 61] and has been reproduced by a single oral virus exposure in older animals [63] .

FIV shows similar tissue tropism to HIV including a replication in T lymphocytes [52, 64-66] , macrophages [45, 47, 55, 67] , B cells [49, 68] , megakaryocytes [69] , monocytes [70] and cells of the nervous system including astrocytes and microglia [53, 71-75] . *In vitro* FIV studies indicate that both CD4+ and CD8+ T cells are susceptible to productive virus infection [64, 76] . In contrast to primate lentiviruses, FIV does not use CD4 [77-79] for entry however the receptor CD134 has recently been shown to be

required in conjunction with CXCR4 for entry [80] . The fact that CD134 is preferentially expressed on activated CD4+ T cells helps to explain the paradox of CD4+ tropism by FIV in the absence of CD4 binding. Questions still remain however surrounding the reason for B cell and CD8 tropism by FIV, since very little or no CD134 has been detected on these cell subsets in cats [81] . Details of the CD134/CXCR4 relationship in FIV have not been determined, such as whether binding to one of the receptors induces a conformational change resulting in increased affinity for the other receptor as in the CD4/CXCR4 binding of T-cell tropic HIV-1.

Shared usage of CXCR4 by both feline and human immunodeficiency viruses

FIV and HIV-1 are the only lentiviruses that cause selective loss of CD4+ T-cell subsets and AIDS in naturally infected host species. Abundant experimental data suggests that the mechanism of virus entry for HIV and FIV is highly conserved:

(1) Entry of both FIV and HIV into cells involves interaction of the V3 loop of the viral envelope gp120 glycoprotein with the CXCR4 or CCR5 receptor [82-86] . The C-type lectin DC-SIGN binds both HIV [87-89] and FIV [90] and has been shown to enhance transmission of HIV. Both FIV and HIV use cell-surface heparans [91-93] as binding receptors to enhance infection. Other coreceptors for HIV have been described [6, 10, 94-96] however their importance in HIV-1 infection has not been clearly established.

(2) Cell-culture-adapted strains of both FIV and HIV demonstrate change from non syncytium-inducing to syncytium-inducing phenotype which correlates with an increase

in net charge of the V3 loop of the envelope glycoprotein [97, 98] . Human cells which naturally express the CXCR4 receptor and nonpermissive human cells transfected with human CXCR4 receptor will undergo syncytium formation with FIV infected feline cells [99, 100] .

(3) A series of bicyclam analogues including AMD 3100 have been shown to specifically bind the CXCR4 receptor and competetively inhibit binding of both HIV-1 [101-109] and FIV [82, 110, 111] *in vitro*. AMD 3100 has also been shown to efficiently block cell-surface-expressed HIV-1 envelope-induced apoptosis in uninfected cells [112].

CXCR4 distribution in human tissues

X4 receptor expression has been demonstrated in several cell types including T cells [113-119] , B cells [114, 115, 120] , monocytes [114, 115, 121] , macrophages [113, 122] , CD 34+ and CD 38+ hemopoietic progenitor cells of bone marrow [121, 123-127] , mature dendritic cells [128] , neurons and microglia [129] , thymocytes [123] , endothelial cells of heart, brain, lung and colon [130-134] and individual cells within the uterine and cervical mucosa [135, 136] as well as on colonic epithelial cells [137, 138] . Methods used for detection of chemokine receptors in human cell phenotypes include CXCR4 and phenotypic labeling by two color flow cytometry [114, 119, 138] , immunohistochemistry [136, 138, 139] , and RT PCR [126, 138] .

CXCR4 receptor distribution in feline tissues

Fewer published studies are available concerning the distribution of feline chemokine receptors. CXCR4 has been identified on feline PBMC [110, 140] , Crandell feline kidney (CrFK) cells [99, 110, 141] feline T-lymphoma cell lines [141] and thymocytes [111] . In fresh PBMC CXCR4 colabels strongly with B cell (B220) and monocyte (CD14) markers but colabels poorly with T cell markers in contrast to expression patterns found in human T lymphocytes. However feline T cells have a high capacity for CXCR4 expression as evidenced by their ability to upregulate CXCR4 with time in culture [110] .

Effects of chemokine receptor blockade on HIV and FIV infection

The alpha chemokine SDF-1α has demonstrated effective in vitro in inhibition of CXCR4-utilizing strains of HIV [7, 142] as well as many strains of FIV [110, 111, 141, 143] . The beta chemokines RANTES and MIP1-α inhibited R5 utilizing strains of HIV-1 [144] in vitro but failed to inhibit FIV [141] infection. The fact that FIV can infect AH927 cells transfected with CXCR4 but not CCR5 provides further evidence that R5 is not a coreceptor for the virus [145] .

The anti-CXCR4 antibody 12G5 inhibited HIV infection in vitro [146] and inhibited syncytium formation between FIV infected feline cells with human cells in culture. However there are no published studies demonstrating inhibition of FIV infection using an anti-CXCR4 antibody.

AMD3100 is a low molecular weight CXCR4-specific antagonist that demonstrates potent antiviral activity in vitro against both HIV and FIV. Inhibitory concentration (IC_{50}) of AMD 3100 for Crandell feline kidney cells and feline thymocytes in culture are 14ηg/ml and 62ηg/ml respectively [111] . *In vitro* data have demonstrated

no cytotoxicity in human or feline blood cells at concentrations > 250μg/ml [104] and the drug is well tolerated in cats at doses up to 1 mg/kg BID SQ x 30 days and 2mg/kg BID SQ for 7 days (see chapter 2) .

Dissertation research

The goal of this research was to investigate the in vivo distribution of the CXCR4 chemokine receptor and determine the relationship of CXCR4 expression to feline immunodeficiency virus infection. Feline tissues were surveyed by both immunohistochemistry and in situ hybridization to determine anatomic distribution of CXCR4 positive cells within lymphoid tissues. To determine whether a correlation exists between CXCR4 expression and FIV infection, CXCR4 positive and negative T and B cell fractions from acutely infected cats were purified by fluorescence activated cell sorting and assayed for provirus by real time PCR. To further investigate the role of the receptor in FIV infection, antiviral studies were conducted in vitro and in vivo using the CXCR4-specific antagonist AMD3100.

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CHAPTER ONE

POTENT INHIBITION OF FELINE IMMUNODEFICIENCY VIRUS BY CXCR4-BINDING BICYCLAMS

ABSTRACT

Feline immunodeficiency virus (FIV) has been shown to use the CXCR4 chemokine receptor for entry into peripheral blood mononuclear cells (PBMC) in vitro, similar to T-cell tropic strains of human immunodeficiency virus type1 (HIV-1) and HIV-2. Here we explore the ability of human recombinant chemokines, multiple cyclam compounds, and anti-CXCR4 antibody to block FIV infection in feline PBMC. The prototypical CXCR4-binding bicyclam AMD3100 (1,1'-[1,4-Phenylenebis(methylene)]-bis-1,4,8,11-tetraazacyclotetradecane Octahydrochloride), inhibited infection of PBMC by primary FIV isolates representing the three major virus clades (A,B,C) when present prior to infection (IC_{50} < 0.3μg/ml) and downregulated FIV infection when added up to 7 days post-infection. Other cyclam molecules tested (JW 367, TAB I135, TAB I381) had little or no inhibitory effect on FIV infection. Inhibition of FIV by brief exposure of feline PBMC to human recombinant SDF-1α was less reliable than AMD3100 and varied

widely between pooled blood samples. Prolonged incubation (14 hr) with SDF-1α resulted in enhanced infection of feline PBMC, likely the result of CXCR4 upregulation. AMD3100 completely blocked binding of anti-CXCR4 monoclonal antibody 44717, however the antibody was unable to inhibit FIV infection indicating antibody interaction with the receptor outside the viral binding site. The ability of the CXCR4-specific ligands SDF1-α and AMD3100 to inhibit or block infection by primary FIV isolates further supports that CXCR4 is a major receptor for FIV in PBMC. FIV may therefore serve as a valuable model for therapeutic interventions targeting lentivirus-CXCR4 interactions.

BACKGROUND

HIV-1 requires binding of virion envelope glycoprotein gp120 to both CD4 and one of the seven–transmembrane, G-protein-coupled chemokine receptors for cellular entry [1] [2, 3] . Two major coreceptors, CCR5 and CXCR4, have been identified each of which have an affinity for different HIV isolates. CCR5 is the primary coreceptor utilized by macrophage tropic (non syncytia-inducing) strains of HIV-1 (R5 HIV-1) during initial infection and throughout the early asymptomatic phase. Random mutations occur over time altering viral affinity for host chemokine receptors [4-7] . Eventually CXCR4 utilizing T-cell tropic (syncytia-inducing) strains (X4 HIV-1) are produced and it is this adaptation to the use of the CXCR4 receptor that corresponds with accelerated virus replication and rapid T-cell depletion leading to AIDS [4, 8-10] . Some strains of HIV (R5X4 HIV-1) are able to use both receptors with comparable efficiency [11] .

FIV uses CXCR4 as a primary receptor similar to T-cell tropic HIV [12-16] . Recent evidence demonstrates that primary strains of FIV also require CD134 [17, 18] as a primary receptor for productive infection, roughly analogous to the requirement of CD4 and chemokine receptor binding by HIV-1. FIV demonstrates similar tissue tropism to HIV including replication in T-lymphocytes [19-23] , macrophages [24-27] , B-cells [20, 28], monocytes [29] , and cells of the nervous system including astrocytes and microglia [16, 30-32]. Cell entry for both FIV and HIV-1 involve interaction of the gp120 V3 loop with the CXCR4 or CCR5 receptor [12, 33-37] . Cell-culture-adapted strains of FIV and HIV demonstrate change from non-syncytium inducing to syncytium inducing phenotype, which correlates with an increased net charge of the V3 loop [12, 38, 39] . Indicative of the similarity of the human and feline CXCR4, human cells which naturally express CXCR4 and nonpermissive human cells transfected with human CXCR4 form syncytia with FIV-infected feline cells [12, 40] .

Antiretroviral activity of polycyclic compounds was first described by Meruelo et al in 1988 [41] . Treatment of a mouse thymoma cell line prior to infection with either Friend leukemia virus (FV) and radiation leukemia virus (RadLV) with the compounds hypericin and pseudohypericin (present in plants of the hypericum family) resulted in marked reduction in RT activity in the supernatant. More recently AMD3100, a small molecular weight cyclam compound, has demonstrated potent antiretroviral activity through selective antagonism of the CXCR4 chemokine receptor [42, 43] . A series of bicyclam analogues including AMD3100 have been shown to specifically bind the CXCR4 receptor and competetively inhibit binding of both HIV-1 [44, 45] and FIV [43] *in vitro* with inhibitory concentrations similar between FIV and HIV [43] . These experiments suggest a close evolutionary relationship between HIV and FIV in their

mechanism of infection involving CXCR4 receptor interactions and could therefore serve as a valuable model for the study of lentivirus-CXCR4 interaction.

The natural CXCR4 ligand SDF-1 has been shown to effectively block infection by T-tropic strains of HIV-1 [46, 47] and similarly inhibits infection of CrFK by cell culture adapted [48] and many primary strains [13, 49] of FIV. However evidence for SDF-1 blockage of FIV in primary T cells is less clear. Some reports show potent inhibition of naturally occurring strains of FIV using SDF-1α in feline PBMC [13, 49] while others demonstrate no antiviral effect [43, 48] . Possible reasons for the inconsistency of antiviral effect using SDF-1 include virus strain specific differences, target cell type as well as differences in protocols or chemokine reagents. There is no commercial resource for feline SDF-1 currently available and while human SDF-1α shares 95.5% homology to feline [50] there may be decreased binding avidity of human recombinant SDF compared with the native ligand. Furthermore the affects of SDF-1 in CrFK have been shown to depend on incubation time with the ligand. Brief exposure of Crandell feline kidney cells (CrFK) to human recombinant chemokine SDF-1α inhibited entry of FIV, although overnight incubation with hSDF1-α before FIV exposure actually enhanced infection [48] . This effect is thought to be a result of upregulation of CXCR4 on the cell surface with prolonged exposure to hSDF1-α. Furthermore CXCR4 expression demonstrates considerable variation between individual blood samples [51, 52] and a remarkable ability for upregulation with storage [53] or culture with exposure to concanavalin A and IL-2 [13, 54] which may further alter chemokine binding kinetics in these studies.

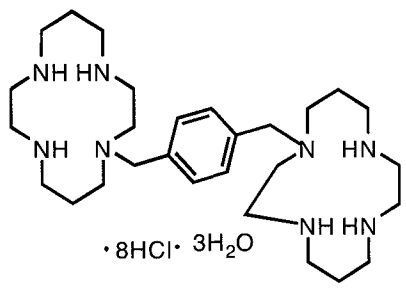
Unlike chemokines, bicyclams can bind chemokine receptors without initiating a signal cascade including intracellular calcium that could have potentially detrimental effects in vivo [55] . Additionally the bicyclam drug AMD3100 has been shown to have negligible in vitro cytotoxicity [44] and does not elicit an immune response when injected parenterally. However, in phase II clinical trials research was halted on the drug due to side effects including cardiac arrhythmias and thrombocytopenia occurring in a subset of study participants [56] . Some of these side effects may be due to alteration of calcium metabolism and new bicyclam analogues are being tested in hopes of increasing bioavailability as well as eliminating toxic side effects.

Here we investigate the ability of multiple CXCR4-specific ligands (SDF-1α, AMD 3100, and anti-CXCR4 mAb) to interact with feline CXCR4 and block or control infection of feline PBMC by primary FIV isolates. FIV subtypes A, B and C, up to 17.8 to 26.2 percent divergent in Env variable regions V3, V4 and V5 [57] , were examined to further assess the effect of CXCR4 ligands in blocking infection by different FIV subtypes.

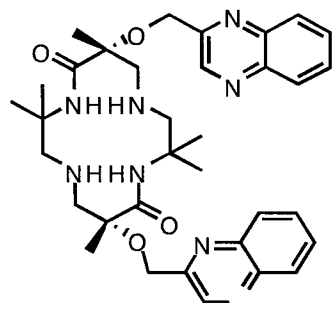
METHODS

Cyclams and chemokines

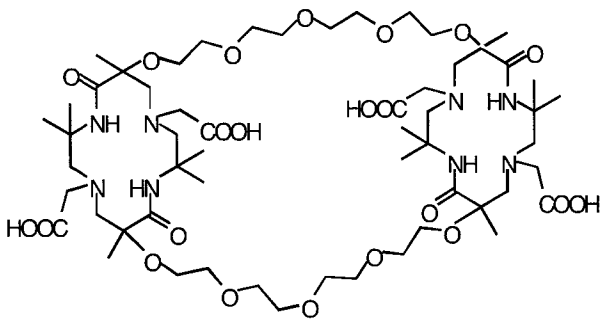
The cyclam compounds (Fig. 1.1) AMD3100, JW 367, TAB I135, and TAB I381 were synthesized at Colorado State University Department of Chemistry by Dr. Lou Hegedus et al using methods previously described [58-60] . The human recombinant chemokines SDF-1α, SDF-1β, RANTES, MIP-1α were purchased from Peprotech Inc., Rocky Hill, NJ.



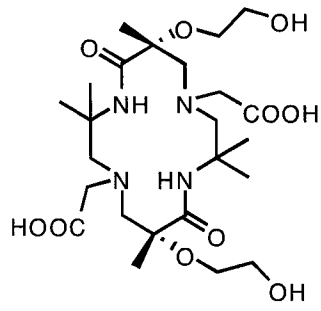
AMD3100



JW 367



TAB I135



TAB I381

Figure 1.1: Chemical structures of cyclam compounds assayed in vitro.

Virus inocula

The cell-free cell culture virus inoculum was obtained by coculture of infected peripheral blood mononuclear cells from FIV-A-Ppr, B-2542 or C-Pgmr infected cats with naïve PBMC. Briefly, eight week old SPF cats were IV inoculated with a minimum of10^8 RNA copies FIV-A, B or C isolate capture stock plasma. At 3 weeks p.i. blood was collected and PBMC were separated by Ficoll-Hypaque gradient centrifugation. Cells were then co-cultured for 3 weeks with naïve Con A stimulated donor cells incorporating weekly supernatant harvests, stored in aliquots at liquid nitrogen temperatures. The

supernatants were frozen in aliquots and titrated for infectivity by virus isolation coculture.

In vitro antiviral assays using chemokines, cyclam compounds and anti CXCR4 antibody

PBMC from 3 specific-pathogen-free cats were recovered from blood by Ficoll-Hypaque gradient centrifugation and resuspended at 2 x 10^6/ml in RPMI1640 medium supplemented with 20% fetal bovine serum, 2% glutamine, 0.5% 2-mercaptoethanol, and 1% Pen-strep and 100 IU hrIL-2/ml. PBMC ($2x10^5$) were added to triplicate wells of a 96 well plate and stimulated with 5μg/ml concanavalin A (Con A). After 3 days in culture PBMC were exposed for 15 minutes to different concentrations (0.04-9.6 μg/ml) of cyclam compounds at 37°C and subsequently infected with FIV isolates representing clades A,B and C (FIV-A-Ppr, B-2542 and C-Pgmr) at >100% TCID. The cells were washed after 24 hours of incubation with virus and the compound was replaced at its original concentration. Supernatants were collected at days 4, 7, 10, and 13. One group received no bicyclam replacement after the initial dose and others received bicyclam doses on days 4 or 7 post infection. Cultures were maintained with partial media changes every 3 days for 16 days.

Chemokine assays were performed in like manner. The effect of brief versus prolonged exposure to SDF-1α was examined by either exposing cells to SDF-1α only once just prior to infection or with overnight incubation.

To investigate whether an anti-CXCR4 antibody inhibits FIV infection of feline PBMC in vitro, cultured PBMC were treated with 5-40μg/ml of anti-CXCR4 mAb 44717 (R&D systems Inc., Minneapolis MN) for 30 min. and exposed overnight to FIVC.

p24 antigen ELISA

Productive in vitro infection was assessed by testing supernatants with a p24 gag antigen capture ELISA described by Dreitz et al [61]. Optical density (OD), measured by absorbance at A_{450} (reference A_{570}), was recorded with a Dynatech 5000 MR™ microplate reader (Dynatech Corp. Chantilly, VA). Positive reactions were defined as those with a minimum OD of 0.1 and at least twice that of the negative control supernatant run in tandem. Statistical analysis for viral inhibition studies was performed using an unpaired two tailed students T-test comparing optical density readings between triplicate samples from treatment groups versus untreated FIV infected cells (positive control).

Flow cytometric analysis

PBMC or cells harvested from lymph nodes were collected and mitogen stimulated for 3 days. Cells were then washed and incubated with different concentrations of AMD3100 for 5 minutes prior to addition of mouse anti-human CXCR4 MAb 44717. The cells were incubated for 1 hour at 4°C, washed twice then incubated with Cy3 conjugated sheep anti-mouse mAb (Sigma Corp., St. Louis, MO) for 30 min at 4°C. The cells were analyzed by flow cytometry (Coulter EPICS XL-MCL).

Inhibition of antibody binding by AMD3100

PBMC were isolated as described above. After 3 days in culture, Con A and IL-2 stimulated cells were washed and treated with either 10μg/ml, 25μg/ml, 50μg/ml

AMD3100 or an equal volume of media alone 15 minutes prior to exposure to anti-CXCR4 mAb 44717 (R&D Systems, Inc.).

RESULTS

SDF1-α inhibition of FIV in feline PBMC

In order to determine the ability of the natural CXCR4 ligand SDF1-α to inhibit FIV infection we performed an in vitro antiviral assay in feline PBMC using the chemokine. Because feline SDF1-α is not commercially available we utilized the human recombinant analogue which has been shown to bind with high affinity to feline CXCR4 [48]. PBMC were exposed to the chemokine for either 15 minutes or overnight prior to exposure to FIV. Brief SDF1-α exposure resulted in viral inhibition while prolonged incubation of PBMC with SDF1-α resulted in enhancement of virus production as measured by p24 gag antigen capture ELISA of supernatants (Fig. 1.2). Similar assays performed using the β-chemokines MIP1-α and RANTES failed to inhibit virus (data not shown).

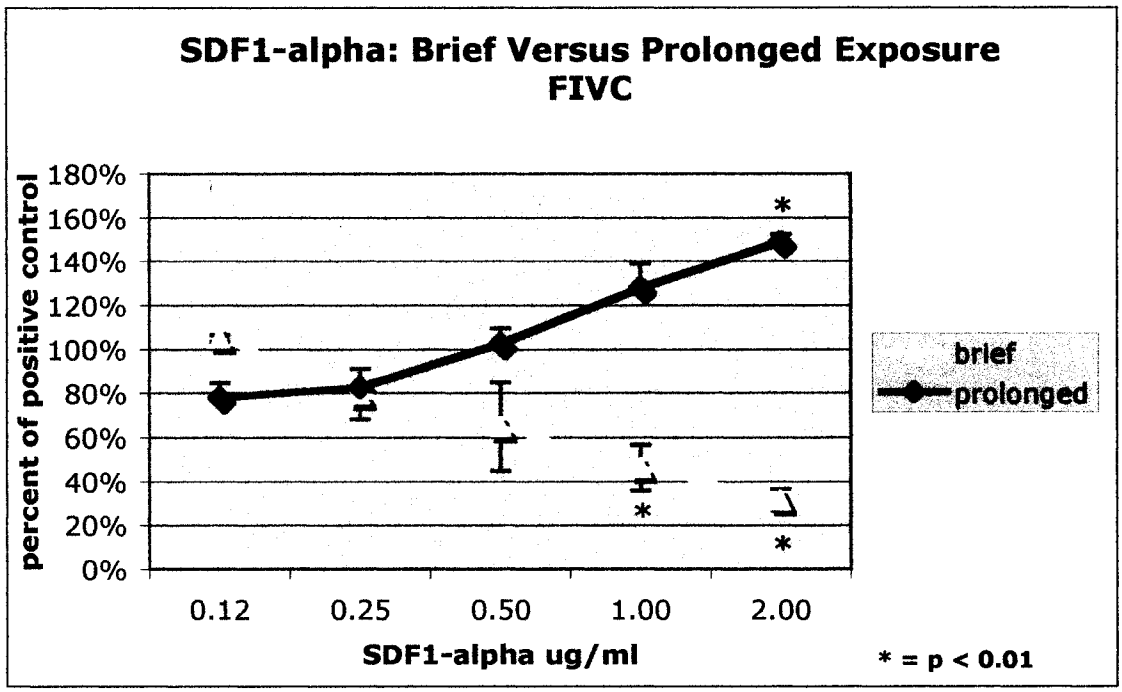


Figure 1.2: p24 gag ELISA, day 13 post-infection, pooled PBMC from three cats. Brief exposure to SDF-1α (a) at 1-2.0 μg/ml inhibited FIV-C-Pgmr infection whereas prolonged incubation (b) resulted in enhancement of infection. Results are means of triplicate wells; representative experiment [n=3].

AMD3100 inhibition of FIV infection in feline PBMC

In order to determine the efficacy and potency of AMD3100 in blocking FIV infection, feline PBMC were exposed to the compound at concentrations ranging from 0.3 to 9.6 μg/ml either 15 minutes prior to addition of virus (FIV A, B or C) (Fig. 1.3) or on day 3 or 7 post infection (Fig. 1.4). AMD3100 potently inhibited all three virus clades in a dose dependent manner and demonstrated effective viral inhibition in cells treated as late as 7 days post-infection at doses as low as 0.3μg/ml. A single dose was somewhat less effective than 2 consecutive doses given 12 hours apart. The drug demonstrated similar potency when tested across different clades of FIV. None of the additional cyclam compounds tested showed significant inhibition of FIV-C (Fig. 1.5).

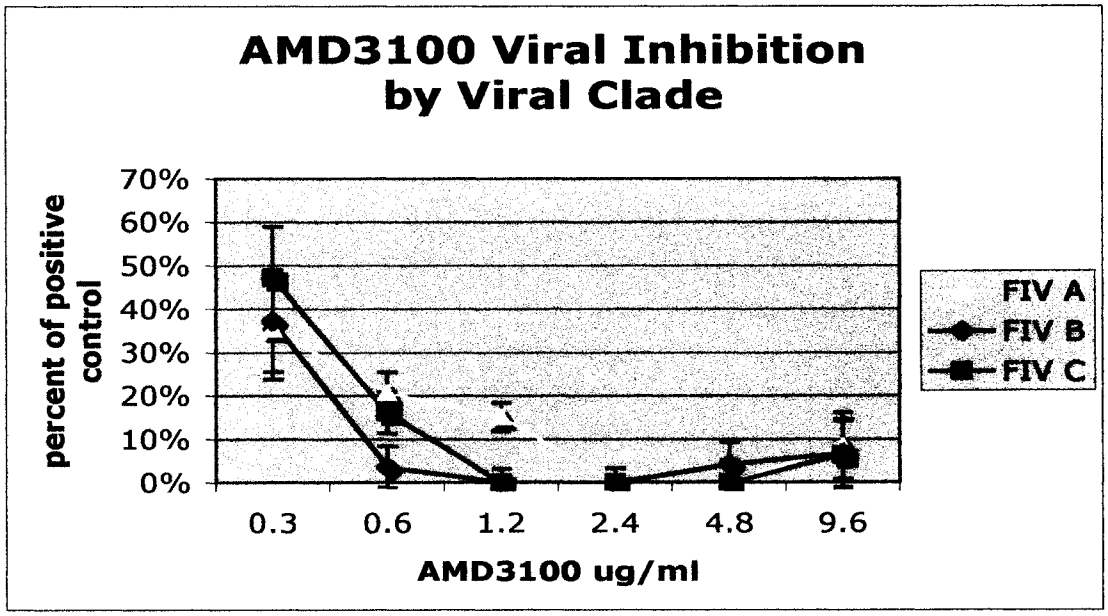


Figure 1.3: p24 gag ELISA, day 13 post-infection, pooled PBMC from three cats. AMD3100 inhibited FIV in a dose dependent manner and with equal potency across three clades. Complete inhibition of virus was seen at 2.4 μg/ml. All concentrations between 0.3-9.6 μg/ml demonstrated statistically significant virus inhibition ($p < 0.01$). Results are means of triplicate wells.

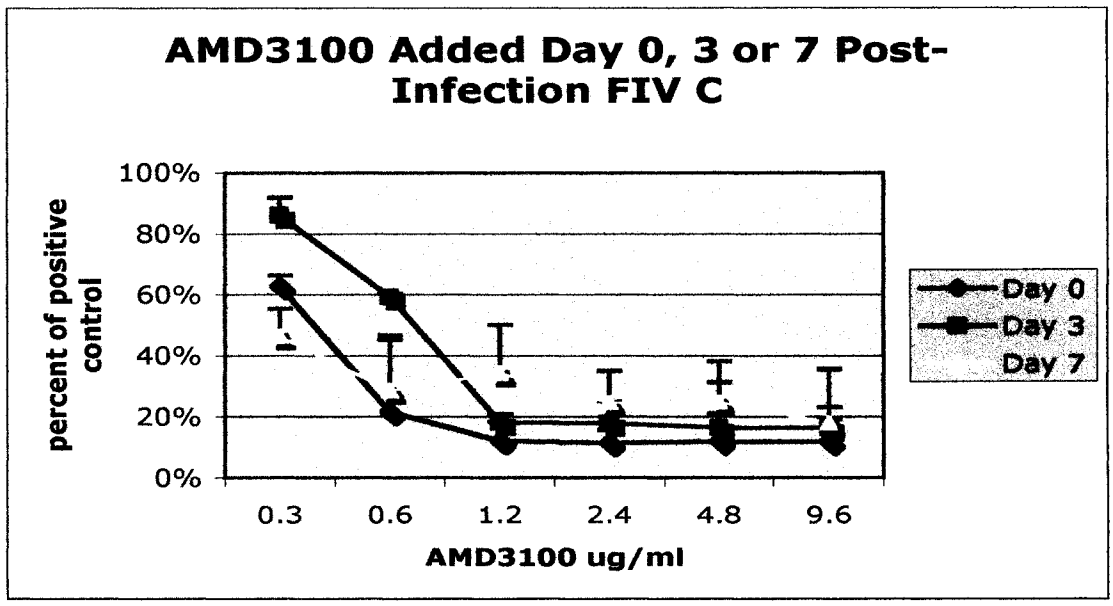


Figure 1.4: p24 gag ELISA day 13 post-infection, pooled PBMC from three cats. AMD3100 inhibited virus at similar levels whether the drug was added prior to virus or up to 7 days post-infection. Each of the concentrations resulted in statistically significant viral inhibition ($p \leq 0.03$). Results are means of triplicate wells.

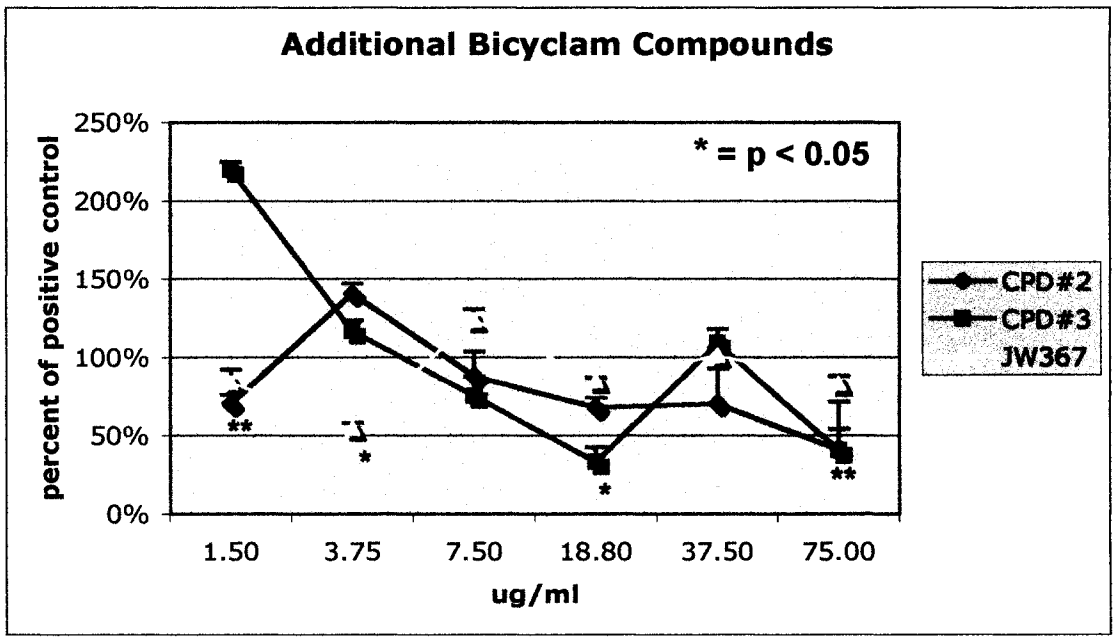


Figure 1.5: p24 gag ELISA day 13 post-infection FIV-C-Pgmr, pooled PBMC from three cats. Additional cyclam compounds had either weak or no antiviral effect. Results are means of triplicate wells.

Inhibition of X4-specific monoclonal antibody binding by AMD3100

In order to determine the specificity of AMD3100 to the CXCR4 receptor an antibody-AMD3100 competition assay was performed. The anti-human CXCR4 mAb 44717 binds to the second extracellular loop of the human CXCR4 and crossreacts with feline CXCR4. AMD3100 added to PBMC 5 minutes prior to antibody completely inhibited antibody binding at concentrations as low as 125μg/ml, as measured by flow cytometry (Fig. 1.6 a). The compound had no effect on anti-CD4 MAb binding to HOSCD4 cells or anti-CXCR5 MAb binding to HOSR5 cells (Fig. 1.6 b, c).

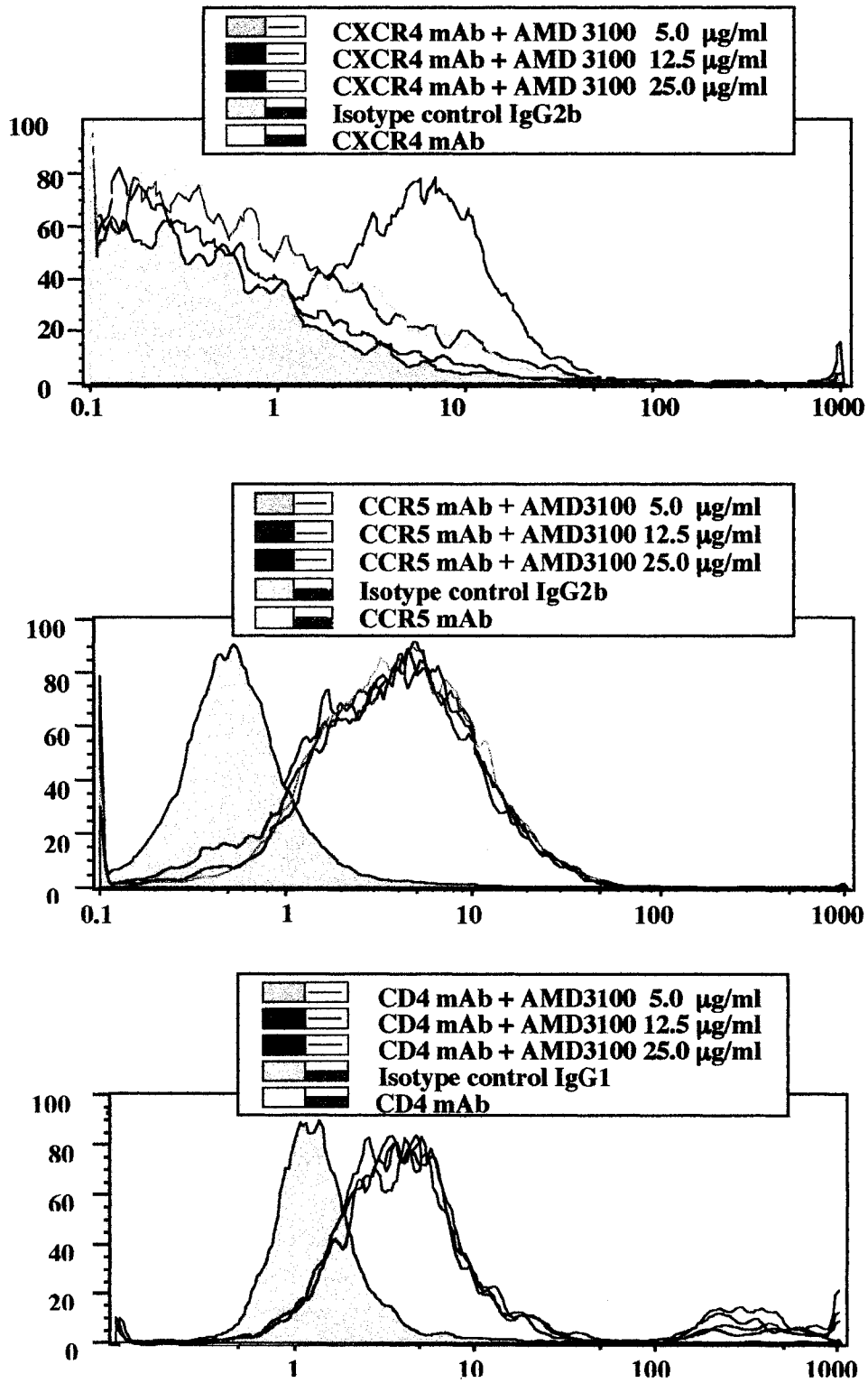


Figure 1.6: AMD3100 inhibited binding of the CXCR4 specific mAb 44717 in feline PBMC (**a**) but had no affect on binding of CCR5 mAb to HOSR5 cells (**b**) or a CD4 mAb to HOSCD4 cells (**c**). Assays represent pooled blood samples from three cats.

Next we tested the ability of the anti-CXCR4 mAb 44717 to inhibit FIV infection of PBMC in vitro. The antibody failed to achieve significant inhibition of virus at concentrations up to 40μg/ml (Fig. 1.7).

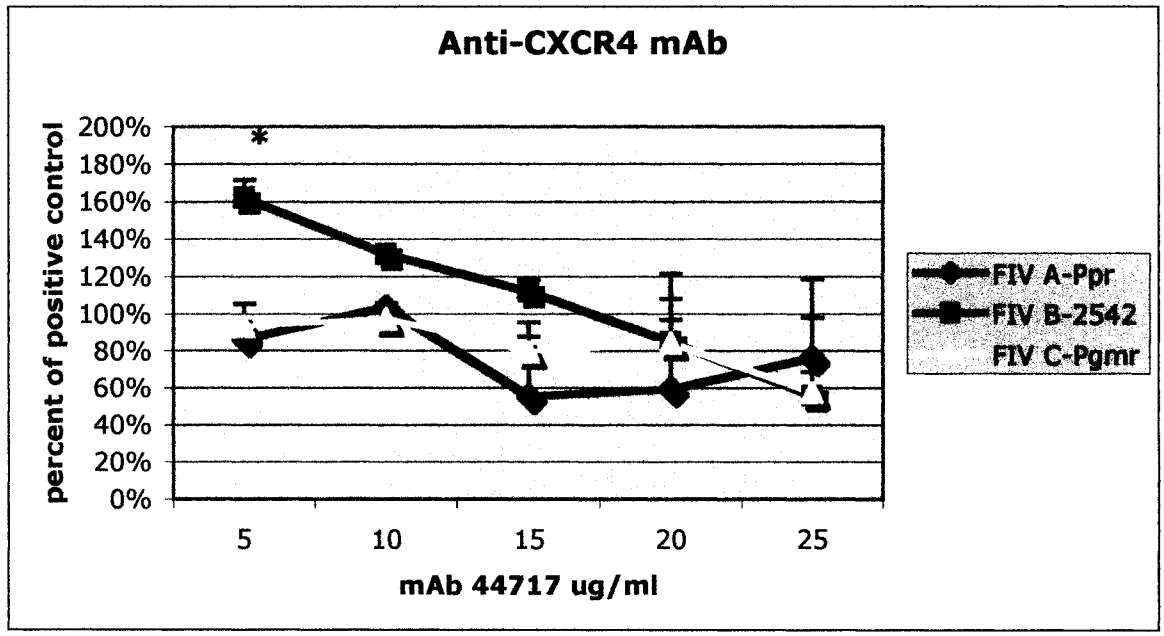


Figure 1.7: p24 gag ELISA, feline PBMC day 13 post-infection. The anti-CXCR4 monoclonal antibody 44717 failed to reduce FIV p24 antigen release. While slightly lower optical density readings were obtained with FIV-A-Ppr they were not statistically significant. Results are means of triplicate wells.

DISCUSSION

The requirement for CXCR4 for viral entry has been shown to be roughly analogous between SI-inducing, T-tropic strains of HIV and several primary isolates and molecular clones of FIV. CXCR4 receptor binding by FIV representing three naturally occurring clades was selectively and potently inhibited by the bicyclam drug AMD3100. Viral inhibition was seen with addition of bicyclam up to 7 days post infection and with an IC_{50} of $< 0.3\mu g/ml$ ($p \leq 0.03$). As expected inhibition of virus after 7 days infection

was partial and likely the result of inhibition of second and third round infection through blockage of the receptor. Similar results were reported by Egberink et al [43] with potent inhibition of FIV infection of CrFK and feline thymocytes using AMD3100.

AMD3100 binding is thought to occur by electrostatic forces and hydrogen bonding between the positively charged bicyclam molecule and negatively charged amino acids Asp^{171}, Asp^{262} and Glu^{288} located in a binding pocket formed between extracellular loops IV, VI and VII respectively [62, 63] . Given such simple interaction with relatively few amino acids it would seem likely that AMD3100 should interact more nonspecifically with other cell surface molecules. However among the G-protein coupled 7-transmembrane receptor family the orientation of this negatively charged amino acid motif appears to be unique to CXCR4 [62] and studies have demonstrated the lack of AMD3100 interaction with any other chemokine receptors [42] . Our experiments demonstrated blockage of anti-X4 mAb 44717 to feline PBMC with the compound and no effect on anti-CD4 or CCR5 mAb binding to CD4 or CCR5 providing further evidence of the compounds specificity for the CXCR4 chemokine receptor. Of course many cell surface molecules are present beyond chemokine receptors and the handful of additional receptors that have been studied with regard to AMD3100 interactions. While potential interactions of AMD3100 with other surface molecules may ultimately have no affect on viral binding or entry the possibility cannot be entirely ruled-out. If we assume that AMD3100-cell interactions are completely CXCR4-specific then the fact that the drug successfully inhibits infection by both primary and laboratory adapted strains of FIV representing all major clades suggests that CXCR4 is absolutely essential for productive infection by FIV.

By comparison results of antiviral studies using human recombinant or human synthetic SDF-1α (both shown to avidly bind feline CXCR4 expressed on CrFK [48]) have been much less consistent in the feline system. SDF1-α potently inhibited infection of CRFK by the CRFK adapted strains of FIV-113cr and FIV-Petaluma [13, 48] but failed to inhibit infection of feline thymocytes by the thymocyte-specific strain FIV-113th [43] and showed no inhibitory activity against FIV-PET or FIV-GL8 in Mya-1 cells [48]. Human recombinant SDF-1 inhibited viral replication for four divergent FIV isolates of subtypes A, B and D in a feline T-lymphocyte cell line [49] and short peptides derived from SDF1-α inhibited viral infection of feline PBMC by several naturally occurring FIV strains [13] . SDF-1 antiviral studies have been further confounded by the observation that exposure time to the chemokine can dramatically alter the antiviral effect [48].

One of the more compelling reasons for variability observed in SDF-1 antiviral studies is the possibility that viral strains may interact with slightly different regions of the feline CXCR4 receptor thus allowing for FIV binding in the presence of bound chemokine. There is also evidence that CXCR4 can exist in antigenically distinct conformations that may be cell type dependent [64] . The receptor has been shown to commonly form of heterodimers with CD4 as well as homodimers and possibly higher order structures both basally and in response to ligand binding [65] . Furthermore cell type dependent differences in rate of receptor cycling may result in weaker viral inhibition by SDF-1 [66].

In our hands SDF-1 demonstrated a much more variable antiviral effect between assays (data not shown) when compared with AMD3100 suggesting that SDF-1 may be

more sensitive to inter-assay variability. Aside from cell type and virus clade related differences, variations in assay conditions including temperature and presence of divalent cations have been shown to greatly affect SDF-1 binding. SDF-1α binding is reduced by 58% at 4°C [66] and addition of 10mM of the common anticoagulant EDTA to media inhibits SDF binding by 68% [66] . While its effect may be conditional, SDF-1 has demonstrated the ability to block infection of cells in vitro by multiple FIV clades whereas human recombinant CCR5 ligands MIP-1α and RANTES have no antiviral effect [48] .

Our flow cytometry experiments using mAb 44717 demonstrate CXCR4 expression on feline PBMC and that AMD3100 completely blocks anti-CXCR4 mAb binding at concentrations as low as 125μg/ml. Interestingly the same antibody blocked by AMD3100 was itself unable to inhibit infection of PBMC by FIVC. This is in contrast to studies using the anti-CXCR4 mAb 12G5 to inhibit T-cell tropic HIV-1 infection in vitro [67] and is most likely explained by antibody binding to an area of the feline receptor that does not interfere with virus binding. Alternatively mAb 44717 may interact with only a subset of CXCR4 isoforms leaving other receptors available for viral attachment.

These studies demonstrate the importance of CXCR4 as a primary receptor for three separate FIV isolates representing subtypes A, B and C in feline PBMC in vitro.

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CHAPTER TWO

IN VIVO EFFICACY OF THE BICYCLAM (AMD3100) ANTIVIRAL IN EXPERIMENTAL FELINE IMMUNODEFICIENCY VIRUS INFECTION

ABSTRACT

Specific pathogen free (SPF) cats were treated subcutaneously with the bicyclam drug AMD3100 (1-1*-[1,4-phenylenebis(methylene)]-bis(1,4,8,11-tetraazacyclotetradecane) at a dose of 1.0 mg/kg twice daily just prior to and for 30 days following intravenous (IV) or oronasal (ON) challenge with a pathogenic primary strain of feline immunodeficiency virus C (FIVC). Statistically significant reductions in mean DNA proviral concentrations ($p < 0.05$) were observed in blood of IV inoculated-AMD3100-treated cats on weeks 2, 5, 7 and 9 concordant with a partially protective effect on CD4 numbers. Interestingly, neither AMD3100 antiviral activity nor a sparing of CD4 numbers could be detected in cats challenged oronasally. Likewise one week of AMD3100 treatment (2.0 mg/kg twice daily) was not effective in reducing proviral loads in cats chronically infected with FIV (22 weeks post-inoculation). We hypothesize that

the differences in the efficacy of the bicyclam based on route of challenge could be attributable to the relatively higher concentration of drug present in the blood compartment versus the mucosal compartment at the time of challenge. These findings suggest that AMD3100 is an active inhibitor of FIV infection when virus and drug are in the same compartment, however sufficient inhibitory concentrations may not be achieved outside the plasma compartment leading to failure of the drug to inhibit viral infection in tissues. In contrast with in vitro antiviral studies, the antiviral effect of AMD3100 was only marginally effective and may indicate a reduced role for CXCR4 and/or the capacity for alternate receptor use by FIV in vivo.

BACKGROUND

Feline immunodeficiency virus (FIV) causes an immune deficiency syndrome in cats characterized by a progressive drop in CD4+ T cell counts similar to AIDS caused by human immunodeficiency virus (HIV) [1-4] . FIV requires the X4 chemokine receptor for entry into peripheral blood mononuclear cells (PBMC) in vitro, similar to T-cell tropic strains of HIV-1 and HIV-2 [5, 6] . There is recent evidence that at least some primary and cell culture adapted strains of FIV require both CD134 and CXCR4 for entry into cells [7, 8] . This is roughly analogous to the CD4/chemokine receptor paradigm of HIV where for most strains neither receptor is sufficient alone to impart susceptibility but rather both are required for entry. As in HIV-1[9, 10], blockade of CXCR4 by its natural ligand SDF-1α resulted in inhibiton of FIV infection in vitro [6, 11-13] . The conserved use of CXCR4 between the two lentiviruses for cellular attachment makes the FIV model

an attractive system for conducting prospective in vivo studies using therapeutics targeting the CXCR4 receptor.

AMD3100 is a low-molecular-weight bicyclam compound with potent in vitro antiviral activity against several primary and laboratory adapted strains of FIV [6, 13, 14] as well as T-cell tropic strains of HIV-1 [15] . While the possibility of some cell surface interaction other than CXCR4 cannot be excluded, there is strong evidence that the compound acts as a selective antagonist of the chemokine receptor . AMD3100 competitively inhibited binding of gp120 [16, 17] , SDF-1α [18] and monoclonal antibodies specific to CXCR4[18] and prevented syncytium formation between FIV Env expressing cells and feline cells expressing CXCR4 in culture [14] . Evidence of the compound's selectivity include lack of inhibition of antibody binding to other chemokine receptors such as human CCR5 or feline CD4 or CD8 [13] , lack of inhibition of HIV-1 or HIV-2 infection mediated by coreceptors CCR5, CCR3, US28, BOB or BONZO [19] , and no effect on calcium signaling induced by ligands of CCR1 through CCR9, or CXCR1 through CXCR3 [20]. While blockade of CXCR4 in cell culture results in potent inhibition of both cell culture adapted and primary strains of FIV the relative importance of CXCR4 to FIV infection in vivo has been difficult to study. Thus the use of a prospective study using the feline model may offer insights into the role of CXCR4 in FIV infection in vivo.

MATERIALS AND METHODS

Animals and sampling

AMD3100 toxicity study:

As a prelude to the antiviral study a toxicity study was performed to determine the maximum tolerated dose of AMD3100 in cats. Six age matched specific pathogen free cats were treated twice daily for 30 days with subcutaneous injections of with either 0.5 or 1.0mg/kg AMD3100. Daily monitoring for adverse drug reactions and weekly hemograms and clinical chemistry data were performed to detect signs of toxicity.

AMD3100 antiviral study (acute infection):

Intravenous inoculation: Eleven 6-8 month-old cats were anesthetized with ketamine-acepromazine and inoculated intravenously with an infectious dose of an acute phase plasma pool of FIV-C-Pgmr. Six cats were treated with AMD3100 at 1.0 mg/kg while 5 control cats received an equivalent volume of saline. Oronasal inoculation: Ten 12-14 week-old kittens were anesthetized with ketamine-acepromazine and challenged oronasally with an infectious dose of FIV-C-Pgmr once daily for two days. Five cats were treated with AMD3100 at 1.0 mg/kg while 5 control cats received an equivalent volume of saline. Cats were treated 90 minutes prior to challenge and then twice daily for 30 days.

AMD3100 antiviral study (chronic infection):

At 22 weeks post (IV) inoculation, five 10-12 month-old FIVC positive control cats from the previous study were given 2.0 mg/kg AMD3100 twice daily for 7 days.

Virus stock

FIV in vivo stocks were produced by in vivo acute phase plasma collection. Briefly, eight week old SPF cats were IV inoculated with a minimum of10^8 RNA copies FIV-C-Pgmr isolate capture stock plasma. At 3 weeks post-inoculation, plasma was centrifuged from heparinized blood, aliquoted, and stored in liquid nitrogen.

Hematology

Heparinized and EDTA blood was collected at weeks 0, 1, 2, 3, 5, 7, 9, 12 and 17 post-inoculation and analyzed for complete blood count and flow cytometric analysis of CD4+ and CD8+ T cell subsets by as described by Dean et al. [21] Lymphocyte subset analysis was performed with a Coulter EPICS Profile II flow cytometer (Coulter Electronics, Hialeah, FL). Absolute cell numbers were calculated from the total lymphocyte count.

Antibody ELISA

FIV-specific antibody from serum was detected by a sandwich enzyme-linked immunosorbent assay (ELISA) modified from a method described by Dreitz et al [22] . Briefly, 2x dilutions of serum were aliquoted across 98-well microtiter plates coated with whole pelleted FIV. Plasma antibody binding was carried out at room temperature for 1

hour. Plates were washed and wells overlaid with peroxidase-labeled secondary antibody solutions containing isotype-specific goat anti-cat antibodies to IgG-γ, IgM-μ (Kikegaard & Perry Laboritories, Gaithersburg, MD) or IgA-α (Bethyl Laboratories, Montgumery, TX). Secondary antibody solutions were diluted 1:500. Optical densities (OD) were measured by adsorbance at A_{450} (reference A_{570}), were recorded using a Dynatech 5000MR™ microplate reader (Dynatech Corp., Chantilly, VA). Positive reactions were defined as those with a minimum OD of 0.1 and at least twice that of negative control serum run in tandem.

Real Time DNA PCR

DNA was extracted from whole blood using Qiagen DNA extraction mini kit (Qiagen, Valencia, CA). DNA concentration was measured using a Multiskan Spectrum 1500 spectrophotometer (Thermo Lab Systems, Franklin, MA). The 25μl PCR mixtures contained 5μl standard or sample, 0.5 ml (400 nM) of each primer, 0.2 ml (80nM) of probe, 6.3ul DNAse free water, and 12.5 μl TaqMan Universal PCR Master mix containing 10mM Tris-HCl (pH 8.3), 50mM KCl, 5mM MgCl2, 300uM each of dATP, dCTP, and dGTP, 600uM dUTP, 0.625U of AmpliTaq Gold DNA polymerase, and 0.25U uracil N-glycosylase (UNG) per reaction. FIVC gag primer and probe sequences were as follows: forward 5'-ACT CAC CCT CCT GAT GGT CCT A-3', reverse 5'-TGA GTC AGC CCT ATC CCC ATT A-3') and probe FAM-5'-ACC ATT GCC ATA CTT CAC TGC AGC CG-3'-TAMRA. FIV C gag plasmid DNA was cut with the restriction enzyme BamHI and repurified.

A standard curve was generated for each plate with serial dilutions of FIV C gag plasmid DNA (10^9, 10^6, 10^2) with 1X TE and 40 ng/ml salmon testes DNA (Sigma) as a DNA carrier. Samples and standards were run in triplicate on 96-well plates using the Biorad iCycler. Polymerase activation and amplification were performed using the following protocol: 2 minutes at 50°C, 10 min. at 90°C 45 cycles at 95°C for 15 seconds, 60°C for 1 minute.

Statistical Analysis

Values for PCR provirus and hematology were log transformed to normalize residuals and equalize variances. The analysis of variance was a 2 factor repeated design. Group was the between subjects effect and week was the repeated measures effect. Two tailed T-tests were computed at each week to compare the two groups. Error bars are presented as 1/2 least significant difference (LSD).

RESULTS

AMD3100 toxicity assay

A marked leukocytosis was observed at 90 minutes post treatment with AMD3100. Neutrophil and lymphocyte numbers were significantly elevated compared with hematologic values from both placebo treated cats (Fig. 2.1) as well as age-matched controls. The effect was transient and otherwise no signs of toxicity were indicated by physical exam and clinical chemistry. Leukocytosis secondary to AMD3100 treatment was previously reported in humans and is currently being investigated as a method for

atraumatically harvesting CD34+ bone marrow stem cells in conjunction with G-CSF [23] . The leukocytosis is thought to result from a CXCR4 blockade on cells of the bone marrow disrupting the local chemotactic effect of SDF-1α resulting in release of cells into the peripheral blood.

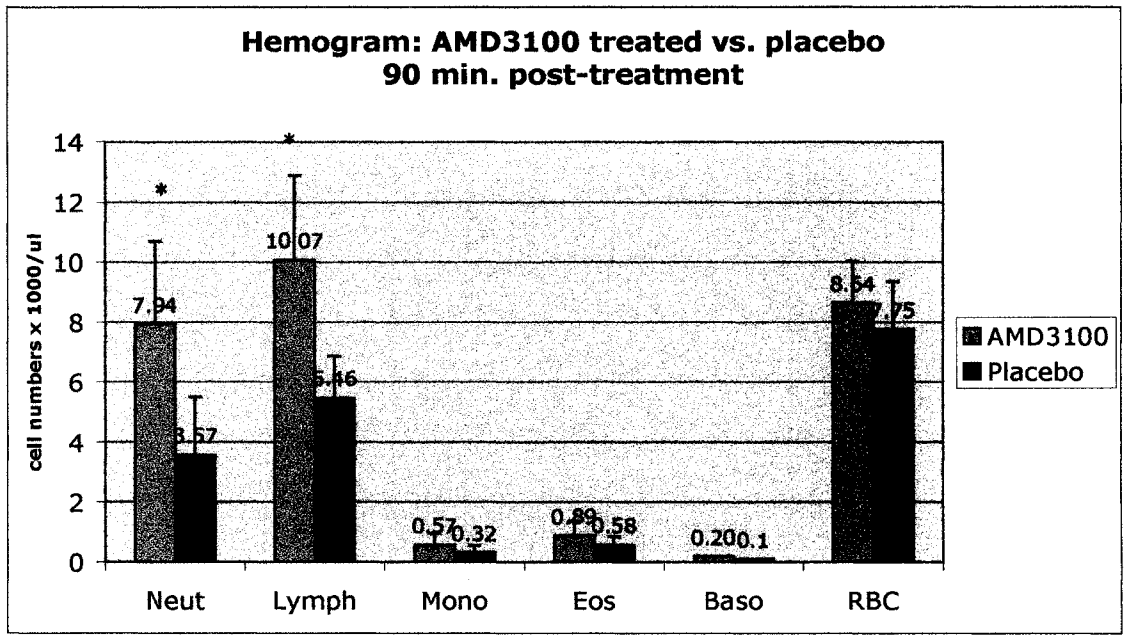


Figure 2.1: AMD3100-induced leukocytosis. Significant transient elevations of neutrophils and lymphocyte numbers occurred at 90 minutes post treatment.

Blood proviral loads

AMD3100 treatment resulted in statistically significant decrease ($p < 0.05$) in proviral loads in IV inoculated cats on weeks 2, 5, 7 and 9 (Fig. 2.2). Reduction in provirus ranged from 56% (week 3) to 94% (week 5) and averaged 77% (percent of untreated FIVC infected controls) over the first 12 weeks of the study. By 17 weeks post-inoculation viral loads were similar between treatment and control groups.

No viral inhibition in PBMC was seen in cats challenged oronasally at any time point (Fig 2.3). The AMD3100 treated group had a 6-fold increase in viral loads at week

one post challenge that was not statistically significant and attributable primarily to one cat (#4118) with a blood proviral concentration of 2.6 x 10^4 copies per million PBMC.

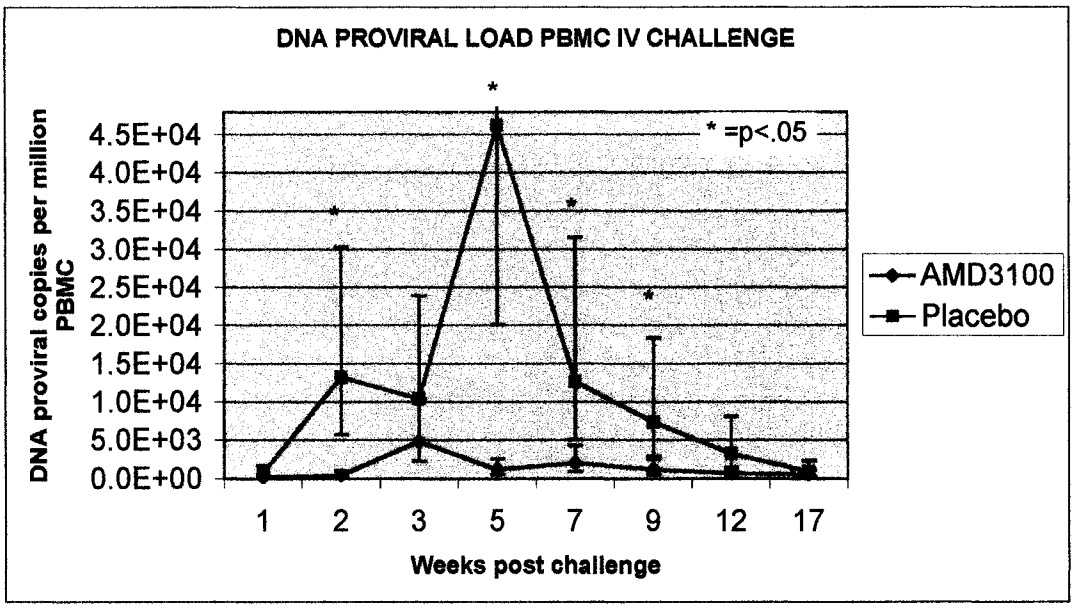


Figure 2.2: AMD3100 reduced PBMC proviral concentration in cats challenged via intravenous route as measured by DNA real time PCR for FIV gag. Inhibition was statistically significant ($p < 0.05$) at weeks 2, 5, 7 and 9 post- challenge.

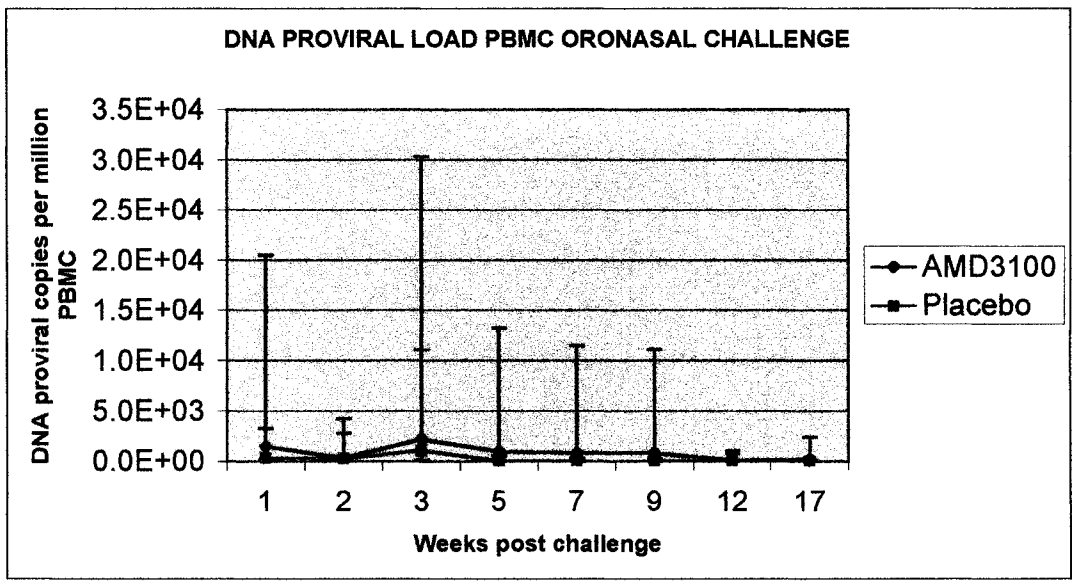


Figure 2.3: AMD3100 failed to inhibit PBMC proviral loads in cats inoculated oronasally. Peak proviral loads were reduced with mucosal inoculation compared with IV inoculation.

CD4+ and CD8+ Tcell- flow cytometry

Sham treated cats experienced a greater reduction in CD4+ T cell numbers after IV challenge than AMD3100 treated cats that was statistically significant ($p < 0.01$) on weeks 2, 5, 7 and 9 (Fig. 2.4) with the treated group averaging 86% higher CD4 counts between weeks 2 and 9. CD8 numbers were also increased slightly at week 2 ($p < 0.01$) in the IV challenged bicyclam treated group (Fig. 2.5). This group also experienced increased CD4/CD8 ratios on weeks 3,5,7,9 and 12 ($p < 0.05$) (Fig 2.6). By comparison oronasally inoculated cats had no significant difference in CD4 numbers and a decrease in CD4:8 ratio in AMD3100 treated group at one time point (week 9, $p < 0.05$ data not shown).

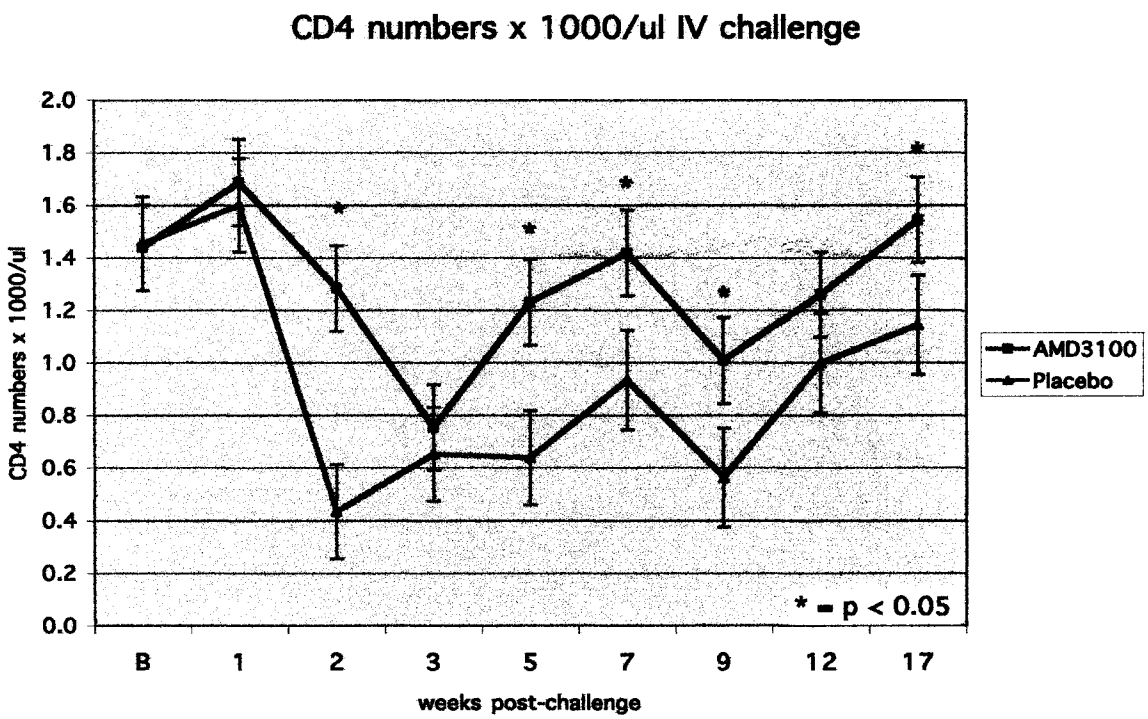


Figure 2.4: AMD3100 had a partially protective effect on CD4 numbers in the IV inoculated group at weeks 2,5,7,9 and 17 post challenge.

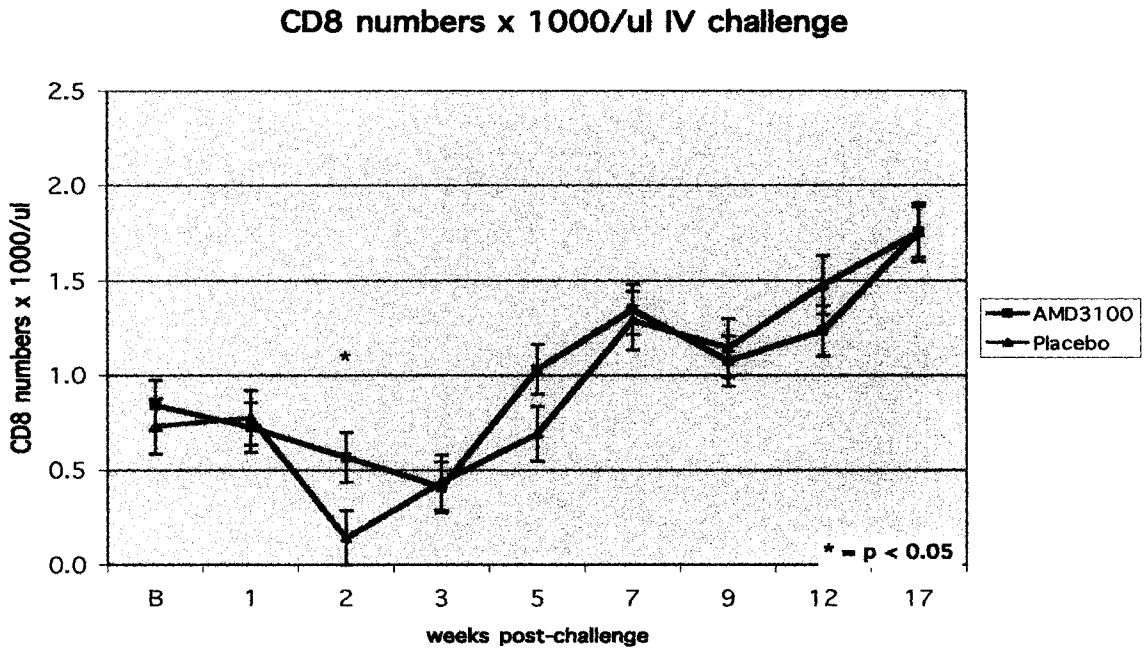


Figure 2.5: CD8 numbers dropped over the first 2 to 3 weeks then rebounded to levels much higher than baseline by 17 weeks post inoculation. With the exception of week two there was no difference in CD8 numbers between AMD3100 treated and sham treated cats.

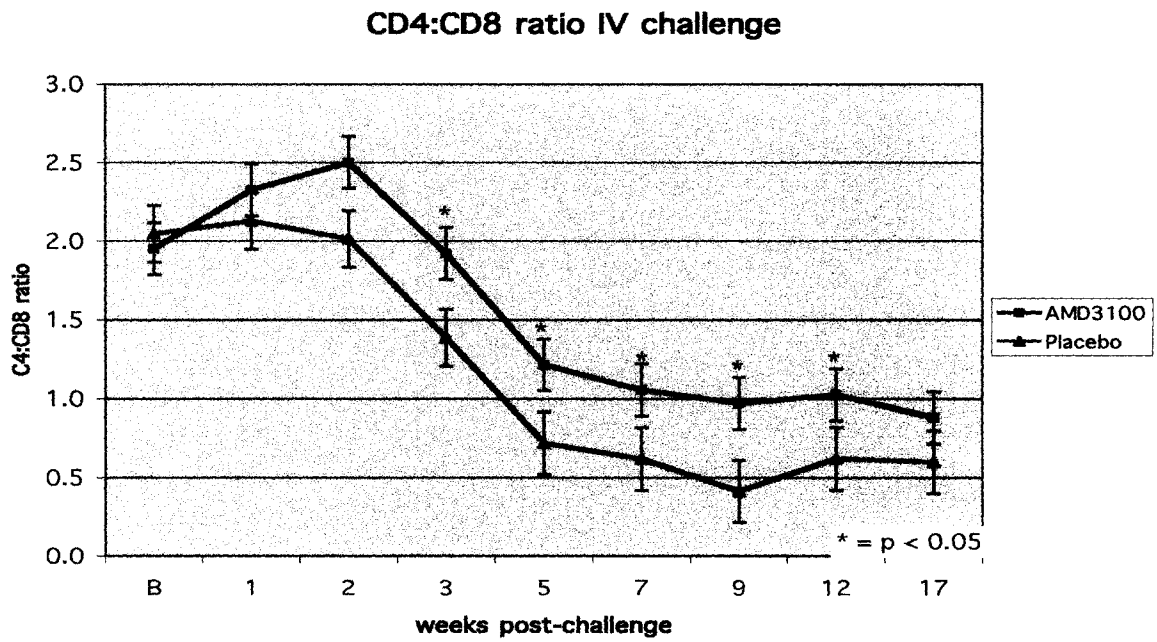
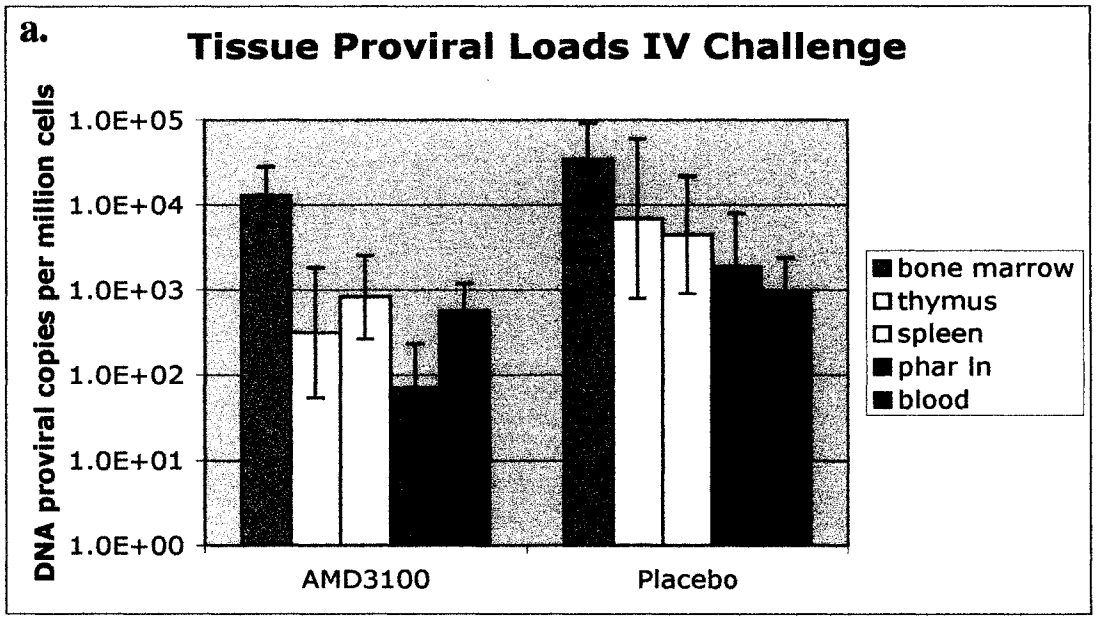


Figure 2.6: CD4:CD8 ratios decreased in all cats but were significantly higher in AMD3100 treated cats over weeks 3-12 primarily due to higher CD4 numbers in this group.

Infected cats from all groups experienced a slight decrease in CD8+ T cells after challenge until week 2 or 3 when CD8 numbers began to rebound and steadily increase through the end of the study. CD8 numbers at week 17 increased an average 79% in placebo cats. The phenomenon of CD8 cell expansion has been described in both FIV [24] and HIV [25, 26] infection and is attributable primarily to expansion of virus specific cytotoxic T-cells.

Tissue provirus

At the termination of the study (6 mo p.i.) cats were euthanised and tissues (bone marrow, thymus, pharyngeal lymph node and spleen) were assayed for FIV provirus by real time DNA PCR (Fig 2.7). Although provirus was reduced in all tissues examined from IV inoculated AMD3100 treated cats the difference was not statistically significant.



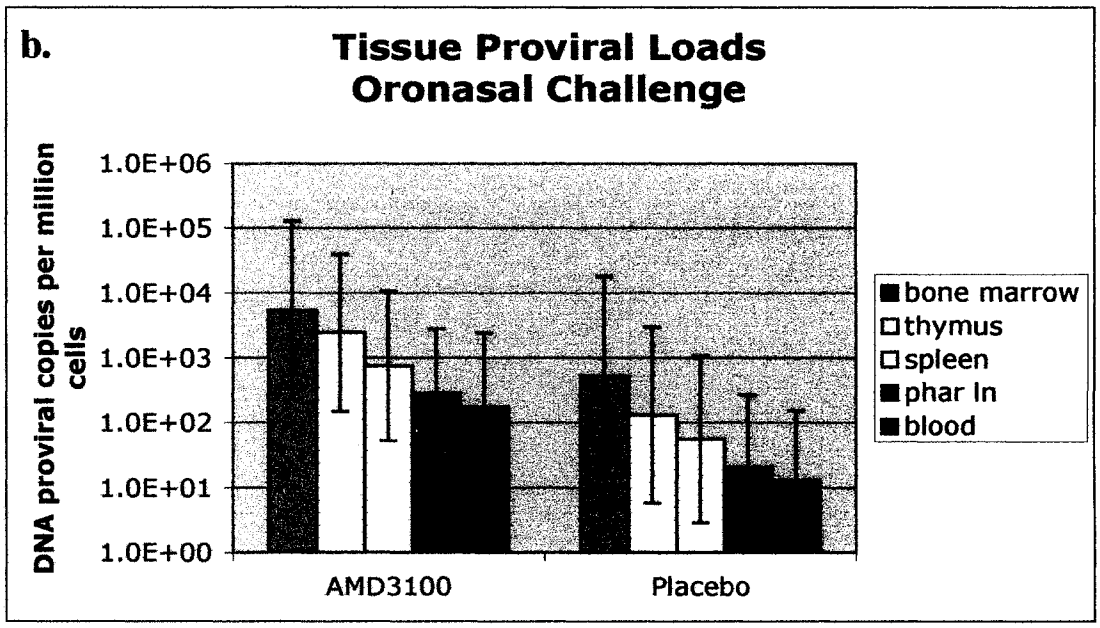


Figure 2.7: Real time DNA PCR performed on tissues at 6 months post-inoculation. There was no significant difference in tissue proviral loads between AMD3100 treated and sham treated controls with either intravenous (**a**) or oronasal (**b**) inoculation.

DISCUSSION

Here we have demonstrated the activity of the bicyclam drug AMD3100 in reducing blood proviral loads and preventing CD4 loss in FIV infection when given prior to intravenous virus challenge. However, the drug failed to inhibit infection in cats inoculated mucosally and had no antiviral effect in chronically infected cats. The latter results in particular are in contrast with in vitro studies with AMD3100 demonstrating consistent potent inhibition of FIV. This contrast may reflect reduced tissue distribution of AMD3100 or an alternate receptor used by FIV in vivo.

Previous pharmacokinetic studies in cats demonstrated blood levels of AMD3100 above 90% of the in vitro EC50 for greater than 10 hr with a single 0.5mg/kg subcutaneous injection [27] . Doubling the dose to 1.0 mg/kg in our study was likely to have provided prolonged antagonism of CXCR4, however without pharmacokinetic data we cannot be sure that sufficient plasma concentrations were achieved for a full 12 hours between treatments. Furthermore while compartmental modeling has been attempted there is no published data regarding actual tissue distribution of AMD3100. Given the drugs lipophilicity it has been speculated that distribution is likely to occur from plasma proteins to body fat [28] . It is likely that greatly reduced concentration of the drug are achieved in the mucosal compartment and lymphoid tissues compared with the plasma. Hence, we postulate that the reason for failure to inhibit virus with mucosal challenge was lack of sufficient drug concentration within mucosal tissues allowing for large numbers of cells to become infected and infection to spread among cells outside the

blood compartment. Indeed the thymus, bone marrow and lymph node contained significantly higher proviral loads than blood suggesting more active viral replication in those tissues. In addition to poor drug distribution in tissues, lack of antiviral effect in chronic infection may also reflect relatively low viral replication at that time. The antiviral activity of AMD3100 is through blocking new viral entry events. Thus, in the face of very low and decreasing viral loads at 22 weeks post challenge, the treatment period may need to be extended to see a reduction in provirus.

While the half life of retroviruses are relatively short (< 24 hours at 37°C extracellularly under ideal pH conditions [29, 30]) it is possible that FIV could distribute to lymphoid tissues within hours after inoculation potentially escaping higher drug concentrations in plasma. Indeed, studies of acute SIV infection have demonstrated viral particles disseminated to lymphoid tissues as soon as 18 hours post mucosal inoculation [31]. Assuming a requirement for CXCR4 binding by FIV, the only avenue for virus escape would be through diffusion into an extravascular compartment where drug concentrations may be lower or through binding CXCR4 receptors in the blood compartment approaching 12 hr post treatment when drug concentrations are lowest. It is more difficult to explain the difference in antiviral effect with route of viral inoculation between weeks three and five. It is likely that by that time the virus would have become disseminated throughout the body regardless of route of inoculation. It is puzzling why proviral loads were not also reduced by the drug during the same viremic phase in oronasally inoculated cats. One possible explanation is that the major antiviral effect occurred at the time of intravenous viral inoculation and that once the virus was able to escape the blood compartment and gain access to lymphoid tissues where drug

concentrations are possibly lower. Differences in viral loads at week five would then be only a reflection of earlier antiviral effects.

The possibility of drug resistance with prolonged antiviral use must also be considered. Thus far viral escape mutants have not been demonstrated in animals undergoing long-term AMD3100 treatment, however, this would seem an unlikely explanation for the failure of the initial course of bicyclam in the present study. Future studies to determine whether escape mutants occurred with long term AMD3100 treatment include virus isolation from plasma of treated in cats. Sensitivity to AMD3100 will be assayed in vitro and compared with virus from untreated cats as well as original virus stock to monitor for drug resistance.

It is possible that a higher dose of AMD3100 could be more effective in inhibiting FIV infection in vivo. Due to limited resources, our toxicity study was designed with only 2 cohorts and based the lower dose (0.5 mg/ml) on a previous report of toxicity (hypermagnesemia) in the feline with doses above 0.5mg/kg [27] . However we did not detect an elevation in blood magnesium in our study even with a 1.0 mg/kg dose. Additionally we did not identify any other side effects that have been reported using similar doses in other species (sedation, spasms dyspnea, diarrhea, tachycardia, and thrombocytopenia [28, 32] implying that the feline may be more resistant to AMD3100 induced toxicity.

Here we have demonstrated inhibition of a primary strain of FIV in vivo by a CXCR4 chemokine receptor antagonist. The effects of the drug were much less potent compared with in vitro studies and were dependent upon route of inoculation. While

these results suggest a role for CXCR4 in FIV infection they also raise the possibility of alternate receptor use by FIV in vivo.

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CHAPTER THREE

TISSUE DISTRIBUTION OF FELINE IMMUNODEFICIENCY VIRUS, CXCR4 AND PHENOTYPE

ABSTRACT

Here we describe several methods for detection of feline immunodeficiency virus (FIV) and CXCR4 in tissues and cell suspensions as well as a method for simultaneous detection of CXCR4, cell phenotype and FIV provirus. FIV was detectable by immonocytochemistry and flow cytometry in cultured Crandell feline kidney cells (CrFK) using several monoclonal antibodies. FIV staining was possible in infected lymph node frozen sections using the anti-FIV gag monoclonal antibody (mAb) PAK3-2C1 but low sensitivity limited virus detection to acutely infected lymphoid follicles. Real time DNA PCR provided enhanced sensitivity of FIV detection and was used to measure proviral loads across multiple tissues in acute and chronic infection. To obtain enhanced FIV detection in tissues we employed real time DNA PCR to measure provirus across multiple tissues in acute and chronic infection. CXCR4 positive cells were most concentrated in lymphoid follicles as assayed by both immunohistochemistry and in situ

hybridization in lymphoid tissues. CXCR4 labeling by flow cytometry was most consistent and could be blocked by the CXCR4 specific antagonist AMD3100. To colocalize FIV with CXCR4 and cell phenotype, fluorescence activated cell sorting (FACS) for CXCR4, CD4, CD8 and CD21 was coupled with real time DNA PCR to measure proviral burdens.

BACKGROUND

The pathogenesis of FIV is quite similar to that of human immunodeficiency virus (HIV). Early transmucosal infection of dendritic cells and T cells [1] is followed by an acute viremia that coincides with a rapid decline in CD4 T cell numbers. The disease then progresses into a chronic phase with reduced viremia and a slower progressive decline in CD4 T cells [2-5] . The terminal phase of FIV infection is characterized by suppression of hemic elements (particularly CD4+ T lymphocytes) and recrudescence of plasma viremia resulting in immunological decompensation and increased susceptibility to opportunistic infection [6-10] . FIV shows similar tissue tropism to HIV including a replication in T lymphocytes [6, 11-13] , macrophages [3, 5, 14, 15] , B cells [10, 16] , megakaryocytes [17] , monocytes [18] and cells of the nervous system including astrocytes and microglia [7, 19-23] . *In vitro* FIV studies indicate that both CD4+ and CD8+ T cells are susceptible to productive virus infection [11, 24] . In contrast to primate lentiviruses, FIV does not use CD4 [25-27] to infect cells however the receptor CD134 has been shown to be required in conjunction with CXCR4 for entry [28, 29] . The events surrounding the mechanism of FIV interaction with these two surface

receptors are currently unknown such as whether (analogous to HIV) binding to one receptor induces a conformational change allowing for subsequent receptor binding.

Entry of both FIV and HIV into cells involves interaction of the V3 loop of the viral envelope gp120 glycoprotein with the X4 or R5 receptor [30-33] , the C-type lectin DC-SIGN [34-38] as well as cell surface heparans [39] . These similarities make FIV a particularly useful model for the study of interactions of T-cell tropic HIV-1 and cell surface (co)receptors. While much is known about FIV cell tropism and coreceptor usage in vitro there is little information correlating the distribution of virus in vivo with cell phenotype or coreceptor use. The importance of CXCR4 or CD134 expression in vivo and its correlation with FIV infection is currently unknown. Here we have investigated multiple anti-human CXCR4 and phenotype antibodies as well as numerous anti-FIV antibodies for their usefulness in ICC, IHC and flow cytometry and describe a method for concurrent detection of CXCR4, FIV and cell phenotype in cell suspensions using a combination of fluorescence activated cell sorting with real time PCR. We then used the most reliable methods developed in these studies to compare tissue proviral loads in acute versus chronic infection and in animals infected by intravenous versus oronasal routes.

MATERIALS AND METHODS

Animals and tissue processing

CXCR4 studies

Two adult SPF naive cats from Colorado State University breeding colony were anesthetized with ketamine/acepromazine and euthanized by intravenous pentobarbital sodium injection. Pharyngeal, axillary and mesenteric lymph nodes were collected and either immediately placed in OCT (Sakura Finetek USA Inc, Torrance, CA) and snap frozen in liquid nitrogen or placed in STF fixative (Streck Labororatories Inc. La Vista, NE) for use in immunohistochemistry. A subset of tissues was also placed in cold RPMI 1640 (Invitrogen Corp. Grand Island, NY) supplemented with 20% fetal calf serum, homogenized, washed in phosphate buffered saline (PBS) and used for flow cytometry.

FIV studies

Three 11wk. old cats from a specific pathogen free (SPF) breeding colony maintained at Colorado State University (Fort Collins, CO) were inoculated intravenously with >100% tissue culture infectious dose (TCID) of acute phase plasma pools of FIVC-Pgmr. Three weeks post inoculation the 3 cats and one age matched naïve control were anesthetized with ketamine/acepromazine and euthanized by intravenous pentobarbital sodium injection. Pharyngeal, submandibular and mesenteric lymph nodes, thymus, spleen, intestine and liver were collected in formalin, ethanol, and Streck (a

proprietary coagulative fixative) and portions of these tissues were snap frozen in OCT for use in immunohistochemistry. At the same time one half of each lymph node was immediately homogenized and suspended in cold RPMI media for CXCR4 and cell phenotype analysis by flow cytometry.

Proviral distribution studies

Six 6-8 month old cats were anesthetized with ketamine-acepromazine and inoculated intravenously with >100% TCID an acute phase plasma pool of FIV-C-Pgmr. Ten 3-4 month cats were anesthetized with ketamine-acepromazine and inoculated oronasally with >100% TCID of FIV-C-Pgmr once daily for two days.

CXCR4 immunocytochemistry

PBMC from specific-pathogen-free cats were recovered from blood by Ficoll-Hypaque gradient centrifugation and either analyzed fresh or resuspended at 2 x 10^6/ml in RPMI 1640 medium supplemented with 20% fetal bovine serum, 2% glutamine, 0.5% 2-mercaptoethanol, and 1% Pen-strep and 100 IU hrIL-2/ml and stimulated with 5μg/ml concanavalin A. Following 3 days of culture cells were added to triplicate wells at $2x10^5$/well of a 96 well round bottom plate, washed in PBS and blocked with 3% goat gamma globulin for 15min. Cells were incubated with 1:50 anti CXCR4 antibody mAb 44717 (R&D Systems Inc, Minneapolis, MN)) for one hour at room temperature in presence or absence of the CXCR4 antagonist AMD3100 followed by goat anti mouse-Cy3 conjugate (Sigma, St Louis, MO) at 1:100 at room temperature for 30 min. Samples

were washed twice and centrifuged at 900-1000 x 10min with PBS between each incubation step. Live cell suspensions were placed onto slides with coverslips and analyzed using an Olympus BX60 fluorescent microscope microscope and digital images captured.

CXCR4 immunohistochemistry

Streck fixed paraffin embedded lymphoid tissues were sectioned, deparaffinized by brief heat treatment and rehydrated through xylene and a series of graded alcohols, then washed in TENT solution (0.05 M Tris, pH7.4, 0.001 M EDTA sodium, 0.15 M NaCL, 0.05% Tween). Antigen retrieval was performed by microwave treatment or boiling at 100°C for 5 minutes in citrate Antigen Unmasking Solution (Vector Laboratories, Burlingame, CA). Slides were allowed to cool to room temperature and washed in TENT solution. Endogenous peroxidases were inactivated by immersing the slides in a Coplin jar with 3% H_2O_2 in phosphate buffered saline (PBS, pH 7.4) for 5 minutes on an orbital rocker with agitation. Sections were blocked for 15 minutes with 5% goat serum in TNB (TSA Systems, NEN, Boston, MA) blocking buffer. Mouse anti-human CXCR4 mAb 44717 crossreactive with feline CXCR4 diluted to 1:200 was applied and slides were incubated for 2 hours in a humidified chamber at 37°C. Sections were washed with TENT and horseradish peroxidase conjugated goat anti-mouse antibody was applied at a dilution of 1:100 for 30 minutes at room temperature. Slides were washed and signal was amplified using the tyramide signal amplification (TSA Fluorescence Systems, NEN) using tyramide conjugated to Cy3 fluorochrome and performed according to manufactures instructions. Following fluorescent antibody

labeling, cell nuclei were stained with 1μg/ml 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma, St. Louis, MO). Sections were dehydrated through graded alcohols to xylene mounted with Vectashield anti-fade medium (Vector) and coverslipped. Histologic sections were examined using an Olympus BX60 fluorescent microscope microscope and digital images captured.

CXCR4/cell phenotype dual fluorescence immunohistochemistry

Following CXCR4 staining using methods described above slides using phenotype antibodies of mouse origin were incubated with unlabeled goat anti-mouse IgG whole molecule antibody at 1:50 to saturate any of the remaining mouse CXCR4 antibody. Slides were then washed in TENT and incubated with anti-phenotype antibody (1:50, 1:100 and 1:200) at room temperature for one hour. Slides were washed in TENT and incubated for 30 minutes with secondary antibody (1:50) at room temperature. Phenotype antibodies used include: T cells; polyclonal rabbit anti-CD3 (Sigma), macrophages; Mac 387 (Serotec), B cells; anti-murine CD45R/B220 (Pharmingen), dendritic cells; p55 (DakoCytomation). Secondary antibodies include: Fluorescein isothiocyanate (FITC) or R-phycoerythrin (PE) conjugated goat anti-rabbit or goat anti-mouse IgG whole molecule antibody (Sigma).

CXCR4 in situ hybridization

A partial feline CXCR4 cDNA clone (p104) isolated from a feline T-cell line 3201 cDNA library and cloned into vector pCR2.1 (Invitrogen) was a kind gift from Dr. Jim Mullins. The insert was sequenced and revealed one predicted amino acid change as

compared to feline CXCR4 Genebank sequence U63558 consisting of a Lys to Arg change in an intracellular region between TM V and TM VI. A CXCR4 riboprobe was synthesized and digoxigenin labeled by Lofstrand Labs Limited (Gaiothersburg, MD).

Tissue sections were deparaffinized and rehydrated as described for IHC. Slides were incubated with 5 mM levamisole for 20 minutes, washed with 1X SSC buffer (Sigma, St Lois, MO), incubated in 0.2 N HCL for 20 minutes, and washed again with SSC buffer. Following endogenous peroxidase blocking with 5% H_2O_2 in methanol for 30 minutes sections were digested with 10 μg/ml proteinase K (PK) in buffer containing 10mM Tris (pH 7.4) and 2 mM CaCl2 for 5 minutes at 37°C. Digestion was stopped with 0.1 M glycine in PBS. The slides were then incubated in 0.1 M triethanolamine-0.25% acetic anhydride solution for 10 minutes, washed in 2X SSC, incubated in 0.1 M Tris (pH 7.4)-0.1 M glycine solution for 15 minutes, and washed in 2X SSC. Prehybridization was performed at 50°C for 15 minutes with hybridization solution containing 50% deionized formamide, 1X SSC, 1X Denhardt's solution, 5 mM NaPO4 (pH 6.8), 0.1% sodium dodecyl sulfate, 250 μg.ml of salmon sperm DNA, 5% dextran sulfate, and 250μg/ml tRNA (all Sigma, St. Louis, MO). Hybridization solution containing 50 ng digoxigenen-labeled CXCR4 antisense or nonsense probe per slide was applied and slides were coverslipped. Sections were heated to 65°C for 10 minutes, chilled for 5 minutes on ice, and hybridized overnight in a humidified chamber at 50°C. Following hybridization, slides were washed with 4X SSC-50% formamide for 1 hour at 53°C and then 2X SSC for 2 minutes. An Rnase mixture (1 unit/ml Rnase T1 and 20 μg/ml Rnase A (Sigma, St. Louis, MO) for 30 minutes at 37°C) was used to digest excess probe and wash steps were repeated. Slides were blocked for 1 hour in a buffer

containing 5% horse serum, 2.5% sheep serum, 2.5% goat serum, 0.3% Tween 20, 1% Boehringer blocking agent (Boehringer Mannheim, Indianapolis, IN), 100 mM Tris (pH 7.4), and 150 mM NaCl. Slides were blocked for an additional 30 minutes in TNB blocking buffer (TSA™ Systems, NEN Life Science Products, Boston, MA).

For chromagen signal detection, a sheep anti-digoxigenin peroxidase conjugated antibody (Roche Molecular Biochemicals, Indianapolis, IN) diluted1:100 in TNB buffer was applied for 30 minutes at room temperature. The sections were washed in TNT buffer (0.1 M Tris-Hcl pH 7.5, 0.15 M NaCL, and 0.05% Tween 20) three times for 5 minutes each. Signal was amplified with tyramide using the TSA™ Plus DNP System (NEN Life Science Products, Boston, MA) according to the manufacturer's directions. Briefly, the tyramide-DNP reagent, diluted 1:50 in NEN diluent was applied to the slides for 15 minutes. After three washes with TNT buffer, the alkaline phosphatase-conjugated anti-DNP antibody provided in the kit, diluted 1:50 to 1:100 in TNB buffer, was applied for 30 minutes. After three more washes with TNT buffer, the sections were incubated with 5-bromo-4-chloro-3-indolyl phosphate (BCIP)-nitroblue tetrazolium (NBT) substrate (Amresco, Solon, OH) for 10-20 minutes in the dark. Following development sections were washed in TNT buffer, counterstained with nuclear fast red (Vector Laboratories, Burlington, CA), dehydrated in graded alcohol, cleared in xylene and mounted with Cytoseal XYL (Stephens Scientific, Kalamazoo, MI).

CXCR4 flow cytometry

PBMC from specific-pathogen-free cats were recovered from blood by Ficoll-Hypaque gradient centrifugation and either analyzed fresh or resuspended at 2 x 10^6/ml in

RPMI1640 medium supplemented with 20% fetal bovine serum, 2% glutamine, 0.5% 2-mercaptoethanol, and 1% Pen-strep and 100 IU hrIL-2/ml and stimulated with 5μg/ml concanavalin A. Either fresh or cultured cells were added to triplicate wells at $2x10^5$/well of a 96 well round bottom plate, washed in PBS and blocked with 3% goat gamma globulin for 15min. Cells were incubated with 1:50 anti CXCR4 mAb 44717 for one hour at room temperature followed by goat anti mouse-Cy3 conjugate at 1:100 at room temperature for 30 min. Cells were washed twice and centrifuged at 900-1000 x 10min with PBS between each incubation step. The samples were then placed into 5 ml tubes and analyzed by flow cytometry. CXCR4/phenotype colabeling studies were performed using phycoerythrin (PE) conjugated CXCR4 mAb 44717P and either B cell mAb B220FITC (Serotec) or anti feline T-cell CD5FITC (Southern Biotech).

CXCR4 fluorescence-activated cell sorting

Following overnight incubation at 37°C in RPMI supplemented with 20% fetal bovine serum, cells were washed and suspended in buffered saline containing 2% heat inactivated fetal bovine serum and incubated with allophycocyanin (APC) conjugated anti-CXCR4 44717, FITC-conjugated anti feline CD4 and CD8 antibodies (Southern Biotech) and phycoerythrin-conjugated anti B-cell antibody (Serotec) crossreactive with dog and cat (Serotec) or isotype matched control antibodies. Samples were incubated at 4°C in the dark for five hours then resuspended and sorted using a DakoCytomation MoFlo high performance cell sorter. Gates were set based on isotype controls and 500,000 events were collected for the majority subsets. For rare cell fractions a minimum of 10,000 cells were collected.

FIV immunocytochemistry

FIVA persistently infected and uninfected control CrFK were placed in eight well chamber well culture slides in media containing minimum essential medium (MEM), 10% FBS, 2% glutamine and 1% Pen Strep and allowed to proliferate to confluent monolayers. Infection status of positive and negative control cells was confirmed by p24 antigen ELISA on culture supernatant. Cells were washed twice with phosphate buffered saline (PBS) and fixed for ten minutes at room temperature with 100% each of ethanol, methanol, acetone, or 2% paraformaldehyde. Paraformaldehyde-fixed samples were further permeabilized using Caltag permeabilization solution (Caltag Laboratories, Burlingame, CA). Slides were washed again with PBS and incubated with serial dilutions of either PAK3-2C1, SU1-30, DF10, or 51G11 for 1 hr. at room temperature followed by incubation with sheep anti-mouse-Cy3 conjugated antibody (Sigma) at 1:50 concentration for 30 minutes.

FIV frozen section IHC

Tissues from acutely infected cats were placed into molds containing OCT embedding compound and snap frozen in liquid nitrogen. Tissues were sectioned at -20°C on a cryostat and placed immediately into cold ethanol for 5 minutes then air dried for 15 minutes at room temperature before being stored at -70°C before use in IHC. Slides were thawed and washed with wash buffer (DakoCytomation, Carpinteria, CA) prior to incubation for 5 minutes with 5% sheep serum. Slides were then incubated with serial dilutions of either PAK3-2C1, SU1-30, DF10, or 51G11 for 1 hr. at room

temperature followed by incubation with lymph node adsorbed sheep anti-mouse-Cy3 conjugated antibody (Sigma) at 1:50 concentration for 30 minutes.

FIV paraffin embedded sections

Formalin, Streck, and ethanol fixed tissues from acutely infected cats (FIVC-Pgmr) or pelleted CRFK (FIVA-2546 and naive) or PBMC (FIVC-Pgmr and naïve) were sectioned, deparaffinized by brief heat treatment and rehydrated through xylene and a series of graded alcohols, then washed in TENT solution (0.05 M Tris, pH7.4, 0.001 M EDTA sodium, 0.15 M NaCL, 0.05% Tween). Slides were incubated for 15 minutes with 5% goat serum followed by either PAK3-2C1, SU1-30, DF10, vif , 51G11 or a cocktail of all of the above. Lymph node adsorbed sheep anti mouse conjugated to Cy3 secondary antibody was used. Antigen retrieval was performed on a subset of slides for each antibody including microwave treatment or boiling at 100°C for 5-15 minutes in buffers of ph 6, 7 or 10.

FIV flow cytometry

CrFK FIV-A-2546 positive or naïve cells were trypsinized from their culture flasks, washed twice with PBS. 5 x 10^5 cells were plated into a 96 well round bottom plate in triplicate. Cells were fixed in 75% ETOH for 10 minutes, washed in PBS and blocked with 3% goat gamma globulin for 15min. Cells were incubated with 1:50 of the primary antibody (PAK3-2C1, SU1-30, p24 or 51G11) for one hour at room temperature then incubated with goat anti mouse-Cy3 conjugate at 1:100 at room temperature for 30 min. Cells were washed twice and centrifuged at 900-1000 x 10min with PBS between

each incubation step. The samples were then placed into 5 ml tubes and analyzed by flow cytometry. Additionally thymus and pharyngeal lymph nodes from 3 acutely infected (FIV-C-Pgmr) cats and one naïve cat were homogenized and suspended in cold RPMI then washed twice in PBS. Cells were plated and stained for FIV and analyzed by flow cytometry as described above.

Quantitative real time PCR

Real time quantitative DNA PCR adapted from a method by Pedersen et al was performed for each cell fraction. DNA was extracted using the QIAmp DNA blood Mini Kit (Qiagen, Valencia, CA). The 25μl PCR mixtures contained 5μl standard or sample, 0.5 ml (400 nM) of each primer, 0.2 ml (80nM) of probe, 6.3ul DNAse free water, and 12.5 μl TaqMan Universal PCR Master mix containing 10mM Tris-HCl (pH 8.3), 50mM KCl, 5mM MgCl2, 300uM each of dATP, dCTP, and dGTP, 600uM dUTP, 0.625U of AmpliTaq Gold DNA polymerase, and 0.25U uracil N-glycosylase (UNG) per reaction. FIV-C gag primer and probe sequences were as follows: forward 5'-ACT CAC CCT CCT GAT GGT CCT A-3', reverse 5'-TGA GTC AGC CCT ATC CCC ATT A-3') and probe FAM-5'-ACC ATT GCC ATA CTT CAC TGC AGC CG-3'-TAMRA. FIV C gag plasmid DNA was cut with the restriction enzyme BamHI and repurified.

A standard curve was generated for each plate with serial dilutions (10^9, 10^5, 10^2 of FIV-C gag plasmid DNA with 1X TE and 40 ng/ml salmon testes DNA (Sigma) as a DNA carrier. Samples and standards were run in triplicate on 96-well plates using the Biorad iCycler. Polymerase activation and amplification were performed using the

following protocol: 2 min. at 50°C, 10 min. at 90° C 45 cycles at 95° C for 15 seconds, 60° C for 1 minute.

RESULTS

CXCR4 immunocytochemistry

In order to determine the ability of the anti-human CXCR4 antibody to crossreact with feline cells, cultured feline PBMC were stained with anti CXCR4 mAb 44717 either in presence or absence of the CXCR4 specific receptor antagonist AMD3100. Staining of AMD3100 treated cells was greatly reduced compared with untreated PBMC (Fig. 3.1).

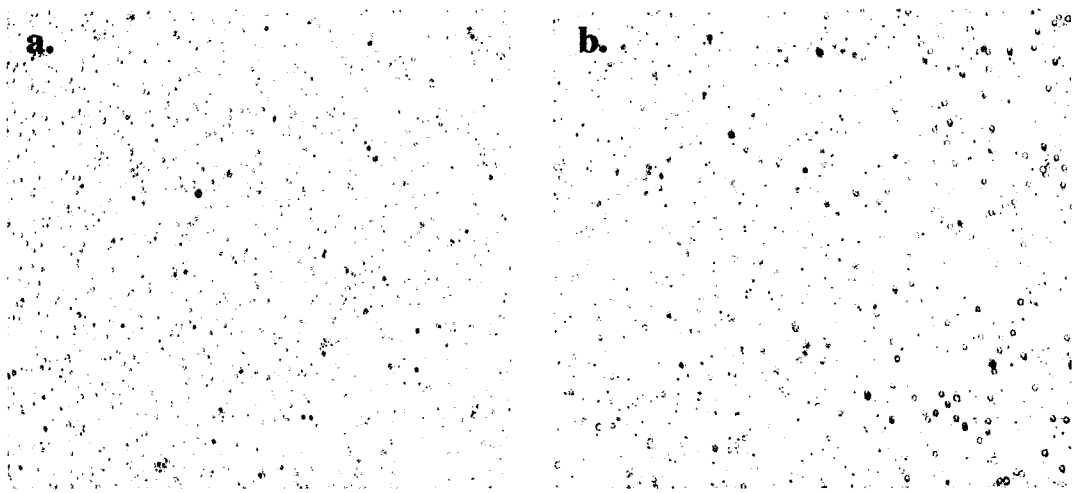

Figure 3.1: PBMC wet mounts. PBMC were stained with anti-CXCR4 mAb 44717 in the presence (a) or absence (b) of the CXCR4 specific receptor antagonist AMD3100. CXCR4 ---PE (red). PBMC---brightfield overlay.

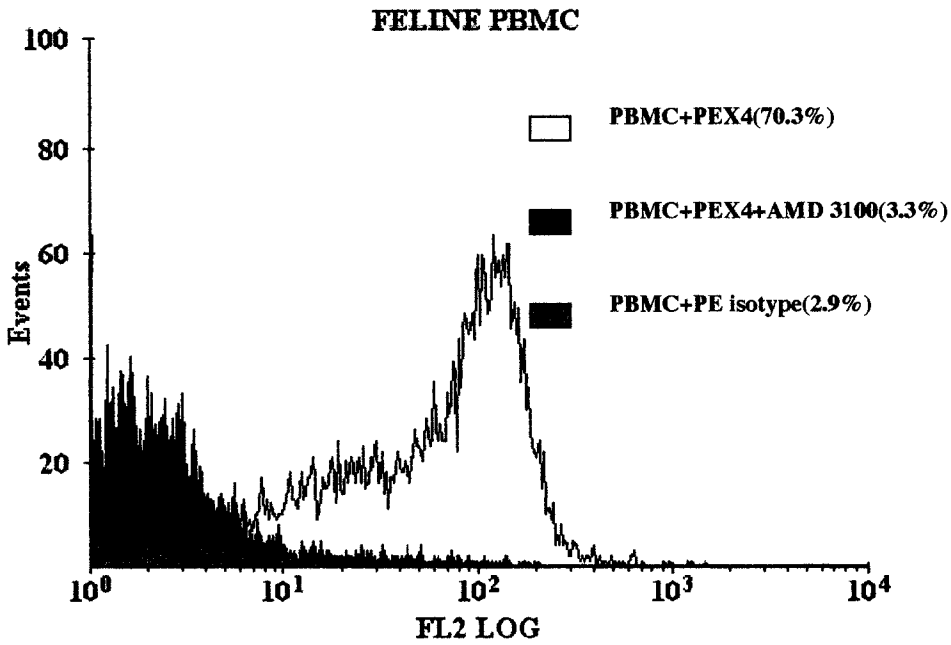

Figure 3.2: Flow cytometric analysis of cells from fig. 3.6. Anti-CXCR4 antibody 44717 is inhibited by the CXCR4 specific antagonist AMD3100.

CXCR4 flow cytometry

Feline PBMC used for immonocytochemistry studies were also analyzed by flow cytometry. Similar to results obtained by ICC, the addition of AMD3100 resulted in strong dose related inhibition of antibody binding (Fig. 3.2). CXCR4 expression was greatly upregulated by culturing cells at 37°C in the presence of Con A and IL-2. Maximum upregulation occurred by 3 days in culture (Fig 3.3) after which expression began to decrease.

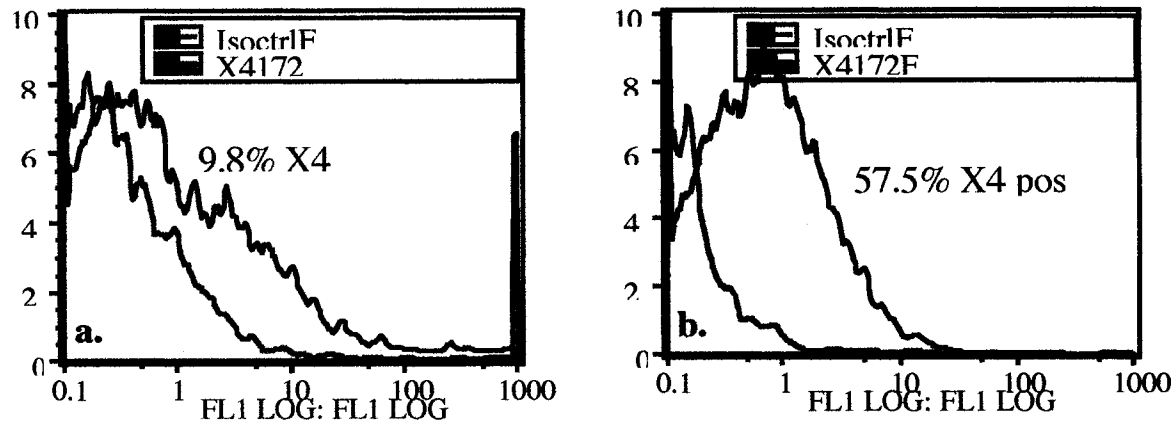

Figure 3.3: Flow cytometric analysis of CXCR4 expression on PBMC from fresh blood (**a**) compared with PBMC cultured with Con A and Il-2 for 3 days (**b**).

CXCR4 immunohistochemistry

In order to compare immunohistochemical and flow cytometric methods of CXCR4 detection pharyngeal lymph nodes from 2 naive cats were sampled and each lymph node was dissected longitudinally into 2 samples. One half of each lymph node was homogenized and examined for CXCR4 expression by flow cytometry while the opposite half was fixed in Streck tissue fixative for 24 hours, paraffin embedded, sectioned and examined by immunohistochemistry. Both samples were stained with anti-CXCR4 antibody 44717. A similar proportion of cells stained positive by each method (Fig. 3.4). The distribution of positive cells as assessed by immunohistochemistry was more concentrated throughout lymphoid follicles and germinal centers with fewer cells staining in paracortical regions and medulla (Fig 3.5). In contrast to results in flow cytometry and immunocytochemistry, AMD3100 failed to inhibit antibody binding in tissue sections.

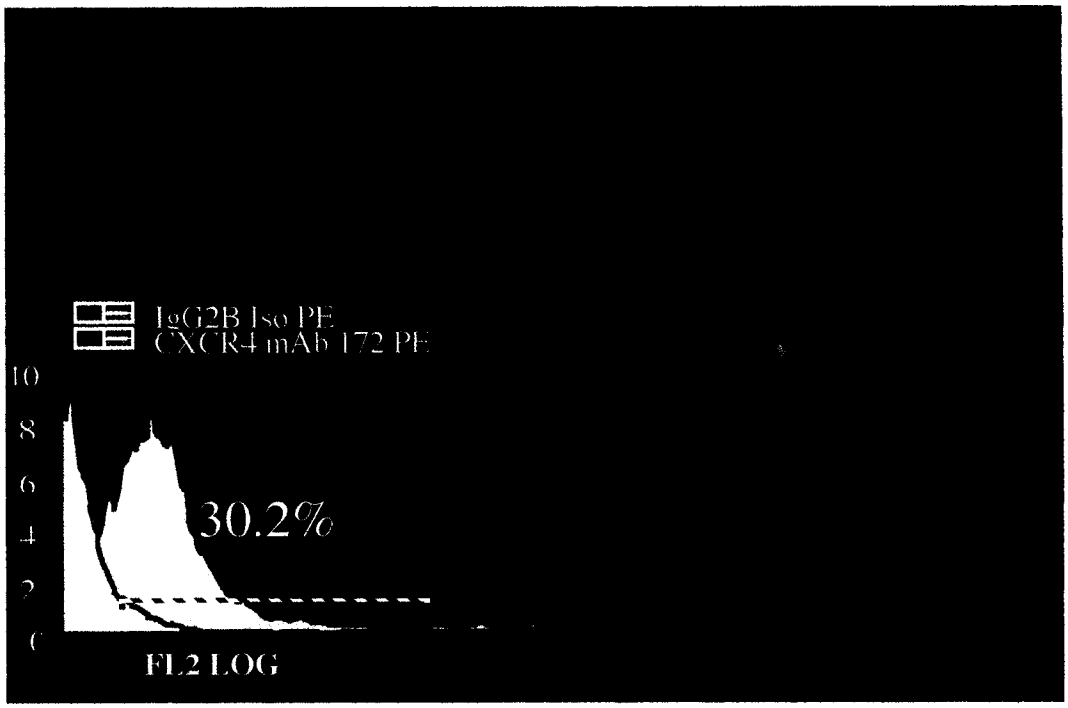

Fig 3.4: Immunohistochemistry and flow cytometry, pharyngeal lymph node. Half of a pharyngeal lymph node was fixed and stained for CXCR4 antigens (green). The other half of the same lymph node was homogenized, stained with the same antibody and analyzed by flow cytometry (inset histogram). Similar proportions of CXCR4 positive cells (approximately 30%) were detected using each method in this lymph node. IHC: CXCR4---FITC, nuclei---DAPI.

CXCR4/phenotype dual immunohistochemistry

In order to colocalize CXCR4 with cell phenotype fixed tissue sections were labeled with CXCR4 followed by either CD3, B220, Mac387, or p55. Although a very small number of CD3 positive cells in the gut colabeled with CXCR4 expression largely did not correspond with any of these phenotypes (Fig. 3.5).

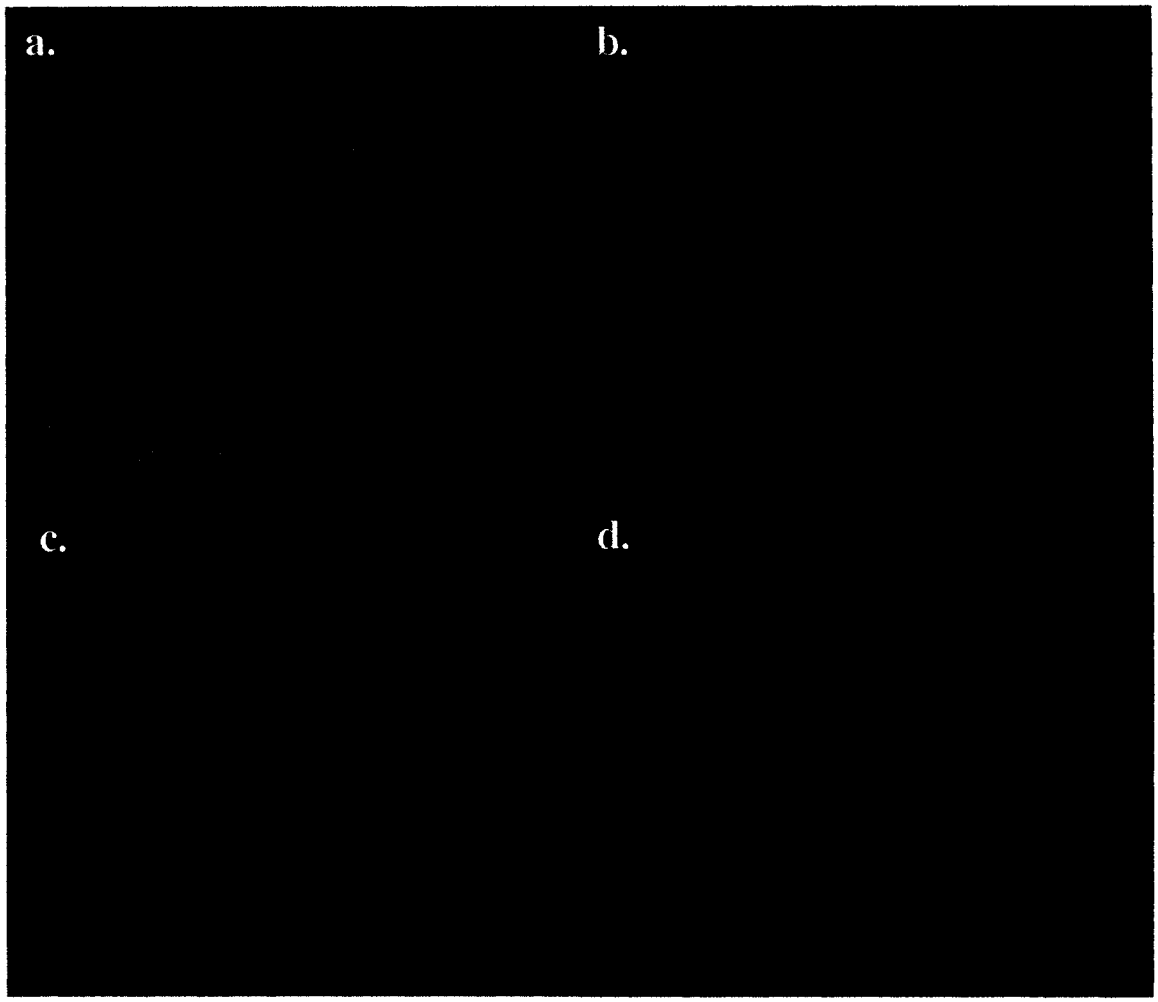

Figure 3.5: Dual fluorescence immunohistochemistry, lymph nodes and intestine. CXCR4 did not colabel with B220 (lymph node 200x) (**a**) CD3 lymph node 200x) (**b**), Mac 387 intestine 200x) (**c**) or p55 lymph node 100x) (**d**) in Streck fixed tissue sections. CXCR4---Cy3 (red), cell phenotype---FITC (green), nuclei---DAPI (blue).

CXCR4 in situ hybridization

Numerous CXCR4 positive cells were demonstrated throughout lymph node sections using RNA in situ hybridization. Similar to IHC detection, positive cells were most prominent in the follicular dark zone (Fig 3.6). An equivalent pattern of CXCR4 expression has been described in mice and other species [40, 41]. No staining occurred

in the lymph node when an irrelevant riboprobe was applied at the same concentration (not shown).

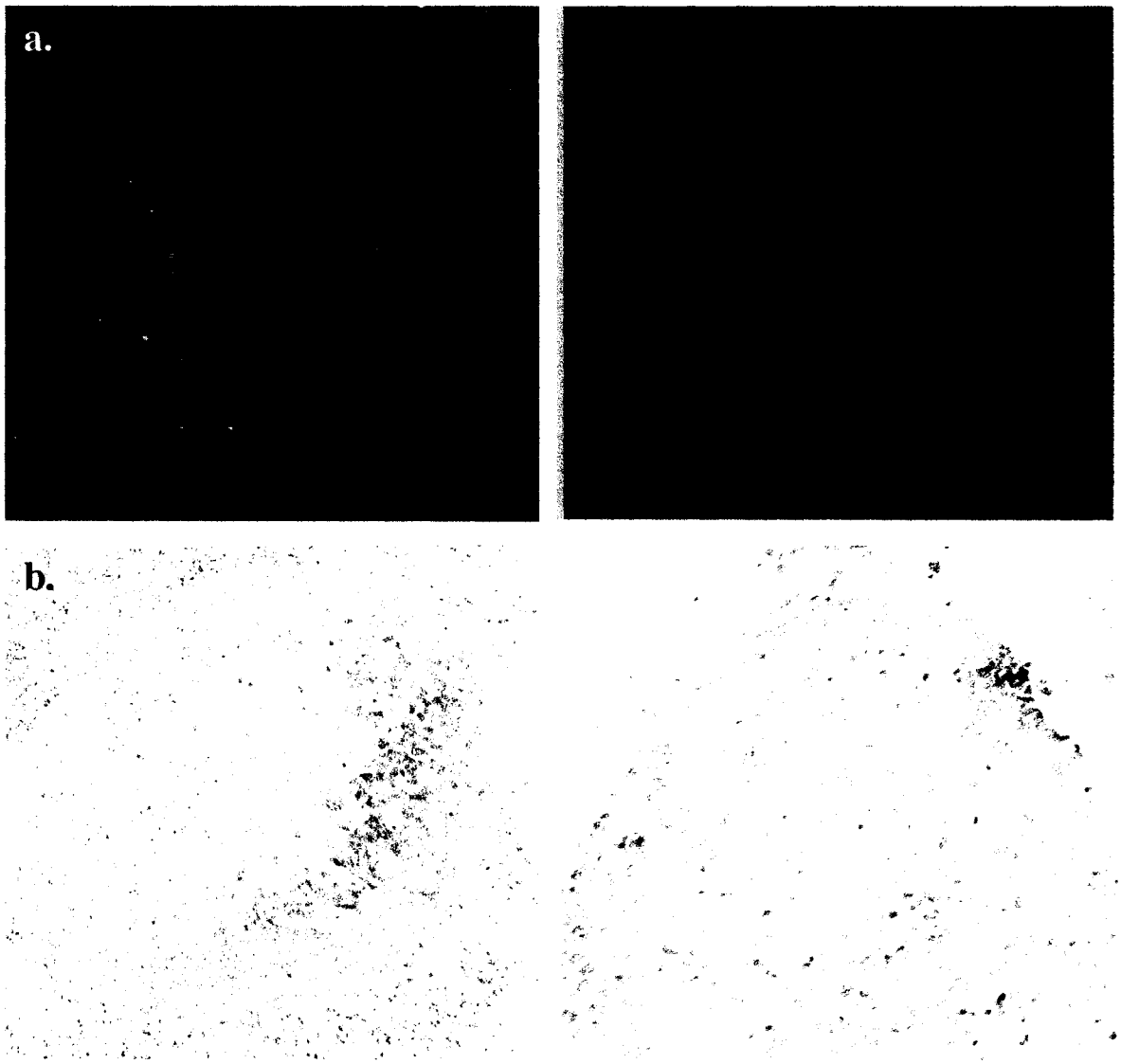

Figure 3.6: Comparison of CXCR4 distribution by fluorescence immunohistochemistry (**a**) versus chromagen in situ hybridization (**b**) demonstrates a similar pattern. There is a follicular pattern of distribution with a concentration of positive cells in an area on the hilar side of the follicle corresponding with the follicular dark zone. Corresponding sections labeled with irrelevant antibody or riboprobe are displayed in the right hand column.

Analysis of CXCR4 expression and cell phenotype flow cytometry

Tissue cell homogenates were colabeled for both CXCR4 and phenotype expression. In fresh uncultured cell homogenates from pharyngeal lymph nodes 84% of CXCR4+ cells were positive for the B-cell marker B220, whereas only 7% of CXCR4+ cells colabeled for the T cell marker CD5 (Fig 3.7). This is in agreement with observations by others in feline cells but in contrast to human CXCR4 expression patterns where CXCR4 colabels strongly with T cell markers [42] .

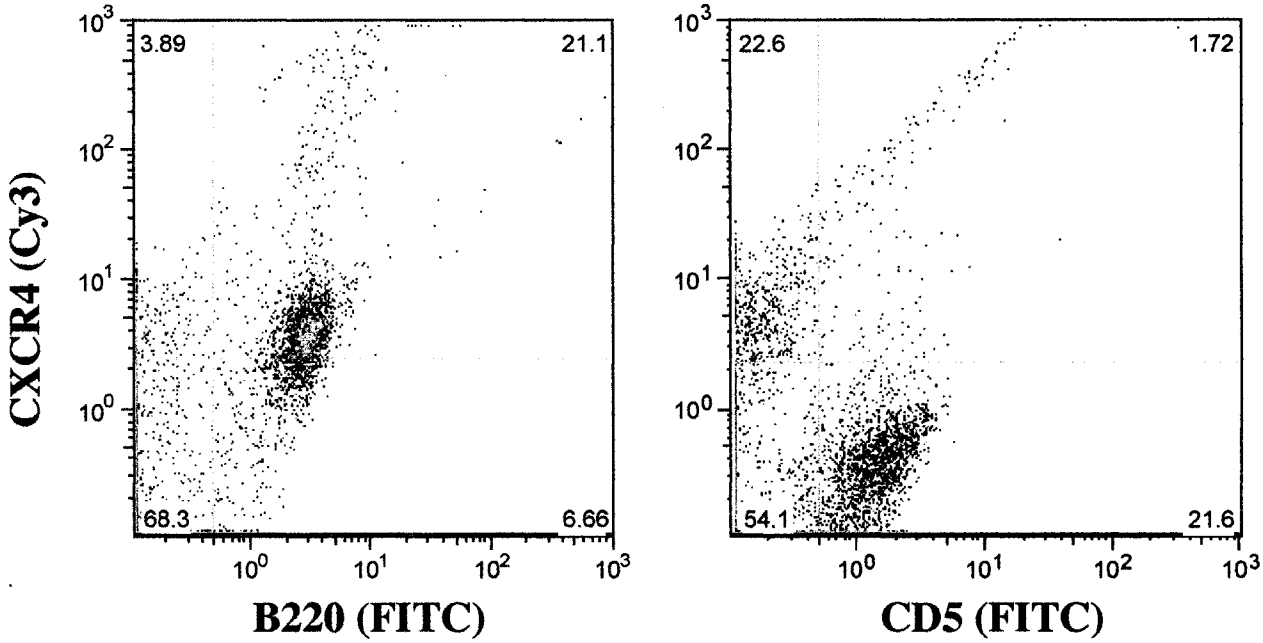

Figure 3.7: Flow cytometric analysis of lymph node homogenate. 84% of CXCR4 positive cells labeled for B cell antigen (B220), whereas only 7% of cells colabeled with T cell antigen (CD5). Lymph nodes from three different cats were analyzed all with similar results. Quadrant gates were set based on isotype controls.

FIV detection in CrFK monolayers

FIV from persistently infected (FIV-A-2546) Crandell feline kidney cell (CrFK) monolayers was successfully detected with the FIV gag mAbs PAK3-2C1, DF10 and 51G11. The technique was successful when cells were fixed and permeabilized with ethanol, methanol, acetone or when treated with paraformaldehyde and permeabilized using the Caltag fixation permeabilization kit. Viral detection was not possible with paraformaldehyde fixation alone. Nearly all inoculated CrFK stained positively (Fig 3.8); syncytia were frequently the most intensely staining cells. Only very faint background staining was apparent in naïve CrFK monolayers. Intensity of FIV antigen staining decreased slightly with prolonged culture.

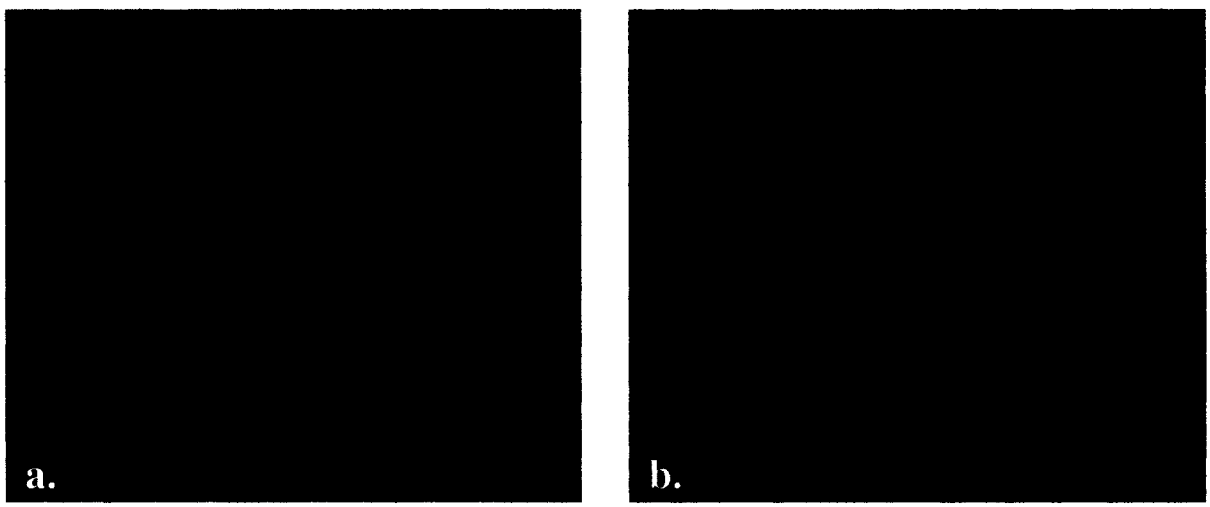

Figure 3.8: Naïve (**a**) or FIVA 2546 infected (**b**) ETOH-fixed CRFK cell monolayers stained with mAb PAK3-2C1.

FIV detection in CrFK and Mya-1 cells by flow cytometry

Suspensions of the same FIV-A-2456 infected CrFK were analyzed by flow cytometry. The vast majority of cells stained positively for FIV Gag by both methods (Fig. 3.9, a). In order to determine the ability of PAK3-2C1 to recognize gag antigens from a different FIV clade, Mya-1 cells at 5 days post infection with FIV-C-Pgmr were stained using the same protocol. A discrete peak representing an FIV positive population was detected but represented a smaller proportion of the cultured cell population as compared to CrFK (Fig 3.9, b).

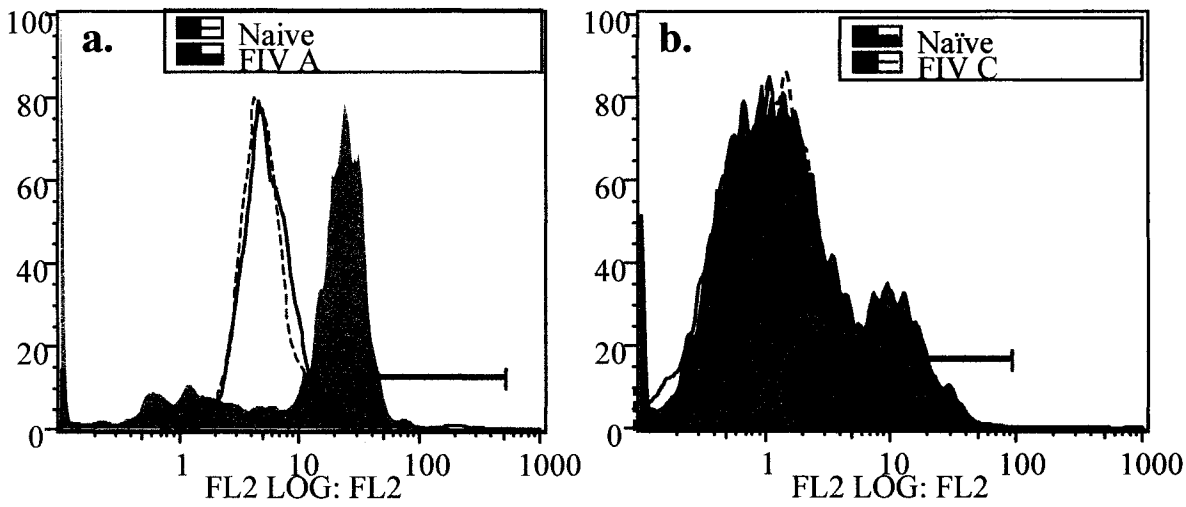

Figure 3.9: Flow cytometric analysis of ETOH-fixed FIV positive (FIVA 2546) and negative CRFK (**a**). Staining of FIV-C-Pgmr infected Mya-1 cells (**b**) demonstrates crossreactivity of the anti-gag antibody PAK3-2C1 with other clades. Dashed lines indicate isotype control antibody staining in FIV positive cells.

FIV immunostaining in paraffin embedded CrFK pellets

In order to determine the effect of processing on FIV antigens a subset of the same FIVA 2456- infected CrFK were pelleted, fixed with ethanol and paraffin embedded. The embedding process resulted in both increased background staining in

negative CrFK (Fig. 3.10, a) and weaker staining in FIV positive CrFK (Fig. 3.10, b). A similar study using pelleted PBMC from culture resulted in abundant background staining and no visible difference in staining between positive and negative samples (data not shown).

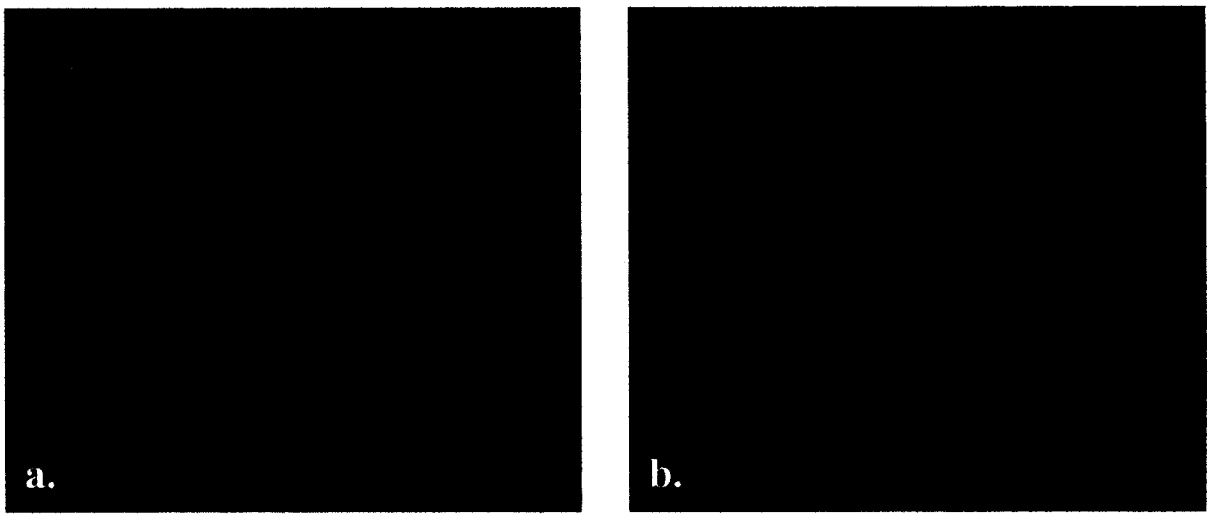

Figure 3.10: Naïve (a) or FIV-A-2546 infected (b) ethanol fixed cells from the same samples shown above were pelleted, paraffin embedded, sectioned and stained with mAb PAK3-2C1. Sample processing resulted in a marked increase in background staining and poor definition of cytoplasmic detail.

FIV immunostaining in paraffin embedded tissue sections

Background staining was prominent in ethanol fixed paraffin embedded tissue sections as evidenced by excessive staining of cells in naïve tissues (Fig. 3.11). The background staining was attributable in large part to nonspecific binding of the primary antibody as evidenced by greatly reduced background in naïve controls when the secondary antibody was used alone. Neither dilution of the primary antibody with subsequent tyramide amplification or antigen retrieval reduced nonspecific background staining.

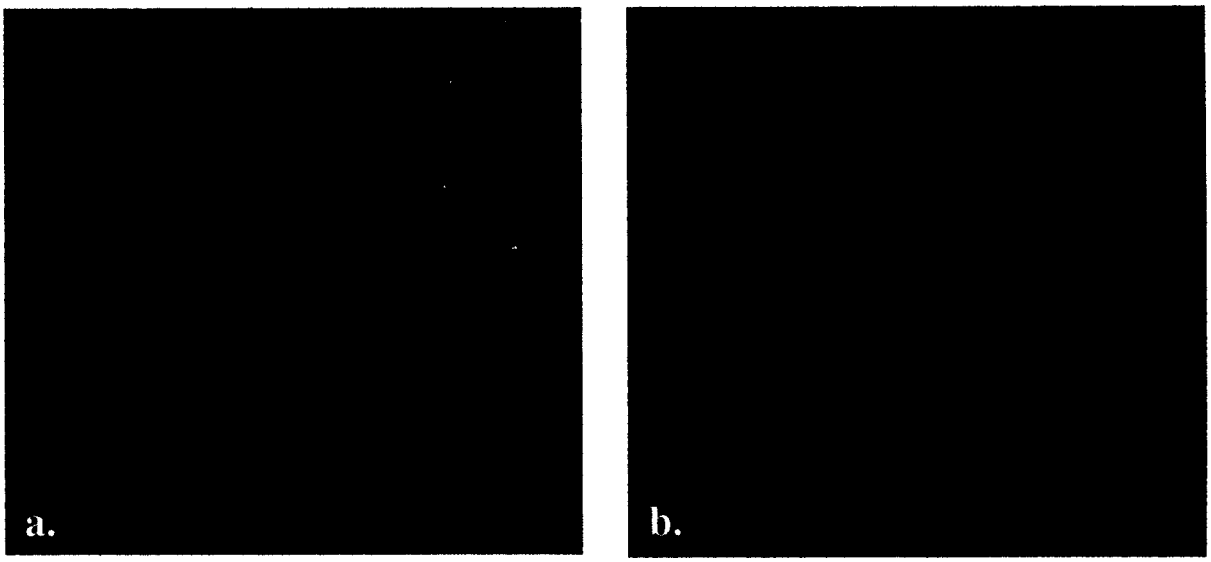

Figure 3.11: Fluorescence immunohistochemistry, ETOH-fixed paraffin embedded lymph node. Both the acutely infected (FIV-C-Pgmr) lymph node (**a**) and naïve lymph node (**b**) have multiple brightly staining cells. While positive tissues routinely had greater numbers of positively staining cells, excessive nonspecific staining in negative control tissues prevented reliable interpretation.

FIV immunostaining in frozen sections

Due to the inability to sufficiently reduce background staining in paraffin embedded tissues a method of FIV immunodetection was developed using frozen sections. While background staining occurred in frozen sections it was primarily attributable the secondary antibody as evidenced by abundant background staining equally as pronounced in the absence of primary antibody or when an isotype control was used as the primary antibody. Background staining was greatly reduced by overnight adsorption of the secondary antibody with homogenized cells from a naïve feline lymph node (Fig. 3.12). This method yielded satisfactory results with tissues from acutely infected cats; however, FIV could not be detected in chronically infected tissues.

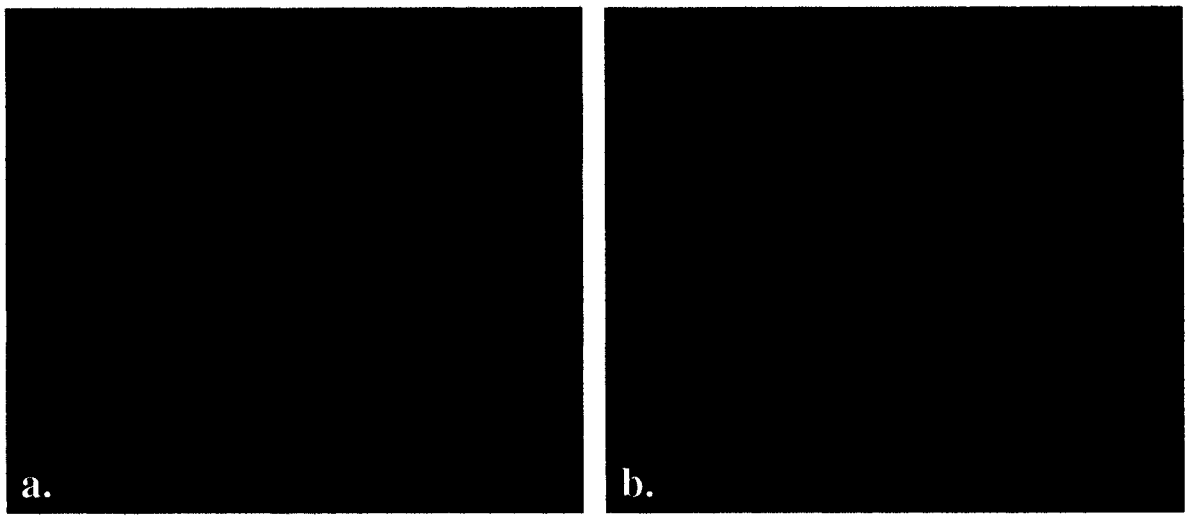

Figure 3.12: Fluorescence immunohistochemistry, ETOH-fixed frozen section, lymph node. FIV antigens (red) are detectable within an acutely infected (FIVC-Pgmr) lymphoid follicle (**a**) whereas minimal background staining occurred in the naïve lymph node (**b**).

FIV detection in lymphoid tissue homogenates by flow cytometry

Having demonstrated effective labeling of FIV in CrFK by flow cytometry, the same method was employed to detect FIV in lymphoid tissues from acutely infected cats. Lymphoid tissues from the same acutely infected cats (high proviral loads confirmed by real time DNA PCR of PBMC) used for frozen section immunolabeling were homogenized ethanol fixed and permeabilized and stained with PAK3-2C1. No significant difference in staining was detected by flow cytometric analysis between infected and control tissues (Fig. 3.13).

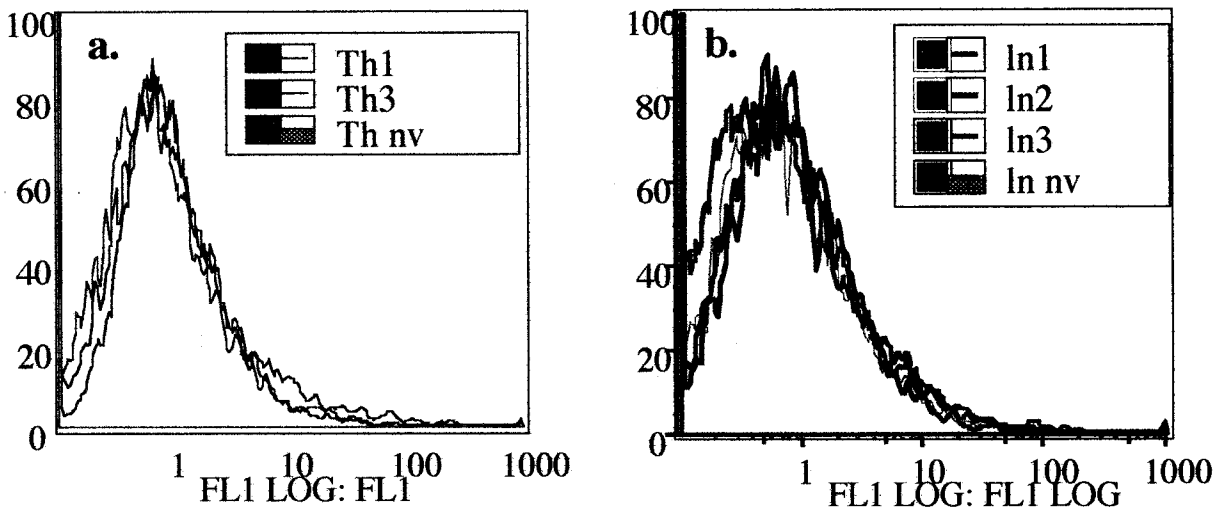

Figure 3.13: Flow cytometric analysis of ETOH-fixed cell suspensions from thymus (**a**) and pharyngeal lymph node (**b**) from acutely infected (FIVC-Pgmr). Infected tissues were indistinguishable from naïve tissues suggesting lack of sensitivity in detection of small numbers of infected cells.

FIV provirus detection by Real time DNA PCR

Due to low sensitivity of FIV immonodetection and incompatibility with other phenotype detection protocols we abandoned immunolabeling techniques in favor of more reliable FIV detection by real time DNA PCR.

We determined tissue proviral loads in cohorts of FIV infected cats in both acute (3-4 weeks p.i.) and chronic infection (6 months p.i.). Tissues for analysis included thymus, pharyngeal lymph node, bone marrow and blood. The thymus was the major reservoir in acute infection (Fig. 3.14, a, $p < 0.05$) cats with an average proviral concentration of 3.41×10^5 copies per million cells whereas the bone marrow had the greatest proviral load in chronic infection (Fig 3.14,b, $p < 0.05$) averaging 3.9×10^4 copies per million cells. Route of inoculation (oronasal versus intravenous) made no

difference in proviral distribution across multiple tissues measured at 6 months p.i. (Fig 3.15).

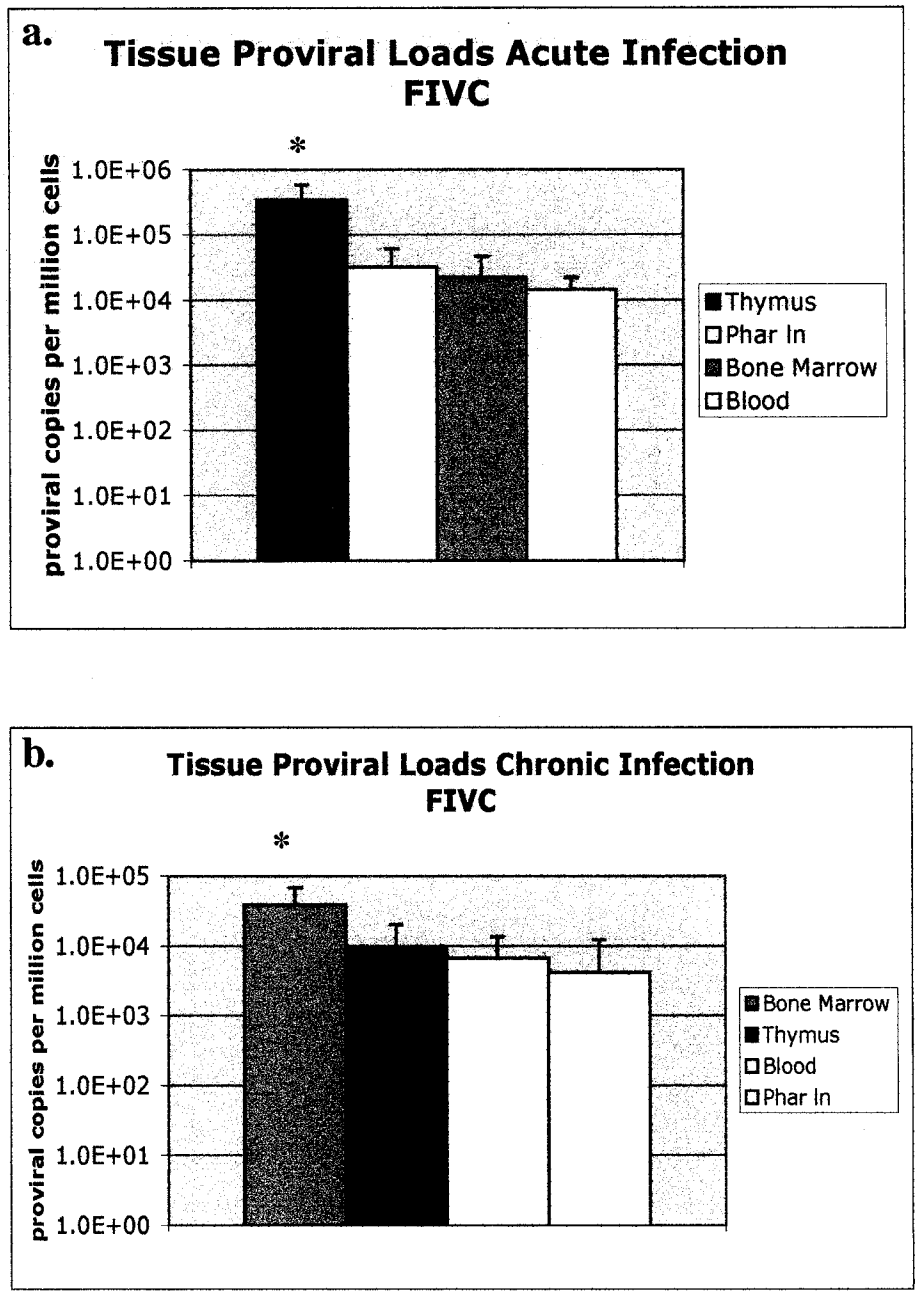

Figure 3.14: Real time DNA PCR FIV provirus. Tissue proviral distribution in acute (**a**) versus chronic (**b**) infection. Thymus contained the greatest proviral burden in acute infection while bone marrow was the greatest viral reservoir in chronic infection.

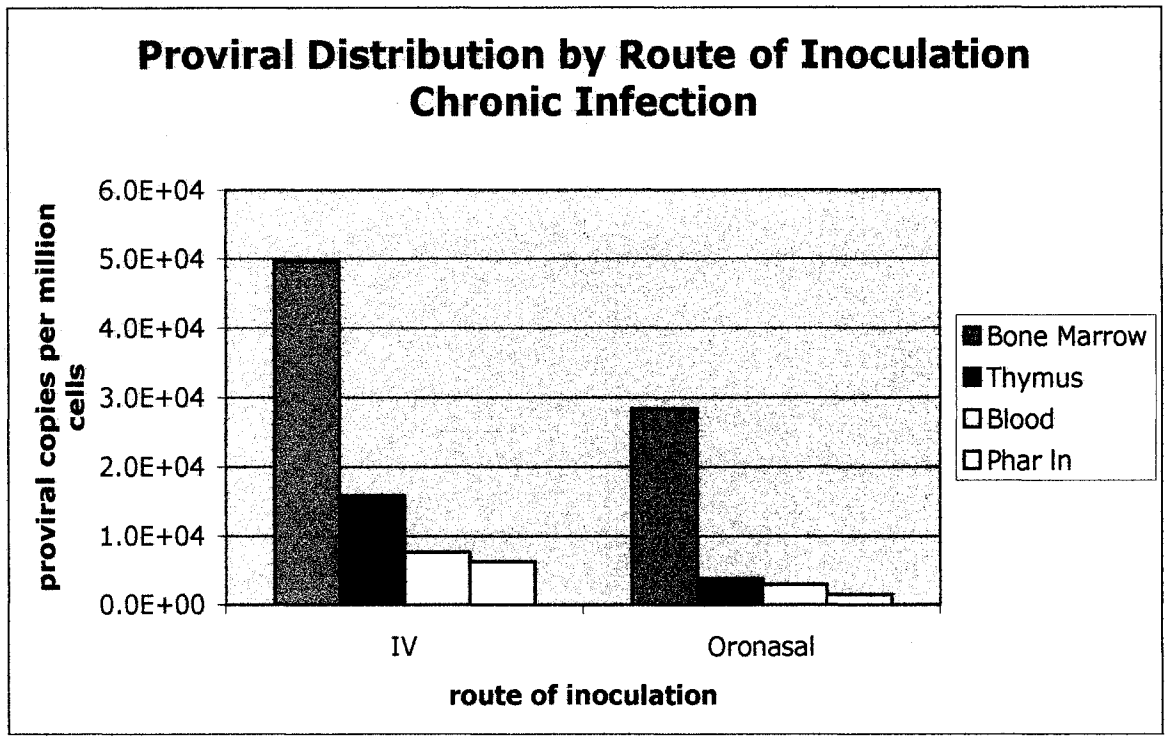

Figure 3.15: Real time DNA PCR FIV provirus. Proportions of provirus within tissues were similar regardless of route of inoculation.

	IHC-frozen	IHC-fixed	FACS	ISH	Real Time PCR
CXCR4	good	good	**excellent**	good	n/a
FIV	good	poor	poor	good	**excellent**
Cell phenotype	n/a	excellent	**excellent**	n/a	n/a

Figure 3.16: Methods employed for simultaneous detection of CXCR4, cell phenotype and FIV in tissues or tissue cell suspensions. The combination of cell sorting for CXCR4 and phenotype followed by real time PCR detection of FIV provirus was most sensitive and consistent (outlined in red). n/a = not attempted.

DISCUSSION

As a general approach to these studies we began by identifying cell types as controls for antibody screening. Antibodies that performed well in the relatively antigen rich and background free cell culture and monolayer environment were then tested in tissue sections using an array of fixation and antigen retrieval methods. With little data regarding CXCR4 distribution in feline tissues and evidence for wide receptor distribution in other species, we had no resource for feline positive and negative control tissues for these studies. We bypassed this limitation through the use of binding competition assays demonstrating dose dependent inhibition of CXCR4 mAb 44717 by the CXCR4-specific antagonist AMD3100 in both the human osteosarcoma cell line (HOSX4) and in feline PBMC by flow cytometry and immunocytochemistry. Antibody screening by IHC revealed CXCR4 expression that was particularly prominent in the dark zone of lymphoid follicles. This distribution corresponded with CXCR4 RNA detection by ISH and is consistent with reports of CXCR4 distribution in other species [40, 41, 43] . Using dual immunohistochemistry we identified only rare colabeling of CXCR4 with T cell marker CD3 and failed to demonstrate coexpression of B-cell, dendritic cell or macrophage (B220, P55, or mac387) markers with CXCR4. Furthermore AMD3100 failed to block CXCR4 antibody binding in tissue sections casting doubt on the specificity of the antibody in fixed tissue sections. It is possible that the CXCR4 epitope could be altered by the coagulative fixative (Streck) such that epitopes recognized by the antibody are preserved and regions necessary for AMD3100 binding are lost. Alternatively fixation may induce conformational changes to the receptor allowing for both AMD3100 and the anti-CXCR4 mAb to bind simultaneously.

We identified several monoclonal antibodies that were able to detect FIV gag or gp120 antigen by flow cytometry and in CrFK monolayers (PAK3-2C1, DF10, 51G11, SU1-30) and in acutely infected lymph node frozen sections (PAK3-2C1). The fact that FIV signal was strong in CRFK, weaker in Mya-1 and undetectable in PBMC or homogenized lymphoid organs indicates low sensitivity, a common problem when using monoclonal antibodies directed towards a single epitope in the presence of relatively small amounts of antigen. Similarly, PAK3-2C1 detected FIV antigen in acutely but not chronically infected infected lymph nodes even when amplification techniques were applied. Cocktails of monoclonal antibodies did not improve detection by flow cytometry or IHC (data not shown). In future studies purification of cell subsets prior to FIV staining may provide infected cell subsets (i.e. CD4+) sufficiently concentrated for virus detection.

In addition to investigating the relationship of FIV and CXCR4, we examined relative FIV proviral loads across tissues both at different time points of infection and with different routes of inoculation. The thymus contained the greatest proviral concentration ($p < 0.05$) in acute infection in agreement with previous studies [44] . However, by 6 months p.i. the major viral reservoir became bone marrow ($p < 0.05$). The shift in proviral proportions was due primarily to a decreased proviral load in the thymus while bone marrow proviral levels remained relatively stable. Decreased thymic proviral concentrations may well reflect loss of cellular targets in that compartment over time. CD4/CD8 ratios have been shown to decline significantly due to loss of CD4 positive target cells within the thymus [45] . Additionally the virus-induced thymic cell loss was likely occurring against a backdrop of natural age-related thymic involution. It would be

interesting to compare viral loads of T and B cell subsets in acute versus chronic infection. While studies from our lab and others have demonstrated higher proviral burdens in T cell subsets in acute infection (see chapter 4) it is likely that a large proportion of measured provirus in chronic infection is harbored in thymic B cells within prominent follicles that form late in infection [16, 45, 46] .

While intravenous FIV inoculation achieved higher peak proviral loads both in blood (see chapter 2) and tissues than mucosal challenge, route of inoculation did not affect the relative proviral distribution between tissues in chronically infected cats. This was somewhat expected considering that by 6 months post challenge virus would have gone through numerous rounds of replication and redistributed throughout the body following the viremic phase.

Our goal was to characterize the importance of CXCR4 expression in FIV infection by colabeling cells for CXCR4, FIV and cell phenotype. After developing methods for identification of each of the markers we set out to combine them. The chart above (Fig. 3.16) displays successful methods employed for each marker with bold arrows indicating strong/ effective labeling and a dashed line indicating weak or conditional labeling. Due to weak antigen detection and the need for cell permeabilization, FIV staining by immunolabeling methods was abandoned in favor of real time DNA PCR for detection of FIV provirus. While presence of provirus is not indicative of productive infection, it does indicate viral entry and reflects the cells ability to display the correct receptors for entry. The method proved to be a consistent and sensitive method for FIV detection and was compatible with successful immunolabeling techniques for cell sorting that had been developed for CXCR4 and cell phenotype.

Fluorescence activated cell sorting of CXCR4 and lymphoid cell phenotypes coupled with real time DNA PCR detection of FIV was used to perform the studies described in chapter 4 of this thesis.

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CHAPTER FOUR

CXCR4 EXPRESSION, LYMPHOID CELL PHENOTYPE, AND FELINE IMMUNODEFICIENCY VIRUS INFECTION

ABSTRACT

Primary isolates of FIV have been shown to require binding to CD134 in conjunction with CXCR4 to productively infect IL-2 dependent T-cell cell lines in culture. While numerous in vitro studies have demonstrated the importance of the CXCR4 chemokine receptor in the attachment and entry phase of feline immunodeficiency virus (FIV) infection little is known about the in vivo role of CXCR4. Here we investigate the role of CXCR4 in vivo using purified cell populations from lymphoid tissues, bone marrow and peripheral blood from acutely infected cats (FIV-C-Pgmr). CXCR4 positive and negative T and B cell fractions were purified by fluorescence-activated cell sorting (FACS) and examined for FIV proviral concentration by real time polymerase chain reaction (PCR). There was no significant difference in proviral concentrations between CXCR4 positive and negative cell fractions. Roughly

half of FIV infected cells expressed detectable levels of CXCR4 by flow cytometry. Of the tissues examined the thymus had the highest proviral burden. Inadequate B cell numbers in thymus and bone marrow precluded estimation of provirus in these subsets however higher proviral loads were detected in T-cells than B-cells in lymph node and blood. CXCR4 expression was highest in lymph node both in cell numbers and mean fluorescence intensity (MFI, $p < 0.02$). Here we have demonstrated a CXCR4-negative FIV provirus-positive population of cells in multiple feline tissues in vivo. While the possibility of limited antibody crossreactivity and FIV mediated CXCR4 downregulation cannot be excluded, the data suggest the possibility of CXCR4 dependent and independent modes of viral entry for FIV-C-Pgmr in susceptible cell subsets.

BACKGROUND

FIV causes an AIDS like syndrome in cats similar to HIV that is characterized by progressive CD4+ cell loss. FIV shows similar tissue tropism to HIV including a replication in T lymphocytes [1-4] , macrophages [5-8] , B cells [9, 10] , megakaryocytes [11] , monocytes [12] and cells of the nervous system including astrocytes and microglia [13-18] . In vitro FIV studies indicate that both CD4+ and CD8+ T cells are susceptible to productive virus infection [2, 19] . In contrast to primate lentiviruses, FIV does not use CD4 [20-22] for entry however the receptor CD134 has been shown to be required in conjunction with CXCR4 for entry [23] . CD134 expression has been shown to be highest in CD4 positive T-cell subsets and may account for depletion of CD4 positive cells in FIV infection even though CD4 itself is not a receptor for FIV.

Abundant experimental data suggests that the mechanism of virus attachment and entry for HIV and FIV is highly conserved. Entry of both FIV and HIV into cells involves interaction of the V3 loop of the viral envelope gp120 glycoprotein with the X4 or R5 receptor [24-27] . Both viruses have demonstrated the ability to use DC-SIGN [28-31] and cell surface heparans [32] to enhance binding affinity. Cell-culture-adapted strains of both FIV and HIV demonstrate change from non-syncytium inducing to syncytium inducing phenotype which correlates with an increase in net charge of the V3 loop of the envelope glycoprotein [33, 34] . Human cells which naturally express the X4 receptor and nonpermissive human cells transfected with human X4 receptor will undergo syncytium formation with FIV infected feline cells [35, 36] . A series of bicyclam analogues including AMD 3100 have been shown to specifically bind the X4 receptor and competitively inhibit binding of both HIV-1 [37-45] and FIV [46-48] *in vitro.*

There are still questions however with regard to the exact mechanism of FIV cell attachment and entry in vivo and whether in vitro models are reflective of what occurs in vivo. For example, in addition to CD4+ CD134+ cells, FIV has also been shown to infect CD8+ T cells, B cells and macrophages that express little or no CD134 [49] . Also while AMD3100 provides potent and consistent inhibition of FIV in vitro, studies using very high doses of the compound have had minimal antiviral activity in vivo (our observations) [50] .

In order to better characterize the role of CXCR4 in clinical FIV infection we have measured CXCR4 expression and FIV proviral loads in tandem in tissues and lymphoid cell subsets from acutely infected cats. In pilot studies we identified a broad

range of CXCR4 expression between cats in peripheral blood, a phenomenon previously reported in cats and humans [51, 52] . Particularly problematic were cats with extremely low CXCR4 expression such that sufficient total cell numbers could not be obtained by cell sorting to assay for provirus [53] . Another problem we faced was the possibility of underestimation of CXCR4 expression due to virus induced receptor downregulation. While several studies have demonstrated no effect of HIV or FIV infection on CXCR4 expression on cell lines in vitro [51, 54] , there is strong evidence for CXCR4 receptor downregulation with HIV infection in vivo [55-57] . Thus, in order to enhance detection of the CXCR4 positive cell populations in the face of potentially altered CXCR4 expression post-infection, we developed a method to rapidly upregulate CXCR4 in feline cells allowing for collection of cells with high capacity for CXCR4 expression rather than only those with high expression at the time of necropsy.

MATERIALS AND METHODS

Animals and sampling

Six specific pathogen free cats were anesthetized with ketamine-acepromazine and inoculated intravenously with > 100% TCID of FIV-C-Pgmr. Heparinized and EDTA blood was collected at 2 weeks post challenge to monitor infection status. Necropsy was performed on six cats at 3 to 4 weeks post inoculation (p.i.). Thymus, bone marrow, pharyngeal lymph node and PBMC from whole blood were collected and homogenized by passage through a 40μm tissue sieve. In order to determine a protocol for optimal enhancement of CXCR4 expression, cells were cultured overnight under a

variety of conditions including storage of cells in either EDTA, citrate, or heparin at 4°C or cultured in RPMI 1640 (Gibco), 2% glutamine and 20% fetal bovine serum (FBS) alone or with phorbol ester 12-myristate 13-acetate (PMA) and ionomycin. Following culture cells were analyzed by flow cytometry for CXCR4 expression.

Fluorescence-activated cell sorting

Following overnight incubation in media consisting of RPMI 1640, 2% glutamine and 20% fetal bovine serum, cells were washed and suspended in buffered saline containing 2% heat inactivated fetal bovine serum and incubated with allophycocyanin (APC) conjugated anti-CXCR4 44717 mAb crossreactive with feline (R&D systems), FITC-conjugated anti feline CD4 and CD8 (Southern Biotech, Birmingham, AL) and phycoerythrin (PE) conjugated anti B-cell antibody clone CA2.1D6 crossreactive with cat (Serotec) or isotype matched control mAbs. Samples were incubated at 4°C in the dark for five hours then resuspended and sorted using a Dakocytomation MoFlo cell sorter. Gates were set based on isotype controls and 500,000 events were collected for the majority subsets. For rare cell fractions a minimum of 10,000 cells were collected. Purity greater than 94% for CXCR4 and 83% for T and B cell subsets was achieved.

Quantitative real time PCR

Real time quantitative DNA PCR adapted from a method by Pedersen et al. was performed for each cell fraction. DNA was extracted using the QIAmp DNA blood Mini Kit (Qiagen, Valencia, CA). The 25μl PCR mixtures contained 5μl standard or sample, 0.5 ml (400 nM) of each primer, 0.2 ml (80nM) of probe, 6.3ul DNAse free water, and

12.5 μl TaqMan Universal PCR Master mix containing 10mM Tris-HCl (pH 8.3), 50mM KCl, 5mM MgCl2, 300uM each of dATP, dCTP, and dGTP, 600uM dUTP, 0.625U of AmpliTaq Gold DNA polymerase, and 0.25U uracil N-glycosylase (UNG) per reaction. FIVC gag primer and probe sequences were as follows: forward 5'-ACT CAC CCT CCT GAT GGT CCT A-3', reverse 5'-TGA GTC AGC CCT ATC CCC ATT A-3') and probe FAM-5'-ACC ATT GCC ATA CTT CAC TGC AGC CG-3'-TAMRA. FIV C gag plasmid DNA was cut with the restriction enzyme BamHI and repurified.

A standard curve was generated for each plate with serial dilutions of FIV C gag plasmid DNA (10^9, 10^6, 10^2) with 1X TE and 40 ng/ml salmon testes DNA (Sigma) as a DNA carrier. Samples and standards were run in triplicate on 96-well plates using the Biorad iCycler. Polymerase activation and amplification were performed using the following protocol: 2 min. at 50°C, 10 min. at 90°C 45 cycles at 95°C for 15 seconds, 60°C for 1 minute.

Results

CXCR4 upregulation

We had previously demonstrated increased CXCR4 expression on PBMC by culturing cells at 37°C in the presence of Con A and interleukin-2 (IL-2) for 3 days. While this method results in marked upregulation of CXCR4, IL-2 exposure selects for T cells at the expense of the other lymphocyte subsets that are eventually lost. In an attempt to enhance CXCR4 expression without alteration of cell subsets we cultured or stored cells briefly (overnight) under a variety of conditions including storage of cells in either EDTA, citrate, or heparin at 4°C or cultured at 37°C with PMA and ionomycin or RPMI and fetal bovine serum alone. Of these techniques, overnight incubation in RPMI

and fetal bovine serum at 37°C provided the greatest enhancement of CXCR4 expression with minimal cell loss (Fig. 4.1). This method resulted in less than 5.0% cell death as assessed by trypan blue staining.

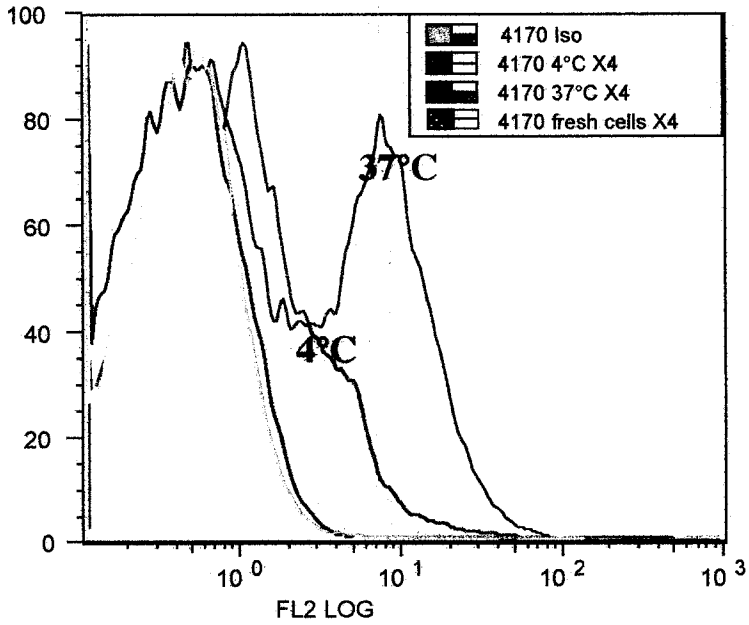


Figure 4.1: CXCR4 upregulation in feline PBMC with overnight incubation in media at 4°C and 37°C. Marked upregualtion occurred at 37°C.

CXCR4 expression in tissues and lymphoid cell subsets

In most tissues gates for negative (R1) and positive fractions (R2) were set slightly apart such that an ambiguous population with marginal CXCR4 expression was not collected. Figure 4.2 displays histograms demonstrating CXCR4 staining across different tissues in three cats. There was significantly greater CXCR4 expression in lymph node than in any other tissue examined both in total numbers of cells as well as in fluorescence intensity ($p < 0.02$) indicating a greater concentration of surface expression (Fig. 4.3). There was no difference statistically in CXCR4 expression among the other tissues.

A greater proportion of T cells expressed CXCR4 compared to B cells in all tissues with 45% of T cells and 23% of B cells staining positive throughout all tissues. Interestingly there was a population cells positive for both T and B cell antigens (Fig. 4.4) that occurred almost exclusively in the CXCR4 positive fraction of blood and lymph node, likely the result of nonspecific crossreactivity of the B cell antibody with a subset of T cells.

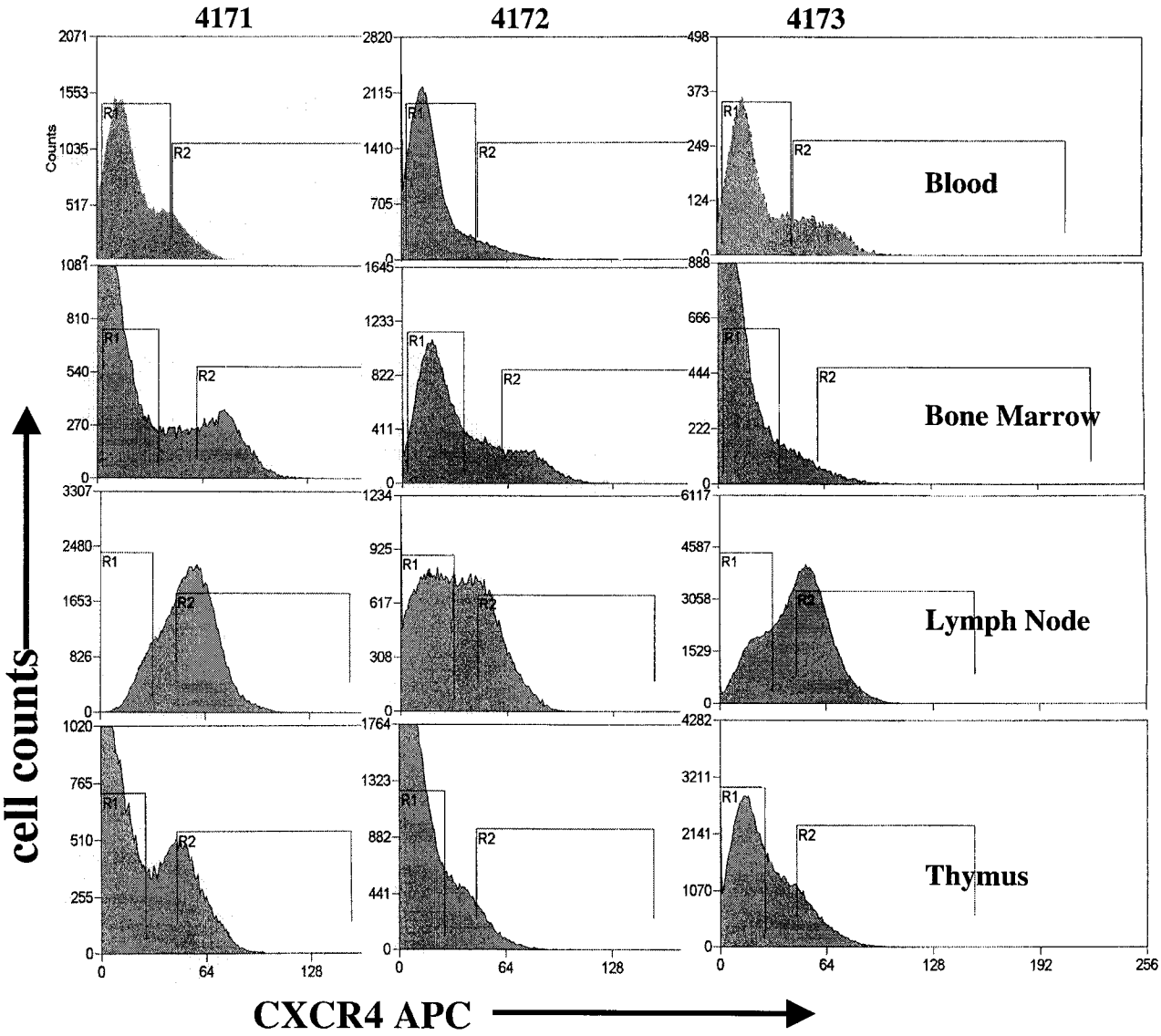
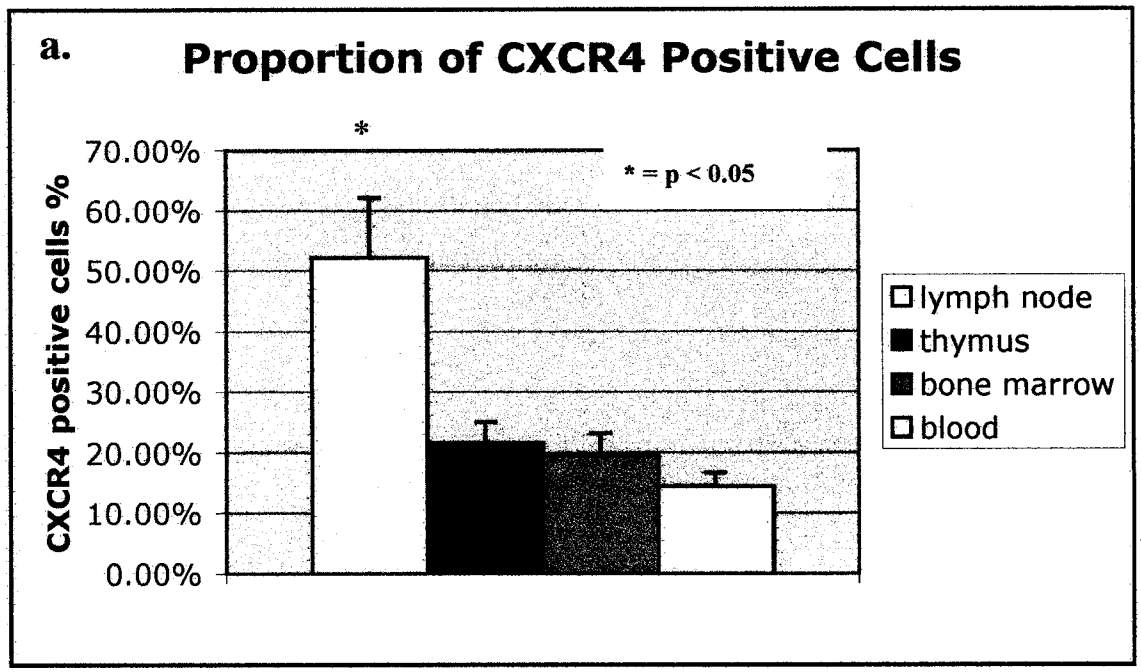


Figure 4.2: Histograms from three cats comparing CXCR4 expression across tissues. Significantly higher CXCR4 expression occurred in the lymph node.



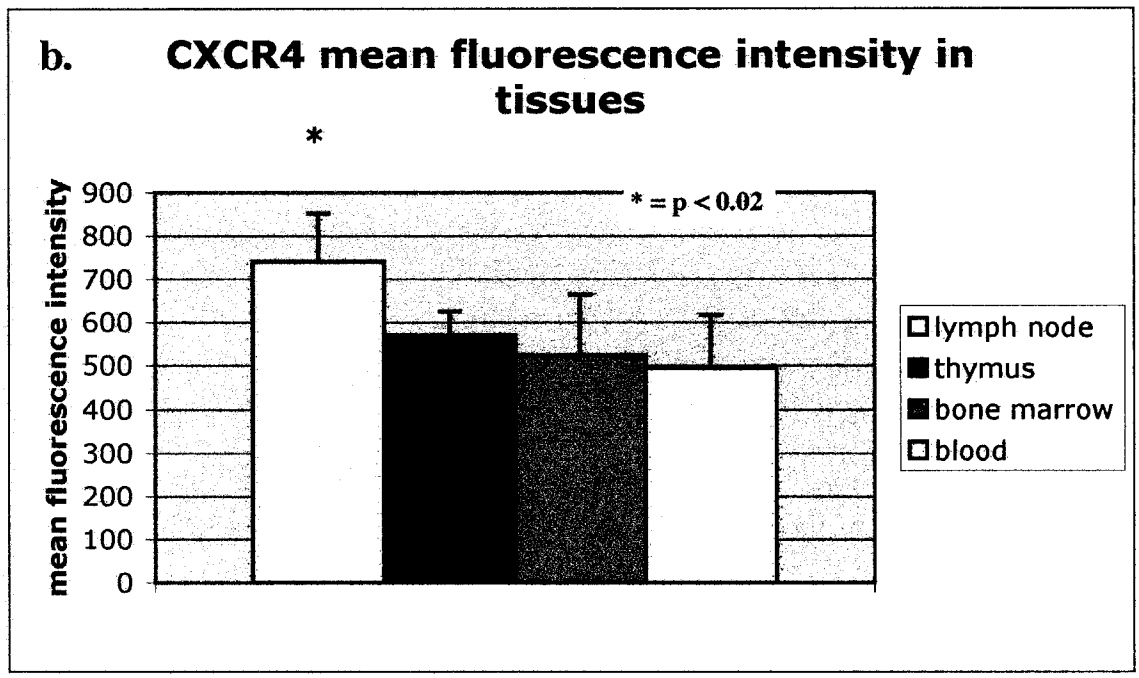


Figure 4.3: The lymph node had the highest level of CXCR4 expression both in terms of total cell numbers (**a**) as well as mean fluorescence intensity (**b**), n = 6.

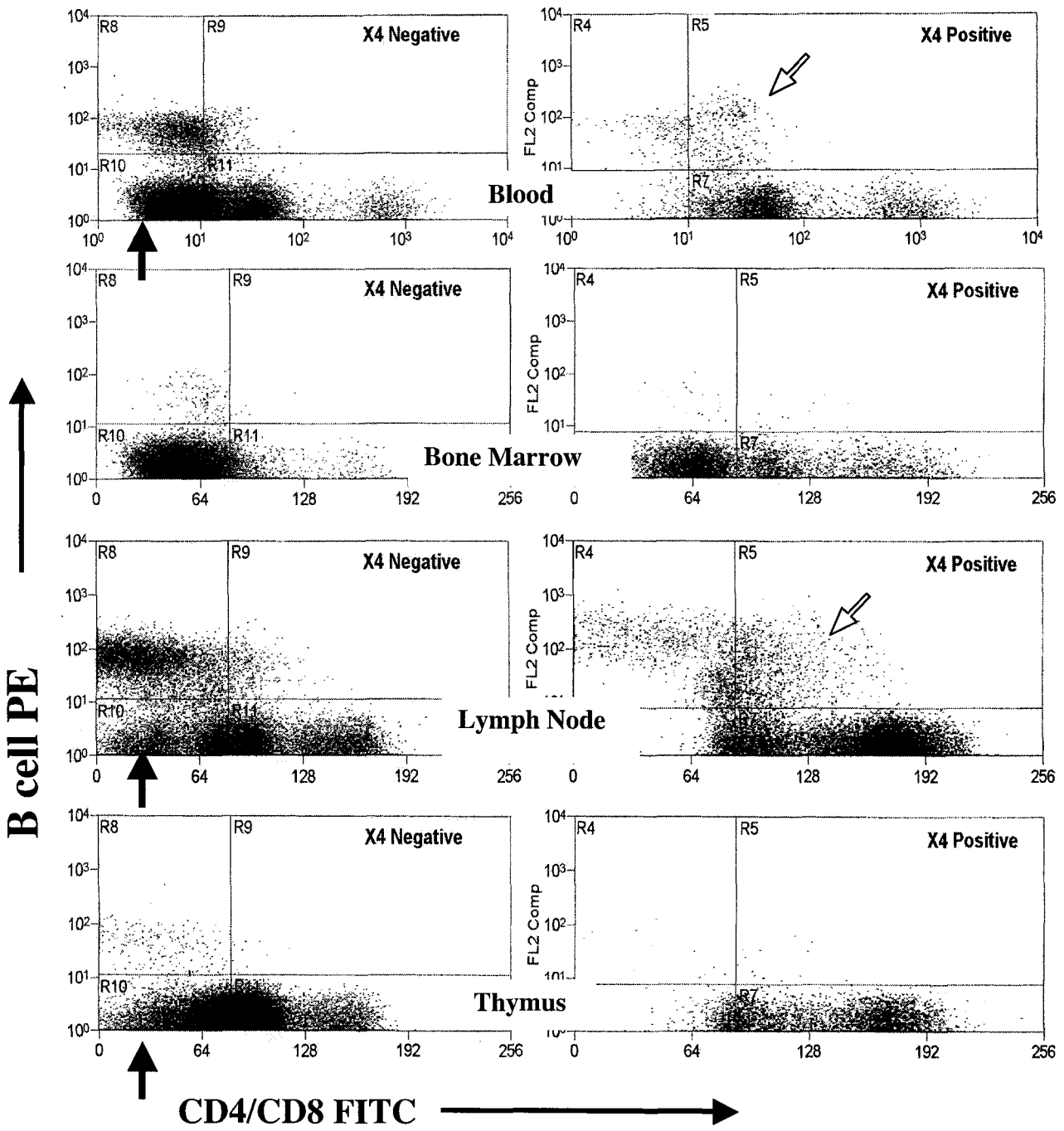


Figure 4.4: Flow cytometric analysis comparing CXCR4 positive and negative lymphoid cell subsets. Very few B cells were present in thymus and bone marrow compared to lymph node and blood. A small cluster of cells stained with both B and T cell markers and is most prominent in the CXCR4 population within blood and lymph node (open arrows). Within the CXCR4 population nearly all cell stain for either B or T cell markers, while the CXCR4 negative population has a large non-B non-T cell population (solid arrows).

DNA proviral levels in tissues

Unfractionated samples were examined to determine proviral loads between tissues. The thymus contained significantly greater proviral loads when compared with each of the other tissues ($p < 0.05$) (Fig. 4.5). There was no difference in proviral levels between bone marrow, lymph node and blood.

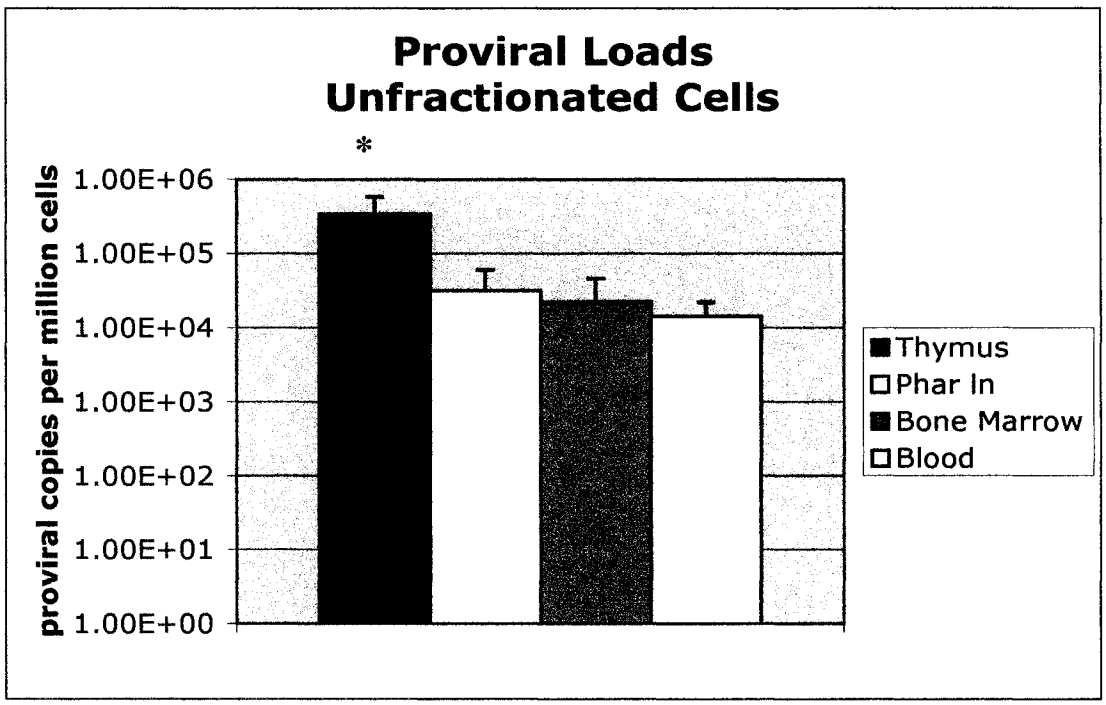


Figure 4.5: FIV Real time DNA PCR average proviral loads in unfractionated cells, n=6. Proviral burdens were greatest in thymus. There was no statistically significant difference in proviral loads between lymph node, bone marrow and blood.

DNA proviral levels in CXCR4 positive and negative subsets

B cells were not collected from the thymus and bone marrow due insufficient numbers for DNA extraction however the bone marrow contained a large population of cells negative for both T and B cell markers. CXCR4 positive and negative subsets of these cells had no statistically significant difference in proviral loads.

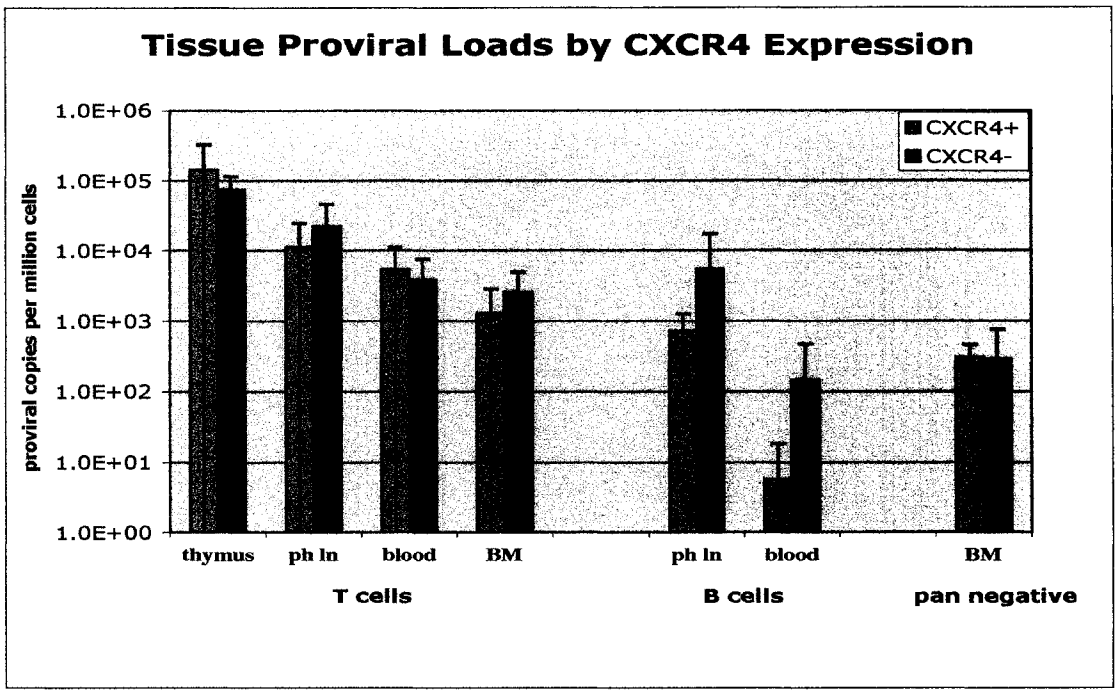


Figure 4.6: Real time DNA PCR FIV provirus, n=6. There was no difference in proviral concentrations between CXCR4 positive and negative subsets for any tissue examined. A population of bone marrow cells negative for both B and T cell markers contained a substantial proviral burden.

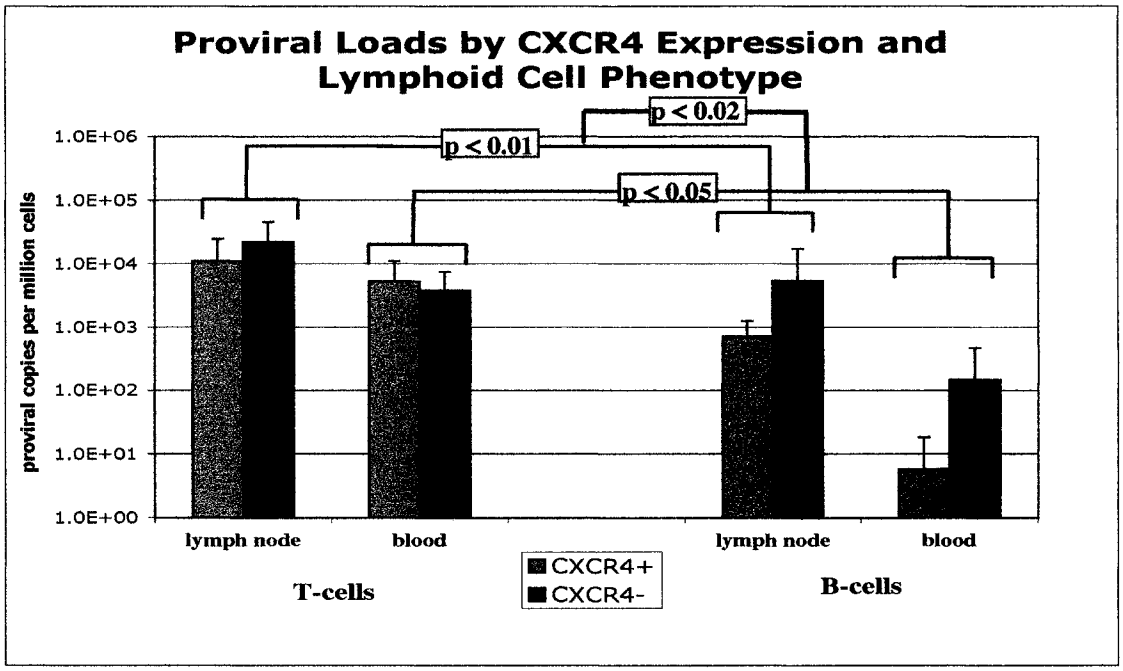


Figure 4.7: Real time DNA PCR FIV provirus, n = 6. Proviral loads were significantly higher in T cell populations compared with B cells in lymph node and blood.

DISCUSSION

Using a combination of cell sorting and real time DNA PCR we identified a population of CXCR4-negative, FIV provirus-positive cells from acutely infected feline tissues. This is surprising given the large amount of in vitro data demonstrating the requirement of CXCR4 expression on cells to render them permissive to FIV infection. The most obvious explanation for this discrepancy would be the possibility of alternate receptor use by FIV-C in vivo. It is possible that the body presents a wider variety of options for virus entry than what is available in cell culture systems and that the virus may be able to select from several receptors for binding and entry. There is certainly precedence for lentiviral evolution towards diverse receptor usage. Numerous strains of HIV and SIV have demonstrated the ability to use alternate coreceptors such as CCR3 [58, 59] , CCR8 [60] , GPR1 [61-63] , GPR15 [61, 63] , CXCR6 [64, 65] , Apj [66, 67] , and RDC1 [68, 69] . Arguing against this scenario are studies demonstrating complete inhibition of primary FIV strains by the CXCR4-specific antagonist AMD3100. It is possible that FIV may use CXCR4 to infect certain cell subsets and that an alternate mode of entry is used for others. Indeed, the antiviral effect of the CXCR4-specific antagonist AMD3100 appeared to be much less potent compared with its effect in vitro (refer to chapter 2) suggesting the possibility of an alternate mechanism of infection in vivo possibly in addition to CXCR4 use.

The disparity between CXCR4 expression and FIV infection may also be a reflection of lentiviral inhibition of CXCR4 expression in a manner that is not overcome by our upregulation protocol. While in vitro studies with FIV [51] and HIV have frequently demonstrated no effect of lentiviral infection on CXCR4 expression, actual

clinical data suggest CXCR4 downregulation in vivo [55-57] . Issues relating to antibody specificity may also result in inaccurate assessment of the FIV/CXCR4 relationship. Studies of antibody-receptor interactions involving human CXCR4 demonstrate that different antibodies interact with a different subset of CXCR4 receptors [70, 71] . These studies suggest that different conformations of CXCR4 are present in vivo that may be cell type dependent. If this is the case, then it is possible that only a fraction of CXCR4 positive cells are recognized by mAb 44717 making it difficult to accurately colocalize the epitope with FIV.

Within lymph node and blood, T cells contained significantly higher proviral burdens than B cells. These results are in agreement with a previous study by Dean et al. describing proviral burdens in lymphocyte subsets of blood and lymph node in acute FIV infection (4 weeks p.i.) [9]. Also in agreement with these studies, we noted similar proviral burdens between blood, lymph node and bone marrow indicating that with the exception of thymus, provirus measured in blood can roughly estimate body-wide proviral concentrations in acute infection.

The vast majority of CXCR4 positive cells also labeled for T or B cell markers whereas there was a much larger population of unstained cells in the CXCR4 negative population. This indicates that CXCR4 is expressed primarily upon T and B cells in feline tissues. As expected there was a large population of cells within the bone marrow that stained for neither B nor T cell markers. In a previous study we had found bone marrow to be a major reservoir for FIV particularly in chronic infection (see chapter 3). Thus we were interested in collecting this population to examine proviral loads in bone marrow cell subsets. With no native or crossreactive antibody to feline bone marrow

subsets, we resorted to sorting a non-lymphocyte fraction to assay for provirus. The proviral loads in this population (primarily myeloid and erythroid progenitors) were slightly lower than the T cell population but are indicative of a susceptible nonlymphoid population within the marrow. Given this evidence and the fact that acute FIV infection is associated with neutropenia, studies to determine susceptibility of bone marrow cell subsets are warranted.

It is not surprising that the lymph node cells demonstrated high CXCR4 expression given that the receptor is associated with cell homing and is necessary for normal organization of lymphoid architecture [72] . In addition to there being a greater proportion of CXCR4 positive cells within the lymph node, an increased concentration of receptors per cell was also present as reflected by a significantly higher mean fluorescence intensity compared with other tissues. Interestingly bone marrow cells expressed CXCR4 at similar levels to thymus and blood. This was surprising given evidence for the receptors importance in leukocyte homing within the bone marrow. Bone marrow cell subsets in people have been shown to have high CXCR4 expression [73, 74] and a transient leukocytosis with 4 fold increase in circulating CD34+ cells can be achieved following administration of the CXCR4 antagonist AMD3100 [75].

These studies have confirmed the role of the thymus as the major target tissue in acute FIV infection and raise new questions as to the role of the bone marrow cell subsets in FIV infection. Further study is needed to investigate FIV tropism in bone marrow and to further characterize the role of CXCR4 in FIV in vivo.

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