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In Vivo CXCR4 Expression, Lymphoid Cell Phenotype, and Feline Immunodeficiency Virus Infection

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Abstract

Primary isolates of feline immunodeficiency virus (FIV) appear to require binding to CD134 in conjunction with CXCR4(X4) to infect IL-2 dependent T-cell derived cells in culture. However, much less is known about the role of X4 for infection of cells *in vivo*. To investigate the correlation between X4 expression and FIV infection in cats acutely infected with FIV-C-Pgmr we used high speed fluorescence activated cell sorting and realtime PCR to co-analyze cell phenotypes from lymph node, thymus, bone marrow and blood for FIV infection and X4 expression. X4 expression was greatest in lymph node, both in frequency and in mean fluorescence intensity. The thymus demonstrated a higher proviral burden in X4+ thymic T cells (~14% in X4+ thymic T cells and 7% in X4- cells) whereas, proviral loads were similar between X4+ and X4- cell populations in all other tissues examined. Assuming a minimum of one proviral copy per cell, a maximum of ~50% of FIV-positive cells were X4+. The highest fraction of FIV-infected X4- cells was present in bone marrow. Regardless of X4 status, proviral loads were higher in lymph node and blood T cells than in B cells. These studies provide both a positive association between X4 expression and FIV infection and introduce the probability that X4- independent infection occurs in other target cells *in vivo*.

Keywords

feline immunodeficiency virus; CXCR4; tissue tropism; in situ hybridization

Introduction

FIV, like HIV, causes a fatal immunodeficiency syndrome characterized by progressive CD4 + T cell loss. FIV and HIV also have similar tissue tropism including T lymphocytes

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Conflict of Interest Statement

None of the authors has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the paper entitled "In Vivo CXCR4 Expression, Lymphoid Cell Phenotype, and feline Immunodeficiency Virus Infection".

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(Yamamoto et al., 1988; Novotney et al., 1990; Brown et al., 1991; Callanan et al., 1994;), macrophages (Brunner and Pedersen, 1989; Bach et al., 1994; Beebe et al., 1994; Parodi et al., 1994), B cells (English et al., 1994; Dean et al., 1996), megakaryocytes (Beebe et al., 1992), monocytes (Dow et al., 1999), astrocytes and microglia (Dow et al., 1992; Coughlan et al., 2000; Nakagaki et al., 2001; Johnston et al., 2002; Hein et al., 2003). In addition, both CD4⁺ and CD8⁺ T cells are susceptible to productive FIV infection (Brown et al., 1991; Willett et al., 1991). In contrast to primate lentiviruses, FIV does not employ CD4 (Hosie et al., 1993; de Parseval et al., 1997; Willett et al., 1997) for cell entry, using instead the receptors CXCR4 (X4) and CD134 (de Parseval et al., 2004; Shimojima et al., 2004). CD134 expression is high in CD4-positive T-cell subsets and may help explain the depletion of CD4 positive cells in FIV infection without the use of CD4 as a receptor.

Abundant data suggest that the mechanism of virus attachment and entry is highly conserved among lentiviruses. For both FIV and HIV, cell entry by the vast majority of strains involves interaction of the Env V3 loop with the X4 or CCR5 receptor (Endrich and Gehring, 1998; Rabehi et al., 1998; Hung et al., 1999; Stanfield et al., 1999). Both viruses have demonstrated ability to use DC-SIGN (Lee et al., 2001; Lin et al., 2001; Soilleux et al., 2001; de Parseval et al., 2004) and cell surface heparans (de Parseval and Elder, 2001) to enhance binding affinity. Both demonstrate change from non-syncytium-inducing to syncytium-inducing phenotype, which correlates with increase in V3 loop charge (Verschoor et al., 1995; Cocchi et al., 1996). Human cells that express CXCR4 undergo syncytium formation with FIV-infected feline cells (Willett et al., 1997; Poeschla and Looney, 1998). Bicyclam analogues, in particular the analogue designated AMD 3100, have been shown to specifically bind X4 and competitively inhibit binding of both HIV-1 (De Clercq et al., 1992; De Clercq et al., 1994; Schols et al., 1997; Donzella et al., 1998; Labrosse et al., 1998; Bridger et al., 1999; Este et al., 1999; Schols, 1999; Labrosse et al., 2003) and FIV (Egberink et al., 1999; Richardson et al., 1999; Garg et al., 2004) *in vitro*.

Whether *in vitro* models are reflective of what occurs *in vivo* is less clear. For example, in addition to CD4⁺ CD134⁺ cells, FIV also has been shown to infect CD8⁺ T cells, B cells and macrophages that express little or no CD134 (de Parseval et al., 2004). Also, while AMD3100 provides potent and consistent inhibition of FIV *in vitro*, the efficacy of its antiviral activity *in vivo* remains much less certain (Hartmann, 2002; Troth et al., unpublished data).

To better understand the role of X4 in FIV infection *in vivo* we studied the relationship of X4 expression and FIV provirus in tissue and lymphoid cell subsets of acutely infected cats. Our results point both to the association of X4 and FIV in certain cell subsets but also to potential X4-independent infection in other cell populations.

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. At 2 weeks post inoculation (p.i.) heparinized and EDTA blood was collected to monitor infection status. At 3 to 4 weeks p.i. necropsy was performed on six cats. Thymus, bone marrow, pharyngeal lymph node and PBMC from whole blood were collected and single cell suspensions prepared by passage through a 40µm tissue sieve.

Enhancement of X4 expression

Upregulation of X4 surface expression in feline cell suspensions was achieved by overnight culture (Bermejo, M. et al.). Cells were placed in a culture medium of RPMI (Gibco), 2%

glutamine and 20% fetal bovine serum and cultured overnight at 37°C. CXCR4 expression in feline PBMC was compared by FACS analysis following overnight culture in identical culture media at different temperatures. Figure 1 demonstrates marked X4 upregulation with culture at 37°C compared with 4°C. Overnight culture at 37°C resulted in less than 5.0% cell death as assessed by trypan blue staining.

Virus inoculum

To avoid *in vitro* attenuation, FIV stocks used were produced by *in vivo* passage of FIV isolate FIV-C-Pgmr (Sodora, D. et al., 1994). Briefly, eight-week-old SPF cats were inoculated intravenously (IV) with 10⁸ RNA copies of FIV-C-Pgmr. At week 3 post-inoculation (PI), plasma was collected from heparinized blood, aliquoted, and stored in liquid nitrogen.

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-X4 44717 mAb crossreactive with feline (R&D systems), FITC-conjugated anti feline antigen CD4 and CD8 (Southern Biotech, Birmingham, AL) and phycoerythrin (PE) conjugated anti B-cell antibody clone CA2.1D6 crossreactive with feline antigen (Serotec) or isotype matched control mAbs. Samples were incubated at 4°C in the dark for five hours then resuspended and sorted using a MoFlo flow cytometer (DakoCytomation, Fort Collins, CO) using an Innova 300 Argon laser emitting at 488nm and an Innova 70C Argon/Krypton laser emitting at 635nm. 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. Typical cell purity following cell sorts were 92.0%, 91.8% and 95.5% for CXCR4, T cell and B cell subsets respectively (Fig 2).

FIV real time DNA PCR

Real time quantitative DNA PCR adapted from a method by Pedersen et al. (Pedersen N.C. et al., 2001) 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 excised with BamHI and repurified.

A standard curve was generated for each plate with serial dilutions of FIV-C *gag* plasmid DNA (10⁹, 10⁶, 10²) with 1X TE and 40 ng/ml salmon testes DNA (Sigma) as a carrier. Samples and standards were analyzed in triplicate on 96-well plates using an iCycler (Biorad). Polymerase activation and target amplification were performed using the following protocol: 2 minutes at 50°C, 10 minutes at 90°C 45 cycles at 95°C for 15 seconds, 60°C for 1 minute.

Results

X4 expression in tissues and lymphoid cell subsets

In most tissues gates for X4- (R1) and X+ fractions (R2) were set such that an ambiguous population with marginal X4 expression was not collected. Fig 3 displays histograms

demonstrating X4 staining across different tissues in three cats. X4 expression was significantly greater in lymph node than in other tissues, as measured both by cell number (Fig. 4a) and fluorescence intensity ($p < 0.02$) (Fig. 4b). No difference in X4 expression was detected among the other tissues examined.

A greater proportion of T cells (mean = $45 \pm 16\%$) than B cells (mean = $23 \pm 23\%$) expressed X4 in all tissues (data not shown).

FIV proviral levels in tissues

Unfractionated tissue homogenates were examined to determine proviral loads in or among tissues. The thymus contained a significantly greater proviral load than all other tissues ($p < 0.05$) (Fig. 4c). Proviral levels in bone marrow, lymph node and blood were not significantly different.

FIV proviral levels in CXCR4 positive and negative subsets

Surprisingly, similar proviral concentrations were detected among X4+ and X4- subsets in all tissues examined (Fig. 4d). The apparent difference in proviral loads observed in blood B cells was not statistically significant ($p = 0.39$). B cells were not analyzed in thymus and bone marrow due to insufficient numbers for DNA extraction. As anticipated, bone marrow contained a large population of cells negative for both T and B cell markers, presumably representing myeloid and erythroid cell precursors. The X4+ and X4- populations were sorted. In neither the X4+ nor the X4- subsets of these non T/non B cell fractions was the proviral load significantly different from those in the B or T cell marking populations.

Proviral levels in T and B cell subsets

T cell subsets in lymph node ($p < 0.01$) and blood ($p < 0.05$) contained significantly higher proviral loads than B cell subsets in both compartments (data not shown).

Discussion

Using combined cell sorting and PCR we identified populations of X4+/FIV+ cells in multiple hemolymphatic tissues of cats. While the presence of the X4+/FIV+ population was anticipated given the *in vitro* data demonstrating the requirement of X4 expression for cell infection, less anticipated was the number of X4- cells also harboring FIV DNA. The most obvious explanation for this discrepancy would be the possibility of an X4- independent pathway for infection of some cell populations *in vivo*. A wider variety of cofactors and options to mediate virus entry are likely present *in vivo* versus culture systems. Indeed, strains of HIV and SIV have demonstrated evolution towards diverse (co)receptor usage such as CCR3 (Choe et al., 1996; Doranz et al., 1996), CCR8 (Rucker et al., 1997), GPR1 (Farzan et al., 1997; Edinger et al., 1998; Shimizu et al., 1999), GPR15 (Farzan et al., 1997; Edinger et al., 1998), CXCR6 (Alkhatib et al., 1997; Deng et al., 1997), Apj (Choe et al., 1996; Edinger et al., 1998), and RDC1 (Shimizu et al., 1999; Willey et al., 2003). Additionally, the antiviral effect of the X4-specific antagonist AMD3100 *in vivo* was much less potent compared with its effect *in vitro* (Troth, unpublished data), suggesting an alternate mechanism of infection. In these studies only marginal decreases in proviral loads were seen in cats following AMD3100 treatment versus nearly complete inhibition of virus in PBMC *in vitro*. While *in vitro* studies with FIV (Willett et al., 2003) and HIV (Sheeter et al., 2003) have demonstrated no effect of lentiviral infection on X4 expression, clinical data exist suggesting downregulation *in vivo* (Nicholson et al., 2001; Ruibal-Ares et al., 2004; Shalekoff et al., 2001). Thus, while attempts were made to reduce temporal variability in X4 expression in the present study via *in vitro* CXCR4 upregulation, it is possible that post-infection down regulation of X4 expression may have occurred. A third possible caveat could be the breadth of X4 antibody reactivity. Human X4

studies have demonstrated variable antibody interactions with different subsets of X4 receptors, suggesting that different conformations of X4 are present *in vivo* that may be cell type dependent (Baribaud et al., 2001; Lapham et al., 2002). Thus, it is possible that not all feline X4+ cells were recognized by mAb 44717. Even if some or all of the above complications are present however, our results suggest that X4 may not be an obligate co-receptor for FIV cell infection *in vivo*.

The vast majority of X4+ cells labeled for T or B cell markers, whereas the X4- population contained a much larger proportion of non-T non-B cells. In particular, the bone marrow contained a large fraction of FIV+ cells that bore neither B nor T cell markers. This parallels our finding in another recent study in chronically infected cats wherein we found that bone marrow was a major reservoir of FIV+ cells, particularly in chronic infection (Troth, unpublished data). In the absence of native or hetero-species cross-reactive antibodies to define feline bone marrow subsets, we were limited to sorting and assaying non-lymphocyte marrow cell fractions. The proviral loads in this latter population (primarily myeloid and erythroid progenitors), while slightly lower than the T cell populations, were substantial, indicative of a nonlymphoid FIV cell reservoir population in the marrow. Interestingly, marrow cells expressed X4 at levels no greater than thymus or blood. This was surprising given the relatively high X4 expression that has been identified in human bone marrow subsets (Rosu-Myles et al., 2000a; Rosu-Myles et al., 2000b). Treatment with X4 antagonist AMD3100 induces a transient leukocytosis in humans (Liles et al., 2003), presumably a result of the compound interfering with X4- mediated leukocyte homing to the bone marrow. These findings, along with the association of FIV infection with neutropenia, warrant additional examination of bone marrow cells as targets and/or reservoirs of FIV and other lentiviruses.

Lymph node and blood T cells contained significantly higher FIV proviral burdens than did B cells. This evidence of FIV T cell tropism within lymphoid cell subsets is consistent with the previous work of Dean et al. (Dean et al., 1996) using somewhat different methodology to examine a clade A FIV isolate (FIV-PET). We also noted similar proviral burdens in blood, lymph node and bone marrow, indicating that with the exception of thymus, blood proviral levels estimate body-wide viral burdens.

In summary, the present study demonstrates a positive association between X4 expression and FIV infection *in vivo*. However, the results also suggest the existence of an X4- independent mode of cell infection *in vivo*. The study further confirms the thymus as the major target in acute FIV infection and points to the bone marrow as a previously under-appreciated target and reservoir for FIV infection.

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FIG. 1. CXCR4 upregulation in feline PBMC by incubation in media at 4°C and 37°C. This protocol was employed to enhance detection of all X4 constitutively expressing cells.

FIG. 2.

Cell purity following FACS. CXCR4 (172-APC), T (CD4/8 cocktail-FITC), and B (CA2.1D6-PE) cells sorted from peripheral blood mononuclear cell suspensions by FACS followed by analysis for purity. Greater than 91% purity was achieved for each phenotype. (Shaded histogram = peripheral blood cell population presort, solid line = post-sort cell populations, dashed line = isotype control antibody).

FIG. 3. CXCR4 expression in feline tissues. CXCR4 expression as compared in four different tissues from three cats (4171, 4172, 4173). Highest CXCR4 expression occurred in the lymph node ($p < 0.05$), however substantial CXCR4 expression was also detected in blood, lymph node and thymus.

FIG. 4.

Relationship between X4 expression and FIV infection in tissues (n=6). Lymph node demonstrated the highest CXCR4 expression both in terms of (a) numbers of positive cells ($P < 0.05$) as well as number of receptors per cell indicated by (b) mean fluorescence intensity ($P < 0.02$). (c) Proviral loads were highest in the thymus compared with lymph node despite lower CXCR4 expression. (d) Some differences in proportions of FIV positive cells were observed in X4 positive and negative subsets of T and B cells, however none were statistically significant. Greater proviral loads were detected in T cells than B cells within pharyngeal lymph node (ph ln) and blood ($P < 0.02$).