Mutations in the *CXCR4* gene cause a population shift in the feline lymphoma cell line 3201 surviving feline immunodeficiency virus infection

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Abstract

The feline 3201-S cell line was established from original 3201 cell populations that survived the productive infection of feline thymic lymphoma 3201 cells with feline immunodeficiency virus (FIV). The 3201-S cells are free of FIV DNA and are refractory to FIV reinfection. In addition, while the cells express CXCR4, a co-receptor for FIV infection, they are unresponsive to the CXCR4 ligand SDF-1α. The 3201-S cells encode distinct mutations in the *CXCR4* gene. Thus, it appears that 3201 cells are heterogeneous, consisting of phenotypically diverse populations resulting from genetic mutations. In this mini-review, possible mechanisms of how the 3201-S cells could restrict FIV infection and not respond to SDF-1α are described and discussed.

Keywords: Feline immunodeficiency virus; 3201; 3201-S; CXCR4; Sequence analysis

1. Introduction

The development of appropriate animal models of acquired immunodeficiency syndrome (AIDS) is critical to better understand the pathogenesis of lentivirus-induced immune deficiency, which is etiologically linked to human immunodeficiency virus type 1 (HIV-1) (Levy 1993). Only two animal lentiviruses have thus far been implicated in AIDS-like diseases, feline immunodeficiency virus (FIV) (Pedersen et al. 1987) and simian immunodeficiency virus (SIV) (Letvin et al. 1985). The use of SIV in macaques provides an opportunity to evaluate a primate lentivirus within a species closely related to humans. A major concern, however, is the relative shortage of non-human primates available for infectivity trials and for safety and efficacy studies on candidate vaccines and antiviral drugs. The advantages of a feline model of AIDS are not only the similarities to human AIDS, but also the practicality and cost compared with the use of non-human primates (Pedersen et al. 1989). As with HIV, FIV primarily targets $CD4^+$ T cells, but instead of CD4, it uses CD134, a T cell activation and co-stimulatory molecule, as a primary receptor (de Parseval et al. 2004, Shimojima et al. 2004). AIDS-like immunodeficiency in its natural host species is caused by a progressive depletion of $CD4^+$ T cells (Pedersen et al. 1989). The feline animal model thus provides the research community with a well-established research species that is easy handle. Moreover, FIV has the advantage that a high level of biocontainment at laboratory facilities is not required, thereby representing an alternative and a valuable model for HIV/AIDS research.

It has been described earlier that the feline lymphoma cell line 3201 (Snyder et al. 1978) was highly susceptible to the cytopathic effects of FIV-Petaluma strain. Moreover, 3201 cells that survived FIV infection established a chronic FIV-producer cell line (3201/FIV) during a chase period of 2 months (Tochikura et al. 1990). Recently, a similar experiment was repeated, and it was found that the

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3201/FIV cell line was established consistent with our earlier observations, but the frequency of FIV antigen-positive cells detected by indirect immunofluorescence assay (IFA) decreased gradually over time during further incubation. At 86 days postinfection (dpi), none of the FIV-surviving cells that were designated as 3201-S were positive for viral antigens (Tochikura et al. 2010). This is of interest, considering that 3201-S cells have not yet been characterized.

2. Establishment of 3201 cells surviving FIV infection

2.1 Disappearance of FIV expression during culture

After the exposure of 3201 cells to cell-free culture fluids derived from the 3201/FIV cell culture, continuous growth of the infected cells and production of FIV were observed (Tochikura et al. 2010); however, the frequency of FIV antigen-positive cells decreased gradually over time during further incubation. When the surviving cells were evaluated at 86 dpi for their ability to be a reservoir of FIV, neither infectious virus nor FIV antigen-expressing cells were detected by IFA, even after treatment with IUdR or phorbol myristate acetate, a well-known inducer of retroviruses (data not shown) (Besmer et al. 1975, Brunner & Pedersen 1989). The non-virus-producing survivor cells, here referred to as 3201-S, were indistinguishable in morphology and growth rate from mock-infected 3201 cells (Tochikura et al. 2010).

To determine if FIV DNA was present in the FIV-surviving 3201-S cells after exposure to FIV, samples were examined by nested polymerase chain reaction (PCR) (Hohdatsu et al. 1996, 1998). DNA isolated from cell cultures derived from virus-producing 3201/FIV cells showed the 329-bp and 873-bp products specific to FIV *gag* and *env*, respectively, whereas no specific bands were detected in samples from 3201-S or mock-infected 3201 cells (Figure 1).

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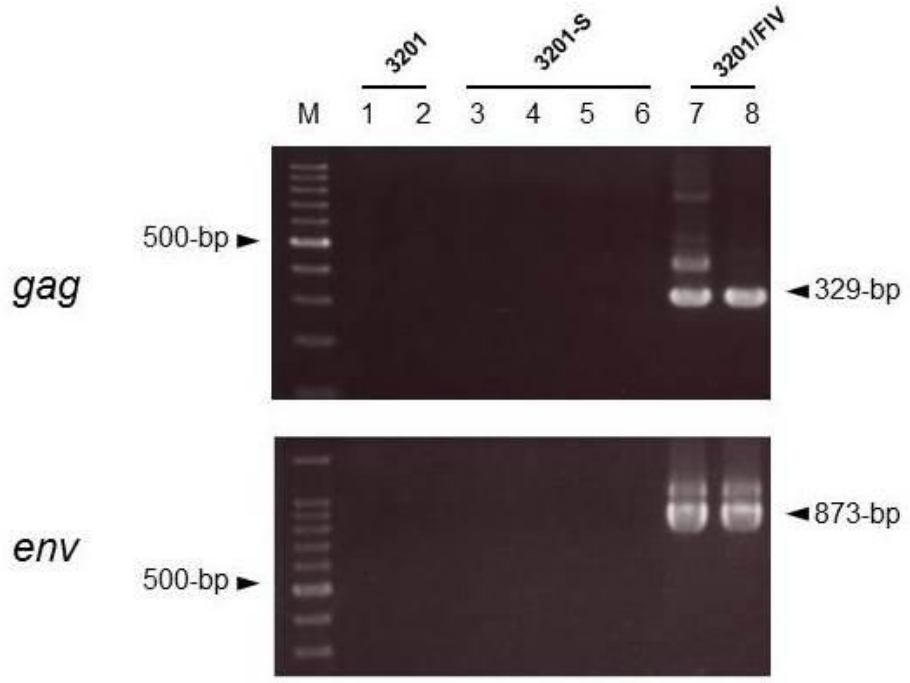

Fig 1. Agarose gel showing the results of PCR amplification of DNA for the FIV *gag*-, and *env*- *gene* regions obtained from the indicated cells. Lanes: M, DNA size marker (100-bp ladder) ; 1 and 2 (duplicate), mock-infected 3201cells ; 3 - 6 (quadruplicate), 3201-S cells ; 7 and 8 (duplicate), 3201/FIV cells.

Figure 1. Agarose gel showing the results of PCR amplification of DNA for the FIV gag-, and env- gene regions obtained from the indicated cells. A nested PCR was used to amplify the FIV *gag* or *env* gene sequences as described previously (Hohdatsu et al. 1996,1998). The positions of bands of 329 bp and 873 bp corresponding to *gag* and *env*, respectively, are shown. Lanes: M, DNA size marker (100-bp ladder); 1 and 2 (duplicate), mock-infected 3201 cells; 3 to 6 (quadruplicate), 3201-S cells; 7 and 8 (duplicate), 3201/FIV cells.

2.2. Absence of CD134 expression in 3201 cells

CD134 has been reported to be expressed in peripheral blood mononuclear cells (PBMCs) and interleukin-2-dependent T cell lines and to represent a primary target of FIV (Shimojima et al. 2004). Thus, an

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analysis was undertaken to determine whether CD134 expression may be involved in FIV infections of 3201 and 3201-S cells. Consistent with previous findings that 3201 cells lack CD134 expression (de Parseval et al. 2004), the binding of anti-CD134 monoclonal antibody (MAb) (Affinity BioReagents, Golden, CO, USA), detected by flow cytometry and indicated as mean fluorescence intensity (MFI), was observed on activated PBMCs used as a positive control but not on 3201 or 3201-S cells (more than a 5-fold difference in MFI, data not shown), confirming that CD134 is not likely to contribute to FIV infection of 3201 cells.

2.3. Presence of CXCR4 expression in 3201 and 3201-S cells

Similar to HIV-1, FIV utilizes CXCR4, a member of the chemokine group of the G-protein-coupled receptor family, as a virus-entry co-receptor for infection. There is evidence of shared chemokine receptor use between HIV-1 and FIV (Willett et al. 1997). CXCR4 is a seven transmembrane protein with an extracellular N-terminus, three extracellular loops (ECLs), three intracellular loops (ICLs), and an intracellular C-terminal tail (Juarez et al. 2004). Although the second extracellular loop (ECL2) has been reported to determine its function as a co-receptor for FIV (Willett et al. 1998), details of the mechanism of FIV entry into cells remain to be elucidated.

It has been shown that 3201 cells express relatively high levels of CXCR4, used by FIV for infection (Endo et al. 2000, Hosie et al. 1998). The cell-surface expression of CXCR4 on 3201, 3201-S, and 3201/FIV cells was next evaluated by flow cytometry using anti-CXCR4 44717 MAb (R&D Systems, Minneapolis, MN, USA). As shown in Figure 2, a marked shift was seen in the fluorescence histogram of anti-CXCR4 stained cells compared with the background (FITC-conjugated secondary antibody alone), indicating the presence of cell surface CXCR4 proteins on 3201 cells.

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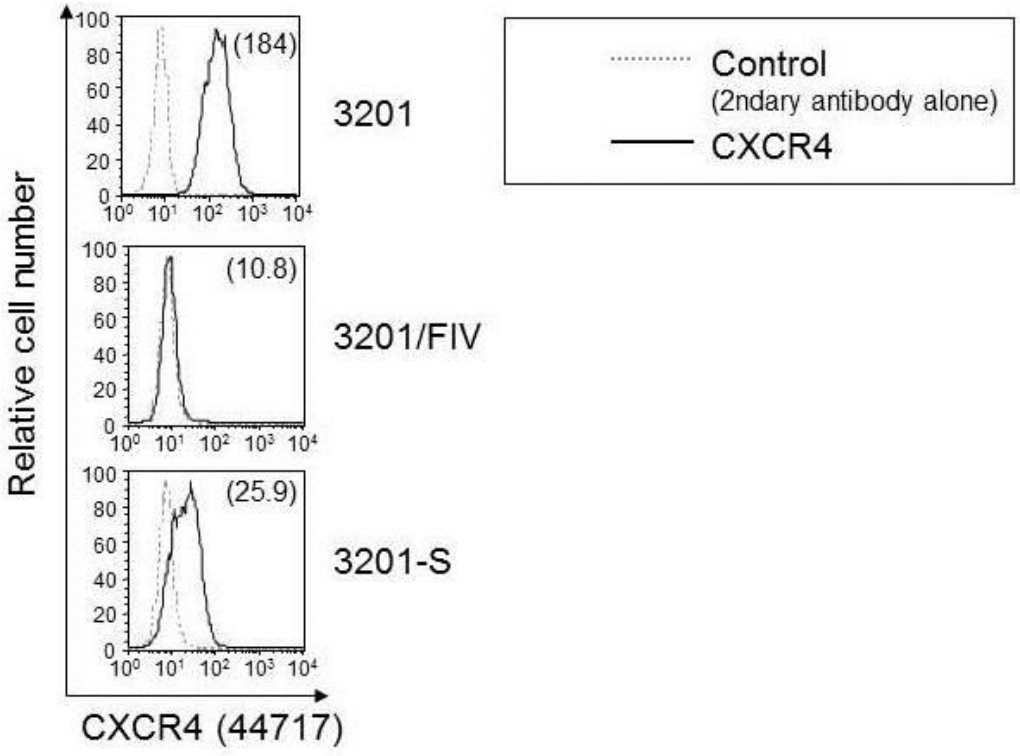

Fig 2. Flow cytometric analysis of CXCR4 expression on 3201, 3201-S, and 3201/FIV cells. Mean fluorescence intensity (MFI) is indicated in parentheses.

Figure 2. Flow cytometric analysis of CXCR4 expression on 3201, 3201-S, and 3201/FIV cells. Cells were stained with anti-CXCR4 44717 MAb, followed by FITC-conjugated anti-IgG (bold line). For all panels, data are shown as relative cell number (y-axis) plotted against relative fluorescence intensity (x-axis). The background staining is the signal derived from incubation of the cells with the FITC-conjugated secondary antibody only (dashed line). Mean fluorescence intensity (MFI) is indicated in parentheses.

The amount of CXCR4 was markedly reduced in the FIV-infected 3201 cells, with more than 90% of the cells positive for FIV antigens. This finding is in agreement with a report that the surface expression of CXCR4 was markedly reduced on Crandell feline kidney (CRFK) cells after FIV infection, presumably due to either a down-regulation of CXCR4 expression or the elimination of CXCR4-expressing cells from the culture (Hosie et al. 1998).

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Of particular interest is that approximately 45% of the cells in the 3201-S culture were recognized by the MAb, albeit with a low intensity, compared with almost 100% and 2% of the cells in the 3201 and 3201/FIV controls, respectively (data not shown). This will be discussed later.

2.4. Demonstration of the presence of FIV DNA in FIV-reinfected 3201-S cells

To ascertain the susceptibility of 3201-S cells to FIV reinfection, the cells were exposed to the culture supernatant of 3201/FIV cells at a M.O.I. of 0.04. Viral antigens of FIV were first detected in the parental 3201 cells by IFA as early as 3 dpi, and the frequency of detection increased gradually with time reaching more than 90% within a week (data not shown), whereas 3201-S cells remained negative for the FIV antigen indistinguishably from mock-infected 3201 cells during a 3-month culture period (data not shown). To further confirm the failure of FIV to reinfect 3201-S cells, the DNA was extracted at 5, 15, and 30 dpi from 3201-S cells that had been reinfected with FIV and analyzed for the presence of FIV DNA by PCR amplification (Hohdatsu et al. 1996, 1998). Interestingly, FIV DNA was detected by nested PCR at 5 dpi in the 3201-S cells reinfected with FIV, the copy number determined by real-time PCR being as little as 4 to 7 per 10^6 cells, whereas FIV-infected 3201 cells were positive for FIV DNA throughout the experiment (Table 1). However, the viral genome in the FIV-reinfected 3201-S cells was no longer detectable at the subsequent testing points, indicating that FIV infection of 3201-S cells is inefficient (less than 1/1000) compared with that of parental 3201 cells.

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Table 1 Detection of FIV DNA in the indicated cells after infection or reinfection with FIV

Cell	Assay type	Days after infection (3201) or reinfection (3201-S)			
		0	5	15	30
3201	Nested PCR [a]	–	+	+	+
	Real-time PCR [b]	0	6,210	47,043	18,274
		0	5,905	45,772	22,559
3201-S	Nested PCR	–	+	–	–
	Real-time PCR	ND	7	ND	ND
		ND	4	ND	ND

[a] +, positive result; –, negative result in nested PCR assay.
[b] Result of real-time PCR assay is expressed as copy number per 10^6 cells.
Experiments were carried out in duplicate.
ND, not done.

2.5. CXCR4 is not functional on 3201-S cells.

The activation of calcium flux in CXCR4-expressing human cells is related to HIV infection, and as reported, stimulation of CXCR4 by its ligand SDF-1α increases calcium flux in several HIV- susceptible cell types (Ichiyama et al. 2003, Sloane et al. 2005). Since it became interesting to reveal whether 3201-S cells will respond to SDF-1α through calcium flux, a calcium mobilization experiment was conducted to determine whether the CXCR4 expressed by 3201-S cells is a functional receptor. As shown in Figure 3, SDF-1α, whose concentration used in this study has previously been shown to stimulate CXCR4 to near maximal levels (Ichiyama et al. 2003), clearly increased the intracellular Ca^{2+} level in the parental 3201 cells. In contrast, SDF-1α did not stimulate the response in 3201-S cells, suggesting that CXCR4 is not functionally active on 3201-S cells.

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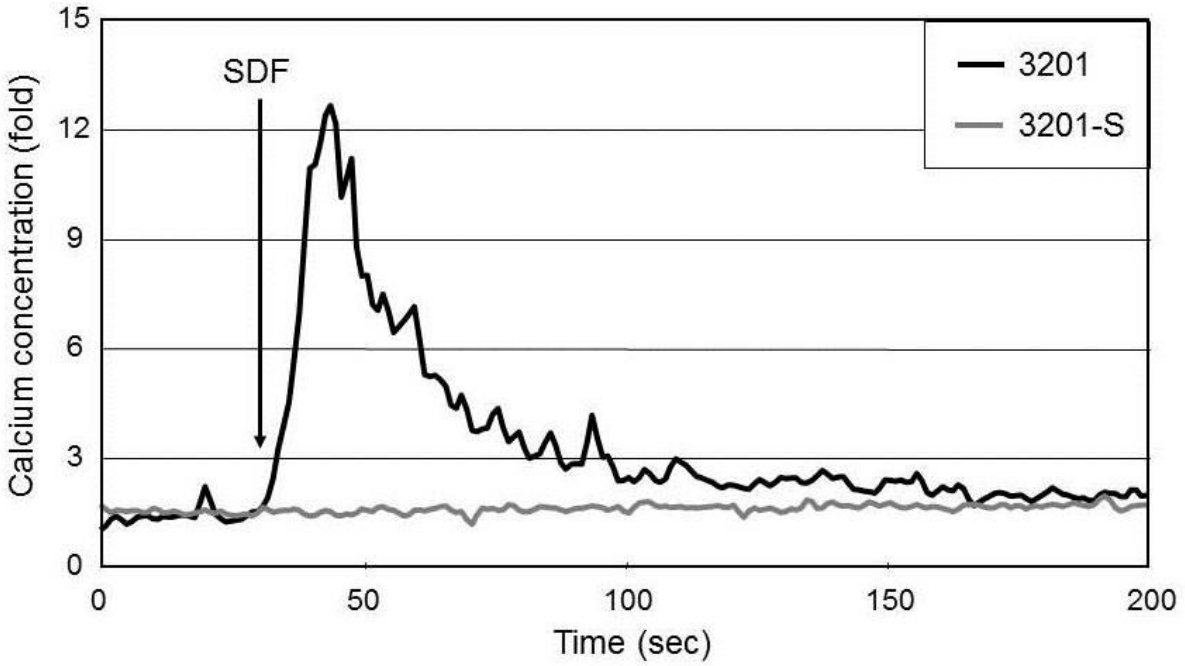

Fig 3. Comparison of SDF-1 responsiveness in 3201 and 3201-S cells.

Figure 3. Comparison of SDF-1α responsiveness in 3201 and 3201-S cells. Cells were loaded with Indo-1 AM (1μM) and incubated in RPMI 1640 medium for 30 min at 37°C in 5% CO_2, followed by stimulation at the indicated time point (arrow) with 1μg/ml SDF-1α. The intracellular Ca^{2+} concentrations were calculated as described previously (Ichiyama et al. 2003).

2.6. Sequence analysis of the *CXCR4* gene

In order to better understand the nature of CXCR4 dysfunction in 3201S cells and their resistance to sustained FIV infection, the nucleotide sequences coding for the *CXCR4* gene in the two 3201 and 3201-S cell lines were examined by PCR (Tochikura & Morimoto 2014). The nucleotide sequences coding for the *CXCR4* gene in both 3201 and 3201-S cell lines showed a common 15 bp deletion in the *CXCR4* genes compared with a published sequence (GenBank accession no. U67558). The deletion spanned amino acid residues 227 to 231 in a region corresponding to ICL3, which is associated with the domain required for Gi-dependent signaling (Juarez et al. 2004) (Figure 4). Since 3201 cells showed a flux of Ca^{2+} in response to

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SDF-1α and susceptibility to FIV infection, the residues Leu-Ser-His-Ser-Lys do not appear to be essential for the biological function of the CXCR4 protein.

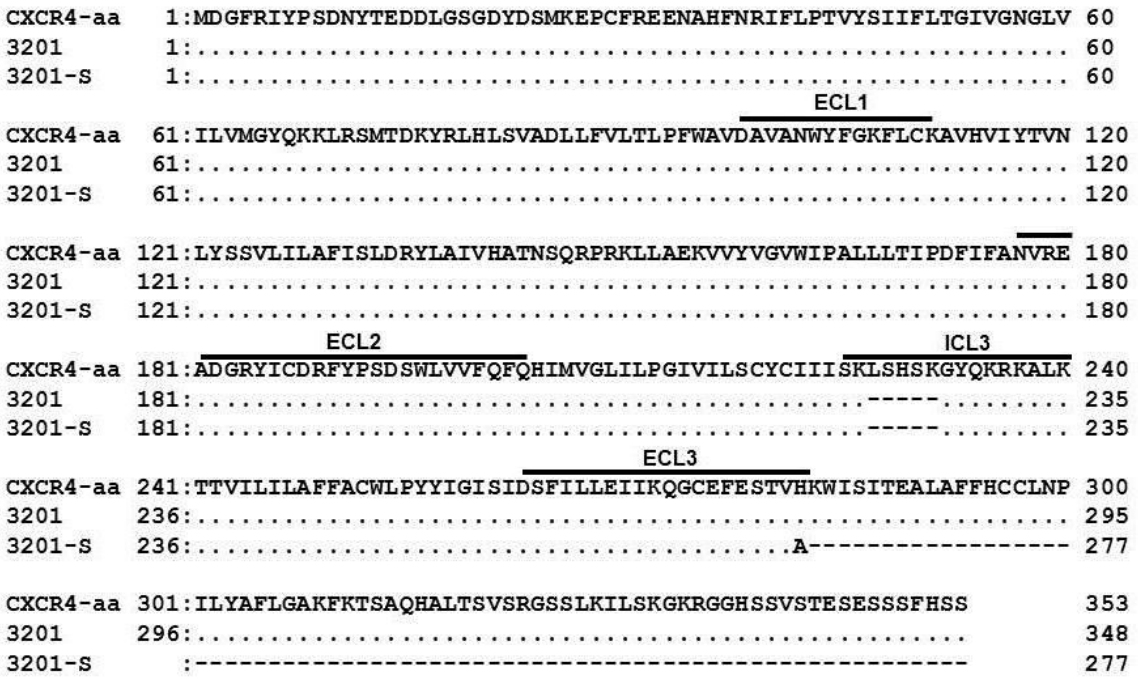

Fig.4. Comparison of the predicted amino acid sequences of feline *CXCR4* in 3201 and 3201-S cells.

Figure 4. Comparison of the predicted amino acid sequences of feline *CXCR4* in 3201 and 3201-S cells. Numbers indicate the position of the amino acid. The character ". " represents an identical amino acid, and "- " represents a residue that has been deleted. ICL3, intracellular loop 3; ECL1, extracellular loop 1; ECL2, extracellular loop 2;ECL3, extracellular loop 3.

More importantly, insertion of a thymidine base within the ECL3 region at the position between nucleotides 831 and 832 in the *CXCR4* gene of 3201-S cells was observed. This insertion causes a nonsense frame shift, leading to an early stop codon, TAA, and thus resulting in truncation of the CXCR4 protein (Figure 5). A predicted histidine residue (position 277) in 3201 cells was replaced by an alanine residue in 3201-S cells. The *CXCR4* gene in 3201-S cells expresses a putatively

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truncated protein of 277 amino acids, whereas the *CXCR4* gene in parental 3201 cells encoded a protein of 348 amino acids (Figure 4).

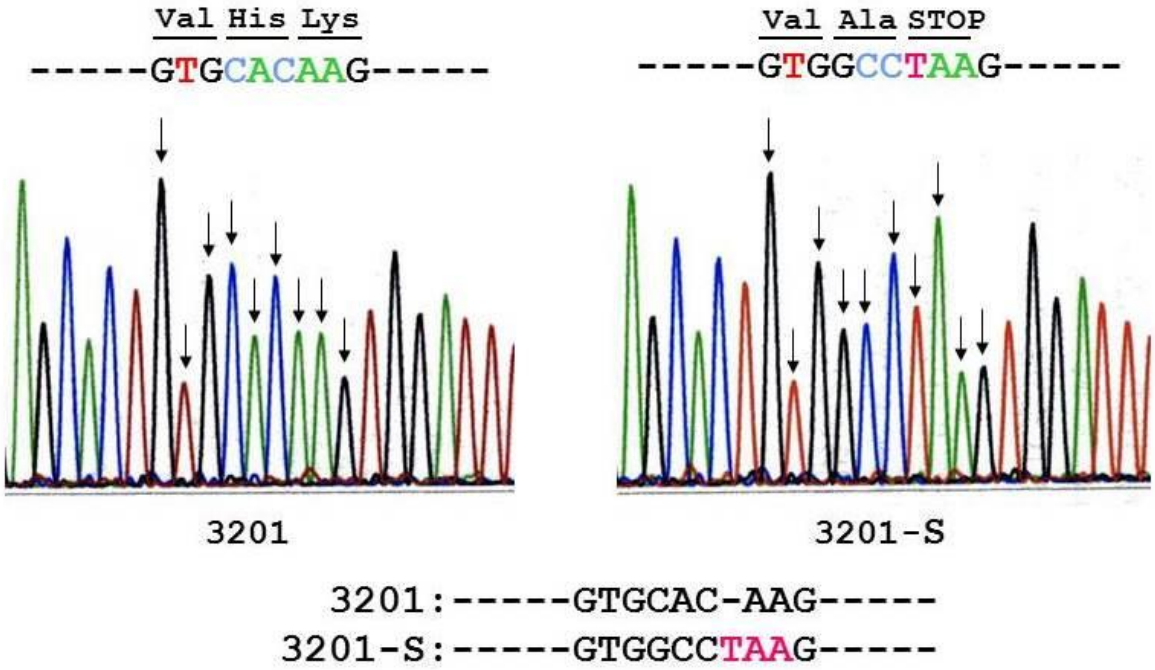

Fig.5. *CXCR4* mutation by PCR and DNA sequencing.

Figure 5. ***CXCR4*** **mutation identified by polymerase chain reaction and direct DNA sequencing.** The sequence difference is caused by the addition of a T in 3201-S cells, which generates a mutated amino acid sequence causing a translational stop.

3. Possible mechanisms of differential CXCR4 expression and function in 3201 cells

The presence of FIV DNA in the 3201-S cells at 5 dpi, albeit at an extremely low frequency, suggests that 3201-S cells are reinfectable by FIV, as determined by the PCR assay; however, FIV did not spread in 3201-S cells, as demonstrated by the lack of any persistent presence of viral DNA (Table 1). Calcium flux experiments showed that 3201 cells, but not 3201-S cells, responded to SDF-1α with a rapid mobilization of calcium, suggesting that CXCR4 in the parental 3201 cells is

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functionally active and is able to couple to G-protein signaling mechanisms (Murphy 1994), as opposed to 3201-S cells whose CXCR4 receptor is likely to be dysfunctional. The interpretation of these results is that the original 3201 cells grew as heterogeneous populations prior to FIV infection. One population, representing the majority of the original culture, expressed a functional CXCR4 and supported FIV infection, but the others, designated as 3201-S, comprising an extremely small number of the original culture, did not confer susceptibility to FIV infection, possibly due to insufficient levels of CXCR4 expression. After the productive infection of the original 3201 cells, FIV-infected cells accompanied by viral antigens (major populations in the original 3201 cell culture) were probably eliminated from the culture due to cytopathic effects, and a small percentage of the cells, such as 3201-S cells, survived to replace the original population during the cultivation.

4. Conclusions

It appears that the original 3201 cell populations can be genetically heterogeneous in the phenotype of the expressed CXCR4, and in this heterogeneity, a few surviving 3201-S cells emerged as a consequence of the differences in susceptibility to FIV. Thus, 3201-S cells represent the first unique subpopulation of $CXCR4^+$ T cells that are resistant to FIV replication.

It is suggested that the truncated form of the CXCR4 protein (frame shift-induced deletion of the last 71 amino acids) may still exhibit conformational structures required for FIV entry, even though this mutated CXCR4 is very weakly expressed on the surface of the cells. In contrast, the truncated CXCR4 seems to have lost its function as a chemokine receptor, since 3201-S cells did not respond to SDF-1α. This finding is indicative for the co-receptor function of CXCR4 being independent of its function in chemokine signaling.

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These findings provide new insights into the natural history of FIV infection, and further characterization of the CXCR4-surface envelope protein interaction may provide valuable information for planning future antiviral approaches against FIV infection.

Conflict of interest

None of the authors have had any financial or personal relationships with people or organizations that could inappropriately influence their work.

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