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An early defect in primary and secondary T cell responses in asymptomatic cats during acute feline immunodeficiency virus (FIV) infection

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SUMMARY

As in HIV infection of humans, cats infected with FIV are particularly susceptible to secondary infection by opportunistic pathogens, suggesting an impaired ability to elicit an effective immune response against foreign antigens. In order to investigate the development of immunity in FIV-infected cats, we have used an autologous culture system to directly measure priming of naive $CD4^+$ T cells to soluble protein antigen, *in vitro*. Using this assay, we showed previously that cats infected with FIV for several months had significantly reduced primary proliferative responses. We have now examined cats before infection, and at varying times after infection with FIV, to determine how soon after infection this defect in T cell priming was evident, compared with other quantitative and qualitative measurements of lymphocyte function. Our results showed a progressive decline in immune function in asymptomatic cats during the acute stage of infection with FIV. Primary T cell responses were most sensitive and a significant reduction in proliferation of naive T cells to foreign antigen occurred 5 weeks after infection, despite normal blastogenesis to T cell mitogens and normal $CD4^+/CD8^+$ ratios at these times. Whilst lymphocyte proliferation to T cell mitogens was unaffected throughout, a significant reduction in proliferation to a B cell mitogen occurred from week 8 onwards. $CD4^+/CD8^+$ ratios fell significantly from week 13 onwards, and proliferation of the memory T cell population to a recall antigen was significantly impaired later, from week 19 onwards. The defect in the priming of naive T cells to foreign antigen early after infection may be important in determining susceptibility to secondary infections.

Keywords feline immunodeficiency virus AIDS T lymphocyte priming

INTRODUCTION

FIV is a lentivirus which is associated with the development of an immunodeficiency syndrome in cats [1–3]. The pathogenesis of the disease in cats shows many clinical and pathological similarities with HIV infection in humans [1], suggesting that the FIV-infected cat may be useful as a small animal model for human AIDS.

It has been possible to stage FIV infection into distinct stages comparable to those in HIV-1 infection [4]. Experimental infection of cats is followed by an acute phase which lasts several weeks to months. This is followed by an asymptomatic carrier (AC) phase and later progression to ARC and AIDS. AIDS in FIV infection is associated with chronic clinical syndromes which arise following infection by several opportunistic secondary pathogens [5–7]. The mechanism of the susceptibility to infection by secondary pathogens is not known. As in HIV infection, studies have shown a progressive decline in immune function through the clinical phases of FIV infection [4], such as reduced numbers of circulating $CD4^+$ T cells, inverted $CD4^+/CD8^+$ ratios, and depressed lymphocyte function, as measured by mitogen blastogenesis [8–10].

In our previous studies we developed an autologous culture system to measure directly the priming of feline T cells to a soluble protein antigen, *in vitro* [11], without a requirement for *in vivo* priming. Priming was antigen-specific and resulted in the expansion of T cells bearing surface CD4, with increased secretion of feline T cell growth factors. Our studies have shown that cats infected with FIV for up to 27 months are impaired in their ability to prime naive $CD4^+$ (T helper) cells to a T-dependent antigen which they have not encountered previously, *in vivo*. Impairment of primary proliferative responses was most marked in cats which had shown clinical signs of immunosuppression at some time during infection, although there was no correlation between duration of infection and the magnitude of the primary response. We have suggested that the susceptibility of cats in late stage FIV infection to secondary infection by opportunistic pathogens may be a result of a defect in T cell priming to the infectious agent.

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However, there is some evidence that cats in primary phase FIV infection are more susceptible to secondary bacterial infection [12], and less able to develop effective specific immunity following vaccination against, or exposure to, other feline pathogens [13]. The purpose of this study was to investigate whether *in vitro* abnormalities in lymphocyte function are present in the primary phase of FIV infection. For this purpose, we have studied T cell priming in asymptomatic cats, at varying times during the acute phase of FIV infection. In addition, proliferation to secondary (recall) antigens, mitogen blastogenesis responses, and $CD4^+/CD8^+$ lymphocyte ratios, were examined.

MATERIALS AND METHODS

Animals

Specific pathogen-free (SPF) cats were bred and housed in the departmental animal facility. All cats were preimmunized with human serum albumin (HSA) by subcutaneous (s.c.) immunization with 1 mg/ml HSA in Quil A/saline, three times at weekly intervals. A group of five of these female cats were uninfected controls (cats 1–5). Another group of six female cats (cats 6–11) were infected by s.c. inoculation of FIV-infected culture fluid (cell-free), derived from mononuclear cells infected with the FIV isolate, T637, isolated in this laboratory. All cats were aged between 18 and 25 months at the start of the study.

Cats became positive for serum anti-FIV antibody (anti-p24) by week 4 post-infection (p.i.), as measured using a commercial enzyme immunoassay kit (Petchek; IDEXX, Portland, ME). Blood samples were taken from the cats for haematology at varying times over the period weeks 2–44 p.i.

Antigens and mitogens

HSA (Sigma Chemical Co., Poole, UK) and Keyhole limpet haemocyanin (KLH; Calbiochem-Behring, La Jolla, CA) were used as secondary (recall) and primary antigens respectively. Both were dialysed extensively against PBS and filter sterilized as stock solutions of 10 mg/ml. Final culture concentrations used were 200 μg/ml HSA and 40 μg/ml KLH.

The B cell mitogen lipopolysaccharide (LPS; from *Escherichia coli*, serotype 0127:B8; Sigma), and the T cell mitogens, pokeweed mitogen (PWM), and concanavalin A (Con A) (Sigma) were used. Final culture concentrations used were 50 μg/ml LPS, 1 μg/ml PWM and 1 μg/ml Con A.

Cell preparation and culture

Blood was collected by jugular venepuncture into sodium citrate solution (Sigma) and diluted with Hank's balanced salt solution (modified HBSS; Flow Laboratories, Irvine, UK). Peripheral blood mononuclear cells (PBMC), comprising 80% lymphocytes, 17% monocytes and 3% polymorphonuclear cells, were prepared by centrifugation over layered density gradients, as previously described [11].

Priming of naive feline $CD4^+$ T cells to soluble protein antigen was measured using an autologous culture system previously described by us [11]. PBMC were cultured in Iscoves medium (Flow), supplemented with 4 mM L-glutamine (Gibco Europe Ltd., Paisley, UK), 100 U/ml penicillin and 100 μg/ml streptomycin (Imperial Laboratories, Andover, UK) and 5×10^{-5} M 2-mercaptoethanol (Sigma). Autologous cat serum, taken before infection with FIV and stored at $-20°C$, was used at a concentration of 2%. No heterologous serum additives were used. PBMC for mitogen blastogenesis and secondary proliferative cultures were prepared as above.

Cultures for mitogen blastogenesis and secondary proliferative responses contained $1{\cdot}0 \times 10^6$ cells/ml in 0·2 ml volumes in U-bottomed 96-well microtitre plates (Flow). Primary cultures contained $1{\cdot}2 \times 10^6$ cells/ml in 2 ml volumes in 24-well plates (Falcon; Becton Dickinson, Lincoln Park, NJ). All cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2.

Triplicate 0·2 ml microtitre cultures were harvested at either day 3 or day 4 from mitogen blastogenesis and secondary cultures respectively. Three 0·1-ml samples were removed from 2-ml primary cultures over days 6–11 of culture. Samples were pulsed with 1 μCi/well of ^{3}H-thymidine (Amersham International, Amersham, UK), for 6 h and then harvested onto glass fibre mats using a multi-sample cell harvester (Skatron-AS, Lier, Norway). Incorporation of ^{3}H-thymidine into DNA was measured by liquid scintillation counting (LKB-Wallac, Turku, Finland). All results are expressed as mean ct/min with s.d. of the mean of replicates.

Measurement of $CD4^+/CD8^+$ lymphocyte ratios in peripheral blood

Indirect fluorescent flow cytometry was performed on leucocytes isolated by density gradient separation (Lymphocyte Separation Medium, density 1·077 g/ml; Flow). Cells were labelled with MoAbs Fe17 and FT2, which recognize the feline homologues of CD4 and CD8 respectively [14,15] followed by a flouroscein-conjugated goat anti-mouse IgG antibody (whole molecule; Sigma). Immunofluorescent staining of cells was detected by flow cytometry (Coulter Corp., Hialeah, FL). Granular cells were excluded from analysis by gating on 90° light scatter. Percentages of $CD4^+$ and $CD8^+$ lymphocytes were determined using Overtons subtraction (Coulter).

Detection of FIV in primary culture supernatant

Eight male and female SPF cats, aged 12–35 months, which had been infected with Bristol FIV isolate T637 for 6–39 months by either s.c. or i.p. routes, were studied for the possibility of virus reactivation during culture with priming antigen for 11 days. Four out of the eight cats had impaired primary proliferative responses to KLH. Samples of primary culture supernatant were removed at day 11 from cultures of PBMC with either KLH or no antigen control. Cells were pelleted by centrifugation at 13000 *g* for 15 min, and 0·1 ml samples of supernatant were tested for the presence of FIV p24 antigen using a commercial antigen enzyme immunoassay (Petchek; IDEXX).

Statistical analysis

Statistical comparisons were made using the Mann–Whitney *U*-test.

RESULTS

Haematology

All erythrocyte parameters were within normal range in both uninfected and FIV-infected cats over the period of investigation (weeks 2–44 p.i.). Total and differential leucocyte counts were normal in the uninfected cats over this period. Two of the infected cats developed leucocytosis, due to increased neutrophils, early after infection (total leucocyte counts of $44{\cdot}4 \times 10^9/l$

Table 1. Enumeration of lymphocyte subsets: mean values

Week p.i.	Absolute numbers × $10^9/l$		Percentage of total lymphocytes		$CD4^+/CD8^+$ ratio
	$CD4^+$	$CD8^+$	$CD4^+$	$CD8^+$	
Uninfected cats ($n=5$)					
0	1·25 (0·21)*	0·76 (0·15)	41·7 (3·33)	25·4 (4·00)	**1·7 (0·35)**
4	0·78 (0·19)	0·62 (0·12)	37·1 (5·41)	29·4 (2·67)	**1·2 (0·44)**
7	0·72 (0·08)	0·58 (0·14)	34·2 (3·21)	27·7 (2·45)	**1·2 (0·35)**
8	0·79 (0·19)	0·66 (0·18)	31·9 (3·61)	26·9 (2·50)	**1·1 (0·41)**
13	0·80 (0·21)	0·67 (0·17)	31·0 (2·40)	29·8 (3·51)	**1·1 (0·28)**
22	1·00 (0·15)	0·75 (0·22)	34·4 (3·79)	25·8 (2·64)	**1·3 (0·43)**
FIV-infected cats ($n=6$)					
0	1·12 (0·33)*	0·65 (0·13)	41·4 (5·37)	24·1 (2·99)	**1·8 (0·28)**
4	1·02 (0·26)	0·75 (0·24)	34·3 (6·80)	25·0 (3·10)	**1·4 (0·46)**
7	1·19 (0·37)	1·21 (0·35)	29·9 (1·64)	30·4 (9·68)	**0·9 (0·26)**
8	1·04 (0·41)	1·43 (0·56)†	29·1 (2·78)	39·8 (3·11)†	**0·6† (0·13)**
13	0·95 (0·34)	1·20 (0·35)†	30·0 (1·56)	35·7 (2·77)	**0·7‡ (0·14)**
22	1·12 (0·27)	1·58 (0·37)†	28·3 (2·53)	39·9 (3·51)†	**0·6‡ (0·14)**

* (±s.d.).
† $P=0·05$–$0·10$.
‡ $P=0·01$–$0·025$.

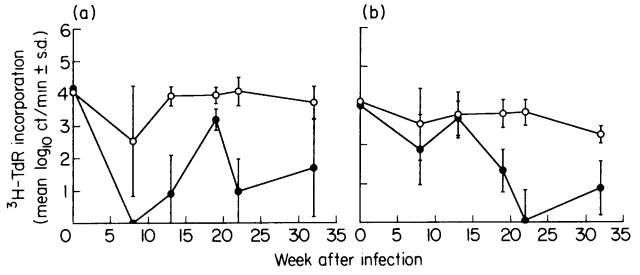

Fig. 1. (a) Mitogen blastogenesis to lipopolysaccharides (LPS) in uninfected and FIV-infected cats, at varying times after infection. Triplicate cultures containing $1·0 \times 10^6$/ml peripheral blood mononuclear cells (PBMC) with 50 μg/ml LPS were sampled at day 4. Triplicate cultures of PBMC with no antigen were controls. Blastogenesis was expressed as the mean ct/min of test triplicates − the mean ct/min of control triplicates. (b) Secondary proliferative response to recall antigen human serum albumin (HSA), in uninfected and FIV-infected cats, at varying times after infection. Triplicate cultures containing $1·0 \times 10^6$/ml PBMC with 200 μg/ml HSA were sampled at day 4. (Blastogenesis was expressed as above.) ○, Uninfected cats; ●, FIV-infected cats.

and $32·9 \times 10^9/l$ and neutrophil counts of $40·8 \times 10^9/l$ and $27·6 \times 10^9/l$, in cats 11 and 8 respectively, at weeks 2–3 p.i.). Total and differential leucocyte counts were within normal range in all the FIV-infected cats from week 4 p.i. onwards.

Atypical cells were observed occasionally in blood samples taken from the FIV-infected cats over weeks 7–19 p.i. (range $0·21 \times 10^9/l$–$0·08 \times 10^9/l$). These atypical cells (proplasmacytes) were seen in all infected cats at some time, but never observed in the uninfected cats.

$CD4^+/CD8^+$ lymphocyte ratios

The mean $CD4^+/CD8^+$ lymphocyte ratio in peripheral blood of the FIV-infected cats fell from week 8 p.i. and was significant from week 13 p.i. onwards ($P=0·01$–$0·025$) (Table 1). The reduction in the ratio did not appear to be due to a loss of $CD4^+$ cells but more likely a consequence of increasing proportions of $CD8^+$ cells at these times, since at weeks 8 and 22 the percentages of $CD8^+$ cells were significantly higher in the FIV-infected cats than in uninfected cats ($P=0·05$–$0·1$ at weeks 8 and 22 respectively) (Table 1). In addition, there was an increase in total $CD8^+$ lymphocytes, but not in $CD4^+$ lymphocytes, in the peripheral blood of FIV-infected cats at weeks 8–22 p.i. ($P=0·05$–$0·025$). At the other weeks of testing, there was no significant difference between the percentage of $CD4^+$ cells and $CD8^+$ cells in uninfected and FIV-infected cats.

Mitogen blastogenesis

There was no significant difference between lymphocyte blastogenesis to the mitogens Con A and PWM in FIV-infected cats and uninfected control cats, at any week of testing (weeks 0–32 p.i.).

A significant reduction (fall of approximately 2–3 $\log_{10}$) in lymphocyte blastogenesis to LPS in the FIV-infected cats, compared with uninfected controls, was detected when cats were examined at week 8 p.i. and at subsequent weeks of testing ($P=0·025$ at week 8, $P=0·005$–$0·01$ at weeks 13–22, and $P=0·05$–$0·1$ at week 32 (Fig. 1a).

Secondary (recall) proliferative responses to HSA

Proliferation to HSA in both uninfected and FIV-infected cats, immunized to HSA before infection, was measured at varying times after infection. Secondary responses to HSA were significantly impaired in the FIV-infected cats, compared with uninfected controls, from week 19 p.i. onwards (a reduction of approximately 0·5–3 $\log_{10}$) ($P=0·005$–$0·01$ at weeks 19 and 22 p.i., $P=0·05$ at week 32) (Fig. 1b).

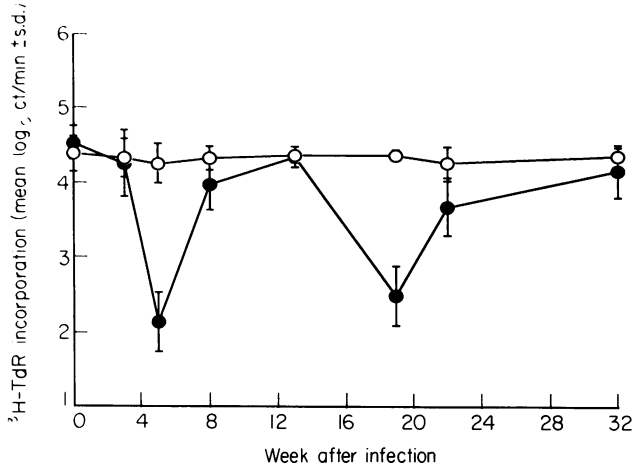

Fig. 2. Primary proliferative response to keyhole limpet haemocyanin (KLH) in uninfected and FIV-infected cats at varying times after infection. Primary cultures of $1{\cdot}2 \times 10^6$/ml peripheral blood mononuclear cells (PBMC) from non-immune cats contained 40 μg/ml KLH or no antigen control. Triplicate samples were taken at days 6–12 of culture inclusive. Proliferation was expressed as the mean ct/min of the test triplicate, at day of peak cell proliferation, − the mean ct/min of the triplicate no antigen control. ○, Uninfected cats; ●, FIV-infected cats.

Primary proliferative responses to KLH

A reduction in the primary proliferative responses to a T-dependent antigen, KLH, in FIV-infected cats compared with the uninfected control cats, of approximately 2 $\log_{10}$, was detected, early after infection at week 5 ($P = 0{\cdot}005$–$0{\cdot}01$) (Fig. 2). This reduction was less marked at week 8 p.i. ($P = 0{\cdot}05$–$0{\cdot}1$) and by week 13 p.i. primary responses to KLH in the infected cats did not differ from the uninfected controls. However, by weeks 19 and 22, the primary proliferative response to KLH in FIV-infected cats had again fallen by 2 $\log_{10}$ ($P = 0{\cdot}01$ and $P = 0{\cdot}025$–$0{\cdot}05$ at weeks 19 and 22 respectively). Later, at week 32 p.i., there was no significant difference between infected and uninfected cats in their ability to become primed to KLH antigen.

The median day of peak proliferation in the primary cultures was calculated for the uninfected and infected groups of cats. At most weeks of testing, peak proliferation to KLH occurred on the same day of culture, for both infected and uninfected cats. Median values of peak proliferation ranged from day 8–11 for FIV-infected cats and from day 8–10·5 for uninfected cats. This indicated that the kinetics of the primary responses was not altered in FIV-infected cats.

In a separate experiment, eight FIV-infected cats, four with impaired primary proliferation, and four with normal primary proliferative responses, were studied for the possibility of virus reactivation in the primary cultures. Primary culture supernatants taken from wells containing either no antigen (control) or antigen, KLH, at day 11 of culture, were all negative for the presence of FIV p24 antigen, as measured by enzyme immunoassay. This inability to demonstrate FIV antigen indicates that culture for 11 days with antigen, KLH, did not cause the reactivation of virus from infected cat peripheral blood cells.

DISCUSSION

The ability of feline lymphocytes to recognize both recall antigen, and antigen which had not been encountered previously *in vivo*, was measured in FIV-infected cats during the acute phase of infection, when they appear to be particularly susceptible to secondary infections [12,13]. CD4$^+$/CD8$^+$ lymphocyte ratios and mitogen blastogenesis were used to assess the general immune status of cats.

CD4$^+$/CD8$^+$ lymphocyte ratios were significantly reduced in the FIV-infected cats at week 13 p.i. and after. This reduction was due to an increase in the number and percentages of CD8$^+$ cells, perhaps in response to acute FIV infection. There was no alteration in CD4$^+$ cell numbers. This contrasts with our studies of cats infected with FIV for longer periods (up to 27 months) where an inversion of the CD4$^+$/CD8$^+$ ratio was due to a selective loss of the CD4$^+$ cells [11]. Other studies have also shown a reduction in the CD4$^+$/CD8$^+$ ratio early after FIV infection [16]. Our findings indicate that infection with FIV has an early direct effect upon T cell subsets in the cat.

Whilst it has been shown that cats in AC, ARC and AIDS phases have defective blastogenesis to T cell mitogens [4,8–11], blastogenesis to Con A and PWM was not impaired in cats in acute phase FIV infection, in this study. This suggests that T cell function, as assessed by these parameters, was normal at this stage of infection. Interestingly, B cell function, as assessed by lymphocyte blastogenesis to LPS, was impaired as early as week 8 p.i. Previously, we have shown that blastogenesis to LPS is more severely reduced than blastogenesis to T cell mitogens, in cats infected with FIV for longer periods [11]. Thus, as in HIV-1 infection, it appears that B cell functional defect may precede defects in T cell function [18,19]. It is noteworthy that feline B cells have been identified as a major reservoir of FIV provirus [20]. We have also noted that FIV-infected cats have elevated serum immunoglobulin G levels [21], indicating a B cell activation. The impairment of the B cell system in these cats may be a consequence of persistently activated B cells becoming refractory to further stimulation from mitogens, as suggested for B cell function abnormalities in HIV [22–24].

However, the significance of impaired blastogenesis to mitogens is unclear. Activation of lymphocytes by mitogens occurs via cell surface carbohydrate residues and does not have the same requirements for accessory cell function and accessory signals as antigen-induced proliferation. Thus, in addition to mitogen blastogenesis responses, all cats were immunized before infection with HSA, and antigen-specific secondary responses to HSA were measured at varying times after infection. Proliferation to HSA in FIV-infected cats was impaired at week 19 p.i. onwards. Thus, whilst mitogen-driven T cell proliferation is normal in acute FIV infection, there is a defect in conventional, T cell receptor-mediated proliferation to soluble antigen by the memory T cell pool. This may reflect defective presentation of antigen to, and/or defective inter- or intracellular signalling by T cells. Activation induced by ligation of the T cell antigen receptor is mediated via a specific protein-tyrosine kinase which is not associated with signalling via cell surface receptors involved in activation by mitogens [25]. A diminished T cell proliferative response to soluble recall antigens has been recognized in patients with AIDS [26,27], and interestingly, also in asymptomatic HIV-1-infected individuals during the first 3 months after seroconversion [28].

In addition to secondary responses to a recall antigen, we have investigated *in vitro* priming of CD4$^+$ T cells to a soluble protein antigen which had not been encountered previously *in vivo*. It is noteworthy that primary proliferation was impaired at

weeks 5–8 p.i., before haematological abnormalities and significant alteration in the $CD4^+/CD8^+$ ratio. This observation suggests that infection with FIV severely affects the ability of the cat to mount a primary immune response, before quantitative changes in the $CD4^+$ lymphocyte population.

The kinetic profile of the primary response in FIV-infected cats over weeks 0–32 p.i. showed a rise and fall over time. This response may mimic viraemia during the acute viral infection and it is noteworthy that isolation of virus from the blood of FIV-infected cats is easier during the initial phase of infection than during the asymptomatic phase [21]. In order to examine the possibility that FIV might be reactivated during culture with antigen for 11 days, supernatants from primary culture were removed and tested for the presence of FIV p24 antigen. No viral p24 antigen was detected in primary culture supernatants removed at day 11 from cultures of PBMC from FIV-infected cats with and without impaired primary proliferative responses. Thus, impairment of primary proliferation did not appear to be as a result of viral infection and cell death in culture. The presence of KLH in culture supernatant does not affect the detection of p24 antigen since p24 cannot be detected even after washing and re-culturing for 12–36 h in the absence of KLH (data not included). Moreover, addition of increasing concentrations (40–640 μg/ml) of KLH to known weakly positive samples does not affect the sensitivity of the assay (data not shown).

Therefore, during acute phase FIV infection, there is a qualitative defect in the recognition of both primary and secondary antigens, which occurs before any quantitative defect in the T cells. It is noteworthy that the impairment in proliferative responses is progressive, with primary responses most sensitive and being impaired first, then secondary responses being affected later. Proliferation to T cell mitogens is impaired in cats infected for longer periods [11]. The differences in susceptibility to impairment between primary and secondary responses may reflect the different requirements for the activation of naive and memory T cell populations. T cell priming results in an increase in the level of expression of a number of antigen-independent adhesion molecules [29]. Probably as a result of these changes, primed T cells are stimulated more readily than naive T cells. Thus, any functional defect in the T cells as a consequence of FIV infection would be expected to be evident first in the naive cell populations.

A further consequence of the changes in T cell phenotype which occur upon priming is that primed, memory T cells can be stimulated by a wider variety of MHC class II bearing cell types, whilst it has been suggested that dendritic cells are the only class II bearing cells able to initiate a primary response in naive T cells [30,31]. Therefore, our observations could also result from a progressive defect in accessory cell function in FIV-infected cats. It is noteworthy that FIV is known to infect and replicate in macrophages as well as $CD4^+$ and $CD8^+$ T cells [32,33]. Furthermore, evidence is accumulating that accessory cell function is impaired early in HIV-1 infection of humans [34,35]. Thus, the nature of this defect in FIV infection needs to be investigated.

The inability to effectively prime $CD4^+$ T helper cells during the acute phase of FIV infection may explain the susceptibility to opportunistic secondary pathogens [12,13], and the impaired humoral response following vaccination [13] in acute phase infection. Moreover, there have been reports of human patients with concomitant infections within the first weeks after infection with HIV, before full seroconversion, indicating a similar susceptibility occurs very early in HIV infection [36]. These findings have obvious implications for vaccine strategies.

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