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Infection with feline immunodeficiency virus is followed by the rapid expansion of a $CD8^+$ lymphocyte subset

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SUMMARY

Lymphocyte subset analysis was performed on specific pathogen-free cats infected with feline immunodeficiency virus (FIV). As early as 4 weeks post-infection a sharp rise in lymphocytes expressing the feline CD8 marker (fCD8) occurred. No changes were observed in feline $CD4^+$ ($fCD4^+$) cell number throughout this period. The expanded subset displayed reduced expression of the fCD8 marker ($fCD8^{low}$) compared to the control population ($fCD8^{high}$). Dual-labelling revealed increased levels of major histocompatibility complex (MHC) class II antigens on the $fCD8^{low}$ subset compared to the $fCD8^{high}$ subset. The $fCD8^{low}$ population appeared to persist as it was detected in cats which had been infected for a period of 18 months and which displayed significantly reduced numbers of $fCD4^+$ lymphocytes. The data suggest that infection with FIV induces rapid alterations in the lymphocyte profile of the cat characterized by the expansion of a $fCD8^+$ lymphocyte subset. The persistence of this population throughout the course of infection suggests that the early events in FIV infection may be of importance in the pathogenesis of the disease.

INTRODUCTION

Infection with feline immunodeficiency virus (FIV) leads to an immune dysfunction characterized by a gradual depletion of circulating $fCD4^+$ lymphocytes.[1-3] The striking similarity between FIV- and human immunodeficiency virus (HIV)-induced disease has led to the suggestion that the two viruses may have similar pathogenic mechanisms. The opportunity to examine the interaction of FIV with the immune system at the earliest stage of infection is of particular importance as this is rarely possible in HIV-infected individuals.

The expansion of a population of T suppressor/cytotoxic cells has been described during HIV infection, expanding populations being defined on separate occasions as $CD8^+$/$HLA\text{-}DR^+$, $CD8^+/CD45RA/CD38^+$ and $CD8^+/CD11^+$.[4-6] The population is thought to represent an activated T-cell subset which is triggered by infection and which persists as the immune system fails to eradicate the virus from the circulation. The altered lymphocyte profile occurs at a time when little or no change in circulating $CD4^+$ T-cell numbers has occurred, the lymphocyte subset being significantly increased in asymptomatic individuals. Although the significance of this population in the pathogenesis of HIV infection is unknown, suppression of HIV-specific and non-specific cytotoxicity has been demonstrated and has been attributed to the $CD8^+/CD11^+$ subset.[6]

In previous studies of FIV infection the expansion of a population of $fCD8^+$ lymphocytes was noted in long-term-infected cats, which is not observed in uninfected cats or cats infected with feline leukaemia virus (FeLV). The subset can be distinguished from normal $fCD8^+$ lymphocytes by a characteristically low expression of the fCD8 marker ($fCD8^{low}$). Preliminary evidence suggests that this population expresses increased levels of major histocompatibility complex (MHC) class II antigens. In this study the development of the atypical lymphocyte subset during the early phase of FIV infection is examined.

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MATERIALS AND METHODS

Animals

Specific pathogen-free cats were used in all experiments. Infection with FIV was by intraperitoneal inoculation of 10 infectious units of FIV-Glasgow-8 isolate which had been titrated both *in vitro* and *in vivo*. FeLV infection was by oronasal inoculation with 1×10^6 focus forming units of FeLV-Glasgow-A. Long-term-infected cats had been infected for 18 months at the time of analysis.

Antibodies

Anti-feline CD4 (vpg34), anti-feline CD8 (vpg9), and anti-MHC class II B-chain (vpg3) were produced as described previously.[7] Anti-feline CD4 (Fel7)[8] and CD8 (FT2)[9] were generously provided by C. Ackley (Birmingham, AL). The fCD8-vpg9 epitope is co-expressed with both the FT2 and

OKT8 epitopes. Typically vpg9 stains 15·0 ± 5·0% of peripheral blood lymphocytes (PBL) compared to 15·7 ± 5·7% with the FT2 antibody ($n = 9$). Double staining of PBL with the vpg9 and FT2 antibodies yields a single homogeneous population. Expression of the vpg9 epitope is mutually exclusive with expression of the fCD4 marker on feline T cells. vpg9 and FT2 have an identical antigen distribution and intensity of expression on lymphocytes, normal thymus, lymph node and reactive cell lines. Estimates of $fCD8^+$ cell number with vpg9 were also corroborated throughout each experiment with OKT8 (cross-reactive anti-human CD8; ATCC, Rockville, MD). vpg9 was conjugated to fluorescein isothiocyanate (FITC) for use in two-colour analyses.

Flow cytometry

Peripheral blood mononuclear cells (PBMC) were isolated from samples of EDTA anti-coagulated blood by whole-blood lysis and processed for flow cytometry. Primary antibodies were detected with FITC-conjugated $F(ab')_2$ fragment of rabbit anti-mouse IgG (Sigma Chemical Company Ltd, Poole, U.K.). In two-colour analyses primary antibody was detected using phycoerythrin (PE)-conjugated $F(ab')_2$ fragment of rabbit anti-mouse IgG (Sigma) and after washing, the FITC-conjugated secondary antibody added. Analyses were performed on a FACStar flow cytometer (Becton Dickinson, Cowley, U.K.), 10,000 events being collected by live-gating from a plot of forward scatter versus 90° side scatter. The cytometer was calibrated for each analysis using unconjugated, FITC-conjugated and PE-conjugated microbead standards (Flow Cytometry Standards Corporation/Becton Dickinson) as per manufacturer's instructions. Electronic compensation for cross-leakage of signal between fluorescence detectors was employed and control samples of feline lymphocytes which had been labelled with isotype-matched antibody against an irrelevant antigen used to set analysis gates and to confirm the accuracy of the fluorescence compensation.

Diagnosis of seroconversion

Sera were tested by immunoblot analysis as described previously.[10] Virus isolation was performed by concanavalin A (Con A) stimulation of PBL isolated from each cat. Heparinized blood was diluted 1:3 in phosphate-buffered saline (PBS) and spun through Lymphoprep (Nycomed Pharma AS, Oslo, Norway) gradient centrifugation medium. PBMC were collected from the interface and washed in two changes of PBS. Cells were seeded at 1×10^6/ml in RPMI-1640 medium supplemented with 2 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin (all from Gibco BRL, Paisley, U.K.), 5×10^{-5} M 2-mercaptoethanol (Sigma), 100 IU/ml recombinant human interleukin-2 (IL-2) (kindly provided by J. Nunberg, Cetus Corporation, Emeryville, CA) and 10% foetal bovine serum (FBS) (Tissue Culture Services, Botolph Claydon, U.K.). Con A (Sigma) was added to a final concentration of 7·5 μg/ml and the culture was incubated at 37°. Presence of virus was detected by the appearance of syncytia and ballooning degeneration in the culture, and confirmed by p24 ELISA (Petcheck FIV antigen test, Idexx Corporation, Portland, ME). Where cell numbers were low, Con A-stimulated cultures were inoculated into a culture of the feline T-helper cell line Q201, which is highly susceptible to infection with FIV.[7]

RESULTS

Lymphocyte subset analysis in long-term-infected animals

$fCD4^+$ and $fCD8^+$ lymphocyte subsets were quantified in the peripheral blood of two groups of age-matched cats which were either uninfected or had been infected 18 months previously with FIV (Table 1). FIV-positive cats had significantly reduced $fCD4^+$ cell numbers (691 ± 130/mm^3 compared to the control value of 1450 ± 460/mm^3: $P < 0.05$, Student's t-test). The high inter-cat variation in lymphocyte number and severity of immunodeficiency is reflected in the large sample variance. FIV-positive cats had $fCD4^+$ lymphocyte numbers ranging from 46/mm^3 to 938/mm^3, i.e. from overt lymphopaenia to apparently normal. Although circulating $fCD8^+$ lymphocyte numbers decreased from 1410 ± 530/mm^3 in the control group to 1100 ± 210/mm^3 in the FIV-positive group this reduction was not statistically significant.

Expression of the fCD8 marker could be divided into $fCD8^{low}$ and $fCD8^{high}$ (Fig. 1). Analysis gates were placed around the $fCD8^{low}$ and $fCD8^{high}$ subsets and the respective subsets were quantified. While the $fCD8^{high}$ subset fell from 1250 ± 470/mm^3 in the control group to 740 ± 180/mm^3 in the FIV-positive group, the $fCD8^{low}$ subset showed a significant expression from 158 ± 60/mm^3 in the control group to 352 ± 60/mm^3 in the FIV-positive group ($0.05 < P < 0.1$). A preliminary examination of fCD8 expression in three cats which had been infected with FeLV for approximately 24 months and which were persistently viraemic revealed similar numbers of $fCD8^{low}$ cells to the control group ($fCD8^{low}$ subset 141 ± 35/mm^3 and $fCD8^{high}$ subset 1710 ± 107/mm^3), even although these cats consistently presented as mildly lymphophilic (data not shown). To investigate the origin of the $fCD8^{low}$ subset expression of the marker was examined in a single group of cats before and after FIV infection.

Early changes in circulating lymphocyte subsets following FIV infection

The effect of FIV infection on circulating lymphocyte numbers in a group of 12 age-matched cats was analysed. Expression of lymphocyte surface markers was examined over a 2-month period prior to infection and for approximately 4 months after infection. Although lymphocyte numbers were found to fluctuate prior to infection this reflected variations in total lymphocyte number, the fCD4:fCD8 quotient being preserved (Fig. 2).

Table 1. T-lymphocyte subsets in specific pathogen-free cats 18 months post-infection with FIV. Peripheral blood T-lymphocyte subsets from uninfected ($n = 4$) and infected ($n = 10$) cats were quantified by flow cytometry and absolute cell numbers calculated from the lymphocyte count. Cell numbers are expressed as cells/mm^3 ± 1SE. Statistical comparison of uninfected versus infected was performed using Student's t-test. * Significant $P < 0.05$; ** significant $P < 0.10$; ^{NS}not significant.

Cell subpopulation	Uninfected	Infected
fCD4	1452 ± 459	691 ± 130*
fCD8	1406 ± 532	1100 ± 214^{NS}
$fCD8^{low}$	158 ± 61	352 ± 59**
$fCD8^{high}$	1247 ± 472	740 ± 181^{NS}

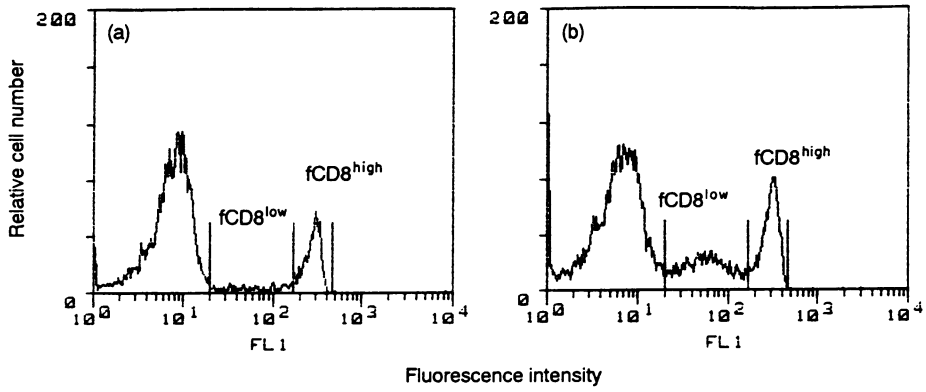


Figure 1. Appearance of a population of cells staining weakly for fCD8 in cats following infection with FIV. Flow cytometric profiles of cell number versus fluorescence intensity from a typical uninfected (a) and infected (b) cat. On lymphocytes from the uninfected cat fCD8 expression is restricted to a single homogenous population ($fCD8^{high}$) whereas on lymphocytes from the FIV-infected cat fCD8 is heterogeneously expressed with many cells expressing low levels of the marker ($fCD8^{low}$).

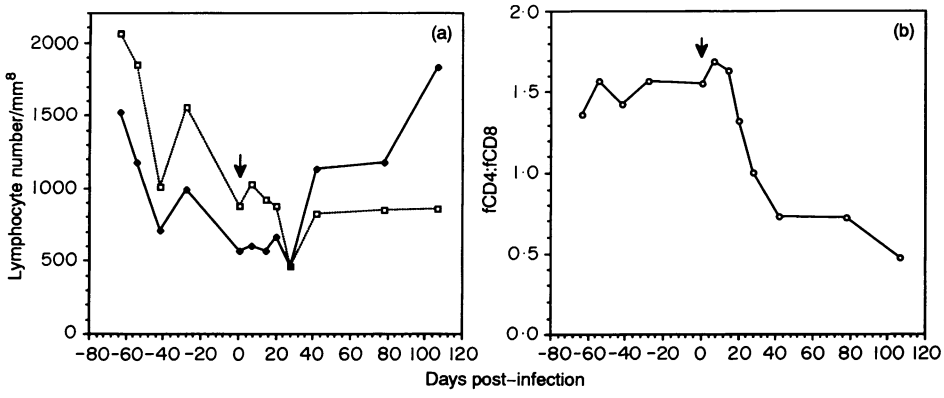


Figure 2. T-lymphocyte subsets in a population of age-matched specific pathogen-free cats ($n = 11$) before and after infection with FIV. Leucocytes were separated from EDTA anti-coagulated blood by whole blood lysis and lymphocyte subsets quantified by flow cytometry. Cell numbers were then calculated from the lymphocyte count and the group mean calculated. Surface marker expression was analysed for 2 months prior to infection to act as an in-built experimental control, on the day of challenge (arrowed), and followed for 3 months post-infection. Although the absolute lymphocyte number was variable (a) the fCD4:fCD8 quotient remained constant at approximately 1·5 (b). Three weeks post-challenge the fCD4:fCD8 quotient became inverted due to an expansion of the $fCD8^+$ subset: fCD4 (□); fCD8 (◆).

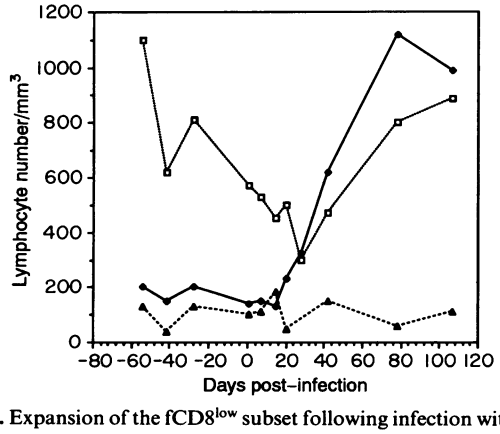
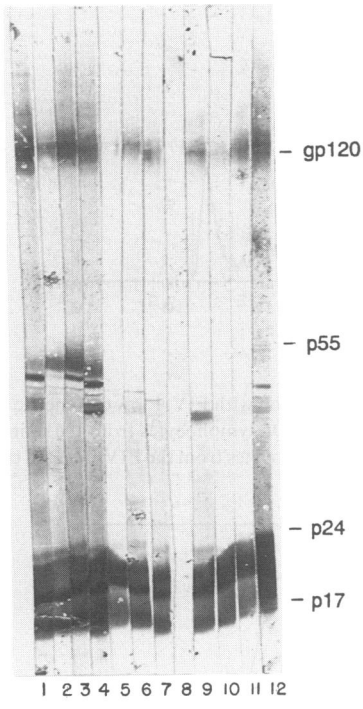


Figure 3. Expansion of the $fCD8^{low}$ subset following infection with FIV. fCD8 expression classified into 'low' and 'high' as illustrated (see Fig. 1). Prior to infection the $fCD8^{low}$ subset showed little variation from a mean of $174 \pm 30/mm^3$. Three weeks post-infection the $fCD8^{low}$ subset began to expand rapidly reaching a maximum of $1120 \pm 30/mm^3$ by day 78 post-infection. A single cat did not become infected (uninfected) and confirms the low variation in the size of the $fCD8^{low}$ subset in the normal cat. $fCD8^{high}$ (□); $fCD8^{low}$ (◆); $fCD8^{low}$ (uninfected) (▲).

Following inoculation with FIV, 11/12 cats seroconverted and became positive for the presence of cell-associated virus. A single cat (Fig. 3—uninfected) did not become productively infected (virus isolation negative and no seroconversion) reflecting the use of a challenge dose close to the minimum infectious dose (although referred to as 'uninfected' this cat may well have been latently infected; this however was not established). Infection triggered a sharp rise in the number of $fCD8^+$ cells leading to an inversion of the fCD4:fCD8 quotient. The increase in $fCD8^+$ lymphocyte numbers following infection was a result of the expansion of the $fCD8^{low}$ subset (Fig. 3). Three weeks post-infection the $fCD8^{low}$ subset expanded rapidly achieving a maximum by day 78 post-infection. Compared to the preinfection baseline this expansion represents an increase of 500% and is highlighted by comparison with the single cat (cat 8—uninfected) which failed to seroconvert and in which $fCD8^{low}$ levels remained constant. During the preinfection period $fCD8^{low}$ cells showed little fluctuation with a mean of $174 \pm 14/mm^3$ (mean of four consecutive analyses). By day 78 post-infection this had risen significantly to $1120 \pm 30/mm^3$ ($P < 0{\cdot}001$). Although the $fCD8^{high}$ subset varied in size prior to infection this was in accordance with previous reports of age,



Subject	1	2	3	4	5	6	7	8	9	10	11	12
Day virus positive	15	15	15	15	15	20	28	—	28	15	15	15

Figure 4. Diagnosis of seroconversion in cats inoculated with FIV. Sera from challenge cats were tested by immunoblot for reactivity against viral components. By day 78 post-infection all cats except cat 8 demonstrated reactivity against either p17, p24, gp120 or precursor p55. Subject 8 did not become productively infected and was negative by both immunoblot and virus isolation. Virus was also isolated from Con A-stimulated peripheral blood lymphocytes. The day on which virus was first isolated is shown.

diurnal and immune-status related variations in lymphocyte number. Similarly, while the mean $fCD8^{high}$ number rose post-infection to $800 \pm 120/mm^3$ this level was not significantly different from the preinfection mean of $774 \pm 82/mm^3$ and cannot be said to be different from regular fluctuations in lymphocyte number. The size of the $fCD8^{low}$ subset, however, appears to remain constant irrespective of alterations in total $fCD4^+$ and $fCD8^+$ cell number.

Relationship between immunophenotype and detection of virus by immunoblot and *in vitro* isolation

The onset of the rise in $fCD8^+$ cells and the development of infection were compared. The presence of cell-associated virus preceded the expansion of the $fCD8^+$ population. All cats became positive for the presence of cell-associated virus before the expansion of the $fCD8^{low}$ population by immunoblot analysis. All virus-positive cats showed a reaction against core proteins p17 and p24, envelope glycoprotein gp120 or the *gag* precursor p55. Cat 8 did not seroconvert and remained virus negative throughout the course of the study.

Immunophenotype of the $fCD8^{low}$ population

Lymphocytes were double-labelled with fCD8 and anti-MHC class II (vpg3). The $fCD8^{low}$ population expressed increased levels of MHC class II compared to expression on the $fCD8^{high}$ subset (Fig. 5). MHC class II expression on feline lymphocytes is constitutive with B cells having higher levels of expression than T cells.[11] This leads to a characteristic bimodal distribution when analysed by flow cytometry, the localization of $fCD8^+$ lymphocytes to the left-hand side (lower expression) of the main MHC class II-positive population confirming this finding. Analysis of the mean MHC class II expression on $fCD8^{low}$ and $fCD8^{high}$ suggests that the former express higher levels (Table 2). In the long-term infection group (control and infected) MHC class II expression fell in fluorescence channel 137 ± 2 for $fCD8^{low}$ cells and 119 ± 3 for $fCD8^{high}$ cells (significant

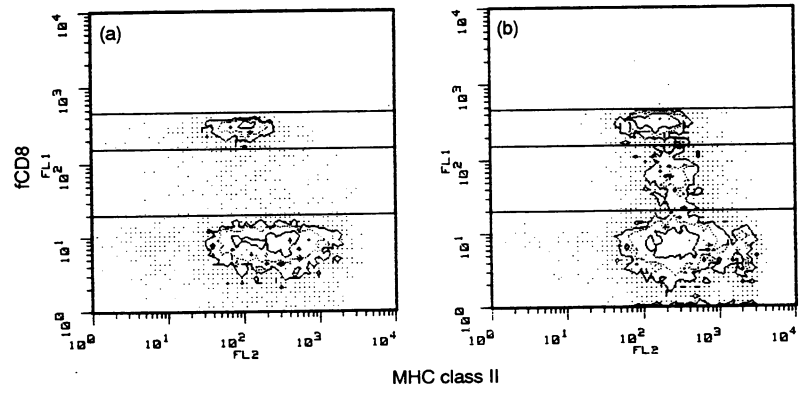


Figure 5. MHC class II expression on $fCD8^+$ lymphocytes from a cat infected with FIV (b) and a cat which did not become productively infected (a) following challenge, both cats from the short-term time–course experiment as illustrated in Fig. 3. Analysis was performed at day 78 post-challenge. Lymphocytes were labelled with anti-feline MHC class II followed by PE-conjugated $F(ab')_2$ fragment of sheep anti-mouse IgG. The cells were then incubated with FITC-conjugated anti-fCD8. In the uninfected cat (a) the majority of fCD8 expression is restricted to a single homogeneous $fCD8^{high}$ population. In contrast, the infected cat is remarkable by the appearance of a population of $fCD8^{low}$ cells. This population expresses higher levels of MHC class II than the $fCD8^{high}$ population.

Table 2. MHC class II expression on the fCD8^{low} and fCD8^{high} T-cell subsets of peripheral blood lymphocytes from the cats in the long-term (both uninfected and infected) and short-term infection studies. Analysis gates were placed around each subset and the mean MHC class II expression calculated using the Consort C30 software package, results being converted from a logarithmic fluorescence intensity value to a linear fluorescence channel number. Data were then analysed for significant difference (Wilcoxon signed-ranks, * significant $P<0{\cdot}001$). The data illustrate that MHC class II expression on the fCD8^{low} subset was significantly higher than on the fCD8^{high} subset. Results are expressed as mean fluorescence channel ± SE

Study group	MHC class II expression on fCD8^{low} subset	MHC class II expression on fCD8^{high} subset
Long-term infection	137 ± 2	119 ± 3*
Short-term infection	161 ± 3	140 ± 12*

$P<0{\cdot}001$). Similarly, in the short-term infection group fCD8^{low} expression peaked at channel 161 ± 3 compared to channel 140 ± 12 for the fCD8^{high} group (significant $P<0{\cdot}001$). In this study the flow cytometric analyses only permitted the comparison of levels of MHC class II expression on lymphocyte subsets within each sample. It is hoped that in future studies it will be possible to analyse whether infection itself leads to increased MHC class II expression. At present it can only be concluded that increased T-cell MHC class II expression does occur and that it is due to the expansion of the fCD8^{low} population, this population constitutively expressing higher levels of MHC class II but being present at low levels in the normal cat.

DISCUSSION

The immunophenotype of feline lymphocytes was investigated immediately after infection with FIV. These studies indicate that the development of cell-associated viraemia is followed by appearance of a population of cells staining weakly for feline CD8. It is not known whether the fCD8^{low} population represents epitope masking, reduced epitope density or altered epitope conformation. Similar low density fCD8 expression on human lymphocytes has been described previously.[12] Expansion of the fCD8^{low} population was not detected in uninfected cats, those which had been immunologically stimulated by vaccination (not shown), or cats in the viraemic phase of FeLV infection.

The fCD8^{low} population expanded rapidly after infection, as early as 4 weeks post-infection. The expansion of this population was preceded by the development of cell-associated viraemia in all cases. The data suggest that the presence of virus in peripheral blood triggers the expansion of this lymphocyte subset. The fCD8^{low} subset was detected 18 months post-infection suggesting that the immunological stimulus for the expansion of this lymphocyte subset remains throughout infection.

To characterize the fCD8^{low} population further, peripheral blood lymphocytes were double stained with anti-MHC class II antibody. The fCD8^{low} population displayed up-regulation of MHC class II expression in comparison with normal fCD8^{+} cells (fCD8^{high}). The fCD8^{low} population also reacted with the feline pan T-cell marker 43-pan T[13] (data not shown) suggesting that the cells are indeed T cells. However, as the 43-pan T marker has yet to be fully characterized the possibility remains that the cells may belong to the equivalent of a CD3^{-} CD8^{+} natural killer (NK)-associated population as observed in man.

Although all feline T cells constitutively express MHC class II molecules,[11] the fCD8^{low} population appeared to express increased levels suggesting that they may belong to an activated T-cell subset expanding in response to the presence of the virus. The mechanism behind the rapid expansion of this subset is unknown but as it occurs specifically in the fCD8^{+} subset, may have profound implications for the pathogenesis of FIV infection. Suppressor T cells have been observed to undergo expansion in HIV-infected individuals, with a CD8^{+}/CD11^{+} subset being evident in asymptomatic patients. Interestingly the increase in fCD8^{low} cells following FIV infection is similar to the rise in CD8^{+}/CD11^{+} and HLA-DR^{+}/CD8^{+} cells following HIV infection. In this experiment fCD8^{low} cells represented 2·98 ± 0·8% of peripheral blood mononuclear cells prior to infection and rose to 17·31 ± 9·5% at 72 days post-infection. A recent study reported levels of 3–5% CD8^{+}/CD11^{+} cells in normal individuals to 10–45% CD8^{+}/CD11^{+} cells in HIV-positive asymptomatic individuals.[6] Similarly, HLA-DR^{+}/CD8^{+} cells constitute 3–8% of CD8^{+} cells in seronegative individuals compared to 20–30% of CD8^{+} cells in HIV-seropositive individuals.

It is unlikely that the fCD8^{low} population represents a unique lymphocyte subpopulation induced by FIV infection but rather that lentivirus infection induces a persistent stimulation of this population. The CD8^{+} CD38^{+} population expands in humans following infection with Epstein–Barr virus and cytomegalovirus but diminishes within 1–2 months, apparently coinciding with regression of the infection.

This study demonstrates that major alterations in immune function may occur very early in infection, a period which can rarely be investigated in HIV infection of man. Although enhanced cytotoxic or suppressor T-cell activity has yet to be demonstrated in these cats, reports have suggested the presence of suppressor cell activity following FIV infection.[14] It has, however, been demonstrated that the fCD8^{low} population expresses increased levels of MHC class II antigens, a phenomenon associated with T-cell activation in man.

The expansion of a subpopulation of feline CD8^{+} lymphocytes during FIV infection suggests that FIV and HIV may share a similar pathogenesis. The accessibility of study of the early events following FIV infection in the cat may hold the key to the broader understanding of lentivirus-induced immunodeficiency.

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