# The effect of FIV infection on $CD4^+$ and $CD8^+$ counts in periradicular lesions

**D. F. Levine[1], D. E. Witherspoon[1], J. L. Gutmann[1], M. E. Nunn[2], J. T. Newman[3] & A. M. Iacopino[4]**

Departments of [1]Restorative Sciences, [2]Public Health Sciences and [3]Biomedical Sciences, Baylor College of Dentistry, Texas A & M University System Health Science Center, Dallas, TX; and [4]Division of Prosthodontics, Marquette University, School of Dentistry, Milwaukee, WI, USA

## Abstract

**Levine DF, Witherspoon DE, Gutmann JL, Nunn ME, Newman JT, Iacopino AM.** The effect of FIV infection on $CD4^+$ and $CD8^+$ counts in periradicular lesions. *International Endodontic Journal,* **34**, 586–593, 2001.

**Aim** The purpose of this study was to elucidate whether a decrease/increase in T-cell populations is present in the development of periradicular disease in the immunocompromised feline model.

**Methodology** Eight cats were immunosuppressed with steroids prior to infection with feline immunodeficiency virus (FIV). Another eight cats, age and sex matched littermates, were monitored and tested at equivalent periods of time and served as uninoculated, seronegative controls. Periradicular lesions were induced using local bacterial inoculations into the pulp of the canine teeth and assessed after one- and four-week periods, corresponding to the acute and chronic stages of the periradicular disease. Block sections were obtained and specimens were prepared for H & E and immunohistochemical staining for $CD4^+$ and $CD8^+$ receptors. Cells were quantified using a computer imaging system and data analysed using generalized estimating equation (GEE) regression models.

**Results** Significantly lower $CD4^+$ counts and $CD4^+/CD8^+$ ratios were observed at all time periods in the periradicular region of the FIV group ($P = 0.0006$). No significant difference in $CD8^+$ counts was observed between the two groups. In both groups there was a significant difference in the $CD4^+$ counts between one week and baseline, and 1 week and 4 weeks. There was no significant difference between baseline and 4 weeks for either group.

**Conclusion** FIV infection reflected decreased $CD4^+$ counts at the periradicular level, however, inflammation and progression of the lesion, appeared to be comparable to the non-immunocompromised controls.

**Keywords:** CD4, CD8, FIV, HIV, periradicular lesion.

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## Introduction

A developing periradicular lesion represents a dynamic interplay between the immune system and infecting bacteria. The primary cell composition of periradicular lesions is T lymphocytes, B lymphocytes, plasma cells, macrophages, polymorphonuclear leucocytes, and mast cells (Stashenko *et al.* 1992), in which macrophages and lymphocytes constitute 80% of the inflammatory cells (Lin *et al.* 1973, Polliack *et al.* 1973, Sullivan *et al.* 1974, Farber 1975). Egress of bacteria and bacterial by-products (antigens) from the root canal system attract phagocytic macrophages into the periradicular tissues. These macrophages remove antigens from the periradicular region and present them to specific T or B lymphocytes. The interaction between the various immunocompetent cells ultimately determines the structural changes in the periradicular bone (Milia *et al.* 1996).

Mononuclear phagocytes (tissue macrophages and blood monocytes) and $CD4^+$ T lymphocytes are primary targets of the human immunodeficiency virus (HIV). However, mononuclear phagocytes are more resistant to virus-induced apoptosis, and therefore, provide a reservoir

Correspondence: Débora F. Levine c/o David E. Witherspoon, Baylor College of Dentistry, Graduate Endodontics (Rm. 335), 3302 Gaston Ave., Dallas, TX 75246 USA (fax: +214 828 8209; e-mail: dewspoon@tambcd.edu).

of ongoing viral replication (Bornemann *et al*. 1997). T cells exert a regulatory influence over macrophage-induced activities (Barbul & Regan 1995). Thereby, infection of the T cells can alter a variety of the macrophages' immunological functions, such as cytokine production (Bornemann *et al*. 1997), chemotaxis, accessory cell function, and microbicidal activity (Ho *et al*. 1994).

The net result of HIV infection is decreased levels of $CD4^+$ cells in peripheral blood. It is unclear whether or not the decrease in peripheral blood $CD4^+$ T lymphocytes is accompanied by a consistent corresponding increase of $CD8^+$ T lymphocytes, as seen in some HIV cases (Ginaldi *et al*. 1997, Evans *et al*. 1998).

The $CD4^+$ T lymphocytes predominate in the acute stages of periradicular pathosis development. As the lesions progress to a more chronic state, increasing numbers of $CD8^+$ T lymphocytes are present (Stashenko *et al*. 1992, Stashenko *et al*. 1994, Kawashima *et al*. 1996). The effect of the decrease in blood levels of $CD4^+$ cells in the HIV population on the periradicular disease process is unknown.

In feline immunodeficiency virus infection (FIV), which closely simulates HIV infection, there are also conflicting reports with regard to the $CD4^+/CD8^+$ inversion in blood (Bendinelli *et al*. 1995). In some instances, decreased levels of $CD4^+$ cells in peripheral blood are accompanied by a compensating increase in $CD8^+$ T lymphocytes levels in FIV (Ackley *et al*. 1990, Bishop *et al*. 1992, Walker *et al*. 1994, Bendinelli *et al*. 1995) during the asymptomatic stage as observed in HIV. At the periradicular level, however, there is no information regarding the levels and mechanisms of cell population modulation in the immunosuppressed population.

The present study is designed to elucidate whether a decrease/increase in T cell populations is present in the development of periradicular disease in the immunocompromised feline model.

## Materials and methods

The development of an immunocompromised host model, described elsewhere (Levine 2000), enabled the comparative assessment of periradicular disease processes in cats with feline immunodeficiency virus (FIV) versus immunocompetent, seronegative controls. The project was approved by the Institutional Review Board Baylor College of Dentistry-TAMUS and all animals were cared for and housed by the Baylor College of Dentistry Animal Research Unit. Briefly, eight adult male cats were immunosuppressed by using steroids (Methylprednisolone acetate; The Upjohn Company, Kalamazoo, MI, USA), and infected with the FIV (Infectious clone FIV-PPR; provided by personnel at the College of Veterinary Medicine, Texas A & M University, College Station, TX, USA). Complete blood counts were performed biweekly for 2 months, and then monthly for the following 8 months. Four enzyme-linked immunosorbent assay (ELISA) tests were performed for the detection of FIV antigen in the laboratory samples, and results were correlated with the blood counts. Eight, age- and sex-matched littermates not given the virus were monitored and tested at equivalent periods of time and served as controls to the FIV/HIV animal model.

For experimental manipulation, animals were sedated by intramuscular injections of 0.5 mL of Ketaset/PromAce (Ketamine HCl/Acepromazine maleate; Fort Dodge Laboratories, Inc., Fort Dodge, IA, USA) at a 10 : 1 ratio (100 $mgmL^{-1}$ 10 $mgmL^{-1}$ vials). Intraoral anaesthesia was obtained by maxillary and mandibular infiltration of 1 mL of 2% lidocaine containing 1 : 100 000 epinephrine (Astra USA, Inc., Westborough, MA, USA).

Maxillary and mandibular canine teeth were allocated randomly to either a one-week infection (two teeth) or a four-week infection (two teeth) group. To achieve infection, two-thirds of the crowns of the canine teeth were removed with a high-speed fissure bur and the canals were exposed. Plaque was inoculated into the canals with a new size 25 K-file, a uniform size cotton pellet was placed in the access, and cavities were sealed with Ketac-Silver (Aplicap, ESPE, Seefeld, Germany) to ensure an anaerobic bacterial population. One carnassial's (cat maxillary premolar) tooth, which was not inoculated, was used as a control. Hence, each cat had one-week inoculated canines (two teeth), four-week inoculated canines (two teeth), and one control tooth when sacrificed.

### Tissue removal and processing

Animals were sedated by intramuscular injections of 0.5 mL of Ketaset/PromAce (Ketamine HCl/Acepromazine maleate; Fort Dodge Laboratories, Inc., Fort Dodge, IA, USA) at a 10 : 1 ratio (100 $mgmL^{-1}$ 10 $mgmL^{-1}$ vials). Right and left common carotid arteries and external jugular veins were surgically exposed and the common carotid arteries were cannulated with the tubing of a positive pressure perfusion system. Approximately 20 mL of 20% potassium chloride solution was injected into the external jugular vein to fibrillate the heart. Following overdose, the carotid artery was perfused with 1–1.5 L of 10% phosphate buffered formalin at a pressure of 120 mmHg, for the purpose of internal fixation.

Butorphanol tartrate (Torbugesic SA; Fort Dodge Laboratories, Inc, Fort Dodge, IA, USA) was used as an analgesic prior to perfusion.

Block sections of the teeth and surrounding bone were removed using a Stryker autopsy saw, and placed into 10% formalin solution for further fixation. The specimens were then placed in 0.5 mol $L^{-1}$ EDTA at 4°C for demineralization. Demineralization was determined complete when radiographed sections demonstrated the absence of radiopaque structures. Block specimens were dehydrated in alcohol infused with Paraplast Plus Paraffin (Monojet, St. Louis, MO, USA) using the Technicon (Technicon Instruments Corporation, Tarrytown, NY, USA). The block sections were embedded in paraffin wax and serially sectioned at 5.0–7.0 mm using a microtome (Leitz, Rockleigh, NJ, USA). Selected specimens closest to the foramen were used as follows: one section each for Hematoxylin and Eosin (H & E) procedures, monoclonal antibodies to $CD4^+$ T cells, monoclonal antibodies to $CD8^+$ T cells, and two sections for negative controls.

### Immunohistochemical staining

Prior to staining, the sections were deparaffinized in xylene and rehydrated in descending concentrations of alcohol, tris-buffered saline (TBS) and 3% hydrogen peroxide ($H_2O_2$) to remove endogenous peroxidase activity. Primary antibodies were diluted 1 : 5 for the $CD4^+$ and 1 : 20 for the $CD8^+$ in TBS. Tissue sections were isolated with a hydrophobic slide marker (PAP Pen, Research Products International Corp, Mount Prospect, IL, USA) and using the Vectastain Universal quick kit (Vector Laboratories Inc., Burlingame, CA, USA) slides were incubated with the primary antibody at room temperature for 45 min following incubation with blocking serum, rinsed in TBS and incubated with the secondary biotinylated antibody for 15 min at room temperature, then rinsed. Sections were then incubated in streptavidin/peroxidase complex working solution, rinsed, and incubated in peroxidase substrate solution (DAB Substrate kit for Peroxidase, Vector Laboratories) until desired stain (all rinses performed were done for 5 min). Finally, sections were dehydrated in ascending concentrations of alcohol, cleared in xylene and mounted using the liquid coverslip Acrymount™ (Statlab Medical Products, Lewisville, TX, USA).

### Evaluation of tissue sections

Photomicrographs were taken with a bright field microscope in the region of the periradicular lesion of three random fields at a magnification of 40× for each of the $CD4^+$ and $CD8^+$ stained specimens (Figs 1–3). For the H & E, two random fields were taken at a magnification of 10× (Fig. 4). All images were standardized, being made

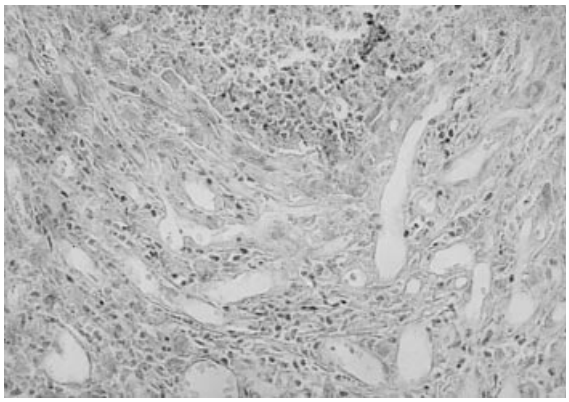

**Figure 1** Immunohistochemical staining for $CD4^+$ cells (40×).

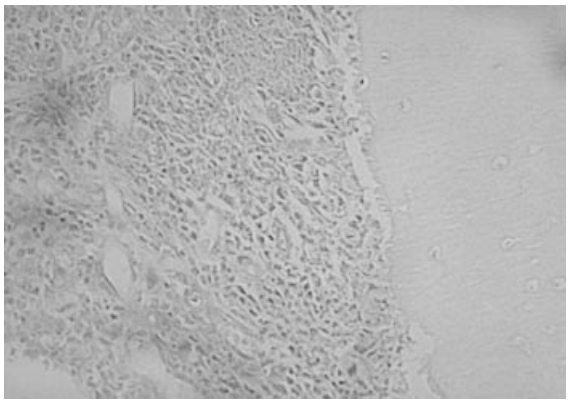

**Figure 2** Immunohistochemical Staining for $CD8^+$ cells (40×).

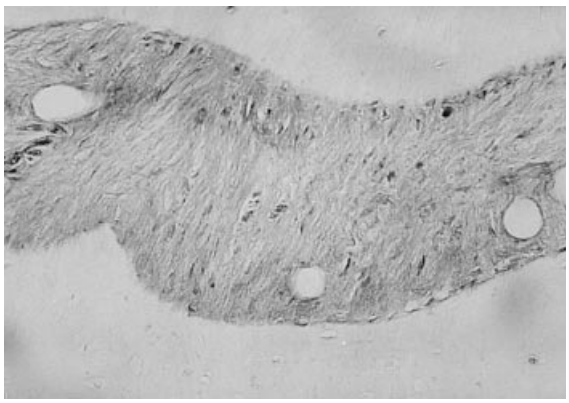

**Figure 3** Immunohistochemical staining for $CD4^+$ cells on a control tooth (40×).

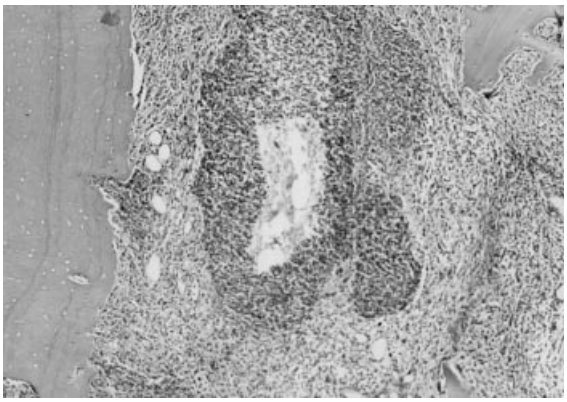

**Figure 4** H & E staining (10×).

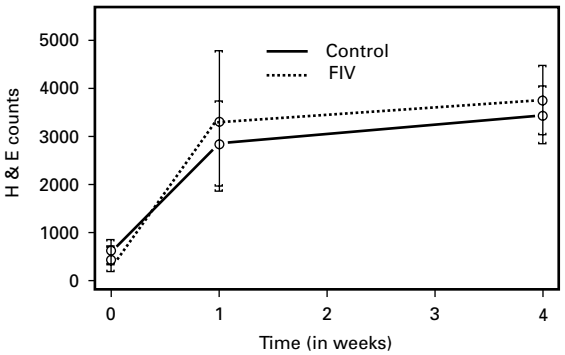


**Figure 5** H & E counts by group and time (in weeks).

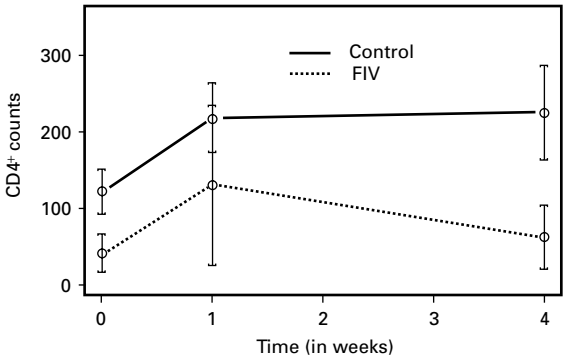


**Figure 6** $CD4^+$ counts by group and time (in weeks).

with the same film, light saturation, and exposure time. The images were then scanned (600 dpi) into a computer system and analysed with the MetaMorph Imageing System (Version 4.0 for Microsoft Windows 95™, Universal Imageing Corporation, West Chester, PA, USA). Briefly, the computer software allows cell selection by setting the threshold parameter according to size and optical density of the cells to be counted. These parameters were established separately for $CD4^+$ and $CD8^+$ cells. The reading was initially repeated to verify the reading. This establishes a reliable and uniform reading method for each section (Wood *et al.* 1997, Ying *et al.* 1999, Wilson *et al.* 2000). For $CD4^+$ and $CD8^+$ quantification, stained cells were selected according to size and density of one section and applied to all. For the H & E stained sections all inflammatory cells were selected.

### Statistical analysis

All statistical comparisons between groups over time were made using the method of generalized estimating equation (GEE) with an exchangeable working correlation assumed. For the count data (i.e. $CD4^+$ counts, $CD8^+$ counts, and H & E counts), a log link was also utilized. SAS statistical software (SAS version 8.0, SAS Institute, Cary, NC, USA) was used for all the statistical analyses.

## Results

Two cats from the FIV-group died before the experimental procedures were performed. Summary statistics for H & E counts, $CD4^+$ counts, $CD8^+$ counts, and $CD4^+$ to $CD8^+$ ratios for control and FIV-infected cats at each time-point are given in Table 1. The summary statistics were computed by first taking averages by cat and then compiling the summary statistics with the cat as the unit of measurement for each cat there were three fields for each of two teeth. In order to test the effect of time (0 weeks, 1 week, and 4 weeks) and group (control and FIV) simultaneously on H & E counts, $CD4^+$ counts, $CD8^+$ counts, and the ratio of $CD4^+$ to $CD8^+$ counts, GEE regression models were fitted assuming a Poisson distribution.

From the GEE model for H & E counts, it was found that there was a significant effect over time. Specifically, the H & E counts increased exponentially over time ($P < 0.0001$) in both groups (Fig. 5). However, there was not a significant difference between the H & E counts for the control cats and the H & E counts for the FIV-infected cats when taking time into account ($P = 0.4678$).

As for $CD4^+$ counts, a significant effect over time was found with this effect being non-linear in nature, and the effect over time was different for the two groups of cats (Fig. 6). Specifically, both group $CD4^+$ counts for baseline (week 0) were not significantly different from $CD4^+$ counts for week 4 ($P = 0.1737$), $CD4^+$ counts for week 1

**Table 1** Summary statistics for H & E counts, $CD4^+$ counts, $CD8^+$ counts, and $CD4^+/CD8^+$ ratios

| | *n* | Mean | SD | Median | Min | Max |
|---|---|---|---|---|---|---|
| H & E count | | | | | | |
| *Control* | | | | | | |
| 0 weeks | 8 | 594.8 | 429.8 | 632.8 | 45.5 | 1337.5 |
| 1 week | 8 | 2986.7 | 1521.8 | 2936.0 | 845.5 | 5472.5 |
| 4 weeks | 8 | 3395.1 | 883.0 | 3028.1 | 2702.5 | 5159.3 |
| *FIV* | | | | | | |
| 0 weeks | 6 | 432.6 | 350.9 | 339.0 | 147.0 | 1096.0 |
| 1 week | 6 | 2920.6 | 2066.0 | 3036.6 | 151.0 | 5387.3 |
| 4 weeks | 6 | 3561.9 | 900.4 | 3283.6 | 2687.5 | 5023.5 |
| $CD4^+$ count | | | | | | |
| *Control* | | | | | | |
| 0 weeks | 7 | 124.4 | 42.8 | 138.3 | 46.7 | 168.0 |
| 1 week | 8 | 233.1 | 84.8 | 226.2 | 131.7 | 378.3 |
| 4 weeks | 8 | 221.6 | 88.8 | 203.4 | 110.2 | 343.8 |
| *FIV* | | | | | | |
| 0 weeks | 6 | 40.8 | 33.8 | 38.2 | 2.7 | 86.3 |
| 1 week | 5 | 164.3 | 168.9 | 90.2 | 8.3 | 404.0 |
| 4 weeks | 5 | 59.2 | 58.2 | 30.5 | 0.7 | 125.7 |
| $CD8^+$ count | | | | | | |
| *Control* | | | | | | |
| 0 weeks | 8 | 30.2 | 43.8 | 8.8 | 1.0 | 112.0 |
| 1 week | 7 | 144.8 | 91.5 | 179.8 | 54.3 | 295.3 |
| 4 weeks | 8 | 95.0 | 74.1 | 57.8 | 24.0 | 211.3 |
| *FIV* | | | | | | |
| 0 weeks | 6 | 50.2 | 36.7 | 48.7 | 15.3 | 115.7 |
| 1 week | 5 | 124.0 | 58.6 | 100.0 | 81.7 | 226.7 |
| 4 weeks | 6 | 163.4 | 93.4 | 218.3 | 39.3 | 232.0 |
| $CD4^+/CD8^+$ ratio | | | | | | |
| *Control* | | | | | | |
| 0 weeks | 7 | 40.7 | 36.7 | 26.9 | 4.2 | 102.6 |
| 1 week | 7 | 26.1 | 45.7 | 10.1 | 0.5 | 127.5 |
| 4 weeks | 8 | 15.9 | 17.9 | 7.4 | 0.8 | 45.9 |
| *FIV* | | | | | | |
| 0 weeks | 6 | 1.9 | 2.4 | 1.2 | 0.1 | 6.6 |
| 1 week | 5 | 2.0 | 2.6 | 1.1 | 0.1 | 6.6 |
| 4 weeks | 5 | 0.6 | 0.7 | 0.4 | 0.0 | 1.7 |

*$n$ = number of cats (two teeth per cat each with three fields viewed).

were significantly different from $CD4^+$ counts for week 4 ($P = 0.0092$), and $CD4^+$ counts for baseline were significantly different from $CD4^+$ counts for week 1 ($P < 0.0001$). In addition to the effect over time, there was a significant difference in the time effect between the two groups of cats. Specifically, the FIV cats demonstrated a significantly greater decrease in $CD4^+$ counts from week 1 to week 4 compared to the control cats ($P = 0.0354$). This difference in the effect of the $CD4^+$ counts over time between the two groups can also be seen by looking at the summary statistics for $CD4^+$ counts by week for the two groups in Table 1. The control cats show an increase in $CD4^+$ counts from baseline to week 1 that remains more or less stable with similar $CD4^+$ counts obtained at week 4. However, the FIV-infected cats show an increase in $CD4^+$ counts from baseline to week 1 that drops back down to almost as low a level as the $CD4^+$ counts at baseline after 4 weeks (Fig. 6). In addition to the effect over time in general and the effect over time by group, there was a significant difference in $CD4^+$ counts by group ($P = 0.0006$). Specifically, the FIV-infected cats had significantly lower $CD4^+$ counts than the control cats.

From the GEE model for $CD8^+$ counts, the only significant effect was a rise in $CD8^+$ counts from baseline to 1 week ($P < 0.0001$) for both groups (Fig. 7). The $CD8^+$ counts for 1 week and 4 weeks were not significantly different ($P = 0.5967$) in either group. Furthermore, there was not a significant difference in $CD8^+$ counts between the control cats and the FIV-infected cats ($P = 0.2609$).

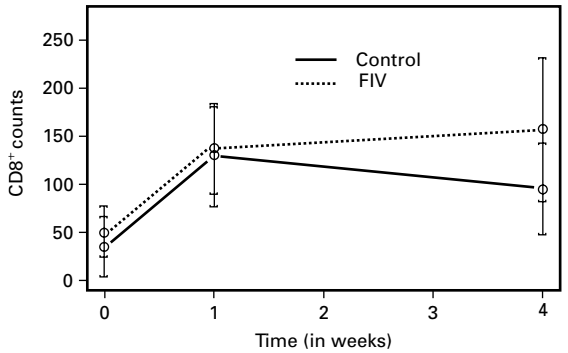


**Figure 7** $CD8^+$ counts by group and time (in weeks).

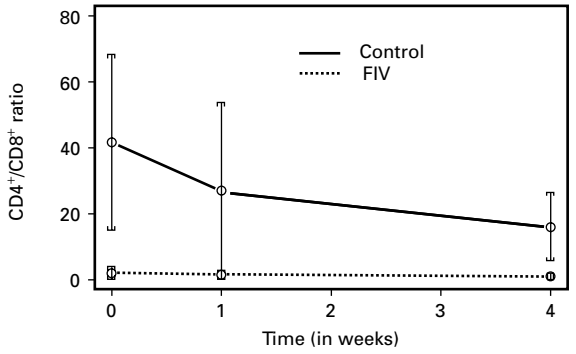


**Figure 8** $CD4^+/CD8^+$ ratios by group and time (in weeks).

There was a significant effect over time in the ratio of $CD4^+$ counts to $CD8^+$ counts, in the GEE model, that was non-linear in nature (Fig. 8). Specifically, both groups (FIV and control) had a significant decrease in the ratios of $CD4^+$ to $CD8^+$ counts from baseline to 4 weeks ($P < 0.0001$), but there was not a significant difference between the ratios for baseline and 1 week ($P = 0.5027$) or between the ratios for 1 week and 4 weeks (0.3468) for either group. There was also a significant difference in the ratio of $CD4^+$ to $CD8^+$ counts between the control cats and the FIV-infected cats ($P < 0.0001$). Specifically, the ratios were much lower for the FIV-infected cats at every time-point compared to the control cats.

When assessing possible associations amongst H & E counts, $CD4^+$ counts, $CD8^+$ counts, and the ratio of $CD4^+$ to $CD8^+$ counts, GEE regression models showed there was no significant association between H & E counts and $CD4^+$ counts ($P = 0.7139$). There was, however, a significantly positive association between H & E counts and $CD8^+$ counts ($P = 0.0099$), and a significant inverse association between H & E counts and the ratio of $CD4^+$ to $CD8^+$ counts ($P < 0.0001$).

## Discussion

Experimental models with immunological deficiencies are useful to explain processes that occur at the tissue level. This study specifically assessed the periradicular disease process. This can help elucidate not only immunological mechanisms and the specific factors involved that might be occurring in the infected group with associated immunological disorders, but also helps to make understandable the mechanisms that occur in normal individuals.

Pedersen *et al.* (1987) described the FIV. In this study, an FIV cat model was established in which periradicular lesions were induced in FIV and non-FIV infected cats. Infection of cats with FIV demonstrates a disease process that closely resembles that of HIV infection and its progression to AIDS, including the development of opportunistic infections and haematological disturbances (Barlough *et al.* 1991, Bendinelli *et al.* 1995).

Some samples were lost during processing of the material, thereby the animals were analysed as a unit so that all samples carried the same weight during analysis. The reason for using the Poisson distribution for these data was because the data were count data that are generally more amenable to the log link used when assuming a Poisson distribution. Log transformations are useful when evaluating count data, since the outcomes are strictly non-negative in nature. The reason for fitting a GEE model was to account for the clustered nature of the data since multiple teeth were observed within each cat over time. This accounts for the lack of independence of sites within each cat as well as the collection of data over time. An exchangeable working correlation structure was assumed for the GEE models that were fit.

This feline model represented accurately what would be expected in an $HIV^+$ individual, decreased levels of $CD4^+$ counts were observed at the periradicular level in the FIV-infected cats compared to the controls. This would be expected since the FIV retrovirus has special affinity for the $CD4^+$ receptor molecule (similar to HIV) that is situated on the surface of T-helper lymphocytes. This, in turn, led to a significant decrease in the $CD4^+/CD8^+$ ratios of the FIV cats compared to the non-FIV cats at all time periods.

The control data also reflect the processes occurring in non-immunosuppressed hosts. Stashenko & Yu (1989) and Stashenko *et al.* (1992), describe the predominance of T-helper (TH) cells over T-suppressor (TS) cells during lesion expansion, which was referred to as the active stage of the disease. However, when lesion expansion had slowed, the TH/TS ratio was reversed, which correlated

to lesion stabilization and a chronic stage of the disease. In this model, $CD4^+/CD8^+$ ratios, which have been associated with TH/TS ratios ($CD4^+$ = TH and $CD8^+$ = TS), also had a significant effect over time. A tendency for the $CD4^+/CD8^+$ ratio to decrease was observed from the one-week to the four-week lesion in both groups, although not statistically significant due to the large standard deviations caused by individual variations.

The fact that T lymphocytes and macrophages are major components of periradicular lesions, with the latter also being infected with FIV, it was expected that some alteration would be present in the lesion development. However, as demonstrated by H & E, no difference was encountered in degree of inflammation and progression of the lesion. This agrees with studies that question the contribution of T lymphocytes to the pathogenesis of periradicular lesions despite their numerous presence (Fouad 1997, Waterman *et al*. 1998). These studies use immunodeficient rodents to delineate the development of periradicular lesions in response to pulp exposures. They demonstrate comparable development of lesions in normal animals and immunodeficient animals, suggesting that non-specific immune mechanisms probably play a role in the development of periradicular lesions.

As for the $CD8^+$ cells, these were also increased from baseline levels to lesion induction (one-week) in both groups, but there was no statistically significant difference between groups. The helper function of T cells has been associated with the $CD4^+$ surface receptor, and the $CD8^+$ receptor has been associated with the cytotoxic/suppressor function. However, helper T cells are found in both subsets as well as cytotoxic and suppressor cells (Naor 1992). These $CD8^+$ cells may be involved in the development of periradicular lesions in the FIV group through unknown mechanisms or by replacing the function of those cells lost to the infection. This could explain the tendency for $CD8^+$ lymphocytes to remain elevated throughout the development of the lesion in the FIV group. In addition, a positive correlation was found between inflammation over time, as reflected by H & E counts, and $CD8^+$ counts, which could also be associated with the chronicity of the lesion. In agreement with this concept are findings suggesting that both $CD4^+$ and $CD8^+$ subsets of T cells are involved in bone resorption, and that $CD8^+$ T cells are not as important when they coexist with $CD4^+$ T cells in non-immunosuppressed hosts (Hara *et al*. 1998), as seen by the sustained high levels of $CD4^+$ cells over time in the control group with a decrease in the $CD8^+$ cells, but still above the baseline level. Therefore, $CD4^+$ cells sustained their influence slightly longer in the control group. However, both subsets influence bone resorption by activating macrophages, which would explain the increased levels of $CD8^+$ cells for longer periods in the FIV group due to the decreased number of $CD4^+$ cells.

Immunohistochemical methods have the advantage of showing cellular distribution, however, cell composition of lesions may vary according to the field examined because of the variable cell distribution in periradicular lesions. While sections evaluated in this study were those closer to the apical foramen, the lesion was not examined *in toto*.

## Conclusion

Progression of a periradicular lesion from an acute phase to a chronic phase in FIV infection resulted in a significantly greater decrease in the number of $CD4^+$ cells found within the lesion. However, this did not result in a change (compared to non-infected controls) in lesion development and progression as assessed by standard histological techniques (H & E).

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