

ABSTRACT

HOWARD, KRISTINA ELAINE. Mucosal immunopathogenesis of feline immunodeficiency virus (FIV). (Under the direction of Dr. Mary Jo Burkhard)

Human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) have profound effects on the structure, function and leukocyte populations of the gastrointestinal tract. Intestinal tract changes have been identified in SIV-infected macaques as early as 1-2 weeks following experimental infection, and in HIV-infected humans within months of infection. FIV is an established model of HIV immune dysfunction but the mucosal pathogenesis of FIV infection has not been well studied. In the present study, we (1) identify the normal phenotype of feline small intestinal leukocyte populations; (2) characterize changes occurring in the gastrointestinal immune system of acutely and chronically FIV-infected cats, and (3) address the impact that different inoculum types (cell-associated versus cell-free virus) may have in early immune alterations.

The methodology for isolation of intraepithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL) was optimized to provide samples of high yield and purity for use in phenotypic and functional assays. We found the phenotype IEL, LPL and Peyer's patches of cats to be similar to that reported in other species, except for the expression of CD5 and MHC II. Profound loss of IEL and mesenteric lymph node cells was observed in persistent FIV infection. In addition, we detected significant immune dysregulation of the medial iliac lymph node that may involve aberrant homing of CD4⁺ T-cells. Loss of IEL was associated with the induction of apoptosis that occurred in a significant proportion of IEL one day after inoculation with cell-associated but not cell-free FIV. In addition to differential induction of

apoptosis, we also identified significant differences in phenotype of leukocyte populations sampled very early after inoculation with cell-associated versus cell-free FIV in every tissue examined.

Thus, immune dysfunction clearly occurs in the mucosal immune system of cats infected with FIV. Furthermore, these findings expand the current knowledge of lentiviral pathogenesis by demonstrating that changes occur much earlier than has been previously reported, and that inoculum type profoundly influences the early immune dysregulation observed.

**MUCOSAL IMMUNOPATHOGENESIS
OF FELINE IMMUNODEFICIENCY VIRUS (FIV)**

by

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DEDICATION

This dissertation is dedicated to my husband, Michael D. Howard, for his continual support, understanding and encouragement; and to Gina Ridgway, who gave me the idea in the first place.

BIOGRAPHY

Kristina Elaine Howard was born in Philadelphia, Pennsylvania. As a child she aspired to be a veterinarian and had a great love for science, but somewhere along the way she detoured into the world of business. The first of many detours in her life was following graduation from Northeast High School in 1982, when she decided to postpone college for a year to attend at the Erasmus Grasser Gymnasium in Munich Germany. During that year, she lived with her grandmother, learned how difficult homework could be – especially calculus in another language, and became fluent in German. When she returned to the U.S., she attended Temple University and majored in accounting and statistics, earning her Bachelor of Business Administration in 1986. Following graduation, she worked for Coopers & Lybrand, and passed the Pennsylvania Certified Public Accounting exam in 1987. She worked as an auditor until 1991, when she left public accounting to continue her career in corporate accounting. She joined Himont, USA in 1991 and worked as a senior accountant and systems analyst, later taking on financial reporting responsibilities for plant, property and equipment until 1996.

During her tenure in accounting Kristina reconnected with her desire to pursue a career in science. To that end, in 1993 she began taking the necessary prerequisite science classes in the evening at various community colleges and universities until they were completed. Kristina's interest in immunology was spawned during her introductory biology classes combined with her interest in diseases affecting companion animals. She decided to pursue a goal of becoming a biomedical researcher, and chose to attend veterinary school to obtain comprehensive medical training. During veterinary school Kristina met Michael Howard, whom she would eventually marry. She graduated from the Virginia-Maryland Regional

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1. INTRODUCTION

Feline immunodeficiency virus (FIV) is a lentivirus of the family Retroviridae. It was discovered in cats in 1986 (124) and is the feline homologue to human immunodeficiency virus (HIV). FIV is more phylogenetically distant from HIV and simian immunodeficiency virus (SIV), but shares many structural, biochemical and genomic characteristics with HIV, as well as the ability to induce disease naturally in its host species (13). Although FIV uses OX40 (CD134), not CD4 as a primary receptor (148), it still targets CD4+ T-cells preferentially to other lymphocyte subsets. The co-receptor for FIV is CXCR4, whereas HIV can utilize either CXCR4 or CCR5 based on stage of infection and viral isolate (13). FIV can be efficiently transmitted by parenteral, oral, vaginal or rectal exposure using either cell-associated or cell-free inoculums (8, 12, 16, 44, 107, 114, 116). Transmission of cell-associated FIV across mucosal surfaces is at equal or greater efficiency than cell-free FIV (8, 16). Once the virus enters, it initially localizes to the thymus, central nervous system, lymph nodes and gastrointestinal mucosa and targets T lymphocytes, dendritic cells and macrophages (7, 30, 114, 135, 164). FIV causes progressive immune compromise in domestic cats with a disease course that mimics the disease observed in humans with HIV. Disease course in cats includes an initial viremia, emergence of CD8+ CTL activity associated with decreased viral load, loss of CD4+ T cells, inversion of the CD4/CD8 ratio, and eventual AIDS-like syndrome with opportunistic infections and neoplastic disorders (27, 29, 35, 36, 65, 163).

Recent studies examining mucosal pathogenesis in HIV-infected humans (11, 103) indicate significant similarity to that previously described in the SIV model (57-59, 75, 171, 173).

Whether the FIV model bears this resemblance is unknown. Remaining unanswered are the very early immunologic changes following FIV infection, gastrointestinal and mucosal tropism, and whether exposure to infected cells alters the mucosal pathogenesis as compared to cell-free virus. This dissertation will examine mucosal pathogenesis of FIV infection in cats by addressing the following specific aims:

- 1) What is the normal phenotype of leukocytes in the feline small intestine?
- 2) Identify acute and chronic features of mucosal pathogenesis in the FIV model.
- 3) What impact does inoculum type play in acute immune changes in peripheral versus mucosal sites?
- 4) Does apoptosis play a role in early FIV mucosal pathogenesis?

2. LITERATURE REVIEW

A) Mucosal Immune System.

1) Overview

The mucosal immune system represents the largest lymphoid compartment in mammals and has many components including the oral and nasal cavities, lungs, gastrointestinal tract, and genito-urinary tract (40, 83). Mucosa-associated lymphoid tissue (MALT) refers to the inductive sites of organized lymphoid tissue throughout the mucosal immune system where antigens are sampled from mucosal surfaces. MALT contains several compartments including the gut-associated lymphoid tissue (GALT), which is defined as Peyer's patches, isolated lymphoid follicles, and the appendix in humans (10). Effector sites are located in the epithelium and lamina propria of the intestine, and include intraepithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL) respectively. Although the lymph nodes that drain these tissues are important in immune responses, antigen sampling occurs at mucosal surfaces and immunity relies on interactions between the epithelium, resident leukocytes, and tolerance of commensal microbionota (108).

2) Intestinal immunity

The digestive tract in the strictest sense includes all parts from the oro-pharynx to the rectum of mammals. The focus of this dissertation is the small intestine, which

includes duodenum, jejunum and ileum. The intestines secrete water, digestive enzymes, immunoglobulins, mucus, and bicarbonate ions typically from crypt cells, whereas absorption occurs in the villus cells. The duodenum and proximal jejunum principally absorb minerals, vitamins, and products of digestion including carbohydrates, proteins and lipids. The distal jejunum and ileum absorb water, vitamins such as B₁₂, and any remaining digestive content (137). Macroscopic lymphoid follicles (LF) typically 0.75 cm x 1.25 cm in size, can be found throughout the feline small intestine, but tend to occur with greater frequency in the middle to distal sections. The Peyer's patches (PP) in cats tend to be concentrated in the ileum, which is typically only a few centimeters in length. Feline PP are generally large aggregates of lymphoid tissue that can comprise the entire circumference of the ileum, and typically range from 3-6 cm in total length (63), (K. Howard, unpublished observations). PP and LF are the primary inductive sites of the small intestine, with the mesenteric lymph nodes (MLN) draining the lymphatics of the small intestine as an additional inductive site (108).

The mucosal surface of the gastrointestinal tract is covered by a layer of epithelial cells, called enterocytes. The total surface of the intestine is covered by villi, which are microscopic projections into the lumen of the gut. New enterocytes are generated from the crypt region of the villi, whereas those on the tip of the villi are more mature cells. The epithelium of the intestine is replaced more rapidly than any other surface in the body, renewing itself in approximately three days in adult mammals. Nestled between enterocytes are leukocytes called intraepithelial lymphocytes (IEL). By definition, IEL are almost exclusively lymphocytes. Below the enterocytes is a

basement membrane that creates a distinct border with the lamina propria. Lamina propria lymphocytes (LPL) comprise the majority of leukocytes found in this compartment; however, other leukocytes may also be seen. Leukocytes found in the epithelium and lamina propria are the primary effector cells of the intestine but differ substantially in their function and phenotype (108, 137). PP are lymphoid aggregates located under a unique epithelia comprised of enterocytes and specialized microfold or M cells. M cells are lymphoepithelial cells that take up a wide range of antigens and play an important role in antigen sampling (115). However, questions remain as to antigen presenting ability of M cells. Some studies have shown that M cells do not express MHC II, suggesting that M cells simply transport antigen to underlying antigen presenting cells (APC) (108). Processed antigens are presented to naïve B cells and T cells in Peyer's patches, which then exit PP through efferent lymphatics to arrive in the MLN. Naïve T- and B-cells then mature, exit the lymph node, and travel through the blood stream to the lamina propria as mature effector cells. Mature lymphocytes can either remain in the lamina propria or migrate to the epithelium as IEL. Most evidence to date indicates that these mature effectors remain in these sites once present in the mucosa (10, 108, 115).

3) Leukocytes in the gastrointestinal tract

a. IEL

Intraepithelial lymphocytes are one of the most unique populations of leukocytes identified to-date. IEL are predominantly CD8+ T-cells (60-80%) with a diverse variety of receptor configurations (84, 89, 165). Typically 5-15% of IEL are CD4+,

and very few, if any, B-cells or plasma cells are present (84, 89, 165). Another unique population is CD3⁺CD8⁻CD4⁻, or double negative T-cells (DN), which comprise 5-15% of IEL (84, 89, 165). The CD8 IEL population is different from the peripheral immune system in several respects. In most species the percentage of TCR $\gamma\delta$ versus the more common TCR $\alpha\beta$, ranges from 25- 65% of IEL (84, 89, 165, 172). Also common is the CD8 $\alpha\alpha$ homodimer, which may be co-expressed with CD4 in some species (172). Thus, IEL are a heterogeneous population.

IEL secrete cytokines, are cytolytic regardless of TCR isotype, and proliferate poorly in response to mitogenic stimulation (162). That which differentiates IEL are the functions they perform, MHC specificity, and their site of origin prior to entering the epithelium. Hayday and colleagues reviewed IEL and proposed two classes of IEL based not only on receptor specificity, but on current perceived function (56). “Type a” IEL are TCR $\alpha\beta$ ⁺CD8 $\alpha\beta$ ⁺, antigen-specific, absent in MHC class Ia deficient mice, and believed to be initially primed in the inductive tissue of the gut prior to homing to the epithelium (5). This group has been previously named “thymus-dependent” IEL. “Type b” IEL represent many unconventional phenotypes including TCR $\alpha\beta$ ⁺CD8 $\alpha\alpha$ ⁺, TCR $\gamma\delta$ ⁺CD8 $\alpha\alpha$ ⁺, TCR $\gamma\delta$ ⁺CD4⁻CD8⁻ (double negative). They are not restricted by conventional MHC specificity (39, 161), do not appear to circulate via lymphatics (5) and do not appear to arise from PP precursors (111). Type b IEL are believed to have important roles in the maintenance of intestinal epithelium (77), surveillance for malignancies (48, 52, 97) and immune regulatory function, and have previously been referred to as “thymus-independent” IEL. Despite the work already done to better understand IEL, there are still many

unanswered questions with respect to their origin, effector functions and regulatory abilities.

b. LPL

The lamina propria (LP) is similar to lymph node with regard to the distribution of B- and T-cells and presence of APC. CD4⁺ T-cells are present in greater numbers than CD8⁺ T-cells with typical CD4:CD8 ratios ranging from 1.5 – 3:1 dependent on the species in question. Also present in the LP are B-cells, plasma cells, dendritic cells, and macrophages. Plasma cells are responsible for the secretion of antibodies into the gut lumen, while B-cells, dendritic cells and macrophages can present antigen that is absorbed from the intestinal lumen. Although the general phenotype of LP leukocytes may resemble peripheral blood or a lymph node, most similarities end there. CD4⁺ T-cells in the LP typically have a memory phenotype (142, 143) and are at a higher activation state indicated by the presence of CD25 and other costimulatory molecules (93). They exist in what could be considered a “resting” memory state because they have greater thresholds that must be met to reactivate them than lymphocytes isolated from blood or lymph node (115). LP T-cells, as well as IEL, proliferate poorly in response to mitogenic stimulation, or anti-CD3 stimulation, but produce significant quantities of cytokines when stimulated by their cognate antigen (69-71). Both LPL and IEL can however, be stimulated to proliferate via the CD2 pathway, albeit not with the strength of response typically observed from peripheral T-cells (49, 139). While LP CD8⁺ T-cells display less receptor diversity than found in CD8⁺ IEL, they are typically also of a memory/activated phenotype and are cytolytic in function.

c. Other leukocytes

B-cells in the lamina propria are generally low in number, as most have differentiated to become IgA producing plasma cells. Plasma cells vary in frequency between species, but typically range from 10-30% of leukocytes found in the LP (9, 37, 38). Dendritic cells and macrophages are both found in low numbers in the LP (64, 95, 110, 121-123, 138), and are believed to be responsible for antigen sampling and removal of apoptotic epithelial cells, respectively (115).

4) Mucosal leukocyte trafficking

Integrin expression modulates leukocyte trafficking. Adhesion molecules bind to receptors on the vascular endothelium, through which cells gain entry to the intestine (85, 96, 174). It should be noted that there are many different integrins. The combination of expression patterns results in access to specific sites. High expression of $\alpha 4\beta 7$ integrin typically targets migration of leukocytes to the LP, whereas high levels of $\alpha E\beta 7$ (CD103) directs IEL to migrate adjacent to epithelial cells. The primary ligands for $\alpha 4\beta 7$ and $\alpha E\beta 7$ are MAdCAM-1 and E-cadherin respectively. Lymphocytes enter inductive sites such as PP through high endothelial venules (HEV), whereas they enter the LP through blood vessels in the mucosa. Leukocytes exit these sites through efferent lymphatics to the mesenteric lymph node, and upon migration out of the lymph node return to the blood stream via the thoracic duct (136). Few lymphocytes in the LP or IEL express CD62L (84, 89), which typically is found on naïve lymphocytes and helps them gain entry into lymph nodes. Entry into the MLN can require concurrent expression of both CD62L and $\beta 7$ integrin (175),

which is unique to this lymph node. A recent study suggests that the location in which a lymphocyte is activated “programs” the expression of homing molecules thereafter, e.g. lymphocytes activated in peripheral lymph nodes do not traffick to mucosal sites following activation (17).

Identifying the homing and trafficking of cells is important for understanding the pathogenesis of disease. It is also critical to consider homing/trafficking when developing vaccines and judging their potential efficacy at distant sites. However, much is still unknown with regard to specific entry signals for many lymph nodes and effector sites in the mucosal immune system.

Much debate currently exists on the level of trafficking that occurs in the “common” mucosal immune system between different compartments such as the oral/nasal cavity, gut, lungs, and reproductive tract. Vaccine studies in mice clearly show that immunization of certain mucosal sites induces antibody production at other mucosal sites, but not all (153, 154). And immunization via certain routes, e.g. oral versus nasal, is inherently better for production of antibodies in the gut versus reproductive tract (82). However, by definition, vaccine studies address preemptive induction of immune responses to prevent infection. In contrast, pathogenesis studies assessing trafficking evaluate an unimmunized animal to determine the sites a pathogen affects, as well as to determine whether lymphocyte homing is altered as compared to either control subjects or subjects infected with unrelated pathogens.

B) Mucosal Pathogenesis of AIDS.

1) HIV

Before a complete understanding of the association between HIV, AIDS, and its associated enteropathy was established, a disease syndrome was described in Africa, nicknamed “slim disease” by locals, to describe the chronic wasting syndrome experienced by those affected (147). At the same time, literature describes an acquired immune deficiency syndrome (AIDS) that was associated with enteropathy (32, 78, 94, 134). These are the earliest reports of intestinal pathogenesis of HIV. A substantial body of literature has since described intestinal manifestations of HIV in humans, however, until recently, very few reports addressed acutely infected individuals or the effect of HAART therapy over time on the LP of the intestine or colon, in a significant number of study participants.

Worldwide, the most frequent route of HIV transmission is mucosally via genital surfaces, with a lesser proportion of infections associated with injecting drug use (1, 2, 73, 101). The mechanisms associated with transmission and dissemination at mucosal surfaces in humans have not been elucidated, but animal models indicate that either cell-associated or cell-free virus can be transmitted with equal efficiency (16). LP CD4⁺ T-cells are known to be an initial target of HIV as they display a permissive phenotype, including CD45RO⁺, CCR5⁺ and CXCR4⁺ (128). As infection of humans is typically not detected for weeks to months following transmission, few data are available to assess early pathogenesis. During the asymptomatic phase of

HIV infection, there is profound depletion of CD4+ LPL in all regions of the small intestine and colon with increases in CD8+ T-cells (22, 88, 144, 177). Alterations in the microscopic appearance of the intestinal villi occur rapidly after infection and include villous atrophy, initial crypt cell proliferation followed by a constant state of hypoproliferation unless secondary infections occur (25, 78, 94, 168, 169). It is suspected that the loss of immune subsets is in part responsible for the remodeling of the intestinal architecture (90, 91, 146). Deficiencies in enterocyte maturation (168), enzymatic activity (168), capability to absorb nutrients (20, 21, 74, 78), SIgA production (72, 80) and fat malabsorption (129) have all been identified in the absence of secondary infection. Diarrhea and other symptoms associated with small intestinal dysfunction have been identified in otherwise asymptomatic HIV patients, with clinical signs being unassociated with opportunistic pathogens (129). Patients with secondary infections or those later in disease course can experience more severe clinical signs including cachexia and malnutrition associated with the wasting syndrome of AIDS patients (22, 51, 87, 167). These signs are observed more frequently in HIV patients from locations such as sub-Saharan Africa as compared to those from industrialized nations (23, 92, 130, 133). Moreover, even with low viral loads in peripheral blood, HIV RNA, DNA or p24 antigen can be easily identified in the intestine of infected patients (131). It has also been observed that progressive weight loss and chronic diarrhea are common clinical manifestations of HIV (25, 78), and the gastrointestinal tract is frequently the site of opportunistic infections in later stages of AIDS (4, 19, 23, 33).

Perturbations in other leukocyte populations have been identified in the intestine, including selective expansion of certain $\gamma\delta$ T-cell subsets, concurrent with the loss of other $\gamma\delta$ T-cell subsets (127), or overall increased numbers of TCR $\gamma\delta$ IEL in the duodenum of HIV patients as compared to control subjects (113). Also decreased in the intestine are NK cells (145), which is hypothesized to be associated with increased incidence of tumors in late-stage HIV infection.

Cytokine assessment from leukocytes in either the small intestine or colon has been limited due to the size of biopsy that can be obtained for testing. Consequently, cytokine expression has typically been evaluated by measuring gene expression using rt-PCR or in-situ hybridization on whole tissue samples rather than purified leukocytes restimulated with specific antigen, which is typical for PBMC or splenocytes. From these studies we know that expression of IL-1 β , TNF- α , IFN- γ , and IL-6 are increased, while IL-10 and IL-13 are decreased (79, 102, 132, 155) in the gastrointestinal tract of HIV infected humans. Clearly, further work in elucidating alterations in the cytokine milieu is necessary.

Recent studies in HIV-infected subjects conclusively show early involvement of distal intestine and colonic CD4+ LPL in the pathogenesis of disease (11, 103). Brenchley and colleagues found that ileal PP and LPL were preferentially depleted of CD4+ T-cells at all stages of HIV infection compared to PBMC and inguinal lymph node, including subjects who had been infected for less than nine months. Mehandru et al evaluated colonic CD4+ LPL and showed significant CD4+ T-cell depletion in almost a dozen newly diagnosed patients (1-2 months post-infection). In addition, these patients were followed longitudinally through the duration of highly active

antiretroviral therapy (HAART) and it was found that even commencement of HAART therapy within four weeks of infection did not restore CD4+ LPL, nor did years of therapy in chronically infected patients. Similar CD4+ LPL depletion was seen in a limited number of acutely HIV-infected patients using tissue from the proximal jejunum (53). In contrast, these authors also demonstrated maintenance or a slight increase in the numbers of CD4+ LPL in three long-term HIV-infected nonprogressors (LTNP), indicating that the maintenance of mucosal CD4+ T-cells may be a correlate of protection. Clearly, these reports show the gastrointestinal immune system is affected during primary HIV infection, and based on new reports, may be more significantly affected than the immune dysregulation described for the peripheral immune system.

2) SIV

SIV is a retrovirus that is present in non-pathogenic form in approximately thirty species of African monkeys (125). It is now known that HIV-1 evolved from SIV in chimpanzees (41, 55), and that SIV_{mac} and HIV-2 evolved from SIV in sooty mangabeys (42, 60). The SIV model utilizes species, such as Indian and Chinese macaques that do not naturally experience infection from this virus, and infection can result in rapid disease pathogenesis. Many isolates of SIV result in disease progression in infected macaques that bears strong resemblance to “rapid progressors” in humans infected with HIV. One isolate, SIV_{smm}PBj14, causes acute, severe enteropathy that typically results in death of infected macaques within weeks

of infection (54, 118). However, this isolate appears to have a tropism for the intestine that is much more pronounced than other isolates of SIV (117).

A significant body of literature describes the pathogenesis of SIV in both peripheral and mucosal sites of macaques. SIV disease progression in mucosal sites includes the acute loss of lamina propria CD4⁺ T cells early in infection (75, 100, 149, 171, 173), and pathology similar to that observed in HIV (58, 86, 158). Data suggest that localization of SIV to the intestine is due to the presence of activated memory lymphocytes in the lamina propria (171, 173). The intestinal immune system of affected macaques has also been shown to have high levels of viral replication (75, 99, 149, 173) and is a viral reservoir in chronic infection (57, 59). While macaques with AIDS have significantly greater numbers of SIV-infected cells distributed throughout the small intestine including IEL and the submucosa, fewer SIV-infected cells are detected in macaques with subclinical infection, and these cells are distributed primarily in the lamina propria and lymphoid follicles of the small intestine (59). It is also noteworthy that significant pathology is evident in the lamina propria within two weeks of infection, even following parenteral exposure (98, 149). This suggests that effects of SIV on the mucosal immune system are observed regardless of initial route of exposure.

As in HIV, villus atrophy, inflammatory infiltrates and abnormal digestive enzyme activity are seen in macaques infected with SIV by either genital or parenteral routes and these changes were observed as early as three months post-infection (58). Crypt hyperplasia has also been described (75, 177), although this diverges from HIV pathology. Absorption and enzymatic activity have also been examined in macaques and a

majority of animals exhibited decreased absorption of D-xylose or sucrase activity at two weeks post-infection. In most animals, these alterations persist over time without the development of diarrhea or opportunistic infections (57). Other measures of intestinal function that have been shown to be dysregulated at early time points include vitamin D and β -carotene concentrations (75).

Immunologic alterations include the lack of production of SIV-specific SIgA from early through chronic infection (141) and increased expression of homing and activation markers in rectal lamina propria and rectal lymphoid follicles (170). A study contrasting pathogenic versus non-pathogenic SIV clones found increased distribution and frequency of SIV-infected cells throughout the intestine following infection with the pathogenic clone versus the non-pathogenic clone as well as increased expression of several cell adhesion molecules including VLA- α 4, LFA-1 α , MAC-1 α , ICAM-1, and β ₂ integrin (157). Consequences of altered integrin expression could include inappropriate extravasation to tissues and/or abnormal trafficking.

CD4 T-cell depletion has been shown in IEL of SIV infected macaques as early as two weeks post-infection (98, 100), however, in macaques many CD4⁺ IEL also express CD8 $\alpha\alpha$ homodimers, so it is unclear which receptor is more significant in mediating this loss. These reports also show an early expansion of CD8 $\alpha\beta$ IEL that produce increased amounts of IFN- γ and MIP-1 β . These cytokines are important in the activation and recruitment of leukocytes to areas of infection and inflammation. Gene expression profiling of intestinal tissue at early time points following SIV infection identified dysregulated expression of genes associated with proliferation and

cell cycle but increased expression of innate, cell-mediated and humoral response genes in macaques that maintained higher levels of LP CD4 T-cells indicating a role for immune containment (45).

These data indicate that mucosal SIV pathogenesis has many similarities to those observed in HIV and underscore that significant alterations can be found at early time points. However, given that the SIV-macaque model does not induce a natural infection, and typically induces rapid progression of disease, it is unclear whether the observations made at one- and two-weeks post-infection can be directly correlated with the time course of alterations that occur in HIV.

3) FIV

FIV is an important natural animal model of infection (13). However, few studies to-date have addressed the effects of FIV in the gastrointestinal immune system. There are no reports that describe the isolation of IEL or LPL with phenotypic or functional evaluation of purified populations following FIV infection at any stage of disease. This makes comparisons with HIV and SIV mucosal pathogenesis sparse. And while some reports have assessed proviral DNA burden and viral RNA quantification in IEL, or rectal antibody production (16, 156), effector function and phenotypic changes during the course of FIV infection have not been addressed. Phenotypic assessment to-date has been limited to the evaluation of a small number of cell surface receptors using in-situ hybridization in the intestine (18). Other studies have evaluated the mesenteric lymph node (MLN) through virus isolation, DNA PCR, CTL assay, or cytokine profile analysis (26, 62, 114). However, while the MLN

is an important site, it is an inductive site, and data obtained from the MLN does not necessarily address effector function of lymphocytes from the gastrointestinal mucosa. The absence of studies focusing on the gastrointestinal tract and its lymph nodes inherently limits our understanding of mucosal FIV pathogenesis and its use as an animal model for HIV.

C) Influence of Viral Inoculum Type and Route of Transmission

1) HIV Transmission

HIV is primarily transmitted through sexual contact, and in lesser numbers through injecting drug use (1). A majority of children are infected by their mothers' during childbirth or through nursing (1). These data indicate that mucosal transmission produces a majority of infections, followed by injection transmission. An important question still to be answered is what form of virus is responsible for these transmissions. Infectious HIV can be identified in reproductive secretions as infected cells or cell-free virus. It is important to determine which of these forms is responsible for transmission, as it would aid not only vaccine research, but also development of topical preventive pharmaceuticals in the absence of a vaccine.

Many studies have attempted to determine the presence of HIV-1 proviral DNA and RNA in the cell-associated and/or cell-free fractions of cervicovaginal secretions (CVS) and semen (24, 28, 31, 151, 152, 159, 160, 176). Correlations between plasma viral load and viral load in genital secretions have been identified (50, 112), however

it is increasingly apparent that inconsistent correlation exists between the presence of cell-associated virus in genital secretions and indicators of infection/progression such as plasma viremia or CD4+ T-cell counts in blood (3, 43, 67, 68, 151). Reports suggest that HAART does not reduce HIV shedding in genital secretions to the same degree it reduces or eliminates plasma viremia and circulating infected PBMC (34, 67).

Epidemiological and virus variant studies suggest that HIV may be more efficiently transmitted during sexual exposure by cell-associated virus (126, 178). Direct inoculation of 300 HIV-infected cells into the endocervical canal of a chimpanzee has resulted in infection (47), suggesting cell-associated transmission of HIV is possible. It has also been demonstrated that increased cellularity of semen is more closely associated with transmission of HIV (61, 126). Other investigators suggest that the initial transmission of HIV may be more closely linked to the presence of cell-associated rather than cell-free virus in vertical and heterosexual transmission (119, 166, 178). Also supporting transmission of cell-associated HIV is the presence of cellular HIV-1 DNA and/or RNA but low or undetectable infectious cell-free HIV in CVS (81, 140, 151). Given these findings, more work targeted at elucidating whether differences in pathogenesis occur based upon alternate inoculum type is necessary.

2) Transmission of cell-associated or cell-free virus in animal models

Investigations into SIV transmission are typically conducted using cell-free inoculums when mucosal routes are employed for infection. This results from cell-

associated SIV inefficiently crossing the mucosa of macaques (106, 150). Some investigators have used the rectal or intravenous route to infect or challenge macaques, but often cell-free SIV is employed in these studies as well (171). Other concerns related to the simian model include the use of very high doses of cell-free virus with certain SIV isolates to achieve infection via the vaginal route, or the use of hormones to artificially thin the mucosa thereby enabling infection (100, 104-106, 120).

Although mice cannot be used to study HIV pathogenesis and vaccination in models other than those employing SCID-hu mice, they are used to study transmission of cell-associated and cell-free virus through the vaginal mucosa. And while treatment with hormones is necessary in some instances, both cell-associated and cell-free HIV vaginally inoculated can infect a SCID-hu mouse (46). Further studies suggest that cells may traffic from the mucosa rather than infecting other cells at the mucosal surface. Foreign lymphocytes inoculated into the cervicovaginal mucosa can be detected in the draining iliac lymph nodes within 24 hours in 34 of 36 mice evaluated by immunohistologic staining of tissues (66). Studies utilizing other virus models have shown that cell-free Herpes simplex virus injected into the skin of mice caused T-cell activation in the draining lymph node 4-6 hours post-injection as a result of antigen presentation, not the presence of virus (109). Application of a topical agent to alter the vaginal epithelium prevents transmission of cell-associated HIV in SCID-hu mice, underscoring the fact that cell-associated virus may need to migrate through the epithelium to infect the host. (76). These reports emphasize the importance of inoculum type in efforts to prevent HIV transmission.

Cats are normally infected by FIV as a result of bites and scratches in non-experimental models. However, numerous studies have shown effective transmission of FIV parenterally, orally, vaginally, and rectally using either cell-associated or cell-free inoculums (8, 12, 15, 16, 44, 107, 114, 116). Infection occurs with equal or greater efficiency when using cell-associated versus cell-free inoculums at equivalent doses, using different isolates of FIV representing multiple clades (6, 12, 14-16). Some of these reports demonstrated differences following acute infection as a result of inoculum type and/or route of infection. Following cell-free versus cell-associated mucosal infection, cats inoculated with cell-free virus demonstrated more rapid seroconversion (16). Clade dependent differences were identified in another report based on route of inoculation. Specifically, a clade B variant induced peak viremia more rapidly following i.v. exposure as compared to vaginal infection, whereas a clade A variant induced peak viremia more rapidly following vaginal infection. This report also identified more rapid loss of CD4+ T-cells from PBMC following i.v. infection independent of the clade utilized or levels of peak viremia (15). Another report showed early differences in antibody production based on route of initial infection (14). However, as has been shown in SIV, the long-term course of infection becomes similar regardless of route of exposure or inoculum type. Overall, these data suggest the FIV model is well suited to discern differences based upon inoculum type and route of infection.

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**Methodology for isolation and phenotypic characterization
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Abstract

Critical assessment of intestinal immune responses requires the ability to characterize leukocytes from different anatomic locations as leukocytes from inductive sites such as Peyer's patches and lymphoid follicles vary significantly from their effector counterparts, intraepithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL). This study describes (1) methods developed to isolate specific intestinal leukocyte populations with high yield and purity, (2) difficulties encountered in establishing a panel of monoclonal antibodies to assess phenotype, and (3) the phenotypic characterization of effector and inductive sites in the feline small intestine. We found that the phenotypic distribution of feline intestinal leukocytes was similar to that found in other species such as humans, macaques and mice. The majority of IEL were CD5+ T-cells with less than 7% B-cells. CD8+ T-cells comprised approximately 60% of the IEL with roughly half displaying CD8 α homodimers. Approximately 10% of IEL were CD4+ T-cells. In the LPL, CD4+T-cells predominated at 42%, with 33% CD8+ T-cells and 10% B-cells. As would be expected, B-cells predominated in Peyer's patches with 40% B-cells, 28% CD4+ T-cells and 20% CD8+ T-cells. Increased MHCII expression was found in the Peyer's patches as compared to the IEL and LPL. B7.1 expression was significantly higher in mucosal leukocyte populations as compared to organized lymphoid tissue in the periphery with expression detected on 65% of IEL and 53% of LPL. Plasma cells were found in all regions of small intestine examined with greater numbers in lamina propria and Peyer's patches. Lymphoblasts were only identified in inductive tissue. In general, no differences were found between the phenotype of mucosal leukocyte populations from specific pathogen free or random source cats. However, the percentage of CD4+CD25+ T-cells was significantly greater in both IEL and LPL from

random source animals. This study provides techniques and a baseline from which future studies of the feline intestinal immune system can be conducted.

Keywords: feline, small intestine, IEL, LPL, Peyer's patches, phenotype

1. Introduction

The intestinal immune system is composed of inductive and effector sites that can be identified by anatomic location. Unlike the peripheral immune system, the intestinal immune system lacks afferent lymphatics and antigen sampling occurs at the mucosal barrier of the gut. Peyer's patches (PP) and lymphoid follicles (LF), which are grossly visible in the cat, are inductive sites where initial activation of immune responses can occur in situ, without first trafficking to a lymph node. Efferent lymphatics permit migration of cells to lymph nodes draining the gastrointestinal tract. Lymphocytes activated in the gut or draining lymph nodes express homing markers that allow them to exit the lymph node and enter effector sites including the intestinal epithelium and lamina propria via high endothelial venules (HEV) from the blood stream. Previous studies have demonstrated important differences in lymphocyte phenotype based on anatomic location, e.g. duodenum vs. jejunum or PP in humans, macaques and cats (Spencer et al., 1986; Schieferdecker et al., 1992; Lundqvist et al., 1995; Veazey et al., 1997; Waly et al., 2001). In addition, understanding the distribution of inductive and effector cells within the small intestine encourages selection of appropriate samples to answer questions regarding immune responses.

The gut is a primary site of infection and disease pathogenesis for human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) (Heise et al., 1993; Veazey et al., 1998; Brenchley et al., 2004; Mehandru et al., 2004). Thus, there is a critical need to assess intestinal immune responses in HIV infected humans and animal models. SIV studies have shown loss of activated/memory CD4⁺ T-cells in the intestinal lamina propria within weeks of infection, prior to systemic immune alterations becoming evident (Heise et

al., 1994; Smit-McBride et al., 1998; Veazey et al., 1998; Kewenig et al., 1999). In a recently published report on HIV pathogenesis in the gastrointestinal tract, the authors identified a profound loss of CD4+ T-cells within the intestine at all stages of disease (Brenchley et al., 2004). Another study found that while peripheral immune populations rebound when patients are on highly active antiretroviral therapy (HAART), the gastrointestinal immune system does not appear to rebound to a pre-infection state, even if anti-viral therapy is initiated very early following infection or with years of therapy (Mehandru et al., 2004). These studies indicate that immunopathologic changes are significant and long lasting, and have implications for early therapeutic intervention as well as development of vaccines that target the mucosal immune system.

Feline immunodeficiency virus (FIV) infection of cats is an important animal model of HIV, however, reports that assess feline intestinal immune responses during FIV infection are few. Prior studies on the normal feline intestinal immune system include phenotypic evaluation by immunohistochemistry (Waly et al., 2001), or by a combination of flow cytometry and immunohistology (Roccabianca et al., 2000). While both studies provide some insight into the phenotype of the leukocytes present in the feline intestine, their results are limited in scope, partially due to the prior scarcity of commercially available immunophenotyping reagents. Furthermore, a reliable method for isolating intraepithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL) of sufficient purity and yield for phenotypic evaluation and functional assays was still lacking.

Studies to date in FIV that assess aspects of the gastrointestinal immune system typically have evaluated limited parameters such as viral load, proviral DNA burden, or rectal antibody production (Burkhard et al., 1997; Stokes et al., 1999), but not effector function or

phenotypic changes. Other studies have evaluated the mesenteric lymph node (MLN) through virus isolation, DNA PCR, CTL assay, or cytokine profile analysis (Hosie and Flynn, 1996; Dean and Pedersen, 1998; Obert and Hoover, 2002), but the MLN is an inductive site, and while an important site, does not address effector function of the gastrointestinal mucosa.

The objectives of this study included the development of techniques to isolate feline intestinal immune populations of high yield and purity, identification of an optimal panel of monoclonal antibodies for FACS analysis, phenotypic characterization of small intestinal leukocytes in each anatomic compartment, and determination of any phenotypic differences in the normal intestinal immune system between typical research cats and an outbred population of cats. This study provides techniques and a baseline from which future studies of feline intestinal immune system can be conducted to answer questions associated with variety of infectious, inflammatory, and neoplastic conditions.

2. Materials and Methods

2.1. Animals

Three specific pathogen free (SPF) cats, group housed and obtained from Liberty Labs (Liberty, NY) were used. Age at the time of euthanasia was approximately two years. All SPF cats were maintained in accordance with the standards set by the Institutional Animal Care and Use Committee. The remaining ten cats used were healthy adult cats, aged one to three years, obtained at a local animal shelter, hereinafter referred to as random source (RS) cats. Random source cats were euthanized due to shelter retention policies, not resulting from

illness or for the purpose of this study. All cats were sedated with Telazol® (Fort Dodge Animal Health, Overland, KS), physically examined ante-mortem and found to be clinically normal at the time of euthanasia. Two random source cats were found to have tapeworms following euthanasia and were included as their results were similar to the other cats tested. No other macroscopic gastrointestinal parasites were found in any of the cats, although fecal examination for microscopic parasites was not performed. All random source cats were tested for feline leukemia virus (Felv) and FIV (SNAP FIV/Felv Test, IDEXX Laboratories, Westbrook, ME) and found to be negative. A total of five male and five female random source cats were evaluated.

2.2. Antibodies

Monoclonal antibodies (mAb) used for cell surface staining were directly conjugated to either fluorescein isothiocyanate (FITC), phycoerythrin (PE), biotin, peridinin chlorophyll protein (PerCP), Spectral Red (R-PE-Cy5.5), PE-Oyster 665 (PE-O) or allophycocyanin (APC). Alternatively, primary antibodies were detected using directly labeled secondary antibodies. A summary of antibodies used in this study is presented in Table 1. MAb clone 3.357 was conjugated using PhycoLink® RPE-Tandem-665™ Conjugation Kit (Prozyme, San Leandro, CA) to PE-Oyster 665 in our laboratory. Mouse anti-cat B-cell mAb, clone 8F9 and MHC II FITC, 42.3H2 (Rideout et al., 1990) were generous gifts from P. Moore (UC Davis, CA). Clone 8F9 was conjugated to FITC using standard protocols, and PE-O as described above in our laboratory. Secondary antibody F(ab')₂ fragment goat anti-mouse IgG PE (#115-116-146) was used with anti-CD45, and donkey anti-mouse IgG (H&L) FITC

(#715-116-150) was used with anti-CD3. Isotype control mouse IgG1_κ-PE was purchased from BD Biosciences.

2.3. Sample collection and processing

Cranial mesenteric lymph node (MLN) and the entire small intestine, from duodenum to terminal ileum, were removed, the small intestine was measured, and both tissues were processed immediately post-euthanasia. Total intestinal length averaged 132 cm, with a range of 107-165 cm. Approximately 2.5 cm from the proximal, middle and distal small intestine were snap-frozen for histologic evaluation. It should be noted that the feline ileum is very short (typically 5-8 cm), and is made up almost entirely of Peyer's patches (Hudson and Hamilton, 1993). PP were removed for separate processing, and 30 cm sections of proximal (including duodenum and proximal jejunum) and distal (jejunum only) small intestine were removed. All grossly visible lymphoid follicles from the entire small intestine were removed by excision with scissors and combined with PP for processing. Representative tissue sections were collected at each processing step and preserved in 10% buffered formalin to histologically assess tissue digestion.

2.4. Mesenteric lymph node processing

Cranial mesenteric lymph node (MLN) was disassociated using scissors and ground on #40 mesh screens. The sample was centrifuged at 400 x g for 10 minutes at 25°C. Pellets were washed twice in 1x PBS, resuspended in LBT medium (Stevens et al., 2004) and counted. In addition, while optimizing monoclonal antibody staining for this study, MLN were also

processed as described for IEL, to control for processing as a source of poor reactivity for some mAb tested.

2.5. Intraepithelial lymphocyte isolation

The protocol used was a modification of a previously reported method (Burkhard et al., 1997). Briefly, 30 cm sections of intestine were flushed with wash medium a minimum of three times prior to removal of lymphoid follicles. Wash medium consisted of 1x PBS (Mediatech, Herndon, VA) supplemented with 20% fetal bovine serum (Hyclone, Logan, UT), 4mM L-glutamine (Gibco, Grand Island, NY), 2mM penicillin-streptomycin (Mediatech), 10 µg/ml gentamicin (Mediatech), and 2.5 µg/ml amphotericin B (Cambrex, East Rutherford, NJ). After lymphoid follicles were removed, the intestine was cut open longitudinally and the section was cut into 0.5 cm strips. The strips were placed in a 250 ml Ehrlenmayer flask with 40 ml spin medium containing 2mM EDTA (Sigma-Aldrich, St. Louis, MO) and 2mM dithiothreitol (DTT) (Sigma-Aldrich, St. Louis, MO). Spin medium consisted of 1x Hanks Salt Solution, without Mg⁺, Ca²⁺, or phenol red (Mediatech) supplemented with 20% fetal bovine serum (Hyclone, Logan, UT), 4mM L-glutamine (Gibco), 2mM penicillin-streptomycin (Mediatech), 10 µg/ml gentamicin (Mediatech), and 2.5 µg/ml amphotericin B (Cambrex). Sections were stirred vigorously on a Thermolyne Nuova II hot plate/stirrer at 800-1,000 rpm for 30 minutes at 37°C. Following stirring, the tissue was strained from the supernatant using a #40 basket style sieve. The supernatant was centrifuged at 1,000 x g for 15 minutes at 16°C and the supernatant was carefully removed from the pellet by pipetting. The pellet was resuspended in 10-15 ml of 30% Percoll,

vortexed vigorously to mix, and then strained through autoclaved cheesecloth into a 50 ml conical tube. After straining, the total volume of 30% Percoll was increased to 35 ml and then vortexed. Using a 14 gauge, 10 cm teat cannula, 12 ml of 70% Percoll was underlaid and then centrifuged for 30 minutes at 400 x g at 25°C. Iso-osmotic Percoll is defined as 90.8% Percoll (Sigma-Aldrich, St. Louis, MO) and 9.2% 10x Hanks Salt Solution (Gibco) with sodium bicarbonate added according to manufacturer's instructions. All Percoll solutions were made with iso-osmotic Percoll and 1x Hanks Salt Solution, (catalog numbers 21-022-CV and 21-021-CV, Mediatech), such that the ending concentration of iso-osmotic Percoll was 30%, 44%, 50%, 67% or 70%. Following centrifugation, the 30/70-interface layer was collected, washed twice in wash medium and cells were counted.

2.6. Lamina propria lymphocyte isolation

LPL were isolated by continued processing of intestinal strips described for IEL in 2.4. Tissue strips were resuspended in spin medium containing 2mM EDTA and stirred for 30 minutes at 37°C on a Thermolyne Nuova II hot plate/stirrer at 800-1,000 rpm. The supernatant was discarded and the intestinal strips were digested twice in succession using 24 ml spin medium and 6 ml Dispase II (Roche Applied Science, Indianapolis, IN) on an orbital shaker at 37°C at 200 rpm for 90 minutes. Each supernatant was collected and centrifuged at 1000 x g for 15 minutes at 16°C. Following centrifugation of the first digestion, the pellet was resuspended in wash medium and placed on ice until the second fraction was ready for Percoll gradient separation. The first fraction was centrifuged for 10 minutes at 400 x g at 16°C and supernatant discarded prior to resuspension in Percoll. The second fraction was

centrifuged at 1000 x g for 15 minutes at 16°C and supernatant removed prior to resuspension in Percoll. After both digestions were completed, each pellet was resuspended separately in 5 ml 44% Percoll, then underlaid successively with 5 ml 50% and 4 ml 67% Percoll in a 15 ml conical tube. Gradients were centrifuged at 400 x g for 30 minutes at 25°C. Following centrifugation, the 44/50 and 50/67 interfaces were collected and combined. They were washed twice in wash medium and cells counted.

2.7 Peyer's Patches and lymphoid follicle processing

Peyer's patches (PP) were defined as macroscopic lymphoid tissue present in the ileum. Lymphoid follicles (LF) were defined as macroscopic lymphoid tissue, typically 1-2 cm in length and 1 cm width found in the jejunum and duodenum of the small intestine. These tissues were identified, excised with scissors and cut into 0.5 cm strips. Leukocytes in the epithelium (hereinafter referred to as PP/LF epithelium) were removed by sequential processing with EDTA and DTT as performed for IEL in 2.5, with only the first fraction used for phenotyping. The organized lymphoid tissue underlying the epithelium (hereinafter referred to as PP/LF), was digested using sequential processing with Dispase II, as was performed for LPL in 2.6.

2.8. Cytospin preparation and counting

An aliquot of 3.0×10^5 freshly isolated cells was placed in PBS containing 50% FBS and spun in a cytospin cassette using a Cytospin 2 machine (Shandon Inc., Pittsburgh, PA) at 500 rpm for 5 minutes at room temperature. After air-drying, slides were stained with the Hema 3 Stat Pack (Fisher Diagnostics, Middletown, VA) and coverslip affixed with Permount (Fisher

Scientific, Pittsburgh, PA). A differential count of 100 cells was completed for every site sampled for each cat. Cells were classified as lymphocytes, large granular leukocytes, plasma cells, macrophages, eosinophils, lymphoblasts, neutrophils, or epithelial cells. Only those cell types for which an average of greater than one per 100 counted cells are shown in Table 2.

2.9. Flow cytometric staining

Phenotype of freshly isolated cells was determined using three- and four-color flow cytometry. Briefly, 1.0×10^6 freshly isolated cells were incubated with no more than two directly conjugated mAb at a time for 20 minutes at 4°C. Cells were washed with cold PBS containing 0.5mM EDTA and centrifuged at 300 x g for 10 minutes at 16°C. This procedure was repeated two to three times based on the number of mAb used for each aliquot. Cells requiring staining with Streptavidin-PECy5 or Streptavidin-PerCP were completed individually following all other mAb required for the sample. Samples were analyzed immediately on a FACScan (BD Immunocytometry Systems) flow cytometer, or fixed using 2% paraformaldehyde and analyzed on a FACS Calibur (BD Immunocytometry Systems) flow cytometer. The gated region represents CD45+ cells, from which 15,000 events were typically collected. Analysis of flow cytometric data was completed using BD CellQuest software and Summit software, ver.3.3 (Dako Cytomation, Fort Collins, CO).

2.10. Statistical analysis

Statistical analysis was completed using an unpaired t-test, with Welch's correction where appropriate, in GraphPad InStat version 3.05 (GraphPad Software, San Diego, CA).

3. Results

3.1. Isolation of intraepithelial and lamina propria leukocytes

Histological evaluation of intestinal tissues demonstrated preferential release of intraepithelial followed by lamina propria leukocytes during processing (Fig. 1). Following two 30-minute agitations, initially with EDTA and DTT, then with EDTA alone, the basement membrane was apparent with no remaining epithelium and no breach into the lamina propria (Fig. 1B). Subsequent enzymatic digestions with Dispase II resulted in complete elimination of the basement membrane and disruption of a significant portion of the lamina propria. While an additional digestion may have liberated the remaining leukocytes, sufficient yield was obtained following two digestions to permit timely completion of functional and phenotypic assays. The histologic results shown are representative of all sections processed and evaluated.

3.2. Purity of isolated populations

To confirm the purity of isolated leukocytes, an aliquot of each sample was stained with anti-CD45 (a pan-leukocyte marker) and analyzed by FACS. Mean purity by site ranged from 99.4% to 99.6% for IEL (Table 3), 98.2% to 99.1% for LPL (Table 4), and over 99%

for all regions associated with PP/LF (Table 5). Fig. 2 demonstrates the typical gating scheme for epithelial and lamina propria leukocytes from each location based on forward and side scatter, with the percent CD45+ cells in the gated region. As indicated by side scatter vs CD45 gating, an increased number of large, granular LPL were identified as compared to IEL. These data confirm that the populations isolated using the techniques developed are highly purified intestinal leukocytes.

To morphologically classify purified intestinal leukocytes, differential counts were performed on cytopsin preparations (Fig. 3, Table 2). As might be expected based on phenotypic characterization in other species (Parrott et al., 1983; Lundqvist et al., 1995), lymphocytes comprised a greater proportion of the leukocytes obtained from epithelial as compared to lamina propria isolations. Lymphoblasts were only identified in the regions associated with PP/LF. An increased number of plasma cells were identified in these regions as well.

3.3. Cell yield by site

Viable cell counts were performed to determine total leukocyte yield from each cell compartment and each intestinal segment for both SPF and random source cats. No statistically significant differences were found between SPF and random source cats regardless of site (Fig. 4). Average yields from proximal IEL ranged from 2.05×10^7 to 4.22×10^7 , while distal IEL provided typically greater yield ranging from 4.39×10^7 to 6.84×10^7 . Our finding of greater yield in the distal compartments of the small intestine agrees with the histologic report from Waly et al showing increased density of IEL in the jejunum and ileum

as compared to the duodenum (Waly et al., 2001). The IEL yields represent the product of one digestion with EDTA and DTT, and yield could be increased if the second digestion with EDTA had been processed rather than discarded. Our experience is that the second fraction of IEL processing produces approximately 50% of the yield obtained initially with similar purity and cell distribution (K. Howard, unpublished observations). Mean proximal LPL yields ranged from 3.51×10^7 to 5.81×10^7 , while mean distal LPL yields ranged from 3.10×10^7 to 5.95×10^7 . In order to ensure sufficient yield was obtained to complete phenotypic characterization, PP were combined with grossly visible lymphoid follicles (LF). Initial processing to remove leukocytes in the epithelium overlaying organized lymphoid tissues (PP/LF epithelium) produced average yields ranging from 2.70×10^7 to 3.30×10^7 and the organized lymphoid tissue below the epithelium (PP/LF) had average yields ranged from 3.17×10^7 to 4.57×10^7 . Overall, each compartment yielded sufficient leukocytes so that phenotypic characterization and functional assays could be performed as a component of FIV pathogenesis or vaccine studies.

3.4. Determination of optimal antibody panel for phenotyping of intestinal leukocytes

Initial studies of intestinal leukocyte phenotyping suggested that alternate staining patterns and reactivity existed as compared to peripheral leukocytes. In order to determine that processing did not play a role in sub-optimal antibody reactivity, we processed MLN cells using EDTA and DTT as described for IEL. We did not identify differences in antibody reactivity such as poor reactivity and lack of clear separation from negative populations that were noted for IEL and LPL (data not shown). Therefore, numerous mAb, including alternate

conjugations of the same mAb, were tested on IEL and LPL to determine optimal antibody panels, with minimum cross-reactivity between the mAb used on each sample.

Several mAb including Southern Biotechnology's mouse anti-cat CD8 (clone fCD8) and NCSU's anti-CD4 (clone 30-A) displayed poor separation from the negative cell pool. To demonstrate this staining pattern, anti-CD4 (clone 30A), which separates poorly, is compared to anti-CD4, clone 3-4F4. As shown in Figure 5a, panel a, clone 3-4F4 generated a more distinct separation of CD4 positive and negative populations when compared with clone 30A.

A number of mAb were found to not label intestinal leukocytes at all. One example was a mAb routinely used to identify B-cells in cats, anti-canine CD21 (clone CA2-1D6). Staining patterns are shown for mesenteric lymph node (MLN) and PP (Fig 5a, panel b). Despite the fact that over 99% of the cells shown in the PP sample are CD45 positive, no staining was noted for PP with this clone, although MLN appeared normal. After trying numerous mAb reported to react with feline B-cells we found that only rat anti-mouse B220 (clone RA3-6B2), (Fig. 5a, panel b) and anti-feline B-cell mAb (clone 8F9), (data not shown) were consistently effective in identifying B-cells in this compartment. B-cell identity was confirmed with two-color analysis of MLN and LPL using different combinations of each mAb including B220, clone 8F9, clone CA2-1D6, as well as anti-feline IgG (data not shown). Another mAb that caused similar difficulties was anti-CD3. Although an anti-CD3 antibody that identified feline leukocytes was found, it was not possible to combine CD3 staining with any other surface mAb (data not shown). When combined with surface receptor staining, the anti-CD3 signal was lost regardless of the secondary mAb used, whether it had been primarily conjugated, or using alternate staining/fixation techniques.

In the case of CD5, a cluster of bright staining cells was evident (MFI=1488), however, unlike peripheral lymphocytes, we also noted dim expression (MFI=117) on IEL and LPL (Fig. 5b, panel a). For those mAb that did identify cell-surface receptors on intestinal leukocytes appropriately, we noted that different conjugations of the same antibody, from the same supplier, could yield differences in staining. Monoclonal antibody anti-CD4, clone 3-4F4, demonstrated such differences. If the PE conjugate was used, a uniform population with obvious separation was evident, whereas the FITC conjugated mAb frequently identified marked low-level reactivity (data not shown). This reactivity may be low-level expression of CD4 on CD8⁺ T-cells, monocytes or eosinophils but could also be due to FcR binding. Three- and four-color FACS analysis showed this low-level CD4 expression occurred on T-cells as well as non-T-cells, but experiments were not conducted to conclusively determine the cause of this pattern. CD8 β , clone 1.117 also showed differential expression based upon the conjugate used. When conjugated to either APC or FITC, but not PE, CD8 $\alpha\beta$ were easily identified from CD8 $\alpha\alpha$ T-cells (Fig. 5b, panel b). This pattern did not change regardless of different conjugations of CD8 α being used in combination (data not shown), suggesting that the lack of intensity of CD8 β PE may not have been affected by other fluorochromes.

3.5. IEL Phenotype

Expression of cell surface markers between SPF and random source cats are reported in the tables, but were not statistically different in any of the sites evaluated, except for the expression of CD25. Mean CD4 expression was slightly higher in proximal IEL, ranging from 11% to 23%, while distal IEL averaged 8% to 12% (Table 3). CD8 α expression

averaged 52% to 60% proximally, while distally it averaged 62% to 66%. CD8 β was present on 28% to 35% of epithelial lymphocytes. These sites had CD4:CD8 ratios that as expected were well below 1:1. Mean CD8 $\alpha\alpha$ T-cell expression was 17% to 35%, and may include $\gamma\delta$ T-cells, however this could not be verified due to unavailability of mAb for feline TCR $\alpha\beta$ or TCR $\gamma\delta$.

Using CD5PE, we were able to identify CD5+ bright versus dim expression on lymphocyte populations (Fig. 5c). T-cells were distinguished from B-cells using anti-B220 or feline B-cell mAb, clone 8F9. The majority of CD5+bright cells were either CD4+ or CD8 α +, whereas many B-cells were CD5+dim (Fig. 6). Some of the CD5+dim cells were not identified as CD4+, CD8 α +, or B-cells. Between 4-6% of CD5+bright cells were also B220 positive. Using mAb clone 8F9, B-cells were identified in very small numbers in the proximal and distal IEL ranging from 3-8%, B220 indicated a greater number of B-cells in these locations, ranging from 8-14%, however, the difference between these mAb may indicate that B220 is identifying a subset of activated T-cells. However, it should be noted that although B-cells have been reported to be rare in the epithelium, evaluation of cytopins confirmed the presence of small numbers of plasma cells, which are terminally differentiated B-cells. Monocytes were present, and in agreement with cytopsin results, they tended to be 2% or less of the leukocytes isolated from this region (Table 3).

MHCII expression was highly variable, but was found on T-cells as is observed in PBMC of cats (Rideout et al., 1990). In proximal and distal IEL, MHCII expression averaged 18% to 43%, and was markedly lower than LPL or PP/LF regions (Tables 3-5). MHCII was predominantly expressed on B-cells and CD5+bright T-cells (data not shown). Levels of L-selectin detected were generally low, averaging 4-11%. B7.1 (CD80) expression on IEL was

surprisingly high, and was typically identified on a majority of CD8 α + T-cells (Table 3, and Fig. 6).

Interestingly, the only statistically significant difference between SPF and random source cats in IEL was the expression of CD25, the IL-2R α chain. Random source cats had statistically significant increases in CD25 expression compared to SPF cats in both the proximal and distal IEL (Fig. 7 and data not shown). CD4+CD25+ expression in IEL was negligible (Fig. 6).

3.6. LPL Phenotype

The only statistical differences identified between SPF and random source cats in lamina propria were in expression of B7.1 and CD25 (Table 4). Overall, proximal and distal LPL both contained CD4+ T-cells in numbers that resembled those found in feline PBMC or LN, with CD4:CD8 ratios that averaged 1.26:1 to 1.96:1. As expected, CD8 α , CD8 β , and CD8 $\alpha\alpha$ expression were significantly lower than that observed in the proximal and distal IEL. CD8 α expression averaged 27-37% in proximal and distal LPL. The proportion of CD8+ T-cells that expressed CD8 $\alpha\alpha$ or CD8 β was similar to that found in the IEL, however, absolute numbers of these subsets were decreased due to the smaller number of total CD8+ T-cells present in the LPL.

In both proximal and distal LPL, the percentage of cells expressing CD5+bright averaged 74%-78%, and CD5+dim averaged from 14-20% (Table 4, Fig. 6). B-cells in the lamina propria tended to express CD5+dim, if CD5 was expressed at all (Fig. 6). Monocytes were few, averaging less than 4% in each compartment evaluated and were consistent with

cytospin results (Table 2). MHCII expression trended higher than observed in the IEL regions, however, it was not significantly greater (Table 4). L-selectin expression was low, ranging from 2-12% (Table 4).

Although there was no difference in the expression of B7.1 (CD80) in the distal LPL, the proximal LPL revealed a statistically significant difference ($p=0.036$) between SPF and random source cats, with random source cats demonstrating higher levels of expression (59.2% mean vs. 41.7% mean). In both SPF and random source cats there was a lower proportion of B7.1 expressed on CD8 α + T-cells as compared to the IEL. B7.1 expression was found on approximately 50% of the CD4+ and CD8 α + T-cells. The phenotype of the remaining B7.1 expressing leukocytes was not determined (Fig. 6 and data not shown).

Again, the major statistically significant difference between SPF and random source cats in the LPL was the expression of CD25. Random source cats had statistically significant increases in CD25 expression compared to SPF cats in both the proximal and distal LPL (Fig. 7). CD4+CD25+ expression represented the majority of CD25+ cells in both LPL locations (Fig. 6).

3.7. PP/LF phenotype

As PP/LF are inductive sites, their phenotype varied from that observed in IEL and LPL. While the PP/LF epithelium averaged 35%-43% CD8 α + T-cells, and 17-19% CD4+ T-cells, it contained numerous B-cells, averaging 27-43% (Table 5). CD5+ bright expression was decreased, averaging 46-59%, likely due to decreased numbers of T-cells. MHCII expression was highly variable, averaging 28-58% in the PP/LF epithelium.

PP/LF had a phenotype resembling that from a lymph node, in that the CD4:CD8 ratio averaged 1.16 – 1.9, and 38-43% of cells isolated were B-cells (Table 5). PP/LF also exhibited much lower levels of CD5+bright staining. MHCII expression was greater than any other intestinal leukocyte population, averaging 52-67%.

4. Discussion

The primary objective of this study was to identify and/or develop a method of isolating IEL and LPL that would provide consistently high yields with excellent purity for phenotypic characterization and functional assays. Numerous techniques have been described in multiple species for the isolation of leukocytes from the epithelium and lamina propria of the small intestine (Mosley and Klein, 1992; Veazey et al., 1997; Gonsky et al., 2000; Van Damme et al., 2001; Mennechet et al., 2002). Initially, we utilized previously described techniques (Kearsey and Stadnyk, 1996; Roccabianca et al., 2000) but were unable to obtain large quantities of highly purified leukocytes using those techniques as described. Therefore, we modified a previously described technique for IEL isolation (Burkhard et al., 1997), and developed a new technique for LPL isolation based in part on a technique previously described for processing human intestinal samples (Melgar et al., 2002).

The methods described here yielded 2.05×10^7 to 6.84×10^7 IEL from a 30 cm section of intestine. Additionally, IEL were available for assays within approximately three hours of sampling, and had excellent viability. Similarly, LPL yields ranged from 3.10×10^7 to 5.95×10^7 from only two 90-minute enzymatic digestions. Thus, significant numbers of LPL were available within eight hours of sampling for use in assays. Certainly additional leukocytes

can be obtained by processing a larger segment of intestine or through utilizing/processing all fractions. Several modifications in previously published methods for IEL and LPL purification were made to enhance viability. For IEL, short agitations (30-minute) and lower concentrations of EDTA resulted in excellent IEL viability. With regard to LPL, we found shorter digestions (90-minutes), with the addition of fresh Dispase following each digestion enhanced further digestion and leukocytes already liberated did not undergo further damage to their receptors. Additionally, the use of Dispase, a type IV collagenase and fibronectinase, considered to be a very mild proteolytic enzyme (Stenn et al., 1989) also helped maintain yield and viability. Better success with the techniques was observed when the strips of small intestine did not exceed 0.5 cm in width, and it was critical to make certain that tissue incubations never exceeded 37°C.

As purity of the isolated cell populations was equally as important as overall yield, anti-CD45 was used to identify leukocytes from every sample isolated (Fig. 2). Use of anti-CD45 has been shown to be a reliable measure of purity in other studies focusing on intestinal leukocyte isolation (Solano-Aguilar et al., 2000). An arbitrary cutoff of at least 95% CD45+ leukocytes in the gated region was used to determine which samples would be included in the phenotypic characterization. IEL were significantly easier to isolate than LPL, with only one random source cat sample from the proximal IEL not meeting the 95% criteria. Overall, epithelial leukocyte purity averaged over 99%. Isolating highly purified LPL was more challenging, and consequently purity of gated cells averaged 98-99%. Of the thirteen cats processed, four random source cats did not meet the minimum 95% purity in proximal LPL, whereas only three random source cats did not meet this criterion in the distal LPL or PP/LF. Cytospin preparations of isolated populations also demonstrated significant purity of isolated

samples with greater heterogeneity of leukocytes observed in cells isolated from the lamina propria as compared to the epithelium. These data are consistent with a previously published report of the immunohistologic homogeneity of IEL and heterogeneity of LPL in the cat (Waly et al., 2001). Microscopic evaluation of cytopsin preparations is useful but given that epithelial cells can be very difficult to distinguish from leukocytes cytologically, CD45 analysis by flow cytometry is the gold standard to determine purity (Ebert and Roberts, 1995). Ebert and Roberts demonstrated that intestinal epithelial cells may stain positively or demonstrate in vitro reactivity that mimics certain types of leukocytes. The defining feature that indicated they were epithelial was the lack of CD45 expression.

Some mAb commonly used for immunophenotypic analysis of feline PBMC and LN did not label IEL and LPL. To minimize negative effects of processing each step was optimized, and the IEL processing method was tested on MLN cells to confirm that the processing technique had not affected our results. Additionally, it was found that use of FBS, even at v/v 5% or less, caused receptors to be masked, so all washing for flow cytometric analysis was done with cold PBS containing EDTA. Despite the modifications made, patterns of sub-optimal reactivity with a number of mAb were still evident. After testing several mAb clones for each antigen, a useful panel of mAb was identified for phenotypic analysis of IEL and LPL. Possible causes of the poor staining observed with some of the mAb/conjugates used include: steric hindrance caused by use of large fluorochromes, damage to epitopes during processing, alternate isoform expression within mucosal sites, decreased density of surface receptors in mucosal sites, or potential lack of receptor expression in the cells being tested.

We identified distinct CD5⁺ bright and dim leukocytes within the epithelial and lamina propria compartments. The range of CD5 expression in both IEL and LPL are in contrast to

reports in human and murine studies that show uniform CD5 expression, restricted to T-cells, with lower fluorescence intensities (Fig 5b, panel a) (Trejdosiewicz et al., 1989; Lefrancois, 1991). Possible explanations for this dichotomy may include a species-specific variation in felines or that this pattern exists in humans and mice but has not been identified. CD5 low/intermediate/high has been reported in studies assessing development in the thymus, and following chronic antigenic stimulation in PBMC and LN (Azzam et al., 1998; Azzam et al., 2001; Stamou et al., 2003). No reports were identified that show the range of CD5 expression in an individual sample as identified in this study. It is known that CD5 can be up- or downregulated in response to TCR/BCR signaling (Brossard et al., 2003), and that CD5 can have an inhibitory or activating effect on cell signaling in different circumstances (Lozano et al., 2000). Given the information currently available on CD5 expression and signaling, it is possible that the high level of CD5 expression demonstrated for many T-cells in the feline small intestine could indicate a regulatory role for many of the lymphocytes, with CD5 directing activation or lack thereof.

The second major goal of this study was to determine the phenotype of leukocytes in the different compartments of the small intestine. Ascertaining the normal phenotype of different regions of the feline small intestine is integral to further studies using FIV as an animal model for HIV, and the study of inflammatory conditions and gastrointestinal lymphosarcoma which are commonly recognized intestinal diseases in cats (Roth et al., 1990; Hart et al., 1994; Willard, 1999).

This study shows similarities with reported phenotypic populations in the IEL and LPL of humans, mice and macaques. In the epithelium, we report a low proportion of CD4+ T-cells, with a preponderance of T-cells expressing CD8 α in both the proximal and distal intestine.

CD8 β was identified on approximately half of total CD8⁺ T-cells, indicating the other half express CD8 $\alpha\alpha$ homodimers. These observations are consistent with results from studies in humans and mice (Lefrancois, 1991; Lundqvist et al., 1995). What is unclear is the proportion of T-cells expressing TCR $\alpha\beta$ or TCR $\gamma\delta$, which have been shown to vary significantly by species (Trejdosiewicz et al., 1989; Lefrancois, 1991; Veazey et al., 1997). This question cannot be addressed at this time as no antibodies have been found that either recognize or cross-react to these receptors in cats.

Similar to a study in macaques, very low percentages of B-cells were identified in the IEL population (Veazey et al., 1997). Several reports using immunohistochemistry (IHC) to identify B-cells suggest they do not exist in the epithelial layer. However, as previously described, IHC may have been unable to identify low numbers of this cell type. This finding is supported by the appearance of an intact basement membrane, leading to the possibility that either a small number of B-cells are present in the IEL of cats, or microscopic lymphoid follicles may be present as has been reported in other species (Moghaddami et al., 1998; Hamada et al., 2002). In addition, we did identify plasma cells on cytopsin, and although they would not be identified with standard B-cell markers, their presence suggests that some B-lymphocytes may also be normal in feline IEL.

Expression of activation markers such as B7.1 (CD80) and CD25 were not evaluated in many studies that originally assessed phenotype in humans and mice as they were either not identified or their importance was not recognized when these studies were completed. B7.1 is expressed by APC and can be upregulated on activated T-cells in the periphery. We found that expression on IEL and LPL was much higher than in the periphery, ranging from 40-70%, indicating that a significant proportion of the T-cells present were activated. CD25

expression yielded the only consistently significant difference between SPF and random source cats phenotype in both IEL and LPL. Studies in humans and macaques show that CD25 expression can approach 25% of LPL (Zeitz et al., 1988; Makita et al., 2004) which agrees with data from this study showing that random source cats average 22-24% CD25+ T-cells. However, random source cats were significantly different from SPF cats in expression of this receptor. SPF cats had very low expression of CD25, leading one to suspect increased antigenic diversity of the diet as well as increased exposure to intestinal bacteria and parasites may play a role. The CD4+CD25+ T-cell phenotype has been associated with regulatory T-cells (Mittrucker and Kaufmann, 2004; Nelson, 2004). Regulatory T-cells are believed to be involved in the maintenance of tolerance to self-antigen as well as commensal organisms found in the gastrointestinal tract (Toms and Powrie, 2001). T-regulatory cells typically express IL-10 and TGF- β proteins and the transcription factor Foxp3 (Jonuleit and Schmitt, 2003). While the CD4+CD25+ T-cells identified in this study were not tested for these features, given data from other species (Maloy et al., 2003; Makita et al., 2004), it is reasonable to suspect that they may be involved in maintenance of tolerance in the feline small intestine.

The LPL compartment was similar to macaques in that the CD4:CD8 ratio was typically less than 2:1. The number of CD4+ T-cells was slightly lower, and CD8+ T-cells were slightly higher than observed in humans, which accounts for the slightly lower CD4:CD8 ratio observed in feline LPL. B-cells were found in low numbers in the lamina propria, averaging 3-13% in the proximal and distal intestine. CD5+B220+ cells were identified in small numbers in both the IEL and LPL. Possible identities for these lymphocytes include B1 B-cells as identified in the mouse, or activated T-cells as B220 (CD45R) has been shown to

be up-regulated on a small subset of activated T-cells. However, further analysis was not completed within this study to identify them conclusively. Plasma cells were identified on cytopins from lamina propria isolations, however we were unable to conclusively identify them using flow cytometry because feline antibodies are not currently available for the limited group of cell-surface receptors used to identify them. B-cells and plasma cells are typically found in the lamina propria of humans and mice, therefore we believe further phenotypic characterization is warranted if new antibodies become available.

The Peyer's patches of cats were similar to data reported from other species, in that a large number of B-cells were identified (average of 38-43%), with T-cells responsible for a large proportion of the other leukocytes present. The CD4:CD8 ratio was consistent with lamina propria isolations. As expected, a heterogeneous population of leukocytes was present, indicated by monocytes, plasma cells and occasional lymphoblasts on cytopin preparations. An interesting feature of cats is the presence of large, grossly visible lymphoid follicles, which have been reported in other domestic animal species (Nickel et al., 1979). Given the phenotype identified for PP/LF regions, Peyer's patches and any isolated lymphoid follicles should be removed when isolating effector cells from the epithelium and lamina propria, as IEL and LPL phenotype would be altered. This study combined Peyer's patches and lymphoid follicles to ensure adequate yield for phenotypic analysis. Therefore it should be noted that the phenotype reported here may be different from that found if primary and secondary lymphoid tissue from different regions of the small intestine were analyzed separately.

In conclusion, we demonstrate in this study that leukocytes of high purity and yield can be isolated from the epithelium and lamina propria to conduct studies of the feline small

intestine. We also show that the phenotype identified is consistent with that found for these compartments in humans, indicating that it can be used as an animal model for various diseases including HIV. Further work in phenotyping the feline small intestine is warranted once additional antibodies become available to distinguish important subsets such as $\gamma\delta$ T-cells.

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Figure Legends:

Figure 1. Isolation of intraepithelial and lamina propria leukocytes from the small intestine. Shown from left to right, normal feline small intestine prior to processing (A), after removal of the epithelium; arrows point to areas where denuded basement membrane of the villi can be visualized (B), and following two digestions with Dispase II, little lamina propria remains undigested (C). Results are representative of multiple sections; original magnification was 100x.

Figure 2. Purity of leukocytes isolated assessed by FACS analysis. Panels on the left show typical forward and side scatter patterns for cells isolated from the indicated locations. The middle panels show the percent of CD45 positive cells in the gates outlined on the left. The right panels show differences in size and granularity of the CD45 positive population using CD45 and side scatter, with an arrow indicating increased size and granularity of leukocytes from the lamina propria as compared to those from the epithelium. Data shown is representative of epithelial and lamina propria isolations in this study. All flow cytometric data presented in this study was gated as shown in this figure.

Figure 3. Cytospin preparations from epithelial and lamina propria isolations. Typical lymphocytes isolated from intestinal epithelium (A). Lamina propria isolation showing small lymphocytes, plasma cells, a monocyte and an eosinophil (B). Photomicrographs are representative of cytopins evaluated for each animal. Original magnification was 400x.

Figure 4. Leukocyte yield by anatomic location and cat source. Leukocytes from IEL or PP epithelium are shown in (A), and LPL or PP in (B). Cellular yields were determined immediately following isolation. Mean is indicated by the symbol with standard deviation shown as error bars. No statistical differences were identified between SPF and random source cats within each anatomic location.

Figure 5a. Poor separation and lack of staining by different monoclonal antibodies. In panel A, the left panel shows clear distinction in MLN using mAb clone 30A, the middle panel using LPL does not show clear separation from negative cells, and the right panel shows another CD4 clone (3-4F4) clearly differentiating CD4+ T-cells from the negative cell pool. Panel B demonstrates the inability of a commonly used monoclonal antibody to identify B-cells. Canine CD21, clone 1D6, is commonly used to identify feline B-cells and in the left panel shows staining in mesenteric lymph node (MLN). The middle panel shows a typical result when used to identify B-cells in feline Peyer's Patches (PP). The right panel shows B-cells identified in PP with anti-B220.

Figure 5b. Unexpected staining patterns observed during study optimization. CD5 PE shows a range of expression from bright to dim in feline intestinal leukocytes (A). CD8 β (clone 1.117) exhibits almost twice the number of positive cells when conjugated to APC versus PE (B). Each pair of samples is from the same cat and cell sample, freshly stained and analyzed immediately following staining.

Figure 6. Phenotypic analysis of IEL and LPL. Representative plots are shown for the monoclonal antibodies indicated. Percentages in the plots are from the data presented in each plot, and are similar to the mean values from Tables 3 and 4. Gating scheme used is as shown in figure 2, with CD45 positive cells gated for phenotypic evaluation.

Figure 7. Comparison of CD25 positive lymphocytes by location and cat source. Individual data points for distal IEL and distal LPL comparing cat source are shown. An unpaired t-test was performed with significance at $p < 0.05$.

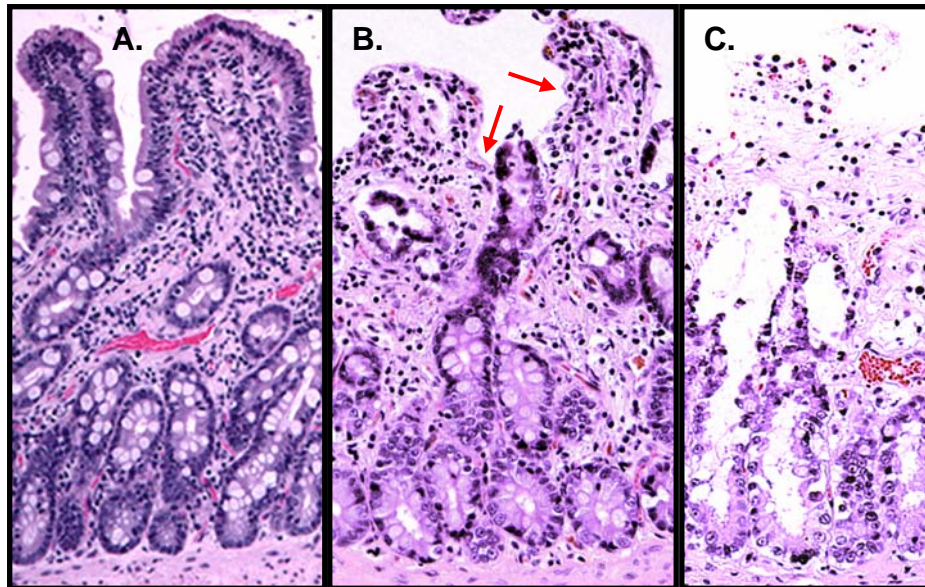


Figure 1. Isolation of intraepithelial and lamina propria leukocytes from the small intestine.

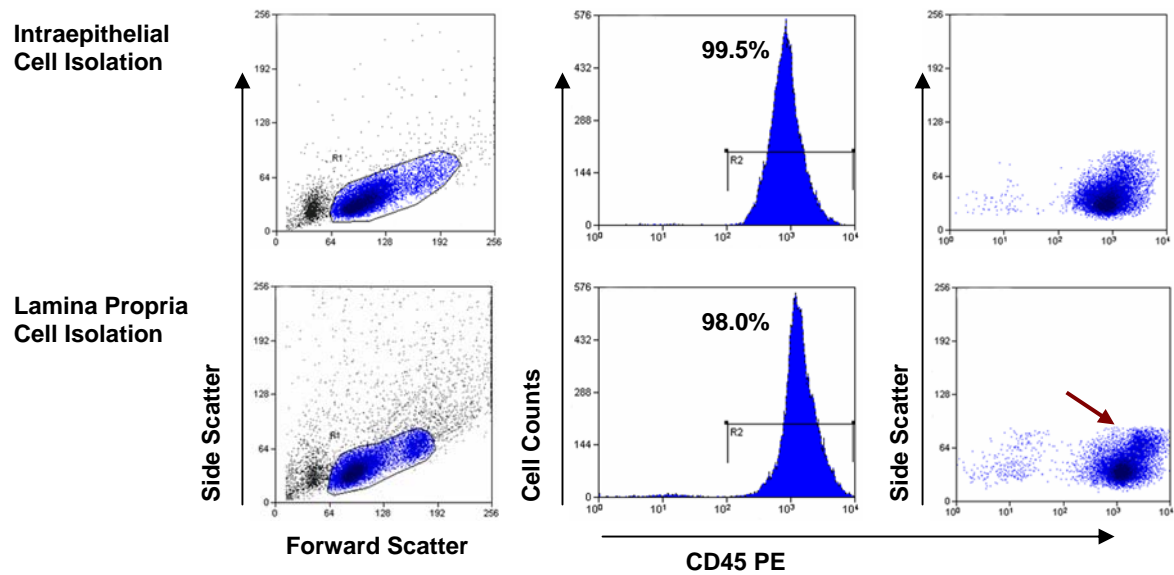


Figure 2. Purity of leukocytes isolated assessed by FACS analysis.

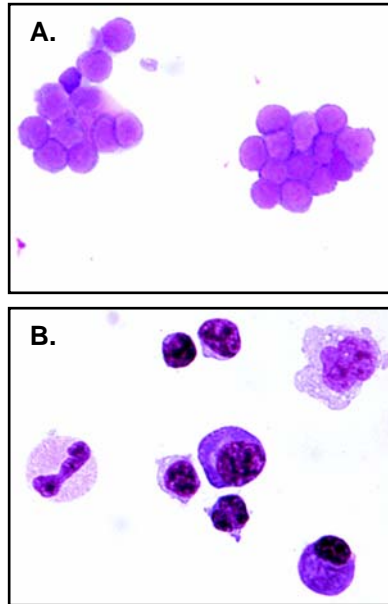
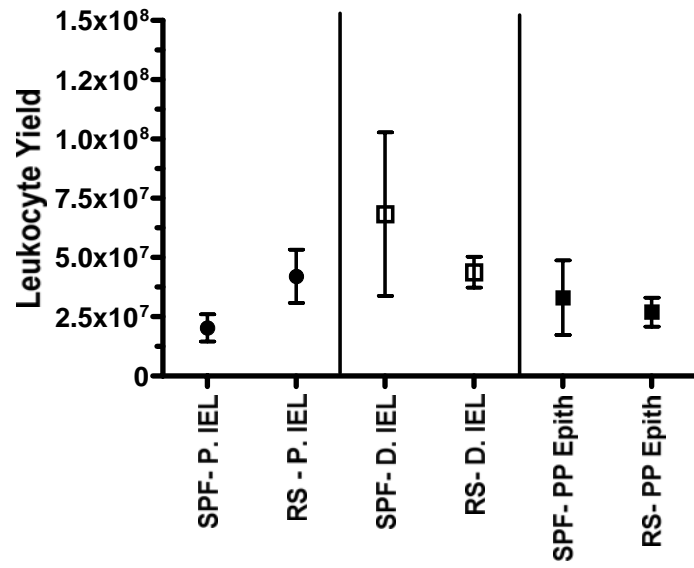


Figure 3. Cytospin preparations from epithelial and lamina propria isolations.

A.



B.

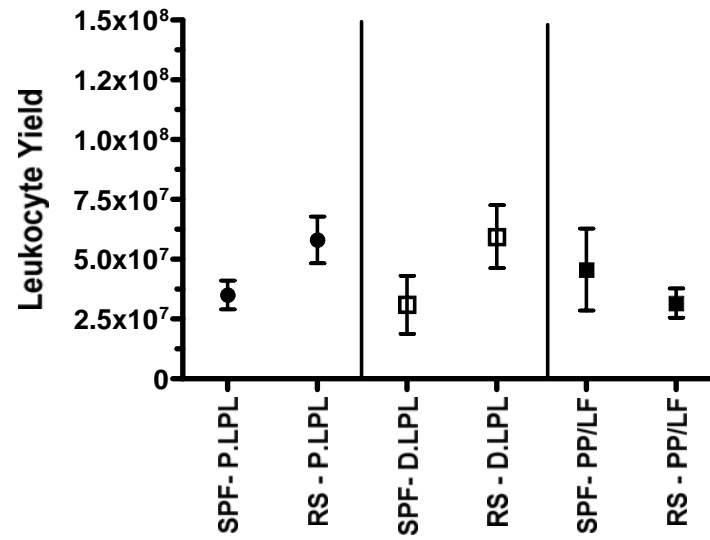


Figure 4. Leukocyte yield by anatomic location and cat source.

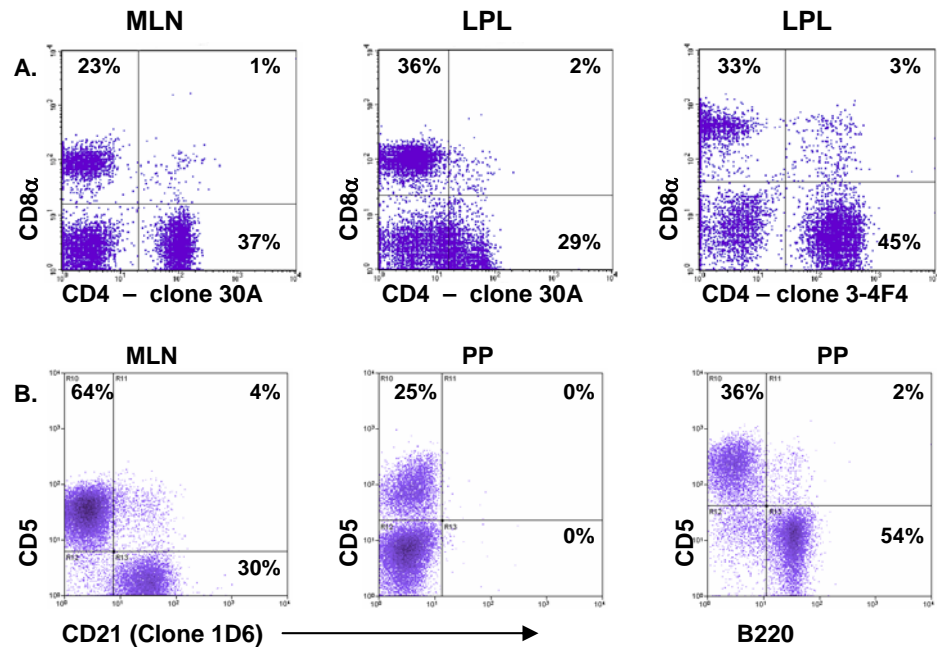


Figure 5a. Poor separation and lack of staining by different monoclonal antibodies.

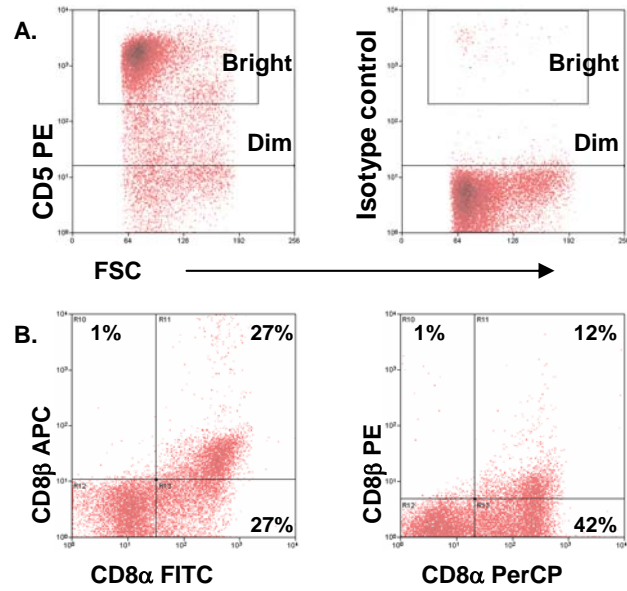


Figure 5b. Unexpected staining patterns observed during study optimization.

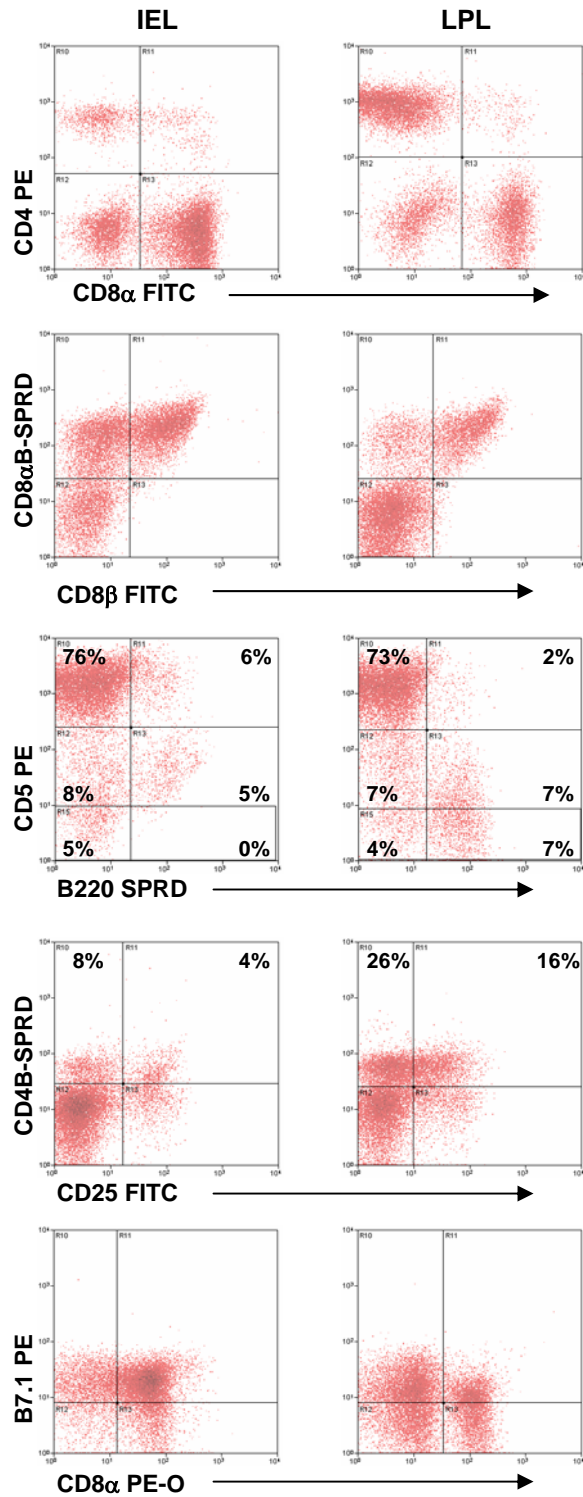


Figure 6. Phenotypic analysis of IEL and LPL.

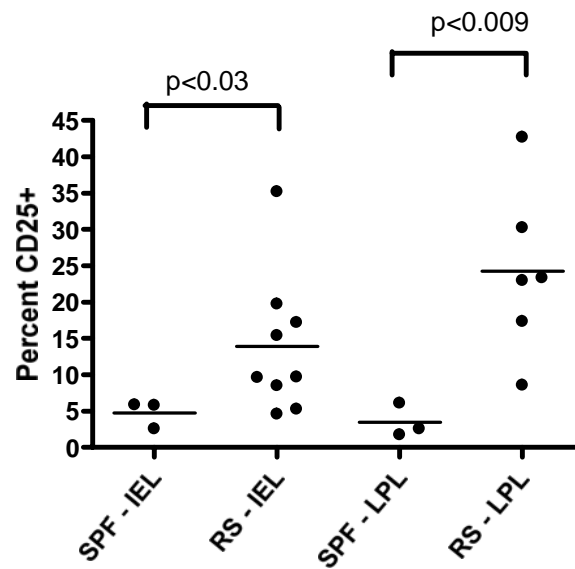


Figure 7. Comparison of CD25 positive lymphocytes by location and cat source.

Table 1
Monoclonal antibodies used in flow cytometric evaluation of feline intestinal leukocytes

Antigen	Conjugation	Clone	Specificity	Source
CD3 ϵ	unconjugated	F7.2.38	mouse anti-human	Dako
CD4	FITC, PE, biotin	3-4F4	mouse anti-cat	Southern Biotechnology
CD4	FITC	30A	mouse anti-cat	Clinical Immunology Laboratory, NCSU
CD5	PE, biotin	F43	mouse anti-cat	Southern Biotechnology
CD8 α	FITC, PE, biotin, PE-O	3.357	mouse anti-cat	Clinical Immunology Laboratory
CD8 β	FITC, PE, APC	1.117	mouse anti-cat	Clinical Immunology Laboratory
CD14	PE	TUK4	mouse anti-human	Dako
CD21	FITC, PE	CA2-1D6	mouse anti-canine	Serotec, Raleigh, NC
B-cells	FITC, PE-O	8F9	mouse anti-cat	Peter Moore, UC Davis
CD25	FITC	9F23	mouse anti-cat	Clinical Immunology Laboratory, NCSU
CD45	unconjugated	WC45a	mouse anti-cat	Serotec, Raleigh, NC
CD62L	PE	SK11	mouse anti-human	BD Biosciences
B220	FITC, SPRD	RA3-6B2	rat anti-mouse	Southern Biotechnology
B7.1	PE	B71.66	mouse anti-cat	Clinical Immunology Laboratory, NCSU
MHC II	FITC	42.3H2	mouse anti-cat	Peter Moore, UC Davis
Streptavidin	SPRD	NA	NA	Southern Biotechnology
Streptavidin	PerCP	NA	NA	BD Biosciences
Streptavidin	APC	NA	NA	BD Biosciences
F(ab) ₂ IgG	PE	catalog # 115-116-146	goat anti-mouse	Jackson Immunoresearch Laboratories
IgG (H&L)	FITC	catalog # 102-095-003	goat anti-cat	Jackson Immunoresearch Laboratories
F(ab) ₂ IgG	FITC, PE	catalog # 715-116-150	donkey anti-mouse	Jackson Immunoresearch Laboratories

Table 2
Cytospin analysis from each anatomic location evaluated (%)

	Lymphocytes	LGL ^a	Plasma Cells	Eosinophils	Monocytes	Lymphoblasts
Proximal epithelium	87	2	4	2	4	0
Distal epithelium	93	2	2	0	3	0
PP ^b /LF ^c epithelium	86	2	9	0	3	1
Proximal LP ^d	76	2	14	5	3	0
Distal LP ^d	83	2	7	4	4	0
PP ^b /LF ^c	81	1	11	3	2	2

Differential counts of cytopins are summarized for all cats in the study, regardless of source. Results shown are mean percentages. Fewer than one epithelial cell or neutrophil were counted on average from each tissue location, and are not included in the summary.

^alarge globular lymphocytes

^bPeyer's patches

^clymphoid follicles

^dlamina propria

Table 3
Phenotype comparison of epithelial leukocytes

Population	Proximal IEL ^a		Distal IEL ^a	
	SPF	RS	SPF	RS
CD45	99.5	99.4	99.6	99.5
CD4	11.5	22.8	8.4	12.1
CD8 α	60.7	51.7	65.6	62.0
CD8 β	30.3	35.1	28.0	35.0
CD8 α + β -	31.2	17.4	35.3	27.2
CD4+ CD8+	5.2	3.3	3.6	4.4
CD4/CD8 ratio	0.19	0.50	0.13	0.20
B cells (8F9)	2.6	7.5	5.5	7.6
B220 (CD45R)	8.3	13.7	14.1	10.3
CD5 Total	88.7	91.7	94.9	92.4
CD5 bright	63.2	69.9	71.0	70.4
CD5 dim	25.6	21.7	23.9	21.9
CD5 brt/B220+	4.3	5.5	5.8	3.9
MHCII	34.6	25.1	43.3	18.5
CD62L	4.1	10.9	10.5	4.9
B7.1	66.4	64.1	71.1	61.7
CD25	4.8*	13.9*	3.1*	9.5*

Significant differences in expression between SPF and random source (RS) cats are noted with (*), identified by an unpaired t-test, with $p < 0.05$. SPF cats, $n=3$.

RS cats, proximal IEL $n=9$; distal IEL, $n=10$.

^amean percent of gated population expressing indicated marker

Table 4
Phenotype comparison of lamina propria leukocytes

Population	Proximal LPL ^a		Distal LPL ^a	
	SPF	RS	SPF	RS
CD45	98.2	98.8	98.7	99.1
CD4	42.9	45.1	40.4	41.1
CD8 α	36.9	27.3	36.1	35.2
CD8 β	18.5	17.8	19.6	19.3
CD8 α + β -	19.3	10.1	18.2	13.7
CD4+ CD8+	4.2	1.5	3.7	2.8
CD4/CD8 ratio	1.40	1.96	1.26	1.28
B cells (8F9)	3.3	10.0	13.3	13.4
B220 (CD45R)	5.8	10.8	13.4	16.2
CD5 Total	92.1	90.6	91.5	94.0
CD5 bright	78.1	73.6	77.3	73.8
CD5 dim	14.0	17.1	14.2	20.2
CD5 brt/B220+	2.2	3.5	4.4	3.2
MHCII	48.2	38.4	60.6	43.1
CD62L	2.1	12.4	5.0	2.5
B7.1	41.7*	59.2*	53.5	54.3
CD25	3.5*	24.2*	5.0*	22.2*

Significant differences in expression between SPF and random source (RS) cats are noted with (*), identified by an unpaired t-test, with $p < 0.05$. SPF cats, $n=3$. RS cats, proximal LPL $n=6$; distal LPL, $n=7$.

^amean percent of gated population expressing indicated marker

Table 5.
Phenotype comparison of Peyer's patches/lymphoid follicles

Population	PP/LF Epithelium ^{a,b}		PP/LF ^{a,c}	
	SPF	RS	SPF	RS
CD45	99.5	99.4	99.0	99.0
CD4	19.2	17.1	26.1	30.9
CD8 α	34.6	43.4	21.7	18.9
CD8 β	ND	27.8	ND	12.4
CD8 α + β -	ND	19.6	ND	6.5
CD4+ CD8+	3.2	3.6	1.9	1.4
CD4/CD8 ratio	0.54	0.64	1.16	1.91
B cells (8F9)	38.7	26.9	38.6	43.3
B220 (CD45R)	43.0	26.9	38.8	41.4
CD5 Total	68.5	90.6	84.6	90.6
CD5 bright	46.3	59.4	53.0	49.5
CD5 dim	22.2	31.2	31.4	36.0
CD5 brt/B220+	4.0	5.0	1.6	2.8
MHCII	57.8	28.1	67.5	52.0

No significant differences in expression between SPF and random source (RS) cats were noted using an unpaired t-test, with $p < 0.05$. SPF cats, $n=3$.

RS cats, PP/LF epithelium $n=10$; PP/LF, $n=7$.

^amean percent of gated population expressing indicated marker

^bleukocytes isolated from the epithelium overlaying organized lymphoid tissue

^cleukocytes isolated from organized lymphoid tissue

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**Significant immune dysregulation occurs in IEL
and mucosal lymph nodes of FIV-infected cats
with alterations found as early as one day post-infection**

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ABSTRACT

Feline immunodeficiency virus (FIV) is the feline lentiviral homolog to human immunodeficiency virus (HIV) that induces similar pathology and disease progression in cats to that seen in humans infected with HIV. Studies of HIV infected humans and simian immunodeficiency virus (SIV) infected macaques have identified profound alterations in the gastrointestinal mucosa including significant depletion of CD4+ T-cells within weeks of infection. However, most studies have focused on lamina propria lymphocytes or the entire mucosa. In this study, we used the FIV animal model to focus on very early mucosal changes occurring one-day post-infection and compare them to alterations found in chronically infected cats. Furthermore, this study specifically examines intraepithelial lymphocytes (IEL), a unique effector population comprised primarily of CD8+ T-cells found in the epithelium of the mucosa, as well as the mesenteric (MLN) and medial iliac (ILN) lymph nodes.

Reduced IEL yield and concurrent phenotypic alteration was evident as early as one day following FIV infection. These changes not only persisted, but also grew in magnitude over the course of FIV infection. In contrast, while IEL were acutely depleted and MLN chronically depleted, cellularity of the ILN increased significantly. The expansion of lymphocyte numbers and delayed inversion of the CD4/CD8 ratio suggest that the ILN may be a site of abnormal lymphocyte homing. Thus, very early events following FIV infection predict or mirror the changes found in persistently infected cats, and suggest that the immediate immunologic response post-infection may be more relevant than previously perceived.

INTRODUCTION

The gastrointestinal immune system contains a significant proportion of the immune cells found in the body and is unique in its organization and functional components as compared to the peripheral immune system (1, 34). Inductive sites include Peyer's patches and lymphoid follicles within the intestine and the mesenteric lymph node (MLN), which drains the intestine. Leukocytes traffic into the intestine via high endothelial venules rather than afferent lymphatics as found in lymph nodes. Furthermore, in the intestine, antigen can be presented to naïve lymphocytes in situ. The major effector cells include lamina propria lymphocytes (LPL) and intraepithelial lymphocytes (IEL). Leukocytes of the lamina propria typically have a CD4:CD8 ratio and B-cell:T-cell ratio similar to that found in lymph nodes, however, plasma cells in the lamina propria primarily secrete IgA (39). IEL are unique in that they are substantially CD8+, and typically cytolytic without requiring restimulation to exert their function (30, 33). IEL are believed to be important in the maintenance of intestinal homeostasis, including the epithelium, and have immune surveillance functions with respect to infection and oncogenesis (IEL reviewed by Hayday et al (19)). Because of the significant changes in LPL found early in the course of HIV and SIV infection, most work to-date has been directed toward LPL with little focus on IEL.

The mucosal pathogenesis of SIV is well established. In SIV-infected macaques, a significant number of CD4+ LPL of an activated and/or memory phenotype are depleted within the first few weeks of infection (22, 24, 41, 46). Furthermore, these alterations precede evidence of peripheral immunologic perturbations (22, 24, 41, 46). Recent findings show that significant and long lasting changes are observed in the gastrointestinal tract of

human patients infected with HIV. They include the depletion of CD4+ LPL early in the course of HIV infection with a failure to rebound to pre-infection levels in the small intestine (2). Even with early or long term highly active anti-retroviral therapy (HAART) treatment, others have demonstrated that colonic CD4+ LPL do not rebound despite stabilization of CD4+ T-cell counts systemically (32).

Feline immunodeficiency virus (FIV) is an important natural animal model of infection (5). While few studies have evaluated pathogenesis of FIV in the gastrointestinal mucosa, we have previously shown that maintenance of CD4+ and CD8+ IEL populations in the small intestine correlates with vaccine protection, corroborating the immunologic importance of the gastrointestinal mucosa (43). Further examination of mucosal FIV pathogenesis in a preliminary study suggested phenotypic alterations were present in IEL populations as early as one-day following FIV infection.

In this study, phenotypic changes were assessed following acute (one-day post-infection) and persistent FIV infection in the mesenteric and medial iliac lymph nodes and IEL. We found very early phenotypic changes and lower IEL cellularity following FIV infection that mirrored changes found in persistently infected cats. In addition, we identified acute and chronic alterations in cellularity and phenotype of mucosal lymph nodes that drain the gastrointestinal and reproductive tracts. These results suggest that the very early immunologic events following FIV infection may be important in the outcome of lentiviral pathogenesis.

MATERIALS AND METHODS

Animals, viruses, and inoculums. Twenty-six specific pathogen free (SPF) cats were obtained from Liberty Labs (Liberty, NY). Cats were group housed and cared for in accordance with the standards of the American Association of Accreditation of Laboratory Animal Care and within all guidelines of the Institutional Animal Care and Use Committee. Of the total, five were controls, four were acutely FIV-infected, and seventeen were persistently FIV-infected. The challenge virus was NCSU₁, a FIV pathogenic sub-group A molecular clone. Acutely infected cats had been challenged by vaginal inoculation with 2.0×10^5 FIV-infected feline PBMC, and euthanized 24 hours post-inoculation. Persistently infected cats had been challenged intravenously with cell-free virus and terminated approximately 10-16 months post-infection. Chronically FIV-infected cats had an average PBMC provirus burden of 2.7×10^4 copies/ 10^6 PBMC, with an average CD4:CD8 ratio of 0.97:1. At time of euthanasia all cats ranged from seven months to three years of age. Prior studies in FIV showed no significant differences in long-term disease course based on route of inoculation after the animals are persistently infected (6, 7).

Sample Collection and Processing. Cranial mesenteric lymph node, medial iliac lymph node and distal jejunum were harvested at necropsy. Lymph nodes were disassociated using mesh screens, washed twice and counted. Briefly, IEL were isolated from a 25 cm section of distal jejunum. The intestine was flushed with wash medium (PBS supplemented with 20% FBS, 4mM L-glutamine, 2mM penicillin/streptomycin, 1% nystatin and 10 μ g/ml gentamicin) and cut into 0.5 cm strips following excision of Peyer's patches and lymphoid follicles. Cut intestinal sections were stirred vigorously for 30 minutes at 37°C in spin medium (Hanks

balanced salt solution without Ca^{2+} or Mg^{+} supplemented with 20% FBS, 4mM L-glutamine, 2mM penicillin/streptomycin, 1% nystatin, 10 $\mu\text{g/ml}$ gentamicin 2mM EDTA and 2mM dithiothreitol). The supernatant was collected then centrifuged at 1000 x g for 15 minutes at 16°C. The supernatant was removed, the pellet resuspended in 30% Percoll (Amersham Biosciences, Piscataway, NJ), then underlaid with 70% Percoll and centrifuged at 400xg for 30 minutes at 25°C. Following centrifugation, the 30/70 interface layer was collected, washed twice in wash medium and counted. All lymph node and IEL samples were processed by the same individual (K. Howard), to eliminate potential variability in yield due to processing technique.

Flow Cytometric Analysis. Monoclonal antibodies (mAb) used for cell surface staining were directly conjugated to either fluorescein isothiocyanate (FITC) or phycoerythrin (PE), except for anti-cat CD45 that was unconjugated. Serotec (Raleigh, NC) antibodies included anti-cat CD45 (clone WC45a) and anti-canine CD21 FITC (clone CA2-1D6). Antibodies obtained from Southern Biotechnology (Birmingham, AL) included anti-cat CD4 FITC and PE (clone 3-4F4) and anti-cat CD5 PE (clone F43). Anti-human CD62L PE (clone SK11) was purchased from BD Biosciences (San Jose, CA). The following mAb were purchased from the Clinical Immunology Laboratory (North Carolina State University, Raleigh, NC): anti-cat CD4 FITC (clone 30A), anti-cat CD8 α FITC and PE (clone 3.357), and anti-cat CD8 β FITC and PE (clone 1.117). Secondary antibody F(ab')₂ fragment goat anti-mouse IgG PE (#115-116-146, Jackson ImmunoResearch Laboratories; West Grove, PA) was used with anti-CD45. MLN, ILN, and IEL samples (1x10⁶ cells) were stained with the monoclonal antibodies described and analyzed with a FACScan flow cytometer (Becton Dickinson, San Jose, CA) using CellQuest software (Becton Dickinson, San Jose, CA).

Statistical Analysis. Statistical analysis was completed using a one-way analysis of variance (ANOVA) with a Tukey-Kramer multiple comparison post-test or nonparametric ANOVA (Kruskal-Wallis test) with Dunn's multiple comparison post-test. The choice of test was dictated by testing the assumptions necessary for parametric methods, such that if a parametric method was not appropriate, non-parametric testing was used. Post-test results that demonstrated significant differences between individual groups are indicated in the figure or table. All analyses were completed using GraphPad InStat version 3.05 (GraphPad Software, San Diego, CA).

RESULTS

Alterations in leukocyte yield. To determine whether quantifiable changes in lymph node and IEL yield occurred, mucosal lymph nodes and distal jejunum were processed and leukocyte yield assessed in control, acutely FIV-infected and chronically FIV-infected cats. Morphologic size of lymph nodes and cellularity of each site differed dramatically following FIV exposure. Significant differences in yield were identified by ANOVA for each site, with $p < 0.01$ for ILN and MLN and $p = 0.03$ for IEL (Figure 1).

IEL yields decreased following acute FIV-infection, and further decreased in chronically infected cats such that yields were reduced by over 58% from control cats ($p < 0.05$). In contrast, ILN yield was 48% higher in acutely infected cats, and increased further in chronically infected cats to 534% of control ($p < 0.01$). MLN cellularity was transiently higher following acute infection, but decreased in chronically FIV-infected cats to 46% of that seen in control cats.

Lymphocyte subsets in IEL. To determine whether the lowered IEL yield was due to perturbations in a specific subset of lymphocytes, we ascertained IEL phenotype for control, acutely and chronically FIV-infected cats. A significant reduction in yield of CD8 α T-cells was identified in both acutely and chronically FIV-infected cats when compared to controls ($p < 0.001$) (Figure 2a). In addition to the lowered percentage of CD8 α^+ T-cells, the mean fluorescence intensity (MFI) of this population decreased markedly in acute and chronic FIV infection (Figure 2b), indicating decreased density of the CD8 α receptor on the surface of IEL following infection.

CD8 β expression was also assessed. A similar reduction in the percent of cells expressing CD8 β was identified in control vs. acutely infected cats ($p < 0.05$), and in control vs. chronic cats, ($p < 0.01$) (Figure 3a). Alterations in MFI were transient during the acute stage of infection, as shown by a rebound to control levels in chronic FIV infection (Figure 3b).

Both CD5 bright and CD5 dim expression were dramatically altered on IEL following acute and chronic FIV-infection. CD5 bright expression was significantly lower in chronically infected cats ($p < 0.01$). In contrast, the expression of CD5 dim was higher in acute ($p < 0.05$) and chronic infection ($p < 0.001$) (Figure 4a). Notably, the staining pattern for this cell surface marker changed markedly over the course of FIV infection. A representative overlay shows control MFI=1694 for CD5, but following acute infection or chronic infection, MFI decreases dramatically to 327 and 270 respectively (Figure 4b). In addition, an atypical wide range of expression intensity for CD5 following FIV infection is observed, which was less pronounced in acutely infected cats, but typical for chronically FIV-infected cats in this study.

Table 1 presents IEL phenotype data by percent expression, and by yield for each cell surface receptor. Absolute yield by cell surface receptor was determined so that alterations in phenotype could be assessed based on the actual number of leukocytes collected.

Although no significant change in the percent of IEL expressing CD4 was identified, significantly lower yield of CD4 cells was identified in chronically infected cats ($p < 0.05$). Not only was the relative percentage of CD8 α , CD8 β and CD5+bright lower, but the absolute yield was also significantly different following FIV infection ($p < 0.05$). Differences in total CD5 expression were identified between control and chronically infected cats when evaluated by absolute yield ($p < 0.05$), whereas CD5 dim expression was only significantly different based on relative percentage. No significant changes in CD4:CD8 ratio or CD62L expression were found in IEL.

Lymphocyte subsets in MLN. As IEL are an effector site, the next question was whether the associated draining lymph node, an inductive site for the intestine, would exhibit changes that correlated with those observed in IEL. Therefore, we assessed the relative phenotype and yield by cell surface receptor in the mesenteric lymph node.

Significant changes were detected in the relative percentage of cells expressing CD4 and the absolute yield of CD4+ cells. Interestingly, the percentage and number of CD4+ T-cells was transiently higher following acute infection, but was lower in chronic FIV infection (Table 2). The percent of both CD8 α and CD8 β expressing T-cells in the MLN tended to be lower at one-day post infection (Figures 2a, 3a), but was higher in chronically infected cats with levels similar or greater than that observed in controls (Table 2, Figures 2a, 3a). The combined changes of CD4 and CD8 expression resulted in a significantly lower CD4:CD8 ratio ($p < 0.05$) in chronically FIV-infected cats. Other significant reductions in yield were

noted from acute to chronic FIV infection in CD62L ($p < 0.01$), CD5 ($p < 0.01$), and CD21 ($p < 0.01$) expressing cells.

Lymphocyte subsets in ILN. To determine whether the immunologic perturbations present in the IEL and MLN correlated with changes in a mucosal lymph node not associated with the intestine, we assessed the relative phenotype and yield by cell surface receptor in the medial iliac lymph node, which drains the reproductive tract of cats.

The CD4:CD8 ratio was not inverted in the ILN of chronically infected cats; however, a significant decrease was found when compared to control ($p < 0.05$) or acutely infected cats ($p < 0.001$) (Table 3). Similar to the MLN, the CD4:CD8 ratio was significantly higher in the ILN following acute infection ($p < 0.001$). Although CD8 α and CD8 β expression were both significantly higher in chronically infected cats ($p < 0.05$), the maintenance of the CD4:CD8 ratio appears to be a result of an influx of CD4 cells even though their relative percentage was lower ($p < 0.001$) (Figs. 2a, 3a). The absolute number of CD4 T-cells in ILN increased from 5.05×10^7 in control cats to 2.68×10^8 in chronically infected cats ($p < 0.05$) suggesting a redistribution of lymphocytes to this lymph node (Table 3).

Following acute infection, greater numbers of naïve lymphocytes were identified based on an increase in CD62L expression ($p < 0.05$). Although the percent of CD62L+ lymphocytes decreased to control levels in chronic cats, the overall number was significantly higher ($p < 0.05$), consistent with increased cellularity of the ILN. Despite a lack of change in the percentage of CD5+ and CD21+ lymphocytes, there were significant increases in absolute numbers in chronically infected cats as compared to controls ($p < 0.05$).

DISCUSSION

The findings of this study show that immune dysregulation is evident in IEL as early as one day following FIV infection. These changes not only persist but, in many cases, increase in magnitude over the course of FIV infection. Further, we show that while IEL are acutely depleted and MLN chronically depleted, the ILN is potentially a site of abnormal lymphocyte homing given the significant increase in cellularity and delayed inversion of CD4:CD8 ratio.

Lower IEL yields were characterized by significantly fewer CD8 α and CD8 β expressing cells, and by a dramatic decrease in MFI of the remaining CD8 α T-cells. Given that CD8 α and CD8 β expression was lowered by 24% and 20% respectively following acute infection versus control cats, it is tempting to assume that the IEL were CD8 $\alpha\beta$. However, other possibilities exist including down-regulation of CD8 β expression with greater reductions in CD8 $\alpha\alpha$ T-cells. Mattapallil et al have reported a significant depletion of CD8 $\alpha\alpha$ CD4⁺ T-cells as early as 2 weeks post-SIV infection in macaques (31). However, they did not observe this reduction at one week post-infection. It is possible that given different routes of inoculation (i.v. versus vaginal), and virus inoculum (cell-free versus cell-associated), the very early pathogenesis in the intestine is altered. We previously noted in a pilot study that IEL yields were lower at multiple early time points including one, two and four weeks following vaginal challenge with cell-associated FIV (K. Howard, unpublished results).

The early and sustained decrease in MFI of CD8 α on IEL indicates the density of this cell-surface receptor is decreased. Lowered MFI of CD8 α has been detected in several systems. Co-culture of PBMC with HIV-Rev or Nef significantly decreased MFI of CD8 α (23). In

SIV-infected macaques, gene expression of CD8 α , measured by microarray, is decreased at least two-fold at two and six weeks post-infection (14). In celiac disease, patients with active disease have decreased expression intensity of CD8 on their IEL as compared to treated patients, suggesting the intensity of expression correlates with disease control (9). Alterations in cell receptor expression in combination with the significant reduction in IEL yield could contribute to the intestinal dysfunction observed in AIDS. IEL perform vital functions in the maintenance of intestinal epithelium, surveillance for malignancies, and regulation of intestinal immune responses (15, 16, 19, 25). This study did not address the potential pathologic consequences associated with significant perturbations that were identified in IEL populations, however, numerous studies of SIV-infected macaques have identified early pathologic alterations in the gastrointestinal mucosa (20, 21, 24, 49). In addition, AIDS enteropathy has been well-described and includes malabsorption of nutrients, villous atrophy, epithelial hypoproliferation, opportunistic infections, and malignancies (40). Clinical signs such as diarrhea can occur without evidence of opportunistic infection, while weight loss and malnutrition usually occur later in disease (26, 45). It is possible that significant alterations in mucosal leukocyte populations, as were identified in IEL in this report, could begin the cascade of events leading to this clinical syndrome.

We have previously reported the expression of CD5^{bright} and CD5^{dim} lymphocytes in both IEL and LPL of the cat (Howard et al, submitted). This is in contrast to reports in humans and mice showing relatively uniform expression of CD5 in IEL with antibodies used to date, typically restricted to TCR $\alpha\beta$ CD8 $\alpha\beta$ IEL (27, 29, 44). However, it has been shown in mice that CD5 expression can be up-regulated on CD8 $\alpha\beta$ +CD5⁻ IEL when stimulated *in vitro* in an antigen specific manner and that housing conditions (SPF vs. conventional) affect

expression of CD5 on IEL (35). In this study, we found that the number of IEL expressing CD5 was markedly reduced and the MFI of CD5 bright expression was noticeably lower following FIV-infection. The decreased proportion of CD5 bright expressing cells was temporally correlated with significant expansion of a population of CD5 dim expressing IEL.

CD5 has positive and negative affects on the regulation of cell signaling (28). Chronic immune stimulation by low levels of antigen results in increased CD5 expression and anergic CD8 T-cell responses (42). Increased CD5 expression appears to negatively regulate signaling by interfering with the immunological synapse and cell:cell interactions (4, 18, 37). Given the significant decrease in expression observed in IEL following FIV-infection, it is possible that decreased CD5 expression could indicate a loss of regulatory function and result in hyper responsiveness in this important effector site.

It was anticipated that significant alterations would be observed in the mesenteric lymph node, given that it drains the intestine and is a key early site of viral replication (7, 36). Previous work has also shown changes in the MLN prior to alterations being detected in blood of FIV-infected cats (43). In this study, we found a significantly lower percentage of cells expressing CD4, and reductions in the CD4:CD8 ratio (less than 1:1) in chronically infected cats. Other significant changes were observed as a result of the significant decrease in MLN cellularity in chronically infected cats. Despite lower total lymph node cellularity, the percentage of CD8 α T-cells was higher. We hypothesize that this relative expansion of CD8+ T-cells likely indicates a CD8+ T-cell response to FIV-infection similar to the homing of HIV-specific cytotoxic CD8 T-cells to areas of active virus replication. (3). This hypothesis is supported by the identification of FIV in the MLN by 5-7 days post-infection (36) and disseminated active viral replication in the MLN by 22 weeks post-infection (13).

The ILN was also examined to compare intestinal results to another mucosal lymph node. It is often overlooked due to its location adjacent to the major blood vessels in the caudo-dorsal abdomen. This lymph node was chosen as acutely infected cats were inoculated vaginally, and previous results showed early viral dissemination to ILN following vaginal inoculation (36), suggesting the likelihood of observing early changes in phenotype at this site. We observed a substantial influx of CD62L⁺ lymphocytes into the ILN of acutely infected cats. The transiently higher CD62L expression at one-day post-infection in the ILN may indicate increased homing to this lymph node, which serves as the draining lymph node for this route of inoculation. It has been reported that SIV and HIV infection up-regulates integrin expression on lymphocytes, including CD62L, $\alpha E\beta 7$ and $\alpha 4\beta 7$ on lymphocytes (12, 17, 47). However, given that we found alterations one-day post exposure, well before significant viral replication can be detected, direct infection seems an unlikely cause of higher expression of CD62L in our study.

The most surprising result was the >500% higher yield of ILN, coupled with the maintenance of a CD4:CD8 ratio exceeding 1:1. A substantial number of CD4⁺ T-cells were found in this lymph node, even in chronically infected cats, as well as a full log increase in the number of CD8⁺ T-cells. Because of its location, this lymph node is typically only evaluated in terminal animal studies, thus little information is available for comparison. While this study was not designed to evaluate lymphocyte homing, the substantial increase in ILN cellularity supports theories of aberrant T-cell homing in HIV infection (10). A study which tracked autologous, indium-labeled T-cells in HIV-infected patients found abnormally rapid localization to lymph nodes and bone marrow as compared to that seen in control or hepatitis B infected individuals (11). A report from the SIV model also indicates the

likelihood of increased migration and sequestration rather than outright destruction of CD4+ T-cells (38). It has been suggested that the lymph node in which a lymphocyte is initially activated “programs” that lymphocyte to express integrins directing it to similar tissues (mucosal vs. peripheral) (8). Differences in cytokine milieu between mucosal and systemic lymph nodes have also been implicated in preferential lymphocyte proliferation at sites consistent with the original cytokine environment (48). However, the chronically FIV-infected cats in this study were infected intravenously, yet there was still clear sequestration of CD4+ T-cells in ILN, suggesting altered homing regardless of infection route. The significant increase in ILN cellularity and maintenance of CD4+ T-cells indicates that more focus should be directed to evaluation of mucosal lymph nodes in animal models that cannot readily be studied in human subjects. Altered homing to mucosal lymph nodes may in fact be important in the pathogenesis of HIV/FIV.

This study is unique because it evaluates acute (24-hour) infection versus control and persistently infected cats. Our data indicate that significant immunologic changes in the mucosa and draining lymph nodes take place well before significant viral replication occurs. To fully understand the pathogenesis of HIV infection, it is clear events occurring immediately post-exposure will need to be more closely examined.

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Figure Legends:

Figure 1. Total leukocyte yield shown by tissue for control, acutely FIV-infected and chronically FIV-infected cats. Acute infection=one-day post-infection, chronic infection=greater than one year post-infection. Data represent mean yield for (A) intraepithelial lymphocytes (IEL), (B) mesenteric lymph node (MLN) and (C) medial iliac lymph node (ILN) with error bars representing SD. ANOVA post-test results are shown for each tissue, with * indicating $p<0.05$, and ** $p<0.01$.

Figure 2. Changes in expression of CD8a following acute or chronic FIV infection. Relative expression of CD8a by tissue site (A). Representative histogram plots are shown for IEL, with changes in mean fluorescence intensity (MFI), gray filled = control, black line = acute, gray line = chronic (B). ANOVA post-test results for each tissue are shown where significant, with * indicating $p<0.01$, and ** $p<0.001$.

Figure 3. Changes in expression of CD8b following acute or chronic FIV infection. Mean expression of CD8b by location (A). Representative histogram plots are shown for IEL, with changes in mean fluorescence intensity (MFI) indicated; gray filled = control, black line = acute, gray line = chronic. ANOVA post-test results for each tissue are shown where significant, with * indicating $p<0.05$, and ** $p<0.01$.

Figure 4. Changes in expression of CD5 on IEL following acute or chronic FIV infection. Mean expression of CD5 by location (A). Representative histogram plots are shown, with changes in mean fluorescence intensity (MFI) indicated; gray filled = control, black line = acute, gray line = chronic. ANOVA post-test results for each CD5 subset are shown if significant, with * indicating $p<0.05$ and ** $p<0.01$.

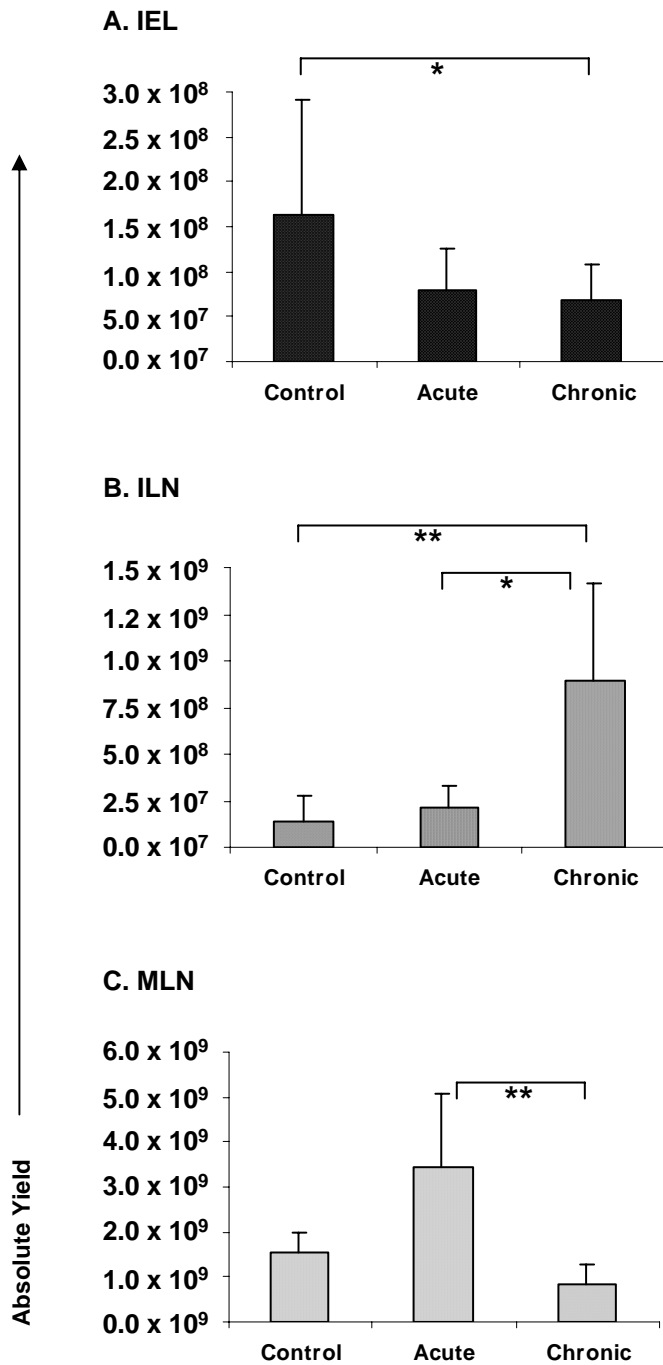


Figure 1. Total leukocyte yield shown by tissue for control, acutely FIV-infected and chronically FIV-infected cats.

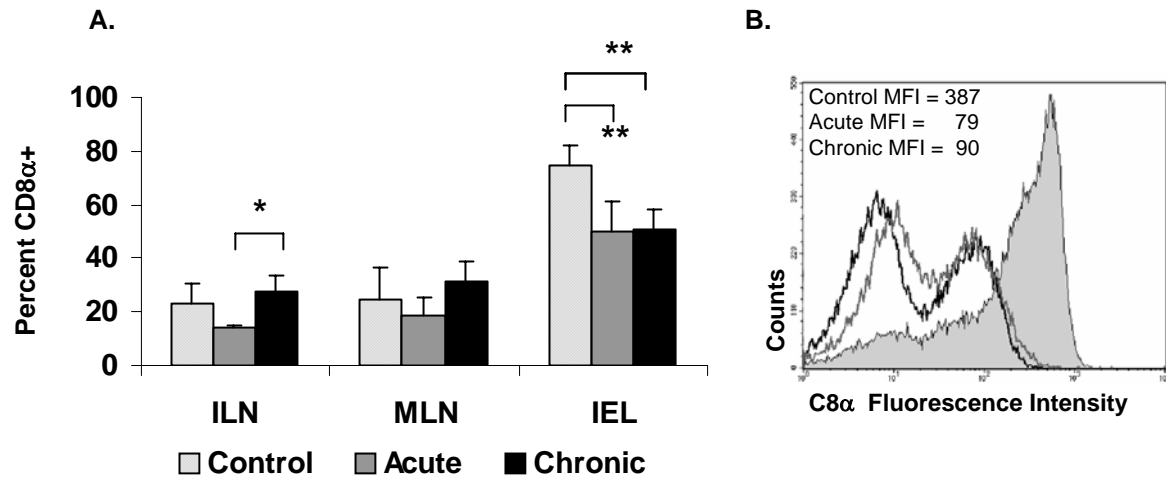


Figure 2. Changes in expression of CD8α following acute or chronic FIV infection.

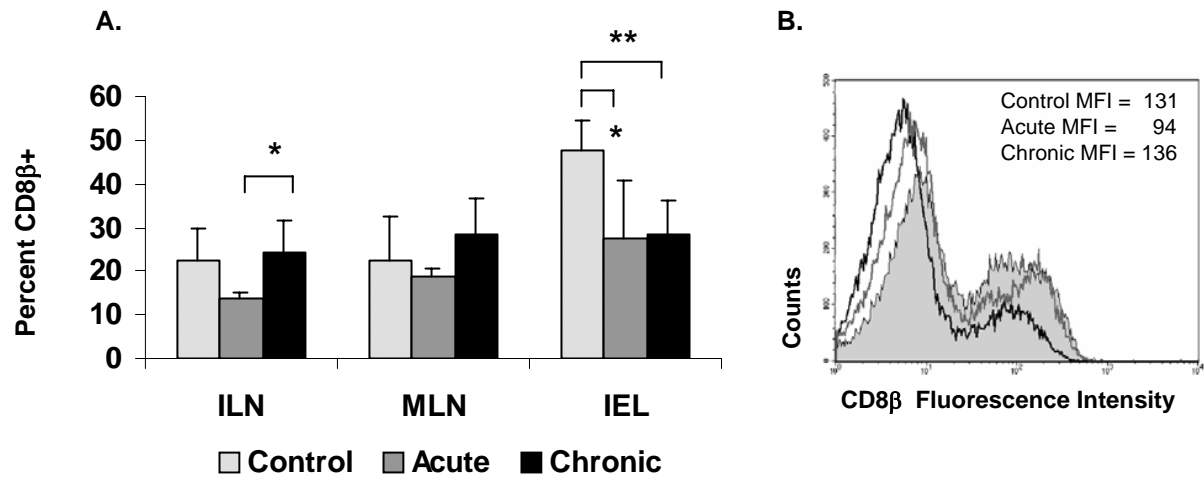


Figure 3. Changes in expression of CD8 β following acute or chronic FIV infection.

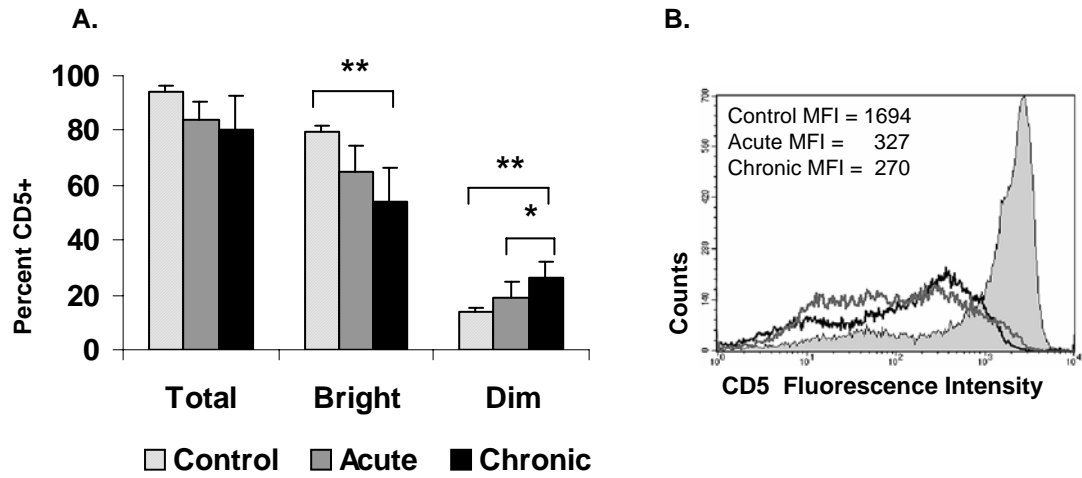


Figure 4. Changes in expression of CD5 on IEL following acute or chronic FIV infection.

Table 1. Phenotypic changes observed in IEL following acute and chronic FIV infection.

CD	Percent Expression (SD) ^a			Absolute Yield ^b		
	Control	Acute	Chronic	Control	Acute	Chronic
CD4	8.8 (3.7)	9.6 (3.0)	8.0 (3.4)	1.43x10 ⁷ †	7.71x10 ⁶	5.44x10 ⁶ †
CD8 α	74.4 (7.6)*†	50.2 (11.1)*	50.7 (7.8)†	1.21x10 ⁸ *†	4.03x10 ⁷ *	3.45x10 ⁷ †
CD8 β	47.7 (6.8)*†	27.6 (13.1)*	28.4 (7.7)†	7.78x10 ⁷ *†	2.22x10 ⁷ *	1.93x10 ⁷ †
CD4/CD8 ratio	0.12 (0.05)	0.19 (0.06)	0.16 (0.08)	NA ^c	NA	NA
CD62L	15.6 (15.0)	5.6 (2.8)	9.2 (7.2)	2.54x10 ⁷	4.50x10 ⁶	6.26x10 ⁶
CD5 total	93.9 (2.6)	83.8 (7.0)	80.4 (12.0)	1.53x10 ⁸ †	6.73x10 ⁷	5.47x10 ⁷ †
CD5 bright	79.8 (1.6)†	64.9 (9.9)	53.9 (12.6)†	1.30x10 ⁸ †	5.21x10 ⁷	3.67x10 ⁷ †
CD5 dim	14.1 (1.4)†	18.9 (6.2)§	26.5 (5.5)†§	2.30x10 ⁷	1.52x10 ⁷	1.80x10 ⁷

Statistically significant differences from ANOVA post-test results are noted as follows: acute vs. control (*), chronic vs. control (†), and acute vs. chronic (§).

^aData presented is the mean expression as determined by FACS analysis, with standard deviation shown in parenthesis.

^bAbsolute yield for each cell-surface marker was calculated based on the yield obtained for each individual cat, multiplied by percentage expression, then averaged.

^cNot applicable

Table 2. Phenotypic changes observed in mesenteric lymph node following acute and chronic FIV infection.

CD	Percent Expression (SD) ^a			Absolute Yield ^b		
	Control	Acute	Chronic	Control	Acute	Chronic
CD4	36.4 (2.1)	39.8 (10.7)§	28.2 (6.1)§	5.54x10 ⁸	1.37x10 ⁹ §	2.31x10 ⁸ §
CD8 α	24.8 (11.6)	18.5 (7.0)	31.6 (7.4)	3.78x10 ⁸	6.36x10 ⁸	2.59x10 ⁸
CD8 β	22.6 (9.9)	18.9 (1.8)	28.6 (8.3)	3.44x10 ⁸	6.50x10 ⁸ §	2.34x10 ⁸ §
CD4/CD8 ratio	2.01 (1.58)	2.41 (1.04)§	0.93 (0.26)§	NA ^c	NA	NA
CD62L	45.3 (24.4)	55.3 (7.9)	37.7 (8.9)	6.90x10 ⁸	1.90x10 ⁹ §	3.09x10 ⁸ §
CD5	60.2 (18.5)	54.9 (10.1)	55.3 (15.5)	9.17x10 ⁸ *	1.89x10 ⁹ *§	4.53x10 ⁸ §
CD21	38.7 (18.2)	49.0 (11.9)	43.4 (14.7)	5.89x10 ⁸ *	1.68x10 ⁹ *§	3.55x10 ⁸ §

Statistically significant differences from ANOVA post-test results are noted as follows: acute vs. control (*), chronic vs. control (†), and acute vs. chronic (§).

^aData presented is the mean expression as determined by FACS analysis, with standard deviation shown in parenthesis.

^bAbsolute yield for each cell-surface marker was calculated based on the yield obtained for each individual cat, multiplied by percentage expression, then averaged.

^cNot applicable

Table 3. Phenotypic changes observed in the medial iliac lymph node following acute and chronic FIV infection.

CD	Percent Expression (SD) ^a			Absolute Yield ^b		
	Control	Acute	Chronic	Control	Acute	Chronic
CD4	35.6 (6.0)	45.2 (8.7)§	29.8 (4.9)§	5.05x10 ⁷ †	9.51x10 ⁷	2.68x10 ⁸ †
CD8 α	23.4 (7.1)	14.1 (0.9)§	27.8 (5.5)§	3.32x10 ⁷ †	2.97x10 ⁷ §	2.50x10 ⁸ †§
CD8 β	22.3 (7.3)	13.9 (1.3)§	24.4 (7.0)§	3.16x10 ⁷ †	2.92x10 ⁷ §	2.19x10 ⁸ †§
CD4/CD8 ratio	1.67 (0.65)*†	2.94(0.53)*§	1.13(0.29)†§	NA ^c	NA	NA
CD62L	39.7 (19.0)*	62.1 (6.3)*§	36.1 (11.1)§	5.63x10 ⁷ †	1.31x10 ⁸	3.25x10 ⁸ †
CD5	61.8 (8.5)	60.0 (9.8)	57.6 (9.0)	8.76x10 ⁷ †	1.26x10 ⁸ §	5.18x10 ⁸ †§
CD21	36.2 (5.6)	48.0 (13.8)	44.3 (9.0)	5.13x10 ⁷ †	1.01x10 ⁸	3.98x10 ⁸ †

Statistically significant differences from ANOVA post-test results are noted as follows: acute vs. control (*), chronic vs. control (†), and acute vs. chronic (§).

^aData presented is the mean expression as determined by FACS analysis, with standard deviation shown in parenthesis.

^bAbsolute yield for each cell-surface marker was calculated based on the yield obtained for each individual cat, multiplied by percentage expression, then averaged.

^cNot applicable

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Phenotypic differences occur very early following vaginal FIV infection in peripheral and mucosal immune sites of cats and changes observed are dependent on whether the inoculum is cell-associated or cell-free virus

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ABSTRACT

The majority of HIV infections are a result of mucosal transmission, typically through contact with genital secretions. It has been established that both cell-associated and cell-free virus are present in cervicovaginal secretions and semen. However, the relative impact each form of virus has on actual transmission of HIV is unclear. The feline immunodeficiency virus (FIV) model is an excellent model to compare differences between cell-associated and cell-free transmission, as cats can be experimentally infected with both cell-associated and cell-free inoculum at equal efficiency without hormonal manipulation. This study compares differences between cell-associated and cell-free vaginal infection in systemic and mucosal sites at one day post-inoculation. It identifies distinctly different phenotypic changes between cell-associated and cell-free vaginally inoculated cats. Alterations were found in all tissue sites examined, and suggest that initial recognition of infection and antigen presentation differ between these inoculums, and that changes are not confined to the vaginal mucosa and draining lymph node.

INTRODUCTION

In 2004, approximately five million new human immunodeficiency virus (HIV) infections occurred, with the majority being transmitted through sexual contact (1). Numerous studies have established the presence of HIV-1 RNA and proviral DNA in the cell-associated and cell-free fractions of cervicovaginal secretions (CVS) and semen, however, there is relatively little information regarding the role of infected cells and cell-free virus in the sexual transmission of HIV (59, 60, 62, 63, 69). Clear correlations have been found between plasma viremia and cell-free viral load in genital secretions of HIV-infected individuals (19, 41). However, it is increasingly apparent that correlations between cell-associated virus in genital secretions and standard peripheral markers of infection such as plasma viremia are inconsistent (2, 15, 25, 26, 59). Other dichotomies exist such as identification of cellular HIV-1 DNA and/or RNA in CVS, with low or undetectable infectious cell-free HIV in the same sample (30, 51, 59). While highly active antiretroviral therapy (HAART) decreases plasma viremia and numbers of infected circulating peripheral blood mononuclear cells (PBMC), it does not appear to reduce HIV shedding in genital secretions to the same degree (14, 25). Several reports suggest that vertical or heterosexual transmission of HIV may be more closely linked to the presence of cell-associated rather than cell-free virus, underscoring the need to understand whether mucosal transmission or pathogenesis differs between cell-associated and cell-free virus (44, 64, 70).

The simian immunodeficiency virus (SIV) model has been used extensively to evaluate mucosal transmission and pathogenesis. However, macaques are difficult to infect vaginally

with cell-associated virus (18, 58), and although cell-free SIV is commonly used to infect macaques vaginally, infection by some isolates requires large doses of virus or hormonal manipulation to thin the vaginal mucosa (34-36, 45). Thus, while several routes of investigation suggest that differential pathogenesis may occur based on inoculum type, this has not been evaluated due to limitations of the SIV model.

Feline immunodeficiency virus (FIV) is an important animal model for HIV (7). It is not only a natural animal model, but numerous studies have shown effective experimental transmission of FIV parenterally, orally, vaginally, and rectally using either cell-associated or cell-free inoculums (4, 6, 8, 9, 16, 37, 42, 43). Cell-associated FIV has been shown to infect mucosal surfaces at equal or greater efficiency than cell-free FIV (4, 9), providing an excellent model to dissect differences in mucosal pathogenesis based on inoculum type.

Also understudied are the very early immunologic events post-infection. Immune changes have been characterized at one, two and four weeks following SIV infection (17, 29, 32, 33, 56), however, the initial week following infection, which is typically associated with innate and early adaptive immune responses, has not been reported. Assessment of early viral replication has shown that the inoculation route does impact the location and speed by which FIV (42) and SIV (22, 31) can be detected by in-situ hybridization techniques. However, the phenotype and function of immune cells can be altered by cytokine release, cell activation and lymphocyte trafficking. Thus, immune responses may not correlate with virus localization. Mucosal transmission of cell-associated and cell-free FIV have been compared with differences identified in tissue burden (9), however, very early immunologic differences were not addressed. Given findings in HIV and SIV, which suggest that lymphocytes of the

mucosal immune system are an early and persistent target for infection, it is critical to understand the very early alterations in the mucosal and systemic immune systems.

Here, we show significant alterations in the phenotype of mucosal and peripheral immune cells one-day after vaginal FIV inoculation. Furthermore, the type and localization of changes in cells of the immune system differed markedly depending on whether exposure was to cell-free virus or infected cells.

MATERIALS AND METHODS

Animals, viruses, and inoculums. Twenty-four specific pathogen free (SPF) sexually mature female cats were obtained from Liberty Labs (Liberty, NY). Cats were group housed and cared for in accordance with the standards of the American Association of Accreditation of Laboratory Animal Care and within all guidelines of the Institutional Animal Care and Use Committee. Of the total, six were vaginally inoculated with cell-associated FIV, seven were inoculated with cell-free FIV and control animals included four naïve, four animals inoculated with media alone and three inoculated with uninfected heterologous feline PBMC. The challenge virus was NCSU₁, a FIV pathogenic sub-group A molecular clone. Following sedation with Telazol, cats were vaginally inoculated with 2.0×10^5 FIV-infected heterologous feline PBMC or with cell-free virus. Cell-free virus was given at an equivalent dose to cell-associated inoculums based on the results of co-culture assay with a permissive CD4⁺ cell line. Naïve cats were not given any inoculum. Media and cell only control cats were sedated with Telzaol and vaginally inoculated with either RPMI or 2.0×10^5 uninfected heterologous

feline PBMC. Age at the time of euthanasia ranged from six to thirteen months of age. Results of early immune changes were assessed at twenty-four hours post-inoculation.

Sample Collection and Processing. Peripheral blood mononuclear cells (PBMC), peripheral (prescapular or popliteal) lymph node (PLN), mesenteric lymph node (MLN), medial iliac lymph node (ILN) and distal jejunum were harvested at necropsy. Briefly, blood was collected in tubes containing EDTA and PBMC were isolated using Histopaque density centrifugation (9). Lymph nodes were disassociated using mesh screens and the resulting pellets were washed and counted. Intraepithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL) were isolated from a 25 cm section of distal jejunum. To isolate IEL, the intestine was flushed with wash medium (PBS supplemented with 20% FBS, 4mM L-glutamine, 2mM penicillin/streptomycin, 2.5µg/ml amphotericin B and 10µg/ml gentamicin) and cut into 0.5cm strips following excision of Peyer's patches and lymphoid follicles. Cut intestinal sections were stirred vigorously for 30 minutes at 37°C in spin medium (Hanks balanced salt solution supplemented with 20% FBS, 4mM L-glutamine, 2mM penicillin/streptomycin, 2.5µg/ml amphotericin B and 10µg/ml gentamicin) with 2mM EDTA and 2mM dithiothreitol added. The supernatant was collected then centrifuged at 1000xg for 15 minutes at 16°C. The supernatant was removed, the pellet resuspended in 30% Percoll (Amersham Biosciences, Piscataway, NJ), then underlaid with 70% Percoll (Amersham Biosciences) and centrifuged at 400xg for 30 minutes at 25°C. Following centrifugation, the 30/70 interface cell layer was collected, washed twice in wash medium and counted. LPL were isolated by continuing processing of intestinal strips from IEL isolations. Following collection of the supernatant for IEL isolation, an additional spin of the strips for 30 minutes at 37°C in spin medium with 2mM EDTA added completely removing

any remaining IEL. The supernatant was discarded and the intestinal strips were digested twice, in succession, in spin medium with 20% Dispase II (Roche Applied Science, Indianapolis, IN) on an orbital shaker at (200 rpm) for 90 minutes at 37°C. Each supernatant was collected and centrifuged at 1000xg for 15 minutes at 16°C. After both digestions were completed, pellets were resuspended in 44% Percoll (Amersham Biosciences), then underlaid successively with 50% and 67% Percoll (Amersham Biosciences) layers. Gradients were centrifuged at 400xg for 30 minutes at 25°C. Following centrifugation, the 44/50 and 50/67 interfaces were collected and combined. They were washed twice in wash medium and counted.

Flow Cytometric Analysis. Monoclonal antibodies (mAb) used for cell surface staining were directly conjugated to either fluorescein isothiocyanate (FITC), phycoerythrin (PE), biotin, peridinin chlorophyll protein (PerCP), Spectral Red (R-PE-Cy5.5), PE-Oyster 665 (PE-O) or allophycocyanin (APC). Alternatively, primary antibodies were detected using directly labeled secondary antibodies. A summary of antibodies used in this study is presented in Table 1. MAb clone 3.357 was conjugated using PhycoLink[®] RPE-Tandem-665[™] Conjugation Kit (Prozyme, San Leandro, CA) to PE-Oyster 665 in our laboratory. Mouse anti-cat B-cell mAb, clone 8F9 was a generous gift from P. Moore (UC Davis, CA). Clone 8F9 was conjugated to FITC using standard protocols and to PE-O as described above in our laboratory. Secondary antibody F(ab')₂ fragment goat anti-mouse IgG PE (#115-116-146) was used with anti-CD45 and anti-CD1a.

Phenotype of freshly isolated cells was determined using three- and four-color flow cytometric analysis. Briefly, 1.0×10^6 freshly isolated cells were incubated with no more than two directly conjugated mAb at a time for 20 minutes at 4°C. Cells were washed with

cold PBS containing 0.5mM EDTA and centrifuged at 300 x g for 10 minutes at 16°C. This procedure was repeated two to three times based on the number of mAb used for each aliquot. Cells requiring staining with Streptavidin-PECy5 or Streptavidin-PerCP were completed individually following all other mAb required for the sample. Samples were analyzed immediately on a FACScan (BD Immunocytometry Systems) flow cytometer, or were fixed using 2% paraformaldehyde and analyzed on a FACS Calibur (BD Immunocytometry Systems) flow cytometer. Typically, 15,000 events were collected from the gated region. Analysis of flow cytometric data was completed using BD CellQuest software.

Statistical Analysis. Statistical analysis was completed using a one-way analysis of variance (ANOVA) with a Tukey-Kramer multiple comparison post-test. Post-test results that demonstrated significant differences between individual groups are indicated in the figure or table. All analysis was completed using GraphPad InStat version 3.05 (GraphPad Software, San Diego, CA).

RESULTS

Results from a prior study showed significant alterations in the medial iliac lymph node, mesenteric lymph node and IEL one day after vaginal inoculation with cell-associated FIV (Howard, K. Ph.D. Dissertation, Chapter 2). In order to understand the role the type of inoculum played in these changes, we compared phenotypic changes in mucosal versus peripheral sites in naïve, media, uninfected cell, cell-associated FIV or cell-free FIV inoculated cats at twenty-four hours post-inoculation.

Phenotypic changes in the draining lymph node. The ILN drains the reproductive tissues of the cat, and it was anticipated that if alterations were identified between inoculum types, it would be in this site. Significant changes were identified by ANOVA testing in CD4, CD8, B-lymphocyte, and CD1a expression (Table 2).

There was no difference in the percent of CD4⁺ T-cells in the ILN of naïve cats or those inoculated with media alone. In contrast, the percentage of ILN CD4⁺ T-cells was higher following inoculation with uninfected cells, cell-associated FIV ($p < 0.05$) and cell-free FIV when compared to control ($p < 0.01$). While not statistically significant, there was a higher percent of CD4⁺CD8⁺ T-cells following inoculation with uninfected cells, cell-associated FIV and cell-free FIV. The CD4⁺CD8⁺ cells appeared to be CD8⁺ T-cells that had upregulated expression of CD4, based on dim expression of CD4 and bright expression of CD8 (data not shown). Differences were detected in the mean expression of CD8 by ANOVA, and cell-free FIV inoculated cats trended toward a higher percentage of CD8⁺ T-cells; however, post-test analysis was not significant. The proportion of B-cells was significantly lower in cell-free inoculated cats ($p < 0.01$). CD1a expression was higher in the ILN of cats inoculated with either cell-free or cell-associated FIV. However, while CD1a expression was increased as might be observed with an influx of dendritic cells in cell-free inoculated cats, expression in cell-associated inoculated cats was marked and more suggestive of upregulated expression on non-dendritic cell types. Several other cell-surface markers, including CD5 ($p < 0.066$), B7.1 ($p < 0.098$) and CD57 ($p < 0.106$), trended toward altered expression in cell-free versus control cats.

Alterations in phenotype in other mucosal sites. The next question addressed was whether mucosal sites not associated with the route of exposure would show evidence of phenotypic changes. Results from a prior study showed early changes in IEL as well as MLN, therefore, we assessed both sites as well as LPL.

Unlike ILN, CD4⁺ T-cells were not altered in the MLN following either type of inoculum (Table 3). In addition, no significant changes were detected in the percentage of MLN CD8⁺ T-cells. CD62L expression was markedly lower in cell-free inoculated cats ($p=0.054$). Similar to the ILN, CD1a expression was significantly higher following vaginal inoculation with cell-associated virus, but not with cell-free virus.

Consistent with our previous findings, the percentage of CD8 α ⁺ IEL was significantly lower as compared to controls following cell-associated, but not cell-free, vaginal FIV infection (Table 4). As seen in the MLN, expression of CD1a was significantly higher in IEL following cell-associated infection, with an increase of lesser magnitude observed following cell-free infection. Expression of B-cell markers was significantly higher following cell-free inoculation ($p<0.01$), concurrent with lower B-cell marker expression in the MLN. Expression of B7.1 was markedly lower, but with variability in both virus-infected groups ($p=0.075$).

Changes in CD4 expression on LPL differed in direction from controls based on the inoculum (Table 5). LPL CD4 expression was lower in cell-associated inoculated cats but higher in cell-free inoculated cats. Similarly, expression of CD25 on LPL was significantly lower in cats inoculated with cell-associated, but not cell-free FIV, ($p<0.05$), and was predominantly reduced on cells also expressing CD4 ($p=0.059$) (Table 5). Similar to ILN and MLN, CD1a expression on LPL was also higher following cell-associated inoculation. Other

variables such as CD5 bright, CD5 dim, and B7.1 all trended toward altered expression in either cell-associated or cell-free inoculated cats, but typically had p-values~0.10.

Phenotypic changes in blood and peripheral lymph nodes. Peripheral lymph nodes and PBMC were evaluated to determine whether mucosal inoculation with cell-free or cell-associated FIV would induce systemic changes and if they would correlate with findings in the mucosa and draining lymph nodes. In Table 6, phenotypic changes in PBMC are shown. Significant changes were identified in expression of CD8, B-cell, and CD14 markers. CD8 expression was lower in cell-associated inoculated cats, but higher in cell-free inoculated cats ($p<0.05$). A higher percentage of B-cells were seen in cell-associated inoculated cats with decreased numbers in cell-free inoculated cats when compared to controls ($p<0.001$). Finally, CD14 expression was significantly lower in cats infected with cell-free but not cell-associated virus ($p<0.05$). While CD1a expression was higher in each inoculum type compared to controls, it was not significant due to greater variability in each group.

Peripheral lymph nodes had few changes, but repeated patterns identified in most other tissues (Table 7). Consistent with changes seen in PBMC, numbers of CD8 T-cells were lower following cell-associated inoculation and higher following cell-free inoculation ($p<0.05$). Other trends were present in PLN, but significance ranged from $p=0.06$ for alterations in B-cell expression to $p=0.09$ for CD1a and CD62L expression.

DISCUSSION

This study evaluated six different sites for phenotypic changes following vaginal inoculation with cell-associated FIV or cell-free FIV, as compared to control cats exposed to uninfected cells, media alone, or unexposed. We show that cell-associated FIV and cell-free FIV induce distinctly different early phenotypic changes 24 hours post-inoculation, and that these changes are not confined to the lymph node draining the site of inoculation or the mucosa in general.

This is the first study to dissect acute differences resulting from cell-associated versus cell-free vaginal infection. While data support that the majority of HIV-1 infections occur via sexual transmission, it is not known whether infections result from cell-associated virus, cell-free virus or a combination of both. Studies of epidemiological cohorts (47), virus variant transmission (70), and vertical transmission (44, 64) suggest that cell-associated HIV may be more frequently responsible for transmission of infection than cell-free HIV. A number of authors have found that cell-associated HIV can persist in genital secretions despite low plasma viral loads or HAART therapy (2, 25, 26, 44, 64). Associations between plasma viremia and viral RNA in genital secretions are abundant (19, 41), however, direct correlations between cell-associated and cell-free virus are not always consistent (2, 12, 15). These studies underscore the need to determine whether differences exist in the early pathogenesis of cell-associated and cell-free virus, as these findings could influence the early responses that must be induced by a potential vaccine.

To-date, few studies have examined the differences between cell-associated and cell-free virus *in vivo*, although explant and *in vitro* experiments using epithelial cell lines of varying

origin have been reported (38, 39). One reason for the lack of *in vivo* studies addressing inoculum type in vaginal transmission is the poor infectivity of cell-associated virus via vaginal exposure in the SIV model (34, 36, 45, 58). In addition, although macaques can be infected vaginally by cell-free SIV and SHIV, not all isolates have been shown to be infective by this route and others require high doses or hormonal manipulation of the epithelium to be infective (34, 36, 45, 58). In contrast, cats can be experimentally infected by either cell-associated or cell-free virus through vaginal, rectal, oral or parenteral transmission (4, 6, 8, 9, 16, 37, 42, 43). Furthermore, transmission of cell-associated or cell-free virus isolates of several different clades has been demonstrated without hormonal manipulation (4, 6, 8, 9).

The most striking finding of this study was that changes in phenotype were identified in all tissues examined at 24 hours post-exposure. In addition, the majority of changes were specific to an inoculum type (e.g. cell-associated or cell-free virus) indicating that the virus inoculum plays a significant role in the early pathogenesis following vaginal infection. One alteration that was consistent between inoculum types was a significant increase in the percent of CD4⁺ T-cells present in the draining lymph node (ILN). This may have important implications for overall pathogenesis, as CD4⁺ T-cells are the primary target for FIV viral entry through the OX-40 (CD134) receptor (52).

Conversely, the percentage of CD4⁺ T-cells in the lamina propria was lower in three of four cats inoculated with cell-associated FIV. This finding is intriguing because several studies have identified depletion of LPL one to two weeks following vaginal or parenteral cell-free SIV infection (29, 56, 65). This may suggest that CD4⁺ LPL depletion is even more rapid following cell-associated infection and would present challenges to vaccine

development. The concurrent decrease in the percentage of CD4+CD25+ LPL in cell-associated inoculated cats ($p=0.059$) may indicate a decrease in the proportion of regulatory T-cells in the lamina propria. The decrease in CD4+CD25+ LPL could also suggest preferential susceptibility to FIV infection, as a recent study found that CD4+CD25+ T-cells were significantly more susceptible to FIV infection as compared to CD4+CD25- T-cells (28).

The percentage of CD8+ T-cells was decreased in IEL, LPL, PBMC and PLN in cell-associated infected cats when compared to controls. While the decreased proportion of CD8 T-cells may rebound later in the course of infection, we have previously shown that the percentage of CD8+ IEL remains lowered in chronically FIV-infected cats (Howard, K. Ph.D. Dissertation, Chapter 2). In contrast, the percentage of CD8+ T-cells was higher in PBMC and lymph nodes of cell-free infected cats. Cytotoxic CD8+ T-cells are critical in the reduction of initial plasma viremia, and important in control of HIV, SIV, and FIV replication in vivo and are typically identified in PBMC and lymph nodes (3, 5, 27, 50, 68). CD8+ T-cells found in the epithelium of the intestine (IEL) are cytolytic, but differ from peripheral CD8+ T-cells in that they have important roles in regulation and homeostasis of the intestinal epithelium (20). Thus, the persistently lowered proportion of CD8+ IEL identified in acutely and chronically FIV-infected cats may contribute to intestinal dysfunction associated with AIDS enteropathy.

The percent of cells expressing CD1a was approximately 6-7% higher following cell-free infection; however, substantial increases in the percent of cells expressing CD1a were found following cell-associated infection as compared to controls. The marked increase in CD1a expression was not anticipated, and likely represents up-regulation on other cell types as the

percentage of T-cells, B-cells, and CD1a+ cells exceeds 100% of leukocytes present following cell-associated infection. CD1 expression has been shown on feline thymocytes and dendritic cells (66) and can also be up-regulated on other cell types (54). We did not identify which cell types expressed CD1a and it is possible that either T- or B-cells were responsible for the increases observed. Supporting T-cell expression of atypical receptors are data that show a proportion of feline T-cells express MHCII, and that FIV infection significantly increases expression levels of MHCII (48, 49). The proportion of cells expressing CD1 following cell-free FIV infection is more consistent with dendritic cell expression rather than the potentially aberrant expression detected following cell-associated infection. CD1 has been shown to present atypical antigen, specifically non-protein antigens such as lipids found in *Mycobacterium* (61). Of interest are several reports in HIV and SIV indicating dysregulation of CD1 expression, leading to decreased antigen presentation (11, 53, 57). Given the role of CD1 molecules in atypical antigen presentation, and dysregulated CD1 expression reported in HIV and SIV infection, it is possible that the significant alterations in CD1a expression observed in this study could represent early immune dysregulation.

Following cell-free infection, the percentage of cells expressing B-cell markers was lower in PBMC and most lymph nodes. However, a curious increase in B-cells was found in IEL of cell-free infected cats. B-cells are uncommon in IEL so this may represent either abnormal homing of B-cells or expression of a B-cell surface marker by other leukocytes.

Also of interest was a trend of lowered B7.1 (CD80) expression in each mucosal site evaluated following vaginal exposure to cell-free virus. CD80 is typically expressed as a co-stimulatory molecule by APC in activating naïve lymphocytes, but can also be expressed by

activated lymphocytes to down-regulate an ongoing immune response through ligation of CTLA-4 on other lymphocytes (67). This may represent down-regulation of CD80 normally found in these sites, or it could represent an influx of naïve leukocytes not expressing CD80.

CD14 expression in PBMC was also higher following cell-free infection, potentially indicating trafficking of APC in the peripheral circulation to enter tissues in response to viral infection. However, we lack availability of an additional marker in the feline system to confirm dendritic cell expression and maturation.

The phenotypic changes observed following FIV infection are intriguing and suggest widespread immune activation shortly after challenge. Given the magnitude and number of alterations identified in multiple sites, many of which were distant from the site of inoculation, it seems unlikely that the processing and transfer of antigen from the cervicovaginal mucosa would induce the changes observed in this study in 24 hours. Investigations using Herpes simplex virus found that early immune changes could be identified in the draining lymph node 4-6 hours following cutaneous inoculation. However, these immune changes were the result of antigen presentation and were not due to the presence of virus in the tissue examined (40). In the study described here, we used two different types of inoculum derived from the same virus stocks, with similar ability to infect CD4+ target cells in vitro. Both inoculums were administered at the vaginal mucosa. If antigen presentation was the initial event that led to mucosal and systemic immune responses, it seems that the early immune responses would be more similar than different. Yet, we found the immune changes to be markedly dichotomous. This raises additional questions as to the mechanism(s) by which cell-associated virus causes infection following genital exposure.

Studies in SIV suggest that following cell-free exposure, the virus is captured by dendritic cells and transported to the regional lymph node (22, 23). Several hypotheses exist for infection by cell-associated virus but none have been confirmed. Virus may be passed from the donor inoculum to epithelial cells and transcytosed to dendritic cells and macrophages (13, 21, 55) as has been shown in vitro. Alternately, infected cells may migrate through the epithelium to directly infect target cells in the mucosa that may then traffic to draining lymph nodes. There is support for this hypothesis as HIV-infected monocytes can traverse the epithelium in an in vitro culture system and mouse model system (10, 46). Finally, it may be that infected cells may pass through the epithelium and traffic to organized lymphoid tissue themselves.

We hypothesize that, similar to cell-free virus, cell-associated virus crosses the cervicovaginal mucosa, but in contrast to cell-free virus, is not captured by dendritic cells for transmission to the lymph node. Instead the infected cells are able to traffic for a period of time after inoculation and migrate to lymph nodes and possibly other mucosal sites such as the intestinal mucosa. In support of this hypothesis are findings in which labeled allogeneic leukocytes, inoculated into the vagina of mice, can be identified in histologic sections of the cervicovaginal mucosa and draining lymph node 24 hours later (24). Whether the same holds true of FIV- or HIV-infected cells remains to be determined. Our results clearly indicate that further study is necessary to identify how cell-associated virus is transmitted, how transmission differs from cell-free virus, and whether the initial trafficking differs based on the inoculum type. Such information will be necessary to construct a successful AIDS vaccine and topical interventions.

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Table 1
 Monoclonal antibodies used in flow cytometric evaluation of feline leukocytes

Antigen	Conjugation	Clone	Specificity	Source
CD1a	unconjugated	Fe1.5F4	mouse anti-cat	Peter Moore, UC Davis
CD4	FITC, PE, biotin	3-4F4	mouse anti-cat	Southern Biotechnology
CD5	PE, biotin	F43	mouse anti-cat	Southern Biotechnology
CD8 α	FITC, PE, biotin, PE-O	3.357	mouse anti-cat	Clinical Immunology Laboratory, NCSU
CD14	PE	TUK4	mouse anti-human	Dako
CD21	FITC, PE	CA2-1D6	mouse anti-canine	Serotec, Raleigh, NC
B-cells	FITC, PE-O	Fe2.8F9	mouse anti-cat	Peter Moore, UC Davis
CD25	FITC	9F23	mouse anti-cat	Clinical Immunology Laboratory, NCSU
CD45	unconjugated	WC45a	mouse anti-cat	Serotec, Raleigh, NC
CD57	FITC			BD Biosciences
CD62L	PE	SK11	mouse anti-human	BD Biosciences
B220	FITC, SPRD	RA3-6B2	rat anti-mouse	Southern Biotechnology
B7.1	PE	B71.66	mouse anti-cat	Clinical Immunology Laboratory, NCSU
Streptavidin	SPRD	NA	NA	Southern Biotechnology
Streptavidin	PerCP	NA	NA	BD Biosciences
Streptavidin	APC	NA	NA	BD Biosciences
F(ab) ₂ IgG	PE	catalog # 115-116-146	goat anti-mouse	Jackson Immunoresearch Laboratories

Table 2
Phenotypic comparison of cells from the medial iliac lymph node

	Uninfected		FIV-infected	
	Control ^{a,b}	Cells-only ^a	Cell-associated ^a	Cell-free ^a
CD4 *	37.2 (7.3) #†	48.8 (1.1)	47.9 (5.8) #	51.4 (6.7)†
CD8 α *	20.5 (7.5)	25.1 (1.9)	20.0 (5.0)	29.0 (5.8)
CD4+ CD8+	1.3 (0.3)	2.9 (0.8)	2.5 (1.2)	2.4 (0.6)
CD4/CD8 ratio	2.0 (0.7)	2.0 (0.2)	2.5 (0.7)	1.8 (0.3)
B cells *	38.9 (12.1)†	39.4 (4.6)	33.0 (10.1)	18.2 (8.6)†
CD5	62.7 (8.3)	68.5 (4.6)	69.0 (8.5)	75.5 (9.3)
CD14	1.3 (0.2)	ND	1.7 (0.4)	0.6 (0.4)
CD57	28.9 (7.6)	31.4 (5.7)	34.8 (8.9)	39.5 (10.6)
CD62L	36.3 (24.0)	28.9 (27.6)	39.2 (24.4)	13.2 (4.7)
CD1a *	1.9 (1.1) #	ND	29.5 (19.3) #§	6.2 (4.2) §
CD25	23.7 (10.9)	35.7 (0.8)	24.5 (14.7)	22.2 (9.0)
CD4+ CD25+	9.1 (2.9)	22.1 (1.3)	12.5 (5.9)	13.6 (5.9)
B7.1	19.1 (8.8)	16.7 (4.8)	12.6 (7.6)	9.8 (3.6)

Significant differences between groups as determined by ANOVA are noted with (*), post-test results with significant differences ($p < 0.05$) are indicated as follows: control vs. CA (#), control vs. CF (†), and CA vs. CF (§).

^aMean percent (standard deviation) of gated population expressing indicated marker.

^bControl animals were either naïve ($n=4$) or inoculated with media ($n=4$); results were combined as no differences were identified in their results.

ND = not done

Table 3
Phenotypic comparison cells from the mesenteric lymph node

	Uninfected		FIV-infected	
	Control ^{a,b}	Cells-only ^a	Cell-associated ^a	Cell-free ^a
CD4	36.7 (4.3)	45.3 (2.5)	40.4 (5.7)	41.9 (4.5)
CD8 α	24.3 (11.1)	28.1 (2.2)	26.0 (4.8)	33.8 (4.8)
CD4+ CD8+	1.6 (1.4)	2.8 (1.2)	2.7 (2.4)	2.5 (0.8)
CD4/CD8 ratio	1.9 (1.1)	1.6 (0.1)	1.6 (0.4)	1.3 (0.2)
B cells	39.2 (14.4)	23.0 (1.0)	39.5 (9.5)	26.2 (7.7)
CD5	59.4 (14.1)	71.9 (5.7)	62.8 (7.5)	73.1 (7.5)
CD14	1.0 (0.3)	ND	1.6 (0.3)	0.9 (0.9)
CD57	24.0 (9.1)	27.5 (7.9)	32.5 (8.6)	34.5 (9.6)
CD62L	44.9 (21.2)	20.3 (17.0)	34.1 (27.1)	9.1 (2.9)
CD1a *	2.4 (1.1) #	ND	40.3 (17.1) #§	7.3 (5.3) §
CD25	20.1 (10.2)	28.2 (16.0)	21.0 (14.4)	14.2 (3.3)
CD4+ CD25+	7.4 (3.5)	12.1 (6.4)	10.5 (6.2)	9.5 (1.7)
B7.1	18.5 (11.4)	13.4 (8.2)	12.9 (4.8)	9.1 (5.2)

Significant differences between groups as determined by ANOVA are noted with (*), post-test results with significant differences ($p < 0.05$) are indicated as follows: control vs. CA (#), control vs. CF (†), and CA vs. CF (§).

^aMean percent (standard deviation) of gated population expressing indicated marker.

^bControl animals were either naïve (n=3) or inoculated with media (n=4); results were combined as no differences were identified in their results.

ND = not done

Table 4
Phenotypic comparison of small intestinal intraepithelial leukocytes

	Uninfected		FIV-infected	
	Control ^{a,b}	Cells-only ^a	Cell-associated ^a	Cell-free ^a
CD4	8.0 (3.1)	13.9 (3.2)	15.3 (10.3)	10.5 (2.4)
CD8 α *	71.0 (10.1) #	56.3 (2.5)	53.2 (9.8) #	66.9 (8.7)
CD4+ CD8+	2.4 (0.3)	ND	5.1 (4.6)	3.6 (1.1)
CD4/CD8 ratio	0.1 (0)	0.2 (0.1)	0.3 (0.2)	0.2 (0)
B cells *	3.9 (1.8) §	7.1 (7.7)	10.9 (6.9)	18.3 (3.1) §
CD5+ total	92.3 (2.7)	87.6 (4.9)	90.9 (4.5)	92.1 (5.2)
CD5+ bright	73.7 (8.0)	70.4 (9.0)	65.8 (13.8)	72.6 (8.9)
CD5+ dim	18.6 (6.7)	17.2 (5.7)	25.1 (11.3)	20.1 (4.5)
CD57	4.1 (0.8)	2.7 (1.3)	4.0 (2.5)	5.8 (3.6)
CD62L	5.5 (2.4)	5.3 (1.9)	6.3 (1.7)	6.5 (3.0)
CD1a *	1.5 (1.4) #	0.5 (0.1) ○	40.6 (0.2) #§○	7.0 (2.8) §○
CD25	7.4 (2.4)	8.7 (2.3)	6.1 (2.4)	5.1 (1.4)
CD4+ CD25+	2.1 (1.5)	3.6 (0.5)	2.9 (1.4)	2.2 (0.8)
B7.1	53.1 (19.8)	21.8 (10.2)	38.7 (17.3)	31.9 (11.8)

Significant differences between groups as determined by ANOVA are noted with (*), post-test results with significant differences ($p < 0.05$) are indicated as follows: control vs. CA (#), control vs. CF (†), CA vs. CF (§), cells-only vs. CA or CF (○).

^aMean percent (standard deviation) of gated population expressing indicated marker.

^bControl animals were either naïve (n=3) or inoculated with media (n=4); results were combined as no differences were identified in their results.

ND = not done

Table 5
Phenotypic comparison of small intestinal lamina propria leukocytes

	Uninfected		FIV-infected	
	Control ^{a,b}	Cells-only ^{a,b}	Cell-associated ^{a,b}	Cell-free ^{a,b}
CD4	49.1 (4.3)	44.1 (5.5)	37.0 (14.7)	55.8 (5.8)
CD8 α	30.4 (11.0)	25.4 (5.2)	20.2 (7.5)	25.8 (3.3)
CD4+ CD8+	3.4 (1.5)	1.7 (0.6)	2.0 (0.8)	3.2 (1.6)
CD4/CD8 ratio	1.8 (0.8)	1.8 (0.1)	2.0 (0.9)	2.2 (0.5)
B cells	ND	ND	6.0 (2.7)	2.7 (0.9)
B220 (CD45R)	ND	ND	12.1 (10.7)	7.3 (3.2)
CD5+ total	85.7 (16.3)	81.4 (4.1)	78.2 (17.6)	91.2 (2.1)
CD5+ bright	78.0 (14.8)	71.6 (3.0)	56.5 (16.7)	78.1 (3.1)
CD5+ dim	7.9 (1.5)	9.8 (1.0)	21.7 (8.7)	16.4 (4.9)
CD14	3.5 (1.6)	2.1 (1.4)	4.7 (3.1)	1.3 (0.8)
CD57	7.3 (3.9)	1.3 (0.4)	4.8 (2.3)	4.0 (0.6)
CD62L	2.2 (0.9)	ND	3.7 (2.0)	ND
CD1a	ND	ND	49.7 (5.2)	ND
CD25 *	29.5 (6.3) #	13.9 (5.4)	9.8 (6.2) #	18.4 (3.8)
CD4+ CD25+	16.6 (3.1)	7.6 (4.4)	5.5 (3.8)	12.3 (3.4)
B7.1	33.8 (0.4)	34.0 (4.9)	39.6 (4.8)	23.1 (11.6)

Significant differences between groups as determined by ANOVA are noted with (*), post-test results with significant differences ($p < 0.05$) are indicated as follows: control vs. CA (#), control vs. CF (†), and CA vs. CF (§).

^aMean percent (standard deviation) of gated population expressing indicated marker.

^bControl (n=2), cells-only (n=2), cell-associated (n=4), and cell-free (n=3).

ND = not done

Table 6
Phenotypic comparison of peripheral blood mononuclear cells

	Uninfected		FIV-infected	
	Control ^{a,b}	Cells-only ^a	Cell-associated ^a	Cell-free ^a
CD4	38.6 (6.9)	45.6 (12.3)	39.4 (8.9)	44.5 (8.8)
CD8 α *	21.3 (12.4)	18.7 (7.8)	14.4 (4.3) §	26.1 (3.6) §
CD4+ CD8+	1.7 (0.8)	3.0 (2.8)	2.6 (3.8)	1.5 (0.5)
CD4/CD8 ratio	2.2 (1.0)	2.6 (1.0)	3.0 (1.3)	1.7 (0.3)
B cells *	21.8 (6.6)	24.0 (12.6)	34.9 (6.4) §	11.6 (8.2) §
B220 (CD45R)	ND	ND	32.0 (1.8)	29.9 (7.5)
CD5	66.2 (15.5)	64.1 (21.7)	55.8 (10.0)	74.5 (7.3)
CD14 *	14.4 (5.9) †	7.7 (5.8)	9.4 (2.8)	4.9 (3.8) †
CD57	25.1 (15.1)	29.0 (9.0)	27.1 (8.0)	38.5 (9.1)
CD62L	56.7 (16.1)	50.9 (16.9)	66.3 (17.9)	46.5 (8.7)
CD1a	3.6 (1.8)	13.3 (15.7)	11.8 (6.6)	6.6 (5.7)
CD25	27.6 (4.6)	19.8 (9.7)	24.3 (8.4)	20.4 (11.1)
CD4+ CD25+	7.7 (3.8)	11.7 (11.0)	8.9 (3.7)	8.8 (4.1)
B7.1	19.6 (5.4)	14.9 (5.5)	19.2 (11.0)	18.3 (6.5)

Significant differences between groups as determined by ANOVA are noted with (*), post-test results with significant differences ($p < 0.05$) are indicated as follows: control vs. CA (#), control vs. CF (†), and CA vs. CF (§).

^aMean percent (standard deviation) of gated population expressing indicated marker.

^bControl animals were either naïve ($n=2$) or inoculated with media ($n=3$); results were combined as no differences were identified in their results.

ND = not done

Table 7
Phenotypic comparison of cells from peripheral lymph nodes

	Uninfected		FIV-infected	
	Control ^{a,b}	Cells-only ^a	Cell-associated ^a	Cell-free ^a
CD4	42.9 (12.5)	54.6 (8.0)	46.3 (8.4)	52.3 (5.7)
CD8 α *	19.8 (7.1) †	24.2 (5.4)	18.3 (4.1) §	28.9 (3.9) †§
CD4+ CD8+	1.7 (1.3)	3.4 (1.6)	1.8 (0.9)	2.5 (0.6)
CD4/CD8 ratio	2.3 (0.6)	2.4 (0.7)	2.7 (0.9)	1.8 (0.4)
B cells	34.2 (16.3)	26.4 (18.0)	36.6 (10.9)	18.2 (6.4)
CD5	63.6 (18.5)	74.3 (8.8)	69.1 (9.6)	76.2 (7.1)
CD14	3.0 (2.9)	ND	ND	0.5 (0.4)
CD57	30.1 (6.3)	27.0 (5.8)	35.0 (8.7)	39.6 (9.9)
CD62L	36.6 (22.0)	17.4 (17.2)	32.2 (20.3)	11.9 (3.9)
CD1a	3.5 (1.1)	11.8 (14.0)	18.1 (12.3)	6.6 (5.7)
CD25	21.7 (6.0)	27.1 (14.3)	19.9 (13.1)	21.9 (8.6)
CD4+ CD25+	9.6 (2.9)	17.6 (10.5)	10.4 (6.2)	12.7 (4.4)
B7.1	15.8 (9.7)	11.3 (6.1)	9.5 (4.5)	12.2 (4.2)

Significant differences between groups as determined by ANOVA are noted with (*), post-test results with significant differences ($p < 0.05$) are indicated as follows: control vs. CA (#), control vs. CF (†), and CA vs. CF (§).

^aMean percent (standard deviation) of gated population expressing indicated marker.

^bControl animals were either naïve (n=2) or inoculated with media (n=3); results were combined as no differences were identified in their results.

ND = not done

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Vaginal inoculation with cell-associated but not cell-free FIV induces acute apoptosis of intraepithelial lymphocytes (IEL)

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ABSTRACT

Infection with human immunodeficiency virus (HIV) or simian immunodeficiency virus (SIV) results in an early depletion of CD4⁺ lamina propria lymphocytes (LPL) throughout the intestine, well before changes can be detected in other organized lymphoid tissue. However, little is known about the pathogenesis of HIV in systemic and mucosal sites immediately following transmission. Use of animal models, such as the feline immunodeficiency virus (FIV) model, provides an opportunity to examine the very early events following infection that cannot be addressed in human subjects.

We have previously identified marked depletion of CD8⁺ intraepithelial lymphocytes (IEL) in FIV-infected cats 24 hours after vaginal transmission. In this study, we evaluated the mechanism of IEL depletion. We found that the depletion was due to apoptosis and only occurred following vaginal infection with cell-associated but not cell-free FIV. Apoptosis occurred only in IEL, and not in LPL, PBMC or lymph nodes. CD8 α T-cells represented the primary cell type undergoing apoptosis. These findings indicate that early differences in pathogenesis exist between cell-associated and cell-free vaginal inoculums, and that significant, early depletion of IEL may play a role in early intestinal dysfunction found in HIV infected patients.

INTRODUCTION

Human immunodeficiency virus (HIV) is primarily transmitted by mucosal exposure (1), but regardless of exposure route has profound effects on the structure, function and leukocyte populations of mucosal sites including the gastrointestinal tract (2, 4, 6, 24, 31, 32, 38, 40). Loss of CD4 T-cells in the lamina propria, villous atrophy, dysfunctional enzyme secretion and nutrient uptake, diarrhea, and tumor development are seen at all stages of HIV infection in the absence of secondary pathogens. Similar changes have been reported in macaques experimentally infected with simian immunodeficiency virus (SIV) (16-18, 39). As the mucosa is the largest lymphoid compartment of the immune system, understanding the mucosal pathogenesis of HIV is critical.

The gastrointestinal immune system is comprised of inductive sites, such as Peyer's patches and lymphoid follicles, and effector sites including the lamina propria and epithelium of the intestine (27). The intestinal epithelium contains a diverse population of leukocytes, primarily CD8⁺ T-cells, bearing cell-surface markers not typically observed in peripheral immune sites (reviewed by A. Hayday (15)). Intraepithelial lymphocytes (IEL) bear either TCR $\alpha\beta$ or TCR $\gamma\delta$. IEL can express CD8 α as a homodimer (CD8 $\alpha\alpha$), CD8 $\alpha\beta$, CD4 alone or in conjunction with CD8, or no CD4 or CD8 receptor at all. IEL can be further characterized by function. The first group includes TCR $\alpha\beta$ CD8 $\alpha\beta$ ⁺ and TCR $\alpha\beta$ CD4⁺ T-cells that recognize foreign antigen via classic MHC interactions. The second group includes more atypical lymphocytes populations such as TCR $\alpha\beta$ CD8 $\alpha\alpha$ ⁺, TCR $\gamma\delta$ CD8 $\alpha\alpha$ ⁺, and TCR $\gamma\delta$ CD4-CD8⁻ T-cells which can recognize self-antigen via unconventional MHC

interactions. These cells perform many functions including helping to maintain epithelial homeostasis and tumor surveillance.

It has been shown that upregulation of CD8 α on CD8 α β T-cells plays a role in the development of immunological memory (23). IEL are also known to be activated and/or memory T-cells that are acutely susceptible to activation-induced cell death as a result of their surface receptors and activation state (33, 37). Taken together, these studies indicate an important role for CD8⁺ IEL in the maintenance of mucosal immune function and further support the need to understand the effects of HIV infection on IEL populations.

Feline immunodeficiency virus (FIV) is a natural animal model of HIV, with affected cats experiencing symptoms and disease progression similar to those observed in humans infected with HIV. These include an initial viremia, emergence of CD8⁺ CTL activity associated with decreased viral load, loss of CD4⁺ T cells, inversion of the CD4/CD8 ratio, and eventual AIDS-like syndrome with opportunistic infections and neoplastic disorders (7-10, 21, 35). In prior studies (Howard, K. Ph.D. dissertation, Chapter 2), IEL yield was markedly lower following vaginal inoculation with cell-associated but not cell-free FIV. Lowered IEL yield was associated with a significant reduction in the number of intestinal epithelial CD8 α , CD8 β and CD5 T-cells. This study investigates whether the previously observed acute depletion of IEL is due to apoptosis and if lymphocytes in locations other than the intestinal epithelium are affected.

MATERIALS AND METHODS

Animals, viruses, and inoculums. Twenty-seven specific pathogen free (SPF) sexually mature female cats obtained from Liberty Labs (Liberty, NY) were group housed and cared for in accordance with the standards of the American Association of Accreditation of Laboratory Animal Care and guidelines of the Institutional Animal Care and Use Committee. At the time of the study, animals ranged from six to thirteen months of age and were assigned to one of three groups, that were vaginally inoculated with cell-associated FIV (n=8), cell-free FIV (n=8) or controls (n=11), following sedation with Telazol. The challenge virus was NCSU₁, a FIV pathogenic sub-group A molecular clone. For cell-associated infection, cats were inoculated with either 2.0×10^5 FIV-infected heterologous feline PBMC (n=6), or 2.0×10^5 FIV infected CD4⁺ FCD4E-cells (a feline T-cell line, n=2). For cell-free infection, cats were inoculated with 2.53×10^4 TCID₅₀, a tissue culture equivalent dose to cell-associated allogeneic PBMC inoculum (n=6), or with cell-free virus at a dose ten times greater than the TCID₅₀ of those inoculated with FIV infected CD4⁺ FCD4E-cells (high-dose, n=2). The amount of cell-free virus given was based on a co-culture assay with a permissive CD4⁺ cell line to determine TCID₅₀ and then equilibrated to the infectivity of the cell-associated inoculum. Controls were either uninoculated (n=3), inoculated with medium only (n=3); inoculated with 2.0×10^5 uninfected heterologous feline PBMC (n=3) or 2.0×10^5 uninfected feline CD4⁺ FCDE-cells (n=2). Results of early immune changes were assessed at twenty-four hours post-inoculation.

Sample Collection and Processing. Peripheral blood mononuclear cells (PBMC), peripheral (prescapular or popliteal) lymph node (PLN), mesenteric lymph node (MLN),

medial iliac lymph node (ILN) and distal jejunum were harvested at necropsy and processed as previously described (5). Briefly, blood was collected in tubes containing EDTA and PBMC were isolated using Histopaque density centrifugation. Lymph nodes were disassociated using mesh screens; resulting pellets were washed and counted. Intraepithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL) were isolated from a 25 cm section of distal jejunum. To isolate IEL, the intestine was flushed with wash medium (PBS supplemented with 20% FBS, 4mM L-glutamine, 2mM penicillin/streptomycin, 2.5µg/ml amphotericin B and 10µg/ml gentamicin) and cut into 0.5cm strips following excision of Peyer's patches and lymphoid follicles. Cut intestinal sections were stirred vigorously for 30 minutes at 37°C in spin medium (Hanks balanced salt solution supplemented with 20% FBS, 4mM L-glutamine, 2mM penicillin/streptomycin, 2.5µg/ml amphotericin B and 10µg/ml gentamicin) with 2mM EDTA and 2mM dithiothreitol added. The supernatant was collected and then centrifuged at 1000xg for 15 minutes at 16°C. The supernatant was removed, the pellet resuspended in 30% Percoll (Amersham Biosciences, Piscataway, NJ), then underlaid with 70% Percoll (Amersham Biosciences) and centrifuged at 400xg for 30 minutes at 25°C. Following centrifugation, the 30/70 interface cell layer was collected, washed twice in wash medium and counted. LPL were isolated by continued processing of intestinal strips from IEL isolations. Following collection of the supernatant for IEL isolation, an additional spin for 30 minutes at 37°C in spin medium with 2mM EDTA added was performed to completely remove any remaining IEL. The supernatant was discarded and the intestinal strips were digested twice, in succession in spin medium with 20% Dispase II (Roche Applied Science, Indianapolis, IN) on an orbital shaker at 37°C at 200 rpm for 90 minutes. Each supernatant was collected and centrifuged at 1000xg for 15 minutes at 16°C. After both

digestions were completed, pellets were resuspended in 44% Percoll (Amersham Biosciences, Piscataway, NJ), then underlaid successively with 50% and 67% Percoll (Amersham Biosciences) layers. Gradients were centrifuged at 400xg for 30 minutes at 25°C. Following centrifugation, the 44/50 and 50/67 interfaces were collected, combined and washed twice in wash medium and counted.

Cryopreservation of Tissue Samples. Immediately following euthanasia, collected tissue samples were maintained on ice, cut into sections, placed in tissue cassettes containing OCT medium and frozen using liquid nitrogen cooled isopentane. Frozen cassettes were maintained at -80°C where they remained until being processed into sections.

Phenotypic Analysis. Monoclonal antibodies (mAb) used for cell surface staining were directly conjugated to phycoerythrin (PE), except for anti-cat CD45 that was unconjugated. Serotec (Raleigh, NC) antibodies included anti-cat CD45 (clone WC45a) and anti-canine CD21 PE (clone CA2-1D6). Antibodies obtained from Southern Biotechnology (Birmingham, AL) included anti-cat CD4 PE (clone 3-4F4) and anti-cat CD5 PE (clone F43). The following mAb were purchased from the Clinical Immunology Laboratory (North Carolina State University, Raleigh, NC): anti-cat CD8 α PE (clone 3.357), and anti-cat CD8 β PE (clone 1.117). Secondary antibody F(ab')₂ fragment goat anti-mouse IgG PE (#115-116-146) was used with anti-CD45, and was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

Annexin V Analysis. Annexin V was purchased as part of an apoptosis detection kit, Annexin-V-Fluos staining kit (Roche Applied Science). 7-amino-actinomycin-D (7-AAD), (Sigma) was evaluated in FL3, and compensated to ensure no staining appeared in FL2. 7-

7-AAD, in conjunction with Annexin V-Fitc, was used for three-color phenotypic identification of apoptotic cell populations.

Apoptosis identification in freshly isolated cells was determined using three-color flow cytometry. Briefly, 1.0×10^6 freshly isolated cells were incubated with PE conjugated monoclonal antibodies for 20 minutes at 4°C. Cells were washed with cold PBS containing 0.5mM EDTA and centrifuged at 300 x g for 10 minutes at 16°C. When secondary antibody was used for anti-CD45 staining, this procedure was repeated. After phenotypic staining was complete, Annexin V and 7-AAD were prepared in buffer solution according to manufacturer instructions, and incubated with the samples for 10-15 minutes. Samples were analyzed immediately on a FACScan (BD Immunocytometry Systems) flow cytometer. To ensure that samples were not stained longer than necessary, 7-AAD/Annexin V were added to one tissue at a time and analyzed before proceeding to the next tissue sample. Typically, 15,000 events were collected from the gated region. Analysis of flow cytometric data was completed using BD CellQuest software.

Caspase Activation Analysis. Detection of activated caspases was performed using a commercial kit (CaspGLOW™ Red Active Caspase Staining Kit; Biovision, Mountain View, CA), which detects activated caspases 3, 8 and 9. Staining was completed according to manufacturer instructions and analyzed on a FACScan (BD Immunocytometry Systems) flow cytometer. Positive control was prepared with naïve, cultured cells 8 hours prior to caspase testing using camptothecin (Sigma). Camptothecin is a potent inhibitor of topoisomerase I and is used to induce apoptosis in a dose-dependent manner *in vitro*.

DNA Fragmentation Analysis. DNA strand breaks in tissue sections were detected using terminal deoxynucleotidyl transferase (TdT) or TUNEL assay with a commercial kit (In Situ

Cell Death Detection Kit, AP; Roche Applied Science) according to manufacturer instructions. TdT signal was converted using Vector Red Substrate (Vector Laboratories, Burlingame, CA), and counterstained with Vector Hematoxylin QS (Vector Laboratories). After slides were dry, coverslips were mounted with VectaMount Permanent Mounting Media (Vector Laboratories) and sealed with clear nail polish. At least two different sections were stained for each tissue analyzed.

Statistical Analysis. Statistical analysis was completed using a one-way analysis of variance (ANOVA) with a Tukey-Kramer multiple comparison post-test or nonparametric ANOVA (Kruskal-Wallis test) with Dunn's multiple comparison post-test. The choice of test was dictated by testing the assumptions necessary for parametric methods, such that if a parametric method was not appropriate, non-parametric testing was used. Post-test results that demonstrated significant differences between individual groups are indicated in the figure or table. All analysis was completed using GraphPad InStat version 3.05 (GraphPad Software, San Diego, CA).

RESULTS

IEL cell death following vaginal inoculation of cell-associated but not cell-free FIV. To investigate differences in the pathogenesis of cell-associated and cell-free FIV, cats were vaginally inoculated with cell-free FIV or FIV-infected CD4⁺ T-cells (FCD4E-cells, a T-cell line) and examined at 1, 3, 5, 7, 14, 28, and 56 days post-infection. FIV NCSU₁ cell-associated and cell-free inoculums were equilibrated using serial dilution co-culture with susceptible CD4⁺ T-cells.

To determine whether abnormal levels of cell death occurred at early time points following FIV infection, cell death was measured using 7-amino actinomycin D (7-AAD), a slightly membrane-permeable nucleic acid dye that is GC selective, and can be assessed by flow cytometry. Positive 7-AAD staining dye indicates that cell membrane permeability has been altered, allowing the dye to enter, which is typically associated with apoptotic or necrotic cells. Changes in cell death were not seen in lymph nodes lymphocytes; however, higher levels of cell death were detected in IEL at several time points (Figure 1). For cats inoculated with cell-free FIV, 7-AAD staining of IEL was maximal at 7 days post-infection with approximately 25% of cells in the live lymphocyte gate positive for 7-AAD. In contrast, cell-associated inoculum induced higher levels of cell death in IEL at one day post-inoculation, with an average of 75% of cells in the live lymphocyte gate positive for 7-AAD. Cell death was also measurable at days 3, 14 and 28 post-infection with mean values for 7-AAD+ IEL exceeding 25% at each of these time points (Figure 1).

Apoptosis assessment twenty-four hours after vaginal FIV inoculation. To determine whether the cell death observed was due to necrosis or apoptosis, and to determine whether exposure to the cell line alone might be responsible for the previous observations, we vaginally inoculated two additional cats with FIV-infected FCD4E-cells and two control cats with an equal number uninfected CD4+ E-cells. Annexin V and 7-AAD were measured in multiple tissues; these reagents are more specific for apoptosis in combination than alone. Annexin V binds to phosphatidylserine residues that are exposed when regions of the cell membrane turn inside out during apoptosis. Apoptotic cells were defined as Annexin V positive. Vaginal inoculation with cells alone caused no increase in apoptosis in any tissue evaluated. However, consistent with our preliminary data, inoculation with FIV-infected

FCD4E-cells resulted in increased levels of apoptosis in IEL at 24 hours post-infection (Table 1).

Despite the *in vitro* equilibration of the cell-associated and cell-free inoculums, *in vivo* infectivity could still result in the differences in the induction of apoptosis seen. To address this, we inoculated two cats with ten times the dose of cell-free virus given in our preliminary study. Increased apoptosis was not observed in any of the tissues examined (Table 1).

To evaluate the possibility that the FIV-infected cell line was responsible for observed apoptosis, we determined whether FIV-infected heterologous leukocytes could induce early IEL apoptosis. PBMC from a persistently FIV-infected cat were isolated and super-infected *in vitro* to obtain an inoculum with a high proportion of FIV-infected cells. Six cats were vaginally inoculated with FIV-infected PBMC. Similar to our results following exposure to FIV-infected CD4E cells, significant apoptosis was detected in IEL, but not other tissues at 24 hours post-inoculation.

Additionally, lamina propria lymphocytes from four cats inoculated with cell-associated virus were evaluated for apoptosis using Annexin V and 7-AAD. Similar to that seen for lymph node lymphocytes, increased apoptosis was not detected in lamina propria lymphocytes (data not shown).

Typical flow cytometric results are shown for peripheral lymph node and IEL in Figures 2 and 3, respectively. For each plot the lower right quadrant represents early apoptosis, whereas the upper right quadrant depicts late apoptosis and necrotic cells are shown in the upper left quadrant. In peripheral lymph node, lymphocytes from a naïve, control cat showed the highest levels of apoptosis as compared to lymphocytes from all other inoculum types (Figure 2). Significantly increased apoptosis was identified only in IEL of cell-associated

inoculated cats, with a large proportion of Annexin V and 7-AAD double positive IEL consistent with late apoptosis (Figure 3).

Caspase activation as a mechanism for apoptosis. To confirm the apoptosis identified using Annexin V and 7-AAD, and potentially identify a mechanism for IEL apoptosis, caspase activation was measured in lymphocytes from control, cell-associated and cell-free inoculated cats. Caspase activation was tested for caspase 3, 8 and 9 using commercial kit that employs a dye that irreversibly binds activated caspases. No significant changes in caspase activation were identified in PBMC or cells from the peripheral, medial iliac, or mesenteric lymph nodes (Figure 4). Consistent with Annexin V and 7-AAD staining, caspase activation was significantly increased in the IEL of cats inoculated with allogeneic FIV-infected PBMC. In contrast, but consistent with Annexin V results, caspase activation was lower in the IEL from cats inoculated with cell-free FIV as compared to control cats (Figure 4).

Induction of apoptosis in situ. The possibility was recognized that the increased levels of apoptosis observed could in some way be associated with processing of IEL. To address this, apoptosis was measured in tissues sections using DNA nick-end labeling (TUNEL assay) to confirm that apoptosis began occurring in situ, and not as a result of processing. A positive TUNEL result is visualized by red staining in the nuclei.

Representative samples of distal jejunum from cats inoculated with medium alone, FIV-infected PBMC and cell-free virus are shown in Figure 5. Multiple IEL are identified with arrows in each section, however only in the section from the cell-associated inoculated cat are red-staining IEL nuclei evident, indicating a TUNEL positive reaction. Apoptosis in the intestine appeared to be specific to the IEL population as, consistent with Annexin V and 7-

AAD staining of cells from cats inoculated with cell-associated virus, only rare TUNEL+ LPL were identified in small intestinal sections from any of the inoculum types.

Phenotype of apoptotic IEL. We have previously shown reduced IEL yields at one-day post-FIV infection (Howard, K. Ph.D. Dissertation, Chapter 2). Specifically, those results were consistent with a lowered yield of CD8 α as well as CD8 β IEL. To determine if apoptosis could be responsible for the depletion of CD8 IEL previously reported, three-color flow cytometry was used to phenotype apoptotic leukocytes. We consistently identified approximately 65% of CD8 α staining IEL as apoptotic (Figure 6). A lower proportion of CD5+bright IEL were apoptotic, averaging approximately 50%, whereas only 28% of CD4+ IEL appeared to undergo apoptosis. Identification of apoptotic CD8 β + IEL was inconclusive as the monoclonal antibody used demonstrated poor separation in IEL populations analyzed.

DISCUSSION

These results indicate that significant apoptosis occurs in IEL one day following vaginal inoculation with cell-associated but not cell-free FIV. Further, we show that this very early apoptosis is confined solely to the IEL, as increased cell death was not detected in PBMC, lymph nodes or LPL at the same sampling time point. Finally, it was also demonstrated that apoptosis occurred in situ, and that apoptosis was not the result of tissue processing.

IEL are a unique population of lymphocytes involved in host defense, immune surveillance, and homeostasis of the intestinal epithelium (15). They differ from LPL in their TCR specificity and phenotype, with LPL more closely resembling the phenotype seen in lymph node lymphocytes. IEL are effector lymphocytes that respond to antigen (13, 36),

produce cytokines (22, 28), and have potent cytotoxic function *ex vivo* without restimulation (25, 26). IEL are also critical for helping to maintain homeostasis of the intestinal epithelium. Significant depletion of IEL, such as we detected immediately following cell-associated FIV infection, could affect the epithelium profoundly and play a role in the changes observed as part of AIDS enteropathy. Although we were unable to thoroughly phenotype the IEL undergoing apoptosis, they were identified as predominantly CD8 α +. In order to better understand the early impact of IEL depletion, identification of cells as TCR $\alpha\beta$ CD8 $\alpha\alpha$ +, TCR $\gamma\delta$ CD8 $\alpha\alpha$ + or TCR $\alpha\beta$ CD8 $\alpha\beta$ + would prove helpful. However, antibodies to feline TCR $\gamma\delta$ and TCR $\alpha\beta$ are currently unavailable.

Apoptosis, also referred to as programmed cell death, represents organized elimination of cells from the body without the induction of inflammation. Two general pathways can be responsible for the induction of apoptosis: external signaling through cell-surface receptors, known as death receptors, or internal signal pathway alterations resulting in mitochondrial cytochrome C release (19, 30). Given that IEL in cats express high levels of B7.1 and are likely of a memory/activated phenotype (Howard, K. Ph.D. Dissertation, Chapter 1), they, like IEL in other species, could express higher levels of cell death receptors and be more susceptible to apoptosis. Another possibility is that alteration in cytokine milieu could occur following cell-associated FIV infection and induce cell-death signaling.

Caspases are cysteine proteases that normally exist in inactive form in cells and upon activation are cleaved and become active in the organized disassembly of cells. Various caspases are activated depending upon the initial apoptotic signal, although caspase-independent pathways have also been identified (3, 19). This study identified the activation of caspases 3, 8, or 9 in IEL of cats inoculated with cell-associated FIV. Each is involved in a

variety of external and internal apoptotic signaling pathways. Identification of activated caspases does support the occurrence of apoptosis, but further studies will be required to determine whether external signaling through death receptors or internal signaling through mitochondrial pathways is responsible.

These results showed that cell-associated but not cell-free FIV induced IEL apoptosis 24 hours after vaginal inoculation with FIV. However, it is still possible that apoptosis of IEL occurs with exposure to cell-free virus at a later time point or gradually over time. Consistent with this hypothesis, we have previously identified significantly lower numbers of CD8⁺ IEL in chronically infected cats that had been inoculated intravenously with cell-free FIV (Howard, K. Ph.D. Dissertation, Chapter 2).

Several lines of evidence support the transmission of cell-associated HIV across mucosal surfaces (29, 41), including studies of discordant partners, virus variant analysis, epidemiological analysis, ex vivo experiments using tissue explants and cervical transmission in a chimpanzee model (12, 14, 20, 29, 41). If mucosal transmission of HIV is due, even in part, to cell-associated virus, our findings of rapid apoptosis in IEL following exposure to cell-associated virus indicate it is critical to examine cell-associated virus in pathogenesis and vaccine development studies. Furthermore, it may be necessary to specifically examine IEL populations rather than evaluating whole intestinal tissue digests. In an FIV vaccine trial, lack of phenotypic alteration in the IEL of vaccinated cats correlated with absence of clinical disease, inability to detect plasma viremia, and inability to detect proviral DNA in tissues (34). This suggests that maintenance of IEL populations may be an important measure of protection.

In summary, these data show that apoptosis occurs acutely in IEL following vaginal exposure to cell-associated FIV. This change occurs in situ and, while the mechanism has not been elucidated, caspases 3, 8, and/or 9 appear to be involved in apoptosis signaling. These results underscore the need to assess very early exposure time points and different inoculum types in an immunologically competent system such as an animal model to clearly define the pathogenesis of mucosally transmitted HIV.

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Figure Legends:

Figure 1. Assessment of cell death in multiple tissues using 7-AAD. Mean percentages are shown for peripheral lymph node (PLN), medial iliac lymph node (ILN), mesenteric lymph node (MLN), and intraepithelial lymphocytes (IEL) for cats vaginally inoculated with either cell-associated FIV or cell-free FIV. Cell death was assessed at days post-inoculation shown. Time point 0 represents control cats (n=4). Total n=2 for each time point. No IEL were obtained for cell-free day 14.

Figure 2. Detection of apoptosis using 7-AAD and Annexin V in peripheral lymph node. Cats were either uninoculated (A), or vaginally inoculated with allogeneic PBMC (B), cell-free FIV (C), or FIV-infected allogeneic PBMC (D) and assessed one day post-inoculation by FACS. The lower right quadrant represents early apoptosis, the upper right quadrant represents late apoptosis, and the upper left quadrant contains dead cells. Histograms are representative of cats tested.

Figure 3. Detection of apoptosis using 7-AAD and Annexin V in intraepithelial lymphocytes. Cats were either uninoculated (A), or vaginally inoculated with allogeneic PBMC (B), cell-free FIV (C), or FIV-infected allogeneic PBMC (D) and assessed one day post-inoculation by FACS. The lower right quadrant represents early apoptosis, the upper right quadrant represents late apoptosis, and the upper left quadrant contains dead cells. Histograms are representative of cats tested.

Figure 4. Caspase activation measured in tissue sites by FACS. Activation of caspases was measured using commercial kit by flow cytometry. (A) Overlay histogram shows IEL from a cat inoculated with cell-associated FIV (gray shaded), lymphocytes with apoptosis induced as a positive control (purple line) and ILN cells from the same animal as IEL (black line). (B) Summary data are shown as mean percent with error bars (SD) of cells positive for caspase activation in PBMC, IEL, peripheral, medial iliac and mesenteric lymph nodes, by type inoculum at one-day post-inoculation. Significant differences as compared to other inoculum types within a tissue type are indicated (*) where $p < 0.05$.

Figure 5. In-situ apoptosis assessment using TUNEL staining of cryopreserved histologic sections. Using a commercial kit, TUNEL staining was assessed in tissue sections from distal jejunum of cats vaginally inoculated with media only (A), cell-associated FIV (B), or cell-free FIV (C) at 24 hours post-inoculation. Arrows indicate intraepithelial lymphocytes, with red-staining indicating positive TUNEL results. Sections are representative of each experimental group. Magnification was 400x.

Figure 6. Phenotype of apoptotic IEL populations. Shown are the percentages of IEL staining positive for CD4, CD8 α and CD5+bright (gray bars), and the corresponding apoptotic proportion for each cell surface marker (purple bars). Mean percentages with standard deviation are shown for IEL from cell-associated FIV inoculated cats.

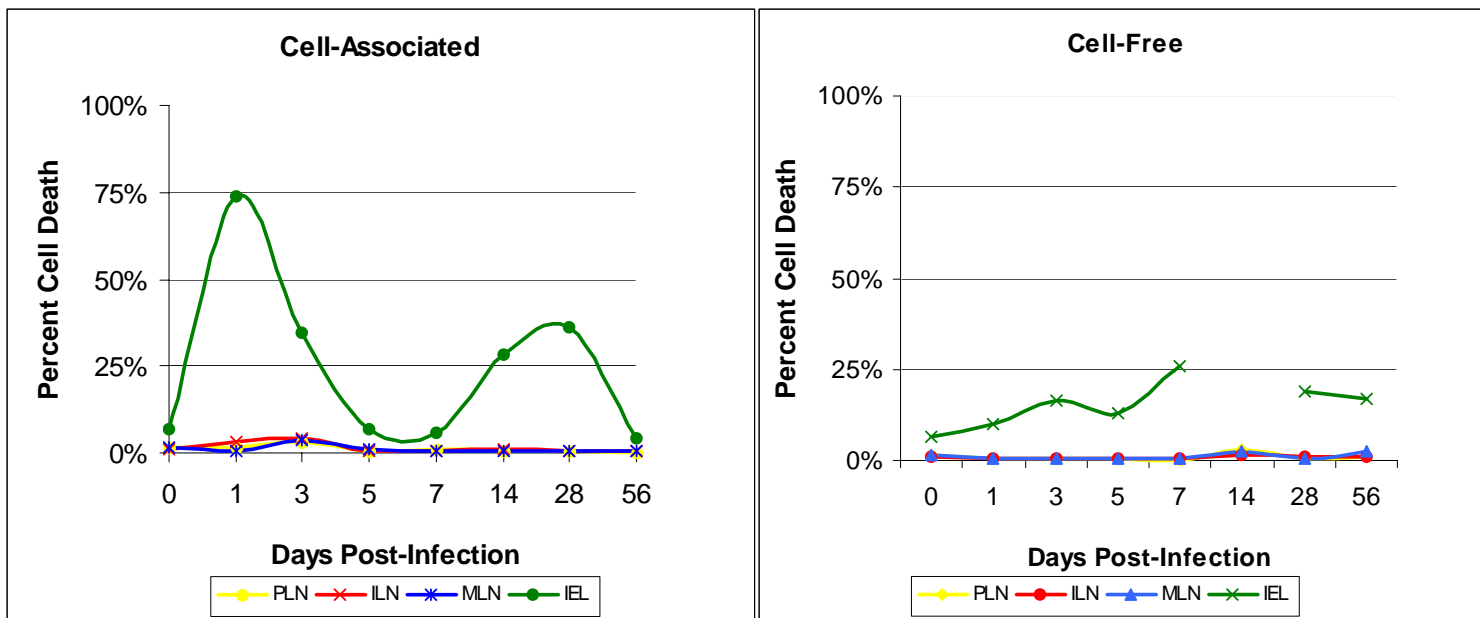


Figure 1. Assessment of cell death in multiple tissues using 7-AAD.

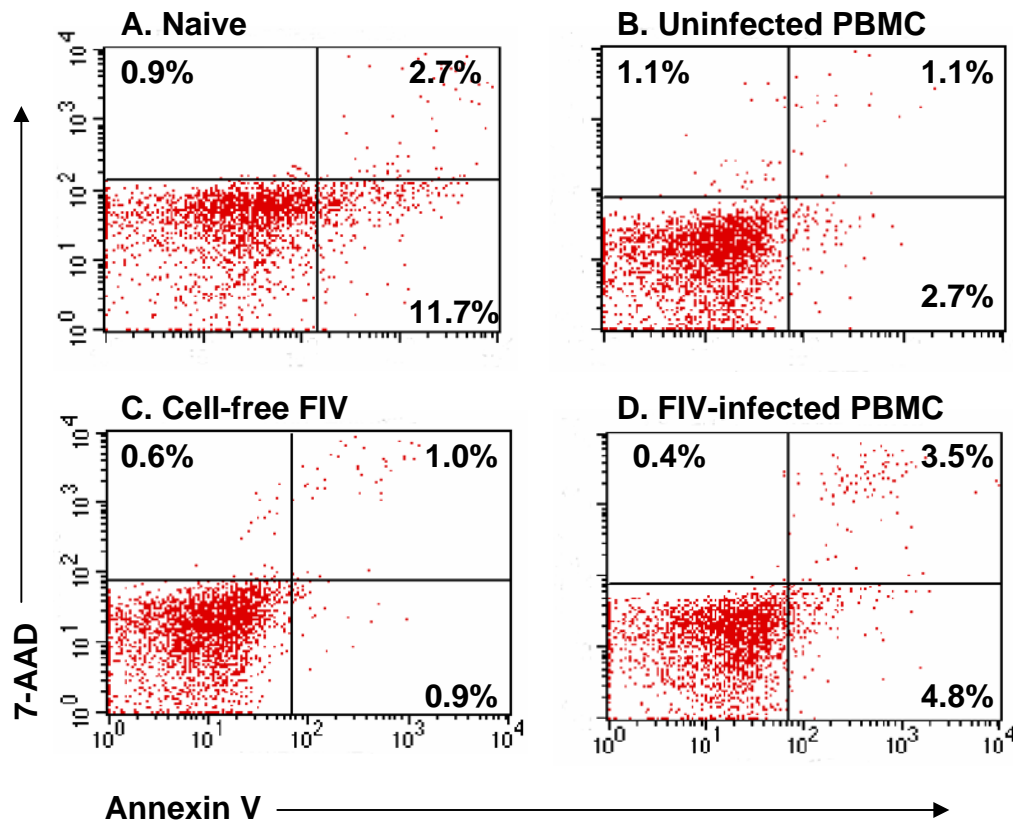


Figure 2. Detection of apoptosis using 7-AAD and Annexin V in peripheral lymph node.

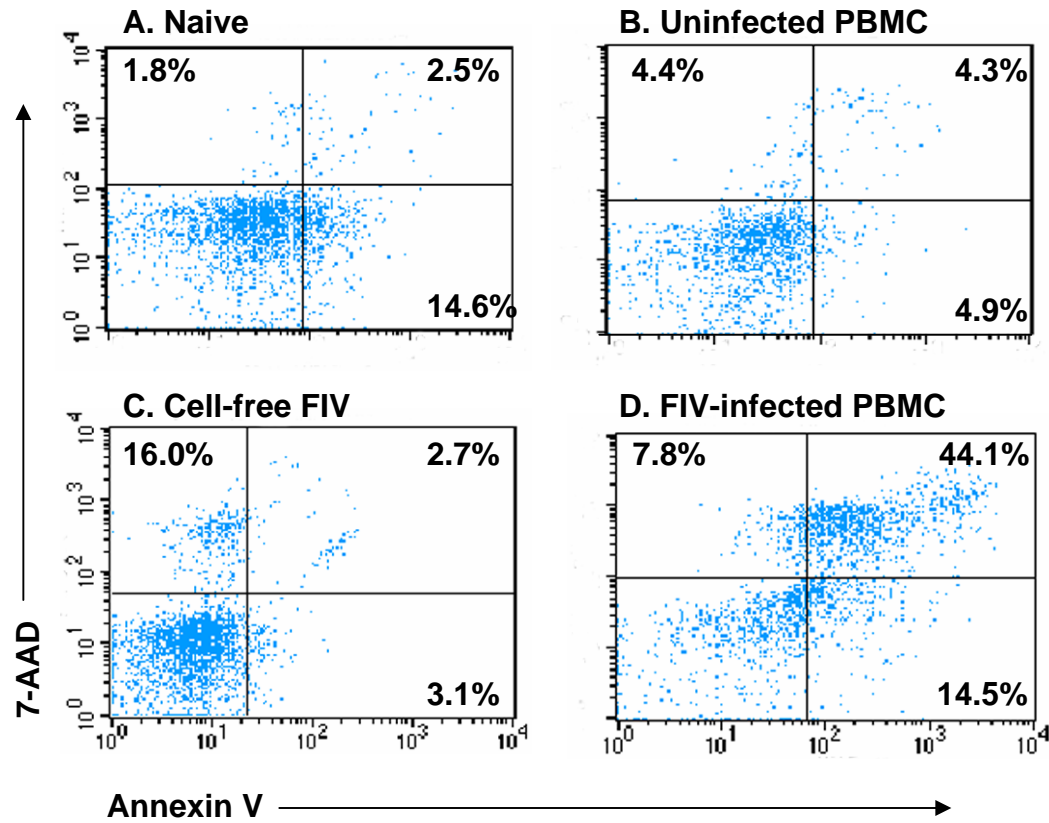
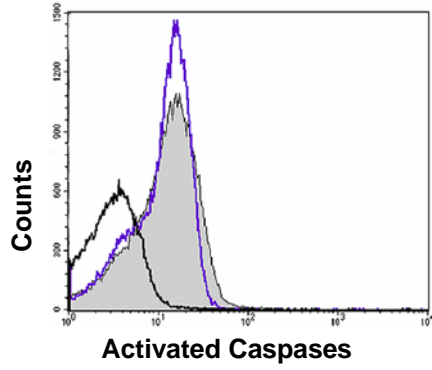


Figure 3. Detection of apoptosis using 7-AAD and Annexin V in intraepithelial lymphocytes.

A.



B.

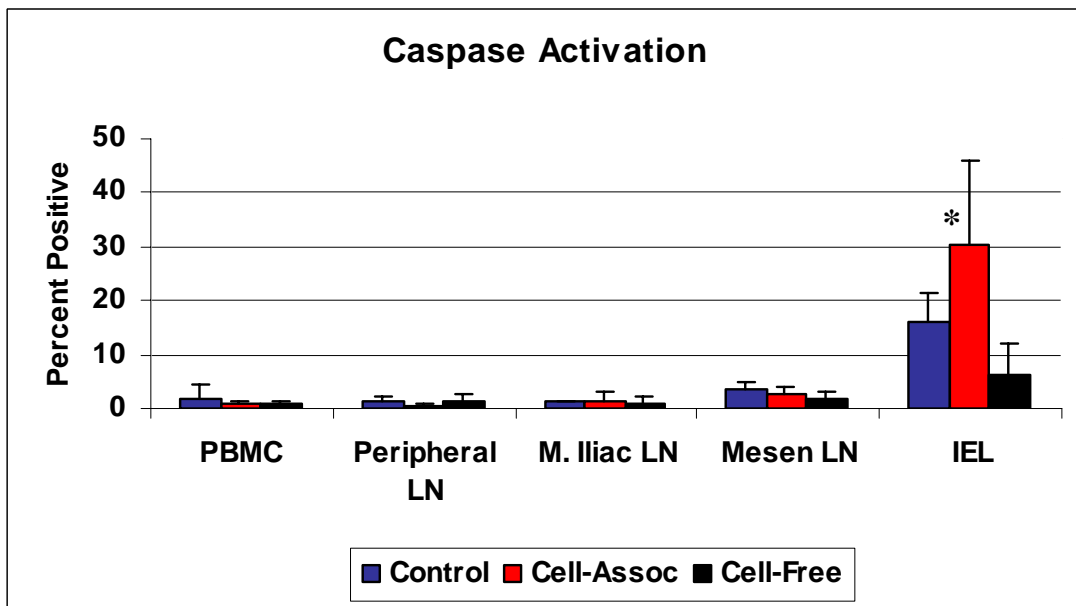


Figure 4. Caspase activation measured in blood and tissue lymphocytes.

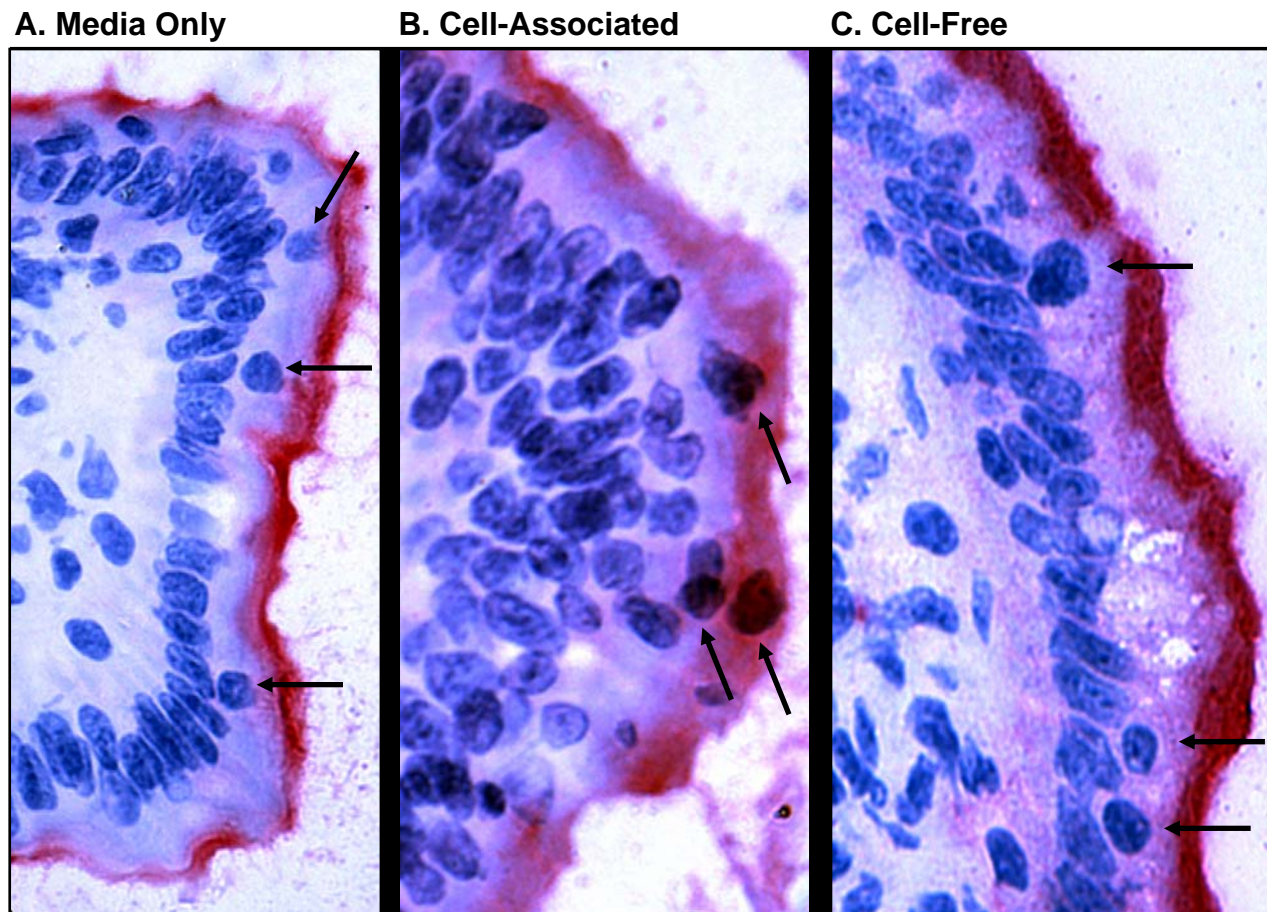


Figure 5. In-situ apoptosis assessment using TUNEL staining of cryopreserved histologic sections.

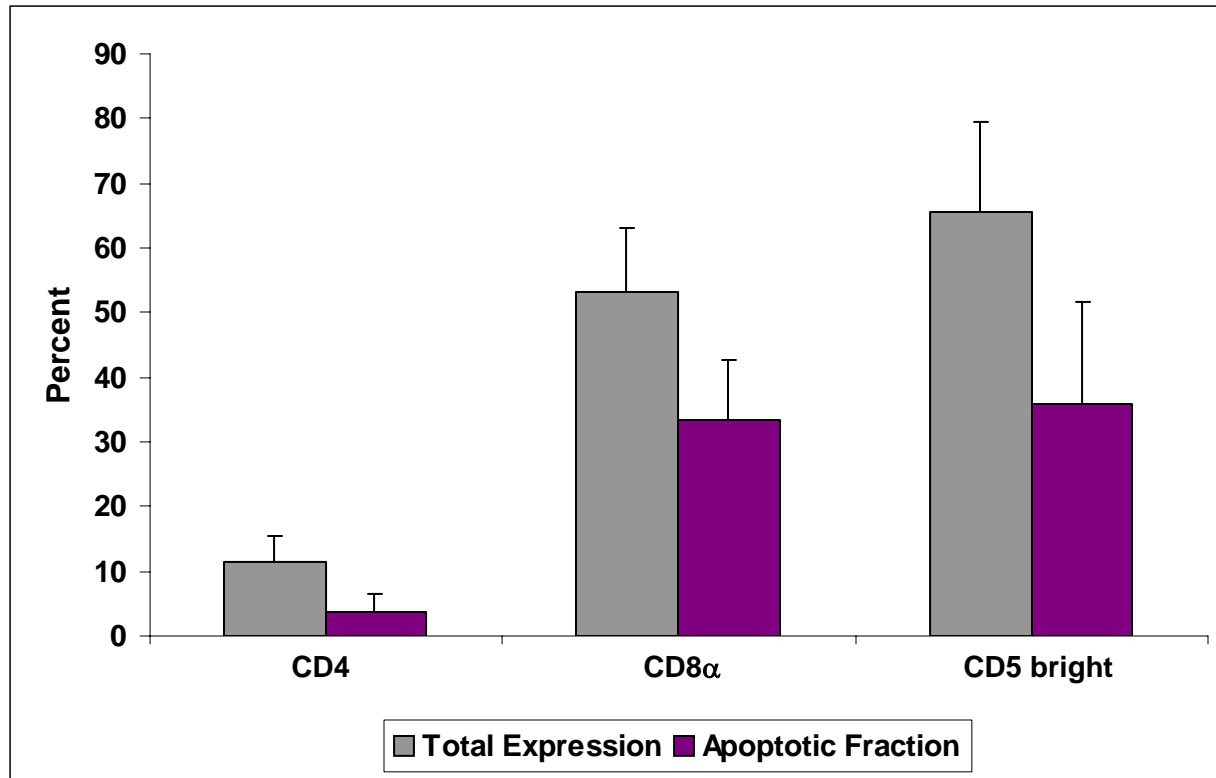


Figure 6. Phenotype of apoptotic IEL populations.

Table 1. Summary of apoptotic leukocytes determined by dual staining with Annexin V and 7-AAD.

Tissue	Control	CD4+ cell line		Allogeneic PBMC ^b		Cell-free	
	Naïve/Media	FIV infected	Cells-only	FIV infected	Cells-only	Equal dose	High dose
PBMC	7.8 ^a	5.6	1.1	2.9	6.1	4.6	0.5
Peripheral LN	15.1	11.2	1.4	4.3	7.8	5.4	2.2
Mucosal LN ^c	10.6	10.4	1.8	4.5	8.8	6.2	6.0
IEL ^d	13.2	31.0*	1.1	38.7*	14.1	12.8	6.9

^aMean percent of live gated leukocyte population with positive staining for apoptosis.

^bPeripheral blood mononuclear cells.

^cMesenteric and medial iliac lymph node data were combined as no differences were noted between these sites.

^dIntraepithelial lymphocytes.

Statistically significant differences are noted with (*) as compare to control, cell-only and cell-free groups, p<0.05.

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8. SUMMARY AND CONCLUSIONS

This study addressed the following questions:

- 1) What is the normal phenotype of leukocytes in the feline small intestine?
- 2) What are features of acute and chronic mucosal pathogenesis in the FIV model?
- 3) What impact does inoculum type play in acute immune changes seen in peripheral versus mucosal sites?
- 4) Does apoptosis play a role in early FIV mucosal pathogenesis?

These questions were answered as follows:

- 1) Feline intestinal leukocytes have phenotypes similar to those found in other species such as humans, macaques and mice in that IEL are predominantly CD8+ T-cells, LPL have CD4:CD8 ratios similar to those found in lymph nodes, and Peyer's patches contain a significant number of B-lymphocytes, with more CD4+ than CD8+ T-cells.
- 2) Immune dysregulation is evident in IEL as early as one day following FIV infection, and that these changes not only persist, but increase in magnitude over the course of FIV infection. Further, we show that while IEL are acutely depleted and MLN chronically depleted, the ILN is potentially a site of abnormal lymphocyte homing given the significant increase in cellularity and delayed inversion of CD4:CD8 ratio.

- 3) Cell-associated FIV and cell-free FIV induce distinctly different early phenotypic changes 24 hours post-inoculation, and these changes are not confined to the lymph node draining the site of inoculation.
- 4) Significant apoptosis occurs in IEL one day following vaginal inoculation with cell-associated but not cell-free FIV. Additionally, very early apoptosis is confined solely to the IEL, as increased cell death was not detected in PBMC, lymph nodes and LPL at the same sampling time point. Finally, it was also demonstrated that apoptosis occurred in situ, and that apoptosis was not the result of processing of tissues.

Despite the significant findings of these studies, much is still unknown and some findings will require further investigation. Some interesting questions raised from these results include when do the differences in the initial immune response between the cell-associated and cell-free virus become similar? How quickly are CD4+ LPL depleted in cell-free inoculated cats? How does the gastrointestinal tract respond if cats are challenged intravenously rather than vaginally? And also of interest is when and where effector T-cells arise, and does this differ based on the route or type of inoculum?

This study raises issues that may be of concern in the development of vaccines to HIV. If significant changes occur as early as one day after infection, what is the best way to induce an effective immune response? It also directs further work to understanding the role of inoculum in transmission and early pathogenesis.

IEL Isolation Protocol

- 1) Remove intestine and cut into segments approx. 10-12" long, flush with Wash media 2-3 times into a waste bottle; squeeze out excess fluid between flushes.
- 2) Open longitudinally, cut into 0.5 cm strips, excising Peyer's Patches/lymphoid follicles prior to cutting into strips.
- 3) Place intestinal strips in a 50ml conical tube, and fill with Wash media, gently inverting the tissue to bring mucus to the surface layer. Once tissues have settled on the bottom, remove aqueous portion and repeat two times. (eliminates mucus)
- 4) Place the washed intestinal fragments, that have had most of free media removed, into a 200ml Erlenmeyer flask and add 40 ml of Spin Media containing a concentration of 2mm DTT and 2mm EDTA, and an autoclaved stir bar.
- 5) Place on a heated stir plate and stir vigorously at 37°C for 30 minutes. (Do not allow the temperature to exceed 37°C!)
- 6) Strain the tissue fragments using an autoclaved basket style sieve, draining the aqueous portion into a 200ml beaker. Then transfer to a 50 ml conical tube.
- 7) Centrifuge at 1000G for 15 minutes at 16°C.
- 8) Pipette off the supernatant, since the mucus layer contains lymphocytes. Add 10-15ml 30% Percoll. Vortex vigorously for 5-10 seconds, or until completely homogenous.
- 9) Using a 10ml pipette, pass the cell suspension through a piece of autoclaved cheesecloth into a new 50ml conical tube. Do not force it through the cheesecloth, use gravity only! If it is very thick you may need to add more 30% Percoll and/or repeat using fresh pieces of cheesecloth.
- 10) Remaining volume should be increased to 35ml total volume of 30% Percoll, and then 12 ml of 70% Percoll underlaid.
- 11) Centrifuge at 400G for 30 minutes at room temp, no brake.
- 12) Remove the top layer of mucus and debris. With a new pipette, collect the 30/70 interface and transfer it to a new 50 ml conical tube..
- 13) Bring the volume up to ~45ml using Wash media and centrifuge at 400G for 10 minutes at 20°C. Wash again using 10-15 ml Wash media.
- 14) Pour off the supernatant and resuspend in approx. 2-4ml, based on pellet size. (Maintain cells cold until used.)
- 15) Count cells.

Lamina Propria Lymphocyte Isolation Protocol

- 1) Remove intestine and cut into segments approx. 10" long, flush with Wash media 2-3 times.
- 2) Open longitudinally, cut into 0.5 cm strips, excising Peyer's Patches/lymphoid follicles prior to cutting into strips.
- 3) Place intestinal strips in a 50ml conical tube, and fill with Wash media, gently inverting the tissue to bring mucus to the surface layer. Once tissues have settled on the bottom, remove aqueous portion and repeat two times.
- 4) Place the washed intestinal fragments, that have had most of free media removed, into a 200ml Erlenmeyer flask and add 40 ml of Spin Media containing a concentration of 2mM DTT and 2mM EDTA, and an autoclaved stir bar.
- 5) Place on a heated stir plate and stir vigorously at 37°C for 30 minutes. (Do not allow the temperature to exceed 37°C!)
- 6) Strain the tissue fragments using an autoclaved basket style sieve, from the aqueous portion into a 200ml beaker and transfer to a 50 ml conical tube. (The supernatant will be centrifuged and continue in IEL processing.) Take the tissue fragments and stir, place them back into the Erlenmeyer flask, adding 30ml Spin media and 1mM concentration of EDTA. Spin vigorously again for 30 minutes at 37°C. (Do not allow the temperature to exceed 37°C!)
- 7) Discard the supernatant (or process if you need more IEL's), and add 24ml Spin media with 6ml Dispase (20% Dispase). Shake constantly at 37°C, preferably in an orbital shaker, for 1½ hours. (Do not allow the temperature to exceed 37°C!)
- 8) Strain the tissue fragments using an autoclaved basket style sieve. The supernatant will be centrifuged, and the tissue fragments will be digested again as in step 7.
- 9) Centrifuge the resulting supernatant at 1000G for 15 minutes at 16°C. Resuspend in 10ml Wash media and place resuspended pellet on ice until second digestion is complete.
- 10) Following completion of the second digestion, centrifuge supernatant as in step 9, and resuspend Percoll (below, step 11) The previously collected fraction (sitting on ice) must be spun down at 400G for 10 minutes at 25°C.
- 11) Pour off the wash media and resuspend each pellet in 5 ml 44% Percoll in a 15ml conical tube. Mix well and then carefully underlay 5ml of 50% Percoll followed by 4ml of 67% Percoll. (To decrease enterocyte contamination, the 44% Percoll mixture can be strained through cheesecloth, as in the IEL isolation, prior to underlaying 50% and 67% Percoll.)
- 12) Centrifuge at 400G for 30 minutes at room temp, no brake.
- 13) Remove the top layer of mucus and debris. With a new pipette, collect the 44/50 interface and then collect the 50/67 interface combining them in one 50 ml conical tube.
- 14) Bring the volume up to ~45ml using Wash media and centrifuge at 400G for 10 minutes at 20°C. Wash again using 10-15 ml Wash media.
- 15) Pour off the supernatant and resuspend in approx. 2-3ml, based on pellet size. (Maintain cells cold until used.)
- 16) Count cells.

Mucosal Culture Media

Reagent	Stock	100ml	200ml	500ml
RPMI 1640(with phenol red)	Cellgro (17-105-CV)	76 ml	152 ml	380 ml
20% Fetal Bovine Serum-heat inactivated	Supplied and tested by UNC	20 ml	40 ml	100 ml
1% Penicillin/ Streptomycin	Gibco-BRL (15140-122 10,000u/ml Penicillin/ 10,000ug/ml streptomycin	1ml	2 ml	5 ml
2% L-glutamine	Gibco-BRL (25030-081) 200mM	2ml	4 ml	10 ml
1% Nystatin (or Fungizone)	Gibco-BRL (15340-052) 10,000 units/ml	1 ml	2 ml	5 ml
10ug/ml gentamicin	Gibco-BRL (15710-064) 10mg/ml	100 ul	200 ul	500 ul

Wash Media

REAGENT	VENDOR	100ml	200ml	500ml
10x PBS, pH 7.2	Gibco-BRL (70013-032)	10 ml	20 ml	50 ml
Distilled water for tissue culture	Gibco-BRL (15230-162)	66 ml	132ml	330 ml
20% Fetal Bovine Serum (heat inactivated)	Supplied and tested by UNC	20ml	40 ml	100 ml
1% Penicillin/Streptomycin	Gibco-BRL (15140-122 10,000u/ml Penicillin/ 10,000ug/ml streptomycin	1ml	2 ml	5 ml
2% L-glutamine	Gibco-BRL (25030-081) 200mM	2ml	4 ml	10 ml
1% Nystatin (or Fungizone)	Gibco-BRL (15340-052) 10,000 units/ml	1 ml	2 ml	5 ml
10ug/ml Gentamicin	Gibco-BRL (15710-064) 10mg/ml	100 ul	200 ul	500 ul

Spin Media (no EDTA or DTT added when initially prepared)

REAGENT	VENDOR	100ml	200ml	500ml
1x Hanks Balanced Salt Solution (w/o phenol red, Ca ²⁺ , or Mg ⁺)	Gibco-BRL (14175-095)	76 ml	152ml	380 ml
20% Fetal Bovine Serum (heat inactivated)	Supplied and tested by UNC	20ml	40 ml	100 ml
1% Penicillin/Streptomycin	Gibco-BRL (15140-122)	1ml	2 ml	5 ml
2% L-glutamine	Gibco-BRL (25030-081) 200mM	2ml	4 ml	10 ml
1% Nystatin (or Fungizone)	Gibco-BRL (15340-052)	1 ml	2 ml	5 ml
10ug/ml Gentamicin	Gibco-BRL (15710-064)	100 ul	200 ul	500 ul

PERCOLL solutions

		Iso-osmotic Percoll	70%	67%	50%	44%	30%
REAGENT	VENDOR	100ml	100ml	50ml	50ml	50ml	100ml
10x Hanks Balanced Salt Solution* (w/o phenol red, Ca ²⁺ , or Mg ⁺)	Gibco-BRL	9.2ml	N/A	N/A	N/A	N/A	N/A
1x Hanks Balanced Salt Solution (w/o phenol red, Ca ²⁺ , or Mg ⁺)	Gibco-BRL (14175-095)	N/A	N/A	16.5ml	N/A	28ml	70ml
1x Hanks Balanced Salt Solution (with phenol red, no Ca ²⁺ or Mg ⁺)	Gibco-BRL (12399-010)	N/A	30ml	N/A	25ml	N/A	N/A
Percoll	Sigma (P-1644)	90.8ml	N/A	N/A	N/A	N/A	N/A
Iso-osmotic Percoll	(As prepared in column 1)	N/A	70ml	33.5ml	25ml	22ml	30ml

*sodium bicarbonate added according to manufacturer instructions before use.

Note: All Percoll solutions, including iso-osmotic Percoll, should be made fresh and used within several days of preparation. Only the 10x HBSS that has been adjusted with sodium bicarbonate can be maintained at 4°C for extended periods of time.