

ABSTRACT

HOOD, SYLVIA FAYE. Monocyte-derived DCs from FIV+ Peripheral Blood Induce Greater CD8+ T cell Proliferation than those from Uninfected Animals. (Under the direction of Dr. J. Fogle, Dr. P. Hess, Dr. S. Laster and Dr. S. Clarke.)

Dendritic cells (DCs) are antigen-presenting cells that have been utilized to enhance CD8+ memory T cell mobilization in response to pathogen-associated peptides for enhancement of vaccine efficacy. CD8+ T cells are responsible for the elimination of viruses during acute viral infection and control of viremia during chronic viral infection. For lentiviral infections such as HIV and FIV, dendritic cell vaccines may be useful in boosting CD8+ T cell function. We asked if there was a difference in DC capacity to stimulate CD8+ T cell proliferation in uninfected control cats when compared to chronically infected FIV+ cats. In this study, we generated DCs from peripheral blood monocytes in vitro by sorting based on forward versus side scatter and treating cells with IL-4 and GM-CSF over the course of 6 days, adding LPS to stimulate maturation after the first 72 hours. We confirmed the identity and maturation status of cells by cytologic examination. We then assessed differences in CD8+ T cell proliferation in the presence of sorted monocytes, immature dendritic cells (iDCs), and mature dendritic cells (mDCs). We demonstrate that CD8+ T cell proliferation is enhanced in the presence of DCs from FIV+ animals. The average relative proliferative indices for CD8+ T cells cultured with monocytes, iDCs, and mDCs were increased by 47.5%, 54.1%, and 50.9% respectively for FIV+ samples over FIV- samples. Additional research is required to ascertain whether this characteristic is related to the hyper-functionality of DCs during chronic immune stimulation or expansion of the FIV-specific CD8+ memory population in culture.

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Monocyte-derived DCs from FIV+ Peripheral Blood Induce Greater CD8+ T cell
Proliferation than those from Uninfected Animals

by
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DEDICATION

This thesis is dedicated to my grandfather Larry Guinn for his support throughout my education and his encouragement to take life by the horns and realize my own dreams and ambitions.

BIOGRAPHY

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ABBREVIATIONS

AIDS: Acquired immunodeficiency syndrome

APC: Antigen presenting cell

ARC: AIDS-related complex

CCR(#): Chemokine receptor

CCL(#): Chemokine ligand

CD(#): Cluster of differentiation

CFSE: Carboxyfluorescein succinimidyl ester

ConA: Concanavalin A

CTL: Cytotoxic T lymphocyte

CXCR(#): Chemokine receptor

DAP-12: DNAX activation protein of 12 kDa

DCs: Dendritic cells

DC-SIGN: Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing

Non-integrin

EIAV: Equine Infectious Anemia Virus

ELISA: Enzyme-linked immunosorbent assay

Env: Envelope protein

ER: Endoplasmic reticulum

FAIDS: Feline Acquired Immunodeficiency Syndrome

FDCs: Follicular dendritic cells

FIV: Feline immunodeficiency virus

FSC: Forward scatter

GM-CSF: Granulocyte macrophage colony stimulating factor

Gag: Group specific antigen

gp120: Envelope glycoprotein 120

HIV: Human immunodeficiency virus

ICAM: Intercellular Adhesion Molecule

iDCs: Immature dendritic cells

IL(#): Interleukin

INF: Interferon

LPS: Lipopolysaccharide

LTR: Long terminal repeat

mDCs: Mature dendritic cells

MHC: Major histocompatibility complex

MIP: Macrophage inflammatory protein

Nef: Negative Regulatory Factor

OrfA: Open reading frame A

PAMPs: Pathogen-associated molecular patterns

PBMCs: Peripheral blood mononuclear cells

pDCs: Plasmacytoid dendritic cells

PLGA: Poly(D,L-lactide-co-glycolide)

Pol: DNA polymerase

RANTES: Regulated upon Activation, Normal T Expressed and presumably Secreted

RPI: Relative Proliferative Index

SIV: Simian Immunodeficiency Virus

SPF: Specific pathogen free

SSC: Side scatter

TAP: Transporter associated with antigen processing

Tar: Trans-activation response element

Tat: Trans-Activator of Transcription

TCID₅₀: Tissue culture infectious dose 50

TCR: T cell receptor

TDF: Tenofovir disoproxil fumarate

Th2: T helper 2

TLR(#): Toll like receptor

TNF α : Tumor necrosis factor alpha

VIF: viral infectivity factor

Vpr: Viral protein R

Vpu: Viral Protein Unique

LITERATURE REVIEW

1.1 Dendritic cells: immunological significance.

Dendritic cells (DCs), which arise from CD34⁺ hematopoietic progenitors in the bone marrow, make up 1% of cells in the peripheral blood but serve a variety of vital roles in the immune system [1, 2]. Certain subsets of DCs can directly stimulate B cells to make antibody or drive their differentiation towards plasma cells [2, 3]. For example, follicular dendritic cells (FDCs) are directly involved in the maintenance of activated B cells, and interstitial dendritic cells are known to induce differentiation of naïve B-cells [2, 3]. DCs also play a role in the generation of Treg cells and the maintenance of tolerance and aid in the release of cytokines to stimulate antiviral processes, pro-inflammatory responses, and T cell development [4, 5, 6, 7, 8]. One of the most important functions of dendritic cells in the immune system is their antigen-presenting capacity [1, 2]. Their role in antigen-dependent stimulation of T cell responses is critical for immunity under normal conditions. In the case of infection by human immunodeficiency virus (HIV) and similar lentiviruses, their trafficking of antigens to the lymph nodes may be manipulated as a catalyst for systemic infection [9].

Dendritic cells acquire antigen from a variety of sources, including exogenously and endogenously, and migrate to the lymph nodes to induce activation of T cells [10, 11]. Immature DCs possess a high capacity for antigen processing and a low concentration of surface molecules associated with T cell activation, but upon maturation, they become potent presenters of antigen and up-regulate expression of major histocompatibility complex

(MHC), co-stimulatory molecules for formation of the immunological synapse, and chemokine receptors for migration to and entry of the lymphoid organs [5, 10]. Maturation follows introduction to a variety of pathogen-associated molecular patterns (PAMPs), cytokines, and T cell-derived signals [5]. Dendritic cells are unique among the antigen presenting cells in the number of MHC molecules (10-100 times higher than monocytes and B cells) that they are able to present to induce T cell clustering and activation [3].

Migration of DCs from the periphery to the lymph nodes is made possible through PAMP and cytokine-induction of activation, augmenting the chemotactic responses of cells to the lymphoid areas [12]. Cytokines such as tumor necrosis factor and IL-1 and chemokines such as MIP-1 α (ligand for CCR1), RANTES (ligand for CCR1), and MIP-3 α (ligand for CCR6) may all play a role in the migration of DCs [1, 3, 13]. Most importantly, migrating DCs up-regulate CCR7 to respond to MIP-3 β and CCL21. Since these are attractants for both mature DC (mDC) and T cells, they may help to bring them together in the lymphoid organs [3]. Expression of chemokine receptors is not sufficient for migration, however, and requires amplification by signaling intermediates such as DAP-12 [14]. In addition, CD40 plays a special role in migration, since its weak signaling allows the expression of CCR7 and associated chemotaxis, but strong signaling, usually only present in lymphoid areas, immobilizes the cells for increased interaction with T cells and induces cytokine-signaling [14]. This lack of mobilization triggers the formation of tightly packed clusters of cells for stimulation. Probing from their immobilized position allows DCs to contact up to 5,000 T cells per hour [15].

Once in lymphoid areas, DCs interact with CD4⁺ T cells and CD8⁺ T cells to induce immune activation through MHC and co-stimulatory molecules, which interact with the T cell receptor (TCR) and other factors at the site of the immunological synapse [16]. MHC class I, which interacts with CD8⁺ T cells is generally thought to express endogenous antigens, while MHC class II, which interacts with CD4⁺ T cells presents exogenous products, but the two systems cross talk, allowing presentation of exogenous peptides by MHC class I and endogenous peptides by MHC class II. This action is important for the priming of CD8⁺ T cells against pathogens [5, 9, 17, 18, 19, 20, 21, 22]. This cross-presentation is typically dependent on proteasome processing and antigen escape from endosomes (perhaps triggered by the incomplete merging of compartments) and loading onto MHC I following transporter associated with antigen processing (TAP) transport to the endoplasmic reticulum (ER), although fusion of phagosomes with the ER has also been suggested as a method of transport [17, 18]. MHC I interacts with TAP by tapasin, and the antigen stabilizes the complex as it is transported to the cell surface by the golgi apparatus [17, 18]. This process is especially important since CD8⁺ T cell production of cytokines, such as INF γ and TNF α , is involved in directing the immune response against intracellular pathogens [5]. Dendritic cells are unique in the efficiency with which they are capable of cross-presenting antigens acquired exogenously, including allogeneic peptides, tumor antigens, and viral components from infected apoptotic cells [5]. They also display a strong phagocytic ability, contributing to their role as an excellent stimulator of CD4⁺ and CD8⁺ T cell responses [9]. In the case of HIV in particular, DCs are capable of presenting acquired

antigen from infected dead cells through both MHCI and MHCII, leading to efficient priming of both CD4⁺ and CD8⁺ T cell subsets [9]. In addition, their expression of C-type lectins increases their probability of uptake of many kinds of pathogens, enhancing the presentation of viral peptides to CD8⁺ T cells [17]. In the case of HIV, it has been demonstrated that this presentation occurs even with virions lacking the ability to actively replicate within DCs upon phagocytosis [9]. Mice depleted in CD11C⁺ dendritic cells were severely handicapped in their ability to elicit CD8⁺ T cell responses against active infection by *L. monocytogenes*, demonstrating the importance of DCs for the purpose of CD8⁺ T cell priming [22]. These basic elements of DC function provide a framework via which the role of DCs in HIV pathogenesis may be assessed.

2.1 Dendritic cells: Role in HIV pathogenesis

HIV is a lentivirus that replicates in human CD4⁺ T cells and other cell types such as macrophages and DCs that exhibit surface expression of CD4, causing immune dysfunction and death by opportunistic infections [23]. When HIV enters the body at the mucosal barrier, some of the first cells that it makes contact with are DCs. HIV readily travels within DCs after initial internalization of virus particles [9].

Dendritic cells acquire HIV antigens and virions for presentation via a number of mechanisms. DCs can sample HIV antigens from the debris of necrotic cells and apoptotic cells, providing a more potent antigen source as their concentrated cytosolic contents have not yet been expelled [9, 21, 24]. DC can also acquire pieces of membrane and cytoplasm from living neighbors by use of scavenger receptors in a process known as nibbling [19, 25].

DC-SIGN and other C-type lectins also provide a mechanism of virion acquisition by DCs [26, 27, 28, 29]. DC-SIGN has a high affinity for HIV-1 gp120, but it is not the only C-type lectin that interacts with the virus, with other examples including BDCA-2 on plasmacytoid DCs (pDCs) and CD23 on monocyte-derived DCs. C-type lectins also bind to Ebola virus, mycobacterium tuberculosis, and helicobacter pylori among other pathogens [27]. This interaction is also observed in Simian Immunodeficiency Virus (SIV) infection [30]. In the case of HIV in particular, attachment of virus to C-type lectins and ferrying to the lymph nodes may increase interaction of virions with T cells when DCs and T cells come into contact [27]. In addition, DCs sampling through tight epithelium may capture HIV-1 via gp120 DC-SIGN interaction, providing a mechanism by which virus can cross epithelial barriers [31]. DC-SIGN causes internalization of virus into non-lysosomal compartments, and while many of the virions are degraded, a sizeable portion remains infectious. Some of these may be directly transferred over the infectious synapse to CD4+ T cell hosts [32, 33, 34].

Once DCs form a synapse with T cells, the virus traffics to the site of contact between the two cells [35, 36]. The cytoskeleton is important for the formation of immunological synapse. Levels of DC-SIGN at the interface are not significantly higher than in the distal regions of the cell. In spite of this, there are significantly more cells in which over 90% of virus particles are trafficked to the immunological synapse between T cells and DCs, and in a portion of DCs, all of the virus is recruited to the contact point, providing a site for viral

transmission[37]. Viral gp120 may then bind to CCR5 and CXCR4 co-receptors along with CD4 to transmit viral RNA to T cells [38].

Active infection of dendritic cells may also aid in the transfer of virus from DCs to T cells [39]. In human monocyte-derived dendritic cells, HIV can replicate for up to 45 days, providing a reservoir for transmission. It is interesting to note that HIV-2, which infects DCs much less readily than HIV-1 also has a lower pathogenicity [40]. The difference in viral ability to bind CCR5 on DCs contributes to this difference [41, 42]. HIV-infection causes down-regulation of CD4, CD1a, and MHC class 1 on DCs, but does not affect the number of MHC class II complexes and co-stimulatory molecules required for formation of an infectious synapse with CD4⁺ T cells [43]. Thus, even low-grade infection of DCs is able to trigger spikes in infection upon cell migration to the lymph nodes. In vitro infection of T cells upon co-culture with infected DCs is vigorous [44]. It appears that immature dendritic cells (iDCs) allow active replication, which is not usually observed in mDCs until they form syncytia with T cells [45]. Other lentiviruses such as SIV, also appear to use active infection of DCs to their advantage [30].

Two broad categories of transport are recognized: trans-infection and cis-infection [46]. In trans-infection, de novo production of virus does not occur, and the DC functions solely as a transport system for HIV [46]. When DCs and T cells interact using the usual components of the immunological synapse, they inadvertently create an infectious synapse for HIV to cross [28, 40]. In addition, endocytosed HIV can also gain access to endosomal multivesicular bodies, allowing it to be exocytosed from the cell in vesicles called exosomes

[47, 48]. Exosomes are capable of presenting MHC molecules on their surface as well as B7-1, B7-2, and ICAM-1, for interaction and fusion with T cells [49,50].

In contrast, cis-infection of DCs incorporates active replication of virus in dendritic cells with eventual budding of virus particles or formation of syncytia [46]. The extent of cis-infection in vivo is still not completely understood, and this mechanism is not required for dissemination of virus. However, it is likely that both trans-infection and cis-infection contribute to viral transmission [46, 51].

HIV infection also lowers the number of DCs as a whole in circulation [52, 53, 54, 55]. This decline is multi-factoral. Direct killing by viral infection may contribute [40]. For example, HIV Vpr, a 14 kDA viral protein, alters cellular proliferation and differentiation and increases apoptosis of infected cells [56]. Indirect contributions to DC decline are also present during HIV infection. Lowered IFN α production, related to viral suppression of TLR7 and TLR8 activation on plasmacytoid dendritic cells (pDCs), impedes the differentiation of myeloid DCs from monocytes or other precursors [57, 58]. In addition, HIV Tat, a viral transcriptional transactivator, stimulates monocytes to produce anti-inflammatory IL-10 and programmed cell death 1 against cells such as DCs [59]. Chronic immune activation may also induce tolerance of dendritic cells to additional stimulus [59]. Finally, up-regulation of CCR7 on DCs during infection may cause more of them to be located in the lymph nodes than the peripheral blood, indicating a change in bodily location as a contribution to the decline of DC in circulation [60].

The quality of retained DC populations is critical for maintenance of immunity. The stimulatory capacity of DCs is vital for the upkeep and expansion of T cell populations during chronic infection. For this reason, the impact of lentiviral infection on DC function is an important consideration. During chronic HIV infection, myeloid DCs retain their ability to produce IL-10, IL-12, and β -chemokines whether the patient is being treated with antiretroviral therapy or experiencing AIDS and may even exhibit hyperactivity in their production of pro-inflammatory cytokines such as IL-12, IL-15, and IL-18 in the acute stages of infection [10,11, 59, 61]. In particular, the status of IL-12 production by DCs throughout infection is critical for induction of T helper cells, so the continued capacity to generate this cytokine is of special importance [59]. However, potential phenotypic and functional alterations to DCs resulting from active HIV infection are controversial. In spite of relocation of DCs within the body during chronic infection, some studies have indicated little difference in the morphology of DCs or their ability to induce T cell activation, while others have suggested that viral infection may inhibit DC maturation [59, 61]. These facts are seemingly contradictory, as mature dendritic cells (mDCs) are more potent activators of T cell activation than immature dendritic cells (iDCs). The unaltered cytokine profiles of monocyte-derived dendritic cells from HIV-infected individuals has interesting implications, especially when compared to cytokine imbalance observed in PBMCs from the same patients, highlighting possible directions of therapeutic approach [61]. Comparable IL-12, IL-10, and β -chemokine expression and similar concentrations of co-stimulatory molecules suggest conserved stimulatory abilities, which are further supported by maintained capacity

for allogeneic stimulation [61]. If dendritic cells remain potent stimulators of T cell activation in the face of chronic lentiviral infection, monocyte-derived DCs could provide an antigen-presenting source for stimulation of naïve T cell responses in infected patients for immunotherapy [61]. The expansion of T cell populations recognizing viral peptides provides a potential mechanism for immune control, and this response is dependent on dendritic cell quality.

In summary, dendritic cells are associated with a variety of immune mechanisms for HIV transport and transmission to T cells that may or may not involve infection of the antigen-presenting cells. HIV also lowers antiviral response and dendritic cell numbers to its own advantage, though it may not alter the cytokine expression of these cells.

3.1 Feline Immunodeficiency Virus: A model for HIV

Feline Immunodeficiency Virus (FIV) is an HIV-related lentivirus found in cats that generates immune dysfunction culminating in an acquired immunodeficiency syndrome (AIDS) [62]. It has been utilized as an alternative to SIV for modeling HIV infection. All three of these viruses are lentiviruses, non-oncogenic retroviruses that are distinguished by persistent infection coupled with long incubation periods [63]. In order to assess the usefulness of FIV as a model organism, its commonalities to HIV must be assessed, and its utility compared to SIV must be scrutinized.

HIV and FIV are both incapable of surviving for long periods outside of the host, so these viruses require transmission through wounds or a mucosal layer. While in HIV, sexual transmission is a common route of transfer, in FIV, the virus is preferentially transmitted in

saliva to bite wounds [62] FIV is present in seminal fluid from infected animals and can be used to transfer virus experimentally, but natural venereal transmission is less well-documented [64]. In general, more virus is required for mucosal transmission in comparison to transmission via bite wounds [62].

FIV shares traits with HIV and many other lentiviruses. It is composed of an envelope with Env proteins that are important in receptor interactions, a lipid bilayer, and a core containing Pol and Gag proteins. Internally, matrix, capsid, and nucleocapsid components are all present. Its provirus contains two long terminal repeats (LTRs) just as HIV does. In addition, it performs the elaborate alternate splicing seen in many lentiviruses [62]. Inaccuracies in the replication process are prevalent in both HIV and FIV and result in many new mutations and opportunities for drug evasion [65]. FIV and HIV share many common genomic traits (Figure 1) [66]. FIV does not encode the Vpr, Vpu, and Nef genes present in HIV and also lacks a Tat/Tar system for regulating viral gene transcription, opting instead to regulate via a gene product called OrfA that may be homologous to Vpr in HIV (Table 1). FIV and HIV proteases are very similar at the crystallographic level, but are most conserved in an amino acid sequence located in the binding pocket [67]. Indeed, FIV and HIV proteases can both partially cleave products from each other [67]. FIV has been used for the development of protease inhibitors that have shed light on HIV substrate sensitivities. The similar structures of both viruses in their cores, Gag proteins, and matrix elements provide a good system for establishing possible intervention methods, especially since well-preserved traits are more likely to be important for viral replication and transmission [67].

The viral infectivity factor (VIF), env, and pol genes of the two viruses also share commonalities and encode for similar products, even responding to the same inhibitors [67]. The two viruses both depend upon proper cleavage of Gag-Pol in the production of new virus particles, shedding light on another possible avenue for inhibitor development [67]. FIV has also been utilized to model binding to CXCR4 [67].

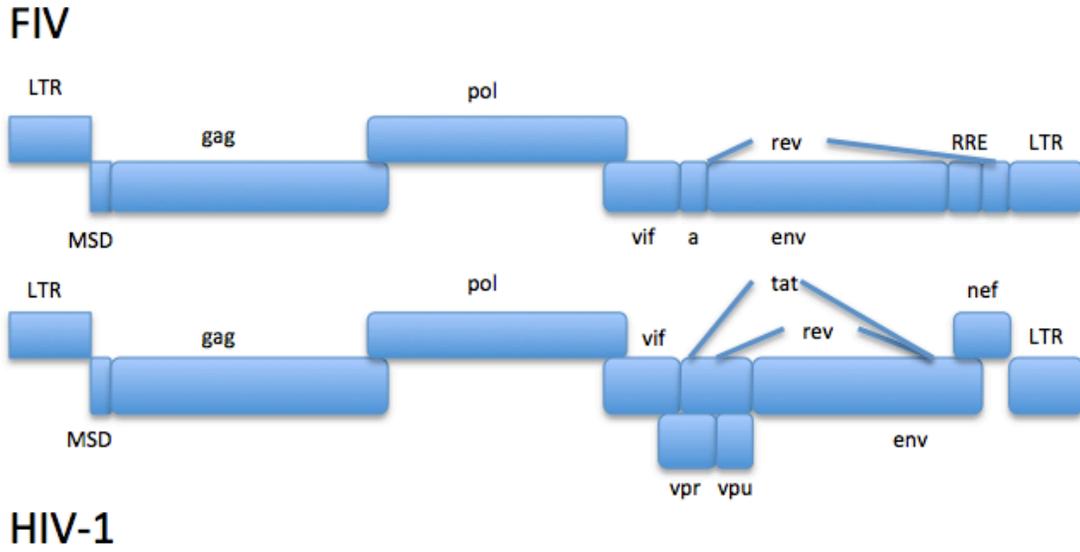


Figure 1. Comparison of FIV and HIV-1 genomic organization [66].

Table 1. Functions of HIV viral proteins with roles analogous to FIV OrfA.

		Function
HIV	Vpr	Involved in nuclear import of viral RNA and proteins, stimulates virus transcription, regulates apoptosis of infected cells, can generate cell cycle arrest [68].
	Vpu	Contributes to membrane permeabilization and aids in the release of new viral particles [69].
	Nef	Interferes with signal transduction pathways, alters expression of membrane proteins [70].
	Tat/Tar	Tat is an activator of transcription and translation that binds to TAR, an RNA structure at the 5' end of the viral mRNA [71].
FIV	OrfA	May be homologous for Vpr, shares functions with Vpr and Tat. Important for RNA post-transcriptional modification and protein ubiquitination. Like TAT, it is an activator of transcription and translation, but it does not bind to a TAR element. Like Vpr, it is involved in cell-cycle arrest and viral release from cells [72].

FIV infects a variety of tissues and cell types over the course of infection. At one week post-infection, FIV can be isolated from all lymphoid tissues and the salivary glands, with the highest concentration of viral DNA and RNA detected in the bone marrow, thymus, and lymph nodes [62]. By three weeks post-infection, all nonlymphoid tissues also yield positive cultures, with high concentrations found in the interstitium [62]. While the virus is present in high quantities in the bone marrow early on in infection and is capable of infecting megakaryocytes, it does not appear to do so efficiently. It can, however, infect follicular dendritic cells and macrophages [62]. In spite of the plasticity of FIV for infecting various cell types, 75% of virus-positive cells isolated from acute infection are T lymphocytes, a trend also observed in HIV infection [62]. FIV, like HIV requires T cell infection and activation for productive infection [62, 67]. However, while FIV may preferentially infect CD4+ T cells, it also infects B cells and CD8+ T cells because it does not utilize CD4 as a primary binding receptor, instead binding to CD134 and co-receptors such as CXCR4 [67]. HIV, in contrast, binds CD4 and a co-receptor such as CCR5 or CXCR4 [73]. In spite of this, FIV is inhibited by some of the same anti-viral compounds used against HIV, such as AMD3100 (plerixafor), which blocks binding to CXCR4 [67].

The clinical progression of infection is also similar between HIV and FIV with a short acute phase lasting just a few weeks and an asymptomatic period lasting years [62]. Cats acutely infected with FIV demonstrate enlarged lymph nodes and following a long asymptomatic phase, weight loss and AIDS-related complex (ARC), characterized by chronic secondary infections [62]. Feline Acquired Immunodeficiency Syndrome (FAIDS), like

AIDS, is characterized by severe secondary infections, opportunistic infections, tumors, neurologic abnormalities, and leukopenia [62]. Leukopenia, which is present in the majority of cats with FAIDS, may further exacerbate opportunistic infections, making them resistant to treatment [62]. In advanced HIV infection, leukopenia may be present in up to 85% of patients [74].

Several abnormalities are observed in FIV+ infected animals. Over the course of infection, CD4+ T cells begin to decline, while CD8+ T cell populations are expanded, leading to an inversion of the CD4+/CD8+ T-lymphocyte ratio observed in uninfected animals [62]. The IgG concentration in the serum and saliva are also elevated along with high concentrations of immune complexes in the blood stream. Numbers of IL-2 receptors on both CD4+ and CD8+ T cells are also lowered [62]. As in HIV infection, the lymph nodes become hyperplastic in the early stages of infection coupled with hypercellularity with associated expansion of the T cell zone. This status is reduced during the asymptomatic phase, and by the final stages of infection, lymph node depletion is present [62, 67]. Plasma cell concentrations in the lymph node germinal centers gradually increase over the course of infection. Atrophy of the thymus, kidney abnormalities, oral lesions with gingivitis, villous atrophy, degenerative cardiomyopathy, and lesions in other organs such as the liver may also be present [62]. Brain lesions observed in infected animals are also similar to those observed in AIDs patients [62]. As in humans, the age of the infected individual plays a role in virus pathogenesis [62]. The rate of progression to AIDS is positively correlated with age [75].

FIV also generates dysfunction in T cells and DCs similar to that observed during HIV infection. Both HIV-infected patients and FIV-infected cats display high frequencies of apoptotic T cells in the lymph nodes, related to increase of programmed cell death [76]. As with HIV, CD8+ T cells aid in the maintenance of the asymptomatic phase [77]. CD8+ T cells suppress the synthesis of FIV mRNA. This lowers not only the production of new viral particles but also the expression of viral proteins on the surface of CD4+ T cells. The combined effect of reduced CTL activity against CD4+ T cells and decreased plasma viremia serves to maintain the CD4+ T cell population in vivo [78]. In addition, feline dendritic cells may become infected at a low rate [79, 80]. The virus is capable of transferring from dendritic cells to CD4+ T cells, and as with HIV, the combined effect of viral transfer with activation of T cells by DCs across the immunological synapse results in increased viral replication in infected T cells [80]. As with HIV, DCs from FIV-infected cats express lower amounts of CD1a, a maturation marker, indicating inhibition of DC maturation [81]. CD11b, which is involved in leukocyte adhesion and migration, is up-regulated on DCs and may be related to chronic immune activation [81].

There are pros and cons for using FIV as a model over SIV. Both of the viruses share morphological, biochemical, and genetic commonalities with HIV that make them useful models. However, FIV is not infectious to humans, and cats, unlike rhesus macaques, are a readily available animal for research and easy to house. In contrast, SIV is more closely related to HIV and shares more of its nucleotide sequence, however the length of the genome of all three lentiviruses is similar, and the gene sequence codes for many of the same

components [62, 65, 67]. SIV, like HIV, binds to CD4 as its main receptor and CCR5 as a co-receptor [82].

SIV infection in rhesus macaques of Indian or Chinese origin is a common model for HIV pathogenesis [82]. Disease progression in these organisms mirrors that in humans with decline in CD4⁺ T cell numbers, immune suppression, an increase in opportunistic infections, and later, an AIDS-like syndrome [83]. However, the two macaque model organisms, despite being of the same species, display variant susceptibility to SIV. For example, in response to SIV strain mac239, Chinese rhesus macaques display much higher antibody titers and a >50% CD4/CD8 ratio not observed in Indian macaques [82]. In addition, neutropenia during acute infection is more advanced in Indian macaques than Chinese macaques, leading to increased viral susceptibility [82].

SIV is similar enough to HIV that viable chimeric virus can be established for analysis of HIV proteins in vivo [82]. Also, the gastrointestinal tract is a major site of replication for both HIV and SIV, leading to a loss of memory CD4⁺ T cells in this area that can be modeled using infected rhesus macaques [82]. A variety of vaccine and cytokine-based therapies have been modeled using SIV; for example, tenofovir disoproxil fumarate (TDF), an antiretroviral drug currently being used to treat patients, was able to control infection of Chinese rhesus macaques by chimeric virus [82]. As with HIV and FIV, levels of SIV-specific CD8⁺ T cells contribute to immune control, with rapid expansion of virus occurring upon depletion of this population of cells [82]. SIV also provides an excellent

model for immune escape by virus, as mutation can make it resistant to established CD8+ T cell epitopes [82].

In short, HIV and FIV share similarities in structure and protein composition, immune interaction, and disease course. While SIV has some advantages over FIV as a disease model, FIV provides an alternative outlet for illuminating pathogenesis, improving antiviral therapies, and developing potential vaccines.

4.1 T cell proliferation: Immunological Significance

T cell activation and proliferation have important immunological effects for controlling infection by triggering the maturation of B cells and directly killing infected cells. CD8+ T cells in particular hold special importance in HIV for their ability to detect and eliminate infected cells. [65]. Understanding of T cell immune regulation provides a framework via which one can better assess the role of lymphocyte function and dysfunction during HIV pathogenesis. Dendritic cells activate and expand CD8+ T cells, and differences in proliferation between naïve and memory populations are important qualities for the preservation of immunity.

The development of CD8+ lymphocyte responses can be categorized into three phases: expansion of cells followed by resolution of infection, population decline, and maintenance of long-term memory lymphocytes [82]. The continuous production of effector cells throughout infection and preservation of clones are essential for immune function [84]. Antigen-specific CD8+ T cells are capable of inducing apoptosis in cells presenting recognizable peptides. Cytotoxic T lymphocytes (CTLs) usually kill their targets, but they

also form an immunological synapse with them over which they can transmit granules and pick up parts of the target membrane [85, 86]. The more membrane that a CD8⁺ T cell picks up from subsequent targeted killings, the more likely it is to be targeted itself, providing a mechanism for controlling the CTL response [85, 86]. CD8⁺ T cells present at the mucosal site of infection help to fight HIV upon first invasion, and many virus-specific epitopes of CD8⁺ memory T cells are produced over the course of chronic immune activation in order to control and suppress viral replication and expansion [31, 87].

Dendritic cells form synapses with CD8⁺ T cells for subsequent activation [5]. While, MHC class I presentation is usually involved with presentation of endogenous peptides, cross-talk occurs between the MHC class I and MHC class II pathways, allowing presentation of exogenous peptides to CD8⁺ T cells [5, 17, 18]. Antigen escape from endosomes, fusion of endosomes with the ER, and proteasome-processing may all contribute to this cross-presentation of antigen [17, 18]. Antigen loading to MHC class I and transport to endosomal compartments is termed the vacuolar pathway, while antigen leakage into the cytosol and subsequent transfer for MHC class I processing is termed the cytosolic pathway [17, 88]. The cytosolic pathway contributes to the majority of cross-presentation [88]. Incomplete fusion of phagosomal vesicles may contribute to antigen escape [18]. MHC class I molecules, which assemble in the ER, bind antigenic segments of 8-10 amino acids in length. TAP translocates peptides to the ER, where peptides binding occurs. Tapasin facilitates the exchange of peptide from TAP to MHC class I molecules. Then, MHC class I molecules are ferried through the Golgi apparatus to the plasma membrane [89]. Following

uptake of FIV by DCs, TAP may bind to FIV viral proteins that have entered the cytosol after compartmental escape, carrying the peptides to the ER for processing and formation of MHC class I complexes [5, 17, 18, 89]. DCs and CD8⁺ T cells then communicate across an immunological synapse [5].

Relatively low numbers of DCs are needed to elicit proliferation and differentiation of T cells [90]. The specificity of interaction of peptide-MHC class I complexes with lymphocytes determines the duration of interaction between the two cells [91]. T cells search for the synapses that form optimal interaction, shifting from one antigen-presenting cell to the next [92]. A long enough period of interaction must occur to activate the T cell, but the interaction must not be strong enough to limit the dissociative ability, since the maximum amount of proliferation and cytotoxic activation will occur when DCs can contact as many cells as possible [4, 92]. Intimate cross-talk between DCs and T cells is required for full activation of T cell response with cytokine production and co-stimulation enhancing the proliferation of target T cells, though the minimum requirement for partial activation and proliferation of T cells is an intact membrane with MHC and co-stimulatory molecules [16, 93]. Once committed to proliferation, CD8⁺ T cells acquire cytotoxic functions and proliferate rapidly in response to IL-2 [92, 94].

There are many differences in naïve CD8⁺ T cells and antigen-specific memory CTLs. For example, immediate degranulation upon antigen encounter is a feature of memory and not of naïve CD8⁺ T cells [95]. Proliferation of naïve CD8⁺ T cells is dependent upon interaction with certain TCR ligands, whereas memory populations can divide in the absence

of MHC binding [96, 97]. However, TCR triggering in memory populations is efficiently coupled to signal transduction pathways, which boost proliferation. Thus, memory T cells require only a fraction of the stimulation time of naïve T cells [92].

Central memory, effector memory, and effector CD8⁺ T cells are all antigen-specific subsets. CD45RA⁺ CCR7⁺ cells make up the naïve subset [84]. Central memory cells are negative for CD45RA but positive for CCR7 allowing them to respond to CCL21 in secondary lymphoid organs; like naïve cells, they lack effector function. Cells negative for CD45RA and CCR7 are termed effector memory cells [84]. The lack of CCR7 prevents efficient transport to secondary lymphoid organs, indicating a peripheral role, and they exhibit rapid production of TCR-induced IFN- γ and perforin-containing granules [84]. The effector population, which is CD45RA⁺ and CCR7⁻ also elicits rapid production of IFN- γ and stimulates perforin formation [84]. All of these cell types are closely tied and in some cases involved in each other's production and maintenance.

Memory T cells have rapid turnover, so chronic stimulation may insure their survival. Re-exposure to antigen and stimulation with cytokines (memory CD8⁺ T cells have increased concentrations of cytokine receptors for this purpose) may play a role in this population maintenance. IL-15, for example, is a strong stimulator of CD8⁺ T cells. Type-1 interferon production triggered by a variety of PAMPs increases IL-15 production and subsequent proliferation of CD8⁺ T cells [98]. Indeed, IL-15 is purported to support the expansion of antigen specific CD8⁺ T cell populations during immune response to infection [96]. IFN- γ , IL-12, and IL-18 also induce CD8⁺ T cell proliferation. IL-2 is a mediator of

clonal expansion. Stimulation of T cell receptors (TCRs) readied memory populations for IL-2 stimulation for 7-10 subsequent cell cycles [84]. Interestingly, however, IL-2 stimulation also generates an apoptotic response following repetitive ligation of the cell's TCR, a process known as activation-induced cell death (AICD). CD27 and other co-receptors such as CD28 are also involved in IL-2-dependent expansion of populations [84]. While antigen specific responses trigger proliferation, bystander stimulation during an infection may also activate T cells, even though it is not antigen specific. This may play a role in background proliferation of memory CD8+ T cells [98]. In addition, it is likely that during persistent infection the production of CD8+ effector T cells is dependent on the continued recruitment of new naïve T cells to boost clonal populations and not just on the maintenance of memory populations [84].

In the case of HIV, CD8+ T cell expansion contributes to control of infection in several different ways. As previously mentioned, CD8+ T cells have the capacity to kill virus infected cells. In addition, CD8+ T cells can inhibit the replication of virus in CD4+ T cells [65]. The expansion of antigen-specific lymphocyte populations is associated with positive clinical status [65]. Early containment of the virus in infected individuals is associated with expansion of CTL populations; the importance of this control was demonstrated by rapid progression of SIV infection in monkeys upon blocking of the CD8+ T cell response [65]. Indeed, it appears that mutation away from CD8+ T cell recognition in the later stages of infection contributes to dramatic increase in viral replication [65].

DC-based immunotherapy or vaccines for boosting virus-specific CD8⁺ T cell responses have potential to utilize DC stimulation of T cells to augment HIV therapy [5, 99]. AIDS is associated with a high diversity of CD8⁺ T cell epitopes, so the DC therapeutic approach may aid patients by driving a more focused CD8⁺ T cell response [87]. Antigen-pulsed DCs are short-lived in vivo because they are targeted for elimination by activated CTLs following multiple treatments [100]. In addition, the high mutation rate of lentiviruses can help them to escape established CD8⁺ T cell epitopes by subtle alteration of specific vital antigens recognized by memory populations [5, 87]. For example, a mutation in the Gag CM9 epitope of SIV leads to CD8⁺ T cell evasion and immune decline [87]. Mutation in the HLA-B27-restricted KK10 epitope in late infection is associated with progression to AIDS in humans [87]. It is also important to consider that DCs administered intravenously often preferentially migrate to the lungs, liver, and spleen as opposed to the lymphoid nodes, so alternative methods of inoculation, such as intradermal administration, may need to be utilized. There may also be a risk for autoimmune activation [5]. However, the utilization of DCs to produce memory CD8⁺ T cell populations to effectively combat infection could prove extremely useful for the vaccination against diseases like HIV, in which direct administration of the virus is not recommended. In DC-based vaccination studies against SIV, researchers have already been able to contain lentivirus within the secondary lymphoid organs and reduce viral replication [101]. DC-based therapy against HIV in a recent human study demonstrated a decrease in plasma viremia and an increase in HIV-1 specific T cell responses compared to unvaccinated patients [102]. The therapy was well accepted by

patients, with few side effects. However, out of twelve human DC immunotherapy clinical trials published as of early 2013, half have failed to show any benefit [102]. These trials may have failed because they did not maximize MHC class I presentation by DCs. This is important because recent findings have demonstrated new ways of boosting cross-presentation such as with immune complexes, heat shock proteins, or nanoparticles [103, 104, 105].

In summary, the role of T cell proliferation and expansion is immunologically important and may prove useful tool for combating HIV. Understanding DC-stimulation of T cell activation and differences in naïve and memory response provides platforms via which one can manipulate the body's defense systems for possible therapeutic and vaccination approaches against HIV.

1. INTRODUCTION

Feline immunodeficiency virus infection in cats is an experimental model that is employed to answer questions about HIV pathogenesis and to assess potential vaccine and therapeutic approaches, including those incorporating DCs. DC presentation of lentiviral antigen to T cells serves an important role in immune control of infection. CD8⁺ T cells are especially critical for controlling viral replication, as this population eliminates infected T cells and DCs. In the case of vaccination, the goal is to quickly eliminate virally infected cells before further spread can occur, for example, by killing infected DCs in route to the lymph nodes. In the case of DC immunotherapy, the goal is to boost virus-specific CD8⁺ T cell numbers for better control of infection.

Before producing HIV-specific CD8⁺ T cells from naïve populations for DC-based vaccination or boosting existing memory populations for DC immunotherapy, one must initially analyze the base-line proliferation of CD8⁺ T cells from infected and naïve hosts in co-culture with DCs, since viral infection may alter the proliferation rate of T cells or affect the function or stimulation efficiency of the DCs being applied in co-culture. The functional capacity of DCs may greatly impact T cell activation. All of these elements must be assessed in order to maximize therapeutic efficacy. In this study, we sought to compare proliferation of CD8⁺ T cells in the presence of monocytes, immature dendritic cells (iDCs), and mature dendritic cells (mDCs) generated from peripheral blood mononuclear cells (PBMCs) of healthy and FIV-infected animals. We hypothesized that the chronically stimulated immune system of lentivirally-infected cats might yield hypersensitive DCs that are better stimulators

of a T cell response. Therefore, we proposed that DCs from FIV-infected cats would stimulate greater autologous CD8⁺ T cell proliferation when compared to those from FIV-cats.

Dendritic cells from HIV-infected patients have been demonstrated to remain largely functionally intact. Huang et al demonstrate that HIV-1 infected patients on combination anti-retroviral therapy exhibit DCs capable of stimulating a potent T cell response, with mDCs stimulating better than iDCs [10]. Chougnet et al. demonstrate that in AIDS patients there is no defect in DC production of IL-12, IL-10, and β -chemokines, and cells from healthy and infected patients demonstrated similar morphology, indicating that DCs from chronically infected immune environments may remain phenotypically and functionally intact [61].

Feline DCs can be generated from monocytes isolated and sorted from the peripheral blood and cultured with granulocyte macrophage colony stimulating factor(GM-CSF) and IL-4 [24, 106, 107, 108, 109]. The resulting population can be matured by stimulation with lipopolysaccharide (LPS), which is a component of gram-negative bacterial cell walls. Monocytes may also differentiate into macrophages, so there may also be some contaminating macrophages in the populations.

The monocytes, immature dendritic cells, and mature dendritic cells obtained via cell sorting and subsequent treatment with GM-CSF and IL-4 were co-cultured at a 1:1 ratio with autologous CD8⁺ T cells to assess differences in APC stimulation of CD8⁺ T cell response in FIV⁺ and naïve cats. We utilized carboxyfluorescein succinimidyl ester (CFSE) for

analysis of CD8⁺ T cell proliferation. CFSE, a cell-staining, fluorescent dye partitions itself evenly among daughter cells upon cell division, providing a route via which one may ascertain the division history of cells under certain stimuli by flow cytometry [110, 111].

We show that under co-culture conditions, CD8⁺ T cells from infected and healthy individuals, demonstrate similar proliferative abilities in the presence of IL-2. However, CD8⁺ T cells exhibit distinct proliferation profiles when co-cultured with autologous monocytes, iDCs, and mDCs. T cells from FIV-infected animals proliferate more in the presence of all three cell-types when compared with those from FIV negative cats. This may be related to chronic-immune activation, or perhaps, if cultured monocytes from FIV⁺ cats have already phagocytosed FIV antigens or are exhibiting low grade infection, they are more efficient activators of CD8⁺ memory T cell expansion.

2. MATERIALS AND METHODS

2.1 Cats

Specific pathogen free (SPF), 6 month-old, male cats were purchased from Liberty Research, Inc. (Waverly, NY). FIV+ animals were infected with 5×10^5 TCID₅₀ of cell-free viral supernatant as previously described [109, 110]. Cats that had been infected for 1-3 years were classified as chronically infected. FIV+ animals (n=6) were housed separately from FIV- animals (n=7).

2.2 PBMC Isolation and Cell Sorting

Whole blood was collected in heparinized containers by jugular venipuncture. Peripheral blood mononuclear cell isolation was performed on samples prior to staining for cell sorting. Cells were surface immunophenotyped with anti-CD14 (anti-CD14 FITC: Serotec MCA1568F), anti-CD5 (Clone 572, Feline anti-CD5 APC- M. Tompkins) and anti-CD8 (Clone 357, Feline anti-CD8 PE- M. Tompkins) prior to sorting for monocytes and CD8+ CD5+ T lymphocytes. A Moflo DakoCytomation high speed cell sorter was used to sort cells. Cells were gated based on FSC vs SSC characteristics. Monocytes were isolated by forward versus side scatter, as the desired population was located within a cluster of CD14+ cells without much overlap with CD5+ T lymphocytes. CD5 and CD14 were used to confirm the monocyte gate with most (90%) of the CD14+ cells inside and most (90%) of the CD5+ cells outside the gate (Figure 2). CD8+ T cells were sorted from a CD8+CD5+ population of cells.

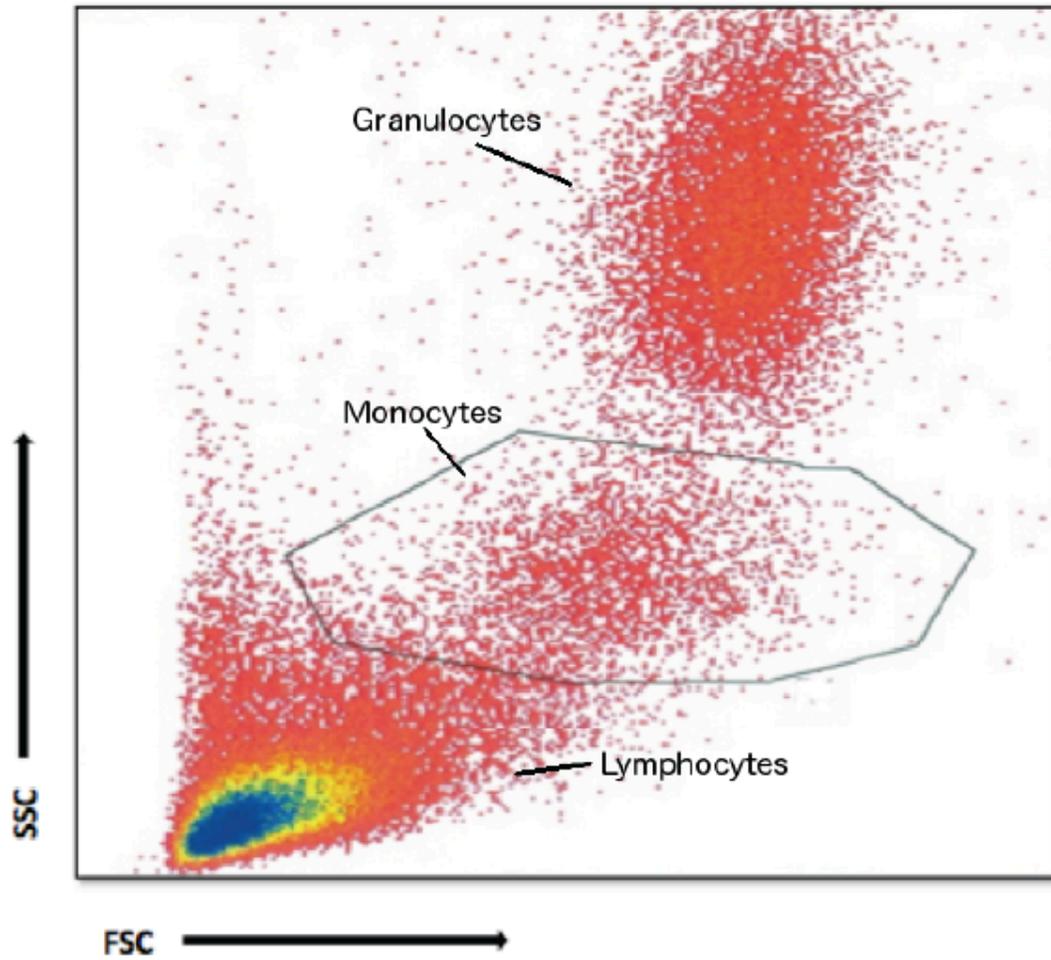


Figure 2. Sorting gate for monocytes. Monocytes were gated by typical FSC vs. SSC characteristics. PBMCs were surface immunophenotyped with anti-CD14 and anti-CD5 prior to sorting, to confirm the monocyte gate with most (90%) of the CD14+ cells inside and most (90%) of the CD5+ cells outside of the gate.

2.3 Monocyte Culturing and Cell Stimulus

Sorted monocytes or CD8⁺ T cells were cultured in RPM1 medium containing 15% FBS, 4mM L-glutamine, 1% non-essential amino acids, 50µg/mL Gentamicin, 1% Penicillin Streptomycin, 2% Hepes, 1% Sodium pyruvate and 50 µM β-mercaptoethanol. All cells were cultured at 2.5x10⁶ cells per mL of culture media in a Costar 48 well plate (1x10⁶ cells in 400 µL per well). CD8⁺ T cells were cultured with 1U/mL IL-2. After 24 hours, wells of monocytes were washed. To one-third of wells, fresh media was added, while to two-thirds of wells media with 1µg/mL IL-4 (Catalogue # 984-FL R&D Systems) and 1µg/mL GM-CSF (Catalogue # 987-FL R&D Systems) was administered. After 48 additional hours, 50µL of media containing 1U/mL IL-2 was added to T cell wells. Fresh media was added to unstimulated monocyte wells. To stimulated monocyte wells, 1µg/mL IL-4 and 1µg/mL GM-CSF were added in 50 µL of media along with 50 µg/mL LPS in half of these wells to induce maturation.

After 72 additional hours of culture, monocyte wells were either scraped in preparation for surface-expression staining, viability analysis, or Diff-quick staining or washed in preparation for co-culture without removing from the dish. T cells were removed from wells, counted, and set aside for CFSE-staining and co-culture.

2.4 Dendritic Cell Characterization and Viability Assays

Monocytes, iDCs, and mDCs were surface immunophenotyped with anti-MHC II Biotin (Serotec MCA2723), anti-MHC I FITC (Washington State B072001/cell line H58A), anti-CD14 FITC (Serotec MCA1568F), anti-B7-1 PE (BD Pharmingen 553769) and anti-

CD5 APC(In house 572) and shaken in the dark for 15 minutes. For cell viability assays, samples were stained with Annexin V APC (BD Biosciences 550424) and Propidium Iodide (Roche Diagnostics 10969300). Samples were assessed via flow cytometry using a Becton Dickson FacsCalibur. Unstained samples were used to assess auto-fluorescence. For cell surface characterization, clusters of CD5+ cells were gated out of the analysis.

2.5 Cytologic Examination

Diff-quick staining was used to characterize monocyte, iDC, and mDC populations at the completion of culture. Samples were centrifuged in a microcentrifuge prior to pipetting onto microscope slides. After drying, slides, were fixed with Diff-quick fixative reagent containing triarylmethane dye and methanol and stained with Diff-quick solution 1 containing xanthene dye, pH buffer and sodium azide and Diff-quick solution 2 containing thiazine dye and pH buffer.

2.6 Autologous Co-culture/Proliferation Assay

CD8+ T lymphocytes were removed from culture and treated with CFSE using the CellTrace CFSE Cell Proliferation Kit (Molecular Probes C34554) according to the manufacturer's instructions. Dendritic cell wells were washed thoroughly without removing the cells. Since 30,000-50,000 DCs were typically harvested, 40,000 autologous T cells were added to each co-culture well with 400 uL media (see recipe in section 2.3), and 1U/mL IL-2. Controls included CD8+ T cells plated with 5 µg/mL ConA and 1U/mL IL-2 and CD8+ T cells plated with 1 µg/mL IL-4, 1 µg/mL GM-CSF and 1 U/mL IL-2. Samples were incubated at 37°C for 96 hours. Cells were gently scraped from wells and assessed via flow

cytometry (Becton Dickson FacsCalibur). Proliferation was assessed via ModFit LT software. The CFSE positive population was gated to remove antigen-presenting cells from the analysis. Control groups were used to establish the position of the cells that have not divided (parent peak) before assessing the proliferative properties of experimental groups.

2.7 Statistical analysis

A Student's T-test was used for pair-wise comparisons of relative proliferative indices. Differences were considered to be significant at $p < 0.05$. Relative proliferative indices were determined by dividing sample proliferative indices by those of the control. The proliferative index is defined as the total number of divisions divided by the number of cells that went into division; cells that did not replicate once were not included in the analysis [114]. This differs from the division index, which assesses the average number of cell divisions that a cell in the parent population underwent, including those cells that did not divide at all [115]. The negative control population was used to establish the approximate location of the parent peak, which defines the population of cells that did not perform any divisions. By employing Gaussian curves based on the halving of fluorescent intensity from the parent peak, Mod-Fit software establishes a distribution of proliferation [114].

3. RESULTS

3.1 Dendritic Cell Characterization

Immature dendritic cells were generated by treating sorted monocytes with IL-4 and GM-CSF. GM-CSF acts on early bone marrow precursors of the myeloid lineage and drives expression of MHC-II, B7-1, and CD40 on monocytes, which can differentiate into DCs [116, 117, 118]. IL-4 is produced during TH2-polarizing immune responses and works with GM-CSF in vitro to promote monocyte maturation into DCs [114]. Monocytes treated with GM-CSF and IL-4 exhibit typical DC morphology with MHC I and II, co-stimulatory molecules such as B7, and CD40 expression, and they also effectively cluster T cells for antigen-presentation [106, 119].

Mature dendritic cells were then generated from iDCs by treating with the pathogen-associated molecular pattern LPS. To ascertain the successful differentiation of our populations, we first sought to characterize the cell surface profiles of the monocyte, iDC, and mDC populations obtained (n=10-14).

Freer et al. previously defined feline monocytes, iDCs, and mDCs by their expression of MHC class I, MHC class II, B7-1, and CD14 [120]. Monocytes have moderate expression of both MHC molecules with no B7-1 expression and high CD14 expression. Immature DCs have increased MHC expression with low B7-1 and CD14 expression. Mature DCs have the highest MHC expression with high B7-1 and high CD14 expression. Both classes of major histocompatibility complex are characteristic of antigen-presenting populations. They are expected to be at their highest concentration on mature antigen-presenting populations

capable of effectively inducing T cell activation, so the concentration of these surface complexes increases following differentiation to the dendritic cell profile and again following maturation. B7-1 on mature dendritic cells binds to CD28 on target T cells; monocytes are negative for this surface molecule, while expression on DCs increases dramatically upon maturation. CD14, a co-receptor for TLR4, is most highly expressed on monocyte and mature dendritic cell populations with lower concentrations on the surface of immature populations [120].

In order to determine whether or not we had successfully generated immature and mature dendritic cells in culture, we addressed whether our populations displayed similar cell surface phenotypes to those defined by Freer et al [120]. The characteristic cell surface expression trends were not observed in the profiles of our isolated monocyte, iDC, and mDC populations. Indeed, little difference could be observed in expression of any of the cell surface molecules between cell types (Figure 3), and there was little difference in the profiles of cells from uninfected and infected cats. There are several explanations for this observation. Since DCs do not typically divide in culture and experience a half-life of a few days, some cell death is expected over the course of 6 days of stimulation; the cell surface profile of apoptotic cells is much more variable than that of healthy cells [121]. In addition, not all of the antibodies utilized were feline-specific, which may contribute to irregularities in binding. We also did not use the same antibodies that Freer et al. employed for their analysis, so differences in binding affinity may be present. Freer et al.'s report suggested that

they experimented with a variety of culture conditions to optimize DC viability, since the lack of proliferation and rapid half-life in DC cultures can critically impact output.

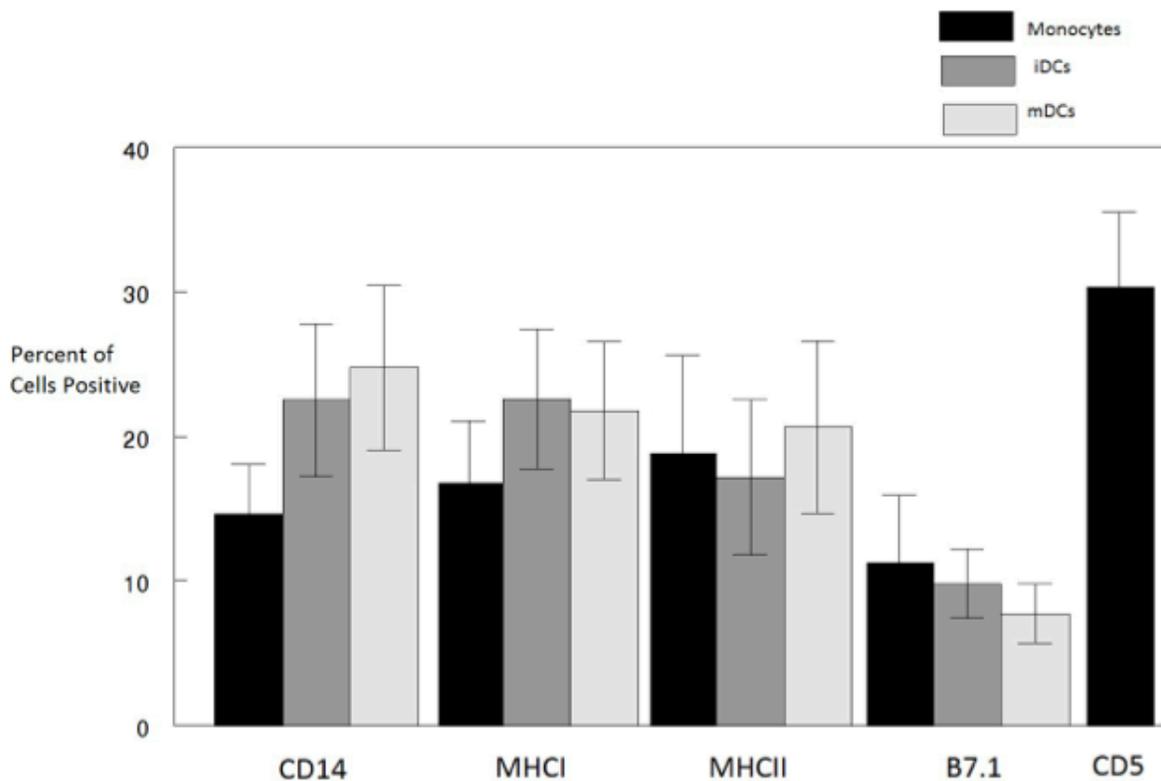


Figure 3. Cell surface profiles of monocytes, iDCs, and mDCs obtained by stimulating a population of monocytes isolated by sorting for forward versus side scatter (90% of CD5+ cells not within gate and 90% of CD14+ cells within gate) (n=10-14). Expression was determined via flow cytometry after establishing an exclusion gate for clustered populations of CD5+ cells. FIV+ and FIV- cats are grouped together as no significant differences were observed between the two.

3.2 Cell populations exhibit monocyte and dendritic cell morphology

Additional methods were required for determination of differentiation status. Cytologic examination was employed to assess physical characteristics of the monocyte, iDC, and mDC populations.

The population of PBMCs sorted according to the forward and side scatter region shown in Figure 1 was also assessed microscopically prior to culture (Figure 4). Monocytes, here visible as large mononuclear cells with crisp edges, were larger than lymphocytes. Granulocytes, such as neutrophils, contained many darkly stained granules. The population sorted by FSC vs SSC cells was approximately 20-30% monocytes, 10-15% lymphocytes, and 40-60% granulocytes (neutrophils). Non-adherent lymphocytes were removed by washing on day two of culture, while short-lived granulocytes do not survive the entire week of culture.

Monocytes with their crisp cell borders and rounded or bean shaped nuclei differ in morphology from iDCs and mDCs. Less well-defined cell borders and increased vacuolation are characteristics of dendritic cells, and these traits become more defined with maturation. It is important to consider that these features exist on a continuum. The monocytes, iDCs, and mDCs all demonstrated these characteristic profiles, with monocytes displaying sharper cell membranes, iDCs demonstrating hair-like dendrites, and mDCs presenting advanced vacuolation (Figure 5).

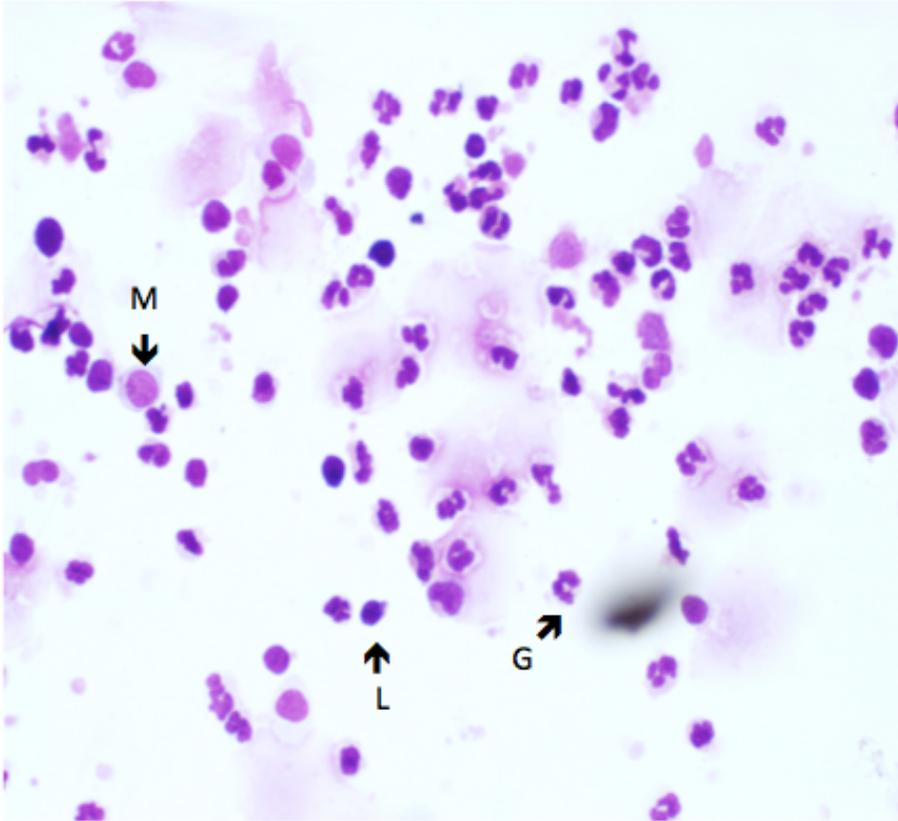
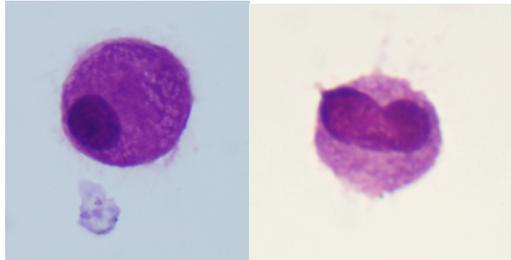
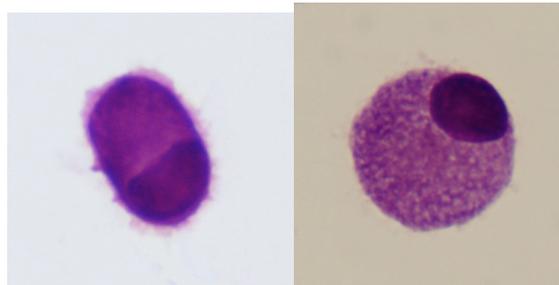


Figure 4. Sorted population characteristics prior to culture (50x). The Diff-quick-stained sample above represents sorted cells from an FIV- cat prior to culture. Monocytes **M**, lymphocytes **L**, and granulocytes (neutrophils) **G** are marked for reference. Monocytes made up 20-30% of the population, neutrophils made up 40-60% of the population, and lymphocytes made up 10-15% of the population.

A.



B.



C.

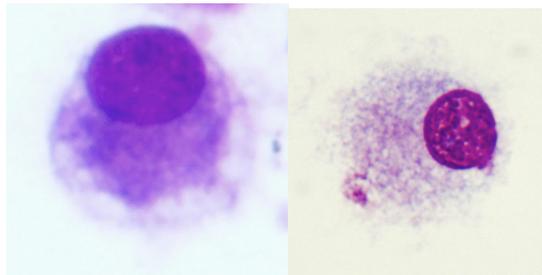


Figure 5. Monocytes, iDCs, and mDCs after culture (100x). The representative Diff-quick stained cells above were taken from cultures of monocytes (A), iDCs (B) and mDCs(C) from FIV- (left) and FIV+(right) populations. The images are documented from two representative cats, one FIV- and one FIV+. Monocytes have more defined cell edges. iDCs have increased vacuolation and less well-defined cell edges. mDCs display advanced vacuolation and much less well-defined cell edges due to dendrite projection. These characteristics exist on a continuum, so some cells may appear less activated than others.

3.3 Cell viability

The rapid-half life of DCs in culture generates culture conditions in which various stages of cell death are present. The cell surface profiles that we observed differed from those reported by Freer et al. [120], so we asked whether a sizeable portion of our analyzed cells were undergoing apoptosis. The apoptotic status of cells is important because it can affect their surface staining profiles, the viability of monocytes, iDCs, and mDCs from FIV+ and uninfected cats was assessed following the 6-day stimulation. Annexin V an indicator of early apoptosis and propidium iodide, an indicator of late apoptosis were utilized to determine the survivability of cells in culture.

The rate of cell death did not vary greatly amongst cell types from animals with the same infection status. When comparing cells from FIV+ animals and uninfected animals, there were fewer cells from infected animals that could be classified as healthy (negative for both Annexin V and Propidium iodide), but the differences were not significant for any of the cell types analyzed. The lack of significance may be due to variability. A higher portion of cells from FIV+ populations could be classified as early apoptotic (Figure 6, Table 2), which is consistent with the observation that immune cells from FIV-infected cats may be more susceptible to in vitro apoptosis in vitro [70, 122, 123]. In FIV-infected cats, B7.1-expressing cells become apoptotic after chronic exposure to CTLA4-expressing cells [70, 122, 123].

Since such a large portion of the cultured cells fell within some state of apoptosis, it is likely that this factor contributed to the variability of expression of cell-surface molecules.

Since DCs do not typically divide in culture and experience a half-life of a few days, over the course of 6 days of stimulation, a substantial amount of cell death is observed [121]. Should surface profile assessments be repeated, gating for apoptotic cells and removing them from the analysis might provide a more accurate depiction of the cell surface profiles.

Table 2. Survivability of monocytes, iDCs, and mDCs from FIV+ and FIV- animals in culture. Viable cells do not stain with Annexin V or propidium iodide. Early apoptotic cells stain positively for Annexin V but not propidium iodide, while late apoptotic cells stain positively for both. Debris (propidium iodide + and Annexin V -) was not included in the analysis.

Monocytes		Viable	Early Apoptotic	Late Apoptotic
	FIV-	52.86 +/- 8.43	25.29 +/- 4.74	21.84 +/- 3.81
	FIV+	38.19 +/- 13.17	42.21 +/- 13.39	29.03 +/- 8.51
iDCs		Viable	Early Apoptotic	Late Apoptotic
	FIV-	53.11 +/- 12.27	25.12 +/- 7.73	21.76 +/- 5.06
	FIV+	34.25 +/- 6.75	33.10 +/- 8.50	32.65 +/- 5.04
mDCs		Viable	Early Apoptotic	Late Apoptotic
	FIV-	38.53 +/- 14.32	32.02 +/- 8.53	29.44 +/- 6.10
	FIV+	32.16 +/- 0.37	33.30 +/- 10.04	34.53 +/- 10.00

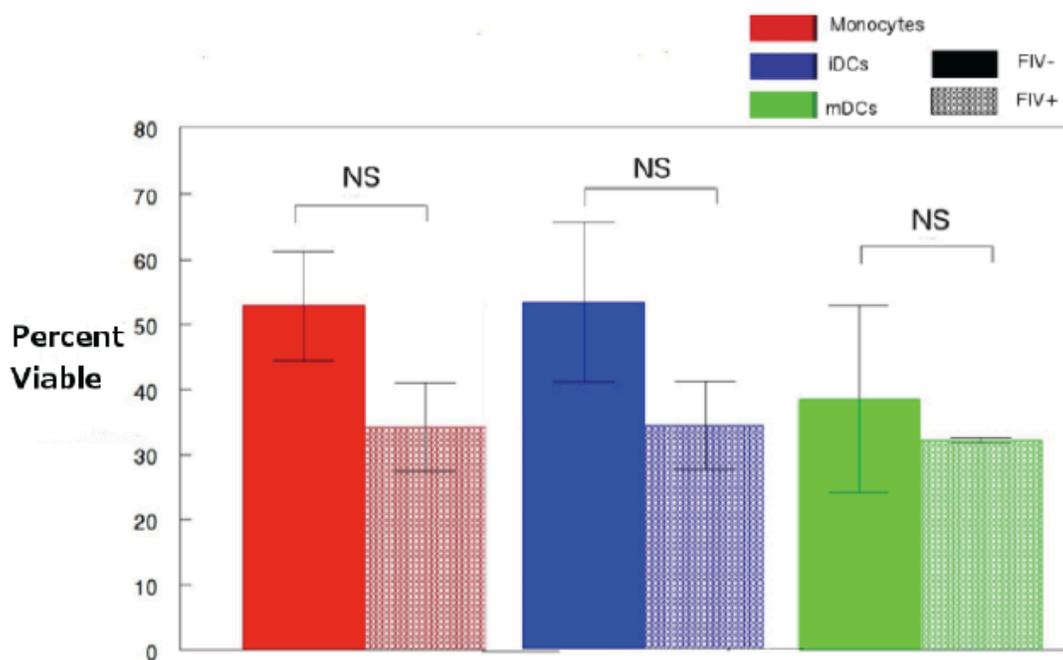


Figure 6. Comparison of healthy populations of APCs from FIV+ and FIV- cats. Viable cells do not stain with Annexin V or propidium iodide. Debris (propidium iodide + and Annexin V -) was not included in the analysis. Lack of significance may be due to variability. Lowered viability of cells from lentivirally-infected subjects has been documented by other groups [70,119, 120].

3.4 Co-culture

Upon confirming that our cell populations exhibited morphological traits characteristic of the desired cell types, we evaluated their antigen-presenting functions to assess differences in stimulation of CD8⁺ T cell proliferation between chronically infected and naïve cats. We asked whether or not DCs from FIV⁺ cats were better or worse stimulators of T cell response than those from FIV⁻ cats. The DCs were not externally introduced to any FIV antigens in order to examine their stimulatory ability in a viral antigen-independent manner. Viral antigen-specific proliferation of CD8⁺ T cells was not a direct focus in this context, as we were focusing primarily on the stimulatory capacity of DCs to stimulate a response, regardless of the memory status of the T cells with which they were being co-cultured. We hypothesized that DCs from FIV⁺ cats possess a superior stimulating capacity under conditions of chronic immune activation. To address this question, CD8⁺ T cells, treated with CFSE, and DCs were cultured together at a 1:1 ratio.

CFSE, a fluorescent cell-staining dye partitions itself evenly among daughter cells upon cell division, providing a way to visualize the division history of stimulated cells using flow cytometry [110, 111, 124]. CFSE diffuses into cells where it is cleaved by intracellular esterases to generate fluorescence. CFSE remains fairly stable within cells, though some may be lost after culturing for long periods of time, producing a small amount of decay in fluorescence signal that is proliferation-independent but consistent across cultures [110, 111]. In general, halving of CFSE fluorescence is due to cell division, with the contents being evenly distributed between daughter cells [111]. While high concentrations of CFSE may

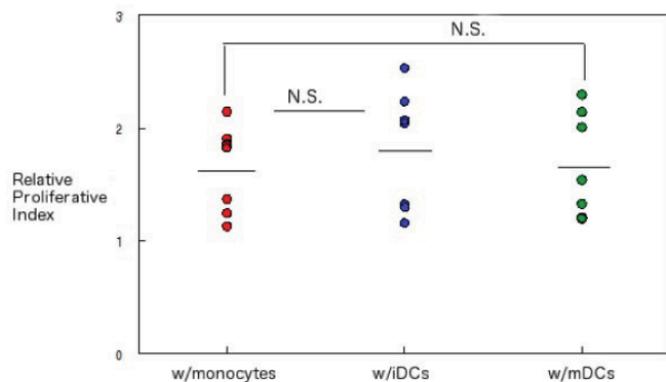
affect the survivability of populations, the doses employed in the present study are not reported to have a significant impact on cell viability [111, 125].

After 96 hours, the CD8⁺ T cells were removed from the wells and division assessed by CFSE dilution. There was no significant difference in the ability of monocytes, iDCs, and mDCs to stimulate CD8⁺ T cell proliferation. This trend was observed in cells from both infected and uninfected cats (Figure 7). The size of the population of CFSE-expressing CD8⁺ T cells is variable between samples. However, variable population sizes do not impact the calculation of the proliferation indices [126].

However, there was a significant difference in the ability of monocytes, iDCs, and mDCs from infected and uninfected cats to stimulate CD8⁺ T cell proliferation (Fig 8). The average relative proliferative indices for autologous CD8⁺ T cells cultured with monocytes, iDCs, and mDCs were higher by 47.5%, 54.1%, and 50.9% respectively for FIV⁺ samples over FIV⁻ samples. There was no significant difference in the ability of CD8⁺ T cells from either source to proliferate in the presence of IL-2 and ConA (Figure 8). This observation suggests that some element of the antigen-presenting cell/T cell interaction is contributing to these differences in proliferative ability. CD8⁺ T cells from both FIV⁺ and FIV⁻ cats proliferate more after co-culture of the T cells with DCs than after stimulation with IL-2 plus Con A or cytokines (IL-2, IL-4, GM-CSF) (Figure 9). Additional investigation is required to ascertain whether or not this activation results from heightened activity or factors intrinsic to the CD8⁺ population from in FIV⁺ cats (since they have the potential to display an FIV-specific response).

A.

Proliferation of CD8+ T cells from FIV- cats upon co-culture with autologous monocytes, iDCs, and mDCs



B.

Proliferation of CD8+ T cells from FIV+ cats upon co-culture with autologous monocytes, iDCs, and mDCs

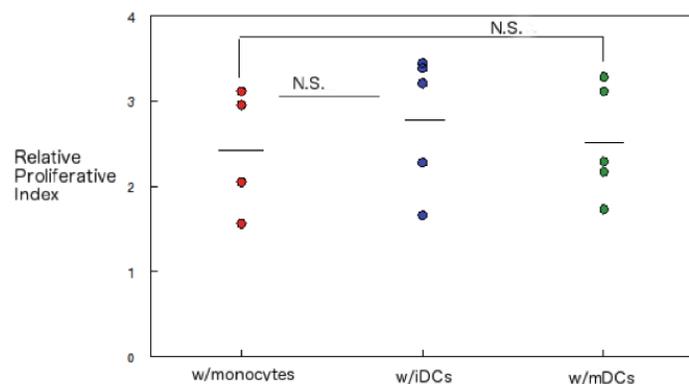
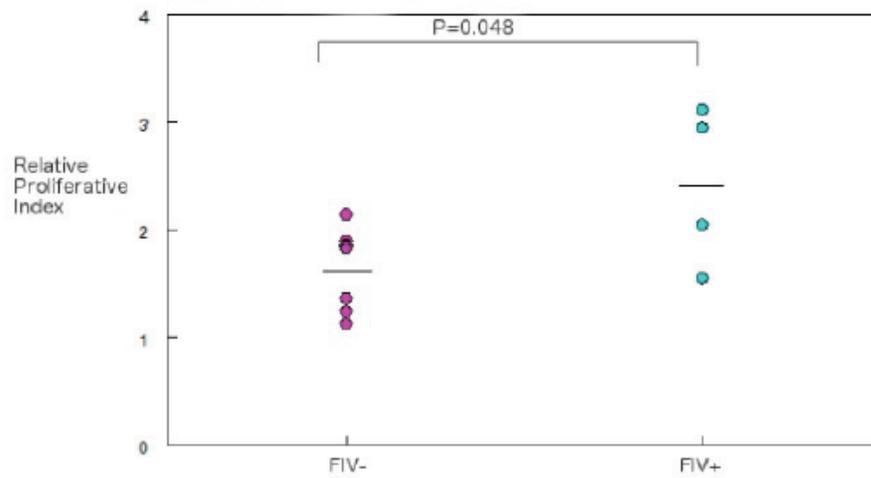


Figure 7. Proliferation of CD8+ T cells from FIV- cats and FIV+ cats upon co-culture with autologous monocytes, iDCs, and mDCs. Panel A represents FIV- cats while panel B represents FIV+ cats. Relative proliferative indices were determined by dividing sample proliferative indices by those of the control. No significant difference in ability to induce proliferation was observed between monocytes, iDCs, or mDCs from either population (n=6 FIV- cats and n=5 FIV+ cats).

Figure 8. Differences in proliferation of CD8⁺ T cells from FIV⁺ or FIV⁻ cats. Proliferation of CD8⁺ T cells upon co-culture with autologous monocytes (A), iDCs (B), and mDCs (C) from FIV⁺ or FIV⁻ cats was assessed. Relative proliferative indices were determined by dividing sample proliferative indices by those of the control. CD8⁺ T cells from FIV⁺ cats proliferate much more readily than their counterparts from FIV⁻ cats in the presence of monocytes, iDCs, and mDCs from the same animal. (n=6 FIV⁻ cats and n=5 FIV⁺ cats).

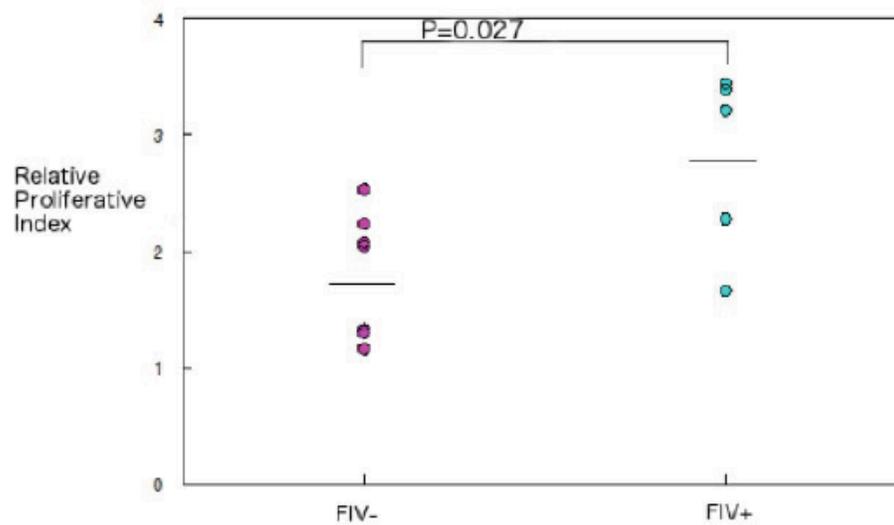
A.

Proliferation of CD8+ T cells upon co-culture with autologous monocytes from FIV+ and FIV- cats



B.

Proliferation of CD8+ T cells upon co-culture with autologous iDCs from FIV+ and FIV- cats



C.

Proliferation of CD8+ T cells upon co-culture with autologous mDCs from FIV+ and FIV- cats

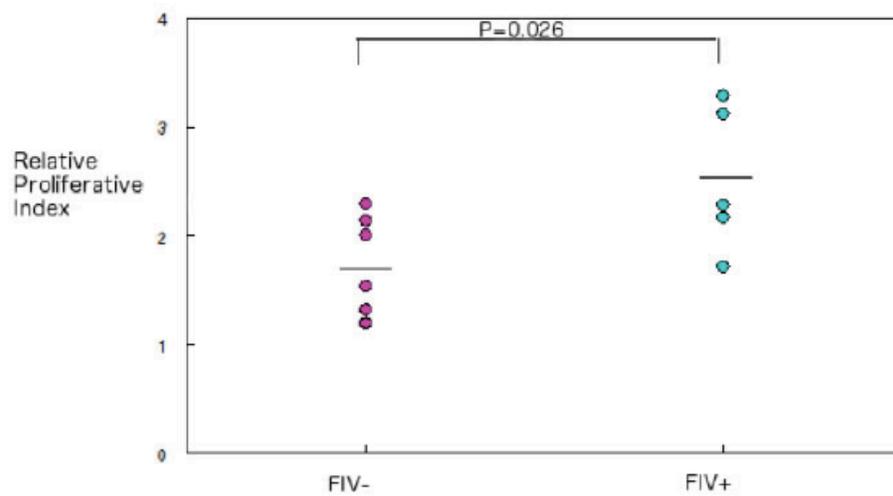
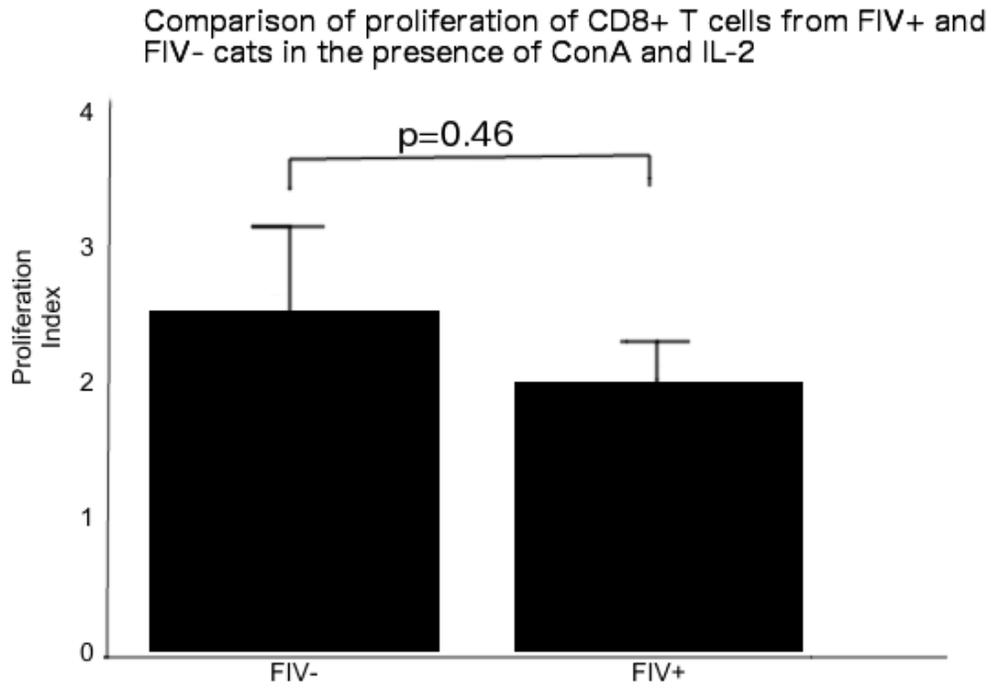
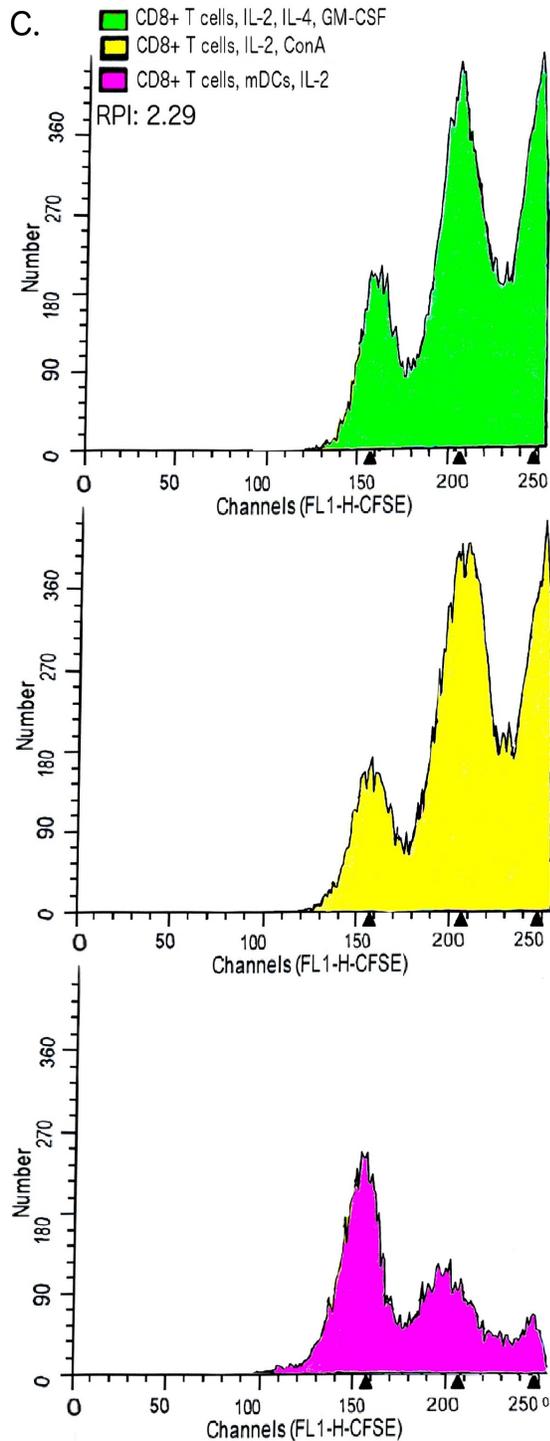
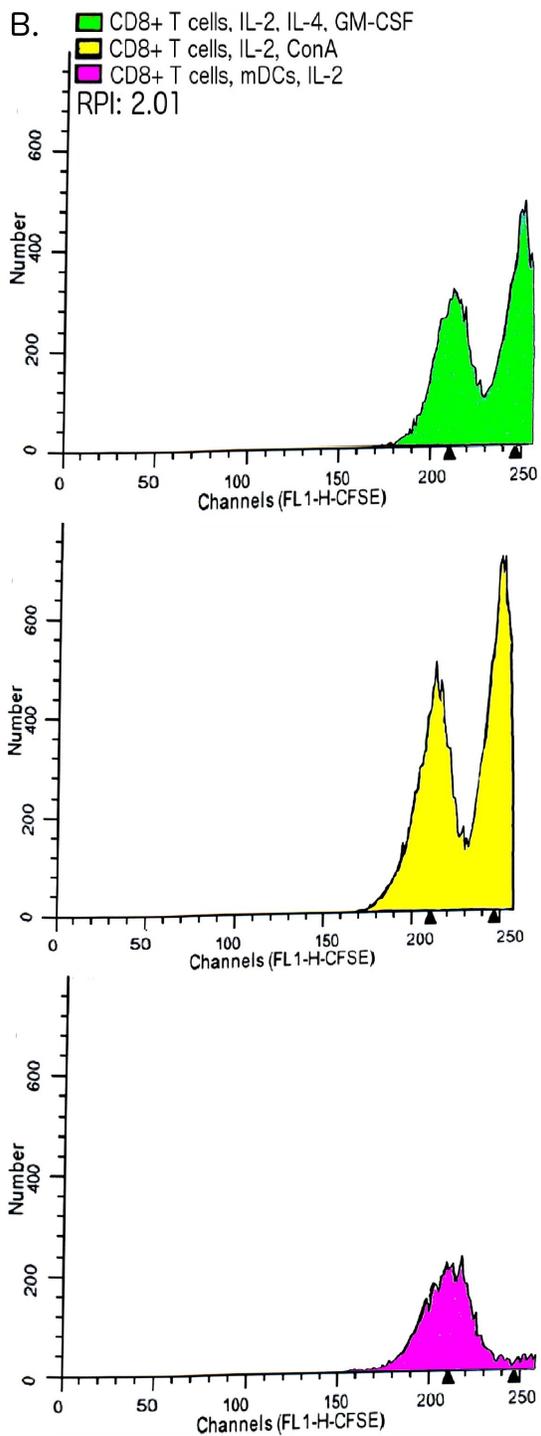


Figure 9. Differences in proliferation of CD8⁺ T cells from FIV⁻ and FIV⁺ animals are related to the interaction between these populations with autologous DCs and not with differences in intrinsic proliferative ability. A. CD8⁺ T cells from FIV⁺ and FIV⁻ cats cultured in the presence of stimulatory factors (ConA, IL-2) do not exhibit differences in proliferative output. B-C. Representative trials from FIV⁻ populations (B) and FIV⁺ (C) populations demonstrate the importance of the APC-T cell interaction for advanced proliferation. CD8⁺ T cells cultured in the presence of cytokines used to induce differentiation of monocytes to DCs (- control) exhibited similar proliferation patterns to those cultured in the presence of stimulatory factors. Relative proliferative indices (RPIs) were determined by dividing sample proliferative indices by those of the control.

A.





4. DISCUSSION

Here we sorted monocytes from peripheral blood mononuclear cells (PBMCs) for differentiation into immature and mature dendritic cell populations using IL-4 and GM-CSF for the former and IL-4, GM-CSF, and LPS for the latter. The identity and characteristics of these populations were confirmed via cytology, with vacuolation and dendrite production increasing with subsequent activation from monocyte to iDC to mDC profile. This method proved to be the most effective way to characterize the cells, as the rapid half-life of DCs in culture generated cells in both early and late apoptotic states, which impacted cell surface marker expression profiles. However, an analysis of phagocytic ability fluorophore-labeled beads could also have been employed to differentiate between iDC and mDC populations since the phagocytosis rate should be much lower in the mature population. In addition, since living cells may be distinguishable from apoptotic cells by FSC and SSC, this information from the viability analysis could be reapplied to the surface phenotype analysis to distinguish apoptotic populations from viable ones and assess the true impact of cell death on surface marker expression.

Using sorted monocytes and differentiated iDCs and mDCs in co-culture with CFSE-stained CD8⁺ T cells, we demonstrated increased proliferation of CD8⁺ T cells in the presence of antigen-presenting cells from FIV-infected cats. These findings support our hypothesis that DCs from FIV-infected cats would stimulate greater autologous CD8⁺ T cell proliferation when compared to those from FIV- cats. The lack of difference in proliferative ability of CD8⁺ T cells from infected and uninfected animals in the presence of stimulatory cytokines suggests that some element of the interaction between APCs and CD8⁺ T cells

contributed to the increased activation. It is possible that during chronic infection, antigen-presenting cells develop an increased proliferative ability. However, it is also possible that monocytes isolated for use in this study had already acquired FIV antigens, in which case, rapid expansion of the memory CD8⁺ T cell population would be observed in co-culture. Normally, memory cells limit their own expansion, but the presence of DC presenting antigen associated with MHC class I molecules stimulates their proliferation [125]. Further research is required to elucidate to what extent the increase in CD8⁺ T cell proliferation in chronically infected subjects is viral antigen-independent.

There was no significant difference in the ability of monocytes, iDCs, and mDCs to induce proliferation in either FIV⁺ or FIV⁻ models. This observation is interesting, considering that immature dendritic cells are reputed to have a much lower cell surface concentration of MHC-complexes and co-stimulatory molecules necessary for stimulation [120]. However, upon considering the range of monocyte to dendritic cell morphology as a continuum, it becomes apparent that viewing the groups as wholly distinctive and concrete is probably inaccurate. There will be monocytes that display a partially activated profile, even without additional stimulation, especially after a week of culture with granulocytes undergoing cell-death in the same wells. Similarly, the iDC group contains cells at various states of activation. One would also expect that the mDC population still contains a portion of cells retaining an immature profile. Thus, a lack of significant difference in stimulatory ability is not entirely surprising. In vivo the main circulating monocyte subpopulation migrates to sites of inflammation and infection. Upon activation, some of these cells shift

towards a DC profile and migrate to the lymph nodes to present acquired antigen to T cells, maturing in the process. Thus, a mechanism for generation of DCs from monocytes is present in vivo and is dependent on activation of cells [126].

Dendritic cells from HIV-infected patients have been demonstrated to remain largely functionally intact with similar cytokine profiles to those from uninfected patients [10, 61]. The role that DCs play in stimulation of memory expansion may also contribute to the differences in proliferation of CD8⁺ T cells observed in our study. Cockburn et al. demonstrated that DCs are vital for boosting of CD8⁺ memory expansion by driving stimulation above the threshold of an established self-check system established by CD8⁺ memory T cells to limit expansion [127]. Other antigen-presenting cell types, while capable stimulators of effector function, were less capable in boosting memory expansion, possibly because of the unique ability of dendritic cells to cross-present exogenous antigen [17, 18, 127]. Indeed, Tanchot et al. demonstrate the importance of MHC class I-TCR interaction for survival of CD8⁺ memory T cells; the memory population is much smaller following inhibition of MHC class I [128]. This is important to consider since DCs are the primary antigen-presenting cell that displays external antigen, such as viral peptides, on MHC-I.

CD8⁺ T cell/DC interactions are vital for maintenance of immunity and control of infection. The use of DCs pulsed with antigen to amplify the body's response to pathogen-specific proteins during vaccination is one possible application of this relationship [51, 90]. The strong co-stimulatory ability of DCs makes them a better tool for T cell activation than peptide alone [128]. The formation of CD8 memory populations is important because CD8⁺

T cells are capable of halting viral replication in CD4⁺ T cells and can kill infected cells. Thus, the clinical status of HIV-infected individuals is associated with their CD8⁺ T cell levels [65]. In addition, DC-based immunotherapy in the late stages of SIV was observed to amplify SIV-specific CD8⁺ T cell responses, pointing to dendritic cells as a therapeutic agent as well as a tool for vaccine application [99]. Individuals immunized with HIV-peptide-pulsed DCs exhibited antigen-specific CD8⁺ T cells that constituted up to 40% of patient memory cells [5]. A portion of this population remains present long-term, though the majority collapses quickly due to the short-half life of the DC stimulators in vivo. Initial results from DC-vaccination against SIV were promising, with controlling of the virus within the secondary lymphoid organs. Subsequently, levels of viral RNA and cell-associated viral DNA were lowered [101]. However, Freer et al., in a DC-based immunization study show little impact on disease course, in spite of increased viral antibody titers and the presence of HIV-specific proliferation of PBMCs [129]. Out of twelve human DC immunotherapy clinical trials published as of early 2013, half have failed to show positive HIV-viral responses to immunization [102].

However, novel methods to enhance cross-presentation of exogenous antigen onto MHC class I molecules may boost the stimulatory ability of DCs for vaccine and immunotherapy applications. Fc receptors bind immune complexes of antigen and IgG with very high affinity, allowing more antigen product to be internalized and ultimately delivered to the cytosol where transport to the ER and loading onto MHC class I molecules may occur [103]. Another method of boosting cross-presentation involves employing heat shock

proteins as chaperones of antigen into DCs. Heat shock proteins may protect peptides from degradation in proteasomes once complexes are internalized, allowing more peptide to be directed to the ER for processing [104]. Nanoparticles, such as poly(D,L-lactide-co-glycolide) (PLGA), may form antigen-complexes that aid in antigen escape from endosomes upon endocytosis [105]. In all three of these methods, transport of antigen into the ER for MHC class I processing is enhanced. Since MHC class I on DCs is important for stimulation of CD8⁺ lymphocyte TCRs, increasing the expression of MHC class I-peptide complexes of interest could increase the efficacy of DC-based immunotherapy techniques. While methods for loading DCs with antigens are not a focus of this paper, it is important to note that DC-based immunotherapy techniques should not be rejected without first attempting to optimize antigen-presentation levels for improved viral responses. Our research highlights the special nature of the DC-T cell reaction in the context of lentiviral infection and may indicate that DCs from chronically infected animals retain their stimulatory function and perhaps even exhibit hyperstimulatory abilities, a factor that should be considered in the context of DC immunotherapy.

In short, DC-based vaccination and DC-based therapeutic measures may provide a mechanism for effectively amplifying or directing CD8⁺ T cell response against HIV and similar retroviruses. This study assessed the ability of CD8⁺ T cells to proliferate in response to monocyte, iDC, and mDC stimulus. While CD8⁺ T cells in co-culture with autologous DC from FIV⁺ animals demonstrated significantly higher proliferation than those of control animals, additional research is required to ascertain whether or not this property is

related to T cell factors such as the ability of memory populations to expand rapidly and/or to DC characteristics relate to chronic immune stimulation.

5. FUTURE DIRECTIONS

By removing memory T cell populations from analysis, we would be able to analyze the proliferative abilities of naïve CD8⁺ T cells alone to ascertain whether or not CD8⁺ memory T cell expansion is sufficient to explain the higher levels of proliferation observed in co-cultures of cells from FIV⁺ compared to FIV⁻ cats. Antigen-specific tetramers of MHC molecules provide a tool for isolating memory populations [32, 130, 131, 132]. MHC class I tetramers have been used to isolate antigen-specific CD8⁺ T cells directly from peripheral blood; similarly, MHC class II tetramers have been utilized for isolation of antigen-specific CD4⁺ T cells [32, 130, 131]. This method has proven useful for observation of a variety of viral-specific cells, but has also been applied to CD8⁺ T cells that recognize tumor-specific antigens [32, 131]. The ability to isolate CD8⁺ T cells specific for certain FIV epitopes would aid in distinguishing DC-stimulation of memory CD8⁺ T cell responses from the endogenous stimulatory ability of DCs under chronic immune activation that we have proposed.

In addition, if isolated monocytes were virally infected at a level that might impact memory CD8⁺ T cell proliferation, viral RNA levels could be assessed by q-PCR and compared to those from cultures with known viral levels. Very low levels of infection are not expected to greatly impact CD8⁺ T cell proliferation. This provides an alternative mechanism for differentiating between viral antigen-independent and viral antigen-dependent DC stimulation to explain why T cells proliferated significantly more in the presence of autologous DCs from chronically infected cats.

In theory, only a very small percentage of T cells should proliferate in response to the MHC-complexes of co-cultured DCs, as the interactions are not antigen-specific. However, antigen-presenting cells produce pro-inflammatory cytokines for the activation of T cells, and this activity may be more pronounced in cells from chronically activated immune systems. The content of IL-12 and other pro-inflammatory cytokines in the culture media could be assessed via enzyme-linked immunosorbent assay (ELISA).

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