DISSERTATION

MODIFICATION OF THE INNATE IMMUNE RESPONSE DURING FELINE IMMUNODEFICIENCY VIRUS INFECTION

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY TRACY L. LEHMAN ENTITLED MODIFICATION OF THE INNATE IMMUNE RESPONSE DURING FELINE IMMUNODEFICIENCY VIRUS INFECTION BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

MODIFICATION OF THE INNATE IMMUNE RESPONSE DURING FELINE IMMUNODEFICIENCY VIRUS INFECTION

Lentiviruses such as the human immunodeficiency virus (HIV) and the feline immunodeficiency virus (FIV) have successfully evolved to both use and subvert the host innate and adaptive immune responses to establish long-term infections. Investigation into the mechanisms lentiviruses use to overcome host immune response allows the development of potential therapies and elucidates the intricacies of the immune response. Dendritic cells are professional antigen presenting cells that are intricately involved in innate immune responses and in coordinating the adaptive immune response. However, these same cells have been implicated in initial lentiviral infection, transfer of infection to other cells of the immune system, and alteration of the immune response to allow chronic and progressive infection of the host. To better understand the effects of lentiviral infection on myeloid dendritic cells (mDC), we used the FIV model and bone marrow-derived mDC to evaluate differences in growth, phenotype, and function. We found that chronic FIV infection did not affect mDC growth in culture,

phenotype, or maturation as assessed by CD11c, MHC class II, CD80, and CD1a and ability to uptake dextran particles. However, mDC from FIV-infected cats were found to have significantly decreased ability to stimulate proliferation of allogeneic CD4+ T cells in the mixed leukocyte reaction. To begin a mechanistic examination of FIV-induced alteration of mDC function, we examined cytokine responses to Toll-like receptor (TLR) ligands and CD40L. We documented changes in the ratio of the immunoregulatory cytokines IL12 and IL10 in response to select TLR ligands and CD40L, which could result in impaired immune responses, impaired T cell interactions, and enhanced viral survival. Having identified alterations in DC function with FIV infection, we attempted to augment the antiviral effects of mDC by supplementing IL-12 levels *in vivo* using an adenoviral vector. Consistent with the known complexity of the immune response, increased IL12 levels proved toxic and thereby failed to be a viable means of enhancing the innate immune response to lentiviral infection. Our research documents functional changes induced in bone marrow-derived mDC by chronic FIV infection and provides a means of further investigation into the development, mechanisms, and therapies for those changes.

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DEDICATION

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INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1)

The lentivirus human immunodeficiency virus type 1 (HIV-1) has caused a global pandemic and is estimated to be responsible for greater than 25 million deaths since 1981 [1]. In 2007 the UNAIDS/WHO estimated that 33 million people were living with HIV, 2.7 million people became newly infected, and 2.0 people died from AIDS related deaths despite major improvements in access to retroviral treatment [1]. The scientific community has been successful in developing several effective anti-retroviral therapies that slow the progression of HIV, but these medications require long-term therapy and medical care and come with great expense and side-effects. Development of alternate treatment strategies and prevention methods is central to the successful control of the HIV epidemic [1]. Early interactions between HIV and the host innate immune response have been found to determine infection and progression of disease [2]. Consequently, significant interest and investigation has been directed towards the possible use of therapeutic dendritic cell (DC) vaccines as an alternative treatment strategy. However, DC and other trial therapies have not proven as successful as early research had suggested. Improved understanding of the mechanisms used by lentiviruses to subvert

the innate immune response would provide valuable insight into potential methods to prevent infection or suppress disease progression.

Feline immunodeficiency virus

The feline immunodeficiency virus causes a disease syndrome in its natural host with clinical phases analogous to those seen with HIV in humans [3-14]. Feline immunodeficiency virus can be transmitted by blood and crosses the oral, vaginal, and rectal mucosa to establish systemic infection, crosses the placenta to establish fetal infection, and concentrates in the milk of lactating queens [15-22]. Feline immunodeficiency virus has been shown to use the receptors CXCR4 and CD134 for virus cell entry analogous to the use of CXCR4 and CD4 by primate lentiviruses [23-25]. Dendritic cells and T lymphocytes are early targets of FIV infection as has been found for both HIV and simian immunodeficiency virus (SIV) infections [26-30]. Infected mucosal DC traffic to lymphoid tissues where FIV replication expands rapidly in T cells, and the virus subsequently disseminates to other tissues within a week of inoculation [30]. Initial infection is characterized by flu-like illness, lymphadenopathy, and a decline in CD4+ T lymphocytes [5, 9, 31-33]. After infection a prolonged asymptomatic latent phase often follows during which viral replication is down-regulated and a progressive loss of CD4+ T cells and other hemolymphatic cells occurs [34-36]. Terminal disease occurs due to immunological exhaustion when plasma viral load again increases and accelerated loss and suppression of cells such as CD4+ T cells leads to increased susceptibility to infection [14, 37]. The strength of the early innate immune response to FIV and HIV is perhaps the most important feature in determining the course of infection and disease

progression. As the feline analog of HIV, FIV infection thereby provides an excellent model in which the immune response to a lentivirus can be studied.

Dendritic cells

Dendritic cells are considered to be the most potent of profession antigen presenting cells (APCs) and link the innate and subsequent cell-mediated immune responses. Dendritic cells are continuously produced from hematopoietic stem cells in the bone marrow and are divided into subsets based on phenotype, function, and tissue localization in some species [38-40]. Due to a lack of reagents, DC subsets have not yet been defined in the cat, although they are suspected to be similar to those found in other species. Dendritic cells migrate to the skin and other tissues to become Langerhans cells or interstitial immature DC [41]. Myeloid and plasmacytoid DC, distinguished by surface expression of CD11c, are suspected to be more directly involved in innate immunity against microbes than Langerhans and interstitial DC. These DC subsets circulate through the body moving from bone marrow through the vasculature to non-lymphoid tissues, lymphatics, and draining lymph nodes, patrolling for pathogens and directing the immune response to pathogens [41]. Toll-like receptors (TLRs) on the surface of immature DC allow them to rapidly respond to pathogens and undergo maturation.

Dendritic cell maturation involves functional and phenotypic changes and is necessary for effective T cell stimulation [42]. Mature DC lose endocytic activity, increase surface expression of MHC I and MHC II and adhesion and co-stimulatory molecules including CD40, CD54, CD80, CD86, and secrete pro-inflammatory cytokines

such as interleukin-1 (IL-1), IL-6, IL-12, IL-18, and IL-23 [41, 43]. Mature DC express CCR7 and migrate in response to chemokines to draining lymph nodes where they induce primary T cell-mediated immune responses [41]. Binding of CD40 ligand (CD40L) on the DC surface with CD40 on T cells results in further DC maturation and enhanced immunoregulatory cytokine production [44, 45]. Cytokines produced by myeloid and plasmacytoid DC promote induction of Th1, Th2, and regulatory responses by T cells in response to TLR recognition of pathogens. Molecules derived from pathogens and T cell signals tend to promote DC IL-12 production and a Th1 response. Anti-inflammatory molecules such as IL-10, TGFβ, PGE2, and corticosteroids tend to inhibit DC maturation and IL-2 production and induce Th2 or regulatory T cell responses. The actual DC response generated by a particular molecule is complex. Current thought suggests that the type of T cell response generated by a DC is determined more by the type of activation signal and activation status of the DC than the lineage of the DC [46-49]. The complete delineation of DC subsets and their functions in different species is still under investigation [50].

Due to their essential function in directing immune responses, DC are a prime target for pathogen manipulation. For example, *Mycobacterium tuberculosis* has been found to use the DC-specific receptor DC-SIGN to impair DC maturation and to induce production of the anti-inflammatory cytokine IL-10 [51]. The herpes simplex virus also uses DC-SIGN as an attachment receptor on immature DC to enhance DC infection and transmission of the virus to permissive target cells [52]. Measle virus-infected DC have been found to develop immunosuppressive and cytotoxic activities as they inhibit T cell proliferation, induce activated T cell apoptosis, and undergo FasL-dependent apoptosis

[53]. Pathogen-induced manipulation of DC function would potently interfere with host immune development and potentially explain the suppressed host immune response to pathogens such as HIV.

Dendritic cells and lentiviral infection

Dendritic cells predominate on mucosal surfaces and are one of the first targets of lentiviruses [30, 54]. Lentiviral infection has been shown to alter DC growth, maturation, and function in favor of viral proliferation and spread although these changes are not fully understood and conflicting evidence exists [2, 55-57]. Lentiviruses likely induce changes in DC both through direct effects of viral infection and through virus-induced alterations in cytokine production [58]. Infected DC traffic to local lymphoid tissue where the virus can be efficiently transmitted and expanded in its primary T cell targets [2]. Binding of HIV-1 gp120 to the DC-specific C-type lectin DC-SIGN plays a key role in the dissemination of HIV-1 by DC. Recent data demonstrates that ligation of DC-SIGN alters the balance of subsequent TLR-mediated cytokine production to favor IL-10 production [59]. Exposure to HIV-1 gp120 *in vitro* leads to abnormal DC maturation and function characterized by decreased IL-12 production and allostimulatory capacity [60]. HIV-infected monocyte-derived DC produce HIV gp120, which impairs normal CD4+ T cell function, and the DC were found to be poor stimulators of allogeneic CD4+ T cell proliferation and IL-2 production [61]. Alterations in DC-T cell interactions may be due to virus-induced interference with CD40L-CD40 interactions between DC and T cells [62-64]. HIV-1-infected DC up-regulate cell surface markers but have altered cytokine production in response to CD40 ligand stimulation [63].

Lentiviral-induced DC dysfunction, particularly late in infection, may inhibit efforts to use DC in viral therapy [56, 65]. Despite the importance of DC in stimulating an immune response, only two studies to date have been able to show any positive response to therapeutic DC vaccination in lentiviral infections [66, 67]. The mechanisms behind the failure of DC therapies remain undetermined. Additionally, the cause for decreased numbers of DC and possible altered DC function in HIV infection has not been fully explored. Although it has been postulated that failure in development and function of DC from CD34+ stem cells may occur with HIV infection, the majority of research in HIV to date has focused on peripheral DC [68]. Little is known about bone-marrow derived DC in HIV infection. Continued investigation of the mechanisms of DC dysfunction induced by lentiviral infection and methods to alter such effects are warranted.

Toll-like receptors and lentiviral infection

Toll-like receptors (TLRs) are pattern recognition receptors that recognize conserved pathogen-derived patterns of specific bacterial, fungal, or viral molecules and trigger the expression of different cytokines and co-stimulatory molecules. Thirteen mammalian TLRs have been identified to date. Double-stranded RNA from viruses stimulates TLR 3 leading to the production of interferon-alpha (IFNα) and IFNβ and the activation of immature DC [69, 70]. Toll-like Receptor 7 recognizes ssRNA and synthetic anti-viral compounds, TLR 9 recognizes CpG DNA motifs, and TLR 2 and TLR 4 recognize the bacterial cell wall products peptidoglycan and lipopolysaccharide

(LPS) respectively [71]. Myeloid DC have been found to express TLRs 1-9, while plasmacytoid DC predominantly express TLR 7 and 9 [72-75].

In response to cell and environmental signals, TLRs and other cell-surface receptors initiate highly variable signaling cascades that converge on the mitogen-activated protein kinases (MAPKs). The five distinct groups of MAPKs that have been identified in mammals then direct cytokine production and pathogen immune responses [76-78]. Increasingly, HIV-1 and other viruses have been found to alter expression or function of MAPKs and other downstream products such as interferon regulatory factors (IRFs) and NF-κB [79-84].

The identification of viruses, including HIV-1, which appear to interfere with TLR-directed APC responses underscores their importance in viral immune reactions [85, 86]. Feline immunodeficiency virus infection has been found to alter TLR expression in feline cell lines, although DC were not investigated [87]. Because of their role in altering the immune response, TLRs are under investigation as potential means of enhancing vaccination efficacy or immune modulating therapy in lentiviral infections such as HIV [88, 89].

Interleukin-12 and lentiviral infection

Interleukin-12 (IL-12) is an important regulatory cytokine that enhances T cell proliferation and cytotoxic T cell activity, partially through induction of type 1 cytokines including IL-2 and IFNγ [90]. Interleukin-12 is produced by DC and other APC in response to protozoal, bacterial, and viral pathogens. In response to IL-12, T and NK cells produce interferon-γ (IFNγ) and tumor necrosis factor-α (TNFα) and inhibit the

production of the inhibitory cytokines IL-4 and IL-10 [91]. Interferon-γ in turn recruits and activates other APC to produce a local response to the pathogen. Interleukin-12 induces the differentiation of naïve CD4+ T cells to Th1 cells to produce more long-standing and generalized effects through a strong cell-mediated response. Interleukin-12 production can be inhibited by IL-10 [92].

Alterations in IL-12 have been noted in several disease processes, potentially aiding in immune evasion. Bone marrow-derived DC in contact with myeloma tumor cells produce less IL-12, which is, in part, due to increased IL-10 production [93]. Providing adenovirus-expressed IL-12 to mice abrogated the Th2-type T cell response induced by *Leishmania major* infection [94]. Decreased levels of IL-12 are predictive for progression to AIDS in HIV infection, and HIV-infected individuals produce sub-normal levels of IL12 relatively early after infection [95, 96]. Blood mononuclear cells from HIV-infected patients produce one-fifth the IL-12 of control cells, and the decreased IL-12 production correlates with decreased CD4+ T cell number and T-cell reactivity [96, 97]. As potent producers of IL-12 and one of the initial targets of HIV infection, DC likely play a key role in the virus-induced alteration of IL-12 levels. Dendritic cell IL-12 production was decreased when co-cultured with CD4+ T cells exposed to HIV envelope proteins, possibly due to viral-induced impairment of CD40L expression by the T cells [64]. HIV infection of DC decreased IL-12 production, up-regulated IL-10 production, and significantly inhibited DC activation of antigen-specific memory and cytotoxic-T-lymphocyte responses [98]. However, Beuria *et al.* did not find impaired IL-12 production in an HIV-infected human DC hybridoma cell line that showed an inability to stimulate allogenenic T cells [99]. Adding IL-12 to HIV+ cells *in vitro* has been shown

to restore allostimulatory capacity and responsiveness of cells to antigen and to block mitogen- and antigen-stimulated T cell death [60, 100-102]. Although significant IL-12 alterations are known to occur with HIV infection, the role of DC in establishing these changes and their significance on DC function is not yet clear.

Dissertation research

The goal of this research was to investigate the effects of feline immunodeficiency virus (FIV) infection on myeloid dendritic cells (mDC) and their role in the innate and subsequent initiation of the adaptive immune response. Feline bone marrow-derived mDC were assessed by growth, phenotype, maturation status, and function to determine changes induced by chronic FIV infection. Cytokine responses to Toll-like receptor ligands and CD40 ligation of the mDC were assessed by real time PCR to evaluate effects of chronic FIV infection on the ability of the mDC to respond to pathogens and T cell interactions. Global effects of FIV-induced altered mDC cytokine responses were evaluated through *in vivo* adenoviral IL-12 augmentation in cats just prior to FIV infection. This research has already directed further focused studies into the mechanisms of lentiviral effects on mDC and evaluation of preventative and therapeutic strategies.

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CHAPTER ONE

CHRONIC FELINE IMMUNODEFICIENCY VIRUS INFECTION ALTERS BONE MARROW-DERIVED MYELOID DENDRITIC CELL FUNCTION

ABSTRACT

As dendritic cells (DC) are potent antigen presenting cells integrating the innate and cell-mediated immune responses to infection, DC-lentivirus interactions could be fundamental to the persistent immune dysfunction that marks feline immunodeficiency virus (FIV), simian immunodeficiency virus (SIV), and human immunodeficiency virus (HIV) infections. We have developed methods to generate large numbers of feline myeloid DC (mDC) from bone marrow precursors, which can be collected via needle biopsy and differentiated *in vitro* in the presence of feline granulocyte macrophage colony stimulating factor. Utilizing serial bone marrow (BM) collections, we have compared mDC generated from naïve and chronically FIV-infected cats to determine

whether differences in BM mDC growth, phenotype, maturation, or function are present. These studies have shown that feline BM-derived mDC from FIV-infected cats: (a) appear to be productively infected by FIV and (b) are impaired in their ability to stimulate CD4+ T cell proliferation, as measured by allogeneic mixed leukocyte reaction (MLR). This mDC dysfunction does not appear to be associated with altered *ex vivo* mDC growth or maturation as determined by enumerating CD11c and MHC class II double positive cells, measuring expression levels of CD11c, CD80, CD1a, and MHC class II, and measuring endocytic capacity. The impaired ability to stimulate T cells appears to be specific to the DC-T cell interaction (immunological synapse) as T cells from the MLR assay proliferate with subsequent mitogenic stimulation. Given that the mDC are differentiated from BM precursors, these results suggest that FIV infection of myeloid precursors may impart long-lasting functional defects in the differentiated progeny. Further studies into the mechanisms of FIV-induced DC impairment are ongoing.

BACKGROUND

Considered to be the most potent of the antigen presenting cells, dendritic cells (DC) link the innate and cell-mediated responses of the mammalian immune system. Dendritic cells were first described in the late nineteenth century and have been the focus of intense study for both their role in the immune response and for their potential therapeutic uses. Dendritic cells are continuously produced from hematopoietic stem

cells in the bone marrow (BM) and are often divided into subsets based on phenotype, function, and tissue localization [1-3]. Some DC migrate to the skin and other tissues to become Langerhans cells or interstitial immature DC [4]. Myeloid and plasmacytoid DC, distinguished by surface expression of CD11c, are suspected to be more directly involved in innate immunity against microbes than Langerhans and interstitial DC. These DC subsets circulate through the body in an immature state moving from BM through the vasculature to non-lymphoid tissues, lymphatics, and draining lymph nodes, patrolling for pathogens and directing the immune response to pathogens [4].

Once immature DC encounter presentable antigen, the DC undergo maturation, which involves functional and phenotypic changes and is necessary for effective T cell stimulation [5]. Mature DC lose endocytic activity, increase surface expression of MHC I and MHC II for antigen presentation and adhesion and co-stimulatory molecules including CD40, CD54, CD80, CD86 which enhance their ability to stimulate T cells. Mature DC also upregulate expression of the chemotactic receptor CCR7 and migrate in response to chemokines to the spleen or draining lymph nodes where they induce primary T cell-mediated immune responses [4]. Mature DC secrete pro-inflammatory cytokines such as IL-1, IL-6, IL-12, IL-18, and IL-23 [4, 6]. Binding of CD40 ligand (CD40L) on the DC surface with CD40 on T cells results in further DC maturation and enhanced immunostimulatory cytokine production [7, 8]. Cytokines produced by myeloid and plasmacytoid DC promote induction of Th1, Th2, and regulatory responses by T cells in response to the pattern recognition of pathogens by Toll-like receptors on the DC. Current thought suggests that the type of T cell response generated by a DC may be determined more by the type of activation signal and activation status of the DC rather

than the DC lineage as significant plasticity between DC lineages has been found [9-12]. The complete delineation of DC subsets and their functions in different species is still under investigation [13].

Due to their essential function in directing immune responses, DC are a prime target for pathogen manipulation. Pathogen-induced manipulation of DC function would potently interfere with host immune development and has been postulated to explain the suppressed immune response to many pathogens and viruses including the lentivirus human immunodeficiency virus (HIV). Lentiviral infection likely induces changes in DC both through direct effects of viral infection and through virus-induced alterations in cytokine production [14]. In fact, lentiviral infection has been shown to alter DC growth, maturation, and function in favor of viral proliferation and spread although these changes are not fully understood and conflicting evidence exists [15-18].

Dendritic cells predominate on mucosal surfaces and are one of the first cell targets of lentiviruses [19, 20]. Infected DC traffic to local lymphoid tissue where the virus can be efficiently transmitted and expanded in its primary T cell targets [15]. Lentivirus infection of DC has also been found to have primary affects on DC and their function. Binding of HIV-1 gp120 to the DC-specific C-type lectin DC-SIGN plays a key role in the dissemination of HIV-1 by DC. Recent data demonstrates that ligation of DC-SIGN alters the balance of subsequent TLR-mediated cytokine production to favor IL10 production [21]. Exposure to HIV-1 gp120 *in vitro* has been shown to cause abnormal DC maturation and function characterized by decreased IL12 production and allostimulatory capacity [22]. HIV-infected monocyte-derived DC produce HIV gp120, which impairs normal CD4+ T cell function, and the DC were found to be poor

stimulators of allogeneic CD4+ T cell proliferation and IL2 production [23]. Alterations in DC-T cell interactions due to suspected virus-induced interference with CD40L-CD40 interactions between DC and T cells have also been identified [24-26]. Human immunodeficiency virus-1-infected DC up-regulate cell surface markers but have altered cytokine production in response to CD40 ligand stimulation [25]. Dendritic cells infected with HIV-1 have also been found to become cytotoxic and induce apoptosis in infected and uninfected CD4+ T cells *in vitro* [27].

Lentiviral-induced DC dysfunction, particularly late in infection, may inhibit efforts to use DC in viral therapy [17, 28]. Despite the importance of DC in stimulating an immune response, only two studies to date have been able to show any positive response to therapeutic DC vaccination in lentiviral infections [29, 30]. The mechanisms behind the failure of DC therapies remain undetermined. Additionally, the cause for decreased numbers of DC and possible altered DC function in HIV infection has not been fully explored. Although it has been postulated that failure in development and function of DC from CD34+ stem cells may occur with HIV infection, the majority of research in HIV to date has focused on peripheral DC [31]. Little is known about BM-derived DC in HIV infection.

Feline immunodeficiency virus (FIV) produces a disease strikingly similar to HIV-1-associated AIDS with clinical phases analogous to those of HIV [22-43]. The feline FIV model provides unique opportunities to study mDC derived directly from bone marrow progenitor cells at all stages of disease as well as to implement subsequent therapeutic interventions. In this study we used the FIV model to evaluate feline BM-

derived mDC for changes in characteristics and function induced by chronic lentiviral infection in cats.

METHODS

Animals

Bone marrow aspirates and blood samples were collected from specific pathogen-free (SPF) cats aged 4 to 96 months from a colony maintained at Colorado State University (Fort Collins, CO) in accordance with Animal Care and Use Committee Regulations.

Virus and virus quantification

Feline immunodeficiency virus infection was induced by intraperitoneal injection of 500ul pooled plasma from FIV-PG (clade C) infected-cats. The FIV p26 Gag antigen ELISA was used on peripheral blood mononuclear cells and culture supernatants to detect viral replication. Protocol for the ELISA was as published by Dreitz *et al.* [44]. Real-time DNA PCR was used to detect proviral load using primers and probes adapted from those developed by Leutenegger *et al.* [45]. Cellular DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA). PCR reactions were performed in a 25µl volume containing 12.5µl TaqMan Universal PCR Mastermix (Applied Biosystems), 400nM of each primer (MWG Biotech), 80nM of probe (MWG Biotech), and 5µl of sample DNA or plasmid FIV DNA standard. Real-time PCR was performed

on an iCycler iQ Real-time PCR Detection System (Bio-rad). Starting quantities of viral DNA were extrapolated from the plasmid DNA standard curve.

Sample collection

Blood was collected via jugular venipuncture and bone marrow was collected from the proximal humerus of specific pathogen free and chronic FIV-infected cats. Mononuclear cells were separated from the bone marrow or blood immediately after collection using ficoll-hypaque (Histopaque-1077, Sigma-Aldrich, St. Louis, MO) density gradient centrifugation. Collected bone marrow mononuclear cells (BMMC) were either plated immediately or frozen at –70°C at a concentration of 1 x 10^7 cells/ml in freezing media consisting of 10% DMSO (Fisher Scientific, Fairlawn, NJ), 45% fetal bovine serum (FBS, Atlanta Biologicals, Norcross, GA), and 45% LBT media containing RPMI-1640 medium (Sigma-Aldrich) supplemented with 20% FBS (Atlanta Biologicals), L-Glutamine 2mM (Invitrogen, Carlsbad, CA), Penicillin 100U/ml (Invitrogen), Streptomycin 100ug/ml (Sigma-Aldrich), and 2ME 50uM (Sigma-Aldrich).

Cell culture

Bone marrow mononuclear cells (BMMC) were cultured at an initial concentration of 5 x 10^6/ml in six-well plates at 37°C in 5% CO_2 for 10 days in LBT media consisting of RPMI 1640 media with Glutamax (Sigma-Aldrich) with 15% FBS (Atlanta Biologicals, Norcross, GA), penicillin (100U/ml; Invitrogen), streptomycin (100ug/ml; Sigma-Aldrich), and 2-mercaptoethanol (50uM; Sigma-Aldrich). Fifty percent of the media was changed and 100ng/ml recombinant feline granulocyte-

macrophage colony stimulating factor (rfGM-CSF; R&D Systems, Minneapolis, MN) was added to the media every other day. Culture wells were divided after six days to prevent overcrowding.

Cell phenotype

Cell phenotype and co-stimulatory molecule expression was determined by flow cytometry. Surface markers on mDC were labeled using commercially available directly conjugated antibodies to MHC class II (clone Tu39, BD Pharmingen). The Zenon Alexa Fluor APC kit (Molecular Probes, Eugene, OR) was used according to manufacturer's instructions to label unconjugated antibodies to CD80 (B7.1, kindly provided by Dr. W. Tompkins, NCSU, Raleigh, NC) and CD1a (Fe1.5F4, Serotec). CD11c (CA11.6A1, Serotec) was labeled using secondary anti-mouse antibodies (715-116-150, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) to allow for multicolor flow cytometry (Dako Cytomation).

Myeloid DC phenotype was determined on unsorted cells. Cells were stained after 0, 3, 5, 7, and 10 days of culture. Harvested cells were collected by centrifugation for 10 minutes at 1000 r.p.m., washed with fluorescence-activate cell-sorting (FACS) buffer (PBS, 1% fetal bovine serum, 0.1% sodium azide), and blocked with serum for 30 minutes. The cells were then incubated consecutively with antibodies and conjugates for 30 minutes at 4°C. The cells were washed after each incubation and then washed twice with FACS buffer prior to analysis or fixation with 2% paraformaldehyde (30 minutes at 4°C). Fixed cells were washed and stored in FACS buffer at 4°C until analysis. Samples

were run on a Dako-Cytomation flow cytometer and analyzed using Summit software (Dako).

Endocytic uptake

Endocytic ability of the mDC was measured by uptake of TRITC-dextran (Invitrogen) with a molecular weight of 3000. Cells were cultured as above for 5, 8, or 10 days and then cultured an additional 48 hours with media or a maturation cocktail (PGE-2 10^{-6}M, Sigma; TNFα 5ng/ml, R&D Systems; IL-6 20ng/ml, R&D Systems; and IL-1β 10ng/ml, R&D Systems) [46]. The cells were harvested, collected by centrifugation for ten minutes at 1000 r.p.m., and incubated in LBT media on ice for one hour. TRITC-dextran (0.2mg/ml) was then added to the samples immediately prior to incubation for exactly one hour at 37°C. Control samples were treated similarly but incubated on ice for exactly one hour. Cells were then washed twice in ice-cold PBS, and fluorescence was determined by flow cytometry (Dako). Data was collected for percent positive cells and MFI. An ice control was run with each sample, and data was analyzed as fold change from ice control (reading at 37°C - reading at ice, divided by reading at ice) to remove individual sample background variability.

Mixed leukocyte reaction

The ability of mDC to stimulate T cell proliferation was evaluated by mixed leukocyte reaction (MLR) using Carboxyfluoroscein succinimidyl ester (CFSE) to document cellular proliferation. After ten days in culture, cells were stained for MHC class II (Tu39, BD PharMingen, San Jose, CA) and unconjugated CD11c (CA11.6A1,

Serotec, Raleigh, NC) with allophycocyanin-conjugated affinipure goat anti-mouse IgG secondary (115-135-205, Jackson ImmunoResearch, West Grove, PA) and sorted by high-speed flow cytometry (Dako Cytomation, Fort Collins, CO). CD4+ T cells from naïve SPF allogeneic donors were isolated from PBMC using antibody to feline CD4 (3-4F4, Southern Biotech, Birmingham, AL) and immunomagnetic beads (Miltenyi Biotec, Auburn, CA) [47]. The CD4+ T cells were then labeled using the CellTrace CFSE Cell Proliferation Kit (C34554, Molecular Probes, Eugene, OR) following the manufacturer's instructions. 2×10^5 CFSE-labeled CD4+ T cells were cultured in duplicate in 96-well round-bottomed tissue culture plates (Falcon, Franklin Lakes, NJ) with 2.5×10^4 purified DC for five days at 37°C. Samples were then harvested and analyzed by flow cytometry (Dako).

In all MLR experiments, concanavalin A (conA; 5μg/ml, Sigma-Aldrich) was added to T cell only wells at Day 0 to confirm proliferative ability of the T cells. Concanavalin A, or media as a control, was also added to some co-culture wells at Day 5, and the cells were cultured for an additional forty-eight hours prior to analysis.

Percent T cell apoptosis in the MLR wells was assessed using annexin V (Molecular Probes) according to manufacturer's instructions. Cells were harvested from MLR wells on Day 3, 5, and 7 of co-culture, stained with annexin V, and analyzed immediately by flow cytometry (Dako).

Statistical analysis

Statistical analysis was performed using a statistical software package available with Microsoft Excel and StatView software (SAS Institute). Parametric data was

analyzed using a two-tailed Student's t-test. The Wilcoxon-Mann-Whitney test was used to analyze non-parametric data. Statistical significance was assumed if the p value was less than 0.05.

RESULTS

Bone marrow yields and cell growth

Routine bone marrow aspirates from cats yielded 1-20 x 10^7 bone marrow mononuclear cells (BMMC) per cat per collection. Cells demonstrated equivalent growth whether freshly obtained or viably frozen at -70°C for up to a year (data not shown). A notable decline in total cell number occurred approximately 3-5 days into the culture period, likely due to death of non-dendritic cell (DC) hematopoietic precursors (Figure 1.1A). Cell numbers then increased over the remaining culture period to yield adequate numbers of myeloid DC (mDC) for subsequent experiments. Myeloid DC yield was approximately 60% of BMMC input numbers at the end of the 10 day culture period. There was no difference in mDC yield from naïve and chronically-FIV-infected cats after 10 days in culture (Figure 1.1B).

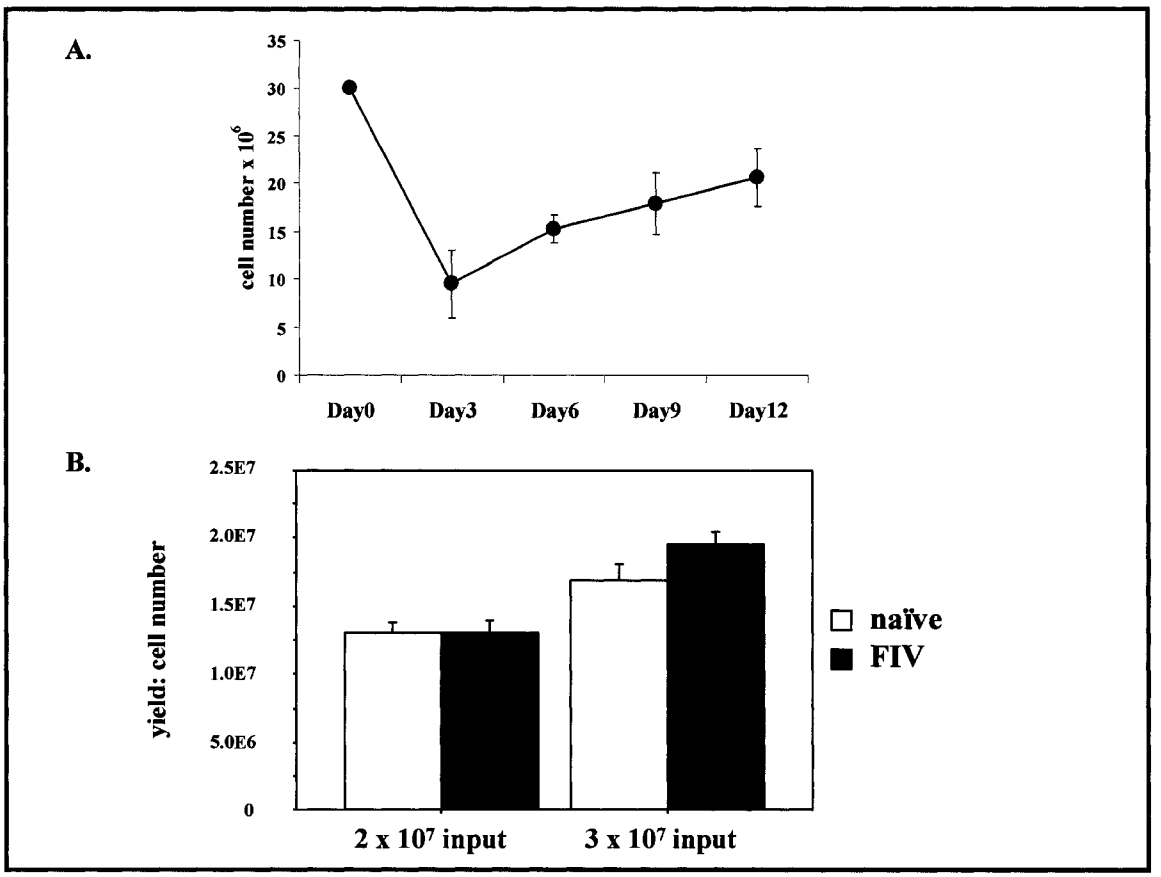

Figure 1.1: Bone marrow-derived myeloid DC (mDC) growth was unaltered by FIV infection status. A) Bone marrow mononuclear cells cultured in LBT media and rfGM-CSF showed an initial decline in numbers at approximately Day 3 of culture. After Day 5, cells numbers increased over the remaining culture period. B) FIV infection status did not affect mDC culture yield when cells were cultured for ten days with LBT media and rfGM-CSF ($p>0.05$; $n>100$ cultures for each group).

Regardless of FIV status, BMMC cultured for ten days with rfGM-CSF showed properties consistent with mDC. The cells were non-adherent and showed characteristic processes seen with mDC (Figure 1.2).

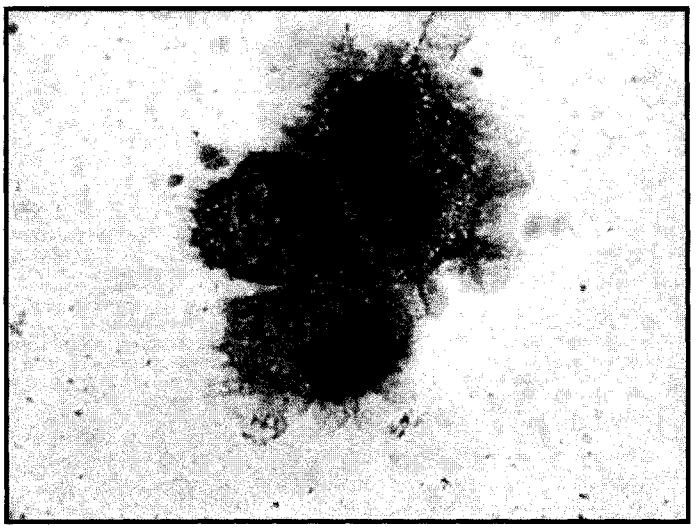

Figure 1.2: Representative photomicrograph of bone marrow-derived myeloid DC after ten days in culture with LBT media and rfGM-CSF (100x). Cells were non-adherent and demonstrated prominent dendrites as is characteristic of DC.

Virus quantification

Feline immunodeficiency virus infection was confirmed in all cats used as mDC donors in this study. Proviral loads were at stable levels consistent with chronic FIV infection in these cats (mean: 4888 copies/10^6 PBMC, range: 235-24,184). Levels of FIV p26 Gag antigen in culture supernatants were found to increase during the ten day culture period in cultures from chronically FIV-infected cats (data not shown). Supernatants from wells containing mDC from naïve cats were consistently negative for FIV p26 Gag antigen (data not shown).

Cell phenotype

Phenotypic evaluation was performed using flow cytometry to measure percent expression (Figure 1.3A-C) and MFI (Figure 1.4A-C) of the surface markers CD11c, MHC class II, CD80, and CD1a of the mDC during the culture period.

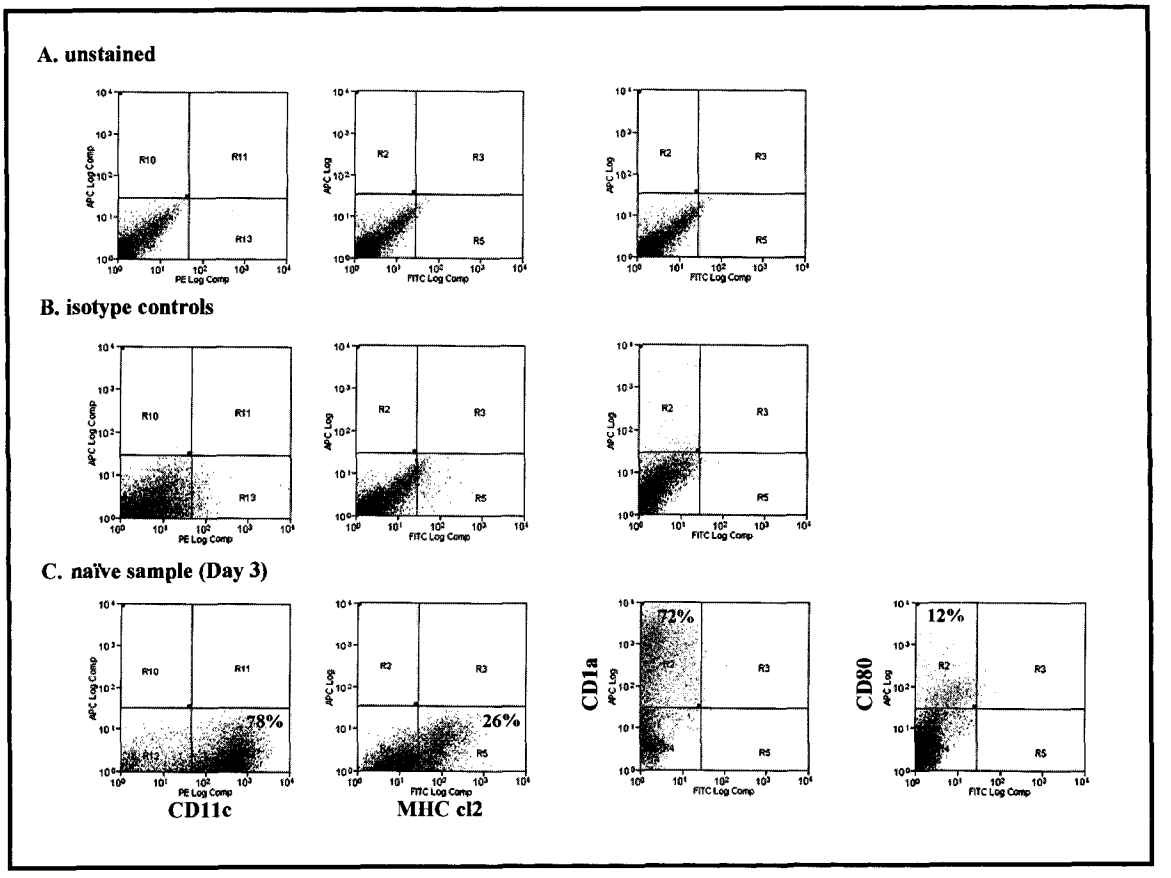

Figure 1.3: Percent expression of CD11c, MHC class II, CD1a, and CD80 was determined on bone marrow-derived myeloid DC (mDC) using flow cytometry. Unstained cells (A) and isotype controls (B) were used to set gates for analysis. C) Representative percent expression histograms from a naïve mDC sample after three days in culture.

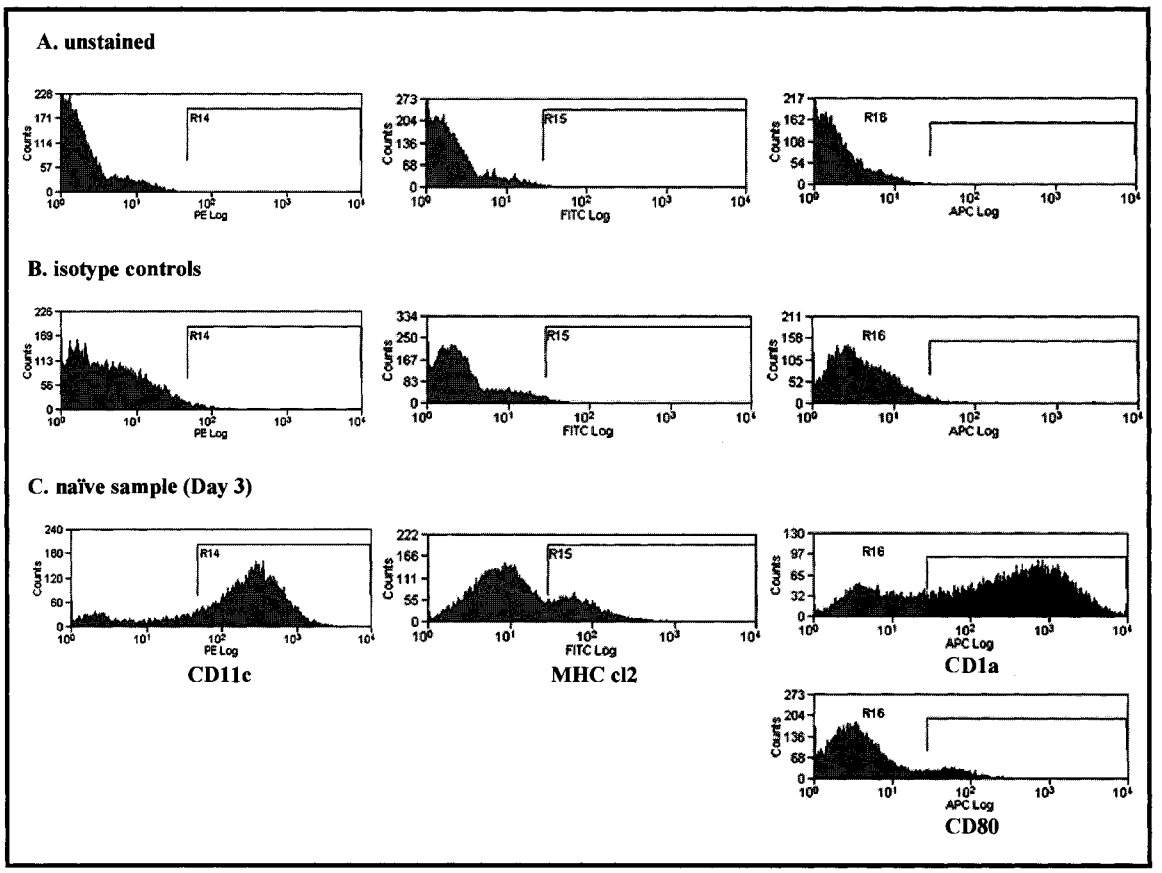

Figure 1.4: Mean fluorescence intensity (MFI) of CD11c, MHC class II, CD1a, and CD80 was determined on bone marrow-derived myeloid DC (mDC) using flow cytometry. Unstained cells (A) and isotype controls (B) were used to set gates for analysis. C) Representative MFI histograms from a naïve mDC sample after three days in culture.

The phenotype data showed significant individual variation, especially for mean fluorescence intensity. A steady increase in the proportion of CD11c and MHC class II double-positive cells was noted during the culture period, corresponding to an increased percentage of MHC class II expression (Figure 1.5A-B). Percent CD11c expression peaked by Day 3 of culture and remained high although the MFI continued to gradually increase over the culture period. The MFI of MHC class II and CD80 remained relatively low and unchanged throughout the culture period. Percent expression of CD80 increased

at culture Day 5 and CD1a increased at culture Day 3, and then both markers maintained peak levels over the remaining culture period. The peak percentage and MFI of CD1a remained relatively higher than CD80 after culture Day 3 (Figure 1.5A-B). No significant differences in percent or MFI expression of CD11c, MHC class II, CD80, or CD1a were found in mDC from naïve versus FIV-infected cats during the ten day culture period (Figure 1.6A-F).

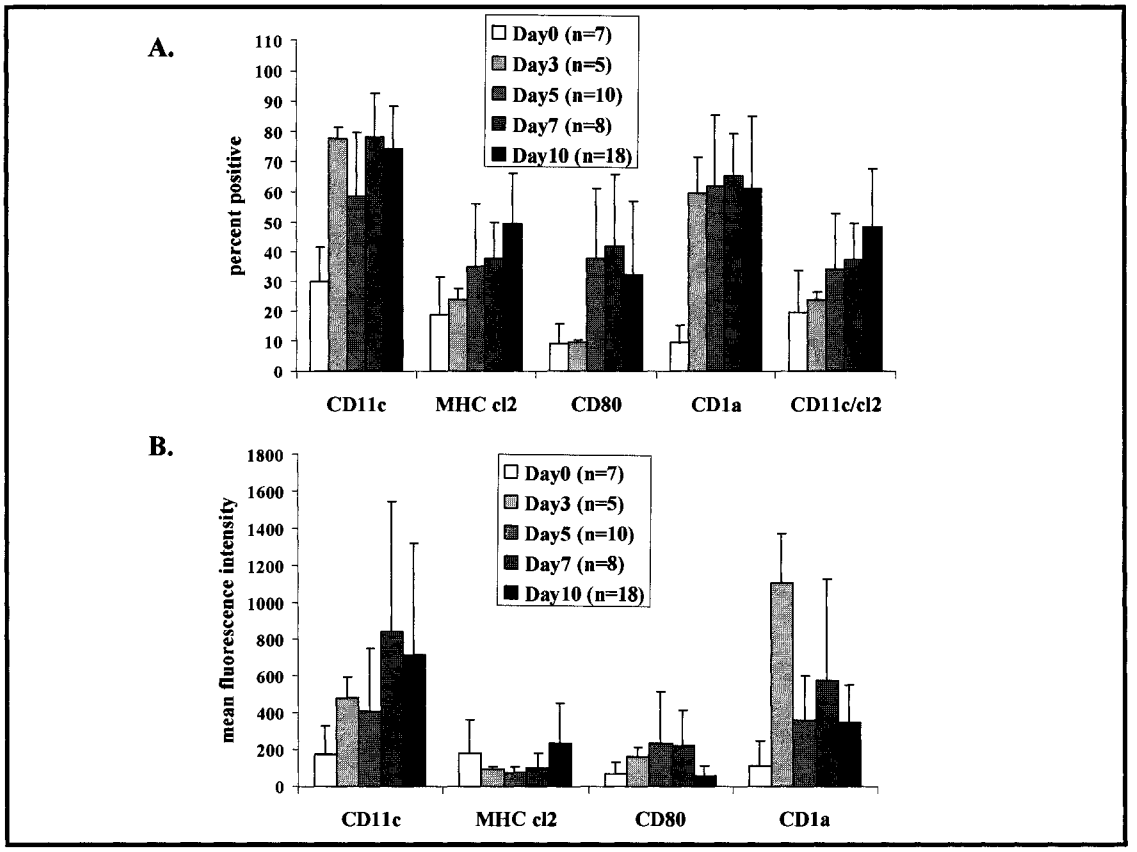

Figure 1.5: Expression of CD11c, MHC class2, CD80, and CD1a in feline bone marrow-derived myeloid DC was measured by flow cytometry at various time points during the ten day culture period. Samples were assessed for both percent positive (A) and mean fluorescence intensity (B). Data from naïve cats is depicted; error bars denote standard deviation.

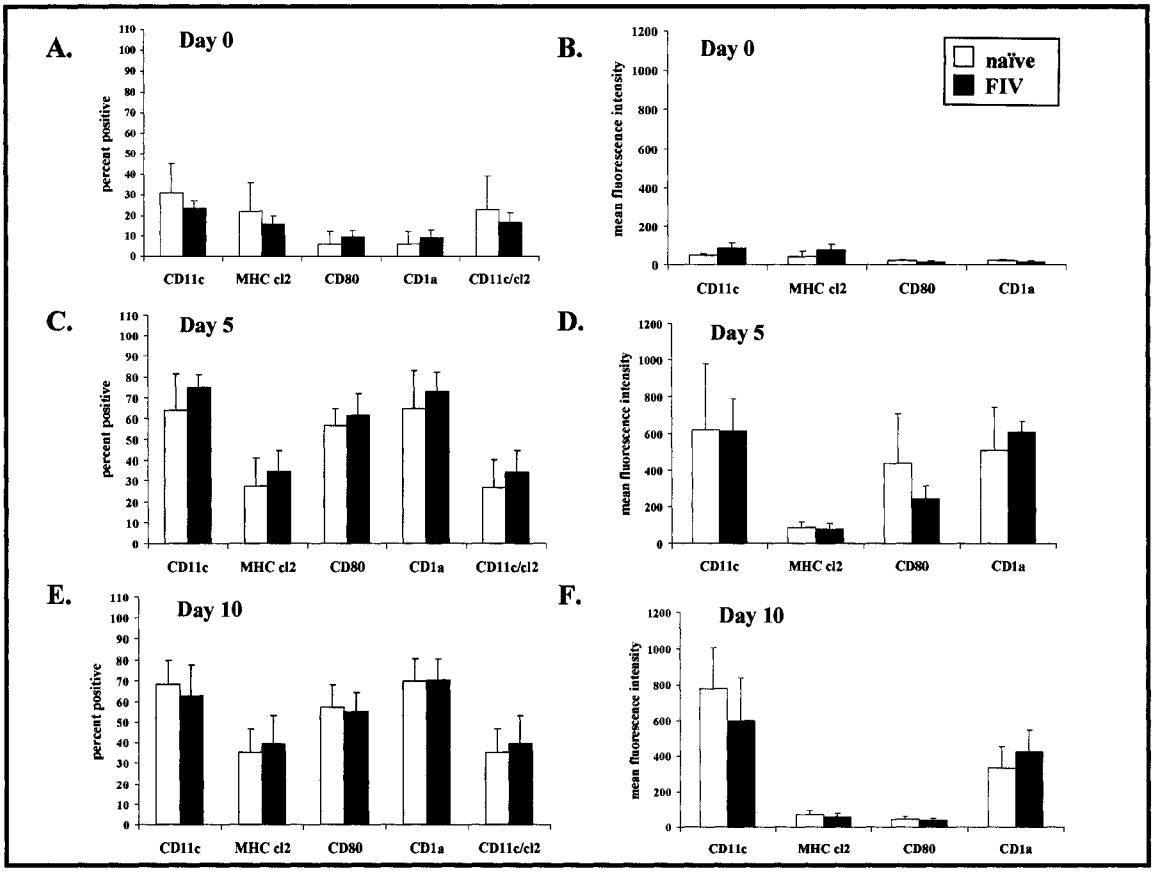

Figure 1.6: Comparison of percent positive and mean fluorescence intensity of CD11c, MHC cl2, CD80, and CD1a in bone marrow-derived myeloid DC from naïve and FIV-infected cats did not show any significant differences related to FIV infection status during the ten day culture period. Representative data after cultures for Day 0 (A and B), Day 5 (C and D), and Day 10 (E and F) is depicted; n=4-5 per group; error bars denote standard deviation.

Endocytic uptake

Effects of chronic FIV infection on mDC endocytic capacity were measured using TRITC-dextrans uptake. Previous studies have found that endocytic capacity of DC is higher in immature cells and decreases with DC maturation, and we would expect our cells to have decreased endocytic uptake after exposure to maturation cocktail. Cells were assessed at day 7, 10, and 12 of culture with and without 48 hour exposure to a

maturation cocktail. All mDC showed the expected trend to decreased endocytic uptake after exposure to maturation cocktail (Figure 1.7A-F). Cells from FIV-infected cats showed a trend for a greater decrease in dextrans uptake after exposure to cocktail than mDC from naïve cats, however no statistically significant differences were found in the ability of BM-derived mDC from naïve or chronically-FIV-infected cats to uptake dextrans at any of the measured time points (Figure 1.7A-F).

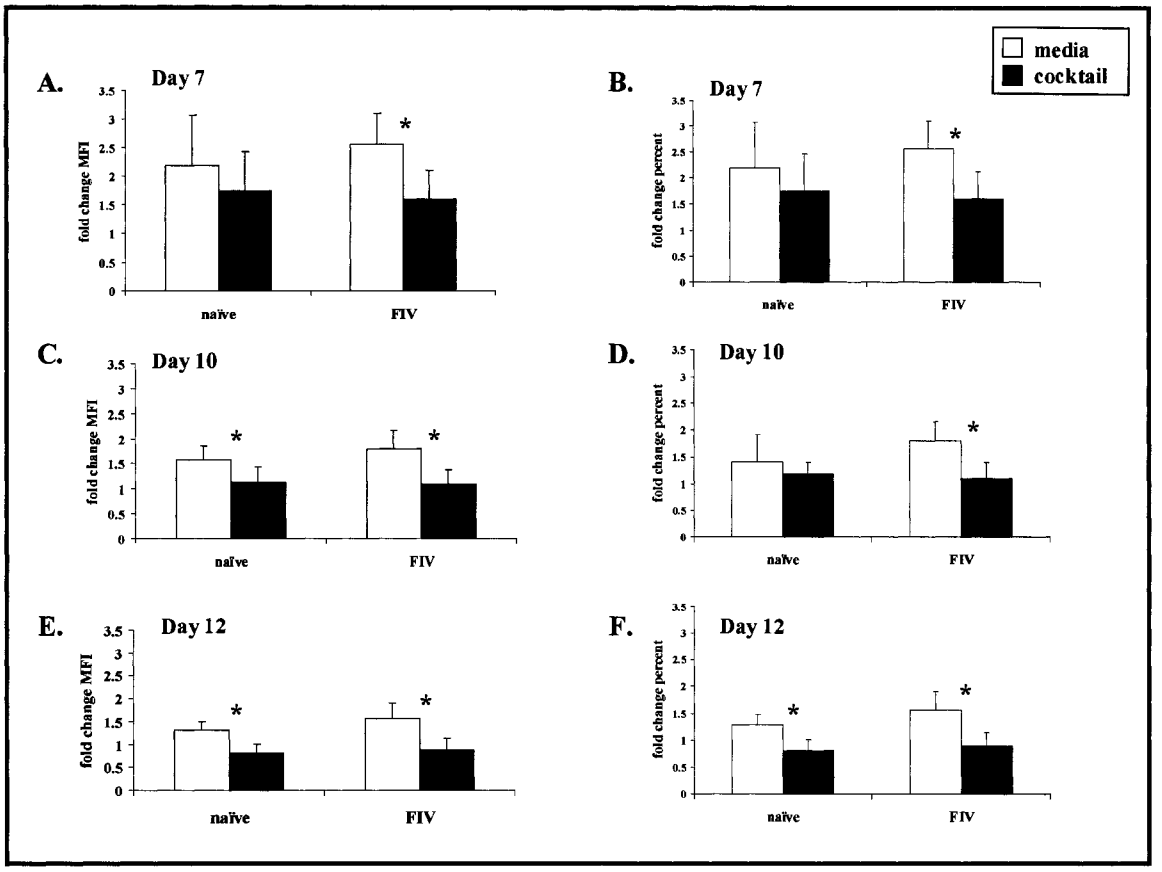

Figure 1.7: Endocytic capacity of bone marrow-derived myeloid DC (mDC) as measured by TRITC-dextrans uptake was not affected by FIV infection status. Endocytic uptake was measured by percent positive and mean fluorescence intensity (MFI) of TRITC-dextran after culture for 7 (A and B), 10 (C and D), and 12 (E and F) days with media (white bars) or a maturation cocktail (black bars) added for the final 48 hours. Data represented as fold change over sample controls (37°C-ice/ice). Error bars denote standard deviation; n=6 for all samples. * indicates statistically significant differences between media and cocktail ($p<0.05$, Student's t-test).

Mixed leukocyte reaction

Bone marrow-derived mDC function was assessed by mixed leukocyte reaction. Myeloid DC were co-cultured with allogeneic CD4+ T cells and assessed by flow cytometric analysis of CFSE staining. The mDC were harvested at Day 10 of culture and sorted by high-speed flow cytometry for co-expression of MHC class II and CD11c. Purity of the mDC samples was confirmed by flow cytometry to be consistently greater than 95% (data not shown). Allogeneic CD4+ T cells were sorted from PBMC by immunomagnetic beads. Purity of the T cells was analyzed by flow cytometry and found to be greater than 80% in all instances (data not shown).

Bone-marrow-derived feline mDC from naïve cats were able to stimulate strong allogeneic CD4+ T cell proliferation after five day co-culture (Figure 1.8A). Contrastingly, mDC obtained from chronically-FIV-infected cats were not able to induce allogeneic CD4+ T cell proliferation above baseline (Figure 1.8B). The average percent CD4+ T cell proliferation as measured by flow cytometric analysis of CFSE staining was significantly lower when the cells were co-cultured with mDC from chronically FIV-infected cats (Mann-Whitney $p=0.002$, Figure 1.8C). Addition of the T cell mitogen concanavalin A to the co-cultures at Day 5 induced significant T cell proliferation in all co-cultures, indicating that the T cells co-cultured with mDC from FIV-infected cats were able to proliferate when exposed to an effective stimulus (Figure 1.9). Annexin V staining on Day 3, 5, and 7 did not indicate any difference in apoptosis in the CFSE-stained T cells cultured with mDC from naïve or chronically FIV-infected cats (Figure 1.10).

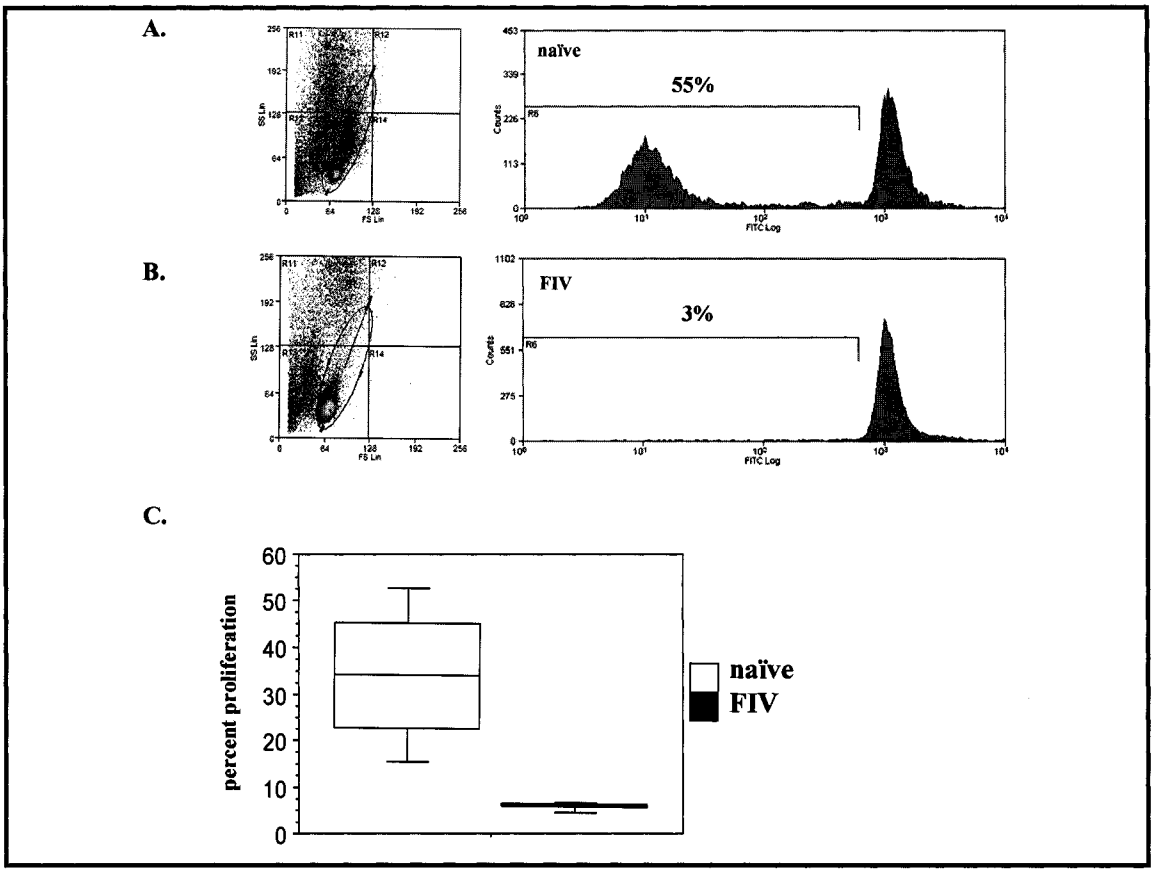

Figure 1.8: Allogeneic CD4+ T cells co-cultured for five days with bone marrow-derived myeloid DC (mDC) from FIV-infected cats failed to proliferate as compared to T cells co-cultured with mDC from naïve cats. A) and B) Representative forward and side scatter plots and FITC-CFSE histograms for T cell proliferation when the cells were co-cultured for five days with mDC from naïve (A) and FIV-infected (B) cats. C) Average percent CD4+ T cell proliferation as measured by flow cytometric analysis of CFSE staining was significantly lower when the cells were co-cultured with mDC from FIV-infected cats as compared to mDC from naïve cats (Mann-Whitney p=0.002, naïve n=8, FIV n=6).

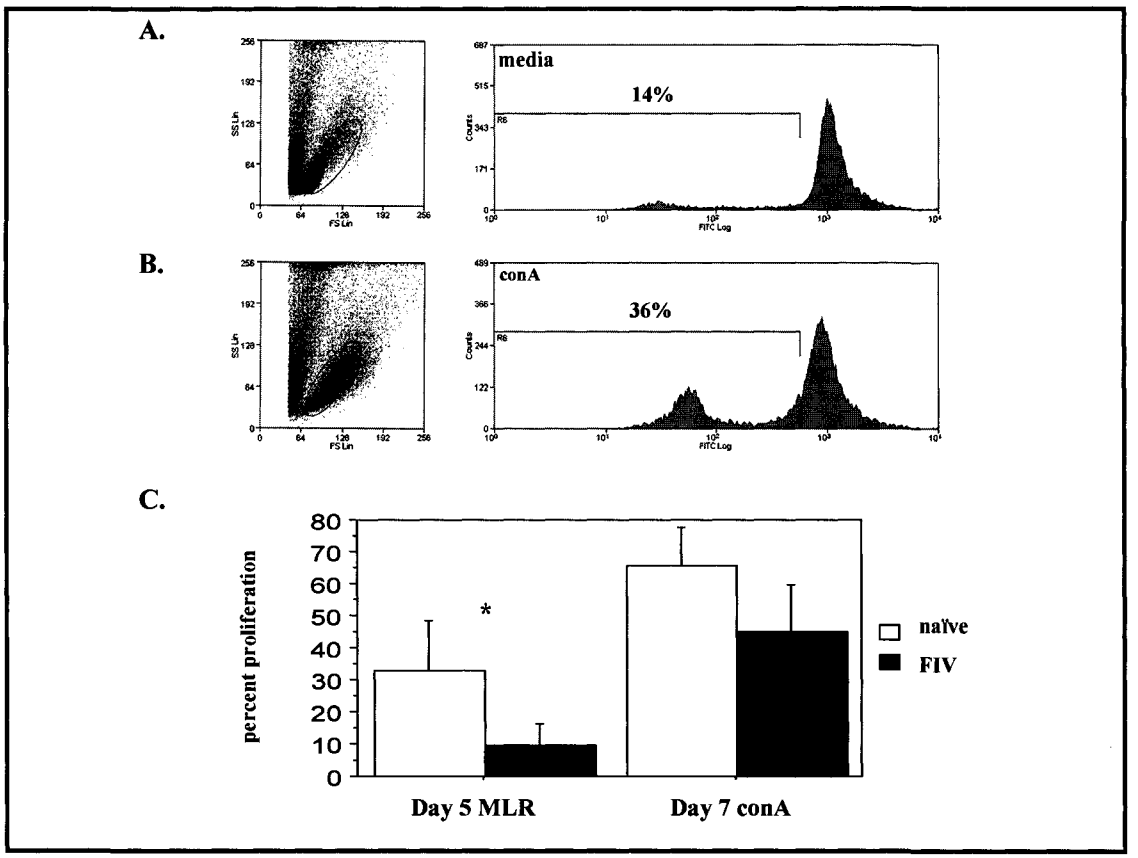

Figure 1.9: Allogeneic CD4+ T cells incubated with bone marrow-derived myeloid DC (mDC) from FIV-infected cats can proliferate maximally with subsequent mitogenic stimulation. CFSE-stained CD4+ T cells were co-cultured with mDC for five days and then media or conA (5μg/ml) was added to the co-culture wells for an additional 48 hours. Stimulation with conA increased CD4+ T cells proliferation in co-cultures with mDC from FIV-infected cats compared to media controls. A) and B) Representative forward and side scatter plots and FITC-CFSE histograms for T cell proliferation when the cells were cultured for 48 hours with media (A) and conA (B). C) Average percent proliferation as measured by flow cytometric analysis of CFSE staining was significantly increased in CD4+ T cells stimulated with conA for 48 hours (Day 7 conA) after co-culture with mDC from both naïve and FIV-infected cats as compared to Day 5 MLR levels ($p<0.05$, Student's t-test; naïve n=6, FIV n=6).

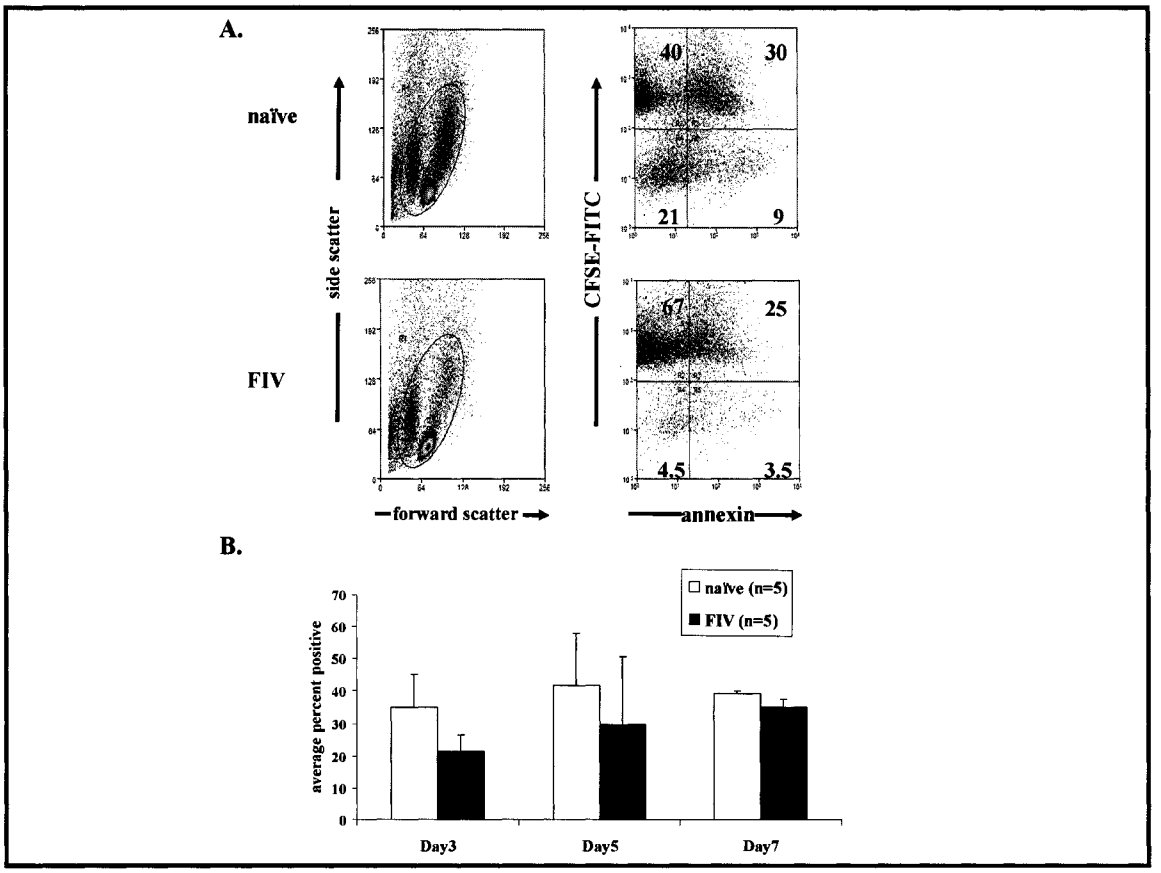

Figure 1.10: FIV infection status did not affect percent apoptosis of allogeneic CFSE-stained CD4+ T cells co-cultured with bone marrow-derived myeloid DC (mDC). Apoptosis was measured by flow cytometry in all CD4+ T lymphocytes in wells from the mixed leukocyte reaction using annexin V staining. A) Representative forward and side scatter plots showing CD4+ T cell gates and CFSE-FITC and annexin V pacific blue histograms used to assess percent apoptosis in T cells co-cultured with mDC from naïve and chronically FIV-infected cats. B) The percentage of CD4+ T lymphocytes that expressed annexin V was not significantly different in cells cultured with mDC from naïve or FIV-infected cats for 3, 5, or 7 days. Error bars denote standard deviation.

DISCUSSION

The current scientific literature provides conflicting evidence as to the effects of the lentivirus HIV-1 on DC, focusing mainly on peripheral DC. To exploit the unique

strengths of the FIV model for the study of DC dysfunction induced by lentivirus infection, we sought to define the effects of FIV on bone marrow-derived myeloid DC (mDC). In our experiments we document the existence of significant differences in mDC obtained from the bone marrow of chronically FIV-infected cats as compared to mDC from naïve cats.

To complete the desired experiments, we successfully developed methods to generate large numbers of feline mDC from bone marrow precursors. The cells generated from feline bone marrow mononuclear cells and used in our experiments were consistent with mDC. The cells had marked dendritic processes, were non-adherent in culture, and cell cultures showed up to 99% purity for CD11c and MHC class II co-expression after ten days. Percent surface expression of CD11c was high after short-term culture and remained high over the culture period. Although not used in the comparison data, our mDC also had high expression of CD11b, which has been observed previously on both murine and feline BM-derived DC [48, 49]. Percentage of cells expressing MHC class II and CD80 (B7.1) increased during the culture period as would be expected with greater percentage of mDC in the cultures over time. Expression of CD1a also increased around Day 5 of culture and remained elevated during the remainder of the culture period corresponding to the increase in cell number after the suspected loss of non-DC cells around Day 3. Unlike some human DC studies where CD1a expression has been correlated with DC immaturity, previous evaluation of peripheral monocyte-derived feline DC noted that CD1a expression was high in both immature and mature feline DC [50]. We were limited in our ability to further assess phenotype of the cells by the paucity of currently available feline antibodies.

Immature DC use macropinocytosis and mannose-receptor uptake to concentrate antigen in the MHC class II compartment, whereas mature DC downregulate their phagocytic activity and focus on presentation of acquired antigen [6]. In accordance with expected immature mDC endocytic capacity, bone marrow-derived mDC were able to uptake dextran particles. Exposure to a maturation cocktail decreased the uptake of dextrans by the cells. The effect of maturation on endocytic capacity noted in the cells may have been more significant if we had used LPS, which has recently been shown to induce greater maturation in feline DC [50].

Finally, bone marrow-derived mDC from naïve cats were able to stimulate significant allogeneic CD4+ T cell proliferation in the mixed leukocyte reaction (MLR), a hallmark of DC function. As the cells were able to stimulate an MLR without addition of maturation cocktail, our culture system and/or the high-speed flow sorting performed to purify mDC used in the MLR likely induced some maturation of the cells [50]. Further characterization of the maturation status of the cultured bone marrow-derived mDC was not performed in this study.

Utilizing cultured bone marrow-derived mDC, we compared the mDC generated from naïve and chronically FIV-infected cats to determine whether FIV infection induced differences in mDC growth, phenotype, maturation, or function. Although decreased levels of circulating DC and decreased viability of cultured blood-derived mDC have been seen with HIV infection, we did not find any significant differences in growth of the bone marrow-derived mDC over the ten day culture period related to infection status of the donor cats [51]. Recent evaluations of DC in chronic HIV infection suggest that the

decrease in circulating DC may be due to relocation to lymphoid tissues rather than virus-induced cell death [52].

Virus-associated changes in cell maturation and function would be expected to be represented by altered surface marker expression. Kawamura *et al.* found that DC expression of CD4, CD1a, and MHC class I decreased twelve days after HIV infection [23]. In this study chronic FIV infection did not induce significant changes in mDC phenotype based on percent expression and mean fluorescence intensity of CD11c, MHC class II, CD80, and CD1a. Similarly, Freer *et al.* did not find an effect of acute *in vitro* FIV infection on upregulation of MHC class II and CD80 in monocyte-derived DC in response to LPS stimulation [50]. Although similar to other studies of feline DC and including MHC class II and the co-stimulatory molecule CD80, the list of phenotypic markers we measured is far from exhaustive, and differences might be present for other molecules. Unfortunately, previous studies have not found cross-reactivity to DC-SIGN or the anti-human CD83 antibodies on feline monocyte-derived DC [50]. As has been seen in a similar system, significant variability in the expression levels of the measured surface markers was present between samples, which would mask small differences in phenotype that might have biological significance [49]. Collection of cells at different stages of differentiation and maturation, and variable rates of subsequent differentiation and maturation are postulated to lead to a less than uniform population when assessed by surface marker expression. Although our cells were from chronically-infected animals, were handled uniformly and cultured for a ten day period, some variability in the differentiation and maturation state likely existed in the samples.

Human immunodeficiency virus has been implicated in altered DC maturation status with subsequent functional consequences [16, 18, 22, 53]. Using phagocytic capacity as a measure of DC maturation, we did not find any significant differences related to infection status of the donor cats on phagocytic ability of the mDC with or without maturation. There was a trend towards greater decrease in dextran uptake with exposure to a maturation cocktail in mDC from FIV-infected cats, suggesting that the mDC from FIV-infected cats were less mature than cells from naïve cats causing them to respond more to the maturation cocktail. However, the trend did not reach statistical significance.

Although chronic FIV infection did not appear to significantly alter bone marrow-derived mDC growth, phenotype, or maturation as measured by dextrans uptake, a significant effect of FIV infection on bone marrow-derived mDC function was uncovered in the MLR. Bone marrow-derived mDC from FIV-infected cats failed to induce significant stimulation and proliferation of allogeneic CD4+ T cells in the MLR. Maturation status of the cells could influence the ability of the mDC to stimulate T cells as immature DC are less able to induce T cell proliferation than mature DC presumably due to lower expression of MHC class II and co-stimulatory molecules such as CD80 and CD83 [54, 55]. Although no differences were seen in MHC class II and CD80 expression or endocytic capacity of the cells prior to the MLR, it is possible that cell handling and purification techniques used in setting up the MLR, which induced functional maturation in the naïve cells, failed to induce similar changes in the mDC from FIV-infected cats.

Inhibition of DC maturation and subsequent immunostimulatory function by HIV-1 and SIV are one proposed mechanism of AIDS-related immunodeficiency [16, 22, 51, 53]. Human immunodeficiency virus-1 replicates in human immature DC, and the HIV-infected mDC have been found to subsequently fail to mature or induce an MLR [51, 56]. Freer *et al.* recently reported that feline monocyte-derived DC maturation is not hampered or induced by FIV infection or exposure and that the infection status of monocyte-derived DC does not appear to influence their ability to stimulate an MLR after LPS-induced maturation [57]. In their study Freer *et al.* evaluated peripheral monocyte-derived DC infected *in vitro* with a clade A virus and had a low level of productively infected DC. We used a clade C virus and chronic *in vivo* infection of cells and saw an increase in p26 Gag antigen over the course of the culture period suggesting a higher level of infectivity in our cells. The percentage of bone marrow-derived mDC infected with FIV was not determined in this study but may be relevant to the different results seen in the two studies. Differences in innoculum, virus concentration, and duration of infection have all been implicated as causes for the contradictory results obtained in studies of HIV effects on mDC function [58].

The frequency of HIV-1 infection of blood mDC is low, and it has been suggested that the effects seen on blood mDC with HIV-1 infection are therefore the result of increased susceptibility and effects of virus on immediate progenitors of the blood DC in the bone marrow [53]. Hematopoietic progenitor cell colony growth and differentiation is known to be inhibited in long-term bone marrow cultures of HIV positive patients [59-61]. HIV-induced alterations in the microenvironment and cytokine dysregulation may cause the observed suppression, especially as hematopoietic growth factors such as GM-

CSF, erythropoietin, and stem cell factor have been reported to reduce HIV-related cytopenias [62, 63]. It has been thought that CD34+ progenitor cells are resistant to HIV infection although *in vitro* infection of the cells has been reported [64, 65]. Our model using bone marrow-derived mDC is ideally created to further evaluate the commonly-identified functional defect in T cell stimulation seen in mDC with viral infection and the possible effects on their progenitor cells in the bone marrow.

Dendritic cells can be infected *in vitro* by HIV, SIV, and FIV [53, 57, 66]. The p26 levels measured in supernatants of our mDC from FIV-infected cats increased during the culture period implying persistent and productive infection of the cells as infectious lentivirus captured by DC does not survive more than 24 hours *in vitro* [67]. Feline immunodeficiency virus-infected monocyte-derived feline DC have been shown to infect activated PBMC efficiently *in trans*, which could affect the ability of T cells to proliferate in co-cultures with infected DC [57]. Previous attempts in our laboratory to decrease viral transmission from mDC to T cells with anti-retroviral agents uniformly impaired the MLR reaction in all cultures. Therefore, to evaluate whether the lack of T cell proliferation in our MLR experiments was due to effects of FIV infection on the T cells rather than mDC dysfunction, we exposed the T cells to the T cell mitogen conA. The T cells co-cultured with mDC from FIV-infected cats were able to proliferate maximally with conA stimulation. As HIV-1 has been associated with increased CD4+ T cell apoptosis, we also evaluated the T cells co-cultured with mDC from FIV-infected cats for percent apoptosis [27]. The T cells co-cultured with mDC from FIV-infected cats did not show increased apoptosis as measured by annexin V staining. Consequently, the failure of CD4+ T cell proliferation when co-cultured with mDC from chronically

FIV-infected cats appears to be a failure of the mDC to activate the T cells and/or adjacent DC.

Similar findings of decreased DC-induced T cell proliferation have been found with other viruses including HIV. Monocyte-derived DC from hepatitis C virus (HCV) patients were poor activators of CD4+ T cells in mixed lymphocyte reactions [68]. The DC were found instead to stimulate proliferation of regulatory T cells (Tregs), which limit proliferation of HCV-specific lymphocytes and indicates a possible role for mDC in expansion of Tregs to promote chronic infection of HCV patients [68]. Granelli-Piperno *et al.* similarly postulated that HIV-1-infected monocyte-derived DC can elicit IL-10 production and T cell regulation although they were unable to detect IL-10 in later studies [53, 56]. We did not evaluate our cultures for the presence of Tregs or cytokine levels in this study. The immunoregulatory enzyme indoleamine 2,3-dioxygenase (IDO) can block T cell activation and has also been implicated in the altered function observed in virally-infected DC [69]. Although not measured in this study, further investigation into the role of IDO in FIV-induced bone marrow-derived mDC dysfunction has been initiated.

Knight *et al.* found that DC from asymptomatic HIV patients caused only low levels of stimulation of allogeneic lymphocytes in the MLR but lymphocytes from the same patients were able to respond to normal allogeneic DC [70]. They postulated that the failure of T cell stimulation by DC in HIV infection represented an incapacity of the DC to transfer antigenic signals to other DC. The ability of our allogeneic T cells to respond to mitogenic stimulation supports the idea that lentiviral infection impairs the ability of DC to transfer antigenic signals at the immunological synapse. Granelli-

Piperno *et al.* have suggested that HIV-1 interferes with direct T cell stimulation by DC by selecting less immunostimulatory forms of DC or that HIV-1 blocks DC maturation [53]. They found that CD83 and MHC class II expression was decreased in HIV-1-infected mDC [53]. Due to a lack of cross-reactivity and feline reagents, we were unable to measure CD83 expression on our feline mDC but MHC class II expression was not affected by FIV status. In their role as APCs, DC not only function to activate lymphocytes to respond to antigen but they can induce tolerance to the presented antigen depending on maturation state and the stimulatory signals they receive [71]. Further investigation into the maturation state of BM-derived mDC and the effects of maturation status on mDC function are warranted.

As is a common practice in DC studies, we had to culture our mDC in order to obtain enough cells to conduct our experiments. A known complication of the DC culture system is that the culture medium, cytokine exposure, and source of the cells can induce differences in their development [57, 72-74]. The specific phenotype and maturation state of the cells used in these experiments is likely a product of our collection and culture system and may not be representative of the *in vivo* state of mDC in cats. We used only the cytokine GM-CSF in our cultures, which is used to increase mobilization, differentiation, and function of DC. In other studies granulocyte macrophage colony-stimulating factor has not been found to cause significant changes in DC function as assessed by MLR [71]. Our model system capitalized on the ability to collect repeated large samples of cells that encountered FIV over time in their natural host and cellular environment. In comparing cells from naïve and chronically FIV-infected cats that were

treated identically, we believe we have uncovered true differences that exist between the cells. The *in vivo* significance of those differences is currently unknown.

In this study we found that chronic *in vivo* FIV infection induced functional changes in BM-derived mDC that impaired their ability to stimulate allogeneic CD4+ T cell proliferation. The functional defect may be associated with the DC-T cell interaction as measures of mDC growth, phenotype, and maturation were unaffected. Given that the mDC are differentiated from BM precursors, these results suggest that FIV infection of myeloid precursors may impart long-lasting functional defects in their differentiated progeny. Further studies into the mechanisms of FIV-induced mDC impairment are ongoing.

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CHAPTER TWO

BONE MARROW-DERIVED DENDRITIC CELL CYTOKINE PRODUCTION IS ALTERED WITH TOLL-LIKE RECEPTOR AND CD40 LIGATION DURING CHRONIC FELINE IMMUNODEFICIENCY VIRUS INFECTION

ABSTRACT

Impaired dendritic cell (DC) function is thought to be central to human immunodeficiency virus-associated immunodeficiency. In this study, we examined the effect of chronic feline immunodeficiency virus (FIV) infection on DC cytokine production in response to microbial and T cell stimulation. Cytokine production after either Toll-like receptor (TLR) or CD40 ligation in bone marrow-derived DC (BM-DC) was measured in naïve and chronically FIV-infected cats. BM-DC were stimulated with ligands to TLR 2, 3, 4, 7, and 9 or co-cultured with 3T3 cells expressing feline CD40L. Toll-like receptor 4 and 9 ligation in BM-DC from infected cats resulted in a significant decrease in the IL-12/IL-10 ratio. Conversely, TLR 7 ligation produced a significant

increase in the IL-12/IL-10 ratio in BM-DC from infected cats. No difference was noted with TLR3 ligation. RNA expression levels of TLR 2, 3, 4, 7, and 9 were not significantly altered by FIV infection. CD40 ligation significantly elevated both IL-10 and IL-12 mRNA production but did not alter the IL-10/IL-12 ratio. Chronic FIV infection alters the ratio of immunoregulatory cytokines produced by BM-DC in response to certain pathogen-derived signals which is likely relevant to the increased risk of opportunistic infections seen in lentiviral infection.

BACKGROUND

During a successful immune response, a pathogen must be recognized and appropriate cytokines must be produced by antigen presenting cells (APC), including dendritic cells (DC). Toll-like receptors (TLRs) recognize conserved molecules on pathogens and play a central role in this process inducing the maturation and activation of DC [1]. Mature, activated DC then stimulate T cells, providing a link between the innate and adaptive immune response. Thirteen TLRs have been described in mammalian cells in recent years [2]. TLRs 1, 2, 4, 5, and 6 are expressed on the cell surface while TLRs 3, 7, 8, and 9 are located within endosomes, although cell type and maturation state can change the expression and localization of TLRs [2]. These TLRs recognize pathogen associated molecular patterns (PAMPS) and induce a complex signaling cascade within the cells that results in the production of cytokines. Unique among the known TLRs, the

signaling cascade induced by TLR 3 ligation does not use the Toll/IL-1 receptor (TIR) domain-containing adaptor MyD88.

Many pathogens, including viruses, have evolved mechanisms that evade or use TLR-driven host defense mechanisms to avoid immune clearance. Respiratory syncytial virus induces TLR 4 signaling resulting in destructive inflammation of the respiratory epithelium [3]. The retrovirus mouse mammary tumor virus (MMTV) uses TLR 4-induced IL-10 production to escape the immune response [4]. Human immunodeficiency virus exploits TLR 2 ligation by concurrent mycobacterial infection to up-regulate viral replication [5]. Feline immunodeficiency (FIV) infection has been shown to result in altered TLR expression on lymphocyte subsets, although the functional significance of this finding is not yet clear [6].

Dendritic cells predominate on mucosal surfaces and are one of the first targets of lentiviruses such as HIV, SIV, and FIV [7, 8]. Lentiviral infection has been shown to alter DC growth, number, maturation, and function in favor of viral proliferation and spread although these changes are not fully understood and conflicting evidence exists [9]. Lentiviruses likely induce changes in DC both through direct effects of viral infection and through virus-induced alterations in cytokine production [10].

Feline immunodeficiency virus is a lentivirus that produces disease strikingly similar to AIDS with clinical phases analogous to those of HIV [11, 12]. Feline immunodeficiency virus provides a model of HIV infection in which the components of the immune response to a lentivirus and the resulting changes induced by such a virus can be studied. We used feline bone marrow-derived DC (BM-DC) expanded with granulocyte-monocyte colony stimulating factor (GM-CSF) to investigate TLR

expression and signaling in cats chronically infected with FIV. Bone marrow mononuclear cells (BMMC) grown with GM-CSF have been shown to be myeloid dendritic cells (mDC), and bone marrow yields greater numbers of DC with fewer macrophages than PBMC (our unpublished data) [13, 14]. Bone marrow-derived mDC have high MHC class II and co-stimulatory molecule expression and are efficient at initiating primary immune responses. Myeloid DC produce IL-12 and direct cell-mediated immune responses, both of which are altered in HIV infected individuals [15, 34].

We also investigated the ability of BM-DC from FIV-infected cats to respond to T cell-derived signals necessary for amplification of the immune response. Interactions between the CD40 surface receptor on DC and CD40 ligand (CD40L, also known as CD154) on CD4+ T cells have been shown to be crucial for the induction of a cell-mediated immune response. Viruses such as measles virus have been found to interfere with CD40-induced DC maturation as well as TLR signaling [16, 17]. Conflicting research exists relative to HIV. Kawamura *et al.* have reported that in vitro HIV-1 infection impairs DC capacity to stimulate $CD4^+$ T-cells but that HIV-1-exposed DC secrete increased amounts of IL-12 p70 after stimulation with soluble CD40L [18]. Alternatively, Smed-Sorensen *et al.* have shown that HIV infection impairs DC IL-12 p70 production in response to CD40L [19].

In the present work, we demonstrate that FIV infection alters the ratio of pro- and anti-inflammatory cytokine production by BM-DC in response to certain TLR ligands. Feline immunodeficiency virus also affects the levels of both IL-10 and IL-12 produced by BM-DC in response to CD40-CD40L interactions, but the overall ratio of the two

cytokines remains unchanged keeping the pro- and anti-inflammatory cytokine balance intact.

METHODS

Animals

Twenty-one naïve specific-pathogen free (SPF) cats and 15 SPF cats which had been infected with the molecular clone FIV-C36 for 12-18 months were used in this study. All animals were housed and treated in accordance with Colorado State University Animal Care and Use Committee approved protocols.

Cells

Bone marrow was collected from the proximal humerus of specific pathogen free and chronic FIV-infected cats. Bone marrow mononuclear cells (BMMC) were isolated using Ficoll-histopaque gradient centrifugation (Sigma-Aldrich, St. Louis, MO) and stored in liquid nitrogen until use. Bone marrow mononuclear cells were cultured at an initial concentration of 5 x 10^6/ml in six-well plates at 37°C in 5% CO_2 for 10 days in LBT media consisting of RPMI 1640 media with Glutamax (Sigma-Aldrich) with 15% FBS (Atlanta Biologicals, Norcross, GA), penicillin (100U/ml; Invitrogen), streptomycin (100ug/ml; Sigma-Aldrich), and 2-mercaptoethanol (50uM; Sigma-Aldrich). Fifty percent of the media was changed and 100ng/ml recombinant feline granulocyte-macrophage colony stimulating factor (rfGM-CSF; R&D Systems, Minneapolis, MN)

was added to the media every other day. After ten days in culture, cells were stained for MHC class II (Tu39, BD PharMingen, San Jose, CA) and CD11c (CA11.6A1, Serotec, Raleigh, NC) and sorted using immunomagnetic beads (Miltenyi Biotec, Auburn, CA). Purity of the samples was confirmed by flow cytometry to be consistently greater than 95%.

TLR Expression

Purified BM-DC were lysed with Trizol (Gibco, Grand Island, NY), and the total RNA was extracted. cDNA was made using the iScript cDNA kit (BIO-RAD, Hercules, CA). Real time qPCR was performed to quantify RNA levels of TLRs 2-9 in the laboratory of Dr. Greg Dean at North Carolina State University as previously described [6].

Cytokine Production via TLR Stimulation

Purified BM-DC were allowed to recover at 37°C overnight in fresh LBT media with rfGM-CSF (R&D, 100ng/ml) after sorting. The cells were then stimulated for six hours with one of the following TLR ligands: Poly(I:C) (25ug/ml; InvivoGen, San Diego, CA), ultrapure LPS (1ug/ml; InvivoGen), Loxoribine (100μM; InvivoGen), Pam2CSK4 (75ng/ml; InvivoGen), or E. coli ssDNA (10ug/ml; InvivoGen) . All TLR ligands have been tested by the manufacturer to exclude non-specific TLR signaling. After six hours, the cells were lysed with Trizol (GIBCO), and the RNA was extracted. cDNA was made using the iScript cDNA synthesis kit (BIO-RAD). Real-time PCR for cytokines interleukin-12 (IL-12), IL-10, IL-6, tumor necrosis factor-alpha (TNFα), and interferon

alpha (IFNα) was performed as previously described [20]. The mRNA levels of cytokines from stimulated cells were compared to those from unstimulated cells in a relative quantitative assay using 18s RNA to normalize for the quantity of input RNA and the $2^{\Delta\Delta CT}$ method was used to determine the relative expression of the genes of interest.

Cytokine Production via CD40/CD40L Interaction

3T3 cells expressing feline CD40L (3T3.CD40L) or a control 3T3 cell line not expressing CD40L, both generously provided by Margaret Hosie (University of Glasgow), were grown to approximately 80% confluency in a 24 well plate at 37°C and 5% CO_2 [21]. 3T3 cells were cultured in DMEM (GIBCO) with 10% FBS (Atlanta Biologicals), 2% glutamine (Invitrogen), and 1% penicillin/streptomycin (Invitrogen). For cell selection purposes, 400ug/ml G418 sulfate (Invivogen) was added to the media for CD40L-expressing 3T3 cells. Purified BM-DC from seven naïve cats and six FIV-C infected cats were allowed to rest overnight in fresh LBT media with rfGM-CSF (R&D, 100ng/ml). The media was removed from the 3T3 cells which did or did not express CD40L, and 1.5×10^5 BM-DC in 1ml of LBT media was added per well. After six hours of culture, the cells were lysed with Trizol (GIBCO), and the RNA was extracted according to the manufacturer's instructions. Cytokine mRNA levels were measured as above. Cytokine RNA production in BM-DC exposed to 3T3.CD40L cells was expressed as an increase over that of BM-DC exposed to 3T3 cells.

Statistical analysis

The Kolmogorov Smirnov test was performed to determine whether the data was normally distributed. The Student's t-test was performed with a $p<0.05$ considered significant.

RESULTS

High cell purity obtained by sorting

Flow cytometric analysis of magnetic bead and high-speed flow cytometry sorting methods based on MHC class II and CD11c expression showed greater than 90% and generally greater than 95% purity for all samples prior to stimulation and/or analysis (data not shown). The purified population of cells stimulates a robust mixed leukocyte response when derived from naïve animals (data not shown).

No difference in TLR mRNA expression in feline BM-DC

Bone marrow-derived mDC from 6 infected and 8 naïve cats were analyzed for expression of TLR 1, 2, 3, 4, 5, 7, 8, and 9 mRNA. Feline BM-DC were found to express all of the TLRs but had low expression of TLR 3, 5, and 9. There was no significant difference in expression of the measured TLRs between infected and naïve cats (Figure 2.1) although there was a trend for increased expression of TLR 2 in infected BM-DC ($p=0.069$, Student's t-test).

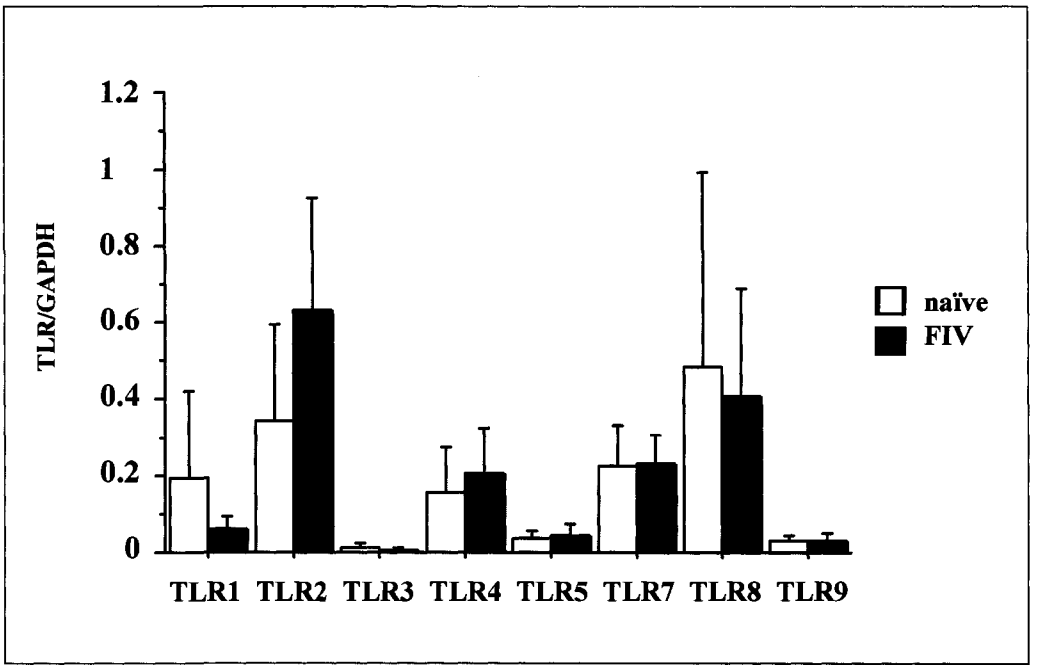

Figure 2.1: TLR mRNA expression is not altered in BM-DC from FIV-infected cats. BM-DC were cultured for ten days in the presence of rfGM-CSF (100ng/ml) and then purified for CD11c and MHC class II expression. Purified BM-DC were lysed with Trizol (GIBCO), and the RNA was extracted. Real time PCR was performed to quantify RNA levels of TLRs 2, 3, 4, 5, 7, and 9. Results are shown as the mean and standard deviation of cells from eight naïve cats and six FIV-infected cats. There were no statistically significant differences in expression levels as determined by Student's t-test.

IL-12/IL-10 ratio is altered with FIV infection

Toll-like receptor ligands for TLR 2, 3, 4, 7, and 9 were used to test cytokine mRNA production in BM-DC from a minimum of five naïve and five FIV-infected cats. Regardless of TLR expression level, all five ligands induced expected cytokine responses in BM-DC from both FIV infected and naïve cats. No significant difference between the two groups was seen for any of the individual cytokines, i.e. IL-6, IL-10, IL-12, TNFα, and IFNα (data not shown). However, looking at individual animals, the IL-12/IL-10

ratio was significantly different between the two groups for TLR 4, 7, and 9 ligation. Toll-like receptor 4 and 9 ligation resulted in significantly lower IL-12/IL-10 ratios in FIV-infected cats compared to naïve cats ($p<0.05$, Student's t-test; Figure 2.2). Toll-like receptor 2 showed a trend for the same change but was not statistically significant. Toll-like receptor 7 showed the opposite effect in that infected cats produced a significantly higher IL-12/IL-10 ratio than naïve cats ($p<0.05$, Student's t-test; Figure 2.2). Toll-like receptor 3 ligation did not result in any differences in the IL-12/IL10 ratio between naïve and infected animals.

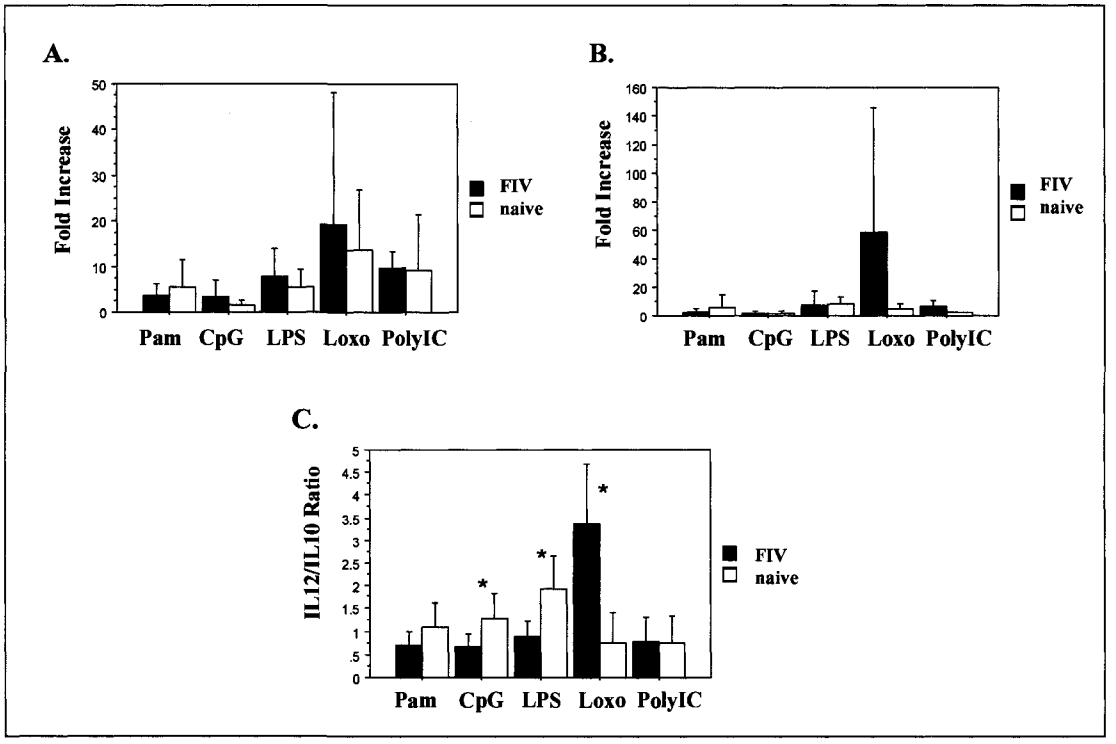

Figure 2.2: There were no statistical differences in the relative induction of IL-10 (A) or IL-12 RNA (B) when DC were stimulated with any of the TLR ligands but the average individual cat IL-12/IL-10 ratio (C) was significantly decreased with TLR 4 and 9 ligation and increased with TLR 7 ligation in BM-DC from FIV-infected cats (* indicates $p<0.05$ by Student's t-test). BM-DC were cultured for ten days in the presence of rfGM-CSF (100ng/ml) and then purified for CD11c and MHC class II expression. Purified BM-DC were cultured for six hours with a TLR ligand to TLR 2, 3, 4, 7, or 9. Real time PCR for the cytokines IL-12, IL-10 was performed. 18s RNA was used to normalize input RNA, and the $2^{-\Delta\Delta CT}$ method was used to determine the relative expression of the genes of interest.

CD40L-induced cytokine production is altered in FIV infection

Cytokine mRNA was measured for IL-6, IL-10, IL-12, TNFα, and IFNα in BM-DC co-cultured with 3T3 cells expressing feline CD40L. No cytokine production was seen in 3T3 cells alone. Increases were seen in IL-6, IL-10, IL-12, and TNFα during co-culture of the BM-DC with the CD40L-expressing cells as compared to BM-DC co-cultured with native 3T3 cells (Figure 2.3A). The CD40L-induced production of both IL-

10 and IL-12 were significantly increased in BM-DC from FIV-infected cats as compared to naïve cells ($p=0.038$ and $p=0.036$ respectively, Student's t-test; Figure 2.3A). Despite the individual increases in IL-12 and IL-10, the resultant ratio of IL-12/IL-10 was unchanged between naïve and infected BM-DC ($p=0.94$, Student's t-test; Figure 2.3B).

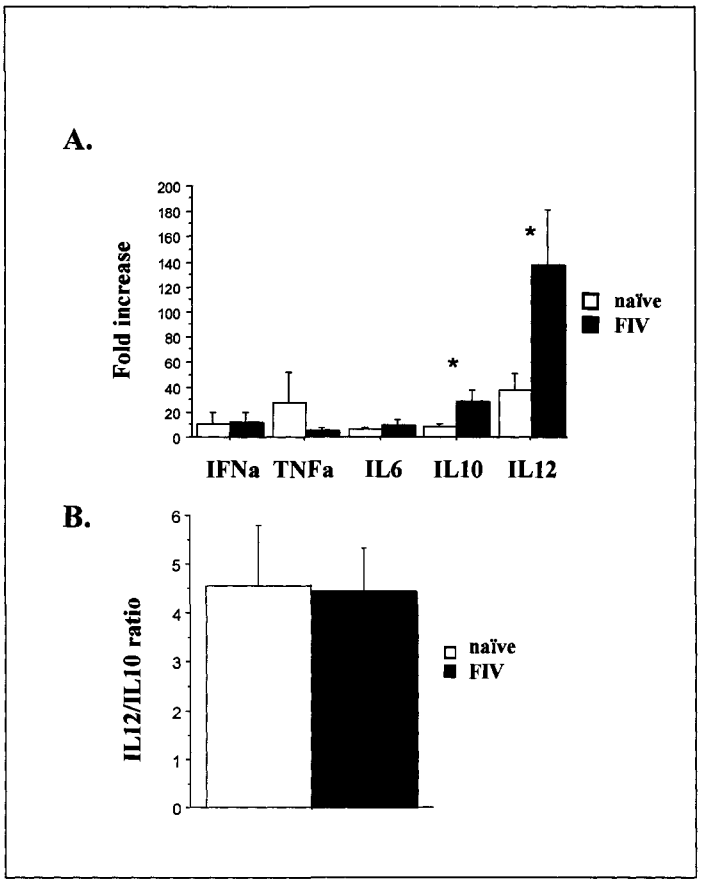

Figure 2.3: Bone marrow-derived mDC (BM-DC) from FIV-infected cats produce relatively more IL-10 and IL-12 when exposed to CD40L than BM-DC from naïve cats yet the ratio of IL12 and IL10 induction remains unchanged. Purified BM-DC from 7 naïve cats and 6 FIV-C infected cats were cultured for six hours with 3T3 cells, which did or did not express CD40L. After six hours, the cells were lysed, and the RNA was extracted. Cytokine mRNA was measured for IL-6, IL-10, IL-12, TNFα, and IFNα by real-time PCR. Cytokine mRNA production in BM-DC exposed to 3T3 cells expressing CD40L is expressed as an increase over that of BM-DC exposed to 3T3 cells that do not express CD40L. A) Exposure to CD40L induced the expression of IL-6, IL-10, IL-12, TNFα and INFα mRNA in feline BM-DC. Bone marrow-derived mDC from FIV infected cats produce more IL-10 and IL-12 mRNA than cells from naïve cats ($p<0.05$, Students t-test). B) No significant change was noted in the IL-12/IL-10 ratio between cells from naïve and FIV-infected cats ($p>0.05$, Student's t-test).

DISCUSSION

Lentiviruses subvert the normal immune response to establish long-term infections despite or because of the efforts of DC and other immune effector cells. Dendritic cells are suspected to become dysfunctional during HIV infection, although the exact mechanism(s) of this dysfunction are not yet clearly defined. There is evidence that HIV and other pathogens interfere with the normal function of TLRs and to use the TLRs to promote infection [5, 16, 22]. The ability of DC to successfully interact with and stimulate T cells during lentiviral infection has also come into question. We sought to determine if chronic FIV infection results in altered DC cytokine production in response to TLR stimulation and/or the CD40-CD40L DC:T-cell interaction, thereby providing further insight into lentiviral affects on DC function.

Dendritic cell subsets have yet to be clearly defined in cats, yet our feline BM-DC are CD11c+, CD11b+, CD1a+ and MHCII high, which is consistent with myeloid origin, and murine BM-DC grown with GM-CSF have been shown to develop into mDC [13]. The retained expression of CD14 on feline DC is consistent with data from other laboratories [14, 23]. Similar to human mDC, we found that feline BM-DC grown with GM-CSF express high levels of TLR 2 and 8, moderate levels of TLR 1, 4, and 7, and low levels of TLR 3, 5, and 9 [24, 25]. We did not find a correlation between the amount of TLR RNA expression and the overall magnitude of the cytokine response to individual TLR ligands. Although it has been reported that TLR expression correlates with cytokine production, measurement of RNA expression levels of TLRs may not accurately predict their activity. Toll-like receptor 4 and TLR9 expression is low in immature human mDC

yet the cells are able to respond strongly to LPS and specific CpG motifs suggesting either an alternate pathway or high efficiency of the receptor [24, 26, 27]. There were no significant differences in TLR expression between BM-DC from naïve or from chronic FIV-infected cats. The high variability in TLR expression observed among individual cats has also been noted for human donors [28].

When cytokine production was measured, we found that BM-DC from FIV-infected cats were able to produce similar increases in IL-12, IL10, TNFα, IL-6, and IFNα mRNA as BM-DC from naïve cats in response to individual stimulation of the five TLRs we tested (TLRs 2, 3, 4, 7, and 9). There was considerable variation in the overall magnitude of cytokine responses among individual animals, which is not surprising in an outbred population. Recent work indicates that people can be classified as low or high responders based on the magnitude of cytokine production in response to LPS stimulation [29].

To account for the variability in the overall magnitude of cytokine response between individual animals, we examined the balance of cytokines that each animal produced. We found that the ratio of IL-12 and IL-10 in individual cats was significantly altered in FIV-infected vs. naïve control cats. In particular, simulation of TLR 4 and TLR 9 shifted the cytokine balance towards IL-10 in BM-DC from FIV-infected cats whereas stimulation of TLR 7 shifted the balance towards IL-12 in the same animals. Concurrently, the MyD88-independent pathway stimulated by ligation of TLR 3 was unaffected. Early DC production of IL-12 is critical to initiating an effective cell-mediated immune response by stimulating T-cell or NK cell production of interferon-gamma (IFNγ) [30]. Increases in IL-10 production can interfere with DC activation of

the adaptive immune response by decreasing IL-12 production, DC surface MHC class II expression, antigen presentation, and impairing DC maturation through inhibition of co-stimulatory molecule expression [30-32]. Decreased co-stimulatory molecule expression and impaired DC maturation have been associated with development of a type 2 cytokine response, regulatory T cells, and tolerance [33, 34].

Many pathogens have been shown to alter this early DC cytokine balance which may inhibit immune clearance. Myeloid DC from patients infected with Hepatitis C virus (HCV) produce higher amounts of IL-10 in response to TLR 3 ligation with poly-IC [35]. Measles virus, which causes immunosuppression and enhanced susceptibility to microbial insults, has been found to suppress IL-12 synthesis through TLR 4 [16]. The gram-negative bacteria *Bordetella bronchiseptica,* which establishes a persistent infection in the murine respiratory tract, expresses virulence factors which have been shown to decrease IL-12 production in BM-DC and to induce a semi-mature phenotype in these same cells [36]. Recent work demonstrates that a soluble extract from the eggs of the helminth *Schistosoma mansoni*, a cause of the chronic and often debilitating disease schistosomiasis, inhibits conventional human monocyte-derived DC maturation and decreases IL-12, IL-6, and TNF-alpha production in response to TLR 3 and 4 ligands [37].

A selective impairment in the cytokine response to the ligation of TLR 4 or 9 could be instrumental in the pathogenesis of many of the opportunistic infections seen during AIDS. Secondary bacterial infections are the leading cause of death in HIV-infected patients. Gram negative organisms such as Salmonella spp. and Pseudomonas aeuroginosa are commonly documented in AIDS patients and are recognized by TLR 4

[38, 39]. Human herpes virus infections are associated with significant morbidity in HIV-infected individuals and the generation of an effective immune response to both Herpes simplex and cytomegalovirus has been shown to be dependent on an intact DC TLR 9 signaling pathway [40, 41]. Toll-like receptor 4 has been shown to be critical in controlling other AIDS-defining illnesses, including disseminated mycobacterial infection, and candidiasis [42, 43]. Feline immunodeficiency virus-infected cats often succumb to disseminated bacterial infections, isolation of Candida albicans has been reported with more frequency in FIV-infected cats and rare feline cases of disseminated mycobacterial infection have been associated with FIV infection [44, 45]. Further work using whole organisms would be required to more directly test this theory of altered DC-mediated pathogen response.

Interestingly, TLR 7 stimulation of DC from FIV-infected cats resulted in the opposite pattern of cytokine expression favoring the production of the pro-inflammatory cytokine IL-12 over IL-10. Toll-like receptor 7 agonists have been shown to preferentially induce IL-12 production from circulating murine CD11c+ mDC leading to subsequent T cell Th1 cytokine polarization [46]. Measles virus infection of DC has recently been shown to selectively impair TLR 4-mediated IL-12 production while resulting in the enhanced production of IL-12 after TLR 7 ligation [16]. Human immunodeficiency virus ssRNA binding to TLR 7 and 8 on latently-infected promonocytic cells results in the production and release of HIV virions despite the fact that it appears to have an anti-viral effect on acutely infected cells [47]. Signaling through TLR 7 may be preserved and even augmented in lentiviral infections if it plays a necessary role in regulating viral replication and latency. These results are of particular

interest given the use of selective TLR 7 agonists in pre-clinical trials of several infectious diseases and argue that preferential TLR 7 stimulation may be of benefit in chronic lentiviral infections to both stimulate IL-12 production and reactivate latent viral reservoirs during treatment.

CD40-CD40L ligation induces up-regulated DC surface expression of MHC class I and II and the co-stimulatory molecules CD80 and CD86 and enhanced production of cytokines such as IL-12 and IL-15, which are important for T-cell growth and polarization [48, 49]. We found that BM-DC from FIV-infected and naïve cats induced equivalent IL-6, TNFα, and IFNα mRNA production in response to CD40-CD40L ligation. The production of both IL-10 and IL-12 was found to be significantly increased in BM-DC from FIV-infected cats, which lead to maintenance of the balance of IL-12 and IL-10 seen in naïve cats. The in vivo significance of a relative increase in the amount of IL-10 and IL-12 mRNA but maintenance of the same pro- and anti-inflammatory cytokine balance is as yet unknown. Zhang *et al.* have shown that co-culture of DC with HIV-exposed CD4+ T cells resulted in decreased DC IL-12 production, but the decrease was due to decreased CD40L expression by the T cells, not DC dysfunction [50]. Other work has shown that bulk culture of HIV-exposed DC revealed no differences in CD40-CD40L-induced maturation status or IL-12 production yet, in these same experiments isolation of the small number of infected DC showed that they were unable to produce IL-12p70 [19]. We are currently unable to specifically isolate FIV-infected DC to evaluate them in a similar fashion. The exact mechanisms by which FIV and HIV affect the CD40-CD40L interaction and their in vivo significance remain to be defined.

The relative balance of DC IL-12 and IL-10 production can determine the outcome of an intracellular infection, and it appears that pathogens including FIV have evolved ways to exploit this balance. The selective alteration of TLR-induced DC cytokine responses may allow FIV to evade immune clearance, predispose the host to AIDS-defining secondary infections, and play a role in regulating viral latency. Feline immunodeficiency virus would not be the first virus known to induce both pro- and anti-inflammatory cytokines during the course of infection. Isolating the exact signaling modification(s) induced by FIV in these cells would shed light on the viral mechanisms of immune alteration and possibly help identify target areas of intervention for lentiviral infections. Further studies to identify the exact mechanism involved in alteration of the TLR-induced signaling pathways are ongoing.

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CHAPTER THREE

IN VIVO ADENOVIRAL ADMINISTRATION OF IL-12 DURING ACUTE FELINE IMMUNODEFICIENCY VIRUS INFECTION

ABSTRACT

Interleukin-12 is an important pro-inflammatory cytokine that stimulates interferon-γ and tumor necrosis factor-α production and enhances cytotoxicity through activation of NK and cytotoxic T lymphocytes. Interleukin-12 stimulates Type 1 immune responses resulting in inflammatory responses to pathogens, viruses, and tumor cells. The role of IL-12 and cytokine balance in inflammation and disease including trauma, sepsis, and SIRS continues to be documented although our understanding remains incomplete. Recombinant IL-12 has been under investigation for use in viral infections, psoriasis, and cancer therapies. Recombinant adenovirus vectors expressing cytokines are powerful immunomodulatory agents, and an adenovirus expressing murine IL-12, AdMEMIL-12, has been developed. We sought to evaluate the physiological response to increased IL-12 levels and the safety and efficacy of the IL-12-expressing adenovirus in

cats while monitoring alterations in viral kinetics during acute feline immunodeficiency virus (FIV) infection as a measure of therapeutic benefit. AdMEMIL-12 or the control AdLacZ was administered intraperitoneally to specific pathogen free cats 24 hours prior to intraperitoneal infection with FIV. Four cats were given AdMEMIL-12 alone. Complete blood count, serum chemistry, CD4/CD8 ratio, intracellular glutathione, nitric oxide, proviral load, circulating and tissue cytokine protein and mRNA levels, and histopathology were evaluated in the cats. Nine out of the ten AdMEMIL-12-treated cats developed life-threatening side effects including progressive anemia, thrombocytopenia, neutropenia, expansion of an unidentified cell population, biochemical abnormalities, and tissue injury. The AdLacZ-treated cats developed no side effects. Although the use of an adenoviral vector appears to be safe in cats, the administration of IL-12 by this method failed to be a viable therapeutic option due to toxicity.

BACKGROUND

The cytokine response of the host immune system is intricately associated with infection and course of disease for many viruses including lentiviruses. Clearance of viruses is supported by a Type 1-dominate cytokine response, including interferon gamma (IFNγ), tumor necrosis factor alpha (TNFα), and interleukin-12 (IL-12). Type 2 cytokines, such as IL-10, antagonize the production and effects of Type 1 cytokines and promote immune tolerance. Human immunodeficiency virus (HIV) is a lentivirus that subverts the host immune response to establish chronic infection. Human

immunodeficiency virus is associated with a progressive loss of Type 1 responses and an increase in Type 2 responses [1]. Cells from HIV-positive persons produce less IL-12 than naïve cells, and infected persons with higher levels of IL-10 have been shown to have the most severely compromised T helper cell function [2-4]. Similarly in feline immunodeficiency virus (FIV), IL-10 has been found to dominate the response in lymphoid tissue CD4+ and CD8+ lymphocytes within the first four weeks after mucosal infection [5]. By as yet undefined mechanisms HIV-1 infection is known to interfere with gene transcription of IL-12, which highlights the potential benefit of supplementing IL-12 in lentiviral-infected individuals [6]. Addition of IL-12 *in vitro* has been shown to restore Type 1 function to peripheral blood mononuclear cells (PBMC) from HIV-positive persons [7]. In Rhesus macaques with simian immunodeficiency virus (SIV), Ansari *et al* showed that IL-12 administration leads to lower viral loads, lower proviral DNA levels, and a marked increase in the disease-free period [8].

Recombinant IL-12 has been under investigation for use in viral infections and cancer therapies for almost a decade. IL-12 strongly stimulates Type 1 immune responses through stimulation of NK and NKT cells, induction of IFNγ production by T cells, and subsequently has been shown to increase immune responses to pathogens, viruses, and tumor cells [9]. However, IL-12 therapies generally require multiple injections due to the short half-life of recombinant proteins *in vivo*, and IL-12 has been noted to have a small therapeutic window with potential significant toxicities [10-14]. Methods of surpassing the need for multiple injections and avoiding the toxicity issues have been under investigation. Recombinant adenovirus vectors expressing cytokines act as powerful immuno-modulatory agents, and an adenovirus expressing murine IL-12,

AdMEMIL-12, has been developed [10, 15]. Mice injected intraperitoneally with the vector displayed serum IL-12 levels that increased proportionately with the amount of virus administered. IL-12 production *in vivo* caused a dose-dependent increase in splenic and lung NK cell activity [15]. Mice given a single intramuscular injection of the adenoviral vector had a peak IL-12 muscle expression at Day 1 that provided protection from *Leishmania major* infection [10].

As FIV provides an excellent model of HIV that can be utilized to study early effects of therapies on viral kinetics, use of the adenoviral IL-12 vector in cats was investigated in our laboratory. Pilot studies in our laboratory using single-dose systemic AdMEMIL-12 showed mild, transient, non-clinical toxicity in cats. The current study was therefore designed to evaluate the effects of using the adenoviral vector to increase systemic IL-12 levels at the time of FIV infection. Our hypothesis was that the increased IL-12 levels would enhance cell-mediated viral clearance. Despite the earlier pilot studies, nearly all the AdMEMIL-12-treated cats developed fatal toxicities. The observed clinical, hematologic, biochemical, immunologic, and histopathologic changes associated with adenoviral murine IL-12 administration in cats are discussed in this paper.

METHODS

Animals

Six specific pathogen free (SPF) cats were used in the pilot study. The cats were divided into two groups: three cats received AdMEMIL-12 and three cats received

AdLacZ. Fifteen seven-month old SPF cats were used for the current study. The animals were housed separately in an AAALAC International Accredited animal facility. The cats were divided into three study groups: group one received AdMEMIL-12 only (n=4), group two received AdLacZ and FIV (n=5), and group three received AdMEMIL-12 and FIV (n=6).

Blood and bone marrow collection

Blood was collected from each cat following approved ACUC procedures. The cats were sedated with ketamine and acepromazine for blood collection. Four milliliters heparinized blood was collected for peripheral viral and cytokine monitoring as described below. Peripheral blood mononuclear cells (PBMC) were isolated from the samples by ficoll-hypaque (Histopaque-1077, Sigma-Aldrich) density gradient centrifugation. Cells were stored at -70°C until analysis. Five hundred microliters of blood anticoagulated with EDTA was collected for immediate determination of complete blood count (analyzed in a veterinary diagnostic laboratory), CD4/CD8 lymphocyte ratio, and intracellular glutathione concentration. One milliliter of whole blood was used for serum separation and biochemical analysis (analyzed in a veterinary diagnostic laboratory) and cytokine assays. Samples were collected at Day 0, 1, 2, 3, and Day 4, 5, or 6 post-adenovirus administration depending on the clinical condition of the cat.

Bone marrow aspirates were collected from the humerus of cats at study termination immediately following humane euthanasia. Slides were made immediately after collection of the bone marrow and were review by a board certified veterinary clinical pathologist.

Histopathology

Tissues were collected immediately post-euthanasia from all six cats in the AdMEMIL-12/FIV group, three cats in the AdMEMIL-12 only group, and two cats in the AdLacZ/FIV group. Samples were collected from the bone marrow, liver, kidney, spleen, lung, small intestine, and mesenteric lymph nodes for histopathologic assessment. Tissues were stored in formalin until processing. The slides were evaluated by a board certified veterinary pathologist who was blinded to the study conditions.

Adenovirus administration

AdMEMIL-12 and AdLacZ were generously donated by Dr. Hitt. Construction and characterization of the Ad5 vectors has been previously described [15]. The Ad5IL-12 vector contains an expression cassette for the p35 subunit of IL-12 in the E1 region, and the IL-12 p40 subunit in the E3 region. For each of the adenoviruses, 1 x 10^9 PFUs were suspended in 1 x PBS to a total volume of 500uls and injected intraperitoneally using a 22gauge needle. The cats were sedated with ketamine and acepromazine (administered subcutaneously) for the procedure. No complications were associated with any of the injections. The adenovirus injection date was designated Day 0.

FIV administration

Twenty-four hours after administration of the adenovirus, 500ul of pooled plasma from FIV clade C-infected cats was injected intraperitoneally to the designated groups.

There were no complications associated with any of the injections. The FIV administration date was designated Day 1.

Viral quantification

Proviral load was determined using real-time DNA PCR. Methods including primers and probes were adapted from those developed by Leutenegger *et al.* [16]. Cellular DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA). PCR reactions were performed in a 25μl volume containing 12.5μl TaqMan Universal PCR Mastermix (Applied Biosystems), 400nM of each primer (MWG Biotech), 80nM of probe (MWG Biotech), and 5μl of sample DNA or plasmid FIV DNA standard. Real-time PCR was performed on an iCycler iQ Real-time PCR Detection System (Bio-rad). Starting quantities of viral DNA were extrapolated from the plasmid DNA standard curve.

Cytokine assays

Feline serum IFNγ levels were determined using a Feline IFNγ ELISA kit (R&D Systems, Minneapolis, MN) according to manufacturer's instructions. Serum murine IL-12 levels were determined using murine IL-12 ELISA reagents purchased from R&D Systems according to the protocol provided by the manufacturer. The clone used for the capture antibody was 30517.

Circulating and tissue cytokine production of the cytokines IL-12, IL-10, IFNγ, IFNα, and TNFα were measured using quantitative RT-PCR techniques. The amplification conditions for feline primer:probe systems were a modification of

procedures developed by Leutenegger *et al.* [17]. RNA was extracted from 5 x 10^6 PBMC or approximately 100mg of tissue for quantitative real-time cytokine RNA PCR using the Trizol™ extraction method (Invitrogen) according to manufacturer's instructions. cDNA was generated using the Bio-rad iScript cDNA Synthesis kit (Bio-rad, Hercules, CA) and subjected to RT-PCR in a 25μl volume containing 12.5μl of 2X TagMan One Step RT-PCR Master Mix (Applied Biosystems, Foster City, CA), 400nM of each primer (MWG Biotech, High Point, NC), 80nM of probe (MWG Biotech), and 2μl sample cDNA. Real-time PCR was performed on an iCycler iQ Real-time PCR Detection System (Bio-rad). Final quantification was performed using the comparative C_T (threshold cycle) method with relative quantification to 18s RNA. The C_T for the target amplicon (IL-10, IL-12 p40, TGFα, IFNγ, IFNα) and the C_T for the internal control (18s RNA) were determined for each sample. Differences in the C_T for the target and the C_T for the internal control, called ΔC_T, were calculated to normalize for differences in the amount of total nucleic acid added to each reaction and the efficiency of the RT step. For peripheral blood samples, the amount of target, normalized to the internal control was calculated by $2^{\Delta\Delta CT}$ with quantities of cytokine expressed as an n-fold difference relative to control samples [17]. Control samples were not available for tissues so comparison between groups was made using ΔC_T.

CD4/CD8 ratio and relative intracellular glutathione

EDTA anti-coagulated whole blood was used for CD4/CD8 ratio determination and to quantify relative intracellular glutathione as previously described [18-20]. Briefly, 30 microliters of EDTA blood was stained for feline CD4 (3-4F4, Southern Biotech,

Birmingham, AL) and CD8 (fCD8, Southern Biotech), and the red blood cells were lysed. The percentage of positively stained cells was determined by flow cytometry (Dako, Fort Collins, CO), and cell numbers were calculated using data from complete blood counts performed on the same samples. The samples were then incubated at room temperature in the dark with monochlorobimane (mBCl, Molecular Probes) to a final concentration of 40μM for exactly 20 minutes. The mean fluorescence intensity (MFI) of mBCl, which becomes fluorescent when conjugated with reduced glutathione (GSH), was then determined by flow cytometry (Dako).

Cell identification by flow cytometry

Cell identification was performed using antibodies to the surface markers CD4, CD8, CD14, and CD21. Expression was quantified by flow cytometry (Dako or Coulter).

Nitric oxide quantification

Concentration of nitric oxide (NO) in heparinized plasma samples was determined using a nitric oxide detection kit (Assay Designs, Ann Arbor, MI) according to manufacturer's instructions. Venous blood was collected, centrifuged, and the plasma was stored at -70°C until analysis. Due to the transient and volatile nature of NO, measurements are made of two stable breakdown products, i.e. nitrate (NO_3) and nitrite (NO_2), by photometric methods. The technique involves the enzymatic conversion of nitrate to nitrite by nitrate reductase followed by colourimetric detection of nitrite as a colored axo dye product of the Griess reaction. Determination of the total of both NO

products in the sample is made by conversion of all sample nitrate into nitrite, followed by the determination of the total concentration of nitrite in the sample.

Statistical analysis

Statistical analyses were performed using StatView and Excel (Student's t-test). The SAS/STAT® 9.2 Proc GLIMMIX computer software was used to run repeated measures analysis of variances on the relative intracellular glutathione data and nitric oxide data. The raw data was assessed for Gaussian distribution and a plot of the residuals versus predicted value was examined graphically to assess variance of the residuals. A pair-wise comparison of days or groups using Tukey-Kramer adjustment to control for error rates was then used to test for significant differences in the adjusted P values, reported as T-K p. Skewed data was log transformed prior to analysis.

RESULTS

Clinical symptoms

Cats administered AdLacZ remained clinically normal throughout the study period and showed no complication from adenovirus administration for greater than two years post-injection. All of the cats in the two groups receiving AdMEMIL-12 became clinically ill three days post-adenoviral injection. The cats were notably lethargic with pale mucous membranes that correlated with a progressive anemia. All of the cats that received AdMEMIL-12 except one cat in the AdMEMIL-12-only group had progressive

clinical signs of lethargy, anorexia, pallor, icterus, and tachypnea requiring humane euthanasia on or before Day 5 of the study. Due to the dramatic and unexpected onset of clinical signs, cats in the AdMEMIL-12-only group required euthanasia on Day 4, cats in the AdMEMIL-12/FIV group were euthanized on Day 4 or 5 with blood samples available only from the 3 cats euthanized on Day 5, and two control cats in the AdLacZ/FIV group were euthanized on Day 6. The one non-progressive AdMEMIL-12 cat recovered rapidly and showed no long-term clinical or biochemical complications.

Hematological changes

In the pilot study, cats receiving AdMEMIL-12 were noted to have a transient decrease below normal values in red blood cell counts, platelets, and neutrophils by Day 5 post adenovirus administration that rebounded to normal by Day 15 (Figure 3.1A, B). Pilot study cats treated with AdLacZ did not show similar changes. None of the cats in the pilot study showed any clinical signs of the decreased red blood cell, platelet, or neutrophil counts.

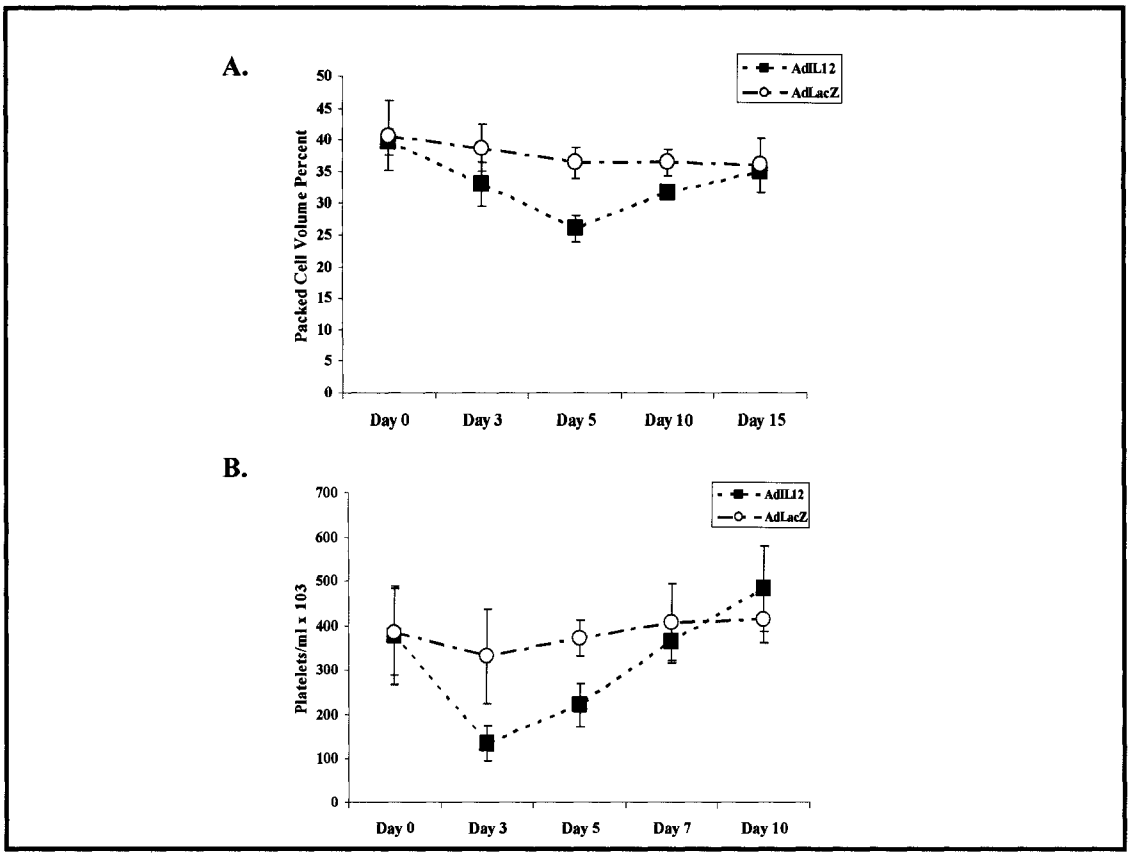

Figure 3.1: Pilot study cats receiving a single intraperitoneal dose of AdMEMIL-12 showed a transient decrease in red blood cell count as measured by packed cell volume (A) and a transient decrease in platelet count (B) that rebounded by Day15 post-injection without any clinical signs. Error bars denote standard deviation.

In the current study, red blood cell counts (packed cell volume, PCV) decreased significantly during a forty-eight hour period in cats receiving AdMEMIL-12 but not in the AdLacZ/FIV cats (Figure 3.2A, $p<0.006$, Student's t-test). At the time of euthanasia, all AdMEMIL-12-treated cats were anemic with an average PCV less than 16% in the AdMEMIL-12-only cats, who were euthanized on Day 4, and 7% in the AdMEMIL-12/FIV cats, who were euthanized on Day 5 (Figure 3.2A). No change in PCV was noted in the AdLacZ/FIV cats during the study period.

Platelet counts significantly decreased by Day 2 in cats receiving AdMEMIL-12 and continued to decline until study termination (Day 4 or 5) when compared to AdLacZ/FIV cats (Day 2: $p < 0.001$, termination day: $p<0.005$; Student's t-test, Figure 3.2B). The AdMEMIL-12/FIV cats had an average platelet count of 37,000/μl, and the AdMEMIL-12 only cats had an average platelet count of 52,000/μl at study termination compared to 274,000/μl in the AdLacZ/FIV group (normal range: 200,000-500,000 platelets/μl).

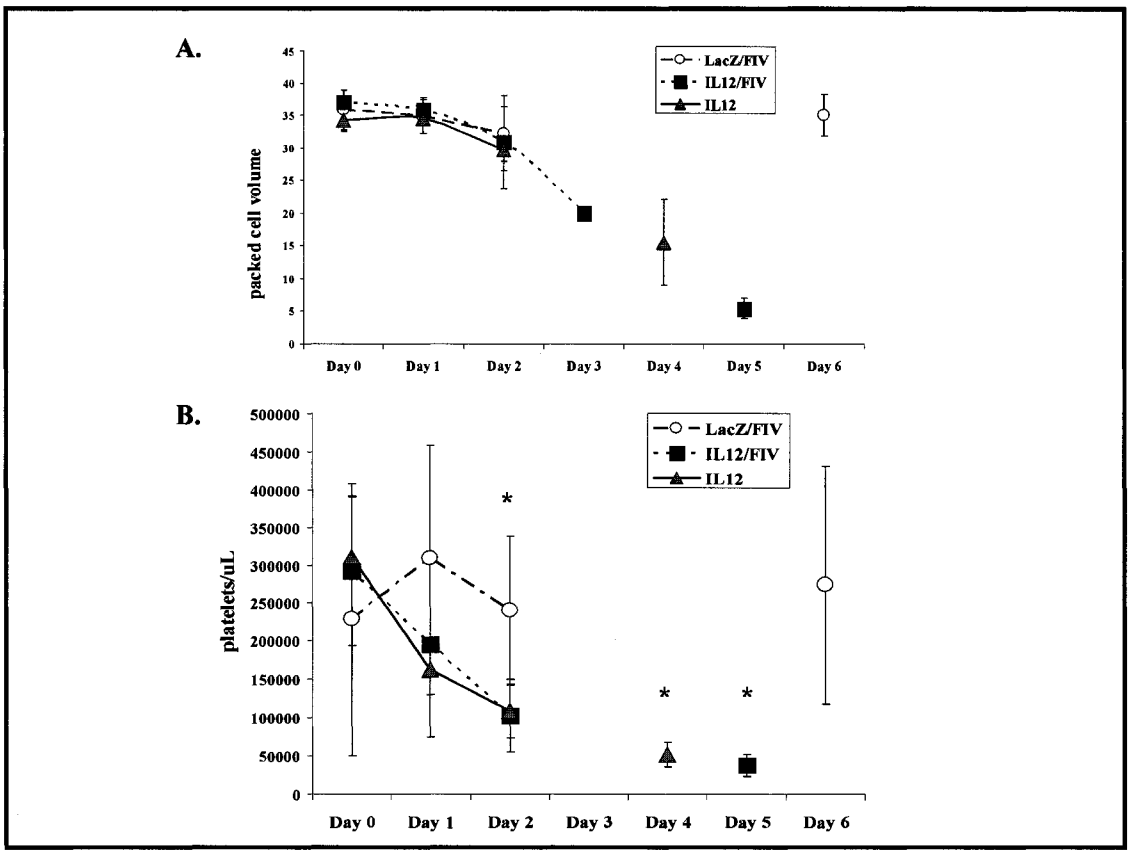

Figure 3.2: Adenoviral administration of IL-12 to cats resulted in significant anemia and thrombocytopenia. A) Packed cell volume decreased significantly during a 48 hour period in cats receiving AdMEMIL-12 but not in the AdLacZ/FIV cats ($p<0.006$, Student's t-test). B) Platelet counts significantly decreased in cats receiving AdMEMIL-12 by Day 2 ($p<0.001$) and continued to decline until study termination (Day 4 or 5, $p<0.005$) when compared to AdLacZ/FIV cats. Error bars indicate standard deviation.

White blood cell (WBC) count in cats receiving AdMEMIL-12 trended toward decline over the first three days of the study but notably increased by Day 4 (Figure 3.3A). The decline in WBC at Day 2 correlated with to a significant decrease in neutrophils seen in cats receiving AdMEMIL-12 when compared to AdLacZ/FIV cats ($p<0.03$; Student's t-test; Figure 3.3B). The WBC increase at study termination correlated to the expansion of an undetermined cell type seen on blood smears and in the bone marrow. The cells were large with basophilic granules suggestive of NK cells (Figure 3.3C). Consistent with human NK cell phenotype, the cells were negative for CD4, CD8, and CD21 (data not shown). Specific markers to identify feline NK cells are not currently available. These cells were present in peripheral blood on Day 4 even in the one surviving AdMEMIL-12-only cat. CD4/CD8 T cell ratio in the cats did not change significantly over the study period (data not shown).

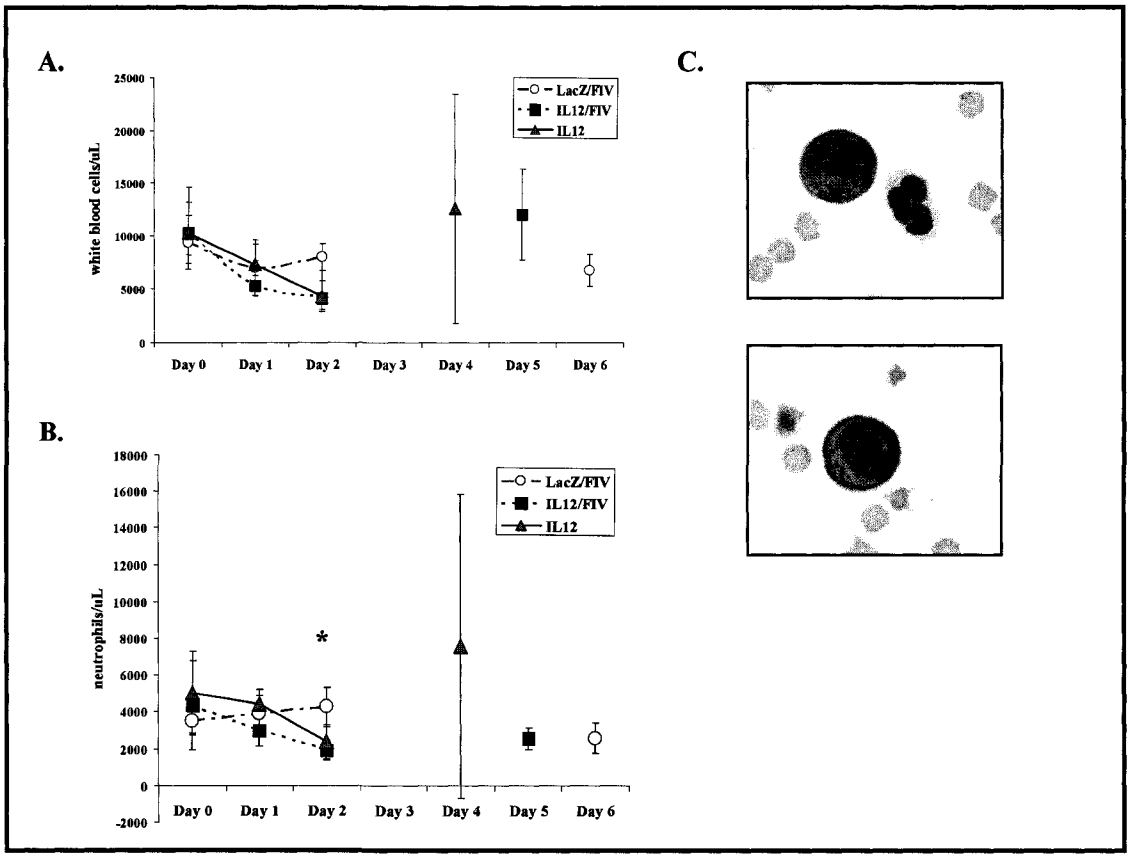

Figure 3.3: The white blood cell count of cats receiving IL-12 initially decreased and then rebounded by study termination due to expansion of an unidentified cell type. A) WBC counts decreased at Day2 in AdMEMIL-12 treated cats corresponding to a significant decreased in neutrophils (B, $p<0.03$, Student's t-test) but then rebounded by Day4; no change was seen in WBC counts in animals treated with AdLacZ. Error bars indicate standard deviation. C) Representative photomicrographs of unidentified cells responsible for increased white blood cell count after study Day 4 in AdMEMIL-12-treated cats. Cells were found in the peripheral blood and bone marrow and were noted to be large with prominent basophilic granules.

Bone marrow cytology

Bone marrow aspirates were taken at the time of euthanasia (study termination) and were reviewed by a board-certified veterinary clinical pathologist. All samples had bone marrow particles of the appropriate overall cellularity for the age of the animals. AdLacZ/FIV-treated cats had normal bone marrow cytology consisting of both myeloid

and erythroid precursors present at all stages and progressing to maturity in an orderly fashion. The myeloid:erythroid cell (M:E) ratio was normal in these animals (range: 1.5-1.8, Figure 3.4A). Bone marrow aspirates from the AdLacZ/FIV cats also had adequate megakaryocytes, and small lymphocytes represented less than five percent of nucleated cells.

All AdMEMIL-12-treated cats had erythroid precursors present at all stages and progressing to maturity in an orderly fashion. However, the myeloid series in these cats was moderately to severely left-shifted with a paucity of band forms and late stage neutrophil precursors and slightly increased numbers of myeloblasts and promyelocytes. The resultant M:E ratio in the AdMEMIL-12 cats was significantly decreased compared to AdLacZ cats (range: 0.33-0.90, $p=0.002$, Student's t-test; Figure 3.4A-B). AdMEMIL-12 cats had adequate megakarytocytes but a higher percentage of small lymphocytes (10-26% of nucleated cells) were noted compared to samples from AdLacZ/FIV cats. As seen on blood smears, large granular lymphocytes with basophilic granules were noted in the bone marrow aspirates of AdMEMIL-12 cats. Erythrophagocytosis was also noted in these samples (Figure 3.5).

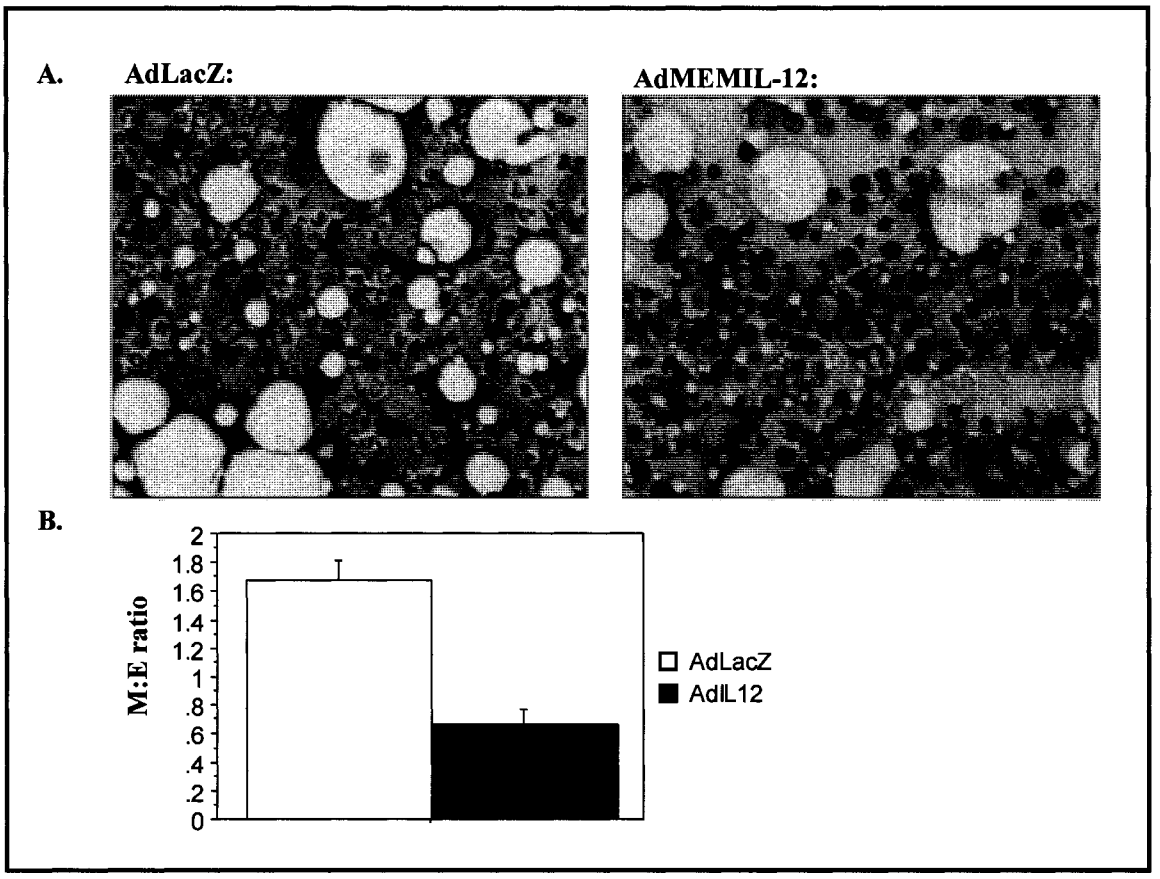

Figure 3.4: Terminal bone marrow aspirates from AdMEMIL-12-treated cats show a significantly decreased myeloid:erythroid (M:E) ratio. A) Representative photomicrographs of bone marrow aspirates from AdLacZ/FIV and AdMEMIL-12-treated cats. B) The M:E ratio was significantly decreased in AdMEMIL-12-treated cats ($p=0.002$, Student's t-test); AdLacZ n=2, AdMEMIL-12 n=6. Error bars denote standard deviation.

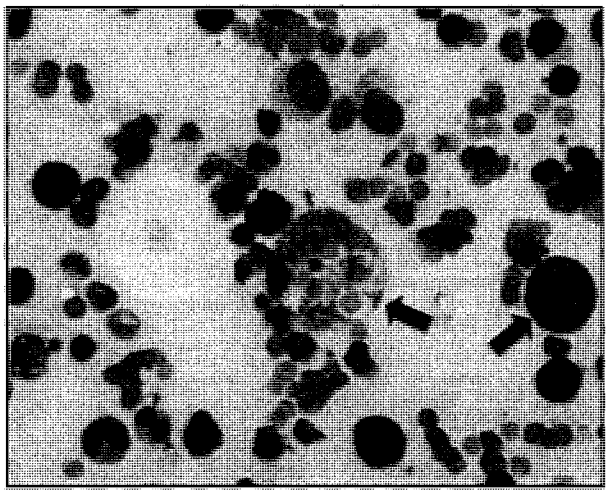

Figure 3.5:
Photomicrograph showing the presence of increased numbers of large granular lymphocytes with basophilic granules and erythrophagocytosis in the bone marrow of AdMEMIL-12-treated cats.

Biochemical changes

Biochemical profiles were performed on serum samples from the study cats. All but three study cats had increased phosphorus levels (range: 4.8-15.6 mg/dL; normal: 2.2-6.5 mg/dL) suspected to be due to increased bone turnover in young animals. Additional elevations in BUN (range: 25-75 mg/dL; normal: 16-35 mg/dL), magnesium (range: 2.8-5.5 mg/dL; normal: 1.8-3.0 mg/dL), potassium (range: 3.5-14.8 mEq/L: normal 3.5-5.2 mEq/L), total bilirubin (range: 3.8-12.9 mg/dL; normal: 0-0.2 mg/dL), creatinine kinase (CK) (range: 388-1895 IU/L; normal: 60-350 IU/L), alanine transferase (ALT) (range: 116-3170 IU/L; normal: 25-120 IU/L), and aspartate aminotransferase (AST) (range: 134-3055 IU/L; normal: 12-46 IU/L) were noted on most serum chemistry profiles performed on Day 4 or 5 from cats that received AdMEMIL-12. Decreases were seen in glucose (range: 8-125 mg/dL; normal: 69-136 mg/dL), creatinine (range: 0.5-1.4 mg/dL;

normal: 1-2.3 mg/dL), total protein (range: 3.7-5.5 gm/dL; normal: 6-8.7 gm/dL), albumin (range: 2.1-3.0 gm/dL; normal: 2.8-4.2 gm/dL), globulin (range: 1.6-2.5 gm/dL; normal: 2.8-5.3 gm/dL), cholesterol (range: 60-109 mg/dL; normal: 70-250 mg/dL), sodium (range: 141-146 mEq/L; normal 148-158 mEq/L), chloride (range: 112-121 mEq/L; normal 114-124 mEq/L), and bicarbonate (range: 2.7-12.3 mEq/L; normal: 13-22 mEq/L) in the AdMEMIL-12 treated cats. The notable exception to the above biochemical changes was the one AdMEMIL-12 cat that survived. The surviving cat's serum chemistry was normal except for a mild increase in total bilirubin (0.4 mg/dL; normal: 0-0.2 mg/dL) and a mild decrease in total protein (5.7 gm/dL; normal: 6-8.7 gm/dL) correlating with a mildly low globulin level (2.5 gm/dL; normal: 2.8-5.3 gm/dL). All cats receiving AdMEMIL-12 with the exception of the one surviving cat had icteric serum. Other than the expected elevations in phosphorus, serum profiles done on the cats receiving AdLacZ/FIV had relatively normal serum biochemical profiles (Table 3.1).

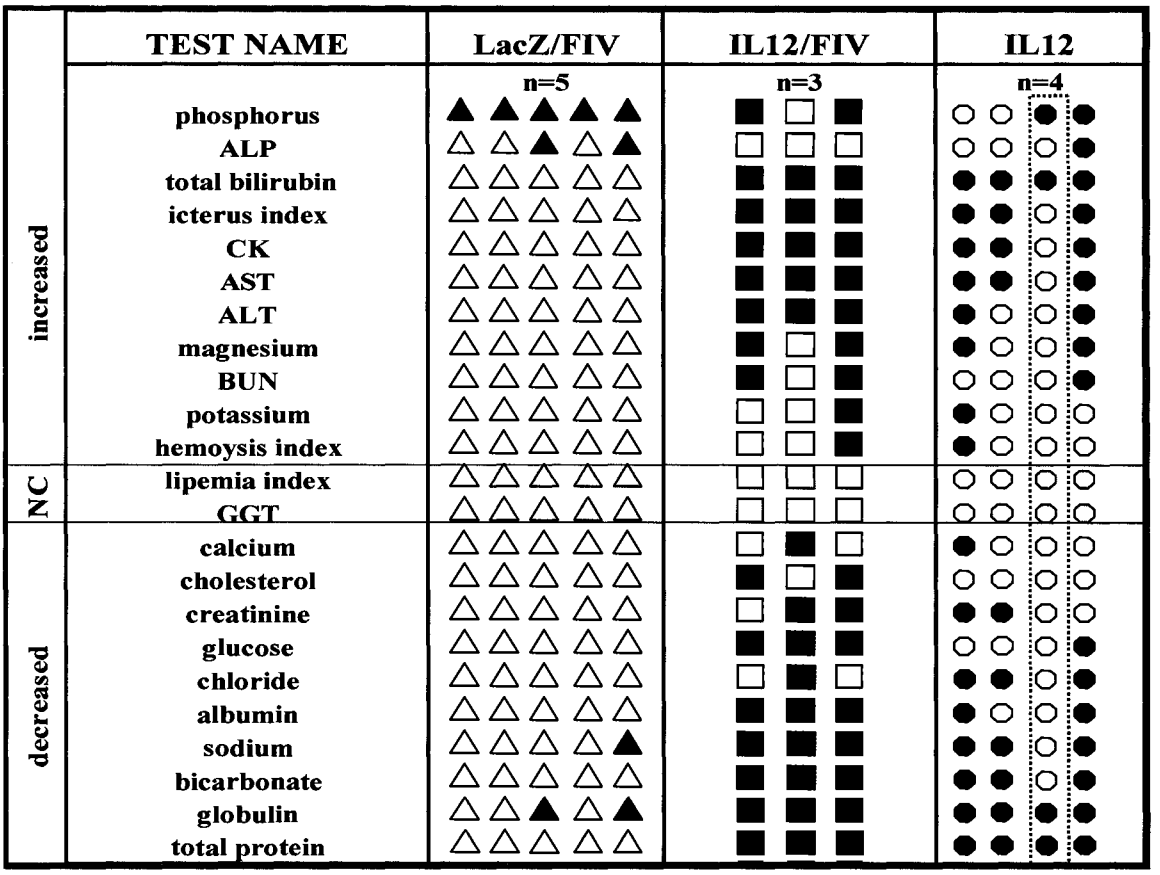

Table 3.1: Administration of adenoviral IL-12 to cats resulted in rapid development of multiple significant biochemical changes. Serum biochemical results from study termination day are depicted. Data was only available for three of the five AdMEMIL-12/FIV cats. Horizontal data denotes values from each cat; dotted box denotes surviving IL-12 cat. Black filled symbols represent abnormal values; white symbols indicate normal values. ALP = alkaline phosphatase; CK = creatinine kinase, AST = aspartate aminotransferase, ALT = alanine transferase, BUN = blood urea nitrogen, GGT = gamma-glutamyl transpeptidase, NC = no change.

Relative intracellular glutathione levels

Relative intracellular glutathione levels were measured in EDTA blood in all study cats at each time point as a measure of oxidative capacity. The raw data was assessed for Gaussian distribution and a plot of the residuals versus predicted value was examined graphically to assess variance of the residuals. A pair-wise comparison using

Tukey-Kramer adjustment was then applied to test for significant differences in mBCl mean fluorescence intensity (MFI) between Days 0, 1, 2, and study termination day for CD4+ and CD8+ T lymphocytes and polymorphonuclear leukocytes (PMNs), assuming significance at T-K adjusted $p < 0.05$. Data for AdMEMIL-12/FIV and AdLacZ/FIV groups were compared together. Data for the AdMEMIL-12 only cats was run and analyzed separately.

As expected, PMNs had significantly higher levels of GSH and therefore also had more mBCl signal than lymphocytes (Figure 3.6A-C). Polymorphonuclear leukocytes from AdMEMIL-12/FIV cats showed a significantly lower level of GSH at study termination than AdLacZ/FIV cats (T-K $p=0.048$; Figure 3.6C). CD4+ and CD8+ T lymphocytes showed similar changes (T-K $p=0.002$ and $p=0.004$, respectively, Figure 3.6A-B). In the CD4+ and CD8+ T lymphocytes, the between group difference was associated with an increase in relative GSH levels in CD4+ and CD8+ T lymphocytes from AdLacZ/FIV cats at study termination (Day 6) when compared with Day 2 levels (T-K $p=0.008$ and $p=0.014$ respectively, Figure 3.6A-B). A similar increase was not seen in the study termination values (Day 5) from AdMEMIL-12/FIV cats.

Samples from AdMEMIL-12 only cats were run separately and log transformed prior to analysis. In these cats, CD4+ and CD8+ T lymphocytes and PMNs underwent similar and significant changes in relative intracellular GSH content following IL-12 administration: a progressive and rapid decline from Day 1 down to almost zero at study termination on Day 4 (Day2 to Day4 T-K $p<0.001$ for all cell types, Figure 3.6D).

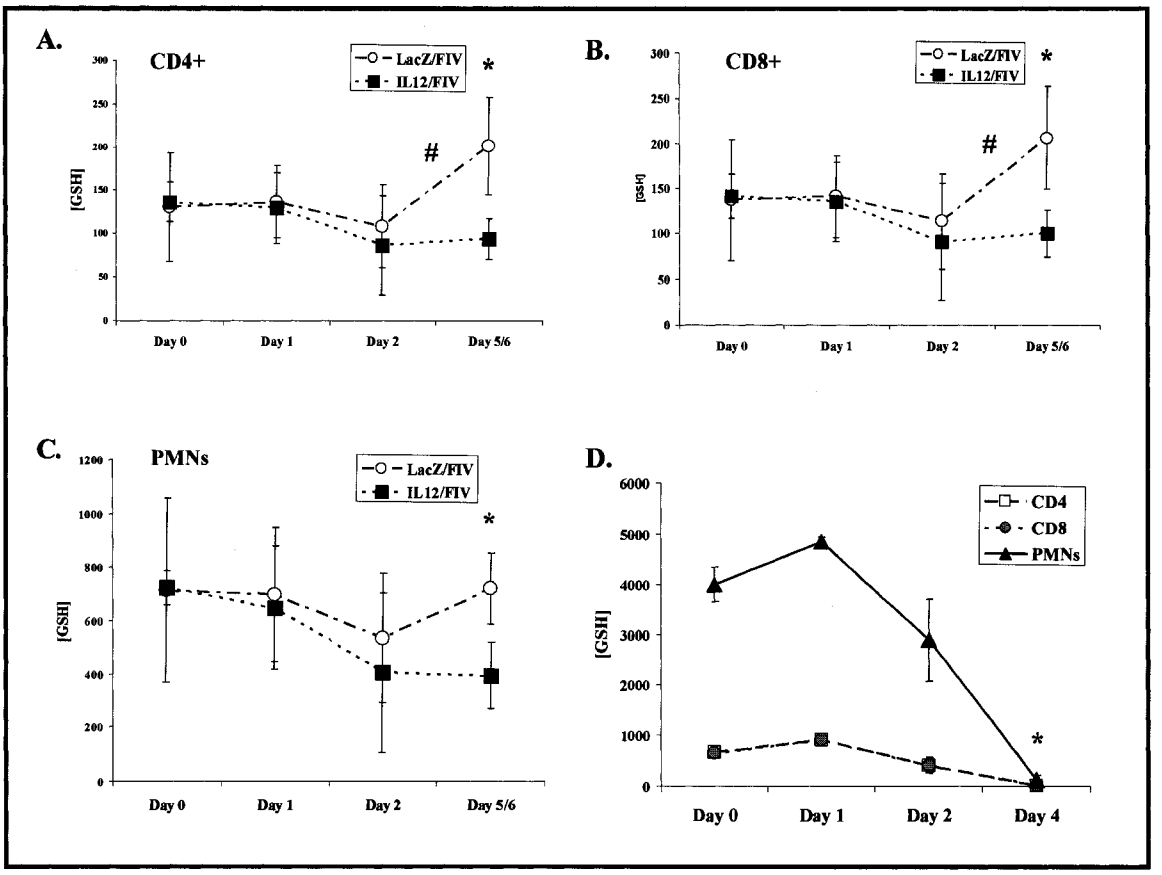

Figure 3.6: Significant differences were found in relative intracellular glutathione levels (GSH) with AdMEMIL-12 administration. Comparing AdMEMIL-12/FIV and AdLacZ/FIV-treated groups, CD4+ T lymphocytes (A) showed a significant increase in GSH at study termination day in AdLacZ/FIV cats (# T-K p=0.008) that did not occur in AdMEMIL-12/FIV cats resulting in a significant between group difference in terminal GSH levels (*, T-K p=0.002); a similar pattern was seen with CD8+ T lymphocytes (B: #, T-K p=0.014; *, T-K p=0.004); peripheral blood mononuclear cells (PMNs) also showed a significant difference between groups at study termination (C: *, T-K p=0.048). D). CD4+ and CD8+ T lymphocytes and PMNs from AdMEMIL-12 only cats underwent similar and significant changes in relative intracellular GSH content following IL-12 administration, showing a rapid decline after Day 1 down to almost zero just prior to death (*, Day2 to Day4 T-K p<0.001 for all cell types). Error bars denote standard deviation.

FIV DNA PCR

PCR for FIV DNA was negative on all samples during the study period, indicating that no cat had integrated provirus by the time of study termination.

Cytokine assays: RT-PCR

Effects of IL-12 administration on levels of IL-12, IL-10, IFNγ, IFNα, and TNFα mRNA were measured in circulated PBMC throughout the study period and in tissues collected at study termination. No significant differences from baseline levels were noted in circulating PBMC mRNA for the cytokines IL-12, IL-10, IFNγ, IFNα, and TNFα from any of the study animals at any time point (data not shown).

Differences in cytokine mRNA production were measured in mesenteric lymph node (LN), spleen, and liver tissues from the cats collected at the time of euthanasia, corresponding to Day 4, 5, or 6 of the study depending on the clinical condition of the animals. Data from AdMEMIL-12/FIV and AdMEMIL-12-only cats was not significantly different except in IL-12 mRNA in the spleen, which was significantly increased in AdMEMIL-12-only cats when compared to AdMEMIL-12/FIV cats ($p=0.03$, Student's t-test, data not shown). Compared to AdLacZ/FIV-treated animals, AdMEMIL-12 treatment resulted in significant increases in IL-12, IL-10, and TNFα mRNA in the liver ($p<0.02$, Student's t-test; Figure 3.7). No significant changes were noted in IFNγ or IFNα mRNA levels in the liver or in mRNA levels of any of the measured cytokines (IL-12, IL-10, IFNγ, IFNα, TNFα) in the spleen or mesenteric LN (data not shown).

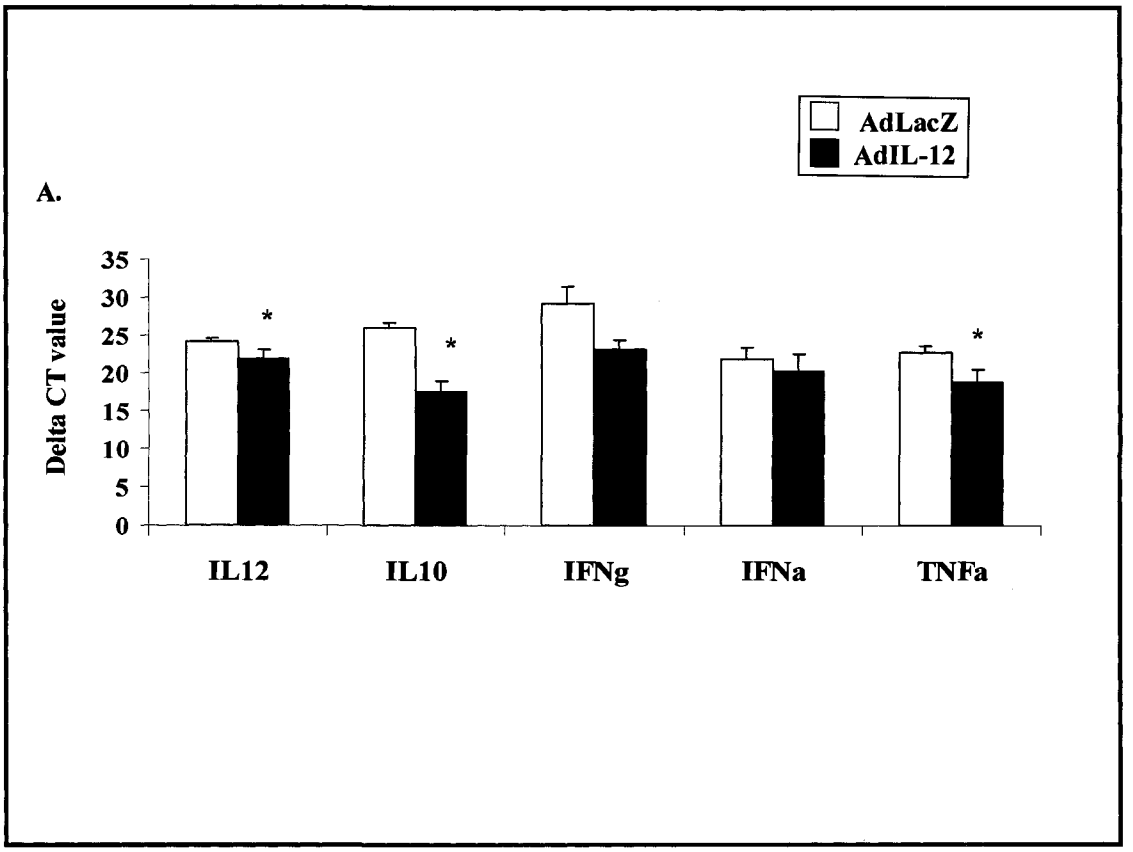

Figure 3.7: Tissue cytokine mRNA levels from AdMEMIL-12-treated cats showed significant increases in IL-12, IL-10, and TNFα the liver when compared to levels in cats administered AdLacZ/FIV. AdMEMIL-12 administration significantly increased mRNA levels of IL-12, IL-10, and TNFα mRNA in the liver ($p=0.018$, $p=0.00003$, and $p=0.007$ respectively, Student's t-test). Differences measured as comparison of Delta CT values between AdLacZ and all AdMEMIL-12-treated cats. Delta CT is the difference in threshold cycle between the sample and the housekeeping gene 18s, giving an inverse relationship to mRNA quantity in the sample. Error bars indicate standard deviation.

Serum cytokine levels

Serum levels of feline IFNγ and murine IL-12 were measured in all study cats. All cats that received AdMEMIL-12 had significant increases in murine IL-12 within twenty-four hours of adenovirus administration (Day 1), which remained significantly elevated through Day 2 when compared to Day 0 values and when compared to

AdLacZ/FIV-treated cats ($p<0.04$, Student's t-test; Figure 3.8A). The murine IL-12 levels increased substantially for all AdMEMIL-12/FIV cats at study termination (Day 5) and for two of the four AdMEMIL-12 cats (Day 4), although the increase was not statistically significant unless all AdMEMIL-12 samples were combined ($p=0.02$, Student's t-test). Although all animals that received AdMEMIL-12 were given the same dose, the serum murine IL-12 levels varied significantly between individual animals at all time points but particularly at termination day (range: 2,536-181,395 pg/ml).

Interleukin-12 induces IFNγ production by T cells, and increases in serum IFNγ levels were anticipated in AdMEMIL12-treated cats. Serum IFNγ levels remained at baseline in all animals until Day 4 of the study. Although there was significant variability between samples in the level of IFNγ (range: 8.57 to 1,180 pg/ml), serum IFNγ levels were significantly elevated by study termination day in all but two cats that received AdMEMIL-12 (Figure 3.8B). The two AdMEMIL-12-treated cats that did not show increased IFNγ were both in the AdMEMIL-12-only group, which was sampled on Day 4, one being the surviving cat. Serum IFNγ levels were significantly higher at study termination in AdMEMIL-12/FIV cats compared to AdLacZ/FIV-treated cats ($p=0.048$, Student's t-test; Figure 3.8B). Grouping all AdMEMIL12-treated cats showed a similar statistically significant difference in IFNγ levels when compared with AdLacZ/FIV cats at study termination ($p=0.03$, Student's t-test). Cats that did not receive AdMEMIL-12 had no significant increase in IFNγ during the study period.

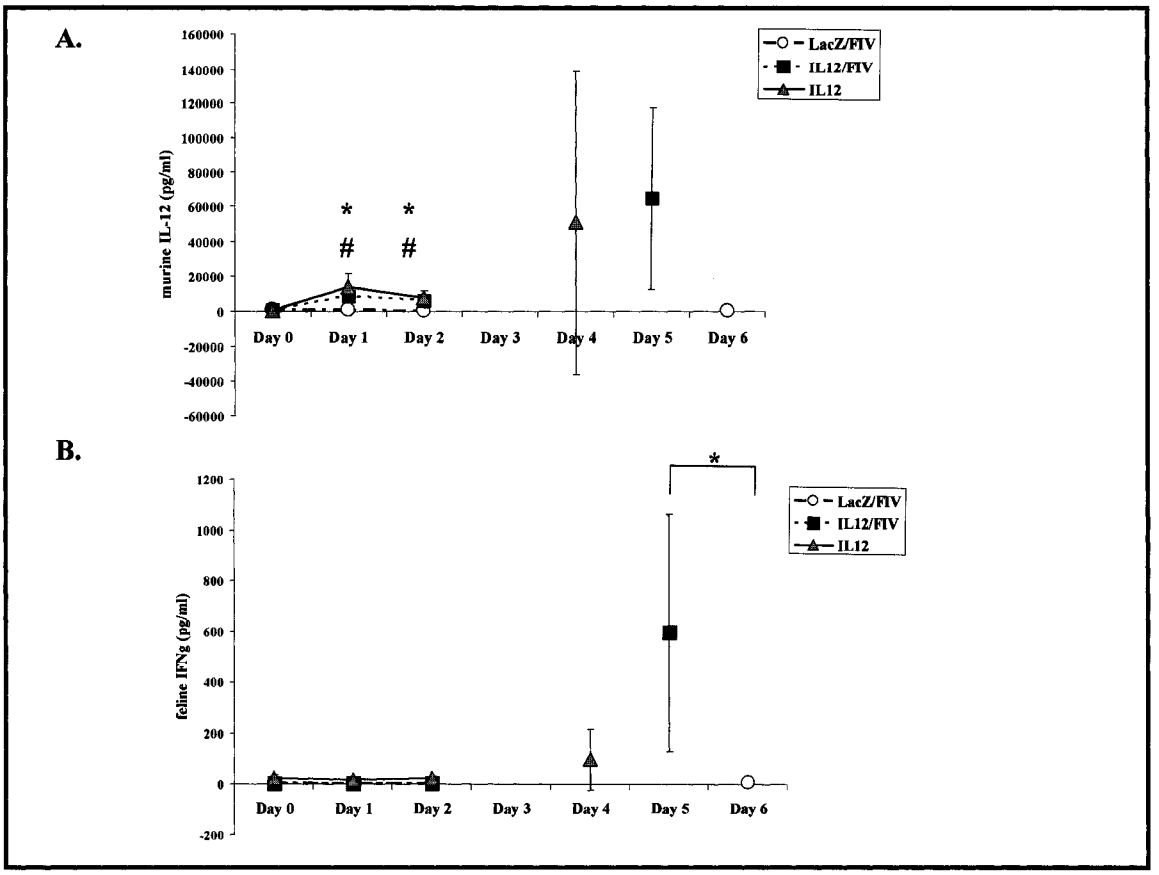

Figure 3.8: Adenoviral administration of murine IL-12 rapidly increased serum murine IL-12 levels, which was followed by an increase in serum feline IFNγ levels. A) Serum murine IL-12 levels were significantly increased on Day 1 and Day 2 in cats receiving AdMEMIL-12 when compared to Day 0 levels (*, p=0.04) and levels from AdLacZ/FIV cats (#, p=0.04, Student's t-test). B) Feline serum IFNγ levels increased in most cats receiving AdMEMIL-12 at study termination (Day 4 or 5) but remained at baseline in cats receiving AdLacZ/FIV. The increased IFNγ levels reached statistical significance in the AdMEMIL-12/FIV group ($p<0.05$, Student's t-test). Error bars denote standard deviation.

Nitric oxide levels

Nitric oxide was measured in heparinized plasma samples from the study cats. Nitric oxide (NO) is known to be induced by pro-inflammatory cytokines including IL-12 and IFNγ as well as by viruses and is increased in some patients infected with HIV-1. Nitric oxide is thought to inhibit excessive amplification of Th1 cells by IL-12 and IFNγ.

The raw data was assessed for Gaussian distribution and a plot of the residuals versus predicted value was examined graphically to assess variance of the residuals. Data was skewed and therefore log transformed prior to analysis. A pair-wise comparison using Tukey-Kramer adjustment was then applied to test for significant differences. Plasma NO levels from AdMEMIL-12/FIV cats were significantly lower than levels from AdLacZ/FIV cats only on Day 1 (T-K $p=0.018$). Plasma NO levels from AdMEMIL-12 only cats were significantly higher than AdMEMIL-12/FIV cats only on Day 2 (T-K $p=0.004$). The only between group difference in NO levels was in the AdMEMIL-12 only cats as Day 4 NO levels were just significantly higher than values on Day 0 (T-K $p=0.049$, Figure 3.9). Nitric oxide levels from AdMEMIL-12/FIV and AdLacZ/FIV were not measured on Day 4.

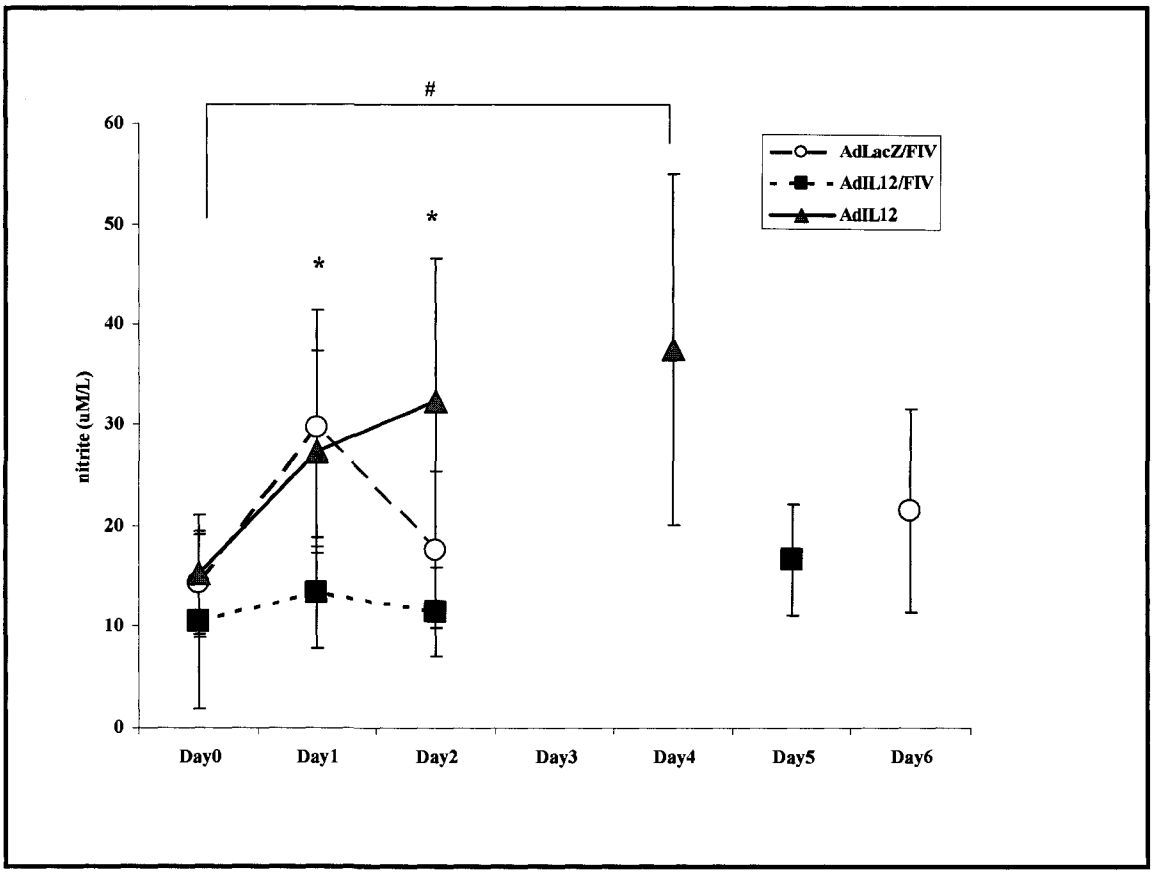

Figure 3.9: Plasma nitric oxide (NO) levels measured as total converted nitrite (uM/L) did not show any consistent statistically significant differences between or within groups over the course of the study. Nitric oxide levels were higher at Day4 when compared to Day0 in the AdIL-12 only group (#, T-K p=0.049). On Day1, NO levels in AdIL-12/FIV cats were significantly lower than levels in AdLacZ/FIV cats (*, T-K p=0.018), and on Day2, AdIL-12/FIV NO levels were lower than AdIL-12 only cats (*, T-K p=0.004). Error bars denote standard deviation.

Histopathology

Tissues were collected at the time of euthanasia. Grossly, the cats that received AdMEMIL-12 were pale and icteric at euthanasia. Samples from the liver, spleen, mesenteric lymph node, lung, kidney, and small intestine were evaluated for histopathological changes.

In all animals given AdMEMIL-12, regardless of FIV infection status, the liver and spleen appeared to be the main target organs for histopathological changes. There was a tendency to greater severity of tissue changes in the FIV-infected cats, which were collected approximately 24 hours later than the AdMEMIL-12 only tissues. The livers appeared with multifocal to bridging and coalescing, severe degeneration and necrosis of hepatocytes with a midzonal to centrolobular distribution (Figure 3.10C). There was individualization of hepatocytes and disruption of the hepatic plates, accompanied by bile stasis, multifocal hemorrhage in some animals and pronounced erythrophagocytosis by Kupffer cells in all but a few animals. In one AdMEMIL-12/FIV cat (4216) there was severe, diffuse coagulation necrosis of the hepatic parenchyma. Additionally, in all these cats there was multifocal mild infiltration of macrophages and fewer lymphocytes and plasma cells in the portal tracts, and diffusely there was accumulation and retention of monocytes and macrophages in the sinusoids.

Splenic changes in the AdMEMIL-12 administered animals comprised severe lymphocyte depletion of the white pulp, affecting the periarteriolar sheaths as well as the follicles, and loss of the histiocytic sheaths associated with the penicillar arterioles (Figure 3.10B). In contrast, large numbers of hemosiderin-laden macrophages were scattered throughout the severely congested red pulp.

The mesenteric lymph nodes from the AdMEMIL-12-administered cats, regardless of FIV-infection status, showed variable lymphocyte depletion, mainly affecting the paracortical zones and to a lesser degree the follicles (Figure 3.10A). The trans-endothelial lymphocyte migration in post-capillary venules was decreased, while the medullary cords and sinuses often were packed with macrophages displaying

erythrophagocytosis. Frank lymphadenitis with multifocal areas of cell necrosis and infiltration of neutrophilic granulocytes was present in one animal (4216).

The only other change noted in some of the AdMEMIL-12-administered cats was a moderate to marked increase in airway, interstitial and intravascular macrophages in the lungs. Apart from mild peritonitis in one AdLacZ/FIV animal, all tissues from this group of cats were histologically unremarkable (Figure 3.10D-F).

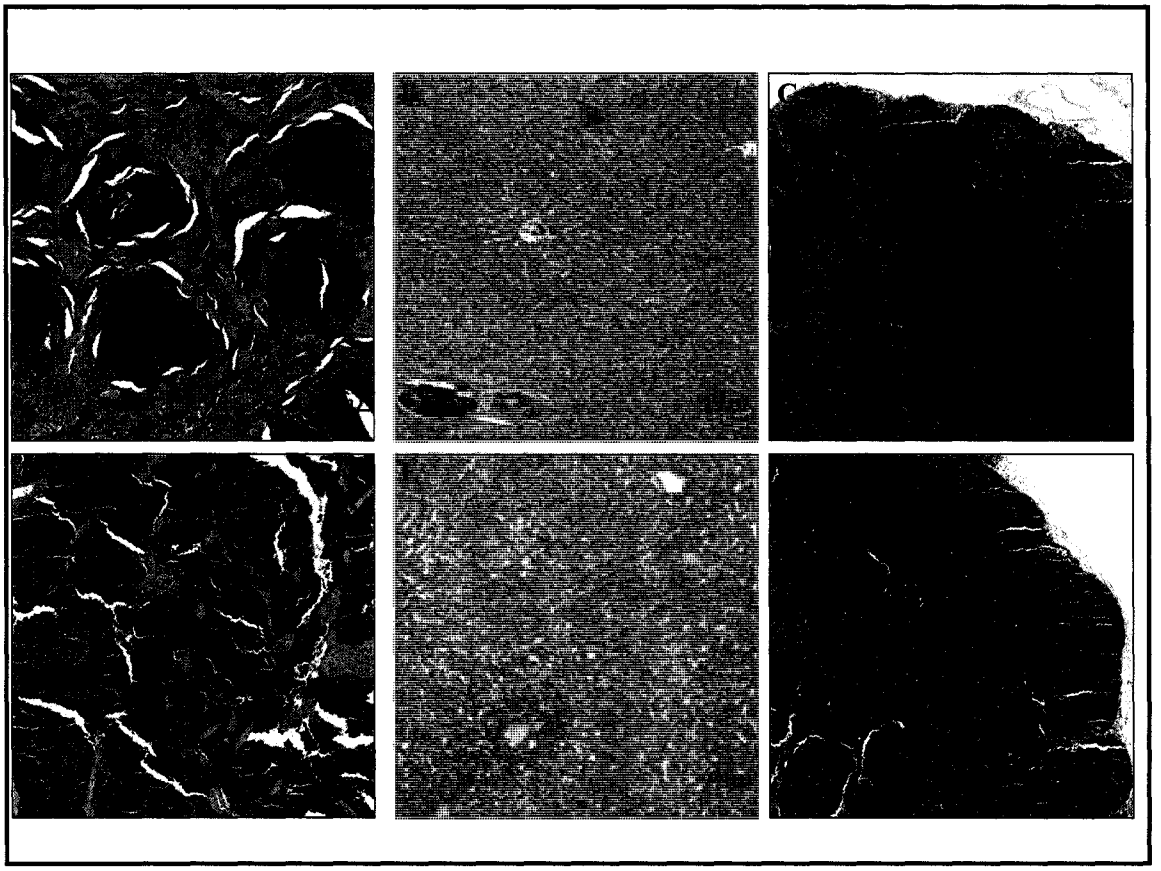

Figure 3.10: Representative photomicrographs of significant tissue changes in AdMEMIL-12 tissues: lymphocyte depletion of the white pulp and severe red pulp congestion in the spleen (D), hepatocellular necrosis (E), and lymphocyte depletion in the mesenteric lymph nodes (F). Figures A, B, and C show spleen, liver, and mesenteric lymph node tissues, respectively, from cats receiving AdLacZ.

DISCUSSION

Interleukin-12 has a wide range of biological activity including stimulating the proliferation and activation of cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells and inducing the production of a number of cytokines that drive induction of Th1 responses and cellular immunity. These factors and the identification of decreased IL-12 levels in patients with HIV make IL-12 an attractive therapy for lentiviral infections. Unfortunately, administration of IL-12 has often been associated with significant toxicity. The toxicity associated with IL-12 has largely been dependent on schedule and route of administration, although genetic or epigenetic variability in animals and people has also been suggested [21-23].

An adenoviral vector expressing murine IL-12 was developed with the intent of decreasing the toxicity of IL-12. Adenoviral vectors are themselves associated with toxicity and have been reported to induce cytotoxicity within minutes. The early cytotoxicity, which is characterized by acute production of proinflammatory cytokines, is vector transcription-independent and is mediated in part by viral particle interaction with components of the host innate immune response. Immediate-early toxicity includes hepatotoxicity, coagulopathy, further amplification of inflammatory cytokines and chemokines, and may last for hours to days after vector application [24]. Delayed toxicity has also been noted with adenoviral vectors, arising several days after adenoviral administration and likely mediated by the host adaptive immune response to de novo production of antigens [24-25]. Determination of a patient's immune tolerance to adenoviral vectors is difficult due to the existence of substantial pharmacogenetic variation in the human population [24].

Adenoviral vectors have induced excessive, lethal innate immune responses in baboons and in a human [26-27]. Empty viral capsids with no genomic DNA were shown to induce a substantial release of inflammatory cytokines from human peripheral blood mononuclear cells *in vitro* [28]. Experiments using high doses of adenovirus have found resultant TNFα upregulation, although some prior studies have failed to find significant TNFα induction in response to adenoviral administration. Quantification of TNFα in the serum does not necessarily reflect the status of membrane-bound TNFα or soluble TNFR production, both of which may influence the magnitude of innate immune responses [24, 26].

A pilot study performed in our laboratory using the AdMEMIL-12 vector in cats resulted in mild, resolving toxicity when a single 1 x 10^9 PFU dose was administered intraperitoneally. The current study was designed to use the AdMEMIL-12 at the same dose and route of administration used in the pilot study to assess the effects of increased IL-12 on early viral kinetics of the feline immunodeficiency virus (FIV) in cats. The AdMEMIL-12 unexpectedly induced fatal toxicity in all but one of the cats within five days of administration, regardless of FIV infection status. The control group receiving AdLacZ and FIV showed no acute or chronic signs of toxicity. Failure of the AdLacZ/FIV-treated group to show adverse reactions demonstrates the significance of the IL-12 rather than the adenovirus vector as the cause of the observed toxicity, assuming that the dose of adenovirus was truly equal in both groups.

Due to the rapidity and severity of toxicity, the influence of IL-12 on acute FIV infection remains undetermined. A significant difference in IL-12 mRNA in the spleen was detected between AdMEMIL-12/FIV and AdMEMIL-12 only groups suggesting that

early FIV infection may down-regulate IL-12 production in some tissues. However, AdMEMIL-12/FIV-treated cat samples were collected approximately 24 hours later than samples from AdMEMIL-12 only treated cats, complicating the evaluation of possible viral effects with a significant time difference. Consistent with previous studies of FIV in our laboratory, viral levels were not yet detectable in any of the cats by the time the study was terminated.

Recombinant IL-12 has previously been noted to induce multiple adverse side effects when given systemically, although the mechanism of these effects has not been fully documented [11, 21, 23, 29-30]. Fatigue, pyrexia, nausea, infection, metabolic acidosis, hepatic toxicity, hyperglycemia, hypoaluminemia, hematological toxicity including transient lymphopenia, monocytopenia, thrombocytopenia, neutropenia, and hemolytic anemia, and death have all been reported with systemic IL-12 administration [23, 31-35]. Mice dosed with IL-12 have additionally shown extramedullary hematopoiesis, muscle wasting, splenomegaly, gastrointestinal mucosal injury, pulmonary edema, and pleural and peritoneal effusions as well as death [21, 36-37]. Higher doses of IL-12 are associated with increased toxicity and immunosuppression [38-39]. The observed toxicity profiles for IL-12 and the tolerated doses have been reported to vary according to the route and schedule of treatment as well as the species and strain of animal studied [31, 38-39]. In our study, we unexpectedly saw toxicities similar to those reported in other studies and species with IL-12 administration. The most dramatic clinical consequences of the IL-12 administration in our study animals were hematological, with dramatic hemolytic anemia, thrombocytopenia, and hepatic toxicity.

Interleukin-12 toxicity has been correlated with serum levels of IFNγ in some studies and has been noted to be dependent on T cells, NK cells, and macrophages [23, 40-41]. Although IL-12 toxicity has been correlated with serum IFNγ levels in other studies, not all animals developing AdMEMIL-12-induced toxicity had elevated IFNγ levels based on serum ELISA. Serum IFNγ levels were only noted to increase at study termination day rather than correlating with the development of clinical symptoms of toxicity. In a human IL-12 trial, no difference in peripheral blood IFNγ mRNA was seen over the first 72 hours [32]. We did not see significant changes in PBMC IFNγ mRNA in this study. The terminal serum IFNγ level of three AdMEMIL-12-treated cats, however, was well above the 506pg/ml level seen in people who developed dose-limiting toxicity after being injected intra-tumorally with 300ng/ml of rhIL-12. Although the animal with the most significant histopathological changes had the second highest serum IFNγ level, the serum level of IFNγ or murine IL-12 could not otherwise be correlated to level of tissue changes. Similarly, no correlation was found between IFNγ expression or in IL-10, IL-12p35, or IL-12p40 expression between IL-12-treated and control human patients [32]. Serum IFNγ was correlated with the level of liver IFNγ mRNA in AdMEMIL-12-treated cats suggesting that serum IFNγ levels may be most indicative of liver activation. Sensitivity limits of the feline IFNγ ELISA and the variability of response between animals could have masked biologically significant changes in IFNγ levels prior to study termination. Although not evident in the tissues we evaluated, levels of IFNγ may also have been significantly altered in tissues that did not correlate with serum levels.

Chen *et al.* has noted that animals exhibiting signs of toxicity were those that failed to return rapidly to the physiological levels of pro-inflammatory factors [42]. The one surviving cat in our study that received AdMEMIL-12 showed a significant (greater than 15-fold) increase in murine IL-12 levels on Day 1. The murine IL-12 level then continued to decrease through study termination day, although the IL-12 levels at study termination were still greater than three-fold higher than baseline for the cat. This surviving cat did not show elevations in serum IFNγ during the study. An additional cat in the AdMEMIL-12 only group followed a similar magnitude of serum murine IL-12 levels both at baseline and at Day 1 and a similar lack of increase in serum IFNγ as the surviving cat, yet this cat developed lethal toxicity. Although the murine IL-12 levels in the cat that developed lethal toxicity decreased between Day 1 and Day 2 similar to the surviving cat, the IL-12 levels failed to decrease between Day 2 and Day 4, remaining seven-fold higher than baseline. Interestingly, the AdMEMIL-12 cat that developed lethal toxicity did not show any significant variations in tissue mRNA from other AdMEMIL-12-treated cats. Tissues from the surviving cat were not examined. Since both of these cats received identical treatment and had extremely similar initial responses to AdMEMIL-12 administration, the development of lethal toxicity would appear to correlate with the 48-hour longer exposure to higher levels of IL-12. Interleukin-12p35 was found to be increased in humans receiving IL-12 at 24 hours but normalized by 72 hours [32]. Mice successfully injected intramuscularly with AdMEMIL-12 showed a peak in IL-12 expression on Day 1 followed by a dramatic decline two days later [10]. In this study, all AdMEMIL-12-treated cats that developed lethal toxicity had sustained or

significantly increased levels of murine IL-12 from Day 2 through Day 4 and 5 (study termination).

Hematotoxicity was a significant part of the fatal toxicity seen in the AdMEMIL-12 treated cats. As has been seen in other reports of IL-12 toxicity, the cats developed severe hemolytic anemia within days of AdMEMIL-12 treatment. The mechanism underlying the IL-12-induced hemolytic anemia has not been fully determined. Significant erythrophagocytosis was noted in the bone marrow of the IL-12-treated cats as well as in macrophages in the liver and mesenteric LN suggesting that clearance of red cells played a large role in the anemia. Hemophagocytosis by macrophages is a phenomenon associated with several diseases including hemolytic anemias, metabolic diseases, malignancies, and infection-induced hemophagocytic syndromes. Although the mechanism of anemia and hemophagocytosis in these diseases has not been definitively determined, hypercytokinemia secondary to uncontrolled T cell activation and IFNγ activation of macrophages and monocytes are suspected to play a significant role in some cases [43-44]. Additionally, the splenic and liver phagocytic system is known to be activated by IFNγ and TNFα and has been speculated to reduce the life-span of circulating blood cells with IL-12 toxicity in mice [36]. Tumor necrosis factor-α mRNA was increased in the liver of AdMEMIL-12-treated cats.

Although initially thought to be a hematopoietic growth factor, IL-12 can cause myelosuppression *in vivo* due to stimulation of IFNγ [36, 45]. Interleukin-12-associated suppression of bone marrow hematopoiesis likely did not play a role in the AdMEMIL-12-associated anemia due to the time frame and severity of symptoms, and serum IFNγ levels was not correlated with level of anemia in the cats [40]. Bone marrow cytology

done at study termination did not show obvious changes in the erythroid cell line of cats treated with AdMEMIL-12.

Slide autoagglutination was noted in two AdMEMIL-12-treated cats suggesting a possible immune component of the red cell clearance in these animals. Red cell agglutination occurs secondary to antibody and/or compliment deposition on the cells. Although we were not able to measure levels of circulating antibody in the study cats due to lack of a positive control, the rapid development of the anemia argues against antibody-induced mechanisms unless the effects of circulating auto-antibodies were somehow enhanced due to IL-12 administration. Interleukin-12 administration has been shown to induce differentiation of Th1 autoreactive cells and exacerbate autoimmune disease [46-47]. Gollob *et al.* postulated that increases in NK cells, which are induced by IL-12 and express Fc receptors, could increase the sensitivity of the cells to even low levels of a low affinity erythrocyte antibody generated secondary to IL-12 [33]. Gately *et. al.* observed an increase in the numbers of NK cells and B cells in the spleen and NK cells and T cells in the liver in normal mice administered IL-12 whereas the number of Th cells in these sites remained essentially unchanged [48]. These mice developed a marked splenomegaly as has also been seen in other studies [48-49]. A human patient with renal cell cancer who developed hemolytic anemia with twice-weekly intravenous rhIL-12 also developed splenomegaly [33]. In contrast, we saw a decrease in lymphocytes in the liver and spleen, and splenomegaly was not noted. The development of severe anemia despite a lack of splenomegaly in the AdMEMIL-12-treated cats in this study does not support the postulation that the hemolysis or the thrombocytopenia seen with IL-12 toxicity are due to hypersplenism [33]. The definitive cause of the severe,

rapid development of hemolytic anemia in AdMEMIL-12-treated cats is undetermined although activation of macrophages, the phagocytic systems of the liver and spleen, and possible effects of increased NK cells are potential mechanisms. Additionally, complement factors and regulatory proteins have been associated with control of IL-12 production and pathogenesis of autoimmune hemocytopenias including anemia, thrombocytopenia, and lymphopenia [50-51]. Although we did not measure levels of complement factors or the expression of complement regulatory proteins in the study cats, the presence of autoagglutination in some cats treated with AdMEMIL-12 suggests a potential role for the complement system in the observed anemia.

In addition to anemia, the AdMEMIL-12 treated cats in this study developed changes in their white blood cell count and differential cell distribution. The AdMEMIL-12-treated cats had significant neutropenia on Day 2 that resolved within the two to three days before study termination. A transient neutropenia was also seen in cats during the pilot study conducted in our laboratory. Bone marrow cytology taken at study termination (Day 4-6) showed a decreased myeloid:erythroid (M:E) ratio in the cats treated with AdMEMIL-12 due to a left shift in the myeloid series created by a loss of mature cells. A left-shifted myeloid series indicates intense peripheral consumption or destruction of neutrophils in the AdMEMIL-12-treated cats. In a human patient receiving twice-weekly IV rhIL-12, severe neutropenia was associated with bone marrow agranulocytosis and a preponderance of large granular lymphocytes in the peripheral blood [33]. In the 2001 Gollob *et. al.* study, one patient was noted to develop an increase in circulating large granular lymphocytes, a third of which were identified as NK cells and elevations in NK cells has been seen in other IL-12 studies [33, 52]. Other

investigators have also identified persistent high proliferation of NK cells as integral to the toxicity seen by IL-12 therapy [42]. The AdMEMIL-12-treated cats in this study were noted to have an expansion of circulating large cells with basophilic granules at study termination that resulted in an increase in the total white blood cell count of the cats. Although we were unable to definitively characterize these cells due to a lack of feline antibodies, surface markers and cell morphology were consistent with NK cells. Expansion of the NK cell population would be expected in response to IL-12 in these cats, and the cells were not seen in the AdLacZ treated cats.

Transient lymphopenia is seen in humans treated with IL-12 intravenously [52]. The observed IL-12-induced lymphopenia in humans is caused by a redistribution of peripheral blood lymphocytes to extravascular sites, such as lymph nodes [32]. The AdMEMIL-12-treated cats in this study did not develop a significant lymphopenia, and histopathological results in this study do no support such a re-distribution in cats. In fact, lymphocyte depletion in the spleen and lymph nodes was noted in AdMEMIL-12-treated cats, perhaps due to movement of the cells to the vasculature. Increases in macrophages in both the liver and spleen were noted, however, as has been seen in mice treated with IL-12 [36]. Induction of TNFα, which was noted in the liver of IL-12-treated cats, is speculated to be involved in the observed cellular recruitment [36].

Significant thrombocytopenia was noted secondary to AdMEMIL-12 treatment in study animals. Systemic administration of recombinant adenoviral vectors has been associated with dose-dependent platelet reduction in different animal models and human patients, and mild thrombocytopenia is among the typical manifestations of naturally occurring wild-type adenoviral infections [24, 53]. The exact mechanism of the

development of thrombocytopenia after recombinant adenoviral vector administration has not yet been extensively investigated. Direct damage or activation of intravascular endothelial cells with subsequent platelet sequestration by the reticuloendothelial system has been proposed [24]. Although the cats received an adenoviral vector, the significant thrombocytopenia seen in the AdMEMIL-12-treated cats in this study appears to be due to the effects of the IL-12 rather than the adenovirus as the AdLacZ-treated cats did not develop thrombocytopenia. The thrombocytopenia was significant within 48 hours of receiving the AdMEMIL-12 and continued to decline until study termination. Bone marrow cytology did not show decreases in megakaryocytes, and necropsy did not support platelet consumption as a cause for the thrombocytopenia, again implicating more direct effects of IL-12 as the cause for the toxicity. There is evidence that activation of platelet caspases induced by TNFα causes thrombocytopenia [24, 54]. Although there was a trend for increased PBMC TNFα mRNA, the change was not statistically significant. Levels of TNF-α mRNA in the liver were consistently higher in AdMEMIL-12 cats than levels from AdLacZ cats, although not the case in the spleen or mesenteric lymph nodes. The specific mechanism of IL-12-induced thrombocytopenia in this study remains to be determined.

The main organs affected by AdMEMIL-12 administration were the liver, spleen, and lymph nodes. Systemically administered adenovirus type 2- and 5-derived vectors are hepatotropic and over 90% of systemically administered adenoviral vectors have been found to move to and be expressed in the liver [55]. Adenoviruses are known to induce hepatotoxicity, coagulopathy, and amplification of inflammatory cytokines and chemokines that can last for hours to days after vector application [24]. Although the

adenovirus itself does not appear to be the cause for the liver insult in these cats, the hepatotropism of the vector is likely partly responsible for the liver being the target organ in the AdMEMIL-12-treated cats. As in previous studies using AdLacZ, the AdLacZ/FIV group did not have any evidence of liver toxicity either on serum chemistry or histopathological assessment [56]. Only the cats receiving IL-12 showed evidence of liver toxicity with mild to severe increases in ALT, AST, and total bilirubin as well as hepatocellular necrosis.

Interleukin-12 itself and through induction of IFNγ, TNFα, and IL-18 can cause necrosis of hepatocytes [57]. Interleukin-12 and TNFα mRNA were both elevated in liver tissue of AdMEMIL-12-treated cats. Some of the noted elevation in IL-12 may have been due to increases in the pro-inflammatory cytokine IL-23, which shares the IL-12 p40 subunit. Both IL-12 and IL-23 induce IFNγ production [58]. Release of radicals such as nitric oxide (NO) from IFNγ- and TNFα-mediated activated macrophages is one proposed mechanism of the hepatocyte injury, although NO is also thought to inhibit excessive amplification of Th1 cells by IL-12 and IFNγ and has been under investigation as a preventative therapy for transplant-associated ischemia and reperfusion liver injury [36, 59]. We did not see any changes in plasma NO concentrations consistent with IL-12 administration in this study. Interleukin-12, when combined with IL-18, has also been postulated to cause impairment of hepatic microcirculation, which would cause hepatocellular necrosis as a result of ischemia and inhibition of oxidative phosphorylation in the liver [60]. Interleukin-18 levels were not measured in this study, and the possible role of IL-18 induction by IL-12 in the observed toxicity is unknown. Although preferential adenoviral delivery of IL-12 to the liver and the potential for IL-12-induced

liver damage seems likely, we cannot rule out the potential for anemia-related ischemic liver injury in the AdMEMIL-12-treated cats.

Changes to the spleen and mesenteric lymph nodes in the AdMEMIL-12-treated cats were mainly associated with lymphocyte depletion and erythrophagocytosis. The cause for the lymphocyte depletion is suspected to be due to peripheral migration as the cats in this study did not develop a lymphopenia as has been noted with IL-12 administration in other studies [32, 52]. Unlike the liver, the spleen and mesenteric LN from AdMEMIL-12-treated cats did not show any significant changes in the mRNA of the five measured cytokines, IL-12, IL-10, IFNγ, IFNα, and TNFα, when compared to AdLacZ cats. The difference in cytokine mRNA expression in the liver as compared to the spleen and mesenteric lymph nodes likely resulted from the hepatotrophic nature of the adenovirus.

The intracellular thiol redox status of cells is a central component in both the control of and response to changes in cytokine production. Intracellular reduced glutathione (GSH) plays an important role in regulating formation of the IL-12 p75 heterodimer, IL-12 secretion from alveolar macrophages, LPS-induced IL-12 production in human monocytes, and pro-inflammatory cytokine production by antigen-presenting cells [61-65]. Using mBCl and flow cytometry we found that administration of AdMEMIL-12 to cats led to a rapid and significant decrease in intracellular GSH content in CD4+ and CD8+ T cells and peripheral blood mononuclear cells. The decreased intracellular GSH was not due to adenovirus administration as AdLacZ/FIV-treated cats had significantly higher relative GSH levels than AdMEMIL-12/FIV-treated cats at study termination. A decrease in glutathione was similarly seen in splenic cells from mice

given RadIL-12 [39]. Decreased glutathione is consistent with NO-mediated oxidative stress, and glutathione levels have been shown to be involved in Th1-associated cytokine production [39, 66]. Oxidative stress has also been shown to influence activation of T and B cells through pathways involving thioredoxin and NF-κB [67]. A severe reduction in intracellular antioxidant defenses induced by AdMEMIL-12 administration may be partially responsible for the IL-12 toxicity seen in these cats and possibly the inhibition of CD8+ T cell responses seen in other studies [38]. Further studies to more completely evaluate the involvement of oxidative stress in feline disease and FIV are ongoing.

All animals in this study and the successful pilot study conducted in our laboratory received what was believed to be the same dose of adenovirus from the same stock, but the two studies had vastly different outcomes. As the cats were all from an inbred specific pathogen breeding colony, the likelihood that the cats had significant genetic alterations or epigenetic variability causing highly variable gene transduction levels and subsequent differences in serum murine IL-12 levels and toxicity is low [23]. The endogenous cytokine balance in the individual animal has been proposed as a cause for variable response to similar doses of IL-12 [38]. Human PBMC treated with the same concentration of rhIL-12 had wide variability in IFNγ production, and doses of IL-12 successfully used in anti-tumor models elicit toxicity in viral infection models [23, 38]. In this study individual animals within the same group were noted to have significantly different mRNA and serum cytokine levels, which could support the theory that even closely-related, clinically-healthy animals are in different cytokine balance states at any given time. The specific "cytokine state" of the animal may dictate the magnitude of response to insult and exogenous cytokine administration. In a study evaluating a similar

and unexpected severe human toxicity to IL-12 doses that had previously proven to be safe, giving a single injection of IL-12 before consecutive daily dosing was found to protect mice and cynomolgus monkeys from acute IL-12-induced toxicity. The protection included decreased mortality and was associated with an attenuated IFNγ response, perhaps indicating that adjustment of the immune response and cytokine balance was necessary to prevent toxicity [14]. Down-regulation of the immune response to repeated exposure to IL-12 has been reported [68]. Coincidentally, the one surviving cat that received AdMEMIL-12 had the highest baseline IFNγ level of all the cats in the study. Measurement of the level of other feline cytokines was inhibited by a lack of feline reagents.

Interleukin-12 holds significant promise as an antiviral and anti-tumor therapy but continues to be associated with variable toxicity in several models, particularly with systemic therapy as would be necessary for HIV treatment. The fatal toxicity seen in some cats treated systemically with murine IL-12 using an adenoviral vector supports the theory and the literature demonstrating that there is variability between species, strains, and individual animals in their response to IL-12. Such individual variation and possible severity of toxicity may limit the usefulness of this cytokine in clinical systemic therapies unless dosages can be individualized, for example, based on gene transduction. Alternate methods of overcoming the toxicity seen with IL-12 such as desensitization, intranasal administration, adoptive transfer of exposed T cells or genetically altered fibroblasts, and use of individual subunit vaccines have shown some promise in other models [22, 69-72]. Although this study showed fatal toxicities in cats treated intraperitoneally with a single dose of adenoviral-delivered murine IL-12, the similarity in the responses of the cats to

reported human studies using IL-12 underscores the value of the feline model as a means of evaluating potential lentiviral therapies. Further investigations into the effects of altered cytokine balance on lentiviral infection are on-going.

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