DISSERTATION

THE PIVOTAL ROLE OF DENDRITIC CELLS IN FELINE IMMUNODEFICIENCY VIRUS INFECTION

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY SUSANNE WENDY SPRAGUE ENTITLED THE PIVOTAL ROLE OF DENDRITIC CELLS IN 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

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Feline immunodeficiency virus (FIV) is the feline counterpart to human immunodeficiency virus. Dendritic cells (DC) are one of the first cell types encountered during lentivirus infections. The studies set forth in this dissertation initiated investigations into the interactions of FIV and DC. Accordingly, objectives addressed here were: (1) to develop methods to culture feline DC; (2) to evaluate the susceptibility of feline DC to FIV infection and determine whether virus is transferred to $CD4^+$ T cells; and (3) to assess the feasibility of prophylactic autologous DC/inactivated FIV vaccination.

Feline dendritic cells were cultured from blood-derived monocytes. Cell surface markers of feline DC were identified and compared with those of other species and contrasted with those of feline macrophages (MØ) to establish that the cells cultured were DC. This work established the system for subsequent FIV/DC interaction studies.

DC proved to be minimally susceptible to FIV infection. Nevertheless, the virus-exposed DC were able to transfer infection effectively to autologous $CD4^+$ T

cells. Transfer of infection was evident whether $CD4^+$ T cells were added to FIV-exposed and washed DC immediately or after DC were re-cultured for two days to allow for degradation of captured and unbound inoculum virus. While resting $CD4^+$ T cell-DC co-cultures supported low level replication, replication in activated $CD4^+$ T cell-DC co-cultures was robust. Thus, FIV has devised a facile means to establish infection through the inherent interaction of DC and T cells with antigens.

Finally, an initial study was conducted to determine whether feline DC pulsed with chemically inactivated FIV plus synthetic cytosine-phosphate guanosine dinucleotides (CpG ODN) as adjuvant might induce protection against subsequent virulent FIV challenge. This autologous feline DC/FIV immunization did not protect recipient cats from FIV challenge. No differences were detected in FIV proviral loads, $CD4^+$ T cell numbers, or anti-FIV humoral and cell-mediated immune responses between vaccinated vs. control animals, indicating a need for refinement of this vaccine strategy in future pursuits.

The studies described here serve a basis for further work defining the role of dendritic cells in FIV infection and immunity.

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DEDICATION

This dissertation is dedicated to my husband, Martin David and my son, Alexandre David. Martin not only gave tireless support and encouragement for my work, but he and Alexandre helped to teach me the importance of balance in family life and research.

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INTRODUCTION

Elusive control of Human Immunodeficiency Virus (HIV-1) Infection:

Human Immunodeficiency virus (HIV-1), the causative agent of acquired immunodeficiency syndrome (AIDS), has killed more that 25 million people since it was first recognized in 1981. And, although new antiretroviral drugs have decreased the incidence of AIDS among HIV-1 infected individuals, there were more people infected with HIV-1 (40.3 million) in 2005 than ever before[1]. One of the most singular aspects of HIV-1 is the inability of the immune response to terminate viral replication and eliminate the virus despite an easily detectable and persistent immune response. Clearly, this is the key to why control of HIV-1 has been elusive. Different approaches are needed to control the spread of this pernicious virus.

Dendritic Cells (DC) in the study of lentivirus infections:

DC are critical to the initial activation and recruitment of T cells during immune responses[2,3]. Possessing the unique ability to activate naïve T cells, these cells are critical to the immunologic bridge between innate and adaptive immunity[2,4,5]. However, DC have also been shown to be targets of HIV-1 and

SIV infections[6-9]. Therefore studying the pivotal interactions of DC with lentiviruses is central to a better understanding of the immune control needed to combat HIV-1 infections. Here we employ feline immunodeficiency virus (FIV), the feline counterpart of HIV-1, to explore lentivirus/DC interactions.

FIV infection as model for HIV-1 infection:

FIV was first isolated in 1986[10] and like HIV-1, causes fatal immune dysfunction characterized by progressive depletion of $CD4^+$ T lymphocytes[11,12]. As with HIV-1 infection, acute FIV infection is down regulated but not extinguished during a long asymptomatic phase of infection characterized by continued $CD4^+$ T cell depletion and immune system compromise, which ultimately terminates in fatal opportunistic infections or cancer[13,14]. We have shown that the early cell tropism and progression of FIV infection is similar to that in HIV-1 infection, i.e. infection of mucosal DC followed by T cells in regional lymphoid tissues[15]. The studies comprising this dissertation show that the interplay of DC with FIV and $CD4^+$ T cells *in vitro* is similar to that of human and macaque DC with HIV-1 and SIV, respectively. These studies are also extended to explore whether autologous DC exposed to inactivated FIV can confer protection from infectious FIV challenge since; (1) neutralizing antibodies and $CD8^+$ cytotoxic T cells (CTLs) appear to be the main correlates of immune protection in acute viral infections, (2) DC can induce Ag-specific cellular immunity and (3) therapeutic DC vaccines that induce CTL responses have been shown to decrease viral loads in HIV-1 and SIV infected individuals[16-19] In

addition, a recent correlate of immunity in the successful yellow fever vaccine, 17D, has been the activation of multiple DC subsets via several different toll-like receptors (TLRs)[20].

DC Biology:

Dendritic cell progenitors arise in bone marrow and traffic to sites of antigen entry where they differentiate into immature DC that lack high expression of MHC class II and costimulatory molecules[21], in contrast to their matured or activated counterparts. However, via mannose receptor-mediated endocytosis, macropinocytosis, and phagocytosis, immature DC can efficiently capture antigens[22,23]. After antigen capture and in the presence of infection and/or inflammation-induced signals, DC mature and migrate to lymphoid tissues where they facilitate powerful T cell stimulation while losing efficient antigen capturing abilities[24,25]. DC migration is mediated in a large part by CCR7 in response to ligands CCL19 and CCL21 in lymphoid organs[26,27], though it is evident that a complex coordination of signals is required for migration to occur[28-30]. As with all antigen-presenting cells, DC can present antigen (Ag) in the context of both MHC I and MHC II, however, DC also have the ability to present exogenous Ags on MHC I molecules via a mechanism termed cross-presentation[31]. In addition to these features, the uniquely potent stimulatory capacity of DC can be explained at least in part by studies demonstrating that newly formed MHC II-peptide complexes move with CD86 costimulatory molecules during lysosomal transport and deposit as stable clusters on the DC plasma membrane, selectively

concentrating TCR ligands and costimulatory molecules for T cell contact[32-34]. In addition, endosomally-derived vesicles termed exosomes are secreted from immature and mature DC with different functional properties. Although mature DC appear to secrete fewer exosomes than immature DC, immune activation by mature DC-derived exosomes has been shown to be stronger[35,36]. That is, unlike immature DC-derived exosomes, mature DC-derived exosomes; (1) can transfer naïve T cell activation to inefficient activators such as B cells, (2) are more able to activate naïve T cells in vivo, and (3) express much higher amounts of MHC II, B7.2, and ICAM-1 critical for naïve T-cell priming[36].

Details elucidating how Ags are processed and presented in DC are continually expanding. For example, it was recently shown that toll-like receptors (TLRs) determine the T-cell receptor (TCR) ligands derived from phagocytosed contents within phagolysosomes. That is, when both bacteria and apoptotic cells were phagocytosed, TCR ligands were generated from the bacteria, but not from the TLR-deficient apoptotic cells, demonstrating a mechanism in which self antigens can be distinguished from microbial antigens[37]. DC function appears also to be influenced by the strength and persistence of CD40 signaling on DC. Specifically, a weak and transient signal induced migratory DC with increased CCR7 expression, whereas a strong and sustained signal induced IL-12p70 production[38]. In addition, MAP kinases were shown to play a significant role in these functional differences given that weak CD40 signals induced extracellular stress-related kinase-1/2 (ERK-1/2)-dependent IL-10 expression and enhanced

DC migration whereas stronger signals induced p38 kinase-dependent IL-12 production and blocked DC migration[38,39].

DC subsets and plasticity

Dendritic cell subset delineation is ever changing. Currently defined are two blood and bone marrow-derived DC, myeloid DC (mDC) and plasmacytoid DC (pDC) and two skin DC subsets described in humans-- Langerhans cells (LC) and interstitial (dermal) DC[40]. More DC subsets have been described in mice including additional tissue phenotypes, however, this discussion will concentrate on human and mouse DC subsets most pertinent to our studies.

mDC and pDC are morphologically similar but have phenotypic and functional distinctions. mDC express myeloid cell surface markers whereas pDC lack expression of these markers[41,42]. mDC respond primarily to bacteria via toll-like receptor (TLR)-2 and TLR-4 by producing TNF-α, IL-6 and IL-12, whereas pDC respond mainly to viruses via TLR-7 and TLR-9 inducing copious amounts of type 1 interferons[43,44]. Where it was once theorized that myeloid DC arose from the common myeloid progenitor (CMP) and plasmacytoid DC arose from the common lymphoid progenitior (CLP), it is now less certain. In mice, it was shown that both DC subtypes can arise from either the CMP or the CLP that express fms-like tyrosine kinase 3 (Flt-3), and that it is Flt-3 expression that determines the potential for precursor cells to differentiate into DC[45]. In a demonstration of DC plasticity, bone marrow plasmacytoid DC from mice infected with lymphocytic choriomenigitis virus converted to myeloid DC after 4 days in culture with Flt3L

and that conversion was at least partially mediated by type 1 interferons[46]. Langerhans cells of the epidermis have recently been shown to arise from monocyte precursors in bone marrow under inflammatory conditions *in vivo*, but it is apparent that under steady-state conditions, LC are maintained locally by dermal-resident $CD14^+$ cells[47-49]. Dermal DC are believed to be derived from myeloid DC precursors in blood[50]. Finally, DC used in most studies to date, including this dissertation research are generated from peripheral blood monocytes using in vitro culture with granulocyte monocyte- colony stimulating factor (GM-CSF) and interleukin-4 (IL-4)[51-53] or from or bone marrow cells using stem cell factor (SCF), GM-CSF and tumor necrosis factor-α (TNF-α)[54,55].

DC and tolerance:

Dendritic cells are not only capable of initiating powerful immunity, but can induce immunologic tolerance both centrally and peripherally[56,57]. Where it was once considered that immature DC were immunologically silent, more recent information revealed that immature DC activated naïve T cells inducing tolerance instead of immunity[58,59]. Tolerance can occur either by a deletional route or by the induction of Ag-dependent regulatory T cells[57,60,61]. It is believed that the main induction of peripheral tolerance is in the steady state with uptake of self-antigens through dying cells. The endocytic receptor, DEC-205, has been shown to be involved in tolerance. DEC-205 is able to efficiently uptake soluble protein and dying cells and present exogenous Ags through cross-presentation and in three independent studies uptake via this receptor induced deletion of T

cells[57,60,62]. However, when an agonistic CD40-antibody was delivered to mature the DC along with Ag targeting the DEC-205 receptor, immunity by both $CD4^+$ and $CD8^+$ T cells was initiated[63]. Where immature DC have been implicated as a major player in peripheral immune tolerance, a role for mature DC has been suggested. In a study by Sporri, et al., mature DC exposed to LPS or CpG were able to induce $CD4^+$ T helper cell responses, however, the same cells exposed only to indirect inflammatory signals induced $CD4^+$ T cell clonal expansion without differentiation to the effector phenotype[64]. In support of this phenomenon of tolerance by mature DC, in the model of cross-presentation, monocye-derived DC pulsed with apoptotic cells and then matured with cytokines only induced a priming effect to $CD8^+$ T cells if there was contact with $CD4^+$ T cells through CD40 ligation. If $CD4^+$ T cells were absent, then $CD8^+$ T cells became tolerogenic[65].

DC and Immunodeficiency-inducing lentivirus infections:

Although the role of DC in the immunopathogenesis of HIV-1 has not been fully elucidated, there is evidence to suggest that mucosal DC (i.e. Langerhans' cells) may be the initial cells infected[66]. In fact, within 60 minutes of intravaginal SIV exposure, $CD1a^+$ LC and $p55^+$ DC comprised the majority of cells productively infected in the vaginal epithelium, lamina propria and cervix by 18 hours post infection[8]. mDC and pDC have important co-receptors for HIV-1 binding and entry, namely CD4, CXCR4 and CCR5[67-69] and they selectively capture macrophage-tropic strains of HIV-1 which predominate during viral

transmission[67,70,71]. C-type lectin receptors (CLRs) have also been shown to be important in the binding and transmission of HIV-1 and SIV[72,73]. DC-specific ICAM-3-grabbing (DC-SIGN) receptor, the most studied CLR, has been shown to present HIV-1 in its native form to T cells, facilitating infection in *trans* of CD4/CCR5 positive T cells[72] and in cooperation with CD4 and CCR5, Lee et al. has demonstrated *cis* enhancement of de novo DC infection[74]. Whereas DC-SIGN has been shown to enhance HIV-1 and SIV infection of T cells[72,75], other work has suggested that this receptor has less importance for infection and transmission than once thought[76-78]. In fact, langerin and mannose receptor (MR), other CLRs expressed on epidermal LC and dermal DC, respectively, have been shown to bind HIV gp120 *in vitro* and may be more likely to be active in the initial DC-virus contact during transmission of lentivirus infections[79].

While HIV-1 and SIV have been shown to actively replicate in DC-T cell co-cultures[80-82] and *in vivo*[7], productive infection of blood-derived DC from infected humans and macaques has rarely been demonstrated[8,83]. Paradoxically, there is evidence pointing to DC being main targets and mediators of lentivirus infection. First, progressive loss of mDC and pDC associated with increasing viral loads has been demonstrated in HIV-1 infected individuals[84]. pDC loss was directly correlated with decreased interferon-α (IFN-α) production and $CD4^+$ T cell counts[85-87], whereas mDC loss was mainly correlated with higher viral loads[87,88]. Interestingly, pDC, but not mDC concentrations were restored during antiretroviral treatment[87]. Second, recent studies have demonstrated *ex vivo* infection of both mDC and pDC by HIV-1[67,89,90], and in contrast to *in vivo*

studies, *ex vivo* infection of pDC was associated with increased IFN-α production[89,90]. This discrepancy may indicate a difference between the interactions of pDC and HIV-1 during initial infection and later stages of infection when pDC are being depleted. Third, subpopulations of DC in the intestinal tissues of acutely and long-termed infected macaques were found to be infected with SIV[9]. Finally, HIV-1 Gag protein was detected in DC containing multinucleated syncytial cells of adenoid and tonsillar lymphoepithelium closely juxtaposed to $CD4^+$ T cells from HIV-1 infected individuals[6,7].

The intricacies of initial DC-lentivirus interactions and subsequent transmission to $CD4^+$ T cells are still being defined. However, several recent *in vitro* studies have begun to reveal more details. First, both immature and mature DC pulsed with inactivated or infectious SIV took up virus into clathrin-coated pits and while only a few intact viral particles localized peripherally in immature DC, several intact viral particles were found perinuclearly in large phagocytic compartments in mature DC[91]. In another study it was shown that upon HIV-1 exposure to DC, virus rapidly moved to DC-$CD4^+$ T cell synapses after the addition of $CD4^+$ T cells. In addition, the initial transfer of virus to $CD4^+$ T cells was transient in that HIV-1 decayed within 24 hours, however, there was a second transfer of infection to $CD4^+$ T cells only by immature DC and this required de novo replication of HIV-1 in the immature DC[92]. Finally, it was demonstrated that after HIV-1 exposure, immature DC endocytosed virus and then rapidly released viral particles with exosomes into the extracellular environment where $CD4^+$ T cell infection ensued. This research group also

found that these exosomes were much more infectious than cell-free virus particles[93].

Although prophylactic DC vaccines have been unsuccessful to date, a few therapeutic DC vaccines in SIV and HIV-1 have generated interest[18,19,94]. All of the latter studies demonstrated decreased proviral loads with production of HIV or SIV-specific CD4 and CD8 responses for as long as one year using either chemical or heat inactivated virus-pulsed DC.

Conclusions:

Clearly these studies have helped to define the role that DC play during initial contact with lentiviruses and during established infections. While our understanding of these interactions increases, it seems clear that a difficult and complex pattern is unfolding. That is, the very cells that are needed to initiate an immune response during infection are being targeted for infection and propagation. And while DC are still able to stimulate immune responses once infected[95], it is these very responses that lentiviruses thrive on for propagation. It will be a challenge to design a way to harness the immune system to fight these viruses while blocking DC-virus interactions.

The studies laid forth in this dissertation have set the stage for and completed some analysis of DC function in FIV infection. This work establishes similarities in the interplay between DC and FIV that parallel other immunodeficiency-inducing lentiviruses and serve as a basis for future DC-FIV blocking studies as well as prophylactic and therapeutic vaccine trials.

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CHAPTER 1

CULTURE AND CHARACTERIZATION OF FELINE MONOCYTE-DERIVED DENDRITIC CELLS

ABSTRACT

To gain insight into the role of dendritic cells (DC) in feline immunodeficiency virus (FIV) infection and immunity, methods were developed to culture feline myeloid DC from $CD14^+$ monocytes with a combination of recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) and interleukin-4 (rhIL-4). These cells were compared with feline macrophages (MØ), cultured in the presence of rhGM-CSF. As with DC in other species, feline DC showed uniformly high MHC class II expression, moderate B7.1 expression, potent induction of the allogeneic mixed leukocyte reaction (MLR), and moderate uptake of fluorescein isothiocyanate-dextran (FITC-DX) in the endocytic assay. In comparison with feline MØ, DC showed higher expression of MHC class II, similar expression of B7.1, CD14, CXCR4 and CD1a, and lower expression of CD11b. When placed on alcian blue-treated

glass slides, DC spread out less than MØ and had characteristic fine cytoplasmic processes instead of the broader pseudopodia of MØ. Basal IL-12 mRNA expression and FITC-DX uptake were greater in DC than in MØ. Unlike feline DC, feline MØ exhibited a dose-dependent suppressive effect in the MLR. Feline DC propagated *in vitro* should prove useful in the development of DC-mediated vaccination and therapy for infectious and neoplastic feline diseases. Additionally, MØ cultured with GM-CSF provide a potential means of studying the mechanism of immunosuppression in cats.

INTRODUCTION

Dendritic cells (DC) are a diverse group of antigen-presenting cells (APC) that are critical in the induction, expansion and regulation of immune responses[1]. "Immature" myeloid DC lie at sites of antigen entry, where they are poorly immunogenic but adept at capturing antigens through receptor-mediated endocytosis, macropinocytosis and phagocytosis [2,3]. When primed with antigen, DC undergo "maturation" and traffic to T-cell areas of lymphoid tissues inducing high levels of co-stimulatory and MHC class II surface molecules; this facilitates powerful T-cell stimulation [4]. In addition, DC in a steady state, can silence self-reactive T cells by uptake and presentation of self-antigens [5].

For these reasons and others, a vaccine strategy centered on education of DC to initiate a potent early immune response would seem logical. In fact, immunity stimulated by DC-based vaccination strategies in mice have protected

against some neoplastic and infectious agents [6,7]. In addition, simian-immunodeficiency virus (SIV) and human immunodeficiency virus (HIV-1)-pulsed DC, when re-infused into macaque and human donors, respectively, elicited sustained immune responses and resulted in a decreased viral burden after infectious virus challenge [8-10].

As part of a study on feline immunodeficiency virus (FIV), the feline analogue of human immunodeficiency virus (HIV-1), we describe a method for culturing feline myeloid DC from blood monocytes. The method, which is similar to but not identical with feline DC culture methods published elsewhere[11,12], is intended to be used in the production of these cells for the purpose of assessing dendritic cell FIV infectibility (Chapter 2) and immunizing cats against FIV (Chapter 3). There also includes a description of the culture of a subset of macrophages (MØ) able to induce suppression in the mixed leukocyte reaction.

MATERIALS AND METHODS

Animals and Sampling

Peripheral Blood Mononuclear Cells (PBMCs) were isolated from blood of specific-pathogen-free (SPF) cats aged 12 to 24 months from a colony maintained at Colorado State University (Fort Collins, CO) in accordance with Institutional Animal Care and Use Committee Regulations.

DC and MØ Culture

Heparinized blood (40 ml) was collected from each cat anaesthetized with ketamine (20 mg/kg, subcutaneously; Fort Dodge Animal Health, Fort Dodge, IA) and acepromazine (0.05 mg/kg, subcutaneously; VEDCO Inc., St Joseph, MO). PBMCs were isolated over a ficoll-hypaque gradient (Histopaque-1077; Sigma-Aldrich, St Louis, MO) and centrifuged at room temperature for 30 min at 650 ***g***. $CD14^+$ monocytes (4-6% of PBMCs on average) were isolated with anti-human CD14 magnetic beads, according to manufacturer's instructions (Miltenyi Biotec, Auburn, CA), and cultured at a density of $1 - 1.5 \times 10^6$ cells/ml in 24-well plates with RPMI-1640 (Sigma-Aldrich) containing 10% fetal bovine serum (FBS; Atlanta Biologicals, Norcross, GA), 100 U/ml penicillin (Invitrogen, Carlsbad, CA), 100 µg/ml streptomycin (Invitrogen), 2mM glutamine (Invitrogen) and 50µM 2-mercaptoethanol (Sigma-Aldrich) (complete RPMI). The cultures were supplemented with human recombinant granulocyte-macrophage colony-stimulating factor (hrGM-CSF; 1000 U/ml) (R & D Systems, Minneapolis, MN) and human recombinant interleukin-4 (hrIL-4; 1000 U/ml) (R & D Systems). Cytokine additions and 50% media exchanges were performed every other day for 6-7 days. On day 6 or 7, the non-adherent cells were harvested for analysis by washing with warmed medium to prevent detachment of adhered cells. Feline MØ were cultured from $CD14^+$ monocytes in DMEM (Sigma-Aldrich) containing 10% FBS, 100U/ml penicillin, 100µg/ml Streptomycin, 2mM glutamine (Invitrogen) and 50µM 2-mercaptoethanol for 6–7 days. This solution was supplemented once with hrGM-CSF (1000 U/ml) on the first day of culture. After

6-7 days some cultures were exposed to a maturation stimulus for an additional 2 days. Maturation stimuli included Lipopolysaccharide (LPS, 1.5µg/ml; Sigma-Aldrich), Polyinosinic acid, polycytidylic acid (Poly I:C, 50µg/ml; Sigma-Aldrich), monocyte-conditioned media (MCM), or a cocktail of cytokines (CKT) including interleukin-1 beta (hrIL-1β, 10ng/ml; R and D systems), prostaglandin E-2 (PGE-2, 1 x 10-6 M; Sigma-Aldrich), tumor necrosis factor alpha (hrTNF-α, 10ng/ml; R and D systems) and interleukin-6 (hrIL-6, 10mg/ml; R and D systems).

Cell Morphology

Nonadherent cells from either feline DC or feline MØ cultures were centrifuged on to glass slides (Cytocentrifuge, Shandon Cytospin 2; Thermo Electron Corporation, Waltham, MA) at 200 rpm for 3 min. Cells were then stained with Wright-Giemsa stain (Wright's stain, EM Science, Gibbstown, NJ; Giemsa stain, Gad Rad, San Juan Capistrano, CA) for bright field microscopy and phase contrast imaging. Alternatively, 2 x 10^4 cells in 35 µl of serum-free medium were placed on 8–mm-diameter well slides (American Master*Tech Scientific, Lodi, CA) pre-coated with alcian blue (8Gx; Sigma-Aldrich), 1 mg/ml. The alcian blue coating method was performed as described by [13] and cell morphology was assessed by phase contrast imaging. DC and macrophages from four different animals were compared.

Flow Cytometry

Phenotypic expression of feline DC and MØs was evaluated by flow cytometry with several monoclonal antibodies (Abs) either specific for or cross-reactive with, feline cells (Table 1). Briefly, 2-4 x 10^5 cells were washed in cold phosphate-buffered saline (PBS) containing 2% FBS and 0.2 g/L sodium azide (PBS/Az) and labeled with a optimal dilution of Ab for 20 min at 4°C. The cells were then washed three times with PBS/Az; for unconjugated Abs, the cells were treated with fluorescein isothiocyanate (FITC)-goat anti-mouse IgG1 (Southern Biotec, Atlanta, GA) for an additional 20 min. They were washed again before being analyzed with a Coulter flow cytometer (EPICS XL-MCL; Beckman Coulter, Miami, FL). Isotype-matched mouse Igs were used as controls. Propidium iodide was used to exclude dead cells from analysis and gating of large cells for analysis was determined by scatter properties. Data were analyzed with FlowJo™ software (Tree Star, Inc., San Carlos, CA). Results are reported as histograms from an individual experiment and the average geometric mean fluorescence intensity (GMFI) +/- standard deviation (SD) of Ab-labeled cells from the large cell gated population minus isotype Ab-labeled cells from at least four different experiments using four different animals. Statistical analysis was performed on the GMFIs with the Student's *t*-test.

Mixed Leukocyte Reaction (MLR)

Feline T cells were positively selected from PBMCs by magnetic cell sorting (Miltenyi Biotec). PBMC Fc-receptors were blocked with normal goat

serum (Jackson ImmunoResearch, West grove, PA), followed by labeling with unconjugated feline Pan T Ab (clone CT843, a gift from Dr N. Gengozian, University of Tennessee [14]) and incubation with goat anti-mouse beads (Miltenyi Biotec). Positive selection of T cells by this method resulted in a purity of 95-98% and the majority of the cells were viable by trypan blue exclusion (data not shown). Feline DC or MØ were added in graded doses to 2 x 10^5 allogeneic T cells in 96-well flat bottomed tissue culture plates (Falcon, Franklin Lakes, NJ). As controls, DC, MØ and T cells were placed in wells alone at concentrations of 5 x 10^3/well, 5 x 10^3/well, and 2 x 10^5/well, respectively. Cultures were incubated for 8 days at 37°C with addition of 1μCi of ^{3}H-Thymidine ([^{3}H]-TdR)/well) for the final 16-18 h of culture as was determined optimal and correlates with previously published mixed lymphocyte cultures in cats[15]. Triplicate cultures were performed for each DC: T-cell ratio. T-cell proliferation was measured by [^{3}H]-TdR uptake as quantified with a Wallac 1450 Microbeta liquid scintillation counter (PerkinElmer Life Sciences, Boston, MA) and statistical analysis was performed with the student's *t*-test. Results are presented as the mean of three experiments plus the standard deviation between experiments. Three animals were used to derive DC and MØ and a fourth animal was used to derive T cells.

Real-time Polymerase Chain Reaction (PCR) for IL-12 mRNA Expression

Non-adherent feline DC or MØ were harvested from one well of a 24-well plate after 7 days in culture. Cells were lysed and mRNA was extracted with TRIzol reagent according to manufacturer's instructions (Invitrogen). mRNA

samples were stored at –70°C until used. Reverse transcription was performed on 2µg of DNase-treated RNA (Invitrogen) by means of the iSCript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Real-time PCR for feline IL-12 p40 mRNA cytokine expression was performed with previously published primers [16] and an iCycler iQ™ (Bio-Rad). Relative quantification of IL-12 was determined by the $2^{-\Delta\Delta C_T}$ method [17], comparing DC expression with that of MØ. Glyceraldehye-3-phosphate dehydrogenase (GAPDH) was used as a recorder gene to normalize IL-12 signals and MØ were treated as controls. Results were compiled from four different experiments with different animals.

FITC-dextan Uptake

DC or MØ (5×10^4) in 50 µl of RPMI containing 10% FBS were added to 1.5 ml Eppendorf tubes and incubated on ice for 30 min. DC or MØ were then incubated with 10 µg/ml FITC-dextran (FITC-DX, Mr = 40,000; Molecular Probes, Eugene, OR) at 37°C or 4°C (control tubes) for 30 min. Cells were washed four times with 500 µl cold RPMI containing 1% FBS and re-suspended in 200 µl of flow buffer (PBS/Az). Propidium iodide was used to exclude dead cells from analysis and gating of large cells for analysis was determined by scatter properties. Fluorescence was detected by flow cytometry and analyzed with FlowJo software (TreeStar,Inc.). The data shown were from one animal and are representative of three experiments conducted with three different animals. Statistical analysis was determined from the GMFI of FITC uptake at 37°C minus

uptake at 4°C as compiled from at least three different cats using the student's t-test.

RESULTS

Culture Yield and Morphology of Feline DC and MØ Propagated In Vitro

Culture of 5-6 x 10^6 $CD14^+$ monocytes for 6– 7 days in the presence of hrIL-4 and hrGM-CSF generated an average of 2.2 x 10^6 DC (large cells were counted and ranged from between 6.3 x 10^5 to 4.8 x 10^6) from 40 ml of blood. MØ yields were slightly greater, with an average of 3.6 x 10^6 MØ (range, 1.4 x 10^6 to 6.3 x 10^6) generated from 40 ml of blood. Feline DC were characterized by an abundance of fine cytoplasmic processes as demonstrated on cytocentrifuged preparations by both bright field and phase contrast imaging (Fig.1.1 A, B). Morphological differences between DC and MØ were also apparent on alcian blue-coated slide preparations. MØ were larger, with flatter and more widely spread cytoplasmic pseudopodia visible by phase contrast microscopy (Fig. 1.1 D, arrowheads). DC, by contrast, were smaller, with many fine cytoplasmic projections (Fig. 1.1 C, arrows).

Phenotypic Analysis of Feline DC vs Feline MØ by Flow Cytometry

Available feline-specific or cross-reactive Abs used to assess immunophenotypic markers of feline DC versus feline MØ are listed in Table1. Feline DC MHC class II expression was consistently 3-4 fold greater than that of

feline MØ (P = 0.0467). DC and MØ differed (P = 0.0126) in respect of CD11b expression, with moderate expression in ≤75% of feline DC and moderate to high expression in ≥ 96% of feline MØ. Expression of several surface molecules - B7.1, CXCR4, CD14 and CD1a - was similar on DC and MØ (Fig. 1.2), although B7.1 expression tended to be slightly higher on DC. Neither feline DC nor MØ expressed CD4, CD8 or CD21 (data not shown).

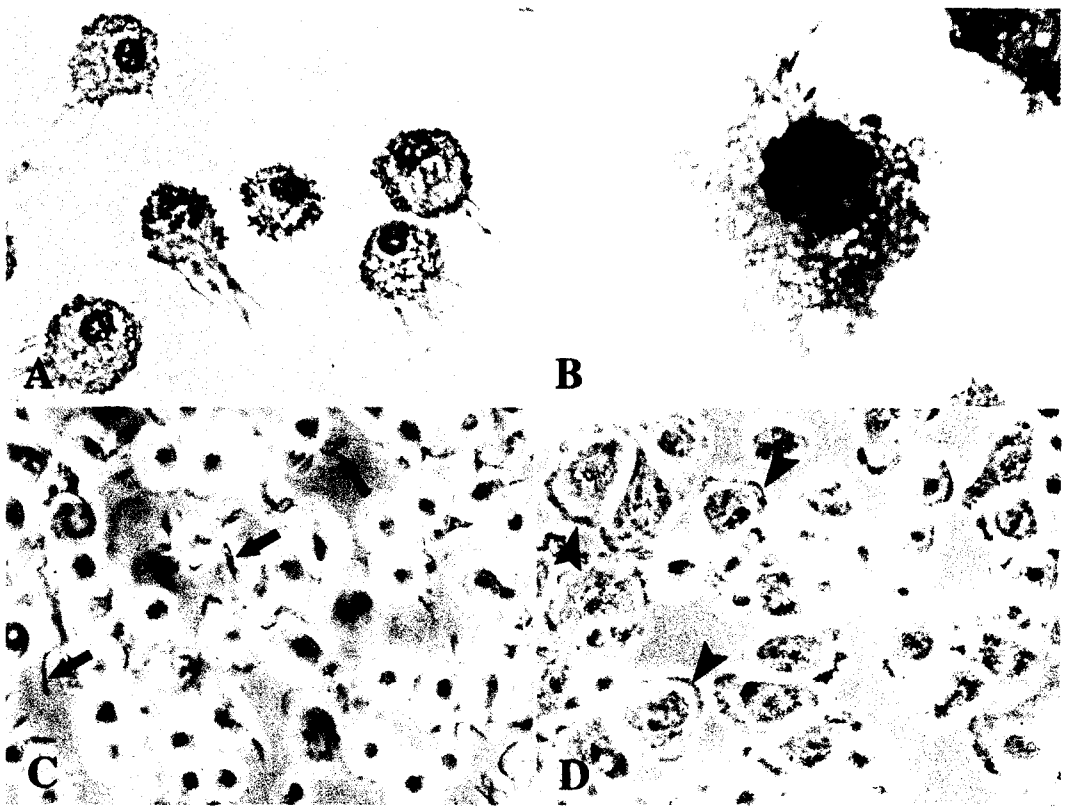


Fig. 1.1 A-D. Morphology of feline DC cultured with rhIL-4 and rhGM-CSF, and feline MØ cultured with rhGM-CSF only. DC had visible processes after cytocentrifugation as shown by (A) phase contrast, x 40, and (B) bright field illumination, x 100. Incubation on alcian blue-coated glass slides resulted in morphological differences between DC and MØ. Note (C) the fine cytoplasmic processes on the DC (arrows), x 40, and the flattened, broader pseudopodia of the MØ (arrowheads), x 40. Results are representative of four different experiments.

Functional Properties of Feline DC and MØ

Feline DC induced marked stimulation of allogeneic T cells at all DC:T cell ratios examined (1:30, 1:60 and 1:120; Fig. 1.3). By contrast, feline MØ had an inhibitory effect at the lower ratios of 1:30 and 1:60, demonstrating a statistical difference in T cell stimulation between DC and MØ at these ratios (P = 0.027 and 0.009, respectively). However, at a ratio of 1:120 the stimulatory capacity of cells from MØ cultures approached that of cells from DC cultures, resulting in a less significant difference (*P* = 0.386) (Fig. 1.3).

Table 1

Antibodies used in immunophenotypic labeling of feline DC and MØ

Monoclonal antibody*	Species	Source	Clone	Dilution	Reference
HLA-DR, DP, DQ	Human	Pharmingen+	Tü39	1 in 20	Pharmingen (2000)
CD14	Human	Caltag#	Tük 4	1 in 20	Willet *et al.* (2003)
CXCR4	Human	R & D Systems	47717	1 in 5	Hosie *et al.* (1998)
CD1a	Feline	Leukocyte Ag Laboratory§	FE1.5F4	1 in 5	Woo and Moore *et al.* (1997)
CD11b	Canine	Leukocyte Ag Laboratory	CA11.6A1	1 in 5	Broderson *et al.* (1998)
B7.1	Feline	Dr W. Tompkins	B7.1.66	1 in 10	Tompkins *et al.* (2002)

*All mAbs were generated in mouse.

+Pharmingen, San Jose CA.

#Caltag, Burlingame CA.

§Peter Moore, University of California Davis.

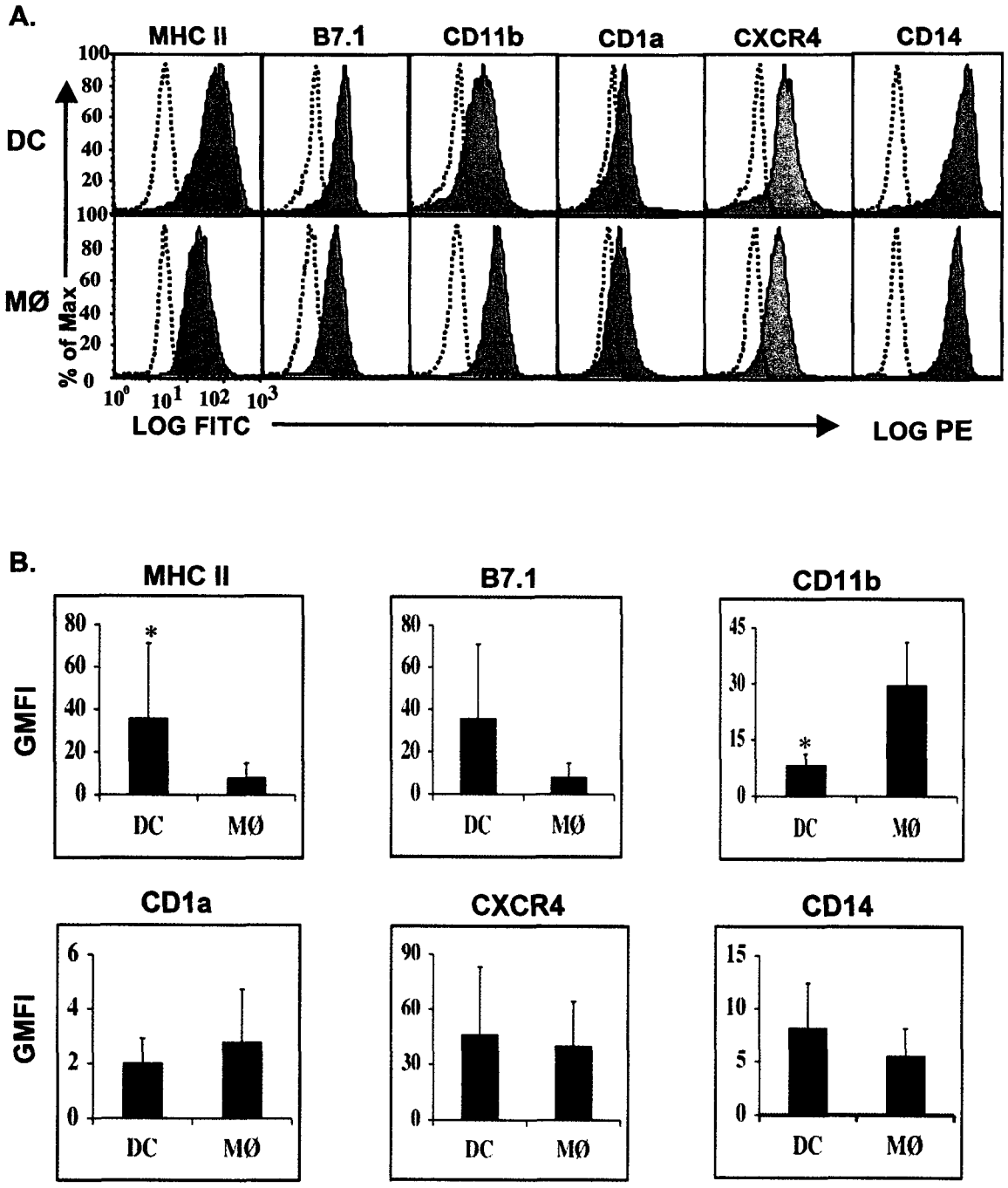
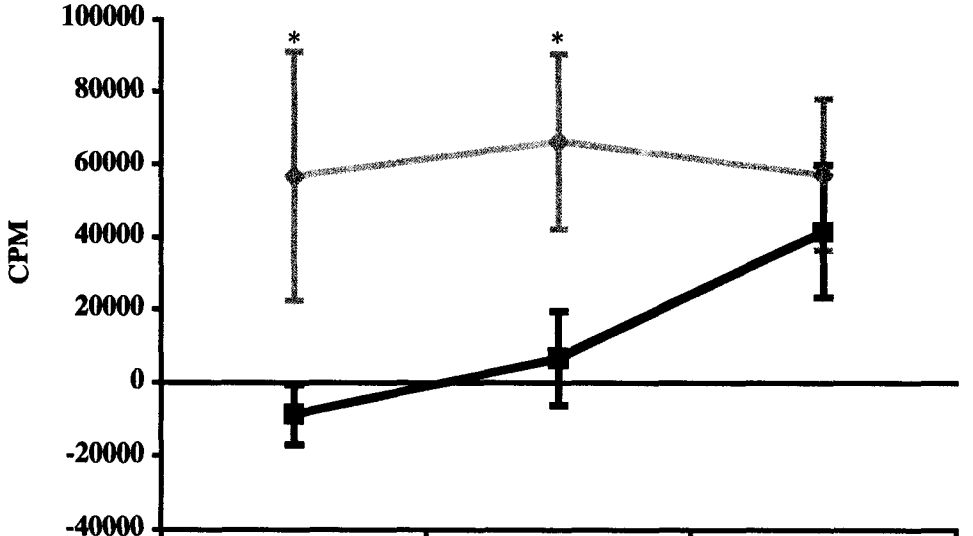


Fig. 1.2 A, B. Comparative phenotypic expression of feline DC vs feline MØ (MØ). (A) Data are presented as histograms of Ab labeling on large cell gated populations. (B) The average geometric mean fluorescence intensity (GMFI) ± SD of Ab labeling, combining at least four different experiments using four different animals. Asterisks (*) denote statistical differences in Ab expression between feline DC and MØ.



	1:30	1:60	1:120
DC	56803	66527	57293
MØ	-8584	6744	41617

cells. Cells were incubated for 8 days adding [^{3}H]-TdR for the final 12-16 h of culture. Data are expressed as the mean cpm ± SD at each DC: T cell ratio, subtracting background T cell and APC [^{3}H]-TdR uptake, and combining results from three separate experiments. Asterisks (*) denote statistical differences in stimulatory activity between feline DC and feline MØ (MØ).

In addition, IL-12 p40 mRNA expression in feline DC was on average 9-fold higher than that of MØ cultured in the presence of GM-CSF alone, as assessed by real time PCR (Fig. 1.4).

Endocytic Activity of Feline DC vs MØ

Immature DC take up antigen in a variety of ways and cellular uptake of FITC-DX occurs via receptor-mediated endocytosis. After 30 min at 37°C, FITC-DX fluorescence in feline DC was greater than in MØ cultured in the presence of GM-CSF. Moreover, there was even less FITC-DX uptake in MØ cultured without GM-CSF, amplifying the difference between DC and MØ (Fig. 1.5).

However, no statistical differences were found between DC and MØ cultured with (p = 0.114) or without GM-CSF (p = 0.0998) when comparing the GMFI of FITC fluorescence (Fig 1.6).

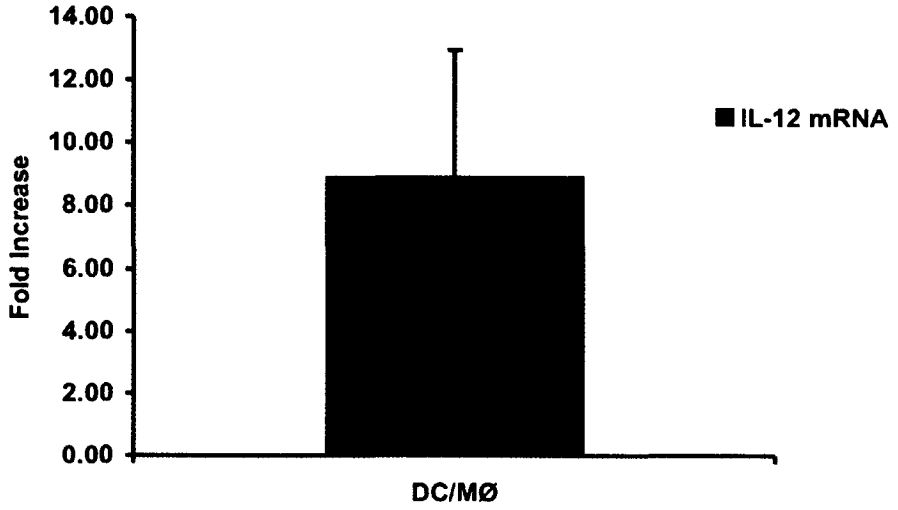


Fig. 1.4. IL-12 p40 mRNA expression in feline DC vs. feline MØ (MØ). DC or MØ were harvested after 7 days of culture. Cells were lysed, mRNA was extracted, and real-time PCR for IL-12 mRNA expression was performed. Data is expressed the fold increase in expression of IL12 mRNA in DC vs. MØ and is compiled from 4 cats.

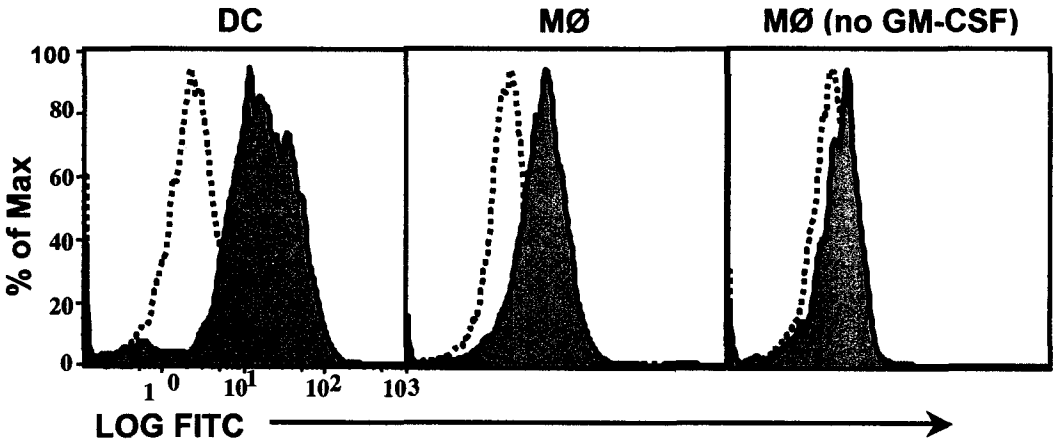


Fig. 1.5. Endocytic uptake of FITC-DX (10 µg/ml) of feline DC vs feline MØ (MØ), cultured with or without rhGM-CSF after 30 min at 37° C (orange filled area) versus 4° C (dotted lines). Washed cells were resuspended in flow buffer and fluorescence intensity of gated large cells was measured by flow cytometry. Data are from one animal but are representative of three experiments.

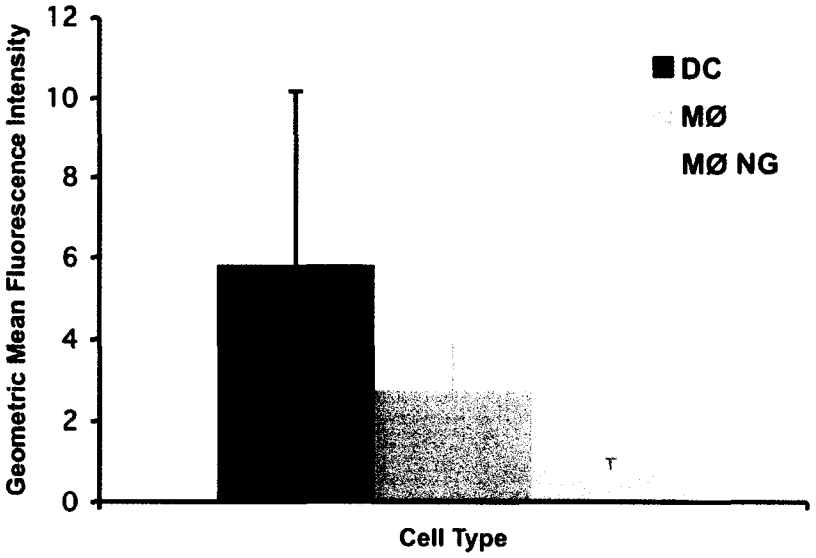


Fig 1.6. Geometric mean fluorescence intensities of FITC fluorescence comparing feline DC vs feline MØ cultured with (MØ) or without GM-CSF (MØ NG) in the FITC-dextran uptake assay. Data is presented as the average fluorescence + SD compiled from at least three different cats.

Attempts to detect maturation in DC cultures

Multiple different stimuli including LPS, CKT, MCM and Poly I;C (detailed in materials and methods) were employed in an attempt to mature feline DC without apparent success, i.e. no phenotypic or functional differences between treated vs. untreated immature DC were detected. Included for clarity is an MLR comparing untreated DC (IM DC) to treated DC (M DC) compiled from three different experiments using different maturation protocols (Fig. 1.7). In addition, we tested several maturation markers that define human and macaque DC, such as (Pharmingen), CD83 (Beckman–Coulter, Fullerton, CA), p55 (Dako, Carpinteria, CA), CCR7 (R and D sytems), however, these antibodies were not cross-reactive with feline DC.

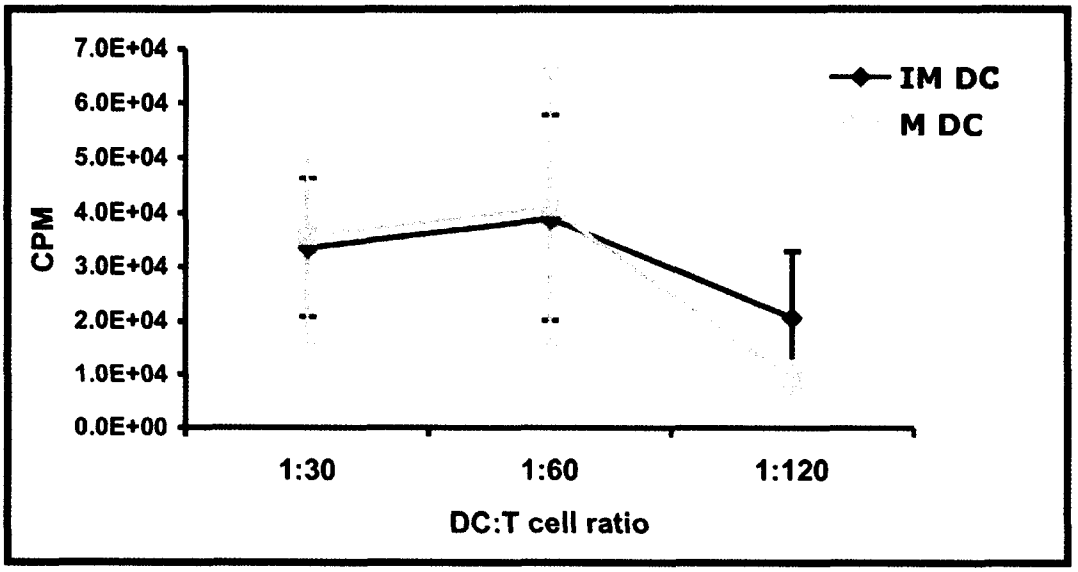


Fig. 1.7. Proliferative response of allogeneic T cells to day 7 feline untreated immature DC (IM DC) or maturation reagent treated DC (M DC) as shown by the MLR. Graded doses of IM DC or M DC were added to 2 x 10^5 T cells. Cells were incubated for 8 days adding [^{3}H]-TdR for the final 12-16 h of culture. Data are expressed as the mean cpm ± SD at each DC: T cell ratio, subtracting background T cell and APC [^{3}H]-TdR uptake, and combining results from three separate experiments. IM DC were treated with various maturational stimuli including Poly I:C, CKT and LPS. No statistical differences were seen between the groups.

DISCUSSION

Feline DC can be generated by culturing $CD14^+$ blood monocytes with hrIL-4 and GM-CSF, with methods similar to those used to culture human and macaque DC [13,18,19]. Feline IL-4 is now available commercially (R & D systems), but this study revealed no difference in culture results between feline and human IL-4 (data not shown). We previously demonstrated functional reactivity of human IL-4 with feline monocytes [20]. Likewise, Bienzle *et al.*, (2003) used human IL-4 to culture feline DC by methods that differed from those used here.

Feline DC cultured from $CD14^+$ monocytes resembled immature monocyte-derived DC of human beings and macaques [13,19] in having uniformly high MHC class II expression, moderate B7.1 expression and potent stimulatory function in the MLR, and in showing a moderate uptake of FITC-DX in the endocytic assay. CD14 is expressed on feline DC, as also observed in macaques and pigs [18,19,21-23]. CD11b, part of an adhesion molecule (CD11b/CD18, Mac-1) present on cells of myeloid lineage, is moderately expressed on feline DC as on immature myeloid DC of cats and other species [11-13,24]. CD1a expression on $CD14^+$ - derived DC appears to be lower than on feline DC cultured by adherence from PBMCs or bone marrow (BM) and similar to feline DC cultured in autologous plasma, which may reflect culture of different DC subsets or different culture conditions [11,12,25,26]. It is noteworthy that CXCR4, a chemokine receptor important in HIV-1 and FIV infections [27,28], is detectable on feline DC, as on more mature human and macaque DC [13,29]. This reflects either a difference between the feline and primate systems or that the feline DC are somewhat activated, resulting in higher CXCR4 expression.

MØ cultures (GM-CSF only) contained some non-adherent cells that resembled DC morphologically (data not shown). When placed on alcian-blue coated glass slides, however, these cells flattened and spread, revealing a morphology differing from that of non-adherent cells in DC cultures (GM-CSF + IL-4). Feline MØ and DC nonetheless shared similarities in expression of CD14, CD1a and CXCR4. Phenotypically, DC were distinguished by higher MHC class II and lower CD11b expression. B7.1 expression was similar on DC and MØ,

thus in accordance with observations on human DC and MØ cultured with GM-CSF with vs. without IL-4 [26]. Feline CD1a expression has been well characterized as a DC/Langerhans cell (LC) marker in feline tissue, with a distribution similar to that of its homologue in other species [30,31]. CD1a expression on MØ in this study was similar to that reported for human MØ cultured in the presence of GM-CSF [26]. The presence of CD1a may indicate that some DC-like cells can be cultured in GM-CSF alone, as has been demonstrated with human monocytes cultured with GM-CSF in serum-free media [32].

Feline DC were also distinguishable from MØ by functional studies. DC had a much greater capacity than GM-CSF-cultured MØ to stimulate an allogeneic MLR at DC:T cell ratios of 1:30 and 1:60. Surprisingly, at a ratio of 1:120 T cells, MØ appeared to be as stimulatory as DC. The suppressive activity of the MØ at higher ratios was both curious, and helpful in emphasizing the difference between DC and MØ in these cultures. Dose-dependent inhibition of allogeneic T-cell proliferation by MØ has been described [33,34] and shown to be mediated by exposure to MØ colony-stimulating factor (M-CSF) [35]. MØ produce the enzyme indoleamine 2,3-dioxygenase (IDO) when exposed to interferon (IFN)-γ and CD40L, which are produced by activated T cells [34]. IDO then catabolizes tryptophan, an essential amino acid required for T-cell cycle progression [36]. In addition, "upregulation" of M-CSF occurs in monocytes cultured in the presence of GM-CSF [37], as in the present study. M-CSF-stimulated MØ also produce cytokines (IL-1 and GM-CSF) that enhance the DC-mediated MLR[38]. The possible presence of some DC in our GM-CSF-stimulated

MØ cultures might explain the enhanced T-cell proliferation by MØ at the 1:120 ratio. At ratios of 1:30 and 1:60, this effect may be inhibited by dose-dependent MØ suppressive factors.

The higher relative IL-12 p40 mRNA expression in feline DC vs MØ is consistent with the MLR data. Basal IL-12 p40 mRNA expression in DC and monocytes is low [39-41], although, to our knowledge, no comparisons between MØ cultured with GM-CSF and DC cultured with GM-CSF and IL-4 have been reported. The higher expression of IL-12 mRNA in feline DC is consistent with these cells being well primed to prompt IFN-γ production by T cells and natural killer (NK) cells in response to pathogens [40,42].

Immature DC are highly specialized to capture antigen in a variety of ways. Feline DC have a much greater capacity than MØ cultured without GM-CSF to endocytose FITC-DX. This accords with reports for PBMC- and BM-generated feline DC and human monocyte-derived DC [2,11,43]. Feline MØ cultured with GM-CSF had an intermediate capacity to take up FITC-DX. Although there are no specific reports of increased endocytic capacity in MØ treated with GM-CSF, this might be expected because (1) GM-CSF upregulates the phagocytic capacity and phorbol 12-myristate 13-acetate (PMA)-induced H_2O_2 release of murine MØ[44], (2) GM-CSF upregulates FCγRII on MØ[26], and (3) GM-CSF augments monocyte phagocytic function against mycobacteria in patients with HIV-1 infection [45].

In conclusion, this study demonstrated that immature feline DC cultured from $CD14^+$ monocytes had characteristics similar to, albeit not identical with DC

in other species, and that many of these characteristics were phenotypically and functionally different from those of feline MØ. The methods described are of value for producing feline DC suitable for vaccine or immunotherapy studies on inducing cell-mediated immunity against infectious and neoplastic diseases. The study also demonstrated that MØ cultured with GM-CSF have stimulator:responder ratio-dependent suppressive and stimulatory effects in the MLR. This dichotomy of effects emphasizes the complicated nature of immunological regulation and provides an additional stimulus for studying the mechanism of immunosuppression in cats. In addition, if MØ at low concentration enhance DC stimulation in the MLR, as we propose, this might have important implications in situations where MØ and DC reside in low numbers relative to T cells, as in many mucosal and all lymphoid tissues.

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CHAPTER 2

SUSCEPTIBILITY OF DENDRITIC CELLS TO FELINE IMMUNODEFICIENCY VIRUS INFECTION

ABSTRACT

To elucidate the initial interaction of dendritic cells (DC) with feline immunodeficiency virus (FIV), we designed experiments to examine the *in vitro* susceptibility of DC to FIV with subsequent spread of infection to $CD4^+$ T cells. Using previously characterized feline monocyte-derived DC, we found that after pulsing DC with FIV, FIV DNA was detected at low concentrations after 7 days in culture in all samples. FIV DNA was amplified significantly when resting $CD4^+$ T cells were added immediately to FIV-pulsed and washed DC, however, amplification was intensified several log-fold if Con A and IL-2 activated $CD4^+$ T cells were added. In support of these findings, FIV p26 capsid antigen was detected in DC-activated $CD4^+$ T cell co-cultures with a steady increase in viral protein noted to 7 days post-infection, but was undetectable in the lesser infected DC or DC-resting $CD4^+$ T cell co-cultures. To further characterize the

mechanism by which DC transfer FIV infection to $CD4^+$ T cells, FIV-pulsed and washed DC were incubated for 2 days to allow for degradation of input virus and replication of new virus particles within DC before adding activated $CD4^+$ T cells for an additional 5 days. This procedure did not alter the ability of DC to infect activated $CD4^+$ T cells, suggesting FIV replication did occur in DC. Taken together, these studies demonstrate similarities between feline DC-FIV interactions and those in other species, and reaffirms the pivotal role for DC in mucosal immunodeficiency virus infections.

INTRODUCTION

Dendritic cells (DC) are ideally placed to play a central role in immunodeficiency virus infections and appear to be one of the first cells to encounter mucosally transmitted lentiviruses. Current evidence indicates that SIV is passed from DC to $CD4^+$ T cells, wherein virus amplification then occurs [1-4]. In HIV-1 and SIV, conjugates of cutaneous DC and T cells have been the site of virus amplification *in vitro* [5,6], and it has been demonstrated *in vivo* that HIV-1 replication is enhanced in syncytia derived from DC in lymphoepithelium of the tonsil and the T-cell rich adenoid mucosa [7,8]. Exactly how DC serve to amplify lentivirus infection is obviously important and thus remains the subject of current research. I*n vitro* studies involving exposure of DC to HIV-1 have yielded varying results, postulated to relate to: (1) different DC subsets used (i.e. blood vs. cultured DC), (2) different DC isolation methods (i.e. antibody

purification vs. metrimazide gradient), (3) different methods of viral detection (i.e. electron microscopy (EM) vs. PCR) and (4) different virus isolates [9-12]. What seems more clear is that, HIV-1 amplification occurs in DC-T cell conjugates [6,13,14]. Recent work has questioned whether HIV-1 and SIV are captured by vs. replicated in DC, and has demonstrated that lentivirus transfer to T cells occurs in two phases with an immediate transfer of virus via C-type lectin receptor endocytosis in immature and mature DC followed by a more efficient and stable transfer of virus by immature DC only that requires de novo virus replication in DC [15,16].

FIV is the feline counterpart of HIV infection, and in order to study the interaction of FIV with DC, we and others have cultured DC from feline monocytes and shown them to be similar to myeloid DC of other species [17-19]. Here we examine whether feline monocyte-derived DC can uptake, replicate, or transfer FIV to $CD4^+$ T cells. We report that FIV infection of DC is minimal, however, transfer and amplification of virus in activated autologous $CD4^+$ T cells is robust. We also provide evidence that de novo replication of FIV likely occurred in these DC, supporting our presumption that these cells are of an immature phenotype.

MATERIALS AND METHODS

Animals and sampling

Peripheral Blood Mononuclear Cells (PBMC) were isolated from blood of specific-pathogen-free (SPF) cats (age 6 to 24 months) from a colony maintained at Colorado State University (Fort Collins, CO) in accordance with Institutional Animal Care and Use Committee Regulations. Blood was collected from the jugular vein under ketamine/acepromazine anesthesia.

Generation of FIV supernatants

The cell-free virus inoculum (8.89×10^4 $TCID_{50}$/ml) was generated from co-culture of naïve feline PBMC with PBMC from a cat intravenously infected with FIV clade C. Infectivity of the supernatants was determined by titration and aliquots were frozen at -70°C.

Monocyte Isolation and DC Culture

Dendritic cells (DC) were cultured from $CD14^+$ monocytes as described in chapter one. Since purity of DC cultures was needed for the FIV infection studies, 6-7 day old DC were harvested and positively selected from contaminating lymphocytes using magnetic columns (Miltenyi Biotec, Auburn, CA) and a cross-reactive canine CD11c monoclonal antibody (MAb) (Leukocyte

Ag Laboratory, University of California, Davis). Briefly, cells were blocked with 200µl goat serum (Sigma-Aldrich), washed with PBS + 0.5% BSA and incubated with CD11c Ab (20µl /10^6 cells) for 20 min. at 4°C. Cells were washed again with PBS + 0.5% BSA and incubated with goat α-mouse beads (20µl/10^6 cells) and placed over a magnetic column according to manufacturer's instructions (MS column, Miltenyi Biotec). $CD4^+$ T cells were isolated from the negative fraction of the $CD14^+$ selection and incubated with phycoerythrin (PE) conjugated anti-feline CD4 (Southern Biotec) at 0.5 µl/10^7 cells for 20 min at 37 °C. Cells were washed and incubated with anti-PE beads (Miltenyi Biotec) according to manufacturer's directions and placed over a magnetic column (LS, Miltenyi Biotec). The positive fraction was collected, cells were counted and then either frozen in liquid nitrogen for resting $CD4^+$ T cells or were cultured in complete RPMI in the presence Concanavalin A (Con A; 5µg/ml) and IL-2 (100U/ml) for activated $CD4^+$ T cells. Con A was added immediately to the cultures and replenished every 2 – 3 days with one half media changes and IL-2 (100U/ml) was added after the first 3 days and replenished with media changes.

Flow Cytometry

Phenotypic expression of $CD11c^+$ purified feline DC was evaluated by flow cytometry as described in chapter one. DC purity was assessed using anti-feline CD4, anti-feline CD8 (Southern Biotec) and a cross-reactive anti-human CD21 (Pharmingen). Isotype-matched mouse Igs were used as controls. Cells were resuspended in flow buffer (PBS + 0.5% NaAz + 2% FBS), propidium

iodide was used to exclude dead cells from analysis and gating of large cells for analysis was determined by scatter properties using a Coulter Counter (EPICS XL-MCL; Beckman Coulter, Miami, FL). Data was analyzed with FlowJo™ software (Tree Star, Inc., San Carlos, CA).

Inoculation of DC with FIV

Between 3 – 7 x 10^5 DC were placed into 15ml centrifuge tubes (BD Falcon, Franklin Lakes, NJ) in 500µl complete RPMI. Cells were infected with 8-9 x 10^3 50% tissue culture infective doses ($TCID_{50}$). DC were incubated for 2 hours at 37°C and then washed 4 times to remove unbound virus. DC were cultured at 6.0 x 10^5 cells/ml in 200µl complete RPMI in 96-well flat-bottomed plates (BD Falcon) for an additional 7 days in the presence of hrGM-CSF (1000U/ml) and hrIL-4 (1000U/ml). To some wells $CD4^+$ resting T cells or activated $CD4^+$ T cells were added immediately. In some experiments, DC were re-cultured for 2 days before adding activated $CD4^+$ T cells to assess if infection was from de novo FIV replication within DC. One-half of the media was changed and cytokines were replenished every other day during the culture period.

Real-time DNA PCR for FIV

DNA was extracted from DC or DC-T cells after 7 days in culture using a QIAamp Mini Kit (Qiagen Inc., Chatsworth, CA). Quantitative real-time PCR (qPCR) was performed using previously published FIV $C_{PGammar}$ primers and

probe sequences [20] and an iCycler iQ™ (Bio-Rad). qPCR was performed using the 2X TaqMan Universal PCR Master Mix (Applied Biosciences, Foster City, CA) consisting of 10mM Tris-HCl (pH 8.3), 50mM KCl, 5mM MgCl2, 300uM each of dATP, dCTP, and dGTP, 600uM dUTP, 0.625U of AmpliTaq Gold DNA polymerase, and 0.25U uracil N-glycosylase (UNG) per reaction. Reactions were in a total of 25µl and consisted of 12.5µl Mastermix, 5µl of sample or standard, 400nmM of each primer and 80nM of probe. Thermal cycling conditions were 2 minutes at 50°C to induce enzymatic activity of UNG, 10 minutes at 95°C to reduce UNG activity and to activate AmpliTaq Gold DNA, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. A standard curve was generated with 10-fold serial dilutions of FIV C gag plasmid DNA with 1X TE and 40 ng/ml salmon testes DNA (Sigma-Aldrich) as a DNA carrier for each run. Calculation of FIV C DNA viral copies was calculated from these curves and statistical analysis was performed on paired samples using the Wilcoxon signed-rank test using Prism version 4.0b software for Macintosh, copyright 2004 (GraphPad, Inc. San Diego, CA).

FIV p26 enzyme-linked immunosorbent assay

Productive infection of DC or DC-T cell co-cultures was assessed by the FIV capsid antigen (p26) capture enzyme-linked immunosorbent assay (ELISA) described by Dreitz et al. [21]. Optical densities, measured by absorbance at 450 nm, were recorded with a Dynatech 5000MRTM microplate reader (Dynatech Corp., Chantilly, VA.). A minimum optical density of 0.1, that was at least twice

the value of negative control supernatants run in parallel, defined the positive reactions. Data was compiled from at least seven different experiments using at least 5 different animals. Statistical analysis was performed using the Student's *t* test.

RESULTS

DC purification for infection studies

DC recovery using $CD11c^+$ selection was approximately 51% ± 10% and purity was between 97 – 99.5% as measured by scatterplot gating and lack of $CD4^+$ and $CD8^+$ staining for T cells and CD21 staining for B cells (Fig 2.1).

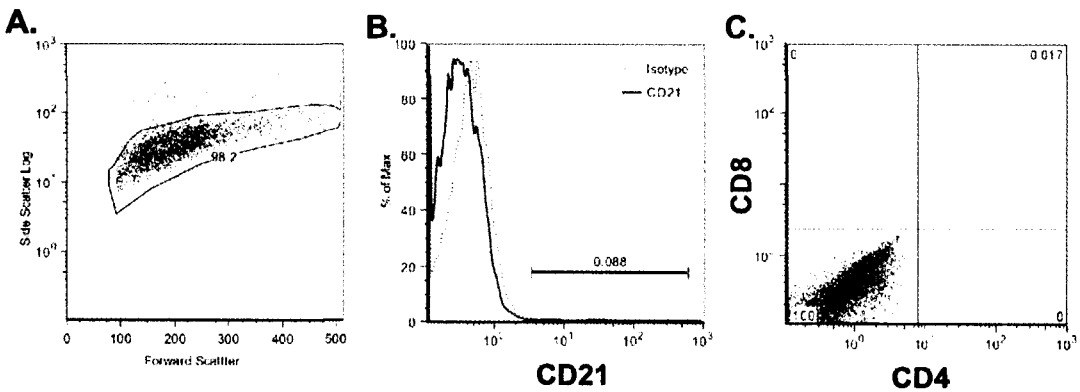

Fig 2.1. Scatterplot (A) demonstrates relative purity in size and complexity of cells. No CD21 (B), CD4 or CD8 (C) expression was found on purified feline DC. Results are from one animal and are representative of at least 10 different experiments.

FIV DNA loads in DC and DC-$CD4^+$ T cell co-cultures

FIV DNA loads were lower in 7-day cultured FIV-pulsed DC, than in paired co-cultures in which $CD4^+$ resting T cells were added to FIV–pulsed DC

(p = 0.0537). When activated $CD4^+$ T cells were added immediately to FIV pulsed and washed DC, viral DNA increased several log fold over cultured DC alone (p = 0.0156) or co-cultured DC plus resting $CD4^+$ T cells (p= 0.0156) (Fig. 2.2).

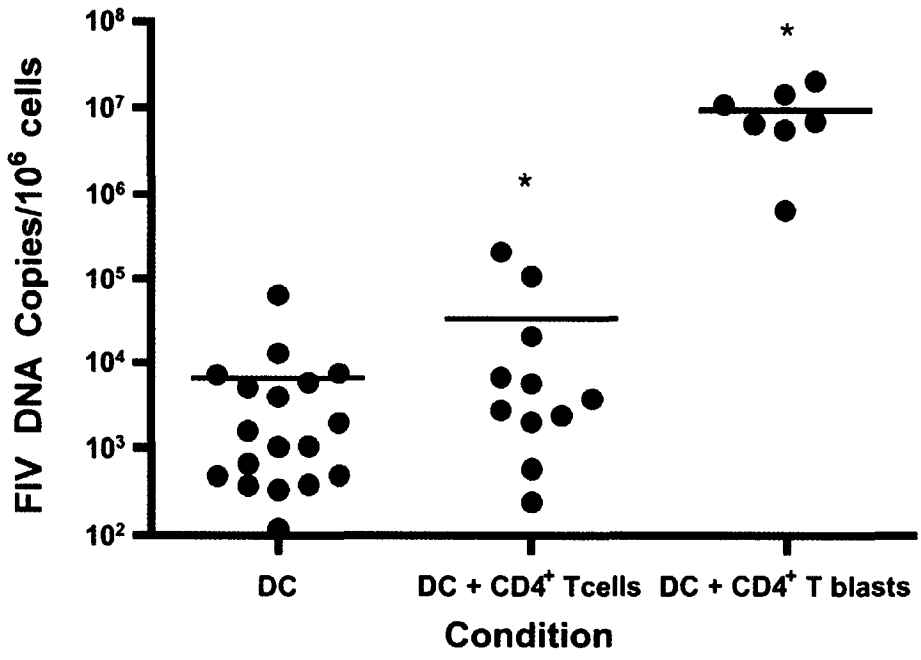

Fig 2.2. Comparison of FIV viral DNA loads in FIV-pulsed DC cultured alone, with resting $CD4^+$ T cells or with Con A / IL-2 activated $CD4^+$ T cells ($CD4^+$ T blasts) for 7 days at 37°C. Asterisks (*) denote statistical differences between DC cultured alone and DC-$CD4^+$ T cell co-cultures.

FIV p26 capsid antigen (Ag) correlates with viral DNA

FIV p26 capsid Ag in supernatants harvested from DC cultures and DC-$CD4^+$ resting T cell co-cultures could not be detected by capture ELISA (Fig. 2.3). Only when Con A and IL-2 activated $CD4^+$ T cells were added to the DC was Ag detected, thereafter a steady increase in viral protein was noted through 7 days post-infection. This data correlates with the several log-fold lower FIV

DNA viral loads in DC and DC-CD4$^+$ resting T cell co-cultures compared with DC-activated CD4$^+$ T cell co-cultures. Statistically significant differences distinguished FIV protein expression in DC cultures (5 days post-infection (PI) $p = 0.0302$; 7 days PI, $p < 0.0001$) and DC-CD4$^+$ resting T cells co-cultures (5 days PI, $p = 0.0353$; 7 days PI, $p < 0.0001$) vs. DC-activated CD4$^+$ T cell cultures.

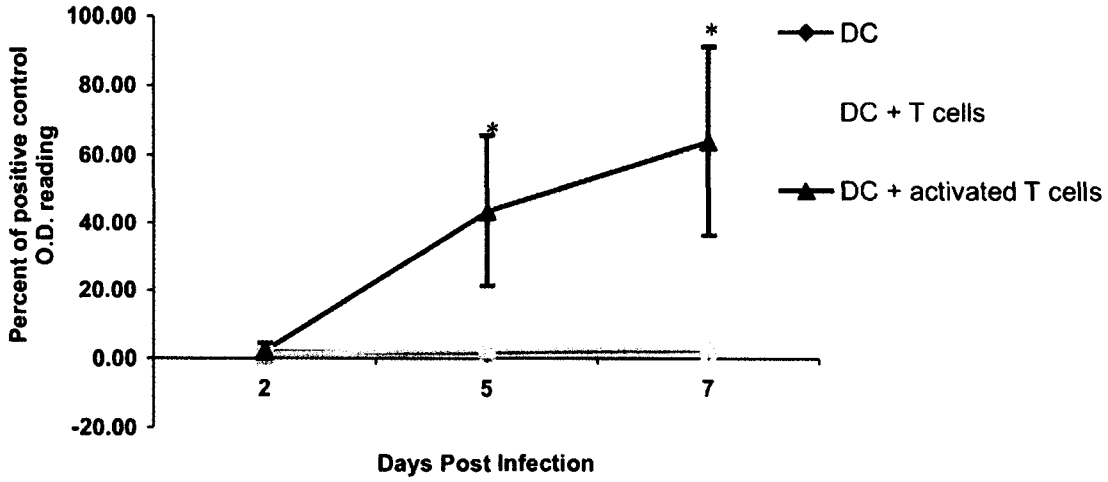

Fig. 2.3. FIV p26 capsid Ag in culture supernatants of FIV-pulsed DC cultured alone, with resting CD4$^+$ T cells (T cells) or with Con A and IL-2 activated CD4$^+$ T cells (activated T cells). Data are presented as the averages $\pm$ SD from at least 7 compiled experiments using at least 5 different animals. Asterisks (*) denote statistical differences between DC cultured alone and DC-resting CD4$^+$ T cell co-cultures vs. DC-activated CD4$^+$ T cell co-cultures.

DC infection of activated CD4$^+$ T cells arises from de novo FIV replication in DC

Infectious lentivirus does not survive more than 24 hours[16] *in vitro*. Therefore, to determine if transfer of infection was induced by de novo replication of FIV within DC, FIV-pulsed and washed DC were cultured for an additional 48 hours prior to adding activated T cells. As seen in figure 2.4,

amplification of viral DNA and gag p26 was still apparent when Con A / IL-2 stimulated T cells were added 48 hours after initial FIV-DC exposure, strongly suggesting that de novo replication of FIV occurred. Amplification of replication was not as marked as when activated $CD4^+$ T cells were added immediately (Fig. 2.2), however, the decreased time of co-culture (5 days vs. 7 days) could explain this discrepancy. Statistical analysis revealed a trend toward significance (p = .0625) between DC cultured alone or with activated $CD4^+$ T cells.

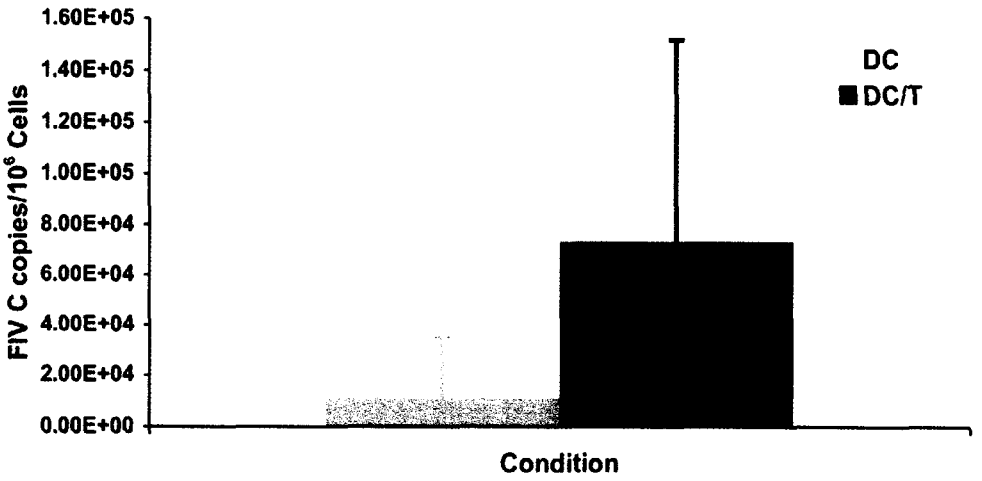

Fig 2.4 FIV viral DNA amplification in DC cultures or DC-activated $CD4^+$ T cell co-cultures using real-time PCR. DC were re-cultured for 2 days before adding the T cells and cultures then harvested 5 days later. Data is presented as the average + SD from 3 different experiments.

FIV p26 capsid antigen is undetectable, but correlates with low FIV DNA levels

FIV p26 capsid Ag was undetectable in culture supernatants, which correlated with the lower viral DNA loads seen in these samples compared to

the samples in which activated $CD4^+$ T cells were added immediately to FIV-pulsed DC.

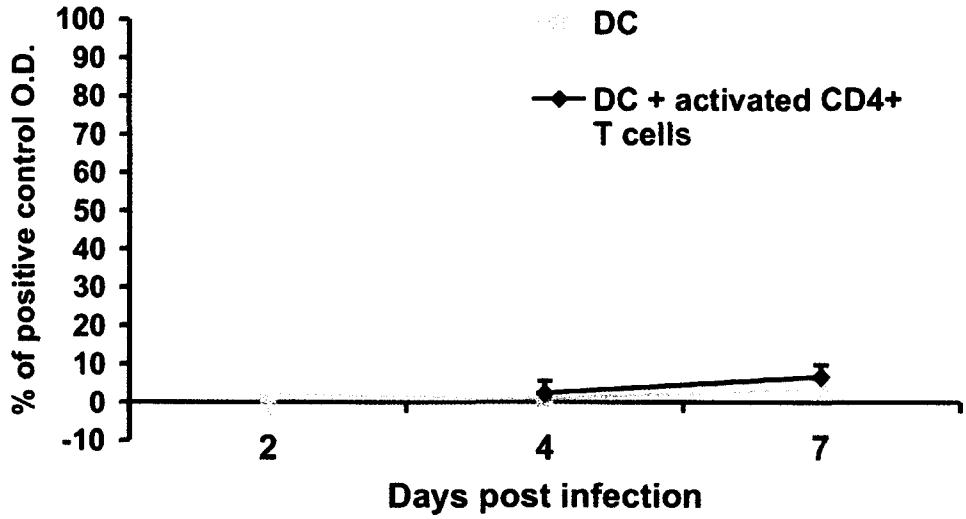

Fig. 2.5. FIV p26 capsid Ag in culture supernatants of FIV-pulsed DC cultured alone or with Con A and IL-2 activated $CD4^+$ T cells. DC were pulsed with FIV, washed and then re-cultured alone 2 days before addition of activated CD4+ T cells. Data is presented as the average $\pm$ SD from 3 different experiments.

DISCUSSION

The exploitation of DC by lentiviruses is likely the pivotal event in the undermining of the immune response by lentiviruses[16,22-25]. In this study, we examined the susceptibility of feline DC to FIV infection, as a comparative model of lentivirus infections. The low to moderate viral DNA loads found in DC exposed to FIV for 2 hours, then washed and re-cultured for 7 days parallel results seen in immature human DC infections with HIV-1 [16,26]. In a few of the animals DC viral DNA loads were higher than expected. This could have been

due to lymphocyte contamination, however, lymphocytes were not appreciably detected by flow cytometry or light microscopy. Likewise, these instances of apparent greater DC FIV susceptibility may also represent individual variation among cats. Measurement of viral DNA immediately after pulsing DC with FIV or after re-culture of DC for 2 days would be helpful in determining whether the low levels of viral DNA were truly evidence of virus replication, however, due to the limited number of DC available, these experiments were not performed. When resting $CD4^+$ T cells were added to the FIV-exposed and washed DC followed by co-culture for 7 days, slight amplification of viral DNA was detected. While it has been shown that highly purified resting $CD4^+$ T cells cannot be infected with HIV-1 *in vitro* [27,28], modest enhancement of virus replication was noted in DC-$CD4^+$ T cell co-cultures[29]. In addition, it has been shown that IL-4, which was added to our co-cultures, enhances replication in both purified resting $CD4^+$ T cells and in DC-$CD4^+$ T cell co-cultures [29,30]. The marked amplification of viral DNA loads observed following co-culture of activated $CD4^+$ T cells with FIV-pulsed DC correlates with previous *in vitro* and *in vivo* studies demonstrating amplified HIV and SIV production in activated $CD4^+$ T cells [4,12,13].

The lack of detectable FIV capsid protein and low to moderate viral DNA levels in DC cultured alone and in resting $CD4^+$ T cell-DC co-cultures is consistent with the results of a study by Messmer et al. demonstrating the absence of SIV p27 Ag in immature DC cultured alone and lack of detectable levels in resting $CD4^+$ T cell-immature DC co-cultures until cells had been in culture for more than 7 days [31]. The low viral DNA levels in the resting $CD4^+$ T

cell-DC co-cultures could reflect lack of viral DNA integration into genomic DNA [28] and/or a block in reverse transcription, phemomena demonstrated in HIV-1 infection of resting $CD4^+$ T cells [27]. Blocks in reverse transcription could be due to the presence of the enzymatically active, low-molecular-mass cytidine deaminase, APOBEC3G, that has been shown to inhibit HIV-1 infection in cells by a yet undetermined cytidine deaminase-independent mechanism [32], and/or the presence of Murr1, a gene product that was shown to inhibit HIV-1 replication in resting $CD4^+$ T cells [33]. FIV p26 capsid Ag was detected by 5 days after addition of activated $CD4^+$ T cells to DC cultures. This result was as expected, since IL-2-stimulated cellular activation and proliferation was shown to be necessary for productive HIV-1 infection [27,29,34].

The means by which DC transfer infection to T cells remains controversial. In order to test the possibility that the transfer of FIV from feline DC to $CD4^+$ T cells was by uptake of DC-replicated or endocytosed virus particles vs. transfer of input virus bound to or endocytosed by DC, we re-cultured FIV-pulsed DC for 48 hours before addition of activated $CD4^+$ T cells. FIV infection of $CD4^+$ T cells remained unobstructed and productive infection ensued. And although FIV DNA levels were lower and p26 capsid Ag was undetectable in these DC cultures compared to those in which activated $CD4^+$ T cells were added immediately, correlation may be compromised by the shorter DC-T cell co-culture time when addition of $CD4^+$ T cells was delayed. While it is not known with certainty that the originally pulsed virus was completely degraded in the 48-hour time period, de novo replication of FIV in these

immature DC presents the most likely scenario for viral transfer to $CD4^+$ T cells [16]. In addition, we attempted to block infection using the reverse transcriptase inhibitor, zidovidine (AZT), however, results were not interpretable due to toxicity of DC when AZT was used at anti-viral concentrations. Our provisional conclusions are that immature feline DC transfer FIV infection to $CD4^+$ T cells by immediate transfer of virus via exosomes and/or through endolysosomal pathways and that delayed transfer of virus occurs via de novo viral replication in DC, thus revealing parallel observations with HIV and immature human DC [16,25].

In summary, we demonstrate that FIV is able to capitalize on initial DC-$CD4^+$ T cell interactions to transmit infection to amplifying $CD4^+$ T cells, consistent with observations made with HIV-1 and SIV. While it may not be surprising that the interactions between different species immune deficiency viruses with the innate immune system would be similar, that these interactions are conserved illustrates the importance of understanding the mechanisms at work in this early stage of pathogenesis. That these lentiviruses are remarkably efficient in transferring infection to $CD4^+$ T cells after DC capture reinforces concentration on mucosal vaccination and microbicide strategies to containing and prevent these persistent and deadly infections.

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CHAPTER 3

AT-2 INACTIVATED FIV-PULSED DENDRITIC CELLS FAILED TO PROTECT CATS AGAINST INFECTIOUS FELINE IMMUNODEFICIENCY VIRUS CHALLENGE

ABSTRACT

To gain insight into the ability of monocyte-derived dendritic cells (DC) to induce a prophylactic vaccine, we designed a pilot study in which DC were pulsed with 2,2'–dithiodipyridine (aldrithiol-2, AT-2) inactivated feline immunodeficiency virus (FIV) and then injected subcutaneously. Since these dendritic cells were considered to be of an "immature" phenotype, *in vitro* tested synthetic cytosine-phosphate guanosine dinucleotides (CpG ODN) were used as an adjuvant to induce maturation *in vivo*. All cats were susceptible to FIV challenge and no differences in FIV viral DNA loads were seen between the groups. Hematology pre- and post-challenge was similar and antibody responses were similar between the groups. Pre-challenge proliferative and interferon-gamma (IFN-γ) responses to AT-2 inactivated FIV *in vitro* were undetectable suggesting the lack of a systemic response to the inactivated virus.

In conclusion, DC vaccination using the protocol described did not provide protection from infectious FIV challenge.

INTRODUCTION

Over 40 million adults and children currently live with HIV/AIDS and approximately 4.9 million new HIV infections were documented in 2005 [1]. Development of an effective HIV-1 vaccine has been stymied by failure of the immune response to effectively contain and extinguish lentivirus infections such as HIV-1. Dendritic cells (DC) are critical to initial antigen processing and T cell activation during the early immune response [2,3]. There is evidence that mucosal DC (i.e. Langerhans' cells) are the initial cells infected by SIV. DC traffic SIV from the mucosa to the nearest lymphoid organ, where infection may amplify and disseminate [4] or possibly as in some cases of HIV-1 infection, can be contained before even an antibody response can be generated[5]. Therefore, it appears likely that DC are pivotal in early viral immune containment vs. viral replication and dissemination.

Given the above, a vaccine strategy centered on education of DC to initiate a potent early immune response would seem logical, if not ideal. In fact, immunity stimulated by DC-based vaccination strategies in mice have protected against some neoplastic and infectious agents [6,7]. In addition, SIV-pulsed DC, when re-infused into macaque donors, elicited immune responses and

decreased viral burden after SIV challenge [8]. A similar response was elicited by HIV-1-pulsed DC in chronically infected humans [9].

The most critical questions regarding the role of DC in immunity to HIV-1 infection can only be addressed with *in vivo* immunization/challenge studies. FIV, a lentivirus of cats that induces similar infection kinetics and clinical disease course to HIV-1 [10-13], was used in this study.

2,2'-dithiodipyridine (aldithiol-2 [AT-2]; Aldrich, Milwaukee, WI) chemically inactivates retroviruses by modification of internal proteins with conformational and functional preservation of viral surface proteins including the immunogenic envelope proteins [14,15]. Vaccination with non-adjuvanted AT-2 inactivated-SIV induced humoral immunity and suppressed RNA viral copy numbers after homologous, but not heterologous infectious challenge [16]. In addition, DC pulsed with AT-2 inactivated-SIV have induced $CD4^+$ and $CD8^+$ immune reponses *in vitro* [17]. We have, therefore, employed this methodology to assess immunogenicity of inactivated FIV when presented by dendritic cells.

Bacterial cytosine-phosphate guanosine oligodinucleotides (CpG) are unmethylated motifs of bacterial DNA that induce potent immune responses in vertebrates. Synthetic CpG oligodinucleotides (CpG ODN) have been developed as vaccine adjuvants with promising results. Immature DC injected with synthetic CpG ODN have shown upregulation of costimulatory molecules and amplified production of IL-12 *in vivo*, i.e., demonstrating a maturation phenotype [18]. Furthermore, vaccine studies in mice using synthetic CpG as adjuvants have shown heightened immune responses [19,20] to HIV-1 that protected mice against

mucosal viral challenge [21,22]. Therefore, we hypothesized that CpG would mature feline DC *in vivo* and enhance specific immune response to FIV DC vaccination. Here we report our initial pilot study using this model to assess the efficacy of antigen-primed DC in generating immunity against FIV infection.

MATERIALS AND METHODS

Animal groups and sampling

Six-month old specific pathogen-free cats were maintained at Colorado State University and bled every four weeks for culture of dendritic cells, as described in Chapter one methods. Three groups of three cats received subcutaneous inoculations of either: (1) DC pulsed with AT-2 inactivated FIV, (2) DC pulsed with PBS + 1% BSA, or (3) AT-2 inactivated FIV without DC four times prior to infectious FIV challenge. Subcutaneous inoculations were chosen due to successful induction of immune responses in macaques and humans inoculated with Ag-pulsed DC subcutaneously [8,9,23,24]. All animals also received CpG ODN as an adjuvant to try and mature DC *in vivo* through activation of plasmacytoid DC. Subcutaneous FIV challenge occurred two weeks after the last DC inoculations followed by hematology analysis and viral infection monitoring every two weeks until termination of the study at 10 weeks post challenge.

Cell Isolation

PBMC were isolated over a ficoll gradient and $CD14^+$ cells were positively selected as described in chapter one. CD14-depleted PBMC were used for proliferation and enzyme-linked immunospot (ELISPOT) assays prior to challenge.

Virus inactivation and infectivity monitoring

AT-2 inactivation of FIV was performed by Dr. Jeffrey Lifson (NCI, Bethesda, MD) as published for HIV [14]. Co-culture of naïve PBMC to assess FIV inactivation was performed in our laboratory. Con A stimulated cells were plated at a density of 1×10^6 cells/well in a 24-well plate. AT-2 inactivated FIV B-2542 was added at the following dilutions: 1:2, 1:4, 1:10, 1:20, 1:200, and control (naïve supernatant). Naïve PBMC or media were added bi-weekly and supernatants were collected twice weekly for four weeks. An FIV p26 Ag capture ELISA was used to assess the presence of viral protein.

DC pulsing with inactivated FIV

DC pulsing with AT-2 inactivated FIV was adapted from published methods in macaques [17]. Briefly, 7-day cultured "immature" DC were incubated with 5µg/ml of AT-2 inactivated FIV viral protein for 1 hour at 37°C, washed once with PBS and injected subcutaneously. Control DC were pulsed with PBS + 1% BSA under the same conditions. The concentration of virus used was

extrapolated from previously published work in SIV ([17] and personal communication, Melissa Robbiani).

In vitro CpG testing prior to in vivo assays

A novel type C CpG ODN (C274) able to stimulate B cells and induce both interferon (IFN) -γ and IFN-α production in human and macaques was provided by Dr. J. Marshall [25-27]. CpG-C ODN C274 (5-TCGTCGAACGTTCGAGATGAT) or control non-CpG ODN C661 (5-TGCTTGCAAGCTTGCAAGCA) were tested for functionality by proliferation of PBMC or purified B cells and by induction of IFN-α and IL-12 mRNA expression.

B cell isolation included Fc receptor blockage with goat serum (Sigma), receptor binding with cross-reacting anti-human CD21 Ab (BD/Pharmingen) and antibody (Ab) binding with goat anti-mouse beads (Miltenyi Biotec). All incubations were 20min at 4°C, followed by washes with PBS + 0.5% bovine serum albumin (Roche Diagnostics, Indianapolis, IN) and positive selection with magnetic columns according to manufacturer's instructions (Miltenyi Biotec).

PBMC (2×10^5/well) or B cells (2×10^4/well) were added to 96-well plates (Falcon) with 200µl RPMI-10. CpG (C274) or non-CpG (C661) ODNs were added in triplicate at optimally determined concentrations of 2, 4, and 8µg/ml. RPMI-10 and Con A (5µg/ml) were added as negative and positive controls, respectively. Cultures were incubated for 3 days at 37°C with addition of 1µCi of ^{3}H-Thymidine ([^{3}H]-TdR)/well) for the final 18-24 h of culture as determined

optimal by earlier experiments (data not shown). Culture duration Proliferation was measured by [^{3}H]-TdR uptake as quantified with a Wallac 1450 Microbeta liquid scintillation counter (PerkinElmer Life Sciences) and statistical analysis was performed with the student's *t*-test. Results are presented as the mean of two (PBMC) to three experiments (B cells) plus the standard deviation among experiments.

For feline IFN-α and IL-12 real-time PCR mRNA assays, previously published primers were used [28,29]. 4 X 10^6 PBMC were treated with 8μg/ml CpG or non-CpG ODN for 12 hours at 37°C. RPMI-10 was used as a negative control. Cells were harvested, mRNA was extracted and cytokine real-time PCR was performed as described in chapter one. Relative quantification of IL-12 and IFN-α was determined by the $2^{-\Delta\Delta C_T}$ method [30], comparing C274 treatment to medium or C661 treatment. Glyceraldehye-3-phosphate dehydrogenase (GAPDH) was used as a recorder gene to normalize IL-12 and IFN-α signals and macrophages were treated as controls. Results were compiled from two different experiments with 4 and 5 different animals for IL-12 and IFN-α, respectively.

FIV Real-Time DNA PCR:

Real-time DNA PCR for FIV DNA viral loads was performed on DNA extracted from DC-T cell co-cultures after FIV inactivation and on feline PBMC using methods operative in our laboratory modified from Leutenegger, et al. [31]. Primers specific for clade FIV-B and an iCycler iQ™ (Bio-Rad) were used and PCR was performed as described in Chapter 2. Input viral DNA was

extrapolated from an FIV plasmid DNA standard curve and viral copies were calculated as FIV copies/10^6 PBMC using the published formula [31]:

$$\text{Copy number}/10^6 \text{ PBMC} = \frac{SQ^* \times 10^6}{(\mu g/\mu l \text{ DNA} \times 5\mu l)(10^6 pg/\mu g)/(6pg \text{ DNA/cell})}$$

(*SQ = copy number generated from the standard curve)

FIV antibody ELISA

FIV Ab was determined using a whole FIV ELISA [32,33]. Pelleted FIV (740ng/well) was diluted in 0.01M Borate buffer with 0.5% deoxycholic acid and adsorbed in 96-well microtiter plates at 4°C overnight. Plates were washed with NTE buffer (.05 M Tris, 0.001M EDTA, .05 M NaCl, 0.2% Tween-20, 20.45g/L NaCl) and blocked with NTE buffer plus 2% dried milk for 2 hours at room temperature. Plates were washed again and stored dessicated at 20°C. Heparinized plasma samples were serially diluted in duplicate in 100µl of ELISA Ab Diluent (NTE buffer, 2% bovine serum albumin (BSA), 5% FBS, 0.5% Triton X-100) for 45 minutes. Peroxidase conjugated goat anti-cat IgG (Cappel, Organon Teknika Corp., Durham, NC) was diluted 1:5000 in ELISA Ab Diluent and added at 100µl per well for 30 minutes for detection of Ab. Plates were developed with 3, 3' 5, 5' tetramethylbenzidine (Kirkegaard & Perry Laboratories, Inc.) for 10 minutes, reactions were stopped with H_2SO_4 and optical densities (OD) were performed at 450nm. Titers were expressed as the inverse of the highest plasma dilution that produced an optical density greater than or equal to twice that of naïve SPF cat

serum. Validity of Ab titers was confirmed by comparing OD readings from Ab + background to Ab – background [34]. Using linear regression, slopes of the lines were generated and compared (Excel 2004 for Mac, version 11.0). If the difference between the slopes of the lines (ΔS) was less than 0.1 then the lines were considered equal and the result was considered valid [31].

FIV-specific T cell proliferation

FIV-specific T cell proliferation was determined after *in vitro* stimulation with AT-2-inactivated virus on pre-challenge vaccinated cat CD14-depleted PBMC. CD14-depleted PBMC were used due to constraints of available blood. Briefly, $CD14^-$ PBMC were plated at 2×10^5 cells/well and exposed to 2.5μg/ml or 5μg/ml AT-2-inactivated FIV. Three-day cultures were pulsed with 1μCi [^{3}H]TdR during the last 18-24 hours, and [^{3}H]TdR incorporation was quantified using a beta counter (Wallac). Alternatively, PBMC were exposed to media with no virus to rule out stimulation by cellular proteins. Proliferation indices were calculated as proliferation of AT-2 treated wells over media-treated wells. To determine optimal timing and concentration for proliferation studies, PBMC from FIV infected cats were used. Proliferation responses and proliferative indices of 3 vs. 6 day-cultured PBMC were compared and 1μCi [^{3}H]TdR was added to cultures during the last 18-24 hours.

Interferon-γ Enzyme-Linked Immunospot Assay

Secretion of IFN-γ by FIV-specific $CD8^+$ T cells was measured using the feline IFN-γ enzyme-linked immunospot (ELISPOT) assay (R and D systems) on pre-challenge vaccinated cats. 2×10^6 $CD14^-$ PBMC were incubated with either 5.0μg/ml or no AT-2-FIV in 1ml RPMI-10 for 24 hours at 37°C. Cells were harvested, washed and $CD8^+$ T cells were enriched using the following protocol. Cells' Fc receptors were blocked with goat serum (Sigma), receptors were labeled with anti-feline CD4 (Serotec) and anti-human CD21 (BD/Pharmingen) and Ab labeling was bound with goat anti-mouse beads (Miltenyi Biotec). The $CD8^+$ fraction was seeded at a concentration of 1×10^5 $CD8^+$ T cells/well in 200μl media. T cell response capacity was confirmed with 5ng/ml stapylocoocal enterotoxin B (SEB; Toxin Technology, Sarasota, FL) and background response was monitored with media alone. Cells were incubated for 24 hours at 37°C and the assay was developed according to manufacturer's instructions (R and D systems). IFN-γ spot forming cells (SFCs) were enumerated using an inverted light microscope.

Hematology and Lymphocyte Subset Determination

White blood cell (WBC) counts were determined on the Coulter counter (Beckman Coulter) and lymphocyte concentrations were calculated from manual differential counts. Packed cell volumes (PCVs) were determined by standard methods. T cell subset analysis was performed using established methods and feline CD4 and CD8 monoclonal antibodies [35]. Subset percentages were

determined using a Coulter flow cytometer (EPICS XL-MCL) and absolute counts were calculated from the total lymphocyte counts.

DC and CpG Vaccination

Between 1.9 x 10^5 and 4.4 x 10^6 AT-2 inactivated FIV or PBS + 1% BSA pulsed DC and 150μg CpG C274 were admixed, divided and injected subcutaneously. Injections were given into the right or left dorsoscapular and lumbosacral regions, rotating sides every four weeks. Cats receiving AT-2 inactivated FIV + CpG without DC received 100μg of viral protein, a concentration reported to be immunogenic in an SIV vaccine trial [15].

Mucosal FIV Challenge

Two weeks after the last DC vaccination, cats were inoculated by subcutaneous exposure with 0.5ml containing 10 animal infectious doses (AID) of cell-free infectious FIV (clade B, 2542 strain).

Statistical Analyses

Statistically significant differences in proliferative responses or mRNA cytokine expression induced by stimulatory versus non-stimulatory CpG or no CpG was determined using the Student's t-test. Statistical analysis comparing hematology parameters, antibody production and DNA viral between the three different vaccine groups was measured by repeated-measures analysis of

variance (ANOVA) two-way comparisons and Bonferroni post hoc tests. Statistical significance was considered to have occurred when a *P* value was <0.05. All statistical analyses were performed using Prism version 4.0b software for Macintosh, copyright 2004 (GraphPad, Inc. San Diego, CA).

RESULTS

FIV Inactivation by AT-2

Inactivation of FIV was assessed by virus-PBMC co-culture followed by FIV p26 capsid Ag ELISA (data not shown). After co-culture for 28 days, no FIV p26 signal could be detected.

Effect of type C CpG ODN on feline PBMC and B cells in vitro

As a step toward *in vivo* studies, we determined whether type C CpG ODN (C274) would induce activation of PBMC and B cells *in vitro*. PBMC or B cells were pulsed with varying concentrations of CpG C274 or non-CpG C661. C274 induced moderate proliferation in PBMC, and lower levels of proliferation in B cell cultures. However, PBMC proliferation indices were significant when compared to a non-stimulatory ODN C661 at all concentrations (2μg/ml $p = 0.0014$; 4μg/ml $p = 0.0005$; 8μg/ml $p = 0.007$) (Fig. 3.1). B cell proliferative indices were significant at 4μg/ml CpG ($p = 0.0025$), but not at 8μg/ml CpG ($p = 0.1013$). C274 induced interferon-alpha (IFN-α) mRNA expression in PBMC from two animals (4078 and 4003) when compared to C661 induction. When comparing

C274 to medium treatment, no difference was noted. IL-12 mRNA expression induced by C274 was not different than that induced by C661 or medium (Fig. 3.2).

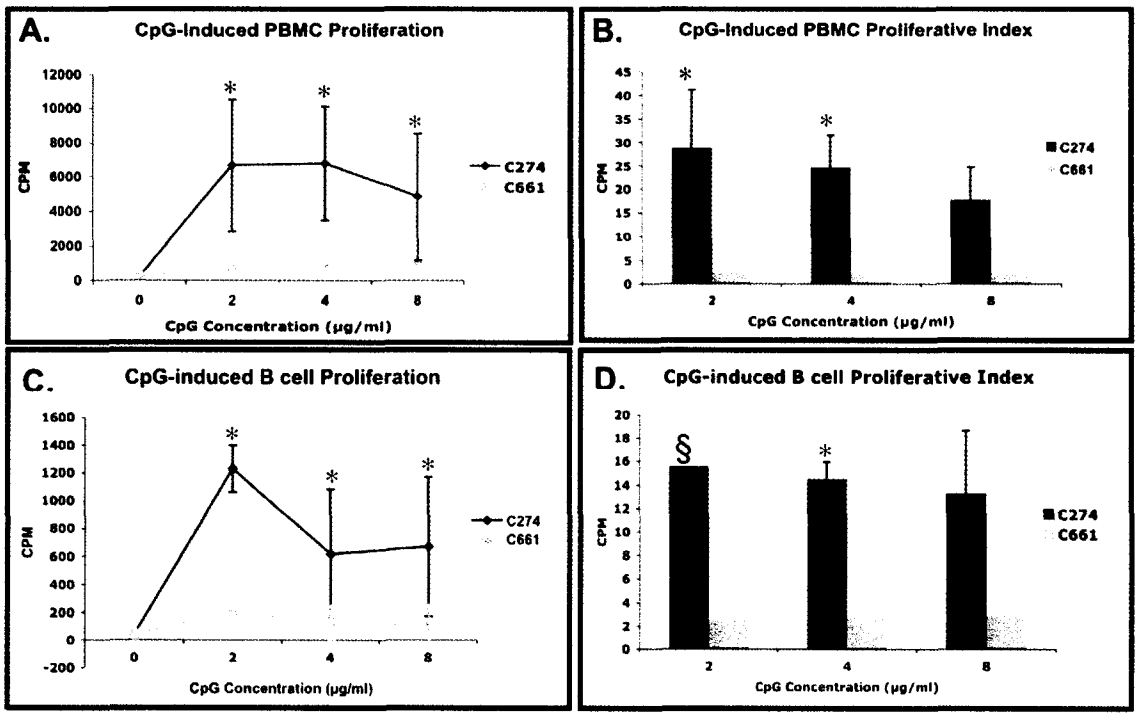

Fig 3.1. Proliferative responses and proliferative indices of PBMC (A, B) and B cells (C, D) in response to a type C immunostimulatory CpG ODN (C274) vs. a non-stimulatory control ODN (C661). Asterisks (*) denote statistical differences between C274 and C661 induced proliferation and proliferative indices. Data is presented as averages plus SD combining at least 3 different experiments, except in (D) where only one experiment was performed at the 2µg/ml concentration (§).

DC vaccination and hematology

Hematologic data remained within normal limits for all animal groups during four months of blood sampling and DC vaccinations (Fig. 3.3). There were no differences among groups as assessed by repeated-measures ANOVA (WBC $p = 0.9846$; RBC $p = 0.8172$; PCV $p = 0.3309$; Lymphocyte $p = 0.9503$).

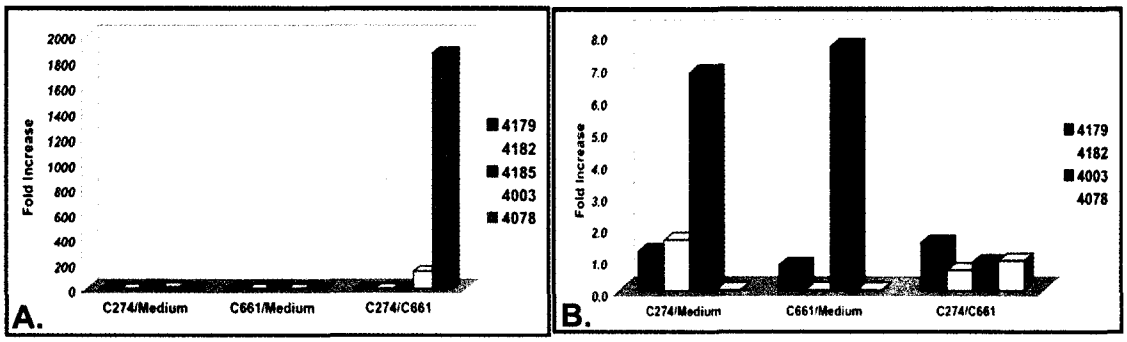

Fig 3.2. IFN-α (A) and IL-12 mRNA (B) expression in PBMC pulsed with Type C CpG ODN. Data is expressed as the fold increase of cytokine expression when PBMC were pulsed with stimulatory CpG C274 versus non-stimulatory CpG C661 or medium.

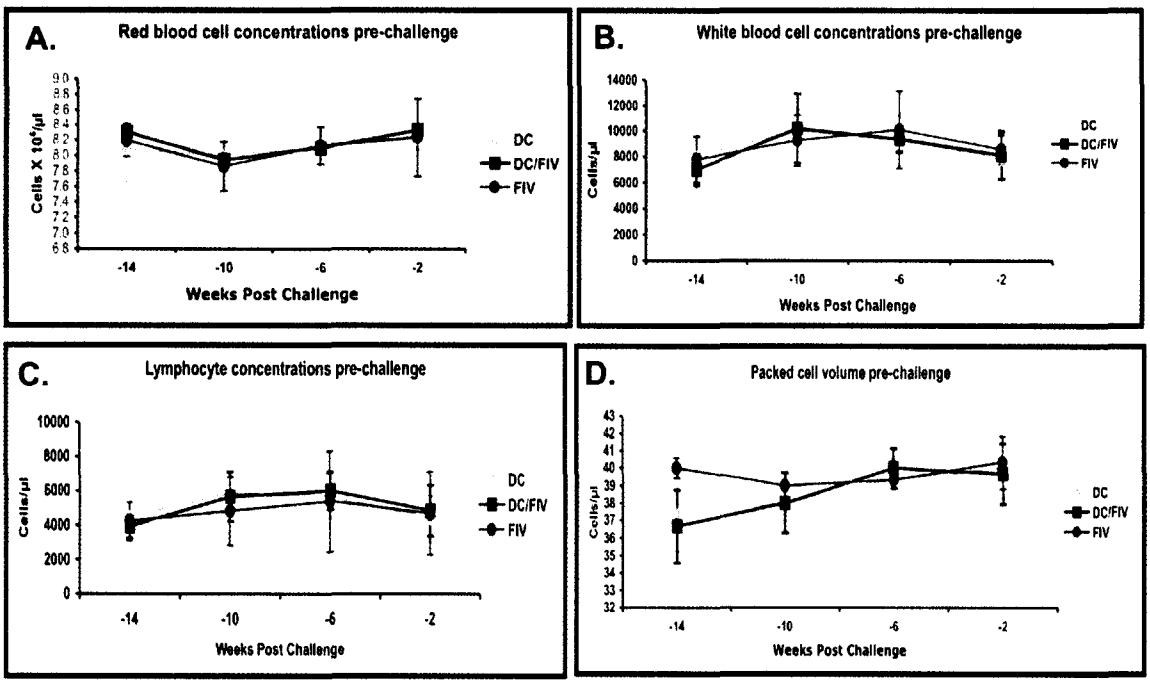

Fig. 3.3. White blood cell (A), red blood cell (B), lymphocyte (C) and packed cell volume (D) averages ± standard deviations during four months of blood sampling and DC vaccination. No statistical difference was seen among groups for any parameter measured.

DC vaccination and lymphocyte subset analysis

No significant changes in CD4 counts, CD8 counts or CD4/CD8 ratios were seen during four months of blood sampling and DC vaccinations (Fig. 3.4). There were no differences among groups as assessed by repeated-measures ANOVA ($CD4^+$ $p = 0.9776$; $CD8^+$ $p = 0.8831$; $CD4^+/CD8^+$ $p = 0.9318$).

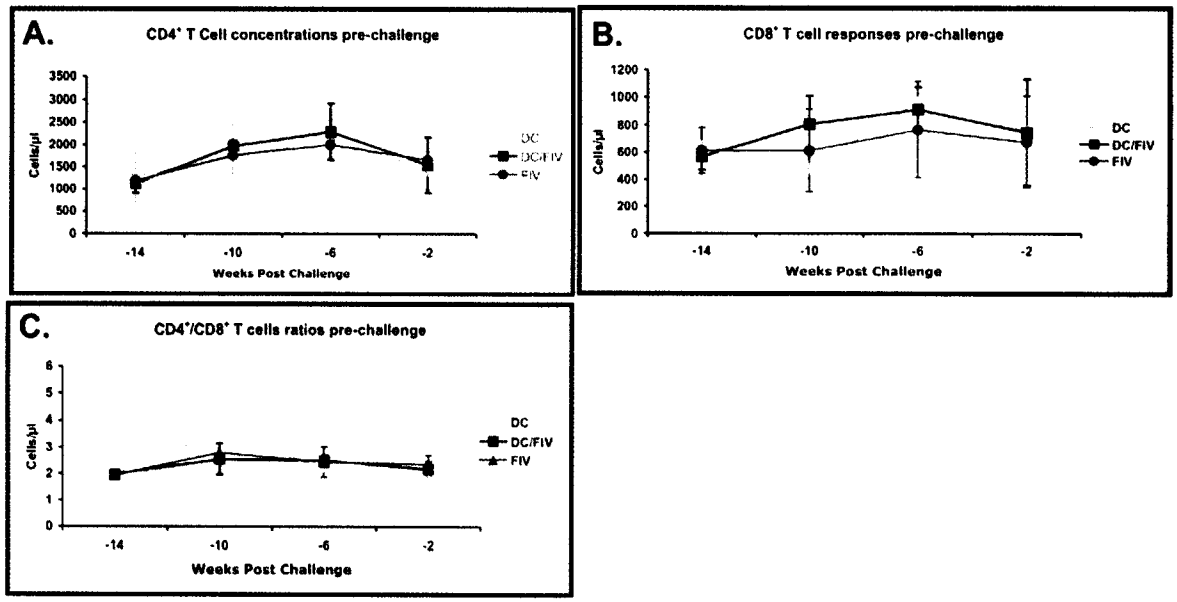

Fig. 3.4. $CD4^+$ (A) and $CD8^+$ T (B) cell averages and CD4/CD8 ratios (C) ± standard deviations during four months of blood sampling and DC vaccination. No statistical difference was seen among groups.

FIV Antibody Responses

FIV Ab production was detected after the first vaccination in all groups, and although not statistically significant ($p = .1740$), there appeared to be a trend toward greater antibody production in the animals receiving DC (Fig. 3.5). However, subsequent western blot analyses revealed that much of this antibody response was against bovine serum albumin (BSA).

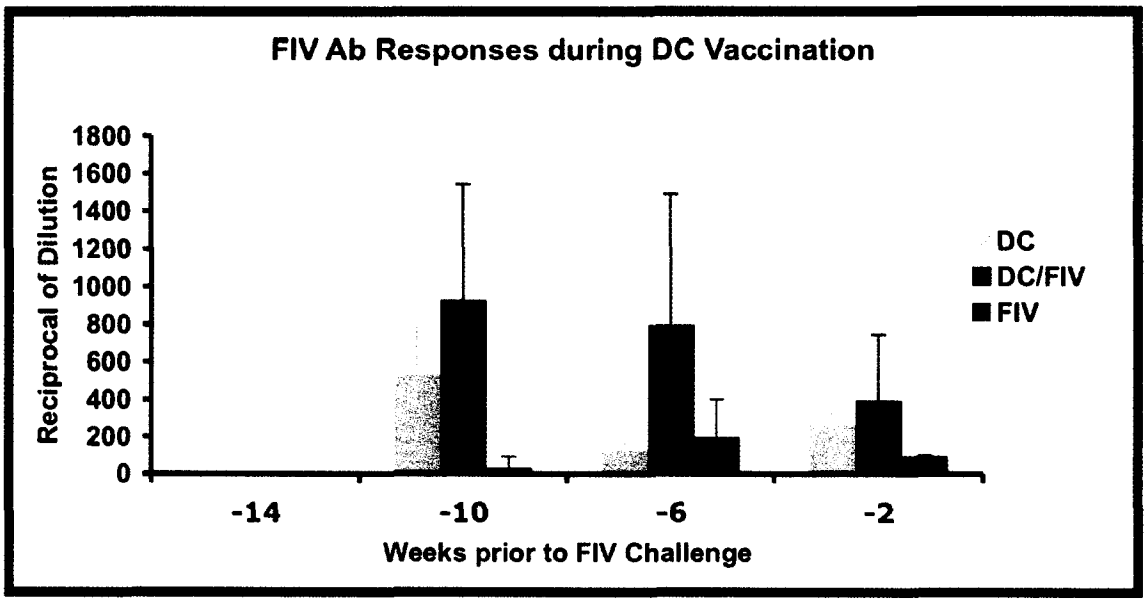

Fig. 3.5. FIV Ab responses during four months of blood sampling and DC vaccination. Results are expressed as the average + SD of the reciprocal of dilution of the highest Ab titer detected at a given sampling period. No statistical differences were detected among the groups at each time point.

Optimization of proliferative responses to AT-2 FIV

The concentration of target AT-2 inactivated FIV to use in and optimal timing for proliferation assays was determined using antibody-positive FIV-infected cats as positive controls (Fig 3.6). Although proliferation is greater at the 6-day incubation timepoint, background was higher making proliferative indices lower at this time compared with the day-3 incubation timepoint. The optimal concentration of AT-2 FIV was determined to be between 1µg/ml and 5µg/ml.

FIV proliferative responses

No proliferative responses were seen in PBMC during DC vaccinations using CD14-depleted responder PBMC (Fig. 3.7).

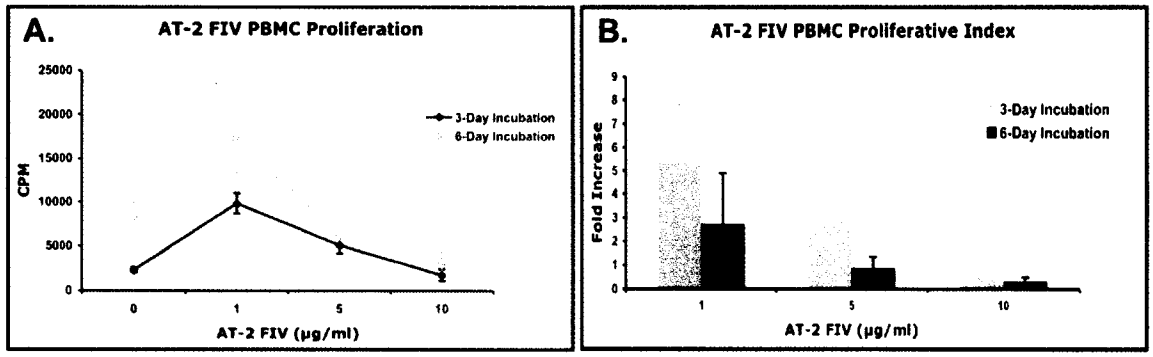

Fig. 3.6. Proliferation responses (A) and proliferative indices (B) of PBMC from FIV infected cats comparing 3-day incubation to 6-day incubation timepoints. Data are presented as the average ± SD (A) or + SD (B) of three different cats.

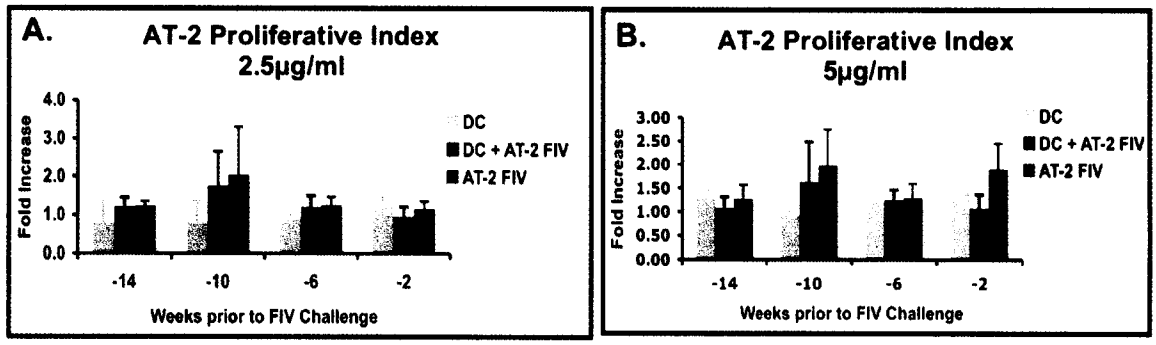

Fig. 3.7. CD14-depleted PBMC proliferative responses to AT-2 inactivated FIV during four months of blood sampling and DC vaccination. Results are expressed as proliferative indices in response to either 2.5µg/ml (A) or 5.0µg/ml (B) of AT-2 FIV vs. medium alone.

Interferon-γ responses to FIV in vitro

IFN-γ release by $CD8^+$ T cells was not detected after stimulation of CD14-depleted PBMC with 5 µg/ml AT-2 inactivated FIV for 24 hours (data not shown). The AT-2 FIV concentration used was determined from titrations performed on PBMC from FIV-infected cats (data not shown) and from published work in SIV[15].

Responses of AT-2-FIV-DC-vaccinated cats to infectious FIV challenge

Hematologic Data

Slight decreases in average white blood cell counts, average red blood cell counts and packed cell volumes were noted post challenge, whereas lymphocyte numbers remained stable (Fig. 3.7). There were no differences among groups as assessed by repeated-measures ANOVA (WBC $p = 0.9053$; RBC $p = 0.5051$; PCV $p = 0.7839$; Lymphocyte $p = 0.9358$).

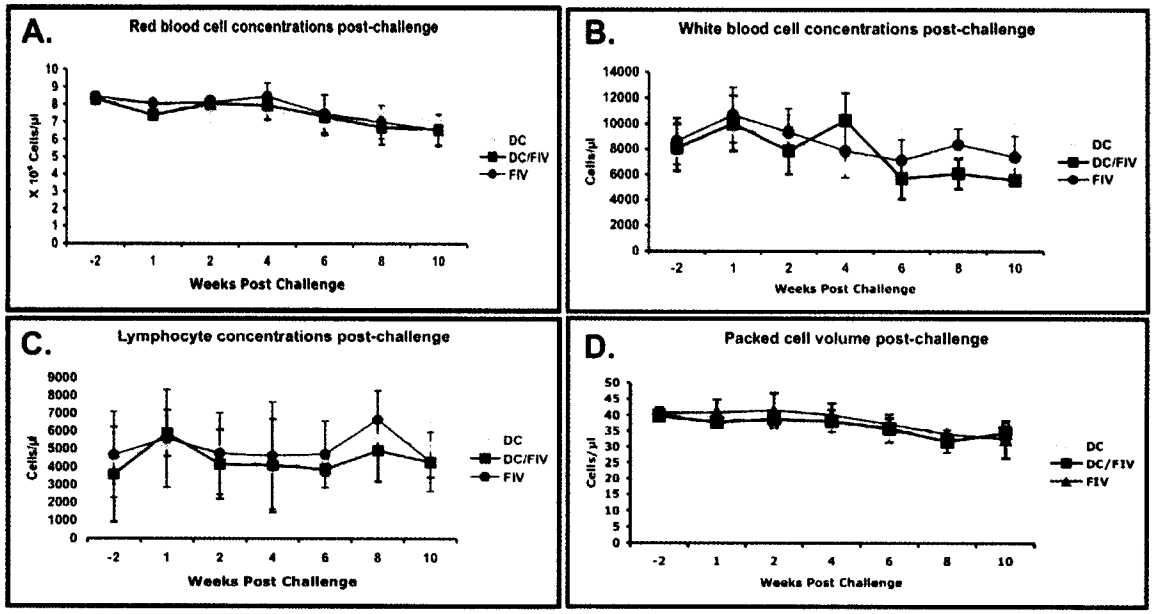

Fig. 3.8. Red blood cell (A), white blood cell (B), lymphocyte (C) and packed cell volume (D) averages ± standard deviations post FIV challenge. No statistical difference was evident among groups for any parameter measured.

CD4/CD8 subset analysis

A characteristic decrease in $CD4^+/CD8^+$ ratio was seen by 4 weeks post-challenge in all groups. This decline was caused by a decrease in $CD4^+$ T cells by 4 weeks and an increase in $CD8^+$ T cells (Fig. 3.8). There were no

differences among groups as assessed by repeated-measures ANOVA ($CD4^+$ p = 0.9472; $CD8^+$ p = 0.7887; $CD4^+/CD8^+$ p = 0.7980).

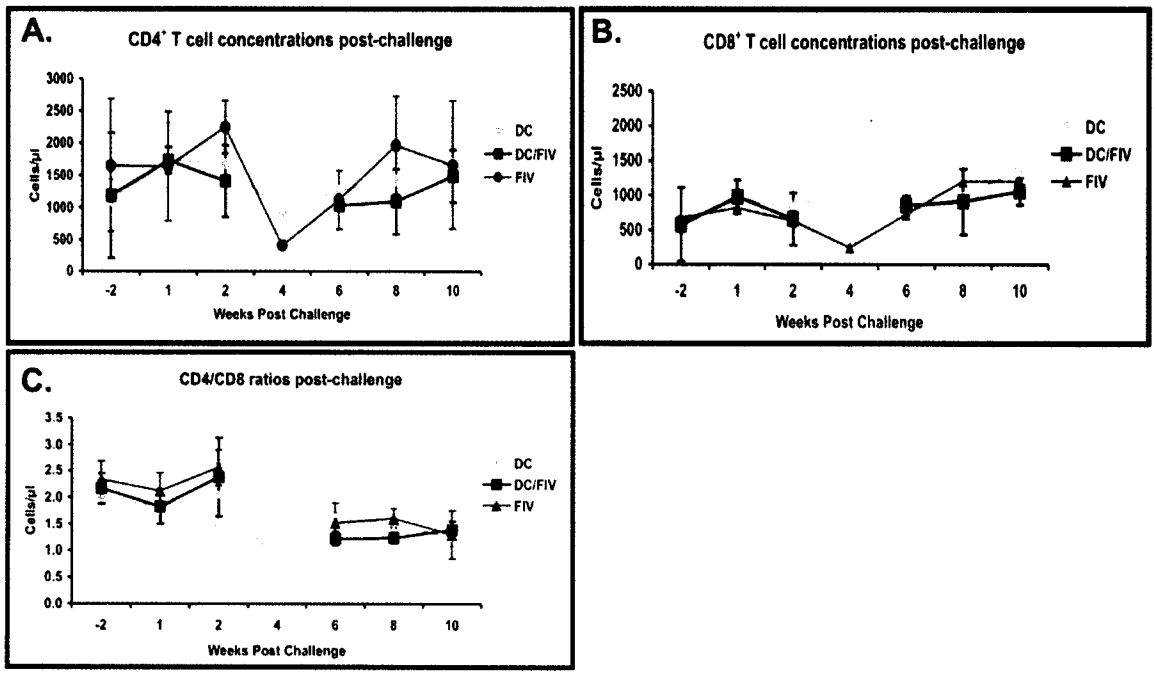

Fig 3.9. $CD4^+$ (A) and $CD8^+$ (B) T cell average concentrations and CD4/CD8 ratios (C) ± standard deviation post-FIV challenge. No statistical difference was seen among the groups.

FIV Antibody Responses Post Challenge

FIV Ab responses steadily increased to 10 weeks post challenge and termination of the study (Fig. 3.9). One animal each in the DC + AT-2 FIV group and the AT-2 FIV group had Ab responses higher than the other cats in the groups. This correlated with higher FIV DNA viral loads in these animals. Overall, there was no statistical difference among vaccine groups (p = 0.6395).

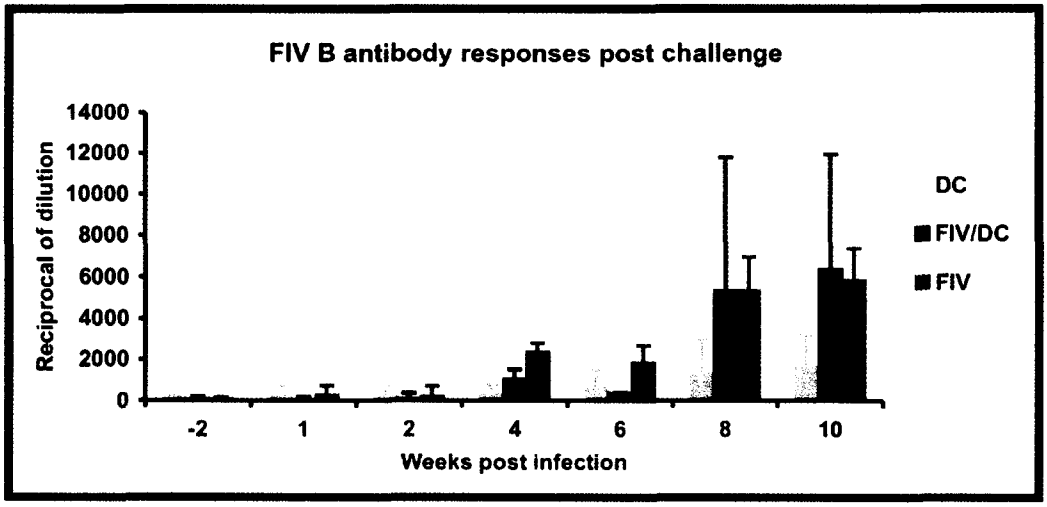

Fig 3.10. FIV Ab responses post challenge. Results are expressed as the averages + SD of the reciprocal of the dilution of the highest Ab titer detected at a given sampling period. No statistical differences were detected among groups at each time point.

FIV DNA viral loads post challenge

FIV DNA viral loads began to increase at 4 weeks post challenge and remained steady from 6 to 10 weeks post challenge (Fig 3.10). One animal in each in the DC + AT-2 FIV group and the AT-2 FIV group had a much higher viral loads than the other cats in the group. No statistical differences were found among the vaccine groups (p = 0.8463).

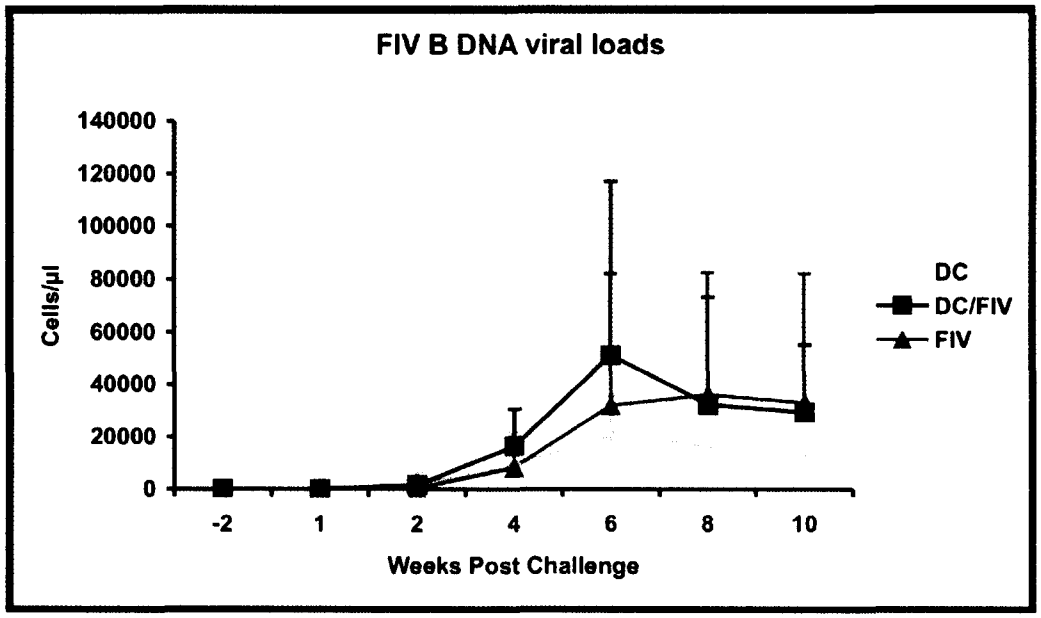

Fig. 3.11. FIV B DNA viral loads post challenge as measured by real-time DNA PCR. Data is presented as group averages + SD.

DISCUSSION

We used a type C CPG ODN as an adjuvant in the DC immunization studies in an attempt to mature the injected DC *in vivo* since it has been shown that plasmacytoid DC activated myeloid DC in vitro in response to inactivated HIV-1 *in vitro* [36]. To validate the *in vivo* use of this CpG, we examined immune responses *in vitro* and found stimulation of PBMC and B cells vs. non-CpG C661 using similar concentrations as used in humans and macaques [25,27,37]. PBMC stimulation indices were similar or better than those induced by other types of CpG in cats [38,39]. However, there was no difference in IFN-α or IL-12 mRNA expression when C274 induction was compared to no induction, in contrast to cytokine induction by this CpG in macaques and humans [27,40]. The higher fold

increase in IL-12 mRNA expression induced by C274 (cat 4078) compared to no induction was likely insignificant since the non-stimulatory control CpG C661 induced similar levels and the other cats did not respond likewise. In addition, the marked increases in IFN-α mRNA expression induced by C274 in two cats when compared to C661, but not to medium alone is perplexing and suggests that C661 is actually suppressive. However, no instances of suppressive effects by non-stimulatory CpG could be found. Generally it can be said that species differences observed for CpG and cytokine induction make the significance of the *in vitro* results uncertain. For example, IL-12 p40 protein was induced in mouse PBMC in response to CpG, but was negligible in human and macaque cells [41-44]. And, a control GpC that was found to be non-stimulatory in mice was subsequently found to be stimulatory in pigs [45,46].

AT-2-DC immunization did not produce adverse clinical reactions nor perturb hematologic indices, consistent with DC vaccine studies in humans [23,47]. In addition, FBS-induced systemic anaphylaxis to fetal bovine serum was a concern [48], however, no adverse effect was seen.

No significant anti-FIV antibody titer was produced by DC immunization. The trend toward greater Ab production observed in the AT-2-FIV-DC and DC alone immunization groups vs. the AT-2 FIV alone group proved to be a result of BSA in the DC-containing inoculums.

Neither CD14-depleted PBMC proliferative nor $CD8^+$ IFN-γ responses to AT-2 inactivated FIV were achieved by vaccinations. It is possible that $CD14^+$ cell depletion interfered with the ability of cells to proliferate in response to

inactivated virus, since SIV IFN-γ responses were variably blunted after removal of $CD14^+$ cells in macaques [17]. It is also possible that the animals simply failed to generate a systemic cell-mediated immune response (discussed below).

Examination of the FIV DNA viral loads revealed no difference among groups with one outlier from each the DC + AT-2 and AT-2 FIV groups. Although small group sizes made statistical analysis problematic, it can be inferred that there was truly no difference and that DC + AT-2 FIV vaccination had no greater ability to induce protection than AT-2 FIV or DC used alone. Without a no CpG adjuvant group, inferences about the effectiveness or lack thereof of CPG *in vivo* cannot be made.

Post-challenge antibody responses were variable among cats and were slightly greater in the DC + AT-2 inactivated FIV group. Overall, however, Ab responses were more likely related to DNA viral load than to immunization status.

There are several possibilities as to why the inactivated FIV-DC vaccine did not provide protection from challenge. First, as demonstrated in Chapters 1 and 2, immature to semi-mature DC were used in the vaccinations, and although reports suggested DC do not have to be matured *in vitro* for effective immunization, there are also reports that suggest differently. For instance, immature DC injected into macaques trafficked to local lymph nodes where they matured [49]. Also, immature DC injected into subcutaneously into skin treated with a topical immunostimulant, generated immune responses [50]. However, immature DC vaccination has also been shown to induce a self-limited silencing

of $CD8^+$ effector T cell function by regulatory T cells that is cell-contact-dependent and largely independent of IL-10 [47,51].

Secondly, the CpG injected subcutaneously may not have been effective at inducing immune activation of resident pDC since it is unknown how many pDC reside in this location. However, pDC numbers are likely quite low since only few were found in the dermal layer of human skin [52]. Also, since differential activation of DC subsets by CpG have been demonstrated in humans vs. mice [53,54], and CpG activation of mDC and pDC in cats is unknown, we cannot be sure that the CpG used in this study had an effect on feline DC. In previous studies, we were unable to demonstrate feline myeloid DC maturation after exposure to a different CpG (data not shown)

Third, antibodies made to FBS and GM-CSF used in DC cultivation may have also impeded generation of FIV-specific immune responses. For example, we demonstrated that animals receiving DC in this study likely developed antibody to BSA. This is supported by induction of antibodies to FBS and BSA in human subjects vaccinated with FBS-exposed DC [48] and induction of protective tumor antibodies upon tumor challenge in mice vaccinated with DC cultivated in FBS in the absence of tumor Ags, but not with DC cultivated in serum-free media[55]. However, no studies could be found that suggested DC were deleted *in vivo* secondary to generation of BSA Ab. In addition, if residual human GM-CSF was present on the injected DC, neutralizing Ab may have been induced as was shown in macaques injected multiple times with human GM-CSF [56].

Fourth, DC migration into lymph nodes may have been impeded for several reasons. First, immature DC, as used in this study, do not express chemokines required for lymph node migration [57] and were less able to migrate compared to mature DC in an *in vivo* migration study [58]. In the same migration study, DC injected subcutaneously, as in our study, were three times less able to migrate to draining lymph nodes compared to intradermally injected DC [58]. And finally, interleukin-4 (IL-4), used as a growth factor in our cultures, has been shown to down-regulate anaphylotoxin C5a receptors, impairing DC migration to sites of inflammation [59].

Fifth, a question can be raised as to the affect of lymphocyte contamination on the survivability of DC after injection. It has recently been shown that DC genetically modified to express GFP presented antigenic peptides to autologous contaminating lymphocytes that then eradicated the DC via a perforin-mediated pathway. So it is possible that once injected, the DC matured, but were then eliminated by contaminating cytotoxic T lymphocytes (CTLs) *in vivo* [60].

Sixth, DC may have been functionally altered by exposure to inactivated FIV since it has been shown that immature DC exposed to AT-2 inactivated HIV-1 or its gp120 protein, had phenotypic changes characteristic of mature DC without the increase in interleukin-12 (IL-12) production and allostimulatory capacity typical of mature DC [61]. However, immunity to FBS on FBS-cultured DC may again be the culprit for the functionally altered DC since a similar SIV

study by Frank et al. found AT-2 inactivated SIV-pulsed mature DC cultured with 1% human plasma could stimulate both $CD4^+$ and $CD8^+$ T cells [17].

Finally, the inactivated viral pulse concentration may have been too low. Using 5μg of viral protein per immunization vs. the extrapolated dose of $5\mu g/10^5$ DC may have provided insufficient stimulus [17]. *In vitro* studies assessing the uptake of AT-2 inactivated FIV by DC and subsequent immune stimulation to $CD4^+$ and CD8 T^+ cells would have helped to estimate an approximate *in vivo* dose, however, due to time constraints these studies were not performed.

As with all negative observations, it is difficult to predict their significance to the field of autologous DC vaccination. Further studies employing mature DC, different adjuvants, different methods of inoculation (i.e. intradermal vs. subcutaneous), DC cultivated in autologous or pooled feline plasma/serum, and more animal groups will be needed before this approach can be appropriately assessed for prophylactic vaccination of lentivirus infections.

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