DISSERTATION

APPLICATIONS OF FELINE IMMUNODEFICIENCY VIRUS AS A MODEL TO STUDY HIV PATHOGENESIS

Submitted by

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ABSTRACT

APPLICATIONS OF FELINE IMMUNODEFICIENCY VIRUS
AS A MODEL TO STUDY HIV PATHOGENESIS

Feline immunodeficiency virus (FIV) is a naturally-occurring retrovirus that infects domestic and non-domestic feline species, and produces progressive immune depletion that eventually results in an acquired immunodeficiency syndrome (AIDS). While it is accepted that FIV is primarily transmitted by biting, few studies have evaluated FIV oral infection kinetics and transmission mechanisms over the last 20 years. Modern quantitative analyses applied to natural FIV oral infection could significantly further our understanding of lentiviral oral disease and transmission. In Chapter 1 of this dissertation, I characterized FIV salivary viral kinetics and antibody secretions to more fully document oral viral pathogenesis. The results of this research demonstrate that (i) oral lymphoid tissues serve as a site for enhanced FIV replication, resulting in accumulation of FIV particles and FIV-infected cells in saliva, and (ii) failure to induce a virus-specific oral mucosal antibody response, and/or viral capability to overcome inhibitory components in saliva may perpetuate chronic oral cavity infection. Most importantly, these results provide a model of oral FIV pathogenesis and suggest alternative diagnostic modalities and translational approaches to study oral HIV infection.

Feline immunodeficiency virus and human immunodeficiency virus (HIV) utilize parallel modes of receptor-mediated entry. The FIV surface glycoprotein (SU) is an important vaccine target for induction of virus neutralizing antibodies, and autoantibodies to the FIV binding receptor (CD134) block FIV infection *ex vivo;* highlighting the potential for immunotherapies which utilize anti-receptor antibodies to block viral infection. In Chapter 2 of this dissertation, I immunized cats with soluble CD134, recombinant FIV-SU protein, and/or CD134+SU complexes prior to challenge with FIV to determine if vaccination with CD134-SU complexes

could induce protection against FIV infection. Immunization induced production of anti-CD134 and anti-SU antibodies in vaccinated cats, and purified anti-CD134 and anti-SU antibodies significantly inhibited FIV infection *in vitro.* However, no vaccine combination protected cats from FIV infection *in vivo,* and vaccination induced high titers of antibodies directed at vaccine by-products relative to target antigens. The results of this research reinforce the need to monitor components of vaccine preparations, and emphasize that vaccination may induce proliferation of susceptible target cells and enhancement of heat-labile serum components that counteract neutralizing antibodies.

Feline immunodeficiency virus induces lifelong infection in cats and may result in a spectrum of immunodeficiency-related diseases. Both prednisolone and cyclosporine A (CsA) are commonly used clinically to treat lymphoproliferative and immune-mediated diseases in cats, but the impact of these compounds on FIV infection has not been well documented, and their understanding immunomodulatory effects on FIV replication and persistence is critical to guide safe and effective use of these therapies in FIV infected cats. In Chapter 3 of this dissertation, I administered immunosuppressive doses of prednisolone or CsA to cats chronically infected with FIV and monitored alterations in hematological parameters and FIV viral/proviral loads in response to therapy. Interestingly, both treatments caused (i) acute increases in CD4+ lymphocytes, (ii) increased FIV viremia, and (iii) significant alterations in cytokine expression that favored a shift toward a Th2 response. The results of this research highlight the potential for immunosuppressive drug-induced perturbation of FIV replication and underscores the need for consideration of chronic viral infection status when prescribing immunomodulatory medications.

Mucosal immune dysfunction, bacterial translocation, systemic immune activation, and chronic inflammation are well-documented features of chronic HIV infection. Despite the success of combinational antiretroviral therapy (cART) in diminishing HIV viral replication and prolonging immune function, a multitude of systemic and local manifestations of HIV infection

persist, including the development of chronic inflammation (periodontitis and gingivitis). Commonly used animal models for studying HIV pathogenesis, including SIV/SHIV infections of non-human primates (NHPs) or HIV infections in humanized mice, do not reliably incite oral lesions. In contrast, gingivitis and periodontitis are primary clinical signs associated with untreated natural and experimental FIV-infection, and are principal attributes of this model that may be exploited to investigate pathogenic mechanisms involved in the perturbation of the oral immune system and microbial environment. Therefore, in Chapter 4, I present the findings of a pilot study in which I investigated changes in the oral microbiota and oral immune system during FIV infection, and further, outline the potential for the feline model of oral AIDS manifestations to elucidate pathogenic mechanisms of HIV-induced oral disease. By assessing FIV-associated changes in clinical status, oral microbiota, local and systemic viral burden, and immune profile under such treatment protocols, future studies implementing the feline model of lentiviral-induced oral disease may provide a cornerstone to expand our understanding of the complex interactions between HIV infection, oral immune dysfunction, and the perturbations to the oral microbiota that occur in the context of HIV infection.

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INTRODUCTION

Feline Immunodeficiency Virus

Feline immunodeficiency virus (FIV) is a naturally-occurring retrovirus that infects domestic and non-domestic feline species, and produces progressive immune depletion that eventually results in an acquired immunodeficiency syndrome (AIDS) [1-10]. A member of the Lentivirus genus within the Retroviridae family, much has been learned about FIV since it was first described in 1987, particularly in regard to its application as a model to study the closely-related lentivirus, human immunodeficiency virus (HIV) [8-12]. The FIV virion is approximately 100nm in diameter, spherical, and contains two identical strands of positive-sense RNA in its 9400-base genome, which is tightly associated with the nucleocapsid protein (p7) and a t-RNA^{lys} bound to each RNA molecule, serving as a primer for negative strand transcription [11-14]. This ribonucleoprotein complex, along with viral enzymes involved with replication and maturation (protease, reverse transcriptase (RT), integrase (IN), and dUTPase), are enclosed within a core of capsid protein (CA, p24), and surrounded by a shell of matrix protein (MA, p14) and outer lipid bilayer [11-13]. Viral envelope glycoproteins (gp) are embedded within the outer lipid bilayer, and include the surface (SU, gp95) and transmembrane (TM, gp40) subunits, which are cleaved from a 130-150kDa membrane-bound precursor protein, glycosylated, and non-covalently anchored within the envelope in a trimeric form [11-13, 15].

The genomic structure of FIV consists of three primary open reading frames (ORFs), *gag, pol,* and *env*, which are flanked by two long-terminal repeats (LTR) and accompanied by numerous small ORFs containing regulatory and accessory genes such as *vif, rev, and orfA*. FIV *gag* encodes the Gag polyprotein, which is cleaved by viral protease to form the three mature proteins, MA, CA, and NC, and is necessary to achieve formation of mature virus particles [11, 16, 17]. Pol polyprotein, the primary product of the FIV *pol* gene, contains 4 important enzymes involved in virus replication and maturation: protease (PR), reverse

transcriptase (RT), integrase (IN), and dUTPase (DU) [11]. Viral PR facilitates the cleavage of Gag and Pol polyproteins into functional enzymatic or structural proteins, while DU catalyzes the hydrolysis of dUTP to dUMP, thus minimizing misincorporation of potentially mutagenic dUTP into host DNA [11, 18, 19]. FIV RT is an RNA-dependent DNA polymerase involved in the reverse transcription of viral genomic RNA into a double-stranded copy of proviral DNA (cDNA), which is subsequently integrated into host cellular DNA via the mature IN enzyme, which contains three functional domains: an N-terminal domain, a central catalytic core, and a C-terminal domain [20-22]. The FIV Env polyprotein, a 130-150 kDa product of the *env* gene, is proteolytically cleaved within the Golgi apparatus into two mature, glycosylated proteins, SU (gp95) and TM (gp40), which play a significant role in virion attachment and entry into target cells [11, 12].

The FIV genome contains one regulatory gene (*rev*) and two accessory genes (*vif* and *orfA*). FIV *rev* encodes Rev, a nucleolar polyprotein that binds to the Rev Response Element (RRE) to allow export of partially spliced and unspliced viral RNA transcripts out of the nucleus with the help of the nuclear export protein, exportin-1 [11, 12, 23]. The FIV Vif protein, is crucial to FIV replication and involved in counteraction of host defense mechanisms such as APOBEC3, a cellular protein that exerts an antiviral effect by deamination of cytosine to uracil during viral replication, resulting in degradation of synthesized minus-strand DNA [11, 12, 24]. FIV Vif thus counteracts APOBEC3 by targeting the host protein to the E3 ubiquitin ligase complex, which is subsequently degraded by the proteasome [11, 12, 24]. The FIV OrfA protein is encoded by the accessory gene *orfA*, and has been shown to transactivate transcription of the FIV genome from the FIV LTR, localizes in the nucleus and causes cell cycle arrest at G2 in infected cells, and may be involved in late steps of virion formation and the early steps of virus infectivity, although the precise role of OrfA is still undetermined [11, 12, 25-28]. Interestingly, OrfA has been shown to downregulate expression of the viral receptor for FIV (CD134) on the surface of cells, as well as E2 ubiquitin-conjugating enzymes and a ubiquitin-protein ligase [12,

29, 30]. These potential functions of OrfA may have implications which aid in viral dissemination by preventing surface interactions with budding virions, and limit degradation of viral proteins by host cell ubiquitin ligase mechanisms, respectively.

FIV requires an initial interaction with a primary binding receptor for infection, and binds to host cells through a high-affinity interaction of the envelope SU protein (gp95) with the CD134 surface molecule present in high amounts on CD4+ lymphocytes and monocytes/macrophages [31-35]. This interaction induces a conformational change in the SU protein, which then exposes a cryptic epitope in the V3 loop of Env; the binding site necessary for binding with the entry (co) receptor CXCR4 [34-36]. Binding of the V3 loop exposes the serpentine region of TM (gp40), which results in the formation of a hairpin structure that allows fusion with the cell membrane and subsequent cell entry [35-37]. However, as infection progresses, the production of neutralizing antibodies by the host increases the need for FIV to escape selective pressures, As a result, the cell tropism of FIV begins to change, as new viral variants arise which exhibit a decreased dependence on CD134 and increased ability to infect cells that express CXCR4 with limited CD134, such as naïve B cells and CD8+ T cells [2, 3, 38]. Thus, this expanded cell tropism results in a vast increase in the number of target cells susceptible to infection, which subsequently causes severe immunodepletion and clinical manifestations associated with AIDS-induced disease.

FIV as a molecular analogue to HIV

The structural and sequence organization of FIV is very similar to HIV, which is also a member of the lentivirus genus [11]. HIV is morphologically characterized by a spherical virion that is roughly 120nm in diameter, and contains a diploid genome composed of two copies of single stranded, positive-sense RNA that is packaged with nucleocapsid (p7) and accessory proteins (protease, reverse transcriptase, integrase) [39]. Like FIV, the ribonucleoprotein complex at the heart of the HIV virion is contained within a dense core of Capsid protein (CA,

p24) and surrounded by a spherical shell of Matrix protein (MA, p17)[39]. Mature Env glycoproteins, SU (gp120) and TM (gp 41), are anchored within the external lipid bilayer, and play significant role in cell entry through binding to host cell receptors. HIV also requires an initial interaction with a primary binding receptor for infection, and utilizes analogous modes of receptor-mediated entry as FIV utilizing chemokine co-receptors [40-42]. However, in lieu of CD134, HIV utilizes CD4 as primary binding receptor and CCR5 as its primary co-receptor, although HIV is also able to utilize CXCR4 [40, 41]. Much like FIV, HIV binds to CD4+ target cells through a high-affinity interaction with the CD4 receptor that induces a conformational change in the envelope glycoprotein gp120, subsequently exposing the binding sites necessary for chemokine co-receptor binding (CXCR4 or CCR5) and subsequent fusion with the cell membrane.

The HIV genome encodes three primary polyproteins, Gag, Pol, and Env, as well as the regulatory protein, Rev, and accessory protein, Vif – all of which exhibit similar functions to FIV [11, 12, 39]. However, in addition to these, HIV also contains genes that encode additional accessory proteins involved in viral maturation, replication, and survival [39]. These include: Tat (p16/p14), a viral transcriptional activator, Vpr (p10-15), a promoter of nuclear localization and inhibitor of cell division (cell cycle arrest at G2/M), Vpu (p16), a promotor of extracellular release of viral particles, Nef (p27-25), a downregulator of CD4 and MHC I expression, Vpx (p12-16), a Vpr homolog present in HIV-2 (absent in HIV-1), and Tev (p28), a tripartite tat-env-rev protein [39]. FIV OrfA shares many similarities with several HIV accessory proteins, including the transcriptional transactivation activity of Tat, nuclear localization and cell cycle arrest functions of Vpr, and downregulation of cell surface receptor ability of Nef [27-29, 39]. This indicates that although FIV differs slightly in terms of accessory gene structure, the accessory proteins expressed during FIV infection exhibit many of the same functions as observed during HIV infection, highlighting the potential for FIV to provide insight into molecular mechanisms of lentiviral infection.

Clinical disease syndromes of FIV

FIV is associated with a variety of clinical syndromes that predominately occur secondary to immunodepletion, including cachexia, anterior uveitis, chronic rhinitis, gingivostomatitis and periodontitis, encephalitis and neurologic dysfunction, and lymphoma [1, 4, 9, 43-53]. The acute phase of FIV infection, lasting approximately 4-8 weeks, is characterized by a sharp increase in CD4+ T lymphocytes that are accompanied by high levels of FIV viral RNA and proviral DNA in circulation [4, 8, 54]. These hematologic changes are typically accompanied by mild to moderate clinical sings which include pyrexia, lethargy, and peripheral lymphadenopathy [4, 54, 55]. Following a prolonged asymptomatic phase, during which the levels of circulating virus remains stable and integrated provirus establishes a reservoir of latently infected target cells, there is progressive decline of CD4+ T lymphocytes and other immunocytes, resulting in functional immunodeficiency and susceptibility to opportunistic infections [6, 13, 36, 56].

During FIV infection, loss of CD4+ T lymphocytes is directly attributable to a viral-induced cytopathic effect, in addition to an increase in FIV-specific CD8-mediated programed cell death, lack of thymic regeneration, and spontaneous apoptosis in response to decreased cytokine support [10, 13, 57, 58]. As a result, the most frequent clinical disease syndromes associated with FIV infection manifest as a direct consequence of immune depletion and dysfunction, such as oral opportunistic infection (gingivitis, stomatitis, and periodontitis), immune-mediated glomerulonephritis, chronic rhinitis, and dermatitis [47, 48, 51, 52, 59, 60]. Oral opportunistic infections are prevalent in a high proportion of FIV-infected cats, and frequently present as erythematous, inflammatory lesions along the gingival margin (gingivitis), multifocal areas of necrotizing inflammation within the gingival sulcus or periodontal ligament (periodontitis), or ulcerative inflammatory lesions along the buccal mucosa, hard palate, or soft palate (stomatitis) [52, 61-63]. Changes in the salivary/oral microbiota have been increasingly associated with FIV infection, and shifts in the proportion of opportunistic pathogens in saliva of

FIV-infected cats have been associated with the development of oral inflammatory lesions [61, 64]. Similarly, FIV-infected cats frequently present severe, necrotizing and/or ulcerative inflammatory lesions (dermatitis) due to opportunistic infection with various bacterial, fungal, protozoal, and parasitic etiologies, including mycobacteriosis, leishmaniasis, toxoplasmosis, and dermatophytosis [48, 59, 65, 66]. Upper respiratory disease is also a frequently finding in FIV-infected cats, and may occur in conjunction with concurrent viral, bacterial, or fungal infections [4, 47, 67, 68].

FIV-induced renal disease is also observed in both experimentally and naturally infected cats, and includes pathologic changes which include glomerulonephritis, proteinuria, protein tubular casts and tubular microcysts, as well as diffuse interstitial inflammatory infiltrates [60, 69]. Mesangial widening with glomerular and interstitial amyloidosis is also observed in kidneys of FIV-infected cats, and when evaluated in the context of another frequent finding during FIV infection, hypergammaglobulinemia, indicate the potential for immune complex deposition to occur within the glomerulus as a result of chronic antigenic stimulation and immune activation [60, 70, 71]. Interestingly, FIV is also associated with the occurrence of various neoplastic diseases, which most frequently manifests in the development of lymphoma in infected cats [7, 72]. Collectively, these two disease syndromes highlight direct consequences of viral-induced immune dysfunction that arise in response to prolonged viral infection.

Neurologic disease is an important manifestation of FIV infection, and affected cats may present with either central nervous system (CNS) or peripheral nervous system (PNS) involvement [46, 49, 50, 73, 74]. In the PNS, FIV induces significantly increased numbers of CD3+ T cells and macrophages in dorsal root ganglia, and infected cats exhibit pronounced changes in epidermal nerve fiber densities [73, 75]. FIV enters the CNS during the acute stages of infection and is present within the brain and cerebral spinal fluid [46, 49, 76]. The primary neuropathogenic effect of FIV infection within the CNS manifests as infiltration and accumulation of perivascular lymphocytes and macrophages (encephalitis), activation of

microglial cells and astrocytes (gliosis), and occasional neuronal loss with myelin degeneration [46, 49, 50, 76, 77]. This infiltration of inflammatory cells and consequences associated with immune activation within the CNS frequently results in clinically apparent neurologic deficits and gradual decline in CNS function, functionally manifesting as abnormal stereotypic motor behaviors, anisocoria, increased aggression, prolonged latencies in brainstem evoked potentials, delayed righting and pupillary reflexes, decreased nerve conduction velocities, and deficits in cognitive-motor functions [78-81].

FIV as a model to study HIV pathogenesis

Immune dysfunction

The primary immunodeficiency of FIV, a gradual and progressive decline in CD4+ T lymphocytes, is a hallmark feature of both natural and experimental infection, and perhaps the most fundamental feature to parallel HIV infection. During both FIV and HIV infection, CD4+ lymphocyte numbers decline over an extended asymptomatic phase, and is associated with an increase in activated CD8+ lymphocytes that have antiviral activity [82-85]. The net effect of this event is a decrease in the ratio of CD4+ cells to CD8 + cells (CD4:CD8), and is used as a clinical indicator of immunosuppression in both FIV and HIV infected patients [84-86]. Additionally, several studies have shown that FIV induces defects in immune function similar to HIV, such as a decreased proliferation response of T lymphocytes in response to mitogens, a deficit in the humoral immune response, and dysregulation of cytokine expression [10, 11, 56].

Clinical manifestations of FIV and HIV-induced immune dysfunction also include aberrant proliferative response and immune function. Neoplasia, primarily the development of large B-cell lymphoma, is frequently observed in HIV patients, and this pathologic finding is paralleled in FIV-infected cats during late stages of infection [7, 72, 87]. Furthermore, a significant complication of HIV infection, systemic immune activation, is likewise implicated in FIV and results in several mutual clinical manifestations such as lentiviral-induced periodontitis,

meningoencephalitis, and vaccine-induced enhancement of infection (outlined in detail below) [88-90].

Neurologic dysfunction

Previous studies have shown that both FIV and HIV enter the central nervous system (CNS) at acute stages of infection, either via trafficking of infected monocytes and lymphocytes, or by penetration of free virus across the blood-brain or blood-CSF barriers [49, 91-96]. Once present in the CNS, both FIV and HIV infection spread to microglia and astrocytes, which then serve as a reservoir for latent viral persistence [45, 49, 95-97]. Although multinucleated giant cells are rarely observed in the CNS during FIV infection, the fundamental neuropathologic finding of encephalitis is well-documented in both HIV and FIV infected patients, and resultant proliferation and activation of these cells (gliosis) is associated with neurodegenerative processes such as myelin degradation and neuronal injury/loss [46, 49, 74, 77, 98]. Thus, the clinical manifestations associated with neuropathology of FIV are likewise observed in HIV infection, and because of this, FIV has been repeatedly used as a model to investigate the pathogenesis of dementia and cognitive-motor processing deficits in AIDS patients. *In vitro* models of FIV have been useful to expand our understanding of role of calcium dysregulation and neural dysfunction during lentiviral infection, and have provided a unique system for the development neuroprotective treatments such as neurotrophin ligands, which prevent the delayed accumulation of intracellular calcium and decreased cytoskeletal damage of neuronal dendrites [49, 99]. Furthermore, because of the low natural prevalence and slow clinical course associated with lentiviral-induced neurologic dysfunction, experimental *in vivo* studies have been developed in the FIV model which accelerate neuropathogenesis (neonatal inoculation, inoculation with neurovirulent strains, direct intracranial inoculation), allowing increased opportunity to evaluate viral kinetics of CNS infection, neurovirulence determinants, and the

potential for novel treatments designed to decrease neurocognitive defects during HIV infection [76, 80, 99, 100].

The use of neurovirulent strains of FIV has also allowed for the investigation of neuropathogenic effects on the peripheral nervous system (PNS) as a model of HIV distal symmetric polyneuropathy (DSP), demonstrating rapid onset of peripheral neuropathy in FIV infected cats with axonal injury, macrophage activation, and detection of virus within the nerve [73, 101]. Indeed, FIV infection results in pathological events in the PNS that are very similar to HIV, including increased numbers of CD3+ T lymphocytes and activated macrophages in skin and dorsal root ganglia (DRGs) that are associated with increased expression of the pro-inflammatory cytokines, as well as changes in epidermal nerve fiber densities, indicative of axonal and myelin degeneration [73, 75]. Additionally, FIV has been useful in the evaluation of the neurotoxicity of antiretroviral toxic neuropathy (ATN), due to mitochondrial dysfunction associated with nucleoside analogue reverse transcriptase (NRTI) inhibitor treatment. Thus, FIV has the potential to expand our understanding of the role of the immunopathology and progression of neuropathy in FIV-infected cats.

SIV models of neuropathogenesis have widely been regarded as the premier model to study HIV-associated neurologic dysfunction (HAND), and has elucidated many mechanisms of neuroAIDS development such as acute CNS infection and the importance of monocyte/macrophage activation in driving CNS lesions [102-105]. Recently, the SIV model of neuroAIDS has been adapted to study peripheral neuropathy, and significant advances have been made that have implicated macrophages within dorsal root and trigeminal ganglia as a source of viral maintenance, in addition to their role in neuronal loss and neuronophagia [106, 107]. These findings are coupled with additional studies that have defined impaired mitochondrial function in distal axons which are more pronounced in ART-treated animals, indicating the potential for antiretroviral-mediated mitochondrial toxicity [108]. However, the SIV model of HAND is most commonly employed in rhesus macaques, and the sequelae of SIV

infection in an unnatural host presents unique disadvantages in studying the progression of neurologic disease, chiefly manifested as rapid progression to AIDS and increased severity of CNS inflammation which amplify pathology compared to HIV-infected humans [104, 105]. Furthermore, NHP studies are also limited by increased zoonotic risk to researchers, high cost associated with animal care and housing, the low number of animals available for research, and the potential for co-infection with a wide array of other pathogens, including rhesus rhadinovirus (RRV), lymphocryptovirus (LCV), simian cytomegalovirus (CMV), simian foamy virus (SFV), simian virus 40 (SV40), and rhesus papillomavirus (RhPV) [109, 110]. While it is impractical to presume that the FIV model could replace the SIV model of neuroAIDS, the caveats of using non-human primates to study pathogenesis of lentiviral-induced neurologic dysfunction present an important opportunity for the FIV model to supplement the repertoire of current investigational methodologies. As FIV infection presents a safer and more economical lentiviral model that more accurately recapitulates neuroAIDS progression in HIV-infected humans, such applications such as evaluation of ART-induced neurotoxicity, neurofibrillary tangle development, and calcium homeostasis during viral infection are at the forefront of advancing our understanding of HIV-associated neurologic dysfunction [46, 49].

Vaccine development

Considerable effort has been directed at the development of an anti-HIV vaccine strategy that can produce protective immunity in humans, and this effort has been paralleled in regard to FIV. A commercially available, whole inactivated virus vaccine containing two FIV subtypes (Fel-O-Vax FIV®) is currently licensed for use in the United States, and various reports have described virus neutralization and cellular immunity in a significant proportion of study animals [111-113]. However, the efficacy of this vaccine is still under debate, as recent studies and field evaluations have reported that the vaccine does not confer immunity against certain FIV strains (ie: FIV_{GL8}), and that the neutralizing antibody response and protective rate may be low in

certain cat populations (i.e. protection is not conferred to certain virulent recombinant strains of FIV) [114-117]. Other attempts at FIV vaccine development have either failed to induce protective immunity against FIV infection, or have resulted in increased susceptibility to infection via antibody-dependent enhancement or general immune activation [118-123].

The development of an anti-HIV vaccine has been impeded by a wide variety of similar complications, such as lack of efficacy or unanticipated side effects, as well as increased susceptibility to infection via analogous mechanisms of FIV vaccine enhancement (antibody-dependent viral enhancement or general immune activation) [124-130]. Indeed, vaccine-induced enhancement of viral infection has been previously reported in a large number of HIV studies [131-134], and has been shown to occur via antibody-dependent or antibody-independent mechanisms of complement activation [135-142], as well as an increase in general immune activation and/or expansion of lymphoid target cells [143-147]; features that have also been observed in FIV studies [118-123]. However, despite these setbacks in lentiviral vaccine development, there are many similarities in the disease course of HIV and FIV infection, and the use of the FIV model to circumvent these may have great potential to provide a translational model for the development of novel immunotherapies to protect from HIV infection in humans.

Traditionally, non-human primate (NHP) models have been at the forefront of anti-HIV vaccine development due to the similarities of SIV and HIV, and have revealed several promising vaccine targets such as *nef*-deleted SIV (which protects from wild-type SIV infection) and broad neutralizing antibodies utilizing chimeric SHIVs that express the HIV-1 envelope glycoprotein [148-151]. However, the successful outcome of these methods to prevent HIV infection in humans has been significantly impeded by various causes, such as restrictions on the use of live-attenuated HIV-1 in humans, as well as difficulty in producing a sufficiently efficacious neutralizing antibody response by vaccination [149]. Alternatively, various humanized mouse models have played a vital role in elucidating key aspects of the immune response to HIV, primarily through use of generally immunocompromised mice engrafted with

reconstituted human immune system tissues such as human fetal thymus and liver (scid-*hu-Thy/Liv*) or peripheral blood lymphocytes (scid-*hu-PBL*) [152]. These models have been used for key studies in HIV immunopathogenesis, including mechanisms of CD4+ T-cells loss, antiretroviral therapy response, and passive immunization with monoclonal antibodies to HIV envelope protein (and testing of *Env*-based vaccines) [110, 152-156]. However, because only certain parts of the human immune system can be reconstituted in humanized mouse models, interactions between the introduced human cells and the murine immune system cannot be evaluated in these hosts, nor the effects of HIV infection in non-hematopoietic tissues [152]. Although FIV is not as molecularly similar to HIV as the NHP model of SIV, and may not be as economical at the humanized mouse model, it nevertheless represents the sole prospect to fully evaluate the immune response during natural lentiviral infection. Furthermore, the availability of a commercially-available vaccine in cats with efficacy against FIV may provide important clues to improving the efficacy of anti-HIV vaccines, and the elucidation of the mechanisms associated with vaccination failure in analogous FIV and HIV models of immunotherapy may provide key insights into improving the efficacy of lentiviral vaccines.

HIV-induced oral disease

Oral manifestations of HIV are exhibited through various disease syndromes such as Oral Candidiasis (OC, “thrush”), Linear Gingival Erythema (LGE), Necrotizing Ulcerative Gingivitis (NUG), and Necrotizing Ulcerative Periodontitis (NUP) [157-159]. Despite the success of combinational antiretroviral therapy (cART) in diminishing HIV viral replication and prolonging immune function, lesions associated with systemic and local immune activation and opportunistic oral infections persist in HIV-infected patients [157, 160-162]. Furthermore, therapies used to treat HIV infection and HIV-induced oral disease have limited success, and treatment strategies do not eliminate HIV in persistently infected oral tissues [157, 163]. Previous studies have shown that CD4+ T-cells are rapidly and severely depleted from the

intestinal mucosa following HIV infection due to direct effects of targeted virus infection and virus-induced Fas-mediated apoptosis, resulting in loss of mucosal integrity and a reduced capacity to control potential pathogens at mucosal surfaces - thereby triggering local and systemic pro-inflammatory responses [88, 164-166]. Based upon the analogous microenvironments of the oral and gastrointestinal mucosa, the same effects of viral-induced immunosuppression is predicted to occur in the oral cavity, resulting in a chronic cycle of immune stimulation, leukocyte recruitment, and target cell infection that produces HIV-induced oral disease lesions [157, 167].

The FIV model is particularly well suited for studies of HIV-associated oral disease, as it not only parallels HIV in its structural, biochemical, and immunological properties, but it is also the only naturally occurring lentivirus to predictably induce oral lesions in its natural host, the domestic cat [1, 4, 9, 10, 61, 62]. Non-human primate (NHP) models of HIV do not reliably cause oral disease and are limited by zoonotic risk to researchers, high cost associated with animal care and housing, the low number of animals available for research, while humanized mouse models of HIV lack both the prevalence of oral lesions and the presence of tonsillar structures similar to humans [110, 168-170]. In contrast, FIV oral manifestations are common in naturally- and experimentally-infected cats [52, 61, 62], and the range of lesions seen include gingivitis, periodontitis, and feline chronic gingivostomatitis [62], with striking similarities to LGE, NUG, and NUP lesions noted in untreated HIV patients [1, 4, 83, 157, 171-174]. Furthermore, opportunistic infections detected in HIV-positive individuals are paralleled in feline oral disease syndromes [65, 175-184], and feline tonsillar tissues (palatine, pharyngeal, and lingual tonsils) are analogous to those in humans [169]. Coupled with recent advances in new generation cART protocols available for use in cats [185-188], the domestic cat model of FIV presents an easily manipulated animal model to evaluate drivers of immune dysfunction and microbial dyscrasias during HIV infection using a controlled *in vivo* study design.

Dissertation Research

The research presented in this dissertation builds upon the background outlined above and the potential for feline immunodeficiency virus to serve as a model to study HIV pathogenesis and viable therapeutic targets. Although it is accepted that FIV is primarily transmitted by biting, few studies have evaluated FIV oral infection kinetics and transmission mechanisms over the last 20 years. Furthermore, recent studies in HIV prove that oral transmission can occur, and that saliva from infected individuals contains significant amounts of HIV RNA and DNA. Therefore, in Chapter 1, I applied modern quantitative analyses to characterize FIV salivary viral kinetics and antibody secretions in order to investigate the pathogenesis of oral FIV infection and further our understanding of lentiviral oral disease and transmission. The results of this research demonstrate that (i) oral lymphoid tissues serve as a site for enhanced FIV replication, resulting in accumulation of FIV particles and FIV-infected cells in saliva, and (ii) failure to induce a virus-specific oral mucosal antibody response, and/or viral capability to overcome inhibitory components in saliva may perpetuate chronic oral cavity infection. Most importantly, these results provide a model of oral FIV pathogenesis and suggest alternative diagnostic modalities and translational approaches to study oral HIV infection.

Because FIV and HIV utilize parallel modes of receptor-mediated entry, the ability of neutralizing antibodies to the FIV binding receptor (CD134) to block FIV infection ex vivo presents a unique opportunity for the development of anti-HIV immunotherapies which utilize anti-receptor antibodies to block viral infection. In Chapter 2, I immunized cats with soluble CD134, recombinant FIV-SU protein, and/or CD134+SU complexes prior to challenge with FIV to determine if vaccination with CD134-SU complexes could induce protection against FIV infection. Immunization induced production of anti-CD134 and anti-SU antibodies in vaccinated cats, and purified anti-CD134 and anti-SU antibodies significantly inhibited FIV infection *in vitro.* However, no vaccine combination protected cats from FIV infection *in vivo* and vaccination induced high titers of antibodies directed at vaccine by-products relative to target antigens. The

results of this research reinforce the need to monitor components of vaccine preparations, and emphasize that vaccination may induce proliferation of susceptible target cells and enhancement of heat-labile serum components that counteract neutralizing antibodies.

Both HIV and FIV induce lifelong infection in their respective hosts, and may result in wide a spectrum of immunodeficiency-related opportunistic diseases. Both prednisolone and cyclosporine A (CsA) are commonly used clinically to treat lymphoproliferative and immune-mediated diseases in these patients, but the impact of these compounds on infection has not been well documented, and their understanding immunomodulatory effects on viral replication and persistence is critical to guide safe and effective use of these therapies. Therefore, in Chapter 3, I administered immunosuppressive doses of prednisolone or CsA to cats chronically infected with FIV and monitored alterations in hematological parameters and FIV viral/proviral loads in response to therapy. Interestingly, both treatments caused (i) acute increases in CD4+ lymphocytes, (ii) increased FIV viremia, and (iii) significant alterations in cytokine expression that favored a shift toward a Th2 response. The results of this research highlight the potential for immunosuppressive drug-induced perturbation of FIV replication and underscores the need for consideration of chronic viral infection status when prescribing immunomodulatory medications.

As a result of my graduate studies, I developed a strong interest in the capacity for FIV to serve as an animal model to study HIV-induced opportunistic disease. Commonly used animal models for HIV, including SIV/SHIV infections of non-human primates (NHPs) or HIV infections in humanized mice, do not reliably incite oral lesions. In contrast, gingivitis and periodontitis are primary clinical signs associated with untreated natural and experimental FIV-infection, and are principal attributes of this model that may be exploited to investigate pathogenic mechanisms involved in the perturbation of the oral immune system and microbial environment. Therefore, in Chapter 4, I present findings of a pilot study in which I investigated changes in the oral microbiota and oral immune system during FIV infection, and outline future

directions and research goals for my career investigating the pathogenic mechanisms of HIV-induced oral disease using the FIV model. By assessing FIV-associated changes in clinical status, oral microbiota, local and systemic viral burden, and immune profile under such treatment protocols, future studies implementing the feline model of lentiviral-induced oral disease may provide a cornerstone to expand our understanding of the complex interactions between HIV infection, oral immune dysfunction, and the perturbations to the oral microbiota that occur in the context of HIV infection.

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CHAPTER 1. PATHOGENESIS OF ORAL FIV INFECTION[1]

Summary

Feline immunodeficiency virus (FIV) is the feline analogue of human immunodeficiency virus (HIV) and features many hallmarks of HIV infection and pathogenesis, including the development of concurrent oral lesions. While HIV is typically transmitted via parenteral transmucosal contact, recent studies prove that oral transmission can occur, and that saliva from infected individuals contains significant amounts of HIV RNA and DNA. While it is accepted that FIV is primarily transmitted by biting, few studies have evaluated FIV oral infection kinetics and transmission mechanisms over the last 20 years. Modern quantitative analyses applied to natural FIV oral infection could significantly further our understanding of lentiviral oral disease and transmission. We therefore characterized FIV salivary viral kinetics and antibody secretions to more fully document oral viral pathogenesis. Our results demonstrate that: (i) saliva of FIV-infected cats contains infectious virus particles, FIV viral RNA at levels equivalent to circulation, and lower but significant amounts of FIV proviral DNA; (ii) the ratio of FIV RNA to DNA is significantly higher in saliva than in circulation; (iii) FIV viral load in oral lymphoid tissues (tonsil, lymph nodes) is significantly higher than mucosal tissues (buccal mucosa, salivary gland, tongue); (iv) salivary IgG antibodies increase significantly over time in FIV-infected cats, while salivary IgA levels remain static; and, (v) saliva from naïve Specific Pathogen Free cats inhibits FIV growth *in vitro.* Collectively, these results suggest that oral lymphoid tissues serve as a site for enhanced FIV replication, resulting in accumulation of FIV particles and FIV-infected cells in saliva. Failure to induce a virus-specific oral mucosal antibody response, and/or viral capability to overcome inhibitory components in saliva may perpetuate chronic oral cavity infection. Based upon these findings, we propose a model of oral FIV pathogenesis and suggest alternative diagnostic modalities & translational approaches to study oral HIV infection.

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Background

Feline immunodeficiency virus (FIV) is a naturally occurring lentivirus of domestic cats and non-domestic feline species that is genetically, structurally, and biochemically similar to human immunodeficiency virus (HIV), thereby providing a valuable animal model for studying HIV infection [1-6]. While HIV transmission typically occurs via parenteral or transmucosal venereal contact, oral transmission of HIV via receptive oral intercourse, breast feeding, and human bites has been well-documented, especially in the presence of a disrupted oral mucosal barrier (ie: epithelial ulceration), inflammation, and/or concurrent oral infections [7-15]. These alternative modes of HIV transmission represent a significant health concern to at-risk individuals, including recipients of oral intercourse, infants of HIV-positive mothers, and dental health professionals. Additionally, recent studies have shown that both HIV viral RNA and proviral DNA are detectable in saliva of infected individuals, and salivary RNA levels are correlated with levels in circulation; facts that have been largely overlooked in studies of HIV pathogenesis [16, 17]. Furthermore, HIV antibodies in saliva may lend themselves to noninvasive diagnostic methodologies via rapid detection of saliva specific antibodies, and HIV therapies that incorporate antiviral properties of saliva have been considered, including the use of inhibitory molecules as components of commercial lubricants [14, 18-23].

HIV, Simian Immunodeficiency Virus (SIV), and FIV have all been shown to target lymphocytes of the alimentary tract early during viral infection, resulting in long term consequences for mucosal immune function [24-28]. Several studies have linked lentiviral-induced gastrointestinal mucosal insult to chronic immune activation associated with infection via translocation of bacteria or bacterial antigens [25, 29-32]. It is feasible that the oral cavity, with its rich lymphoid tonsillar structures, is similarly impacted by lentiviral infection, and may serve as important reservoir for lentiviral persistence [27]. Unlike primate lentiviral infections, which are primarily considered to be transmitted venereally, during maternal-offspring interactions, or via exchange of blood products, FIV is believed to be primarily transmitted from

cat to cat via bite wounds during antagonistic or mating interactions. However, vertical transmission of FIV has been experimentally shown via colostrum and milk, and may provide an appropriate model to study mother-to-offspring transmission of HIV [33, 34]. Additionally, FIV periodontitis/gingivitis is a hallmark of both naturally occurring and experimental FIV infections, and characteristic lesions of FIV gingivitis are similar to those frequently reported for HIV-associated dental disease, such as linear bands of erythema along the gingival margin and ulcerative to necrotizing lesions within the gingival and buccal mucosa [1, 6, 35-44]. Despite the fact that FIV associated gingival disease is widely diagnosed, very few studies have assessed mechanisms of FIV salivary excretion, transmission, and pathogenesis [42, 45]. Further, recent reports document that FIV infection is poorly or rarely transmitted among cats in multi-cat households, calling into question whether FIV salivary transmission is limited by innate immune barriers in stable social groups [46].

Studies of FIV salivary transmission and pathogenesis of oral disease have the potential to reveal sites of lentiviral replication and persistence, mechanisms of salivary excretion, and oral lentiviral immune responses and diseases - thereby expanding our knowledge of the pathogenesis of FIV disease and mechanisms of orally transmitted pathogens. Although it is widely accepted that FIV is primarily transmitted by biting, and much less efficiently during social grooming, few studies have evaluated FIV oral infection kinetics and transmission mechanisms over the last 20 years. Modern quantitative analyses applied to natural FIV oral infection could significantly further our understanding of the temporal events that occur during FIV oral infection pathogenesis, and may thus aid in the design of improved diagnostics, vaccines and vaccine modalities, choice of adjuvants, and design and delivery of antiviral agents for FIV. As salivary excretion and kinetics of HIV infection is also understudied, identification of similarities and differences between FIV and HIV oral pathogenesis can also ultimately enhance our understanding of HIV-associated oral disease.

Previous studies of FIV oral transmission and pathogenesis have sought to characterize viral excretion mechanisms by detection of saliva FIV RNA, DNA or antibodies, or though isolation of virus in saliva or oral tissues [42, 44, 45, 47, 48]. However, because much of this work was performed before the development of quantitative analyses, there has been limited understanding of the temporal events that take place in the oral cavity during FIV infection. To address these knowledge gaps, we opportunistically evaluated salivary transmission characteristics in cats intravenously inoculated with a well-characterized immunopathogenic strain of FIV (FIV_{C36}) [36, 49]. Blood, saliva, and tissue samples from 18 cats enrolled in a clinical trial were evaluated for viral RNA and proviral DNA by real-time quantitative PCR (qPCR) analysis, in addition to other clinical and hematologic parameters associated with disease progression. Additionally, quantification of total IgA/IgG and FIV-specific IgA/IgG antibodies in saliva was performed using microsphere immunoassay (MIA), and histologic changes in tissues were assessed. Saliva from naïve SPF (specific pathogen free) cats was incubated with a viral stock of FIV_{C36} and tested for capacity to inhibit viral growth in Crandall feline kidney (CRFK) cell cultures. Coincident studies evaluating hematologic and clinical features of infection in these same cats allowed comparisons of oral and peripheral characteristics of viral infection [50, 51].

This study represents the first quantitative analysis of FIV RNA and DNA in saliva and oral tissues of FIV-infected cats, evaluated in conjunction with quantification of anti-FIV IgG and IgA antibodies in saliva and assessment of concurrent alterations in the oral mucosa to provide a comprehensive assessment of the pathogenic events that contribute to the development of oral FIV infection. Overall, our findings characterize sites of FIV replication and persistence, and reveal that oral cavity viral kinetics and antibody responses mirror that of the periphery; suggesting that immunological and tissue level barriers are ineffective at inhibiting FIV viral replication and excretion at the oral mucosal surface.

Materials and Methods

Ethics Statement

This study was approved by the Colorado State University Institutional Animal Care and Use Committee; 09-064A-01 - New Therapies for Retroviral Diseases. Colorado State University's animal care program is licensed by the United States Department of Agriculture (USDA), accredited by Association for Assessment and Accreditation of Laboratory Animal Care (AAALA) International, and holds an Office of Laboratory Animal Welfare (OLAW) assurance (A3572-01). In accordance with the approved IACUC protocol, if any animal exhibited significant clinical abnormalities (vomiting, diarrhea, lethargy, refusal of food > 24 hours, labored breathing, dehydration, >5% weight loss from one week to the next), a complete physical exam with additional bloodwork was performed. In the case that a study animal exhibited severe discomfort or symptoms listed above which worsened and did not respond to symptomatic therapy, humane euthanasia was to be performed and followed by a complete necropsy. Time points for euthanasia of study animals was determined by previously established time intervals to assess acute FIV infection, or at the discretion of a clinical veterinarian based upon the severity of any associated clinical signs and/or response to symptomatic therapy. Humane euthanasia procedures were conducted by phenobarbital overdose in accordance with IACUC protocols and American Veterinary Medical Association (AVMA) Guidelines for the Euthanasia of Animals. Prior to euthanasia, all study animals were anesthetized by intramuscular injection of ketamine (20mg/kg) and acepromazine (2mg/kg) to minimize animal suffering and distress. No animals died without euthanasia during this study as a result of experimental procedures. All study animals were monitored daily by animal care personnel for development of clinical signs of FIV infection. Any animals exhibiting clinical signs either associated with FIV infection or other untoward condition were evaluated and treated as prescribed by the clinical veterinarian. One animal developed clinical signs at week 6 post-infection, and was humanely

euthanized at the discretion of the attending veterinarian. All other animals were euthanized at the end of the experimental study.

In vivo protocol

Twenty-four, 8-11 week old, specific pathogen free (SPF) cats, procured from Cedar River Laboratories, Mason City, IA, and the Andrea D. Lauerman Specific Pathogen Free Feline Research Colony, Fort Collins, CO, were housed within barrier rooms in accordance with Colorado State University (CSU) IACUC-approved protocols at a CSU AAALAC-international accredited animal facility. All animals were part of an anti-retroviral protocol, and were acclimated to the facility for 2 weeks prior to initiation of the study. At day 0, eighteen cats were intravenously inoculated with 1ml of a 1:10 dilution of a previously characterized FIV_{C36} viral stock that is acutely immunopathogenic and induces reproducible high titer viremia [37, 49]. Six additional cats were sham inoculated as negative controls. Over the course of the study, 12 of the FIV-infected cats received experimental anti-retroviral treatment, while 6 FIV-infected cats and the 6 sham-inoculated cats received no anti-retroviral treatment and served as positive and negative controls, respectively.

Evaluation of viral RNA and DNA in blood and saliva

Blood and saliva samples were collected from cats at 7-day intervals, beginning at 15 days post-infection and ending at 92 days post-infection. Blood samples were obtained as previously described [50]. Saliva was obtained from under the tongue and cheek pouches of each cat using sterile cotton swabs, which were immediately broken off into 1.5ml microcentrifuge tubes containing 200µL of RNAlater Solution (Ambion, Austin, TX) and stored at -20°C. At processing, stored swabs were thawed at room temperature, vortexed vigorously for 1 min, and centrifuged at 400 x g for 1 min. To collect saliva from the swab tip, swabs were inverted using sterile forceps, placed back into microcentrifuge tubes, spun at 2000 rpm for 2 min, and then discarded, leaving the saliva/RNAlater solution in the microcentrifuge tube.

Viral RNA was extracted from saliva using an RNAqueous total RNA isolation kit (Ambion, Austin, TX), according to manufacturer's instructions. Samples were eluted in 50µL and ethanol precipitated overnight at -20°C (2.5 vol 100% ethanol, 0.1 vol 3M sodium acetate, and 1.0µL glycogen). Precipitated RNA was pelleted at 18,000 x g for 20 min at 4°C and re-suspended in 20µL of RNA Storage Solution (Ambion, Austin, TX). RNA from each sample was converted to cDNA using the RETROscript reverse transcription kit (Ambion, Austin, TX). The total volume of extracted RNA was transferred into two 20µL reactions and converted using random decamer primers and following manufacturer's instructions for reverse transcription without heat denaturation of RNA. FIV-C was detected by qPCR in triplicate using an iQ5 thermocycler (Bio-Rad, Hercules, CA) with reaction components, cycling parameters, and FIV-C primers and probes as previously described [52, 53]. To quantitate viral copy number in each reaction, a six-point standard curve was generated by diluting FIV-C virus stock in a 10-fold dilution series into RNAlater solution. Each dilution was then extracted and converted to cDNA as described above, and assigned a copy number value based on comparison to a FIV C gag plasmid standard curve ranging from 10^5 to 10^{-1} copies per reaction. A Ct threshold was set according to the run data for each plate, and Ct values greater than those of negative controls were included in the analysis. The resulting copy number data for each sample was analyzed using Prism 4 (GraphPad Software, La Jolla, CA). Triplicate values for each sample were averaged, and calculated to determine viral copies per mL saliva. Standard error was calculated for each treatment group at each time-point. Viral RNA was extracted from blood and quantified by qPCR as previously described [50].

Proviral DNA was extracted from saliva of all cats for time-points 43 and 64 days post-inoculation using a DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) and a user-adapted protocol for purification of total DNA from animal saliva [54]. Samples were eluted in 100µL, and then ethanol precipitated (2.5 vol 100% ethanol, 0.1 vol 3M sodium acetate, and 1.0µL glycogen) overnight at -20°C. Precipitated DNA was then pelleted at 18,000 x g for 20 min at

4°C and re-suspended in 20µL H_2O. FIV-C provirus was quantitated by qPCR in duplicate using an iQ5 thermocycler (Bio-Rad, Hercules, CA), FIV-C primers and probe, and FIV-C plasmid standards as previously described [52, 53]. Proviral copy number was then normalized to copies per 10^6 cells by quantitating genomic DNA using real-time qPCR targeted to feline GAPDH. A GAPDH plasmid standard curve was prepared as previously described [55] and the following primers were used for qPCR: GAPDH-F forward primer, 5'-AAGGCTGAGAACGGGAAAC -3'; GAPDH-R reverse primer, 5'-CATTTGATGTTGGCGGGATC– 3'. GAPDH qPCR for each sample was set up in 25 µl using the following reaction components: 2 µl sample DNA, 8.5 µl de-ionized water, 1 µl GAPDH-F, 1 µl GAPDH-R, and 12.5 µl SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA). Reaction mixtures were denatured at 95°C for 30 seconds and then followed by a two-step reaction cycle protocol of 40 cycles that alternated between 95°C for 5 seconds and 60°C for 10 seconds. Proviral DNA was extracted from blood, quantified by qPCR, and normalized to a GAPDH standard curve to determine the number of cell equivalents per DNA sample. Proviral copy number per cell was calculated as previously described [49, 50].

To confirm the presence of infectious FIV particles in saliva of infected cats, duplicate cell cultures consisting of GFox cells (CrFK cells overexpressing CD134) were established in 96-well plates at 20,000 cells/well and allowed to attach at 37°C overnight [53, 56]. GFox cell cultures were maintained at 37°C and 5% CO_2, in 240 µl of culture medium composed of Dulbecco's modified Eagle's medium (DMEM) with GlutaMAX-1, 10% fetal bovine serum (FBS), and 1x penicillin-streptomycin (10,000 U/liter penicillin and 10,000 µg/liter streptomycin), as well as 1 µg/ml of Fungizone® (Amphotericin B; Life Technologies) [57]. At day 0, 10 µl of saliva from 5 FIV-infected and 1 sham-inoculated (uninfected) cats (collected as previously described) were added to the previously established Gfox cell culture, bringing the total volume to 250 µl (1:25 dilution of saliva). A parallel set of wells containing Gfox cells without virus was incubated with media only (negative control). Cell cultures were then incubated at 37°C for 12 hours, at

which point all culture media was removed from each well, discarded, and replaced with 250 µl of fresh culture media. GFox cells were visually inspected at days 7, 10, 14, 21, and 28 post-inoculation by inverted light microscopy for evidence of cell growth, attachment, syncytial cell formation, detachment, and cell death. At days 7, 14, 21 and 28 post-inoculation, 125 µl of supernatant was removed from each well, frozen at -80°C, and replaced with 125 µl of fresh culture media. The supernatant collected from each well and each time point was then assayed for the detection of FIV p26 antigen by capture ELISA using previously described protocols [58]. For each treatment, absorbance (Abs) was measured at 450 nm and a background threshold value was established by calculating the mean Abs of FIV-negative (naïve) saliva plus 3 standard deviations. All Abs values above this threshold were considered positive.

Evaluation of viral RNA and DNA in oral tissues

DNA was extracted from frozen necropsy tissues using a DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA), and eluted in 200µl AE buffer. All samples were adjusted to 20ng/µl using 1X TE buffer and FIV-C provirus was quantitated by qPCR using previously described reaction components, cycling parameters, and FIV-C primers and probes [52, 53]. Proviral copy number within these tissues was quantitated using a standard curve with 1:10 serial dilutions of FIV-C gag plasmid into 1X TE buffer, ranging from 10^5 copies to 10^1 copies per reaction. Resulting proviral copy numbers were normalized to copies per 10^6 cells based on the total amount of DNA present in the reaction (100ng) as previously described [49].

RNA was extracted from frozen necropsy tissues using an RNeasy Mini Kit (Qiagen, Valencia, CA) and tissue homogenizer (MP Biomedicals, Solon, OH). Superscript II (Invitrogen), random primers (Invitrogen), and RNase Out (Invitrogen) were used to synthesize cDNA by reverse transcription. Real-time qPCR quantification of viral RNA was then performed on an iQ5 thermocycler (Bio-Rad, Hercules, CA) using previously described reaction

components, cycling parameters, and FIV-C primers and probes [52, 53]. Viral RNA was normalized to GAPDH expression as previously described [46] using the ΔΔCT method [59].

Histological evaluation

Necropsy was performed on sixteen FIV-inoculated cats at 92 days post inoculation; one sham-inoculated cat was coincidently necropsied to provide FIV negative control tissues. Palatine tonsils, retropharyngeal lymph nodes, submandibular salivary glands, tongue and buccal mucosa were collected. Necropsy tissues were then halved and placed into either 1ml tubes and frozen at -80°C, or into standard tissue cassettes that were then fixed in 10% neutral-buffered formaldehyde for 24 hours prior to trimming and processing for histology. Five µm paraffin sections were collected onto charged slides (Superfrost; Colorado Histo-Prep, Fort Collins, CO), and one slide of each tissue was stained with hematoxylin and eosin (H & E) for microscopic examination. Tissues were scored using the following criteria: 0 = no apparent pathology/change, 1 = minimal change (minimally increased numbers of small lymphocytes, plasma cells, macrophages, and/or mast cells), 2 = mild change (mild inflammation, edema, and/or parafollicular expansion, secondary follicle formation, and presence of tingible body macrophages within lymph nodes), 3 = moderate change (as previously described, but more moderately extensive), 4 = marked changes (as previously described, but with severe inflammation, edema, and/or lymphoid reactivity)

Quantification of IgA and IgG antibodies in saliva

Saliva from FIV-infected and sham-inoculated cats was evaluated for circulating antibodies at days 22, 36, 57, and 71 post-inoculation. Total IgA and IgG from saliva were quantified using microsphere immunoassay (MIA) protocols involving conjugation of magnetic microspheres with IgA or IgG capture antibodies [60]. Following conjugation protocols, a hemocytometer was used to determine microsphere concentrations and protein coupling was confirmed via incubation of microspheres with primary antibodies and/or PE-conjugated

detection antibodies [51]. Successful coupling of antibody to microspheres was determined by a median fluorescence intensity (MFI) of >2,000. Saliva samples from FIV-infected and negative control cats were diluted 1:100 and 1:1000 in assay buffer for detection of IgG and IgA, respectively. These samples were then incubated in duplicate with approximately 2,500 conjugated beads per well in untreated, round-bottom 96-well plates [51, 60]. Total IgG and IgA antibody concentrations were calculated from an 8-point standard curve (2-fold dilution series, run in duplicate) using the MFI obtained from ≥100 microspheres per analyte per well (Bio-Plex™ Manager 5.0). Reagent concentrations, volumes, incubation times, acceptable standard recovery, and data analysis were performed as previously described [51, 60].

FIV-specific antibodies were detected using microsphere immunoassay (MIA) protocols involving conjugation of magnetic microspheres with FIV-specific capsid (CA) or envelope surface glycoprotein (SU_{C36}-Fc) recombinant proteins [51, 60]. Following conjugation protocols, a hemocytometer was used to determine microsphere concentrations, and protein coupling was confirmed via incubation of microspheres with primary antibodies and/or PE-conjugated detection antibodies [51]. Successful coupling was determined by a median fluorescence intensity (MFI) of >2,000. All saliva samples from FIV-infected and negative control cats were diluted 1:10 in assay buffer and then incubated in duplicate with approximately 2,500 conjugated beads per well. All samples were assayed in conjunction with FIV-C and naïve reference samples diluted 1:50 in assay buffer, as well as four diluent control wells per experiment. The MFI was calculated from ≥100 microspheres per analyte per well (Bio-Plex™ Manager 5.0) and then used for data analysis. All reagent concentrations, volumes, incubation times, acceptable standard recovery, and data analysis were as previously described [51, 60].

In vitro salivary inhibition of viral infection

Approximately 80µl of saliva was obtained from each of 6 healthy, non-infected SPF cats, and was pooled and used in same day *in vitro* experiments. Duplicate cell cultures

consisting of GFox cells (CrFK cells overexpressing CD134) were established in 24-well plates at 180,000 cells/well and allowed to attach at 37°C overnight [53, 56]. GFox cell cultures were grown at 37°C in 5% CO_2 in culture medium composed of Dulbecco's modified Eagle's medium (DMEM) with GlutaMAX-1, 10% fetal bovine serum (FBS), and 1x penicillin-streptomycin (10,000 U/liter penicillin and 10,000 µg/liter streptomycin), as well as 1 µg/ml of Fungizone® (Amphotericin B; Life Technologies) [57]. At day 0, 50,000 $TCID_{50}$ of FIV_{C36} was incubated with varying dilutions of saliva (1:100, 1:50, or no saliva (positive control)) in 1 ml of fresh culture media for 1 hour at 37°C [61]. Following incubation, infected media was pipetted onto Gfox cells plated in 1 ml of culture media, bringing the total volume in each well to 2 ml. Cell cultures were then incubated at 37°C for 12 hours, at which point all culture media was removed from each well, discarded, and replaced with 2ml of fresh culture media. At days 4, 6, 8 and 10 post-inoculation, 1ml of supernatant was removed from each well, frozen at -80°C, and replaced with 1 ml of fresh culture media. The supernatant collected from each well and each time point was then assayed for the detection of FIV p26 antigen by capture ELISA, measured at an absorbance of 450nm in 96-well flat bottom plates as previously described [58]. Percent inhibition was calculated from mean absorbance values (Abs) using the formula $[(X - Y)/X] \times 100$, where X is fraction of cells infected in the absence of saliva (positive control) and Y is the fraction of cells infected in the presence of the saliva (1:100 or 1:50 saliva) [62]. For each plate, a parallel set of wells containing cells without virus was incubated with corresponding dilutions of saliva (1:100, 1:50, or no saliva (negative control)). GFox cells were visually inspected at days 4, 6, 8, and 10 post-inoculation by inverted light microscopy for evidence of cell growth, attachment, syncytial cell formation, detachment, and cell death.

Statistical Analyses

Kruskal–Wallis test, Pearson correlations, ANOVA, and repeated-measures ANOVA (RM-ANOVA) were used to compare differences in salivary viral and/or proviral load among FIV-

infected individuals, between sample type (saliva, plasma/PBMC), for each tissue individually, and between tissue types (lymphoid versus mucosal). RM-ANOVA was used to assess antibody responses over time and treatment after log_{10}-transformation. For *in vitro* experiments, RM-ANOVA with multiple comparisons was used to evaluate differences in mean absorbance values and percent inhibition among treatment groups over time. For all significant results, pair-wise comparisons were made by post-hoc analysis. Analyses were conducted in R (http://www.r-project.org/) or using GraphPad Prism 6.0 software (La Jolla, CA). P-values < 0.05 were considered significant.

Results

FIV viral RNA and proviral DNA is present in saliva of infected cats

Cats infected with FIVC36 experienced plasma viremia, CD4/CD8 inversion and other clinical signs typically associated with acute pathogenic FIV infection [50]. Quantitative PCR analysis of saliva from FIV-infected cats revealed levels of viral RNA equivalent to that of plasma, with a trend for salivary viral load to be higher than plasma viremia over time **(**source p=0.119, interaction p=0.165, **Fig 1.1A)**. Although present in lower quantities, saliva from FIV-infected cats also contained detectable quantities of proviral DNA **(Fig 1.1B)**. Concurrent analysis of housekeeping gene GAPDH in saliva indicated that cellular DNA was abundant in saliva, was used as a proxy to determine cell equivalents per ml of saliva (range $3.19x10^5$-$8.72x10^6$ cells/ml), and allowed us to calculate proviral DNA copies per saliva cell equivalent. FIV DNA levels tended to be approximately 10-fold lower in saliva than in peripheral PBMCs, although this trend did not differ over time (source p=0.024, interaction p=0.214). Interestingly, when DNA proviral and RNA viral loads were normalized to the number of copies per 1ml of saliva, the ratio of FIV RNA to DNA in saliva of infected cats was significantly higher over time than in the peripheral circulation, suggesting the oral cavity is an important site for persistent and enhanced FIV replication relative to peripheral circulation **(**source p=0.001, interaction p=0.036, **Fig 1.1C)**.

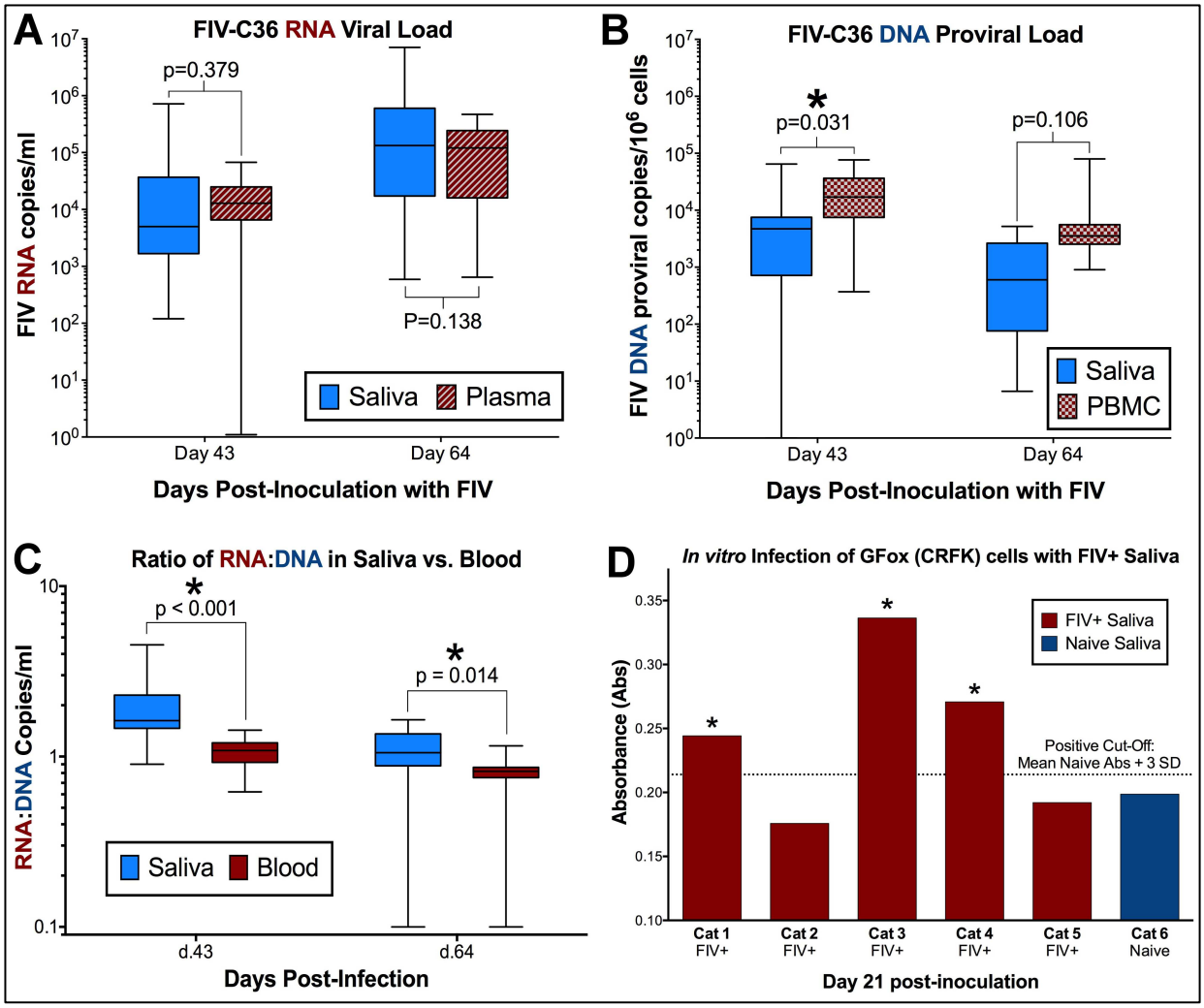

Fig 1.1. FIV viral RNA and proviral DNA are detected in saliva of infected cats. (A) Mean FIV viral RNA in saliva of infected cats is greater than observed in plasma (Mean: 446,000 copies/ml saliva, 85,100 copies/ml plasma). There is a trend (p=0.165) for salivary viral load to be higher than plasma over the course of the study (RM-ANOVA). **(B)** Proviral DNA is present in saliva of infected cats, although levels tended to be 10-fold lower than circulating PBMC (p=0.214; RM-ANOVA). **(C)** The ratio of FIV RNA to DNA is significantly higher in saliva than circulating levels in blood over the entire study (p=0.036) and at each time point measured (day 43, p=<0.001; day 64, p=0.014; RM-ANOVA with multiple comparisons). **(D)** Saliva from FIV-infected cats contains infectious FIV virus, as evidenced by FIV replication in GFox (CRFK) cells and the production of FIV viral particles following inoculation of saliva (day 21 post-inoculation, positive Cutoff = Mean Naïve Abs + 3*SD).

FIV is recovered from saliva of infected cats

The presence of infectious virus in saliva was confirmed by FIV p26 ELISA of tissue culture supernatants collected from GFox cells incubated for 21 days with saliva from 5 FIV-infected cats collected at 28 days post-FIV-infection **(Fig 1.1D)** [56]. Cells from infected cultures also exhibited characteristic cytopathic effect at this timepoint. This collection point correlated

with high plasma viremia in FIV-infected cats [50], and confirms results reported by Matteucci et al. in 1993 [45] that infectious FIV is present in saliva of infected cats. FIV-negative saliva collected from sham-inoculated (naïve) cats was included as a negative control.

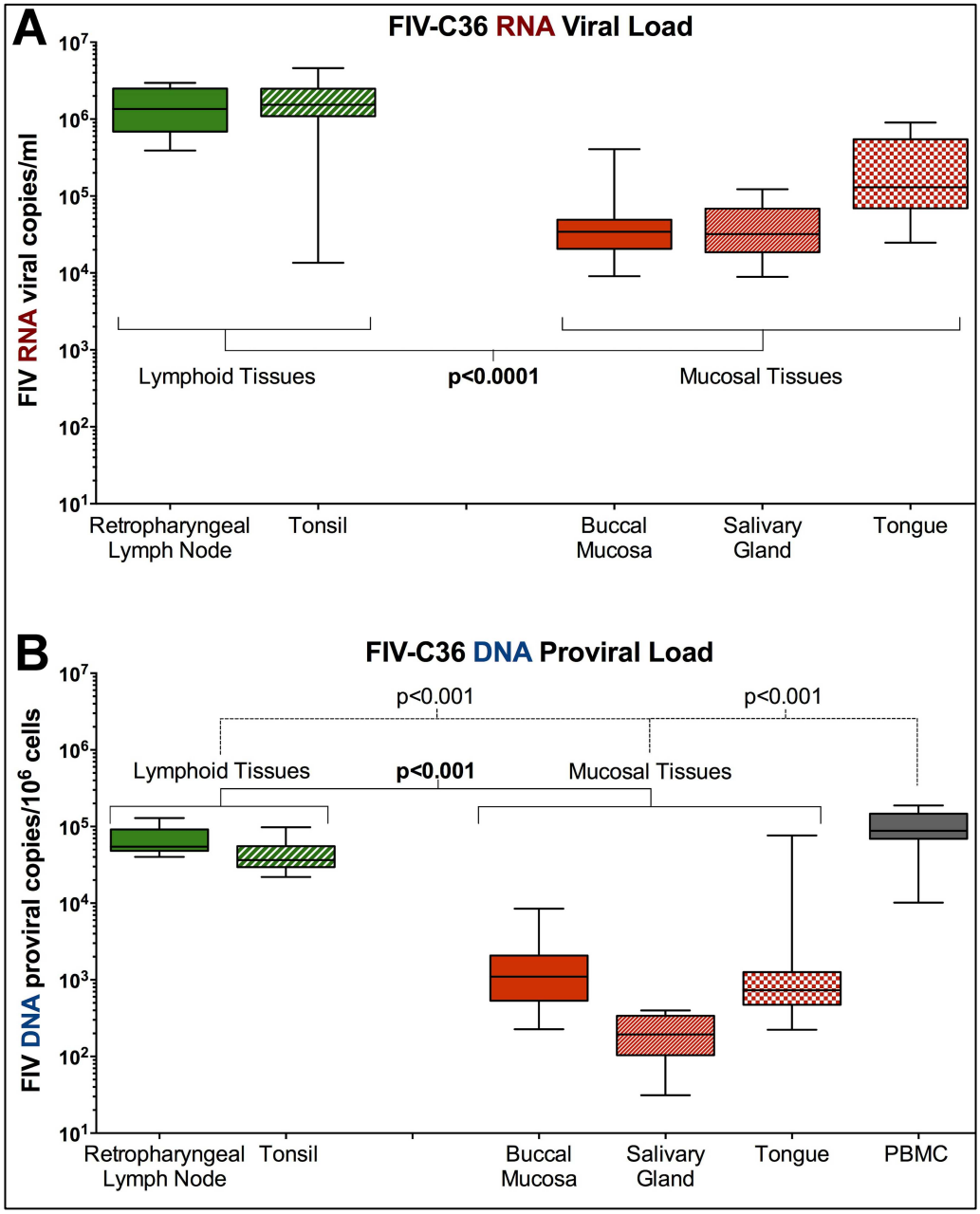

Fig 1.2. FIV RNA and DNA in oral lymphoid tissues is significantly higher than levels in non-lymphoid oral tissues. (A) FIV *RNA* levels in oral lymphoid tissues (retropharyngeal LN and tonsil) are significantly higher than non-lymphoid oral tissues (tongue, buccal mucosa, salivary gland) ($p<0.0001$), suggesting that oral lymphoid tissues serve as the site of viral replication and release into saliva. **(B)** FIV *proviral DNA* levels in oral lymphoid tissues are not as high as in circulating PBMC, but are significantly greater than in non-lymphoid tissues ($p<0.0001$), indicating that lymphoid organs may serve as oral reservoirs of FIV latency and persistence.

FIV viral and proviral loads are higher in oral lymphoid tissues

The retropharyngeal lymph node, tonsil, tongue, buccal mucosa, and salivary gland of FIV-infected cats contained appreciable quantities of viral RNA **(Fig 1.2A)**. FIV RNA present within oral lymphoid tissues (retropharyngeal lymph node, tonsil) was significantly higher than oral mucosal tissues ($p<0.001$; tongue, buccal mucosa, salivary gland). Proviral DNA was also detected in all oral tissues, and oral lymphoid tissues contained significantly more FIV DNA than oral mucosal tissues ($p<0.001$) **(Fig 1.2B**), however, the quantity of FIV DNA in oral lymphoid tissues and mucosal tissues was lower than in circulating PBMC ($p<0.001$). Viral RNA and proviral DNA were not detected in the saliva or oral tissues from naïve animals (data not shown).

FIV infection induces pathological changes in oral tissues

All tissue sections from a sham-inoculated control animal were histologically unremarkable (**Fig 1.3A**). Of the 16 FIV-infected animals, the retropharyngeal lymph node (n=10) (**Fig 1.3B**) and the palatine tonsil (n=11) (**Fig 1.3C**) exhibited mild to moderate lymphoid hyperplasia, characterized by multifocal enlarged germinal centers with thin mantle zones and a frequent “starry-sky” appearance due to numerous tingible body macrophages. Eight FIV-infected individuals exhibited mild (n=7) to moderate (n=1) lymphoplasmacytic and histiocytic glossitis. In three of these animals, the submucosa was multifocally expanded by mild to moderate numbers of mast cells (**Fig 1.3D**). Additionally, 4 FIV-infected animals had a mild, multifocal, lymphoplasmacytic stomatitis of the buccal mucosa, with scattered mast cell infiltration observed in the same 3 animals in which the tongue was similarly affected (**Fig 1.3E**). Small numbers of scattered small lymphocytes and plasma cells were occasionally observed at the periphery and surrounding individual acini of the salivary glands in 7 of the FIV-infected cats (**Fig 1.3F)**. Overall, the degree of histologic change in oral lymphoid tissues (retropharyngeal lymph node and tonsil) was significantly higher than in non-lymphoid tissues (tongue, buccal

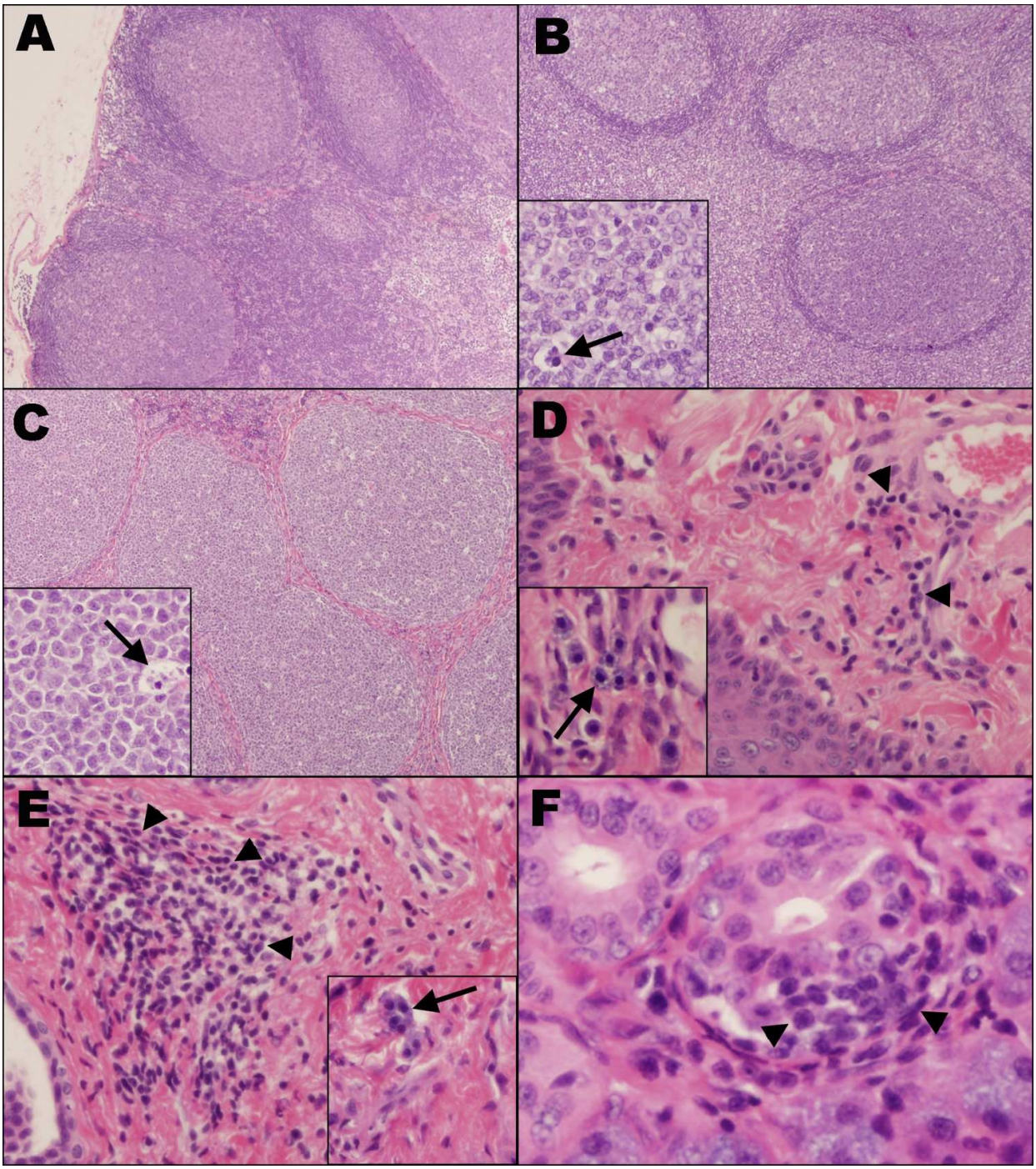

Fig 1.3. FIV induces mild to moderate pathology in oral tissues. (A) Retropharyngeal lymph node. Sham-inoculated control, HE stain, 40x. **(B)** Retropharyngeal lymph node and **(C)** palatine tonsil from FIV-infected cats exhibit moderate lymphoid hyperplasia with multifocally enlarged germinal centers and thin mantle zones, 40x. Higher magnification (insets, 200x) demonstrates tingible body macrophages (arrows). HE stain. The submucosa of the tongue **(D)** and buccal mucosa **(E)** are multifocally infiltrated by small to moderate numbers of small lymphocytes and plasma cells (arrows, 100x), as well as small numbers of scattered mast cells (arrows, inset, 200x). HE stain. **(F)** Submandibular salivary gland. Minimal numbers of small lymphocytes and plasma cells multifocally surround acini (arrowheads). HE stain, 400x.

mucosa, and salivary gland; p<0.001**, Fig 1.4)**. Individually, the degree of histologic change in the retropharyngeal lymph node (p<0.001), palatine tonsil (p<0.001), and tongue (p=0.002) was significantly greater than in the salivary gland, and there were more histologic changes in the retropharyngeal lymph node compared to the buccal mucosa (p<0.001) **(Fig 1.4)**.

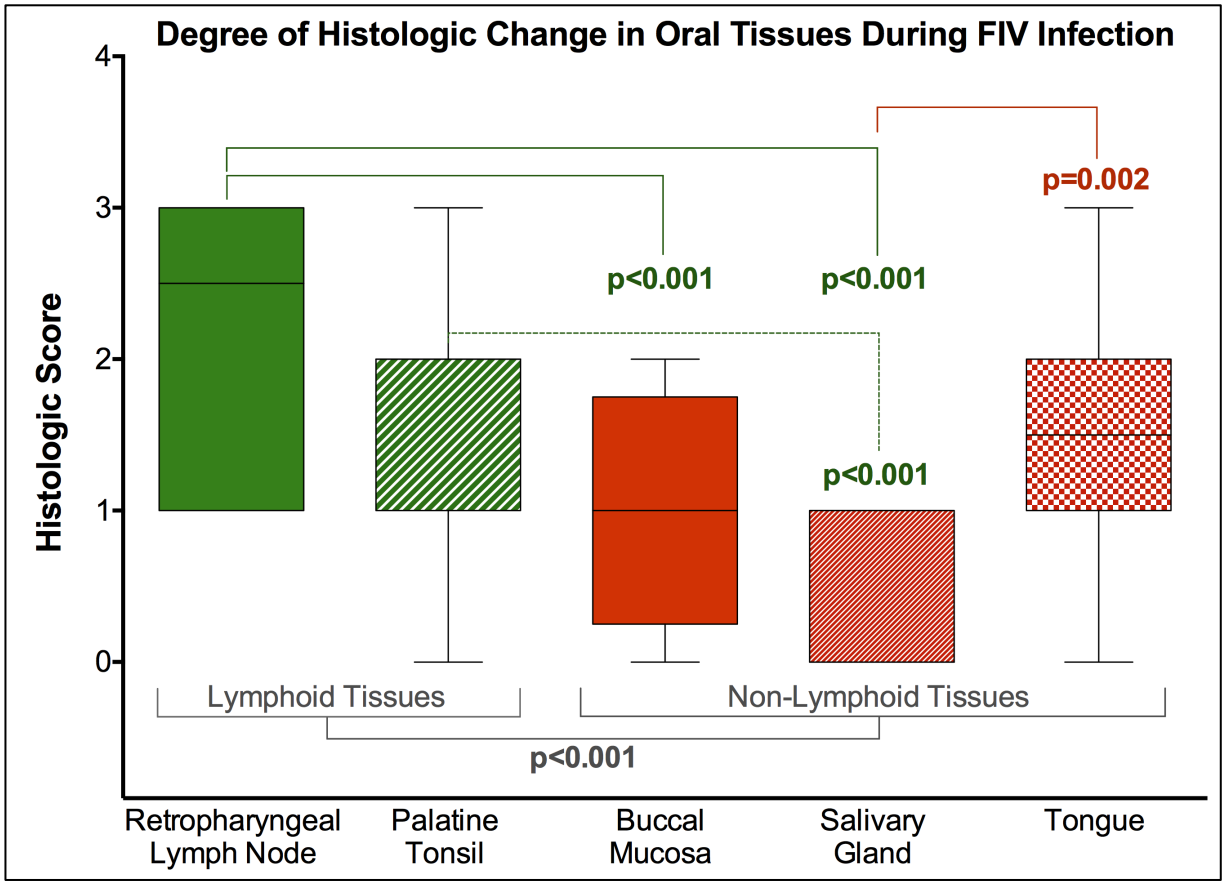

Fig 4. Oral lymphoid tissues exhibit a greater degree of histologic change than non-lymphoid tissues. The degree of histologic change in oral lymphoid tissues (retropharyngeal lymph node and tonsil) was significantly higher than in non-lymphoid tissues (tongue, buccal mucosa, and salivary gland) (p<0.001). The degree of histologic change was significantly greater in the retropharyngeal lymph node (p<0.001), palatine tonsil (p<0.001), and tongue (P=0.002) than in the salivary gland. A greater degree of histologic change was also observed in the retropharyngeal lymph node compared to the buccal mucosa (p<0.001) (ANOVA with Tukey test for multiple comparisons).

FIV-specific IgG increases in saliva of FIV+ cats, while IgA remains static

Saliva samples were collected from FIV-infected and negative control cats at four time points, and total IgA and IgG antibodies from saliva were quantified by microsphere immunoassay (MIA). The concentration of total IgG in FIV-infected cat saliva ranged from 0.001 to 0.037mg/ml over the course of infection, while the total IgA concentration in FIV-infected cat saliva ranged from 0.005 to 0.026mg/ml. There was a slight trend for total salivary IgG to be increased in FIV-infected cats compared to naive individuals (treatment $p=0.216$), however, total IgA antibody levels did not differ in saliva from FIV-infected and sham-inoculated cats (treatment $p=0.999$) **(Fig 1.5)**. FIV-specific anti-SU and anti-CA IgG antibodies were consistently detected in saliva of FIV-infected cats and increased significantly over time (anti-SU interaction $p<0.001$; anti-CA interaction $p<0.05$, **Fig 1.6A-B)**. Although anti-SU IgA antibodies were elevated in saliva from FIV-infected cats **(**treatment $p<0.05$, **Figure 1.6C)**, they did not increase over time compared to the background of uninfected saliva (interaction $p=0.569$). Furthermore, anti-CA IgA antibodies were not significantly elevated in FIV-infected saliva (treatment $p=0.809$, **Fig 1.6D**).

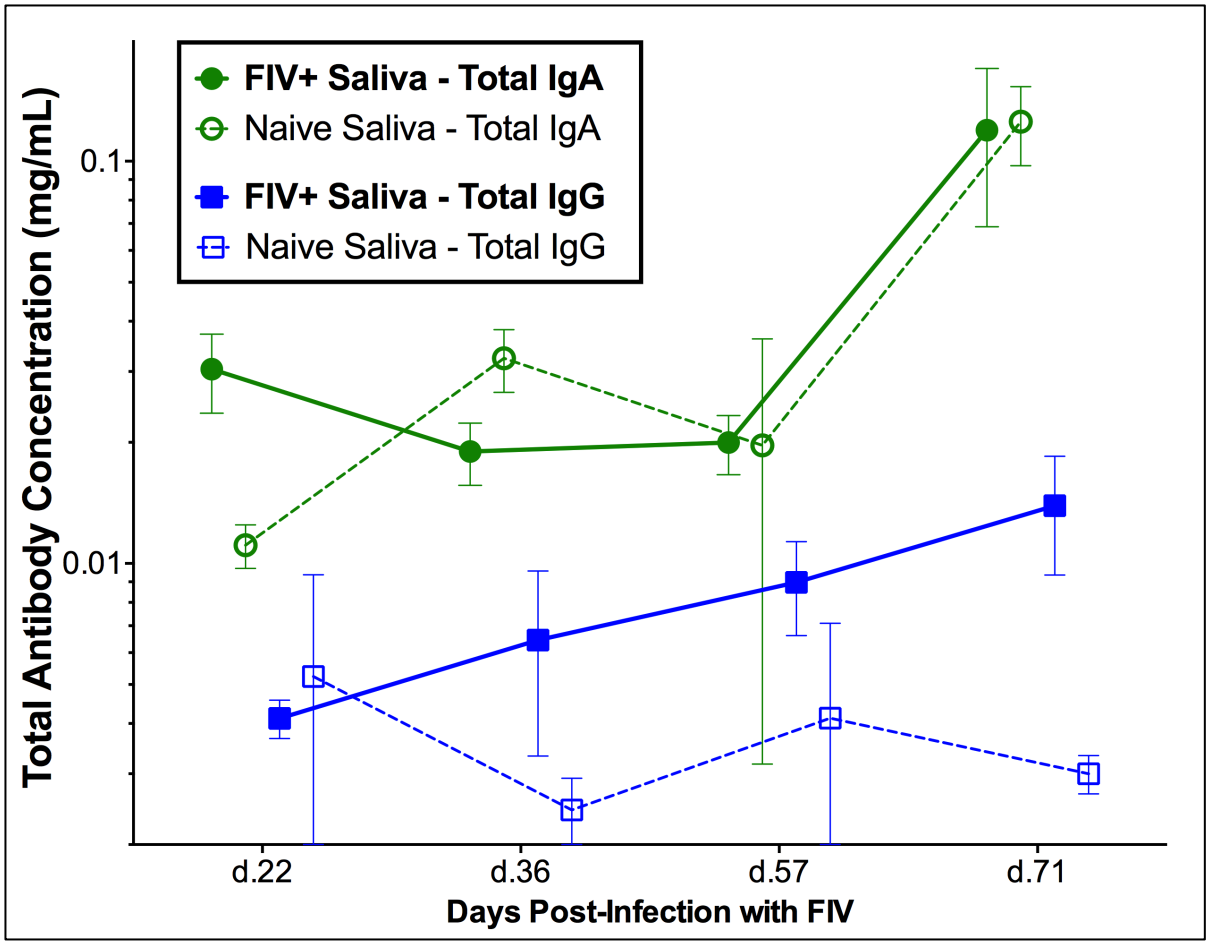

Fig 1.5. Limited IgA mucosal antibody response during FIV infection. There is a trend for salivary IgG concentrations to be slightly increased in FIV-infected cats following infection (solid blue line/squares) (mean ±SE; p=0.216, RM-ANOVA). Salivary IgG remained constant in naïve animals (blue dashed line/clear squares). Salivary IgA concentrations did not differ significantly between FIV-infected cats (solid green line/circles) and naïve cats (dashed line/clear circles) over time (p=0.969), but as expected, levels of IgA were higher in saliva than IgG.

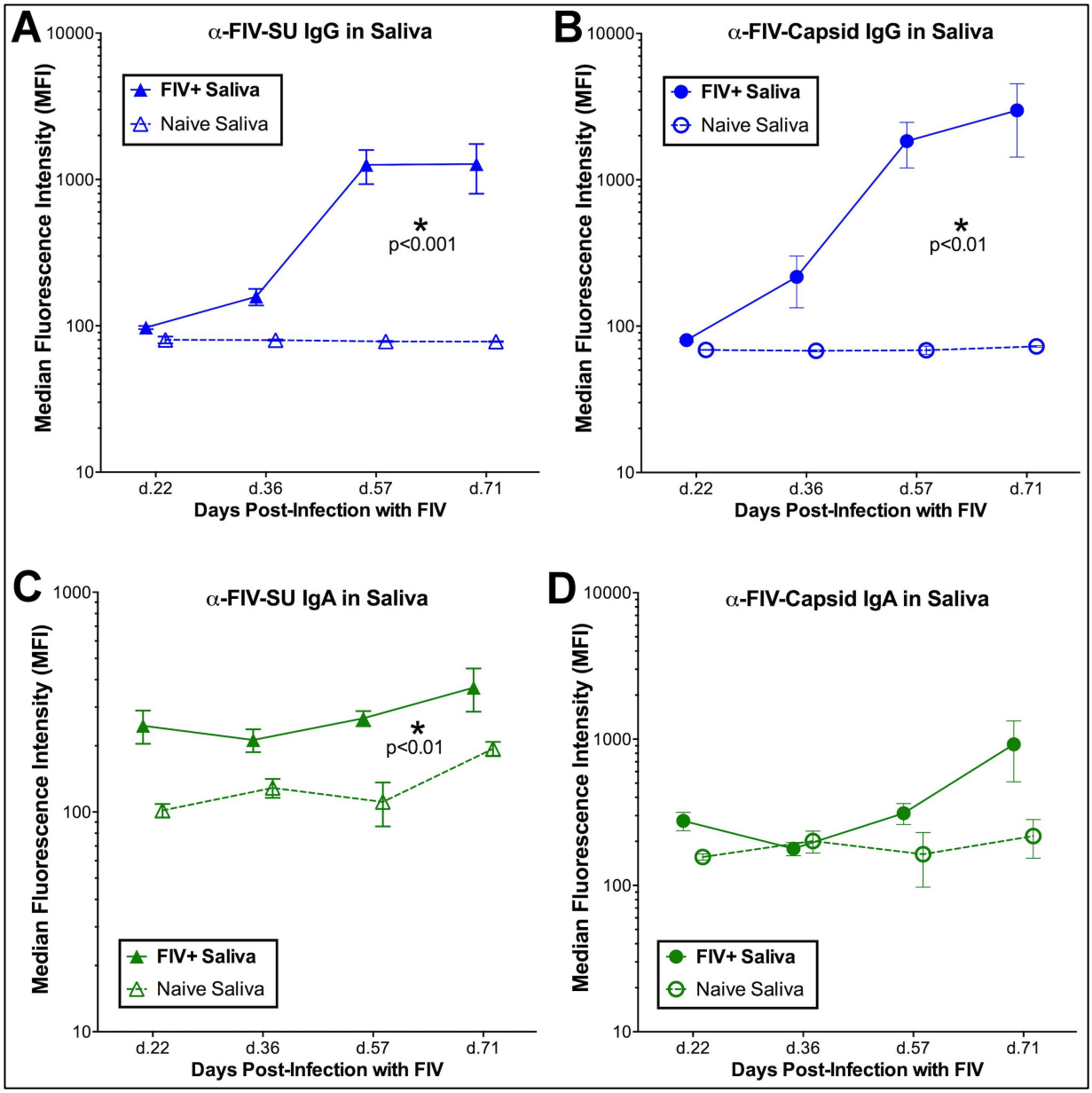

Fig 1.6: FIV specific IgG and IgA antibodies are detected in saliva of infected cats. (A) Anti-SU IgG and **(B)** anti-CA IgG antibody levels in saliva of FIV infected cats (solid blue lines) were significantly elevated (mean ±SE; treatment $p<0.01$ and $p<0.001$, respectively,) compared to naïve animals (dotted blue lines) and increased significantly over time compared to naïve saliva (interaction anti-CA: $p<0.05$; anti-SU: $p<0.001$; RM-ANOVA). **(C)** Anti-SU IgA antibody levels in saliva of FIV infected cats (solid green lines) were significantly elevated compared to naïve animals (dotted green lines)(treatment $p<0.01$), but did not increase significantly over time compared to naïve saliva (interaction $p=0.569$; RM-ANOVA). **(D)** No difference in salivary anti-CA IgA antibody levels was observed between FIV-positive animals and naïve animals.

Saliva exhibits an inhibitory effect on FIV infection in vitro

Naïve cat saliva significantly inhibited FIV_{C36} growth in GFox cells (CrFK cells overexpressing feline receptor CD134) over time, indicated by lower FIV p26 ELISA absorbance values in the presence of saliva **(**interaction p<0.001, **Fig 1.7A)**. Percent viral inhibition compared to no-saliva controls differed significantly over time, peaking at 8 days post-inoculation and declining slightly thereafter **(**interaction p<0.05, **Fig 1.7B)**.

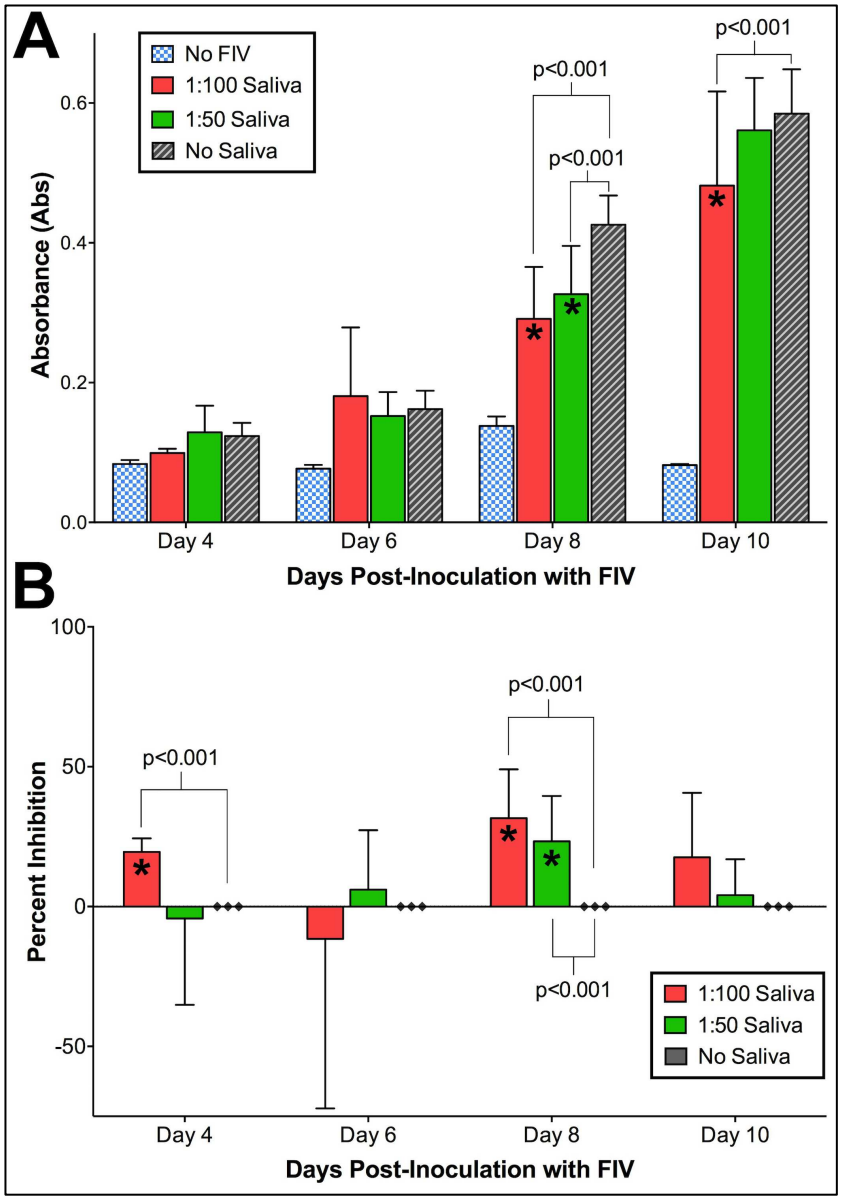

Fig 1.7. Naïve cat saliva inhibits FIV replication. FIV_{C36} was incubated with 1:50 or 1:100 dilutions of naïve cat saliva in duplicate and inoculated onto CRFK cultures as described in the text. A 'No Saliva' virus-only positive control represented 100% FIV growth as measured by ELISA absorbance. **(A)** Mean

ELISA absorbance values increased for all treatments except for virus-negative control (blue-checkered bars). Absorbance values for FIV pre-incubated with saliva at both 1:100 and 1:50 dilutions were significantly lower ($p<0.001$; RM-ANOVA) than the no saliva control (gray-striped bars), indicating a lower FIV replication rate in the presence of saliva. **(B)** Analysis of percent inhibition over time revealed a significant inhibitory effect with saliva treatments differing over time ($p<0.05$; RM-ANOVA) and at individual time points (days 4, 8 post-inoculation) relative to the 'No Saliva' virus-only positive controls.

Discussion

Our findings document that: (1) FIV infection predominates in oral lymphoid tissues versus mucosal and salivary gland, resulting in mild to moderate histologic pathology **(Figs 1.2-1.4,)**, and provide evidence of a site of viral persistence and chronic immune dysregulation; (2) FIV viral RNA ratio to proviral load, a surrogate for viral replication rate, is significantly higher in saliva than in plasma/circulating PBMC **(Fig 1.1C)**, suggesting enhanced FIV replication occurs at a site where viral shedding is thought to take place; (3) though FIV-specific IgG antibodies increase in saliva during infection, secretion of FIV-specific IgA in saliva is impaired **(Figs 1.5-1.6)**, suggesting one potential mechanism for chronic oral infection, the development of opportunistic oral disease, and transmission of FIV via oral secretions; and, (4) saliva from uninfected SPF cats harbors *in vitro* anti-viral properties **(Fig 1.7),** which may be an important mechanism for limiting 'passive' FIV transmissions among FIV-infected and uninfected cats in stable social structures.

Collectively, these data provide a descriptive analysis of viral, immunological, and pathogenic features of oral FIV infection, and highlight a continuum between salivary and peripheral viral kinetics that is represented in **Fig 1.8**. Initial events of FIV infection occur systemically, resulting in peripheral lymphocyte infection, viremia, and an anti-FIV antibody response in which IgG antibodies predominate over IgA [39, 51, 52, 63]. During HIV and SIV infection, CD4+ T-cells are rapidly infected and severely depleted from the intestinal mucosal surface, resulting in loss of mucosal integrity, reduced capacity to control potential pathogens at mucosal surfaces, and subsequent triggering of pro-inflammatory responses [25, 26, 28, 29, 31, 32]. Comparatively, we demonstrate that FIV exhibits a significant tropism for oral lymphoid

tissues, which may potentially serve as initial sites for circulating virus to infect resting T-lymphocytes and dendritic cells **(Fig 1.8A)**. As an extension of the digestive tract, similar effects of viral-induced immunosuppression may likely occur at the oral mucosa, resulting in a chronic cycle of lymphocyte depletion, microbial translocation, immune stimulation, and leukocyte recruitment and consequent infection **(Fig 1.8B),** as evidenced by FIV accumulation and histologic changes in oral tissues.

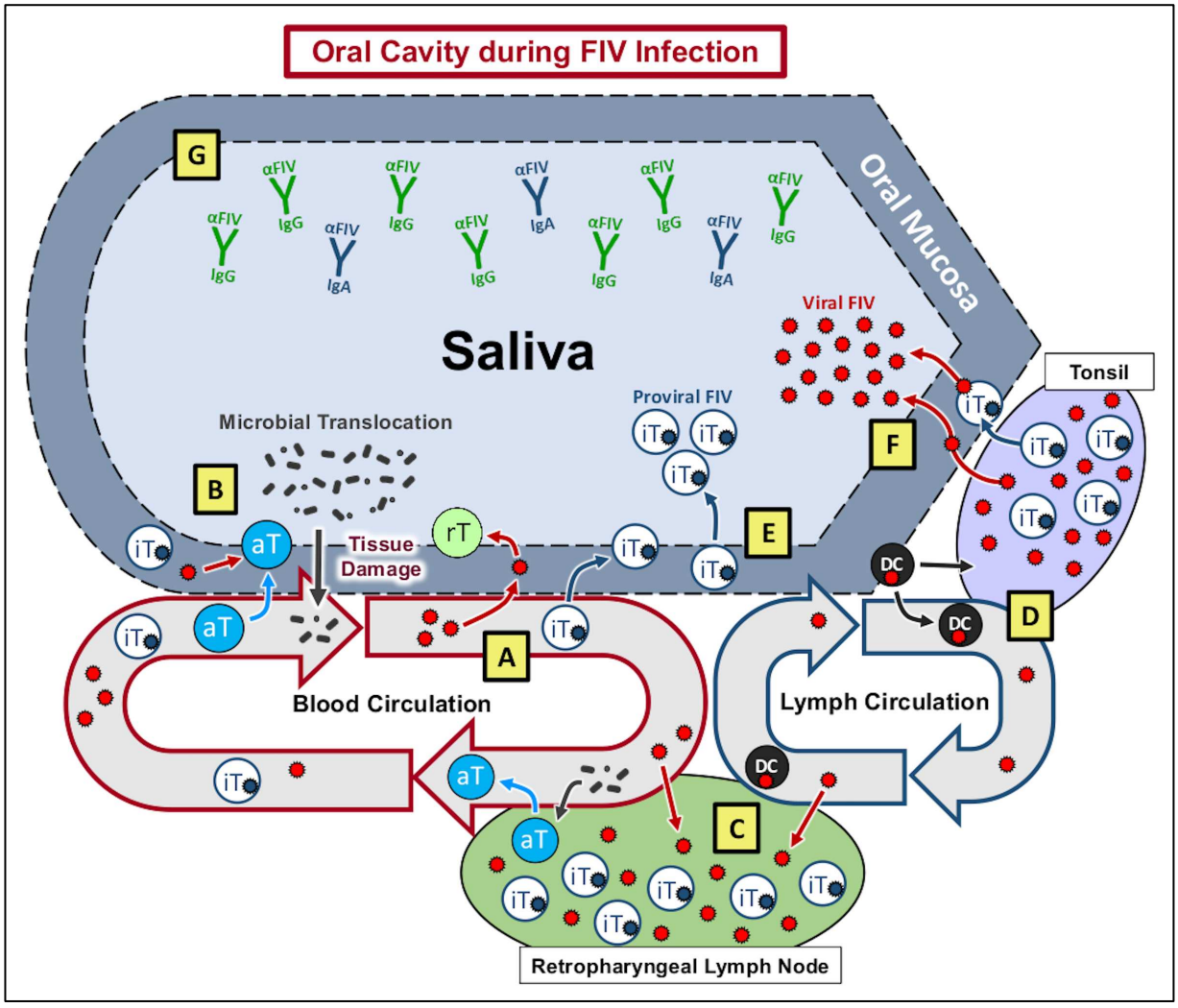

Fig 1.8. Proposed model of oral FIV pathogenesis. Similar to the gastrointestinal tract, FIV exhibits a tropism for oral tissues, providing a pathway for circulating virus to extravasate and infect resting T-lymphocytes (rT) and dendritic cells (DC). **(A)** As documented in the intestinal mucosa, infection and depletion of resting mucosal lymphocytes in the oral mucosa may cause inflammation, damage mucosal barriers, and subsequent translocation of oral microbes; resulting in lymphocyte activation and recruitment to oral mucosal tissues. **(B)** Mucosal injury initiates a chronic cycle of immune activation and provides a renewable source for target cell infection by recruiting susceptible cells to the site of injury. Peripheral FIV-infected cells traffic to oral lymphoid tissue via lymphatics **(C)** or direct migration to tonsils

(D) resulting in antigen presentation, T-lymphocyte activation (aT), and infection of resident leukocytes, establishing a reservoir of persistent FIV replication in latently infected T-lymphocytes (iT) in oral lymphoid tissues. **(E)** FIV infected cells are likely shed from oral mucosal sites into saliva, resulting in the presence of salivary proviral DNA. Cells within oral lymphoid tissues have enhanced FIV replicative activity, resulting in a higher ratio of FIV viral RNA to proviral DNA than noted in the peripheral circulation. **(F)** FIV RNA in saliva may be achieved by direct release of virus particles from infected cells into saliva via the intimate association of tonsillar lymphoid tissue with the oral mucosa. **(G)** While FIV specific IgG responses are detected in saliva, anti-FIV IgA antibodies are not specifically enhanced, allowing FIV virus and infected cells to persist in saliva at high levels.

FIV virus particles and/or infected cells traffic to oral lymphoid tissues via circulation **(Fig 1.8C)** or by direct migration to the tonsils from the oral mucosa **(Fig 1.8D)**, subsequently infecting resident leukocytes and establishing a reservoir of persistent FIV replication in latently infected T-lymphocytes within oral lymphoid tissues. Viral RNA and proviral DNA are also detected in non-lymphoid oral tissues, but at a much lower quantity than in lymphoid tissues, indicating that these sites (tongue, buccal mucosa, salivary gland) are not a primary site of FIV persistence. However, accumulation of proviral DNA in these tissues suggests that passive migration of FIV-infected cells may occur from the periphery to non-lymphoid oral tissues, which may then perpetuate FIV-infection at these sites.

Although detectable, the quantity of FIV DNA is lower in saliva than in circulating PBMCs, and reflects the concept that the FIV-infected cells being shed into saliva may be shed via the oral mucosal epithelium in lieu of oral lymphoid reservoirs **(Fig 1.8E),** which contain larger amounts of FIV proviral DNA. In contrast, FIV RNA appears later in saliva than in plasma [63], but salivary FIV RNA concentration is equivalent to plasma, and FIV RNA is present in high quantities in oral lymphoid tissues (**Fig 1.1)**. Our finding that FIV RNA is proportionally higher (compared to proviral load) in saliva than in plasma suggests that the oral cavity is a preferential site for viral RNA transcription. The observed predilection of FIV for oral lymphoid tissues indicates that these tissues may serve as an important reservoir and primary source of viral replication and shedding of viral RNA into the saliva **(Fig 1.8F)**. Systemic hyperplasia of lymphoid structures is a prominent feature of FIV infection, and the histologic changes observed support an analogous impact on oral lymphoid tissues; thus supplying an ample source of target

cells for persistent FIV replication [39, 64-66]. Moreover, the anatomic distribution of the palatine, paraepiglottic, pharyngeal and lingual tonsils provides an intimate association with the overlying oral mucosa, and highlights the potential for these organs to serve as sites of extrusion of FIV into saliva through trafficking of infected cells across the adjacent mucosal epithelium [67].

IgA represents the primary mucosal antibody that limits numbers of mucosa-associated bacteria and prevents bacterial penetration of host tissues. This is best demonstrated by IgA deficiency, which results in increased penetration of symbiotic bacteria into the host tissues and consequent inflammation [68-71]. In the oral cavity and gastrointestinal tract, differentiated plasma cells secrete IgA, which then transcytoses across the epithelial layer to the apical surface of the epithelium where it works to crucially maintain homeostasis through luminal compartmentalization of intestinal bacteria [68]. However, during FIV infection, IgG antibodies increase in saliva and in the peripheral circulation of infected cats [51], but the IgA antibody response remains static at both of these sites. Furthermore, FIV-specific IgA antibodies against capsid are undetectable in saliva, and anti-SU IgA does not increase over time. In contrast, both anti-SU and anti-CA IgG antibodies increase significantly in saliva during FIV infection **(Figs 1.6B and 1.8G)**. These results indicate a failure to mount an effective mucosal antibody response during FIV infection, which may perpetuate oral mucosal viral infection as well as changes in the oral microbiome, thus contributing to microbial translocation and a cycle of immune dysfunction.

Currently, FIV infection is diagnosed clinically by either lateral-flow serum ELISA or microwell, and western blot and immunofluorescent antibody (IFA) assays are frequently used as confirmatory tests following positive ELISA results [72]. Unfortunately, these assays require blood samples to screen for serum FIV-specific antibodies, which may be impractical to acquire in intractable patients, or during field operations to capture and control the feral cat population. The presence of anti-FIV IgG antibodies in saliva suggests that a reliable alternative diagnostic

assay can be developed based upon saliva sampling, and may allow for methods to differentiate between vaccinated and un-vaccinated cats as previously suggested by Wood et al. [51]. Microsphere immunoassay technology or other sensitive antibody detection assays may be of great use in clinical applications to test for FIV in a less invasive manner, as these assays only require passage of an oral swab along the interior of the oral cavity. Furthermore, optimization to detect FIV in saliva may be directly adapted to test human saliva for HIV without the need for blood sampling, thus increasing the capacity to screen large numbers of patients in endemic areas.

Results of this study demonstrate that uninfected feline saliva contains a component with anti-FIV properties, a feature that may have implication for FIV transmission in natural settings. Prolonged contact between animals is typically required for transmission of FIV, and feral male cats are most at risk for FIV infection. Numerous studies have indicated that horizontal transmission through casual contact such as grooming is extremely inefficient and rarely occurs in the absence of biting, i.e. amongst cats maintained indoors or in stable groups [47, 73-75]. Our findings suggest that cats which groom each other may be less susceptible to FIV transmission via fomites and cat to cat oral contact because of unidentified innate factors in saliva, which inhibit infection via the oral route. While experimental transmission studies have documented potential for oral transmission by inoculation of virus into the oral cavity [76, 77], these studies provided an artificially high dose of concentrated virus, which would be an unusual situation to encounter in a natural transmission setting.

Previous studies in humans infected with HIV have demonstrated that significant anti-viral activity in human saliva is conferred by numerous cofactors and immune modulators, such as anti-HIV antibodies, defensins, thrombospondin-1, proline-rich proteins, salivary agglutinin, and secretory leukocyte protease inhibitor (SLPI) [14, 15]. It is possible that naïve feline saliva may confer anti-viral activity *in* vivo by similar mechanisms and effector molecules. However, the capacity of FIV to be transmitted via saliva despite its potential inhibitory properties may

indicate alterations in the composition of saliva in infected cats, or perhaps other viral-induced mechanisms by which FIV may overcome these inhibitory effects. Further investigation is warranted to determine whether feline saliva contains inhibitory molecules similar to those in human saliva and how the composition of feline saliva changes following FIV infection.

Although HIV transmission typically occurs via parenteral or transmucosal venereal routes, recent epidemiological studies have provided definitive evidence that HIV can be transmitted by receptive oral intercourse, and occasional cases of transmission by biting have been documented [7-13]. Furthermore, significant quantities of both HIV viral RNA and proviral DNA have been detected in saliva of infected individuals, with significant correlations between salivary and plasma RNA levels [16, 17]. Oral HIV transmission occurs less frequently than in FIV transmission, likely due to the absence of aggressive biting in human populations versus cat populations [7, 11, 21, 78]. The use of SIV animal models has provided further proof that oral transmission of primate lentiviral infections is possible, and experimental SIV studies have helped to understand the oral immune response during infection [13, 79-81]. Moreover, retroviral-induced oral disease continues to affect a high proportion of individuals despite the success of highly active antiretroviral therapy (HAART), and is a common manifestation of both HIV and FIV infection [38, 41-43, 76]. Oral lesions are not typically observed during SIV infection and may highlight significant limitations of the SIV model to study HIV-induced oral disease [82, 83]. In contrast, FIV produces oral lesions in cats that closely resemble Linear Gingival Erythema and Necrotizing Ulcerative Gingivitis in humans with HIV-induced disease, and opportunistic microorganisms detected in saliva of HIV-positive individuals (*Candida albicans, Fusobacterium sp., Streptococcus sp., Prevotella sp., Campylobacter sp.,* and *Porphyromonas gingivalis*) are also implicated in feline oral disease [1, 6, 31, 38, 39, 41-43, 47, 84-88]. Indeed, shifts in oral microbial structure during HIV and FIV infection have been increasingly linked to disease phenotypes, and the use of a feline animal model is a pragmatic solution to assess the impact of novel therapeutic strategies [1, 25, 38, 41, 84, 86, 89, 90]. An

effective anti-HIV vaccine or prophylactic therapeutic agent that will cure or prevent HIV infection will likely need to function at the mucosal surface, and FIV provides a relevant animal model that might be exploited to specifically assess oral mucosal lentiviral disease and interventions [13, 91, 92].

The results of this study provide new evidence for unique pathogenic features of oral FIV infection and suggest mechanisms that overcome host resistance. Manipulation of constituents of saliva, inhibition of viral replication at oral lymphoid sites, or enhancement of anti-viral IgA production may represent novel therapeutic interventions to reduce or eliminate oral FIV infection, as well as the potential to treat HIV-induced oral disease. Our results additionally provide a plausible hypothesis linking site-specific viral replication, mucosal immune deficiency, and salivary inhibition to the natural transmission and infection cycle of FIV.

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CHAPTER 2. NOVEL VACCINATION STRATEGIES IN FIV

Summary

Feline immunodeficiency virus (FIV) is the feline analogue to human immunodeficiency virus (HIV) and utilizes parallel modes of receptor-mediated entry. The FIV surface glycoprotein (SU) is an important vaccine target for induction of virus neutralizing antibodies, and autoantibodies to the FIV binding receptor (CD134) block FIV infection *ex vivo;* highlighting the potential for immunotherapies which utilize anti-receptor antibodies to block viral infection. To determine if vaccination with CD134-SU complexes could induce protection against FIV infection, cats were immunized with soluble CD134, recombinant FIV-SU protein, and/or CD134+SU complexes prior to challenge with FIV. *In vivo* generation of anti-CD134 and anti-SU IgG antibodies was measured by microsphere immunoassay (MIA), and neutralizing ability of antibodies and antibody-containing serum fractions was quantified *in vitro*. Post-challenge FIV RNA and DNA levels and hematologic alterations were measured for evidence of vaccinal success. Immunization induced production of anti-CD134 and anti-SU antibodies in vaccinated cats, and purified anti-CD134 and anti-SU antibodies significantly inhibited FIV infection *in vitro.* However, no vaccine combination protected cats from FIV infection *in vivo,* and neat serum from vaccinated cats enhanced FIV growth *in vitro*. Prior to challenge, CD134+SU vaccinated cats exhibited an enhanced CD4:CD8 ratio, and vaccination induced high titers of antibodies directed at vaccine by-products relative to target antigens. Results suggest that vaccination against viral and cryptic receptor epitopes yields neutralizing antibodies that synergistically inhibit FIV infection *in vitro*. Factors that may have contributed to vaccine failure include: (1) Heat-labile serum factors that enhance viral replication, (2) changes in circulating target cell populations and/or vaccine-induced immunomodulation, and (3) weak immunogenicity of neutralizing epitopes compared to off-target vaccine components. Results reinforce the need to monitor

vaccine components, and emphasize that vaccination may induce expansion of target cells and enhancement of heat-labile serum components that counteract neutralizing antibodies.

Background

Feline immunodeficiency virus (FIV) is a naturally-occurring lentivirus that is genetically similar to human immunodeficiency virus (HIV) and shares many immunopathogenic features of HIV infection [1-6]. Like HIV, FIV primarily infects and replicates within CD4+ T cells, and is characterized by progressive depletion of CD4+ T lymphocytes and an AIDS-like syndrome during natural infection of domestic cats [7-9]. Both lentiviruses require an initial interaction with a primary binding receptor for infection, and utilize analogous modes of receptor-mediated entry via the chemokine co-receptor, CXCR4 [10-12]. HIV binds to CD4+ target cells through a high-affinity interaction with the CD4 receptor that induces a conformational change in the envelope glycoprotein gp120 to expose binding sites necessary for chemokine co-receptor binding (CXCR4 or CCR5) and subsequent fusion with the cell membrane **(Figure 2.1A)** [10, 11]. FIV utilizes CD134 as primary binding receptor, and studies have demonstrated that binding of the CD134 receptor alters the conformation of FIV envelope protein gp95 (SU) in a similar fashion to that which occurs in the CD4/ HIV gp120 interaction, to promote high affinity binding with the entry receptor CXCR4 **(Figure 2.1A)** [13, 14].

Since the discovery of FIV, considerable effort has been directed at the development of safe vaccine strategies that can produce protective immunity in cats. A commercially available, whole inactivated virus vaccine containing two FIV subtypes (Fel-O-Vax FIV®) is currently licensed for use in the United States, and various reports have described virus neutralization and cellular immunity in a significant proportion of study animals [15-17]. However, the efficacy of this vaccine is still under debate, as recent studies and field evaluations have reported that the vaccine does not confer immunity against certain FIV strains (ie: FIV_{GL8}), and that the neutralizing antibody response and protective rate may be low in certain cat populations (i.e.

protection is not conferred to certain virulent recombinant strains of FIV) [18-21]. Other attempts at FIV vaccine development have either failed to induce protective immunity against FIV infection, or have resulted in increased susceptibility to infection via antibody-dependent enhancement or general immune activation [22-27]. Thus, the development of novel immunotherapies aimed at preventing viral entry may have great potential to increase the level of protection against naturally-occurring FIV infection.

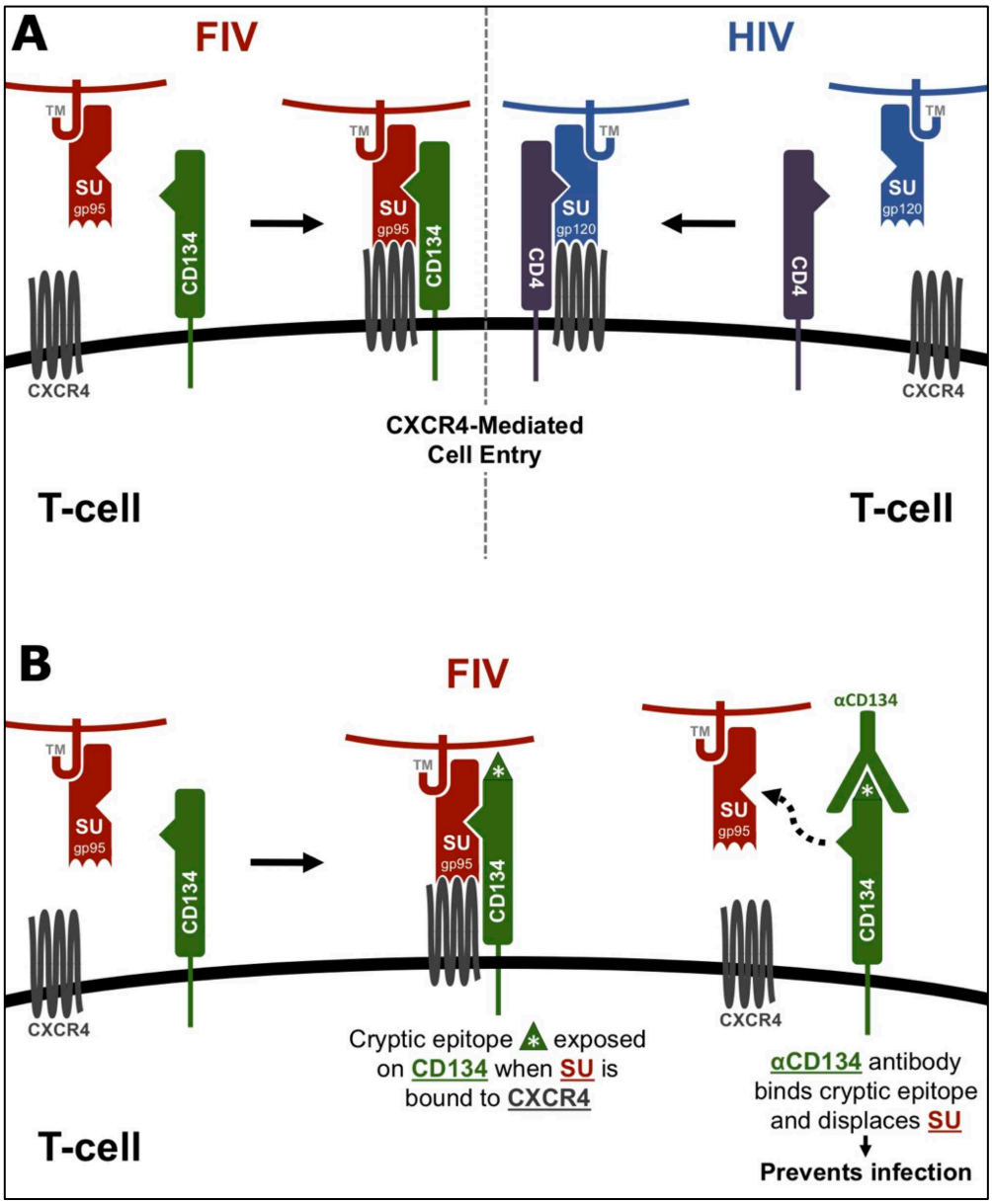

Figure 2.1. (A) FIV and HIV utilize analogous modes of receptor-mediated cell entry. HIV binds to CD4+ target cells through a high-affinity interaction with the CD4 receptor, inducing a conformational change in the envelope glycoprotein gp120 (SU) that exposes the CXCR4 co-receptor binding site and

subsequent fusion with the cell membrane. FIV utilizes a primary receptor CD134, and similar to HIV, binding of FIV Env to the CD134 receptor alters the conformation of envelope glycoprotein gp95 surface (SU) component to facilitate CXCR4 co-receptor binding, and viral entry. **(B) Anti-CD134 antibodies prevent FIV infection in the presence of viral glycoproteins.** Binding of FIV SU to CD134 induces a conformational change in the receptor and exposes a cryptic epitope that results in anti-CD134 generation. Anti-CD134 antibody binding to CD134 cause a second conformational change in the CD134 receptor that displaces SU from the cell surface, inhibiting FIV infection [28].

Previous studies by Grant et al. [28] reported that antibodies to the CD134 receptor (anti-CD134) and the viral surface glycoprotein (anti-SU) are expressed in a high proportion of chronically FIV-infected cats, and that increased levels of anti-CD134 are correlated with lower viral loads and improved health status in these animals [28]. Furthermore, anti-CD134 antibodies purified from serum of infected cats have been shown to exhibit significant neutralizing activity and are able to block FIV infection *ex vivo* [28]. This activity is related to binding of FIV SU to CD134 on the target cell, inducing a conformational change that exposes a cryptic epitope of CD134 that is recognized and blocked by feline anti-CD134 [28]. Binding of anti-CD134 autoantibodies to CD134 causes a second conformational change in the CD134 receptor and results in displacement of SU from the cell surface **(Figure 2.1B),** demonstrating an active contribution of anti-receptor antibody responses to controlling viral infection [28]. Parallel mechanisms of cell entry are also observed in HIV infection, whereby neutralizing epitopes are exposed following interaction of SU with the CD4 binding receptor [29-33]. Thus, the analogous modes of receptor-mediated viral entry that exist between FIV and HIV likely reflect common immunological pressures in the two hosts, and may indicate a convergent pathway for the development of strategies to compromise the virus' ability to escape immune surveillance [2].

Such propensity for anti-CD134 and anti-SU antibodies to block FIV infection *ex vivo*, coupled with the increased survival of cats expressing high levels of anti-CD134 antibodies, highlights the potential for novel immunotherapies which utilize anti-receptor antibodies to protect from viral infection. In the present study, we assessed the potential for immunization with soluble CD134 and FIV-SU complexes (CD134+SU) to induce a neutralizing antibody response

and protection against FIV infection in domestic cats. Our results demonstrate that immunization with soluble CD134 and recombinant SU protein induces production of anti-CD134 and anti-SU antibodies, and that these antibodies significantly inhibit FIV infection *in vitro*. However, vaccinated cats became infected following FIV challenge, and vaccination altered circulating cell populations with potential consequences for subsequent infection. Furthermore, antibodies were generated against irrelevant antigens in the vaccine preparation at levels that significantly exceeded antibodies produced in response to target antigens. This study highlights potential targets and requirements for vaccine optimization for anti-receptor and anti-immunodeficiency virus development.

Materials and methods

Vaccine Design

Two vaccine trials were conducted to test whether addition of Fe-CD134 antigen to the vaccine preparation would augment the efficacy of a vaccine that contained only FIV-Env. *Phase I* vaccine trial included immunization with soluble CD134-huFc and FIV-PPR-SU-huFc immunoadhesins, either alone or together as a complex. These proteins were expressed in and purified from Chinese hamster ovary (CHO) cells as previously described [34], and the human Fc tag, in-frame with either protein, served as a means to purify the proteins using Staphylococcus Protein A-Sepharose [13, 35]. *Phase II* vaccine trial employed soluble, His-tagged CD134 and FIV-PPR-SU derived by expression and purification from human 293S T cells**.** Soluble feline CD134 (aa28-215) with a C-term 6X histidine tag, and FIV-PPR-SU (aa170-601) were cloned into pCMV mammalian expression vectors (Life Technologies, Carlsbad, CA) and transfected into human 293S T cells using Lipofectamine 2000 (Life Technologies). His-CD134 (CD134-293S) was purified by nickel chromatography and untagged PPR- SU (PPR-SU-293S) was purified using Galanthus lectin-sepharose (Vector Laboratories, Burlingame, CA)

from supernatants of 293S cells grown in defined serum-free medium (FreeStyle medium, Invitrogen).

In vivo protocols

This study was approved by the Colorado State University Institutional Animal Care and Use Committee; 14-4872A - Molecular Characterization of FIV. Colorado State University's animal care program is licensed by the United States Department of Agriculture (USDA), accredited by Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International, and holds an Office of Laboratory Animal Welfare (OLAW) assurance (A3572-01). All animal experiments complied with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). Prior to experimental procedures, all study animals were anesthetized by intramuscular injection of ketamine (20mg/kg) and acepromazine (2mg/kg) to minimize animal suffering and distress. All study animals were monitored daily by animal care personnel for development of clinical signs of FIV infection and observed by clinical veterinarians. No animals exhibited clinical signs associated with FIV infection or any other untoward conditions during vaccine and challenge experiments.

Phase I. Fifteen (15), 8-11 week-old specific pathogen free (SPF) cats (Andrea D. Lauerman Specific Pathogen Free Feline Research Colony, Fort Collins, CO) were housed within barrier rooms in accordance with Colorado State University (CSU) IACUC-approved protocols at a CSU AAALAC-international accredited animal facility, and were acclimated to the facility for 2 weeks prior to the initiation of the study. An outline of the study design for Phase I is presented in **Figure 2.2A**. At weeks 0, 4, and 8, all cats were subcutaneously inoculated with 1ml of vaccine composed of Dulbecco's phosphate-buffered saline (D-PBS; Life Technologies Corporation, Grand Island, NY), 5mg of Alum (aluminum hydroxide, an adjuvant licensed for use in cats that induces significant humoral vs cellular immune responses), and one of the following

immunogens: (1) sham-vaccine (D-PBS and Alum only)(**sham** group; n=5); (2) PPR-SU-huFc peptide-immunoadhesion (**SU**-huFc group; n=5); and (3) FIV-PPR-SU-huFc peptide-immunoadhesion complexed with soluble CD134 (**CD134+SU**-huFc group; n=5)). At week 12, all fifteen cats were intravenously inoculated with 75,000 infectious units of FIV_{PPR} (1ml of a viral stock solution with a $TCID_{50}$ titer of $1X10^{5.87}$). Blood samples were obtained for all cats at 7-day intervals using previously established protocols, beginning at week 0 and ending at week 24 [36, 37].

Phase II. Twelve (12), 8-11 week-old SPF cats (Andrea D. Lauerman Specific Pathogen Free Feline Research Colony, Fort Collins, CO) were housed as previously described above in accordance with CSU IACUC-approved protocols. An outline of the study design for Phase II is presented in **Figure 2.2B**. At weeks 0, 4, and 8, eight (8) cats were subcutaneously inoculated with 1ml vaccine composed of Dulbecco's phosphate-buffered saline (D-PBS; Life Technologies Corporation, Grand Island, NY), 5mg of Alum, and 100μg of soluble **CD134**-293S. The remaining 4 cats were inoculated with a sham vaccine composed of D-PBS and 5mg Alum. At weeks 12 and 17, four cats that had previously received the vaccine containing soluble CD134-293S were instead inoculated with a vaccine composed of D-PBS, 5mg of Alum, and 100ug PPR-SU-293S complexed with CD134-293S (**CD134+SU**-293S group; n=4). The **CD134**-293S group (n=4) and **sham** group (n=4) were vaccinated as previously described at weeks 12 and 17 **(Figure 2.2B)**. Blood samples were collected from all cats using previously established protocols at 7-day intervals, beginning at week 0 and ending at week 24 [36, 37]. At week 20, all twelve cats were intravenously inoculated with 75,000 infectious units of FIV_{PPR} (1ml of a viral stock solution with a $TCID_{50}$ titer of $1X10^{5.87}$).

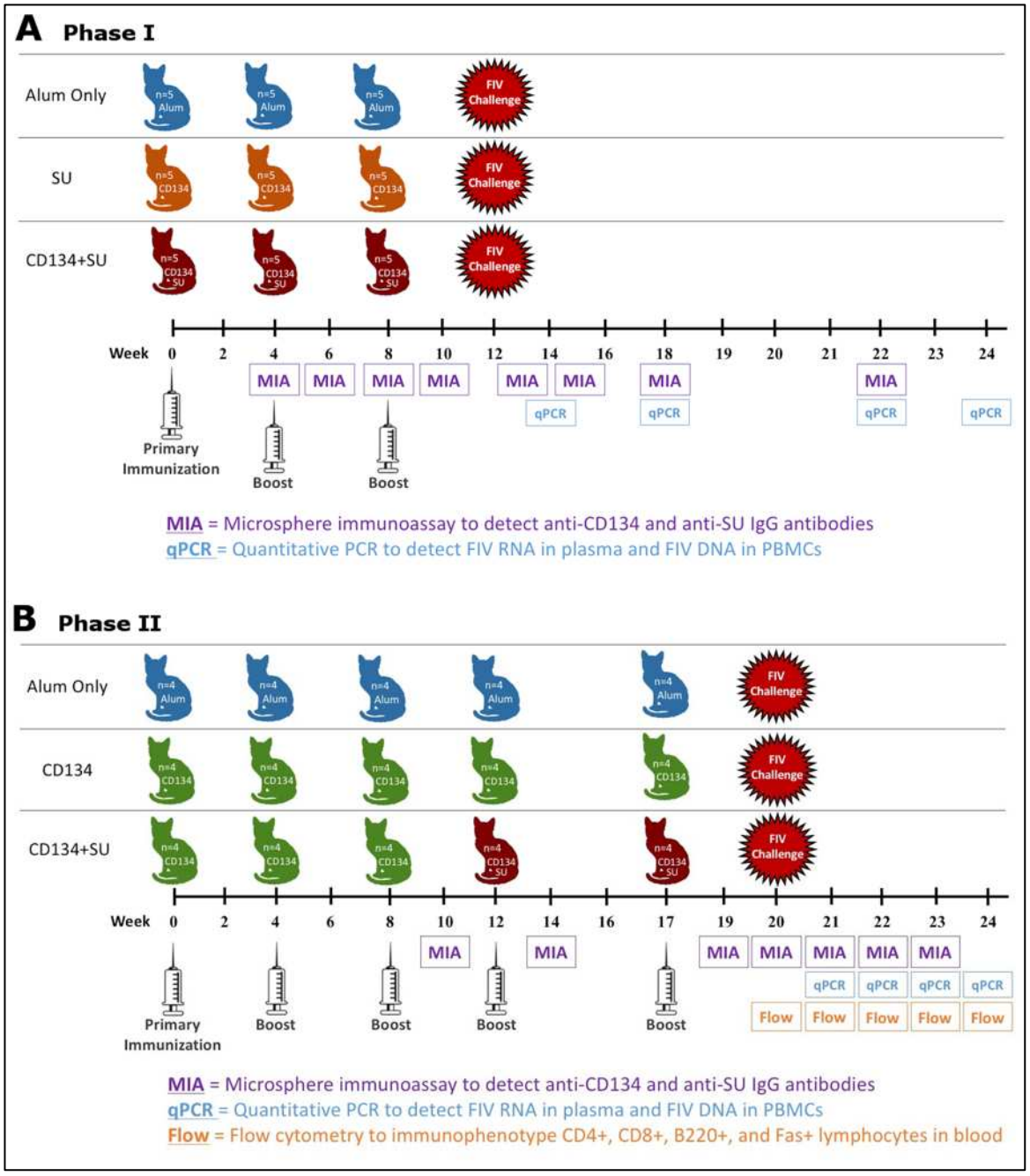

Figure 2.2. (A) Phase I vaccine study design. **(B)** Phase II vaccine study design.

Quantification of vaccine-specific IgG antibody production

Evaluation of vaccine-specific antibodies was performed using previously established microsphere immunoassay (MIA) protocols involving conjugation of carboxylated magnetic microspheres (MagPlex® Microspheres, Luminex, Austin, TX) with FIV glycoprotein (SU_{PPR}) and soluble CD134 recombinant proteins (rProteins) [38, 39]. Following conjugation protocols, a

hemocytometer was used to determine microsphere concentrations, and protein coupling was confirmed via incubation of microspheres with primary antibodies and/or PE-conjugated detection antibodies [38]. Successful coupling was defined by a median fluorescence intensity (MFI) of >2,000. All samples from FIV-infected and negative control cats were diluted 1:50 in assay buffer and then incubated in duplicate with approximately 2,500 conjugated beads per well. All samples were assayed in conjunction with FIV-A and naïve reference samples diluted 1:50 in assay buffer, as well as four diluent control wells per experiment. The MFI was calculated from ≥100 microspheres per analyte per well (Bio-Plex™ Manager 5.0) and then used for data analysis. All reagent concentrations, volumes, incubation times, acceptable standard recovery, and data analysis were as previously described [38, 39].

Phase I. Plasma samples from all study animals collected at weeks 4, 6, 8, 10, 13, 15, 18, and 22 **(Figure 2.2A)** were evaluated by MIA to detect anti-CD134 and anti-SU IgG antibodies. To detect antibodies specific to CD134 and/or FIV-PPR-SU, and to limit interference and detection of background antibodies specific to the huFc tag used to purify the ***Phase I*** vaccine immunoadhesion peptides, alternate immunoadhesion molecules without huFc were purified from **293S** cells (**CD134-293S** and **FIV-PPR-SU-293S**) and conjugated to microspheres for use in all MIA assays involving samples from ***Phase I*** of this study **(Figure 2.3)**. Microspheres conjugated to huFc rProtein and albumin were included with all samples and served as internal controls. Additionally, samples were analyzed in parallel using microspheres conjugated to huFc-purified FIV-PPR-SU (**FIV-PPR-SU-huFc)** to assess background levels of anti-huFc IgG generated in response to vaccination. Levels of anti-huFc were estimated by subtracting levels of anti-SU IgG (as detected by SU-293S-conjugated microspheres) from the anti-huFc/anti-SU background levels detected with SU-huFc-conjugated microspheres **(Figure 2.3)**.

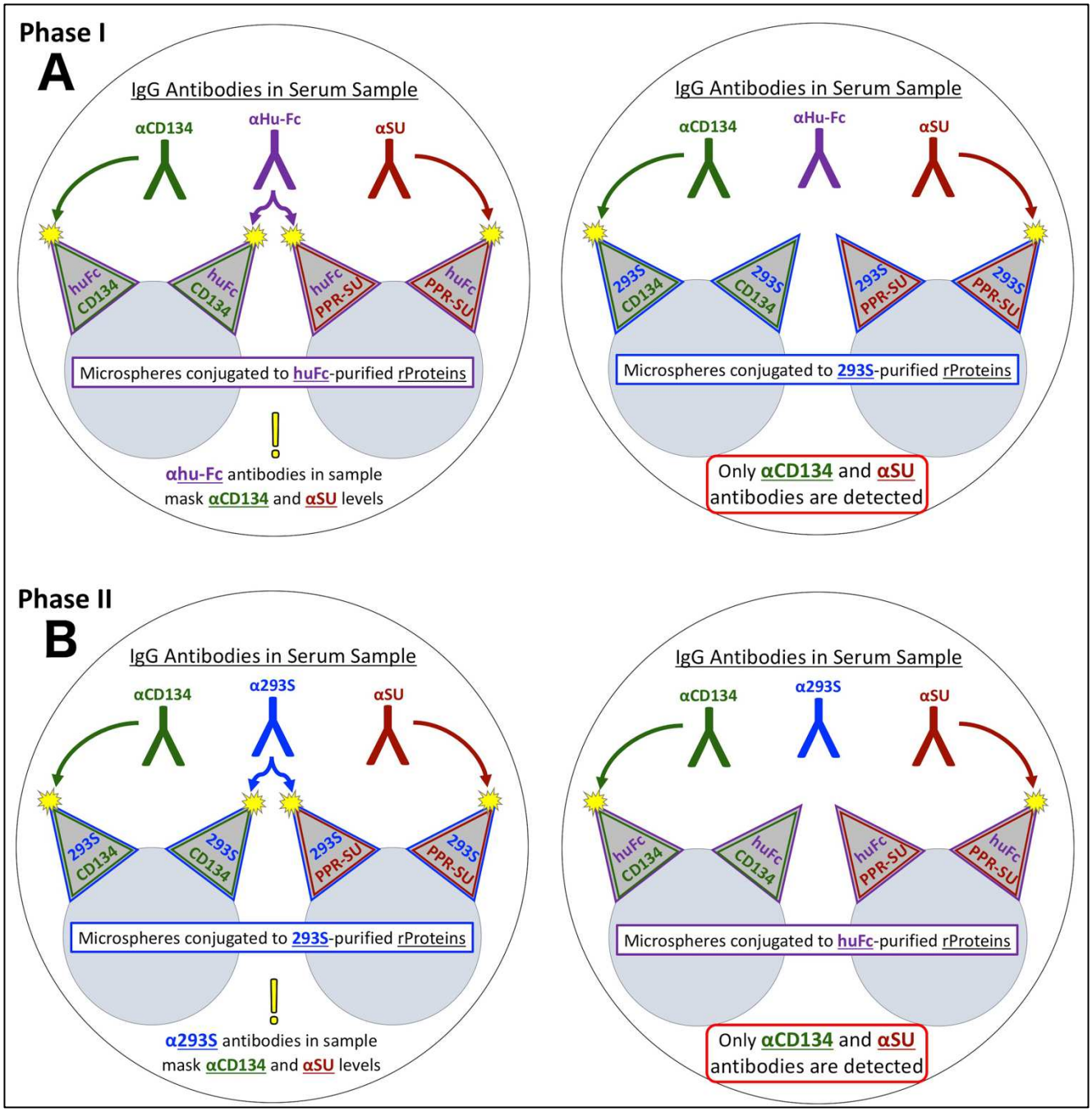

Figure 2.3. Microsphere immunoassay differentiates antibodies to specific vaccine antigens from irrelevant antibodies (anti-huFc, Phase I, and anti-293S Phase II). **(A)** Serum from cats vaccinated with huFc-derived CD134 and SU in Phase I was analyzed using microspheres coupled to soluble CD134 and recombinant SU purified in 293S cells in order to detect anti-CD134 and anti-Su IgG antibodies. **(B)** Phase II serum (cats vaccinated with 293S-derived CD134 and SU) was analyzed using huFC-purified soluble CD134 and recombinant SU. See text for additional details.

Phase II. Vaccine-specific antibodies were detected in plasma samples of study animals at weeks 10, 14, and 19-23 **(Figure 2.2B)** as described above. Similar to *Phase I*, SU and CD134

rProtein produced in CHO cells from *Phase I* (**huFc-purified**; **CD134-huFc** and **FIV-PPR-SU-huFc**) were conjugated to microspheres and used in all MIA assays involving samples from ***Phase II*** of this study to detect antibodies specific to CD134 and/or FIV-PPR-SU, and to limit interference and detection of background antibodies specific to vaccine by-products associated with the 293S-purified SU and CD134 **(Figure 2.3)**. All samples were analyzed in parallel using microspheres conjugated to 293S-purified CD134 and FIV-PPR-SU to assess background levels of anti-293S IgG generated in response to vaccination. The presence of anti-293S antibodies was confirmed by capture ELISA at an absorbance of 450nm in 96-well flat bottom plates coated overnight 293S cell supernatant at a concentration of 10µg/ml diluted in 100µl of 0.1M Carbonate Buffer (7.5 g/L Sodium Bicarbonate, 2.0 g/L Sodium Carbonate, pH ~9.5). Immunodepleted serum samples were diluted 1:25 in ELISA diluent and incubated for 2 hours at room temperature. Each well was then washed 5 times (TEN buffer + 0.2% Tween 20) and then incubated with Cappel™ horseradish peroxidase (HRP)-conjugated goat anti-cat IgG (MP Biomedicals, Santa Ana, CA) diluted 1:5000 in ELISA diluent with 5% mouse sera for 1 hour at room temperature. Each well was then washed 5 times and incubated for 10 minutes with 3, 3', 5, 5' tetramethyl benzidine (TMB) substrate and peroxidase (Biolegend, San Diego, CA) at room temperature before adding 2.5N H_2SO_4. Photometric measurements of absorbance were then recorded for each plate at 450nm as previously described [40].

Detection and quantification of FIV viral RNA and proviral DNA in blood

Blood samples collected during Phase I and Phase II were analyzed by real-time polymerase chain reaction (PCR) analysis to quantify FIV proviral DNA and FIV*gag* RNA at timepoints illustrated in **Figure 2.2**. Plasma was isolated from EDTA-treated whole blood following centrifugation and frozen at −70°C until processing. Viral RNA was extracted from 140µl plasma using a QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA) according to manufacturer's instructions. Viral RNA from each sample was converted to cDNA using Superscript II

(Invitrogen) in individual reactions with random hexamers (Invitrogen) and then treated with RNase Out (Invitrogen) prior to real-time PCR quantification. Peripheral blood mononuclear cells (PBMC) from all cats were purified on a Histopaque (Sigma, St. Louis, MO) gradient, washed, pelleted, and then frozen at −80°C. Proviral DNA was extracted from PBMCs using a DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) prior to real-time PCR quantification.

Real-time PCR reactions were performed on a CFX96™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA) to detect and quantify FIV proviral DNA in PBMCs and FIV *gag* RNA in plasma using previously described FIV-A primers and probes [41], and an iTaq™ Universal Probes Supermix (Bio-Rad, Hercules, CA) containing an antibody-mediated hot-start iTaq DNA polymerase. Copy number of viral RNA in plasma was calculated as previously described [37, 42], implementing a standard curve generated by diluting FIV-PPR virus stock in naïve cat plasma and analyzed by reverse-transcriptase quantitative PCR as outlined above. To quantify proviral DNA in PBMCs, a real-time PCR standard curve was generated from serial dilutions of feline PBMCs from 1000 to $5x10^6$ subjected to real time PCR for the cellular house-keeping gene, Glyceraldehyde-3-Phosphate Dehydrogenase (GADPH) as previously described [42, 43]. Resulting proviral copy numbers were normalized to copies per 10^6 cells based on the total amount of DNA present in the reaction (100ng).

Hematologic analyses.

Phase II. Complete blood counts (CBC) and serum biochemistry analysis were performed for all blood samples in Phase II by the CSU Veterinary Diagnostic Lab (CSU-VDL). Blood was collected from all cats prior to the study to establish baseline values, then at each time point indicated in **Figure 2.2B**. At weeks 20-24, the percentage of cells positive for CD4, CD8, Fas, and B220 surface antigens was determined by incubating 30µl of EDTA-treated blood from each cat in 96-well round-bottom plates with 0.6µl of RPE-labeled anti-feline CD4 (Southern Biotech; clone 3-4F4), FITC-labeled anti-feline CD8 (Southern Biotech; clone fCD8), PE/Cy7-labeled

anti-feline CD45R/B220 (Biolegend; clone RA3-6B2), and APC/Cy7-labeled anti-feline Fas/TNFRSF6 (R&D Systems; clone 431006) mouse monoclonal antibodies diluted in FACS buffer (5% BSA, 0.1% sodium azide in PBS). Following incubation for 30 min in the dark at room temperature, red blood cells (RBCs) were lysed, and stained cells were fixed using a Beckman Coulter Q-Prep work station with 600 µl of 0.1% Formic Acid, 270 µl of 0.06 M Na_2CO_3 anhydrous, 0.25 M NaCl, 0.25 M Na_2SO_3, and 90 µl 1% wt/vol paraformaldehyde in 1×PBS. Flow cytometry was performed on a Coulter Gallios (Beckman Coulter Inc, Brea, CA) and results were analyzed using FlowJo® software (FlowJo, Ashland, OR). Immunophenotype cell counts were calculated as previously described [37, 42] and compared with CBC data to evaluate changes in circulating immunophenotype over the course of vaccination and subsequent FIV infection. All CD4, CD8, and CD45R/B220 antibodies were directly labeled by the manufacturer. Anti-Fas antibody was unlabeled but subsequently conjugated to APC/Cy7 using a APC/Cy7® Labeling Kit (Abcam).

In vitro antibody inhibition and enhancement of viral replication.

Phase II. Duplicate cell cultures consisting of GFox cells (CrFK cells overexpressing CD134) [44, 45] were established in 48-well plates at 40,000 cells/well and allowed to attach at 37°C overnight. GFox cell cultures were grown at 37°C and 5% CO_2 in 250µl of culture medium composed of Dulbecco's modified Eagle's medium (DMEM) with GlutaMAX-1, 10% fetal bovine serum (FBS), and 1x penicillin-streptomycin (10,000 U/liter penicillin and 10,000 µg/liter streptomycin), as well as 1 µg/ml of Fungizone® (Amphotericin B; Life Technologies) [46]. At day 0, 10µl of FIV_{PPR} stock (containing 50,000 infectious units) was incubated for 1 hour at 37°C with 230µl of culture media and 10µl of whole serum from sham vaccinated, CD134 vaccinated, or CD134+SU vaccinated cats collected at week 19 (1 week pre-FIV challenge). Following incubation, infected media was then added to cell culture plates, bringing the total volume to 500µl (1:50 serum/FIV). Duplicate negative control (1:50 serum only, no FIV) and positive

control (FIV only, no serum) wells were included for each sample. At days 4, 6, 8, and 10 post-inoculation, 200µl of supernatant was removed from each well, frozen at -80°C, and replaced with 200µl of fresh culture media. At day 10, the supernatant collected from each well and each time point was assayed by a previously described capture ELISA protocol to detect FIV p26 antigen at an absorbance of 450nm in 96-well flat bottom plates [40]. Percent inhibition was calculated from mean absorbance values (Abs) using the previously described formula $((X - Y)/X) \times 100$, where X is fraction of cells infected in the absence of serum (virus only positive control) and Y is the fraction of cells infected in the presence of various serum treatments [47].

To elucidate the discrete effects of anti-SU, anti-CD134, and anti-293S antibodies generated *in vivo* in response to immunization, serum from vaccinated cats collected at week 19 (1 week pre-FIV challenge) was immunodepleted of anti-CD134 and anti-SU antibodies via serial passages over Actigel ALD agarose bead resin (Sterogene, Carlsbad, CA) that had been coupled to either CD134-huFc or FIV-PPR-SU-huFc rProteins per manufacturer's instructions. Anti-CD134 and anti-SU immunodepleted serum was then filtered through a microcentrifuge column by centrifugation at 8,000 rpm for 5 minutes. Vaccinated cat serum was immunodepleted of anti-293S antibodies by incubating 5mg of acetone-powdered 293S cells with 500µl of serum at 4°C overnight with gentle agitation, followed by centrifugation at 8,000 rpm for 5 minutes and pipet recovery of the immunodepleted supernatant. Immunodepleted samples and a subset of whole serum were depleted of complement by heat inactivation (56°C for 30 minutes). Immunodepletion of anti-CD134, anti-SU, and anti-293S antibodies was confirmed by capture ELISA (as previously outlined) at an absorbance of 450nm in 96-well flat bottom plates coated overnight with either CD134-huFc, FIV-PPR-SU-huFc, or 293S cell supernatant at a concentration of 10µg/ml diluted in 100µl of 0.1M Carbonate Buffer (7.5 g/L Sodium Bicarbonate, 2.0 g/L Sodium Carbonate, pH ~9.5). ELISA absorbance values indicated

statistically significant depletion of all targeted antibodies (Two-tailed t tests; anti-CD134 $p<0.0001$; anti-SU $p=0.005$; anti-293S $p=0.0002$).

Duplicate cell cultures consisting of GFox cells (CrFK cells overexpressing CD134) [44, 45] were established in 48-well plates as described above. At day 0, 10µl of FIV_{PPR} stock (containing 50,000 infectious units) was added to 480µl of fresh culture media along with 10µl (1:50 dilution) of various combinations of immunodepleted serum, with the contents of each well as follows: (1) No FIV (Negative Control), (2) FIV only (positive control), (3) Heat-treated CD134 vaccinated serum (to inactivate complement) (4) Heat-treated CD134+SU vaccinated serum (5) anti-SU serum, (6) anti-293S serum, and (7) anti-CD134 serum. Following incubation for 1 hour at 37°C, infected media was pipetted onto duplicate GFox cell cultures and incubated at 37°C for 12 hours, at which point all culture media was removed from each well, discarded, and replaced with 500µl of fresh culture media. GFox cells were visually inspected daily by inverted light microscopy for evidence of cell growth, attachment, syncytial cell formation, detachment, and cell death. At days 6, 8, and 10 post-inoculation, 200µl of supernatant was removed from each well, frozen at -80°C, and replaced with 200µl of fresh culture media. At day 10, the supernatant collected from each well and each time point was assayed by FIV p26 ELISA and the Abs value used to calculate percent inhibition as previously outlined.

To further identify the individualized effects of vaccine-produced antibodies in serum of immunized cats, previously absorbed anti-SU and anti-CD134 antibodies from each serum sample were recovered from agarose bead resins by elution with Pierce™ IgG Elution Buffer per manufacturer's instructions. Elutions containing purified anti-CD134 or anti-SU IgG were pooled separately and then stored in 100µl of D-PBS at 4°C. Duplicate GFox cell cultures were established in 48-well plates as previously outlined, and at day 0, 10µl of FIV_{PPR} stock (containing 50,000 infectious units) was combined with either 10µl or 5µl (1:50 or 1:100 dilution) of either purified antiCD134 or anti-SU IgG, and added to sufficient culture media to bring the total volume to 500µl. Duplicate negative control (no virus) and positive control (virus only)

wells were included for each sample. Following incubation for 1 hour at 37°C, infected media was pipetted onto GFox cells and incubated at 37°C for 12 hours, at which point all culture media was removed from each well, discarded, and replaced with 500µl of fresh culture media. At days 5, 7, and 9 post-inoculation, 200µl of supernatant was removed from each well, frozen at -80°C, and replaced with 200µl of fresh culture media. At day 10, the supernatant collected from each well and each time point was assayed by FIV p26 capture ELISA protocol and percent inhibition was calculated from mean absorbance values (Abs) as previously outlined.

Statistical Analyses

All analyses were conducted in the program R v3.0.2 (www.r-project.org) using the 'stats' package or using GraphPad Prism 6.0 software (La Jolla, CA). P-values < 0.05 were considered significant. Repeated measures ANOVA was utilized to evaluate the difference in viral RNA and proviral DNA (copies/ml) among the three vaccine groups (Naïve, CD134, CD134+SU) over time. Viral RNA, proviral DNA, and were log transformed to achieve normality prior to analysis. Repeated measures ANOVA was utilized to evaluate differences in anti-CD134, anti-PPR-SU, and anti-Capsid IgG antibodies in serum from the three vaccine groups (Naïve, CD134, CD134+SU) over time, and was also used to evaluate differences in FIV replication (Absorbance) and Percent Inhibition among the vaccinated groups over time. Logically, inhibition was not analyzed for the negative and positive groups owing to all values being 1 or 0 respectively.

Results

Anti-CD134 and anti-FIV-SU antibodies generated in vivo prior to viral challenge.

Phase I. Serum samples from weeks 4, 6, 8, 10, 13, 15, 18, and 22 were tested by microsphere immunoassay (MIA) to detect anti-SU and/or anti-CD134 IgG antibodies in cats vaccinated with FIV-PPR-SU-huFc or a combination of CD134+SU-huFc; results of which are presented in **Figure 2.4**. Increased levels of anti-CD134 antibodies were detected as early as

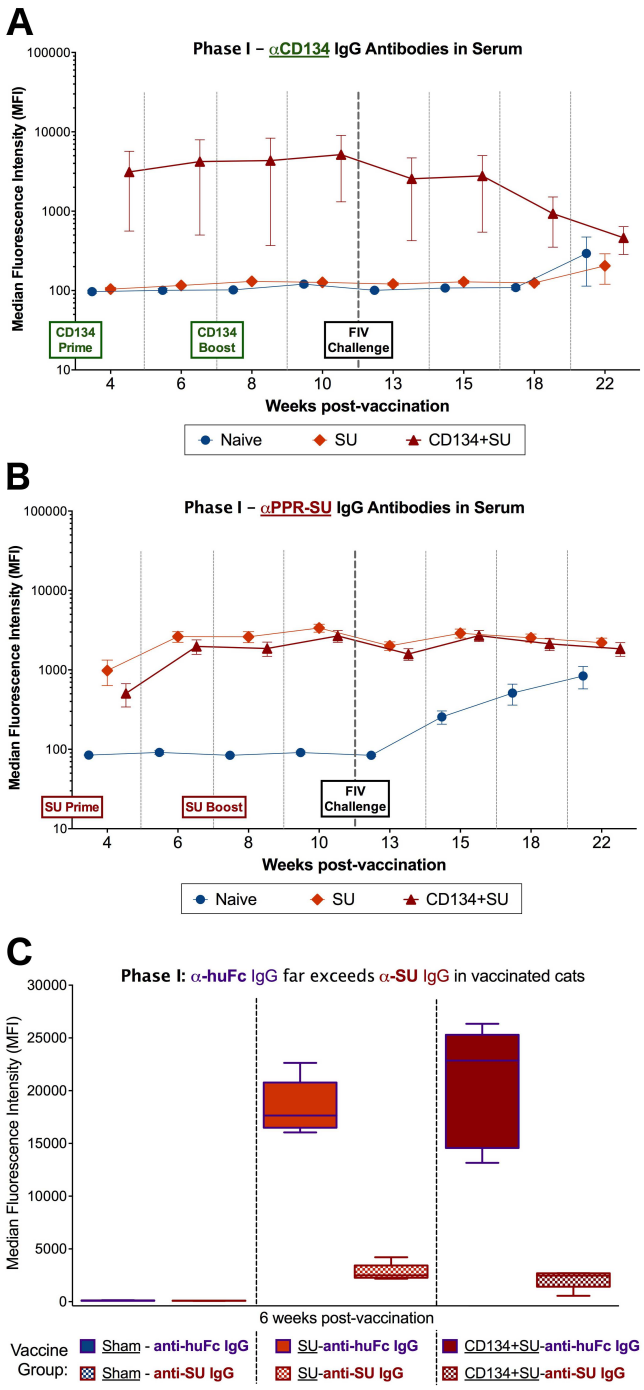

Figure 2.4. Anti-CD134, anti-SU, and irrelevant antibodies (α-huFc) are elicited during Phase I vaccination. (A) Microsphere immunoassay (MIA) detected anti-CD134 IgG antibodies in CD134+SU vaccinated cats beginning at week 4 post-vaccination and peaking at 10 weeks post-vaccination (3 weeks after final CD134 boost) **(B)** Anti-SU IgG was detected by MIA in SU vaccinated and CD134+SU vaccinated cats beginning at week 6 post-vaccination and these levels differed significantly over time compared to Sham-vaccinated controls ($p<0.0001$). **(C)** MIA measured IgG antibodies against huFc tag used to purify soluble CD134 and PPR-SU rProteins in Phase I vaccine preparation. Anti-huFc IgG levels (indicated by MFI) significantly exceeded levels of anti-SU and anti-CD134+SU ($p<0.0001$). Relative concentration of antibody response to huFc was estimated to be 10-fold higher than anti-SU specific IgG response (as described in text).

week 4post-vaccination in CD134+SU-huFc vaccinated cats and were significantly elevated at week 19 (1 week pre-FIV infection) compared to background levels (treatment p=0.014), but decreased slightly after infection **(Figure 2.4A)**. As expected, anti-CD134 IgG antibodies were not detected in serum from SU-huFc vaccinated or sham vaccinated study animals prior to FIV-infection **(Figure 2.4A)**.

Increased levels of anti-SU IgG were detected in SU-huFc vaccinated and CD134+SU-huFc vaccinated cats beginning at week 4 post-vaccination **(Figure 2.4B)**, and these levels differed significantly over time when compared to background levels (interaction p<0.0001). Anti-SU IgG antibody levels remained elevated in all SU-huFc and CD134+SU-huFc vaccinated animals after FIV infection. Anti-SU IgG antibodies were not detected in sham vaccinated cats prior to FIV infection, but increased slightly following intravenous viral inoculation as expected **(Figure 2.4B)**. When serum samples were evaluated for antibodies against the huFc tag used to purify the soluble CD134 and PPR-SU rProteins in the vaccine preparation, significantly increased levels of anti-huFc IgG were detected in SU-huFc and CD134+SU-huFc vaccinated cats (p<0.0001) which often exceeded anti-SU IgG by more than 10-fold **(Figure 2.4C)**.

Phase II. Serum samples from weeks 10, 14, and 19-23 were tested by MIA to detect anti-CD134 and anti-SU IgG antibodies in cats vaccinated with soluble CD134 and PPR-SU rProteins purified from 293S cells. Anti-CD134 IgG antibodies were detected in serum from CD134 vaccinated cats beginning at week 14 post-vaccination (6 weeks pre-FIV infection) and increased significantly over time compared to sham vaccinated background levels (interaction p=0.003) **(Figure 2.5A)**. Similarly, anti-CD134 IgG was detected in CD134+SU vaccinated animals beginning at week 10 post-vaccination (10 weeks prior to FIV infection), but the anti-CD134 IgG response was not as robust in this vaccine group and did not differ significantly from background levels (interaction p=0.135) **(Figure 2.5A)**.

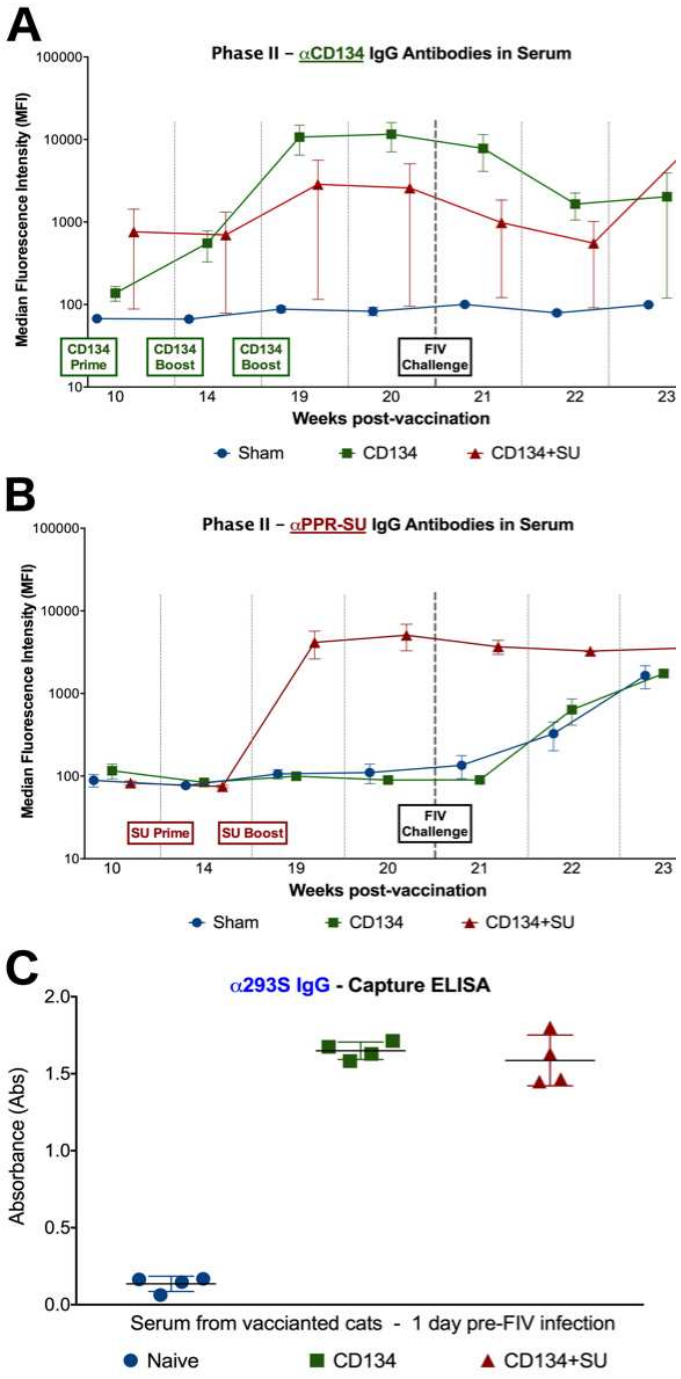

Figure 2.5. Phase II vaccination scheme boosts vaccine-specific antibody response *in vivo*, but irrelevant antibodies are still present. (A) MIA detected significant quantities of anti-CD134 IgG antibodies in serum from CD134 and CD134+SU vaccinated cats beginning at week 14 post-vaccination (6 weeks pre-FIV infection) which increased significantly over time in CD134 vaccinated cats compared to sham vaccinated background levels ($p=0.003$). **(B)** Anti-SU IgG antibodies were detected in CD134+SU vaccinated cats by MIA at 1 week prior to FIV challenge (2 weeks after final SU boost), and antibody levels increased significantly over time compared to controls ($p=0.001$). **(C)** Capture ELISA detected high levels of anti-293S antibodies in serum of both CD134 vaccinated ($p<0.0001$) and CD134+SU vaccinated ($p<0.0001$) cats immunized with the 293S-purified CD134 and/or FIV-SU vaccine construct compared to sham vaccinated cats.

Anti-SU IgG antibodies were detected in CD134+SU vaccinated cats beginning at week 19 (1 week prior to FIV infection), and antibody levels increased significantly over time compared to the background levels (interaction p=0.001) **(Figure 2.5B)**. As expected, anti-SU antibodies were not detected in sham vaccinated or in CD134 vaccinated cats prior to FIV infection, but increased slightly over time after intravenous viral inoculation. Similar to *phase I*, high levels of anti-293S antibodies were detected by ELISA in serum of cats vaccinated with 293S-purified s oluble CD134 and/or PPR-SU rProteins **(Figure 2.5C)**. Specifically, serum from CD134 and CD134+SU vaccinated cats exhibited significantly elevated absorbance (Abs) values compared to serum from sham vaccinated cats (interaction, $p<0.0001$), indicating that significant *in vivo* antibody production occurred in response to the 293S cell products used in vaccine production.

FIV challenge

Phase I. At week 12 post-vaccination, study animals were intravenously inoculated with FIV_{PPR} as described above. Plasma samples from all study animals in all vaccine groups had detectable FIV plasma RNA by week 2 post-FIV infection **(Figure 2.6A)**. Moreover, FIV RNA levels were significantly elevated at week 2 in SU-huFc vaccinated ($p<0.001$) and CD134+SU-huFc vaccinated ($p<0.05$) study animals when compared to sham vaccinated animals **(Figure 2.6A),** suggesting a transient enhancement of FIV infection in these vaccine groups. PBMC FIV proviral DNA was detected in all study animals by week 2 post-FIV infection, and proviral load peaked at week 8 post-infection **(Figure 2.6B)**. No significant differences in FIV DNA proviral loads were detected between vaccine groups over time.

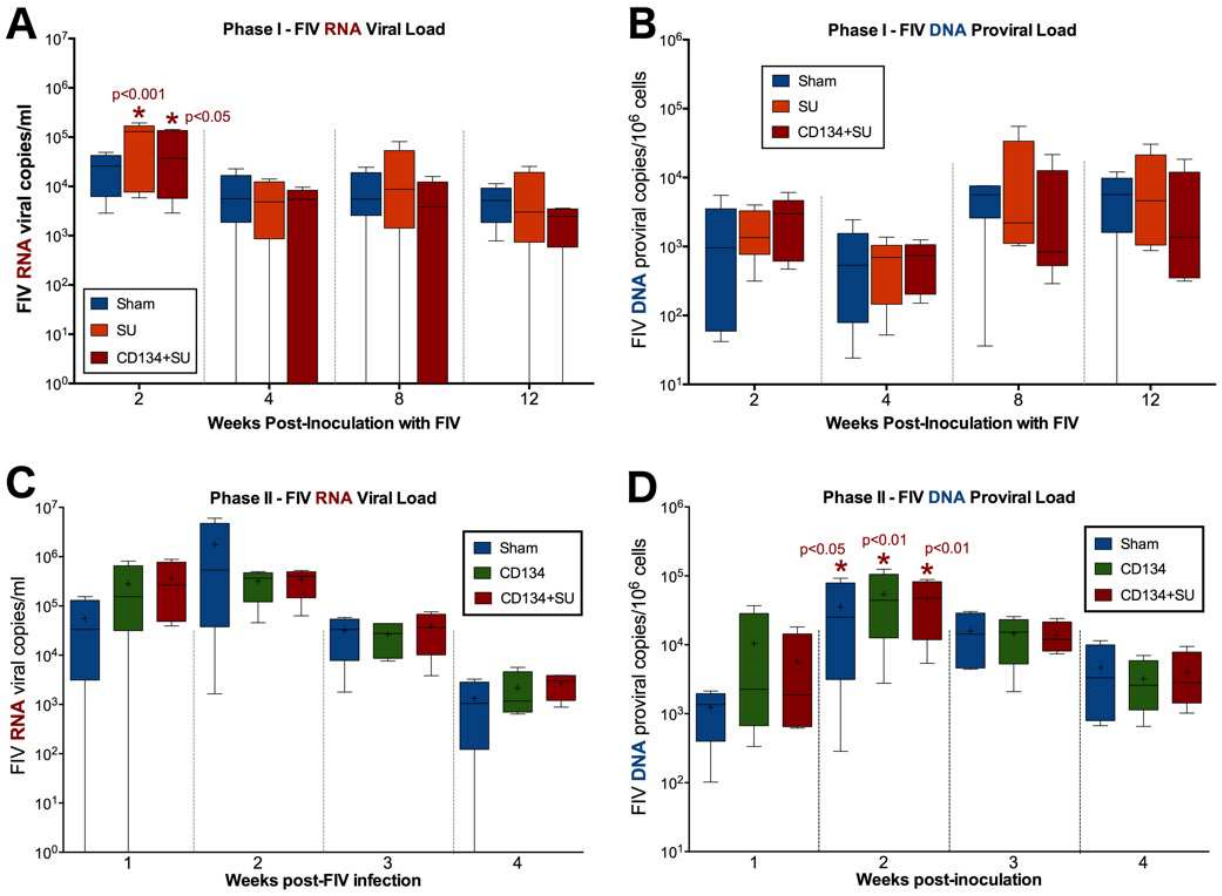

Figure 2.6. CD134 and/or SU vaccinated cats were not protected against FIV challenge. (A) All *Phase I* study animals demonstrated positive viremia by week 2 post-FIV infection, and FIV RNA levels were significantly elevated at week 2 in SU vaccinated ($p<0.001$) and CD134+SU vaccinated ($p<0.05$) cats; suggesting a transient enhancement of FIV infection. **(B)** Proviral DNA was detected in PBMCs of all *Phase I* study animals by week 2 post-FIV infection, but did not differ between vaccine groups over time. **(C)** All *Phase II* study animals demonstrated FIV RNA in plasma by week 2 post-infection. FIV RNA levels decreased significantly over time in all groups (time $p<0.001$), however, no significant differences were observed between vaccine groups over time during this phase. **(D)** Proviral DNA was detected in PBMCs of all *Phase II* study animals at week 1 post-FIV infection, and were significantly elevated above baseline in all study groups by week 2 post-inoculation. Proviral DNA levels did not differ over time or between vaccine groups during Phase II.

Phase II. At week 20 post-vaccination, study animals were intravenously inoculated with FIV_{PPR} as detailed above. Quantitative PCR analysis of plasma detected FIV RNA in most study animals within 1 week of FIV inoculation **(Figure 2.6C)**. All CD134+SU vaccinated cats demonstrated FIV RNA in plasma by week 1 post-FIV infection, and all sham and CD134 vaccinated cats were positive for FIV RNA by week 2 post-infection. Although the level of viremia (FIV RNA) decreased significantly over time in all groups (time $p<0.001$, **Table 2.1**), no

significant differences in FIV RNA levels were observed between vaccine groups. PBMC proviral DNA was detected in all study animals at week 1 post-FIV infection **(Figure 2.6D)** and was significantly elevated above baseline in all study groups by week 2 post-inoculation. However, proviral loads did not differ significantly between groups over time in this phase of the study. Results indicate that vaccination with CD134 or CD134+SU complexes as prepared in either trial does not provide protection from FIV infection *in vivo*.

Table 2.1. No significant differences in vaccine treatment are observed over time in Phase I or Phase II.

Quantitative PCR	**Viral RNA**			**Viral DNA**		
	F	df	**P**	F	df	P
Phase I						
Time	12.53	3	**<0.001**	14.28	3	**<0.001**
Interaction (Time*Treatment)	1.959	9	0.0742	0.4282	9	0.911
Phase II						
Time	14.368	1	**<0.001**	0.255	1	0.615
Interaction (Time*Treatment)	1.852	2	0.164	0.598	2	0.552

Purified anti-CD134 and anti-SU antibodies inhibit FIV replication in vitro

Phase II. Prior to FIV infection, serum was collected from sham, CD134 and CD134+SU vaccinated cats and incubated directly with FIV_{PPR} prior to *in vitro* inoculation. Additionally, serum from CD134+SU vaccinated cats was heat-treated (to inactivate complement) and depleted of anti-SU, anti-CD134, and/or anti-293S antibodies to evaluate the individual effects of these components on FIV replication *in vitro*. Results of these experiments are summarized in **Table 2.2**. Surprisingly, whole serum from both CD134 and CD134+SU vaccinated cats significantly enhanced FIV replication *in vitro*, as indicated by significantly higher FIV p24 ELISA absorbance values and decreased percent inhibition below threshold **(Figure 2.7A)**. In contrast, heat-inactivated serum depleted of anti-CD134 and anti-293S antibodies (containing only anti-SU antibodies) significantly inhibited FIV_{PPR} replication in GFox cell culture, as evidenced by significantly lower FIV p24 ELISA absorbance values and calculated percent inhibition compared to the FIV-only positive control **(Figure 2.7B).** Similarly, serum containing

only anti-CD134 antibodies inhibited FIV growth in vitro on day 6 of culture **(Figure 2.7B)**. Interestingly, a significant and sustainted inhibitory effect was observed at all timepoints utilizing heat-treated serum from CD134+SU vaccinated cats, in which complement was inactivated but contained all antibodies generated *in vivo (*anti-SU, antiCD134, and anti-293S). In contrast, no inhibitory effect was observed in wells treated with heat-treated serum from CD134 vaccinated

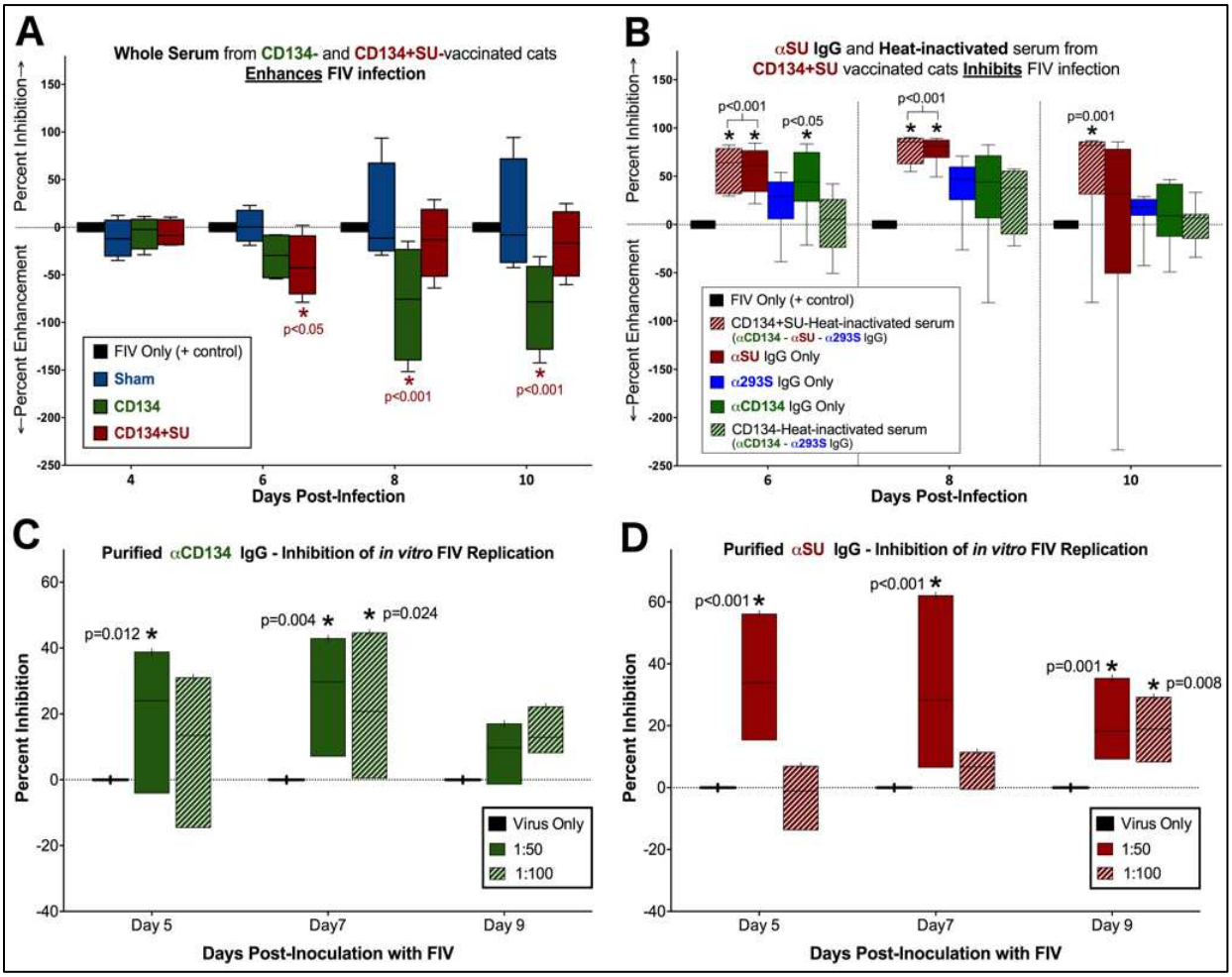

Figure 2.7. Anti-CD134 and anti-SU antibodies significantly inhibit FIV replication *in vitro*. **(A)** Whole serum from CD134 and CD134+SU vaccinated cats produced significant enhancement of FIV replication in GFox cells (CD134 p=0.0005; CD134+SU p=0.047), indicated by significantly decreased percent inhibition compared to FIV only infections. Sham vaccinated serum did not significantly inhibit infection. **(B)** Antibody fractions representing anti-CD134, anti-SU, and anti-293S were enriched or depleted from serum as described in the text, and analyzed for ability to inhibit in vitro viral infection. Significant inhibitory effects were observed with purified anti-SU IgG and heat-inactivated serum containing anti-SU, anti-CD134, and anti-293S IgG **(Table 2.2)**. Enriched anti-CD134 **(C)** and anti-SU **(D)** antibodies recovered from serum of CD134 and CD134+SU vaccinated cats demonstrated significant inhibition of FIV replication *in vitro* **(Table 2.3)**, confirming the individual capacity to inhibit FIV infection reported by Grant et al. [28].

cats (complement-inactivated serum with anti-CD134 and anti-293S IgG). Serum depleted of anti-CD134 and anti-SU, but still containing anti-293S (ie., contaminating) antibodies from CD134+SU vaccinated cats had a negligible effect of FIV replication, signaling that these irrelevant antibodies were not a cause of inhibition/neutralization interference *in vitro*. Importantly, enhancing effects were not observed for any of the heat-treated samples,

Table 2.2. Anti-SU IgG fraction neutralizes in vitro FIV infection whereas whole serum enhances infection. Whole serum from CD134 and CD134+SU vaccinated cats significantly enhanced FIV replication *in vitro*. Heat inactivation of whole serum from CD134+SU vaccinated cats resulted in *in vitro* inhibition, suggesting enhancing factors in whole serum are heat-labile. Anti-SU antibodies generated in CD134+SU vaccinated cats exhibit significant individual or combined inhibitory effects on FIV replication following heat-treatment to remove heat-labile proteins in serum, while serum enriched for anti-CD134 and anti-293S fractions neither inhibited or enhanced in vitro infection.

Treatment	*Factors Removed from Serum*	Immune Factors Retained in Serum	Effect on FIV Replication *in vitro*
Whole Serum **Sham group**	*N/A*	**Complement** & heat labile factors	*N/A*
Whole Serum **CD134 group**	*N/A*	**Complement** & heat labile factors **anti-CD134 IgG** **anti-293S IgG**	**Enhancement**
Whole Serum **CD134+SU group**	*N/A*	**Complement** & heat labile factors **anti-SU IgG** **anti-CD134 IgG** **anti-293S IgG**	**Enhancement**
Heat-treated serum **Sham group**	***Complement*** *(inactivated)* *Heat labile factors (inactivated)*	*N/A*	*N/A*
Heat-treated serum **CD134 group**	***Complement*** *(inactivated)* *Heat labile factors (inactivated)*	**anti-CD134 IgG** **anti-293S IgG**	*N/A*
Heat-treated serum **CD134+SU group**	***Complement*** *(inactivated)* *Heat labile factors (inactivated)*	**anti-SU IgG** **anti-CD134 IgG** **anti-293S IgG**	**Inhibition**
Serum enriched for anti-SU	***Complement*** *(inactivated)* *Heat labile factors (inactivated)* **anti-CD134 IgG** **anti-293S IgG**	**anti-SU IgG**	**Inhibition**
Serum enriched for anti-CD134	***Complement*** *(inactivated)* *Heat labile factors (inactivated)* **anti-SU IgG** **anti-293S IgG**	**anti-CD134 IgG**	*N/A*
Serum enriched for anti-293S	***Complement*** *(inactivated)* *Heat labile factors (inactivated)* **anti-SU IgG** **anti-CD134 IgG**	**anti-293S IgG**	*N/A*

regardless of antibody content, indicating that significant enhancement of FIV replication *in vitro* may occur as a result of heat-labile proteins such as complement.

Purified anti-CD134 and anti-SU antibodies from serum of vaccinated cats were used to further evaluate the individual capacity of these antibodies to inhibit FIV replication in GFox cells **(Table 2.3)**. Analysis of mean absorbance values demonstrated that both purified anti-CD134 IgG ($p<0.01$) and anti-SU IgG ($p<0.04$) significantly inhibited FIV replication *in vitro*. Post-hoc analysis revealed that both 1:50 and 1:100 dilutions of purified fractions of anti-CD134 **(Figure 2.7B)** and anti-SU **(Figure 2.7C)** were able to significantly inhibit FIV.

Table 2.3. Purified anti-CD134 and anti-SU IgG antibodies neutralize FIV infection *in vitro*. Anti-SU and anti-CD134 antibodies from CD134+SU vaccinated cat serum were adsorbed onto agarose bead resins and recovered with Pierce™ IgG Elution Buffer. Elutions containing purified anti-CD134 or anti-SU IgG were then inoculated onto GFox cells infected with FIV_{PPR} stock. Results demonstrate that both purified anti-CD134 and anti-SU antibodies significantly inhibit FIV replication independently **(Figure 2.7B-C)**, but not in the presence of heat-labile elements in serum (such as complement).

Treatment	Immune Factors Present	Effect on FIV Replication *in vitro*
Whole Serum **CD134+SU group**	**Complement** & heat labile factors **anti-SU IgG** **anti-CD134 IgG** **anti-293S IgG**	**Enhancement**
Purified anti-SU	**anti-SU IgG**	**Inhibition**
Purified anti-CD134	**anti-CD134 IgG**	**Inhibition**

Vaccinated cats exhibit divergent immunologic responses

In ***Phase II***, phenotype analysis of circulating immunocytes was performed to determine whether: (1) vaccine-associated changes in peripheral blood immunophenotype at the time of inoculation may have contributed to vaccine failure; or (2) vaccination protocols altered post-FIV challenge peripheral blood immune profile. Prior to FIV challenge, CD8+, CD4+, and B220+

lymphocytes were significantly elevated in CD134+SU vaccinated cats compared to CD134 vaccinated cats ($p<0.03$, $p<0.04$, $p<0.03$, respectively) **(Figure 2.8A-C).** Average numbers of CD4+ and B220+ lymphocytes also tended to be slightly higher in CD134+SU vaccinated cats than in sham vaccinated cats, but this finding was not statistically significant **(Figure 2.8A, 2.8C).** Additionally, there was a trend ($p=0.081$) for CD8+ lymphocytes to be decreased in CD134 vaccinated cats compared to sham vaccinated cats prior to FIV challenge **(Figure 2.8B)**. In CD134+SU vaccinated cats, the CD4:CD8 ratio prior to FIV challenge was correlated with early proviral load ($p=0.05$, $R^2=0.89$) **(Figure 2.8D)**, indicating that increased viral integration may be associated with decreased numbers of CD4+ lymphocytes relative to CD8+ cells

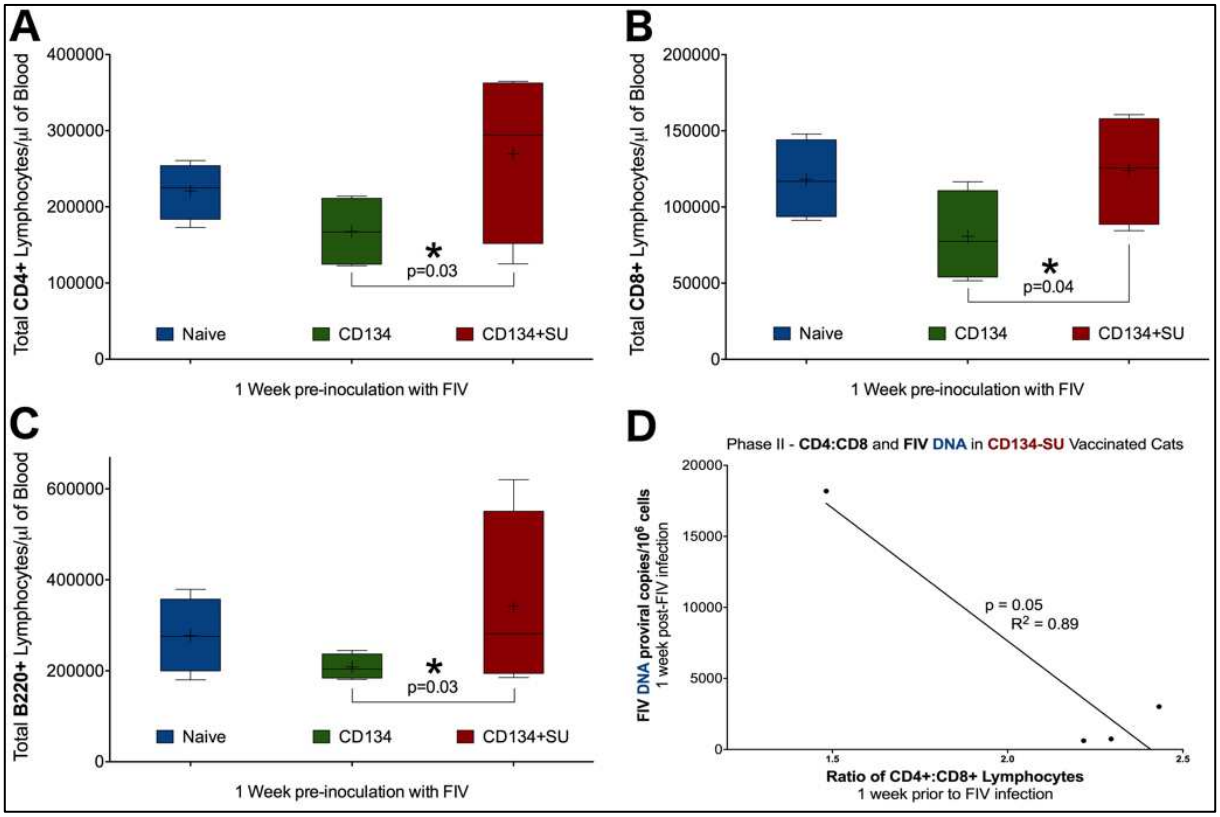

Figure 2.8. Immunization results in alterations in lymphoid immunophenotype in vaccinated cats. Prior to FIV challenge in Phase II studies, CD8+ **(A)** CD4+ **(B)**, and B220+ **(C)** lymphocytes were significantly elevated in CD134+SU vaccinated cats compared to CD134 vaccinated cats ($p<0.03$, $p<0.04$, $p<0.03$, respectively). CD4+ and B220+ cell levels were slightly higher in CD134+SU vaccinated cats compared to Sham vaccinated cats prior to FIV challenge, and there was a trend for CD8+ cells to be decreased ($p=0.081$) in CD134 vaccinated cats. **(D)** In CD134+SU vaccinated cats, decreased CD4:CD8 ratios prior to FIV challenge correlated with higher proviral DNA loads during acute stages of FIV infection, indicating that changes induced in circulating T cell populations by vaccination-associated immunostimulation may predispose to enhanced susceptibility to viral infection.

Discussion

The development of a partially effective commercial FIV vaccine, coupled with previous findings that anti-receptor autoantibodies are associated with better clinical outcomes, suggests that co-immunization with viral and receptor antigens might augment vaccine effectiveness. However, despite the promising results of *in vivo* production of neutralizing antibodies, cats in this study were not protected against homologous FIV infection, and occasionally exhibited transient enhanced infection (as evidenced by increased FIV RNA levels post challenge). Several studies in FIV vaccine design have resulted in enhanced susceptibility to infection rather that protection [22-24, 48]. These complications are frequently paralleled in SIV and HIV vaccine development [48-54] and have impeded progress in vaccine efficacy by a variety of mechanisms, including antibody-dependent viral enhancement or general immune activation [22-27]. Accordingly, we performed several further analyses in this study in attempt to elucidate the mechanism for vaccine failure, including: (1) potential contribution of anti-SU or anti-CD134 vaccine induced antibodies to enhanced FIV-replication; (2) assessment of alterations in circulating PBMC induced by vaccination (potentially in response to immune activation) which might render vaccinated cats more susceptible to viral challenge via expanded target cell population; (3) evaluation of antibodies generated in response to irrelevant antigens present in the vaccine preparation that could contribute to enhancement; and, (4) magnitude of relevant (ie anti-SU, anti-CD134) versus irrelevant (ie anti-huFc, anti-293S antigen) antibody responses. By determining which aspects of vaccination contribute to failure of this trial, future efforts can be modified to avoid pitfalls of this study.

Primary results of this study confirm that vaccination with soluble CD134-SU complexes induces *in vivo* production of both anti-SU and anti-CD134 antibodies, and when purified from serum of vaccinated cats, both antibodies are independently effective at neutralizing FIV infection in tissue culture. However, even in the presence of circulating neutralizing antibodies, cats challenged with homologous FIV virus were not protected against infection *in vivo*, and in

some cases, succumbed to transient enhanced infection in the presence of SU rProtein. Correspondingly, *in vitro* analysis of whole serum from CD134+SU vaccinated cats indicated significantly enhanced viral infection in cell culture **(Figure 2.7A)**, but when heat-treated to inactivate complement and other heat-labile elements, serum containing anti-CD134 and anti-SU antibodies exhibited significant inhibitory effects. Indeed, heat-inactivated serum from CD134+SU (but not CD134) vaccinated cats significantly inhibited *in vitro* FIV replication at all time points evaluated, indicating a sustained inhibitory effect when both anti-CD134 and anti-SU antibodies were present **(Figure 2.7B)**. These findings highlight the potential for a synergistic mechanism of viral inhibition between anti-CD134 and anti-SU antibodies produced in response to vaccination with CD134-SU complexes, but only in the absence of heat-labile serum factors.

Complement enhancement of viral infection has been previously reported in HIV [55-58] and SIV [59-61] studies, and may occur via both antibody-dependent and antibody-independent mechanisms of complement activation [62-69]. Similar consequences of complement activation may be responsible for the enhancement effects noted in this study. For example, immunization with anti-receptor complexes may induce complement activation *in vivo*, resulting in viral opsonization or deposition of antibody-complement complexes on the cell to provide prolonged receptor contact and subsequent fusion within the cell membrane [48, 56, 70]. Interestingly, non-heat-treated whole serum from sham vaccinated cats did not enhance FIV-replication *in vitro* **(Figure 2.7A)**, despite the presence of serum complement in treated wells. This suggests that *in vivo* complement activation may not occur in the absence of soluble CD134 or recombinant SU protein, and that the presence of anti-receptor complexes in vaccinated cats may in fact influence the activation of complement and enhancement of FIV replication *in vitro*. Other heat-labile factors, such as cytokines, chemokines, growth factors, and adhesion molecules, are also present in serum and may similarly contribute to enhanced replication of FIV *in vitro* [71], particularly if the vaccination protocol has altered the immune landscape. Additionally, because sham vaccinated whole serum did not cause enhancement effects like

CD134 and CD134+SU vaccinated whole serum, it is reasonable to assume that activation of such viral enhancing elements in this study are likely attributable to vaccination with soluble CD134, recombinant SU protein, and/or purification by-products (huFc or 293S cells). Future studies will be directed at elucidating the role that complement activation and other heat-labile serum components play in the interference of the anti-receptor antibody response during viral infection.

An additional factor to consider is the disproportionate antibody response generated in vaccinated cats against the vaccine by-products; huFc and 293S cell contaminants co-purified with the recombinant proteins. Microsphere immunoassay results demonstrated a substantial quantity of anti-Fc antibodies in *Phase I* vaccinated cats, as well as a marked anti-293S antibody response in *Phase II* animals. While *in vitro* experimental results indicated that these antibodies did not directly interfere with or enhance FIV infection, it is possible that their production may have indirectly interfered with vaccine efficacy by compromising the neutralizing antibody response. Based upon the magnitude of antibodies generated to these antigens (huFc and 293S), the proportion of coincident anti-CD134 and anti-SU antibodies produced in response to the targeted immunogens was on average 10-fold lower, indicating that the bulk of antibody production was directed at these “irrelevant” antigens instead of their intended target. As such, the potential level of anti-CD134 and anti-SU antibody necessary for protection from FIV infection was likely never attained *in vivo*, thus preventing the current immunization strategy from providing a neutralizing antibody response strong enough to prevent infection in vaccinated cats. Moreover, the potential for these foreign antigens to activate complement and other heat-labile immune response factors may have contributed to the transient enhancement effects observed *in vivo,* and the magnitude of the antibody response to such antigens may account for the *in vitro* enhancement effects observed in cats vaccinated with anti-receptor complexes. Lastly, it is possible that exposure to foreign/irrelevant antigens may have driven antibody production toward a predominant IgG1 response rather than a more protective IgG2a response.

The potential for this may be subsequently avoided through use of non-alum based adjuvants such as Ribi, which enhance IgG2a production and the Th1 response, as well as production of protective interferon-gamma [72-74]. Future vaccine design methods will incorporate the use of more highly-purified immunoadhesion peptides which lack purification by-products, thereby limiting the potential for interference in neutralizing antibody production and innate immune activation by irrelevant antigens.

In SIV and HIV immunization studies, much of the capacity to enhance susceptibility to infection has been attributed to an increase in general immune activation and/or expansion of lymphoid target cells, and this feature has also been observed in FIV studies [25, 54, 75-79]. Flow cytometry analysis of lymphoid immunophenotypes in *Phase II* identified significant variations in CD4+, CD8+, and B220+ lymphocytes among CD134 and CD134+SU treatment groups prior to infection (**Figure 2.8)**, and a positive correlation was observed between the CD4:CD8 ratio and acute DNA proviral load in CD134+SU immunized cats. Collectively, these results indicate that an acute, vaccine-induced increase in CD4+ lymphocytes relative to CD8+ cells in CD134+SU immunized cats may contribute to viral integration and the establishment of FIV infection. However, no significant differences in immunophenotype prior to infection were observed between sham vaccinated cats and either CD134 or CD134+SU vaccinated cats in *Phase II* of this study, suggesting that alterations in circulating immunophenotype due to vaccination with anti-receptor complexes may not play a major role in vaccine failure.

Alternatively, it is possible that vaccination with alum (aluminum hydroxide), a widely-used immunopotentiating reagent frequently used in vaccine preparations to boost humoral immunity [80-82], resulted in increased amounts of target cells and general immune activation in this study, thus priming the immune system for infection prior to FIV inoculation. However, sham vaccinated cats also received alum during this study, and no evidence of viral enhancement was observed in these animals *in vivo* **(Figure 2.6)** or through use of their serum *in vitro* **(Figure 2.7)**. These results suggest that any enhancing effect observed in SU-huFc

vaccinated cats of Phase I and CD134+SU vaccinated cats of Phase II was less dependent upon the use of alum, and implicate the immunogen among these groups: SU rProtein. The direct indictment of SU as a sole cause of vaccine failure is, however, unlikely considering the following evidence: (1) all CD134 vaccinated cats in Phase II became infected by week 1 post-infection, yet these animals were not immunized with SU, and did not generate an anti-SU antibody response prior to infection; and, (2) purified anti-SU antibodies from CD134+SU vaccinated cats significantly *inhibited* FIV replication *in vitro,* substantiating that the presence of these antibodies elicit protective effects against FIV infection as previously reported [28].

In summary, the results of this study identify specific obstacles to overcome in the development of anti-receptor antibody immunization, and highlight potential targets for optimization and *in vivo* study design. Immunization with soluble receptors (CD134) and viral surface glycoprotein (FIV-SU) elicits *in vivo* production of neutralizing antibodies (anti-CD134 and anti-SU IgG) in vaccinated cats, both of which significantly inhibit FIV replication *in vitro*. Immunization with anti-receptor complexes did not prevent FIV infection, though efficacy may have been negatively affected by a substantially disproportionate antibody response to vaccine by-products that likely compromised an effective neutralizing antibody response. Importantly, the role of heat-labile factors, including complement, in serum of vaccinated cats may play a role in the interference of anti-receptor antibody function and vaccine efficacy, and vaccination protocols need to be developed that do not enhance populations of circulating cells with enhanced susceptibility to FIV infection. Collectively, these findings illustrate several key features of the receptor-mediated immunogenic response in cats, and suggests that alteration of immune landscape during vaccination may improve efficacy in the development of a lentiviral vaccine.

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CHAPTER 3. IMMUNOMODULATORY THERAPY DURING FIV INFECTION

Summary

Feline immunodeficiency virus (FIV) induces lifelong infection in cats and may result in a spectrum of immunodeficiency-related diseases. Both prednisolone and cyclosporine A (CsA) are commonly used clinically to treat lymphoproliferative and immune-mediated diseases in cats, but the impact of these compounds on FIV infection has not been well documented, and their understanding immunomodulatory effects on FIV replication and persistence is critical to guide safe and effective use of these therapies in FIV infected cats. To fill this knowledge gap, FIV-infected cats or age-matched controls were administered immunosuppressive doses of prednisolone (2mg/kg) or CsA (5mg/kg) for 28 days in a cross-over experiment. Alterations in hematological parameters and FIV viral loads were monitored by flow cytometry and quantitative PCR. Both prednisolone and CsA induced acute and transient increases in FIV proviral load and viremia, and significant differences in lymphocyte phenotype were observed between FIV-infected and naïve cats treated with CsA and prednisolone. Both treatments caused: (1) acute increases in CD4+ lymphocytes; (2) increased FIV viremia; and, (3) significant alterations in cytokine expression that favored a shift toward a Th2 response. Antiretroviral therapy inhibited CsA-induced viremia, but not proviral load, indicating CsA treatment induces new viral replication but does not result in complete reactivation of latently infected cells. Results from this study highlight the potential for immunosuppressive drug-induced perturbation of FIV replication and underscores the need for consideration of chronic viral infection status when prescribing immunomodulatory medications.

Background

Feline immunodeficiency virus (FIV) is a naturally occurring lentivirus of domestic cats that produces progressive immune depletion resulting in an AIDS-like syndrome [1-10]. Similar

to human immunodeficiency virus (HIV), FIV-infected cats frequently develop secondary or opportunistic infections as a consequence of viral-induced immune dysfunction, including anterior uveitis, chronic rhinitis, gingivostomatitis and periodontitis, encephalitis and neurologic dysfunction, and lymphoma [10-19]. While antiretroviral therapy (ART) has demonstrated modest success at controlling FIV infection *in vivo,* the capacity of ART to alleviate symptoms and control the development of secondary disease syndromes has been limited [9,20-22]. Adjunct immunosuppressive therapies may be prescribed to ameliorate symptoms of secondary diseases associated with FIV infection, or unrelated inflammatory conditions that might occur in FIV infected cats. Two commonly used compounds in this category are prednisolone and cyclosporine A (CsA), but the effects of these compounds on FIV infection kinetics are unknown [23-32]. Thus, there is a critical need for a better understanding of the immunomodulatory effects of these drugs on FIV replication and persistence in order to guide safe and effective therapies during FIV infection.

While prednisolone and CsA differ distinctly in regard to pharmacokinetics and mechanism of action, they produce similar therapeutic effects that are mediated primarily through modulation of interleukin 2 (IL-2) expression [32-38]. IL-2 is a pro-inflammatory cytokine with many regulatory functions during the development and differentiation of T-lymphocytes [39,40]. Additionally, IL-2 expression plays a central role in the proliferation and activation of effector and memory T-lymphocytes, and both prednisolone and CsA exhibit a similar impact on this important regulatory pathway [39,40]. The primary immunomodulatory effects of prednisolone are achieved through binding of glucocorticoid receptors, followed by nuclear translocation and binding of glucocorticoid response elements (GRE) within target genes [36-38]. By this mechanism, prednisolone inhibits gene transcription of inflammatory genes, causing suppression of IL-2 expression that results in decreased T-lymphocyte proliferation [36-38]. Alternatively, CsA binds to the intracellular protein receptor cyclophilin and inhibits the phosphatase activity of calcineurin, a critical component of the T-cell activation pathway [32-35].

By blocking calcineurin activity, CsA inhibits the translocation of the cytosolic component of the nuclear factor of activated T cells (NF-AT), resulting in suppression of IL-2 gene promoter/enhancer function and consequent inhibition of T-lymphocyte proliferation [32-35]. Thus, although they utilize divergent pharmacokinetic pathways, these compounds produce a similar immunomodulatory effect by decreasing proliferation and activation of T-lymphocytes.

Because FIV predominately targets CD4+ T-lymphocytes during infection in domestic cats [1-4,6,8], it is possible that immunosuppressive doses of prednisolone and CsA could broadly reduce lymphocyte proliferation and activation in FIV-infected cats; thereby decreasing the number of target cells for FIV infection and as a result, reduce FIV viral and proviral loads in circulation. Conversely, the immunosuppressive effects of these compounds could weaken the anti-FIV immune responses that suppress FIV replication during the chronic phase of infection, resulting in increases in viral loads. Thus, to determine the impact of prednisolone and CsA on FIV replication kinetics, chronically-infected SPF cats were administered immunosuppressive doses of prednisolone or CsA in a cross-over study design. Following a wash-out period, CsA was administered prior to antiretroviral therapy (ART - zidovudine + lamivudine) to assess the effects of immunomodulation on ART efficacy, and to determine whether CsA might result in activation of viral replication. Hematological parameters were monitored by flow cytometry, and compared with changes in circulating FIV RNA and DNA levels as detected by quantitative PCR (qPCR). Shifts in circulating lymphocyte immunophenotype and virologic parameters were compared with peripheral cytokine levels quantified by microsphere immunoassay (MIA) to assess changes in innate immune function induced by drug treatment. We determined that both prednisolone and CsA induced acute and transient increases in FIV proviral DNA level and FIV viremia, which correlated with acute increases in lymphocyte subsets during treatment. This finding implies that acute changes in circulating lymphocytes increase FIV transcription, resulting in transient increases in FIV viral load. Moreover, significant alterations in cytokine expression that favored a shift toward a Th2 response were detected in both prednisolone and

CsA treated, FIV-infected cats. Finally, (iv) ART therapy abrogated CsA-induced FIV viremia, but did not affect FIV proviral loads, suggesting CsA induces FIV replication but does not drive all latent genomes into an actively replicating state (which would subsequently be diminished by ART). Collectively, these results document unexpected effects of both prednisolone and CsA during FIV-infection, and highlight the need to assess the long-term immunopathogenic consequences of these immunomodulatory compounds.

Materials and Methods

In vivo protocols

This study was approved by the Colorado State University Institutional Animal Care and Use Committee; 14-4872A. Colorado State University's animal care program is licensed by the United States Department of Agriculture (USDA), accredited by Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International, and holds an Office of Laboratory Animal Welfare (OLAW) assurance (A3572-01). All animal experiments complied with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). Prior to experimental procedures, all study animals were anesthetized by intramuscular injection of ketamine (20mg/kg) and acepromazine (2mg/kg) to minimize animal suffering and distress. All study animals were monitored daily by animal care personnel for development of clinical signs of FIV infection and observed by clinical veterinarians.

An outline of the study design is presented in **Figure 3.1.** Twelve (12), 8-11 week-old SPF cats (Andrea D. Lauerman Specific Pathogen Free Feline Research Colony, Fort Collins, CO) were housed as previously described above in accordance with CSU IACUC-approved protocols, and were acclimated to the facility for 2 weeks prior to initiation of the study. All animals were part of an unrelated study and had been infected with 75,000 infectious units of FIV_{PPR} (1ml of a viral stock solution with a $TCID_{50}$ titer of $1X10^{5.87}$) approximately 9 months (35

weeks) prior to enrollment in this protocol. A group of age-matched, uninfected SPF cats (n=12) served as negative controls.

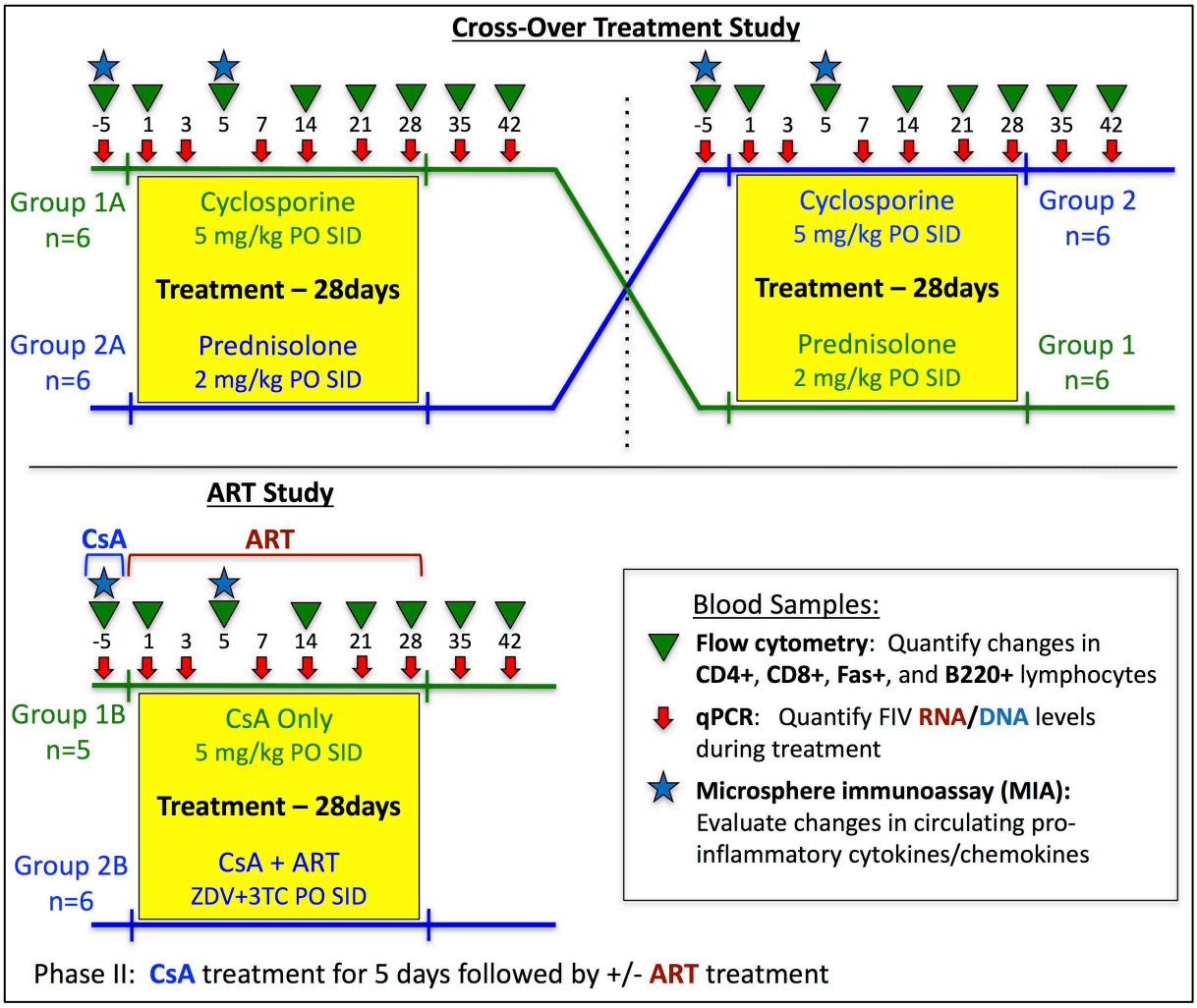

Figure 3.1. **Study design**. *Cross-over treatment study.* Twelve cats were divided into 2 groups (n=6) and treated with either ATOPICA® for Cats (cyclosporine A; 5 mg/kg PO SID) or prednisolone (2 mg/kg PO SID) for 28 days. Following a 6-week wash-out period, groups were alternated so that each cat received the opposite treatment for 28 days. *ART study*. Following a 6-week wash-out period at the end of the *cross-over treatment study*, Group 1B (n=5) received CsA for 5 days, after which they received no further treatment. Group 2B (n=6) received CsA for 5 days, after which CsA treatment was terminated. Group 2B then received antiretroviral therapy (ART +CsA) for 28 days (zidovudine 10 mg/kg + lamivudine 50 mg/kg, PO BID). See text for additional details.

Cross-over treatment study. FIV-positive cats were divided into 2 groups (n=6, randomized for previous vaccine treatment, gender, and litter) and treated as follows: Group 1A - ATOPICA® for Cats (cyclosporine oral solution, Novartis) at 5 mg/kg PO SID for 28 days; Group 2A - prednisolone at 2 mg/kg PO SID for 28 days. Following this treatment protocol, all cats were

subjected to a wash-out period of 6 weeks where no treatments were administered. During this period, plasma drug concentration was evaluated every 2 weeks. Blood samples were collected at days -7, 1, 3, 5, 7, 10, 14, 21, 28, 35, 42 of Phase I for qPCR, CBC, and flow cytometry analysis using previously established protocols [10,41] **(Figure 3.1)**. Following the 6-week wash-out period, drug treatment protocols for each cat group were treated with the alternate therapy prescribed during phase I of the study. Blood samples were collected as previously described for qPCR, CBC, and flow cytometry analysis **(Figure 3.1).** For the cross-over study, uninfected, age-matched negative control cats (n=12) were included, divided into 2 groups (n=6 each), and administered either ATOPICA® (CsA, 5 mg/kg PO SID) or prednisolone (2 mg/kg PO SID) as previously described for FIV-infected cats.

ART study. FIV-positive cats were again divided into 2 randomized groups and treated as follows: Group 1B - (CsA Only, n=5) CsA at 5 mg/kg PO SID for 5 days prior to day 0; Group 2B – (CsA + ART, n=6) CsA at 5 mg/kg PO SID for 5 days prior to day 0, followed by zidovudine (ZDV) 10 mg/kg + lamivudine (3TC) 50 mg/kg, PO BID for 28 days **(Figure 3.1)**. Blood samples were collected for qPCR, CBC, and flow cytometry analysis as outlined in **Figure 3.1** [10,41].

Hematologic analyses

Complete blood counts (CBC) and serum biochemistry analysis were performed for all blood samples in both the cross-over treatment study and the ART study by the CSU Veterinary Diagnostic Lab (CSU-VDL). Blood was collected from all cats prior to the study to establish baseline values, then at each time point indicated in **Figure 3.1**. The percentage of cells positively labeld by CD4, CD8, Fas, and B220 surface antigens was determined by incubating 30µl of EDTA-treated blood from each cat in 96-well round-bottom plates with 0.6µl of RPE-labeled anti-feline CD4 (Southern Biotech; clone 3-4F4), FITC-labeled anti-feline CD8 (Southern Biotech; clone fCD8), PE/Cy7-labeled anti-feline CD45R/B220 (Biolegend; clone RA3-6B2), and APC/Cy7-labeled anti-feline Fas/TNFRSF6 (R&D Systems; clone 431006) mouse monoclonal

antibodies diluted in FACS buffer (5% BSA, 0.1% sodium azide in PBS). Following incubation for 30 min in the dark at room temperature, red blood cells (RBCs) were lysed, and stained cells were fixed using a Beckman Coulter Q-Prep work station with 600 µl of 0.1% Formic Acid, 270 µl of 0.06 M Na_2CO_3 anhydrous, 0.25 M NaCl, 0.25 M Na_2SO_3, and 90 µl 1% wt/vol paraformaldehyde in 1×PBS. Flow cytometry was performed on a Coulter Gallios (Beckman Coulter Inc, Brea, CA) and results were analyzed using FlowJo® software (FlowJo, Ashland, OR). The percentage of lymphocytes positive for each marker was evaluated over time and compared to baseline values and naïve control data to compare alterations in lymphocyte immunophenotype in response to treatment and in the presence of FIV infection. Immunophenotype cell counts were calculated as previously described [41,42] and compared with CBC and qPCR data to evaluate changes in circulating immunophenotype compared to FIV viral and proviral loads over the course of the study and at individual time points. All CD4, CD8, and CD45R/B220 antibodies were directly labeled by the manufacturer. Anti-Fas antibody was unlabeled but later conjugated to APC/Cy7 using a APC/Cy7® Labeling Kit (Abcam).

Quantification of FIV viral RNA and proviral DNA in blood

Blood samples collected during the cross-over study and the ART study were analyzed by real-time polymerase chain reaction (PCR) analysis to quantify FIV proviral DNA and FIV*gag* RNA at time points illustrated in **Figure 3.1**. Plasma was isolated from EDTA-treated whole blood following centrifugation and frozen at −70°C until processing. Viral RNA was extracted from 140µl plasma using a QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA) according to manufacturer's instructions. Viral RNA from each sample was converted to cDNA using Superscript II (Invitrogen) in individual reactions with random hexamers (Invitrogen) and then treated with RNase Out (Invitrogen) prior to real-time PCR quantification. Peripheral blood mononuclear cells (PBMC) from all cats were purified on a Histopaque (Sigma, St. Louis, MO)

gradient, washed, pelleted, and then frozen at −80°C. Proviral DNA was extracted from PBMCs using a DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) prior to real-time PCR.

Real-time PCR reactions were performed on a CFX96™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA) to detect and quantify FIV proviral DNA in PBMCs and FIV *gag* RNA in plasma using previously described FIV-A primers and probes [43], and an iTaq™ Universal Probes Supermix (Bio-Rad, Hercules, CA) containing an antibody-mediated hot-start iTaq DNA polymerase. Copy number of viral RNA in plasma was calculated as previously described [41,42], implementing a standard curve generated by diluting FIV-PPR virus stock in naïve cat plasma and analyzed by reverse-transcriptase quantitative PCR as outlined above. To quantify proviral DNA in PBMCs, a real-time PCR standard curve was generated from serial dilutions of feline PBMCs from 1000 to $5x10^6$ subjected to real time PCR for the cellular house-keeping gene, Glyceraldehyde-3-Phosphate Dehydrogenase (GADPH) as previously described [42,44]. Resulting proviral copy numbers were normalized to copies per 10^6 cells based on the total amount of DNA in the reaction (100ng). Repeated measures ANOVA (RM-ANOVA) with multiple comparisons was used to evaluate differences in FIV RNA and DNA loads among treatment groups over time and at individual time points. Analyses were conducted using GraphPad Prism 6.0 software (La Jolla, CA) and p-values < 0.05 were considered significant.

Evaluation of peripheral cytokine expression during immunomodulatory therapy

Plasma samples collected at days -5 (pre-treatment) and day 5 (post-treatment) of the cross-over study **(Figure 3.1)** were analyzed by a commercially available MILLIPLEX® MAP Feline Cytokine/Chemokine Magnetic Bead Panel (fluorophore-conjugated microspheres, Millipore) per manufacturer's instructions. Briefly, 25ul from each triplicate sample was combined into one sample per animal per sample. 50ul of each combined sample was incubated with a composite panel of microspheres coupled with capture antibodies to INFγ, IL-

1β, IL- 2, IL-8, IL- 10, MCP-1,TNFα , Fas, SDF-1, SCF, RANTES, PDGF-BB, KC, IL-18, IL-13, IL-12 (p40), IL-6, IL-4, GM-CSF, and Flt-3 ligand. Following incubation with biotinylated secondary antibodies and streptavidin-conjugated phycoerythrin (PE), soluble cytokine molecules were detected in each sample using a Luminex® 200™ detection system. Final analyte concentration was calculated using manufacturer-provided standard curves for each analyte and Bio-Plex™ Manager 5.0 software (Bio-Rad). RM-ANOVA with multiple comparisons was used to evaluate differences in cytokine concentration among treatment groups over time and at individual time points. Analyses were conducted using GraphPad Prism 6.0 software (La Jolla, CA) and p-values < 0.05 were considered significant.

Results

Prednisolone & CsA induce acute and transient increases in circulating FIV RNA and DNA

Cross-over treatment study. Quantitative PCR analysis of plasma detected FIV RNA in study animals prior to treatment with both prednisolone and CsA, and at all time points during and following treatment **(Figure 3.2)**. Changes in FIV viral copies per ml of plasma were compared over time to detect changes in RNA viral load during treatment by RM-ANOVA. Although FIV RNA viral loads did not differ over time between treatment groups in this study, significant differences in FVA RNA were detected at individual time points when compared to pre-treatment FIV RNA viral loads. Levels of FIV RNA were significantly elevated in plasma of

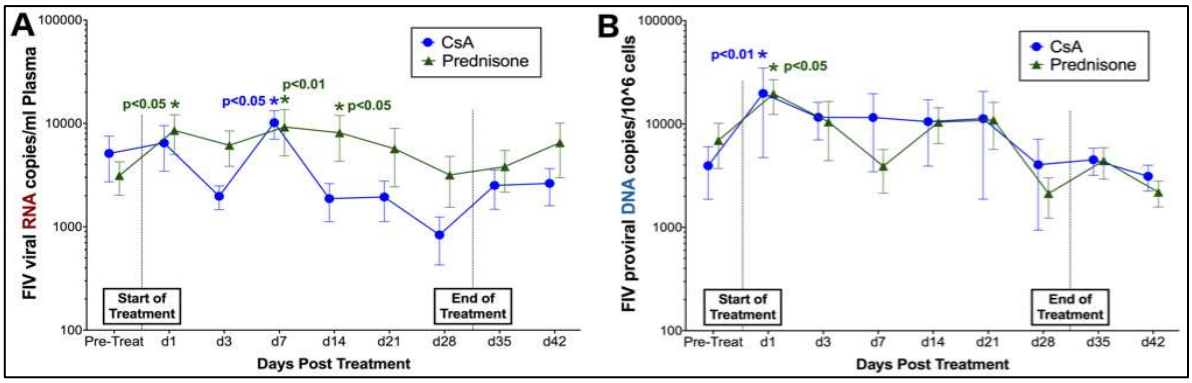

Figure 3.2. Prednisolone and CsA induce acute and transient increases in FIV RNA and DNA. **(A)** Prednisolone-treated cats exhibited significantly increased levels of FIV RNA at days 1, 7, and 14 compared to pre-treatment measurements, while CsA-treated cats had increased FIV RNA at day 7 post-

treatment. **(B)** Both prednisolone and CsA-treated cats exhibited significantly increased levels of FIV DNA at 1 day post-treatment.

prednisolone-treated cats at days 1, 7, and 14 during treatment, and increased at day 7 of CsA treatment **(Figure 3.2A)**. Similarly, FIV proviral DNA loads did not differ between treatment groups over time, however, levels of FIV DNA were significantly increased in PBMCs of both prednisolone and CsA-treated cats after one day of treatment **(Figure 3.2B).**

ART study. Cats were treated with CsA prior to antiretroviral therapy (ART) to assess whether CsA induced viremia can be inhibited by antiretroviral therapy (ART) replication. Quantitative PCR was used to evaluate changes in FIV RNA and DNA viral and proviral loads over time in response to therapy. As previously observed, cats receiving CsA treatment (CsA Only) exhibited a significant transient increase in FIV RNA at day 5 post-CsA treatment (p=0.013) **(Figure 3.3A)**. While cats treated with CsA + ART exhibited a slight decrease in FIV RNA levels at day 5 (compared to pre-treatment levels), this finding was not statistically significant. However, FIV RNA levels increased after day 5 in cats treated with CsA and then ART, resulting in significantly increased viral loads at day 21 of ART (p=0.002) **(Figure 3.3A).** Interestingly, acute and transient increases in FIV proviral DNA were observed in cats treated with both CsA Only (day 5, p=0.004; day 14, p=0.005) and CsA followed by antiretroviral therapy (CsA+ART) (day 1, p=0.011; day 5, p=0.002) **(Figure 3.3B).**

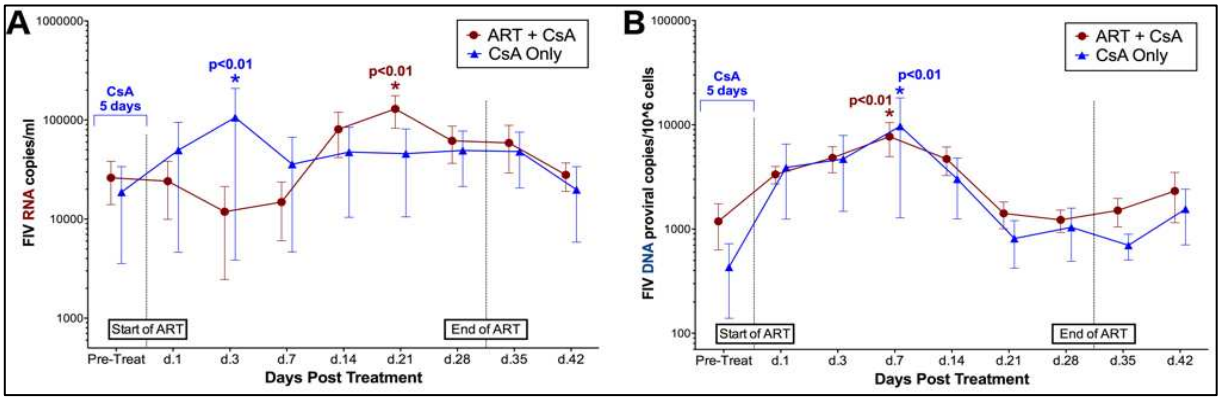

Figure 3.3. CsA induces recurrent increases in FIV RNA and DNA. (A) Cats treated only with CsA (CsA Only) exhibit a transient increase FIV RNA at day 3 post-treatment. FIV-infected cats pre-treated with CsA exhibit a slight decrease in FIV RNA at days 3 and 7 of antiretroviral treatment, but viral loads are significantly increased by day 21. **(B)** Cats treated with CsA alone and CsA+ART exhibit significantly increased levels of FIV DNA at day 7 post-CsA treatment.

Peripheral cytokine expression is altered by prednisolone and CsA during FIV infection

Circulating cytokine and chemokines were quantified in plasma by microsphere immunoassay (MIA) at day -5 (pre-treatment) and day 5 (post-treatment) to detect changes in cytokine expression in FIV-infected cats treated with prednisolone or cyclosporine A. Overall, FIV-infected cats treated with CsA exhibited increased levels of Fas ($p<0.001$), IL-4 ($p=0.001$), SDF-1 ($p=0.004$), IL13 ($p=0.013$), and IL-8 ($p=0.013$) compared to baseline levels, while levels of Flt-3L ($p=0.002$), IL-12 p40 ($p=0.008$), and SCF ($p=0.022$) were significantly decreased **(Figure 3.4A)**. Similar findings were observed in FIV-infected cats treated with prednisolone,

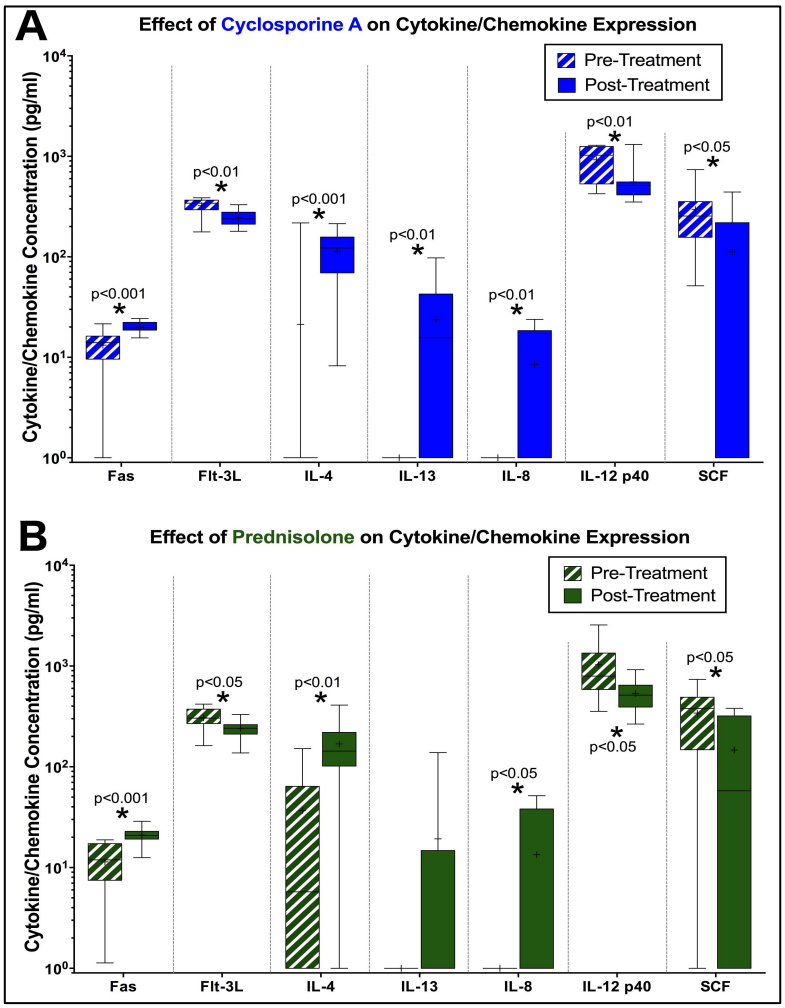

Figure 3.4. FIV-infected cats treated with CsA and prednisolone exhibit alterations in circulating cytokines. (A) FIV-infected cats exhibit increased levels of Fas, IL-4, IL-13, and IL-8 at 5 days of CsA treatment, while levels of Flt-3L, IL-12 p40, and SCF were decreased compared to pre-treatment levels. **(B)** Prednisolone treated cats with FIV exhibit similar changes in cytokine expression (increased Fas, IL-4, IL-8 and decreased Flt-3L, IL-12 p40, SCF) at 5 days of treatment.

which exhibited increased levels of Fas (p<0.001), Il-4 (p=0.002), and IL-8 (p=0.049) compared to baseline data, while levels of Flt-3L (p=0.019), IL-12 p40 (p=0.018), and SCF (p=0.022) were comparatively decreased **(Figure 3.4B)**.

FIV-infected cats exhibit divergent lymphocyte immunophenotypes with CsA and prednisolone Cross Over Treatment Study. Flow cytometry was used to evaluate changes in the percentage of lymphocytes positive for CD4, CD8, Fas, and B220 surface antigens over time in FIV-infected and naïve cats. Changes in FIV-infected cats were compared to baseline values and data from naïve control cat samples to compare alterations in lymphocyte

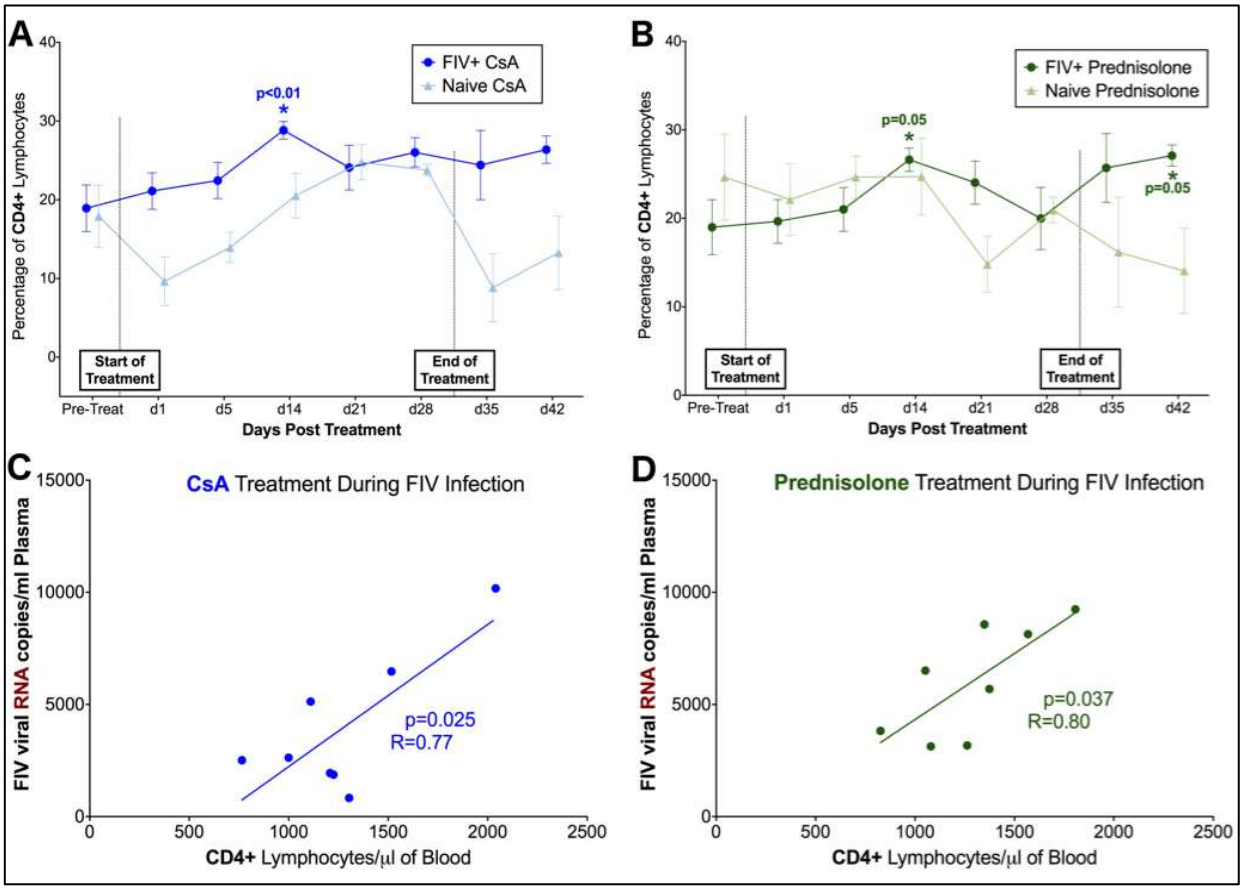

Figure 3.5. CsA and prednisolone induce a transient increase in CD4+ lymphocytes in FIV-infected cats that correlates with increased FIV viral load. CD4+ lymphocytes are significantly increased in FIV-infected cats at day 14 of CsA treatment **(A)**, and at day 14 of prednisolone treatment **(B)**. Increased levels of CD4+ lymphocytes correlate with increased FIV RNA in CsA treated cats **(C)** and prednisolone-treated cats **(D).**

immunophenotype in response to treatment and in the presence of FIV infection. Overall, the percentage of CD4+ lymphocytes differed significantly over time in FIV-infected cats treated with both CsA (interaction, p=0.05) and prednisolone (interaction, p=0.03) when compared to FIV-negative control cats. Post-hoc analyses indicated that FIV-infected cats exhibited transient increases in CD4+ lymphocytes at 14 days of treatment with both CsA (p=0.004) and prednisolone (p=0.05) when compared to baseline data (pre-treatment)**(Figure 3.5A-B)**. When compared to FIV viral and proviral loads, a significant positive correlation was observed between numbers of CD4+ lymphocytes and circulating copies of FIV RNA in CSA (p=0.025) and prednisolone treated cats (p=0.037) **(Figure 3.5C-D)**. Although CD4+ lymphocytes were slightly decreased at day 1 of CsA treatment in naïve control animals, this change was not significant, and the percentage of CD4+ lymphocytes did not differ over time during CsA and prednisolone treatment in control animals.The percentage of B220+ lymphocytes also differed significantly over time in FIV-infected cats treated with CsA (interaction, p=0.02) and prednisolone (interaction, p=0.02) compared to naïve controls. However, in contrast with changes in CD4+ lymphocytes, FIV-infected cats exhibited significant decreases in B220+ after 28 days of treatment with CsA (p=0.033-0.013) and after 21 days of prednisolone (p=0.034-0.005) **(Figures 3.6A and 3.7A)**.

Although CD8+ lymphocytes tended to be slightly lower in FIV-infected cats treated with CsA when compared to naïve controls **(Figures 3.6B and 3.7B)**, these findings were not significant, and no significant differences over time were observed when compared to baseline levels. Similarly, Fas+ lymphocytes did not differ between FIV-infected and naïve cats treated with CsA **(Figure 3.6C)**. However, the percentage of Fas+ lymphocytes differed significantly over time in FIV-cats treated with prednisolone (p=0.037) compared to naïve controls, and post-hoc analysis revealed transient increases in Fas+ lymphocytes at 14 days (p=0.004) and 21 days (p=0.016) of prednisolone treatment, followed by a significant spike in Fas+ cells with cessation of treatment (day 35, p<0.0001) **(Figure 3.7C)**. The percentage of CD8+, B220+, and

Fas+ lymphocytes did not differ over time during CsA and prednisolone treatment in naive control animals.

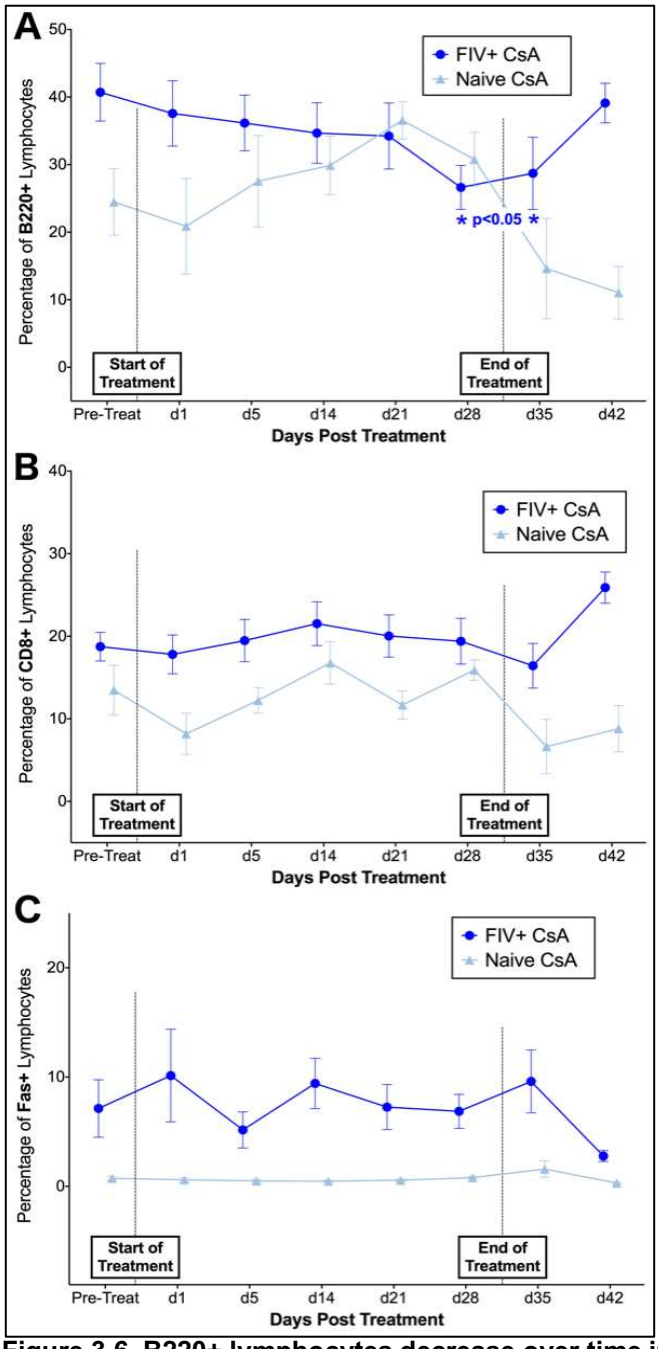

Figure 3.6. B220+ lymphocytes decrease over time in FIV-infected cats treated with CsA. **(A)** B220+ lymphocytes are significantly decreased in FIV-infected cats after 28 days of treatment with CsA. **(B-C)** While CD8+ and Fas+ lymphocytes tended to be slightly lower in FIV-infected cats treated with CsA when compared to naïve controls, these findings were not significant, and no significant differences over time were observed when compared to baseline levels.

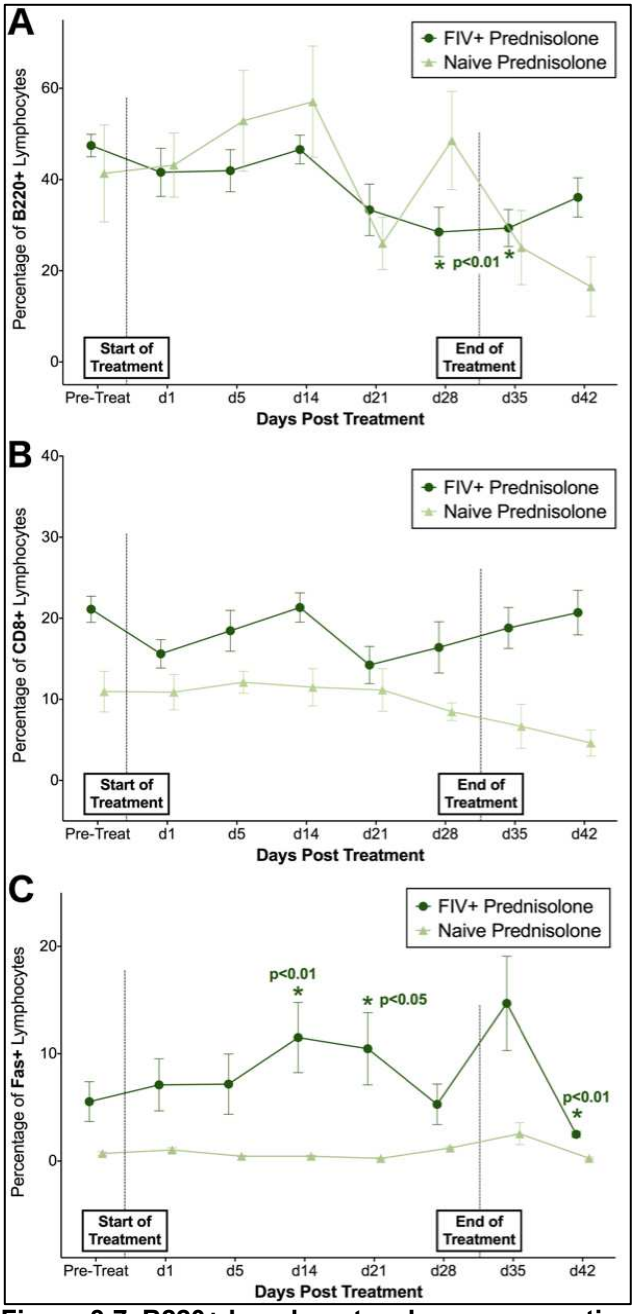

Figure 3.7. B220+ lymphocytes decrease over time in FIV-infected cats treated with prednisolone. **(A)** B220+ lymphocytes are significantly decreased in FIV-infected cats after 28 days of treatment with prednisolone. No significant changes in CD8+ lymphocytes are observed in FIV-infected cats during Phase I **(B)**. However, Fas+ lymphocytes are significantly increased at days 14 and 21 of prednisolone treatment in FIV-infected cats **(D)**, although this population of lymphocytes significantly decreases with cessation of treatment (day 42).

ART study. Flow cytometry was used to evaluate changes in the percentage of lymphocytes positive for CD4, CD8, Fas, and B220 surface antigens over time in FIV-infected in response to treatment with CsA with or without antiretroviral therapy (CsA only, CsA+ART). During the ART

study, no significant differences were observed in response to 5 days of CsA treatment, or in response to 28 days of ART **(Figure 3.8A-D)**.

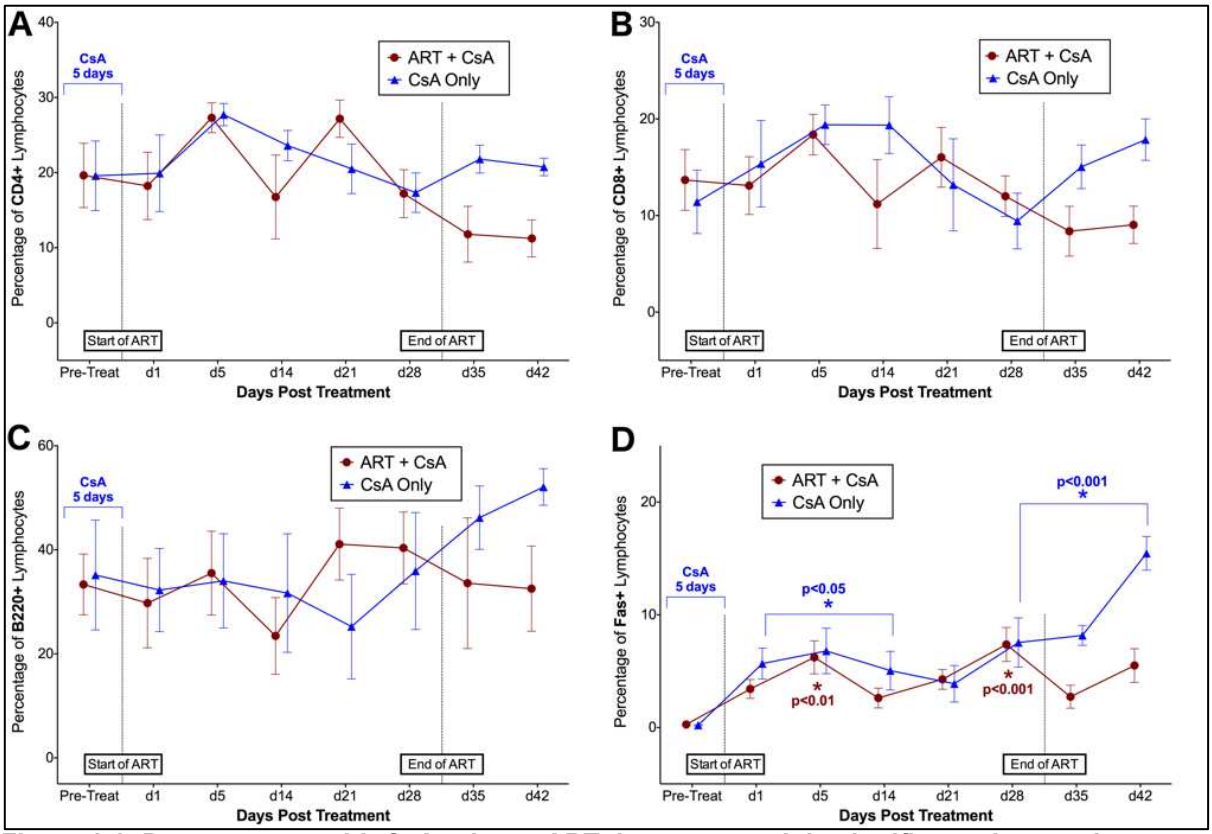

Figure 3.8. Pre-treatment with CsA prior to ART does not result in significant changes in lymphocyte immunophenotype. No significant differences between **(A)** CD4+, **(B)** CD8+, **(C)** B220+, or **(D)** Fas+ lymphocytes are observed following 5 days of CsA Treatment (CsA Only group, n=5; CsA+ART group, n=6) or with 28 days or antiretroviral therapy (CsA+ART group, n=6).

Discussion

The results of this study represent unexpected and previously undocumented effects of prednisolone and cyclosporine A during chronic FIV infection, and highlight important complications to consider when using immunosuppressive therapy to treat opportunistic disease in FIV-infected cats. Because prednisolone and CsA exhibit well-documented, dose-dependent immunosuppressive effects via inhibition of IL-2 mediated T-lymphocyte proliferation and activation [32-34,36,37], it was expected that treatment with these immunomodulatory agents in FIV-infected cats would result in broad reduction of circulating lymphocytes necessary for FIV-infection and persistence; fundamentally reducing the capacity for FIV to infect and replicate

within appropriate target cells. However, immunosuppressive doses of CsA and prednisolone in FIV-infected cats failed to elicit an inhibitory effect on T lymphocytes and FIV replication in this study, and in fact, exacerbated FIV replication rates in conjunction with acute increases in CD4+ lymphocytes and alterations in the inflammatory cytokine profiles of FIV-infected cats. While an unequivocal cause for the discrepancy in the anticipated therapeutic effect is not immediately apparent, correlative increases in FIV viral load and CD4+ T lymphocytes suggest that direct interplay between these factors may account for such unanticipated outcomes.

Perhaps the most important findings of this study are the acute and transient increases in FIV RNA and DNA loads during CsA and prednisolone treatment, as well as the positive correlation of FIV RNA levels with that of CD4+ lymphocytes. This direct relationship suggests that both CsA and prednisolone may not only prevent CD4+ lymphocyte depletion during FIV infection, but may also improve CD4+ counts, albeit to the detriment of enhanced viral propagation. Indeed, the fact that CD4+ lymphocytes increase in response to CsA and prednisolone treatment indicates that these immunomodulatory agents may cause an increase in circulating target cells, which thereby increase levels of FIV infection and replication within naïve effector T-cells, and thus contradict the expected therapeutic effect of these drugs and our intended outcome. While prednisolone has not previously been documented to induce such changes in FIV-infected cats, one prior study demonstrated increased FIV viral loads in plasma at 4 weeks (28 days) of CsA treatment, which transiently subsided as observed in the present cohort of FIV-infected cats [45]. However, increased viral isolation and RNA viral loads have been well-documented in HIV-infected humans treated with both prednisolone and CsA, and these drug-induced changes are frequently associated with increased CD4+ lymphocytes [46-48], suggesting a causative relationship that provides latent virus with the means to reinitiate infection of naïve T-cells. Such interactions may therefore account for the corresponding transient increase in FIV proviral DNA, likely mediated through successive integration and viral maintenance within this renewed source or target cells.

In accordance with these findings, we subsequently investigated the potential for an innovative immunomodulatory approach to compliment antiretroviral therapy. We hypothesized acute increases in FIV replication induced by CsA might result in highly effective anti-FIV therapy induced by selected nucleoside analogue reverse transcriptase inhibitors (NARTIs), zidovudine and lamivudine (ART), previously shown to be effective *in vivo* at reducing FIV viral loads in chronically infected cats [21]. Because NARTIs exert their primary therapeutic effect during active viral replication through competitive inhibition and reduction in the activity of reverse transcriptase [49-51], we theorized that CsA-mediated induction of an enhanced replicative state might improve the efficacy of antiretroviral therapy. We pre-treated all FIV-infected cats with CsA prior to day 0, after which CsA treatment ceased and a subset of cats were then administered ART for 28 days. As previously observed in the cross-over treatment study, cats that received CsA treatment only (no ART at day 0) exhibited an identical transient increase in FIV RNA viral load, confirming that CsA treatment induces enhancement of FIV replication in infected cats. Interestingly, a slight reduction in viral load was observed in FIV-infected cats receiving CsA then ART, although this change was not significant when compared to pre-treatment levels. In retrospect, it is likely that this trend may have continued if CsA treatment had been continued in conjunction with ART, rather than terminating CsA treatment at day 0. Because ART diminished viral replication in CsA + ART treated cats compared to cats that received CsA only, it is likely that the CsA-mediated increase in viral load was due to new viral replication/viral activation. The subsequent rise in FIV RNA levels by day 21 of ART may likely reflect the withdrawal of CsA from the treatment protocol, which effectively terminated the activated state of viral replication that was conducive to improving ART efficacy. Future studies will assess whether prolonged combinational therapy with CsA + ART can sustain this antiviral effect.

Appreciable changes in lymphoid immunophenotype also extended to B220+ lymphocytes, which decreased significantly over time during the course of treatment with both

CsA and prednisolone. B220 is a heavily glycosylated isoform of CD45R, and a surface antigen expressed in immature and mature naïve B-cells, as well as other lymphocyte subsets such as activated T-cells and dendritic cells [52,53]. It is well documented that at high doses, prednisolone broadly decreases both T and B lymphocytes, but at lower doses, prednisolone produces selective depletion of B lymphocytes [54,55]. It is possible that although we selected a higher, immunosuppressive dose of prednisolone, the dose may have not been high enough to suppress both T and B lymphocytes in our FIV-infected cohort, thus accounting for the selective depression of B220+ cells and not CD4+ T cells. In contrast, cyclosporine A is known to be primarily selective for T lymphocytes, producing less inhibitory effects on their B lymphocyte counterparts [56-58]. However, certain subsets of B lymphocytes, such as those responding to thymus independent (TI) antigens, have been shown to be sensitive to CsA treatment, while primed (or activated) T lymphocytes are resistant [58]. Previous studies have shown that FIV is capable of directly activating T lymphocytes, either by primary virus infection or chronic antigenic stimulation [59-61], while not affecting the function of B cells recognizing T-independent antigens [8,62]. In this present study, the predisposing condition of FIV-infection may therefore account for the decreased efficacy of CsA to reduce CD4+ lymphocytes in circulation, and provide cause for the selective depletion of CsA-sensitive subpopulation of B lymphocytes. Future studies should assess the activation status and antibody response to T-dependent and T-independent antigens during prednisolone and CsA treatment to elucidate the divergent mechanisms of action during lentiviral infection.

Prednisolone and CsA are both well-known inducers of apoptosis in lymphocytes [63-66]. Interestingly, Fas expression, a marker of apoptosis in lymphocytes was significantly elevated in prednisolone treated cats, but not in CsA-treated cats. Glucocorticoid and CsA induced apoptosis occurs primarily via caspase-dependent activation, and predominately occurs independent of the FasL/Fas system [38,64,66]. Fas-induced apoptosis may occur in viral-infected cells via binding of FasL on CD8+ T cells, and may be upregulated in this study as a

result of increased infection of CD4+ lymphocytes in prednisolone and CsA treated animals [67]. However, the cause for increased Fas expression on lymphocytes during prednisolone treatment but not CsA treatment is unknown and likely multifactorial due to overlying viral infection. Future studies will investigate other markers of apoptosis (i.e. caspase activation) and lymphocyte activation to elucidate mechanisms of apoptosis during concurrent lentiviral infection and immunosuppressive therapy.

Similar to the expression of surface markers detected by flow cytometry, microsphere immunoassay detected significant increases in soluble Fas expression in FIV-infected cats treated with both CsA and prednisolone. Increased levels of apoptosis in CD4+ T-cells and CD19+ B-cells have been well-described during HIV infection, and have been correlated with viral RNA levels in plasma and a reduction in these important lymphocyte subsets [68,69]. Interestingly, such a correlation was not observed in this study, however, CD4+ lymphocytes were observed to increase in response to CsA and prednisolone treatment, and may have counterbalanced any viral-induced decrease in lymphocyte numbers in the face of continued CD8-mediated apoptosis. Increases in Il-4 was also observed in both treatment groups, in addition to increased IL-13 observed in CsA-treated cats infected with FIV. Interleukin 4 (IL-4) is produced predominately by T helper 2 (Th2) cells, as well as smaller T cell subsets such as Natural Killer (NK) T cells and γ/δ T cells, and primarily confers anti-inflammatory effects on Th1 cells, macrophages, and interferon gamma (IFNγ) production, as well as plasma cell differentiation, IgE class switching, and differentiation of antigen-stimulated naive T cells into the Th2 subset [70-73]. IL-13 exhibits similar effects on the inhibition of inflammatory cytokines, and is functionally related to IL-4 [74]. Increased levels of these anti-inflammatory cytokines have been previously associated with HIV infection [75-77], indicating a shift to a predominant Th2 subtype during infection, but these effects have not been well studied in cats with FIV. Furthermore, prior studies have demonstrated that both prednisolone and CsA treatment are

associated with decreases in IL-4 expression, yet in this study, circulating IL-4 concentrations increased in response to treatment [78-81]. The cause for such a shift in cytokine expression in response to CsA or prednisolone treatment is not apparent, but may be due to underlying viral infection. If FIV infection produces a shift to a Th2 response as observed in HIV, it is possible that the pharmacological effect of these compounds may have stimulated differentiation of these CD4+ lymphocytes along the Th2 pathway, thus increasing expression of these associated cytokines. Future studies will evaluate additional surface markers and transcription factors to establish the role of CD4+ T cell development and differentiation during FIV infection.

FIV-infected cats treated with both prednisolone and CsA also exhibited increased levels of circulating IL-8 when compared to baseline levels (pre-treatment). IL-8 is pro-inflammatory cytokine that is primarily involved in neutrophil chemotaxis, but also exhibits chemotactic activity against T cells and basophils [82]. Previous studies have shown that both prednisolone and CsA exhibit significant inhibitory effects on IL-8 production [83-85], but in this study, IL-8 was significantly increased following treatment. Studies in HIV have demonstrated significant increases in circulating levels of IL-8 in plasma of infected patients, and have shown that IL-8 plays a significant role in stimulating HIV replication [86-88]. It is therefore reasonable to assume that IL-8 may exhibit similar kinetics during FIV-infection, and that the increased rate of FIV replication during prednisolone and CsA treatment may account for the corresponding increase in IL-8 expression during treatment with these immunomodulatory agents.

Contrary to the above findings, FIV-infected cats treated with prednisolone and CsA exhibited a significant decrease in the expression of several pro-inflammatory cytokines, such as IL-12 p40, Flt-3L, and SCF-1. IL-12 p40 is secreted by active macrophages and is critical in the production of IFNγ and the induction of Th1 cells [89]. Prednisolone and cyclosporine may directly suppress IL-12 p40 expression and likely account for the decreased levels in this study [90-92], however previous studies have also shown that HIV-infected patients have impaired IL-12 p40 secretion, and the contribution of FIV infection and replication may likewise play a role in

this study as well [93]. Flt-3L and SCF play an important role in hematopoiesis and function by increasing lymphocyte proliferation by activating hematopoietic progenitors [94-97]. Decreased Flt-3L and SCF has been well-documented during prednisolone and CsA treatment, which most likely account for decreased levels of these cytokines, yet the inhibition of these inducers of cell proliferation was not sufficient to cause a decrease in CD4+ lymphocytes in FIV-infected cats [98-100]. While the exact mechanism is not clear in this present study, it is likely that CD4+ lymphocyte increases occurred independently of these stimulatory cytokines during treatment with CsA and prednisolone in FIV-infected cats. Unfortunately, levels of IL-2 were undetectable is nearly all FIV-infected cats treated with prednisolone and CsA, which prevented evaluation of IL-2-mediated changes in hematologic parameters. Future studies will be focused on the optimization and extended analysis of this pivotal cytokine the determine its role during prednisolone and CsA treatment in FIV-infected cats.

While the findings presented here demonstrate a paradoxical outcome of a therapeutic regimen designed to improve clinical FIV infection, these results provide new insight into the use of these commonly prescribed immunomodulatory agents. In summary, both prednisolone and CsA induce acute and transient increases in the percentage of CD4+ lymphocytes in FIV-infected cats, which directly correlate with increased FIV RNA viral loads. FIV proviral DNA levels also exhibit a similar, transient increase following prednisolone and CsA treatment, and pre-treatment with CsA in FIV-infected cats has the potential to reduce viral loads when combined with nucleoside analog reverse transcriptase inhibitors through induction of an active state of viral replication conducive to competitive inhibition and reduced reverse transcriptase activity. Furthermore, evidence suggests treatment with these immunomodulatory agents during FIV infection may perpetuate a shift toward a Th2-mediated inflammatory response with concurrent reduction in mediators of a pro-inflammatory Th1 response. Future studies will investigate prolonged combinational therapy with CsA + ART to determine the potential for sustained decrease in FIV viral loads, as well as evaluation of other innate mediators of T

lymphocyte proliferation during immunomodulatory therapy. Overall, these results highlight the potential for drug-induced perturbation of FIV infection and underscore the need for more information regarding immunopathologic consequences of therapeutic agents on concurrent viral infections.

CHAPTER 3 REFERENCES

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CHAPTER 4. FIV AS A MODEL TO STUDY LENTIVIRAL-INDUCED ORAL DISEASE

Summary

HIV infection results in well-documented changes to gut-associated lymphoid tissue (GALT), but few studies have evaluated whether similar mucosal immunological dysfunction occurs in the oral cavity. Studies of these phenomena have been hampered by lack of a suitable animal model, as commonly used animal models for HIV (SIV/SHIV infections of non-human primates and HIV infections in humanized mice) do not reliably result in oral disease. In contrast, FIV results in immunological dysfunctions and opportunistic infections similar to HIV-AIDS, including periodontal and oral inflammatory disease that occurs in more than 50% of untreated, FIV-infected animals. As a result of previous work described in this dissertation, I have developed a strong interest in (i) investigating the role of the oral microbiome and immune function in the development of lentiviral-induced oral disease (CHAPTER 1), as well as (ii) the investigation of new modalities of ART that might be more efficacious in treating this refractory condition in HIV patients (CHAPTER 3). Thus, in Chapter 4 of this dissertation, I conducted a pilot study to assess FIV-associated changes in clinical status, oral microbiota, local and systemic viral burden, and immune profile as a model to study HIV-induced oral disease development. Results of this pilot study demonstrate that FIV infection of domestic cats is associated with: (1) oral microbiota dysbiosis, with a marked loss of microbial diversity during FIV-associated periodontitis; (2) changes in salivary cytokine levels, even in the absence of FIV clinical oral disease; and, (3) systemic markers of immune stimulation, signaling local and peripheral immune activation, and suggesting bacterial translocation. These findings highlight the potential for the feline model of lentiviral-induced oral disease to expand our understanding of the complex interactions between HIV infection, oral immune dysfunction, and the perturbations to the oral microbiota that occur in the context of HIV infection.

Background

Mucosal immune dysfunction, bacterial translocation, systemic immune activation, and chronic inflammation are well-documented features of chronic HIV infection. Despite the success of combinational antiretroviral therapy (cART) in diminishing HIV viral replication and prolonging immune function, a multitude of systemic and local manifestations of HIV infection persist. For example, cART is associated with a decrease in the occurrence of many oral manifestations of HIV, yet a subset of oral infections and chronic periodontitis persist in HIV-infected individuals [1-6]. Furthermore, HIV infection precipitates alterations of the oral microbiome and secretory and cellular components of the oral cavity during infection, and these changes are not restored by cART [2]. The oral cavity, as an immunologically active extension of the gastrointestinal tract, may be an important site for HIV replication and persistence, yet this aspect of HIV disease has not been closely studied, and the relationship between viral-induced immune dyscrasias, oral dysbiosis, and the chronic inflammatory lesions have not been well established [8-11]. Gaps in our understanding of the drivers of HIV-associated oral dysbiosis, mediators of chronic oral and systemic inflammation, and association with clinical oral disease underscore the need for studies to investigate the pathogenesis and relationship of these alterations.

HIV-induced deficits in gut-associated lymphoid tissue (GALT) are associated with bacterial byproduct translocation resulting in chronic systemic immune activation [1, 3, 14-22]. Conversely, the role of oral mucosal associated lymphoid tissue (MALT) in promoting HIV-associated chronic inflammation has not been comprehensively evaluated. cART protocols may not eliminate HIV persistence in oral tissues, and underlying chronic immune activation associated with HIV infection may promote oral dysbiosis [2, 23-25]. Few studies have characterized perturbations in the microbial ecology of the oral cavity during HIV infection, and it is unknown whether bacterial translocation and disruption of oral MALT contributes to persistent systemic immune activation in HIV-infected patients [1, 3, 15-17, 26]. Measuring the impact of

cART on oral microbiome composition, secretory and cellular oral immune parameters, or systemic inflammation during HIV infection is untenable as untreated control groups cannot ethically be studied; thus, the use of an animal model to evaluate these associations is a critical component of furthering translational studies.

Commonly used animal models for HIV, including SIV/SHIV infections of non-human primates (NHPs) or HIV infections in humanized mice, do not reliably incite oral lesions [27, 28]. Feline immunodeficiency virus (FIV) is the feline analog of HIV, and the only non-primate lentivirus to cause AIDS in its natural host. The molecular biology of FIV is strikingly similar to HIV, and the FIV animal model has been employed for many studies relevant to HIV, including development of anti-retroviral drugs, determination of the role of regulatory T cells during lentiviral infections, modeling for HIV latency, and for studies of lentiviral vaccine development [29-36]. Importantly, gingivitis and periodontitis are primary clinical signs associated with untreated natural and experimental FIV-infection, and are principal attributes of this model that may be exploited to investigate pathogenic mechanisms involved in the perturbation of the oral immune system and microbial environment [37, 38].

Oral manifestations of HIV infection, including opportunistic infections (OI) and immunodeficiency associated periodontal lesions, were estimated to occur in a large proportion of adult HIV patients prior to cART [2, 26, 39-46]. Fortunately, cART has significantly decreased many HIV oral manifestations, especially candidiasis and necrotizing gingivitis [2, 26, 47-56]. In fact, oral manifestations of HIV are now considered a clinical hallmark of cART failure, and/or diagnosis of these conditions may be the first indication of HIV infection [2, 26, 57]. Despite progress, the incidence of periodontitis may be increased in HIV patients beyond the general population, and increases in some OI, notably HPV, has been observed [58-60]. Advances in high throughput sequencing technologies have revealed that microbial shifts occur in the oral cavity during HIV infection, even in the presence of cART [16, 26]. The recognition that the microbiome contributes to health and disease states, and the complex interplay between oral

mucosal integrity, microbial colonization, co-infections, and mucosal immunity suggests microbiome composition may play an important role in HIV pathogenesis [61].

Most cats with FIV are not treated with cART, and oral manifestations are common in naturally and experimentally infected cats [37, 62, 63]. These manifestations are frequently characterized by periodontitis, and feline chronic gingivostomatitis [37] that exhibit striking similarity to lesions noted in untreated HIV patients (**Figure 4.1**). [2, 7, 64-69]. Additionally, opportunistic microorganisms detected in saliva of HIV-positive individuals are similarly implicated in feline oral disease (*C. albicans, Fusobacterium sp., Streptococcus sp., Prevotella sp., Campylobacter sp.,* and *Porphyromonas gingivalis*) [4, 16, 70-78], indicating that the impact of FIV on feline oral microbial populations may parallel changes observed in HIV infection.

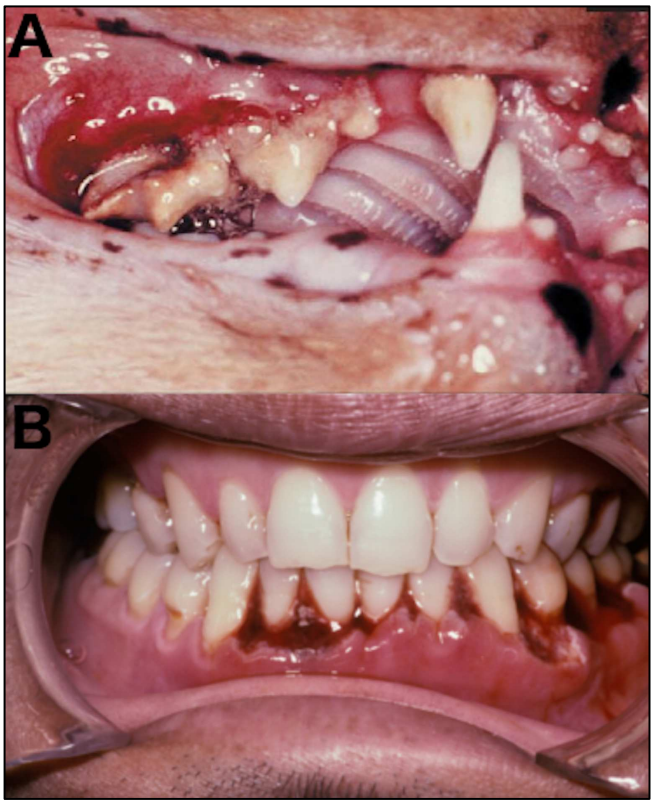

Figure 4.1. **Periodontal disease and gingivostomatitis are common manifestations of untreated FIV and HIV infection.** FIV-infected cats exhibit ulcerative and necrotizing gingivitis and stomatitis **(1A)** resembling Necrotizing Ulcerative Gingivitis (NUG) in HIV-infected humans **(1B)** Reprinted from [7].

Previous studies have shown that HIV and FIV induce characteristic depletion of circulating CD4+ lymphocytes, resulting in global immunosuppression, and that widespread loss

of lymphocytes o at the surface of the gastrointestinal tract mucosa results in compromised local immune function [1, 19-22, 79-81]. This change is correlated with increased populations of opportunistic pathogens and shifts in microbiota composition [1, 14, 19]. Oral dysbiosis likely occurs by a similar mechanism during acute HIV and FIV infection, and immunodeficiency-associated shifts in the oral microbiota presumably result in activation of immunocytes and perturbation of inflammatory mediators; thus contributing to oral lesion development in affected individuals [1, 14, 19]. As such, an animal model designed to investigate *in vivo* kinetics and immunopathologic mechanisms contributing to oral disease during lentiviral infection may provide critical information for the development of more effective strategies to manage chronic impacts of HIV-infection.

Material and Methods

FIV alterations of oral microbiota

Saliva was collected from the sublingual area and ventral cheek pouches from naïve juvenile SPF cats (12-14 month-old) and age-matched cats infected with FIV (PPR strain) of 8 months duration (n=5/group). FIV_{PPR} is a relatively apathogenic strain of FIV that typically results in mild self-limiting gingivitis and/or periodontitis during acute infection [82]. Animals did not have overt, visual signs of clinical periodontitis at the time of sampling. FIV-infected and naïve SPF animals were maintained on a similar diet, and similar anatomic regions were swabbed from all animals at the same time of day. DNA was extracted as previously described [83], and amplicon sequencing was performed using illumina MiSeq to generate paired-end 2x250bp sequences of the hyper-variable region 4 (V4) of the 16S rDNA. Data were normalized using cumulative sum scaling [84], and used to construct a nonmetric multidimensional scaling 3D plot (bioinformatics analysis conducted by University of Missouri Metagenomics Center (MUMC) and Z. Abdo, DMIP, CSU).

FIV alterations of oral cytokine expression

Saliva was collected from naïve and FIV-infected cats without detectable oral lesions (as described above; n=5/group), and analyzed by a commercially available MILLIPLEX® MAP Feline Cytokine/Chemokine Magnetic Bead Panel (fluorophore-conjugated microspheres, Millipore) per manufacturer's instructions. Briefly, 50ul of saliva (diluted 1:3 in assay buffer) was incubated with a composite panel of microspheres coupled with capture antibodies to INFγ, IL-1β, IL- 2, IL-8, IL- 10, MCP-1,TNFα , Fas, SDF-1, SCF, RANTES, PDGF-BB, KC, IL-18, IL-13, IL-12 (p40), IL-6, IL-4, GM-CSF, and Flt-3 ligand. Following incubation with biotinylated secondary antibodies and streptavidin-conjugated phycoerythrin (PE), soluble cytokine molecules were detected in each sample using a Luminex® 200™ detection system. Final analyte concentration was calculated using manufacturer-provided standard curves for each analyte and Bio-Plex™ Manager 5.0 software (Bio-Rad). One-way ANOVA with multiple comparisons was used to evaluate differences in cytokine concentration among treatment groups. Analyses were conducted using GraphPad Prism 6.0 software (La Jolla, CA) and p-values < 0.05 were considered significant.

Innovative cART Regimen

Triplicate cell cultures consisting of GFox cells (CrFK cells overexpressing CD134) [82, 85] were established in 96-well plates at 20,000 cells/well and allowed to attach at 37°C overnight. GFox cell cultures were grown at 37°C and 5% CO_2 in 100µl of culture medium composed of Dulbecco's modified Eagle's medium (DMEM) with GlutaMAX-1, 10% fetal bovine serum (FBS), and 1x penicillin-streptomycin (10,000 U/liter penicillin and 10,000 µg/liter streptomycin), as well as 1 µg/ml of Fungizone® (Amphotericin B; Life Technologies) [86]. At day 0, 9µl of FIV_{C36} stock (containing 50,000 infectious units) was incubated for 1 hour at 37°C with culture medium and varying concentrations of Dolutegravir (DTG), Tenofovir disoproxil fumarate (PMPA), and Emtricitabine (FTC) in a ratio previously utilized in *in vivo* studies in SIV-infected macaques (see

Figure 4.4) (total volume 100µl) [12, 13]. Following incubation, infected media was then added to cell culture plates, bringing the total volume to 200µl. Triplicate negative control (no FIV, cART only: 10uM DTG, 80uM PMPA, 100uM FTC) and positive control (FIV only, no cART) wells were included for each sample. At days 4, 6, 8, and 11 post-inoculation, 120µl of supernatant was removed from each well, frozen at -80°C, and replaced with 120µl of fresh culture media. At day 11, the supernatant collected from each well and each time point was assayed by a previously described capture ELISA protocol to detect FIV p26 antigen at an absorbance of 450nm in 96-well flat bottom plates [87]. Percent inhibition was calculated from mean absorbance values (Abs) using the previously described formula $((X - Y)/X) \times 100$, where X is fraction of cells infected in the absence of cART (FIV only positive control) and Y is the fraction of cells infected in the presence of various cART treatments [88].

Results

FIV alterations of oral microbiota

Significant differences were detected in the oral microbiota composition of FIV-infected cats relative to naïve animals (**Figure 4.2A**). Normalized data were tested using the Zero Inflated Gaussian model implemented in the R package from metagenomeSeq [89] to identify the putative OTUs driving differences between FIV+ and FIV- cats. Significant log-fold change in abundance in 12 genera were noted between groups at the 0.1 level of significance after correction for multiple testing (**Figure 4.2B**). One FIV-positive cat developed moderate to severe erythematous gingivitis during the course of infection and saliva was collected and analyzed as described above. Upon analysis of saliva, this individual demonstrated a dramatically altered microbiome population with >95% operational taxonomic units (OTUs) corresponding to the genus *Moraxellaceae* (**Figure 4.2C**, MUMC and Z. Abdo, DMIP, CSU).

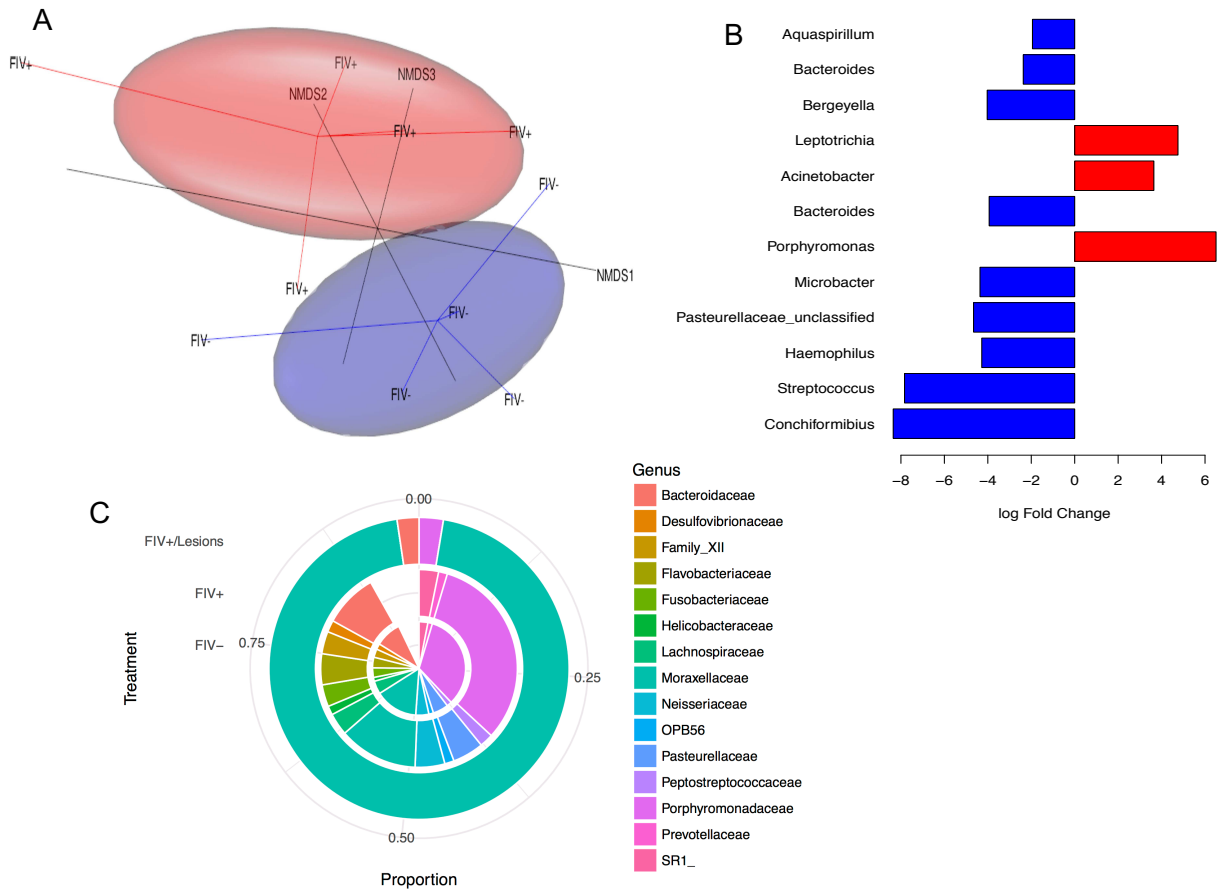

Figure 4.2: Salivary microbiome alterations during FIV infection. **(A)** 3D Nonmetric Multidimensional Scaling (NMDS) separates clusters of FIV- and FIV+ cat microbiome samples**.** Ovals represent the 90% confidence ellipsoids around the centroid of the clusters (FIV+ = red; FIV- = blue). **(B)** OTUs with significant log-fold change in abundance between FIV+ and FIV- cats at the 0.1 level of significance (after correcting for multiple testing). The list on the left shows the genera of each of these OTUs. Red indicates over representation of that OTU in the FIV+ cats. **(C)** FIV+ cat with clinical gingivitis/periodontitis with near monoculture of *Moraxellaceae.*

FIV alterations of oral cytokine expression

Circulating cytokine and chemokines were quantified in saliva of naïve and FIV-infected SPF cats (n=5 per group) by microsphere immunoassay (MIA) to detect changes in innate immune parameters in the presence of FIV infection. At a single time point with small group sizes in cats without gingivitis, FIV-infected cats exhibited trends for decreased levels of IFNγ (p=0.149), IL-8 (CXCL8, p=0.108), IL-1B (p=0.103), and IL-12p40 (0.096) compared to naïve cats, while levels of IL-2 (p=0.184) and KC (CXCL1, p=0.101) tended to be slightly decreased (**Figure 4.3**).

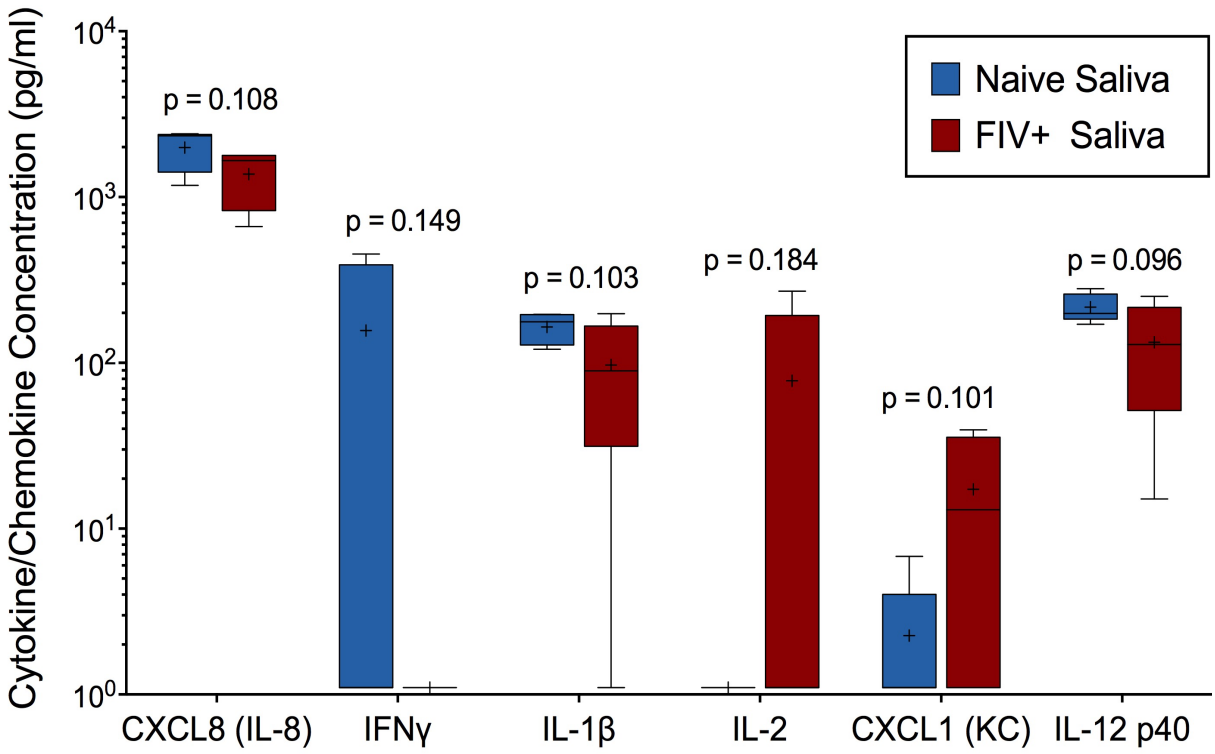

Figure 4.3. FIV-infected cats show trends towards oral cytokine expression differences. Saliva of FIV-infected cats without clinical oral lesions and saliva from naïve animals (n=5/group) was tested by MIA for cytokines as described. IL-8, IFNγ, IL-1b, and IL-12 p40 tended to be decreased in FIV positive cats while CXCL1 and IL-2 tended to be higher in FIV infection when analyzed by pairwise t test.

Innovative cART Regimen

The ability of Dolutegravir, Tenofovir, and Emtricitabine to inhibit FIV replication was tested on GFox cells *in vitro* in the presence of 50,000 infectious units of FIV_{C36}. When used in combination (cART), Dolutegravir (DTG), Tenofovir disoproxil fumarate (PMPA), and Emtricitabine (FTC) exhibited a significant inhibitory effect for all drug concentrations over time (p=0.0003; RM-ANOVA), even at the lowest tested drug concentrations (**Figure 4.4**).

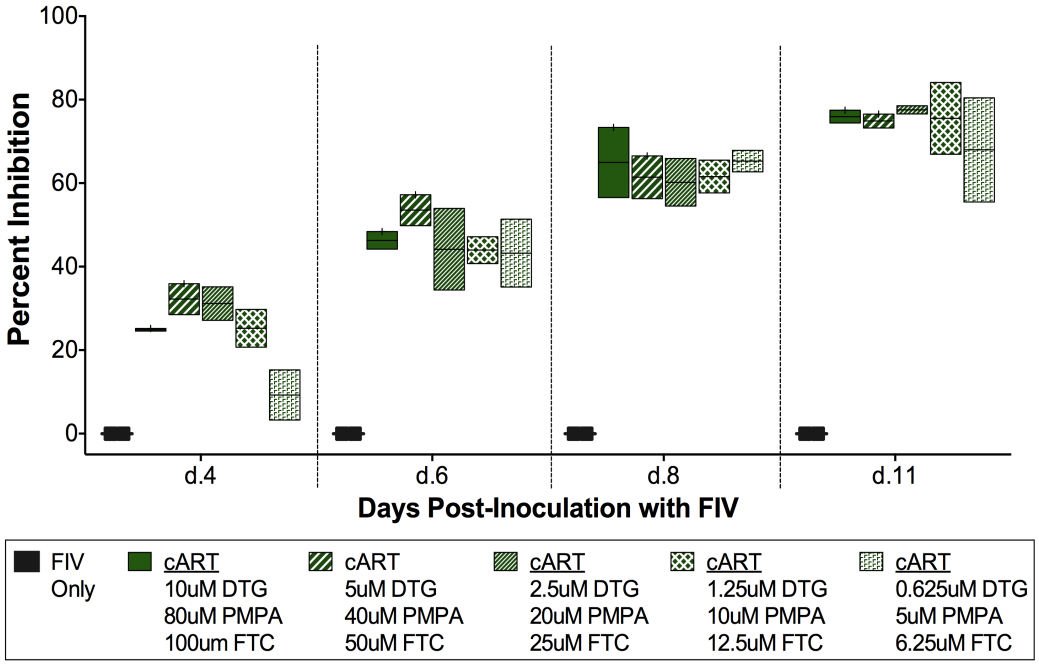

Figure 4.4. Combination antiretroviral therapy (cART) strongly inhibits FIV infection *in vitro,* even at low concentrations. FIV_{C36} was incubated with varying concentrations of Dolutegravir (DTG), Tenofovir disoproxil fumarate (PMPA), and Emtricitabine (FTC) in a ratio utilized in *in vivo* studies [12, 13] and tested for inhibition on GFOX cells. "No cART" FIV-only positive control represented 100% FIV growth as measured by ELISA absorbance at 450nm. Analysis of percent inhibition over time revealed a significant inhibitory effect for all drug concentrations over time (p=0.0003; RM-ANOVA), even at the lowest tested drug concentrations.

Discussion

Results of this pilot study highlight the potential to develop relevant therapeutic interventions in an animal model that closely recapitulates HIV-AIDS oral cavity dysfunction, with the goal of translating findings to HIV oral infections. As noted previously, many features of FIV oral cavity disease parallel findings in humans with HIV. The work outlined in Chapter 1 of this dissertation documents that FIV oral cavity infection is concentrated in oral lymphoid tissues [38], and results of these preliminary studies denote changes in salivary cytokine expression during FIV infection, indicating viral-induced alterations in innate immune function (**Figure 4.3**). Furthermore, these preliminary results highlight significant alterations in the microbiota of FIV-infected cats, and document a striking shift to a near monoculture of *Moraxellaceae* in one case of FIV-associated moderate to severe gingivitis using 16s rRNA metagenomics analysis (**Figure 4.2**). Because HIV and FIV infections are both associated with perturbations in the oral

microbiota and chronic immune dysfunction [2-4, 14-17, 70-74, 77], future studies will be directed at defining perturbations in oral microbiota and oral/systemic inflammation during treatment with an innovative cART protocol.

While cART has been successful at limiting some oral manifestations of HIV, it has not completely eliminated oral pathologies [2, 16, 90], and has only been marginally successful in cats with FIV (CHAPTER 3). The relationship between cART therapy and oral microbial shifts during HIV infection is unknown and impossible to study in HIV patients. Dolutegravir, Tenofovir, and Emtricitabine have recently been used as safe and effective therapies in SIV infection [12, 13], and are convenient as they can be applied via daily injection. The efficacy and pharmacokinetics of these individual or related compounds has been documented in FIV-infected cats [91-94], however, their combined effect *in vivo* has yet to be evaluated. The preliminary results presented above indicate a profound synergistic effect *in vitro*, even at the lowest concentration utilized in this study (**Figure 4**). Collectively, this data indicates that such a cART regimen will also be highly effective against FIV infection *in vivo*. By comparing FIV naïve cats to cats with FIV infections treated with cART or placebo, the FIV model may provide means to assess the impact of active viral replication on oral dysbiosis, viral-induced oral cavity and MALT immune dysfunctions, and systemic parameters; thus providing a basis for understanding how cART impacts oral microbial ecology and downstream pathologies [23, 62, 95].

The persistence of HIV infection and periodontitis in patients on cART indicates that ancillary treatments specifically directed at restoring the normal oral microbiota in conjunction with cART may improve HIV periodontal progression and decrease systemic immune activation [16, 23, 24, 26]. Feline dental disease is currently managed by comprehensive dental treatment consisting of hand and ultrasonic scaling, identical to techniques used in humans [96, 97]. Probiotic supplementation has been successful in early studies as an adjuvant for treating periodontitis in people, and similar commercial oral probiotics products are available for management of feline oral conditions [98-100]. Thus, the application of comprehensive dental

cleaning with probiotic treatments in the feline model has the potential to assess the impact of local therapy for restoring oral homeostasis during lentiviral infection, and may increase our understanding of the progression and/or resolution of FIV-induced oral lesions and oral microbiome in the presence and absence of cART. Results from such studies may help to guide the development of potentially effective strategies for management of HIV oral infections, and may also provide means to match MALT related changes to chronic systemic inflammation.

The findings presented in this chapter indicates that FIV-infection produces aberrant cytokine expression in saliva of infected cats. IL-8, IFNγ, IL-1B, and IL-12 p40 are critical for innate and adaptive immunity against viral and bacterial infections, neutrophil function, and lymphocyte proliferation and differentiation. Deficiencies in these cytokines could facilitate oral dysbiosis through reduced immunocyte function and inability to maintain normal oral flora [101-103]. IL-2 promotes proliferation of both CD4+ and CD8+ T cells, and local increases in IL-2 may therefore promote lymphocyte activation and inflammation as a result of bacterial overgrowth [104]. KC (CXCL1) is secreted by macrophages, neutrophils, and epithelial cells at sites of inflammation, and functions as a major neutrophilic chemoattractant [105, 106]. Increases in this pro-inflammatory cytokine may likely represent a consequence of innate immune stimulation in response to opportunistic bacterial overgrowth. Collectively, these preliminary results suggest that FIV mediated immune dysfunction may result in oral dysbiosis, perpetuating FIV associated oral disease through secondary immune activation and lymphocyte proliferation. Most importantly, these findings represent an opportunity to investigate the role of immune dysfunction in the development of lentiviral induced oral disease, and highlight the potential for immunomodulatory therapy to attenuate systemic and local immune activation through restoration of oral homeostasis by chronic inflammation during FIV and HIV infection.

Type I interferons (IFN-α, IFN-β, and IFN-ω) are expressed during viral infection and exhibit antiviral and anti-proliferative properties [107, 108]. The mechanism for Type 1 IFN inhibition of HIV (and other viruses) has been linked to changes in expression of Interferon-

stimulated genes which are activated soon after IFN exposure [109, 110]. While IFN-α has been shown to inhibit HIV infection *in vitro*, its therapeutic use *in vivo* has been associated with disease progression during late infection and limited treatment effect on HIV-induced oral disease [111, 112]. IFN-ω is a monomeric glycoprotein secreted by virus-infected leukocytes structurally related to IFN-α and IFN-β [107]. In contrast to other Type I Interferons, IFN-ω exerts strong immunomodulatory effects by stimulating Natural Killer cell activity, enhancing expression of MHC-I, and inhibiting lymphocyte proliferation [107]. IFN-ω has also been reported to be a potent inhibitor of HIV infection *in vitro*, but *in vivo* therapeutic potential in human patients has not been evaluated [108]. Alternatively, recombinant feline interferon omega (rFeIFN-ω) has been shown *in vivo* to decrease the severity of oral disease in FIV-infected cats and influence innate immunity to reduce pro-inflammatory stimuli [113-115]. Therefore, studies aimed at assessing the outcomes of IFN-ω therapy on FIV oral disease in the presence and absence of cART have great potential to elucidate anti-inflammatory mechanisms and potential for adoption as an agent for HIV-associated oral disease.

In summary, the results of pilot study demonstrate that similar to HIV, FIV infection of domestic cats is associated with (1) oral microbiota dysbiosis with marked loss of microbial diversity during lentiviral-associated periodontitis; (2) changes in salivary cytokine levels, even in the absence of FIV clinical oral disease; and, (3) systemic markers of immune stimulation, signaling local and peripheral immune activation, and suggesting bacterial translocation. These findings identify key pathways leading to changes in oral homeostasis during HIV infection using an analogous animal model, and highlight the potential of the FIV model to guide approaches to manage local and systemic inflammation and dysbiosis in HIV patients.

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CONCLUSIONS

Over the course of my graduate studies and as presented in this dissertation, I have investigated several aspects of feline immunodeficiency virus (FIV) with a focus on its applications as a model to study HIV. Because FIV produces progressive immune depletion that eventually results in an acquired immunodeficiency syndrome (AIDS) similar to HIV infection in humans, and because the occupational risks of working with cats and FIV are considerably less than non-human primates with SIV or SHIV, the feline model of AIDS has great potential as a safer and more economical alternative to NHP studies. In Chapter 1 of this dissertation, I characterized FIV salivary viral kinetics and antibody secretions to more fully document oral viral pathogenesis. The results of this research demonstrate that (i) oral lymphoid tissues serve as a site for enhanced FIV replication, resulting in accumulation of FIV particles and FIV-infected cells in saliva, and (ii) failure to induce a virus-specific oral mucosal antibody response, and/or viral capability to overcome inhibitory components in saliva may perpetuate chronic oral cavity infection. While it is accepted that FIV is primarily transmitted by biting, few studies have evaluated FIV oral infection kinetics and transmission mechanisms, and such modern quantitative analyses applied to natural FIV oral infection help to further our understanding of lentiviral oral disease and transmission. Most importantly, these results provide a model of oral FIV pathogenesis and suggest alternative diagnostic modalities and translational approaches to study HIV pathogenesis, particularly oral manifestations of HIV infection.

In Chapter 2 of this dissertation, I immunized cats with soluble CD134, recombinant FIV-SU protein, and/or CD134+SU complexes prior to challenge with FIV to determine if vaccination with CD134-SU complexes could induce protection against FIV infection. Immunization induced production of anti-CD134 and anti-SU antibodies in vaccinated cats, and purified anti-CD134 and anti-SU antibodies significantly inhibited FIV infection *in vitro.* However, no vaccine

combination protected cats from FIV infection *in vivo* and vaccination induced high titers of antibodies directed at vaccine by-products relative to target antigens. Because FIV and HIV utilize parallel modes of receptor-mediated entry, and because autoantibodies to FIV-SU and the CD134 binding receptor are able to block FIV infection *ex vivo,* it was optimistic that vaccination with anti-receptor antibodies could provide a novel immunotherapy to block viral infection *in vivo,* and that this innovative vaccine modality might be adapted to preventative strategies in HIV. However, due to the failure of this vaccine strategy to induce protection from infection *in vivo,* the results presented in Chapter 2 of this dissertation reinforce the need to monitor components of vaccine preparations, and emphasize that vaccination may induce proliferation of susceptible target cells and enhancement of heat-labile serum components that counteract neutralizing antibodies.

In Chapter 3 of this dissertation, I administered immunosuppressive doses of prednisolone or CsA to cats chronically infected with FIV and monitored alterations in hematological parameters and FIV viral/proviral loads in response to therapy. Similar to HIV, FIV-infected cats frequently develop secondary or opportunistic infections as a consequence of viral-induced immune dysfunction, including anterior uveitis, chronic rhinitis, gingivostomatitis and periodontitis, encephalitis and neurologic dysfunction, and lymphoma. Both prednisolone and cyclosporine A (CsA) are commonly used clinically to treat lymphoproliferative and immune-mediated diseases in these patients, but the impact of these compounds on infection has not been well documented, and their understanding immunomodulatory effects on viral replication and persistence is critical to guide safe and effective use of these therapies. In this study, both prednisolone and CsA treatments caused (i) acute increases in CD4+ lymphocytes, (ii) increased FIV viremia, and (iii) significant alterations in cytokine expression that favored a shift toward a Th2 response. These results highlight the potential for immunosuppressive drug-induced perturbation of FIV replication and underscores the need for consideration of chronic viral infection status when prescribing immunomodulatory medications.

In Chapter 4, I presented the findings of a pilot study in which I investigated changes in the oral microbiota and oral immune system during FIV infection. Because gingivitis and periodontitis are primary clinical signs associated with untreated natural and experimental FIV-infection, these principal attributes have great potential to elucidate pathogenic mechanisms involved in the perturbation of the oral immune system and microbial environment. Since other commonly used animal models for studying HIV pathogenesis, such as SIV/SHIV infections in NHPs or HIV infections in humanized mice, do not reliably incite oral lesions, the FIV model of lentiviral-induced oral disease presents a unique opportunity to investigate the development of this disease syndrome and to test the efficacy of novel immunomodulatory therapies that may have translational applications during HIV infection. Such studies aimed at evaluating FIV-associated changes in the oral microbiota, local and systemic virologic parameters, and immune dysfunction under novel treatment protocols may provide an innovative approach in the future to expand our understanding of the complex interactions between HIV infection, oral immune dysfunction, and the perturbations to the oral microbiota that occur in the context of HIV infection.