ABSTRACT

SIMOES, RITA DE CASSIA. Investigation of Natural Killer Cells in Chronically Feline Immunodeficiency Virus Infected Cats. (Under the direction of Dr. Gregg A. Dean).

Human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV), and feline immunodeficiency virus (FIV) are members of the genus *Lentivirus* and the family Retroviridae that cause immune dysregulation that eventually results in acquired immunodeficiency syndrome (AIDS). The host defense against viral infection is complex and involves interplay between the innate and adaptive immune response. NK cells play a significant role against invading microorganism by directly killing infected cells without prior sensitization, in addition to shaping both the innate and adaptive immune response through the production of cytokines. Significant abnormalities of NK cell have been observed throughout the course of HIV-1 infection. The importance of NK cells in HIV-1 infection has been corroborated by studies showing that certain combinations of killer immunoglobulin-like receptors (KIR) and MHC class I molecules correlate with a slower progression of HIV-1 disease. Although several studies have described the mechanisms by which NK cells modulate the immune response, the exact mechanisms by which NK cell responses are regulated by other cells is still unclear. Regulatory T cells (Treg) are key players in the maintenance of peripheral tolerance and restraining inflammatory responses. They control the function of CD4+ and CD8+ T cell, B cells, and dendritic cells. In addition, recent reports have described that Treg cells also suppress NK cell functions. Strong evidences suggest that Treg cells and NK cells have important roles in HIV-disease pathogenesis. Treg cells have been described in the domestic cat, and the immunosuppression exerted by these cells is enhanced during FIV infection. In the present
studies, we probed the innate immune response in chronically FIV-infected cats with the well characterized opportunistic pathogen *Listeria monocytogenes* (Lm). Using the FIV/cat/Lm model we addressed the effects of chronic FIV infection on the NK cell response and also sought to determine the role played by Treg cells in the NK cell response to Lm in the context of lentiviral infection.

We found that when compared to SPF-control cats, NK cells from FIV-infected cats have a constitutive higher level of proliferation that is counter-balanced by increased apoptosis. Upon challenge with Lm, NK cells of FIV-infected cats fail to traffic to lymph nodes, have a lower proliferative response, and show a minimal increase in perforin expression. We also observed a reduced number of DC in the LN of FIV infected cats with a higher MHC II expression level, indicating a heightened immune activation. Similarly, NK and NKT cells from FIV-infected cats displayed higher levels of the activation marker CD25⁺. Treg cell depletion led to increased expression of perforin and granzyme A by NK and NKT cells from SPF-control cats, indicating that Treg cells exert some control on these cell populations, but other factors and/or cell populations besides Treg cells must also be responsible for suppression of the NK cell response. Based on our results, it is still possible that Treg cells may indirectly reduce efficient activation of NK cells during lentiviral infection.

We propose that NK cell defect is one of the consequences of chronic lentiviral infection. We found no evidence of a direct negative effect on NK cells by Treg cells in vivo. Whether there is an indirect effect of Treg cells on NK cell function and/or whether the relationship between these two cell types is different at the time of lentivirus infection and during the acute phase of disease, remains to be determined.
Investigation of Natural Killer Cells in Chronically Feline Immunodeficiency Virus Infected Cats

by
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A dissertation submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Comparative Biomedical Sciences

Raleigh, North Carolina
2012

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DEDICATION

To my husband, Rafaelo, who supported me each step of the way with love and patience. I could not have done it without you.

A special feeling of gratitude to my mother, for her support and motivation throughout my life.

To the loving memory of my father, who taught me to believe in myself.
BIOGRAPHY

Rita was born in the city of Jundiaí, in São Paulo, Brazil. Daughter of Dagoberto and Araci Munarolo Simões, she was the youngster, with two siblings, Rogério and Rosane Simões. Rita’s interest in veterinary medicine goes as far as she can remember, and her interest on becoming a veterinarian only increased while she was in high school. Rita started attending the Universidade Federal de Viçosa to pursue her degree in Veterinary Medicine in 1995, in the city of Viçosa, in Minas Gerais, Brazil. In 1996, as a veterinary student, Rita was awarded a research scholarship and joined Dr. Marcelo J. Vilella’s lab to work with cancer research until her graduation from vet school, in 2000. During the same period, she met her future husband Rafaelo. After moving back to her hometown and working for a little over six months in a private small animal practice, she realized she really missed doing research and applied to graduate school. She and her husband moved to the city of Londrina, in Paraná, Brazil. There, she started her Master Degree studies in Microbiology at the Universidade Estadual de Londrina. She studied and characterized a virulence factor from avian E. coli and its use as a potential vaccine, until receiving her M.Sc. in 2003. In April of 2003, she joined her husband in Raleigh, NC, after they lived more than one year apart. In 2004, after initial adaptation with the new language, customs and all the red tape with working authorization, she started working as a Research Specialist and Laboratory Manager for ArrayXpress Inc., a biotech company incubated at the Centennial Campus North Carolina State University (NCSU). She worked close to the company’s CSO Dr. Len van Zyl with microarray technology, and they became good friends. Dr. van Zyl always motivated her on
pursuing a Ph.D., they would frequently discuss science during coffee break. Enjoying her work with research, but missing her connections with veterinary medicine, Rita decided she should find a job at the College of Veterinary Medicine at NCSU. In early 2005, she joined Dr. Prema Arasu’s lab as a research specialist and was delighted to work again with animal research, with focus in the relationship of hookworm infection and allergy – the so called ‘hygiene hypothesis’. Once again, she found herself discussing research all the times, became friends with her new boss, who also motivated her to attend the Immunology Journal Club and apply to the PhD program at NCSU. In 2007 she decided to apply for admittance to the Comparative Biomedical Sciences Program at NCSU, she joined the laboratory of Dr. Gregg A. Dean where she spent the last five years.
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1. INTRODUCTION

Human immunodeficiency virus (HIV) is the causative agent of acquired immunodeficiency syndrome (AIDS) and is a major global public health threat, causing considerable morbidity, mortality, and negative socioeconomic impact. HIV infection is considered pandemic by the World Health Organization (WHO), affecting approximately 35 million people worldwide. Feline immunodeficiency virus (FIV) infection of cats is clinically and immunologically very similar to HIV infection in people and is a well established animal model of HIV and AIDS. Both HIV and FIV-infection have been reported to negatively impact the innate immune compartment. More specifically, it has been known for a long time that NK cells are dysfunctional during all the stages of HIV disease, from acute infection to development of AIDS. Interest about this cell population resurfaced after recent discovery of NK cell in helping to modulate the adaptive immune response, and the undisputable impact of killer immunoglobulin-like receptors (KIR) and human leukocyte antigen (HLA) molecules on the outcome not only of HIV-1 disease progression in infected people, but also on HIV-1 protection in highly exposed populations. Relatively little is known about the effects of chronic lentivirus infection on the innate immune system or the contribution of this compartment to disease progression and AIDS, and the majority of the studies have been done in vitro and using mainly NK cells from the blood compartment. More recently, studies have suggested that Treg cells may play a role in NK cell steady state as well as its activation and function during an immune response. Our laboratory has demonstrated that Treg depletion in chronically FIV-infected cats can improve
the cats adaptive immune response. However, whether Treg depletion are harmful or helpful to the innate immune response of feline hosts during chronic HIV infection is unknown. Because the innate immune responses are complex and multifactorial, involving a number of different cell types and soluble mediators such as cytokines and chemokines, its study on HIV-1 infected patients is not feasible. This dissertation explores the in vivo innate immune response against *Listeria monocytogenes*, an opportunistic pathogen, in chronically FIV infected cats. The following specific aims are addressed:

1. Determine the nature and mechanism of in vivo NK cell response in chronically FIV-infected cats after subcutaneous challenge with the opportunistic pathogen *Listeria monocytogenes*.
2. Determine whether in vivo depletion of CD25+ Treg cells improves or diminishes the NK cell response to *Listeria monocytogenes*. 
2. LITERATURE REVIEW

A. FIV Infection as a Model System for HIV

1. HIV Models Overview

Since the discovery of human immunodeficiency virus (HIV) as the etiological agent for acquired immunodeficiency syndrome (AIDS) in the early 1980s [1-3], the need for a suitable animal model to study HIV immunopathogenesis and testing of potential strategies of intervention was crucial. Attempts to experimentally infect different animal species with HIV-1 were not successful, and the few non-human primates where seroconversion and transient lymphadenopathy after HIV-1 inoculation were reported – chimpanzees, gibbons and pig-tailed macaques – did not show the same immunopathogenesis and/or AIDS-like syndrome during the course of infection [4-6], limiting their use as animal models to study HIV-1 infection.

Almost immediately after the emergence of HIV/AIDS, lentiviruses became the focus of intense research. They have been divided into five subgroups restricted to a single mammalian order or family [7]. Particular interest was given to simian immunodeficiency viruses (SIV), isolated from non-human primates presenting AIDS-like disease and/or lymphoma shortly after the discovery of HIV [8-11]. Several macaque species, including cynomolgus, pig-tailed, and rhesus macaques, were experimentally infected with SIV and developed immunopathological responses comparable to HIV/AIDS in humans. Shortly after the discovery of HIV and SIV, Pedersen and colleagues described for the first time the feline immunodeficiency virus
(FIV), a naturally occurring lentivirus that causes AIDS-like disease in domestic cats [12]. Given that FIV disease pathogenesis proved to be very similar to HIV-1 infection in humans, the fact that specific pathogen free (SPF) cats were readily available, cheaper and easier to handle and maintain when compared to monkeys, the group correctly predicted the importance of the FIV as a model system for HIV [12]. More recently, humanized mice have emerged as potential models to address important questions in HIV/AIDS immunopathogenesis and therapeutics/prevention [13,14]. However, this model has not been completely characterized and more information about the immunopathogenesis of HIV within this system needs further clarification in order to realize the full potential of this model.

While no single animal model captures all aspects of HIV-1 infection in humans, the FIV/cat model has many features required for studying the immunopathogenic mechanisms that could lead to the development of a more effective treatment and/or a vaccine against HIV.

2. FIV Genome

Feline immunodeficiency virus belongs to the family Retroviridae, subfamily Lentivirinae in the Lentivirus genus and like HIV-1 has a worldwide distribution [15,16]. Although FIV is phylogenetically closer to the lentiviruses of small ruminants than to human and non-human primates [17], FIV shares many genomic, structural and biochemical features with HIV [18,19]. The FIV genome is roughly 9,400 nucleotides long, with the genes gag, pol, and env flanked by long terminal repeats (LTRs) similar to
all members of the Retroviridae family, including HIV. Despite similarities, there are also significant genomic differences between FIV and HIV genetic components [17,20,21].

As with all lentiviruses, reverse transcription, the first step of retroviral replication cycle [22,23], requires a tRNA_{lys} isoacceptor for the reverse transcriptase in order to initiate first strand synthesis [24-26]. Full length and spliced mRNA transport is regulated by Rev, but in FIV both the Rev responsive element (RRE) and the second coding exon are located 3’ of env whereas in HIV it overlaps the transmembrane (TM) polyprotein coding region.

In contrast to HIV, FIV does not encode vpr, vpu or nef genes and also lacks a viral gene transcription regulatory system Tat/Tar [17,27]. Lack of tat and the transactivation response (TAR) element lead to significant differences in FIV transactivation. FIV does encode a small gene product expressed along with Rev, called ORF-A (or ORF-2), a tat-like gene that encodes a viral transactivator essential for productive growth of FIV in T cells and its expression is followed by an increase in translation of gene products driven by the FIV LTR [27-30]. ORF-A defective FIV has reduced pathogenicity that positively correlates with lower replication efficiency as showed in experiments where cats were infected with FIV and/or ORF-A-defective FIV [31-34]. Gemeniano and colleagues demonstrated that ORF-A has the ability to influence cell cycle as well as virus release from the cells, playing a similar role as Vpr in HIV [35]. These findings suggest that ORF-A may be able to accumulate functions of more than one gene product missing from FIV compared to HIV.
FIV Pol comprises the same genes that are common with HIV-1 (protease, reverse transcriptase, and integrase genes), however it also encodes a gene for deoxyuridined pyrophosphatase (DU), an enzyme that is found on all non-primate lentiviruses [36,37]. The primary role of DU is preventing uracil incorporation into FIV DNA by limiting the concentration of dUTP [32,38]. FIV lacking DU do not replicate successfully in cells that are not dividing, such as macrophages [37]. Since rapidly dividing cells express high levels of endogenous DU, DU-defective FIV can productively infect and replicate in these cells [37]. It is still unclear how HIV, which lacks DU, avoids uracil incorporation in viral DNA. Studies suggest that Vpr may play a role in excision of misincorporated uracil by association with an enzyme responsible for excision of uracil misincorporated into DNA [39,40], and also based on the fact that Vpr defective HIV and DU defective FIV cannot replicate in macrophages [41,42].

The viral infectivity factor (Vif) is encoded by all the lentiviruses but equine infectious anemia virus (EIAV). Vif is responsible for regulating viral infectivity in both FIV and HIV-1 and is essential for productive infection of lymphocytes [43]. FIV Vif shows significant amino acid sequence divergence [39], but has a similar size and the prerequisite functional consensus sequence SLQ(Y/F)LA is conserved [43]. After infection with Vif-defective FIV, viral antigens can be detected in lymphoid tissues, but can barely be detected in blood, suggesting a reduced ability of virus replication and dissemination [31,44].
3. FIV Receptors

HIV uses the CD4 molecule as its primary binding receptor and further interaction with chemokine coreceptors is required in order to trigger conformational changes and fusion of the virion with cellular membranes for subsequent virus entry [45]. While the CD4 molecule was shown to be the primary receptor of HIV during the mid-1980’s, the primary receptor of FIV has only recently been unveiled. Since 1993 it has been known that CD4 was neither used as a primary receptor nor as a coreceptor by FIV [46-48]. Shimojima and colleagues [49] reported for the first time that the activation marker CD134, also called OX40, is the primary FIV binding receptor, which was later confirmed by others [50,51]. The chemokine receptor CXCR4 is essential for virus entry and is used as a coreceptor by both, FIV and HIV [52-54]. Additionally, some FIV strains were reported to infect CXCR4+ cells in the absence of CD134 [52,55,56]. Preliminary reports have suggested that some FIV strains could use the chemokine receptor CCR5 as a coreceptor [56-58], but further studies are needed in order to confirm this observation. The ability of FIV to target CD4+ T cells in vivo without using CD4 as its primary receptor was clarified by de Parseval and colleagues [50] who demonstrated that expression of CD134, a member of the tumor necrosis factor receptor family, is upregulated on activated CD4+ T cells. Similar to HIV, interaction between the binding receptor (CD134) and the virus results in conformational changes of SU and consequently promotes high affinity binding to CXCR4 [55].

During the course of HIV-1 infection there is a switch from CCR5 to CXCR4 coreceptor tropism [59,60] in ~50% of infected patients [61]. Coreceptor switching is
associated with faster CD4+ T cell loss and progression to AIDS [61], suggesting that coreceptor switch plays a role in HIV pathogenesis. Similarly, FIV cell tropism changes as disease progresses and expression of CD134 and/or CXCR4 by dendritic cells, B cells, CD8+ T cells, and macrophages is upregulated, increasing the number and range of target cells permissive of virus infection [50,62-64].

Together, these studies show parallels of viral entry mechanisms between FIV and HIV, suggesting that these viruses result in a similar immunological response in both hosts, thus offering a valuable tool to study HIV immunopathogenesis.

4. FIV Immunopathogenesis Overview

The FIV/cat model is a well established animal model for the study of HIV-1 infection in humans. Like HIV, FIV transmission can occur both horizontally and vertically. Horizontal transmission occurs primarily during fighting through direct contact with contaminated blood via biting and exposed lesions [65,66], but it can also be transmitted through saliva [67], and semen [68]. Vertical transmission occurs in utero, intrapartum [69,70], and through colostrum and/or milk [71,72]. FIV pathogenesis is very similar to that in HIV infection, where infected cats develop a short acute phase presenting a very high viremia that peaks 2-3 weeks post infection, with transient symptoms such as low grade pyrexia and lymphadenopathy [18,73]. The acute stage is followed by an expansion of the CD8+ T cell subpopulation [74-76], and a considerable reduction in plasma viremia due to both cytotoxic [77-80] and non-cytotoxic [81-83] cell mediated mechanisms. CTL responses against Gag, Pol, and Env epitopes as well as
whole FIV are present [77,79,80]. Non-cytotoxic CD8+ antiviral activity is present in the blood, peripheral lymphoid tissue, and thymus following experimental FIV infection [81,84-86], and correlates with reduction in virus burden [81,83,86,87]. Down regulation of virus replication marks the onset of the asymptomatic period, which can last for several years and is characterized by a progressive immune dysfunction with a substantial loss of CD4+ T cells – also a hallmark of HIV infection, altered cytokine profiles, and reduction of T cell proliferative responses to both mitogens and FIV recall antigens [88-92]. The combination of increased numbers of CD8+ T cells and depletion of CD4+ T cells leads to an inverted CD4:CD8 T cell ratio in FIV-infected cats [92], another hallmark observed during HIV infection in humans [93]. CD4+ T cell depletion and inversion of CD4:CD8 ratio are independent of the inoculation route, with systemic, mucosal, or vertical transmission leading to the same outcome [69,72,81,94]. The strong T-helper 1 response during early stages of FIV infection steadily declines as disease progresses [90], the ability to produce naive T cells is lost, and the naive T cell pool is replaced by memory/activated L-selectin- CD4+ and CD8+ T cells (adhesion+, integrin+, and MHC-II+) [82,95].

Compared to the T cell compartment, B-cell dysfunction is not as remarkable, affecting mainly the primary antibody responses to T-dependent antigens [92,96]. FIV-specific antibodies can be detected as early as 2 weeks after infection and typically is sustained throughout all the stages of infection [72,97-99]. The presence of FIV neutralizing antibodies is observed soon after the production of CTL, providing an additional layer of immunity to FIV [31], while the absence of antibody production in
FIV-infected cats leads to a rapid progression to AIDS and death of the animal [72,100,101,101]. Infected cats often develop hyperactive virus-specific B-cell responses, with high levels of serum IgG, similar to HIV-1 infected patients [102-104].

HIV-1 infection in humans is associated with reduction of type 1 (Th1) cytokine production, such as IL-2, IL-12, and IFN-γ [105-107]. However, there is still controversy whether these changes correspond to a shift to type 2 (Th2) cytokines [108-110]. In contrast, changes in cytokine expression and production are well described throughout the course of FIV infection. Studies have shown a reduction of IL-2 and IL-12 and increased levels of IL-10, IFN-γ, and TNF-α expression in lymph nodes, spleen, and thymus [111,112]. Elevated serum levels of IL-1, IL-6 and TNF-α are observed in FIV-infected cats, and a positive correlation is seen between these cytokine levels and presence of clinical signs [113,114]. It is believed that the innate and cell mediated immune defects observed in FIV-infected cats are partially due to cytokine dysregulation, leading to an ineffective immune response against opportunistic infections. FIV-infected cats challenged with *Toxoplasma gondii* showed increased levels of IFN-γ, and IL-10, but displayed suppressed levels of IL-2, IL-6 and IL-12 [112,115]. In another well established challenge model, FIV-infected cats probed with *Listeria monocytogenes* presented similar cytokine dysfunction, with FIV-infected cats unable to control the initial *L. monocytogenes* burden [111]. Interesting, locally delivered IL-15 increased the number of NK cells and improved *L. monocytogenes* clearance [111,116], suggesting not only that FIV infection leads to NK cell dysfunction, but more importantly, this functional defect could potentially be rescued *in vivo*. 
B. Innate Immune Response During Lentivirus Infection

1. Overview

Viruses are pathogens that exploit the host’s cell biological processes and machinery for their survival and replication. The host defense against viral infection is complex and involves interplay between the innate and adaptive immune response. Dendritic cells (DC), monocytes/macrophages and NK cells represent important innate immune cells that not only control the initial viral replication but also determine the type and strength of the adaptive immune responses through their effector and regulatory functions. Based on its critical role during the initial viral control and in shaping the adaptive immune response, it is clear that an appropriate innate response is the basis of the host’s ability to efficiently fight infection and ultimately survive. In order to succeed, viruses have evolved to overcome the innate immune response. Although the hallmark of HIV-1 infection is a progressive decline of CD4+ T cell immunity, a profound dysfunction is also observed in the innate immunity compartment, including NK cells [117], NKT cells [118], and DC populations [119-121]. Reports have shown a dichotomy played by innate immunity in controlling HIV-1 infection and in contributing to the immunopathogenesis of disease, with recent studies implicating the innate immune compartment as a key player on chronic immune activation leading to disease progression and AIDS.
2. Type I interferons

Type I interferons (IFN-α/β) are innate cytokines that display several antiviral mechanisms against a wide range of viruses and have the ability to block viral replication at different steps of the virus life cycle [122,123]. In addition, type I IFNs regulates immune activation and apoptosis, which combined with their antiviral properties contribute to a degree of HIV resistance in host cells at sites of virus exposure. On the other hand, type I IFN are potent immunostimulatory cytokines that trigger activation and maturation of several cell populations such as NK cells, DCs, macrophages and T cells [124], resulting in a local immune activation that could lead to a detrimental outcome due to increased susceptibility to HIV infection. Since type I IFNs are produced following HIV infection and at later stages of infection, one can speculate that these cytokines may also have a detrimental effect throughout HIV infection. An important antiviral activity of type I IFNs includes upregulation of cellular restriction factors, which block cross-species transmission of lentiviruses [125]. The host-encoded restriction factors APOBEC3 (A3, apolipoprotein B mRNA-editing polypeptide 3) display important antiviral mechanisms [126], and HIV has evolved to offset these restriction factors by encoding accessory genes [127,128]. In a similar way, FIV encoded Vif protects the virus against APOBEC3 restriction from host cells [129]. The observation that HIV-infected cells barely upregulate the production of type I IFNs suggests the importance of this innate cytokine not only by their intrinsic antiviral effects [128], but also the potent antiviral activity of the restriction factors. Reports have suggested that during chronic HIV-1 infection, type I IFNs may contribute to disease pathogenesis by promoting
apoptosis of both infected and uninfected cells through both TRAIL and Fas/FasL pathways [130-133].

Plasmacytoid dendritic cells (pDC) are the most potent IFN type I producing cells. pDC are found mainly in peripheral blood and lymphoid tissues and produce copious amounts of IFN-α after encounter with either DNA and RNA viruses [134]. Both cell-free HIV and HIV-infected cells are capable of stimulating IFN-α production by pDC [135,136]. IFN-α production by pDC occurs after viral uptake by these cells and subsequent binding of viral DNA to TLR9 [137] and/or viral ssRNA to TLR7 [138]. pDC also produce IFN-α in the absence of virus replication by sampling portions of virus-infected live cells [139], bypassing IFN-α blockade by viral proteins. Early studies showed that IFN-α production was compromised in HIV-infected patients. In these studies, PBMC from AIDS patients had a defective production of IFN-α in response to HSV, and this reduction of IFN-α correlated with the subsequent development of opportunistic infections by these patients [140,141]. After the phenotypic characterization of the pDC, several groups observed a reduction of this cell population in PBMC from HIV-infected people [142-144]. Feldman and colleagues [142] proposed that HIV leads to a functional defect in pDC, based on the fact that pDC from patients with high viremia and low CD4⁺ cell counts produced less IFN-α after in vitro stimulation with HSV than non-infected or HIV-infected patients with low viremia and higher CD4⁺ cell counts. Additional evidence of the protective role played by pDC was suggested by Almeida and colleagues [119], who reported normal levels of pDC within HIV-infected patients who are long-term nonprogressors.
It is clear that the role played by IFN-α during the course of HIV-1 infection is still controversial. While some studies correlate a faster disease progression with a reduced number of pDC as well as defective production of IFN-α, other studies show that chronic production of IFN-α has a detrimental effect in chronic HIV-infected patients. Indeed, early trials of IFN-α therapy showed no beneficial effects while others showed a negative outcome in HIV-1 infected patients [145]. Most likely fine tuning of the IFN type I system is crucial to control HIV replication and disease progression, and both overproduction or underproduction can play a detrimental role in disease outcome.

3. Conventional Dendritic cells

The adaptive immune response against viral infections is orchestrated by T and B cells expressing antigen-specific receptors. Activation and differentiation of T and B cells take place within the secondary lymphoid organs through an interplay between components of both the innate and adaptive immune system, including antigen-presenting cells (APC). DCs are probably the most important type of APC, displaying the broadest range of antigen presentation, effectively linking innate recognition of invading pathogens to the generation of an appropriate type and strength of adaptive immune response [146]. Immature conventional DCs reside in essentially every tissue, where they act as sentinels for invading pathogens. During viral invasion, upon encounter with foreign antigens, pathogen associated molecules (PAMPs) are recognized by DC germline-encoded pattern recognition receptors (PRRs), triggering profound functional changes that culminate with the shift from antigen processing to presentation. Once
activation occurs, DC expression of the inflammatory chemokine receptors CCR1, CCR2, CCR5, CCR6 and CXCR1 are downregulated, whereas CCR7 is upregulated, allowing DCs to follow a CCL21 and CCL19 gradient towards the draining lymph node [147-152]. Upon arrival of activated DC, a rapid increase in lymph node size is observed as a consequence of increased influx mediated by chemokines and a decreased efflux mediated by the signaling of sphingolipid sphingosine-1-phosphate (S1P) [153,154]. Once in the lymph node, the activated DC completes its maturation, upregulating MHC and T cell co-stimulatory molecules such as CD40, B7.1 and B7.2 [146]. Mature DCs recruit CXCR3+CD62L+ NK cells to draining lymph node in a CXCL9 and CXCL10 dependent manner, where they prime and activate NK cells through cell-to-cell contact dependent signals and soluble mediators such as IL-15 (membrane-bound and soluble), IL-12 and type I IFNs. [146,155-157]. Activated NK cells produce inflammatory cytokines such as IFN-γ and TNF-α, and the crosstalk between DCs and NK cells influence the full maturation of both cell populations. HIV-1 infected patients show a reduced number of circulating DCs, and an inverse correlation between DC number and plasma viremia has been reported [158]. The decrease in DC is observed throughout HIV-1 infection, from the acute to the chronic phases [158,159]. There are several theories why DC numbers are reduced during HIV-1 infection. One theory is that DC are infected by HIV-1, however the evidence is conflicting [158,160,161], and even if they indeed are infected, the extremely low frequency would not account for the circulating DC decrease observed in HIV-1 patients. Another theory would be a reduction caused by indirect mechanisms, such as abnormal production of IFN-α observed in HIV-1 patients interfering with DC
differentiation from precursors [162], and increased levels of apoptosis compared to DCs from healthy individuals [163]. A more convincing explanation is that circulating DCs are redistributed and accumulate within the secondary lymphoid organs of patients with HIV [164-166]. Several reports have suggested that during HIV-1 infection, DCs that accumulate in the lymph nodes are not functional and present a semi-mature phenotype [121,167-169]. Semi-mature DCs are characterized by upregulation of costimulatory molecules in the absence of production of inflammatory cytokines, such as TNF-α, IL-6 and IL-12, which are responsible for induction of T cell responses and suppression of T regulatory cells [170,171]. Thus semi-mature DCs may play an important role in the defective innate and adaptive immune responses observed in HIV-1 patients, including failure to suppress T regulatory cells. The exact mechanisms that lead to DC impairment are still unclear.

4. **Natural Killer cells**

NK cells are important innate immune lymphocytes that display effector and regulatory functions. NK cells play a significant role against invading microorganism by directly killing infected cells without prior sensitization, in addition to shaping both the innate and adaptive immune response by the production of cytokines [156,172,173]. In healthy humans, NK cells make up about 10% of total blood lymphocytes, and can be divided into two heterogeneous populations that differ phenotypically and functionally. CD56\textsuperscript{hi} CD16\textsuperscript{low} CD25\textsuperscript{high} NK cells, account for approximately 10% of all circulating NK cells, produce high level of cytokines and are poorly cytotoxic. The
CD56<sup>dim</sup>CD16<sup>+</sup> population represents about 90% of circulating NK cells, produces lower levels of cytokines but display potent cytotoxicity due to constitutive expression of cytolytic granules and upregulation of CD16 [174]. This population is considered to be phenotypically and functionally mature. Indeed, the CD56<sup>bright</sup> population can undertake the CD56<sup>dim</sup> cytotoxic features in the presence of IL-2 and IL-12, or IL-15 by upregulating granzyme and perforin as well as CD16 [175], suggesting that the CD56<sup>bright</sup> population gives rise to a more differentiated NK cell population [176,177]. Interaction of NK cells and DCs influence the innate immune response and shape the type and strength of the adaptive immune response [174,178-181]. NK cells are recruited to draining lymph nodes through soluble factors produced by activated DCs as previously mentioned. Once they both co-localize, cross-talk between NK cells and DCs occurs through cell-to-cell contact and cytokine production. DC-derived IL-12 and IL-18 and membrane bound IL-15 promote optimal production of NK-derived IFN-γ, creating a positive feedback loop which sequentially triggers a Th1 immune response [147,182]. NK cell function is regulated by the balance between inhibitory and activating signals. Natural cytotoxicity receptors (NCR) such as NKp30, NKp44, and NKp46 and the stress ligand recognition receptor NKG2D have been identified as major activating receptors [183]. Inhibitory receptors (iNKRs) are classified as killer immunoglobulin-like receptors (KIRs) and immunoglobulin-like receptors, both are members of the immunoglobulin superfamily and are c-type lectin receptors [184]. The iNKRs recognize mainly, but not exclusively, the major histocompatibility complex (MHC) class I molecules.
NK cells expressing CD94/NKG2A receptor and lacking the killer Ig-like receptors (KIR) for self-HLA class I molecules are responsible for the NK cell mediated editing of DCs, where immature DCs are killed by activated NK cells while mature DCs are resistant to killing. DC editing is a very important NK cell effector function and assures not only the quality of mature DCs but also controls the intensity of the adaptive immune response, contributing to an adequate immune response against foreign antigens while preventing potential damage caused from an excessive immune response. In turn, DCs also modulate NK effector functions by means of cytokines as previously mentioned. The important role played by both DCs and NK cells in modulating the outcome of the adaptive immune response show how critical the integrity of this arm of the immune response is by protecting against infection, such as lentivirus and opportunistic infections, and preventing tissue damage by controlling the intensity and duration of the immune response.

There are several hypotheses as to how NK cell recognition of HIV-infected cells occurs. Indirect recognition after CD16 cross-linking and/or direct recognition mediated by a yet unknown receptor. NK cell responses to HIV peptides have been reported, but whether these responses are triggered by direct or indirect recognition is still unclear [185]. Several reports describing NK cell dysfunction in patients suffering from AIDS were published prior to the discovery and confirmation of HIV as the causative agent of AIDS. These groups showed that NK cells from AIDS patients with persistent opportunistic infections displayed a diminished cytotoxicity against target cells when compared to healthy controls [186-189]. NK cell defects are observed throughout the
course of HIV-1 infection, appearing soon after infection and continuing as disease progresses. During HIV-1 infection, overall circulating NK cell numbers do not change drastically, however a shift in NK cell phenotype is observed, with fewer CD56+ NK cells and increased numbers of anergic CD56- cells being observed [190,191]. Kottilil and colleagues observed an inverse correlation between viremia levels and suppression of HIV replication by NK cells [192], and there are several possible explanations for this phenomenon. Circulating NK cells in HIV-1 infected patients show a reduced cytotoxicity, and it could be caused by the decreased NK cell expression of granzyme and perforin observed in patients [193]. In addition, abnormal expression of numerous activating and inhibitory NK cell receptors have been shown and may be a consequence of persistent viremia and chronic immune stimulation. De Maria and colleagues [194] reported an association between reduced in vitro cytotoxicity and a decreased expression of NCRs by NK cells from HIV-1 patients. In contrast, a study has shown that upregulation of NKp44 during HIV-1 infection directs NK cell cytotoxicity against CD4+ T cells, contributing to the loss of uninfected CD4+ T cells observed in HIV-1 patients [195]. It is also believed that chronic immune stimulation leads to incomplete NK cell activation, culminating in NK cell exhaustion and anergy [196]. HIV-1 immunopathogenesis leads to immunossupression and consequently patients become highly susceptible to opportunistic infections. Finally, a small population of NK cells expressing both CD4+ and chemokine receptors were shown to be infected in vitro, suggesting that this cell population could work as a viral reservoir [197,198]. However, this is very unlikely to explain the broad effects of HIV-1 virus on NK cell function.
Despite the initial correlation of NK cell dysfunction with the onset of AIDS, and confirmation that HIV-1 infection profoundly affects NK cells from the very initial stages of infection, the original interest in this innate immune cell population dissipated for nearly two decades. Interest in studying NK cells during HIV-1 infection was reinvigorated after two critical breakthroughs. First was the understanding that innate immunity plays a major role on the outcome of the adaptive immune response, as previously mentioned. Second was the indisputable association of KIRs and HLA molecules with the outcome of HIV-1 infection and disease shown by genetic and functional studies. The link between expression of KIR and HLA molecules by NK cells and HIV-1 disease outcome was first described by Martin and colleagues [199]. They showed that HIV-1 infected patients considered slow progressors expressed a particular combination of KIR3DS1 with HLA-Bw4-80I alleles, whereas patients expressing only one or neither of these molecules showed a rapid immune deterioration and progression to AIDS. Subsequent studies supported the initial findings, showing that NK cells expressing this specific KIR/HLA combination not only increase during HIV-1 infection, but also efficiently suppress viral replication in vitro [200,201]. Boulet and colleagues [202,203] have also shown that the combination of KIR3DS1 with HLA-Bw4-80I alleles influences the resistance to HIV-1 infection. They showed that the percentage of this specific KIR/HLA in HIV-1 highly exposed uninfected individuals was much higher than in the HIV-1 infected patients. The mechanisms by which these molecules influence both infection and disease progression remains unclear, and there is no evidence of a direct interaction between KIR3DS1 and HLA-Bw4-80I [204,205].
Despite the advances in the past decades, the mechanisms underlying NK cell dysfunction as well as protection against HIV-1 infection are not completely understood, and in vivo data is lacking. Because in vivo studies with human subjects are not feasible, the use of animal models is necessary in order to address these questions. Our laboratory has shown that FIV-infected cats are more susceptible to the opportunistic bacterium *Listeria monocytogenes*, as evidenced by a delayed clearance of the infection within the draining lymph node [111]. Furthermore, when treated with an array of cytokines locally delivered to the draining lymph node, IL-15 was the only one that significantly increased bacterial control in infected cats, and this was associated with an increase in NK cell numbers [116]. This is in concordance with a study where in vitro stimulation with IL-15 restored the function and proliferative activity of NK cells from patients with HIV or HCV [206]. These studies suggest that NK cell function may be restored, and the defect could be, in part, due to inefficient stimulation from semi-immature DCs as well as suppression by the expanded regulatory T cells.

C. T regulatory cells

1. Overview

The concept that T cells could suppress the immune response was first proposed in 1970 by R.K. Gershon [207]. In this early publication, the authors demonstrated that populations of T cells, named suppressor T cells, were capable of inhibiting both cytotoxic T cell responses as well as antibody production by B cells. During the following decade it was believed that suppression by T cells was mediated by antigen-
specific soluble factors that contained I-J determinants [208] localized within the murine MHC [209-212]. However, this hypothesis was proved wrong after the cloning and sequencing of T cell receptors failed to identify an I-J gene within the MHC [213]. The subsequent description of T helper 1 (Th1) and T helper 2 (Th2) populations in mice [214,215] and humans [216] led researchers to conclude that suppression was a phenomenon of the activity of cytokines and the concept of suppressor T cells was abandoned. The comment by Green and Webb [217] summarizes the general feeling that lasted for the subsequent decade: “There is little doubt that the ‘S’ word (suppression, as in ‘suppressor T cells’) is the nearest thing to a dirty word we have in cellular immunology. Its use is considered by some (not all) to be synonymous with over interpretation of scanty data and phenomenology (the ‘P’ world) bordering on the mystical”.

Researchers started to reconsider the existence of a T cell subpopulation capable of suppressing the immune responses after studying several mouse models of autoimmune diseases. It was observed that autoreactive T cells frequently escaped thymic depletion and once in the periphery these cells could have 2 fates: remain non-responsive for long periods of time, or become activated thus triggering autoimmunity. So the pursuit for the T suppressor cell restarted, and several studies attempted to unveil this cell population by depleting various subpopulations of T cells. Finally, 25 years after R.K. Gershon first hypothesized the existence of suppressor T cells, Sakaguchi and colleagues [218] described a subset of CD4+ T cells that constitutively express the α-chain of the IL-2 receptor (CD4+CD25+ cells) as responsible for peripheral tolerance. In
this study they inoculated BALB/c athymic nude mice with CD25+ depleted CD4+ cells suspension from BALB/c nu/+ mice. BALB/c nude mice that received the CD4+CD25- transplant developed autoimmune disease, while reconstitution with CD4+CD25+ cells prevented development of autoimmune disease in a dose-dependent manner. Since the identification of the CD4+CD25+T cell population, researchers renewed their interest in the suppressor T cells, now renamed as T regulatory cells (Treg). Shortly after Sakaguchi’s description of murine Treg cells, the human counterpart was described [219,220]

Because Treg and T helper cells share several surface markers, such as CD25, B7, CTL-4 and GITR, phenotyping Treg cells can be challenging. The high affinity IL-2 receptor alpha (CD25) is constitutively expressed by Treg cells and is essential for their regulatory function [221,222]. During the course of an inflammatory disease, Treg cells are stimulated by IL-2 produced by activated lymphocytes and are then capable of damping the inflammatory response in a negative feedback loop [223,224].

The most studied T regulatory cells are: CD4+CD25+ T cells, called natural Treg cells; and pathogen-induced, also called adaptative, Treg cells. The natural Treg cell population was the first to be described, they originate in the thymus and play a very important role in maintaining peripheral self tolerance [223]. Activation of natural CD4+CD25+ Treg cells occurs through the interaction between self peptides and MHC, and once activated natural Treg cells suppress nearby T and B cells in a non-specific way [225,226]. Adaptive CD4+CD25+ Treg cells can be derived from either CD4+CD25- or CD4+CD25+ T [227-229], and are very important in modulating the immune response to
pathogenic microorganisms such as viruses, bacteria, fungi, and intracellular parasites, preventing excessive inflammation that could be deleterious to the host [230]. Because both natural and adaptive Treg cells are phenotypically and functionally very similar, it is not possible to readily differentiate these two populations.

The intracellular forkhead or winged helix family transcription factor (FOXP3) is the best characterized marker of regulatory T cells. Chatila and colleagues first described a mutation in the Foxp3 gene as the cause of the human genetic disease immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) [231]. This disease is caused by generalized immune dysregulation and autoimmunity. Soon after another group described a mutation in the gene Foxp3 as the cause of disease in the mutant mouse strain scurfy [232], characterized by cell mediated lymphoproliferative disease in a similar way to IPEX in humans. A critical link was established between FOXP3 and Treg cells when researchers studying scurfy mice found a defect within the CD4⁺CD25⁺ compartment. That FOXP3 is specifically expressed in Treg cells and are required for Treg development and function, has been demonstrated by different groups independently. Fontenot and colleagues [233] used the mutant mouse strain scurfy in their studies, while Khattri and colleagues [234] developed a new mouse strain Foxp3-deleted (Foxp3⁻/⁻), which causes the same syndrome as observed in the scurfy mice. Both groups demonstrated the absence of Treg cells, and despite the presence of CD25⁺ cells, these CD4⁺CD25⁺ cells were able to proliferate and did not suppress immune response in vitro. In addition, Fontenot and colleagues were able to prevent disease by transferring CD4⁺CD25⁺ cells into neonatal scurfy mice, indicating that FOXP3 is required for
functional Treg cells. FOXP3 expression occurs at later stages of Treg differentiation and involves signaling through IL-2 and TGF-β receptors [235]. TGF-β is a critical mediator used by Treg cells to suppress CD4+ and CD8+ effector T cells [236,237]. FOXP3 then interacts with NFAT and both associate with the IL-2 promoter region [235]. It has also been demonstrated that FOXP3 upregulates the expression of Treg-associated surface genes by binding to the high affinity interleukin 2 receptor (CD25), Cytotoxic T Lymphocyte Antigen-4 (CTL-4) and Glucocorticoid-induced TNF Receptor (GITR) promoter regions [238]. Binding of Treg-associated molecules on CD4+CD25+FOXP3+ cells with their cognate receptors on CD4+ T effector cells downregulates IL-2 production and inhibits proliferation in effector cells [239], thus helping Treg regulate the immune response intensity and prevent autoimmunity [240,241]. More recently, Yamaguchi and colleagues [242] identified the Folate Receptor 4 (FR4) as a specific marker of murine Treg cells, but a definitive marker of human and other model species are still not identified. The majority of the studies that characterized the role of FOXP3 Treg cell development and function were carried out using mouse models. It is important to mention that significant differences have been reported in humans and another animal models. Several studies have questioned if all Treg cells are FOXP3+ or all FOXP3+ are Treg cells [reviewed in [243]]. FOXP3 expression in both humans and cats are not limited to Treg cells [244-246]. In humans, FOXP3 can also be transiently expressed at low levels by conventional CD4+ T cell during activation without causing any level of suppression [245-247]. Treg cells also
express cytotoxic granules and are capable of promoting suppression by deleting T effector cells and B lymphocytes in a perforin/granzyme manner [248-250]

2. **Treg cells in Lentiviruses infection**

As previously mentioned, the most relevant alteration present during lentiviral (HIV/SIV/FIV) infection is the lack of an effective immune response against not only the virus but also to opportunistic pathogens, as well as a generalized immune activation. To date, the exact mechanisms that lead to these alterations are still unknown. The renewed interest in Treg cells and the identification of their role in other infections led researchers to investigate a possible role played by regulatory cells in HIV-induced immune dysfunction. If Treg cells play a detrimental role in HIV infection by suppressing T cell response allowing viral persistence, or if they play a beneficial role by controlling the already heightened immune activation remains unclear despite the extensive body of literature published to date. The reality is probably some place in between, as these two roles are nonexclusive and may occur during different stages of viral infection.

Several groups have published data that corroborates he deleterious role of Treg cells in HIV pathogenesis. Weiss and colleagues [251] observed an increase in circulating Treg cells in HIV-infected patients, and showed that these cells were fully functional, capable of suppressing T cell responses to HIV antigens as well as secreting IL-10 and TGF-β. Another group showed that despite the fact that Treg cell frequency was not altered, HIV-specific T cells from untreated HIV patients failed to respond to HIV antigens, and anti-HIV responses were enhanced after Treg cell depletion [252].
Tsunemi and colleagues [253] found an increased level of Treg cells in non-treated viremic HIV patients compared to both healthy donors and HIV-infected patients with undetectable plasma viremia. In both HIV-infected groups a negative correlation with Th1 response and a positive correlation with Th2 response was observed, and a negative correlation between Treg cell numbers and CD4$^+$ T cell counts was observed within the viremic cohort, suggesting that increased Treg frequencies in PBMC indicates a polarization towards Th2 immune response and loss of CD4$^+$ T cells. Nielsson and colleagues [254] compared HIV-infected patients and SIV-infected macaques with progressive and non-progressive disease. They reported an increased number of Treg cells and a reduction of cytokine secretion by CD8$^+$ T cells in the lymphoid tissue of progressors when compared to non-progressors and these finding were not correlated with immune activation. *In vitro* studies have suggested that exposure of human Treg cells to HIV not only upregulates FOXP3 expression by these cells [255], but also upregulates expression of CCR5, increasing susceptibility to HIV infection [256] and promoting survival signals that inhibit apoptosis, thus leading to a selective expansion of Treg cells [254]. Treg cells have also been identified studied in the FIV/cat model, with several studies published by the Tompkins group [257,258,259]. They showed that Treg cells freshly isolated from lymph nodes of acutely and chronically FIV-infected cats upregulated the expression of activation molecules and were capable of suppressing CD4$^+$ T helper cell proliferation and cytokine production, indicating that Treg cells are activated *in vivo* during FIV infection [257,258]. In another study they reported
suppression of CD8⁺ T cell immune response by Treg cells during both the acute and chronic stages of FIV infection [259].

Contradicting the detrimental effect of Treg cells during lentiviral infection, several studies have suggested that Treg cells play a protective role during lentiviral infection by limiting the activation of T cells, preventing T cell dysfunction and depletion. Oswald-Ritcher and colleagues [256] showed a decrease in circulating Treg cell number in HIV-infected patients compared to healthy individuals, supporting a negative correlation between Treg number and T cell activation and a positive correlation between Treg cell number and CD4⁺ T cell count, which suggests that loss of Treg cells could potentially contribute to a heightened immune activation, CD4⁺ T cell loss and consequently progression to AIDS. Another cohort study showed a decrease in Treg cell numbers in untreated HIV-infected patients, which strongly correlated with T cell activation [260]. Because the correlation between Treg cells and activation was stronger than with either viremia or CD4⁺ loss, the authors suggested that loss of Treg cells is likely responsible for the heightened immune activation observed in untreated patients.

Kinter and colleagues showed an increase in Treg cells in HIV patients compared to healthy controls, and these cells were able to suppress T cell proliferation as well as cytokine production. They also observed that HIV-infected patients with the strongest Treg immunosuppressive activity in vitro had the lowest levels of plasma viremia and higher CD4⁺ T count, while the opposite was observed in patients with weak Treg activity which was associated with more advanced disease. Mikkelsen and colleagues [261] have shown an improved antiviral T cell response in chronic FIV-infected cats after
Treg cell depletion, suggesting suppression of FIV-specific T cell response by these cells. Importantly, depletion did not result in increased viral load or increasing inflammatory cytokine production. In addition, depleted animals were capable of mounting a robust humoral response against a new antigen, leading the authors to hypothesize that a Treg cell depletion strategy for therapeutic vaccination could facilitate induction of novel immune responses during HIV infection.

Based on all the contradictory work published to date, it remains unclear whether the detrimental role or a beneficial role of Treg cells prevail during the course of lentiviral infection. It seems that Treg cell suppression reaches its maximum during early infection and slowly dissipates as disease progresses. This is supported by the observation that after depletion, Treg cells rebound faster in acutely FIV-infected cats [262] than in chronically infected cats [261].

3. Role of Treg Cells in Innate Immune Response

Dendritic cells were the first innate immune cell to be shown to communicate with Treg cells. Researchers demonstrated that *in vitro* coculture of Treg cell and DC led to suppression of DC and this culminated with a tolerogenic immune response [263-265]. DC suppression by Treg cells was mediated by a CTLA-4-dependent mechanism [265,266] that seems to involve binding to B7 molecules on DC [266-269]. Treg cells downregulated expression of costimulatory molecules and rendered DC incompetent to present antigens [263,264]. In addition, studies have suggested that DC play a role in Treg cell expansion [270,271].
Shimizu and colleagues [272] suggested for the first time that NK cell effector functions could be suppressed by Treg cells. They showed that normal splenocytes depleted of Treg cells were capable of eliciting an effective immune response against syngeneic tumors in a Balb/c nude mice model for leukemia, while transfer of splenocytes not depleted of Treg cell had no effect on tumor control. They attributed the lytic function to NK cells activated by IL-2 expressed by CD4+ effector T cells in the absence of Treg cells. In a seminal paper by Guiringhelli and colleagues [273], the direct effects of Treg cells on human blood NK cells (CD3−CD56+ cells) was shown. The authors initially showed an inverse correlation between NK cells and Treg cells in cancer patients. Then, using ex vivo experiments, they demonstrated that both NK cell cytotoxicity and IFN-γ production is suppressed by Treg cells in a contact-dependent manner mediated by membrane bound TGF-β. Similar findings were shown with murine Treg/NK cell experiments, where Treg cells suppressed NK cell-dependent killing and this effect was also mediated by TGF-β [274]. There is evidence that Treg cell suppression of NK cells by membrane-bound TGF-β occurs through inhibition of NKG2D expression in both human and mouse NK cells [273,274]. Human NK cell cytotoxicity against K562 cells was blocked by Treg cells and restored after addition of anti-TGF-β monoclonal antibodies. In mice, Treg cells were capable of specifically inhibiting NKG2D-dependent killing, and adoptive transfer of TGF-β-defective Treg cells did not result in NK cell inhibition. These studies suggest a common mechanism involving NKG2D and TGF-β by which Treg cells regulate NK cell function both in vivo and in vitro.
There is also evidence that Treg cells are involved in the control of NK cells in a steady state. Scurfy mice display abnormal NK cell proliferation compared to control animals, and this is attributed to a lack of functional Treg cells [273]. Similar results were observed in mice treated with anti-CD25 antibodies and cyclophosphamide during transient Treg depletion [275,276]. Evidence that NK cells accumulate in peripheral lymphoid tissue in the absence of Treg cells was reported by Kim and colleagues [277]. Using FOXP3-DTR mice, they reported a several fold increase in NK cell numbers in lymph nodes and spleen of Treg-depleted mice compared to control animals. Giroux and colleagues [278] suggested that under steady state Treg cells, through a short interaction with NK cells, prevent the generation of mature NK cells in the lymph node. Another group found the increased number of NK cells in lymph nodes of mice with either a reduction or complete depletion of their Treg cells was a result of increased proliferation as measured by BrdU incorporation [275,276]. In this study it was suggested that Treg cells interfere with DC/NK crosstalk, demonstrating that NK cells were activated by IL-15-expressing DC, as no proliferation was observed in IL-15Rα-/- mice or DC-depleted mice, and this was inhibited in the presence of Treg cells [275,276]. So it seems plausible that Treg cells not only control NK cell functions after stimulation, but also the NK/DC cross talk in a steady state. Lund and colleagues [279] showed that Treg cells also play a role in controlling infection. Using the FOXP3-DTR mouse model, they showed that after vaginal herpes simplex virus infection both Treg and T helper cells accumulated in the draining lymph nodes as well as at the site of infection. Treg depletion led to a faster progression of disease compared to non-depleted mice, implying
that Treg cells play a role in controlling infection. They also showed that in the absence of Treg cells, NK cell, DC, and T helper cell numbers were reduced in the vaginal tract but increased in the draining lymph nodes. An increase in proinflammatory cytokines was also observed in the draining lymph nodes of Treg-depleted animals. They concluded that Treg cells help regulate chemokine production at the site of infection and facilitate homing of immune cells, enhancing mucosal protection.

D. Listeria monocytogenes as an innate immune probe

The Gram-positive facultative intracellular bacterium Listeria monocytogenes was isolated by several groups during the 1920s from a wide range of animal species [280-283]. The pivotal role played by cellular immunity in controlling L. monocytogenes infection was first demonstrated by Mackaness and colleagues in the early 1960s [284], and shortly this intracellular pathogen was recognized as a powerful tool to study both innate and adaptive immunity. Since then, the use of L. monocytogenes infection has been key to the unveiling of several fundamental paradigms in the immune response, while also helping to elucidate the molecular mechanisms involved in host-pathogen interactions. Macrophages are the main cells infected by L. monocytogenes, however fibroblasts, hepatocytes, epithelial, endothelial and dendritic cells have been reported to be infected [285-289]. After L. monocytogenes adhesion and invasion into the host cells, they escape from the phagosome to the cytosol by disrupting the phagosomal membranes through secretion of a cytolytic, pore forming protein, the virulence factor listeriolysin O (LLO) [289-291]. Upon entry into the cytoplasm bacteria thrive and replicate in the host cells cytosol. Interestingly, passage
through the phagosomal compartment appears to be critical for the expression of genes involved in cytolitic replication based on the observation that direct microinjection of bacteria into the cytosol results in defective bacterial replication [292,293].

*Listeria monocytogenes* triggers a multifactorial immune response that involves both the innate and adaptive immune compartments, involving macrophages, neutrophils, dendritic and natural killer cells, CD4+ and CD8+ T cells, and a number of cytokines [294,295]. Innate immune responses are triggered after initial infection and are essential for bacterial clearance and host survival [296], and immediately after cytosol invasion, transcription of NK-κB and IFN regulatory factor 3 (IRF3), culminating in immune activation and cytokine production [297]. Several cytokines are necessary for an effective immune response against *L. monocytogenes*. After bacterial entry, expression of chemokines and pro-inflammatory cytokines, such as IL-1, IL-6, IL-12, IL-18, KC/MIP-2, and TNF-α, are upregulated and are fundamental in the initiation of an effective innate immune response [297,298]. These inflammatory cytokines and chemokines recruit neutrophils and NK cells, and are involved in priming IFN-γ production by NK cells. IFN-γ plays a pivotal role during the innate immune response as it mediates macrophage activation, upregulates costimulatory molecules involved in activation of adaptive immune response, induces production of IL-12, and suppresses the immunesuppressive cytokine, IL-10 [286,299-301].

Studies of the mouse-*Listeria* model employing nude mice and severe combined immunodeficiency (SCID) mice showed the efficiency of the initial innate immune response to *L. monocytogenes* [302,303]. Despite the absence of T and B cells, mice infected with *L. monocytogenes* show a significant resistance against the initial stages of
infection, although they are unable to completely clear infection. Early control of infection is attributed to IFN-γ production by NK cells and subsequent macrophage activation [304]. Both IFN-γ and TNF-α are key cytokines during the initial response against *L. monocytogenes*, and mice lacking these cytokines and/or their receptors deteriorate rapidly [286,299-301,305-307].

The fact that both the microbiological aspects and the innate immune response mounted against *L. monocytogenes* are extremely well characterized makes this a very attractive model to study the interactions between the immune system and an opportunistic intracellular pathogen.

E. References


In Vivo Assessment of Natural Killer Cell Responses during Chronic Feline Immunodeficiency Virus Infection

Published in the PLoS ONE Journal

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A. Abstract

Accumulating evidence suggests that natural killer (NK) cells may have an important role in HIV-1 disease pathogenesis; however, in vivo studies are lacking. Feline immunodeficiency virus (FIV) infection of cats provides a valuable model to study NK cell function in vivo. The immune response against *Listeria monocytogenes* (Lm) is well characterized, allowing its use as an innate immune probe. We have previously shown that locally delivered IL-15 can improve Lm clearance in FIV-infected animals, and this correlated with an increase in NK cell number. In the present study, chronically FIV-infected and SPF-control cats were challenged with Lm by unilateral subcutaneous injection next to the footpad and then treated with 5-bromo-2’-deoxyuridine (BrdU). The Lm draining and contralateral control lymph nodes were evaluated for NK, NKT, CD4+ and CD8+ T cell number, proliferation, apoptosis, and NK cell function. *Listeria monocytogenes* burden was also assessed in both control and Lm draining lymph nodes. NK, NKT, CD4+ T and CD8+ T cells in the Lm-challenged lymph node of FIV-infected cats did not increase in number. In addition, after Lm challenge, NK cells from FIV-infected cats did not increase their proliferation rate, apoptosis was elevated, and perforin expression was not upregulated when compared to SPF-control cats. The failure of the NK cell response against Lm challenge in the draining lymph node of FIV-infected cats correlates with the delayed control and clearance of this opportunistic bacterial pathogen.
B. Introduction

Natural killer (NK) cells are part of the innate immune compartment and are considered the first line of defense against obligate intracellular pathogens and transformed cells. Recent studies have shown the importance of NK cells as a bridge between innate and adaptive immune responses, and that in collaboration with other innate immune cells they help modulate the type and strength of the adaptive immune response (reviewed in [1]). Several studies have suggested the NK cell response during the course of HIV-1 infection is compromised. Significant abnormalities in NK cell phenotype, function and number have been reported during HIV-1 infection [2,3]. Mechanisms have been proposed to explain the NK cell defect in HIV-1 infection, including reduction of T cell-derived IL-2, induction of apoptosis, and modulation of MHC class I receptors by NK cells [4,5]. Furthermore, the importance of NK cells in HIV-1 infection has been corroborated by studies showing that certain combinations of killer immunoglobulin-like receptors (KIR) and MHC class I molecules correlate with a slower HIV-1 disease progression [6], while HIV-1 exposed healthy subjects show enhanced NK cell function [7]. Although there is convincing evidence supporting the importance of NK cells during the course of HIV-1 infection, the exact mechanisms underlying NK cell dysfunction are unknown. Since investigating the dynamics of the NK cell response in lymph nodes (LN) of HIV-infected or healthy people in response to a microorganism challenge is not feasible, we used the feline immunodeficiency virus (FIV) model to study HIV/AIDS. FIV infection of cats is clinically and immunologically similar to HIV-1 in people [8-10], providing a valuable animal model to investigate the consequences of lentivirus infection on the innate immune response. Because the innate
immune response to *Listeria monocytogenes* (Lm) is well understood (reviewed in [11]), we used this intracellular pathogen to probe the innate immune system in order to investigate the effects of chronic FIV infection on NK cell function. We previously reported that FIV-infected cats have an impaired innate response that fails to gain initial control of bacterial replication prior to the adaptive immune response [12]. We also demonstrated that locally delivered IL-15, a cytokine known to activate and stimulate NK cell proliferation, cytolytic activity, and cytokine and chemokine production, significantly restored innate immune function as measured by Lm clearance [13]. Here, we show that compared to SPF-control cats, NK cells from chronically FIV-infected cats have a constitutively higher level of proliferation that is counter-balanced by increased apoptosis. Upon challenge with Lm, NK cells of FIV-infected cats fail to traffic to lymph nodes, have a lower proliferative response, and show a minimal increase in perforin expression.

C. Results

**Innate immune control of Lm is impaired in chronically FIV-infected cats**

We have previously shown that chronically and acutely FIV-infected cats have an impaired innate immune response to the intracellular pathogen Lm [12,13]. Here we showed that 3 days post-Lm challenge, chronically FIV-infected animals had a greater number of Lm colony-forming units per LN than SPF-control cats (64,280 ± 31,253; 5,318 ± 3,878 CFU/LN respectively, mean ± SEM). No bacterial colonies were recovered from the contralateral control LN regardless of FIV status (data not shown). Plasma viremia from chronic FIV-infected cats ranged from 471 to 5121 copies/mL, and FIV proviral load ranged between 245
and 7345 per 1 x 10^6 PBMC (data not shown). These results confirmed that the innate immune response against Lm in cats with chronic FIV infection is diminished compared to SPF-control animals.

**Chronic FIV infection is associated with decreased NK, NKT, CD4^+ T and CD8^+ T cell numbers in Lm challenged LN**

We previously reported that FIV-infected cats had a delayed and blunted enlargement and follicle formation in draining LN after Lm challenge as compared to SPF-control cats [12,13]. To further investigate this, LN were removed on day 3 after Lm challenge and the total number of cells was determined. Lm-challenged LN from SPF-control cats contained a significantly greater number of cells as compared to control LN (p < 0.01), but no such difference was observed in chronic FIV-infected cats (Figure 1A). These results show a quantitative difference in cellular response to Lm challenge by FIV-infected versus SPF-control cats.

To further characterize the reduced number of lymphocytes in the Lm draining lymph nodes of FIV-infected cats we determined the total cell number of NK cells (CD3^-CD56^+), NKT cells (CD3^+CD56^+), CD4^+ T cells (CD3^+CD4^+), CD8^+ T cells (CD3^+CD8^+), regulatory T cells (CD4^+CD25^+FOXP3^+) and Langerhans cells (CD1a^+) in LN of FIV-infected and SPF-control cats by flow cytometry. No differences in total NK cell numbers were observed between SPF-control and FIV-infected cats in control LN (Figure 1B). However, while SPF-control cats showed a 10-fold increase in NK cell number from Lm-challenged LN compared to control nodes, no such increase was observed between LN of FIV-infected animals (Figure
This suggests FIV infection may affect NK cell recruitment, death and/or proliferation. SPF-control cats had a significant increase ($p < 0.05$) in NKT, CD4$^+$ and CD8$^+$ T cell numbers in Lm-challenged nodes as compared to control LN (Figures 1C, D, E). Again, no such differences were seen between the nodes from FIV-infected cats. Interestingly, there were no differences in Langerhans cell number between challenged and control nodes or between FIV-infected and SPF-control cats (Figure 1F). We also found no difference in CD4$^+$CD25$^+$FOXP3$^+$ cell number between LN, independent of the FIV status (data not shown). The percentages of the different cell populations observed within the control and Lm-challenged lymph nodes of both SPF-control and FIV-infected cats are shown in Figure S1A-E. While NK (Figure S1A) and CD8$^+$ T cells (Figure S1D) cells showed a similar trend as seen with the absolute number of cell results, no differences were observed between the other cell populations analyzed. Flow cytometric gating strategies and representative plots are shown in Figures 2A and 2B, respectively.

Both FIV-infected and SPF-control cats were challenged subcutaneously with Lm and whole blood was collected at day 3 after challenge at the same time as LN collection. Circulating CD4$^+$ T cell number was significantly lower in FIV-infected cats compared to SPF-control cats (Figure 3A). No differences were seen between circulating CD8$^+$ T cell or total lymphocyte numbers (Figure 3B-C), whereas NK cell numbers in the blood compartment were lower in FIV-infected cats but did not reach significance ($p = 0.1$, Figure 3D). Percentagewise, SPF-control cats had twice as many circulating CD4$^+$ T cells when compared to FIV-infected animals (Figure S2A) while FIV-infected cats had 1.5 more CD8$^+$
T cells (Figure S2B) than SPF-control cats, and no differences were observed in circulating NK cells between both groups of cats (Figure S2C).

**NK cell proliferation differs depending on the compartment analyzed**

Increased lymphocyte proliferation and turnover of T, B, and NK cells is characteristic of pathogenic lentivirus infection [14,15]. We asked whether the proliferation rate of lymphocyte subpopulations in FIV-infected cats would increase in response to an immune challenge. Proliferation of NK cells, and CD4+ and CD8+ T cells, was measured by the in vivo incorporation of BrdU [16] as well as intranuclear Ki-67 expression [17].

Cell proliferation was assessed in control and challenged LN, as well as in PBMC. In FIV-infected cats, a higher number of NK cells incorporated BrdU and expressed Ki-67 in control and challenged LN as compared to LN from SPF-control cats (Figure 4A). However, SPF-control animals had a significantly greater increase in NK cell proliferation in response to Lm when compared to the contralateral control LN (p < 0.01), while no significant difference in response to Lm was observed between the nodes of FIV-infected cats (Figure 4A). CD4+ T cell proliferation, as indicated by either BrdU incorporation or Ki-67 expression, was increased in the control but not the challenged LN of FIV-infected cats as compared to SPF-control cats (Figure 4B). In contrast to the NK cell response, CD4+ T cell proliferation in response to Lm was increased in both SPF-control and FIV-infected cats (Figure 4B). Similar results were observed with the CD8+ T cell population (Figure 4C). There was a significant difference in total lymphocyte proliferation between FIV-infected and SPF-control cats in control and challenged nodes (p < 0.01, Figure 4D). Total
lymphocyte proliferation was significantly increased (4 and 6 folds, Ki67 and BrdU, respectively) in challenged nodes of SPF-control cats compared to control nodes while no such difference was observed between lymph nodes of FIV-infected cat (Figure 4D). Similar results were observed regarding the percentage of proliferation for the different cell populations analyzed (Figure S3).

Peripheral blood was collected 3 days after Lm challenge from both SPF-control and FIV-infected cats. In the blood compartment, 2 times more NK cells from SPF-control cats incorporated BrdU than in FIV-infected cats (Figure 5A). CD4$^+$ T cell proliferation did not differ between FIV-infected and SPF-control animals (Figure 5B), while CD8$^+$ T cell and total lymphocyte proliferation were significantly increased (2 and 6 fold, respectively) in FIV-infected cats (Figures 5C and 5D). Similar results were seen when the percentage of proliferation within the circulating cells was analyzed (Figure S4).

**Lymphocytes from FIV-infected cats undergo apoptosis at a higher rate**

To reconcile the overall trend of lower lymphocyte numbers in the face of greater proliferation, we investigated whether cells were undergoing apoptosis as indicated by AnnexinV binding. FIV-infected cats had a higher number of apoptotic NK cells in the Lm-challenged LN as compared to SPF-control cats; however no difference was observed between the control nodes (Figure 6A). There was a trend that did not reach statistical significance of more CD4$^+$ T cells undergoing apoptosis in control and challenged LN from FIV-infected cats (Figure 6B). CD8$^+$ T cells apoptosis was greater in control and challenged LN (7 and 10 fold respectively) of FIV-infected as compared to SPF-control cats (Figure
Similarly, the apoptotic rate of the total lymphocyte population was 3.5 fold higher in FIV-infected than in SPF-control cats in both control and Lm-challenged LN (Figure 6D). Percentagewise, a similar trend seen in absolute number of cells was observed (Figure S5A-C), with the exception of AnnexinV+ within the total lymphocyte population from CLN and Lm-LN (Figure S5D), where no differences were observed between SPF-control and FIV-infected animals. Interestingly, in the blood compartment, no differences in apoptosis were observed for any cell type investigated (data not shown).

**Fewer NK cells from FIV-infected cats produce perforin.**

As an indicator of NK cell functionality, we determined the number of NK cells expressing perforin, IFN-γ and granzyme A after ex vivo stimulation with IL-2. While a significantly higher number of Lm-challenged NK cells from SPF-control cats produced perforin after in vitro stimulation compared to FIV-infected cats (Figure 7A), we found no differences between FIV-infected and SPF-control cats with regard to expression of IFN-γ (Figure 7B) or granzyme A (Figure 7C).

**D. Discussion**

The effect of HIV-1 infection on NK cells and the role of NK cells in HIV-1 control and immunopathogenesis have been the focus of increasing interest. Several findings point to a central role of NK cells in protection against HIV-1 and disease progression [6,7,18], and evidence suggests this cell population can be profoundly impaired during all the stages of HIV-1 infection in humans [2,3,19]. However, the mechanisms of NK cell dysfunction
observed in HIV-1 patients are not fully understood and the impact on the overall immune response in vivo is unknown. In this study, using the FIV/cat model of HIV infection, we demonstrated that in vivo NK cell number, proliferation and apoptosis are abnormal in FIV-infected cats in response to challenge with the intracellular pathogen, *Listeria monocytogenes*.

We have previously shown that the immune defect in FIV-infected cats in response to Lm infection occurs during the first four days after challenge and is characterized by a delayed cellular response in the draining lymph node and lack of control of bacterial growth [13]. However, FIV-infected cats are eventually able to clear the infection, presumably due to the T cell response, suggesting a defective innate immune response against Lm. We also have demonstrated that local delivery of IL-15 rescues the early response of FIV-infected cats to Lm, which correlates with increased NK cells in the lymph node. The biological activities of IL-15 include NK cell activation, proliferation, and increased cell survival [20].

*Listeria* infection is known to induce a multifactorial immune response that initially controls and eventually eliminates the bacterium. Early bacterial control does not seem to rely on T cells, since mice with severe combined immunodeficiency (SCID) control the initial infection as effectively as normal animals, yet ultimately fail to clear infection [21]. NK cells are thought to be an important component of the innate immune response to Lm through their production of IFN-γ induced by IL-12 and IL-18 [22]. Recently, athymic nude rats lacking T cells but with functional NK cells were shown capable of early control while rats depleted of NK cells were not [23]. Similarly, a previous study demonstrated that NK cell depletion led to a higher Lm burden in the draining lymph nodes after mice were
subcutaneously inoculated [24]. Some studies have questioned the role played by NK cells during Lm infection. Teixeira and colleagues [25] showed that depletion of NK1+ cells in B6 mice led to a decreased Lm burden in the spleen, and soon after Takada and colleagues [26] confirmed this finding and suggested that γδ T cells play a role in the improved clearance of Lm infection. Despite the conflicting data demonstrating the importance of NK cells on the initial control of infection, it is a consensus that after Lm entry, NK cells are recruited from the blood to spleen and liver, and/or lymph nodes (depending on the route of inoculation), and an increase in NK cell activity during the first days of Lm infection is observed.

Challenge of SPF-control cats with Lm resulted in a 4-fold increase in draining lymph node cellularity characterized by significant increases in NK cells, CD4+ T cells and CD8+ T cells. Lymphocytes are recruited to the draining lymph node upon arrival of activated dendritic cells (DC) that produce chemokines, and this combined with a decrease in the efferent flow of lymph results in an increased cell number within the node [27-29]. DC also play a pivotal role in stimulating NK cell function and proliferation by means of cell-to-cell contact and cytokine production, including IL-15 and IL-12 [30]. The crosstalk between NK cells and DC following pathogen invasion is a key event in the induction of innate and adaptive immune responses [31]. One of the sites of DC-NK cell interaction is the secondary lymphoid tissue where activated NK cells produce cytokines, such as IFN-γ, that prime DC to produce IL-12 that in turn promotes further NK cell maturation as well as induction of CTL response [32,33]. We evaluated the Langerhans cell (LC) population and found no quantitative difference between FIV-infected and SPF-control cats, suggesting that the defect in cell number is not due to reduced numbers of LC migrating to the draining LN. As we did
not evaluate LC activation status or other DC populations, we cannot exclude the possibility that LC/DC dysfunction might play a role. It has been shown that plasmacytoid and myeloid DC are reduced in the LN of HIV-1 patients [3]. Another possible explanation is the altered expression of homing markers. Previous work showed NK cells from SIV-infected macaques upregulate the expression of the gut-homing marker α4β7 while CCR7 expression, a LN-trafficking marker, is downregulated [34]. Recently, a study showed NK cells from HIV-infected patients had reduced expression of the homing receptors CXCR1 and CX3CR1 [35], again suggesting that NK cell migration to LN may be compromised.

The substitution of thymidine with BrdU into the DNA of the dividing cells ensures restricted labeling of dividing cells. BrdU is not only retained in cells that have undergone division in the presence of BrdU but it is also passed to daughter cells after free BrdU has been cleared from the blood. However, there are also limitations to the use of BrdU, such as the uncertainty of BrdU distribution among tissues and the bioavailability in different species after administration. In order to confirm the data generated by in vivo BrdU labeling, we also utilized the nuclear protein Ki-67, a reliable marker of mitosis since it is expressed during mitosis and it has a short half-life, providing a snapshot of the dividing cells at a given time. As no ideal method of detection of proliferation exists, assessing the same data with more than one technique make it more reliable. In this study, we observed that for the majority of the analyses, both techniques showed very similar results; albeit a significant difference was occasionally observed with BrdU incorporation and not with Ki-67 staining, or vice-versa, the trends were consistent.
The proliferation rate of T cells and NK cells in the control and Lm-draining lymph nodes of FIV-infected cats was significantly greater compared to that of SPF-control animals. In the case of CD4⁺ and CD8⁺ T cells, this was counter-balanced by higher levels of apoptosis, but this was not true for NK cells, which showed a similar level of apoptosis regardless of FIV status. Under conditions of Lm challenge, the proliferative rate of T cells further increased in FIV-infected and uninfected cats indicating T cells were responsive to the immune challenge. Thus, overall while constitutive levels of proliferation and apoptosis of T cells were greater in FIV-infected cats, the T cell proliferative and apoptotic rates in response to the Lm challenge were similar to those of SPF-control cats. In contrast, the NK cell response was disparate between FIV-infected and control groups. NK cells from FIV-infected cats did not proliferate significantly in response to Lm challenge and did not down-regulate apoptosis as was observed in SPF-control cats. A higher constitutive turnover of NK and T cells has been shown in SIV-infected macaques [14,36] and HIV-infected patients [37]. The rate of cell turnover has been correlated with plasma viremia [14,37,38], and is most likely a consequence of generalized immune activation [39,40]. The lack of increased LN cellularity upon challenge of FIV-infected cats with Lm might suggest that cell turnover increased as proliferation increased. Although the present study was not designed to measure the turnover rate of cells, it did provide a snapshot of proliferation and apoptosis at a critical point during the innate immune response against Lm, revealing a disparate response between T cells and NK cells in FIV-infected and uninfected cats.

We showed that NK cells from FIV-infected cats failed to upregulate expression of perforin compared to SPF-control cats. NK cells are known for their ability to eliminate
virus-infected or transformed cells by secreting pre-formed granules containing perforin and the serine protease granzymes, which combine to promote apoptosis of target cells [41]. Perforin is an essential component of cytotoxic cells and unlike granzymes, does not have any functional redundancy [42]. Furthermore, it has been shown that perforin knockout mice have an impaired ability to control Lm infection [43]. Previous reports have demonstrated that NK cells from chronic HIV-1 patients have low perforin expression and this is associated with NK cell anergy [44]. Based on our findings, we speculate that due to lower perforin production, NK cells from FIV-infected cats may not be capable of efficiently killing infected or transformed cells in vivo.

The observations that the NK cell response in FIV-infected cats was defective at the level of number, proliferation, apoptosis and function, combined with our previous observations that exogenous IL-15 rescued NK cell number and bacterial clearance, lead us to speculate that a qualitative or quantitative IL-15 deficiency may underlie the impaired innate immune response of FIV-infected cats to Lm challenge. This remains to be proven but if true, the spotlight would turn to the sources of IL-15: dendritic cells and macrophages. Given the general lack of response in the LN of FIV-infected cats, it seems plausible that there may be a more basic defect in the innate immune response, possibly at the dendritic cell level.
E. Material and Methods

Ethics Statement

Animals were housed and cared for in accordance with Association for the Assessment of Laboratory Animal Care standards and Institutional Animal Care and Use Committee guidelines.

Animals, virus and bacterial inoculum, and BrdU administration

Twenty-one specific-pathogen-free (SPF) cats were purchased from Liberty Labs (Liberty, Waverly, NY). Eleven neutered males and 2 females were infected with $5 \times 10^5$ FIV cell-associated NCSU1 virus [45] by intravenous route at 20 weeks of age, and were considered chronically infected 12 months post-inoculation. Control cats included 3 neutered males and 5 females. A dose of $2.5 \times 10^5$ cfu Lm was subcutaneously injected proximal to either the right metatarsal or metacarpal footpad of FIV-infected and control cats. A 20 mg/mL stock solution of 5-bromo-2’-deoxyuridine (BrdU) (Sigma, St. Louis, MO) was prepared by adding 1 x PBS (Gibco, Life Technologies, Grand Island, NY). All cats received 30 mg/kg BrdU, intraperitoneally, on days 0, 1 and 2 post-Lm inoculation.

Sample collection, processing, and bacterial quantification

Whole blood and either popliteal or cervical LN were collected 3 days post-Lm challenge. Peripheral blood mononuclear cells (PBMC) were isolated as previously described [46]. Blood for plasma isolation, complete blood counts, and leukocyte differentials was collected in EDTA tubes. Plasma was isolated by centrifugation and
aliquots were stored at -80°C. Popliteal LN biopsies were performed on anesthetized cats while cervical LN were harvested at necropsy. Contralateral LN were harvested and used as controls. LN were weighted, bisected, and a portion was homogenized and cultured for Lm quantification as previously described [13], with the exception that LN homogenate was plated on BHI agar. The remaining portion of the LN was processed into single cell suspension as previously described [47] for flow cytometry or functional assay.

**Immunophenotyping**

PBMC and LN cells (10^6 cells) were labeled with the following monoclonal antibodies. Anti-CD3 (NZM1) [48] was used unconjugated, anti-CD4 (30 A) [49] was conjugated to Pacific-Blue (Invitrogen, Life Technologies, Grand Island, NY), anti-CD25 (9F23) was conjugated to FITC using standard protocols, anti-CD8α (3.357) [49] was conjugated either to PerCP (ProZyme, Hayward, CA) or FITC, and anti-CD1a (Fel 5f4, Dr. Peter Moore, UC Davis) was conjugated to APC (ProZyme). Anti-CD4-PE (3-4F4; Southern Biotech, Birmingham, AL) and CD56-APC or PECy7 (HCD56; BioLegend, San Diego, CA) were purchased. Intracellular FOXP3 staining was performed using FOXP3-APC antibody as previously described [50]. Intranuclear BrdU staining was performed with BD Biosciences BrdU staining buffers and anti-BrdU-FITC antibody (3D4) according to manufacturer’s recommendations, with Ki-67-PE (B56; BD) staining performed at the same time. AnnexinV-Pacific Blue (Invitrogen) was used according to manufacturer’s instructions. Staining and flow cytometric analysis were performed as described [51], with at least 500,000 gated events collected for each sample.
**Functional assessment of NK cells**

The ability of ex vivo NK cells to produce perforin, granzyme A and IFN-γ was assessed using a FACS-based assay. Whole cell suspensions from LN were cultured overnight with and without 50 U/mL IL-2 in RPMI 1640 medium supplemented with 10% fetal bovine serum (Gibco), penicillin-streptomycin (10 IU/mL and 10 µg/mL respectively; Gibco), L-glutamine (4 mM; Invitrogen), sodium pyruvate (1 mM, Gibco), HEPES (15 mM, Gibco) and 2-mercaptoethanol (Gibco) and used as effector cells. Heterologous target cells were isolated from the spleen of a random source cat using anti-CD21 (1D6, Dr. Peter Moore) and anti-mouse Ig magnetic beads (Miltenyi Biotec Inc, Auburn, CA), and confirmed to be >95% B-cells. Cells were co-cultured at an effector:target ratio of 50:1. Culture of effector cells without target was also assessed. Cells were cultured for 4h, with monensin (BioLegend) to a final concentration of 1 x added after 1h. Following culture, staining was completed as previously described [52] for NK cell surface receptors and intracellular production of perforin (DG9-PE, BioLegend), granzyme (CB9-Pacific Blue, BioLegend) and IFN-γ (E6D4A5-APC). Samples were analyzed using a BD LSRII flow cytometer, with a minimum of 500,000 gated events collected per sample.

**Viral Parameters**

Quantitative real-time one-step reverse transcriptase (RT)-PCR assays were performed to detect viral RNA in plasma using NCSU1 FIV-gag specific primers and probe as previously described [50] (range of detection 10^1 to 10^5 copies/mL). FIV copies/mL plasma ranged between 471 and 5121 in FIV-infected cats (data not shown).
FIV proviral load was determined by real-time PCR using specific FIV-gag and CCR5 primers and probes as previously described [53,54]. The limit of detection is ≤ 10 copies of FIV per 1 µg DNA. Proviral copies per 1 x 10⁶ cells ranged from 245 to 7345 in FIV-infected cats (data not shown).

**Statistical analysis**

Comparison between control and FIV-infected cats was performed by Mann-Whitney U test and Wilcoxon Signed-Rank test was used to compare Lm-challenged and control nodes between animals with the same FIV status. Means with standard errors (SEM) are reported. Significance was defined as $P \leq 0.05$ and analyses were performed using GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA).

**Acknowledgements**

We thank Alora LaVoy, Julie M. Long and Lin Zhang for expert technical assistance.
F. Figures

Figure 1. Chronic FIV infection is associated with decreased lymphocyte numbers in Lm challenged LN. Chronically FIV-infected and SPF-control cats were challenged with $2.5 \times 10^5$ cfu *Listeria monocytogenes* subcutaneously proximal to either the right metatarsal or metacarpal footpad. After 3 days, the local draining lymph node and the contralateral control nodes were removed, processed into a single cell suspension, assessed by trypan blue dye exclusion, and the total number of cells per lymph node was determined. The absolute cell number for lymphocyte subpopulations was calculated based on the percent of gated lymphocytes determined by flow cytometric analysis and total number of lymphocytes from the LN. (A) Total cell number, (B) Absolute number of NK cells (CD3^−CD56^+), (C) Absolute number of NKT cells (CD3^+CD56^+), (D) Absolute number of CD4^+ T cells (CD3^+CD4^+), (E) Absolute number of CD8^+ T cells (CD3^+CD8^+), and (F) Absolute number of Langerhans cells (CD1a^+). Columns represent mean ± standard error of the mean (SEM). FIV-infected (black columns) and SPF-control animals (white columns). Statistical significance was determined between control and Lm challenged LN. Statistical analysis was performed using Wilcoxon Signed-Rank test. * indicates $P<0.05$, ** indicates $P<0.01$. SPF-control cats n=8, FIV-infected cats n=13.
Figure 2. **Flow cytometry gating strategy and representative plots.** Flow cytometry gating strategy and representative plots from control lymph node cells of a SPF-control cat (CD4$^+$ and CD8$^+$ T and NK gatings) and from control lymph node of a FIV-infected cat (CD4$^+$CD25$^+$ and FOXP3 gatings). (A) The lymphocyte population was gated based on side and forward scatter (inner gate). Lymphocyte subpopulations were gated based on expression of CD3, CD4, CD8, CD56, CD25 and FOXP3. A broader gating strategy of the total cell population (outer gate) was used to identify the CD1a$^+$ subpopulation. (B) Representative dot plots are shown for AnnexinV$^+$ cells within total, CD4$^+$ and CD8$^+$ T cells, and NK cells. Representative contour plots of BrdU$^+$ and Ki67$^+$ cells within total, CD4$^+$ and CD8$^+$ T cells, and NK cells from an FIV-infected cat are shown.
Figure 3. Effect of FIV infection on PBMC subpopulations. Whole blood was collected at the time of lymph node biopsy 3 days after the Lm challenge. The absolute cell number for lymphocyte subpopulations was calculated based on the percent of gated lymphocytes determined by flow cytometric analysis multiplied by lymphocyte absolute number. (A) Absolute number of CD4⁺ T cells, (B) Absolute number of CD8⁺ T cells, (C) Absolute number of PBMC, (D) Absolute number of NK cells. Columns represent mean ± SEM. FIV-infected (black columns) and SPF-control animals (white columns). Statistical significance was determined between control and Lm challenged LN. Statistical analysis was performed using Mann-Whitney U test. ** indicates *P*<0.01. SPF-control cats n=8, FIV-infected cats n=13.
Figure 4. Effect of FIV infection on lymph node cell proliferation. Cell proliferation was assessed by BrdU incorporation and expression of the nuclear antigen Ki-67. The absolute number of proliferating lymphocyte subpopulations was calculated based on the percent of gated lymphocytes that either incorporated BrdU or expressed Ki-67, determined by flow cytometric analysis, and the total number of lymphocytes from the LN. (A) Absolute number of proliferating NK cells, (B) Absolute number of proliferating CD4⁺ T cells, (C) Absolute number of proliferating CD8⁺ T cells, (D) Absolute number of proliferating cells. Columns represent mean ± SEM. FIV-infected (black columns) and SPF-control animals (white columns). Statistical significance was determined between control and challenged LN, and between FIV-infected and SPF-control cats. Statistical analysis was performed using Wilcoxon Signed-Rank test and Mann-Whitney U test. * indicates \( P < 0.05 \), ** indicates \( P < 0.01 \), *** indicates \( P < 0.001 \). SPF-control cats \( n=8 \), FIV-infected cats \( n=13 \).
Figure 5. Effect of FIV infection on peripheral blood mononuclear cell (PBMC) proliferation. Whole blood was collected at the time of lymph node biopsy 3 days after the Lm challenge. Cell proliferation was assessed by BrdU incorporation and expression of the nuclear antigen Ki-67. (A) Absolute number of proliferating NK cells, (B) Absolute number of proliferating CD4^+ T cells, (C) Absolute number of proliferating CD8^+ T cells, (D) Absolute number of proliferating cells. Columns represent mean ± SEM. FIV-infected (black columns) and SPF-control animals (white columns). Statistical significance was determined between FIV-infected and SPF-control cats. Statistical analysis was performed using Mann-Whitney U test. * indicates P<0.05. SPF-control cats n=8, FIV-infected cats n=13.
Figure 6. Lymphocyte subpopulations from FIV-infected cats undergo apoptosis at a higher rate. Apoptosis was assessed by AnnexinV+ staining followed by flow cytometric analysis. The absolute number of AnnexinV+ lymphocyte in each phenotypic subpopulations was calculated by determining the percent of each subpopulation by flow cytometric analysis and then multiplying by the total number of lymphocytes in the LN. (A) Absolute number of AnnexinV+ NK cells, (B) Absolute number of AnnexinV+ CD4+ T, (C) Absolute number of AnnexinV+ CD8+ T cells, (D) Absolute number of AnnexinV+ lymphocytes. Columns represent mean ± SEM. FIV-infected (black columns) and SPF-control animals (white columns). Statistical significance was determined between FIV-infected and SPF-control cats. Statistical analysis was performed using Mann-Whitney U test. * indicates $P<0.05$, ** indicates $P<0.01$. SPF-control cats n=5, FIV-infected cats n=6.
Figure 7. Fewer NK cells from FIV-infected cats produce perforin. Whole cell suspensions from lymph nodes were cultured overnight with IL-2. Cells were then co-cultured for 4 h with target cells at an effector:target ratio of 50:1, with monensin added after the first hour of incubation. Cells were stained for cell surface receptors CD3 and CD56 and for intracellular perforin, IFN-γ and granzyme A. Samples were immediately analyzed by flow cytometry. Columns represent mean ± SEM. FIV-infected (black columns) and SPF-control (white columns). Statistical significance was determined between FIV-infected and SPF-control cats. Statistical analysis was performed using Mann-Whitney U test. * indicates P<0.05. SPF-control cats n=6, FIV-infected cats n=12.
Supplemental Figure S1. Relative percentages of LN cell populations after Lm challenge. Chronically FIV-infected and SPF-control cats were challenged with $2.5 \times 10^5$ cfu *Listeria monocytogenes* subcutaneously proximal to either the right metatarsal or metacarpal footpad. After 3 days, the local draining lymph node and the contralateral control node were removed, processed into a single cell suspension, assessed by trypan blue dye exclusion and the total number of cells per lymph node was determined. The percentages of lymphocyte subpopulations were determined by flow cytometric analysis. (A) Percent of NK cells, (B) Percent of NKT cells, (C) Percent of CD4$^+$ T cells, (D) Percent of CD8$^+$ T cells, and (E) Percent of Langerhans cells. Columns represent mean and standard error of the mean (SEM). FIV-infected (black columns) and, SPF-control animals (white columns). Statistical significance was determined between control and Lm challenged LN. Statistical analysis was performed using Wilcoxon Signed-Rank test. SPF-control cats n=8, FIV-infected cats n=13.
Supplemental Figure S2. Effect of FIV infection on PBMC subpopulations. Chronically FIV-infected and SPF-control cats were challenged with 2.5 x 10^5 cfu *Listeria monocytogenes* subcutaneously proximal to either the right metatarsal or metacarpal footpad. Whole blood was collected 3 days after challenge. The percentages of lymphocyte subpopulations were determined by flow cytometric analysis. (A) Percent of CD4^+^ T cells (B) Percent of CD8^+^ T cells, (C) Percent of NK cells. Columns represent mean ± SEM. FIV-infected (black columns) and SPF-control animals (white columns). Statistical significance was determined between control and Lm challenged LN. Statistical analysis was performed using Mann-Whitney U test. * indicates P<0.05. SPF-control cats n=8, FIV-infected cats n=13.
Supplemental Figure S3. Effect of FIV infection and Lm challenge on lymph node cell proliferation. Cell proliferation was assessed by BrdU incorporation and expression of the nuclear antigen Ki-67. The percent of proliferating lymphocyte subpopulations was calculated based on the percent of gated lymphocytes that either incorporated BrdU or expressed Ki-67, determined by flow cytometric analysis and are shown within a given cell subpopulation. (A) Percent of proliferating NK cells, (B) Percent of proliferating CD4+ T cells, (C) Percent of proliferating CD8+ T cells, (D) Percent of total lymphocyte proliferating cells. Columns represent mean ± SEM. FIV-infected (black columns) and, SPF-control cats (white columns). Statistical significance was determined between control and challenged LN, and between FIV-infected and SPF-control cats. Statistical analysis was performed using Wilcoxon Signed-Rank test and Mann-Whitney U test. * indicates $P<0.05$, ** indicates $P<0.01$, *** indicates $P<0.001$. SPF-control cats n=8, FIV-infected cats n=13.
Supplemental Figure S4. Effect of FIV infection on peripheral blood mononuclear cell (PBMC) proliferation. Whole blood was collected at the time of lymph node biopsy 3 days after the Lm challenge. Cell proliferation was assessed by BrdU incorporation and expression of the nuclear antigen Ki-67 and are shown within a given cell subpopulation. (A) Percent of proliferating NK cells, (B) Percent of proliferating CD4⁺ T cells, (C) Percent of proliferating CD8⁺ T cells, (D) Percent of proliferating total lymphocytes. Columns represent mean ± SEM. FIV-infected (black columns) and SPF-control animals (white columns). Statistical significance was determined between FIV-infected and SPF-control cats. Statistical analysis was performed using Mann-Whitney U test. * indicates P<0.05. SPF-control cats n=8, FIV-infected cats n=13.
Supplemental Figure S5. Lymphocyte subpopulations from FIV-infected cats undergo apoptosis at a higher rate. Apoptosis was assessed by AnnexinV staining followed by flow cytometric analysis. The percent of AnnexinV$^+$ cells in lymphocyte subpopulations was determined by flow cytometric analysis. (A) Percent of AnnexinV$^+$ of NK cells, (B) Percent of AnnexinV$^+$ of CD4$^+$ T cells, (C) Percent of AnnexinV$^+$ of CD8$^+$ T cells, (D) Percent of AnnexinV$^+$ of total lymphocytes. Columns represent mean ± SEM. FIV-infected (black columns) and, SPF-control animals (white columns). Statistical significance was determined FIV-infected and SPF-control cats. Statistical analysis was performed using Mann-Whitney U test. * indicates $P<0.05$, ** indicates $P<0.01$. SPF-control cats n=5, FIV-infected cats n=6.
G. References


Effects of T Regulatory Cell Depletion on NK cell Response against *Listeria monocytogenes* in Feline Immunodeficiency Virus Infected Cats

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A. Abstract

Regulatory T cells (Treg) are key players in the maintenance of peripheral tolerance, preventing autoimmune diseases and restraining chronic inflammatory diseases. They control the function of CD4+ and CD8+ effector T cell, B cells, and dendritic cells (DC). In addition, recent reports have described CD4+CD25+ Treg cell suppression of NK cell functions. Strong evidence suggests that Treg cells and NK cells have important roles in HIV-disease pathogenesis; however, in vivo studies investigating the interplay between these two cell populations during a lentiviral infection are lacking. Lentivirus-induced CD4+CD25+ Treg cells were first described in feline immunodeficiency virus (FIV)-infected cats. In the present study, we used the FIV/cat/Listeria monocytogenes (Lm) model to study the interplay between Treg cells and NK cells during a lentivirus infection. Because the immune response to Lm is well characterized, we used this opportunistic pathogen to probe the innate immune response in cats. We demonstrated that anti-feline CD25 monoclonal antibody (mAb) therapy depletes Treg cells from the lymph nodes of FIV-infected and SPF-control cats. Lymph node (LN) cellularity after Treg depletion was dependent on the FIV status but no correlation between Treg cell depletion, LN cellularity and control of Lm burden was observed. NK and NKT cell proliferation increased in response to Lm challenge was observed within the FIV-infected depleted group, while DC numbers and expression of CD80 and MHC II did not change in FIV-infected cats. We observed a reduced number of DC on the LN of FIV-infected cats with a higher MHC II expression level, indicating a heightened immune activation regardless the challenge with Lm. Similarly, NK and NKT cells from FIV-infected cats displayed higher levels of the activation marker CD25 in both
CLN and Lm-LN. Overall Treg cell depletion did not affect DC number or activation status or expression of CD25 by NK cells and NKT cells regardless of FIV status. Treatment with anti-feline CD25 mAb led to a decrease in AnnexinV⁺ cells from SPF-control cats, but not in FIV-infected cats. Treg cell depletion led to increased expression of perforin and granzyme A by NK and NKT cells from SPF-control cats, indicating that Treg cells exert a control on these cell populations; while no changes were observed in FIV-infected cats. In conclusion, there was no improvement in Lm clearance after removal of Treg cells during chronic FIV infection, reducing the likelihood that there is a direct negative effect by Treg cells on NK cells in vivo. It appears that although Treg cell suppress NK cell in vivo under normal conditions, other suppressor factors besides Treg cells contribute to NK cell reduced activity. Whether there is an indirect effect of Treg cells on NK cell function and/or whether the relationship between these two cell types is different at the time of lentivirus infection and during the acute phase of disease, remains to be determined.
B. Introduction

Natural killer (NK) cells are a key cellular component of the innate immune system and are considered the first line of defense against virally infected and neoplastic cells [1-3]. In addition to their ability to directly kill target cells, NK cells can secrete cytokines that help modulate the type and strength of the adaptive immune response [2-4]. Several studies have shown that NK cells participate in dendritic cell (DC) maturation [5], generation of cytotoxic T lymphocytes (CTL) and memory T cells [6,7] as well as promoting T helper cell type-1 (Th1) responses [8,9]. While several studies have described the mechanisms by which NK cells modulate the immune response, the exact mechanisms by which NK cell responses are regulated by other cells is still unclear. Regulatory T cells (Treg) are important immunomodulatory cells responsible for maintaining immune system homeostasis and tolerance to self-antigens [10,11].

Treg cells are phenotypically characterized by expression of the CD4 molecule and the IL-2 receptor α (IL-2Rα), as well as the transcription factor FOXP3 [12]. Treg cells are activated through the T-cell receptor and exert suppressive effects through a direct cell-to-cell interaction that induces anergy in antigen-specific T cells [13]. Treg cells are key players in controlling the intensity of immune response mediated inflammation and have been shown to suppress antigen-specific CD4⁺ and CD8⁺ T cell responses against allografts, tumors, and a wide range of infectious agents. Lentivirus-induced CD4⁺CD25⁺ Treg cells were first described in FIV-infected cats [14,15]. FIV is a naturally occurring lentivirus infection of cats, which causes a chronic progressive disease resulting in an AIDS-like disease [16-18] similar to that observed in HIV-1 infected people. Treg cells seem to be
activated in vivo as a result of chronic immune stimulation caused by lentivirus infection in people [19-21] and cats [15]. We have shown that treatment of cats with mouse anti-feline CD25 monoclonal antibody preferentially depletes the CD4+CD25 high Treg population [22] resulting in improved antiviral T cell response in chronically FIV-infected cats [23].

Suppression of NK cells by Treg cells was first suggested by Shimizu and colleagues when they described an increased NK cell activity against tumor after the removal of CD4+CD25+ T cells [24]. It was later shown that NK cell suppression by Treg cells required cell-to-cell contact and was mediated by membrane bound TGF-β [25]. Several reports have shown lentivirus infection profoundly compromises NK cell responses. HIV-1 infected patients present abnormalities in NK cell phenotype, function and number [26,27].

To determine whether Treg cells impact the innate immune response during HIV-1 disease we used the well established FIV/cat model. We employed the intracellular opportunistic pathogen Listeria monocytogenes to probe the innate immune response in FIV infected cats. We previously reported that FIV-infected cats have an impaired innate response that fails to gain initial control of bacterial replication prior to the adaptive immune response [28]. We also showed that locally delivered IL-15, a cytokine known to activate and stimulate NK cells, significantly restored innate immune function in FIV-infected animals as measured by Lm clearance [29]. In addition, we know that NK cells from FIV-infected cats display a heightened level of proliferation and apoptosis, and display a defective response to Lm compared to the NK cell response in SPF-control cats [30].

In the present study we sought to determine whether in vivo Treg cell depletion using anti-feline CD25 monoclonal antibody prior to innate immune challenge with Lm in the
FIV/cat/Listeria model would alter bacterium clearance and innate immune responses. We observed a reduced number of DC in the LN of FIV-infected cats with a higher MHC II expression level, indicating a heightened immune activation regardless of the challenge with Lm. Similarly, NK and NKT cells from FIV-infected cats displayed higher levels of the activation marker CD25$^+$ in both control lymph nodes (CLN) and Listeria draining lymph nodes (Lm-LN). Treatment with anti-feline CD25 led to a decrease in AnnexinV$^+$ cells from SPF-control cats, but not in FIV-infected cats. Treg cell depletion led to increased expression of perforin and granzyme A by NK and NKT cells from SPF-control cats, indicating that Treg cells exert a control on these cell populations; while no changes were observed in FIV-infected cats, suggesting that other factor and/or cell populations besides Treg cells also exert suppression of the NK cell response. Based on our results, it is still possible that Treg cells may reduce efficient activation of NK cells during lentiviral infection.
C. Results

Anti-CD25 mAb treatment depletes Treg cells from lymph nodes of cats and challenge with Lm does not alter the depletion profile

We have previously reported that anti-CD25 monoclonal antibody treatment depletes Treg cells in naive SPF-control cats [22] and chronically FIV-infected cats [23] without disturbing the immune cell profile. In addition, Treg depletion led to an overall improvement in specific anti-FIV as well as novel adaptive immune responses in chronically FIV-infected cats [23]. Here we sought to verify if in vivo Treg depletion would modulate the innate immune response against an opportunistic pathogen in chronically infected cats. Twelve chronically FIV-infected cats and 12 SPF-control cats were divided into 4 groups of 6 cats each and injected with either anti-CD25 mAb or isotype control antibody. In previous work we showed that maximum Treg cell depletion occurred 10 days after anti-CD25 mAb administration [22,23], therefore we decided to probe the innate immune response at this time point. *Listeria monocytogenes* was injected subcutaneously next to the foot pad 10 days post-treatment with anti-CD25 mAb. Three days later both the draining LN (Lm-LN) and the contralateral control LN (CLN) were removed. The CD4^+^CD25^{high} population was reduced between 90% and 98% in CLN and Lm-LN of both SPF-control and FIV-infected cats (Figure 1A) 3 days after Lm challenge (13 days after anti-CD25 mAb treatment). The absolute number of CD4^+^CD25^{high} cells expressing the intranuclear transcription factor FOXP3 was similarly reduced between 89% and 95% (Figure 1B). FOXP3 is associated with the Treg suppressive phenotype in mice, human and cats [31-33].
**Viral burden is unaffected by anti-CD25 mAb treatment**

Similar to previously described results [23], FIV plasma viremia was not altered by either anti-CD25 mAb or isotype control mAb treatment (data not shown). Challenge with Lm led to a 6-fold increase in plasma viremia in the CD25-depleted group compared to viremia immediately prior to Lm treatment ($p = 0.02$). Although viremia in the isotype control group increased after Lm challenge, the difference was not significant ($p = 0.06$). The anti-CD25 mAb treated group showed a higher viral burden compared to the isotype control group without reaching significance ($p = 0.06$). Plasma viremia in the isotype control group was $19.61 \pm 9.37$, $14.18 \pm 6.65$, $57.17 \pm 29.59$ copies/mL (days -10, 0 and 3 post-Lm challenge respectively) and $28.03 \pm 7.37$, $36.5 \pm 9.27$, $223.64 \pm 87.30$ copies/mL in the anti-CD25 treated group (Figure 1C).

Proviral load in PBMC was not altered by anti-CD25 mAb and isotype control mAb treatment, or by Lm challenge (36930.09± 14364.94, 36540.5± 11804.43, 59661.74± 17086.4; days -10, 0 and 3 post-Lm challenge respectively) and anti-CD25 mAb treated group (58043.93± 21215.49, 75811.98± 18458.69, 90156.11± 24666.28 days -10, 0 and 3 post-Lm challenge respectively) (Figure 1C). No differences on proviral load within the CLN and Lm-LN cells were observed at 3 days post-Lm challenge between Treg cell depleted (30124.67± 7095.017 and 55328.24± 19830.77 CFU/LN; CLN and Lm-LN respectively) and control groups (25969.4± 6511.044, 23298.36± 8271.519; CLN and Lm-LN respectively) (Figure 1C).
**Lm clearance is not improved by anti-CD25 antibody treatment**

We have previously shown that FIV-infection impairs the initial control of Lm infection compared to SPF-control cats [28,29]. Here we confirmed our previous observations and showed that depletion of CD4⁺CD25⁺ cells did not result in a more effective clearance of Lm in either FIV-infected or SPF-control animals (Figure 2). There was a trend of higher bacterial recovery from Lm-LN of FIV-infected cats treated with isotype control mAb compared to the CD25-depleted group, however this difference did not reach significance ($p = 0.1$).

**Effect of CD25 depletion on total LN cellularity is dependent on FIV status**

We have previously reported that upon Lm challenge the draining LN of SPF-control cats increases in size (weight), whereas in FIV-infected cats this was not the case [28]. Here we extended that observation to show that the total cell number is lower in the draining LN of FIV-infected animals as compared to SPF-controls after Lm challenge (Figure 3). A dichotomy was observed in the CD25 depleted groups depending on FIV status. While treatment with anti-CD25 mAb led to a decrease in cellularity in SPF-control animals in both CLN and Lm-LN, the opposite was observed in FIV-infected animals (Figure 3), where cellularity increased in consequence of anti-CD25 mAb treatment. More important, CD25 depletion led to an increased cellularity in response to Lm similar to that observed in SPF-control cats.
NK and NKT cell proliferative profile of FIV-infected cats is altered after treatment with anti-CD25 mAb

We have previously shown that the proliferative rate of total lymphocytes and NK cells from LN of SPF-control cats increases in response to Lm challenge, while no changes in proliferation are observed in chronically FIV-infected cats [30]. We have also shown that CD25 depletion improves antiviral responses in chronically FIV-infected cats without increasing the production of proinflammatory cytokines by T cells [23]. Here we sought to determine if Treg cell depletion alters the proliferative profile in FIV-infected cats in response to an immune challenge. Proliferation of total lymphocytes, NK and NKT cells was measured by the in vivo incorporation of BrdU [35] as well as intranuclear Ki-67 expression [36]. Cell proliferation was assessed in CLN and Lm-LN, 3 days after Lm challenge. Treg cell depletion did not alter overall proliferation regardless of the FIV status (Figure 4A). Interestingly, Treg cell depletion in FIV-infected cats resulted in increased proliferation of NK cells in response to Lm challenge, rendering a response similar to SPF-control cats (Figure 4B). Treg depletion also led to a similar increase in NKT cells proliferation in response to Lm (Figure 4C).

**Treg cell depletion results in a reduced number of AnnexinV⁺ cells in SPF-control cats**

We previously showed that FIV infection led to an increased number of AnnexinV⁺ lymphocytes in both CLN and Lm-LN when compared to SPF-control cats [30]. We observed the same result in the present study (Figure 5A). Treg cell depletion led to a reduction in AnnexinV⁺ total lymphocytes, NK cells and NKT cells in both CLN and Lm-LN
(Figures 5A, B and C), while FIV-infected cats displayed a heightened level of AnnexinV+ cells that remained unchanged after depletion. This suggests that although CD4+CD25+ cells seem to promote apoptosis in cats with intact immune responses, this does not seem to be the case in chronically FIV-infected cats, where depletion of Treg cells did not change the apoptotic rate of any population analyzed (Figures 5A, B and C).

**Treg depletion does not affect DC percentage or activation status**

We sought to determine if Treg cell depletion would alter the number or activation status of CD1a+ or CD11c+ dendritic cells. We did not see a difference in percentage of CD1a+ (Figure 6A) nor CD11c+ (data not shown) between isotype control and anti-CD25 mAb treated groups regardless of FIV status. Although a slight increase in CD1a+ percentage was observed in CLN and Lm-LN of FIV-infected cats after CD25 depletion, this difference was not significant (Figure 6A). Depletion did not affect expression levels of CD80 (B7.1) on CD1a+ cells (Figure 6B), nor expression of MHCII by CD11c+ cells (Figure 6C). Overall, frequency of CD1a+ was between 1.5 and 3 fold higher in SPF-control cats compared to FIV-infected cats (Figure 6A). A higher percentage of CD11c+ cells from FIV-infected cats upregulated the expression of MHCII regardless of Lm challenge or Treg cell depletion status (Figure 6C).
Treg depletion did not alter the levels of NK cells and NKT cells expressing the activation marker CD25; while depletion of Treg cells promoted changes in NK/NKT cell expressing perforin or granzyme A and in CD8+ T cells expressing CD62L

We investigated whether NK and NKT cells upregulated the expression of CD25+ and if Treg depletion had an effect on the regulation of this activation marker. The same clone used to deplete CD25+ cells was used to detect cell populations expressing this activation marker. We have previously demonstrated that in vivo anti-CD25 mAb treatment does not prevent its subsequent accurate identification [23]. Treatment with anti-CD25 mAb did not alter the pool of NK and NKT cells expressing CD25+ regardless of FIV status (Figures 7A and B). More than twice as many NK and NKT cells from FIV-infected cats expressed CD25+ compared to NK and NKT cells from SPF-cats, suggesting a higher activation level of these cell populations during chronic FIV infection.

We observed that in SPF-control cats the expression of the CD62+ was upregulated after Lm challenge, while cells from FIV-infected did not show an increase in expression of this lymph node homing marker (Figures 8A-D). Anti-CD25 treatment led to a ~50% increase in the percentage of CD8+ T cell expressing CD62L in both CLN and Lm-LN (Figure 8C), and this was the only cell population analyzed where depletion affected expression of CD62L (Figures 8A, B and D).

In order to assess cell functionality, we determined the number of NK and NKT cells expressing perforin and granzyme A after ex vivo stimulation with IL-2. An increased number of NK and NKT cells from the anti-CD25 treated group expressed perforin and
granzyme A in SPF-control cats, while no difference was observed in the FIV-infected cats (Figures 9A-D).
D. Discussion

Three key immunological features of lentiviral infection are the lack of an effective immune response against the virus, susceptibility to opportunistic pathogens, and generalized immune activation. The underlying mechanisms that lead to these immunologic alterations are incompletely understood. It is, however, well known that Treg-mediated suppression can render the host immune system unable to clear several chronic infections [36]. During chronic HIV-1, SIV, or FIV infection Treg cells are capable of suppressing in vitro antiviral responses [15,37-39]. Our lab has shown that Treg suppression of antiviral responses is also exerted in vivo during chronic FIV infection [23] and an improved antiviral response was observed after the transient depletion of CD25+ Treg cells. Treg cells thus play a detrimental role in lentiviral infection by suppressing immune responses allowing viral persistence and opportunistic infections, but if they play a beneficial role by controlling the destructive immune activation remains unclear. The net effect of these contradictory roles is likely variable during different stages of viral infection. Recently, several in vitro and in vivo experiments showed that NK cell function after stimulation is suppressed by Treg cells [24,25,40-42]. The relevance of the Treg-NK cell interactions in pathological conditions has been primarily studied in tumor models and patients with cancer, where inhibition of Treg cells improved the efficacy of NK cell-based therapy [40]. In vivo NK cell suppression by Treg cells occurs through direct inhibition of NKG2D- mediated cytotoxicity, most likely by membrane bound and soluble TGF-β. Interestingly, IL-10 produced by Treg cells does not seem to alter NK cell function [41]. We and others have described significant abnormalities in NK cell phenotype, function and number during the course of lentiviral infection.
Since it is now clear that NK cells play an important role in determining the type and strength of the adaptive immune response, there has been interest in developing immunotherapeutic approaches that target this particular arm of the innate immune system. It has been shown that the NK cell response to plasmid DNA is suppressed by Treg cells, and removal of Treg restored NK cell function and led to a stronger CD8\(^+\) T cell response [43]. To date no in vivo study has been performed to investigate the interaction between Treg cells and NK cells in the context of HIV-1 infection. In previous work we have shown that the innate immune response in FIV-infected cats is defective [28,29]. We also have shown that locally delivered IL-15 rescued bacterial clearance in FIV-infected cats to levels comparable to those of SPF-control cats, and also promoted an increase in NK cell numbers [29].

In the present study we employed the well characterized FIV/cat/\textit{Listeria monocytogenes} (Lm) model to investigate the in vivo significance of Treg cell activity in an innate immune response during chronic lentivirus infection. We sought to determine whether in vivo Treg cell depletion during chronic FIV infection might allow an improved innate immune response against the opportunistic pathogen Lm. Infection with Lm is known to induce a multifactorial immune response that initially controls and eventually eliminates the bacterium. Early bacterial control mechanism does not rely on T cells, however a T cell response is required for bacterial clearance as demonstrated by T cell deficient mice that are able to control but not clear Lm infection [44]. It seems that NK cell production of IFN-\(\gamma\) after stimulation with IL-12 and IL-18 plays an important role in Lm control [45]. Several studies have shown that NK cell-depleted mice and rats fail to control initial Lm infection resulting in a higher bacterial burden [46,47], and in normal animals, infected with Lm, NK
cells are recruited from the blood to the spleen, liver and/or lymph nodes and increased NK cell activity is observed during the first days of Lm infection.

As we have previously reported, treatment mouse anti-feline CD25 mAb resulted in depletion of Treg cells as measured by the frequency of CD4⁺CD25^{high} as well as CD4⁺CD25^{high}FOXP3⁺ cells [22,23]. While treatment of chronically FIV-infected cats with anti-CD25 mAb or isotype control mAb did not affect viral burden [23], an increase in plasma viremia three days after Lm challenge was seen presumably due to transient activation of the immune system. Such an association of a transient increase in viremia with opportunistic infections is well described in chronic HIV-1 infection [48]. Interestingly, while anti-CD25 mAb treatment increased the total number of cells in the Lm-LN of FIV-infected cats, the opposite was observed in the SPF-control group, where a decrease in total cell number was observed. Furthermore, the increase in cellularity did not result in more efficient control of Lm infection by FIV-infected cats nor did the decreased cellularity affect Lm control in Lm-LN from SPF-control cats 3 days after the challenge. In mice chronically infected with the retrovirus Friend Virus, increased cellularity was observed in the LN and spleen after Treg depletion [49]. Similar results were observed in a mouse model of herpes simplex virus (HSV), where Treg depleted mice infected with HSV had increased cellularity in the virus draining lymph nodes, but reduced cellularity at the site of infection [50]. Thus, in FIV infected cats, there was no improvement in listericidal competence despite successful depletion of Treg. A plausible explanation for this observation could be that NK cells require activating from other cells that were not rescued by Treg cell depletion. Mature DC prime and activate NK cells through cell-to-cell contact dependent signals and soluble mediators.
such as IL-15 (membrane-bound and soluble), IL-12 and type I IFNs [5,51-53]. Results presented here and our previous observation that locally delivered IL-15 can rescue the ability of FIV-infected cats to control initial Lm infection [29], suggests impaired NK cell function may be due to a defective DC population. Indeed, we observed an overall decrease in percentage of CD1a+ cells (Langerhans cells) [56] in the lymph nodes of FIV-infected cats. SPF-control cats also showed an increased percentage of Langerhans cells expressing the costimulatory molecule CD80 (B7.1) after challenge with Lm, whereas upregulation of CD80 was not observed in FIV-infected cats, suggesting an incomplete activation/maturation of DC in response to pathogens during chronic FIV-infection. Additionally, CD11c+ cells from FIV-infected cats showed upregulated MHC II compared to SPF-control cats. It is known that HIV-1 infection results in upregulation of MHC II [57], that may be a dysfuntional immature form of MHC II [58]. Overall Treg cell depletion did not affect DC number or activation status regardless the FIV status.

As previously mentioned, generalized immune activation is one of the hallmarks of HIV-1 infection. Heightened lymphocyte proliferation is reported to be one of the consequences of high levels of immune activation during HIV-1 infection [59,60]. A higher constitutive turnover of NK and T cells has been described during SIV [61,62], HIV [63,64]. We have also reported increased constitutive cell proliferation during chronic FIV-infection in cats that does not increase upon Lm challenge, suggesting that FIV-infection not only drives cell proliferation but also render these cells unresponsive to further stimuli [30]. Treg cells suppress proliferation and function of a wide range of cells including conventional CD4+ and CD8+ T lymphocytes [41,65,66], B cells [67], dendritic cells (DC) [68],

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monocytes/macrophages [69], natural killer T (NKT) cells [70] and natural killer cells [40,71].

In the present study, the absence of CD25+ Treg in FIV-infected cats led to an increase in NK and NKT cell proliferation in response to Lm challenge, similar to that observed in SPF-control cats independent of Treg cell status. Treg depletion or Lm challenge did not alter the expression of the high-affinity interleukin 2 receptor (CD25) on NK or NKT cells. The IL-2Rα (CD25) is considered an activation marker in NK and NKT, and can predict the potential of these cell populations to proliferate [72]. In agreement with the reported generalized immune activation triggered by lentiviral infection, NK and NKT cells from FIV-infected cats show upregulated expression of CD25. There is no evidence that these cell populations were depleted by anti-CD25 treatment since expression of CD25 by NK and NKT cells from FIV-infected cats was the same regardless of the mAb treatment. The differential expression of multiple surface adhesion molecules mediates the trafficking of lymphocytes to inflamed sites. Resting lymphocytes home to infected lymph nodes by expressing L-Selectin (CD62L) which binds to vascular addressins such as GlyCAM-1 and MadCAM-1 in draining lymph nodes [73], and upon activation, CD62L is downregulated. Our results showed that expression of CD62L increased in response to bacterial challenge only in SPF-control cats. Depletion of CD25+ Treg cells led to an increased expression of CD62L molecule in CD8+ T cells in FIV-infected animals, although the increased expression was observed not only in the Lm-LN but also in the CLN, suggesting that even though apparently more naive CD8+ T cells are arriving to the LN, this is not apparently in response specifically to Lm infection.
Consistent with our previous findings, FIV infection is associated with a higher constitutive percentage of cells undergoing apoptosis, however this was unaffected by Treg cell depletion. In contrast, we observed an overall reduction in apoptosis after Treg depletion in LN cells from SPF-control cats. Thus, under normal conditions, apoptosis appears to be driven at least in part by Treg cells presumably through the perforin/granzyme pathway [74,75]. Whether this mechanism of killing by Treg cells is intact or is simply superfluous given the other apoptotic mechanisms associated with FIV/HIV remains to be determined.

Finally, we showed that the percentage of NK and NKT cells expressing perforin and granzyme A increased after Treg depletion in SPF-control cats, and no changes were observed in expression of these functional markers by NK and NKT cells from FIV-infected animals. Taken together, these results raise the question of whether Treg cells function normally in the chronic stage of lentivirus infection. Thus it appears that although Treg cell suppress NK cell in vivo under normal conditions, other suppressor factors besides Treg cells contribute to NK cell reduced activity.

In conclusion, there was no improvement in Lm clearance after removal of Treg cells during chronic FIV infection, reducing the likelihood that there is a direct negative effect by Treg cells on NK cells in vivo. Whether there is an indirect effect of Treg cells on NK cell function and/or whether the relationship between these two cell types is different at the time of lentivirus infection and during the acute phase of disease, remains to be determined.
E. Material and Methods

Ethics Statement

All experimental manipulations and protocols were approved by North Carolina State University Institutional Animal Care and Use Committee. Animals were housed and cared for in accordance with standards established in the Animal Welfare Act and Guide for the Care and Use of Laboratory Animals.

Animals, viral inoculum, and monoclonal antibody administration

A total of 24 specific-pathogen-free (SPF) female cats were purchased from Liberty Labs (Liberty, Waverly, NY) and group housed. A group of 12 cats between 16 and 18 weeks of age were infected with $3.75 \times 10^5$ cell-associated and $9.75 \times 10^4$ TCID-50 cell-free FIV NCSU$_1$ virus [77]. Cell-free and cell associated virus inocula were mixed immediately prior to administration and each animal received half the dose by intravenous and half by intravaginal routes. Cats were considered chronically infected after 1 year post-FIV inoculation. Control animals included 12 female SPF cats. CD25 (9F23) [78] and mouse anti-yellow fever antigen (YFA; CRL-1689, ATCC, Manassas, VA) monoclonal antibodies (mAB) were purified and certified mycoplasm- and endotoxin-free (Leinco, St. Louis, MO). A total of 6 FIV-infected and 6 SPF-control cats were treated with 9 mg/Kg anti-feline CD25 mAb intraperitoneally (i.p.), and 6 FIV-infected and 6 SPF-control cats were treated with 9 mg/Kg anti-YFA mAb i.p. as an isotype control mAb (IgG2a, κ light chain).
**Listeria monocytogenes inoculum and BrdU administration**

After 10 days of treatment with either anti-feline CD25 or anti-YFA mAb, FIV-infected and SPF-control cats were challenged with *Listeria monocytogenes*. A total of 100 µL of 2.5 x 10^6 cfu/mL of Lm was subcutaneously injected proximal to both the right metatarsal and metacarpal footpad. All animals were given 100 µL of PBS injected proximal to both the left metatarsal and metacarpal footpad of FIV-infected and SPF-control cats (sham-inoculated control sites). FIV-infected and SPF-control cats received 30 mg/Kg BrdU (Sigma, St. Louis, MO) by intraperitoneal route on days 0, 1 and 2 post-Lm inoculation.

**Sample collection and processing, and Lm quantification**

Blood for complete blood counts, leucocyte differentials, and plasma isolation was collected in Vacutainer tubes (BD) containing EDTA. Plasma was isolated by centrifugation and aliquots were stored at -80ºC. Cervical and popliteal Lm-draining LN and their contralateral control LN (sham-infected) were harvested at necropsy. LN were weighted, bisected, and a portion was processed into single cell suspension as previously described [80] for phenotypic analysis or functional assays. The remaining portion was homogenized and cultured for bacterial quantification as previously described [29], with the exception that LN homogenate was plated on BHI agar.

**Immunophenotyping**

At least 1 x 10^6 cells were labeled with the following monoclonal antibodies for flow cytometric analysis. Antibodies against feline CD3 (NZM1) was used unconjugated [81],
anti-CD4 (30A) [82] was conjugated either to Pacific-Blue (Invitrogen, Molecular Probes, Carlsbad, CA) or R-Phycoerythrin (ProZyme, San Leandro, CA), anti-CD8 (3.357) [82] was conjugated either to PerCP (Prozyme) or to FITC using standard protocols. All the above mentioned antibodies were purified from hybridoma supernatants in our lab. Anti CD80-PE (B7.1.66) was generously provided by Dr. Mary Tompkins of North Carolina State University [83]. Anti-CD1a (Fel.5F4) was provided by Dr Peter Moore of the University of California at Davis [84] and conjugated to APC (Prozyme). Anti- CD25 (9F23) was conjugated to FITC. Anti-CD56-APC (HCD56; BioLegend, San Diego, California), unconjugated anti-MHCII (PF8J-9B; Serotec, Raleigh, NC) and secondary anti-mouse IgG2-PECy7 (Southern Biotech, Birmingham, AL), anti- CD11c-A488 (BU15; Serotec), anti-CD62L-PE (SK11; BD Biosciences, San Diego, CA) were purchased. Anti-mouse IgG3 was purchased from Jackson Immunoresearch (West Grove, PA), conjugated to Pacific Orange (Invitrogen, Molecular Probes) and used for secondary detection of anti-CD3 (NZM1). AnnexinV-Pacific Blue (Invitrogen) was used according to manufacturer’s instructions. Intracellular FOXP3 staining was performed using FOXP3 staining buffers and FOXP3-APC (FJK-16s; eBiosciences, San Diego, CA) as previously described[23].

Intranuclear BrdU staining was performed with BD Biosciences BrdU staining buffers and anti-BrdU-FITC antibody (3D4; San Diego, CA) according to manufacturer’s recommendations, with Ki-67-PE (B56, BD Biosciences) staining performed at the same time. Staining was performed as previously described [23] with at least 500,000 gated events collected for each sample. Flow cytometric data analysis was performed using FlowJo 7.6.5 (TreeStar, Ashland, OR). Gating strategy has been described elsewhere [30].
Perforin and Granzyme A production by NK cells

Production of perforin and granzyme B by ex-vivo NK and NKT cells was assessed using a FACS-based assay. Whole cell suspensions from LN were incubated ON at 37°C and 5% CO₂ in the presence of 50U/mL IL-2 in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin-streptomycin (10 IU/mL and 10 µg/mL respectively), GlutaMax (4mM), sodium pyruvate (1 mM), HEPES (15 mM) and 2-mercaptoethanol (all Life Technology). The next day, monensin (BioLegend) was added to a final concentration of 1 x and cells were incubated for 3 hours. After incubation, cells were washed in PBS (Life Technologies, Gibco) and surface staining was performed using anti-CD56-APC (HCD56; BioLegend), feline anti-CD3 (NZM1) unconjugated. Mouse anti-IgG3 (Jackson Immunoresearch) conjugated to Pacific Orange (Invitrogen, Molecular Probes) was used for secondary detection of anti-CD3. Cells were resuspended in 100 µL of a 4% paraformaldehyde solution at 4°C for 15 minutes. After fixation, cells were washed 2 x with PBS and resuspended in BD Perm/Wash buffer for 15 minutes on ice under agitation. Following permeabilization, cells were washed and stained with perforin conjugated to A488 (DG9, BioLegend) and granzyme A (CB9-Pacific Blue). Samples were analyzed using a BD LSRII flow cytometer, with a minimum of 500,000 gated events collected per sample. Flow cytometric data analysis was performed using FlowJo 7.6.5 (TreeStar, Ashland, OR). Gating strategy has been described elsewhere [30].
Viral Parameters

Quantitative real-time one-step reverse transcriptase (RT)-PCR assays were performed on a Bio-Rad MyiQ™ PCR detection system (Hercules, CA). QIAamp viral RNA mini kit (Qiagen, Valencia, CA) was used to extract plasma RNA. Detection of plasma viremia in RNA samples was performed using NCSU FIV gag specific primers as previously described

Quantitative real-time one-step reverse transcriptase (RT)-PCR assays were performed to detect viral RNA in plasma using NCSU FIV-gag specific primers and probe as previously described range [23]. Bio-Rad MyiQ™ optical system software v2.0 was used to generate a standard curve and viral RNA copies/mL was calculated. Range of detection was between $10^1$ to $10^5$ copies/mL.

FIV proviral load from PBMC and LN cells was determined by real-time PCR using specific FIV-gag and CCR5 primers and probes as previously described [23], with a limit of detection $\leq 10$ copies of FIV per 1 µg DNA. Shortly, DNA from PBMC and LN cells were extracted with the DNeasy tissue kit (Qiagen) and 0.5 µg DNA sample or standard was used per reaction.

Statistical analysis

Data were compared by 1-way ANOVA with Tukey’s post-test. Statistics were calculated using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA).
Acknowledgements

We thank Janet Dow, Susan Lankford, Alora LaVoy, Julie M. Long and Lin Zhang for expert technical assistance.
F. Figures

Figure 1. Anti-CD25 mAb treatment depletes Treg cells from lymph nodes of cats and challenge with Lm does not alter the depletion profile. Groups of six chronically FIV-infected or SPF-control cats received 9 mg/Kg anti-CD25 mAb (9F23) or 9 mg/Kg isotype control mAb (CRL-1689; IgG2a, κ light chain) i.p. on day -10. All animals were challenged with 2.5 x 10^5 cfu Listeria monocytogenes subcutaneously proximal to the right metatarsal and metacarpal footpad. After 3 days, the local draining lymph node and the contralateral control nodes were removed, processed into a single cell suspension, assessed by trypan blue dye exclusion, and the total number of cells per lymph node was determined. The absolute cell number for lymphocyte subpopulations was calculated based on the percent of gated lymphocytes determined by flow cytometric analysis and total number of lymphocytes from the LN. Whole blood sample for PBMC and plasma viremia was collected on days -10, 0 and 3 post-Lm challenge. To detect FIV provirus in PBMCs and LN cells, quantitative real-time PCR was performed on extracted DNA samples. To detect plama viremia quantitative real-time RT-PCR was performed on extracted RNA samples from plasma. A) Representative dot plots of LN cells isolated on day 3 post-Lm challenge of one FIV-infected cat receiving anti-CD25 mAb and one FIV-infected cat receiving isotype control mAb are shown. B) CD4^+CD25^{high}FOXP3^+ LN cell number was calculated based on the percent of gated lymphocytes determined by flow cytometric analysis and total number of lymphocytes from the LN. Anti-CD25 mAb treated cats (open bars) and isotype control mAb treated cats (hatched bars) are shown. C) Plasma viremia was detected on days -10, 0, and 3; provirus was detected in PBMC and LN cells on days -10, 0 and 3 post-Lm infection. Statistical analysis was performed using 1-way ANOVA with Tukey’s post-test. N=6 for each treatment group.
Figure 2. **Lm clearance is not improved by anti-CD25 antibody treatment.** The number of Lm colony-forming units per LN is shown at day 3 after challenge. Chronically FIV-infected cats had a significant greater number of colony-forming units per LN than did SPF-control cats. Anti-CD25 mAb treated cats (open bars) and isotype control mAb treated cats (hatched bars) are shown. Statistical analysis was performed using Mann-Whitney U test. SPF-control cats n=6, FIV-infected cats n=6.
Figure 3. Effect of CD25 depletion on total LN cellularity is dependent on FIV status. Groups of six chronically FIV-infected or SPF-control cats received anti-CD25 or isotype control mAb i.p. on day -10. All animals were challenged with 2.5 x 10^5 cfu *Listeria monocytogenes* subcutaneously proximal to the right metatarsal and metacarpal footpad. Chronically, after 3 days, the local draining lymph node and the contralateral control nodes were removed, processed into a single cell suspension, assessed by trypan blue dye exclusion, and the total number of cells per lymph node was determined. Anti-CD25 mAb treated cats (open bars) and isotype control mAb treated cats (hatched bars) are shown. Statistical analysis was performed using 1-way ANOVA with Tukey’s post-test. N=6 for each treatment group.
Figure 4. NK and NKT cell proliferative profile of FIV-infected cats is altered after treatment with anti-CD25 mAb. Cell proliferation was assessed by BrdU incorporation and expression of the nuclear antigen Ki-67. The percent of gated lymphocytes that either incorporated BrdU or expressed Ki-67, determined by flow cytometric analysis. (A) Percent of proliferating total lymphocytes, (B) Percent of proliferating NK cells, (C) Percent of proliferating NKT cells. Anti-CD25 mAb treated cats (open bars) and isotype control mAb treated cats (hatched bars) are shown. Statistical analysis was performed using 1-way ANOVA with Tukey’s post-test. N=6 for each treatment group.
Figure 5. Treg depletion results in a reduced number of AnnexinV⁺ cells in SPF-control cats. Apoptosis was assessed by AnnexinV staining followed by flow cytometric analysis. The percent of AnnexinV⁺ cells in lymphocyte subpopulations was determined by flow cytometric analysis. (A) Percent of AnnexinV⁺ of NK cells, (B) Percent of AnnexinV⁺ of CD4⁺ T cells, (C) Percent of AnnexinV⁺ of CD8⁺ T cells. Anti-CD25 mAb treated cats (open bars) and isotype control mAb treated cats (hatched bars) are shown. Statistical analysis was performed using 1-way ANOVA with Tukey’s post-test. N=6 for each treatment group.
Figure 6. Treg depletion does not affect DC percentage or activation status. Expression of CD80 by CD11c+ cells and expression of MHC II by CD1a+ cells was determined by flow cytometric analysis. (A) Percent of CD1a+ cells, (B) Percent of CD80+ of CD1a+ cells, (C) Percent of MHC II of CD11c+ cells. Anti-CD25 mAb treated cats (open bars) and isotype control mAb treated cats (hatched bars) are shown. Statistical analysis was performed using 1-way ANOVA with Tukey’s post-test. N=6 for each treatment group.
Figure 7. Treg depletion does not alter the levels of NK and NKT cells expressing the activation marker CD25. Chronically FIV-infected and SPF-control cats were challenged with $2.5 \times 10^5$ cfu *Listeria monocytogenes* subcutaneously proximal to the right metatarsal and metacarpal footpad. After 3 days, the local draining lymph node and the contralateral control node were removed and processed into a single cell suspension. The percentages of lymphocyte subpopulations were determined by flow cytometric analysis. (A) Percent of NK cells expressing CD25, (B) Percent of NKT cells expressing CD25. Anti-CD25 mAb treated cats (open bars) and isotype control mAb treated cats (hatched bars) are shown. Statistical analysis was performed using 1-way ANOVA with Tukey’s post-test. N=6 for each treatment group.
Figure 8. Chronic FIV-infection led to NK cells to upregulate the expression of CD62L. Chronically FIV-infected and SPF-control cats were challenged with $2.5 \times 10^5$ cfu *Listeria monocytogenes* subcutaneously proximal to the right metatarsal and metacarpal footpad. After 3 days, the local draining lymph node and the contralateral control node were removed and processed into a single cell suspension. The percentages of lymphocyte subpopulations were determined by flow cytometric analysis. (A) Percent of total cells expressing CD62L, (B) Percent of CD4$^+$ cells expressing CD62L, (C) Percent of CD8$^+$ cells expressing CD62L, (D) Percent of NK. Anti-CD25 mAb treated cats (open bars) and isotype control mAb treated cats (hatched bars) are shown. Statistical analysis was performed using 1-way ANOVA with Tukey’s post-test. N=6 for each treatment group.
Figure 9. Effect of CD25 depletion on expression of perforin and granzyme A by NK and NKT is dependent on FIV status. Whole cell suspensions from lymph nodes were cultured overnight with IL-2. 1 X monensin was added and the cells were incubated for 3 hours. Cells were stained for cell surface receptors CD3 and CD56 and for intracellular perforin and granzyme A. Samples were immediately analyzed by flow cytometry. Anti-CD25 mAb treated cats (open bars) and isotype control mAb treated cats (hatched bars) are shown. Statistical analysis was performed using 1-way ANOVA with Tukey’s post-test. N=6 for each treatment group.
G. References


5. APPENDIX

A. FIV Acute Infection

Material and Methods

Animals, viral inoculum, and monoclonal antibody administration

A total of 24 specific-pathogen-free (SPF) female cats were purchased from Liberty Labs (Liberty, Waverly, NY) and group housed. A group of 12 cats between 16 and 18 weeks of age were infected with $3.75 \times 10^5$ cell-associated and $9.75 \times 10^4$ TCID-50 cell-free FIV NCSU1 virus [1]. Cell-free and cell associated virus inocula were mixed immediately prior to administration and each animal received half the dose by intravenous and half by intravaginal routes. Control animals included 12 female SPF cats.

Sample collection and processing

Blood for immunophenotyping was collected using Vacutainer tubes (BD) containing EDTA. Samples were collected from alternate groups of 6 FIV-infected or SPF-control cats on weeks 0, 1, 2, 3, 4, 6, 8, 12, 16, and 24 post-FIV infection. Peripheral blood mononuclear cells (PBMC) were isolated as previously described [2].

Immunophenotyping

At least $1 \times 10^6$ cells were labeled with the following monoclonal antibodies for flow cytometric analysis. Antibodies against feline CD3 (NZM1) was used unconjugated, anti-
CD4 (30A) was conjugated to Pacific-Blue (Life Technologies, Invitrogen, Molecular Probes, Carlsbad, CA), anti-CD8 (3.357) was conjugated to PerCP (Prozyme). All the above mentioned antibodies were purified from hybridoma supernatants in our lab. Anti-CD25 (9F23) was conjugated to FITC using standard techniques. Feline anti-CD4-PE (3-4F4; Southern Biotech, Birmingham, AL), anti-CD56-APC and –PECy7 (HCD56; BioLegend, San Diego, California), and Ki-67-PE and –FITC (B56, BD Biosciences) were purchased. Anti-mouse unconjugated (conjugated to Pacific Orange using Life Biotechnologies, Invitrogen, Molecular Probes kit) and conjugated to IgG3-FITC were purchased from Jackson ImmunoResearch (West Grove, PA), and used for secondary detection of anti-CD3 (NZM1). AnnexinV-Pacific Blue (Invitrogen) was used according to manufacturer’s instructions. Staining was performed as previously described [2] with at least 500,000 gated events collected for each sample. Flow cytometric data analysis was performed using FlowJo 7.6.5 (TreeStar, Ashland, OR).

**Statistical analysis**

Comparison between control and FIV-infected cats was performed by Mann-Whitney U test and Wilcoxon Signed-Rank test was used to compare Lm-challenged and control nodes between animals with the same FIV status. Means with standard errors (SEM) are reported. Significance was defined as $P \leq 0.05$ and analyses were performed using GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA).
Figures

Figure 1. Effect of FIV infection on PBMC subpopulations. SPF cats were infected with 3.75 x 10^5 cell-associated and 9.75 x 10^4 TCID-50 cell-free FIV NCSU1 virus. Whole blood was collected on weeks 0, 1, 2, 3, 4, 6, 8, 12, 16, and 24 post-FIV infection. The percentages of lymphocyte subpopulations were determined by flow cytometric analysis. (A) Percent of NK cells (B) Percent of NKT, (C) Percent of CD4^+ T cells, (D) Percent of CD8^+ T cells, and (E) Percent of CD4^+ CD25^+ cells. Columns represent mean ± SEM. FIV-infected (gray columns) and SPF-control animals (white columns). Statistical significance was determined between FIV-infected and SPF-control cats. Statistical analysis was performed using Mann-Whitney U test. * indicates P<0.05, ** indicates P<0.01, *** indicates P<0.001. SPF-control cats n=6, FIV-infected cats n=6.
Figure 2. Percentage of lymphocyte subpopulations undergoing apoptosis. SPF cats were infected with 3.75 x 10^5 cell-associated and 9.75 x 10^4 TCID-50 cell-free FIV NCSU1 virus. Whole blood was collected on weeks 0, 1, 2, 3, 4, 6, 8, 12, 16, and 24 post-FIV infection. Apoptosis was assessed by AnnexinV staining followed by flow cytometric analysis. The percent of AnnexinV⁺ cells in lymphocyte subpopulations was determined by flow cytometric analysis. (A) Percent of AnnexinV⁺ of NK cells, (B) Percent of AnnexinV⁺ of NK T cells, (C) Percent of AnnexinV⁺ of CD4⁺ T cells, and (D) Percent of AnnexinV⁺ of CD8⁺ T cells. Columns represent mean ± SEM. FIV-infected (gray columns) and SPF-control animals (white columns). Statistical significance was determined between FIV-infected and SPF-control cats. Statistical analysis was performed using Mann-Whitney U test. * indicates P<0.05, ** indicates P<0.01. SPF-control cats n=6, FIV-infected cats n=6.
Figure 3. Percentage of proliferating lymphocyte subpopulations. SPF cats were infected with $3.75 \times 10^5$ cell-associated and $9.75 \times 10^4$ TCID-50 cell-free FIV NCSU1 virus. Whole blood was collected on weeks 0, 1, 2, 3, 4, 6, 8, 12, 16, and 24 post-FIV infection. Cell proliferation was assessed by the expression of the nuclear antigen Ki-67 and are shown within a given cell subpopulation. (A) Percent of proliferating NK cells, (B) Percent of proliferating NKT cells, (C) Percent of proliferating CD4$^+$ T cells, (D) Percent of proliferating CD8$^+$ T cells, (E) Percent of proliferating CD4$^+$CD25$^+$ cells. Columns represent mean ± SEM. FIV-infected (gray columns) and, SPF-control animals (white columns). Statistical significance was determined between FIV-infected and SPF-control cats. Statistical analysis was performed using Mann-Whitney U test. * indicates $P<0.05$, ** indicates $P<0.01$, *** indicates $P<0.001$. SPF-control cats n=6, FIV-infected cats n=6.
References


B. Treg depletion during chronic FIV infection

Material and Methods

Antibody treatment, *Listeria monocytogenes* inoculum and BrdU administration

Cats were considered chronically infected after 1 year post-FIV inoculation. CD25 (9F23) and mouse anti-yellow fever antigen (YFA; CRL-1689, ATCC, Manassas, VA) monoclonal antibodies (mAB) were purified and certified mycoplasm- and endotoxin-free (Leinco, St. Louis, MO). A total of 6 FIV-infected and 6 SPF-control cats were treated with 9 mg/Kg anti-feline CD25 mAb intraperitoneally (i.p.) , and 6 FIV-infected and 6 SPF-control cats were treated with 9 mg/Kg anti-YFA mAb i.p. as an isotype control mAb (IgG2a, κ light chain). After 10 days of treatment with either anti-feline CD25 or anti-YFA mAb, FIV-infected and SPF-control cats we challenged with *Listeria monocytogenes*. A total of 100 µL of 2.5 x 10^6 cfu/mL of Lm was subcutaneously injected proximal to both the right metatarsal and metacarpal footpad. All animals were given 100 µL of PBS injected proximal to both the left metatarsal and metacarpal footpad of FIV-infected and SPF-control cats (sham-inoculated control sites). FIV-infected and SPF-control cats received 30 mg/Kg BrdU (Sigma, St. Louis, MO) by intraperitoneal route on days 0, 1 and 2 post-Lm inoculation.

Sample collection and processing

Blood for complete blood counts, leucocyte differentials, and plasma isolation was collected in Vacutainer tubes (BD) containing EDTA. Plasma was isolated by centrifugation
and aliquots were stored at -80°C. Cervical and popliteal Lm-draining LN and their contralateral control LN (sham-infected) were harvested at necropsy. LN were weighted, bisected, and a portion was processed into single cell suspension for phenotypic analysis or functional assays.

**Cytotoxicity Assay**

Cytotoxicity was assessed using total cells from CLN and Lm-LN incubated with or without 50U/IL-2 at 37°C, 5% CO₂ ON as effector cells. K562 cells were used as target cells and were stained with 200 mM Cell Trace Blue (Life Technologies, Invitrogen, Grand Island, NY) as per manufacturer protocol. Cells were plated into a 96-well plate at 50:1, 25:1, 12:1, 6:1 and 3:1 effector to target (E:T) ratios and after a brief spin the plates were incubated at 37°C, 5% CO₂ for 2h. After incubation, cells were washed with AnnexinV buffer and stained with AnnexinV-A488 and 7AAD (Life Technologies, Invitrogen) as per manufacturer protocol. AnnexinV and 7AAD positive cells within the K562-PB⁺ gating were assessed immediately after staining using a BD LSRII flow cytometer.

**Functional assessment of NK cells**

The ability of ex vivo NK cells to produce perforin, granzyme A and IFN-γ was assessed using a FACS-based assay. Whole cell suspensions from LN were cultured overnight with and without 50 U/mL IL-2 in RPMI 1640 medium supplemented with 10% fetal bovine serum (Gibco), penicillin-streptomycin (10 IU/mL and 10 µg/mL respectively; Gibco), L-glutamine (4 mM; Invitrogen), sodium pyruvate (1 mM, Gibco), HEPES (15 mM,
Gibco) and 2-mercaptoethanol (Gibco) and used as effector cells. The myelogenous leukemia cell line K562 was used as target cells. Cells were co-cultured at an effector:target ratio of 50:1. Culture of effector cells without target was also assessed. Cells were cultured for 4h, with monensin (BioLegend) to a final concentration of 1 x added after 1h. Following culture, staining was completed for NK cell surface receptors and intracellular production of perforin (DG9-A488, BioLegend), granzyme (CB9-Pacific Blue, BioLegend) and IFN-γ (E6D4A5-APC). Samples were analyzed using a BD LSRII flow cytometer, with a minimum of 500,000 gated events collected per sample.

**Statistical analysis**

Data were compared by 1-way ANOVA with Tukey’s post-test. Means with standard errors (SEM) are reported. Significance was defined as $P \leq 0.05$ and analyses were performed using GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA).
Figure 1. Lymph node weight. Control lymph nodes (CLN) and *Listeria monocytogenes* draining lymph nodes (Lm-LN) from chronically FIV-infected cats and SPF-control cats were harvested 3 days post-Lm infection. Harvested LN were cleaned and weighted. Columns represent mean ± SEM. FIV-infected (gray columns), SPF-control (white columns), anti-CD25⁺ depleted (hatched column), and isotype control treated cats (open column). Statistical significance was determined between FIV-infected, SPF-control cats, CD25⁺ depleted and isotype control treated cats. Statistical analysis was performed using 1-way ANOVA with Tukey’s post-test.
Figure 2. Depletion of Treg cells by anti-CD25 mAb treatment. Groups of six cats each received 9 mg/kg anti-CD25 mAb (9F23) or 9 mg/kg isotype control mAb (YFA, CRL-1689/IgG2a, κ light chain, ATCC, Manassas, VA) i.p. on day -10. Lymphocytes were labeled to detect surface CD4 and CD25 by flow cytometry. PBMCs within gated lymphocytes was determined on days -10, 0 and 3 post-Lm challenge and multiplied by the absolute number of peripheral blood lymphocytes. Absolute CD4⁺CD25<sup>high</sup> cell numbers are reported relative to day -10 absolute numbers, which have been normalized to 100. Means ± SE are shown. Statistical significance was calculated compared to isotype control group. ** indicates p < 0.01.
Figure 3. Percentage of circulating lymphocyte subpopulations. SPF cats were infected with 3.75 x 10^5 cell-associated and 9.75 x 10^4 TCID-50 cell-free FIV NCSU1 virus. Cats infected for at least a year were considered chronically infected. Whole blood was collected on days -10, 0, and 3 post-Lm infection. The percentages of lymphocyte subpopulations were determined by flow cytometric analysis. (A) Percent of CD4^+CD25^+ cells (B) Percent of CD4^+CD25_{high} cells, (C) Percent of FOXP3^+ cells within CD4^+CD25_{high} cells, (D) Percent of FOXP3^+ cells within CD4^+CD25^+ cells. Columns represent mean ± SEM. FIV-infected (gray columns), SPF-control animals (white columns), CD25 depleted animals (dashed column), and isotype control antibody (opened column). Statistical analysis was performed using 1-way ANOVA with Tukey’s post-test. * indicates P<0.05, ** indicates P<0.01.
Figure 4. Absolute number of circulating lymphocytes. Whole blood was collected on days -10, 0 and 3 post-Lm infection. The absolute cell number for lymphocyte subpopulations was calculated based on the percent of gated lymphocytes determined by flow cytometric analysis multiplied by lymphocyte absolute number. (A) Absolute number of CD4⁺CD25⁺ cells (B) Absolute number of CD4⁺CD25<sup>high</sup> cells, (C) Absolute number CD4⁺CD25<sup>high</sup> FOXP3<sup>+</sup> cells, (D) Absolute number of CD4⁺CD25<sup>+</sup> FOXP3<sup>+</sup> cells. Columns represent mean ± SEM. FIV-infected (gray columns), SPF-control animals (white columns), CD25 depleted animals (dashed column), and isotype control antibody (opened column). Statistical analysis was performed using 1-way ANOVA with Tukey’s post-test. * indicates P<0.05, ** indicates P<0.01. Statistical analysis was performed using 1-way ANOVA with Tukey’s post-test.
Figure 5. Percent of circulating lymphocytes. SPF cats were infected with $3.75 \times 10^5$ cell-associated and $9.75 \times 10^4$ TCID-50 cell-free FIV NCSU₁ virus. Cats infected for at least a year were considered chronically infected. Whole blood was collected on days -10, 0, and 3 post-Lm infection. The percentages of lymphocyte subpopulations were determined by flow cytometric analysis. (A) Percent of NK cells (B) Percent of NKT cells, (C) Percent of CD4⁺ T cells, (D) Percent of CD8⁺ T cells. Columns represent mean ± SEM. FIV-infected (gray columns), SPF-control animals (white columns), CD25 depleted animals (dashed column), and isotype control antibody (opened column). Statistical analysis was performed using 1-way ANOVA with Tukey’s post-test. * indicates $P<0.05$, ** indicates $P<0.01$. 


Figure 6. Absolute number of circulating lymphocytes. Whole blood was collected on days -10, 0, and 3 post-Lm infection. The absolute cell number for lymphocyte subpopulations was calculated based on the percent of gated lymphocytes determined by flow cytometric analysis multiplied by lymphocyte absolute number. (A) Absolute number of NK cells (B) Absolute number of NKT cells, (C) Absolute number CD4⁺ T cells, (D) Absolute number CD8⁺ T cells, and (E) Absolute number of total lymphocytes. Columns represent mean ± SEM. FIV-infected (gray columns), SPF-control animals (white columns), CD25 depleted animals (dashed column), and isotype control antibody (opened column). Statistical analysis was performed using 1-way ANOVA with Tukey’s post-test. * indicates $P<0.05$, ** indicates $P<0.01$. 

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Figure 7. Percent of circulating lymphocytes undergoing apoptosis. SPF cats were infected with 3.75 x 10^5 cell-associated and 9.75 x 10^4 TCID-50 cell-free FIV NCSU1 virus. Cats infected for at least a year were considered chronically infected. Whole blood was collected on days -10, 0, and 3 post-Lm infection. The percent of AnnexinV+ cells in lymphocyte subpopulations was determined by flow cytometric analysis. (A) Percent of AnnexinV+ cells of NK cells, (B) Percent of AnnexinV+ cells of NKT cells, (C) Percent of AnnexinV+ cells of CD4+ T cells, (D) Percent of AnnexinV+ cells of CD8+ T cells. Columns represent mean ± SEM. FIV-infected (gray columns), SPF-control animals (white columns), CD25 depleted animals (dashed column), and isotype control antibody (opened column). Statistical analysis was performed using 1-way ANOVA with Tukey’s post-test. * indicates P<0.05, ** indicates P<0.01.
Figure 8. Absolute number of circulating lymphocytes undergoing apoptosis. Whole blood was collected on days -10, 0, and 3 post-Lm infection. The absolute cell number for lymphocyte subpopulations was calculated based on the percent of gated lymphocytes determined by flow cytometric analysis multiplied by lymphocyte absolute number. (A) Absolute number of AnnexinV+ NK cells (B) Absolute number of AnnexinV+ NKT cells, (C) Absolute number AnnexinV+ CD4+ T cells, (D) Absolute number of AnnexinV+ CD8+ T cells, and (E) Absolute number of AnnexinV+ total lymphocytes. Columns represent mean ± SEM. FIV-infected (gray columns), SPF-control animals (white columns), CD25 depleted animals (dashed column), and isotype control antibody (opened column). Statistical analysis was performed using 1-way ANOVA with Tukey’s post-test. * indicates P<0.05, ** indicates P<0.01.
Figure 9. Percent of proliferating PBMC. SPF cats were infected with 3.75 x 10^5 cell-associated and 9.75 x 10^4 TCID-50 cell-free FIV NCSU1 virus. Cats infected for at least a year were considered chronically infected. Whole blood was collected on days -10, 0, and 3 post-Lm infection. The percent of Ki67^+ cells in lymphocyte subpopulations was determined by flow cytometric analysis. (A) Percent of Ki67^+ cells of NK cells, (B) Percent of Ki67^+ cells of NKT cells, (C) Percent of Ki67^+ cells of CD4^+ T cells, (D) Percent of Ki67^+ cells of CD8^+ T cells. Columns represent mean ± SEM. FIV-infected (gray columns), SPF-control animals (white columns), CD25 depleted animals (dashed column), and isotype control antibody (opened column). Statistical analysis was performed using 1-way ANOVA with Tukey’s post-test. * indicates P<0.05, ** indicates P<0.01.
Figure 10. Absolute number of proliferating PBMC. Whole blood was collected on days -10, 0, and 3 post-Lm infection. The absolute cell number for lymphocyte subpopulations was calculated based on the percent of gated lymphocytes determined by flow cytometric analysis multiplied by lymphocyte absolute number. (A) Absolute number of Ki67+ NK cells (B) Absolute number of Ki67+ NKT cells, (C) Absolute number Ki67+ CD4+ T cells, (D) Absolute number of Ki67+ CD8+ T cells, and (E) Absolute number of Ki67+ total lymphocytes. Columns represent mean ± SEM. FIV-infected (gray columns), SPF-control animals (white columns), CD25 depleted animals (dashed column), and isotype control antibody (opened column). Statistical analysis was performed using 1-way ANOVA with Tukey’s post-test. * indicates $P<0.05$, ** indicates $P<0.01$. 
Figure 11. Cytotoxicity assay. Cytotoxicity was assessed using total cells from CLN and Lm-LN incubated with or without 50U IL-2 at 37°C, 5% CO₂ ON as effector cells. K562 cells were used as target cells and were stained with 200 mM Cell Trace Blue (Life Technologies, Invitrogen, Grand Island, NY) as per manufacturer protocol. Cells were plated into a 96-well plate at 50:1, 25:1, 12:1, 6:1 and 3:1 effector to target (E:T) ratios and after a brief spin the plates were incubated at 37°C, 5% CO₂ for 2h. After incubation, cells were washed with AnnexinV buffer and stained with AnnexinV-A488 and 7AAD (Life Technologies, Invitrogen) as per manufacturer protocol. AnnexinV and 7AAD positive cells within the K562-PB⁺ gating were assessed immediately after staining using a BD LSRII flow cytometer. Statistical analysis was performed using 1-way ANOVA with Tukey’s post-test. (A) SPF-control animals, IL-2 treated showing mean ± SEM, (B) SPF-control animals, IL-2 treated showing mean only, (C) FIV-infected animals, IL-2 treated showing mean ± SEM, and (D) FIV-infected, IL-2 treated showing mean only.
Figure 12. Cytotoxicity assay. Cytotoxicity was assessed using total cells from CLN and Lm-LN incubated with or without 50U/IL-2 at 37°C, 5% CO₂ ON as effector cells. K562 cells were used as target cells and were stained with 200 mM Cell Trace Blue (Life Technologies, Invitrogen, Grand Island, NY) as per manufacturer protocol. Cells were plated into a 96-well plate at 50:1, 25:1, 12:1, 6:1 and 3:1 effector to target (E:T) ratios and after a brief spin the plates were incubated at 37°C, 5% CO₂ for 2h. After incubation, cells were washed with AnnexinV buffer and stained with AnnexinV-A488 and 7AAD (Life Technologies, Invitrogen) as per manufacturer protocol. AnnexinV and 7AAD positive cells within the K562-PB⁺ gating were assessed immediately after staining using a using a BD LSRII flow cytometer. Statistical analysis was performed using 1-way ANOVA with Tukey’s post-test. A) SPF-control animals, no IL-2 showing mean ± SEM, (B) SPF-control animals, no IL-2 treated, showing mean only, (C) FIV-infected animals, no IL-2 treated showing mean ± SEM, and (D) FIV-infected, no IL-2 treated showing mean only.
Figure 13. Effect of CD25 depletion on expression of IFN-γ by NK and NKT cells. LN were harvested 3 days post-Lm infection. Whole cell suspensions from lymph nodes were cultured overnight with and without IL-2 and with and without target cells (K562). 1 X monensin was added after 1 h incubation with target cells and the cells were further incubated for 3 hours. Cells were stained for cell surface receptors CD3 and CD56 and for intracellular IFN-γ. Samples were immediately analyzed by flow cytometry. (A) Percent of NKT cells expressing IFN-γ in the presence of IL-2 and absence of target cells, (B) Percent of NKT cells expressing IFN-γ in the absence of IL-2 and target cells, (C) Percent of NKT cells expressing IFN-γ in the presence of IL-2 and target cells, (D) Percent of NKT cells expressing IFN-γ in the absence of IL-2 and presence of target cells, (E) Percent of NK cells expressing IFN-γ in the presence of IL-2 and absence of target cells, (F) Percent of NK cells expressing IFN-γ in the absence of IL-2 and target cells, (G) Percent of NK cells expressing IFN-γ in the presence of IL-2 and target cells, (H) Percent of NK cells expressing IFN-γ in the absence of IL-2 and presence of target cells. Statistical analysis was performed using 1-way ANOVA with Tukey’s post-test. Mean ± SEM are shown.
Figure 14. Effect of CD25 depletion on expression of Perforin by NK and NKT cells. LN were harvested 3 days post-Lm infection. Whole cell suspensions from lymph nodes were cultured overnight with and without IL-2 and with and without target cells (K562). 1 X monensin was added after 1 h incubation with target cells and the cells were further incubated for 3 hours. Cells were stained for cell surface receptors CD3 and CD56 and for intracellular Perforin. Samples were immediately analyzed by flow cytometry. (A) Percent of NK (left) and NKT (right) cells expressing Perforin in the presence of IL-2 and target cells, (B) Percent of NK (left) and NKT (right) cells expressing Perforin in the absence of IL-2 and target cells, and (C) Percent of NK (left) and NKT (right) cells expressing Perforin in the absence of IL-2 and presence of target cells. Statistical analysis was performed using 1-way ANOVA with Tukey’s post-test. Mean ± SEM are shown.
Figure 15. Effect of CD25 depletion on expression of Granzyme A by NK and NKT cells. LN were harvested 3 days post-Lm infection. Whole cell suspensions from lymph nodes were cultured overnight with and without IL-2 and with and without target cells (K562). 1 X monensin was added after 1 h incubation with target cells and the cells were further incubated for 3 hours. Cells were stained for cell surface receptors CD3 and CD56 and for intracellular Granzyme A. Samples were immediately analyzed by flow cytometry. (A) Percent of NK (left) and NKT (right) cells expressing Granzyme A in the presence of IL-2 and target cells, (B) Percent of NK (left) and NKT (right) cells expressing Granzyme A in the absence of IL-2 and target cells, and (C) Percent of NK (left) and NKT (right) cells expressing Granzyme A in the absence of IL-2 and presence of target cells. Statistical analysis was performed using 1-way ANOVA with Tukey’s post-test. Mean ± SEM are shown.
Figure 16. Absolute number and percent of CD8α+, CD69+, B220+, and CD57+ NK cells. SPF cats were infected with 3.75 x 10^5 cell-associated and 9.75 x 10^4 TCID-50 cell-free FIV NCSU1 virus. Cats infected for at least a year were considered chronically infected. CLN and Lm-LN were collected 13 days after antibody treatment and 3 days post-Lm infection. The percent of cells in lymphocyte subpopulations was determined by flow cytometric analysis. Statistical analysis was performed using 1-way ANOVA with Tukey’s post-test. (A) Absolute (left) and percent (right) of CD8α+ of NK cells, (B) Absolute (left) and percent (right) of CD69+ of NK cells, (C) Absolute (left) and percent (right) of B220+ of NK cells, and (D) Absolute (left) and percent (right) of CD57+ of NK cells. Statistical analysis was performed using 1-way ANOVA with Tukey’s post-test. Columns represent mean ± SEM. FIV-infected (gray columns), SPF-control animals (white columns), CD25 depleted animals (dashed column), and isotype control antibody (opened column).
Figure 17. Absolute number and percent of CD8α⁺, CD69⁺, B220⁺, and CD57⁺ NKT cells. SPF cats were infected with 3.75 x 10⁵ cell-associated and 9.75 x 10⁴ TCID-50 cell-free FIV NCSU₁ virus. Cats infected for at least a year were considered chronically infected. CLN and Lm-LN were collected 13 days after antibody treatment and 3 days post-Lm infection. The absolute number of proliferating lymphocyte subpopulations was calculated based on the percent of gated lymphocytes determined by flow cytometric analysis, and the total number of lymphocytes from the LN. (A) Absolute (left) and percent (right) of CD8α⁺ of NKT cells, (B) Absolute (left) and percent (right) of CD69⁺ of NKT cells, (C) Absolute (left) and percent (right) of B220⁺ of NKT cells, and (D) Absolute (left) and percent (right) of CD57⁺ of NKT cells. Statistical analysis was performed using 1-way ANOVA with Tukey’s post-test. Columns represent mean ± SEM. FIV-infected (gray columns), SPF-control animals (white columns), CD25 depleted animals (dashed column), and isotype control antibody (opened column).
Figure 18. Absolute number of NK and NKT cells expressing CD25\(^+\) and CD62L\(^+\) surface molecules. SPF cats were infected with 3.75 x 10\(^5\) cell-associated and 9.75 x 10\(^4\) TCID-50 cell-free FIV NCSU\(_1\) virus. Cats infected for at least a year were considered chronically infected. CLN and Lm-LN were collected 13 days after antibody treatment and 3 days post-Lm infection. The absolute number of proliferating lymphocyte subpopulations was calculated based on the percent of gated lymphocytes determined by flow cytometric analysis, and the total number of lymphocytes from the LN. (A) Absolute number of CD25\(^+\)NK cells, (B) Absolute number of CD25\(^+\)NKT cells, (C) Absolute number of CD\(^+\)NK cells, and (D) Absolute number of CD57\(^+\)NK or NKT cells. Columns represent mean ± SEM. Statistical analysis was performed using 1-way ANOVA with Tukey’s post-test. FIV-infected (gray columns), SPF-control animals (white columns), CD25 depleted animals (dashed column), and isotype control antibody (opened column).
Figure 19. DC activation. SPF cats were infected with $3.75 \times 10^5$ cell-associated and $9.75 \times 10^4$ TCID-50 cell-free FIV NCSU1 virus. Cats infected for at least a year were considered chronically infected. CLN and Lm-LN were collected 13 days after antibody treatment and 3 days post-Lm infection. The percent of cells in lymphocyte subpopulations was determined by flow cytometric analysis. The absolute number of proliferating lymphocyte subpopulations was calculated based on the percent of gated lymphocytes determined by flow cytometric analysis, and the total number of lymphocytes from the LN (A) Absolute number of CD1a$^+$ cells (left) and percent of CD1a$^+$ cells (right), (B) Absolute number of CD1a$^+$CD80$^+$ cells (left) and percent of CD80$^+$ of CD1a$^+$ cells, and (C) Absolute number of CD1a$^+$MHCII$^+$ cells (left) and percent of MHCII$^+$ of CD1a$^+$ cells. Statistical analysis was performed using 1-way ANOVA with Tukey’s post-test. Columns represent mean ± SEM. FIV-infected (gray columns), SPF-control animals (white columns), CD25 depleted animals (dashed column), and isotype control antibody (opened column).
Figure 20. DC activation. SPF cats were infected with $3.75 \times 10^5$ cell-associated and $9.75 \times 10^4$ TCID-50 cell-free FIV NCSU1 virus. Cats infected for at least a year were considered chronically infected. CLN and Lm-LN were collected 13 days after antibody treatment and 3 days post-Lm infection. The percent of cells in lymphocyte subpopulations was determined by flow cytometric analysis. The absolute number of proliferating lymphocyte subpopulations was calculated based on the percent of gated lymphocytes determined by flow cytometric analysis, and the total number of lymphocytes from the LN (A) Absolute number of CD11c$^+$ cells (left) and percent of CD11c$^+$ cells (right), (B) Absolute number of CD11c$^+$CD80$^+$ cells (left) and percent of CD80$^+$ of CD11c$^+$ cells, and (C) Absolute number of CD11c$^+$MHCII$^+$ cells (left) and percent of MHCII$^+$ of CD11c$^+$ cells. Statistical analysis was performed using 1-way ANOVA with Tukey's post-test. Columns represent mean ± SEM. FIV-infected (gray columns), SPF-control animals (white columns), CD25 depleted animals (dashed column), and isotype control antibody (opened column).
Figure 21. Lymphocyte subpopulations from CLN and Lm-LN. Chronically FIV-infected and SPF-control cats were challenged with 2.5 x 10^5 cfu Listeria monocytogenes subcutaneously proximal to either the right metatarsal or metacarpal footpad 10 days after antibody treatment. After 3 days, the local draining lymph node and the contralateral control nodes were removed, processed into a single cell suspension, assessed by trypan blue dye exclusion, and the total number of cells per lymph node was determined. The absolute cell number for lymphocyte subpopulations was calculated based on the percent of gated lymphocytes determined by flow cytometric analysis and total number of lymphocytes from the LN. (A) Absolute number (left) and percent (right) of NK cells (CD3^−CD56^+), (B) Absolute number (left) and percent (right) of NKT cells (CD3^+CD56^+), (C) Absolute number (left) and percent (right) of CD4^+ T cells (CD3^+CD4^+), and, (D) Absolute number (left) and percent (right) of CD8^+ T cells (CD3^+CD8^+). Columns represent mean ± standard error of the mean (SEM). FIV-infected (black columns) and SPF-control animals (white columns). Statistical analysis was performed using 1-way ANOVA with Tukey’s post-test. Columns represent mean ± SEM. FIV-infected (gray columns), SPF-control animals (white columns), CD25 depleted animals (dashed column), and isotype control antibody (opened column).
Figure 22. CD4$^+$ and CD8$^+$ T cell proliferation. Cell proliferation was assessed by BrdU incorporation and expression of the nuclear antigen Ki-67. The percent of gated lymphocytes that either incorporated BrdU or expressed Ki-67 was determined by flow cytometric analysis. (A) Percent of proliferating CD4$^+$ T cells and, (B) Percent of proliferating CD8$^+$ T. Columns represent mean ± standard error of the mean (SEM). FIV-infected (black columns) and SPF-control animals (white columns). Statistical analysis was performed using 1-way ANOVA with Tukey’s post-test. Columns represent mean ± SEM. FIV-infected (gray columns), SPF-control animals (white columns), CD25 depleted animals (dashed column), and isotype control antibody (opened column).
Figure 23. BrdU⁺ lymphocytes. Cell proliferation was assessed by BrdU incorporation. The absolute number of proliferating lymphocyte subpopulations was calculated based on the percent of gated lymphocytes determined by flow cytometric analysis, and the total number of lymphocytes from the LN. (A) Absolute number of BrdU⁺ NK cells, (B) Absolute number of BrdU⁺ NKT cells, (C) Absolute number of BrdU⁺ CD4⁺ T cells, (D) Absolute number of BrdU⁺ CD8⁺ T cells, and (E) Total lymphocytes BrdU⁺. Columns represent mean ± standard error of the mean (SEM). FIV-infected (black columns) and SPF-control animals (white columns). Statistical analysis was performed using 1-way ANOVA with Tukey’s post-test. Columns represent mean ± SEM. FIV-infected (gray columns), SPF-control animals (white columns), CD25 depleted animals (dashed column), and isotype control antibody (opened column).
Ki67+ lymphocytes. Cell proliferation was assessed by the expression of the nuclear antigen Ki-67. The absolute number of proliferating lymphocyte subpopulations was calculated based on the percent of gated lymphocytes determined by flow cytometric analysis, and the total number of lymphocytes from the LN. (A) Absolute number of Ki67+ NK cells, (B) Absolute number of Ki67+ NKT cells, (C) Absolute number of Ki67+ CD4+ T cells, (D) Absolute number of Ki67+ CD8+ T cells, and (E) Total lymphocytes Ki67+. FIV-infected (black columns) and SPF-control animals (white columns). Statistical analysis was performed using 1-way ANOVA with Tukey’s post-test. Columns represent mean ± SEM. FIV-infected (gray columns), SPF-control animals (white columns), CD25 depleted animals (dashed column), and isotype control antibody (opened column).
Figure 25. Lymphocyte apoptosis. Apoptosis was assessed by AnnexinV staining followed by flow cytometric analysis. The absolute number of cells undergoing apoptosis was calculated based on the percent of gated lymphocytes determined by flow cytometric analysis, and the total number of lymphocytes from the LN. (A) Absolute number of AnnexinV$^+$ NK cells, (B) Absolute number of AnnexinV$^+$ NKT cells, (C) Absolute number of AnnexinV$^+$ CD4$^+$ T cells, (D) Absolute number of AnnexinV$^+$ CD8$^+$ T cells, and (E) Total lymphocytes AnnexinV$^+$. Columns represent mean ± standard error of the mean (SEM). FIV-infected (black columns) and SPF-control animals (white columns). Statistical analysis was performed using 1-way ANOVA with Tukey’s post-test. Columns represent mean ± SEM. FIV-infected (gray columns), SPF-control animals (white columns), CD25 depleted animals (dashed column), and isotype control antibody (opened column).
**Figure 26. T cell apoptosis.** Apoptosis was assessed by AnnexinV staining followed by flow cytometric analysis. The percent of AnnexinV$^+$ cells in lymphocyte subpopulations was determined by flow cytometric analysis. (A) Percent of AnnexinV$^+$ of CD4$^+$ T cells, and (B) Percent of AnnexinV$^+$ of CD8$^+$ T cells. Columns represent mean ± standard error of the mean (SEM). FIV-infected (black columns) and SPF-control animals (white columns). Statistical analysis was performed using 1-way ANOVA with Tukey’s post-test. Columns represent mean ± SEM. FIV-infected (gray columns), SPF-control animals (white columns), CD25 depleted animals (dashed column), and isotype control antibody (opened column).
6. DISSERTATION SUMMARY

The studies described in this dissertation span over five years and were designed to fill in knowledge gaps regarding the NK cell response and the role that T regulatory T (Treg) cells exert on NK cells during chronic lentiviral pathogenesis in a natural host. Investigations of NK cell activity in HIV-1 infected patients are restricted to in vitro assays or in vivo descriptive and correlative studies, the latter mainly from the peripheral blood mononuclear cell (PBMC) compartment. Since investigating the dynamics of the NK cell response in lymph nodes (LN) of HIV-infected or healthy people in response to opportunistic pathogens is not feasible, we sought to better define the dynamics of NK cell response during the chronic phase of lentiviral infection using the feline immunodeficiency virus (FIV)/cat model of HIV/AIDS in people. We previously described a model to study the innate immune response in FIV-infected cats [1]. Because the innate immune response to Listeria monocytogenes (Lm) is well understood, this intracellular pathogen was used to probe the innate immune system in order to investigate the effects of chronic FIV infection on innate immune function. In this study, FIV-infected cats or SPF-control cats were probed with the opportunistic pathogen Listeria monocytogenes. Listeria monocytogenes was delivered subcutaneously and the draining LN (Lm-LN) was removed at several time-points between 0 and 13 days post-Lm infection. We found that FIV-infected cats failed to control initial bacterial replication compared to SPF-control cats. Our laboratory has also described that among several locally delivered cytokines, only IL-15 rescued the innate immune response of FIV-infected cats against Lm, which correlated with increased NK cells in the lymph node.
The biological activities of IL-15 include NK cell activation, proliferation, and increased cell survival.

In our first study, we used chronically FIV-infected cats and SPF-cat. Animals were considered to be chronically infected after one year post-FIV NCSU1 infection. We probed the innate immune response of FIV-infected or SPF-control cats with Lm. All animals received intraperitoneal injections of 5-bromo-2’-deoxyuridine (BrdU), a thymidine analog that incorporates into the DNA of proliferating cells, on days -2, -1 and 0 post-Lm infection. Three days post-Lm infection, the Lm-LN, the contralateral control LN (CLN) and blood were collected. Challenge of SPF-control cats with Lm resulted in a 4-fold increase in draining lymph node cellularity characterized by significant increases in NK cells, CD4+ T cells and CD8+ T cells, while FIV-infected cats failed to respond to bacterial challenge. We evaluated the Langerhans cell (LC) population and found no quantitative difference regardless of the FIV status. The constitutive proliferation rate of T cells and NK cells in the lymph nodes of FIV-infected cats was significantly greater compared to that of control animals. In the case of CD4+ and CD8+ T cells, this was counter-balanced by higher levels of apoptosis, but this was not true for NK cells, which showed a similar level of apoptosis regardless of FIV status. Under conditions of Lm challenge, the proliferative rate of T cells further increased in FIV-infected and uninfected cats indicating T cells were responsive to the immune challenge. Thus, overall while constitutive levels of proliferation and apoptosis of T cells were greater in FIV-infected cats, the T cell response to the Lm challenge was similar to that of control cats. In contrast, the NK cell response was disparate between FIV-infected and control groups. NK cells from FIV-infected animals did not proliferate
significantly in response to Lm challenge and did not downregulate apoptosis as was observed in SPF-control cats. Using ex vivo assays we showed that NK cells from FIV-infected cats failed to upregulate expression of perforin compared to SPF-control cats. The most relevant conclusion from this study is that lentiviral infection leads to a compromised in vivo NK cell response against an opportunistic pathogen.

In our next study, we sought to determine if Treg cells influence the innate immune response against an opportunistic pathogen during the chronic phase of a lentiviral infection using the FIV/cat/Lm model. Animals were treated intraperitoneally either with feline anti-CD25 mAb or an isotype control mAb. Ten days after mAb therapy, cats were challenged with either Lm or PBS, injected subcutaneously, and received the first BrdU dose intraperitoneally, followed by two BrdU doses given on consecutive days. After 3 days, Lm-LN and CLN were removed. We have shown that treatment of cats with anti-CD25 preferentially depletes the CD4^+CD25^{high} Treg population [3] resulting in improved antiviral T cell responses in chronically FIV-infected cats [4]. In the present study treatment with mouse anti-feline CD25 mAb resulted in depletion of Treg cells as measured by the frequency of CD4^+CD25^{high} as well as CD4^+CD25^{high}FOXP3^+ cells. Neither treatment with anti-CD25 mAb or isotype control mAb affected viral burden. Interestingly, while anti-CD25 mAb treatment increased the total number of cells in the Lm-LN of FIV-infected cats, the opposite was observed in the SPF-control group, where a decrease in total cell number was observed. Furthermore, the increase in cellularity did not result in more efficient control of Lm infection by FIV-infected cats nor did the decreased cellularity affect Lm control in Lm-LN from SPF-control. We observed an overall decrease in the percentage of CD1a^+ cells
(Langerhans cells) in the lymph nodes of FIV-infected cats. SPF-control cats also showed an increased percentage of Langerhans cells expressing the costimulatory molecule CD80 (B7.1) after challenge with Lm, whereas upregulation of CD80 was not observed in FIV-infected cats, suggesting an incomplete activation/maturation of DC in response to pathogens during chronic FIV-infection. Additionally, CD11c+ cells from FIV-infected cats showed upregulated MHC II compared to SPF-control cats. Overall Treg cell depletion did not affect DC number or activation status regardless the FIV-status. In the present study, the absence of CD25+ Treg in FIV-infected cats led to an increase in NK and NKT cell proliferation in response to Lm challenge, similar to that observed in SPF-control cats independent of Treg cell status. The absence of CD25+ Treg in FIV-infected cats led to an increase in NK and NKT cell proliferation in response to Lm challenge, similar to that observed in SPF-control cats independent of Treg cats status. Consistent with our first study, FIV infection was associated with a higher constitutive percentage of cells undergoing apoptosis, however this was unaffected by Treg cell depletion. In contrast, we observed an overall reduction in apoptosis after Treg depletion in LN cells from SPF-control cats. Thus, under normal conditions, apoptosis appears to be driven at least in part by Treg cells. Finally, we showed that NK and NKT cells expressing perforin and granzyme A increased after Treg depletion in SPF-control cats, and no changes were observed in expression of these functional markers by NK and NKT cells from FIV-infected animals. Taken together, these results raise the question of whether Treg cells function normally in the chronic stage of lentivirus infection. In conclusion, there was no improvement in Lm clearance after removal of Treg cells during
chronic FIV infection, reducing the likelihood that there is a direct negative effect by Treg cells on NK cells in vivo.

Taken together, we can conclude from these studies that NK cell response in FIV-infected cats was defective at the level of number, proliferation, apoptosis and function. These findings combined with our previous observations that exogenous IL-15 rescued NK cell number and bacterial clearance, lead us to speculate that a qualitative or quantitative IL-15 deficiency may underlie the impaired innate immune response of FIV-infected cats to Lm challenge. This remains to be proven but if true, the spotlight would turn to the sources of IL-15: dendritic cells and macrophages. Given the general lack of response in the LN of FIV-infected cats, it seems plausible that there may be a more basic defect in the innate immune response, possibly at the dendritic cell level. It appears that although Treg cell suppress NK cells in vivo under normal conditions, other suppressor factors besides Treg cells contribute to reduced NK activity. Whether there is an indirect effect of Treg cells on NK cell function and/or whether the relationship between these two cell types is different at the time of lentivirus infection and during the acute phase of disease, remains to be determined.

Despite the significant findings of these studies, much is still unknown and some findings will require additional investigation. It would be valuable to perform a study where animals were treated with systemic IL-15, a multifunctional cytokine that plays a role in innate immune responses and is critical for NK cell ontogeny, differentiation, proliferation, cytotoxicity, and cytokine production. Dendritic cells represent an important source of IL-15 and increased IL-15 production has been correlated with a better HIV-1 disease outcome.
Further studies are necessary in order to evaluate IL-15 production by DC during HIV infection and to assess the therapeutic potential of exogenous IL-15.
References


