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Analysis of regulatory T cell activation markers in feline immunodeficiency virus (FIV)-infected and control placenta samples from early and late term pregnancy

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ANALYSIS OF REGULATORY T CELL ACTIVATION MARKERS IN FELINE IMMUNODEFICIENCY VIRUS (FIV)-INFECTED AND CONTROL PLACENTA SAMPLES FROM EARLY AND LATE TERM PREGNANCY

By

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Mother-to-child transmission of HIV is the leading cause of pediatric AIDS; however, mechanisms by which HIV compromises pregnancy are not understood. CD4+CD25+ T-regulatory (Treg) cells play a role in pregnancy maintenance. RNA from early and late gestation placentas and fetuses from FIV-infected and control cats were probed for expression of FIV gag and Treg markers CD25, FOXP3, and CTLA4, using real time reverse-transcriptase (RT)-PCR. High rates of vertical transmission and reproductive failure were detected in early and late pregnancy. In control animals, both FOXP3 and CTLA4 expression decreased with gestational stage, indicating a natural decline in Tregs. Expression of FOXP3 and CTLA4 was decreased at early gestation in FIV-infected queens and a trend toward decreased expression of CD25, FOXP3, and CTLA4 in placentas from FIV-infected queens producing non-viable pregnancies was observed as well. Our results suggest that FIV infection may alter placental Treg function and adversely affect pregnancy outcome.

DEDICATION

I would like to dedicate my research to my parents, the late Tommy L. Lockett and Rose M. Lockett, for being a never ending source of strength and support.

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CHAPTER I

REVIEW OF PERTINENT LITERATURE

The AIDS epidemic remains a global scourge despite effective antiretroviral therapy. In the United States alone, more than 56,000 new HIV infections occur each year (NIAID, 2008). In 2007, there were an estimated 33 million people living with HIV globally, and more than 420,000 children were newly infected with HIV. Pediatric infections represented 16% of new HIV infections; more than 1,100 new HIV infections of children occur daily (UNAIDS, 2008). Mother-to-child transmission (MTCT) of HIV is the leading cause of pediatric AIDS cases worldwide, accounting for more than 90% of pediatric infections (CDC, 2008). As pre- and perinatal services to prevent mother-to-child transmission have expanded, the annual number of new HIV infections among children, worldwide, has declined since 2002. With the use of antiretroviral therapy by pregnant women and cesarean delivery of their babies, MTCT can be reduced to less than 1% (NIAID, 2008). However, without antiretroviral therapy, one-fourth to one-third of HIV-infected women will transmit the virus to their babies.

HIV infection may contribute to compromised pregnancy and reproductive failure. Adverse pregnancy outcome, including increased levels of miscarriage, stillbirth, pre-term delivery, and low birth weight was significantly increased in HIV-infected women from the U.S., Europe, Asia, and Africa (Kumar et al., 1995; D'Ubaldo et al., 1998; Goldstein, 2000; Anderson, 2001; Rollins et al., 2007). Langston et al (1995)

evaluated miscarriage rates in a group of HIV-infected women from the United States and reported that spontaneous fetal loss occurred in 14/124 pregnancies ranging from 8 to 32 weeks gestation. HIV was detected in 7/12 of the aborted fetal tissues, four of which were 18 weeks gestation and younger, and three of which were older than 32 weeks. On the other hand, other studies revealed no differences in reproductive outcome between HIV seropositive and negative women (Massad et al., 2004).

FIV as a model for HIV

FIV and HIV are both T lymphotropic lentiviruses that belong to the family *Retroviridae*. Like HIV, FIV uses CXCR4, a chemokine receptor, as a co-receptor, but the two viruses differ in their primary receptor. Whereas HIV uses CD4 as its primary receptor, FIV uses CD134 (OX40), a T cell activation marker (Shimojima et al., 2004). These receptors are used for viral adherence and penetration into the host cell by fusion. FIV binds to the receptors via its 95 Kd surface glycoprotein (gp95) (Bendinelli et al., 1995). Gp95 binds to CD134, causing a conformational change that allows gp95 to bind to CXCR4. A hydrophobic fusion peptide of the 40 Kd transmembrane glycoprotein (gp40) is inserted into the membrane of the cell, consequently causing the fusion of the viral and cellular membranes. The viral capsid is internalized into the cell while the viral envelope remains associated with the cellular membrane.

FIV was first isolated from a group of domestic cats in Petaluma, California in 1986 (Pedersen et al., 1987), and its similarity to HIV was quickly recognized. Feline disease caused by FIV infection is similar to that produced in HIV-infected humans in both clinical outcome and stages of disease progression (Diehl et al., 1995). Importantly,

this is the only animal model system for HIV wherein immunodeficiency results from infection of the natural host. Thus, the FIV-infected cat is a suitable small animal model for HIV-induced immunodeficiency in humans.

There are three stages of feline disease progression: acute, latent, and AIDS-like stage. The acute stage, occurring four to six weeks after infection, is characterized by an initial burst of virus replication and a high circulating virus load (Diehl et al., 1995). This stage is accompanied by non-specific signs such as fever, depression, swollen lymph nodes, gingivitis, and susceptibility to skin or intestinal infections. Further progression of the disease leads to the latent or subclinical stage, which can last for many years. In this stage, there are no signs of disease but the virus slowly weakens the host immune system, as CD4+ T cells are progressively depleted (Bendinelli et al., 1995). The final or AIDS-like stage occurs as a result of immunosuppression. Depletion of CD4+ T cells results in inversion of the CD4: CD8 T cell ratio (Garg et al., 2004). During this final phase, the host is more prone to chronic infections of the oral cavity and upper respiratory tract, opportunistic diseases that cause serious illness, and cancer. The ultimate outcome is usually death.

Shimojima et al (2004) reported that CD4+ T lymphocyte depletion is due to syncytium formation that occurs when an FIV-infected T cell expressing FIV envelope (Env) glycoprotein on its surface comes in contact with an adjacent cell expressing CD134 and CXCR4. Garg et al (2004) reported that Env binding to CD134 can induce apoptosis in non-infected cells and that cell death is also related to an increase in the expression of FIV co-receptor, CXCR4.

The FIV-infected cat also provides an excellent model for MTCT. Like HIV, FIV can be transplacentally transmitted (O'Neil et al., 1996). Transplacental transmission of FIV occurs readily during experimental infection with some FIV isolates, producing frequent reproductive failure (Boudreaux et al., 2009; O'Neil et al., 1996; Rogers and Hoover, 1998; Weaver et al., 2005). High rates of MTCT were reported using FIV isolates from clades A, B, and C (O'Neil et al., 1996; Rogers and Hoover, 1998). Rogers and Hoover (1998) reported that in utero infection rate increased from 0% at 3 weeks gestation to 60% at nine weeks in FIV-B-2542-infected queens. We detected provirus in 21/22 offspring (95%) delivered by cesarean section from infected queens during week 8 of gestation (Weaver et al., 2005) and viral RNA in 12/14 fetuses (86%) delivered during week 3-4 gestation (Boudreaux et al., 2009) using the same isolate.

Placental Immunity and Pregnancy Maintenance

Pregnancy is a natural phenomenon requiring the maternal immune system to tolerate the presence of the semi-allogeneic fetus while maintaining a normal immune response to protect the mother from infection. The placental immune response must be precisely balanced to accomplish this feat. Placental immunomodulation requires the production of an assortment of cytokines which aid in the maintenance of pregnancy (Denison et al., 1998). T-helper 2 (Th2) cytokines are favored at the maternal-fetal interface. For example, IL-10 is a potent immunomodulatory cytokine whose expression is associated with a successful pregnancy outcome (Denison et al., 1998). This cytokine is among the Th2 cytokines favored at the maternal-fetal interface. Th2 cytokines promote the development of a humoral immune response and suppress cell-mediated immunity induced by Th1 cytokines, which could lead to fetal death and abortion.

Dysregulation of cytokine expression can adversely affect pregnancy outcome. For instance, TNF-α is commonly produced during late pregnancy, possibly stimulating uterine contractions to induce labor. If produced during early pregnancy, this cytokine enhances the chances of spontaneous abortion by inducing natural killer cell activity. However, cytokine expression that did not fit the typical Th1/Th2 dichotomy was found at the maternal-fetal interface of mice. For example, inflammatory (IL-12, IL-15) and anti-inflammatory (IL-11, IL-13) cytokines were identified at the maternal-fetal interface in mice at various stages of pregnancy (Zourbas et al., 2001). Bates et al (2002) reported that in-vitro stimulated PBMCs from pregnant women produced more IL-10 and significantly less TNF-α and IFN-γ than non-pregnant women. This group also reported that IL-10 was more markedly increased and TNF-α and IFN-γ expression was further reduced in women with recurrent pregnancy loss.

Cytokine production in the human placenta is affected by HIV infection. Increased expression of pro-inflammatory cytokines, such as IL-1β, IFN-γ, and TNF-α is associated with primary immune response to HIV infection, whereas levels of IL-2 and IL-4 are absent or barely detectable in the peripheral blood (Graziosi et al., 1996). The expression of IL-1β, IL-6, and TNF-α was elevated in HIV-infected placental trophoblasts compared to control placentas (Lee et al., 1997). Furthermore, HIV infection often produces increased expression of IFN-γ (Graziosi et al., 1996) and decreased IL-2 production which contributes to progression to AIDS (Legrand et al., 2006).

CD4+CD25+ T Regulatory Cells

CD4+CD25+ T regulatory cells (Tregs) are immunosuppressive, thereby maintaining immune system homeostasis and tolerance to self antigens. They play a major role in the suppression of graft rejection and innate mucosal immunity. Tregs that are thymus derived and directed towards self antigens are known as naturally occurring Tregs, while Tregs derived from mature T cells in peripheral organs after antigenic stimulation are called adaptive Tregs.

Tregs are characterized by the expression CD25, forkhead box P3 transcription factor (FOXP3), and cytotoxic T lymphocyte antigen 4 (CTLA4). CD25 represents the alpha chain of the interleukin 2 receptor (IL-2Rα). It is upregulated on naïve T cells after antigenic stimulation and differentiation into adaptive Tregs. However, CD25 expression is not restricted to Tregs. Activated T cells, such as CD8+ T cells (Woo et al., 2001), and B cells express CD25 after antigenic stimulation (Lowenthal et al., 1985). FOXP3 is a transcriptional activator that plays a major role in the regulation of Treg function and differentiation (Zheng and Rudensky, 2007). Sustained expression of FOXP3 is necessary to program cells for Treg function (Gavin et al., 2007). Like CD25, it is expressed by naturally occurring Tregs and can be upregulated upon activation and differentiation of CD4+CD25- T cells to adaptive Tregs. FOXP3 was also detected in small amounts in CD4+CD25-, CD8+, and CD21+ lymphocytes in the cat (Lankford et al., 2008), but it was expressed much more abundantly in CD4+CD25+ T cells. CTLA4 is also an activation marker expressed by adaptive Tregs and naturally occurring Tregs. B7 receptors on antigen presenting cells initially engage the CD28 receptor on CD4+ T cells, transducing a signal that upregulates the production of IL-2. CTLA4 is induced

approximately 24-48 hours after activation of the T cell (Krummel and Allison, 1996). It outcompetes CD28 for B7 binding (Tompkins and Tompkins, 2008) and acts as a negative regulator of T cell activation by preventing cell proliferation, cell cycle progression, and IL-2 production (Krummel and Allison, 1996).

CD4+CD25+ T cells comprise 5-10% of the total T cell population in the blood of normal rodents and humans (Vahlenkamp et al., 2004). Their means of immunosuppression is either through the release of cytokines, such as IL-4 and IL-10 (Thornton and Shevach, 1998) or through cell-contact dependent transcriptional down-regulation of IL-2 (Quack et al., 2001; Shevach, 2002; Thornton and Shevach, 2000). CD4+CD25+ Tregs suppress CD4+ and CD8+ T cell responses, e.g. proliferation and cytokine production, to HIV antigens in infected individuals (Kinter et al., 2007a; Kinter et al., 2007b). These mechanisms of suppression are cell-contact dependent and cytokine independent; and they support the idea that CD4+CD25+ Tregs specific for HIV antigens may contribute to the general immunosuppression during HIV infection (Vahlenkamp et al., 2005). Another means by which Tregs suppress immune activation is via modulation of tryptophan catabolism (Fallarino et al., 2003). Indoleamine 2,3-dioxygenase (IDO), a tryptophan-catabolizing enzyme produced by antigen presenting cells, suppresses T cell proliferation by the depletion of tryptophan (Mellor et al., 2001; Munn et al., 1998). IDO activation is dependent upon IFN-γ expression by dendritic cells and CTLA4 expression by Tregs (Fallarino et al., 2003).

Tregs and Pregnancy Maintenance

Tregs play a crucial role in pregnancy maintenance. T cells with similar phenotype and function as adult Tregs were detected in umbilical venous blood from healthy newborn infants (Ng et al., 2001). Human fetal T cells are highly responsive to antigenic stimulation in the absence of CD4+CD25+ Tregs (Michaelsson et al., 2006). Mold et al. (2008) reported that depletion of fetal Tregs resulted in a significant increase in proliferation of fetal T cells against maternal APCs. The increase in fetal T cell proliferation provided evidence that fetal T cells are not deficient at responding to maternal alloantigen, but rather, their function is actively suppressed by fetal Tregs. While fetal Tregs allow fetal tolerance of maternal alloantigens (Mold et al., 2008), placental Tregs allow maternal tolerance of the semi-allogeneic fetus (Michaelsson et al., 2006; Zenclussen et al., 2005) in both the mouse model and in humans. In the human decidua, CD4+CD25+ T cells comprise approximately 10% of CD4+ T cells during early pregnancy (Chao et al., 2002) and 14% of CD4+ T cells at term (Heikkinen et al., 2004). Successful pregnancy is generally associated with higher levels of activated Tregs in both the periphery and the decidua (Sasaki et al., 2004; Somerset et al., 2004; Zhu et al., 2005). Others report expansion of Tregs in the periphery from early to mid-gestation, followed by a decrease to pre-pregnancy levels at term (Somerset et al., 2004). A concomitant decrease in CD4+CD25(high) and increase in CD4+CD25(low) T cells was associated with induction of labor in mice (Zhao et al., 2007).

Feline Tregs and FIV Infection

Feline Tregs are characterized most definitively by co-expression of CD4, CD25, and FOXP3. These cells comprise 5 to 10% of the total CD4+ T cells in the blood and 20 to 30% of lymph node CD4+ T cells and express many phenotypic and functional characteristics of their human and murine counterparts. They are anergic, do not produce IL-2, are responsive to LPS, and are capable of suppressing CD4+CD25- T cells (Vahlenkamp et al., 2004).

CD4+CD25+ T cells support productive replication of FIV. While both CD4+CD25+ and CD4+CD25- T cells may be latently infected with FIV, the state of activation of these cells dictates whether or not productive infection occurs. CD4+CD25+ T cells stimulated with low levels of IL-2 support FIV replication. However, stimulation of latently-infected CD4+CD25- T cells with potent mitogens such as concanavalin A or high levels of IL-2 is required to promote productive infection, correlating with upregulation of CD25 expression in these cells (Joshi et al., 2004). This pattern of replication parallels that of HIV, which also infects both CD4+CD25+ and CD4+CD25- T cells, but in the absence of additional activation, only the CD25+ T cells produce viral protein. Reasons for preferential FIV replication in these regulatory T cells were revealed by Joshi et al (2005). This group found that CD4+CD25+ T cells expressed higher levels of CXCR4 and CD134 than CD4+CD25- T cells, and FIV entry showed a positive correlation with levels of CXCR4 expression. Moreover, CD4+CD25+ T cells constitutively expressed transcriptional activators which bind the FIV long terminal repeat (LTR) and enhance viral replication. CD4+CD25- T cells constitutively expressed very low levels of the transcriptional activators, all of which

were up-regulated by concanavalin A stimulation. Expression of co-stimulatory molecules B7.1, B7.2, and CTLA4 on blood or lymph node Tregs is upregulated in FIV-infected animals (Vahlenkamp et al., 2004).

Cell death is related to an increase in the expression of CXCR4, and the binding of FIV envelope glycoprotein to CD134 can induce apoptosis in non-infected cells (Garg et al., 2004). These findings correlate with the depletion of CD4+ T cells after infection and the inversion of the CD4:CD8 ratio. Moreover, they also relate to Treg susceptibility to FIV infection, since these cells express the FIV primary and coreceptors (Shimojima et al., 2004). Joshi et al (2005) reported that CD4+CD25+ T cells express more CXCR4 and CD134 than CD4+CD25- T cells, and therefore, CD4+CD25+ T cells are more susceptible to FIV binding and entry. Likewise, Human CD4+CD25+ T cells support the replication of HIV more efficiently than CD4+CD25- T cells (Chou et al., 1997). It has been proposed that Tregs are depleted in HIV infection, and that this depletion, along with viremia, stimulates CD4+ and CD8+ T cell activation (Eggena et al., 2005). This immune activation may lead to increased apoptosis of T-helper cells or increased viral replication and infection (Eggena et al., 2005). Consequently, this type of activation silences the host immune system and gives way to the progression of the disease.

Significance and Purpose

Pediatric AIDS remains an important component of the AIDS pandemic, and MTCT accounts for more than 90% of pediatric infections (CDC, 2008). Moreover, transplacentally-acquired HIV infections frequently result in poor pregnancy outcome, including increased rates of miscarriage and low birth weight babies (Anderson, 2001;

D'Ubaldo et al., 1998; Goldstein, 2000; Kumar et al., 1995; Rollins et al., 2007). Yet, despite more than two decades of research, mechanisms by which HIV compromise pregnancy remain poorly understood. Using the FIV-infected cat model, we demonstrated very high rates MTCT and reproductive failure at both early and late pregnancy. Moreover, our data indicate that placental inflammation may be associated with poor pregnancy outcome (Weaver et al., 2005). Given the importance of placental Tregs in pregnancy maintenance reported for both human and murine systems, we believe feline placental Tregs may be implicated in the cat system as well. Therefore, we hypothesized that FIV infection in pregnant cats alters placental Treg population dynamics in the placenta, thereby causing aberrant placental inflammation that potentiates vertical transmission and adversely affects reproductive outcome. Our aim was to use the cat model to provide an early glimpse into possible mechanisms by which lentiviruses, such as HIV, compromise pregnancy and induce reproductive failure.

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CHAPTER II

EXPRESSION OF REGULATORY T CELL (TREG) ACTIVATION MARKERS IN PLACENTAL TISSUES FROM EARLY AND LATE PREGNANCY IN THE FELINE IMMUNODEFICIENCY VIRUS (FIV)-INFECTED CAT

Abstract

Regulatory T cells (Tregs) support pregnancy maintenance by suppressing placental inflammation, while diminished placental Treg function may accompany reproductive failure. Experimental FIV infection results in frequent vertical transmission and increased pregnancy failure in the cat. The mechanism of reproductive compromise is unknown. We hypothesized that FIV infection alters placental Treg population dynamics and function, potentiating vertical transmission and reproductive failure. RNA collected from early and late gestation placentas and fetuses from FIV-infected and control cats was probed for expression of FIV gag and the Treg markers CD25, FOXP3, and CTLA4, using real time reverse-transcriptase (RT)-PCR. A high rate of placental and fetal infection and frequent reproductive failure was detected at early and late pregnancy. Expression of FOXP3 and CTLA4 was higher in early gestation placentas from control cats. FIV infection significantly reduced expression of FOXP3 at early, but not late pregnancy. At late pregnancy, CTLA4 was expressed to higher levels in infected placentas. No significant changes in CD25 expression occurred between FIV-infected and control placentas at early or late pregnancy. There was no significant difference in

the Treg marker expression between viable and non-viable pregnancies in infected cats, although a trend toward decreased expression of CD25 and FOXP3 accompanied non-viable pregnancy. The detection of Treg markers in feline placental tissues provides the first evidence of feline placental Tregs and suggests that such cells diminish as pregnancy progresses. These cells may be depleted or rendered less functional by viral infection, but their role in pregnancy maintenance is unclear.

Introduction

Mother-to-child transmission of HIV is the leading cause of pediatric AIDS worldwide (UNAIDS, 2008). Adverse pregnancy outcome, including increased levels of miscarriage, stillbirth, pre-term delivery, and low birth weight was significantly increased in HIV-infected women from the U.S., Europe, Asia, and Africa (Kumar et al., 1995; D'Ubaldo et al., 1998; Goldstein, 2000; Anderson, 2001; Rollins et al., 2007), yet mechanisms by which HIV compromises pregnancy are not understood.

FIV naturally produces a syndrome culminating in feline AIDS and terminal susceptibility to opportunistic infections and cancer, similar to progression to AIDS in HIV-infected humans. Transplacental transmission of FIV occurs readily during experimental infection with some FIV isolates, producing frequent reproductive failure (O'Neil et al., 1996; Rogers and Hoover, 1998; Weaver et al., 2005; Boudreaux et al., 2009). Thus, the FIV-infected cat provides a useful small animal model for HIV pathogenesis and MTCT.

Tregs, characterized by the expression of the alpha chain of the interleukin 2 receptor (IL-2Rα or CD25), forkhead transcription factor (FOXP3) and cytotoxic T

lymphocyte antigen 4 (CTLA4), are immunosuppressive, maintaining immune system homeostasis and tolerance to self antigens. They play a major role in the suppression of graft rejection and innate mucosal immunity (Maloy et al., 2003). These cells also play a crucial role in pregnancy maintenance. In both the mouse model and in humans, placental Tregs allow maternal tolerance of the semi-allogeneic fetus (Zenclussen et al., 2005; Michaelsson et al., 2006), while fetal Tregs allow fetal tolerance of maternal alloantigens (Mold et al., 2008). In the human decidua, Tregs comprise approximately 10% of CD4+ T cells during early pregnancy (Chao et al., 2002) and 14% of CD4+ T cells at term (Heikkinen et al., 2004). A successful pregnancy is associated with higher levels of activated Tregs in both the periphery and the decidua (Sasaki et al., 2004; Somerset et al., 2004; Zhu et al., 2005). During normal human pregnancy, CD4+CD25^{high}CTLA4 Treg cells increase, but decrease to pre-pregnancy levels if pregnancy fails (Sasaki et al., 2004).

Feline Tregs are characterized most definitively by co-expression of CD4, CD25, and FOXP3 (Lankford et al., 2008). Like human and murine Tregs, feline Tregs comprise 5-10% of the total CD4+ T cells in the blood and 20-30% of lymph node CD4+ T cells and express many of the same phenotypic and functional characteristics (Vahlenkamp et al., 2004). These cells support productive replication of FIV. Joshi et al (2005) found that CD4+CD25+ T cells express higher levels of CXCR4 and CD134 than CD4+CD25- T cells, and FIV entry positively correlates with levels of CXCR4 expression. In addition, CD4+CD25+ T cells constitutively express transcriptional activators which bind the FIV long terminal repeat (LTR), enhancing viral replication.

Expression of co-stimulatory molecules B7.1, B7.2, and CTLA4 on blood or lymph node Tregs is upregulated in FIV-infected animals (Vahlenkamp et al., 2004).

We previously reported very high rates of MTCT and frequent reproductive failure in FIV-infected queens at both early and late pregnancy (Weaver et al., 2005; Boudreaux et al., 2009). Increased expression of two inflammatory cytokines accompanied pregnancy failure in the FIV-infected cat (Weaver et al., 2005). Moreover, successful pregnancy was accompanied by higher levels of CD134 and CXCR4 expression (Scott et al., 2008), suggesting that cells bearing these markers, such as Tregs, may be important to pregnancy maintenance. The data indicate that FIV infection results in enhanced inflammation in the feline placenta. We hypothesize that FIV infection in pregnant cats alters placental Treg population dynamics in the placenta, thereby causing aberrant placental inflammation that potentiates vertical transmission and possibly reproductive failure. Both placentas and fetal/kitten tissues were evaluated for viral infection. Expression of the Treg markers CD25, FOXP3 and CTLA4 was quantified in random sections of placental tissues obtained from FIV-infected and control queens producing viable and non-viable offspring at early and late pregnancy. Both FOXP3 and CTLA4 expression decreased with gestational stage in control animals, indicating a possible natural decline in Tregs from early to late pregnancy. We found a very high rate of viral infection in both placentas and offspring, accompanied by significant or near significant decreases in FOXP3 and CTLA4 expression at early gestation in FIV-infected queens. Although not significant, there was a trend toward decreased expression of CD25, FOXP3, and CTLA4 in placentas from non-viable pregnancies. Collectively, the

data suggest that FIV infection may alter placental Treg function, thereby adversely affecting pregnancy outcome.

Materials and Methods

Animals and Virus

Two groups of twenty, reproductively mature, specific pathogen-free animals (SPF), obtained from a commercial cattery, were used to evaluate early and late pregnancy. Ten queens from each study group were inoculated intravenously with a feline plasma pool containing approximately 1.3×10^4 copies/ml of FIV-B-2542, originally provided by Dr. Edward A. Hoover (Rogers and Hoover, 1998). The other ten queens in each group were used as controls. All procedures involving the cats were performed with approval of the Mississippi State University Institutional Animal Care and Use Committee.

Tissue Collection

Following confirmation of FIV infection using PCR and serology, the cats were allowed to breed naturally with SPF males. Pregnancies were confirmed by ultrasonography and terminated at week 3-4 (early) or week 8 (late). The time of FIV inoculation to delivery ranged from approximately 9.5 to 13.5 months (mean 11.14 months) for the early gestation study and 4.7 to 14.1 months (mean 9.5 months) for the late gestation study. Kittens from both FIV-infected and control queens were delivered by cesarean section. The placentas were collected from the gestational sacs, labeled by the number of the queen, and given an alphabetical designation. Fetuses and placentas

were collected, snap frozen in liquid nitrogen or formalin fixed, then stored at -80°C (Weaver et al., 2005).

RNA Isolation

RNA was isolated from early and late term placental tissues using TRIzol Reagent (Invitrogen, Corp. Carlsbad, CA). According to the manufacturer's instructions, the tissues was submerged in 1 ml TRIzol Reagent and homogenized. Homogenates were supplemented with 0.2 ml chloroform and allowed to sit for 5 min at an ambient temperature (20–25°C). Samples were covered and vigorously shaken for 15 s. The resulting mixture was allowed to sit at room temperature for 15 min. Samples were then centrifuged at 12,000 x g for 15 min. Next, the colorless upper aqueous phase containing RNA was transferred to a fresh tube. RNA was precipitated from the aqueous phase with the addition of 0.5 ml isopropanol and incubated at room temperature for 10 min. The RNA precipitate was then pelleted by centrifugation at 12,000 x g for 8 min, and the supernatant was removed. The RNA pellet was washed by vortexing with 1 ml of 75% ethanol and centrifuged at 12,000 x g for 5 min. The ethanol wash was removed, and the RNA pellet was air-dried for 5 min. After drying, the RNA pellet was dissolved in 100 µl of RNase-free water and incubated for 15 min in a 55–60 °C water bath. RNA concentrations were determined using a NanoDrop 1000 (Thermo Scientific, Waltham, MA) and frozen at -80^{o}C.

Primer and Probe sets

Primers for CTLA4 and CD25 (IL-2Rα) were designed using cytokine mRNA sequences for the cat and human obtained from the National Center for Biotechnology

Information (NCBI) and aligned using the European Molecular Biology Laboratory-European Bioinformatics Institute (EMBL-EBI) Clustal W alignment tool. The human mRNA was BLAST searched against the human genome to locate the exon/intron boundaries and used to find the homologous boundaries in the feline sequences. Real-time PCR (Taqman) primer design (Genscript) was used to design primer/probe sets targeting CTLA-4 and CD25. Each target sequence was entered in the software with semicolons to indicate the boundaries. The primer/probe sets were compared and the best set was chosen according to its amplicon size, length, and GC%. The target probes were 5' labeled with the reporter dye FAM (6-carboxyfluorescein) and 3' labeled with the quencher dye TAMRA (6-carboxytetramethylrhodamine). β-actin was 5' labeled with the reporter dye HEX (hexachloro-6-carboxyfluorescein) and 3' labeled with the quencher dye TAMRA. Primer/probe sets (Table 2.1) for FOXP3 (Lankford et al., 2008), β-actin (Scott et al., 2008), FIV gag (Weaver et al., 2005), CTLA4, and CD25 were obtained commercially (MWG-BIOTECH, Inc., High Point, NC).

Table 2.1 Sequences of Primers and Probes used in Real Time RT-PCR

Receptor	Primer	Sequence (5'→3')
Gag	Sense	GTATGATCGTACTCATCCTCCTGAT
	Anti-sense	TCTACATTGCATTCTGGCTGGT
	Probe	AGACCACTGCCCTACTTCACTGCCG
β-actin	Sense	GACTACCTCATGAAGATCCTCACG
	Anti-sense	CCTTGATGTCACGCACAATTTCC
	Probe	ACAGTTTCACCACCACCGCCGAGC
FOXP3	Sense	GCCTGCCACCTGGAATCAAC
	Anti-sense	GTGTGCTGGGGCTTGGGA
	Probe	CAGTGCTGGCTCCCTGGACACCCA
CD25	Sense	CCACGTGACAGAACTGTGTG
	Anti-sense	GTGCCCGTCTTGTATGTGAG
	Probe	CCGCCAGATATCCAACACGCC
CTLA4	Sense	TCTCCAAAGGGATGCATGT
	Anti-sense	CCCATATTCACACACGAAGC
	Probe	CTGGCCAGCACCACTGCAGG

Real Time Reverse-Transcriptase (RT)-PCR

Gene expression was determined using TaqMan real time RT-PCR targeting FOXP3, CTLA4, CD25, FIV gag, and the housekeeping gene β-actin. Each reaction contained 12.5 µl of the commercial reaction mix, 0.5 µl of ThermoscriptTM Plus/Platinum1 Taq Mix (Invitrogen), 1 µl of forward and reverse β-actin primers (7.5 pmol/µl), 1 µl of forward and reverse target primers (10 pmol/µl), 1 µl of the respective probe (100 fmol/µl), and 30-50 ng/µl RNA. Real time RT-PCR was run on an iCycler (BioRad Laboratories, Valencia, CA) as follows: 50°C, 30 min; 95°C, 5 min; 45 x (95°C, 15 s; 60°C, 1 min). For every placental RNA sample, parallel reactions were run in duplicate. Serially-diluted RNA from control cats was used to generate a standard curve. The standard curves were used to normalize for differences in PCR efficiency. Differences in the amount of template RNA in each reaction were corrected by the cycle threshold (Ct) value for β-actin. For assurance that the integrity of RNA isolated from frozen early and late gestation tissues was equivalent, mean Ct values for β-actin, obtained by amplifying multiple replicas from 35 early placental samples and 31 late placental samples, were compared. β-actin mean Ct values for the two sets of tissues differed by 0.25 Ct.

Statistical Analysis

Statistical comparisons of mean Ct values between groups were done using single-factor ANOVA (Microsoft Excel-VISTA) at the 95% confidence level.

Results

Pregnancy Outcome

The effect of FIV infection on pregnancy outcome at early and late gestation was reported previously (Weaver et al., 2005; Boudreaux et al., 2009), and the data are summarized in Table 2.2. Briefly, at late gestation, fetal non-viability was 60% (15/25 fetuses) in FIV-infected queens and 3.2% (1/31) in control queens; differences in non-viable offspring produced between the two groups were significant ($p=0.04$). Two infected queens did not conceive after repeated attempts at breeding. At early gestation, fetal non-viability of 22.2% (6/27) in infected queens and 4.7% (2/43) in control animals. The difference in the number of viable fetuses between the infected and control groups, nearly 50% fewer in infected cats, was significant ($p=0.02$). Although three times higher in the infected group, fetal non-viability failed to reach the traditional level of significance ($p>0.05$). Two infected queens did not conceive as a result of pyometra or ovarian cysts; another developed right horn pyometra but developed three fetuses in the left horn. Two control cats did not become pregnant for unknown reasons. Fetal non-viability included resorptions and developmentally-arrested fetuses.

Virus Detection in Early and Late Term Placental and Fetal Samples

Real time RT-PCR was used to confirm the presence of viral RNA in placentas and fetuses collected from FIV-infected queens at early (Figure 2.1a) and late (Figure 2.1b) pregnancy. Limits of detection were reported (Boudreaux et al., 2009). At early gestation, all placentas (17/17; 100%) and 12/14 fetuses (85.7%) tested were positive for viral RNA. No accompanying fetal tissue could be collected from placentas 5111 C,

8035 D, and 1893 R because fetuses were resorbed (Figure 2.1a). Fetus 8035 C (a non-viable fetus) had the second highest level of viral RNA, based on Ct value, while no viral RNA was detected in littermates 8035 A (a viable fetus) and B (a non-viable fetus), showing variability even within a single litter. The detection of vertical transmission of FIV at this early stage of gestation was a novel finding.

Viral RNA expression was detected in most of the placental samples (10/17) obtained from the late term FIV-infected queens (Figure 2.1b). Viral RNA was not detected in one sample from queens 9746 and 9730 and four samples from queen 13226. Although we were unable to detect viral RNA from all samples, provirus was detected in 21/22 (95.5%) kittens and 14/15 (93.3%) placentas tested using standard PCR targeting a 293 bp region of FIV gag gene followed by Southern blotting (Weaver et al., 2005).

Expression of Treg Markers in Feline Placentas at Early and Late Gestation

Tregs can be identified by the expression of CD25, FOXP3, and CTLA4. We analyzed the expression of these markers in early and late term control placental tissues to look for evidence of normal Treg dynamics in placental tissue as gestation progresses (Figure 2.2). Evaluating placentas from only viable pregnancies, the higher level of expression of all three markers occurred at early pregnancy. While the slightly lower level of CD25 expression at late pregnancy did not reach the level of significance ($p=0.10$), FOXP3 ($p=0.006$) and CTLA4 ($p=0.015$) expression was significantly lower at late pregnancy.

The effect of FIV infection on Treg marker expression at early and late gestation was determined (Figure 2.3). There was no difference in CD25 expression between

control and infected tissues at either early (p=0.51) or late (p=0.48) gestation (Figure 2.3a). At early gestation, FOXP3 expression was higher in control than infected placentas (p=0.004), but FOXP3 did not differ between control and infected cats at late pregnancy (p=0.85) (Figure 2.3b). A trend toward higher CTLA4 expression in control placentas at early pregnancy did not reach the traditional level of significance (p=0.078). Interestingly, expression of this marker was significantly elevated in infected placentas at late pregnancy (p=0.009) (Figure 2.3c).

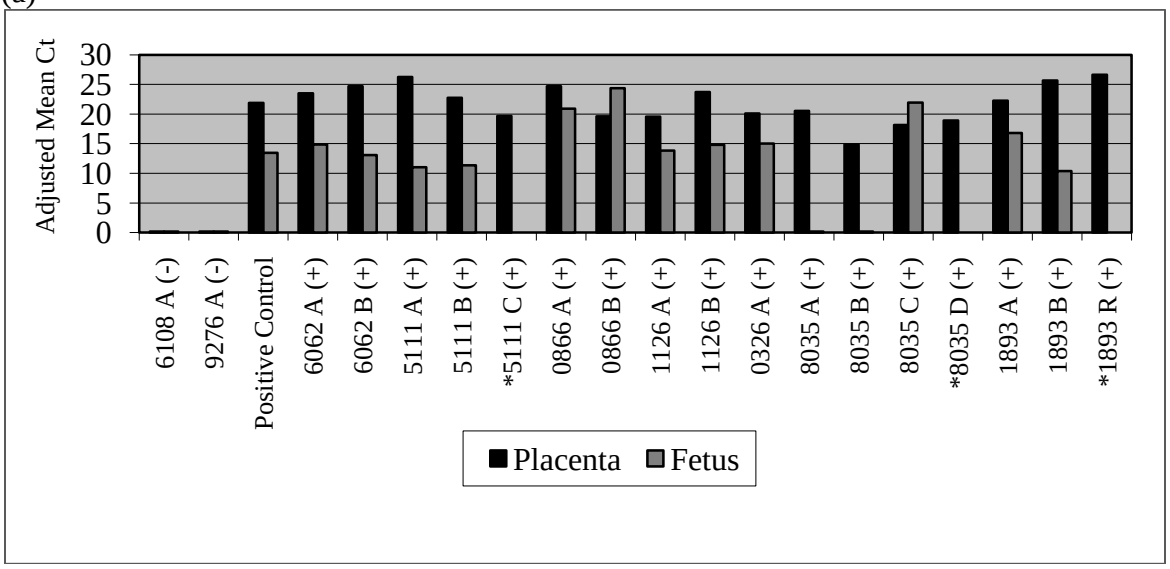
Treg marker expression in viable versus non-viable pregnancies was compared in early and late term infected placentas (Figure 2.4). No significant difference in the placental expression of CD25 was found between viable and non-viable pregnancies in infected cats in either early (p=0.34) or late term tissues (p=0.34) (Figure 2.4a). Likewise, the expression of FOXP3 did not differ significantly between viable and non-viable pregnancies in infected cats at both early (p=0.39) and late pregnancy (p=0.74) (Figure 2.4b). However, a trend toward decreased expression of both markers in placentas from non-viable pregnancies was detected at both early and late term. Placental expression of CTLA4 was very similar between viable and non-viable pregnancies at early (p=0.84) and late (p=0.69) gestation (Figure 2.4c).

Table 2.2 Pregnancy Outcome of the Study Groups

Early Pregnancy					
Queen Number	FIV Status	Viable Kittens	Arrested Fetuses	Fetal Resorptions	Samples Tested (Viable/Non-Viable)
6108	-	4	0	0	2/0
7284	-	4	0	0	2/0
8059	-	6	0	0	2/0
2779	-	7	1	0	2/1
3550	-	4	0	0	2/0
9276	-	5	0	1	2/1
8291	-	4	0	0	2/0
0373	-	7	0	0	2/0
6062	+	3	0	0	2/0
5111	+	2	1	0	2/1
0866	+	5	1	0	1/1
1126	+	4	0	0	2/0
0326	+	1	0	0	1/0
8035	+	1	1	2	1/3
1893	+	5	1	0	2/1
Late Pregnancy					
Queen Number	FIV Status	Viable Kittens	Arrested Fetuses	Fetal Resorptions	Samples Tested (Viable/Non-Viable)
9522	-	3	0	0	2/0
9581	-	2	1	0	2/0
9746	-	6	0	0	2/0
9801	-	6	0	0	2/0
13634	-	3	0	0	1/0
13668	-	2	0	0	2/0
13671	-	4	0	0	2/0
9674	+	1	0	0	1/0
9730	+	2	1	2	2/0
9745	+	2	0	4	2/4
9806	+	3	0	0	1/0
9809	+	1	0	0	1/0
9810	+	1	0	0	1/0
9813	+	1	0	2	1/0
13226	+	0	5	0	1/5

* RNA was tested using real time RT-PCR. In early pregnancy, fetal non-viability was 4.7% (2/43) in control queens and 22% (6/27) in FIV-infected queens. The difference in the number of viable fetuses between the two groups was significant (p=0.02). In late pregnancy, fetal non-viability was 3.2% (1/31) in control queens and 60% (15/25) in FIV-infected queens. The difference in fetal non-viability at late gestation was significant (p=0.04).

(a)



(b)

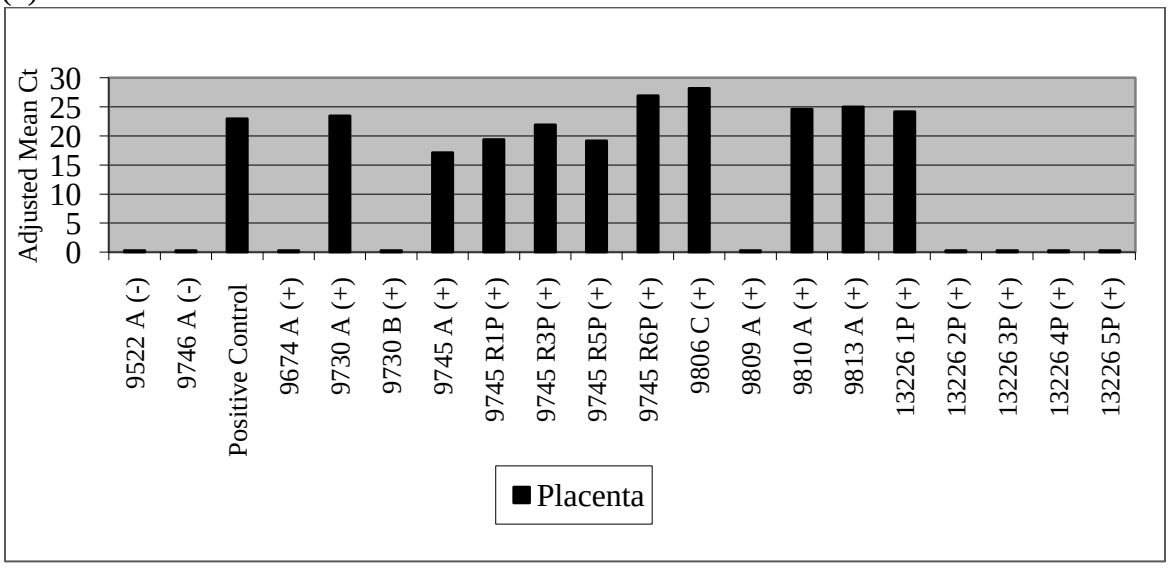


Figure 2.1 Detection of FIV in Early and Late Gestation Tissues

TaqMan real time RT-PCR analysis of FIV gag gene expression in placentas and corresponding fetuses from early (week 3-4) pregnancy and placentas from late (week 8) pregnancy in FIV-infected cats. (a) Viral mRNA was amplified from all placental samples (14 of 14 samples). FIV gag was detected in 12 of 14 in all fetal samples. The asterisks indicate that no fetal tissue was collected from placenta 5111 C, 8035 D, and 1893 R due to resorption. (b) Viral RNA was detected in 10 of 17 placental samples from the late term FIV-infected queens. Bars represent adjusted mean Ct values (50 - mean Ct)

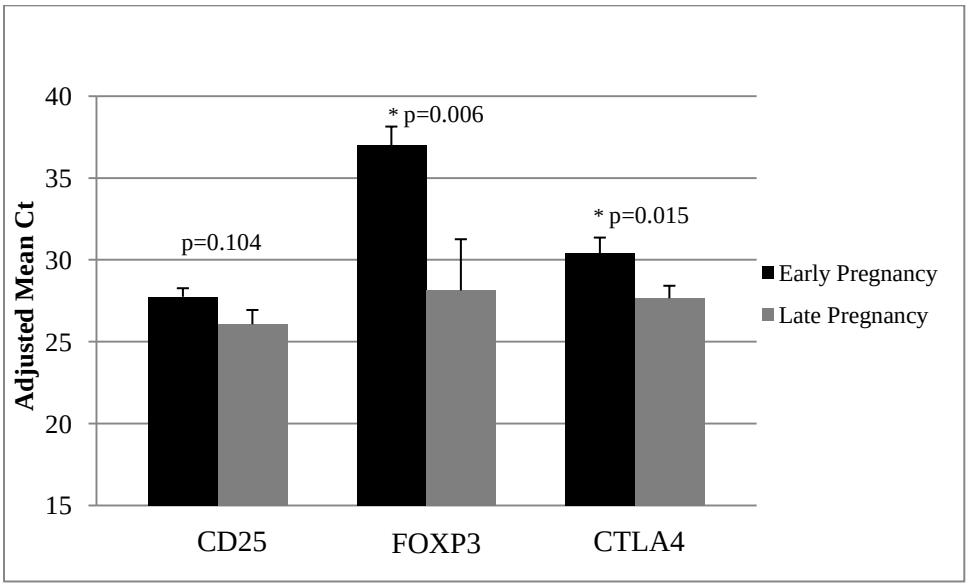


Figure 2.2 Relative Expression of Placental Treg Markers in Control Cats

TaqMan real time RT-PCR analysis of placental expression of Treg markers CD25, FOXP3, and CTLA4 in early gestation control (n=18) versus late gestation control (n=13) samples. Bars represent adjusted mean Ct values (50 - mean Ct), and error bars represent standard errors of the mean. P values obtained from single-value ANOVA are noted. *P values < 0.05 were considered significant.

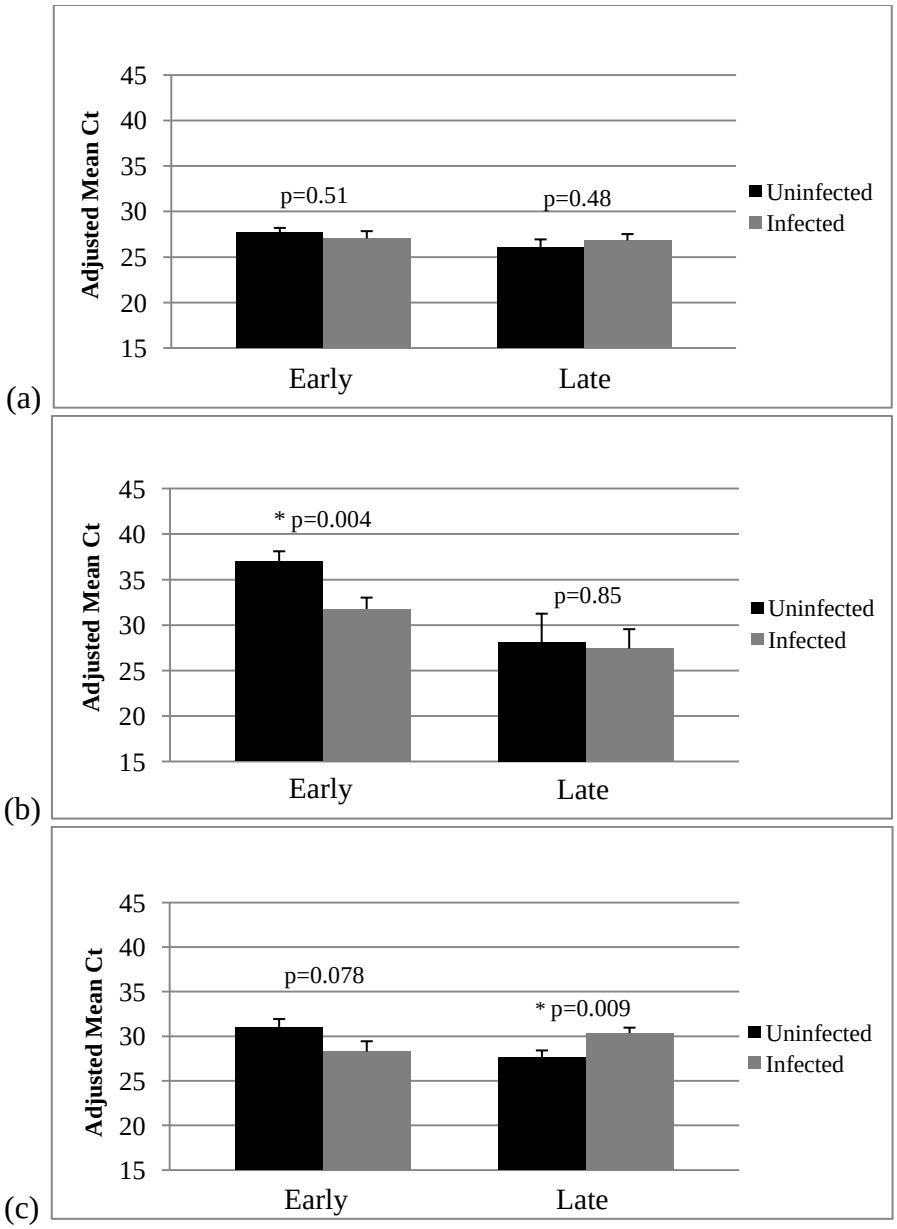


Figure 2.3 Relative Expression of CD25, FOXP3, and CTLA4 in Feline Placentas

TaqMan real time RT-PCR analysis of placental expression of Treg markers CD25, FOXP3, and CTLA4 was performed using control and infected placentas at early and late pregnancy. Placental samples were evaluated as follows: infected (n=17) versus control (n=18) at early pregnancy; and infected (n=18) versus control (n=13) at late pregnancy. Bars represent the adjusted mean Ct values (50 - mean Ct), bracketed by standard errors of the mean. P values obtained from single-value ANOVA are noted. *P values < 0.05 were considered significant. (a) CD25 (b) FOXP3 (c) CTLA4.

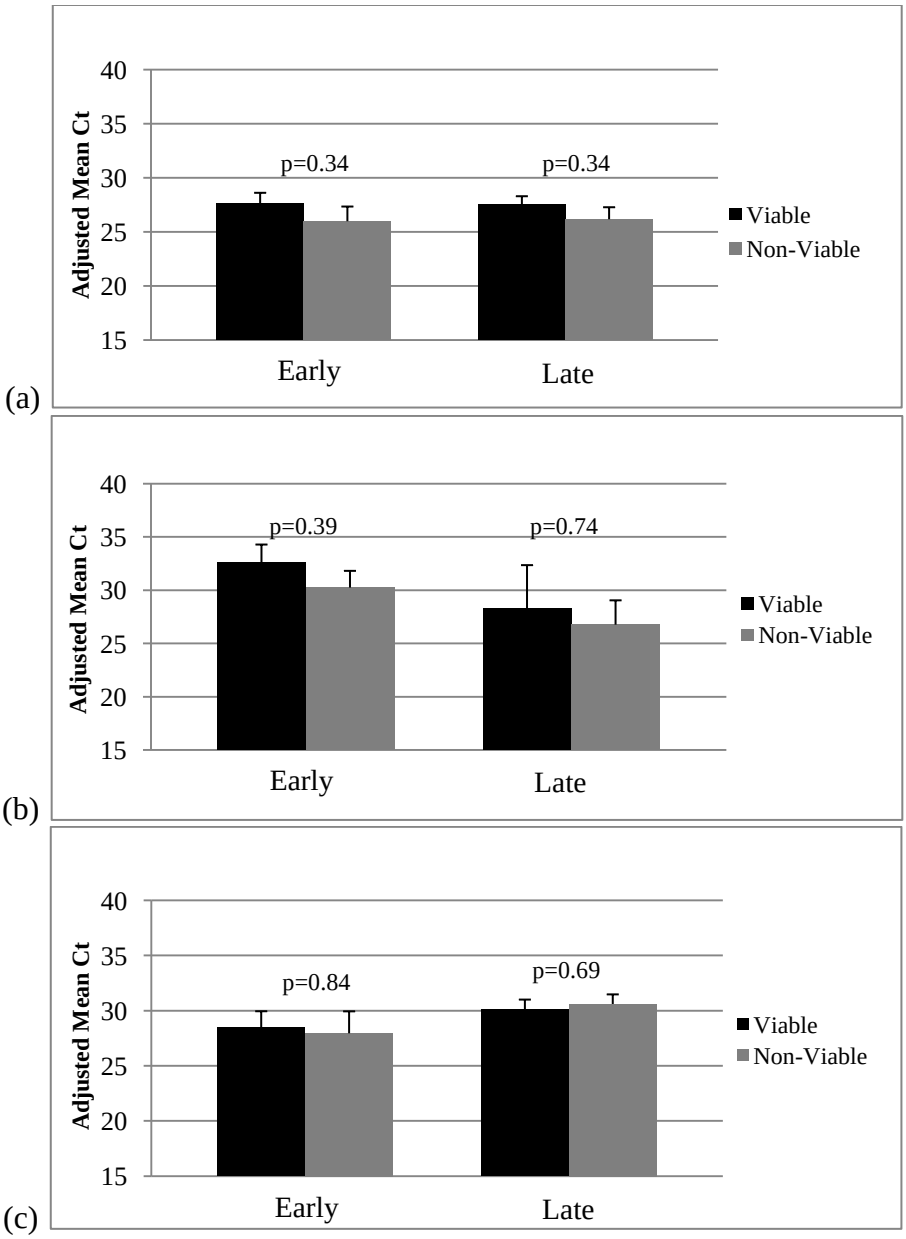


Figure 2.4 Relative Expression of CD25, FOXP3, and CTLA4 in Placentas from FIV-infected Cats

Relative expression of the Treg markers in early and late term infected placentas producing viable versus non-viable fetuses. The placental samples were evaluated as follows: infected cats producing viable offspring (n=11) versus infected cats producing non-viable offspring at early pregnancy (n=6); and infected cats producing viable offspring (n=9) versus infected cats producing non-viable offspring at late pregnancy (n=9). Bars represent adjusted mean Ct values, bracketed by standard errors of the mean. *P values < 0.05 were considered significant. (a) CD25, (b) FOXP3, (c) CTLA4.

Discussion

Successful pregnancy is generally associated with higher levels of activated Tregs in both the periphery and the decidua of humans and mice (Sasaki et al., 2004; Somerset et al., 2004; Zhu et al., 2005). Expansion of Tregs in the periphery from early to mid-gestation, followed by a decrease to pre-pregnancy levels at term was reported (Somerset et al., 2004). A concomitant decrease in CD4+CD25(high) and an increase in CD4+CD25(low) T cells was associated with induction of labor in mice (Zhao et al., 2007). Expansion of the placental Treg population from early to late pregnancy was reported in humans (Chao et al., 2002; Heikkinen et al., 2004).

Feline Tregs are routinely characterized by the co-expression of CD4, CD25, FOXP3, and CTLA4. However, CD25 expression is not restricted to Tregs. Activated T cells, such as CD8+ T cells (Woo et al., 2001), and B cells express CD25 after antigenic stimulation (Lowenthal et al., 1985). FOXP3 is a transcriptional activator that plays a major role in the regulation of Treg function and differentiation (Zheng and Rudensky, 2007). It is expressed by naturally occurring Treg cells and can be upregulated upon activation and differentiation of CD4+CD25- T cells to adaptive Treg cells. In the cat, FOXP3 was also detected in small amounts in CD4+CD25-, CD8+, and CD21+ lymphocytes (Lankford et al., 2008), but it was expressed much more abundantly in CD4+CD25+ T cells. Sustained expression of FOXP3 is necessary to program cells for Treg function (Gavin et al., 2007). CTLA4 is an activation marker expressed by adaptive Treg cells and naturally occurring Tregs. CTLA4 outcompetes CD28 for B7 binding (Tompkins and Tompkins, 2008) and acts as a negative regulator of T cell activation by

preventing cell proliferation, cell cycle progression, and IL-2 production (Krummel and Allison, 1996).

Our previous studies suggest a role for placental inflammation in reproductive failure in the FIV-infected cat model (Weaver et al., 2005). Coupling this preliminary information with knowledge that FIV is preferentially tropic for Tregs, we suspected that FIV infection may alter placental Treg function, allowing placental inflammation, potentiating maternal-fetal transmission, and compromising pregnancy. To lay the groundwork to address this question, we quantified Treg markers CD25, FOXP3, and CTLA4 in early and late term placental tissues from control and FIV-infected animals, detected placental and fetal infection, and documented reproductive outcome among our animals.

MTCT occurred efficiently in chronically-infected queens by week 3-4 gestation, and fetal non-viability in infected queens, including resorbed and arrested fetuses, was 22.2% compared to 4.7% in control queens (Boudreaux et al., 2009). The detection of viral RNA in a non-viable fetus (8035 C) but not a viable fetus from the same litter (8035 A), suggests that fetal death was not a direct result of fetal infection. Such findings allow a role for FIV-induced placental immunopathology in pregnancy failure. Efficient MTCT occurred in chronically-infected queens that delivered at late gestation (Weaver et al., 2005). Fetal non-viability in infected queens, including resorbed and arrested fetuses was 60%, compared to 3.2% in control queens (Weaver et al., 2005). Enhanced reproductive failure in FIV-infected cats was consistent with reports by others using the same or different FIV isolate (Rogers and Hoover, 1998).

We first examined placental Treg marker expression in normal, control cats producing viable offspring at early and late gestation to determine whether Treg populations change over the course of pregnancy. The slightly higher level of expression of CD25 at early gestation did not significantly differ from that of late gestation. However, significantly higher levels of expression of FOXP3 and CTLA4 were found at early pregnancy. The data suggest that cell populations expressing these Treg markers may be normally diminished or less activated as pregnancy progresses. A decrease in number or function of Tregs may allow increased inflammation late in gestation, which is a normal precursor to parturition (Challis et al., 2009).

FIV infection altered the expression of Treg markers in the placenta. At early gestation, there was no difference in the expression of CD25 between control and infected placentas. However, FIV infection resulted in significantly decreased expression of FOXP3 and decreased expression of CTLA4 that approached, but did not reach, significance ($p=0.078$). The data suggest that at early pregnancy, Tregs may be depleted or rendered less functional by viral infection, resulting in the early fetal loss that we reported previously (Boudreaux et al., 2009). The presence of viral RNA in early placenta supports this idea, although the cells harboring the virus were not specifically identified. At late gestation, expression of CD25 and FOXP3 did not differ between control and infected placentas. A possible explanation for this finding may be that natural diminution of Treg activation at late pregnancy (Figure 2) may render these cells less vulnerable to the impact of viral infection. In contrast, FIV infection caused more CTLA4 to be expressed at this time period. Although constitutively expressed on Tregs, CTLA4 is also expressed on other T cell populations 24-48 hours after activation

(Krummel and Allison, 1996; Tompkins and Tompkins, 2008). Perhaps expression of this marker late in infection in FIV-infected cats was attributable to activation of non-regulatory T cells, coinciding with increased inflammation.

Finally, we wanted to determine if alterations in the putative Treg population had any effect on pregnancy outcome. Although differences in expression did not reach traditional significance ($p>0.05$), the obvious trend was lower level of expression of CD25 and FOXP3 in placentas from non-viable pregnancies at both early and late pregnancy, while the mean Ct values for CTLA4 between the two groups were nearly identical at both time points.

An obvious limitation of this study is that Treg populations were not directly enumerated nor was RNA extracted from these precise populations for gene expression analyses. Rather, RNA was isolated from random placental specimens containing unknown quantities of Tregs. Because even subtle changes in gene expression may be significant in terms of function, dilution of RNA from irrelevant cell populations may have masked important information. Thus, in future studies, viral RNA will be isolated from microdissected, labeled Tregs for gene expression analyses.

The value of the present study is that it provides the first evidence of feline placental Tregs. It also demonstrates that FIV infection alters placental expression of Treg markers, possibly leading to compromised pregnancy. The data provide an early glimpse into a possible mechanism for lentivirus-induced reproductive failure, clearly revealing the need for further study of these processes using the FIV-infected cat model.

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CHAPTER III

SUMMARY

MTCT accounts for more than 90% of pediatric HIV-infections (CDC, 2008). With preventative measures, such as antiretroviral therapy and cesarean delivery, MTCT can be reduced to less than 1% (NIAID, 2008). Previously, we reported very high rates of MTCT and frequent reproductive failure in FIV-infected queens at both early and late pregnancy, and increased expression of two inflammatory cytokines accompanied pregnancy failure in the FIV-infected cat (Weaver et al., 2005). These results suggest that FIV infection results in enhanced inflammation in the feline placenta. Furthermore, we reported that higher levels of CD134 and CXCR4 expression accompanied successful pregnancy (Scott et al., 2008), suggesting that cells bearing these markers, such as Tregs, may be important to pregnancy maintenance. Tregs suppress inflammation, and FIV is preferentially tropic for Tregs. Thus, we suspected that FIV infection may alter placental Treg function, allowing placental inflammation which may potentiate maternal-fetal transmission and/or compromise pregnancy.

In Chapter II of this thesis, we reported the first evidence of feline placental Tregs. Our detection of Treg markers in the feline placenta establishes a noteworthy similarity between the feline and human reproductive systems and strengthens the FIV-infected cat as a practical model for both HIV pathogenesis and MTCT. We found very high rates of vertical transmission and reproductive failure at both early and late

pregnancy in infected queens. A comparison of placentas from control queens producing viable fetuses from early and late gestation was performed to look for changes in Treg populations during the course of normal pregnancy. Expression of FOXP3 and CTLA4 was higher in early gestation placentas from control cats. The data suggest that cells expressing those Treg markers are diminished as pregnancy progresses from early to late term, which may allow the natural increase in inflammation during late pregnancy. FIV infection altered the Treg marker expression in the feline placenta. Although no significant change in CD25 expression occurred between FIV-infected and control placentas at early or late pregnancy, FIV infection resulted in reduced expression of FOXP3 and CTLA4 at early pregnancy and increased expression of CTLA4 at late pregnancy. These results suggest that, at early pregnancy, Tregs may be depleted or rendered less functional by viral infection. However, at late pregnancy, the natural reduction of Treg activation may render these cells less vulnerable to the impact of viral infection. Finally, we compared placentas from viable and non-viable pregnancies from FIV-infected cats to determine if alterations in the putative Treg population had any effect on pregnancy outcome. We report no significant difference in Treg marker expression, although a trend toward decreased expression of CD25 and FOXP3 accompanied non-viable pregnancy. Together, our data demonstrate that FIV infection alters placental expression of Treg markers, coincident with adverse pregnancy outcome, although a causal relationship between Treg function and FIV-induced reproductive failure was not clearly established. These results suggest a direction for further research into the mechanisms by which lentiviruses, such as HIV, compromise pregnancy, a direction that points toward placental Tregs.

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APPENDIX A

IMMUNOHISTOCHEMISTRY OF FORMALIN-FIXED PLACENTAL TISSUES FROM EARLY GESTATION

Introduction

CD4+CD25+ Tregs are immunosuppressive T cells that play a major role in the suppression of inflammation, graft rejection, and pregnancy maintenance. In both the mouse model and in humans, placental Tregs allow maternal tolerance of the semi-allogeneic fetus (Michaelsson et al., 2006; Zenclussen et al., 2005). Several groups have reported that successful pregnancy is associated with higher levels of activated Tregs in both the decidua and the periphery (Sasaki et al., 2004; Somerset et al., 2004; Zhu et al., 2005). In the human decidua, CD4+CD25+ T cells comprise approximately 10% of CD4+ T cells during early pregnancy (Chao et al., 2002) and 14% of CD4+ T cells at term (Heikkinen et al., 2004). Expansion of Tregs in the periphery from early to mid-gestation followed by a decrease to pre-pregnancy levels at term was also reported (Somerset et al., 2004).

Transplacentally-acquired HIV infections frequently result in poor pregnancy outcome, including increased rates of miscarriage, stillbirth, pre-term delivery, and low birth weight babies (Kumar et al., 1995; D'Ubaldo et al., 1998; Goldstein, 2000; Anderson, 2001; Rollins et al., 2007). Yet, mechanisms by which HIV compromises pregnancy are poorly understood. We previously reported high rates of MTCT and frequent reproductive failure in FIV-infected queens at both early and late pregnancy (Weaver et al., 2005; Boudreaux et al., 2009). Moreover, our data indicate that placental inflammation may be associated with poor pregnancy outcome (Weaver et al., 2005). Taking into consideration the importance of placental Tregs in pregnancy maintenance reported for both human and murine systems, we believe feline placental Tregs may be implicated in the cat system as well. Therefore, our purpose was to identify Treg

populations (CD4+CD25+ T cells) in early and late gestation, formalin-fixed placental tissues from both FIV-infected and control queens using immunohistochemistry (IHC) and confocal microscopy. Early gestation, formalin-fixed placental tissues were sectioned and subjected to IHC procedures using both monoclonal and polyclonal antibodies to detect T cell markers. Despite variations in antibody treatments and blocking procedures, non-specific antibody binding was an unresolvable problem that precluded our ability to identify Tregs, specifically, in placental sections.

Materials and Methods

Tissue Preparation

Formalin-fixed placental tissues were sectioned to a thickness of 4 µm using a cryostat and placed on poly-L-lysine-coated, RNAse-free slides. Sections were allowed to air dry for 1h or overnight. After drying, the sections were washed twice, 5 min each, in wash buffer (Tris-HCl containing 0.5% Tween). To reverse the protein cross-linking caused by formalin fixation, antigen retrieval was performed by placing each slide in 0.05% citraconic anhydride (2-methylmaleic anhydride) for 45 min at 98°C.

Immunohistochemistry (IHC)

After antigen retrieval, the sections were washed three times, 5 min each, and treated for 30 min with a variety of blocking solutions to identify one that would best minimize non-specific antibody binding. These blocking solutions included serum-free block (2% BSA and 8% DMSO in 100 mL PBS containing 200 µl Triton X-100), blotto (5% non-fat dry milk in wash buffer), 5% bovine serum albumin (BSA), and 5% mouse,

horse, or rabbit serum, according to the primary antibody used. We also used reduced L-Glutathione, mouse Fc block, and purified cat IgG. The primary antibodies used in these experiments targeted T cell markers CD4, CD8, CD25, and FOXP3 and are described in Table 1. Primary antibodies were diluted to 1:10,000, added to the sections, and slides were incubated for 30 min at 37°C on a slide warmer. Sections were then washed three times, 5 min each, in wash buffer. If a labeled primary antibody was used, the slides were then incubated with a DNA stain, DAPI (4,6-diamidino-2-phenylindole), for 5 min, then washed three times, 5 min each. If an unlabeled primary antibody was used, the sections were incubated with the secondary antibody (rabbit anti-goat IgG whole molecule conjugated with FITC), diluted 1:1,000, and placed on a slide warmer at 37°C for 30 min. After three, 5 min washes to remove unbound secondary antibody, the slides were incubated with DAPI for 5 min, and washed again. Slides were mounted with a drop of SHUR/Mount (Triangle Biochemical Sciences) and coverslipped.

Confocal Microscopy

Each section was observed using a confocal microscope (Zeiss LSM 510). Fluorescent detection was done at a wavelength of 521nm for FITC, 570nm for R-PE, and 461 for DAPI. Differential inference contrast (DIC), a transmission mode, was used to enhance the contrast of the transparent sample and give a three dimensional picture of the sections.

Table A.1 Antibodies used in Immunohistochemistry

Target	Antibody Description	Label	Source
CD4	Mouse anti-feline CD4 (clone 3-4F4)	None	Southern Biotech
	Mouse anti-feline CD4 (clone 3-4F4)	R-PE	Southern Biotech
	Goat anti-feline CD4 (polyclonal) (Product no. AF2597)	None	R&D Systems
CD8	Mouse anti-feline CD8 (clone fCD8)	None	Southern Biotech
	Mouse anti-feline CD8 (clone fCD8)	FITC	Southern Biotech
	Goat anti-feline CD8 (polyclonal) (Product no. AF2598)	None	R&D Systems
CD25	Mouse anti-feline CD25	FITC	Dr. Gregg Dean*
FOXP3	Mouse anti-mouse/rat FOXP3 (Product no. 11-5773-80)	FITC	eBioscience
Goat IgG	Mouse anti-goat IgG (Product no. F7367)	FITC	Sigma Aldrich

* North Carolina State University
Note: R-PE = R-phycoerthrin
FITC = fluorescein isothiocyanate

Results

Monoclonal Antibodies

Primary, monoclonal antibodies to CD4, CD8, CD25, and FOXP3 were used to quantify CD4+ and CD8 + T cell populations within the placental tissues and to identify Tregs (CD4+CD25+FOXP3+) within the T cell population (Figure A.1). Unfortunately, we were unable to either quantify T cells or distinguish the Treg population within the sections because all antibodies bound non-specifically. A number of variations in antibody treatment were performed to eliminate non-specific binding. Those treatments included varying the dilution of the antibodies (10 fold dilutions starting at 1:100), diluting the antibody with blocking solutions, and varying the incubation time and temperature (30 min at 37°C or 1 h at room temperature). Although our problem was not resolved, we found that diluting the monoclonal antibodies to 1:10,000 and incubating them on the sections for 30 min at 37°C provided good fluorescent labeling.

Blocking Solutions

We treated the placental sections with a variety of blocking solutions to identify one that would best minimize non-specific antibody binding. Sections were blocked for 30 min, 1 h, and overnight with serum-free block, blotto, BSA, and mouse, horse, and rabbit serum. We found that serum-free block best reduced the background noise seen in the confocal images. It did not, however, eradicate the non-specific binding seen with the monoclonal antibodies.

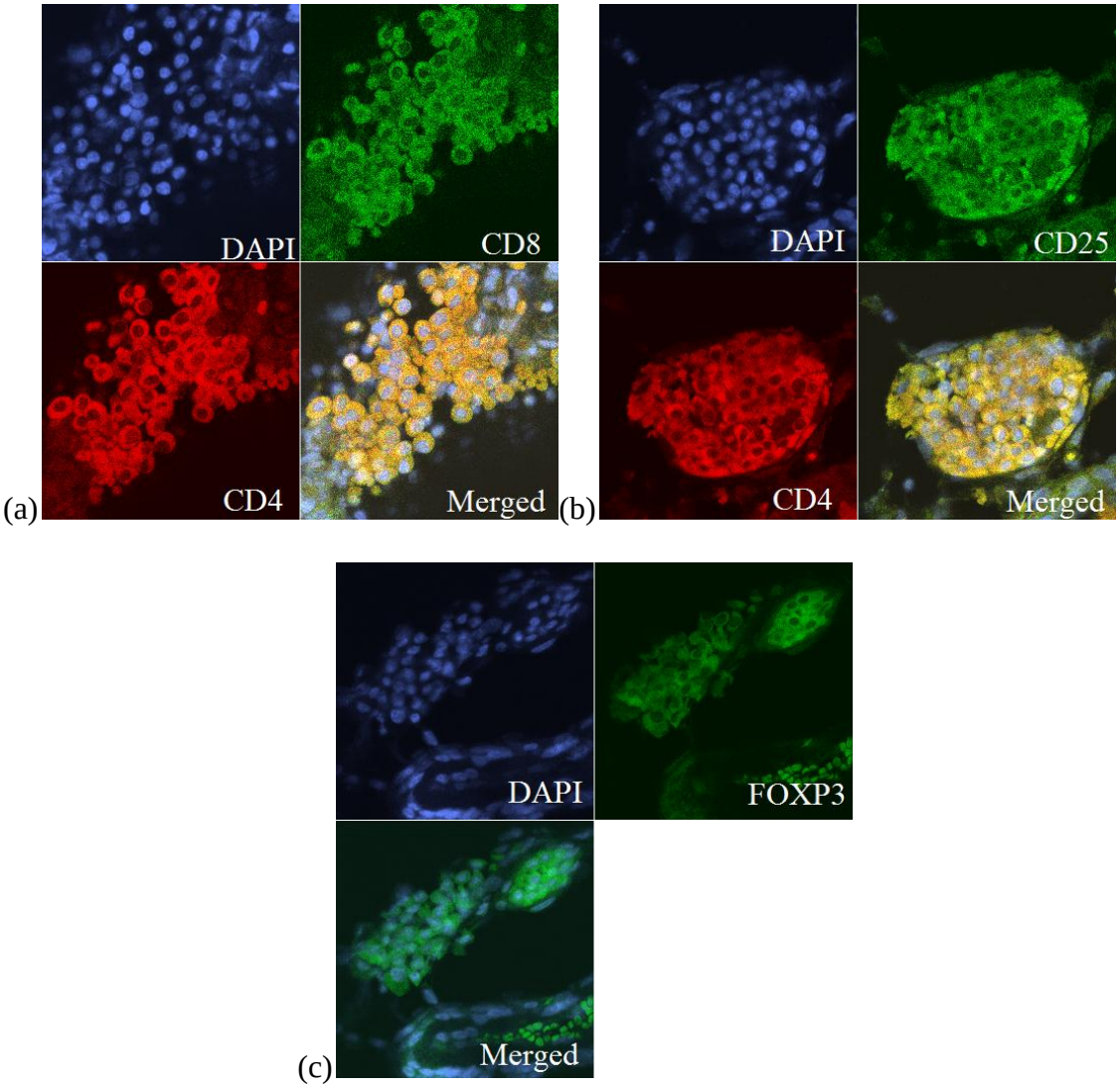


Figure A.1 Immunohistochemistry of Early Gestation, Formalin-Fixed Placenta Tissues from Queen 6108 using Mouse, Monoclonal Antibodies to T cell Markers as the Primary Antibody

Antibodies were conjugated as follows: anti-CD4-R-PE (red); anti-CD8-FITC (green); anti-CD25-FITC (green). All sections were stained with the nuclear stain DAPI (blue). (a) Placenta C was coincubated with monoclonal antibody to CD4 and CD8. Bottom right panel shows merged images. (b) Placenta B was coincubated CD4 and CD25. Bottom right panel shows merged images. (c) Placenta B was incubated with FOXP3. Bottom left panel shows merged images. Magnification 1500X.

Polyclonal Antibodies

After unsuccessful IHC using monoclonal antibodies and various blocking solutions, we used primary, polyclonal antibodies to quantify CD4+ and CD8+ T cell populations within the formalin-fixed placental tissues. Incubation with a primary and secondary antibody proved inconclusive (Figure A.2a). In order to determine if non-specific binding remained, we incubated placental sections with secondary antibody only (Figure A.2b). Secondary antibody reacted with the tissues, showing non-specificity of antibody binding. We then attempted the same variations in antibody treatment used with the monoclonal antibodies. Good fluorescent labeling was produced with the secondary antibody diluted at 1:10,000 and incubated on the sections for 30 min at 37°C.

Reduced L-Glutathione (GSH)

Reduced L-Glutathione (GSH) is a thiol reactive compound that binds antibody sulfhydryl (SH) residues (Rogers et al., 2006). We used this block in an attempt to prevent unwanted disulfide bridges between the thiol groups on antibodies and placental tissues which would cause a non-specific interaction. Antibodies were pre-treated with diluted GSH at concentrations of 0, 3, 6, 9, or 30 mM, and incubated on ice for 1h before being added to the placental sections. The same concentrations of GSH were used to block the sections. Although GSH reduced some non-specific binding of the monoclonal antibodies (Figure A.3a), it did not reduce non-specific secondary antibody binding (Figure A.3b).

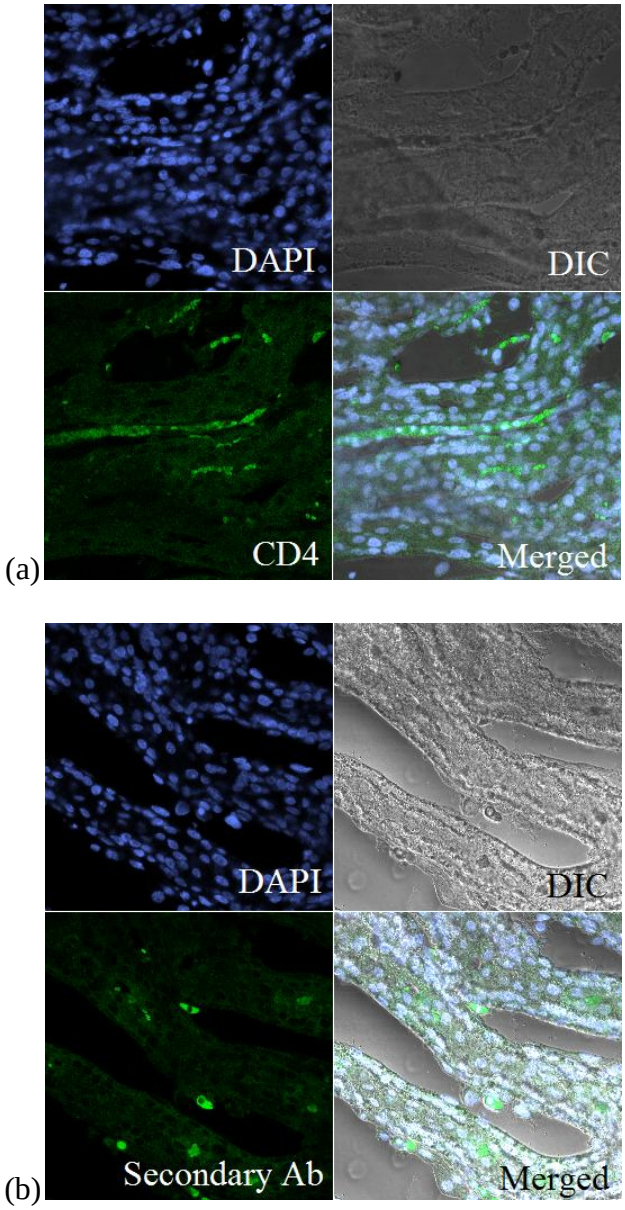


Figure A.2 Immunohistochemistry of Early Gestation, Formalin-Fixed Placenta Tissues from Queen 0373 using Goat, Polyclonal Antibody to CD4 as the Primary Antibody

Secondary antibody was conjugated with FITC (green). Sections were stained with DAPI (blue). (a) Placenta C was incubated with anti-CD4 and secondary antibody and (b) placenta B was incubated with secondary antibody only (green). Both sections were incubated with the DNA stain, DAPI. Bottom right panels show merged images. Top right panels show the image in transmission mode. Magnification 1500X.

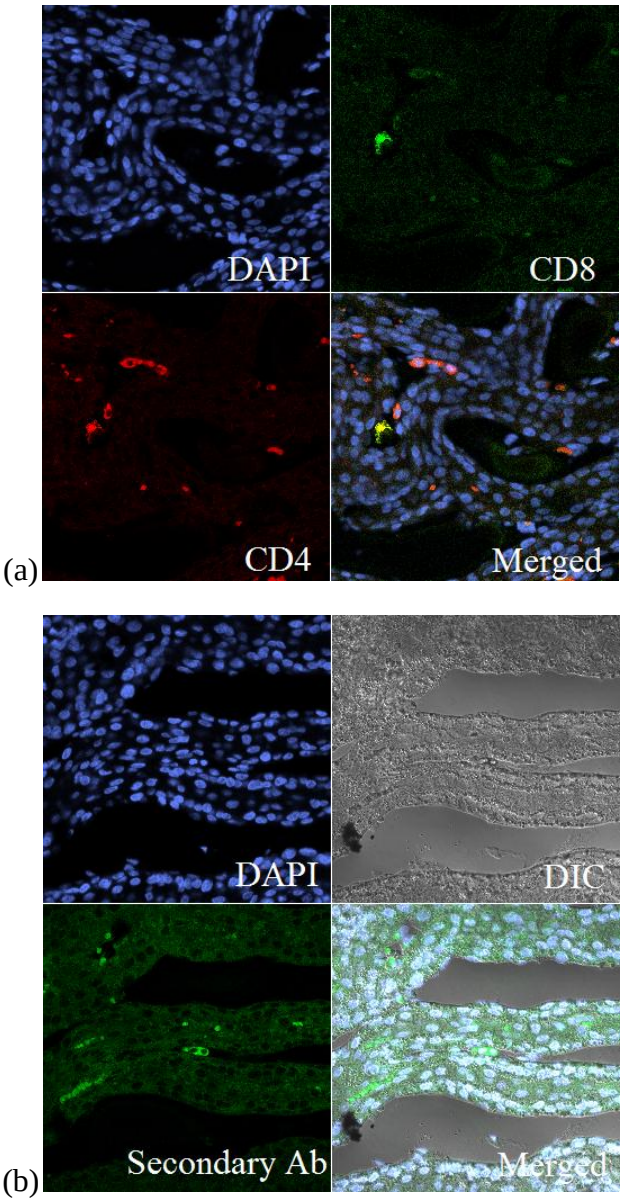


Figure A.3 Immunohistochemistry of Early Gestation, Formalin-Fixed Placenta Tissues from Queen 0373 using Reduced L-Glutathione

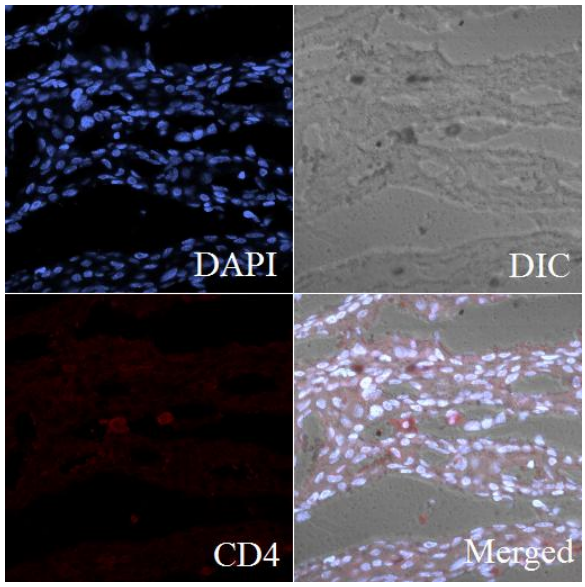
Placental sections were blocked with various dilutions of reduced glutathione before incubating with primary antibody. (a) Placenta C was coincubated with primary, monoclonal antibodies (anti-CD4-R-PE; red and anti-CD8-FITC; green) and blocked with 3mM of glutathione. (b) Placenta B was incubated with secondary antibody only (green) and blocked with 6mM of glutathione. Top right panel shows the image in transmission mode. Both sections (a and b) were incubated with DAPI (blue). Bottom right panels show merged images. Magnification 1500X.

Mouse Fc Block

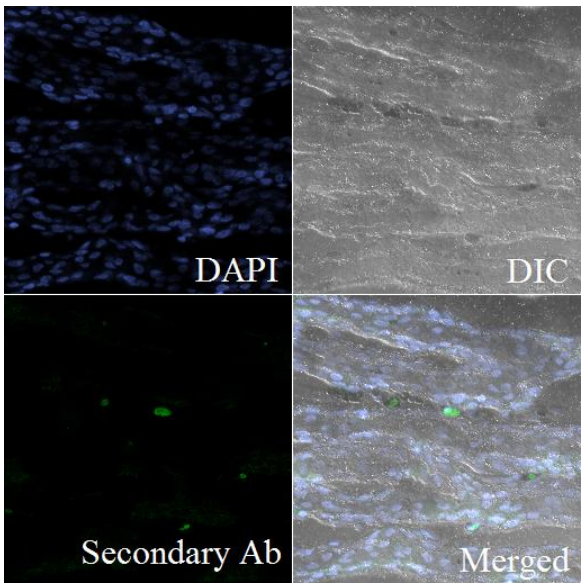
Mouse Fc block was used in an attempt to block the Fc receptors, known to be present on placental tissues (Simister et al., 1996), to which the antibodies may bind non-specifically. Sections were incubated for 5 min at room temperature or 4°C with mouse Fc block at a concentration of 1µg/mL (Figure A.4). This blocking protocol alleviated much of the background noise seen on the confocal images but it did not stop either the monoclonal antibody (anti-CD4) (Figure A.4a) or secondary antibody (Figure A.4b) from binding non-specifically.

Purified Cat IgG

Honig et al (2005) reported difficulty performing IHC in human placental tissues due to non-specific reactivity of monoclonal antibodies of IgG2b isotype to placental tissues. Knowing that this problem was resolved by blocking specimens with human IgG, we attempted to reproduce this blocking procedure using purified feline IgG. Sections were incubated with the purified feline IgG for 30 min at room temperature followed by incubation with monoclonal antibodies for 30 min at 37°C. This treatment did not eliminate non-specific binding if antibodies (data not shown).



(a)



(b)

Figure A.4 Immunohistochemistry of Early Gestation, Formalin-Fixed Placenta Tissues from Queen 0373 using Mouse Fc Block

Placental sections were blocked with mouse Fc block before incubation with primary antibody. (a) Placenta F was incubated with primary, monoclonal antibody anti-CD4-R-PE or (b) secondary antibody only. Both sections were incubated with DAPI. Bottom right panels show merged images. Top right panels show the image in transmission mode. Magnification 1500X.

Discussion

Using the FIV-infected cat model, we demonstrated very high rates of MTCT and reproductive failure at both early and late pregnancy (Weaver et al., 2005; Boudreaux et al., 2009). Moreover, our data indicate that placental inflammation may be associated with poor pregnancy outcome (Weaver et al., 2005). With the knowledge that placental Tregs allow maternal tolerance of the semi-allogeneic fetus in both the mouse model and in humans (Zenclussen et al., 2005; Michaelsson et al., 2006), we believe feline placental Tregs may be implicated in the cat system as well. Our aim was to identify, specifically, Treg populations (CD4+CD25+ T cells) in early and late gestation, formalin-fixed placental tissues from both FIV-infected and control queens using IHC and confocal microscopy.

Early gestation, formalin-fixed placental tissues were sectioned and stained using both monoclonal and polyclonal antibodies. Although all antibodies bound non-specifically, we found good fluorescent labeling when the antibodies were diluted 1:10,000 and sections were incubated for 30 min at 37°C. Several blocking solutions were tested to identify one that would eliminate non-specific binding. Unfortunately, we were unable to eliminate non-specific binding completely. We did, however, find that serum-free block, reduced L-Glutathione (GSH), and mouse Fc block did the best at alleviating excess background noise seen on the confocal images.

Interestingly, non-specific binding did not occur uniformly on all cells in the specimen. It was readily apparent by comparing patterns of DAPI staining with patterns of antibody reactivity, that only subpopulations of placental cells were bound by the

antibodies. However, when an antibody was reactive with cells, those same cells were bound by all other antibodies. The reason for this finding is unknown at the present time.

Other investigators reported difficulty in performing IHC in human placental tissues due to non-specific reactivity of monoclonal antibodies of IgG2b isotype to placental tissues (Honig et al., 2005). The problem was resolved by blocking specimens with human IgG. However, our attempt to reproduce this blocking procedure using purified feline IgG failed to eliminate the non-specific reactivity.

The problems that we encountered with non-specific antibody binding in feline placental tissues were not adequately corrected, and thus, these studies were not pursued beyond those presented herein, due to lack of funds to support further experimentation. A priority of future research will be to quantify feline placental Tregs as we determine a role for these cells in FIV-induced placental immunopathology and reproductive compromise. The value of the present study is that it provides essential knowledge with regard to usage of antibodies and blocking agents, antigen retrieval, and IHC and confocal microscopy techniques to provide an efficient starting point for additional experimentation.

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