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Peripheral and Placental Immunology in the Feline Immunodeficiency Virus (FIV)-Infected Cat Model

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PERIPHERAL AND PLACENTAL IMMUNOLOGY IN THE FELINE IMMUNODEFICIENCY VIRUS (FIV)-INFECTED CAT MODEL

By

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PERIPHERAL AND PLACENTAL IMMUNOLOGY IN THE FELINE

IMMUNODEFICIENCY VIRUS (FIV)-INFECTED CAT MODEL

By

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We are using the feline immunodeficiency virus (FIV)-infected cat to model HIV mother-to-child-transmission (MTCT). Vertical transmission of either virus may result not only in infected offspring, but also failed pregnancy.In HIV infections, maternal hematological and virological parameters predict MTCT.We hypothesized that such parameters would likewise be predictors of FIV vertical transfer. We inoculated ten cats with FIV-B-2542; 10 cats were uninoculated. Cats were allowed to breed naturally. Fetuses were delivered at approximately week 3 (early) gestation by cesarean section. Fetal and placental tissues were collected.Blood samples were collected from the day of inoculation through delivery. We quantified CD4:CD8 T cell ratios, proviral load, and plasma viremia, and monitored seroreactivity to FIV proteins in longitudinal sera from both groups of cats. We documented clinical and reproductive outcome. The infected group produced reduced litter size and more failed pregnancies; CD4:CD8 ratios were depressed by 3.5 months p.i.Proviral DNA was detected in 14 of 14 (100%) placentas tested and 12 of 14 (86%) fetuses. However, the parameters assessed

were not predictive of reproductive outcome and suggested a role for placental immunopathology in compromised pregnancy.Regulatory T cells (Treg) are anti-inflammatory and essential in maintaining pregnancy.Th17 cells are pro-inflammatory and associated with pregnancy failure. The activation of these cell populations is regulated by the cytokines TGF-β and IL-6. We hypothesized that placental immunology may result from altered dynamics of these cell populations.Using immunofluorescence confocal microscopy to measure Treg and Th17 markers FoxP3 and RORγ, respectively, we quantified these cells in placental specimens from FIV-infected and control cats at early and late (week 8) gestation.Significantly higher levels of RORγ were measured in FIV-infected placentas at early pregnancy; these cells co-localized at the maternal-fetal interface. We quantified the expression of Treg immunomodulators by quantitative PCR, noting higher expression of TGF-β in infected queens.A positive correlation of RORγ with IL-6 occurred in control placentas, as predicted, but not in infected placentas.Collectively, the data suggest that an inflammatory placental microenvironment at early pregnancy in infected queens may result, in part, from dysregulation of the Treg/Th17 balance.

DEDICATION

This work is dedicated in loving memory of my grandmothers, Ruby Mae Borne Boudreaux and Thelma Cavalier Boudreaux, and my grandfather, Harold Joseph Boudreaux. You have instilled in me life's secrets to success.

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CHAPTER I

REVIEW OF PERTINENT LITERATURE

Women are the fastest-growing newly HIV-infected population in the United States. Since the onset of the epidemic in 1985, the percentage of women diagnosed with HIV increased from ~7% to 25% (CDC, 2009). In 2009, 42,959 people were infected with HIV in 40 U.S. states and 5 U.S. dependent areas; 9,973 were women and 166 were children under the age of 13. Of the 166 children, 131 of those cases (79%) were a result of perinatal transmission (CDC, 2009). Effective antiretroviral therapy in the U.S. has reduced mother to child transmission (MTCT) to approximately 2% of pregnancies, and pediatric AIDS represents about 1% of all AIDS cases (CDC, 2009). Globally, there are 33.3 million people living with HIV, 15.9 million are women, and 370,000 are children (UNAIDS, 2010). MTCT is the most important means of pediatric infection, accounting for more than 90% of infections (CDC, 2009). The number of new pediatric infections has reached 2.6 million (UNAIDS, 2010). In addition, HIV infection of pregnant women often results in poor outcome, including low-birth-weight babies, preterm delivery, and enhanced incidence of spontaneous abortions (D'Ubaldo et al., 1998; Kumar, Uduman, and Khurranna, 1995; Langston et al., 1995).

The FIV-infected Cat as a Model for HIV Pathogenesis

The FIV-infected cat is an excellent small animal model for HIV pathogenesis and perinatal transmission. FIV and HIV-1 share many similar characteristics as members of the family *Retroviridae,* genus *Lentivirus*. A spherical to ellipsoid core encapsidates a dimeric, positive-stranded, polyadenylated RNA genome 9,200 base pairs in length.The genome organization is similar to other lentiviruses with three large open reading frames (ORF), gag, pol, and env. Core proteins encoded in the gag ORF share antigenic determinants with other members of this genus as a result of the high degree of genetic conservation in the gag region (Olmsted et al., 1989). Amino acid alignments between the highly conserved gag regions of FIV and HIV-1 reveal 40% homology. There are common accessory genes, viral infectivity factor (vif), rev response element (rev), and OrfA involved in regulation of viral gene expression and viral replication.

A difference between the two viruses is host cell tropism. FIV has a broader cell tropism in vivo, including both CD4+ and CD8+ T cells, monocytes/macrophages, B cells, astrocytes, and microglia (Brown et al., 1991; Brunner and Pedersen, 1989; Dow, Dreitz, and Hoover, 1992; English et al., 1993). The primary binding receptor utilized by FIV is CD134, an activation marker on T cells (de Parseval et al., 2004; Shimojima et al., 2004). Both FIV and HIV use the chemokine receptor CXCR4 as a co-receptor for entry into the host cell (Richardson et al., 1999; Willett, Cannon, and Hosie, 2002; Willett et al., 1997).

Natural infection differs between the two viruses.The seroprevalence of FIV ranges from 3-15% of domestic cats to nearly 100% of some wild felid species. FIV is mainly transmitted through bite wounds. Therefore, in domestic cats, infection is most frequent in free-roaming males. The potential for bite transmission was confirmed by the detection of antigen in saliva (Bendinelli et al., 1995).

Clinical Consequences of Infection

The clinical outcome of FIV infection in the cat closely parallels that occurring in the HIV-infected human.FIV-induced disease progresses in stages similar to those occurring in the HIV-infected human (Bendinelli et al., 1995). The acute phase of disease occurs weeks to months after initial infection and is manifested as mild symptoms that typically are not fatal. On a few occasions there have been reports of deaths at this stage of infection, but a causal relationship between viral infection and the deaths of the animals was not determined. Symptoms that may occur during the acute phase include lymphadenopathy, mild pyrexia, dullness, depression, anorexia, acute diarrhea, conjunctivitis, dermatitis, gingivitis, and mild upper respiratory symptoms. Progression of the disease leads to the asymptomatic phase in which the symptoms diminish, and the disease remains clinically silent for a period up to 2 years. The silencing of symptoms during the asymptomatic phase does not correspond to viral clearance, as the virus can be isolated from peripheral blood mononuclear cells, plasma, and saliva during this stage. The AIDS-related complex (ARC) stage occurs with the start of chronic secondary infections of the

oral cavity, upper respiratory tract, and other body sites. Symptoms occurring during this phase include weight loss, lymphadenopathy, fever, hematologic abnormalities, alopecia, and pruritus. Some disorders of the nervous system and kidneys may appear. Feline AIDS is characterized by an increase in the severity of the chronic secondary infections. Neoplastic and neurologic disorders may occur, and unlike the ARC stage, incurable opportunistic infections of the oral cavity and upper respiratory tract caused by *Staphylococcus*sp, *Pseudomonas* sp., *Streptococcuscanis*, and other aerobic and anaerobic bacteria may occur. Progressive weight loss and anemia occur, followed by a rapid clinical decline which can cause death in less than a year.

Viral Load in FIV Infection

High viral RNA loads frequently are detected in peripheral blood mononuclear cells, serum, and plasma during the acute and terminal stages of an FIV infection, while viral loads decline during the asymptomatic phase. Peak viremia may occur at 1-3 weeks post infection(Diehl et al., 1995; Diehl et al., 1996). Disease progression depends on the immune competence of each individual animal; therefore, cats that have rapidly-developing immunodeficiency have a plasma viral RNA load 1 to 2 logs higher than those cats that can withstand the disease for long periods of time (Diehl et al., 1995).

In some cases where FIV-specific antibody reactivity was compared to PCR, animals were seropositive but PCR negative due to the amount of fluctuation in viremia in the early stages post infection. Fluctuation in proviral loads occurring during the course of infection were ascribed to a decrease in

infected CD4+ T cells targeted by cytolytic T lymphocytes (CTLs) and to apoptosis (Ohkura et al., 1997).

The timing of virus expression in animal tissues was evaluated by comparing tissues for the presence of FIV provirus and viral RNA. Provirus was detected in various lymphoid tissues 35 days post infection, but viral RNA was found in the thymus only after 70 days post infection (Ohkura et al., 1997). Infected cells emerging from infected primary lymphoid tissues replenish the supply of infected cells in the peripheral circulation, thereby maintaining provirus within the circulatory system.

Maternal Correlates of Lentiviral Vertical Transmission

Maternal virological and hematological factors including plasma viremia and CD4+ T cell counts influence HIV vertical transfer. HIV-1 targets CD4+T cells, dendritic cells, and monocytes/macrophages. The viral outer-envelope glycoprotein gp120 binds to CD4+ (primary receptor) and a cell specific chemokine receptor (co-receptor), leading to fusion of the viral and cellular membranes and entry of the virus into the targeted cell (Douek et al., 2002). The migratory nature of these cell populations may be responsible for transport of the virus to other tissues.

Plasma viremia is essential to vertical transmission. In one case study, a woman became seropositive for HIV at 34 weeks pregnancy with a viral load 402,000 copies/ml at this time. She was treated with antiretroviral drugs, which decreased the viral loadto 744 copies/ml by week 38. Although her plasma

viremia was still at this level at 8 days prior to delivery, her baby was infected at delivery(Moses et al., 2008).

Phuapradit et al. (1999) documented transmission of HIV-1 in one out of nine fetuses with a maternal viral load greater than or equal to 100,000 copies/ml compared to one out of thirty-two with less than 100,000 copies/ml. High virus titer and low CD4+ count predispose vertical transmission (Blanche et al., 1994; Tovo et al., 1994;Weiser et al., 1994). Assessing maternal CD4/CD8 T-cell ratios is an effective method for predicting potentially infected infants (Read, 2007; Shearer et al., 2007). A South African study showed risk of MTCT by 8 weeks in a population of 962 infants correlating with maternal CD4+ T cell counts (Rollins et al., 2007). Maternal CD4+ T cell counts of 500 cell/ml or more correlated with 38 (9%) infections, 201-500 cell/ml correlated with 76 (18%) infections, and 200 cell/ml or less correlated with 27 (25%) infections (Rollins et al., 2007).

Similar to HIV infections, CD4+ T cell counts in FIV infected cats are inversely related to viral load. O'Neil, et al. (1996) evaluated CD4+ counts and vertical transmission rate. A transmission rate of 88% was seen in queens with CD4+ counts less than 200 cells per ul. Queens with higher counts showed a 39% transmission rate (O'Neil, Burkhard, and Hoover, 1996). In both HIV and FIV infections, MTCT seems to increase when CD4+ T-cell counts fall below 200 cell/ul(O'Neil, Burkhard, and Hoover, 1996).

Hematological Dynamics of Viral Infection

Humans infected with HIV show a depletion of CD4+ T cells as a consequence of virus-mediated destruction of these cells (Deeks et al., 2004;

McCune, 2001; Yates et al., 2007). At each stage of infection, HIV-specific memory CD4+ T cells contain more HIV proviral DNA than other memory CD4+ T cells (Douek et al., 2002), potentially explaining the selective loss of these cells during infection and consequent loss of immunological control of HIV infection. Activated memory T cells, especially those expressing CCR5, are much more vulnerable to lysis by cytotoxic T lymphocytes than resting memory T cells that are CCR5-, leading to a persistent HIV infection (Liu and Roederer, 2007). During early HIV infection, CD4+ and CD8+ T cell activation correlates with the level of viremia, and the rate of CD4+ T cell depletion can be predicted by the activation of CD8+ T cells (Deeks et al., 2004). Infected individuals may develop AIDS rapidly when higher plasma viral loads are present due to the inability of the immune system to replenish destroyed CD4+ lymphocytes (John W. Mellors, 1997).

Maternal CD4+ counts were found to be inversely related to HIV-1 in the cervix, vagina, and breast milk. FIV infects many cell types including CD4+ and CD8+ T lymphocytes, B lymphocytes, and macrophages (Frey, Hoover, and Mullins, 2001).FIV provirus was detected in peripheral blood CD4+ and CD8+ T cells as early as two weeks post infection (Dean et al., 1996).

A feature of infection is the depletion of circulating CD4+ T cells in two different phases of the infection, the first being the primary phase where the largest decline is detected. Shortly thereafter, the depletion becomes more gradual (Bendinelli et al., 1995). Activated CD4+ T cells are depleted largely during the acute phase of an FIV infection due to the expression of the receptor CD134 (Shimojima et al., 2004; Willett, Cannon, and Hosie, 2003). As CD4+ T

cells are depleted, FIV modifies the immune system by activating more CD8+T cells (Paillot et al., 2005).

FIV infections result in a distinctly inverted CD4:CD8 T cells ratio with the depletion of CD4+ cells (Garg, Joshi, and Tompkins, 2004). Song and colleagues (1992) reported that the CD4:CD8 T cell ratio in peripheral blood mononuclear cells dropped below 1.0 by twenty weeks of infection and remained low throughout the course of infection(Song et al., 1992). An inversion of the T cell ratio is further mediated by the differentiation of CD8+ cells into TNF-α+cells which mark themselves for apoptosis, die, and eventually re-establish their population, while CD4+ T cells apparently are not readily replenished (Paillot et al., 2005). Garg et al (2004) reported that apoptosis in activated peripheral blood mononuclear cells is dependent upon binding of the envelope glycoprotein to CXCR4, but not CD134, and that fusion induced by gp41 is essential to this process.

Rate and Timing of MTCT

Vertical transmission of HIV occurs mainly at late gestation and/or at the time of delivery, corresponding to rupture of maternal membranes (Fang et al., 1995; Garcia et al., 1999;Phuapradit et al., 1999). However, early in utero transmission was indicated by the detection of HIV in 8 week fetal tissue (Courgnaud et al., 1991; Lewis et al., 1990) and by the frequent detection of the virus in tissues from electively-aborted, 16- to 24- week fetuses (fetuses less than 16 weeks were not examined) from asymptomatic, HIV-infected women (Courgnaud et al., 1991). Langston, et al (Langston et al., 1995) detected HIV in

four miscarried fetuses that were 18 weeks gestation and younger and in three fetuses that were older than 32 weeks gestation. Collectively, these data indicate that early in utero transmission may be more frequent than recognized.

Rogers and Hoover (Rogers and Hoover, 1998) evaluated the timing of transplacental transmission of FIV-B-2542 in chronically-infected queens, reporting that in utero infection rate increased with progressing gestational age. The virus was detected in 0/16 kittens delivered at 3 weeks gestation, 1/19 (5%) kittens delivered at 5 weeks gestation, 6/16 (38%) kittens delivered at 7 weeks gestation, and 9/15 (60%) kittens delivered at nine weeks gestation. Virus load and hematological parameters in the queens did not vary substantially with gestational age, suggesting that placental or fetal maturation may predispose the timing of in utero infection. Using the same isolate, we detected proviral FIV in at least one tissue from 21 of 22 offspring delivered by cesarean section from infected queens during the eighth week of gestation, a 95% in utero transmission rate. Fourteen of 15 placentas were virus positive, and provirus was frequently detected in blood, brain, liver, bone marrow, spleen, and thymus (Weaver et al., 2005a). Using cats inoculated with either FIV-B-2542 or FIV-AB-2771 a minimum of four months prior to breeding, O'Neil, et al. (O'Neil, Burkhard, and Hoover, 1996) detected FIV infection in 42 of 83 fetuses (51%) at term delivery and in 7 foster-nursed kittens by 6 months of age. The latter group of kittens apparently wasinfected late in gestation or during delivery. At 9 weeks gestation, in utero transmission rates of 67% (8 of 12 fetuses) and 91% (10 of 11 fetuses) occurred in cats infected with FIV-A-Pet and FIV-C-Pgmr, respectively. Viral load and tissue distribution varied with different isolates, and viral sequestration in

fetal tissues frequently occurred in the absence of detectable viremia. Infected fetuses frequently were detected even where virus could not be detected in the placenta (Rogers and Hoover, 2002).

Reproductive Outcome in Lentiviral Infections

HIV infection may contribute to compromised pregnancy and reproductive failure. The CDC reported an estimated six to seven thousand HIV-positive women give birth each year in the U.S. (CDC, 2008). Up to 370 HIV-infected infants are born in the U.S. with 5000 cumulative deaths from perinatally acquired AIDS (CDC, 2008). Evaluating miscarriage rates in a cohort of HIV-infected women from the United States, spontaneous fetal loss occurred in 14 of 124 pregnancies (11.3%) ranging from 8 to 32 weeks gestation, approximately three times the rate of loss in the general population at comparable gestational stages(Langston et al., 1995). HIV was detected in 7 of 12 (58%) of these aborted fetal tissues. A retrospective study of HIV-infected Italian women revealed a 67% increase in the risk of spontaneous abortions in that group (D'Ubaldo et al., 1998). Significantly increased rates of spontaneous abortion and other indicators of compromised pregnancy were noted in a group of HIV-infected Indian women(Kumar, Uduman, and Khurranna, 1995). In a population of 1449 HIV- infected South African pregnant women, there were 13 (0.90%) spontaneous abortions, 5 (0.35%) antenatal deaths, and 63 (4..35%) stillbirths (Rollins et al., 2007). HIV infection is associated with low-birth-weight babies (Goldstein, 2000) and pre-term delivery (Anderson, 2001). Pre-term delivery appears to correlate with immune suppression (less than 14% CD4+ T cells) in

HIV-infected women (Cohan, 2003). No differences in reproductive outcome between HIV seropositive and negative womenwere reported in other studies(Massad et al., 2004).

FIV infection in the pregnant queen may result in pregnancy perturbation. In our study of vertical transmission using FIV-B-2542-infected queens, average litter sizes delivered during the eighth week of gestation were 2.7 kittens/litter and 3.8 kittens/litter in FIV-infected and control queens, respectively (Weaver et al., 2005b). Fetal non-viability was 60% (15 of 25 offspring) in the infected group and 3.2% (1 of 31 offspring) in the control group. Fetal resorptions and fetuses developmentally arrested early in pregnancy comprised the non-viable offspring, indicating that perturbation occurred early in pregnancy. In a separate study using FIV-B-2542, 11 cats were infected that produced 25 viable and 18 non-viable kittens (Allison and Hoover, 2003). Arrested fetal development, stillbirth, and abortion associated with in utero FIV-CSU-2771 infections were reported (O'Neil et al., 1995). Nine of 17 of the kittens (53%) were either stillborn or dead within 48 hours of birth in that study. O'Neil et al. infected a group of 13 queens with either of two clinical isolates FIV-B-2542 and FIV-AB-2771. From the 13 queens, 83 kittens were produced with 51% of the kittens testing FIV positive at birth (O'Neil, Burkhard, and Hoover, 1996). Viability was 70% with 58 kittens and non-viability was 30% with 25 non-viable kittens (O'Neil, Burkhard, and Hoover, 1996). Collectively, these data demonstrate the deleterious effect of FIV infection on pregnancy and indicate that compromised pregnancy in the FIV-infected cat may parallel pregnancy loss that occurs in HIV-infected women.

The immunology of pregnancy

The phenomenon of immune tolerance to the fetal allograft is not fully understood. Nobel Laureate Peter Medawar began the field of pregnancy immunology in 1953 with three hypotheses in an attempt to explain the suppression of the maternal immune system and tolerance to the fetus: (a) physical separation of mother and fetus; (b) antigenic immaturity of the fetus; and (c) immunologic inertness of the mother(Medawar, 1953). Wegmann and Raghupathy refuted these hypotheses in 1980 with the Th1/Th2 paradigm (Raghupathy, 1997; Wegmann et al., 1993). The paradigm established the idea that up regulation of Th2 anti-inflammatory cytokines and suppression of Th1 pro-inflammatory cytokines correlates with a successful pregnancy (Lim et al., 2000; Raghupathy, 1997; Raghupathy, 2001; Raghupathy et al., 1999; Raghupathy et al., 2000).

Immunoregulatory cytokines produced at the maternal-fetal interface are essentialfor a successful pregnancy, and this site favors, but is not limited toTh2 responses (Mellor, 2000). Suppression of the inflammatory cytokines is necessary in the early stages of pregnancy because Th1 cytokines induce placental inflammation, leading to pre-eclampsia, pre-term delivery, and/or spontaneous abortion (Lim et al., 2000; Makhseed et al., 2000;Makhseed et al., 2001). Currently, the paradigm is challenged with new evidence revealing the contributions of innate and adaptive immunity to regulatory mechanisms essential for pregnancy (Chaouat et al., 2004; Lim et al., 2000; Saito et al., 2010; Sargent, Borzychowski, and Redman, 2006; Trowsdale and Betz, 2006).

The inflammatory cytokine IL-18, which causes increased IFN-γproduction by T cells and NK cells, was expressed at lower levels in abortion prone mice (Ostojic et al., 2003).Other murine studies show both inflammatory and anti-inflammatory cytokines present at the maternal-fetal interface (Zourbas et al., 2001)and lower inflammatory cytokine levels of IL-12 in the decidual and peripheral leukocytes from spontaneous abortion patients as compared to patients experiencing a normal pregnancy (Zenclussen, 2006). Mellor and Munn stated that the uterus may be "preconditioned" to acceptthe fetal allograft as dramatic changes in cytokine balance are likely to be minimal at mucosal surfaces and lymph nodes draining the maternal-fetal unit (Mellor, 2000).

The mechanisms of transplacental transmission and pregnancy perturbationassociated with lentiviral infections are unclear. Placental inflammation in HIV-infected women was hypothesized to contribute to transplacental transfer as a result of abnormal cytokine expression (Shearer et al., 1997). Disruption of cytokine expression in placental trophoblasts caused by the virus may contribute to inflammation of the placenta and transplacental transfer (Shearer et al., 1997).

Using the FIV-infected cat model, parameters of lentiviral placental inflammation can be analyzed. We found no significant differences in the expression of placental inflammatory or anti-inflammatory cytokines between infected and control cats at week eight gestation (Weaver et al., 2005a). However, in nonviable pregnancies, inflammatory cytokine expression was increased (Weaver et al., 2005a). In addition, expression of CXCR4 and CD134 was detected at differing levels in infected and control populations of viable and

nonviable fetuses (Scott et al., 2008). The surface expression of these markers suggested that cells bearing these receptors play a role in pregnancy maintenance. Recently, we found evidence of a significant decrease in the number of regulatory T cells in infected versus control whole placental tissue at early pregnancy (Lockett et al., 2010). The inflammatory cytokine IL-6 was also found to be significantly up regulated in whole placental tissues at early pregnancy (Scott et al., 2011). Our collective data support a pro-inflammatory placental microenvironment at early pregnancy.

Regulatory T cells and pregnancy

Natural regulatory T cells were first characterized in mice when a small percentage ofCD4+ T cells expressing the alpha-chain of the interleukin-2 receptor, CD25, were depleted causing autoimmune disease (Sakaguchi et al., 1995). Regulatory T cells comprise 5-10% of peripheral CD4+ T cells and suppress autoimmune diseases, graft rejection, and innate mucosal immunity (Maloy and Powrie, 2001; Maloy et al., 2003). CD25 is not always consistently expressed and can be down-regulated to CD4+CD25- upon activation; however, these cells can potentially remain suppressor cells (Chen et al., 2005). Forkhead transcription factor family (FoxP3) and cytotoxic T lymphocyte antigen 4 (CTLA4) are both considered activation markers for T regs. The X chromosome encodes FoxP3 and a mutation causes X-linked autoimmune disease (Brunkow et al., 2001). FoxP3 is a unique marker that was identified when normally suppressing CD4+CD25+ cells were not suppressive in scurfy mice upon mutation on the X

chromosome causing autoimmune disease (Li et al., 2008). Nearly all current reports identify Tregs by their expression of FoxP3.

Tregs are immunosuppressive via two mechanisms, either by a cell-contact-dependent manner or by release of cytokines (Akenasy, 2008). The release of cytokine IL-4, IL-10 and transforming growth factor (TGF)-β inhibit the proliferation of T cells by suppression of IL-2 production (Thornton and Shevach, 1998).

T regs suppress immune activation by modulation of tryptophan catabolism via CTLA-4 expression(Fallarino et al., 2003). CTLA-4 is an inhibitory cell surface protein that out competes CD28 for binding to B7, a co-stimulatory molecule, on antigen presenting cells. The enzyme used for catabolism of tryptophan, indoleamine2,3-dioxygenase (IDO), is produced by antigen presenting cells and trophoblasts to suppress T cell proliferation by the depletion of tryptophan(Mellor, 2000). Dendritic cell IDO production is dependent upon the activation marker CTLA-4 expression by Treg cells and INF-γ expression by dendritic cells (Fallarino et al., 2003). IFN-γ production increases CTLA-4 expression on human decidual Treg cells which up-regulates IDO (Miwa et al., 2005). Mice treated with increased concentrations of an IDO inhibitor showed a dose dependent increase of fetal allograft rejection (Miwa et al., 2005). Inflammation at the maternal-fetal interface resulted in failed pregnancies where IDO expression was reduced in DCs and monocytes (Miwa et al., 2005).

Several studies have correlated increased number of activated Treg cells in the periphery and decidua with successful pregnancies, while a reduced number of Treg cells accompany failed pregnancies (Sasaki et al., 2004; Zhu,

2005). Treg cells from normal pregnant mice were adoptively transferred to abortion prone mice preventing fetal rejection (Zenclussen, 2006). Only Tregs previously exposed to paternal alloantigens were found to have regulatory activity that is needed to prevent miscarriage (Zenclussen, 2006). These Treg populations can cross the placenta from the maternal to fetal side to reside in lymph nodes with the ability to suppress auto-reactivity to the fetus (Mold et al., 2008). This response has been shown to be induced in utero and assist in regulating infant immune responses after birth (Mold et al., 2008).

Feline lentiviral infection and T regulatory cells

Feline T reg cells are also characterized by FoxP3 expression. Typically, CD4+CD25+ T cell populations express FoxP3; however, in the cat, small populations of CD4+CD25-, CD8+, and CD21+ lymphocytes also express FoxP3. The population of feline Treg cells comprises 5-10% of the circulating CD4+ lymphocytes and 20-30% of lymph node CD4+ cells. This Treg population has an IL-2-deficient anergic phenotype that is responsive to LPS and capable of suppressing Con A-stimulated CD4+CD25- cells (Joshi et al., 2004).

CD4+CD25+ regulatory T cells are infected and activated during acute FIV infection (Mexas et al., 2008). Higher viral mRNA levels were found in CD4+CD25+ cells than CD4+CD25- suggesting increased viral replication or infectivity early after infection. These cells remain activated throughout the course of infection (Mexas et al., 2008). CD4+CD25+ T regulatory cells in acute FIV infection up-regulated FoxP3 and TGF-β; furthermore, increased levels of FoxP3 mRNA production were detected by intracellular protein expression

(Mexas et al., 2008). It is still uncertain if natural regulatory T cells play a major role in lentiviral pathogenesis of the virus or if the induced Tregs from naïve CD4+ T cells have a larger contribution.

Th17 cells and pregnancy

Th17 cells differentiate from CD4+ T cells activated through antigenic stimulation by IFN-γ or IL-4. These cells are responsible for secreting the cytokine IL-17 and express transcription factor RORγ. Upon activation, Th17 cells have pro-inflammatory properties that induce the expression of several cytokines and chemokines, including IL-6, IL-8, IL-21, IL-22, IL-17, TNFα, CXCL1 and CXCL10. IL-17 is important for recruitment of cell types responsible for the elimination of gram-negative and fungal infections and can dictate the recruitment of CD4+ Th1 cell response to control the infection (Bettelli, Korn, and Kuchroo, 2007).

IL-17 has also been detected in murine studies with known autoimmune diseases such as multiple sclerosis, rheumatoid arthritis, and psoriasis (Bettelli, Korn, and Kuchroo, 2007). Mice deficient in the IL-23 cytokine, also responsible for the proliferation of Th17 cells, had decreased production of IL-17 and developed resistance to autoimmune diseases. Levels of IFN-γ did not differ between deficient and non-deficient mice; however, with the decreased levels of IL-17, recruitment of CD4+ T cells for a Th1 response may be affected. Regulation of the T cell response results in an inverse relationship between T regulatory cells exhibiting anti-inflammatory properties and Th17 cells exhibiting pro-inflammatory properties (Bettelli, Korn, and Kuchroo, 2007).

Transforming growth factor–β (TGF-β) is responsible for the induction of FoxP3 transcription factor expressed in T regulatory cells. With the unveiling of Th17 cells, a role for TGF-β in the differentiation of Th17 cells was also identified. The immune system has an intricate cytokine system in place for the regulation of expression of these two cell types. TGF-β causes the proliferation of both Treg and Th17 cells from a common progenitor. T regulatory cells and Th17 cells have an inverse relationship controlled by the cytokine IL-6. During inflammation, IL-6 production inhibits the induction of FoxP3 and, thus, the generation of T regulatory cells. In the presence of both IL-6 and TGF-β, Th17 cells proliferate. Thus, IL-6 plays a prominent role in regulating a pro- or anti-inflammatory immune response, a role that may be critical in placental immunology and reproductive outcome. Our recent data showed a significant increase in the level of IL-6 in early FIV-infected placental tissues, suggesting an inflammatory microenvironment that may cause pregnancy failure (Scott et al., 2011).

Several studies have demonstrated an increase in both Th17 cells and IL-17 in the peripheral blood and in the decidual tissues of unexplained recurrent spontaneous abortion (URSA) patients as compared to non-pregnant women (Liu et al., 2011; Toldi et al., 2011) (Liu et al., 2010; Toldi et al., 2011). These data indicate a role for Th17 in local pregnancy maintenance that occurs in parallel with the systemic response (Liu et al., 2011).

Significance and Purpose

The FIV-infected cat is a well-accepted small animal model for HIV pathogenesis. We are using the FIV-infected cat to study immunopathology occurring in the placenta as a consequence of viral infection that may result in vertical transfer of the virus and/or reproductive failure. The first part of this project examines the consequences of early FIV infection that occur in the peripheral circulation of the queen. The significance of this research is that it may provide insight into maternal serological, immunological, and virological correlates that predispose vertical transfer and/or predict reproductive outcome. Thus, we hypothesized that detectable serological, immunological, and virological consequences accompany early maternal FIV infection, possibly identifying maternal correlates of infection that are predictive of pregnancy outcome. The objectives of part 1 were to: 1) quantify CD4+ and CD8+ T cell population dynamics in blood samples collected from FIV infected and control cats; 2) determine serological reactivity to viral proteins in longitudinal serum samples; 3) quantify viremia in longitudinal blood samples; and 4) evaluate FIV status, clinical consequences, and pregnancy outcome in queens.

The immunology of the placental environment must be precisely controlled for pregnancy to be successful. Among the many leukocyte populations that are involved in the placental immune response, two cell populations are emerging as particularly relevant, Tregs and Th17 cells.Tregs suppress inflammation by inhibiting activation of immune cells. Tregs areimportant in the maintenance of pregnancy by allowing maternal tolerance of the semi-allogeneic fetus. FIV replicates preferentially in and activates this cell population, potentially

suppressing antiviral immunity and allowing chronic FIV infection to be established. Tregs are present in the feline placenta and likely play an immunosuppressive role.Th17 cells are a newly-recognized population of inflammatory T cells whose role in reproductive failure and placental infection in the human has recently come to light. There are no current reports of the involvement of these cells in feline gestation. Altered function of both cell populations resulting from FIV infection may produce placental immunopathology, predisposing MTCT and/or reproductive failure. The second part of this study focuses mainly on Treg dynamics in the feline placenta. We hypothesized that FIV infection significantly impacts Treg dynamics and activation in early and late-term placental tissues from viable and non-viable pregnancies, predisposing MTCT and/or reproductive failure.

The following objective were addressed in part 2 of this study: 1) determine the placental Treg population density in FIV-infected versus control cats; 2) evaluate placental Treg activation in the study populations at early and late pregnancy by measuring the expression of Treg products and cell surface markers; 3) quantify virus load in these cells.

Likewise, we recently recognized the potential role for Th17 cells in pregnancy and hypothesized that FIV infection impacts Th17 dynamics in the same tissues. A preliminary objective was to determine whether these cells exist in feline placentas and to quantify expression of the definitive Th17 marker, RORγ.

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CHAPTER II

MATERNAL HEMATOLOGICAL AND VIROLOGICAL CHARACTERISTICS DURING EARLY FELINE IMMUNODEFICIENCY VIRUS (FIV) INFECTION OF CATS AS PREDICTORS OF FETAL INFECTION AND REPRODUCTIVE OUTCOME AT EARLY GESTATION

Boudreaux, C. E., N. N. Lockett, et al. (2009). Maternal hematological and virological characteristics during early feline immunodeficiency virus (FIV) infection of cats as predictors of fetal infection and reproductive outcome at early gestation. Veterinary Immunology and Immunopathology 131(3-4): 290-297.

Abstract

The FIV-infected cat is a small animal model for HIV mother-to-child transmission (MTCT) because the two lentiviruses are biologically related and produce similar clinical syndromes. Both viruses are vertically transmissible and may negatively impact reproductive outcome. Maternal hematological and virological parameters are predictors of MTCT in HIV-infected women. Our purpose was to determine whether similar maternal characteristics during early pregnancy in FIV-infected cats influence pregnancy outcome. We inoculated ten cats with FIV-B-2542; 10 cats were uninoculated.We quantified longitudinal CD4:CD8 T cell ratios, proviral load, and plasma viremia, monitored longitudinal serostatus, and documented clinical and reproductive outcome during early pregnancy. Inoculated queens were seropositive and provirus positive by week 4

post infection (p.i.). CD4:CD8 ratios were depressed in the infected group by month 3.5 p.i. Proviral load was variable in the animals throughout the course of infection; plasma viremia was below the level of detection in all animals. Reduced litter sizes and increased fetal demise occurred in infected queens. Viral RNA, but not proviral DNA, was detected in representative placentas (14 of 14; 100%) and fetuses (12 of 14; 86%) collected from infected queens. However, maternal virological and hematological characteristics did not correlate either positively or negatively with reproductive outcome.

Introduction

Globally, more than 420,000 children were newly infected with HIV in 2007, representing 16% of new HIV infections (UNAIDS, 2008). MTCT accounts for more than 90% of pediatric infections (CDC, 2008). In addition, HIV infection of pregnant women often results in poor outcome, including low-birth-weight babies, preterm delivery, and enhanced incidence of spontaneous abortions (D'Ubaldo et al., 1998; Kumar et al., 1995; Langston et al., 1995).

Maternal virological and hematological factors including plasma viremia and CD4+ T cell counts influence HIV vertical transfer. MTCT typically accompanies a decrease in the maternal CD4+ T cell population, resulting from virus-mediated destruction of these cells (Deeks et al., 2004; McCune, 2001; Yates et al., 2007), and high maternal plasma virus load (O'Donovan et al., 2000).

The FIV-infected cat provides an excellent small animal model of HIV MTCT because the viruses share many common genetic and biological features

and produce very similar clinical syndromes in their respective hosts (Willett et al., 1997). FIV is readily transmitted from mother-to-child under experimental conditions, resulting in pregnancy outcomes similar to those of HIV-infected pregnant women.

A high rate of vertical transmission of FIV occurs late in gestation in experimentally-infected cats with frequent reproductive failure (Allison and Hoover, 2003a; O'Neil et al., 1996; Rogers and Hoover, 1998, 2002; Weaver et al., 2005). We hypothesized that maternal virological and hematological characteristics occurring during early pregnancy in the FIV-infected cat may be predictive of pregnancy outcome. Our objectives were to: 1) quantify T cell population dynamics occurring in the peripheral circulation of queens during early FIV infection; 2) confirm and quantify FIV infection in longitudinal blood samples; and 3) determine virus-induced reproductive outcome during early pregnancy. We report reduced fecundity and increased fetal loss during early gestation in the infected group. Viral RNA, but not provirus, was detected in placentas and fetuses. The CD4:CD8 T cell ratio declined significantly in the infected group within 3.5 months p.i. However, individual CD4:CD8 T cells ratios neither positively nor negatively correlated with pregnancy outcome. Plasma viremia was below detectable limits at all time points in all cats, but cats were provirus positive and seropositive within four weeks p.i. and remained positive throughout the duration of the experiment. Maternal hematological and virological correlates of reproductive outcome were not clearly identified in this study.

Materials and Methods

Animals and virus

Cats were female, reproductively mature, specific pathogen-free (SPF) animals (*Felis domesticus*), obtained from a commercial cattery. Physical evaluation of cats, including respiration, pulse rate, weight, and body condition, was done two weeks prior to inoculation by trained veterinary staff. Ten cats were inoculated intravenously with 1 cc of a feline plasma pool containing approximately 1 x 10^4 copies per ml of FIV-B-2542 (Weaver et al., 2005); ten cats were uninoculated controls. All animals were evaluated by caretakers on a daily basis, and veterinary care was administered appropriately. Whole blood (15 ml) was collected into Vacutainer7 tubes at biweekly to monthly intervals until delivery for the collection of serum, plasma, and peripheral blood leukocytes (PBLs). Following confirmation of infection by PCR and serology, described below, queens were allowed to breed naturally with SPF males. The time of FIV inoculation until delivery ranged from approximately 9.5 months to 13.5 months (mean 11.14 months). Fetuses were delivered by cesarean section immediately after pregnancies were confirmed by ultrasonography, at week 3-4 gestation. Infected queens were euthanized following delivery using Beuthanasia at 1 cc per 10 lb body weight. Control cats were spayed and released for adoption after recovery. Fetuses, placentas, and amniotic fluids were collected from all animals. Tissues were snap frozen in liquid nitrogen or fixed in formalin. Tissues and amniotic fluids were frozen at -80C. Animal protocols were approved by the Institutional Animal Care and Use Committee of Mississippi State University.

Flow cytometric analysis of CD4+ and CD8+ T cell populations

Mouse anti-feline monoclonal antibodies to CD4 and CD8 conjugated with phycoerythin (PE) and fluorescein isothiocyanate (FITC) (Southern Biotech, Birmingham, AL), respectively, were diluted 1:20 in PBS. Whole blood was sampled at 250 µl, and red cells were lysed with ACK lysing buffer (Quality Biological, Inc., Gaithersburg, MD). All samples were washed with PBS and centrifuged at 400 x g for 5 min using a Beckman TJ-6 centrifuge. Samples were double stained with 40 µl of each diluted antibody and incubated 15 min at room temperature in the dark. Flow cytometry was performed using FACSCalibur (BD Biosciences, San Jose, CA); 10,000 events were collected.

Whole virus purification

Supernatant and cell lysate collected from FIV-infected T cell cultures (MCH 5-4, kindly provided by Dr. John Elder) were centrifuged at 11,750 x g in a GSA centrifuge at 4^{o}C for 20 min to remove cellular debris. The clarified supernatant was centrifuged at 23,000 x g for 90 min at 4^{o}C using an SW27 rotor and a Beckman L8-80M Ultracentrifuge. The supernatant was discarded, and the tubes were inverted and allowed to drain. Pellets containing virus were resuspended in 200 µl of PBS and stored at -80^{o}C. Real time reverse transcriptase (RT)-PCR, described below, was used to confirm FIV RNA in these preparations.

SDS-PAGE and Western blot

Viral proteins from the purified virus preparations were resolved under reducing conditions on 12% SDS-polyacrylamide gels. Electrophoresis was

done using a Mini Protein II system (Bio-Rad Laboratories, Hercules, CA), according to standard procedure (Laemmli, 1970).

Proteins were trans-blotted onto nitrocellulose membranes (Micron Separations Inc., Westborough, MA) using the Trans Blot SD Semi-Dry Transfer cell (Bio-Rad) and Towbin buffer containing methanol (25mM Tris, 192mM glycine, 20% methanol, pH 8.3). The nitrocellulose was cut into vertical strips for use in Western blots.

Longitudinal serum samples (diluted 1:50) were evaluated by Western blot according to established protocol (Whetstone, 1991). The secondary antibody (diluted 1:1000) was goat anti-cat IgG peroxidase (MP Biomedicals, Solon, OH). Blots were developed using a solution of 0.02 g 4-chloronapthol dissolved in 5 ml of methanol, 25 ml 0.05 M Tris (pH 6.8), and 12 :l hydrogen peroxide. Positive and negative control sera, obtained from FIV-B-2542 inoculated and control cats from a previous study (Weaver et al., 2005), were included in all Western blot assays.

ELISA to detect FIV-specific antibody in serum

Longitudinal sera from the inoculated queens were evaluated for seroconversion using the SNAP FIV/FeLV Combo Test Kit (IDEXX Laboratories, Westbrook, ME), according to kit instructions. Sera collected at week 2 p.i and thereafter were evaluated until seroconversion was detected.

ELISA to detect FIV p24 antigen in plasma

Plasma samples collected at three different time points p.i. were evaluated for FIV p24 antigen using the Petcheck Feline Immunodeficiency Virus Antigen

Test Kit (IDEXX Laboratories), according to kit instructions. Absorbance values at 650 nm (A_{650}) for plasma samples and positive and negative controls (supplied in kit) were measured using a plate reader. The cutoff value was calculated based on mean values for negative controls. Samples with A_{650} values below the cutoff value were considered negative.

Purification of DNA and RNA from feline plasma, PBLs, and tissues

TRIzol Reagent (Invitrogen, Carlsbad, CA) was used to fractionate RNA and DNA from PBLs and sections of placental and fetal tissues. RNA was purified from the aqueous phase as described (Scott et al., 2008). DNA was further purified from the organic phase using the DNEasy Kit (Qiagen, Valencia, CA). RNA was isolated from plasma using the QIAampViral RNA Mini Kit (Qiagen). DNA and RNA concentrations were determined using a NanoDrop 1000 (Thermo Scientific, Waltham, MA) and frozen at -80^{o}C. Viral RNA to be used as standard was isolated from purified, whole virus, using the QIAamp Viral RNA Mini Kit (Qiagen), treated with RNase-free DNAse, and quantified to estimate copy number.

Detection of provirus in PBLs and placental and fetal tissue using PCR

FIV provirus was amplified from PBLs and feline tissues using standard, nested PCR targeting a 293 bp region of the FIV gag gene (Allison and Hoover, 2003a, b). DNA obtained from FIV-B-2542 infected MCH 5-4 cells was used as a positive control in PCR analyses. Reaction conditions were reported (Weaver et al., 2005). The limit of detection was previously determined to be 1 to 10 copies of target (Allison and Hoover, 2003a, b).

Quantification of FIV provirus by limiting dilution PCR

A two-fold dilution series of DNA isolated from PBLs at three time points p.i. was done to determine the end point dilution for detection of FIV provirus, using PCR to detect the 293 bp gag proviral fragment. It was assumed that the highest positive dilution of DNA contained at least one copy of provirus. Virus load was estimated as copies per microgram of DNA.

Detection of FIV RNA in placentas, fetuses, and maternal plasma using TaqMan real time RT-PCR

Real time TaqMan Primers and probes targeting the FIV gag gene and β-actin were obtained commercially (MWG-BIOTECH, Inc., High Point, NC). Sequences (Scott et al., 2008; Weaver et al., 2005) and 5' and 3' probe labels (Scott et al., 2008) were previously reported. The real-time RT-PCR, targeting FIV gag in placental, fetal, and plasma RNA, was done using an iCycler (Bio-Rad) according to established parameters, and normalized Ct values were determined (Scott et al., 2008). To determine the limit of detection, five-fold dilutions of the RNA standard in DEPC-treated water (ranging from 1:5 to 1:1.95 x 10^6) were subjected to the real time RT-PCR protocol to achieve a standard curve. The limit of detection was 2 x 10^4 copies/µl RNA.

Statistical analyses

Statistical evaluation of T cell populations was done using single-factor ANOVA (Microsoft Excel-XP, Redmond, WA).

Results and Discussion

Routine physical examination of control queens began at their arrival and continued throughout the breeding period, which ranged from 5 months to 9.5 months (mean 7 months) after experimentation began. No noteworthy abnormalities were revealed. Several abnormalities were noted among the FIV-infected group. Within approximately 6 weeks p.i., cat 6850 developed sudden dehydration and fever which was unresponsive to antibiotic therapy. The cat was euthanized at month 2 p.i. Cat 0326 developed conjunctivitis at month 3 p.i. which responded well to antibiotic treatment. Three cats, 6062, 5587, and 3779 developed reproductive problems, discussed below.

Maternal hematological and virological characteristics are known to influence maternal-fetal transmission of HIV. High levels of plasma viremia and depressed CD4+ T cells positively correlate with fetal infection (O'Donovan et al., 2000). We evaluated these predictors of early in utero transmission during the first year of FIV infection in the cat model of MTCT.

For both control and infected groups, the mean percentages of CD4+ and CD8+ T cells were determined at each time point sampled, and CD4:CD8 T cell ratios were calculated. Longitudinal CD4:CD8 ratios for individual infected cats are shown in Figure 2.1. The CD4:CD8 ratio for the control group was calculated from the collective CD4+ and CD8+ T cell percentages of all control cats measured. With the exception of cat 5111, whose CD4:CD8 ratio increased, FIV infection caused a decreased T cell ratio in all cats. However, T cell dynamics were not predictive of pregnancy outcome. The mean CD4:CD8 ratio was significantly depressed in the infected group by month 3.5 p.i. (P=0.03), and it

remained depressed at later sampling intervals. The decreased T cell ratio was a result of depletion of the CD4+ T cell population in the infected group by month 3.5 p.i., while no significant changes were observed in the CD8+ T cells in the infected group.

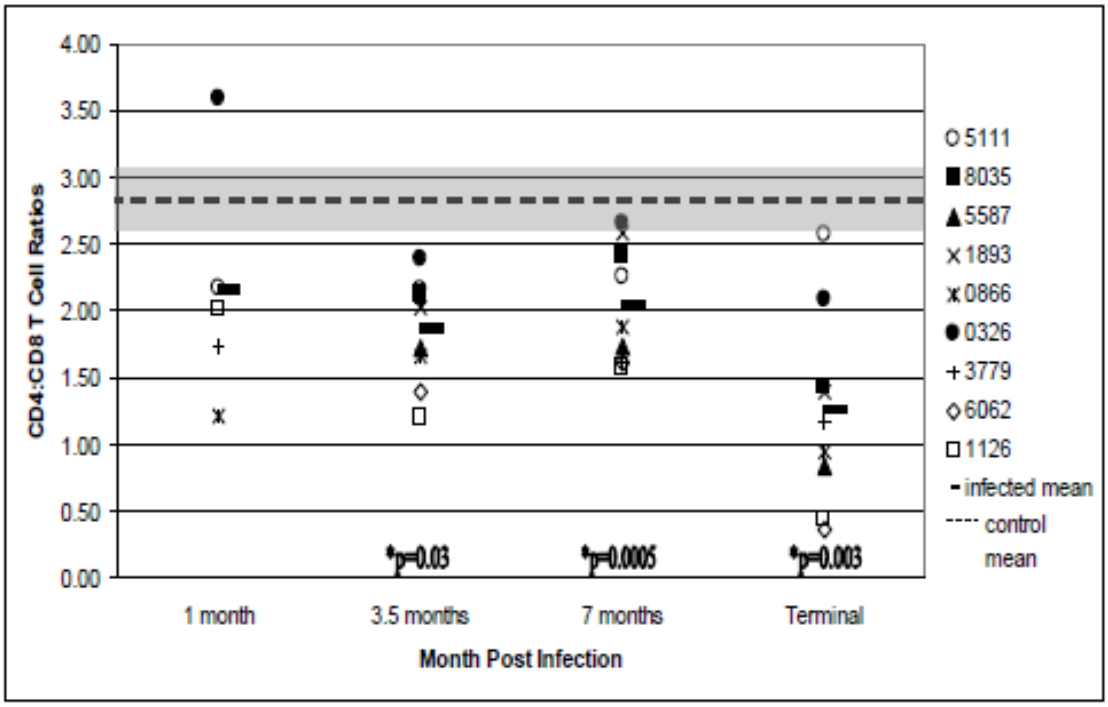


Figure 2.1 CD4:CD8 T cell ratios in longitudinal blood samples from FIV-infected cats.

The mean CD4:CD8 ratio for control cats, determined by compiling data from all sample periods, is included for comparison (dashed line at 2.75 flanked by the standard error of the mean, shaded in grey). Individual cat numbers are shown in the legend at right. CD4+ and CD8+ T cells were quantified from whole blood samples collected at regular times points p.i. The data are plotted beginning at 1 month p.i. and representative time points are shown through termination. Although the T cell ratios fluctuated in individual animals over time, by delivery and/or euthanasia at 9 to 12 months p.i., a decline in the CD4:CD8 T cell ratio had occurred in all animals except cat 5111. CD4:CD8 ratios were significantly different from the control by 3.5 months p.i. and at subsequent time points ($P < 0.05$).

The mean CD4:CD8 T cell ratio determined for control cats in this study was 2.7, falling within the normal range for cats (Dean et al., 1991; Novotney et al., 1990). In a previous study, the CD4:CD8 T cell ratio in peripheral blood dropped below 1.0 by twenty weeks of FIV infection and remained low throughout the course of infection (Song et al., 1992). The depletion in the CD4+ T cell population was explained in part by the virus's preferential infection of activated CD4+ T cells, which most abundantly express CD134.

By week 4 p.i. all animals were weakly seropositive for FIV p24-specific antibody, as determined by ELISA (Table 1). Nearly all cats remained seropositive with increasing intensity of seroreactivity at subsequent time points until termination/delivery (Figure 2.2). FIV provirus was detected in PBLs of all infected animals at week 4 p.i. Animals remained provirus positive throughout the duration of the study (Figure 2.2).

Table 2.1 Maternal Hematology, Virology, and Pregnancy Outcome in Control and Infected Queens

Cat number	FIV Serostatus*	Plasma Viremia**	CD4:CD8 Ratio (Terminal)	Viable Fetuses	Non-viable Fetuses	Reproductive complications
3550	-	nt	2.19	4	0	
6108	-	nt	2.68	4	0	
7824	-	nt	1.91	4	0	
8291	-	nt	1.44	4	0	
8059	-	nt	3.90	6	0	
2779	-	nt	2.29	7	1	
5373	-	nt	3.30	7	0	
9276	-	nt	1.82	5	1	
4102	-	nt	3.25	0	0	Unknown uterine abnormality
9307	-	nt	2.70	0	0	Failed to conceive
Total Fetuses				41	2	4.7% non-viability
0326	+	-	2.09	1	0	
8035	+	-	1.43	1	3	
5111	+	-	2.57	2	1	
0866	+	-	0.95	5	1	
1893	+	-	1.39	5	1	
6062	+	-	0.37	3	0	Right horn pyometra
1126	+	-	0.43	4	0	
5587	+	-	0.84	0	0	Pyometra
3779	+	-	1.18	0	0	Ovarian cysts
6850	+	-	N/A	0	0	Euthanized before mating
Total Fetuses				21	6	22.2% non-viability

* Sera from all inoculated queens were tested by ELISA and Western blot;all queens were positive by week 4 p.i.

**Plasma was tested by real time RT-PCR for viral RNA and by FIV p24 ELISA for viral antigen.

nt = Not tested

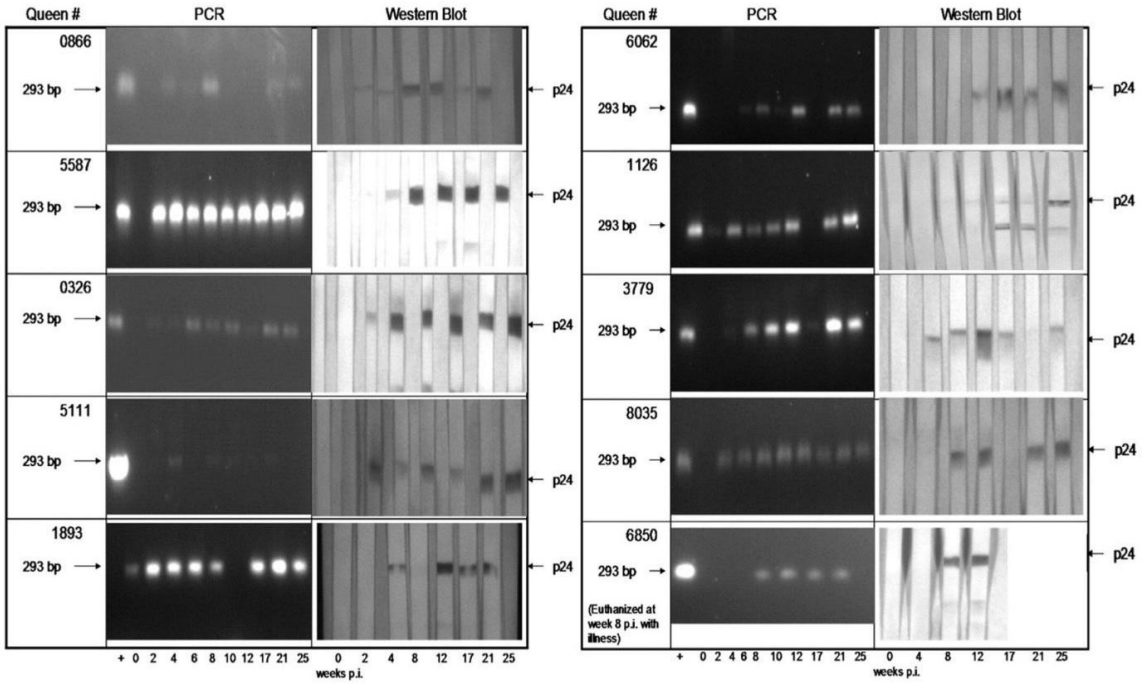


Figure 2.2 Detection of FIV provirus and FIV p24-specific antibodies in longitudinal blood samples from FIV infected cats.

Left panels: FIV provirus was detected by standard PCR targeting a 293 bp region of the gag gene. PCR products were resolved on 1% agarose gels. By week 4 week p.i., the virus was detected in all infected animals. (+) shows the PCR product amplified from DNA purified from cultured MCH 5-4 cells infected with FIV-B-2542. Right Panels: Western blot analysis of longitudinal serum samples (diluted 1:50) was performed following resolution of purified FIV proteins on 12% SDS-PAGE gels and transfer to nitrocellose membranes. Positive and negative controls (not shown) were sera obtained from FIV infected and uninfected cats from a previous study. FIV p24 is the major immunodominant viral protein, and seroreactivity was most consistent toward this protein. Seroconversion was detected by week 4 p.i. and persisted until termination.

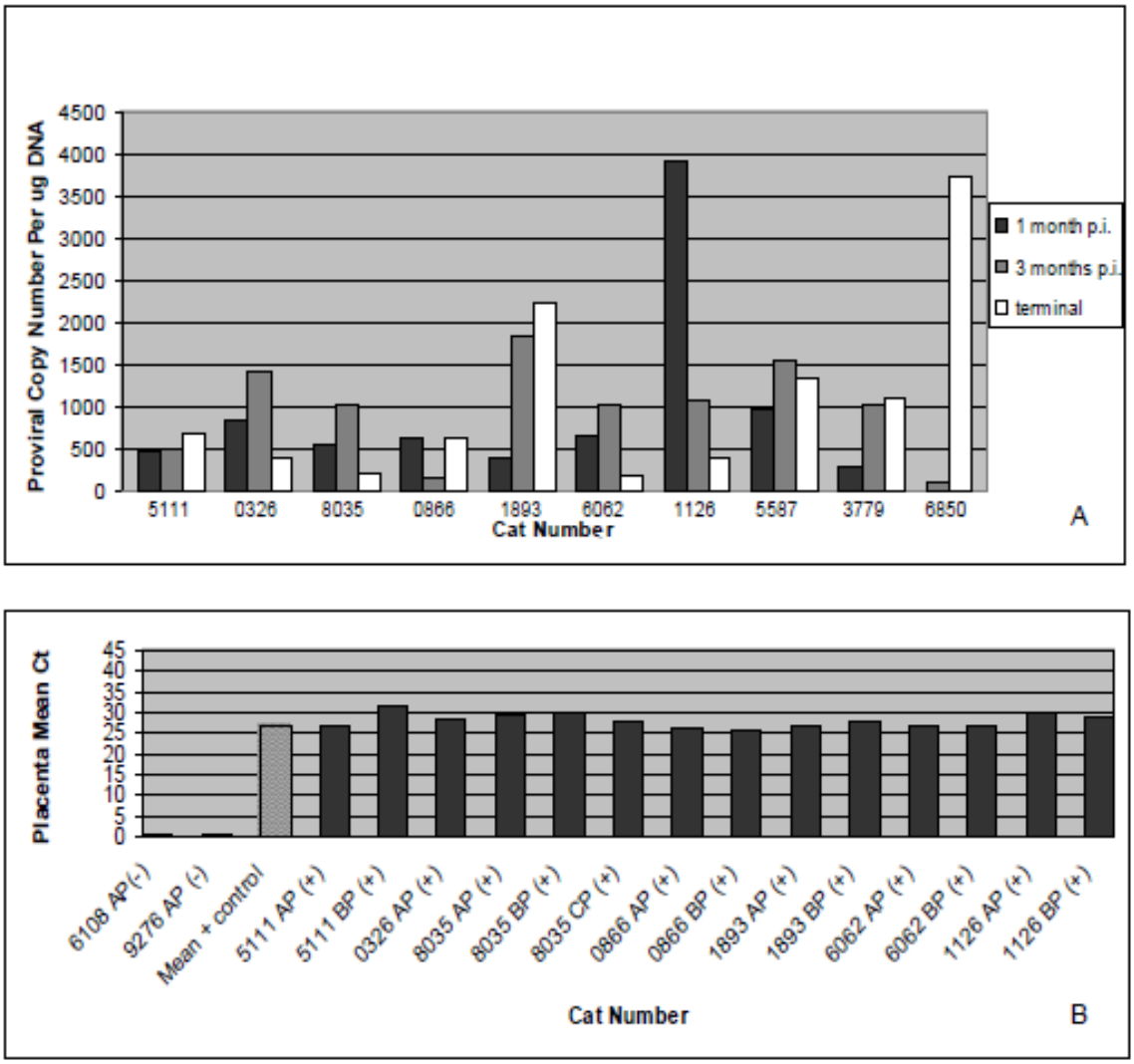
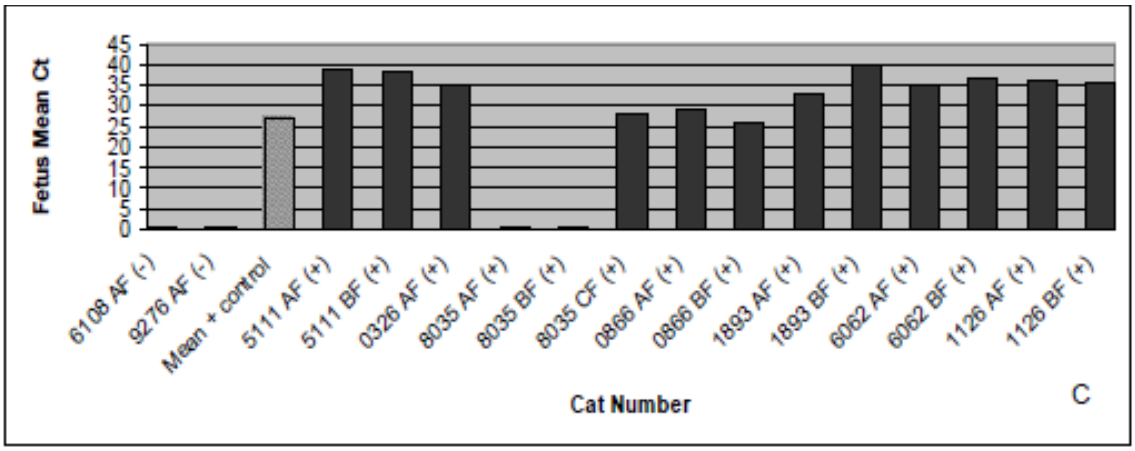


Figure 2.3 Determination of FIV proviral copy number in longitudinal blood samples and relative expression of viral RNA in placentas and fetuses from FIV infected cats.



2.3 (continued)

(A) Proviral copy number was determined using limiting dilution PCR targeting a 293 bp region of the FIV gag gene. Proviral load is reported as copies/:g DNA at 1 and 3 months p.i. and terminal time points. (B) Placental and (C) fetal expression of FIV RNA was determined using TaqMan real time RT-PCR targeting the FIV gag gene. Mean Ct values were determined from duplicate samples. Ct values are inversely related to viral RNA load. Placentas or fetuses from two uninoculated cats (6108 and 9276) were included as negative controls. Mean positive control (striped bar) represents mean Ct values from 10 placental samples from 6 FIV positive cats from a previous study.

Proviral loads, estimated as copy number per microgram of DNA, were determined at months 1 and 3 p.i. and at delivery and/or euthanasia (Figure 2.3A). By 1 month p.i., proviral loads varied among infected queens, ranging from a low of 80 copies/μg DNA to greater than 3900 copies/μg DNA.

Interestingly, the proviral load of cat 6850, euthanized due to unresolvable infection at month 2 p.i, was lowest of any cat at 1 month p.i. but increased more than any other animal, a 41-fold increase by month 2 p.i (Figure 2.3A). This increase in proviral load represented a 7-fold greater increase than in the cat producing the next highest increase in provirus.

Proviral copy number in peripheral blood was neither positively nor negatively correlated with pregnancy outcome. Queen 1893 produced the

largest litter size, 5 viable fetuses and 1 non-viable fetus (Table 2.1), yet this animal had the highest proviral load (> 2200 copies/µg DNA) of any reproductively-successful animal (Figure 2.3A). On the other hand, queen 8035 produced the greatest amount of fetal damage (3 non-viable fetuses; 1 viable fetus) (Table 2.1), but this animal had among the lowest terminal proviral loads (Figure 2.3A).

Two animals who failed to conceive, cats 5587 and 3779, had among the highest proviral loads (> 1000 copies/µg DNA) at the time of euthanasia (Figure 2.3A). While it is tempting to speculate that FIV infection caused the infertility of these two cats, reproductive tissues from these two cats were not evaluated for viral infection. Moreover, two control cats also failed to conceive after repeated attempts at breeding. Consequently, the role that FIV infection may have played in their reproductive pathologies is unclear.

The variability in proviral load that we observed is not uncommon in FIV infection. Fluctuation in proviral loads during the course of infection due to a decrease in infected CD4+ T cells was reported by others who determined that CD4+ T cell depletion occurred as a result of targeting by cytolytic T lymphocytes and apoptosis (Ohkura et al., 1997).

Using real time TaqMan RT-PCR targeting FIV gag RNA, fourteen representative placentas and their corresponding fetuses were evaluated for viral infection (Figure 2.3B and C). With the exception of queen 0326, who produced only one offspring, two to three placentas per queen were evaluated (Figure 2.3B). Fourteen of fourteen placentas (100%) and twelve of fourteen fetuses (86%) were positive. A comparison of mean Ct values provided a means to

determine relative gene expression from each sample. Negative control tissues yielded no fluorescent signal (Ct = 0); positive control tissue yielded a mean Ct value of 27. Twelve of fourteen placentas were strongly positive (Ct < 30), while two placentas (5111 B, 1126 A) gave a moderate reaction (Ct 31.4 and 30.1, respectively). On the other hand, only three fetal specimens were strongly positive (8035 C, 0866 A, and 0866 B) (Figure 2.3C). The remaining samples were either moderately to weakly positive (Ct >30) or negative (8035 A and B). It was interesting to note that two of three placentas from cat 8035 (A and B) were negative, while the third placenta (8035 C) was strongly positive. Samples B and C from this animal were placentas corresponding to arrested fetuses, while sample A came from a viable fetus. Also, fetus 0866 A was viable while 0866 B was a resorption. Both of these fetal samples were strong positives. While copy number was not quantified in these samples, these data suggest that the virus load in placental or fetal tissue did not determine pregnancy outcome.

Proviral DNA was not detected in placental or fetal tissues using standard, nested PCR analysis. These findings indicate that our calculated real time RT-PCR detection limit (2 x 10^4 copies/µl) is an underestimation of assay sensitivity, probably due to co-purification of cellular RNA in our viral RNA standard, which confounds calculation of viral copy number. Others (Rogers and Hoover, 1998) were unable to detect fetal infection prior to 5 weeks of gestation but detected infection in 96% of placentas at early, intermediate, and late stages of gestation, using standard, nested PCR. MTCT occurring in HIV-infected women is most frequent during late stages of pregnancy and at delivery (Brossard et al., 1995; Chouquet et al., 1999).

FIV p24 antigen was not detected in any of the plasma samples collected from infected cats at any time point p.i. Likewise, viral RNA was not amplified from plasma samples from infected cats at any time period. Spiking plasma with standard viral RNA resulted in amplication, proving that reaction conditions were appropriate. Thus, plasma viremia was below the level of detection in all animals. Maternal plasma viral RNA loads were measured previously in FIV-B-2542-infected queens at 3, 5, 7, and 9 weeks pregnancy. Plasma viral RNA loads below the detection threshold (1000 copies/ml) occurred in nearly half the queens, regardless of pregnancy stage, and only one animal had a plasma viral RNA load above the threshold value (Rogers and Hoover, 1998).

Pregnancy outcome in individual control and infected queens is reported (Table 1). Eight control cats produced 41 viable and 2 non-viable fetuses (4.7% non-viability), an average of 5.4 offspring/litter. Two control animals failed to conceive. An undiagnosed uterine abnormality was noted upon ultrasonography of one of these animals (4102). The other cat, 9307, showed no evidence of reproductive pathology. Both of these animals were otherwise clinically normal. Seven infected queens produced 21 viable and 6 non-viable fetuses (22.2% non-viability), an average of 3.9 offspring/litter. Infected cats 5587 and 3779 failed to conceive after repeated matings, due to pyometra and ovarian cysts, respectively. Cat 6062, who produced 3 viable fetuses in the left uterine horn, had right horn pyometra at delivery. 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 ($P>0.05$).

High rates of fetal non-viability in full or near-full term FIV-infected cats were reported elsewhere (O'Neil et al., 1995; Weaver et al., 2005), but rates of fetal demise in FIV-infected cats at this early stage of pregnancy have not been reported.

These same maternal hematological and virological characteristics were not evaluated by us at late pregnancy, where high rates of fetal infection and increased reproductive failure occurred (Weaver et al., 2005). However, other investigators found that these maternal markers did not change significantly with gestational age, although MTCT correspondingly increased (Rogers and Hoover, 1998).

In summary, maternal correlates of HIV vertical transmission, including peripheral blood provirus load, plasma viremia, and depressed CD4+ T cell populations, neither positively nor negatively correlated with fetal infection or pregnancy outcome in the FIV-infected cat model at 3-4 weeks gestation. Our data, coupled with those reported previously (Rogers and Hoover, 1998), indicate that the experimentally-infected cat may not adequately model these particular maternal correlates of HIV MTCT. However, it is clear that early FIV infection reduced fecundity, compromised reproductive outcome, and resulted in a high rate of MTCT during early weeks of pregnancy. The inability to correlate fetal infection with reproductive outcome suggests an indirect mechanism of fetal damage, such as virus-induced placental pathology or pathology of other reproductive tissues. We previously reported preliminary evidence for a role for placental inflammation in compromised pregnancy in the FIV-infected cat (Scott

et al., 2008; Weaver et al., 2005). The effect of FIV infection on placental immune parameters at early and late pregnancy is currently ongoing.

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CHAPTER III

TH17/TREG POPULATIONS AT EARLY AND LATE TERM GESTATION IN FELINE IMMUNODEFICIENCY VIRUS (FIV) INFECTED AND UNINFECTED PLACENTAL TISSUE

Abstract

The T cell immune response plays an important role in the maintenance of pregnancy. Regulatory T cell (Treg) populations, an immunosuppressive population of CD4+ T cells, are essential in maintaining the anti-inflammatory placental microenvironment that protects the semi-allogeneic fetus from the maternal immune response. Th17 cells are a pro-inflammatory CD4+ T cell population involved in autoimmune disease and the protective response to extracellular microbes. Low levels of Tregs, and conversely, abnormally high levels of Th17 cells in the placenta are associated with reproductive failure in humans. We previously found evidence of a pro-inflammatory placental microenvironment in feline immunodeficiency virus (FIV)-infected cats at early pregnancy and a high rate of reproductive failure in these animals. We hypothesized that FIV infection in the pregnant cat causes altered placental Treg and Th17 cell populations, possibly allowing lentivirus-induced placental inflammation and frequent fetal demise. We quantified the expression of Treg marker FoxP3 and Th17 marker RORγin placental samples from FIV-infected and control queens at early and late term gestation by measuring fluorescence

intensity by confocal microscopy. We detected significantly higher levels of RORγ in FIV-infected placentas at early pregnancy, indicating that the viral infection caused increased numbers of Th17 cells to be localized in the placenta at this stage of pregnancy. These data shed additional light on the mechanism of FIV-induced inflammation in the feline placenta.

Introduction

The phenomenon of immune tolerance to the fetal allograft is not fully understood. Paternal antigens expressed by the embryo must be tolerated by the maternal immune system for pregnancy to be successful. Maternal tolerance is accomplished, in part, by the expression of cytokines and other immunoregulatory molecules at the maternal-fetal interface which modulate appropriate placental immunology (Kwak-Kim et al., 2010; Mellor, 2000). The Th1/Th2 paradigm, which assumed that Th2 (anti-inflammatory) cytokines dominate the maternal-fetal interface during most of pregnancy while Th1 (pro-inflammatory) cytokines are suppressed, has been recently succeeded by a Th1/Th2/Th17 and regulatory T cell paradigm (Saito et al., 2010) which allows for a cytokine milieu during various stages of pregnancy which does not fit the traditional Th1/Th2 model.

Tregs are an immunosuppressive T cell population characterized phenotypically by the surface expression of CD4 and CD25 (the alpha chain of the IL-2 receptor) and intranuclear expression of the transcriptional activator FoxP3. Tregs facilitate an anti-inflammatory response with the release of IL-10 and TGF-β which suppress expression of such inflammatory cytokines as IL-17.

Their role in pregnancy maintenance was recently recognized. An increased number of Tregs were reported in the circulation and decidua at normal pregnancy, while a reduced number of Treg cells accompanied failed pregnancies (Sasaki et al., 2004; Yang et al., 2008;Zhu, 2005).

Feline CD4+CD25+ Tregs were previously described (Joshi et al., 2004). These cells express both intranuclear FoxP3 and surface TGF-β. Their function parallels that of human and murine Tregs (Joshi et al., 2004). Furthermore, FIV replicates preferentially in the cells during acute infection, producing high levels of viral RNA which correspond to peak plasma viremia (Mexas et al., 2008).

Th17 cells are a pro-inflammatory population of T cells that differentiate from CD4+ T progenitor cells in the presence of TGF-β and IL-6. Among other pro-inflammatory cytokines and chemokines, these cells secrete the cytokine IL-17 and express transcription factor RORγ. (Bettelli, Korn, and Kuchroo, 2007). The role of these cells in human pregnancy is under investigation. Lower population numbers of Th17 cells were associated with normal pregnancies while increased numbers corresponded to spontaneously aborted fetuses (Liu et al., 2011). Increased levels of IL-17 in the periphery caused decreased immunosuppressive ability of Tregs, a finding which was speculated to lead to increased spontaneous abortions (Wang et al., 2010). Collectively, the data suggest an inverse relationship between Tregs and Th17 during gestation. Feline Th17 cells have not yet been described.

We are using the FIV-infected cat to model lentivirus-induced placental immunopathology. We reported that FIV infection results in significantly increased reproductive failure which corresponds to an infection-induced, pro-

inflammatory placental microenvironment, particularly at early pregnancy. Our previous report (Lockett et al., 2010) suggests that infection may reduce the population of placental Tregs at early pregnancy, partially explaining the pro-inflammatory state of the tissue. In the present study we hypothesized that FIV infection in the pregnant cat causes altered placental Treg and Th17 cell population dynamics, possibly allowing placental inflammation as a result of infection and frequent fetal demise. We quantified the expression of Treg marker FoxP3 and Th17 marker RORγin placental samples from FIV-infected and control queens at early and late term gestation by immunofluorescence confocal microscopy. Th17 cells were significantly increased in infected placentas at early, but not late pregnancy. A negative correlation in FoxP3 versus RORγ expression was predicted because Tregs and Th17 cells are inversely related. However, a significant negative correlation of Tregs and Th17 cells was evident only in control animals late in pregnancy. A positive correlation of these cells approached, but did not reach significance in FIV infected tissues at early pregnancy. Collectively, the data provide the first evidence that a Treg/Th17 imbalance may occur in early pregnancy in the FIV-infected cat, supporting our prior evidence of a virus-induced, pro-inflammatory placental microenvironment at early pregnancy.

Materials and Methods

Animals and Virus

Cats were female, reproductively mature, specific pathogen-free (SPF) animals (*Felis domesticus*), obtained from a commercial cattery. Ten cats were

inoculated intravenously with 1 cc a feline plasma pool containing FIV-B-2542 at approximately 1 x 10^4 copies per ml; ten cats were uninoculated controls. Whole blood (15 ml) was collected into Vacutainer® tubes at biweekly to monthly intervals until delivery of kittens. Serum, plasma, and peripheral blood leukocytes (PBLs) were collected. Confirmation of infection was performed by standard PCR and serology (Boudreaux et al., 2009; Weaver et al., 2005). Queens were allowed to breed naturally with SPF males. Fetuses were delivered by cesarean section immediately after pregnancies were confirmed by ultrasonography at week 3-4 gestation (early term) or at 8 weeks gestation (late term). 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. Fetal and placental tissues were collected from all animals, snap frozen in liquid nitrogen, and frozen at -80˚C. Infected animals used were euthanized following delivery. Control cats were spayed and released for adoption after recovery. Animal protocols were approved by the Institutional Animal Care and Use Committee of Mississippi State University. The placental tissues used in this study are shown in Table 1.

Detection of Virus in Tissue

Placental tissues were evaluated for FIV provirus using standard PCR or for viral RNA targeting FIV gag using qPCR according to the published protocols (Lockett et al., 2010; Weaver et al., 2005).

Immunofluorescent staining

Snap frozen placental tissues were embedded in OCT (Tissue-Tek®, Sakura Finetek, Torrance, CA.) and sectioned to a thickness of 4um using a cryostat. Five random sections for each placenta collected from the maternal/fetal interface were placed on poly-L-lysine coated slides and allowed to dry. Tissues were fixed in acetone (100%) for 15 minutes at room temperature and then treated with 0.01% Triton-X-100 to permeabilize the tissue. Sections were blocked with feline IgG (0.1mg/ul) and 5% non-fat milk, and incubated at 4^0C for at least 1 hour. Sections were incubated with the primary antibodies rabbit polyclonal antibody to RORγ (abcam; dilution 1:500), and rabbit polyclonal antibody to FoxP3 (abcam; dilution 1:500) for 2h at 4^oC. Secondary antibody, goat anti-rabbit IgG (H+L) fluorescein conjugated (CHEMICON, Temecula, CA, 1:500), was applied and incubated 45 minutes to 1h at 4^oC. Parallel sections were treated with isotype-matched control antibodies (Dako North America Inc., Carpinteria CA.) conjugated with appropriate fluorochromes to assure that reactivity was not a result of non-specific binding. Sections were mounted with Vecta shield containing DAPI (Vector Laboratories, Inc.).

Confocal Microscopy

The fluorescence intensity was acquired using a Zeiss LSM 510 Confocal Laser Scanning Microscope (Carl Zeiss Microimaging, Inc) with an Inverted Zeiss Axiovert 200 M Light microscope and a plan apochromat 40 X/1.30 NA objective lens. A DAPI/fluorescein filter set was used in single channel mode imaging. Excitation wavelengths of 405nm and 488nm were used and Band Pass (BP) Emission wavelengths of 420-480 nm (Blue) and Long Pass (LP) 505nm (Green)

were acquired at 1024x1024 pixel format for imaging purposes. Fluorescence intensity was collected from 10 randomly selected fields of view per section. Background fluorescence was acquired for each sample using the secondary antibody.

Statistical Analysis

Statistical analyses of mean fluorescence intensity for targeted cell populations from (FoxP3 and RORγ) between FIV control and infected cats were done using single-factor ANOVA. Spearman Rank Order Correlation(http://www.wessa.net/rankcorr.wasp) was used to determine the correlation between FoxP3 and RORγ. Differences were considered significant at $p \leq 0.05$.

Results

FIV infection status of placental tissues

FIV provirus or viral RNA was detected in all placental tissues tested (Lockett et al., 2010; Weaver et al., 2005). Placental samples and infection status are listed in Table 3.1.

Quantification of Treg and Th17 markers at early and late gestation

FoxP3 and RORγ expressing cells were detected at the maternal-fetal interface of representative samples following labeling with primary polyclonal antibody to the two proteins and FITC-conjugated secondary antibody (Fig 3.1). Negative control reactions (3.1, top panel) showed minimal background fluorescence, validating the specificity of the primary antibodies. The

fluorescence intensity of FoxP3 and RORγ was visibly higher in the infected group as compared to the controls (3.1, middle panel). The intranuclear expression of FoxP3 and RORγ was evident when fluorescently-labeled cells were stained with DAPI (3.1, bottom panel), identifying those cells as Tregs or Th17 cells, respectively.

The cell populations were quantified by measuring the mean fluorescence intensity of the respective cellular markers. There was no significant difference in the Treg population between infected and control groups at either early ($p=0.34$) or late ($p=0.29$) gestation (Fig 3.2A). At early gestation the fluorescence intensity for RORγ was significantly higher in the infected than the control group ($p=0.003$), suggesting a greater population of Th17 cells in this group. No significant differences ($p=0.21$) in the Th17 population at late gestation were evident (Fig 3.2B).

Table 3.1 Placental samples included in confocal analysis.

Placenta Number Early Pregnancy	Queen FIV Status	Placental FIV Status	Fetus*	Placenta Number Late Pregnancy	Queen FIV Status	Placental FIV Status	Fetus*
7824A	-	-	V	13671 AP	-	-	V
7824B	-	-	V	13671 DP	-	-	V
7824C	-	-	V	13668 AP	-	-	V
2779A	-	-	V	13668 BP	-	-	V
2779E	-	-	V	9746 AP	-	-	V
6108A	-	-	V	9746 DP	-	-	V
8291A	-	-	V	9801 AP	-	-	V
8291B	-	-	V	9801 BP	-	-	V
9784B	-	-	V	9674 AP	+	+	V
6062B	+	+	V	9809 A	+	+	V
6062C	+	+	V	13226 1P	+	+	NV
8035B	+	+	NV	13226 2P	+	+	NV
1893B	+	+	NV	9703 R1	+	+	NV
1893C	+	+	V	9813 R1	+	+	NV
1126A	+	+	V	9813 R2	+	+	NV
1126D	+	+	V				
5111A	+	+	V				
5111C	+	+	NV				
0866B	+	+	V				

*Viable (V), Nonviable (NV)

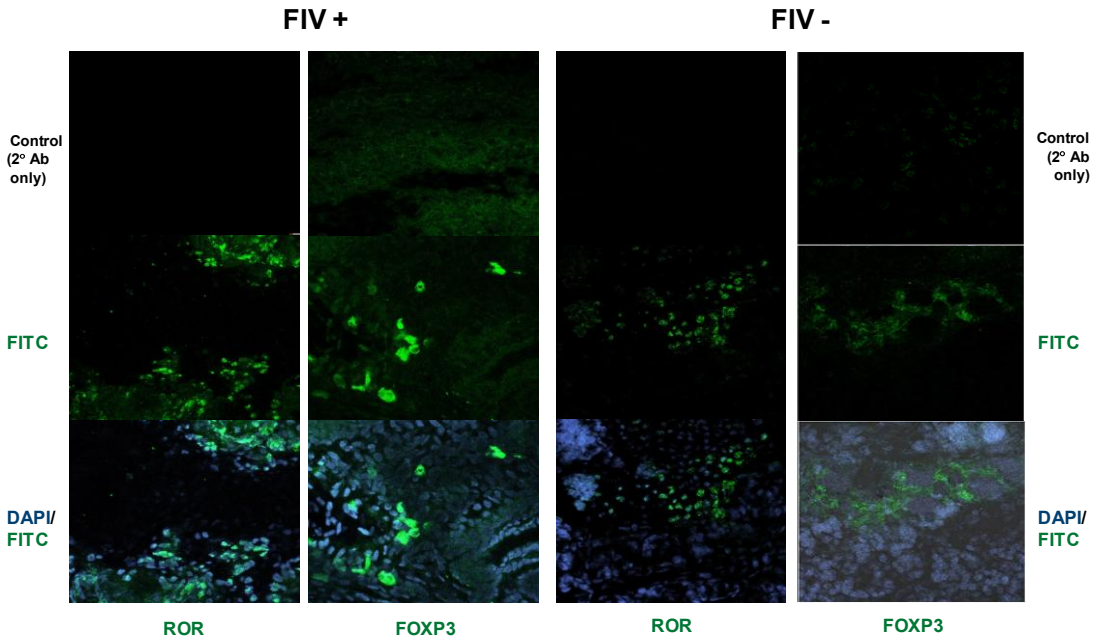
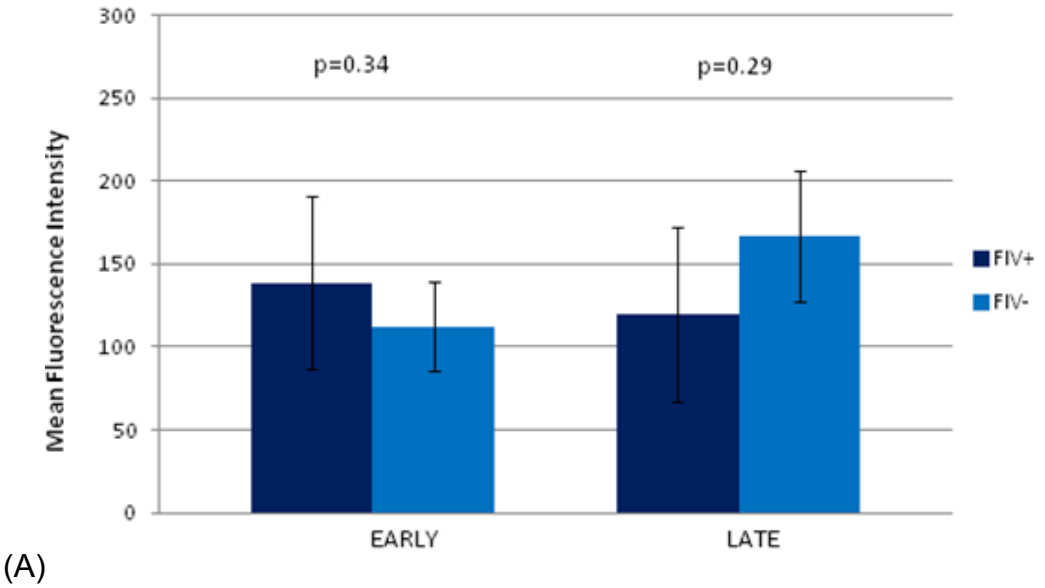
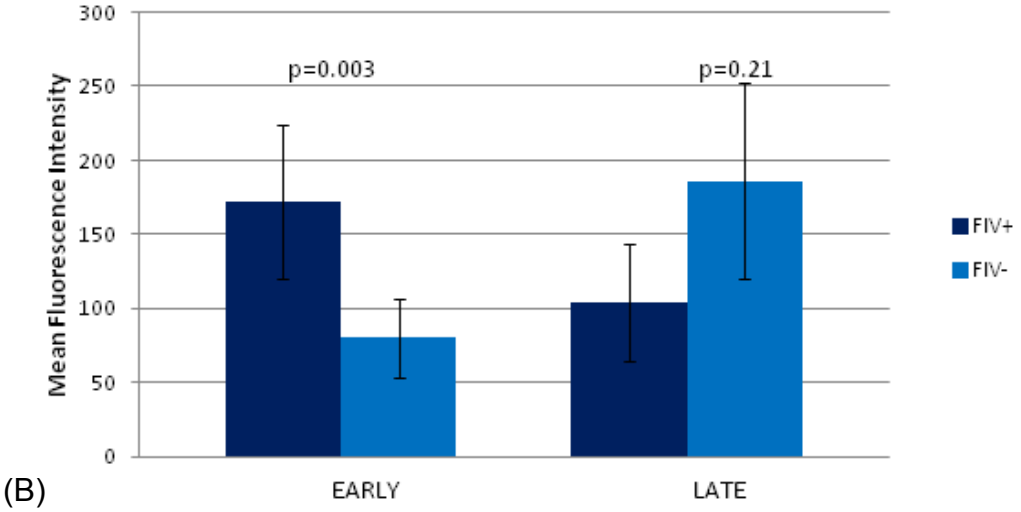


Figure 3.1 Immunofluorescence labeling of RORγ and FoxP3 at the maternal fetal interface of tissue from representative infected and control cats.

Negative controls included no primary antibody (top panels).RORγ and FoxP3 (green) were detected using polyclonal rabbit anti-FoxP3 or anti-RORγ antiserum followed by goat anti-rabbit IgG (H + L) fluorescein conjugate (middle panels). Cells were counterstained with DAPI (blue) (bottom panels). Cells were viewed by confocal laser scanning microscopy using a 40x oil immersion objective.



(A)



(B)

Figure 3.2 Quantification of Treg (A) and Th17 (B) cell populations at the maternal fetal interface.

Mean fluorescence intensity of FoxP3 and RORγ, which label Treg and Th17 cells, respectively, were measured at early and late term gestation in both control and infected cats. Values are bracketed by standard error of the mean. Samples were evaluated as follows: infected (n=11) versus control (n=9) at early gestation; and infected (n=7) versus control (n=8) at late gestation. P values < 0.05 were considered significant.

Correlation of RORγ and FoxP3 at early and late gestation

RORγ and FoxP3 expression neither positively nor negatively correlated in either FIV positive or control cats at early gestation (3.3A and B), although a positive trend approached significance (p=0.069) in FIV positive cats at this stage. At late gestation the RORγ:FoxP3 correlation was not significant in FIV positive cats, but the negative correlation of RORγ:FoxP3 was significant (p=0.05) in control animals at late pregnancy (3.4A and B), indicating a normal reciprocal relationship in these cell populations at this stage of pregnancy.

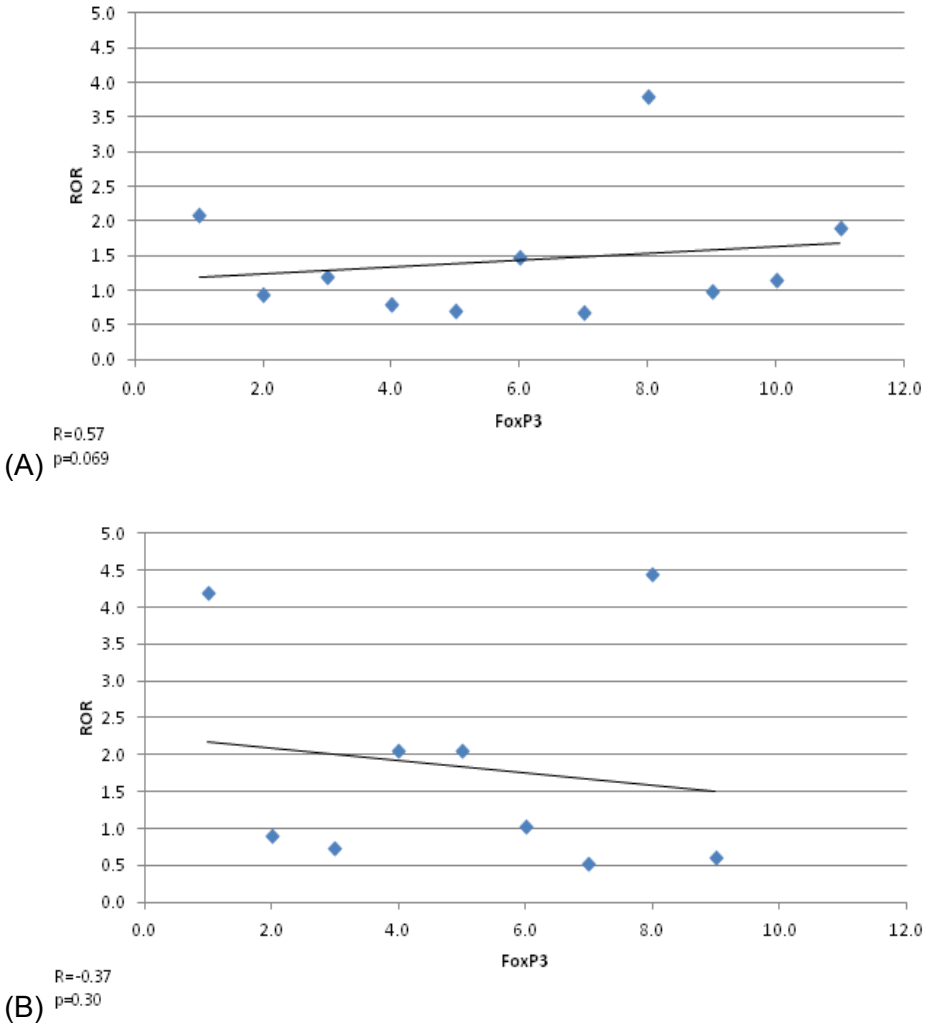


Figure 3.3 Correlation of RORγ to FoxP3 in infected (A) and control (B) cats at early gestation.

Analysis was performed using Spearman Rank Order Correlation. P values <0.05 were considered significant.

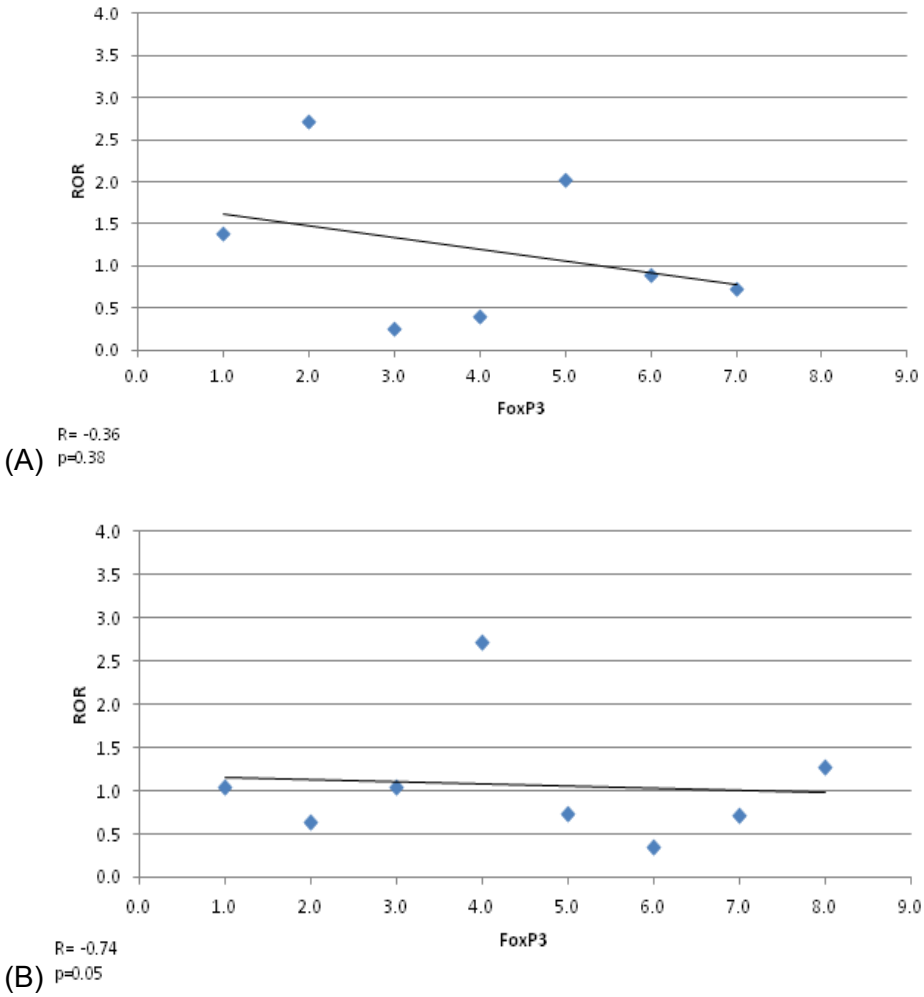


Figure 3.4 Correlation of RORγ to FoxP3 in infected (A) and control (B) cats at late gestation.

Analysis was performed using Spearman Rank Order Correlation. P values <0.05 were considered significant.

Discussion

During normal human pregnancy, Treg populations expand in the blood and deciduum as pregnancy progresses, while the Treg population decreases in failed pregnancies (Sasaki et al., 2004). In HIV-positive women, the Treg population in blood was unchanged between the first and second trimester (Kolte et al., 2011). No data are available to evaluate the influence of HIV infection on

placental Treg populations in women. We are evaluating placental Treg populations in the FIV-infected cat model. Previously, we used qPCR to detect expression of Treg markers in sections excised from whole placentas. Lower levels of FoxP3 and CTLA4 expression occurred in placentas of FIV-infected cats as compared to control cats at early pregnancy, indicating a possibly reduced population of Tregs in infected animals at this stage (Lockett et al., 2010). In the present study we used confocal microscopy to identify and quantify these cells specifically in immuno-labeled tissues based on fluorescence intensity. We were unable to detect significant differences in FoxP3 expression between FIV infected and control cats at either early or late pregnancy.

Knowledge of the role of Th17 cells in pregnancy is newly emerging. Significantly higher numbers of Th17 cells were detected in the decidua of patients who spontaneously aborted as compared to patients with normal pregnancies (Liu et al., 2011; Tomi, Nishimura, and Nakashima, 2011). IL-17 was localized in both cytotrophoblasts and syncytiotrophoblasts of normal, early pregnancy patients and those exhibiting unexplained recurrent spontaneous abortions (URSA), although IL-17 was significantly high in URSA patients. Cells other than lymphocytes also expressed IL-17 at the maternal-fetal interface of URSA patients (Liu et al., 2011).

TGF-β normally drives the differentiation of both Tregs and Th17 cells from progenitor CD4+ T cells. Generation of Th17 cells requires the additional presence of IL-6 and IL-21 (Deenick, 2007). In an inflammatory environment, Tregs can convert into Th17 cells (Xu et al., 2007; Zheng, Wang, and Horwitz, 2008). We found that FIV infection alters cytokine expression in the placenta.

Among these cytokines, IL-6 was significantly increased in FIV-infected cats at early pregnancy. A positive correlation between viral load and IL-6 at late pregnancy was reported (Scott et al., 2011). Those data indicate a placental microenvironment conducive to Th17 dominance in the FIV-infected cat model, and in the present study, Th17 cells were significantly increased in placentas from FIV-infected cats at early, but not late pregnancy. These data explain, in part, the pro-inflammatory placental microenvironment that we previously speculated (Scott et al., 2011).

Treg and Th17 cell populations are normally negatively correlated with reciprocal developmental pathways for inflammation and immune regulation (Xu et al., 2007; Zheng, Wang, and Horwitz, 2008). We detected a significant negative correlation of FoxP3 and RORγ expression at late pregnancy in the control group. A trend toward a negative correlation between the two populations in control placentas at early pregnancy and in the FIV-infected group at late pregnancy did not reach the level of significance. A trend toward a positive correlation approached, but did not reach significance ($p=0.069$) in the FIV positive, early pregnancy group.

These data lead us to speculate that Th17 cells may arise from and contribute to the localized inflammatory microenvironment that occurs in the placentas of FIV-infected queens at early pregnancy. An increased presence of Th17 cells in the uterine cavity has previously been suggested due to the non-sterility of the cavity and the contribution of Th17 cells to the protective immune response to extracellular microbes (Saito et al., 2010). The Th17-induced pro-inflammatory placental microenvironment resulting from HIV infection in women

and in our FIV-infected cat model may pose a dilemma for the developing fetus by causing an imbalance of regulatory and protective cytokines that results in the enhanced pregnancy failure that occurs in these lentiviral infections.

In summary, our data indicate a significant increase in the Th17 cell population in early-term placentas from infected feline queens. This result is consistent with our previous reports of a pro-inflammatory placental microenvironment at this stage of pregnancy in FIV-infected queens and an enhanced rate of reproductive failure in this group of cats. Currently, immunomodulator profiles from laser capture microdissected Treg and Th17 cells and adjacent placental microenvironments are being evaluated in the FIV-infected cat model. These profiles may illuminate the functional effects of viral infection on these immunologically-important placental cell populations.

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CHAPTER IV

EVIDENCE FOR PLACENTAL TREG IMMUNOMODULATORS IN THE FIV INFECTED CAT MODEL

Abstract

Treg cells are important in the maintenance of pregnancy and are preferentially infected and activated during acute FIV infection.TGF-β promotes initial differentiation of both Tregs and Th17 cells from a common progenitor. IL-6 promotes further progression to the Th17 phenotype and arrests further differentiate of Tregs. Thus, Tregs and Th17 cells should be have an inverse relationship controlled by IL-6. Disruption in expression of the cytokine potentially perturbs the balance in the two cell populations. We previously found evidence of a pro-inflammatory placental environment with decreased levels of FoxP3 and increased levels of IL-6 in the placentas of FIV-infected cats at early pregnancy with a high rate of reproductive failure. We hypothesized that FIV infection in pregnant cats causes alteration in placental Treg populations and increased inflammation, potentially allowing transplacental transmission of the virus and frequent damage to the fetus. We showed the localization of Treg and Th17 cells at the maternal-fetal interface and quantified the expression of Treg modulators FoxP3, CD134, CXCR4, IL-10, and TGF-β in placental samples from FIV-infected and control queens at early pregnancy by qPCR. We correlated the expression of IL-6 and RORγ for the characterization of Th17 cells. We detected

significantly higher levels of TGF-β in infected queens, indicating dysregulation in both Treg and Th17 cell populations. In control cats the expression of IL-6 and RORγ was positively correlated as predicted, but this relationship was disrupted in infected animals.These data are consistent with our previous reports of a pro-inflammatory placental microenvironment at this stage of pregnancy in FIV-infected queens that may include perturbation of Th17 cell dynamics.

Introduction

Feline Tregs, characterized by detection of intracellular expression of FoxP3, arepreferentially infected and activated during acute FIV infection (Mexas, Fogle et al. 2008). The expression of both FoxP3 and TGF-β are up-regulated in Tregs of infected animals (Mexas, Fogle et al. 2008). Tregs and Th17 cells differentiate from a common T helper progenitor cell. TGF-β causes the proliferation of both Treg and Th17 cells (Mangan, Harrington et al. 2006)(Li, Wan et al. 2007). During inflammation, enhanced production of IL-6 inhibits the induction of FoxP3, halting the generation of Tregs, and activates the expression of RORγ, driving the proliferation of Th17 cells (Bettelli, Korn et al. 2007). Th17 cells further enhance the inflammatory response by releasing the cytokines IL17, IL-6, TNFα, and IL-22. Thus, IL-6 levels dictate a pro- or anti-inflammatory cellular response and an inverse relationship between Tregs and Th17 cells (Bettelli, Korn et al. 2007). Disruption in cytokine expression potentially perturbs the balance in the two cell populations.

Cytokines at the maternal-fetal interface play an essential role in the maintenance of pregnancy. This site favors, but is not limited to, an anti-

inflammatory environment during early to mid-pregnancy (Mellor 2000). Suppression of inflammatory cytokines is necessary in the early stages of pregnancy because inflammation may cause pre-eclampsia, pre-term delivery, and/or spontaneous abortion (Lim, Odukoya et al. 2000; Makhseed, Raghupathy et al. 2000;Makhseed, Raghupathy et al. 2001). Late in pregnancy inflammation is necessary to induce parturition (Guerin, Prins et al. 2009). A significant decrease in the Treg population in the decidua during late pregnancy allows an inflammatory response to precede parturition, an important factor in the expulsion of the full term fetus (Guerin, Prins et al. 2009).

Several investigators have correlated increased numbers of activated Treg cells in the periphery and decidua with successful pregnancies, while a reduced number of Tregs accompany failed pregnancies (Sasaki, Sakai et al. 2004; Zhu 2005). The inflammatory cytokine IL-17, a product of Th17 cells, was found to localize in cytotrophoblast and syncytiotrophoblast cells in the deciduas of unexplained recurrent spontaneous abortions (URSA) and normal early pregnant women, but IL-17 was significantly higher in the URSA group (Liu, Wu et al. 2011). This finding suggests that Th17 cells may play a role in pregnancy failure. IL17 levels were also significantly elevated in the peripheral blood of these patients, providing evidence for a concurrent systemic response (Liu, Wu et al. 2011). Interestingly, progesterone induces the production of several anti-inflammatory cytokines, including TGF-β from decidual cells (Nakamura 2009). Thus, decreased levels of progesterone, which accompany failed pregnancy (Nakamura 2009), may affect the differentiation of these two cell populations.

The myriad of contributors to immunological imbalance and consequent failed pregnancy that may occur during pregnancy are becoming increasingly evident. However, the complexity confounds the ability to define the causal relationship between aberrant placental immunology and perturbed pregnancy. The need for additional study is apparent; yet, the inability to obtain human tissues at will to conduct these investigations highlights the value of an animal model.

We have used the FIV- infected cat model to evaluate parameters of lentivirus-induced placental inflammation. Our previous data suggest a pro-inflammatory placental microenvironment at early pregnancy in the infected cat, based on the ratio of pro- to anti-inflammatory cytokines, expression of IL-6 (Scott, Boudreaux et al. 2011), and the likely decreased population of Tregs (Lockett, Scott et al. 2010). We detected and quantified FoxP3 and RORγ-expressing cells in whole placental sections using immunofluorescence confocal microscopy (Chapter III); yet, the location of these cells in the tissues was unclear. Therefore, the first objective of this study was to localize the two cell populations by labeling parallel sections with either FoxP3 or RORγ-specific antibody and comparing both to a parallel specimen immunolabeled with anti-relaxin antibody. As relaxin is produced by trophoblasts, its presence demarcates the maternal-fetal interface. Both FoxP3 and RORγ colocalized with relaxin. The second objective was to provide molecular evidence of Treg function in whole placentas. Thus, the expression of Treg markers or products were evaluated, including FoxP3, CD134, CXCR4, IL-10, and TGF-β. We report a significant decrease in TGF-β expression in the infected group, but no other

differences were detected between the two groups. Finally, we correlated expression of IL-6 (Scott, Boudreaux et al. 2011)with RORγ.As an enhanced level of IL-6 is indicative of inflammation, its expression should be inversely correlated to Treg function and positively correlated with Th17 function. In control cats the expression of IL-6 and RORγ was positively correlated as predicted, but this relationship was disrupted in infected animals. Collectively, the data suggest that FIV-induced immunopathology may include perturbation of Th17 cell dynamics at early pregnancy.This report supports our prior evidence of a virus-induced, pro-inflammatory placental microenvironment at early pregnancy.

Materials and Methods

Animals and Virus

Cats were female, reproductively mature, specific pathogen-free (SPF) animals (*Felis domesticus*), obtained from a commercial cattery. Ten cats were inoculated intravenously with 1 cc a feline plasma pool containing FIV-B-2542 at approximately 1 x 10^4 copies per ml; ten cats were uninoculated controls. Whole blood (15 ml) was collected into Vacutainer® tubes at biweekly to monthly intervals until delivery of kittens. Serum, plasma, and peripheral blood leukocytes (PBLs) were collected. Confirmation of infection was performed by standard PCR and serology (Boudreaux, Lockett et al. 2009). Queens were allowed to breed naturally with SPF males. Fetuses were delivered by cesarean section immediately after pregnancies were confirmed by ultrasonography at week 3-4 gestation (early term). The time of FIV inoculation to delivery ranged

from approximately 9.5 to 13.5 months (mean 11.14 months) for early-gestation. Fetal and placental tissues were collected from all animals, snap frozen in liquid nitrogen, and frozen at -80˚C. Infected animals used were euthanized following delivery. Control cats were spayed and released for adoption after recovery. Animal protocols were approved by the Institutional Animal Care and Use Committee of Mississippi State University. The placental tissues used in this study are shown in Table 4.1.

Table 4.1 Placental Samples Included in Treg Expression Analysis.

Fetus Number	Queen FIV Status	Placental FIV Status	Fetus Outcome *
8059A	-	-	V
8059B	-	-	V
8059C	-	-	V
8059D	-	-	V
6108A	-	-	V
6108B	-	-	V
6108C	-	-	V
7824A	-	-	V
7824B	-	-	V
7824C	-	-	V
6062A	+	+	V
6062B	+	+	V
8035A	+	+	V
8035C	+	+	NV
1126A	+	+	V
1126B	+	+	V
5111B	+	+	V
5111C	+	+	NV
0866A	+	+	V
0866B	+	+	NV

*Viable (V), Nonviable (NV)

Purification of RNA from feline placental tissues and conversion to cDNA

Random sections of tissue were obtained from whole placenta. TRIzol Reagent (Invitrogen, Carlsbad, CA) was used to purify RNA from these tissues. RNA was purified from the aqueous phase as described (Scott, Burgess et al. 2008). Concentrations were determined using a NanoDrop 1000 (Thermo Scientific, Waltham, MA). Purified RNA was reverse transcribed using High Capacity cDNA kit (Applied Biosystems). Reverse transcription reactions (20ul) were performed using the following protocol: 25C for 10min, 37C for 120min, 85C for 5sec.

Quantification of Cytokines and Treg Markers by Real time PCR

Complementary DNA obtained from the High Capacity cDNA reactions were used in gene expression analysis. Primetime assays (Integrated DNA Technologie, Coralville, Iowa) were used to evaluate the expression levels of FoxP3, CD134, CXCR4, IL-10, and TGF-B, along with the housekeeping gene β-actin. Primer/probe combinations for use in real time analysis are listed in Table 4.2. Probes for target genes were labeled at the 5' and 3' ends with 6-carboxyfluorescein (FAM)- and Iowa Black ® FQ, respectively. Simplex reactions were performed with a target gene and the housekeeping gene using an ABI thermocycler with the following protocol: Step 1. 50C, 2 min. and 95C, 10min; Step 2. 40 cycles (95C for 15sec and 60C for 1min.). Each reaction well contained 10ul of the 2X Taqman gene expression master mix, 1 ul of 20X TaqMan gene expression assay and approximately 6ug of cDNA. For every placental RNA sample, parallel reactions were run in duplicate on separate plates for each gene. Serially-diluted, pooled RNA from control cats was used to

generate a standard curve for simplex reactions.Standard curves were used to calculate the correction coefficient for each target. Differences in the amount of template cDNA in each reaction were corrected by the cycle threshold (Ct) value for B-actin. Comparisons of Treg markers between infected versus uninfected placentas were analyzed statistically using ANOVA. Correlation analyses were done using an online Spearman rank correlation calculator (http://www.wessa.net/rankcorr.wasp). Differences were considered significant at $p \leq 0.05$.

Table 4.2 Primer/Probe Sequences for Prime Time Assays.

Gene	Probe/Primer	Sequence (5'-3')
β-actin	Probe	/56-FAM/ATG GAG AAG /ZEN/ATC TGG CAC CAC ACC TT/3IABkFQ/
	Forward	ATC GAA CAC GGC ATT GTC ACC AAC
	Reverse	AGT CAT CTT CTC ACG GTT GGC CTT
TGF-β	Probe	/56-FAM/AGC AAT AAT /ZEN/TCC TGG CGC TAC CTC AGC A/3IABkFQ/
	Forward	AGC ACG TGG AGC TGT ACC AGA AAT
	Reverse	TCC AGT GAC ATC AAA GGA CAG CCA
FoxP3	Probe	/56-FAM/AGC CTA CAC /ZEN/AAA TGC TTT GTG CGG GT/3IABkFQ/
	Forward	GGT TCA CAC GCA TGT TTG CCT TCT
	Reverse	ACT CGA ATT CAT CCA CGG TCC ACA
CD134	Probe	/56-FAM/AAG CAG AGA /ZEN/TGC ACA CCC ACG CAG GA/3IABkFQ/
	Forward	TGC ACA CAG TGC AAC CAG AGA AGT
	Reverse	ACA GTC GAC TCC ACG ATC GTA ACC
CXCR4	Probe	/56-FAM/ACT GGC ATA /ZEN/GTG GGC AAT GGA TTG GT/3IABkFQ/
	Forward	TGA CTC CAT GAA GGA ACC CTG CTT
	Reverse	AGG TCT GCC ACA GAT AGG TGC AAT
IL-10	Probe	/56-FAM/ACC AGG TCC /ZEN/TTG CTG GAG GAC TTT AA/3IABkFQ/
	Forward	TTT CAA ACC AAG GAC GAG CTG CAC
	Reverse	AAC TGG ATC ATC TCG GAC AAG GCT

Localization of Tregs and TH17 cells in Placental Tissues using Immunohistochemistry (IHC)

An IHC protocol developed by our laboratory for detection of specific cell populations in frozen feline placentas sections (Scott and Wallace, 2011) was used to localize Treg and Th17 cells. Rabbit polyclonal antibodies (abcam,1:500) to FoxP3 and RORγ were used to identify Treg and Th17 cells, respectively. The maternal-fetal interface was determined using a relaxin antibody (1:500) (a gift from Dr. Peter Ryan, Mississippi State University, Department of Animal Science). Parallel sections were stained sequentially for Relaxin, FoxP3, RORγ, and a universal negative control. Tissues were inclubated with ready to use secondary antibody, goat anti-rabbit IgG, poly-HRP (Chemicon International Inc., Temecula, CA.) and developed with 3,3'-diaminobenzidine (DAB, Invitrogen). Tissues were counterstained with Mayer hematoxylin and dehydrated.

Results

Localization of Tregs and Th17 Cells at the Maternal-fetal Interface

Treg and Th17 cells were detected by their dark brown intranuclear staining. These cells were localized by comparing parallel sections immunolabeled for relaxin (4.1A), FoxP3 (4.1B), and RORγ (4.1C). A universal negative control specimen (4.1D) did not label, negating the possibility of non-specific binding of the secondary antibody.

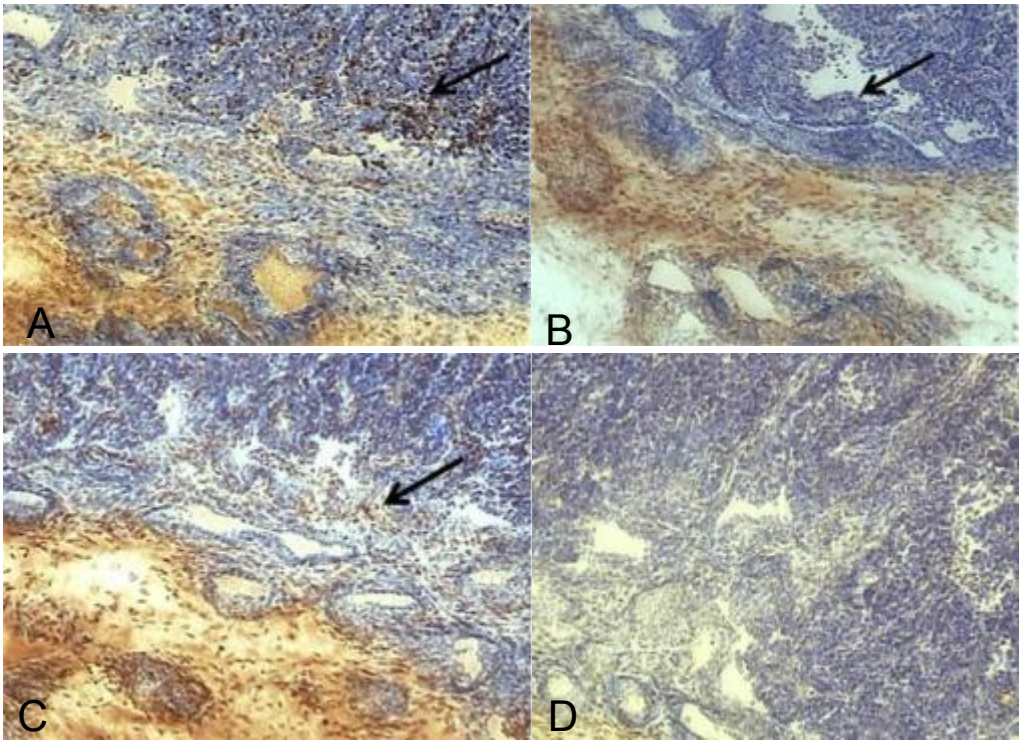


Figure 4.1 Immunohistochemical Localization of Relaxin, FoxP3, and RORγ at the Maternal-Fetal Interface of a Representative Early Term Placental Sample.

Relaxin localized in the placental trophoblast at the maternal-fetal interface (A). FoxP3 and RORγ co-localized at the maternal-fetal interface (B,C). A universal negative control was included to assure no nonspecific binding occurred (D). (20X magnification)

Expression of Treg Markers and Cytokines in Placental Tissues

Real time PCR targeting markers that characterize Treg cells in feline placentas from control and FIV-infected cats was performed (4.2). TGF-β expression was significantly decreased ($p=0.05$) in the infected group at early pregnancy. Differences in expression between placentas from control and infected animals did not reach the level of significance for any other immunomodulator evaluated.

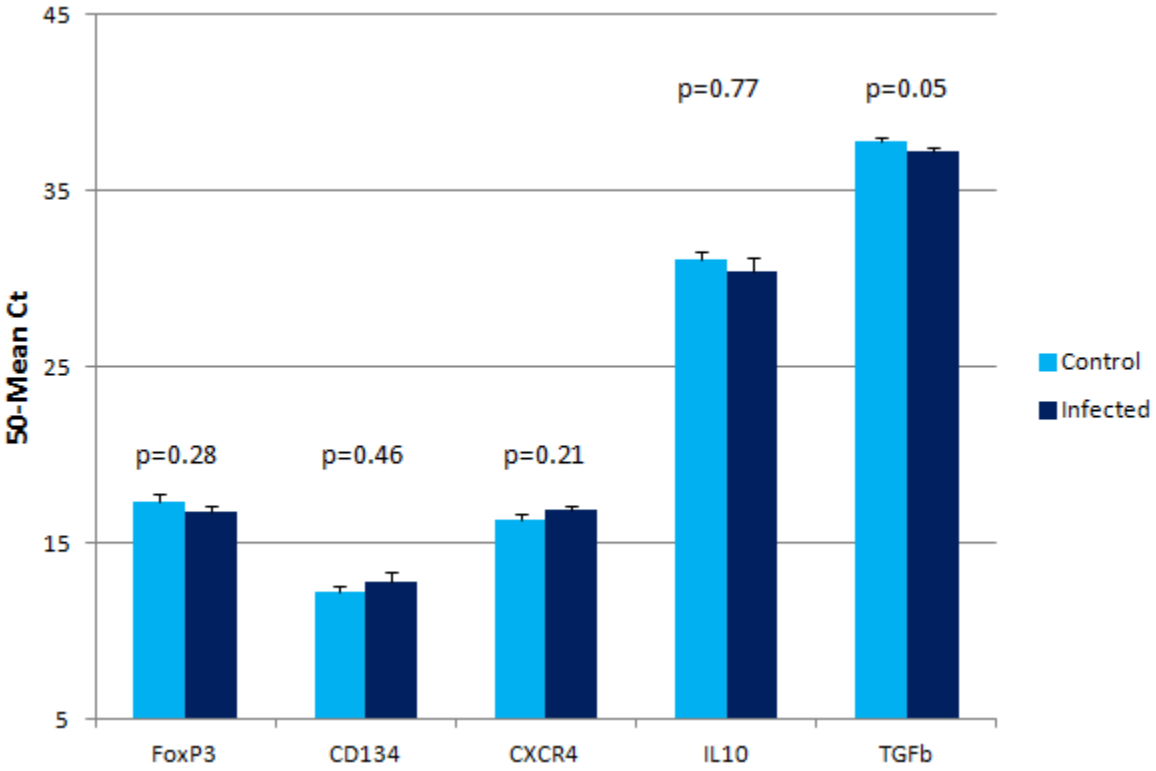


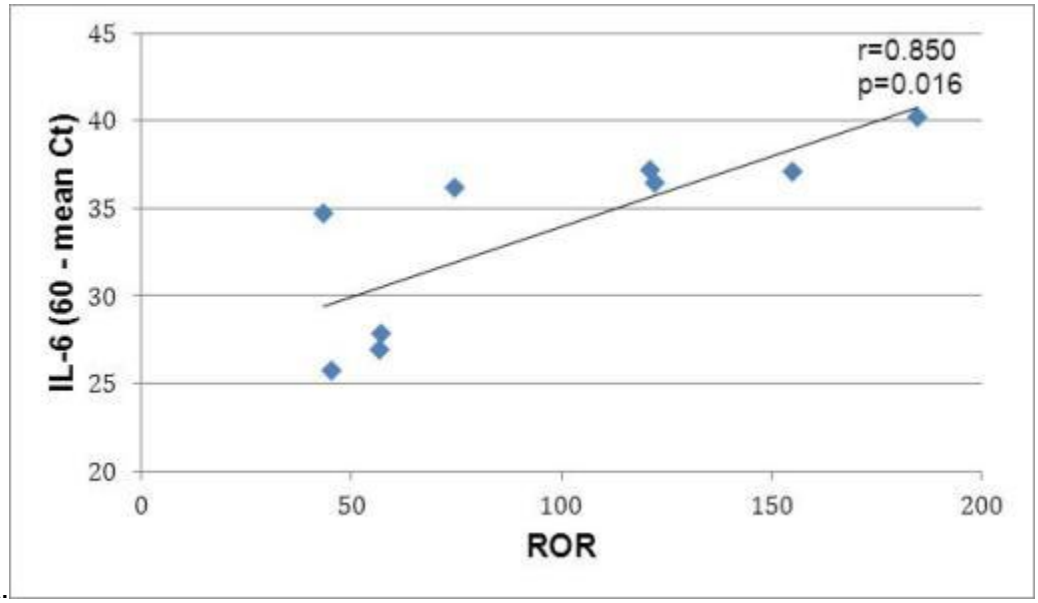
Figure 4.2 Real time qPCR was used to quantify expression of Treg markers or products.

FoxP3, CD134, CXCR4, IL10, and TGF-β expression was compared between control (n=10) and infected (n=10) tissues at early pregnancy. Bars represent mean Ct values subtracted from a negative endpoint (50-mean Ct), bracketed by standard errors of the mean. P values were obtained from single factor ANOVA. Values of $p<0.05$ were considered significant.

Correlation of RORγ and IL-6 Expression in Placentas

A positive correlation between RORγ and IL-6 was observed in the early term control group, as predicted (p=0.016) (4.3A). RORγ and IL-6 were neither positively nor negatively correlated in the infected group, indicating infection-induced disruption in the expected relationship between IL-6 and Th17 cells (4.3B).

A.



B.

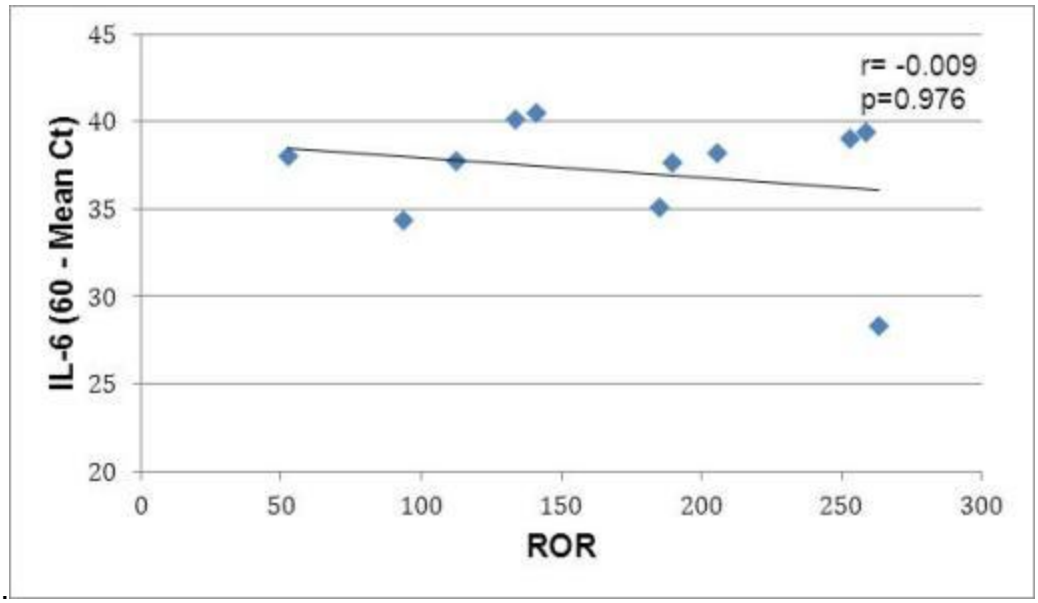


Figure 4.3 The expression of IL-6 and RORγ were correlated in control (n=10) and infected (n=10) placental tissues at early gestation.

The data were analyzed using Spearman Rank Order Correlation. P values <0.05 were considered significant.

Discussion

Pregnancy requires precise regulation of the placental immune response for successful fetal development and delivery to occur. The inverse relationship

between Treg and Th17 cells is essential. Elevated Tregs and reduced Th17 cells are favored early in fetal development and continue throughout most of pregnancy, promoting an anti-inflammatory environment. TGF-β and IL-6 facilitate the proliferation of Tregs and Th17 cells by activating their intranuclear transcription factors FoxP3 and RORγ, respectively. In this study, we used placental tissues from the FIV-infected cat and control animals to begin to evaluate how these cell populations may be involved in lentivirus-induced placental inflammation.

Tregs accumulate in the human decidua during early, normal pregnancy (Guerin, Prins et al. 2009),while Th17 cells are depressed. The ratio of the cells is often inverted in cases of failed pregnancy or placental infection (Liu, Wu et al. 2011)(Zhang 2011 AJRI) illustrating the relevance of the balance in these cell populations to pregnancy outcome. As there was no information concerning a role for Tregs and Th17 cells in the feline placenta, it was important to demonstrate their presence and location within this tissue. We did so by comparing parallel sections immunolabeled for expression of FoxP3 and RORγ to sections labeled for relaxin. Relaxin is a protein hormone produced by decidual cells that causes cervical ripening, pelvic elasticity, and induction of labor late in human pregnancy, in part by inducing the expression of key inflammatory cytokines from decidual cells (Bryant-Greenwood et al. 2007). In the cat, relaxin is known to be expressed by trophoblasts (Klonisch). Thus, expression of relaxin can be used to demarcate the maternal-fetal interface. We found that Tregs and Th17 cells co-localize at the maternal-fetal interface in the cat.

The balance of cytokine modulators at the maternal-fetal interface is critical for successful pregnancy to occur. Proliferation of Tregs depends on the continuous production of TGF-β, an anti-inflammatory cytokine, by the Treg population. In this study, a significant decrease in TGF-β in placentas from the infected group was detected, possibly explaining the decrease in the placental Treg population in infected cats at early pregnancy that we reported previously (Lockett, 2010). However, Th17 cells are likewise dependent upon TGF-β to initiate their differentiation from progenitor T cells. Yet, IL-6 is required to drive those cells further toward their Th17 phenotype. We previously reported significantly increased levels of IL-6 expression in early-term placentas (Scott, Boudreaux et al. 2011). In chapter III, we report a trend toward a positive correlation between Treg and Th17 populations at early pregnancy in infected cats (Chapter III), while a negative correlation is the norm. Herein, a positive correlation of IL-6 and RORγ expression in early, control placentas was shown, while this expected correlation was perturbed in infected tissues. These data provide evidence that altered expression of key cytokines may contribute to Treg/Th17 imbalance in infected animals.

In summary, our data confirm the presence of Treg and Th17 cell populations at the maternal-fetal interface of feline placentas. A virus-induced decrease in TGF-β in early-term placentas was evident. Along with our previous reports of increased IL-6 expression (Scott, 2011) and altered Treg/Th17 populations in early-term placentas from FIV-infected cats (Lockett, 2010; Chap 3), the data support a pro-inflammatory placental microenvironmentat this stage of pregnancy in FIV-infected queens. Collectively, the data suggest that FIV-

induced immunopathology may include perturbation of Th17 cell dynamics at early pregnancy. Currently, modulators of Th17 cells are being evaluated in whole placental tissue to help elucidate the interaction between immune responses and pregnancy failure.

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CHAPTER V

SUMMARY

The biological similarities of HIV and FIV, the clinical parallels of infection in their respective species, and the potential for vertical transmission of both viruses illustrate the value of the FIV-infected cat as a small animal model for HIV pathogenesis and perinatal transmission. As in HIV-infected women, vertical transmission of FIV can result in poor pregnancy outcome. Reasons why viral infection compromise pregnancy are not completely understood, but we suspect that placental immunopathology associated with viral infection may be a contributing factor. Thus, we are using the FIV-infected cat to study vertical transmission and lentivirus-inducedplacental immunopathology.

Maternal hematological and virological factors are known to influence vertical transmission in HIV-infected women. We hypothesized that similar parameters would be associated with fetal infection in the cat model. In chapter II of this dissertation, we reported reduced fecundity and increased fetal loss during early gestation in the infected group. Viral RNA, but not provirus, was detected in placentas and fetuses. The CD4:CD8 T cell ratio declined significantly in the infected group within 3.5 months p.i. However, individual CD4:CD8 T cells ratios neither positively nor negatively correlated with pregnancy outcome. Plasma viremia was below detectable limits at all time points in all cats, but cats were provirus positive and seropositive within four

weeks p.i. and remained positive throughout the duration of the experiment. Fetal infection did not directly correlate with reproductive outcome. On an individual cat basis, neither maternal hematology nor virologycould be correlated with vertical transmission or reproductive outcome as is evident in HIV-infected women.

The inability to correlate fetal infection with reproductive outcome suggests an indirect mechanism of fetal damage, such as virus-induced placental pathology. We previously reported preliminary evidence for a role for placental inflammation in compromised pregnancy in the FIV-infected cat (Scott et al., 2008; Weaver et al., 2005). We hypothesized the placental Treg and Th17 cells were altered by FIV infection. In chapter III, we quantified the expression of Treg marker FoxP3 and Th17 marker RORγin placental samples from FIV-infected and control queens at early and late term gestation by measuring fluorescence intensity by immunofluorescence confocal microscopy. We detected significantly higher levels of RORγ in FIV-infected placentas at early pregnancy, indicating that the viral infection may have caused increased numbers of Th17 cells to be localized in the placenta at this stage of pregnancy. This result is consistent with our previous reports of a pro-inflammatory placental microenvironment at this stage of pregnancy in FIV-infected queens coinciding with an elevated rate of reproductive failure in this group of cats.

In chapter IV, we showed the localization of Treg and Th17 cells at the maternal-fetal interface and quantified the expression of Treg modulators FoxP3, CD134, CXCR4, IL-10, and TGF-β in placental samples from FIV-infected and control queens at early pregnancy by qPCR. We correlated the expression of IL-

6 and RORγ, used to identify Th17 cells. We detected significantly higher levels of TGF-β in infected queens, indicating the potential for dysregulation of both Treg and Th17 cell populations. In control cats the expression of IL-6 and RORγ was positively correlated as predicted, but this relationship was disrupted in infected animals.These data indicate that Th17 cell dynamicsare altered in FIV-infected queens, possibly contributing to reproductive failure in this group of cats.

Collectively, the data support a pro-inflammatory placental microenvironmentat early pregnancy in FIV-infected queens. From a mechanistic viewpoint, we provide evidence that FIV-induced immunopathology may result from perturbation of the Treg/Th17 cell balance at early pregnancy. Currently, laser capture microdissection of both Treg and Th17 cell populations is underway to quantify expression of immunomodulators specific to those cell populations to determine the impact of viral infection on their function.These expression profiles may illuminate the functional effects of viral infection on these immunologically-important placental cell populations.

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APPENDIX A

MEASUREMENT OF GENE EXPRESSION IN REGULATORY T CELLS MICRODISSECTED FROM PLACENTAL TISSUES FROM FIV-INFECTED AND CONTROL CATS

Abstract

Regulatory T cells (Tregs) in the placenta play an essential role in pregnancy maintenance. In the FIV-infected cat model of mother-to-child transmission of lentiviruses, infection results in reduced fecundity and increased reproductive failure. Previous studies using whole placental specimens indicate that Treg populations may be altered in the FIV-infected cat. Therefore, we hypothesized that viral infection alters placental Treg function. The objective of this study was to analyze gene expression from these cells specifically. Placental tissues were collected from FIV-infected and control cats at week three (early) and week eight (late) pregnancy, cryosectioned, and labeled for expression of the Treg marker FoxP3 using immunohistochemistry (IHC). Tregs were collected from the tissue using laser capture microdissection (LCM), RNA was isolated and subjected to cDNA synthesis, and cDNA was analyzed by real time PCRtargeting specific Treg markers and β-actin.We were unable to amplify any gene product, including β-actin. The data proved that RNA degradation occurred, likely during tissue manipulation for IHC or exposure to the laser during LCM.Techniques to preserve the integrity of the RNA during these manipulations must be developed before this method of Treg gene expression analysis will be useful. These experiments are currently ongoing.

Introduction

The mechanisms of transplacental transmission and pregnancy perturbationassociated with lentiviral infections are unclear. Placental inflammation in HIV-infected women was hypothesized to contribute to

transplacental transfer as a result of abnormal cytokine expression (Shearer et al., 1997). Disruption of cytokine expression in placental trophoblasts caused by the virus may contribute to inflammation of the placenta and transplacental transfer (Shearer et al., 1997). Immunoregulatory cytokines produced at the maternal-fetal interface are essential for successful pregnancy(Mellor, 2000).

T regulatory cells (Tregs) are immunosuppressive by the release of cytokine IL-4, IL-10 and transforming growth factor (TGF-β) inhibiting the proliferation of T cells by suppression of IL-2 production (Thornton and Shevach, 1998). Several studies have correlated an increased number of activated Tregs in the periphery and decidua with successful pregnancies, while a reduced number of Tregs accompanies failed pregnancies (Sasaki et al., 2004; Zhu, 2005).

Feline CD4+CD25+ T cells (feline Tregs) are infected and activated during acute FIV infection (Mexas et al., 2008), producing higherviral mRNA levels than CD4+CD25- T cells. During acute FIV infection, these cells remain activated throughout the course of infection (Mexas et al., 2008). Feline Tregs up-regulate expression of FoxP3 and TGF-β. Increased levels of FoxP3 mRNA were detected by intracellular protein expression (Mexas et al., 2008). We localized Treg cells at the maternal-fetal interface (Chapter IV). In the present study, we hypothesized that FIV infection in pregnant cats causes altered placental Treg function,potentially contributing to transplacental transmission of the virus and damage to the fetus.The objective of this study was to analyze gene expression from these cells collected from early- and late-term placental tissues from FIV-infected and control cats. Following identification of the cells in cryosectioned

placentas by IHC, cells were removed from the sections using laser capture microdissection. RNA was extracted from the cells, subjected to cDNA synthesis, and analyzed using real time PCR to analyze expression of key Treg markers. These experiments were unsuccessful due to degradation of RNA.

Materials and Methods

Immunohistochemistry (IHC) for FoxP3 labeling

An IHC protocol developed by our laboratory for detection of specific cell populations in frozen feline placentas sections (Scott et al., 2011) was modified and used to label Treg cells (Table A1). Rabbit polyclonal antibodies (abcam, Cambridge, MA, 1:500) to FoxP3 were used to identify Treg cells. A universal negative control antibody was used to stain parallel sections. Tissues were incubated with ready-to-use secondary antibody, goat anti-rabbit IgG, poly-HRP (Chemicon International Inc., Temecula, CA.) and developed with 3,3'-diaminobenzidine (DAB, Invitrogen, Carlsbad, CA). Tissues were counterstained with Mayer hematoxylin and dehydrated for laser capture microdissection.

Table A.1 Immunohistochemistry protocol used to stain sections from frozen feline placental tissue for LCM and RNA extraction.

Step	Procedure	Duration
1	Fix with acetone at room temperature	10 min
2	Air dry	5 min
3	Quench with H_2O_2 in methanol	30 min
4	Wash in 0.05 M Tris-HCl	2 X 5 min
5	Block with Feline IgG in antibody diluent*	1 hr
6	Wash in 0.05 M Tris-HCl	2 X 5 min
7	Block with 5% (wt/vol) nonfat dry milk in Tris buffer	1 hr
8	Wash in 0.05 M Tris-HCl	2 X 5 min
9	Incubate with rabbit polyclonal antibody to FoxP3 in antibody diluents*	1 hr
10	Wash in 0.05 M Tris-HCl	2 X 5 min
11	Incubate with goat anti-rabbit secondary antibody at room temperature	45 min
12	Wash in 0.05 M Tris-HCl	2 X 5 min
13	Incubate with DAB (3,3'-diaminobenzidine)	15 min
14	Wash by dipping in distilled DEPC* H_2O	3 X 3 sec
15	Counterstain with Mayer hematoxylin	5 min
16	Wash by dipping in DEPC H_2O	3 X 3 sec
17	Blue hematoxylin by dipping in 37 mM ammonia	10 X 2 sec
18	Dehydrate: 75% EtOH 95% EtOH 100% EtOH Xylene	 30 sec 30 sec 30 sec 5 min
19	Dry in fume hood	5 min
20	Transfer to LCM in a desiccated slide holder	

*0.005 M Tris-HCl + 1% bovine serum albumin

*Diethylpyrocarbonate

*Precautionary measures were taken to ensure RNase free working environment.

Laser Capture Microdissection

Treg cells from IHC prepared samples were dissected by laser capture microdissection (Veritas Imaging System, Sunnyvale, CA). Cells were cut and captured by LCM and extracted onto the surface of the cap (CapSure ™ Macro LCM Caps, Applied Biosystems, Carlsbad, CA) (1). The caps were incubated at 45C for 30min in 50µl of extraction buffer (PicoPure™ RNA Isolation Kit, Applied Biosystems, Carlsbad, CA) to remove cells. Samples were frozen at -80C until RNA extractions were performed.

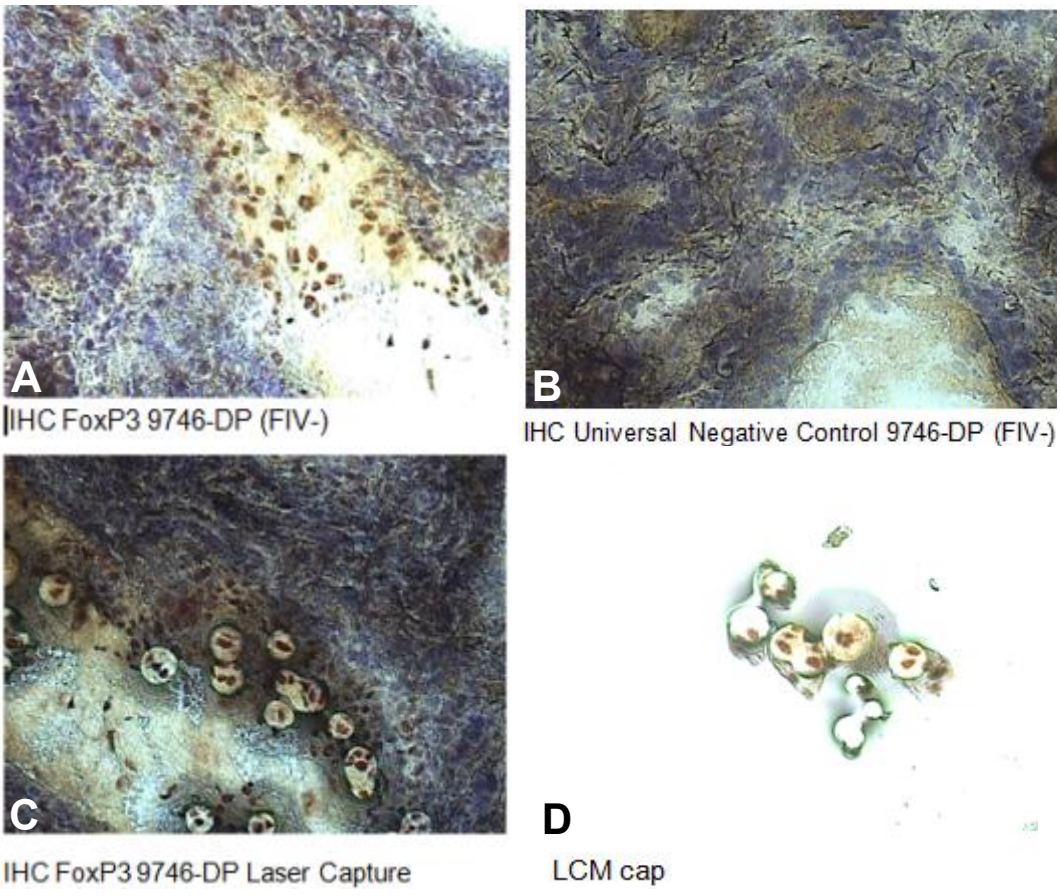

Figure A.1 Immunohistochemical staining of FoxP3 in late term placental samples from a representative control cat, 9746.

FoxP3 + cells localized at the maternal-fetal interface (A). Negative control (B). FoxP3 + cells marked for dissection (C). Dissected cells collected on a cap (D).

RNA Extraction and Complementary DNA (cDNA) Synthesis

RNA was extracted from the cells using Pico Pure (Applied Biosystems, Carlsbad, CA). RNA was converted to cDNA as previously described (Chapter IV). Concentrations of cDNA were determined using a NanoDrop 1000 (Thermo Scientific, Waltham, MA).

Agarose Gel Electrophoresis

RNA degradation was determined using a 1% agarose gel in an RNAse free working environment (http://www.flychip.org.uk/rna_qc.php).

Real time PCR

cDNA was used in real time reactions using Prime Time assays as previously described in Chapter IV.

Results

Real Time PCR

We were unable to amplify product using primer/probe combinations for either or target and housekeeping genes (A2).

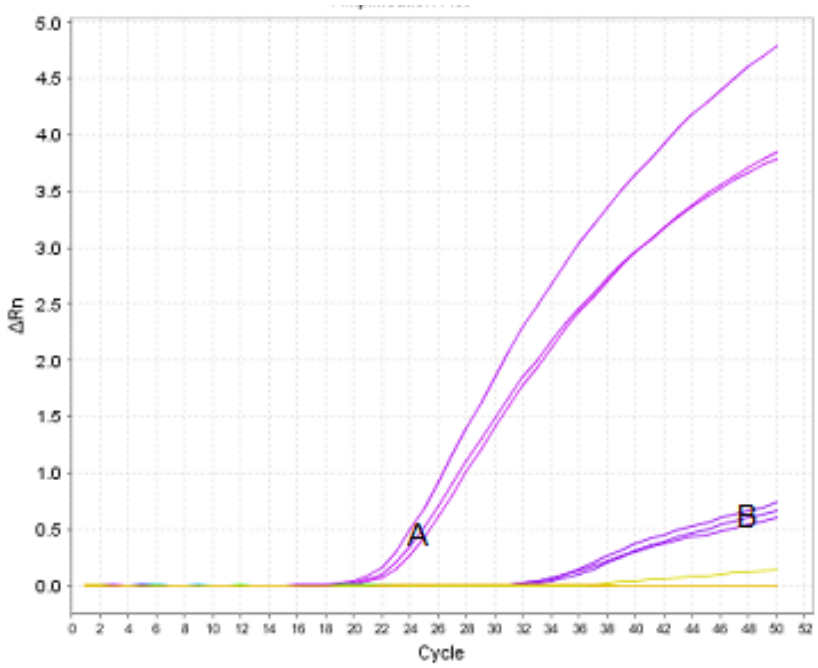

Figure A.2 Amplification curves from gene expression analysis following conversion of placental RNA to cDNA.

Products from whole placentas are shown in purple. Products from microdissected cells are shown in yellow. (A. β-actin, B. FoxP3)

Gel Electrophoresis

A comparison of RNA migration on a 1% RNA gel showed intact RNA from whole placental tissues. RNA extracted from microdissected cells was below the level of detection(A3).

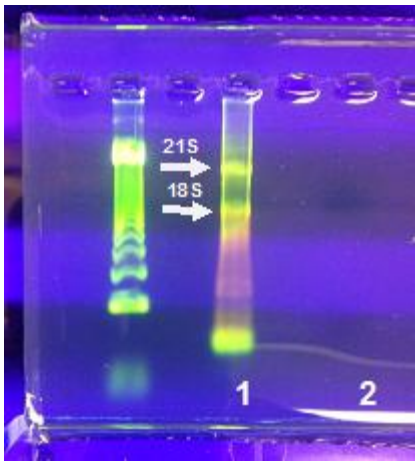

Figure A.3 1% agarose gel of RNA extracted from whole tissue (1) and microdissected cells (2).

Conclusions

The data indicate that RNA degradation occurred, likely during tissue manipulation for staining and microdissection of the Treg population. There are several steps in the protocol that may result in RNA degradation. The following changes in protocol may improve the integrity of isolated RNA: 1) Extended storage of cells extracted from the cap should be avoided. The isolatedRNA should be immediately reverse transcribed to cDNA and stored in the more stable state. The previous protocol did not proceed to RNA extraction until all dissected cells were collected and stored at -80C. It is possible that RNA degradation occurred during this longer-term storage. 2) Collecting larger clusters of cells as opposed to individual cells may increase RNA yield. 3) Using RNA Later(Qiagen, Valencia, CA) during initial collection of the cells may improve RNA yield. This product protects the tissue sample from degradation by RNAses during the storage period. A very similar LCM procedure developed in our lab (Scott et al., 2011)was used successfully to collect larger sections,

trophoblast populations, from placental tissues. Therefore, we believe adjustments to our protocol will allow successful microdissection and gene expression analyses of Treg popoulations as well. This work is currently underway.

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