5-1-2010

Immunomodulator expression in trophoblasts from the feline immunodeficiency virus (FIV)-infected cat as a contributor to placental immunopathology and reproductive failure at early- and late-term pregnancy

Veronica Lynn Scott

IMMUNOMODULATOR EXPRESSION IN TROPHOBLASTS FROM THE FELINE IMMUNODEFICIENCY VIRUS (FIV)-INFECTED CAT AS A CONTRIBUTOR TO PLACENTAL IMMUNOPATHOLOGY AND REPRODUCTIVE FAILURE AT EARLY- AND LATE-TERM PREGNANCY

By

Veronica Lynn Scott

A Dissertation
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
in Biological Sciences
in the Department of Biological Sciences

Mississippi State, Mississippi

May 2010

IMMUNOMODULATOR EXPRESSION IN TROPHOBLASTS FROM THE FELINE IMMUNODEFICIENCY VIRUS (FIV)-INFECTED CAT AS A CONTRIBUTOR TO PLACENTAL IMMUNOPATHOLOGY AND REPRODUCTIVE FAILURE AT EARLY- AND LATE-TERM PREGNANCY

By

Veronica Lynn Scott

Approved:

Karen S. Coats Associate Professor of Biological Sciences (Director of Dissertation)	Donald N. Downer Professor Emeritus of Biological Sciences (Committee Member)
Dwayne A. Wise Professor of Biological Sciences (Committee Member)	Shane C. Burgess Professor and Associate Dean College of Veterinary Medicine (Committee Member)
Peter Ryan Professor of Animal and Dairy Sciences (Committee Member)	Phillip D. Nelson Visiting Professor of Immunology Western University of Health Sciences (Committee Member)
Gary N. Ervin Associate Professor of Biological Sciences (Graduate Coordinator)	Gary L. Myers Dean of the College of Arts and Sciences

Name: Veronica Lynn Scott

Date of Degree: May 1, 2010

Institution: Mississippi State University

Major Field: Biological Sciences (Microbiology)

Major Professor: Karen S. Coats

Title of Study: IMMUNOMODULATOR EXPRESSION IN TROPHOBLASTS FROM THE FELINE IMMUNODEFICIENCY VIRUS (FIV)-INFECTED CAT AS A CONTRIBUTOR TO PLACENTAL IMMUNOPATHOLOGY AND REPRODUCTIVE FAILURE AT EARLY- AND LATE-TERM PREGNANCY

Pages in Study: 186

Candidate for Degree of Doctor of Philosophy

Mother-to-child transmission (MTCT) of HIV accounts for more than 90% of pediatric infections worldwide, yet the mechanism of vertical transfer remains unknown. The feline immunodeficiency virus (FIV)-infected cat is a cost-effective, small-animal model of HIV pathogenesis and MTCT, which produces a high rate of reproductive failure and fetal infection in litters delivered at early- and late-term gestation. Our previous data suggest that FIV infection may dysregulate placental cytokines and compromise pregnancy. We hypothesized that FIV-infection may cause dysregulation of placental cytokine expression, and aberrant expression of these cytokines may potentiate inflammation and transplacental infections. The purpose of this project was to evaluate feline placental immunopathology at the whole and cellular levels during early- and late-term gestation to understand how lentiviruses may perturb placental immune parameters. To determine whether placentas were vulnerable to FIV infection, we quantified the

expression of the FIV receptors, CD134 and CXCR4, in RNA extracted from late-term placental tissue. We found higher expression of CD134 and CXCR4 in placentas from successful pregnancies. To evaluate relative cytokine expression in randomly-sampled, whole placental specimens, we quantified representative pro- and anti-inflammatory cytokines and a chemokine. IL-6 and IL-12p35 were increased in early-gestation, FIV-infected queens; IL-6 was increased in late-gestation, FIV-infected queens. To evaluate placental immunopathology at the cellular level, we developed a novel immunohistochemistry method to identify trophoblastic cells selectively. Trophoblasts were collected using laser capture microdissection, and RNA was extracted from captured cells. We detected expression of several anti- and pro-inflammatory cytokines and the chemokine receptor CXCR4 (the FIV co-receptor) in trophoblasts at both stages of gestation. However, we failed to detect expression of other cytokines and CD134, the FIV primary receptor. FIV infection slightly lowered expression of all cytokines at both early and late pregnancy, although only the decrease in IL-5, from early pregnancy, and IL-4 and IL-12p35, from late pregnancy, reached significant levels. Fetal non-viability was associated with decreased trophoblast expression of IL-4, IL-6, IL-12p35, and CXCR4 at early gestation and decreased expression of IL-4, IL-12p35, IL-12p40 at late gestation. Collectively, these data indicate that FIV infection negatively impacts pregnancy outcome and alters placental immunomodulation.

DEDICATION

TO GOD BE THE GLORY!!! I would like to first give thanks and honor to my Lord and Savior, Jesus Christ, without whom none of this would have been possible! I thank Him for blessing me with two wonderful parents, Rev. and Mrs. David A. Scott. I am so grateful for the love, support, encouragement, guidance, and prayers you all gave me throughout my life and graduate career. I dedicate this work to you. Thank you, Mom and Dad!

ACKNOWLEDGEMENTS

Matriculation through graduate school requires a village of people offering their assistance, guidance, and support. There are several people that I would like to thank for being a part of my village. I thank my family, friends, and the family of Peter's Rock Temple C.O.G.I.C. for loving and praying me through the tough times. I am grateful for all of the research assistance given to me by the faculty and staff of the Department of Basic Sciences in the College of Veterinary Medicine. I would also like to thank the Alliance for Graduate Education in Mississippi (AGEM) and Dr. William A. Person for the financial support and graduate programs that assisted me in my matriculation through graduate school. I thank Leslie A. Shack for instructing me about quantitative PCR. I thank Kylie Nash and Will Gray III for staying with me in the laboratory during the early morning hours while I completed my experiments. I am grateful to have had wonderful and supportive lab partners, Crystal E. Boudreaux, Brittany T. Clay, and Nikki N. Lockett. And last but certainly not least, I would like to thank my graduate committee, Dr. Karen S. Coats, Dr. Donald N. Downer, Dr. Dwayne A. Wise, Dr. Shane C. Burgess, Dr. Peter Ryan, and Dr. Phillip D. Nelson for all of the guidance you all provided throughout my graduate program. I am grateful to Dr. Karen S. Coats for giving me the opportunity to earn a graduate degree and serving as my major professor. Thank you for motivating and challenging me throughout my collegiate and graduate career.

TABLE OF CONTENTS

CHAPTER Page

CHAPTER Page

LIST OF TABLES

TABLE Page

LIST OF FIGURES

FIGURE Page

FIGURE Page

FIGURE Page

CHAPTER I

REVIEW OF PERTINENT LITERATURE

The AIDS pandemic remains a global health concern despite prevention strategies and effective antiretroviral drugs. In 2008, there were an estimated 33.4 million people living with HIV, 15.7 million of which were women (UNAIDS/WHO, 2009a). Of the HIV-infected, pregnant women living in low to middle income countries, less than 45% received anti-viral therapy. Thirty-two percent of infants born to infected, untreated women received antiviral treatment (UNAIDS/WHO, 2009a). In 2008, children accounted for 430,000 new infections with HIV and most were the result of mother-to-child transmission (MTCT) (WHO, 2009). In the United States, MTCT is the most common source of all AIDS cases in children and results in 100–200 infected infants annually (CDC, 2007). Children who are infected *in utero* have a more rapid progression to AIDS and generally become symptomatic during the first year of life. In the absence of treatment, 50% of HIV-infected newborns will succumb to the disease before 24 months of age (UNAIDS/WHO, 2009b). Fortunately, the use of antiviral drugs by HIV-infected, pregnant women in the United States has reduced the number of perinatally acquired HIV infections by approximately 90% since 1990 (CDC, 2010). Moreover, the estimated number of pediatric AIDS cases (defined as AIDS occurring in children under the age of 13 years) has decreased 64% from 2002 to 2006 in the U.S. (CDC, 2006).

However in non-industrialized nations access to antiretroviral treatment is limited, and without therapy pregnant women are more likely to transmit the virus to their babies.

The Feline Immunodeficiency Virus (FIV)-infected Cat as a Model for HIV-infections

Discovery of the FIV-infected cat research model

The feline immunodeficiency virus is a lentivirus affecting domestic cats that causes a clinical course of infection similar to that of HIV. There is evidence of seroreactivity to FIV in cat sera dating as far back as 1968 (Shelton et al., 1990). The virus was first isolated in 1986 from cats with clinical features of an AIDS-like complex in a cattery in California (Pedersen et al., 1987). In 1987, the laboratory of Neils Pederson first described the feline immunodeficiency virus (Pedersen et al., 1987). The clinical description of this virus led Pederson and coworkers to realize the potential for FIV to serve as a model for HIV. Since then, the FIV-infected cat has become the most utilized small animal model for HIV biomedical research, allowing the study of lentivirus biology including viral transmission and pathogenesis, host immune responses, and vaccine development (Willett et al., 1997). Moreover, the FIV-infected cat is the only animal model for HIV that utilizes a lentivirus that infects its natural host.

Cellular receptors used by FIV and HIV

FIV and HIV are T-lymphotropic lentiviruses in the *Retroviridae* family. FIV causes a natural infection of domestic cats that resembles HIV-1 infection in pathogenesis

and disease progression. Both HIV and FIV are characterized by progressive depletion of the CD4+ T cell population and fatal opportunistic infections. Although both viruses use the same co-receptor, the chemokine receptor CXCR4, FIV does not utilize the HIV primary receptor, CD4 (de Parseval et al., 2004a; Shimojima et al., 2004). The primary FIV receptor is CD134 (OX40), a T cell activation and co-stimulatory molecule (Shimojima et al., 2004). The virus binds these receptors via its surface glycoprotein, gp95 (de Parseval and Elder, 2001). The cellular tropism of FIV broadens with disease progression, consistent with the expression of CD134 (Shimojima et al., 2004), and includes helper T cells, regulatory T cells, B cells, and others. Progression to disease in HIV-1 infections is correlated with the ability to bind CXCR4 (Connor et al., 1997; Lu et al., 1997). Similarly, susceptibility to infection with FIV is directly related to cellular expression of CXCR4, levels of which were shown to be the limiting factor in productive infection of feline cells (de Parseval et al., 2004b; Willett et al., 2002). Despite the use of different primary receptors, the shared usage of CXCR4 as a cellular co-receptor by HIV and FIV establishes similarities in cell tropism between the two lentiviruses, thus reiterating the usefulness of the FIV-infected cat model.

Modes of FIV transmission

FIV is primarily transmitted via contact with infectious saliva in bite wounds (Pedersen et al., 1987). Mucosal transmission of either cell-free or cell-associated virus can be established following application to vaginal and rectal tissue under experimental conditions (Bishop et al., 1996; Burkhard et al., 1997). Virus was detected in semen during acute FIV infection (Jordan et al., 1999). Horizontal transmission of FIV was

demonstrated when FIV-negative queens became infected after artificial insemination with semen from asymptomatic, chronically-infected toms (Jordan et al., 1998). There is no evidence of transplacental transfer of FIV occurring in natural infection (Callanan et al., 1991; Ueland and Nesse, 1992). However, vertical transmission of FIV can be readily achieved under experimental conditions with certain FIV isolates (Allison and Hoover, 2003; Boudreaux et al., 2009; O'Neil et al., 1995; O'Neil et al., 1996; Rogers and Hoover, 1998, 2002; Weaver et al., 2005).

Feline vs. Human Placentation

The placenta, an endocrine organ of fetal origin, is responsible for metabolic exchange between the mother and the developing fetus, and produces hormones that are responsible for pregnancy maintenance and fetal growth. Placentation varies between species and is classified based upon distribution of chorionic villi and the quantity of placental layers that separate maternal and fetal blood. Humans, primates, and rodents have a hemochorial placenta in which the chorionic epithelium is directly apposed to maternal pools of blood. Therefore, maternal blood products (nutrients, gases, and possibly viruses) move through three tissue layers and enter the fetal circulation via chorionic capillaries. Cats possess an endotheliochorial placenta in which the endometrial epithelium and the underlying interstitium are completely eroded. Thus, maternal blood products are exchanged through five tissue layers to reach the fetal circulation via chorionic capillaries. In addition, human and feline placentation also differs in the distribution of chorionic villi, the functional portion of the fetal placenta. Humans possess a discoid placenta in which the chorionic villi are arranged on

contiguous discs that border the endometrium and provide a site for nutrient and waste exchange. Cats have a zonary placenta which consists of three zones; a transfer zone, a pigmented zone, and a nonvascular zone, the allantochorion. The transfer zone is the site for nutrient and waste exchange and forms around the conceptus. The pigmented zone is located on the ends of the transfer zone and contains maternal blood clots and hemorrhages. The allantochorion is a poorly vascularized, transparent zone located on the chorion distal end and functions in the adsorption of materials from the uterine lumen. Placental chorionic villi are composed of trophoblastic cells (cytotrophoblasts and syncytiotrophoblasts). These trophoblasts are fetal-derived cells of epithelial origin that cover the blastocyst and form the chorion. Syncytiotrophoblasts comprise the outer layer of the trophoblast that contact and form attachment with the maternal endometrium (Senger, 2003). The cytotrophoblasts form the inner layer of the trophoblast between the syncytiotrophoblasts and the chorionic villous capillaries. In human pregnancy, these trophoblast layers form a barrier between maternal and fetal blood (Lagaye et al., 2001) and are likely to be infected if vertical transfer of the virus occurs (Douglas et al., 1991). Our data (Boudreaux et al., 2009; Weaver et al., 2005) and the data of others (Allison and Hoover, 2003; O'Neil et al., 1995; O'Neil et al., 1996; Rogers and Hoover, 1998, 2002) show that like HIV, FIV is transmissible by vertical routes, despite differences in placentation between cats and humans. Thus, the feline model is suitable for the study of lentiviral intrauterine transmission.

Pregnancy Outcome in HIV and FIV infections

HIV infection of pregnant women may perturb pregnancy and negatively impact reproductive outcome. Spontaneous fetal death was reported in a cohort of HIV-infected American women (Langston et al., 1995). In that study, fetal loss occurred in 14 of 124 (11.3%) pregnancies evaluated during 8 to 32 weeks gestation. A 67% increase in the risk of spontaneous abortions occurred in a study of HIV-infected Italian women (D'Ubaldo et al., 1998). Similarly, spontaneous abortion and poor reproductive outcome was observed in symptomatic, HIV-infected Indian women (Kumar et al., 1995). Reproductive failure was also reported in HIV-infected, pregnant women receiving the highly active antiretroviral therapy (HAART) in urban Nigeria (Olagbuji et al., 2009). In that study, HIV-infected women were more likely to have intrauterine growth restriction, preterm labor, and low birth weights when compared to HIV-negative controls.

Transmission of FIV occurs frequently *in utero*, often resulting in reproductive failure (Boudreaux et al., 2009; Rogers and Hoover, 1998; Weaver et al., 2005). In previous reports by our laboratory (Boudreaux et al., 2009; Weaver et al., 2005) and others (O'Neil et al., 1996) FIV-B-2542-infected queens transmitted the virus to more than 50% of their offspring. Boudreaux et al. (2009) reported that fetal non-viability (defined as fetal resorptions or developmentally-arrested fetuses) at early gestation was 4.7% (2/43 fetuses) in control cats and 22.2% (6/27 fetuses) in FIV-infected cats. We also reported high rates of fetal non-viability at late gestation (Weaver et al., 2005). In that report, fetal non-viability was 3.2% (1/31 fetuses) in control cats and 60% (15/25 fetuses) in FIV-infected cats. O'Neil et al. (1995) reported higher incidences of fetal non-viability associated with acutely-infected versus chronically-infected queens (53%

and 30%, respectively), and the rate of *in utero* FIV-infection increased with gestational age (Rogers and Hoover, 1998). However, we detected a high rate of fetal infection by week 3–4 gestation with 12 of 14 fetuses positive for viral RNA (Boudreaux et al., 2009). Therefore, compromised reproductive outcome is a feature showed by FIV and HIV. Clearly, the use of the feline model may elucidate the mechanisms of lentiviral transplacental transfer and the impact of infection on pregnancy outcome.

The Immunology of Pregnancy

The immunology of pregnancy remains an inexplicable phenomenon and continues to be a topic of great debate among reproductive biologists. The human embryo and fetus are semi-allografts, and it is unclear why this allogeneic tissue is not rejected during gestation. Immunoregulatory molecules such as cytokines and chemokines are responsible for maternal tolerance of embryo and fetal allografts, embryo implantation, endometrial development, the development of fetal placentation, and the regulation of maternal cell-mediated and humoral immune responses against infection (Bennett et al., 1998; Challis et al., 2009; Clark and Chaouat, 1989; Robertson et al., 1994; Saito, 2001). The cytokines and chemokines that modulate pregnancy are found in syncytio- and cytotrophoblastic cells, decidual epithelium and stroma, chorionic and amnionic membranes, and Hofbauer cells (Wilczynski, 2005). Wegmann et al. (1993) proposed that a Th1/Th2 cytokine paradigm exists during normal pregnancy in which successful outcome is biased toward a Th2 (anti-inflammatory) immune response with a suppressed Th1 (pro-inflammatory) immune response at the maternal-fetal interface. The production of Th2 cytokines aids in the maintenance of pregnancy by

downregulating Th1 cytokines, thereby preventing the formation of an inflammatory microenvironment in the placenta (Challis et al., 2009). Th1 and Th2 cytokines are mutually antagonistic (Mosmann and Coffman, 1989; Wegmann et al., 1993). The altered expression of the Th1 cytokine, IL-12 (a pro-inflammatory cytokine), and the Th2 cytokine, IL-10 (an anti-inflammatory cytokine), is a clear indicator of Th1:Th2 immune shifts and cytokine dysregulation (Challis et al., 2009; Costeas et al., 2004; Romagnani, 1994; Wegmann et al., 1993). Cytokine dysregulation in the placenta may result in poor reproductive outcome, including spontaneous abortion, preterm labor, preeclampsia, and intrauterine growth restriction (Challis et al., 2009; Orsi and Tribe, 2008). Cytokine dysregulation in the murine model was reported (Chaouat et al., 1995; Clark et al., 1998). In those studies, spontaneous abortion occurred in pregnant CBA/J x DBA/2 mice when administered exogenous Th1 cytokines (IL-2, TNF-α, and IFN-γ). However, spontaneous abortion in pregnant CBA/J x DBA/2 mice was prevented when mice were administered antibodies against Th1 cytokines or given the exogenous Th2 cytokines (IL-10, GM-CSF, and IL-13) (Chaouat et al., 1995; Clark et al., 1998). Thus, it appears that Th1 immunity is associated with fetal loss and that successful murine pregnancy is biased towards a Th2 immune response. Similarly, cytokine dysregulation in human pregnancy and subsequent spontaneous fetal loss was reported (Daher et al., 2004; Hill et al., 1995; Lim et al., 2000; Makhseed et al., 2001; Marzi et al., 1996; Raghupathy et al., 1999; Raghupathy et al., 2000). In those studies, women with recurrent spontaneous abortions expressed higher levels of Th1 cytokines, IFNγ (Daher et al., 2004; Hill et al., 1995; Lim et al., 2000; Marzi et al., 1996; Raghupathy et al., 1999; Raghupathy et al., 2000), TNF-α (Daher et al., 2004; Raghupathy et al., 2000), and IL-2 (Hill et al., 1995; Marzi et al.,

1996; Raghupathy et al., 2000) and decreased levels of Th2 cytokines, IL-10 (Hill et al., 1995; Marzi et al., 1996; Raghupathy et al., 1999; Raghupathy et al., 2000) , IL-6 (Lim et al., 2000; Raghupathy et al., 1999; Raghupathy et al., 2000), and IL-4 (Hill et al., 1995; Marzi et al., 1996; Raghupathy et al., 2000) when compared to normal pregnant women. Therefore it appears that, like the murine model, normal human pregnancy is biased towards Th2 cytokine production and that Th1-type immunity is associated with reproductive failure.

On the other hand, the Th1/Th2 paradigm of pregnancy has been challenged (Bates et al., 2002; Chaouat, 2003; Chaouat et al., 2004a, b; Chaouat et al., 2002; Wilczynski, 2005). Chaouat et al. (2002) suggested that this paradigm is an 'oversimplification' after evaluating Th1/Th2 cytokine expression at the maternal-fetal interface of mice. For example, there was greater expression of IL-18, a Th1-type cytokine that is responsible for the induction of IFN-γ, in the deciduas and placentas of non-abortion-prone mice (CBA x BALB/c mating) than in abortion-prone mice (CBA x DBA/2 mating) (Ostojic et al., 2003). Bates et al. (2002) reported increased production of IFN-γ and decreased production of IL-10 and IL-4 in peripheral blood mononuclear cells (PBMCs) of normal pregnant women when compared to women with recurrent pregnancy loss (RPL). The data repudiated the hypothesis that Th1 immunity is associated with fetal loss. Moreover, in that report, there was no significant difference in the expression of IFN-γ, IL-10, or IL-4 in women with a history of RPL who subsequently aborted when compared to RPL women with successful pregnancy outcomes. Interestingly, the concentrations of TNF-α in PBMCs were similar in RPL women with successful pregnancy outcomes and normal pregnant women. In addition,

RPL women who subsequently aborted expressed lower concentrations of TNF-α when compared to normal pregnant women. Other investigators have reported reduced production of IFN-γ in uterine irrigation fluid of women with a history of recurrent spontaneous abortions (Inagaki et al., 2003), challenging the hypothesis of cell-mediated immunity as a detriment to pregnancy. However, the specific placental cells contributing to immunopathology were not evaluated in those reports and it is unclear whether infection was a contributor to reproductive failure.

The production of placental immunomodulators such as cytokines augment HIV expression (Kedzierska and Crowe, 2001), and HIV infections can dysregulate cytokine networks in placental tissues (Lee et al., 1997; Shearer et al., 1997). Aberrant expression of cytokines may result in placental inflammation, and the presence of inflamed placental membranes is likely to facilitate viral transplacental transfer (Shearer et al., 1997). Placental trophoblastic cells can be infected with HIV (Lee et al., 2001; Lee et al., 1997; Vidricaire et al., 2003; Zachar et al., 2002). Lee et al. (1997) evaluated the role of placental inflammatory cytokine expression in vertical transfer of HIV. In that report, the expression of IL-1β, IL-6, and TNF-α was evaluated in unstimulated and LPS-stimulated trophoblast cell cultures from HIV-infected and uninfected women. Placental trophoblasts from HIV-infected placentas expressed significantly higher amounts of IL-1β, IL-6, and TNF-α than placentas from uninfected women. The data indicate that HIV-infection during pregnancy results in increased cytokine expression and that a pro-inflammatory microenvironment (an environment potentially conducive to vertical transfer) exists in HIV-infected placentas. Mechanisms of maternal-fetal transcytosis of HIV were reported (Lagaye et al., 2001; Parry et al., 2006; Vidricaire et al., 2004).

Vidricaire et al. (2004) reported that HIV virions were internalized by polarized, human trophoblastic JAR cells *in vitro* using endocytic host cell machinery. The endocytosed virus was trafficked from the apical pole of cells, transported in a vesicular system, and released intact at the basolateral pole. This process of membrane trafficking could allow the virus to traverse the placenta and infect fetal trophoblastic cells. In addition, expression of pro-inflammatory mediators such as TNF-α contributed to transcytosis of HIV across trophoblastic cell layers (Parry et al., 2006). In that study, transcytosis of HIV from infected peripheral blood mononuclear cells was upregulated by TNF-α. Moreover, the presence of TNF-α did not perturb cellular viability or confluency as other investigators reported (Garcia-Lloret et al., 1996; Yui et al., 1994). Although maternal-fetal membrane trafficking clearly plays a role in HIV transplacental transfer, the affect of aberrant placental immunomodulator expression on MTCT and reproductive outcome remains undefined.

Placental Cytokine and Chemokine Expression in the FIV-infected Cat

Using the FIV-infected cat as a model for lentiviral transplacental transmission of HIV, we evaluated how infection might impact the expression of placental immunomodulators. Weaver et al. (2005) reported no significant difference in the expression of pro-inflammatory cytokines (IFN-γ and IL-1β), or the anti-inflammatory cytokine (IL-10) between infected and control cats. However, increased placental expression of IFN-γ and IL-1β was associated with non-viable pregnancies. The data suggest that, similar to HIV, FIV infections during pregnancy may result in aberrant cytokine production and reproductive failure. Recently, we hypothesized that placental

expression of CD134 and CXCR4 may render the placenta vulnerable to FIV infection, possibly facilitating efficient vertical transmission of FIV, and impact pregnancy outcome (Scott et al., 2008). Using real-time one-step reverse transcriptase-PCR, we quantified the relative expression of CD134 and CXCR4 mRNA from term placentas of three groups of cats: uninfected queens producing viable offspring, experimentally-infected queens producing only viable offspring, and experimentally-infected queens producing viable offspring among mostly non-viable fetuses. Placentas from FIV-infected queens producing litters of only viable offspring expressed more CD134 and CXCR4 mRNA than those from uninfected queens, suggesting that infection may cause upregulation of the receptors. On the other hand, placentas from FIV-infected cats with non-successful pregnancies expressed similar levels of CD134 mRNA and slightly less CXCR4 mRNA than those from uninfected queens. Thus, it appears that cells expressing these receptors may play a role in pregnancy maintenance.

Significance and Purpose

Although antiretroviral therapy is effective in reducing MTCT of HIV, pregnancy remains at-risk among the population of under-developed nations where antiretroviral therapy is not accessible. We are interested in understanding the mechanisms by which HIV crosses the placenta to establish fetal infection and/or compromise pregnancy. Using the FIV-infected cat model, we demonstrated frequent reproductive failure in litters delivered at early- and late-gestation by cesarean section (Boudreaux et al., 2009; Weaver et al., 2005). Vertical transmission of FIV occurred in nearly all pregnancies, and FIV was detected in the tissues of both viable and non-viable kittens and fetuses.

Litter size was larger in uninfected cats and incidences of fetal non-viability occurred at high rates in fetuses delivered at both early- and late-gestation among FIV-infected queens. To assess a potential role for the placental immune response in vertical transmission and reproductive failure, we evaluated a variety of immune parameters in placentas harvested at early- and late-gestation. We were particularly interested in whether inflammation contributes to pregnancy outcome. We evaluated placental expression of selected pro-inflammatory (Th1) and anti-inflammatory (Th2) cytokines, along with FIV receptors. Th1 and Th2 cytokine expression did not differ significantly in the placentas of infected and uninfected cats. Th1 cytokine expression was significantly increased in placentas of FIV-infected, resorbed fetuses in comparison to placentas from viable pregnancies (Weaver et al., 2005). These data parallel some of the immunological abnormalities that occur in placentas of women with compromised pregnancy, including HIV-infected pregnant women who spontaneously abort (Behbahani et al., 2000; Courgnaud et al., 1991; D'Ubaldo et al., 1998; Kumar et al., 1995; Langston et al., 1995; Moussa et al., 2001). We concluded that FIV infection causes abnormalities that perturb pregnancy, probably early in gestation, leading to compromised reproduction and fetal death. Whether this outcome is a result of virus-induced placental pathology or direct viral attack on the developing fetus remains unknown. The specific cell populations expressing the potentially harmful cytokines were not identified (Weaver et al., 2005). In the present study, we hypothesized that FIV infections may cause dysregulation of trophoblastic cytokine expression, and aberrant expression of these cytokines may potentiate inflammation and transplacental infections. Our aim was to use the FIV-infected cat model to evaluate placental immunopathology at the cellular level, during

both early- and late-gestation, to begin to understand mechanistically how lentiviruses perturb the placenta and are transmitted transplacentally, leading to fetal infection and/or compromised pregnancy.

References

Allison, R.W., Hoover, E.A., 2003, Covert vertical transmission of feline immunodeficiency virus. AIDS Res Hum Retroviruses 19, 421-434.

Bates, M.D., Quenby, S., Takakuwa, K., Johnson, P.M., Vince, G.S., 2002, Aberrant cytokine production by peripheral blood mononuclear cells in recurrent pregnancy loss? Hum Reprod 17, 2439-2444.

Behbahani, H., Popek, E., Garcia, P., Andersson, J., Spetz, A.L., Landay, A., Flener, Z., Patterson, B.K., 2000, Up-regulation of CCR5 expression in the placenta is associated with human immunodeficiency virus-1 vertical transmission. Am J Pathol 157, 1811-1818.

Bennett, W.A., Lagoo-Deenadayalan, S., Stopple, J.A., Barber, W.H., Hale, E., Brackin, M.N., Cowan, B.D., 1998, Cytokine expression by first-trimester human chorionic villi. Am J Reprod Immunol 40, 309-318.

Bishop, S.A., Stokes, C.R., Gruffydd-Jones, T.J., Whiting, C.V., Harbour, D.A., 1996, Vaginal and rectal infection of cats with feline immunodeficiency virus. Vet Microbiol 51, 217-227.

Boudreaux, C.E., Lockett, N.N., Chemerys, D.N., Clay, B.T., Scott, V.L., Willeford, B., Brown, T., Coats, K.S., 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. Vet Immunol Immunopathol 131, 290-297.

Burkhard, M.J., Obert, L.A., O'Neil, L.L., Diehl, L.J., Hoover, E.A., 1997, Mucosal transmission of cell-associated and cell-free feline immunodeficiency virus. AIDS Res Hum Retroviruses 13, 347-355.

Callanan, J.J., Hosie, M.J., Jarrett, O., 1991, Transmission of feline immunodeficiency virus from mother to kitten. Vet Rec 128, 332-333.

CDC 2006. HIV/AIDS Surveillance Report: Cases of HIV infection and AIDS in the United States and Dependent Areas.

CDC 2007. Mother-to-Child (Perinatal) HIV Transmission and Prevention. In CDC HIV/AIDS Fact Sheet (Department of Health and Human Services, USA).

CDC, 2010, Racial/ethnic disparities among children with diagnoses of perinatal HIV infection - 34 states, 2004-2007. MMWR Morb Mortal Wkly Rep 59, 97-101.

Challis, J.R., Lockwood, C.J., Myatt, L., Norman, J.E., Strauss, J.F., 3rd, Petraglia, F., 2009, Inflammation and pregnancy. Reprod Sci 16, 206-215.

Chaouat, G., 2003, Innately moving away from the Th1/Th2 paradigm in pregnancy. Clin Exp Immunol 131, 393-395.

Chaouat, G., Assal Meliani, A., Martal, J., Raghupathy, R., Elliott, J.F., Mosmann, T., Wegmann, T.G., 1995, IL-10 prevents naturally occurring fetal loss in the CBA x DBA/2 mating combination, and local defect in IL-10 production in this abortion-prone combination is corrected by in vivo injection of IFN-tau. J Immunol 154, 4261-4268.

Chaouat, G., Ledee-Bataille, N., Dubanchet, S., Zourbas, S., Sandra, O., Martal, J., 2004a, Reproductive immunology 2003: reassessing the Th1/Th2 paradigm? Immunol Lett 92, 207-214.

Chaouat, G., Ledee-Bataille, N., Dubanchet, S., Zourbas, S., Sandra, O., Martal, J., 2004b, TH1/TH2 paradigm in pregnancy: paradigm lost? Cytokines in pregnancy/early abortion: reexamining the TH1/TH2 paradigm. Int Arch Allergy Immunol 134, 93-119.

Chaouat, G., Zourbas, S., Ostojic, S., Lappree-Delage, G., Dubanchet, S., Ledee, N., Martal, J., 2002, A brief review of recent data on some cytokine expressions at the materno-foetal interface which might challenge the classical Th1/Th2 dichotomy. J Reprod Immunol 53, 241-256.

Clark, D.A., Chaouat, G., 1989, What do we know about spontaneous abortion mechanisms? Am J Reprod Immunol 19, 28-37.

Clark, D.A., Chaouat, G., Arck, P.C., Mittruecker, H.W., Levy, G.A., 1998, Cytokine-dependent abortion in CBA x DBA/2 mice is mediated by the procoagulant fgl2 prothrombinase [correction of prothombinase]. J Immunol 160, 545-549.

Connor, R.I., Sheridan, K.E., Ceradini, D., Choe, S., Landau, N.R., 1997, Change in coreceptor use coreceptor use correlates with disease progression in HIV-1--infected individuals. J Exp Med 185, 621-628.

Costeas, P.A., Koumouli, A., Giantsiou-Kyriakou, A., Papaloizou, A., Koumas, L., 2004, Th2/Th3 cytokine genotypes are associated with pregnancy loss. Hum Immunol 65, 135-141.

Courgnaud, V., Laure, F., Brossard, A., Bignozzi, C., Goudeau, A., Barin, F., Brechot, C., 1991, Frequent and early in utero HIV-1 infection. AIDS Res Hum Retroviruses 7, 337-341.

D'Ubaldo, C., Pezzotti, P., Rezza, G., Branca, M., Ippolito, G., 1998, Association between HIV-1 infection and miscarriage: a retrospective study. DIANAIDS Collaborative Study Group. Diagnosi Iniziale Anomalie Neoplastiche AIDS. Aids 12, 1087-1093.

Daher, S., de Arruda Geraldes Denardi, K., Blotta, M.H., Mamoni, R.L., Reck, A.P., Camano, L., Mattar, R., 2004, Cytokines in recurrent pregnancy loss. J Reprod Immunol 62, 151-157.

de Parseval, A., Chatterji, U., Sun, P., Elder, J.H., 2004a, Feline immunodeficiency virus targets activated CD4+ T cells by using CD134 as a binding receptor. Proc Natl Acad Sci U S A 101, 13044-13049.

de Parseval, A., Elder, J.H., 2001, Binding of recombinant feline immunodeficiency virus surface glycoprotein to feline cells: role of CXCR4, cell-surface heparans, and an unidentified non-CXCR4 receptor. J Virol 75, 4528-4539.

de Parseval, A., Ngo, S., Sun, P., Elder, J.H., 2004b, Factors that increase the effective concentration of CXCR4 dictate feline immunodeficiency virus tropism and kinetics of replication. J Virol 78, 9132-9143.

Douglas, G.C., Fry, G.N., Thirkill, T., Holmes, E., Hakim, H., Jennings, M., King, B.F., 1991, Cell-mediated infection of human placental trophoblast with HIV in vitro. AIDS Res Hum Retroviruses 7, 735-740.

Garcia-Lloret, M.I., Yui, J., Winkler-Lowen, B., Guilbert, L.J., 1996, Epidermal growth factor inhibits cytokine-induced apoptosis of primary human trophoblasts. J Cell Physiol 167, 324-332.

Hill, J.A., Polgar, K., Anderson, D.J., 1995, T-helper 1-type immunity to trophoblast in women with recurrent spontaneous abortion. Jama 273, 1933-1936.

Inagaki, N., Stern, C., McBain, J., Lopata, A., Kornman, L., Wilkinson, D., 2003, Analysis of intra-uterine cytokine concentration and matrix-metalloproteinase activity in women with recurrent failed embryo transfer. Hum Reprod 18, 608-615.

Jordan, H.L., Howard, J.G., Bucci, J.G., Butterworth, J.L., English, R., Kennedy-Stoskopf, S., Tompkins, M.B., Tompkins, W.A., 1998, Horizontal transmission of feline immunodeficiency virus with semen from seropositive cats. J Reprod Immunol 41, 341-357.

Jordan, H.L., Liang, Y., Hudson, L.C., Tompkins, W.A., 1999, Shedding of feline immunodeficiency virus in semen of domestic cats during acute infection. Am J Vet Res 60, 211-215.

Kedzierska, K., Crowe, S.M., 2001, Cytokines and HIV-1: interactions and clinical implications. Antivir Chem Chemother 12, 133-150.

Kumar, R.M., Uduman, S.A., Khurranna, A.K., 1995, Impact of maternal HIV-1 infection on perinatal outcome. Int J Gynaecol Obstet 49, 137-143.

Lagaye, S., Derrien, M., Menu, E., Coito, C., Tresoldi, E., Mauclere, P., Scarlatti, G., Chaouat, G., Barre-Sinoussi, F., Bomsel, M., 2001, Cell-to-cell contact results in a selective translocation of maternal human immunodeficiency virus type 1 quasispecies across a trophoblastic barrier by both transcytosis and infection. J Virol 75, 4780-4791.

Langston, C., Lewis, D.E., Hammill, H.A., Popek, E.J., Kozinetz, C.A., Kline, M.W., Hanson, I.C., Shearer, W.T., 1995, Excess intrauterine fetal demise associated with maternal human immunodeficiency virus infection. J Infect Dis 172, 1451-1460.

Lee, B.N., Hammill, H., Popek, E.J., Cron, S., Kozinetz, C., Paul, M., Shearer, W.T., Reuben, J.M., 2001, Production of interferons and beta-chemokines by placental trophoblasts of HIV-1-infected women. Infect Dis Obstet Gynecol 9, 95-104.

Lee, B.N., Ordonez, N., Popek, E.J., Lu, J.G., Helfgott, A., Eriksen, N., Hammill, H., Kozinetz, C., Doyle, M., Kline, M., Langston, C., Shearer, W.T., Reuben, J.M., 1997, Inflammatory cytokine expression is correlated with the level of human immunodeficiency virus (HIV) transcripts in HIV-infected placental trophoblastic cells. J Virol 71, 3628-3635.

Lim, K.J., Odukoya, O.A., Ajjan, R.A., Li, T.C., Weetman, A.P., Cooke, I.D., 2000, The role of T-helper cytokines in human reproduction. Fertil Steril 73, 136-142.

Lu, Z., Berson, J.F., Chen, Y., Turner, J.D., Zhang, T., Sharron, M., Jenks, M.H., Wang, Z., Kim, J., Rucker, J., Hoxie, J.A., Peiper, S.C., Doms, R.W., 1997, Evolution of HIV-1 coreceptor usage through interactions with distinct CCR5 and CXCR4 domains. Proc Natl Acad Sci U S A 94, 6426-6431.

Makhseed, M., Raghupathy, R., Azizieh, F., Omu, A., Al-Shamali, E., Ashkanani, L., 2001, Th1 and Th2 cytokine profiles in recurrent aborters with successful pregnancy and with subsequent abortions. Hum Reprod 16, 2219-2226.

Marzi, M., Vigano, A., Trabattoni, D., Villa, M.L., Salvaggio, A., Clerici, E., Clerici, M., 1996, Characterization of type 1 and type 2 cytokine production profile in physiologic and pathologic human pregnancy. Clin Exp Immunol 106, 127-133.

Mosmann, T.R., Coffman, R.L., 1989, Heterogeneity of cytokine secretion patterns and functions of helper T cells. Adv Immunol 46, 111-147.

Moussa, M., Roques, P., Fievet, N., Menu, E., Maldonado-Estrada, J.G., Brunerie, J., Frydman, R., Fritel, X., Herve, F., Chaouat, G., 2001, Placental cytokine and chemokine production in HIV-1-infected women: trophoblast cells show a different pattern compared to cells from HIV-negative women. Clin Exp Immunol 125, 455-464.

O'Neil, L.L., Burkhard, M.J., Diehl, L.J., Hoover, E.A., 1995, Vertical transmission of feline immunodeficiency virus. Semin Vet Med Surg (Small Anim) 10, 266-278.

O'Neil, L.L., Burkhard, M.J., Hoover, E.A., 1996, Frequent perinatal transmission of feline immunodeficiency virus by chronically infected cats. J Virol 70, 2894-2901.

Olagbuji, B.N., Ezeanochie, M.C., Ande, A.B., Oboro, V.O., 2009, Obstetric and perinatal outcome in HIV positive women receiving HAART in urban Nigeria. Arch Gynecol Obstet.

Orsi, N.M., Tribe, R.M., 2008, Cytokine networks and the regulation of uterine function in pregnancy and parturition. J Neuroendocrinol 20, 462-469.

Ostojic, S., Dubanchet, S., Chaouat, G., Abdelkarim, M., Truyens, C., Capron, F., 2003, Demonstration of the presence of IL-16, IL-17 and IL-18 at the murine fetomaternal interface during murine pregnancy. Am J Reprod Immunol 49, 101-112.

Parry, S., Zhang, J., Koi, H., Arechavaleta-Velasco, F., Elovitz, M.A., 2006, Transcytosis of Human immunodeficiency virus 1 across the placenta is enhanced by treatment with tumour necrosis factor alpha. J Gen Virol 87, 2269-2278.

Pedersen, N.C., Ho, E.W., Brown, M.L., Yamamoto, J.K., 1987, Isolation of a T-lymphotropic virus from domestic cats with an immunodeficiency-like syndrome. Science 235, 790-793.

Raghupathy, R., Makhseed, M., Azizieh, F., Hassan, N., Al-Azemi, M., Al-Shamali, E., 1999, Maternal Th1- and Th2-type reactivity to placental antigens in normal human pregnancy and unexplained recurrent spontaneous abortions. Cell Immunol 196, 122-130.

Raghupathy, R., Makhseed, M., Azizieh, F., Omu, A., Gupta, M., Farhat, R., 2000, Cytokine production by maternal lymphocytes during normal human pregnancy and in unexplained recurrent spontaneous abortion. Hum Reprod 15, 713-718.

Robertson, S.A., Seamark, R.F., Guilbert, L.J., Wegmann, T.G., 1994, The role of cytokines in gestation. Crit Rev Immunol 14, 239-292.

Rogers, A.B., Hoover, E.A., 1998, Maternal-fetal feline immunodeficiency virus transmission: timing and tissue tropisms. J Infect Dis 178, 960-967.

Rogers, A.B., Hoover, E.A., 2002, Fetal feline immunodeficiency virus is prevalent and occult. J Infect Dis 186, 895-904.

Romagnani, S., 1994, Lymphokine production by human T cells in disease states. Annu Rev Immunol 12, 227-257.

Saito, S., 2001, Cytokine cross-talk between mother and the embryo/placenta. J Reprod Immunol 52, 15-33.

Scott, V.L., Burgess, S.C., Shack, L.A., Lockett, N.N., Coats, K.S., 2008, Expression of CD134 and CXCR4 mRNA in term placentas from FIV-infected and control cats. Vet Immunol Immunopathol 123, 90-96.

Senger, P.L., 2003, Pathways to Pregnancy and Parturition, 2nd Edition Edition. Current Conceptions, Inc., Pullman, WA, 368 p.

Shearer, W.T., Reuben, J., Lee, B.N., Popek, E.J., Lewis, D.E., Hammill, H.H., Hanson, I.C., Kline, M.W., Langston, C., 1997, Role of placental cytokines and inflammation in vertical transmission of HIV infection. Acta Paediatr Suppl 421, 33-38.

Shelton, G.H., Grant, C.K., Cotter, S.M., Gardner, M.B., Hardy, W.D., Jr., DiGiacomo, R.F., 1990, Feline immunodeficiency virus and feline leukemia virus infections and their relationships to lymphoid malignancies in cats: a retrospective study (1968-1988). J Acquir Immune Defic Syndr 3, 623-630.

Shimojima, M., Miyazawa, T., Ikeda, Y., McMonagle, E.L., Haining, H., Akashi, H., Takeuchi, Y., Hosie, M.J., Willett, B.J., 2004, Use of CD134 as a primary receptor by the feline immunodeficiency virus. Science 303, 1192-1195.

Ueland, K., Nesse, L.L., 1992, No evidence of vertical transmission of naturally acquired feline immunodeficiency virus infection. Vet Immunol Immunopathol 33, 301-308.

UNAIDS/WHO 2009a. AIDS Epidemic Update 2009. Joint United Nations Programme on HIV/AIDS.

UNAIDS/WHO 2009b. Towards Universal Access. Joint United Nations Programme on HIV/AIDS.

Vidricaire, G., Imbeault, M., Tremblay, M.J., 2004, Endocytic host cell machinery plays a dominant role in intracellular trafficking of incoming human immunodeficiency virus type 1 in human placental trophoblasts. J Virol 78, 11904-11915.

Vidricaire, G., Tardif, M.R., Tremblay, M.J., 2003, The low viral production in trophoblastic cells is due to a high endocytic internalization of the human immunodeficiency virus type 1 and can be overcome by the pro-inflammatory cytokines tumor necrosis factor-alpha and interleukin-1. J Biol Chem 278, 15832-15841.

Weaver, C.C., Burgess, S.C., Nelson, P.D., Wilkinson, M., Ryan, P.L., Nail, C.A., Kelly-Quagliana, K.A., May, M.L., Reeves, R.K., Boyle, C.R., Coats, K.S., 2005, Placental immunopathology and pregnancy failure in the FIV-infected cat. Placenta 26, 138-147.

Wegmann, T.G., Lin, H., Guilbert, L., Mosmann, T.R., 1993, Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a TH2 phenomenon? Immunol Today 14, 353-356.

WHO 2009. Mother-to-child transmission of HIV. Programmes and projects. HIV/AIDS Topics.

Wilczynski, J.R., 2005, Th1/Th2 cytokines balance--yin and yang of reproductive immunology. Eur J Obstet Gynecol Reprod Biol 122, 136-143.

Willett, B.J., Cannon, C.A., Hosie, M.J., 2002, Upregulation of surface feline CXCR4 expression following ectopic expression of CCR5: implications for studies of the cell tropism of feline immunodeficiency virus. J Virol 76, 9242-9252.

Willett, B.J., Flynn, J.N., Hosie, M.J., 1997, FIV infection of the domestic cat: an animal model for AIDS. Immunol Today 18, 182-189.

Yui, J., Garcia-Lloret, M., Wegmann, T.G., Guilbert, L.J., 1994, Cytotoxicity of tumour necrosis factor-alpha and gamma-interferon against primary human placental trophoblasts. Placenta 15, 819-835.

Zachar, V., Fink, T., Koppelhus, U., Ebbesen, P., 2002, Role of placental cytokines in transcriptional modulation of HIV type 1 in the isolated villous trophoblast. AIDS Res Hum Retroviruses 18, 839-847.

CHAPTER II

EXPRESSION OF CD134 AND CXCR4 mRNA IN TERM PLACENTAS FROM FIV-INFECTED AND CONTROL CATS

(Published in *Veterinary Immunology and Immunopathology* 2008; 123, 90 – 96)

Abstract

Feline immunodeficiency virus (FIV) causes a natural infection of domestic cats that resembles HIV-1 in pathogenesis and disease progression. Feline AIDS is characterized by depression of the CD4+ T cell population and fatal opportunistic infections. Maternal-fetal transmission of FIV readily occurs under experimental conditions, resulting in infected viable kittens and resorbed or arrested fetal tissues. Although both FIV and HIV use the chemokine receptor CXCR4 as a co-receptor, FIV does not utilize CD4 as the primary receptor. Rather, CD134 (OX40), a T cell activation antigen and co-stimulatory molecule, is the primary receptor for FIV. We hypothesized that placental expression of CD134 and CXCR4 may render the placenta vulnerable to FIV infection, possibly facilitating efficient vertical transmission of FIV, and impact pregnancy outcome. The purpose of this project was to quantify the relative expression of CD134 and CXCR4 mRNA from the term placentas of three groups of cats: uninfected queens producing viable offspring, experimentally-infected queens producing

only viable offspring, and experimentally-infected queens producing viable offspring among mostly non-viable fetuses. Total RNA was extracted from term placental tissues from all groups of cats. Real-time one-step reverse transcriptase-PCR was used to measure gene expression. The FIV receptors CD134 and CXCR4 were expressed in all late term feline placental tissues. Placentas from FIV-infected queens producing litters of only viable offspring expressed more CD134 and CXCR4 mRNA than those from uninfected queens, suggesting that infection may cause upregulation of the receptors. On the other hand, placentas from FIV-infected cats with non-successful pregnancies expressed similar levels of CD134 mRNA and slightly less CXCR4 mRNA than those from uninfected queens. Thus, it appears that cells expressing these receptors may play a role in pregnancy maintenance.

Introduction

Globally, the estimated number of people living with HIV is 39.5 million, 2.3 million of which are children under the age of 15 years (UNAIDS/WHO, 2006). In the United States pediatric AIDS cases (defined as AIDS occurring in children under the age of 13 years) represent 1% of the total number of AIDS cases, with maternal-fetal transmission of the virus accounting for more than 93% of pediatric infections (CDC, 2005). Children who are infected *in utero* have a more rapid progression to AIDS and generally become symptomatic during the first year of life. In addition, HIV infection of pregnant women often results in poor outcome, including low birth weight babies, pre-term delivery, and an increased incidence of spontaneous abortions (D'Ubaldo et al., 1998; Kumar et al., 1995; Langston et al., 1995). Specific placental cell populations are

permissive to productive HIV infection, probably contributing to transplacental transfer of virus or virus-infected cells across the placenta leading to fetal infection (Al-Harthi et al., 2002; Arias et al., 2003; David et al., 1992; McGann et al., 1994). Although HIV infection clearly has the potential to negatively impact fetal and neonatal health, the mechanism and timing of HIV vertical transmission to the fetus is not yet clear.

FIV causes a natural infection of domestic cats that resembles HIV-1 infection in pathogenesis and disease progression. Feline AIDS is characterized by progressive depletion of the CD4+ T cell population and fatal opportunistic infections. Although both viruses use the same co-receptor, the chemokine receptor CXCR4, FIV does not utilize CD4 as the primary receptor (de Parseval et al., 2004a; Shimojima et al., 2004). The primary FIV receptor is CD134 (OX40), a T cell activation and co-stimulatory molecule (Shimojima et al., 2004). The virus binds these receptors via its surface glycoprotein, gp95 (de Parseval and Elder, 2001). Previous studies demonstrated that CXCR4 expression is essential to viral entry and that levels of CXCR4 on the host cell were the limiting factor in productive virus infection and controlled virus spread and cytopathogenicity (de Parseval et al., 2004b).

Vertical transmission of FIV occurs commonly *in vitro* (Allison and Hoover, 2003; O'Neil et al., 1996; Weaver et al., 2005). We recently reported frequent reproductive failure in FIV-B-2542-infected cats delivered late in gestation by cesarean section (Weaver et al., 2005). While preliminary evidence of immunopathology was identified in the study, the interaction between the virus and its cellular receptors was not analyzed. In the present study, we hypothesized that placental expression of CD134 and CXCR4 may facilitate efficient vertical transmission of FIV and impact pregnancy

outcome. Our objective was to quantify the relative expression of CD134 and CXCR4 mRNA from term placentas of experimentally-infected and control queens. We report that the FIV receptors CD134 and CXCR4 were expressed in late term feline placental tissues and that altered placental expression of CD134 and CXCR4 was dependent upon FIV-status of queens and appeared to be correlated with pregnancy outcome.

Materials and Methods

Animals and virus

All procedures utilizing cats (*Felis domesticus*) were performed with approval of the Mississippi State University Institutional Animal Care and Use Committee. As previously reported (Weaver et al., 2005), cats were reproductively mature, specific-pathogen-free (SPF) animals of less than 12 months old and obtained from a commercial cattery. Ten cats were inoculated intravenously with 1 ml of a plasma pool containing approximately 1.33×10^4 copies/ml of FIV-B-2542 originally provided by Dr. Edward Hoover (Rogers and Hoover, 1998). Ten cats were sham inoculated with 1 ml of pooled normal plasma. Infection was confirmed within 6 weeks p.i. by detection of FIV provirus by PCR and for seroconversion by ELISA, and cats were allowed to naturally breed with SPF toms. Toms used to breed uninfected females were never exposed to infected females and vice versa. Breeding was observed, dates were recorded, and pregnancy was confirmed by palpation and ultrasonography.

Cesarean delivery and tissue harvest

Kittens were delivered during week 8 gestation (approximately day 56) by cesarean section. Each kitten or fetus was removed with the associated placental membranes. All placental tissues were snap frozen in liquid nitrogen and stored at –80°C (Weaver et al., 2005).

Generation of study groups

The relative expression of CD134 and CXCR4 mRNA in term placental tissue from FIV-negative (Group 1) and FIV-positive cats were evaluated. The infected cats were subdivided into two groups, one of which consisted of cats who delivered only viable offspring (Group 2), the other of which consisted of cats who delivered a majority of non-viable offspring (Group 3). Due to the paucity of placental tissues associated with fetal resorption, placentas sampled from Group 3 cats 9730 and 9813 were taken from placentas associated with their viable fetuses. Pregnancy outcome study groups are listed in Table 2.1.

Detection of FIV provirus in placental tissues

FIV provirus was detected in placental DNA samples using PCR targeting the FIV gag gene, followed by Southern hybridization as described (Weaver et al., 2005).

Table 2.1

Pregnancy Outcome of the Study Groups

Group	Queen Number	FIV Status	Viable kittens	Arrested fetuses	Fetal resorptions
1	9522	–	3	0	0
	9746	–	6	0	0
	9801	–	6	0	0
2	9806	+	3	0	0
	9809	+	1	0	0
	9810	+	1	0	0
3	9730	+	2	1	2
	9813	+	1	0	2
	13226	+	0	5	0

Placental tissues were taken from each associated queen according to the following groupings—Group 1: FIV(–) queens producing viable offspring; Group 2: FIV(+) queens producing litters of only viable offspring; Group 3: FIV(+) queens producing viable offspring among majority non-viable litters.

Detection of antibody to FIV by ELISA

FIV was detected in feline plasma from both groups of FIV-infected queens using the SNAP Combo FeLV Ag/FIV Antibody Test Kit (Idexx Laboratories, Westbrook, ME) according to the manufacturer's instructions. Positive results were scored when the

sample produced a blue spot in the appropriate position on the membrane. A sample from a seronegative cat was included as a negative control in a parallel reaction.

RNA extraction

RNA was isolated from 10 randomly sectioned placental tissue samples from 9 cats using TRIzol Reagent (Invitrogen, Corp. Carlsbad, CA) according to the manufacturer's instructions. Briefly, cells were lysed in 1 ml TRIzol Reagent. Homogenates were allowed to sit for 5 min at ambient temperature (20–25°C) and supplemented with 0.2 ml chloroform. Samples were covered and vigorously shaken for 15 s. The resulting mixture was allowed to sit at ambient temperature (20–25°C) for 15 min. Samples were centrifuged at 12,000 x g for 15 min at 4°C. Following centrifugation, the colorless upper aqueous phase containing RNA was transferred to a fresh tube. RNA was precipitated from the aqueous phase with the addition of 0.5 ml isopropanol and incubated at ambient temperature for 10 min. The RNA precipitate was pelleted by centrifugation at 12,000 x g for 8 min at 4°C, and the supernatant was removed. The RNA pellet was washed by vortexing with 1 ml of 75% ethanol and centrifuged at 12,000 x g (5 min, 4°C). The ethanol was removed, and the RNA pellet was air-dried for 5 min. After drying, the RNA pellet was dissolved in 100 µl of RNase-free water and incubated for 15 min in a 55–60°C water bath. The resulting RNA was stored at –80°C until use.

Real-time reverse transcriptase-PCR

Cytokine mRNA sequences for the cat and other comparative mammals (human, dog, cow, and pig) were obtained from the National Center for Biotechnology Information (NCBI) and aligned using the European Molecular Biology Laboratory-European Bioinformatics Institute (EMBL-EBI) ClustalW alignment tool. The human mRNA was blast-searched against the human genome to locate the exon/intron boundaries. These boundaries were used to find the homologous boundaries in the feline sequence. We used Beacon Designer (PREMIER Biosoft) to design primer/probe sets (Table 2.2) targeting the feline receptors CD134 and CXCR4, and the internal control gene (β-actin). All PCR amplicons spanned an intron-exon junction. The target probes were 5' labeled with the reporter dye FAM (6-carboxyfluorescein) and 3' labeled with the quencher dye TAMRA (6-carboxytetramethylrhodamine). The probe for the housekeeping gene was 5' labeled with the reporter dye HEX (hexachloro-6-carboxyfluorescein) and 3' labeled with the quencher dye TAMRA. Primers and probes were obtained commercially (MWG-BIOTECH, Inc., High Point, NC). Primers and probes used to quantify FIV targeted the FIV gag gene (Weaver et al., 2005).

The real-time reverse transcriptase-PCR (rtRT-PCR) used an iCycler (BioRad Laboratories, Valencia, CA): 50°C, 30 min; 95°C, 5 min; 45 X (95°C, 15 s, 60°C, 1 min). Each reaction contained 12.5 µl of the commercial reaction mix, 0.5 µl of Thermoscript™ Plus/Platinum® *Taq* Mix, 1 µl of forward and reverse β-actin primers (7.5 pmol/µl), 1 µl of forward and reverse target primers (10 pmol/µl), 1 µl of the respective probe (100 fmol/µl), ~0.06–0.27 µg RNA. For every placental RNA sample,

Table 2.2

Sequences of PCR Primers and TaqMan Probes Specific for FIV Receptors and the Internal Control mRNA

Receptor	Primer	Sequence (5'-3')	Length	Accession	Probe sequence (5'-3')
CD134	Sense	CAGGTTATGGGATGGAGAGTCG	22	AY738589	TGACCAGGACACCAAGTGCCTCCAGTG
	Anti-sense	TGCAAGGCTCGTAGTTCACG	20		
CXCR4	Sense	AAGGCAGTCCATGTCATCTACAC	23	AJ009816	ACCTCTACAGCAGTGTCCTCATCCTGGC
	Anti-sense	AGACCACCTTTTCAGCCAACAG	22		
β-actin	Sense	GACTACCTCATGAAGATCCTCACG	24	AB051104	ACAGTTTCACCACCACCGCCGAGC
	Anti-sense	CCTTGATGTCACGCACAATTTCC	23		

parallel reactions were performed in replicated triplicate reactions on separate plates for each receptor. Real-time RT-PCR to quantify FIV gag was carried out as described previously (Weaver et al., 2005). Serially diluted, pooled RNA from uninfected cats was used to generate a standard curve for both duplex-partner amplicons. The standard curves were used to normalize for differences in PCR efficiency between duplex partners and between sample plate runs. Differences in the amount of template RNA in each reaction were corrected by the cycle threshold (Ct) value for β-actin. Normalized samples were divided by the calibration generating the relative expression levels.

Statistical analysis

Statistical evaluation of litter sizes and fetal viability were done using single-factor ANOVA (Microsoft Excel-XP, Redmond, WA). To perform statistical analysis of receptor expression, the mean normalized values for the control samples and each set of treated samples (FIV-infected placentas) was done by using Monte Carlo resampling

simulation with 500 iterations (Simon, 1997). Confidence intervals were calculated (α = 0.05) based on fold differences for each of the control versus treated comparisons.

Results

Pregnancy outcome

The pregnancy outcome data (Table 2.1) for FIV-infected and control queens was previously reported (Weaver et al., 2005), and these data were used to generate three study groups containing three randomly selected placental tissues from FIV-infected and control queens. Control cats produced a total of 15 viable kittens, a mean litter size of 5 kittens/litter. Infected queens with litters of only viable offspring produced 5 viable kittens, a mean litter size of 1.6 kittens/litter. Infected queens producing viable offspring from majority non-viable litters had a total of 13 kittens, 3 of which were viable (23%) and 10 of which were non-viable (77%). The mean litter size was 4.3 kittens/litter. Non-viable offspring were either arrested during early development (n = 6) or fetal resorptions (n = 4). Differences in litter sizes between the three groups were significant (p = 0.05).

Detection of FIV infection

FIV provirus was detected in all but one of the placental specimens from FIV-infected cats, as reported (Weaver et al., 2005). We failed to amplify FIV provirus from the placenta of animal 9809, although infection of the corresponding kitten was confirmed by amplification of FIV provirus from kitten bone marrow. FIV infection of the inoculated queens was confirmed serologically.

Expression of CD134 and CXCR4 in placental tissues

The relative expression of CD134 and CXCR4 mRNA in near term placental tissue was quantified, and data for the three groups of queens were compared as follows: control queens producing viable offspring (Group 1) versus FIV positive queens producing litters of only viable offspring (Group 2) and FIV positive queens producing viable offspring among majority non-viable litters (Group 3) versus Group 1. Confidence intervals were calculated ($\alpha = 0.05$) for each of the control versus treated comparisons (Fig. 2.1). If the mRNA expression between two sample sets was the same, the relative expression was 100%.

The mRNA for FIV receptors CD134 and CXCR4 was expressed in late term placental tissues from all groups (Fig. 2.1A and B). Group 2 queens expressed more CD134 (mean 261%; lower and upper confidence intervals 235% and 290%, respectively) and CXCR4 (mean 327%; lower and upper confidence intervals 287% and 371%, respectively) mRNA compared to placentas from Group 1 (Fig. 2.1A). Groups 1 and 3 queens expressed similar levels of CD134 (mean 107%; lower and upper confidence intervals 100% and 114%, respectively) mRNA, while Group 1 expression of CXCR4 (mean 118%; lower and upper confidence intervals 107% and 130%, respectively) mRNA was slightly higher than that of Group 3 queens (Fig. 2.1B).

Relative expression of FIV RNA in placentas

The relative expression of FIV between the two groups of infected cats was quantified using rtRT-PCR targeting the FIV gag gene. There were no differences in the levels of FIV expression between the two groups ($p > 0.05$).

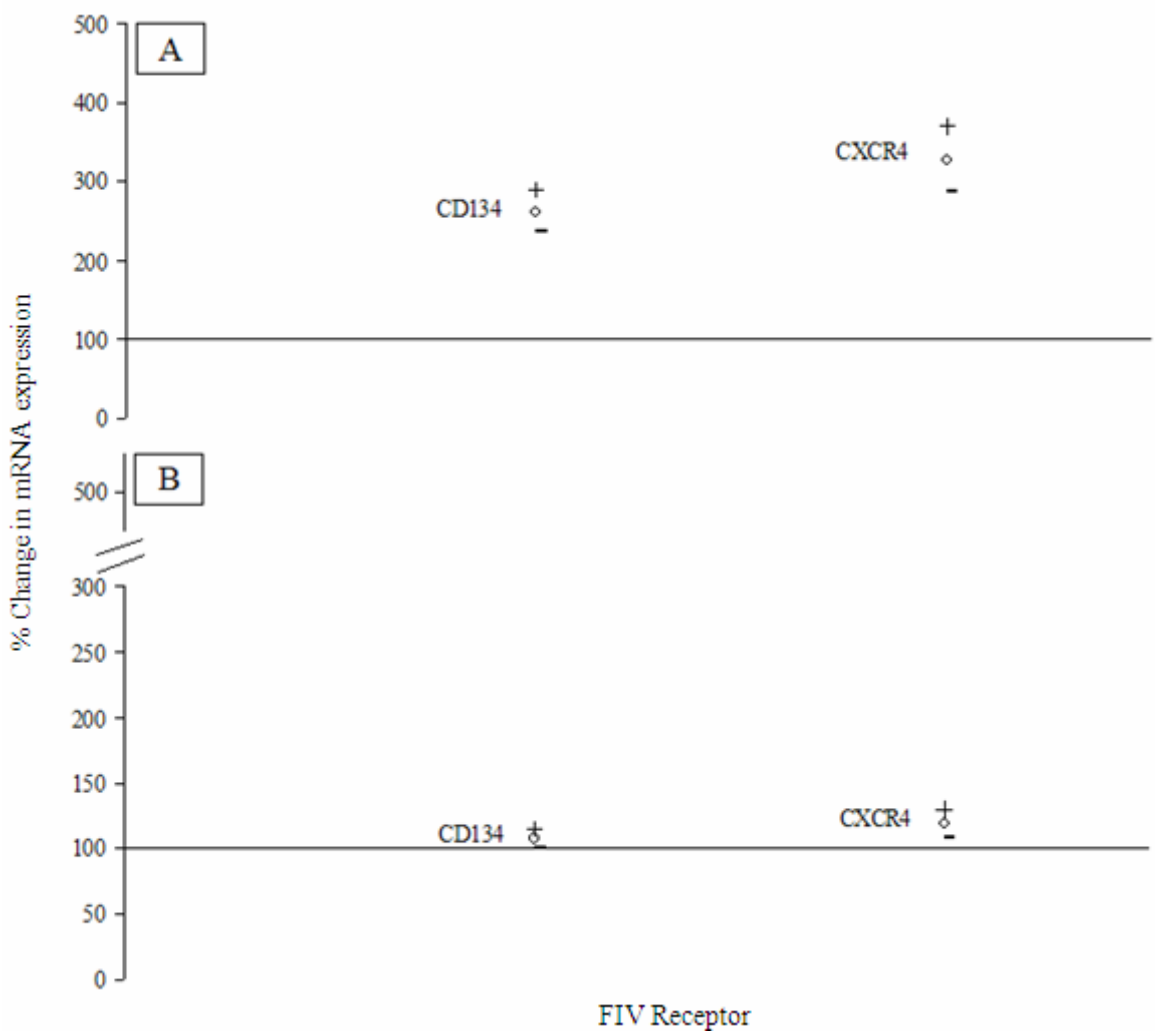

Figure 2.1 Percentage Change in FIV Receptor mRNA Expression in Control vs. Treated Comparisons

(A) Percentage change in CD134 and CXCR4 mRNA in placentas from infected queens producing litters of viable offspring (Group 2) compared to control cats (Group 1). (B) Percentage change in CD134 and CXCR4 mRNA in placentas from Group 1 cats compared to infected queens producing viable offspring among majority non-viable litters (Group 3). Results are shown as 95% upper (+) and lower (–) confidence intervals around the mean (○). Relative expression was 100% if the mRNA expression between the two sample sets was the same.

Discussion

Transmission of FIV *in utero* often results in reproductive failure (Rogers and Hoover, 1998; Weaver et al., 2005). O'Neil et al. (1996), reported no significant difference in the litter sizes between chronically and acutely infected queens (51% and 47%, respectively), however, higher incidences of fetal non-viability were associated with acutely infected versus chronically infected queens (53% and 30%, respectively) (O'Neil et al., 1995). FIV-B-2542-infected queens transmitted the virus to more than 50% of their offspring (O'Neil et al., 1996; Weaver et al., 2005) and the rate of *in utero* infection increased with gestational age (Rogers and Hoover, 1998). Despite the evidence supporting efficient *in utero* transmission, the mechanisms by which the virus traverses the placenta and enters the fetal circulation have not been described.

The interaction between the virus and its binding receptor(s) establishes cell tropism and is the first event in the virus replicative cycle, yet little is known about the expression of FIV receptors at the feline maternal-fetal interface. HIV and FIV utilize CD4 (Dalgleish et al., 1984; Klatzmann et al., 1984) and CD134 (de Parseval et al., 2004a; Shimojima et al., 2004), as their primary receptors, respectively. HIV-1 selectively targets CD4-bearing cells, resulting in their depletion. However, FIV has a cell tropism that broadens with disease progression, consistent with the expression of CD134 (Shimojima et al., 2004). HIV and FIV share usage of a common co-receptor, the chemokine receptor CXCR4. Progression to disease in HIV-1 infections is correlated with the ability to bind CXCR4 (Connor et al., 1997; Lu et al., 1997). Similarly, susceptibility to infection with FIV is directly related to cellular expression of CXCR4, levels of which have been shown to be the limiting factor in productive infection of feline

cells (de Parseval et al., 2004b; Willett et al., 2002). Despite the use of different primary receptors, the shared usage of CXCR4 as a cellular co-receptor by HIV and FIV establishes similarities in cell tropism between the two lentiviruses, thus reiterating the usefulness of the FIV-infected cat as a model for lentivirus-induced immunopathology at the maternal-fetal interface.

The expression of CXCR4 at both the protein and mRNA levels in the human placenta was described by Kumar et al. (2004), who reported that CXCR4 was expressed to two-fold higher levels in early versus late human placentas. In addition, the expression of CXCR4 was shown in all layers of the human placenta including the trophoblasts, stroma, and endothelium (Bustamante et al., 2005). In a previous study, abundant CXCR4 transcripts were detected in late term feline placentas, but analysis of placental CXCR4 expression was limited to a comparison of levels in FIV-infected versus control queens using single-factor ANOVA. The levels of CXCR4 expression between the two groups were virtually identical ($p = 0.98$), indicating that FIV did not affect expression of this chemokine receptor late in gestation (Weaver et al., 2005).

In the present study, we used real-time RT-PCR to quantify the relative expression of CD134 and CXCR4 mRNA in placental samples from three study groups: FIV negative (control) queens producing viable offspring (Group 1), FIV positive queens producing litters of only viable offspring (Group 2), and FIV positive queens producing viable offspring among majority non-viable litters (Group 3). Statistical analysis of the mean normalized Ct values for the control versus treated comparisons was done by using Monte Carlo resampling simulation (Simon, 1997) because the number of placental samples obtained from queens was limited due to pregnancy failure in FIV-infected

queens. Monte Carlo random resampling analysis results in accurate estimates with fewer replications and is especially useful in the estimation of unpaired data sets where sample numbers are small and parametric statistics are more prone to error. Furthermore, confidence intervals for small sample sizes with a limited range of values can be calculated using random sampling (Chu and Ette, 2005).

We report that CD134 and CXCR4 mRNA was expressed in all placental samples and that CXCR4 was the more abundantly expressed receptor. To our knowledge, this is the first report describing the expression of CD134 at the feline maternal-fetal interface. Placentas from Group 2 cats expressed higher percentages of CD134 and CXCR4 mRNA than those of the controls (Group 1) (Fig. 2.1A). Other investigators reported that upregulation of CD134 and CXCR4 on CD4+ T cells occurs as a consequence of T cell activation, enhancing the effector function of the T cells (de Parseval et al., 2004a; Joshi et al., 2005), and that increased surface expression of CXCR4 on CD4+ T cell populations results in increased susceptibility of these cells to FIV infection (Joshi et al., 2005). Although we have not evaluated placental T cells directly, upregulation of CD134 and CXCR4 in the placental tissues may provide evidence of FIV-induced T cell activation in these tissues.

Unlike the Group 2 queens, the FIV-infected queens producing non-viable fetuses (Group 3) expressed placental CD134 mRNA at a similar level to that of Group 1 (controls), while control queens expressed slightly more CXCR4 mRNA than Group 3 queens. Collectively, these results may implicate a role for cells bearing these receptors in feline pregnancy maintenance.

We reported previously (Weaver et al., 2005) that FIV was detectable in 93% of placentas examined. A comparison of relative viral RNA expression between the two groups of infected cats evaluated in the present study revealed no significant difference in viral expression between the two. The expression late in gestation of both the primary receptor (CD134) and co-receptor (CXCR4) utilized by FIV may explain the frequent placental infection. Further investigation is needed to clarify the role of cells bearing these receptors in vertical transmission of the virus and to determine whether poor pregnancy outcome is a direct result of viral infection of the fetus or an indirect result of virus-induced immunopathology.

Acknowledgements

We thank Dr. Edward A. Hoover, Colorado State University, for generously providing the infectious plasma pool containing FIV-B-2542. We are grateful to the veterinary staff at the College of Veterinary Medicine, Mississippi State University, for assistance with animal care and surgery. We thank Crystal E. Boudreaux for performing ELISAs. This project was supported by the National Institutes of Health (2R15AI048419-02A1) and (3R15AI048419-02A1S1).

References

Al-Harthi, L., Guilbert, L.J., Hoxie, J.A., Landay, A., 2002, Trophoblasts are productively infected by CD4-independent isolate of HIV type 1. AIDS Res Hum Retroviruses 18, 13-17.

Allison, R.W., Hoover, E.A., 2003, Covert vertical transmission of feline immunodeficiency virus. AIDS Res Hum Retroviruses 19, 421-434.

Arias, R.A., Munoz, L.D., Munoz-Fernandez, M.A., 2003, Transmission of HIV-1 infection between trophoblast placental cells and T-cells take place via an LFA-1-mediated cell to cell contact. Virology 307, 266-277.

Bustamante, S., Garcia, Y., Garrido, H., Bethencourt, S., Tovar, R., Ponce, L., Corado, J., Mora-Orta, S., 2005, CXCR-4 AND CCR-5 expression in normal term human placenta. Invest Clin 46, 25-35.

CDC 2005. HIV AIDS Surveillance Report Cases of HIV infection and AIDS in the United States and Dependent Areas

Chu, H.M., Ette, E.I., 2005, A random sampling approach for robust estimation of tissue-to-plasma ratio from extremely sparse data. Aaps J 7, E249-258.

Connor, R.I., Sheridan, K.E., Ceradini, D., Choe, S., Landau, N.R., 1997, Change in coreceptor use coreceptor use correlates with disease progression in HIV-1--infected individuals. J Exp Med 185, 621-628.

D'Ubaldo, C., Pezzotti, P., Rezza, G., Branca, M., Ippolito, G., 1998, Association between HIV-1 infection and miscarriage: a retrospective study. DIANAIDS Collaborative Study Group. Diagnosi Iniziale Anomalie Neoplastiche AIDS. Aids 12, 1087-1093.

Dalgleish, A.G., Beverley, P.C., Clapham, P.R., Crawford, D.H., Greaves, M.F., Weiss, R.A., 1984, The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. Nature 312, 763-767.

David, F.J., Autran, B., Tran, H.C., Menu, E., Raphael, M., Debre, P., Hsi, B.L., Wegman, T.G., Barre-Sinoussi, F., Chaouat, G., 1992, Human trophoblast cells express CD4 and are permissive for productive infection with HIV-1. Clin Exp Immunol 88, 10-16.

de Parseval, A., Chatterji, U., Sun, P., Elder, J.H., 2004a, Feline immunodeficiency virus targets activated CD4+ T cells by using CD134 as a binding receptor. Proc Natl Acad Sci U S A 101, 13044-13049.

de Parseval, A., Elder, J.H., 2001, Binding of recombinant feline immunodeficiency virus surface glycoprotein to feline cells: role of CXCR4, cell-surface heparans, and an unidentified non-CXCR4 receptor. J Virol 75, 4528-4539.

de Parseval, A., Ngo, S., Sun, P., Elder, J.H., 2004b, Factors that increase the effective concentration of CXCR4 dictate feline immunodeficiency virus tropism and kinetics of replication. J Virol 78, 9132-9143.

Joshi, A., Garg, H., Tompkins, M.B., Tompkins, W.A., 2005, Preferential feline immunodeficiency virus (FIV) infection of CD4+ CD25+ T-regulatory cells correlates both with surface expression of CXCR4 and activation of FIV long terminal repeat binding cellular transcriptional factors. J Virol 79, 4965-4976.

Klatzmann, D., Champagne, E., Chamaret, S., Gruest, J., Guetard, D., Hercend, T., Gluckman, J.C., Montagnier, L., 1984, T-lymphocyte T4 molecule behaves as the receptor for human retrovirus LAV. Nature 312, 767-768.

Kumar, A., Kumar, S., Dinda, A.K., Luthra, K., 2004, Differential expression of CXCR4 receptor in early and term human placenta. Placenta 25, 347-351.

Kumar, R.M., Uduman, S.A., Khurranna, A.K., 1995, Impact of maternal HIV-1 infection on perinatal outcome. Int J Gynaecol Obstet 49, 137-143.

Langston, C., Lewis, D.E., Hammill, H.A., Popek, E.J., Kozinetz, C.A., Kline, M.W., Hanson, I.C., Shearer, W.T., 1995, Excess intrauterine fetal demise associated with maternal human immunodeficiency virus infection. J Infect Dis 172, 1451-1460.

Lu, Z., Berson, J.F., Chen, Y., Turner, J.D., Zhang, T., Sharron, M., Jenks, M.H., Wang, Z., Kim, J., Rucker, J., Hoxie, J.A., Peiper, S.C., Doms, R.W., 1997, Evolution of HIV-1 coreceptor usage through interactions with distinct CCR5 and CXCR4 domains. Proc Natl Acad Sci U S A 94, 6426-6431.

McGann, K.A., Collman, R., Kolson, D.L., Gonzalez-Scarano, F., Coukos, G., Coutifaris, C., Strauss, J.F., Nathanson, N., 1994, Human immunodeficiency virus type 1 causes productive infection of macrophages in primary placental cell cultures. J Infect Dis 169, 746-753.

O'Neil, L.L., Burkhard, M.J., Diehl, L.J., Hoover, E.A., 1995, Vertical transmission of feline immunodeficiency virus. Semin Vet Med Surg (Small Anim) 10, 266-278.

O'Neil, L.L., Burkhard, M.J., Hoover, E.A., 1996, Frequent perinatal transmission of feline immunodeficiency virus by chronically infected cats. J Virol 70, 2894-2901.

Rogers, A.B., Hoover, E.A., 1998, Maternal-fetal feline immunodeficiency virus transmission: timing and tissue tropisms. J Infect Dis 178, 960-967.

Shimojima, M., Miyazawa, T., Ikeda, Y., McMonagle, E.L., Haining, H., Akashi, H., Takeuchi, Y., Hosie, M.J., Willett, B.J., 2004, Use of CD134 as a primary receptor by the feline immunodeficiency virus. Science 303, 1192-1195.

Simon, J.L., 1997, Resampling: The New Statistics, 2nd Edition. Resampling Stats, Inc., Arlington, VA, 420 p.

UNAIDS/WHO 2006. AIDS Epidemic Update 2006. Joint United Nations Programme on HIV/AIDS.

Weaver, C.C., Burgess, S.C., Nelson, P.D., Wilkinson, M., Ryan, P.L., Nail, C.A., Kelly-Quagliana, K.A., May, M.L., Reeves, R.K., Boyle, C.R., Coats, K.S., 2005, Placental immunopathology and pregnancy failure in the FIV-infected cat. Placenta 26, 138-147.

Willett, B.J., Cannon, C.A., Hosie, M.J., 2002, Upregulation of surface feline CXCR4 expression following ectopic expression of CCR5: implications for studies of the cell tropism of feline immunodeficiency virus. J Virol 76, 9242-9252.

CHAPTER III

PLACENTAL CYTOKINE DYSREGULATION IN EARLY- AND LATE-TERM FIV-INFECTED FELINE PLACENTAS

Abstract

Typically, successful pregnancy is biased towards a Th2 immune response and a suppressed Th1 immune response at the maternal-fetal interface early in pregnancy, while Th1 immunity dominates late in pregnancy prior to the onset of labor. HIV infection can alter placental cytokine patterns, but the implications for vertical transmission and pregnancy outcome are unresolved. The FIV-infected cat is a model for HIV transplacental transfer due to biological and clinical similarities. We found very high rates of fetal infection and increased reproductive failure in the offspring of FIV-infected queens delivered at early (week 3-4) and late (week 8) gestation. We hypothesized that dysregulation of placental cytokine expression occurs in FIV-infected queens, and aberrant expression of these cytokines may potentiate inflammation, promote vertical transmission, and impact pregnancy outcome. The purpose of this project was to quantify the expression of representative pro-inflammatory cytokines (IL-6, IL-12p35, and IL-1β), the anti-inflammatory cytokine (IL-10), and the chemokine (SDF-1α) in early- and late-term placental tissues and evaluate whether immunomodulator expression is related to fetal FIV infection and pregnancy outcome. Total RNA was extracted from

placental tissues, and real-time one-step reverse-transcriptase-PCR was used to measure gene expression. Immunomodulator mRNA was expressed in all early- and late-term placental tissues. With the exception of IL-12p35, patterns of cytokine expression in control animals were as we predicted, Th2 cytokine mRNAs (IL-10 and IL-6) were higher at early gestation and decreased by late gestation, while pro-inflammatory mediator mRNAs (IL-1β and SDF-1α) were lower at early gestation and increased by late gestation. We report that FIV-infection during early gestation was associated with increased expression of two pro-inflammatory cytokines, IL-6 and IL-12p35, and decreased expression of IL-10. At late gestation, IL-6 expression was significantly increased while IL-1β and SDF-1α were decreased. Only IL-6 placental expression was consistently affected by viral infection, increasing in FIV-infected cats at both gestational stages. Placentas from non-viable fetuses produced decreased SDF-1α and IL-12p35. While the impact that these immunopathological findings may have on fetal infection and reproductive failure are not yet known, it is clear that FIV infection alters the placental cytokine microenvironment.

Introduction

Placental cytokines and chemokines are immunoregulatory molecules that are responsible for pregnancy maintenance. These immunomodulators are critical for embryo implantation, endometrial development (Saito, 2001), and the development of fetal placentation (Clark and Chaouat, 1989). Successful pregnancy is biased towards a Th2 immune response with a suppressed Th1 response at the maternal-fetal interface

(Challis et al., 2009; Costeas et al., 2004; Raghupathy, 1997; Wegmann et al., 1993). Th1 and Th2 cytokines are mutually antagonistic (Mosmann and Coffman, 1989; Wegmann et al., 1993). The production of Th2 cytokines aids in the maintenance of pregnancy by downregulating Th1 cytokines, thereby preventing the formation of an inflammatory microenvironment in the placenta (Challis et al., 2009). The altered expression of the Th1 cytokine, IL-12 (a pro-inflammatory cytokine), and the Th2 cytokine, IL-10 (an anti-inflammatory cytokine), is a clear indicator of Th1:Th2 immune shifts and cytokine dysregulation (Challis et al., 2009; Costeas et al., 2004; Romagnani, 1994; Wegmann et al., 1993). Cytokine dysregulation in the placenta may result in poor reproductive outcome, including spontaneous abortion, preterm labor, preeclampsia, and intrauterine growth restriction (Challis et al., 2009; Orsi and Tribe, 2008).

Perinatal transfer of HIV (defined as HIV transfer from mother-to-child during pregnancy, labor and delivery, or breastfeeding) accounts for more than 90% of pediatric infections worldwide (CDC, 2005). In the United States, mother-to-child transmission (MTCT) is the most common source of all AIDS cases in children and results in 100-200 infected infants annually (CDC, 2005, 2007). The rate of disease progression in infants is rapid and about 50% of infected newborns will succumb to the disease before 24 months of age (UNAIDS/WHO, 2009). In addition, HIV infection of pregnant women often results in reproductive failure, including low birth weight babies, preterm delivery, and an increased incidence of spontaneous abortions (D'Ubaldo et al., 1998; Kumar et al., 1995; Langston et al., 1995). Although it is evident that HIV has the potential to

negatively impact reproductive outcome, the mechanisms of viral transplacental transfer and pregnancy perturbation associated with infection remain undefined.

HIV expression is augmented by the production of placental immunomodulators such as cytokines (Kedzierska and Crowe, 2001), and HIV infections can dysregulate cytokine networks in placental tissues (Lee et al., 1997; Shearer et al., 1997). Aberrant expression of cytokines may result in placental inflammation, and the presence of inflamed placental membranes is likely to facilitate viral transplacental transfer (Shearer et al., 1997).

The feline immunodeficiency virus (FIV) causes a natural infection of domestic cats that is clinically similar to HIV-1. Vertical transmission of FIV occurs *in vitro*, producing infected offspring (Allison and Hoover, 2003; Boudreaux et al., 2009; O'Neil et al., 1996; Weaver et al., 2005). Frequent reproductive failure in the FIV-infected cat parallels the increased incidence of spontaneous abortion in HIV-infected women. Therefore, the FIV-infected cat model is useful for the study of lentiviral transplacental transmission.

We previously reported frequent reproductive failure in litters delivered at late gestation by cesarean section in FIV-infected cats (Weaver et al., 2005). In that report, vertical transmission of FIV occurred in nearly all pregnancies, and FIV was detected in tissues of both viable and non-viable kittens and fetuses. We found that Th1 and Th2 cytokine expression did not differ significantly in the late-term placentas of infected and uninfected cats, although the trend was toward increased expression of Th1 cytokines and decreased expression of the Th2 cytokine (Weaver et al., 2005). However, Th1 cytokine

expression was significantly increased in placentas of FIV-infected, resorbed fetuses in comparison to other placentas. Although we detected preliminary evidence of reproductive failure at late gestation, pro-inflammatory cytokine expression in early-term placental tissue was not evaluated.

We hypothesized that dysregulation of placental cytokine expression occurs in FIV-infected queens, and aberrant expression of these cytokines may potentiate inflammation and impact pregnancy outcome. Our objectives were to determine the expression of representative pro-inflammatory cytokines (IL-6, IL-12p35, and IL-1β), the anti-inflammatory cytokine (IL-10), and the chemokine (SDF-1α) in early- and late-term placental tissues and evaluate whether immunomodulator expression is related to FIV infection and pregnancy outcome. We report that FIV-infection during early gestation was associated with increased expression of two pro-inflammatory cytokines, IL-6 and IL-12p35, and FIV-infection during late gestation was associated with increased expression of IL-6.

Materials and Methods

Feline placental tissues

All procedures utilizing cats (*Felis domesticus*) were performed with approval of the Mississippi State University Institutional Animal Care and Use Committee. As previously reported (Boudreaux et al., 2009; Weaver et al., 2005), cats were reproductively mature, specific-pathogen-free (SPF) animals of less than 12 months of

age when obtained from a commercial cattery. Ten cats were inoculated intravenously with 1 ml of a plasma pool containing approximately 1.33 x 10^4 copies/ml of FIV-B-2542, provided by Dr. Edward A. Hoover (Rogers and Hoover, 1998). Uninfected cats served as normal controls. Infection was confirmed by detection of FIV provirus by PCR and for seroconversion by ELISA. All queens were allowed to naturally breed with SPF toms. Breeding was observed and pregnancy was confirmed by ultrasonography.

Kittens were delivered by cesarean section during week 3 of gestation (early-term) (Boudreaux et al., 2009) and week 8 of gestation (late-term) (Weaver et al., 2005) from FIV-B-2542-infected and uninfected queens. Placentas were collected under sterile conditions using the following dissection procedure. The uteri were removed and individual fetal membranes were collected. Following rinses with sterile PBS, membranes were incised with a sterile scalpel, and fetuses and placentas were collected. Placental tissues were snap frozen in liquid nitrogen and cryopreserved at -80°C. The effect of FIV infection on pregnancy at early- and late-term gestation was previously reported (Boudreaux et al., 2009; Weaver et al., 2005). For this study, we evaluated selected placental samples from both early- and late-term pregnancy, including placentas from both viable and non-viable pregnancies (Table 3.1).

RNA extraction

RNA was purified from frozen placental tissues of FIV-B-2542-infected and uninfected queens at early gestation (n = 27 and n = 42, respectively) and late gestation (n = 23 and n = 24, respectively), using TRIzol Reagent (Invitrogen, Corp. Carlsbad, CA)

according to the manufacturer's instructions. The RNA extraction procedure was performed as previously reported (Scott et al., 2008).

Table 3.1

Tissues Included in the Study of Placental Immunomodulator Expression

Early Gestation			
FIV status	Viable kittens	Arrested fetuses	Fetal resorptions
FIV(-)	41	0	1
FIV(+)	21	2	4
Late Gestation			
FIV status	Viable kittens	Arrested fetuses	Fetal resorptions
FIV(-)	24	0	0
FIV(+)	9	5	9

Primer design of immunomodulators

Cytokine and chemokine mRNA sequences for the cat and human were obtained from the National Center for Biotechnology Information (NCBI) and aligned using the European Molecular Biology Laboratory-European Bioinformatics Institute (EMBL-EBI) ClustalW alignment tool. The human mRNA was blasted against the human genome to locate the exon/intron boundaries. These boundaries were used to find the homologous boundaries in the feline sequence. We used Beacon Designer (PREMIER Biosoft) to design primer/probe sets targeting the representative pro-inflammatory cytokines (IL-6, IL-12p35, and IL-1β), an anti-inflammatory cytokine (IL-10), a chemokine (SDF-1α), and the internal control gene (β-actin). All PCR amplicons spanned an intron-exon junction. The target probes were 5' labeled with the reporter dye FAM (6-

carboxyfluorescein) and 3' labeled with the quencher dye TAMRA (6-carboxytetramethylrhodamine). The probe for the housekeeping gene was 5' labeled with the reporter dye HEX (hexachloro-6-carboxyfluorescein) and 3' labeled with the quencher dye TAMRA. Primers and probes were obtained commercially (MWG-BIOTECH, Inc., High Point, NC). Sequences of primers and probes used in these studies are shown in Table 3.2.

Real-time reverse transcriptase-PCR

The real-time reverse transcriptase-PCR (rtRT-PCR) used an iCycler (BioRad Laboratories, Valencia, CA): 50°C, 30 min; 95°C, 5 min; 45 X (95°C, 15 s; 60°C, 1 min). Each reaction contained 12.5 µl of the commercial reaction mix, 0.5 µl of Thermoscript™ Plus/Platinum® *Taq* Mix, 1 µl of forward and reverse β-actin primers (7.5 pmol/µl), 1µl of forward and reverse target primers (10 pmol/µl), 1 µl of the respective probe (100 fmol/µl), and 180–300 ng RNA. For every placental RNA sample, parallel reactions were performed in triplicate for each gene. Standard curves were generated from serially diluted, pooled RNA from uninfected cats and used to normalize for differences in PCR efficiency and RNA template as previously described (Scott et al., 2008).

Table 3.2

Sequences of PCR Primers and TaqMan Probes Specific for Feline Immunomodulators and the Internal Control mRNA

Gene	Primer	Sequence (5'–3')	Length	Accession	Probe sequence (5'–3')
IL-10	Sense	ACTTTCTT**TC**AAACCAAGGACGAG	24	AF060520	TCTCGGACAAGGCTTGGCAACCCA
	Anti-sense	GGCATCACCTCCTCCAAATAAAAC	24		
IL-6	Sense	GTGTGACAACTATAACAAATGTGAGG	26	L16914	CAAGGAGGCACTGGCAGAAAACAACCT
	Anti-sense	GTCT**CCT**GATTGAACCCAGATTG	23		
IL-12p35	Sense	ACACCAAGCCCAGGAATGTTC	21	U83185	AACCACTCCCAAACCCTGCTGCGA
	Anti-sense	TGG**CCT**TCTGAAGCGTGTTG	20		
IL-1β	Sense	ATTGTGGCTATGGAGAAACTGAAG	24	M92060	TTTGCCTGCTCACAACCCCTCCAG
	Anti-sense	T**CT**TCTTCAAAGATGCAGCAAAAG	24		
SDF-1α	Sense	GCTACAGATGTCCTTGCCGATTC	23	AB011965	TCGAGAGCCACGTTGCCAGAGCCA
	Anti-sense	TCTTCAGCCTCG**CCAC**GATC	20		
β-Actin	Sense	GACTACCTCATGAAGATCCTCACG	24	AB051104	ACAGTTTCACCACCACCGCCGAGC
	Anti-sense	CCTTGATGTCACGCACAATTTCC	23		

Intron-exon junctions are in bold and underlined.

Statistical analysis

Statistical evaluation of litter sizes and fetal viability from early- and late-term gestation were previously reported (Boudreaux et al., 2009; Weaver et al., 2005, respectively). Statistical analysis of immunomodulator expression for early- and late-term control and FIV-B-2542-infected placental samples were done using single-factor ANOVA (Microsoft Excel-XP, Redmond, WA).

Results

Expression of immunomodulators in placental tissues

Immunomodulator mRNA was expressed in all early- and late-term placental tissues. To determine normal feline placental expression of the immunomodulators between the two stages of pregnancy, control placentas from early- and late-term pregnancies were compared. There were significant differences in expression of all immunomodulators in control placentas. Control placentas from early gestation expressed more IL-10 ($p = 1.57E-05$) , IL-6 ($p = 0.00024$) and IL-12p35 ($p = 1.27E-15$) mRNA than placentas from late gestation (Fig. 3.1). However, control placentas from late gestation expressed more IL-1β ($p = 7.6E-19$) and SDF-1α ($p = 8.68E-16$) mRNA than placentas from early gestation (Fig. 3.1).

To determine the effect of FIV infection on placental cytokine expression, FIV-infected and control placentas from both early- and late-term gestation were compared. At early gestation, FIV-infection resulted in increased expression of the pro-

inflammatory cytokine mRNAs, IL-6 ($p = 0.001$) and IL-12p35 ($p = 0.0005$), decreased expression of the anti-inflammatory cytokine mRNA, IL-10 ($p = 0.0007$), and had no effect on the mRNA expression of IL-1β ($p = 0.57$) and SDF-1α ($p = 0.90$) (Fig. 3.2). At late gestation, FIV-infection resulted in increased mRNA expression of the pro-inflammatory cytokine IL-6 ($p = 8.865E-05$), decreased expression of the pro-inflammatory cytokines IL-1β ($p = 0.0002$) and SDF-1α ($p = 0.004$), and had no effect on mRNA expression of IL-10 ($p = 0.609$) and IL-12p35 ($p = 0.289$) (Fig. 3.3).

To determine whether cytokine expression was related to fetal viability, placentas from FIV-infected, viable pregnancies were compared to placentas from FIV-infected non-viable pregnancies for both early- and late-term gestation. Of all immunomodulators evaluated, only IL-12p35 and SDF-1α had significant differences in expression when infected viable placentas were compared to infected non-viable placentas (Fig. 3.4 and 3.5, respectively). Differences in expression of IL-12p35 between early infected viable placentas and non-viable placentas did not reach the level of significance ($p = 0.06$) (Fig. 3.4A). At late-term gestation, infected viable placentas expressed more IL-12p35 ($p = 4.04E-06$) mRNA than infected non-viable placentas (Fig. 3.4B). Infected viable placentas expressed more SDF-1α mRNA than infected non-viable placentas at both early- ($p = 0.017$) and late-term ($p = 0.0008$) gestation (Fig. 3.5A and B, respectively).

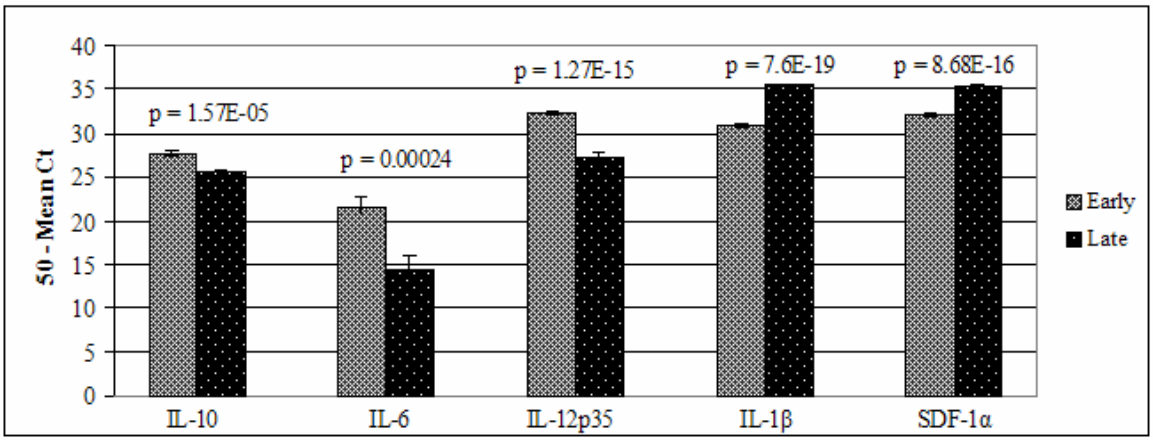

Figure 3.1 Relative Expression of Immunomodulators in Control Cats

Real-time RT-PCR analysis of normal feline placental expression of pro-inflammatory cytokines (IL-6, IL-12p35, and IL-1β), the anti-inflammatory cytokine (IL-10), and the chemokine (SDF-1α) in early-term uninfected placentas (n = 42) versus late-term uninfected placentas (n = 24). Bars represent mean Ct values substracted from a negative endpoint (50 - mean Ct), bracketed by standard errors of the mean. P values obtained from single factor ANOVA are noted. Alpha = 0.05.

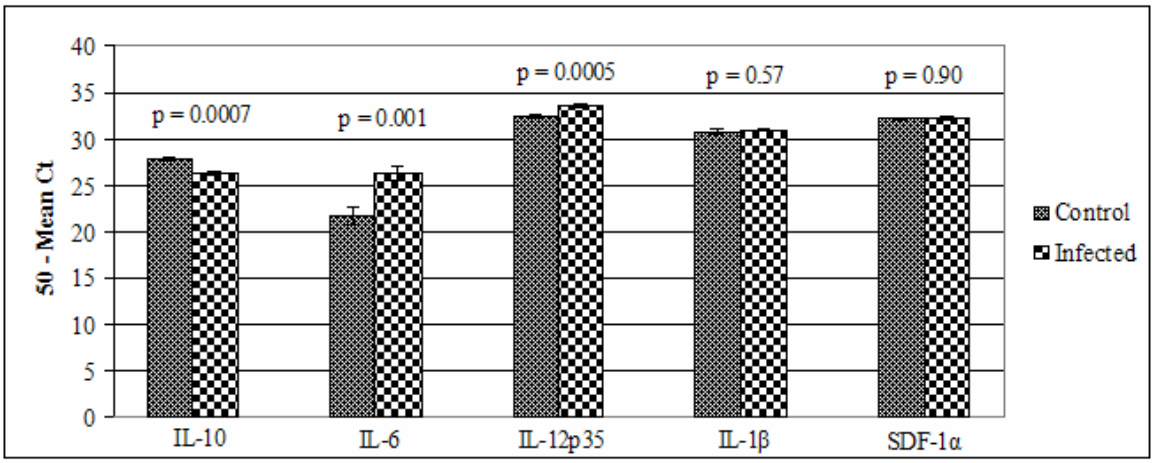

Figure 3.2 Relative Expression of Immunomodulators in Early-Term Feline Placentas

Real-time RT-PCR analysis of feline placental expression of pro-inflammatory cytokines (IL-6, IL-12p35, and IL-1β), the anti-inflammatory cytokine (IL-10), and the chemokine (SDF-1α) in early-term FIV-infected (n = 27) versus uninfected (control) (n = 42) placentas. Bars represent mean Ct values substracted from a negative endpoint (50 - mean Ct), bracketed by standard errors of the mean. P values obtained from single factor ANOVA are noted. Alpha = 0.05.

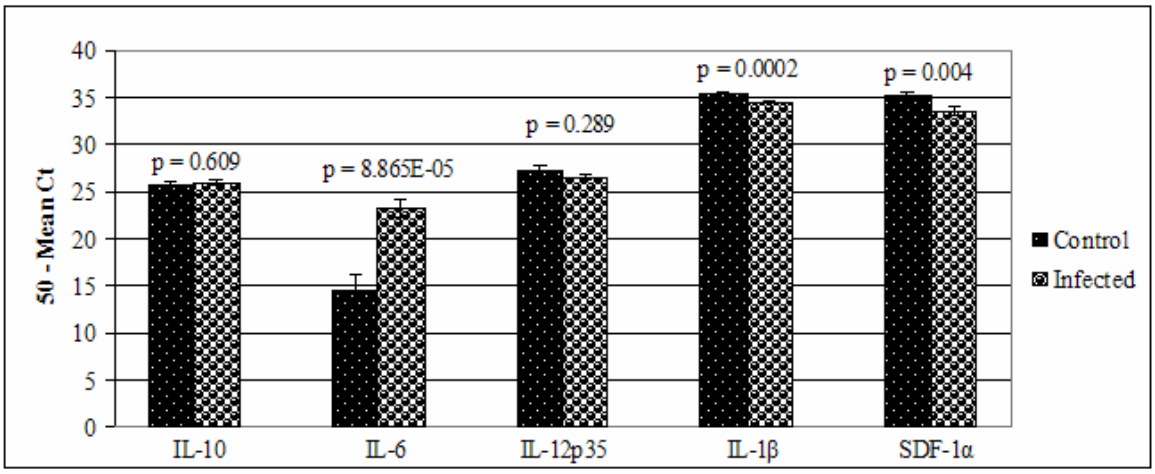

Figure 3.3 Relative Expression of Immunomodulators in Late-Term Feline Placentas

Real-time RT-PCR analysis of feline placental expression of pro-inflammatory cytokines (IL-6, IL-12p35, and IL-1β), the anti-inflammatory cytokine (IL-10), and the chemokine (SDF-1α) in late-term FIV-infected (n = 23) versus uninfected (control) (n = 24) placentas. Bars represent mean Ct values substracted from a negative endpoint (50 - mean Ct), bracketed by standard errors of the mean. P values obtained from single factor ANOVA are noted. Alpha = 0.05.

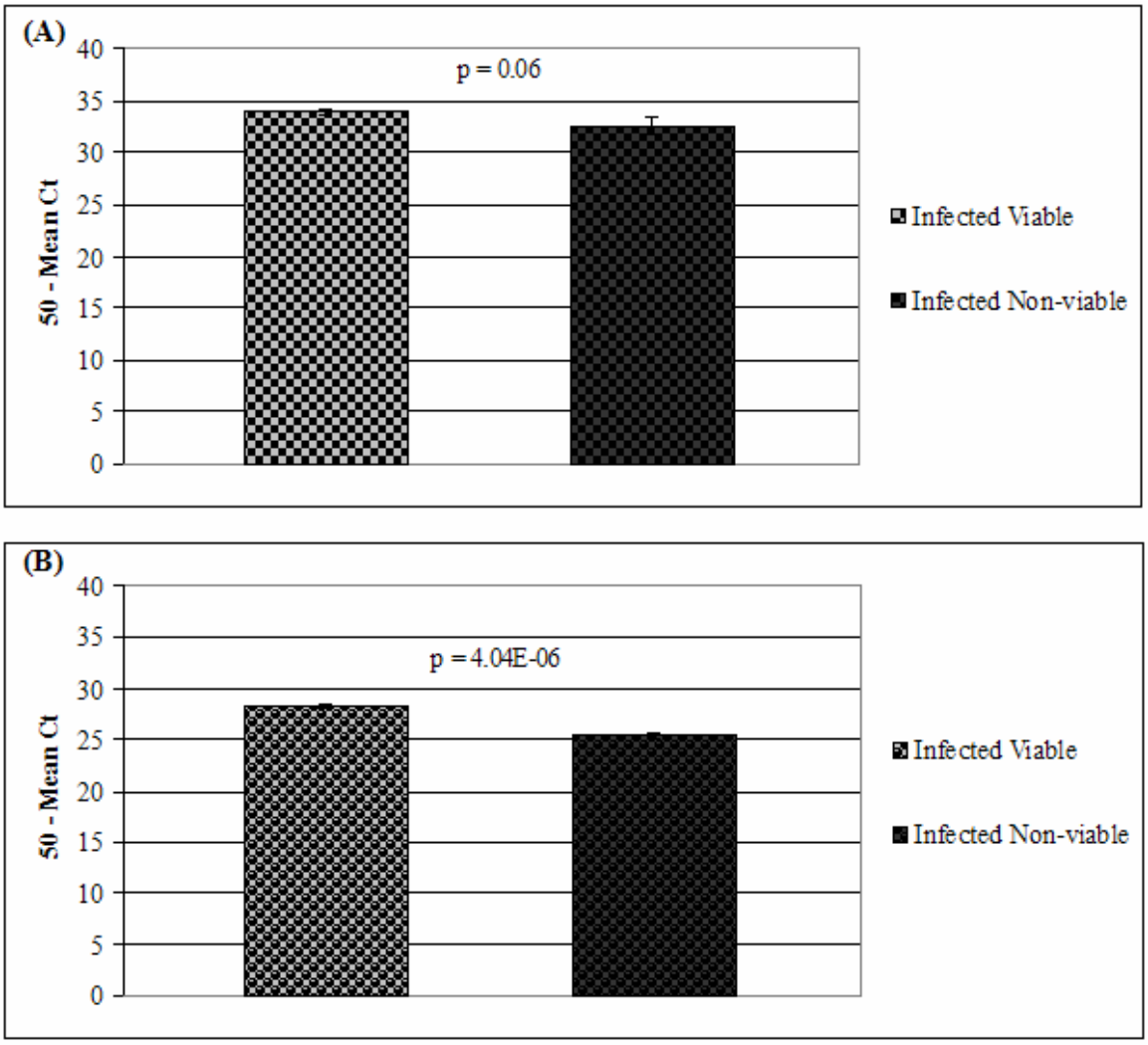

Figure 3.4 Relative Expression of IL-12p35 in Placentas from FIV-infected Cats

Comparison of IL-12p35 expression in placentas from viable and non-viable pregnancies in FIV-infected cats. (A) early placentas from viable pregnancies (n = 21) versus non-viable pregnancies (n = 6); (B) late-term placentas from viable pregnancies (n = 9) versus non-viable pregnancies (n = 14). Bars represent mean Ct values substracted from a negative endpoint (50 - mean Ct), bracketed by standard errors of the mean. P values obtained from single factor ANOVA are noted. Alpha = 0.05.

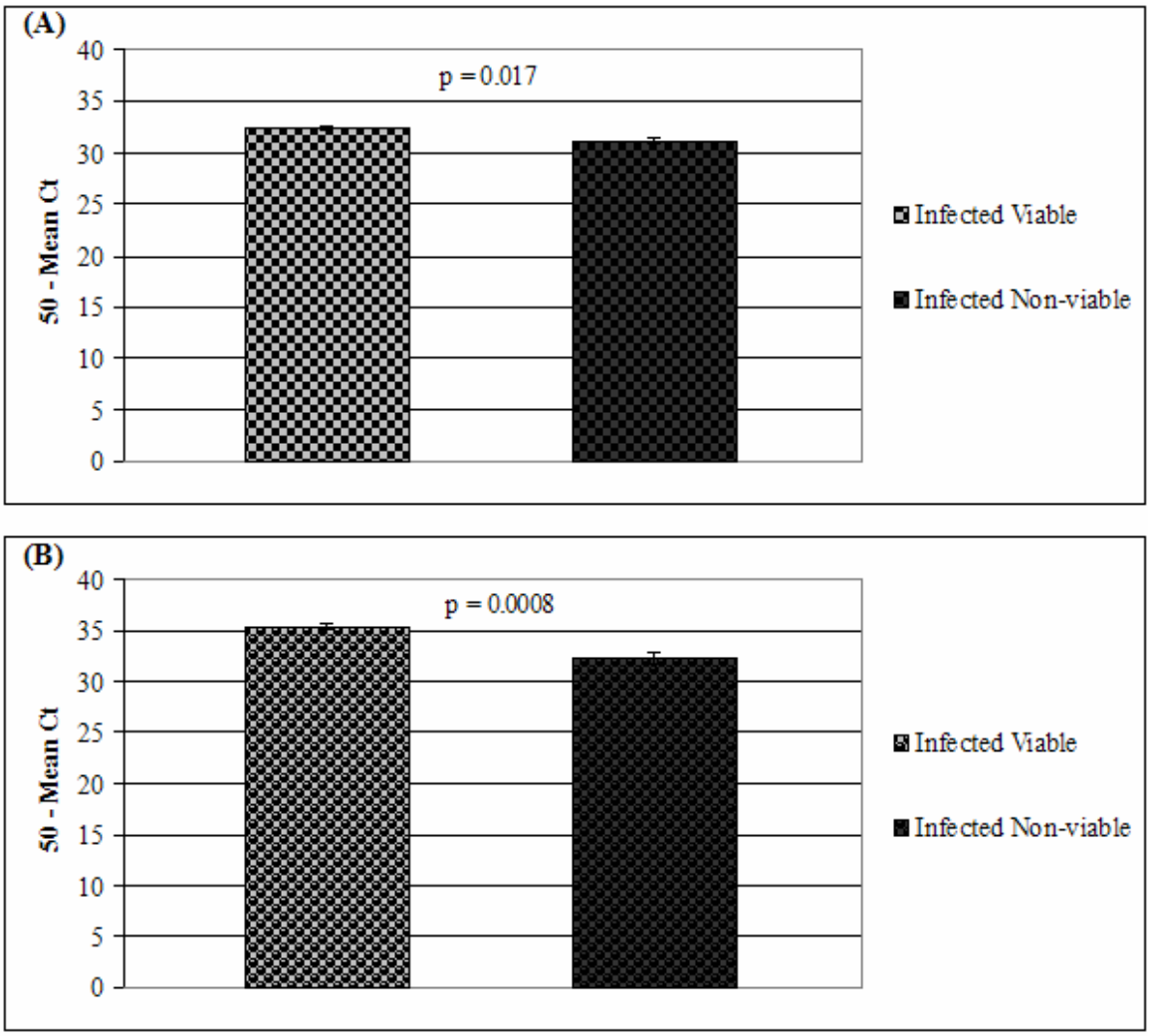

Figure 3.5 Relative Expression of SDF-1α in Placentas from FIV-infected Cats

Analysis of fetal viability and expression of the chemokine (SDF-1α) in placentas obtained from (A) early-term FIV-infected viable (n = 21) versus infected non-viable (n = 6) pregnancies and (B) late-term FIV-infected viable (n = 9) versus infected non-viable (n = 14) pregnancies. Bars represent adjusted mean Ct values and error bars represent standard errors of the mean. P values obtained from single factor ANOVA are noted. Alpha = 0.05.

Discussion

HIV infection of pregnant women may perturb pregnancy and negatively impact reproductive outcome. Langston et al. (1995) reported spontaneous abortions in HIV-infected women with fetal loss occurring in 11.3% (14/124) of pregnancies evaluated. A 67% increase in the risk of spontaneous abortions occurred in a study of HIV-infected Italian women (D'Ubaldo et al., 1998). Similarly, spontaneous abortion and poor reproductive outcome was observed in symptomatic, HIV-infected Indian women (Kumar et al., 1995). Reproductive failure was also reported in HIV-infected, pregnant women receiving the highly active antiretroviral therapy (HAART) in urban Nigeria (Olagbuji et al., 2009). In that study, HIV-infected women were more likely to have intrauterine growth restriction, preterm labor, and low birth weights when compared to HIV-negative controls.

Similar to HIV, transmission of FIV occurs frequently *in utero* under experimental conditions, resulting in reproductive failure (Boudreaux et al., 2009; Rogers and Hoover, 1998; Weaver et al., 2005). Therefore, the FIV-infected, pregnant queen is a useful model to study lentiviral transplacental transfer and the impact of infection on pregnancy outcome. In a previous study by our laboratory (Boudreaux et al., 2009), fetal non-viability at early gestation was 4.7% in control cats and 22.2% in FIV-infected cats. We also reported high rates of fetal non-viability at late gestation (Weaver et al., 2005). O'Neil et al. (1995) reported higher incidences of fetal non-viability associated with acutely-infected versus chronically-infected queens (53% and 30%, respectively), and the rate of *in utero* FIV-infection increased with gestational age (Rogers and Hoover, 1998).

Despite the evidence supporting efficient *in utero* transmission of FIV, the mechanisms by which the virus traverses the placenta and affects fetal viability have not been elucidated.

In typical, normal pregnancy, Th2 (anti-inflammatory) cytokines are favored at the maternal-fetal interface at early pregnancy, then a shift toward Th1 (pro-inflammatory) cytokine dominance occurs during late pregnancy in preparation for the onset of labor (Challis et al., 2009). The anti-inflammatory environment suppresses the maternal cell-mediated immune response in the placenta, allowing maternal tolerance of the semi-allogeneic fetus (Lim et al., 1998, 2000; Raghupathy, 1997; Raghupathy et al., 1999; Raghupathy et al., 2000). No such information has been published for the cat. In the present study, we used real-time RT-PCR to quantify the expression of representative pro-inflammatory cytokines IL-6 (which is also a Th2 cytokine), IL-12p35, and IL-1β, the Th2 cytokine IL-10, and the chemokine SDF-1α in early- and late-term feline placental tissues. To determine the normal pattern of expression, we first compared data obtained from early and late placentas from normal cats only. We detected immunomodulator mRNA in all placental tissues from both early- and late-term gestation. The two Th2 cytokines were expressed to higher levels at early pregnancy and two pro-inflammatory mediators (IL-1β and SDF-1α) were expressed to higher levels at late gestation. The expression of only IL-12p35 failed to match the predicted pattern. The pattern of expression of IL-6 was similar to that reported by Moussa et al. (2001) who also found that IL-6 was expressed to higher levels in first trimester human chorionic villi than later in pregnancy. IL-6 is a pleiotropic cytokine produced by

trophoblasts, macrophages, and Th2 cells. IL-6 regulates expression of human chorionic gonadotropin (HCG) from trophoblasts by activating IL-6 receptor-mediated signal transduction (Masuhiro et al., 1991). Therefore, its expression during early pregnancy is essential. Whether this cytokine plays a role in feline reproductive hormone expression is unknown.

HIV infection alters cytokine expression in placental tissue. Lee et al. (1997) reported increased levels of IL-1β, IL-6, and TNF-α in trophoblasts isolated from HIV-infected human term placentas collected immediately after delivery. On the other hand, other investigators have reported decreased espression of IL-6 and other inflammatory cytokines and β-chemokines in HIV-infected term placentas (Moussa et al., 2001). The dysregulation of placental cytokines in both HIV (Shearer et al., 1997; Weetman, 1999) and FIV (Weaver et al., 2005) infections was associated with increased incidence of reproductive failure. Shearer et al. (1997) suggested that HIV infection causes altered expression of maternal and fetal Th1 and Th2 cytokines, leading to fetal rejection and spontaneous abortion. Moreover, increased expression of Th1 cytokines and decreased expression of a Th2 cytokine (IL-10) was associated with spontaneous abortion and low-birth weight infants born to HIV-infected women (Weetman, 1999). In a comparison of cytokine expression in placentas collected from viable versus non-viable pregnancies, we found elevated expression of inflammatory cytokines interferon (IFN)-γ and IL-1β and increased Th1/Th2 ratios (IL-1β/IL-10) in placentas from resorptions, indicating increased placental pro-inflammatory cytokine expression was associated with failed pregnancy (Weaver et al., 2005). Reports on the effect of HIV-1 on reproductive

hormone expression is limited, but maternal serum human chorionic gonadotropin (HCG) and α-fetoprotein levels were increased in HIV-infected women at delivery, correlative with increased viral load and decreased CD4+ T cell counts (Gross et al., 2003).

To determine how FIV infection might impact expression of these cytokines, we compared their expression in placentas obtained from both control and infected animals at early and late pregnancy. At early gestation, FIV infection increased the expression of the pro-inflammatory cytokines IL-6 and IL-12p35, decreased the expression of the anti-inflammatory cytokine IL-10, and had no effect on IL-1β and SDF-1α. While the source of IL-12 in the placenta was not defined, it is known to be a product of activated macrophages and a potent inducer of the Th1 response and NK cell activation (Sutterwala and Mosser, 1999). Collectively, the data point toward a virus-induced pro-inflammatory microenvironment at early gestation, which is generally not supportive of fetal survival. Given the increased rate of reproductive failure that we found at early and late gestation, it appears that early placental immunopathology may have been a contributor. At late gestation, FIV infection increased the expression of the pro-inflammatory cytokine IL-6, decreased the expression of the pro-inflammatory cytokines IL-1β and SDF-1α, and had no effect on the expression of IL-10 and IL-12p35. While we do not know the function that IL-6 plays in the feline placenta, its consistently-increased expression in infected placentas at early and late term suggests it may be a key cytokine in virus-induced placental immunopathology. Other investigators reported increased levels of IL-6 (Lawrence et al., 1995; Ohashi et al., 1992) and IL-12 (Dean and Pedersen, 1998) in FIV-infected, symptomatic cats. In addition, Lawrence et al. (1995) reported that

increased production of IL-6, IL-1, and tumor necrosis factor (TNF) was associated with impaired immune responses and clinical symptoms of feline AIDS.

We predicted that we would detect evidence of a pro-inflammatory microenvironment in placentas from infected, non-viable pregnancies versus those of infected, viable pregnancies. We did not see this pattern. Only IL-12p35 and SDF-1α expression differed between viable and non-viable placentas. IL-12p35 was expressed to lower levels in late, non-viable placentas than viable ones, and SDF-1α was expressed to lower levels in both early and late non-viable placentas, and the data were highly significant. This pattern is opposite our prediction, and we do not yet have an explanation for this finding.

The key to understanding the relevance of these data is to identify the placental or decidual cells that are a source of these cytokines. Trophoblasts are fetal-derived cells that produce numerous cytokines that regulate placental function (Bennett et al., 1996; Bennett et al., 1998; Guilbert et al., 1993; Roth et al., 1996; Wilczynski, 2005). Maternal regulatory T cells (Tregs), located in the deciduum, produce cytokines that suppress inflammation (Maloy et al., 2003). Both of these cell populations can be infected by HIV, and feline Tregs were shown to support the replication of FIV (Joshi et al., 2005; Joshi et al., 2004). In the event that these cell populations were infected with FIV in our study, then viral infection leading to depletion or altered function of these cells may explain abnormal cytokine patterns. Our laboratory is currently exploring the role of trophoblasts and placental Tregs in lentivirus-induced immunopathology and pregnancy failure.

Acknowledgements

We thank Dr. Shane C. Burgess and Leslie A. Shack for laboratory facility use and quantitative PCR advisement. We are grateful to Dr. Edward A. Hoover, Colorado State University, for providing the FIV-B-2542 inoculum. We thank the veterinary staff at the College of Veterinary Medicine, Mississippi State University for assistance with animal surgical care. This project was supported by the National Institutes of Health (2R15AI048419-02A1) and (3R15AI048419-02A1S1).

References

Allison, R.W., Hoover, E.A., 2003, Covert vertical transmission of feline immunodeficiency virus. AIDS Res Hum Retroviruses 19, 421-434.

Bennett, W.A., Lagoo-Deenadayalan, S., Brackin, M.N., Hale, E., Cowan, B.D., 1996, Cytokine expression by models of human trophoblast as assessed by a semiquantitative reverse transcription-polymerase chain reaction technique. Am J Reprod Immunol 36, 285-294.

Bennett, W.A., Lagoo-Deenadayalan, S., Stopple, J.A., Barber, W.H., Hale, E., Brackin, M.N., Cowan, B.D., 1998, Cytokine expression by first-trimester human chorionic villi. Am J Reprod Immunol 40, 309-318.

Boudreaux, C.E., Lockett, N.N., Chemerys, D.N., Clay, B.T., Scott, V.L., Willeford, B., Brown, T., Coats, K.S., 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. Vet Immunol Immunopathol 131, 290-297.

CDC 2005. HIV AIDS Surveillance Report Cases of HIV infection and AIDS in the United States.

CDC 2007. Mother-to-Child (Perinatal) HIV Transmission and Prevention. In CDC HIV/AIDS Fact Sheet (Department of Health and Human Services, USA).

Challis, J.R., Lockwood, C.J., Myatt, L., Norman, J.E., Strauss, J.F., 3rd, Petraglia, F., 2009, Inflammation and pregnancy. Reprod Sci 16, 206-215.

Clark, D.A., Chaouat, G., 1989, What do we know about spontaneous abortion mechanisms? Am J Reprod Immunol 19, 28-37.

Costeas, P.A., Koumouli, A., Giantsiou-Kyriakou, A., Papaloizou, A., Koumas, L., 2004, Th2/Th3 cytokine genotypes are associated with pregnancy loss. Hum Immunol 65, 135-141.

D'Ubaldo, C., Pezzotti, P., Rezza, G., Branca, M., Ippolito, G., 1998, Association between HIV-1 infection and miscarriage: a retrospective study. DIANAIDS Collaborative Study Group. Diagnosi Iniziale Anomalie Neoplastiche AIDS. Aids 12, 1087-1093.

Dean, G.A., Pedersen, N.C., 1998, Cytokine response in multiple lymphoid tissues during the primary phase of feline immunodeficiency virus infection. J Virol 72, 9436-9440.

Gross, S., Castillo, W., Crane, M., Espinosa, B., Carter, S., DeVeaux, R., Salafia, C., 2003, Maternal serum alpha-fetoprotein and human chorionic gonadotropin levels in women with human immunodeficiency virus. Am J Obstet Gynecol 188, 1052-1056.

Guilbert, L., Robertson, S.A., Wegmann, T.G., 1993, The trophoblast as an integral component of a macrophage-cytokine network. Immunol Cell Biol 71 (Pt 1), 49-57.

Joshi, A., Garg, H., Tompkins, M.B., Tompkins, W.A., 2005, Preferential feline immunodeficiency virus (FIV) infection of CD4+ CD25+ T-regulatory cells correlates both with surface expression of CXCR4 and activation of FIV long terminal repeat binding cellular transcriptional factors. J Virol 79, 4965-4976.

Joshi, A., Vahlenkamp, T.W., Garg, H., Tompkins, W.A., Tompkins, M.B., 2004, Preferential replication of FIV in activated CD4(+)CD25(+)T cells independent of cellular proliferation. Virology 321, 307-322.

Kedzierska, K., Crowe, S.M., 2001, Cytokines and HIV-1: interactions and clinical implications. Antivir Chem Chemother 12, 133-150.

Kumar, R.M., Uduman, S.A., Khurranna, A.K., 1995, Impact of maternal HIV-1 infection on perinatal outcome. Int J Gynaecol Obstet 49, 137-143.

Langston, C., Lewis, D.E., Hammill, H.A., Popek, E.J., Kozinetz, C.A., Kline, M.W., Hanson, I.C., Shearer, W.T., 1995, Excess intrauterine fetal demise associated with maternal human immunodeficiency virus infection. J Infect Dis 172, 1451-1460.

Lawrence, C.E., Callanan, J.J., Willett, B.J., Jarrett, O., 1995, Cytokine production by cats infected with feline immunodeficiency virus: a longitudinal study. Immunology 85, 568-574.

Lee, B.N., Ordonez, N., Popek, E.J., Lu, J.G., Helfgott, A., Eriksen, N., Hammill, H., Kozinetz, C., Doyle, M., Kline, M., Langston, C., Shearer, W.T., Reuben, J.M., 1997, Inflammatory cytokine expression is correlated with the level of human immunodeficiency virus (HIV) transcripts in HIV-infected placental trophoblastic cells. J Virol 71, 3628-3635.

Lim, K.J., Odukoya, O.A., Ajjan, R.A., Li, T.C., Weetman, A.P., Cooke, I.D., 1998, Profile of cytokine mRNA expression in peri-implantation human endometrium. Mol Hum Reprod 4, 77-81.

Lim, K.J., Odukoya, O.A., Ajjan, R.A., Li, T.C., Weetman, A.P., Cooke, I.D., 2000, The role of T-helper cytokines in human reproduction. Fertil Steril 73, 136-142.

Maloy, K.J., Salaun, L., Cahill, R., Dougan, G., Saunders, N.J., Powrie, F., 2003, CD4+CD25+ T(R) cells suppress innate immune pathology through cytokine-dependent mechanisms. J Exp Med 197, 111-119.

Masuhiro, K., Matsuzaki, N., Nishino, E., Taniguchi, T., Kameda, T., Li, Y., Saji, F., Tanizawa, O., 1991, Trophoblast-derived interleukin-1 (IL-1) stimulates the release of human chorionic gonadotropin by activating IL-6 and IL-6-receptor system in first trimester human trophoblasts. J Clin Endocrinol Metab 72, 594-601.

Mosmann, T.R., Coffman, R.L., 1989, Heterogeneity of cytokine secretion patterns and functions of helper T cells. Adv Immunol 46, 111-147.

Moussa, M., Roques, P., Fievet, N., Menu, E., Maldonado-Estrada, J.G., Brunerie, J., Frydman, R., Fritel, X., Herve, F., Chaouat, G., 2001, Placental cytokine and chemokine production in HIV-1-infected women: trophoblast cells show a different pattern compared to cells from HIV-negative women. Clin Exp Immunol 125, 455-464.

O'Neil, L.L., Burkhard, M.J., Hoover, E.A., 1996, Frequent perinatal transmission of feline immunodeficiency virus by chronically infected cats. J Virol 70, 2894-2901.

Ohashi, T., Goitsuka, R., Watari, T., Tsujimoto, H., Hasegawa, A., 1992, Elevation of feline interleukin 6-like activity in feline immunodeficiency virus infection. Clin Immunol Immunopathol 65, 207-211.

Olagbuji, B.N., Ezeanochie, M.C., Ande, A.B., Oboro, V.O., 2009, Obstetric and perinatal outcome in HIV positive women receiving HAART in urban Nigeria. Arch Gynecol Obstet.

Orsi, N.M., Tribe, R.M., 2008, Cytokine networks and the regulation of uterine function in pregnancy and parturition. J Neuroendocrinol 20, 462-469.

Raghupathy, R., 1997, Th1-type immunity is incompatible with successful pregnancy. Immunol Today 18, 478-482.

Raghupathy, R., Makhseed, M., Azizieh, F., Hassan, N., Al-Azemi, M., Al-Shamali, E., 1999, Maternal Th1- and Th2-type reactivity to placental antigens in normal human pregnancy and unexplained recurrent spontaneous abortions. Cell Immunol 196, 122-130.

Raghupathy, R., Makhseed, M., Azizieh, F., Omu, A., Gupta, M., Farhat, R., 2000, Cytokine production by maternal lymphocytes during normal human pregnancy and in unexplained recurrent spontaneous abortion. Hum Reprod 15, 713-718.

Rogers, A.B., Hoover, E.A., 1998, Maternal-fetal feline immunodeficiency virus transmission: timing and tissue tropisms. J Infect Dis 178, 960-967.

Romagnani, S., 1994, Lymphokine production by human T cells in disease states. Annu Rev Immunol 12, 227-257.

Roth, I., Corry, D.B., Locksley, R.M., Abrams, J.S., Litton, M.J., Fisher, S.J., 1996, Human placental cytotrophoblasts produce the immunosuppressive cytokine interleukin 10. J Exp Med 184, 539-548.

Saito, S., 2001, Cytokine cross-talk between mother and the embryo/placenta. J Reprod Immunol 52, 15-33.

Scott, V.L., Burgess, S.C., Shack, L.A., Lockett, N.N., Coats, K.S., 2008, Expression of CD134 and CXCR4 mRNA in term placentas from FIV-infected and control cats. Vet Immunol Immunopathol 123, 90-96.

Shearer, W.T., Reuben, J., Lee, B.N., Popek, E.J., Lewis, D.E., Hammill, H.H., Hanson, I.C., Kline, M.W., Langston, C., 1997, Role of placental cytokines and inflammation in vertical transmission of HIV infection. Acta Paediatr Suppl 421, 33-38.

Sutterwala, F.S., Mosser, D.M., 1999, The taming of IL-12: suppressing the production of proinflammatory cytokines. J Leukoc Biol 65, 543-551.

UNAIDS/WHO 2009. Towards Universal Access. Joint United Nations Programme on HIV/AIDS.

Weaver, C.C., Burgess, S.C., Nelson, P.D., Wilkinson, M., Ryan, P.L., Nail, C.A., Kelly-Quagliana, K.A., May, M.L., Reeves, R.K., Boyle, C.R., Coats, K.S., 2005, Placental immunopathology and pregnancy failure in the FIV-infected cat. Placenta 26, 138-147.

Weetman, A.P., 1999, The immunology of pregnancy. Thyroid 9, 643-646.

Wegmann, T.G., Lin, H., Guilbert, L., Mosmann, T.R., 1993, Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a TH2 phenomenon? Immunol Today 14, 353-356.

Wilczynski, J.R., 2005, Th1/Th2 cytokines balance--yin and yang of reproductive immunology. Eur J Obstet Gynecol Reprod Biol 122, 136-143.

CHAPTER IV

A NOVEL IMMUNOHISTOCHEMICAL ASSAY TO DETECT TROPHOBLASTS IN FELINE PLACENTA

Abstract

The placenta, a fetal endocrine organ, is composed of a subpopulation of trophoblasts, cytotrophoblasts and syncytiotrophoblasts. Trophoblastic populations can be distinguished based upon their expression of cytokeratin. The purpose of this project was to develop an immunohistochemistry (IHC) method to identify trophoblasts selectively in frozen feline placental tissue using antibodies specific for cytokeratin. We used the mouse monoclonal antibody anti-human pan-cytokeratin AE1/AE3 and the detection system DAKO EnVision System, HRP. We encountered non-specific immunoreactivity that could not be eliminated with altered blocking methods. The non-specific reactivity was attributed to the goat anti-mouse/rabbit IgG peroxidase polymer included in the kit. Alternatively, we used a polyclonal rabbit anti-cow cytokeratin wide spectrum screening (WSS) antibody with goat anti-rabbit IgG polyclonal secondary antibody to detect cytokeratin in feline placental tissue. This IHC procedure eliminated non-specific immunoreactivity while specifically labeling cytokeratin. This new approach provides an innovative IHC method to identify trophoblasts in feline placenta.

Introduction

The placenta, an endocrine organ of fetal origin, is responsible for metabolic exchange between the mother and the developing fetus (Senger, 2003). This organ produces hormones that are responsible for pregnancy maintenance and fetal growth (Senger, 2003). Trophoblastic cells (cytotrophoblasts and syncytiotrophoblasts) are a population of cells that comprise the placental chorionic villi. These trophoblasts are fetal-derived cells of epithelial origin that cover the blastocyst and form the chorion, the fetal portion of the placenta (Senger, 2003). Syncytiotrophoblasts comprise the outer trophoblast layer, contacting and forming attachment with the maternal endometrium (Senger, 2003). The cytotrophoblasts form the inner layer of the trophoblast between the syncytiotrophoblasts and the chorionic villous capillaries. Placental trophoblasts express unusual immunological markers, including HLA-G and other Class I molecules that regulate immune suppression (Kovats et al., 1990; Szekeres-Bartho, 2002) and cytokines that regulate immune function (Baergen et al., 1994; Moussa et al., 2001). The immunological characteristics of trophoblastic cells contribute to the immunological privilege of the placenta, promoting fetal tolerance and successful pregnancy.

As a part of an ongoing study of feline immunodeficiency virus (FIV) vertical transmission and virus-induced placental immunopathology, we were interested in the impact of viral infection on gene expression by trophoblasts. Our initial goal was to develop an immunohistochemistry method to identify trophoblasts in feline placental tissue based on their expression of cytokeratin intermediate filaments. We attempted to detect cytokeratin antigens using the mouse anti-human pan-cytokeratin monoclonal

antibody AE1/AE3 and DAKO EnVision System, HRP kit, which utilizes a goat anti-mouse/rabbit IgG peroxidase-labeled polymer as a secondary antibody. However, in feline placentas this system produced non-specific reactivity which could not be eliminated with modification of protocol. Thus, we developed an innovative IHC procedure utilizing a polyclonal rabbit anti-cow cytokeratin wide spectrum screening (WSS) antibody paired with goat anti-rabbit Poly-HRP secondary antibody to detect cytokeratin in feline placental tissue. This procedure resulted in specific staining of cytokeratin in feline trophoblasts, eliminating non-specific antibody binding.

Materials and Methods

Feline placental tissues

All procedures utilizing cats (*Felis domesticus*) were performed with approval of the Mississippi State University Institutional Animal Care and Use Committee. Cats used in this investigation were part of an ongoing study of FIV-B-2542 (Rogers and Hoover, 1998) transplacental transmission. Cats were reproductively mature, specific-pathogen-free (SPF) animals of less than 12 months of age when obtained from a commercial cattery. Uninfected cats served as normal controls. All queens were allowed to breed naturally with SPF toms. Breeding was observed and pregnancy was confirmed by palpation and ultrasonography.

Kittens were delivered by cesarean section during week 8 of gestation (late-term) (Weaver et al., 2005). Placentas were collected under sterile conditions using the

following dissection procedure. The uteri were removed and individual fetal membranes were collected. Following rinses with sterile PBS, membranes were incised with a sterile scalpel, and fetuses and placentas were collected. Placental tissues were snap frozen in liquid nitrogen and cryopreserved at -80°C. Tissues from control cats only were used in the following procedures.

Histological evaluation of placental tissues

OCT-embedded placental sections from a late-term control queen were fixed in acetone for 10 min and allowed to dehydrate for 1 min. Following dehydration, routine hematoxylin and eosin (H&E) staining was performed to observe histology as follows. Placental tissues were stained with Mayer's hematoxylin (Sigma, St. Louis, MO) for 15 min and washed under tap water for 20 min. Sections were counterstained with eosin for 2 min and dehydrated sequentially in two changes of 95% and 100% EtOH, 2 min each. After a 1 min dehydration at room temp, sections were cleared twice with 2 min xylene rinses, and mounted with Permount (Fisher Scientific, Waltham, MA).

Basic immunohistochemistry procedure

All IHC procedures were performed using the following basic staining protocol with modifications only to endogenous peroxidase quenching, serum pretreatments, and antibody incubations. Frozen placental tissues were cryosectioned to 4 μm and placed onto poly-L-lysine coated slides (Lab Scientific, Inc., Livingston, NJ). Sections were allowed to air dry at room temp overnight. Dried sections were fixed in acetone for 10

min at room temp and allowed to air dry completely. To quench endogenous peroxidase activity, sections were blocked with the commercial peroxidase blocking reagent (DAKO EnVision; Dako Corp., Carpinteria, CA) for 10 min and washed in a bath of Tris-buffer (TB; 0.05 M Tris-HCl, pH 7.0-7.6). The primary antibody or negative control reagent was diluted 1:50 in TB containing 1% bovine serum albumin (BSA). Sections were incubated with either the mouse anti-human pan-cytokeratin monoclonal antibody AE1/AE3 (Dako Corp.) or the mouse universal negative control reagent (Dako Corp.) for 10 min. Following the incubation of the primary antibody or the negative control reagent, sections were washed in separate baths of TB. Sections were then incubated with the goat anti-mouse/rabbit IgG peroxidase-labeled polymer (DAKO EnVision, HRP) for 10 min and washed in a fresh bath of TB. Sections were incubated with the buffered substrate solution containing 3,3'-diaminobenzidine (DAB) chromogen (Dako Corp. or Invitrogen, Carlsbad, CA) for 10 min and washed in a bath of distilled water for 1 min. Sections were then counterstained with Mayer's hematoxylin (Sigma) for 5 min and rinsed in fresh, distilled water for 1 min. The counterstain was blued by dipping sections 10 times in a bath of 37 mM ammonia and rinsing sections in a fresh bath of distilled water. Sections were dehydrated in 75%, 95%, and 100% EtOH for 30 sec and xylene for 5 min. Immunoreactivity was detected microscopically.

Endogenous peroxidase quenching and tissue blocking

Dehydrated sections were blocked by sequential pretreatment with both mouse and goat serum (1:100) for 10 min each. Blocking serum was removed from the slides,

and unrinsed sections were quenched with peroxidase blocking reagent (DAKO EnVision, HRP). Alternatively, dehydrated sections untreated with sera were quenched for 30 min with a solution of 2 ml 30% H_2O_2 (Sigma) and 400 ml methanol (Champion et al., 2003). Quenched sections were blocked with 5% (w/v) non-fat dry milk in TB (Blotto) for 1 hr 15 min.

Modified IHC Procedure

Sections quenched with peroxidase blocking reagent were incubated with mouse antibodies (as previously described) using the following conditions: no antibody, primary antibody only, and secondary antibody only. Sections quenched with H_2O_2 in methanol and blocked with Blotto were incubated with the polyclonal rabbit anti-cow cytokeratin wide spectrum screening (WSS) antibody (Dako Corp.) or rabbit universal negative control reagent (Dako Corp.) and secondary goat anti-rabbit, Poly-HRP (Chemicon International, Inc., Temecula, CA).

Antibodies

All antibodies used in these assays are shown in Table 4.1.

Table 4.1

Antibodies Used in Immunohistochemistry

Antibody	Clone / Reagent Type	Dilution(s)	Source	Catalog Number
Monoclonal mouse anti-human cytokeratin clones AE1/AE3	Monoclonal / Primary Antibody	1:50	Dako Corporation (Carpinteria, CA)	Code N1590
N-Universal negative control mouse	Negative Control Reagent	1:50	Dako Corporation (Carpinteria, CA)	Code N1698
Goat anti-mouse/rabbit IgG peroxidase-labeled polymer (L.V. DAKO EnVision System, HRP)	Secondary Antibody	Ready-to-use	Dako Corporation (Carpinteria, CA)	Code K1392
Polyclonal rabbit anti-cow cytokeratin, wide spectrum screening (WSS)	Polyclonal / Primary Antibody	1:50 or 1:100	Dako Corporation (Carpinteria, CA)	Code Z0622
Goat anti-rabbit IgG, Poly-HRP, Ampli-HRP	Polyclonal / Secondary Antibody	Ready-to-use	Chemicon International, Inc. (Temecula, CA)	AP342P
N-Universal negative control rabbit	Negative Control Reagent	1:100	Dako Corporation (Carpinteria, CA)	Code N1699

Results

Histological evaluation of placental tissues

Hematoxylin and eosin staining of the placental tissue revealed the location of trophoblasts and provided confirmation that tissue integrity was not compromised during cryopreservation and frozen storage (Fig. 4.1).

Immunohistochemistry

The results of all IHC methods are listed in Table 4.2.

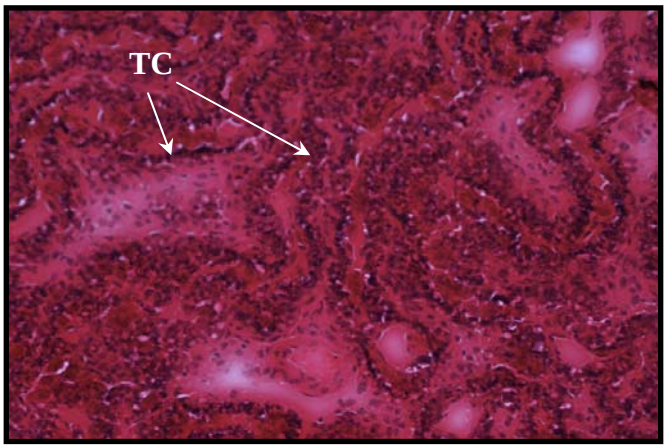

Figure 4.1 Histological Evaluation of Late-Term Feline Placenta

Placental tissues were cryosectioned to a thickness of 4 μm, fixed in acetone, and dehydrated. Sections were stained with hematoxylin and eosin. The location of trophoblastic cells (TC) (arrows) is shown. Magnification: 100X.

IHC using AE1/AE3 mouse monoclonal antibodies and the DAKO EnVision, HRP kit

We used the mouse anti-human pan-cytokeratin monoclonal antibody AE1/AE3 and a negative control reagent with the DAKO EnVision System, HRP kit to detect cytokeratin in acetone-fixed, frozen placental tissues. Immunolabeling revealed similar immunoreactivity in tissues treated with the mouse monoclonal antibody AE1/AE3 and the negative control reagent (Fig. 4.2), indicating non-specific labeling in our IHC procedure.

Parallel placental sections were stained for cytokeratin using serum pretreatments and modified antibody conditions. Cytokeratin labeling did not occur in sections subjected to the protocol in the absence of both primary and secondary antibody or sections that were treated with only the primary mouse monoclonal (Fig. 4.2C and 4.2D, respectively), suggesting that endogenous peroxidase activity was not the cause of non-specific labeling. Sections that were treated with the goat anti-mouse/rabbit IgG peroxidase polymer alone had concentrated areas of non-specific labeling (Fig. 4.2E), indicating that this secondary antibody was non-specifically reactive.

Novel immunohistochemistry technique using alternative antibodies

To overcome problems with non-specific immunoreactivity, alternative antibody pairs were used to target placental tissue sections. Sections treated with the polyclonal rabbit anti-cow cytokeratin primary antibody (1:50 or 1:100) followed by secondary antibody developed strong, specific binding to cytokeratin (Fig. 4.3A). Sections treated with either secondary antibody alone (Fig. 4.3B) or with rabbit universal negative control antibody followed by secondary antibody (Fig. 4.3C) did not stain with chromogen.

Table 4.2

Summary of Methods Used to Detect Cytokeratin Antigens in Feline Placental Tissues

Quenching/ Blocking Agent	Primary (1°) Antibody	Secondary (2°) Antibody	Result
Peroxidase blocking reagent (DAKO EnVision)	Mouse universal negative control (1:50) (Dako)	Goat anti-mouse/rabbit IgG peroxidase polymer (DAKO EnVision)	Non-specific binding
Peroxidase blocking reagent (DAKO EnVision)	Mouse monoclonal AE1/AE3 (1:50) (Dako)	Goat anti-mouse/rabbit IgG peroxidase polymer (DAKO EnVision)	Non-specific binding
Mouse and goat sera; peroxidase blocking reagent (DAKO EnVision)	NONE	NONE	No staining
Mouse and goat sera; peroxidase blocking reagent (DAKO EnVision)	Mouse monoclonal AE1/AE3 (1:50) (Dako)	NONE	No staining
Mouse and goat sera; peroxidase blocking reagent (DAKO EnVision)	NONE	Goat anti-mouse/rabbit IgG peroxidase polymer (DAKO EnVision)	Non-specific binding
H_2O_2 in methanol; Blotto	NONE	Goat anti-rabbit IgG, Poly-HRP (Chemicon)	No staining
H_2O_2 in methanol; Blotto	Polyclonal rabbit anti-cow cytokeratin (1:50) (Dako)	Goat anti-rabbit IgG, Poly-HRP (Chemicon)	Strong, specific antigen binding
H_2O_2 in methanol; Blotto	Rabbit universal negative control (1:100) (Dako)	Goat anti-rabbit IgG, Poly-HRP (Chemicon)	No staining
H_2O_2 in methanol; Blotto	Polyclonal rabbit anti-cow cytokeratin (1:100) (Dako)	Goat anti-rabbit IgG, Poly-HRP (Chemicon)	Strong, specific antigen binding

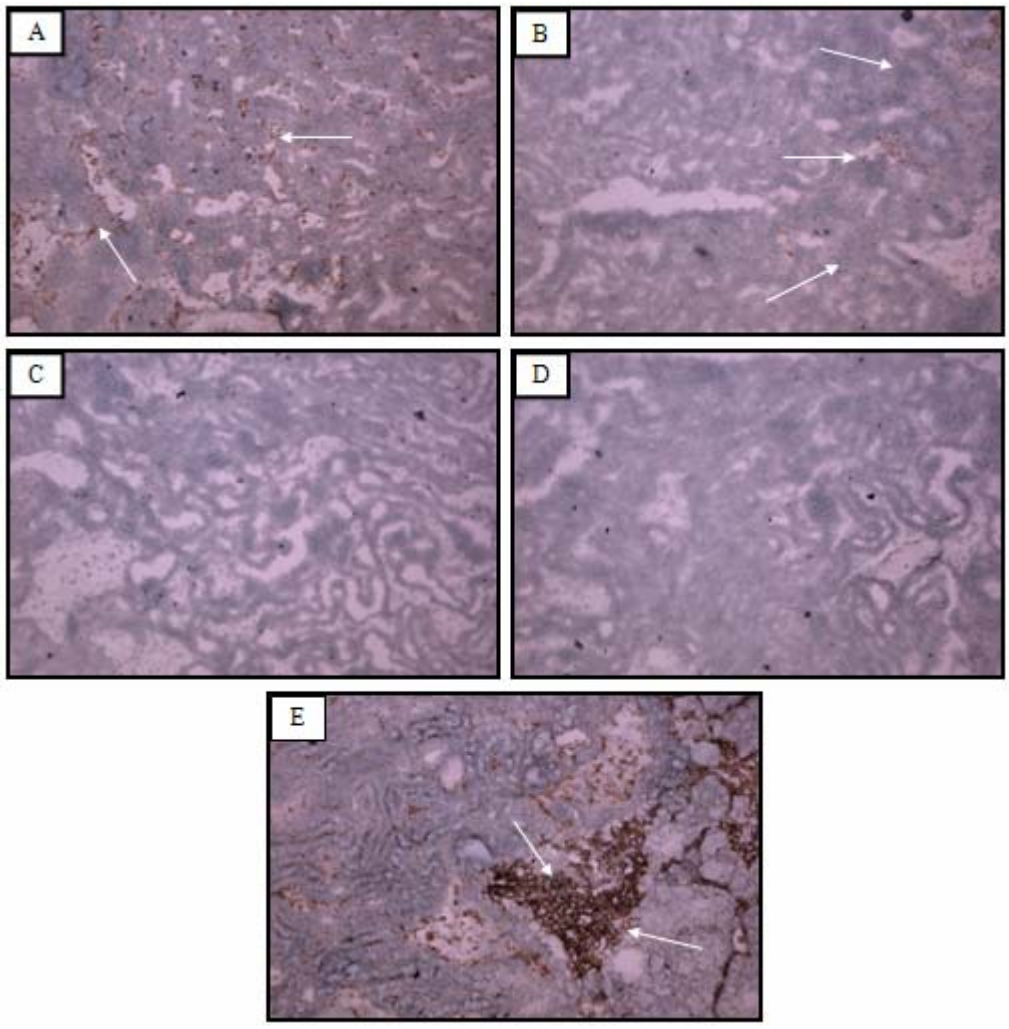

Figure 4.2 Immunohistochemistry using AE1/AE3 Mouse Monoclonal Antibodies and the DAKO EnVision, HRP kit

Placental tissues were cryosectioned to a thickness of 4 μm, fixed in acetone, and dehydrated. Immunostaining of late-term feline placental tissue sections using mouse monoclonal antibodies and the EnVision kit resulted in non-specific immunoreactivity. Arrows show chromogen staining. Magnification: 100X. (A) Sections labeled with the mouse universal negative control reagent and (B) sections labeled with the mouse monoclonal antibody AE1/AE3 followed by the secondary antibody goat anti-mouse/rabbit IgG peroxidase polymer stained with the chromogen. (C) Unlabeled sections and (D) sections treated only with mouse monoclonal AE1/AE3 did not stain. (E) Sections labeled only with secondary antibody goat anti-mouse/rabbit IgG peroxidase polymer produced strong, non-specific staining.

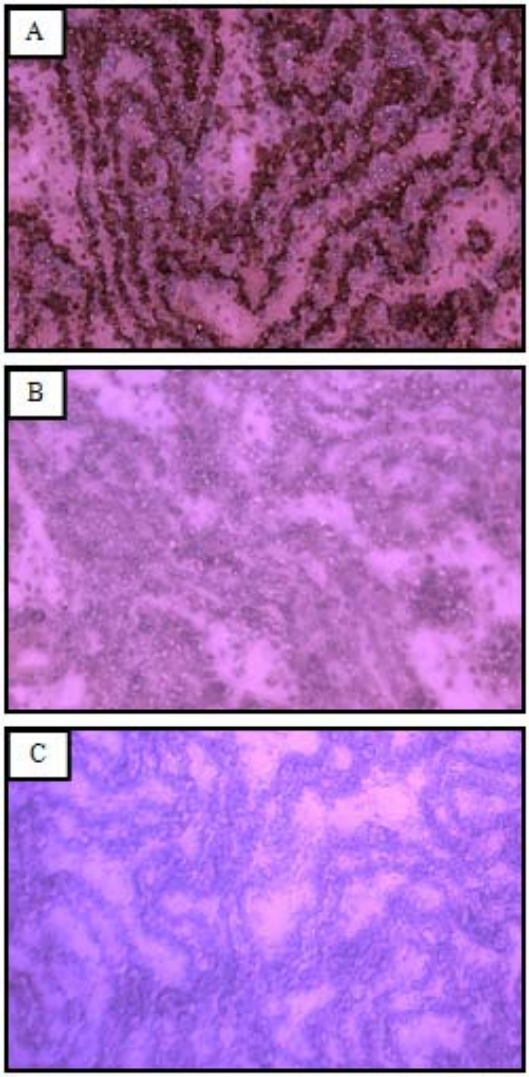

Figure 4.3 Immunohistochemistry using Alternative Antibodies

Placental tissues were cryosectioned to a thickness of 4 μm, fixed in acetone, and dehydrated. Immunohistochemical staining of late-term feline placental tissue using polyclonal antibodies produced specific immunoreactivity to cytokeratin. Magnification: 100X. (A) Sections stained with the polyclonal antibody rabbit anti-cow cytokeratin followed by secondary antibody goat anti-rabbit, Poly-HRP resulted in strong immunoreactivity to cytokeratin (brown stain). (B) Sections stained with only secondary antibody goat anti-rabbit, Poly-HRP did not stain with chromogen. (C) Sections stained with the rabbit universal negative control antibody and secondary goat anti-rabbit, Poly-HRP did not stain with chromogen.

Discussion

To determine the role of trophoblasts in FIV-induced placental immunopathology, it was important for us to identify trophoblasts in feline placental tissue by IHC. To identify these cells we targeted cytokeratin, a marker for trophoblasts. We began our investigation by using the mouse anti-human pan-cytokeratin monoclonal antibody AE1/AE3 with a traditional IHC kit (DAKO EnVision, HRP). The use of anti-cytokeratin monoclonal antibodies to identify trophoblastic cellular populations with immunohistochemistry has been described in detail in both the human and feline placenta (Joling et al., 1996; Kadyrov et al., 2007; Muhlhauser et al., 1995; Walter and Schonkypl, 2006). Mulhauser et al. (1995) identified villous and extravillous human trophoblast populations throughout pregnancy in frozen sections using monoclonal antibodies directed against cytokeratin. Recently, anti-cytokeratin monoclonal antibodies were used to evaluate the distribution of cytokeratin intermediate filaments in feline fetal chorionic lamellae (Walter and Schonkypl, 2006). However, our immunostaining revealed non-specific immunoreactivity in feline placental tissues labeled with the mouse monoclonal AE1/AE3 and goat anti-mouse/rabbit IgG peroxidase polymer which we were unable to resolve with altered blocking techniques.

Non-specific reactivity of the goat anti-mouse/rabbit IgG peroxidase polymer was demonstrated by reactivity to placental protein in a western blot assay (data not shown). Likewise, we attributed non-specific immunoreactivity in our IHC procedure to this secondary antibody. This DAKO EnVision, HRP kit component appears to be an unreliable IHC reagent for labeling feline placental tissue. Although we did not

specifically pinpoint the reliability of the monoclonal antibody AE1/AE3 in our tissues, this antibody failed to bind cytokeratin reliably in a majority of normal feline epithelial tissues in a previous report (Peaston et al., 1992).

Honig et al. (2005) reported non-specific immunoreactivity in IHC of human placental tissues when attempting to identify dendritic cells by immunolabeling with mouse anti-CD83 monoclonal antibodies and HLA-G by immunolabeling with specific mouse monoclonal antibody. In that study, non-specific immunoreactivity occurred when mouse isotype IgG2b was used. Non-specific binding of mouse antibody IgG2b isotype to endothelial cells in human chorionic villi was prevented by blocking tissues with purified human IgG, which contained a mixture of antibody isotypes. Although the reason for non-specific immunoreactivity was unproven, it was attributed to inappropriate antibody binding to placental Fc receptors with an affinity for the mouse immunoglobulin Fc domain. Interestingly, the placentas of both humans and mice possess hemochorial placentation, similar mechanisms of IgG transport from mother to fetus, and an "antigen-independent" affinity for antibody (Honig et al., 2005; Lyden et al., 2001; Simister, 2003).

Likewise, we have encountered non-specific immunoreactivity of a panel of mouse monoclonal and some polyclonal antibodies to feline placenta in IHC and immunofluorescence procedures (data not shown). Here, our approach to eliminate this problem was to use a rabbit polyclonal antibody to bovine cytokeratin and secondary goat anti-rabbit, Poly-HRP, along with adjusted quenching and blocking procedures. These modifications resulted in specific cytokeratin labeling. An alternative approach that has

been shown effective in avoiding Fc receptor non-immune adherence is the use of Fab or F(ab')2 portions of the IgG molecule rather than the whole IgG molecule in immunoassays (Ramos-Vara, 2005). We have not attempted IHC in feline tissues with these fragmented antibodies.

Our data confirm that like human placental tissue, the feline placenta presents a unique challenge to the use of immunolabeling for detection of placental antigens. Our innovative approach to feline placental immunohistochemistry provides a useful alternative method for specifically targeting feline cytokeratin antigen and may provide a basis for targeting other placental antigens with appropriate immunoreactivity.

Conclusions

Non-specific immunoreactivity of feline placental tissues presents a challenge when attempting immunoassays to identify specific antigen in these tissues. We developed an innovative immunohistochemistry procedure utilizing a wide spectrum rabbit polyclonal antibody and goat anti-rabbit secondary polyclonal antibody to label cytokeratin specifically in feline trophoblasts and eliminate non-specific immunoreactivity. Our novel IHC protocol is detailed in Table 4.3.

Table 4.3

Immunohistochemistry Protocol for Trophoblast Staining in Frozen Feline Placental Tissues

Step	Procedure	Duration
1	Fix with room temperature acetone	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 5% Blotto	1 hr 15 min
6	Wash in 0.05 M Tris-HCl	2 x 5 min
7	Incubate with rabbit anti-cow 1° Ab in 0.05 M Tris-HCl + 1% BSA	Overnight at 4°C
8	Wash in 0.05 M Tris-HCl	2 x 5 min
9	Incubate with goat anti-rabbit 2° Ab at room temperature	1 hr
10	Wash in 0.05 M Tris-HCl	2 x 5 min
11	Incubate with DAB	15 min
12	Wash by dipping in dH_2O	3 x 3 sec
13	Counterstain with Mayer's Hematoxylin	5 min
14	Wash by dipping in dH_2O	3 x 3 sec
15	Blue hematoxylin by dipping in 37 mM ammonia	10 x 2 sec
16	Dehydrate:	
	- 75% EtOH	30 sec
	- 95% EtOH	30 sec
	- 100% EtOH	30 sec
	- Xylene	5 min
	- Dry in fume hood	5 min
17	Mount in Permount	

Acknowledgements

We thank Dr. Edward A. Hoover, Colorado State University, for generously providing the infectious plasma pool containing FIV-B-2542. We thank Dr. William Bennett, University of Mississippi Medical Center, for providing technical advice, laboratory facilities, and antibodies. We are grateful to the histology staff in the Department of Pathobiology and Population Medicine at the College of Veterinary Medicine, Mississippi State University, for generously providing the polyclonal rabbit anti-cow cytokeratin WSS antibody and assistance with staining. We appreciate Brittany T. Clay for assistance with histology. We thank the laboratories of Dr. Jeffery Eells and Dr. Nikolay Filipov, College of Veterinary Medicine, Mississippi State University, for assistance with microscopy and photography. We thank Dr. Donna Gordon, Department of Biological Sciences, Mississippi State University, for assistance with photography. This project was supported by the National Institutes of Health (2R15AI048419-02A1) and (3R15AI048419-02A1S1).

References

Baergen, R., Benirschke, K., Ulich, T.R., 1994, Cytokine expression in the placenta. The role of interleukin 1 and interleukin 1 receptor antagonist expression in chorioamnionitis and parturition. Arch Pathol Lab Med 118, 52-55.

Champion, E.E., Glazier, J.D., Greenwood, S.L., Mann, S.J., Rawlings, J.M., Sibley, C.P., Jones, C.J., 2003, Localization of alkaline phosphatase and Ca2+-ATPase in the cat placenta. Placenta 24, 453-461.

Honig, A., Rieger, L., Kapp, M., Dietl, J., Kammerer, U., 2005, Immunohistochemistry in human placental tissue--pitfalls of antigen detection. J Histochem Cytochem 53, 1413-1420.

Joling, P., Broekhuizen, R., de Weger, R.A., Rottier, P.J., Egberink, H., 1996, Immunohistochemical demonstration of cellular antigens of the cat defined by anti-human antibodies. Vet Immunol Immunopathol 53, 115-127.

Kadyrov, M., Garnier, Y., Gantert, M., Kramer, B.W., Kaufmann, P., Huppertz, B., 2007, Cytokeratin antibodies as differential markers of trophoblast and fetomaternal syncytial plaques in the sheep placentome. Placenta 28, 1107-1109.

Kovats, S., Main, E.K., Librach, C., Stubblebine, M., Fisher, S.J., DeMars, R., 1990, A class I antigen, HLA-G, expressed in human trophoblasts. Science 248, 220-223.

Lyden, T.W., Robinson, J.M., Tridandapani, S., Teillaud, J.L., Garber, S.A., Osborne, J.M., Frey, J., Budde, P., Anderson, C.L., 2001, The Fc receptor for IgG expressed in the villus endothelium of human placenta is Fc gamma RIIb2. J Immunol 166, 3882-3889.

Moussa, M., Roques, P., Fievet, N., Menu, E., Maldonado-Estrada, J.G., Brunerie, J., Frydman, R., Fritel, X., Herve, F., Chaouat, G., 2001, Placental cytokine and chemokine production in HIV-1-infected women: trophoblast cells show a different pattern compared to cells from HIV-negative women. Clin Exp Immunol 125, 455-464.

Muhlhauser, J., Crescimanno, C., Kasper, M., Zaccheo, D., Castellucci, M., 1995, Differentiation of human trophoblast populations involves alterations in cytokeratin patterns. J Histochem Cytochem 43, 579-589.

Peaston, A.E., Higgins, R.J., Naydan, D.K., Sokol, K.A., 1992, Evaluation of commercially available antibodies to cytokeratin intermediate filaments and laminin in normal cat pinna. J Vet Diagn Invest 4, 306-311.

Ramos-Vara, J.A., 2005, Technical aspects of immunohistochemistry. Vet Pathol 42, 405-426.

Rogers, A.B., Hoover, E.A., 1998, Maternal-fetal feline immunodeficiency virus transmission: timing and tissue tropisms. J Infect Dis 178, 960-967.

Senger, P.L., 2003, Pathways to Pregnancy and Parturition, 2nd Edition Edition. Current Conceptions, Inc., Pullman, WA, 368 p.

Simister, N.E., 2003, Placental transport of immunoglobulin G. Vaccine 21, 3365-3369.

Szekeres-Bartho, J., 2002, Immunological relationship between the mother and the fetus. Int Rev Immunol 21, 471-495.

Walter, I., Schonkypl, S., 2006, Extracellular matrix components and matrix degrading enzymes in the feline placenta during gestation. Placenta 27, 291-306.

Weaver, C.C., Burgess, S.C., Nelson, P.D., Wilkinson, M., Ryan, P.L., Nail, C.A., Kelly-Quagliana, K.A., May, M.L., Reeves, R.K., Boyle, C.R., Coats, K.S., 2005, Placental immunopathology and pregnancy failure in the FIV-infected cat. Placenta 26, 138-147.

CHAPTER V

HISTOLOGICAL EVALUATION OF FROZEN FELINE PLACENTAL TISSUES AND IMMUNOHISTOCHEMICAL LOCALIZATION AND COLLECTION OF PLACENTAL TROPHOBLASTS

Introduction

Maternal-fetal transmission of the human immunodeficiency virus (HIV) frequently results in infected babies or miscarriage (D'Ubaldo et al., 1998; Kumar et al., 1995; Langston et al., 1995). HIV-infections may dysregulate placental cytokines and induce inflammation and transplacental infections (Salazar-Gonzalez, 1997; Sinicco, 1993). The feline immunodeficiency virus (FIV) is a lentivirus in cats that resembles HIV in biology and clinical disease. FIV infection of queens results in frequent reproductive failure (Allison and Hoover, 2003; Boudreaux et al., 2009; Weaver et al., 2005). Th1 cytokine expression is increased in placental tissues from resorbed pregnancies (Weaver et al., 2005), yet the specific placental cells contributing to immunopathology have not been identified. We are interested in evaluating the expression of representative placental cytokines in cells known to produce numerous immunomodulators during gestation, the trophoblasts. We hypothesized that aberrant immunomodulator expression in trophoblasts contributes to placental immunopathology, possibly facilitating vertical transfer of FIV and/or reproductive failure. The purpose of

this project was to histologically evaluate the integrity of frozen feline placental tissues and selectively identify and collect placental trophoblasts from early- and late-term FIV-infected and non-infected queens.

Materials and Methods

Animals and virus

Cats were reproductively mature, specific-pathogen-free (SPF) animals (*Felis domesticus*) obtained from a commercial cattery. Experimental cats were inoculated intravenously with a feline plasma pool containing FIV-B-2542. Infection was confirmed within 6 weeks by detection of FIV provirus by PCR and for seroconversion using the PetChek® FIV Antibody Test Kit (IDEXX Laboratories, Inc., Westbrook, ME). The cats were allowed to naturally breed with SPF toms. Breeding was observed and pregnancy was confirmed by palpation and ultrasonography.

Placental tissue harvest

Kittens were delivered by cesarean section during week 3-4 of gestation (early-term) and week 8 gestation (late-term) from FIV-infected and control queens. Placentas were collected under sterile conditions using the following dissection procedure. The uteri were removed and individual fetal membranes were collected. Following rinses with sterile PBS, membranes were incised with a sterile scalpel, and placentas were collected. Placental tissues were snap frozen in liquid nitrogen and cryopreserved at

-80°C. Eight placental tissues each from both early- and late-term queens were used in this study.

Histological evaluation of placental tissues

OCT-embedded placental sections from all tissues were fixed in acetone for 10 min and allowed to dehydrate for 1 min. Following dehydration, routine hematoxylin and eosin (H&E) staining was performed to observe histology. Sections were mounted with Permount (Fisher Scientific, Waltham, ME) and viewed microscopically using the Veritas™ Microdissection Instrument (Arcturus Bioscience, Inc., MountainView, CA).

Immunohistochemistry (IHC) to detect trophoblasts

Snap frozen placental tissues were embedded in OCT, sectioned to a thickness of 10 μm using a cryostat, and placed on RNase-free glass membrane slides (Molecular Devices, Sunnyvale, CA). Sections were fixed with acetone at room temperature (20–25ºC). Endogenous peroxidase activity in dehydrated sections was quenched with 400 ml methanol / 2 ml 30% H_2O_2 solution. Quenched sections were washed in 0.05 M Tris-HCl buffer (TB) (pH 7.0–7.6). To prevent non-specific immunological binding, sections were blocked with 5% (w/v) non-fat dry milk in TB (Blotto) and washed in 0.05 M Tris-HCl buffer (pH 7.0–7.6). Trophoblasts were stained with a polyclonal rabbit anti-cow cytokeratin wide spectrum screening (WSS) antibody (Dako Corp., Carpinteria, CA). A universal negative rabbit antibody (Dako Corp.) was used as the negative control reagent. The primary antibody and negative control dilutions were prepared in 0.05 M Tris-HCl

buffer, pH 7.0–7.6, containing 1% bovine serum albumin. After an overnight incubation at 4°C, sections were washed with 0.05 M Tris-HCl buffer. Sections were incubated with a ready-to-use goat anti-rabbit IgG, poly-HRP immunohistochemistry amplification reagent (Chemicon International, Inc., Temecula, CA) for 1 hr at room temperature (20–25ºC). After washing in 0.05 M Tris-HCl buffer (pH 7.0–7.6), sections were incubated with the buffered substrate-DAB chromogen solution (Invitrogen Corp., Carlsbad, CA) for 15 min, rinsed in deionized, diethylpyrocarbonate (DEPC)-treated H_2O, and counterstained with Mayer's hematoxylin. In order to successfully capture trophoblasts, sections were dehydrated in 75% , 95%, and 100% EtOH. Sections were then placed in xylene until laser capture microdissection was performed.

Laser capture microdissection (LCM) to capture trophoblasts

Following immunohistochemical detection in placental specimens, trophoblasts were microdissected using the Veritas™ Microdissection Instrument (Arcturus). Microdissection was performed using the Capture IR laser (Power: 70 mW; Pulse: 2500 μsec; and Intensity: 200 mV) and UV Cutting laser (Laser spot size: ~8.0 μm and Laser power: 7.0 mW). Similar sized cellular fields of ~2.00 mm^2 were collected onto CapSure Macro LCM Caps (Arcturus). All sections and capture areas were photographed using the dissection instrument.

Results

All H&E, IHC, and LCM results are shown in Figures 5.1–5.32. Routine hematoxylin and eosin staining of the placental tissue produced the typical result, cells with blue nuclei and pink cytoplasm. In addition, H&E staining of the placental tissue provided confirmation that tissue integrity was not compromised during frozen storage. IHC using the universal negative rabbit antibody and goat anti-rabbit IgG, poly-HRP IHC amplification reagent did not result in intracellular staining. However, trophoblasts were clearly identified after IHC staining with the polyclonal rabbit anti-cow cytokeratin wide spectrum screening (WSS) antibody and goat anti-rabbit IgG, poly-HRP IHC amplification reagent. Stained trophoblasts were demarcated and removed using laser capture microdissection.

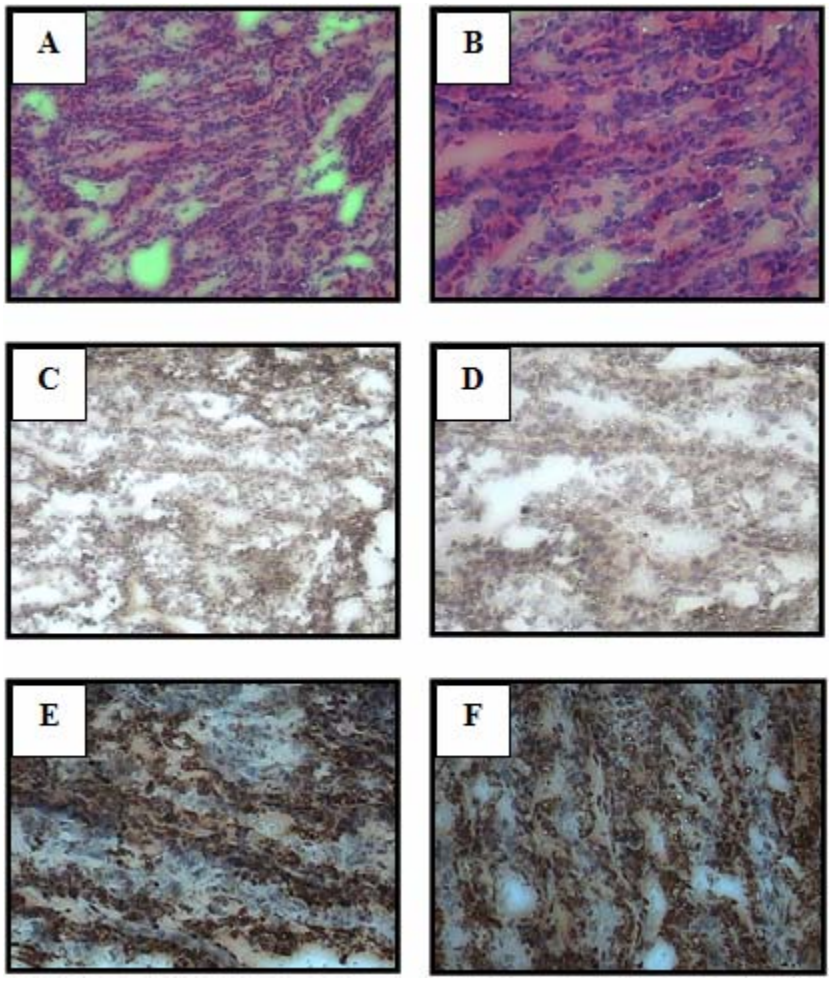

Figure 5.1 Histological Evaluation and Immunohistochemical Localization of Placental Trophoblasts from Early-Term FIV-negative Queen 2779 Placenta B

Placental tissues were cryosectioned to a thickness of 10 μm, fixed in acetone, and dehydrated. Sections were stained with hematoxylin and eosin and photographed using 10X and 20X Veritas instrument objectives (A and B, respectively). Sections were stained with a universal negative rabbit antibody and goat anti-rabbit IgG, poly-HRP immunohistochemistry amplification reagent and photographed using 10X and 20X Veritas instrument objectives (C and D, respectively). Sections were stained with the polyclonal rabbit anti-cow cytokeratin WSS antibody and goat anti-rabbit IgG, poly-HRP and photographed using the 20X Veritas instrument objective (E and F). Cytokeratin-positive trophoblasts are evident by the brown staining (E and F).

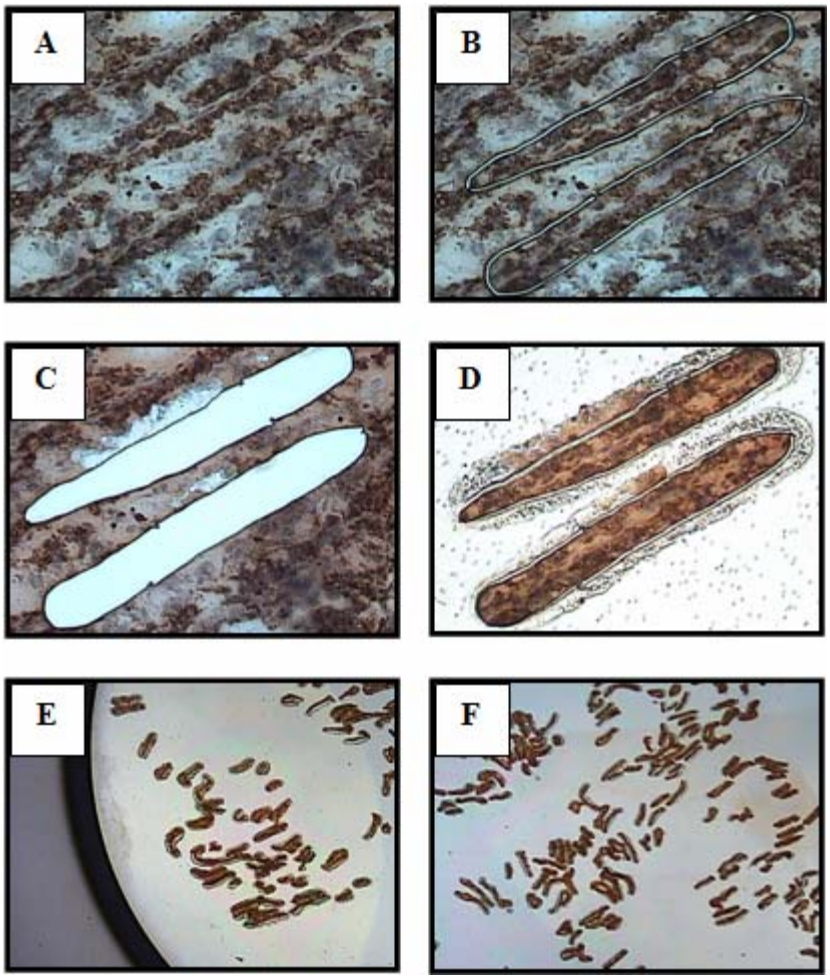

Figure 5.2 Laser Capture Microdissection of IHC Stained Placental Trophoblasts from Early-Term FIV-negative Queen 2779 Placenta B

Cytokeratin-positive placental trophoblasts are evident by the brown staining. (A) Trophoblasts were selectively identified. (B) Areas of trophoblast-rich populations were cut and captured using the UV cutting laser and Capture IR laser, respectively. (C) Selected trophoblasts were removed from the tissue area. (D) Trophoblastic cells were attached to the cap. (E and F) Cap images of acquired trophoblasts using the 2X Veritas instrument objective. (A–D) Photography was performed with the 20X Veritas instrument objective.

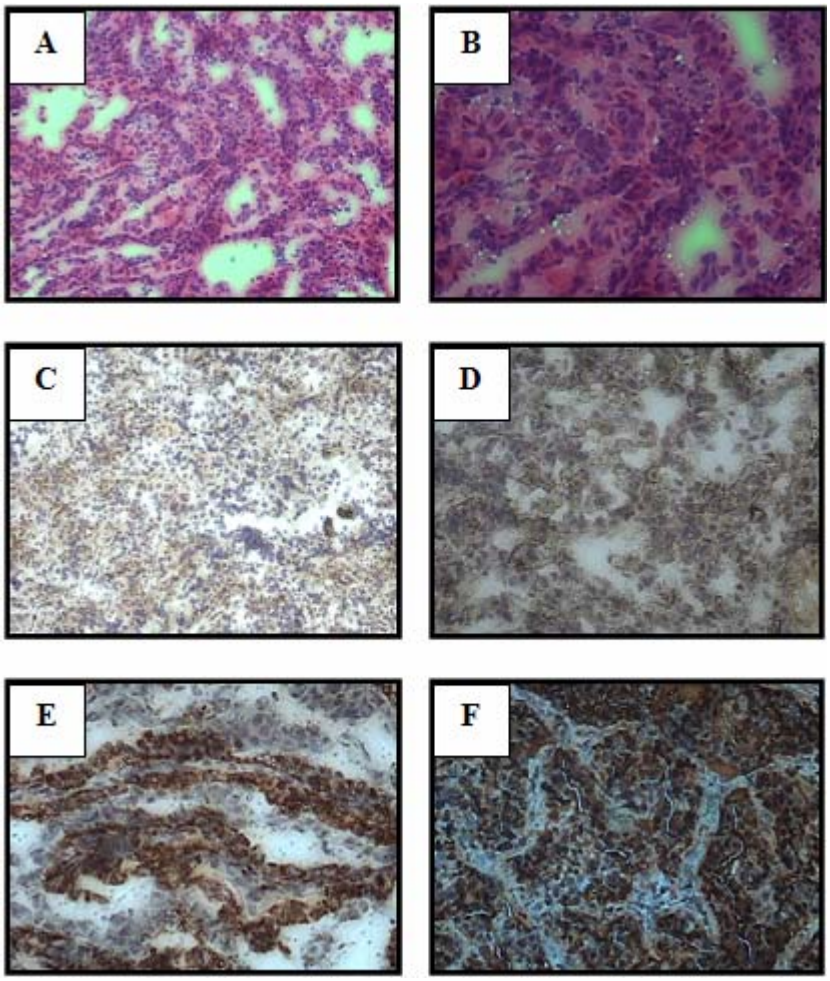

Figure 5.3 Histological Evaluation and Immunohistochemical Localization of Placental Trophoblasts from Early-Term FIV-negative Queen 2779 Placenta D

Placental tissues were cryosectioned to a thickness of 10 μm, fixed in acetone, and dehydrated. Sections were stained with hematoxylin and eosin and photographed using 10X and 20X Veritas instrument objectives (A and B, respectively). Sections were stained with a universal negative rabbit antibody and goat anti-rabbit IgG, poly-HRP immunohistochemistry amplification reagent and photographed using 10X and 20X Veritas instrument objectives (C and D, respectively). Sections were stained with the polyclonal rabbit anti-cow cytokeratin WSS antibody and goat anti-rabbit IgG, poly-HRP and photographed using the 20X Veritas instrument objective (E and F). Cytokeratin-positive trophoblastic cells are evident by the brown staining (E and F).

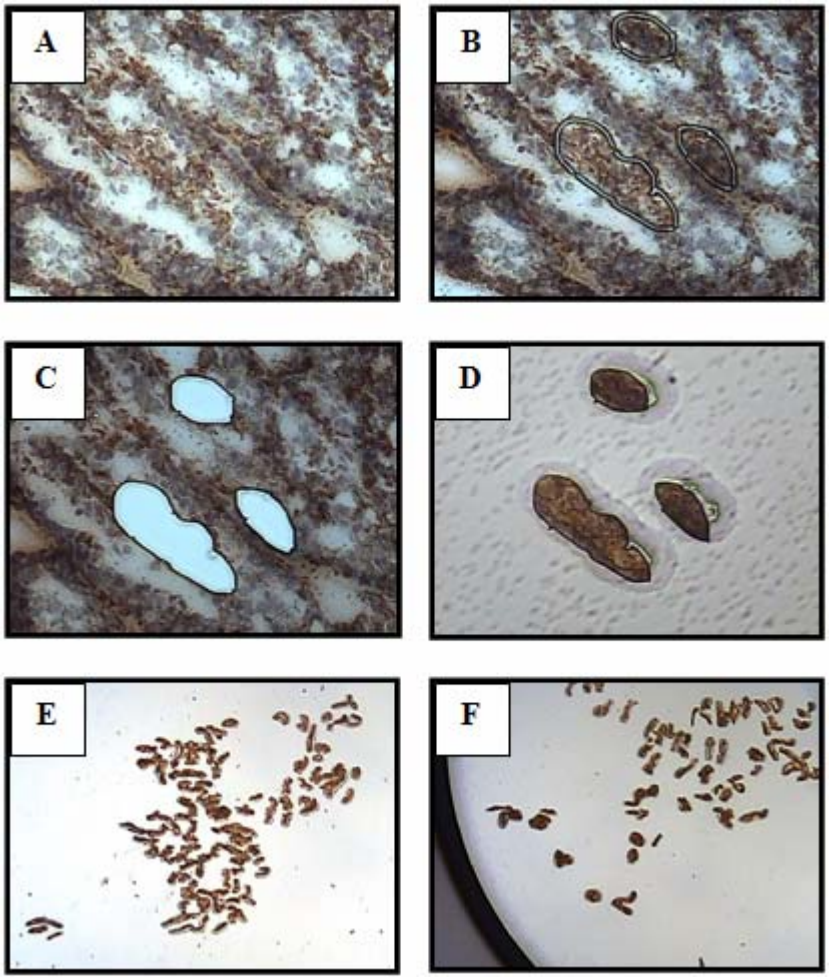

Figure 5.4 Laser Capture Microdissection of IHC Stained Placental Trophoblasts from Early-Term FIV-negative Queen 2779 Placenta D

Cytokeratin-positive placental trophoblasts are evident by the brown staining. (A) Trophoblasts were selectively identified. (B) Areas of trophoblast-rich populations were cut and captured using the UV cutting laser and Capture IR laser, respectively. (C) Selected trophoblasts were removed from the tissue area. (D) Trophoblastic cells were attached to the cap. (E and F) Cap images of acquired trophoblasts using the 2X Veritas instrument objective. (A–D) Photography was performed with the 20X Veritas instrument objective.

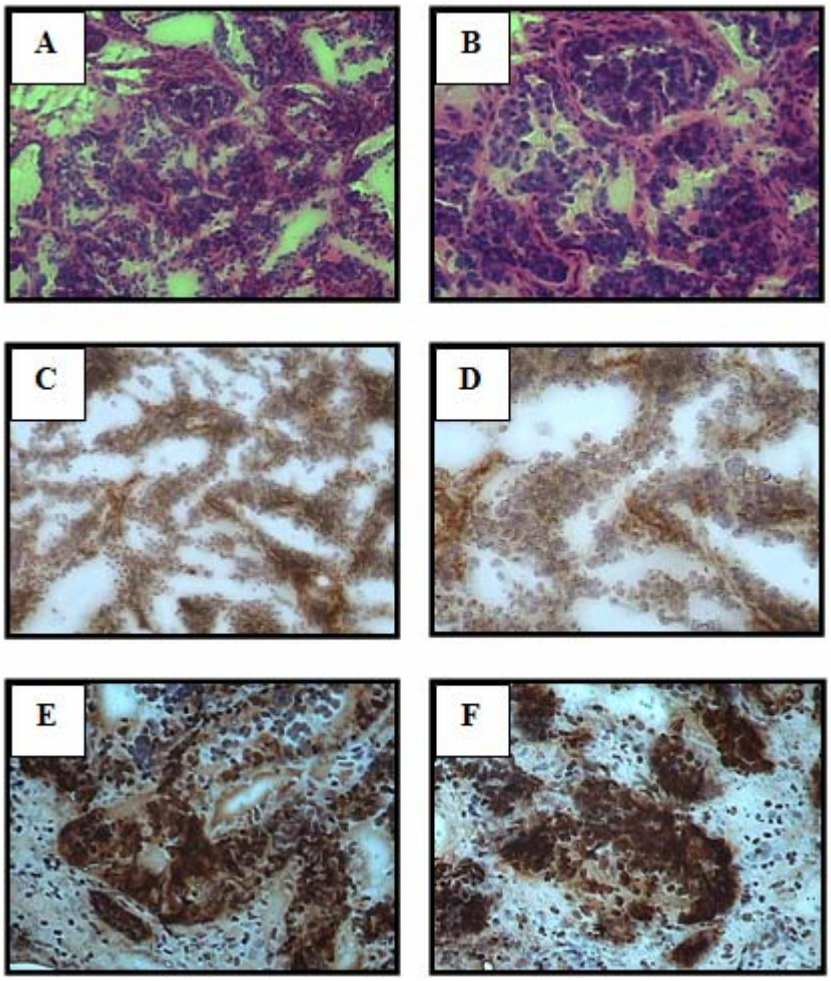

Figure 5.5 Histological Evaluation and Immunohistochemical Localization of Placental Trophoblasts from Early-Term FIV-negative Queen 2779 Resorption E

Placental tissues were cryosectioned to a thickness of 10 μm, fixed in acetone, and dehydrated. Sections were stained with hematoxylin and eosin and photographed using 10X and 20X Veritas instrument objectives (A and B, respectively). Sections were stained with a universal negative rabbit antibody and goat anti-rabbit IgG, poly-HRP immunohistochemistry amplification reagent and photographed using 10X and 20X Veritas instrument objectives (C and D, respectively). Sections were stained with the polyclonal rabbit anti-cow cytokeratin WSS antibody and goat anti-rabbit IgG, poly-HRP and photographed using the 20X Veritas instrument objective (E and F). Cytokeratin-positive trophoblastic cells are evident by the brown staining (E and F).

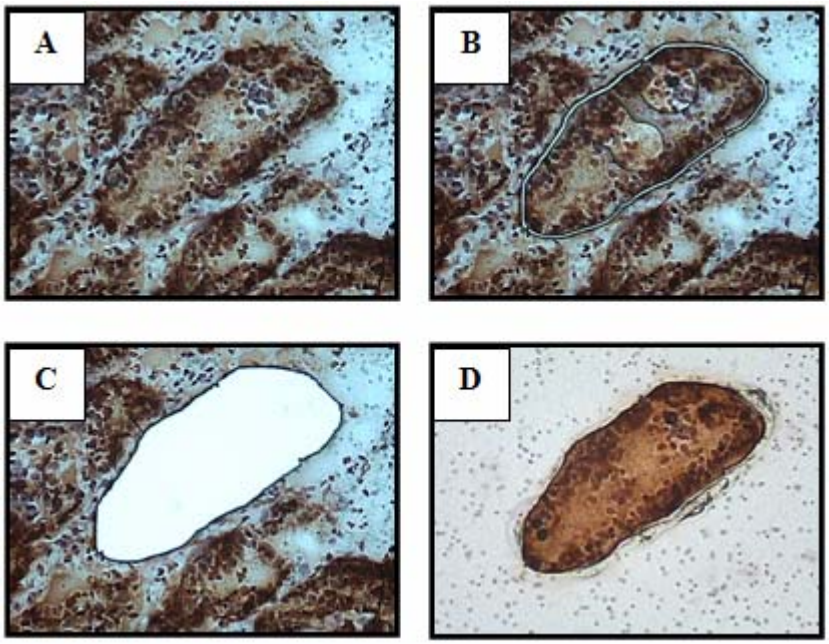

Figure 5.6 Laser Capture Microdissection of IHC Stained Placental Trophoblasts from Early-Term FIV-negative Queen 2779 Resorption E

Cytokeratin-positive placental trophoblasts are evident by the brown staining. (A) Trophoblasts were selectively identified. (B) Areas of trophoblast-rich populations were cut and captured using the UV cutting laser and Capture IR laser, respectively. (C) Selected trophoblasts were removed from the tissue area. (D) Trophoblastic cells were attached to the cap. All photography was performed with the 20X Veritas instrument objective.

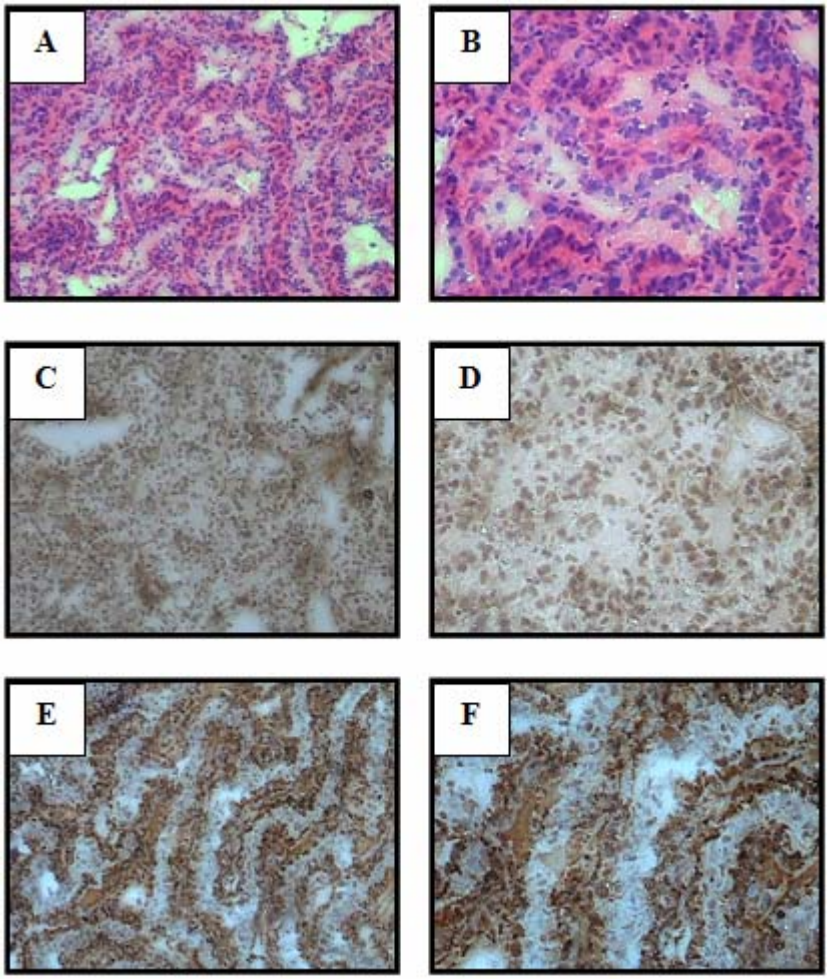

Figure 5.7 Histological Evaluation and Immunohistochemical Localization of Placental Trophoblasts from Early-Term FIV-positive Queen 0326 Placenta A

Placental tissues were cryosectioned to a thickness of 10 μm, fixed in acetone, and dehydrated. Sections were stained with hematoxylin and eosin and photographed using 10X and 20X Veritas instrument objectives (A and B, respectively). Sections were stained with a universal negative rabbit antibody and goat anti-rabbit IgG, poly-HRP immunohistochemistry amplification reagent and photographed using 10X and 20X Veritas instrument objectives (C and D, respectively). Sections were stained with the polyclonal rabbit anti-cow cytokeratin WSS antibody and goat anti-rabbit IgG, poly-HRP and photographed using the 10X and 20X Veritas instrument objectives (E and F, respectively). Cytokeratin-positive trophoblastic cells are evident by the brown staining (E and F).

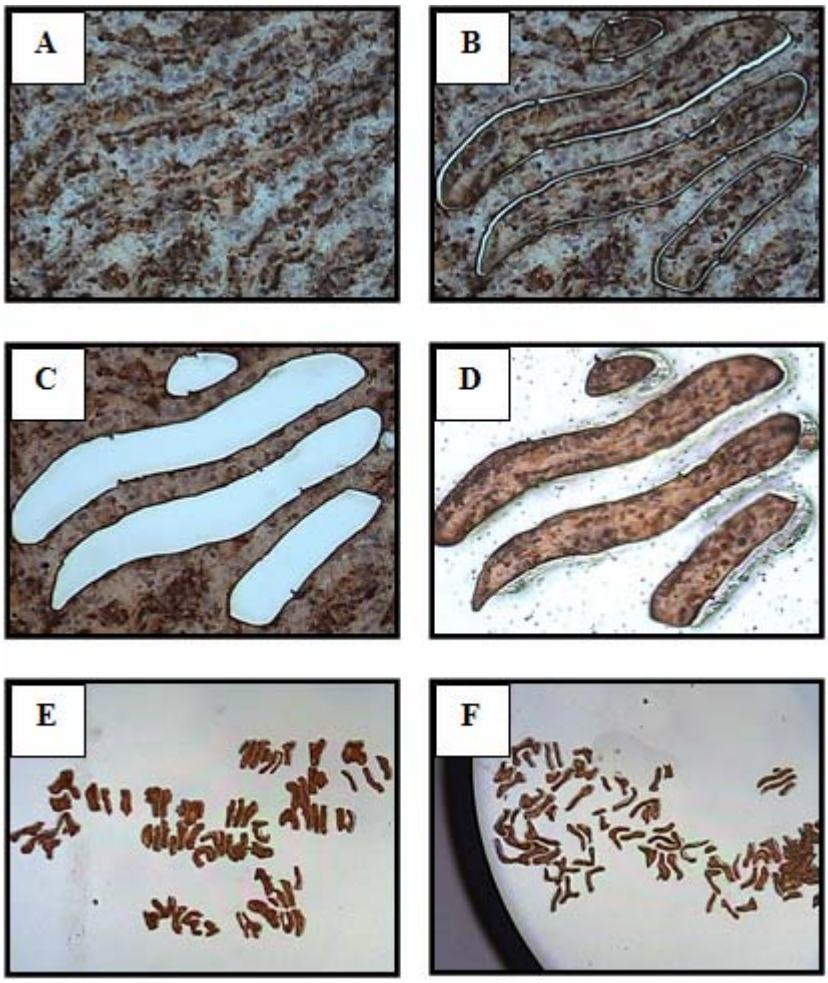

Figure 5.8 Laser Capture Microdissection of IHC Stained Placental Trophoblasts from Early-Term FIV-positive Queen 0326 Placenta A

Cytokeratin-positive placental trophoblasts are evident by the brown staining. (A) Trophoblasts were selectively identified. (B) Areas of trophoblast-rich populations were cut and captured using the UV cutting laser and Capture IR laser, respectively. (C) Selected trophoblasts were removed from the tissue area. (D) Trophoblastic cells were attached to the cap. (E and F) Cap images of acquired trophoblasts using the 2X Veritas instrument objective. (A–D) Photography was performed with the 20X Veritas instrument objective.

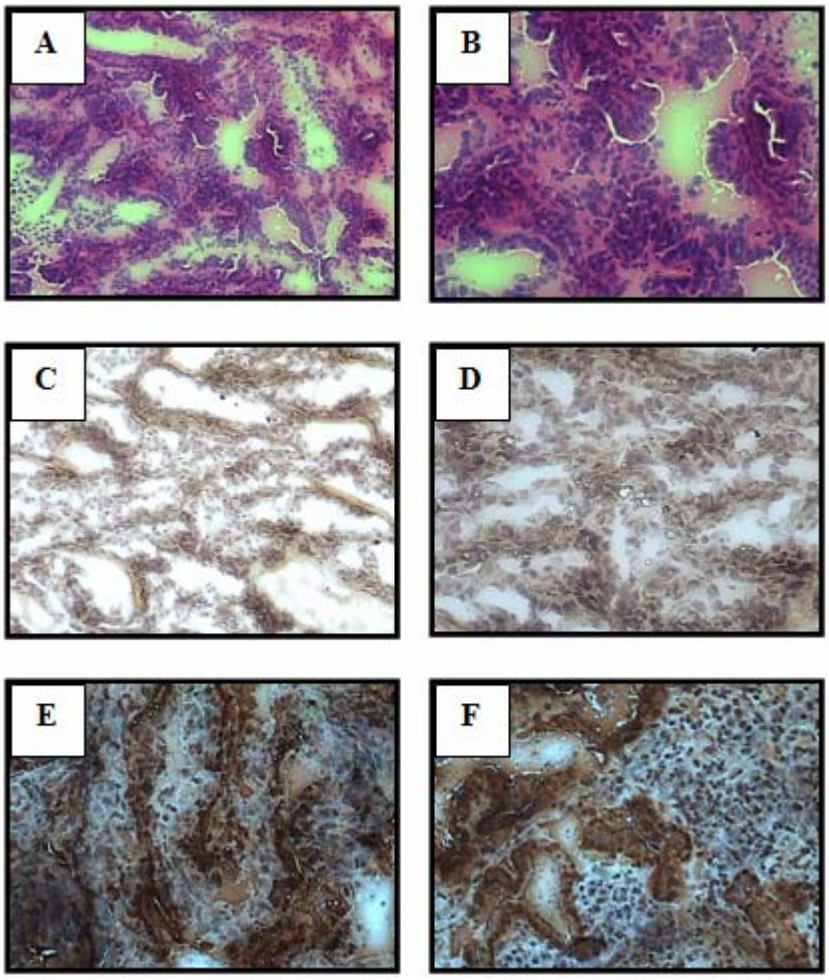

Figure 5.9 Histological Evaluation and Immunohistochemical Localization of Placental Trophoblasts from Early-Term FIV-positive Queen 8035 Arrested Placenta B

Placental tissues were cryosectioned to a thickness of 10 μm, fixed in acetone, and dehydrated. Sections were stained with hematoxylin and eosin and photographed using 10X and 20X Veritas instrument objectives (A and B, respectively). Sections were stained with a universal negative rabbit antibody and goat anti-rabbit IgG, poly-HRP immunohistochemistry amplification reagent and photographed using 10X and 20X Veritas instrument objectives (C and D, respectively). Sections were stained with the polyclonal rabbit anti-cow cytokeratin WSS antibody and goat anti-rabbit IgG, poly-HRP and photographed using the 20X Veritas instrument objective (E and F). Cytokeratin-positive trophoblastic cells are evident by the brown staining (E and F).

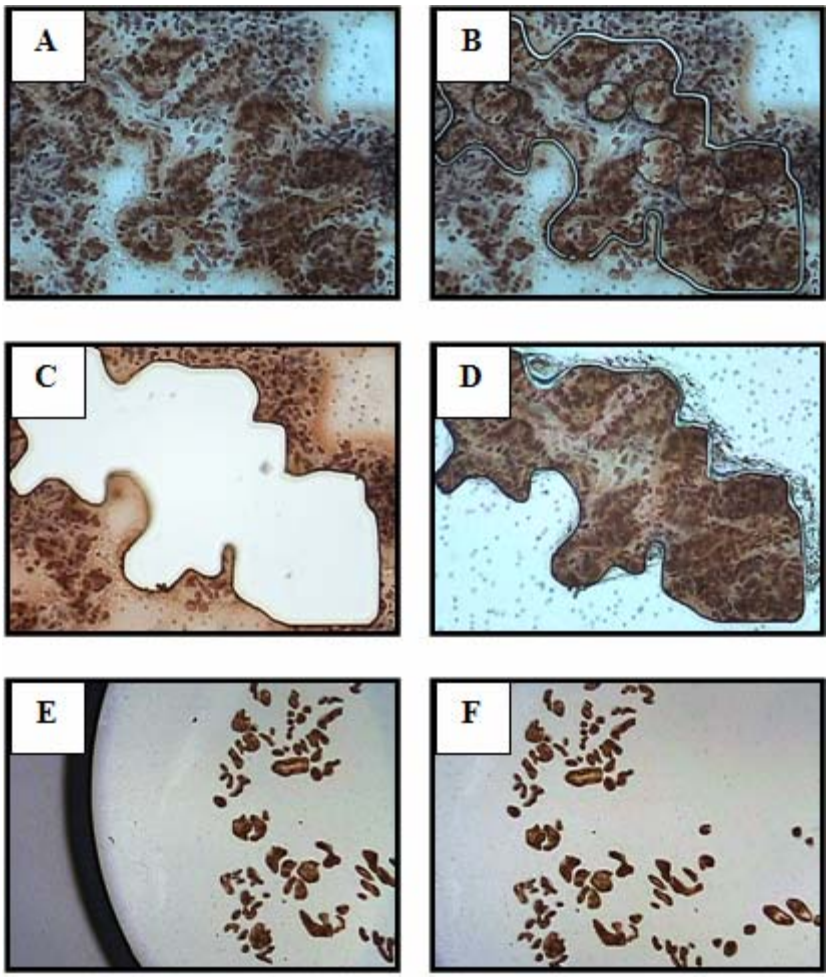

Figure 5.10 Laser Capture Microdissection of IHC Stained Placental Trophoblasts from Early-Term FIV-positive Queen 8035 Arrested Placenta B

Cytokeratin-positive placental trophoblasts are evident by the brown staining. (A) Trophoblasts were selectively identified. (B) Areas of trophoblast-rich populations were cut and captured using the UV cutting laser and Capture IR laser, respectively. (C) Selected trophoblasts were removed from the tissue area. (D) Trophoblastic cells were attached to the cap. (E and F) Cap images of acquired trophoblasts using the 2X Veritas instrument objective. (A–D) Photography was performed with the 20X Veritas instrument objective.

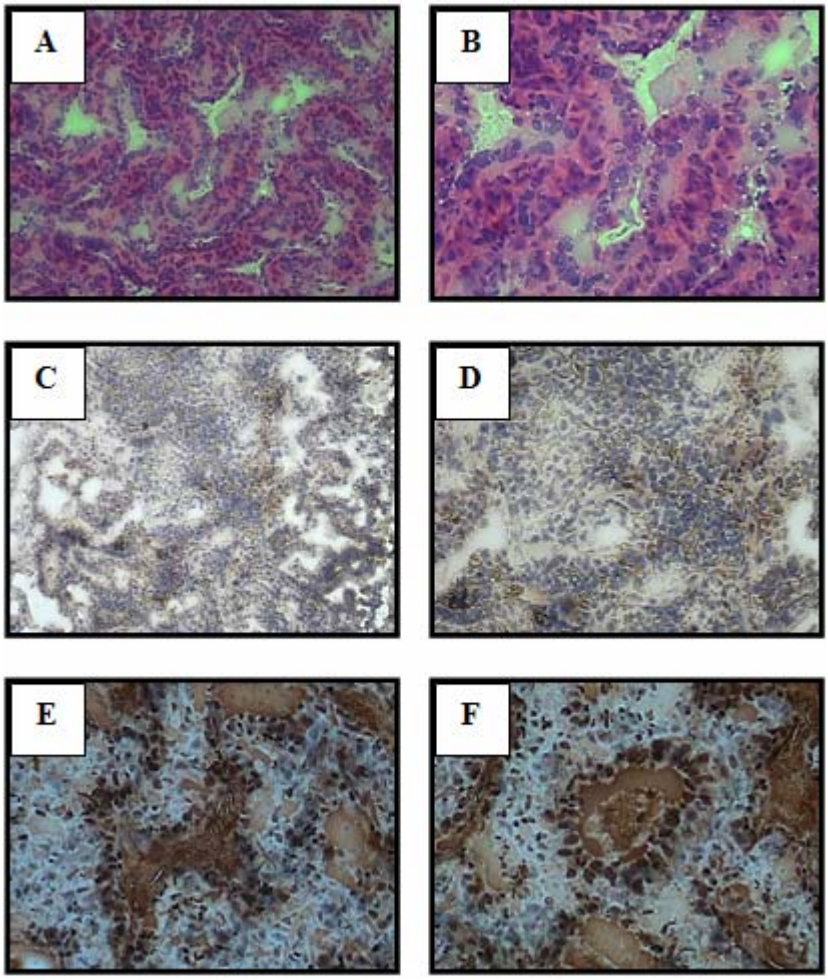

Figure 5.11 Histological Evaluation and Immunohistochemical Localization of Placental Trophoblasts from Early-Term FIV-positive Queen 8035 Arrested Placenta C

Placental tissues were cryosectioned to a thickness of 10 μm, fixed in acetone, and dehydrated. Sections were stained with hematoxylin and eosin and photographed using 10X and 20X Veritas instrument objectives (A and B, respectively). Sections were stained with a universal negative rabbit antibody and goat anti-rabbit IgG, poly-HRP immunohistochemistry amplification reagent and photographed using 10X and 20X Veritas instrument objectives (C and D, respectively). Sections were stained with the polyclonal rabbit anti-cow cytokeratin WSS antibody and goat anti-rabbit IgG, poly-HRP and photographed using the 20X Veritas instrument objective (E and F). Cytokeratin-positive trophoblastic cells are evident by the brown staining (E and F).

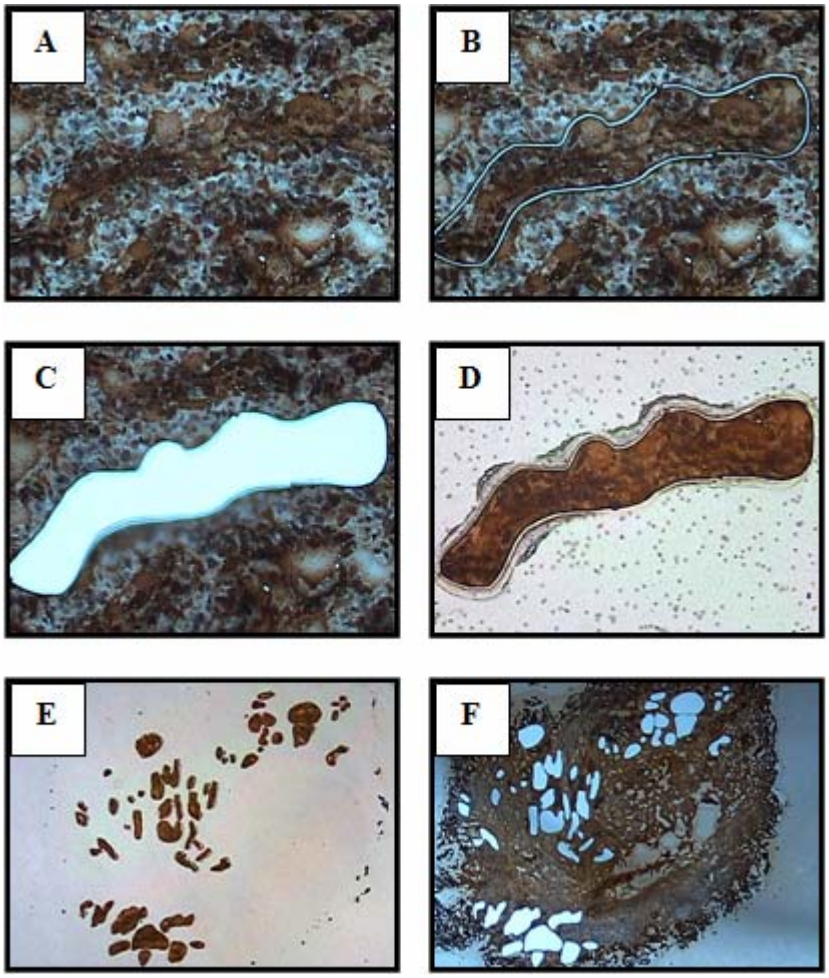

Figure 5.12 Laser Capture Microdissection of IHC Stained Placental Trophoblasts from Early-Term FIV-positive Queen 8035 Arrested Placenta C

Cytokeratin-positive placental trophoblasts are evident by the brown staining. (A) Trophoblasts were selectively identified. (B) Areas of trophoblast-rich populations were cut and captured using the UV cutting laser and Capture IR laser, respectively. (C) Selected trophoblasts were removed from the tissue area. (D) Trophoblastic cells were attached to the cap. (E) Cap image of acquired trophoblasts. (F) Tissue area remaining after cellular extraction. (A–D) Photography was performed with the 20X Veritas instrument objective. (E and F) Photography was performed with the 2X Veritas instrument objective.

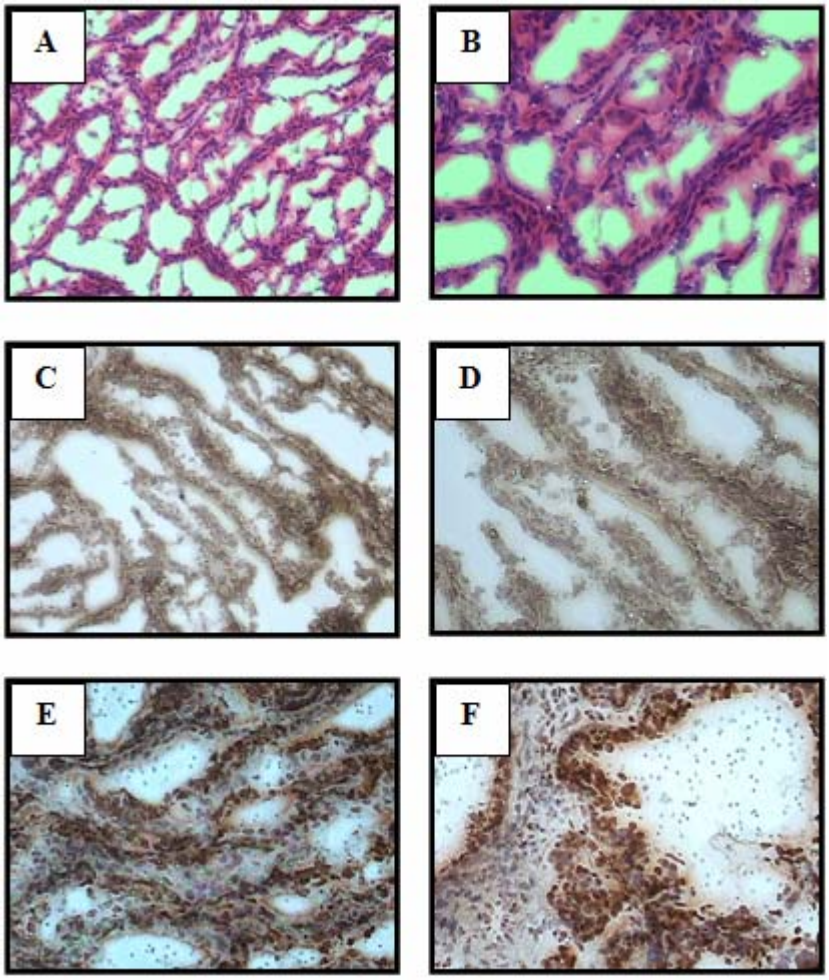

Figure 5.13 Histological Evaluation and Immunohistochemical Localization of Placental Trophoblasts from Early-Term FIV-positive Queen 0866 Placenta A

Placental tissues were cryosectioned to a thickness of 10 μm, fixed in acetone, and dehydrated. Sections were stained with hematoxylin and eosin and photographed using 10X and 20X Veritas instrument objectives (A and B, respectively). Sections were stained with a universal negative rabbit antibody and goat anti-rabbit IgG, poly-HRP immunohistochemistry amplification reagent and photographed using 10X and 20X Veritas instrument objectives (C and D, respectively). Sections were stained with the polyclonal rabbit anti-cow cytokeratin WSS antibody and goat anti-rabbit IgG, poly-HRP and photographed using the 20X Veritas instrument objective (E and F). Cytokeratin-positive trophoblastic cells are evident by the brown staining (E and F).

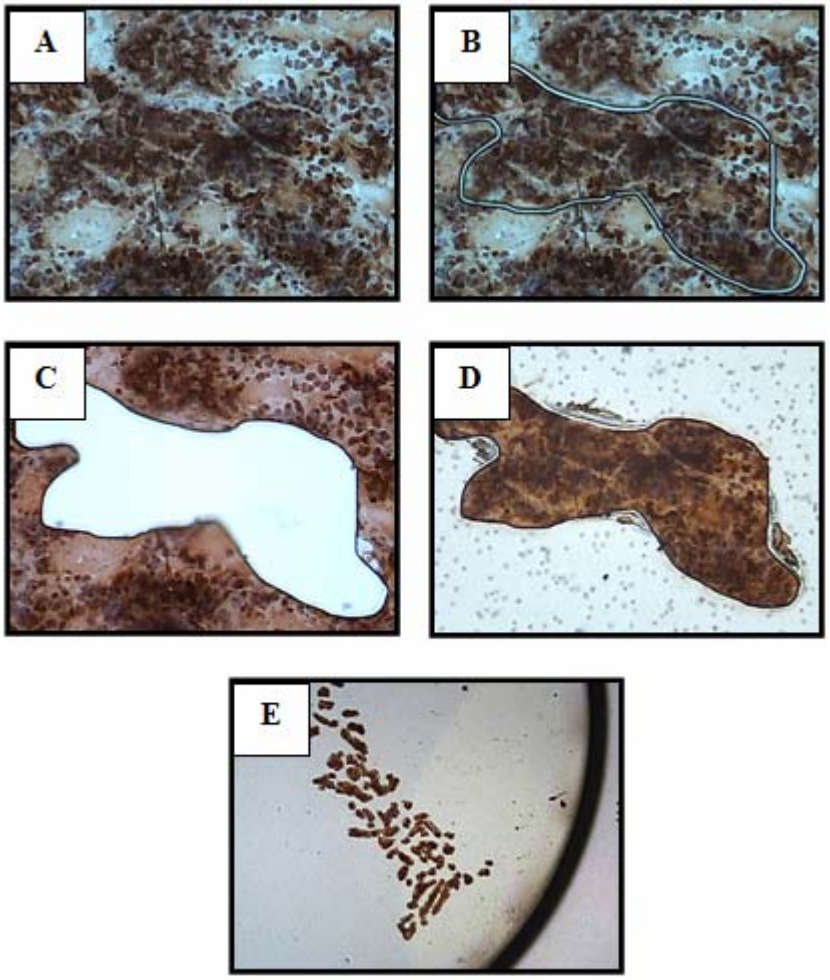

Figure 5.14 Laser Capture Microdissection of IHC Stained Placental Trophoblasts from Early-Term FIV-positive Queen 0866 Placenta A

Cytokeratin-positive placental trophoblasts are evident by the brown staining. (A) Trophoblasts were selectively identified. (B) Areas of trophoblast-rich populations were cut and captured using the UV cutting laser and Capture IR laser, respectively. (C) Selected trophoblasts were removed from the tissue area. (D) Trophoblastic cells were attached to the cap. (E) Cap image of acquired trophoblasts using the 2X Veritas instrument objective. (A–D) Photography was performed with the 20X Veritas instrument objective.

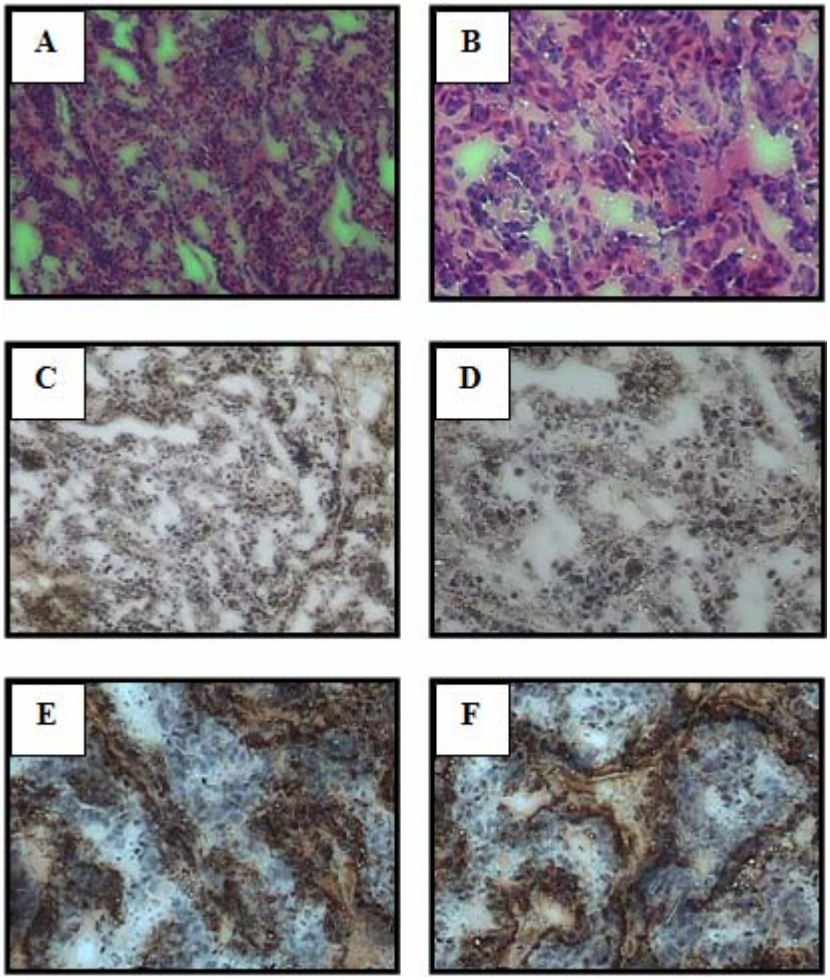

Figure 5.15 Histological Evaluation and Immunohistochemical Localization of Placental Trophoblasts from Early-Term FIV-positive Queen 0866 Placenta B Resorption

Placental tissues were cryosectioned to a thickness of 10 μm, fixed in acetone, and dehydrated. Sections were stained with hematoxylin and eosin and photographed using 10X and 20X Veritas instrument objectives (A and B, respectively). Sections were stained with a universal negative rabbit antibody and goat anti-rabbit IgG, poly-HRP immunohistochemistry amplification reagent and photographed using 10X and 20X Veritas instrument objectives (C and D, respectively). Sections were stained with the polyclonal rabbit anti-cow cytokeratin WSS antibody and goat anti-rabbit IgG, poly-HRP and photographed using the 20X Veritas instrument objective (E and F). Cytokeratin-positive trophoblastic cells are evident by the brown staining (E and F).

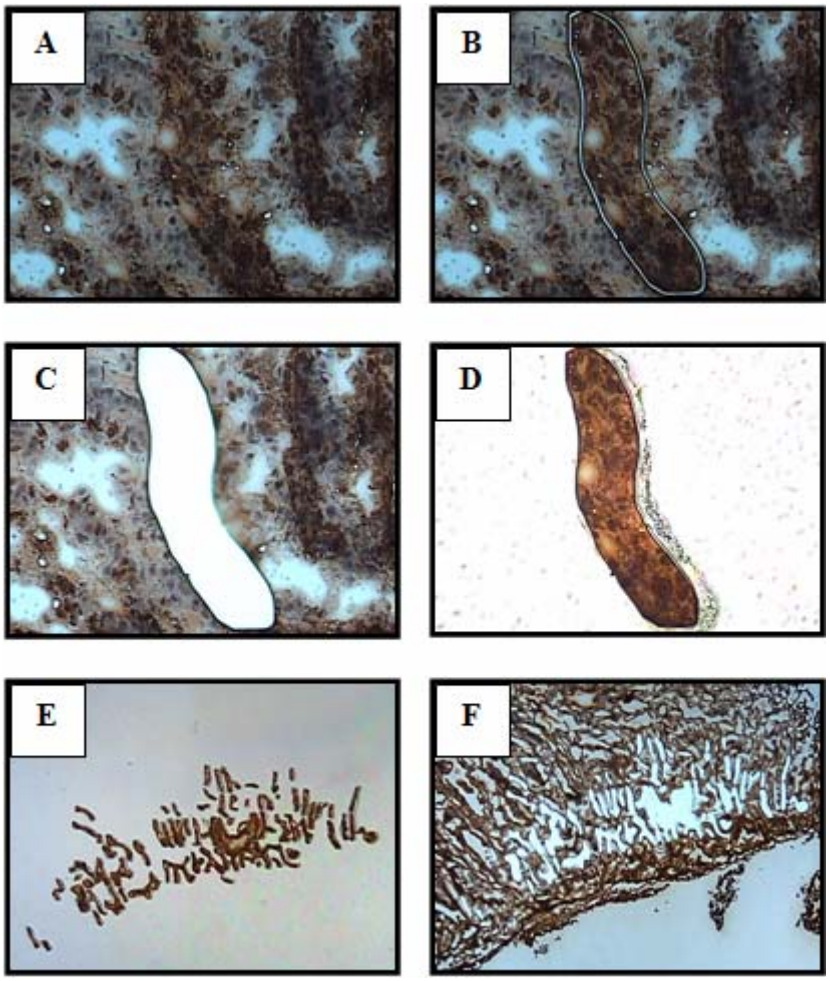

Figure 5.16 Laser Capture Microdissection of IHC Stained Placental Trophoblasts from Early-Term FIV-positive Queen 0866 Placenta B Resorption

Cytokeratin-positive placental trophoblasts are evident by the brown staining. (A) Trophoblasts were selectively identified. (B) Areas of trophoblast-rich populations were cut and captured using the UV cutting laser and Capture IR laser, respectively. (C) Selected trophoblasts were removed from the tissue area. (D) Trophoblastic cells were attached to the cap. (E) Cap image of acquired trophoblasts. (F) Tissue area remaining after cellular extraction. (A–D) Photography was performed with the 20X Veritas instrument objective. (E and F) Photography was performed with the 2X Veritas instrument objective.

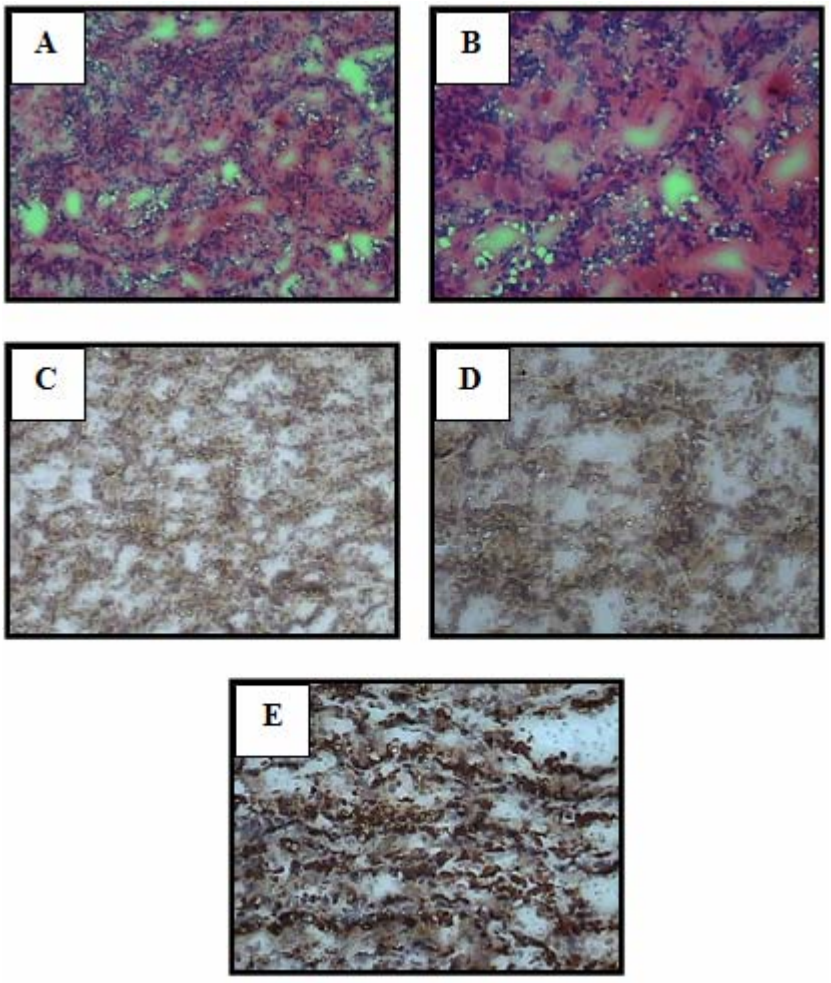

Figure 5.17 Histological Evaluation and Immunohistochemical Localization of Placental Trophoblasts from Late-Term FIV-negative Queen 9581 Placenta A

Placental tissues were cryosectioned to a thickness of 10 μm, fixed in acetone, and dehydrated. Sections were stained with hematoxylin and eosin and photographed using 10X and 20X Veritas instrument objectives (A and B, respectively). Sections were stained with a universal negative rabbit antibody and goat anti-rabbit IgG, poly-HRP immunohistochemistry amplification reagent and photographed using 10X and 20X Veritas instrument objectives (C and D, respectively). Sections were stained with the polyclonal rabbit anti-cow cytokeratin WSS antibody and goat anti-rabbit IgG, poly-HRP and photographed using the 20X Veritas instrument objective (E). Cytokeratin-positive trophoblastic cells are evident by the brown staining (E) .

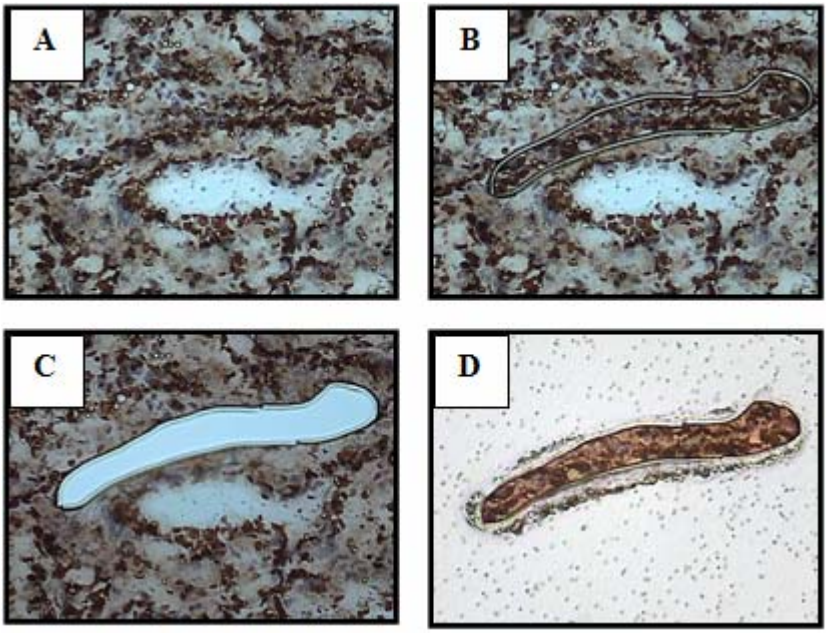

Figure 5.18 Laser Capture Microdissection of IHC Stained Placental Trophoblasts from Late-Term FIV-negative Queen 9581 Placenta A

Cytokeratin-positive placental trophoblasts are evident by the brown staining. (A) Trophoblasts were selectively identified. (B) Areas of trophoblast-rich populations were cut and captured using the UV cutting laser and Capture IR laser, respectively. (C) Selected trophoblasts were removed from the tissue area. (D) Trophoblastic cells were attached to the cap. All photography was performed with the 20X Veritas instrument objective.

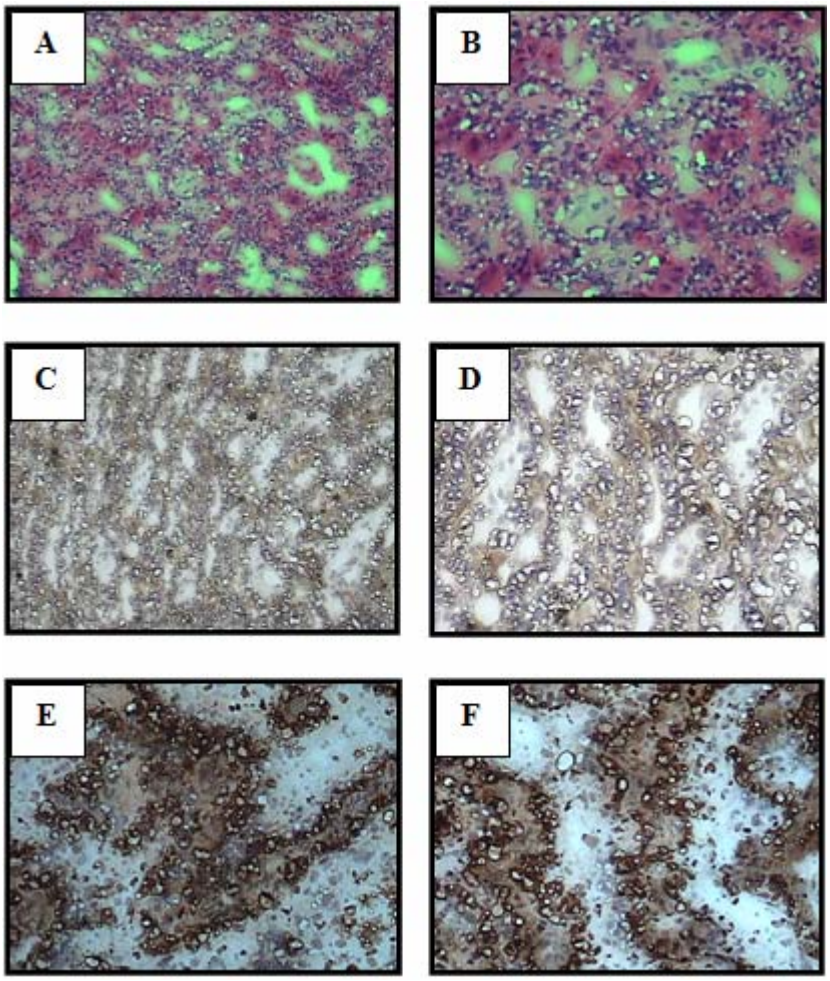

Figure 5.19 Histological Evaluation and Immunohistochemical Localization of Placental Trophoblasts from Late-Term FIV-negative Queen 9581 Placenta B

Placental tissues were cryosectioned to a thickness of 10 μm, fixed in acetone, and dehydrated. Sections were stained with hematoxylin and eosin and photographed using 10X and 20X Veritas instrument objectives (A and B, respectively). Sections were stained with a universal negative rabbit antibody and goat anti-rabbit IgG, poly-HRP immunohistochemistry amplification reagent and photographed using 10X and 20X Veritas instrument objectives (C and D, respectively). Sections were stained with the polyclonal rabbit anti-cow cytokeratin WSS antibody and goat anti-rabbit IgG, poly-HRP and photographed using the 20X Veritas instrument objective (E and F). Cytokeratin-positive trophoblastic cells are evident by the brown staining (E and F).

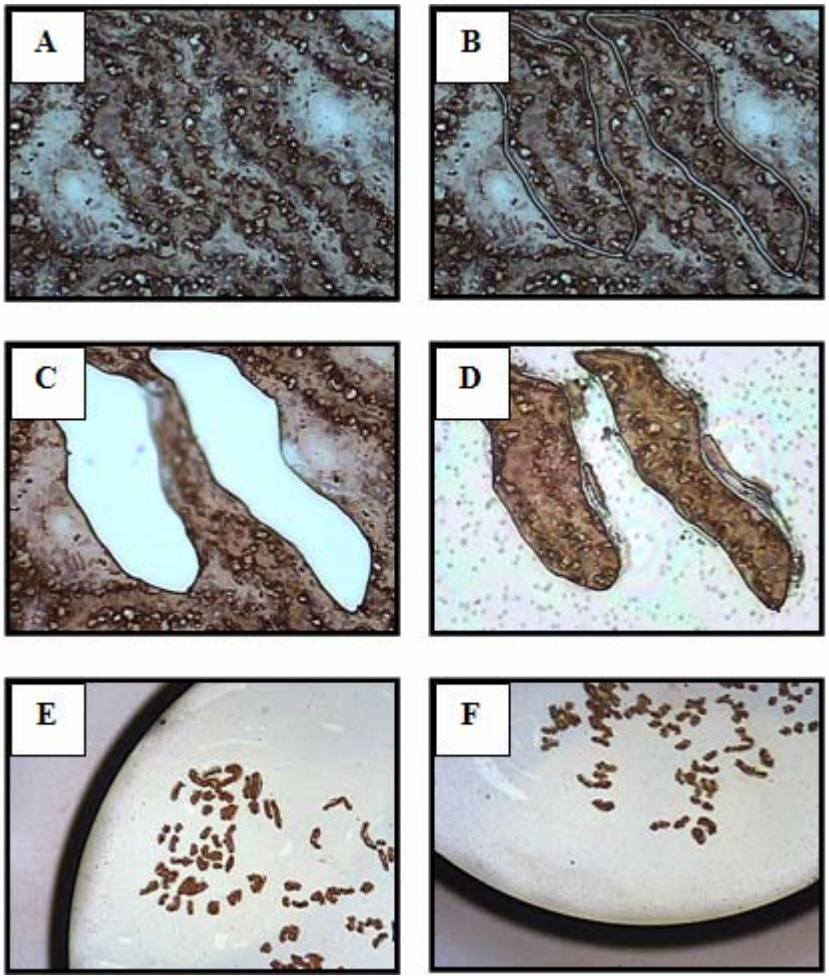

Figure 5.20 Laser Capture Microdissection of IHC Stained Placental Trophoblasts from Late-Term FIV-negative Queen 9581 Placenta B

Cytokeratin-positive placental trophoblasts are evident by the brown staining. (A) Trophoblasts were selectively identified. (B) Areas of trophoblast-rich populations were cut and captured using the UV cutting laser and Capture IR laser, respectively. (C) Selected trophoblasts were removed from the tissue area. (D) Trophoblastic cells were attached to the cap. (E and F) Cap images of acquired trophoblasts using the 2X Veritas instrument objective. (A–D) Photography was performed with the 20X Veritas instrument objective.

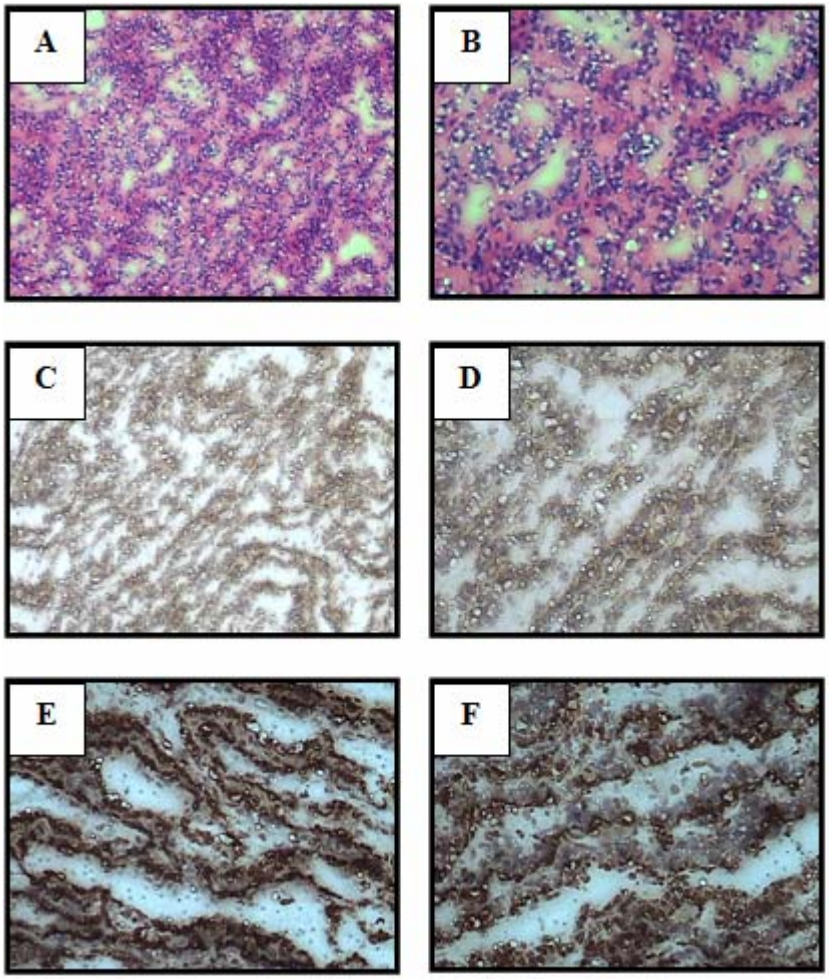

Figure 5.21 Histological Evaluation and Immunohistochemical Localization of Placental Trophoblasts from Late-Term FIV-negative Queen 9746 Placenta C

Placental tissues were cryosectioned to a thickness of 10 μm, fixed in acetone, and dehydrated. Sections were stained with hematoxylin and eosin and photographed using 10X and 20X Veritas instrument objectives (A and B, respectively). Sections were stained with a universal negative rabbit antibody and goat anti-rabbit IgG, poly-HRP immunohistochemistry amplification reagent and photographed using 10X and 20X Veritas instrument objectives (C and D, respectively). Sections were stained with the polyclonal rabbit anti-cow cytokeratin WSS antibody and goat anti-rabbit IgG, poly-HRP and photographed using the 20X Veritas instrument objective (E and F). Cytokeratin-positive trophoblastic cells are evident by the brown staining (E and F).

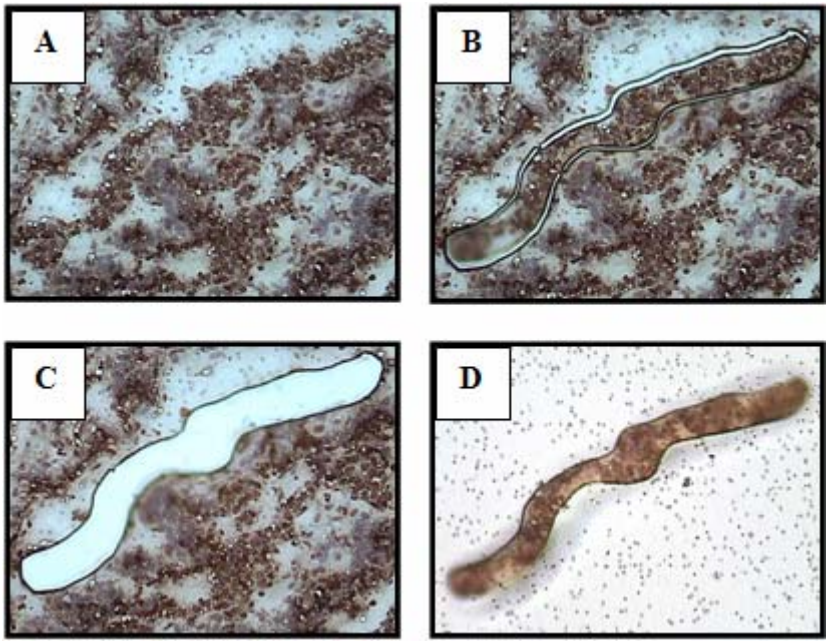

Figure 5.22 Laser Capture Microdissection of IHC Stained Placental Trophoblasts from Late-Term FIV-negative Queen 9746 Placenta C

Cytokeratin-positive placental trophoblasts are evident by the brown staining. (A) Trophoblasts were selectively identified. (B) Areas of trophoblast-rich populations were cut and captured using the UV cutting laser and Capture IR laser, respectively. (C) Selected trophoblasts were removed from the tissue area. (D) Trophoblastic cells were attached to the cap. All photography was performed with the 20X Veritas instrument objective.

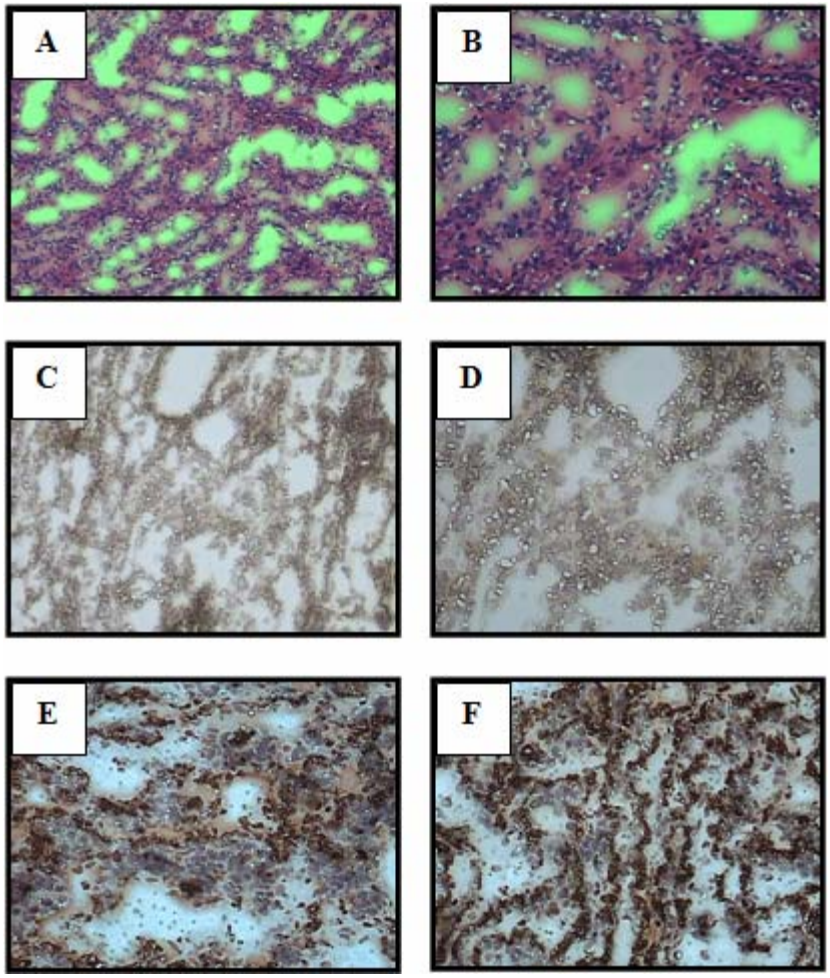

Figure 5.23 Histological Evaluation and Immunohistochemical Localization of Placental Trophoblasts from Late-Term FIV-positive Queen 9730 Placenta B

Placental tissues were cryosectioned to a thickness of 10 μm, fixed in acetone, and dehydrated. Sections were stained with hematoxylin and eosin and photographed using 10X and 20X Veritas instrument objectives (A and B, respectively). Sections were stained with a universal negative rabbit antibody and goat anti-rabbit IgG, poly-HRP immunohistochemistry amplification reagent and photographed using 10X and 20X Veritas instrument objectives (C and D, respectively). Sections were stained with the polyclonal rabbit anti-cow cytokeratin WSS antibody and goat anti-rabbit IgG, poly-HRP and photographed using the 20X Veritas instrument objective (E and F). Cytokeratin-positive trophoblastic cells are evident by the brown staining (E and F).

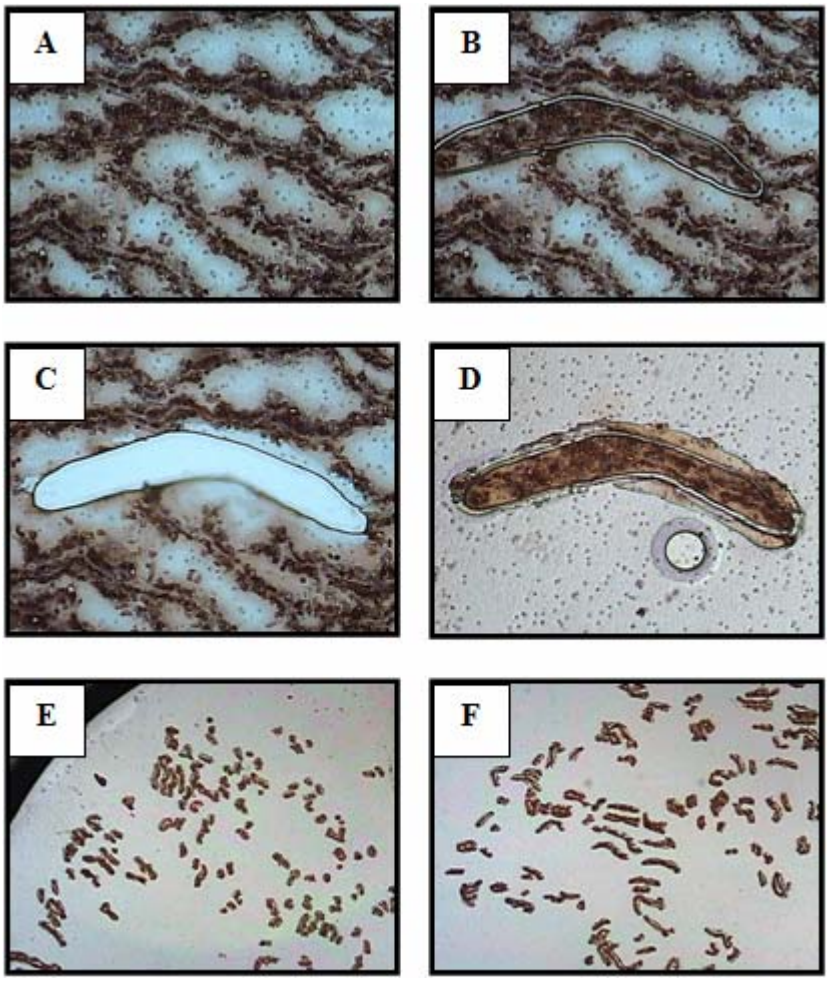

Figure 5.24 Laser Capture Microdissection of IHC Stained Placental Trophoblasts from Late-Term FIV-positive Queen 9730 Placenta B

Cytokeratin-positive placental trophoblasts are evident by the brown staining. (A) Trophoblasts were selectively identified. (B) Areas of trophoblast-rich populations were cut and captured using the UV cutting laser and Capture IR laser, respectively. (C) Selected trophoblasts were removed from the tissue area. (D) Trophoblastic cells were attached to the cap. (E and F) Cap images of acquired trophoblasts using the 2X Veritas instrument objective. (A–D) Photography was performed with the 20X Veritas instrument objective.

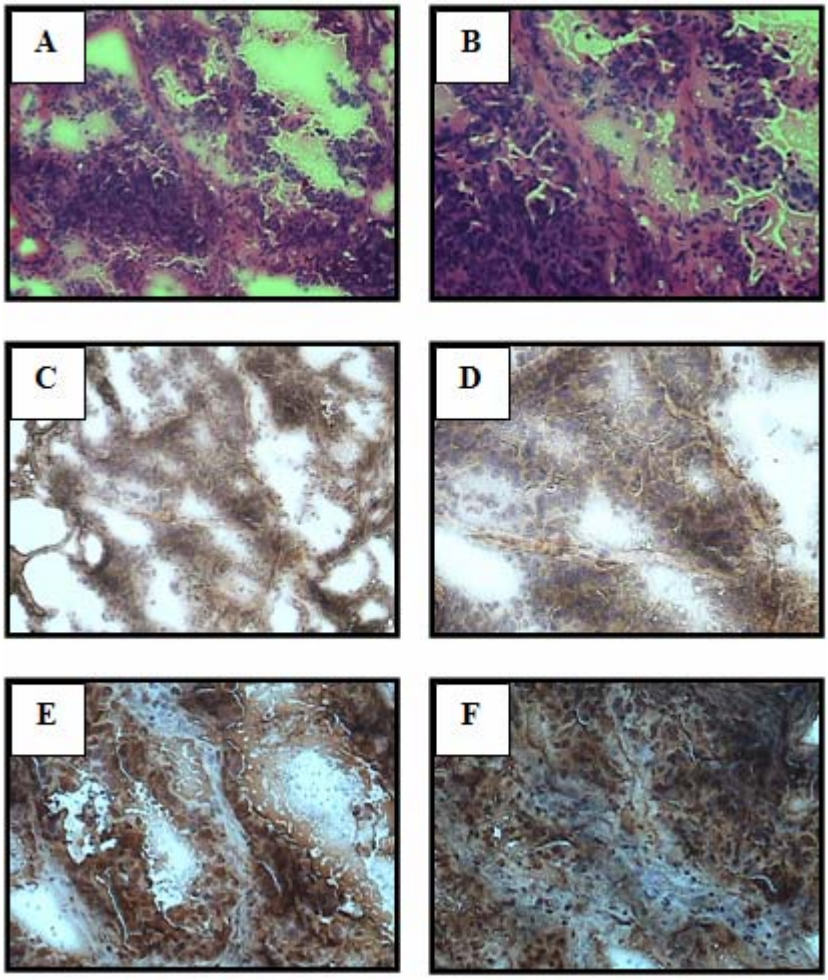

Figure 5.25 Histological Evaluation and Immunohistochemical Localization of Placental Trophoblasts from Late-Term FIV-positive Queen 9730 Placenta Resorption 1

Placental tissues were cryosectioned to a thickness of 10 μm, fixed in acetone, and dehydrated. Sections were stained with hematoxylin and eosin and photographed using 10X and 20X Veritas instrument objectives (A and B, respectively). Sections were stained with a universal negative rabbit antibody and goat anti-rabbit IgG, poly-HRP immunohistochemistry amplification reagent and photographed using 10X and 20X Veritas instrument objectives (C and D, respectively). Sections were stained with the polyclonal rabbit anti-cow cytokeratin WSS antibody and goat anti-rabbit IgG, poly-HRP and photographed using the 20X Veritas instrument objective (E and F). Cytokeratin-positive trophoblastic cells are evident by the brown staining (E and F).

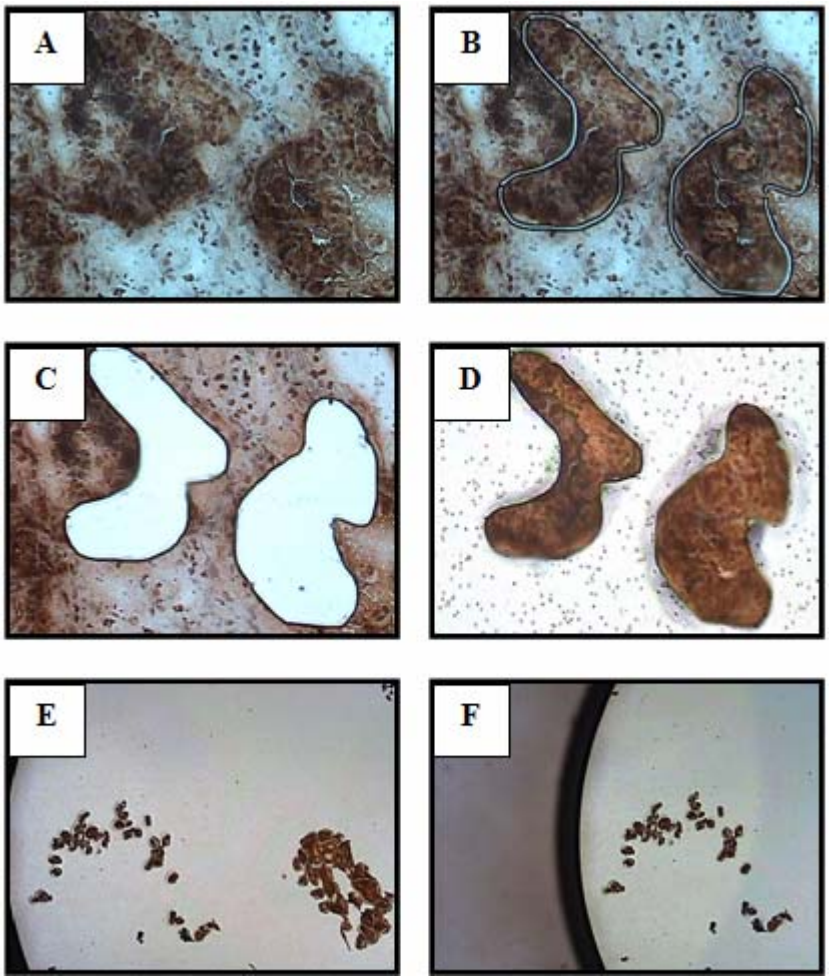

Figure 5.26 Laser Capture Microdissection of IHC Stained Placental Trophoblasts from Late-Term FIV-positive Queen 9730 Placenta Resorption 1

Cytokeratin-positive placental trophoblasts are evident by the brown staining. (A) Trophoblasts were selectively identified. (B) Areas of trophoblast-rich populations were cut and captured using the UV cutting laser and Capture IR laser, respectively. (C) Selected trophoblasts were removed from the tissue area. (D) Trophoblastic cells were attached to the cap. (E and F) Cap images of acquired trophoblasts using the 2X Veritas instrument objective. (A–D) Photography was performed with the 20X Veritas instrument objective.

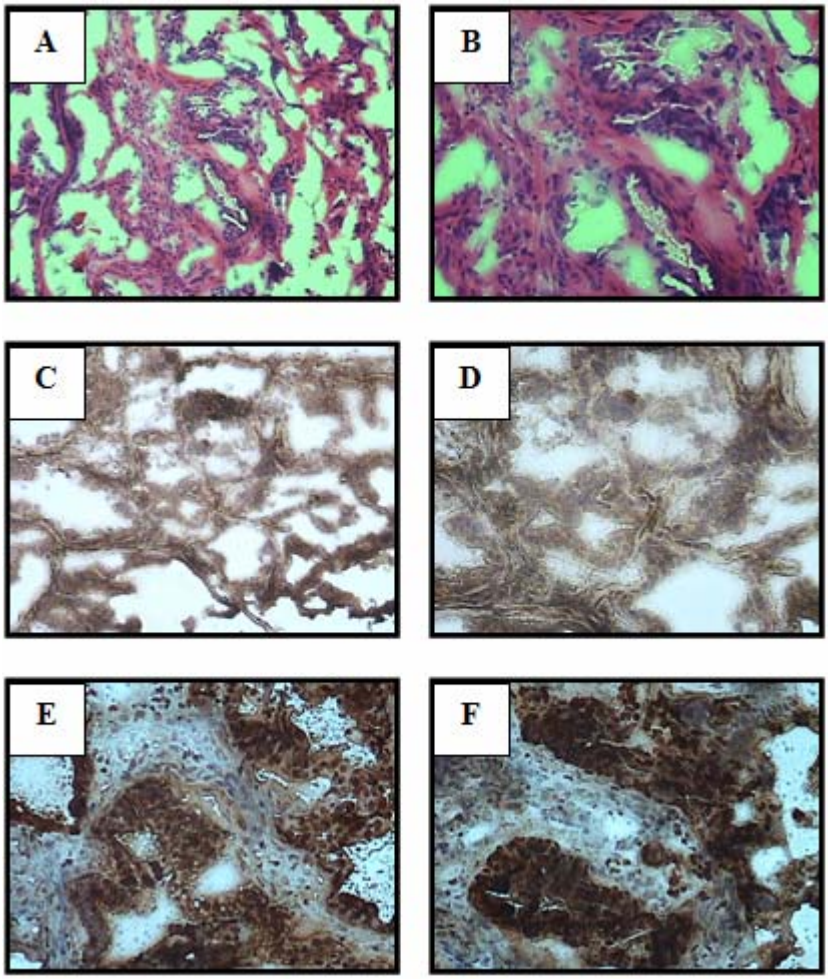

Figure 5.27 Histological Evaluation and Immunohistochemical Localization of Placental Trophoblasts from Late-Term FIV-positive Queen 9730 Placenta Resorption 3

Placental tissues were cryosectioned to a thickness of 10 μm, fixed in acetone, and dehydrated. Sections were stained with hematoxylin and eosin and photographed using 10X and 20X Veritas instrument objectives (A and B, respectively). Sections were stained with a universal negative rabbit antibody and goat anti-rabbit IgG, poly-HRP immunohistochemistry amplification reagent and photographed using 10X and 20X Veritas instrument objectives (C and D, respectively). Sections were stained with the polyclonal rabbit anti-cow cytokeratin WSS antibody and goat anti-rabbit IgG, poly-HRP and photographed using the 20X Veritas instrument objective (E and F). Cytokeratin-positive trophoblastic cells are evident by the brown staining (E and F).

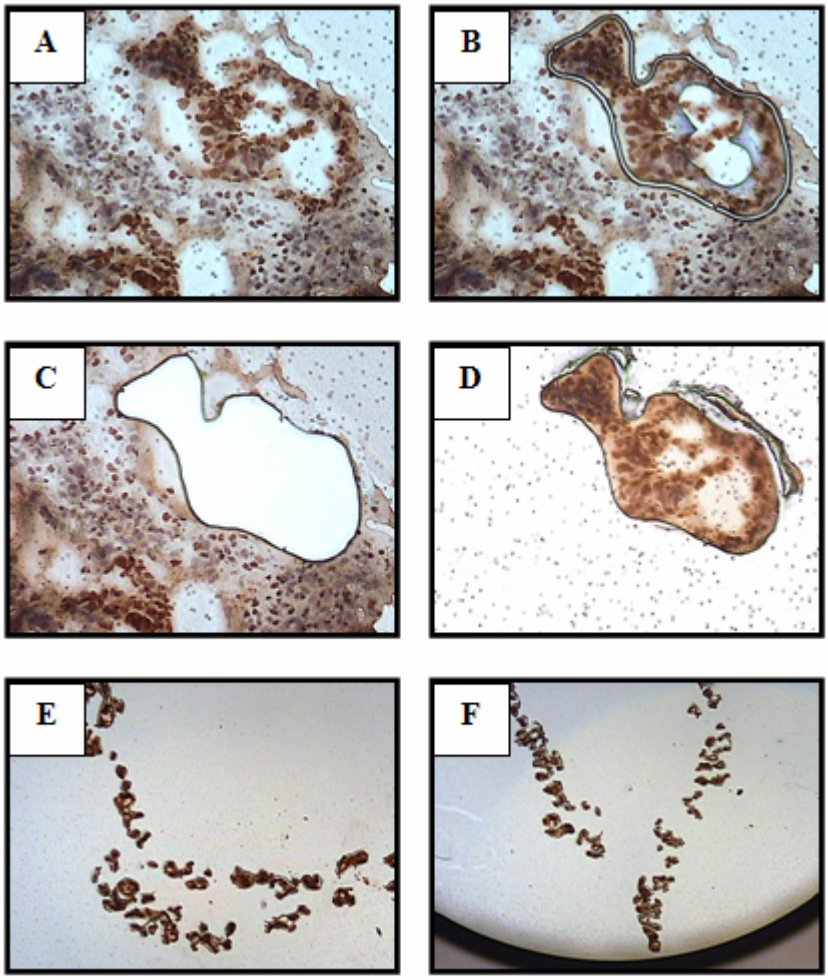

Figure 5.28 Laser Capture Microdissection of IHC Stained Placental Trophoblasts from Late-Term FIV-positive Queen 9730 Placenta Resorption 3

Cytokeratin-positive placental trophoblasts are evident by the brown staining. (A) Trophoblasts were selectively identified. (B) Areas of trophoblast-rich populations were cut and captured using the UV cutting laser and Capture IR laser, respectively. (C) Selected trophoblasts were removed from the tissue area. (D) Trophoblastic cells were attached to the cap. (E and F) Cap images of acquired trophoblasts using the 2X Veritas instrument objective. (A–D) Photography was performed with the 20X Veritas instrument objective.

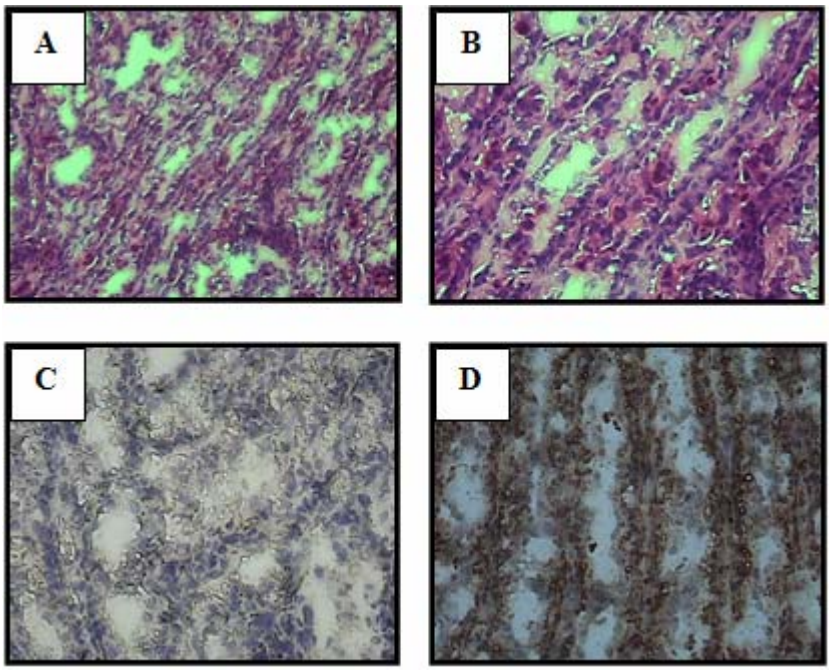

Figure 5.29 Histological Evaluation and Immunohistochemical Localization of Placental Trophoblasts from Late-Term FIV-positive Queen 13226 Arrested Placenta 3

Placental tissues were cryosectioned to a thickness of 10 μm, fixed in acetone, and dehydrated. Sections were stained with hematoxylin and eosin and photographed using 10X and 20X Veritas instrument objectives (A and B, respectively). Sections were stained with a universal negative rabbit antibody and goat anti-rabbit IgG, poly-HRP immunohistochemistry amplification reagent and photographed using the 20X Veritas instrument objective (C). Sections were stained with the polyclonal rabbit anti-cow cytokeratin WSS antibody and goat anti-rabbit IgG, poly-HRP and photographed using the 20X Veritas instrument objective (D). Cytokeratin-positive trophoblastic cells are evident by the brown staining (D).

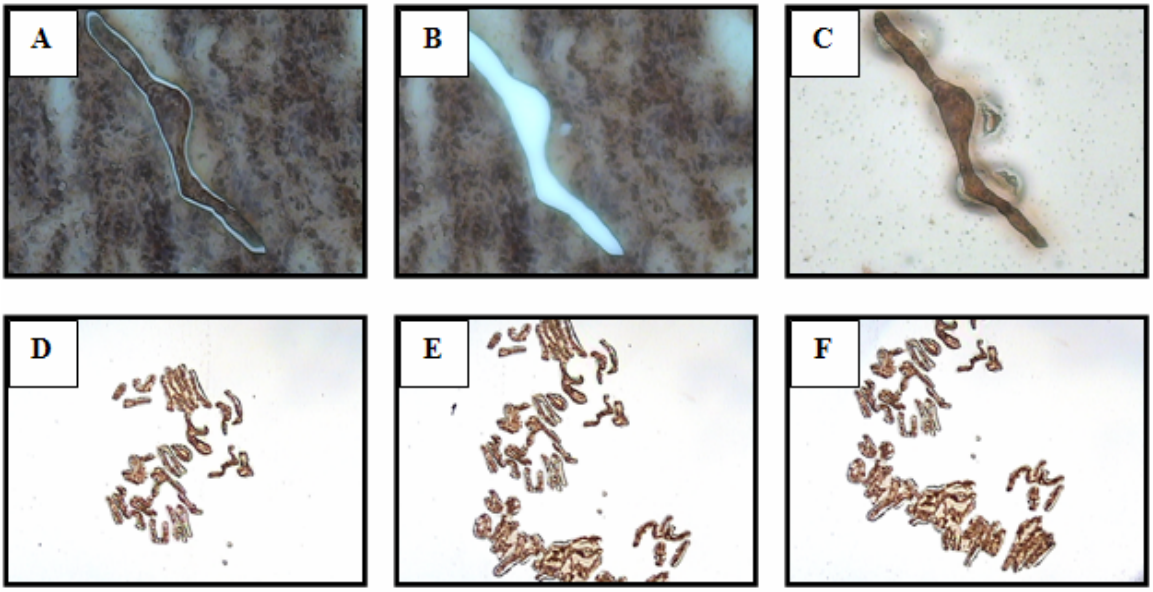

Figure 5.30 Laser Capture Microdissection of IHC Stained Placental Trophoblasts from Late-Term FIV-positive Queen 13226 Arrested Placenta 3

Cytokeratin-positive placental trophoblasts are evident by the brown staining. (A) Trophoblasts were selectively identified and areas of trophoblast-rich populations were cut and captured using the UV cutting laser and Capture IR laser, respectively. (B) Selected trophoblasts were removed from the tissue area. (C) Trophoblastic cells were attached to the cap. (D–F) Cap images of acquired trophoblasts using the 2X Veritas instrument objective. (A–C) Photography was performed with the 20X Veritas instrument objective.

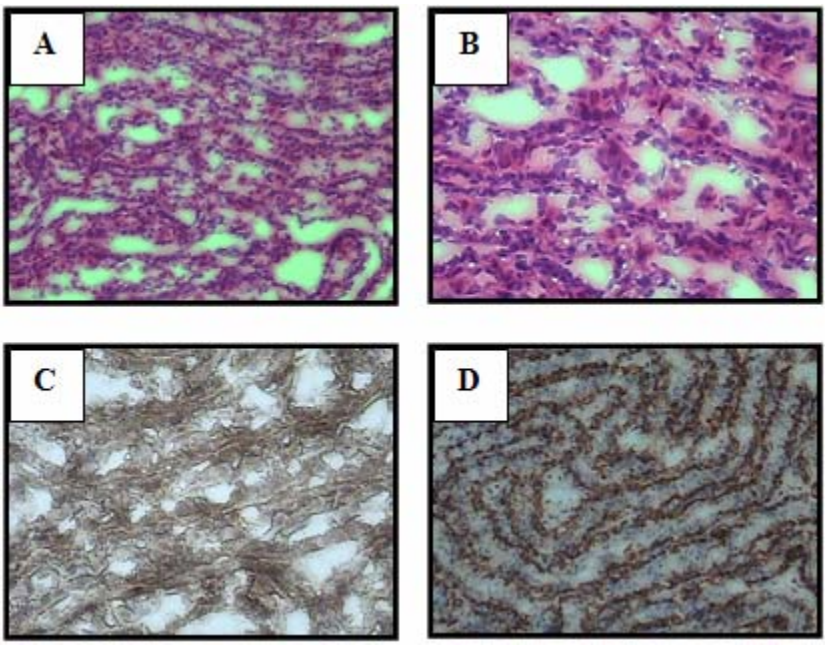

Figure 5.31 Histological Evaluation and Immunohistochemical Localization of Placental Trophoblasts from Late-Term FIV-positive Queen 13226 Arrested Placenta 4

Placental tissues were cryosectioned to a thickness of 10 μm, fixed in acetone, and dehydrated. Sections were stained with hematoxylin and eosin and photographed using 10X and 20X Veritas instrument objectives (A and B, respectively). Sections were stained with a universal negative rabbit antibody and goat anti-rabbit IgG, poly-HRP immunohistochemistry amplification reagent and photographed using the 20X Veritas instrument objective (C). Sections were stained with the polyclonal rabbit anti-cow cytokeratin WSS antibody and goat anti-rabbit IgG, poly-HRP and photographed using the 10X Veritas instrument objective (D). Cytokeratin-positive trophoblastic cells are evident by the brown staining (D).

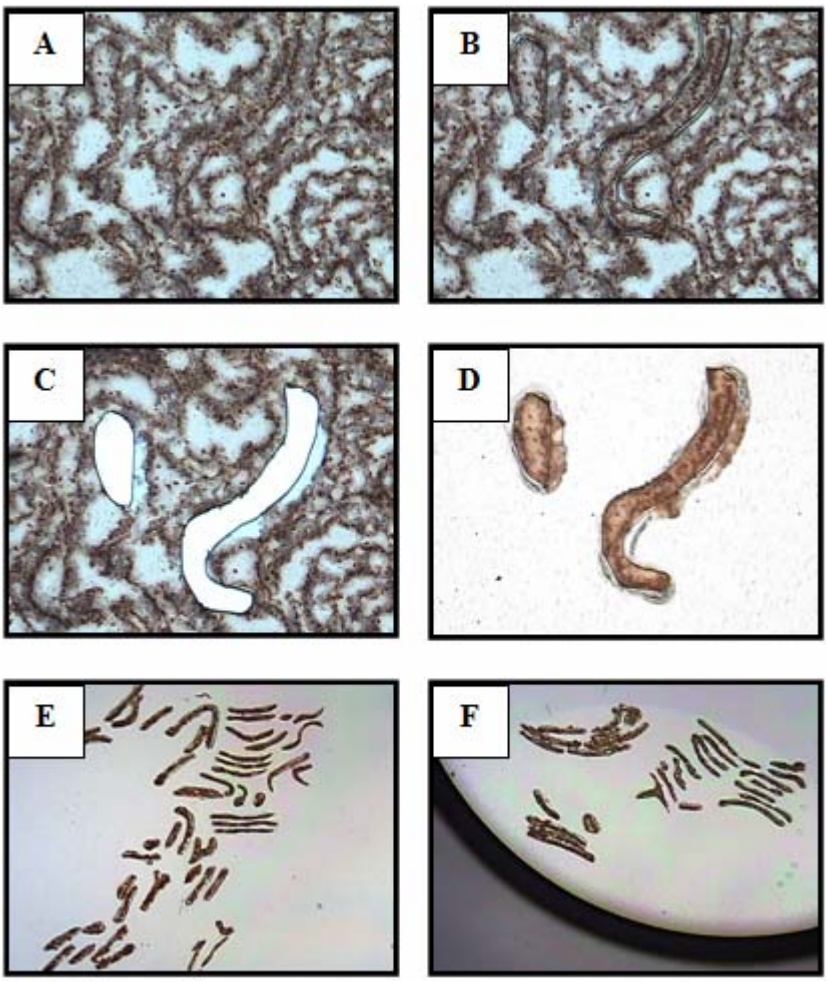

Figure 5.32 Laser Capture Microdissection of IHC Stained Placental Trophoblasts from Late-Term FIV-positive Queen 13226 Arrested Placenta 4

Cytokeratin-positive placental trophoblasts are evident by the brown staining. (A) Trophoblasts were selectively identified. (B) Areas of trophoblast-rich populations were cut and captured using the UV cutting laser and Capture IR laser, respectively. (C) Selected trophoblasts were removed from the tissue area. (D) Trophoblastic cells were attached to the cap. (E and F) Cap images of acquired trophoblasts using the 2X Veritas instrument objective. (A–D) Photography was performed with the 10X Veritas instrument objective.

Conclusion

Similar to HIV, FIV infections may cause dysregulation of trophoblastic cytokine expression, and aberrant expression of these cytokines may potentiate inflammation and transplacental infections. Although an early report by our lab indicated that inflammatory cytokine expression is upregulated in placentas where reproductive failure occurred, the specific cell populations expressing the cytokines were not identified (Weaver et al., 2005). Here we report that hematoxylin and eosin staining of FIV-B-2542-infected and non-infected samples resulted in stained cells with blue nuclei and pink cytoplasm and confirmed that tissue integrity was not compromised during frozen storage. Trophoblastic cells were selectively identified in all placental samples using immunohistochemistry. Trophoblastic cells were demarcated and removed using laser capture microdissection. Future studies will involve nucleic acid extraction from collected trophoblastic cells. This nucleic acid will be used in real-time RT-PCR to measure gene expression.

References

Allison, R.W., Hoover, E.A., 2003, Covert vertical transmission of feline immunodeficiency virus. AIDS Res Hum Retroviruses 19, 421-434.

Boudreaux, C.E., Lockett, N.N., Chemerys, D.N., Clay, B.T., Scott, V.L., Willeford, B., Brown, T., Coats, K.S., 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. Vet Immunol Immunopathol 131, 290-297.

D'Ubaldo, C., Pezzotti, P., Rezza, G., Branca, M., Ippolito, G., 1998, Association between HIV-1 infection and miscarriage: a retrospective study. DIANAIDS Collaborative Study Group. Diagnosi Iniziale Anomalie Neoplastiche AIDS. Aids 12, 1087-1093.

Kumar, R.M., Uduman, S.A., Khurranna, A.K., 1995, Impact of maternal HIV-1 infection on perinatal outcome. Int J Gynaecol Obstet 49, 137-143.

Langston, C., Lewis, D.E., Hammill, H.A., Popek, E.J., Kozinetz, C.A., Kline, M.W., Hanson, I.C., Shearer, W.T., 1995, Excess intrauterine fetal demise associated with maternal human immunodeficiency virus infection. J Infect Dis 172, 1451-1460.

Salazar-Gonzalez, J.F., O. Martinez-Maza, N. Aziz, et. al. , 1997, Relationship of plasma HIV-RNA levels and levels of TNF-alpha and immune activation products in HIV infection. Clin. Immunol. Immunopathol. 84, 36-45.

Sinicco, A., A. Biglino, M. Sciandra, et. al. , 1993, Cytokine network and acute primary HIV-1 infection. AIDS. 7, 1167-1172.

Weaver, C.C., Burgess, S.C., Nelson, P.D., Wilkinson, M., Ryan, P.L., Nail, C.A., Kelly-Quagliana, K.A., May, M.L., Reeves, R.K., Boyle, C.R., Coats, K.S., 2005, Placental immunopathology and pregnancy failure in the FIV-infected cat. Placenta 26, 138-147.

CHAPTER VI

IMMUNOMODULATOR EXPESSION IN TROPHOBLASTS FROM THE FIV-INFECTED CAT

Abstract

Maternal-fetal transmission of the feline immunodeficiency virus (FIV) occurs commonly in queens experimentally infected with FIV-B-2542. Fetal infection with FIV has been detected by 3–4 weeks gestation. Reproductive failure frequently occurs in the FIV-infected cat, resulting in resorptions or developmentally-arrested fetuses. The incidence of reproductive failure in the FIV-infected cat parallels the incidence of spontaneous abortion in HIV-infected women. Immune homeostasis of cytokines and chemokines in the placenta is necessary for successful pregnancy. Successful pregnancy is typically biased towards a Th2 humoral immune response (anti-inflammatory) with a suppressed Th1 cell-mediated immune response (pro-inflammatory) at the maternal-fetal interface. Dysregulation of cytokines during pregnancy often results in fetal death. Placental trophoblasts produce cytokines and chemokines that modulate pregnancy and may play a role in maternal tolerance of embryo and fetal allografts. We hypothesized that FIV infections may cause dysregulation of trophoblastic cytokine expression, and aberrant expression of these cytokines may potentiate inflammation and transplacental infections. The purpose of this project was to quantify the expression of Th1 cytokines

(TNF-α, IFN-γ, IL-1β, IL-2, IL-12p35, IL-12p40, and IL-18), the Th2 cytokines (IL-4, IL-5, IL-6, and IL-10), the cytokine GM-CSF, the primary FIV receptor CD134, the chemokine SDF-1α, the chemokine receptor CXCR4, and FIV gag in placental trophoblasts from early- and late-term pregnancy. We used a cytokeratin marker in immunohistochemistry to selectively identify trophoblastic cellular populations. Identified trophoblastic cells were collected using laser capture microdissection and total RNA was extracted from the captured cell populations. Reverse transcription-PCR was used to amplify cDNA from the RNA samples. Real-time quantitative PCR was used to measure gene expression. We were able to detect only IL-4, IL-5, IL-6, IL-1β, IL-12p35, IL-12p40, and CXCR4 in feline placental trophoblasts from early- and late-term pregnancy. We found expression of pro- and anti-inflammatory cytokines at both time points during gestation. We report increased immune function of trophoblasts late in pregnancy. While we did not find that increased expression of pro-inflammatory cytokines accompanied reproductive failure, FIV-infection did alter expression of some cytokines in trophoblasts, and reproductive failure was accompanied by down-regulation of both pro- and anti-inflammatory cytokines. FIV gag was detected in only one trophoblast specimen, and CD134 was not detected. Thus, feline placental trophoblasts do not appear to be easily infected by FIV and trophoblastic immunomodulation is influenced by the surrounding placental microenvironment rather than direct viral infection.

Introduction

Globally, the estimated number of people living with HIV is 33.4 million, 2.1 million of which are children (UNAIDS/WHO, 2009a). The pediatric AIDS pandemic causes 1,200 new HIV infections daily (UNAIDS/WHO, 2009a), with mother-to-child transmission (MTCT) of the virus accounting for 90% of pediatric infections (CDC, 2008). In the United States, MTCT is the most common cause of pediatric AIDS cases (defined as AIDS occurring in children under the age of 13 years) and results in 100–200 infected infants annually (CDC, 2007). Children who are infected *in utero* have a more rapid progression to AIDS and generally become symptomatic during the first year of life. In the absence of antiviral treatment, 50% of HIV-infected newborns will succumb to the disease before 24 months of age (UNAIDS/WHO, 2009b). Moreover, transplacental transfer of HIV results in poor reproductive outcomes, including low birth weight babies, preterm delivery, and an increased incidence of spontaneous abortions (D'Ubaldo et al., 1998; Kumar et al., 1995; Langston et al., 1995). Although HIV has the potential to negatively impact fetal and neonatal health, the mechanisms of HIV vertical transmission and pregnancy perturbation associated with infection remain undefined.

Immunoregulatory molecules such as cytokines and chemokines are responsible for pregnancy maintenance and aid in the maternal tolerance of embryo and fetal allografts and the regulation of maternal cell-mediated and humoral immune responses against infection (Bennett et al., 1998; Challis et al., 2009; Clark and Chaouat, 1989; Robertson et al., 1994; Saito, 2001). Successful pregnancy is typically biased towards a Th2 humoral immune response (anti-inflammatory) with a suppressed Th1 cell-mediated

immune response (pro-inflammatory) at the maternal-fetal interface (Challis et al., 2009; Costeas et al., 2004; Raghupathy, 1997; Wegmann et al., 1993). Due to the mutually antagonistic nature of Th1 and Th2 cytokines, an inflammatory microenvironment in the placenta is prevented with enhanced humoral immunity during early pregnancy (Challis et al., 2009; Mosmann and Coffman, 1989; Wegmann et al., 1993). However, the balance of cytokines is shifted during late pregnancy and a Th1 immune response dominates to initate labor and the expulsion of the fetus and fetal membranes. A clear indicator of Th1:Th2 immune shifts is the altered expression of the Th1 cytokine, IL-12 (a pro-inflammatory cytokine), and the Th2 cytokine, IL-10 (an anti-inflammatory cytokine). Moreover, the altered expression of Th1 and Th2 cytokines during pregnancy may result in cytokine dysregulation (Challis et al., 2009; Costeas et al., 2004; Romagnani, 1994; Wegmann et al., 1993). Cytokine dysregulation in the placenta may compromise pregnancy and cause fetal death (Challis et al., 2009; Orsi and Tribe, 2008).

During gestation, Th1 and Th2 cytokines are produced in the decidual epithelium and stroma, chorionic and amnionic membranes, and trophoblastic cells (Wilczynski, 2005). Placental trophoblastic cells (cytotrophoblasts and syncytiotrophoblasts) are fetal-derived cells of epithelial origin that form the placental chorionic villi. These trophoblastic cells contact and form attachment with the maternal endometrium and serve as a barrier between maternal and fetal blood supplies in human pregnancy (Lagaye et al., 2001). Vertical transfer of HIV likely results in productive viral infection of these trophoblast layers (Douglas et al., 1991; Vidricaire et al., 2003). The expression of HIV is augmented by placental cytokines and chemokines (Kedzierska and Crowe, 2001), and

HIV infections can dysregulate cytokine networks in placental tissues (Lee et al., 1997; Shearer et al., 1997). Aberrant expression of cytokines may result in placental inflammation, and the presence of inflamed placental membranes is likely to facilitate viral transplacental transfer (Shearer et al., 1997).

The feline immunodeficiency virus (FIV) causes a natural retroviral infection of domestic cats with a clinical course of infection similar to that of HIV. Feline AIDS is characterized by progressive depletion of CD4+ T cells culminating in fatal opportunistic infections. Vertical transmission of FIV occurs commonly under experimental conditions, resulting in viable and non-viable infected offspring (Allison and Hoover, 2003; Boudreaux et al., 2009; Weaver et al., 2005), similar to pregnancy outcome in HIV-infected women. Therefore, the FIV-infected cat is a useful small animal model for the study of lentiviral transplacental transfer.

Using the FIV-infected cat model, we demonstrated frequent reproductive failure in litters delivered at early- and late-gestation by cesarean section (Boudreaux et al., 2009; Weaver et al., 2005). Placental and fetal infection occurred in nearly all FIV-infected pregnancies, and fetal non-viability was frequent and appeared to occur during early pregnancy. Th1 and Th2 cytokine expression did not differ significantly in the late-term placentas of infected and uninfected cats, although the trend was toward increased expression of Th1 cytokines (IFN-γ and IL-1β) and decreased expression of the Th2 cytokine (IL-10). Th1 cytokine expression was significantly increased in placentas from FIV-infected, resorbed fetuses in comparison to other placentas. Early-term placentas were not evaluated in that study (Weaver et al., 2005).

In Chapter III, we reported that FIV-infection during early- and late-term gestation affects immunomodulator expression. However, the specific cell populations expressing the targeted immunomodulators were not identified. In the present study, we hypothesized that FIV infections may cause dysregulation of trophoblastic cytokine expression, and aberrant expression of these cytokines may potentiate inflammation and transplacental infections. Our objective was to quantify the expression of Th1 cytokines (TNF-α, IFN-γ, IL-1β, IL-2, IL-12p35, IL-12p40, and IL-18), the Th2 cytokines (IL-4, IL-5, IL-6, and IL-10), the cytokine GM-CSF, the primary FIV receptor CD134, the chemokine SDF-1α, the chemokine receptor CXCR4, and FIV gag in trophoblasts microdissected from early- and late-term placental tissues. We were able to detect only IL-4, IL-5, IL-6, IL-1β, IL-12p35, IL-12p40, and CXCR4 in feline placental trophoblasts from early- and late-term pregnancy. We report altered trophoblast function late in pregnancy. We did not detect expression of the FIV primary receptor CD134 and our failure to detect FIV gag indicated that trophoblasts were not infected by the virus. Therefore, aberrant trophoblast function is most likely a result of an altered microenvironment occurring in FIV-infected placental tissue surrounding the trophoblasts, rather than a direct consequence of trophoblast infection.

Materials and Methods

Animals and virus

All procedures utilizing cats (*Felis domesticus*) were performed with approval of the Mississippi State University Institutional Animal Care and Use Committee. As previously reported (Boudreaux et al., 2009; Weaver et al., 2005), cats were reproductively mature, specific-pathogen-free (SPF) animals of less than 12 months of age when obtained from a commercial cattery. Ten cats were inoculated intravenously with 1 ml of a feline plasma pool containing approximately 1.33×10^4 copies/ml of FIV-B-2542 originally provided by Dr. Edward Hoover (Rogers and Hoover, 1998). Ten cats were sham inoculated with 1 ml of pooled uninfected plasma and served as normal controls. Infection was confirmed within 6 weeks p.i. by detection of FIV provirus by PCR and for seroconversion using the PetChek® FIV Antibody Test Kit (IDEXX Laboratories, Inc., Westbrook, ME). All queens were allowed to naturally breed with SPF toms. Toms used to breed uninfected females were never exposed to infected females and vice versa. Breeding was observed and pregnancy was confirmed by palpation and ultrasonography.

Kittens were delivered by cesarean section during week 3 gestation (early-term) (Boudreaux et al., 2009) and week 8 gestation (late-term) (Weaver et al., 2005). Placentas were collected under sterile conditions using the following dissection procedure. The uteri were removed and individual gestational sacs were collected. Following rinses with sterile PBS, sacs were incised with a sterile scalpel, and fetuses and

placentas were collected. Placental tissues were snap frozen in liquid nitrogen and cryopreserved at -80°C. The effect of FIV infection on fetal viability at early- and late-term pregnancy was previously reported (Boudreaux et al., 2009; Weaver et al., 2005). For this study, we evaluated eight selected placental samples from both early- and late-term gestation, including placentas from viable and non-viable pregnancies. Due to the poor placental development at 3 weeks gestation, placentas from early-term pregnancies were collected from tissue connecting the embryo to the umbilical cord. All placental tissues used in this study are listed in Table 6.1

Immunohistochemistry (IHC) to detect trophoblasts

All IHC procedures were performed using RNase-free, sterile conditions. Snap frozen placental tissues were embedded in OCT, sectioned to a thickness of 10 μm using a cryostat, and placed on RNase-free glass membrane slides (Molecular Devices, Sunnyvale, CA). Sections were fixed with acetone at room temperature (20–25ºC). Endogenous peroxidase activity in dehydrated sections was quenched with 400 ml methanol / 2 ml 30% H_2O_2 solution. Quenched sections were washed in 0.05 M Tris-HCl buffer (TB) (pH 7.0–7.6). To prevent non-specific antibody binding, sections were blocked with 5% (w/v) non-fat dry milk in TB (Blotto) and washed in 0.05 M Tris-HCl buffer (pH 7.0–7.6). Trophoblastic cells were stained with a polyclonal rabbit anti-cow cytokeratin wide spectrum screening (WSS) antibody (Dako Corp., Carpinteria, CA). A universal negative rabbit antibody (Dako Corp.) was used as the negative control reagent. The primary antibody and negative control dilutions were prepared in 0.05 M Tris-HCl

buffer, pH 7.0–7.6, containing 1% bovine serum albumin. After an overnight incubation at 4°C, sections were washed with 0.05 M Tris-HCl buffer. Sections were incubated with a ready-to-use goat anti-rabbit IgG, poly-HRP immunohistochemistry amplification reagent (Chemicon International, Inc., Temecula, CA) for 1 hr at room temperature (20–25ºC). After washing in 0.05 M Tris-HCl buffer (pH 7.0–7.6), sections were incubated with the buffered substrate-DAB chromogen solution (Invitrogen Corp., Carlsbad, CA) for 15 min, rinsed in deionized, diethylpyrocarbonate (DEPC)-treated H_2O, and counterstained with Mayer's hematoxylin. To successfully capture trophoblastic cells, sections were dehydrated in 75% , 95%, and 100% EtOH. Sections were then placed in xylene until laser capture microdissection was performed.

Laser capture microdissection (LCM) to capture trophoblasts

Following immunohistochemical detection in placental specimens, trophoblasts were microdissected using the Veritas™ Microdissection Instrument (Arcturus Bioscience, Inc., MountainView, CA). Microdissection was performed using the Capture IR laser (Power: 70 mW; Pulse: 2500 μsec; and Intensity: 200 mV) and UV Cutting laser (Laser spot size: ~8.0 μm and Laser power: 7.0 mW). Similar-sized cellular fields of ~2.00 mm^2 were collected onto CapSure Macro LCM Caps (Arcturus). All sections and capture areas were photographed using the dissection instrument (Chapter V).

RNA purification and quantification from microdissected tissues

RNA was extracted from microdissected trophoblastic cells of FIV-B-2542-infected and uninfected queens at early gestation (n = 5 and n = 3, respectively) and late gestation (n = 5 and n = 3, respectively), using the PicoPure RNA Isolation Kit (Arcturus). All RNA samples were treated with DNase to remove contaminating genomic DNA. RNA was quantified using the NanoDrop™ 3300 Fluorospectrometer (Thermo Fisher Scientific, Inc. Waltham, MA). All RNA was stored at -80°C until use.

Reverse transcription-polymerase chain reaction (RT-PCR) to generate cDNA

Reverse transcription-polymerase chain reaction (RT-PCR) used a MJ Mini Personal Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA): 25°C, 10 min; 37°C, 120 min; 85°C, 5 sec; 4°C, hold. cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Inc., Foster City, CA) according to the manufacturer's instructions. Briefly, each reverse transcription reaction was performed in a final reaction volume of 20 µl containing 2 µl of the 10X RT buffer and RT random primers, 0.8 µl of 25X dNTP, 1 µl of Multiscribe Reverse Transcriptase and RNase inhibitor, 3.2 µl of RNase-free water, and 10 µl of ~30–800 pg RNA template. All cDNA was stored at -20°C until use.

Primer design of feline immunomodulators, FIV receptors, and the internal control

All primer design methods were previously reported (Scott et al., 2008) and sequences of PCR primers and TaqMan probes specific for feline immunomodulators,

FIV receptors, and the internal control mRNA are listed in Table A.1. All reference feline primers and probes used in this study are listed in Table A.2.

Evaluation of expression of immunomodulators using TaqMan real-time PCR

The real-time PCR (rtPCR) used an iCycler (Bio-Rad): 50°C, 2 min; 95°C, 10 min; 60 X (95°C, 15 s; 60°C, 1 min). Each reaction contained 10 µl of the 2X TaqMan® Universal PCR Master Mix (Applied Biosystems), 1 µl of forward and reverse primers (10 pmol/µl), 1 µl of the respective probe (100 fmol/µl), and ~2–4 µg cDNA. For every placental cDNA sample, parallel reactions were performed in triplicate simplex reactions on separate plates for each gene. Due to the paucity of cDNA concentrations associated with cellular extractions, serially-diluted and pooled whole tissue RNA from representative uninfected cats (Chapter III) was used to generate standard curves for the internal control and target gene amplicons. Each titration simplex reaction contained 6.25 µl of the 2X Thermoscript™ reaction mix, 0.25 µl of Thermoscript™ RT/Platinum® *Taq* Mix (Invitrogen), 1 µl of forward and reverse primers (10 pmol/µl), 1 µl of the respective probe (100 fmol/µl), and five-fold diluted 2 µg RNA as starting titration template. The standard curves were used to normalize for differences in PCR efficiency between the internal control and target genes and between sample plate runs. Differences in the amount of template cDNA in each reaction was corrected by the cycle threshold (Ct) value for β-actin. Normalized samples were divided by the calibration, generating the relative expression levels.

Statistical analysis

Statistical evaluation of fetal viability from early- and late-term gestation were previously reported (Boudreaux et al., 2009; Weaver et al., 2005). Statistical analysis of immunomodulator expression for early- and late-term control and FIV-B-2542-infected trophoblasts were done using single-factor ANOVA (Microsoft Excel-XP, Redmond, WA) followed by the two independent sample Wilcoxon rank sum test (SOCR Analysis, University of California, Los Angeles, CA) .

Results

Expression of immunomodulators in placental trophoblasts

Immunomodulator mRNAs for IL-4, IL-5, IL-6, IL-1β, IL-12p35, IL-12p40, and CXCR4 were expressed in all early- and late-term placental trophoblasts. We were unable to detect TNF-α, IFN-γ, IL-2, IL-18, IL-10, GM-CSF, CD134, and SDF-1α in trophoblasts from either stage of pregnancy. We detected Gag in only one late-term trophoblastic sample that was associated with a fetal resorption (Fig. 6.1).

To determine normal feline placental trophoblastic expression of the detected immunomodulators between the two stages of pregnancy, data obtained from control placentas from early- and late-term were compared. There were significant differences in expression of all immunomodulators except IL-5 ($p = 0.135$) in control placental trophoblasts between the two stages of pregnancy. Trophoblasts from control cats at late gestation expressed more IL-4 ($p = 0.025$), IL-6 ($p = 1.36E-08$), IL-1β ($p = 0.001$),

IL-12p35 ($p = 0.001$), IL-12p40 ($p = 0.002$), and CXCR4 ($p = 0.006$) mRNA than trophoblasts from early gestation (Fig. 6.2).

To determine the effect of FIV infection on placental trophoblastic cytokine expression, FIV-infected and control trophoblasts from both early- and late-term gestation were compared. At early gestation, FIV-infection resulted in decreased expression of the anti-inflammatory cytokine IL-5 ($p = 0.038$) mRNA and had no effect on the mRNA expression of the anti-inflammatory cytokine IL-4 ($p = 0.335$), the pro-inflammatory cytokines IL-6 ($p = 0.276$), IL-1β ($p = 0.096$), IL-12p35 ($p = 0.060$), IL-12p40 ($p = 0.319$), or the chemokine receptor CXCR4 ($p = 0.199$) (Fig. 6.3). At late gestation, FIV-infection resulted in decreased mRNA expression of the anti-inflammatory cytokine IL-4 ($p = 0.021$) and the pro-inflammatory cytokine IL-12p35 ($p = 0.021$) and had no effect on the mRNA expression of IL-5 ($p = 0.0135$), IL-6 ($p = 0.178$), IL-1β ($p = 0.108$), and CXCR4 ($p = 0.111$) (Fig. 6.4). Decreased expression of the pro-inflammatory cytokine IL-12p40 ($p = 0.057$) approached significance (Fig. 6.4).

To determine whether cytokine expression was related to fetal viability, placental trophoblasts from FIV-infected, viable pregnancies were compared to trophoblasts from FIV-infected, non-viable pregnancies for early-term gestation. Due to the low concentration of trophoblast RNA associated with microdissection and subsequent lack of detection of some cytokine mRNA in some infected viable and infected non-viable trophoblasts, we were unable to statistically compare infected, viable trophoblasts to infected, non-viable trophoblasts for late-term gestation. In addition, we were only able to make statistical comparisons of IL-4, IL-5, IL-6, IL-1β, and IL-12p35 for early-term

infected, viable and infected, non-viable trophoblasts. At early gestation, there were no significant differences in immunomodulator expression when trophoblasts from infected, viable pregnancies were compared to trophoblasts from infected, non-viable pregnancies (Fig. 6.5).

To determine whether cytokine expression was related to fetal viability, regardless of infection, trophoblasts from all viable pregnancies were compared to trophoblasts from all non-viable pregnancies for both early- and late-term gestation. At early gestation, trophoblasts from non-viable pregnancies expressed significantly less IL-4 ($p = 0.032$), IL-6 ($p = 0.048$), IL-12p35 ($p = 0.001$), and CXCR4 ($p = 0.004$) mRNA than those of viable pregnancies. The decreased IL-1β expression in non-viable pregnancies approached, but did not reach the level of significance ($p = 0.052$) (Fig. 6.6). At late gestation, trophoblasts from non-viable pregnancies expressed significantly less IL-4 ($p = 0.021$), IL-12p35 ($p = 0.021$), and IL-12p40 ($p = 0.038$) than trophoblasts from viable pregnancies (Fig. 6.7).

Table 6.1

Feline Placental Tissues Included in the Study of Trophoblast Immunomodulator Expression

Queen number and placental identification	Pregnancy term	FIV status	Fetal viability
2779 Placenta B	Early	–	Viable
2779 Placenta D	Early	–	Viable
2779 Placenta E	Early	–	Non-viable; fetal resorption
0326 Placenta A	Early	+	Viable
8035 Placenta B	Early	+	Non-viable; arrested fetus
8035 Placenta C	Early	+	Non-viable; arrested fetus
0866 Placenta A	Early	+	Viable
0866 Placenta B	Early	+	Non-viable; fetal resorption
9581 Placenta A	Late	–	Viable
9581 Placenta B	Late	–	Viable
9746 Placenta C	Late	–	Viable
9730 Placenta B	Late	+	Viable
9730 Resorb 1	Late	+	Non-viable; fetal resorption
9730 Resorb 3	Late	+	Non-viable; fetal resorption
13226 Placenta 3	Late	+	Non-viable; arrested fetus
13226 Placenta 4	Late	+	Non-viable; arrested fetus

Placental tissues were identified based upon the experimental number of the delivering queen and given an alphabetical or numerical designation based on order of harvest. Trophoblastic cells were extracted from placental tissues taken from each associated queen.

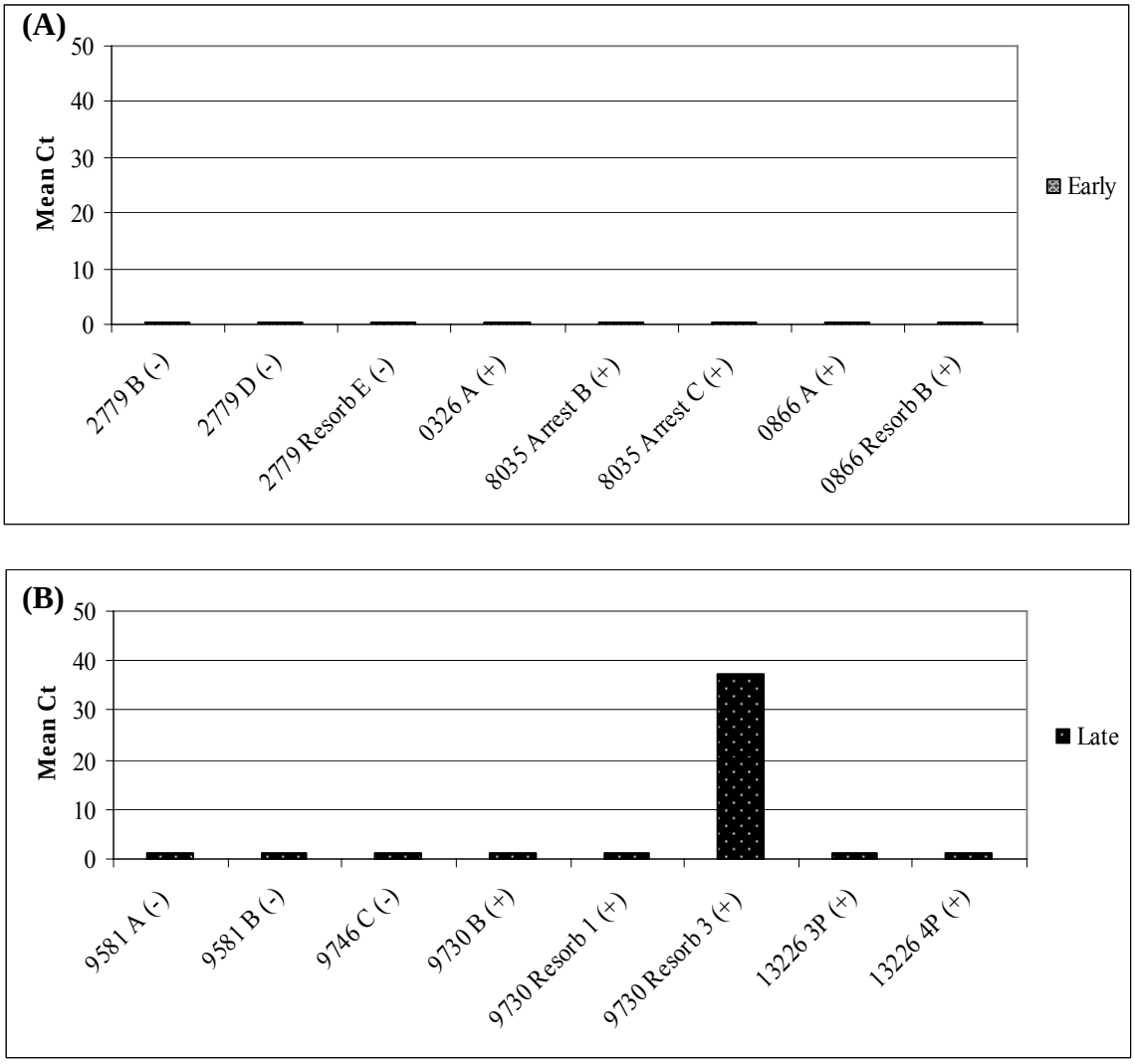

Figure 6.1 Detection of FIV in Trophoblasts from Early- and Late-Term Feline Placentas

Real-time RT-PCR analysis of FIV gag gene expression in feline placental trophoblasts from all pregnancies. (A) early-term placental trophoblastic samples (n = 8); (B) late-term placental trophoblastic samples (n = 8). Bars represent mean Ct values.

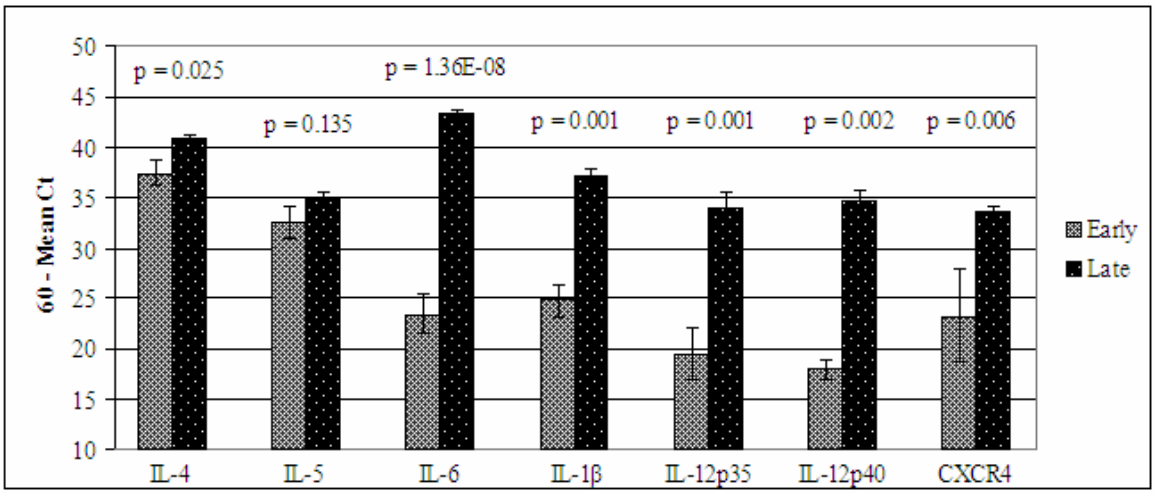

Figure 6.2 Relative Expression of Immunomodulators in Trophoblasts from Control Cats

Real-time RT-PCR analysis of normal feline placental trophoblastic expression of the pro-inflammatory cytokines (IL-6, IL-1β, IL-12p35, and IL-12p40), the anti-inflammatory cytokines (IL-4 and IL-5), and the chemokine receptor (CXCR4) in early-term uninfected placentas (n = 3) versus late-term uninfected placentas (n = 3). Bars represent mean Ct values substracted from a negative endpoint (60 - mean Ct), bracketed by standard errors of the mean. P values obtained from single factor ANOVA and Wilcoxon rank sum test are noted. Alpha = 0.05.

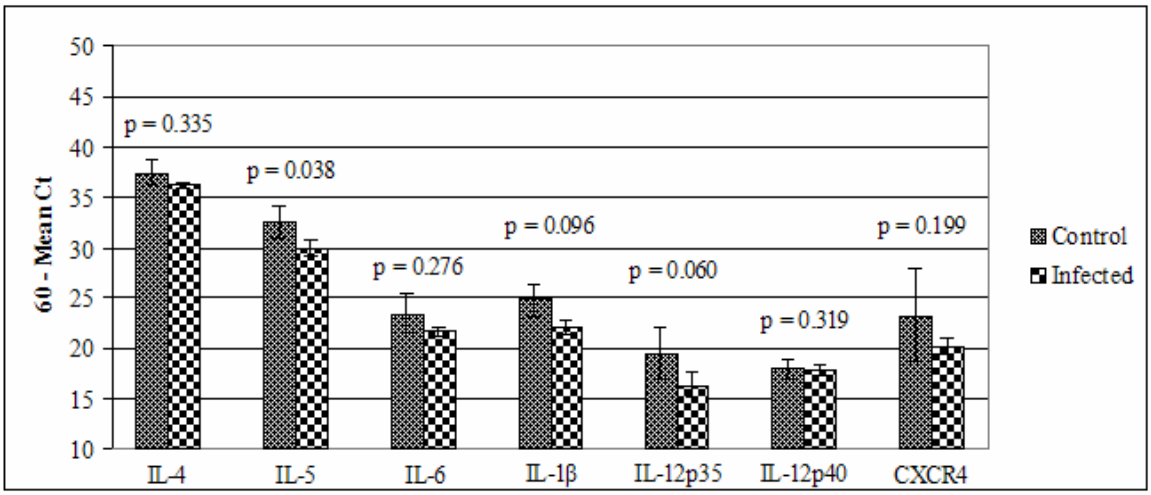

Figure 6.3 Relative Expression of Immunomodulators in Trophoblasts from Early-Term Feline Placentas

Real-time RT-PCR analysis of feline placental trophoblastic expression of the pro-inflammatory cytokines (IL-6, IL-1β, IL-12p35, and IL-12p40), the anti-inflammatory cytokines (IL-4 and IL-5), and the chemokine receptor (CXCR4) in early-term FIV-infected (n = 5) versus uninfected (control) (n = 3) placentas. Bars represent mean Ct values substracted from a negative endpoint (60 - mean Ct), bracketed by standard errors of the mean. P values obtained from Wilcoxon rank sum test are noted. Alpha = 0.05.

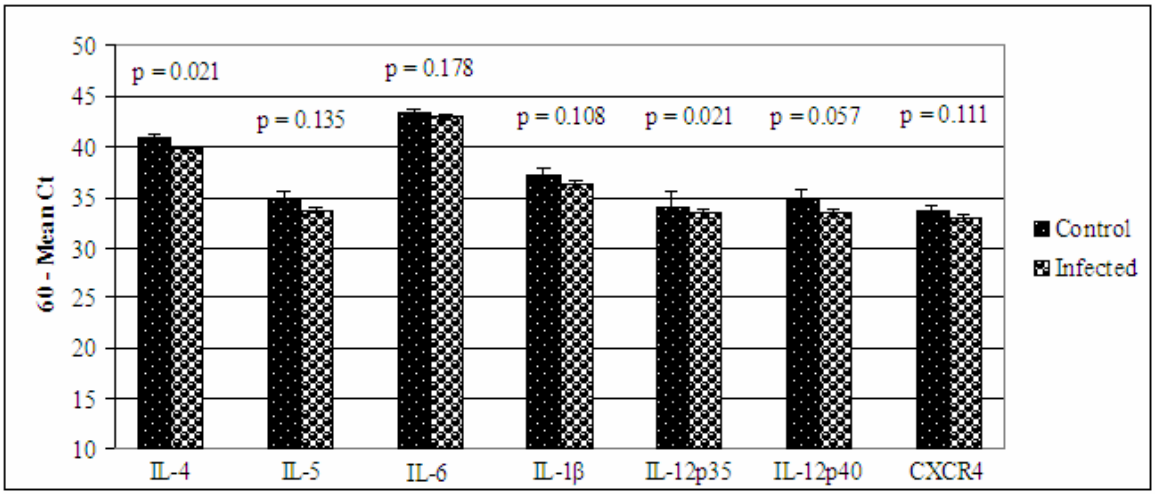

Figure 6.4 Relative Expression of Immunomodulators in Trophoblasts from Late-Term Feline Placentas

Real-time RT-PCR analysis of feline placental trophoblastic expression of the pro-inflammatory cytokines (IL-6, IL-1β, IL-12p35, and IL-12p40), the anti-inflammatory cytokines (IL-4 and IL-5), and the chemokine receptor (CXCR4) in late-term FIV-infected (n = 5) versus uninfected (control) (n = 3) placentas. Bars represent mean Ct values substracted from a negative endpoint (60 - mean Ct), bracketed by standard errors of the mean. P values obtained from Wilcoxon rank sum test are noted. Alpha = 0.05.

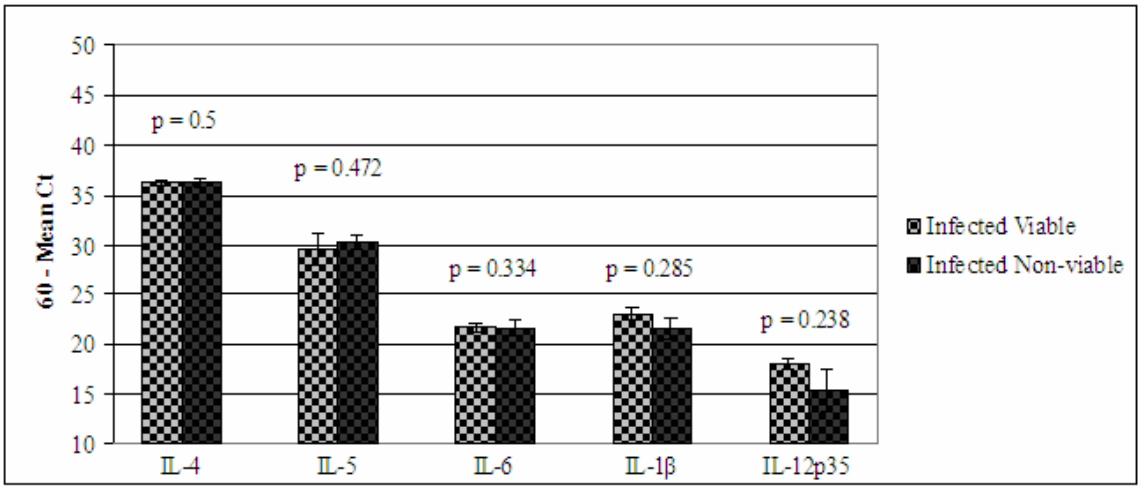

Figure 6.5 Relative Expression of Immunomodulators in Trophoblasts from Early-Term FIV-Infected Feline Placentas

Real-time RT-PCR analysis of trophoblastic expression of the pro-inflammatory cytokines (IL-6, IL-1β, and IL-12p35) and the anti-inflammatory cytokines (IL-4 and IL-5) in placentas from FIV-infected viable (n = 2) versus FIV-infected non-viable (n = 3) pregnancies. Bars represent mean Ct values substracted from a negative endpoint (60 - mean Ct), bracketed by standard errors of the mean. P values obtained from Wilcoxon rank sum test are noted. Alpha = 0.05.

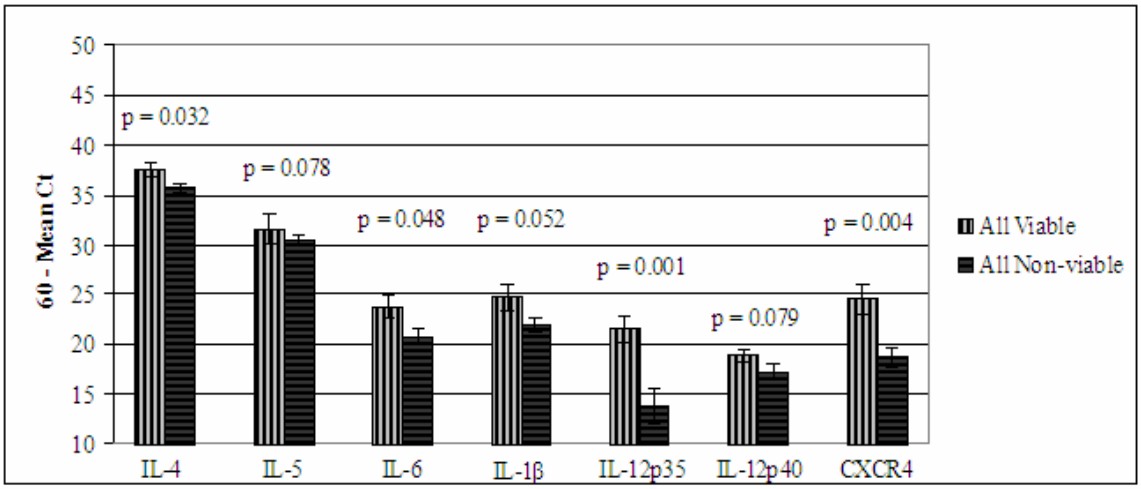

Figure 6.6 Relative Expression of Immunomodulators in Trophoblasts from Early-Term Feline Placentas from Viable and Non-viable Pregnancies

Real-time RT-PCR analysis of trophoblastic expression of the pro-inflammatory cytokines (IL-6, IL-1β, IL-12p35, and IL-12p40), the anti-inflammatory cytokines (IL-4 and IL-5), and the chemokine receptor (CXCR4) in early-term placentas from all viable (n = 4) versus all non-viable (n = 4) pregnancies. Bars represent mean Ct values substracted from a negative endpoint (60 - mean Ct), bracketed by standard errors of the mean. P values obtained from Wilcoxon rank sum test are noted. Alpha = 0.05.

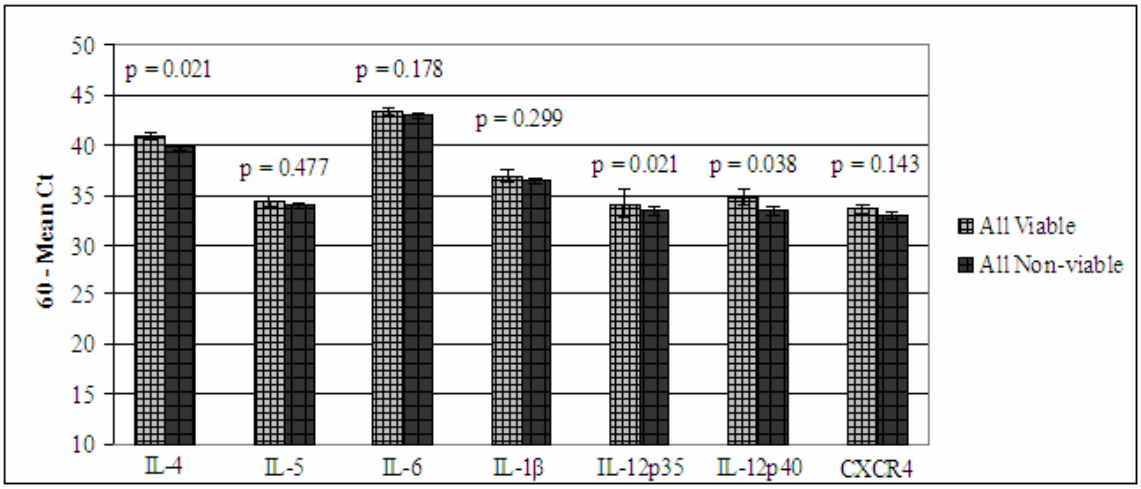

Figure 6.7 Relative Expression of Immunomodulators in Trophoblasts from Late-Term Feline Placentas from Viable and Non-viable Pregnancies

Real-time RT-PCR analysis of trophoblastic expression of the pro-inflammatory cytokines (IL-6, IL-1β, IL-12p35, and IL-12p40), the anti-inflammatory cytokines (IL-4 and IL-5), and the chemokine receptor (CXCR4) in late-term placentas from all viable (n = 4) versus all non-viable (n = 4) pregnancies. Bars represent mean Ct values substracted from a negative endpoint (60 - mean Ct), bracketed by standard errors of the mean. P values obtained from Wilcoxon rank sum test are noted. Alpha = 0.05.

Discussion

HIV infection of pregnant women contributes to reproductive failure. Langston et al. (1995) reported spontaneous fetal death in 14 of 124 (11.3%) HIV-infected pregnancies during 8 to 32 weeks gestation. In addition, HIV-infected, pregnant women receiving the highly active antiretroviral therapy (HAART) were more likely to have complications during pregnancy and poor reproductive outcomes including intrauterine growth restriction, preterm labor, and low birth weights when compared to HIV-negative women (Olagbuji et al., 2009).

Similar to HIV, reproductive failure and fetal loss is associated with FIV-infection. Vertical transfer of FIV occurs commonly under experimental conditions producing some viable offspring among majority non-viable litters (Allison and Hoover, 2003; Boudreaux et al., 2009; Weaver et al., 2005). Using the FIV-infected, pregnant queen to evaluate compromised reproduction resulting from infection, we reported vertical transfer of FIV-B-2542 at early- and late-term pregnancy (Boudreaux et al., 2009; Weaver et al., 2005). In reports by our laboratory (Boudreaux et al., 2009; Weaver et al., 2005) and others (O'Neil et al., 1996), FIV-B-2542-infected queens transmitted the virus to more than 50% of their offspring. Boudreaux et al. (2009) reported that fetal non-viability (defined as fetal resorptions or developmentally-arrested fetuses) at early gestation was 4.7% (2/43 fetuses) in control cats and 22.2% (6/27 fetuses) in FIV-infected cats. We also reported high rates of fetal non-viability at late gestation (Weaver et al., 2005). In that report, fetal non-viability was 3.2% (1/31 fetuses) in control cats and 60% (15/25 fetuses) in FIV-infected cats. In addition, higher incidences of fetal non-

viability associated with acutely-infected versus chronically-infected queens (53% and 30%, respectively) was reported (O'Neil et al., 1995). Therefore, the FIV-infected, pregnant queen is an acceptable small animal model for the study of lentiviral transplacental transfer and pregnancy perturbation associated with infection.

The immunology of pregnancy is an inexplicable phenomenon, and the role of immunoregulatory molecules such as cytokines and chemokines in maintaining immune homeostatis during pregnancy remains unclear. Wegmann et al. (1993) proposed that successful pregnancy is biased toward a Th2 (anti-inflammatory) immune response with a suppressed Th1 (pro-inflammatory) immune response at the maternal-fetal interface. This hypothesis was validated when spontaneous abortion occurred in pregnant CBA/J x DBA/2 mice when administered exogenous Th1 cytokines (IL-2, TNF-α, and IFN-γ). However, spontaneous abortion in pregnant CBA/J x DBA/2 mice was prevented when mice were administered antibodies against Th1 cytokines or given the exogenous Th2 cytokines (IL-10, GM-CSF, and IL-13) (Chaouat et al., 1995; Clark et al., 1998). Similarly, women with recurrent spontaneous abortions expressed higher levels of Th1 cytokines, IFN-γ (Daher et al., 2004; Hill et al., 1995; Lim et al., 2000; Marzi et al., 1996; Raghupathy et al., 1999; Raghupathy et al., 2000), TNF-α (Daher et al., 2004; Raghupathy et al., 2000), and IL-2 (Hill et al., 1995; Marzi et al., 1996; Raghupathy et al., 2000) and decreased levels of Th2 cytokines, IL-10 (Hill et al., 1995; Marzi et al., 1996; Raghupathy et al., 1999; Raghupathy et al., 2000) , IL-6 (Lim et al., 2000; Raghupathy et al., 1999; Raghupathy et al., 2000), and IL-4 (Hill et al., 1995; Marzi et al., 1996; Raghupathy et al., 2000) when compared to normal pregnant women.

Therefore it appears that, like the murine model, normal human pregnancy is biased towards Th2 cytokine production and that Th1-type immunity is associated with reproductive failure.

In a previous report by our laboratory (Weaver et al., 2005), we evaluated expression of representative Th1 cytokines (IL-1β and IFN-γ) and a Th2 cytokine (IL-10) in late-term placental tissues from FIV-infected and control cats. Although we did not detect significant differences in the expression of Th1 and Th2 cytokines, there was a trend towards increased Th1 cytokine expression and decreased Th2 cytokine expression. Moreover, when cytokine expression was evaluated in placentas from all viable versus all non-viable pregnancies, there was increased expression of IL-1β and IFN-γ and no significant difference in the expression of IL-10. However, we were not able to evaluate early-term placental expression of these cytokines.

In Chapter III of this study, we evaluated the expression of representative pro-inflammatory cytokines (IL-6, IL-12p35, and IL-1β), the anti-inflammatory cytokine (IL-10), and the chemokine (SDF-1α) in early- and late-term placental tissues. We found that with the exception of IL-12p35, patterns of cytokine expression in control animals were as we predicted, Th2 cytokine mRNAs (IL-10 and IL-6) were higher at early gestation and decreased by late gestation, while pro-inflammatory mediator mRNAs (IL-1β and SDF-1α) were lower at early gestation and increased by late gestation. However, we did not evaluate the specific cell populations responsible for the production of cytokines and chemokines during pregnancy.

In this study, we evaluated feline placental immunopathology associated with lentiviral infection by studying cytokine expression in trophoblastic cells. By evaluating cytokine production in trophoblasts, we predicted that we would detect evidence of a pro-inflammatory microenvironment in the placentas associated with FIV infection and non-viable pregnancies. However, we did not see this pattern. We report expression of IL-4, IL-5, IL-6, IL-1β, IL-12p35, IL-12p40, and CXCR4 in feline placental trophoblasts at both time points during feline gestation. Increased expression of pro-inflammatory cytokines associated with reproductive failure was not evident. The relative expression of all trophoblastic immunomodulators increased in uninfected cats as gestation progressed, indicating increased immune function of trophoblasts at late pregnancy. At early gestation, FIV infection and non-viability was associated with decreased expression of all cytokines. However, only IL-5 (an anti-inflammatory cytokine) was significantly decreased in FIV-infected queens at early gestation. In addition, when immunomodulator expression in early-term placental trophoblasts from all viable pregnancies were compared to all non-viable pregnancies, non-viability was associated with significantly decreased expression of the anti-inflammatory cytokine IL-4 and two pro-inflammatory cytokines (IL-6 and IL-12p35). The low concentrations of IL-4 and IL-6 in early-term placental trophoblasts parallels the IL-4 and IL-6 expression patterns found in the endometrial tissue and deciduas of women with frequent implantation failures (Stewart-Akers et al., 1998) and recurrent abortions (Piccinni and Romagnani, 1996). IL-4 is a Th2 cytokine that is mainly involved in the peri-implantation period of placental development (Wilczynski, 2005). Together with transforming growth factor-β

(TGF-β), IL-4 mediates the process of trophoblast invasion during human placentation (Irving and Lala, 1995; Klentzeris, 1997; Piccinni et al., 2000a; Piccinni et al., 2000b). Moreover, IL-4 and IL-6 are responsible for the angiogenesis of trophoblastic villi (Jauniaux et al., 1996). In addition, IL-12 (a pro-inflammatory cytokine produced by macrophages) can impede the depth of trophoblast invasion by inducing trophoblast apoptosis (Bennett et al., 1996; Marzusch et al., 1997). Therefore, the decreased expression of IL-4, IL-6, and IL-12p35 may indicate that trophoblastic infiltration is hindered during early-term feline gestation, and the resulting lack of oxygen and nutrient supply from the uterus contributes to fetal non-viability. At late gestation, FIV infection and non-viability was associated with decreased expression of all cytokines. However, only IL-4 and IL-12p35 were significantly down-regulated in placental trophoblasts from infected queens. In addition, when immunomodulator expression in late-term placental trophoblasts from all viable pregnancies were compared to placental trophoblasts from all non-viable pregnancies, IL-4, both IL-12 genes, and CXCR4 were depressed. The low expression of IL-4 and IL-12p35 associated with non-viability parallels the expression of these immunomodulators at early-term gestation. However, the non-viability at late-term gestation suggests that the immunoregulatory molecules (IL-4 and IL-12p35) impact fetal viability, especially at early feline gestation.

We failed to detect the primary receptor for FIV (CD134) in any trophoblastic sample from early- or late-term pregnancy, and we were able to detect FIV in only one late-term placental sample from a fetal resorption. Therefore, direct viral infection of trophoblasts apparently did not affect trophoblast function. Perhaps the altered placental

microenvironment of the surrounding infected tissue indirectly affects trophoblast function. To our knowledge, this is the first report describing lentivirus-induced placental immunomodulation in feline trophoblasts.

Our data do not support the Th1/Th2 paradigm of pregnancy proposal by Wegmann et al. (1993). We found expression of pro-inflammatory and anti-inflammatory cytokines at both time points during feline gestation. We were unable to associate the production of pro-inflammatory cytokines with the occurrence of reproductive failure. Others have reported patterns of cytokine expression that also challenge the paradigm. Bates et al. (2002) reported increased production of IFN-γ and decreased production of IL-10 and IL-4 in peripheral blood mononuclear cells (PBMCs) of normal pregnant women when compared to women with recurrent pregnancy loss (RPL), repudiating the hypothesis that Th1 immunity is associated with fetal loss. Moreover, in that report, there was no significant difference in the expression of IFN-γ, IL-10, or IL-4 in women with a history of RPL who subsequently aborted when compared to RPL women with successful pregnancy outcomes. Interestingly, the concentrations of TNF-α in PBMCs were similar in RPL women with successful pregnancy outcomes and normal pregnant women. In addition, RPL women who subsequently aborted expressed lower concentrations of TNF-α when compared to normal pregnant women (Bates et al., 2002).

We report a disparity in trophoblastic immunomodulator expression between placentas from HIV-infected women and the FIV-infected cat. Lee et al. (1997) reported cultured placental trophoblasts from HIV-infected women expressed significantly higher

amounts of IL-1β, IL-6, and TNF-α than placentas from uninfected women. These data indicate that HIV-infection during pregnancy results in increased cytokine expression and that a pro-inflammatory microenvironment (an environment potentially conducive to vertical transfer) exists in HIV-infected placentas. However, we did not see this trend in the animal model. In this report, we demonstrated increased immune function of trophoblasts late in gestation. However, this cellular population does not appear to support the replication of FIV. Therefore, there may be other immune cells that contribute to immunopathology, such as regulatory T cells (Tregs), which were shown to support the replication of FIV (Joshi et al., 2005; Joshi et al., 2004). Our laboratory is currently investigating feline Tregs in lentivirus-induced immunopathology and pregnancy failure.

Acknowledgements

We thank Dr. Jeffery Eells, College of Veterinary Medicine, Mississippi State University, for providing technical advice, laboratory facilities, and microscopy supplies. We are grateful to Dr. Janet R. Donaldson, Department of Biological Sciences, Mississippi State University for providing RT-PCR advisement, laboratory facilities, and reagent use. We thank Dr. Shane C. Burgess and Leslie A. Shack for laboratory facility use and quantitative PCR advisement. We are grateful to Dr. Edward A. Hoover, Colorado State University, for providing the FIV-B-2542 inoculum. We thank the veterinary staff at the College of Veterinary Medicine, Mississippi State University for assistance with animal surgical care. This project was supported by the National Institutes of Health (2R15AI048419-02A1) and (3R15AI048419-02A1S1).

References

Allison, R.W., Hoover, E.A., 2003, Covert vertical transmission of feline immunodeficiency virus. AIDS Res Hum Retroviruses 19, 421-434.

Bates, M.D., Quenby, S., Takakuwa, K., Johnson, P.M., Vince, G.S., 2002, Aberrant cytokine production by peripheral blood mononuclear cells in recurrent pregnancy loss? Hum Reprod 17, 2439-2444.

Bennett, W.A., Lagoo-Deenadayalan, S., Brackin, M.N., Hale, E., Cowan, B.D., 1996, Cytokine expression by models of human trophoblast as assessed by a semiquantitative reverse transcription-polymerase chain reaction technique. Am J Reprod Immunol 36, 285-294.

Bennett, W.A., Lagoo-Deenadayalan, S., Stopple, J.A., Barber, W.H., Hale, E., Brackin, M.N., Cowan, B.D., 1998, Cytokine expression by first-trimester human chorionic villi. Am J Reprod Immunol 40, 309-318.

Boudreaux, C.E., Lockett, N.N., Chemerys, D.N., Clay, B.T., Scott, V.L., Willeford, B., Brown, T., Coats, K.S., 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. Vet Immunol Immunopathol 131, 290-297.

CDC 2007. Mother-to-Child (Perinatal) HIV Transmission and Prevention. In CDC HIV/AIDS Fact Sheet (Department of Health and Human Services, USA).

CDC 2008. Cases of HIV Infection and AIDS in the United States and Dependent Areas, 2006.

Challis, J.R., Lockwood, C.J., Myatt, L., Norman, J.E., Strauss, J.F., 3rd, Petraglia, F., 2009, Inflammation and pregnancy. Reprod Sci 16, 206-215.

Chaouat, G., Assal Meliani, A., Martal, J., Raghupathy, R., Elliott, J.F., Mosmann, T., Wegmann, T.G., 1995, IL-10 prevents naturally occurring fetal loss in the CBA x DBA/2 mating combination, and local defect in IL-10 production in this abortion-prone combination is corrected by in vivo injection of IFN-tau. J Immunol 154, 4261-4268.

Clark, D.A., Chaouat, G., 1989, What do we know about spontaneous abortion mechanisms? Am J Reprod Immunol 19, 28-37.

Clark, D.A., Chaouat, G., Arck, P.C., Mittruecker, H.W., Levy, G.A., 1998, Cytokine-dependent abortion in CBA x DBA/2 mice is mediated by the procoagulant fgl2 prothrombinase [correction of prothombinase]. J Immunol 160, 545-549.

Costeas, P.A., Koumouli, A., Giantsiou-Kyriakou, A., Papaloizou, A., Koumas, L., 2004, Th2/Th3 cytokine genotypes are associated with pregnancy loss. Hum Immunol 65, 135-141.

D'Ubaldo, C., Pezzotti, P., Rezza, G., Branca, M., Ippolito, G., 1998, Association between HIV-1 infection and miscarriage: a retrospective study. DIANAIDS Collaborative Study Group. Diagnosi Iniziale Anomalie Neoplastiche AIDS. Aids 12, 1087-1093.

Daher, S., de Arruda Geraldes Denardi, K., Blotta, M.H., Mamoni, R.L., Reck, A.P., Camano, L., Mattar, R., 2004, Cytokines in recurrent pregnancy loss. J Reprod Immunol 62, 151-157.

Douglas, G.C., Fry, G.N., Thirkill, T., Holmes, E., Hakim, H., Jennings, M., King, B.F., 1991, Cell-mediated infection of human placental trophoblast with HIV in vitro. AIDS Res Hum Retroviruses 7, 735-740.

Hill, J.A., Polgar, K., Anderson, D.J., 1995, T-helper 1-type immunity to trophoblast in women with recurrent spontaneous abortion. Jama 273, 1933-1936.

Irving, J.A., Lala, P.K., 1995, Functional role of cell surface integrins on human trophoblast cell migration: regulation by TGF-beta, IGF-II, and IGFBP-1. Exp Cell Res 217, 419-427.

Jauniaux, E., Gulbis, B., Schandene, L., Collette, J., Hustin, J., 1996, Distribution of interleukin-6 in maternal and embryonic tissues during the first trimester. Mol Hum Reprod 2, 239-243.

Joshi, A., Garg, H., Tompkins, M.B., Tompkins, W.A., 2005, Preferential feline immunodeficiency virus (FIV) infection of CD4+ CD25+ T-regulatory cells correlates both with surface expression of CXCR4 and activation of FIV long terminal repeat binding cellular transcriptional factors. J Virol 79, 4965-4976.

Joshi, A., Vahlenkamp, T.W., Garg, H., Tompkins, W.A., Tompkins, M.B., 2004, Preferential replication of FIV in activated CD4(+)CD25(+)T cells independent of cellular proliferation. Virology 321, 307-322.

Kedzierska, K., Crowe, S.M., 2001, Cytokines and HIV-1: interactions and clinical implications. Antivir Chem Chemother 12, 133-150.

Klentzeris, L.D., 1997, The role of endometrium in implantation. Hum Reprod 12, 170-175.

Kumar, R.M., Uduman, S.A., Khurranna, A.K., 1995, Impact of maternal HIV-1 infection on perinatal outcome. Int J Gynaecol Obstet 49, 137-143.

Lagaye, S., Derrien, M., Menu, E., Coito, C., Tresoldi, E., Mauclere, P., Scarlatti, G., Chaouat, G., Barre-Sinoussi, F., Bomsel, M., 2001, Cell-to-cell contact results in a selective translocation of maternal human immunodeficiency virus type 1 quasispecies across a trophoblastic barrier by both transcytosis and infection. J Virol 75, 4780-4791.

Langston, C., Lewis, D.E., Hammill, H.A., Popek, E.J., Kozinetz, C.A., Kline, M.W., Hanson, I.C., Shearer, W.T., 1995, Excess intrauterine fetal demise associated with maternal human immunodeficiency virus infection. J Infect Dis 172, 1451-1460.

Lee, B.N., Ordonez, N., Popek, E.J., Lu, J.G., Helfgott, A., Eriksen, N., Hammill, H., Kozinetz, C., Doyle, M., Kline, M., Langston, C., Shearer, W.T., Reuben, J.M., 1997, Inflammatory cytokine expression is correlated with the level of human immunodeficiency virus (HIV) transcripts in HIV-infected placental trophoblastic cells. J Virol 71, 3628-3635.

Lim, K.J., Odukoya, O.A., Ajjan, R.A., Li, T.C., Weetman, A.P., Cooke, I.D., 2000, The role of T-helper cytokines in human reproduction. Fertil Steril 73, 136-142.

Marzi, M., Vigano, A., Trabattoni, D., Villa, M.L., Salvaggio, A., Clerici, E., Clerici, M., 1996, Characterization of type 1 and type 2 cytokine production profile in physiologic and pathologic human pregnancy. Clin Exp Immunol 106, 127-133.

Marzusch, K., Buchholz, F., Ruck, P., Handgretinger, R., Geiselhart, A., Engelmann, L., Dietl, J., 1997, Interleukin-12- and interleukin-2-stimulated release of interferon-gamma by uterine CD56++ large granular lymphocytes is amplified by decidual macrophages. Hum Reprod 12, 921-924.

Mosmann, T.R., Coffman, R.L., 1989, Heterogeneity of cytokine secretion patterns and functions of helper T cells. Adv Immunol 46, 111-147.

O'Neil, L.L., Burkhard, M.J., Diehl, L.J., Hoover, E.A., 1995, Vertical transmission of feline immunodeficiency virus. AIDS Res Hum Retroviruses 11, 171-182.

O'Neil, L.L., Burkhard, M.J., Hoover, E.A., 1996, Frequent perinatal transmission of feline immunodeficiency virus by chronically infected cats. J Virol 70, 2894-2901.

Olagbuji, B.N., Ezeanochie, M.C., Ande, A.B., Oboro, V.O., 2009, Obstetric and perinatal outcome in HIV positive women receiving HAART in urban Nigeria. Arch Gynecol Obstet.

Orsi, N.M., Tribe, R.M., 2008, Cytokine networks and the regulation of uterine function in pregnancy and parturition. J Neuroendocrinol 20, 462-469.

Piccinni, M.P., Maggi, E., Romagnani, S., 2000a, Role of hormone-controlled T-cell cytokines in the maintenance of pregnancy. Biochem Soc Trans 28, 212-215.

Piccinni, M.P., Romagnani, S., 1996, Regulation of fetal allograft survival by a hormone-controlled Th1- and Th2-type cytokines. Immunol Res 15, 141-150.

Piccinni, M.P., Scaletti, C., Maggi, E., Romagnani, S., 2000b, Role of hormone-controlled Th1- and Th2-type cytokines in successful pregnancy. J Neuroimmunol 109, 30-33.

Raghupathy, R., 1997, Th1-type immunity is incompatible with successful pregnancy. Immunol Today 18, 478-482.

Raghupathy, R., Makhseed, M., Azizieh, F., Hassan, N., Al-Azemi, M., Al-Shamali, E., 1999, Maternal Th1- and Th2-type reactivity to placental antigens in normal human pregnancy and unexplained recurrent spontaneous abortions. Cell Immunol 196, 122-130.

Raghupathy, R., Makhseed, M., Azizieh, F., Omu, A., Gupta, M., Farhat, R., 2000, Cytokine production by maternal lymphocytes during normal human pregnancy and in unexplained recurrent spontaneous abortion. Hum Reprod 15, 713-718.

Robertson, S.A., Seamark, R.F., Guilbert, L.J., Wegmann, T.G., 1994, The role of cytokines in gestation. Crit Rev Immunol 14, 239-292.

Rogers, A.B., Hoover, E.A., 1998, Maternal-fetal feline immunodeficiency virus transmission: timing and tissue tropisms. J Infect Dis 178, 960-967.

Romagnani, S., 1994, Lymphokine production by human T cells in disease states. Annu Rev Immunol 12, 227-257.

Saito, S., 2001, Cytokine cross-talk between mother and the embryo/placenta. J Reprod Immunol 52, 15-33.

Scott, V.L., Burgess, S.C., Shack, L.A., Lockett, N.N., Coats, K.S., 2008, Expression of CD134 and CXCR4 mRNA in term placentas from FIV-infected and control cats. Vet Immunol Immunopathol 123, 90-96.

Shearer, W.T., Reuben, J., Lee, B.N., Popek, E.J., Lewis, D.E., Hammill, H.H., Hanson, I.C., Kline, M.W., Langston, C., 1997, Role of placental cytokines and inflammation in vertical transmission of HIV infection. Acta Paediatr Suppl 421, 33-38.

Stewart-Akers, A.M., Krasnow, J.S., Brekosky, J., DeLoia, J.A., 1998, Endometrial leukocytes are altered numerically and functionally in women with implantation defects. Am J Reprod Immunol 39, 1-11.

UNAIDS/WHO 2009a. AIDS Epidemic Update 2009. Joint United Nations Programme on HIV/AIDS.

UNAIDS/WHO 2009b. Towards Universal Access. Joint United Nations Programme on HIV/AIDS.

Vidricaire, G., Tardif, M.R., Tremblay, M.J., 2003, The low viral production in trophoblastic cells is due to a high endocytic internalization of the human immunodeficiency virus type 1 and can be overcome by the pro-inflammatory cytokines tumor necrosis factor-alpha and interleukin-1. J Biol Chem 278, 15832-15841.

Weaver, C.C., Burgess, S.C., Nelson, P.D., Wilkinson, M., Ryan, P.L., Nail, C.A., Kelly-Quagliana, K.A., May, M.L., Reeves, R.K., Boyle, C.R., Coats, K.S., 2005, Placental immunopathology and pregnancy failure in the FIV-infected cat. Placenta 26, 138-147.

Wegmann, T.G., Lin, H., Guilbert, L., Mosmann, T.R., 1993, Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a TH2 phenomenon? Immunol Today 14, 353-356.

Wilczynski, J.R., 2005, Th1/Th2 cytokines balance--yin and yang of reproductive immunology. Eur J Obstet Gynecol Reprod Biol 122, 136-143.

CHAPTER VII

SUMMARY

Pediatric AIDS is an integral part of the global HIV/AIDS pandemic. In the United States, pediatric AIDS cases (defined as AIDS occurring in children under the age of 13 years) represent 1% of the total number of AIDS cases (CDC, 2005), with mother-to-child transmission (MTCT) of the virus accounting for 90% of pediatric infections (CDC, 2008). Children who are infected *in utero* have a more rapid progression to AIDS and generally become symptomatic during the first year of life. In the absence of antiviral treatment, 50% of HIV-infected newborns will succumb to the disease before 24 months of age (UNAIDS/WHO, 2009). Moreover, transplacental transfer of HIV results in poor reproductive outcomes, including low birth weight babies, preterm delivery, and an increased incidence of spontaneous abortions (D'Ubaldo et al., 1998; Kumar et al., 1995; Langston et al., 1995).

Using the FIV-infected cat as a model for lentiviral transplacental transfer, we previously demonstrated frequent reproductive failure in litters delivered at early- and late-term gestation by cesarean section (Boudreaux et al., 2009; Weaver et al., 2005). Vertical transmission of FIV occurred in all pregnancies and FIV was detected in the tissues of both viable and non-viable kittens and fetuses. Fetal non-viability was associated with early pregnancy, and litter size was larger in uninfected cats. In addition,

increased placental expression of Th1 cytokines was associated with FIV-infected, resorbed fetuses, suggesting that FIV infection may dysregulate placental cytokines during late-term gestation and compromise pregnancy (Weaver et al., 2005). However, neither early-term placental tissue nor the specific placental cell populations responsible for cytokine production were evaluated. Thus, we hypothesized that FIV infections may cause dysregulation of placental cytokine expression, probably early in gestation, and aberrant expression of these cytokines may potentiate inflammation and transplacental infections.

In Chapter II of this dissertation, we reported the first evidence of the FIV primary receptor, CD134 at the feline maternal-fetal interface. Expression of CD134 and the FIV co-receptor, CXCR4 was observed in all late-term placental tissues. The presence of these receptors may render the placenta vulnerable to FIV infection, possibly facilitating efficient vertical transmission of FIV, and impact pregnancy outcome. We found that placentas from FIV-infected queens producing litters of only viable offspring expressed more CD134 and CXCR4 mRNA than those from uninfected queens, suggesting that infection may cause upregulation of the receptors. On the other hand, placentas from FIV-infected cats with non-successful pregnancies expressed similar levels of CD134 mRNA and slightly less CXCR4 mRNA than those from uninfected queens. Thus, it appears that cells that express these receptors may play a role in pregnancy maintenance. A separate study in our laboratory is currently examining whether regulatory T cells, which express both markers and are preferentially infected by FIV, may contribute to pregnancy outcome in the FIV-infected cat.

In Chapter III, we evaluated cytokine dysregulation in placental tissues from early- and late-term pregnancy. We quantified the expression of representative pro-inflammatory cytokines (IL-6, IL-12p35, and IL-1β), the anti-inflammatory cytokine IL-10, and the chemokine SDF-1α in early- and late-term placental tissues and evaluated whether immunomodulator expression was related to fetal FIV infection. With the exception of IL-12p35, patterns of cytokine expression in control animals were as we predicted, Th2 cytokine mRNAs (IL-10 and IL-6) were higher at early gestation and decreased by late gestation, while pro-inflammatory mediator mRNAs (IL-1β and SDF-1α) were lower at early gestation and increased by late gestation. We report that FIV-infection during early gestation was associated with increased expression of two pro-inflammatory cytokines, IL-6 and IL-12p35, and decreased expression of IL-10, supporting the idea that a virus-induced inflammatory microenvironment occurred at early pregnancy. At late gestation, IL-6 expression was significantly increased while IL-1β and SDF-1α were decreased. Only IL-6 placental expression was consistently affected by viral infection, increasing in FIV-infected cats at both gestational stages. IL-6 is a multi-functional cytokine produced by both immune cells and trophoblasts that promotes HCG production in early human pregnancy. However, the function of IL-6 in feline gestation is unknown. Placentas from non-viable fetuses produced significantly decreased SDF-1α and IL-12p35, but no other differences in cytokine expression were detected. SDF-1α is the chemokine ligand for CXCR4. The decreased expression in non-viable pregnancies correlate with decreased CXCR4 expression in the same cats discussed above. While the impact that these immunopathological findings may have on

fetal infection and reproductive failure are not yet known, we found that FIV infection clearly alters the placental cytokine microenvironment.

In Chapter IV, we report a novel feline placental immunohistochemistry method for specifically targeting feline cytokeratin antigens and selectively labeling placental trophoblasts. We found that like human placental tissue, the feline placenta presents a unique challenge to the use of immunolabeling for detection of placental antigens. Similar to immunohistochemistry studies involving human placental tissue, we encountered problems with non-specific immunoreactivity using traditional IHC methods. After we determined that immunoreactivity could not be eliminated with altered blocking methods, we developed an innovative immunohistochemistry procedure utilizing a wide spectrum rabbit polyclonal antibody and goat anti-rabbit secondary polyclonal antibody to label cytokeratin specifically in feline trophoblasts and eliminate non-specific binding. Specifically labeling placental trophoblasts was necessary for us to determine the role of trophoblasts in FIV-induced placental immunopathology.

In order to continue our evaluation of feline trophoblasts and their role in FIV-induced immunopathology, in Chapter V we evaluated the integrity of frozen feline placental tissues using hematoxylin and eosin (H&E) staining. We found that hematoxylin and eosin staining of FIV-B-2542-infected and uninfected samples resulted in stained cells with blue nuclei and pink cytoplasm and confirmed that tissue integrity was not compromised during frozen storage. We also identified and collected placental trophoblasts from early- and late-term FIV-infected and uninfected queens using our novel IHC procedure and laser capture microdissection.

In Chapter VI, we reported the first evidence of placental immunomodulator expression in feline trophoblasts. We evaluated cytokine dysregulation in placental cells known to produce cytokines and chemokines that modulate pregnancy, the trophoblasts. We suspected that FIV infections may cause dysregulation of trophoblastic cytokine expression at early- and late-term pregnancy, and aberrant expression of these cytokines may potentiate inflammation and transplacental infections. We quantified the expression of Th1 cytokines (TNF-α, IFN-γ, IL-1β, IL-2, IL-12p35, IL-12p40, and IL-18), the Th2 cytokines (IL-4, IL-5, IL-6, and IL-10), the cytokine GM-CSF, the primary FIV receptor CD134, the chemokine SDF-1α, the chemokine receptor CXCR4, and FIV gag in trophoblasts from early- and late-term pregnancy. We were able to detect only IL-4, IL-5, IL-6, IL-1β, IL-12p35, IL-12p40, and CXCR4 in feline placental trophoblasts from early- and late-term pregnancy. We found expression of these pro-inflammatory and anti-inflammatory cytokines at both time points during feline gestation. We report increased immune function of trophoblasts late in gestation. Only IL-5 (a Th2 cytokine) expression was altered (decreased) in FIV-infected queens at early gestation. However, when immunomodulator expression at early gestation was compared for all viable pregnancies versus all non-viable pregnancies (including both control and infected animals), IL-4 (a Th2 cytokine), IL-6 (a pleiotropic, multi-functional cytokine), and IL-12p35 (a pro-inflammatory cytokine) were all down-regulated. At late gestation, IL-4 and IL-12p35 were down-regulated in placentas from infected queens, and when all viable and all non-viable pregnancies were compared at this stage, IL-4, both IL-12 genes, and CXCR4 were depressed. While we did not find that increased expression of

pro-inflammatory cytokines accompanied reproductive failure as we had predicted, FIV infection did alter expression of some cytokines in trophoblasts, and reproductive failure was accompanied by down-regulation of both pro- and anti-inflammatory cytokines. Interestingly, FIV gag was detected in only one trophoblast specimen, and CD134 was not detected. Thus, feline trophoblasts do not appear to be easily infected by FIV. Therefore, it is likely that altered immune or physiological function within the surrounding placental microenvironment influence trophoblast function, rather than direct viral infection of these cells.

References

Boudreaux, C.E., Lockett, N.N., Chemerys, D.N., Clay, B.T., Scott, V.L., Willeford, B., Brown, T., Coats, K.S., 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. Vet Immunol Immunopathol 131, 290-297.

CDC 2005. HIV AIDS Surveillance Report Cases of HIV infection and AIDS in the United States and Dependent Areas.

.

CDC 2008. Cases of HIV Infection and AIDS in the United States and Dependent Areas, 2006.

D'Ubaldo, C., Pezzotti, P., Rezza, G., Branca, M., Ippolito, G., 1998, Association between HIV-1 infection and miscarriage: a retrospective study. DIANAIDS Collaborative Study Group. Diagnosi Iniziale Anomalie Neoplastiche AIDS. Aids 12, 1087-1093.

Joshi, A., Garg, H., Tompkins, M.B., Tompkins, W.A., 2005, Preferential feline immunodeficiency virus (FIV) infection of CD4+ CD25+ T-regulatory cells correlates both with surface expression of CXCR4 and activation of FIV long terminal repeat binding cellular transcriptional factors. J Virol 79, 4965-4976.

Joshi, A., Vahlenkamp, T.W., Garg, H., Tompkins, W.A., Tompkins, M.B., 2004, Preferential replication of FIV in activated CD4(+)CD25(+)T cells independent of cellular proliferation. Virology 321, 307-322.

Kumar, R.M., Uduman, S.A., Khurranna, A.K., 1995, Impact of maternal HIV-1 infection on perinatal outcome. Int J Gynaecol Obstet 49, 137-143.

Langston, C., Lewis, D.E., Hammill, H.A., Popek, E.J., Kozinetz, C.A., Kline, M.W., Hanson, I.C., Shearer, W.T., 1995, Excess intrauterine fetal demise associated with maternal human immunodeficiency virus infection. J Infect Dis 172, 1451-1460.

UNAIDS/WHO 2009. Towards Universal Access. Joint United Nations Programme on HIV/AIDS.

Weaver, C.C., Burgess, S.C., Nelson, P.D., Wilkinson, M., Ryan, P.L., Nail, C.A., Kelly-Quagliana, K.A., May, M.L., Reeves, R.K., Boyle, C.R., Coats, K.S., 2005, Placental immunopathology and pregnancy failure in the FIV-infected cat. Placenta 26, 138-147.

APPENDIX A

FELINE-SPECIFIC PRIMER DESIGN FOR THE QUANTIFICATION OF CYTOKINE mRNA IN FELINE TROPHOBLASTS

Abstract

We used the FIV-infected cat to model HIV-induced placental immunopathology, detecting increased inflammatory cytokine expression from resorbed fetal tissue. We hypothesize that FIV dysregulates cytokine expression in trophoblasts, potentiating inflammation and *in utero* infections. The purpose of this project was to design feline-specific primers that span intron-exon junctions to target mRNA of placental immunomodulators. The mRNA sequences for the cat and comparative mammals were obtained from the National Center for Biotechnology Information (NCBI) and aligned using the European Bioinformatics Institute (EBI) ClustalW alignment tool. Human mRNA was blasted against the human genome to find exons, which were used to identify homologous exons in the feline sequence. Feline mRNA sequences were loaded into Beacon Designer, and primer/probe sets were selected in which one of the primers spanned the junction. We evaluated the linear regressions and amplification efficiencies of each duplex partner using standard curves generated from real-time RT-PCR. We found variations between the standard curve slope values for β-actin and the target genes, an unexpected result and an indicator of non-optimal amplification efficiency. However, all R^2 values were close to 1.0, suggesting that our dilution of template was an accurate predictor of the cycle threshold value. Therefore, the designed primer/probe sets are optimized to quantify feline placental cytokine mRNA.

Introduction

The feline immunodeficiency virus (FIV)-infected cat is a well accepted animal model to study HIV-induced placental immunopathology. Maternal-fetal transmission of FIV readily occurs under experimental conditions, resulting in infected viable kittens and resorbed or arrested fetal tissues (Allison and Hoover, 2003; Boudreaux et al., 2009; O'Neil et al., 1996; Weaver et al., 2005). In a previous report by our lab (Weaver et al., 2005), increased inflammatory cytokine expression was associated with resorbed fetal tissue. We hypothesize that FIV dysregulates cytokine expression in placental tissue, potentiating inflammation and *in utero* infections. The purpose of this project was to design feline-specific primers that span intron-exon junctions to target mRNA of placental immunomodulators.

Materials and Methods

Primer design of immunomodulators and receptors

Representative pro-inflammatory and anti-inflammatory cytokines and chemokines involved in the maintenance of pregnancy, the receptors for FIV, and the internal control gene were selected for primer design. Cytokine mRNA sequences for the cat and other comparative mammals (human, dog, cow, and pig) were obtained from the National Center for Biotechnology Information (NCBI) and aligned using the European Molecular Biology Laboratory-European Bioinformatics Institute (EMBL-EBI) ClustalW alignment tool. The human mRNA was blast-searched against the human genome to

locate the exon/intron boundaries. These boundaries were used to find the homologous boundaries in the feline sequence. Beacon Designer (PREMIER Biosoft) was used to design primer/probe sets targeting the representative pro-inflammatory cytokines (IL-1β, IL-12p35, and IL-12p40), Th2 cytokines (IL-6 and IL-10), the chemokine (SDF-1α), the chemokine receptor and FIV co-receptor (CXCR4), the co-stimulatory molecule and FIV primary receptor (CD134), and the internal control gene (β-actin). All PCR amplicons spanned intron-exon junctions. The target probes were 5' labeled with the reporter dye FAM (6-carboxyfluorescein) and 3' labeled with the quencher dye TAMRA (6-carboxytetramethylrhodamine). The probe for the housekeeping gene was 5' labeled with the reporter dye HEX (hexachloro-6-carboxyfluorescein) and 3' labeled with the quencher dye TAMRA. The best primer/probe set for each gene of interest was chosen based upon primer length, melting temperature, and GC content. Primers chosen were between 9–40 bases in length, had melting temperatures (Tm) between 58–60°C, and a GC content of 40–80%. Probes selected were between 9–40 bases in length, had melting temperatures that were 10°C higher than the primer Tm, and a GC content of 20–80%. All amplicons were between 50–150 bp in length. Primers and probes were obtained commercially (MWG-BIOTECH, Inc., High Point, NC).

Reference primer and probe sets

Primer/probe sets for IL-2 (Taglinger et al., 2008), IL-4 (Taglinger et al., 2008), IL-5 (Taglinger et al., 2008), IL-18 (Foley et al., 2003), IFN-γ (Leutenegger et al., 1999),

GM-CSF (Kipar et al., 2006), TNF-α (Kipar et al., 2001), and FIV gag (Weaver et al., 2005) were obtained commercially (MWG-BIOTECH, Inc., High Point, NC).

Generation of standard curves

Real-time reverse transcriptase-PCR (rtRT-PCR) targeting β-actin and feline genes in placental RNA was done using an iCycler (BioRad Laboratories, Valencia, CA) according to established parameters (Scott et al., 2008). To determine the linear range and amplification efficiencies of the targeted genes, pooled placental RNA from all early- and late-term uninfected cats (described in Chapter III) was serially diluted and amplified in triplicate reactions. The resulting cycle threshold (Ct) values for each two-fold dilution were used to generate standard curves for each duplex-partner amplicon and the linear regression was calculated. Standard curves for FIV gag were previously generated (Boudreaux et al., 2009; Weaver et al., 2005).

Results

Feline-specific primer design

Feline placental immunomodulator-specific primers that spanned intron-exon junctions were successfully designed. Designed primer and probe sets are listed in Table A.1. Reference primer and probe sets are listed in Table A.2.

Linear regressions and amplification efficiencies of β-actin and feline cytokine cDNA

Standard curves generated to determine the linear regressions (R^2 values) and amplification efficiencies (slopes) of each duplex partner are shown in Figures A.1–A.4. All R^2 values and slopes for each target gene are listed in Tables A.3–A.6. Variation between the standard curve slope values for β-actin and the target genes indicated non-optimal amplification efficiencies. However, all R^2 values were close to 1.0, suggesting that all points on the standard curve were in a straight line and that our dilution of template was an accurate predictor of the cycle threshold value. In other words, the standard curves show that as template was diluted, higher Ct values were generated. This result indicates that our designed primer/probe sets were efficiently optimized to quantify placental cytokine mRNA.

Table A.1

Sequences of PCR Primers and TaqMan Probes Specific for Feline Cytokines, FIV Receptors, and the Internal Control mRNA

Gene	Primer	Sequence (5'–3')	Length	Accession	Probe sequence (5'–3')
β-Actin	Sense	GACTACCTCATGAAGATCCTCACG	24	AB051104	ACAGTTTCACCACCACCGCCGAGC
	Anti-sense	CCTTGATGTCACGCACAATTTCC	23		
IL-1β	Sense	ATTGTGGCTATGGAGAAACTGAAG	24	M92060	TTTGCCTGCTCACAACCCCTCCAG
	Anti-sense	T**CT**TCTTCAAAGATGCAGCAAAAG	24		
IL-6	Sense	GTGTGACAACTATAACAAATGTGAGG	26	L16914	CAAGGAGGCACTGGCAGAAAACAACCT
	Anti-sense	GTCT**CCT**GATTGAACCCAGATTG	23		
IL-10	Sense	ACTTTCTT**TC**AAACCAAGGACGAG	24	AF060520	TCTCGGACAAGGCTTGGCAACCCA
	Anti-sense	GGCATCACCTCCTCCAAATAAAAC	24		
IL-12p35	Sense	ACACCAAGCCCAGGAATGTTC	21	U83185	AACCACTCCCAAACCCTGCTGCGA
	Anti-sense	TGG**CCT**TCTGAAGCGTGTTG	20		
IL-12p40	Sense	GAAGTACACAGTGGAGTGTCAGG	23	U83184	CAGTGCCTGCCCGGCTGCCG
	Anti-sense	GGTTTG**AT**GATGTCCCTGATGAAG	24		
CD134	Sense	**CAGG**TTATGGGATGGAGAGTCG	22	AY738589	TGACCAGGACACCAAGTGCCTCCAGTG
	Anti-sense	TGCAAGGCTCGTAGTTCACG	20		
CXCR4	Sense	AAGGCAGTCCATGTCATCTACAC	23	AJ009816	ACCTCTACAGCAGTGTCCTCATCCTGGC
	Anti-sense	AGACCACCTTTTCAGCCAACAG	22		
SDF-1α	Sense	GCTACAGATGTCCTTGCCGATTC	23	AB011965	TCGAGAGCCACGTTGCCAGAGCCA
	Anti-sense	TCTTCAGCCTCG**CCAC**GATC	20		

Intron-exon junctions are in bold and underlined.

Table A.2

Reference Sequences of PCR Primers and TaqMan Probes Specific for Feline Gene mRNA

Gene	Primer	Sequence (5'–3')	Product Length (bp)	Probe sequence (5'–3')	Reference
IL-2	Sense Anti-sense	ACGGTTGCTTTTGAATGGAG CAATTCTGTGGCCTTCTTGG	97	CCCCAAACTCTCCAGGATGCTCA	Taglinger et al., 2008
IL-4	Sense Anti-sense	CCCCTAAGAACACAAGTGACAAG CCTTTGAGGAATTTGGTGGAG	100	TTCTGCAGAGCCACAACCGTGC	Taglinger et al., 2008
IL-5	Sense Anti-sense	TGCTTCTGCATTTGAGTTTG CAGCCTATTCATGGGACTTTG	90	TGGCAGAAACATAGGCAGCCCC	Taglinger et al., 2008
IL-18	Sense Anti-sense	GGAGATCAACCTGTGTTTGAGGAT GATGGTTACTGCCAGACCTCTAGTG	105	ATTCTGACTGTACAGATAATGCACCCCGGAC	Foley et al., 2003
Gag	Sense Anti-sense	GTATGATCGTACTCATCCTCCTGAT TCTACATTGCATTCTGGCTGGT	–	AGACCACTGCCCTACTTCACTGCCG	Weaver et al., 2005
GM-CSF	Sense Anti-sense	AATGAAACGGTAGAAGTCGTCTCTG CGTACAGCTTTAGGTGAGTCTGCA	–	TTGACCCTGAGGAGCCGAATTGCC	Kipar et al., 2006
IFN-γ	Sense Anti-sense	TGGTGGGTCGCTTTTCGTAG GAAGGAGACAATTTGGCTTTGAA	–	CATTTTGAAGAACTGGAAAGAGGAGAGTGATAAAACAAT	Leutenegger et al., 1999
TNF-α	Sense Anti-sense	CTTCTCGAACTCCGAGTGACAAG CCACTGGAGTTGCCCTTCA	74	TAGCCCATGTAGTAGCAAACCCCGAAGC	Kipar et al., 2001

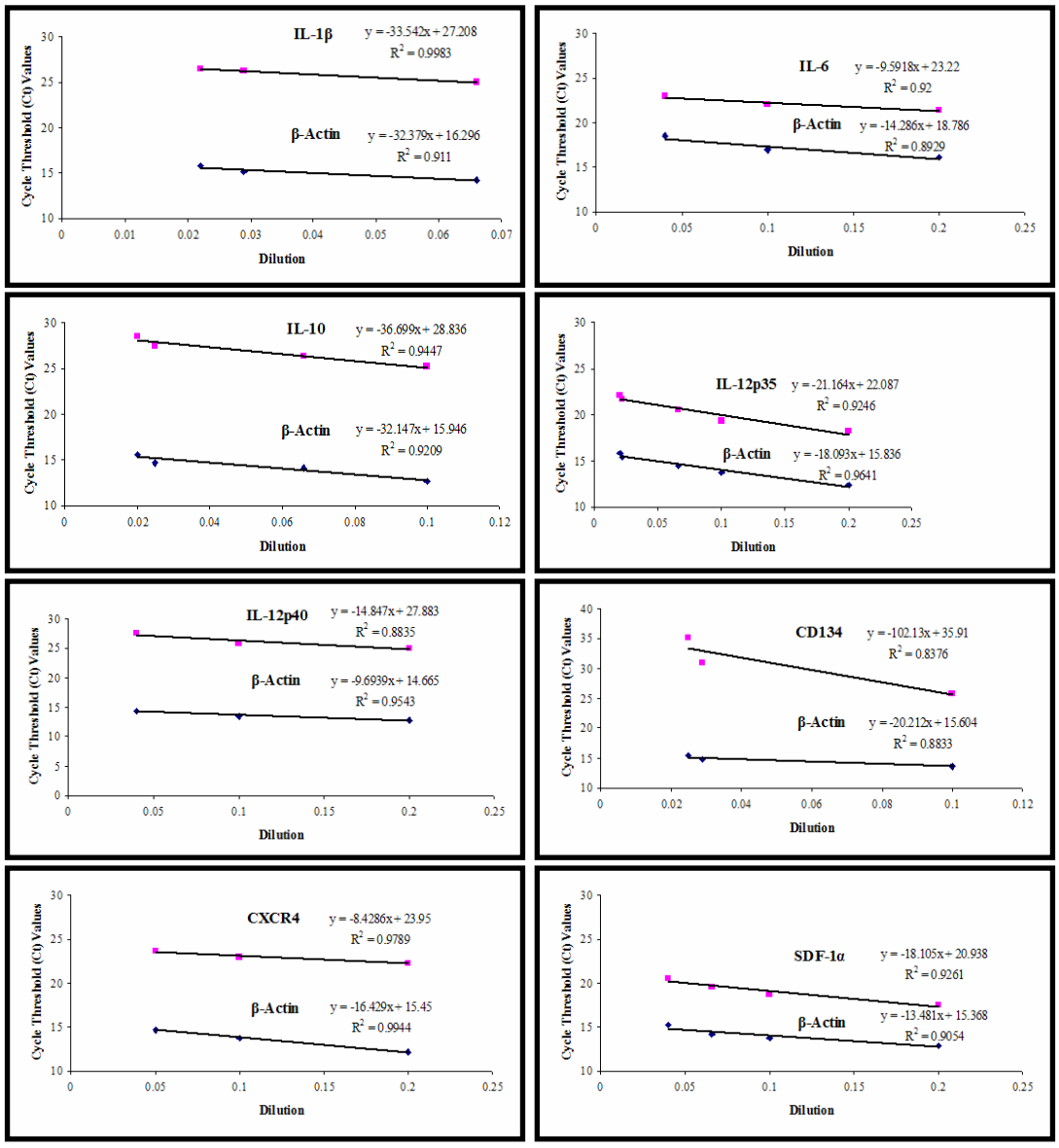

Figure A.1 Standard Curves Generated Using Designed Primer/Probe Sets for the Quantification of Feline Placental Cytokine mRNA from Early Gestation

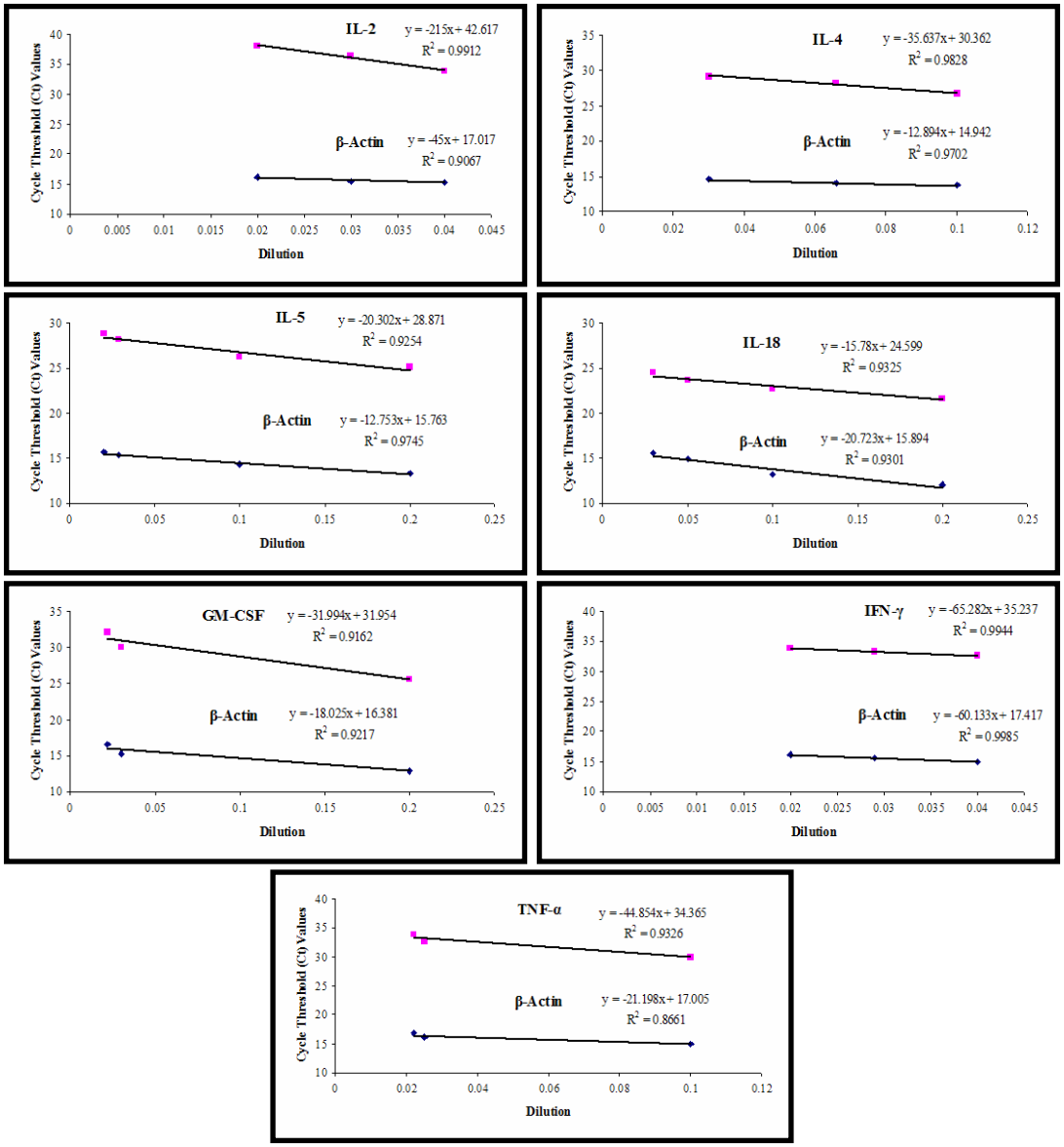

Figure A.2 Standard Curves Generated Using Reference Primer/Probe Sets for the Quantification of Feline Placental Cytokine mRNA from Early Gestation

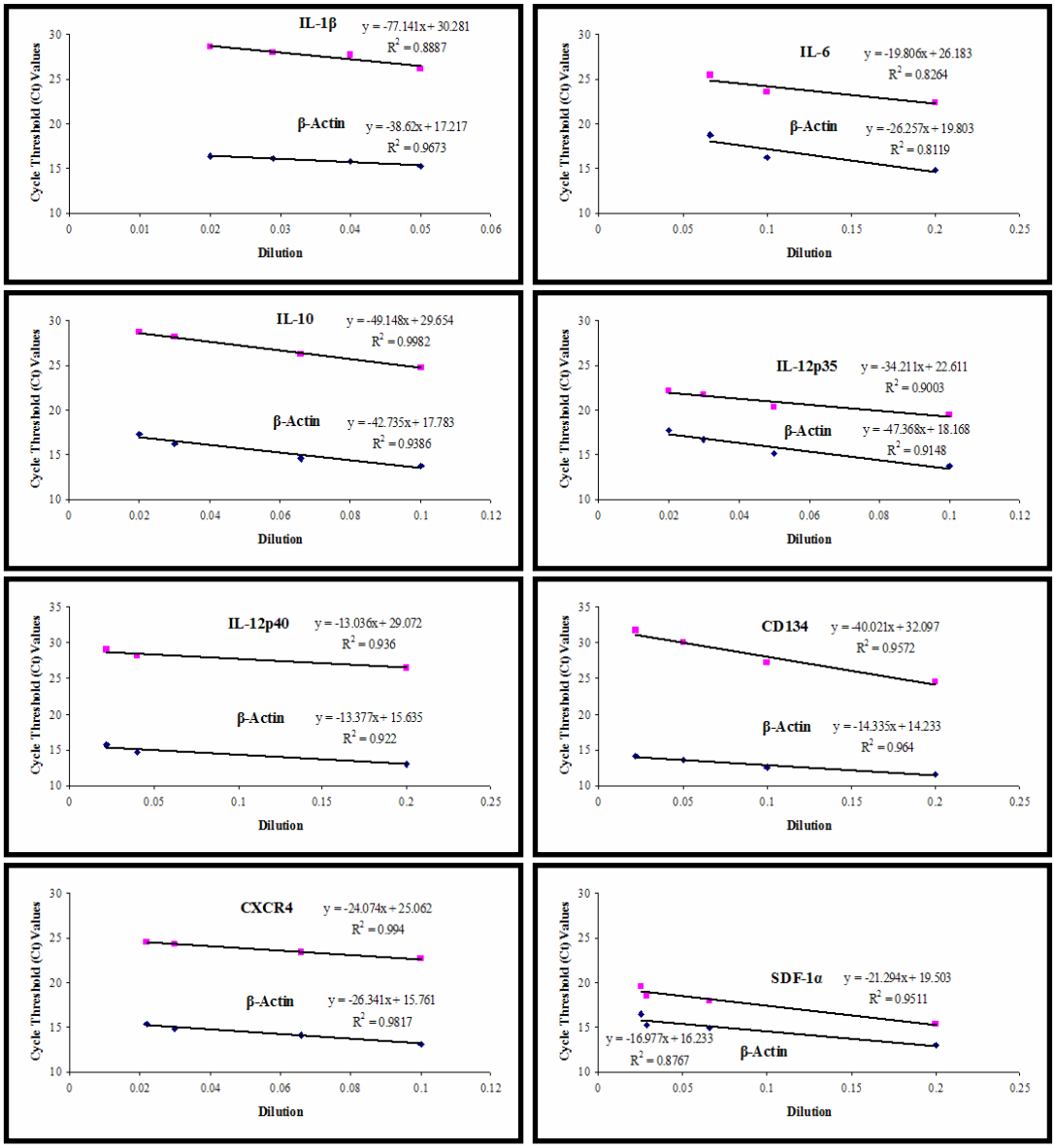

Figure A.3 Standard Curves Generated Using Designed Primer/Probe Sets for the Quantification of Feline Placental Cytokine mRNA from Late Gestation

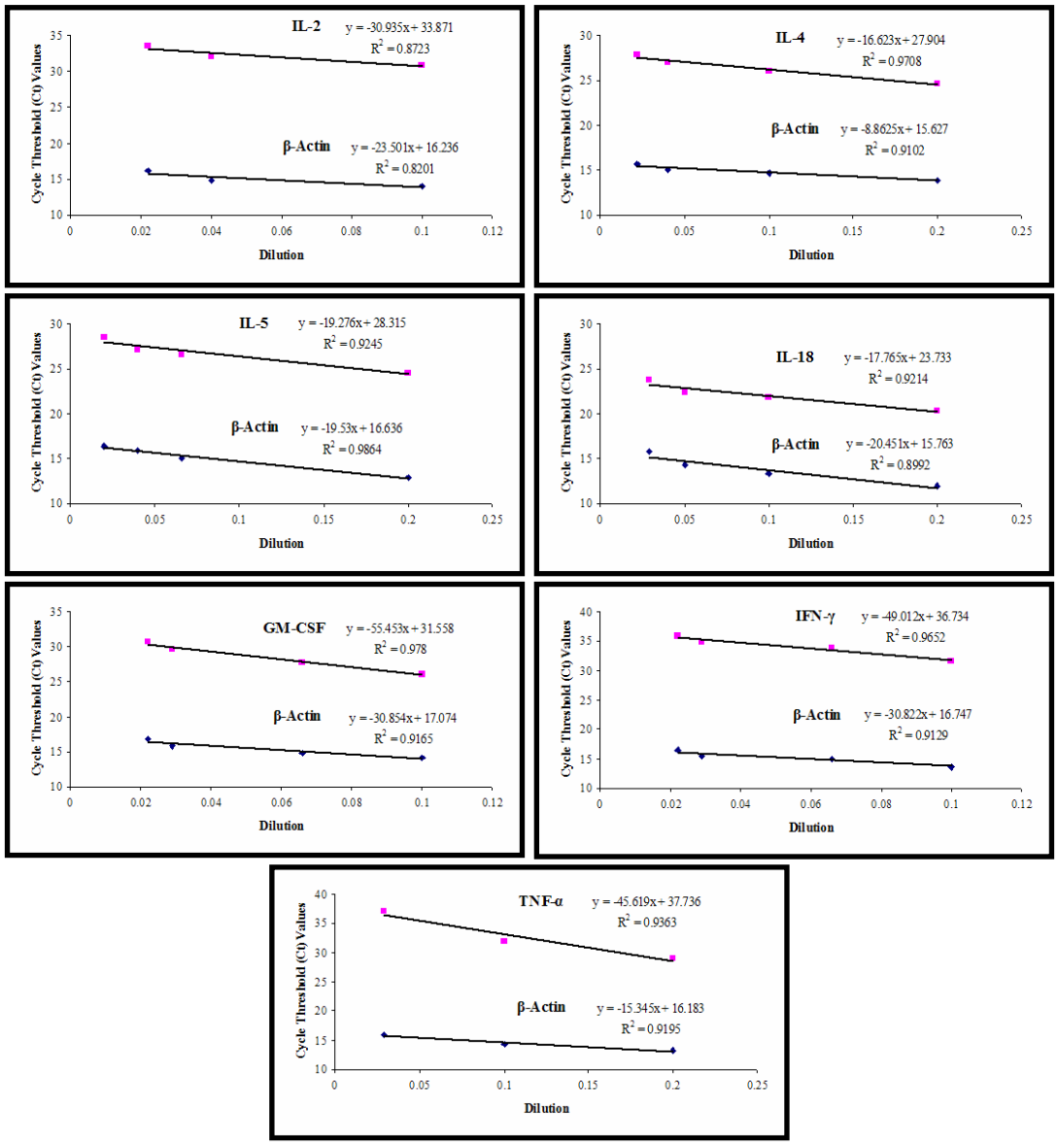

Figure A.4 Standard Curves Generated Using Reference Primer/Probe Sets for the Quantification of Feline Placental Cytokine mRNA from Late Gestation

Table A.3

Slopes and R^2 Values of the Standard Curves Generated Using Designed Primer/Probe Sets for the Quantification of Feline Placental Cytokine mRNA from Early-Term Gestation

Gene	IL-1β	IL-6	IL-10	IL-12p35	IL-12p40	CD134	CXCR4	SDF-1α
Slope	-33.542	-9.5918	-36.699	-21.164	-14.847	-102.13	-8.4286	-18.105
R^2	0.9983	0.92	0.9447	0.9246	0.8835	0.8376	0.9789	0.9261

Table A.4

Slopes and R^2 Values of the Standard Curves Generated Using Reference Primer/Probe Sets for the Quantification of Feline Placental Cytokine mRNA from Early-Term Gestation

Gene	IL-2	IL-4	IL-5	IL-18	GM-CSF	IFN-γ	TNF-α
Slope	-215	-35.637	-20.302	-15.78	-31.994	-65.282	-44.854
R^2	0.9912	0.9828	0.9254	0.9325	0.9162	0.9944	0.9326

Table A.5

Slopes and R^2 Values of the Standard Curves Generated Using Designed Primer/Probe Sets for the Quantification of Feline Placental Cytokine mRNA from Late-Term Gestation

Gene	IL-1β	IL-6	IL-10	IL-12p35	IL-12p40	CD134	CXCR4	SDF-1α
Slope	-77.141	-19.806	-49.148	-34.211	-13.036	-40.021	-24.074	-21.294
R^2	0.8887	0.8264	0.9982	0.9003	0.936	0.9572	0.994	0.9511

Table A.6

Slopes and R^2 Values of the Standard Curves Generated Using Reference Primer/Probe Sets for the Quantification of Feline Placental Cytokine mRNA from Late-Term Gestation

Gene	IL-2	IL-4	IL-5	IL-18	GM-CSF	IFN-γ	TNF-α
Slope	-30.935	-16.623	-19.276	-17.765	-55.453	-49.012	-45.619
R^2	0.8723	0.9708	0.9245	0.9214	0.978	0.9652	0.9363

Discussion

FIV may dysregulate placental immunology during pregnancy, potentiating inflammation and transplacental infections. Evaluating the expression of immunomodulators in placental tissue is thereby an effective method to determine the presence of an inflammatory microenvironment in the placenta. In this study, we designed feline-specific primers that targeted the mRNA of immunomodulators involved in the maintenance of pregnancy. We evaluated the linear regressions and amplification efficiencies of each duplex partner using standard curves generated from real-time RT-PCR. We found variations between the standard curve slope values for β-actin and the target genes which is normally a predictor of non-optimal amplification efficiency. Other investigators have reported optimal amplification efficiencies from similar slope values for feline cytokine genes and the internal control mRNA (Kipar et al., 2001). Although we do not have an explanation for the variation of slope values between β-actin and the target genes, all R^2 values were close to 1.0, suggesting that our dilution of template was an accurate predictor of the cycle threshold value. Therefore, we conclude that all primer/probe sets amplified the cDNA of the feline immunomodulators and are efficiently optimized for use in the quantification of placental cytokine mRNA. These primer/probe sets were used in quantitative PCR for the measurement of cytokine mRNA in feline placental trophoblasts.

References

Allison, R.W., Hoover, E.A., 2003, Covert vertical transmission of feline immunodeficiency virus. AIDS Res Hum Retroviruses 19, 421-434.

Boudreaux, C.E., Lockett, N.N., Chemerys, D.N., Clay, B.T., Scott, V.L., Willeford, B., Brown, T., Coats, K.S., 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. Vet Immunol Immunopathol 131, 290-297.

Foley, J.E., Rand, C., Leutenegger, C., 2003, Inflammation and changes in cytokine levels in neurological feline infectious peritonitis. J Feline Med Surg 5, 313-322.

Kipar, A., Leutenegger, C.M., Hetzel, U., Akens, M.K., Mislin, C.N., Reinacher, M., Lutz, H., 2001, Cytokine mRNA levels in isolated feline monocytes. Vet Immunol Immunopathol 78, 305-315.

Kipar, A., Meli, M.L., Failing, K., Euler, T., Gomes-Keller, M.A., Schwartz, D., Lutz, H., Reinacher, M., 2006, Natural feline coronavirus infection: differences in cytokine patterns in association with the outcome of infection. Vet Immunol Immunopathol 112, 141-155.

Leutenegger, C.M., Mislin, C.N., Sigrist, B., Ehrengruber, M.U., Hofmann-Lehmann, R., Lutz, H., 1999, Quantitative real-time PCR for the measurement of feline cytokine mRNA. Vet Immunol Immunopathol 71, 291-305.

O'Neil, L.L., Burkhard, M.J., Hoover, E.A., 1996, Frequent perinatal transmission of feline immunodeficiency virus by chronically infected cats. J Virol 70, 2894-2901.

Scott, V.L., Burgess, S.C., Shack, L.A., Lockett, N.N., Coats, K.S., 2008, Expression of CD134 and CXCR4 mRNA in term placentas from FIV-infected and control cats. Vet Immunol Immunopathol 123, 90-96.

Taglinger, K., Van Nguyen, N., Helps, C.R., Day, M.J., Foster, A.P., 2008, Quantitative real-time RT-PCR measurement of cytokine mRNA expression in the skin of normal cats and cats with allergic skin disease. Vet Immunol Immunopathol 122, 216-230.

Weaver, C.C., Burgess, S.C., Nelson, P.D., Wilkinson, M., Ryan, P.L., Nail, C.A., Kelly-Quagliana, K.A., May, M.L., Reeves, R.K., Boyle, C.R., Coats, K.S., 2005, Placental immunopathology and pregnancy failure in the FIV-infected cat. Placenta 26, 138-147.