INVASION AND SURVIVAL:

IMMUNOLOGICAL STUDIES

OF THE EQUINE CHORIONIC GIRDLE

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INVASION AND SURVIVAL: IMMUNOLOGICAL STUDIES OF THE EQUINE CHORIONIC GIRDLE

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Successful mammalian pregnancy requires precise modulation of normal biological processes to ensure the survival of the feto-placental tissues while preserving the good health of the mother. The original research presented in this dissertation encompasses studies in both of these areas. The first part of this work addresses the long-standing question as to how feto-placental tissues, specifically invasive trophoblast, survive in the hostile environment of the maternal immune system. The Antczak laboratory previously developed an ectopic trophoblast transplant system to facilitate studies of the maternal immune response to invasive trophoblast in the horse. Single transplants of horse chorionic girdle into the vulvar mucosa of non-pregnant horse recipients were found to have lifespans and physiologic effects upon recipients similar to endometrial cups in a normal horse pregnancy. The current studies first expanded the trophoblast transplant system to include multiple rather than single transplants in individual recipients. The objective of this was to examine ectopic trophoblast survival in an immunologically primed as compared to naïve recipient. These serial transplants had lifespans similar to the single transplants, suggesting that the mechanisms protecting the trophoblast from immune destruction are initiated by the trophoblast itself. A series of transplants then was performed using Jenny donkeys as recipients. In each donkey recipient, primary transplants had lifespans of similar length to the horse-to-horse transplants, with second

and subsequent transplants having progressively shorter lifespans. This pattern suggests that the horse-to-donkey transplants were subjected to a classic destructive memory response.

The second body of work addresses the question as to how normal placentation disrupts maternal anatomy and physiology without causing adverse effects. This work was initiated with the discovery of interleukin 22 (*IL22*) mRNA in day 34 chorionic girdle using gene expression array analysis. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) then revealed that *IL22* mRNA was rapidly upregulated in the chorionic girdle between days 32 and 35 of pregnancy. This represented the first identification of *IL22* expression by a non-immune cell type. Interleukin 22 receptor RA1 (*IL22RA1*) mRNA was identified in pregnant endometrium, and in situ hybridization localized the mRNA to both the luminal and glandular epithelia. A monoclonal antibody was then generated against horse IL22. Mice were immunized with a fusion protein containing recombinant horse IL4 and IL22. Murine splenic cells were fused with SP 2/O myeloma cells to generate hybridoma cell lines. A monoclonal antibody to equine IL22 was identified. IL22 protein was then successfully detected in day 35 chorionic girdle. Knowing that IL22 in other biological systems has a role in preserving the integrity of mucosal surfaces and in epithelial cell proliferation, it was hypothesized that IL22 produced from the chorionic girdle binds receptor in the endometrium, repairing the damage caused by trophoblast invasion and initiating endometrial gland development.

BIOGRAPHICAL SKETCH

Margaret Mary Brosnahan (Peggy) was born in Pittsburgh, PA on April 3, 1965, where she lived until the age of three. Following two years in Glen Ellyn, IL, her family moved to Cheshire, CT where she remained until departing for college. Peggy grew up the sole horse lover in her family. She became fascinated with horses at the age of five when left alone in a library she happened upon an evolutionary biology book showing illustrations of the oddly adorable *Eohippus*, the modern horse, and everything in between.

Peggy attended Bates College in Lewiston, ME receiving a B.A. in History in 1987. She graduated *Magna Cum Laude* with departmental honors in History, and was inducted into Phi Beta Kappa. Peggy then spent several years as a project manager with Andersen Consulting prior to attending veterinary school at Tufts University. She received her D.V.M. in 2002 and went on to complete an internship in Large Animal Medicine and Surgery at the University of Minnesota. After two years in private equine ambulatory practice, she then completed a residency in equine medicine and an M.S. in Veterinary Biomedical Sciences at Oklahoma State University. There she also was inducted into the veterinary honor society, Phi Zeta.

In 2008, Peggy came to Cornell University as a post-doctoral associate in the laboratory of Dr. Nikolaus Osterrieder. She applied to the graduate program during that year, and completed her Ph.D. in the laboratory of Dr. Douglas Antczak at the Baker Institute for Animal Health in 2014.

Dedicated to the memory of my mother, Margaret Patricia Foley Brosnahan,

and to my father, Brian Patrick Brosnahan.

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CHAPTER 1

INTRODUCTION

Overview

Mammalian pregnancy requires modulation of normal biological processes to preserve the good health of the mother while ensuring the survival of her offspring. The precarious situation of the fetus was described over sixty years ago by Sir Peter Medawar in his seminal definition of the immunological "problems" of pregnancy (Medawar, 1953). The genetically distinct conceptus exists in an environment where under non-pregnant conditions foreign tissue is swiftly destroyed by the maternal immune system. Survival of the fetus to term depends upon immunologic modifications that to this day are not fully defined (Jiang et al., 2014; Nancy and Erlebacher, 2014; Schumacher et al., 2014).

The growing fetus also demands sustenance from maternal nutrient stores, and in some species auxiliary endocrine support. The vehicle for this assistance is the placenta. Placentation is by design an invasive process, causing disruption and remodeling of maternal tissues. Cells of the trophoblast lineage are key players in this process. Though the extent of trophoblast invasion varies across species, maternal tissues affected may include uterine epithelium, stroma, myometrium and blood vessels (King, 1993; Pijenenborg et al., 2011a). Dysregulation of trophoblast invasion may result in conditions harmful to the mother such as pre-eclampsia (Young et al., 2010), morbidly adherent placenta (Abuhamad, 2013) or gestational trophoblastic disease (Seckl et al., 2010). In the horse, dysregulation of the life cycle of invasive trophoblast can result in a condition known as persistent endometrial cups. While not fatal to the mare, this can result in loss of fertility (Crabtree et al., 2012).

The fetus therefore requires a dampening of the maternal immune response to prevent its destruction and a fully developed placenta to facilitate nutrient acquisition and to perform other support functions. Ongoing welfare of the mother demands an immune system that retains the ability to protect her from pathogens, and mechanisms to allow an appropriate degree of trophoblast invasion while still containing the boundaries of this tissue.

These two themes define the body of work presented in the following chapters (Figure 1.1). Chapters 2 through 4 address the question of trophoblast survival within the hostile environment of the maternal immune system using a method for ectopic invasive trophoblast transplantation. Chapters 5 through 7 focus on the discovery of interleukin 22 (*IL22*) mRNA in the chorionic girdle, leading to the hypothesis that IL22 acts upon receptors in the endometrium to mitigate damage caused by chorionic girdle invasion and to facilitate glandular development. The remainder of this chapter summarizes research in pertinent areas including the biology of equine invasive trophoblast, the ectopic trophoblast transplant system, mechanisms of maternal tolerance and the role of IL22 in mucosal protection. The final conclusions are summarized in Chapter 8.

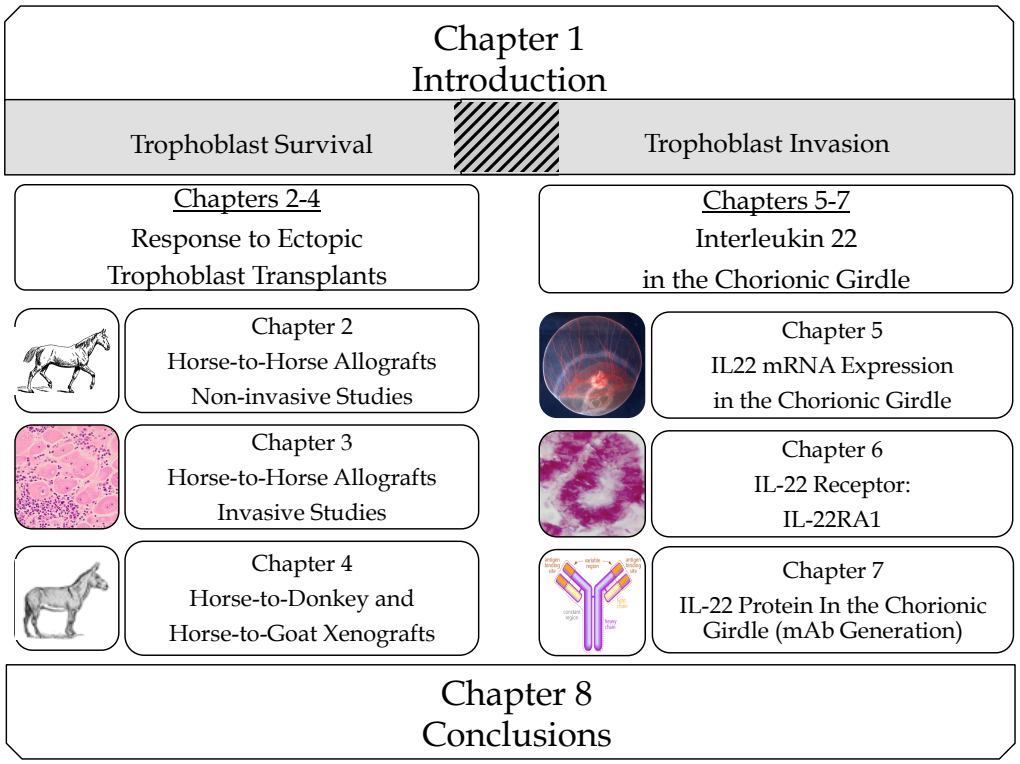

Figure 1.1 Schematic of dissertation contents. This schematic illustrates the two major themes of the research presented within this dissertation, trophoblast survival and trophoblast invasion, and depicts the organization of each chapter under these themes.

Biology of the Chorionic Girdle and Endometrial Cups

From an historical perspective, studies in the horse have made an important contribution to the field of reproductive immunology. Horse pregnancies are characterized by a late implantation that allows investigators to obtain invasive trophoblast tissue from approximately day 30 to 36 of gestation. Mares mount both humoral and cellular responses to histo-incompatible pregnancies, allowing comprehensive immunological studies of the maternal response to pregnancy (Noronha and Antczak, 2010).

The endometrial cup cells originate from the invasive trophoblast of the chorionic girdle, a structure unique to equids. The cells arise in a band around the circumference of the conceptus

near day 27 and by day 32 they begin terminal differentiation into the binucleate cells that produce equine chorionic gonadotrophin (eCG) until approximately day 120 of a normal pregnancy (Wooding et al., 2001). The function of the eCG is to stimulate secondary ovulations in the mare to maintain adequate progesterone in the early phases of pregnancy (Allen and Stewart, 1981). Around day 36 of pregnancy the chorionic girdle cells depart from the conceptus and migrate through the endometrium. Endometrium is actively destroyed by the trophoblast via phagocytosis during this process. The endometrium re-epithelializes over the nascent endometrial cups, and they become in essence small fetal allografts completely surrounded by maternal tissue (Allen et al., 1973).

Invasion of the chorionic girdle into the maternal endometrium elicits an immune response from the dam. This is referred to as the endometrial cup reaction, and occurs in both the humoral and cellular immune compartments (Lunn et al., 1997). Paradoxically, at the time of invasion the chorionic girdle cells express both maternally and paternally derived major histocompatibility complex class I (MHC-I) molecules (Donaldson et al., 1994; Donaldson et al., 1990). By day 44 to 45 of pregnancy MHC-I has been down-regulated and is no longer detectable on the endometrial cup cells (Donaldson et al., 1992). In normal, MHC-incompatible horse pregnancies, the maternal immune response to trophoblast invasion is characterized by a strong cytotoxic antibody response to antigens of the sire (Antczak et al., 1984). There is evidence that these antibodies are generated against paternal MHC-I molecules expressed by the invading chorionic girdle (Antczak et al., 1984; Crump et al., 1987). Anamnestic antibody responses that are stronger and occur sooner, but not prior to trophoblast invasion, are observed in subsequent pregnancies of the same antigenic character (Kydd et al., 1982).

A cellular response to trophoblast invasion is also observed. This occurs in both MHC-matched and MHC-mismatched pregnancies. Maternal immune cells rapidly surround the endometrial cups, but do not destroy them. Infiltrating cells include primarily CD4+ T-lymphocytes with smaller numbers of CD8+ T-lymphocytes. Rare B-cells have been noted. Eosinophils have been identified at the end stages of the endometrial cup lifespan, though their function is uncertain (Grunig et al., 1995). Molecular evidence has also been found for natural killer cells (Noronha et al., 2012) and T-regulatory cells (de Mestre et al., 2010) at the site of endometrial cup development.

In the horse the maternal immune response to pregnancy has been described as a "split tolerance" in which an intact humoral immune system responds by generating cytotoxic antibodies against paternal antigens expressed on the invasive trophoblast, while immune cells migrate to the site of trophoblast invasion but fail to destroy the foreign cells in their midst (de Mestre et al., 2010). After approximately 80 days of producing eCG, the endometrial cups die or are destroyed, and slough from the endometrium. The mechanisms that allowed the endometrial cup cells to live, and those that result in their eventual destruction, remain largely unknown.

The Ectopic Trophoblast Transplant System

The studies presented in Chapters 2 through 4 utilize a novel system of ectopic trophoblast transplantation to investigate both the systemic and local response to invasive trophoblast outside of the context of pregnancy. This system takes advantage of several unique characteristics of pregnancy in the horse. These include the ease of isolating invasive trophoblast between days 30 and 36, just prior to its migration through the endometrium; the strong humoral and cellular

immune responses to trophoblast; the well-defined lifespan of the invasive trophoblast; and the ability to measure this lifespan non-invasively by monitoring serum equine chorionic gonadotrophin (eCG) concentration in the recipient.

The survival of endometrial cups for a period of 80 days during normal horse pregnancy is impressive when considered in the context of conventional allograft rejection. Understanding the immunological events that allow survival of the trophoblast allograft is critical. To this end the system for transplanting chorionic girdle tissue to an ectopic site was developed. In the original studies of ectopic trophoblast transplants, chorionic girdle was dissected from horse conceptuses and transplanted to an ectopic site in an unrelated, non-pregnant mare. It was found that the transplanted cells maintained a similar lifespan and exerted a similar effect upon the recipient as is observed during the events of trophoblast invasion and endometrial cup development in normal pregnancy. Equine chorionic gonadotrophin (eCG) was measurable in the recipients' serum for a median of 75 days. The eCG appeared to have a physiologic effect upon the reproductive cycle of the recipient, and serum progesterone concentrations remained increased. A cytotoxic antibody response to donor sire antigens was measurable in recipients' serum. Biopsies of the transplant sites showed cellular infiltrates similar to those that surround endometrial cups (Adams and Antczak, 2001; de Mestre et al., 2011).

A challenge to complete understanding of the mechanisms of maternal tolerance is the complexity and redundancy of these mechanisms. The ectopic trophoblast transplant studies effectively remove the influences of physical sequestration within the uterus and the hormonal priming of pregnancy. The findings of the ectopic transplant experiments suggest that the

mechanisms that enable survival of the horse invasive trophoblast are independent of these two factors. Survival of the trophoblast at an ectopic site occurs in spite of the *in vitro* observation that equine trophoblast can in fact be a target of activated cytotoxic T-cells (Baker et al., 2000). This differs from other species such as the mouse in which destruction of ectopic trophoblast by natural killer cells was taken as evidence that situation within the pregnant uterus is critical for trophoblast survival (Erlebacher et al., 2002).

Mechanisms of Maternal Tolerance

In his original work, Sir Peter Medawar identified three mechanistic categories as to how the fetus might be protected from destruction by the maternal immune system: physical sequestration within the uterus, lack of antigenicity of the fetus, and unresponsiveness of the maternal immune system (Medawar, 1953). Progress made over sixty years of research has shown nuances of these to hold true, yet also has revealed the complexity of this problem and the redundancy of its solutions. While comparative studies in reproductive immunology reveal common themes, they also highlight the diverse mechanisms that have evolved to ensure fetal survival and suggest that in many ways maternal tolerance is a very active as opposed to passive process.

Down-regulation of fetal antigenicity is in fact a mechanism used in some species, notably the human, to mitigate the maternal response to pregnancy. This involves modification of trophoblast surface molecules, specifically major histocompatibility class I (MHC-I) molecules. Human trophoblast cells typically bear non-classical MHC-I molecules that do not incite a strong maternal response (Hemberger, 2013; Ristich et al., 2005). Expression of the non-classical human leukocyte antigen G (HLA-G) is restricted to the extravillous trophoblast; villous

trophoblast does not express histocompatibility antigens. The HLA-G molecule possesses several unique characteristics relative to classical leukocyte antigen molecules, including greatly decreased polymorphism, modifications in cellular trafficking and a small peptide binding repertoire. These characteristics are thought to render HLA-G unable to present antigen, thus providing a measure of protection to the fetus (Apps et al., 2008).

As described briefly above, the pattern of MHC-I expression on horse trophoblast is quite different. The invasive trophoblast cells of the chorionic girdle express high levels of conventional MHC-I, both maternal and paternal. The MHC-I molecules are most highly expressed around days 32 through 36 of pregnancy, paradoxically just at the time the trophoblast cells are to invade the endometrium (Donaldson et al., 1994; Donaldson et al., 1990; Kydd et al., 1991). Down-regulation occurs by day 44, with mature endometrial cups being devoid of MHC-I (Donaldson et al., 1992). This pattern of MHC-I expression in equine trophoblast raises questions that have not yet been answered. The relatively rapid down-regulation of MHC-I after trophoblast invasion might explain why in a first pregnancy the trophoblast evades destruction. However, the questions remain as to why invading trophoblast in second and subsequent pregnancies of similar antigenic character are not immediately destroyed, and what is the ultimate mechanism of destruction of the mature endometrial cup cells that lack MHC-I. Exposure of foreign fetal antigens to the maternal immune system nonetheless seems counterintuitive. One hypothesis has been suggested that exposure to MHC-I antigens early in pregnancy is a first step in preparing for eventual expulsion of the placenta (Rapacz-Leonard et al., 2014).

Maternal immune response to pregnancy can take place in either the humoral or cellular compartments of the immune system, or sometimes both. The generation of cytotoxic antibodies against paternal antigens is a defining characteristic of the maternal response to MHC-mismatched pregnancy in the horse. Anamnestic responses are observed in subsequent pregnancies (Antczak et al., 1984; Kydd et al., 1982). These antibodies exert no adverse effects upon the pregnancy, and their function, if any, is unknown. Observations on cytotoxic anti-HLA antibodies in humans have varied with parity, the timing of testing relative to pregnancy and the sensitivity of the testing method. These antibodies have been detected in 30% to 54% of women in first pregnancies, and up to 74% of women having more than two pregnancies (Densmore et al., 1999; Masson et al., 2013; Morin-Papunen et al., 1984; Regan et al., 1991). Recent studies have suggested that the presence of these antibodies in early to mid gestation may be associated with chronic chorioamnionitis (Lee et al., 2011) and spontaneous preterm delivery (Lee et al., 2013). One meta-analysis identified no significant effects of anti-HLA antibodies on pathologies of pregnancy, but also noted the heterogeneous nature of existing studies (Lashley et al., 2013).

Cellular responses play an important role in maternal immune response to pregnancy, though the character of these responses varies greatly with species. As with the differences noted above in structure and function between HLA-G and classical HLA-I molecules, the phenotype of a cell at the maternal-fetal interface may differ from its phenotype at other locations in a way that is favorable to the success of the pregnancy. For example, the decidual natural killer cell is a predominant type of cell at the maternal-fetal interface in humans. Natural killer cells outside the context of pregnancy show cytotoxic activity under many pathologic conditions. They can identify and kill cells that have a decreased expression of MHC-I on their surface, typically

tumor cells and cells that are infected with virus (Campbell and Hasegawa, 2103). This cytotoxic activity is not a prominent feature of uterine NK cells, which play an important role in other functions such as spiral artery remodeling in early human pregnancy (Hemberger, 2013; Kopcow et al., 2005). Molecular evidence for natural killer cells has been identified in the cellular infiltrates surrounding equine endometrial cups (Noronha et al., 2012). Though it might be expected that NK cells could play a role in the ultimate destruction of the MHC-I devoid endometrial cup cells, a definitive role for these cells in equine pregnancy is unknown.

The local cellular immune response to pregnancy in the horse is unique in that it is composed primarily of T-lymphocytes (Grunig et al., 1995). Both CD4+ and CD8+ T-lymphocytes are present in abundance, differing greatly from observations in mice and humans. In mice only 3% of leukocytes in the decidua on embryonic day 8.5 are T-cells, with CD4+ and CD8+ cells present in equal numbers (Nancy and Erlebacher, 2014). Furthermore, in mice epigenetic regulation of T-cell chemokines in decidual cells inhibits the ability of maternal effector T-cells to migrate to the decidua (Nancy et al., 2012). In humans, less than 20% of decidual leukocytes are T-cells. The T-cell population may include a greater proportion of CD8+ T-cells. A definitive role for T-cells in healthy or abnormal pregnancies is uncertain (Erlebacher, 2013; Nancy and Erlebacher, 2014).

Studies in the horse have shown that chorionic girdle cells are not inherently resistant to destruction by CD8+ cytotoxic T-lymphocytes (Baker et al., 2000). Cytotoxic T-lymphocyte (CTL) activity rather is systemically dampened during pregnancy in both mares and Jenny donkeys carrying intra-species pregnancies (Baker et al., 1999). The decreased CTL activity is

not restricted to cells bearing the MHC-I antigens of the sire, but extends to cells of other MHC haplotypes (Noronha and Antczak, 2012). This same effect is not observed in horses carrying interspecies (mule) pregnancies (Baker et al., 1999).

While comparative studies in maternal tolerance highlight the diverse mechanisms that can contribute to fetal survival, there is some common ground. One of these areas is the proposed role for regulatory T-lymphocytes in maternal tolerance. Research in mice has shown a role for CD4+ CD25+ T-regulatory cells in protecting the pregnancy. Abortion was prevented in these studies using T-regulatory cell therapy (Zenclussen et al., 2006; Zenclussen et al., 2005). Other studies have demonstrated that T-regulatory cells during pregnancy show specificity to fetal antigens. They also generate memory pools during first pregnancies that may impact the state of later pregnancies (Rowe et al., 2012).

In humans, circulating T-regulatory cells are increased in pregnant women (Somerset et al., 2004), and decreases in these cells have been associated with a variety of complications in pregnancy (Jiang et al., 2014). T-regulatory cells of the CD4+ CD25+ FoxP3+ phenotype have been identified in equine endometrial cups (de Mestre et al., 2010). A decreased number of these cells in peripheral blood has been associated with early pregnancy loss in mares (Aurich et al., 2013).

A discussion of maternal tolerance is not complete without consideration of the role of endocrine hormones. In the horse, the primary hormones of pregnancy include progesterone and equine chorionic gonadotropin (eCG). While research into the immunomodulatory functions of eCG is

limited, both progesterone and human chorionic gonadotrophin (hCG) are known to have both direct and indirect effects on the actions of many immune cell types including T-regulatory cells and uterine NK-cells (Hunt et al., 1998; Lee et al., 2012; Schumacher et al., 2014; Schumacher et al., 2013).

Trophoblast Invasion, Interleukin 22 and the Maintenance of Epithelial Integrity

While one critical component of a successful pregnancy is the modulation of maternal immune response to ensure survival of the fetus, of equal importance is the successful remodeling of uterine anatomy to accommodate feto-placental development without compromising maternal health. Blastocyst implantation and trophoblast invasion both disrupt maternal tissue, though the extent of this disruption is species-specific. Trophoblast by definition is one of two cell types that make up the blastocyst. These cells go on to form part of the extra-embryonic placenta (Pfeffer and Pearton, 2012). During the peri-implantation period, some types of trophoblast cells develop an invasive phenotype, with the eventual extent of trophoblast invasion and associated tissue remodeling varying with species and type of placentation.

The human hemochorial placenta is perhaps the most invasive of all species. Implantation in the human is interstitial, involving destruction of endometrial epithelium and subsequent regeneration of this tissue over the blastocyst. Decidualization results in alterations in the extracellular matrix of the endometrium, spiral arteries and the inner myometrium (Pijenenborg et al., 2011a). Similar observations have been made in the chimpanzee and the lowland gorilla (Pijenenborg et al., 2011b; Pijenenborg et al., 2011c). Less invasive forms of trophoblast are seen in domestic species such as the ruminants and pigs. The binucleate trophoblast cells of the

ruminant placenta have migratory properties and can form syncytia with endometrial epithelial cells but do not deeply invade or phagocytose maternal tissues (Igwebuike, 2006; Wooding et al., 1996). Equids are unique in that although their diffuse, epitheliochorial placentation generally is considered to be non-invasive, invasion of the endometrium by the chorionic girdle involves phagocytosis of the endometrium by the trophoblast, and subsequent re-epithelialization of the endometrium (Allen et al., 1973).

Gene expression array analysis identified significant upregulation of interleukin 22 (*IL22*) mRNA in the invasive trophoblast of the equine chorionic girdle (Brosnahan et al., 2012). IL22 belongs to the interleukin 10 (IL10) family of cytokines (Trivella et al., 2010) and plays a role in mucosal immunity and the maintenance and repair of epithelia (Aujla and Kolls, 2009; Maloy and Kullberg, 2008; Ouyang and Valdez, 2008; Sugimoto et al., 2008). Since first being described (Dumoutier et al., 2000; Xie et al., 2000) IL22 has been documented exclusively in immune cells including T-helper subsets (Th17, Th22), natural killer cells, and bovine γδ T-cells (Colonna, 2009; Ma et al., 2010; Maloy and Kullberg, 2008; Sonnenberg et al., 2011; Trifari and Spits, 2010). The primary target of IL22 is a transmembrane, heterodimeric receptor composed of IL22RA1 and IL-10R2 (Wolk et al., 2010). IL22RA1 is expressed on epithelial surfaces, including respiratory (Aujla et al., 2008) and digestive tracts, and skin (Wolk et al., 2004). Binding of IL22R1 by IL22 activates transcription factors STAT3, STAT1 or STAT5 (Lejeune et al., 2002) and regulates genes associated with innate immunity (Liang et al., 2006) and cellular differentiation, migration and survival (Boniface et al., 2005; Radaeva et al., 2004).

Trophoblast invasion can significantly alter the integrity of maternal tissues. Structures including the endometrium, myometrium and blood vessels are phagocytosed, digested and remodeled at various stages of pregnancy contingent on the species in question. Molecules such as proteases, metalloproteinases, angiogenic factors and cytokines are known to play a role in the remodeling process, as well as immune cells such as uterine natural killer cells, macrophages and dendritic cells (Hemberger, 2013; Yeh et al., 2012). Considering the invasive character of the chorionic girdle and the known functions of IL22 in other biological systems, it is possible that the cytokine interleukin 22 falls into this category of molecules, playing a role in remodeling the equine endometrium following trophoblast invasion.

Summary

The following chapters focus on the unifying concept that pregnancy imparts modifications to maternal physiology to ensure the survival of both the fetus and the mother. The genetically distinct feto-placental tissues are shielded from destruction by the maternal immune system. Simultaneously, the disruption of maternal reproductive anatomy during placental development is controlled such that the mother is not harmed. Chapters 2 through 4 present a series of experiments that used the ectopic trophoblast transplant system to advance towards an understanding of the mechanisms that protect the trophoblast from immune destruction. Research presented in Chapters 5 through 7 investigates the expression of interleukin 22 in the invasive trophoblast of the equine chorionic girdle, and generates a hypothesis for its possible actions upon the endometrium in early equine pregnancy. Chapter 8 summarizes and integrates the findings in each of these areas, and sets the stage for future discoveries.

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CHAPTER 2

SERIAL ECTOPIC TROPHOBLAST TRANSPLANTS:

NON-INVASIVE STUDY OF HORSE-TO-HORSE ALLOGRAFTS

Abstract

Ectopic transplants of invasive horse trophoblast into fully allogeneic, major histocompatibility complex class I (MHC-I) incompatible recipient mares survive and function with lifespans similar to those of endometrial cups in normal pregnancy. The transplants induce both humoral and cellular immune responses upon first exposure. There have been no investigations of immunologic memory in this experimental system. This study tested the hypothesis that repeated exposure to ectopic trophoblast will alter the immune status of recipients in a way that will change the lifespan of successive grafts. Trophoblast lifespans could become progressively longer as a result of tolerance induced by chronic exposure or shorter because of a destructive memory response. Alternatively, the trophoblast may control its own fate via an intrinsic cellular mechanism resulting in an unchanged lifespan. To investigate this hypothesis, four mares received two or four successive MHC-I incompatible transplants from conceptuses sired by the same homozygous stallion over one breeding season. Serum eCG, progesterone, and antibody responses were monitored in the recipients. There was no evidence for decreasing transplant lifespans over time even though recipients made anamnestic cytotoxic antibody responses after each transplant. Two of the mares received two additional transplants the following breeding season. Median transplant lifespan was not altered relative to the first breeding season. These results ruled out the hypothesis that ectopic trophoblast would succumb more rapidly to a destructive memory response during second and subsequent transplant events. These data suggest that tolerogenic mechanisms preventing rejection of primary transplants remain capable of protecting closely spaced second, third and fourth transplants of the same antigenic character, and that the mechanisms may be initiated by the trophoblast itself. A break in exposure did not change this; lifespans of the fifth and sixth transplants were not different than earlier transplants.

Introduction

The success of mammalian reproduction implies that mechanisms protecting the fetal allograft from destruction by the maternal immune system persist through multiple pregnancies. Since Peter Medawar's seminal definition of the immunological "problems" of pregnancy over sixty years ago (Medawar, 1953) researchers have worked to define these mechanisms. Considerable progress has been made, but the complexity and redundancy of factors protecting the fetus present a challenge (Erlebacher, 2013a; Erlebacher, 2013b; Petroff, 2011).

Studies in the horse historically have made an important contribution to the characterization of maternal immune responses to pregnancy. The horse has been a useful species for these studies due in part to the late implantation stage of equine pregnancy. This enables the easy acquisition of intact horse conceptuses, including the invasive trophoblast of the chorionic girdle, up to day 36 of pregnancy (Noronha and Antczak, 2010). Mares that are pregnant with histo-incompatible fetuses mount robust humoral and cellular immune responses to the invasive trophoblast, enabling studies of both compartments of the immune system (Lunn et al., 1997).

The study of the maternal immune response to pregnancy in the horse centers on the endometrial cup reaction (Lunn et al., 1997). The endometrial cup cells originate from the invasive trophoblast of the chorionic girdle, a structure unique to equids. At approximately day 36 the chorionic girdle cells depart from the conceptus, penetrate the endometrial epithelium, and migrate through the endometrial stroma. The cells terminally differentiate to form the binucleate, eCG-producing cells of the endometrial cups (Allen et al., 1973). At the time of invasion, the chorionic girdle cells express both maternal and paternal MHC-I molecules on their

cell surface (Donaldson et al., 1994; Donaldson et al., 1992; Donaldson et al., 1990). The mare's immune system mounts a response to the invading trophoblast (Lunn et al., 1997). The nature of this response has been described as a "split tolerance" in which an intact humoral immune system responds appropriately by generating cytotoxic antibodies to paternal antigens while T-lymphocytes surround the invading cells but fail to reject the foreign tissue (de Mestre et al., 2010). The endometrial cups go on to survive for approximately 80 days within the maternal endometrium (Lunn et al., 1997). The immunologic events of early equine pregnancy differ from those of human pregnancy in which the extravillous trophoblast expresses non-classical human leukocyte antigen (HLA) class I HLA-G molecules that do not incite a strong maternal response, as well as HLA-C and HLA-E. The villous trophoblast does not express HLA molecules of any type (Apps et al., 2008; Hemberger, 2013; Ristich et al., 2005; Trowsdale and Betz, 2006).

A system for transplanting horse trophoblast to an easily accessible ectopic site in recipient mares has facilitated reproductive immunology studies in the horse even further. Single ectopic transplants of chorionic girdle tissue into histo-incompatible, non-pregnant mares produced physiological and immunological effects similar to a normal pregnancy. Equine chorionic gonadotropin (eCG) was measurable in the recipient mares' serum for a median of 75 days, progesterone remained high, and cellular and humoral responses to the transplant occurred as in normal equine pregnancy (Adams and Antczak, 2001; de Mestre et al., 2011). The success of this system provided a valuable insight into the mechanisms of maternal tolerance in the horse: the invasive trophoblast in this species can survive and function as a full allograft outside of the pregnant uterus.

These studies did not establish that the mechanisms protecting the ectopic trophoblast are identical to the mechanisms at work in pregnancy. Furthermore, because only one transplant was performed in each maiden recipient, there is a possibility that survival of the transplant was a consequence of the recipient's naïve immune system and that ectopic trophoblast would not survive multiple times in a single recipient as would trophoblast in a normal multiparous mare. The primary objective of the current study was to examine the trophoblast transplant model of maternal tolerance in the context of immunologic memory. The hypothesis tested in this study was that the immunologic priming of the recipient's immune system by a first transplant would result in a change of lifespan in second and subsequent transplants. This change could be a longer lifespan due to regulatory T-lymphocyte memory (Aurich et al., 2013; de Mestre et al., 2010; Rowe et al., 2012) or other mechanisms of induced tolerance resulting from prolonged antigen exposure (Schietinger and Greenberg, 2014), or a decrease in lifespan due to a destructive memory response. Alternatively, there could be no change in trophoblast lifespan. This may suggest that the trophoblast cells possess an intrinsic mechanism that controls their lifespan.

Materials and Methods

Animals

Horses used in this study were part of the Cornell Equine Genetics Center herd. Recipient mares used for all serial trophoblast transplants were reproductively mature, maiden animals lacking the Equine Leukocyte Antigen (ELA) A3 haplotype. Mares that donated conceptuses were of various breeds, ages and ELA haplotypes. The donor stallion was a single male homozygous for

the A3/A3 haplotype. Animal care and experimental design were in compliance with protocols approved by the Cornell University Institutional Animal Care and Use Committee (IACUC).

Breeding, Conceptus Recovery and Transplantation Technique

Donor mares were impregnated via artificial insemination with fresh semen from the donor stallion. Conceptuses were recovered at day 34 by non-invasive uterine lavage using aseptic technique as previously described (Antczak et al., 1987). Transplants were performed in a manner consistent with a method previously described (de Mestre et al., 2011). Briefly, after recovery the entire chorionic girdle was dissected, minced, loaded into a tuberculin syringe with 1 mL phosphate buffered saline and penicillin/streptomycin and injected aseptically into the vulvar mucosa of the recipient. Serial transplants were placed on alternating sides of the vulva. In two instances (the fourth and fifth transplants in the six-transplant recipients), a single girdle was split between the two recipients.

Sampling

Blood samples were drawn via jugular venipuncture into serum tubes from recipient mares three times per week until eCG was no longer measurable in their serum. Blood was allowed to clot, tubes were centrifuged and serum stored at -20° C until processing. Blood from the donor stallion was drawn as needed into heparinized tubes and processed immediately for use in the cytotoxicity assays.

eCG and Progesterone Quantifications

Serum eCG and progesterone were quantified using commercially available ELISA kits (PMSG and Progesterone ELISAs, DRG International, Springfield, NJ) according to manufacturers instructions. Plates were read on a Multiskan Ex and data were analyzed with Ascent 2.6 software (ThermoFisher Scientific, Waltham, MA).

Lymphocyte Microcytotoxicity Assay

Lymphocytes from the donor stallion were isolated from heparinized venous blood.

The presence of cytotoxic antibodies in recipient serum was determined using a lymphocyte microcytotoxicity assay as previously described (Antczak et al., 1982). Briefly, serum from recipients was plated in microtiter plates (Robbins Scientific, Mountain View, CA) in two-fold dilutions. Serum was incubated with lymphocytes from the donor stallion and rabbit complement (Pel-Freez, Lot #PF29521, Brown Deer, WI). Eosin dye was added and the wells were fixed with formalin. Wells were scored as percent cell killing indicated by dye exclusion. Wells showing greater than 50% killing were considered positive. Titers were called as the highest dilution with a positive result. Each sample was tested at least twice.

Calculation of Trophoblast Lifespan

The presence of eCG in the recipients' serum was considered an indicator of live and functioning trophoblast. Transplant lifespan was calculated as previously described (de Mestre et al., 2011). Briefly, the definitive end point of a transplant was considered to be the point at which eCG was at least twice baseline, and with a drop of less than 50% in the previous six days. Ideal timing of a second or later transplant was considered to be when the eCG from the previous transplant

approached baseline. In three instances transplants were performed early (prior to eCG from the previous transplant reaching baseline) due to the availability of conceptuses. In these cases the shortened transplant was considered "Incomplete" for the purposes of lifespan calculation. For transplants that closely approached but did not reach baseline, the more conservative (i.e. slightly shortened) minimum lifespan was used.

Graphing and Analysis

Data were analyzed and graphs were created using Prism 6 for Mac OS X (GraphPad Software, La Jolla, CA).

Results

Overview

These studies were undertaken to determine if ectopic trophoblast transplant lifespans became longer, shorter, or remained unchanged when the recipient's immune system was primed by previous exposure. Donor mares first were bred to a stallion that was homozygous for the ELA-A3 haplotype. The conceptus was flushed on day 34 and the chorionic girdle transplanted into the vulvar mucosa of a recipient mare that did not possess the ELA-A3 haplotype. Once transplanted, the trophoblast tissue was left undisturbed and monitored non-invasively with three serum-based assays. The hormones eCG and progesterone were used as indicators of trophoblast lifespan and physiologic effect respectively. A lymphocyte microcytotoxicity assay measured production of antibody against donor sire antigen, assessing recognition of the transplant by the recipient's immune system and generation of a humoral response. When eCG approached baseline, the next transplant was performed in that recipient. In three of the 18 transplants,

timing was slightly early due to conceptus availability, and eCG from the previous transplant had not returned to baseline. Transplants for which eCG did not return to baseline were considered "Incomplete". These transplants are clearly indicated in Figure 2.3 in the section on lifespan analysis, and were excluded from the calculations. A schematic diagram (Figure 2.1) illustrates the experimental approach.

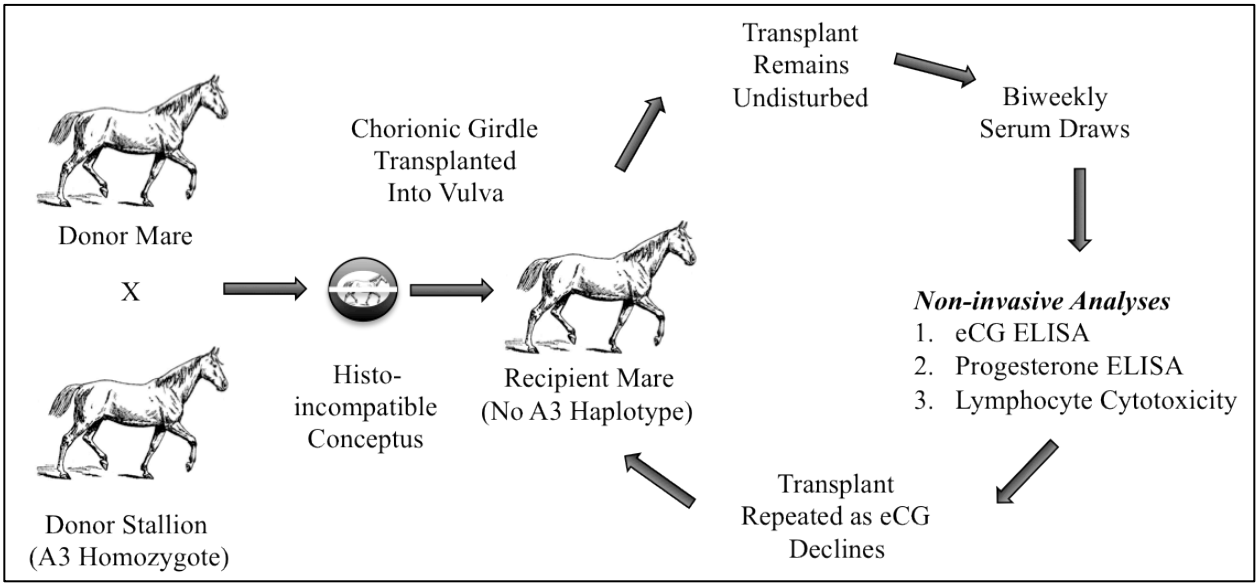

Figure 2.1 Schematic of the non-invasive horse-to-horse trophoblast transplant approach. Chorionic girdle was obtained from a 34-day horse conceptus and transplanted into the vulvar mucosa of an unrelated, histo-incompatible recipient mare. Transplants were monitored non-invasively by analyzing recipients' serum for eCG, progesterone, and antibody production. When eCG approached baseline, the next transplant was performed.

A total of 18 trophoblast transplants were performed in four mares (Table 2.1). A single stallion homozygous for the ELA-A3 haplotype was the sire of all conceptuses recovered for this study. Each mare received two or four transplants over one breeding season to evaluate transplant lifespans during sustained trophoblast exposure. Two of these mares received two additional transplants the following breeding season after a break in exposure of two (mare 3876) or five (mare 4084) months. Equine chorionic gonadotropin, progesterone and antibody production

were tracked from the time the first transplant was placed until eCG from the last transplant was no longer detectable.

Table 2.1 Recipient and Donor Mares Used in Trophoblast Transplant Experiments

			Season 1 Transplants (Donor Mare/MHC)				Season 2 Transplants (Donor Mare/MHC)	
Recipient	Recipient MHC	Age	1	2	3	4	5	6
3958	A2/A10	4	3725 A2/?	4065 A19/?				
3875	A2/?	3	3640 A3/?	3640 A3/?	3640 A3/?	3725 A2/?		
3876	A2/?	3	3640 A3/?	4069 A1/?	3725 A2/?	3729 A3/A3	3875 A2/?	3908 A2/A2
4084	A5/A9	5	3957 A1/A3	3957 A1/A3	3492 A2/?	3729 A3/A3	3875 A2/?	3725 A2/?

Results from Breeding Season One

Summary Data for Individual Recipients

The complete data sets for each individual mare are shown in the graphs below (Figures 2.2.1-2.2.4). These figures align the eCG (A), progesterone (B) and cytotoxicity titers (C) from the transplants performed in each mare during breeding season one. Dashed, vertical, red lines indicate each transplant, with the transplant number, experimental day, and month and date of the transplant provided above. Quarterly month and day are indicated below the X-axis to provide information on seasonality.

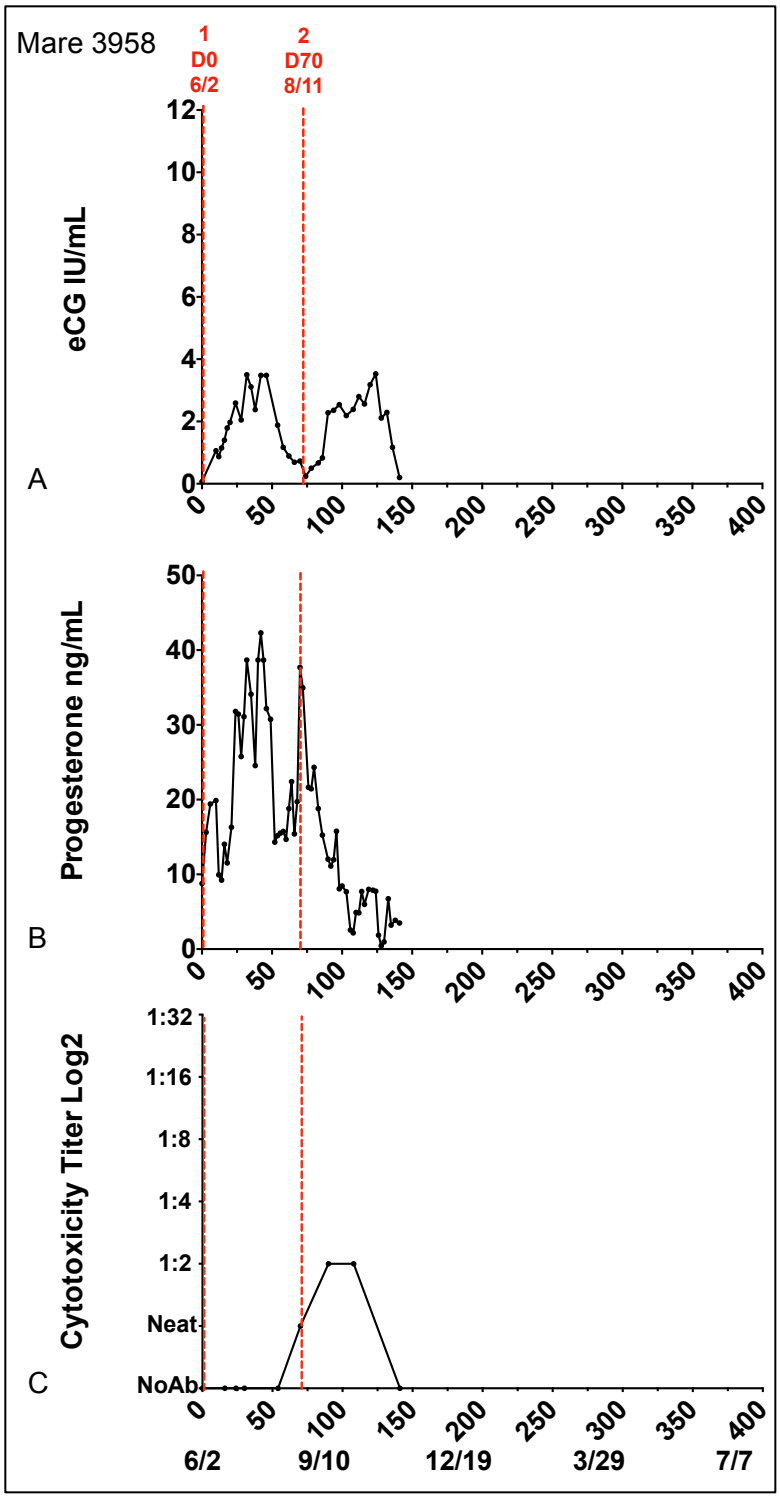

Figure 2.2.1 Mare 3958, summary eCG, progesterone and antibody data from breeding season one. The detection of eCG (A) in this recipient's serum corresponds with persistently high progesterone (B) for the life of two transplants. The mare's antibody response (C) to the first transplant was slow, and her peak titer was the lowest of the four recipients in breeding season one.

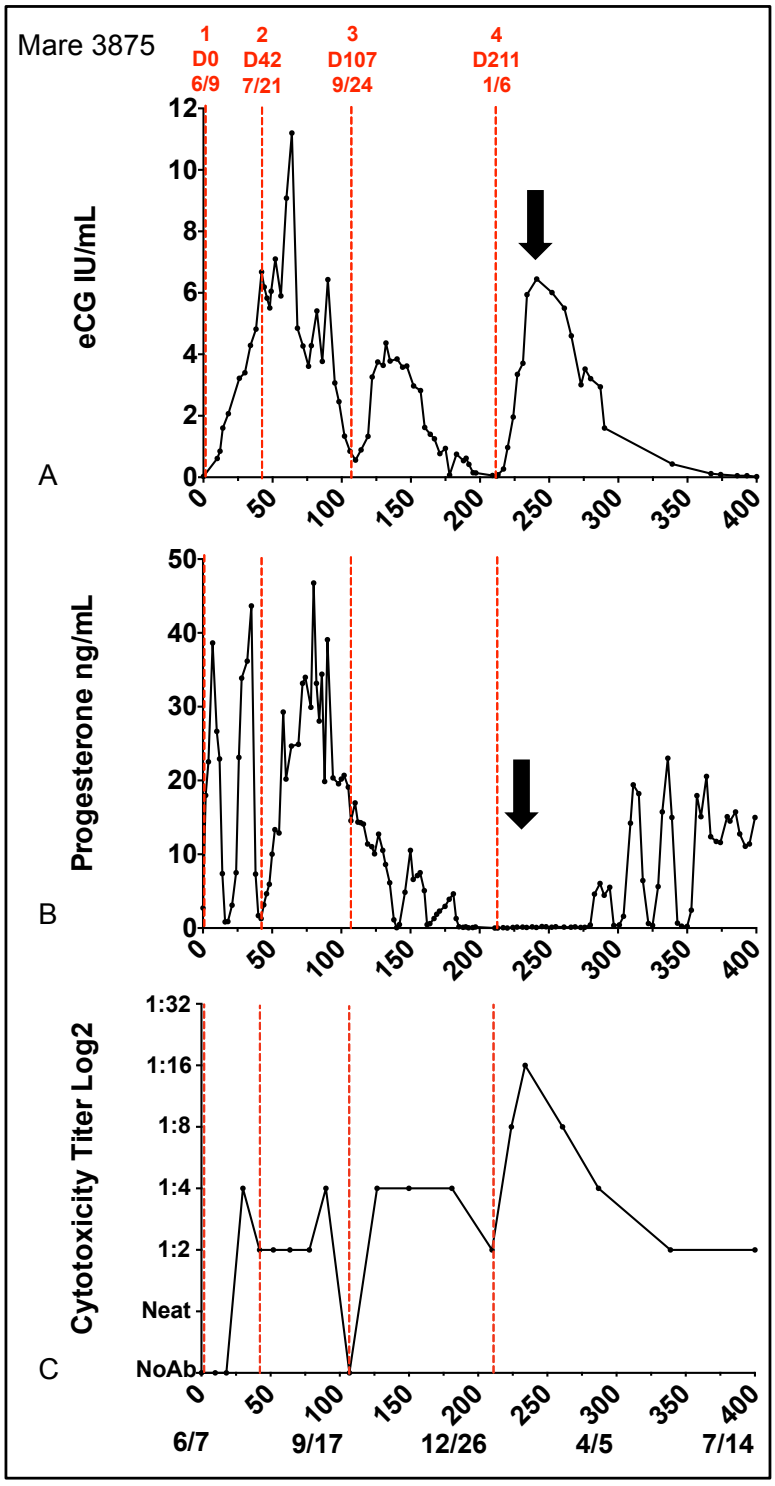

Figure 2.2.2 Mare 3875, summary eCG, progesterone and antibody data from breeding season one. Of note in this mare is transplant four, which produce the longest duration eCG curve (A, arrow) in the study. This transplant was performed during a period of anestrus in which progesterone (B, arrow) remained undetectable for a period of time before and after the transplant. Increases in cytotoxic antibodies (C) were observed following each transplant. Transplant 1 in this mare was removed from analysis due to eCG not returning to baseline.

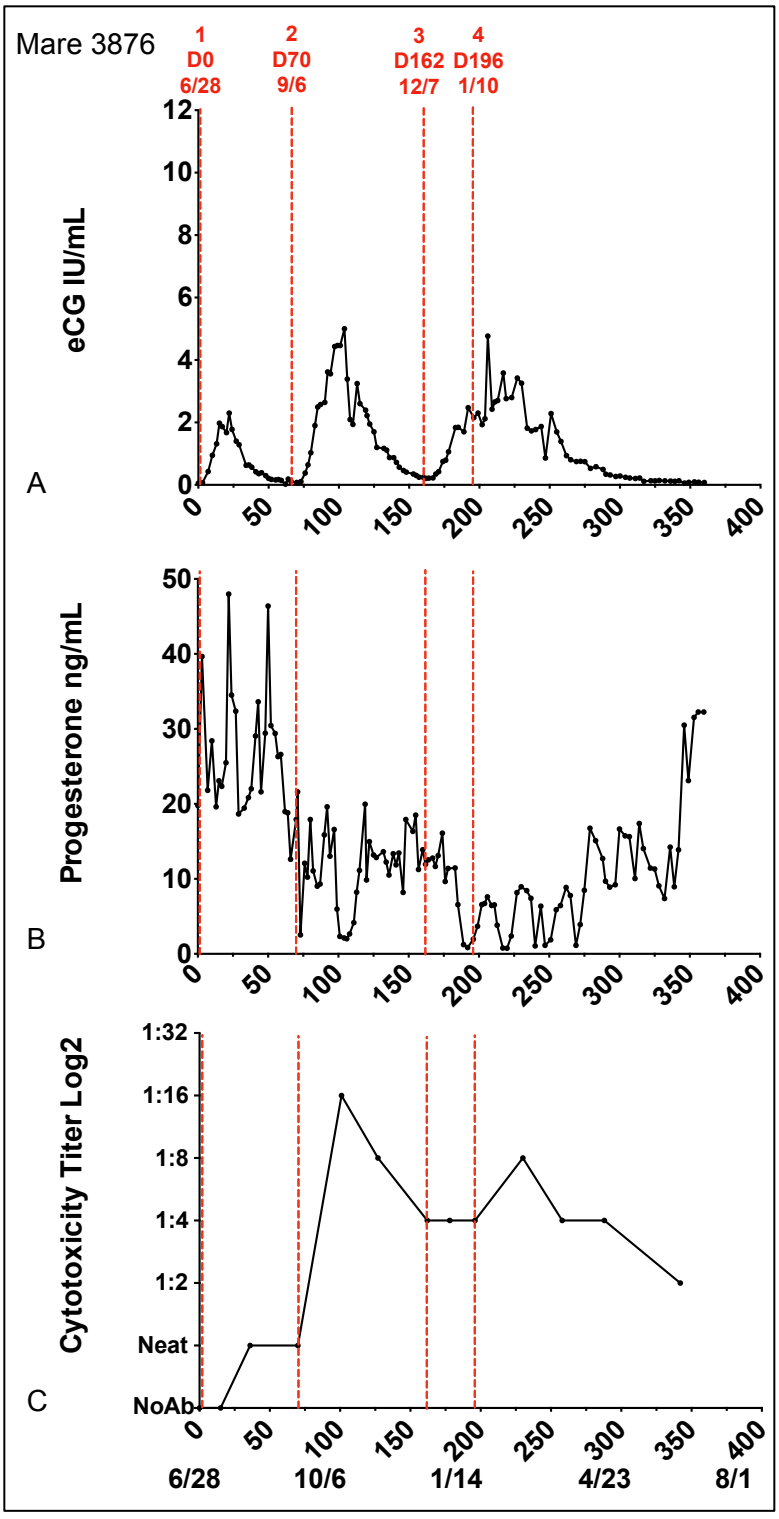

Figure 2.2.3 Mare 3876, summary eCG, progesterone and antibody data from breeding season one. Serum eCG (A) for transplant 3 did not return to baseline so this transplant was not included in the lifespan analysis. Progesterone (B) was less consistently increased following transplants performed in winter months. An antibody response (C) was not observed after transplant 3, possibly due to the short interval between transplants 3 and 4.

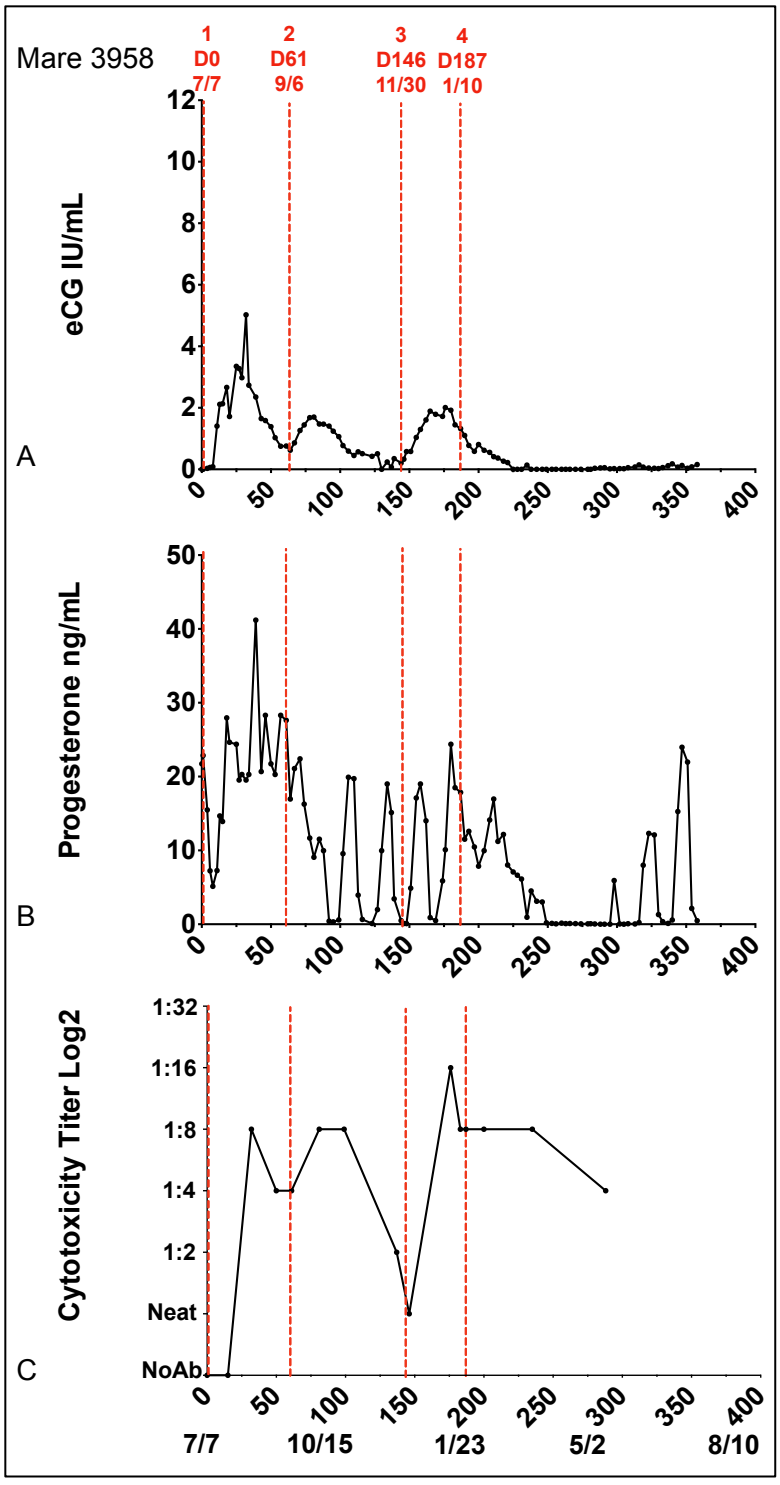

Figure 2.2.4 Mare 4084, summary eCG, progesterone and antibody data from breeding season one. Serum eCG (A) for transplant 3 did not return to baseline so this transplant was not included in the lifespan analysis. eCG peaks were lower in this mare than in the others. Progesterone (B) levels were inconsistently increased. The lack of increase in both eCG peak and cytotoxicity titer following transplant 4 suggests a possible technical failure of this transplant.

Median horse-to-horse trophoblast lifespans did not decrease over time.

Measurable eCG in recipients' serum was used as an indicator of living trophoblast. Four maiden mares received two or four transplants over a period of 141 to 338 days for a total of 14 transplants performed over the first breeding season. Lifespans for completed transplants were calculated from serum eCG curves (Figure 2.3). Calculated lifespans were variable within and among individual mares (Table 2.2), ranging from 38 to 156 days, with a median lifespan for all transplants of 71 days. Lifespan increases from the first to the last completed transplant in three individual mares ranged from 1 to 91 days. One mare (4084) showed a decrease from first to last transplant of 23 days, though an increase of 24 days was observed between the first and second. Median lifespans of sequential transplants did not decrease over time, but showed a trend toward increasing (Figure 2.4).

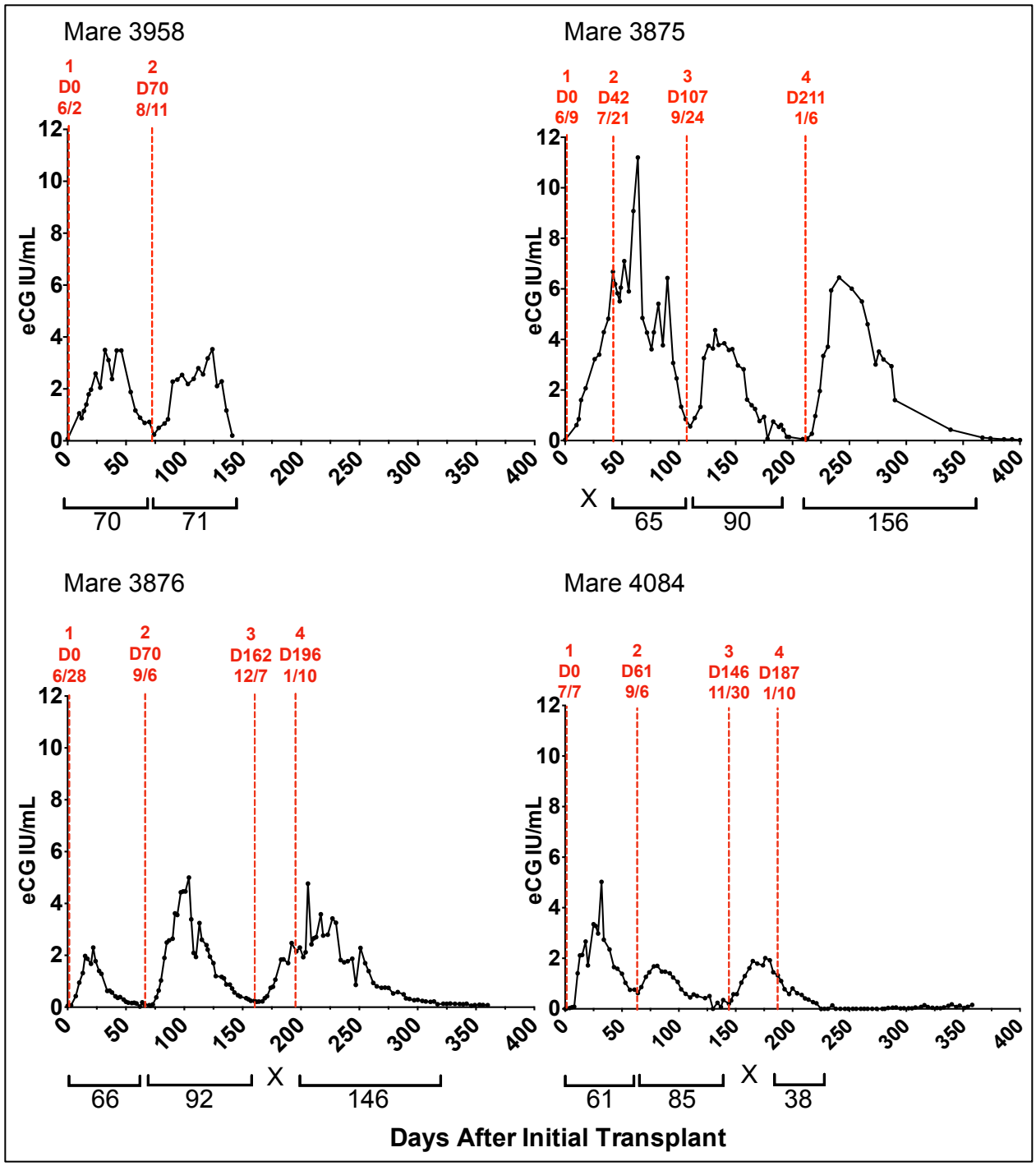

Figure 2.3 Breeding season one: trophoblast transplant lifespans calculated from eCG curves. The eCG curves shown in Figures 2.2.1 through 2.2.4 are presented again with the lifespan determinations detailed below the X-axis. The three transplants that did not return to baseline prior to the next transplant were not included in the analysis; these are marked with "X". The transplant number is given above, along with the day of current season experiment and transplant date.

Table 2.2 Breeding Season One: Trophoblast Transplant Outcome Summary

Recipient Number	Transplant Number	Lifespan
3958	1	70
	2	71
3875	1	Incomplete
	2	65
	3	90
	4	156
3876	1	66
	2	92
	3	Incomplete
	4	146
4084	1	61
	2	85
	3	Incomplete
	4	38
Overall Median		**71**

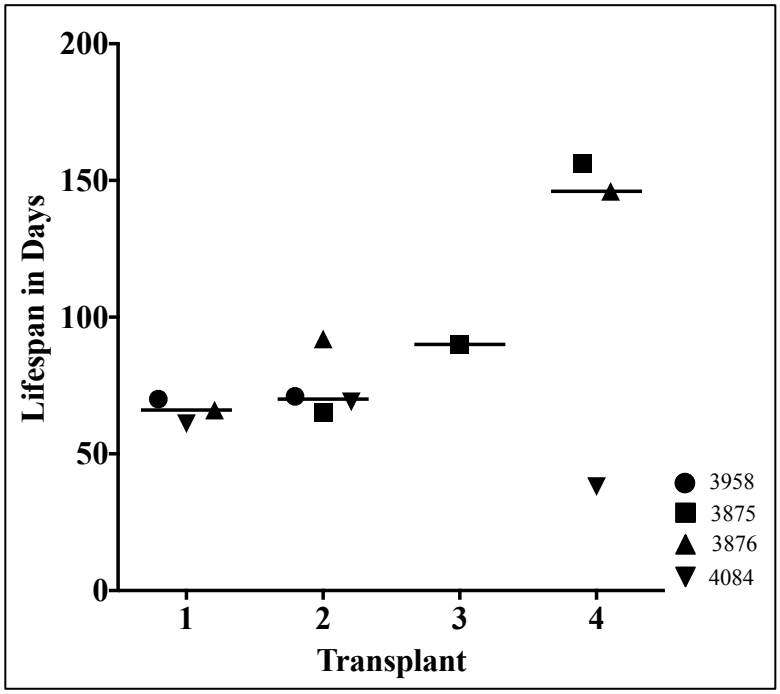

Figure 2.4 Breeding season one: trophoblast transplant lifespans. Calculated lifespans of completed serial trophoblast transplants are shown. The horizontal bar at each time point represents the median lifespan. Transplants designated "Incomplete" were excluded from the analysis (see Table 2.2).

Serial transplants survived repeatedly in the presence of anti-donor sire cytotoxic antibodies. Non-invasive monitoring of recipient mares allowed estimation of humoral immune responses to the transplants. The lymphocyte microcytotoxicity assay was used to track the development of recipient cytotoxic antibodies against donor sire MHC-I (Figure 2.5). All recipient mares were free of cytotoxic antibodies to ELA A3 at the start of the experiment. A rise in recipient antibody titer to paternal antigen following a transplant indicated that the transplanted tissue was recognized by the recipient's immune system and that the recipient's immune system effectively mounted a humoral response to the foreign tissue.

Following 12 of 14 transplants, recipients showed rises in cytotoxic antibody titers against paternal MHC-I antigens. The two remaining transplants (Mare 3876, transplant 3 and Mare 4084, transplant 4) resulted in continuation of a high titer without a rise. With only one exception (transplant 3 in mare 3875) all recipients still had cytotoxic antibodies present at the time of transplants two and beyond.

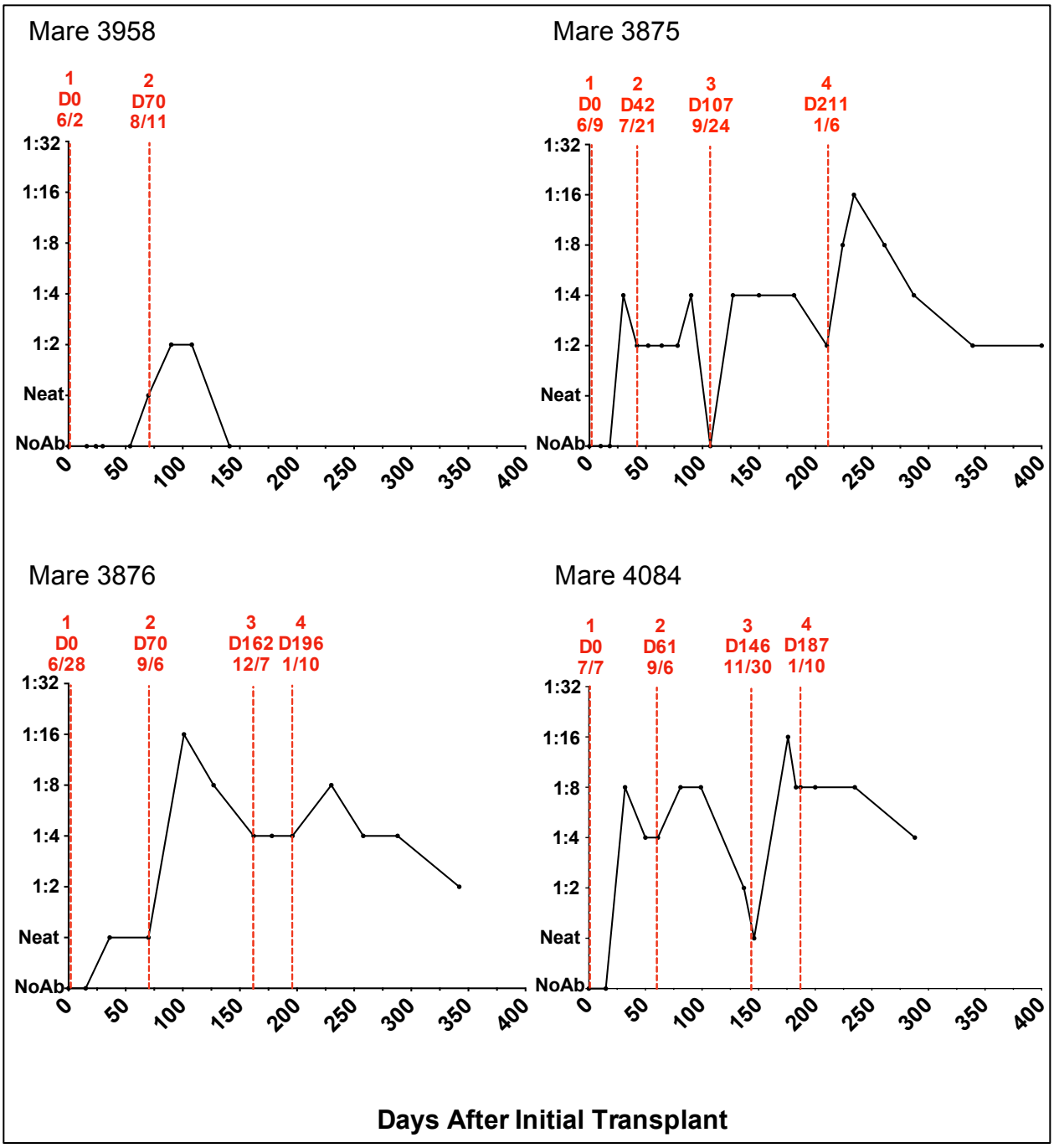

Figure 2.5 Breeding season one: lymphocyte microcytotoxicity titers after serial trophoblast transplants. The cytotoxic antibody titers shown in Figures 2.2.1 through 2.2.4 are presented for comparison between recipients. All recipients had negative baseline titers. Titers were sustained throughout the experiment with the exception of one return to baseline in mare 3875. Recipients' immune systems recognized and mounted humoral immune responses to serial transplants as evidenced by increases in antibody titer in 12 out of 14 transplants. The date of the transplant is given above, with the day of current season experiment, and the transplant date.

Trophoblast transplants had inconsistent physiologic effects.

Sustained post-transplant increases in serum progesterone concentration (lengthened interovulatory periods) as would occur in a normal pregnancy were taken to indicate that the transplant had a physiologic effect upon the recipient's reproductive system. Serum progesterone was monitored for the duration of the serial transplants (Figure 2.6). Following six of the 11 complete transplants, recipients maintained high serum progesterone for the entire lifespan of the transplant with no ovulations occurring (Table 2.3). One other transplant was followed by high serum progesterone for 58 days of a 71-day lifespan. Four transplants were followed by a nearly normal cycle pattern of mean 21-30 days.

A preliminary observation may be made that seasonality and stage of the recipient's cycle appeared to be external factors that influenced whether or not the transplant exerted a physiologic effect on the recipient. Transplants were more likely to exert a physiologic effect (i.e. mean interovulatory periods showed a trend towards being longer) during the normal equine breeding season (Figure 2.7). A single transplant performed during anestrus was unable to stimulate ovulation in the recipient mare, and the period of anestrus continued for the life of the transplant (Mare 3875, transplant 4, refer to Figure 2.2.2).

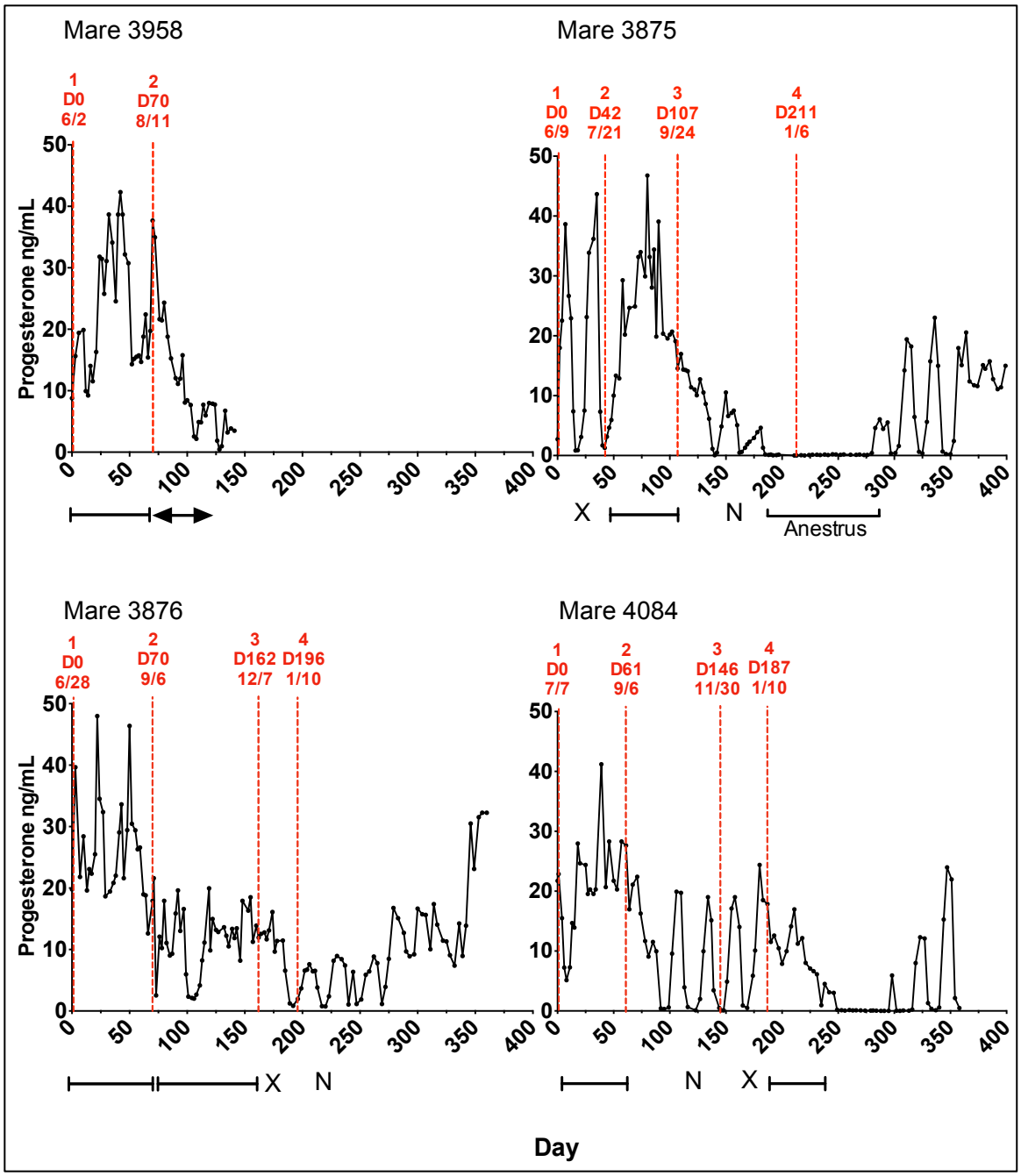

Figure 2.6 Breeding season one: serum progesterone concentrations. Progesterone was measured by ELISA for the duration of the experiments. Sustained serum progesterone indicated that a transplant was exerting a physiologic effect upon the recipient. Seven transplants were physiologically effective for all (bar) or most (arrow) of their lifespan. X indicates the three incomplete transplants and N indicates transplants followed by normal cycles. The number of the transplant is given above, along with the day of current season experiment and the transplant date.

Table 2.3 Interovulatory[a] Periods: Breeding Season One

Recipient Number	Transplant Number	Month	Lifespan	P4 at Transplant	I/O Periods	Mean I/O Length	Days of Anestrus
3958	1	JUN	70	8.8	<1	70	0
	2	AUG	71	37.7	1	58	0
3875	1	JUN	Incomplete	--	--	--	--
	2	JUL	65	1.3	<1	65	0
	3	SEP	90	14.5	3	26	12
	4	JAN	156	0.0	3	21	69
3876	1	JUN	66	19.8	<1	70	0
	2	SEP	92	17.9	<1	92	0
	3	DEC	Incomplete	--	--	--	--
	4	JAN	146	2.0	4+[b]	30	0
4084	1	JUL	61	21.7	<1	61	0
	2	SEP	85	27.6	3	25	0
	3	NOV	Incomplete	--	--	--	--
	4	JAN	38	17.9	<1	48	0

[a] = ovulation defined as a progesterone drop to ≤1 ng/mL

[b] = the last I/O period continued beyond the end of the TT lifespan; days of increased progesterone were averaged in as if an I/O

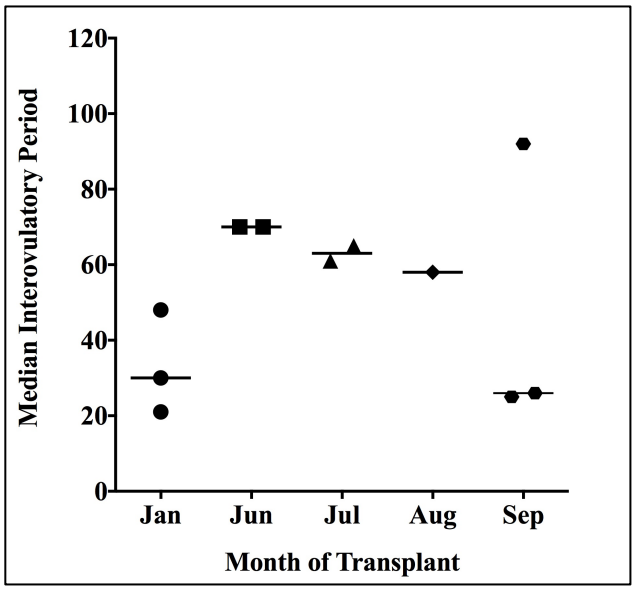

Figure 2.7 Median interovulatory periods by month of trophoblast transplant. Median interovulatory periods tended to be greater (i.e. transplants had a physiologic effect) when transplants were performed during the normal equine breeding season. Transplants designated Incomplete are excluded from the analysis.

Results for Breeding Season Two

Summary Data for Individual Recipients

Two of the mares (3876 and 4084) receiving four transplants in the first breeding season then received two additional transplants during the second breeding season. The objective of this study was to determine if there would be a change in transplant lifespan following a break in exposure. Non-invasive analysis was performed as in the first breeding season (Figure 2.8).

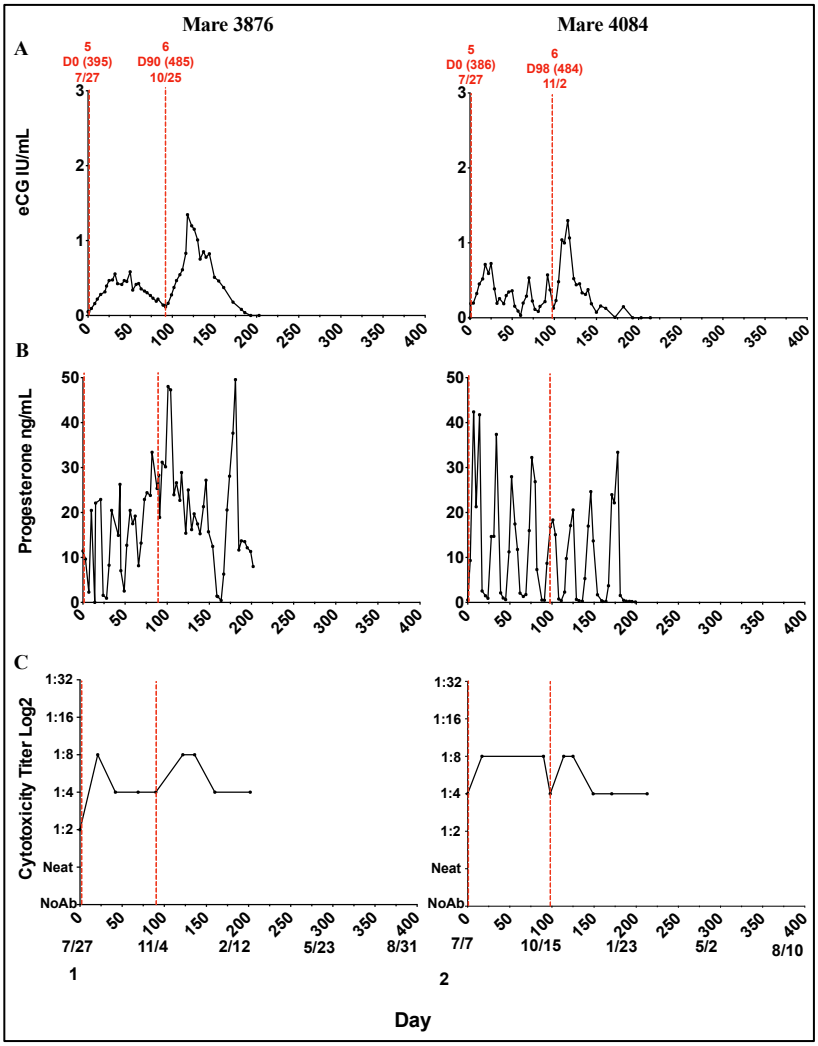

Figure 2.8 Mares 3876 and 4084, summary of eCG, progesterone and antibody data from breeding season two. Serum eCG (A 1-2), progesterone (B 1-2) and lymphocyte cytotoxicity titers (C 1-2) were tracked for the duration of the experiment. Vertical, dashed red lines indicate each transplant. The date of the transplant is given above, with the day of current season experiment, (day from first transplant, first season), and transplant date. The quarterly month and day are provided under the bottom X-axis to indicate seasonality.

Median Lifespan from Breeding Two Season Was Comparable to Breeding Season One

The four transplants performed in breeding season two had lifespans within the range of those in breeding season one (Figure 2.9). Although the median lifespan of transplant six was slightly less than transplant five (Figure 2.10), the median lifespan for all transplants performed in the second breeding season was 71 days (Table 2.4). Although transplant lifespans were comparable across breeding seasons, serum eCG peaks in breeding season two were notably lower than those produced by transplants performed during the breeding season one (Figure 2.11).

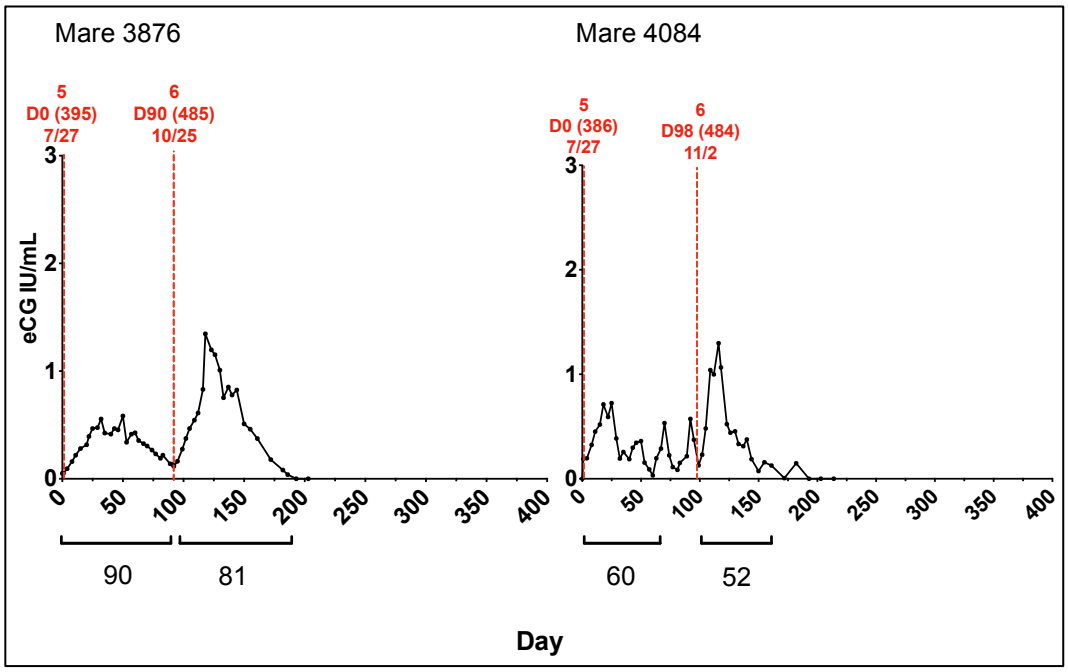

Figure 2.9 Breeding season two: trophoblast transplant lifespans calculated from eCG curves. Lifespans of transplants in the second breeding season were similar to those in the first. eCG peaks were noticeably lower, remaining under 2 IU/mL. The transplant number along with day of current experiment, (day from first transplant, first season), and transplant date.

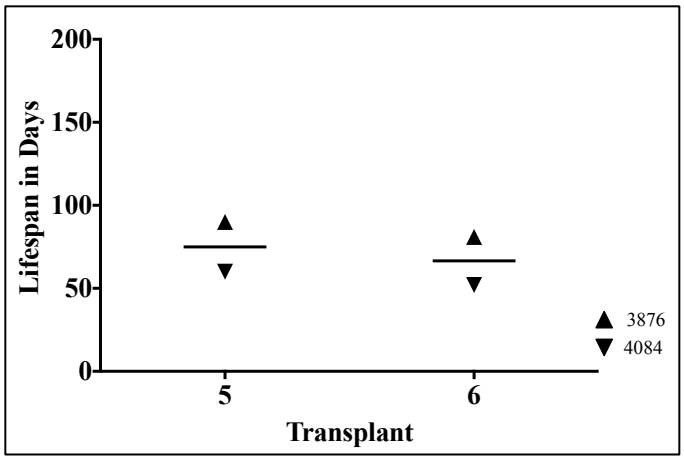

Figure 2.10 Breeding season two: trophoblast transplant lifespans. Trophoblast transplant lifespans from the four transplants performed during breeding season two. The horizontal lines indicate the median transplant lifespans.

Table 2.4 Trophoblast Transplant Outcome Summary: Breeding Season Two

Recipient Number	Transplant Number	Lifespan
3876	5	90
	6	81
4084	5	60
	6	52
Overall Median		**71**

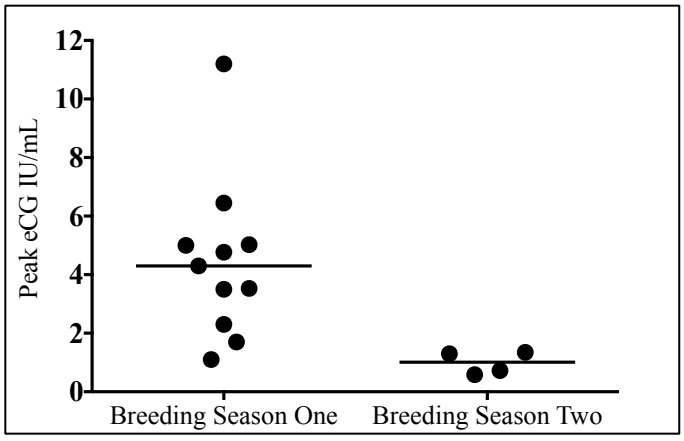

Figure 2.11 Breeding season two: eCG peaks. eCG peaks from transplants performed during the first and second breeding seasons. There was a trend toward lower eCG peaks in breeding season two.

Transplants survived in the presence of pre-existing antibodies and incited immune responses. Both mares that received transplants during the first breeding season had positive antibody titers to the donor sire antigen at the beginning of the second breeding season. All four transplants performed in the second breeding season induced humoral immune responses in the recipients as evidenced by increased cytotoxic antibody titers (Figure 2.12).

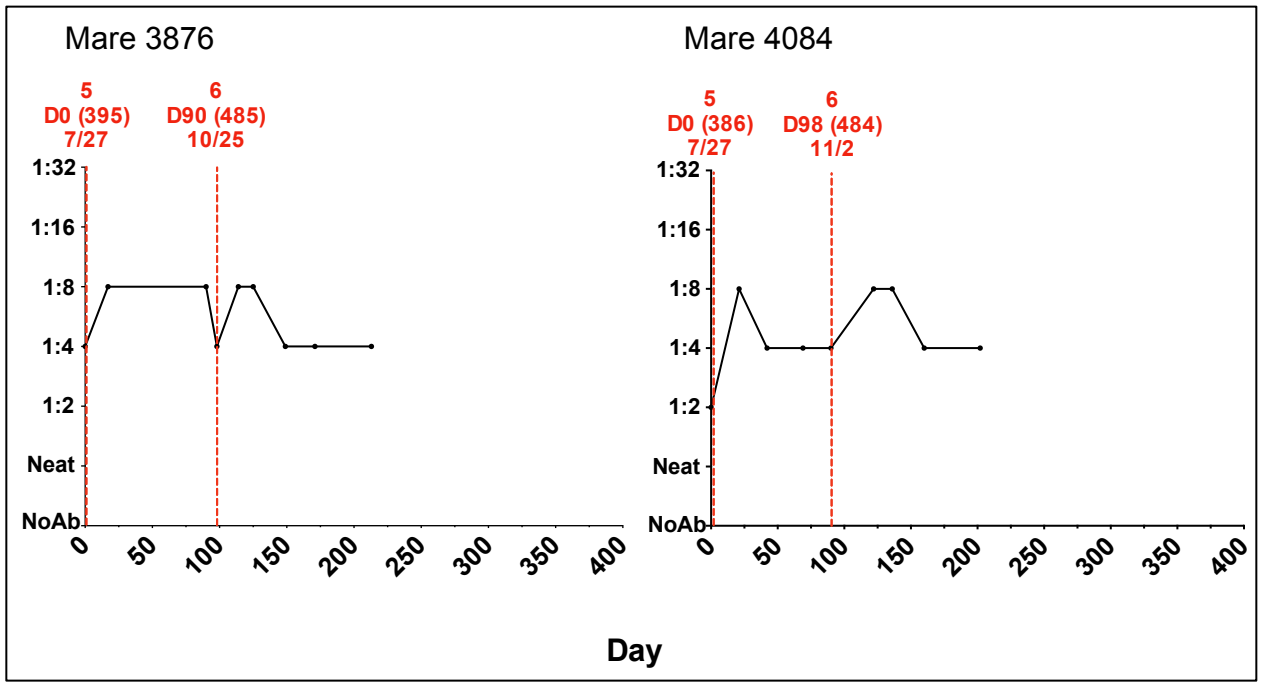

Figure 2.12 Lymphocyte microcytotoxicity titers: breeding season two. Both mares receiving transplants during the second breeding season had positive baseline titers. Both mares mounted humoral immune responses to transplants performed following the break in exposure. The transplant number is indicated above along with day of current experiment, (day from first transplant, first season), and transplant date.

Transplants during the second breeding season did not have a consistent physiologic effect. During the second breeding season only one transplant (mare 3876, transplant 6) was followed by sustained progesterone for nearly the entire transplant lifespan (Figure 2.13). The remaining

transplants were followed by nearly normal estrus cycles. No periods of anestrus were observed (Table 2.5).

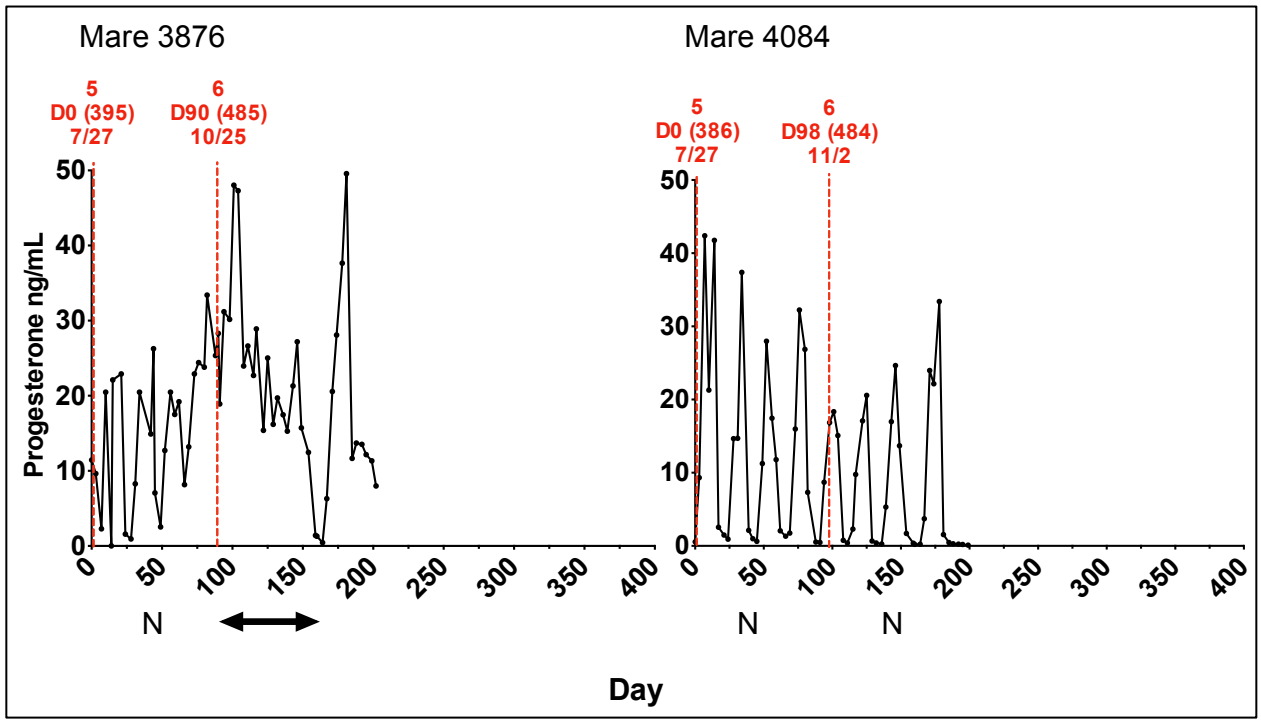

Figure 2.13 Serum progesterone concentrations: breeding season two. Sustained high serum progesterone indicates a transplant that is physiologically effective upon the recipient. One transplant (arrow) was followed by high serum progesterone for nearly the entire lifespan. Following the remaining three (N) recipients continued to cycle nearly normally.

Table 2.5 Interovulatory[a] Periods: Breeding Season Two

Recipient ID	TT	Month	Lifespan	P4 at TT	I/O Periods	Mean I/O Length	Days of Anestrus
3876	5	JUL	90	11.4	3[b]	30	0
	6	OCT	81	18.9	1[c]	74	0
4084	5	JUL	60	0.56	4[d]	22	0
	6	NOV	52	16.9	3	23	0

[a] = ovulation defined as a progesterone drop to ≤1 ng/mL
[b] = the last I/O period continued beyond the end of the TT lifespan, only days of elevated progesterone until the next TT were counted.
[c] = eight days of the subsequent I/O period were included in the transplant lifespan, but as this was a partial cycle it was excluded from the calculation
[d] = an additional 23 day cycle overlapped transplants 5 and 6; this was counted in transplant 6

Discussion

This study investigated the hypothesis that priming a recipient's immune system with an initial ectopic trophoblast transplant would alter the lifespans of subsequent transplants. Second and subsequent serial transplants could have longer lifespans resulting from a protective memory response or chronic antigenic exposure, or shorter lifespans resulting from a destructive memory response. The primary finding of this study was that the median lifespan of second and subsequent trophoblast transplants does not become shorter than that of first transplants. There was no difference in the median transplant lifespans from the first and second breeding seasons, with both being 71 days. Furthermore, this is similar to the 75 day median lifespan reported in a study of single transplants (de Mestre et al., 2011).

These findings first suggest that survival of an initial ectopic trophoblast transplant is not due only to a naïve recipient immune system. There is evidence for a memory response with second and subsequent transplants in the form of cytotoxic antibodies measurable in recipient serum, but this does not result in earlier destruction of the transplanted tissue. Second, the serial trophoblast transplants also survived without the protection of the uterus and the hormonal priming of early pregnancy, further suggesting that the mechanisms preventing immune destruction may be initiated by the trophoblast itself.

In the original trophoblast transplant experiments performed by Adams and de Mestre, recipient mares were maidens possessing no cytotoxic antibodies to donor sire MHC-I (Adams and Antczak, 2001; de Mestre et al., 2011). Survival of a single, ectopic trophoblast transplant in a maiden recipient or of the endometrial cups in a primigravida mare therefore possibly could be

explained by a naïve recipient or maternal immune system. Although the chorionic girdle cells express paternal as well as maternal MHC-I at the time of transplantation (day 34), and at the time of invasion in normal pregnancy (day 36), the MHC-I molecules then are down-regulated between days 41 to 44. (Donaldson et al., 1994; Donaldson et al., 1992; Donaldson et al., 1990). This effectively removes the target of the cytotoxic antibodies, providing one possible reason why the trophoblast can continue to survive in an immunologically hostile environment.

MHC-I down-regulation can not explain the success of second and subsequent pregnancies in which MHC-I-bearing trophoblast invades the maternal tissue of a dam with pre-existing cytotoxic antibodies. The presence of pre-existing antibodies against donor antigens typically results in hyperacute rejection of the foreign tissue in conventional organ transplantation (Mengel et al., 2012), but many studies show this does not hold true in the context of equine pregnancy. Cytotoxic antibodies persisted in mares following first pregnancies, and anamnestic responses were observed in subsequent pregnancies with sires of the same MHC haplotype (Antczak et al., 1984). Strong secondary antibody responses occurred whether the dam's immune system was primed by a first pregnancy or by a skin allograft from the sire, and no changes were observed in endometrial cup biology in these pregnancies (Adams et al., 2007).

In each of the cases above the secondary responses occurred in a pregnant mare, with trophoblast situated in the uterus. In the current studies these protective elements were removed. Serial trophoblast transplants repeatedly induced measurable antibody production in recipients. Second and subsequent transplants were placed into recipients possessing cytotoxic antibodies against donor sire MHC-I in all but one instance. Although these experiments took place outside of the

context of pregnancy, the presence of pre-existing antibody did not result in more rapid destruction of the transplanted tissue, which continued to survive and function for a period of time comparable to single ectopic transplants and normal endometrial cups.

The non-invasive trophoblast transplant model does not permit direct evaluation of the cellular response to the ectopic tissue. However, it is likely that in addition to an anamnestic antibody response, a T-cell memory response also is initiated at the time of the first transplant. It is clear that the cellular memory is not destructive in nature, but possibly could be protective. T-regulatory cells have been shown to play a role in maternal tolerance in mice. These T-regulatory cells generate memory pools following first pregnancies, and have been shown to have specific affinity to fetal antigens (Rowe et al., 2012). T-regulatory cells likely play a significant role in fetal tolerance in human pregnancy as well (Santner-Nanan et al., 2009; Somerset et al., 2004). In the horse, T-regulatory cells have been identified surrounding endometrial cups in normal equine pregnancy (de Mestre et al., 2010), and mares suffering early pregnancy loss show low levels of T-regulatory cells in their peripheral blood (Aurich et al., 2013). Although T-regulatory cells were not investigated in this study, it is possible that they contributed to trophoblast survival. It is further possible that memory T-regulatory cells contributed to the trend toward increasing transplant lifespans observed in the closely spaced transplants in the first breeding season. Alternatively, this trend may have been due to anergy, exhaustion or senescence from chronic antigenic exposure (Schietinger and Greenberg, 2014). Regardless of mechanism, this effect was small and was not sustained, as it was not observed in transplants performed during the second breeding season following the interruption in antigen exposure.

In addition to the presence of T-regulatory cells, the observation that transplant lifespans remained stable over time could also be explained by a dampening of T-cell effector functions as has been documented in pregnant mares. Cytotoxic T-lymphocyte (CTL) responses in recipients may be systemically blunted as is observed in normal pregnancies. The chorionic girdle cells are not inherently resistant to destruction by cytotoxic T-lymphocytes, and in vitro studies have demonstrated that equine invasive trophoblast can be lysed by CD8+ cytotoxic T-lymphocytes first primed by exposure to paternal lymphocytes if the lymphocytes are obtained from non-pregnant mares (Baker et al., 2000). Co-culture with invasive trophoblast or conditioned media diminished proliferation of naïve lymphocytes and changed the cells' cytokine profile. However, this effect too was abrogated if the lymphocytes were first stimulated before exposure to trophoblast.

In contrast to the in vitro studies, cytotoxic T-lymphocyte reactivity against paternal antigens is decreased in pregnant mares (Baker et al., 1999), a phenomenon that is antigen-independent, and unlike the cytotoxic antibody response occurs in MHC-I compatible as well as incompatible matings (Noronha and Antczak, 2012). The recipient mares in this study were not pregnant and therefore should not have had any modulation of their immune systems prior to the transplants. Cytotoxic T-lymphocyte activity was not evaluated in the recipients, but if T-cell effector function was dampened it would again suggest that the trigger for this level of tolerance may have originated from the trophoblast itself.

The prolonged survival of serial ectopic trophoblast transplants in non-pregnant recipients with primed immune systems suggests that the signal to initiate tolerance originates from the

trophoblast itself, though the nature of the signal is unknown. Immunomodulatory molecules of trophoblast origin have been identified in both humans and mouse models. One of these is CD200, a molecule found in both murine and human trophoblast. CD200 is thought to be involved in the development of T-regulatory cells, and may play a role in T-cell inhibition via the indoleamine pathway (Clark et al., 2003; Clark et al., 2001; Gorczynski et al., 2005; Wang et al., 2014; Yu et al., 2008) . Another such molecule is macrophage inhibitory cytokine-I, produced by human trophoblast. This molecule is hypothesized to shift dendritic cells to a tolerogenic phenotype (Segerer et al., 2012). Galectin-1 has been identified in human extravillous trophoblast, and promotes fetal tolerance by both inhibiting effector T-cell activity and promoting T-regulatory cell development (Ramhorst et al., 2012). Investigation of these and other molecules with the potential to control lymphocyte function (Dong et al., 2008) in the horse could add additional insight into ectopic trophoblast transplant survival.

Although there was some variation in transplant lifespans within and between mares in both seasons, the lifespan of this tissue gravitated towards approximately 70 to 80 days as observed in normal pregnancy. The fourth transplant in mare 4084 was an outlier with a lifespan of only 38 days (Figure 2.3, Table 2.2). However, this transplant also did not result in a rise in antibody titer. The reason for this is uncertain, though it could have been due to a technical failure of the transplant rather than aberrant immunologic activity.

A secondary outcome of this study was the generation of endocrinologic data (progesterone and eCG) that spanned multiple breeding seasons. The original purpose for collecting this data was to use as readouts for trophoblast transplant lifespan (eCG) and physiologic effect

(progesterone). Unexpectedly, the duration of these studies through consecutive breeding seasons in conjunction with the seasonally anestrus mare reproductive cycle resulted in some interesting, though very preliminary, observations on the role of these hormones in trophoblast survival and reproductive cycle manipulation.

Studies of single trophoblast transplants showed that the transplants are able to exert a physiologic effect upon the recipient's reproductive cycle. The physiologic effect of trophoblast transplants was variable in the current study. The single transplant studies suggested that when transplants are placed into a mare already in diestrus (i.e. mimicking the hormonal environment of normal pregnancy), there is a greater likelihood of physiologic effect upon the recipient (de Mestre et al., 2011), but that trend did not hold true in this study. The long-term nature of this study in conjunction with the seasonally anestrus nature of the mare estrous cycle likely accounted for most of this variability. Transplants showed a greater likelihood of physiologic effect during the normal equine breeding season, and when placed during anestrus did not initiate cycling in the recipient. Only one of the four transplants showed a physiologic effect during the second breeding season. This was possibly due to the very low levels of eCG produced by those transplants; serum eCG concentrations remained below 2.0 IU/mL. In studies of persistent endometrial cups, serum eCG of 1.67 IU/mL has been reported as a threshold for estrus cycle disruption (Crabtree et al., 2012; Huber et al., 1993).

These very low eCG concentrations produced by transplants in the second breeding season were an unexpected observation. A similar observation has been made in normal pregnancies in which mares were bred to the same stallion for up to five successive pregnancies. In some of

those mares eCG decreased with each parity from the third to the fifth. In those mares progesterone was not measured, but pregnancies were carried to term indicating adequate levels of eCG and progesterone produced in the context of normal pregnancy (Wilsher and Allen, 2011), whereas in the transplants the eCG was generally not adequate to inhibit the estrus cycle.

Although the reason for measuring progesterone in this study was as an indicator of the physiologic effect of the transplants, the duration of the study in conjunction with the seasonally anestrous nature of the mare reproductive cycle provided an intriguing though very preliminary observation on the role of progesterone in transplant survival. Critical hormones of pregnancy, including progesterone and human chorionic gonadotropin (hCG), are important modulators of immune cell function in humans in addition to their purely endocrine roles. These hormones can influence the actions of many immune cell types both directly and indirectly. They have proposed roles in the biology of T-regulatory cell and uterine NK-cell development, and inhibit macrophage migration into the pregnant mouse uterus (Hunt et al., 1998; Lee et al., 2012; Schumacher et al., 2014; Schumacher et al., 2013). Studies investigating the immunomodulatory properties of progesterone and equine chorionic gonadotropin in equine pregnancy are lacking, and a definitive role for these hormones in maternal tolerance has not been established in the horse.

Though the 34-day priming of the recipients' immune systems that would occur in a normal equine pregnancy is absent in the context of the trophoblast transplants, in most cases both hormones are present in temporal proximity to the transplant. The one exception to this was the fourth transplant in mare 3875. This transplant was placed in January at a time when the mare

already had been in anestrus for a period of 12 days. There was no detectable progesterone in her serum at this time. Following the transplant she remained in anestrus for another 69 days. In spite of this, the lifespan of that transplant was the longest in the study at 156 days. Although this timing occurred in only a single transplant, it suggests that progesterone may not be a requirement for trophoblast transplant survival. Furthermore, it raises the possibility that a lack of exposure to progesterone could contribute to an increased lifespan. Since the primary function of the chorionic girdle cells is to induce secondary follicles to produce enough progesterone to maintain the pregnancy, lack of exposure to progesterone could in theory interfere with a feedback loop. Invasive chorionic girdle cells have been found not to express progesterone receptor (Wilsher et al., 2011) so this would have to occur via another indirect mechanism. Alternatively, this long lifespan could be an effect secondary to chronic exposure from the three previous transplants. Progesterone deficiency in ovariectomized rats has been shown to alter the composition of circulating T-cell populations (Leposavic et al., 2014). This does not appear to hold true in normal mares during anestrus (Ferreira-Dias et al., 2005), but might affect recipient immune response to an ectopic trophoblast transplant in a way not examined by this single study.

In summary, the serial trophoblast transplant experiments tested the hypothesis that immune priming of the recipient would affect the lifespan of the transplant. The results indicated that immune priming of the recipient does not result in a shorter transplant lifespan, and may result in a longer lifespan if the transplants are closely spaced. The mechanisms behind this are uncertain, but likely are initiated by the trophoblast itself, and involve a complex interaction of immunologic and endocrine components.

Acknowledgements

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CHAPTER 3

SERIAL ECTOPIC TROPHOBLAST TRANSPLANTS:

INVASIVE STUDY OF HORSE-TO-HORSE ALLOGRAFTS

Abstract

Studies in equine reproductive immunology demonstrated that horse invasive trophoblast transplanted to an ectopic site in an unrelated, histo-incompatible, non-pregnant mare survives and functions for a length of time similar to a normal horse pregnancy. When the transplanted tissue was biopsied periodically through day 28, immunohistochemical analysis indicated that the expression of trophoblast markers and major histocompatibility class I (MHC-I), and the production of equine chorionic gonadotrophin (eCG), showed patterns similar to endometrial cups in normal horse pregnancy. Furthermore, the cellular infiltrates at the transplant site were similar to the cell populations observed surrounding normal endometrial cups. Later studies of single transplants documented trophoblast marker by immunohistochemistry up to 56 days, but did not examine the surrounding cells. The objective of this study was to determine if repeated trophoblast transplants in a single mare would result in alterations to the physical characteristics of the trophoblast itself, or of the surrounding cellular infiltrates when compared to endometrial cups in a normal pregnancy. A maiden mare received three serial trophoblast transplants that were biopsied at 10, 40 and 50 days post-transplant. The recipient's serum was analyzed for eCG and antibodies against donor sire MHC-I. Immunohistochemistry was used on biopsied tissue to characterize changes in the transplanted cells and the nature of the cellular infiltrates. Healthy trophoblast was identified out to day 50 in the third transplant in spite of the presence of cytotoxic antibodies. Identification of MHC-I on trophoblast biopsied at day 10 suggests a delay in MHC-I down-regulation relative to normal pregnancy. Transplants biopsied at days 40 and 50 showed increased numbers of eosinophils in the cellular infiltrates relative to the day 10 biopsy, suggesting that eosinophils may be associated with mature trophoblast transplants as observed in late stage endometrial cups.

Introduction

The focus of many reproductive immunology studies in the horse has been the endometrial cup reaction, which involves both a humoral and cellular immune response by the dam to the invading chorionic girdle cells. The humoral response is characterized by the appearance of alloantibodies against paternal major histocompatibility class I (MHC-I) in the serum of the dam, and occurs in all histo-incompatible pregnancies. The cellular response involves the rapid infiltration of leukocytes to the site of trophoblast invasion, and is seen in both MHC-I-compatible and MHC-I-incompatible pregnancies. In spite of this robust immune response to the genetically distinct trophoblast, the cells survive and function for approximately 80 days in a normal horse pregnancy (Lunn et al., 1997).

The endometrial cup reaction in the horse begins shortly after the chorionic girdle cells invade the endometrium on day 36 of pregnancy. The chorionic girdle cells at this time express both maternal and paternal MHC-I, and remain positive for MHC-I until day 45 of pregnancy (Donaldson et al., 1992; Kydd et al., 1991). The invading cells immediately begin to produce equine chorionic gonadotropin (eCG) and continue to do so until approximately day 120 of pregnancy (Lunn et al., 1997). The local cellular response to the foreign tissue is characterized primarily by CD4+ and CD8+ T-lymphocytes, with rare B-cells and eosinophils appearing during the later stages (Grunig et al., 1995).

The abundance of CD4+ and CD8+ T-lymphocytes differs markedly from what is observed in mice and humans. In mice only 3% of leukocytes in the decidua on embryonic day 8.5 are T-cells, with CD4+ and CD8+ cells present in equal numbers (Nancy and Erlebacher, 2014). In

humans, the predominant cell type in the decidua is the uterine natural killer (NK) cell with less than 20% of leukocytes being T-cells. The T-cell population may include a greater proportion of CD8+ T-cells. A definitive role for CD4+ and CD8+ T-cells in healthy or abnormal pregnancies is unclear (Erlebacher, 2013; Nancy and Erlebacher, 2014). Uterine NK cells in humans lack the cytotoxic phenotype of peripheral blood NK cells, and instead engage in more specialized functions such as spiral artery remodeling in early human pregnancy (Hemberger, 2013; Kopcow et al., 2005). Molecular evidence for natural killer cells has been identified in equine endometrial cups, but a significant functional role for these cells is not known (Noronha et al., 2012b).

A system for transplanting chorionic girdle cells to an ectopic site in an unrelated, non-pregnant mare has been established to facilitate the study of interactions between invasive trophoblast and the maternal immune system in the horse. When the transplant recipient is a maiden mare, the ectopic trophoblast cells show a similar lifespan and comparable physiologic effects as endometrial cups in a normal horse pregnancy. Biopsies of transplanted tissue have indicated that cellular infiltrates at least through day 28 are comparable to the leukocytes that surround normal endometrial cups (Adams and Antczak, 2001; de Mestre et al., 2011). As presented in Chapter 2, additional non-invasive studies of the trophoblast transplant system have established that the median lifespans of second and subsequent transplants in a single mare do not change relative to first transplants. This indicates that the chorionic girdle cells can survive when the recipient immune system is primed to donor sire MHC-I. The objective of the present study was to further characterize the serial trophoblast transplant model by direct examination of

transplanted tissue and cellular infiltrates at several different time points throughout the lifespan of the transplant.

Materials and Methods

Animals

Animals used in this study were members of the Cornell Equine Genetics Center herd. The recipient mare used for the biopsy experiments was a reproductively mature, maiden horse lacking the Equine Leukocyte Antigen (ELA) A3 haplotype. Donor mares were of various breeds, ages and ELA haplotypes. The donor sire was a Thoroughbred stallion homozygous for the A3 ELA haplotype. Animal care and experimental design were in compliance with protocols approved by the Cornell University Institutional Animal Care and Use Committee (IACUC).

Breeding, Conceptus Recovery and Transplantation Technique

Donor mares were impregnated via artificial insemination with fresh semen from the donor stallion. Conceptuses were recovered at day 34 by non-invasive uterine lavage as previously described (Antczak et al., 1987). Transplants were performed in a manner consistent with a method previously described (de Mestre et al., 2011). Briefly, after recovery the entire chorionic girdle was dissected, minced, loaded into a tuberculin syringe with 1 mL phosphate buffered saline and penicillin/streptomycin and injected aseptically into the vulvar mucosa of the recipient. Serial transplants were placed on alternating sides of the vulva.

Sampling

Blood samples were drawn via jugular venipuncture into serum tubes from recipient mares three times per week until eCG was no longer measurable in their serum. Blood was allowed to clot, tubes were centrifuged and serum stored at -20° C until processing. Blood from the donor stallion was drawn as needed into heparinized tubes and processed immediately for use in the cytotoxicity assays.

Biopsies

Transplant biopsies were performed under sedation (xylazine 0.4 mg/kg or romifidine 40 µg/kg IV) and a local block (1 to 2 mL of 2% lidocaine). Tissue was surgically excised from the location of transplantation and placed on ice until processing. The excision site was sutured and monitored until healed. Tissue sections were embedded (O.C.T. Compound, Sakura, Torrance, CA) and frozen at -80 ° C until processing. Slides (Superfrost Plus, Fisher Scientific, Pittsburgh, PA) were sectioned at 6 µm, fixed for 10 minutes in cold acetone and stored at -20 ° C until processing.

eCG Quantifications

A commercially available ELISA kit (PMSG ELISA, DRG International, Springfield, NJ) was used to quantify the recipient's serum eCG. Plates were read on a Multiskan Ex and data were analyzed with Ascent 2.6 software (ThermoFisher Scientific, Waltham, MA).

Lymphocyte Microcytotoxicity Assay

Lymphocytes from the donor stallion were isolated from heparinized venous blood.

The presence of recipient antibody to donor sire MHC-I antigen was determined using a lymphocyte microcytotoxicity assay as previously described (Antczak et al., 1982). Briefly, serum from recipients was plated in microtiter plates (Robbins Scientific, Mountain View, CA) in two-fold dilutions. Dilutions were carried out until a negative test occurred. Serum was incubated with lymphocytes from the donor stallion and rabbit complement (Pel-Freez, Lot #PF29521, Brown Deer, WI). Eosin dye was added and the wells were fixed with formalin. Wells were scored as percent cell killing indicated by dye exclusion. Wells showing greater than 50% killing were considered positive. Titers were called as the highest dilution with a positive result. Each sample was tested at least twice.

Immunohistochemistry

All steps in this assay took place at room temperature in a humidity chamber. Slides were incubated with primary antibody (Table 3.1) diluted in TBS (0.5M Tris base, 1.55M NaCl) for 30 minutes and then washed three times in TBS. Slides were blocked for 15 minutes with 10% normal goat serum. Second stage antibody (HRP conjugated goat anti-mouse or anti-rat IgG, Jackson ImmunoResearch, West Grove, PA) was diluted with 10% normal goat serum and applied for 30 minutes. Slides were again washed three times in TBS and incubated for 25 minutes with aminoethyl carbazole (AEC Kit, Life Technologies, Grand Island, NY). Slides were rinsed in TBS and then water, and counterstained for 5 minutes with hematoxylin (Gill's Formulation #2, Fisher Scientific, Pittsburgh, PA). Coverslips then were mounted (Glycergel, Dako, Agilent Technologies, Santa Clara, CA).

Table 3.1 Monoclonal Antibodies Used in Serial Horse-to-Horse Trophoblast Transplant Experiments

Target	Antibody ID	Species	Isotype	Reference
Horse Trophoblast	102.1	Mouse	IgG1	(Oriol et al., 1989)
CD4	HB61A	Mouse	IgG1	(Kydd et al., 1994)
CD8	HT14A	Mouse	IgG1	(Kydd et al., 1994)
eCG	67.1	Rat	IgG2a	(Oriol et al., 1991)
MHC-I	CZ3	Mouse	IgG1	(Kydd et al., 1994)
B-cell	2.1	Mouse	IgG1	(Kydd et al., 1994)
NKp46	4F2	Mouse	IgG1	(Noronha et al., 2012a)
Negative Control	Anti -canine Parvovirus	Mouse, Rat	IgG1	(Parrish et al., 1982)

Results

The objective of this study was to determine if repeated trophoblast transplants in a single mare would result in alterations to the physical characteristics of the trophoblast itself, or of the surrounding cellular infiltrates when compared to endometrial cups in a normal pregnancy. Three histo-incompatible transplants were performed in a maiden mare (Table 3.2), and each was then biopsied at a different time point. Serum from the mare was monitored for eCG and antibodies against the donor sire's lymphocytes. Immunohistochemical analysis was performed on tissue biopsies to identify trophoblast surface molecules and eCG production and to characterize the surrounding cell populations (Figure 3.1).

Table 3.2 Animals Used in Serial Trophoblast Transplant Experiments

			Transplants Donor Mare/MHC Donor Sire/MHC		
Recipient	Recipient MHC	Age	1[a]	2[b]	3[c]
4069	A1/?	8	3640 A3/?	3099 A2/A2	3957 A1/A3
			3474 A3/A3	3474 A3/A3	3474 A3/A3

[a] biopsied at 10 days
[b] biopsied at 40 days
[c] biopsied at 50 days

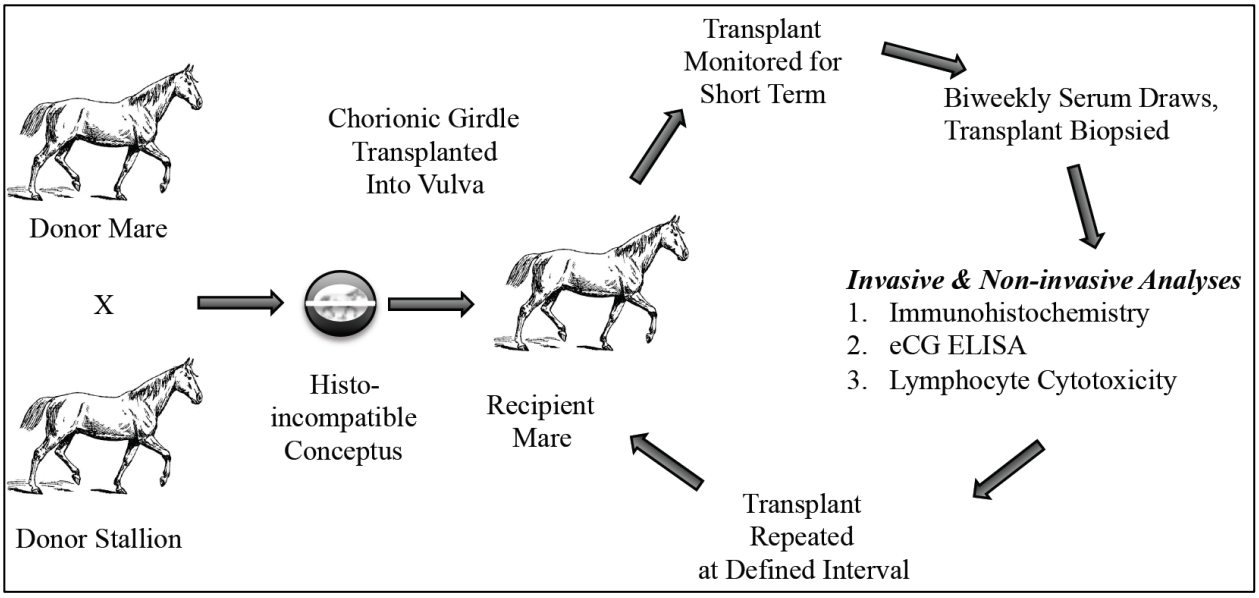

Figure 3.1 Schematic of biopsied horse-to-horse trophoblast transplants. Chorionic girdle was obtained from a 34-day horse conceptus and transplanted into the vulvar mucosa of an unrelated, histo-incompatible recipient mare. The transplants were monitored non-invasively for a defined period of 10, 40 or 50 days by analyzing recipient serum for eCG and antibody production. The tissue was then biopsied and the transplanted tissue and surrounding recipient leukocytes were characterized using immunohistochemistry.

Serial trophoblast transplants express trophoblast marker and down regulate MHC-I

Immunohistochemical analysis of all three biopsies showed large areas of tissue expressing a trophoblast marker. At the time of the first transplant, the recipient's serum had no cytotoxic antibodies against the donor sire lymphocytes. MHC-I was present on trophoblast in the day 10 biopsy. The detection of cytotoxic antibodies against donor sire MHC-I by the time of the first biopsy indicates that the transplant was recognized by the recipient's immune system and a humoral response occurred. At the time of the second and third transplants the recipient still had serum antibodies present. Biopsies showed trophoblast devoid of MHC-I, but increases in antibody titer indicated an anamnestic response (Figure 3.2).

Serial trophoblast transplants continue to secrete eCG throughout their lifespan

Secretion of eCG by the ectopic trophoblast is indicative of a transplant that is surviving and functioning. This was evaluated by two methods in the biopsy experiments. Serum eCG was measured by ELISA, and results indicated that all three transplants produced sufficient eCG to be detected in the peripheral blood of the recipient. Immunohistochemistry also identified eCG in cells at all three time points, indicating that serum eCG at the later time points resulted from continued secretion of eCG (Figure 3.3).

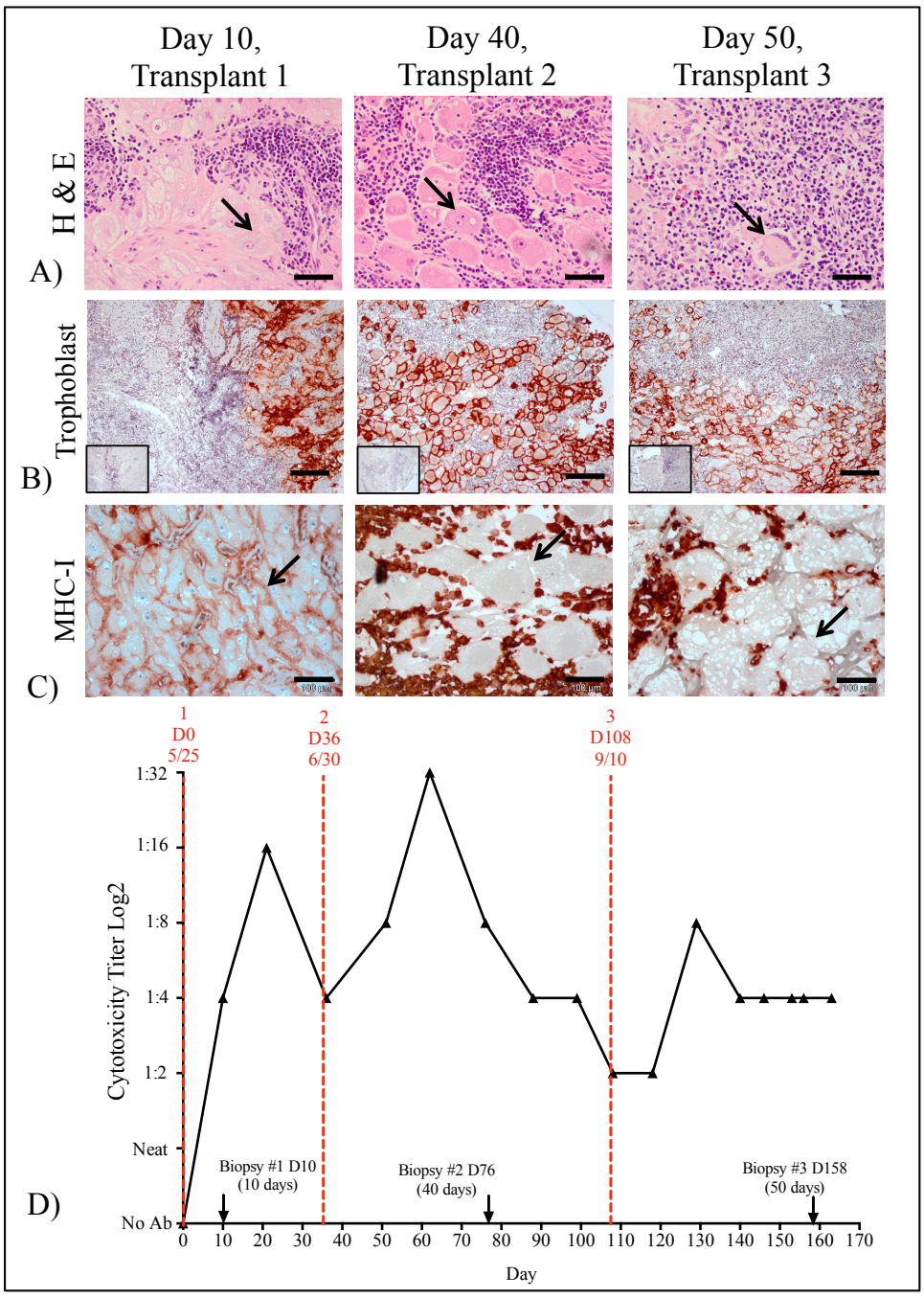

Figure 3.2 Immunohistochemistry identifying trophoblast surface molecules on serial transplant biopsies. Formalin-fixed, hematoxylin and eosin stained sections (A) of serial trophoblast transplants show binucleate chorionic girdle cells (arrows) surrounded by recipient's leukocytes. Immunohistochemistry using an anti-trophoblast antibody (B) confirms the presence of equine trophoblast in the biopsied tissue at all three time points (negative control shown in inset). MHC-I expression (C) is seen on the surface of trophoblast cells at day 10 (arrow). As expected, the later biopsies showed that MHC-I was present on the infiltrating lymphocytes but not on the trophoblast itself (arrows). Note that transplant days 10, 40 and 50 correspond to endometrial cup days 44, 74 and 84 respectively. The recipient mounted a humoral immune response against all three transplants (D).

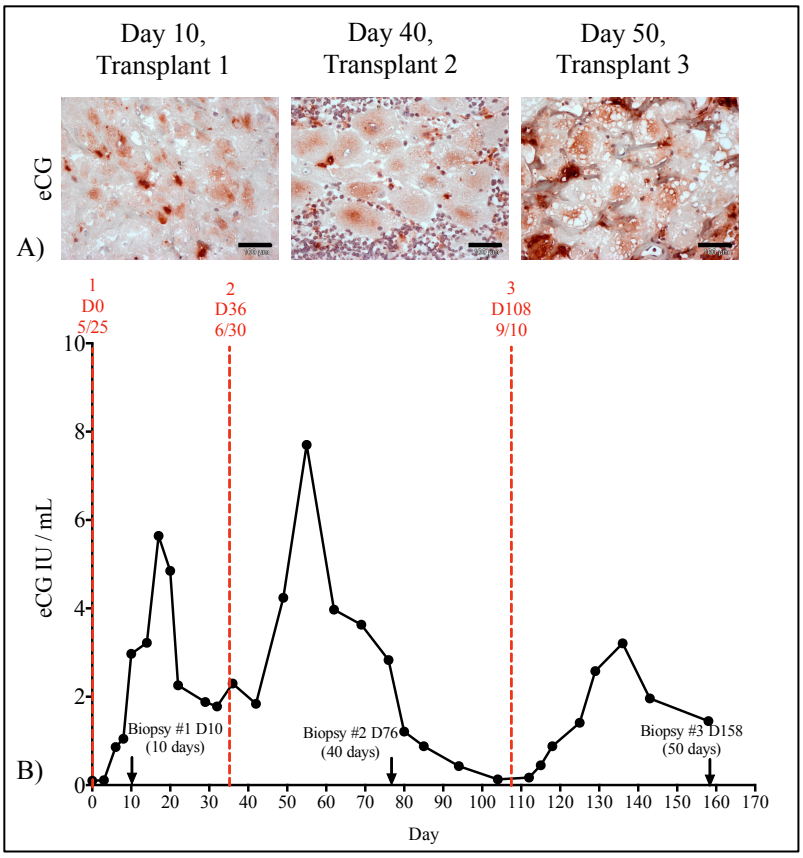

Figure 3.3 Identification of eCG in recipient's serum and in trophoblast transplant biopsies. eCG was identified at all stages of the serial transplant lifespans using immunohistochemistry on tissue (A) and an ELISA on serum (B). Transplants are indicated by dashed red lines, with biopsies indicated by arrows. The transplant number, day of experiment and month and day of transplant are shown above.

Cellular infiltrates surrounding serial transplants were predominantly CD4+ lymphocytes.

Each biopsy performed was analyzed using a panel of antibodies against lymphocyte markers including CD4, CD8, a B-cell marker, and an NK cell marker, while the distinctive equine eosinophils were identified by cytology. In all three biopsies, trophoblast was surrounded by large numbers of CD4+ and CD8+ lymphocytes. B-lymphocytes and natural killer cells were not identified in any of the biopsies. Eosinophils were present in abundance by days 40 and 50 (Figure 3.4). On sections stained with antibodies against CD4 and CD8, lymphocytes were counted in three non-overlapping, contiguous, high power fields to either side of the trophoblast transplant (Figure 3.5). The ratio of CD4+ to CD8+ cells was calculated (Table 3.3).

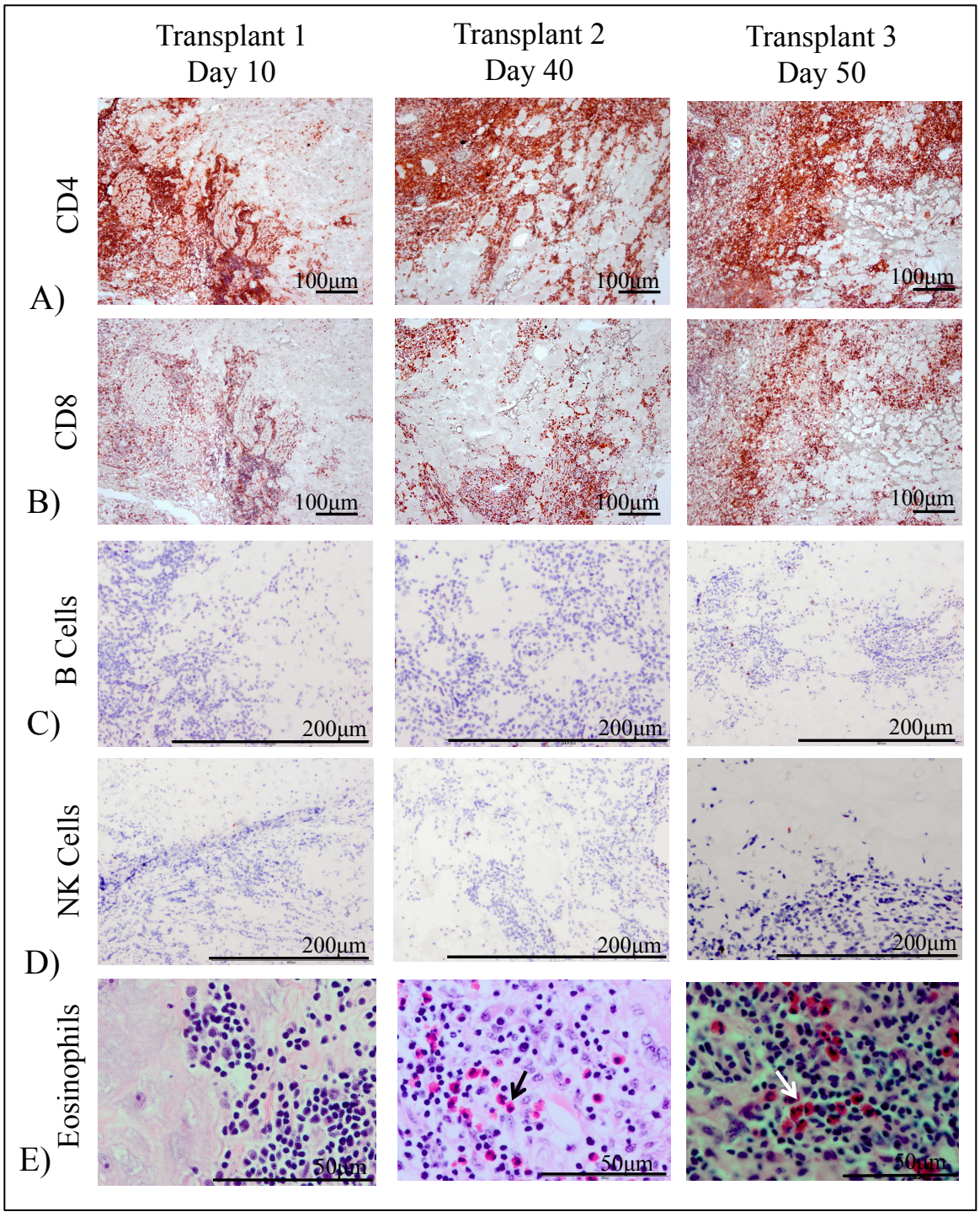

Figure 3.4 Characterization of cellular infiltrates in serial trophoblast transplant biopsies. Immunohistochemistry identified populations of CD4+ (A) and CD8+ (B) T-lymphocytes. Neither B-lymphocytes (C) nor natural killer cells (D) were identified at any time point. Eosinophils, identified by their eosinophilia and characteristic grape-cluster appearance (E, arrows) were not seen in the day 10 biopsy, but were readily observed at both later time points.

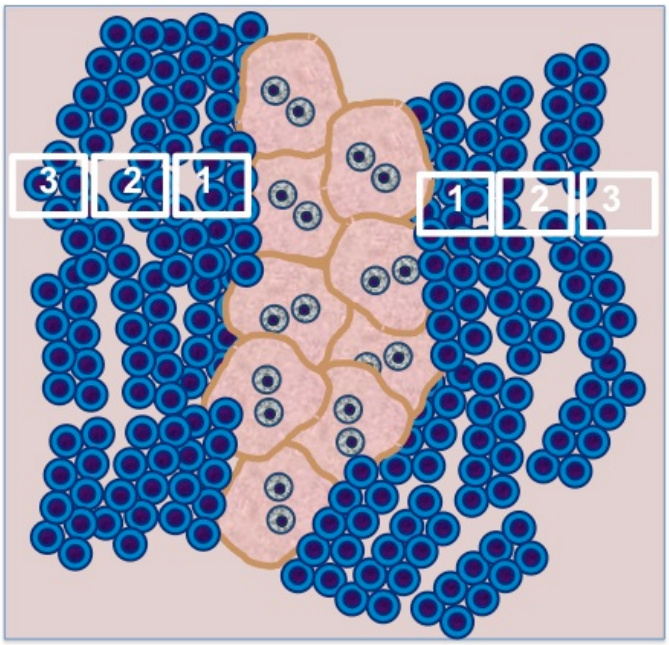

Figure 3.5 Schematic illustrating how lymphocyte populations were counted for biopsied trophoblast transplants. Three adjacent, non-overlapping high-power fields were counted on either side of the chorionic girdle tissue. Cell counts in fields labeled 1, 2 and 3 were averaged.

Table 3.3 CD4/CD8 Ratios in Serial Transplant Biopsies Compared to Previous Studies

Tissue	Range	Field 1	Field 2	Field 3	Overall
Day 10 Serial Biopsy	--	1.5	2.8	5.1	2.8
Day 40 Serial Biopsy	--	1.2	1.4	1.4	1.3
Day 50 Serial Biopsy	--	1.2	1.5	1.4	1.3
Single Transplants[1]	1.75-2.26				
Forming Cups[2]	1.34-5.61				
Mature Cups[2]	1.32-3.83				
Dying Cups[2]	1.48-4.51				

[1] (Adams and Antczak, 2001)
[2] (Grunig et al., 1995)

Discussion

The results of this study suggest that serial ectopic trophoblast transplants may show changes in developmental milestones, but that local immune responses are similar to endometrial cup sites of comparable age. In normal equine pregnancy, MHC-I expression by invasive trophoblast is down-regulated by approximately day 44 of pregnancy. The eventual down-regulation of MHC-I is thought to be independent of external factors such as the influence of the pregnant uterus (Donaldson et al., 1994; Donaldson et al., 1992; Kydd et al., 1991), and to be controlled at the level of transcription (Maher et al., 1996). Studies in cultured chorionic girdle cells and single trophoblast transplant biopsies suggest that while this down-regulation still occurs, it may be delayed in an extra-uterine environment (Adams and Antczak, 2001; Donaldson et al., 1992).

In this study the transplant biopsied at day 10, corresponding to a day 44 endometrial cup, still showed obvious expression of MHC-I. Previous studies of single trophoblast transplants identified some MHC-I expression out to 28 days post transplant, corresponding to a 62 day endometrial cup (Adams and Antczak, 2001). In this study the two transplants biopsied at days 40 and 50, corresponding to day 75 and 85 endometrial cups, showed no expression of MHC-I. This suggests that MHC-I molecules on serial trophoblast transplants are eventually completely down-regulated, but possibly over a longer time frame than endometrial cups in normal pregnancy. This may indicate that a maternal signal or a signal from the conceptus is required for timely down-regulation of MHC-I. In the two transplants that were biopsied on the later days, early increases in cytotoxic antibody titers in the recipient's serum indicated an immune response by the recipient, demonstrating immune recognition of the transplant.

In normal horse pregnancies, eCG production continues for a period of approximately 80 days, while in single trophoblast transplants eCG is measurable in recipient's serum for a median of 75 days (de Mestre et al., 2011). In these serial transplant biopsy experiments, eCG was identified both by ELISA in serum and by immunohistochemistry. The identification of eCG within the transplanted trophoblast cells supports the conclusion that the cells are alive and still actively producing eCG.

In horses the major population of cells at the interface of the invasive trophoblast and the maternal tissues are CD4+ and CD8+ T-lymphocytes. In endometrial cups from normal equine pregnancies, early cups had a CD4+ to CD8+ ratios of 2.48 to 2.75 while the ratios for later stages ranged from 1.67 to 1.98 (Grunig et al., 1995). In single transplants, biopsies taken at on days 7, 14, 21, and 28 had CD4+ to CD8+ ratios ranging from 1.69 to 2.26 (Adams and Antczak, 2001). In the current study, CD4+ to CD8+ ratios in the serial transplants ranged from 2.8 at 10 days into the first transplant to 1.3 at day 40 and 50 on the second and third transplants. CD4+ to CD8+ ratios are similar in normal equine pregnancy and single and serial transplants, however the ranges overlap such that drawing conclusions from this data is difficult. Additional information may be gained from further identification of subpopulations such as T-regulatory cells, a subset identified in both CD4+ and CD8+ T-lymphocytes (Vinay and Kwon, 2010; Zenclussen et al., 2006).

In normal equine pregnancy B-lymphocytes and eosinophils are present in small numbers in dying endometrial cups (Grunig et al., 1995). B-lymphocytes were not detected at any stage in the serial transplant, though eosinophils were readily visible on the day 40 and 50 biopsies. The

role of eosinophils in the life cycle of endometrial cups and trophoblast transplants is uncertain. In rodents eosinophils are present in the uterus, particularly during estrus, and may play a role in uterine preparation for pregnancy (Rothenberg and Hogan, 2006). In mice lacking interleukin 5, known to be an eosinophil chemo-attractant, eosinophil numbers were reduced but the tissue distribution was unchanged, and pregnancy and parturition were not adversely affected (Robertson et al., 2000). In one study of sub-fertile female dogs, eosinophilic endometritis was significantly associated with fetal death. A potential role for the eosinophils as a cause for the fetal loss was uncertain (Gifford et al., 2014). Unlike human decidua where uterine natural killer cells are the predominant cell type, (Hemberger, 2013; Kopcow et al., 2005) there were no natural killer cells identified at any biopsy time point in the horse-to-horse experiments.

These data taken together provide additional evidence that invasive trophoblast can survive and function in an ectopic site in a recipient with a primed immune system. Cytologic examination of the biopsies showed morphologically normal binucleate cells. Immunohistochemistry allowed visualization of MHC-I molecules and eCG, correlating with the findings on serum analysis. There was some evidence for a delay in the down-regulation of MHC-I, but the biology of the trophoblast did not otherwise appear to change over time.

Acknowledgements

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CHAPTER 4

XENOGENEIC ECTOPIC TROPHOBLAST TRANSPLANTS

Abstract

Horses and donkeys can interbreed to produce hybrid mules and hinnies, and embryo transfer experiments have demonstrated that donkeys can successfully carry horse embryos to term. Though some differences in placentation have been observed in these inter- and extra-species pregnancies, chorionic girdle development and endometrial cup formation are at least adequate to support pregnancy, and in the cases of hinny pregnancies and horse-to-donkey embryo transfers they are exceptionally well developed. By contrast, the dampening of systemic cytotoxic T-lymphocyte activity as observed in intra-species equine pregnancies does not occur in inter-species mule pregnancy. When invasive trophoblast (chorionic girdle) isolated from a horse conceptus is transplanted to an ectopic site in a non-pregnant mare, the cells survive and function for a similar length of time as within the uterus in normal pregnancy. This study used a xenogeneic approach to the ectopic trophoblast system, testing the hypothesis that horse invasive trophoblast transplanted to an ectopic site in a Jenny donkey would show an altered lifespan. Alternatively the transplant could have a lifespan and function comparable to the horse-to-horse trophoblast transplants. Chorionic girdles from day 34 conceptuses were transplanted to the vulvar mucosa of Jenny donkeys. The first transplant into each Jenny donkey had a lifespan comparable to the horse-to-horse transplants, but unexpectedly subsequent transplants were rapidly destroyed. The observed pattern was suggestive of a destructive immunologic memory response. Immunohistochemical analysis of transplant biopsies was consistent with trophoblast destruction. Unexpectedly large clusters of eosinophils were observed. These findings indicate that the immunological mechanisms protecting invasive trophoblast in inter- and extra-species pregnancies and intra-species ectopic transplants are not effective in xenogeneic ectopic transplants.

Introduction

Within the genus *Equus*, viable offspring may result from many combinations of parentage (intra-, inter- or extra- species) using natural breeding, artificial insemination or embryo transfer. Species within the genus *Equus* can interbreed to produce hybrid animals. The best known of these are matings between the horse (*E. caballus*) and donkey (*E. asinus*) that produce the hybrids mule (female horse x male donkey) and hinny (female donkey x male horse) (Short, 1975; Short, 1997). There are some differences in placental structure and function in pregnant mares and donkey Jennies carrying mule and hinny pregnancies, and these appear to be influenced by the genotype of the sire. In pregnancies where the sire is a horse (intra-species horse and inter-species hinny) the chorionic girdle is wide, resulting in larger endometrial cups and higher serum eCG concentration in the dam. Conversely, when the sire is a Jack donkey (intra-species donkey and inter-species mule), the girdle is narrow, endometrial cups are shallow and eCG levels are low (Allen, 1975).

Embryo transfer is a common procedure in many breeds of horse (Hinrichs, 2013); a mare can readily carry to term offspring that is not her own but of the same species (i.e. a full allograft, as compared to the partial allograft of normal pregnancy). When horse embryos were transferred to donkey recipients, endometrial cups became well-developed and normal intrauterine pregnancies occurred (Allen et al., 1987; Allen et al., 1993). When mule embryos were transferred to donkey recipients, the endometrial cups were more robust and eCG production was greater as compared to when the mule embryo developed in a mare. Although the sire of the mule embryo was a Jack donkey, the endocrine profiles and endometrial cup histology from Jenny donkeys carrying mule embryos were more similar to normal hinny pregnancies. This suggested that the uterine

environment also may influence endometrial cup biology (Allen et al., 1993). Although immunologic factors are hypothesized to account for some of the observed variations in endometrial cup size and eCG production (Allen et al., 1993) in each of these types of pregnancy endometrial cup lifespan and function was adequate to carry the pregnancies to term.

Although these inter- and extra-species pregnancies can be successful, differences in maternal responses to pregnancy have been noted. In mares and Jennies carrying foals of their own species, cytotoxic T-lymphocyte (CTL) activity is reduced. MHC-compatible and MHC-incompatible pregnancies both produce this effect. Furthermore, the decreased CTL activity is not restricted to cells bearing paternal MHC-I molecules but instead extends to cells of other haplotypes (Baker et al., 1999; Noronha and Antczak, 2012). In horses carrying mule foals, the diminished CTL activity is not observed (Baker et al., 1999).

Experiments in which invasive trophoblast (chorionic girdle) isolated from a horse conceptus was transplanted to an ectopic site in a non-pregnant, histo-incompatible recipient mare suggested that mechanisms enabling the chorionic girdle cells to evade immune destruction remain intact outside of the pregnant uterus. This holds true when the recipient's immune system is naïve to (Adams and Antczak, 2001; de Mestre et al., 2011) or primed to (refer to Chapter 2) an MHC-incompatible donor sire. The results of the horse-to-horse trophoblast transplant studies taken in the context of the reproductive success of inter- and extra- species equine pregnancies raised the question as to whether horse chorionic girdle would survive in an ectopic site in a donkey recipient. The major objective of this study was to test the hypothesis that horse chorionic girdle transplanted to an ectopic site in an immunologically naïve or primed

Jenny donkey would have a different lifespan than that observed in a horse-to-horse chorionic girdle transplant. The lifespan could be shorter due to failure of mechanisms that protect trophoblast in intra-species pregnancies and those that may protect horse-to-horse transplants such as the diminished CTL activity. The robust development of invasive trophoblast in hinny and horse-to-donkey embryo transfers suggests that a longer lifespan may be possible. Alternatively, there may be no difference between the lifespans of the horse-to-donkey and horse-to-horse transplants. A secondary hypothesis was also tested: that horse chorionic girdle transplanted to a distantly related species, the domestic goat, would be immediately destroyed as would be expected for any xenograft.

Materials and Methods

Animals

Animal care and experimental design were in compliance with a protocol approved by the Cornell University Institutional Animal Care and Use Committee (IACUC). All animals in this study belonged to the Cornell Equine Genetics Center. Three reproductively mature female donkeys (Jennies) and one boer-cross female goat (doe) were used as trophoblast transplant recipients. Conceptus donor mares were of various breeds and ages. Two Thoroughbred stallions homozygous for Equine Leukocyte Antigen (ELA) haplotype A2 or A3 were used as donor stallions.

Conceptus Recovery and Transplantation

Donor mares were impregnated via artificial insemination from one of the two stallions. Conceptuses were recovered at day 34 by non-invasive uterine lavage and the chorionic girdle

transplants were performed based upon a previously described method (Antczak et al., 1987; de Mestre et al., 2011). Briefly, after conceptus recovery the chorionic girdle was dissected, minced, and loaded into a 1 mL tuberculin syringe suspended in phosphate buffered saline with penicillin and streptomycin. The chorionic girdle fragments were then injected aseptically into the vulvar mucosa of the recipient Jenny or doe.

Sampling

Blood samples were drawn from recipients three times per week via jugular venipuncture into serum tubes. Blood was collected until eCG was no longer measurable in recipient serum. Blood was allowed to clot, then tubes were centrifuged and serum was stored at -20° C until processing. Blood from the donor sires required for lymphocyte cytotoxicity assays was drawn as needed into heparin tubes, then processed and used immediately.

Transplant biopsies were performed under sedation (xylazine 0.4 mg/kg or romifidine 40 μg/kg IV for Jennies; xylazine 0.1 mg/kg IM for the goat) and a local block (1 to 2 mL of 2% lidocaine for Jennies; 0.5-1 mL for the goat). Tissue was surgically excised from the location of transplantation and placed on ice until processing. The excision site was sutured and monitored until healed. Tissue sections were embedded (O.C.T. Compound, Sakura, Torrance, CA) and frozen at -80 ° C until processing. Slides (Superfrost Plus, Fisher Scientific, Pittsburgh, PA) were sectioned at 6 μm, fixed for 10 minutes in cold acetone and stored at -20 ° C until processing.

eCG and Progesterone Quantifications

Commercially available ELISA kits (PMSG and Progesterone ELISAs, DRG International, Springfield, NJ) were used to quantify recipients' serum eCG and progesterone. Plates were read on a Multiskan Ex and data were analyzed with Ascent 2.6 software (ThermoFisher Scientific, Waltham, MA).

Lymphocyte Microcytotoxicity Assay

Lymphocytes from the donor stallion were isolated from heparinized venous blood.

The presence of recipient antibody to donor sire antigens was determined using a lymphocyte microcytotoxicity assay as previously described (Antczak et al., 1982). Briefly, serum from recipients was plated in microtiter plates (Robbins Scientific, Mountain View, CA) in two-fold dilutions. Dilutions were carried out until a negative test occurred. Serum was incubated with lymphocytes from the donor stallion and rabbit complement (Pel-Freez, Lot #PF29521, Brown Deer, WI). Eosin dye was added and the wells were fixed with formalin. Wells were scored as percent cell killing indicated by dye exclusion. Wells showing greater than 50% killing were considered positive. Titers were called as the highest dilution with a positive result. Each sample was tested at least twice.

Trophoblast Lifespan Calculation

Detection of eCG in the recipients' serum was considered an indicator of live and functioning trophoblast. Transplant lifespan was calculated as previously described (de Mestre et al., 2011). Briefly, the definitive end point of a transplant was considered to be the point at which eCG was at least twice baseline, and with a drop of less than 50% in the previous six days.

Flow Cytometry

Blood was collected into heparin tubes via jugular venipuncture. Peripheral blood mononuclear cells (PBMCs) were isolated from the blood using a density centrifugation technique previously described (Antczak et al., 1982). Cells were diluted to 1 x 10^6 cells/mL, and 0.5 mL of cells (500,000 cells) and an equal volume of 4% formalin were incubated for 20 minutes at room temperature. The cells were rinsed twice in PBS and resuspended with antibody diluted in flow cytometry buffer (PBS, 0.5% BSA, 0.02% NaAz). Cells were incubated with antibody for 15 minutes at room temperature, rinsed twice, and resuspended in flow cytometry buffer for processing. The flow cytometer used for these experiments was a Beckman-Coulter Gallios. Alexa 488 conjugated anti-horse CD4 and CD8 antibodies were used.

Immunohistochemistry

All steps in this protocol took place at room temperature in a humidity chamber. Slides were incubated with primary antibody (Table 4.1) diluted in TBS (0.5M Tris base, 1.55M NaCl) for 30 minutes and then washed three times in TBS. Slides were blocked for 15 minutes with 10% normal goat serum as indicated. Second stage antibody (HRP conjugated goat anti-mouse or anti-rat IgG , Jackson ImmunoResearch, West Grove, PA) was diluted with 10% normal goat serum and applied for 30 minutes. Slides were again washed three times in TBS and incubated for 25 minutes with aminoethyl carbazole (AEC Kit, Life Technologies, Grand Island, NY). Slides were rinsed in TBS and then water, and counterstained for 5 minutes with hematoxylin (Gill's Formulation #2, Fisher Scientific, Pittsburgh, PA). Coverslips then were mounted (Glycergel, Dako, Agilent Technologies, Santa Clara, CA).

Table 4.1 Monoclonal Antibodies Used in Serial Horse-to-Donkey Trophoblast Transplant Experiments

Target	Antibody ID	Species	Isotype	Reference
Horse Trophoblast	102.1	Mouse	IgG1	(Oriol et al., 1989)
CD4	HB61A	Mouse	IgG1	(Kydd et al., 1994)
CD8	HT14A	Mouse	IgG1	(Kydd et al., 1994)
eCG	67.1	Rat	IgG2a	(Oriol et al., 1991)
MHC-I	CZ3	Mouse	IgG1	(Kydd et al., 1994)
B-cell	2.1	Mouse	IgG1	(Kydd et al., 1994)
NKp46	4F2	Mouse	IgG1	(Noronha et al., 2012)
Negative Control	Anti -canine Parvovirus	Mouse, Rat	IgG1	(Parrish et al., 1982)

Graphing and Analysis

Data were analyzed and graphs were created using Prism 6 for Mac OS X (GraphPad Software, La Jolla, CA).

Results

Overview

The primary objective of these experiments was to test the hypothesis that horse-to-donkey ectopic trophoblast transplants would have an altered lifespan relative to horse-to-horse transplants. Two types of experiments were undertaken to investigate this hypothesis. In the first set of experiments, the ectopic trophoblast transplants underwent long-term, non-invasive monitoring for the duration of the lifespan. Three Jenny donkeys of various reproductive statuses were available for these experiments, and each received one to three transplants. In a

second set of experiments, one donkey (3418) received two additional transplants that were biopsied to be used for immunohistochemical analysis of the local transplant site. A minor objective of this study was to assess chorionic girdle lifespan following transplantation to an ectopic site in a non-equine ungulate; one female goat was used in this experiment (Table 4.2).

Table 4.2 Recipient and Donor Animals Used in Trophoblast Transplant Xenografts

					Donor Mare		Donor Sire	
Recipient	**Species**	**Status**	**MHC**	**Transplant**	**ID**	**MHC-I**	**ID**	**MHC-I**
3374	Donkey	Foaled Donkey	3/4	1	3875	A2/?	3475	A2/A2
4058	Donkey	Maiden	3/3	1	3729	A3/A3	3474	A3/A3
				2	3157	A3/A3	3474	A3/A3
				3	4067	A2/A2	3474	A3/A3
3418	Donkey	Aborted Hinny	1/1	1	3875	A2/?	3475	A2/A2
				2	3725	A2/?	3475	A2/A2
				3	4069	A1/?	3475	A2/A2
				4[1]	3958	A2/A10	3475	A2/A2
				5[2]			3475	A2/A2
2080	Goat	Maiden	--	1	3958	A2/A10	3474	A3/A3

[1] = two sites, biopsied at D7, D14
[2] = Biopsied at D10

Testing of Anti-Equine CD4 and CD8 Antibodies in Donkeys

Prior to using anti-horse CD4 and CD8 antibodies in immunohistochemistry assays on the horse-to-donkey transplant biopsies, the antibodies were tested by flow cytometry experiment on horse and donkey PBMCs. Some variation was observed in total numbers of CD4+ and CD8+ cells. Both cell populations had CD4/CD8 ratios of 1.7 (Figure 4.1).

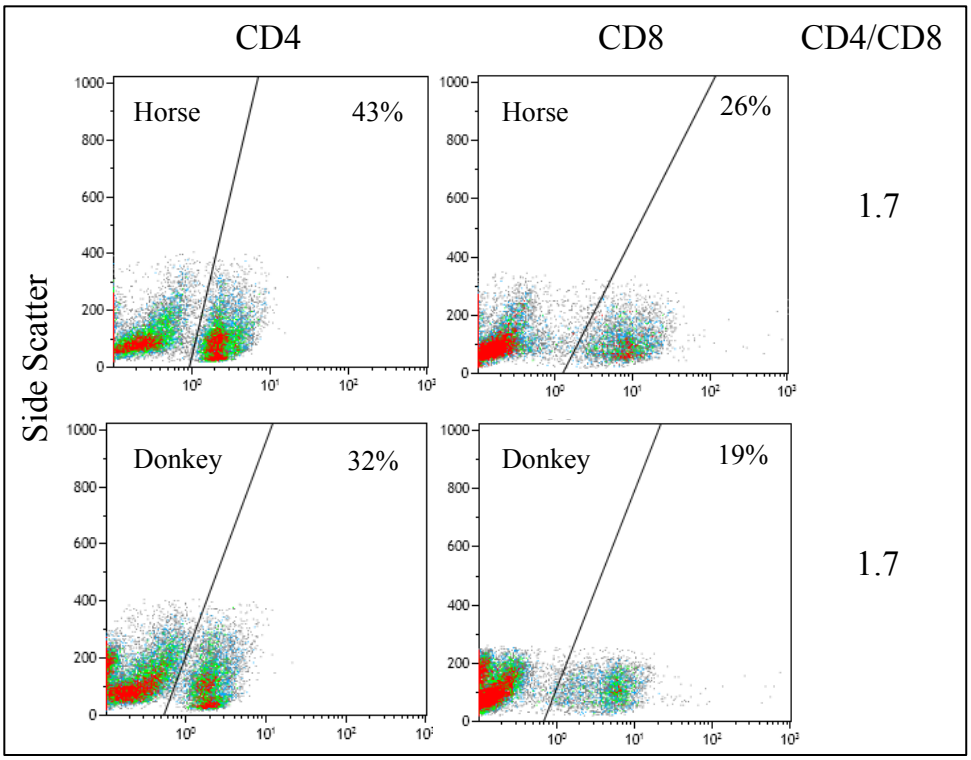

Figure 4.1 Flow cytometry using anti-horse CD4 and CD8 antibodies on horse and donkey PBMCs. Prior to using anti-horse CD4 and CD8 antibodies on horse-to-donkey trophoblast transplant biopsies for the purpose of calculating the CD4+/CD8+ ratio of cellular infiltrates, the antibodies were tested simultaneously on horse and donkey lymphocytes. Similar CD4/CD8 ratios were obtained.

Long Term, Non-invasive Horse-to-Donkey Trophoblast Transplants

In the initial set of horse-to-donkey trophoblast xenografts, the ectopic chorionic girdle tissue was left undisturbed for its entire lifespan. The transplants were monitored non-invasively by analysis of the recipients' serum. Serum concentrations of the pregnancy hormones eCG and progesterone were used as indicators of trophoblast lifespan and physiologic effect respectively. Cytotoxic antibody production against donor sire antigens was measured by a lymphocyte cytotoxicity assay (Figure 4.2).

In the first experiment a single transplant was placed and analyzed in one Jenny (3374). This was followed by a set of serial transplants in which two Jennies (4058 and 3418) received three closely spaced transplants each. Summary data from the long-term transplants aligns the eCG, progesterone and cytotoxic antibody titers for each recipient (Figures 4.3.1, 4.3.2 and 4.3.3). In each graph, the transplants are indicated by vertical, dashed, red lines. The transplant number, experimental day, and month and day the transplant was performed are provided above, and quarterly month and day are provided below the bottom X-axis to provide information on the seasonality of the transplant.

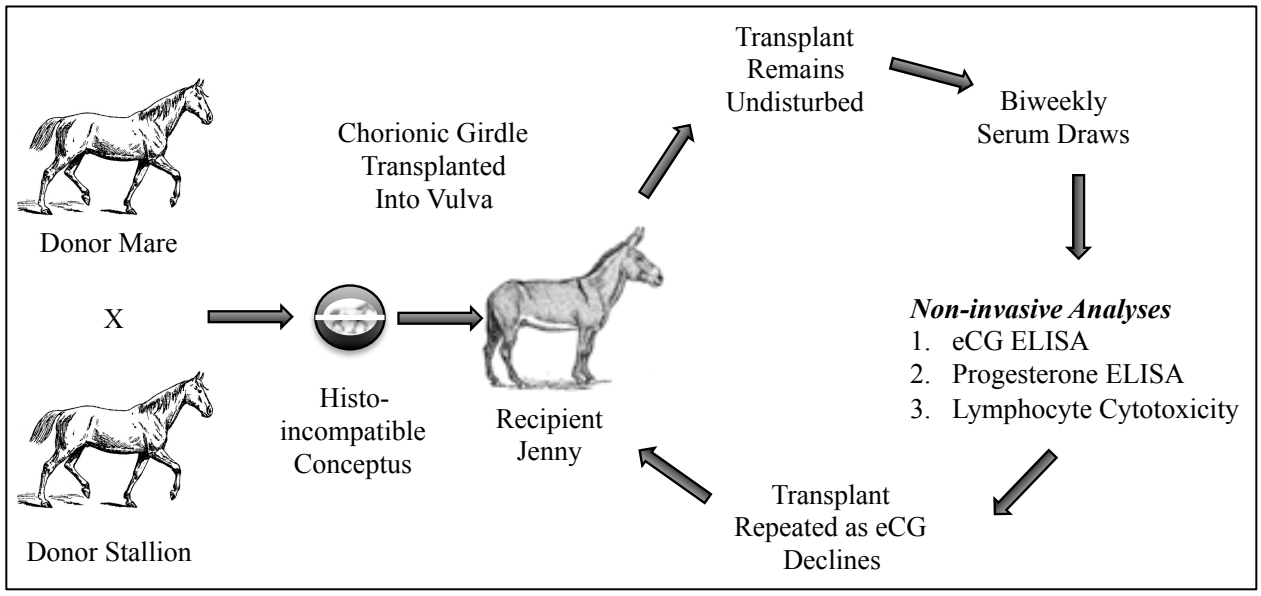

Figure 4.2 Schematic of long-term horse-to-donkey trophoblast transplant protocol. Chorionic girdle obtained from a normal horse pregnancy at day 34 was transplanted into the vulvar mucosa of a Jenny donkey. Transplants were monitored non-invasively via biweekly serum draws. Serum was screened for the pregnancy hormones eCG and progesterone using commercial ELISA assays. A lymphocyte cytotoxicity assay identified recipient antibodies to donor sire antigens. In one of the three Jennies used in this experiment a single transplant was performed. In the other two, second and third transplants were performed when the eCG from the previous transplant approached baseline.

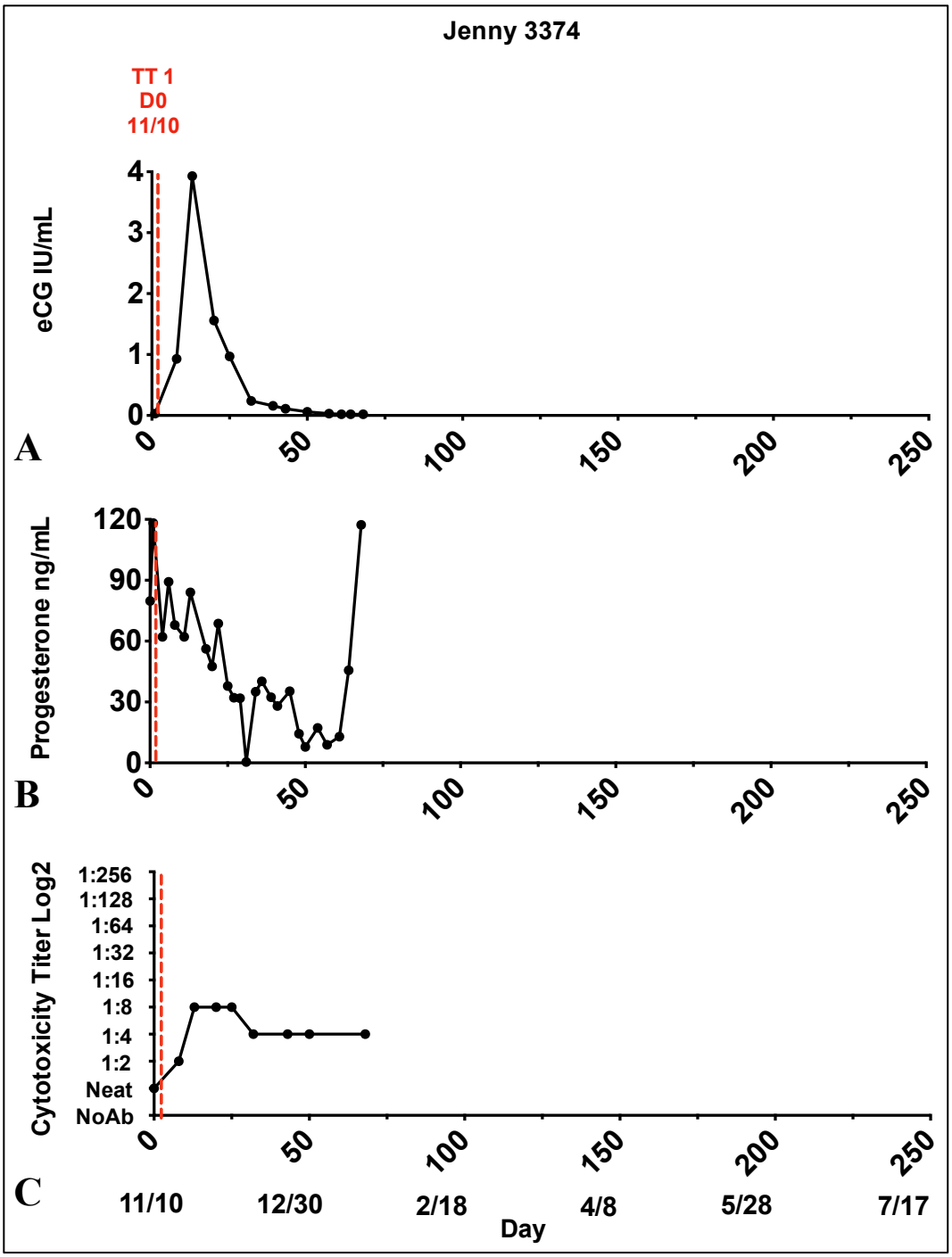

Figure 4.3.1 Summary eCG, progesterone and antibody data from Jenny 3374. Jenny 3374 received the first horse-to-donkey trophoblast transplant. Serum eCG (A) was tracked for the duration of the transplant, and was used to calculate the trophoblast lifespan of 50 days. Progesterone (B) was tracked as a measure of physiologic activity of the transplant. It remained high for the duration of the transplant, suggesting a physiologic effect of the transplant. Lymphocyte cytotoxicity results (C) indicate that this donkey had pre-existing antibodies against donor sire antigens. This was likely from a history of being bred to the donor sire's sire. No pregnancy resulted. An anamnestic response was observed following the transplant.

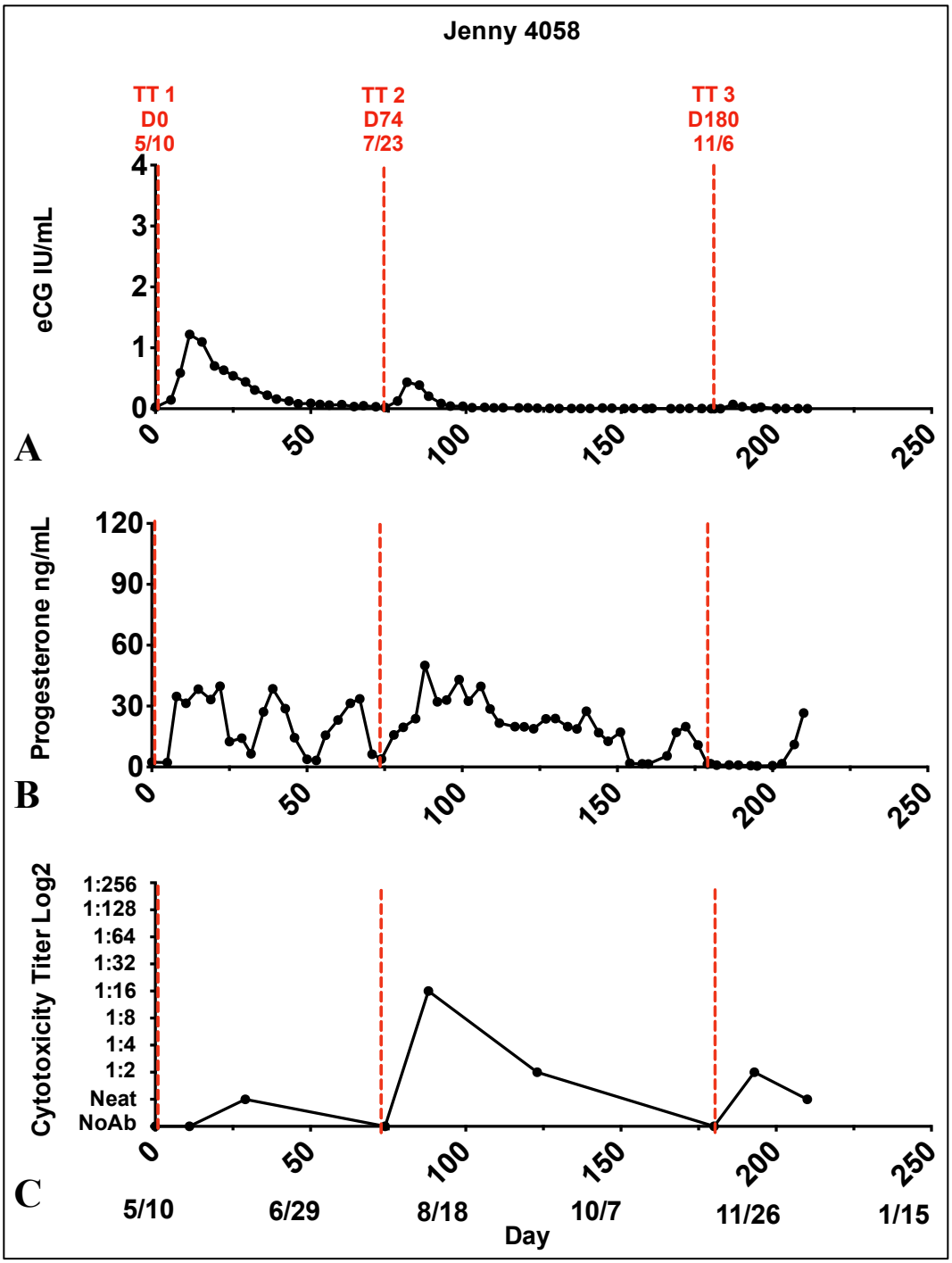

Figure 4.3.2 Summary eCG, progesterone and antibody data from Jenny 4058. Jenny 4058 was a maiden that received three transplants of horse chorionic girdle. Serum eCG (A) was tracked for the duration of the transplants, and was used to calculate trophoblast lifespans. Each successive transplant had a shorter lifespan than the previous one. Progesterone (B) was tracked as a measure of physiologic activity of the transplants; no definitive pattern was observed. Lymphocyte cytotoxicity results (C) indicate that the recipient's immune system was naïve to donor sire antigens. The donkey mounted a primary immune response following the first transplant, and responded to each transplant thereafter. It is of note that this recipient's cytotoxic antibodies returned to baseline following each transplant.

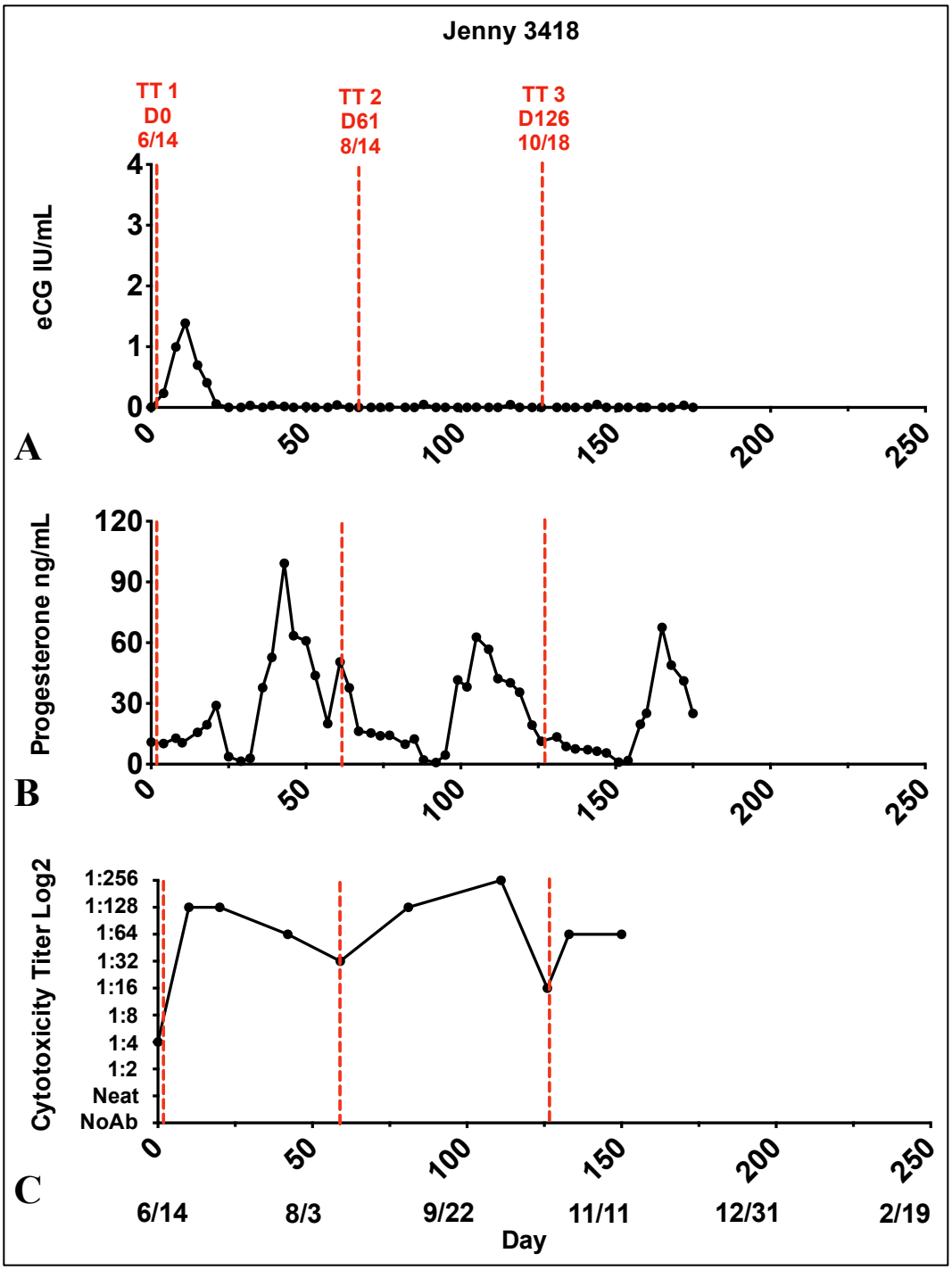

Figure 4.3.3 Summary eCG, progesterone and antibody data from Jenny 3418. Jenny 3418 had a history of aborting an early hinny pregnancy. Serum eCG (A) was tracked for the duration of the transplants, and was used to calculate trophoblast lifespans. Only the first transplant survived long enough to produce detectable eCG. Progesterone (B) was tracked as a measure of physiologic activity of the transplants. No clear pattern was observed. Lymphocyte cytotoxicity results (C) indicate that this recipient had a baseline titer to donor sire antigens. An anamnestic response occurred following each transplant.

Lifespans of Horse-to-Donkey Xenografts Became Shorter With Repeated Transplantation

The single ectopic trophoblast transplant performed in Jenny 3374 represented the first horse-to-donkey invasive trophoblast xenograft. The transplant was monitored non-invasively until eCG was no longer detectable in the recipient's serum. The calculated lifespan of the transplanted trophoblast was 50 days, within the range observed for horse-to-horse trophoblast transplants (Figure 4.4 A).

Following this initial transplant, two sets of three serial horse-to-donkey transplants were performed. In Jenny 4058 (Figure 4.4 B), the first transplant had a lifespan of 60 days, again within the range observed for horse-to-horse transplants. Unexpectedly, the second transplant survived for only 25 days. Following the third transplant eCG was detected above baseline for only 6 days, suggesting rapid destruction of the graft. In the Jenny that had a history of aborting an early hinny pregnancy (3418), the first transplant had a very short lifespan of only 17 days. The second and third transplants in this Jenny did not survive long enough to produce eCG detectable in the recipient's serum (Figure 4.4 C).

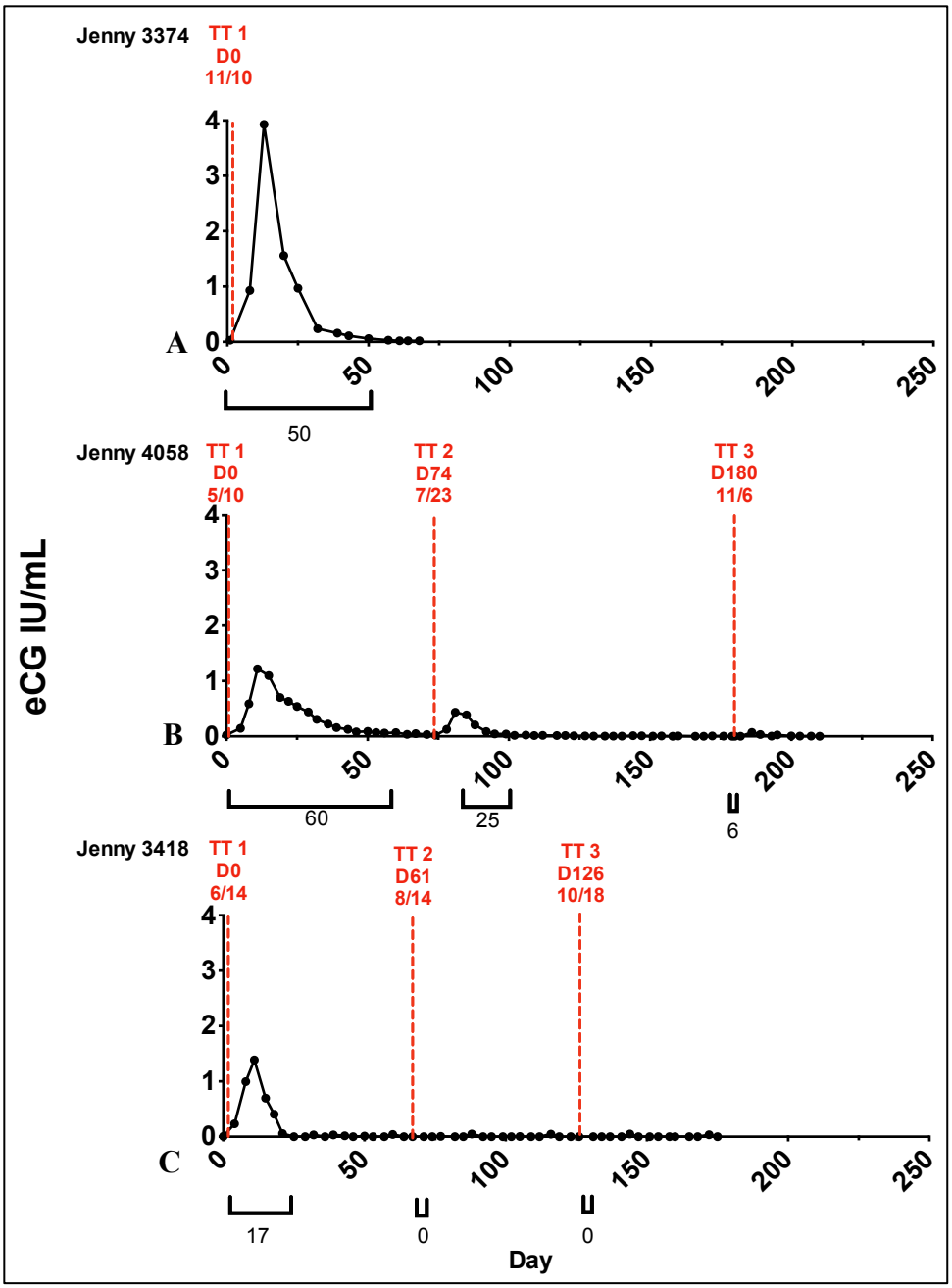

Figure 4.4 Lifespans of horse-to-donkey trophoblast xenografts calculated from serum eCG curves. Jenny 3374 (A) received the first trophoblast xenograft; this had a lifespan comparable to the horse-to-horse transplants at 50 days. Three serial transplants then were performed in Jennies 4058 (B) and 3418 (C). In both of these recipients the second and third transplants had markedly decreased to non-existent lifespans relative to the first transplant. This pattern is suggestive of a conventional destructive immunologic memory response.

To summarize, three Jenny donkeys received one to three xenografts of horse chorionic girdle. In all three recipients, the first transplant survived for a length of time comparable to the horse-to-horse ectopic trophoblast transplants. In the two donkeys that received second and third transplants, the lifespans of these later transplants were markedly decreased. The median lifespans for each transplant ordinal show a clear trend towards decreasing (Figure 4.5).

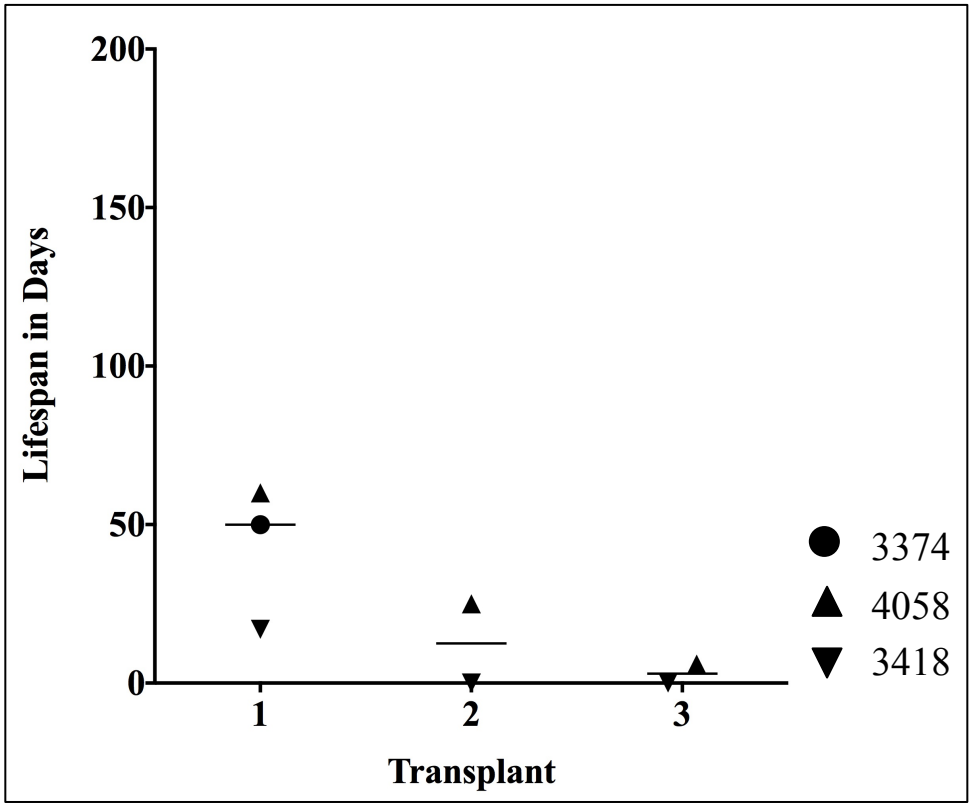

Figure 4.5 Median horse-to-donkey trophoblast transplant lifespans. The lifespans of all seven horse-to-donkey transplants from the three donkey recipients are graphed by ordinal, with the median lifespan of each ordinal indicated by a horizontal bar. Median lifespans of serial trophoblast xenografts show a trend toward decreasing.

Horse-to-Donkey Trophoblast Xenografts Elicit Humoral Responses in the Recipients

A lymphocyte cytotoxicity assay identified recipient antibodies against donor sire antigens. Two Jennies, 4474 and 3418, had positive baseline cytotoxic antibody titers (Figure 4.6 A, C). Jenny 3418 was known to have aborted an early hinny pregnancy. Jenny 3374 had a history of being bred to the sire of the donor sire of the conceptus used for her transplant, though a pregnancy did not result from these breedings. Each of these recipients showed an anamnestic antibody response to the three transplants.

Jenny 4058 (Figure 4.6 B) was a young maiden who had no cytotoxic antibodies to the donor sire at the start of the experiment. This recipient mounted humoral immune responses to each transplant, but it is of note that following each transplant cytotoxic antibodies became undetectable in her serum.

Considering the two Jennies that received three serial transplants, Jenny 3418 with the baseline titer of 1:4 and known exposure to horse antigens through an inter-species pregnancy showed a short lifespan for the initial transplant, and a more rapid decrease in lifespans of subsequent transplants than the maiden Jenny 4058 with a negative baseline titer. Table 4.3 summarizes the lifespan and cytotoxic antibody titers for the seven horse-to-donkey xenografts.

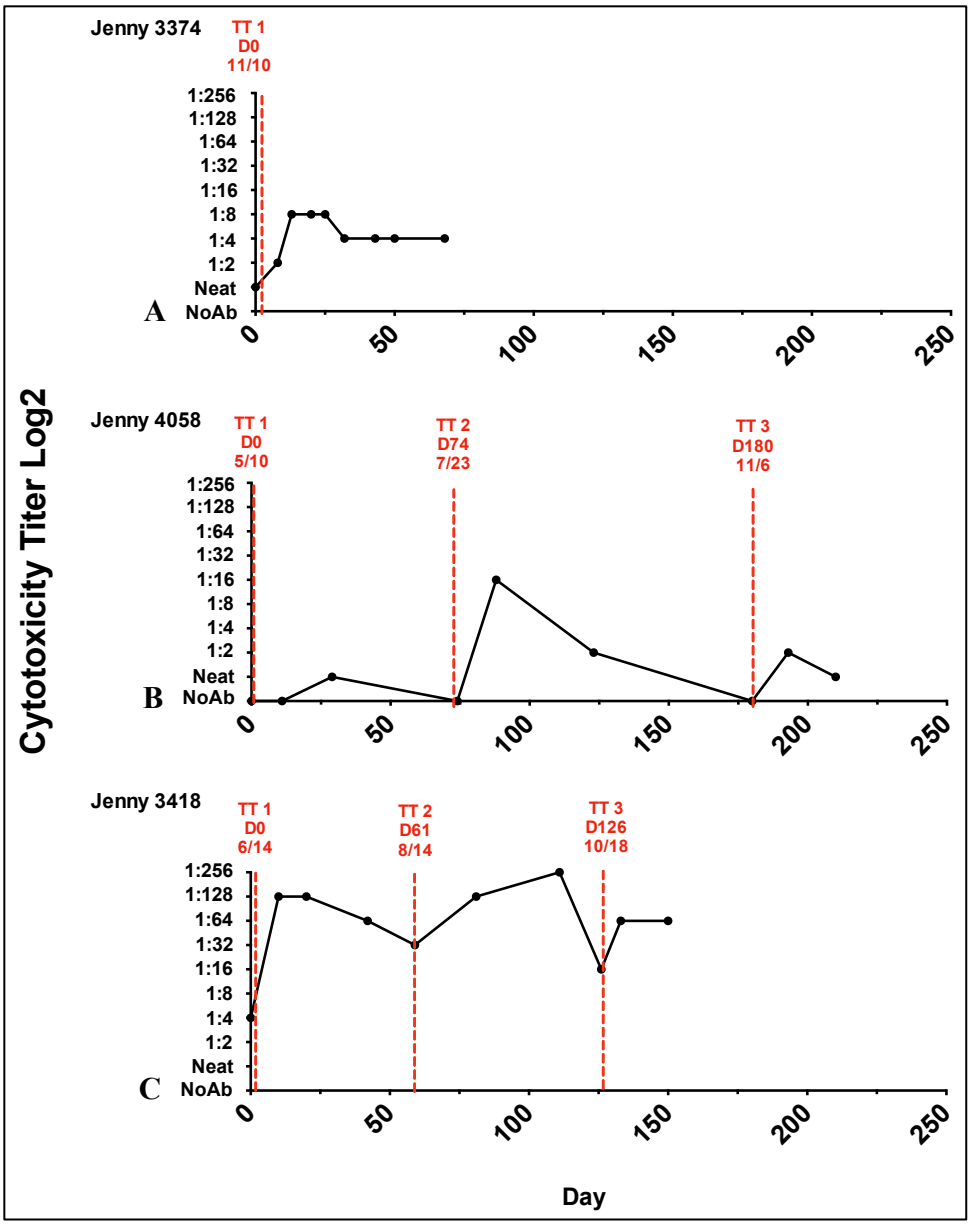

Figure 4.6 Lymphocyte cytotoxicity titers from horse-to-donkey trophoblast xenografts. Two Jennies (A and C) showed baseline positive titers to donor sire antigens. Jenny 3418 was known to have aborted a hinny pregnancy. Jenny 3374 had a history of being bred to the sire of the donor sire, though no pregnancy resulted. Jenny 4058 (B) was a young maiden with a negative baseline titer. All transplants produced increases in cytotoxic antibodies in the recipients.

Table 4.3 Summary of Horse-to-Donkey Transplant Lifespans and Antibody Titers

Recipient ID	Transplant	Titer at Transplant	Lifespan
3374	1	Neat	50
4058	1	0	60
	2	0	25
	3	0	6
3418	1	1:4	17
	2	1:32	0
	3	1:16	0

Horse-to-donkey xenografts did not show a predictable physiologic effect on the recipients. Recipients' serum progesterone levels were monitored for the duration of the transplant experiments (Figure 4.7). The biological function of eCG is to stimulate the development of secondary luteal structures in the ovaries of pregnant dams. The progesterone produced by the secondary corpora lutea supports the early phases of pregnancy. A sustained level of serum progesterone (increased interovulatory periods) in a recipient following an ectopic trophoblast transplant is considered evidence that the transplant is influencing the reproductive cycle of the recipient. A definitive relationship between serum eCG and serum progesterone was not observed in these experiments. Nearly normal cycles were observed in the presence of persistently increased serum eCG (Figure 4.7 A, refer also to Figure 4.3.1), while extended periods of high progesterone were observed during periods when little or no eCG was detectable in the recipient's serum (Figure 4.7 B, Transplant 2 and Figure 4.7 C, Transplant 1; refer also to Figures 4.3.2 and 4.3.3). Table 4.4 summarizes the relationship between transplant lifespans and recipients' serum progesterone and interovulatory periods.

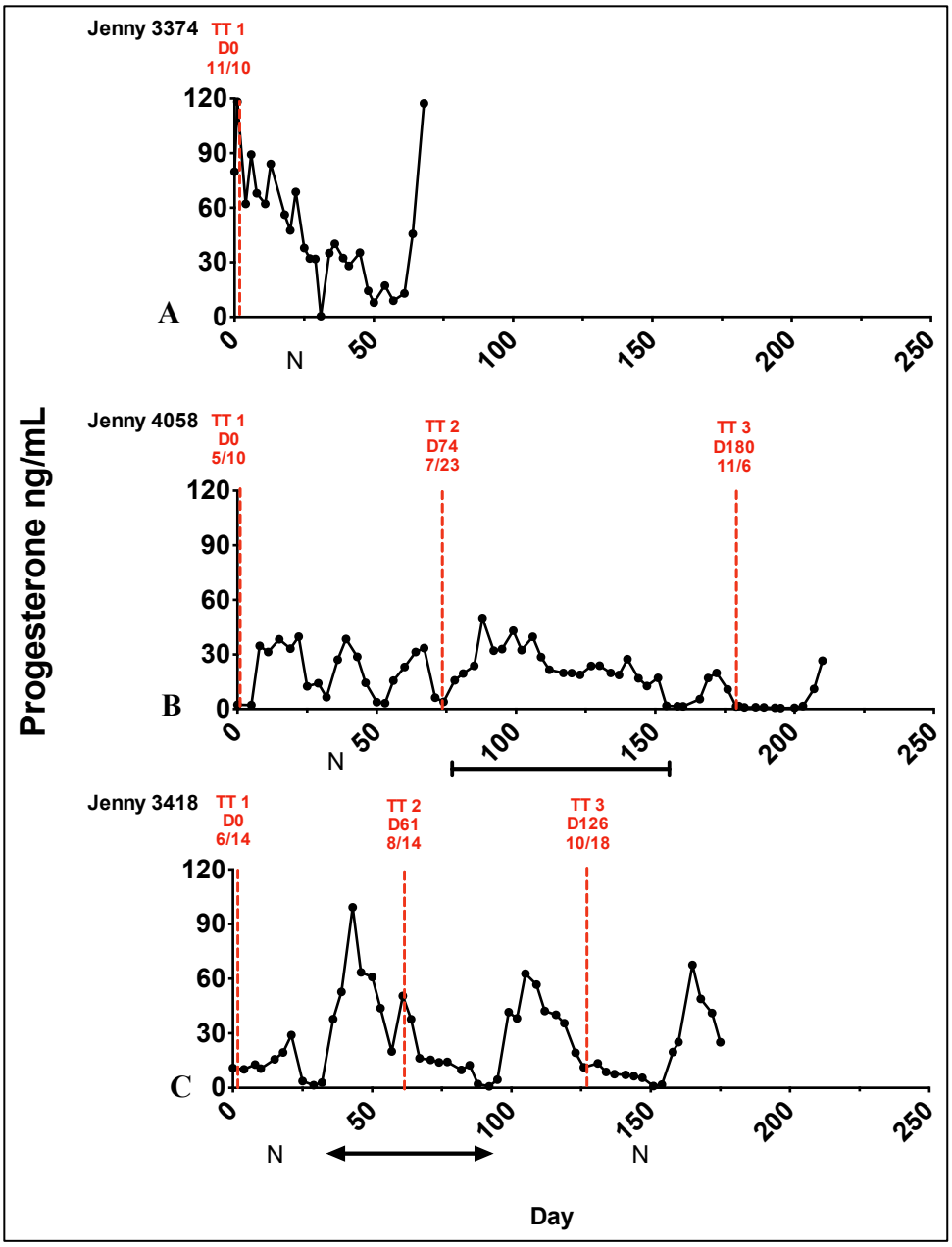

Figure 4.7 Serum progesterone concentrations following horse-to-donkey trophoblast xenografts. Serum progesterone did not follow a predictable pattern following the horse-to-donkey xenografts. A nearly normal interovulatory period was observed in Jenny 3374 following the single transplant, while the high progesterone curves for Jenny 4058 (B) and 3418 (C) did not correlate with the eCG curves of each transplant. Jenny 4058 (B) continued to cycle following a transplant with a normal lifespan of 60 days, while a 25-day transplant corresponded to 80 days of high progesterone (bar). Jenny 3418 had a 17 day first transplant with no eCG detected following the second transplant, yet high progesterone was recorded in the interim (arrow).

Table 4.4 Serum Progesterone and Interovulatory[a] Periods After Trophoblast Xenografts

Recipient	No.	Month	Lifespan	Baseline Progesterone	I/O Periods	Mean I/O Length	Days of Anestrus
3374	1	NOV	50	79	1	31	0
4058	1	MAY	60	2.3	3	25	0
	2	JUL	17	3.9	2	53	0
	3[b]	NOV	6	1.6	0	0	6
3418	1[b]	JUN	17	10.8	1+	29+	--
	2[b,c]	AUG	0	50.5	--	31+	--
	3[c]	OCT	0	11.2	--	25+	--

[a] = ovulation defined as a progesterone drop to ≤1 ng/mL
[b] = the last I/O period continued beyond the end of the TT lifespan
[c] = no eCG detectable during this interval

Horse-to-donkey trophoblast transplants with biopsies

Additional horse-to-donkey trophoblast transplants were performed in which the ectopic chorionic girdle was biopsied at intervals for histologic examination of the transplanted tissue and surrounding immune cells (Figure 4.8). One Jenny (3418) that had previously received a set of three transplants then received a fourth transplant that was biopsied at days seven and fourteen, and then a final transplant that was biopsied at day ten (Table 4.2).

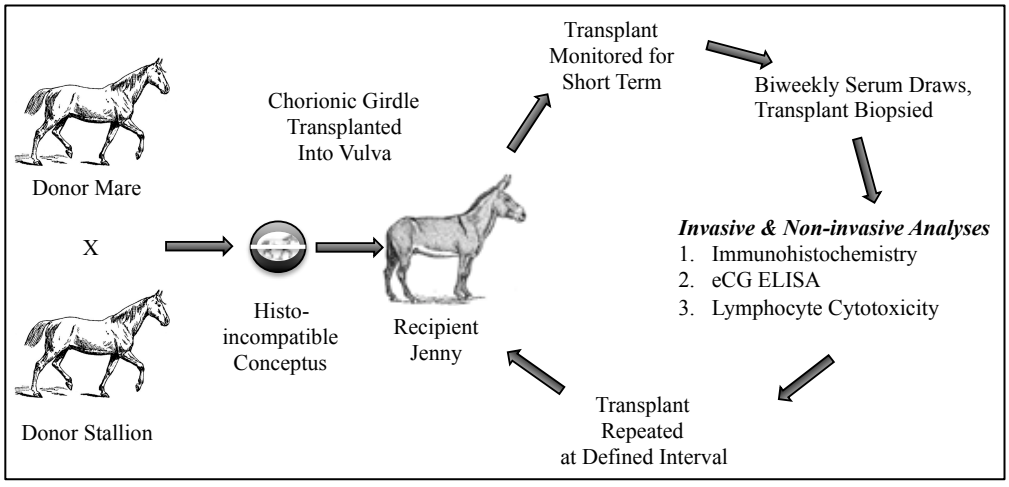

Figure 4.8 Schematic of horse-to-donkey trophoblast transplants with biopsies. Chorionic girdle from normal horse conceptuses was injected into the vulvar mucosa of a non-pregnant recipient Jenny. Transplants were monitored non-invasively with serum analyses until biopsies were performed at day 7 and day 14 on the first transplant, and day 10 on the second transplant.

Serum eCG and cytotoxic antibody analysis in trophoblast xenografts targeted for biopsy

Jenny 3418 received two additional transplants approximately one year after the initial three. In the first transplant to be biopsied (her fourth transplant overall) the horse chorionic girdle was divided in half and injected into two sites. One site was biopsied on day seven, and the other on day 14. A fifth transplant was performed and biopsied on day 10. The Jenny's serum was analyzed for eCG and cytotoxic antibodies. As observed following the second and third transplants in this Jenny, eCG was never detected in her serum (Figure 4.9, refer to Figure 4.3.3 A). At the start of the biopsy experiments, the recipient still had cytotoxic antibodies present from the previous long-term transplants. She showed an anamnestic antibody response to both the fourth and fifth transplants (Figure 4.9). The implication of this is that like the second and third transplants in this recipient, the fourth and fifth transplants were rapidly destroyed.

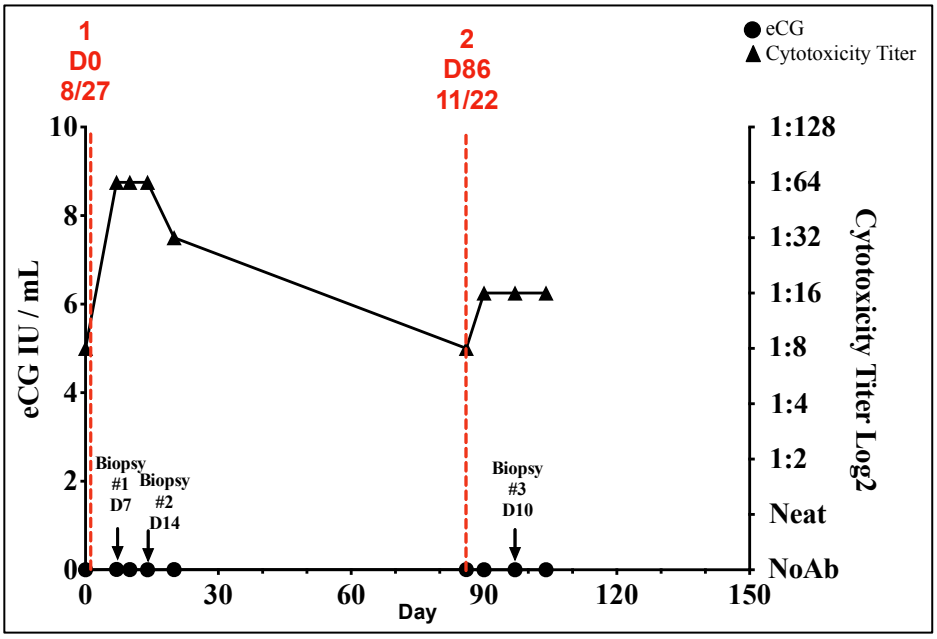

Figure 4.9 Serum analyses of horse-to-donkey trophoblast transplants undergoing biopsies. eCG (circles) was not identified in recipient's peripheral blood after either transplant. A lymphocyte cytotoxicity test showed that cytotoxic antibodies persisted from the previous experiments; the baseline titer (triangles) was determined to be 1:8. The recipient mounted an anamnestic antibody response after each transplant.

Immunohistochemical analysis of biopsies suggests early destruction of transplanted tissue

These horse-to-donkey trophoblast transplants were biopsied to evaluate the condition of the transplanted tissue and the infiltrating immune cells. Immunohistochemical analysis of the day seven biopsy using a monoclonal antibody against equine trophoblast (102.1) showed only small, fragmented particles of trophoblast tissue (Figure 4.10 A) surrounded by immune cells. This contrasts sharply with the large, binucleate cells visible in a day ten biopsy from a horse-into-horse transplant (Figure 4.10 B). Trophoblast was not visualized at all on the day 14 biopsy, or on a subsequent transplant biopsied on day ten (data not shown).

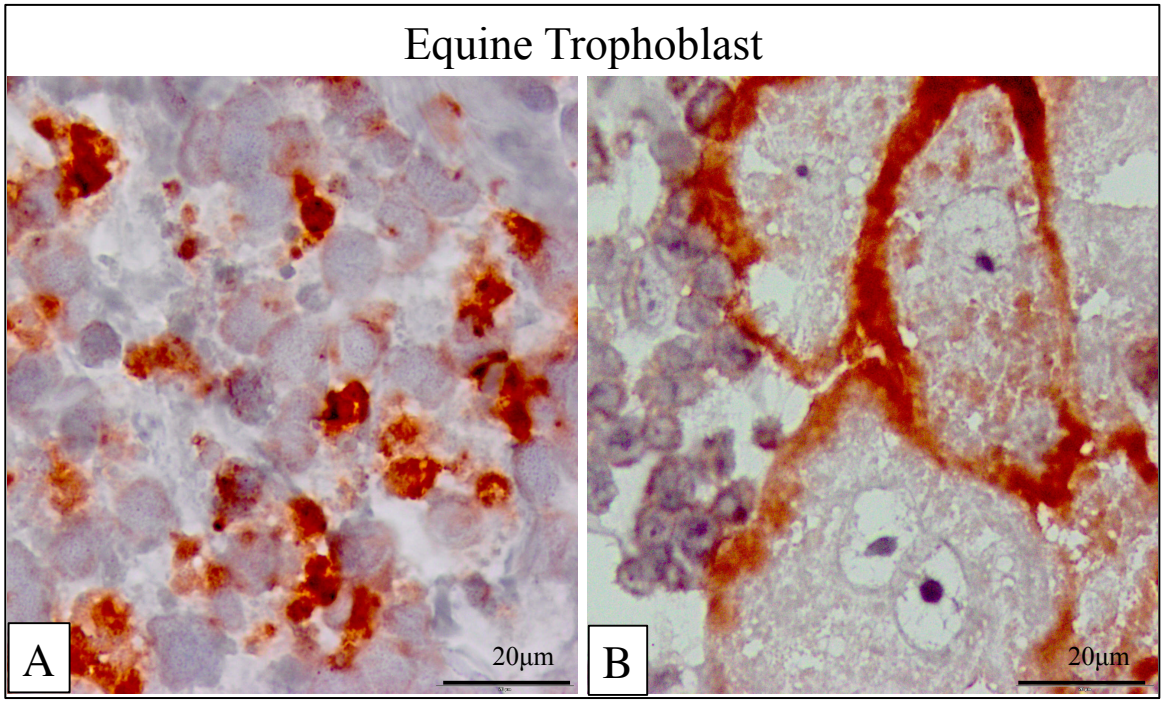

Figure 4.10 Immunohistochemical analysis using an antibody (102.1) against an equine trophoblast marker on trophoblast transplant biopsies. Frozen sections of biopsies from a day 7 horse-to-donkey trophoblast transplant (A) and a day 10 horse-to-horse transplant using an aminoethyl carbazole-based protocol. Trophoblast detected in the xenograft (A) consists of cell fragments surrounded by immune cells. This contrasts sharply with the large, binucleate cells visible in a day 10 biopsy of a horse-into-horse transplant (B). In the horse-to-horse section, immune cells are primarily located at the periphery of clusters of trophoblast cells. Tissues are counterstained with hematoxylin.

Xenograft biopsies were also evaluated for evidence of eCG secretion (Figure 4.11). Although no eCG was detected in the serum of the recipient, fragments of eCG positive cells were observed within the cellular infiltrates at the site where the chorionic girdle tissue was injected (Figure 4.11). This suggests that although eCG-containing cells were injected into the recipient, they did not survive long enough to secrete adequate eCG to be detected in the recipient's peripheral blood.

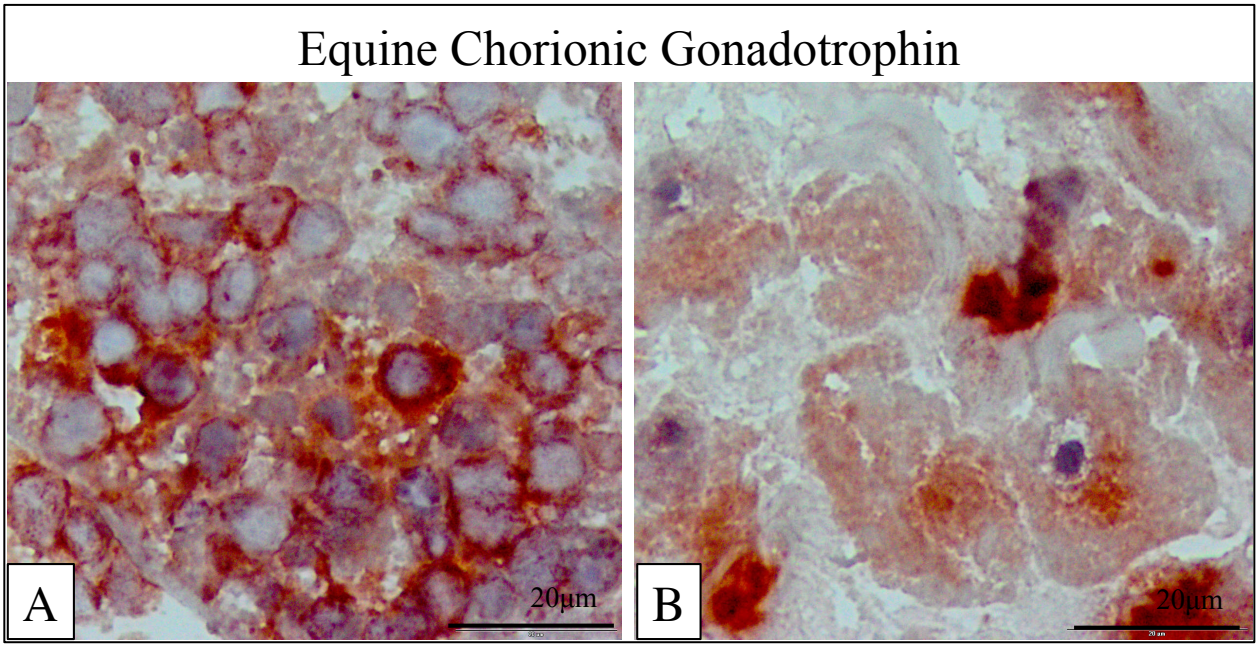

Figure 4.11 Immunohistochemical analysis using an antibody against equine chorionic gonadotrophin (67.1) on trophoblast transplant biopsies. Frozen sections of biopsies from a day 7 horse-to-donkey trophoblast transplant (A) and a day 10 horse-to-horse transplant using an aminoethyl carbazole-based protocol. eCG-positive areas of the xenografts biopsy (A) are seen diffusely within the area of cellular infiltration. By comparison, eCG positive areas in the horse-to-horse biopsies (B) are primarily observed within the binucleate cells. Tissues are counterstained with hematoxylin.

Local cellular infiltrates at the horse-to-donkey trophoblast xenograft site

A panel of available monoclonal antibodies was used on frozen sections from a day 7 biopsy to characterize the cellular infiltrates at the transplant site. Both CD4+ and CD8+ cells were observed surrounding the remnants of the trophoblast tissue. B-cells were not detected. Rare

natural killer cells were observed. Unexpectedly, hematoxylin and eosin staining of formalin fixed sections from a day 7 xenograft showed large clusters of cells cytologically consistent with equine eosinophils (Figure 4.12).

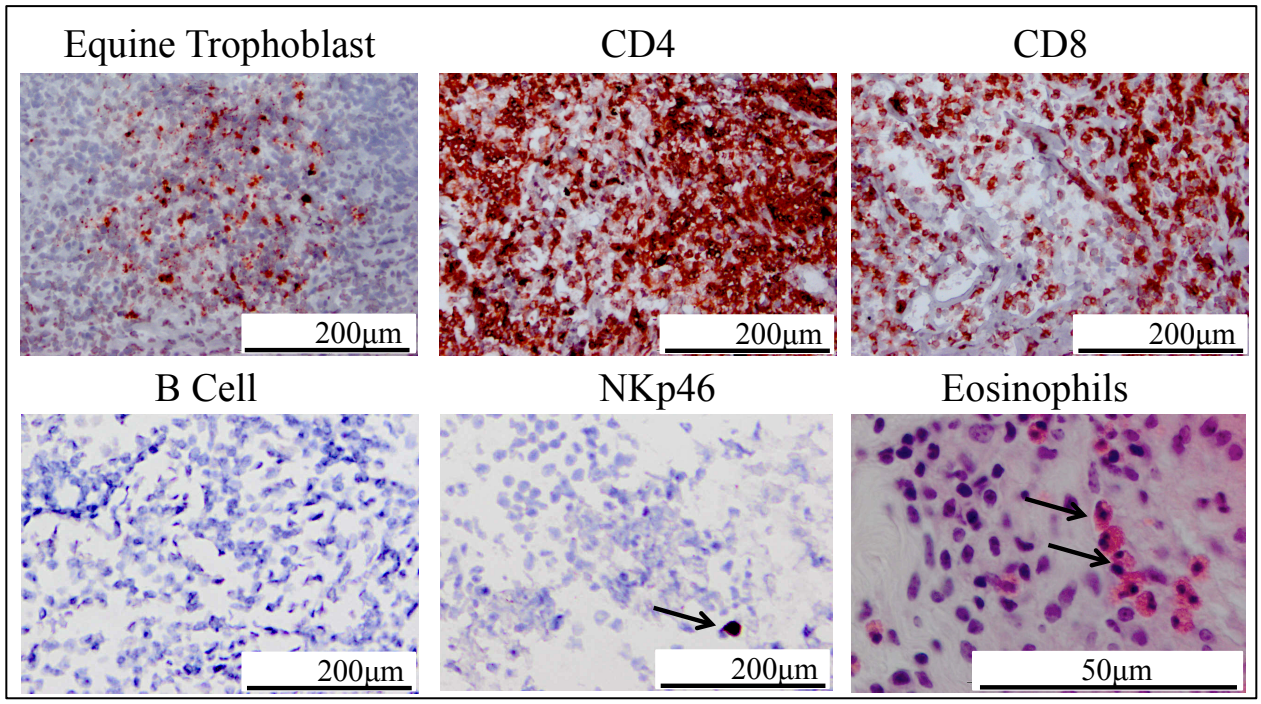

Figure 4.12 Analysis of cellular infiltrates in a day 7 horse-to-donkey transplant using frozen and fixed sections. Serial sections of a day seven horse-to-donkey trophoblast transplant biopsy show small remnants of trophoblast tissue (A) using an anti-trophoblast antibody (102.1) surrounded by CD4+ (B) and CD8+ (C) lymphocytes (antibodies HB61A and HT14A respectively). B-lymphocytes were not identified with antibody 2.1. Rare natural killer cells (arrow) were observed using a monoclonal antibody to horse NKp46 (4F2). All immunohistochemistry sections were counterstained with hematoxylin. Hematoxylin and eosin stained formalin fixed sections show large clusters of eosinophils (arrows).

A ratio of CD4+ to CD8+ cells was calculated for comparison with cellular infiltrates identified around normal endometrial cups and horse-to-horse ectopic trophoblast transplants. Cells were counted manually in three adjacent, non-overlapping zones (Figure 4.13). This was performed in three locations, and counts were averaged for each location. An overall CD4+ to CD8+ ratio of 1.6 was calculated (Table 4.5).

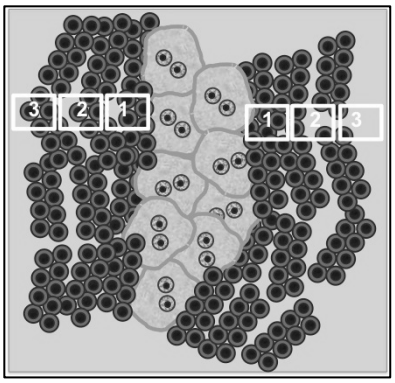

Figure 4.13 Schematic illustrating how lymphocyte populations were counted for biopsied trophoblast transplants. Three adjacent, non-overlapping high-power fields were counted on either side of the observed chorionic girdle tissue. This was performed in three locations on each side of the trophoblast tissue. Cell counts in fields labeled 1, 2 and 3 were averaged.

Table 4.5 CD4/CD8 Ratios of Cellular Infiltrates in Horse-to-Donkey Transplant Biopsies

Biopsy	1	2	3	Overall
Day 7	1.3	1.6	1.9	1.6

Horse-into-goat trophoblast transplants are rapidly destroyed

The overall pattern of the horse-to-donkey trophoblast transplant lifespans was unexpected. The progressively shorter lifespans of each successive transplant was suggestive of a destructive memory response. To help determine whether this pattern might be unique to horse trophoblast xenografts performed within the genus *Equus*, a final experiment was performed in which horse chorionic girdle was transplanted to the vulvar mucosa of a non-equine ungulate, a domestic goat. A single doe was used in this study (Table 4.2). Because of the anticipated rapid destruction of the xenograft, the chorionic girdle tissue was divided in two, and half was transplanted on each side of the doe's vulva. One side was biopsied at five days post-transplant, and the other at 22 days (Figure 4.14).

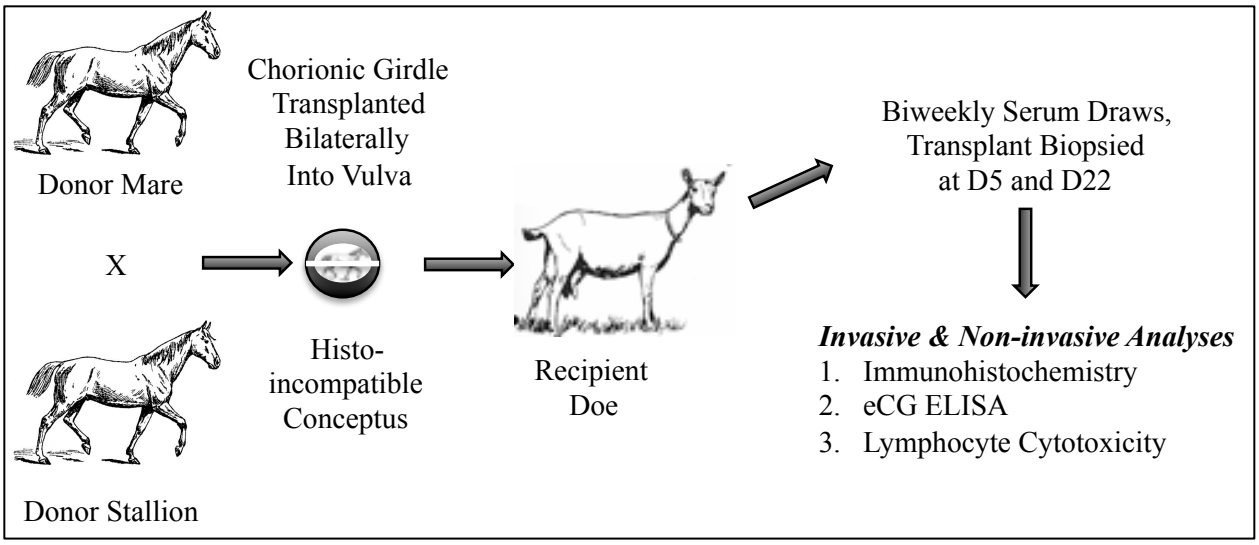

Figure 4.14 Schematic of horse-to-goat trophoblast transplant biopsies. Chorionic girdle from normal horse conceptuses was injected into the vulvar mucosa of a non-pregnant recipient doe. Because of the anticipated rapid destruction of the xenografts, the chorionic girdle tissue was divided in half, with one half transplanted on either side of the vulva. One side was biopsied on day 5, and the other on day 22.

Analysis of the recipient goat's serum did not detect eCG (Figure 4.15). The recipient goat, along with another control goat of the same age, was found to have pre-existing antibodies against the donor sire. The recipient mounted an anamnestic response to the transplant (Figure 4.15). An unusual observation was made in that a second increase in cytotoxic antibody titer was observed at the time of the day 22 biopsy. Trophoblast was not identified in the biopsied tissue (not shown).

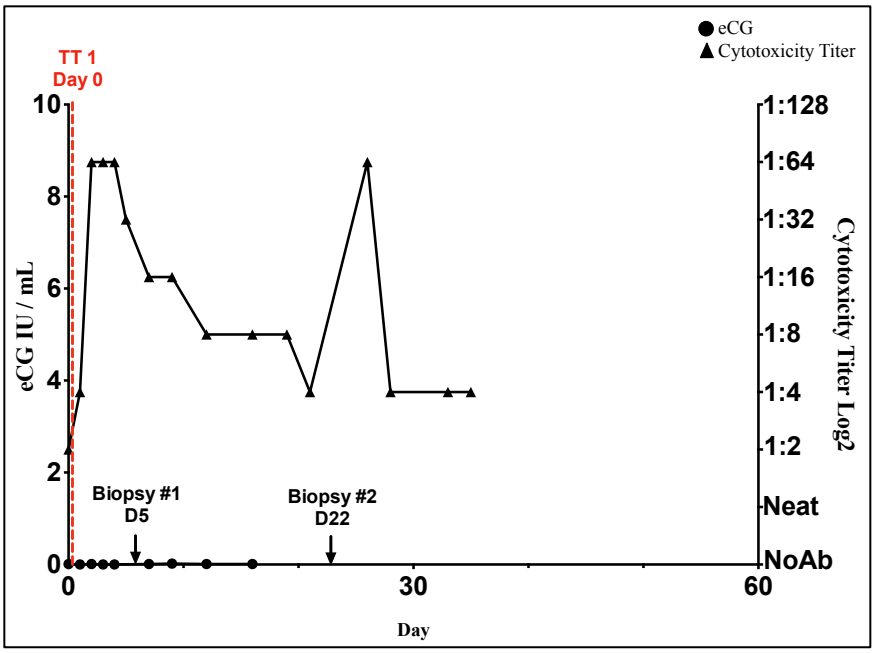

Figure 4.15 Serum analyses of horse-to-goat ectopic trophoblast transplants undergoing biopsies. eCG (circles) was not identified in recipient's peripheral blood after either transplant. The recipient had a pre-existing cytotoxic antibody titer (triangles) of 1:4. An anamnestic response was observed following the transplant. The second rise in cytotoxic antibody titer corresponds to the time of the day 22 biopsy.

Discussion

The primary objective of this study was to test the hypothesis that horse-to-donkey chorionic girdle xenografts in immunologically naïve or primed Jenny donkeys would have lifespans that are different (longer or shorter) than the lifespans of horse-to-horse chorionic girdle allografts. Two historical observations served as the basis for this hypothesis. First was the success of inter- and extra-species pregnancies in which chorionic girdle that was genotypically horse in whole or in part developed within a donkey uterus. In normal hinny pregnancies and in pregnancies established by transfers of mule embryos to donkey recipients, endometrial cups are well developed and serum eCG concentrations in the dam are high (Allen et al., 1987; Allen et al., 1993). Second, and in contrast to this, the dampening of CTL activity observed in mares and jennies carrying intra-species pregnancies does not occur in mares carrying mule pregnancies

(Baker et al., 1999). These observations provide rationale for lifespans that could be possibly longer or shorter than the horse-to-horse transplants. Alternatively, the horse-to-donkey transplants could have lifespans similar to the 70 to 75 days observed for the horse-to-horse transplants allografts (de Mestre et al., 2011), suggesting that the trophoblast may dictate its own lifespan.

The results of seven horse-to-donkey trophoblast xenografts clearly showed shortening of the transplant lifespans. The pattern of transplant lifespans observed was not one of immediate destruction as might be expected in hyperacute rejection of conventional xenografts (Cooper et al., 1988), nor was it the very consistently normal lifespans observed in horse-to-horse trophoblast transplants. Instead, initial transplants showed lifespans similar to horse-to-horse allografts while second and third transplants had strikingly decreased lifespans, a pattern consistent with a classic destructive memory response.

These observations raise the question as to how the transplanted trophoblast tissue is destroyed. One possibility is antibody-mediated destruction. It has long been known that in normal, MHC-mismatched equine pregnancies dams produce strong cytotoxic antibody responses to antigens of the sire (Antczak et al., 1984). These antibodies are generated against paternal MHC-I molecules expressed by the invading chorionic girdle (Antczak et al., 1984; Crump et al., 1987). Mares receiving ectopic trophoblast allografts also generate cytotoxic antibodies to donor sire antigens (Adams and Antczak, 2001). Anamnestic antibody responses are observed in both normal pregnancies (Kydd et al., 1982) and when invasive trophoblast is transplanted to an ectopic site two or more times in an individual mare (Chapter 2). Establishing new pregnancies

or transplanting invasive trophoblast to an ectopic site in mares with pre-existing cytotoxic antibodies does not result in more rapid destruction of endometrial cups or trophoblast allografts (Chapter 2). A definitive role for these antibodies in the eventual death of endometrial cups or allografts has not been established.

The role of cytotoxic antibodies against paternal HLA in human pregnancy is equally unclear. Up to three-quarters of women who have had more than two pregnancies test positive for these antibodies. Interpretation of these findings is difficult as existing studies have varied with parity of subjects, the timing of testing relative to pregnancy and the sensitivity of the testing method. (Densmore et al., 1999; Masson et al., 2013; Morin-Papunen et al., 1984; Regan et al., 1991). Individual studies have suggested that the presence of these antibodies in early to mid gestation may be associated with chronic chorioamnionitis (Lee et al., 2011) and spontaneous preterm delivery (Lee et al., 2013). However, a meta-analysis identified no significant effects of anti-HLA antibodies on pathologies of pregnancy, although this analysis also noted the variability of methodologies used in existing studies (Lashley et al., 2013).

In the current study, baseline cytotoxic antibody titers for the three recipients included a negative titer, positive titer on neat serum presumed to have been initiated by previous breeding to the sire of the donor sire, and a titer of 1:4 initiated by early abortion of a hinny pregnancy. All xenografts resulted in early increases in cytotoxic antibody titers. Transplants into the maiden donkey (4058) had lifespans of 60, 25 and 0 days. The single transplant into the donkey (3374) with the low titer and history of exposure to horse semen had a lifespan of 50 days. The initial transplant into the donkey (3418) with the highest titer was very short relative to horse-to horse

transplants. The following two transplants, performed when cytotoxic antibodies were still high, appeared to be destroyed immediately. Although the sample is small, the pattern suggests that the presence of pre-existing antibodies may correlate with more rapid destruction of the graft. Interestingly, in horse-into-donkey embryo transfers that resulted in live foal births, antibodies to paternal MHC-I were not detected until just before term (Kydd et al., 1982).

It is of note that the second and third transplants in the maiden donkey (4058) were performed at a time when the cytotoxic antibody titer had returned to baseline, yet the transplants were still rapidly destroyed. This raises the question as to the target of the cytotoxic antibodies.

Studies in donkeys carrying hinny pregnancies suggest that alloantibodies are in fact generated against MHC-I molecules as observed in the horse (Baker et al., 2001). In other species such as the sheep and goat, humoral immune responses have been ruled out as a cause for failure of xenogeneic embryo transfers and hybrid pregnancies (Oppenheim et al., 2000).

In this study the results of lymphocyte cytotoxicity assays using recipient serum and donor sire lymphocytes indicate that cytotoxic antibodies are in fact made against molecules expressed on the donor sire lymphocytes, quite possibly MHC-I. Rapid destruction of secondary transplants in the absence of pre-existing antibodies suggests the possibility that antibodies are also raised against paternal antigens present on the trophoblast tissue, but not on lymphocytes.

In Jenny 4058 the fact that the cytotoxic antibody titers returned to baseline more rapidly than in the others was of interest. The two other donkey recipients maintained positive titers from breeding contact and pregnancy years earlier, but those events as well as the transplants conducted in this study involved sires homozygous for the A2/A2 ELA haplotype. Jenny 4058

received chorionic girdle from conceptuses sired by the A3/A3 homozygous stallion. Previous research using horse-to-horse embryo transfer demonstrated that the cytotoxic antibody titers against the A2 haplotype in MHC-mismatched pregnancies are stronger and more persistent than those against the A3/A3 haplotype (Crump et al., 1985). It is possible that this effect carries across species.

Alternatively, the shortened trophoblast transplant lifespans may have been caused by a cell-mediated rather than antibody-mediated process. Previous studies have demonstrated that equine trophoblast in vitro can be killed by primed cytotoxic T-lymphocytes, so the trophoblast tissue is not inherently resistant to killing (Baker et al., 2000). Cytotoxic T-lymphocyte (CTL) activity is reduced in mares and Jennies carrying intra-species pregnancies. This holds true for mares carrying both histo-compatible and histo-incompatible pregnancies, and the dampening of CTL activity is not restricted to cells bearing paternal MHC-I molecules. Rather, it extends to cells of other haplotypes (Baker et al., 1999; Noronha and Antczak, 2012). The same effect is not observed in horses carrying mule foals (Baker et al., 1999). One possible explanation for the differences in transplant lifespan observed between the serial horse-to-horse and horse-to-donkey experiments may be that CTL is reduced in intra-species ectopic trophoblast transplants as in normal pregnancy, but not in inter-species transplants. The CTL response has not been evaluated in horse or donkey recipients of ectopic trophoblast transplants at this time.

Examination of the cellular infiltrates at the site of a day seven biopsy of horse-to-donkey ectopic trophoblast transplant produced some interesting observations, though not a definitive reason for the rapid destruction of the second and subsequent transplants. The overall CD4+ to

CD8+ ratio in the horse-to-donkey transplant biopsies was similar to horse-to-horse transplants and normal endometrial cups (Adams and Antczak, 2001; Grunig et al., 1995). The ratio of cells at different distances from the remaining girdle tissue showed a more even distribution, suggesting perhaps a more rapid infiltration of CD8+ cells.

Rare natural killer (NK) cells were identified at the transplant site. Outside of the context of pregnancy, NK cells target other cells that show decreased expression of MHC-I, most notably tumor cells and cells that are infected with virus (Campbell and Hasegawa, 2103). In human pregnancy, decidual NK cells play a role in functions such as spiral artery remodeling and do not show the same cytotoxic phenotype as peripheral blood NK cells (Hemberger, 2013; Kopcow et al., 2005). Natural killer cells were not investigated in the original, single horse-to-horse trophoblast transplant studies (Adams and Antczak, 2001), but were not identified in serial horse-to-horse transplants at day 10, 40 or 50 (Chapter 3). Natural killer cells are unlikely to place a role in the destruction of the horse-to-donkey trophoblast transplants for several reasons. First, they appeared in biopsied tissue only rarely. Second, the horse-to-donkey biopsies appear to have been destroyed prior to the time MHC-I would have been down-regulated. Finally, the MHC-I molecules expressed on the trophoblast transplants would have been those of the donor sire and dam, and thus even if present would likely have been recognized as foreign.

The presence of clusters of eosinophils in the day 7 biopsy was unexpected. In normal horse pregnancy eosinophils are found in small numbers in dying endometrial cups (Grunig et al., 1995), and small numbers of eosinophils were seen on the day 40 and 50 biopsies of serial horse-to-horse transplants (Chapter 3). A specific role for eosinophils in the life cycle of endometrial

cups and trophoblast transplants has not been defined. Eosinophils are common in the rodent uterus, particularly during estrus. They are thought to have a function in preparation of the uterus for pregnancy (Rothenberg and Hogan, 2006). Mice lacking the eosinophil chemoattractant interleukin 5 showed reduced numbers of eosinophils but an unchanged tissue distribution of these cells. Pregnancy and parturition in these animals were not adversely affected (Robertson et al., 2000). Eosinophilic endometritis was significantly associated with fetal death in one study of sub-fertile female dogs, but a role for the eosinophils as a cause for the fetal loss not established (Gifford et al., 2014). The possibility of a role for eosinophils in the early demise of the horse-to-donkey is intriguing. It is uncertain at this time if the eosinophils are present in association with dying trophoblast or as part of the donkey's response to the presence of foreign antigen.

This study did not evaluate lymphocyte sub-populations beyond the CD4+ and CD8+ classifications. In some species including the horse a role has been proposed for CD4+, CD25+ T-regulatory cells in maternal tolerance (Aurich et al., 2013; de Mestre et al., 2010; Rowe et al., 2012; Zenclussen et al., 2006). The distribution of T-regulatory cells in ectopic trophoblast transplants has not been thoroughly examined, but it is possible that a failure of this mechanism in xenogeneic transplants could contribute to transplant demise. This would not likely explain the observation that the initial transplants had lifespans akin to those of the horse-to-horse transplants, but may explain why the second and subsequent transplants had lifespans that were greatly decreased.

Recipients' serum progesterone concentrations following the horse-to-donkey trophoblast transplants did not show a predictable response as did those in single horse-to-horse transplants (de Mestre et al., 2011). Recipients' reproductive cycles did not always respond to circulating eCG following transplants, and in some progesterone remained high long after eCG levels became undetectable. Previous studies have shown that donkeys carrying hinny conceptuses produce much higher levels of progesterone than other equine pregnancies (Sheldrick et al., 1977). The seemingly random pattern of progesterone response to circulating eCG could represent species differences in sensitivity to eCG on a molecular level, such as through receptor numbers or distribution, or binding affinities.

In summary, the horse-to-donkey trophoblast transplants produced strikingly different results than the horse-to-horse transplants. The lifespans of initial horse-to-donkey transplants were similar to the horse-to-horse transplants, but second and subsequent transplants were greatly reduced. This suggests that the immunomodulatory mechanisms that protect horse chorionic girdle from immune destruction in an intra- or extra-species intrauterine pregnancy do not remain intact when the tissue is located at an ectopic site.

It remains unknown as to how allogeneic trophoblast in second and subsequent pregnancies evades immune destruction for 80 days, and the mechanism of eventual destruction is also unknown. The protective mechanism appears to remain intact in horse-to-horse trophoblast transplants (de Mestre et al., 2010), and the survival of these transplants suggests that the signal to initiate protection may originate within the trophoblast itself. The rapid destruction of horse-to-donkey transplants likely represents a failure of this protective mechanism.

Acknowledgements

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CHAPTER 5

INTERLEUKIN 22 IS EXPRESSED BY THE INVASIVE TROPHOBLAST OF THE EQUINE (*Equus caballus*) CHORIONIC GIRDLE[1]

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[1]Brosnahan MM, Miller DC, Adams M, Antczak DF. (2012). IL-22 is expressed by the invasive trophoblast of the equine (Equus caballus) chorionic girdle. Journal of Immunology, 188, 4181-4187.

Abstract

The invasive trophoblast cells of the equine placenta migrate into the endometrium to form endometrial cups, dense accumulations of trophoblast cells that produce equine chorionic gonadotropin (eCG) between days 40 and 120 of normal pregnancy. The mechanisms by which the trophoblast cells invade the endometrium while evading maternal immune destruction are poorly defined. A gene expression microarray analysis performed on placental tissues obtained at day 34 of gestation revealed a greater than 900-fold upregulation of mRNA encoding the cytokine interleukin 22 in chorionic girdle relative to non-invasive chorion. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) assays were used to verify high expression of *IL22* in chorionic girdle. Additional qRT-PCR analysis showed a striking increase in *IL22* mRNA expression in chorionic girdle from days 32 to 35, and an absence of *IL22* expression in other conceptus tissues. Bioinformatic analysis and cDNA sequencing confirmed the predicted length of horse *IL22*, which carries a 3' extension absent in *IL22* genes of humans and mice but present in the cow and pig. Our discovery of *IL22* in the chorionic girdle is a novel finding, as this cytokine has been previously reported in immune cells only. IL22 has immunoregulatory functions, with primary action on epithelial cells. mRNA of IL22 receptor *IL22R1* was detected in pregnant endometrium at levels similar to other equine epithelia. Based upon these findings we hypothesize that IL22 cytokine produced by the chorionic girdle binds IL22R1 on endometrium, serving as a mechanism of fetal-maternal communication by modulating endometrial responses to trophoblast invasion.

Introduction

The mechanisms that enable feto-placental tissues to evade destruction by the maternal immune system are a longstanding focus of scientific investigation. In the decades since Medawar proposed the "fetus as allograft" model, reviewed by Billington (Billington, 2003), research has implicated a complex communication between trophoblast, maternal immune cells and endometrium. Examples include production of IL4 and IL10 (Saito, 2000) HLA-G, IDO (Wicherek, 2008) and complement regulatory proteins (Xu et al., 2000) by trophoblast, expression of RCAS1 by endometrium (Wicherek, 2008) and FoxP3+ regulatory T cells at sites of trophoblast invasion (de Mestre et al., 2010).

Migration and endometrial invasion are attributes of trophoblast cells in many species, with the binucleate equine chorionic girdle (CG) cells being one example of this invasive phenotype (Hoffman and Wooding, 1993). Deep invasion of trophoblast is thought to have played a role in human evolution by facilitating development of the human brain, yet this process also brings increased risk of immune-related placental dysfunction and diseases such as preeclampsia (Carter, 2011). Some immunomodulatory molecules (e.g. galectins) are proposed to have evolved in tandem with specific forms of placentation (Carter, 2011; Than et al., 2009). Trophoblast cells may also be novel sources of molecules produced by immune cells in adult organisms, as in the production of macrophage migration inhibitory factor by human villous cytotrophoblasts (Arcuri et al., 1999).

Using gene expression array analysis comparing invasive and non-invasive equine trophoblast we identified novel production of the immunomodulatory cytokine interleukin 22 (*IL22*) by CG

cells just prior to their migration through the endometrium to form the binucleate, chorionic gonadotropin-producing endometrial cups (Allen et al., 1973). IL22 is a member of the interleukin 10 (IL10) family of cytokines (Trivella et al., 2010) and is involved in mucosal immunity and the maintenance and repair of epithelia (Aujla and Kolls, 2009; Maloy and Kullberg, 2008; Ouyang and Valdez, 2008; Sugimoto et al., 2008). Since its first description in 2000 in human and mouse T-cells (Dumoutier et al., 2000; Xie et al., 2000). IL22 has been documented exclusively in immune cells including T-helper subsets (Th17, Th22), natural killer cells, and bovine γδ T-cells (Colonna, 2009; Ma et al., 2010; Maloy and Kullberg, 2008; Sonnenberg et al., 2011; Trifari and Spits, 2010).

IL22 acts upon a heterodimeric receptor composed of its primary target IL22R1 and IL10R2 (Wolk et al., 2010). This receptor is expressed on epithelial surfaces, including respiratory (Aujla et al., 2008) and digestive tracts, and skin (Wolk et al., 2004). Binding of IL22R1 by IL22 activates transcription factors STAT3, STAT1 or STAT5 (Lejeune et al., 2002) and regulates genes associated with innate immunity (Liang et al., 2006) and cellular differentiation, migration and survival (Boniface et al., 2005; Radaeva et al., 2004). A second receptor, IL22R2, is a soluble binding protein thought to block downstream functions of IL22 (Kotenko et al., 2001; Xu et al., 2001). This study presents our initial microarray finding of *IL22* expression by CG cells, substantiates and expands upon this using qRT-PCR and bioinformatics, and identifies potential targets expressing *IL22R1* mRNA.

Materials and Methods

Animals

Mares of various breeds, ages and parity were bred by artificial insemination to Thoroughbred stallions using techniques previously described (Adams and Antczak, 2001). All horses were owned by the Cornell Center for Equine Genetics and maintained in a herd setting. Procedures were performed in accordance with an animal care and use protocol approved by the Institutional Animal Care and Use Committee (IACUC) of Cornell University.

Tissue Preparation

Equine conceptuses were recovered by uterine lavage using a procedure previously described (Antczak et al., 1987). Individual components including chorionic girdle, chorion, allantochorion, yolk sac, bilaminar omphalopleure and fetus (Figure 5.1) were isolated using a dissecting microscope. Endometrium was obtained with biopsy forceps following the conceptus recovery. Tissues were snap frozen in liquid nitrogen within one-half hour of collection. Epithelium and skin were obtained from animals of mixed age and breed euthanized in the Cornell University Hospital for Animals due to orthopedic conditions but free of other diseases. Tissues were put on ice post mortem and snap frozen in liquid nitrogen within one hour of collection. All samples were stored at -80°C until processing. Peripheral blood lymphocytes were isolated from heparinized jugular venous blood using a density centrifugation technique previously described (Antczak et al., 1982). Stimulated lymphocytes were prepared in coculture with 2.5 μg/ml pokeweed mitogen using a procedure described previously (Flaminio and Antczak, 2005).

RNA Isolation and cDNA Synthesis

RNA was isolated from samples using commercial kits (Qiashredder; RNeasy or RNeasy Plus Mini, Qiagen, Valencia, CA). All samples used in expression array experiments had RNA Integrity Numbers (RINs) above 9.0, with most between 9.8 and 10.0, as determined by an Agilent Bioanalyzer (Agilent, Santa Clara, CA).

RNA samples isolated with the RNeasy kit were treated with DNase I (Invitrogen, Carlsbad, CA) while those isolated with RNeasy Plus were put over a genomic DNA removal column. First strand cDNA synthesis was performed using M-MLV Reverse Transcriptase (USB, Cleveland, OH) following the manufacturer's protocol. All cDNA samples used in this study showed amplification of horse-specific β-2 microglobulin and no evidence of gDNA contamination.

Equine Expression Arrays

Two gene expression arrays developed on the Agilent (Agilent Technologies, Santa Clara, CA) platform were used, a commercial 4x44k array ("Agilent" array) and an 8x15k custom designed product ("Cornell" array). For the Cornell array, horse sequences were obtained from three NCBI databases. Predicted reference sequences (RefSeqs) from the first assembly of the horse whole genome sequence (http://www.broad.mit.edu/mammals/horse) were combined with all horse UNIGENE entries, resulting in 24,628 sequences. All 37,316 available horse expressed sequence tag (EST) sequences were combined with the RefSeq and UNIGENE entries to provide a transcriptome file for the eArray pipeline.

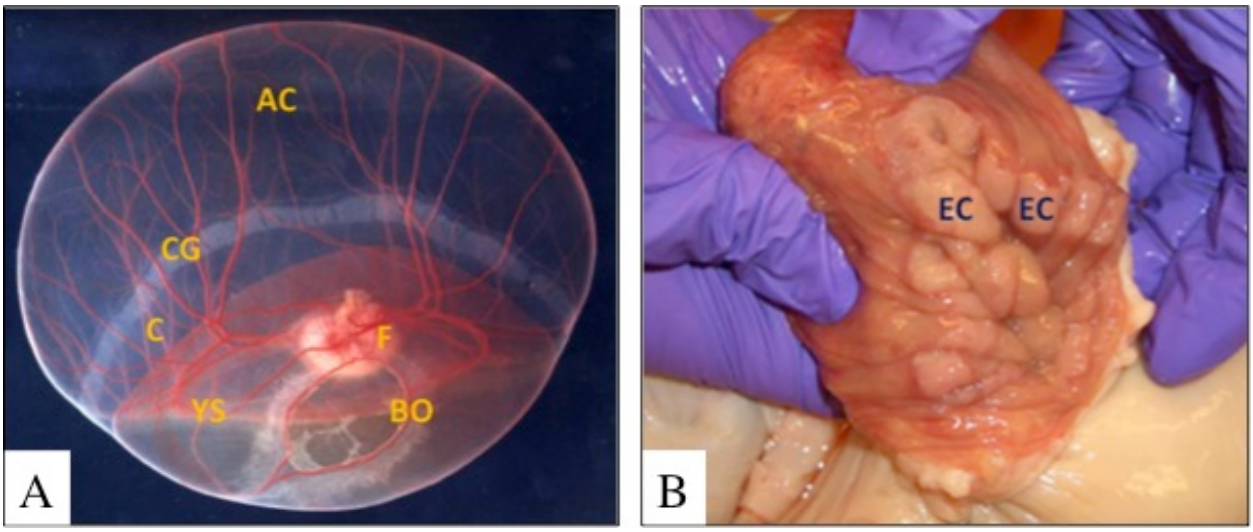

Figure 5.1 Gross specimens of horse conceptus and endometrial cups *in situ*. A) Day 34 horse conceptus showing chorionic girdle (CG) as a distinct pale band encircling the conceptus, chorion (C), allantochorion (AC), yolk sac (YS), bilaminar omphalopleure (BO) and fetus (F). The chorionic girdle cells begin migrating from the conceptus through the endometrium at days 36 to 38 to form B) the equine chorionic gonadotropin-producing endometrial cups (EC), shown here within the maternal endometrium at day 44 of gestation in a post-mortem specimen.

Horse specific mRNA sequences were uploaded into the Agilent eArray custom microarray design pipeline (https://earray.chem.agilent.com/earray/) and duplicate sequences were filtered out. One probe was designed for each input sequence. Each probe received an Agilent quality score of 1 (highest) to 4 (lowest) and a cross hybridization potential score of 0 to 1. 14,307 probes with quality/cross hybridization scores of 1/0, 1/1, or 2/0 were selected for the Cornell array.

Array Experiments

RNA was amplified linearly and Cy3 labeled using the Ambion Amino Allyl MessageAmp™ II kit (Applied Biosystems, Foster City, CA), then hybridized to both the Cornell and Agilent array slides using standard techniques. Data files were analyzed using GeneSpring GX11 software (Agilent). Samples were quantile normalized, and poorly performing probes were filtered out. P-values for fold changes were calculated using paired t-tests. Results were exported into Excel (Microsoft, Redmond, WA, USA) files.

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR) Assays

qRT-PCR assays used SYBR Green technology (Applied Biosystems, Foster City, CA) run on an Applied Biosytems 7500 Fast Real Time PCR instrument. Standard curves were generated using known copy numbers of a plasmid with a target gene-containing fragment. Primer sequences were designed with Primer3 (Rozen and Skaletsky, 2000) and are shown in Table 5.1. SCAMP3 was used as a housekeeping gene.

Table 5.1 Primer Sequences Used for Amplification of Equine Products in RT-PCR Assays

Gene	Reaction	Forward 5'-3'	Reverse 5'-3'
IL22[1]	RT-PCR	ATGGCCACCCTGCAGAAAT	TTAGTTATCCATTTTCCAGCTTTTCTC
β-2M	RT-PCR	TATGTCTCTGGGTTCCATCC	GATGCTGGTTAGAGGTCTCG
IL22	qRT-PCR	CACAGATGTTCGGCTCATTG	GTAAGGCTGGAATCGGTCAG
IL22R1	qRT-PCR	TTCCAACCTGGTCCAAAGAG	CACCTGAGAAGGCGTAGGTC
IL22R2	qRT-PCR	AGCTCACAGGGTCATTGAGG	CTCAGGCCGGTAGATTTCAG
CD3	qRT-PCR	GGCCTCATCCTGGCTATCAC	CCCAGATTCCGTGTAGTTTCTC
SCAMP3	qRT-PCR	CTGTGCTGGGAATTGTGATG	ATTCTTGCTGGGCCTTCTG

[1] Primer set used for cloning of the entire coding sequence

Cloning and Sequencing of the Equine IL22 Expressed Gene Sequence

IL22 was amplified by RT-PCR from mitogen-stimulated equine lymphocytes. The PCR product was purified using QIAquick PCR Purification kit (#28106) and cloned using the pcr4bluntTOPO vector transfected into *E. coli.* Plasmid DNA was purified using QIAprep Spin Miniprep (#27106), digested with EcoRI and run on 1.5% agarose. Clones with bands at 570 bp were sequenced using the Sanger technique. Data was analyzed using Geneious Pro 5.0.4 (Biomatters, Ltd.) (Drummond et al., 2010) and Mega 5.05 (Felsenstein, 1985; Saitou and Nei, 1987; Tamura et al., 2011; Zuckerkandl and Pauling, 1965).

Results

IL22 is Expressed by Equine Chorionic Girdle Cells Just Prior to Endometrial Invasion

Global gene expression was compared between invasive (chorionic girdle) and non-invasive (chorion) trophoblast (Figure 5.1) from day 34 conceptuses. Four chorionic girdle and chorion pairs were tested on the Cornell array, with three of the same pairs also tested on the Agilent array. Over 300 genes were differentially expressed between the two forms of horse trophoblast (Miller & Antczak, in preparation).

Our most striking result was high expression of *IL22* in chorionic girdle relative to chorion, observed on all six probes used on both arrays. A single *IL22* probe on the Cornell array showed a 904-fold upregulation while five *IL22* probes on the Agilent array had fold changes ranging from 665 to 1,039 with a mean of 856 (Table 5.2; array results deposited in GEO, reference number GSE35743). The novelty of our observation called for further exploration of *IL22*

expression, the equine *IL22* gene, and the receptor components of the *IL22* system in the equine conceptus and endometrium.

To verify and expand upon our initial microarray finding, temporal expression of *IL22* was quantified in 15 chorionic girdle – chorion pairs from days 32, 33, 34, and 35 of gestation using qRT-PCR. These points encompass the time when the chorionic girdle is first grossly visible until the cells begin migrating into the endometrium. *IL22* mRNA was present in chorionic girdle on day 32 and increased rapidly through day 35 (Figure 5.2). Expression remained negligible in chorion and in trophectoderm from conceptuses recovered on days 15, 21, 25 and 30, prior to a grossly observable chorionic girdle (data not shown). To evaluate spatial expression of *IL22* three day 34 equine conceptuses were dissected into their main component tissues: chorionic girdle, chorion, allantochorion, yok sac, bilaminar omphalopleure, and fetus (Figure 5.1). Only the chorionic girdle expressed high levels of *IL22* mRNA (Figure 5.3).

IL22 is produced by lymphocytes (Wolk et al., 2010) prompting us to use stimulated horse lymphocytes as a proxy for comparison to assess physiologic relevance of the *IL22* levels in chorionic girdle. Microarray analysis of lymphocyte mRNA produced hybridization signal intensities on the *IL22* probes comparable to those recorded for chorionic girdle (Table 5.2), and qRT-PCR analysis showed that peak *IL22* expression in a day 35 chorionic girdle exceeded that of stimulated lymphocytes (Figure 5.4).

To establish that chorionic girdle samples were free from T-lymphocyte contamination that could be the source of *IL22*, the samples were evaluated for T-lymphocyte marker *CD3*. *CD3* was not

detected in chorionic girdle from days 32, 33, 34 or 35 while resting horse lymphocytes had 5,153 copies of *CD3* per 10 ng mRNA (data not shown).

Table 5.2 Expression Array Results for *IL22* and the Receptors in Chorionic Girdle, Chorion and Lymphocytes

Gene	Tissue of Interest	Comparison Tissue	Array	Probes	Fold Change	p-Value	Tissue of Interest Signal
IL22	Chorionic Girdle	Chorion	Cornell 8x15	Single	903.72	0.0007	12.23
			Agilent 4x44	Mean of 5	856.76	0.016	12.31
	Stimulated Lymphocytes	Resting Lymphocytes	Cornell 8x15	Single	628.36	0.02	14.23
			Agilent 4x44	Mean of 5	2029.14	0.044	14.31
IL22R1	Chorionic Girdle	Chorion	Cornell 8x15	Single	-1.38	0.02	8.03
			Agilent 4x44	Single	-1.49	0.03	8.88
	Stimulated Lymphocytes	Resting Lymphocytes	Cornell 8x15	Single	-1.13	n/s	8.71
			Agilent 4x44	Single	-1.05	n/s	8.78
IL22R2	Chorionic Girdle	Chorion	Cornell 8x15	Single	1.04	n/s	3.70
			Agilent 4x44	Single	1.74	n/s	4.29
	Stimulated Lymphocytes	Resting Lymphocytes	Cornell 8x15	Single	435.32	n/s	11.33
			Agilent 4x44	Single	631.32	0.05	14.04

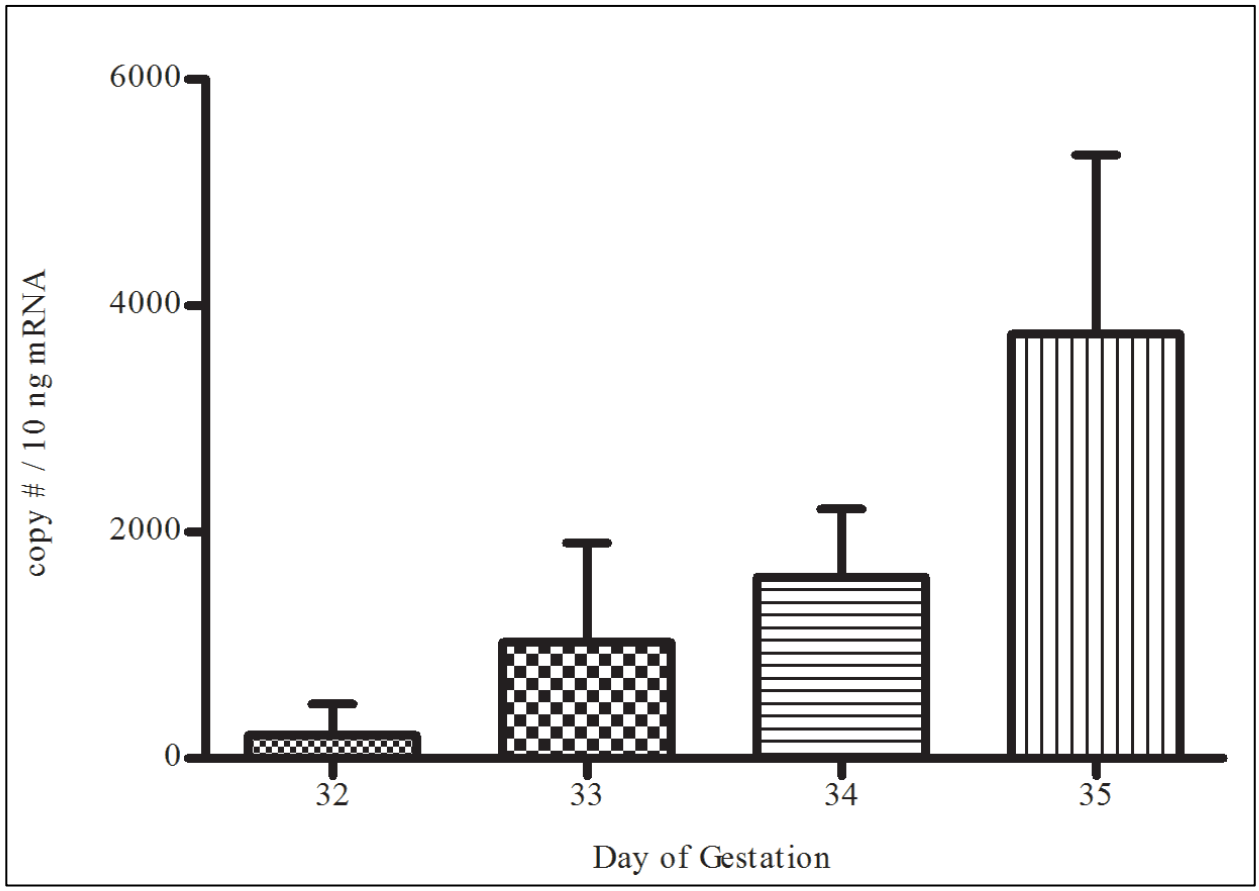

Figure 5.2 Temporal analysis of *IL22* expression in the chorionic girdle. Chorionic girdle samples from days 32 (n=3), 33 (n=3), 34 (n=5) and 35 (n=4) show *IL22* expression increasing dramatically just prior to invasion of the endometrium. Copy numbers represent means of two separate qPCR experiments run in triplicate for each sample. Chorion membrane from each conceptus also was tested (data not shown, mean = 3.7 copies/10 ng mRNA, range = 0-30 copies/10 ng mRNA). Trophectoderm from days 15, 21, 25, and 30 taken from the approximate area where the chorionic girdle arises also showed negligible expression of *IL22* (data not shown, n = 6, mean = 3.7 copies/10 ng mRNA, range = 0-10 copies/10 ng mRNA).

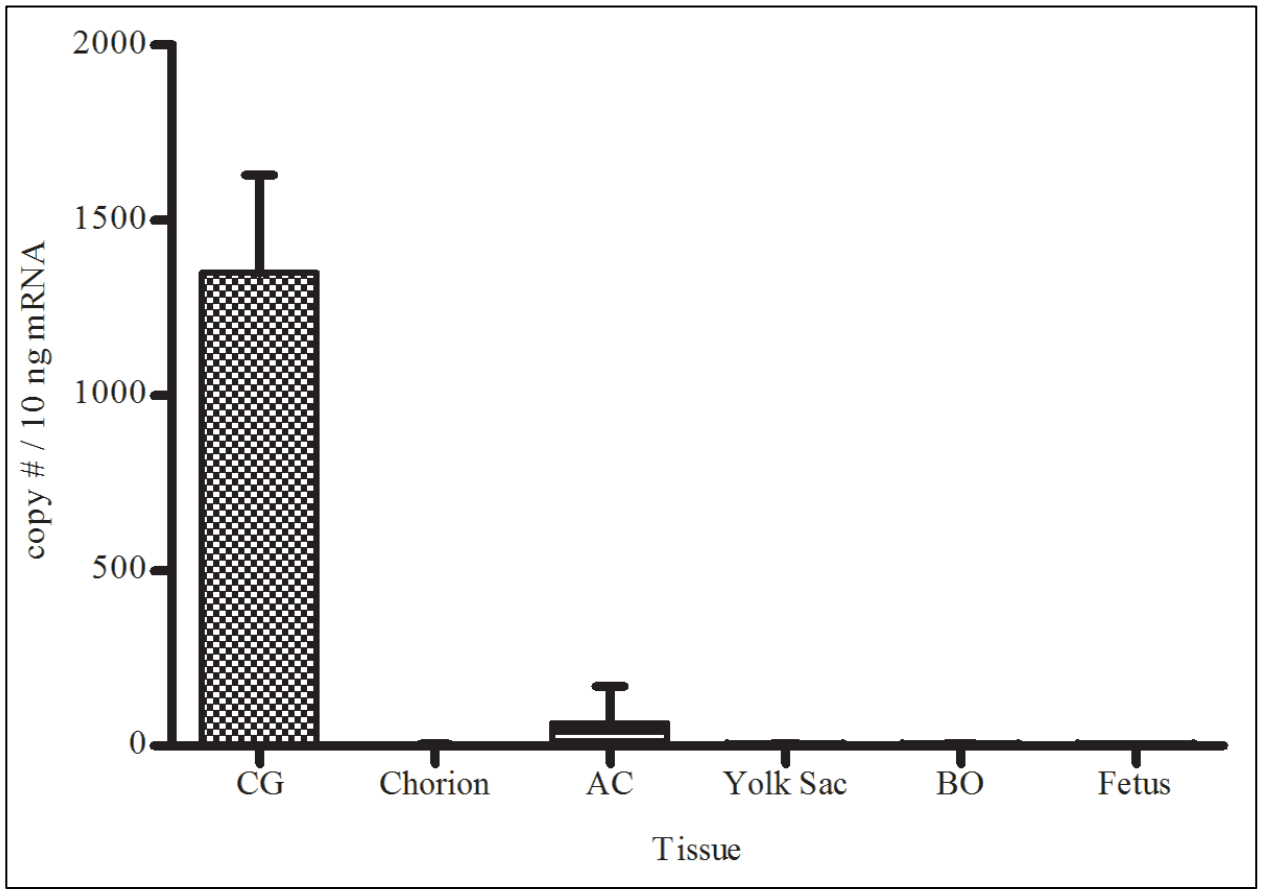

Figure 5.3 Spatial analysis of *IL22* expression in the day 34 horse conceptus. Additional tissues from three day 34 conceptuses were analyzed to assess the spatial pattern of *IL22* mRNA expression, including allantochorion (AC), yok sac, bilaminar omphalopleure (BO), and fetus. The high expression of *IL22* in the horse conceptus is restricted to the chorionic girdle. Samples were run in triplicate.

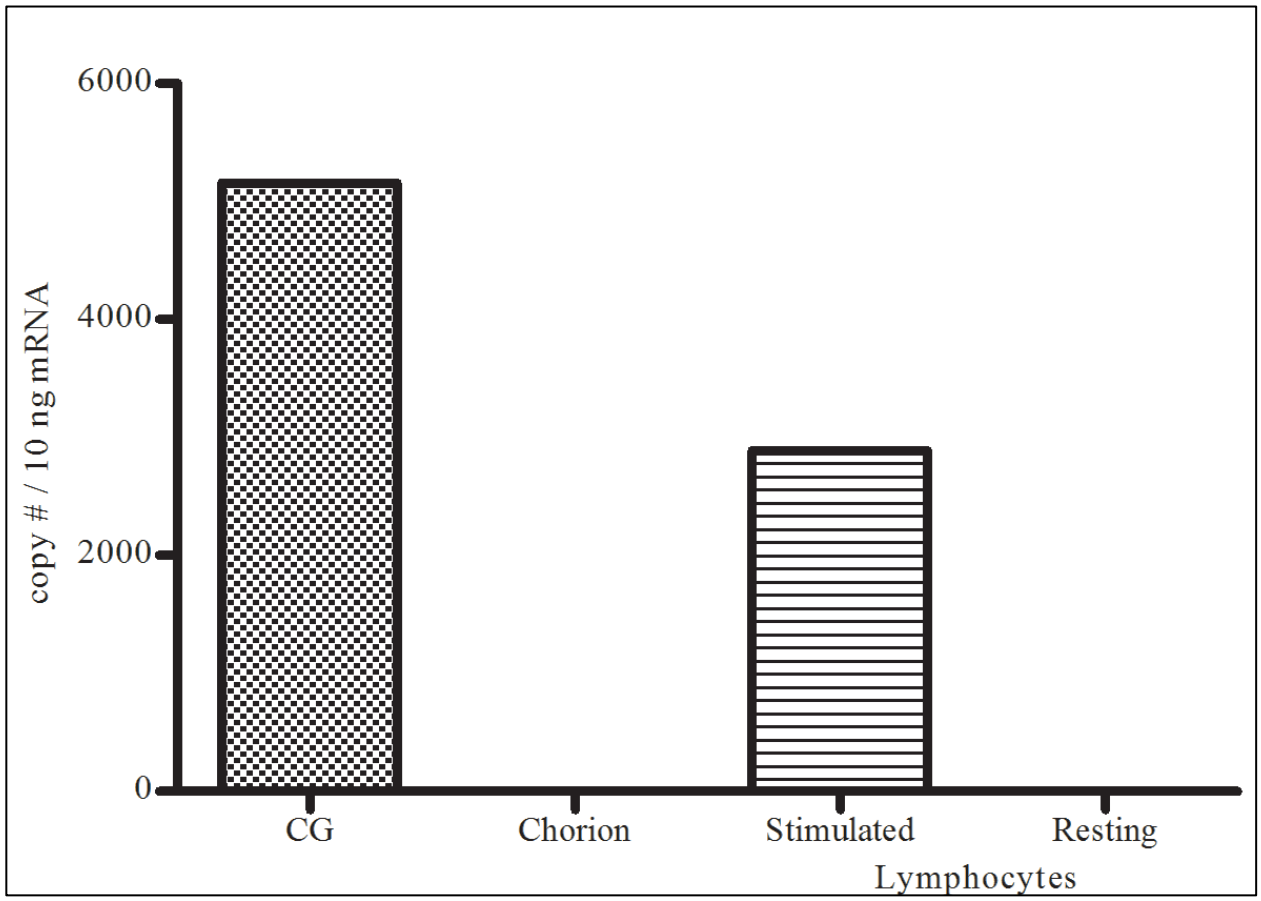

Figure 5.4 *IL22* Expression in day 35 chorionic girdle and chorion, and horse lymphocytes. qPCR assays demonstrate that peak production of *IL22* in the chorionic girdle exceeds that of stimulated equine lymphocytes. Data represents individual samples run in triplicate.

Horse IL22 is Similar to Other Species in Gene Structure and Sequence

We explored whether the unusual expression of equine *IL22* in trophoblast might relate to peculiarities in its molecular structure or genomic environment. We confirmed the expressed coding sequence of equine *IL22* (Figure 5.5) and found that alignment of the predicted protein with other species reveals a 3' extension of 10 amino acids relative to the human, mouse and dog but also observed in the cow and pig (Figure 5.6).

The full equine IL22 amino acid sequence showed greatest identity with the dog (*Canis familiaris*, 76%), followed by human (*Homo sapiens,* 75%) pig (*Sus scrofa,* 73%), mouse (*Mus musculus,* 69%) and cow (*Bos taurus,* 64%). Slightly higher sequence identities were observed if the 3' extension is excluded (Table 5.3). A tree diagram further illustrates the relationships among the horse IL22 protein sequence and the IL22 sequences of these five other species (Figure 5.7). The predicted protein structure of the translated sequence is α-helical as in other species (Figure 5.8) (Ma et al., 2010; Wolk et al., 2010).

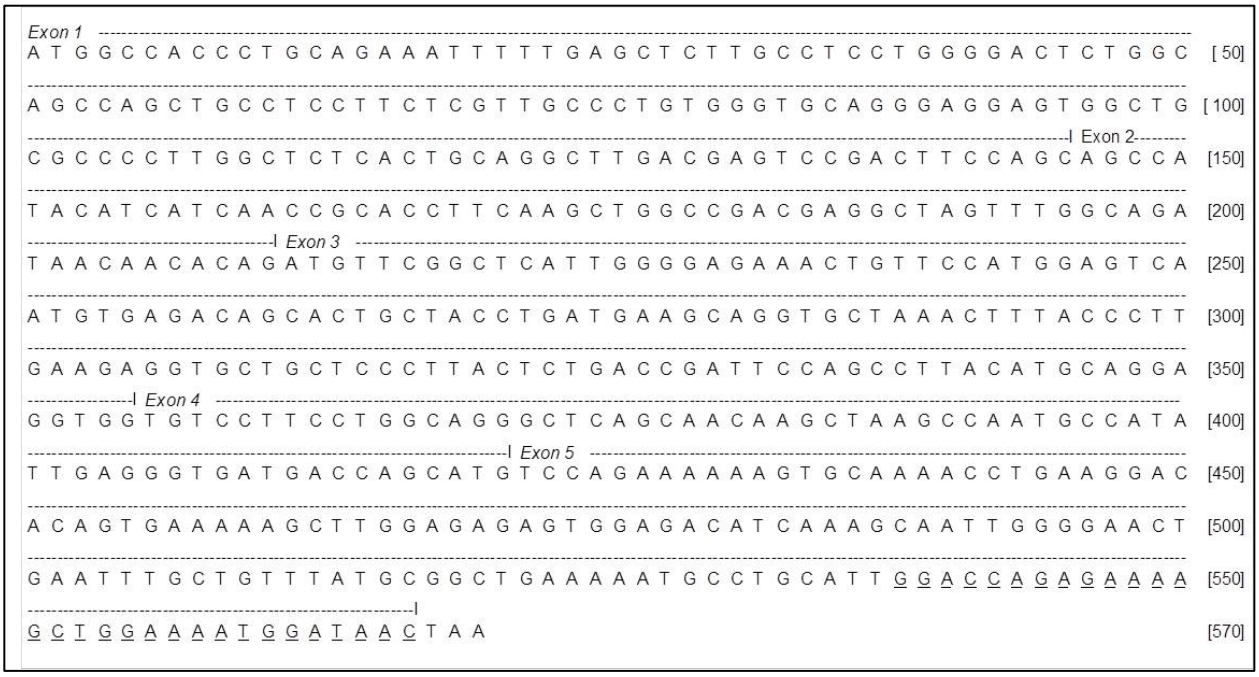

Figure 5.5 Nucleotide sequence of horse *IL22*. The coding sequence of horse *IL22* is shown as determined from mRNA isolation and complementary DNA synthesis. Five coding exons are labeled, and nucleotides producing the 10-amino-acid 3' extension peculiar to ungulate *IL22* are underlined.

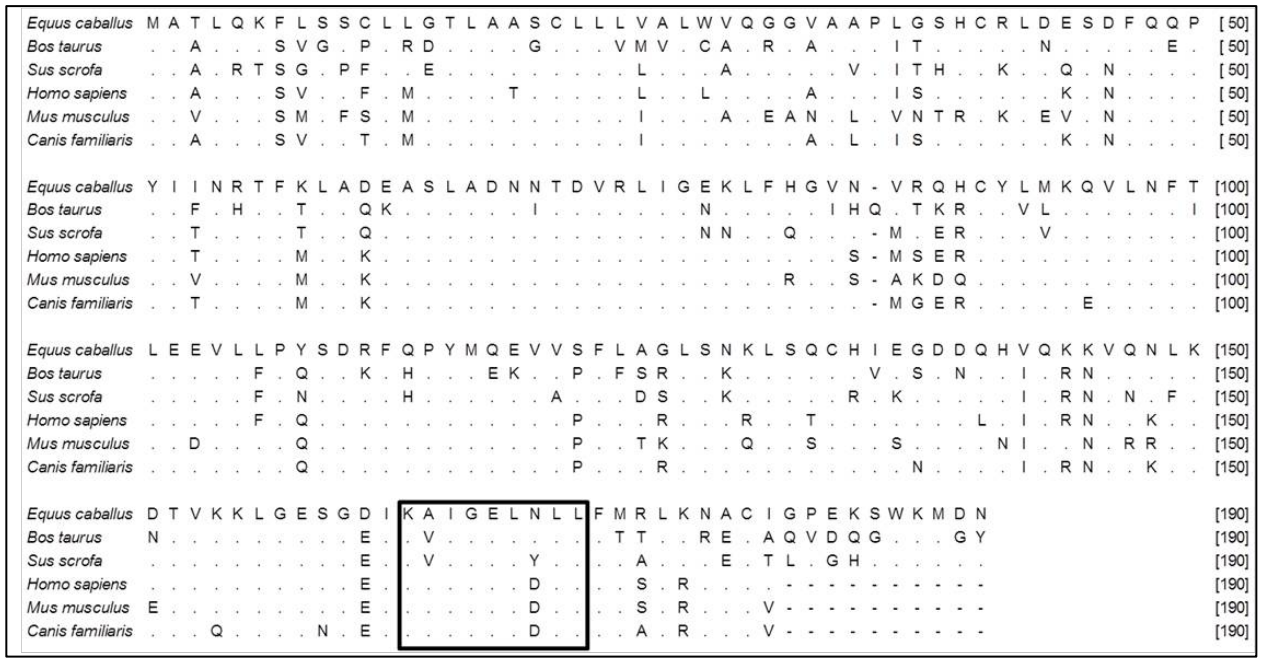

Figure 5.6 Comparative alignment (ClustalW) of IL22 from the domestic horse and other species. Horse IL22 shows a 3' extension also observed in bovine and predicted porcine IL22, but not in other species. Consensus is indicated by "." and "-" indicates a gap. The sequence within the box represents the non-interferon IL10 family signature. (*Equus caballus* RefSeq: XP_001491804; *Bos taurus* GenBank: DAA29799; *Sus scrofa* RefSeq: XP_001926191.1; *Homo sapiens* GenBank: AAQ8924.1; *Mus musculus* GenBank: AAI16236.1; *Canis lupus familiaris* RefSeq: XP_538274.1) Analysis performed with MEGA5.

Table 5.3 Amino Acid Sequence Identity Between Equine IL22 With and Without the 3' Extension and Other Species[1]

	Equus caballus (full sequence)	*Equus caballus* (short sequence)
Homo sapiens	75%	79%
Mus musculus	69%	73%
Canis familiaris	76%	80%
Bos taurus (full sequence)	64%	--
Bos taurus (short sequence)	--	66%
Sus scrofa (full sequence)	73%	--
Sus scrofa (short sequence)	--	73%

[1] Analysis performed with Geneious 5.0.4

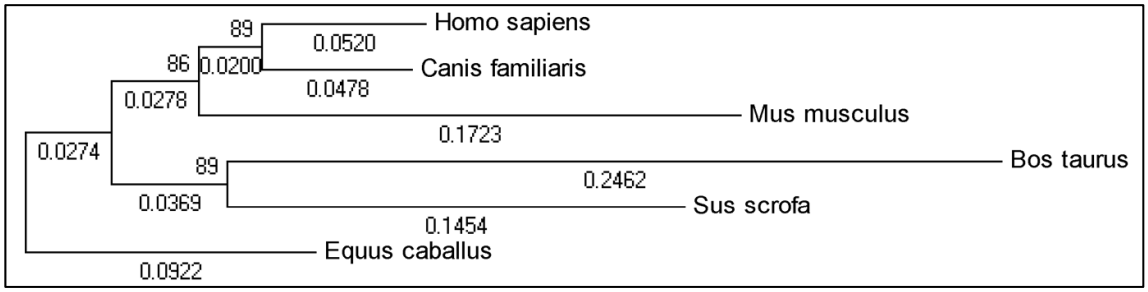

Figure 5.7 Tree diagram depicting the relationship between the horse IL22 protein sequence and IL22 from selected other species. The tree was generated in MEGA5 using the Neighbor-Joining method and rooted on the horse. The optimal tree with the sum of branch length = 0.86793050 is shown. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The tree is drawn to scale. Branch lengths (next to the branches) represent evolutionary distances computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated. There were a total of 179 positions in the final dataset.

Figure 5.8 Predicted secondary structure of horse IL22. The predicted amino secondary structure shows that horse IL22 is primarily alpha-helical as in other species. Legend: = α-helix, = β-sheet, = turn, = coil. The predicted structure of the 3' extension is also alpha-helical. Analysis performed with Geneious 5.0.4.

Equine *IL22* is located on equine chromosome 6 and contains 5 coding exons. The genomic landscape of *IL22* is similar to that of human and mouse, with *IFNG*, *IL26* and *MDM1* in close proximity (Figure 5.9) (Wade CM, 2009). *In silico* analysis of the promoter regions of the horse and several other species was performed. Species included in the analysis have varying forms of placentation, including human (discoid hemochorial), dog (zonary endotheliochorial), cow (cotyledonary synepitheliochorial) and pig (diffuse epitheliochorial, similar to the mare). The human *IL22* promoter sequence was obtained from the Transcriptional Regulatory Element Database (TRED, http://rulai.cshl.edu/cgi-bin/TRED/tred.cgi?process=home). TRED promoter sequence ID 9992 was searched against the horse (*Equus caballus*, TaxID 9796), dog (*Canis familiaris*, TaxID 9615), cow (*Bos taurus*, TaxID 9913) and pig (*Sus scrofa*, TaxID 9823) genomes using the NCBI Basic Local Alignment Search Tool (BLAST, http://blast.ncbi.nlm.nih.gov) discontiguous megaBLAST and default parameters. The sequence

aligned only to regions overlapping the predicted *IL22* gene in all species (horse: E value 0.0, sequence identity 80%; dog: E value $3e^{-51}$, sequence identity 84%; cow: E value $2e^{-160}$, sequence identity 78%; pig: E value $3e^{-36}$, sequence identity 85%). Canonical regulatory elements conserved in all species included a TATA box (TATAWAWN) sequence. An initiator sequence (motif YYANWYY) was present with an identical sequence in the horse, cow and pig. The same motif was present in the human with a single nucleotide difference, and it did not appear in the dog. CCAAT box motifs did not show conserved locations.

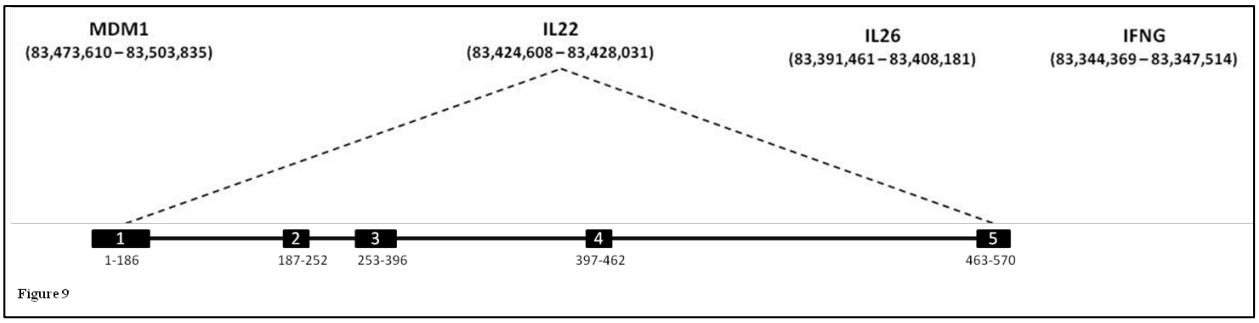

Figure 5.9 Exon structure and genomic landscape of *IL22* on equine chromosome 6. The horse *IL22* gene is located on equine chromosome 6 and contains 5 coding exons. This diagram shows the position of the *IL22* genomic sequence on chromosome 6 and distribution of the coding sequence across exons (bottom numbers). Neighboring genes include *MDM1*, *IL26* and *IFNG* as are found in other species.

A computer-based transcription factor analysis of the promoter regions using AliBaba2 (http://www.gene-regulation.com/pub/programs/alibaba2/intro.html), TFSearch (http://www.cbrc.jp/htbin/nph-tfsearch) and SignalScan (http://www-bimas.cit.nih.gov/cgi-bin/molbio/signal) revealed STATX and AP-1 sites in all species examined (Figure 5.10).

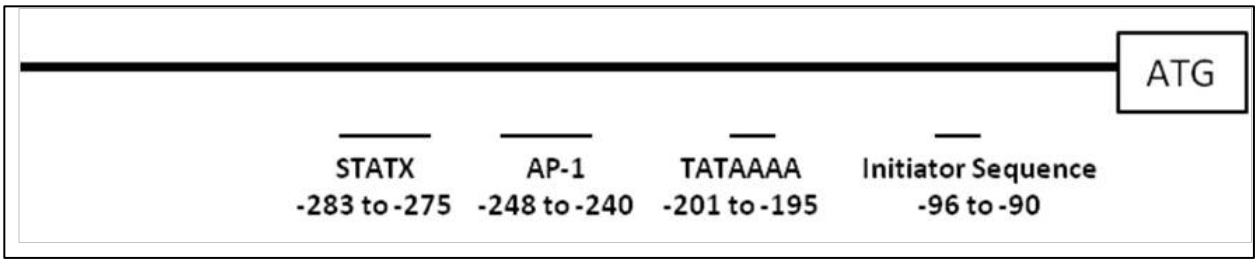

Figure 5.10 Schematic of upstream regulatory elements in equine *IL22*. This schematic shows putative regulatory elements identified using *in silico* analysis of the equine *IL22* promoter region. The sites are conserved with other mammalian species having various types of placentation, including the human, dog, pig and cow. An identical initiator sequence is present in the horse, pig and cow. The same motif is present in the human with a single nucleotide difference, and is not apparent in the dog.

IL22R1 is Expressed in Endometrium at Levels Similar to Other Equine Epithelial Tissues

We investigated potential targets of CG IL22 by assaying for receptor mRNA in adjacent tissues. qRT-PCR assays did not identify *IL22R1* in CG or other conceptus tissues with the exception of chorion (Figure 5.11A), as observed on the initial microarray (Table 5.2). Assays were then run on endometrial tissue along with epithelium from the equine respiratory and gastrointestinal tracts and skin for comparison. *IL22R1* mRNA was detectable in day 32 and day 34 pregnant endometrium at levels similar to epithelium (Figure 5.11B) where it is expected to be found (Wolk et al., 2004; Wolk and Sabat, 2006). qRT-PCR analysis of conceptus tissues for *IL22R2* showed minimal expression in fetal tissues (data not shown). CG and chorion showed no expression of this molecule, with 2,803 copies per 10 ng mRNA in stimulated equine lymphocytes, consistent with our original microarray finding (Table 5.2).

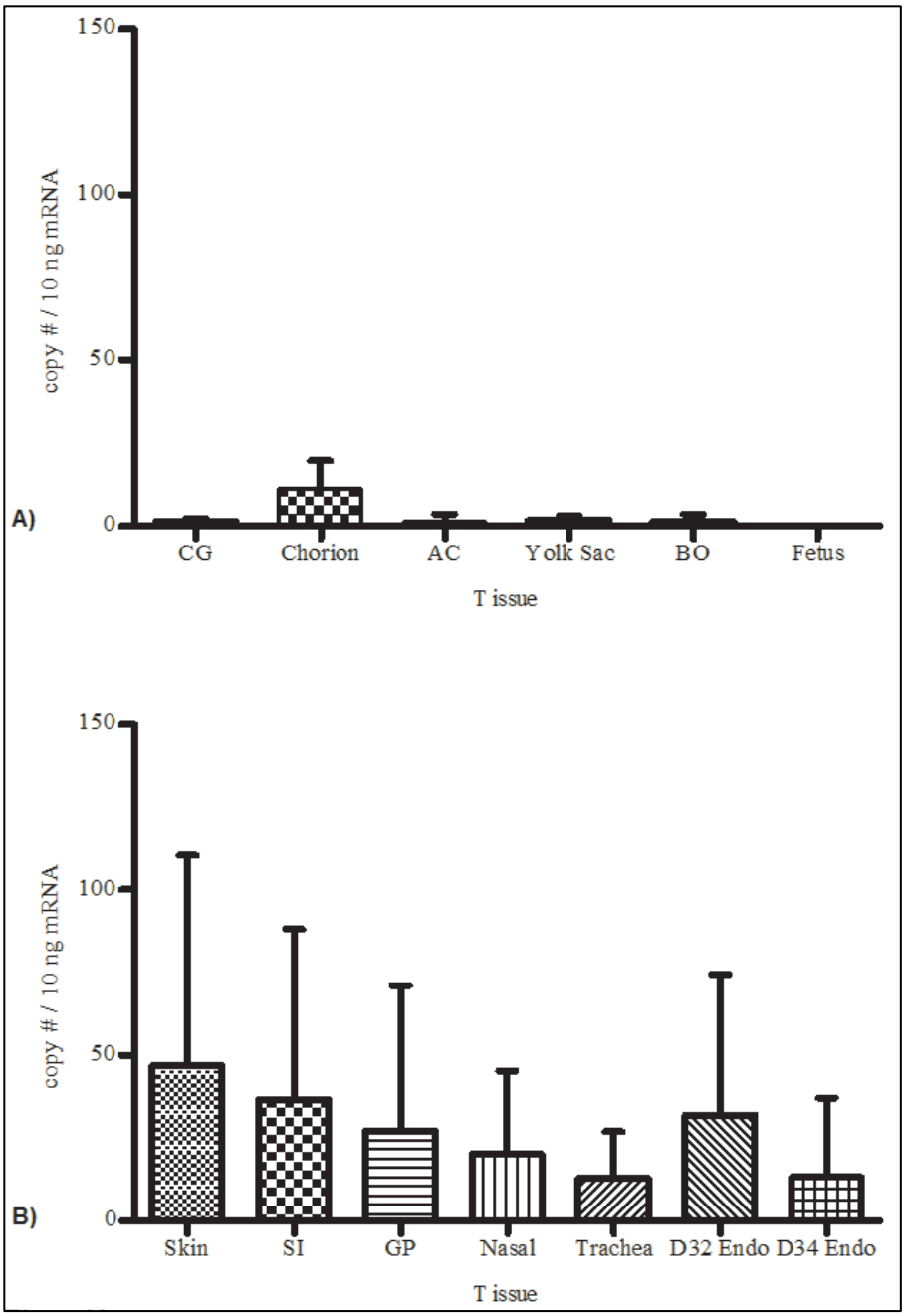

Figure 5.11 *IL22R1* expression in the equine conceptus. *IL22RA1* expression assessed by qRT-PCR in 3 day 34 equine conceptuses (A) and in other equine epithelial tissues (B). Epithelial samples included endometrium from days 32 (n = 2) and 34 (n = 8) of pregnancy, skin (n = 6), nasal mucosa (n = 6), tracheal mucosa (n = 6), guttural pouch mucosa (GP, n = 4) and small intestine (SI, n = 2). Samples were run in triplicate.

Discussion

Our discovery that *IL22* is expressed by the chorionic girdle cells of the equine trophoblast is the first clear evidence for production of this cytokine by a non-immune cell type. IL22 was first identified in lymphocytes of the mouse and the human (Dumoutier et al., 2000; Xie et al., 2000) and subsequently in other lymphoid populations (Commins et al., 2008; Eyerich et al., 2010; Ma et al., 2010; Torchinsky and Blander, 2010; Trifari and Spits, 2010; Wolk K et al., 2006; Wolk et al., 2010). Attempts to identify IL22 in non-hematopoietic cells were unsuccessful (Wolk et al., 2010). Taken together our data provides the first clear evidence for expression of *IL22* by a non-immune cell type. We show consistently high expression of *IL22* in CG using both gene expression arrays and qRT-PCR assays. Rapid upregulation of *IL22* just prior to endometrial invasion and restriction of expression to CG suggest a specific physiologic function for IL22 at this stage of placental development. An *IL22* mRNA level in CG comparable to that found in stimulated lymphocytes supports the physiologic relevance of our finding.

Expression of *IL22* in CG is a novel example of trophoblast co-opting an existing immunoregulatory gene for a physiologic need specific to placentation. A role for IL22 has been implicated in human reproduction but in these instances is produced by uterine natural killer cells (Male et al., 2010). Our finding may represent a mechanism specific to endometrial cup biology in equids or more broadly to epitheliochorial placentation. It is clear from the pattern of expression we observed that *IL22* mRNA is detectable for a brief interval, and possibly the appropriate samples have not been evaluated in other species.

Though the structure and coding sequence of equine *IL22* are similar to other species, a notable characteristic of the predicted equine IL22 protein is its extra ten amino acids relative to the human, mouse and dog. The longer amino acid sequence was considered unique in cows by researchers investigating bovine IL22 (Ma et al., 2010). We demonstrate that this extension is also present in the horse and pig. It is of note that the three species having the 3' extension do not share the greatest sequence identity of the IL22 protein (refer to Table 5.3 and Figure 5.7). This raises questions as to the conservation of function associated with this extension. These three species do however share identical regulatory motifs in their upstream regions. In some reproductive hormones, modifications to c-terminal extensions affect receptor affinity (Bousfield et al., 1996). Additional investigation is necessary to determine if this modification has functional significance in ungulate IL22.

Current understanding of *IL22* regulation is based largely upon Th17 and other immune cell pathways, known to involve STATX, AP-1, RORγt, IL6, IL23, AhR and other molecules (Ma et al., 2010; Schraml et al., 2009; Torchinsky and Blander, 2010). Of these only AhR showed significant expression in CG on our original array. STATX and AP-1 motifs were identified in the promoter regions of the horse and other species examined in our *in silico* analysis. Further research must be undertaken to define the mechanisms upstream and downstream of *IL22* expression in the CG.

Binding of IL22R1 activates STAT pathways that regulate proteins involved in antimicrobial defense, cell differentiation and cell migration (Wolk K et al., 2006). In vitro studies using reconstituted human epithelium indicate a role for IL22 in the healing of epithelial surfaces

(Boniface et al., 2005). The overlap of physiologic processes involved in CG cell formation of the endometrial cups and IL22 functions in other cell types allows plausible hypotheses for IL22 function in the CG cells. From early specialized CG cell development on day 25 to final formation of the endometrial cups by day 42, CG cells undergo extensive differentiation and migration. Mononuclear cells terminally differentiate into binucleate, equine chorionic gonadotropin-producing cells of the functional endometrial cup and migrate through the endometrial epithelium and into the stroma (Allen et al., 1973; Enders and Liu, 1991; Gerstenberg et al., 1999). The presence of *IL22* in the CG and *IL22R1* in the maternal endometriim lends itself to the hypothesis that IL22 facilitates re-epithelialization of the endometrium after trophoblast migration. IL22 also may be involve in the uterine innate immune system. IL22 upregulates antimicrobial proteins such as β-defensins at mucosal surfaces (Wolk K et al., 2006; Wolk et al., 2004). Maternal – fetal cooperation in immune response to infection has been documented previously (Guleria and Pollard, 2000; Koga et al., 2009). Localized disruption of endometrium at the site of trophoblast invasion could be a route for infection, counteracted by stimulation of antimicrobial molecules. Finally, the presence of *IL22R1* in chorion could indicate a role for IL22 in the development of the girdle itself.

IL22R2 has been identified in various tissues including human placenta and lymphatic tissues. It has multiple splice variants in other species, and *in vivo* functions that remain undefined (Wolk and Sabat, 2006). A role for IL22R2 in equine placentation is not immediately evident.

In summary, our data establishes dramatic *IL22* expression by a non-immune cell type, the invasive trophoblast of the equine chorionic girdle. We hypothesize that IL22 from the chorionic

girdle cells facilitates equine placental development by initiating repair of the endometrial epithelium at the sites of trophoblast invasion.

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CHAPTER 6

LOCALIZATION OF INTERLEUKIN 22 RECEPTOR (IL22RA1)

IN HORSE ENDOMETRIUM

Abstract

Previous research identified novel expression of interleukin 22 (*IL22*) mRNA in chorionic girdle from a normal day 34 horse pregnancy. Endometrium and chorion were the only tissues in proximity to the chorionic girdle found to express mRNA for the IL22 target receptor *IL22RA1*. Based upon existing knowledge of a role for IL22 in the maintenance of epithelial integrity, and the aggressive nature of chorionic girdle invasion, IL22RA1 on the endometrium was considered to be the most likely target of IL22 originating from the chorionic girdle. The objective of this study was to localize the *IL22RA1* mRNA to specific cell populations within mare endometrium using RNA in situ hybridization. A minor objective was to perform a comparative bioinformatic analysis on the predicted IL22RA1 protein sequence. Five species in addition to the horse were included in this analysis. A seven amino acid region was found to be absent in the human, but present in the horse and five other species examined. Eight of twelve residues known to be critical for binding IL22 were conserved in all species. Samples of pregnant and non-pregnant endometrium were analyzed via in situ hybridization. Strong expression of *IL22RA1* was detected in glandular and luminal epithelium, and probes hybridized to pregnant endometrium more intensely than to non-pregnant endometrium. This finding supports the hypothesis that IL22 of chorionic girdle origin may bind receptor on the endometrial epithelium, repairing the damage caused by chorionic girdle cell invasion. The presence of *IL22RA1* in glandular epithelium further suggests a role for IL22 in endometrial gland development in early pregnancy.

Introduction

Interleukin 22 (IL22) is a cytokine most commonly produced by a variety of immune cell types, including T-helper cell populations and natural killer cells (Rutz et al., 2013). The primary target of IL22 is the transmembrane receptor IL22RA1. This heterodimeric receptor is located on epithelial cells, keratinocytes and other specialized cells of the liver, pancreas and kidney. Binding of IL22 to IL22RA1 upregulates genes involved in cellular proliferation, differentiation and migration, thereby preserving the integrity of the epithelium in the face of infectious, mechanical or chemical trauma (Hoegl et al., 2010; Rutz et al., 2013; Schieermann et al., 2013; Wolk et al., 2006).

Previous work using gene expression arrays and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) detected *IL22* mRNA in the invasive trophoblast of the equine chorionic girdle. Gene expression was highly upregulated in chorionic girdle between days 32 and 35 of pregnancy (Brosnahan et al., 2012). *IL22RA1* mRNA was identified only in the maternal endometrium and the chorion but not in other tissues in proximity to the chorionic girdle (Brosnahan et al., 2012).

At approximately day 36 of normal equine pregnancy, chorionic girdle cells penetrate the endometrial epithelium by phagocytosis and migrate through the stroma in conjunction with the endometrial glands prior to coalescing and forming the mature, equine chorionic gonadotrophin-secreting endometrial cups (Allen et al., 1973). Based upon existing knowledge of the protective mechanisms of IL22 secreted by immune cells in proximity to epithelial surfaces, and this aggressive invasion of maternal endometrium by the chorionic girdle cells, we hypothesized that

IL22 secreted by the chorionic girdle cells binds IL22RA1 receptor on the endometrium, initiating repair of damage caused by chorionic girdle cell invasion. The primary objective of this study was to use in situ hybridization to localize *IL22RA1* mRNA to specific cell populations within the endometrium. A secondary objective was to perform a comparative bioinformatic analysis of IL22RA1. A previous bioinformatic analysis of the cytokine IL22 showed that the predicted protein of the horse includes a 10 amino acid extension at the 3' end (Brosnahan et al., 2012). This was found to be present in other ungulates including the cow (Ma et al., 2010) and pig, but not in the human, mouse or dog (Brosnahan et al., 2012). A functional significance for the extension is unknown. The current bioinformatics analysis was undertaken to determine if structural changes peculiar to equids or ungulates might also be present in the receptor.

Materials and Methods

Animals

Mares used in this study were of various breeds and ages. Stallions used for insemination included two Thoroughbreds homozygous for equine leukocyte antigens (ELA) A2 or A3. Animals were housed in a pasture setting at the Equine Genetics Center at Cornell University. All procedures were performed in accordance with an animal care and use protocol approved by the Institutional Animal Care and Use Committee (IACUC) of Cornell University.

Breeding, Conceptus Recovery and Endometrial Biopsies

Mares were bred by artificial insemination using techniques previously described (Adams and Antczak, 2001). Equine conceptuses were recovered as previously described using a non-invasive uterine lavage technique (Antczak et al., 1987). Endometrial biopsies from pregnant

mares were obtained using biopsy forceps immediately following conceptus recovery. Biopsies using an identical procedure were also performed on non-pregnant animals. Tissues to be sectioned for in situ hybridization were embedded (O.C.T. Compound, Sakura, Torrance, CA) and stored at -80° C until sectioning. Tissues were sectioned at 6 μm onto RNAase free slides (Superfrost Plus, Fisher Scientific) within 24 hours of in situ hybridization stored at -80° C until final processing.

Generation and Labeling of RNA in situ Hybridization Probes

The primer sequences used to create the in situ hybridization probes to *IL22RA1* were developed using Geneious 6.1.2. (Table 6.1).

Table 6.1 Primer Sequences

Primer Set	Sequence	Product Length	Target Tissue	Anti-Sense Probe	Sense Probe
IL22RA1-F	CAACCTGACCGTGGAGACCG	585	Oral	SP6	T7
IL22RA1-R	GGCTGGAAGGTCAGGACACG		Mucosa		

Primers sets were used in a polymerase chain reaction (PCR) to amplify cDNA synthesized from the mRNA of an equine oral mucosa sample previously shown by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) to express high levels of *IL22RA1* mRNA. The PCR product was run out on 1.5% agarose. The appropriate sized band was excised and the amplification product was purified (QIAQuick Gel Extraction Kit). The purified product then was ligated into a pGEM-T Easy vector according to manufacturer's instructions. The ligation reaction product was transformed into competent *E. coli* and grown on LB-agar with ampicillin. Colonies were picked and grown overnight. Plasmids were isolated (QIAPrep Spin Mini Prep)

and inserts were sequenced by standard Sanger technology at the Cornell University Biotechnology Resource Center. One plasmid with the correct sequence was chosen to use as the in situ probe template. Plasmids were linearized and the labeled RNA probe was synthesized using a commercial kit (Dig RNA Labeling Kit (SP6/T7), Roche Applied Science, Penzberg, Germany).

In Situ Hybridization

In situ hybridization was based upon a methodology previously published (Rosello-Diez et al., 2011). The assay was performed over three days. On the first day slides were fixed in 4% paraformaldehyde (PFA), rinsed with phosphate buffered saline (PBS) and digested for 10 minutes in 10 ug/mL proteinase K in PBS. Slides were again fixed in PFA then treated with 0.1 N hydrochloric acid followed by 0.1 M triethanolamine and acetic anhydride at pH 8.0. Probes were diluted in hybridization solution (50% formamide, 5x saline sodium citrate (SCC) pH 5.5, 1x Denhardt's, 0.1% Tween 20, 0.1% Chaps, 50 ug/ML tRNA), applied to the slides, and incubated overnight in a humidifying chamber in a hybridization oven at 65°C. The following day the slides were rinsed first with a solution of 50% formamide, 5x SCC pH 5.5, 1% SDS in water followed by 50% formamide, 2x SCC pH 5.5, 0.2% SDS in water. Slides were washed in maleic acid, sodium chloride and 0.1% Tween-20 (MABT) and pre-blocked with MABT, 10% sheep serum and 1% blocking reagent for three hours. Slides were then incubated overnight with the anti-Dig-AP at 1:2,000 in MABT and 1% sheep serum, 1% blocking reagent.

On the final day, slides were washed with MABT followed by 100mM Tris HCl pH 9.5, 50 mM MgCl2, 100 mM NaCl, 0.1% Tween-20. Finally slides were incubated in developing solution

(10 mL NTMT with 5 uL nitrotetrazolium blue choride and 5 uL 5-bromo-4-chloro-3-indoyl phosphate disodium salt).

Bioinformatic Analysis

Coding sequences and predicted protein sequences for *IL22RA1* from horse, human, mouse, dog, pig and cow were obtained from the NCBI web site (http://www.ncbi.nlm.nih.gov/gene/). Genomic environment was determined using the UCSC Genome Browser (http://genome.ucsc.edu) and Ensembl (http://useast.ensembl.org). Analysis was conducted using Geneious 6.1.8 (Biomatters, Ltd, Auckland, NZ) and MEGA 5.2.2 (Tamura et al., 2011).

Results

Comparative Bioinformatic Analysis of IL22RA1

Previously a bioinformatic analysis of the cytokine IL22 showed that the predicted protein of the horse includes a 10 amino acid extension at the 3' end. This was found also in the cow (Ma et al., 2010) and pig, but not in the human, mouse or dog (Brosnahan et al., 2012). A functional significance for the extension is unknown. In this study a comparative bioinformatic analysis was performed on the predicted sequences of IL22RA1 from the horse and five other species to determine if notable differences exist. Predicted proteins varied across species in number of amino acids with 574 (human), 578 (dog), 580 (pig) and 581 (cow, horse, mouse). A seven amino acid region at positions 542 through 548 is present in all domestic species examined, but absent in the human (Figure 6.1, box). Nine of twelve amino acid residues determined to be critical for binding to IL22 (Bleicher et al., 2008) were identical across the six species (Figure 6.1, arrows). The horse IL22RA1 protein was most similar to the dog with a percent sequence

identity of 78.1%. The horse protein was least similar to the mouse with a percent sequence identity of 67.5% (Table 6.2). A tree diagram further illustrates the relationships among the horse IL22RA1 protein sequence and the IL22RA1 sequences of five other species (Figure 6.2). The predicted secondary structure of the horse IL22RA1 protein is shown in Figure 6.3.

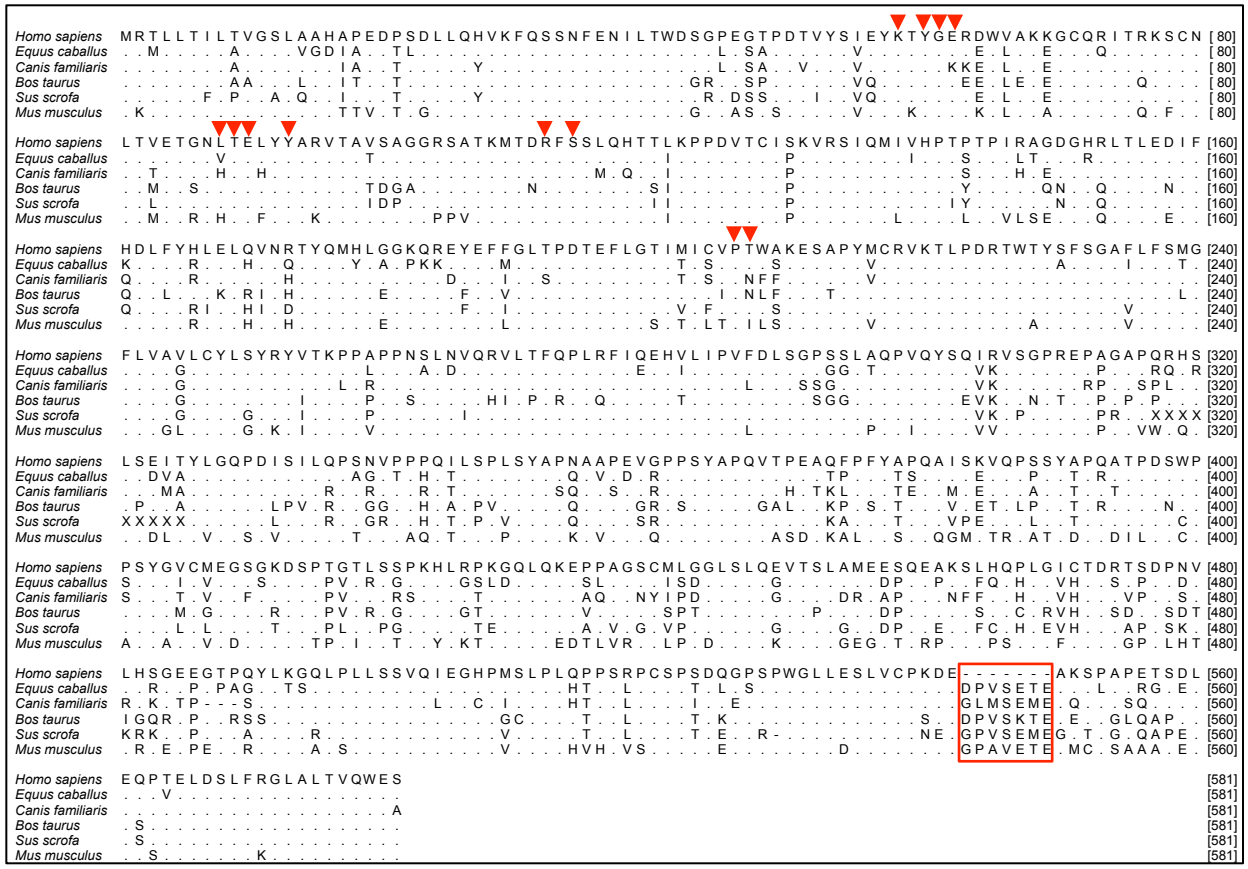

Figure 6.1 Comparative alignment (ClustalW) of IL22RA1 from the domestic horse and five other species. Amino acids from position 542 through 548 are shown within the red box. These seven amino acids are present in all species examined, but not the human. Red triangles indicate critical residues involved in the IL22/IL22RA1 binding site as defined in Bleicher, et al 2008. Consensus is indicated by "." and "-" indicates a gap. (*Equus caballus* RefSeq: XP_001501338.2; *Bos taurus* RefSeq: NP_001029483.1; *Sus scrofa* RefSeq: XP_003360894.1; *Homo sapiens* RefSeq: NP_067081.2; *Mus musculus* RefSeq: NP_839988.1; *Canis lupus familiaris* RefSeq: XP_855113.2) Analysis performed with MEGA5.2 and Geneious 6.1.8.

Table 6.2 Percent Sequence Identity of IL22RA1 Across Species

	Equus caballus	*Bos taurus*	*Sus scrofa*	*Homo sapiens*	*Mus musculus*	*Canis familiaris*
Equus caballus		73.0	75.5	77.3	67.5	78.1
Bos taurus	73		76.5	73.3	64.2	72.6
Sus scrofa	75.5	76.5		76.3	68.7	75.8
Homo sapiens	77.3	73.3	76.3		71.9	77.8
Mus musculus	67.5	64.2	68.7	71.9		68.3
Canis familiaris	78.1	72.6	75.8	77.8	68.3	

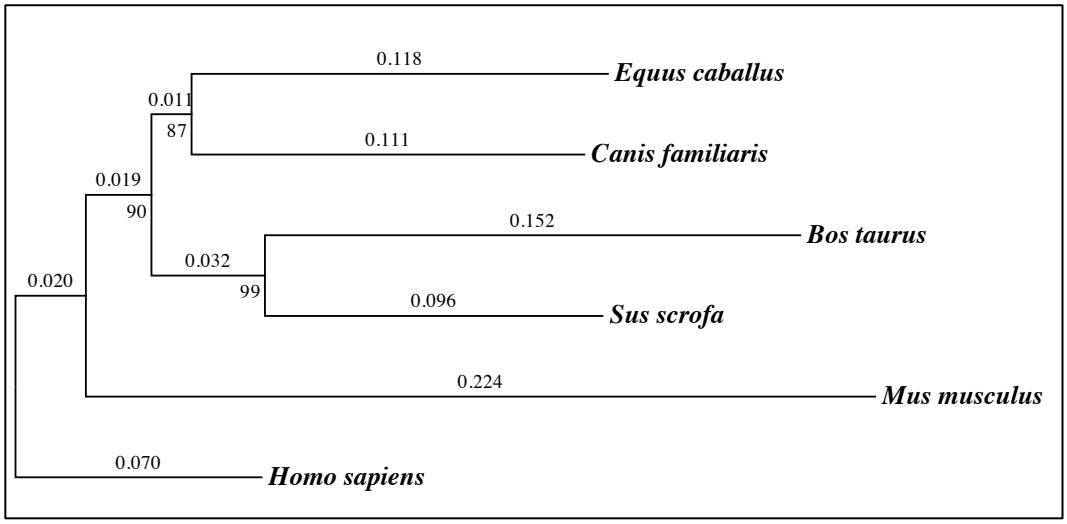

Figure 6.2 Tree diagram depicting the relationship between the horse IL22RA1 protein sequence and IL22RA1 from selected other species. The evolutionary history was inferred using the Neighbor-Joining method rooted on the human. The optimal tree with the sum of branch length = 0.85266926 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The tree is drawn to scale. Branch lengths (next to the branches) represent evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid differences per site. All positions containing gaps and missing data were eliminated. There were a total of 561 positions in the final dataset.

Figure 6.3 Predicted secondary structure of horse IL22RA1. The predicted secondary structure of horse IL22RA1 determined by analysis with Geneious 6.1.8. Legend:

= α-helix, = β-sheet, = turn, = coil.

The gene encoding horse IL22RA1 is located on ECA2 and contains seven coding exons. The gene is flanked by genes encoding an interferon lambda receptor and myomesin 3, a protein found in striated muscle tissue (Figure 6.4).

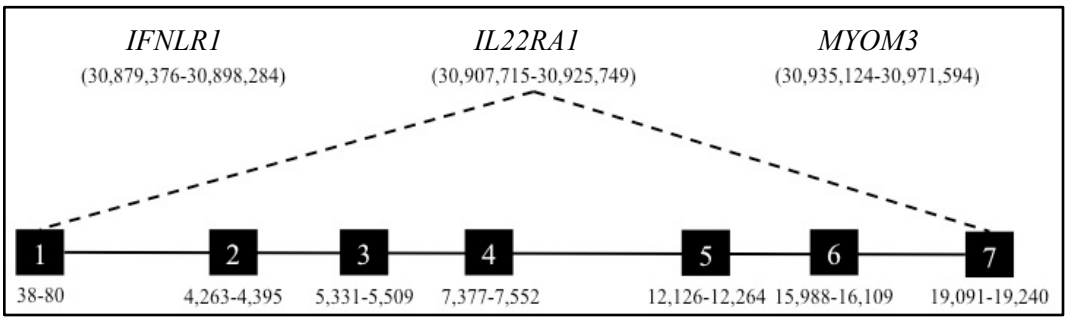

Figure 6.4 Exon structure and genomic landscape of *IL22RA1* on equine chromosome 2. The horse *IL22RA1* gene is located on equine chromosome 2 and contains 7 coding exons. This diagram shows the position of the *IL22RA1* genomic sequence on chromosome 2 and distribution of the coding sequence across exons (bottom numbers). Neighboring genes include IFNLR1 and MYOM3. This same genomic environment is seen in other species included in the analysis.

IL22RA1 mRNA localizes to both the luminal and glandular epithelia of the mare's uterus

IL22RA1 previously was identified by qRT-PCR in both mare endometrium as well as several other equine epithelial tissues (Brosnahan et al., 2012). Unlike the chorionic girdle tissue that can be obtained in pure form from day 32 through day 35, endometrial tissue obtained by biopsy contains multiple cell types. Therefore, mRNA extracted from this tissue is a pooled sample from all cell types present. In order to refine our qRT-PCR finding of *IL22RA1* mRNA in mare endometrium, we performed in situ hybridization to localize the receptor to specific cell types. The *IL22RA1* anti-sense probes hybridized to both the luminal and glandular epithelia of the equine uterus. Hybridization was more intense on endometrium from the day 35 endometrial biopsy (Figure 6.9 A, B, D) as compared to non-pregnant endometrium (Figure 6.9 C, E). The *IL22RA1* sense probes did not hybridize to endometrial tissue (Figure 6.9 F).

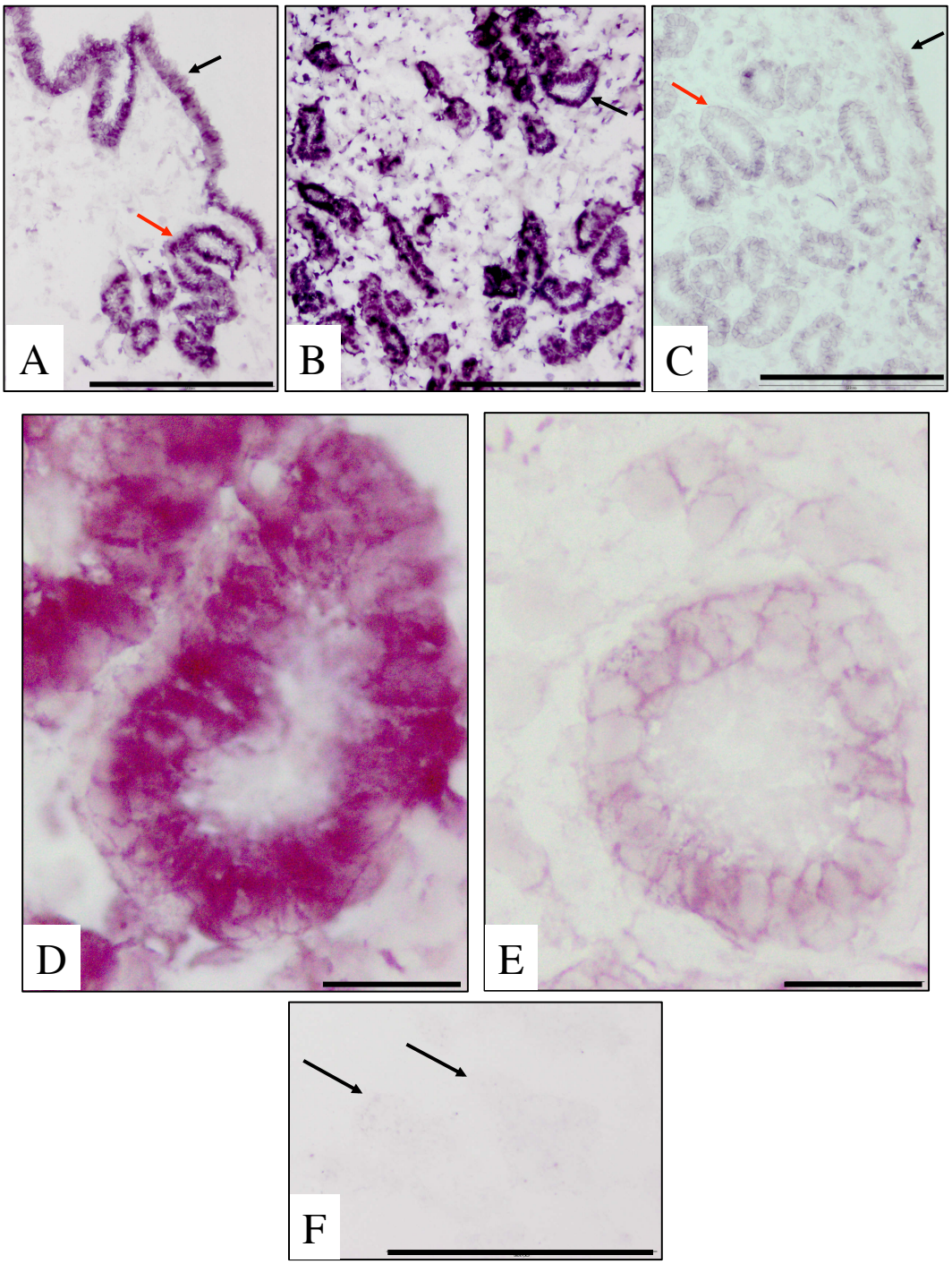

Figure 6.9 In situ hybridization of *IL22RA1* mRNA in pregnant and non-pregnant endometrium. Endometrial biopsies taken on day 35 of pregnancy show very strong hybridization to the *IL22RA1* anti-sense probes (A) at both the luminal (black arrow) and glandular (red arrow) epithelia. Endometrial glands are seen more closely in (B). Non-pregnant endometrium (C) shows a much less intense signal. High power image of an endometrial gland from day 35 (D) and non-pregnant (E) endometrium, again showing much more intense hybridization in the pregnant endometrium. *IL22RA1* sense probes did not hybridize to the day 35 endometrium. Arrows indicates tissue outline (F). Bar = 200uM (A, B, C, F) or 20 uM (D, E).

Discussion

The primary objective of this study was to refine the findings of a previous investigation of *IL22RA1* in the endometrium of the horse (Brosnahan et al., 2012) by localizing *IL22RA1* mRNA to specific cell populations within this tissue. In situ hybridization identified *IL22RA1* mRNA in both the luminal and glandular epithelia of the mare's endometrium, with more intense hybridization to endometrium biopsied from a day 35 pregnancy as compared to non-pregnant endometrium.

Early investigation of the structure and function of the chorionic girdle cells describes their very invasive, destructive nature. At approximately day 36, the chorionic girdle cells attach to the luminal surface of the endometrium and phagocytose through the epithelium. They penetrate the basal lamina, and proceed to invade the endometrial stroma where they terminally differentiate to form the eCG-producing endometrial cups (Allen et al., 1973). In biologic systems involving IL22 produced by immune cells, the cytokine binds receptor IL22RA1 on target epithelial cells. This may result in epithelial cell proliferation, differentiation and migration, mechanisms that repair damage and preserve the integrity of the mucosal barrier. It also may result in upregulation of antimicrobial molecules such as defensins (Rutz et al., 2013; Wolk et al., 2006). The presence of *IL22RA1* mRNA in abundance on the luminal epithelium just at the time of chorionic girdle cell invasion suggests that one possible role of IL22 in horse pregnancy may be to ensure integrity of the endometrial epithelium. Another possible function would be to enhance immune defenses at the maternal – fetal interface by initiating production of molecules such as defensins that provide antimicrobial activity. Antimicrobial peptides including beta-

defensins have been identified at the maternal – fetal interface in humans. These are thought to play a role in protection from intrauterine infection during pregnancy (Kai-Larsen et al., 2014).

Historical observations on the biology of the chorionic girdle also showed that as the girdle cells invade the stroma, they travel in association with the endometrial gland epithelium but without the pattern of phagocytosis and destruction evident at the luminal epithelium (Allen et al., 1973). It is clear from the current set of experiments that *IL22RA1* mRNA is present in abundance in the glandular epithelia in pregnant endometrium. The presumed absence of damage to the glandular epithelia suggests that the action of chorionic girdle IL22 upon the endometrial glands may be different than on the luminal epithelium. In most mammals, but especially in species such as horses, pigs, cows and sheep that experience delayed implantation, endometrial gland secretions are critical for the well-being of the developing conceptus (Gray et al., 2001). In the horse, endometrial glands in proximity to the endometrial cups are observed to experience extensive hypertrophy (Enders et al., 2000). Around the time of chorionic girdle invasion most endometrial glands begin to secrete large amounts of proteins including uteroferrin (Ellenberger et al., 2008). IL22 may be involved in endometrial glandular hypertrophy as it is known to be involved in cellular proliferation. Alternatively, IL22 may be involved in initiating some of the glandular secretions. One study has shown that migrating trophoblast cells have a proliferative effect on endometrial glands in close proximity, and that the nature of secretions from these glands is different than glands more distant from the endometrial cups (Enders et al., 2000).

A comparative bioinformatic analysis provided some additional insight into the biology of IL22RA1. One observation made in this analysis was that a length of seven amino acids is

absent in the human but present in all other species examined. The significance of this is uncertain, but it does not appear to involve the binding interface between IL22 and IL22RA1 that has been identified by other researchers (Bleicher et al., 2008).

One group of researchers has identified the amino acid residues making up the interface between the extracellular binding domain of human IL22RA1 and IL22 cytokine. Of twelve IL22RA1 amino acid residues these researchers found to be directly involved in binding (Bleicher et al., 2008), the analysis performed here showed that eight of these were identical in all species examined. In the horse, 11 of the 12 critical residues were identical to the human with the one exception being a valine substitution for the leucine at position 88. The dog and mouse amino acids also differed at this position, with both possessing a histidine residue. The dog differed from all other species at position 62, and the cow, mouse and dog differed at position 207. This same group of researchers determined that 13 amino acid residues on the IL22 protein are involved in the receptor binding interface. Eleven of the 13 amino acids were conserved across all species examined. At position 53, the horse, mouse and cow differ from the human, whereas the pig and dog are identical to the human. At position 175, the horse, cow and pig possess a lysine in place of an arginine. This analysis suggests that although there appears to be a structural difference in the IL22 cytokine between ungulates and other organisms, the nature of the binding interface between IL22 and IL22RA1 is conserved across species.

The genes in proximity to *IL22RA1* in the horse were identical to other species. IL22RA1 is a type two cytokine receptor. In all species examined *IL22RA1* is located in proximity to one other receptor in this class, the interferon lambda receptor (also referred to as *IL28RA*). Interferon

lambda is a largely pro-inflammatory cytokine (Renauld, 2003). Also in proximity to *IL22RA1* is the gene encoding myomesin 3, a recently described protein identified in striated muscle (Schoenauer et al., 2008).

This study identified the cell types of the horse endometrium that express transmembrane receptor *IL22RA1* using in situ hybridization. These include both the luminal and glandular epithelia of pregnant and non-pregnant endometrium, with receptor mRNA greater in pregnant as compared to non-pregnant endometrium. This data set, though small, supports the hypothesis that IL22 secreted by the chorionic girdle binds IL22RA1 on the maternal endometrium to repair the damage that occurs secondary to trophoblast migration. Unexpectedly, *IL22RA1* mRNA was also identified in the glandular epithelium, expanding the initial hypothesis to include an additional role for IL22 of chorionic girdle origin. IL22 produced by the chorionic girdle may also stimulate proliferation of endometrial glands in preparation for full placentation. A comparative bioinformatic analysis of the primary and secondary structures of IL22RA1 showed that they are similar to other domestic species in regions that are critical to the binding interface between IL22 and IL22RA1. Differences between these domestic species and the human do not appear to involve the binding interface.

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CHAPTER 7

IDENTIFICATION OF INTERLEUKIN 22 PROTEIN

IN THE CHORIONIC GIRDLE

Abstract

Interleukin 22 (IL22) is a member of the interleukin 10 family of cytokines and is involved in the defense and repair of epithelial surfaces. This cytokine is secreted by helper T-cell subpopulations and natural killer (NK) cells in many species. Previous research in the horse provided the first molecular evidence for *IL22* expression by a non-immune cell type: the invasive trophoblast of the chorionic girdle. To identify IL22 protein in the invasive trophoblast cells a monoclonal antibody was generated against horse IL22. Mice were immunized with a recombinant fusion protein of equine IL4 and IL22 and splenic lymphocytes were fused with SP 2/0 myelomma cells to generate hybridoma cell lines. Clones were screened using two ELISA assays and one clone with reactivity to IL22 but not IL4 was identified. Specificity of the monoclonal antibody to the IL22 component of the fusion protein was verified via additional flow cytometry, immunohistochemistry and ELISA assays. The ability of the monoclonal antibody to bind native equine IL22 was demonstrated by flow cytometry and by immunohistochemistry on cytospins of resting and stimulated equine peripheral blood mononuclear cells (PBMCs). Finally, immunohistochemistry using the monoclonal antibody on frozen sections of equine chorionic girdle recovered from day 35 of pregnancy identified IL22 protein in this tissue.

Introduction

Interleukin 22 (IL22) is an interleukin 10 family cytokine involved in the defense and repair of epithelial surfaces (Wolk et al., 2010). Produced primarily by cells of the immune system, including T-helper cells (Th17 and Th22) and NK cells, IL22 plays a role in normal host responses to infectious (Aujla et al., 2008), toxic (Schieermann et al., 2013) and mechanical (Hoegl et al., 2010) insults. IL22 binds two receptors. Transmembrane receptor IL22R1 is expressed on various epithelial cell types (Kotenko et al., 2001). Soluble receptor IL22R2 is less well described, but has been associated with macrophage function in an experimental model of multiple sclerosis (Beyeen et al., 2012) and is produced by dendritic cells (Martin et al., 2014). Dysregulation of IL22 has been implicated in conditions such as psoriasis (Van Belle et al., 2012; Wolk et al., 2006) and graft-versus-host disease (Hanash AM et al., 2012). Research in laboratory animals suggests that manipulation of the IL22 pathway has potential for therapeutic application (Sabat et al., 2014).

Investigation of IL22 in species other than humans and mice has been limited. Researchers have identified IL22 production in bovine gamma delta T-cells (Ma et al., 2010) and a role for IL22 has been suggested in the host response to bovine tuberculosis (Aranday-Cortes et al., 2012). Previous research using molecular techniques has identified *IL22* expression in a novel cell type in the horse, the invasive trophoblast of the chorionic girdle. *IL22* mRNA is rapidly upregulated in this tissue just prior to its invasion of the maternal endometrium (Brosnahan et al., 2012).

The objective of this study was to produce an antibody specific to equine IL22 to document the presence of IL22 protein in the chorionic girdle. This paper describes the generation and

characterization of a monoclonal antibody against equine IL22 using a method previously described (Wagner et al., 2012). This antibody did enable us to identify IL22 protein in the equine chorionic girdle, where we previously had found molecular evidence of *IL22* gene transcription. Availability of an anti-horse monoclonal antibody to IL22 will enable ongoing investigation of the role of IL22 in normal equine immunity and in the equine response to disease.

Materials and Methods

Animals

The Cornell University Institutional Animal Care and Use Committee (IACUC) approved all animal procedures performed in this study. Horses used as donors for equine conceptus tissue and peripheral blood mononuclear cells (PBMCs) belonged to the Cornell University Equine Genetics Center. Horses were maintained on pasture in a herd setting. Balb-c mice were used for the generation of the monoclonal antibody. The mice were housed in the rodent facility at the Baker Institute for Animal Health.

Conceptus Tissue Preparation

Mares were bred by artificial insemination to one of two Thoroughbred stallions using techniques previously described (Adams and Antczak, 2001). Equine conceptuses were recovered at day 35 of pregnancy using non-invasive uterine lavage as previously described (Antczak et al., 1987). Chorionic girdle then was isolated from the conceptus by gross dissection. Tissue to be sectioned for immunohistochemistry was embedded (O.C.T. Compound, Sakura, Torrance, CA) and frozen in isopentane chilled in liquid nitrogen. All tissues were

stored at -80° C until processing. Slides (Superfrost Plus, Fisher Scientific) were prepared with tissue sectioned at 6 μm, fixed for 10 minutes in cold acetone, and stored at -20° C until processing.

Cloning of Equine IL22 and Recombinant Protein Production

Polymerase chain reaction (PCR) primers were designed to amplify the full length coding sequence of equine *IL22*. Primer sequences (Table 7.1) were designed and cloned sequences were analyzed using Geneious 5.6.4 (Biomatters, Auckland, NZ). mRNA was isolated from a day 35 chorionic girdle using commercial kits (Qiashredder; RNeasy, Qiagen, Valencia, CA). First strand cDNA synthesis was performed using M-MLV Reverse Transcriptase (USB, Cleveland, OH) following the manufacturer's protocol. The target sequence was amplified using Pfu DNA polymerase (Stratagene, LaJolla, CA) in a 25 uL PCR reaction (MasterCycler, Eppendorf North America, Hauppauge, NY). The PCR product was gel purified and the extracted product ligated into a pCR4Blunt-TOPO vector (T4 DNA Ligase, Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instruction. The vector was transformed into competent *E. coli* and plated on LB-agar with ampicillin. Individual colonies were grown overnight in LB with ampicillin, plasmid DNA was isolated (QiaPrep Spin Miniprep 27106, Qiagen) and inserts were sequenced by the Cornell Biotechnology Resource Center using standard Sanger technology. A second round of PCR amplification was performed on the plasmid insert excluding the signal sequence and creating BamHI and KpnI overhangs. The PCR product was ligated into a pCR4Blunt-TOPO vector and processed as before, digested with BamHI and KpnI (Invitrogen Life Technologies, Carlsbad, CA), and ligated into a pcDNA3.1 vector (Invitrogen Life Technologies, Carlsbad, CA) downstream of a previously inserted equine

IL4 coding sequence (Wagner et al., 2012). Plasmid was amplified and isolated as before, and linearized using PVUI (New England Biolabs, Ipswich, MA 01938).

Table 7.1 – Primer Sequences

Primer	5' – 3' Sequence	Product
IL22F	ATGGCCACCCTGCAGAAAT	570 bp
IL22R	TTAGTTATCCATTTTCCAGCTTTTCTC	
IL22BamHI-F	GCGGGATCCTGCGCCCCTTGGCTCTCACTGC	484 bp
IL22KpnI-R	GCGGGTACCTTAGTTATCCATTTTCCAGCTTTTC	

Stable Transfection of CHO Cells and Protein Purification

CHO K-1 cells were transfected with the linearized plasmid containing the coding sequence for a recombinant equine IL4/IL22 fusion protein. The transfection was performed using a commercially available kit (GenePorter, Genlantis, San Diego, CA) according to manufacturer's instructions. Cells were selected in culture using G418 (Invitrogen Life Technologies, Carlsbad, CA) and cloned by limiting dilution. Clones were screened for production of the IL4/IL22 fusion protein using ELISA and flow cytometry as previously described (Wagner et al., 2012). The fusion protein was collected using fast protein liquid chromatography with an IL4 affinity column, concentrated by centrifugation and quantified using a BSA Protein Assay kit (Pierce BCA Protein Assay Kit, Bovine Serum Albumin Standards, Thermo Scientific, Rockford, IL 61101). The molecular weight of the purified fusion protein was determined by SDS-PAGE on a 10% non-reducing polyacrylamide gel.

Mouse Immunizations

Immunizations were performed as previously described (Wagner et al., 2003). Briefly, mice were immunized with 50ug of rIL4/IL22 (1.1 ug/uL) by intraperitoneal injection on day 0, then boosted with 25 ug on days 14, 21, 28, 29 and 30. Immunizations on days 0, 14 and 21 contained adjuvant (Gerbu Adjuvant MM, Gerbu Biotechnik, Heidelberg, Germany) at a 1:1 volume ratio. Response to immunization was confirmed by serum titers to equine IL4 using an ELISA assay. Mice were euthanized on day 31.

Myeloma Cell Fusion

Splenic cells were fused with SP2/0 myeloma cells as previously described (Appleton et al., 1989). Briefly, following euthanasia of the mouse the spleen was removed, minced and passed through a sieve. SP2/0 cells and splenic lymphocytes were combined at a 1:2 ratio. The cell mixture was swirled in a beaker of warm water and 1 mL polyethylene glycol and $NaHCO_3$ was added over one minute. A 5 mL aliquot of warm DMEM (DMEM, pen/strep, gentamicin, glutamine, NEAA, Na-PY, 2-ME) was added over three minutes, then a 14 mL aliquot over 1 minute. The volume was brought to 50 mL and cells were rested for five minutes and then pelleted. Cells were diluted to 2×10^6 cells/mL in the DMEM medium with 20% FBS and HAT. Cells were allowed to settle for 5 minutes and incubated at 37° C overnight. Cells were wrapped in plastic wrap after 24 hours.

Hybridoma Cell Screening

Plates were examined for visible clones beginning at ten days post-fusion. Hybridoma cell culture supernatants were screened by ELISA for monoclonal antibody production against the

desired portion of the fusion protein as previously described (Wagner et al., 2012; Wagner et al., 2005). Clones that recognized rIL22/rIL4 but not rIL4/IgG were selected for further study. Three rounds of limiting dilution ensured the monoclonal nature of the clones.

Isolation and Labeling of Monoclonal Antibody

The desired monoclonal antibody was isolated from hybridoma supernatant using a Protein G column (AKTA FPLC, GE Healthcare Bio-Sciences, Pittsburgh, PA). Sample was eluted using a 0.1 M glycine-HCl buffer at pH 2.7. Purified protein was then dialyzed in PBS at 4° C with solution changes every two hours for six hours, then overnight. Aliquots of purified protein were labeled with commercial AlexaFluor 647 (Invitrogen A-20006, Carlsbad, CA) and biotinylation (Ez-Link Sulfo-NHS-Biotin, Thermo Scientific, Rockford, IL) kits according to manufacturer's instructions.

Isolation, Stimulation and Preparation of Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMCs) to be used for flow cytometry were isolated from heparinized jugular venous blood using a density centrifugation technique previously described (Antczak et al., 1982). Cells were suspended at a concentration of 3×10^6 cells/mL then stimulated with pokeweed mitogen (PWM) at 2.5 µg/ml for 24 hours with restimulation for the final four hours using phorbol myristate acetate (PMA) at 25 ng/mL, ionomycin (IO) at 1 µM and brefeldin A at 10 µg/mL to inhibit IL22 secretion (Dr. A. deMestre, personal communication). Cells to be tested via qRT-PCR for the presence of *IL22* mRNA were stimulated with each protocol individually (Flaminio and Antczak, 2005; Wagner et al., 2006).

Pokeweed stimulation was performed in 45% AIMV, 45% RPMI, 10% (v/v) fetal calf serum, 1% (v/v) non-essential amino acids, 2 mM L-glutamine, 50 μM 2-meercaptoethanol, 0.5% (v/v) sodium pyruvate, 1% (v/v) penicillin/streptomycin. Stimulation with PMA/IO was performed in DMEM with 10% (v/v) fetal calf serum, 1% (v/v) non-essential amino acids, 2 mM L-glutamine, 50 μM 2-mercaptoethanol and 50 μg/ml gentamicin.

Cells were harvested for immediate use in flow cytometry or immunohistochemistry or pelleted and frozen at -80°C for subsequent mRNA extraction. Cytospin slides (Superfrost Plus, Fisher Scientific) for immunohistochemistry were prepared by centrifugation of 50 uL PBS with 50,000 cells. Slides were fixed for 10 minutes in cold acetone, and stored at -20° C until processing.

RNA Isolation and cDNA Synthesis

RNA was isolated from PBMCs using commercial kits (Qiashredder; RNeasy, Qiagen, Valencia, CA). RNA samples isolated with the RNeasy kit were treated with DNase I (Invitrogen, Carlsbad, CA). First strand cDNA synthesis was performed using M-MLV Reverse Transcriptase (USB, Cleveland, OH) following the manufacturer's protocol.

CHO Cell Preparation for Immunohistochemistry and Flow Cytometry

CHO K-1 cells for use in flow cytometry were harvested by trypsinization with 0.05% trypsin. Cells were rinsed in medium (HAMs with 5% FBS) and then with phosphate buffered saline. CHO K-1 cells used for immunohistochemical staining were first cultured in the presence of brefeldin A (10 ug/ml) for four hours. CHO K-1 cells used as controls included an untransfected population, and a population transfected with a vector containing the coding sequence for an

rIL4/IL22R2 fusion protein. Cytospin slides (Superfrost Plus, Fisher Scientific) for immunohistochemistry were prepared by centrifugation of 50 uL PBS containing 25,000 cells. Slides were fixed for 10 minutes in cold acetone, and stored at -20° C until processing.

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR) Assays

Quantification of *IL22* mRNA used reagents and techniques previously described (Brosnahan et al., 2012). Briefly, the *IL22* qRT-PCR assays used SYBR Green technology (Applied Biosystems, Foster City, CA) run on an Applied Biosytems 7500 Fast Real Time PCR instrument. Standard curves were generated using known copy numbers of a plasmid with a target gene-containing fragment.

Immunohistochemistry

All steps took place at room temperature in a humidity chamber. Slides were incubated with primary antibody diluted in TBS (0.5M Tris base, 1.55M NaCl) for 30 minutes and then washed three times in TBS. Slides were blocked for 15 minutes with 10% normal goat serum. Second stage antibody (HRP conjugated goat anti mouse IgG, Jackson ImmunoResearch) was diluted with 10% normal goat serum and incubated for 30 minutes. Slides were again washed three times in TBS and incubated for 25 minutes using AEC RED Substrate Kit (Invitrogen). Slides were rinsed in TBS and then water, and counterstained for 5 minutes with hematoxylin. Coverslips then were mounted (Glycergel, Dako, Agilent Technologies, Santa Clara, CA) and slides read.

Flow Cytometry Preparation

After collection cells were rinsed in PBS and diluted to 1 x 10^6 cells/mL. One half mL of cells (500,000 cells) and an equal volume of 4% formalin were incubated for 20 minutes at room temperature. The cells were rinsed twice in PBS and resuspended with antibody diluted in flow cytometry buffer (PBS, 0.5% BSA, 0.02% NaAz; 1% saponin added for intracellular staining). Cells were incubated with antibody for 15 minutes at room temperature, rinsed twice, and resuspended in flow cytometry buffer (without saponin) until processing. The flow cytometer used for all of these experiments was a Beckman-Coulter Gallios.

Results

Generation of a monoclonal antibody to equine interleukin 22 was accomplished using a previously published approach based upon the creation of a recombinant fusion protein (Wagner et al., 2012). The coding sequence of *IL22* was ligated into a vector already containing the coding sequence of equine *IL4*, and the resultant fusion protein was expressed and secreted by CHO K-1 cells (Figure 7.1 A). The protein was purified from cell culture supernatant using an IL4 affinity column. An SDS PAGE gel determined the molecular weight of the fusion protein to be consistent with the expected molecular weight of approximately 40kDa (Figure 7.1 B). Mice were inoculated with the fusion protein, and following euthanasia on day 31 splenic lymphocytes were recovered. These cells were fused with SP2/0 myeloma cells and cloned by limiting dilution.

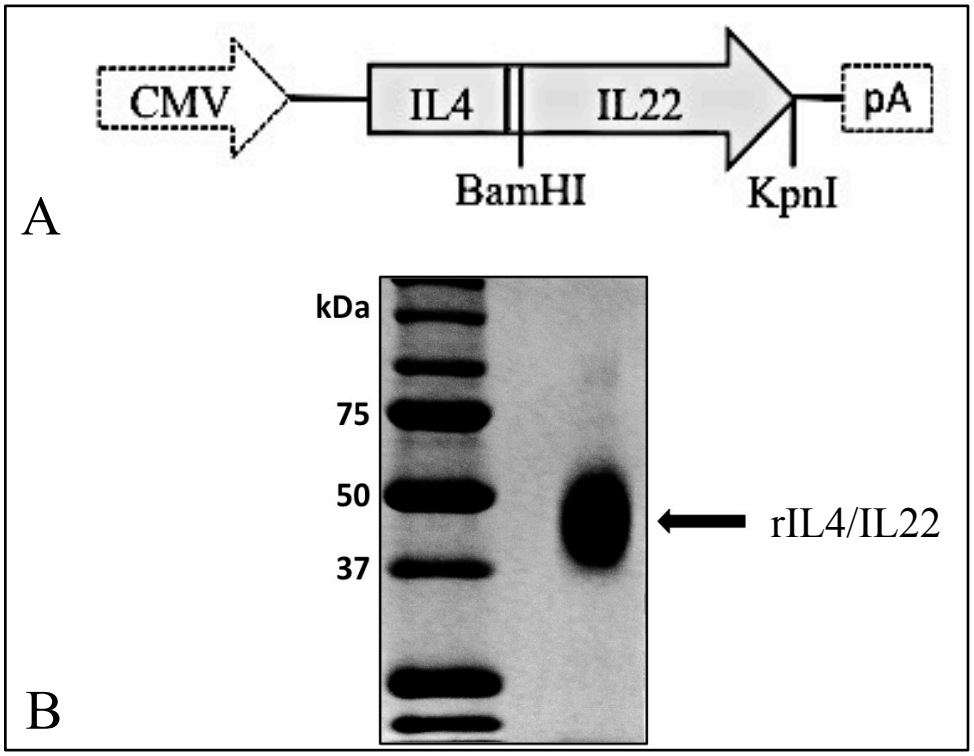

Figure 7.1 Generation and expression of the rIL4/IL22 fusion protein. A) Schematic of the pcDNA 3.1 vector that expressed the equine rIL4/IL22 fusion protein. B) SDS-PAGE gel showing the molecular weight of the fusion protein to be consistent with the expected weight of approximately 40kDa.

When a hybridoma clone colony became grossly visible within a well, hybridoma supernatant was screened to detect the presence of IL22-specific antibody using two ELISA assays (Figure 7.2 A). Briefly, the first ELISA used a goat anti-equine IL4 capture antibody to bind the rIL4/IL22 fusion protein, which in turn binds all antibodies to either component of the fusion protein. Hybridoma supernatants producing a positive (colorimetric) result were then screened on the second ELISA. This assay utilized a goat anti-equine IgG capture antibody to bind a rIL4-IgG fusion protein, which then produced a positive result only on hybridoma supernatants containing antibodies to the IL4 component of the rIL4/IL22 fusion protein. Supernatants with a negative result on the second ELISA were considered to contain anti-equine IL22 antibodies.

One clone specific to the IL22 portion of the fusion protein was identified.

One clone identified as IL22 mAb 15.27 was strongly positive on the first screening ELISA for rIL4/IL22 but not on the second ELISA targeting rIL4/IgG, identifying it as a monoclonal antibody to the IL22 portion of the fusion protein (Figure 7.2 B). The antibody was determined to be an IgG1 isotype (Mouse Monoclonal Antibody Typing Reagents, #098K4823, Sigma-Aldrich, St. Louis, MO). The clone was expanded, and affinity purification of 250 mL of hybridoma supernatant produced 2 mL of mAb 15.27 with a concentration of 3 mg/ml (Pierce BCA Protein Assay Kit, Bovine Gamma Globulin Standards, Thermo Scientific, Rockford, IL). Stocks of hybridoma supernatant and purified mAb were titrated for anti-IL22 activity (Figure 7.3). This affinity-purified antibody was used in subsequent experiments either in purified, unconjugated form or conjugated to a fluorophore or to biotin.

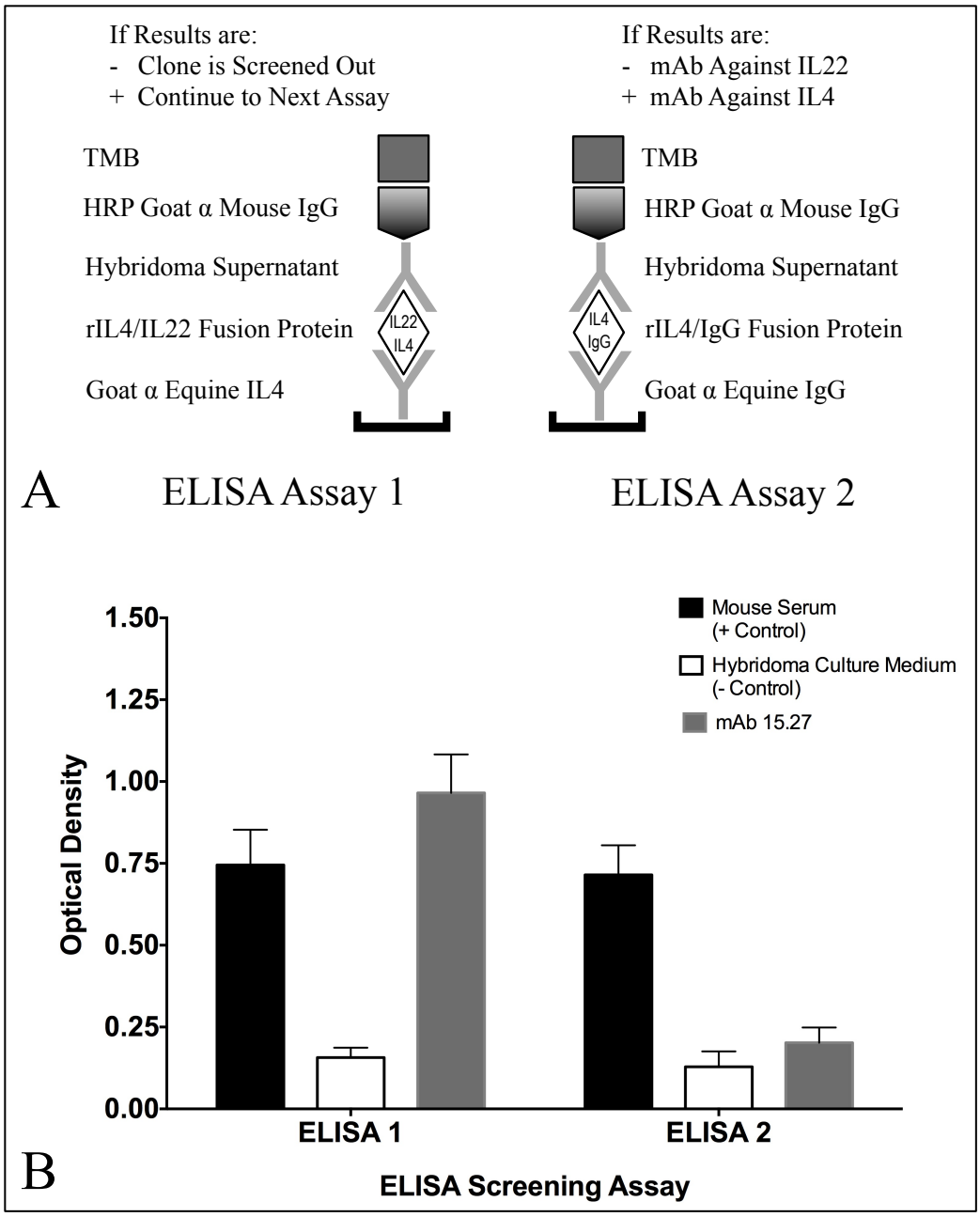

Figure 7.2 Hybridoma screening and identification of anti-equine IL22 monoclonal antibody 15.27. A) Schematic of the two screening ELISAs and possible outcomes. Supernatants with a positive colorimetric result on the first assay and a negative result on the second assay were considered to contain antibodies against equine IL22. B) ELISA results on hybridoma supernatant from clone 15.27 showing strongly positive results on the screening ELISA recognizing equine rIL22/IL4 but not on the ELISA recognizing equine rIL4/IgG. This pattern confirms clonal specificity for the IL22 portion of the fusion protein. Results show the mean of five separate ELISAs. (mAb = monoclonal antibody TMB = tetramethylbenzidine HRP = horseradish peroxidase).

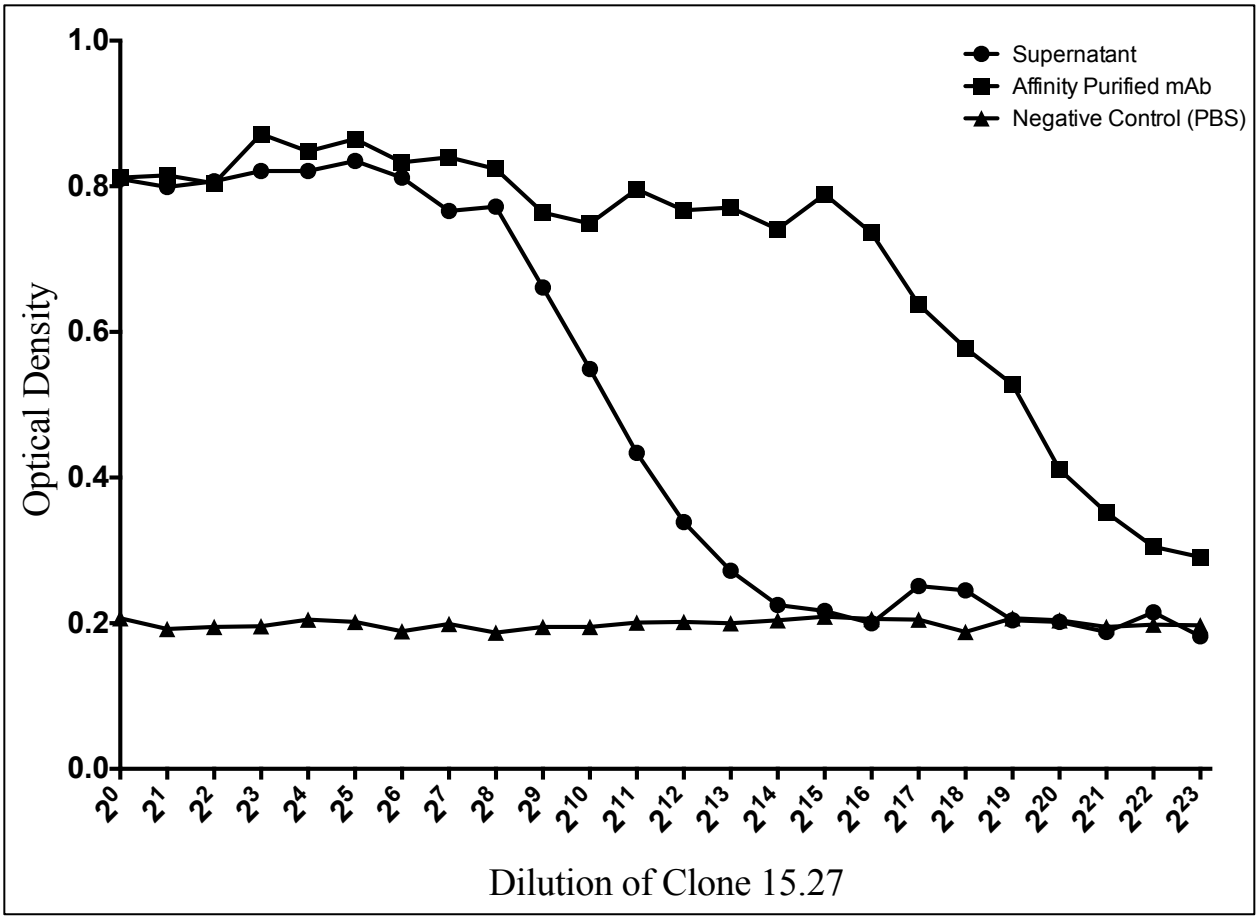

Figure 7.3 Titration of IL22 activity of clone 15.27 hybridoma supernatant and affinity purified antibody. Serial dilutions of hybridoma supernatant (circles) and affinity-purified antibody (squares) were evaluated for anti-IL22 activity using the rIL4/IL22 ELISA. Phosphate buffered saline was used as a negative control (triangles) and as diluent. The affinity-purified antibody (3 mg/mL) was used in subsequent experiments.

Clone 15.27 binds the rIL22/IL4 fusion protein, but not an rIL22RA2/IL4 fusion protein

The initial screening ELISAs identified clone 15.27 as an antibody with affinity to the IL22 and not the IL4 component of the fusion protein. To further test this affinity clone 15.27 was utilized in immunohistochemistry and flow cytometry assays using the rIL4/IL22 transfected CHO K-1 cells as the target. CHO K-1 cells transfected to secrete a different recombinant horse protein (rIL22RA2/IL4) were used as control targets. On flow cytometry, clone 15.27 bound to greater than 90% of CHO cells containing the rIL4/IL22 fusion protein, while an isotype control

antibody against horse NKp46 did not bind to these cells (Figure 7.4A). Flow cytometry was repeated using CHO cells secreting the rIL22RA2/IL4 fusion protein. Neither the anti-NKp46 antibody nor clone 15.27 bound to these cells. An antibody to horse IL22RA2 generated against the rIL22RA2/IL4 fusion protein did bind to these cells (Figure 7.4B). On immunohistochemistry, clone 15.27 bound to cells secreting rIL4/IL22 (Figure 7.4 C) but not untransfected CHO cells (Figure 7.4D) or cells secreting the rIL22RA2/IL4 fusion protein (Figure 7.4E). An isotype control antibody specific for equine trophoblast did not bind the rIL4/IL22 CHO K-1 cells (Figure 7.4 F).

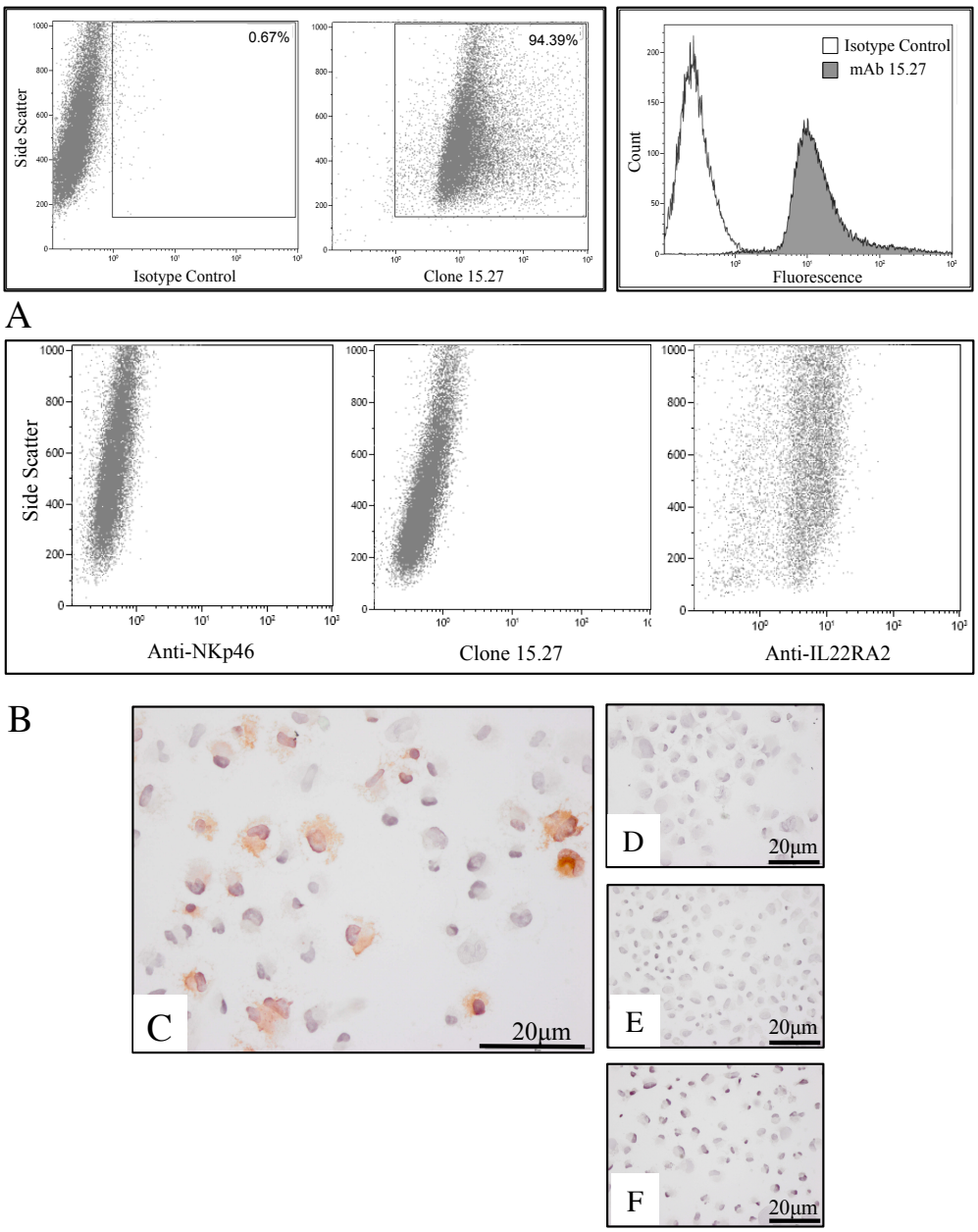

Figure 7.4 Testing of clone 15.27 using flow cytometry with directly labeled antibodies and immunohistochemistry using an amnioethyl carbazole-based protocol. On flow cytometry (A) an isotype control against horse NKp46 labeled with Alexa647 did not bind to CHO cells secreting the rIL22/IL4 fusion protein, while Alexa 647 conjugated clone 15.27 bound to 94% of these cells. Flow cytometry then was performed using the CHO cells stably transfected to secrete an IL22RA2/IL4 fusion protein (B). The anti horse NKp46 antibody did not bind to these cells, nor did clone 15.27. An anti-horse IL22RA2 antibody generated against the fusion protein secreted by these CHO cells did bind to them. Immunohistochemistry was then performed on cytospins of both types of transfected CHO cells along with untransfected cells. Clone 15.27 is shown binding to CHO K-1 cells containing the rIL4/IL22 pcDNA 3.1 vector (C) but not to vector-free CHO K-1 cells (D) or CHO K-1 cells secreting the rIL22RA2/IL4 fusion protein (E). An isotype control specific to equine trophoblast failed to bind the rIL4/IL22 CHO K-1 cells (F). Immunohistochemistry was performed using an aminoethyl carbazole-based protocol. Slides were counterstained with hematoxylin counterstain.

Horse PBMCs express IL22 mRNA when stimulated with PWM or PMA

Previous molecular studies of IL22 showed that mRNA from this cytokine is not expressed in resting PBMCs. When horse PBMCs were stimulated with either pokeweed mitogen (PWM) or phorbol myristate acetate and ionomycin (PMA/IO), *IL22* mRNA was rapidly upregulated (Figure 7.5). This suggested that IL22 protein may be produced by these cells which could serve as a source of native horse IL22 for further testing of clone 15.27.

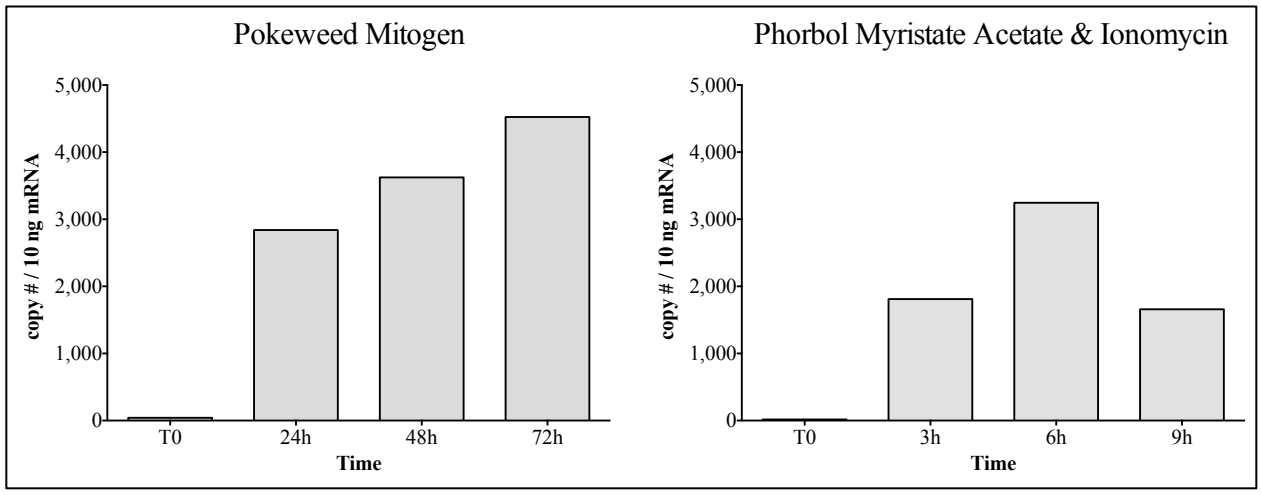

Figure 7.5 qRT-PCR analysis to identify *IL22* mRNA in resting and stimulated horse PBMCs. *IL22* mRNA is undetectable in resting equine PBMCs, but transcripts are upregulated rapidly after 24 hours of stimulation with (A) pokeweed mitogen (mean of three horses) and at three to six hours after (B) phorbol myristate acetate and ionomycin stimulation (mean of two horses).

Clone 15.27 binds native equine IL22 produced by equine peripheral blood mononuclear cells

Peripheral blood mononuclear cells from three horses were stimulated with the combined PWM, PMA and ionomycin protocol in the presence of brefeldin A (Dr. A. deMestre, personal communication) and analyzed via flow cytometry. IL22 was identified in approximately 2 to 5% of the stimulated cells (Figure 7.6).

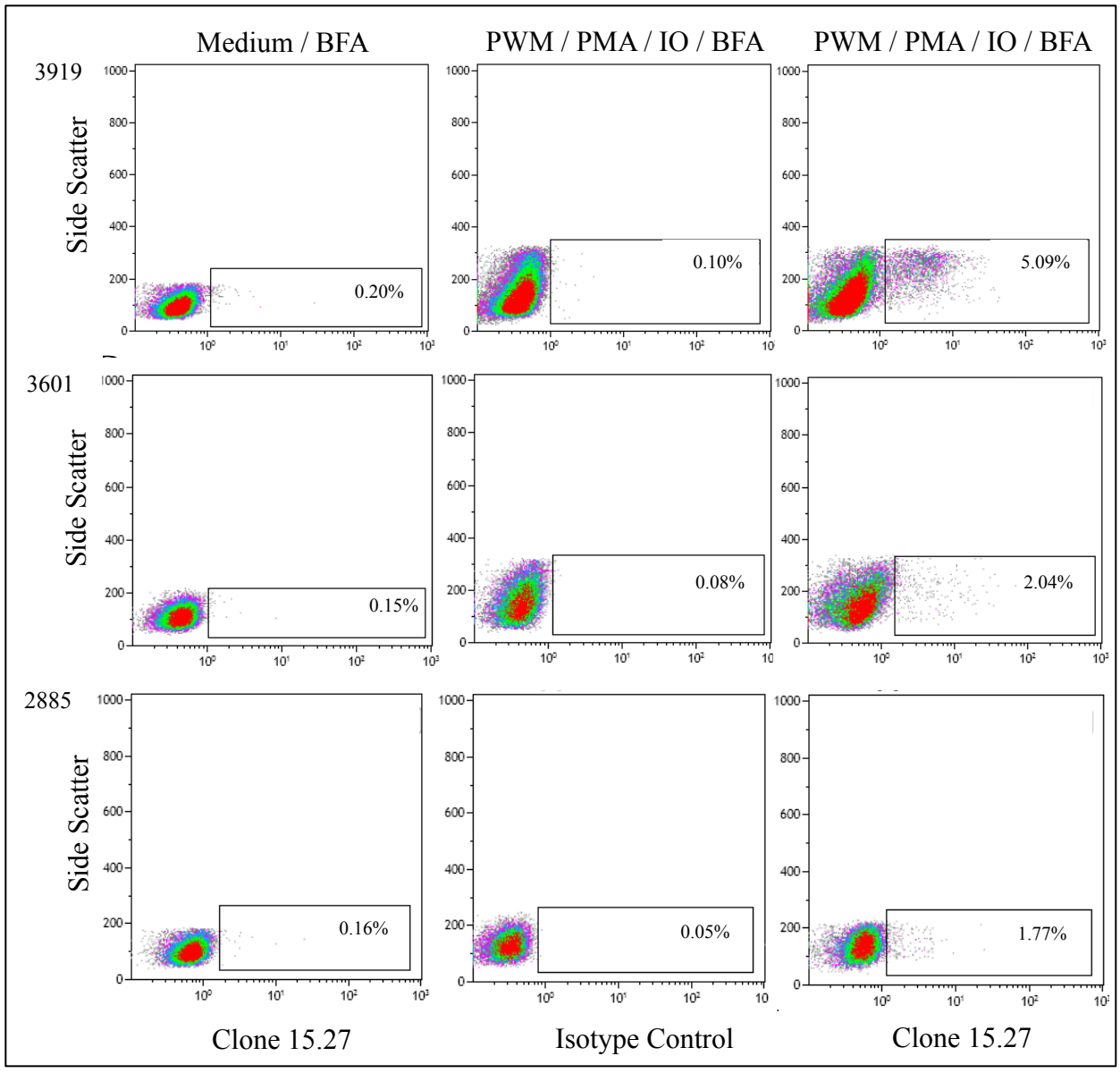

Figure 7.6 Flow cytometry of clone 15.27 on stimulated peripheral blood mononuclear cells from three horses. PBMCs from three horses were stimulated with a combination of PWM, PMA and ionomycin in the presence of brefeldin A. All antibodies were directly labeled with Alexa 647. Clone 15.27 did not label cells incubated in plain medium with brefeldin A, nor did an isotype control label stimulated cells. Clone 15.27 labeled 2 to 5% of stimulated cells. PWM = pokeweed mitogen PMA = phorbol myristate acetate, IO = ionomycin, BFA = brefeldin A.

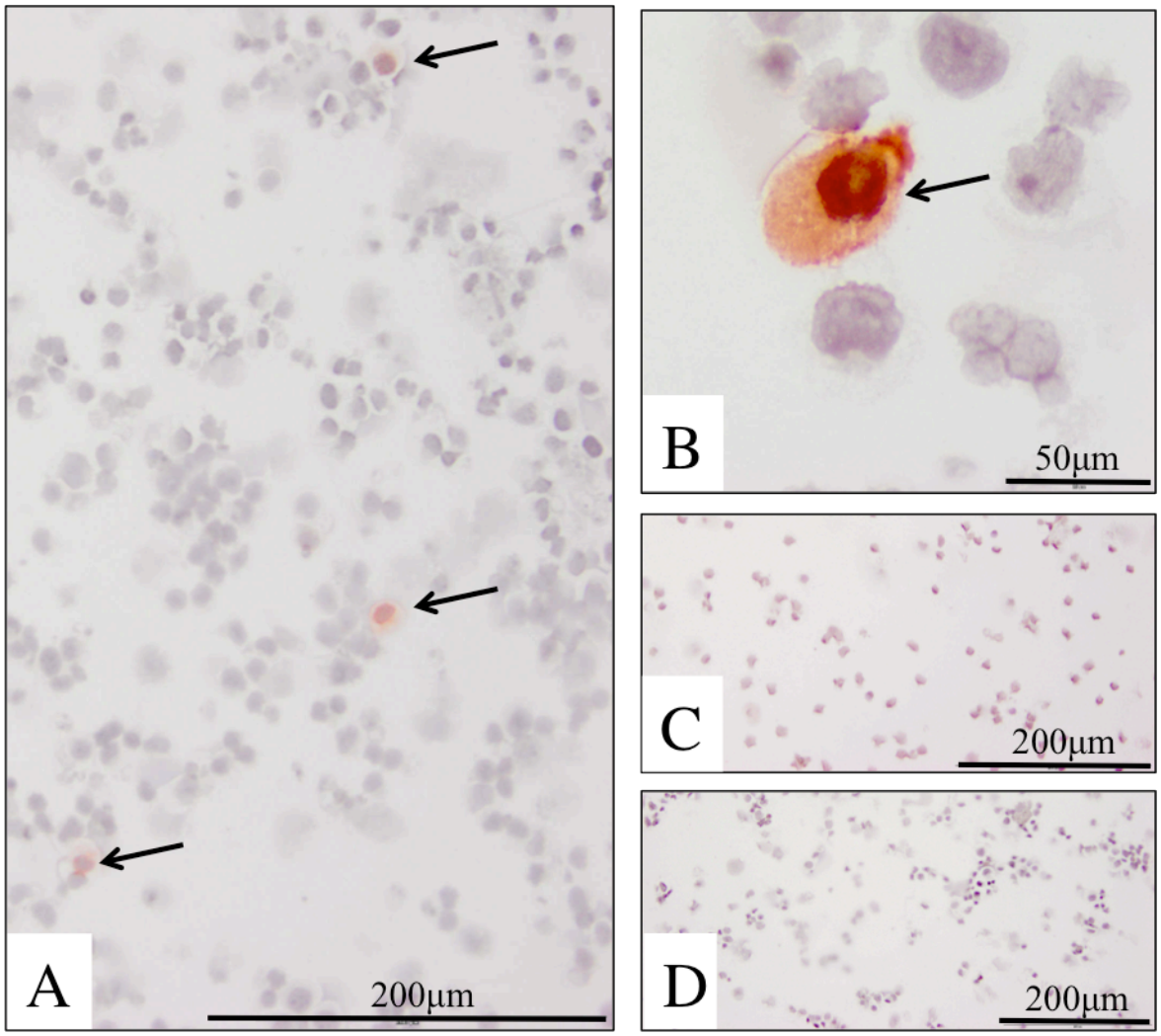

Figure 7.7 Clone 15.27 utilized in an immunohistochemistry assay on cytospins of stimulated peripheral blood mononuclear cells. Cytospin preparations were made from stimulated PBMCs and were used in an aminoethyl carbazole-based immunohistochemistry assay (A) Representative immunohistochemistry slides from one horse showing that clone 15.27 binds stimulated PBMCs (A, B) but not unstimulated PBMCs (C). An isotype control antibody against canine parvovirus did not label stimulated PBMCs (D). Bar = 200 uM Slides are counterstained with hematoxylin.

Clone 15.27 does not cross-react with interleukin 10

Interleukin 22 is a member of the interleukin 10 (IL10) family of cytokines, and shares the IL10 family signature sequence. To ensure that there was no cross reactivity of clone 15.27 to IL10, the antibody was tested against a supernatant containing an rIL10/IgG fusion protein. It was also tested against rIL22RA2/IL4 and rNKp46/IL4-containing supernatants generated using the same protocol used to generate the rIL22/IL4-secreting CHO cells. Clone 15.27 bound the rIL22/IL4 fusion protein, and this activity titrated out as expected. No activity was shown against the rIL10/IgG, rIL22RA2/IL4 or rNKp46/IL4 fusion proteins (Figure 7.8).

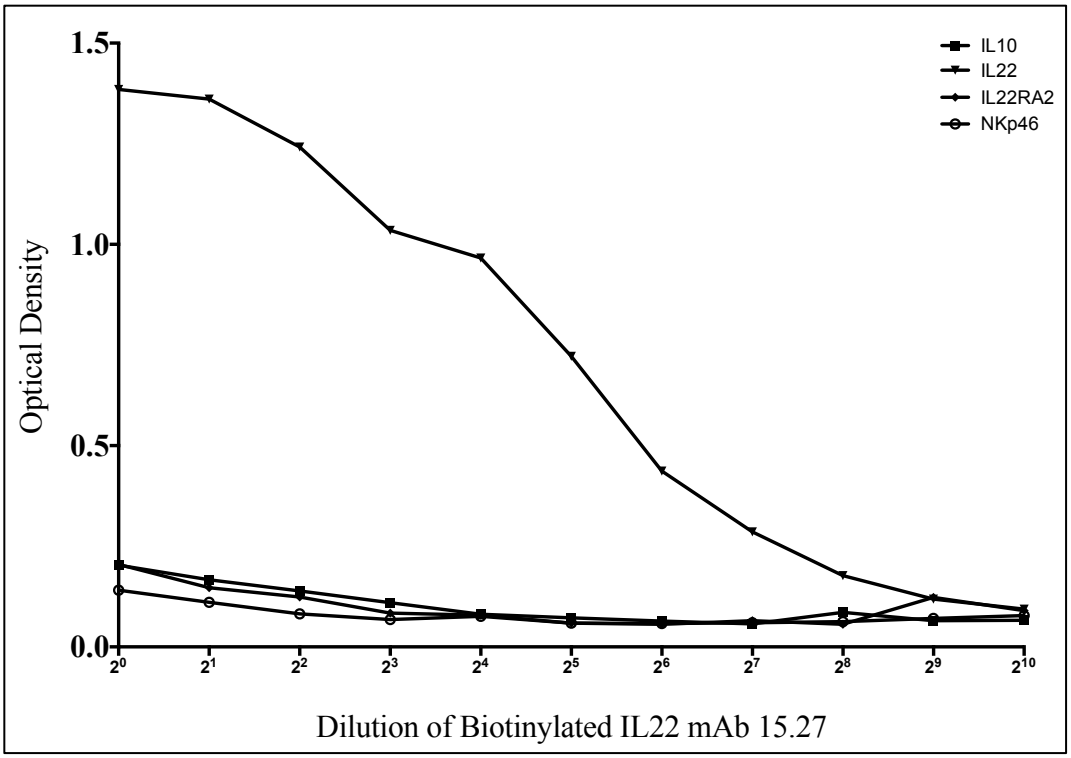

Figure 7.8 ELISA assay using biotinylated clone 15.27 on supernatants containing IL10, IL22, IL22RA2 and NKp46. Twofold dilutions were made of biotinlyated clone 15.27 and tested on supernatants containing the original rIL22/IL4 fusion protein (triangle), fusion proteins made up of rIL4 and horse IL22RA2 (diamond) and NKp46 (circle) and a fusion protein made up of horse IL10 and IgG (square). Clone 15.27 showed strong activity against the supernatant containing the rIL22/IL4 fusion protein, and this activity titrated out with increasing dilution. Negligible activity was observed against any of the other supernatants.

Clone 15.27 identifies the presence of IL22 protein in day 35 chorionic girdle

Previous work with molecular techniques including gene expression arrays and qRT-PCR has shown that *IL22* mRNA is highly upregulated in the invasive trophoblast of the equine chorionic girdle between day 32 and day 35 of a normal equine pregnancy. This occurs just prior to chorionic girdle cell invasion into the endometrium, suggesting a functional role for IL22 in maintaining the integrity of the endometrial epithelium. Flow cytometry and immunohistochemistry using clone 15.27 on a known source of IL22, PBMCs, suggests that the antibody generated to the IL22 component of the rIL4/IL22 fusion protein also binds native equine IL22. Clone 15.27 was used in an immunohistochemistry assay on day 35 chorionic girdle, and identified IL22 diffusely throughout the chorionic girdle (Figure 7.10 A). An isotype control antibody to canine parvovirus failed to label the day 35 girdle sections (Figure 7.10 B).

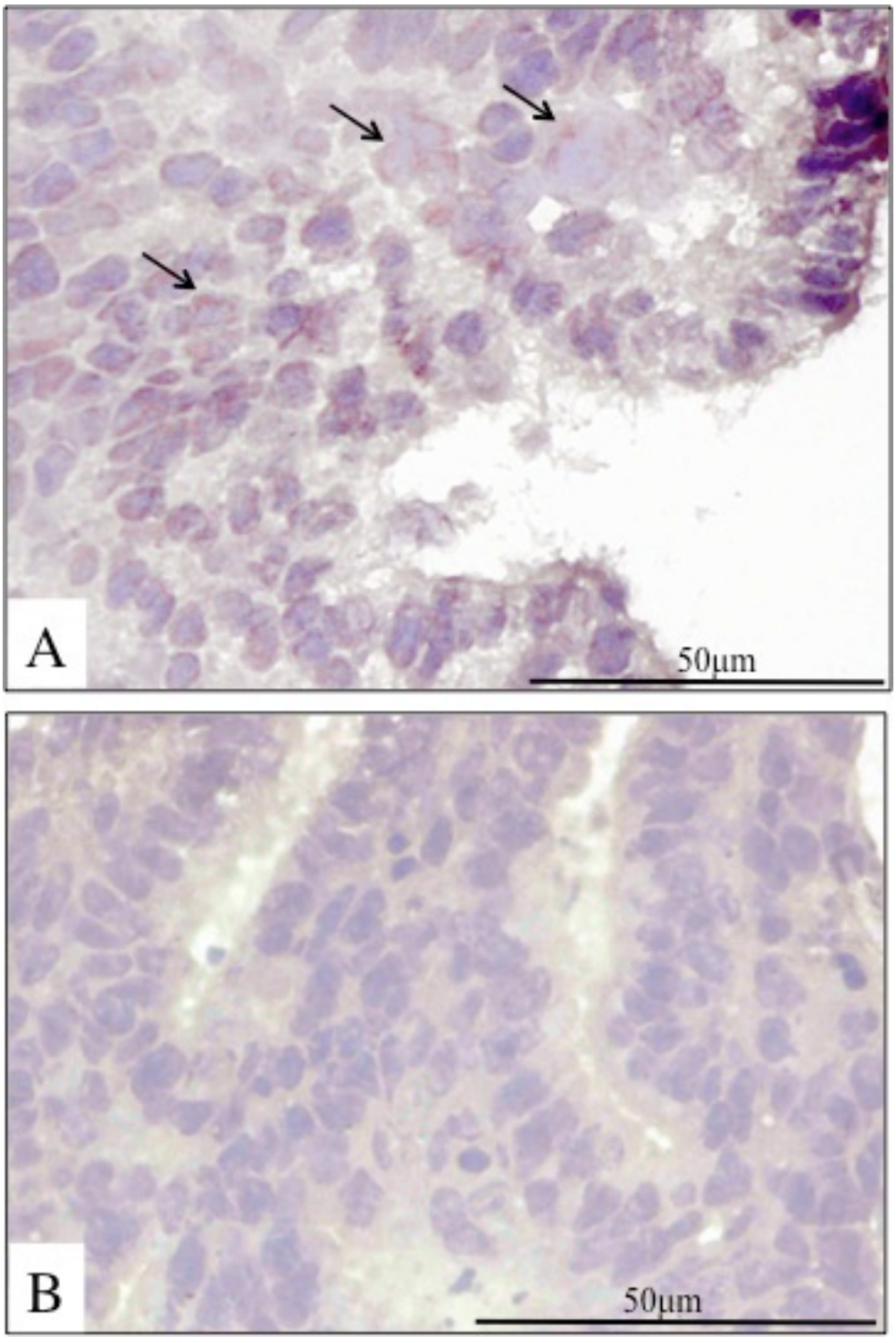

Figure 7.9 Clone 15.27 identifies IL22 protein in chorionic girdle from a day 35 pregnancy.
(A) Immunohistochemistry on frozen sections using an aminoethyl carbazole-based protocol shows IL22 to be diffusely present throughout the day 35 chorionic girdle (arrows). (B) A serial section to (A) stained with an isotype control antibody (mouse anti-canine parvo) shows negative results on the day 35 equine chorionic girdle. Bar = 50 uM. Slides are counterstained with hematoxylin.

Discussion

The primary objective of this study was to further previous molecular research that identified *IL22* mRNA in the invasive trophoblast of the chorionic girdle. This involved first generating a monoclonal antibody against horse IL22, and then using the new reagent to identify IL22 protein in the chorionic girdle. The anti-horse IL22 antibody was generated using a proven protocol (Noronha et al., 2012a; Noronha et al., 2012b; Wagner et al., 2012). Initial screening identified one clone with activity against IL22, and the results of a series of tests including ELISAs, flow cytometry and immunohistochemistry supported this.

Stimulated horse PBMCs were used as a source of native IL22 for the next phase of antibody testing. The IL22 positive cell population in stimulated horse PBMCs was small, ranging from 2 to 5% of total cells. This small population is consistent with what has been observed in other biologic systems. Innate lymphoid cells stimulated with interleukin 23 in the presence of brefeldin A resulted in 6.6% IL22 producing cells (Van Maele et al., 2014). Stimulation of human PBMCs for seven days with a combination of fungal components and PMA and ionomycin with brefeldin A resulted in 9% of CD4+ cells producing IL22 (Gresnigt et al., 2013). A third study quantified IL22 producing cells following stimulation of PBMCs from healthy humans and those affected with rheumatoid arthritis (RA). Following stimulation with PMA and ionomycin in the presence of brefeldin A, 0.54% of total cells from the healthy humans were IL22 positive, while 5.52% of the total cells from the RA humans produced IL22 (Zhao et al., 2013). The detection of 2 to 5% IL22 positive cells in non-specifically stimulated PBMCs from healthy horses is consisted with the range observed in other species.

Interleukin 22 is produced by subpopulations of immune cells in many species. Data available for other domestic large animal species is limited to the cow, where *IL22* mRNA has been detected in both α/β and γ/δ T-cells in response to mitogen stimulation (Ma et al., 2010). More data is available regarding IL22 production in humans and mice. Early on IL22 was identified in CD4+ T-cells and natural killer (NK) cells (Colonna, 2009; Wolk and Sabat, 2006). It is of interest that in humans uterine NK cells produce IL22 during early pregnancy and are proposed to have a role in preservation of mucosal surfaces, although a target receptor was not identified (Male et al., 2010). IL22 has also been identified in innate lymphoid cells (Spits and diSanto, 2011) and more recently in CD4+ cells producing IL22 but not interleukin 17, termed Th22 cells, along with some populations of CD8+ cells (Rutz et al., 2013). Alveolar macrophages in both humans and mice produce IL22 as part of the innate immune response (Hansson et al., 2013) .

In the horse, previous molecular research using gene expression arrays and qRT-PCR made a novel finding of *IL22* mRNA in the invasive trophoblast of the chorionic girdle cells (Brosnahan et al., 2012). Target receptor *IL22R1* mRNA was identified in both luminal and glandular epithelium using qRT-PCR and in situ hybridization (Chapter 6). In biologic systems involving IL22 produced by immune cells, the cytokine binds receptor IL22RA1 on target epithelial cells. This results in epithelial cell proliferation, differentiation and migration, mechanisms that repair damage and preserve the integrity of the mucosal barrier (Rutz et al., 2013; Wolk et al., 2006). Molecular findings in the horse led to the hypothesis that IL22 secreted by the chorionic girdle may bind maternal receptor IL22RA1 and serve as a mechanism of maternal-fetal communication to repair epithelial damage caused by the migrating chorionic girdle cells.

Clone 15.27 used in an immunohistochemistry assay upon a day 35 chorionic girdle revealed the presence of IL22 diffusely throughout the tissue. Although the identification of protein does not give additional clues as to the function of IL22 in the chorionic girdle, its presence throughout the chorionic girdle just at the time of chorionic girdle cell invasion is further supportive of a hypothesis that IL22 produced by the chorionic girdle performs a function similar to lymphoid IL22, preservation of endometrial epithelial integrity.

Acknowledgements

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CHAPTER 8

CONCLUSIONS

Overview

The overall objective of this body of work was to expand the current understanding of maternal-fetal tolerance both in the context of equine pregnancy and from a comparative perspective. Two aspects of maternal tolerance were considered during the course of this research: conventional immunological tolerance as well as physical tolerance of the anatomical changes that occur during pregnancy. These two ways of approaching studies of trophoblast biology historically have made up two paradigms, "trophoblast as allograft" and "trophoblast as tumor" respectively.

The first set of experiments presented in Chapters 2 through 4 focused on the "trophoblast as allograft", considering the question as to how feto-placental tissues survive within the hostile environment of the maternal immune system. These experiments utilized an ectopic trophoblast transplant system and took advantage of an inter-species approach such as has been used historically to enhance studies in equine reproductive immunology. The second set of experiments presented in Chapters 5 through 7 focused on the "trophoblast as tumor", considering the ability of the invasive trophoblast to disrupt normal maternal anatomy without causing adverse effects. These experiments utilized complementary molecular techniques including expression arrays, quantitative reverse transcriptase polymerase chain reaction (qPCR) and RNA in situ hybridization to investigate the presence of the cytokine interleukin 22 (IL22) and its receptor interleukin 22RA1 (IL22RA1) in chorionic girdle and endometrium respectively. A monoclonal antibody was generated against IL22, and the antibody was used to support the molecular findings. A schematic summary of these experiments along with the major findings and conclusions from each line of study is presented in Figure 8.1. The remainder of this chapter

discusses these conclusions in the context of their implications for the field of reproductive immunology.

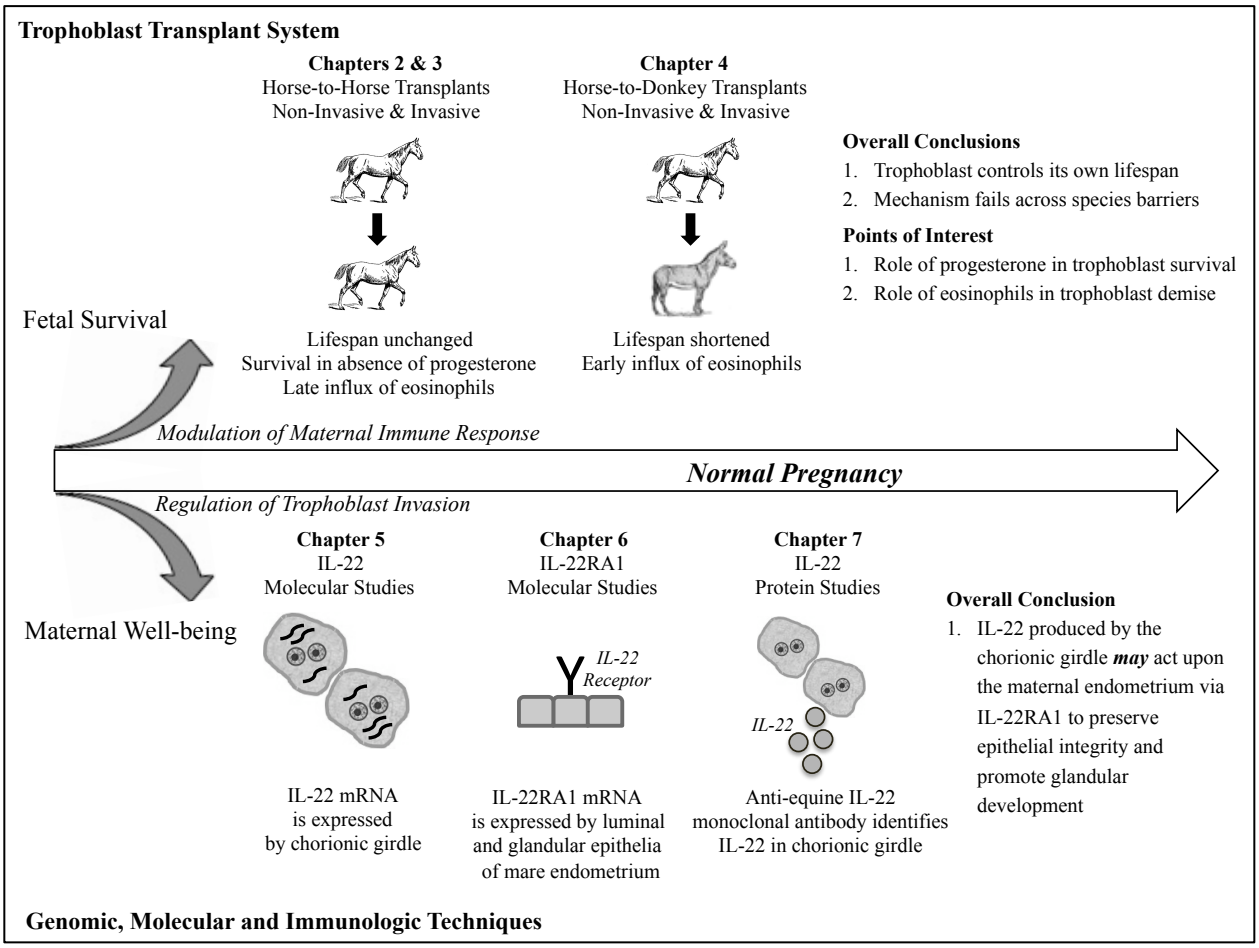

Figure 8.1 Summary of results and conclusions. This schematic summarizes the experimental approach and conclusions for the two lines of research investigated as part of this work. Ectopic trophoblast transplant studies in horses and donkeys suggest that the signal to initiate protection of the trophoblast from immune destruction originates from within the trophoblast itself rather than from the pregnant uterus. Molecular studies revealed the first known expression of *IL22* by a non-immune cell type, the chorionic girdle, and identified a potential target receptor in the endometrium. A monoclonal antibody identified IL22 protein in the chorionic girdle. This suggests an important role for IL22 in early equine pregnancy.

Ectopic Trophoblast Transplants

Since Sir Peter Medawar first framed pregnancy in the context of an "immunological problem" akin to an allograft (Medawar, 1953), investigators have sought to identify at a molecular level the signals that initiate protection of the feto-placental tissues. Challenges to research in this area include the complexity and redundancy of protective mechanisms along with the great diversity in placentation across mammalian species. The work presented in Chapters 2 and 3 provides another piece to this puzzle. The primary conclusion that can be drawn from the horse-to-horse ectopic trophoblast studies is that the signal to initiate the mechanisms that protect the transplanted tissue from host immune destruction originates from the trophoblast itself. This conclusion is based upon data from the present studies viewed within the context of historical data.

The first point to consider is that trophoblast itself is not inherently resistant to killing. In vitro studies have shown that equine trophoblast expressing MHC-I is subject to lysis by CD8+ cytotoxic T-cells primed against allogeneic lymphocytes (Baker et al., 2000). Second, studies of single ectopic trophoblast transplants showed that the tissue could survive in vivo without the protection of the uterus and the hormonal priming of pregnancy (Adams and Antczak, 2001; de Mestre et al., 2011). However, these initial studies of ectopic trophoblast transplants used maiden mares, and only one transplant was performed in each mare. Therefore it was unknown if trophoblast survival was due to the immunologically naïve recipient immune system.

The hypothesis tested in the current study was that immunologic priming of the recipient's immune system by a first transplant would result in a change of the length of lifespan in second

and subsequent transplants. This change could be a longer lifespan due to regulatory T-lymphocyte memory (Aurich et al., 2013; de Mestre et al., 2010; Rowe et al., 2012) or other mechanisms of induced tolerance resulting from prolonged antigen exposure (Schietinger and Greenberg, 2014), or a decrease in lifespan due to a destructive memory response. Alternatively, there could be no change in trophoblast lifespan, suggesting that the trophoblast cells possess an intrinsic mechanism that controls their own lifespan.

The key finding of this study was that chorionic girdle transplanted to an ectopic site in a recipient with a primed immune system did not have a progressively shorter lifespan (Figure 8.2). Survival of the tissue for a period of time comparable to single transplants and normal equine pregnancy suggests that neither physical sequestration within the uterus nor the hormonal priming of pregnancy is prerequisite for survival of the tissue. It is therefore likely that the signal for protection originates from the trophoblast. The small number of mares used in this study precluded a determination as to whether the trend toward increasing lifespan was statistically significant, but this effect was transient as it did not continue into the second breeding season. Transplant lifespans during the second breeding season gravitated back towards the 70 to 80 days observed in previous single transplant studies and normal pregnancies (de Mestre et al., 2011). This may suggest further that the lifespan is predetermined and that the signal to "allow" the ultimate destruction of the tissue also originates from the trophoblast. However, it is clear in these studies that trophoblast lifespan is not decreased in immunologically primed environments.

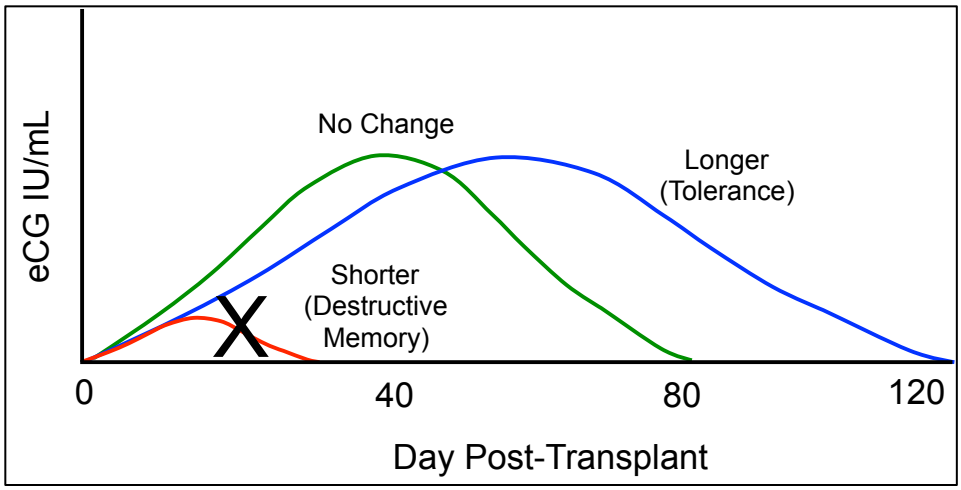

Figure 8.2 Summary of horse-to-horse trophoblast transplant results. This schematic depicts the key finding of the serial horse-to-horse ectopic trophoblast transplants. Lifespans of serial transplants did not decrease over time.

The results of this study leave open the question as to what is the precise molecular signal that initiates immune protection of the trophoblast. Several immunomodulatory molecules of trophoblast origin have been identified, including CD200 (Clark et al., 2003; Clark et al., 2001; Gorczynski et al., 2005; Wang et al., 2014; Yu et al., 2008), MIC-I (Segerer et al., 2012), galectin-1(Ramhorst et al., 2012) and possibly others (Dong et al., 2008). These molecules have not been studied in the horse and could be investigated for a possible role in trophoblast transplant survival.

A role for T-regulatory cells in maternal tolerance has been described in many species including the horse (Aurich et al., 2013; Jiang et al., 2014; Robbin et al., 2011; Rowe et al., 2012; Zenclussen et al., 2006). The ability of equine invasive trophoblast to initiate development of peripheral T-regulatory cells (pTregs) would provide one possible mechanism to ensure survival. Numerous factors are known to be involved in differentiation of lymphocytes to a pTreg phenotype in response to appropriate antigenic stimulation, including cytokines TGF-β and IL-2,

along with transcription factors NF-κB and Nr4a, and regulatory element CNS1within the target cells (Luo and Li, 2013; Zheng et al., 2010). Endocrine hormones including progesterone also play a role in the differentiation of Tregs (Lee et al., 2012). TGF-β has been identified in equine endometrium around the time of implantation, and also within leukocytes surrounding the endometrial cups (Lennard et al., 1995), but has not been investigated in horse trophoblast. Equine invasive trophoblast is known not to produce interleukin 2 (Grunig and Antczak, 1995). The other molecules have not been investigated in the context of equine trophoblast. Of great interest is the observation in the present study that one horse-to-horse transplant survived in the absence of progesterone, suggesting that this hormone is not prerequisite for trophoblast survival. One weakness of the current study was the inability to examine lymphocyte subpopulations at the transplant site, therefore the suspected role for Tregs in transplant survival is unconfirmed.

In contrast to the horse-to-horse transplants, the horse-to-donkey transplants showed a lifespan pattern consistent with a conventional destructive memory response (Figure 8.3). The failure of the horse-to-donkey trophoblast transplants to survive repeatedly suggests that the signal to initiate immune protection of the trophoblast tissue is species-specific at a molecular level. It further confirms that trophoblast can be killed by the immune system and is not inherently resistant to destruction regardless of environment. If success of the horse-to-horse transplants is in fact dependent upon induction of Tregs, the failure of horse-to-donkey transplants may represent an inability of horse trophoblast to initiate differentiation of donkey pTregs. A growing area of research in the field of transplant immunology is focused upon a potential role for Tregs in the protection of xenogeneic transplants (Muller et al., 2009). Future experiments designed to identify mechanisms implicating Tregs in the success of horse-to-horse transplants

and the failure of horse-to-donkey transplants would likely be revealing, with implications for the field of transplant immunology as well as reproductive immunology.

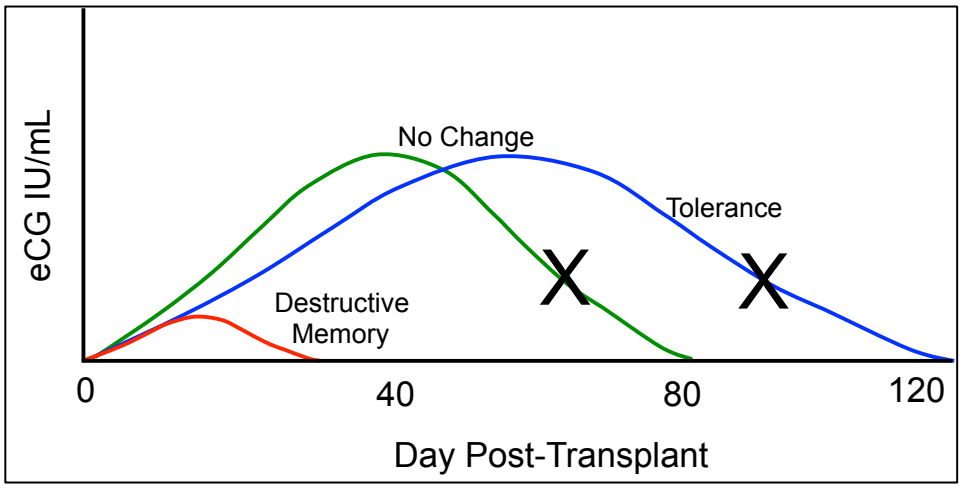

Figure 8.3 Summary of horse-to-donkey trophoblast transplant results. This schematic depicts the key finding of the serial horse-to-donkey ectopic trophoblast transplants. Lifespans of serial transplants showed a pattern consistent with a destructive memory response.

The discovery of eosinophils in the cellular infiltrates associated with late stage degeneration of horse-to-horse transplants as occurs around mature and dying endometrial cups, and with the early death of horse-to-donkey transplants, suggests that they may play a role in trophoblast destruction. Eosinophils are observed at various stages of pregnancy and during the reproductive cycle in other species (Robertson et al., 2000). A definitive role for these cells in equine trophoblast biology is uncertain. The ectopic trophoblast transplant system represents a reliable method for recruitment of eosinophils to a site of local inflammation and may provide a novel system for studies of eosinophil function.

Interleukin 22 in Early Equine Pregnancy

The data presented in Chapters 5 through 7 falls within the "trophoblast as tumor" paradigm. While trophoblast exhibits many characteristics observed in tumor cells, such as invasiveness,

angiogenesis and nutrient acquisition, a major difference between the two is that trophoblast invasion is tightly controlled while tumor invasion is unregulated (Soundararajan and Rao, 2004). As detailed in Chapter 5, this line of work began with the chance discovery on a gene expression array that the interleukin 10-family cytokine interleukin 22 was greater than 900 times upregulated in the invasive chorionic girdle as compared to the non-invasive chorion (Brosnahan et al., 2012). Additional experiments using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) showed that the *IL22* mRNA was rapidly upregulated between days 32 and 35 of normal equine pregnancy just prior to trophoblast invasion. Identification of *IL22* mRNA in the chorionic girdle was a notable finding, as prior to this it had only been documented in immune cell types cells (Colonna, 2009; Ma et al., 2010; Maloy and Kullberg, 2008; Sonnenberg et al., 2011; Trifari and Spits, 2010). Interleukin 22 was of particular interest as it plays a role in mucosal immunity and the maintenance and repair of epithelia (Aujla and Kolls, 2009; Maloy and Kullberg, 2008; Ouyang and Valdez, 2008; Sugimoto et al., 2008). Its upregulation at a time when the invasive trophoblast was poised to phagocytose through the maternal endometrium suggested a physiologic role in maintenance of the endometrial epithelium.

A physiologic effect of interleukin 22 of chorionic girdle origin requires the presence of a target receptor. Interleukin 22 of leukocyte origin acts upon a heterodimeric receptor composed of its primary target IL22R1 and IL10R2 (Wolk et al., 2010). This receptor is expressed on epithelial surfaces, including respiratory (Aujla et al., 2008) and digestive tracts, and skin (Wolk et al., 2004). Binding of IL22R1 by IL22 activates transcription factors STAT3, STAT1 or STAT5

(Lejeune et al., 2002) and regulates genes associated with innate immunity (Liang et al., 2006) and cellular differentiation, migration and survival (Boniface et al., 2005; Radaeva et al., 2004).

mRNA for this primary target receptor for IL22, *IL22RA1*, subsequently was identified in maternal endometrium using qRT-PCR analysis. As detailed in Chapter 6, in situ hybridization was used to localized the *IL22RA1* mRNA to specific cells within the endometrium. *IL22RA1* mRNA was identified in both the luminal and glandular epithelium of mare endometrium. Probe hybridization was stronger on pregnant versus non-pregnant endometrium. This suggested a possible physiologic effect of IL22 from the chorionic girdle upon both the luminal and the glandular epithelium. The presence of IL22 protein in chorionic girdle was confirmed by the generation of a monoclonal antibody against horse IL22 (Chapter 7).

These studies support the hypothesis that the IL22 produced by the chorionic girdle binds receptor on the luminal epithelium of the endometrium to restore its integrity following trophoblast invasion, and binds receptor on the glandular epithelium to initiate proliferation. Current research on IL22 has shown that this cytokine has a restorative effect in many biologic systems following damage from to infectious (Aujla et al., 2008), toxic (Schieermann et al., 2013) and mechanical (Hoegl et al., 2010) causes. Dysregulation of IL-22 has also been shown to play a role in tumor development (Lim and Savan, 2014). Elucidation of the molecular mechanisms of action of IL22 in trophoblast invasion may make a significant contribution to the existing body of knowledge of IL22 in physiologic and pathologic systems.

In summary, the results of this work moved forward the field of equine reproductive immunology in several key ways. Our understanding of the nature of immune evasion by the chorionic girdle has been greatly enhanced. This research suggests that the signal for initiating maternal tolerance originates from the trophoblast, and that it is species specific. The results further suggest that interleukin 22 plays a role in modulating changes to the uterus during early pregnancies. Both of these discoveries provide a new direction for future studies in the field of reproductive immunology.

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