PERIPHERAL AND LOCAL IMMUNE MODULATION DURING EQUINE PREGNANCY

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PERIPHERAL AND LOCAL IMMUNE MODULATION DURING EQUINE PREGNANCY

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The studies in this dissertation were designed to investigate the immunomodulatory changes that occur in the cytotoxic lymphocyte populations of the horse at the systemic and regional levels during early pregnancy.

Pregnant mares demonstrate a reduction in cytotoxic T lymphocyte (CTL) reactivity against target cells from the breeding stallion. I investigated whether this effect is limited to activity against paternal Major Histocompatibility Complex (MHC) antigens, and whether it occurs during MHC compatible pregnancy. Mares carrying MHC incompatible pregnancies demonstrated reduced CTL activity against lymphocytes from horses with unrelated MHC haplotypes in addition to those from the breeding stallion. This effect was also observed in mares carrying MHC compatible pregnancies, suggesting that an antigen-independent mechanism may be responsible for the decrease in CTL reactivity.

I then examined whether natural killer (NK) cells may be present in the uterus among the dense lymphocytic infiltrate that surrounds the placental trophoblasts of the endometrial cups. Using quantitative molecular techniques, I identified the equine homologs of the NK cell marker genes *NKP46*, *CD16*, *CD56*, and *CD94* and found that gene expression of all four markers was significantly higher in lymphocytes isolated from the endometrial cups compared to peripheral blood lymphocytes (PBL) isolated from the same animal on the same day. This provides the first evidence for the existence of NK cells in the equine endometrium during pregnancy. After developing panels of monoclonal antibodies that specifically recognize the equine homologs of NKp46 and CD16, I performed experiments to characterize equine NK cells. The two markers labeled a small percentage of PBL, and were also expressed on a subpopulation of CD3+ cells

in most horses, suggesting that NK cells in the horse may have a CD3+ NK cell phenotype that differs from other species, or that the primary cell type recognized by these markers is the Natural Killer T cell. Immunohistochemical labelling of endometrial cup tissue sections showed that endometrial cup lymphocytes (ECL) consisted of predominantly CD16+ cells, which overlapped with a previously observed CD3+ population. This novel finding suggests that the CD3+ ECL population is also CD16+, and represents a dramatic increase over the small peripheral CD16+ population.

Overall, this study advances knowledge of changes in maternal immunity during pregnancy in the mare and provides a more complete characterization of uterine leukocytes in the horse.

BIOGRAPHICAL SKETCH

Leela was born into a medical family in New Brunswick, NJ in 1971. Just before her second birthday, she moved to the great state of West Virginia, where she grew up among the natural beauty and grandeur of the Appalachian mountains. She spent a large part of her youth on her grandparents' cattle farm amidst a happy assortment of animals. Her lifelong love of horses began at the age of four with her first pony, Peanut.

After attending boarding school in Pennsylvania at Villa Maria High School and Shady Side Academy, she went on to Brown University in Providence, RI where she earned a Sc.B. with Honors in Biochemistry. She performed her undergraduate honors thesis work "Expression of HIV-1 Tat in the yeast two hybrid system" in the laboratory of Dr. Peter Shank. After graduation, she worked in the Cancer Group at Pfizer Central Research in Groton CT, and at Provirus, a biotech company in Maryland, before beginning her veterinary medical education at the Virginia-Maryland Regional College of Veterinary Medicine in Blacksburg, VA. While in vet school, she spent a summer at the International Livestock Research Institute in Nairobi, Kenya as a Geraldine R. Dodge Fellow. Following graduation in 2001, she had the unique experience of participating in the international effort to contain the outbreak of Foot and Mouth Disease that occurred in the United Kingdom the summer of that year.

After 4 years of private practice in the VA/MD/WV region, she began her doctoral training in Immunology at the Cornell University College of Veterinary Medicine. She rotated through the laboratories of Dr. Klaus Osterrieder and Dr. Bettina Wagner before joining Dr. Doug Antczak's group, where she earned a Ph.D. in 2012.

Dedicated to my grandparents, Lawrence and Dorothy O'Donnell

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CHAPTER 1

INTRODUCTION[1]

[1]Noronha, L.E., and Antczak, D.F. (2010). Maternal immune responses to trophoblast: the contribution of the horse to pregnancy immunology. *Am J Reprod Immunol* 64, 231-244. Reprinted with permission from John Wiley & Sons.

Abstract

The horse has proven to be a distinctively informative species in the study of pregnancy immunology for several reasons. First, unique aspects of the anatomy and physiology of the equine conceptus facilitate approaches that are not possible in other model organisms, such as non-surgical recovery of early stage embryos and conceptuses, and isolation of pure trophoblast cell populations. Second, pregnant mares make strong cytotoxic antibody responses to paternal Major Histocompatibility Complex (MHC) class I antigens expressed by the chorionic girdle cells, permitting detailed evaluation of the antigenicity of these invasive trophoblasts and how they affect the maternal immune system. Third, there is abundant evidence for local maternal cellular immune responses to the invading trophoblasts in the pregnant mare. The survival of the equine fetus in the face of strong maternal immune responses highlights the complex immunoregulatory mechanisms that result in materno-fetal tolerance. Finally, the parallels between human and horse trophoblast cell types, their gene expression, and function make the study of equine pregnancy highly relevant to human health. Here, we review the most pertinent aspects of equine reproductive immunology, and how studies of the pregnant mare have contributed to our understanding of maternal acceptance of the allogeneic fetus.

Introduction

In the nearly 60 years since Sir Peter Medawar first compared the fetus to a successful tissue transplant (Medawar, 1953), rigorous study of pregnancy immunology has yielded many insights into the mechanisms of materno-fetal tolerance. The inability to formulate a unifying hypothesis is likely due to the fact that the processes behind maternal acceptance of the fetus are complex, multifactorial, and often compensatory (Aluvihare et al., 2005; Billington, 2003; Croy and Murphy, 2008; Guleria and Sayegh, 2007; Koch and Platt, 2007; Moffett and Loke, 2006; Seavey and Mosmann, 2008; Trowsdale and Betz, 2006; Zenclussen et al., 2007). One approach to move the field forward is to incorporate insights gained from comparative studies of multiple mammalian species (Carter and Enders, 2004; Carter et al., 2006; Enders and Carter, 2004).

For centuries, scientific study of the horse (*Equus caballus*) has contributed to the medical community's understanding of anatomy and physiology (North and National Library of Medicine, 2006). In recent years, studies of equine pregnancy have likewise advanced the fields of reproduction and immunology. As we discuss below, the horse is a natural model for immune recognition of the fetus. The pregnant mare demonstrates a clear immune response to placental alloantigens, thus addressing the central question of whether the mother is immunologically ignorant of, or tolerant to, her gestating fetus.

This review discusses the ways in which the horse has contributed to our understanding of pregnancy immunology and how equine research can advance the field. Here, we focus on the events of early pregnancy, as that is the period when there is abundant evidence for engagement and alteration of the maternal immune response. We first discuss the pertinent anatomic and physiological aspects of early horse pregnancy. We then discuss the concept of materno-fetal tolerance as it pertains to the horse. Finally, we describe resources that make the horse a valuable species for the study of reproductive immunology and address pressing unanswered questions in our understanding of equine pregnancy.

Equine placentation and the endometrial cups

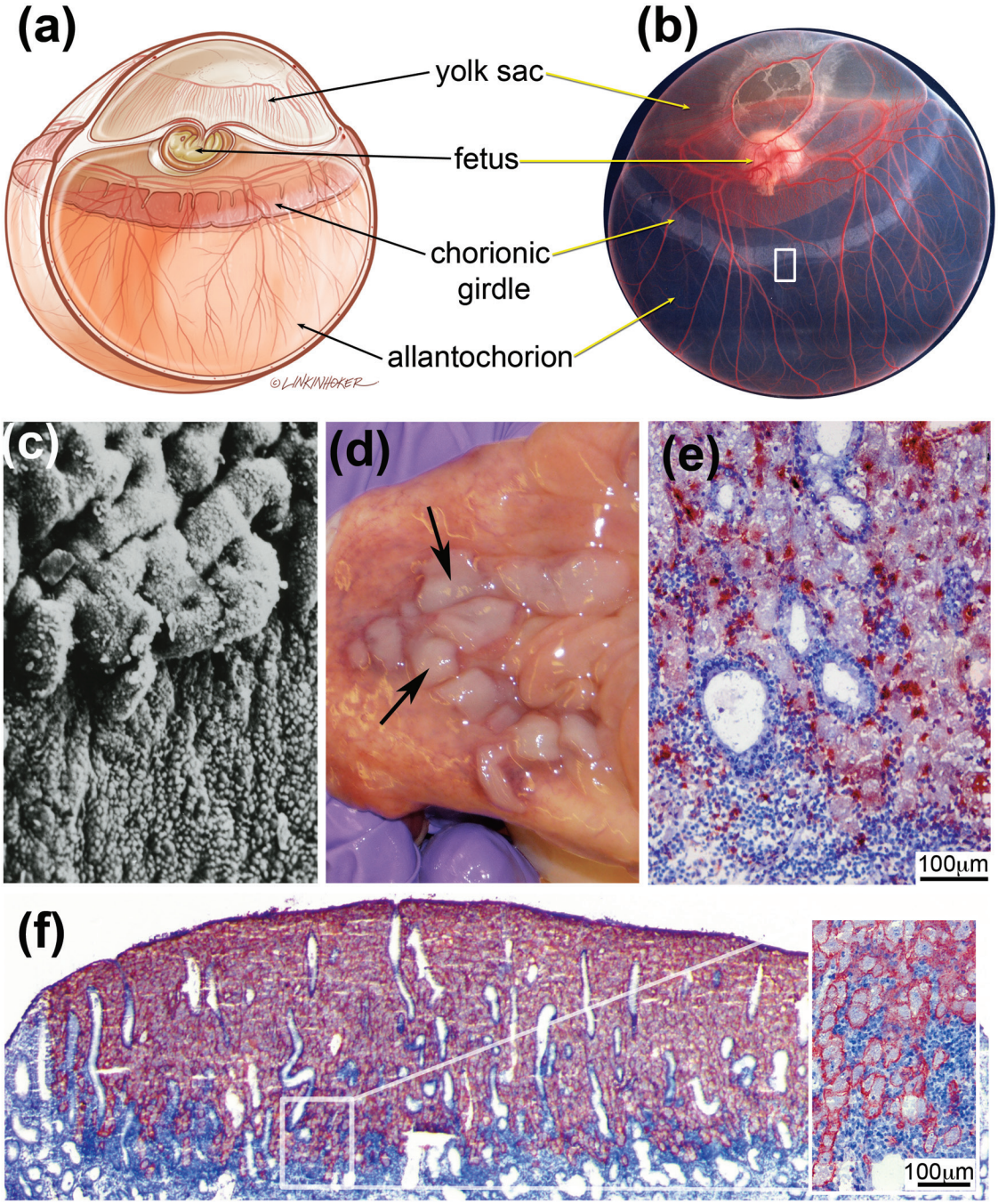
The equine placenta is characterized as diffuse and epitheliochorial, with six intact tissue layers between the maternal and fetal blood supplies (Amoroso, 1952). The majority of the interface between the uterus and placenta is formed by the tight apposition of the endometrial epithelium with the non-invasive trophoblasts of the allantochorion (Samuel et al., 1975). This attachment occurs by the interdigitation of highly branched allantochorion villi with the facing endometrium to form microcotyledons. The microcotyledons, located near capillaries in the maternal and placental tissues, act as the primary units for nutrient exchange between mother and fetus (Samuel et al., 1976). In this regard, the horse is similar to other species with epitheliochorial placentation, such as the pig. However, the equine placenta is distinguished by the specialized, highly invasive trophoblasts of the chorionic girdle.

The chorionic girdle, first described in 1897 (Ewart, 1897), is so named because it forms a circumferential band around the developing conceptus (Figure 1.1a, b). It is first visible at approximately 25 days of gestation, following the fusion of the allantois and chorion which form the allantochorion membrane. At the junction of the allantochorion and the regressing yolk sac, the cells of the chorionic girdle proliferate rapidly, forming a discrete region of pseudo-columnar epithelium with alternating ridges and pits (Figure 1.1c) (Enders and Liu, 1991). By day 36, the chorionic girdle trophoblasts develop an invasive phenotype and are able to penetrate the uterine epithelium and invade the maternal endometrium well into the stromal layer (Allen et al., 1973). Prior to this event, the conceptus is held in place at the base of one uterine horn largely by uterine tension without firm attachment to the endometrium. This very late attachment of the conceptus allows equine embryos and conceptuses from days 7 to 36 to be collected through non-surgical uterine lavage (Adams and Antczak, 2001); a great advantage for the study of the early phases of development of the fetus and placenta.

The cells of the chorionic girdle invade the endometrium like an advancing phalanx, with the leading cells followed closely by subsequent layers of cells (Figure 1.4A). By day 38, girdle invasion is usually complete, and the binucleate girdle cells quickly transform into terminally differentiated, sessile trophoblasts (Figure 1.1e, f) (Wooding et al., 2001). These tightly packed trophoblast cells are grossly visible as discrete plaques of tissue in the superficial endometrium known as endometrial cups (Fig.1d) (Schauder, 1912). The endometrial cup trophoblasts are the sole source of the high concentrations of equine chorionic gonadotropin (eCG) detectable in the blood of pregnant mares between days 40 and 120 of pregnancy (Allen and Moor, 1972; Cole and Saunders, 1935).

eCG has both LH and FSH-like activities and shares functional parallels with human chorionic gonadotropin (hCG) (Stewart, 1976). The primary function of eCG is considered to be its role in the luteinization of secondary ovarian follicles (Allen and Stewart, 2001; Squires and Ginther, 1975). These in turn secrete progesterone which maintains the pregnancy until approximately

Figure 1.1 Overview of the equine chorionic girdle and endometrial cups. Diagram (a) and gross specimen (b) of a day 34 conceptus obtained by uterine lavage. The size of the conceptus is approximately 4 cm in diameter. Note the pale band of chorionic girdle trophoblasts. The white box in panel b represents the region visualized in the scanning electron micrograph (c) of the junction of the allantochorion (bottom) and the chorionic girdle (top; x 200). (d) Gross specimen of endometrial cups shown in situ on the lumenal surface of the endometrium at day 44 of pregnancy. (e) Immunohistochemical labeling of an endometrial cup section from day 44 of pregnancy using a monoclonal antibody to eCG (mAb 67.1) and (f) horse trophoblast (mAb 71.8;


(a)
(b)
yolk sac
fetus
chorionic girdle
allantochorion
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(c)
(d)
(e)
100μm
(f)
100μm

day 100 of the 340-day gestation of the mare, when sufficient progesterone is produced by the placenta proper.

The uterine epithelium re-grows over the cups, severing the connection between the trophoblasts and the conceptus. At the same time, maternal mononuclear leukocytes are recruited into the endometrial stroma around the cups, forming a striking infiltrate at the cup periphery (Figure 1.2a,b) (Allen, 1975). No such accumulation is evident along the interface between the maternal endometrium and the non-invasive allantochorion (Figure 1.2c) (Samuel et al., 1974). Despite the seemingly hostile environment in which the cups exists, they persist *in situ* until their eventual death and desquamation which occurs around days 100 to 120 of pregnancy (Allen, 1979). At this time, eCG production, which peaks at around day 70, precipitously declines (Figure 1.3b) (Allen, 1975; Amoroso, 1952).

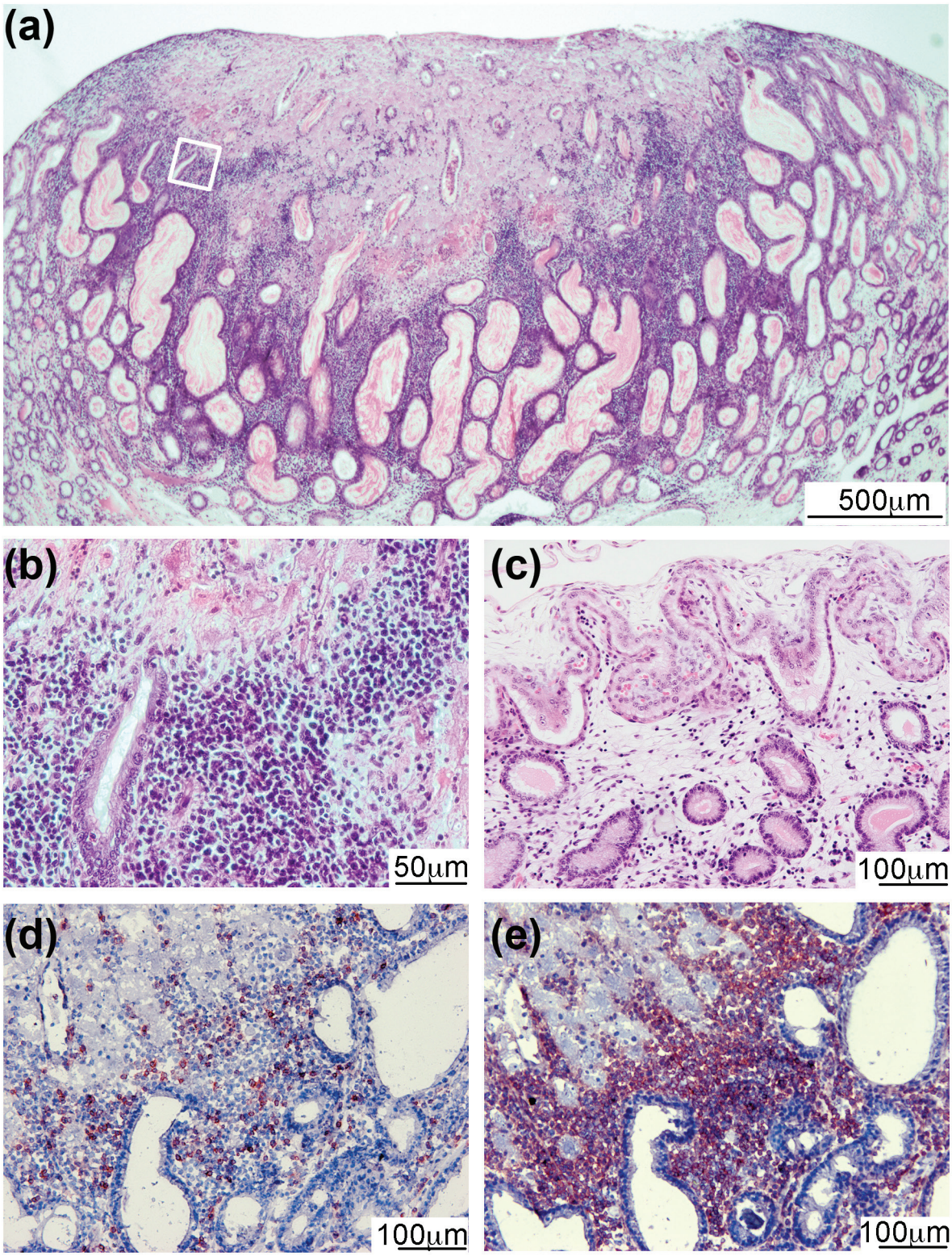
Mechanisms of maternal tolerance to the developing fetus

Studies of maternal immunological tolerance to the developing fetus in several species, including the horse, have identified overlapping and complex mechanisms that have both antigen specific and non-specific effects. These mechanisms can be grouped into three categories: 1) the repression of expression of paternally inherited alloantigens, particularly MHC antigens, by the placenta, 2) systemic alterations in the maternal immune response during pregnancy, and 3) local immune modulation at the placental interface in the uterus.

Regulation of MHC expression on trophoblast cells

The primary foreign antigens expressed by placental tissues are the products of the paternal Major Histocompatibility Complex (MHC) genes. MHC class I and II genes encode the molecules that stimulate rapid and potent cell-mediated and humoral immune responses during conventional allograft rejection. In the various eutherian species that have been studied, expression of MHC molecules by most trophoblast cells is repressed, presumably as strategy to avoid recognition and

Figure 1.2 The local maternal cellular response to the equine endometrial cups. (a-c) H&E stained, formalin fixed tissue sections from day 60 of pregnancy. (a) Low power image of an endometrial cup showing the dramatic accumulation of maternal lymphocytes at the periphery. The white box indicates the trophoblast-lymphocyte interface magnified in (b). (c) Interface between the endometrium and the non-invasive allantochorion in areas away from the endometrial cups, showing interdigitation of microvilli. Note the paucity of leukocytes in the endometrial stroma. (d-e) Immunohistochemical labeling of frozen serial sections of an endometrial cup from day 44 of pregnancy using monoclonal antibodies against equine CD8 and CD4 (HT14A, HB61A, respectively) demonstrating that the maternal leukocytic infiltrate surrounding the trophoblasts is comprised primarily of CD8+ (d) and CD4+ (e) lymphocytes.


(a)
500μm
(b)
50μm
(c)
100μm
(d)
100μm
(e)
100μm

destruction by the maternal immune system. However, in several species, minor subpopulations of trophoblasts paradoxically express some MHC class I molecules.

The trophoblast cells of the horse are unique in the combination of both spatial and temporal regulation of MHC expression they exhibit during placentation. The allantochorion trophoblasts, which comprise the majority of the fetal-maternal interface, do not express MHC class I proteins, although some mRNA can be detected in these cells (Bacon et al., 2002). During a short window in early pregnancy, the trophoblasts of the chorionic girdle and endometrial cups transiently express very high levels of polymorphic MHC class I antigens (Figure 1.3a) of both maternal and paternal origin (Donaldson et al., 1994). Starting at day 30, the chorionic girdle expresses MHC class I genes at levels approximately 10-fold higher than somatic cells, comparable to levels seen in lymphoid tissues (Figure 1.3b) (Bacon et al., 2002). The expression of these allogeneic molecules is maintained during chorionic girdle invasion into the maternal tissues. It remains high until shortly after the cells differentiate into endometrial cup trophoblasts, then drops off to nearly undetectable levels by day 45 (Crump et al., 1987; Donaldson et al., 1992; Donaldson et al., 1990; Kydd et al., 1991; Maher et al., 1996).

The MHC class I antigens of the chorionic girdle induce strong cytotoxic antibody responses in nearly 100% of mares carrying histoincompatible pregnancies (Figure 1.3b) (Antczak et al., 1982; Antczak et al., 1984; Kydd et al., 1982). Antibodies to paternal MHC class I antigens are usually detectable by day 60 in primiparous mares, at levels similar to those induced by allogeneic skin grafts (Adams et al., 2007). Multiparous mares demonstrate evidence of anamnestic responses, with antibodies detectable by day 41, indicating full engagement of the adaptive immune system, including T-lymphocyte help for the strong secondary antibody responses (Adams et al., 2007; Antczak et al., 1984). By comparison, only about 30% of multiparous women develop antibodies to paternal MHC class I antigens (Densmore et al., 1999), and in primiparous women the antibodies are rarely detected before week 28 (Regan et al., 1991). Isolated chorionic girdle trophoblasts are capable of inducing antibody on their own, as has been demonstrated by

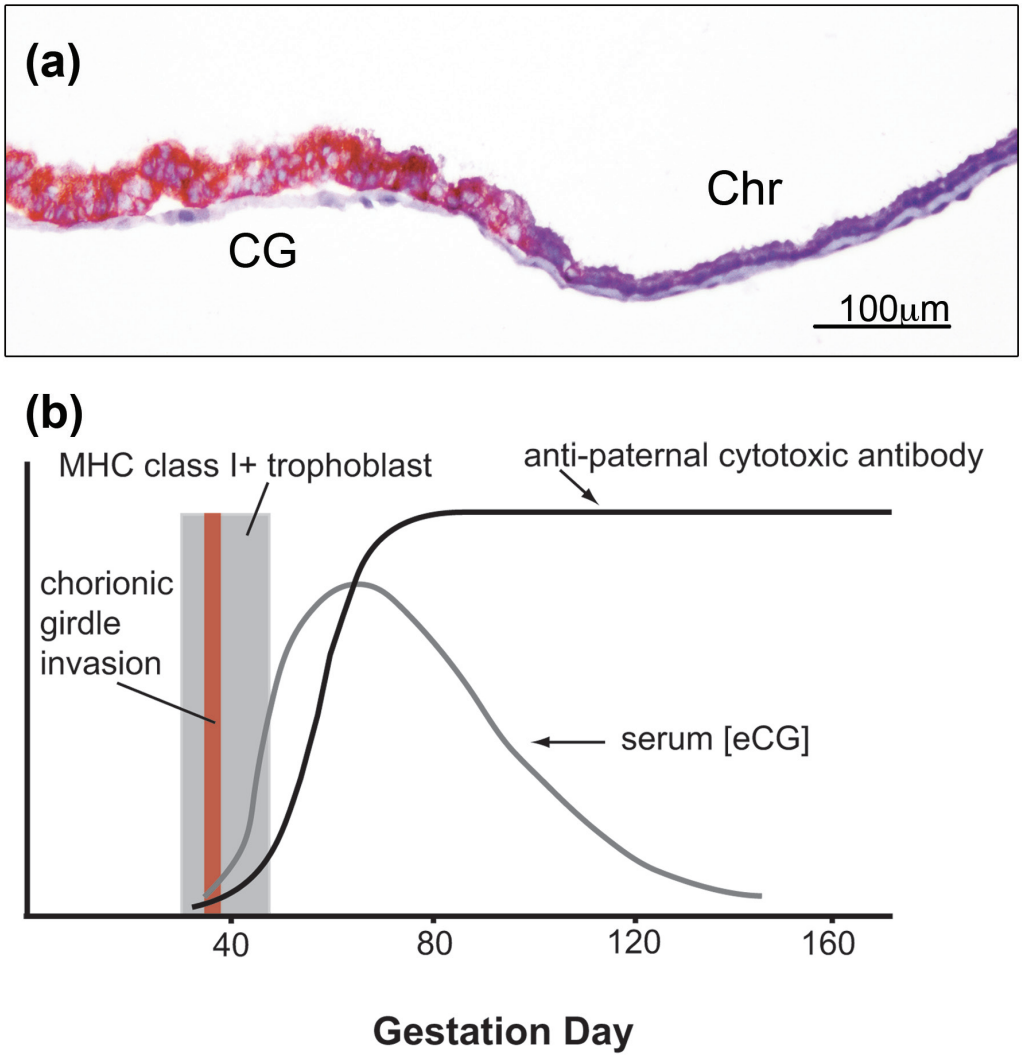


Figure 1.3 Temporal and spatial expression patterns of MHC class I molecules on equine trophoblast. (a) Immunohistochemical labeling of trophoblast dissected from a day 34 conceptus and stained with a monoclonal antibody specific for MHC class I (H58A). Note the negative chorion (Chr) and basement membrane adjacent to and below the strongly positive chorionic girdle (CG) trophoblasts. (b) Schematic illustration of the pattern of cytotoxic antibody against paternal MHC class I antigens generated in early equine pregnancy. Antibody appears shortly after eCG is first detectable in maternal serum. Gray column indicates period in which the chorionic girdle and early endometrial cups express MHC class I antigens (day 30 – 45 of gestation); red column indicates period of trophoblast invasion (days 36-38).

transplantation experiments (Adams and Antczak, 2001; Donaldson et al., 1994). The horse, therefore, more than any other species yet identified, provides incontrovertible evidence for the antigenic capacity of trophoblast cells.

MHC class I antigens are expressed on trophoblast subpopulations in several other species. The invasive extravillous trophoblast cells of the human placenta express MHC molecules from several loci: HLA-C, -E, and –G (Apps et al., 2009). Mouse labyrinthine trophoblasts express paternal MHC class I (Raghupathy et al., 1981). The interplacentomal trophoblasts of the cow express both classical and non-classical MHC class I genes late in pregnancy (Davies et al., 2006). As in other species, MHC class II molecules are not expressed by any equine trophoblast populations (Donaldson et al., 1990; Holtz et al., 2003).

Systemic alterations in maternal immune responses

While the pregnant mare is capable of mounting a robust and reproducible humoral immune response against paternal MHC class I antigens, this is not the case with the cell-mediated immune response. Equine pregnancy appears to induce a state of "split-tolerance" to trophoblast— a situation where one compartment of the immune system responds to an antigen, while another is tolerant (de Mestre et al., 2010; Hunziker et al., 1997; Sprent et al., 1995). In the pregnant mare, this presents as a dramatic allospecific anti-paternal humoral immune response with a simultaneous dampening of certain T cell-mediated responses.

Peripheral blood lymphocytes isolated from pregnant mares demonstrate a reduced capacity to develop into effective cytotoxic T-lymphocytes (CTL) capable of lysing target cells from the breeding stallion (Baker et al., 1999). This reduction in T cell-mediated alloreactivity reverts after parturition or pregnancy termination, and it is not observed in males or non-pregnant females. This phenomenon seems logical, as the formation of anti-paternal cytotoxic cells during pregnancy could be disastrous for the semi-allogeneic fetus. However, a generalized reduction in cell-mediated immunity would make the mother susceptible to certain types of infections. It has

not yet been determined whether the alteration in the CTL activity of pregnant mares is limited to responses against paternal alloantigens. Studies using transgenic mice have demonstrated that peripheral maternal lymphocytes specific for paternal antigens may be inactivated or deleted during pregnancy (Ait-Azzouzene et al., 1998; Jiang and Vacchio, 1998; Tafuri et al., 1995). Studies of infectious diseases in conventional pregnant mice suggest broader antigen-independent mechanisms (Krishnan et al., 1996; Pejcic-Karapetrovic et al., 2007). Likewise, pregnant women appear to experience an increased susceptibility to infections such as *Listeria* and *Toxoplasma* (Avelino et al., 2003; Smith, 1999). While mares are vulnerable to a number of pregnancy-associated abortogenic infections (Ellis et al., 1983; Givens and Marley, 2008; Lunn et al., 2009), it is not clear whether this is due to a general systemic immune tolerance or pregnancy-associated tissue tropism.

The peripheral lymphocyte populations of pregnant mares have demonstrated few significant detectable alterations in phenotype. A modest increase in the number of circulating lymphocytes that express the TH2 cytokine IL-4 has been demonstrated during pregnancy (de Mestre et al., 2010). This finding is consistent with the high levels of paternal alloantibodies observed during pregnancy, as the presence of IL-4 favors a humoral immune response.

Locally acting mechanisms of immune tolerance

The maternal leukocytes that accumulate around the equine endometrial cups represent one of the most dramatic examples of a local cellular immune response to the conceptus. Immunohistochemical labeling of endometrial cup tissues has identified these leukocytes as primarily $CD4^+$ and $CD8^+$ T cells (Figure 1.2d,e) (Grunig et al., 1995). Mechanisms that operate where these maternal immune cells directly encounter placental antigens may dampen their effector activities by creating a local immunosuppressive environment. This strategy seems advantageous in that the systemic maternal responses can remain largely intact to defend against pathogens. Work by multiple groups has demonstrated trophoblast-produced soluble factors that may create such an environment by modulating the proliferation and blastogenesis of maternal lymphocytes. Ex-

tracts from day 80 placenta have been shown to inhibit the proliferation of maternal lymphocytes (Lea and Bolton, 1991), and co-culture of chorionic girdle trophoblasts with maternal lymphocytes caused a decrease in proliferation and a reduction in cytokine production (Flaminio and Antczak, 2005; Flaminio et al., 2004). Also, a >100,000 kDa molecule isolated from culture supernatants of day 20 conceptuses, termed horse conceptus-derived immunosuppressive factor, was found to inhibit lymphocyte proliferation by inhibiting IL-2R expression (Roth et al., 1992). Further investigation of trophoblast-produced immunomodulatory factors is warranted, based upon the important role they play in other species. In humans and mice, trophoblast molecules such as Fas ligand and indoleamine 2,3 dioxygenase (IDO) have been identified as providing protection from T-cell cytotoxicity (Hunt et al., 1997; Mellor and Munn, 2000); and the molecules Crry (mouse) and decay accelerating factor (human) confer protection from the complement cascade (Cunningham and Tichenor, 1995; Xu et al., 2000). hCG has been implicated as immunoregulatory molecule in human pregnancy (Tsampalas et al., 2009); however a study measuring *in vitro* inhibition of equine lymphocyte proliferation did not support such a role for eCG (Lea and Bolton, 1991).

Evidence also exists that the endometrium of the pregnant mare may be a primary source of local immunosuppressive factors. Prostaglandins in culture supernatant from endometrium of pregnant mares were shown to reduce lymphocyte blastogenesis (Watson, 1990; Watson and Zanecosky, 1991). Recently, local populations of regulatory T cells (Tregs) have been identified at the equine materno-fetal interface. The Treg marker FOXP3 has been demonstrated at both the gene and protein levels in the CD4+ cells that surround the endometrial cups (de Mestre et al., 2010). Endometrial cup lymphocytes isolated from day 43-46 of pregnancy showed a 3-fold increase in the number of CD4+FOXP3+ cells compared to peripheral lymphocytes. This is consistent with an increase in Tregs observed during pregnancy in multiple other species (Aluvihare et al., 2004; Oliveira and Hansen, 2008; Sasaki et al., 2007; Tilburgs et al., 2008; Zenclussen et al., 2006). Local regulatory activity by Tregs at the placental interface may be a mechanism by which the early MHC class I+ trophoblast populations are able to resist destruction by the large accumula-

tion of maternal lymphocytes with which they are in contact.

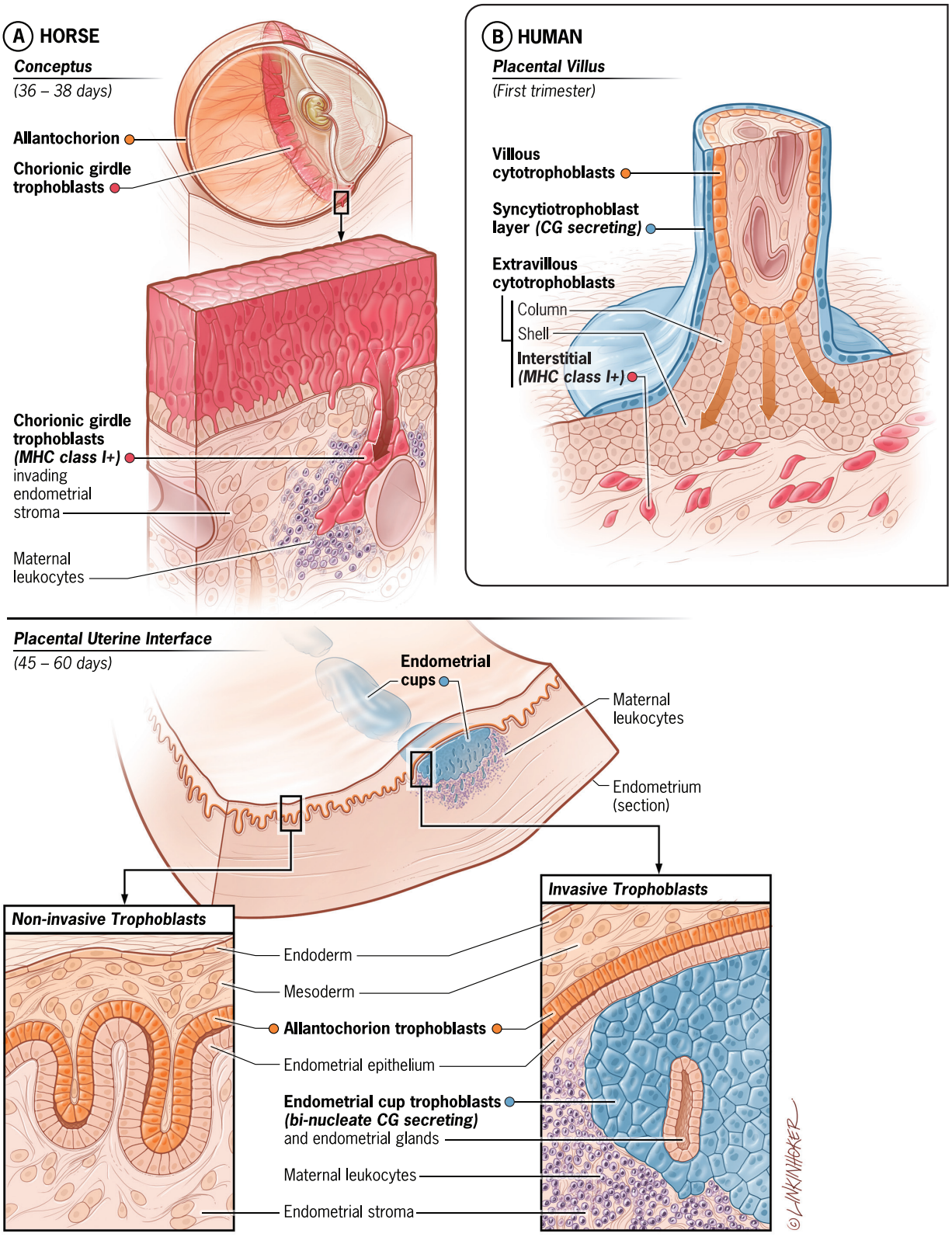
In the same day 43-46 endometrial cup lymphocyte samples, an increase in the number of interferon gamma (IFNG)+ lymphocytes was also observed. This observation initially appears to be in conflict with the traditional dogma of a TH2 bias during successful pregnancy. However, recent studies have implicated IFNG at the materno-fetal interface as having a critical role in human, murine, and porcine pregnancy (Murphy et al., 2009).

Value of the horse in the study of pregnancy immunology

Several aspects of equine pregnancy make its study useful in understanding eutherian materno-fetal interactions. In addition to the aforementioned maternal immune responses that distinguish the horse, its specialized trophoblast populations, combined with advances in assisted reproductive technologies, immunological reagents, and genomic resources, make the horse a uniquely valuable species in the advancement of pregnancy immunology.

The trophoblast populations of human and horse placentas share significant phenotypic similarities. For each of the three principal types of equine trophoblast, there is a human counterpart (Figure 1.4). The basic cell types and essential properties are conserved between these parallel groups, however some functions have been distributed differently. The equine allantochorion trophoblasts correspond to the human villous cytotrophoblasts (Figure 1.4, orange). Both are mononuclear cells with stem cell-like properties that enable them to differentiate into other trophoblast types. They both express low levels of MHC class I mRNA, but not protein (Bacon et al., 2002; Hunt et al., 1990). The allantochorion trophoblasts are the primary mediators of nutrient exchange in the horse, whereas the syncytiotrophoblast layer provides this function in the human placenta. The chorionic girdle trophoblasts are similar to human extravillous trophoblasts (Figure 1.4, red). Both cell types are invasive and migrate into the endometrial stroma. They both express MHC class I antigens from more than one locus, although the genes are not homologous (Donaldson et al., 1994; Hunt et al., 1990; Kovats et al., 1990; Moffett-King, 2002).

Figure 1.4 Comparison of horse and human trophoblast populations. This diagram summarizes the similarities in cell type, gene expression, and function between the three principal horse (A) and human (B) trophoblast types during early pregnancy. Analogous trophoblast populations of the two species are color matched. See text for a detailed comparison. The top of the horse diagram (A) depicts the period surrounding the invasion of the chorionic girdle trophoblasts into the endometrium; the bottom shows after the endometrial cups have been established. The diagram of the human placental villus (B) represents a time during the first trimester when extravillous cytotrophoblasts migrate into the endometrium.


A HORSE
Conceptus
(36 – 38 days)
Allantochorion
Chorionic girdle trophoblasts
Chorionic girdle trophoblasts (MHC class I+) invading endometrial stroma
Maternal leukocytes
B HUMAN
Placental Villus
(First trimester)
Villous cytotrophoblasts
Syncytiotrophoblast layer (CG secreting)
Extravillous cytotrophoblasts
Column
Shell
Interstitial (MHC class I+)
Placental Uterine Interface
(45 – 60 days)
Endometrial cups
Maternal leukocytes
Endometrium (section)
Non-invasive Trophoblasts
Invasive Trophoblasts
Endoderm
Mesoderm
Allantochorion trophoblasts
Endometrial epithelium
Endometrial cup trophoblasts (bi-nucleate CG secreting) and endometrial glands
Maternal leukocytes
Endometrial stroma
©LINKWORKER

Lastly, the equine endometrial cup trophoblasts correspond to the human syncytiotrophoblasts (Figure 1.4, blue). Both cells types are multi-nucleate, sessile, and terminally differentiated. They suppress MHC class I gene expression at the transcriptional level (Hunt et al., 1990; Maher et al., 1996) and secrete chorionic gonadotropins. Only primate and equid species are known to produce placental gonadotropins (Fiddes and Talmadge, 1984; Pierce and Parsons, 1981). Additionally, recent molecular studies have identified transcription factors involved in trophoblast differentiation that are conserved between horse and human placentas (de Mestre et al., 2009).

Another relevant similarity between human and horse pregnancy is the extended gestation length. The mare's 340 day gestation allows adequate time for full engagement and commitment of the adaptive immune system. The resulting anti-paternal humoral immune response observed in nearly all mares carrying histoincompatible pregnancies is easily monitored through measurement of serum antibody levels (Antczak et al., 1982; Antczak et al., 1984).

Up to day 36, the equine conceptus can be recovered non-surgically from the mare's uterus, enabling the collection of pure trophoblasts which can be further investigated *in vitro* (Antczak et al., 1987). The purified trophoblasts can be maintained in culture and driven to differentiate, allowing the opportunity for *in vitro* manipulation of specific populations (de Mestre et al., 2008). Isolated chorionic girdle trophoblast can also be transplanted into non-pregnant recipient mares using a novel *in vivo* system (Adams and Antczak, 2001; de Mestre et al., 2008). The transplanted trophoblasts undergo autonomous terminal differentiation in ectopic sites independent of the physiological state of pregnancy. They stimulate maternal antibody responses and attract T cells to the sites of transplantation, and yet evade immediate destruction by the immune system of the recipients. The trophoblasts also maintain their endocrine capacity and produce eCG (de Mestre et al., 2008).

In addition to the characteristics that make the horse unique as a species in the study of pregnancy immunology, many advantages offered by commonly used animal models apply. The MHC of the horse has been well-characterized using functional and genetic studies (Carpenter

et al., 2001; Ellis et al., 1995; Gustafson et al., 2003; Lazary et al., 1988; Tallmadge et al., 2010; Tallmadge et al., 2005). Horses have been selectively bred for homozygosity at the MHC region, enabling the establishment of MHC-compatible and -incompatible pregnancies to investigate the role of paternal antigens in maternal immune recognition (Adams and Antczak, 2001). Advanced assisted reproductive techniques, such as artificial insemination and embryo transfer, are routinely used in horse breeding. Notably, embryo transfer is performed in thousands of horses every year worldwide with high success rates (Carney et al., 1991), suggesting that the insemination-induced tolerance that plays a role in pregnancy in some species (Robertson and Sharkey, 2001) may be less important in others. Other more advanced techniques such as oocyte transfer, intracytoplasmic sperm injection, and nuclear transfer (cloning), are also successfully used in horse reproduction (Coutinho da Silva, 2008). These techniques are primarily used to generate genetically desirable offspring, but they can also be useful tools in understanding early reproductive events such as fertilization and conception.

Recent advances in equine genomics and immunology have expanded opportunities for the study of pregnancy immunology at the mechanistic level. A 6.8X sequence of the equine genome has been determined and extensively annotated (Wade et al., 2009). Multiple horse-specific expression microarrays have been developed and validated, allowing researchers to investigate the expression of thousands of genes simultaneously (Glaser et al., 2009; Gu and Bertone, 2004; Mienaltowski et al., 2009; Ramery et al., 2009). Molecular advances have also facilitated the development of new horse-specific monoclonal antibodies (Wagner et al., 2006; Wagner et al., 2008a; Wagner et al., 2008b; Wagner et al., 2005) and immune assay technologies (Wagner and Freer, 2009).

Discussion

Our understanding of the mare's immune responses during pregnancy has progressed substantially, but several critical questions still remain. Firstly, why do the chorionic girdle trophoblasts

express such high levels of paternal MHC class I while invading the maternal endometrium? The horse is not unique in this respect— MHC class I expression can be observed in trophoblast populations of other species at various stages of placentation. However, the horse demonstrates the clearest evidence for maternal immune recognition of paternal alloantigens expressed by trophoblast. A proposed role for the expression of HLA molecules by human invasive extravillous trophoblasts is to confer protection from cytotoxic NK cells (Rouas-Freiss et al., 1997). NK cells have been putatively identified in the periphery of the horse (Lunn et al., 1995; Viveiros and Antczak, 1999), but not yet in the uterus. *In vitro*, peripheral equine NK-like lymphokine activate killing (LAK) cells have shown the capacity to lyse differentiated MHC class I negative binucleate chorionic girdle cells (Vagnoni et al., 1996). However, their role *in vivo* has not been determined. Studies of porcine pregnancy have demonstrated that NK cells can be recruited to the uterus of a species with epitheliochorial placentation (Croy et al., 1988). The advent of new reagents to detect equine NK cells should help address this question.

A second pressing question is why and how the endometrial cups are ultimately destroyed after two months of successful evasion of maternal immune effectors. Clusters of $CD4^+$ and $CD8^+$ lymphocytes and inflammatory leukocytes are seen within sections of dying cups (Grunig et al., 1995). Here, in the absence of MHC class I antigen expression it is possible that NK cells could be acting as cytotoxic cells. However it is not clear whether infiltrating immune cells are a primary cause of destruction of the cups or if they simply undergo apoptosis at the end of their natural lifespan.

Evidence for an immunological basis for endometrial cup destruction has been demonstrated by experimental interspecies matings. In a standard MHC incompatible horse mating, there is no change in the lifespan of the cups with multiple pregnancies (Adams et al., 2007). However, when mares are mated to male donkeys to produce mule pregnancies, the cups are destroyed earlier in subsequent pregnancies, suggestive of an anamnestic response (Antczak and Allen, 1984). Lymphocytes from mares carrying mule pregnancies do not demonstrate reduced CTL activity *in*

vitro against cells from the donkey sire (Baker et al., 1999), indicating a failure in the systemic dampening of cell mediated immunity in these interspecies matings.

A more dramatic version of an apparent immune-based destruction of the endometrial cups is seen in the donkey-in-horse pregnancy model. While most females of the genus *Equus* can successfully carry a pregnancy from any of the other species following embryo transfer, only rarely can a horse maintain a transferred donkey embryo (Allen, 1982; Allen et al., 1987). In this situation, the chorionic girdle fails to invade the endometrium of the surrogate mare. No endometrial cups form and there is no detectable eCG in the serum. Large numbers of endometrial leukocytes are seen at the border of the non-invasive allantochorion, which abnormally expresses MHC class I antigens and fails to interdigitate with the maternal endometrium (Antczak and Allen, 1988; Enders et al., 1996; Kydd et al., 1991). Furthermore, these mares carrying embryo transfer donkey conceptuses also appear to demonstrate an anamnestic response; mares that abort one donkey pregnancy abort subsequent pregnancies of this type earlier in gestation (Enders et al., 1996).

The breeding of *in utero* immunotolerized chimeric twins has also lent insights into the role of immune mechanisms in endometrial cup destruction. In an experiment where female horses were bred to their male co-twins, peripheral eCG was detectable through day 220-260, roughly double the normal time-frame (Bouters et al., 1978; Spincemaille et al., 1975). This significantly extended lifespan of the endometrial cups suggests that foreign paternal antigens may play a role in their destruction. With the increased success of equine cloning (Hinrichs, 2006), this question may be further addressed.

Endometrial cup destruction is sometimes delayed, leading to a clinical condition termed “persistent endometrial cups” (Allen et al., 2007; Willis and Riddle, 2005). It can occur in mares that abort after the endometrial cups have formed, and in normal post-partum mares. It has some similarities to post-partum microchimerism seen in women (Gammill and Nelson, 2010). The persistent cups remain active and eCG can be detectable in the sera beyond the usual time-frame.

Consequently, return to estrous cyclicity is delayed (Willis and Riddle, 2005). The persistent cups eventually die, but it is not know why they survive beyond the standard time frame as multiple allografts within a non-pregnant animal. Further study of this phenomenon would be useful in understanding the signals that initiate and terminate maternal tolerance.

In conclusion, the pregnant mare's immune responses to the trophoblast of her developing placenta are fascinating in their complexity. By providing a window into the nature of materno-fetal interactions, the horse has illuminated immunological events not easily detectable in other species. Future studies in equine pregnancy hold great promise in the revelation of more secrets of the materno-fetal immunological relationship.

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CHAPTER 2

SPLIT IMMUNOLOGICAL TOLERANCE TO TROPHOBLAST[1,2]

[1] de Mestre, A., Noronha, L., Wagner, B., and Antczak, D.F. (2010). Split immunological tolerance to trophoblast. *International Journal of Developmental Biology* 54, 445-455. Reprinted with permission from UBC press.

[2]LEN contribution: peripheral cells studies; *FOXP3* cloning and qPCR

Abstract

Split immunological tolerance refers to states in which an individual is capable of mounting certain types of immune responses to a particular antigenic challenge, but is tolerant of the same antigen in other compartments of the immune system. This concept is applicable to the immunological relationship between mother and fetus, and particularly relevant in equine pregnancy. In pregnant mares, antibody responses to paternal foreign Major Histocompatibility Complex class I antigens are robust, while anti-paternal cytotoxic T cell responses are diminished compared to those mounted by non-pregnant mares. Here we compared the distribution of the major lymphocyte subsets, the percentage of lymphocytes expressing Interferon Gamma (IFNG) and Interleukin 4 (IL4) and the level of expression of the immunoregulatory transcription factor FOXP3 between pregnant and non-pregnant mares, and between peripheral blood and the endometrium during pregnancy. In a cohort of mares in which peripheral blood lymphocytes were tested during early pregnancy and in the non-pregnant state, there were only slight changes observed during pregnancy. In contrast, comparison of peripheral blood lymphocytes with lymphocytes isolated from the endometrial cups of pregnant mares revealed striking differences in lymphocyte sub-populations. The endometrial cups contained higher numbers of IFNG+ lymphocytes, and lower numbers of lymphocytes expressing IL4. The endometrial cup lymphocytes also had higher numbers of FOXP3+ cells compared to peripheral blood lymphocytes. Taken together, these results strengthen the evidence for a state of split tolerance to trophoblast, and furthermore define sharp differences in immune reactivity during equine pregnancy between peripheral blood lymphocytes and lymphocytes at the maternal-fetal interface.

Introduction

The paradox of the successful fetus-as-allograft paradigm was first proposed by Peter Medawar in 1953, and hypotheses put forth in that classic paper have generated numerous clinical and experimental studies in the field of pregnancy immunology. Now 55 years later, many strategies and mechanisms have been identified that may explain how the fetus escapes recognition

and destruction by the maternal immune system. These include repression of expression of alloantigens and tissue specific antigens in the placenta, systemic alterations in the character of maternal immune responses during pregnancy, and locally operating mechanisms of trophoblast cells that protect the fetal tissues against destruction by maternal immune effector cells and molecules. Progress in these areas has been evaluated in a number of comprehensive reviews (Billington 2003; Caucheteux et al., 2003; Koch and Platt, 2003; Hunt, 2005; Moffett and Loke, 2006; Trowsdale and Betz, 2006; Zenclussen et al., 2007; Seavey and Mossman, 2008).

In descriptions of the fetal-maternal immunological relationship, the concept of maternal tolerance to the fetus is often used (Mellor and Munn, 2000; Robertson and Sharkey, 2001; Kannellopoulos-Langevin et al., 2003; Aluvihare et al., 2004; Blois et al., 2007). Although immunological tolerance is well understood operationally, tolerance can be achieved by many distinct mechanisms, and not all of them have yet been elucidated. A number of experimental approaches have identified both antigen-specific and non-specific tolerogenic mechanisms operating during pregnancy. In T cell transgenic mice there is evidence for either deletion or inactivation of T cells (Tafuri et al., 1995; Jiang & Vacchio, 1998) or B cells (Ait-Azzouzene et al., 1998, 2001) reactive with paternal Major Histocompatibility Complex (MHC) class I antigens or the H-Y minor histocompatibility antigen. The antigen-specific effects have largely been detected using transgenic mice expressing only a single specificity of T cell receptor, whereas non-specific mechanisms have been detected in normal mice (Krishnan et al., 1996a, b; Aluvihare et al., 2004; Pejcic-Karapetrovic et al., 2007).

It is not clear if and how these two principal types of feto-maternal tolerance are related. Antigen specific mechanisms have the great advantage of leaving the remainder of the mother's immune system intact, allowing her to defend herself against infection during pregnancy. However, they also require that the conceptus express the antigens to which tolerance is induced. The antigen non-specific mechanisms of tolerance do not require information about the specific histocompatibility challenge of the fetus. Their disadvantage, however, is that they might alter the mother's

immune system in ways that would make her more susceptible to certain types of infection during pregnancy. The increased susceptibility of pregnant women to Toxoplasma and Listeria infection may reflect such an untoward effect (Smith, 1999; Avelino et al., 2003).
The mechanisms by which maternal tolerance to the fetus is induced are not yet fully understood, but critical components may include local signals from sperm, seminal fluid, and the developing conceptus, the hormonal state of pregnancy, and in the case of antigen-specific tolerance, expression of MHC molecules by the conceptus in a context that favors tolerance over immunity (Robertson et al., 1997; Robertson & Sharkey, 2001). The detection of expanded numbers of circulating or locally accumulated regulatory T cells (Tregs) in normal mouse (Aluvihare et al., 2004; Zenclussen et al., 2006) and human (Tilburgs et al., 2008) pregnancy provides a framework focused on a CD4+CD25+ FOXP3+ T cell (Ramsdell, 2003; Wood & Sakaguchi, 2003; Nagler-Anderson et al., 2004).

The term 'split tolerance' has two meanings in immunology. In the context of tissue and organ transplantation, it refers to the observation that grafts of some tissues, classically liver, may be accepted by a recipient while grafts of other tissues from the same donor, for example, skin, are rejected (Qian et al., 1997; Chan et al., 2008; Chung et al. 2005; Luo et al., 2007; Matthes et al., 2003). The second and more relevant use of the term for this study has broader implications in immunological tolerance. It refers to states in which an individual is capable of making some types immunological responses to a particular antigenic challenge, but is apparently tolerant to the same antigen from the perspective of other immune system compartments (Sprent et al., 1995; Hunziger et al., 1997; Baker et al., 2001). Although the mechanisms leading to split tolerance are not well understood, we propose that the operational definition as presented may be useful in shaping a new framework for the complex immunological relationship between mother and fetus.

The equine placenta is of the non-invasive epitheliochorial type, with six intact cell layers separating maternal and fetal blood supplies. The principal interface between uterus and

placenta is an interdigitation of endometrial epithelium with allantochorion trophoblast that forms characteristic microvilli (Allen 1975). The trophoblast cells at this interface do not express either MHC class I or MHC class II antigens (Donaldson et al., 1990, 1992; Maher et al., 1996), and thus do not pose an immunological challenge to the mother. However, equids also have a minor subpopulation of invasive trophoblasts that do express MHC molecules as they migrate into the endometrium to form the endometrial cups (Fig. 1). The invasive equine chorionic girdle trophoblasts and the early endometrial cup trophoblast cells express very high levels of polymorphic, paternal and maternal MHC class I antigens during a short window in early pregnancy between days 30 and 45 of gestation (Donaldson et al., 1992, 1994). The level of expression of these MHC class I antigens is similar to that found on lymphocytes and other antigen presenting cells of the immune system, and about 10 fold higher than the level found on other somatic tissues (Bacon et al., 2002).

Virtually 100% of mares carrying MHC incompatible pregnancies mount strong primary or secondary antibody responses to the foreign paternally inherited MHC class I antigens of their fetuses, and the timing of this response is consistent with induction by the MHC class I positive chorionic girdle and early endometrial cup cells (Antczak et al., 1982, 1984). Transplantation of allogeneic trophoblast has demonstrated that the chorionic girdle cells are capable of producing this immunological sensitization on their own (Adams & Antczak, 2001; de Mestre et al., 2008). These observations demonstrate conclusively that the B cell compartment of the pregnant mare's immune system is not tolerized, and suggests that T cells required to 'help' B cells produce antibody may also be activated during pregnancy.

At the level of the fetal-maternal interface, the invading MHC class I positive trophoblasts of the early endometrial cups attract a striking accumulation of maternal CD4+ and CD8+ T lymphocytes around them, but this apparent cellular immune response does not result in immediate destruction of the endometrial cups (Fig. 2) (Grunig et al., 1995). Once the endometrial cups are fully formed, the binucleate, equine chorionic gonadotrophin (eCG)

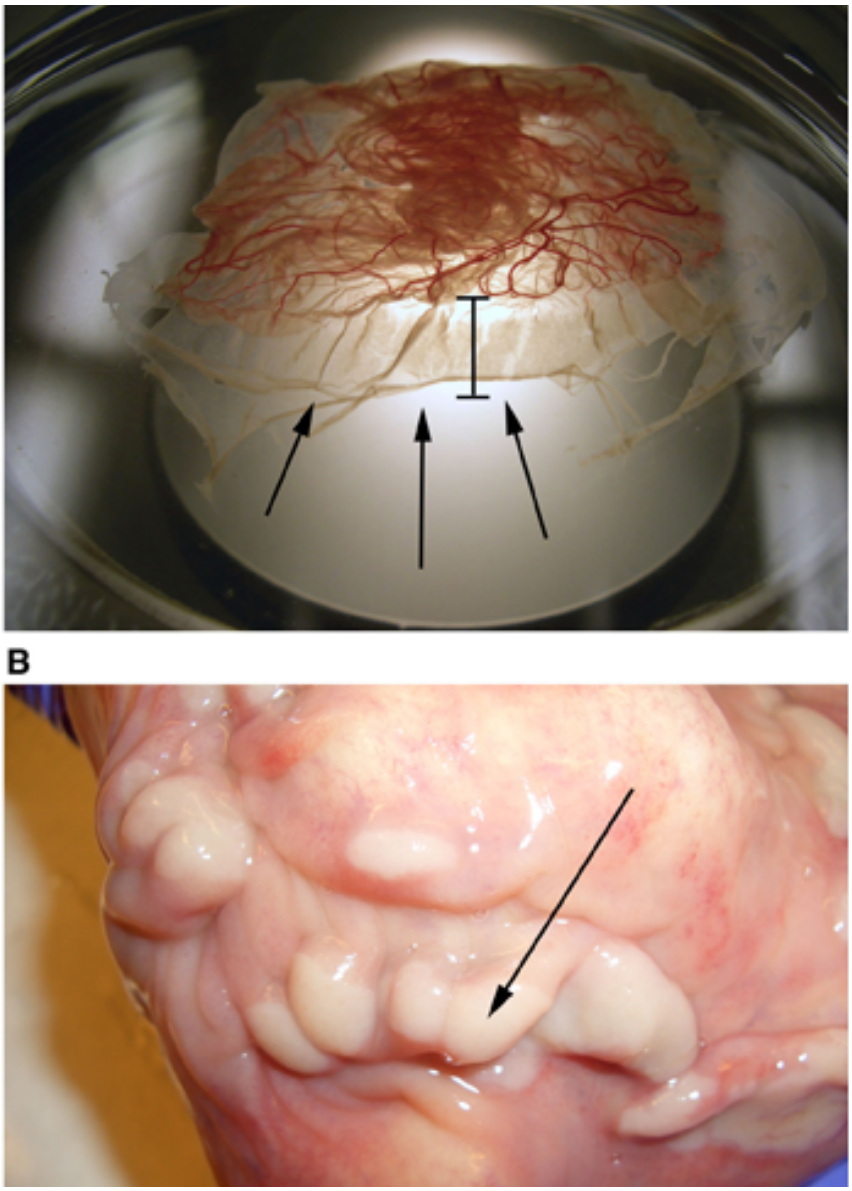
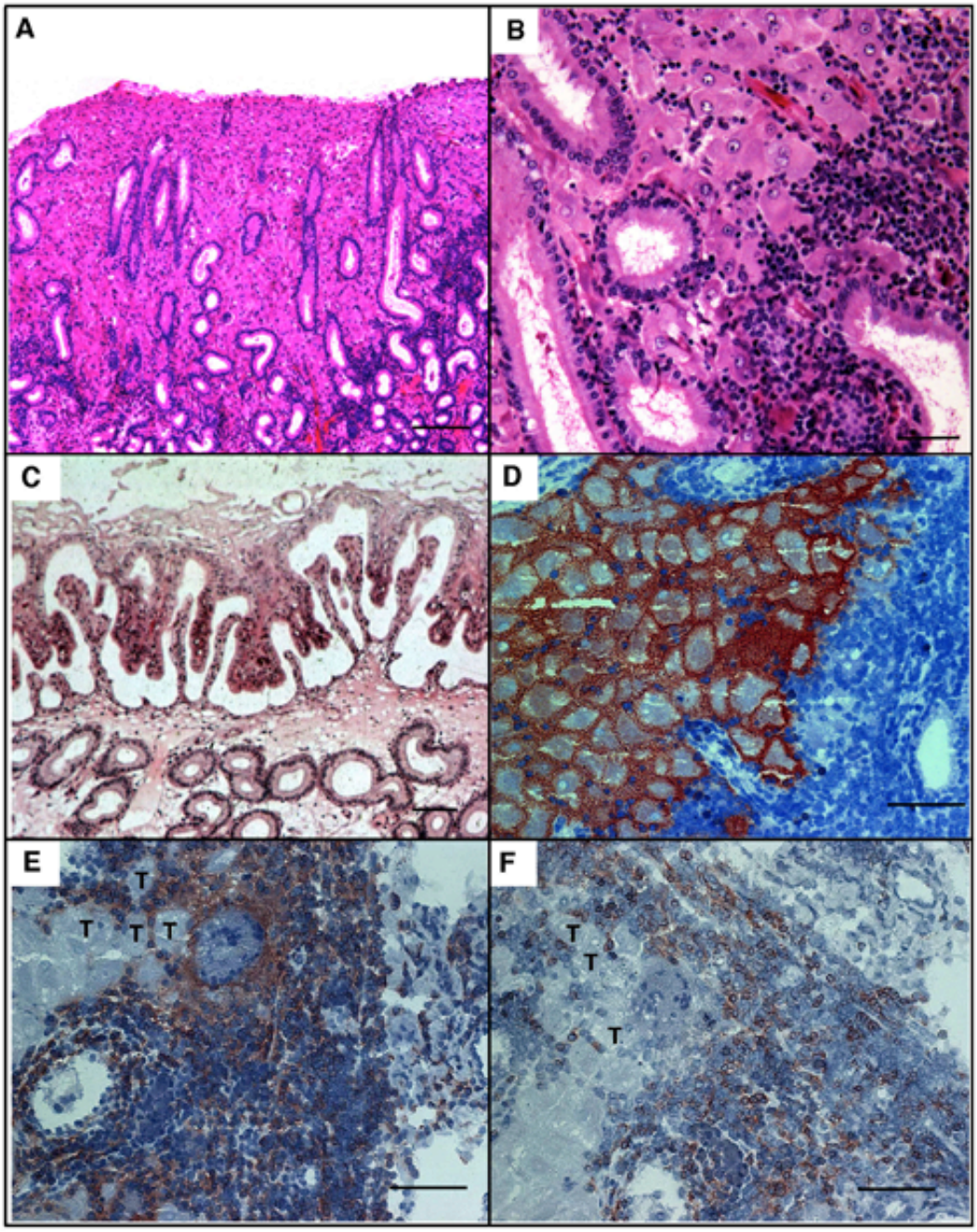


Figure 2.1 Gross specimens of day 34 equine conceptus and endometrial cups from day 45 of gestation. A. Day 34 conceptus showing the horizontal band of invasive trophoblast of the chorionic girdle, demarcated by the vertical bracket and arrows. At day 36-38 of gestation the chorionic girdle cells migrate into the endometrium to form the mature, eCG secreting endometrial cups. Specimen obtained by non-surgical uterine lavage. B. Mature endometrial cups at day 45 of gestation shown in the endometrium. Arrow points to a strip of cups, which average 1 cm in diameter. Specimen obtained at necropsy.

Figure 2.2 Histological images of endometrial cups and endometrium from early equine pregnancy. H&E stained fixed sections: A. Low power image of endometrial cup showing accumulations of maternal lymphocytes concentrated along the periphery of the cup. B. High power image from A., showing aggregations of maternal lymphocytes, endometrial glands, and large binucleate pale staining endometrial cup trophoblast cells. C. Endometrium – allantochorion border. Note the lack of lymphocyte accumulations at the placental-uterine interface. D-F Immunohistochemical labeling of frozen sections of endometrial cups. D. Monoclonal antibody 102.1 (anti-horse trophoblast), showing distinct margin of the endometrial cup. Anti-horse CD4 antibody (E.) and anti-horse CD8 antibody (F.) labeling the respective T cell subsets surrounding endometrial cup trophoblasts (T). Size bar indicates 100μm in all panels except A (400μm). All specimens are from day 43-46 of gestation.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secreting trophoblast cells of the cups down regulate expression of their MHC genes (Donaldson et al., 1992; Maher et al., 1996). Paradoxically, the local lymphocyte-dominated response appears to eventually result in the destruction of the endometrial cups, which is usually complete between days 80 and 120 of the mare's 335 day gestation (Allen 1979).

Earlier work from our group identified a decrease in the capacity of peripheral blood lymphocytes from pregnant mares and jenny donkeys to develop into alloreactive cytotoxic lymphocytes after in vitro culture with irradiated lymphocytes from MHC incompatible mating stallions or jack donkeys (Baker et al., 1999). Thus, the peripheral cytotoxic T cell (CTL) response to paternal alloantigens seems to be impaired during normal equine pregnancy, while the B cell response remains intact. We hypothesized that other systemic differences might exist between peripheral lymphocytes of pregnant and non-pregnant mares, and we therefore examined several variables for this study. We also tested a second hypothesis, that immune reactivity would differ between peripheral and local immune compartments, and here we compared tissue lymphocytes from the endometrium and endometrial cups with peripheral blood lymphocytes obtained on the same day of gestation from pregnant mares.

Materials and Methods

Animals

Adult horses of mixed breeds and ages were used in this research (Tables 1 and 2). Horses were maintained at the Baker Institute for Animal Health, Cornell University. Animal care was performed in accordance with the guidelines set forth by the Institutional Animal Care and Use Committee of Cornell University. Pregnancies were established as previously described (Adams and Antczak, 2001). Major Histocompatibility Complex haplotypes were assigned to the horses based on results of tissue typing using a panel of well-characterized alloantisera that had been validated in international workshops (Lazary et al., 1988).

Table 2.1 Mares used for comparison of immune status in peripheral blood in the pregnant and non-pregnant states.

Mare ID	Mare MHC haplotype#	Mating stallion MHC haplotype#	Gestational day of PBMC isolation
3845	A5 / W16	A2 / A2	31
3837	? / ?	A2 / A2	29
3157	A3 / A3	A2 / A2	30
3638	A7 / ?	A3 / A3	30
2885	A3 / A3	A2 / A2	31
3419	A2 / A5	A3 / A3	32
3099	A2 / A2	A3 / A3	29
3725	A2 / ?	A3 / A3	31
3641	A3 / A19	A2 / A2	31
3820	A19 / W16	A2 / A2	32
3354	A3 / A3	A2 / A2	29
3640	A3 / ?	A2 / A2	30
3493	A2 / ?	A3 / A3	31
2998	? / ?	A3 / A3	30
3821	A6 / W16	A3 / A3	30

MHC types of the horses were determined by a standard lymphocyte microcytotoxicity assay using alloantisera to Equine Leukocyte Antigen (ELA) markers that had been validated in international workshops, as described in the Materials and Methods. Equine MHC haplotypes are designated by the letter A followed by a number. The two mating stallions were purpose-bred MHC homozygotes from the Cornell experimental herd. Mares known to be MHC homozygotes are indicated with the same nomenclature used for the stallions. Mares with one or two “?” designations are either homozygotes for the single defined haplotype they carry, or heterozygotes carrying a haplotype (or two) for which no identifying antisera are available. The undetermined MHC haplotypes were not ELA-A2 or ELA-A3.

Table 2.2 Mares used for endometrial cup lymphocyte isolation and characterization

Mare ID	Mare MHC haplotype#	Mating stallion MHC haplotype#	Gestational day of PBMC and ECL* isolation	Number of cells isolated from endometrial cups
3382	A10 / ?	A3 / A3	46	60.5×10^6
3549	A19 / ?	A3 / A3	43	26.0×10^6
3842	A5 / ?	A2 / A2	45	16.7×10^6
3845	A5 / ?	A3 / A3	44	6.2×10^6
3837	A8 / ?	A3 / A3	44	26.5×10^6
3901	A2 / A19	A2 / A2	45	25.5×10^6

For a description of the MHC typing methods and assignments, see footnote to Table 1.
* ECL: Endometrial Cup Lymphocytes

Tissue and cell preparation

Heparinized samples of venous jugular blood were collected from mares during diestrus, at day 31±2 of pregnancy, or immediately prior to euthanasia, as indicated. Peripheral blood mononuclear cells (PBMC) and peripheral blood lymphocytes (PBL) were isolated using methods described previously (Antczak et al, 1982; Wagner et al., 2008). Endometrial cup lymphocytes (ECL) and endometrial lymphocytes (ENDO L) were isolated using an adaptation of a previously described method for human endometrial lymphocytes (Flynn et al., 1999). Equine uteri were obtained surgically immediately following euthanasia of six mares confirmed by transrectal ultrasonography to be day 43 to day 46 pregnant. One mare was pregnant with twins. The tissue was placed immediately into Hanks Balanced salt solution (Gibco Invitrogen Corp, Carlsbad, CA) supplemented with 5% fetal calf serum (FCS, Hyclone, Logan, Utah). The endometrial cups and approximately 2-3 mm of adjacent endometrium were dissected free of the remaining uterine tissues. The average weight of the endometrial cup tissue was nine grams. Endometrial tissue was collected from a site distal to the endometrial cups. Tissue was minced using scissors, then placed into a enzyme solution containing RPMI medium with 25 mM Hepes (Gibco Invitrogen Corp), 1% FCS, 1% (w/v) bovine serum albumin (Sigma, St Louis, MO), and 35 U/ml DNase (Sigma) and incubated at 37 degrees. After 10 minutes, collagenase (Sigma) was added to the enzyme solution at a concentration of 200 U/ml and the tissue incubated at 37 degrees for an additional 20 minutes. Tissue was then passed through 100 µm and 40 µm cell strainers (BD Biosciences, San Jose, CA). The cell suspension was then washed in phosphate buffered saline (PBS) /0.5% FCS. Cell suspensions were subjected to fractionation using Ficoll-Paque Plus (GE Healthcare, Piscataway, NJ). Lymphocyte enriched cell suspensions were then washed twice in PBS /0.5% FCS. Viability of isolated cells was confirmed using trypan blue exclusion, and found to be greater than 80-90% for all samples. The total number of cells isolated from endometrial cups ranged from 1.1-3.6 x 10^6 cells/g of tissue (Table 2).

Cell Culture and Fluorescent labeling of cells and flow cytometry

ECL, ENDO L and PBMC were either fixed in 2% paraformaldehyde (Sigma) or stimulated with 25 ng/ml phorbol 12-myristate 13-acetate (PMA) and 1 μM ionomycin (IO) in the presence of 10 μg/ml brefeldin A as previously described (Wagner et al., 2008). After 4 hours in culture, the stimulated cells were washed in PBS and fixed. Cells were labeled with monoclonal antibodies to equine cell surface markers CD4 (HB61A, VMRD, Pullman, WA), CD8 (CVS8, Lunn et al, 1998) and CD19 (CZ2.1, Lunn et al, 1998). Cytokine staining was performed using anti-bovine interferon gamma (IFNG) (MorphoSys, AbD Serotec, Oxford, UK) and anti-equine interleukin 4 (IL4) as previously described (Wagner et al., 2005, 2006). CD4, CD8, and IL4 antibodies were conjugated to Alexa dyes (A647 or A488) and anti-bovine IFNG was FITC conjugated by the supplier. For detection of intracellular expression of forkhead box P3 (FOXP3), ECL or PBL were isolated as described above. Freshly isolated cells were labeled with a directly conjugated antibody to equine CD4, followed by fixation, permeabilization, and labeling using a FOXP3 staining kit (eBioscience, San Diego, CA) and a cross reactive PE conjugated antibody to human FOXP3 (clone PCH101, eBioscience) or an IgG2a isotype control antibody (eBioscience) as per the manufacturer's instructions. Immunofluorescence flow cytometry was performed using a BD FACSCalibur (BD, Franklin Lakes, NJ) and data analysis was performed using Flowjo software (Tree Star, Ashland, OR). For statistical comparison of the ECL and PBMC/PBL samples, and pregnant and non-pregnant PBMC, paired two-tailed Student's t tests or Wilcoxon rank sum tests were used with alpha error = 5% using GraphPad Prism software. For statistical comparison of the ECL, PBMC, and ENDO L samples, Krustal-Wallis or Tukey's one-way analysis of variance tests were used with an alpha error = 5% using GraphPad Prism software.

RNA isolation, cDNA synthesis Real Time RT-PCR

ECL and PBMC were isolated as described above. RNA was isolated from 5 x10^6 snap frozen cells, following homogenization by QIAshredder (Qiagen, Valencia, CA), using a RNeasy kit (Qiagen) as directed by the manufacturer. Five hundred nanograms of RNA was treated with

DNase I (Invitrogen, Carlsbad, CA), then first strand cDNA synthesis was carried out using M-MLV Reverse Transcriptase (USB, Cleveland, OH) as per the manufacturer's guidelines. SYBR Green (Applied Biosystems, Shelton, CO) real time RT-PCR reactions for amplification of equine FOXP3 or the housekeeper gene equine ubiquitin-conjugating enzyme E2D 2 (UBE2D2) (de Mestre et al., 2003) mRNA were performed using a ABI PRISM 7700 or 7500 Fast sequence detector (PerkinElmer Life Sciences) in a total volume of 20 µl. Primers were designed over intron / exon boundaries to prevent amplification of genomic DNA. A dissociation curve was performed after each experiment to confirm that a single product was amplified. A standard curve was generated for FOXP3 and UBE2D2 genes using known copy numbers of a plasmid that contained the cDNA specific to the gene. Each FOXP3 sample was first normalized to 7500 copies of UBE2D2. The percentage of CD4+ lymphocytes in an aliquot of each sample was determined by flow cytometric analysis and FOXP3 mRNA expression was normalized to 50% CD4+ lymphocytes. The sequences of the oligonucleotides are FOXP3RT1: TGGCAAATG-GTGTCTGCAA; FOXP3RT2: GCGCTCTGCCCTTCTCATC; UBC1: TGAAGAGAATCCA-CAAGGAATTGA; UBC2: CAACAGGACCTGCTGAACACTG. Changes in expression were analyzed for statistical significance by using a paired two-tailed Student's t test.

Tissue immunohistochemistry

Sections of endometrial cups and endometrium obtained at necropsy were fixed in buffered formaldehyde for conventional histology or transferred immediately to O.C.T. embedding compound (VWR Scientific Products, Willard, OH), snap frozen in an isopentane bath in liquid nitrogen, and then stored at -80 degrees C. Immunohistochemical labeling of frozen sections was performed as previously described (de Mestre et al., 2008).

Results

Prior work had established that lymphocytes from pregnant mares show a decrease in capacity to generate cytotoxic T cells towards the mating stallion compared to the non-pregnant state (Baker

et al. 1999). In the first part of this study, selected additional aspects of the immune status of a cohort of 15 mares was compared during pregnancy and in the non-pregnant state. Jugular blood samples were obtained prior to the establishment of pregnancy, and again at about 30 days of gestation, a stage in which the decrease in CTL reactivity had been readily detected. The composition of the lymphocyte populations, cytokine profiles, and FOXP3 expression were determined in PBMC samples from the mares. All of the mares were mated to produce MHC incompatible conceptuses, using one of two MHC homozygous stallions (Table 1).

Peripheral CD4 and CD8 populations in early pregnancy in the mare

Flow cytometry with equine specific monoclonal antibodies was used to determine the percentage of peripheral blood lymphocytes expressing the T lymphocyte subset markers CD4 or CD8 (Figure 3). The average percentage of CD4+ lymphocytes was 56% in the non-pregnant state, and 54% in the pregnant state, and ranged between 40% and 70% of total lymphocytes.

The average percentage of CD8+ lymphocytes was 16.5% in the non-pregnant state, and 17% in the pregnant state, and ranged between 9% and 23% of total lymphocytes. There were no significant changes in the percentages of CD4+ lymphocytes or CD8+ lymphocytes between the pregnant and non-pregnant states. However, there was a trend towards decreasing CD4+ lymphocytes and increasing CD8+ lymphocytes in the mares during pregnancy. Thus, the ratio of CD4:CD8 was significantly reduced in pregnancy compared to the non-pregnant state.

Changes in cytokine producing lymphocytes in early equine pregnancy

The percentages of peripheral blood lymphocytes expressing IFNG or IL4 were determined using newly characterized monoclonal antibodies reactive with these equine cytokines (Figure 4). In the population of mares under study, the percentage of IFNG+ lymphocytes varied considerably in both the non-pregnant and pregnant groups. In the mares sampled when pregnant, there was a trend towards increases in the percentages of IFNG+ cells in the overall lymphocyte

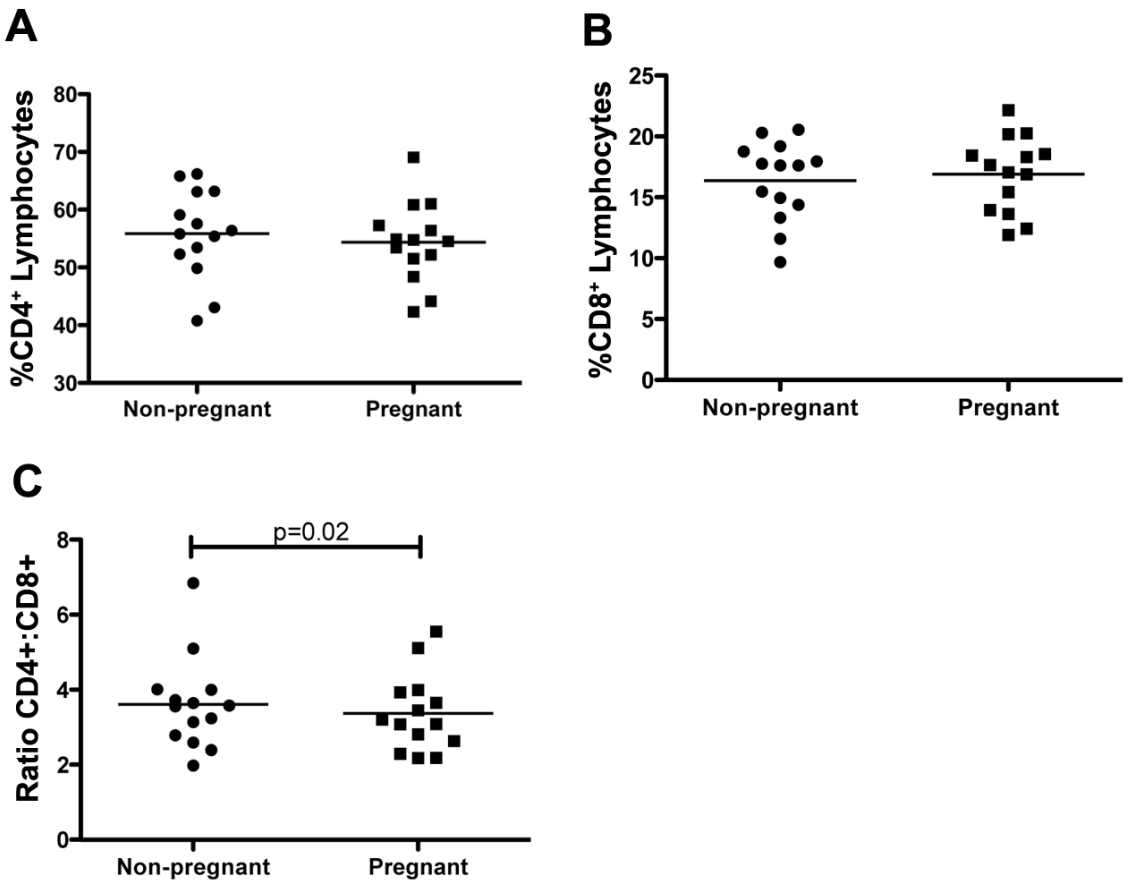


Figure 2.3 Cell surface marker expression by peripheral lymphocytes from mares during the pregnant and non-pregnant states. Flow cytometric analysis of paired samples of PBMC isolated during early pregnancy (days 29-33) or the luteal phase of estrous (n=15). Cells were labeled with monoclonal antibodies to cell surface markers as described in Materials and Methods. A. Percentage of peripheral lymphocytes expressing CD4. B. Percentage of peripheral lymphocytes expressing CD8. C. The ratio of CD4+:CD8+ peripheral lymphocytes.

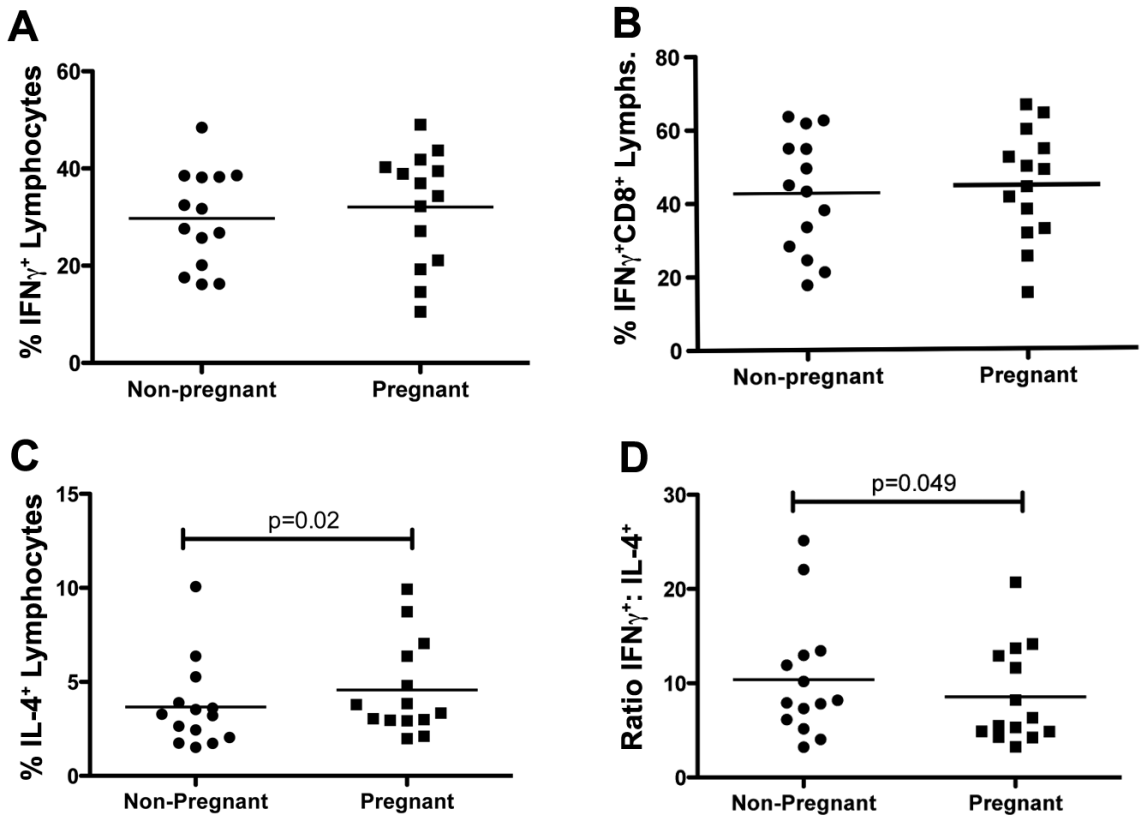


Figure 2.4 Cytokine expression by peripheral lymphocytes from mares during the pregnant and non-pregnant states. Flow cytometric analysis of IFNG and IL4 expression of paired samples of PBMC isolated during early pregnancy (days 29-33) or the luteal phase of estrous (n=15). Cells were stimulated then stained for intracellular cytokines and cell surface markers as described in materials and methods. A. Percentage of peripheral lymphocytes expressing IFNG. B. Percentage of CD8+ peripheral lymphocytes expressing IFNG. C. Percentage of peripheral lymphocytes expressing IL4. D. The ratio of IFNG+:IL4+ peripheral lymphocytes.

population (Fig. 4A) and in the sub-population of CD8+ T cells (Fig. 4 B), although these changes were not statistically significant. However, there was a modest increase in the percentage of IL4+ cells (Fig. 4C) and a decrease in the IFNG:IL4 ratio (Fig. 4D) in the overall lymphocyte population in the mares during pregnancy.

FOXP3 expressing lymphocytes were unchanged during early equine pregnancy

A quantitative RT-PCR assay was used to determine the number of transcripts of the immunoregulatory transcription factor FOXP3 in lymphocyte samples collected from mares in the pregnant and non-pregnant state (Fig. 5). The values were normalized to the percentage of CD4+ lymphocytes detected in the PBMC samples. There was a trend towards an increase in FOXP3 expression in the pregnant group, but the difference was not statistically significant.

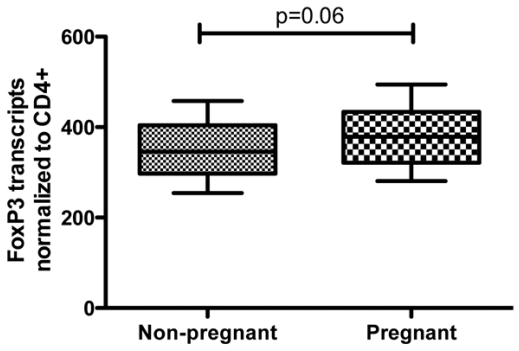


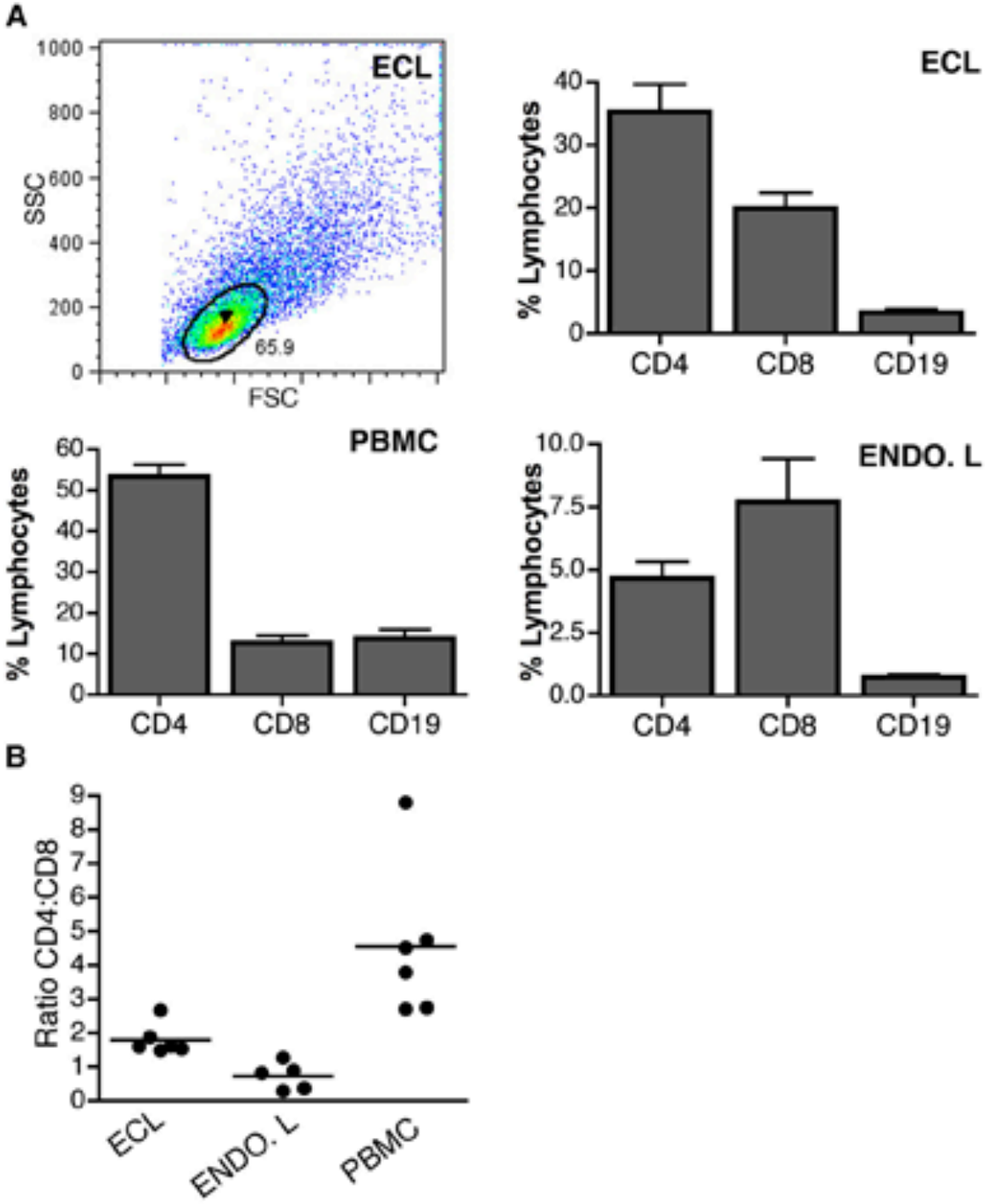
Figure 2.5 FOXP3 expression by peripheral lymphocytes from mares during pregnant and non-pregnant states. Quantitative real-time PCR analysis of FOXP3 expression in paired samples of cDNA from PBMC isolated during early pregnancy (days 29-33) or the luteal phase of estrous (n=15). Absolute numbers of FOXP3+ transcripts were determined and normalized to a housekeeper gene and the number of CD4+ lymphocytes as described in Materials and Meth-

Phenotype of lymphocytes at the equine fetal-maternal interface

The second part of this study compared immune reactivity of equine peripheral blood lymphocytes with tissue lymphocyte populations from the endometrium in samples obtained at necropsy in a group of six pregnant mares between days 43 and 46 of gestation. The endometrial cup reaction is characterized by focal accumulations of CD4+ and CD8+ lymphocytes which are located both within and immediately surrounding the eCG secreting terminally differentiated invasive trophoblast cells. The endometrium located away from the endometrial cups contains only small numbers of leukocytes (Fig. 2C and Grunig et al., 1995). For these investigations we isolated cells from both the endometrial cups and the endometrium. The endometrial cups represent only a small portion of the total endometrial surface (Fig. 1B). On average, 9 grams of endometrial cup tissue was recovered from each uterus. The endometrial cups were dissected from the endometrium by trimming away all but 2-3 mm of adjacent endometrial tissue, and subjected to enzyme mediated digestion. This yielded an average of 38 million cells for analysis. In contrast, about 30 grams of endometrium was typically used for cell isolation, resulting in recovery of only about 10 million total cells.

In order to determine the phenotype of the cells isolated from endometrial tissues, we performed flow cytometric analysis with equine specific monoclonal antibodies. Using gates set for lymphocytes, the percentages of cells expressing equine CD4, CD8, and CD19 were determined and compared to values obtained from peripheral blood samples taken from the mares on the day of necropsy. The cells isolated from the endometrial cups were comprised of 35% CD4+ lymphocytes, 20% CD8+ lymphocytes, and only about 3% B cells. In the endometrial cell population 4.7% of the cells were CD4+, with 7.7% positive for CD8, and less than 1% B cells. The cells isolated from endometrial cups and endometrium contained variable numbers of contaminating non-lymphoid cells. The PBMC populations had an average of 53% CD4+ lymphocytes, 12% CD8+ lymphocytes, and 14% B cells. Although the ratios of CD4+:CD8+ cells appeared different in the three groups, because of high variance in the PBMC group the differences were not

Figure 2.6 Cell surface marker expression of lymphocytes isolated from the equine maternal-fetal interface at day 43-46 of pregnancy. A. Flow cytometric analysis of lymphocytes isolated from endometrial cups (ECL): CD4 n=6, CD8 n=6, CD19 n=4; pregnant endometrium (ENDO. L): n=5; and PBMC: n=6. Cells were labeled for the cell surface markers CD4, CD8, and CD19 as described in Materials and Methods. Upper left panel shows a representative image of forward scatter (FSC) and side scatter (SSC) of ECL and the gate set to analyze the lymphocyte population. A similarly positioned gate was set to analyze ENDO. L and PBMC. B. The ratio of CD4+:CD8+ lymphocytes at the site of endometrial cups (ECL), in pregnant endometrium (ENDO. L.) and in the periphery (PBMC) (n=6).


A
ECL
SSC
FSC
65.9
1000
800
600
400
200
0
% Lymphocytes
40
30
20
10
0
CD4
CD8
CD19
PBMC
60
50
40
30
20
10
0
ENDO. L
10.0
7.5
5.0
2.5
0.0
B
Ratio CD4:CD8
9
8
7
6
5
4
3
2
1
0
ECL
ENDO. L
PBMC

statistically significant (Fig. 6B).

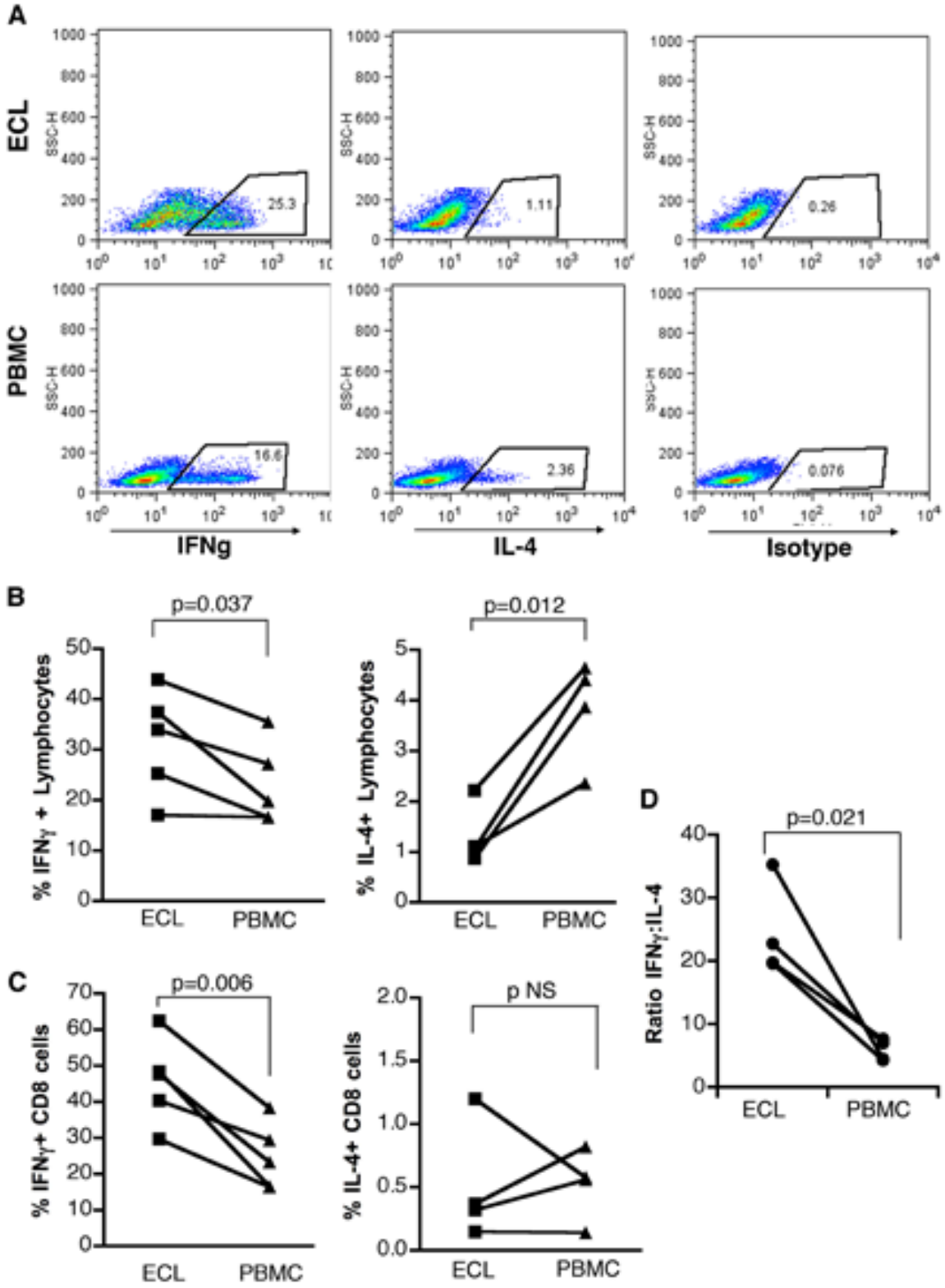
Cytokine expression by lymphocytes at the equine fetal-maternal interface

The percentages of lymphocytes expressing IFNG or IL4 in peripheral blood and in the endometrial cup lymphocyte population were determined in samples paired from the same donor mares (Fig. 7). In every comparison between peripheral blood and endometrial cups, the percentage of IFNG+ lymphocytes was increased and the percentage of IL4+ lymphocytes was decreased in the local compartment in the uterus (Fig. 7 A and B). There was a 36% increase in IFNG+ lymphocytes and a 65% decrease in IL4+ lymphocytes surrounding the endometrial cups (Fig. 7B). In the CD8+ subpopulation, this change was accentuated for IFNG, where there was an 84% increase in IFNG+ lymphocytes around the endometrial cups compared to PBMC (Fig. 7 C). Finally, these changes resulted in an approximately 3-fold increase in the ratio of IFNG to IL4 in the endometrial cups lymphocytes compared to lymphocytes from peripheral blood (Fig. 7 D).

Evidence for regulatory T cells at the equine fetal-maternal interface

A combination of flow cytometry and quantitative RT-PCR assays were used to compare the expression of FOXP3 protein and messenger RNA in paired samples of CD4+ lymphocytes from the endometrial cups and peripheral blood of pregnant mares (Fig. 8). In all cases the expression of FOXP3 was higher in the local lymphocyte population from the uterus compared to peripheral blood, and the levels in the grouped samples did not overlap. There was a 3-fold increase in FOXP3 mRNA expression, which correlated well with the 3.2-fold increase in FOXP3+ cells in the same population. It is likely that these CD4+, FOXP3+ lymphocytes represent equine regulatory T cells.

Figure 2.7 Cytokine expression by lymphocytes at the equine maternal-fetal interface. Flow cytometric analysis of IFNG and IL4 expression by paired samples of endometrial cup lymphocytes (ECL) and PBMC. Cells were stimulated ex vivo for 4 hours with PMA and IO in the presence of Brefeldin A. A. Images of representative dot plots of ECL and PBMC cells isolated from one mare following labeling with IFNG (left panel), IL4 (middle panel) or an isotype control antibody (right panel). Lymphocyte gates were set to analyze cytokine expression by the lymphocyte population. B. Percentage of lymphocytes expressing IFNG (n=5 mares) or IL4 (n=4 mares). C. Percentage of CD8+ lymphocytes surrounding the endometrial cups (ECL) or in the periphery (PBMC) producing IFNG (n=5) or IL4- (n=4). Lymphocyte and CD8 gates were set to analyze the IFNG and IL4 populations. D. The ratio of IFNG+:IL4+ lymphocytes at the site of endometrial cups (ECL) and in the periphery (PBMC) (n=4).


A
ECL
PBMC
SSC-H
25.3
1.11
0.26
16.6
2.36
0.076
IFNg
IL-4
Isotype
B
p=0.037
% IFNγ + Lymphocytes
p=0.012
% IL-4+ Lymphocytes
ECL
PBMC
C
p=0.006
% IFNγ+ CD8 cells
p NS
% IL-4+ CD8 cells
D
p=0.021
Ratio IFNγ:IL-4

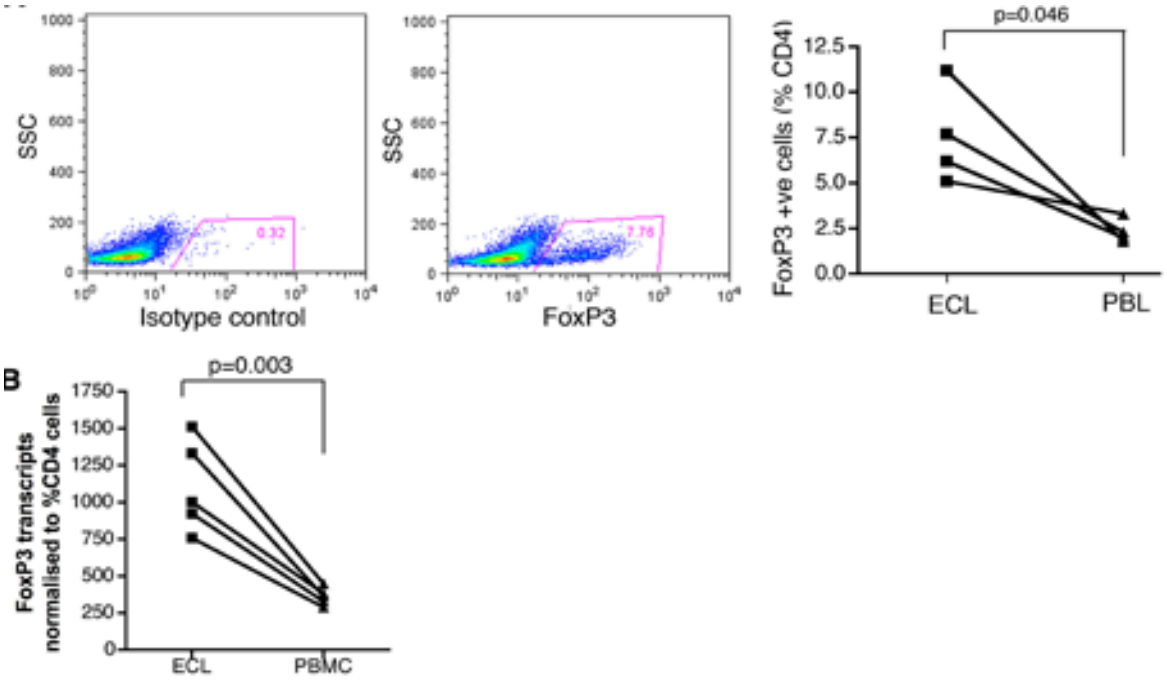


Figure 2.8 Tregs are increased at the equine maternal-fetal interface. A. Flow cytometric analysis of FOXP3 expression in paired samples of lymphocytes isolated from endometrial cups (ECL) and PBL (n=4 mares). Lymphocyte and CD4 gates were set to analyze the FOXP3 population. Representative dot plots of ECL used to calculate the FOXP3 population are shown. B. FOXP3 mRNA expression in paired samples of lymphocytes isolated from endometrial cups (ECL) and PBMC (n=5 mares).

Discussion

This study addressed aspects of two unresolved issues in reproductive immunology. First is the question of systemic changes in maternal immune reactivity during pregnancy, and second is the relevance of those changes in the periphery to local immunological events at the fetal-maternal interface. The pregnant mare is a good subject in which to address these issues because the horse provides the opportunity for examination of peripheral immune responses before and during pregnancy in the same individual, and also for direct comparison of peripheral and local responses in the uterus. In the mare there is an early, robust, and consistent antibody response to the fetus (Antczak et al., 1984), and evidence for diminished capacity to generate cytotoxic lymphocytes reactive against paternal MHC class I antigens during pregnancy (Baker et al., 1999). In a large, well-controlled cohort of mares sampled before and during pregnancy, we detected only slight changes in the character and composition of peripheral blood lymphocytes. In contrast, we measured significant differences in the same variables when comparing the periphery with the endometrium in pregnant mares.

Many physiologic changes accompany the transition from the non-pregnant to the pregnant state, including alterations in cells and molecules of the immune system. These changes may reflect specific maternal immunological recognition of the conceptus (Antczak, 1989), a generalized shift in the character of the maternal immune system that favors the development of the semi-allogeneic fetus (Baker et al., 1999; Krishnan et al., 1996a; Pejcic-Karapetrovic et al., 2007), or specific tolerance to paternal and / or fetal alloantigens (Tafuri et al., 1995; Jiang & Vacchio, 1998; Ait-Azzouzene et al., 1998, 2001; Erlebacher et al., 2007). It is in this context that the term ‘split tolerance to trophoblast’ offers a novel framework for defining the complex immunological relationship between mother and fetus.

At the systemic level represented by the circulating cells of the immune system, there is evidence for changes during pregnancy in several species in the percentages of lymphocytes producing specific cytokines (Faas et al., 2005), in the ratios and numbers of lymphocytes and lymphocyte

subsets (Faas et al., 2005), and in the numbers of regulatory T cells (Aluvihare et al., 2004; Somerset et al. 2004; Saito et al. 2005; Oliviera & Hansen, 2008). In humans, it has been reported that in peripheral blood the percentage of lymphocytes producing IFNG decreases, and the percentage of lymphocytes producing IL4 does not change. In contrast, in rats the percentage of IFNG and IL4 producing lymphocytes during pregnancy did not change, although the total number of circulating lymphocytes was reduced (Faas et al., 2005). In cattle, at day 33-34 of pregnancy no changes in the numbers of CD4, CD8, or gamma-delta T cells were detected compared to the non-pregnant state, but the percentage of CD4+ T cells that also expressed CD25+ (presumed Tregs) was increased (Oliveira and Hansen, 2008). The changes measured in one species are not always identified in studies in other species, but overall, the research cited supports the generality of peripheral immune system alterations during pregnancy. One of the most consistent changes noted in these studies is the increase in regulatory T cells in peripheral blood during pregnancy.

The immune status differences we measured in the periphery between the pregnant and non-pregnant state in mares were not dramatic. Thus, the ratios of CD4+:CD8+ T cells, the percentages of T cells expressing either IFNG or IL4, and the number of FOXP3 transcripts in lymphocytes were not drastically altered during pregnancy. Taken together, our results have not identified immunological pathways that might account for either the decreased capacity of mares to mount CTL responses against MHC class I antigens of the mating stallions (Baker et al., 1999), or the strong antipaternal alloantibody responses characteristic of MHC incompatible pregnancies in the mare (Antczak et al., 1984). These results reinforce the idea that different components of the immune system are affected differentially during pregnancy.

At the local level of the uterus, there is increasing evidence for the accumulation in the endometrium of lymphocytes with the CD4+CD25+FOXP3+ phenotype of Tregs in mice (Aluvihare et al., 2004; Zenclussen et al., 2006) and in humans (Tilburgs et al., 2006; Sasaki et al., 2007). This is consistent with our findings in the mare reported here. We measured increased

numbers of FOXP3+ CD4+ T cells around the endometrial cups compared to peripheral blood (Fig. 8). In the lymphocytes recovered from the endometrial cups, there was also a marked increase in IFNG+ cells in the total population and in the CD8+ subpopulation and a decrease in lymphocytes expressing IL4 (Fig. 7). Our results are in agreement with a recent study that reported high levels of expression of IFNG and undetectable levels of IL4 by human decidual CD8+ lymphocytes (Scaife et al., 2006). Interestingly, our findings, as well as those of Scaife and colleagues in human pregnancy, are in conflict to the traditional dogma that pregnancy is associated with a decrease in the ratio of Th1:Th2 cytokines. Again this highlights the important differences that exist between local and peripheral compartments of the immune system during pregnancy, and furthermore suggests that the cytokine milieu during pregnancy is more complex than previously reported.

The increase in IFNG+ lymphocytes at the fetal-maternal interface may be related mechanistically to the increase in FOXP3. It has been shown that IFNG conditions the development of Tregs that can mediate allograft acceptance in mice (Feng et al., 2008). Five of the six pregnant mares we studied carried MHC incompatible conceptuses, and the sixth carried an MHC compatible conceptus. Previous studies demonstrated that the lymphocyte accumulations around the endometrial cups are not diminished in MHC compatible pregnancies (Allen et al., 1984), and the results presented here suggest that the lymphocyte, cytokine, and FOXP3 profiles are also similar to those of MHC incompatible pregnancies. To our knowledge, our results represent the first description of regulatory T cells in the horse.

The immunological events in equine pregnancy are consistent with a state of split tolerance to trophoblast. The early, robust alloantibody response in mares is induced locally in the uterus by the invasion of the chorionic girdle cells bearing high levels of polymorphic cell surface MHC class I antigens (Antczak et al., 1984; Donaldson et al, 1990, 1992). Mares can generate very high secondary antibody responses in early pregnancy after prior priming in the periphery by skin grafting (Adams et al., 2007), and chorionic girdle cells transplanted to sites outside

the uterus in non-pregnant mares can stimulate alloantibody responses without any additional components of the conceptus (Adams and Antczak, 2001; de Mestre et al., 2008). These results all point to the ability of the invasive trophoblast to induce a local B cell (antibody) response to the fetus in the endometrium.

The involvement of the T cell arm of the immune system in the response to invading trophoblast seems much more complex. The invasion of the endometrium by the chorionic girdle is accompanied by the accumulation of large numbers of CD4+ and CD8+ T lymphocytes around the base of the cups (Fig. 2 and Grunig et al., 1995). An early interpretation of this endometrial cup reaction was that it represented T cell mediated recognition that would result in destruction of the endometrial cup trophoblast cells (Allen 1975, 1979). The discovery that the chorionic girdle expresses high levels of polymorphic paternal MHC class I antigens, while the allantochorion trophoblast does not (Donaldson et al., 1990), suggested a reason why the uterine lymphocyte accumulations of early equine pregnancy were restricted to the area around the endometrial cups, and not the endometrium-allantochorion border.

This solution, however, posed another question. How do the endometrial cup trophoblasts avoid destruction by the surrounding T cells during their normal 50 – 70 day lifespan? Although the invading chorionic girdle and early endometrial cups do express polymorphic MHC class I antigens, these molecules are lost from the cell surface as the cup trophoblast cells mature into their, binucleate, eCG secreting, terminally differentiated state (Donaldson et al., 1992, Maher et al., 1996). This down regulation of MHC class I genes and molecules in the cup cells may extend their lifespan; classical alloreactive cytotoxic T lymphocytes should be unable to kill the mature, MHC class I negative endometrial cup trophoblasts. This would enable to cups to safely secrete the eCG that is necessary to induce the secondary corpora lutea that provide the progesterone needed to maintain equine pregnancy until approximately day 100, when the placenta itself develops the capacity to produce progesterone. The mechanisms that result in the death of the endometrial cup trophoblasts remain elusive: are they killed by an as yet

uncharacterized immune response, perhaps mediated by NK cells, or do they self-destruct, having outlived their usefulness?

Equally intriguing are the mechanisms that prevent T cell mediated destruction of the day 38 - 45 chorionic girdle and early endometrial cup trophoblasts that express MHC class I antigens. In vitro studies demonstrated that equine MHC class I chorionic girdle trophoblasts are susceptible to killing by alloreactive cytotoxic lymphocytes, when the responding lymphocytes are obtained from non-pregnant horses (Baker et al., 2000). Longitudinal studies of the leukocyte response to the endometrial cups revealed that the numbers of T lymphocytes around the cups diminish as the cups mature, and then increase again towards the end of the normal lifespan of the cups (Grunig et al., 1995). This was interpreted as evidence for immunoregulatory events aimed at the T cells surrounding the cups. The molecular and cellular phenotyping studies of endometrial cup lymphocytes reported here strengthen that hypothesis. It may be that CD4+FOXP3+ regulatory T cells in the mare's uterus are recruited to the site of chorionic girdle invasion, where they could act to prevent the pre-mature destruction of the endometrial cups before the cup trophoblasts down regulate their MHC class I antigens and thus become invisible to cytotoxic T cells. Co-cultures of invasive trophoblast cells and peripheral lymphocytes resulted in diminished lymphocyte proliferation to mitogenic stimuli (Flaminio and Antczak, 2005). That model system may be an in vitro correlate of mechanisms operating in and around the endometrial cups.

It is not known if the local regulatory T cells of the endometrial cups are related to the peripheral decrease in CTL capacity in the pregnant mare, but it seems unlikely, because the peripheral lymphocytes of pregnant mares had the same level of FOXP3 expression as lymphocytes from the mares when not pregnant. The systemic, strong antibody responses of equine pregnancy highlight the robust, intact, B cell compartment of the mare during pregnancy and the ability of the mare's immune system to generate serological responses to antigenic stimulation by the conceptus within the uterus. In contrast, the evidence for different peripheral and local regulation of aspects of T cell immunity in the pregnant mare emphasizes the split nature of

tolerogenic mechanisms protecting the equine fetus from destruction by the maternal immune system.

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CHAPTER 3

ANTIGEN-INDEPENDENT MODULATION OF T-CELL REACTIVITY DURING EQUINE PREGNANCY[1]

[1]Noronha, L.E. and Antczak, D.F., Antigen-Independent Modulation of T-Cell Reactivity during Equine Pregnancy. To be submitted to the *American Journal of Reproductive Immunology*.

Abstract

Problem: Pregnant mares demonstrate a reduction in cytotoxic T lymphocyte (CTL) reactivity against cells from the breeding stallion. We investigated whether this effect is limited to activity against paternal MHC antigens, and whether it occurs during MHC compatible pregnancy.

Method of Study: Mares were mated to carry MHC compatible or incompatible pregnancies. CTL activity of these mares when pregnant and non-pregnant was measured against cells from horses with MHC haplotypes unrelated to the mare or breeding stallion.

Results: While carrying MHC incompatible pregnancies, mares demonstrated reduced CTL activity against lymphocytes from third party horses in addition to those from the breeding stallion. This effect was also observed in mares carrying MHC compatible pregnancies.

Conclusions: The decrease in maternal T-cell reactivity characteristic of normal equine pregnancy is not restricted to paternal alloantigen, and occurs during MHC-matched matings. This suggests that antigen-independent mechanisms may be responsible for this reduction in cell-mediated immune activity.

Introduction

The placenta expresses fetal molecules of paternal origin at the maternal-fetal interface and thus represents a source of foreign antigens within a pregnant mother. However, unlike a conventional tissue graft, the semi-allogeneic fetus is not rejected during normal pregnancy. Although the maternal-fetal immunological relationship has been intensively researched, the complex mechanisms that lead to maternal tolerance remain unclear. Due to the obvious evolutionary advantage of reproductive success, it is likely that multiple overlapping processes exist at the placental, fetal, and systemic levels to ensure acceptance of the fetus. The scope of this study focuses on pregnancy-protective changes that occur at the systemic level of the maternal immune response, specifically in the T-cell compartment.

In general terms, modulation of maternal responses to fetal antigens could be the result of a

failure to recognize the fetus (ignorance), or a failure to act upon that recognition (tolerance). There is evidence from multiple species that the maternal immune system is capable of recognizing paternal antigens such as Major Histocompatibility Complex (MHC) and minor histocompatibility (H) antigens. Humans, horses, mice, and rats can develop allospecific antibodies as a result of exposure to paternal MHC during pregnancy or birth (Van Rood, Eernisse et al. 1958; Antczak, Bright et al. 1982; Roe and Bell 1982; Antczak, Miller et al. 1984; Smith, Amsden et al. 1986). Therefore maternal awareness of paternal antigens appears to be intact; in fact, evidence from several groups suggests that maternal recognition of paternal alloantigen is the stimulus for inducing maternal tolerance (Seavey and Mosmann 2006; Robertson, Guerin et al. 2009; Tilburgs, Scherjon et al. 2009; Zenclussen, Thuere et al. 2010).

Systemic maternal tolerance to fetal antigens could be achieved by a generalized modulation in T-cell responses, or one limited to those that respond to paternal antigen. While a reduction in reactivity to paternal alloantigens has obvious benefits to the maintenance of the fetus, a generalized immunosuppression could leave the pregnant mother susceptible to infectious disease. The existence of such an antigen-independent T-cell modulation is suggested by the observation that pregnant women (van der Klooster and Roelofs 1997; Smith 1999; Avelino, Campos et al. 2003) and mice (Luft and Remington 1982; Krishnan, Guilbert et al. 1996; Rowe, Ertelt et al. 2011) display increased susceptibility to intracellular infections such as toxoplasmosis, listeriosis, and salmonellosis. Also, women (Ostensen and Husby 1983) and mice (Waites and Whyte 1987) with cell-mediated auto-immune diseases, such as rheumatoid arthritis, may experience an amelioration of symptoms during pregnancy due to dampened T-cells responses.

Evidence also exists for an antigen-specific modulation of maternal cell-mediated immune responses. CD8+ and cytotoxic T lymphocytes (CTL), the primary effectors of cell-mediated immune responses, are responsible for allograft rejection and are therefore potentially deleterious to pregnancy. Indeed, increases in CD8+ and CTL have been associated with pregnancy loss in

women (Manyonda, Pereira et al. 1993; Yamada 1993; Kotlan, Fulop et al. 2001). Decreases in anti-paternal CD8+ and CTL responses have been observed during pregnancy in several species (Clark and McDermott 1978; Parvin, Isobe et al. 1992; Tafuri, Alferink et al. 1995; Jiang and Vacchio 1998; Baker, Bamford et al. 1999; Erlebacher, Vencato et al. 2007; van Halteren, Jankowska-Gan et al. 2009). In transgenic mouse models, some groups determined that the loss of function was antigen specific, and was the result of a specific clonal deletion (Tafuri, Alferink et al. 1995; Jiang and Vacchio 1998; Erlebacher, Vencato et al. 2007) and/or a functional unresponsiveness (Jiang and Vacchio 1998; Zhou and Mellor 1998; Erlebacher, Vencato et al. 2007) of lymphocytes that recognize paternal antigen.

However, others have observed a less restricted decrease in CTL function during pregnancy. In women, a reduction in CTL activity against viral infections such as Epstein-Barr virus has been observed (Nakamura, Miyazaki et al. 1993). CTL isolated from pregnant mice have shown a reduced ability to lyse cells from a third party, i.e. with MHC antigens not of maternal or paternal origin (Hamilton and Hellstrom 1977; Clark, McDermott et al. 1980; Chaouat, Monnot et al. 1982; Slapsys and Clark 1982; Thomas and Erickson 1986). Some of these results were obtained during syngeneic (MHC compatible) matings, where no foreign paternal MHC antigens were present. Therefore the antigen-specificity of maternal CTL modulation remains unclear.

Pregnancy in the horse has been uniquely informative in better understanding maternal tolerance to the fetus (Noronha and Antczak 2010). Pregnant female horses (mares) demonstrate a clear immunological recognition of the fetus. Nearly all mares carrying MHC incompatible pregnancies generate high cytotoxic alloantibody titers against the MHC antigens of the breeding stallion (Antczak, Bright et al. 1982; Antczak 1984) at levels similar to those resulting from conventional skin allografts (Adams, Oriol et al. 2007). However, pregnant mares demonstrate a reduced ability to generate effective CTL responses against stallion MHC antigens (Baker, Bamford et al. 1999). This effect is transient, and reverts after parturition; and it is not observed in males or non-pregnant females. It is not known whether this effect is specific for, or

dependent upon paternal alloantigens.

The purpose of this study was to use better understand the specificity of this diminished cell-mediated immune response in the pregnant mare. To this end, we utilized a herd of horses bred for homozygosity at the MHC region of the genome (Ellis, Bontrop et al. 2006) in order to generate MHC compatible and incompatible pregnancies. Specifically, our aims were to determine: 1) if the decrease in maternal CTL activity is only observed against paternal MHC antigens, and 2) if it requires a MHC mismatch between the breeding mare and stallion.

Materials and Methods

Animals

Clinically healthy adult horses of mixed breeds and ages were used in these experiments (Table 3.1). Major Histocompatibility Complex haplotypes were assigned to the horses based on results of tissue typing using a panel of well-characterized alloantisera validated

in international workshops, complemented by genomic analysis (Lazary, Antczak et al. 1988; Tseng, Miller et al. 2010). Horses were maintained at the Equine Genetics Center, Baker Institute for Animal Health, Cornell University. Animal care was performed in accordance with the guidelines set forth by the Institutional Animal Care and Use Committee of Cornell University. Mares of various MHC haplotypes were artificially inseminated with fresh semen collected from either a stallion homozygous for the ELA-A2 or -A3 haplotype to generate MHC matched (n=4) or mismatched (n=6) pregnancies. The day of ovulation was determined by daily transrectal ultrasound examination, from which gestational ages were deduced. Fetal viability was confirmed by observing fetal heartbeat via transrectal ultrasonography prior to blood collection.

CTL Assays

Excepting animal #3164 (gestation day 320), matched heparinized venous blood samples were collected from each mare at 31-34 days of pregnancy and before and/or after pregnancy. It

Table 3.1: Summary of mares used for CTL assays.

Mare Accession#	Age (years)	Mare MHC type[1]	Breeding Stallion MHC type[1]	Histocompatibility of Pregnancy[2]	Day of gestation tested
3725	6	A2/?	A3/A3	Incompatible	33
3558	15	A2/A2	A3/A3	Incompatible	31
3819	8	A6/?	A2/A2	Incompatible	34
4066	6	A2/A19	A3/A3	Incompatible	31
4065	9	A19/?	A3/A3	Incompatible	33
3099[3]	17	A2/A2	A3/A3	Incompatible	32
3099[3]	17	A2/A2	A2/A2	Compatible	33
3601	8	A3/A3	A3/A3	Compatible	34
3164	16	A3/A3	A3/A3	Compatible	320
3880	3	A3/A3	A3/A3	Compatible	32

[1]Equine MHC Haplotypes were assigned as described in Materials & Methods. Haplotypes are designated as Equine Leukocyte Antigen (ELA)-AX where X is a number that refers to a haplotype defined by serological +/- molecular tissue typing. Animals assigned "?" carry a haplotype for which no identifying antisera are available, but which has been determined to be distinct from the breeding stallions and third party animals. [2]Pregnancies generated from two animals of the same MHC type are designated as "histocompatible;" those from animals with different MHC types are "histoincompatible." [3]Mare 3099 was mated to generate 2 different pregnancies, separated by 2 months.

was previously determined that there is no difference in CTL activity as a result of previous matings (Baker, Bamford et al. 1999). Peripheral blood mononuclear cells (PBMC) were isolated using density gradient centrifugation as previously described (Wagner, Hillegas et al. 2008). CTL assays were performed as previously described (Baker, Bamford et al. 1999) with modifications as follows. 10^8 PBMC were placed into a T75 flask with 5 x 10^7 stimulator cells that had been irradiated using a cesium source (Gammacell 40; Nordion International, Ottawa, ON, Canada) at a dose of 950 rads. Cultures were maintained in "ARM" medium: 10% FCS (Clontech, Mountain View, CA), 45% AIMV, 45% RPMI-140, 1X NEAA, 0.5X NaPyruvate, 1X Penicillin/Streptomycin, 1X 2-mercaptoethanol (all Gibco, Grand Island, NY). Flasks were incubated upright at 37°C in 5% CO_2 for 7 days. On day 7, the cultures were re-stimulated with freshly prepared irradiated stimulator cells that were plated at half the density of the surviving responder cells. All cultures were stimulated for a total of 10 days. Pokeweed mitogen (Sigma, St. Louis, MO)-stimulated target cells were labeled with ^{51}chromium (Perkin-Elmer, Boston, MA) for 6 hours at 37°C, 5% CO_2. Labeled targets were plated to give effector:target ratios of 100:1, 50:1, 25:1,12.5:1, 6.25:1, and 3.1:1in 150 μl final volume in 96-well round-bottom plates. Spontaneous release control wells contained only target cells and medium. Total release control wells contained 10% Triton X-100 (Sigma) and target cells. The average spontaneous release did not exceed 15% in any assay. All test dilutions and controls were done in triplicate. The assay plates were incubated at 37°C in 5% CO_2 for 4 h and then centrifuged at 1000xg for 5 minutes. Cell supernatants were harvested using Molecular Devices Supernatant Collection System (Sunnyvale, CA). ^{51}Cr activity was measured with a Wallac 1470 gamma counter (Perkin Elmer) as cpm over 2 minutes.

Data Analysis

Percent cytotoxicity was normalized to the spontaneous and total release controls using the following formula: % cytotoxicity = [mean sample release - mean spontaneous release]/[mean total release - mean spontaneous release]. Deviations from Gaussian distribution were tested

for using the Kolmogorov-Smirnov normality test. Non-parametric data were normalized using the simple transformation Y=log(Y) (Figure 3.5). Normalized % cytolysis values at effector:target=100:1 were tested with a repeated-measures (paired) Student's t-test to determine statistical significance ($p<0.05$). All statistics were performed using Graph Pad Prism software, v4.0 (San Diego, CA).

Results

In order to test the antigen specificity of T-cell responses in mares carrying MHC compatible and MHC incompatible pregnancies, specific matings were established between animals based upon their MHC haplotypes (Table 3.1, Figure 3.1). Paired PBMC samples were isolated from the mares while pregnant and non-pregnant. CTL were primed *in vitro* for 10 days, and then tested for cytolytic capacity using a standard quantitative chromium-release cell lysis assay.

CTL Activity Against Third Party MHC Antigens is Reduced During Pregnancy

We first investigated whether the previously observed pregnancy-associated reduction in CTL activity is limited to reactivity against paternal MHC antigens. Six mares of various MHC haplotypes were artificially inseminated with semen from a stallion of a different MHC haplotype in order to establish MHC incompatible pregnancies. PBMC were isolated from the same mares while non-pregnant and during pregnancy. CTL were generated *in vitro* against stimulator cells from: 1) the breeding stallion, and 2) a horse with a MHC haplotype different from both the mare and stallion (third party). These primed CTL were then tested for the ability to lyse cells from the respective stimulator animals as well as control animals.

The baseline activity of CTL isolated from non-pregnant mares was robust and specific for the MHC of the stimulator animals (Figure 3.2). As previously observed, CTL from mares carrying incompatible pregnancies demonstrated a significantly diminished capacity to lyse cells from the breeding stallion with the mean cytolysis decreasing from 30% (NP, non-pregnant) to 5%

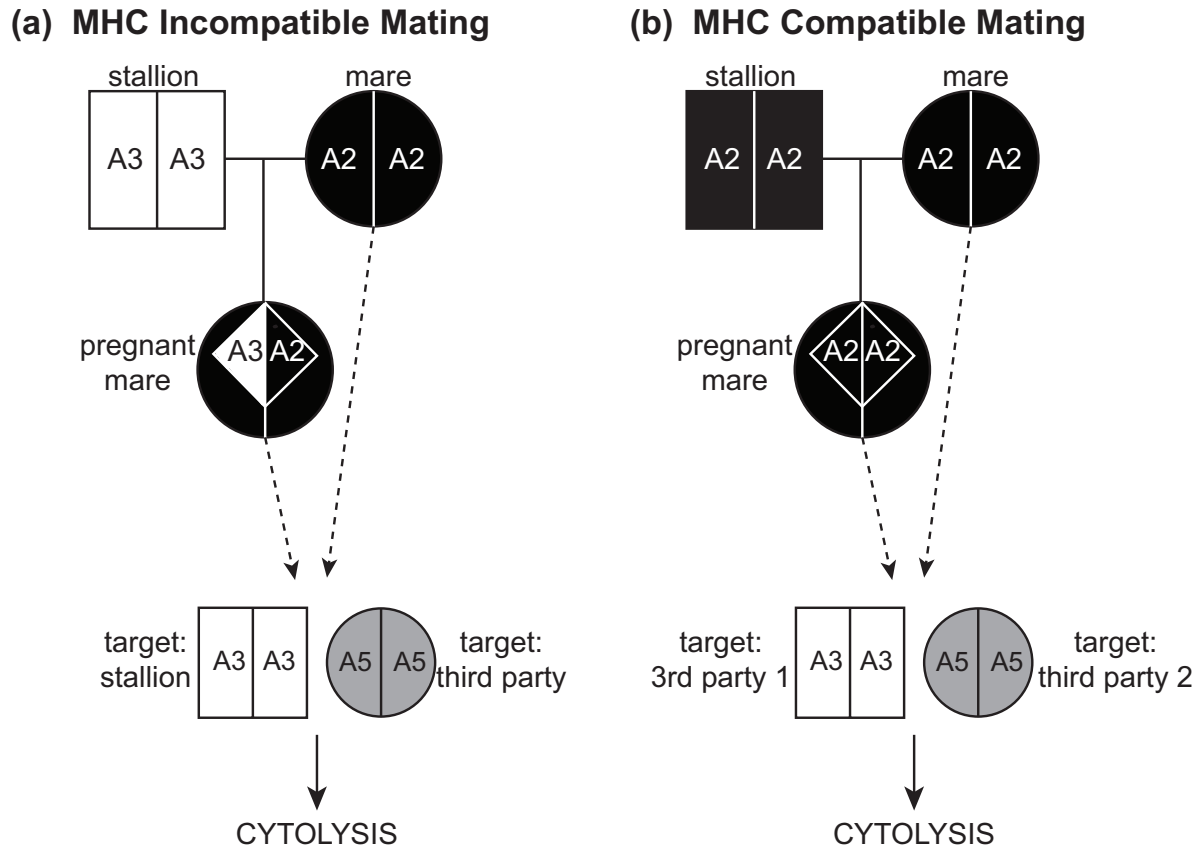

Figure 3.1 Summary diagram of experimental design. Mares were artificially inseminated to generate either (a) MHC-incompatible) or (b) MHC-compatible) pregnancies. PBMC were isolated from the mares during pregnancy, and while non-pregnant (dashed arrows). CTL were differentiated *in vitro* for 10 days and tested for the ability to lyse target cells from the breeding stallion and third party animals. Representative equine leukocyte antigen (ELA) MHC haplotypes are used (i.e. ELA-A2= "A2"); full animal details are listed in Table 3.1.

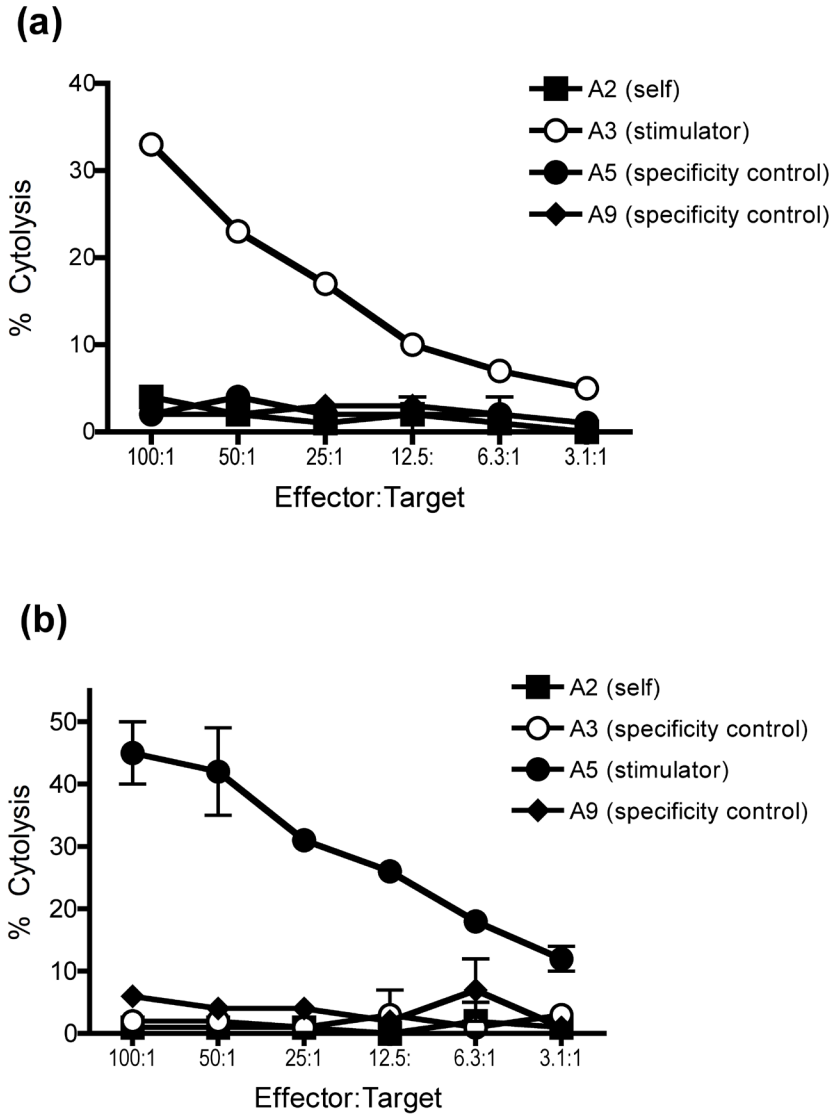

Figure 3.2 CTL activity in non-pregnant mares is robust and specific for the MHC of the stimulator animal. Curves demonstrating cytolysis of target cells at decreasing effector: target cell ratios are shown for Mare #3558, homozygous at the MHC for the ELA-A2 haplotype, stimulated against an A3 (a) and A5 (b) animal during the 10 day in vitro CTL differentiation phase of the assay. Target cells listed as "self" and "specificity controls" are negative controls to demonstrate specific lysis of only stimulator cells.

(P, pregnant) (Figure 3.3). When CTL from these mares were tested for the ability to lyse cells from an unrelated third party individual, a similar decrease from 39% while non-pregnant to 9% cytolysis while pregnant was observed (Figure 3.4).

CTL Activity is Reduced During MHC Compatible Pregnancy

In order to investigate whether a mismatch between maternal and paternal MHC antigens may be required to generate maternal tolerance, we next tested maternal CTL responses during MHC compatible pregnancy.

Four mares homozygous at the MHC region for the ELA-A2 or -A3 haplotype were mated to a homozygous stallion of the same haplotype in order to establish compatible pregnancies. CTL were generated *in vitro* against stimulator cells from two different third party animals with MHC haplotypes distinct from both the stallion and mare. Differentiated CTL were tested for the ability to lyse cells from the third party animals as well as negative controls. CTL from mares pregnant with MHC compatible pregnancies demonstrated a reduced capacity to lyse target cells from male (48% NP, 18% P; Figure 3.5a, b) and female (51% NP, 17% P; Figure 3.5c, d) third party animals.

Discussion

These data expand upon the previous observation that anti-paternal CTL responses are dramatically reduced in pregnant mares (Baker, Bamford et al. 1999). Here, we demonstrate that this reduction occurs independently of paternal MHC antigens on two levels. First, the mare's inability to generate effective CTL is not limited to responses directed against the MHC of the breeding stallion. Second, a disparity between maternal and paternal MHC antigens is not necessary to induce this effect.

Our observations regarding the antigen-independent nature of these changes in CTL activity are consistent with data from both human and mouse studies. In studies using selective breeding

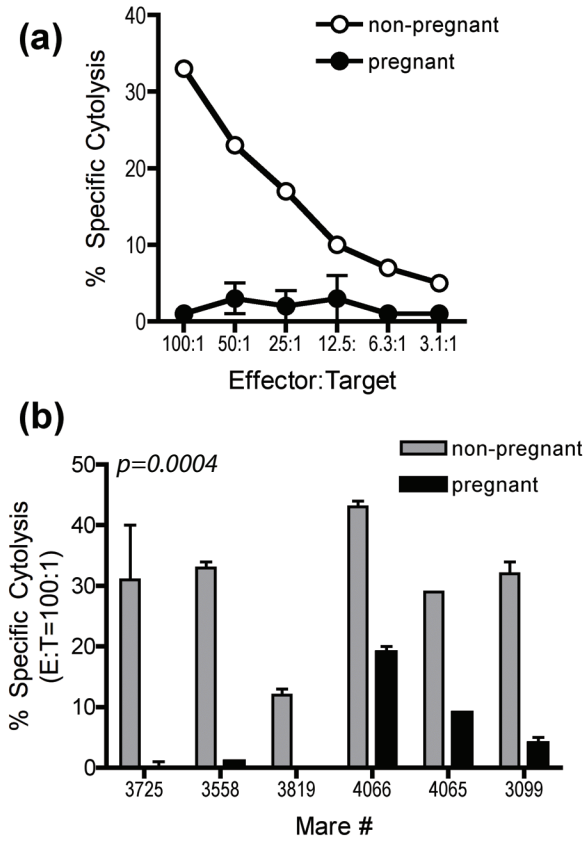

Figure 3.3 The CTL response is reduced against the breeding stallion during MHC incompatible pregnancy. Six mares were tested for the ability to generate effective CTL responses against cells from an MHC-disparate stallion while non-pregnant vs. while pregnant with incompatible pregnancies sired by that stallion. "Effectors" refers to CTL derived from the mares; "targets" refers to ^{51}Cr-labeled lysis-target PBMC from the breeding stallion with either an ELA-A3 or A2 MHC haplotype as indicated in Table 3.1. (a) Curve showing cytolysis at decreasing effector:target cell ratios for CTL collected from mare #3558 while non-pregnant and during pregnancy. (b) Graph showing cytolysis at effector:target=100:1 for six mares.

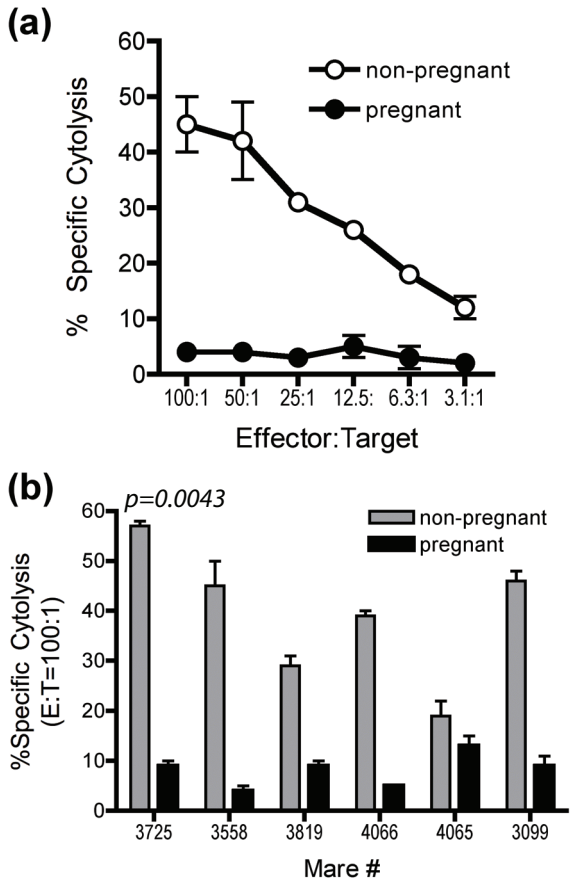

Figure 3.4 The CTL response is reduced against third party cells during MHC incompatible pregnancy. The six mares described in Figure 3.3 were tested for the ability to generate effective CTL responses against cells from an animal (third party) with a MHC haplotype different from both the mare and breeding stallion. "Effectors" refers to CTL derived from the mares; "targets" refers to ^{51}Cr-labeled PBMC from the third party animal with an ELA-A5 haplotype. (a) Curve showing cytolysis at decreasing effector:target cell ratios for CTL collected from mare #3558 while non-pregnant and during pregnancy. (b) Graph showing cytolysis at effector:target=100:1 for six mares.

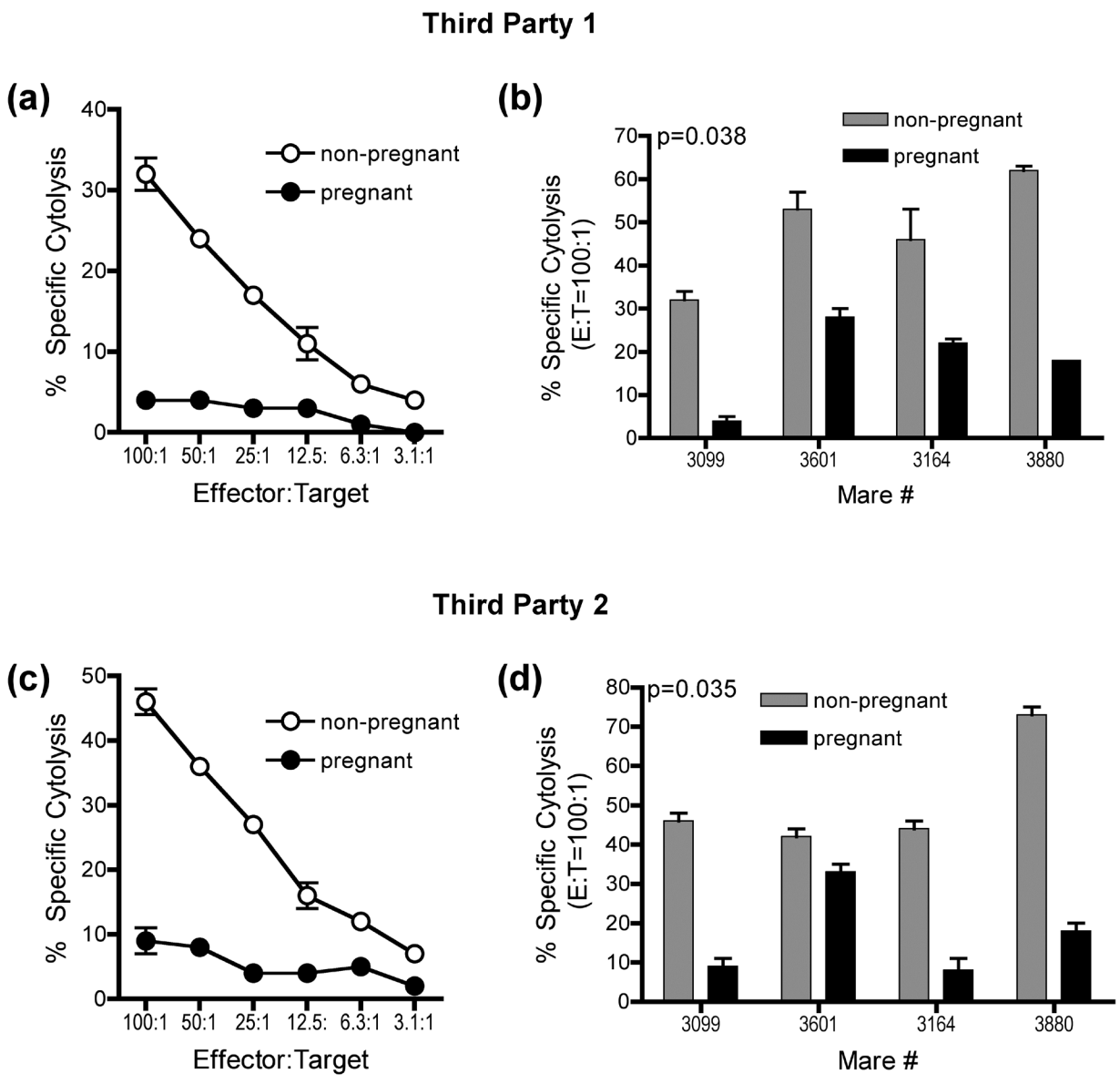

Figure 3.5 The decreased CTL response observed during pregnancy does not require MHC incompatibility between sire and dam. Four mares were tested for the ability to generate effective CTL responses against cells from two different third party animals while non-pregnant vs. while pregnant with MHC compatible pregnancies. Full cytolysis curves at decreasing effector:target cell ratios are shown for mare # 3099. Graphs showing cytolysis at effector:target=100:1 for all four mares. Top: cytolytic activity against cells from a male third party with either an ELA-A2 or A3 MHC haplotype, as appropriate for a mismatch, (a, b). Bottom: activity against cells from a female third party animal with an ELA-A5 haplotype (c, d).

of mice to generate allogeneic (MHC incompatible) pregnancies, results similar to ours were observed— CTL activity against third party MHC target cells was reduced during allogeneic pregnancy (Clark, McDermott et al. 1980; Thomas and Erickson 1986). During human pregnancy, deficits in virus-specific CTL populations have been observed (Nakamura, Miyazaki et al. 1993), demonstrating that CTL responses against some non-paternal antigens may be modulated in pregnant women. Observations in other species also parallel our findings that there is a reduction of maternal CTL reactivity during MHC compatible pregnancies. In multiple mouse studies investigating syngeneic (MHC compatible) pregnancies, reductions in maternal CTL reactivity against third party MHC were demonstrated (Hamilton and Hellstrom 1977; Chaouat, Monnot et al. 1982; Slapsys and Clark 1982; Thomas and Erickson 1986).

If indeed the maternal CTL response is suppressed in an antigen-independent manner, one major concern is that the pregnant mother could have an increased susceptibility to infectious disease. Epidemiological evidence for an equine pregnancy-induced susceptibility to pathogens is not clear. Mares are vulnerable to a number of pregnancy-associated abortogenic infections (Ellis, Bryson et al. 1983; Givens and Marley 2008; Lunn, Davis-Poynter et al. 2009), but this may be due pathogen tissue tropism rather than a general systemic immune tolerance. Equine Herpes Virus Type-1 (EHV-1) is one common equine abortogenic virus that is controlled by CTL responses. It has been observed that seropositive pregnant mares have lower baseline numbers of EHV-1 specific effector CD8+ cells compared to normal healthy adults (Paillot, Daly et al. 2007). However, they were capable of increasing that population in response to infection. A similar situation has been observed in the response of pregnant women to cytomegalovirus (Lissauer, Choudhary et al. 2011). To date, no direct comparison has been made between CTL responses of pregnant and non-pregnant horses and there is not currently sufficient evidence to conclude whether pregnant mares might have a diminished response to pathogens.

It is not yet clear what mechanism is causing the reduction in T cell responses seen here. One explanation is that there could be a pregnancy-associated reduction in CD8+ CTL precursors,

as has been observed in transgenic mouse studies (Tafuri, Alferink et al. 1995). In the horse, no difference in the number of circulating CD8+ lymphocytes has been detected between non-pregnant mares and mares in early and late pregnancy (Agricola, Carvalho et al. 2008; de Mestre, Noronha et al. 2010), or between normal pregnant mares and those that experienced early embryonic death (Krakowski, Krawczyk et al. 2010).

An alternative explanation is that the number of CTL may not change, but their cytolytic activity may be suppressed. One pregnancy-associated factor that can affect immune cells in an antigen-independent manner is the physiological state of pregnancy, i.e. the hormonal milieu. Levels of estrogens and progesterone, which reach extremely high levels in pregnancy, have been implicated in the increased susceptibility to pathogens in human and mice (Beagley and Gockel 2003). In progesterone treated pseudopregnant mice, a modulation in CTL activity has been observed, suggesting that the hormonal environment of pregnancy was sufficient to induce CTL modulation (Slapsys and Clark 1982). In women, CTL cytolysis is nearly absent during the menstrual cycle secretory phase, which is characterized by high levels of estradiol and progesterone, (White, Crassi et al. 1997). Therefore, there is evidence in two different species that hormonal changes alone, in the absence of paternal antigens, can modulate CTL activity. In the horse, there is some evidence for a progesterone-associated increase in neutrophil activity (Watson, Stokes et al. 1987), but no relationship has been demonstrated between reproductive hormones and acquired immunity.

Another potential mechanism of CTL modulation related to hormonal suppression is the production of soluble immunomodulatory factors by the placenta and uterus. In humans and mice, several placenta-derived secreted factors, such as IDO, Fas ligand, soluble HLA-G, and trophoblast microvesicles (Morales, Pace et al. 2003; Pap, Pallinger et al. 2008), have shown evidence for T-cell modulation at local and systemic levels. Maternal endometrial factors such as galectin-1and PD-1, also demonstrate such activity. In the horse, no specific factors have been isolated, but several groups have demonstrated that soluble factors produced by trophoblast and

endometrium can inhibit the proliferation and activity of peripheral maternal lymphocytes in vitro (Watson 1990; Lea and Bolton 1991; Watson and Zanecosky 1991; Roth, White et al. 1992; Flaminio, Yen et al. 2004; Flaminio and Antczak 2005). It is not yet known if these molecules are capable of acting systemically in the pregnant mare.

CTL function may also be modulated by the suppressive activity of other immune cells. Regulatory T cells (Tregs) have been observed to expand during allogeneic and syngeneic mouse pregnancy in an antigen-independent manner, possibly due to the hormones of pregnancy (Aluvihare, Kallikourdis et al. 2004). During horse pregnancy, CD4+FOXP3+ Tregs increases dramatically in the uterus, but no difference in FOXP3 gene expression has been detected between PBMC from mares carrying early pregnancies and non-pregnant mares in the luteal phase of estrous (de Mestre, Noronha et al. 2010). However, because Treg expansion in pregnancy may be an estrogen-dependent event (Polanczyk, Carson et al. 2004; Prieto and Rosenstein 2006), the difference may not have been detectable at these time points which are both progesterone-dominant in the horse. Therefore, while Tregs appear to have a role at the fetal-maternal interface of the horse, it is not known if they contribute to the effect seen here with peripheral CTL populations.

A number of immunomodulatory cytokines produced by T cells and innate immune cells can also reduce CTL activity. IL-4 has been shown to reduce anti-viral CTL responses (Aung, Tang et al. 1999) and protect against CTL-mediated allograft rejection (Gao, Chen et al. 1996). It can also be induced in an antigen-independent manner by estradiol (Hepworth, Hardman et al. 2010) and progesterone (Druckmann and Druckmann 2005). We have previously reported that mares experience an increase in the number of circulating IL-4+ lymphocytes during MHC incompatible pregnancy (de Mestre, Noronha et al. 2010). However, this did not occur during MHC compatible pregnancy (data not shown), and is therefore unlikely to be the cause of the CTL deficiency seen here.

The mechanism of CTL modulation during equine pregnancy is not yet clear. The effect appears

to exist in manner independent of maternal-fetal MHC compatibility. However, the reduction in cytolysis in this study was most pronounced among mares carrying an MHC-incompatible fetus. It is possible therefore that the phenomenon has a multifactorial origin with some amount antigen dependence. The cumulative effect of multiple, less antigenic minor histocompatibility antigens in MHC compatible pregnancies could be sufficient to induce CTL modulation. This is consistent with what has been observed in women carrying MHC matched, H-antigen mismatched fetuses; a combination of a reduction in circulating CTL, and a suppression of remaining CTL by Tregs (van Halteren, Jankowska-Gan et al. 2009).

The collective data from multiple species remains inconclusive regarding the role of paternal antigen in maternal tolerance. Here, we show here that the horse may contribute novel information to further investigations of this highly complex phenomenon.

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CHAPTER 4

MOLECULAR EVIDENCE FOR NATURAL KILLER CELLS IN EQUINE ENDOMETRIAL CUPS[1]

[1]Noronha, L.E., Huggler, K.H., de Mestre, A.M., Miler, D.M., Antczak, D.F., Molecular Evidence for Natural Killer Cells in Equine Endometrial Cups. *Placenta* (submitted)

Abstract

Objectives

To identify equine orthologs of major NK cell marker genes and utilize them to determine whether NK cells are present among the dense infiltration of lymphocytes that surround the endometrial cup structures of the horse placenta during early pregnancy.

Study Design

PCR primers were developed to detect the equine orthologs of *NKP46*, *CD16*, *CD56*, and *CD94*; gene expression was detected in RNA isolated from lymphocytes using standard 2-step reverse transcriptase (RT) PCR and products were cloned and sequenced. Absolute real-time RT-PCR was used to quantitate gene expression in total (n=5), CD3+ (n=4), and CD3- (n=4) peripheral lymphocytes, and invasive trophoblast (n=5). Lymphocytes surrounding the endometrial cups (ECL) of five mares in early pregnancy were isolated and NK marker gene expression levels were assayed by quantitative RT-PCR.

Main Outcome Measures

Absolute mRNA transcript numbers were determined by performing quantitative RT-PCR and comparing values to plasmid standards of known quantities.

Results

NKP46 gene expression in peripheral CD3- lymphocytes was higher than in CD3+ lymphocytes, *CD16* levels were higher in the CD3+ population, and no significant differences were detected for *CD56* and *CD94* between the two groups. Expression of all four NK cell markers was significantly higher in lymphocytes isolated from the endometrial cups of pregnant mares compared to PBMC isolated from the same animal on the same day (*NKP46*, 14-fold higher; *CD94*, 8-fold higher; *CD16*, 20-fold higher; *CD56*, 44-fold higher).

Conclusions

These data provide the first evidence for the expression of major NK cell markers by horse cells and the existence of a previously undetected population of NK cells in the equine endometrium during pregnancy.

Introduction

Placentation in the mare is diffuse, epitheliochorial, and primarily non-invasive. However, the horse placenta contains a population of invasive placental cells called the chorionic girdle trophoblast. These cells of fetal origin express both maternal and paternal polymorphic MHC class I antigen at very high levels (Bacon et al., 2002; Donaldson et al., 1994). During a period in early pregnancy, they invade the uterus of the pregnant mare, differentiate, and organize to form discrete tissue structures in the superficial endometrium known as endometrial cups (Allen et al., 1973). Maternal mononuclear leukocytes are recruited into the endometrial stroma around the cups, forming a dramatic cellular infiltrate at the cup periphery. Our lab has previously identified these leukocytes as primarily CD4+ and CD8+ lymphocytes, most of which are also CD3+ (Grunig et al., 1995). Despite the seemingly hostile environment in which the trophoblast cells of the cups exists, they persist in situ until their eventual death approximately two months later. During this time, the paternal MHC class I antigen expressed on the surface of these trophoblasts is recognized by the maternal immune system and induces a robust humoral immune response in nearly all pregnant mares (Antczak, 1984). It is not clear how the highly antigenic trophoblast cells are able to evade the maternal immune response for such an extended period.

In humans and mice, NK cells are the primary leukocyte population in the decidua during early pregnancy. Despite the implications of their name, they are weakly lytic and promote the establishment and maintenance of pregnancy. In most species, NK cells of the uterus and decidua are distinct from peripheral populations in terms of phenotype and function (Croy et al., 2006). Their role in pregnancy is not completely understood, but they have been implicated in vascular remodeling and facilitation of trophoblast invasion (Harris, 2011; Tabiasco et al., 2006). Changes in NK cell numbers and phenotype have been associated with multiple reproductive disorders in women (Lash and Bulmer, 2011); and NK cell-depleted mice demonstrate aberrant spiral artery development (Croy et al., 2003). Based upon the important role that NK cells play in species with invasive placentae, we wanted to determine whether they are present among the leukocyte infiltration that surround the invasive trophoblast of the equine endometrial cups.

There is some evidence for an NK cell presence at the maternal-fetal interface of the horse. Electron microscopy studies of the equine endometrium during early pregnancy reveal large granular lymphocytes consistent with uterine NK cells (uNK) (Enders and Liu, 1991). But progress in the investigation of equine NK cells has been inhibited by a lack of reagents. Our lab has previously described a population of peripheral lymphocytes that exhibit NK cell-like characteristics by using a cross-reactive monoclonal antibody to a catfish vimentin-like protein shown to identify human NK cells (Viveiros and Antczak, 1999). Also, expression of *LY49* family genes has been detected by screening a horse spleen cDNA library (Bacon et al., 2002). However, to date, none of the primary markers used to phenotype NK cell cells, or methods to detect them, have been described in the horse. In order to explore whether NK cells might play a role at the equine fetal-maternal interface, we identified equine orthologs of four NK cell marker genes and using molecular methods, investigated their expression in the endometrium during early pregnancy.

Materials and Methods

Animals

Horses used in this study were maintained at the Cornell Equine Genetics Center; all procedures were performed in accordance with guidelines established by the Institutional Animal Care and Use Committee of Cornell University. Adult horses of mixed genetic backgrounds, sexes, and ages were used (Table 4.1). Pregnancies were established as previously described (Adams and Antczak, 2001). Major Histocompatibility Complex haplotypes were assigned to horses using serological and genomic methods (Lazary et al., 1988; Tseng et al., 2010).

Cells and Tissues

Peripheral blood mononuclear cells (PBMC) and peripheral blood lymphocytes (PBL) were isolated using density gradient centrifugation with (PBL) or without (PBMC) pre-incubation with carbonyl iron to remove phagocytes. Endometrial cup lymphocytes (ECL) were similarly

Table 4.1 Animals used for leukocyte isolation

Peripheral blood studies

Cornell #	Breed	DOB	Sex	MHC Haplotype[1]
3099	Thoroughbred	1992	Mare	A2/A2
3521	Thoroughbred	2000	Gelding	A2/A2
3903	Thoroughbred	2001	Mare	A9/A9
3639	Pony	2000	Mare	A7/?
3105	Thoroughbred	1992	Gelding	A3/A3

Endometrial cup lymphocyte studies

Cornell #	Breed	DOB	Day of gestation	Mare MHC Haplotype[1]	Mating Stallion MHC Haplotype
3382	Thoroughbred	1995	46	A10/?	A3/A3
3549	Thoroughbred	1994	43	A19/?	A3/A3
3842	Thoroughbred	2000	45	A5/A19	A2/A2
3845	Pony	2004	44	A5/W16	A3/A3
3901	Thoroughbred	2001	45	A2/A19	A2/A2

[1]"*?*" one allele could not be determined using available typing sera.

isolated following surgical dissection and collagenase digestion as previously described (de Mestre et al., 2010). Chorionic Girdle trophoblast were microdissected from conceptuses collected at days 33-34 of pregnancy as previously described (de Mestre et al., 2009). CD3 cell sorting was performed using an AutoMACS cell sorter (Miltenyi Biotec, Auburn, CA) following incubation of PBL with a mouse monoclonal antibody specific for equine CD3 (clone F6G, UC Davis, Davis, CA) and rat anti-mouse IgG1 MicroBeads (Miltenyi Biotec). CD3-depleted and enriched populations were verified by flow cytometry. Depleted populations were a mean 8% CD3+; enriched populations were 91% CD3+. RNA isolation and cDNA synthesis were performed as previously described (de Mestre et al., 2010).

Cloning

Equine *NKP46*, *CD16*, *CD56*, and *CD94* were amplified from horse PBMC cDNA using *Pfu* DNA polymerase (Stratagene, La Jolla, CA), gel purified/extracted, cloned into pCR4Blunt-TOPO vector (Invitrogen, Carlsbad, CA), and sequenced on an Applied Biosystems Automated 3730 DNA Analyzer at the Cornell Life Sciences Center. Sequences were analyzed using the DNAStar software suite.

qPCR

SYBR Green (Applied Biosystems, Carlsbad, CA) real time PCR reactions for amplification of genes listed in Table 4.2, or the housekeeper gene equine ubiquitin-conjugating enzyme E2D 2 (UBE2D2), were performed using an ABI 7500 Fast sequence detector (Applied Biosystems). Primers were designed with Primer3 software (MIT, Cambridge, MA) to cross intron/exon boundaries to prevent amplification of genomic DNA (Table 4.1). A dissociation curve was performed after each experiment to confirm a single product was amplified. A standard curve was generated for all genes using known copy numbers of a plasmid that contained the DNA specific to the gene. Each sample was first normalized to 1.5×10^4 copies of UBE2D2. Data were analyzed using Graph Pad Prism Software. Data sets were checked for normality using the Kolmogorov-Smirnov test (for $n>4$) or normal Q-Q plots (for $n\leq4$). Differences between groups

Table 4.2 Gene sequences and quantitative PCR primers used

Equine Gene	Chromosome location	Genbank Accession #	Quantitative PCR Primers (5'-3')
NCR1 (NKP46)	10:24121252-24125282	JN808451	F: CACCTGGAATGATGAACAAAG R: CCTGGGATGAACTGAGAGG
CD3G	7:26203732-26210259	JN808452	F: GGCCTCATCCTGGCTATCAC R: CCCAGATTCCGTGTAGTTTCTC
FCGR3 (CD16)	5:36222322-36228997	JN795139	F: AGACAGCCCTCTCACCACTC R: GTGCACATGCTTGTTCTTCC
NCAM1 (CD56)	7: 21413392-21712444	JN808450	F: CCGGCATTTACAAGTGTGTG R: GGGTTGGTGCATTCTTGAAC
KLRD1 (CD94)	6:37355730-37361350	JN795140	F: AGAATGGCTCTGCTGTCTCC R: CCCTTGGCAGTCTTCATCC
GCM1	20:50724492-50740140	XM_001503164.1	F: CAACTTCTGGAGGCACGAC R: CGCCTTCTTCATTGCTCTTC

were determined using unpaired (Figs. 3, 4) or paired (Fig. 5) two-tailed Student's t tests, or the Mann-Whitney test for non-parametric data (Figure 4.4C).

Results

Comparative genomics of equine NK cell receptor gene orthologs

We chose the NK cell markers *NKp46*, *CD94*, *CD56*, and *CD16* to investigate equine NK cells based upon their expression patterns in peripheral and uterine NK cells of other species. PCR primers for these genes were designed by analyzing the equine whole genome sequence (WGS) for regions of homology with annotated genes of other species. Full-length (*NKP46*, *CD94*, and *CD16)* and partial (*CD56*) transcripts were amplified from cDNA generated from PBMC RNA derived from the WGS-donor animal maintained in our research herd. Coding sequences (CDS) were translated and aligned with the human, bovine, murine, and porcine protein sequences, as available (Figure 4.1).

Equine *NKP46* was identified on chromosome 10 (ECA10) in a region syntenic with the leukocyte receptor gene complex (LRC) of human chromosome 19 (HSA19), where the *NKP46* gene is located (Colucci et al., 2011). Multiple splice variants were identified, most correlating closely to validated human transcript variants (Figure 4.2). The translated sequence of the dominant *NKp46* isoform shares 54-67% identity with the protein sequences of other selected species (Figure 4.1A, Table 4.3). Conservation of critical protein motifs such as two immunoglobulin (Ig)-like domains, a transmembrane domain containing an arginine required for activation, and cysteines that form stabilizing intramolecular disulfide bonds, suggest a functional capacity of the gene product.

Equine *CD16* was identified on ECA5, clustered with other predicted Fc receptor genes and syntenic to a homologous region on HSA1. The translated sequence shares 37-60% identity with other species (Figure 4.1B, Table 4.3); the two Ig-like domains, stabilizing cysteine residues, and transmembrane domain with a required aspartic acid, are conserved in the equine ortholog.

Figure 1. Multi-species alignments of NK cell marker protein sequences. Amino acid sequences of equine NKp46 (A), CD16 (B), CD94 (C), and CD56 (D) orthologs were determined by translation of cDNA sequences determined from a combination of bioinformatic analysis of the equine WGS and amplification from horse PBMC mRNA by RT-PCR. *Equus caballus* (EC) sequences were aligned with sequences of *Homo sapiens* (HS), *Bos taurus* (BT), *Sus scrofa* (SS) and *Mus musculus* (MM) using Clustal W. Full-length clones were obtained for *NKP46*, *CD16*, and *CD94* genes; a 247 bp partial clone was obtained from *CD56*, as indicated by brackets. Dots represent identities, dashes represent gaps. Dashed boxes represent transmembrane domains and grey bars represent conserved immunoglobulin-like domains. Asterisks highlight highly conserved transmembrane domain charged residues (arginine and aspartic acid) required for association with adaptor molecules responsible for intracellular signaling. Closed boxes represent cysteine residues known to stabilize intramolecular secondary structures. Genbank IDs for sequences used: NP_004820 (HS), NP_899209 (BT), NP_001116615.1 (SS), NP_034876 (MM), NKp46; AAH17865.1 (HS), AAI12757.1 (BT), NP_999556 (SS), NP_034318.2 (MM), CD16; NP_001107868 (HS), NP_001002890 (BT), EDK99936 (MM), CD94; NP_851996.2 (HS), NP_776824.1 (BT), NP_001074914.1 (MM), CD56

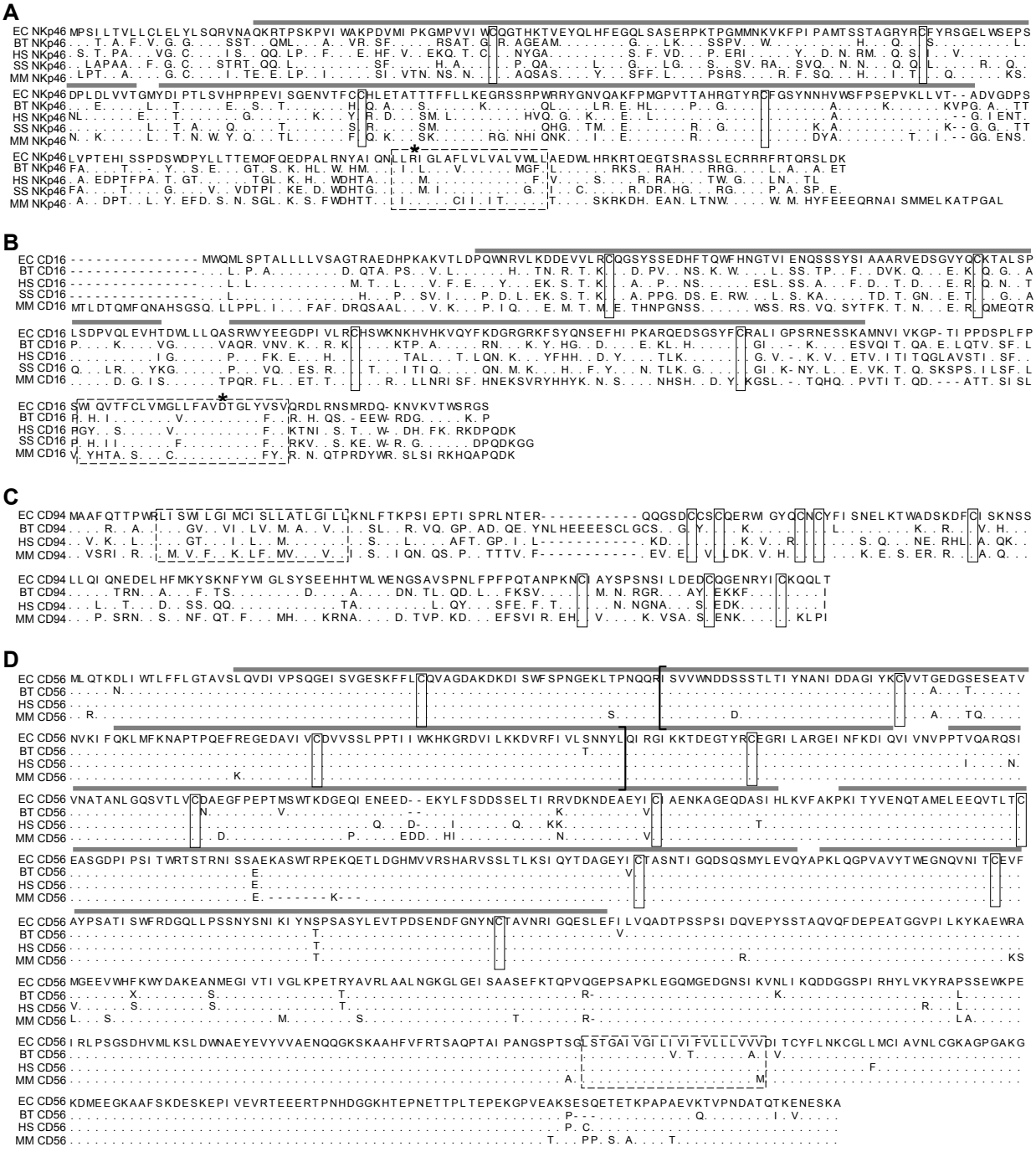
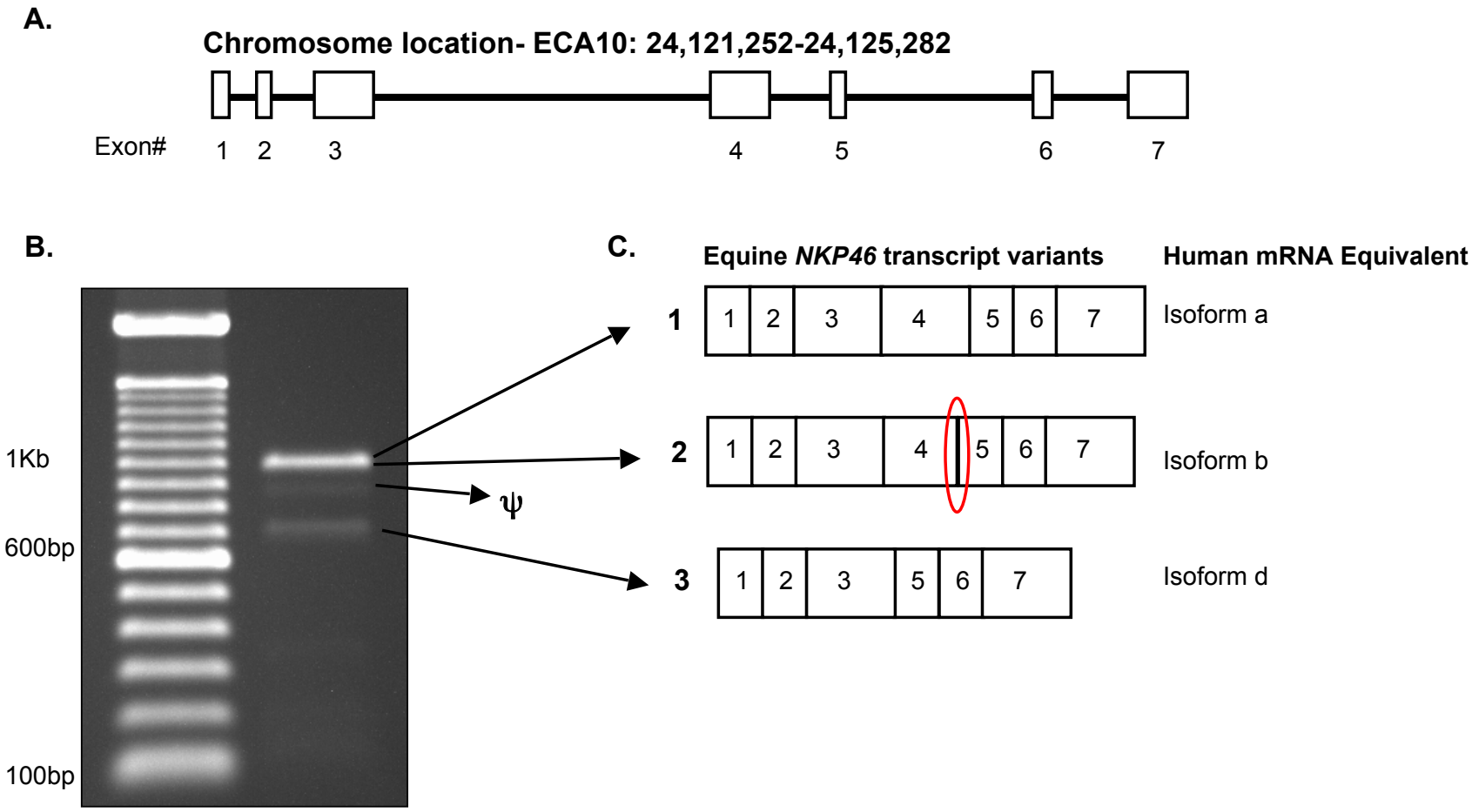

A
EC NKp46
BT NKp46
HS NKp46
SS NKp46
MM NKp46
B
EC CD16
BT CD16
HS CD16
SS CD16
MM CD16
C
EC CD94
BT CD94
HS CD94
SS CD94
MM CD94
D
EC CD56
BT CD56
HS CD56
SS CD56
MM CD56

Figure 4.2 Relation of equine and human *NKP46* splice isoforms. (A) Schematic diagram of *NKP46* genomic structure. (B) Agarose gel of PCR products generated from PBMC cDNA using primers designed to amplify the full-length CDS. Bands were extracted, cloned, and sequenced, yielding 3 transcripts with open reading frames (C). Equine transcript variant 1 is similar to full length human *NKP46* isoform a (NM_004829.5). Variant 2 uses an alternate in-frame splice site at the exon 4-5 boundary, similar to human isoform b (NM_001145457.1). Variant 3 has a deletion of exon 4, corresponding to the loss of one Ig-like domain, similar to the human exon 3 deletion isoform d (NM_001242356.1). The band observed at 900bp is an intron-retention mutant of variant 3 with a premature stop codon (ψ, pseudogene).


A.
Chromosome location- ECA10: 24,121,252-24,125,282
Exon#
1 2 3 4 5 6 7
B.
1Kb
600bp
100bp
ψ
C.
Equine NKP46 transcript variants
Human mRNA Equivalent
1
1 2 3 4 5 6 7
Isoform a
2
1 2 3 4 5 6 7
Isoform b
3
1 2 3 5 6 7
Isoform d

Equine *CD94* was located in a region of ECA6 syntenic with the natural killer gene complex (NKC) of HSA12 (Colucci et al., 2011); and a splice variant conserved between human, horse, and cow was identified (data not shown). The translated dominant *CD94* sequence shares 51-67% identity with other species (Figure 4.1C, Table 4.3). The C-type lectin domain, lack of a cytoplasmic signaling motif, and structure-stabilizing cysteine residues of the human protein are conserved in the equine homolog.

Equine *CD56* was found on ECA7 and demonstrated very high homology with other species at the amino acid level (96-98%). It shared the highest homology of all selected species to the human protein (Table 4.3). Due to its low abundance in PBMC, the full 6kb mRNA was difficult to clone in its entirety. A region spanning predicted exons 2-4 was cloned, sequenced, and used for the studies below.

Table 4.3 Percent amino acid identity of equine NK cell markers with those of other selected species

	NKp46	CD16	CD94	CD56
Homo sapiens	65	60	67	98
Bos taurus	65	58	60	97
Sus scrofa	67	57	N/A	N/A
Mus musculus	54	37	51	96

Expression of equine NK cell receptor genes in peripheral lymphocytes

In order to determine expression levels of NK marker genes in the circulating immune cells of the horse, quantitative PCR was performed on cDNA generated from equine PBL. Lymphocyte expression levels were compared to those from a non-immune tissue: chorionic girdle (CG) trophoblast microdissected from conceptuses recovered at days 33-34 of pregnancy. These invasive cells are the immediate precursors of the endometrial cup trophoblast. In addition to NK marker genes, expression levels for the T-cell marker CD3 gamma (*CD3G*) and the trophoblast marker glial cells missing homologue 1 (*GCM1*) (de Mestre et al., 2009) were used as controls for the two cell types.

Transcripts for the NK markers *CD16, CD94*, and *NKP46* were present in PBL, with *CD16* and *CD94* demonstrating robust expression and *NKP46* exhibiting modest expression (Figure 4.3A, B, D). Trophoblast expressed at most trace amounts of these three genes. *CD56* exhibited barely detectable expression levels in PBL, consistent with the low expression levels of antigen observed on the predominantly $CD56^{dim}$ peripheral NK cells of other species (Fig 3C). Somewhat surprisingly, *CD56* expression was detected in the CG trophoblast. This expression pattern is also observed in human and macaque invasive trophoblast (Golos et al., 2010; Proll et al., 1996). Predictably, *CD3G* demonstrated high expression in lymphocytes, but none in trophoblast (Fig 3E). *GCM1* was highly expressed in trophoblast but absent in PBL (Fig 3E).

We next determined the expression of these NK cell markers in CD3+ and CD3- lymphocyte populations, in order to compare the patterns to those seen in other species. cDNA was generated from peripheral lymphocytes magnetically sorted according to expression of cell-surface CD3 antigen. *NKP46* was expressed at 4-fold higher levels in the CD3-depleted (CD3-) population (Figure 4.4A). This is consistent with the canonical CD3- NKp46+ NK cell phenotype exhibited by most species. *CD94* expression did not differ between the two-lymphocyte groups (Figure 4.4D).

This is not surprising as, in addition to CD3- NK cells, conventional CD3+ lymphocytes and

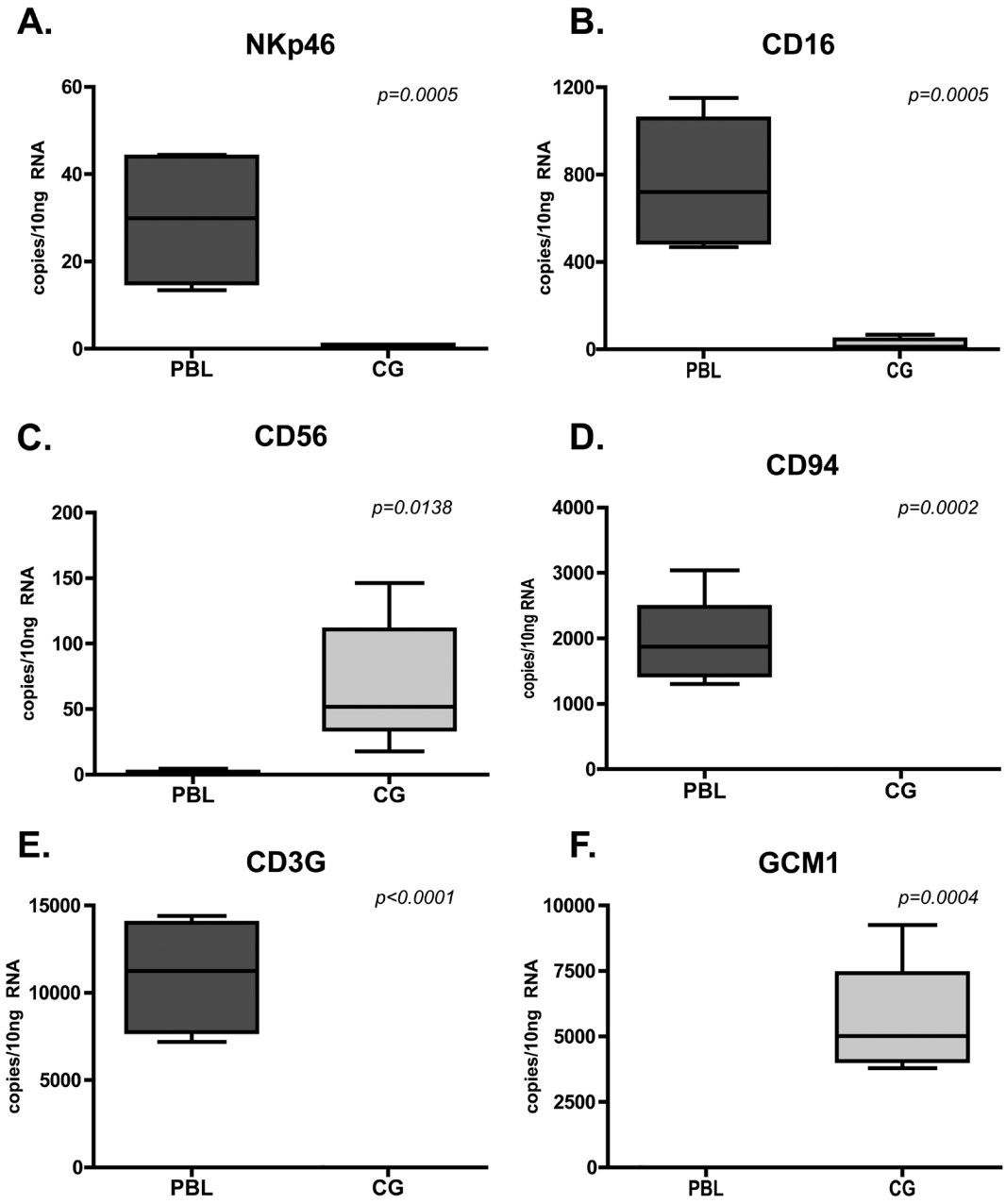


Figure 4.3 Detection of NK cell marker expression in equine peripheral lymphocytes. Expression of equine *NKP46* (A), *CD16* (B), *CD56* (C), and *CD94* (D) was determined using quantitative RT-PCR performed on RNA isolated from PBL and chorionic girdle trophoblast (CG). *CD3G* (D) and *GCM1* (E) expression levels were also measured as controls; n=5.

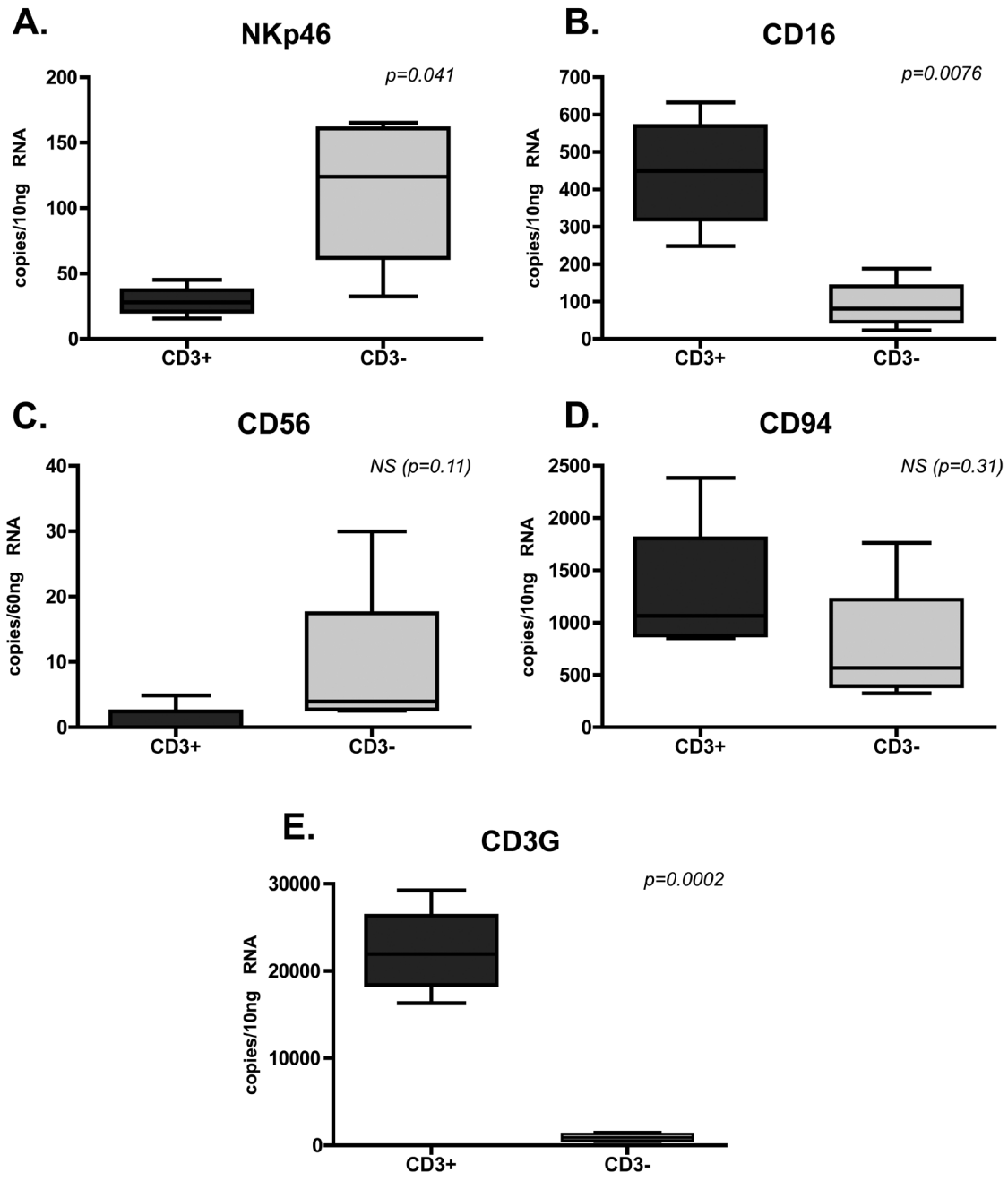


Figure 4.4 Expression of NK cell marker expression in CD3-depleted vs. CD3-enriched lymphocyte populations. PBL were magnetically sorted into CD3 depleted (CD3-) and enriched (CD3+) populations; isolated RNA was analyzed for expression of *NKP46* (A), *CD16* (B), *CD56* (C), *CD94* (D), and *CD3G* (E) using quantitative RT-PCR; n=4.

NK cells can express *CD94*. However *CD16*, which is usually associated with peripheral CD3- NK cells, was 5-fold more highly expressed in the CD3-enriched (CD3+) population (Figure 4.4B). This higher *CD16* expression among the CD3+ cells may therefore represent NKT cells, a CD16+ T lymphocyte subset (Lanier et al., 1985), or a species-related difference in NK cell phenotype. Changes in *CD56* levels were difficult to detect due to its low expression levels in the periphery (Figure 4.3C). After increasing the input cDNA by 6-fold, transcript levels were still low, but we observed a trend toward increased expression in CD3- lymphocytes (Figure 4.4C).

NK cell marker expression is higher in endometrial cup lymphocytes compared to PBMC

Expression of NK cell marker genes was next investigated in the equine uterus during early pregnancy. Lymphocytes were isolated from the endometrial cups of five mares pregnant between days 43 and 46 of gestation. Expression levels of NK markers in these endometrial cup lymphocyte (ECL) samples were compared to PBMC isolated from the same mares just prior to euthanasia.

Expression levels of all NK markers were dramatically increased in ECL samples compared to the paired PBMC (Fig. 5). *NKP46*, which was modestly expressed in the periphery, was expressed at 7-43 fold (mean=14) higher levels in the endometrial cups (Fig. 5A). *CD16,* which was highly expressed in PBL, shows a dramatic 20-fold higher expression in ECL (Fig. 5B). Based upon the slight decrease in *CD3G* expression in the endometrial cups (Fig. 5E), the increase in *CD16* is not likely due to cells like the CD3+CD16+ population seen in the periphery (Figure 4.4B). *CD94*, also highly expressed in the periphery, is 8-fold higher in the ECL (Fig. 5D); again this is unlikely attributable to CD3+ cells. Finally, *CD56*, a marker that is nearly undetectable in the periphery, exhibits a 44-fold increase in the endometrial cup lymphocyte samples (Fig. 5C). Because *CD56* expression was also observed in chorionic girdle trophoblast, we measured *GCM1* in the ECL samples in order to detect contaminating trophoblast cDNA. *GCM1* expression was detected (Fig. 5F), but at levels 1/5 of those in the trophoblast samples (Figure 4.3F) and the difference between ECL and PBMC expression was not statistically

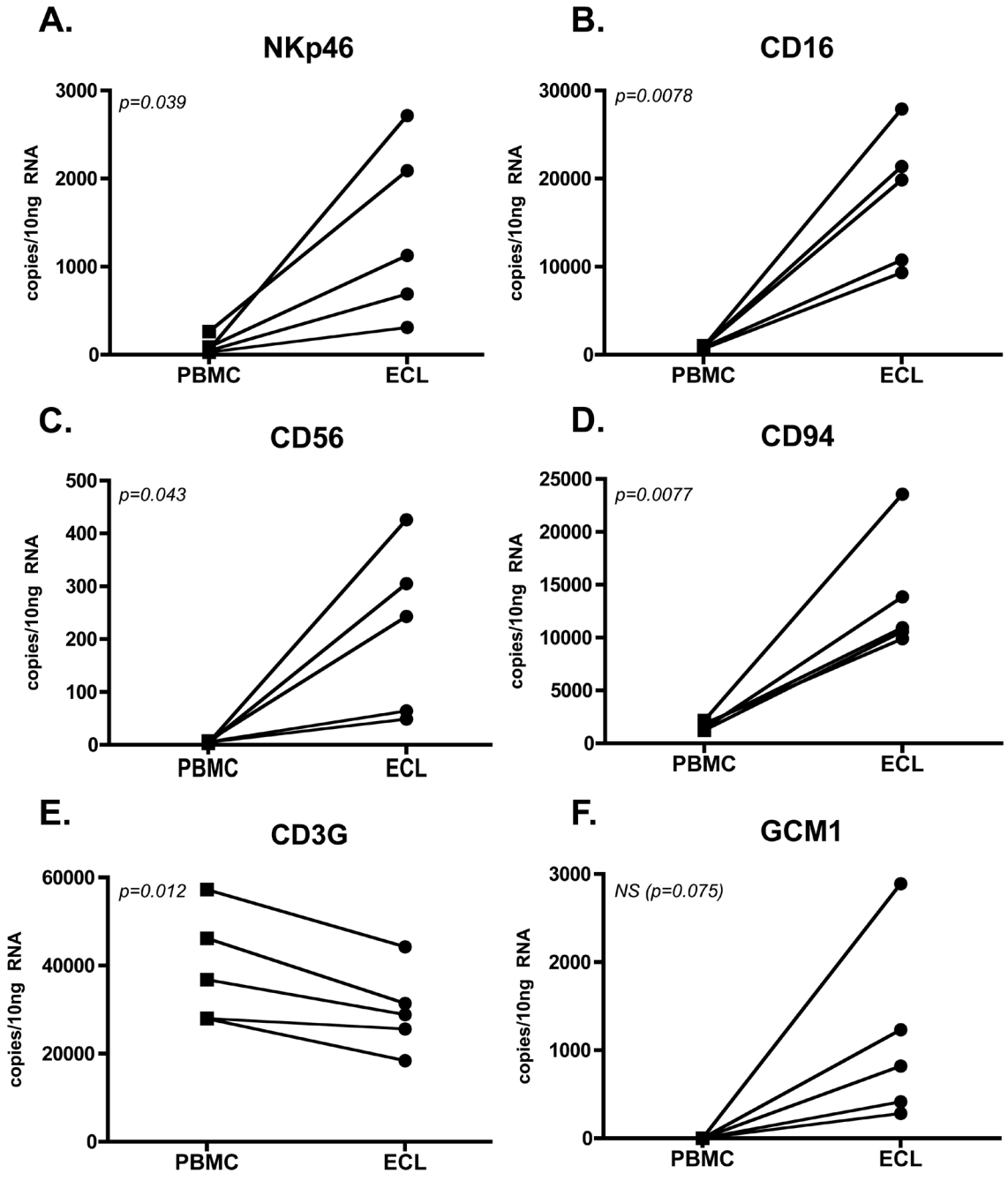


Figure 4.5 Comparison of NK cell marker expression in peripheral and endometrial cup lymphocytes. Paired PBMC and ECL were isolated from five mares pregnant at days 43-46 of gestation. Expression of *NKP46* (A), *CD16* (B), *CD56* (C), *CD94* (D), *CD3G* (E), and *GCM1*(F) were measured using quantitative RT-PCR.

significant. Therefore, contaminating trophoblast cannot account for the dramatic increase in *CD56* expression seen in the ECL samples.

Discussion

Using a highly specific and sensitive quantitative molecular assay, we have demonstrated that the four primary markers used to determine NK cell phenotype in other species are expressed by horse lymphocytes. These genes exhibit conservation of critical structural and functional elements despite the approximately 100 million years of divergence between horses and humans (Figure 4.1). This suggests that they have undergone evolutionary pressure to maintain these functional motifs, and are therefore likely to have biological activity. The conservation of alternative splicing patterns of human and horse NK marker transcripts also seen here (Figure 4.2) is not a broadly observed phenomenon across genomes (Yeo et al., 2005). This suggests an evolutionary pressure at the mRNA processing level, further supporting preservation of gene functionality.

The activating receptor NKp46 is considered to be a specific marker for NK cells in multiple species, and is present on all NK subsets in primates and rodents at stable expression levels (Narni-Mancinelli et al., 2011). Therefore, the dramatic increase of *NKP46* in ECL suggests the presence of lymphocytes with an NK phenotype in the mare endometrium. *CD16*, while not associated with the NK cells found in the decidua during human pregnancy, is expressed in the pregnant uteri of the mouse (Yadi et al., 2008), rhesus monkey (Dambaeva et al., 2011), and pig (Engelhardt et al., 2002).

The expression patterns of equine *CD94* and *CD56* genes seen here are particularly interesting because to date, expression of these molecules at the maternal-fetal interface has only been observed in primates. The CD94 proteins, a co-receptor for non-classical MHC class I molecules, is more highly expressed in the human decidua compared to the periphery (King et al., 2000), as seen here with the equine gene. CD56, perhaps the most important marker of primate decidual NK cells, is minimally expressed in the periphery but highly expressed in the decidua (Kalkunte

et al., 2008), similar to what we observed in the ECL. Neither marker is expressed by mouse uterine NK cells.

The CD56 ^{bright} NK cells of the primate decidua are characterized by a poor cytolytic capacity and the secretion of high levels of IFNg (Kalkunte et al., 2008). Interestingly, we have previously observed an increase in the number of IFNγ+ lymphocytes among the same ECL samples studied here (de Mestre et al., 2010). In order to determine if there is a relationship between CD56 and IFNγ in these ECL, we examined the correlation between *CD56* expression levels and the percent of IFNγ+ lymphocytes in the ECL (Figure 4.6). Of the four data points tested,

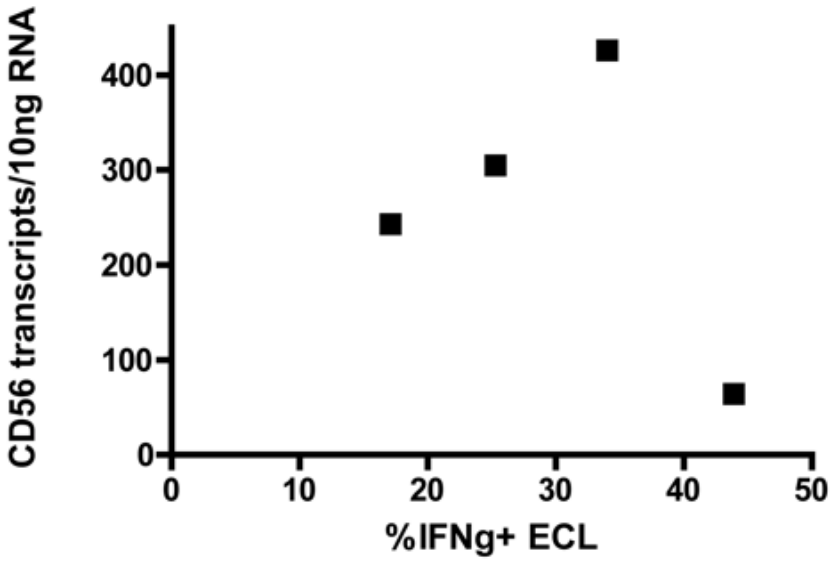


Figure 4.6 Relationship between *CD56* gene expression and IFNγ+ lymphocytes in endometrial cup lymphocytes. ECL samples from four horses were previously assayed for intracellular IFNγ expression by flow cytometry (de Mestre et al., 2010). *CD56* expression was measured in frozen samples of the same cells using qPCR as described in Fig. 5. The number of IFNγ+ ECL was compared to *CD56* gene expression for each sample. The outlier point was obtained from an animal carrying a twin pregnancy (#3382), which may contribute to the dramatic difference in IFNγ+ cells at her maternal-fetal interface. No association was observed with the other cytokines measured (IL-4, IL-10).

three demonstrated a relationship suggesting a strong correlation (r=0.99); the fourth outlying data point was from ECL collected from a twin pregnancy. The sample size was insufficient to determine if a statistically significant relationship exists, but the pattern clearly suggests further investigation is warranted to determine if these two molecules are expressed by the same cells.

The stage of pregnancy studied here, days 43-46, is roughly one week following trophoblast invasion. The presence of NK cells in the equine uterus during this period could help explain the poorly understood phenomenon of paternal class I MHC expression by the chorionic girdle trophoblast. Interaction between uterine NK cell activating receptors and allogeneic paternal MHC I has recently been shown to be important for trophoblast invasion, vascular development, and fetal growth in both humans and mice (Hiby et al., 2010; Madeja et al., 2011). The horse uterus does not show the same spiral artery alteration seen in species with hemochorial placentae, so this uNK-cell function may operate irrespective of placentation in order to facilitate trophoblast invasion and endometrial remodeling. It will be interesting to further explore what role this population of cells plays in equine pregnancy, and what new insights it will lend to our knowledge of evolutionarily-conserved maternal-fetal interactions.

Acknowledgements

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CHAPTER 5

GENERATION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES TO EQUINE CD16[1]

[1]Noronha, L.E., Harman, R.M., Wagner, B., and Antczak, D.F. Generation and characterization of monoclonal antibodies to equine CD16. To be submitted to *Veterinary Immunology and Immunopathology.*

Abstract

The low-affinity Fc receptor CD16 plays a central role in the inflammatory and innate immune responses of many species, but has not yet been investigated in the horse. Using the predicted extracellular region of equine CD16 expressed as a recombinant fusion protein with equine IL-4 (rIL-4/CD16), we generated a panel of mouse monoclonal antibodies (mAbs) that recognize equine CD16. Nine mAbs were chosen for characterization based upon recognition of CD16 in ELISA, but not IL-4. All nine mAbs recognized full-length, cell-surface CD16 expressed as a GFP fusion protein by CHO cells, but not the closely related Fc receptor CD32 expressed in the same system. In flow cytometric analysis with equine peripheral leukocytes, the mAbs labeled cells in the granulocyte, monocyte, and lymphocyte populations in a pattern consistent with other species. Monocytes that were strongly labeled with CD16 mAb 9G5 were also positive for the LPS receptor CD14. Cytospins made with peripheral leukocytes were immunohistochemically labeled and showed mAb recognition of primarily mononuclear cells. ELISA revealed that the nine mAbs can be grouped into three patterns of epitope recognition. These new antibodies will serve as useful tools in the investigation of equine immune responses and inflammatory processes.

Introduction

The recognition of immune complexes is a key initiating event in the establishment of innate immune responses and inflammation. A primary mediator of this process is the membrane glycoprotein CD16 (FcgRIII), a low-affinity receptor for the Fc region of aggregated IgG. Together with CD64 (FcgRI) and CD32 (FcgRII), it forms a family of three closely related Fcg receptors (Ravetch and Kinet, 1991). CD16 is an activating receptor; its effector function is initiated when the extracellular region, comprised of two C2-type immunoglobulin-like domains, binds to the Fc regions of IgG in immune complexes (Lanier et al., 1989; Simmons and Seed, 1988; Trinchieri and Valiante, 1993). Depending on the cell type, activation can lead to phagocytosis, antibody-dependant cytotoxicity (ADCC), release of inflammatory mediators,

or degranulation. A soluble form of CD16 in human plasma has also been shown to bind complement receptors, resulting in the production of inflammatory cytokines (Galon et al., 1996). Mice deficient in CD16 demonstrate impaired NK cell, monocyte, and mast cell effector functions, as well as reduced inflammation-induced pathology (Hazenbos et al., 1996).

In the human, CD16 exists as two isoforms encoded by closely related genes: a transmembrane form (IIIa) and glycosylphosphatidylinositol (GPI)-linked form (IIIb). The extracellular regions of the two proteins are nearly identical and differ by only six amino acids (Ravetch and Perussia, 1989). The transmembrane form is expressed on NK cells, monocytes, macrophages, mast cells, and small T-cell subsets, while the GPI-linked form is expressed exclusively on neutrophils (Ravetch and Kinet, 1991). In non-primate species that demonstrate CD16 expression, no glypiated forms have been identified, and all CD16+ cell populations are thought to express the transmembrane form (Collins et al., 1997; Halloran et al., 1994; Hughes, 1996; Nishimura et al., 2000).

Expression of an equine CD16 ortholog has not yet been demonstrated at the genetic or protein level. Although CD16 molecules of other livestock species have been successfully identified with commercially available cross-reactive antibodies (Boysen et al., 2008; Elhmouzi-Younes et al., 2010), our group and others (Ibrahim and Steinbach, 2007) have not successfully identified such reagents for use with horse cells. Researchers studying equine immunology and inflammation have therefore been limited by an inability to study this fundamental immunoreceptor. Here, we describe the generation of a recombinant equine CD16 protein, and the subsequent development of a panel of CD16-specific monoclonal antibodies that can be used in multiple immunological applications to address this knowledge gap.

Materials and Methods

Recombinant IL-4/CD16 (rIL-4/CD16)

Equine *CD16* was amplified from horse PBMC cDNA using *Pfu* DNA polymerase (Stratagene, La Jolla, CA), gel purified/extracted, cloned into pCR4Blunt-TOPO vector (Invitrogen, Carlsbad, CA), and sequenced on an Applied Biosystems Automated 3730 DNA Analyzer at the Cornell Life Sciences Center; sequence IDs and PCR primers listed in Table 5.1. Sequences were analyzed using the DNAStar software suite. The amplified sequence was identical to a region of equine chromosome 5 that is syntenic to the FcgR region of human chromosome 1. No evidence of a second soluble form of *CD16* was found in the genome. The extracellular domain (bases 52-579 of the coding sequence) was predicted by performing Clustal W alignments with validated *CD16* sequences of other species, and was directionally cloned into a pcDNA3.1 vector (Invitrogen, Carlsbad, CA) 3' to the coding sequence of equine *IL-4* as previously described (Wagner, 2011). CHO K-1 cells were transfected with the linearized vector using the Geneporter2 system (Genlantis, San Diego, CA). Stable transfectants were selectively cultured in G418 (Invitrogen), cloned by limiting dilution, and screened for IL-4 production by flow cytometry and ELISA as previously described (Wagner, 2011). *r*IL-4/CD16 was purified from serum-free supernatant by fast protein liquid chromatography using an anti-IL-4 affinity column as previously described (Wagner, 2011). A fraction of the purified fusion protein was resolved by SDS-PAGE on a 10% non-reducing polyacrylamide gel to determine molecular weight.

Immunization and splenic fusion

Mice were maintained at the Baker Institute for Animal Health rodent facility at Cornell University. Animal care was performed in accordance with the guidelines set forth by the Institutional Animal Care and Use Committee of Cornell University. Immunization was performed as previously described (Wagner et al., 2003). Animal response was measured by monitoring serum titers to equine IL-4 using ELISA. Spleen cells were fused to SP2/0 myeloma

Table 5.1 Sequences of genes and primers used.

Gene	Accession #	Primers (5'-3')
CD16	JN795139	*IL-4 fusion construct*
		F-GGCGGATCCTGGCACACGAGCTGAAGATC
		R-GGCAAGCTTTCAACCTTTAACAATGACGTTCATAGCC
		GFP fusion construct
		F-GGCGCTAGCATGTGGCAGATGCTATCACCAACGG
		R-GGCGGTACCTCAGAGCCCCGGCTCCATGTG
IL-4	GU139701	*GFP fusion construct*
		F-GGCGCTAGCATGGGTCTCACCTACCAACTGATTCCAG
		R-GGCGGTACCTCACACTTGGAGTATTTCTCTTTCATGATCGTCTTTAGC
CD32	JN795138	*GFP fusion construct*
		F-GGCGCTAGC ATGACTATGGAGATCCTGATGTTTCCAAATGTACATC
		R-GGCGGTACCTCTTTGTTTTCAGGGCTCTGGCTCCA

cells as previously described (Appleton et al., 1989). Nascent hybridomas were plated into 96 well tissue culture plates and supernatants from all wells were screened for reactivity to *r*IL-4/CD16 and *r*IL-4/IgG using ELISA, and for cell surface labeling of equine PBMC using flow cytometry. Antibodies which labeled PBMC and detected *r*IL-4/CD16 but not *r*IL-4/IgG were selected for further study. Cultures were cloned by performing three rounds of limiting dilution, measuring sensitivity and specificity of secreted immunoglobulin by ELISA and flow cytometry as above after each round. Mouse immunoglobulin isotypes of secreted antibodies were determined by ELISA (Sigma, St. Louis, MO). Antibodies were purified by fast protein liquid chromatography using a protein G affinity column (GE Healthcare, Piscataway, NJ). Proteins were quantified using a Bradford assay (Bio-rad, Hercules, CA). Selected antibodies were biotinylated using Sulfo-NHS-Biotin (Thermo Fisher Scientific, Waltham, MA).

Antibody screening and ELISA

Cell culture supernatants were screened for mAbs to *r*IL-4/CD16 by ELISA as described previously (Wagner, 2011) and against *r*IL-4/IgG1 (Wagner et al., 2005) to confirm their specificity to CD16. For epitope ELISA, plates were coated with 1ug purified mAbs, washed, then followed with *r*IL-4/CD16 fusion protein. Following washing, 1ug of biotinylated antibodies were then added, followed by streptavidin-HRP. Reactions were developed and analyzed as previously described (Wagner et al., 2006). For mAb 2A2, an additional ELISA was performed where plate-bound fusion protein was incubated with unlabeled antibody prior to adding the biotinylated antibody.

GFP fusion protein expression and flow cytometric analysis

Full-length sequence (minus termination codon) for the equine *CD16*, *CD32*, and *IL-4* genes were PCR amplified using *Pfu* DNA polymerase from equine PBMC cDNA and directionally cloned into the multiple cloning region of pEGFPN1 vector 5' to the *EGFP* gene (PCR primers listed in Table 5.1). CHO-K1 cells were transfected with the vectors using the Geneporter2

system and assayed for protein expression 48 hours post- transfection. Successful expression of GFP was confirmed by fluorescence microscopy and indicated correct reading frame cloning of the fusion protein, as GFP sequence was downstream of the protein of interest. Cells were detached with trypsin and used either fresh or fixed with 2%PFA for 20 minutes. Cells were labeled in FACS buffer [PBS, 0.5%BSA, 0.02%NaN3 with (fixed) or without (fresh) 0.5% saponin)] with mAb supernatants, isotype controls, or anti IL-4 mAb 13G7 (Wagner et al., 2006) followed by DyLight649-conjugated goat-anti-mouse IgG (H+L) $F(ab')_2$ (Jackson Immunoresearch, West Grove, PA). Cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA), and with FlowJo Software (Tree Star, Inc., Ashland, OR).

Leukocyte flow cytometry and immunohistochemistry

Heparinized blood samples were isolated from horses at the Equine Genetics Center, Baker Institute for Animal Health, Cornell University (animal details in Table 5.2). Animal care was performed in accordance with the guidelines set forth by the Institutional Animal Care and Use Committee of Cornell University. Erythrocytes were removed from blood samples using isotonic lysis (0.2% NaCl followed by 1.6% NaCl). Leukocytes were washed and assayed for viability using trypan blue exclusion and phase contrast microscopy. One million fresh cells were labeled with mAb 9G5 and/or a monoclonal antibody recognizing equine CD14 (Kabithe et al., 2010), or a mAb recognizing anti-canine parvovirus (CPV) as an isotype control, and analyzed by flow cytometry. Dead cells were excluded following staining for viability with propidium iodide. Five hundred thousand leukocytes were adhered to a glass slide with a Cytospin centrifuge, fixed in acetone, and labeled with mAbs as previously described (de Mestre et al., 2010).

Results

Expression of rIL-4/CD16 and selection of mAbs to equine CD16

To generate monoclonal antibodies to equine CD16 we employed a system using a recombinant secreted fusion protein made with equine IL-4 and the protein of interest (Wagner, 2011). To

Table 5.2 Animals used for leukocyte isolation

Cornell #	Breed	DOB	Sex	MHC Haplotype[1]
3099	Thoroughbred	1992	Mare	A2/A2
3521	Thoroughbred	2000	Gelding	A2/A2
3958	Thoroughbred	2002	Mare	A2/A10
3880	Thoroughbred	2006	Mare	A3/A3
2885	Thoroughbred	1988	Mare	A3/A3
2955	Thoroughbred	1990	Gelding	A3/A3
3954	MO Fox Trotter	1997	Gelding	A1/A8
3916	Thoroughbred	2002	Mare	A5/A7

[1]Equine Leukocyte Antigen haplotypes assigned by serologic and genomic testing.

create the fusion protein, the predicted extracellular domain of CD16 was PCR-amplified from equine PBMC cDNA and inserted into a mammalian expression vector downstream of equine IL-4 (Figure 5.1A). CHO-K1 cells were stably-transfected with this construct and the secreted fusion protein was purified from the culture medium by fast protein liquid chromatography using an equine IL-4 affinity column. The size of the recovered protein was consistent with the 36.7 kDa predicted molecular weight of *r*IL-4/CD16 (Figure 5.1B). The extracellular domain of equine CD16 contains two predicted N-linked glycosylation sites (N-X-S/T-X), therefore the three bands observed on SDS-PAGE likely represent differentially glycosylated species of the fusion protein (Figure 5.1B, arrowheads). The fusion protein was used to immunize mice, the spleens of which were recovered to generate hybridomas. Media samples from the hybridomas were screened for the ability to recognize the *r*IL-4/CD16 fusion protein in ELISA (Figure 5.1C). Specificity of this recognition was determined by testing the media samples against a *r*IL-4/IgG1 fusion protein (Figure 5.1C). Antibodies that recognized the CD16 fusion protein, but not *r*IL-4/IgG1, were considered CD16 specific. MAbs generated by nine hybridoma clones met these initial screening criteria and were selected for further characterization.

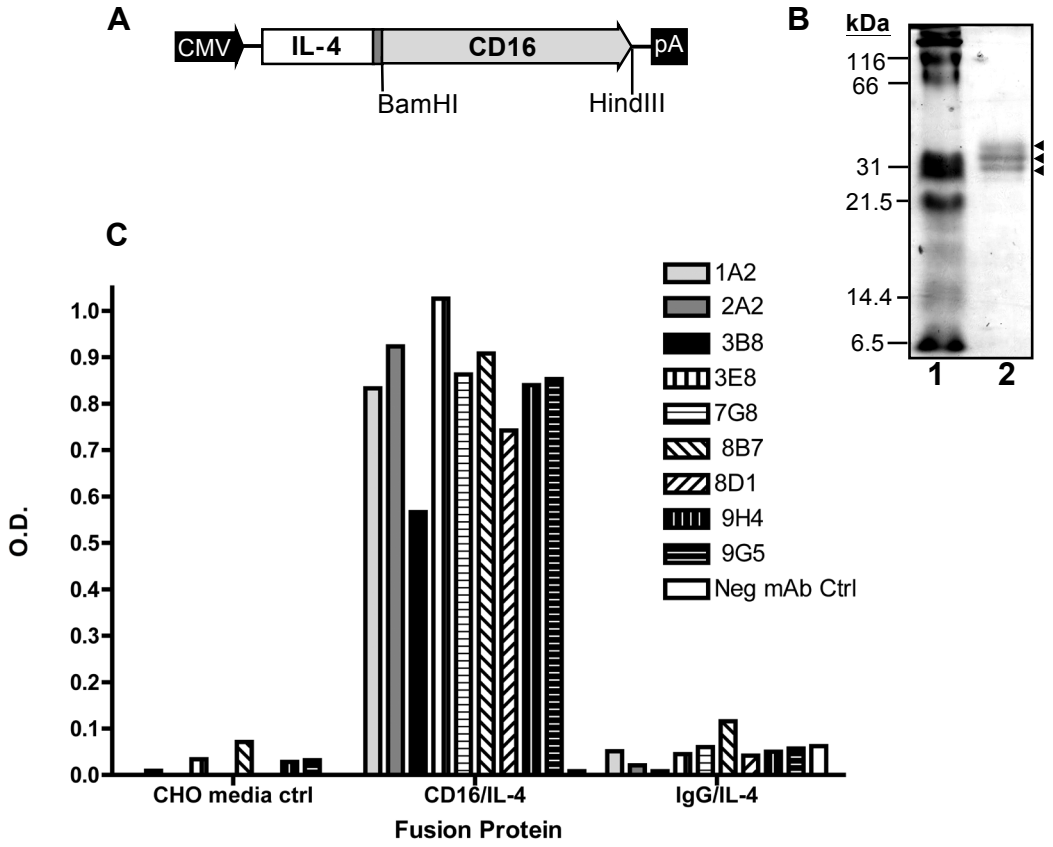


Figure 5.1 Construction and expression of *r*CD16/IL-4, and utilization to identify anti-CD16 hybridomas. (A) The predicted extracellular domain of equine *CD16* was directionally inserted 3' to equine *IL-4* in a pcDNA3.1 expression vector (*CMV*, Human cytomegalovirus immediate-early promoter; *pA*, Bovine Growth Hormone polyadenylation signal). (B) Soluble *r*IL-4/CD16 was expressed in CHO cells, affinity purified, and resolved by SDS-PAGE on a 10% gel under non-reducing conditions (lane 2). Arrowheads denote multiple glycosylation forms. (C) Culture media from hybridomas were tested by ELISA for the ability to recognize *r*IL-4/CD16 and rIL-4/IgG1; *negative mAb control*, anti-CPV.

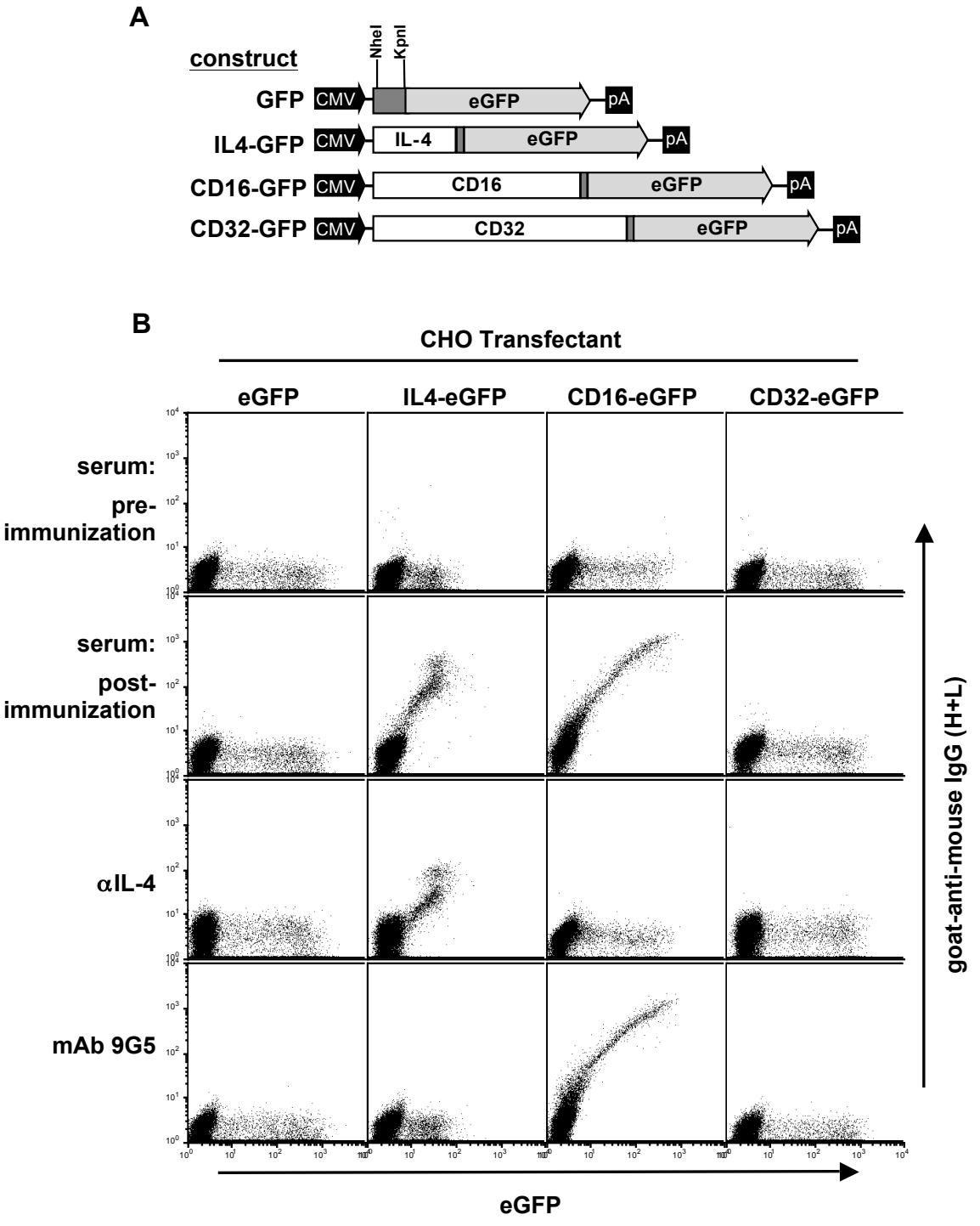
MAbs recognize full-length equine CD16

To test the ability of the nine mAbs to recognize full-length CD16 expressed on the cell surface, the full coding sequence (minus the stop codon) was cloned into the pEGFPN-1 mammalian expression vector upstream of the *EGFP* gene (Figure 5.2A). This allowed membrane expression of CD16 with minimal alterations to surface protein conformation, as eGFP would be expressed on the cytosolic side of the cell membrane. CHO-K1 cells were transfected with the construct and GFP expression was verified by fluorescence microscopy. The cells were labeled with the nine mAbs and analyzed by flow cytometry, where they demonstrated robust recognition of the full-length CD16 protein (Figure 5.2B). Because CD16 is one of three closely related Fc receptors known to have significant sequence similarity in other species, we also tested the specificity of the selected mAbs to CD32, the FcgR with the most predicted similarity to CD16. Equine *CD32* was similarly cloned into pEGFPN-1 and expressed in CHO cells; none of the nine antibodies recognized the CD32 construct in flow cytometric analysis (Figure 5.2B). MAb labeling was performed on fresh cells, so that only native, cell surface protein could be recognized (data not shown) as well as fixed, permeabilized cells, so that control cells producing soluble IL-4-GFP could be included to confirm the lack of mAb reactivity to IL-4 seen in ELISA (Figure 5.2B). All mAbs demonstrated the ability to recognize membrane-expressed CD16 in a specific manner.

MAbs recognize native CD16 on equine leukocytes

We next tested the ability of the antibody panel to recognize native CD16 on intact horse cells. Total equine leukocytes were used so that the granulocyte, monocyte, and lymphocyte populations could be investigated. First, the labeling patterns of the nine mAbs were compared on samples from one animal. The mean fluorescence intensity (MFI), an indicator of relative antigen density, varied between the three cell populations in a pattern that was similar for all mAbs (Figure 5.3A, exceptions noted in Table 5.4). Granulocytes were dimly labeled, most lymphocytes and monocytes were labeled with moderate intensity, and a small population

Figure 5.2 Generation of full-length CD16-, CD32-, and IL4-GFP expression constructs and flow cytometric analysis showing recognition by anti-equine-CD16 mAbs. (A) Full length *CD16*, *CD32*, and *IL-4* coding sequences (minus stop codons) were directionally inserted into the pEGFP-N1 mammalian expression vector 5' to *EGFP* (*CMV*, Human cytomegalovirus immediate-early promoter; *pA*, SV40 polyadenylation signal). (B) CHO cells transfected with fusion constructs were labeled with anti-equine-CD16 mAbs and a fluorescently-labeled anti-mouse immunoglobulin secondary antibody (goat-anti-mouse-IgG (H+L)-647) and analyzed by flow cytometry. MAb 9G5 is shown as representative mAb. Anti-equine-IL-4 mAb (13G7) and sera taken from mouse spleen donor prior to immunization (*pre-immunization*) and at spleen collection (*post-immunization*) were used as controls.


A
construct
NheI
KpnI
GFP
CMV
eGFP
pA
IL4-GFP
IL-4
CD16-GFP
CD16
CD32-GFP
CD32
B
CHO Transfectant
eGFP
IL4-eGFP
CD16-eGFP
CD32-eGFP
serum:
pre-immunization
serum:
post-immunization
αIL-4
mAb 9G5
goat-anti-mouse IgG (H+L)
eGFP

of cells in the monocyte/large lymphocyte size range labeled very intensely, indicating the highest levels of expression. The percent of total leukocytes labeled varied between 1.5 and 7.6 (median=3.0%) and the observed patterns were similar to Figure 5.3A for all mAb.

In order to further investigate the leukocyte populations labeled by these mAbs, cells were isolated from seven additional horses, labeled with representative mAb 9G5, and analyzed by flow cytometry. The lymphocyte, granulocyte, and monocyte/large lymphocyte populations were gated based upon morphology (Figure 5.3B) and analyzed for percent labeled within each group. Individual animal variability of roughly 3- to 4-fold was observed for each cell population. The number of labeled granulocytes varied from 1.2% to 8.3% (median=3.1%); monocytes and large lymphocytes varied from 4.6% to 13.9% (median=8.4%); and lymphoctyes varied from 1.1%-3% (median=2.1%).

In order to visualize the labeled cells, leukocytes were adhered to slides and labeled with the mAbs by immunohistochemistry (Figure 5.3D). No positively stained granulocytes were observed, possibly due to a low receptor density as evidenced by the low MFI observed in the flow cytometry experiments. Among the labeled cells, the most intensely stained ($9G5^{hi}$) had large lymphocyte and monocyte morphologies, while smaller lymphocytes and some monocytes were moderately or dimly stained (9G5lo). These observations are consistent with the MFI values of these populations observed in Figure 5.3A.

The 9G5hi monocyte/large lymphocyte population was further investigated using 2-color flow cytometry. In humans, cows, and sheep, a substantial fraction of CD16+ monocytes are positive for the LPS receptor CD14 (Elhmouzi-Younes et al., 2010; Ziegler-Heitbrock, 2007). To determine whether this is also the case in the horse, leukocytes from 6 horses were labeled with mAb 9G5 and a monoclonal antibody to CD14. Cells in the monocyte/large lymphocyte population were gated and analyzed as above. In all horses, the 9G5hi population was consistently CD14+ (Figure 5.3E).

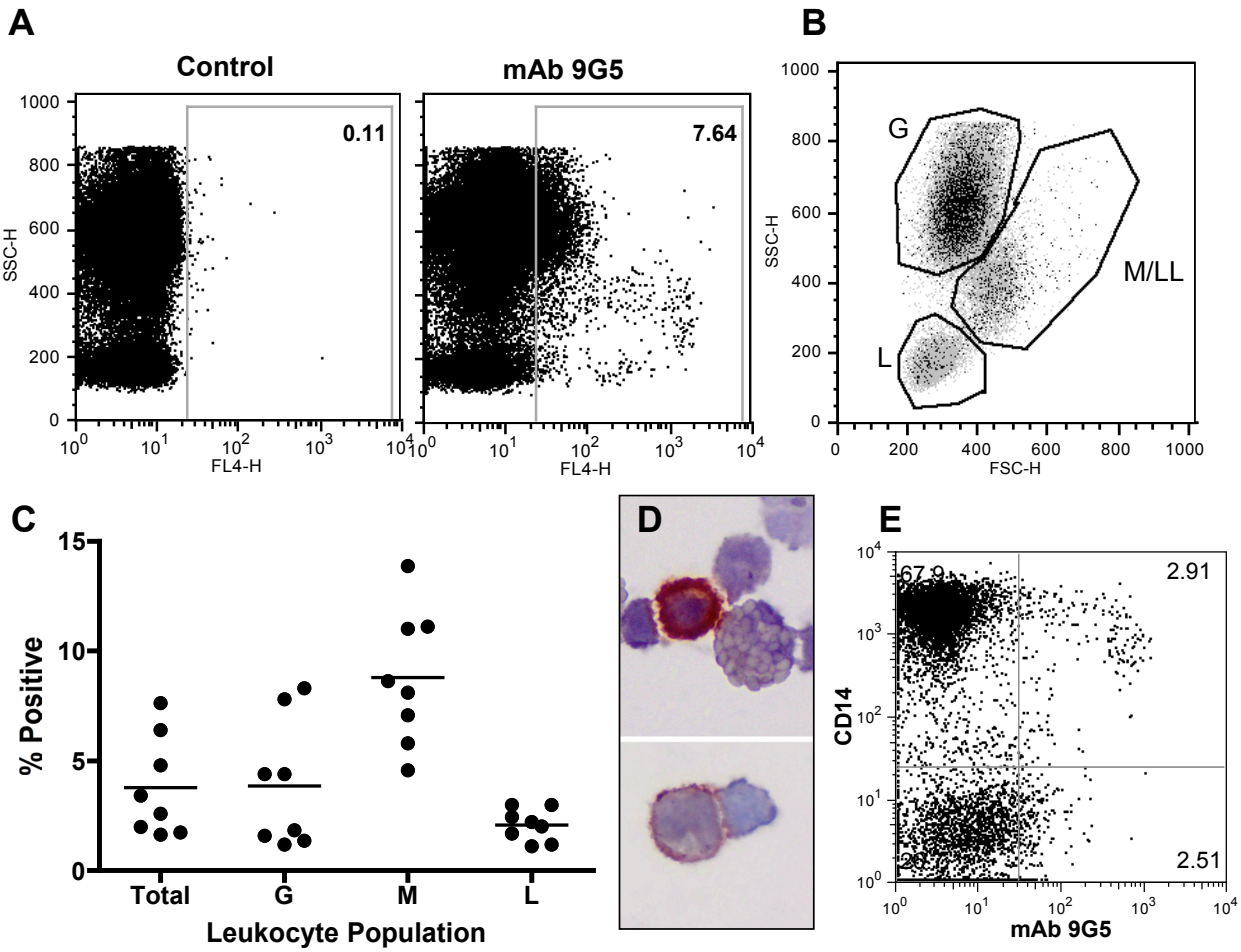


Figure 5.3 Recognition of horse peripheral blood leukocytes by anti-CD16 mAb 9G5.
(A) Isolated leukocytes were labeled with IgG1 control mAb (anti-CPV, left) or clone 9G5 (right) and analyzed by flow cytometry. (B) Dot plot showing morphology of positive staining cells (black) in relation to total cells (grey) and gating strategy for leukocyte populations (*G*, granulocytes; *M/LL*, monocytes/large lymphocytes; *L*, lymphocytes). (C) Graph depicting percentages of 9G5-labeled cell populations for eight horses. (D) Immunohistochemical labeling of peripheral leukocytes with mAb 9G5 (1000X) showing a representative brightly-stained lymphocyte (top) and dimly-stained monocytic cell (bottom). (E) Leukocytes were labeled with mAb 9G5 in conjunction with a mAb recognizing equine CD14. Dot plots show M/LL populations as indicated in B.

MAbs recognize three different epitopes

Finally, the nine mAbs were assayed by ELISA in order to determine if they recognized different antigen epitopes. rIL-4/CD16 was captured using each of the mAbs. Biotinylated mAbs were then applied to determine if the remaining free sites on the fusion protein could be bound, indicating recognition of distinct epitopes (Table 5.3). Based on this assay, the mAbs were assigned to one of three groups of epitope recognition. One mAb, 2A2, appeared to recognize a repeating epitope based upon its ability to bind rIL-4/CD16 already captured by 2A2. To test this, we incubated the captured fusion protein with increasing concentrations of unlabeled 2A2 to saturate remaining epitopes prior to probing with biotinylated 2A2. Binding of the biotinylated antibody was reduced in a dose-dependent manner, indicating that the mAb bound multiple sites on the protein (data not shown).

Table 5.3 ELISA and Epitope Recognition Group Assignments

		2. Biotinylated Antibody									
		1A2	2A2	3B8	3E8	7G8	8B7	8D1	9H4	9G5	Group Assignment
1. Capture Antibody	1A2	■		■		■				■	A
	2A2		*								C
	3B8	■		■		■				■	A
	3E8				■		■	■	■		B
	7G8	■		■		■				■	A
	8B7				■		■	■	■		B
	8D1				■		■	■	■		B
	9H4				■		■	■	■		B
	9G5	■		■		■				■	A

Black: Reduced binding by biotinylated antibody; White: No reduction in binding; *based on additional assays, mAb 2A2 binds a repeating epitope on CD16

Discussion

We determined that the nine mAb characterized here specifically recognize CD16 in multiple assays. In addition to the specificity demonstrated by ELISA and recognition of transfected cells, the consistent pattern observed in flow cytometric analysis of leukocytes indicates recognition of the same cell surface molecule among the numerous immunoreceptors on the leukocyte surface. Some variation in the percentage of labeled leukocytes was observed between mAb. This may be due to factors such as differences in affinity for antigen, variability in antibody concentration within the hybridoma culture media samples, or subtle differences in epitope recognition due to differential glysolyation of leukocyte populations. Some variation was also observed when leukocytes isolated from different horses were labeled. This difference among individuals is not unexpected. We have previously observed that cell surface expression of immunoreceptors can vary markedly among individual horses of mixed genetic backgrounds housed in an outdoor environment (de Mestre et al., 2010). Furthermore, expression of CD16 is known to be up-regulated during inflammation in other species. As the sampled horses are exposed to standard environmental insults, it is reasonable that individual animals may be in different states of normal, sub-clinical inflammation, therefore contributing to variability in CD16 expression.

Comparison of leukocytes among livestock species shows some variations in CD16 expression patterns. In the pig, CD16 is expressed on 13% lymphocytes, 70% monocytes, and >95% neutrophils (Dato et al., 1992). CD16 expression on sheep PBMC is reported at a median 9.1% (7.5-12.6) and 10.2% (8.1-11) for cows, in a pattern similar to Figure 5.3A (Elhmouzi-Younes et al., 2010). To compare our data in context, the percent of labeled equine PBMC was calculated by gating only the mAb 9G5-labelled monocyte and lymphocyte populations, yielding a median 3.7% (1.1-8.1). One explanation for this difference among livestock species may be an age-related difference in CD16 expression. The animals used for the cow and sheep studies were juveniles, while the horses used here were adults over 5 years of age. The difference may also be species-related, which is not surprising as CD16 expression levels vary significantly even

among closely related non-human primates (Rogers et al., 2006). Notably, however, the pattern of equine $CD14^{+}CD16^{+}$ monocytes that we observed here was strikingly similar to that seen in humans (Fingerle et al., 1993). This is particularly interesting considering that mice do not have a monocyte population with this phenotype (Sunderkotter et al., 2004). Therefore, the horse may serve as a useful model species for the study of these cells.

Table 5.4 Summary of αCD16 mAbs

Epitope Group	mAb	Isotype	Application ELISA	Application FACS[1]	Application IHC[4]
A	1A2	IgG1	+	++	++
	3B8	IgG1	+	++	+
	7G8	IgG1	+	+	+[5]
	9G5	IgG1	+	++	++[5]
B	3E8	IgG2b	+	+[2]	+++[5]
	8B7	IgG1	+	+	+
	8D1	IgG1	+	+[3]	+
	9H4	IgG1	+	+	+
C	2A2	IgG1	+	++	++

[1]Labels both fresh and PFA-fixed cells; [2]Labels additional lymphocyte population (common IgG2b artifact with fresh horse cells); [3]Labels a small fraction of granulocytes more brightly; [4]Frozen acetone-fixed cells (and tissues, data not shown); [5]Also labels PFA-fixed cells (other mAbs not tested)

Conclusion

The antibodies described here, summarized in Table 5.4, demonstrate recognition of recombinant CD16 in ELISA and full-length CD16 in flow cytometry. This recognition appears specific for CD16 and does not occur with other structurally-similar immunoreceptors. These antibodies label equine leukocyte populations in a pattern that is consistent with CD16 expression observed in other species. We therefore conclude that this panel of monoclonal antibodies recognizes the equine CD16 antigen in ELISA, flow cytometry, and immunohistochemistry, and will expand our ability to study horse health and disease.

Acknowledgements

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CHAPTER 6

GENERATION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES TO EQUINE NKP46[1]

[1]Noronha, L.E., Harman, R.M., Wagner, B., and Antczak, D.F. Generation and characterization of monoclonal antibodies to equine NKp46. To be submitted to *Veterinary Immunology and Immunopathology.*

Abstract

The immunoreceptor NKp46 is considered to be the most consistent marker of NK cells across mammalian species. Here, we use a recombinant NKp46 protein to generate a panel of monoclonal antibodies that recognize equine NKp46. The extracellular region of equine NKp46 was expressed with equine IL-4 as a recombinant fusion protein (*r*IL-4/NKp46) and used as an immunogen to generate mouse monoclonal antibodies (mAbs). MAbs were first screened by ELISA for an ability to recognize NKp46, but not IL-4, or the structurally related immunoreceptor CD16. Nine mAbs were selected and were shown to recognize full-length NKp46 expressed on the surface of transfected CHO cells as a GFP fusion protein. The mAbs recognized a population of lymphocytes by flow cytometric analysis that was morphologically similar to NKp46+ cells in humans and cattle. In a study using nine horses, representative mAb 4F2 labeled 0.8-2.1% PBL with a mean fluorescence intensity consistent with gene expression data. MAb 4F2+ PBL were enriched by magnetic cell sorting and were found to express higher levels of *NKP46* mRNA than 4F2- cells by quantitative RT-PCR. CD3-depleted PBL from five horses contained a higher percentage of 4F2+ cells than unsorted PBL. Using ELISA, we determined that the nine mAbs recognize three different epitopes. These mAbs will be useful tools in better understanding the largely uncharacterized equine NK cell population.

Introduction

Natural killer (NK) cells serve a vital role in the innate immune response due to their ability to destroy foreign cells, such as virus-infected cells and tumor cells, during a primary encounter. Their cytotoxic activity is determined by a complex interaction of activating and inhibitory cell-surface receptors (Joncker et al., 2009). While many of these receptors can be expressed on multiple cell types, the activating receptor NKp46 (NCR1, CD335) appears to be specific to NK and NK-like cells (Sivori et al., 1997). Thus, NKp46 is currently considered the most reliable identifying marker for NK cells across species (Walzer et al., 2007).

NKp46 is a type-I glycoprotein belonging to the immunoglobulin (Ig) superfamily. NKp46, with NKp30 (NCR3), and NKp44 (NCR2) comprise the natural cytotoxicity receptors (NCRs): activating receptors capable of inducing NK cell mediated cytotoxicity. Although the NCRs have similar cellular functions, NKP46 is structurally distinct from the other two molecules and is located in a different region of the genome (Biassoni et al., 2002). It also appears to be more stably expressed, and is generally present on all resting and activated NK cells (Sivori et al., 1997).

The structure of NKp46 consists of two extracellular C2-type Ig-like domains, a transmembrane region, and a short cytoplasmic tail (Ponassi et al., 2003). The receptor alone cannot transmit an activating signal; it acts by complexing with intracellular signaling molecules, such as CD3ζ and FCeRIγ, which contain immunoreceptor tyrosine-based activation motifs that initiate signal-transduction cascades resulting in NK cell activation (Biassoni et al., 2001). Known ligands that engage NKp46 include viral hemagglutinins and cellular heparan sulfate proteoglycans (Bloushtain et al., 2004; Mandelboim et al., 2001). Based upon the NKp46-mediated cytolysis of tumor cells, additional unidentified cellular ligands are presumed to exist (Halfteck et al., 2009). Indeed, mice that lack NCR1, the murine NKp46 ortholog, are more susceptible to influenza and the growth of some types tumors (Gazit et al., 2006; Halfteck et al., 2009).

NKp46 appears to be well conserved among species and its expression has been identified in primates, mice, rats, cattle, sheep, and pigs (Biassoni et al., 1999; Connelley et al., 2011; De Maria et al., 2001; Jozaki et al., 2010; Sivori et al., 1997; Storset et al., 2003). Monoclonal antibodies (mAbs) have been developed to recognize NKp46 in most of these species, and thus far, its expression is limited to NK or NK-like cytotoxic lymphocyte populations.

Our group has recently described the identification of the equine ortholog of the *NKP46* gene (Noronha, 2012a). It is expressed by lymphocytes, and its sequence contains conserved domains required for protein function. The predicted equine protein shares 65% identity with the human and bovine proteins. This degree of similarity is apparently insufficient to permit cross-reactivity

with anti-human and -bovine NKp46 mAbs, as our attempts to label horse lymphocytes with several have shown a lack of recognition (data not shown). Therefore, using a system we recently employed to develop mAbs to equine CD16 (Noronha, 2012b), we generated a panel of novel mAbs that recognize equine NKp46.

Materials and Methods

Recombinant IL-4/NKP46 (rIL-4/NKP46)

Sequence IDs and PCR primers are listed in Table 6.1. The full-length coding sequence (CDS) of equine *NKP46* was previously cloned and sequenced (Noronha, 2012a). The extracellular domain (bases 62-668 of the CDS) was predicted by performing Clustal W alignments with validated *NKP46* sequences of other species, and was directionally cloned into a pcDNA3.1 vector (Invitrogen, Carlsbad, CA) downstream from the CDS of equine *IL-4* as previously described (Noronha, 2012b; Wagner, 2011). CHO K-1 cells were transfected with linearized IL-4/NKP46 plasmid using the Geneporter2 system (Genlantis, San Diego, CA). Stable transfectants were selectively cultured in G418 (Invitrogen), cloned by limiting dilution, and screened for IL-4 production by flow cytometry and ELISA as previously described (Wagner, 2011). *r*IL-4/NKP46 was purified from serum-free supernatant by fast protein liquid chromatography using an anti-IL-4 affinity column as previously described (Wagner, 2011). 1ug of the purified fusion protein was resolved by SDS-PAGE on a 10% non-reducing polyacrylamide gel to determine molecular weight.

Immunization and splenic fusion

Mice were maintained at the Baker Institute for Animal Health rodent facility at Cornell University. Animal care was performed in accordance with the guidelines set forth by the Institutional Animal Care and Use Committee (IACUC) of Cornell University. Immunization was performed as previously described (Wagner et al., 2003). Animal response was measured

Table 6.1 Sequences of genes and primers used

Gene	Accession #	Primers (5'-3')
NKP46	JN808451	*IL-4 fusion construct* F-GGCGGATCCCCAGAAGCGTACTCCCTCTAAAC R-GGCAAGCTTTCATGAATCAGGAGAAGAGATGTGC *GFP fusion construct* F-GGCGCTAGCATGCCTTCTATACTCACTGTCCTGCTC R-GGCGGTACCTCTTTGTCCAGGGATCTTTGTGTTCTGAAC *qPCR* F-CACCTGGAATGATGAACAAAG R-CCTGGGATGAACTGAGAGG
IL-4	GU139701	*GFP fusion construct* F-GGCGCTAGCATGGGTCTCACCTACCAACTGATTCCAG R-GGCGGTACCTCACACTTGGAGTATTTCTCTTTCATGATCGTCTTTAGC
CD16	JN795139	*GFP fusion construct* F-GGCGCTAGCATGTGGCAGATGCTATCACCAACGG R-GGCGGTACCTCAGAGCCCCGGCTCCATGTG

by monitoring serum titers to equine IL-4 using ELISA. Spleen cells were fused to SP2/0 myeloma cells as previously described (Appleton et al., 1989). Nascent hybridomas were plated into 96 well tissue culture plates and supernatants from all wells were screened for reactivity to *r*IL-4/NKP46 and *r*IL-4/IgG using ELISA, and for cell surface labeling of equine PBMC using flow cytometry. Antibodies that labeled PBMC and detected *r*IL-4/NKP46 but not *r*IL-4/IgG were selected for further study. All hybridoma cultures except mAb 8F9 were cloned by performing three rounds of limiting dilution, measuring sensitivity and specificity of secreted immunoglobulin by ELISA and flow cytometry as above after each round. Mouse immunoglobulin isotypes of secreted antibodies were determined by ELISA (Sigma, St. Louis, MO). Antibodies were purified by fast protein liquid chromatography using a protein G affinity column (GE Healthcare, Piscataway, NJ). Proteins were quantified using a Bradford assay (Bio-rad, Hercules, CA). Selected antibodies were biotinylated using Sulfo-NHS-Biotin (Thermo Fisher Scientific, Waltham, MA).

Antibody screening and ELISA

Cell culture supernatants were screened for mAbs to *r*IL-4/NKP46 by ELISA as described previously (Wagner, 2011) and against *r*IL-4/IgG1 (Wagner et al., 2005) and *r*IL-4/CD16 (Noronha, 2012b) to confirm their specificity to NKP46. For epitope ELISA, plates were coated with 1ug purified mAbs, washed, then followed with *r*IL-4/NKP46 fusion protein in transfected CHO cell supernatant. Following washing, 1ug of biotinylated antibodies was then added, followed by streptavidin-HRP. Reactions were developed and analyzed as previously described (Wagner et al., 2006). For 8F9, purified and biotinylated antibodies were not available; capture antibody was in the form of hybridoma supernatant.

GFP fusion protein expression and flow cytometric analysis

Full-length sequence (minus termination codon) for the equine *NKP46*, *CD16*, and *IL-4* genes were PCR-amplified and cloned into the pEGFPN1 vector as previously described (Noronha,

2012b). CHO-K1 cells were transfected with the vectors using the Geneporter2 system and assayed for protein expression 48 hours post- transfection. Successful expression of GFP was confirmed by fluorescence microscopy and indicated correct reading frame cloning of the fusion protein, as GFP sequence was downstream of the protein of interest. Cells were detached with trypsin and used either fresh or fixed with 2%PFA for 20 minutes. Cells were labeled and analyzed by FACS as previously described (Noronha, 2012b).

Lymphocyte isolation, flow cytometry, and immunohistochemistry

Heparinized blood samples were collected from horses maintained at the Equine Genetics Center, Baker Institute for Animal Health, Cornell University (animal details in Table 6.2). Animal care was performed in accordance with the guidelines set forth by the Cornell University IACUC. Lymphocytes were isolated by incubation with carbonyl-iron followed by density gradient centrifugation as previously described (de Mestre et al., 2010). PBMC were similarly isolated but without use of carbonyl-iron. Cells were assayed for viability using trypan blue exclusion and phase contrast microscopy. For flow cytometry experiments, one million fresh cells were labeled with mAb 4F2 or a monoclonal antibody recognizing anti-canine parvovirus (CPV) as an isotype control. Dead cells were excluded following staining for viability with propidium iodide. Five hundred thousand leukocytes were adhered to a glass slide with a Cytospin centrifuge, fixed in acetone, and labeled with mAbs as previously described (de Mestre et al., 2010).

Magnetic cell sorting and qPCR

CD3 cell sorting was performed using an AutoMACS cell sorter (Miltenyi Biotec, Auburn, CA) following incubation of 10^8 PBL with a mouse monoclonal antibody specific for equine CD3 (clone F6G, UC Davis, Davis, CA) and rat anti-mouse IgG1 MicroBeads (Miltenyi Biotec). CD3-depleted populations were a mean 8% CD3+ as verified by FACS. 4F2 sorting was similarly performed using $5x10^8$ PBL and mAb 4F2. Total RNA isolation and cDNA synthesis were performed as previously described (de Mestre et al., 2010). SYBR Green

Table 6.2 Animals used for lymphocyte isolation

Cornell #	DOB	Sex[1]	Breed[2]	MHC Haplotype[3]
3875	2006	F	TB x pony	A2/?
3908	2007	F	TB	A2/A2
3099	1992	F	TB	A2/A2
3521	2000	MC	TB	A2/A2
3709	2003	M	Pony	A6/?
2885	1988	F	TB	A3/A3
3157	1993	F	TB	A3/A3
4056	2009	MC	TB	A3/A3

[1]*F*, female; *M*, male; *MC*, male castrate. [2]*TB*, thoroughbred. [3]Equine Leukocyte Antigen haplotypes assigned by serologic and genomic testing.

(Applied Biosystems, Carlsbad, CA) real time PCR reactions for amplification of *NKP46* or the housekeeper gene equine ubiquitin-conjugating enzyme E2D 2 (UBE2D2), were performed using an ABI 7500 Fast sequence detector (Applied Biosystems) as previously described (de Mestre et al., 2010). Primers were designed with Primer3 software (MIT, Cambridge, MA) to cross intron/exon boundaries to prevent amplification of genomic DNA. A dissociation curve was performed after each experiment to confirm a single product was amplified. A standard curve was generated for all genes using known copy numbers of a plasmid that contained the DNA specific to the gene. Each sample was first normalized to 1.5×10^4 copies of UBE2D2. Data were analyzed using Graph Pad Prism Software. Data sets were checked for normality using the Kolmogorov-Smirnov test. Differences between groups were determined using a paired two-tailed Student's t tests. Values were considered significantly different at P values <0.05.

Results

Expression of rIL-4/NKp46 and selection of mAbs to equine NKp46

To generate monoclonal antibodies to equine NKp46, we employed a system that we recently used to generate mAbs to equine CD16 (Noronha, 2012b). This method utilizes a recombinant protein made by tagging equine IL-4 to a target antigen (Wagner, 2011). To create this fusion protein, the extracellular domain of NKp46 was predicted by comparing the CDS of *NKP46* to annotated sequences from other species and identifying homologous regions. The extracellular region was PCR-amplified from equine lymphocyte cDNA and inserted into a mammalian expression vector downstream of equine IL-4 (Figure 6.1A); CHO-K1 cells transfected with this construct secreted the soluble protein. Secreted protein was purified from the culture medium by fast protein liquid chromatography using an affinity column designed to capture equine IL-4 (Wagner, 2011). SDS-PAGE of the purified protein showed a molecular weight consistent with 39.9 kDa, the predicted molecular weight of *r*IL-4/NKp46 (Figure 6.1B). The three bands observed on SDS-PAGE likely represent differentially glycosylated species of the fusion protein, as it contains three predicted N-linked glycosylation sites with an N-X-S/T-X motif (Figure 6.1B,

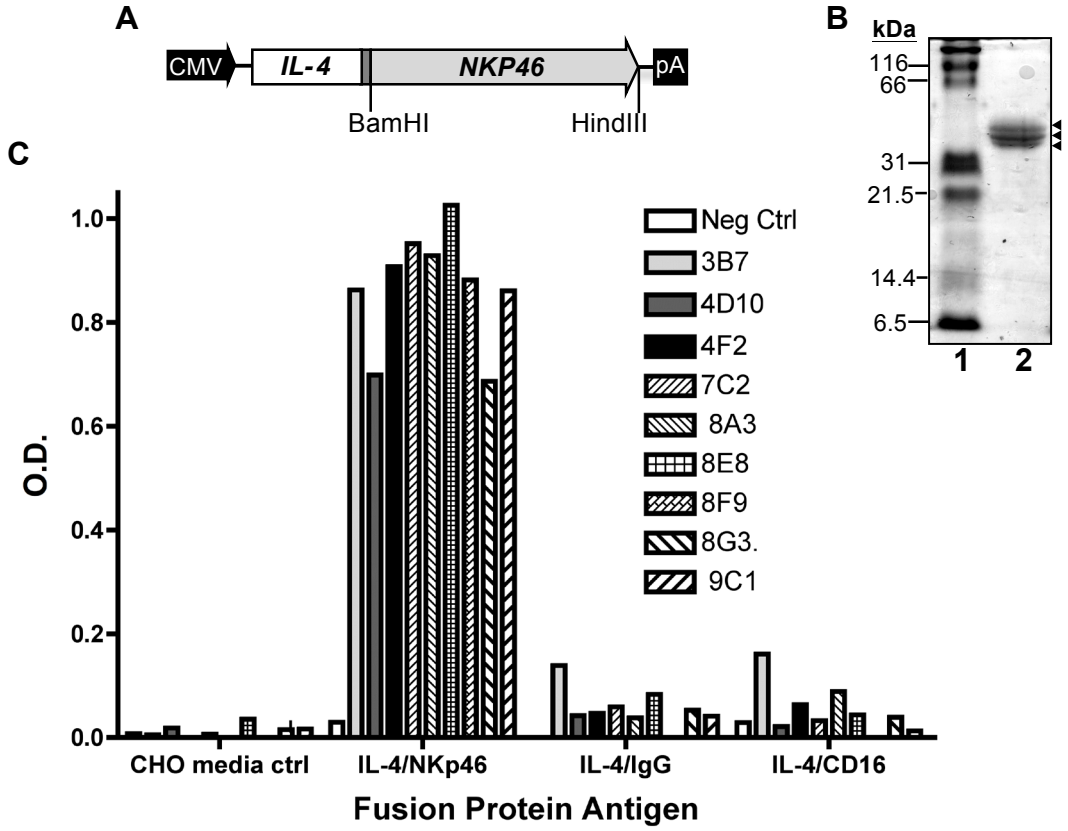


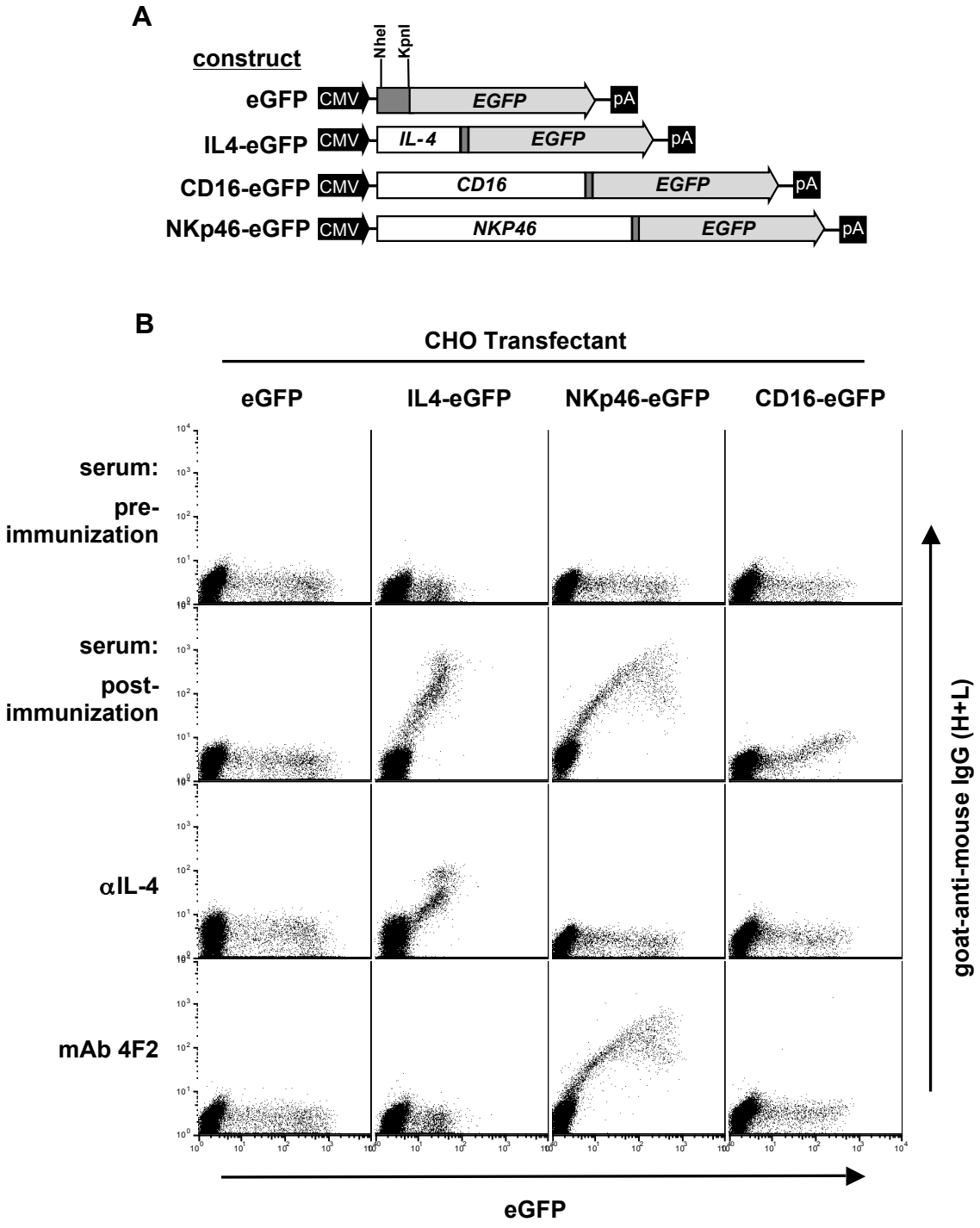
Figure 6.1 Construction and expression of *r*NKp46/IL-4, and utilization to identify anti-NKp46 hybridomas. (A) The predicted extracellular domain of equine *NKP46* was directionally inserted 3' to equine *IL-4* in a pcDNA3.1 expression vector (*CMV*, Human cytomegalovirus immediate-early promoter; *pA*, Bovine Growth Hormone polyadenylation signal). (B) Soluble *r*IL-4/NKP46 was expressed in CHO cells, affinity purified, and resolved by SDS-PAGE on a 10% gel under non-reducing conditions (lane 2). Arrowheads denote multiple glycosylation forms. (C) Culture media from hybridomas were tested by ELISA for the ability to recognize *r*IL-4/NKP46, rIL-4/IgG1, and rIL-4/CD16.

arrowheads). The purified protein was used in combination with an adjuvant to immunize Balb/c mice from which splenocytes were recovered and used to generate hybridomas. Media samples from hybridoma cell clones were screened for the ability to recognize *r*IL-4/NKp46 in ELISA (Figure 6.1C). Specificity of this recognition was determined by also testing the samples against *r*IL-4/IgG1 and *r*IL-4/CD16 fusion proteins (Figure 6.1C). CD16 was used as a specificity control because like NKp46, it has two extracellular C2-type Ig-like domains, and is therefore more structurally similar than the other NCR family members. Antibodies that recognized only the NKp46 fusion protein were considered to be specific for NKp46. Nine hybridoma clones produced mAbs that were selected for further characterization based upon fulfillment of these initial screening criteria.

MAbs recognize full-length equine NKp46

We next wanted to determine if the selected mAbs could recognize NKp46 in its native conformation of the surface of a cell. To test this, full-length CDS (minus the stop codon) of *NKP46* was inserted into the pEGFPN-1 expression vector upstream of the *EGFP* gene (Figure 6.2A). This fusion protein was designed to express NKp46 on the cell surface, with eGFP confined to the cytosolic compartment, thereby mimicking the conformation of NKp46 on NK cells. CHO-K1 cells were transfected with the expression vector, and successful expression was verified by fluorescence microscopy. eGFP fluorecence also verified in-frame cloning of *NKP46*. Transfected cells were then labeled with the nine mAbs and analyzed by FACS. In order to demonstrate specificity of recognition, additional cells were transfected with IL-4- and CD16-eGFP constructs. Labeling was performed with both fresh cells and fixed, permeablized cells, as permeabilization was required to detect intracellular IL-4-GFP. All nine mAbs demonstrated recognition of the NKp46-expressing cells, but not IL-4 (Figure 6.2B). Eight of the nine clones failed to label CD16-transfected cells. One clone, 3B7, showed faint recognition of CD16 in a pattern similar to what was seen with the polyclonal post-immune sera (Figure 6.2B).

Figure 6.2 Generation of full-length NKP46-, CD16-, and IL4-GFP expression constructs and flow cytometric analysis showing recognition by anti-equine-NKP46 mAbs. (A) Full length *NKP46*, *CD16,* and *IL-4* coding sequences (minus stop codons) were directionally inserted into the pEGFP-N1 mammalian expression vector 5' to *EGFP* (*CMV*, Human cytomegalovirus immediate-early promoter; *pA*, SV40 polyadenylation signal). (B) CHO cells transfected with fusion constructs were labeled with anti-equine-NKP46 mAbs and a fluorescently-labeled anti-mouse immunoglobulin secondary antibody (goat-anti-mouse-IgG (H+L)-647) and analyzed by flow cytometry. MAb 4F2 is shown as representative mAb. Anti-equine-IL-4 mAb (13G7) and sera taken from mouse spleen donor prior to immunization (*pre-immunization*) and at spleen collection (*post-immunization*) were used as controls.


A
construct
NheI
KpnI
eGFP
CMV
EGFP
pA
IL4-eGFP
IL-4
CD16-eGFP
CD16
NKp46-eGFP
NKP46
B
CHO Transfectant
eGFP
IL4-eGFP
NKp46-eGFP
CD16-eGFP
serum:
pre-
immunization
serum:
post-
immunization
αIL-4
mAb 4F2
goat-anti-mouse IgG (H+L)
eGFP

MAbs recognize NKp46 on equine lymphocytes

We next tested the ability of the antibody panel to recognize NKp46 on intact horse cells. The mAbs were first tested on isolated PBMC. Labeling was only observed in the lymphocyte population (Figure 6.3); therefore PBL were used for all subsequent analyses. All nine mAbs labeled lymphocytes with a similar pattern (Figure 6.4A). The percent of labeled PBL varied between 1.0 and 1.5 (median=1.25%) among the mAbs on cells from the same horse. PBL were then isolated from eight additional horses, and representative mAb 4F2 was used for labeling. The percent of labeled lymphocytes varied from 0.8%-2.1% (mean=1.3%) among animals (Figure 6.4B).

In order to determine if the cells labeled with mAb 4F2 expressed NKp46, we labeled PBL with mAb 4F2 and separated the cells into positive and negative fractions by magnetic cell sorting. The 4F2-enriched fraction increased from an initial 1.1% to 49.1% 4F2+ cells. cDNA was prepared from these cells as well as the 4F2-depleted (4F2-) fraction, and expression of *NKP46* transcripts was measured by real-time quantitative PCR. *NKP46* expression in the enriched population was 23-fold higher than in the depleted population.

We next wanted to determine if the 4F2+ population demonstrated the typical CD3- phenotype seen in NKp46+ cells of most species. To do this, we examined whether the depletion of CD3+ affected the number of 4F2+ cells. PBL from five horses were labeled with a CD3 mAb and CD3+ cells were depleted by magnetic cell sorting. Unsorted cells and CD3-depleted (CD3-) cells were labeled with mAb 4F2 and the paired samples were compared. The number of 4F2+ cells increased by 2.3-fold following CD3-depletion. The fold-change for the five individual animals ranged from 1.1-3.4.

In order to visualize the morphology of the cell population recognized by these mAbs, PBMC were adhered to slides and labeled with the mAbs by immunohistochemistry (Figure 6.4G). A small population of dimly stained lymphocytes was observed (arrow). The frequency of positive

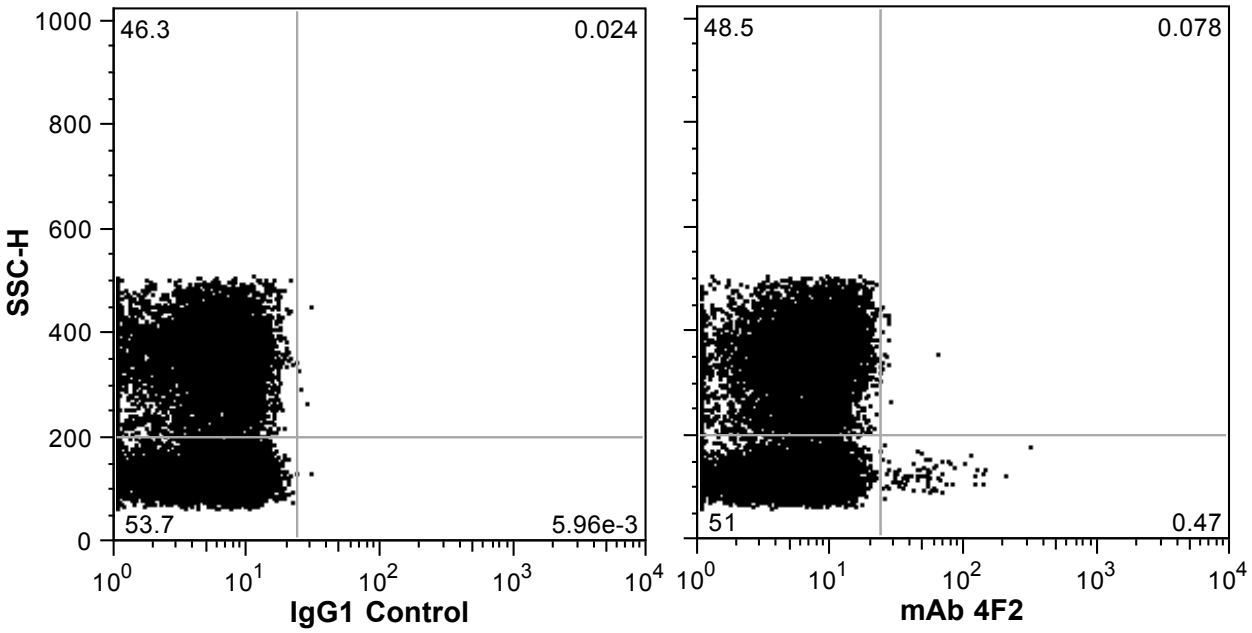
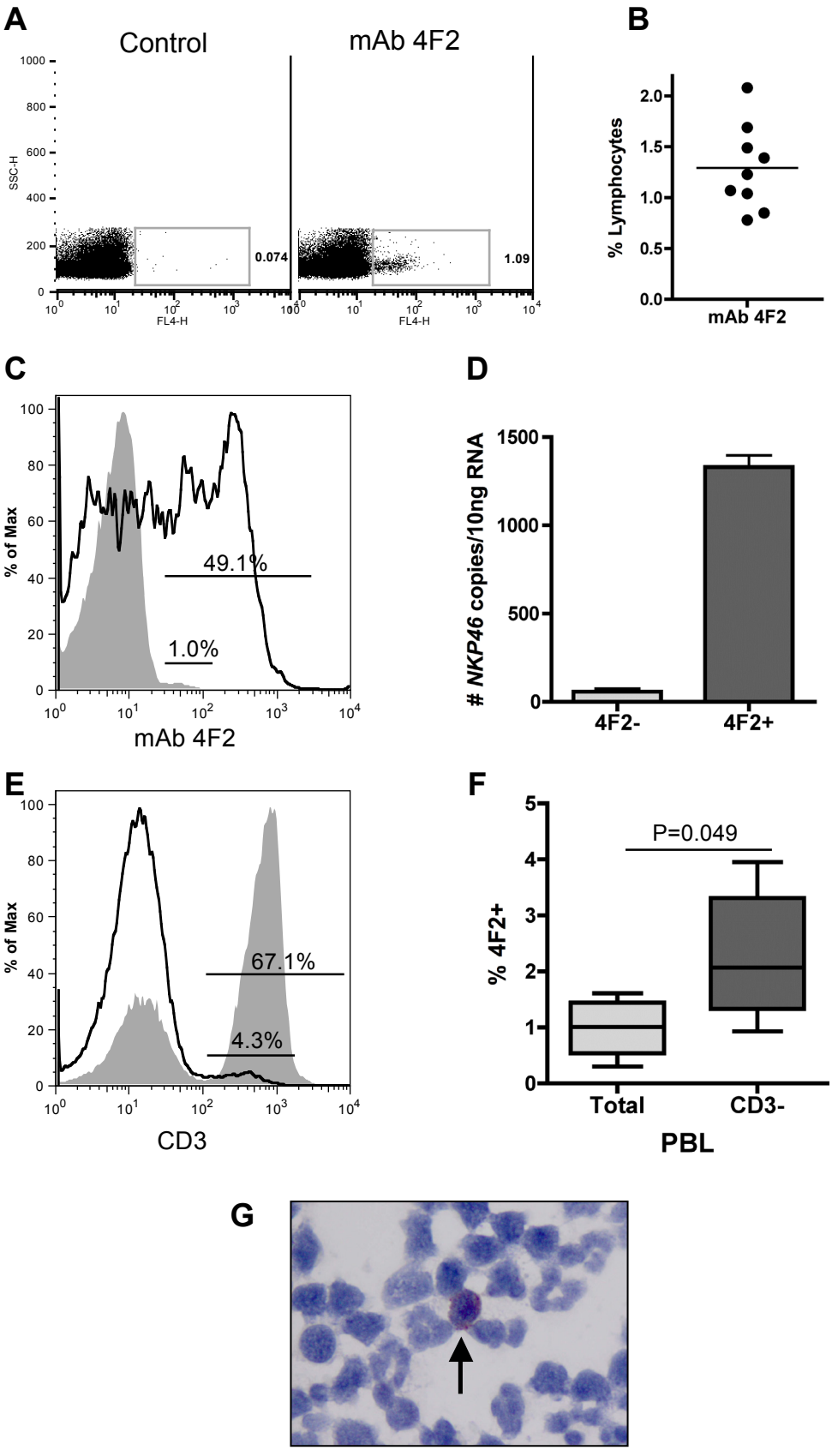


Figure 6.3 Recognition of horse peripheral leukocytes by anti-NKP46 mAb 4F2 is limited to lymphocytes. Isolated PBMC were labeled with IgG1 control mAb (anti-CPV, left) or mAb 4F2 (right) and analyzed by flow cytometry.

Figure 6.4 Recognition of horse peripheral blood lymphocytes by anti-NKP46 mAb 4F2. (A) Isolated lymphocytes were labeled with IgG1 control mAb (anti-CPV, left) or mAb 4F2 (right) and analyzed by flow cytometry. (B) Graph depicting percentages of 4F2-labeled lymphocytes for nine horses. (C) Histogram showing mean fluorescence intensity (MFI) of 4F2-labeled lymphocytes prior to (shaded curve) and following (open curve) enrichment of 4F2+ cells using magnetic cell sorting. Bars reflect the percentage of labeled lymphocytes (lower, pre-enrichment; upper, post-enrichment). (D) Real-time quantitative PCR analysis of *NKP46* transcripts in 4F2-depleted (*4F2*-) and enriched (*4F2*+) lymphocyte populations. (E) Histogram showing MFI of CD3-labeled lymphocytes prior to (shaded curve) and following (open curve) depletion of CD3+ cells using magnetic cell sorting. Bars reflect the percentage of labeled lymphocytes (upper, pre-depletion; lower, post-depletion). (F) Graph comparing percentages of 4F2-labeled lymphocytes of five horses prior to (*total*) and following (*CD3*-) CD3 depletion. (G) Immunohistochemical labeling of PBMC with mAb 4F2 (1000X) showing a representative dimly-stained lymphocyte (arrow).


A
Control
mAb 4F2
SSC-H
FL4-H
0.074
1.09
B
% Lymphocytes
mAb 4F2
C
% of Max
49.1%
1.0%
mAb 4F2
D
NKP46 copies/10ng RNA
4F2-
4F2+
E
% of Max
67.1%
4.3%
CD3
F
P=0.049
% 4F2+
Total
CD3-
PBL
G

cells was approximately 10-fold lower than that observed by flow cytometry.

MAbs recognize three different epitopes

Lastly, the epitope-recognition of the nine mAbs were analyzed by ELISA. Each of the mAbs was first used to capture *r*IL-4/NKp46. Remaining free sites on the recombinant protein were then labeled with a biotinylated version of each mAb in a checkerboard fashion (Table 6.3). Failure to bind by the second antibody indicated a blocked site, and therefore recognition of the same epitope as the "capture" mAb. Using this assay, we determined that the nine mAbs could

Table 6.3 ELISA and Epitope Recognition Group Assignments

		2. Biotinylated Antibody								
		3B7	4D10	4F2	7C2	8A3	8E8	8G3	9C1	Group Assignment
1. Capture Antibody	3B7	■	■	■	■		■	■	■	A
	4D10	■	■	■	■		■	■	■	A
	4F2	■	■	■	■		■	■	■	A
	7C2	■	■	■	■		■	■	■	A
	8A3					■				B
	8E8	■	■	■	■		■	■	■	A
	8F9*									C
	8G3	■	■	■	■		■	■	■	A
	9C1	■	■	■	■		■	■	■	A

Black: Reduced binding by biotinylated antibody; White: No reduction in binding; *8F9 was only used as a capture antibody

be divided into three groups of epitope recognition.

Discussion

Using a combination of ELISA, flow cytometry, and molecular methods, we have demonstrated that the mAbs described here demonstrate specific reactivity with equine NKp46. These mAbs show recognition of recombinant NKp46, as well as native protein on the surface of equine lymphocytes.

The size and granularity of equine leukocytes labeled by these mAbs, as determined by FACS forward and side scatter, was similar to what is seen with NKp46+ cells of humans and cattle (Almeida-Oliveira et al., 2011; Kulberg et al., 2004). However, the intensity of labeling, i.e. the mean fluorescence intensity (MFI) measured by FACS (Figure 6.4A), appears to be lower than in other species. It is unlikely that this relatively dim labeling is due to poor antibody affinity for NKp46 because all of the mAbs have a similar pattern, which cannot be overcome by increasing the antibody concentration. Also, CHO cells transfected with NKp46-eGFP demonstrated a high MFI following labeling. Therefore, it is more likely that the low signal is due to a low antigen density on the cell surface. This is consistent with quantitative RT-PCR studies we have previously reported showing that lymphocytes express a modest level of NKp46 mRNA (Noronha, 2012a). This low level of antigen expression may explain why the number of cells detected by immunohistochemistry (IHC) was less than what was seen with FACS, as colorimetric IHC is a less sensitive assay. While these mAbs appear to be capable of labeling cells in IHC assays, under the conditions here we are only able to visualize the cells expressing the most antigen. More sensitive immunofluorescence microscopy assays may be required to visualize the entire population.

The 1.3% of lymphocytes recognized here in adult horses is less than what is seen in adult humans (7%), but is similar to what is reported for adult cows (1.8-2.6%) (Almeida-Oliveira et al., 2011; Kulberg et al., 2004). It is also lower than what is observed in juvenile sheep (3-16%).

However, this may reflect an age-related effect as a progressive decline in the percentage of NKp46+ cells from birth to adulthood has been observed in some species (Almeida-Oliveira et al., 2011; Kulberg et al., 2004).

Our data clearly support that the mAbs described here recognize the equine NKp46 ortholog on horse cells. However, it is not yet clear if these cells are functional NK cells. As discussed above, NKp46 is generally considered the most reliable identifying marker of NK cells across species. Here, we see that the percentage of mAb 4F2+ PBL increases following depletion of CD3+ cells, which is consistent with the canonical CD3-NKp46+ phenotype used to describe NK cells in most species. However the 2.3-fold increase was not proportional to the 8.4-fold reduction in CD3+ cells, and one animal increased only marginally (1.1-fold). These data suggest that some NKp46+ cells could also be CD3+. Such cells may be NKp46+ NKT cells similar to those recently described in the human and mouse (Yu et al., 2011), or they may represent a species-related difference in the horse. CD3+ natural killer-like cells have previously been observed in lymphoid tissues recovered from severe combined immunodeficiency (SCID) foals that were devoid of conventional lymphocytes (Lunn et al., 1995).

Our group has previously identified equine lymphocytes with an NK cell phenotype using a cross-reactive monoclonal antibody to a catfish vimentin-like protein ("FAM," function-associated molecule) (Viveiros and Antczak, 1999). Those cells constitute a larger percentage of the lymphocyte population (4.5 to 23.3%) than what is seen here, and are all CD3-. Interestingly, in those studies, when CD3+ cells were depleted then labeled with anti-FAM mAb, the increase in presumptive NK cells was roughly 2.6 fold, very similar to the 2.3 fold increase seen here (Figure 6.4F). It is not yet clear what the relationship is between FAM+ cytotoxic cells and the NKp46+ cells we have identified. Multiple overlapping phenotypic subsets are seen among the NK cells of other species (Lanier, 2005), and it is reasonable to think that equine NK cells would be equally complex.

Table 6.4 Summary of aNKp46 mAbs

Epitope Group	mAb	Isotype	Application ELISA	FACS[1]	IHC[2]
A	3B7	IgG1	+[3]	+[3]	+/-
	4D10	IgG1	+	+	+/-
	4F2	IgG1	+	+	+/-
	7C2	IgG1	+	+	+/-
	8E8	IgG1	+	+	+/-
	8G3	IgG1	+	+	+/-
	9C1	IgG1	+	+	+/-
B	8A3	IgG1	+	+	+[4]
C	8F9	IgG1	+	+	+/-

[1]Labels both fresh and PFA-fixed cells; [2]Frozen acetone-fixed cells, "+/-": only brightest 10% of cells detected due to low antigen and limits of assay sensitivity; [3]Slight crossreactivity with CD16 observed in ELISA and FACS with transfected cells; [4]Strongly labels granulocytes in acetone-fixed sections.

Conclusions

The mAbs described here demonstrate the ability to recognize native and recombinant NKp46 in multiple assays (Table 6.4). These new reagents, in conjunction with our recently described antibodies recognizing equine CD16, will allow immunologists to better describe and understand the largely uncharacterized population of equine NK cells. They will also facilitate new study in areas with clinical relevance such as viral immune responses and tumor immunology.

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CHAPTER 7

EXPRESSION OF NK CELL MARKERS ON THE PERIPHERAL AND ENDOMETRIAL CUP LYMPHOCYTES OF THE HORSE

Summary

Natural killer (NK) cells are innate immune cells with constitutive cytotoxic activity. They are often defined by their cell surface marker profile, including the markers CD16 and NKp46. Here, using newly developed monoclonal antibodies specific for the equine homologs of these markers, we investigate their expression in peripheral and endometrial cup lymphocytes. Peripheral lymphocytes were observed to contain a CD16+NKp46+CD4-CD8+LFA-I+MHCII+ population of cells that were CD3+ to varying degrees, depending on the horse. Endometrial cup lymphocytes contained predominantly CD16+ cells, which overlapped with a previously observed CD3+ population. NKp46+ cells were also present among the ECL to a lesser extent. Together, these findings suggest that NK cells in the horse may have a CD3+ NK cell phenotype that differs from other species, or that the dominant cell type recognized by these markers is NKT cells. Furthermore, it appears that the CD3+ cells observed in the dramatic infiltration around equine endometrial cups are also CD16+.

Introduction

Natural Killer cells (NK cells) are vital components of the innate immune system. Among their multiple functions are the abilities to eliminate tumor cells and to confer host protection against pathogens during the gap between exposure and the maturation of acquired immune responses. Activating receptors are critical components in the effector functions of NK cells, so much so that NK cells are often defined by their receptor repertoire. This aim of this study is to utilize recently developed reagents to investigate the expression of two of these receptors, CD16 and NKp46, on equine lymphocytes in order to better understand the largely uncharacterized equine NK cell population.

CD16 (FCGRIII) is a low-affinity Fc receptor present on NK cells, monocytes, and in some species, granulocytes (Boysen et al., 2008; Dato et al., 1992; Elhmouzi-Younes et al., 2010; Fingerle et al., 1993; Rogers et al., 2006). It triggers NK cell mediated lysis primarily by binding cell

surface bound IgG and initiating antibody dependent cellular cytotoxicity (ADCC) (Trinchieri and Valiante, 1993). Our group has recently developed a panel of mouse monoclonal antibodies (mAbs) that specifically recognize equine CD16 (Noronha et al., 2012a). We have also developed a panel of mAbs to the equine homolog of the natural cytotoxicity receptor NKp46 (CD335, NCR1) (Noronha et al., 2012b). This molecule directly activates NK cell lysis through the binding of viral and tumor ligands, and is generally considered to be the most consistent and specific marker of NK cells across species (Walzer et al., 2007). It has been identified on functional NK cells of mice, rats, humans, non-human primates, sheep, and cattle (Biassoni et al., 1999; Connelley et al., 2011; De Maria et al., 2001; Sivori et al., 1997; Storset et al., 2003). In the horse, these two NK cell markers were each found to be expressed on a small population (1-2%) of peripheral equine lymphocytes (Noronha et al., 2012a, b). It is not yet known how these two markers relate to each other or to equine NK cells.

NK cells of the horse have been largely unstudied due to a lack of available reagents. Our group has previously described a population of peripheral lymphocytes that exhibit NK cell-like characteristics by using a cross-reactive monoclonal antibody to a catfish vimentin-like protein shown to identify human NK cells (Viveiros and Antczak, 1999). These cells were observed to be CD3-CD4-CD8-LFA-I+MHCI+. In that study, it was also shown that CD3-depleted peripheral lymphocytes have a higher innate cytotoxic capacity than total lymphocytes. Together these finding suggest that NK cells of the horse may reside in the CD3- lymphocyte population.

More recently, we have shown evidence for a regional accumulation of NK cells in the equine uterus during early pregnancy. Using quantitative RT-PCR, we detected increased gene expression of equine *NKP46*, *CD16*, *CD56*, and *CD94* in the endometrial cups— uterine structures formed in the superficial endometrium by invasive trophoblasts (Allen et al., 1973). Maternal mononuclear leukocytes, which our group has previously identified as being primarily CD3+, CD4+, and CD8+ (Grunig et al., 1995), infiltrate the stroma surrounding the cups and surround the trophoblast cells (reviewed in Noronha and Antczak, 2010). Here, we seek to determine if our newly developed mAbs will detect NK cell marker expression by these endometrial cup lympho-

cytes (ECL).

Materials and Methods

Animals

Horses used in this study were maintained at the Cornell Equine Genetics Center; all procedures were performed in accordance with guidelines established by the Institutional Animal Care and Use Committee of Cornell University. Adult horses of mixed genetic backgrounds, sexes, and ages were used (Table 7.1). Pregnancies were established as previously described (Adams and Antczak, 2001). Major Histocompatibility Complex haplotypes were assigned to horses using serological and genomic methods (Lazary et al., 1988; Tseng et al., 2010).

Cells and Tissues

Lymphocytes were isolated from heparinized blood samples by incubation with carbonyl-iron followed by density gradient centrifugation as previously described (de Mestre et al., 2010). Cells were assayed for viability using trypan blue exclusion and phase contrast microscopy. Sections of endometrial cups (day 46 of pregnancy) and lymph nodes (non-pregnant horse) were obtained at necropsy and transferred immediately to O.C.T. embedding compound (VWR Scientific Products, Willard, OH), snap frozen in an isopentane bath in liquid nitrogen, and then stored at -80^{o}C.

Flow Cytometry and Immunohistochemistry

Peripheral lymphocytes were labeled immediately after isolation or following 18 hours of stimulation with 25 ng/ml phorbol 12-myristate 13-acetate (PMA) and 1 μM ionomycin (IO) in the presence of 10 μg/ml brefeldin A as previously described (Wagner et al., 2008). Cells were labeled with monoclonal antibodies to equine cell surface markers CD3 (Blanchard-Channell et al., 1994), LFA-I CZ3.2, (Zhang et al., 1998), CD16 (Noronha et al., 2012a), NKp46 (Noronha et al., 2012b), CD4 (HB61A, VMRD, Pullman, WA), CD8 (CVS8, Lunn et al, 1998), MHCII

(CZ11, Lunn et al, 1998), CD56 (clone 123C3, Zymed labs), and anti-c-myc (Invitrogen). Cytokine staining was performed on stimulated cells using anti-bovine interferon gamma (IFNγ) (MorphoSys, AbD Serotec, Oxford, UK) and anti-equine interleukin 4 (IL4;Wagner et al., 2006) as previously described (Wagner et al., 2005, 2006). Immunofluorescence flow cytometry was performed using a BD FACSCalibur (BD, Franklin Lakes, NJ) and data analysis was performed using Flowjo software (Tree Star, Ashland, OR). Immunohistochemical labeling of frozen sections was performed as previously described (de Mestre et al., 2008).

Results

CD16 and NKp46 can be co-expressed on equine lymphocytes

Flow cytometric analysis was used to determine the relationship between CD16+ and NKp46+ lymphocyte populations. Lymphocytes were isolated from nine horses and labeled with mAbs recognizing equine NKp46 and CD16. Dot plots representing three of the nine horses are shown in Figure 7.1. In most cases, approximately half of the CD16+ cells were NKp46+, while most NKp46 cells were CD16+. A notable exception is that in the two youngest animals, the ratio of NKp46+ to CD16+ single positive cells was higher.

Horses can express CD16 and NKp46 on CD3+ lymphocytes

We next investigated the relationship between the NK cell markers CD16 and NKp46, and the conventional T cell marker CD3. NK cells in other species are typically described as being CD16+NKp46+CD3-. Lymphocytes were isolated from ten horses and labeled with mAbs recognizing equine CD3, NKp46 and CD16. Dot plots representing three of the nine horses are shown in Figure 7.2. In all but one horse (center), half or more of the CD16+ cells were also CD3+ (Figure 7.2A). The horses showed more variability in expression patterns regarding CD3 on NKp46+ cells (Figure 7.2B). Some showed the classic NKp46+ cell pattern with few CD3+ cells, others had CD3+ cells to varying degrees up to 92% NKp46+CD3+ (right). The pattern for each horse was found to be consistent when experiments were repeated on different days separat-

Table 7.1 Animals used for lymphocyte and tissue isolation

Cornell #	DOB	Sex[1]	Breed[2]	MHC Haplotype[3]
3875	2006	F	TB x pony	A2/?
3908	2007	F	TB	A2/A2
3099	1992	F	TB	A2/A2
3521	2000	MC	TB	A2/A2
3709	2003	M	Pony	A6/?
2885	1988	F	TB	A3/A3
3157	1993	F	TB	A3/A3
4056	2009	MC	TB	A3/A3
3903	2001	F	TB	A9/A9
4056	2009	MC	TB	A3/A3
3105	1992	MC	TB	A3/A3
3419	1998	F	SB x TB	A5/A2

[1]*F*, female; *M*, male; *MC*, male castrate. [2]*TB*, thoroughbred, *SB*, standardbred. [3]Equine Leukocyte Antigen haplotypes assigned by serologic and genomic testing.

ed by 4-6 weeks. This suggests that the phenotype is not the result of a temporary up-regulation of inducible receptors.

CD16+NKp46+ lymphocytes are CD8+

Lymphocytes from five horses were then labeled with CD16 and/or NKp46, and several markers for horse T cells (Figure 7.3, 7.4). Unfortunately, the mAb our group previously used to identify NK-like cells is now manufactured by a different vendor and causes a dose-dependent cell-death when used to label cells (data not shown). Few or no CD16+ and NKp46+ cells were CD4+. Most of the of the NKp46+ and CD16+ populations were each found to be CD8+. Interestingly, three-color analysis showed that these were almost exclusively the double positive NKp46+CD16+ population. The exception was a $CD16^{hi}$ population that was found to be CD8+, CD3-, and NKp46-. We next looked at MHCII, which is found on most horse lymphocytes and antigen presenting cells. All NKp46+ cells and most CD16+ cells were MHCII+ with a mean fluorescence intensity in the center of the positive population. The exception was the $CD16^{hi}$ group, which was also $MHCII^{hi}$. Finally, all cells highly expressed the pan-lymphocyte marker LFA-I, which is a requirement for cytolytic activity in NK cells and CTL of other species (Barber et al., 2004).

CD16+ and NKp46+ lymphocytes express minimal cytokines following pharmacological stimulation

In order to determine if CD16+ or NKp46+ cells produce basal levels of cytokines that might suggest that they have effector functions, we performed *ex vivo* pharmacological stimulation overnight in the presence of a secretion blocker, then labeled extracellular markers and intracellular cytokines and performed three-color flow cytometric analysis (Figure 7.5). We first looked at the canonical NK cell cytokine, IFNγ . There was modest production of IFNγ by CD16+ cells, but essentially none by NKp46+ cells. Because we had observed a CD3+ subset of NK cells that could potentially represent NKT cells, we next looked at IL-4 production, as some

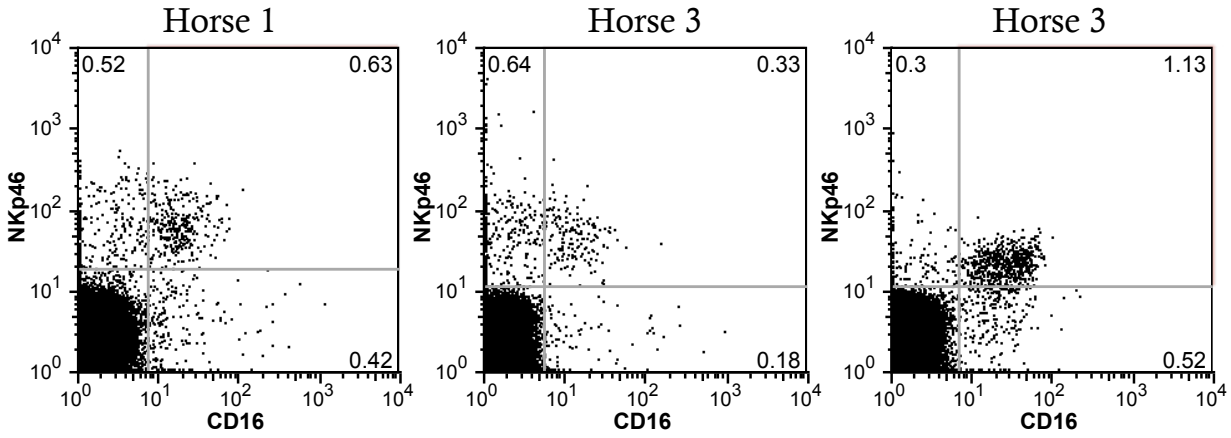


Figure 7.1 Dual labeling of CD16 and NKp46 on peripheral lymphocytes. Flow cytometric analysis of peripheral lymphocytes isolated from three representative normal horses. Cells were labeled with monoclonal antibodies to equine CD16 and NKp46.

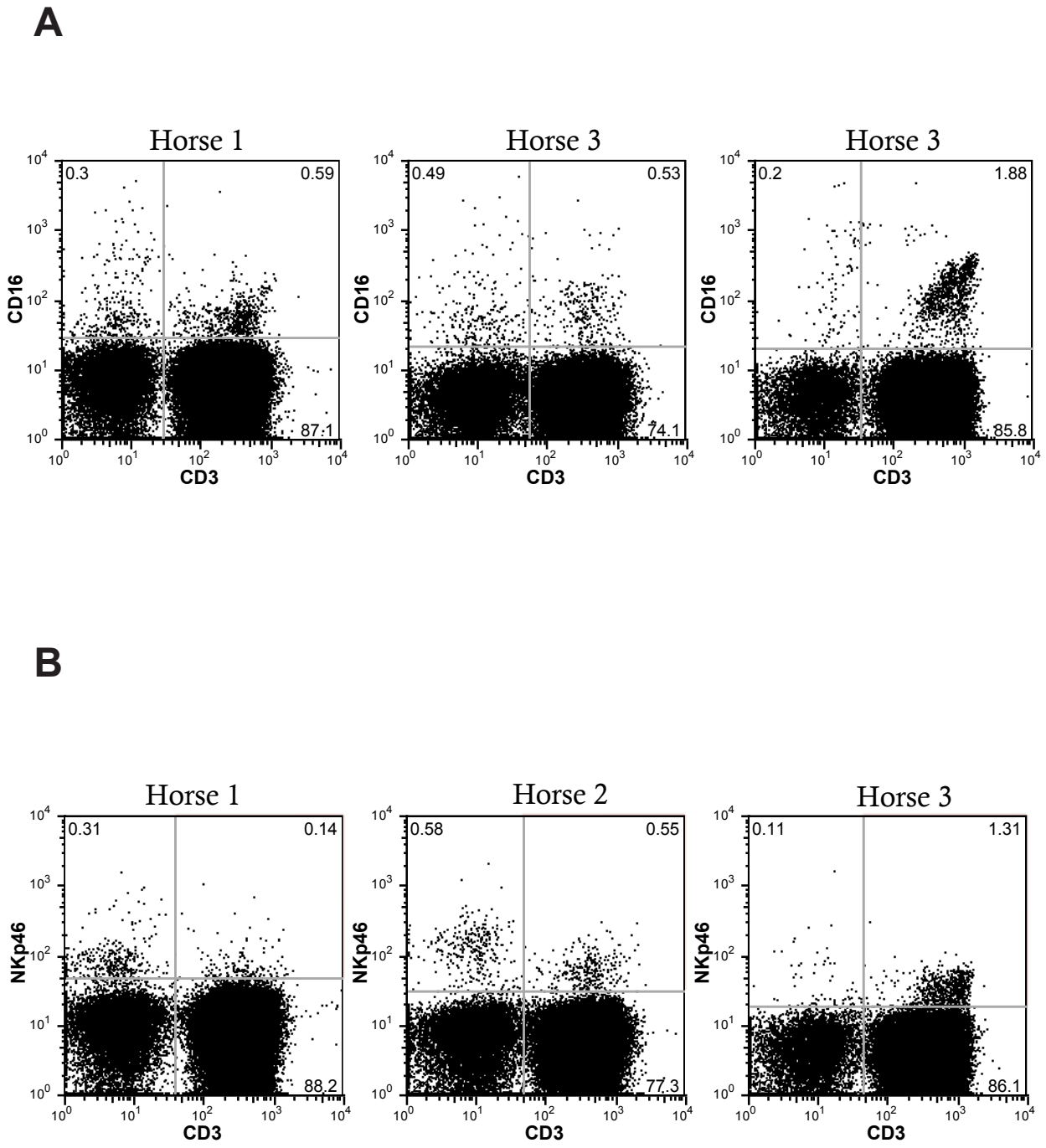
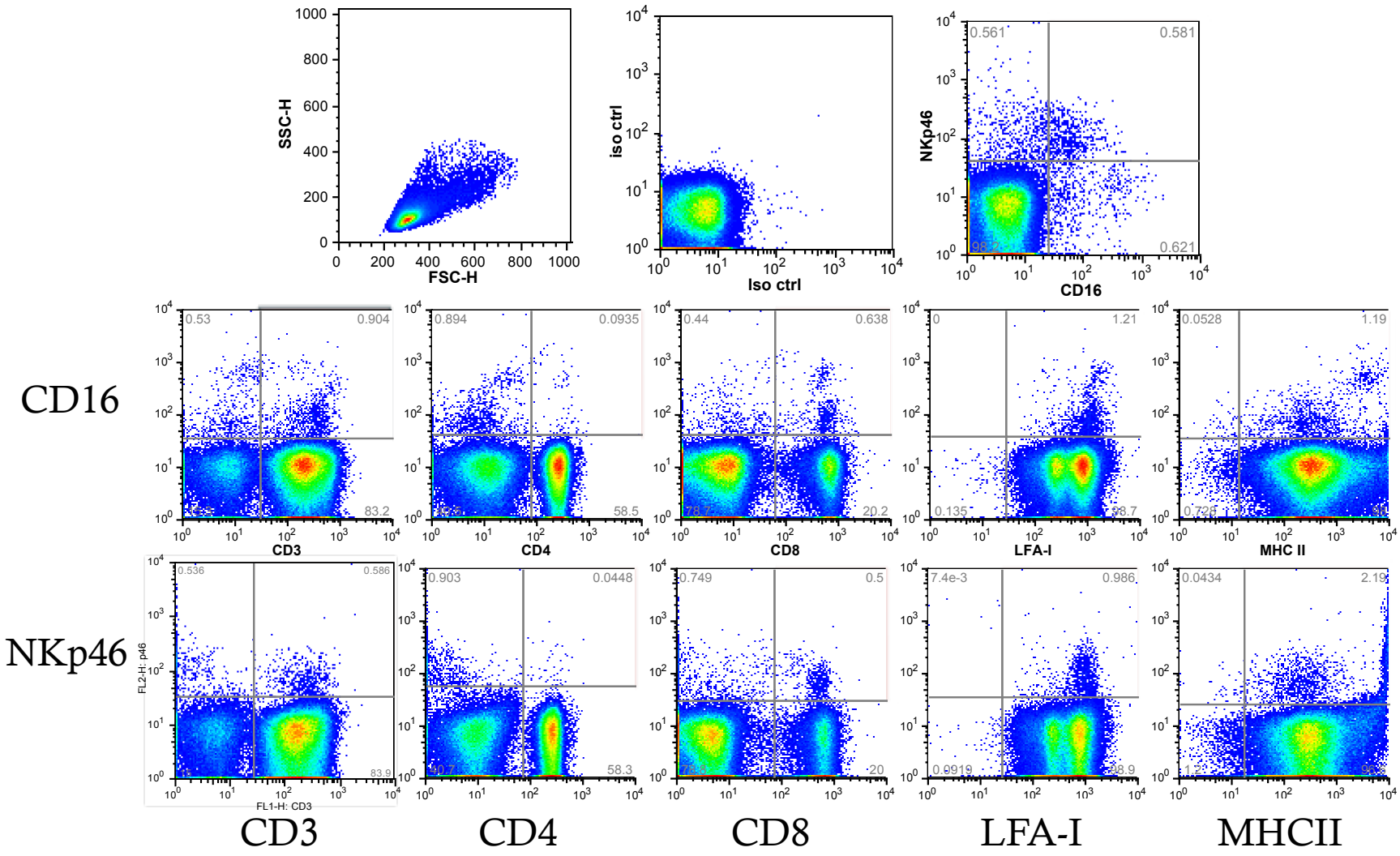


Figure 7.2 Cell surface expression of CD3 and NK cell markers on peripheral lymphocytes. Flow cytometric analysis of peripheral lymphocytes isolated from three representative normal horses. Cells were labeled with monoclonal antibodies to equine CD3 and CD16 (A) or NKp46 (B).

Figure 7.3 Immunophenotyping of peripheral CD16+ and NKp46+ lymphocytes. Flow cytometric analysis of peripheral lymphocytes, representative dot plots of cells isolated from one horse are shown. Cells were labeled with monoclonal antibodies to equine CD16 (middle row) or NKp46 (bottom row) and antibodies to other cell surface leukocyte markers, as indicated. Top row: forward-scattter and side scatter plot showing morphology of cells studied (left), cells stained with 2-color isotype controls (center), and CD16+NKp46+ co-localization for this horse.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CD16
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0.894
0.0935
58.5
0.44
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20.2
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1.21
0.135
58.7
0.0528
1.19
CD3
CD4
CD8
LFA-I
MHC II
0.536
0.586
83.9
0.903
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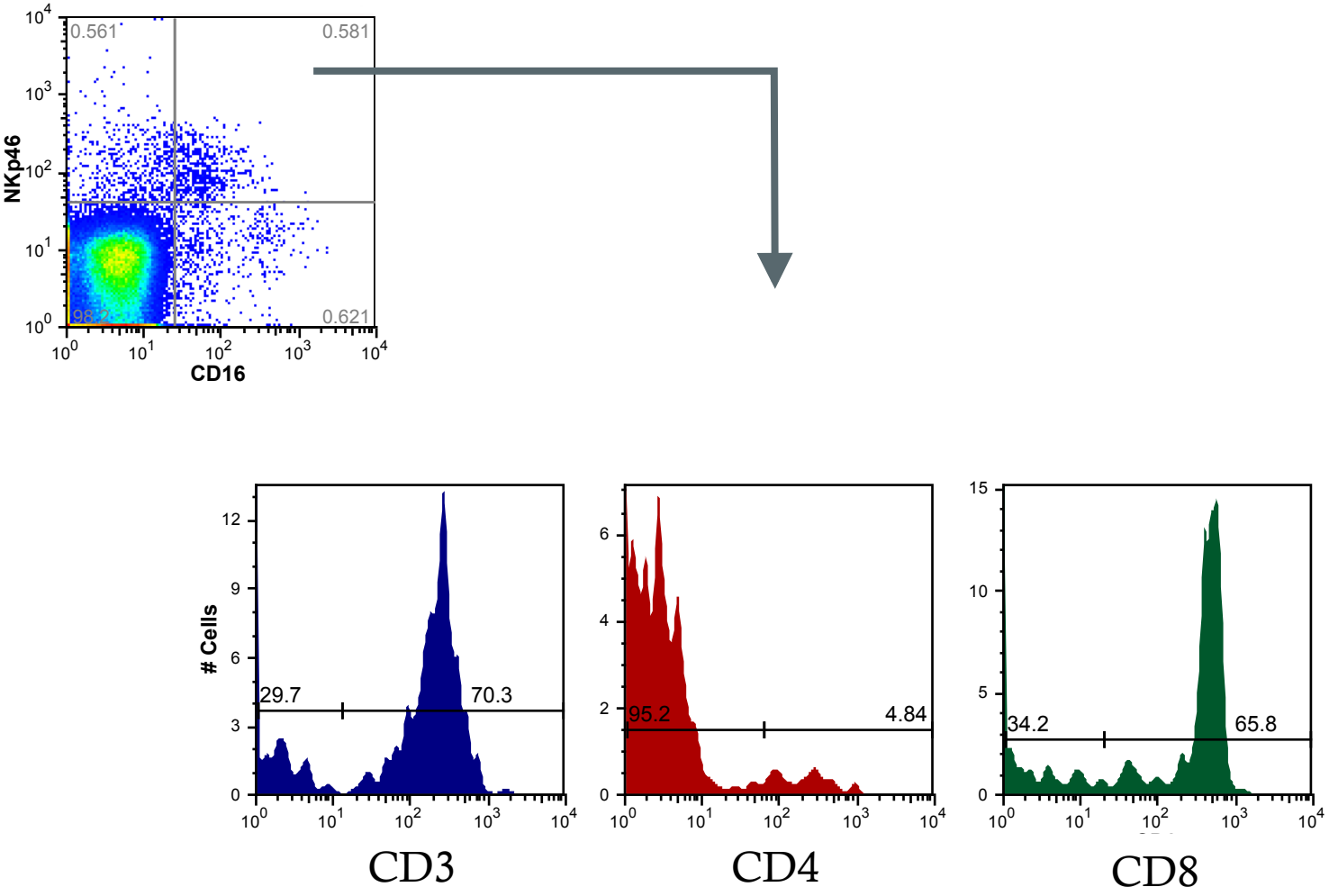
FL2-H: p46
FL1-H: CD3
CD3
CD4
CD8
LFA-I
MHCII

Figure 7.4 Three color labeling with NK cell and T cell markers. Flow cytometric analysis of peripheral lymphocytes, representative dot plots of cells isolated from one horse are shown. Cells were labeled with monoclonal antibodies to equine CD16, NKp46, and antibodies to other T cell markers, as indicated.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CD3
CD4
CD8

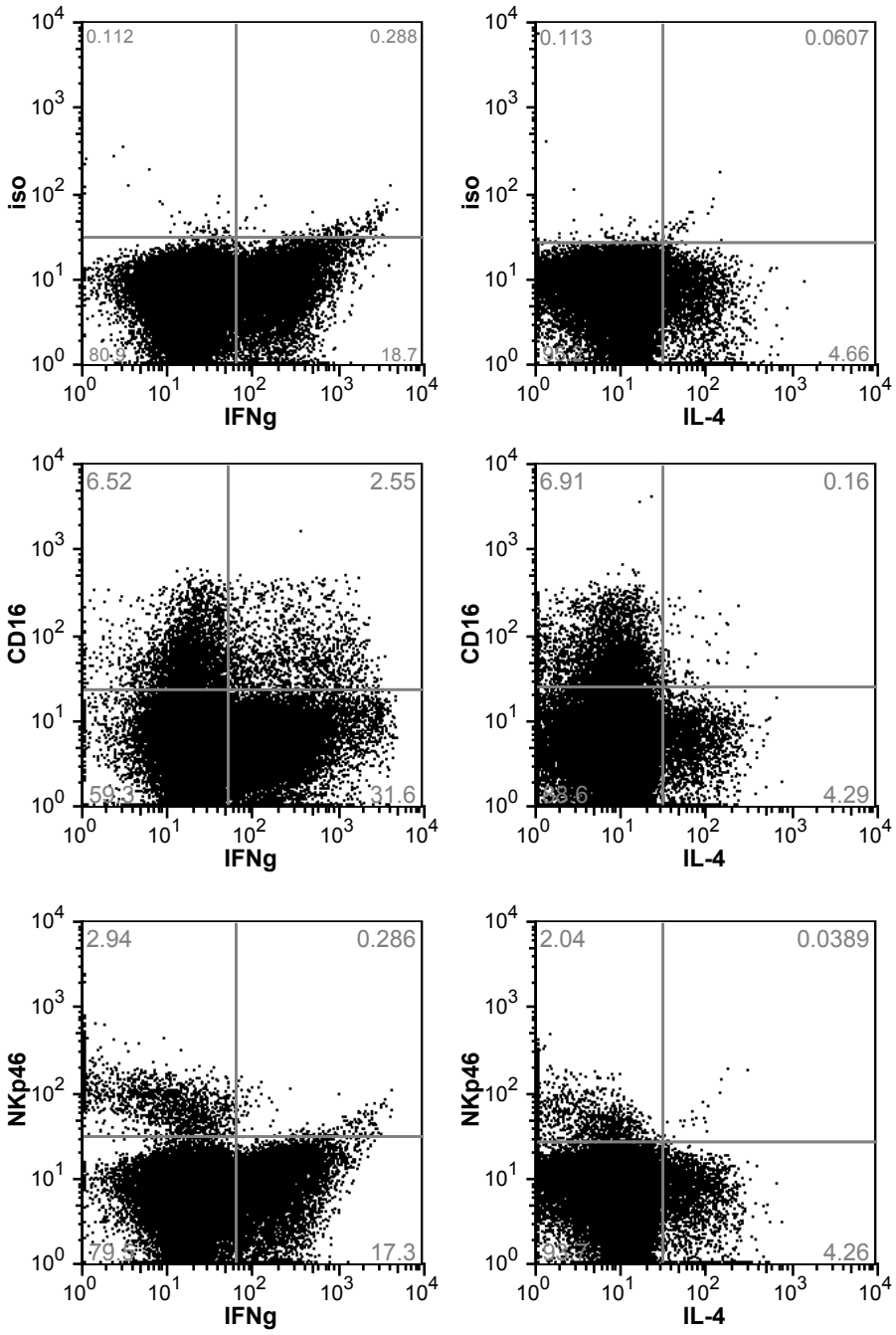


Figure 7.5 Cytokine production in PMA/IO stimulated peripheral CD16+ and NKp46+ lymphocytes. Flow cytometric analysis of IFNγ and IL-4 expression by lymphocytes stimulated *ex vivo* for 4 hours with PMA and 18h in the presence of Brefeldin A. Representative dot plots of cells isolated from one horse following intracellular labeling with IFNγ (left column) or IL-4 (right column).

populations of NKT cells produce IL-4 in other species (Denyer et al., 2006; Subleski and Ortaldo, 2009). Neither the CD16+ or NKp46+ populations produced significant amounts of IL-4.

Distribution of CD16+ and NKp46+ cells in the lymph node

In order to visualize the in situ distribution of cells expressing CD16 and NKp46, we performed immunohistochemical labeling on submandibular lymph nodes taken from a healthy horse (Figure 7.6). Submandibular lymph nodes drain the oral cavity and generally contain activated leukocytes of different types, including NK cells (Martin-Fontecha et al., 2004).

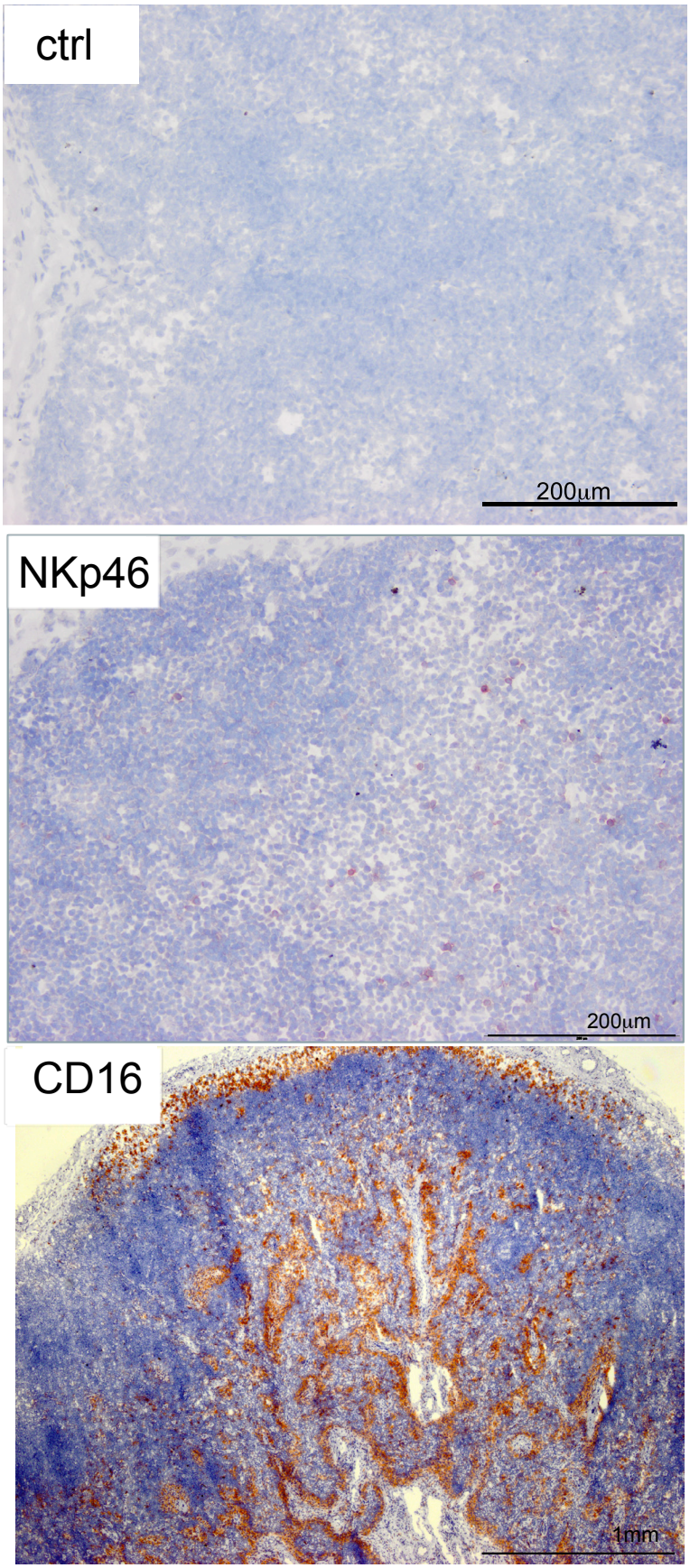
NKp46+ cells were observed to be in the cortical and paracortical regions in a pattern similar to the distribution of NKp46+ cells observed in bovine and ovine lymph nodes (Boysen et al., 2008; Connelley et al., 2011). CD16+ cells demonstrated a very distinctive pattern, lining the subcapsular and trabecular sinuses. We have previously shown that CD16 is expressed on equine monocytes (Noronha et al., 2012a), and this tissue expression pattern is consistent with expression on myeloid lineage APCs, such as macrophages and dendritic cells.

Distribution of NK marker molecules in endometrial cups

As we have recently shown molecular evidence for NK marker gene expression in lymphocytes surrounding the endometrial cups during early pregnancy (Noronha et al., 2012c), we next investigated protein expression of these markers on endometrial cups taken during this time frame. Serial sections of endometrial cups were labeled by IHC with antibodies recognizing NK cell markers and the primary markers of T-cells, B-cells, and monocytes.

The pattern of infiltrating LFA-I+, CD3+, CD4+, and CD8+ cells (Figure 7.7) is similar to what we have observed in past studies (Grunig et al., 1995). Here, we observe that CD16+ cells also exhibit a similar pattern. Among the lymphocyte accumulation directly adjacent to the endometrial cup trophoblasts, the number of CD16+ cells exceeds CD4+ and CD8+, and is comparable to CD3+ cells. Additionally, CD16+ cells continue distally into the endometrial stroma in fairly

Figure 7.6 Distribution of CD16+ and NKp46+ cells in lymph nodes. Frozen sections of sub-mandibular lymph nodes take from healthy adult horses were immunohistochemically labeled with monoclonal antibodies against equine CD16 and NKp46, as indicated. Negative control (top panel), is anti-c-myc.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CD16
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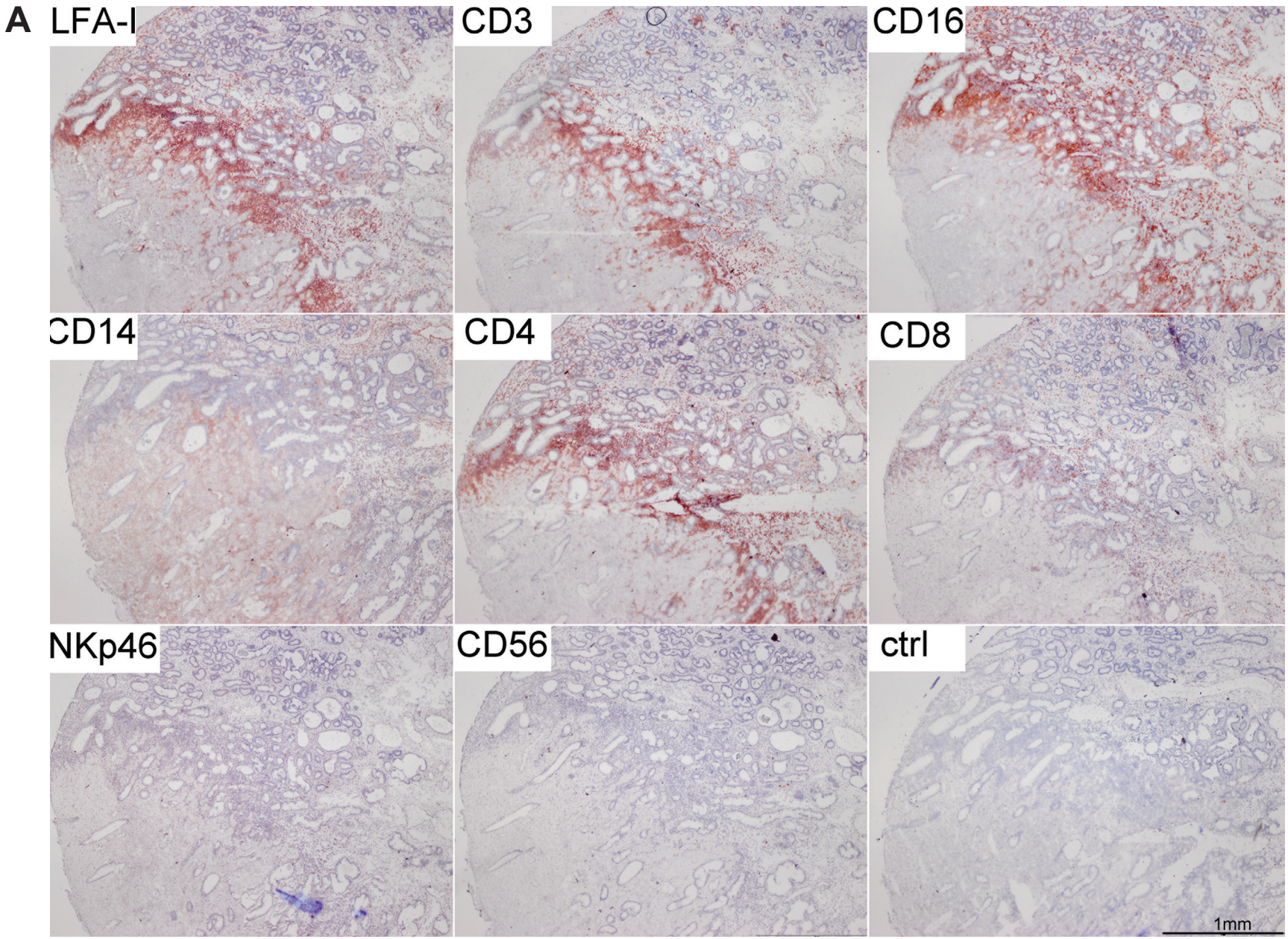
Figure 7.7 Distribution of cells expressing NK cell markers among the cellular response of early endometrial cups. Frozen serial sections of an endometrial cup taken from a horse on day 46 of pregnancy were immunohistochemically labeled with monoclonal antibodies against equine cell surface markers, as indicated. Negative control, anti-c-myc. Tissues are oriented with trophoblasts in lower left corner and endometrium to right. (A) low power (40X) view; (B) higher power (200X) view of central transition zone between endometrial cup and endometrium.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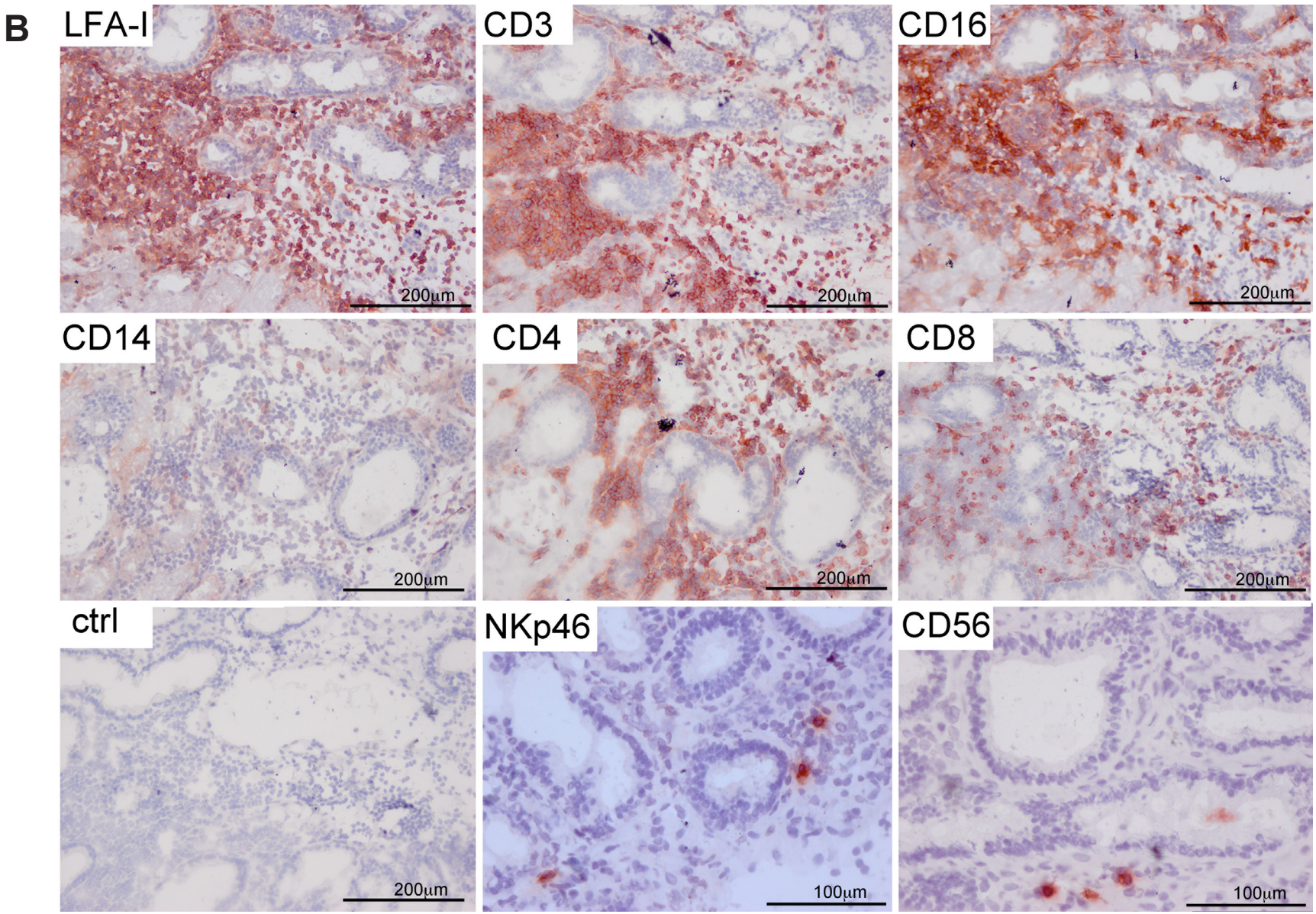
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large numbers, where the T-cell markers do not.

Because many of the markers used here (LFA-I, CD4+, CD8+) can also be expressed on equine cells of the monocyte lineage, we compared the CD16 pattern to that seen with a marker for this population, CD14. Unlike CD16, CD14 was expressed in only a small number of cells among the lymphocytes surrounding the endometrial cup trophoblasts.

Our previous molecular data demonstrated that CD56 and NKp46, which were both expressed in low levels in peripheral lymphocytes, demonstrated a dramatic upregulation of expression in endometrial cup lymphocytes. We next investigated the expression of these molecules on endometrial cup tissue sections.

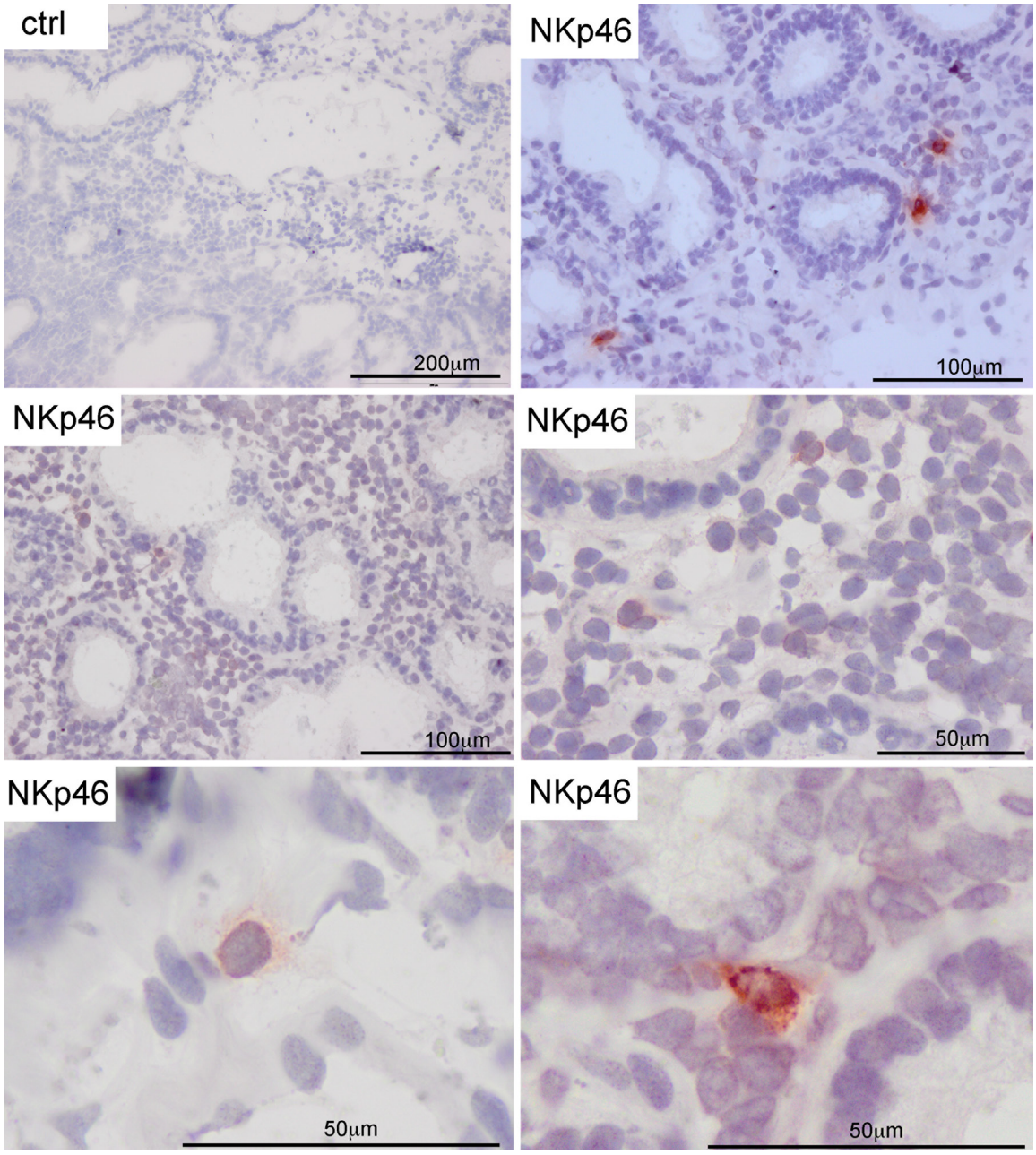
CD56 transcripts are nearly undetectable in peripheral equine lymphocytes, but are expressed at levels 40-fold higher in the ECL. The equine CD56 protein shares 98% amino acid identity with the human protein, so we used a commonly used anti-human CD56 antibody to attempt to detect the equine protein (Figure 7.7). A small number of bright cells were detected among the ECL and none were found in the adjacent endometrium. At first glance, NKp46 appeared to demonstrate the same pattern of a few isolated bright cells. However, many dim cells were also present (Figure 7.8). This staining intensity is consistent with what is seen in peripheral cells, i.e. low antigen density, suggesting that NKp46 may be at the limits of detection of this colorimetric assay. Alternatively, this mAb may not work effectively in immunohistochemical assays.

Discussion

These data describe the expression profile of the common NK cell markers CD16 and NKp46 in equine lymphocytes. We have investigated their expression on peripheral lymphocyte populations, and regional populations in situ. Together these data suggest a unique profile for equine NK cells.

The two NK cell markers studied here, CD16 and NKp46, have some shared expression patterns. They co-localize on the majority of lymphocytes on which they are expressed, they are

Figure 7.8 Close-up view of distribution of NKp46+ cells in early endometrial cups. Frozen serial sections of an endometrial cup taken from a horse on day 46 of pregnancy were immunohistochemically labeled with a monoclonal antibodiy against NKp46. Negative control, anti-c-myc. Center row, closer views of dimly labeled cells. Bottom row, high power views of dimly stained (left) and brightly stained (right) NKp46+ cells in the lymphocyte-dense region of the endometrial cup.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all MHCII+LFA-I+CD4-, and most of the CD16+NKp46+ double positive lymphocytes are also CD8+, a phenotype consistent with the NK cells of species including humans, macaques, rats, pigs, and sheep (Carter et al., 1999; Elhmouzi-Younes et al., 2010; Gerner et al., 2009; Perussia et al., 1983; Torres-Nagel et al., 1992). However, they differ in respect to CD3 expression. The CD16+ cells of virtually all horses tested here were predominantly CD3+. The CD3 pattern in Nkp46+ cells varies significantly between individual animals. While CD16 can be expressed on multiple cell types, NKp46 has been considered to be the most consistent NK cell marker across species. It is typically found on CD3- cells in primates, mice, cows, and rats (Biassoni et al., 2002; Connelley et al., 2011; Sivori et al., 1997; Storset et al., 2004; Westgaard et al., 2004). Here, we see it to a variable extent, sometimes exclusively, on CD3+ cells. One explanation is that these could be NKT cells, which have both NK cell and T cell markers. CD3+NKp46+ NKT cells have been described in humans and mice (Yu et al., 2011) and CD3+CD16+ NKT cells have been found in pigs (Denyer et al., 2006). Another explanation is that this phenotype could be an equine-specific difference. T-cells and NK cells are derived from a common lymphoid progenitor that expresses cytoplasmic CD3ε (the same subunit that the equine mAb recognizes), but go on to lose this marker during maturation (T- cells later regain it) (Plum et al., 1999; Sanchez et al., 1994). Consequently, CD3+ NK cells are the predominant type found in the fetal liver (Phillips et al., 1992). However, cytoplasmic CD3ε has also been observed in adult NK cell clones (Lanier et al., 1992). So perhaps that while the amino acid sequences of the major NK phenotypic molecules have remained conserved between species (Noronha et al., 2012c), the cell ontogeny has not, and the horse NK cell is more primitive in terms of lineage maturation. Other groups have seen evidence of possible equine CD3+ NK cells; CD3+ cells with NK-like activity have previously been observed in foals with severe combined immunodeficiency, a condition that renders them devoid of conventional lymphocytes (Lunn et al., 1995). There, like here, the CD3 profile varied among individuals. Those putative NK cells were also CD4-CD8+LFA-I+MHCII+.

The small CD3+CD16+ phenotype seen in PBL appears to be the rpredominant type among the endometrial cup lymphocytes. CD16+ cells are numerous among the ECL and are clearly

overlapping with the CD3+ population, presenting a cellular profile different than what we had thought. It is somewhat more difficult to make conclusions about the degree to which the smaller CD4+ and CD8+ populations overlap with CD16+. The CD14+ does not appear to exhibit a similar pattern, suggesting that CD16 expression is not due to myeloid cells. CD56 appears to be present in very small numbers; but it is not yet clear if this is an actual representation of and inability to react with sufficient affinity to horse proteins. Likewise, it is difficult to make conclusions about NKp46 expression with the current assay due to its low antigen density and the limitations of colorimetric IHC. NKp46 labels peripheral cells with very low mean fluorescence intensity as measured by FACS. This reflects a low antigen density which is likely the reason that cells appear so dim in IHC assays. The next step in investigating these unanswered questions, and determining co-localization of markers on the ECL is to use a more sensitive assay where more than one marker can be visualized, i.e. fluorescence microscopy.

Regardless of the cell surface phenotype of these cells, the primary question that remains is whether they are NK cells. Ultimately that is not determined by their marker profile, but by their capacity for innate effector function. IFNγ production is considered a good surrogate for cytolytic capacity. The chemical stimulation conditions used here has been shown to work with human peripheral NK cells (Shi et al., 2007), but did not yield significant cytokine production here. Perhaps a more NK cell specific stimulation protocol, such as IL-12 driven stimulation, would be more informative. The gold standard ultimately would be to directly measure cytolytic capacity of these cells, as this function is what defines them. Our group has previously identified cells with innate cytolytic capacity as being CD3-. This does not necessarily conflict with our results here. As we have determined CD3 expression on NKp46+ cells if highly variable among individuals, it may be that the horses in that study had CD3- NK cells. Or it may be that, like in other species, the horse has multiple, sometimes overlapping, NK and NK-like populations.

Conclusions

Here we have made advances toward better understanding the NK and/or NK-like cell population in the horse. A population of NKp46+CD16+CD8+ CD4-LFA-I+MHCII+CD3+/- cells has consistently been identified in the peripheral lymphocytes of multiple horses. These cells share a phenotype with the cells that accumulate in the maternal endometrium during early pregnancy. Their role there is not yet known, but their sheer number makes them an intriguing target for further studies.

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CHAPTER 8

DISCUSSION AND CONCLUSIONS

The aim of this dissertation was to better understand maternal-fetal tolerance during equine pregnancy, with the larger goal of making observations that could help elucidate the phenomenon across species. The ability of a pregnant mother to accept the presence of a genetically foreign fetus is quite remarkable. In parallel situations outside the context of pregnancy, such as organ transplantation, individuals must take immunosuppressive drugs in order to prevent rejection of histocompatible tissues, and even then success is mixed. Indeed, the concept of the "fetal allograft" as a model for understanding pregnancy immunology was developed nearly 60 years ago, and is still relevant today (Medawar, 1953).

The work described here studied changes in the immune system of the mare that occur at both the systemic and regional (uterine) levels. In particular, it focused on the cytotoxic lymphocyte population, as they are the effector cells primarily responsible for tissue rejection. This first part investigated lymphocytes of the acquired immune system such as CD8+ cells and the CD4+ cells that affect their behavior. The second part concentrated on innate cytotoxic cells, the natural killer (NK) cells.

Chapter 1 reviewed the pertinent aspects of equine pregnancy immunology that provided the impetus for the studies in the following chapters. Of particular importance were the following observations previously made by previous members of our group: 1) the invasive chorionic girdle (CG) trophoblast cells of the horse express high levels of allogeneic paternal class I MHC antigens, similar to what is found in immune cells (Bacon et al., 2002; Donaldson et al., 1994); 2) these CG trophoblasts invade the maternal endometrium and recruit large numbers of maternal leukocytes which maintain direct contact with them for several weeks without causing their destruction (Allen, 1975); 3) nearly 100% of pregnant mares develop high titers of cytotoxic antibodies directed against the class I MHC antigens of the mating stallion, thereby demonstrating that trophoblast alloantigens are recognized by the maternal immune system (Antczak, 1984; Antczak et al., 1982); and 4) pregnant mares exhibit a reduced ability to mount an effective systemic cell-mediated immune response against paternal alloantigen, as measured

by peripheral CTL activity against target cells from the mating stallion (Baker et al., 1999). These observations raise many unanswered questions. If a mare clearly recognizes paternal class I MHC, then why does she not mount a deleterious immune response? Why are the endometrial cup trophoblasts not destroyed by the wall of lymphocytes that surround them? If a mother's immune system tolerates paternal antigens, does it tolerate exogenous antigens also? The work in this dissertation was an attempt to address some of these pressing questions.

In Chapter 2, the aims were to determine if the peripheral lymphocytes of the mare are phenotypically different during pregnancy, and whether immune reactivity may differ between the peripheral and regional compartments. We compared peripheral blood lymphocytes (PBL) of pregnant and non-pregnant mares, as well as tissue lymphocytes from the endometrium and endometrial cups (ECL) with PBL obtained on the same day of gestation from pregnant mares.

We did not detect dramatic differences in peripheral lymphocytes during pregnancy. The ratio of CD4:CD8 was reduced in pregnancy compared to the non-pregnant state resulting from a trend towards decreasing CD4+ lymphocytes and increasing CD8+ lymphocytes in the mares during pregnancy. There was a modest increase in the percentage of IL-4+ cells and a decrease in the IFNγ:IL-4 ratio in the overall lymphocyte population in the mares during pregnancy. This finding is consistent with the high levels of paternal alloantibodies observed during pregnancy, as the presence of IL-4 favors a humoral immune response. There was also trend towards an increase in FOXP3 gene expression in the pregnant group, but the difference was not statistically significant.

At the regional level of the endometrial cups, we measured increased numbers of FOXP3+ CD4+ T cells around the endometrial cups compared to peripheral blood. In the lymphocytes recovered from the endometrial cups, there was also a marked increase in IFNγ + cells in the total and CD8+ populations, and a decrease in lymphocytes expressing IL-4. This observation appears to be in conflict with the traditional dogma of a TH2 bias during successful pregnancy (Wegmann et al., 1993). However, the cytokine profiles of lymphocyte subsets has been determined to

be more complex than previously thought (Mosmann et al., 2009). And these results were similar to results from a study of human decidual CD8+ lymphocytes that reported high levels of expression of IFNγ and undetectable levels of IL-4 (Scaife et al., 2006). Furthermore, IFNγ has been implicated at the maternal-fetal interface as having a critical role in human, murine, and porcine pregnancy (Murphy et al., 2009). The results of this chapter also highlighted that significant differences may exist between local and peripheral immune cells during pregnancy.

The aim of the studies in Chapter 3 was to further explore the previously observed deficits in peripheral CTL activity of the pregnant mare, and investigate the role paternal class I MHC antigens. Although pregnant mares demonstrate clear immunological recognition of an allogeneic fetus, they demonstrate a reduced ability to generate effective CTL responses against stallion MHC antigens (Baker et al., 1999). In order to determine the role of paternal alloantigens in this effect, we generated MHC compatible and incompatible horse pregnancies and tested paired PBMC samples isolated from mares while pregnant and non-pregnant for cytolytic capacity. CTL from mares carrying incompatible pregnancies demonstrated a significantly diminished capacity to lyse cells from the breeding stallion as well as cells from an unrelated third party individual. By determining the existence of a reduced ability of pregnant mares to lyse cells from a third party animal, we were then able to test this effect in MHC matched pregnancies. CTL from mares pregnant with MHC compatible pregnancies also demonstrated a reduced capacity to lyse target cells from third party animals. These data demonstrate that the observed reduction of CTL activity occurs independently of paternal MHC antigens on two levels. First, the mare's inability to generate effective CTL is not limited to responses directed against the MHC of the breeding stallion. Second, an MHC incompatibility between mare and stallion is not necessary to induce this effect.

It is not yet clear what mechanism is causing the antigen-independent reduction in T cell responses, but results from Chapter 2 lends some insights. There, no difference was detected in the number of circulating CD8+ lymphocytes between non-pregnant mares and mares in

early pregnancy. Other groups studying late equine pregnancy have gotten similar results (Agricola et al., 2008). This suggests that the mechanism might not be a pregnancy-associated reduction in CD8+ CTL precursors as has been observed in transgenic mouse studies (Tafuri et al., 1995). CTL could also be modulated by the suppressive activity Tregs, which expand in the periphery in other species during allogeneic and syngeneic mouse pregnancy in an antigen-independent manner (Aluvihare et al., 2004). In Chapter 2, we observed only a statistical trend towards an increase in the gene expression of FOXP3 between PBMC from mares carrying early pregnancies and non-pregnant mares in the luteal phase of estrous. However, These samples were taken in early pregnancy, before estrogen rises significantly. As Treg expansion in pregnancy may be estrogen-dependent (Polanczyk et al., 2004; Prieto and Rosenstein, 2006), the difference may have been sufficient to be suppressive, but not enough to be measurable at this stage of equine pregnancy.

Chapter 4 begins the second set of studies— the investigations of equine NK cells. In this chapter, we performed molecular studies looking for evidence of expression of common NK cell phenotypic markers in PBL. We then used this new information to determine whether a previously undetected population of NK cells might exist in the lymphocytes surrounding the endometrial cups. We determined the sequences for the equine orthologs of the NK cell markers *NKP46*, *CD16*, *CD56*, and *CD94*, and that they were expressed in PBL. In the four animals measured, *NKP46* gene expression in peripheral CD3- lymphocytes was higher than in CD3+ lymphocytes. *CD16* levels were higher in the CD3+ population, an unexpected observation that was later corroborated at the protein level by flow cytometry experiments in Chapter 7. Expression of all four NK cell markers was significantly higher in lymphocytes isolated from the endometrial cups of pregnant mares compared to PBMC isolated from the same animal on the same day, suggesting that NK cells may be present in the equine endometrium during pregnancy.

Uterine and decidual NK cell populations are generally characterized as having poor cytolytic capacity, but have the ability to secrete cytokines, often large amounts of IFNγ (Platt and Hunt,

1998; Saito et al., 1993). This is consistent with the finding in Chapter 2 that lymphocytes around the endometrial cups produce elevated amounts of IFNγ . The stage of pregnancy studied here, days 43-46, is roughly one week following trophoblast invasion; therefore it is possible that IFNγ may have a role in tissue remodeling as has been described in other species (Tayade et al., 2007; Zhang et al., 2011). NK cells could also mediate this remodeling via interaction with polymorphic paternal class I MHC as seen in human and mice (Hiby et al., 2010; Madeja et al., 2011). This could help explain the poorly understood phenomenon of paternal class I MHC expression by the chorionic girdle trophoblast at the time of invasion.

In order to progress in our studies of NK cells at the maternal-fetal interface, it was necessary obtain monoclonal antibodies that recognized equine NK cell markers. We obtained several antibodies that recognize these proteins in other species, but none demonstrated suitable cross-reactivity with horse cells. This is not surprising considering that, as we determined in Chapter 4, the homology across species of the NK markers was typically in the 60% range. The exception here was CD56, which was 98% identical to the human protein. The anti-catfish-FAM (vimentin-like cytotoxic cell marker) mAb previously used to identify NK-like cells by our group (Viveiros and Antczak, 1999), was now manufactured by a new company and determined to be unsuitable for experiments. Therefore, we set out to develop novel monoclonal antibodies that recognized equine NK cell markers. The generation and characterization of these antibodies are described in Chapters 5 and 6.

Chapter 5 describes the creation of a panel of mAbs that recognize equine homolog of the low-affinity Fc receptor CD16. These mAbs were created using a recombinant CD16 (rCD16) protein as an immunogen. Using techniques such as ELISA and flow cytometry on rCD16-transfected cells, the mAbs were determined to be specific for CD16. FACS analysis of equine peripheral leukocytes showed a recognition of cells in the lymphocyte (2.1%), large lymphocyte/monocyte (8.4%), and granulocyte (3.1%) populations. The overall number of cells that were labeled was modest (3%), but the pattern of labeling was similar to that seen in sheep and cattle

(Elhmouzi-Younes et al., 2010). A portion of cells in the monocyte gate were also CD14+, a recognized sub-population of CD16+ cells, and looked remarkably similar to the human pattern for these cells (Fingerle et al., 1993).

Chapter 6 describes the generation of a panel of mAbs that recognize the equine homolog of the natural cytotoxicity receptor NKp46. Similar methods were used to characterize the specificity of these mAbs as in Chapter 5. These mAbs labeled 1.3% of lymphocytes and no monocytes or granulocytes. This is roughly 1/10th the population identified using the anti-FAM mAb. Although the percent of positively-labeled lymphocytes was similar to that seen in cows (1-8-2.6; Kulberg et al., 2004), a species where this marker has been well characterized, the labeling appeared to be dim and continuous, as opposed to positive/negative as in other species. Therefore, we performed additional characterization assays on these antibodies. One mAb (4F2) was used as a label for magnetic cell sorting. The enriched and depleted populations were then analyzed for gene expression of *NKP46*. The enriched population had a 23-fold higher *NKP46* expression, indicating that the mAb was in fact recognizing cells that express NKp46 proteins. We then depleted CD3+ cells and found an increase in NKp46+ cells in the CD3- fraction. Interestingly, the depleted fraction was 2.3-fold higher than the total cell population, nearly identical to the 2.6-fold increase seen when the anti-FAM antibody was used (Viveiros and Antczak, 1999). Although these data suggest that on the whole, NKp46+ cells are associated with CD3- cells, it was observed that in one animal the number of NKp46+ cells did not change after CD3 depletion, suggesting that some of the CD3+ cells must be NKp46+. The multicolor FACS experiments in Chapter 7 go on to demonstrate this observation in more detail. Finally, it was observed that when we performed immunohistochemical labeling on cytospins of peripheral blood, the number of cells that were detected were lower than that seen by FACS. Rare bright cells could be observed, but the number of positive cells did not amount to the 1% seen by FACS. Based upon the low mean fluorescence intensity (MFI) seen in FACS analyses, it appears that the antigen density of NKp46 is very low on the surface of lymphocytes. This is consistent with the quantitiative PCR experiments of Chapter 4, which showed peripheral

lymphocytes as having an estimated 1-10 copies per cell. This raises the question of whether the increase in NKP46 transcripts seen among the endometrial cup lymphocytes in Chapter 4 is due to an increase of a few bright cells with a lot of expression, or many dim cells with dim expression.

We attempted to answer this question in Chapter 7 where we used the newly generated NKp46 and CD16 mAbs to investigate peripheral and endometrial cup lymphocyte expression of the NK cell markers (summarized in Figure 8.1 and Table 8.1). We observed that most horses have a distinct population of double positive CD16+NKp46+ cells. CD16+ cells were typically at least half, sometimes all, CD3+. These results were consistent with the unexpected qPCR data in Chapter 4 showing that CD3+ cells had higher levels of CD16 expression. This is a profile not typically seen on NK cells. CD16+CD3+ cells have been observed as a T-cell subset in humans (Lanier et al., 1985), and an NKT population in pigs (Denyer et al., 2006). CD3 was also associated with a variable number of NKp46+ cells, depending on the animal. Some animals had primarily CD3- cells, others had mostly CD3+. The CD3+NKp46+ cells appear to also be CD16+. Again, this is not the typical phenotype of NK cells described in any species. Until recently, there were few reports of NKp46+ NKT cells (Yu et al., 2011) it was considered to be highly specific for NK cells. However, it has long been observed that CD3ε, a marker usually present on only fetal NK cells, may be found in the cytoplasm of adult NK cells, (Lanier et al., 1992). So one possible explanation is that horse cells could be retaining fetal cell surface CD3, or exporting adult cytoplasmic CD3 to the cell surface. Alternatively, this could be a species-related phenotypic variation, similar to the MHCII expression seen on equine T-lymphocytes. Another explanation is that some animals have NKT cells that co-express these markers, while others do not. However, NKT cells are not simply NK cells with CD3, they have a distinct, thymic-derived cell type with a unique type of class I MHC that recognizes CD1d-bound glycolipid molecules. It does not seem like this population should vary from all to none between horses. The CD3+NKp46+CD16+ cells seen here could be tested for gene or protein expression of TCR molecules in order to further investigate this possibility.

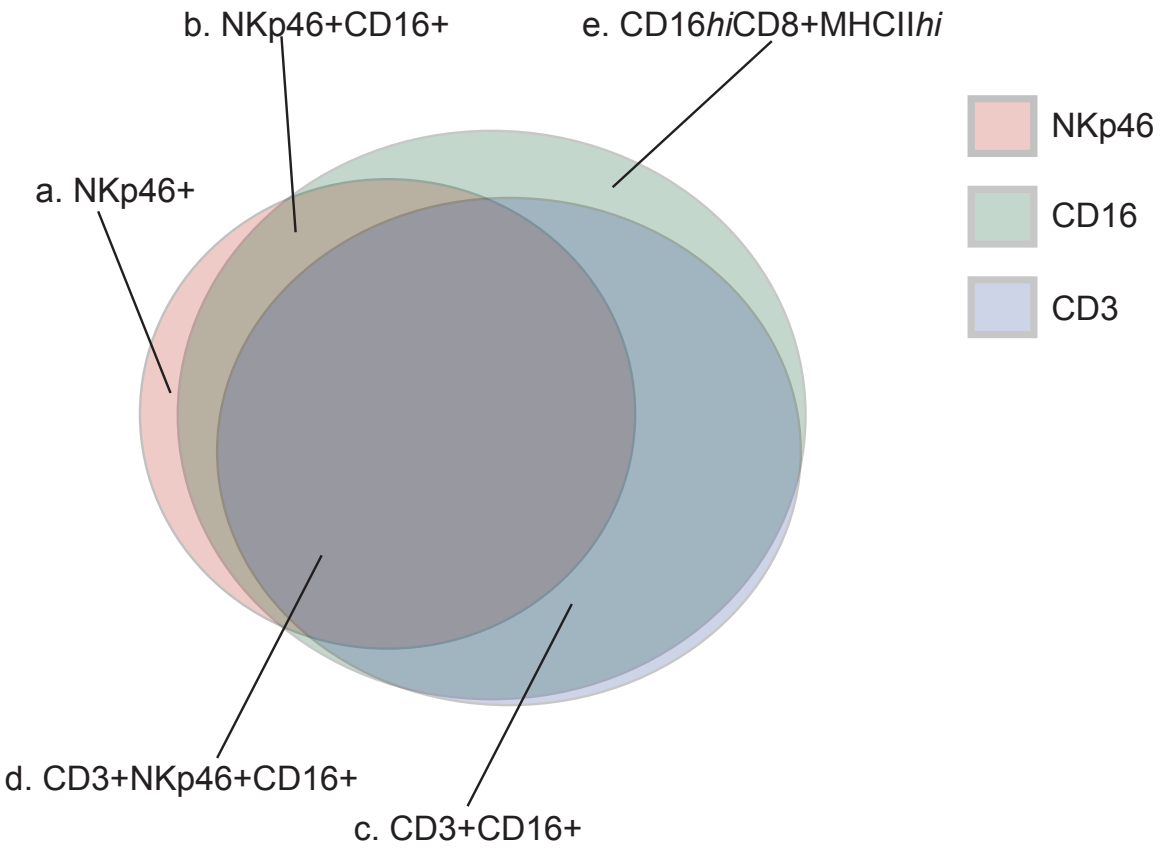

Figure 8.1. Diagram Summarizing current knowledge of CD16+ and NKp46+ lymphocyte phenotypes. Overlap between lymphocyte populations is represented by circle overlays. This is a general representation; the number of CD3+ cells with NK markers varies considerably between horses.

Table 8.1 Summary of current knowledge of CD16+ and NKp46+ lymphocyte phenotypes

a	NKp46+ (single positive)	Uncommon-only seen in youngest animals, seem to have higher MFI
b	NKp46+CD16+	Common
c	CD3+CD16+	Varies between animals, consistent day-to-day
d	CD3+NKp46+CD16+	Varies as product of *c*
e	(CD3-) $CD16^{hi}$CD8+$MHCII^{hi}$	Varies between days?

The lymph node distribution of these markers was consistent with what would be predicted. NKp46 exhibited a pattern seen in cows and sheep (Boysen et al., 2008; Connelley et al., 2011), and CD16 was distributed to areas of the lymph node where CD16+ antigen presenting cells typically reside. In the endometrial cups, there was a large population of CD16+ cells that seemed to overlap with the CD3+ population. This is not surprising based on what was observed in the periphery. It would be interesting to use paired peripheral and endometrial cup samples from the same horse, to see if this CD3+CD16+ population changes between compartments. Also, this would allow us to investigate the CD8+ and CD4+ status of the CD16+ ECL. It is possible that the IFNγ-producing CD8+ cells seen in Chapter 2 could be these cells. NKp46 labeling again yielded many dim cells, as was seen with peripheral blood slides. It is difficult to determine the true number of these cells, but it appears to be higher than the ~1% in PBL. In order to answer these questions about the number of NKp46 cells and their phenotype(s), one could use fluorescence microscopy of tissues sections, or cell isolation as in Chapter 2.

Our new mAbs have been very useful in generating new information about horse lymphocytes, but they have opened up more questions than they have answered. In order to determine what the NKp46+CD16+ cells are, the next step should be to determine if they exhibit behaviors consistent with NK cells. This should include three types of experiments: 1) measure the ability to spontaneously lyse cells that lack class I MHC; 2) measure IFNγ production in response to IL-12 stimulation; and 3) determine if they proliferate in response to IL-15. The other set of experiments that should be performed are the aforementioned assays for TCR expression. This should differentiate them from NKT cells. Information obtained from this group of experiments should allow us to determine if these are in fact, NK cells.

In closing, the experiments here succeeded in advancing our prior knowledge, such as determining the lack of antigen specificity in maternal CTL modulation, and determining that many cells at the maternal-fetal interface are CD16+. They have also opened up many new avenues of study such as characterizing what appears to be a unique NK cell phenotype. It will

be exciting to see where all of these areas lead and ultimately intersect.

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