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Split immunological tolerance to trophoblast

AMANDA DE MESTRE[1], LEELA NORONHA[2], BETTINA WAGNER[3] and DOUGLAS F. ANTCZAK*[,2]

[1]Department of Veterinary Basic Sciences, The Royal Veterinary College, Royal College Street, London, UK, [2]Baker Institute for Animal Health, College of Veterinary Medicine, Cornell University, Ithaca, New York, USA and [3]Department of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine, Cornell University, Ithaca, New York, USA

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.

KEY WORDS: *trophoblast, pregnancy, tolerance, cytokine, lymphocyte*

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 56 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, 2006; 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

Abbreviations used in this paper: CTL, cytotoxic T cell; eCG, equine chorionic gonadotropin; FoxP3, forkhead box P3; IFNG, interferon gamma; IL, interleukin; MHC, major histocompatibility complex; Tregs, regulatory T cells; UBE2D2, ubiquitin conjugating enzyme E2D 2.

***Address correspondence to:** Douglas F. Antczak. Baker Institute for Animal Health, College of Veterinary Medicine, Cornell University, Ithaca, New York 14853 USA. Fax: +1-607-256-5608. e-mail: dfa1@cornell.edu

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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).

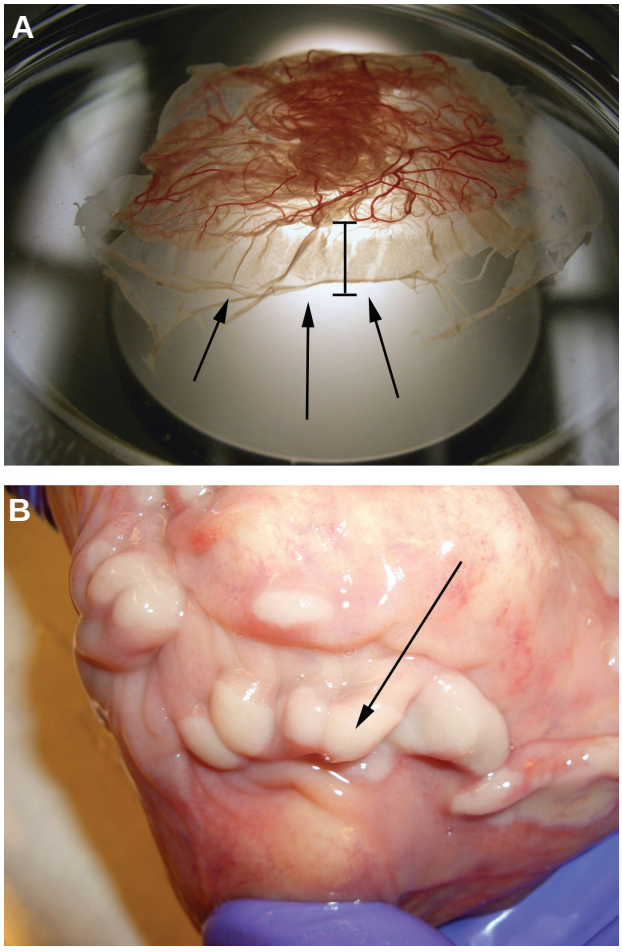


Fig. 1. Gross specimens of day 34 equine conceptus and endometrial cups from day 45 of gestation. (A) *Day 34 conceptus showing the horizontal band of invasive trophoblast of the chorionic girdle, demarcated by the vertical bracket and arrows. At day 36-38 of gestation the chorionic girdle cells migrate 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.*

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, 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; Mathes *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; Hunziker *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,

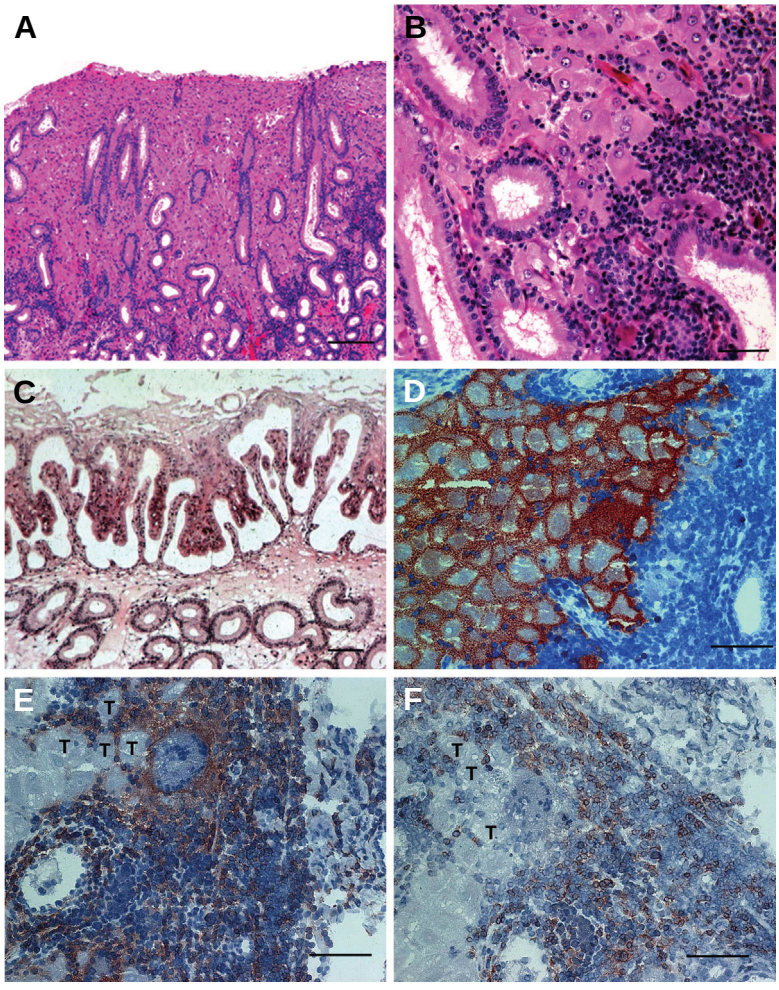


Fig. 2. Histological images of endometrial cups and endometrium from early equine pregnancy. (A-C) *H&E stained fixed sections.* **(A)** *Low power image of endometrial cup showing accumulations of maternal lymphocytes concentrated along the periphery of the cup.* **(B)** *High power image from (A) showing 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 100 μm in all panels except A (400 μm). All specimens are from day 43-46 of gestation.*

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) (Grünig *et al.*, 1995). Once the endometrial cups are fully formed, the binucleate, equine chorionic gonadotrophin (eCG) 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.

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 were 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

TABLE 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	A8 /?	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
3492	A2 /?	A3 / A3	31
2998	? /?	A3 / A3	30
3821	A6 / W16	A3 / A3	30

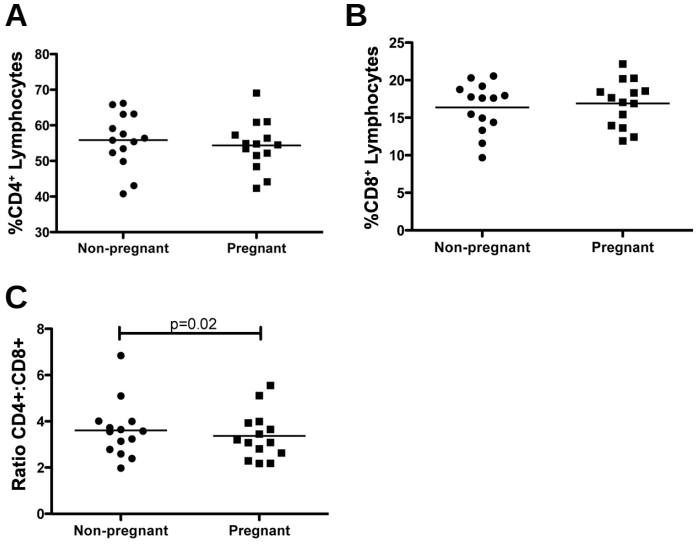
MHC types of the horses were determined by a standard lymphocyte microcytotoxicity assay using alloantisera to Equine Leukocyte Antigen (ELA) markers that had been validated in international workshops, as described in the Materials and Methods. Equine MHC haplotypes are designated by the letter A followed 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.

CD8 (Fig. 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 (Fig. 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 population (Fig. 4A) and in the sub-population of CD8+ T cells (Fig. 4B), 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.



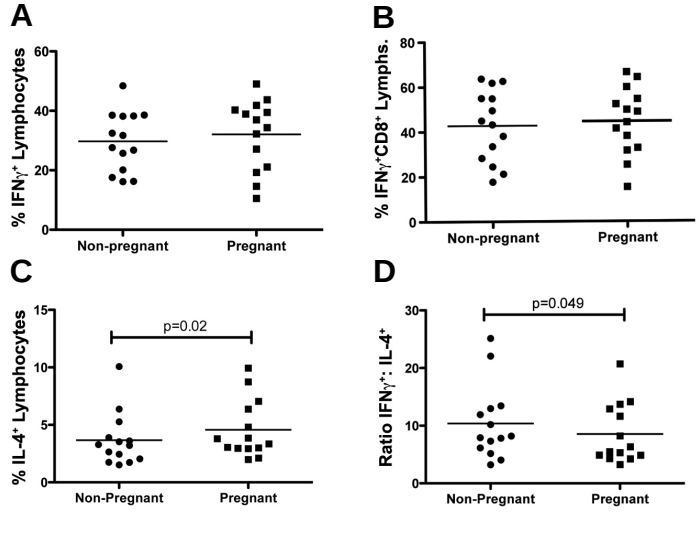


Fig. 3 (Left). Cell surface marker expression by peripheral lymphocytes from mares during the pregnant and non-pregnant states. *Flow cytometric analysis of paired samples of PBMC isolated during early pregnancy (days 29-33) or the luteal phase of estrous (n=15). Cells were labeled with monoclonal antibodies 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.*

Fig. 4 (Right). 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.*

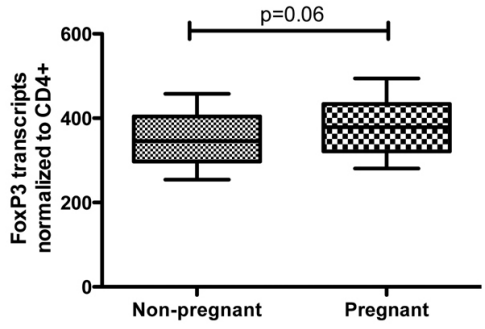


Fig. 5 (Left). ***FOXP3*** **expression by peripheral lymphocytes from mares during pregnant and non-pregnant states.** *Quantitative real-time PCR analysis of* FOXP3 *expression in paired samples of cDNA from PBMC isolated during early pregnancy (days 29-33) or the luteal phase of estrous (n=15). Absolute numbers of* FOXP3+ *transcripts were determined and normalized to a housekeeper gene and the number of CD4+ lymphocytes as described in Materials and Methods.*

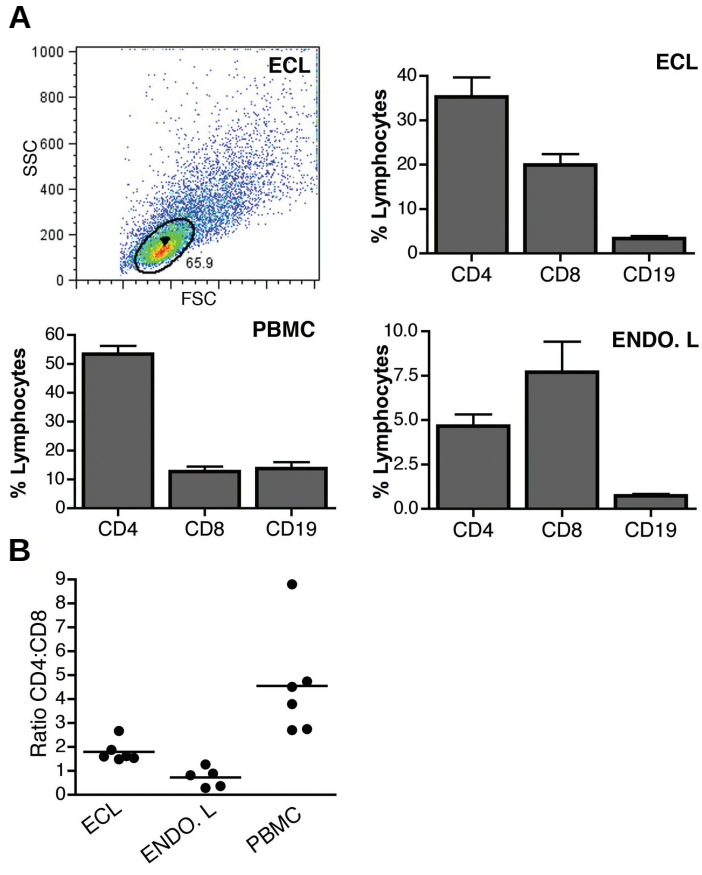


Fig. 6. (Right). Cell surface marker expression of lymphocytes isolated from the equine maternal-fetal interface at day 43-46 of pregnancy. (A) *Flow cytometric analysis of lymphocytes isolated from endometrial cups (ECL): CD4 n=6, CD8 n=6, CD19 n=4; pregnant endometrium (ENDO. L): n=5; and PBMC: n=6. 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).*

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.

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 Grünig *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 the B-cell marker 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 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 popula-

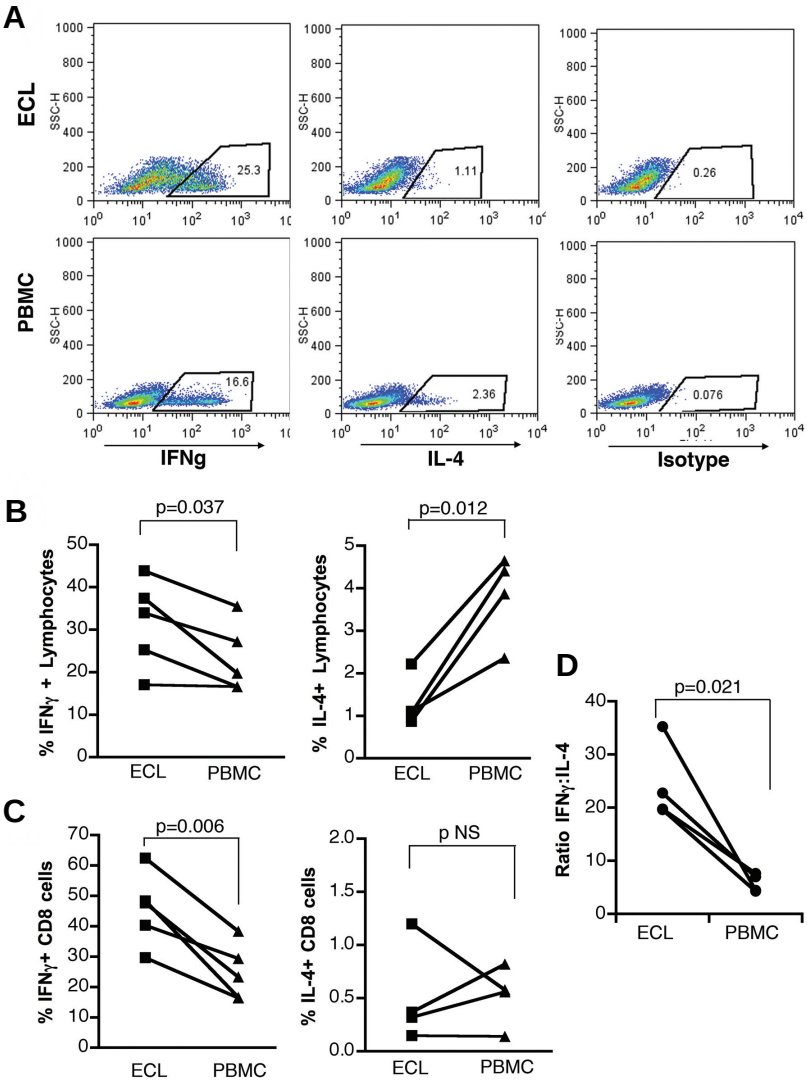


Fig. 7. Cytokine expression by lymphocytes at the equine maternal-fetal interface. *Flow cytometric analysis of IFNG and IL4 expression by paired samples of endometrial cup lymphocytes (ECL) and PBMC. Cells were stimulated* ex vivo *for 4 hours with PMA and IO in the presence of Brefeldin A.* **(A)** *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).*

tion 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.

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

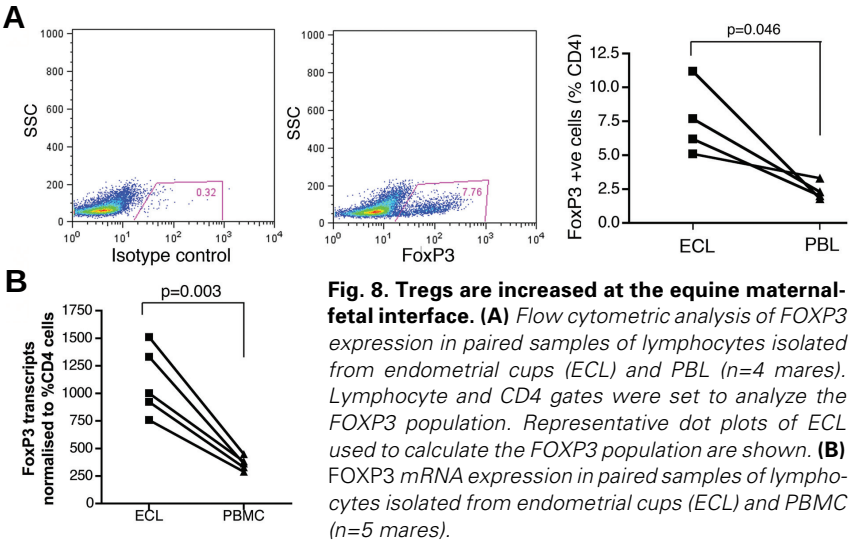


Fig. 8. Tregs are increased at the equine maternal-fetal interface. (A) *Flow cytometric analysis of FOXP3 expression in paired samples of lymphocytes isolated from endometrial cups (ECL) and PBL (n=4 mares). Lymphocyte and CD4 gates were set to analyze the FOXP3 population. Representative dot plots 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).*

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 Grünig *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 the 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 (Grünig *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 premature 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.

Materials & 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).

Tissue and cell preparation

Heparinized samples of venous jugular blood were collected from mares during diestrus, at day 31$\pm$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

TABLE 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 / A19	A2 / A2	45	16.7×10^6
3845	A5 / W16	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 Lymphocyte.

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: TGGCAAATGGTGTCTGCAA;
FOXP3RT2: GCGCTCTGCCCTTCTCATC;
UBC1: TGAAGAGAATCCACAAGGAATTGA;
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).

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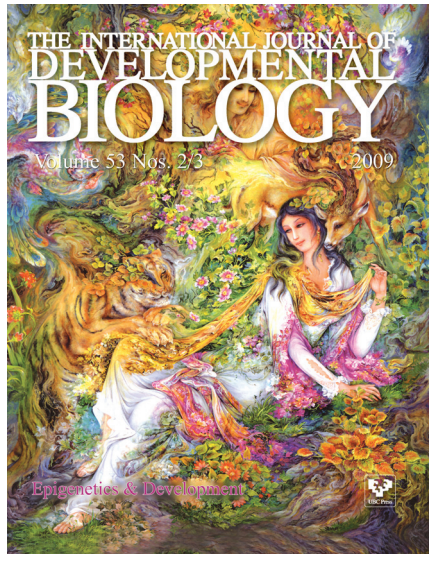

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