THE ROLE OF THE PD-1 IMMUNORECEPTOR IN PREGNANCY

by

Elizabeth Susan Taglauer

Submitted to the graduate degree program in Anatomy & Cell Biology and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Margaret G. Petroff Ph.D.
(Chair)

William H. Kinsey, Ph.D

Katherine F. Roby, Ph.D.

Michael J. Soares, Ph.D.

Patrick E. Fields, Ph.D.

July 7, 2008

Date of Dissertation Defense

The Dissertation Committee for Elizabeth Susan Taglauer certifies that this is the approved version of the following dissertation:

THE ROLE OF THE PD-1 IMMUNORECEPTOR IN PREGNANCY

Committee

Margaret G. Petroff Ph.D.
(Chair)

William H. Kinsey, Ph.D

Katherine F. Roby, Ph.D.

Michael J. Soares, Ph.D.

Patrick E. Fields, Ph.D.

Date Approved: July 11, 2008

Abstract

Successful pregnancy requires the coordination of several maternal systems to accommodate the developing fetus. Of these, the maternal immune system must be carefully modulated as the fetus is immunologically foreign to her leukocytes. In these studies, we propose that the CD28 family of immune receptors functions during pregnancy to control maternal immune reactions. These receptors bind to members of the B7 family of co-stimulatory molecules to influence leukocyte activation, either negatively or positively. One CD28 family member, PD-1, is expressed on T lymphocytes and following interaction with its ligand, B7-H1, prevents T cell activation. This pathway maintains self-tolerance and prevents allograft rejection through mechanisms including modulation of cytokine production, suppression of proliferation or apoptosis induction. Interestingly, B7-H1 is abundantly expressed in the human placenta throughout gestation. Therefore we studied the role of the PD-1 receptor in modulating maternal T cells during pregnancy. Within the human maternal-fetal interface, PD-1 is preferentially expressed on decidual T cells as compared to their counterparts in peripheral blood and B7-H1 inhibits the inflammatory cytokine production of activated decidual T lymphocytes. In addition, through mechanisms possibly involving B7-H1:PD-1 interactions, primary human trophoblast cells induce a regulatory T cell phenotype that can suppress the proliferation of other T cells. Finally, in murine pregnancy, PD-1 controls the accumulation of paternal antigen-specific T cells in the lymph nodes draining the uterus potentially through induction of apoptosis. Overall, the PD-1:B7-H1 pathway

functions at multiple sites through distinct mechanisms to help maintain maternal-fetal tolerance for the ultimate goal of successful pregnancy and healthy fetal development.

Dedication

To my family and scientific mentors

Acknowledgements

I would like to thank the following individuals for their respective contributions to this body of work: first, my incredibly supportive family for giving me the strong base with which I was able to enter into these studies; my former scientific mentors for kindling my pursuit of research; my friends throughout medical and graduate school for their empathy during challenging times; Dr. Joan Hunt and Dr. Joseph Bast for encouraging me to pursue the MD/PhD program; my husband Dr. Amir Khan for his insight, love and constant reassurance; and most importantly, my thesis mentor Dr. Margaret Petroff for providing a perfect balance of encouragement and challenge, without which I would not have accomplished this goal and for being a constant example of intelligence, dedication, and compassion to which I aspire.

Table of Contents

List of Figures

Chapter I:

Chapter II:

Chapter III:

Chapter IV:

Chapter V:

Chapter I

Introduction

Portions of this chapter have been submitted for publication:

Taglauer, E.S., Adams Waldorf, K.M., Petroff, M.G., The hidden maternal-fetal interface: secondary lymphoid organs and maternal-fetal tolerance. *Submitted.*

A. The immunology of pregnancy

1. *Successful pregnancy requires systemic coordination*

The production of offspring in viviparous species is a complex process during which the maternal anatomy and physiology are naturally modified to accommodate one or more developing fetuses for a prolonged period of time. Intricate coordination of several physiological systems is required for delivery of numerous signals and modulation of cellular activity at precise times throughout gestation. While these processes are successfully synchronized in healthy pregnancy, it is not surprising that commonly, one or more processes do not function correctly, thus resulting in infertility and gestational pathologies. Thus, the ability to conceive a child and maintain a successful pregnancy remains a challenge in today's society despite the ongoing development of therapies and clinical protocols to combat this problem. A national survey on the fertility of women in America estimated that 6.1 million women have impaired ability to conceive and that 2.1 million couples experience infertility (Statistics, CDC 1997). In addition, other studies have found that up to 30% of patients with known pregnancies experience miscarriage (Wilcox et al. 1988; Everett 1997).

In order to improve the reproductive health of women, it is essential to understand the biological reasons underlying these unfortunate statistics. Among the systems that are required for successful pregnancy, a potential cause for infertility and fetal loss may involve improper regulation of the maternal immune system. An

imbalance of immunological control during pregnancy can result in miscarriage, pre-eclampsia, or impaired fetal growth (Pearson 2002).

2. *Overview of the immune system*

The immune system is comprised of an intricately connected network of cell populations that ultimately function to mount responses against foreign entities in the body, such as viral or bacterial pathogens. Immune cell, or leukocyte, populations are grouped within two branches of the immune system, innate and the adaptive immunity. For responses against pathogens, cells of the innate immune system recognize bacteria and viruses through a series of pattern recognition receptors that subsequently initiate rapid non-specific inflammatory responses (Takeda et al. 2003). Cells of this type of immunity include natural killer cells, dendritic cells and macrophages. Once activated, these cells communicate with members of the adaptive immune system, which respond in turn by mounting more specific and longer lasting effector responses against pathogens; T and B lymphocytes are the main effector cells of the adaptive immune system.

Communication between the innate and adaptive immune systems occurs through cell-cell interactions and secreted products. Natural killer cells carry out direct effector mechanisms against pathogen-infected cells and produce cytokines that activate other immune cells. In addition, during the initial response to a pathogen, macrophages and dendritic cells process and present antigens to T lymphocytes of the adaptive immune system. Two principal types of T cells, distinguished by their surface expression of CD4 or CD8 molecules, possess different effector functions.

Depending on the nature of the pathogen, $CD4^+$ helper T cells produce cytokines that fall into two broad categories. Th1 cytokines stimulate cellular immune responses and Th2 cytokines initiate humoral immune responses. In Th1 type (cellular) immunity, $CD4^+$ helper T lymphocytes respond to antigen presentation by producing cytokines that activate $CD8^+$ (cytotoxic) T cells, which, following antigen exposure, carry out direct effector mechanisms against pathogen-infected cells. During Th2 (humoral) immune responses $CD4^+$ T cell cytokines activate B lymphocytes, which respond to antigen exposure by producing epitope-specific antibodies against that antigen.

T cells recognize self and non-self antigen only in the context of major histocompatability complex proteins (MHC). These cell surface proteins have a high level of polymorphism, such that each individual possesses a unique repertoire. MHC molecules are categorized into two classes. Class I MHC proteins are found on the surface of nearly all cells in the body and present intracellular antigen to $CD8^+$ T cells including peptides derived from self-proteins and intracellular pathogens. Class II MHC expression is restricted to professional antigen presenting cells, such as macrophages, dendritic cells and endothelial cells, and these molecules present extracellularly derived antigens. In contrast to Class I, Class II MHC proteins present antigen to $CD4^+$ T cells.

Presentation of peptides derived from self proteins serves the function of inducing tolerance to autologous tissues. Immune tolerance is a process by which the T cell repertoire is specifically modified to prevent reactions against self-antigens and self MHC. The ultimate goal of tolerance induction is to prevent T cell reactivity

against autologous tissues and the mechanism of tolerization can include death, anergy or even differentiation of T cells (Hugues et al. 2006; Cook et al. 2007). This can occur "centrally" in the thymus during T cell development or "peripherally" after mature T cells exit the thymus. Central tolerance occurs through deletion of T cells that react inappropriately to self-antigens presented by self-MHC in the thymus. Peripheral tolerance occurs as a result of recognition of self antigen in regional lymph nodes by self-reactive T cells that have escaped tolerance induction in the thymus. Peripheral tolerance can also be developed against certain non-self antigens. For example, mesenteric lymph nodes draining the intestine are a site for induction of oral tolerance against ingested antigens (Kraal et al. 2006) .

The result of central and peripheral tolerance is the absence of reactivity to self antigens and self MHC. Conversely, T cells mount strong cytotoxic responses against non-self MHC. This was realized through a series of experiments involving skin grafts in mice, in which grafts transplanted between inbred strains of mice containing identical MHC profiles (syngeneic) were accepted, while grafts between different strains with different MHC profiles (allogeneic) were rejected (Klein 1986). These studies led to the term “histocompatability” for MHC, as it was discovered that these molecules were responsible for the immune reactions being compatible with self, but not foreign tissue. This concept also became evident in human recipients of kidney transplants or blood transfusions who made antibodies against antigens on the surface of the donor leukocytes, which were identified as the human MHC molecules

and subsequently named human leukocyte antigens (HLA) (Dausset 1954; van Rood 1993).

3. *The immune paradox of pregnancy*

As more understanding was gained about the involvement of MHC/HLA molecules in the role of graft acceptance or rejection, the phenomenon of pregnancy in this context came under consideration. This is an immunological paradox first proposed by Sir Peter Medawar (Medawar 1953) who recognized that due to the genetic disparity of mother and fetus, including MHC, the maternal immune system should reject fetal tissues. Instead, the fetus is accepted for the duration of pregnancy. Medawar further postulated three explanations for the lack of semi-allogeneic fetal rejection: an anatomical separation between mother and fetus, a non-antigenic status of fetal tissues, and a paucity of maternal immune responses during pregnancy. The latter two proposed mechanisms have been supported through numerous studies. However, in humans, acceptance of the fetus may not depend on anatomically separated fetal tissues as the placental cells intimately contact maternal tissues in the gravid uterus throughout gestation.

4. *The maternal-fetal interface*

One of the requirements of viviparous species is delivery of nutrients to the developing fetus throughout gestation. Humans accomplish this goal through hemochorial placentation in which the fetal placenta is attached to the uterus and is

constantly bathed in maternal blood supplied by the uterine arteries throughout gestation. Figure 1.1 illustrates various components of this interface between maternal and fetal tissues. The human placenta is comprised of a network of branching villi containing a core of fetal blood vessels originating from the umbilical artery and veins. These villous blood vessels are covered by two layers of specialized phagocytic cells called trophoblasts (Fig. 1.1 B). The inner trophoblast layer consists of villous cytotrophoblast cells that differentiate and fuse to form syncytiotrophoblast cells constituting the outer layer. These cells take up molecules from the maternal blood and transfer them to the blood vessels within the core of the villi, thus transferring maternal nutrients to fetus throughout gestation. In addition, upon their differentiation, syncytiotrophoblast cells acquire an endocrine function, producing several steroid and peptide hormones critical for the maintenance of pregnancy (Kaufmann 1982). Trophoblast cells also comprise the chorion membrane, one of the layers constituting the extraplacental membranes surrounding the fetus (Fig. 1.1 C). These extraplacental membranes encase the amniotic fluid in which the fetus develops.

Throughout gestation, the placenta and fetal membranes are in contact with maternal decidua, specialized uterine tissue, which is divided into anatomical regions within the gravid uterus. The decidua basalis juxtaposes the placenta, decidua capsularis surrounds the fetal membranes, and decidua parietalis is located on the opposite side of the fetus from the attachment site of the placenta (Fig. 1.1). As the

fetus grows larger and its associated membranes expand throughout pregnancy, the decidua capsularis and parietalis fuse to become one entity (Benirschke et al. 2000).

Within the decidua basalis an additional trophoblast cell type is located. At the points of attachment to the decidua basalis, cytotrophoblasts migrate out from the villi to become extravillous trophoblast cells, initially proliferating to form cell columns and then migrating deeper into decidual tissue. Some extravillous trophoblasts invade maternal uterine spiral arteries, transforming them into low resistance vessels allowing increased blood flow to placental tissues (Fig. 1.1 A). The transformation of spiral arteries ensures increased nutrient delivery to the placenta in elegant congruence with augmented fetal growth as gestation progresses (Harris et al. 2007).

Similar to human pregnancy, mice utilize hemochorial placentation to support their developing fetuses *in utero*. Trophoblast cells within the murine placenta also have access to maternal blood and uterine tissues, thus maintaining intimate contact between mother and fetus (Georgiades et al. 2002). Mice are therefore commonly used as a model to study maternal responses to fetal antigen during pregnancy (Vacchio et al. 1999; Erlebacher et al. 2007).

5. *Decidual leukocytes*

Due to the immunological discrepancy between mother and fetus, the gravid uterus was initially thought to be an immune privileged site, as is observed for other parts of the body, such as the eye, gonads and central nervous system (Simpson 2006). However, several inaugural studies showed that the human maternal-fetal

interface contains multiple populations of leukocytes, from both innate and adaptive branches of the immune system (Fig. 1.1). These cells remain active within the gravid uterus, as they are capable of mounting responses against invading pathogens and commonly, their activity is also directed toward physiological functions during pregnancy.

i. NK cells

Natural killer (NK) cells of the innate immune system are the most abundant population in the gravid uterus within early pregnancy, and decrease in number as gestation continues. Decidual NK (dNK) have a $CD16^{-}$ $CD56^{bright}$ surface phenotype and are less cytotoxic than peripheral blood $CD16^{+}$ $CD56^{dim}$ NK. The main function of dNK at the maternal-fetal interface appears to be production of cytokines and chemokines that can potentiate trophoblast invasiveness and angiogenesis (Hanna et al. 2006; Hanna et al. 2007). Because of this function, dNK play a critical role in decidual artery remodeling during pregnancy as shown through *in vivo* dNK deficient mouse models (Guimond et al. 1997; Ashkar et al. 2000).

ii. Macrophages

Macrophages comprise around 18-25% of decidual leukocytes and remain in relatively constant numbers throughout gestation (Vince et al. 1990). Decidual macrophages (dMΦ) express lower levels of MHC-II and the co-receptor CD86 than peripheral blood monocytes of pregnant and non-pregnant women (Heikkinen et al.

2003). This surface phenotype suggests that dMΦ may be less efficient at presenting antigen and indeed dMΦ may have a decreased capacity to induce T cell responses in comparison with their peripheral blood counterparts (Hunt et al. 1992; Mizuno et al. 1994). The reduced stimulatory capacity of dMΦ could also be attributed to their production of anti-inflammatory cytokines such as IL-10 (Hunt 1994; Heikkinen et al. 2003). In addition, these cells may prevent inflammation at the maternal-fetal interface through phagocytosis of apoptotic trophoblast cells; the dead cells would thus be prevented from releasing their contents, which could stimulate anti-fetal responses (Mor et al. 2006).

iii. Dendritic cells

Dendritic cells are an additional antigen presenting cell in the decidua. Broadly, the functional outcome of interactions between a dendritic cell and a naïve T cell is determined by the activation state of the dendritic cell at the time of interaction. For example, presentation of an antigen by activated or mature dendritic cells can result in T cell priming and induction of an immune response to the corresponding antigen, while presentation of the same antigen by immature, or steady-state dendritic cells can induce antigen-specific T cell tolerance (Steinman et al. 2003). During pregnancy decidual dendritic cells express markers consistent with an immature phenotype (Kammerer et al. 2000; Gardner et al. 2003; Rieger et al. 2004). Thus, they may be readily available to help promote tolerance of the fetal allograft either within the maternal-fetal interface or in the uterine draining lymph nodes.

iv. T lymphocytes

Because T lymphocytes have a central role in graft rejection (Rocha et al. 2003), the interaction of fetal antigen and maternal T lymphocytes was initially thought to be a route for rejection of the semi-allogeneic fetus. However, maternal T cells are among the main immune cell populations at the human maternal-fetal interface, constituting 15-30% of decidual leukocytes in first trimester (Morii et al. 1993; Mincheva-Nilsson et al. 1994; Vassiliadou et al. 1996), and are present in constant or possibly enriched percentages among decidual leukocytes at term (Haller et al. 1993; Sindram-Trujillo et al. 2004). Both $CD4^+$ T helper cells and $CD8^+$ cytotoxic T cells are found in equal abundance relative to total $CD3^+$ T cell population within the decidua (Vassiliadou et al. 1996).

T lymphocytes at the maternal-fetal interface are not entirely quiescent as some express activation markers CD69 and CD25 (Chao et al. 1999; Aluvihare et al. 2004; Tilburgs et al. 2006). Although their functional role is unclear, they are apparently programmed to perform physiological functions. For example, *in vitro* stimulated decidual T cell produce cytokines that can potentiate the invasive capabilities of trophoblast cells (Scaife et al. 2006). In addition $CD4^+CD25^+$ cells play an immunosuppressive role in pregnancy (Saito et al. 2005).

6. *Immunomodulation at the maternal-fetal interface*

During pregnancy, the maternal immune system is controlled through an intiricate network of mechanisms participating in two different types of immune suppression. Within the maternal-fetal interface, there is modulation of ongoing immune activity without compromising the ability to mount responses against infectious pathogens. Within the spleen and uterus-draining lymph nodes, these mechanisms control fetal-antigen specific responses, i.e. induce tolerance.

i. MHC expression

A highly studied immune evasive mechanism at the maternal-fetal interface is the paucity of MHC expression within the placenta. In the human maternal-fetal interface, the majority of trophoblast cells lack the highly polymorphic MHC Class Ia HLA-A, HLA-B and Class II molecules (Petroff 2005a). Overall, the paucity of MHC expression prevents maternal T cell detection of fetal histo-*in*compatability, ensuring that trophoblast cells do not serve as direct targets for anti-fetal responses. However, the class Ia HLA-C molecule is expressed by extravillous trophoblast cells (King et al. 2000), but rather than stimulating anti-fetal immune responses, this molecule may be involved in modulating NK cell activity (Hiby et al. 2004). In addition, extravillous trophoblast cells express MHC Class Ib molecules, HLA-E, F and G (Ishitani et al. 2003). Class Ib molecules are less polymorphic than those of the class Ia family and do not stimulate induce maternal anti-fetal immune reactions. HLA-G in particular has well-characterized immunosuppressive capabilities,

including modification of macrophage and T cell cytokine production *in vitro* (Kanai et al. 2001; McIntire et al. 2004).

ii. Complement regulatory proteins

The complement system is an immune defense mechanism consisting of serum proteins that trigger direct cytotoxicity against target cells through introduction of membrane pores and subsequent cell lysis. Self-tissues are protected from this attack by expression of complement regulatory proteins that control various components of the complement cascade. Trophoblast cells are no exception (Holmes et al. 1990; Hsi et al. 1991), and indeed these proteins are indispensable for fetal survival. Murine embryos lacking the compliment regulatory protein Crry die at mid-gestation due to deposition of maternal compliment (Xu et al. 2000). However, complement-mediated fetal loss occurs in syngeneic pregnancy suggesting that these regulatory proteins provide homeostatic rather than allo-antigen-specific suppression.

iii. The Fas/Fas ligand proteins

Fas is member of the TNFR family, can be highly expressed by activated T cells (Drappa et al. 1993), and upon binding of its ligand (FasL) is known to induce peripheral T cell apoptosis (Singer et al. 1994). Fas-L-expressing human trophoblast cells can be found in close proximity to apoptotic decidual leukocytes *in situ*, and primary trophoblast cells can induce Fas-L-mediated apoptosis of activated lymphocytes *in vitro* (Mor et al. 1998; Coumans et al. 1999). In murine pregnancy,

fetal FasL may responsible for deletion of fetal antigen-specific T cells during late gestation (Vacchio et al. 2005), although interruption of this pathway during pregnancy did not cause fetal rejection even when maternal T cell responses are skewed toward responsiveness major or minor paternal antigens (Rogers et al. 1998; Vacchio et al. 2005).

iv. Indoleamine-2,3 dioxygenase

Indoleamine-2,3-dioxygenase (IDO) is a tryptyphan-catabolizing enzyme that may be involved in the maintenance of T cell tolerance (Mellor et al. 2004). While short-term pharmacological inhibition of IDO resulted in T cell mediated rejection of allogenic fetuses (Munn et al. 1998), genetic deletion of IDO did not result in corresponding fetal rejection (Baban et al. 2004). These studies suggest an inability to prevent maternal T cell responses with the acute loss of IDO while redundant mechanisms may be protecting the fetus during a long-term absence of this molecule.

v. Cytokines

Early examination of cytokines in pregnancy proposed a "Th-2 bias" in which anti-inflamamatory Th2 cytokines are preferentially produced over pro-inflammatory Th1 cytokines to maintain maternal immunological suppression (Wegmann et al. 1993). Indeed, overproduction of Th1or paucity of Th2 cytokines is associated with recurrent spontaneous abortion in humans and fetal loss in mice (Piccinni et al. 1998; El-Shazly et al. 2004; Mjihdi et al. 2004; Kim et al. 2005; Murphy et al. 2005).

However, inflammatory cytokines may also control trophoblast invasion in early pregnancy, and play a role in parturition at term (Pollard et al. 1993; Buonocore et al. 1995; Ashkar et al. 2000; Fukushima et al. 2003; Cohen et al. 2006; Scaife et al. 2006). Thus both Th1 and Th2 cytokines are important during pregnancy, and it appears that a balance Th1/Th2 production is critical in promoting maternal immune tolerance to the fetus while allowing leukocytes to perform physiological functions within the maternal-fetal interface (Wilczynski 2005).

vi. Regulatory T cells

Among the several mechanisms proposed to maintain T cell tolerance in pregnancy, one of the most experimentally robust mechanisms identified to date involves the suppressive activity of $CD4^+CD25^+FoxP3^+$ regulatory T cells (T_{reg}). These cells suppress the activation and proliferation of autologous T cells primarily through contact-dependent mechanisms (Thornton et al. 1998; Sasaki et al. 2004) and are key regulators of autoimmune T cell reactivity (Sakaguchi 2000; Shevach 2002). T_{reg} expand in the peripheral blood and lymph organs of both pregnant women and mice, and preferentially localize to the decidua (Aluvihare et al. 2004; Heikkinen et al. 2004; Sasaki et al. 2004; Somerset et al. 2004; Tilburgs et al. 2006). Further, they are required for the survival of semi-allogeneic fetuses during murine pregnancy models (Aluvihare et al. 2004; Zenclussen et al. 2005; Darrasse-Jeze et al. 2006) and associated with healthy pregnancy humans (Sasaki et al. 2004). This expansion of and functional requirement for T_{reg} highlights the importance of proper

immunological control of maternal T cells during pregnancy, and suggests that mechanisms involved in maintaining self-tolerance may also be utilized to establish maternal-fetal tolerance.

B. The B7 and CD28 families

Another well-established mechanism for modulating T lymphocyte function and maintaining tolerance to self-antigens involves the B7 and CD28 families of immune cell co-receptors (Greenwald et al. 2005). B7 molecules bind to members of the CD28 receptor family to influence lymphocyte activation, either negatively or positively (Greenwald et al. 2005) (Fig. 2). Both the B7 and CD28 families are type I transmembrane proteins classified within the immunoglobulin superfamily. The first members of the B7 family to be characterized, B7-1 and B7-2, are expressed primarily by antigen presenting cells, i.e. macrophages and dendritic cells (Freedman et al. 1987; Freeman et al. 1991; Freeman 1993; Freeman et al. 1993). Both B7-1 and B7-2 bind to CD28, a receptor that is constitutively expressed on T cells (Aruffo et al. 1987; Gross et al. 1990) (Fig. 1.2), the ligation of which is required for optimal T cell activation (Jenkins et al. 1991; Shahinian et al. 1993). Without this signal from CD28, the T cells either die or become anergic. Indeed, the delivery of the MHC signal in the absence of CD28 ligation has been postulated to serve as a mechanism of tolerance (Bretscher 1999). B7-1 and B7-2 also ligate another receptor, CTLA-4, which is present only on activated T cells and plays an important role in down-regulation of an ongoing immune response (Brunet et al. 1987) (Fig. 1.2).

Since the initial characterization of these proteins, five additional B7 proteins and two CD28 family receptors have been identified (Fig. 1.2). Like the function of B7-1 and B7-2, most novel B7 family members can either stimulate or inhibit the immune cells with which they interact, depending on the receptor they bind (Greenwald et al. 2005). With regard to pregnancy, the B7 family members B7-2, B7-H1, B7-DC, B7-H2, and B7-H3 have been mapped at the human maternal-fetal interface (Petroff et al. 2003; Petroff et al. 2005b).

C. The B7-H1:PD-1 pathway

1. *B7-H1: a uniquely expressed B7 family member*

B7-H1 was the third B7 family member to be characterized. Unlike B7-1 and B7-2, B7-H1 can be expressed by both immune and non-immune cells. B7-H1 is found on macrophages, dendritic cells and activated lymphocytes (Liang et al. 2003); outside of the immune system, B7-H1 is expressed by activated endothelial cells but is only found on the parenchymal cells of organs under inflammatory conditions (Iwai et al. 2003; Liang et al. 2003). One major exception is human trophoblast cells, which constituitively express B7-H1 throughout pregnancy (Petroff et al. 2003; Petroff et al. 2005b). Consistent with this finding, the placenta contains higher levels of B7-H1 mRNA when compared to other non-immune organs (Dong et al. 1999). These data suggest a unique requirement for and specific regulation of B7-H1 expression at the maternal-fetal interface.

During pregnancy, B7-H1 expression is low in first trimester and increases in term placental tissues. B7-H1 is correspondingly downregulated *in vitro* by low oxygen tension similar to that observed in early pregnancy (Holets et al. 2006). B7-H1 expression can also be increased by post-translational mechanisms through treatment with epidermal growth factor (EGF), which causes trophoblast differentiation (Petroff et al. 2003). Through regulation by EGF and oxygen, B7-H1 expression is increased as the placenta matures and increased nutrients are delivered to the fetus, which may be a critical period for immunomodulation within the maternal-fetal interface.

While B7-H1 has been well mapped in human pregnancy, its location at the murine maternal-fetal interface remains unclear (Liang et al. 2003; Guleria et al. 2005). B7-H1 mRNA is present in both decidua and placenta (Taglauer and Petroff, unpublished data). However, conflicting reports suggest protein expression on maternal decidual cells or trophoblast cells (Liang et al. 2003; Guleria et al. 2005).

2. *PD-1: the inhibitory receptor for B7-H1*

PD-1 is the receptor for B7-H1 as well as for another B7 family member, B7-DC (Freeman 2000; Latchman et al. 2001) (Fig. 1.2). PD-1 was first identified in T cells undergoing apoptosis and has since been identified on activated T, B, and myeloid cells (Ishida et al. 1992; Agata et al. 1996). The cytoplasmic domain of PD-1 contains two tyrosines that are associated with different intracellular signaling motifs. One is within an intracellular tyrosine inhibitory motif (ITIM), which can

mediate immunoreceptor inhibitory functions (Ishida et al. 1992). The other tyrosine is within an intracellular tyrosine switch motif (ITSM), which can bind either tyrosine or inositol phosphatases to result in different outcomes of receptor signaling (Shlapatska et al. 2001). Studies involving PD-1 chimeric proteins demonstrate that when the PD-1 cytoplasmic domain is activated through antigen stimulation, the ITSM in PD-1 recruits the SHP-2 tyrosine phosphatase. Recruitment of SHP-2 can cause inhibition of lymphocyte activation by de-phosphorylating proteins in the antigen receptor signalling (Okazaki 2001; Chemnitz et al. 2004; Paul A. Saunders 2005). SHP-2 activity ultimately leads to deactivation of signaling molecules including phosphatidylinositol-3 kinase (PI3K), phospholipase C-(PLC) γ2, and external signal-regulated kinase (ERK), all of which are involved in signal transduction pathways carry out diverse functions of cell survival, proliferation, and differentiation of lymphocytes (Okazaki 2001).

3. *Effects of B7-H1: PD-1 interactions at the cellular level*

The effects of B7-H1 and PD-1 interactions were initially studied using *in vitro* culture systems. In these studies, B7-H1 was found to inhibit the T cell cytokine secretion and proliferaion (Freeman 2000; Latchman et al. 2001). Further studies also suggested that B7-H1:PD-1 interactions can also result in apoptosis (Dong et al. 2002; Hori et al. 2006; Muhlbauer et al. 2006). Both $CD4^+$ and $CD8^+$ T cell subsets are susceptible to B7-H1 inhibition, although $CD8^+$ may be more sensitive to B7-H1

(Carter et al. 2002). However other studies have revealed that B7-H1 may preferentially suppress $CD4^+$ T cells during allograft responses (Ito et al. 2005)

T_{reg} also utilize the B7-H1:PD-1 pathway to differentiate or to inhibit of autologous T lymphocyte activation. These cells can express B7-H1, which may mediate T_{reg} suppression of effector $CD4^+$ T cells (Yang et al. 2006; Jinushi et al. 2007). PD-1 has also been identified on T_{reg} (Jinushi et al. 2007) and two recent studies have suggested that B7-H1 signaling may induce the differentiation of these cells outside of the thymus (Krupnick et al. 2005; Beswick et al. 2007).

Other studies have suggested that B7-H1 can also stimulate lymphocytes. Under different *in vitro* conditions, B7-H1 may cause increases in T cell proliferation and secretion of interleukin-10, an anti-inflammatory cytokine (Dong et al. 1999). Interestingly, in mutated B7-H1 proteins with disrupted PD-1 binding ability, stimulatory effects were still observed, suggesting that B7-H1 may work through a different receptor to cause stimulation (Wang et al. 2003). However, this receptor has not yet been identified.

D. Pathological and physiological consequences of B7-H1: PD-1 interactions

The characterization of B7-H1 interactions through PD-1 and the implications of this interaction as a negative regulator of lymphocytes began to draw attention from several areas of research. Roles for B7-H1 and PD-1 have been identified in cancer, infectious disease, autoimmunity, transplantation, and reproduction. Depending on the context, the presence of B7-H1 or PD-1 may have a pathological

role, as in cancer or infectious disease, or a physiologic role, as in self-tolerance, allograft-acceptance and pregnancy.

1. *Tumor biology*

Although it is not normally expressed on parenchymal cells of organs, B7-H1 is constitutively expressed on several tumors of different origin (Dong et al. 2002; Brown et al. 2003; Thompson et al. 2004). In mouse studies, B7-H1 expression on mastocytoma tumor cells can cause apoptosis of tumor-specific T cells (Dong et al. 2002). In addition, the growth of B7-H1-expressing myeloma cells are completely suppressed in PD-1 deficient mice (Iwai et al. 2002). Interestingly, in human clinical studies, the presence of B7-H1 on renal cell carcinomas correlates with aggressive tumors and poor long-term prognosis, also implying that B7-H1 is inhibiting host anti-tumor immune defenses (Thompson et al. 2004; Thompson et al. 2006). Tumors can also evade the immune system through induction of regulatory T cells that could suppress anti-tumor T cell reactions. Interestingly, B7-H1 is expressed by T_{reg} infiltrating into B-cell hodgekins lymphomas, and may mediate T_{reg} suppression of intratumoral T cell proliferation (Yang et al. 2006). Overall, these data suggest that the B7-H1:PD-1 pathway can serve as a mechanism for tumors to escape host immune responses

2. *Infectious disease*

Induction of B7-H1 on antigen presenting cells may also result in decreased immune responses against pathogens. During a parasitic infection, macrophages from

Schistosoma mansoni-infected mice have elevated B7-H1 expression levels and through B7-H1, induce anergy in T cells (Smith et al. 2004). In addition, intestinal epithelial cells infected with *Helicobacter Pylori* bacteria also upregulate B7-H1 following infection. In this model, B7-H1 induced differentiation of T_{reg} that could suppress anti-bacterial T cell responses (Beswick et al. 2007). Other studies have also focused on the role of PD-1 on T cells in infectious disease. In mice, during chronic lymphocytic choriomeningitis virus (LCMV) infection, $CD8^+$ T cells are inhibited through PD-1, culminating in failure of viral clearance (Barber et al. 2006). Consistent with this idea, PD-1-null, adenovirus-infected mice have increased effector T cell proliferation and accelerated viral clearance suggesting the absence of PD-1 may aid in overcoming a viral infection (Iwai et al. 2003).

3. *Autoimmune disease*

Although expression of B7-H1 and PD-1 in tumors and infectious diseases may have a pathological influence, there are clearly also physiologic roles for these proteins. For example PD-1 and B7-H1 have a central role in preventing autoimmune reactions. PD-1 knockout mice develop autoimmune phenotypes that depend on the background strain of mouse. C57BL/6 PD-$1^{-/-}$ mice develop late-onset, lupus-like arthritis and glomerulonephritis (Nishimura et al. 1999), while BALB/c PD-$1^{-/-}$ mice develop severe early-onset dilated cardiomyopathy characterized by autoreactive antibodies against cardiac troponin-1 (Nishimura et al. 2001; Nishimura et al. 2003). The consequences of genetic alteration of PD-1 are also evident in humans where

small nucleotide polymorphisms (SNPs) in the PD-1 gene may be associated with certain autoimmune diseases including systemic lupus erythemous and type I diabetes (Prokunina et al. 2002; Nielsen et al. 2003; Ni et al. 2007). In more recent studies, models utilizing antigen specific T cells demonstrate that PD-1 is involved the induction of self reactive T cell anergy in the periphery (Tsushima et al. 2007), and this inhibitory receptor prevents antigen-specific cytotoxic T cell attack of self-tissues (Martin-Orozco et al. 2006; Keir et al. 2007). Overall these combined results suggest that PD-1 has a critical role in maintaining tolerance of immune cells to self-antigens in peripheral organs.

Unlike PD-1 knockout mice, B7-H1-deficient mice do not spontaneously develop autoimmune disease. However, *in vitro,* $CD8^+$ T cells from B7-$H1^{-/-}$ mice display increased antigen-specific cytotoxic responses when compared to wildtype controls. *In vivo,* B7-H1 knockout mice are more susceptible to experimental autoimmune hepatitis and encephalomyelitis, and diabetes that wild type controls (Dong et al. 2004; Latchman et al. 2004; Keir et al. 2006).

4. *Allograft tolerance*

In addition to preventing autoimmune disease, B7-H1 and PD-1 function to suppress allograft rejection. In allogeneic murine cardiac transplants, B7-H1 and PD-1 are expressed on recipient leukocytes infiltrating the cardiac tissue during rejection (Ozkaynak et al. 2002). This pathway can work either independently or synergistically with other immunosuppressive agents to promote allograft survival

through mechanisms including control of inflammatory cytokine production and T_{reg} cell induction (Ozkaynak et al. 2002; Gao 2003; Tanaka et al. 2007; Wang et al. 2007). Interestingly, B7-H1 expression on donor cardiac allograft tissues may also be critical for preventing rejection (Yang et al. 2008). This is a particularly interesting result because it demonstrates that B7-H1 expressed on non-immune cells can inhibit T cell activation against an allograft, a situation that may be analogous to trophoblast-mediated inhibition of maternal T cells.

5. *Pregnancy*

In our studies, we hypothesize that the function of B7-H1 and PD-1 in pregnancy correlates with results from other fields: the B7-H1:PD-1 pathway may control immune reactions against fetal antigen. This follows directly from observations that B7-H1 is highly expressed on several fetal trophoblast cells throughout gestation, including those invading the uterus and those that are exposed to maternal blood (Petroff et al. 2003; Petroff et al. 2005b).

However, antibody-mediated blockade of maternal B7-H1 in allogeneic but not syngeneic pregnancy resulted in increased fetal resorption which could be reversed through maternal T cell depletion or transfer of B7-H1 expressing regulatory T cells (Guleria et al. 2005; Habicht et al. 2007). B7-H1$^{-/-}$ female mice also had reduced litter size when bred allogeneically (Guleria et al. 2005). These studies suggest that B7-H1 prevents rejection of semi-allogeneic fetuses by controlling maternal T lymphocyte activation.

E. Summary and experimental goals

The inhibitory actions of the B7-H1:PD-1 pathway from the research areas described above have a common theme: various cell types express B7-H1 which then binds to PD-1 on immune cells and results in reduced activation or tolerance. These interactions may contribute to the pathogenesis of tumors and infectious diseases, but also prevent autoimmune disease and promote transplant acceptance. Due to the immunoinhibitory actions of PD-1, as well as the abundance and function of its ligand, B7-H1, at the maternal-fetal interface we hypothesize that this receptor also has an important role in the immunological maintenance of pregnancy. We explored this hypothesis using three series of experiments.

First, we examined the expression and function of the PD-1 receptor in human pregnancy. We collected autologous peripheral blood and decidual tissues from patients at term pregnancy and evaluated PD-1 expression on their T cell subpopulations through flow cytometry. An *in vitro* co-culture system was developed to examine the effects of B7-H1 on cytokine production and cell death in decidual T cells. Within this model, we evaluated suppression of previously activated T cells, which models suppression of ongoing immune activation of these decidual leukocytes.

Second, we examined T_{reg} during human pregnancy. First, we analyzed their distribution within the peripheral blood and decidual tissues at term pregnancy. We also asked whether T_{reg} may be expanded during pregnancy by factors within the

maternal-fetal interface. In particular we examined whether primary trophoblast cells can induce a T_{reg} phenotype from peripheral blood naïve $CD4^+$ T cells.

Third, we performed murine studies to examine PD-1 regulation of maternal T cells *in vivo*. We utilized an adoptive transfer model to evaluate the role of PD-1 on paternal-antigen specific T cells during pregnancy, evaluating immunomodulation against antigen-targeted responses within the maternal lymph node and spleen. In addition we determined whether maternal PD-1 is required for acceptance of the semi-allogeneic fetus.

F. Figures and Legends

Figure 1.1. The maternal-fetal interface. Diagram of the developing human fetus and placenta within the gravid uterus (A) Anchoring placental villous attaching to the decidua basalis. Cytotrophoblasts migrate out from the villous to become extravillous trophoblast cells that reside in the decidua basalis or invade maternal uterine spiral arteries. (B) Placental villi lined with cytotrophoblast (CTB) and syncytiotrophoblast (sTB) layers facing the maternal blood space. (C) Extraplacental membranes comprised in part of tropholbast cells in the chorion membrane covered with decidua capsularis.

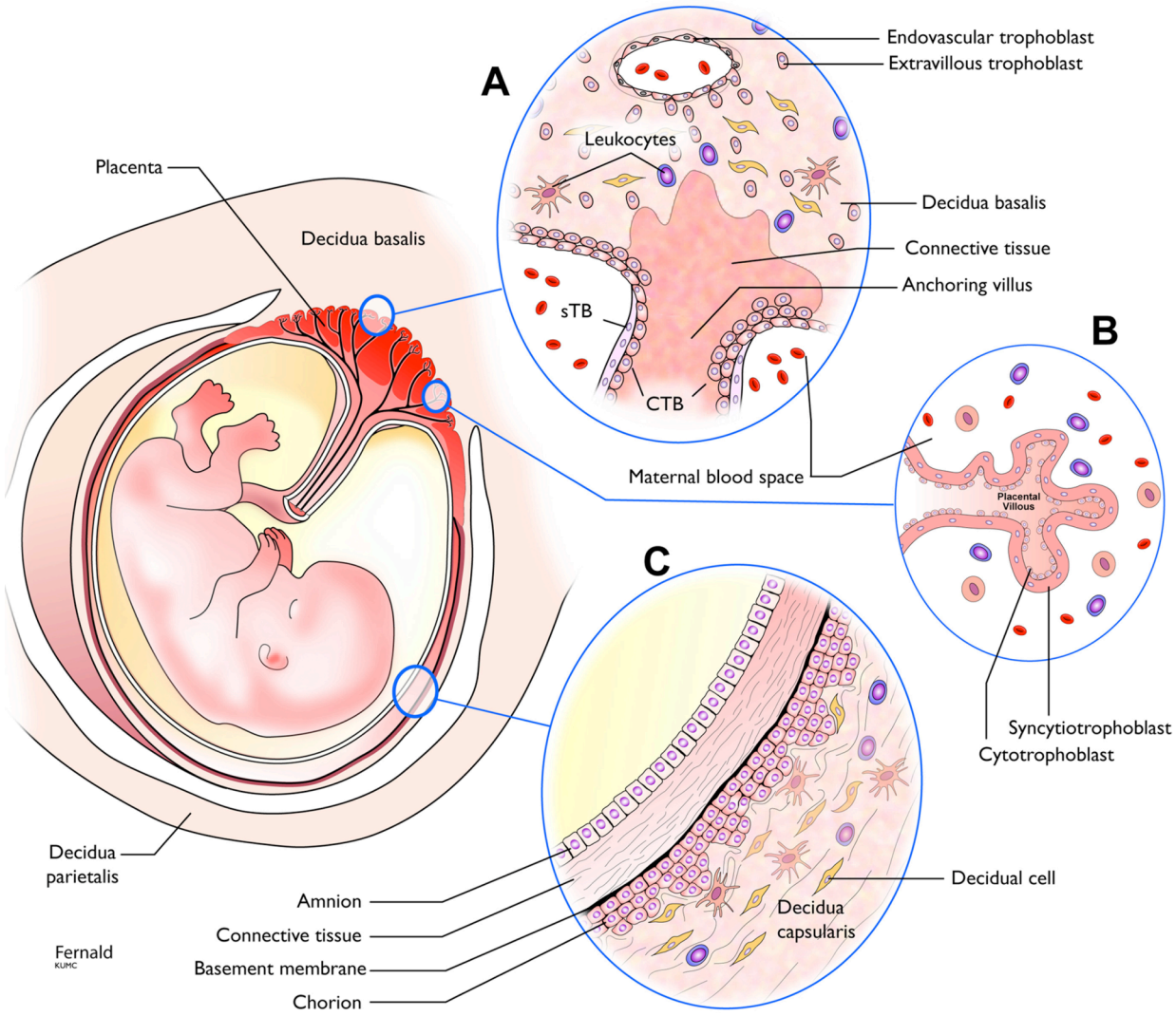
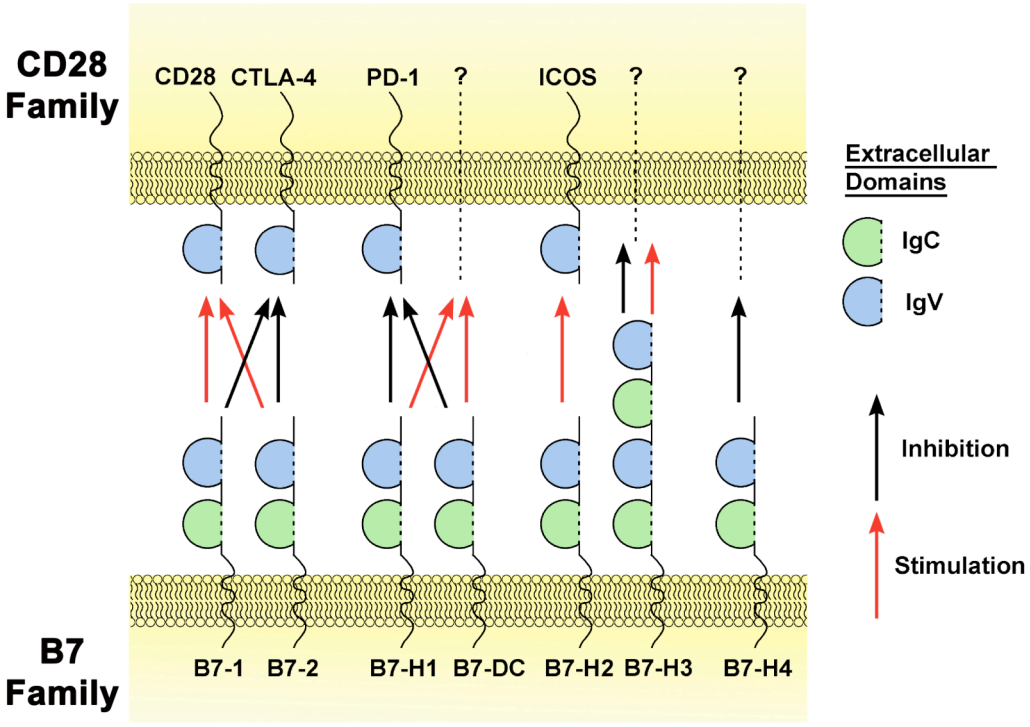

A
Endovascular trophoblast
Extravillous trophoblast
Leukocytes
Decidua basalis
Connective tissue
Anchoring villus
sTB
CTB
Placenta
Decidua basalis
B
Maternal blood space
Placental Villous
Syncytiotrophoblast
Cytotrophoblast
C
Decidual cell
Decidua capsularis
Decidua parietalis
Amnion
Connective tissue
Basement membrane
Chorion
Fernald
KUMC

Figure 1.2. The B7 and CD28 Families

Schematic diagram of B7 and CD28 family members and the functional outcomes of cognate receptor:ligand interactions. Both the families are type I transmembrane proteins classified within the immunoglobulin superfamily. B7 family ligands bind to CD28 family receptors on lymphocytes to influence their activation, either negatively (black arrows) or positively (red arrows).


CD28 Family
CD28
CTLA-4
PD-1
?
ICOS
?
?
Extracellular Domains
IgC
IgV
Inhibition
Stimulation
B7 Family
B7-1
B7-2
B7-H1
B7-DC
B7-H2
B7-H3
B7-H4

Chapter II

Expression and Function of PD-1 at the Human Maternal-Fetal Interface

Taglauer ES, Trickhacheva AS, Slusser JG, Petroff MG. Expression and function of the PDCD1 at the human maternal-fetal interface. (*In press,* Biology of Reproduction DOI:10.1095/biolreprod.107.066324).

A. Abstract

The failure to reject the semiallogenic fetus by maternal T lymphocytes suggests that potent mechanisms regulate these cells. PD-1 is a CD28 family receptor expressed by T cells, and its ligand B7-H1 is strongly expressed by trophoblast cells of the human placenta. In this study, we examined whether human maternal T cells express PD-1. Immunofluorescence examination of uterine tissues revealed PD-1 expression on $CD3^+$ cells was low in non-pregnant endometrium but increased in first trimester decidua and remained elevated in term decidua ($P < 0.05$). In addition, higher relative proportions of term decidual $CD8^{bright}$, $CD4^+$, and regulatory T cells expressed PD-1 in comparison to autologous peripheral blood ($P < 0.05$). Term decidual T cells also expressed full length and soluble PD-1 mRNA isoforms more abundantly than their peripheral blood counterparts ($P \leq 0.05$). We then examined the effects of PD-1:B7-H1 interactions in decidual T cells. Jar choriocarcinoma cells were transfected with B7-H1 and co-cultured with activated decidual $CD4^+$ or $CD8^{bright}$ T cells for 72 hours followed by analysis of supernatant cytokine concentration and decidual T cell apoptosis. As compared to empty vector transfected cells, B7-H1-transfected Jar cells caused a significant suppression of IFN-γ and TNF-α production by $CD4^+$ ($P < 0.05$) but not $CD8^{bright}$ T cells, while B7-H1 had no effect on secretion of IL-10 or T cell apoptosis. These results suggest that the PD-1:B7-H1 pathway functions in modification of the maternal immune system during pregnancy.

B. Introduction

The fetus expresses both maternal and paternal genes, and is therefore partially foreign, or semi-allogenic, in relation to the mother's immune system. Because a central feature of the immune system is to discriminate between self and non-self tissues, it is paradoxical that the semi-allogenic fetus develops in the mother without normally being rejected by her leukocytes. Maternal T cells are among the main immune cell populations at the maternal-fetal interface constituting 15-30% of decidual leukocytes in first trimester (Morii et al. 1993; Mincheva-Nilsson et al. 1994; Vassiliadou et al. 1996), and are present in constant or possibly enriched numbers at term (Haller et al. 1993; Sindram-Trujillo et al. 2004). Because their presence in the decidua puts them in contact with the semi-allogenic trophoblast cells throughout gestation, it has been thought that maternal T cell activity, including cytokine production, is modulated in order to maintain normal pregnancy.

The production and activity of cytokines throughout pregnancy can result in either harmful or beneficial outcomes and thus need to be carefully controlled. For example, an overproduction of interferon (IFN)-γ and tumor necosis factor (TNF)-α may be associated with recurrent spontaneous abortions in humans and fetal loss in mice (Jenkins et al. 2000; Mjihdi et al. 2004; Kim et al. 2005). However, these same cytokines are found in normal decidual and placental tissues, and accumulating evidence suggests physiological roles for these factors in regulating trophoblast invasion, spiral artery modification and placental morphogenesis (Cohen et al. 2006; Scaife et al. 2006; Hanna et al. 2007). While decidual natural killer cells contribute

significantly to the cytokine environment at the maternal-fetal interface (Hanna et al. 2007), decidual T cell cytokine production may also be involved in the normal functions of pregnancy (Scaife et al. 2006). However, because of their central role in allograft rejection (Rocha et al. 2003), maternal T cell activation and cytokine production are likely to be held in balance to promote physiological function and prevent pathological consequences in order to maintain healthy pregnancy.

One mechanism by which maternal T cells may be controlled is through the CD28 family of immune cell co-receptors. These cell-surface proteins transduce either positive or negative signals following ligation of their B7-family ligands. One of these receptors, programmed cell death-1 (PD-1) is induced on activated lymphocytes and negatively regulates T cells through its ligand B7-H1 (also called PD-L1 or CD274) (Ishida et al. 1992; Agata et al. 1996; Freeman et al. 2000; Latchman et al. 2001; Latchman et al. 2004). The suppressive action of PD-1 is also demonstrated though *in vivo* studies where targeted mutation or blockade of PD-1 results in development of spontaneous tissue-specific autoimmune disease (Nishimura et al. 1999; Nishimura et al. 2001) or antigen specific T cell cytotoxicity against peripheral self-tissues (Martin-Orozco et al. 2006; Keir et al. 2007). In addition, this inhibitory receptor promotes allograft acceptance (Ozkaynak et al. 2002; Tanaka et al. 2007; Wang et al. 2007; Yang et al. 2008). PD-1 thus appears to have a key role in maintaining not only immunological self-tolerance to peripheral tissues, but also tolerance to foreign grafts. In addition, five alternatively spliced isoforms of the PD-1 mRNA transcript have recently been identified (Nielsen et al. 2005), including an

isoform (PD-1 Δ Ex3) that lacks a transmembrane domain while retaining an intact B7-H1 binding domain, suggesting that it is a secreted but inactive form of PD-1. Soluble PD-1 has been detected in the synovial fluid and plasma of patients with rheumatoid arthritis and may act as a decoy receptor to prevent B7-H1:PD-1-mediated T cell inhibition (Wan et al. 2006).

Interestingly, trophoblast cells abundantly and constitutively express the PD-1 ligand, B7-H1, throughout pregnancy including those invading the pregnant uterus and those exposed to maternal blood (Petroff et al. 2003; Petroff et al. 2005b). In addition, B7-H1 has been suggested to be necessary for the survival of semi-allogenic fetuses in murine pregnancy (Guleria et al. 2005). The role of PD-1 on T cells in peripheral tolerance and allograft acceptance, together with the abundance of B7-H1 at the maternal-fetal interface suggest an important function for this receptor:ligand complex in the immunological maintenance of pregnancy. The goal of this study was to examine the expression of the PD-1 receptor and its soluble isoforms on T cells at the maternal-fetal interface. In addition, we examined the effect of the PD-1:B7-H1 interactions on maternal T cell subpopulations during pregnancy.

C. Materials and methods

Tissue collection

All tissue samples were collected in accordance with human subjects protocols approved by the University of Kansas Medical Center Institutional Research Board (HSC #3037). Archival mid- (n=1) and late (n=5) secretory phase

endometrial tissues were obtained from the University of Kansas Medical Center tissue bank and staged by a board-certified pathologist. Late secretory phase endometrial samples were identified by pre-decidual changes and variable apical vacuolation of glandular epithelium, characteristic of the time at which implantation occurs. Samples were collected from pre-menopausal women ages 24-49 undergoing surgery for reasons other than uterine pathology; patients were negative for birth control or replacement hormone therapies at the time of biopsy, although intrauterine device status was unknown. First trimester tissues, including decidua from voluntary pregnancy terminations and peripheral blood, were collected from patients at 6-11 weeks of gestation. First trimester decidual samples were collected within the Tissue Collection Core (HD049480) at the University of Chicago. Four out of seven first trimester decidual samples contained decidua basalis and extravillous trophoblast cells.Normal autologous term decidua and peripheral blood were obtained on the day of elective or repeat Caesarian (C)-section. All tissue collected for histology was either snap-frozen in liquid nitrogen and subsequently fixed in 4% paraformaldehyde for 20 minutes after sectioning, or pre-fixed for 4 hours in 4% paraformaldehyde and soaked in 18% sucrose for 18-24 hours prior to freezing. In addition, blood and decidual tissue were processed for lymphocyte isolation as described below.

Immunofluorescence

Ten µm-thick tissue sections were cut on a cryostat and placed in duplicate onto lysine-coated slides. After blocking of non-specific antibody binding sites in 2%

rabbit serum, 50% SuperBlock (Pierce, Rockford, IL), and 0.2% Triton-X-100 (Fisher Scientific, Pittsburgh, PA), tissue sections were incubated with an anti-PD-1 antibody (10μg/mL, clone J116; eBioscience, San Diego, CA) or its isotype control (mouse IgG1κ; BD Pharmingen, San Jose, CA) overnight at 4ºC followed by incubation with Alexa 568 rabbit anti-mouse secondary antibody (Invitrogen, Carlsbad, CA) for one hour at room temperature. Tissues were then incubated with CD3-fluorescein isothiocyanate (FITC) (10μg/mL, clone UCHT-1; BD Pharmingen,) or its isotype (mouse IgG1κ –FITC; eBioscience) at 37ºC for one hour. Slides were coverslipped with Prolong Gold (Invitrogen) and cured overnight before imaging. Immunofluorescence was visualized on a Nikon 90i upright microscope via mercury fluorescent excitation then confirmed via confocal scanning (Nikon C1 series confocal scan head) (Nikon, Melville, NY). Lasers used for emission detection were a 488 Multiline Argon (green IR) and 561 Diode laser (red IR), acquisition via the Nikon EZ-C1 series software (3.60). For quantification of single and double positive cells, images were captured from 10 randomly chosen, non-overlapping viewing fields (40x objective) for each tissue. Double-positive cells were confirmed by comparison of single color scans for each image. Samples from seven different patients were examined for each tissue group.

Isolation of decidual and peripheral blood lymphocytes

Term decidua was obtained by either peeling the fused decidua capsularis/parietalis (decidua) and chorion from the amnion for flow cytometric

studies or by scraping the decidua from the chorion for mRNA and *in vitro* co-cultures. Decidual tissues were then dissociated using 200 U/mL type IV collagenase, 1 mg/mL type1-S hyaluronidase, and 150 µg/mL type IV DNAse in a shaking 37^{o}C water bath (Vince et al. 1990). All enzymes were purchased from Sigma-Aldrich. Both first trimester and term cell suspensions were layered over Histopaque (Sigma-Aldrich) and centrifuged. The mononuclear cell fraction, which included decidual lymphocytes and chorionic trophoblasts, was collected and counted to assess cell viability and yield. For mRNA analysis and *in vitro* assays, the mononuclear cell fraction was collected and plated for 2-3 hours at 37˚C to allow adherence of non-lymphocytes. For preparation of term autologous peripheral blood lymphocytes, samples were diluted in PBS, layered over Histopaque, collected and counted to assess cell viability and yield.

Flow cytometry

Dispersed decidual lymphocytes, chorionic trophoblast cells, and peripheral blood lymphocytes were labeled for specific markers using the following anti-human antibodies from eBioscience: CD4- FITC (clone RPA-T4), CD8- phycoerythrin (PE) (clone RPA-T8), CD25- allophycocyanin (APC) (clone BC96), FoxP3-PE (clone PCH101), PD-1-biotin (clone J116), and B7-H1-biotin (clone M1H1). Biotinylated antibodies were detected using a conjugate of phycoerythrin-Cy5 (PE-Cy5) bound to streptavidin (BD Pharmingen). Fluorophore-conjugated mouse IgG1 isotype controls were used for CD4, CD8, and CD25; PE-conjugated rat IgG2a control was used for

FoxP3 (eBioscience). A minimum of 20,000 lymphocytes were gated based on forward and side scatter characteristics as well as T cell subpopulation marker expression and data were collected for each desired population. Samples were processed on a BD LSRII instrument and analyzed using FACS Diva software (BD Pharmingen).

Fluorescence-activated cell sorting

Peripheral blood mononuclear cells and non-adherent decidual mononuclear cells were labeled for specific markers using the following anti-human antibodies from eBioscience: anti-CD3 allophycocyanin (clone UCHT-1), anti-CD4-fluorescein isothiocyanate (FITC) (clone RPA-T4), anti-CD8- phycoerythrin (PE) (clone RPA-T8). Lymphocytes were sorted into $CD3^+/CD4^+$ or $CD3^+/CD8^{bright}$ populations using a BD FACS Aria using FACS DIVA software (Becton Dickinson). Cell population purity for five samples was analyzed post-sorting and was an average of 96.0 ± 1.22 % for $CD3^+/CD4^+$ cells and 97.2 ± 0.61 % for $CD3^+/CD8^{bright}$ cells.

Isolation, reverse transcription and PCR analysis of RNA

After sorting of specific T cell subpopulations, total cellular RNA was isolated from each group of cells using Trizol (Invitrogen) according to the manufacturer's instructions. Following quantification by spectrophotometry, 1 μg of RNA was reverse transcribed using MMLV reverse transcriptase and oligo-dT primers (Invitrogen) in a 40 μl reaction volume. RNA was also added to a reaction without

MMLV reverse transcriptase as a control to ensure the absence of genomic DNA amplification. PCR amplification of cDNA products was performed using Taq DNA polymerase (Invitrogen) in conjunction with primers (forward: 5' GCGGCCAGGATGGTTCTTA-3' ; reverse: 5'-TACTCCGTCTCGTCAGGA-3'), which correspond to positions 125-143 and 793-811 of the PD-1 mRNA transcript, respectively (GenBank Accession No. NM-005018) (Nielsen et al. 2005). PCR reaction products (20 μL) were subjected to electrophoresis on a 2% 3:1 agarose gel (Amresco, Solon OH) and visualized using ethidium bromide (Sigma-Aldrich) to examine product size. Following visualization, products were excised and sequenced to confirm identity.

Real-time PCR

The following PD-1 isoform-specific primers were used: full length PD-1 (forward: 5' CTCAGGGTGACAGAGAGAAG 3', positions 492-511 of PD-1 mRNA transcript; reverse: 5' GACACCAACCACCAGGGTTT 3', positions 568-587); PD-1 Δ Exon 2 (forward: 5' GGTTCTTAGAGAGAAGGGCA 3', positions 135-144 and 505-515; reverse: 5' AGGGTGACAGGGACAATAGG 3', positions 568-587); PD-1 Δ Exon 3 (forward: 5' AGGGTGACAGGGACAATAGG 3', positions 495-504 and 661-670; reverse: 5' CCATAGTCCACAGAGAACAC 3', positions 720-739); PD-1 Δ Exon 2,3 (forward: 5' TGGTTCTTAGGGACAATAGG 3', positions 135-144 and 661-670; reverse 5' TCTTCTCTCGCCACTGGAAA 3', positions 749-768); and PD-1 Δ Exon 2,3,4 (forward: 5'

TGGTTCTTAGAAGGAGGACC 3', positions 135-144 and 696-705; reverse 5' TCTTCTCTCGCCACTGGAAA, positions 749-768) (Nielsen et al. 2005). β̃actin primers were purchased from Applied Biosystems (Foster City, CA). These primers were used along with Power SYBR Green PCR Master Mix (Applied Biosystems) and 1μL of reverse transcribed RNA. As a negative control, 1μL of water was substituted for cDNA. Reactions were run for 40 cycles in an ABI Prism 7900 Sequence Detection System (Applied Biosystems). Threshold cycle (C_t) values were obtained for each reaction and compared to those of a standard curve generated from decidual T cell cDNA to determine the relative abundance of the amplified product. All values were normalized to relative abundance values of β-actin products. The identity of PD-1 isoform products was confirmed by sequence analysis.

Jar cell transfectants

The full length coding sequence for B7-H1 (Dong et al. 1999) was subcloned into a pcDNA3.1 expression vector (Invitrogen) containing a neomycin resistance cassette. Expression vectors with or without coding sequences were then stably transfected into Jar choriocarcinoma cells, which lack endogenous B7-H1 expression (Petroff et al. 2003). Jar/V (vector only) and Jar/B7 (B7-H1 expressing) cell lines were maintained under selective pressure 300 μg/mL neomycin in RPMI containing 10% FBS and antibiotics. To ensure consistency of the presence (Jar/B7) and absence (Jar/V) of B7-H1 expression, both cell lines were routinely evaluated by flow

cytometry using a PE-conjugated anti-B7-H1 antibody (clone MIH1) and PE-conjugated Mouse IgG1κ isotype control (eBioscience).

Trophoblast/lympocyte co-culture

Transfected Jar cells were irradiated (2000 rad) and plated overnight in neomycin-containing selective medium at a density of 5,000 cells/well in a 48 well tissue culture plate. The following day, Jar cells were washed with fresh culture media to remove neomycin from the culture wells, and sorted $CD4^{+}$ or $CD8^{bright}$ decidual T cells were plated at a density of 275,000 cells/well in 500 μL of culture media containing 3 μg/mL phytohemagglutinin (PHA) (Sigma-Aldrich). Co-cultures were placed at 37ºC for 72 hours, after which cell culture supernatants were collected and stored at -80°C. To evaluate the effect of B7-H1 on cell proliferation in separate co-cultures, sorted decidual T cells were labeled with 5μM of carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen) according to the manufacturers instructions and then placed into culture with transfected Jar cells as described above.

Analysis of cytokine production

Analysis of IFN-γ, TNF-α, interleukin (IL)-2 and interleukin (IL)-10 concentrations in cell culture supernatants was performed using a multiplex Beadlyte® Human Multi-Cytokine Detection System (Upstate/Millipore, Billerica, MA) according to the manufacturer's instructions. Samples were processed on a

Luminex® 200 instrument and data were analyzed using Luminex IS software version 2.3 (Upstate).

Evaluation of apoptosis and proliferation

In experiments evaluating apoptosis or cell proliferation after the 72 hour co-culture, decidual T cells in the supernatant were collected and labeled with APC-conjugated anti-human CD3 antibody (eBioscience), and LIVE/DEAD® Fixable Violet Dead Cell Stain Kit (Invitrogen). For samples to be analyzed for apoptosis, cells were also stained with PE-conjugated Annexin V (BD Pharmingen). All samples were processed on a BD LSRII instrument and analyzed using FACS Diva software (BD Pharmingen). For apoptosis evaluation, $CD3^+$ cells with a $violet^{bright}$ and $Annexin^+$ phenotype were designated as apoptotic. In separate experiments, cell proliferation was quantified by evaluating the CFSE profiles of $CD3^+$ cells.

Statistics

Statistical significance was determined using either a one-way analysis of variance (ANOVA) with a Student-Newman-Keuls post hoc analysis (cell counts from immunohistochemistry) or two-way ANOVA with either a Fisher's LSD (flow cytometry) or Tukey post hoc analysis (semi-quantitative real-time PCR, multiplex cytokine analysis). Differences were considered significant at $\alpha = 0.05$.

D. Results

PD-1 is expressed on decidual T cells throughout pregnancy

Because of the high level of B7-H1 expression on human fetal trophoblast cells, we examined whether $CD3^+$ T cells at the maternal-fetal interface express its cognate receptor, PD-1. Using double fluorescence immunohistochemistry, we examined both PD-1 and CD3 expression in normal secretory phase endometrium (n = 6), first trimester decidua, and term extraplacental membranes and basal plate (n=7 per group) (Fig. 2.1). We then enumerated cells positive for one or both markers (Fig. 2.2), noting that the signal intensity for both CD3 and PD-1 varied within individual sections in all tissues. In agreement with other observations (Haller et al. 1993; Morii et al. 1993; Mincheva-Nilsson et al. 1994; Vassiliadou et al. 1996; Sindram-Trujillo et al. 2004) $CD3^+$ cells were present in secretory phase endometrium as well as first trimester and term decidua (Fig 2.1 A-E). While the number of $CD3^+$ T cells per image did vary (Fig 2.1), the overall number of $CD3^+$ cells counted did not differ significantly between tissue groups ($p = 0.849$).

When examining PD-1 expression, we next observed that $\geq 96\%$ of PD-1^+ cells were also $CD3^+$ (Fig. 1), suggesting that the majority of the PD-1-expressing cells were T cells. Further analysis revealed that the percentage of $CD3^+$ cells expressing PD-1 was low in non-pregnant secretory endometrium (Fig 2.1 A, Fig 2.2), but increased significantly in first trimester decidua (Fig. 2.1 B, Fig. 2.2), with no apparent difference between first trimester decidua parietalis and basalis. The abundance of PD-1^+CD3^+ cells remained elevated in late gestation (Fig. 2.1 C-E; Fig. 2.2), and was significantly higher in term decidua associated with fetal membranes as

compared to first trimester decidua (Fig. 2.2). We also examined PD-1 expression on peripheral blood T cells from patients in first trimester (n = 4,) and term pregnancy (n = 8) by flow cytometry; the proportion of PD-1^+ T cells was low in first trimester blood and did not change significantly in late gestation ($P > 0.05$; Fig. 3 and data not shown). In summary, these data, suggest that PD-1 expression is induced locally on decidual T cells during pregnancy.

PD-1-expressing T cell subpopulations are specifically localized in the decidua

We further examined whether PD-1 expression was specifically induced at the maternal-fetal interface by evaluating decidual and peripheral blood samples from the same patients at term pregnancy. Additionally we examined which T cell subpopulations express PD-1. Figure 2.3 shows the flow cytometric analysis of $CD8^{bright}$ (n=6), $CD4^+$ (n=8), and CD4/CD25/$FoxP3^+$ (T_{Reg}) (n=8) lymphocyte populations in peripheral blood just before normal term C-section delivery and autologous term decidual tissue collected after delivery. Each of these populations was easily identifiable in both peripheral blood and decidua; as expected (Tilburgs et al. 2006), T_{Reg} were more abundant in decidual tissue than in peripheral blood (6.65% vs 0.64% respectively, $P = 0.007$). Further analysis by flow cytometry consistently revealed that cell surface-associated PD-1 was more abundant on decidual T cells than on peripheral blood T cells from the same patient; this was true for each of the three populations examined (Fig. 2.3 A-C). Figure 2.3 D shows the

combined data for all patients, and statistical analysis revealed that differences in PD-1-expressing T cells between decidua and blood were highly significant.

To confirm the specificity of our findings of PD-1 expression on T cells, we stained decidual and peripheral blood samples with antibodies against CD3, CD4, CD8 and a second clone of an anti-PD-1 antibody (clone MIH4, eBioscience). Through flow cytometric analysis, we observed a similar preferential expression of PD-1 in decidual $CD3^+/CD4^+$ and $CD3^+/CD8^{bright}$ T cell populations when compared to peripheral blood (n=2, data not shown). Also, to verify that decidual cell populations examined were resident within the decidua, we routinely labeled cells with an anti-human CD19 antibody (clone HIB19, eBioscience) using peripheral blood as a positive control. As expected (Mincheva-Nilsson et al. 1994), purified decidual lymphocytes completely lacked $CD19^+$ B cells, confirming little or no peripheral blood contamination of decidual tissues (data not shown).

PD-1 mRNA isoforms are preferentially expressed in decidual T cell subpopulations

Because these data suggested that decidual T cells preferentially express the PD-1 surface protein (Fig. 2.2,2.3), we next examined whether term decidual and peripheral blood T cells express different repertoires of PD-1 mRNA isoforms. Conventional RT-PCR analysis of mRNA from peripheral blood and decidual $CD4^+$ or $CD8^+$ T cell subpopulations showed two amplicons, each of which were sequenced and identified as the full length (FL PD-1) and soluble (PD-1 Δ Ex 3) isoforms of the PD-1 mRNA transcript (Fig. 2.4A). In separate experiments using semi-quntatitive

real-time PCR and isoform specific primers, we found that the full length PD-1 mRNA transcript was more abundantly expressed in decidual T cells when compared to autologous peripheral blood T cells at term pregnancy (n = 5) (Fig. 2.4 B). This was true of both $CD4^+$ and $CD8^{bright}$ T cell subpopulations, and was consistent with our observations for PD-1 surface expression in decidual T lymphocytes (Fig. 2.3). The Δ Ex3 PD-1 isoform was also expressed more abundantly in decidual $CD4^+$ and $CD8^{bright}$ T cells. Three other PD-1 mRNA isoforms were detectable by real-time PCR in both peripheral blood and decidual lymphocytes, and although the sequence of each isoform was verified (Nielsen et al. 2005), the abundance of all transcripts was too low to confidently assess quantity (data not shown).

Effects of B7-H1 on term decidual T cells

Because B7-H1 is abundantly expressed on placental trophoblast cells (Petroff et al. 2003; Petroff et al. 2005b) and PD-1 is preferentially expressed by decidual T cells at term pregnancy (Fig. 2.2-2.4), we examined the effects of B7-H1-expressing cells on term decidual T lymphocyte apoptosis, proliferation and cytokine production. We established a co-culture system with B7-H1-transfected Jar choriocarcinoma cells and decidual $CD4^+$ and $CD8^{bright}$ T cells. B7-H1 expression was routinely monitored on control transfected (Jar/V) or B7-H1-transfected (Jar/B7) cells by flow cytometry and found to be highly expressed on the surface of Jar/B7 cells, as it is on primary chorionic trophoblast cells (n=5, Fig. 2.5 A) (Petroff et al. 2003).

When evaluating the influence of B7-H1 on decidual T cell death, Jar/B7 co-cultures, when compared to Jar/V, did not increase the percentage of either $CD4^+$ or $CD8^{bright}$ T cells undergoing apoptosis (n=5, Fig. 2.5 B). Further, decidual T cell proliferation, quantified by CFSE analysis, was similar between Jar/V and Jar/B7 co-cultures (n=2, data not shown). Examination of cytokine production revealed that decidual T cell secretion of IFN-γ and IL-10 was increased in the presence of Jar/V (n=6, Fig. 2.6). However, B7-H1-transfected Jar cells reversed the stimulatory effect on IFN-γ and TNF-α in $CD4^+$ T lymphocytes while having no significant effect on the production of IL-10 from these cells (Fig. 2.6). In contrast, Jar/B7 cells did not significantly influence the secretion of any cytokines from CD8+ T cells (Fig. 2.6). Interleukin(IL)-2 secretion was also evaluated, but its concentration was below detectable limits in all co-cultures examined, suggesting that decidual T cells could not be induced to secrete this cytokine. Additionally, Jar/V or Jar/B7 cells cultured without T cells did not secrete any detectable IFN-γ, TNF-α, IL-10, or IL-2 (n=4, data not shown).

E. Discussion

Through these studies we have identified a novel location and potential function of the CD28 family molecule, PD-1, at the maternal-fetal interface. While we found PD-1 to be expressed on a small percentage of T cells in the secretory phase endometrium, there was a significant increase in PD-1^+ $CD3^+$ cells in first trimester and term decidua (Fig. 2.1, 2.2). This induction of PD-1 expression was specific to

the decidua, as autologous term pregnancy decidual samples contained significantly higher percentages of PD-1^+ T cells than their peripheral blood counterparts (Fig. 2.3). Indeed, peripheral blood samples from patients in both first trimester and term pregnancy contained low percentages of PD-1^+ T cells (Fig. 2.3), similar to non-pregnant peripheral blood (Hatachi et al. 2003). Taken together these data suggest that pregnancy-specific factors up-regulate the expression of this receptor preferentially at the maternal-fetal interface. PD-1 is known to be induced via mechanisms including CD3 stimulation, antigen recognition and hormones (Agata et al. 1996; Isogawa et al. 2005; Polanczyk et al. 2006). Surface PD-1 can be up-regulated on T cells by specific antigen stimulation (Isogawa et al. 2005), thus exposure to fetal antigen may induce PD-1 expression in decidual T cells during pregnancy. Others have reported that PD-1 expression may be under hormonal regulation (Polanczyk et al. 2006), therefore elevated hormone levels throughout gestation could also influence PD-1 expression in the gravid uterus.

The preferential expression of PD-1 receptor by decidual T cells in both first trimester and term pregnancy complements our previous studies in which we observed expression of the its ligand, B7-H1, on trophoblast cell populations throughout gestation and hypothesized that placental B7-H1 serves as a molecular shield against maternal alloresponses to the fetus (Petroff et al. 2003; Petroff et al. 2005b). The expression of PD-1 on both decidual $CD8^{bright}$ and $CD4^+$ T cells in first trimester and term pregnancy samples supports the hypothesis that these cells could be targets for trophoblast B7-H1-mediated regulation during pregnancy. Indeed, in

vitro and in vivo studies have shown B7-H1:PD-1-mediated inhibition of allogeneic immune responses and amelioration of allograft rejection, including anti-fetal responses (Ozkaynak et al. 2002; Latchman et al. 2004; Guleria et al. 2005).

Along with $CD8^{bright}$ and $CD4^{+}$ T cells, PD-1 was preferentially expressed on decidual T_{Reg} (Fig. 3B). PD-1 expression by T_{Reg} has been previously documented (Raimondi et al. 2006), and two recent studies have suggested that B7-H1 expression on parenchymal cells may induce differentiation of naïve $CD4^{+}$ T cells into T_{Reg} outside of the thymus (Krupnick et al. 2005; Beswick et al. 2007). T_{Reg} are a significant population in the decidua, are associated with the success of human pregnancy, and are required for allogenic murine pregnancies (Aluvihare et al. 2004; Heikkinen et al. 2004; Sasaki et al. 2004; Darrasse-Jeze et al. 2006; Tilburgs et al. 2006). While the function of PD-1 on T_{Reg} is unclear, its presence could be a reflection of their differentiation, and raise the interesting possibility that trophoblast B7-H1 may induce naïve $CD4^{+}$ T cells to differentiate into T_{Reg} in order to promote immune tolerance of the fetal allograft.

The preferential expression of PD-1 by decidual $CD4^{+}$ and $CD8^{bright}$ T cells was confirmed at the mRNA level where the full length PD-1 transcript was upregulated in decidual T cells compared to peripheral blood. Interestingly, the PD-1 Δ Ex3 transcript was also more abundantly expressed in decidual T cells (Fig. 2.4 B). Because the PD-1 Δ Ex3 transcript encodes a soluble form of the receptor that may prevent T cell inhibition by acting as a decoy (Wan et al. 2006), its upregulation by decidual T cells was somewhat unexpected. While expression of this isoform may be

a safeguard against over-suppression of decidual T cells in order to maintain their physiological activity (Piccinni et al. 1998; Scaife et al. 2006), it will be interesting to examine PD-1 Δ Ex3 in pregnancy pathologies such as recurrent spontaneous abortion where endometrial/decidual T cells may be activated (Maruyama et al. 1992; Vassiliadou et al. 1999; Quack et al. 2001).

In regard to the function of PD-1 during pregnancy, we also examined whether its ligand, B7-H1, could alter cytokine secretion or apoptosis of decidual T cells as a potential means of modifying maternal immune activation (Freeman et al. 2000; Dong et al. 2002). In co-culture experiments we observed that the Jar/V cells caused increased cytokine production in the decidual T cells (Fig. 2.6), which parallels a previous study showing that Jar cells can induce production of IL-10 and TNF-α in uterine natural killer cells (Rieger et al. 2001). We also observed that B7-H1 expression on Jar cells selectively inhibited secretion of IFN-γ and TNF-α in $CD4^+$ but not $CD8^{bright}$ decidual T cells (Fig. 2.6). $CD4^+$ T helper cells control activation of antigen-presenting cells and $CD8^+$ T cells through cytokine secretion, and therefore it may be particularly important to modify the cytokine repertoire of these cells in order to control maternal anti-fetal immune reactions. The lack of effect of B7-H1 on IL-10 production in either cell type (Fig. 2.6) suggests that B7-H1 is causing selective inhibition, rather than a global suppression, of decidual T cytokines. Consistent with this finding, B7-H1 also did not increase the percentage of T cells undergoing apoptosis (Fig. 2.5 B).

A balance of cytokine production appears to be critical in promoting pregnancy. For example, overproduction of IFN-γ and TNF-α is associated with preterm labor and recurrent spontaneous abortions in humans as well as fetal rejection in mice (El-Shazly et al. 2004; Mjihdi et al. 2004; Kim et al. 2005). On the other hand, the same cytokines may also control trophoblast invasion in early pregnancy, and play a role in parturition at term (Pollard et al. 1993; Buonocore et al. 1995; Ashkar et al. 2000; Fukushima et al. 2003; Cohen et al. 2006; Scaife et al. 2006). Anti-inflammatory cytokines also have a critical role at the maternal-fetal interface as a reduction or absence of IL-10 is associated with chronic human pregnancy failure and potentiation of inflammation-induced murine fetal loss (Piccinni et al. 1998; Murphy et al. 2005; Robertson et al. 2007). The B7-H1:PD-1 pathway may selectively modify decidual T cell cytokine secretion in order to maintain a proper immunological environment at certain times during pregnancy while still allowing for appropriate physiological functions throughout gestation.

In conclusion, these studies identify a novel location of the PD-1 receptor at the maternal-fetal interface, highlight potential targets of B7-H1-mediated regulation and demonstrate that the B7-H1:PD-1 pathway is present and functional for modification of the maternal immune system during pregnancy.

Acknowledgments

The authors would like to thank the following individuals: L. Holets and E. Kharatyan for technical assistance, E. Roach for imaging assistance, J. Ballenger

(Dept. of Pathology) for endometrial samples, J. Vu (Dept. of Obstretics and Gynecology) for patient sample collection, and C. Ober, C. Billstrand, M. Gilliam, L. Bennett, and E. Quansah at the University of Chicago for assistance with collection of first trimester decidual samples. Further resources and services were provided by the KUMC Center for Reproductive Sciences (NICHD), the KUMC Center Flow Cytometry Core (NCRR), the KUMC COBRE program in Cell Development and Differentiation (P20RR024214), the Kansas State University COBRE program in Epithelial Function (P20RR017686), the Kansas INBRE (P20RR016475).

F. Figures and Legends

Figure 2.1. PD-1 expression in endometrial and decidual tissues. Double immuno-fluorescent immunohistochemistry of normal secretory endometrial tissues (A), first trimester decidua (B), term basal plate (C) and term extraplacental membranes (D,E) with antibodies against CD3 (green color), PD-1 (red color). (A-E): Photomicrographs are representative images of CD3 and PD-1 immunolabeling for each group of tissues. (F): representative isotype control image from extraplacental membranes. Arrows indicate double positive cells. Bars, 50 μM.

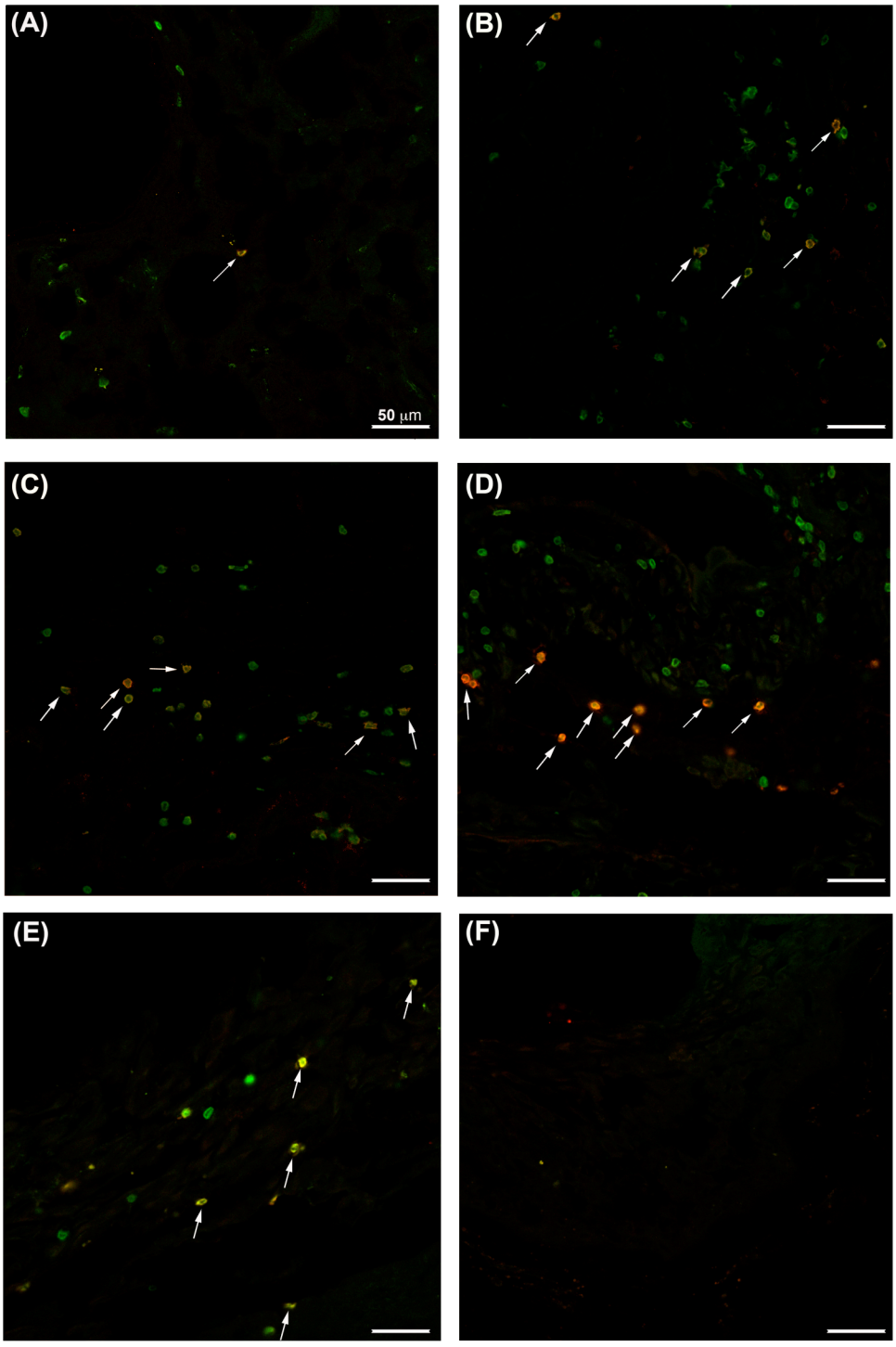

(A)
50 μm
(B)
(C)
(D)
(E)
(F)

Figure 2.2. Quantification of PD-1-expressing T cells in endometrial and decidual tissues. Graph represents mean percentages of $PD\text{-}1^{+}CD3^{+}$ cells in each tissue type (SE, n = 6; FT, TBP, TM, n = 7/group). Bars, SEM. SE, secretory endometrium; FT, first trimester decidua; TBP, term basal plate; TM, term extraplacental membranes. Comparisons for which no *P* value is given were not significantly different ($P > 0.05$).

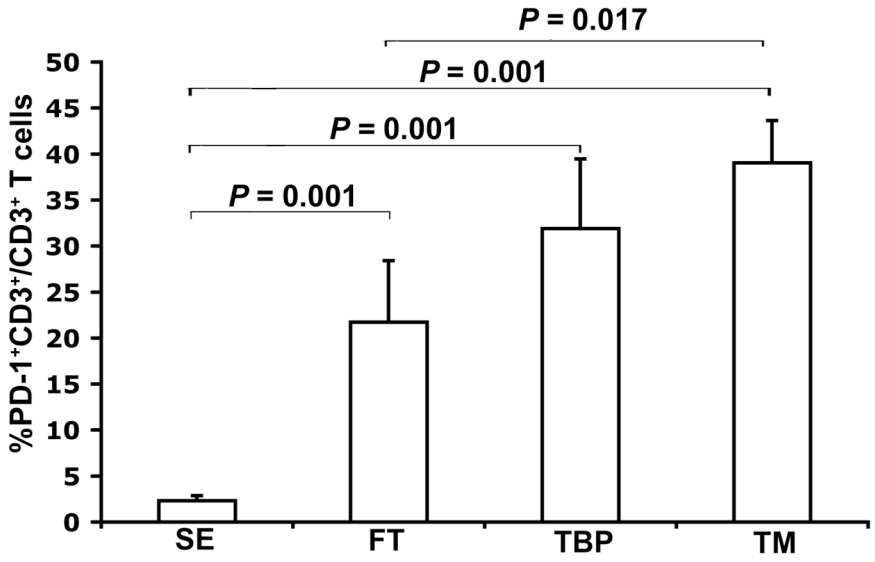

P = 0.017
P = 0.001
P = 0.001
P = 0.001
%PD-1+CD3+/CD3+ T cells
50
45
40
35
30
25
20
15
10
5
0
SE
FT
TBP
TM

Figure 2.3. Flow cytometry of PD-1-expressing T cell subpopulations in term peripheral blood and decidua. Histograms are representative images of flow cytometry in autologous peripheral blood (top panels of A-C) and decidual samples (bottom panels of A-C). Bars represent boundaries of gating for each T cell subpopulation marker (left panels of A-C) and PD-1 (right panels of A-C). Solid lines represent isotype controls for CD4 and CD8 and fluorescence-minus-one controls for PD-1. (A) Left panels: CD8 expression on lymphocytes; right panels: PD-1 expression on $CD8^{Bright}$ populations. (B) Left panels: CD4 expression on lymphocytes; right panels: PD-1 expression on $CD4^+$ population. (C) Upper right quadrant of dot plot represents gated $CD4^+CD25^+FoxP3^+$ population for PD-1 expression analysis. Left panels: CD25 and FoxP3 expression on $CD4^+$ lymphocytes; right panels: PD-1 expression on the $CD4^+CD25^+FoxP3^+$ population. (D) Combined data from a minimum of 6 different patients. Boxes: 10^{th} to 90^{th} percentile values of the data set; solid lines in box: mean value; dotted lines in box: median value; bars: range of values for each data set.

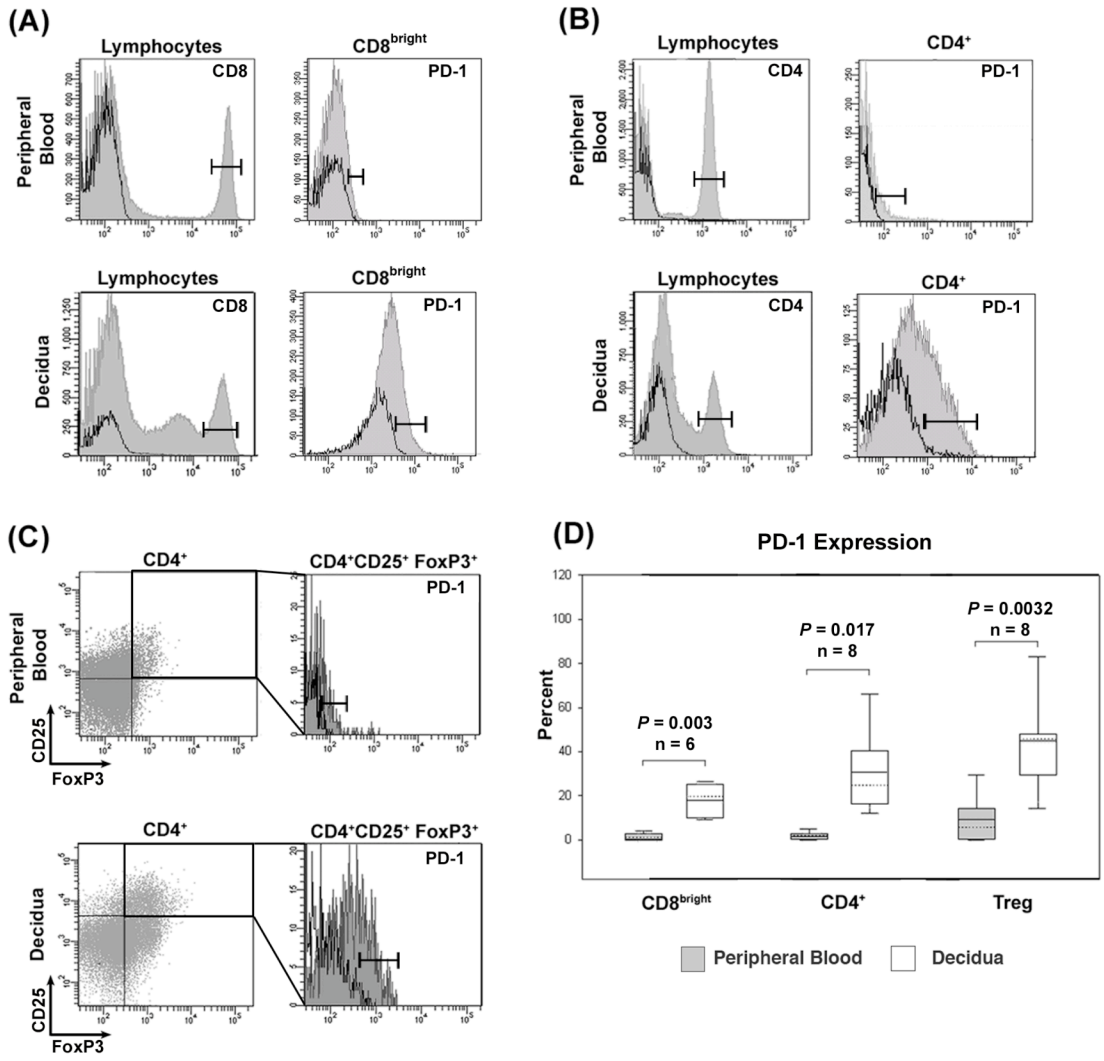

(A)
Lymphocytes
CD8
CD8bright
PD-1
Peripheral Blood
Decidua
(B)
CD4+
CD4
(C)
CD4+
CD4+CD25+ FoxP3+
CD25
FoxP3
(D)
PD-1 Expression
Percent
P = 0.003
n = 6
P = 0.017
n = 8
P = 0.0032
n = 8
CD8bright
CD4+
Treg
Peripheral Blood
Decidua

Figure 2.4. RT-PCR analysis of PD-1 mRNA isoforms in autologous term peripheral blood and decidual T cells. (A) Agarose gel analysis of RT-PCR using PD-1 and b-actin specific primers. PB, Peripheral blood. Dec, Decidua. (B) Graphs represent semi-quantatitive real-time PCR analysis of cDNA (n=5 patients) using PD-1 mRNA isoform-specific primers. Samples were normalized to β-actin expression.

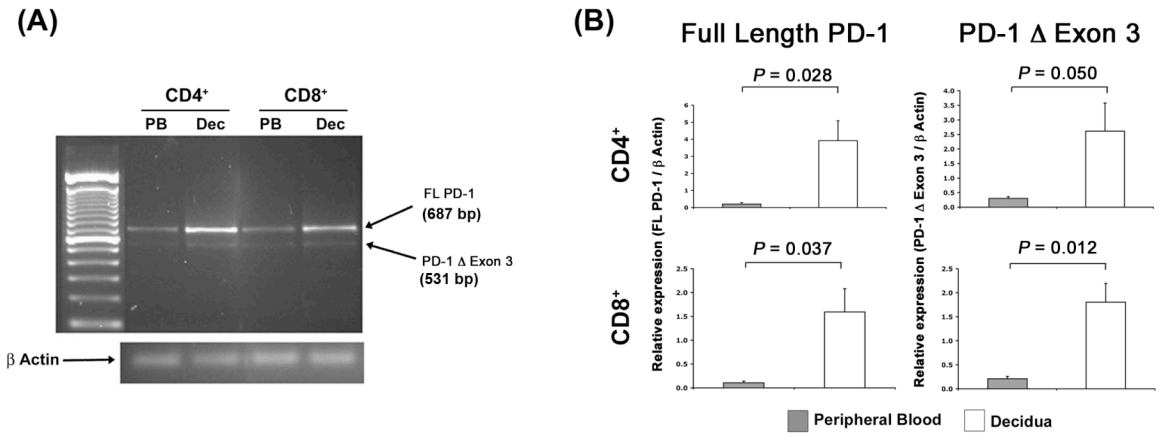

(A)
CD4+
CD8+
PB
Dec
PB
Dec
FL PD-1
(687 bp)
PD-1 Δ Exon 3
(531 bp)
β Actin
(B)
Full Length PD-1
PD-1 Δ Exon 3
P = 0.028
P = 0.050
P = 0.037
P = 0.012
CD4+
CD8+
Relative expression (FL PD-1 / β Actin)
Relative expression (PD-1 Δ Exon 3 / β Actin)
Peripheral Blood
Decidua

Figure 2.5. Analysis of B7-H1-mediated effects on decidual T cell apoptosis. (A) Representative histograms from flow cytometric analysis of B7-H1 expression in control (Jar/V) or B7-H1-transfected (Jar/B7) cells and primary chorionic trohpoblast cells (Primary CTB). (B) Analysis of B7-H1 effects on decidual T cell apoptosis. Graphs represent mean percentages of apoptotic T cells from 6 different patients. Bars, SEM.

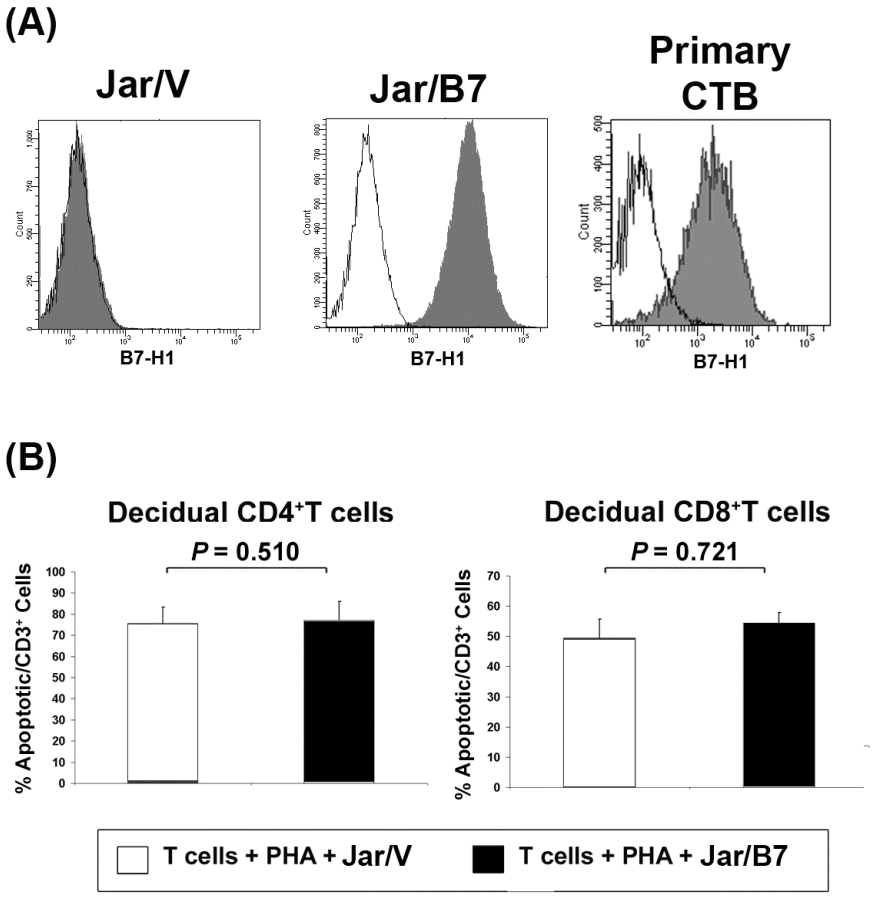
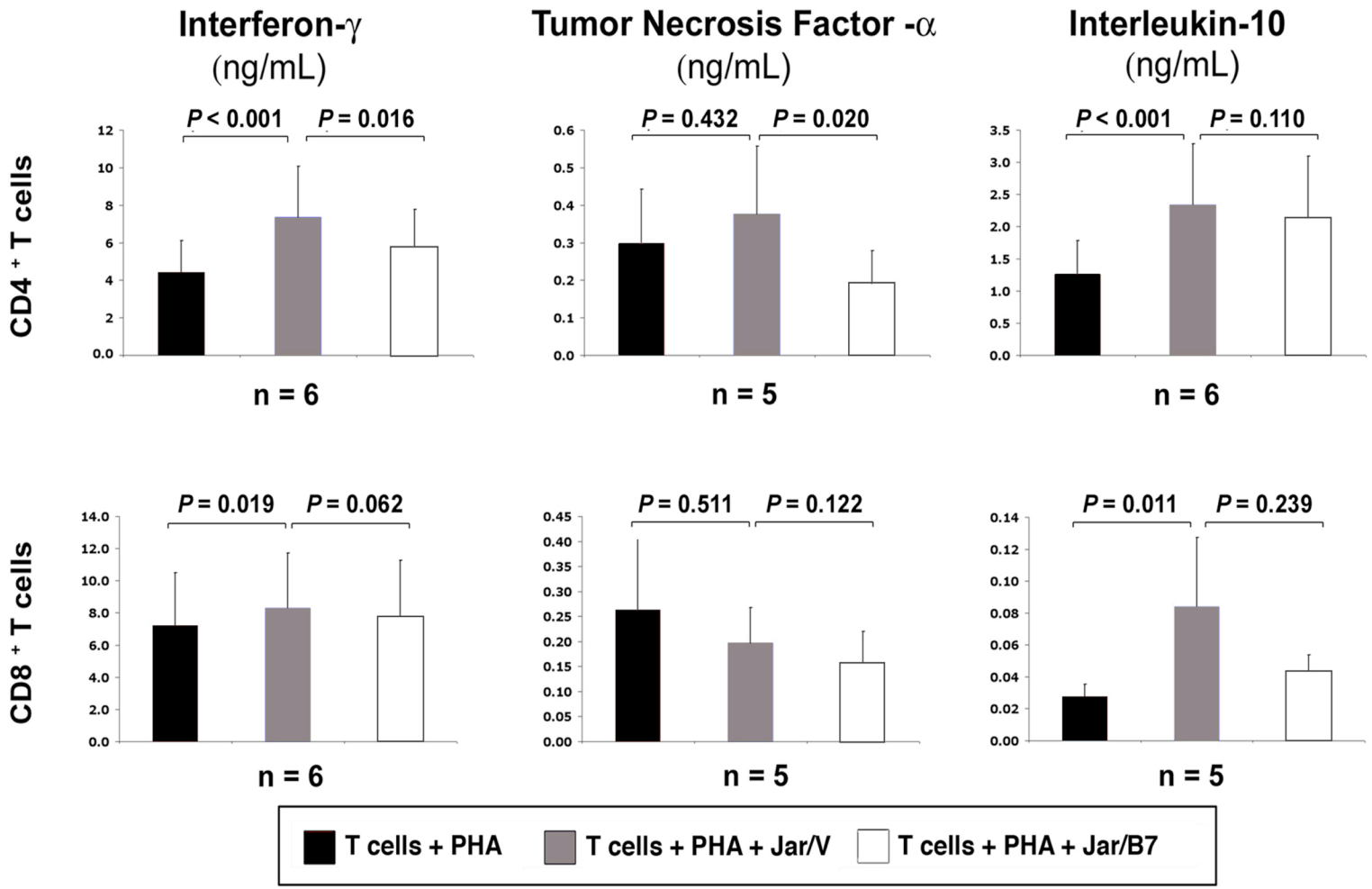

(A)
Jar/V
Jar/B7
Primary CTB
Count
B7-H1
(B)
Decidual CD4+T cells
P = 0.510
Decidual CD8+T cells
P = 0.721
% Apoptotic/CD3+ Cells
T cells + PHA + Jar/V
T cells + PHA + Jar/B7

Figure 2.6. Cytokine assay analysis of B7-H1 mediated effects of decidual T cell cytokine production. Graphs represent mean values of decidual T cell cytokine secretion from a minimum of 5 different patients. Bars: SEM.


Interferon-γ
(ng/mL)
Tumor Necrosis Factor -α
(ng/mL)
Interleukin-10
(ng/mL)
CD4 + T cells
CD8 + T cells
P < 0.001
P = 0.016
P = 0.432
P = 0.020
P < 0.001
P = 0.110
P = 0.019
P = 0.062
P = 0.511
P = 0.122
P = 0.011
P = 0.239
n = 6
n = 5
n = 6
n = 6
n = 5
n = 5
T cells + PHA
T cells + PHA + Jar/V
T cells + PHA + Jar/B7

Chapter III

Human Trophoblast-Mediated Regulatory T cell Differentiation

Taglauer, E.S., Zhao J., Petroff M.G., Human trophoblast mediated expansion of $CD4^{+}CD25^{high}FoxP3^{+}$ regulatory T cells. (*Submitted)*

A. Abstract

Several immunoregulatory mechanisms are in place during pregnancy to protect the semi-allogeneic fetus from the maternal immune system. Of these, the suppression maternal T cell activity by $CD4^+CD25^+$ and $FoxP3^+$ regulatory T cells (T_{reg}) is one of the most potent as they prevent rejection of semi-allogeneic fetuses in murine pregnancy. In humans, T_{reg} are preferentially localized within decidual tissues as compared to peripheral blood, and a reduction in these cells within the decidua is associated with recurrent miscarriage. As the mechanisms responsible for T_{reg} abundance and localization during pregnancy incompletely understood, we asked whether cytotrophoblast cells (CTB) could directly influence expansion of $T_{reg.}$ Peripheral blood naïve $CD4^+$ T cells were co-cultured with CTB followed by flow cytometric analysis of T_{reg} markers. CTB caused a 3-fold induction of the percentage of $CD4^+CD25^{bright}$ $FoxP3^+$ cells ($P < 0.001$, n = 6) after 7-day culture. To test whether a syncytiotrophoblast phenotype altered the expansion of T_{reg}, we also cultured naïve $CD4^+$ T cells with CTB treated with epidermal growth factor (EGF). The percentage of T_{reg} did not differ significantly between co-cultures with untreated and EGF-treated CTB, suggesting that trophoblasts induce T_{reg} expansion regardless of their differentiation status. Re-purified $CD4^+CD25^+$ cells from these cultures were also were capable of suppressing proliferation of autologous $CD4^+$ T cells *in vitro*. These studies raise the possibility that the fetal trophoblast may drive expansion of T_{reg} during pregnancy as a potential means of controlling the maternal immune system.

B. Introduction

Regulatory T cells (T_{reg}), defined by their $CD4^+CD25^+FoxP3^+$ phenotype, are powerful modulators of autologous T cell activity, and are critical in maintaining self-tolerance and preventing autoimmune disease (Sakaguchi 2000; Shevach 2002). These cells suppress T cell activity primarily through contact- dependent mechanisms (Thornton et al. 1998; Sasaki et al. 2004) and can be divided into central and peripheral subclasses based on their developmental origin (Abbas 2003). Central T_{reg} develop within the thymus through a widely accepted mechanism involving T cell receptor (TCR) engagement of self major histocompatability molecules (MHC) and peptides (Jordan et al. 2001; Apostolou et al. 2002; Hsieh et al. 2006). Peripheral T_{reg} mature extra-thymically, through either proliferation of a pre-cursor population or *de novo* differentiation from naïve $CD4^+$ T cells, expanding in peripheral tissues, particularly during pathological conditions involving viral infections and tumorgenesis (Pillai et al. 2007).

Pregnancy is a physiological condition also associated with high numbers of peripheral T_{reg}. These cells localize preferentially to the decidual tissues within the human maternal-fetal interface (Tilburgs et al. 2006) and decidual T_{reg} are significantly reduced in decidual bed biopsies from spontaneously aborted and pre-eclamptic pregnancies (Sasaki et al. 2004; Sasaki et al. 2007). In murine pregnancy, depletion of T_{reg} causes T cell-mediated fetal rejection in allogeneic but not syngeneic fetuses (Aluvihare et al. 2004; Darrasse-Jeze et al. 2006), further confirming the

critical role of these cells in maintaining maternal tolerance to the semi-allogeneic fetus.

The localization of T_{reg} in human pregnancy was recently re-examined (Tilburgs et al. 2008) and $CD4^{+}CD25^{bright}FoxP3^{+}$ cells appear to be less abundant in the peripheral blood of pregnant women compared to non-pregnant controls. The decrease in circulating T_{reg} coupled with a preferential localization of these cells in autologous decidual tissues suggests that decidual T_{reg} are recruited from peripheral blood (Tilburgs et al. 2008). However, the high relative abundance of T_{reg} within the decidua also suggests that other mechanisms may influence their localization within this tissue.

In the current study, we examined whether T_{reg} could also be expanded by factors present within the human maternal-fetal interface. Among the numerous cells within the human placenta and decidua that could contribute to this process, the fetal trophoblast is a strong candidate. These cells express several surface associated and soluble molecules that are known to induce regulatory T cell expansion. For example, B7-H1, a member of the B7 co-stiumlatory family, is highly expressed on numerous trophoblast cell types in the human placenta (Petroff et al. 2003; Petroff et al. 2005b) and may be involved in T_{reg} differentiation in peripheral tissues (Krupnick et al. 2005; Beswick et al. 2007). In addition, the B7-H1 receptor, PD-1 is preferentially expressed on decidual $CD4^{+}$ and T_{reg} populations (Taglauer *et al*, in press). Trophoblast cells also secrete other proteins and hormones that could

influence T_{reg} expansion, including HLA-G, TGF-β and estrogen (Graham et al. 1992; Morales et al. 2003; Pasqualini 2005).

Because trophoblasts express a unique combination of cell surface and soluble factors known to influence T_{reg} proliferation or differentiation, we hypothesized that these cells induce expansion of T_{reg} as a means of enabling immune regulation within the human maternal-fetal interface. To this end, we co-cultured naïve $CD4^+$ T cells with primary cytotrophoblast cells. Following culture, we examined the T cells for expression of T_{reg} markers and evaluated whether they obtained suppressive capabilities.

C. Materials and methods

Tissue collection

All tissue samples were collected in accordance with human subjects protocols approved by the University of Kansas Medical Center Institutional Research Board (HSC #3037). Placental/decidual tissues and peripheral blood were collected from healthy patients on the day of elective or repeat Caesarian (C)-section; peripheral blood was also collected from healthy male donors, ages 25-40.

Lymphocyte and trophoblast isolation

Term decidual lymphocytes were obtained by peeling the fused decidua capsularis/parietalis (decidua) and chorion from the amnion. Decidual tissues were then dissociated using 200 U/mL type IV collagenase, 1 mg/mL type1-S

hyaluronidase, and 150 µg/mL type IV DNAse in a shaking 37°C water bath (Vince et al. 1990). All enzymes were purchased from Sigma-Aldrich. Dissociated cell suspensions were layered over Histopaque (Sigma-Aldrich) and centrifuged. The mononuclear cell fraction was collected and counted to assess cell viability and yield. For preparation of term autologous peripheral blood lymphocytes, samples were diluted in PBS, layered over Histopaque, collected and counted to assess cell viability and yield.

For isolation of primary cytotrophoblast cells, 30-40g villous tissue was dissected, rinsed, and enzymatically digested as described previously (Petroff et al. 2006b). Briefly, minced placental villi were dissociated in three 30-minute stages of using trypsin and DNase. The cell suspension was then layered over a 5-70% stepwise Percoll (Sigma-Aldrich) gradient and centrifuged. The trophoblast fraction was collected and subjected to immunopurification by negative selection over magnetic bead columns coated with anti-HLA class I antibody (W6/32; BioExpress, West Lebanon, NH). Cells were routinely greater than 95% pure as assessed by immunocytochemical staining for cytokeratin-7 (DAKO Corp., Carpinteria, CA).

Co-culture of naïve T cells and primary cytotrophoblasts

For co-culture experiments, peripheral blood naïve $CD4^+$ T cells were isolated by negative selection using a magnetic cell separation kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. Purified cytotrophoblast cells (CTB) were seeded in duplicate into 48 well plates at a density of 5.25×10^5

cells/well in IMDM medium (Sigma-Aldrich, St. Louis, MO) containing 10% FBS and antibiotics. The following day, dead cells were removed by washing with media, and freshly isolated naïve $CD4^+$ T cells were plated at a density of 2.1 x 10^6 cells/well in 500 μL of culture media. Co-cultures were placed at 37°C for 7 days, with fresh medium added every 48 hours (Fig. 3.1A,B). In some experiments, cytotrophoblasts were pre-treated with 10ng/mL epidermal growth factor (EGF) (PeproTech, Rocky Hill, NJ) for 96 hours before addition of naïve $CD4^+$ T cells, and fresh EGF-containing medium was added every 48 hours (Fig. 3.1A). Following culture, cells were collected and analyzed by flow cytometry.

Cell proliferation suppression assay

After seven days in cell culture, $CD4^+CD25^+$ T cells were purified from the co-cultures by negative selection (CD4) and positive selection (CD25) using a magnetic cell separation kit (Miltenyi Biotech). These cells were added to CFSE-labelled autologous naïve $CD4^+$ T cells at a 1:1 or 1:10 ratio. Together, the cells were cultured for 5 days in uncoated plates or plates coated with anti-CD3 (1 μg/mL OKT-3, eBiocience, San Diego, CA) and CD28 (0.05 μg/mLclone CD28.2, eBioscience) antibodies (Fig. 1B).

Flow cytometry

For analysis of T_{reg} in either primary decidual/peripheral blood lymphocytes or CTB co-cultures, cells were labeled for specific markers using the following anti-

human antibodies: CD4-fluorescein isothiocyanate (FITC) (clone RPA-T4), CD25-allophycocyanin (APC) (clone BC96), and FoxP3-PE (clone PCH101). The isotype control antibodies for this staining included fluorophore-conjugated mouse IgG1 isotype controls CD4 and CD25 and PE-conjugated rat IgG2a control for FoxP3. The purity of naïve $CD4^+$ T cell isolations for CTB-co-cultures and T_{reg} suppression assays was evaluated by labeling cells with antibodies against CD4-FITC (clone RPA-T4) and CD45RA-PE (clone HI100), or with corresponding isotype controls FITC-mouse IgG1-FITC and IgG2b-PE. For examination of proliferation within T_{reg} suppression assays, cells were labeled with anti-human CD3-APC (clone UCHT-1) and CFSE profiles of $CD3^+$ responder cells were analyzed. All antibodies were purchased from eBioscience. Cells from each experiment were labeled with LIVE/DEAD® Fixable Violet Dead Cell Stain Kit (Invitrogen). A minimum of 20,000 violet-dim lymphocytes were gated based on forward and side scatter characteristics and the data were collected for each desired population. Samples were processed on a BD LSRII instrument and analyzed using BD FACSDiva™ software (BD Pharmingen, San Jose, CA).

Statistics

Statistical significance was determined using two-way analysis of variance (ANOVA) with either a Fisher's LSD (peripheral blood and decidual T_{reg} flow cytometry) or Student-Newman-Keuls (CTB/naïve T cell co-cultures) post hoc analysis. Differences were considered significant at $\alpha = 0.05$.

D. Results

We first examined the distribution of T_{reg} in the peripheral blood and decidual tissues of pregnant women. Autologous peripheral blood and decidual lymphocytes were collected from patients at term pregnancy and examined for expression of CD4, CD25, and FoxP3 via flow cytometry (Fig. 3.2). We found that decidual $CD4^+$ lymphocytes contained a higher percentage of $CD25^+FoxP3^+$ cells as compared to autologous peripheral blood samples ($P = 0.007$; Fig. 3.2 A,B). These results are consistent with previous reports (Sasaki et al. 2004; Tilburgs et al. 2006; Tilburgs et al. 2008) and suggest a preferential localization of T_{reg} to the maternal-fetal interface in pregnant women.

We next asked whether trophoblast cells could cause expansion of T_{reg}. Peripheral blood naïve $CD4^+$ T lymphocytes were placed into culture with purified primary cytotrophoblast cells (CTB) for seven days followed by flow cytometric analysis of CD4, CD25 and FoxP3 expression (Fig. 3.1 A,B). The purity of naïve $CD4^+$ T cells was monitored by flow cytrometry and averaged 96.5% ± 0.5 SEM $CD4^+CD45RA^+$ cells/total lymphocytes (Fig. 3.1 B). Before co-culture, $CD4^+CD25^{bright}FoxP3^+$ cells were found in very low abundance among purified naïve $CD4^+$ T cells and following culture in medium alone, the percentage of these cells was unaltered ($P = 0.982$) (Fig. 3.3 A,B). However, when naïve T cells were cultured with CTB there was a dramatic increase in the abundance of T_{reg} (Fig. 3.3 A), which

was highly significant in combined data from 6 different patients ($P < 0.001$; Fig. 3B).

Our results suggest that CTB can expand T_{reg} *in vitro*. However, in the placenta, syncytiotrophoblast cells are anatomically closer to the leukocytes in the maternal blood space and phenotypically distinct from CTB (Kaufmann 1982; Benirschke 1994; Georgiades et al. 2002). Therefore we next examined whether syncytiotrophoblast cells could also influence expansion of T_{reg}. To induce the syncytiotrophoblast phenotype, we pre-treated primary CTB with epidermal growth factor (EGF) for 96 hours prior to culture (Morrish et al. 1987). Naïve $CD4^+$ T cells were placed into culture with EGF-treated CTB and EGF was maintained in culture conditions throughout the seven day incubation period (Fig. 3.1 A). Culture in EGF-containing medium alone did not induce a T_{reg} phenotype (Fig 3.3 A). In addition, while abundance of T_{reg} increased in the presence of EGF-treated CTB (Fig. 3.3 A) the percentage of T_{reg} was not significantly different from cultures with undifferentiated CTB ($P = 0.083$; Fig. 3.3 B).

An additional readout of a T_{reg} phenotype is the capability to suppress proliferation of other T cells. We therefore we examined whether CTB-induced could suppress proliferation of $CD4^+$ T lymphocytes. CFSE-labeled naïve $CD4^+$ T cells (responders), were placed into culture with decreasing ratios of $CD4^+$ $CD25^+$ cells from CTB co-cultures (CTB T_{reg}), and stimulated with antibodies against CD3 and CD28 (Fig. 3.1 B). After 5 days, responder cells had undergone up to 5 cell divisions, while unstimulated cells had not proliferated (Fig. 3.4 A). Addition of

CTB-T_{reg} to responder cells resulted in a dose-dependent reduction in proliferation as assessed by evaluation of CFSE profiles (Fig. 3.4 A,B).

E. Discussion

The objective of the current study was to examine the mechanisms responsible for the expansion of T_{reg} in pregnancy. Consistent with previous studies (Tilburgs et al. 2006), we observed that T_{reg} were more abundant among decidual lymphocytes compared to autologous peripheral blood samples (Fig. 3.2). In addition, primary CTB induced expansion of $CD4^+CD25^{high}FoxP3^+$ cells (Fig. 3.3). Purified $CD4^+$ $CD25^+$ T cells from CTB co-cultures suppressed the proliferation of autologous $CD4^+$ T cells responder cells at 1:1 ratio (Fig. 3.4), an effect that was lost at a ratio of 1:10 (T_{reg}:responder). This range is similar to suppressive capacity of human peripheral blood T_{reg} *in vitro* (Tsaknaridis et al. 2003) and suggests that CTB-induced T_{reg} inhibit T cell proliferation in a dose-dependent manner.

Trophoblast-mediated T_{reg} expansion was unaltered by their differentiation status (Fig. 3.3). CTB differentiate to become sTB and upon differentiation these cells upregulate production of hormones including estrogen and progesterone (Simpson et al. 1981; Pepe et al. 1999; Jiang et al. 2005). Like CTB, sTB produce soluble HLA-G and TGF-β (Graham et al. 1992; Morales et al. 2003) and sTB express increased levels of cell surface B7-H1 compared to CTB (Petroff et al. 2003). EGF treatment of CTB in vitro mimics sTB differentiation, recapitulating their syncytial phenotype, hormone production and increased levels of B7-H1 expression (Morrish et al. 1987;

Petroff et al. 2003). Thus in our co-cultures both undifferentiated and EGF-treated CTB express several molecules that may directly influence T_{reg} induction.

Of these, B7-H1 is a strong candidate as its presence on intestinal epithelial cells causes differentiation of human naïve $CD4^+$ T cells into T_{reg} (Beswick et al. 2007). Human decidual $CD4^+$ and $CD4^+CD25^+FoxP3^+$ T cells preferentially express the B7-H1 receptor, PD-1, compared to their counterparts in peripheral blood (Taglauer *et al*, in press). While an EGF-mediated increase in B7-H1 expression on CTB did not cause a significant change in the abundance of T_{reg} (Fig. 3.3), B7-H1 is still expressed by undifferentiated CTB (Petroff et al. 2003). A threshold level of B7-H1 signaling within CTB co-cultures could be sufficient to drive T_{reg} expansion and B7-H1 signaling effects may have reached a plateau within EGF-treated CTB co-cultures, limiting further induction of T_{reg}.

In addition to B7-H1, both CTB and sTB express HLA-G and TGF-β which are also postulated to induce T_{reg} expansion (Graham et al. 1992; Morales et al. 2003). Soluble HLA-G may mediate human bone marrow-derived mesenchmal stem cell induction of T_{reg} (Selmani et al. 2008). However, other evidence suggests that this class Ib molecule does not induce $CD4^+CD25^+FoxP3^+$ T_{reg} (Bahri et al. 2006; Naji et al. 2007), and may instead preferentially induce other regulatory T cell subtypes with a $CD3^+CD4^{lo}$ and $CD3^+$ $CD8^{lo}$ phenotype (Le Rond et al. 2006; Naji et al. 2007). Thus, direct testing of HLA-G within our culture conditions would be necessary to reconcile these data and determine its role in CTB-mediated T_{reg} expansion. The anti-inflammatory cytokine TGF-β may also induce T_{reg} differentiation from TCR

stimulated naïve $CD4^+$ T cells, although this signal may require concomitant signaling through retinoic acid (Mucida et al. 2007).

Trophoblast production of steroid hormones may also influence T_{reg} expansion. Estrogen treatment can cause T_{reg} expansion in murine lymphoid organs and stimulate T_{reg} proliferation *in vitro*; however it is unclear whether concomitant T cell receptor stimulation is required for these effects (Polanczyk et al. 2005; Prieto et al. 2006; Tai et al. 2008). While estrogen is a possible candidate for trophoblast-mediated T_{reg} expansion within the placenta, it seems unlikely that it accounts for the effects we are observing *in vitro,* since both CTB and sTB lack the proper enzymes to independently synthesize estrogen (Pasqualini 2005). Trophoblasts can autonomously convert cholesterol into progesterone (Pasqualini 2005), although the direct role of this hormone in T_{reg} induction remains unclear. In mice, exogenous delivery of progesterone either alone or in conjunction with estradiol did not cause expansion of T_{reg} in secondary lymph organs (Zhao et al. 2007). However, progesterone causes promotion of a Th2 cytokine profile, some of which could favor T_{reg} induction (Arck et al. 2007).

As one or more of these trophoblast expressed molecules could be involved in T_{reg} induction, further analysis utilizing various blocking reagents or recombinant proteins will be necessary to elucidate the molecular mechanisms at work within in our co-cultures. In addition, it is unclear from the current results whether the trophoblast-mediated expansion of T_{reg} is due to proliferation of a pre-cursor population or *de novo* differentiation. Although differentiation from naïve precursors

may be more likely scenario for human peripheral Treg in disease states (Pillai et al. 2007), pregnancy-induced T_{reg} expansion may be through different mechanisms. Indeed, first trimester trophoblast cells cause proliferation of a $CD8^+$ regulatory T cell population (Shao et al. 2005).

Whether through proliferation or differentiation, CTB-induced T_{reg} expansion likely occurs in the absence of TCR stimulation, as MHC class II proteins are not expressed on human trophoblast cells (Murphy et al. 1998). While previous reports propose a requirement for TCR stimulation during central T_{reg} induction (Jordan et al. 2001; Apostolou et al. 2002; Hsieh et al. 2006), others suggest these cells may develop independently of MHC:TCR interactions (Haxhinasto et al. 2007). In addition, during murine pregnancy, peripheral T_{reg} expand in females carrying either syngeneic and or semi-allogeneic fetuses, suggesting that T_{reg} induction can occur through mechanisms independent of fetal antigen and TCR stimulation (Aluvihare et al. 2004). However, T_{reg} expressing the chemokine receptor CCR5, which have a stronger suppressive phenotype than $CCR5^-$ T_{reg}, preferentially accumulate in the gravid uterus of allogeneic pregnancy. Further, these cells exclusively confer maternal tolerance to semi-allogeneic fetuses (Kallikourdis et al. 2007). Thus, comparative analysis of the suppressive capabilities of T_{reg} induced by CTB and those differentiated through other cellular interactions may be informative.

These studies confirm the preferential localization of maternal T_{reg} within decidual tissues and place trophoblast cells among the many factors that may influence T_{reg} expansion. In addition, our co-culture model provides a unique method

to study peripheral T_{reg} induction in the absence of TCR:MHC interactions. Finally, these results raise the interesting possibility that fetal tissues may influence T_{reg} expansion during pregnancy as a means of self-protection against potentially harmful maternal immune activity.

F. Figures and Legends

Figure 3.1. Experimental model. (A) Timeline for co-culture of undifferentiated and EGF-differentiated CTB:naïve T cell co-cultures. *, treatment with either medium or EGF. (B) Schematic diagram of CTB and naïve $CD4^+$ T cells co-culture. Scatter plot is representative of naïve $CD4^+$ T cell purity as assessed through flow cytometry for CD4 and CD45 expression. (C) Schematic diagram of assay to evaluate Treg suppression of autologous CFSE-labeled $CD4^+$ proliferation following stimulation with anti CD3/CD28 plate-bound antibodies.

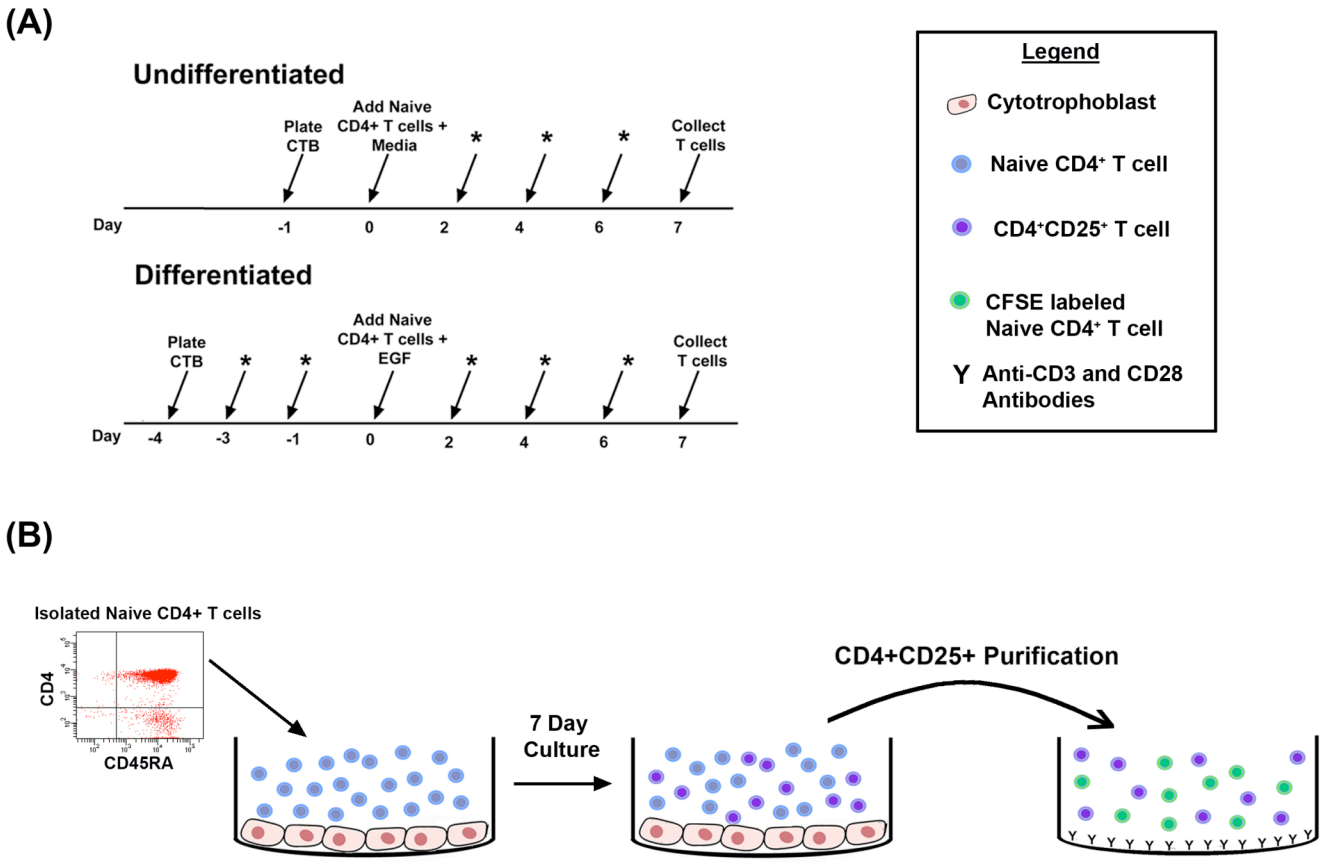
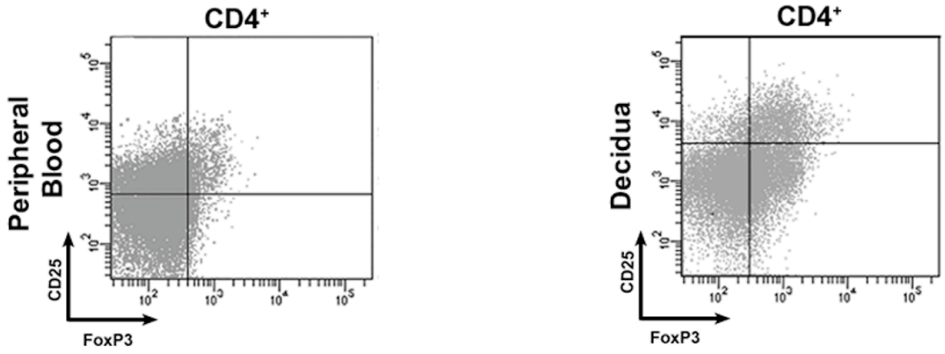

(A)
Undifferentiated
Plate CTB
Add Naive CD4+ T cells + Media
Collect T cells
Day
-1
0
2
4
6
7
Differentiated
Plate CTB
Add Naive CD4+ T cells + EGF
Collect T cells
Day
-4
-3
-1
0
2
4
6
7
Legend
Cytotrophoblast
Naive CD4+ T cell
CD4+CD25+ T cell
CFSE labeled Naive CD4+ T cell
Anti-CD3 and CD28 Antibodies
(B)
Isolated Naive CD4+ T cells
CD4
CD45RA
7 Day Culture
CD4+CD25+ Purification

Figure 3.2. Abundance of T_{reg} in peripheral blood and decidual tissues at term pregnancy. Autologous peripheral blood and decidual lymphocytes were collected on the day of C-section followed by flow cytometric analysis. (A) Representative dot plots of CD25 and FoxP3 expression on $CD4^+$ lymphocytes. (B) Graph represents summarized data from n=6 patients for the percentage of $CD25^+FoxP3^+$ cells out of $CD4^+$ lymphocytes in each tissue. Bars, SEM

(A)


CD4+
Peripheral Blood
CD25
FoxP3
CD4+
Decidua
CD25
FoxP3

(B)

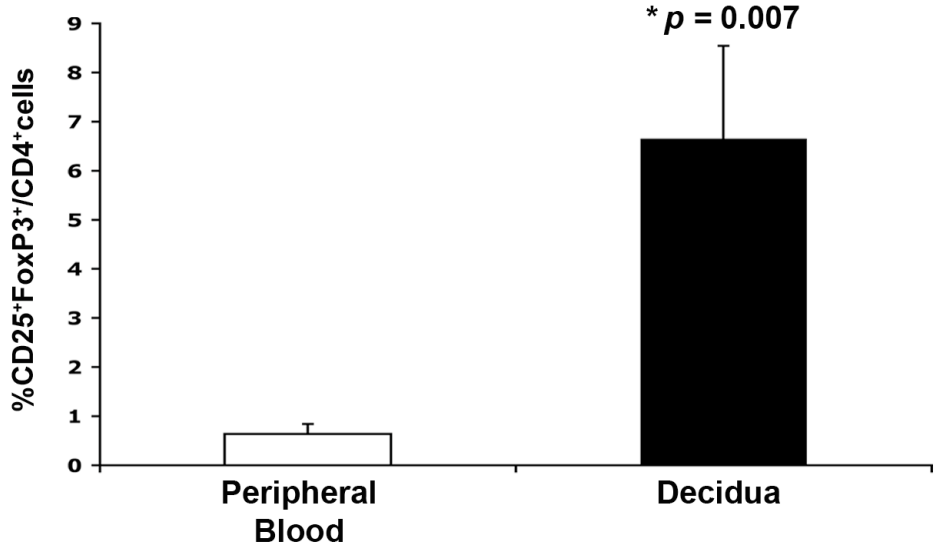

* p = 0.007
%CD25+FoxP3+/CD4+cells
0
1
2
3
4
5
6
7
8
9
Peripheral Blood
Decidua

Figure 3.3. Term CTB induce expansion of T_{reg}. Peripheral blood naïve $CD4^+$ T cells were placed into culture for seven days with either untreated CTB (undifferentiated) or CTB treated for 96 hours with EGF prior to culture (differentiated). EGF was maintained within medium of differentiated culture conditions throughout the incubation period. (A) Representative dot plots of CD25 and FoxP3 expression on $CD4^+$ lymphocytes either before culture, or following culture with undifferentiated or EGF-treated CTB. (B) Graph represents summarized data for the percentage of of $CD25^{high}FoxP3^+$cells out of $CD4^+$ lymphocytes from n=6 different peripheral blood donors cultured with CTB from six different placentas. Bars, SEM. Different letters indicate groups with significantly different values.

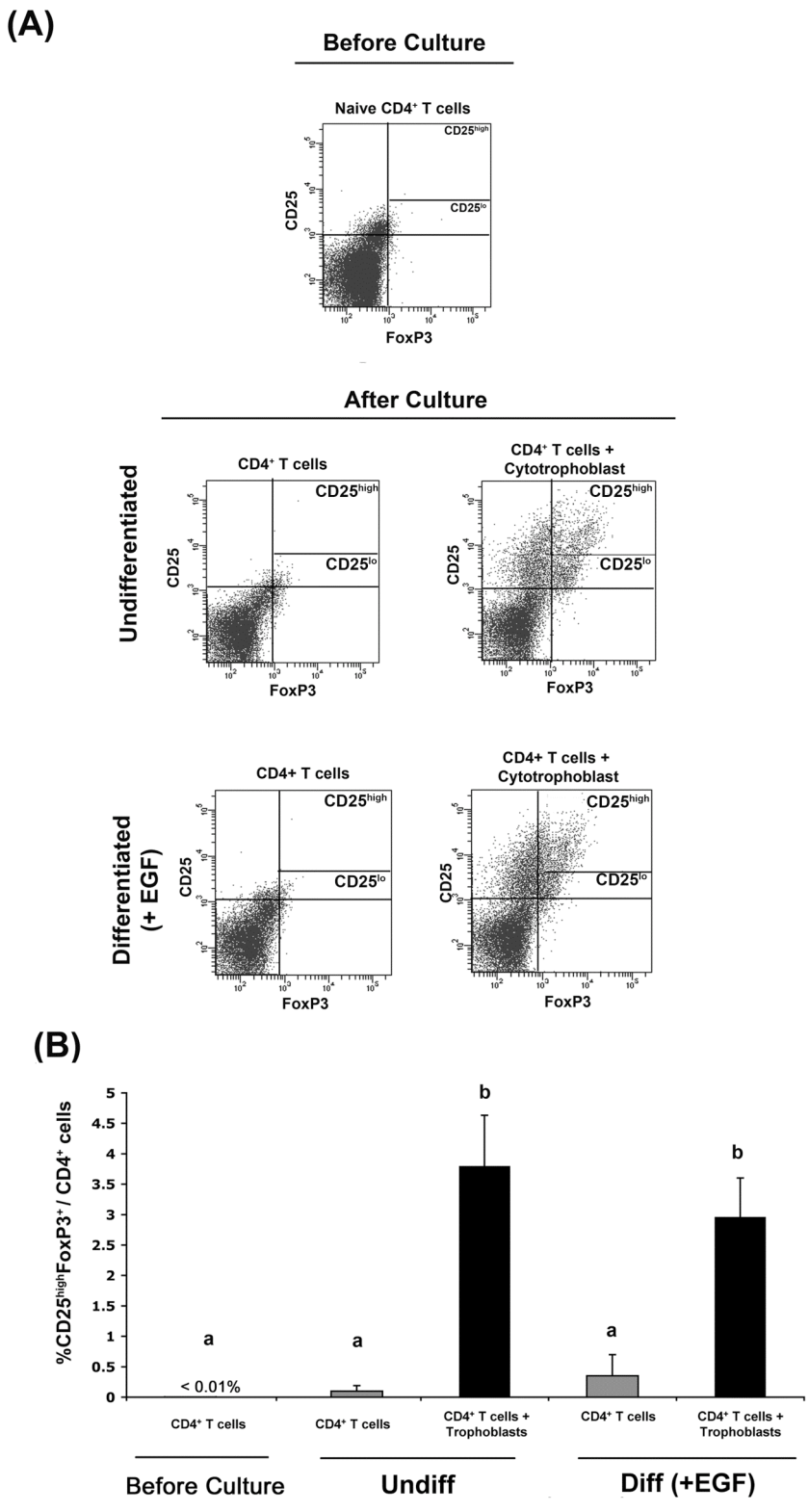
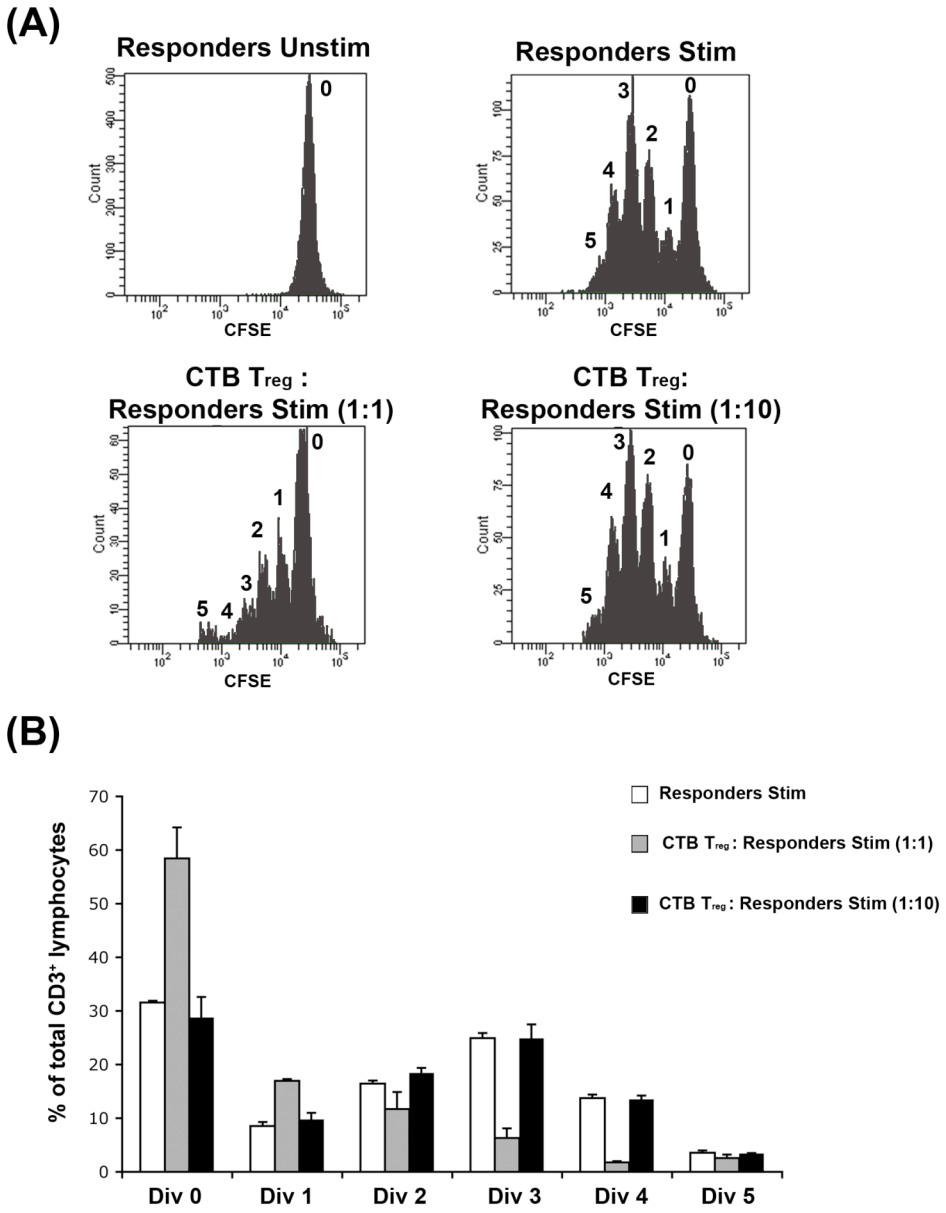

(A)
Before Culture
Naive CD4+ T cells
CD25high
CD25lo
CD25
FoxP3
After Culture
Undifferentiated
CD4+ T cells
CD4+ T cells + Cytotrophoblast
Differentiated (+ EGF)
CD4+ T cells
CD4+ T cells + Cytotrophoblast
(B)
%CD25highFoxP3+ / CD4+ cells
< 0.01%
a
b
CD4+ T cells
CD4+ T cells + Trophoblasts
Before Culture
Undiff
Diff (+EGF)

Figure 3.4. T_{reg} from CTB co-cultures suppress the proliferation of autologous activated $CD4^+$ T cells. $CD4^+CD25^+$ cells were isolated from co-cultures containing cytotrophoblast cells (CTB T_{reg}) and placed into culture with CFSE-labeled autologous naïve $CD4^+$ T cells (responders) stimulated with plate bound anti-CD3 and anti-CD28 antibodies. (A) Representative histograms of CFSE staining in unstimulated (unstim), and stimulated (stim) responder cells with or without CTB T_{reg}. Peaks labeled 0-5 correspond to the number of cell divisions with undivided cells designated as 0. (B) Graph represents summarized data n=1 patient sample plated in duplicate. Div, cell division. Bars, SEM.


(A)
Responders Unstim
Responders Stim
CTB Treg : Responders Stim (1:1)
CTB Treg: Responders Stim (1:10)
Count
CFSE
0
1
2
3
4
5
(B)
% of total CD3+ lymphocytes
Responders Stim
CTB Treg : Responders Stim (1:1)
CTB Treg : Responders Stim (1:10)
Div 0
Div 1
Div 2
Div 3
Div 4
Div 5

Chapter IV

Maternal PD-1 Regulates Accumulation of Paternal Antigen-Specific CD8$^+$ T cells in Pregnancy

Taglauer ES, Yankee TM, Petroff MG. Maternal PD-1 Regulates Accumulation of Paternal Antigen-Specific CD8$^+$ T cells in Pregnancy. (*Submitted)*

A. Abstract

The failure to reject the semiallogeneic fetus suggests that maternal T lymphocytes are regulated by potent mechanisms in pregnancy. The T cell immunoinhibitory receptor, Programmed Death-1 (PD-1), and its ligand, B7-H1, maintain peripheral tolerance by inhibiting activation of self-reactive lymphocytes. Here, we tested the role of the PD-1/B7-H1 pathway in maternal tolerance to the fetus. Antigen-specific maternal T cells both proliferate and upregulate PD-1 *in vivo* at mid-gestation in response to paternal antigen expressed by the fetus. In addition, when these cells carry a null deletion of PD-1, they accumulate excessively in the uterus-draining lymph nodes ($P < 0.001$) without a concomitant increase in proliferation. *In vitro* assays showed that apoptosis of antigen-specific $CD8^{+}$ $PD\text{-}1^{-/-}$ cells was reduced following peptide stimulation, suggesting that the accumulation of these cells in maternal lymph nodes is due to decreased cell death. However, the absence of neither maternal PD-1 nor B7-H1 had detectable effects on birth rate, gestation length, litter size, or pup weight at birth in either syngeneic or allogeneic pregnancies. These results suggest that PD-1 plays a role in inducing apoptosis of paternal antigen-specific T cells during pregnancy, thereby controlling their abundance.

B. Introduction

Over time, several mechanisms have evolved to ensure propagation of viviparous species, including transport of maternal nutrients to the developing fetus *in utero*. A highly effective method for nutrient delivery is through hemochorial placentation in which maternal blood comes into direct contact with fetal trophoblast cells. However, due to species genetic diversity, a paradox exists in which the mother must carry a fetus whose tissues are immunologically distinct from her own. Hemochorial placentation further complicates this paradox by allowing close juxtaposition of semi-allogeneic fetal tissues and the maternal leukocytes in the blood and uterine decidua.

This type of placentation also enables access of fetal antigens to maternal secondary lymph organs through antigen shedding into the maternal blood space surrounding the villous placenta and trafficking to lymphoid organs via maternal antigen presenting cells (reviewed in Taglauer *et al*, submitted). Indeed, fetal antigen can be detected in the maternal spleen and uterus-draining lymph nodes, and antigen-specific T lymphocytes in both organs proliferate in response to fetal tissue during specific timepoints in gestation (Erlebacher et al. 2007). The intimate contact of maternal and fetal tissues and maternal immune recognition of the fetus in secondary lymphoid organs would seem to pose an increased risk for immunological attack against the fetal allograft. However, the reproductive and immune systems likely co-evolved to develop immunosuppressive mechanisms within pregnancy to accommodate this situation, instead allowing the fetus to thrive.

Various immunomodulatory mechanisms are instrumental in controlling maternal leukocytes during pregnancy, including the B7 family of immune cell co-stimulatory molecules (Petroff 2006a). B7 molecules are cell surface proteins belonging to the immunoglobulin superfamily, and they bind to CD28 family receptors on leukocytes to influence their activation, either negatively or positively. One B7 family member, B7-H1, inhibits T lymphocyte activation through engagement of its receptor, PD-1 (Freeman et al. 2000; Latchman et al. 2001), and consequences of B7-H1:PD-1 interactions include suppression of proliferation, alteration of cytokines and induction of apoptosis (Freeman et al. 2000; Latchman et al. 2001; Hori et al. 2006; Muhlbauer et al. 2006; Keir et al. 2007; Zhang et al. 2008). PD-1 is expressed on activated lymphocytes in blood, lymphoid organs and tissues undergoing an immune response (Ishida et al. 1992; Agata et al. 1996).

The suppressive action of PD-1 is demonstrated though *in vivo* studies in which targeted mutation or blockade of PD-1 results in spontaneous tissue-specific autoimmune disease (Nishimura et al. 1999; Nishimura et al. 2001) or T cell cytotoxicity against peripheral self-tissues (Martin-Orozco et al. 2006; Keir et al. 2007). This inhibitory receptor is also involved in preventing allograft rejection (Ozkaynak et al. 2002; Tanaka et al. 2007; Wang et al. 2007; Yang et al. 2008). Similarly, B7-H1-deficient mice are also more susceptible to experimentally induced autoimmune diseases (Dong et al. 2004; Latchman et al. 2004; Keir et al. 2006) and the absence of maternal B7-H1 may facilitate rejection of semi-allogeneic fetuses during pregnancy (Guleria et al. 2005; Habicht et al. 2007). The B7-H1:PD-1

pathway thus appears to have a central function in maintaining immunological self-tolerance in addition to tolerance to foreign grafts, including the fetal allograft.

In this study we investigated the physiological function of maternal PD-1 and B7-H1 in pregnancy using murine T cell receptor transgenic and knockout models. Based on the published evidence for the central role of PD-1 in tolerance to self and foreign tissues, we hypothesized that this receptor has an important function in maternal tolerance of the fetal-allograft.

C. Materials and methods

Mice

PD-$1^{-/-}$ and B7-H$1^{-/-}$ mice on the C57BL/6 (B6) background were gifts from the laboratories of T. Honjo (Kyoto University) and L. Chen (Johns Hopkins University) (Nishimura et al. 1998; Dong et al. 2004) respectively. Wild type (WT) B6, BALB/c, CBA/J, B6-Tg(TcraTcrb)1100Mjb/J (OT-I), and B6-Tg(ACTB-OVA)916Jen/J (Ova-Tg) mice were obtained from Jackson Laboratories (Bar Harbor, ME).

All mice were housed under pathogen-free conditions. For adoptive transfer experiments, PD-$1^{+/-}$ OT-I and PD-$1^{-/-}$ OT-I mice were generated by crossing OT-I with PD-$1^{-/-}$ mice . PD-$1^{-/-}$ and B7-H$1^{-/-}$ mice were genotyped using primers specific for PD-1 (Fwd: ACC AGG AAC TCC CCG TTA GT ; Rev: TAT TTA GGG TGC AGC CTC GT), B7-H1 and neomycin (Dong et al. 2004), and OT-I and OVA-Tg mice were genotyped using protocols from Jackson Laboratories. Surface protein

phenotypes of PD-1$^{-/-}$ and B7-H1$^{-/-}$ mice were confirmed by stimulation of splenocytes with 3ug/mL conconavalin A (Sigma-Aldrich, St. Louis, MO) for 72h followed by flow cytometry. All experimental protocols were approved by the University of Kansas Medical Center Institutional Animal Care and Use Committee.

Breeding experiments and assessment of fecundity

To examine fecundity in the absence of maternal PD-1, virgin WT or PD-1$^{-/-}$ females were bred either syngeneically to B6 (H2b) males or allogeneically to BALB/c (H2d) males. Similarly, virgin WT or B7-H1$^{-/-}$ female mice were mated syngeneically to B6 (H2b) or allogeneically to CBA/J males to examine the role of maternal B7-H1 in pregnancy. Vaginal plugs were evaluated each morning, and the presence of a vaginal plug was designated as gestation day (gd) 0.5. Evaluation of fecundity included enumeration of healthy and resorbed implantation sites on gd 13.5, or examination of gestation length, litter size, pup weight at birth as well as pup weight and male/female ratios at weaning (postnatal day 21).

Adoptive transfer

For adoptive transfer experiments, virgin B6 female mice were bred to either B6 WT males (WT-bred) or OVA-Tg (OVA-bred) males. To obtain OT-I cells for adoptive transfer, splenocytes were isolated from virgin female WT OT-I or PD-1$^{-/-}$ OT-I mice and labeled with 5μM CFSE (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. OT-I surface phenotype was confirmed flow cytometry

prior to transfer in all experiments. $10x10^6$ CFSE-labeled splenocytes were transferred into WT- or OVA-bred recipient female mice on gd10.5 via injection into the tail vein. Injection consistency was monitored by calculating the percentage of undivided ($CFSE^{bright}$) non-antigen-specific cells/total PALN lymphocytes, and did not differ significantly between experimental groups ($P = 0.569$, data not shown). On gd 13.5, PALN and spleen cells from recipient female mice were isolated and subjected to flow cytometric analysis.

In vitro apoptosis and proliferation assays

For analysis of activation-induced apoptosis and proliferation *in vitro*, splenocytes from virgin female WT OT-I or PD-$1^{-/-}$ OT-I mice were isolated and placed into 500 μL total volume in a 24 well plate at density of 1 x 10^6 cells/well with either medium alone or 100ng/mL SIINFEKL peptide (ProImmune, Bradenton, FL). For proliferation analysis, splenocytes were labeled with 5μM CFSE (Invitrogen) prior to culture. At 48 hours (apoptosis assay) or 72 hours (proliferation assay) following plating, cells were collected and analyzed by flow cytometry.

Flow cytometry

Isolated cells from spleen and/or lymph nodes were stained with the following anti-mouse antibodies or reagents: CD8 phychoerythryn (PE)-Cy5 (clone H35-17.2, eBioscience, San Diego, CA), Vα2-PE (clone B20.1; BD Pharmingen, San Jose, CA), Vβ5-fluorescein isothiocyanate (FITC) (clone MH3-2; BD Pharmingen), PD-1-PE

(clone J43, eBioscience), B7-H1-PE (clone MIH5, eBioscience) and Annexin V-PE (BD Pharmingen). In all experiments, cells were labeled with LIVE/DEAD® Fixable Violet Dead Cell Stain Kit (Invitrogen). A minimum of 500,000 lymphocytes for adoptive transfer experiments and 20,000 lymphocytes for *in vitro* apoptosis/proliferation assays were collected in a gate based on forward and side scatter characteristics. All samples were processed on a BD LSRII instrument and analyzed using BD FACSDiva™ (BD Pharmingen).

Statistics

Statistical significance was determined using Kruskal-Wallis one-way analysis of variance (ANOVA) on ranks for *in vitro* analysis of apoptosis, and one-way (ANOVA) for all other experiments. All pairwise multiple comparisons were performed with a Student-Newman-Keuls post-test. Differences were considered significant at $\alpha = 0.05$.

D. Results

PD-1 controls accumulation of paternal antigen-specific T cells during pregnancy.

To determine whether PD-1 influences the fate of paternal antigen-specific T cells. we employed the OVA-OT-I model system, in which OT-I T cell receptor (TCR)-transgeneic T cells recognize the ovalbumin-derived SIINFEKL peptide. B6 WT females were bred to either B6 WT or OVA-Tg males and CFSE-labeled OT-I splenocytes were adoptively transferred into pregnant females mid-gestation (gd

10.5). Three days post-transfer (gd 13.5), cells from uterus-draining lymph nodes and spleens were collected and stained for OT-I T cell receptor markers and PD-1 and analyzed by flow cytometry (Fig. 4.1). In the PALN of females mated to WT males, OT-I cells were rare and did not proliferate (Fig. 4.1a). In contrast, in the PALN of OVA-Tg-mated females, OT-I cells were readily detected and showed significantly higher percentage of proliferating cells (OVA-Tg bred vs WT-bred, $P < 0.01$) while non-antigen specific spleen cells did not proliferate (Fig. 4.1b). In addition, proliferating paternal antigen-specific $CD8^+$ T cells expressed the PD-1 receptor (Fig. 4.1 c). Similar proliferation and PD-1 expression on OT-I cells was observed within the spleens of Ova-Tg mated females (data not shown).

We next examined the effect of PD-1 deficiency during paternal antigen-specific T cell responses by transferring either WT OT-I or PD-$1^{-/-}$ OT-I CFSE-labeled cells into virgin, WT-bred, or OVA-bred female recipients. In the PALN of Ova-Tg bred females, paternal-antigen specific T cells lacking PD-1 were more abundant than WT OT-I cells (Fig. 4.2 a), relative to both total lymphocytes ($P < 0.001$, Fig. 4.2 b) and $CD8^+$ T cells ($P < 0.001$, Fig. 4.2 c). This difference was not observed in WT-bred recipients, as the abundance of both WT-OT-I and PD-$1^{-/-}$ OT-I cells in these mice was similar to virgin females (Fig. 4.2 b,c). Conversely, in the spleens of OVA-Tg bred females, OT-I T cells accumulated regardless of PD-1 expression, particularly relative to endogenous splenic $CD8^+$ T cells (Fig. 4.2 e-g). However, no significant differences in the number of fetal resorption sites in females receiving OT-I WT or OT-I PD-$1^{-/-}$ cells were observed (data not shown).

To determine a potential mechanism for paternal antigen-specific T cell accumulation in the absence of PD-1, we evaluated the proliferation of adoptively transferred cells at gd 13.5. There was no alteration in the percentage of dividing WT OT-I or PD-$1^{-/-}$ OT-I cells in the PALN or spleen of OVA-Tg bred females (Fig 4.2 d,h), suggesting that accumulation was not due to increased proliferation by PD-1-deficient cells. We next examined the possibility that PD-1 limits accumulation of paternal antigen-specific T cells through induction of apoptosis. WT OT-I or PD-$1^{-/-}$ OT-I splenocytes were stimulated *in vitro* with SIINFEKL peptide followed by examination of proliferation and apoptosis using flow cytometry. CFSE-labeled OT-I cells stimulated with SIINFEKL peptide showed no significant difference in proliferation between WT and PD-$1^{-/-}$ cells (Fig. 4.3 a,b), consistent with our observations *in vivo* (Fig. 4.2 d,h). In parallel experiments, decreased percentages of peptide-stimulated PD-$1^{-/-}$ OT-I cells were positive for Annexin V as compared to WT OT-I cells (Fig. 4.3 c,d), suggesting that PD-1 deficient cells are less susceptible to activation induced cell death.

PD-1 is not required for fetal survival during allogeneic pregnancy

Because PD-1 controls the accumulation of paternal antigen-specific T cells, we next examined whether maternal PD-1 is required for tolerance to the semi-allogeneic fetus. C57BL/6 WT and PD-$1^{-/-}$ females were mated with either B6WT or BALB/c males followed by evaluation of birth rate, gestation length, litter size, and offspring neonatal and weaning weights. The absence of PD-1 in PD-$1^{-/-}$ mice was

confirmed through flow cytometry (Fig. 4a). No significant alteration in the birthrate or other gestational or neonatal offspring parameters was observed between syngeneic and allogeneic pregnancies in WT and PD-1$^{-/-}$ mice (Fig. 4.4 b-e). Also, there was no consistent trend in the ratios male and female offspring among the breeding groups examined (data not shown); however weight was increased in offspring born from PD-1$^{-/-}$ mothers, an effect seen in both syngeneic and semi-allogeneic litters (Fig. 4.4 f).

These results were unexpected, given a previous report that maternal tolerance of semi-allogenic fetuses is dependent on the PD-1 ligand, B7-H1 (Guleria et al. 2005). We therefore re-examined whether B7-H1 was critical for the success of allogeneic pregnancy by evaluating the fecundity of female B7-H1$^{-/-}$ mice during syngeneic and allogeneic pregnancy (Fig. 4.5). Our results from the B7-H1$^{-/-}$ female syngeneic or allogeneic crosses were consistent with those observed for PD-1$^{-/-}$ mice, with no obvious changes in gestation rate or offspring viability (Fig. 4.5 b-f). Overall, these data strongly suggest that neither maternal PD-1 nor B7-H1 are required for the viability of semi-allogeneic fetuses.

E. Discussion

The overall goal of these studies was to examine the role of the B7-H1:PD-1 pathway in maternal-fetal tolerance. To address this question, we utilized PD-1$^{-/-}$ and B7-H1$^{-/-}$ mice as well as adoptive transfer of T cells specific for paternal antigen. We found that PD-1 control the accumulation of paternal antigen-specific CD8^{+} T cells in

the PALN possibly through induction of apoptosis. However, there was no effect on the fecundity or offspring survival during allogeneic pregnancy in the absence of either maternal PD-1 or B7-H1.

We investigated the role of PD-1 on T cells in pregnancy using an approach that enables analysis of paternal antigen-specific responses at the cellular level. By adoptively transferring OT-I cells into WT females bred to Ova-Tg males, we found that OT-I cells specific for paternal ovalbumin express PD-1 (Fig. 4.1). Further, OT-I T cells accumulated to a greater extent in the PALN of OVA-bred females only in the absence of PD-1 (Fig. 4.2 a-d), whereas in the spleen OT-I cells accumulated regardless of PD-1 expression (Fig. 4.2 e-h). A previous study showed that during pregnancy, adoptively transferred paternal antigen-specific T cells do not accumulate despite high proliferation in the PALN and spleen, suggesting that these cells are being deleted (Erlebacher et al. 2007). These results are similar to our observations for WT OT-I cells in the PALN (Fig. 4.4). However, accumulation did occur in absence of PD-1, suggesting that this receptor may be involved in the deletion of the paternal-antigen specific T cells. We did not observe decreased accumulation of WT OT-I cells in the spleen by three days post adoptive transfer, suggesting that the kinetics of paternal antigen-specific T cell deletion may differ between the PALN and spleen.

Other reports also propose that paternal antigen specific T cells are deleted within maternal lympohoid organs. $CD8^+$ T cells specific for the paternal antigen H-Y undergo an expansion in both the PALN and spleen during early-mid gestation

followed by a contraction in numbers that continues until term (Vacchio et al. 1999). The interactions of CD28/B7 or Fas/FasL may facilitate deletion of these cells in the spleen during late gestation (Vacchio et al. 2003; Vacchio et al. 2005). While these studies examined endogenous, rather than adoptively transferred, maternal T cells, the current study suggests that PD-1 may be an additional mechanism for controlling accumulation of paternal antigen-specific T cells within the lymph nodes draining the gravid uterus. Continued analysis of these models using mice lacking PD-1 along with Fas/FasL and/or the CD28/B7 proteins would provide insight into whether these pathways work in conjunction to prevent propagation of maternal effector T cell activity during pregnancy.

The effects of PD-1 on OT-I T cells during pregnancy are congruent with previous models where a lack of PD-1 on adopively transferred OT-I cells caused an increased accumulation of these cells in draining lymph nodes of peripheral tissues expressing ovalbumin (Martin-Orozco et al. 2006; Keir et al. 2007). The eventual result of this accumulation was an antigen-specific attack against self-tissues. In our model we did not see an increase in fetal resorption sites following injection of PD-$1^{-/-}$ fetal antigen-specific T cells (data not shown). In these previous studies, the earliest signs of tissue destruction were not evident until approximately 5 days post injection, so the lack of effect on fetal viability seen in our model could be due to insufficient duration of antigenic exposure *in vivo*, as we examined implantation sites only 3 days following transfer of OT-I cells. More likely, however, is that fetal loss by these cells would not occur, given the results of PD-$1^{-/-}$ female allogeneic

pregnancies and apparent redundancy of immunosuppressive mechanisms at the maternal-fetal interface (Fig. 4.4) (Petroff 2005a).

The B7-H1:PD-1 pathway can inhibit T lymphocytes through several mechanisms including control of proliferation, alteration of cytokine production and induction of apoptosis (Freeman et al. 2000; Latchman et al. 2001; Hori et al. 2006; Muhlbauer et al. 2006; Keir et al. 2007; Zhang et al. 2008). Our results support a role for PD-1 in apoptosis of paternal antigen-specific T cells rather than inhibition of proliferation (Fig. 4.2, 4.3). Indeed, the downstream inhibitory effector of PD-1 signaling, SHP-2 phosphatase participates in apoptosis induction and following ligation of PD-1, one net effect of SHP-2 recruitment is downregulation of PI3K and ERK anti-apoptotic pathways (Ballif et al. 2001; Okazaki 2001; Yuan et al. 2005). In addition, PD-1 signaling with concomitant T cell receptor stimulation prevents upregulation of the prosurvival molecule Bcl-2 (Keir et al. 2005). Interestingly, an overexpression of Bcl-2 causes increased accumulation and reduced apoptosis of antigen-specific T cells in tissue-draining lymph nodes (Redmond et al. 2008). In the current study, a lack of PD-1 did not alter the proliferation of OT-I cells either *in vivo* (Fig. 4.2 d,h) or *in vitro* (Fig. 4.3 a,b). However, in the absence of PD-1, a lower percentage of *in vitro* peptide-stimulated OT-I cells were apoptotic (Fig. 4.3 c,d), which parallels the effects of Bcl-2 overexpression. These results point to a role for PD-1 in controlling accumulation of fetus-specific T cells in uterus-draining lymph nodes through induction of apoptosis, which may be mediated by downregulation of Bcl-2.

Several studies have shown that PD-1 is involved in maintaining tolerance to both self and allograft tissues (Martin-Orozco et al. 2006; Keir et al. 2007; Tanaka et al. 2007; Wang et al. 2007). However, the lack of fetal rejection at term pregnancy in PD-$1^{-/-}$ mothers indicates that fetal allograft viability is not dependent on PD-1: B7-H1 interactions (Fig. 4.4). Our results conflict with a previous report in which B7-$H1^{-/-}$ mothers lose tolerance to semi-allogeneic fetuses (Guleria et al. 2005). The inhibitory effects of B7-H1 may be propagated through a receptor other than PD-1 (Dong et al. 2002) , which could reconcile our divergent observations for the PD-$1^{-/-}$ mice. However, we also examined the fecundity of B7-H1 deficient females following syngeneic and allogeneic breeding and found no significant differences in the gestation rate or offspring viability between B6WT and B7-$H1^{-/-}$ mice (Fig. 4.5). The discrepant results between the current and previous analyses of B7-H1 functions during pregnancy draws attention to the differences between the B7-$H1^{-/-}$ mice utilized for each study. The B7-H1-deficient mice in the previous study lack exons 2 and 3 of the B7-H1 gene, which differs from the mice used in the current study which lack exon 1 and majority of exon 2 (Dong et al. 2004). However, both of these strategies delete the exon 2-encoded PD-1 binding site from the B7-H1 transcript (Wang et al. 2003; Dong et al. 2004; Guleria et al. 2005). In addition, the mice in our study lack the leader sequence for B7-H1 (exon 1), precluding B7-H1 from being expressed on the surface of lymphocytes, as confirmed by flow cytometry (Fig. 4.5). However, as the variation in B7-H1 deletion constructs are the main identifiable

difference between these two reports, the specific mechanism(s) for the divergent results from these two studies remains unclear.

While the absence of maternal PD-1 did not affect fetal survival, offspring were significantly heavier at the time of weaning. This effect was seen in both syngeneic and allogeneic pregnancies and may be due to an altered immunologic environment during pregnancy or changes in lactation physiology. PD-1 is known to inhibit pro-inflammatory cytokine production (Freeman et al. 2000; Latchman et al. 2001; Keir et al. 2007), and thus in the absence of PD-1 during pregnancy could propagate an inflammatory environment that may influence body composition later in life. A similar effect was also be observed in mice lacking the anti-inflammatory cytokine IL-10, where fetuses from IL-10 deficient mothers were heavier at birth (White et al. 2004) and recent evidence suggests that obesity in both children and adults can be associated with altered physiology caused by inflammatory processes (Das 2001). In addition, while the role of PD-1 in lactation physiology remains unclear, PD-1 may be involved in homeostatic immune processes during lactation that could have downstream effects on offspring growth.

Overall, these results strongly suggest that neither maternal PD-1 nor its ligand B7-H1 are indispensable for fetal survival during allogeneic murine pregnancy. The current study is consistent with several previous reports in which abrogation of an immunoinhibitory mechanism during pregnancy does not affect survival of the semi-allogeneic fetus (Petroff 2005a). During pregnancy, inflammatory signals are absent during presentation of fetal antigen to maternal T

cells, which could be a broad-spectrum mechanism to ensure maternal-fetal tolerance (Bonney et al. 1998). However, rejection of semi-allogenic fetuses can occur in the absence of regulatory T cells or galectin-1 (Aluvihare et al. 2004; Darrasse-Jeze et al. 2006; Blois et al. 2007), suggesting that tolerance is more than the paucity of maternal immune reactivity simply due to the absence of a danger signal. Further, a functional hierarchy may exist among the network of immunomodulatory pathways present at the maternal-fetal interface. Within this network, the B7-H1:PD-1 pathway may modulate the accumulation of paternal antigen-specific T cells in the uterus draining lymph nodes through a mechanism involving apoptosis and operate in conjunction with other immunotolerizing mechanisms for the ultimate goal of maintaining healthy gestation and fetal development.

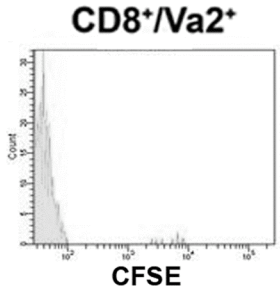
Acknowledgements

The authors would like to thank Joyce Slusser for flow cytometry experimental design and analysis, Stan Fernald for image design, and Ann Trikchacheva and Jie Zhao for technical assistance.

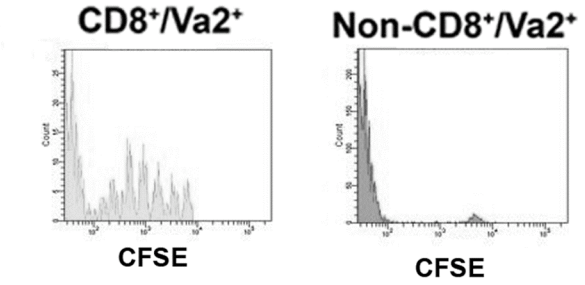
F. Figures and Legends

Figure 4.1. Paternal antigen-specific T cells proliferate and express PD-1 during pregnancy. CFSE-labeled spleen cells from OT-I mice were transferred at gd 10.5 into females bred to ova-transgenic (OVA) or WT males. On gd 13.5, cells from the peri-aortic lymph nodes were stained for OT-I markers and PD-1 and analyzed by flow cytometry. (a,b) Representative dot plots and histograms of $CD8^+/Va2^+$ OT-I cells in the peri-aortic lymph nodes of either WT (a) or OVA bred females (b). (c) Representative dot plot and histogram of PD-1 expression in maternal OT-I cells in the peri-aortic lymph nodes. Empty peak, isotype control. MFI, mean fluorescence intensity. Figure representative of n=4 female recipients.

a.


CD8+/Va2+
Count
CFSE

b.


CD8+/Va2+
Non-CD8+/Va2+
Count
CFSE
Count
CFSE

c.

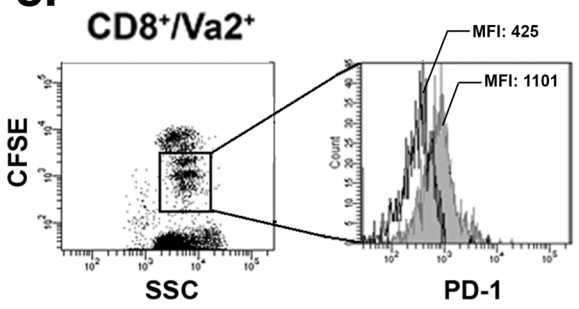

CD8+/Va2+
MFI: 425
MFI: 1101
CFSE
SSC
Count
PD-1

Figure 4.2. PD-1 controls accumulation of paternal antigen-specific T cells in the uterus draining lymph nodes. CFSE labeled splenocytes from WT or PD-$1^{-/-}$ mice were adoptively transferred into virgin, WT-bred or OVA-bred females on gd 10.5. Recipient female peri-aortic lymph nodes and spleens were collected on gd 13.5 and analyzed by flow cytometry. (a,e) Representative dot plots of peri-aortic lymph node (a) and spleen (e) cells stained for CD8 and Vα2. (b,f) Graph represents percentage of OT-I cells out of total peri-aortic (b) or splenic (f) lymphocytes in virgin (n =3), WT bred (n = 3) or OVA bred (n = 4) females. (c,g) Graph represents percentage of OT-I cells out of peri-aortic (c) or splenic (g) $CD8^+$ cells in same groups as described for b and f. (d,h) Graph represents the percentage of OT-I ($CD8^+V\alpha2^+$) cells in the peri-aotric lymph nodes (d) or spleen (h) undergoing more than 3 cell divisions. Different letters denote groups whose difference was statistically significant.

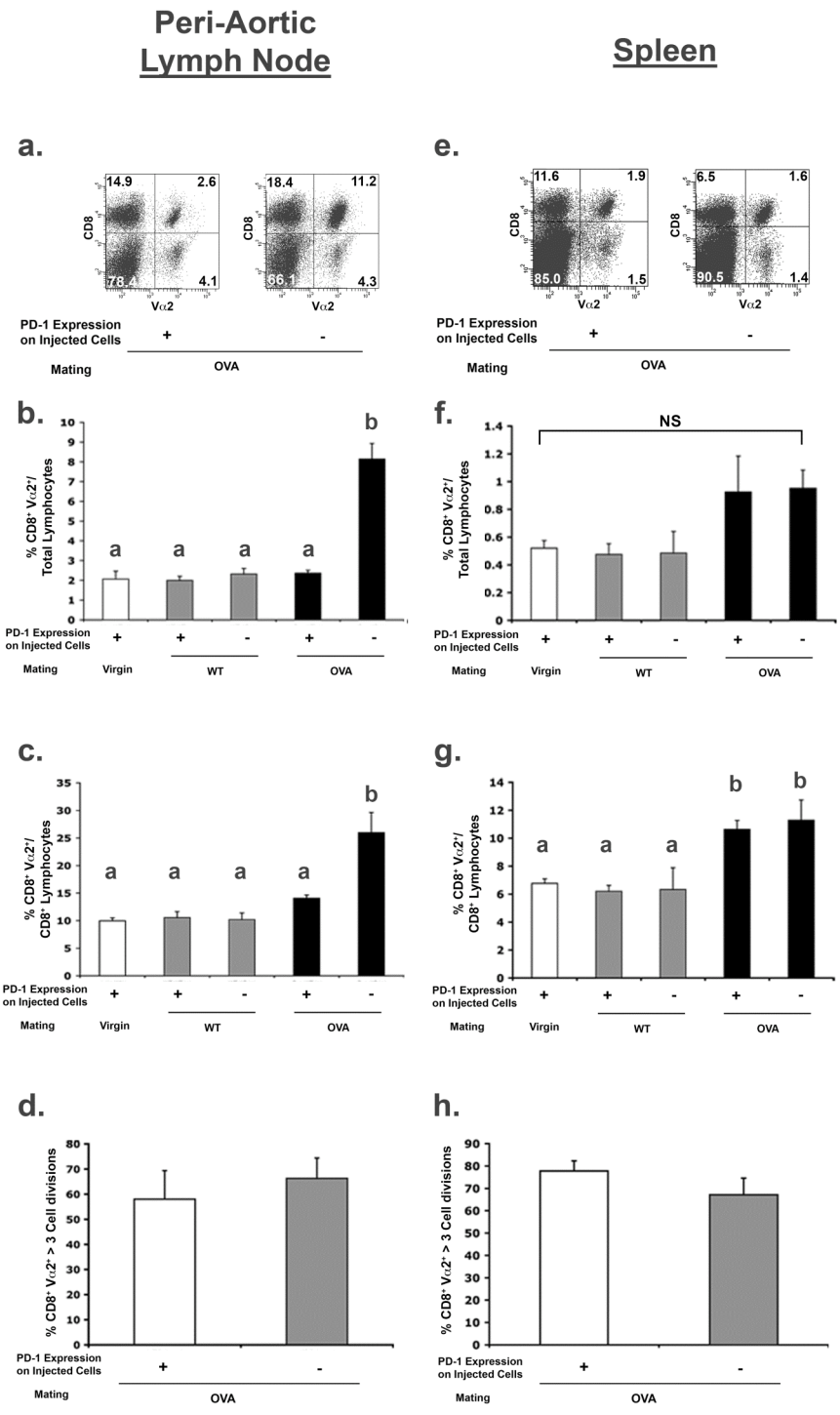

Peri-Aortic Lymph Node
Spleen
a.
14.9
2.6
78.4
4.1
18.4
11.2
66.1
4.3
CD8
Vα2
PD-1 Expression on Injected Cells
+
-
Mating
OVA
e.
11.6
1.9
85.0
1.5
6.5
1.6
90.5
1.4
b.
% CD8+ Vα2+/ Total Lymphocytes
a
a
a
a
b
Virgin
WT
OVA
f.
NS
c.
% CD8+ Vα2+/ CD8+ Lymphocytes
g.
d.
% CD8+ Vα2+ > 3 Cell divisions
h.

Figure 4.3. PD-1 induces apoptosis without altering proliferation in activated antigen-specific T cells. Unlabeled (a,b) or CFSE-labeled (c,d) OT-I WT and PD-$1^{-/-}$ splenocytes were placed into culture with 100ng/mL SIINFEKL peptide or medium at 37^{o} C for a total of 72 hours followed by flow cytometric analysis for OT-I markers (CD8, Vα2) and/or apoptosis markers (Annexin V, Violet Live/Dead Stain). (a) Representative dot plots of $CD8^{+}$ $Va2^{+}$ splenocytes analyzed for apoptosis markers following 48 hour culture. Lines were set based on unstained cells for Violet Live/Dead stain and fluorescence minus one controls for Annexin V stain. (b) Graph represents summarized data from n=4 mice/group. Different letters denote groups whose difference was statistically significant. (c) Representative dot plots of CFSE-labled $CD8^{+}$ $Va2^{+}$ splenocytes splenocytes analyzed for proliferation following 72 hour culture. (d) Graph represents summarized data from n=4 mice/group.

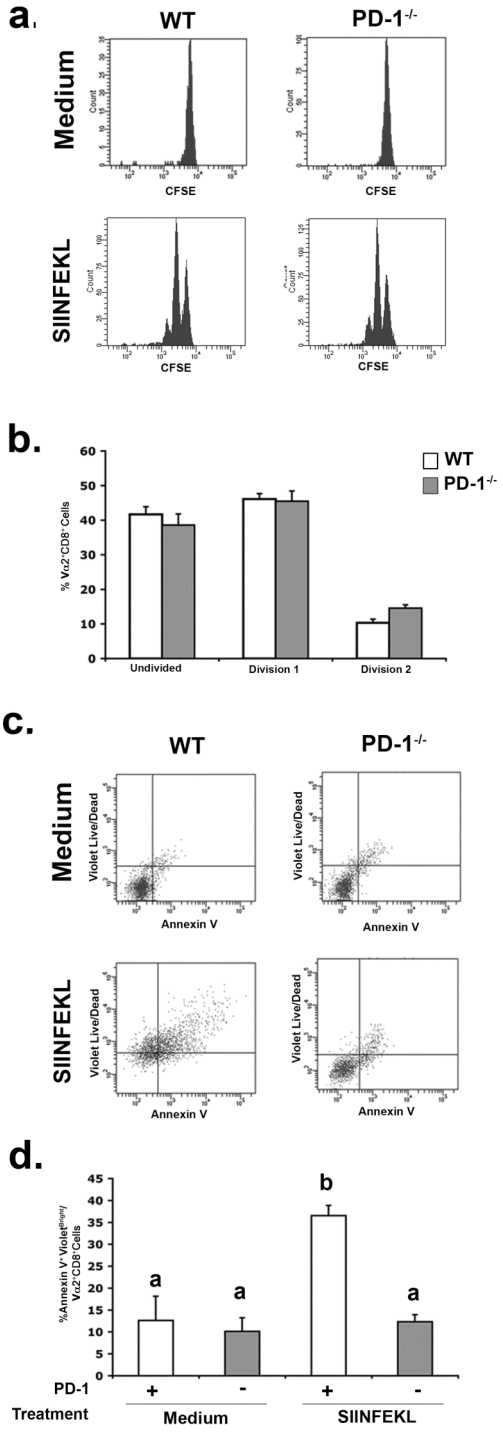

a.
WT
PD-1-/-
Medium
SIINFEKL
Count
CFSE
b.
WT
PD-1-/-
% Vα2+CD8+ Cells
Undivided
Division 1
Division 2
c.
WT
PD-1-/-
Medium
SIINFEKL
Violet Live/Dead
Annexin V
d.
%Annexin V+ Violetlow/ Vα2+CD8+ Cells
a
a
b
a
PD-1
+
-
+
-
Treatment
Medium
SIINFEKL

Figure 4.4. PD-1 is not required for the maintenance of allogeneic pregnancy. (a) Whole splenocytes were stimulated with conconavalin A for 72 hours at 37^{o}C followed by flow cytometric analysis of PD-1. Empty peaks, isotype control. (b) analysis of gestation rate for syngeneic (syn) and allogeneic (allo) matings in WT (syn: n=12, allo: n=10) and PD-1$^{-/-}$ (syn: n=8, allo: n=10); (c-e) analysis of gestation length (c), litter size (d), and fetal weight at birth (e)/weaning (f) in syngeneic (syn) and allogeneic (allo) pregnancies in WT (syn: n=9, allo: n=7) and PD-1$^{-/-}$ (syn: n=5, allo: n=5) females. Different letters denote groups whose difference was statistically significant.

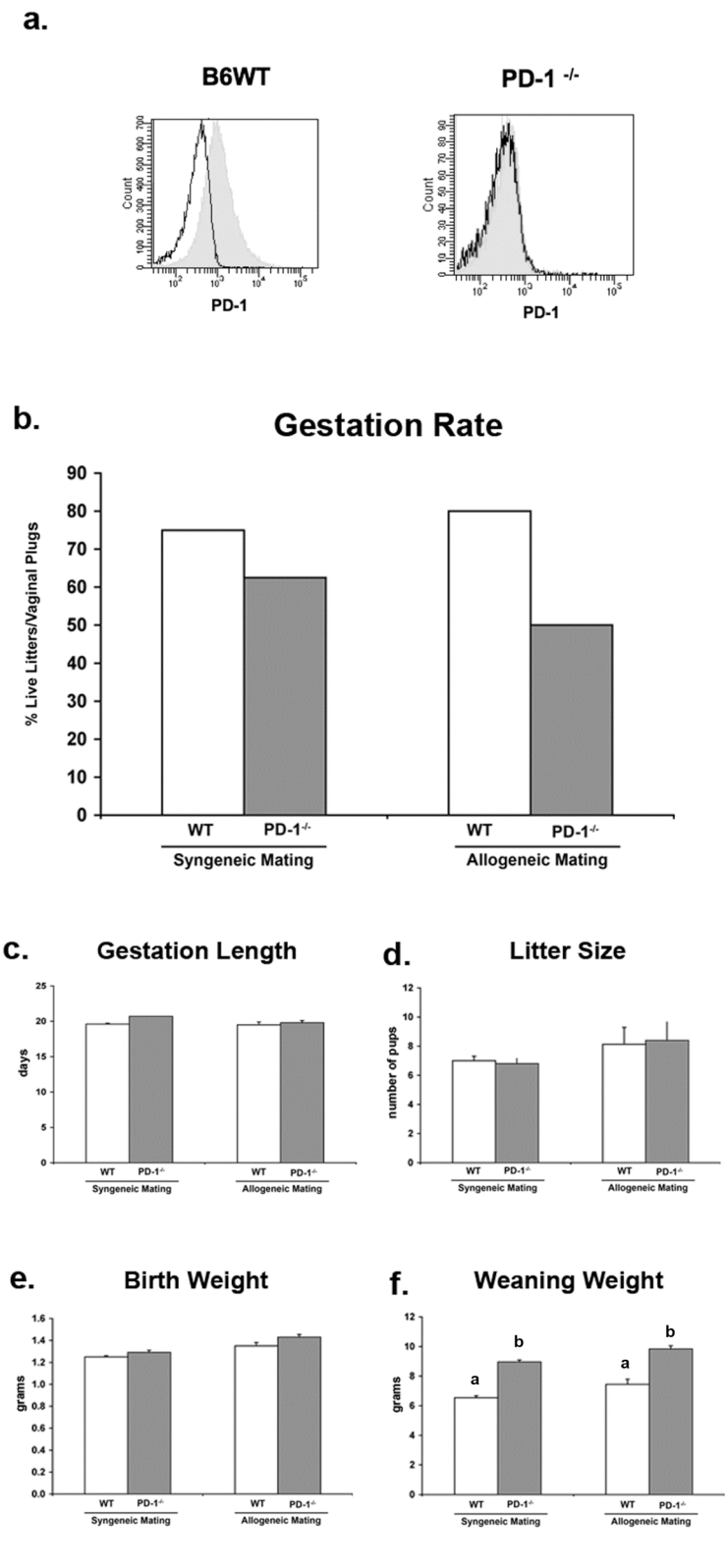
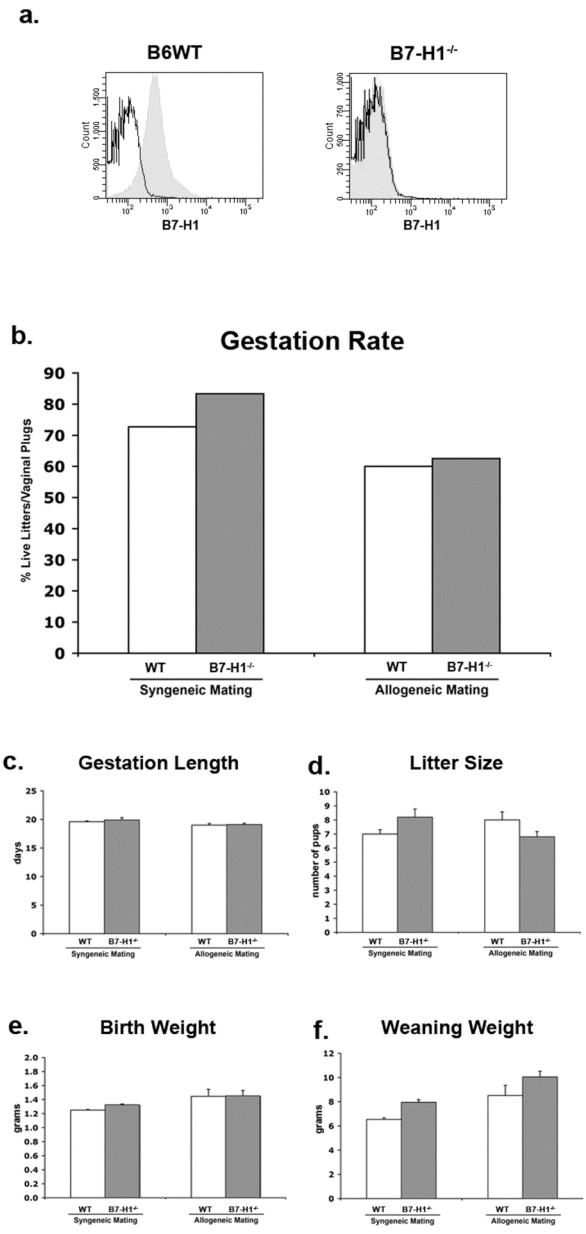

a.
B6WT
PD-1 -/-
Count
PD-1
b.
Gestation Rate
% Live Litters/Vaginal Plugs
WT
PD-1-/-
Syngeneic Mating
Allogeneic Mating
c.
Gestation Length
days
d.
Litter Size
number of pups
e.
Birth Weight
grams
f.
Weaning Weight
grams
a
b

Figure 4.5. B7-H1 is not required for the survival of allogeneic fetuses. (a) Whole splenocytes were stimulated with conconavalin A for 72 hours at 37^{o}C followed by flow cytometric analysis of B7-H1. Empty peaks, isotype control. (b-f) Analysis of gestation rate (b), gestation length (c), litter size (d), and fetal weight at birth (e)/weaning (f) in syngeneic (syn) and allogeneic (allo) pregnancies in WT (syn: n=9, allo: n=4) and PD-1$^{-/-}$ (syn: n=5, allo: n =5) females. No values were significantly different in any of the graphs displayed.


a.
B6WT
B7-H1-/-
Count
B7-H1
b.
Gestation Rate
% Live Litters/Vaginal Plugs
WT
B7-H1-/-
Syngeneic Mating
Allogeneic Mating
c.
Gestation Length
days
d.
Litter Size
number of pups
e.
Birth Weight
grams
f.
Weaning Weight
grams

Chapter V

Conclusions

Portions of this chapter are in preparation for publication:

Taglauer, E.S., Adams Waldorf, K.M., Petroff, M.G., The hidden maternal-fetal interface: secondary lymphoid organs and maternal-fetal tolerance. (*Submitted)*

A. Conclusions

The overall objective of these studies was to add to the existing knowledge about maternal T cell regulation during pregnancy. More specifically, we examined the expression and function the immunoinhibitory receptor PD-1 within multiple sites of the maternal-fetal interface. First we found that the abundance of PD-1-expressing cells increases dramatically within human decidual tissues throughout gestation as compared to non-pregnant endometrium. We then identified that PD-1 is preferentially expressed on maternal T cell subpopulations in the term decidua and that B7-H1 modifies the cytokine repertoire of decidual T cells (Fig. 5.1 A). Next we established that human trophoblast cells induce expansion of regulatory T cells (Fig 5.1 B). Finally we found that PD-1 is required for deletion of paternal antigen-specific T cells in the uterus-draining lymph nodes during pregnancy (Fig 5.1 C). These results identify novel functions of the B7-H1:PD-1 pathway in maternal-fetal tolerance and highlight the importance of examining multiple sites of immune regulation during pregnancy.

Throughout gestation there are numerous sites of interaction between the maternal immune system and fetal tissues (reviewed,Taglauer et al, submitted). One site is between maternal leukocytes in the decidua and blood surrounding the fetal placenta and membranes (Fig 5.1 A.B). Another less well-characterized interface is within the secondary lymph nodes in pregnant females (Fig 5.1 C). Our studies suggest that the B7-H1:PD-1 pathway regulates maternal T cells within both of these interfaces, but through distinct mechanisms. B7-H1 modifies cytokine production

without induction of cell death of decidual $CD4^+$ T cells (Fig. 5.1 A). Cytotrophoblast cells are capable of inducing T_{reg} expansion *in vitro* which may involve pathways including B7-H1 signaling (Fig. 5.1 B). Finally within the uterus-draining lymph nodes, PD-1 limits accumulation of proliferating $CD8^+$ T cells potentially through induction of apoptosis (Fig. 5.1 C). The diverse outcomes of B7-H1:PD-1 interactions within these different models reflects the diverse repertoire of inhibitory mechanisms utilized by this pathway in order to suppress T lymphocytes (Freeman et al. 2000; Latchman et al. 2001; Hori et al. 2006; Muhlbauer et al. 2006; Keir et al. 2007; Zhang et al. 2008). Indeed, following PD-1 ligation, the SHP-2 phosphatase acts on numerous target proteins involved in diverse signaling pathways, congruent with multiple outcomes following B7-H1:PD-1 ligation (Okazaki 2001).

We propose that the varied consequences of B7-H1:PD-1 interactions observed in these studies may function together to create multiple checkpoints for maternal T cell control. During pregnancy, fetal antigen traffics to the lymph node and where it is presented to T cells by maternal APC (Erlebacher et al. 2007). The T cells subsequently respond by proliferating and upregulating activation markers (Erlebacher et al. 2007). PD-1 is also upregulated during this proliferation and upon ligation of B7-H1, its signaling may alter prosurvival/proapoptotic pathways to induce apoptosis of fetal-antigen specific cytotoxic $CD8^+$ T cells thereby preventing their accumulation (Fig. 5.1 C). Our finding that abrogation of this signal alone does not result in immunological attack of fetal tissues suggests that PD-1 works in

conjunction with other suppressive mechanisms to preclude perpetuation of maternal $CD8^+$ T cell effector functions.

In addition to their responses within the uterus-draining lymph nodes, it is clear that some maternal T lymphocytes populate to the uterus following implantation and once there, are not entirely quiescent, as evidenced by their expression of activation markers (Chao et al. 1999). Our studies suggest that B7-H1 on trophoblast cells interacts with PD-1 on decidual T cells to selectively modify their cytokine production, without induction of apoptosis, thereby allowing decidual T cells to carry out potential physiological functions in a controlled manner (Fig. 5.1A). Indeed, an excess of activated decidual T cells appears to be associated with a pathologic outcome of pregnancy, such as recurrent spontaneous abortion (Maruyama et al. 1992; Vassiliadou et al. 1999; Quack et al. 2001). T_{reg} expanded by placental trophoblasts or other mechanisms (Polanczyk et al. 2005; Blois et al. 2007; Kallikourdis et al. 2007) may control proliferation of maternal T cells to regulate their abundance within the decidua (Fig. 5.1B).

This multi-site activity is reflective of how the B7-H1:PD-1 pathway functions to maintain self-tolerance to peripheral tissues. For example, modulation of antigen-specific responses against pancreatic tissues involves B7-H1:PD-1 regulation within both draining lymph nodes and parenchymal tissue cells (Keir et al. 2007). Similar to our observations in murine pregnancy, PD-1 suppresses antigen-specific T cell activation in the pancreatic-draining lymph nodes. In addition, B7-H1-expressing pancreatic islet β cells islet cells provide an additional source of T cell modulation,

analogous to the functions we propose for B7-H1 expressing trophoblast cells in the human placenta.

Self-tolerance has been described as "a robust state with multiple checkpoints that must be overcome before autoimmunity is unleashed" (Hamilton-Williams et al. 2005). The same can be said of tolerance to fetal antigens in pregnancy; indeed, dependence on a single tolerizing mechanism could be catastrophic for species propagation. These studies suggest that B7-H1:PD-1 interactions control maternal T lymphocyte activity at multiple sites through diverse mechanisms. In light of the current results, future analysis of suppressive pathways in pregnancy should consider their activity within multiple sites of interaction between maternal immune cells and fetal cells/antigens.

B. Future Directions

The immune system is composed of several cell types functioning through complex series of signals and cellular interactions. Thus, examination of individual leukocyte populations must be followed with an analysis of their interactions with other components of the immune system. Several studies to date have focused on maternal T cell activation and effector function in response to both major and minor fetal antigens, and several lines of evidence point to a hyporesponsiveness of these cells. However, a critical component of peripheral tolerance is not only the resulting anergy or deletion of T cells, but also the status of the dendritic cell (DC) delivering the antigen against which that T cell must be tolerized. A valuable, yet understudied,

area of research of maternal-fetal tolerance is examination of maternal antigen presenting cells within secondary lymphoid organs during pregnancy. Indeed, DC within lymph nodes draining mucosal immune sites have different, and often uniquely tolerogenic properties compared to systemic lymph nodes (Kraal et al. 2006).

Local microenvironments play an important role in dictating dendritic cell function and phenotype. Pregnancy itself may alter specific properties of dendritic cells within decidua or other tissues through mechanisms including hormones, cytokines, and HLA-G (Steinbrink et al. 2000; Holmes et al. 2003; Seavey et al. 2006; Apps et al. 2007). In addition, DC may also be programmed through ingestion and presentation of foreign antigens associated with dying cells, known mechanism to induce long-lasting T cell tolerance provided that inflammatory signals are absent (Liu et al. 2002). Apoptotic trophoblast cells and debris containing fetal antigens are regularly shed into the maternal circulation during pregnancy, providing exactly the right types of signals to immature maternal dendritic cells for induction of T cell tolerance.

Thus presentation of fetal antigens by maternal DC could be a means to educate the maternal immune system to prevent anti-fetal reactions (Blois et al. 2007). This may be in contrast to pathological pregnancies in which overabundance or imbalance of immunogenic dendritic cells might lead to failure of immunological tolerance of the fetus (Askelund et al. 2004; Huang et al. 2008). Additionally, maternal DC presentation of fetal-derived peptides may change maternal immunologic "self" temporarily during pregnancy (Adams et al. 2007). In fact, co-

presentation of fetal and maternal peptides could lead to transient remission of certain autoimmune diseases during pregnancy (Hench 1938; Kung et al. 1998; Houtchens 2007). Thus, further studies of DC in maternal-fetal tolerance may have broader applications and could provide compelling models for the study of autoimmunity.

C. Overall Significance

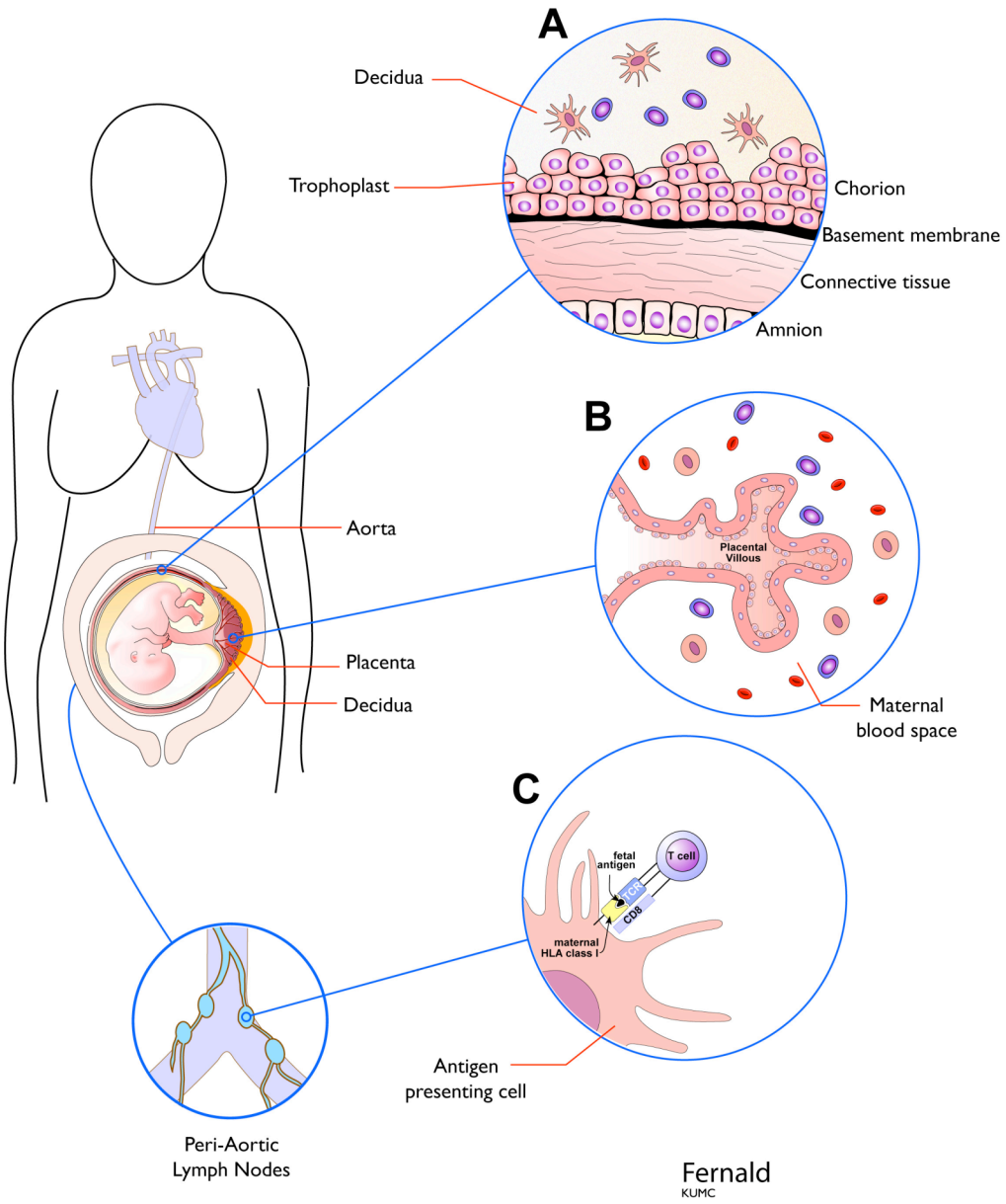
These studies have mapped expression of PD-1 during pregnancy and defined its role in modifying maternal T lymphocyte activity. Improper control of maternal T lymphocyte activation is associated with recurrent spontaneous abortion and pre-eclampsia in humans (Maruyama et al. 1992; Piccinni et al. 1998; Vassiliadou et al. 1999; Quack et al. 2001; Sasaki et al. 2004; Darmochwal-Kolarz et al. 2007; Sasaki et al. 2007; Yang et al. 2007) and allogeneic fetal loss in mice (Aluvihare et al. 2004; Darrasse-Jeze et al. 2006). Thus, the conclusions drawn from our work may begin to provide a basis for novel therapies in these pregnancy pathologies. The B7-H1:PD-1 pathway is also involved in self-tolerance, allograft acceptance, and cancer. Understanding how B7-H1 and PD-1 are naturally recruited during pregnancy to control maternal immune activity may aid in the development of improved treatments for autoimmune disease and transplantation and more effective models for targeting tumor growth.

This research also gives insight into the mechanisms through which the adaptive immune system is properly controlled despite its broad spectrum of functions throughout the body. Because its activity involves several cells and signals

located within numerous tissues, modulation of adaptive immunity may employ a specific repertoire of mechanisms with diverse applications at multiple sites, as we have observed for the B7-H1:PD-1 pathway in pregnancy. Further, our work highlights that the designation of "adaptive" for this branch of the immune system means that it not only adapts to mount specific responses against pathogens, but also can be adapted through immunosuppressive pathways to accommodate physiological events that are beneficial for propagation of the species.

D. Figures and Legends

Figure 5.1 Functions of PD-1 within the multi-faceted maternal-fetal interface. Schematic diagrams showing immunomodulation through PD-1:B7-H1 interactions within the placenta (A,B) and uterus draining lymph nodes (C). (A) PD-1 expressed on decidual T cells may interact with B7-H1 on trophoblast cells interactions within extraplacental membranes to modify their cytokine production. (B) Cytotrophoblast cells in the villous placenta induce regulatory T cells (T_{reg}) (C) B7-H1:PD-1 interactions within the peri-aortic lymph nodes may control accumulation of paternal antigen-specific T cells during pregnancy through induction of apoptosis.


A
Decidua
Trophoplast
Chorion
Basement membrane
Connective tissue
Amnion
B
Placental Villous
Maternal blood space
Aorta
Placenta
Decidua
C
fetal antigen
T cell
CD8
maternal HLA class I
Antigen presenting cell
Peri-Aortic Lymph Nodes
Fernald
KUMC

References

Abbas, A. K. (2003). "The control of T cell activation vs. tolerance." Autoimmun Rev **2**(3): 115-8.

Adams, K. M., Z. Yan, et al. (2007). "The changing maternal "self" hypothesis: a mechanism for maternal tolerance of the fetus." Placenta **28**(5-6): 378-382.

Agata, Y., A. Kawasaki, et al. (1996). "Expression of the PD-1 antigen on the surface of stimulated mouse T and B lymphocytes." Int Immunol. **8**(5): 765-772.

Aluvihare, V. R., M. Kallikourdis, et al. (2004). "Regulatory T cells mediate maternal tolerance to the fetus." Nat Immunol **5**(3): 266-271.

Apostolou, I., A. Sarukhan, et al. (2002). "Origin of regulatory T cells with known specificity for antigen." Nat Immunol **3**(8): 756-63.

Apps, R., L. Gardner, et al. (2007). "A homodimeric complex of HLA-G on normal trophoblast cells modulates antigen-presenting cells via LILRB1." Eur J Immunol **37**(7): 1924-37.

Arck, P., P. J. Hansen, et al. (2007). "Progesterone during pregnancy: endocrine-immune cross talk in mammalian species and the role of stress." Am J Reprod Immunol **58**(3): 268-79.

Aruffo, A. and B. Seed (1987). "Molecular cloning of a CD28 cDNA by a high-efficiency ICOS cell expression system." PNAS **84**(23): 8573-8577.

Ashkar, A. A., J. P. Di Santo, et al. (2000). "Interferon gamma contributes to initiation of uterine vascular modification, decidual integrity, and uterine

natural killer cell maturation during normal murine pregnancy." J Exp Med **192**(2): 259-70.

Askelund, K., H. S. Liddell, et al. (2004). "CD83(+)dendritic cells in the decidua of women with recurrent miscarriage and normal pregnancy." Placenta **25**(2-3): 140-5.

Baban, B., P. Chandler, et al. (2004). "Indoleamine 2,3-dioxygenase expression is restricted to fetal trophoblast giant cells during murine gestation and is maternal genome specific." J Reprod Immunol **61**(2): 67-77.

Bahri, R., F. Hirsch, et al. (2006). "Soluble HLA-G inhibits cell cycle progression in human alloreactive T lymphocytes." J Immunol **176**(3): 1331-1339.

Ballif, B. A. and J. Blenis (2001). "Molecular mechanisms mediating mammalian mitogen-activated protein kinase (MAPK) kinase (MEK)-MAPK cell survival signals." Cell Growth Differ **12**(8): 397-408.

Barber, D. L., E. J. Wherry, et al. (2006). "Restoring function in exhausted CD8 T cells during chronic viral infection." Nature **439**(7077): 682-687.

Benirschke, K. (1994). "Anatomical relationship between fetus and mother." Ann N Y Acad Sci **731**: 9-20.

Benirschke, K. and P. Kaufmann (2000). Pathology of the Human Placenta, Springer.

Beswick, E. J., I. V. Pinchuk, et al. (2007). "Expression of the programmed death ligand 1, B7-H1, on gastric epithelial cells after helicobacter pylori exposure promotes development of CD4(+) CD25(+) FoxP3(+) regulatory T cells." Infect Immun **75**(9): 4334-41.

Blois, S. M., J. M. Ilarregui, et al. (2007). "A pivotal role for galectin-1 in fetomaternal tolerance." Nat Med **13**(12): 1450-7.

Blois, S. M., U. Kammerer, et al. (2007). "Dendritic cells: key to fetal tolerance?" Biol Reprod **77**(4): 590-598.

Bonney, E. A. and P. Matzinger (1998). "Much IDO about pregnancy." Nat Med **4**(10): 1128-9.

Bretscher, P. A. (1999). "A two-step, two-signal model for the primary activation of precursor helper T cells." PNAS **96**(1): 185-190.

Brown, J. A., D. M. Dorfman, et al. (2003). "Blockade of programmed death-1 ligands on dendritic cells enhances T cell activation and cytokine production." J Immunol **170**(3): 1257-1266.

Brunet, J.-F. and P. Golstein (1987). "A new member of the immunoglobulin superfamily-CTLA-4." Nature (328): 267-270.

Buonocore, G., M. De Filippo, et al. (1995). "Maternal and neonatal plasma cytokine levels in relation to mode of delivery." Biol Neonate **68**(2): 104-10.

Carter, L., L. A. Fouser, et al. (2002). "PD-1:PD-L inhibitory pathway affects both CD4(+) and CD8(+) T cells and is overcome by IL-2." Eur J Immunol **32**(3): 634-43.

Chao, K.-H., M.-Y. Wu, et al. (1999). "The expression of killer cell inhibitory receptors on natural killer cells and activation status of CD4+ and CD8+ T cells in the decidua of normal and abnormal early pregnancies." Human Immunol **60**(9): 791-797.

Chemnitz, J. M., R. V. Parry, et al. (2004). "SHP-1 and SHP-2 associate with immunoreceptor tyrosine-based switch motif of programmed death 1 upon primary human T cell stimulation, but only receptor ligation prevents T cell activation." J Immunol **173**: 945-954.

Cohen, M., A. Meisser, et al. (2006). "Involvement of MAPK pathway in TNF-alpha-induced MMP-9 expression in human trophoblastic cells." Mol Hum Reprod **12**(4): 225-32.

Cook, D. N. and K. Bottomly (2007). "Innate immune control of pulmonary dendritic cell trafficking." Proc Am Thorac Soc **4**(3): 234-9.

Coumans, B., O. Thellin, et al. (1999). "Lymphoid cell apoptosis induced by trophoblastic cells: a model of active foeto-placental tolerance." J Immunol Methods **224**(1-2): 185-96.

Darmochwal-Kolarz, D., S. Saito, et al. (2007). "Activated T lymphocytes in pre-eclampsia." Am J Reprod Immunol **58**(1): 39-45.

Darrasse-Jeze, G., D. Klatzmann, et al. (2006). "CD4+CD25+ regulatory/suppressor T cells prevent allogeneic fetus rejection in mice." Immunol Lett **102**(1): 106-9.

Das, U. N. (2001). "Is obesity an inflammatory condition?" Nutrition **17**(11-12): 953-66.

Dausset, J. (1954). "Leukoagglutinins IV. Leukoagglutinins and blood transfusion." Vox Sang **4**: 190.

Dong, H., S. E. Strome, et al. (2002). "Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion". Nat Med **8**(8): 793-800.

Dong, H., G. Zhu, et al. (1999). "B7-H1, a third member of the B7 family, co-stimulates T cell proliferation and interleukin 10 secretion." Nat Med **5**(12): 1365-9.

Dong, H., G. Zhu, et al. (2004). "B7-H1 determines accumulation and deletion of intrahepatic CD8+ T lymphocytes." Immunity **20**(3): 327-336.

Drappa, J., N. Brot, et al. (1993). "The Fas protein is expressed at high levels on CD4+CD8+ thymocytes and activated mature lymphocytes in normal mice but not in the lupus-prone strain, MRL lpr/lpr." Proc Natl Acad Sci U S A **90**(21): 10340-4.

El-Shazly, S., M. Makhseed, et al. (2004). "Increased expression of pro-inflammatory cytokines in placentas of women undergoing spontaneous preterm delivery or premature rupture of membranes." Am J Reprod Immunol **52**(1): 45-52.

Erlebacher, A., D. Vencato, et al. (2007). "Constraints in antigen presentation severely restrict T cell recognition of the allogeneic fetus." J Clin Invest **117**(5): 1399-411.

Everett, C. (1997). "Incidence and outcome of bleeding before the 20th week of pregnancy: prospective study from general practice." BMJ **315**(7099): 32-34.

Freedman, A. S., G. Freeman, et al. (1987). "B7, a B-cell-restricted antigen that identifies preactivated B cells." J Immunol **139**(10): 3260-3267.

Freeman, G. J. (1993). "Cloning of B7-2: a CTLA-4 counter-receptor that costimulates human T cell proliferation." Science **262**(5135): 844-5.

Freeman, G. J., F. Borriello, et al. (1993). "Murine B7-2, an alternative CTLA4 counter-receptor that costimulates T cell proliferation and interleukin 2 production." J Exp Med **178**(6): 2185-2192.

Freeman, G. J., G. S. Gray, et al. (1991). "Structure, expression, and T cell costimulatory activity of the murine homologue of the human B lymphocyte activation antigen B7." J Exp Med **174**(3): 625-631.

Freeman, G. J., A. J. Long, et al. (2000). "Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation." J Exp Med **192**(7): 1027-1034.

Fukushima, K., S. Miyamoto, et al. (2003). "TNFalpha-induced apoptosis and integrin switching in human extravillous trophoblast cell line." Biol Reprod **68**(5): 1771-8.

Gao, W. D., Gulcin; Strom, Terry B.; Li, Xian Chang (2003). "Stimulating PD-1-negative signals concurrent with blocking CD154 co-stimulation induces long-term islet allograft survival." Transplantation **76**(6): 994-999.

Gardner, L. and A. Moffett (2003). "Dendritic cells in the human decidua." Biol Reprod **69**(4): 1438-1446.

Georgiades, P., A. C. Ferguson-Smith, et al. (2002). "Comparative developmental anatomy of the murine and human definitive placentae." Placenta **23**(1): 3-19.

Graham, C. H., J. J. Lysiak, et al. (1992). "Localization of transforming growth factor-beta at the human fetal-maternal interface: role in trophoblast growth and differentiation." Biol Reprod **46**(4): 561-72.

Greenwald, R. J., G. J. Freeman, et al. (2005). "The B7 family revisited." Ann Rev Immunol **23**(1): 515-548.

Gross, J. A., T. St. John, et al. (1990). "The murine homologue of the T lymphocyte antigen CD28. Molecular cloning and cell surface expression." J Immunol **144**(8): 3201-3210.

Guimond, M. J., J. A. Luross, et al. (1997). "Absence of natural killer cells during murine pregnancy is associated with reproductive compromise in TgE26 mice." Biol Reprod **56**(1): 169-179.

Guleria, I., A. Khosroshahi, et al. (2005). "A critical role for the programmed death ligand 1 in fetomaternal tolerance." J Exp Med **202**(2): 231-237.

Habicht, A., S. Dada, et al. (2007). "A link between PDL1 and T regulatory cells in fetomaternal tolerance." J Immunol **179**(8): 5211-9.

Haller, H., O. Radillo, et al. (1993). "An immunohistochemical study of leucocytes in human endometrium, first and third trimester basal decidua." J Reprod Immunol **23**(1): 41-9.

Hamilton-Williams, E. E., A. Lang, et al. (2005). "TLR ligands are not sufficient to break cross-tolerance to self-antigens." J Immunol **174**: 1159-1163.

Hanna, J., D. Goldman-Wohl, et al. (2006). "Decidual NK cells regulate key developmental processes at the human fetal-maternal interface." Nat Med **12**(9): 1065-74.

Hanna, J. and O. Mandelboim (2007). "When killers become helpers." Trends Immunol **28**(5): 201-6.

Harris, L. K. and J. D. Aplin (2007). "Vascular remodeling and extracellular matrix breakdown in the uterine spiral arteries during pregnancy." Reprod Sci **14**(8 Suppl): 28-34.

Hatachi, S., Y. Iwai, et al. (2003). "CD4+ PD-1+ T cells accumulate as unique anergic cells in rheumatoid arthritis synovial fluid." J Rheumatol **30**(7): 1410-9.

Haxhinasto, S., C. Benoist, et al. (2007). "Regulatory T-cell differentiation: Committed to control: a precocious choice?" Immunol Cell Biol **85**(3): 175-6.

Heikkinen, J., M. Mottonen, et al. (2003). "Phenotypic characterization of human decidual macrophages." Clin Exp Immunol **131**(3): 498-505.

Hench, P. (1938). "The ameliorating effect of pregnancy on chronic atrophic infections, rheumatoid arthritis, fibrositis, and intermittent hydroarthrosis." Mayo Clin Proc **13**: 161-167.

Hiby, S. E., J. J. Walker, et al. (2004). "Combinations of maternal KIR and fetal HLA-C genes influence the risk of preeclampsia and reproductive success." J Exp Med **200**(8): 957-65.

Holets, L. M., J. S. Hunt, et al. (2006). "Trophoblast CD274 (B7-H1) is differentially expressed across gestation: influence of oxygen concentration." Biol Reprod **74**(2): 352-358.

Holmes, C. H., K. L. Simpson, et al. (1990). "Preferential expression of the complement regulatory protein decay accelerating factor at the fetomaternal interface during human pregnancy." J Immunol **144**(8): 3099-105.

Holmes, V. A., J. M. Wallace, et al. (2003). "Plasma levels of the immunomodulatory cytokine interleukin-10 during normal human pregnancy: a longitudinal study." Cytokine **21**(6): 265-9.

Hori, J., M. Wang, et al. (2006). "B7-H1-induced apoptosis as a mechanism of immune privilege of corneal allografts." J Immunol **177**(9): 5928-35.

Houtchens, M. (2007). "Pregnancy and multiple sclerosis." Semin Neurol **27**: 434-441.

Hsi, B. L., J. S. Hunt, et al. (1991). "Differential expression of complement regulatory proteins on subpopulations of human trophoblast cells." J Reprod Immunol **19**(3): 209-23.

Hsieh, C. S., Y. Zheng, et al. (2006). "An intersection between the self-reactive regulatory and nonregulatory T cell receptor repertoires." Nat Immunol **7**(4): 401-10.

Huang, S. J., C. P. Chen, et al. (2008). "Pre-eclampsia is associated with dendritic cell recruitment into the uterine decidua." J Pathol **214**(3): 328-36.

Hugues, S., A. Boissonnas, et al. (2006). "The dynamics of dendritic cell-T cell interactions in priming and tolerance." Curr Opin Immunol **18**(4): 491-5.

Hunt, J. S. (1994). "Immunologically relevant cells in the uterus." Biol Reprod **50**(3): 461-6.

Hunt, J. S. and J. W. Pollard (1992). "Macrophages in the uterus and placenta." Curr Top Microbiol Immunol **181**: 39-63.

Ishida, Y., Y. Agata, et al. (1992). "Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death." EMBO J. **11**: 3887-3895.

Ishitani, A., N. Sageshima, et al. (2003). "Protein expression and peptide binding suggest unique and interacting functional roles for HLA-E, F, and G in maternal-placental immune recognition." J Immunol **171**(3): 1376-1384.

Isogawa, M., Y. Furuichi, et al. (2005). "Oscillating CD8+ T cell effector functions after antigen recognition in the liver." Immunity **23**: 53-63.

Ito, T., T. Ueno, et al. (2005). "Analysis of the role of negative T cell costimulatory pathways in CD4 and CD8 T cell-mediated alloimmune responses in vivo." J Immunol **174**(11): 6648-56.

Iwai, Y., M. Ishida, et al. (2002). "Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade." PNAS **99**(19): 12293-12297.

Iwai, Y., S. Terawaki, et al. (2003). "PD-1 inhibits antiviral immunity at the effector phase in the liver." J Exp Med **198**(1): 39-50.

Jenkins, C., J. Roberts, et al. (2000). "Evidence of a TH 1 type response associated with recurrent miscarriage." Fertil Steril **73**(6): 1206-1208.

Jenkins, M. K., P. S. Taylor, et al. (1991). "CD28 delivers a costimulatory signal involved in antigen-specific IL-2 production by human T cells." J Immunol **147**(8): 2461-2466.

Jiang, B. and C. R. Mendelson (2005). "O2 enhancement of human trophoblast differentiation and hCYP19 (aromatase) gene expression are mediated by proteasomal degradation of USF1 and USF2." Mol Cell Biol **25**(20): 8824-33.

Jinushi, M., T. Takehara, et al. (2007). "Natural killer cell and hepatic cell interaction via NKG2A leads to dendritic cell-mediated induction of CD4+ CD25+ T cells with PD-1-dependent regulatory activities." Immunology **120**(1): 73-82.

Jordan, M. S., A. Boesteanu, et al. (2001). "Thymic selection of CD4+CD25+ regulatory T cells induced by an agonist self-peptide." Nat Immunol **2**(4): 301-6.

Kallikourdis, M., K. G. Andersen, et al. (2007). "Alloantigen-enhanced accumulation of CCR5+ 'effector' regulatory T cells in the gravid uterus." Proc Natl Acad Sci U S A **104**(2): 594-9.

Kammerer, U., M. Schoppet, et al. (2000). "Human decidua contains potent immunostimulatory CD83+ dendritic cells." Am J Pathol **157**(1): 159-169.

Kanai, T., T. Fujii, et al. (2001). "Human leukocyte antigen-G-expressing cells differently modulate the release of cytokines from mononuclear cells present in the decidua versus peripheral blood." Am J Reprod Immunol **45**(2): 94-99.

Kaufmann, P. (1982). "Development and differentiation of the human placental villous tree." Bibl Anat(22): 29-39.

Keir, M. E., G. J. Freeman, et al. (2007). "PD-1 regulates self-reactive CD8+ T cell responses to antigen in lymph nodes and tissues." J Immunol **179**(8): 5064-70.

Keir, M. E., Y. E. Latchman, et al. (2005). "Programmed death-1 (PD-1):PD-ligand 1 interactions inhibit TCR-mediated positive selection of thymocytes." J Immunol **175**(11): 7372-9.

Keir, M. E., S. C. Liang, et al. (2006). "Tissue expression of PD-L1 mediates peripheral T cell tolerance." J Exp Med **203**(4): 883-895.

Kim, S., D. Lee, et al. (2005). "Interferon-gamma promotes abortion due to Brucella infection in pregnant mice." BMC Microbiology **5**(1): 22.

King, A., T. D. Burrows, et al. (2000). "Surface expression of HLA-C antigen by human extravillous trophoblast." Placenta **21**(4): 376-87.

Klein, J. (1986). "Seeds of time: fifty years ago Peter A. Gorer discovered the H-2 complex." Immunogenetics **24**(6): 331-8.

Kraal, G., J. N. Samsom, et al. (2006). "The importance of regional lymph nodes for mucosal tolerance." Immunol Rev **213**: 119-30.

Krupnick, A. S., A. E. Gelman, et al. (2005). "Cutting edge: murine vascular endothelium activates and induces the generation of allogeneic CD4+25+Foxp3+ regulatory T cells." J Immunol **175**(10): 6265-6270.

Kung, A. W. C. and B. M. Jones (1998). "A change from stimulatory to blocking antibody activity in Graves' disease during pregnancy." J Clin Endocrinol Metab **83**(2): 514-518.

Latchman, Y., C. R. Wood, et al. (2001). "PD-L2 is a second ligand for PD-1 and inhibits T cell activation." Nat Immunol **2**(3): 261-268.

Latchman, Y. E., S. C. Liang, et al. (2004). "PD-L1-deficient mice show that PD-L1 on T cells, antigen-presenting cells, and host tissues negatively regulates T cells." PNAS **101**(29): 10691-10696.

Le Rond, S., C. Azema, et al. (2006). "Evidence to support the role of HLA-G5 in allograft acceptance through induction of immunosuppressive/ regulatory T cells." J Immunol **176**(5): 3266-76.

Liang, S. C., Y. E. Latchman, et al. (2003). "Regulation of PD-1, PD-L1, and PD-L2 expression during normal and autoimmune responses." Eur J Immunol **33**(10): 2706-2716.

Liu, K., T. Iyoda, et al. (2002). "Immune tolerance after delivery of dying cells to dendritic cells in situ." J Exp Med **196**(8): 1091-7.

Martin-Orozco, N., Y. H. Wang, et al. (2006). "Cutting Edge: programmed death (PD) ligand-1/PD-1 interaction is required for CD8+ T cell tolerance to tissue antigens." J Immunol **177**(12): 8291-5.

Maruyama, T., T. Makino, et al. (1992). "Flow-cytometric analysis of immune cell populations in human decidua from various types of first-trimester pregnancy." Hum Immunol **34**(3): 212-8.

McIntire, R. H., P. J. Morales, et al. (2004). "Recombinant HLA-G5 and -G6 drive U937 myelomonocytic cell production of TGF-beta1." J Leukoc Biol **76**(6): 1220-8.

Medawar, P. (1953). "Some immunological and endocrinological problems raised by the evolution of viviparity in vertebrates." Symp Soc Exp Biol **7**: 320-338.

Mellor, A. L. and D. H. Munn (2004). "IDO expression by dendritic cells: tolerance and tryptophan catabolism." Nat Rev Immunol **4**(10): 762-74.

Mincheva-Nilsson, L., V. Baranov, et al. (1994). "Immunomorphologic studies of human decidua-associated lymphoid cells in normal early pregnancy." J Immunol **152**(4): 2020-32.

Mizuno, M., K. Aoki, et al. (1994). "Functions of macrophages in human decidual tissue in early pregnancy." Am J Reprod Immunol **31**(4): 180-8.

Mjihdi, A., C. Truyens, et al. (2004). "Systemic and placental productions of tumor necrosis factor contribute to induce fetal mortality in mice acutely infected with Trypanosoma cruzi." Exp Parasitol **107**(1-2): 58-64.

Mor, G., L. S. Gutierrez, et al. (1998). "Fas-fas ligand system-induced apoptosis in human placenta and gestational trophoblastic disease." Am J Reprod Immunol **40**(2): 89-94.

Mor, G., R. Romero, et al. (2006). Macrophages and Pregnancy. Immunology of Pregnancy. G. Mor. New York, NY, Sringer Science+Business Media**:** 63-72.

Morales, P. J., J. L. Pace, et al. (2003). "Placental cell expression of HLA-G2 isoforms is limited to the invasive trophoblast phenotype." J Immunol **171**(11): 6215-24.

Morii, T., K. Nishikawa, et al. (1993). "T cell receptors are expressed but down-regulated on intradecidual T lymphocytes." Am J Reprod Immunol **29**(1): 1-4.

Morrish, D. W., D. Bhardwaj, et al. (1987). "Epidermal growth factor induces differentiation and secretion of human chorionic gonadotropin and placental lactogen in normal human placenta." J Clin Endocrinol Metab **65**(6): 1282-90.

Mucida, D., Y. Park, et al. (2007). "Reciprocal Th17 and regulatory T cell differentiation mediated by retinoic acid." Science **317**(5835): 256-60.

Muhlbauer, M., M. Fleck, et al. (2006). "PD-L1 is induced in hepatocytes by viral infection and by interferon-alpha and -gamma and mediates T cell apoptosis." J Hepatol **45**(4): 520-8.

Munn, D. H., M. Zhou, et al. (1998). "Prevention of allogeneic fetal rejection by tryptophan catabolism." Science **281**(5380): 1191-3.

Murphy, S. P., L. D. Fast, et al. (2005). "Uterine NK cells mediate inflammation-induced fetal demise in IL-10-null mice." J Immunol **175**(6): 4084-4090.

Murphy, S. P. and T. B. Tomasi (1998). "Absence of MHC class II antigen expression in trophoblast cells results from a lack of class II transactivator (CIITA) gene expression." Mol Reprod Dev **51**(1): 1-12.

Naji, A., S. Le Rond, et al. (2007). "CD3+CD4low and CD3+CD8low are induced by HLA-G: novel human peripheral blood suppressor T-cell subsets involved in transplant acceptance." Blood **110**(12): 3936-48.

Ni, R., K. Ihara, et al. (2007). "PD-1 gene haplotype is associated with the development of type 1 diabetes mellitus in Japanese children." Hum Genet **121**(2): 223-32.

Nielsen, C., D. Hansen, et al. (2003). "Association of a putative regulatory polymorphism in the PD-1 gene with susceptibility to type 1 diabetes." Tissue Antigens **62**(6): 492-7.

Nielsen, C., L. Ohm-Laursen, et al. (2005). "Alternative splice variants of the human PD-1 gene." Cellular Immunology **235**(2): 109-116.

Nishimura, H., N. Minato, et al. (1998). "Immunological studies on PD-1 deficient mice: implication of PD-1 as a negative regulator for B cell responses." Int. Immunol. **10**(10): 1563-1572.

Nishimura, H., M. Nose, et al. (1999). "Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor." Immunity **11**(2): 141-151.

Nishimura, H., T. Okazaki, et al. (2001). "Autoimmune dilated cardiomyopathy in PD-1 receptor-deficient mice." Science **291**(5502): 319-322.

Nishimura, H., Y. Tanaka, et al. (2003). "Autoantibodies against cardiac troponin I are responsible for dilated cardiomyopathy in PD-1-deficient mice." Nature Med. **9**: 1477-83.

Okazaki, T. (2001). "PD-1 immunoreceptor inhibits B cell receptor-mediated signaling by recruiting src homology 2-domain-containing tyrosine phosphatase 2 to phosphotyrosine." Proc Natl Acad Sci USA **98**: 13866-13871.

Ozkaynak, E., L. Wang, et al. (2002). "Programmed death-1 targeting can promote allograft survival." J Immunol **169**(11): 6546-6553.

Pasqualini, J. R. (2005). "Enzymes involved in the formation and transformation of steroid hormones in the fetal and placental compartments." J Steroid Biochem Mol Biol **97**(5): 401-15.

Paul A. Saunders, et al. (2005). "PD-L2:PD-1 involvement in T cell proliferation, cytokine production, and integrin-mediated adhesion." Eur J Immunol **35**(12): 3561-3569.

Pearson, H. (2002). "Reproductive immunology: Immunity's pregnant pause." Nature **420**(6913): 265-266.

Pepe, G. J. and E. D. Albrecht (1999). "Regulation of functional differentiation of the placental villous syncytiotrophoblast by estrogen during primate pregnancy." Steroids **64**(9): 624-7.

Petroff, M. G. (2005a). "Immune interactions at the maternal-fetal interface." J Reprod Immunol **68**(1-2): 1-13.

Petroff, M. G. (2006a). B7 family molecules in the placenta. Immunology of Pregnancy. G. Mor. New York, NY, Springer Science+Business Media, Inc.**:** 159-166.

Petroff, M. G., L. Chen, et al. (2003). "B7 family molecules are favorably positioned at the human maternal-fetal interface." Biol Reprod **68**(5): 1496-1504.

Petroff, M. G., E. Kharatyan, et al. (2005b). "The immunomodulatory proteins B7-DC, B7-H2, and B7-H3 are differentially expressed across gestation in the human placenta." Am J Pathol **167**(2): 465-473.

Petroff, M. G., T. A. Phillips, et al. (2006b). "Isolation and culture of term human trophoblast cells." Meth Mol Med **121**: 203-217.

Piccinni, M.-P., L. Beloni, et al. (1998). "Defective production of both leukemia inhibitory factor and type 2 T-helper cytokines by decidual T cells in unexplained recurrent abortions." Nat Med **4**(9): 1020-1024.

Pillai, V. and N. J. Karandikar (2007). "Human regulatory T cells: a unique, stable thymic subset or a reversible peripheral state of differentiation?" Immunol Lett **114**(1): 9-15.

Polanczyk, M. J., C. Hopke, et al. (2005). "Enhanced FoxP3 expression and Treg cell function in pregnant and estrogen-treated mice." J Neuroimmunol **170**(1-2): 85-92.

Polanczyk, M. J., C. Hopke, et al. (2006). "Estrogen-mediated immunomodulation involves reduced activation of effector T cells, potentiation of Treg cells, and enhanced expression of the PD-1 costimulatory pathway." J Neurosci Res **84**(2): 370-378.

Pollard, J. K. and M. D. Mitchell (1993). "Tumor necrosis factor alpha stimulates amnion prostaglandin biosynthesis primarily via an action on fatty acid cyclooxygenase." Prostaglandins **46**(6): 499-510.

Prieto, G. A. and Y. Rosenstein (2006). "Oestradiol potentiates the suppressive function of human CD4 CD25 regulatory T cells by promoting their proliferation." Immunology **118**(1): 58-65.

Prokunina, L., C. Castillejo-Lopez, et al. (2002). "A regulatory polymorphism in PDCD1 is associated with susceptibility to systemic lupus erythematosus in humans." Nat Genet **32**(4): 666-9.

Quack, K. C., N. Vassiliadou, et al. (2001). "Leukocyte activation in the decidua of chromosomally normal and abnormal fetuses from women with recurrent abortion." Hum Reprod **16**(5): 949-55.

Raimondi, G., W. J. Shufesky, et al. (2006). "Regulated compartmentalization of programmed cell death-1 discriminates CD4+CD25+ resting regulatory T cells from activated T cells." J Immunol **176**(5): 2808-16.

Redmond, W. L., C. H. Wei, et al. (2008). "The apoptotic pathway contributing to the deletion of naive CD8 T cells during the induction of peripheral tolerance to a cross-presented self-antigen." J Immunol **180**(8): 5275-82.

Rieger, L., A. Honig, et al. (2004). "Antigen presenting cells in human endometrium during the menstrual cycle compared to early pregnancy." J Soc Gynecol Investig **11**(7): 488-493.

Rieger, L., U. Kammerer, et al. (2001). "Choriocarcinoma cells modulate the cytokine production of decidual large granular lymphocytes in coculture." Am J Reprod Immunol **46**(2): 137-143.

Robertson, S. A., A. S. Care, et al. (2007). "Interleukin 10 regulates inflammatory cytokine synthesis to protect against lipopolysaccharide-induced abortion and fetal growth restriction in mice." Biol Reprod **76**(5): 738-48.

Rocha, P. N., T. J. Plumb, et al. (2003). "Effector mechanisms in transplant rejection." Immunol Rev **196**(1): 51-64.

Rogers, A. M., I. Boime, et al. (1998). "Maternal-fetal tolerance is maintained despite transgene-driven trophoblast expression of MHC class I, and defects in Fas and its ligand." Eur J Immunol **28**(11): 3479-87.

Saito, S., Y. Sasaki, et al. (2005). "CD4(+)CD25high regulatory T cells in human pregnancy." J Reprod Immunol **65**(2): 111-20.

Sakaguchi, S. (2000). "Regulatory T cells: Key controllers of immunologic self-tolerance." Cell **101**: 455-458.

Sasaki, Y., D. Darmochwal-Kolarz, et al. (2007). "Proportion of peripheral blood and decidual CD4(+) CD25(bright) regulatory T cells in pre-eclampsia." Clin Exp Immunol **149**(1): 139-45.

Sasaki, Y., M. Sakai, et al. (2004). "Decidual and peripheral blood CD4+CD25+ regulatory T cells in early pregnancy subjects and spontaneous abortion cases." Mol Hum Reprod **10**(5): 347-353.

Scaife, P. J., J. N. Bulmer, et al. (2006). "Effector activity of decidual CD8+ T lymphocytes in early human pregnancy." Biol Reprod **75**(4): 562-567.

Seavey, M. M. and T. R. Mosmann (2006). "Paternal antigen-bearing cells transferred during insemination do not stimulate anti-paternal CD8+ T cells: role of estradiol in locally inhibiting CD8+ T cell responses." J Immunol **177**(11): 7567-78.

Selmani, Z., A. Naji, et al. (2008). "Human leukocyte antigen-G5 secretion by human mesenchymal stem cells is required to suppress T lymphocyte and natural killer function and to induce CD4+CD25highFOXP3+ regulatory T cells." Stem Cells **26**(1): 212-22.

Shahinian, A., K. Pfeffer, et al. (1993). "Differential T cell costimulatory requirements in CD28-deficient mice." Science **261**(5121): 609-612.

Shao, L., A. R. Jacobs, et al. (2005). "Activation of CD8+ regulatory T cells by human placental trophoblasts." J Immunol **174**(12): 7539-7547.

Shevach, E. M. (2002). "CD4+CD25+ suppressor T cells: more questions than answers." Nat Rev Immunol **2**(6): 389-400.

Shlapatska, L. M., S. V. Mikhalap, et al. (2001). "CD150 association with either the SH2-containing inositol phosphatase or the SH2-containing protein tyrosine phosphatase Is regulated by the adaptor protein SH2D1A." J Immunol **166**(9): 5480-5487.

Simpson, E. (2006). "A historical perspective on immunological privilege." Immunol Rev **213**: 12-22.

Simpson, E. R. and P. C. MacDonald (1981). "Endocrine physiology of the placenta." Annu Rev Physiol **43**: 163-88.

Sindram-Trujillo, A. P., S. A. Scherjon, et al. (2004). "Comparison of decidual leukocytes following spontaneous vaginal delivery and elective cesarean section in uncomplicated human term pregnancy." J Reprod Immunol **62**(1-2): 125-37.

Singer, G. G. and A. K. Abbas (1994). "The fas antigen is involved in peripheral but not thymic deletion of T lymphocytes in T cell receptor transgenic mice." Immunity **1**(5): 365-71.

Smith, P., C. M. Walsh, et al. (2004). "Schistosoma mansoni worms induce anergy of T cells via selective up-regulation of programmed death ligand 1 on macrophages." J Immunol **173**(2): 1240-1248.

Somerset, D. A., Y. Zheng, et al. (2004). "Normal human pregnancy is associated with an elevation in the immune suppressive CD25+CD4+ regulatory T-cell subset." Immunology **112**(1): 38-43.

Statistics, (1997). Fertility, family planning, and women's health: new data from the 1995 national survey of family growth. 1995 National Survey of Family Growth, Centers for Disease Control and Prevention/National Center for Health Statistics. **Series 23:** 68.

Steinbrink, K., L. Paragnik, et al. (2000). "Induction of dendritic cell maturation and modulation of dendritic cell-induced immune responses by prostaglandins." Arch Dermatol Res **292**(9): 437-45.

Steinman, R. M., D. Hawiger, et al. (2003). "Tolerogenic dendritic cells." Annu Rev Immunol **21**: 685-711.

Tai, P., J. Wang, et al. (2008). "Induction of regulatory T cells by physiological level estrogen." J Cell Physiol **214**(2): 456-64.

Takeda, K., T. Kaisho, et al. (2003). "Toll-like receptors." Annu Rev Immunol **21**: 335-76.

Tanaka, K., M. J. Albin, et al. (2007). "PDL1 is required for peripheral transplantation tolerance and protection from chronic allograft rejection." J Immunol **179**(8): 5204-10.

Thompson, R. H., M. D. Gillett, et al. (2004). "Costimulatory B7-H1 in renal cell carcinoma patients: Indicator of tumor aggressiveness and potential therapeutic target." PNAS **101**(49): 17174-17179.

Thompson, R. H., S. M. Kuntz, et al. (2006). "Tumor B7-H1 Is associated with poor prognosis in renal cell carcinoma patients with long-term follow-up." Cancer Res **66**(7): 3381-3385.

Thornton, A. M. and E. M. Shevach (1998). "CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production." J Exp Med **188**(2): 287-296.

Tilburgs, T., D. L. Roelen, et al. (2008). "Evidence for a selective migration of fetus-specific CD4+CD25bright regulatory T cells from the peripheral blood to the decidua in human pregnancy." J Immunol **180**(8): 5737-45.

Tilburgs, T., D. L. Roelen, et al. (2006). "Differential distribution of CD4(+)CD25(bright) and CD8(+)CD28(-) T-cells in decidua and maternal blood during human pregnancy." Placenta **27 Suppl A**: S47-53.

Tsaknaridis, L., L. Spencer, et al. (2003). "Functional assay for human CD4+CD25+ Treg cells reveals an age-dependent loss of suppressive activity." J Neurosci Res **74**(2): 296-308.

Tsushima, F., S. Yao, et al. (2007). "Interaction between B7-H1 and PD-1 determines initiation and reversal of T-cell anergy." Blood: blood-2006-11-060087.

Vacchio, M. S. and R. J. Hodes (2003). "CD28 costimulation is required for in vivo induction of peripheral tolerance in CD8 T cells." J Exp Med **197**(1): 19-26.

Vacchio, M. S. and R. J. Hodes (2005). "Fetal expression of Fas ligand is necessary and sufficient for induction of CD8 T cell tolerance to the fetal antigen H-Y during pregnancy." J Immunol **174**(8): 4657-61.

Vacchio, M. S. and S.-P. Jiang (1999). "The fetus and the maternal immune system: pregnancy as a model to study peripheral T-cell tolerance." Crit Rev in Immunol **19**(5-6): 461-80.

van Rood, J. J. (1993). "HLA and I." Annu Rev Immunol **11**: 1-28.

Vassiliadou, N. and J. N. Bulmer (1996). "Quantitative analysis of T lymphocyte subsets in pregnant and nonpregnant human endometrium." Biol Reprod **55**(5): 1017-1022.

Vassiliadou, N., R. F. Searle, et al. (1999). "Elevated expression of activation molecules by decidual lymphocytes in women suffering spontaneous early pregnancy loss." Hum Reprod **14**(5): 1194-200.

Vince, G., P. Starkey, et al. (1990). "Flow cytometric characterization of cell populations in human pregnancy decidua and isolation of decidual macrophages." J Immunol Methods **132**(2): 181-9.

Wan, B., H. Nie, et al. (2006). "Aberrant regulation of synovial T cell activation by soluble costimulatory molecules in rheumatoid arthritis." J Immunol **177**(12): 8844-8850.

Wang, L., R. Han, et al. (2007). "Programmed cell death 1 (PD-1) and its ligand PD-L1 are required for allograft tolerance." Eur J Immunol **37**(10): 2983-90.

Wang, S., J. Bajorath, et al. (2003). "Molecular modeling and functional mapping of B7-H1 and B7-DC uncouple costimulatory function from PD-1 interaction." J Exp Med **197**(9): 1083-1091.

Wegmann, T. G., H. Lin, et al. (1993). "Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a TH2 phenomenon?" Immunol Today **14**(7): 353-6.

White, C. A., M. Johansson, et al. (2004). "Effect of interleukin-10 null mutation on maternal immune response and reproductive outcome in mice." Biol Reprod **70**(1): 123-31.

Wilcox, A. J., C. R. Weinberg, et al. (1988). "Incidence of early loss of pregnancy." N Engl J Med **319**(4): 189-194.

Wilczynski, J. R. (2005). "Th1/Th2 cytokines balance-yin and yang of reproductive immunology." Eur J Obstet Gyne and Reprod Biol **122**(2): 136-143.

Xu, C., D. Mao, et al. (2000). "A critical role for murine complement regulator Crry in fetomaternal tolerance." Science **287**(5452): 498-501.

Yang, H., L. Qiu, et al. (2007). "Proportional change of CD4(+)CD25(+) regulatory T cells in decidua and peripheral blood in unexplained recurrent spontaneous abortion patients." Fertil Steril.

Yang, J., J. Popoola, et al. (2008). "Critical role of donor tissue expression of programmed death ligand-1 in regulating cardiac allograft rejection and vasculopathy." Circulation **117**(5): 660-9.

Yang, Z.-Z., A. J. Novak, et al. (2006). "Intratumoral CD4+CD25+ regulatory T-cell-mediated suppression of infiltrating CD4+ T cells in B-cell non-Hodgkin lymphoma." Blood **107**(9): 3639-3646.

Yuan, L., W. M. Yu, et al. (2005). "SHP-2 phosphatase regulates DNA damage-induced apoptosis and G2/M arrest in catalytically dependent and independent manners, respectively." J Biol Chem **280**(52): 42701-6.

Zenclussen, A. C. (2005). "CD4(+)CD25+ T regulatory cells in murine pregnancy." J Reprod Immunol **65**(2): 101-10.

Zenclussen, A. C., K. Gerlof, et al. (2005). "Abnormal T-cell reactivity against paternal antigens in spontaneous abortion: adoptive transfer of pregnancy-induced CD4+CD25+ T regulatory cells prevents fetal rejection in a murine abortion model." Am J Pathol **166**(3): 811-822.

Zhang, P., D. M. Su, et al. (2008). "Chemopreventive agents induce programmed death-1-ligand 1 (PD-L1) surface expression in breast cancer cells and promote PD-L1-mediated T cell apoptosis." Mol Immunol **45**(5): 1470-6.

Zhao, J. X., Y. Y. Zeng, et al. (2007). "Fetal alloantigen is responsible for the expansion of the CD4(+)CD25(+) regulatory T cell pool during pregnancy." J Reprod Immunol **75**(2): 71-81.