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Maternal immune responses to conceptus signals during early pregnancy in ruminants

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Abstract

Pregnancy is an immunological paradox created by the hospitable interaction between the maternal immune system and the allogeneic conceptus in the gravid uterus. The mother is not immune-suppressed during early pregnancy, nor is the conceptus immune-privileged, shielding itself from maternal immune detection by an immunologically inert placenta. Rather, what is becoming clear is that the conceptus and other endocrine mediators, including progesterone, actively shape the maternal immune response during early pregnancy to facilitate growth and development of a functioning placenta. In this regard, conceptual thinking derived from immune cell – pathogen interactions inadequately describe the unique homeorhetic mechanisms mediating early conceptus-maternal interactions. Nonetheless, evidence is mounting that conceptus signals induce a tolerogenic (Th2) bias in immune cell function at the fetal-maternal interface. This is accompanied by tissue remodeling and angiogenesis facilitated by tissue-resident immune cells that sets the trajectory for placental growth and, ultimately, fetal growth. Perturbations in these interactions, including systemic inflammation and various stressors at the earliest stages of pregnancy can interfere with communication between the conceptus and uterus, reducing conception rates and resulting in poor pregnancy outcomes.

Keywords: conceptus, fertility, immune, pregnancy, uterus.

Introduction

In eutherians, the conceptus must initiate a biochemical dialogue with the mother to establish pregnancy. In ruminants, this dialogue may differ from that in species in which the conceptus is more invasive. Conceptus signaling in ruminants occurs shortly after it enters the uterus (day 4-5 after fertilization) and following hatching from the zona pellucida (day 8-9). The observation that embryos successfully establish when transferred into cyclic cattle as late as 15-16 days after estrus (day 0), but no later, clearly defines when maternal responses to conceptus signaling must be initiated (Betteridge *et al*., 1980). The conceptus has several major "hurdles" to overcome during the peri-implantation period. Among these, the conceptus must block luteolysis to ensure continued progesterone production by the corpus luteum (CL), which maintains endometrial secretory activity and a quiescent uterine environment. Interferon tau (IFNT) produced by the conceptus alters endometrial prostaglandin production and maintains luteal function (Bazer *et al*., 2009). The two remaining challenges for the conceptus are: 1) avoiding attack by maternal immune cells, and 2) inducing endometrial remodeling and angiogenesis to support formation of the epitheliochorial placenta. The conceptus does so, in part, by inducing redistribution of maternal immune cells, and altering their function to ensure the appropriate maternal response to conceptus alloantigens. The conceptus accomplishes this on a background of elevated circulating concentrations of progesterone. Conceptus-induced alterations in immune function also extend to peripheral blood immune cells (PBL) during the very earliest stages of pregnancy recognition (Yankey *et al*., 2001; Han *et al*., 2006; Gifford *et al*., 2007, 2008; Stevenson *et al*., 2007; Green *et al*., 2010; Ott and Gifford, 2010; Ribeiro *et al*., 2014). Clearly, however, much is yet to be learned about the maternal immune response to conceptus signaling.

Medawar's dilemma

Over 50 years ago Sir Peter Medawar proposed three mechanisms whereby the allogeneic conceptus evades attack from the maternal immune system: (1) the gestating mother is immunosuppressed, (2) the conceptus is immunologically inert (e.g. does not express histocompatability antigens), and (3) the placenta creates a barrier to maternal immune cells (Medawar, 1953). Although pregnancy does suppress certain immune functions in the uterus by inducing immunosuppressive molecules such as uterine serpin (Hansen *et al*., 1986; Gottshall and Hansen, 1992; Hansen, 2007), there is little evidence that pregnancy is exclusively immunosuppressive. For example, Natural Killer (NK) cells isolated from the endometrium of early pregnant ewes exhibit lytic activity that is absent in NK cells from blood (Segerson and Beetham, 2000), arguing against broad, nonspecific immunosuppression.

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Evidence against the conceptus being immunologically inert includes the observation that altered expression of major histocompatability (MHC) antigens occurs on trophoblast cells exposed to maternal immune cells (Davies *et al*., 2000, 2006). This is perhaps best exemplified in humans, wherein placental cytotrophoblast cells express nonclassical, monomorphic histocompatibility antigens, HLA-E, -F and -G that bind immunoglobulin-like transcript (ILT) receptors on NK cells, resulting in reduced NK cell-induced cytolysis (Van Mourik *et al*., 2009). Bovine trophoblast cells also express nonclassical (NC1) MHCI molecules on their surface (Davies *et al*., 2006), suggesting analogous mechanisms of immune evasion in this species. The bovine NC1 promoter is activated by interferon gamma (IFNG), interferon tau (IFNT), interleukin (IL) 4 and high concentrations of progesterone (O'Gorman *et al*., 2010; Al Naib *et al*., 2011). Expression of classical MHCI molecules on peri-implantation bovine trophoblast has also been reported (Templeton *et al*., 1987; Low *et al*., 1990), but expression is generally downregulated in early pregnancy (Low *et al*., 1990; Davies *et al*., 2000; Bainbridge *et al*., 2001). Together, these results indicate that expression of nonclassical, monomorphic MHC1 (NC1) could potentially protect the bovine trophoblast from NK cell attack. Lastly, FAS ligand (FASL) is expressed by human and rodent placenta and induces apoptosis of maternal immune cells (Makrigiannakis *et al*., 2008) creating a barrier, or "blinding" the maternal immune system to conceptus alloantigens (Kayisli *et al*., 2003). Whether a similar mechanism occurs in ruminants remains to be determined.

Thus, while none of Medawar's original postulates have individually proven to be solely responsible for the establishment and maintenance of an allogeneic pregnancy, all appear to be involved to some extent during early pregnancy. More importantly, however, they have provided a framework for hypothesis testing that has yielded insight into the accommodations of the maternal immune system that support gestation of an allogeneic conceptus. One hypothesis examined in this review is that the conceptus directly regulates immune function in the endometrium to both protect it from immune rejection and to direct tissue-resident immune cells to participate in formation of the placenta. In this regard, it is necessary to think more broadly about the role of the immune system mouting as a homeorhetic response required to build a functioning placenta rather than focusing on the classical definitions of immune function that derive from studies of host-pathogen interactions.

The Th paradigm of pregnancy-induced immune tolerance

Conceptus signals may drive immune responses toward tolerance and promote placental growth and function. This shift is characterized by reduced expression of cytokines associated with inflammatory (Th1) immune responses, including IFNG, tumor necrosis factor α (TNF), IL2 and increased expression of cytokines that suppress or regulate (Th2) immune responses, including IL4, IL5 and IL10 (Wegmann *et al*., 1993). This theory of a pregnancy bias toward Th2 responses is referred to as the Th1-Th2 shift and has strong support in the literature. However, it oversimplifies the diverse array of immune cell responses typically observed during early pregnancy (Makrigiannakis *et al*., 2008; Wattegedera *et al*., 2008). Furthermore, this terminology primarily relates to immune responses to pathogens. Clearly, pregnancy is a different stimulus for the immune system. For the purposes of this review, the term regulatory will be used in the broad sense to refer to cells expressing Th2, anti-inflammatory or regulatory cytokines and molecules such as arginase-1 (ARG1), prostaglandin E (PGE) and indoleamine 2,3-dioxygenase (IDO) that are associated with immune suppression/resolution. In addition, while these terms may help define the immune response, pregnancy-induced immune changes clearly involve both activation and suppression of immune function.

Uterine immune cells during pregnancy recognition in ruminants

There is little information available describing the changes in immune cell populations during the peri-implantation period in cattle compared to the more extensive description of these cells later in pregnancy and the periparturient period (Nasar *et al*., 2002; Tekin and Hansen, 2002; Oliveira and Hansen, 2008, 2009; Fox *et al*., 2010). Much of this work occurred more than 15 years ago, when cellular morphology alone (Vander Wielen and King, 1984), or antibodies of poor specificity (Lee *et al*., 1988) were used to identify immune cell populations. Moreover, many of these studies were limited by too few animals, or slaughterhouse specimens of uncertain staging, to accurately evaluate uterine immune cell populations. For these reasons, the phenotypes and functions of immune cells present in the uterus during the critical period of conceptus signaling in dairy cattle (days 15-20) are not well defined.

Natural killer cells constitute a possible target of conceptus signaling during early pregnancy in cattle. Natural killer cells express CD335 (NKp46) and the

protein tyrosine phosphatase receptor ($CD45^+$), which is expressed on the vast majority of leukocytes, but express little CD5 (T and certain B cells) and surface immunoglobulins indicative of B cells (Lee *et al*., 1988). Natural killer cells often contain 1-3 large membrane-bound granules and they possess the ability to lyse cells without co-stimulation. Killing activity in the uterus was demonstrated by endometrial cells from early pregnant but not cyclic ewes (Segerson and Beetham, 2000). The response of NK cells to pregnancy is not clear, however. For example, NK killing activity was inhibited by treatment of cells with uterine serpin or with an antibody against the NK function-associated molecule (Tekin and Hansen, 2002). Conversely, treatment of cells with IFNT increased NK killing activity (Tekin and Hansen, 2002). Because of their autonomous nature, NK cells are considered one of the first lines of defense at mucosal surfaces. Interestingly, inappropriate regulation of NK cells contributes to a number of human reproductive disorders (Lash and Bulmer, 2011) and they clearly play an important role in placentation in humans by virtue of their ability to modify maternal spiral arteries and support placental vascular development (Kane *et al*., 2009). Uterine NK-like cells represent a potential target for conceptus signals at the conceptus-maternal interface during early pregnancy in ruminants. Preliminary work from our laboratory demonstrated that cells expressing the NK marker, CD335, increase as a percentage of uterine-resident $CD45^+$ cells at day 17 of pregnancy compared to day 17 of the estrous cycle in dairy heifers. Approximately 50% of these putative NK cells also co-express the CD8 protein characteristic of cytotoxic (CD8) T cells (Ott and Vasudevan, 2014, Department of Animal Science, Pennsylvania State University, University Park, PA, USA, unpublished observation).

Vander Wielen and King (1984) described a distinct population of intra-epithelial lymphocytes (IEL) in the uterus of cyclic cattle whose numbers remained constant across the estrous cycle and early pregnancy up to day 21. The number of these cells then decreased by more than 50% as pregnancy progressed from day 21 to day 27, especially in areas adjacent to the syncitial layer formed between trophoblast giant binucleate cells (BNGC) and maternal uterine epithelium during placental development. Similarly in sheep, cytotoxic ($CD8^+$) T cells were located primarily adjacent to the epithelial layer of the uterus (IEL), whereas helper ($CD4^+$) T cells were found predominantly in the subepithelial stroma (Meeusen *et al*., 1993). In the ewe, approximately 25% of uterine $CD8^+$ cells were $\gamma\delta^+$ IEL and contained 1-3 cytoplasmic granules (Meeusen *et al*., 1993). This cell population represents a potential danger to the developing conceptus because these cells can kill in a non-MHC restricted fashion (Fox *et al*., 2010). Helper ($CD4^+$) T cells direct immune responses by the production of inflammatory (Th1) or regulatory (Th2) cytokines. Cytotoxic ($CD8^+$) T cells both produce and respond to some of these same cytokines and can participate directly in killing. The $\gamma\delta^+$ population of IEL increases in the sheep uterus toward the end of gestation as does their granularity (Meeusen *et al*., 1993), suggesting that they are involved in parturition and shedding of the placenta during the postpartum period. As with NK cells, the cytoplasmic granules of $\gamma\delta^+$ IEL contain perforin and granzyme, further supporting a killing function for these cells (Fox *et al*., 2010). The presence of $\gamma\delta^+$ cells at the conceptus-maternal interface suggests that they could be targets of conceptus signaling. However, there are no reports describing the effects of the conceptus or IFNT on $\gamma\delta^+$ cells in the uterus during early pregnancy in dairy cattle.

Considering the presence of NK, $CD8^+$ and $\gamma\delta^+$ T cells at the fetal/maternal interface, it is clear that regulatory mechanisms must be in place to protect the allogeneic conceptus from attack. Presumably, the progesterone-mediated responses mentioned previously are among these. However, the T helper cell lineage may also play a key role in limiting the immune response, as shown in other species. Regulatory T cells (Treg) express CD25 (IL2 receptor) and the transcription factor forkhead box protein 3 (FoxP3; Seo *et al*., 2009). The suppressor activity of Treg cells is clear in rodent (Forward *et al*., 2010) and human endometrium (Saito *et al*., 2008), and this mechanism may be conserved across placental mammals. Suppressor activity was demonstrated in ruminant endometrial cells isolated by uterine curettage (Segerson *et al*., 1998; Segerson and Beetham, 2000), but the cells that mediated this effect were not described. Oliveira and Hansen (2008) demonstrated increased $CD4^+CD25^+$ cells in the periphery and uterine endometrium during late gestation. However, FoxP3 expression was not measured nor was suppressor activity confirmed. The availability of new antibodies for detecting FoxP3 (Hoek *et al*., 2009; Seo *et al*., 2009; Poole and Pate, 2012) are yielding new insights into Treg function in ruminants, but recent results raise some question about whether $CD4^+$ $CD25^+$ $FoxP3^+$ cells are the only regulatory T cell in cattle. For example, Hoek *et al*. (2009) demonstrated that a subpopulation of $\gamma\delta^+$ cells (Workshop Cluster (WC)1.1^+, $WC1.2^+$, $FoxP3^-$) along with $CD14^+$ cells (monocytes) were responsible for suppressor activity in *ex vivo* assays of PBL in cattle. Both of these cell types also expressed the regulatory cytokine IL10. In addition, they were unable to detect any suppressive activity from $CD4^+CD25^{high}FoxP3^+$ cells. These results are intriguing given the abundance

of $\gamma\delta^+$ T cells, both in the periphery and in the uterus of cattle during early pregnancy. However, they should be cautiously interpreted until confirmed by additional studies.

Studies focusing on immune cell function during the period of maternal recognition of pregnancy in cattle are few. Leung *et al*. (2000) characterized uterine lymphocyte distribution and cytokine mRNA at day 16 of pregnancy in dairy cattle and demonstrated the presence of $CD4^+$ (T cells), $CD21^+$ (B cells) and $CD14^+$ (macrophages, dendritic cells) cells on day 16 of pregnancy. They did not detect differences in the proportions of these cells between cyclic and pregnant heifers on day 16. Interestingly, a large number of $CD14^+$ (myeloid lineage cells) cells were detected in the epithelium and subepithelial stroma (Leung *et al*., 2000). Similarly, $CD14^+$ $CD68^+$ cells (macrophages) are present in the endometrium of cyclic ewes (Tekin and Hansen, 2004; Mansouri-Attia *et al*., 2012), and the number of these cells increased substantially later in pregnancy in the cow and expressed genes associated with a regulatory phenotype including the mannose receptor (MRC1) and CD163 (Oliveira and Hansen, 2008; Oliveira *et al*., 2010). Recent work by Mansouri-Attia *et al*. (2012) also detected myeloid lineage cells in the endometrium and demonstrated that a $CD11c^+$/$CD172a^+$ fraction increased in the endometrium at day 16 of pregnancy in beef heifers, which was accompanied by increased expression of pentraxin 3 (PTX3) a protein involved in angiogenesis and tissue remodeling (Martinez and Gordon, 2014). Dendritic cells (DC) are a key antigen presenting cell that regulates activation status of the immune system. The presence of macrophages and DC at the conceptus-maternal interface may promote conceptus survival by expressing immunoregulatory molecules such as PGE, IL10, IL4, and IDO (Munn *et al*., 1998; Nagamatsu and Schust, 2010; Groebner *et al*., 2011). In preliminary work in our laboratory, the shallow glands of early pregnant dairy heifers contained a greater number of cells expressing MHCII (macrophage and dendritic cells) than those of cyclic heifers (Ott and Kamat, Department of Animal Science, Pennsylvania State University, University Park, PA, USA, unpublished observations). However, the activation status of these myeloid lineage cells is not known.

Partitioning of macrophages into inflammatory (M1) and anti-inflammatory (M2) functional subtypes based upon the production of inflammatory or regulatory molecules, reminiscent of the Th1/Th2 paradigm for lymphocytes, requires that macrophage functional characterization extend beyond surface protein (CD14, MHCII and CD68) identification to determine pregnancy-specific effects (Sica *et al*., 2008; Nagamatsu and Shust, 2010; Oliveira *et al*., 2010). For example, M1 macrophages produce TNF, IL6 and CCL2 (MCP1), whereas M2 macrophages produce arginase-1(ARG1), IL10 (Ohashi *et al*., 2010) and IDO (Groebner *et al*., 2011). However, recent evidence suggests that even this characterization might be too simplistic because there are now at least 3 types of M2 macrophages described in the literature (Martinez and Gordon, 2014). Clearly, effector function assays combined with cytokine expression profiles will help better define macrophage and dendritic cell function in the endometrium. However, there is little information on the activation status of macrophage/DC in the uterus during early conceptus signaling in dairy heifers and cows.

Conceptus signals and immune cell function

The bovine conceptus produces a number of bioactive compounds during early pregnancy including IFNT, pregnancy associated glycoproteins (PAG), placental lactogens (PL) and prostaglandins E_2 and $F_2\alpha$ (PGE & PGF; Hashizume *et al*., 2007; Bazer and Spencer, 2009; Mamo *et al*., 2011; Spencer *et al*., 2013). The role of conceptus-produced prostaglandins PG in ruminants is not clear, however, recent results suggest that these lipid mediators increase expression of interferon stimulated genes (ISG) in the endometrium during the earliest stages of pregnancy, perhaps serving as priming signals in advance of IFNT production (Spencer *et al*., 2013). Pregnancy associated glycoproteins are a large family of aspartic proteinases expressed by the placenta in ruminants and several other species (Hughes *et al*., 2003). The PAG gene family can be partitioned into more ancient members (bPAG-2) and members that duplicated and diverged more recently (bPAG-1). The first PAG described, pregnancy-specific protein B, is a member of the PAG-1 family (PSPB; Butler *et al*., 1982; Wooding *et al*., 2005). The function of PAG during early pregnancy remains an enigma. They apparently do not function as active proteases due to amino acid substitutions in their catalytic domains, however they retain the ability to bind peptides (Wooding *et al*., 2005). Their presence in high concentration at the maternal-fetal interface and in maternal circulation has led some to suggest that PAG play a role in modulating the maternal immune system during pregnancy (Wooding *et al*., 2005). The presence of PAG in maternal blood and milk has been utilized as an effective pregnancy diagnostic in a number of ruminant species (Sasser *et al*., 1986; Green and Roberts, 2006).

It is now clear that conceptus IFNT affects immune function genes both in the uterus and in the

peripheral blood (Ott and Gifford, 2010). The effect on peripheral blood occurs as IFNT exits the uterus via the uterine vein (Bott *et al*., 2010). Microarray analysis revealed that a large number of known interferon stimulated genes were greater in peripheral blood leukocytes from pregnant compared to cyclic cows (Gifford *et al*., 2008; Ott and Gifford, 2014, Department of Animal Science, Pennsylvania State University, University Park, PA, USA, unpublished). This difference occurs as early as days 14-15 of pregnancy in sheep (Yankey *et al*., 2001) and days 16-17 of pregnancy in cattle (Gifford *et al*., 2007; Walker *et al*., 2010). In addition to increased ISG expression in endometrial epithelial, stromal and myometrial cells (Ott *et al*., 1998), there is considerable expression of ISG mRNA in an uncharacterized population of endometrial immune cells (Song *et al*., 2007; Gifford *et al*., 2008). However, the effects of IFNT on immune cell functions at the fetal-maternal interface or in peripheral blood during conceptus signaling have not been determined (Ott and Gifford, 2010). The function of increased ISG in endometrial cells is largely unknown, but recent work by our group indicates that the myxovirus resistance 1 (MX1) protein is involved in the process of unconventional secretion by the uterine epithelium (Racicot and Ott, 2011; Racicot *et al*., 2012).

Type I interferons, including IFNT, promote immunosuppressive functions of immune cells (Johnson and Blalock, 1980; Newton *et al*., 1989; Skopets *et al*., 1992; Tuo *et al*., 1993; Tekin *et al*., 2000), including the induction of regulatory T cells. For instance, IFNT-treated $CD4^+$ cells exhibit regulatory activity by expressing transforming growth factor beta 1 (TGFB1) and IL10 (Mujtaba *et al*., 1997). In addition, IFNT inhibits experimental autoimmune diabetes in a nonobese, diabetic mouse model (NOD; Sobel *et al*. 2008). This inhibition was accompanied by increases in $CD4^+$ $CD25^+$ $FoxP3^+$ cells in the spleens of the mice. The effects of IFNT were postulated to be mediated by dendritic cell-induction of Tregs (Sobel *et al*., 2008). Exposure to IFNT also suppressed antigen-induced increases in $WC1^+$ $\gamma\delta^+$ cells and increased $CD8^+$ $WC1^-$ $\gamma\delta^+$ cells (Tuo *et al*., 1999). $WC1^+$ $\gamma\delta^+$ cells tend to express more pro-inflammatory genes than $WC1^-$ $\gamma\delta^+$ cells (Chen *et al*., 2009). Importantly, the effect of conceptus signaling, and IFNT in particular, on regulatory immune cell phenotypes and functions in the endometrium and peripheral blood of dairy cattle has not yet been determined.

Summary and Conclusions

Clearly, much remains to be learned about the phenotype and function of uterine-resident immune cells during early pregnancy in cattle. Figure 1 describes a working model for the interactions that are postulated to occur during early pregnancy in dairy cattle. Early pregnancy signaling between the conceptus, uterus and peripheral immune system is accomplished under high circulating and even greater local uterine concentrations of progesterone. Progesterone induces expression of immunosuppressive molecules including uterine serpins that regulate aspects of mucosal immunity. Conceptus signals including IFNT, PAG, placental lactogens (PL), prostaglandin E and F (PG) alter gene expression in all uterine cells including epithelial, stromal and myometrial cells. IFNT induces expression of interferon stimulated genes (MX1 & MX2, ISG15, OAS, RTP4) and several cytokines (IL10) and chemokines (MCP2). Many, if not all of these ISG are also increased in peripheral blood leukocytes (PBL), which include T and B cells, neutrophils (PMN) and monocytes. However, the effects of increased ISG expression on immune cell function are not known. Peripheral activation of ISG in PBL suggests that tissue resident immune cells also respond to IFNT, although the details of these responses remain to be determined. Numerous T cells (CD4, CD8 and γδ), NK cells, DC, B cells and macrophages (MΦ) are present at the conceptus-maternal interface during pregnancy recognition. However, surprisingly little is known about how these cells respond to the conceptus. We postulate that conceptus signals increase processes critical for establishment of pregnancy and growth of the placenta including induction of tolerogenic immune regulators (IDO, IL10, ARG1), stimulation of tissue remodeling and angiogenesis (PTX3, VEGF, PGE) and induction of ISG to increase innate immunity. However, we still do not know which cells respond to IFNT or how they respond. Additionally, these interactions are susceptible to perturbation by a number of factors that are known to reduce pregnancy rates in dairy cows including: heat stress, infection, metabolic disease, age, nutrition, genetics and other stressors.

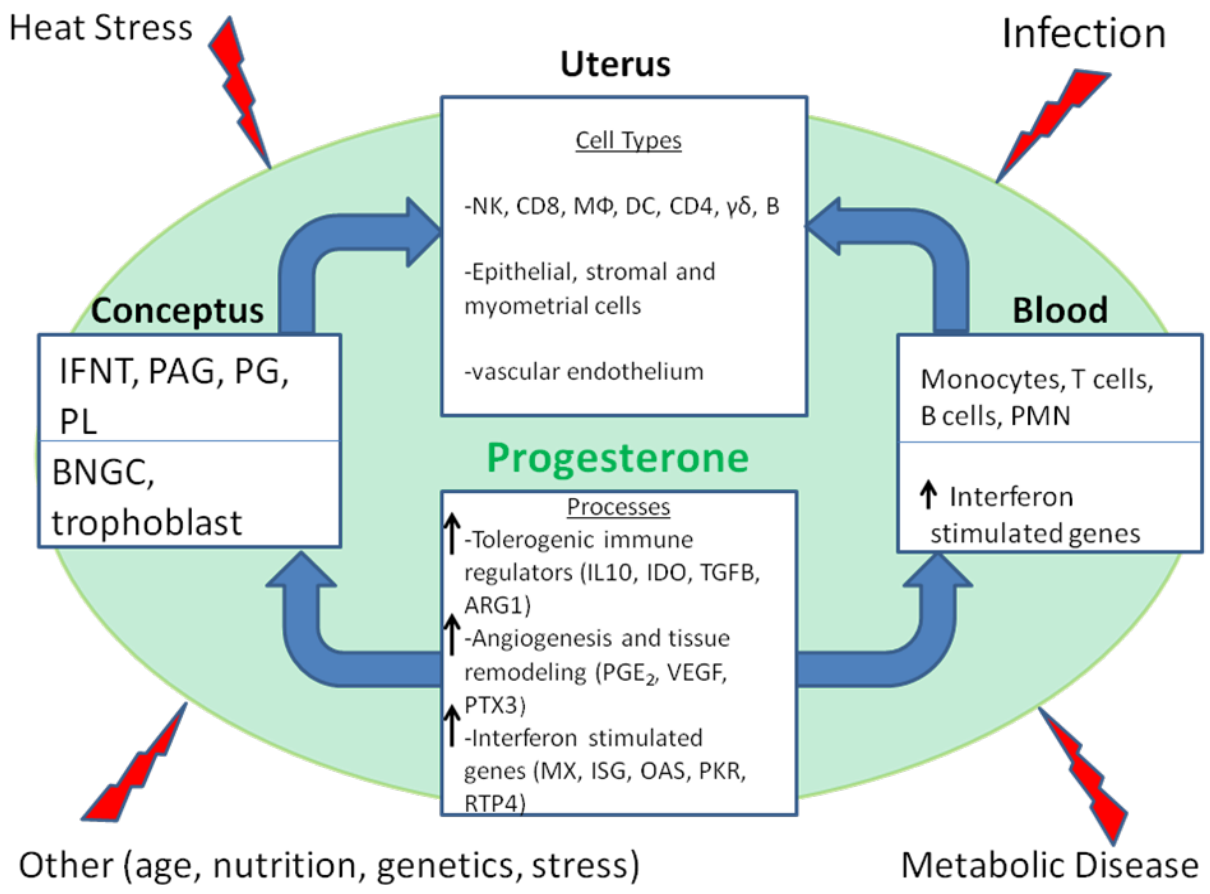

Figure 1. Complex interactions between the conceptus, uterine somatic and immune cells and peripheral blood immune cells during early pregnancy in dairy cows. See text for description.

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