Consequences of interactions between the maternal immune system and the preimplantation embryo in cattle

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Summary
Using the cow, three questions related to the importance of the maternal immune system for the developing embryo are addressed: role of semen-induced inflammation for pregnancy establishment, regulation of preimplantation development by molecules that function as soluble mediators of immune cells, and immunological aspects of embryonic signaling by interferon-τ. Unlike rodents, there is no indication that semen modifies the physiology of the mother in a way that promotes embryonic survival. Bulls without seminal vesicles are fertile, artificial insemination results in pregnancy rates similar to natural mating and intrauterine deposition of seminal plasma at the time of insemination does not improve fertility. Regulatory molecules associated with the immune system can be important for development of the embryo. Among the molecules implicated in embryonic growth and survival are colony stimulating factor 2, which can enhance embryo competence to establish and maintain pregnancy, and tumor necrosis factor α, which induces apoptosis and blocks development. Elongation of the trophoblast beginning at Day 14 of development results in large-scale changes in the transcriptome of the embryo, including many genes involved in immune responses. Signaling by interferon-τ and other embryo-derived molecules does not, however, lead to a large-scale change in accumulation of lymphocytes in the endometrium. In contrast, numbers of macrophages and dendritic cells do increase and there is increased expression of genes associated with macrophage activation. Perhaps these antigen-presenting and immunoregulatory cells play an important role in continued survival and development of the conceptus.

Introduction
The preimplantation period in ruminants is bracketed by two events causing local activation of the immune system. The first, deposition of semen in the reproductive tract at estrus, elicits an inflammatory response characterized by migration of neutrophils into the uterine lumen (Howe & Black 1963, Mattner 1968). Near the end of the preimplantation period, enhanced secretion of the embryonic type 1 interferon, IFNT, and possibly other molecules results in expression of a large number of immune-response genes in the endometrium (Klein et al. 2006, Walker et al. 2010, Cerri et al. 2012, Bauersachs et al. 2012, Forde et al. 2012, Mansouri-Attia et al. 2012). Attachment of the trophoblast to the endometrium begins a few days after IFNT secretion is maximal (King et al. 1981), marking the end of the preimplantation period.
The dynamic changes in local immune function exhibited during early pregnancy beg the question as to whether immune cells resident in the reproductive tract exert beneficial or deleterious effects on the preimplantation embryo. Some interactions between the maternal immune system and the embryo could potentially promote embryonic development. If so, disruption of these interactions caused by maternal dysfunction could be a cause of infertility. Supportive of this idea is the observation that endometrium of cows that established pregnancy after embryo transfer had a higher expression of genes involved in T-and B cell signaling and NF-κB signaling at Day 7 after estrus in the estrous cycle preceding embryo transfer than endometrium of cows that did not establish pregnancy (Ponsuksili et al. 2012). In addition, endometrial expression of several genes involved in immune function were altered in cows with retarded embryos at Day 7 after estrus as compared to cows with embryos at the correct stage of development (Beltman et al. 2013). For example, expression of IL6, IL10, IFNG and IFNA was higher for endometrium from cows with retarded embryos while expression of NFκB1 and IL21 was reduced. It is conceivable that one cause of the reduced developmental competence of embryos produced in vitro (Farin et al. 2006, Hansen et al. 2010) is the lack of exposure of embryos to regulatory signals of immune origin. In other cases, establishment of alternative patterns of immune activation in early pregnancy could potentially harm the embryo. Indeed, endometritis is associated with infertility (Thatcher et al. 2010) and with alteration in endometrial expression of immune-modulatory genes (Hoelker et al. 2012).

In this paper, the cow will be used as a model to address several questions relevant to understanding the importance of the maternal immune system for the developing embryo. In particular, the possible role of semen-induced inflammation for pregnancy establishment will be assessed, the consequences of exposure of the embryo to molecules that function as soluble mediators of immune cells will be highlighted, and the function of changes in expression of immune-related genes in the endometrium caused by IFNT will be speculated upon.

**Inflammatory events induced by semen**

The major role of inflammation caused by semen is likely to be promotion of phagocytic removal of microorganisms and spermatozoa deposited in the reproductive tract coincident with mating. Sperm are antigenic in cattle (Lander et al. 1990) and cows immunized against sperm have been reported to have lower fertility (Menge 1967, 1969). It has been proposed that inflammatory or immune responses to semen also facilitate the establishment and maintenance of pregnancy by modifying the nature of maternal immune responses (Robertson et al. 2013). The best substantiation for this idea comes from studies in the mouse. In this species, seminal plasma has been reported to increase the number of regulatory T cells reactive against paternal antigens (Robertson et al. 2013) and to increase expression in the oviduct for genes that stimulate embryonic development (Lif, Csf2, Il6 and Egf) (Bromfield et al. 2014). Females mated with males lacking seminal vesicles had reduced conception rates, embryos with poor development to the blastocyst stage and low implantation rates, and offspring which experienced sex-dependent alterations in physiological function that persisted into adulthood (Bromfield et al. 2014).

Each year, millions of cattle are bred artificially in a system where the female comes in contact with small volumes of highly-diluted seminal plasma. If semen plays an important role in regulating embryonic development and responsiveness to the maternal immune system, it might be possible to improve fertility and fetal development in cows bred artificially by pharmacological activation of key aspects of the immune response to sperm.
Results in cattle, however, are not supportive of a role for seminal plasma in modulating fertility. Faulkner et al. (1968) evaluated consequences of removal of seminal vesicles on bull fertility. When each of five vesiculectomized bulls was used to breed 8-20 heifers each, conception rates were 40, 71, 75, 75 and 100%. Thus, bulls were generally fertile despite the absence of seminal vesicles. Moreover, the percent of ova fertilized after cows and heifers were inseminated artificially with sperm from the testes, epididymis or ejaculate were 0%, 84% and 94%, respectively (n = 106) (Amann & Griel 1974). Fertility was high even when insemination was performed with epididymal sperm that had not been in contact with semen.

It is not known whether there are differences in the nature of immune changes in the reproductive tract following deposition of sperm in cows bred by natural vs artificial insemination. Differences might even depend on semen extender – egg yolk has been reported to block the ability of seminal plasma to promote binding to neutrophils (Alghamdi et al. 2009). Given the large dilution of seminal plasma for ejaculates prepared for artificial insemination, it is reasonable to assume that the magnitude of immune deviation in the reproductive tract caused by sperm deposition would be less for cows subjected to artificial insemination than for cows bred naturally. In one study, only 43% of cows had detectable neutrophils in cytobrush samples from the endometrium collected at 4 h after artificial insemination (Kaufmann et al. 2009). Despite the differences in volume of seminal plasma deposited in the reproductive tract and the presumed difference in inflammatory response, there were no differences in conception rate between cows bred naturally or via artificial insemination (Landivar et al. 1985, Lima et al. 2009).

Seminal plasma is a rich source of TGFB and this growth factor may be responsible for semen-induced activation or expansion of regulatory T cells during pregnancy in mice (Ingman & Robertson 2009, Robertson et al. 2013). However, Odhiambo et al. (2009) found that neither intrauterine deposition of 0.5 ml seminal plasma nor 40 ng TGFB1 at the time of artificial insemination improved pregnancy rate in cattle (Table 1).

Thus, regardless of the model used, there is no evidence that seminal plasma can enhance fertility in cattle.

Table 1. Effect of intrauterine deposition of seminal plasma on fertility of cows bred by artificial insemination.a,b,c

| Trial number | Cow type | No. of animals | Conception rate (%) | |
|-------------|---------|---------------|---------------------|---|
|             |         |               | Control          | Seminal plasma | TGFB |
| 1-3         | Beef    | 763           | 55.1              | 58.8            | 51.0 |
| 4           | Beef    | 206           | 59.5              | 67.5            | --   |
| 5           | Beef    | 167           | 52.4              | 61.4            | --   |
| 6           | Dairy   | 800           | 33.2              | 37.8            | 36.3 |

a The control treatment was either intrauterine deposition of bovine serum albumin (trials 1-4) or no additional deposition (trials 5-6).
b Differences between treatments were not significant.
c Odhiambo et al. (2009).

Alterations in embryonic development caused by cytokines associated with the immune system

The microenvironment of the embryo is established by a mucosa populated in part by cells that function in the immune system. The oviduct contains lymphocytes, macrophages and mast
cells (Du Bois et al. 1980, Abughrien et al. 2000). During early pregnancy (days 5-13), B cells are abundant in the deep stroma and myometrium of the endometrium, T cells are moderately abundant and located in the shallow stroma near the epithelium, and natural killer cells and δγ-T cells are present in low numbers in the shallow stroma (Oliveira et al. 2013). During the same time period, macrophages and dendritic cells are present in the stroma, with the former cells abundant and the latter cells present in low numbers (Mansouri-Attia et al. 2012).

Epithelial and stromal cells of the endosalpinx and endometrium also participate in innate immunity. Epithelial cells in the endosalpinx of the oviduct express Toll-like receptors TLR2 and TLR4 (Kowsar et al. 2013). At low concentrations, bacterial lipopolysaccharide (LPS) induced expression of TLR4, PTGS2, IL1B, NFKB1, and TNF while, at higher concentrations, LPS induced a different set of genes (TLR2, IL4, IL10 and PTGES) (Kowsar et al. 2013). Endometrial epithelial and stromal cells also express genes encoding for TLR (Davies et al. 2008) and epithelial cells express genes for antimicrobial peptides (Davies et al. 2008). Moreover, LPS increased expression of some of these genes and increased secretion of prostaglandin E2, IL6 and IL8 (Davies et al. 2008, Turner et al. 2014).

There was no effect of pregnancy status at Day 5, 7 or 13 on accumulation of lymphocytes, macrophages or dendritic cells in the endometrium (Mansouri-Attia et al. 2012, Oliveira et al. 2013). It cannot be ruled out, however, that there are local changes in leukocyte accumulation or activation in the reproductive tract at sites nearby to the embryo. Among the embryonic signals that could activate maternal immune cells include IFNT (expressed as early as the 16-cell or morula stage; Lonergan et al. 2003, Yao et al. 2009), IL1B and TNF (Muñoz et al. 2012). Major histocompatibility antigen class I can be detected on the surface of the blastocyst (Templeton et al. 1987, Low et al. 1990, Doyle et al. 2009) and this molecule could also interact with maternal cells after hatching from the zona. There could also be hormonal regulation of immune cells in the endometrium.

Depending upon the characteristics of immune activation, cytokines produced in the reproductive tract could potentially alter embryonic development in a beneficial or adverse manner. That some cytokines can promote embryonic development is indicated by results of experiments in which embryos were transferred Day 7 after estrus into the uterus of cows that received intrauterine deposition of autologous peripheral-blood mononuclear cells on Day 4. Mononuclear cells were cultured for 24 h in the presence of fetal bovine serum before transfer and appeared to become activated based on an increase in transcript abundance for IL1B and several other genes (Ideta et al. 2010a). Transfer of mononuclear cells increased pregnancy rate (Ideta et al. 2010a). Embryos transferred into cows that received intrauterine deposition of blood mononuclear cells also experienced more extensive development at Day 15 of gestation than embryos transferred to control cows (Ideta et al. 2010b). Trophoblast length was greater for embryos recovered from treated cows and a greater percentage of embryos had observable embryonic discs.

There are several cytokines that can improve development of the bovine embryo. The likelihood that an in vitro produced embryo developed to the blastocyst stage was improved by culture with IL1B (Paula-Lopes et al. 1998), LIF (Neira et al. 2010) and CSF2 (de Moraes & Hansen 1997, Loureiro et al. 2009, Neira et al. 2010, Dobbs et al. 2013). Actions of CSF2 to improve development to the blastocyst stage depend upon the overall competence of embryos for development (Dobbs et al. 2013). CSF2 increased blastocyst percent when the percent of control embryos developing to the blastocyst stage was low. When it was high, however, CSF2 decreased the percent of embryos becoming a blastocyst.

Treatment with CSF2 also increases ability of the resultant blastocysts to establish and maintain pregnancy. This action has been shown not only in cattle (Loureiro et al. 2009) but
also in mice (Sjöblom et al. 2005) and humans (Ziebe et al. 2013). Exposure of the bovine embryo to CSF2 beginning at Day 5 of development alters several properties of the embryo that might improve competence to establish pregnancy (Fig. 1). These include alterations of expression of several genes related to differentiation (Loureiro et al. 2011), increased resistance to apoptosis (Loureiro et al. 2011), and an increase in number and pluripotency of cells of the inner cell mass (Loureiro et al. 2009, Dobbs et al. 2013).

Pro-developmental cytokines could be derived from leukocytes resident in the reproductive tract or from epithelial or stromal cells. Immunoreactive CSF2 is present in both endosalpinx and endometrium throughout the estrous cycle; labeling was greatest in epithelial cells (de Moraes et al. 1997, Emond et al. 2004). Similarly, immunoreactive IL1B was found in both epithelial and stromal cells of the bovine oviduct and endometrium (Paula-Lopes et al. 1999).

While some cytokines enhance embryonic development, other products of immune activation compromise survival of the embryo. In particular, several molecules produced

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**Fig. 1.** Actions of CSF2 on the bovine preimplantation embryo. Addition of CSF2 at Day 5 of development changes properties of the resultant morula at Day 6 and blastocyst at Day 7. At Day 6, CSF2 alters gene expression in a way that decreases capacity for apoptosis and canonical WNT signaling as well as alters expression of several other genes involved in differentiation. Moreover, induction of apoptosis is reduced in embryos treated with CSF2. At Day 7, CSF2 increases numbers of cells in the inner cell mass without affecting number of trophectoderm cells. Moreover, isolated clusters of inner cell mass cells are better able to survive passage in a pluripotent state when cultured than cells from control embryos. Additionally, blastocysts treated with CSF2 have increased capacity to continue development to term when transferred to recipients.
as part of the inflammatory response inhibit embryonic development in the preimplantation period, including nitric oxide (Soto et al. 2003a), prostaglandin-F2α (PGF2α) (Scenna et al. 2004), and TNF (Jackson et al. 2012). The action of TNF involves activation of prostaglandin synthesis because it could be blocked by indomethacin (Jackson et al. 2012). In addition, TNF can trigger apoptosis in embryonic cells (Soto et al. 2003b). Findings from the above-mentioned studies suggest that one of the causes of infertility in cows experiencing endometritis is local production of bioactive molecules inhibitory to the embryo.

The dual nature of immune regulation of embryonic survival, with some molecules enhancing embryo competence for development and some molecules being inhibitory, means that the consequences of immune activation are likely to depend on the nature of the signals activating innate or adaptive immune responses as well as on properties of the embryo. Researchers at the University of Bonn conducted two experiments to identify differences in the endometrial transcriptome between embryo transfer recipients in which pregnancy failure or success occurred. In the one experiment, using embryos produced in vivo, among the molecular pathways overrepresented at day 7 in the estrous cycle before transfer for cows that did not establish pregnancy were those for immune response and chemokine activity (Salilew-Wondiomo et al. 2010). This result could be interpreted to mean that overactivation of immune responses in the endometrial were inimical to pregnancy success. In another study, using embryos produced in vitro, endometrium of cows that did not establish pregnancy had lower expression of genes involved in T-and B cell signaling and NF-κB signaling (Ponsuksili et al. 2012). Perhaps, for these embryos, where exposure to maternal cytokines did not occur in vitro, some activation of immune responses in utero was supportive of development. Additional work is needed to understand the characteristics and control of cells producing cytokines in the reproductive tract and the role of those molecules in embryonic development.

Immune consequences of IFNT secretion by the elongated embryo

Prior to rapid elongation of the trophoblast around Day 14-15 of gestation (Betteridge & Fléchon 1988), the presence of the embryo in the reproductive tract causes little if any change in the function of the endometrium. Indeed, through Day 13 of gestation, differences in the transcriptome between endometrium of pregnant and non-pregnant cows were small (Forde et al. 2012) or non-detectable (Forde et al. 2011, Bauersachs et al. 2012). Similarly, there were few differences in populations of immune cells resident in the endometrium (Mansouri-Attia et al. 2012, Oliveira et al. 2013). Coincident with elongation, however, the endometrium undergoes large-scale changes in gene expression; transcript abundance changes for hundreds of genes including many involved in immune function (Walker et al. 2010, Forde et al. 2011, 2012, Bauersachs et al. 2012, Cerri et al. 2012, Mansouri-Attia et al. 2012). For example, Walker et al. (2010) examined differences in gene expression between pregnant and cyclic cows at Day 17 of pregnancy. A total of 1,839 in caruncular tissue and 1,189 genes in intercaruncular tissue were differentially expressed. The four most statistically significant canonical pathways in which these genes were represented were interferon signaling, complement system, role of pattern recognition receptors in the recognition of bacteria and viruses, and antigen presentation.

Changes in endometrial gene expression during Days 15-18 of gestation are caused largely by the increase in IFNT secretion by the trophectoderm that occurs coincident with elongation (Robinson et al. 2006). Most pregnancy-associated changes in endometrial gene expression at this time can be mimicked by intrauterine infusion of human IFNA2 (Bauersachs et al. 2012) or bovine IFNT (Forde et al. 2012). The pattern of gene expression induced by type I IFN is
Interactions between immune cells and the embryo

Interactions between immune cells and the embryo are not identical to pregnancy, however, probably because some actions of IFNT are exerted in conjunction with other regulatory molecules such as placental lactogen, cortisol, prostaglandins and progesterone (Dorniak et al. 2013). In addition, there might be other regulatory molecules secreted by the embryo at this time in pregnancy including those involved in immune function.

Like other type I interferons, IFNT possesses antiviral activity (Alexenko et al. 1997) and the pregnant endometrium around the time of trophoblast elongation exhibits an antiviral state as indicated by upregulation of genes involved in inhibition of viral proliferation (Walker et al. 2010, Bauersachs et al. 2012, Forde et al. 2011, 2012, Cerri et al. 2012) as well as increased activity of the antiviral protein 2’5’ oligoadenylate cyclase (Short et al. 1991). IFNT can also block lymphocyte proliferation (Skopets et al. 1992, Alexenko et al. 1997) but it is not known whether T or B cell function is inhibited in the reproductive tract coincident with IFNT secretion. Some endometrial genes that are upregulated at Day 17 of pregnancy are associated with inhibition of lymphocyte function but others are associated with lymphocyte activation (Fig. 2). Examples of the former include IDO1 (Fallorino et al. 2012), molecules of the FASLG signaling cascade (FAS, FASLG, FADD), which promote lymphocyte apoptosis (Lettau et al. 2011), and the T-cell inhibitory molecule SERPINA14 (Peltier & Hansen 2001). Examples of the latter include IL18, which promotes T cell chemotaxis and maturation of helper T cells (Volin & Koch 2011) and TNFSF13B, which stimulates B cell survival and expansion (Goenka et al. 2014) and T cell activation (Chen et al. 2014). Moreover, despite the upregulation of several chemokine genes in the pregnant endometrium (for example, CCL2, CCL8, CCL11, CXCL2, CXCL9, CXCL10, and CXCL11; Walker et al. 2010, Cerri et al. 2012), changes in accumulation of lymphocytes in the endometrium at this time are minimal. At Day 16 of pregnancy, there was no difference between pregnant and non-pregnant cows in the number of B cells or T cells positive for CD4, CD8 or the δγ T cell receptor (Leung et al. 2000, Oliveira et al. 2013).

The same lack of consistent pattern of regulation is true for NK cells. One function of IFNT

Fig. 2. Changes in gene expression (top) and accumulation of leukocytes (bottom) in the endometrium occurring coincident with trophoblast elongation. Genes in red represent those associated with immune activation, genes in green represent those associated with immune inhibition and genes in gray are unresolved. Lines represent actions of gene products (red, stimulatory; green, inhibitory). The figure is based on results on gene expression from Walker et al. (2010) and Cerri et al. (2012) and results on leukocyte numbers from Mansouri-Attia et al. (2012) and Oliveira et al. (2013).
is to activate lytic activity of NK cells (Tuo et al. 1993, Tekin & Hansen 2002). Evidence is contradictory, though, as to whether there is increased NK cell activity in the pregnant endometrium during trophoblast elongation. Some genes whose expression is elevated in the endometrium of pregnant cows promote NK cell function, for example, *IL15* and *IL18* (Marçais et al. 2013), whereas others are associated with decreased NK cell activity such as *IFITM1* (Yang et al. 2005), *SERPINA14* (Tekin & Hansen, 2002), and *TAP1* (Kalkunte et al. 2009). Oliveira et al. (2013) found that the number of cells in the endometrium at Day 16 that labeled for the NK cell marker Nkp46 was lower for pregnant heifers than cyclic heifers. In particular, there was an increase in number of Nkp46+ cells from Day 13 to 16 in cyclic animals that did not occur in pregnant animals. In contrast, Vasudevan et al. (2013) observed that the proportion of CD45+ leukocytes in Day 17 endometrium that expressed Nkp46 was higher for pregnant heifers as compared to cyclic animals.

Three of the genes that were found by Walker et al. (2010) to be higher in pregnant cows at Day 17 of pregnancy, *IDO1*, *IL7*, and *IL15*, are involved in generation, proliferation and survival of regulatory T cells (Harnaha et al. 2006, Guerin et al. 2009, Fallarino et al. 2012). This subpopulation of T cells plays an important role to induce anergy against fetal antigens in the mother (Rowe et al. 2012). Perhaps IFNT or other embryonic signals released at this time in pregnancy are critical for regulation of maternal regulatory T cells in ruminants. There was, however, no difference between pregnant and cyclic cows in expression of *FOXP3*, a transcription factor associated with regulatory T cells, in the endometrium at Day 16 after estrus (Oliveira et al. 2013).

The situation with respect to macrophages and dendritic cells in the endometrium is more clear because there is increased accumulation of cells positive for macrophage and dendritic cell markers at Day 16 of pregnancy (Mansouri-Attia et al. 2012). Moreover, many genes associated with activation of these antigen-presenting and immunoregulatory cells are upregulated in the endometrium of pregnant cows (see Fig. 2). Heterogeneity exists in the function of various subsets of macrophages and dendritic cells, with some cell populations promoting inflammation and others limiting immune responsiveness (Butcher & Galkina 2012). During mid- and late-gestation, macrophages resident in the endometrium exhibit a pattern of gene expression consistent with cells being differentiated towards the anti-inflammatory M2 phenotype (Oliveira et al. 2010). It will be instructive to determine phenotypes of endometrial macrophages and dendritic cells earlier in pregnancy.

Activation of a massive antiviral response in the pregnant endometrium during the periattachment period distinguishes ruminants from other mammals. IFNT arose in evolution about 36 million years ago after ruminants diverged from other mammals so the gene is restricted to ruminant species (Roberts et al. 2003). The type I *IFN* locus in cattle has undergone much rearrangement and expansion compared to the mouse and human (Walker & Roberts 2009). One possibility is that IFNT was selected in evolution specifically to regulate corpus luteum function. If so, the antiviral and immunoregulatory changes in the endometrium accompanying IFNT secretion may not be important for establishment of pregnancy. It has been argued that generation of trophoblast interferons and accompanying changes in the endometrium is conserved more broadly among mammals (Bazer et al. 2009) but, if so, the characteristics of trophoblast production of interferons and endometrial response to those molecules is much different in non-ruminants than in ruminants (Roberts et al. 2008, Bazer et al. 2009). Roberts et al. (2008) has speculated that the increased expression of IFN response genes in endometrium at specific stages of pregnancy means that species use a variety of conceptus signals to induce a common set of uterine genes.
Conclusion

There is little evidence for recognition of the developing embryo by the mother until it undergoes trophoblast elongation and IFNT secretion. This is true both with respects to immunological and non-immunological recognition. It is possible that there are local changes in the endosalpinx or endometrium in response to the presence of the embryo but at this point such a conclusion is speculative. The lack of maternal immunological response to the embryo does not necessarily mean that regulatory molecules associated with the immune system are not important for development of the embryo. Embryonic growth and survival can be improved by addition of activated lymphocytes to the endometrium (Ideta et al. 2010ab) and competence of the embryo produced in vitro to go to term following embryo transfer can be enhanced by exposure to CSF2 during the morula-to-blastocyst transition (Loureiro et al. 2009). Embryotrophic cytokines may be derived from leukocytes resident in the endometrium or be products of epithelial cells or stromal fibroblasts. Unlike rodents, there is no indication that semen modifies the physiology of the mother in a way that promotes embryonic survival.

The quiescence of the reproductive tract with respect to presence of the embryo is transformed when trophoblast elongation takes place a few days before formation of the first embryo-maternal attachments accompanying placentation. At this point, the transcriptome of the endometrium undergoes large-scale changes and macrophage and dendritic cell numbers increase. The consequences of these changes for maintenance of the corpus luteum have been recognized and described but implications for the immunological relationship between the embryo and mother are only now being elucidated. The endometrial macrophage may be a key target of regulation by IFNT.

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