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Immunoregulatory molecules in human placentas: potential for diverse roles in pregnancy

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ABSTRACT Molecules with immunological functions abound in hemochorial mammalian placentas where maternal blood and tissues are in direct contact with fetal placental cells. For the most part, investigators have focused on the possibility that these molecules are primarily in place for the purpose of preventing maternal immune mechanisms from attacking the genetically different fetal cells. Yet information collected in recent years indicates that these "immunological" mediators may serve other, non-immunological functions in placentas. In this article we discuss two families of these molecules investigated in our and other laboratories, namely the tumor necrosis factor superfamily (TNFSF) and the human leukocyte antigen (HLA) family, and present accumulating evidence for dichotomy of function during gestation.

KEY WORDS: *major histocompatibility antigen, human, placenta, receptor, tumor necrosis family*

Introduction

Reproductive immunologists have long focused on the observation that the maternal-fetal interface is one of the human "immune privileged sites" where active mechanisms of cell destruction achieved by acquired immunity are muted or entirely bypassed. Important changes leading to tolerance of the fetal semiallograft occur in the maternal uterus, particularly modification of the leukocyte residents such that innate rather than adaptive responses are fostered (Bulmer *et al.*, 2010; Hunt, 2006). Yet it is the fetal placenta and extraplacental membranes that mainly provide the unusual array of substances capable of blunting or entirely avoiding the destructive capacities of maternal immune cells, thus protecting the integrity of the fetus.

Trophoblast cells derived from the trophectoderm layer of the blastocyst that form the fetal component of the maternal-fetal interface are the most accessible and therefore the most logical fetal targets for a maternal immunological onslaught. Differentiated subpopulations of these cells are anatomically positioned so as to be in direct contact with maternal blood or cells in the decidua, a tissue modified from the uterine endometrium by pregnancy hormones. Not surprisingly, it is trophoblast cells that must develop effective systems for their own protection against killing by maternal immunologic cells and their products.

Unexpectedly, studies by reproductive immunologists have shown that the protective systems in trophoblast cells employ many of the same immunological attack/defense molecules used by the immune system itself. Some of these are attack substances such as apoptosis-inducing members of the tumor necrosis factor superfamily of ligands (TNFSF) that are responsible for most instances of normal cell death, and some are defense substances such as the HLA class Ib molecules that program immune cells to tolerate foreign antigens.

The question of whether some or all of these placenta-derived molecules normally associated with host defense might have other "non-immunological" functions in reproduction has been virtually ignored as a consequence of the nearly exclusive focus on "immunological" functions. Yet experimental evidence for the concept of functional dichotomy abounds. For example, early studies in our laboratory were the first to reveal that tumor necrosis factor (TNFα is now known as TNF) itself as well as many other TNFSF ligands and receptors (TNFRSF) are transcribed in human placentas (Phillips *et al.*, 1999; Phillips *et al.*, 2001). Many investigators have now built on these original observations, and we and others have reported some of their diverse functions. One example of combined non-immunological and immunological functions in the TNFSF is FasL, a ligand that is best known for its

Abbreviations used in this paper: CTB, cytotrophoblast; HLA, human leucocyte antigen; IGF, insulin-like growth factor; TNF, tumor necrosis factor; TNFSF, TNF superfamily; TNFSFR, TNF superfamily receptor.

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participation in programmed cell death during embryogenesis (Raff, 1997), but when expressed by migrating cytotrophoblast (CTB) cells in human decidua may target immune cells and vascular elements (Abrahams *et al.*, 2004; Ashton *et al.*, 2005), clearing a path to maternal spiral arteries and permitting expansion of the maternal blood supply to the placenta. In mice, FasL expressed by trophoblast cells bordering maternal blood spaces appears to prevent bidirectional cell traffic across the placenta (Hunt *et al.*, 1997). Thus, FasL is clearly a charter member of the group of multitasking placental substances first identified in the immune system.

Expression of immune system-employed ligands in the human placental villous stroma and in villous CTB cells, neither of which is normally exposed to maternal immune cells, is exceedingly common. Expression is frequently a function of cell type and is often related to stage of gestation. The same is true of placental expression of the receptors for these same ligands; receptors for both attack and defense molecules are expressed in placentas in cell type-specific, gestation-related patterns. Collectively, these observations indicate that immunity may not drive expression in all cases.

In this article we focus primarily on evidence for novel functions of certain TNFSF ligands and their receptors expressed in human placentas that we have investigated in our laboratory. These are three apoptosis-inducing ligands, TNF, TRAIL (TNF-related, apoptosis-inducing ligand) and LIGHT (homologous to lymphotoxins, exhibits inducible expression, competes with herpes simplex virus glycoprotein D for HVEM, a receptor expressed by T lymphocytes; also known as TNFSF14), and two -apoptosis-inducing ligands, BAFF (B cell activating factor, BlyS, TALL-1, CD257, TNFSF13B) and APRIL (a proliferation inducing ligand, CD256, TNFSF13). We conclude with comments on the potential dichotomy of function revealed by studies on the HLA class Ib molecule, HLA-G.

The TNF family of ligands and receptors: evidence for dichotomy of function in placentas

Investigations we conducted as a consequence of evaluating the TNFSF and TNFRSF genes identified by J. Ni (Human Genome Sciences) as part of the Human Genome Project led us to uncover expression of multiple TNFSF and TNFRSF transcripts in human placentas (Phillips *et al.*, 1999, 2001, 2003). Some of the ligand:receptor interactions induce apoptotic cell death and others do not. The expression patterns we found and subsequent functional assays led us to the present postulate: TNFSF and TNFRSF members may be contributing to both immune protection of the placenta and to placental development and function.

Ligands and receptors causing cell death

Cell death as a central requirement for organogenesis and functioning of the mature organism is a concept that has had a major impact on research and researchers. In 1997 and 1998 full issues of *Cell* and *Science* focused on apoptosis. Raff (1997) identified two basic types of apoptosis, i.e., programmed cell death and activated cell death. Programmed cell death occurs during embryogenesis and organ regeneration, and directs cells into appropriate developmental pathways. By contrast, activated cell death is a means for eliminating activated immune cells and, therefore, down-regulating an active immune response. This latter pathway has been applied to immune privilege, where activated cells are excluded from certain organs and tissues. Both programmed and activated cell death are highly relevant to the development and function of placentas.

Members of the TNF supergene family of ligands, which number approximately 19 [4-1BBL, APRIL, BAFF, CD27L, CD30L, CD40L, EDA1, EDA2, FasL, GITRL, LIGHT, lymphotoxin alpha (LTα, TNFβ), lymphotoxin α/β (LTαβ), OX40L, RANKL, TL1A, TNF, TWEAK, TRAIL], are the main effectors of apoptosis. It should be noted that the scientific literature contains multiple names for many of the ligands and their receptors, not all of which are given here. The ligands have major homologies to one another and so do their receptors, which may associate with intracellular proteins containing death domains (Raff, 1997; Ashkenazi and Dixit, 1998). The receptors may or may not be ligand-specific; TNF and LTα share a set of receptors (TNF-R1, TNF-R2), LTα1β2 and LIGHT share the LTβR, and LIGHT and LTα share the HSV entry mediator (HVEM). LTα3 also binds TNF-R. TRAIL has five receptors, by far the largest number. Species specificity and interactions of the TNF family of ligands and their receptors have been recently reviewed, with differences reported between the human and mouse (Bossen *et al.*, 2006). Interactions of apoptosis-inducing receptors and their cognizant ligands stimulate a cascade of interactive proteins that ultimately activate certain caspases leading to cleavage of DNA. The cell death cascades are tightly controlled and may be interrupted or retarded by other molecules. Interaction of other TNFSF ligands with their receptors may, in contrast, promote cell proliferation and differentiation.

Placental expression of apoptosis-inducing TNFSF and TNFRSF genes and proteins

Specific placental expression patterns for messages encoding TNFSF ligands capable of inducing cell death identified in our laboratory are listed in Table 1 (Phillips *et al.*, 2001). Overall, mRNAs for these ligands were common, six being identified in first trimester placentas and seven in term placentas. The studies showed that (*i*) message levels in first trimester and term placentas were comparable except that LTα mRNA did not become evident until term and TRAIL mRNA was increased at term, (*ii*) mRNA levels in primary villous CTB cells from term placentas and trophoblastic tumor cells were usually about the same except that TRAIL transcripts were lacking in term CTB, (*iii*) primary term placental macrophages and macrophage tumor cells were similar except that in contrast to tumor cells, primary cells exhibited FasL mRNA and lacked 4-1BBL.

Messages encoding TNFRSF members recognizing cell death-inducing TNFSF ligands are similarly abundant in human placentas, with 13 identified in both first trimester and term placentas (Phillips *et al.*, 2001). Transcripts encoding all six that contain death domains (TNFR1, Fas, DR4, DR5, DR3, DR6), three of the four that induce death but lack death domains [TNFR2, LTβR, HVEM (now known not to signal apoptosis), 4-1BB], and all four decoy/soluble receptors (DcR1, DcR2, OPG, DcR3) were identified (Phillips *et al.*, 2001). 4-1BB was the only receptor lacking throughout. Cell lineage influenced expression; term villous CTB cells did not contain detectable Fas, HVEM or OPG transcripts

whereas term placental macrophages were missing DcR1 and OPG messages.

Although the TNFSF ligand(s) recognized by receptors are, for the most part, known, some are unknown or are very recently identified. DR6 (TNFRSF21) remains an enigma. This orphan receptor is involved in altering the phenotype and secretory profile of dendritic cells by cooperating with MMP-14 (Derosa *et al.*, 2008). Both are synthesized and expressed by tumor cells and are associated with malignancy. DR3 has recently been shown to receive signals through the TNF-like ligand, TL1A, and to have a role in rheumatoid arthritis (Bull *et al.*, 2008). Messages encoding both DR6 and DR3 are found in all placental preparations (Phillips *et al.*, 2001) and might have a role in placental immune defense and/or organ development.

Inhibitors of apoptosis

The cell death cascade is strictly controlled by members of the IAP (inhibitors of apoptosis) family, which include cIAP-1, cIAP-2, XIAP, NAIP, Survivin, Livin and a FLICE-like inhibitory protein, (FLIP), that act on caspases within the cascade. Recent studies from our laboratory have shown that mRNA derived from all of the currently known IAP genes as well as the *FLIP* gene and their encoded proteins are present in early and late gestation placentas, term CTB cells and two choriocarcinoma cell lines, JEG-3 and Jar (Ka and Hunt, 2003). Levels of IAPs and FLIP proteins differ according to gestational stage and also differ between normal and transformed trophoblast cells. Thus, IAP and FLIP proteins appear to have specific roles in placental cell survival that may include protecting normal trophoblast cells from TRAIL and other apoptosis-inducing TNF family members, but may be suborned to protect tumorigenic trophoblast cells. We have also identified FLICE-inhibitory protein (Ka and Hunt, 2006).

Non-apoptosis-inducing TNFSF ligands

Several TNFSF ligands are incapable of inducing cell death via apoptosis (APRIL, BLyS/BAFF, CD30L/CD153, CD40L/CD154, CD27L, OX40L, AITRL). Messages encoding only three of these (APRIL, BAFF, CD30L) have been identified in human placentas; all three are present in both early and late stages of gestation (Phillips 2003). We have also studied nine of their receptors and located mRNAs for CD30, CD40, RANK, OPG, CD27, OX40 and AITR in placentas. Much remains to be learned about these factors, but we have recently documented expression patterns for BAFF, APRIL and their receptors (Langat *et al.*, 2008) as described below which suggest novel functions for these ligand/receptor pairs. Information on other poorly studied TNFSF ligands is turning up in various contexts (Kim *et al.*, 2007)

TABLE 1

MESSENGER RNA ENCODING APOPTOSIS-INDUCING TNFSF LIGANDS

	TNF	*LTα*	*FasL*	*TRAIL*	*TWEAK*	*LTβ*	*LIGHT*	*4-1BBL*
1st T placenta	+*	0	+	+	++	++	+	0
Term placenta	+	+	+/0	+++	++	++	+	0
Trophoblast								
Jar	+	0	0	+++	+	0	0	+
JEG-3	+	0	0	+++	+	0	0/+	+
Term villous CTB	+	+/0	0	0	+/0	0	+**	+
Mononuclear phagocytes								
U937	+++	+/0	0	+	0	+++	+++	+
THP-1	+++	+	0	++	+	+++	+++	+
Term placental macrophages	+++	+	+	+	+	+++	++	0

These mRNAs were detected in first trimester and term placentas, term villous cytotrophoblast cells (villous CTB), trophoblastic tumor cell lines (Jar, JEG-3), myelomonocytic cell lines (U937, THP-1) and term placental macrophages (Phillips *et al.*, 2001). * RT-PCR results: negative, 0; weakly positive, +/0; positive, +; stronger positive signal, ++; strong positive signal, +++. **Originally reported as negative (Phillips *et al.,* 2001) but with more sensitive techniques now known to be positive (Gill *et al.*, 2002; Gill and Hunt, 2004)

TNF

TNF is the flag-bearer of the TNFSF of ligands. This powerful cytokine has been intensively studied for its role in inflammation in many diseases and conditions, including infected pregnancy. The gene for human TNF maps within the major histocompatibility complex on chromosome 6. This cytokine exists as either a cell-bound or soluble homotrimer depending on whether the intact molecule has been cleaved by a metalloproteinase called TACE. Although produced primarily by mononuclear phagocytes, other types of cells, including trophoblast and other human placental cells (Chen *et al.*, 1991), also transcribe and translate *TNF*.

Because of the well described role of TNF in inflammation, it was a considerable surprise to learn that this pro-inflammatory cytokine is produced in normal rat, mouse and human placentas (Chen *et al.*, 1991; Yelavarthi *et al.*, 1991; Hunt *et al.*, 1993; Hunt *et al.*, 1996). In the cycling uteri of women (Hunt *et al.*, 1992) and mice (Roby and Hunt, 1995) evidence has been collected for hormonal regulation of cellular production. Readers particularly interested in TNF, its physiological and pathological roles in female reproduction, are referred to a comprehensive recent review from Haider and Knofler (2008).

Immunological targets of placental TNF

When first identified, trophoblast-produced TNF was postulated to target and kill activated maternal immune cells via TNF-R1 (Hunt *et al.*, 1996; Jerzak and Bischof, 2002), thus participating in the protection of the semiallogeneic fetus and its extraembryonic tissues. However, levels of TNF produced in trophoblast are normally very low. Control of TNF production in trophoblast cells is critical as high levels may cause disease. For example, TNF is high in the CTB cells of intrauterine growth retardation (IUGR) placentas, as shown by Kilani and coworkers (2007), suggesting relationships between excess TNF and this common disease of pregnancy, which is strongly associated with preeclampsia. In yet another context, TNF-specific mRNA has been identified in syncytiotrophoblast (Chen *et al.*, 1991), and syncytiotrophoblast fragments circulating in mothers stimulate maternal production of inflammatory molecules that include TNF. This inflammatory response is believed by many to comprise an underlying cause of preeclampsia (Germain *et al.,* 2007). However, syncytiotrophobast-derived TNF may or may not be a substantial contributory factor.

Non-immunological targets of placental TNF

In humans, although specific message is stored in both placental trophoblast cells and placental macrophages during the course of pregnancy, levels of production of trophoblast TNF protein are extremely low whereas in placental macrophages, levels of protein are low during pregnancy but are strikingly elevated as the process of termination of pregnancy goes forward (Chen *et al.*,

1991). Thus, TNF may have an important role in parturition. Targets in the fetal membranes and/or the maternal endometrium or, more likely, the myometrium, remain to be clearly defined. A recent study from Hayashi *et al.* (2008) showed that although TNF increased in amniotic fluid during labor, soluble TNF receptors did not, suggesting that tipping the balance toward ligand abundance assisted in induction of labor. Consistent with a role for TNF in terminating pregnancy, TNF is almost invariably elevated in instances of infection-associated preterm labor.

Early on, Yui *et al.* (1994) suggested that TNF might assist in bringing fetal capillaries close to the syncytium by killing intervening villous CTB cells, a potential feature of placental development. While it is true that TNF may kill normal trophoblast cells, this cytokine supports growth of trophoblastic tumor cells (Yang *et al.*, 1993), illustrating how normal processes may be subverted during malignancy. Interestingly, as noted above, this powerful cytokine is produced in the cycling uterus as well as the pregnant uterus (Hunt *et al.*, 1992), suggesting that TNF with its ability to regulate many other genes is useful in both fetal and maternal tissues undergoing differentiation.

Protection from endogenous TNF

Production of TNF in normal placentas raises the question of how the placental cells themselves are protected from TNF-mediated apoptosis. Placental cells express TNFR1, an apoptosis-signaling receptor, and TNFR2, a receptor that may mediate apoptotic effects but is less efficient than TNFR1. Austgulen and coworkers were the first to present experimental evidence for a protective mechanism (1993). Their studies show that TNF produced by fetal tissues is very likely to be absorbed by soluble TNF receptors, which are abundant in amniotic fluid. At term, overflow receptors are found in maternal urine.

TRAIL

The human TRAIL gene is located on chromosome 3 at position 3q26 (Golstein, 1997). TRAIL is a type II membrane protein, the carboxy-terminal extracellular domain of which shows clear homology to other TNF family members. Unlike some other TNFSF ligands, the amino-terminal intracellular domain of TRAIL is short and is not conserved between mice and humans. While having many features in common with TNF, TRAIL also differs significantly: transcripts are readily detectable in many normal tissues (spleen, thymus, prostate, lung, placenta) although they are absent in others (brain, liver, testis).

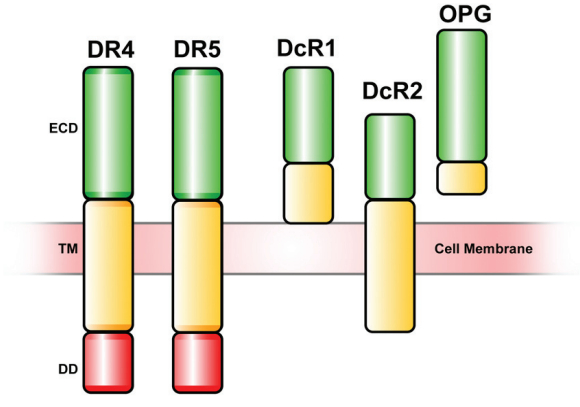

Fig. 1. **Schematic drawing of the five TRAIL receptors.** *Two transmembrane receptors mediate apoptosis using a death domain (DD), DR4 and DR5, two are decoy receptors, DcR1 and transmembrane DcR2, and the fifth, osteoprotegrin (OPG), is a soluble receptor.*

TRAIL-mediated functions are regulated by expression of multiple receptors (Fig. 1). Two receptors transduce apoptotic signals (DR4/TRAIL-R1; DR5/TRICK2/TRAIL-R2) and two operate as decoy receptors (DcR1/TRID/LIT/TRAIL-R3; DcR2/TRUNDD/TRAIL-R4). A fifth receptor, osteoprotegerin (OPG), is a secreted protein. TRAIL clearly has the greatest flexibility of all the known apoptosis-inducing members of the TNF supergene family because of this multiplicity of receptors, which permits fine selection of resistance/susceptibility to killing on an individual cell basis.

Immunological targets of placental TRAIL

Early experiments in our laboratory (Phillips *et al.*, 1999) showed that TRAIL mRNA and protein are readily detected in human placentas, where proteins are localized to both trophoblast and macrophages. Although no experiments were done we postulated that trophoblast-derived TRAIL would protect the placenta from activated circulating or decidual maternal immune cells expressing apoptosis-signaling DR4 or DR5 while being protected from autocrine destruction by decoy receptors.

Discussing this point of how trophoblast TRAIL might function, Jerzak and Bischof (2002) in a later review described TRAIL as a critical component of the array of aggressive (and protective) devices provided to migrating CTB cells in early gestation decidua. The conclusion was that CTB-derived TRAIL killed immune cells, presumably uterine natural killer cells, macrophages and/or certain subpopulations of T lymphocytes (Bulmer *et al.*, 2010), as they migrate toward the spiral arteries. However, this issue is not fully resolved as FasL may also be involved (Abrahams *et al.*, 2004). Harris and coworkers (2007) report that in the BeWo trophoblastic tumor cell line both FasL and TRAIL play active roles in remodeling spiral arteries using their apoptosis-inducing properties, and Ashton *et al.* (2005) have reported that remodeling by the Fas/FasL system involves endothelial cells as targets.

Non-immunological targets of placental TRAIL

TRAIL appears to have several non-immunological functions in placentas. One is assisting in modifying maternal spiral arteries, as mentioned above. Although this activity utilizes the apoptosis-inducing function of TRAIL, the targets may be cells comprising the vasculature (Keogh *et al.*, 2007), not immune cells. In these studies, vascular smooth muscle was identified as the target. The result is expansion of the maternal blood supply required for delivery of nutrients and removal of waste products through the placenta (Keogh *et al.*, 2007).

Identification of TRAIL ligand and receptors within the human placenta suggests that apoptosis might be utilized for modeling and remodeling as the placenta develops and differentiates, with placental macrophages as potential sources of ligand. Trophoblast cells harvested from placental villi displayed transcripts encoding TRAIL receptors DR4 and DR5 that signal apoptosis and also DcR1 and DcR2 soluble receptors that could inhibit cell death pathways. These findings suggest a highly sophisticated paracrine system in placentas for maintaining or diminishing CTB cell viability.

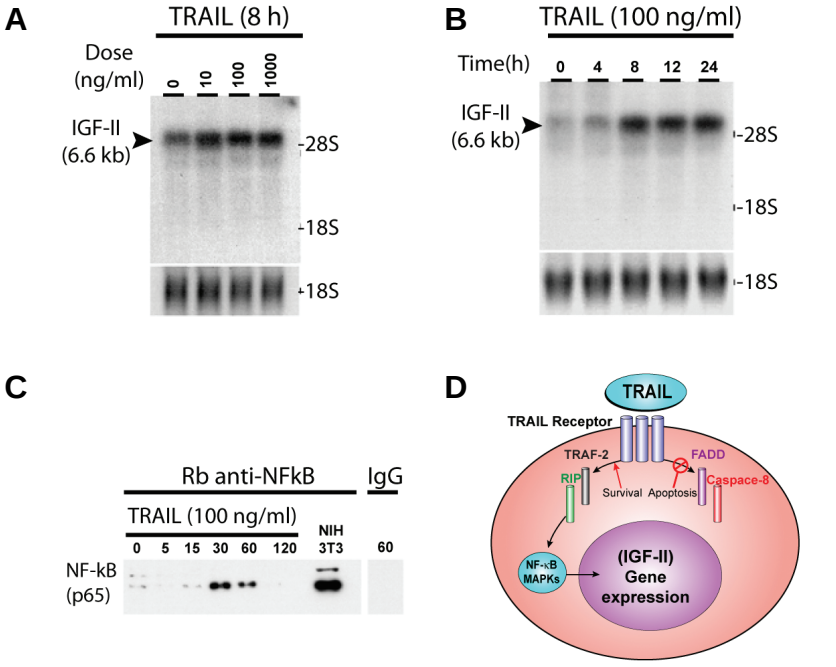

Fig. 2. **TRAIL and induction of *IGF-II* mRNA in villous cytotrophoblast cells**. **(A)** *Levels of* IGF-II *mRNA in term villous cytotrophoblast cells are increased by 10, 100 and 1000 ng/ml of recombinant TRAIL.* 18S *mRNA served as a loading control.* **(B)** *Levels of* IGF-II *mRNA in term villous cytotrophoblast cells exposed to 100 ng/ml of recombinant TRAIL are elevated after 4 h, maximal at 8 h, and continue elevated at 12 and 24 h.* 18S *mRNA served as a loading control.* **(C)** *Induction of NFkB p65 in term villous cytotrophoblast cells following exposure to 100 ng/ml of recombinant TRAIL is maximal at 30 minutes. NIH3T3 cells serve as a positive control and normal IgG provides a negative control.* **(D)** *A schematic drawing depicting a potential pathway by which TRAIL drives increases in* IGF-II *mRNA in term villous cytotrophoblast cells.*

A second potential activity is modifying production of a critical growth factor, IGF-II, by term villous CTB cells. Unreported experiments in our laboratory (Ka, H., and Hunt, J. S.) have provided preliminary evidence in support of the idea that human trophoblast cells undergo stimulation by TRAIL that results in a a dose-dependent elevation of levels of mRNAs encoding this major growth factor (Fig. 2A). Message levels are low in the absence of recombinant TRAIL but peak between eight and 24 h post-exposure to100 ng/ml of this cytokine (Fig. 2B). This is associated with elevation of NFκB mRNA as early as 15 minutes after exposure (Fig. 2C). Potential sources of TRAIL would include placental macrophages (Table I) and decidual stromal cells (Popovici *et al.*, 2000) and could take place via the simplified pathway shown in Figure 2D. Whether IGF-II protein levels are increased remains to be seen; villous CTB cells deliver other species of mRNA and protein to the adjacent syncytiotrophoblast when the two are associated *in vivo*, but in our experience are poorly secretory when cultured *in vitro*.

Protection from endogenous TRAIL

Studies in our laboratory were the first to show that mRNAs encoding all of the TRAIL receptors are expressed in human placentas, with trophoblast cell lines expressing high levels of both the apoptosis signaling receptor, DR5, and the decoy receptor, DcR1 (Phillips *et al.*, 1999; Phillips *et al.*, 2001). The same lines could not be killed by recombinant TRAIL, indicating that, at least in trophoblast tumor cell lines, DcR1 (TRAIL-R3) may protect against apoptosis. More recently, Lonergan *et al.* (2003) have reported that osteoprotegerin, a soluble receptor for TRAIL, as well as the two TRAIL decoy receptors, TRAIL-R3 and -R4, are high in gestational membranes and may protect against the proapoptotic effects of locally produced TRAIL. Thus, all three receptors may be important.

In addition, as mentioned above, trophoblast cells exhibit multiple members of the IAP family, which include cIAP-1, cIAP-2, XIAP, NAIP, Survivin, Livin and a FLICE-like inhibitory protein (FLIP), all of which act on caspases within the apoptosis cascade (Ka and Hunt, 2003). These may also protect trophoblast against endogenous TRAIL-mediated damage.

LIGHT

LIGHT is an apoptosis-inducing member of the TNFSF synthesized in normal human placentas (Gill *et al.*, 2002; Gill *et al.*, 2006). Messenger RNAs encoding LIGHT and all of its receptors, lymphotoxin-β receptor (LTβR), decoy receptor-3 (DcR3) and herpes virus entry mediator (HVEM) are present in both first trimester and term placentas, and proteins have been localized to specific cells by immunohistology. Both ligand and receptors are positioned to assist in placental immune privilege as well as placental development (Fig. 3).

Immunological targets of placental LIGHT

The idea that LIGHT expressed on syncytiotrophoblast assists in protecting the placenta from receptor-bearing maternal immune cells remains a possibility although much work remains to be done. Both LIGHT and all three of its receptors are readily identified on syncytiotrophoblast early and late in pregnancy (Gill *et al.*, 2002; Gill *et al.*, 2006), raising the possibility of cross-talk with maternal immune cells throughout gestation. Although co-expression of LIGHT and one of its receptors, HVEM, reportedly leads to allograft rejection via T cell-Tcell interactions and activation of natural killer cells (Ye *et al.*, 2002; Granger and Rickert, 2003; Fan *et al.*, 2006), the HVEM receptor has been shown in more recent experimentation to have an anti-inflammatory role, particularly when expressed by innate immune cells such as macrophages and their relatives (Steinberg *et al.*, 2007). LIGHT's other membrane bound receptor, LTβR, has been reported as indispensable for recruitment of bone marrow derived dendritic cells to peripheral tissue (Abe *et al.*, 2003). Given that both macrophages and dendritic cells are present in decidua during pregnancy (Kammerer *et al.*, 2003; Bulmer *et al.*, 2010), placental LIGHT expression may influence the maternal immune system through modulation of these central antigen-presenting cells.

Although this question is of considerable interest, it is also important to understand if (*i*) LIGHT and its receptors might have a role(s) in the processes involved in placental develop-

ment and cell differentiation and (*ii*) if HVEM on the syncytiotrophoblast might defend against cytotoxic, LIGHT-expressing circulating maternal immune cells.

Non-immunological targets of placental LIGHT

Studies on the temporal distribution of LIGHT and its receptors in our laboratory strongly suggest a developmental role for this system. In early (6 to 7 weeks) gestation, LIGHT and two of its receptors (LTβR, DcR3) are expressed on villous CTB cells but neither ligand nor receptors is present on stromal cells (Fig. 3) (Gill *et al.*, 2002; Gill *et al.*, 2006). All three molecules are readily identified in stromal cells by gestation week 8, and expression continues to term. In contrast, LIGHT, LTβR, and DcR3 are expressed on CTB cells throughout gestation. On syncytiotrophoblast these are joined by HVEM expression. Therefore, prior to 8 weeks of gestation placental stromal cells are unlikely participants in LIGHT pathways whereas both precursor CTB cells and differentiated syncytiotrophoblast would be fully engaged, with only the syncytiotrophoblast layer that is exposed to maternal blood receiving the extra protection of HVEM. The HVEM/LTβR story is interesting; signaling through HVEM involves TRAF2 and TRAF5, which interact with NFκB (Wu *et al.*, 1999; Rooney *et al.*, 2000), a protective pathway, whereas LTβR signaling involves TRAF3, which interacts with caspases and causes apoptosis. Clearly, multiple ligand/receptor interactions are required to maintain or diminish syncytiotrophoblast viability as required during the various stages of pregnancy.

Identification of LIGHT ligand and receptors within the stroma of the placental villi suggests that, as with FasL in embryonic tissues during development, LIGHT-mediated apoptosis might be utilized for modeling in the placenta.

Protection from endogenous LIGHT

Co-expression of LIGHT and its receptors may lead to either inflammatory or anti-inflammatory conditions (Ye *et al.*, 2002; Granger and Rickert, 2003; Fan *et al.*, 2006; Steinberg *et al.*, 2007). Yet both ligand and all three receptors are readily identified on syncytial trophoblast both early and late in pregnancy (Gill *et al.*, 2002; Gill *et al.*, 2006). Lack of cell death might be traced to expression of HVEM as well as co-expression of the DcR3 decoy receptor on this cell layer. Interestingly, as with the LIGHT system, B7H1, a receptor that has been posited to both stimulate and inhibit lymphocyte activation, is also expressed on syncytiotrophoblast (Petroff *et al.*, 2003). Although positioning on the apical surface of syncytiotrophoblast encourages the idea that these molecules effectively protect against attack by activated, circulating blood lymphocytes, definitive evidence is lacking. More convincing documentation may eventually be reported as both are expressed in mice.

Studies in our laboratory designed to investigate mechanisms protecting a second subpopulation of trophoblast, villous CTB cells, from LIGHT-mediated apoptosis have shown that these cells purified from term placentas are highly resistant to LIGHT-induced apoptosis (Gill and Hunt, 2004). The cells are also resistant to killing by another placental cytokine, interferon-γ (IFN-γ), but together the two cytokines induce apoptosis. Killing is prevented by a decoy receptor fragment (DcR3). Additional studies on two of the cellular inhibitors of apoptosis expressed in CTB cells, cIAP-1 and cIAP-2, indicated that cIAP-2 has an important protective role but that this may be overridden when IFNγ is produced during inflammation (Gill and Hunt, 2004). Collectively, our studies are consistent with the postulate that placental CTB cells are protected from LIGHT-mediated apoptosis by a soluble receptor, DcR3, as well as cIAP-2.

BAFF and APRIL: potential immunological and non-immunological targets

The TNFSF contains two soluble ligands that do not mediate apoptosis but are involved in B lymphocyte development, BAFF and APRIL. Three receptors recognize the two ligands: the exclusive BAFF receptor (BAFF-R, CD268, TNFRSF17) and two receptors that recognize both BAFF and APRIL, TACI (transmembrane-activator-1 and calcium-modulator- and cyclophilin ligand-interactor CD267, TNFRSF13B) and BCMA (B cell maturation antigen, CD269, TNFRSF13C). All but BAFF-R, for which reagents were not available, have been located in human placentas (Phillips *et al.*, 2003). BAFF and APRIL are best known for their roles in stimulating B cell growth and differentiation, as illustrated in Fig. 4. BAFF has been assessed in the sera of pregnant women: Figure 5 shows that BAFF peaks at midgestation (weeks 12 through 18) and declines from week 24. It is not known whether BAFF in maternal sera has arisen in part from the fetal placenta or whether declining levels at term reflect transport into the fetus or diminished placental or maternal contributions. Observations discussed below suggest that It may be possible to address these questions in mice.

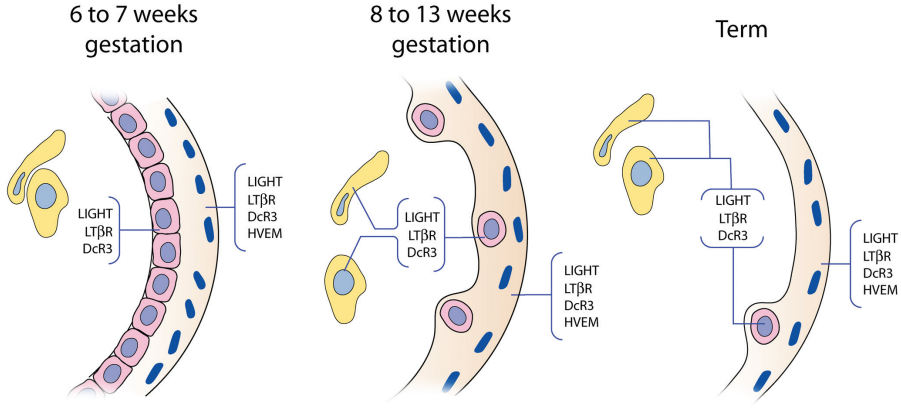

Fig. 3. Localization of LIGHT and its receptors in human placentas. *Gestation-related expression suggests the possibility of autocrine/paracrine impact on placental development and/or function as well as defense against activated immune cells in maternal blood. Stromal cells, yellow; villous CTB cells, pink; syncytiotrophoblast, tan.*

BAFF and APRIL in human placentas

Our experiments showed that both BAFF and APRIL messages and proteins were present and illustrated cell type-specific and gestation-dependent expression patterns. By immunohistology (Phillips *et al.*, 2003), APRIL protein was prominent on the microvilli of syncytiotrophoblast and in the cytoplasm of both syncytiotrophoblast and villous CTB of first trimester placentas

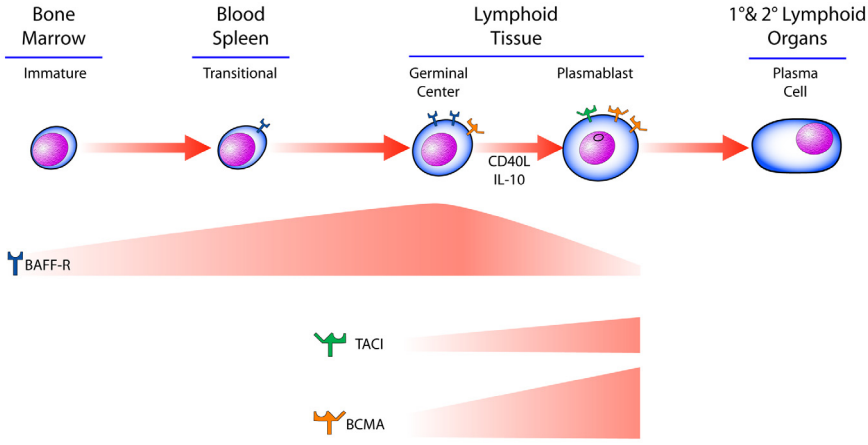

Fig. 4. Pathway by which interaction of BAFF and APRIL with receptors on B lymphocytes drives B cell proliferation and differentiation. *BAFF-R present on transitional B lymphocytes in blood and spleen is shown in dark blue. TACI, which is most prominent on plasmablasts is shown in green and BCMA, a feature of germinal center B lymphocytes and plasmablasts, is shown in orange.*

but was essentially undetectable in term placentas. By contrast, BAFF was prominent in villous CTB cells but was weak in syncytiotrophoblast. As with APRIL, BAFF was more abundant in early than in late gestation placentas. This difference was clearly distinguished in western blots. In this same study, neither BCMA or TACI receptor mRNA was identified although transcripts encoding other non-apoptosis-inducing TNFRSF (CD30, RANK, OPG, CD27, OX40, AITR) were readily detected by semi-quantitative PCR. These data have been verified by Guo *et al*. (2008) and expanded to note that BAFF was decreased in the tissues of women with recurrent spontaneous abortion (RSA).

We concluded from this first series of experiments that placental BAFF and APRIL target in a paracrine manner to B lymphocyte precursors in mothers, assisting them in producing the high quantities of antibody required for delivery into the fetus.

Receptors in human placentas

A new study in our laboratory has revealed that both ligands and receptors are present in human placentas but expression is cell lineage-specific (Langat *et al*., 2008). Placental cells were separated into villous CTB preparations and mesenchymal cell preparations using anti-HLA class I affinity columns and were tested by real-time PCR. Villous CTB cells contained barely detectable levels of BAFF and APRIL transcripts whereas villous mesenchymal cells contained high levels. As for receptors, both Real-Time PCR and immunohistochemistry identified BAFF-R and BCMA mRNA and proteins in villous CTB cells but essentially no TACI. By contrast, villous mesenchymal cells contained readily detectable levels of all three receptors. Repetition of this work by Guo *et al*. (2008) failed to uncover BAFF-R mRNA in either normal or RSA patients; the reason for this discrepancy is not known.

Altogether, our results indicate that although signaling to maternal B cells might take place there is also the possibility that in humans, placental mesenchymal cell BAFF and/or APRIL signal to placental villous CTB cells, a concept illustrated in Fig. 6 A,B. Thus, lineage-specific regulation of placental cell viability, differentiation and/or other activities may be novel functions of these proteins.

BAFF in mouse placenta

Further studies on BAFF in pregnancy may be conducted in mice, as this cytokine can readily be identified in maternal blood by commercial ELISA (Pace, J.L. and Hunt, J.S., unreported results). BAFF is also present in placentas. Unpublished immunohistochemical studies in our laboratory show that at gestation day 8, BAFF protein is strongly expressed in the extraembryonic endoderm (Fig. 7A), by gestation day 10 is present in both trophoblast and embryo (not shown), and at gestation day 14 is readily identified in decidual cells immediately adjacent to the placenta and in glycogen cells of the spongiotrophoblast layer. This cytokine is absent in giant trophoblast cells and large spongiotrophoblast cells (Fig. 7B). Trophoblast cell lines generated in our laboratory also demonstrate BAFF mRNA (Fig. 7E), and might be useful tools for exploring regulation and function. Given the importance of BAFF and APRIL in rheumatoid and other antibody-mediated autoimmune diseases, conducting functional experiments, perhaps in the mouse system where natural mutants and genetically modified strains are readily available, could be highly important to pregnant women.

Placental HLA class Ib expression and potential for dichotomy of function

The discovery of HLA class Ib antigens in placentas generated considerable excitement and raised hopes that the major modulator of maternal-fetal immunological interactions was finally identified. Evidence for this postulate have been supplied in multiple functional studies as reviewed by Hunt *et al*. (2005), Hunt

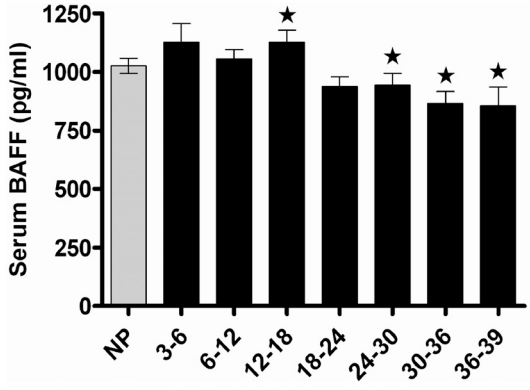

Fig. 5. BAFF levels in normal pregnant women. *Sera from 99 women (156 samples) were tested using a BAFF ELISA assay (R&D Systems, Minneapolis, MN). The figure shows the mean and SEM for nonpregnant (n, 50) first (n, 32; gestation weeks 3 through 12), second (n, 33; gestation weeks 12 through 24) and third trimester (n, 29; gestation weeks 24 through 39) patients. *, P<0.05 against nonpregnant (NP) control.*

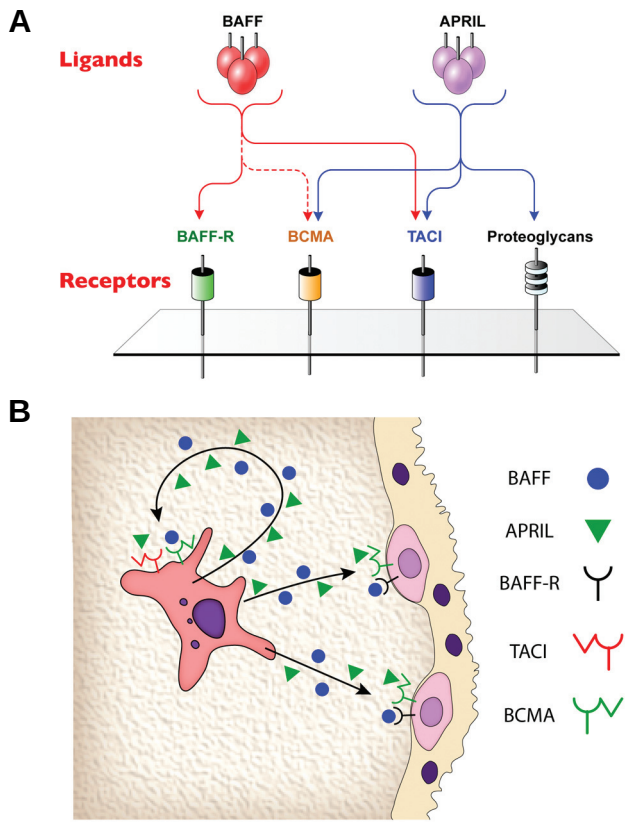

Fig. 6. BAFF and APRIL: potential ligand/receptor interactions in human placentas. **(A)** *Schematic drawing showing known ligand (BAFF, APRIL)/receptor (BAFF-R, TACI, BCMA) interactions.* **(B)** *Potential autocrine and paracrine pathways in human placentas. Stromal cells, dark pink; villous CTB cells, light pink; syncytiotrophoblast, tan.*

(2006) and Carosella *et al.* (2008) and taken to the genetic level by Hviid *et al.* (2003) and others who have correlated allelic differences with isoform choices and common placental pathologies. As a consequence of this excitement, the structures and expression patterns of the HLA genes and antigens expressed in human placentas have been exhaustively investigated over the past decade. Chromosome 6p21.3 is the location of genes encoding both HLA class Ia and Ib antigens. The class Ia antigens as well as the class II antigens are highly polymorphic and stimulate immune rejection when disparate between host and graft, which accounts for the requirement for tissue matching prior to organ transplantation. By contrast, the HLA class Ib genes have very low numbers of alleles. It is this difference that is believed primarily to account for the decision of placental cells to substitute the class Ib antigens for class Ia antigens expressed by nearly all other types of normal cells.

Expression of HLA class Ib antigens in placentas

All three HLA class Ib antigens, HLA-E, -F, and –G, are exhibited by placental cells. HLA-E is expressed on the surface of all cells exhibiting HLA class I molecules, including invasive trophoblasts, because of its unique ability to combine with all other class I molecules as they progress from the endoplasmic reticulum to the cell surface. This HLA class Ib protein appears to be an important regulator of uterine natural killer cells via the CD94-NKG2 receptor family (Sullivan *et al.*, 2007). HLA-F is present in/on cells at the maternal-fetal interface (Ishitani *et al.*, 2003) but its functions remain unknown.

HLA-G and isoform functions

HLA-G is the most thoroughly studied of the placenta-expressed HLA class Ib genes. All of the seven HLA-G mRNAs derived from the single mRNA by alternative splicing are generated in trophoblast cells. These exhibit some degree of redundancy as both HLA-G5 (sG1) and HLA-G6 (sG2) inhibit CD8 expression by lymphocytes (Morales *et al.*, 2003) and homozygosity of a null allele that prevents synthesis of two of the seven isoforms (HLA-G1, HLA-G5) but not the other five fails to interrupt pregnancy (Ober *et al.*, 1998). Whether or not non-redundant functions will be identified remains to be established unequivocally, but in our own studies, recombinant HLA-G5 produced in human embryonic kidney (HEK) cells is a similar but usually more powerful modulator than HLA-G6 produced in the same types of cells (Morales *et al.*, 2003; McIntire *et al.*, 2005).

Expression of inhibitory receptors for HLA-G and functional implications

Immunoregulation is a well documented property of both membrane and soluble forms of HLA-G (reviewed by Hunt *et al.*, 2005; Hunt, 2006; Carosella *et al.*, 2008). Binding to receptors such as the leukocyte immunoglobulin-like receptors (LILR)B1 (CD85j, ILT2, LIR-1), and LILRB2 (CD85d, ILT4, LIR-2), which interfere with immune cell activation, have been implicated in this process. LILRB1 are found on many types of leukocytes whereas LILRB2 are generally believed to be restricted to the myeloid family, which includes monocytes, macrophages and dendritic cells (Allan *et al.*, 2000; Shiroishi *et al.*, 2003). Both receptors have inhibitory effects on immune cell activation, blocking stimulating pathways leading to production of inflammatory cytokines. In the placenta and decidua, villous stromal cells are positive for both receptors (McIntire *et al.*, 2008; Sifers T, Hunt, JS, unreported observations). These data are consistent with an earlier finding from our laboratory showing that highly purified macrophages selected from maternal decidua are positive for both LILRB1 and LILRB2 (Petroff *et al.*, 2002). Possibly, inhibitory receptor expression prevents unwanted activation of macrophages and other immune cells during normal pregnancy.

Yet immunosuppression may or may not be the major function of HLA-G. Experiments reported by Fons *et al.* (2006), who have uncovered another receptor for HLA-G, CD160, on endothelial cells, raise the question of which function of HLA-G is essential to pregnancy, its immune regulatory property or regulation of vasculature. This question is also raised by our recent observation that placental vascular smooth muscle exhibits reactivity with an anti-LILRB2 monoclonal antibody shared with us by Amgen, Inc. (clone M422; McIntire *et al.*, 2008). Although the apparent expression of an inhibitory receptor on placental smooth muscle suggests that HLA-G may program normal development or function of the placental vasculature, the specific isoform is not known and considerable experimentation remains to be done. Nonetheless, the idea that HLA-G

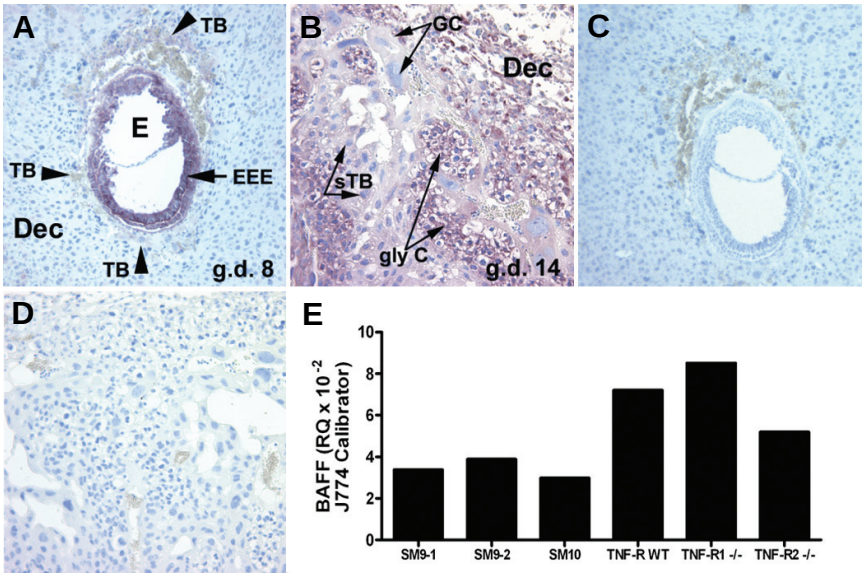

Fig. 7. Identification of BAFF in pregnant C57Bl/6 mice and mouse placental cell lines. (A) *Gestation day 8 implantation site showing weak BAFF protein signals in the extraplacental cone and anti-mesometrial trophoblast, with strong signals in extraembryonic endoderm.* **(B)** *Gestation day 14, interface of placenta and maternal decidua shows positive BAFF signals in cells in the decidua, some or all of which may be migrating trophoblast cells. Glycogen cells are also positive but giant cells and large spongiotrophoblast cells are negative.* **(C,D)** *Isotype controls are negative. Dec, decidua; E, embryo space; EEE, extraembryonic endoderm; GC, trophoblast giant cells; glyC, glycogen cell trophoblast; sTB, spongiotrophoblast; TB, trophoblast. All micrographs, x200 original magnification.* **(E)** *BAFF mRNA in six mouse trophoblast-like cell lines tested by real time PCR. Signals for mouse trophoblast BAFF were quantified against BAFF mRNA in the J774 mouse macrophage cell line (positive control). Cell lines include three derived from Swiss mouse placentas (SM-1, SM-2, SM10), one from a TNF-R-competent wild type mouse placenta (TNF-R WT), one from a TNF-R1-/- mouse placenta and one from a TNF-R2-/- mouse placenta.*

might affect placental vasculature is supported by the study on monkeys where administration of anti-Mamu AG to pregnant monkeys disrupted generation of placental blood vessels (Golos *et al.*, 2010).

Definitive evidence

Evaluating the question of whether or not HLA class Ib antigen production in the preimplantation embryo (Hunt *et al.*, 2006) and/or defined placenta (Hunt *et al.*, 2005; Hunt, 2006) is essential is exceedingly difficult. Discussion continues on expression patterns of HLA-G and its isoforms and their functions, with conflicting viewpoints abounding in the recent scientific literature (Blaschitz *et al.*, 2005; Hunt and Geraghty, 2005; Hunt, 2006; Morales *et al.*, 2007; Apps *et al.*, 2008). Although all authors are striving to arrive at valid principles, some of these reports are based on potentially flawed experimental findings and others on incomplete literature searches. Further, putative homologues of the human genes are expressed only in primates, and the homology between humans and non-human primates is not perfect (Langat *et al.*, 2004). Thus, evidence favoring a central position for HLA-G remains mainly indirect; as with the complement regulatory proteins that protect the placenta from destruction by complement-activating cytotoxic maternal antibodies, the main point in favor of centrality for this gene product is lack of identification of any living person failing entirely to synthesize all isoforms of HLA-G.

Summary

Human placentas and, to a lesser extent, hemochorial placentas in other mammals, produce an unexpectedly rich array of molecules normally associated with the immune system. Extensive investigation has shown that many of these substances drive the localized tolerance required for success of semiallogeneic pregnancy. Yet evidence is mounting for the idea that many of these modulators of maternal-fetal tolerance are established in placental cells to provide other services during the course of gestation. Most convincingly, not only the immune system-associated molecules, but also many of their receptors are expressed by placental cells, indicating that autocrine, juxtacrine and/or paracrine actions may permit novel, unexpected functions of these placental ligands. We and others have considered the possibility that specific apoptosis-inducing cytokines associated with reproduction (TNF, TRAIL, FasL, LIGHT), as well as non-apoptosis-inducing TNFSF ligands, may have new functions in placental cell development and function (Yui *et al.,* 1994; Hunt *et al.,* 1995; Phillips *et al.,* 1999; Jerzak and Bischof, 2002; Phillips *et al.,* 2003; Chen *et al.,* 2004; De Falco *et al.,* 2005; Langat *et al.,* 2008). Here, in an effort to establish a general principle of economy of gene expression and protein production, we present a collection of data correlating production of both apoptosis-inducing and non-apoptosis-inducing substances with governance of placental cell development and function. Definitive experiments to establish what we propose to be functional dichotomy, cannot be performed in humans and are difficult even in genetically modified experimental rodents. Essentially all are embryonic or neonatal lethals. Moreover, as with human leucocyte antigen-G (HLA-G), the substances of interest may be expressed exclusively in the highly sophisticated systems of primates. In summary, many placental products that contribute to immune privilege in pregnancy are likely also to have major roles in driving placental development and programming its functions. Recognition of the power of these "immune molecules" to influence placentas is not only critical to understanding the developmental biology of healthy placentas, but may also be important to the avoidance of therapeutic interventions during pregnancy that would adversely impact the placenta.

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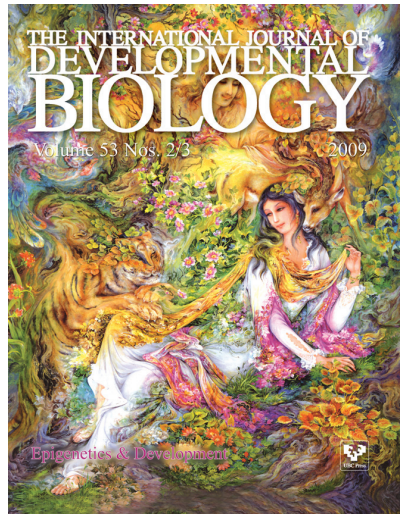

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