A Variety of Opportunities for Immune Interactions During Trophoblast Development and Invasion

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Introduction

In the human, the processes of implantation and placentation are closely linked to each other and involve the direct contact of fetal cells and tissues with maternal cells and tissues. It is not only close contact but there is also invasion of fetal cells into maternal tissues and migration through the extracellular matrix of such tissues. Of course, this unusual interaction of two individuals is extremely tightly regulated, as normal pregnancy should not harm the mother or lead to rejection of the baby. During the course of pregnancy, especially during trophoblast invasion, a number of morphological sites develop and enable an immunological interplay between the two individuals at various levels:

1. Gestational days 6–7 after conception (pc):

   The blastocyst adheres and attaches to the uterine epithelium.

2. Gestational days 7–9 pc: The early invasive syncytiotrophoblast invades into uterine tissues.

3. Gestational day 14 until delivery:

   Extravillous trophoblast (EVT) first invade into the uterine decidua and then reach the myometrium.

4. Gestational week 3 until delivery:

   Subsets of the extravillous trophoblast invade into uterine spiral arteries (endovascular trophoblast) or uterine lands (endoglandular trophoblast).

5. Gestational weeks 4–11:

   First trimester: Only maternal plasma is flowing through the intervillous space of the placenta.

6. Gestational week 11 until delivery:

   After the first trimester: Maternal blood flows into and through the intervillous space of the placenta.
The blastocyst adheres and attaches to the uterine epithelium

The blastocyst develops from the morula and is characterized by the development of the first cell lineage during human embryogenesis, the trophoblast. During the blastocyst stage, mononucleated trophoblast cells (also called trophectoderm cells) set up the outer wall of the blastocyst, leaving space for the embryoblast, also called the inner cell mass, and the cavity of the blastocyst, the blastocoel.

Those trophectoderm cells that are in direct contact to the inner cell mass seem to be the ones that are crucial for attachment of the blastocyst to the uterine epithelium. It appears that attachment of trophectoderm cells covering the blastocoel but not being in contact to the embryoblast takes place when blastocyst adhesion does not occur properly. Further rolling of the blastocyst and attachment of the ‘wrong’ trophectoderm cells have been described in pathological pregnancies arising from in vitro fertilization. This displaced attachment may result in abnormal placental development and may further progress into abnormalities such as abnormal shapes of the placenta and eccentric insertion of the umbilical cord. It seems as if the close interaction between embryoblast, trophectoderm, and uterine epithelial cells is crucial for a correct regulation of the materno-fetal interactions controlling implantation.

Also the cells of the uterine epithelium need to adapt to blastocyst attachment and to go through remarkable ultrastructural changes preceding direct contact with the trophoblast. The apical site of the epithelial cells withdraws the microvillous brush border and builds up flattened projections, called pinopods or uterodomes. Only in the presence of such pinopods, the trophectoderm cells of the blastocyst can definitively adhere and attach to this epithelium.
The early invasive syncytiotrophoblast invades into uterine tissues

At gestational day 6–7 pc, the blastocyst has firmly attached to the uterine epithelium and initiates implantation. At this time point, a fundamental differentiation step of the trophoblast takes place, the development of the multinucleated syncytiotrophoblast. The trophoblast cells that are in direct contact to cells of the uterine epithelium (and maybe only those in direct contact to cells of the embryoblast) syncytially fuse with each other. These first fusion events result in the generation of the first, still oligonucleated syncytiotrophoblast. Those trophoblast cells remaining as mononucleated cells are now termed ‘cytotrophoblast cells’. As the multinucleated syncytiotrophoblast is no longer able to proliferate, the pool of cytotrophoblasts acts as a reservoir of cells. This pool of cytotrophoblasts further generates other trophoblast subtypes in the course of pregnancy as well. At the same time, those cytotrophoblasts located underneath the syncytiotrophoblast serve as the pool to refresh this multinucleated layer. By continuous proliferation and subsequent syncytial fusion, they serve as the basis for growth and preservation of the syncytiotrophoblast throughout pregnancy.5 The extravillous subset of trophoblast cells outside the placental villous tissues and the villous subset of trophoblast acting as epithelial cover of placental villi represent the two major sets of trophoblast cells, different in function, expression profile, and morphology.

Syncytial Fusion and Its Implications for Trophoblast Development

Interestingly, the very early fusion events of the trophectoderm cells are different to trophoblastic fusion events later in pregnancy. At the time of implantation, two mononucleated trophectoderm cells fuse with each other. This may even occur several times during implantation. Later in pregnancy, the trophoblast has further differentiated into the villous trophoblast covering placental villi as a two-layered epithelium and the extravillous trophoblast invading into uterine tissues. The syncytiotrophoblast develops into the outer layer of the villous trophoblast with a pool of mononucleated cytotrophoblasts underneath. Another set of mononucleated cytotrophoblasts leaves the placental parenchyma and becomes EVT. While fusion between two EVT still occurs later in pregnancy, fusion between two neighboring mononucleated villous cytotrophoblasts should no longer take place. Now it is fusion between a mononucleated cytotrophoblast with the overlying syncytiotrophoblast that comes into play.5 If the villous trophoblast fusion occurred between two mononucleated cytotrophoblasts, the feeding of the syncytiotrophoblast would stop at specific sites and result in disintegration of the syncytiotrophoblast. It would also produce a second syncytial layer, which interestingly can be found in the labyrinth of mouse and rat placentas.6

Trophoblast and the Penetration of Monolayered Epithelia

At the time of implantation, penetration of the uterine epithelium by the blastocyst is mandatory for a successful pregnancy.7 Although comprehensive knowledge of the mechanisms involved in implantation in the human is still rare, it seems that also in the human, the early syncytiotrophoblast is the only tissue that is able to penetrate through the uterine epithelium.8 Hence, hindering early syncytial fusion of trophectoderm cells results in blocking implantation and thus pregnancy.

In vitro studies using placental cells and tissues provided further evidence that mononucleated cytotrophoblasts cannot penetrate a living monolayered epithelium. We have used first-trimester villous explants and have confronted them with term ‘amnion or first-trimester decidua’. In both cases, the outgrowing mononucleated EVT were not able to penetrate through an intact living epithelium, even if it is a simple monolayered epithelium similar to the uterine epithelium.9,10

EVT first invade into the uterine decidua and then reach the myometrium

Differentiation of EVT in Cell Columns

At about day 14 pc of pregnancy, cytotrophoblasts have penetrated through the syncytiotrophoblast and, for the first time during pregnancy, directly contact maternal uterine tissues. At this stage, those cells have left the placental parenchyma and are no longer found within the placental villous tissues. This is why they have been termed ‘EVT’.

Between the tips of anchoring villi and decidual tissues, EVT accumulate in column-shaped structures,
called trophoblast cell columns. The cell columns are the source for all EVT invading into uterine tissues. Interestingly, trophoblast proliferation within the cell columns is only found in the proximal part close to the basement membrane of the anchoring villi.\textsuperscript{11,12} The post-proliferative daughter cells that have lost contact to the basement membrane leave the cell cycle and are pushed forward in the cell column because of the proliferation pressure above them. Only when they reach the distal end of the column and come into contact to maternal tissues, the EVT start their invasive journey through the uterus.

Early in pregnancy, the proliferation pressure is quite high, resulting in high cell columns. It seems as if this proliferation is reduced at the end of the first trimester, most probably due to the inflow of maternal blood into the placenta and the parallel increase in placental oxygen at that stage of pregnancy. Cell columns at around mid-gestation (about 20 weeks) show a reduced height, and at term, most of the cell columns have disappeared.

Looking at cell columns in more detail, it becomes obvious that EVT adopt a new phenotype along their way through the cell column.\textsuperscript{13,14} The first differentiation step is leaving the cell cycle as soon as the cell looses contact to the basement membrane of the anchoring villus.\textsuperscript{11,12} This is an important difference of trophoblast invasion compared with malignant tumor cells. EVT show proliferative activity or invasive behavior, but not both at the same time.\textsuperscript{15}

The proliferative EVT as well as their early daughter cells share the expression of integrins that bind to basement membrane-associated matrix proteins, such as collagen IV and laminin. The respective integrins like alpha6/beta4 integrin are present in the cell membranes of those trophoblasts in the proximal part of the cell columns.\textsuperscript{16}

In the part of the cell column below, the EVT still do not show any invasive behavior; however, they further differentiate into various subtypes of extravillous trophoblast. This becomes obvious on the morphological level, on the level of integrin expression, as well as on their routes of invasion. Some of them change their integrin repertoire from ‘basement membrane’ integrins toward ‘interstitial’ integrins such as alpha1/beta1, alpha5/beta1, or alpha-v/beta3/5 integrins.\textsuperscript{16–19} Only when the EVT have reached the distal end of the cell columns, they actively invade into uterine tissues.

Morphological Phenotypes of Extravillous Trophoblast
During their way through the cell columns and also subsequently on their way through the decidual stroma, EVT adopt different phenotypes. From a morphological point of view, a number of different cell types can be distinguished. As morphology is always linked to function, we will try to discuss a function to each morphological phenotype we describe. Additionally, it would be very interesting to characterize the different phenotypes on an immunological background as they differ in terms of contacting maternal cells and invading maternal tissues.

Morphologically, EVT within the decidua and myometrium can be subdivided into three different morphological phenotypes (Fig. 1):

\textbf{Large EVT with a polygonal shape}
These EVT are large cells with a polygonal shape. Their single nucleus is again large but irregularly formed, and in H&E-stained sections, this nucleus displays a very intense staining. Compared with all other extravillous trophoblast subtypes, these large EVT exhibit the strongest staining with antibodies directed against cytokeratin 7. At the same time, they never display staining if tested for proliferation markers such as Ki67 (Mib-1).

The large EVT are the typical trophoblast cells of a term ‘basal plate’. They can also be found along the route of invasion down to the inner third of the myometrium. Throughout pregnancy, the relative number of this subtype increases from 45\% at the end of the first trimester via 69\% at mid-gestation to 89\% at term.\textsuperscript{15} Hence, at the time of delivery, the large EVT are the main morphological phenotype of extravillous trophoblast.

A typical and characteristic feature of the large EVT is their secretion of a specific basement membrane-like extracellular matrix, termed ‘matrix-type fibrinoid’.\textsuperscript{20,21} During their differentiation into the large subtype, these EVT have changed their expression profile of integrins. They do no longer express alpha6/beta4 integrins, but now show expression of integrins such as alpha5/beta1, alpha1/beta1, and alpha-v/beta3/5.\textsuperscript{16,17,19}

The large EVT produce large amounts of their matrix and can easily be found self-embedded in this matrix, especially in the basal plate of a delivered placenta at term. By secreting and embedding, they...
do not only separate from each other but also separate from maternal cells. Clusters of these cells – as seen in the basal plate – are mostly void of any maternal cell. Along the route of invasion, in close vicinity of maternal cells, their self-secreted matrix keeps the large EVT clearly separated from surrounding maternal cells.

Because of the ease of their identification, the large EVT are the most frequently determined EVT. They are known since decades and have earlier been called X-cells. Interestingly, their shape, the secretion profile, and their high numbers in the basal plate do not support the notion that the large EVT are highly invasive. By contrast, it seems as if this subtype of extravillous trophoblast together with its matrix-type fibrinoid may function as a glue to fix the placenta to the uterine wall. From an immunological point of view, these cells do not seem to have much contact to maternal cells. In this respect, their matrix may be of importance as it has been shown that this matrix contains oncofetal antigens such as oncofetal fibronectin and the blood group precursor antigen ‘I’.21,23

Small EVT with a spindle shape

The small EVT display an elongated, sometimes filiform shape with a single, small, and ovoid nucleus and are mostly oriented perpendicular to the uterine wall. Also, the small EVT are negative for proliferation markers, while their immunopositivity for cytokeratin 7 is only moderate. The integrin expression of these cells is restricted to ‘interstitial’ integrins such as alpha5/beta1 and alpha-v-integrins.

The small EVT can mostly be found in early pregnancy from the distal end of cell columns down to the inner third of the myometrium. They are nearly absent in the basal plate of a delivered placenta at term. Their relative numbers decrease during pregnancy from 55% at the end of the first trimester via 31% at mid-gestation to 11% at term. In the placental bed, the small EVT may arrange in small clusters or may be found as single cells invading through the uterine interstitium, producing only small amounts of extracellular matrix, which mostly consists of cellular and oncofetal isoforms of fibronectin.21,23–25

The expression and exposure of ‘interstitial’ integrins in combination with the expression of oncofetal isoforms of fibronectin has been found to be closely linked to invasion and seems to be an essential mechanism of trophoblast invasiveness.21,26,27 Aplin et al.28 have experimentally proven that the interaction between alpha5/beta1 integrins and fibronectins is crucial for trophoblast invasion. Hence, the small EVT seem to represent the highly invasive subtype of extravillous trophoblast that may undergo further differentiation into the large subtype once the cells have reached their final position.

Interestingly, up to now, there are only a few descriptions of the small EVT.15 The reasons for this may be as follows:

1. The small cells display an unexpected shape for a trophoblast.
2. Because of their longitudinal shape, they may only be present as small fragments in tissue sections.
3. The cells are only moderately positive for cytokeratin 7, not as strong as the large polygonal EVT.

On the route of trophoblast invasion from cell columns down to the myometrium, a switch of the trophoblast integrin profile has been described, the so-called integrin switch.17,29 So far, it is hypothesized that the change in integrin expression occurs on single trophoblast cells invading into uterine tissues. However, with the two morphological phenotypes of extravillous trophoblast described previously, another explanation comes into focus. The large EVT do not invade as fast and as deep as the small cells do. Hence, more large cells expressing ‘epithelial’ as well as ‘interstitial’ integrins are present in the area of the basal plate, while the small EVT expressing only ‘interstitial’ integrins prevail in deeper zones of the placental bed. Thus, the integrin switch does not take place on a single cell on its way into uterine tissues, but rather represents the change of morphological phenotypes of extravillous trophoblast along the invasive pathway.

Giant EVT with multiple nuclei

The trophoblast giant cells may contain two to more than ten nuclei of irregular shape and varying size. As a result, their volume is much larger than that of the other two subtypes of extravillous trophoblast, and their diameter is in the range of 50–100 µm.15 The giant cells are mostly located deep in the invasive zone, at the borderline between decidua and myometrium. Hence, they are quite rare in the basal plate of a delivered placenta.
Like all EVT in the placental bed, also the giant cells do not show any immunoreactivity for proliferation markers. Interestingly, this subtype may only display a weak staining for cytokeratin 7; sometimes only a few spots are stained for cytokeratin.

The development of this subtype of extravillous trophoblast seems to be the fusion of mononucleated EVT. Although there is no fusion between two mononucleated villous trophoblasts, there may still be fusion of two mononucleated EVT throughout gestation. An explanation why the giant cells develop in the course of pregnancy is that they may serve in controlling trophoblast invasion. The giant cells are not invasive and remain at their sites until delivery. Hence, fusion of the invasive small EVT with each other or with the large subtype limits the depth of invasion of single EVT.

**Immunological Considerations of Extravillous Trophoblast Invasion**

During the course of pregnancy, EVT invade through the stroma of the decidua and eventually reach the inner third of the myometrium. Along their way through maternal tissues, they come into contact with a variety of maternal immune cells. There exists a very close contact and interaction between EVT and maternal immune cells; however, a detailed knowledge on the interplay between fetal and maternal cells is still missing.

There are a number of facets of EVT that need to be taken into account when discussing the immunological interactions between trophoblasts and maternal cells:

1. EVT are not abnormal or transformed cells, but rather normal healthy cells.
2. EVT have differentiated from trophectoderm cells that developed prior to the development of defined embryonic tissues (blastocyst stage). The trophectoderm developed concurrently with embryonic tissues and hence is a non-somatic cell population.
3. EVT express specific proteins such as oncofetal proteins, for example, oncofetal fibronectin and the blood group antigen precursor ‘i’, products of endogenous retrovirus-encoded DNA regions like the family of syncytins, and unusual major histocompatibility complex (MHC) molecules like the human leukocyte antigens (HLA) HLA-C, HLA-E, and HLA-G.

**Subsets of the extravillous trophoblast invade into uterine spiral arteries (endovascular trophoblast) or uterine glands (endoglandular trophoblast)**

During the course of pregnancy, EVT invade through uterine tissues starting at the distal ends of the cell columns that developed early during the first trimester. The trophoblasts may stay in the decidual stroma, may invade quite deep, and reach the inner third of the myometrium – or they may further invade specific morphological structures in decidua and myometrium: uterine spiral arteries and uterine glands (Fig. 1).

There is a subpopulation of EVT that decide to take a side route of invasion toward uterine arteries and glands. It is not clear so far whether there are two specific populations already from the start when leaving the cell columns or whether some trophoblasts are triggered to change their route while migrating through the uterine stroma.

**Endovascular Trophoblast**

The term ‘endovascular trophoblast’ has been used for only those EVT that reside inside the lumen of spiral arteries, but also for all those trophoblasts found in the walls of such arteries. It needs to be clarified again that this subpopulation needs to go through the uterine stroma first, subsequently reaches the walls of the arteries from the interstitial side, needs to penetrate these walls, and only then it is able to reach the lumen of the arteries. There have been descriptions of pathways where endovascular trophoblasts enter the open lumen of the arteries from the intervillos space. Unfortunately, this hypothesis cannot hold true, as there is no source of extravillous trophoblast anywhere from within the intervillos space.

There are two primary roles of endovascular trophoblast during pregnancy: (i) to block flow of maternal blood cells toward the placenta during the first trimester of pregnancy to ensure a low-oxygen environment during embryonic development in the first 10 weeks of pregnancy and (ii) to transform uterine spiral arteries into large-capacity tubes without vasomotor control of the mother to guarantee adequate blood flow velocities toward the placenta and hence ensure adequate nutrition and oxygenation of the fetus.

Invasion of endovascular trophoblasts into the wall of spiral arteries finally leads to transformation of these vessels. Loss of elastic fibers within the
media and reduced numbers and activity of smooth muscle cells result in dilation of the lumen up to several times the original diameter.14,40

Endoglandular Trophoblast

The uterine endometrium outside pregnancy as well as the decidua during pregnancy is full of uterine glands that release their secretion products into the uterine cavity. These secretion products contain a mixture of proteins, lipids, and carbohydrates similar to other eutherian species and seem to be essential for early embryo development between fertilization and implantation.42 Besides their function as nutritive support of the fetus, the secretion products also seem to play a more functional role during the process of implantation, as MUC-1 and leukemia inhibitory factor (LIF) have been described to be present in these products.43,44

Only recently, it became clear that the secretion products of uterine glands are also important for embryonic development after implantation.42 Burton et al. have been able to visualize such secretion products in the intervillous space of first-trimester placental tissues. These authors described that the clear solution flowing through the intervillous space of a first-trimester placenta is not only composed of blood plasma, but rather also contains secretion products of uterine glands. This has led to the description of histiotrophic nutrition of the developing villi of the first-trimester placenta.41,50,51

The combination of blood plasma and secretion products of uterine glands provides a mixture of nutrients, growth factors, and cytokines to the developing villi of the first-trimester placenta.41,50,51 Plugging of spiral arteries during the first trimester, and hence hindrance of blood cell transport toward the placenta, results in a low-oxygen environment during growth and development of embryo and early placenta. An oxygen partial pressure of <20 mmHg prior to 10 weeks of gestation may affect the critical stages of tissue and organ development of the growing embryo as follows:

1. Reduction of free radicals to protect the fast-growing embryo from teratogenesis mediated by free radicals.
2. Embryonic cells grow much faster under low oxygen concentrations ensuring rapid growth of the embryo.
3. Oxygen diffusion seems to be sufficient during the first trimester of pregnancy, and nutritive support is covered by blood plasma as well as secretion of uterine glands (histiotrophic nutrition).
4. The absence of maternal blood cells from the intervillous space of the first-trimester placenta also keeps away any maternal circulating immune cell from the developing placenta.

After the first trimester: maternal blood flows into and through the intervillous space of the placenta

The dissolution of the endovascular trophoblast plugs at the end of the first trimester results in the onset of maternal blood flow (including blood cells) into the intervillous space of the placenta. The flow of maternal blood cells into the placenta leads to an increase in placental partial oxygen pressure from 20 to 60 mmHg. This increase in placental oxygen may provide a significant stimulus for differentiation of villous as well as extravillous trophoblast.

The onset of maternal blood flow into the intervillous space of the placenta at the end of the first trimester:

only maternal plasma is flowing through the intervillous space of the placenta

Because of endovascular trophoblast plugs within the lumen of invaded spiral arteries, flow of maternal blood (including blood cells) into the intervillous space of the placenta is not established prior to the end of the first trimester. Accordingly, up to gestational weeks ten to twelve, only a clear solution circulates through the intervillous space.49 The solution is composed of blood plasma as an ultrafiltrate through the plugs of the endovascular trophoblast and secretion products of uterine glands.49 The combination of blood plasma and secretion products of uterine glands provides a mixture of nutrients, growth factors, and cytokines to the developing villi of the first-trimester placenta.41,50,51

The onset of maternal blood flow into the intervillous space of the placenta at the end of the first trimester:
trimester finally enables the direct physical contact between maternal circulating immune cells and the placental syncytiotrophoblast.

Conclusions

The development of the placenta from implantation until delivery opens a variety of opportunities for immune interactions, mostly between maternal cells and extravillous trophoblast. It is mostly the trophoblast that comes into direct contact with maternal cells, while contact of other fetal cells with the maternal system is limited. Within the subpopulation of extravillous trophoblast, the interstitial trophoblasts invade into decidua and the inner third of the myometrium; the endovascular trophoblasts invade into the walls of uterine spiral arteries and finally reach the lumen of these vessels, while the endoglandular trophoblasts invade into uterine glands to ensure the histiotrophic nutrition of the embryo during the first trimester of pregnancy. Those trophoblasts come into direct contact with a variety of maternal tissue cells, while the villous syncytiotrophoblast as the outer epithelial cover of placental villi comes into direct contact with circulating maternal cells. It is up to the reproductive immunologists to describe the consequences of such contacts between mother and fetus in terms of their impact on fetal well-being and pregnancy outcome.

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