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Immunological challenges during pregnancy : preeclampsia and egg donation

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Immunological challenges during pregnancy

Preeclampsia and Egg Donation

Marie-Louise van der Hoorn

Immunological challenges during pregnancy

Preeclampsia and Egg Donation

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Immunological challenges during pregnancy; preeclampsia and egg donation

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Introduction

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Immunological paradox

Human pregnancy is an interesting immunological paradox. The fetus is a semi-allograft, carrying paternal and maternal genes but is not rejected by the maternal immune system. The placenta is a key player in maintaining the pregnancy, since this fetus-derived organ is in direct contact with the mother. At this fetal-maternal interface, cells of the mother come in direct contact with cells of the fetus. This thesis describes the results of investigations on the immune regulation at the fetal-maternal interface with emphasis on two immunological challenges during pregnancy. First, preeclampsia, which might be immunologically related to host versus graft disease as seen in solid organ transplantation and second, egg donation (ED) pregnancies, which show that even complete allogeneic fetal allografts can be tolerated by the mother. The immunological mechanisms involved in acceptance of the totally allogeneic fetus in ED pregnancies are not well understood yet. It is possible that it leads to differential immunological regulation. This hypothesis is tested in this thesis. This general introduction will give an overview of placenta development, general immunology, immunology at the fetal-maternal interface, preeclampsia and ED pregnancies.

1. Placenta development

1.1 Placenta

The development of the placenta is essential for fetal growth, development and maintenance of (un)complicated pregnancy. The in growth of the placenta in to the maternal endometrium promotes acceptance of the fetal allograft, and the placenta serves metabolic and endocrine functions. Already at the time of fertilization placental development starts. The placenta develops from fetal derived cells. Around four days after fertilization the blastocyst consists of two cell types: the inner cell mass, which will form the embryo and the trophoblast, which will form the placenta and fetal membranes. During implantation the blastocyst will invade the uterine decidualized epithelium. The stem cells of the placenta are progenitor villous trophoblast cells. They can develop into invasive extravillous trophoblast or into syncytiotrophoblast (Figure 1). The core of the highly branched villi is surrounded by two types of non-invasive trophoblast; the mononuclear cytotrophoblast and, when fused, it forms the multinuclear syncytiotrophoblast which overlies the villi. The syncytiotrophoblast has direct contact with the surrounding floating maternal blood. The syncytiotrophoblast layer does not divide but is able to shed syncytiotrophoblast microparticles, which will enter the maternal blood via the intervillous space [1]. Nutrients in the maternal blood will transport across the two layers of trophoblast in to fetal blood vessels. These fetal blood vessels originate from the umbilical cord arteries, to supply each villus. Waste products and deoxygenated blood are transported in fetal arteries to chorionic villi. The fetal vein carries oxygenated blood and nutrients from the placenta to the fetus. Floating villi are not in contact with the decidua and are surrounded by the maternal blood which is present in the intervillous space. Other villi are attached to the decidua basalis and are called anchoring villi.

Extravillous trophoblast invades the maternal decidua and is thereby responsible for anchoring the placenta to the maternal myometrium. Invasive extravillous cytotrophoblast become either interstitial trophoblast cells or multinucleated placental bed giant cells [2]. These cells interact with decidual cells in the decidua basalis. Furthermore, extravillous cytotrophoblast cells invade the uterine spiral arteries, becoming endovascular trophoblast and partly replacing endothelial cells. This gives the fetus access to the maternal vascular system to assure the supply of oxygen and nutrients. A balance of this invasion is very important; the cells need to invade enough for the anchoring and to receive nutrients, on the other hand over-invasion of trophoblast cells

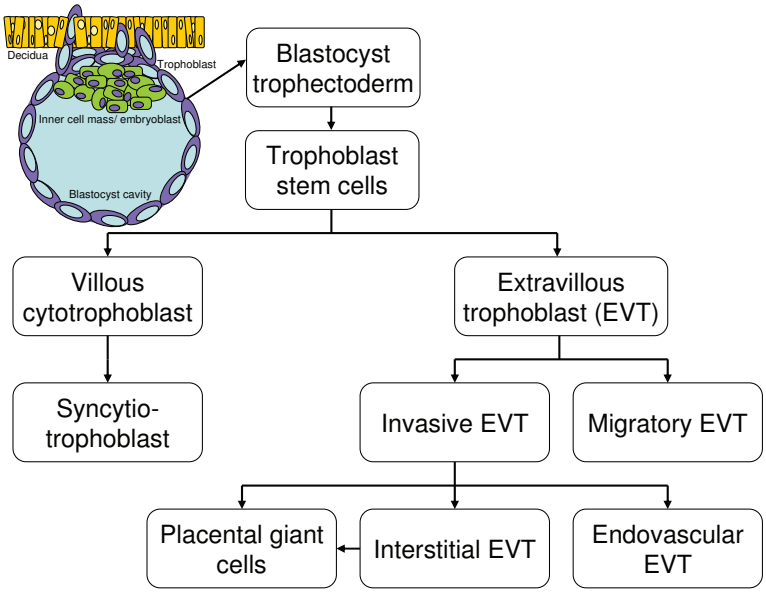

Figure 1 Flowchart of trophoblast development. The trophoblast stem cells differentiate in different trophoblast cells. Migratory EVTs are found in the chorionic plate and cell islands. The syncytiotrophoblast forms a superficial layer facing the intervillous space. EVTs are the basic material for all the non-villous parts of the placenta. In figure 4 the different types of trophoblast are shown in its environment.

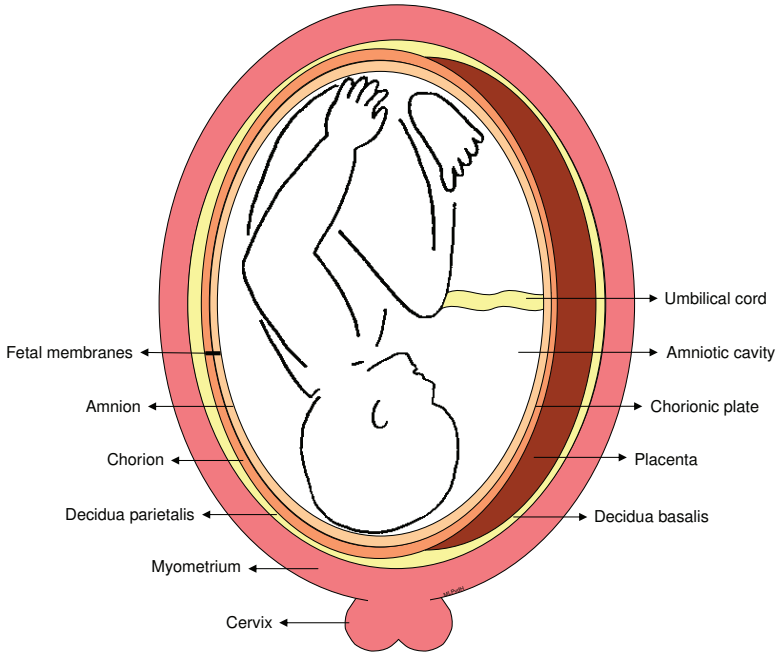

Figure 2 Uterus, placenta and fetal membranes. The fetal membranes consist of the amnion, chorion and the decidua parietalis. This latter layer is adjacent to the maternal myometrium. The placenta consists of the chonionic plate, villi and the decidua basalis which is adjacent to the maternal myometrium. The fetus is connected to the placenta via the umbilical cord.

has to be limited to protect the mother from hazardous complications like placenta accreta. In healthy pregnancies the extravillous cytotrophoblast cells invades as far as the inner third of the myometrium. Failure of this regulation, like inadequate placental invasion, might play a role in preeclampsia and fetal growth restriction. On the other hand, excessive invasion might lead to placenta accreta, a condition in which the placenta is abnormally deep attached in the endometrium and the myometrium. A schematic overview of the placenta and fetal membranes in relation to the fetus is depicted in Figure 2.

1.2 Fetal membranes

The fetal membranes surround and protect the fetus throughout gestation. Their function includes turnover of amniotic fluid and enzymatic activity during the initiation of labor. They are composed of four layers, from fetal to maternal side: amnion, chorion, trophoblast and decidua. The amnion consists of the amniotic epithelium and the amniotic mesoderm. The latter is divided in to the basal membrane, a compact stromal layer and a fibroblast layer. Amnion is adjacent to the chorion which facilitates sliding of the amnion across the chorion. The chorion is composed of the chorionic mesoderm, which includes blood vessels and a basal membrane. The chorion is adjoining the trophoblast layer. These trophoblast cells constitute a population of extravillous trophoblast. The decidual layer forms the maternal component of the membranes. In Figure 3 the layers are schematically shown.

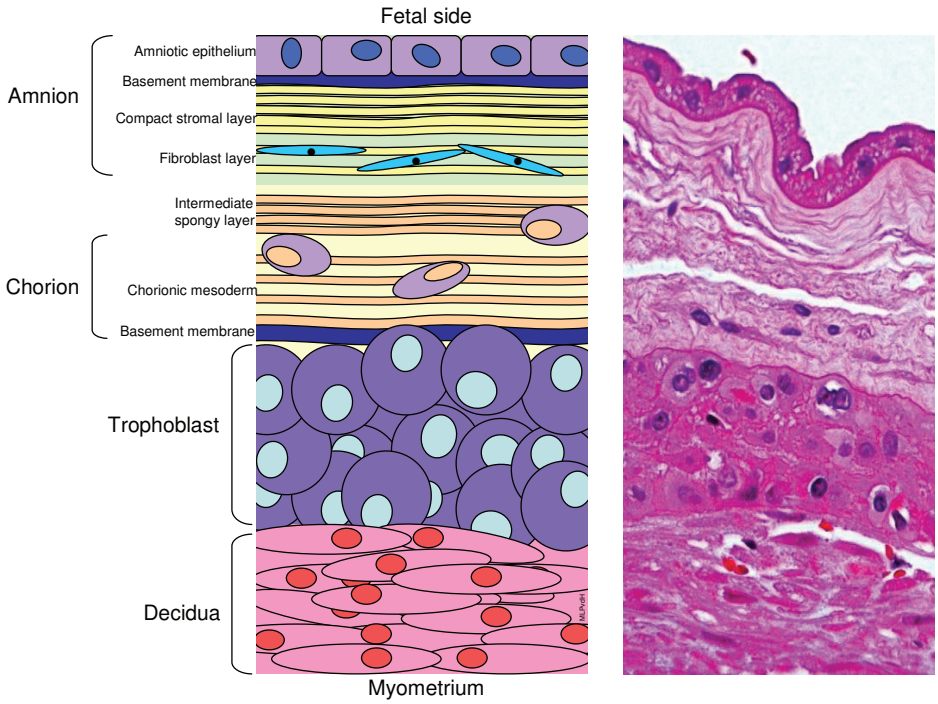

Figure 3 Fetal membranes. The layers of the fetal membranes schematically illustrated at the left panel and a histological picture at the right panel (H&E staining). From the fetal to the maternal side the fetal membranes consists of the amnion, chorion, trophoblast layer and the decidua parietalis.

1.3 Decidua

At term the decidua can be divided in to two parts. The maternal side of the placenta is the decidua basalis (Figure 4). This is the site where implantation has taken place and where the placenta has been developed. Furthermore, upon implantation, this is the first location where fetal-maternal contact takes place. The second part of the decidua is the decidua parietalis. This is the maternal side of the fetal membranes (Figure 3).

The fetus is never in direct contact with maternal tissues. In the decidua fetal and maternal cells come in contact, also referred as the fetal-maternal interface. There are three contact locations. First, the decidua parietalis, the maternal part of the membranes contacts the non-invading trophoblast of the chorion. Second, the decidua basalis (Figure 4), the maternal part of the placenta interacts with invading villous trophoblast and third maternal peripheral blood contacts the syncytiotrophoblast layer during utero-placental circulation.

The investigation of immunological mechanisms at the fetal-maternal interface gives insight in the processes leading to the acceptance of the fetal allograft.

2. Immunology

2.1 Immune system

The immune system protects the human body against diseases by identifying and killing pathogens and tumor cells. In order to function properly the cells of the immune system must distinguish between the own healthy cells and pathogens like virus, bacteria and parasites. The innate immune system attacks pathogens in a non-specific manner. The human immune system is able to adapt over time to recognize pathogens more efficiently and creates immunological memory. This part of the immune system is referred to adaptive or acquired immunity.

2.2 Innate immunity

The innate immune response provides immediate, but non-specific first line of defence against pathogens. The main function is recruitment of immune cells to the sites of infection, through the production of cytokines. Furthermore, it activates the complement cascade, kills pathogens by white blood cells and leads to activation of the acquired immune system by antigen presentation. Upon an infection inflammation is one of the first responses of the immune system. The individual recognizes infection by pain, swelling, redness, heat and a possible dysfunction of the targeted tissue. This occurs because chemokines are produced and attracts neutrophils and macrophages, which then releases cytokines and thereby triggering other parts of the immune system. The complement system refers to a cascade of reactions which eventually helps the immune system to recognize and kill pathogens. Natural killer (NK) cells, mast cells, basophils, eosinophils, macrophages, neutrophils and dendritic cells belong to the innate immune system. Phagocytes (macrophages, neutrophils and dendritic cells) are able to engulf pathogens, which results in the release of cytokines and products that kills the engulfed pathogen. The cells of the innate immune system are able to activate the acquired immune system.

2.3 Acquired immunity

The acquired immune response is highly specific for a particular pathogen improving with successive encounters via memory. T and B cells are involved in the acquired immunity. B cells are involved in the humoral immune response and T cells are involved in the cell mediated immune response. T cells recognize antigens in the complex of the major histocompatibility complex (MHC), presented on the cell surface. When T cells are activated they replicate and these cells can develop in to memory cells. Memory T cells have developed the skills to recognize antigens since they have previously encountered and responded to an antigen in a prior infection. If the pathogen is recognized again throughout life time, this will elicit a faster and a stronger immune response.

The differences between the two immune responses are obvious. The innate immune response is initiated almost immediately after infection, whereas adaptive immunity takes longer to develop. Innate immunity uses generalized and invariant mechanisms to recognize pathogens. Innate immunity is often unable to eradicate the pathogens completely, and it does not provide a stronger immunity to re-infection. In contrast, the adaptive immune response involves specific recognition by highly specific receptors on lymphocytes. This response is powerful enough to eradicate the infection and provides immunological memory. However, both immune responses work together and are able to protect an individual from harmful pathogenic infections. If an individual's immune response does not work properly, this may lead to serious complications. For example immunodeficient patients, who are not able to eradicate an infection are at a higher risk to die upon an infection. On the other hand autoimmune diseases like, diabetes or rheumatoid arthritis, are the result of an immune system which does not work appropriately.

2.4 Human leukocyte antigens

Pathogen recognition requires the ability to distinguish self from non-self. The MHC plays a pivotal role in this process. The MHC is a region of highly polymorphic genes, located in humans on the short arm of chromosome six. The human MHC system is called human leukocyte antigens (HLA). The protein products of the HLA genes are divided in to two major groups: class I and class II. The structure of these proteins is comparable. HLA class I molecules include HLA-A, -B, and -C, which are expressed on all nucleated cells and platelets. HLA class I molecules do not bind to peptides derived from pathogen-derived proteins until the peptides have been transported into the endoplasmatic reticulum. Transport to the endoplasmatic reticulum does not occur until after proteolytic cleavage of the pathogen proteins has occurred in the cytoplasm [3]. Once the peptide has bound a HLA class I molecule, this complex will be transported to the cell surface for the presentation to CD8 T cells (Figure 5) [4]. The HLA class I molecules inspects the intracellular environment. HLA class II molecules includes HLA-DR, -DQ and -DP, they are found an a few specialized cell types; macrophages, dendritic cells and B cells. HLA class II molecules bind pathogen derived peptides in a location inside endocytic vesicles, where the pathogens proteins are present (Figure 5). A peptide will bind to HLA class II molecule and this complex will be transported to the cells surface for the presentation to CD4 T cells [5]. The HLA class II molecules presents peptides derived from proteins from the extracellular environment.

The T cells recognize peptides bound to HLA molecules. To bind specifically the T cell receptor must recognize both the peptide and the part of the HLA molecule surrounding the peptide. This leads to antigen recognition and hence T cell activation. CD4 T cells, also known as T helper cells or regulatory cells, function by secreting cytokines that instruct other cells to acquire effector function. They only recognize antigens presented by HLA class II molecules. CD8 T cells differentiate into cytotoxic effector cells and kill the target cells that they recognize. These cells only recognize antigens presented by HLA class I molecules.

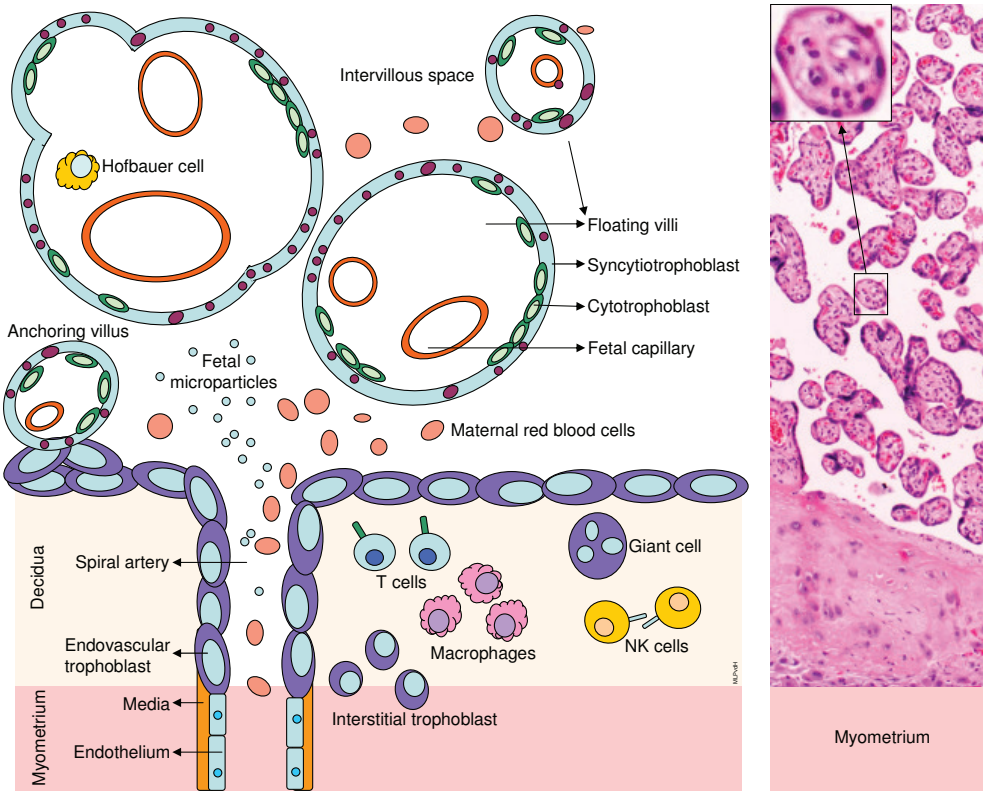

Figure 4 The fetal-maternal interface of the placenta. The left panel schematically illustrate the cells present at the fetal-maternal interface. The villi consist of different cell types and blood vessels. Microparticles are shed from the syncytiotrophoblast layer and enter the maternal blood which surrounds the villi in the intervillous space. The decidua basalis is invaded by different immune cells and spiral arteries. The decidua is adjacent to the maternal myometrium. The right panel shows a histological picture (H&E staining), with an enlargement of a single villus.

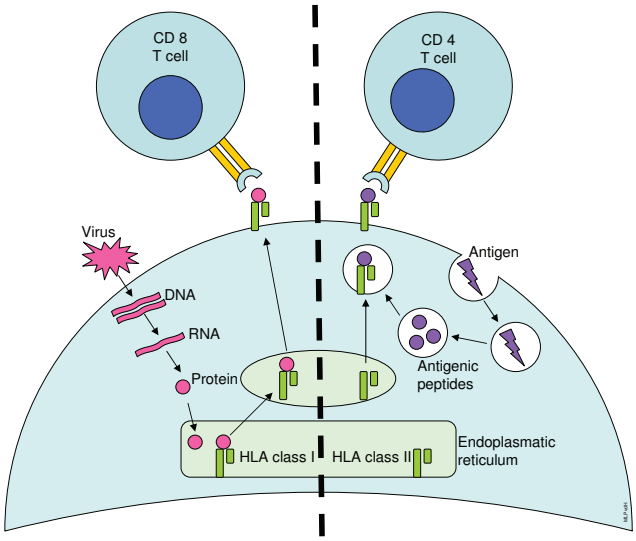

Figure 5 Antigen presentation. Two different routes of antigen presentation by HLA class I and II. On the left side the CD8 T cell is able to recognize peptides presented by HLA class I (HLA-A, -B and -C). The peptides derived from pathogens-derived proteins are processed by the endoplasmatic reticulum and presented by HLA class I molecules. The right side shows a peptide presented by HLA class II (HLA-DP, -DR and -DQ) recognized by CD4 T cells. The peptides are derived from the extracellular environment.

2.5 Cytokines

Cytokines are small proteins secreted by cells to mediate and regulate immune responses, inflammation and hematopoiesis. After an immune stimulus cytokines are produced and secreted, which then will act on a specific membrane receptor. Their expression profile has been used to categorize immune responses and the functional status of the immune system. Many cytokines have been discovered, and the ones relevant for this thesis, are highlighted here.

Interleukin-2 – IL-2 is produced after antigen binding to the T cell receptor. This leads to an expansion of IL-2 receptors on the T cell surface, and leads to growth and differentiation of T cells. Normal pregnancy is characterized by a shift towards type 2 immunity and inhibition of cytotoxic type 1 (IL-2) immune responses. An increased production of IL-2 by peripheral mononuclear cells in preeclampsia has been found [6].

Interleukin-6 – IL-6 has important roles in hematopoiesis, acute phase reactions and immune responses. IL-6 is a pro-inflammatory as well as an anti-inflammatory cytokine. It is produced by T cells and macrophages to stimulate immune responses. It acts as an anti-inflammatory cytokines by inhibiting tumor necrosis factor (TNF)-α and IL-1, and it activates IL-10. In contrast, increased concentrations of IL-6 and other pro-inflammatory (IL-1, TNF-α, and IL-8) cytokines are found in the placentas of pregnancies complicated by pre-term premature rupture of the membranes [7]. Furthermore, IL-6 levels in the amniotic fluid are increased preceding uterine contractions [8].

Interleukin-10 – IL-10 is an immunosuppressive molecule, produced by T cells, macrophages, monocytes and B cells. This cytokine is spontaneously produced in high levels by decidual macrophages [9]. It is a type 2 cytokine and appears to be pregnancy protective [10]. IL-10 is seen as a facilitator of successful pregnancy and alterations of the levels of IL-10 may be related to adverse pregnancy conditions [11]. Decreased villous trophoblast staining of IL-10 has been demonstrated in women with preeclampsia compared to normal pregnancy with correlated gestational age [12,13]. IL-10 administration in abortion prone mice significantly abrogated the incidence of spontaneous fetal loss [14]. IL-10 is produced in a gestational age-dependent manner. In first and second trimester the IL-10 levels are significantly higher. This may suggest that IL-10 is downregulated at term to prepare for the onset of labor programmed by the production of an inflammatory milieu [15]. Furthermore, first trimester missed abortion placental samples showed decreased IL-10 production [16].

Interleukin-17 – Th17 cells, the CD4+ cells that produce pro-inflammatory IL-17, is a recently discovered population involved in the maternal immunomodulation [17,18]. These cells are closely related to regulatory T cells and differentiate upon inflammatory signals whereas conditions that promote tolerance favor generation of regulatory T cells [19]. A balance between Th17 and regulatory T cells might be correlated with successful pregnancy; however the role of Th17 in human pregnancy remains to be investigated more substantially.

Transforming growth factor-β – TGF-β has well described immunosuppressive effects. Already during early pregnancy TGF-β might have an important role since it is involved in implantation of the blastocyst by inducing apoptosis of endometrial cells within the uterus. Decidual TGF-β is proposed to act on uterine NK cells to downregulate their cytotoxicity producing the uterine-specific phenotype [20]. TGF-β can stimulate two distinct receptors and thereby it is able to initiate two different SMAD signaling pathways with opposite effects. The TGF-β/ALK1 pathway induces proliferation and migration, while activation of the TGF-β/ALK5 signaling pathway inhibits these responses. Activation of the TGF-β/ALK5 signaling pathway leads to a cascade of reactions eventually leading to the phosphorylation SMAD2. Therefore SMAD2 mediates the signals of TGF-β and thus regulates several cellular processes such as proliferation, apoptosis, tissue remodeling and differentiation. Detection of phosphorylated SMAD2 reveals TGF-β signaling. Endoglin, a co-receptor of the TGF-β receptor, highly expressed during angiogenesis, is essential for ALK1

signaling. In the absence of endoglin, the TGF-β/ALK5 signaling is predominant and maintains quiescent endothelium. High endoglin expression stimulates the ALK1 pathway and indirectly inhibits ALK5 signaling, thus promoting the activation state of angiogenesis [21]. Endoglin is expressed on trophoblast. Increased serum levels of soluble endoglin are found in pregnancies complicated by preeclampsia [22].

Galectin-1 – Galectin-1 is an immunoregulatory glycan binding protein. Galectin-1 is able to modulate immune cell functions in different manners, for example by blocking the secretion of pro-inflammatory molecules [23], apoptosis of activated T cells [24] and antagonizing T cell activation [25]. Galectin-1 deficient mice show increased rates of fetal loss when compared with wild type controls, and injection of Galectin-1 in to the deficient mice rescued the pregnancy, possibly leading to expansion of IL-10 producing regulatory T cells [26].

Vascular endothelial growth factor – Vascular endothelial growth factor (VEGF) is an angiogenic protein. Membrane-bound fmslike tyrosine kinase 1 (Flt-1) is a receptor for VEGF and placental growth factor (PLGF). A splice variant of Flt-1 is soluble Flt-1 (sFlt-1, also known as sVEGFR-1) which antagonizes the VEGF and PLGF receptor. This soluble form prevents interactions of VEGF and PLGF with the functional membrane bound Flt-1 which thereby leads to endothelial dysfunction. In preeclampsia sFlt-1 is expressed in excessive amounts [27]. Hypoxia is considered to be the trigger for the production of sFlt-1 by villous trophoblast cells. VEGF antagonism by sFlt-1 may cause the clinical manifestations of preeclampsia, such as hypertension and proteinuria [28].

Interferon-γ – IFN-γ is a pro-inflammatory cytokine which plays a critical role in the initiation of endometrial vasculature remodeling, angiogenesis at the implantation side and maintenance of the decidua [29]. Deviations in these pregnancies are thought to lead to gestational complications like preeclampsia and fetal loss [30]. IFN-γ is involved in the innate and adaptive immunity against virus, intracellular bacterial infections and tumor control. It is predominantly produced by NK cells.

3. Immunology at the fetal-maternal interface

The immunological paradox is a medical enigma that has stimulated research for half a century. In the early days four hypotheses were postulated [31]. The first hypothesis was that the fetus lacked immunogenicity. This hypothesis is abandoned since studies showed that the fetus has immunogenic properties [32]. The second hypothesis was based on a possible diminished maternal responsiveness to pregnancy, leading to acceptance of the foreign fetus. Although peripheral changes during pregnancy are described, this hypothesis can not totally hold since this would make the pregnant women susceptible to harmful infections. The third hypothesis reflects the uterus as an immune-privileged site; however this is not a unique characteristic of the uterus since ectopic pregnancies occur. And the fourth hypothesis states that the placenta is an immune barrier. The immune barrier does not reflect a physical barrier, since fetal and maternal cells indeed come in contact at the location known as the fetal-maternal interface. The acceptance of the immunological foreign fetus is mediated by both maternal and fetal mechanisms. Already during implantation immunological adaptations are necessary, maintaining till the end of a successful pregnancy.

3.1 Immune escape mechanisms by trophoblast

HLA expression – Villous trophoblast (syncytiotrophoblast) expresses no HLA antigens on its surfaces. Extravillous trophoblast expresses a very particular set of HLA. Only four types of HLA class I genes are expressed, HLA-C, HLA-E, HLA-F and HLA-G. These HLA molecules may dampen the immune response by interaction with the leukocyte inhibitory receptors (LIR) on uterine NK cells, macrophages and with the T cell receptor on CD8+ cells [33,34]. This interaction blocks the cytotoxicity of these cells. NK cells have been shown to kill cells which lack HLA expression on the cell surface, therefore, by expression HLA molecules, NK cell mediated cytotoxicity is avoided [35]. HLA-G is mostly restricted in expression to the extravillous trophoblast. Class II HLA molecules are completely absent on extravillous trophoblast cells. Hence, the semi-allogeneic fetus is able to evade immune rejection by the maternal immune system.

B7 family – Second, the co-stimulatory molecules of the B7 family are selectively expressed on the trophoblast cells in human placenta. Activation of lymphocytes in circulating maternal blood is repressed by expression of B7H1 which is uniquely expressed on syncytiotrophoblast [36].

IDO – Indoleamine 2,3-diogygenase (IDO) is an enzymatic protein that catabolises tryptophan [37]. T cells are uniquely sensitive to fluctuations of tryptophan, and by the destruction of tryptophan by IDO the T cells become inactivated. IDO is produced by trophoblast cells and thereby this mechanism may contribute to the reduction or inhibition of immune reactions. Furthermore, IDO is as well produced by macrophages in response to IFN-γ.

Th1/Th2 balance – Uncomplicated pregnancy is considered to be an anti-inflammatory condition with predominantly the production of T helper (Th)-2 cytokines. Th1-type reaction in the placenta generates mainly inflammatory responses and correlate with miscarriage. Th2 cytokines are produced at the fetal-maternal interface and can inhibit Th1 responses, improving fetal survival but impairing responses against some pathogens [38]. Th1 cells produce IL-2 and IFN-γ, and Th2 cells synthesise IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13. Furthermore, the human placenta produces immunosuppressive molecules as progesterone, prostaglandin E2, and anti-inflammatory cytokines as IL-4 and IL-10 [33,39]. In this way trophoblast cells are able to influence the Th1/Th2 balance by the production of cytokines and hormones [10].

Complement system – In the placenta, the complement system helps to protect the mother and fetus against the invasion of pathogens. The fetus is protected by the maternal immune system by the expression of complement inhibitors. Trophoblast cells express complement regulatory proteins, which are important to protect the fetal cells because complement activation leads to destruction of the immunologic target [40]. Uncontrolled complement activation is prevented by decay accelerating factor (DAF), membrane cofactor protein (MCP), and CD59 [41].

Furthermore, tumor necrosis factor (TNF) α, Fas ligand (CD95L), TNF related apoptosis inducing ligand (TRAIL) are ligands identified in or on human trophoblast cells which are able to support the pregnancy host defense by supporting the maternal or fetal antibody production [42-44].

The various strategies of immune evasion may result in the acceptance of the fetus. However, despite these mechanisms the maternal immune system is aware of paternal antigens. Microchimerism is the persistence of a small population of foreign cells in another individual. Microchimerism is present between mother and fetus [45]. Therefore, other additional mechanisms are necessary to tolerate allogeneic cells by the maternal immune system. The microparticles which are shed from the syncytiotrophoblast layer lack HLA expression and therefore they will not be attacked by alloreactive T cells. However, the microparticles are able to bind to monocytes and stimulate the production of inflammatory cytokines, making them potential contributors to altered systemic inflammatory responsiveness in pregnancy [46].

3.2 Maternal cells

The decidua is populated by a variety of leukocytes during pregnancy [47,48]. Levels of lymphocytes are relatively low. During implantation the leukocytes mainly consist of NK cells. Macrophages form, after the uterine NK cells, the largest population of decidual leukocytes in early pregnancy (20-30%). Their numbers remain relatively constant throughout gestation [49]. In contrast, the numbers of NK cells decrease during pregnancy being absent at term [50]. This suggests that the innate immune system plays an important role in fetal-maternal immune adjustment. Macrophages as the main cells of the innate immune system are key players in the local regulation of maternal immune responses toward the fetus. The presence of both macrophages and dendritic cells at the fetal-maternal interface permits modulation of the immune response to protect the mother and fetus. Figure 6 summarize the leukocyte densities at the fetal-maternal interface during gestation.

Antigen presenting cells

An antigen has the capacity to trigger the adaptive immune response through several steps. The antigenic particles or proteins must be captured, processed and presented to T cells. These activities are performed by antigen presenting cells (APCs). Three kinds of APCs are defined: B lymphocytes, macrophages and dendritic cells. APCs sample the environment for potentially harmful extracellular particles. They are able to present components of antigenic particles on their cell surface via an intracellular breakdown mechanism. T cells can recognize the membrane bound components. To come in contact with the T cells, APCs transport antigens from the tissues to the peripheral lymphoid organs.

B cells

Only a few B cells can be detected in the endometrium and decidua. Their number does not vary during pregnancy. Uterine B cells are able to respond to antigenic challenges in for example pregnancies complicated with intrauterine infections.

Macrophages

The origin of the macrophages is in the bone marrow where myeloid progenitors differentiate into promonocytes and then into circulating monocytes which migrate transendothelially into the various organs to become macrophages. These macrophages are very effective in presenting antigenic peptides to T cells. They occur in almost all organs of the body. Upon fertilization, macrophages flux into the decidualized endometrium, and are found in close association with trophoblasts populations which secrete chemotactic molecules [51]. Macrophages comprise at least 10% of total decidual leukocytes [52]. In the decidua parietalis the trophoblast cells are scarce and also the macrophages are found in few numbers [50]. Macrophages are pluripotent, especially near the end of pregnancy, therefore it is hypothesized that their relative number increase at the end of gestation [52]. The close association of macrophages and extravillous trophoblast cells suggest an early recognition of fetal tissue by the immune system and a role in placental development, possibly by connection with HLA-G. Two types of macrophages populate the decidua, pro-inflammatory CD163- type 1 macrophages and immune modulatory CD163+ type 2 macrophages. Type 1 macrophages produce high levels of IL-12 and have a T cell stimulating potential. Type 2 macrophages do not have the T cell stimulating potential, do have a phagocytosis potential, and produce high levels of IL-10. Several studies show that decidual macrophages may have an immunoinhibitory function at the fetal-maternal interface since these macrophages are not able to differentiate into dendritic cells. Furthermore, they produce IL-10 and IDO and express low levels of the T lymphocyte co-stimulatory molecules CD80 and CD86 [9]. IL-10 can, by blocking the expression of co-stimulatory molecules on APCs, reduce the T cell activity against the fetus [53].

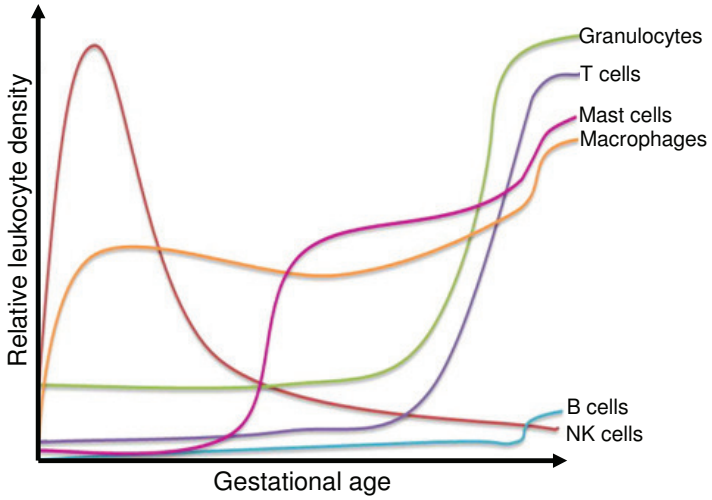

Figure 6 Leukocytes in human decidua. The relative leukocyte density at the fetal-maternal interface and the relation with gestational age is shown. Adjusted from [52].

Dendritic cells

Dendritic cells are closely related to macrophages. Dendritic cells have the power to induce primary immune responses and occur in mucosal sites such as skin, airways, gut and decidua. These cells transform information to the adaptive immune system. They also play a role in the induction of immunological tolerance by regulation of T cell mediated immune responses. Dendritic cells comprise approximately 1-2% of decidual leukocytes.

Two types of dendritic cells are reported in the literature. Myeloid dendritic cells are the major subpopulation of human blood dendritic cells and express the BDCA-1 (CD1c) antigen. These cells are efficient in antigen uptake and presentation. Plasmocytoid (or lymphoid) dendritic cells express the antigen BDCA-2 (CD303) and have the ability to induce T cell differentiation into Th2 cells. Thus dendritic cells are able to modulate the immune system in a stimulatory or tolerogenic way. This makes them suitable cells to exert regulatory functions in pregnancy. Consequently, decreased levels of plasmocytoid dendritic cells can be involved in the impairment of differentiation into Th2 cells in preeclamptic pregnancies. This has been shown in peripheral blood [54]. Furthermore, in the decidua of preeclamptic pregnancies a dense infiltration of immature and mature dendritic cells has been demonstrated [55].

Dendritic cells have several mechanisms to induce immune tolerance in absence of inflammation or infection. First, dendritic cells present antigens in lymph nodes and in response T cells proliferate and are then destroyed. Second, dendritic cells can induce IL-10 production. Dendritic cells express IDO, which is involved in inhibiting T cell proliferation [56]. These mechanisms may operate to prevent maternal T cell activation to the trophoblast. In absence of infection dendritic cells have an immature phenotype. They capture antigens generated by dying, infected or allogeneic cells. The presentation of these antigens to T cells induces antigen-specific T cell tolerance. Antigen capture by dendritic cells in an infectious environment drives dendritic cells to draining lymph nodes. Here the dendritic cells will transform into mature dendritic cells. This cell functions as a potent APC, capable of activating naive and memory helper T cells, cytotoxic T cells and B cells [57]. Relating these different functions to the decidua, immature dendritic cells present fetal antigens from invading trophoblast cells and present these to maternal T cells which are locally in attendance. This interaction induces tolerance to these antigens. However, in the midst of an infection mature dendritic cells are able to capture fetal antigens and migrate to lymph nodes which could result in maternal T cell reactivity to the conceptus.

T cells

The numbers of decidual T cells increase during pregnancy, starting with 5-20% of all CD45+ decidual lymphocytes in early pregnancy samples, till 40-80% at term [58]. Decidual T cells encompass a very heterogenic subset of T cells that include activated CD4+ and effector memory type CD8+ T cells. These activated T cells are found together with T cells subsets that are capable to suppress the decidual lymphocyte response [59]. Suppression of T cells may lead to acceptance of the allograft. Therefore, T cell research has dominated research in the immunology of pregnancy in the past years. Furthermore, T cells play an important role in the immunology after solid organ transplantations. CD4+ T cells can respond directly or indirectly to antigens of the semi-allogeneic allograft. Extravillous trophoblast cells only express HLA-C as the HLA class I molecules, and no HLA class II molecules. Therefore direct presentation is unlikely to be very important. In indirect presentation, allogeneic HLA molecules are taken up and processed by recipient APCs and these processed T cells are presented to recipient T cells in the context of self HLA. In the decidua dendritic cells and macrophages are present to fulfill this role.

Regulatory CD4+CD25bright T cells are present in human decidua in higher numbers compared to peripheral maternal blood [59], suggesting an important role at the fetal-maternal interface. It has been shown that fetus specific CD4+CD25bright T cells are recruited to the maternal decidua where they are able to suppress the local immune response [60]. T cells produce a variety of type 1 and type 2 cytokines and thereby may contribute to the local regulation of the fetus-specific responses within the decidua.

Alterations in the distributions of T cells may lead to pregnancy complications. Decreased numbers of regulatory T cells in peripheral blood have been found in preeclampsia and recurrent spontaneous abortions [61,62]. These results postulate that a sufficient number of regulatory T cells is necessary to maintain an uncomplicated pregnancy. The exact mechanism how regulatory T cells are activated and induce tolerance during pregnancy remains to be elucidated.

NK cells

NK cells are the predominant cell type of the decidua during implantation. Every menstrual cycle uterine NK cells are activated and expanded in to the decidua. High numbers are found in the stroma and clustered around glands and spiral arteries. When trophoblast invasion is complete, after the twentieth week, the number of NK cells will decrease. NK cells interact with extravillous trophoblast cells, this interaction is thought to be essential for the control of implantation [63]. In tubal pregnancies, which are characteristic for excessive trophoblast invasion, NK cells are absent [64]. In preeclampsia abnormal implantation occurs as a result of increased NK cell activity. NK cells express a variety of receptors which are able to recognize HLA class I molecules. Decidual NK cells are different compared to peripheral NK cells. Decidual NK cells express perforin, granzyme A and B and, unlike peripheral NK cells, they contain reduced cytolytic activity to HLA class I negative targets [65], secrete proteins with immunomodulatory potentials [66] and produce angiogenic factors like VEGF and PLGF [67]. Furthermore, decidual NK cells may recognize fetus HLA-C1 and HLA-C2 by the expression of killer immunoglobulin like receptor (KIR) [68].

It seems that mother's immune suppression is restricted to responses directed against the fetus. The fetus as well as the mother is dependent on the maternal immune system during the pregnant state. Even beyond birth the fetus is protected from harmful pathogens by passive immunization by the transfer of maternal antibodies through the colostrum and milk [69].

4. Preeclampsia

Four hypertensive disorders can occur during pregnancy; preexisting hypertension, gestational hypertension, preeclampsia and superimposed preeclampsia [70]. Preexisting hypertension is defined as systolic pressure of higher than 140 mmHg and/or diastolic pressure higher 90 mmHg before pregnancy, present before the 20th week of pregnancy, or persists longer than 12 weeks postpartum. Gestational hypertension refers to elevated blood pressure first detected after 20 weeks of gestation without proteinuria. Some patients with gestational hypertension will develop proteinuria over time and be considered preeclamptic, while others will be diagnosed with preexisting hypertension because of persistent blood pressure elevation postpartum. Preeclampsia refers to the syndrome of new onset of hypertension and proteinuria after 20 weeks of gestation in a previously normotensive woman or worsening hypertension with new onset proteinuria in a woman with preexisting hypertension (superimposed preeclampsia). Additional symptoms include visual disturbances, headache, epigastric pain, thrombocytopenia and abnormal liver function can occur. Preeclampsia occurs in approximately 3 to 14% of all pregnancies worldwide [71,72]. Abnormal placenta development plays a critical role in the pathogenesis of preeclampsia. Immunological factors are postulated to contribute to this abnormal development, since prior exposure to paternal antigens appears to protect against preeclampsia [73,74]. Preeclampsia is only a disease of pregnancy since it is cured after delivery.

The pathogenesis of preeclampsia starts during implantation and occurs before clinical manifestation. In normal pregnancies the spiral arteries are invaded by cytotrophoblasts and these vessels undergo a transformation from small to large leading to facilitated blood flow to the placenta. This remodeling of spiral arteries begins in the first trimester and is completed by 18 to 20 weeks of gestation. In preeclampsia the trophoblast cells do not have the capacity to migrate into the myometrium part of the spiral arteries. This will result in placental hypoperfusion, since the re-modulation of the vessels does not occur [75]. Ischemia and impaired placentation are thought to be the primary events leading to the release of soluble factors that are able to cause systemic endothelial dysfunction resulting in the clinical symptoms of the disease [76].

4.1 Preeclampsia and immunology

Preeclampsia is seen as an immunological disease. It is a disease of primipara and it is thought to occur in multipara with new parternity since previous studies have shown that partner change increased the risk of preeclampsia or hypertension in pregnancy. However, women who change partners often have a longer birth interval, and a longer interval is associated with a higher incidence of preeclampsia [77]. Artificial donor insemination and ED increase the risk of hypertensive disorders in pregnancy. In contrast, there is a protective role of maternal exposure to seminal fluid of her partner during an extended period [74].

Pregnancy related disorders as preeclampsia, abortions or fetal growth restrictions are a major cause of morbidity and mortality of both the mother and fetus. These disorders are related with increased levels of type-1 inflammatory cytokines, decreased levels of type-2 cytokines and macrophages have been found to be aberrantly activated [78].

In the decidua a specialized population of NK cells are present in high numbers at the implantation side. Direct interaction between invading trophoblast and decidual NK cells results in the production of various cytokines [79]. Hereby, NK cells play a direct role in trophoblast invasion and spiral artery remodeling and hereby disturbance of NK cell functions might be involved in the pathogenesis of preeclampsia. The receptors for HLA-C expressed on NK cells are known as KIRs. Every gestation represents a unique couple-specific interaction between fetal trophoblast HLA-C and maternal KIRs [80]. Specific HLA-C – KIR interactions are strongly associated with

preeclampsia; mothers lacking most or all activating KIR (women with the AA genotype) when the fetus possessed HLA-C belonging to the HLA-C2 group, are at a greatly increased risk of preeclampsia [81]. Furthermore, mothers with KIR AA frequencies have an increased risk of affected pregnancies only when the fetus has more group 2 HLA-C genes (C2) than the mother [82].

In normal pregnancy extravillous trophoblasts are located around the spiral arteries. Macrophages are located next to this layer in the stroma of the spiral arteries. In pathological pregnancies the distribution of macrophages is altered. The macrophages are located within and around the spiral arteries. Extravillous trophoblast cells are separated from the arteries. This creates a barrier between the spiral arteries and the invading trophoblast cells and complicates the transformation of spiral arteries [83]. In the normal situation, macrophages enhance trophoblast survival while in the pathologic situation the macrophages induce apoptosis. Aberrantly activated macrophages could contribute to the etiology of preeclampsia, fetal growth restrictions or abortions by disturbing the placental angiogenesis. Macrophages secrete the angiogenic factor VEGF [84]. Low levels of VEGF and PLGF may contribute to the deficiency in placental angiogenesis. The function of VEGF and PLGF can be inhibited by sFlt-1, which is a splice variant of VEGFreceptor 1 (Figure 7). In pregnancies complicated by preeclampsia the level of sFlt-1 is increased and alters the angiogenic activity of macrophages by binding to its receptors [84].

Fetal and placental growth is dependent on an adequate IL-10 production. A decreased IL-10 expression in trophoblast in preeclampsia compared to normal pregnancy has been observed [13]. IL-10 can promote the differentiation of monocytes into macrophages. Since the level of IL-10 is lower in preeclampsia, it is possible that the number of macrophages is also reduced.

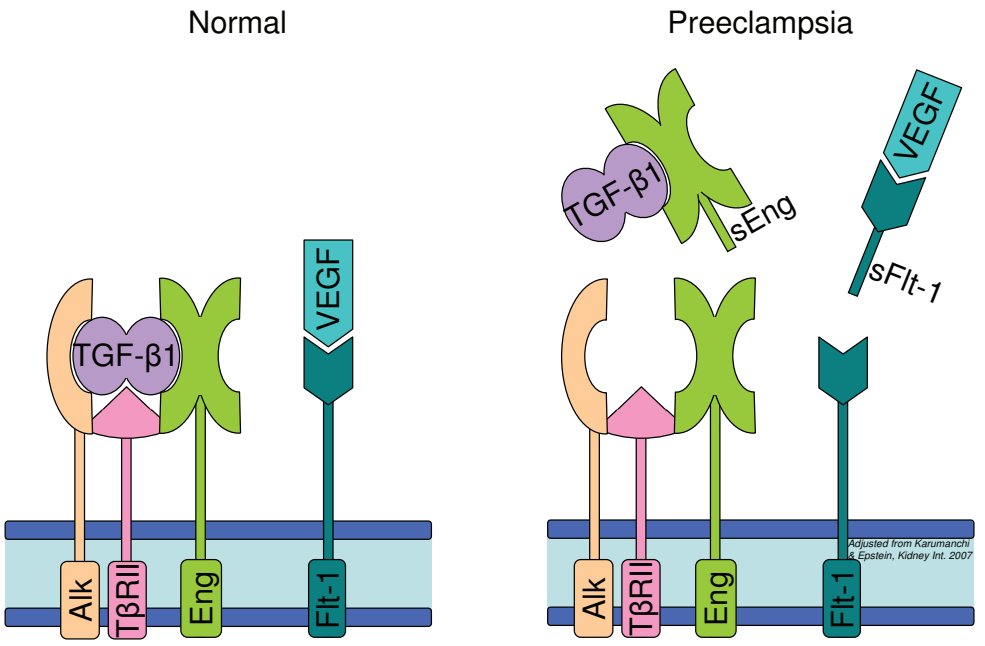

Figure 7 Cytokines involved in preeclampsia. During normal pregnancy vascular homeostasis is maintained by physiological levels of vascular endothelial growth factor (VEGF) and transforming growth factor-β1 (TGF-β1) signaling in the vasculature. In preeclampsia soluble endoglin (sEng) and soluble fmslike tyrosine kinase 1 (sFlt-1) derived from placental tissue are able to inhibit the normal functions of VEGF and TGF-β1, resulting in endothelial dysfunction [88].

Macrophages in the basal plate of the preeclamptic decidua are present in a reduced number compared to normal control decidua [85]. This can be caused by reduced migration of monocytes through the blood vessels into the tissue, or by reduced differentiation after migration of monocytes. However, discrepancy in literature exists whether the number of macrophages in preeclampsia is increased, reduced or unaltered.

Since the risk of preeclampsia is lower in second pregnancies with the same partner, there must be a certain degree of immunological memory. T cells belong to the adaptive immune system, and an activated T cell is able to develop in to memory cells. For this reason, T cells probably have a role in the pathogenesis of preeclampsia. NK cells, macrophages and dendritic cells belong to the innate immunity, which is probably not able to develop immunological memory. However, recently it has been shown that NK cells can demonstrate immunological memory [86,87]. If decidual NK cells are also capable of immunological memory, they might play a role in the pathogenesis of partner specific preeclampsia.

5. Egg donation

ED is the donation of unfertilized eggs to a woman who does not have (appropriate) eggs of herself or to women with genetic disorders. The egg donor receives a hormone treatment followed by an egg retrieval procedure. After retrieval the eggs are fertilized by sperm of the future father. In the meantime the recipient uterus is appropriately prepared to receive the fertilized eggs. After several days the best embryo is transferred in the uterus of the recipient.

The law forbids commercial and anonymous ED in the Netherlands. ED based on non-commercial purposes is allowed. The main reason to perform ED in the Netherlands is premature ovarian failure. This disease is characterized by early onset of ovarian failure by for example radiotherapy, genetic disorders, surgical destruction of the ovaries or an unknown cause. The woman does have a functional uterus. Furthermore, ED is necessary if the ovary can not be reached in an IVF procedure, although women do have correctly functioning ovaries. An additional indication to perform ED is present in women who have a high risk of getting children with high risk genetic disorders. The age on which women gets their first child has increased up till 29.4 years in the Netherlands in 2008. Reasons to postpone pregnancy are the availability of anti-conceptive and better educational and career opportunities for women. Since the year 2000 the number of women who go abroad for egg donation has increased threefold. Couples wishing an anonymous donor or who can not find a (non-profit) egg donor in the Netherlands go abroad. Spain is by far the most popular country, followed by Belgium. Overall, abroad more embryos are transferred per cycle compared to the single embryo transfer in the Netherlands. Although exact numbers are difficult to collect, a maximum of five embryo transfer per cycle has been described in ED pregnancies performed abroad. Multiple pregnancies are potentially hazardous for the gestational carrier and fetuses since they lead to more complications. The costs for ED abroad differ from 3,000 up to 30,000 euro per treatment [89].

Pregnancy conceived after ED, reflects an interesting model to study immunological reactions. ED pregnancies are a result of in vitro fertilization of a donated egg by a relative, or more commonly an unrelated donor. Hereby, neither of the fetal haplotypes matches with the gestational carrier. Progressive knowledge in the field of assisted reproductive technologies and extension of the medical indications leads to an increase of number of ED pregnancies. Nevertheless, it can lead to harmful maternal consequences during pregnancy, which may be related to the allogeneic nature of the fetus. Maternal complications in ED pregnancies include an increased risk of pregnancy induced hypertension, an increased rate of caesarean section deliveries, an increased risk of postpartum hemorrhage and an increased risk of first trimester vaginal bleeding. Although the

maternal complications are higher in ED pregnancies compared to spontaneously conceived pregnancies, there is no increased complication risk for the fetus or newborn [90-93].

5.1 Transplantation and egg donation pregnancies

Since in ED pregnancies the entire fetal genome is allogeneic towards the gestational carrier, immune mechanisms in successful ED pregnancies might be relevant for the induction of immunological tolerance in solid organ transplantation.

Blood transfusions are the most widespread kind of transplantations in clinical medicine. Compared to solid organ transplantation, blood transfusions have less immunological barriers. Recipients and donors are typed and cross-matched for the ABO and the rhesus erythrocyte antigens. If an ABO incompatible organ is transplanted a hyperacute rejection may occur. Anti HLA antibodies may also be present in prospective transplant patients. Blood transfusions or pregnancies are the source of these antibodies. Fetal cells enter the maternal circulation and antibodies against the paternal HLA antigens or in case of ED, the donor HLA antigens. The presence of HLA antibodies is associated with a reduced chance of a live birth [94]. Besides blood transfusion and pregnancy, anti-HLA antibodies can be developed after previous organ transplants.

Acute rejection of the transplanted graft occurs if donor antigen presenting cells carry complexes of donor HLA molecules on their surfaces. In a secondary lymphoid organ they will enter T cell areas and present their antigens towards them. The recipient T cells become activated by specifically binding to the complexes of allogeneic donor HLA. The effector T cells have the capability to attack the transplanted organ. In (ED) pregnancy this type of immunological rejection possibly plays no role, since maternal T cells do not come in contact with fetal antigen presenting cells. In chronic rejection, indirect antigen presentation plays a major role. The recipient's dendritic cells enodcytose HLA class I and II particles from donor cells. The peptides are then presented by the recipient's HLA and CD4 T helper cells may become activated. Indirect presentation possibly plays a role in the immunology of pregnancy. Fetal derived microparticles are present in the maternal bloodstream and might be taken up by maternal antigen presenting cells [46,95] and present them via the indirect pathway to maternal T cells.

In placentas of ED pregnancies severe chronic deciduitis combined with fibrinoid deposition has been observed [96]. These pathological findings are localized in the basal plate of the placenta, the location where the extravillous cytotrophoblast lines with the maternal decidua. This pathological finding is considered to be immunological modulated.

6. Outline of this thesis

The question why the semi-allogeneic fetus is accepted by the immune system of the mother has already risen in 1953 [97]. Medawar was the first to imply the fetus as a semi-allograft. Ever since then much research has been performed in the field of reproductive immunology. However, until today pregnancy remains an immunological paradox and the exact mechanism leading to the acceptance of the semi-allogeneic fetus remains to be elucidated. Although the mechanism is still not yet well understood in normal pregnancies, immunological knowledge of complicated pregnancies might give insight in the underlying mechanisms of tolerance.

The aim of this thesis is to study the immunological mechanisms in uncomplicated, preeclamptic, ED and non donor IVF pregnancies. Preeclampsia and ED are seen as an additional immunological challenge during pregnancy. Since ED pregnancies are characterized by a higher number of HLA mismatches compared with naturally conceived pregnancies, this thesis hypothesizes that differential immune regulation is necessary to maintain pregnancy.

To investigate the immunological mechanism, placentas of uncomplicated, preeclamptic, ED and non donor IVF pregnancies were collected. They were used to study the local immunological mechanisms by immunohistochemistry analysis of the decidua basalis and parietalis. Blood samples of umbilical cord blood and of the mothers of uncomplicated, preeclamptic, ED, and non donor IVF pregnancies were taken and cells were isolated. Those cells were used to simulate peripheral immune responses. The reaction of peripheral cells from uncomplicated, ED, and IVF pregnancies and non pregnant controls up on stimulation with own umbilical cord blood, allogeneic umbilical cord blood and peripheral blood samples was measured by mixed lymphocyte reactions and by cytokine production. The cells were phenotyped using flow cytometry. Of the pregnancies described in this thesis the number of HLA mismatches was calculated.

Chapter 2 investigates the peripheral immune response in uncomplicated pregnancies compared with non pregnant controls. The specific and non-specific maternal immune response was studied. The aim of Chapter 3 is to study macrophages in the decidua of preterm preeclamptic pregnancies compared with uncomplicated preterm control and control pregnancies by immunohistochemistry. Chapter 4 describes two case reports. The first case describes a woman pregnant after IVF suffering from preeclampsia while the fetuses have severe growth retardation. The second case describes an ED pregnancy with preeclampsia without fetal growth retardation. The question is raised whether preeclampsia in ED pregnancy is based on different pathophysiological mechanism.

The focus of the studies described in the Chapters 5 – 7 is on ED pregnancies. Since the fetus in ED pregnancies is fully allogeneic to the gestational carrier, immune mechanisms in successful ED pregnancies might be relevant for the induction of immunological tolerance in solid organ transplantation. This is discussed in Chapter 5. Chapter 6 gives an overview of the clinical and immunological aspects of ED pregnancies. In Chapter 7 ED, non donor IVF and naturally conceived pregnancies are studied. The expression of several cytokines in the placenta and in serum of the patients is investigated. Furthermore, the phenotype of cells in peripheral blood is analyzed and the reactivity of those cells in response to umbilical cord blood of the own or allogeneic umbilical cord blood is studied.

The conclusions of the different chapters are summarized and discussed in Chapter 8.

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Changes in cytokine production and composition of peripheral blood leukocytes during pregnancy are not associated with a difference in the proliferative immune response to the fetus

2

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Abstract

Objective: We analyzed peripheral blood from women at term pregnancy for leukocyte composition, *in vitro* proliferative responses and cytokine production after non- and fetus-specific stimulation.

Methods: Maternal PBMCs were collected and stimulated with umbilical cord blood (UCB) of own child, 3rd-party UCB, non-specific stimulus PHA and anti-CD3 antibody, with non-pregnant females (cPBMC) as control. Nine combinations of patient-child-3rd-party child and control were selected on basis of sharing one HLA-DR antigen. The response of mPBMC upon specific stimulation with fetal antigens was similar to cPBMC.

Results: No differences were found when comparing the maternal response upon stimulation to her own child with stimulation to a control child. Non-specific stimulation with PHA and anti-CD3 antibody did not reveal a difference in proliferation rate between mPBMC and cPBMC. However, mPBMC contained a higher percentage of CD14+ cells ($p=0.001$) and activated T cells (CD25dim, $p<0.0001$), but a lower percentage CD16-CD56bright NK-cells ($p=0.001$) and CD16+CD56+ NK-cells ($p=0.003$). mPBMC produced more IL-6, IL-10 and IL-17 compared to cPBMC ($p<0.05$).

Conclusions: We found differences in lymphocyte composition and cytokine production between mPBMC and cPBMC. These differences did not result in quantitative changes in proliferative responses during pregnancy compared to non-pregnant controls.

Introduction

During pregnancy, semi-allogeneic fetal tissue is directly exposed to the maternal blood since it invades the maternal decidua. This implies a possible attack of fetal tissue by the immune system of the mother. However, the fetus escapes from maternal rejection and is tolerated by the induction of several maternal and fetal mechanisms. In 1953, Medawar suggested several mechanisms to explain this 'immunological paradox of pregnancy' [1]. One of his explanations is based on a diminished maternal responsiveness to pregnancy, leading to acceptance of the foreign fetus. Indeed, the cellular immune response seems to be decreased during pregnancy, reflected by the increased susceptibility to viral infections and specific intracellular pathogens, such as *Listeria monocytogenes* and by the remission of some T-cell mediated autoimmune diseases in pregnancy [2,3]. Other clinical observations including flare-ups of humoral autoimmune diseases in pregnancy like systemic lupus erythematosus [4], suggest a paradoxical activation of other arms of the immune system, including B cells and innate immunity [5].

In fact, there is direct evidence for fetus-specific antigen recognition by the maternal adaptive immune system even during the first trimester exemplified by local lymph node swelling in mice in first pregnancy, a recall flare in the second pregnancy [6] and the formation of anti-paternal antibodies [7]. These antibodies are developed in 10-30% of women against paternal inherited human leukocyte antigens (HLA) of the fetus and can persist for more than 10 years [7]. In pregnancy, there are two ways of maternal sensitization: one locally in the fetal-maternal interface via processing of major histocompatibility complex (MHC) alloantigens by antigen-presenting cells and the second via fetal cell entry in the maternal circulation. This entry can consist of fetal whole cells (microchimerism), syncytiothrophoblast fragments, fetal DNA, and debris from apoptotic cells. The (long-term) consequence of the HLA antibodies is unclear; e.g. the presence of anti-paternal antibodies in patients with recurrent spontaneous abortion is associated with a higher [8] as well as with a reduced success rate [9] on live birth. T-cell allo-reactivity is observed in pregnancy. Primed T cells to paternal HLA antigens and fetus-specific minor histocompatibility complexes, like HY, have been demonstrated in the peripheral blood of pregnant women [10-12]. In addition, recent studies by our group show that the CD4+CD25dim (activated) T-cell population increases in maternal peripheral blood during pregnancy [13].

Pregnancy has long been suggested as a balance of the maternal immune system with a predominance of T helper 2 immunity [4,14,15]. Nowadays, little consensus on this Th1/Th2 shift in peripheral blood in normal human pregnancy exists [14,16,17] and more candidate mechanisms have been proposed to describe immunostimulation and immunoregulation during pregnancy. Saito et al. [18] state that while the Th1/Th2 balance is shifted, Th3 and Tr1 cells, which produce immunosuppressive cytokines TGF-β and interleukin (IL)-10 respectively, regulate the Th1 cell-induced rejection. A specialized subset of T cells, CD4+CD25bright regulatory T cells, regulate overstimulation of either type 1 or type 2 responses [18] and are therefore able to suppress autoimmunity [19]. In addition, recently a regulatory NK cell subset and NKr1 cells, producing IL-10, have been demonstrated which might play an important role in the maternal immune response [18,20,21].

These mechanisms (non-specific or specific for fetal antigens) have been described for complicated pregnancies in which human placental tissue damage was suggested to occur after immune activation [5,22,23]. However, so far specific and non-specific maternal immune responses during normal pregnancy have not been compared to non-pregnant controls. Therefore, we determined the phenotype of different subsets of leukocytes in the peripheral blood of pregnant and non-pregnant women using flow cytometry. We also studied the proliferation capacity and cytokine production of maternal peripheral blood mononuclear cells (mPBMC) in a mixed lymphocyte reaction (MLR) after stimulation with umbilical cord blood (UCB) derived lymphocytes of the own child and lymphocytes of another child (3rd-party UCB). A significant positive correlation was

found between the number of HLA-DR mismatches and the alloreactivity in transplant recipients [24]. Therefore, in this study we used 3rd-party UCB controls with an equal number of HLA class II mismatches compared to the own child.

Material and Methods

Blood samples

Heparinized maternal peripheral blood and UCB was obtained from healthy women after uncomplicated term pregnancy (with a minimal gestational age of 37 weeks, n=50). UCB was obtained directly after cord clamping from the umbilical cord veins. Patients tested in the proliferation experiments were 9 women who delivered by a cesarean section and 2 women who delivered spontaneously. Control PBMC (cPBMC) samples were obtained from age-matched healthy non-pregnant female volunteers (n=30). For each patient-child combination a control was selected on the basis of sharing one HLA-DR antigen with the child. We screened for maternal HLA antibodies and excluded combinations with HLA-DR antibodies. Table 1 shows the HLA-DR typing. Informed consent was obtained from all women. The study was approved by the Ethics Committee of the Leiden University Medical Center.

Blood was layered on a Ficoll Hypaque (LUMC pharmacy; Leiden, The Netherlands) gradient for density gradient centrifugation at room temperature (20min/800g). After centrifugation PBMCs were collected from the interface, washed twice and counted. Part of the cells were fixed with 1% paraformaldehyde and stored at 4°C until time of cell staining for flow cytometry analysis. For proliferation studies the remaining cells were frozen in liquid nitrogen.

Couple	Mother	UCB	3rd-party UCB	Control
1	DR17, DR4	**DR17**, DR15	**DR4**, DR13	DR17, DR4
2*	DR17, DR4	**DR4**, DR13	**DR4**, DR13	DR4 DR11
3	DR1, DR17	DR8, **DR17**	**DR1**, DR15	DR1, DR17
4	DR15, DR16	DR17, **DR16**	DR17, **DR15**	DR15, DR16
5	DR1, DR17	**DR17**, **DR15**	**DR17, DR7**	DR7 DR15
6	DR10, DR13	**DR4, DR13**	**DR7, DR10**	DR4, DR7
7	DR10, DR13	**DR7, DR10**	**DR4, DR13**	DR4, DR7
8	DR15, DR16	**DR17, DR15**	**DR1, DR15**	DR1, DR17
9*	DR4, DR9	**DR4**, DR13	**DR4**, DR13	DR4, DR11
10		**DR4**, DR13	**DR4**, DR13	DR4 DR17
11		**DR17**, DR15	**DR17**, DR7	DR1 DR17

Table 1 HLA-DR typing of mother, own child (UCB), control child (3rd party UCB) and control. Shared antigens are depicted in bold font. Combination 2 and 9 were omitted from the MLR results, since the HLA-DR antigens were similar between own and control child. Therefore, two extra control-child combinations were added with one shared HLA-DR antigen.

Flow cytometry

The following directly conjugated mouse-anti-human monoclonal antibodies were used for four-color immunofluorescence surface staining of the PBMCs: CD45-APC, CD14-FITC, CD19-PE, CD3-PerCP, CD4-APC, CD8-PE, CD16-FITC, CD25-PE, CD28-APC, CD56-PE, CD69-FITC and HLA-DR-FITC (Becton Dickinson, Franklin Lakes, NJ, USA), used in concentrations according to manufactures instructions. Flow cytometry was performed on a FACS Calibur using Cellquest-Pro software (Becton Dickinson). Percentages were calculated within gates set around the lymphocytes (in FCS/SSC dotplot) and the CD45+, CD45+CD3+, CD45+CD3+CD4+, or CD45+CD3+CD8+ fraction. %CD14+ cells were calculated within the CD45+ fraction without a lymphogate. Gating strategies were performed on basis of previous research [13].

Non-specific stimulation

Cultures were established in triplicate in flat-bottomed 96-well plates (Costar, Cambridge, MA, USA). One well contained $1x10^5$ PBMC's as responder cells in 100 µl of culture medium. Culture medium contained RPMI 1640 with 10% human serum and 3 mM L-glutamine. For mitogen stimulation, 100 µl of purified phytohemagglutinin (0.4 mg/ml, PHA) (Welcome, Dartford, UK) was added. For stimulation with CD3 antibody (Ab) the plates were incubated with 50 µl of anti-CD3 (OKT3, Ortho Biotec, Bridgewater, NJ, USA), diluted in PBS at 1 µg/ml concentration per well for 90 minutes at 37°C in a humidified atmosphere of 5% CO_2. Plates were washed twice with PBS before cells were added. Culture medium alone was used as a negative control. Plates were incubated at 37°C in a humidified atmosphere of 5% CO_2 for 3 days. Cultures were pulsed with 20 µCi/well ^{3}H-thymidine diluted in RPMI 1640 medium for the last 8 hours of incubation. Just before pulsing, 100 µl of supernatant was removed from each well and stored at -20°C until further analysis. ^{3}H-thymidine incorporation was measured by liquid scintillation spectroscopy using a betaplate counter (Perkin Elmer, Waltham, MA, USA). The results were expressed as the median counts per minute (cpm) for each triplicate culture.

Specific stimulation in one-way mixed lymphocyte reaction

Mixed lymphocyte cultures (MLR) were set up with 100 µl of $1x10^5$ mPBMC or cPBMC in culture medium added in triplicate wells in a round-bottom 96-well plate (Costar) to 100 µl of (a) $1x10^5$ irradiated (30 Gy) fetal leukocytes of her own child; (b) $1x10^5$ irradiated fetal leukocytes of a third party child or (c) culture medium. Proliferation was measured on day 5 and day 7 by incorporation of ^{3}H-thymidine added during the last 16 hours of culture. Just before pulsing, 100 µl of supernatant was removed from each well and stored at -20°C until further analysis. The results were expressed as the median counts per minute (cpm) for each triplicate culture.

Cytokine Analysis

Harvested supernatants were tested for the following cytokines: IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, IFN-γ, TNF-α, GM-CSF, using a Bio-Plex assay (Bio-Rad Laboratories, Veenendaal, The Netherlands) following manufacturers instructions. Samples were analyzed using a Bio-Plex^{tm} Array Reader with Bio-Plex software.

Statistical Analysis

To determine differences between more than 2 groups an ANOVA was performed. If $p<0.05$, the Mann-Whitney test was performed to compare the phenotype of the different cell-subsets,

the proliferative responses and cytokine production of maternal lymphocytes and control lymphocytes. To compare the proliferative responses of maternal lymphocytes after specific stimulation with lymphocytes of own child and control child, the Wilcoxon signed rank test was performed. For all tests the value of $p<0.05$ was defined as significant.

Results

Phenotypic analysis

To compare the different subsets of leukocytes in the peripheral blood between pregnant and non-pregnant women, we performed a phenotypic analysis using flow-cytometry. No difference was observed in %CD3+ T-cells and %CD19+ B-cells. However, mPBMC contained a significantly lower percentage of CD16-CD56bright NK-cells (p=0.001) and CD16+CD56+ NK-cells (p=0.003) compared to non-pregnant cPBMC (Figure 1a). The %CD14+ monocytes were significantly higher in mPBMC (p=0.001, Figure 1b). Analysis of the different subsets of (CD3+) T-cells revealed no difference in %CD4+ or %CD8+ T-cells (Figure 1c). The activation state of CD3+ T-cells was studied by measuring CD69 expression (early marker of activation), IL-2R expression (CD25) and

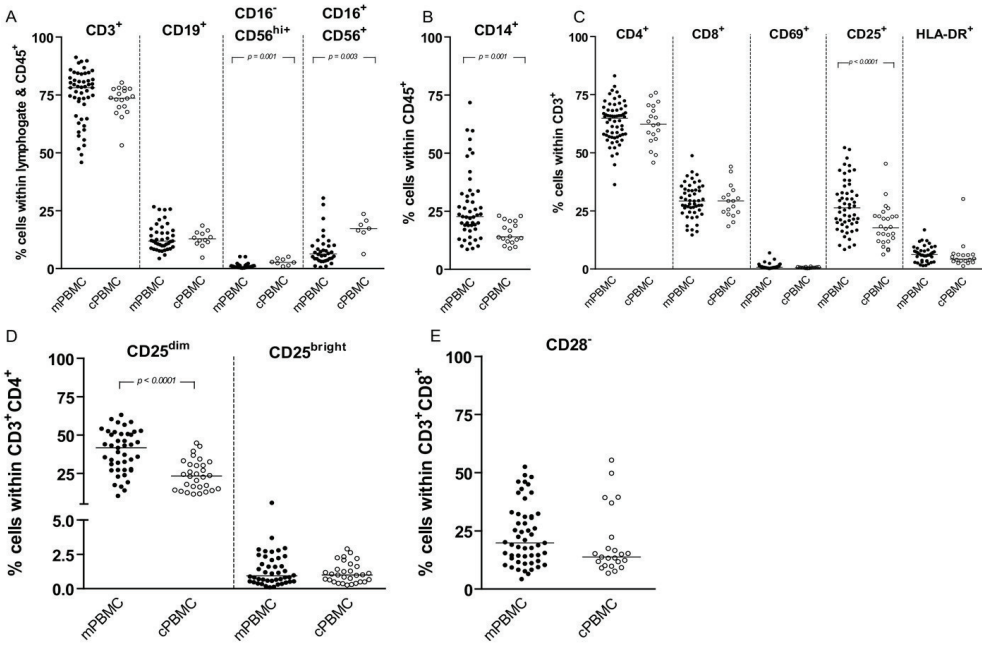

Figure 1 Distribution of different subsets of leukocytes in peripheral blood between pregnant (n=50) and non-pregnant (n=30) women. All lines are median percentages. A. Percentage of CD3+ within lymphogate and CD45+ cells in mPBMC (78.1%) and cPBMC (73.6%), percentage of CD19+ in mPBMC (11.9%) and cPBMC (12.8%), percentage of CD16-CD56hi+ in mPBMC (0.7%) and cPBMC (2.7%), and percentage of CD16+CD56+ in mPBMC (6.3%) and cPBMC (17.3%). B. Percentage of CD14+ within CD45+ cells in mPBMC (22.7%) and cPBMC (14.0%). C. Percentage of CD4+ within CD3+ cells in mPBMC (64.9%) and cPBMC (62.3%), percentage of CD8+ in mPBMC (29.2%) and cPBMC (29.3%), percentage of CD69+ in mPBMC (0.7%) and cPBMC (0.61%), percentage of CD25+ in mPBMC (26.3%) and cPBMC (17.7%), and percentage of HLA-DR+ in mPBMC (6.2%) and cPBMC (4.3%). D. Percentage of CD25dim within CD3+CD4+ cells in mPBMC (41.7%) and cPBMC (23.4%), percentage of CD25bright in mPBMC (0.9%) and cPBMC (1.0%). E. Percentage of CD28- within CD3+CD8+ cells in mPBMC (19.8%) and cPBMC (13.7%).

HLA-DR expression (late marker of activation). mPBMC contained a significant higher percentage of CD3+CD25+ T-cells compared to cPBMC (p<0.0001), no difference in percentage of CD69+, and a slightly higher but not significant increase in percentage HLA-DR+ T cells (p=0.11, Figure 1c). CD4+ T cells which express CD25 can be divided into a CD25dim population (activated phenotype) and a CD25bright population (regulatory phenotype). mPBMC contained a significantly higher percentage of CD4+CD25dim T-cells compared to cPBMC (p<0.0001, Figure 1d). However, there was no difference in percentage of CD4+CD25bright (regulatory) T-cells. The percentage of CD8+CD28- T-cells, another cell population with possible suppressive capacity, was not different from non-pregnant controls (Figure 1e).

Non-specific proliferative response to PHA and anti-CD3

In order to determine the proliferation capacity of mPBMC and cPBMC, cells were stimulated with PHA and anti-CD3 Ab for 3 days. There was no significant difference in proliferation to PHA or anti-CD3 Ab between maternal and control PBMC (p=0.55 vs. p=0.90, Figure 2).

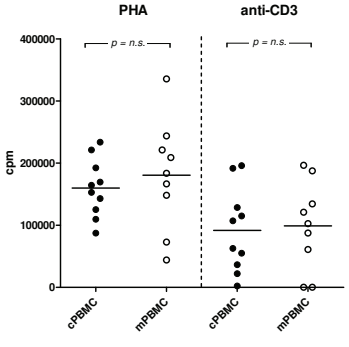

Figure 2 Proliferative response. Proliferative response of maternal PBMC (mPBMC, ○) and non-pregnant control PBMC (cPBMC, ●) upon stimulation with PHA or anti-CD3 antibody at day 3. Median values are depicted by a horizontal line.

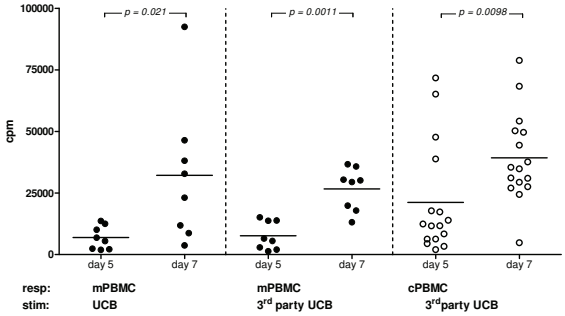

Figure 3 Proliferation of mPBMC to own child or a control child. Proliferation of mPBMC (●) to own child (UCB, left panel) or to a control child (3rd-party UCB, middle panel) measured at day 5 and 7. Proliferation of cPBMC (○) 3rd-party UCB (right panel) measured on day 5 or day 7. Median values are depicted by a horizontal line.

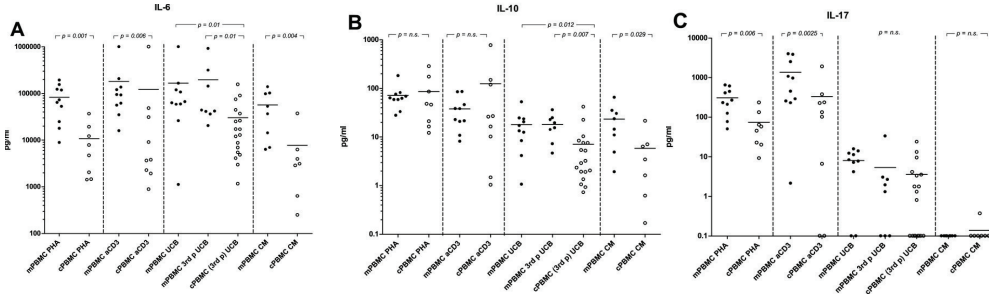

Figure 4 Cytokines in supernatant (pg/ml). IL-6 (**A.**), IL-10 (**B.**) and IL-17 (**C.**) levels in supernatants of mPBMC (●) vs. cPBMC (○) stimulated with PHA, anti-CD3 antibodies, own child (UCB), control child (3rd-party UCB) or culture medium (CM).

Fetus-specific immune response

To determine differences in the maternal immune response to UCB of her own child compared to a 3rd-party UCB, we analyzed proliferative capacity of mPBMC in a MLR. The response of mPBMC after stimulation with cells from the own child (UCB), with a control child (3rd-party UCB), and the response of cPBMC was significantly higher on day 7 compared to day 5 (p=0.021, p=0.001 and p=0.009 respectively), as expected with a normal mixed lymphocyte reaction. A non-parametric one-way ANOVA showed no significant differences between the responses of mPBMC, after stimulation with cells from her own child or control child, and cPBMC, both on day 5 (p=0.11) and on day 7 (p=0.34, Figure 3).

Cytokine production

The cytokine production by mPBMC and cPBMC was measured in the supernatant after co-culture of PBMC with the different stimuli on the fifth day. Only IL-6, IL-10 and IL-17 showed a significant difference between mPBMC and cPBMC responses with stimulation anti-CD3, UCB, or 3rd-party UCB (Table 2). We analyzed the amount of these cytokines (pg/ml) after mixed lymphocyte reaction daily to determine the day of maximum production. For IL-6, IL-10 and IL-17 this maximum was on day 5 (data not shown).

There was no difference in cytokine production by mPBMC when stimulated with the own child (UCB) compared to control child (3rd-party UCB). However many differences were found between mPBMC and cPBMC. mPBMC produced significantly more IL-6 after stimulation with all the non-specific and fetus specific stimuli (Figure 4a). The IL-10 production after allogeneic stimulation was significantly higher in mPBMC compared to cPBMC cultures (Figure 4b). mPBMC produced significantly more IL-17 compared to controls after PHA and aCD3 stimulation (Figure 4c), no differences were observed in IL-17 production after UCB stimulation.

Furthermore in control cultures with control medium alone a significantly higher production of IL-6 and IL-10 was observed in mPBMC compared to cPBMC.

	IL2	IL4	IL5	IL6[a]	IL10	IL12[b]	IL13	IL15	IL17	GM-CSF	IFNγ	TNFα
PHA	-	↓*	↓*	↑**	-	↓	-	-	↑**	-	-	-
aCD3	-	-	-	↑**	-	-	-	-	↑*	-	-	-
UCB	-	-	-	↑*	↑*	-	-	-	-	-	-	-
3p UCB	-	-	-	↑**	↑*	-	-	-	-	-	-	-
CM	-	-	-	↑*	↑*	-	-	-	-	↑*	-	↑*

Table 2 Cytokine production in supernatants of mPBMC versus cPBMC. Cells stimulated with PHA, anti-CD3, own child (UCB), control child (3p UCB) or culture medium (CM). [a] production very high, [b] production very low, - = similar levels, ↓ = decreased in mPBMC, ↑ = increased in mPBMC, *p<0.05, **p<0.01.

Discussion

In this study we examined leukocyte composition, proliferative responses and cytokine production in mPBMC and cPBMC. We observed a significant increased percentage of monocytes and activated T cells (CD3+CD25+) in mPBMC compared to cPBMC. In contrast we observed a decreased percentage of both NK-cell subsets (CD16+CD56+ and CD16-CD56bright) in mPBMC.

No differences between mPBMC and cPBMC were observed in the proliferative responses to anti-CD3, PHA, fetus specific UCB and 3rd-party UCB. However, a significant increase in IL-6, IL-10 and IL-17 was observed in mPBMC compared to cPBMC. No differences between fetus specific and 3rd-party UCB were observed. These data indicate that the maternal peripheral immune response is altered during pregnancy, though these differences do not result in quantitative changes in proliferative responses during pregnancy compared to non-pregnant controls.

The increase in percentage of CD14+ monocytes in pregnant woman versus non-pregnant women confirms an increased production of monocytes or an increased trafficking of the monocytes. Macrophages and monocytes have been reported to be more activated with cell surface marker expression similar to those during systemic sepsis [25,26]. Absolute numbers of circulating NK-cells (CD16+CD56+) have been described to increase in early pregnancy and decrease in late pregnancy when compared to non-pregnant healthy controls [27,28]. We confirm these data by showing a decreased percentage of both NK-cell subsets (CD16+CD56+ and CD16-CD56bright) at term pregnancy in pregnant versus non-pregnant women.

With respect to the acquired immunesystem we found no difference in percentage of CD8+ T-cells, CD4+ T-cells or B-cells in pregnant versus non-pregnant women. Large contradictions between the results of different studies have been described; for CD8+ T-cells an increase [29], no change [27] and even a decrease [30] were found in pregnant women compared to non-pregnant controls. During labor an increase of CD8+ T-cells has been reported [31]. Discrepancies also exist for the CD4 (helper) T-cell subset. Some studies show no change [27,32] whereas others found a decreased percentage in pregnant women [28]. Frequency and counts of B-cells seem to be unaltered during pregnancy [2,28]. These inconsistent findings may be caused by difference in analyzing methods or most likely by differences between patient groups.

We did find a higher percentage of activated T-cells (CD4+CD25dim) in pregnant women compared to non-pregnant controls, and a slightly higher percentage of HLA-DR+ T-cells (p=0.11), confirming earlier studies by our group [13]. These findings provide evidence for activation of the adaptive immune system during pregnancy.

Alterations in the distributions of T cells may lead to pregnancy complications. Decreased numbers of regulatory T cells in peripheral blood have been found in preeclampsia and recurrent spontaneous abortions [23,33]. These results postulate that a sufficient number of regulatory T cells is necessary to maintain an uncomplicated pregnancy. The exact mechanism how regulatory T cells are activated and induce tolerance during pregnancy remains to be elucidated. We found a significantly higher percentage of activated T cells (CD4+CD25dim), but no significant difference between the percentage of CD4+CD25bright in mPBMC compared to cPBMC. Previous studies found a significantly increased CD4+CD25bright T cells fraction in peripheral blood samples of pregnant women [34,35]. This discrepancy might be explained by different time points of maternal blood sampling or due to differences in gating strategies of CD25 expression. We earlier showed that differences in gating strategies might be responsible for different results [13].

HLA-mismatching between maternal and fetal antigens is a possible source of immune activation during pregnancy. The responsiveness to fetal antigens is probably a key factor controlling the activity of the maternal immune system in pregnancy and may influence pregnancy outcome [36]. In this study we do not demonstrate a difference between the maternal peripheral response to

own child UCB and 3rd-party UCB. In contrast to other studies, we used 3rd-party UCB controls with an equal number of HLA-DR mismatches compared to the own child. Since we performed HLA typing before proliferation, we had to use frozen cells, which is a drawback of this study. Our results confirm an earlier study where reactivity of mPBMC to own and unrelated newborn lymphocytes was not different [37]. Steinborn et al. showed reduced responses in MLR to own child compared to control donors [38]. In this study, the cells were obtained from adult volunteers instead from UCB. The observed difference can be explained because fetal antigen presenting cells are less efficient than adult antigen presenting cells.

Our data show that the mother's peripheral immune system has an equal proliferation capacity to cells from her own child as to those from an unrelated control child.

We observed also no differences in cytokine response between stimulation with own child and an unrelated child. However, significant differences in IL-6, IL-10 and IL-17 production between mPBMC and non-pregnant cPBMC were observed. Recently, Visser et al. reviewed the literature on cytokine and chemokine mapping in pre-eclampsia [39], including a few studies on normal pregnancies compared to non-pregnant women. One study described increased serum/plasma levels of IL-6 and TNF-α in pregnant women compared to non-pregnant controls [40]. In cultured PBMC (monocytes stimulated with LPS) no difference was found in IL-1β, IL-6 or TNF-α production [26]. We found an increase in IL-6 production by PBMC from pregnant women compared to non-pregnant controls, either spontaneously, but also after non-specific and allo-specific stimulation. TNF-α production was only higher in supernatant from cells with culture medium alone, which was also seen for IL-6 and IL-10 production. Probably these cytokines are produced by activated monocytes from the maternal peripheral blood. Again this suggests a more activated innate immune system in pregnancy.

We found no difference in IFN-γ levels and a slight decrease in IL-4 after mitogen stimulation. Other studies observed a decrease in numbers of maternal lymphocytes producing IFN-γ [14,41,42] and no difference in producing IL-4 [41,42]. A significantly increased number of PBMC producing IL-4 and unchanged number of cells secreting IFN-γ in the second and third trimester was found by Ekerfelt et al. [43]. These discrepancies in the outcomes of IL-4 and IFN-γ production are possibly due to different methods of stimulation or different methods of measuring cytokine production. In addition, we used PBMCs while other studies analyzed different cell populations.

Furthermore, we found hardly any IL-12 in our supernatants, which may be due to the fact that we used non-separated leukocytes in one culture well (about 20% of CD45+ cells were CD14+) or that the percentage of CD14+ macrophages was too low to be able to detect any IL-12 produced. On IL-12 also contradictory results have been described; Sakai et al. found a decreased production in cultured PBMC (no stimulus) [44] whereas an enhanced production of IL-12 by monocytes was seen (stimulation with endotoxin and IFN-γ) by Sacks et al. [41]. It seems that an increased or decreased production of IL-12 is dependent on the method applied.

In our patients, IL-10 production was increased especially after stimulation with allo-antigens, but also spontaneously. IL-10 is a major T helper cell type 2 or regulatory cytokine produced by T regulatory cells or NK cells. It inhibits T cell activation and production of cytotoxic cytokines (IL-12 and IFN-γ) but stimulates induction of regulatory T cells [3]. Hereby, the Th1 response is suppressed [18]. It is tempting to speculate that Th2 cells do play a role in allo-responses during pregnancy, but IL-10 can also be produced by Th1 cells, macrophages and B cells, not only by Th2 cells. Populations of peripheral blood IL-10-producing NK cells in early pregnancy were increased [45]. Veenstra van Nieuwenhoven et al. also reported a mild increase in the IL-10 production of pregnant peripheral blood NK in the third trimester of pregnancy compared to non-pregnant women [42]; however this increase was not significant. The same group found no change in IL-10 producing T cells after stimulation with PMA and ionomycin (unpublished data).

We observed more IL-17 production after non-specific stimulation, but no difference after allo-specific stimulation. Nakashima et al. also showed no difference in IL-17 production after non-specific stimulation (PMA and ionomycin) of PBMC [46]. Th17 cells, the CD4+ cells that produce pro-inflammatory IL-17, is a recently discovered population involved in the maternal immunomodulation [47,48]. These cells are closely related to regulatory T cells and differentiate upon inflammatory signals whereas conditions that promote tolerance favor generation of Treg [49]. A balance between Th17 and Treg might be correlated with successful pregnancy; however the role of Th17 in human pregnancy remains to be investigated more substantially.

In conclusion, our results demonstrate that in the peripheral circulation, the innate and the acquired immune system are enhanced during pregnancy compared to non-pregnant controls reflected by phenotype of PBMC and *in vitro* cytokine production. However, there is no changed immune response when measuring proliferation capacity. The mother is capable of creating a fine-tuned environment optimal for the fetus to grow but also optimal to maintain adequate immune responses to diseases.

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Differential distribution and phenotype of decidual macrophages in preeclamptic versus control pregnancies

3

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Abstract

Background: Tolerance towards the semi-allogeneic fetus is a complex and basically unrevealed phenomenon. As macrophages are an abundant cell population in the human decidua, changes in distribution or phenotype may be involved in the development of preeclampsia. The aim of this study was to assess the distribution and phenotype of macrophages in preterm preeclamptic, preterm control, and term control placentas.

Methods: Placentas of preterm preeclamptic (n=6), of preterm control (n=5), and of term control pregnancies (n=6) were sequentially immunohistochemically stained for CD14, CD163, DC SIGN and IL-10. The distributions of CD14+, CD163+, DC SIGN+, IL-10+, CD163+/CD14+, DC SIGN+/CD14+ and Flt-1/CD14+ cells were determined by double staining and by digital image analysis of sequential photomicrographs.

Results: CD14 and CD163 expression was significantly increased in preterm preeclamptic decidua basalis compared with preterm control pregnancies (p=0.0006 and p=0.034 respectively). IL-10 expression was significantly lower in the decidua parietalis of preterm preeclamptic pregnancies compared with preterm control pregnancies (p=0.03). The ratio CD163/CD14 was significantly lower in the decidua basalis (p=0.0293) and the ratio of DC SIGN/CD14 was significantly higher decidua basalis (p=<0.0001) and parietalis (p=<0.0001) of preterm preeclamptic compared with preterm control pregnancies. CD14+ macrophages did express Flt-1.

Conclusion: Alterations in distribution and phenotype of macrophages in the decidua of preterm preeclamptic pregnancies compared to control pregnancies may contribute to the pathogenesis of preeclampsia.

Introduction

Maternal immune tolerance towards the semi-allogeneic fetus and placenta is important in uncomplicated human pregnancy. Maternal immune cells at the feto-maternal interface are directly exposed to fetal antigens at three locations [1]. First, the maternal tissue lining the fetal membranes, the decidua parietalis, interact with the trophoblast cells of the chorion. Second, the maternal part of the placenta, the decidua basalis, is infiltrated by invading extravillous trophoblast. Third, after the establishment of the utero-placental circulation, maternal peripheral blood contacts with syncytiotrophoblast. Several mechanisms, some of them implying a special role for macrophages at the three interfaces, have been postulated to promote an immunomodulatory state [2,3].

Macrophages are antigen-presenting cells which account for the second most numerous type of leukocytes in the human decidua [4]. They are mononuclear phagocytotic cells involved in the innate and adaptive immune system. Macrophages promote inflammation by production of inflammatory molecules during an innate immune response and, are able to present antigens to T cells as part of the adaptive immune system. Macrophages may have a role in immunosuppression in the human decidua, as suggested by their ability to suppress a one-way mixed lymphocyte reaction [5]. Furthermore, macrophages express costimulation molecules CD80 and CD86 in low levels and they express indoleamine2,3-dioxygenase, both preventing T lymphocyte activation [6]. An alteration in the quantity or distribution of these cells may be involved in the development of preeclampsia. Preeclampsia is a relatively common but potentially dangerous disorder in human pregnancy, leading to maternal and neonatal morbidity and mortality. It affects 1-7% of nulliparous women who have a three times higher risk than multiparous women [7,8]. The disease is characterized by inadequate transformation of the spiral arteries [9] and generalized maternal sFlt-1-mediated endothelial cell dysfunction [10]. Furthermore, immunologic factors are involved in the pathogenesis of preeclampsia since earlier exposure with paternal antigens decreases the risk of preeclampsia [11,12].

The exact role of macrophages in the human decidua and their function in preeclampsia remains unknown. The numbers of macrophages have been studied by several groups with varying results. A reduction in the number of CD14+ macrophages [13], no alteration [14] and increased numbers of macrophages [15] have been found in decidua from preeclampsia compared to control women. Because of these discrepancies in the literature we intended to study the role and distribution of macrophages in control and preeclamptic decidua. For phenotypic characterization of the macrophage subsets three different markers were tested. CD14, a glycosylphosphatidylinositol-anchored membrane protein, is present on monocytes and macrophages. The macrophage scavenging receptor, CD163 is a mononuclear phagocyte restricted cell surface glycoprotein antigen present on type 2 macrophages (M2 cells) which have been reported to exert an anti-inflammatory function [16]. Gene expression profiling shows that human decidua mainly contains M2 cells, which contribute to the immunosuppressive state favorable to the maintenance of the semi-allogeneic fetus [17]. In contrast to M2 cells, macrophages stimulated with Th1 cytokines polarize toward a pro-inflammatory type 1 macrophages (M1 cells). These cells are able to defend upon utero-placental infections but do not contribute to the tolerance of the fetus [18]. Furthermore, we used the dendritic cell-specific marker ICAM3-grabbing nonintegrin (DC SIGN) for phenotypic characterization. DC SIGN is highly expressed on immature DCs but also present on macrophages in the human decidua [19,20].

In addition we stained the IL-10 and Flt-1 expression by immunohistochemistry in the decidua basalis and parietalis. IL-10 is an immunosuppressive molecule, produced by T cells, macrophages/monocytes and B cells. This cytokine is spontaneously produced in high levels by decidual macrophages [6]. It is a Th2 type cytokine and appears to be pregnancy protective [21]. Decreased villous trophoblast staining of IL-10 has been demonstrated in women with

preeclampsia compared to normal pregnancy with correlated gestational age [22].

Coexpression of CD14 and CD68 as a general macrophage marker, with either CD163, DC SIGN or sFlt was studied to define the phenotype of cells. We determined the number and type of macrophages in decidua of preterm preeclamptic, preterm control, and term control pregnancies and defined the natural polarization of decidual macrophages and alterations of the phenotype of these cells.

Material and Methods

Patient selection

After a pilot study of five preterm preeclamptic and five term control placentas, six preterm preeclamptic, five preterm control and six term control placentas were collected. Criteria for inclusion in the preeclamptic group were presence of hypertension (diastolic blood pressure ≥ 95 mm Hg), proteinuria (> 0.3 gr/l/24 hours) and a gestational age below 34 0/7 weeks. Term placentas were collected from healthy women after normal, uncomplicated pregnancies of 37-42 weeks gestational age. Preterm placentas were collected if delivered before 34 weeks gestational age after an uncomplicated pregnancy without any signs of infection. This group contained a quadruplet of which the placentas were analyzed as separate. The values obtained in the singleton preterm control placenta were in the same range as those observed in the placentas of the quadruplet pregnancy. No significant differences were present between the singleton preterm control placenta and the quadruplet preterm control placentas for the stainings of CD14, CD163 and DC SIGN as well as in the decidua basalis or parietalis (data not shown). Tissue samples were collected within five hours after the time of delivery of the placenta after primary caesarean section or vaginal delivery. The study was approved by the ethics committee of the Leiden University Medical Center (LUMC) and informed consent of every patient was obtained.

Immunohistochemistry

Tissue blocks of the placenta and rolls of fetal membranes were taken at three locations, fixed in 4% formalin and routinely embedded in paraffin. Sequential serial sections (4µm-thick) were cut on adhesive coated glasses and dried overnight at 37°C. Tissue sections were deparaffinized and hydrated by xylene in decreasing alcohol concentration to demi-H_2O. Endogenous peroxidase was blocked with 3% hydrogen peroxide for 20 minutes. After a wash step with demi-H_2O, antigen retrieval was performed by boiling the sections for 10 minutes in citrate buffer (pH 6.0). The slides were cooled down for 20 minutes followed by another wash step. The optimal dilution for each primary antibody was determined in positive decidual tissue selected on the basis of maximal specific reactivity and minimal background staining (Table 1). As a control the primary antibody was replaced by normal serum. The primary antibody was incubated for one hour at room temperature at the appropriate dilutions in PBS with 1% BSA (except for IL-10, which was pre treated with normal goat serum for 30 minutes and incubated overnight). After washing three times in PBS the slides were incubated 30 minutes with Envision (DAKO, North America Inc, USA). Another wash step was followed by 5 minutes incubation with diaminobenzidine (DAB, DAKO Cytomation). Demi-H_2O was used to stop the reaction. The tissue sections were subsequently counterstained with haematoxylin (SIGMA, Switzerland, Steinheim).The slides were mounted in mounting medium (Surgipath Medical Ind., Inc. Richmond) and covered.

Antibody	Isotype	Dilution	Source
CD14	IgG2a	1:200	Novocastra, Newcastle, United Kingdom
CD68	IgG1	1:250	DAKO, North America Inc, USA
CD163	IgG1	1:20	Abcam, Cambridge, United Kingdom
DC SIGN (CD209)	IgG2b	1:4000	Miltenyi Biotecs MACS, Bergisch Gladbach, Germany
IL-10	Polyclonal IgG	1:50	Hycult Biotech, Uden, The Netherlands
Flt-1	Polyclonal IgG	1:250	Santa cruz, Biotechnology Inc, Heidelberg, Germany

Table 1 Antibody characteristics.

Double label immunohistochemistry of CD68 and CD163 or DC SIGN

To determine if cells were double positive for CD68 and CD163 or DC SIGN, besides the use of sequential slides, also double labelling was performed. Extensive investigation showed that the combination of CD14 and CD163 or DC SIGN did not give reliable results. Therefore, CD163 or DC SIGN and CD68, a general and a pan-macrophage marker, double labelling was performed. The sections were deparaffinised in xylene followed by alcohol 100%. Blocking was performed with methanol 0.3% H_2O_2. The sections were rehydrated and rinsed with PBS. The Tris-HCL buffer (pH8.2, 100 mM) was preheated in a water bath at 97°C. The sections were incubated with the buffer for 30 minutes on 97°C, and cooled down for 45 min. on ice. Thereafter sections were incubated with the first antibody (CD163 or DC SIGN), for 1 hour at room temperature and afterwards rinsed with PBS. The sections were incubated with Envision-HRP anti-mouse (DAKO, North America Inc, USA) for 30 minutes and rinsed with PBS. For 7 minutes at room temperature the sections were incubated with Vector NovaRed (Vector Laboratories Inc, Burlingame, USA) and rinsed with PBS. Then the sections were incubated with the second antibody (CD68) for 1 hour, followed by incubation with Rabbit anti-mouse (DAKO) for 30 minutes, APAAP mouse (DAKO) for 30 minutes and with Vector blue (Vector laboratories Inc, Burlingame, USA) for 25 minutes. In between each step the slides were rinsed with PBS. Finally, the sections were dried and covered with mounting medium (Pertex, Histolab Products, Gothenburg, Sweden).

Double label immunohistochemistry of CD14 and Flt-1

Double-immunohistochemistry staining of CD14 and Flt-1 was performed using the DAKO Envision G/2 Doublestain system (code K5361) following the manufactors protocol. Briefly, slides were deparaffinized and hydrated via graded alcohols to demiwater. Heat-induced antigen-retrieval was performed with citrate buffer (pH 6.0) for 20 minutes in a microwave, followed by washing steps in PBS. Endogenous alkaline phosphatase and peroxidase activity was blocked for 5 min by dual endogenous enzyme block. The sections were incubated with primary antibody anti-Flt-1 (dilution 1:250, Santa cruz-316), followed by incubation with Polymer/HRP reagent, using DAB+ as chromogen. Next a blocking step with double stain block reagent was performed. The sections were incubated with the second primary antibody anti-CD14 (dilution 1:200 in 1% BSA/PBS, Novocastra, clone 1F6), afterwards a Rabbit/Mouse LINK was added, followed by incubation with Polymer/AP reagent, using Permanent Red as chromogen. As a control, primary antibodies were replaced with isotype control antibodies to obtain single immunohistochemical staining. Double stained sections were counterstained with haematoxylin.

Quantification of staining

Equivalent fields containing decidua of sequential sections were digitized blinded by study group (Zeiss Axioskop 40, magnification 200x, Zeiss Axiocam MRc 5 camera, 150x150dpi). For every staining of one placenta a total of 15 pictures of the decidua parietalis and 15 of the basalis were taken (3 locations and 5 pictures per location). Only the decidual stroma was selected for evaluation; irrelevant structures like blood vessels and shadows were digitally removed. Using Image-J software [23], the numbers of positive pixels per area were measured indicating the level of expression. The program is able to identify and measure positive cells by setting a threshold. For every staining a macro was made, predefining the threshold of a positive cell. This threshold was independently defined by two observers. Of the 15 pictures the mean and standard deviation of the number of pixels per area were calculated. The CD163/CD14 ratio and the DC SIGN/CD14 ratio were calculated for every side matched pictures. All analyses were performed blinded for the pregnancy group. Placentas included in the preterm preeclamptic group all showed histological characteristics of preeclampsia (increased syncytial knots, chronic villitis, decidual vasculopathy, thickening of trophoblastic basement membrane, and infarction) [24], blindly observed in H&E staining.

Statistical analysis

The total amount of pixels per area for every antibody staining was compared between preterm preeclampsia versus preterm control placentas and preterm control versus term control. Ratios (CD163/CD14 and DC SIGN/CD14) were calculated in order to define the amount of CD163+ and DC SIGN+ cells within the macrophage population. Descriptive statistical analysis was performed using Graph Pad Prism (Graph Pad Software Inc.) and SPSS (SPSS Inc 17). A p value of <0.05 was considered statistically significant. The one way ANOVA and the non-parametric Mann Whitney test were used to identify differences between the data.

Results

Pilot findings and patient characteristics

In a pilot study of 5 other preterm preeclamptic and term control placentas a difference was found in the level of expression of CD14, CD163 and DC SIGN in preterm preeclamptic and term control. A higher expression rate of CD14 and CD163 and a lower expression rate of DC SIGN was found in decidua basalis of preterm preeclamptic placentas compared with term control placentas (data not shown). Because a difference in gestational age in preterm preeclamptic and term control placentas (40 weeks versus 30 weeks respectively, $p=<0.05$) could have an effect on these outcomes, a preterm control group was collected for the current study. Patient characteristics are shown in Table 2. Patients in the preterm preeclampsia group had a significantly lower gestational age, a higher systolic and a higher diastolic blood pressure ($p=<0.05$) compared with term control and preterm control placentas (Table 2). The gestational age of preterm preeclampsia and preterm control group were 33 and 34 weeks respectively ($p=0.033$). The decidua of preterm preeclamptic, preterm control and term control placentas all showed positive cells for the used antibodies. Negative control slides were all negative. In general, the average amount of expression for every antigen is higher in the decidua basalis, compared to the decidua parietalis irrespective of the pregnancy group (Figure 1A-D). The staining location of CD14+, CD163+ and DC SIGN+ cells was in general similar at both locations (decidua basalis and parietalis, Figure 1E).

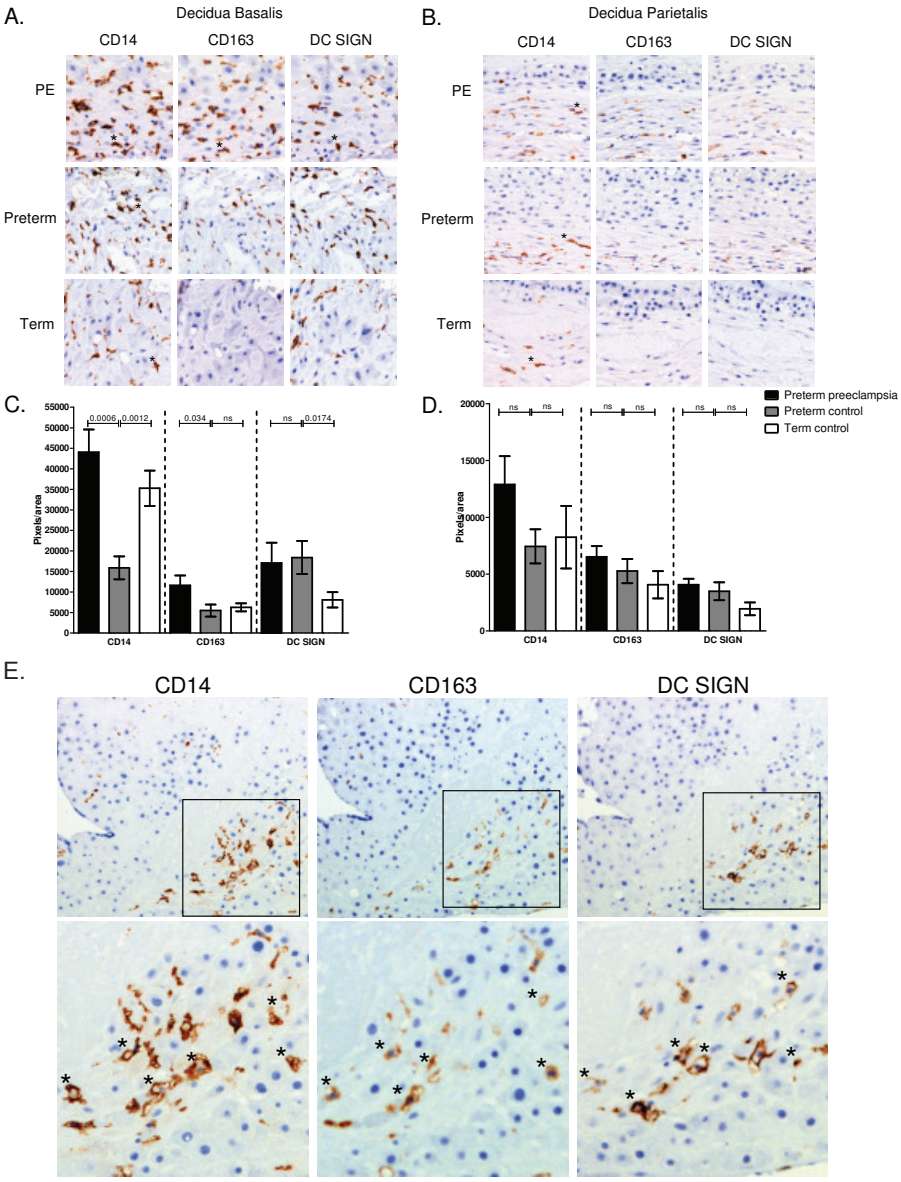

Figure 1 Photomicrographs of sequential sections stained immunohistochemically for CD14, CD163 and DC SIGN of the decidua basalis (A.) and decidua parietalis (B.) (original magnification x400, positive cells are brown, nuclei are stained blue). The upper row shows the staining in preterm preeclamptic (PE) pregnancies. In the decidua basalis more CD14+ and CD163+ staining is present in the preterm preeclampsia group compared with preterm controls, the amounts of DC SIGN staining do not differ between the preterm preeclampsia group and preterm controls. In the decidua parietalis no significant differences are present. Asterisks indicate examples of positive cells. C and D. Graphs illustrating the amount of positive pixels per area in the decidua basalis (**C.**) and parietalis (**D.**) respectively for each antibody in preterm preeclamptic, preterm control or term control placentas. Statistical differences were determined using the non-parametric Mann Whitney test. Values presented as means, the error bars indicate the SEM. **E.** Photomicrographs of sequential sections stained immunohistochemically for CD14, CD163 and DC SIGN (original magnification x200, positive cells are brown, nuclei are stained blue). In the upper panel the same pattern of staining for the three antigens is visible. The lower panel shows a magnification in which asterisks indicate positive cells for CD14, CD163 and DC SIGN.

	Preeclampsia	Preterm	Term	p value*
Maternal age (years)	31±6	28±2.5	30±2.5	ns
Gestational age (weeks)	33±2	34±0.5	39±1.5	<0.05**
Highest systole (mmHg)	185±10	123±2	126±14	<0.05***
Highest diastole (mmHg)	106±11.5	76±7.5	77±12.5	<0.05***
Gravidity	1	2	1	ns
Parity	0	0	0	ns
Medication	4 x anti-hypertensive	no	no	<0.05****

Table 2 Patient characteristics. Plus-minus values are ranges. * One way ANOVA. **One way ANOVA, followed by t-test showed significant differences between the comparisons of all groups (preeclampsia vs term p=<0.0001, preeclampsia vs preterm p=0.033, preterm vs term p=0.0023). ***One way ANOVA, followed by t-test showed significant differences between preeclampsia versus term and preeclampsia versus preterm. ****Kruskal-Wallis test.

Comparison in level of expression of CD14, CD163 and DC SIGN in decidua basalis an parietalis between preterm preeclampsia and preterm control

To compare the phenotype of decidual macrophages of the preterm preeclamptic, term control and preterm control first the expression of the markers CD14 and CD163 were analyzed. The level of expression of CD14 and CD163 was significantly higher in the preterm preeclamptic decidua basalis compared with the decidua basalis of preterm control pregnancies (p=0.0006 and p=0.034 respectively, Figure 1C). No significant differences were present in the level of expression of DC SIGN positive cells in the decidua basalis. In the decidua parietalis no significant differences were present between preterm preeclamptic and preterm control pregnancies for CD14, CD163 or DC SIGN (Figure 1A-D).

Comparison in level of expression of CD14, CD163 and DC SIGN in decidua basalis and parietalis between preterm and term control

As gestational age could have an effect on study outcomes in comparing outcomes of the level of expression in macrophage markers, we also analyzed the differences between the preterm and term control group. Significant differences are present for CD14 and DC SIGN. CD14 expression is significantly lower in the preterm control group compared with the term control group (p=0.0012, Figure 1C). CD163 is significantly higher in preterm control group compared with the term control group (p=0.0174, Figure 1C). In the decidua parietalis no significant differences were present between preterm and term control pregnancies (Figure 1D).

The ratio CD163/CD14 is lower and the ratio DC SIGN/CD14 is higher in preterm preeclamptic decidua basalis, when compared with preterm control pregnancies

In general, the sequential stained slides showed a similar staining pattern for CD14, CD163 and DC SIGN although not all CD14+ cells are positive for CD163 or DC SIGN (Figure 1E). To prove that cells were double positive for CD68 and CD163 or DC SIGN next to the use of sequential slide also double labeling was performed. The double staining of CD68 and CD163 or DC SIGN

confirms that some cells which were positive for a general macrophage marker are as well positive for the M2 marker (Figure 2A and B). To examine the natural polarization of decidual macrophages and alterations of the phenotype the CD163/CD14 and DC SIGN/CD14 ratios of subsequent areas were calculated. Although, the individual level of expression of CD163 is higher in preterm preeclamptic decidua basalis compared with preterm control (Figure 1C), the number of CD163 positive cells in the fraction of CD14 positive cells (CD163/CD14) was significantly lower in preterm preeclamptic decidua basalis compared with preterm control decidua basalis ($p=0.0293$, Figure 3A). By contrast the level of DC SIGN in the fraction of CD14 positive cells (DC SIGN/CD14) was significantly higher in preterm preeclamptic placentas than in preterm control placentas ($p=<0.0001$, Figure 3A). As in the decidua basalis, in the decidua parietalis the ratio DC SIGN/CD14 was significantly higher in preterm preeclamptic and preterm control pregnancies ($p=<0.0001$, Figure 3B). The ratio CD163/CD14 and ratio DC SIGN/CD14 is significantly higher in decidua basalis of preterm controls compared with term controls ($p=0.0190$ and <0.0001 respectively, Figure 3A). In the decidua parietalis the ratio DC SIGN/CD14 is significantly lower in preterm controls compared with term controls ($p=<0.0001$, Figure 3B).

CD14+ macrophages are Flt-1+

As suggested that decidual macrophages are a possible additional source of sFlt-1 production and thereby they could contribute to the pathogenesis of preeclampsia. Therefore we investigated whether macrophages are positive for Flt-1. Double labeling of CD14 and Flt-1 shows that macrophages in the decidua basalis did express Flt-1 (Figure 2C).

Lower expression of IL-10 in decidua parietalis of preterm preeclamptic pregnancies compared with preterm control pregnancies

To functionally characterize cells in the decidua, immunohistochemical staining of IL-10 was performed on placental tissue. The level of expression of IL-10 in preterm preeclamptic decidua parietalis is significantly lower compared with preterm control pregnancies ($p=0.03$). No significant differences were found in the expression of IL-10 in the decidua basalis of preterm preeclamptic, preterm control and term control placentas (Figure 4).

Discussion

This study investigated the phenotype and natural polarization of decidual macrophages by comparing the myeloid cell markers CD14, CD163 and DC SIGN cells in decidua basalis and parietalis of preterm preeclamptic, preterm control, and term control pregnancies using immunohistochemistry and an objective quantification method. We found significantly more CD14+ cells in the decidua basalis in preterm preeclamptic pregnancies compared with preterm control pregnancies. In addition the specific M2 marker CD163, was significantly upregulated in the decidua basalis in preterm preeclamptic pregnancies compared with preterm control pregnancies. Insight of the functional importance of the phenotypic differences in decidual macrophages is limited by lack of M1 markers, and therefore the M2 ratio of CD163/CD14 was used. In the decidua basalis the number of M2 cells (ratio of CD163/CD14) was significantly lower in placentas from preterm preeclamptic pregnancies compared with preterm control pregnancies. The ratio DC SIGN/CD14 was significantly higher in decidua basalis and parietalis of preterm preeclamptic pregnancies compared with preterm control pregnancies. In addition to the preterm control group we compared the term control group with the preterm control group. A significantly lower level of expression of CD14 was present in the decidua basalis of preterm

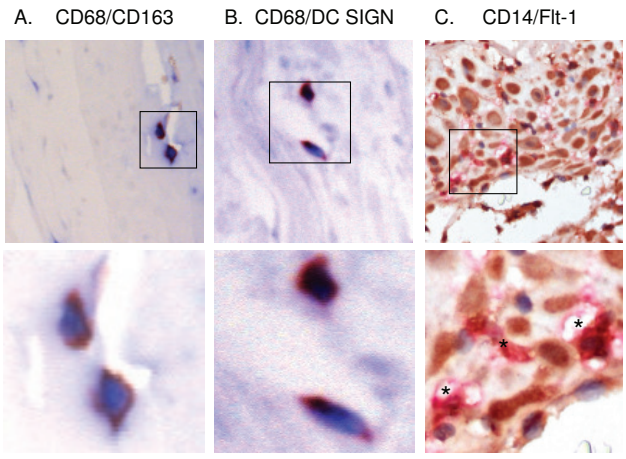

Figure 2 Double staining. A. Example of cells in the decidua parietalis which are double positive for CD68 (blue) and CD163. No nuclear counter staining was used. The pictures in the lower panel show a magnification from the pictures in the upper panel. (Original magnification x400) **B.** Example of cells in the decidua parietalis which are double positive for CD68 (blue) DC SIGN (red). No nuclear counter staining was used. The pictures in the lower panel show a magnification from the pictures in the upper panel. (Original magnification x400.) **C.** Example of cells in the decidua basalis which are double positive for CD14 (red) and Flt-1 (brown). The nuclei are stained blue. The pictures in the lower panel show a magnification from the pictures in the upper panel. Double positive cells are indicated by an asterisk.

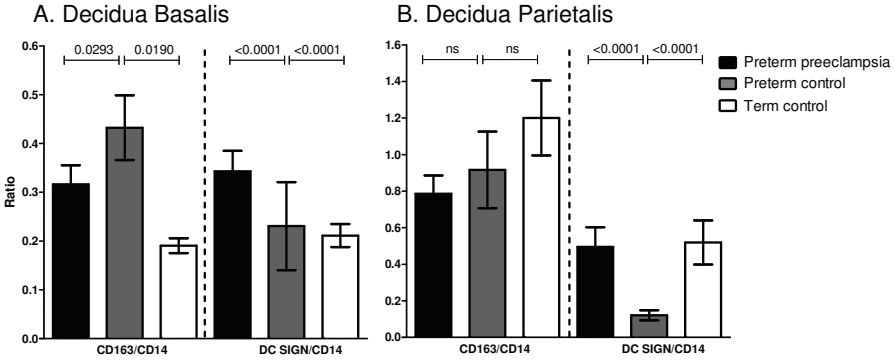

Figure 3 A. Ratio CD163/CD14 and DC SIGN/CD14 calculated from subsequent pictures. The ratio CD163/CD14 is significantly lower (p=0.0293) and the ratio DC SIGN is significantly higher (p=0.0001) in preterm preeclamptic decidua parietalis compared with preterm control pregnancies. **B.** The ratios of CD163/CD14 and DC SIGN/CD14 in the decidua parietalis. The ratio CD163/CD14 is not significantly different and the ratio DC SIGN is significantly higher (p=0.0001) in preterm preeclamptic decidua parietalis compared with preterm control pregnancies.

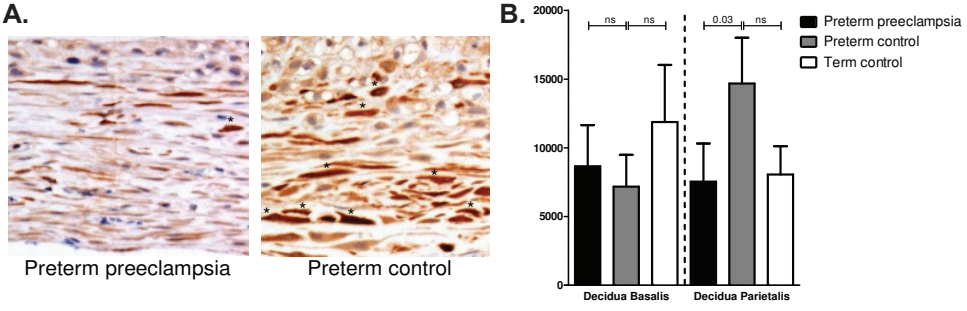

Figure 4 IL-10 results. A. Photomicrographs of sections stained immunohistochemically for IL-10 in preterm preeclamptic and preterm control decidua parietalis (original magnification x400). Asterisks indicate examples of positive cells. **B.** In the decidua basalis no significant differences are present in the amount of IL-10+ cells between preterm preeclamptic, preterm control or term control pregnancies. In the decidua parietalis less IL-10 staining is present in the preterm preeclampsia group compared with preterm controls (p=0.03).

control compared with term controls (p=0.0012). This indicates that it is important to have a gestational age matched control group when investigating macrophages in preterm preeclamptic pregnancies.

The most abundant differences are found in the decidua basalis, and not in the decidua parietalis, which could be explained by the invasion of trophoblast which occurs in the decidua basalis and not in the decidua parietalis.

Maternal tolerance towards the semi-allogeneic fetus is important for an uncomplicated pregnancy. The decidual cell population consists of several immunologic cells and a disturbance in the distribution of phenotype of these cells may lead to pregnancy complications. Macrophages and DCs are present in the human decidua [6,19,25,26] and an alteration of the phenotype and distribution may be involved in the pathogenesis of preeclampsia [27].

The sequential stained immunohistochemical slides showed that in general CD14+ cells can also be DC SIGN+ and CD163+. Our study confirms earlier reports of predominant polarization to M2 macrophages in the term placenta (reviewed by Nagamatsu et al [28]). The amount of CD14+ or CD163+ cells in the decidua basalis were significantly higher in placentas from preterm preeclamptic pregnancies compared with preterm control pregnancies. Severity of preeclampsia could contribute to this higher number and different functionality of macrophages present in the decidua. Therefore, two placentas from most severe cases of preeclampsia (based on the level of diastolic pressure, amount of proteinuria and gestational age) demonstrated the highest number of cells in the decidua basalis.

The number of M2 macrophages in relation to all macrophages (ratio CD163/CD14) was lower in placentas from preterm preeclamptic pregnancies, compared with preterm control pregnancies. To our knowledge, this is the first study that describes a decrease in M2 in the decidua basalis of preterm preeclamptic pregnancies compared to preterm control pregnancies. We speculate that this lower amount of M2 may contribute to the etiology of preeclampsia. Furthermore, we have shown an increase of the ratio of DC SIGN+ cells in placentas from preterm preeclamptic pregnancies. The phenotypic plasticity of myeloid cells such as DCs and macrophages is substantial and a subset distinction is difficult to make. Only a few markers are known which really make the distinction between macrophages and DCs. Gardner et al [19]. already postulated that DC SIGN is present on decidual macrophages but not on decidual DCs. It remains unclear whether this cell subset, also called intermediate antigen presenting cells, is a subset of macrophages or of DCs. CD14+DC SIGN+ cells are reported in other human tissues [29,30], and these cells produce large amounts of proinflammatory cytokines [31]. In line with the study of Gardner et al, our study also shows in general a similar staining pattern between CD14+ and DC SIGN+ cells. The presence of this subset of DC SIGN+ macrophages in the decidua is pregnancy-associated and these cells may play a crucial role for the local immune response. Therefore, alterations in the function and distribution of this cell may result in pathological pregnancies, like preeclampsia which has been shown by Huang et al [32]. Preeclamptic decidua contained an infiltrate of DC SIGN+ cells in contrast to their sparse presence in the decidua of uncomplicated pregnancies. This study also confirms an increased level of DC SIGN expression in preterm preeclamptic decidua compared to preterm control decidua. However, current study relates DC SIGN+ cells with macrophages in stead of DCs because of their co-localization and as shown by double staining. In contrast to our study, Scholz et al found no significant differences between preeclamptic and control placentas in the amount of DC SIGN+ cells using immunohistochemistry [33]. However, they found a higher amount of DC SIGN+ cells in placentas from patients who developed HELLP (hemolysis, elevated liver enzymes, low platelets) syndrome. It is possible that our preterm preeclamptic group is more comparable with the HELLP group of the study of Scholz et al. since our study included only very severe preterm preeclamptic patients with deliveries with a gestational age below 34 0/7 weeks.

In addition of the presence macrophage antigens in the decidua, this study investigated the production of IL-10 in placental tissue. During pregnancy IL-10 is an important cytokine, it plays a role in the prevention of placental rejection. Human pregnancy is a type 2 immune state shown by a shift in cytokine production from type 1 to type 2. This balance is different in preeclampsia in which a decrease in IL-10 compared to the pro-inflammatory cytokines is present. IL-10 is secreted by cytotrophoblast and it can suppress an allogeneic immune response *in vitro* [34]. It is possible that IL-10 may be involved in protecting the semi-allogeneic fetus in normal pregnancy [21]. To our knowledge, only one earlier published study performed IL-10 immunohistochemical staining on placental tissue [22]. Hennesy *et al.* showed a change in IL-10 immunolocalization in term placentas from women with preeclampsia compared to those with a normal pregnancy outcome. They showed a general decrease in cytoplasmic trophoblast villi IL-10 content in preeclamptic pregnancies. Additionally, a decrease in IL-10+ trophoblast cells located in the decidual tissue was present. A lower level of IL-10 in the decidua basalis suggests an impaired protective mechanism of the mother toward the allogeneic fetus in preeclampsia.

Our digital analysis shows that the number of IL-10 positive cells is lower in the decidua parietalis of preeclamptic pregnancies compared to preterm pregnancies. This indicates that there is a difference in defense mechanism between the decidua parietalis and basalis. The decidua parietalis contacts the non-invading trophoblast of the chorion and the decidua basalis interacts with invading villous trophoblast. It seems that the contact between the chorion in the decidua parietalis in preterm pregnancies synthesizes the trophoblast cells to produce IL-10, which does not appear in preeclamptic decidua parietalis. Since this study showed a lower amount of positive IL-10 cells in the decidua parietalis of preeclamptic pregnancies compared with preterm pregnancies, we speculate that a high level of IL-10 is necessary to maintain pregnancy without complications, and that a down regulation of IL-10 produced by the decidua parietalis is a permissive condition for the development of preeclampsia.

Recently, it has been shown in chronic kidney disease that monocytes may be a possible source of sFlt-1 [35]. Increase of sFlt-1 leads to endothelial dysfunction and increased levels have been found in patients with preeclampsia [10,36]. Double labeling immunohistochemical staining of CD14+ and Flt-1 shows that macrophages in the decidua basalis are positive for Flt-1. Since we found an increase of the amount of CD14+ cells in preeclamptic decidua basalis compared with preterm decidua basalis ($p=0.0006$) it is possible that decidual macrophages are responsible for the increased sFlt-1 production which may contribute to the etiology of preeclampsia.

Tolerance of the genetically foreign fetus by the maternal immune system fetus is a complex phenomenon and remains to be elucidated. Multiple mechanisms are involved in maintaining the pregnancy. Localized secretion of immunoregulatory cytokines may prevent immune rejection of the placenta. In addition, the presence of immunomodulatory cells may be important in dampening an inflammatory immune response. Preeclampsia is a state in which the immune system has to work harder to maintain pregnancy. Alterations in immunomodulatory cells in the decidua basalis and parietalis of preterm preeclamptic pregnancies compared to control pregnancies may contribute to the etiology of preeclampsia. The question is whether alterations in the immune system lead to the pathogenesis of preeclampsia or its prevention in subsequent pregnancies.

In conclusion, present study shows that macrophages can be DC SIGN+ as well as CD163+ based upon the double staining and based on the similar staining pattern of these antigens. An increase of CD163+ cells in preterm preeclamptic placentas was found compared with preterm control placentas. However, the total amount of CD14+ cells is also increased in preterm preeclamptic placentas compared with preterm control placentas. The amount of CD163+ cells in the fraction of CD14+ cells is lower in preterm preeclamptic placentas compared with preterm control placentas. Furthermore, this study found an increase in DC SIGN/CD14 myeloid cells in the decidua parietalis and basalis of preterm preeclamptic pregnancies compared with preterm control pregnancies.

This study suggests that further investigation of the distribution and phenotype of macrophages is possibly relevant for further understanding the immunology at the fetal-maternal interface.

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Preeclampsia in non donor IVF and egg donation pregnancy: is there a different pathophysiology?

4

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Abstract

Background: Egg donation (ED) gives women with premature ovarian or other causes of reproductive failure the ability to conceive. This results in a unique pregnancy since the entire fetal genome can be allogeneic to the mother. Two cases of IVF, one after ED and the other non donor IVF, are discussed to demonstrate why we hypothesize a different possibly immunological mechanism as the cause of preeclampsia in ED pregnancies as compared to non donor IVF.

Case 1 describes a 30 year old woman whose pregnancy after non donor IVF resulting in a dizygotic twin was complicated by preeclampsia and intra-uterine growth retardation of both fetuses. The pregnancy was a result of concurrent IVF and spontaneous conception, which is extremely rare.

Case 2 describes a 41 year old woman pregnant after ED of a dizygotic twin whose pregnancy was also complicated by severe preeclampsia. Both fetuses had a normal fetal birth weight.

We suggest a different pathophysiological mechanism of preeclampsia after ED compared with preeclampsia in non donor IVF conception.

Results: ED pregnancies are associated with a higher incidence of pregnancy-induced hypertension and a specific placental pathology. Other perinatal complications, such as intrauterine growth retardation and prematurity are also reported, but the incidence is comparable to conventional IVF. It is known that during pregnancy, both local and systemic immunological changes occur. Possibly, in ED pregnancies these changes are different or more pronounced.

Conclusion: Because of a higher degree of antigenic dissimilarity compared to non donor IVF, ED pregnancies represent an interesting model to study complex immunological interactions, as the allogeneic fetus is not rejected but tolerated by the pregnant woman. Knowledge of the immune system in ED pregnancies might have broader significance, as it may also give insight into immunologic aspects of tolerance in solid organ transplantation.

Introduction

We postulate that preeclampsia in egg donation (ED) pregnancies might have a different pathophysiological mechanism, compared with spontaneously conceived pregnancies. This chapter describes two examples of clinical complications in twin pregnancies resulting from assisted reproductive pregnancies. By describing both cases the differences and similarities become apparent.

Case 1 depicts a pregnancy after a non donor IVF procedure and case 2 illustrates a pregnancy after ED. In case 1 an infertile couple underwent non donor IVF with the transfer of one embryo, however resulting in a dizygotic pregnancy of concurrent IVF and spontaneous conception. Although this is an interesting and rare phenomenon, for the consideration of this chapter the focus is on the possible related development of preeclampsia and intra-uterine growth retardation of both fetuses. The second case discusses a woman pregnant after ED complicated by severe preeclampsia, however without intra-uterine growth retardation in none of the fetuses. By comparing both cases, we hypothesize a possible different pathophysiological immunological mechanism as the cause of preeclampsia in ED pregnancies.

Case 1 Dizygotic twin pregnancy following transfer of one embryo

Introduction

Two case reports suggest spontaneous conception in IVF cycles in which two embryos were transferred resulting in quadruplet pregnancies with different zygosity [1,2]. Although efforts are exerted to reduce the risk of multiple pregnancies, in general no advice against intercourse during the transfer period of an IVF cycle is given as it might have a positive effect on the success rate of IVF procedures [3]. In this case report we discuss a woman who conceived from intercourse and through an embryo transfer in the same menstrual cycle. Already during pregnancy permission was obtained from the couple to publish this report.

Case

A 30 year old nulliparous, ovulatory woman and her 39 year old partner (normospermia) were examined for primary infertility. Her medical history revealed appendectomy after perforation, menorrhagia due to an intramural myoma and a treated PID due to Chlamydia trachomatis. Shortly thereafter, a hysterosalpingogram and a laparoscopy showed a myoma (diameter 8 cm) in the anterior uterine wall and possible tubal obstruction as tube permeability for contrast fluid was absent. The couple was referred to a university center for uterine and possibly tubal surgery. During surgery, directly after myomectomy and myometrium closure whereby the cavum uteri was not opened, different approaches for testing tubal patency showed no tubal patency. An intramural obstruction of both tubes was considered. Seven months after surgery an IVF procedure resulted in 7 fertilized oocytes out of 10 collected. One of them was transferred and ended in a biochemical pregnancy and 5 were suitable for cryopreservation. Three months after the first transfer a 10-cell stage cryo embryo was transferred in a spontaneous cycle at the 16th cycle day, leaving 4 cryopreserved embryos. Ultrasound at 7 weeks gestation revealed a dichorial twin pregnancy with a septum of 6mm. The crown rump lengths were 8.74mm and 8.77mm, both conform a pregnancy duration of 6 weeks and 6 days. Routine ultrasound scan at

19 weeks gestational age showed a severely growth retarded boy (p<3), and a girl with a normal growth pattern (p10-50). The growth retardation of the boy persisted throughout gestation and at 35+6 weeks gestational age the abdominal circumference of boy was below p3, whereas the girl showed an abdominal circumference in between p3 and p10, with an estimated fetal weight of 1,720 g and 2,200 g, respectively. Ultrasound scan of the flows in the artery cerebri media of the boy showed a high end diastolic flow, with brainsparing, indicative of placental insufficiency [4]. The patient developed preeclampsia (high blood pressure and proteinuria) from the 29th week onwards. Throughout gestation the cardiotocograms (CTG) were normal, the fetal movements were good, and the patient had no other clinical complaints, besides gastric acid complaints.

At 36+0 weeks gestation, after spontaneously ruptured membranes, the boy showed signs of fetal distress (a raised basal heart frequency of 175/min combined with decelerations), indicating a caesarean section. The girl was born in cephalic position with an Apgar score of 10 after 1 minute, with a body weight of 2,025g. The boy was born after version and extraction, with an Apgar score of 6 after 1 minute and 7 after 5 and 10 minutes, with a body weight of 1,475g. The boy was admitted to the NICU because of a need for mechanical ventilation and hypoglycemia. The girl developed a mild hyperbilirubinemia without a need for phototherapy. After 9 days the twins were transferred in good condition to a non-academic centre with body weights of 1,550g (boy) and 2,055g (girl). The mother was discharged in good clinical condition three days after the cesarean section.

Human leukocyte antigen (HLA) typing showed that both children are of maternal and paternal origin as both inherited one set of antigen from the mother and the other from the father. By coincidence, the father and mother share 5 HLA antigens (Figure 1A, blue). Macroscopically the placentas were separated (Figure 1B). Microscopically, the dividing fetal membrane showed two amniotic and two chorionic membranes with fused trophoblast (Figure 1C). Fluorescence in situ hybridization (FISH) staining clearly shows that the one of the membranes originates from the girl and the other from the boy (Figure 1D).

Discussion

Ultrasound scan around 20 weeks of gestation was compatible with dizygotic twins as the fetuses had different genders. After birth two separate placentas were identified (Figure 1B) and no vascular anastomoses were present. HLA typing demonstrates that the children are derived from two different oocytes of the same mother (Figure 1A). Histological examination of the dividing fetal membranes showed that this was a diamniotic, dichorionic membrane (Figure 1C). Both findings strongly support dizygosity. Possibility of transfer of two embryos, instead of one, or laboratory mishandling have been considered, but rejected in favor of natural fertilization as the most plausible hypothesis. Four fertilized oocytes are still cryopreserved. The embryo transfer took place in the patients' own cycle, the sperm characteristics were normal and the couple confirmed intercourse without contraception in the period around embryo transfer. An increased rate of monozygotic twin pregnancy is observed after the transfer of one blastocyte [5]. The assumption of monozygosity in cases of twin pregnancies of the same gender could be an overestimation of monozygosity as one of the twins might be conceived after natural conception. Therefore, we hypothesize that dizygotic twin pregnancy following transfer of one embryo occurs more often than is expected.

It is known that hysterosalpingography is of limited use for detecting tubal patency because of its low sensitivity; however its high specificity makes it a useful test for ruling in tubal obstruction [6]. The negative tubal patency test, even after several attempts at laparoscopy, was possibly caused by swollen tissue after myoma surgery. This case report shows that at least one oocyte was able to travel through one of the tubes.

The ideal outcome of IVF is a singleton pregnancy after single embryo transfer, since multiple gestations have a higher risk of complications for the mother and fetus [7]. The couple in this case was not advised against having intercourse without contraception. This case is also an example of a twin pregnancy showing a substantial increased risk of maternal and fetal complications since the mother developed preeclampsia and both children showed severe intra-uterine growth retardation. Interestingly, the HLA typing of the mother and boy is similar, which is possible since the mother and father share one set of HLA antigens. It is assumed that a certain level of HLA mismatches is necessary to develop an uncomplicated pregnancy. We hypothesize that because of the high level of HLA sharing, and therefore the low level of mismatches, the boy has a more severe growth retardation compared to the girl.

Based on this case, we suggest that couples should abstain from intercourse without contraception during an IVF procedure to prevent multiple gestations, which are related to higher maternal and fetal complications.

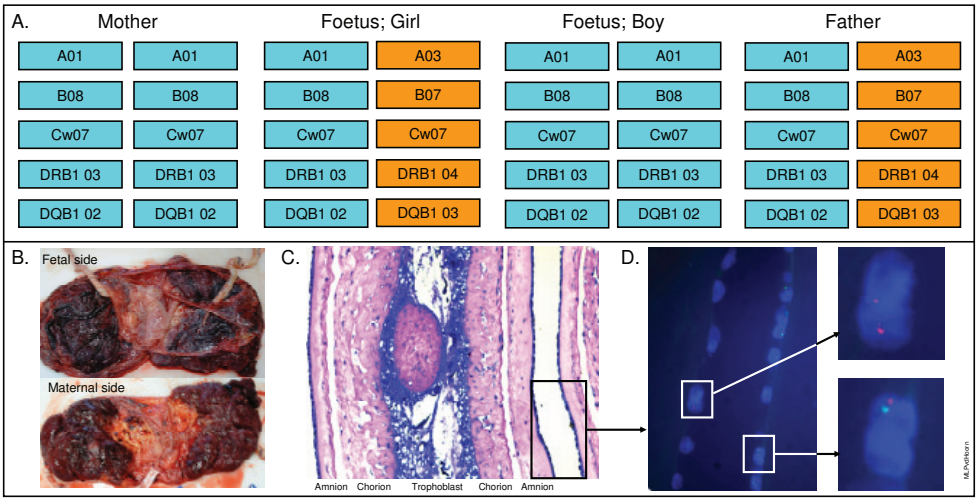

Figure 1 Four evidences strongly suggesting that the twin in this case report was dizygotic. HLA-typing of the mother, both children and father; macroscopic examination of the placenta; histology of the dividing fetal membrane; FISH staining of the X and Y chromosome. **A.** HLA typing of 5 HLA antigens shows that both children inherited one set of antigens from the mother. The other set of HLA antigens is inherited from the father. By coincidence, the father and mother share 5 HLA antigens (blue). **B.** Pictures of the placenta from the fetal side (upper picture) and from the maternal side (lower picture). These pictures show that two separate placentas were present, without vascular anastomoses. **C.** May-Grünwald-Giemsa staining of the dividing fetal membrane showing that the twin was dichorionic and diamniotic. **D.** FISH staining of two adjacent amniotic membranes. Blue stains the cell nucleus, red stains the X-chromosome and green the Y-chromosome. The enlargement clearly shows that one amniotic membrane originates from the girl and the other from the boy.

Case 2 Preeclampsia as complication in egg donation pregnancies: is there a different pathophysiological mechanism?

Introduction

In 1984 the first successful egg donation (ED) pregnancy was described [8]. The initial indication was premature ovarian failure. Nowadays, several indications leading to ED or embryo donations have increased the use of this technique worldwide. Pregnancy after ED is unique since the fetal genome can be entirely allogeneic towards the mother (Figure 2). It is suggested that therefore these pregnancies may result in more, mainly hypertensive, complications in comparison to spontaneously conceived or *in vitro* fertilization (IVF) pregnancies [9,10]. Remarkably, especially the mothers can be afflicted by these severe complications, while in contrast fetal parameters may be completely normal. This article describes a patient, pregnant after ED, suffering from severe preeclampsia, without growth retardation of none of the children.

Case

A 41-year-old primigravida, was pregnant of a dichorial, diamniotic twin pregnancy after ED. The oocytes were donated by the patient her sister. The indication for ED was idiopathic premature ovarian failure. The patient had no specific medical history. At a gestational age of 28 weeks, patient was referred to our academic center, due to a severe, early preeclampsia. Besides a progressive edema in the face and ankles, patient had headache complaints and was nauseous. Patient used methyldopa 500mg 3 times a day.

Patients blood pressure was 130/80 mmHg and there were signs of edema of the legs. From the start of pregnancy patient had gained 40 kilo's of weight. Her fluid balance was positive (400-800 ml per day), while her urine production was decreased: 25-30 ml/h. Reflexes of the limbs were normal.

Blood tests showed the following results (with references values in brackets): hemoglobin 6.9 mmol/l (7.5-10), hematocrit 0.34 l/l (0.37-0.47), thrombocytes $187x10^9$/l (150-450), creatinine 81 µmol/l (44-80), urea 10.7 mmol/l (2.5-7.5), uric acid 0.58 mmol/l (0.14-0.34), ASAT17 U/l (5-30), ALAT 7 U/l (5-34), LDH 355 U/l (100-248). Total protein loss was 2.6 g/24 h. Ultrasound showed of both children normal fetal movements and a normal biometry. Estimated fetal weight of the first child was 1371 grams (p50) and the second child 1275 grams (p30). Doppler ultrasound examination of the umbilical artery was comparable with a normal placenta perfusion.

As maternal blood pressure was acceptable and stable a conservative management was installed. To induce fetal lung maturation betamethason (12 mg 2 times within 48 hours) was given. During hospitalization edema became more prominent and the positive fluid balance increased up till 1 liter/day. Urine production was stable on average 30 ml/day. The blood test result for creatinine, ureum and uric acid became gradually more abnormal with maximum values of 92µmol/l, 14.7 and 0.72 mmol/l respectively. The total loss of protein increased up to 17 grams/24 hours, which made the decision to terminate pregnancy.

Delivery started spontaneously at gestational age 29+1. The first fetus (boy) was born in head position with a birth weight of 1363 grams (p50-75). By primary breech extraction a daughter was born with a birth weight of 1369 grams (p50-75). Both children had normal Apgar scores. Post partum patient recovery was quick. Urine production normalized and the peripheral edema disappeared. In one week 20 kilograms of body weight were lost and the kidney function became

normal again (creatinine 63 µmol/l, ureum 4.9 mmol/l and uric acid 0.4 mmol/l). Two weeks after delivery patient could leave hospital in good condition. Six weeks after delivery patient reported no complaints. Blood pressure was 125/75 mmHg without medication and no proteinuria. Both children were transferred to a non-academic hospital.

Discussion

In the literature several perinatal complications of ED pregnancies as compared to spontaneous and IVF pregnancies have been described. There is an increased incidence of early pregnancy complications (in particular blood loss), but late complications as well [11,12]. Reasons for these higher incidences of complications, although some of them might be explained by the higher incidence of multiplets, are still unidentified. Interestingly, the incidence of pregnancy induced hypertension is significantly higher if the donor of the egg is not related to the recipient, compared to a related donor [11].

HLA incompatibility

Complete HLA-incompatibility refers to a situation whereby the fetal genome is completely different as compared to the genome of the mother. This situation normally does not occur in natural conceived pregnancies. It is suggested that partner choice by women aims at an optimal possible number of HLA-matches and mismatches [12]. Sharing of too many HLA-molecules with the partner might be unfavorable, as this is hypothesized to be related to occurrence of preeclampsia [13,14]. This suggests that preeclampsia in ED pregnancies where the number of mismatches is increased might be based on a different pathophysiological mechanism, compared to preeclampsia in non-ED pregnancies. As shown in our patient, although an increased incidence of hypertensive complications in ED pregnancies has been reported, surprisingly no effect on placental perfusion or birth weight has been demonstrated [15]. The incidence of other perinatal complications as prematurity are similar to non donor IVF pregnancies [15].

Immune response

For an adequate immune reaction associated with normal implantation, maternal (allogeneic) immune recognition needs a certain level of HLA-incompatibility [16]. In ED pregnancies it is possible that this immunological response against fetal and placental tissue is inadequate, which may play a role in the development of specific hypertensive complications. This might be the pathophysiological background for of preeclampsia in ED pregnancies [11]. Indeed, at the fetal-maternal interface of the placentas of ED pregnancies an increased immunological activation and fibrin deposition are found, which resembles graft-versus-host disease after solid organ transplantation [17].

Activation of the immune system may lead to an increased production of certain cytokines and antiangiogenic factors. In a pilot study of pregnancy complicated by preeclampsia it was found that in ED pregnancies a higher amount of soluble fms-like tyrosine kinase (sFlt, an antagonist of the pro angiogenic vascular endothelial growth factor (VEGF)), and soluble endogline (sEng, a co receptor of transforming growth factor (TGF)-β) were found. Serum levels of these substances were determined in our patient (Table 1). Remarkably high levels of sFlt, sEng and TGF-β were found, compared to levels in uncomplicated spontaneously conceived pregnancies. These values are also found severe forms of preeclampsia and are suggested to possibly explain kidney disorders in preeclampsia. The source of sFlt could be, other than the placenta, maternal monocytes. Recent studies showed that monocytes in chronic kidney disease patients also produce sFlt [18]. Post partum the production of sFlt by monocytes will decrease and thereby the kidney function will return back to normal, as in our patient.

Cytokine	Function	Patient	Control
TGF-β (pg/ml)	Immune regulation	45506	158
sEng (ng/ml)	TGF-β co receptor	71	13
sFlt (pg/ml)	Anti-angiogenic VEGF antagonist Antagonist of pro- angiogenic molecules	14298	8396

Table 1 Cytokine levels. Level of specific cytokines in serum (tested by Luminex) of the patient discussed in this case compared with median levels of 51 uncomplicated pregnancies. TGF-β: transforming growth factor-β. sEng: soluble endoglin. sFlt: soluble fms-like tyrosine kinase. VEGF: vascular endothelial growth factor.

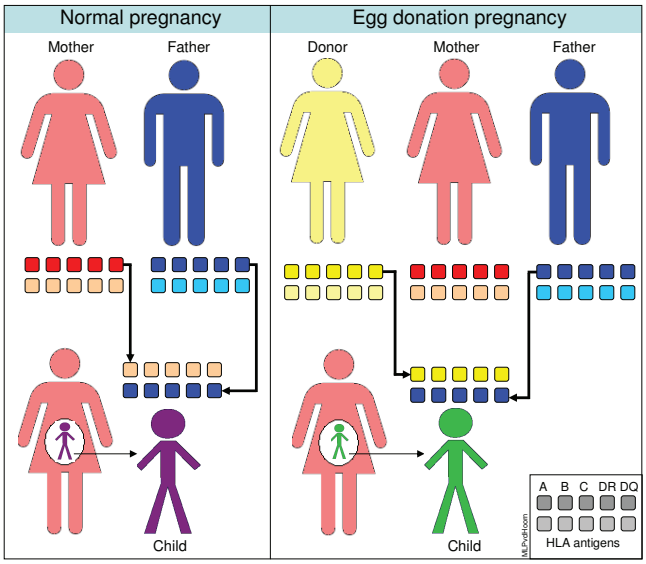

Figure 2 Schematic drawing of the inheritance of the most immunogenic HLA-antigens in a normal and ED pregnancy. A. In a spontaneously conceived (or non donor IVF) pregnancy the child inherits antigens of the father and antigens of the mother. The 5 most immunogenic HLA antigens (HLA-A, -B, -C, -DR and -DQ) are depicted in red for the mother and in blue for the father. The child inherits one set from the mother and one set from the father. Comparing the antigens of the child with the mother a maximum of 5 mismatches is possible. **B.** In an unrelated ED pregnancy no antigens from the mother are present in the fetus. The antigens of the donor are depicted in yellow and the antigens from the father in blue. The set of genes inherited by the child contains no antigens of the mother; therefore, a maximum of 10 mismatches is possible between the mother and the child in an ED pregnancy.

Conclusion

More research is needed for the understanding of underlying mechanism of preeclampsia in ED pregnancies. This might also give insight in the mechanism of occurrence of preeclampsia in non donor pregnancies. This knowledge can also be of significance for other areas of patient care, for example transplantation medicine.

Consideration

Case 1 describes a dizygotic pregnancy of concurrent IVF and spontaneous conception. Although this is an interesting and rare phenomenon, for this discussion the focus was on the development of preeclampsia. Since case 1 describes a dichorial diamniotic pregnancy, conceived by non donor IVF, this pregnancy can be considered as a control pregnancy for the second patient of this chapter. Case 2 is also a dichorial diamniotic pregnancy, however being conceived after ED. Both cases received hormonal treatment for hormonal treatment for endometrium preparation, however only patient 1 received hormonal treatment for oocyte retrieval. Interestingly, patient 1 developed preeclampsia combined with severe growth retardation, while patient 2 developed severe preeclampsia without fetal growth retardation. These findings are illustrating our hypothesis that preeclampsia in ED pregnancies might have a different pathophysiological mechanism, as explained in case 2.

The characteristics of case 1 and case 2 are summarized in Table 2. Figure 3 shows the birth weight of the four children born in the cases, plotted between references values.

	Case 1: IVF	Case 2: ED
Age	35 year	41 year
Gravidity	Primigravida	Primigravida
Choronicity	Dichorial diamniotic	Dichorial diamniotic
Infertility based on	Tubal obstruction	Premature ovarian failure
Onset preeclampsia	29 weeks	28 weeks
Medication	No medication	Methyldopa
Protein loss	0.36 g/24h	2.6 – 17 g/24h
Delivery	36+0	29+1
Mode of delivery	Cesarean section	Spontaneously
Birth weight and gender (percentiles)	1550 g (P<2.3) boy 2025 g (P5-P10) girl	1363 g (P50-P75) boy 1369 g (P50-P75) girl
Number of HLA mismatches	0 (boy) 5 (girl)	5 (boy) 5 (girl)
Placenta weight	980 g	640 g

Table 2 Characteristics of case 1 and case 2.

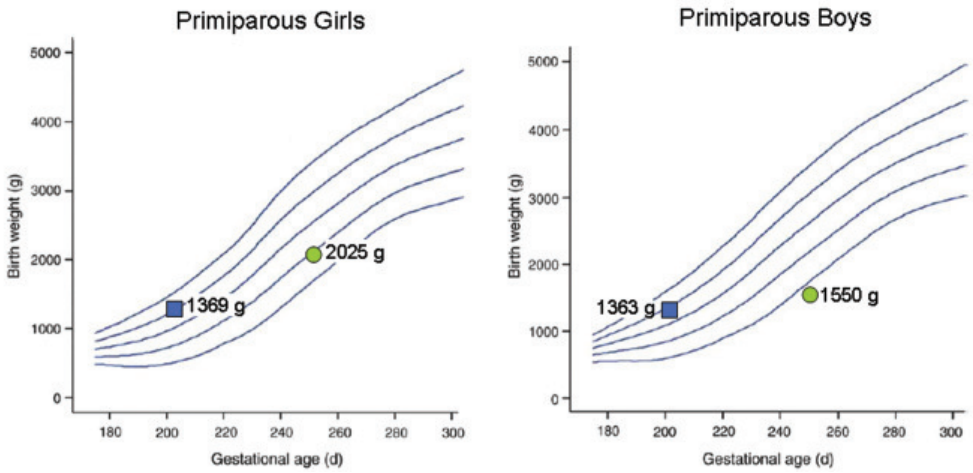

Figure 3 Reference curves (mean and 1 to 2 standard deviations) for boys and girls from primiparous women. The circle indicates case 1; preeclampsia in a non donor IVF pregnancy with severe growth retardation of both fetuses and the square indicated case 2; preeclampsia in an ED pregnancy with normal fetal birth weights. (Curves adapted from [19].)

In case 2 HLA typing of the mother and the boy are identical, which is possible since the mother and father share one set of HLA antigens. It is assumed that a certain level of HLA mismatches is necessary to develop an uncomplicated pregnancy. Partner choice by women seems to aim at an optimal number of HLA matches and mismatches [12]. We hypothesize that because of the high level of HLA sharing, and therefore the low level of mismatches, the boy is more severely growth retarded compared to the girl. Sharing of too many HLA molecules with the partner has also shown to be unfavorable, since this might also be related with the occurrence of preeclampsia [13,14]. In contrast, too many of the same HLA antigens between mother and child may as well be a disadvantage.

It is thought that preeclampsia is the consequence of an unsuccessful attack of the maternal non specific host defense on the implanting blastocyst, resulting in defective implantation resulting in a continuously stimulation of the maternal inflammation response [11]. Control of placentation has an immunological basis, with interaction between maternal and fetal genes. In some of the ED pregnancies there might also be a shorter pre-pregnancy exposure, via sperm, to non-maternal antigens, possibly leading to inadequate immunoprotection of placentation, eventually resulting in preeclampsia.

Further immunological involvement in the pathogenesis of preeclampsia is demonstrated by uterine NK cells and their relation with implantation. NK cells express KIR receptors; HLA is the most important ligand for these receptors. The combination of maternal (inhibitory) KIR AA genotype and fetal HLA-C2 is associated with an increased risk of preeclampsia [20]. The consequence of this interaction is that fetal HLA-C2 will only interact with an inhibitory KIR receptor, resulting in too much inhibition of uterine NK cells. It is thought that this interaction results in inadequate trophoblast invasion and insufficient remodeling of the spiral arteries, which is associated with the causation of preeclampsia. Since this combination has a disadvantage effect in evolution, the frequencies of these genotypes in populations have been investigated. Indeed, populations with a high frequency of the KIR AA genotype, have a low frequency of HLA-C2 and vice versa [20]. As commercial ED is not possible in the Netherlands, many women who are in need of ED go abroad. Hereby, such a population-protective effect might not be present, raising this as a possibility for the increased incidence of preeclampsia in ED pregnancies. The sperm of donors with a C1/C1 genotype is predicted to be safer than sperm of C2/C2 males, since the latter will always results in a fetus expressing C2 [21]. In the future it might become feasible to perform HLA-typing before IVF or ED, the combination of maternal KIR AA, fetal C2 and sperm donors with the C2/C2 genotype should be avoided in order to decrease the risk of preeclampsia. If the fetus has more C2 genes than the mother the risk of getting preeclampsia is two times higher (OR 2.09, 95% CI: 1.24-3.58, p=0.007) [21]. This shows that preeclampsia is not only explained by the combination of KIR genotype and HLA-C genotype; the genotype of mother and both children in case 1 was C1/C1. Even in the presence of the protective fetal phenotype, the patient did develop preeclampsia. In case 2 the mother was C1/C2 and both children were C2/C2. The maternal KIR typing is unknown. It is possible that preeclampsia in case 2 is partly caused by the 'dangerous' C2/C2 phenotype of the fetus.

In this chapter only 2 cases were described. Further investigation of preeclamptic ED placentas is essential to confirm our hypothesis that preeclampsia in ED pregnancies is based on a different pathophysiological mechanism. Because of a higher degree of antigenic dissimilarity, ED pregnancies represent an interesting model to study complex immunological interactions, as even in these pregnancies the allogeneic fetus is not rejected but tolerated by the pregnant woman. Understanding the fetus specific tolerance induction during pregnancy may lead to new insights for the induction of donor specific tolerance also in the transplantation setting.

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Egg donation pregnancy as an immunological model for solid organ transplantation

5

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Abstract

In egg donation (ED) pregnancies the fetus is allogeneic to the gestational carrier. During these ED pregnancies the mother has to cope with a higher degree of antigenic dissimilarity compared with spontaneously conceived pregnancies. At the fetal-maternal interface maternal cells and fetal cells come in close contact. Understanding the immune mechanisms at this fetal-maternal interface gives more insight into the question why the (semi-)allogeneic fetus is accepted and not rejected by the mother. The degree of antigenic dissimilarity in ED pregnancies is comparable with that in solid organ transplantations with HLA mismatched unrelated donors. Therefore, the immunologic interactions between mother and child in successful ED pregnancies may be relevant for the induction of immunological tolerance in solid organ transplantation.

Introduction

The fetus is a semi-allograft expressing both maternal (self) and paternal (non-self) genes. The placenta and fetal membranes are directly exposed to maternal tissue. Therefore during the accomplishment of uncomplicated pregnancy specific, local immune adaptations are necessary at the fetal-maternal interface. As in egg donation (ED) pregnancies the fetus can be fully allogeneic to the mother, ED pregnancies represent an interesting model to study complex immunologic interactions between the fetus and the pregnant women. During these ED pregnancies the mother has to cope with a higher degree of antigenic dissimilarity compared to spontaneously conceived pregnancies. Understanding immune mechanisms involved in successful ED pregnancies can possibly lead to new strategies for the induction of immunological tolerance in human leukocyte antigen (HLA) mismatched solid organ transplantations. To elucidate aspects of ED pregnancies as an immunological model for solid organ transplantation, knowledge of maternal mechanisms during spontaneously conceived pregnancies in the acceptance of the developing fetus and placenta is essential. In this review immunogenetic and immunological similarities between ED pregnancy and transplantation are discussed. In addition an overview of immunological aspects of spontaneous, uncomplicated pregnancy is given, showing why fetal tissues are immunologically tolerated in the maternal host environment.

Egg donation pregnancies

ED pregnancies are a result of in vitro fertilization (IVF) of an oocyte, donated by a related or, more commonly, by an unrelated donor. Hereby, neither of the fetal haplotypes will match with the gestational carrier. Increased knowledge in the field of assisted reproductive technologies, a more liberal interpretation of medical indications and social acceptance of the procedure has led to an ever increasing number of ED pregnancies. Clinically relevant complications in ED pregnancies, presumably related to the allogeneic nature of the fetus, occur more frequently. The literature reports on a higher risk of pregnancy induced hypertension, a higher incidence of cesarean sections, an increased risk of postpartum hemorrhage and more first trimester vaginal bleeding complications [1-5]. Although these maternal complications are higher in ED pregnancies compared to spontaneously conceived pregnancies, they are not associated with an increase in fetal and/or neonatal complications [3,4,6,7]. This suggests that downregulation of the maternal immune response preventing a detrimental maternal immunological response is possible, even in a completely allogeneic situation. Histological findings of ED placentas show some resemblance with a host versus graft rejection phenomenon as seen with solid organ transplantations [8]. Severe chronic deciduitis admixed with fibrinoid deposition has been observed in ED placentas compared with non donor IVF placentas [8]. Histological findings, as chronic deciduitis, found in the basal plate of the placenta where extravillous cytotrophoblast interfaces with the maternal decidua, are thought to resemble immune mediated placenta pathology.

Although the possible maternal complications in ED pregnancies are clearly described, relatively little is known on the underlying immune regulation in ED pregnancies. Research in this field will not only help us to understand the role of the immune system in ED pregnancies but may give insight into strategies to induce immunologic tolerance in HLA mismatched solid organ transplantations.

Blood transfusion

In ED pregnancy, the mother is exposed to foreign cells and antigens, a situation that has some resemblance to blood transfusions and organ transplantation (Figure 1). It is to be conceived that the downregulation of the maternal alloimmune response to the fetus during ED pregnancies needs more adaptation compared to a spontaneously conceived pregnancy. The degree of antigenic dissimilarity (reflected by the number of HLA mismatches) is in general higher in ED pregnancies compared to spontaneously conceived pregnancies. In the transplantation setting the degree of HLA compatibility between the donor and recipient is relevant for graft survival. More mismatches will lead to poorer graft survival [9]. Enhanced graft survival has been observed in kidney transplant recipients who prior to transplantation received a blood transfusion [10]. However, as discussed later, pretransplant blood transfusion can have different immunomodulatory effects as they either activate or suppress the immune system of the recipient.

Downregulation of the immune system by HLA-DR matched blood transfusions

Pretransplant allogeneic blood transfusion has a positive effect on kidney graft survival [10]: patients transfused with one HLA-DR matched transfusion (semi-allogeneic, a situation similar to a normal pregnancy) showed an enhanced kidney [11] and heart [12] transplant survival. No beneficial effect was seen for pretransplant blood transfusion with fully HLA-DR mismatched blood transfusions (a situation similar to ED pregnancies). In addition, HLA alloantibody formation was significantly higher after fully HLA mismatched transfusions compared to one HLA-DR matched transfusions [13]. The immune mechanism suggested to be involved in modulation of alloreactivity by blood transfusion might as well occur during conception and prior exposure to semen [14]. The shared HLA-DR allele is supposed to play a pivotal role in the downregulation of the immune response [15,16] as CD4+ regulatory T cells may recognize an allopeptide in the context of this self HLA-DR on the transfused blood cells. When this allopeptide is shared between the blood donor and organ donor, CD4+ T cells are capable of downregulating all activated T cells involved in graft rejection, leading to an enhanced graft survival. A similar mechanism may play a role during a normal pregnancy or during an ED pregnancy where the fetus shares the HLA class II allele with the mother. However, the situation is different in fully allogeneic ED pregnancies, where the fetus is completely HLA mismatched. It is to be expected that a stronger or different immune regulation is necessary to prevent rejection of the fully allogeneic fetus. Studies in mice demonstrate that the maternal T cell repertoire is aware of paternal antigens during pregnancy, but in healthy pregnancy reactive T cells do not mediate a detrimental anti-fetal immunity [17]. In humans, it has been shown that a distinct subset of HLA-DR+ regulatory T cells is involved in the induction of preterm labor and in the induction of organ rejection after transplantation [18]. All these studies suggest that a HLA-DR match play an important role in the induction of immunological tolerance. Since more HLA mismatches are inherent to ED pregnancies, one can imagine that the higher number of HLA-DR mismatches in ED pregnancies led to more complications. As the allogeneic fetus is able to survive nine months in the uterus, without any additional immunosuppressive medication as is needed in solid organ transplantation, it is likely that a very efficient local and peripheral immune regulation is responsible for such a successful ED pregnancy.

The role of antibody formation

Preceding organ transplantation a screening for HLA antibody is performed as in organ transplantation preformed donor specific HLA antibodies are associated with (hyper) acute graft failure. It is well known that blood transfusions are associated with the induction of HLA alloantibodies. The degree of HLA mismatches determines the immunization; HLA alloantibodies

are formed more frequently after transfusion of donors with two HLA class II mismatches compared to one HLA class II mismatch [11]. Since more than 30% of the women produce HLA antibodies directed against the paternal HLA antigens of the fetus already before delivery [19], it is commonly assumed that during pregnancy the formation of these antibodies is a harmless phenomenon. However, recently it has been shown that HLA alloantibodies are associated with a reduced chance of live birth in patients with recurrent miscarriages [20]. Only 41% of HLA-antibody positive pregnant recurrent miscarriage patients had a live birth compared to 76% of HLA-antibody negative recurrent miscarriage patients. Furthermore, placental abruption is increased in patients with a higher prevalence of HLA class I antibodies whereby the presence of these antibodies possibly serve as a marker for the activation of maternal immune response against the fetus [21]. It remains to be elucidated which role HLA alloantibody formation plays in pregnancy complications. They may either play a role in the etiology or may be a parameter associated with a detrimental immune response by maternal immune cells. Similarly, the induction of donor specific HLA antibodies after kidney transplantation is associated with a higher incidence of chronic rejection [22], although patients with donor specific HLA antibodies may have an excellent graft function for many years [23]. Also here, it is unclear whether the antibodies are the direct cause of the clinical problems.

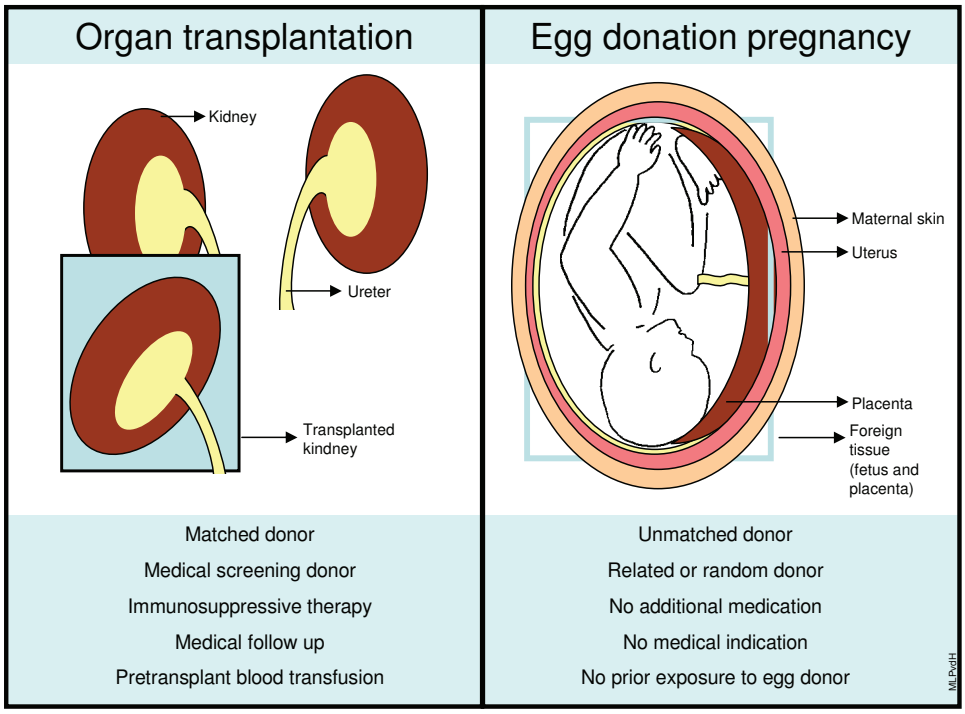

Figure 1 Organ transplantation versus egg donation pregnancy. Schematic overview of the differences in the medical consequences between solid organ transplantation and egg donation pregnancies, while in both situation antigenic dissimilarity is present.

Preeclampsia

Preeclampsia is a syndrome characterized by the newly onset hypertension and proteinuria after 20 weeks of gestation, which disappears after delivery. Immunologic abnormalities, similar to those observed in allograft rejection, have been observed in preeclamptic women [24]. For example, the most dangerous form of preeclampsia is HELLP (hemolysis, elevated liver enzymes and low platelets) which is in addition to hypertension characterized by elevated liver enzymes and low platelet counts and this disease may lead to multi organ failure. Cytopenias and multi organ failure are as well reported in organ transplant rejection [25]. Women with preeclampsia have an increased level of circulating fetal DNA in comparison to controls [26]. Also after organ transplantation a substantial degree of donor lymphocyte chimerism may be present in the recipient [27]. Furthermore, as a host versus graft immune response is stopped by removal of the transplanted organ, also in preeclampsia a rapid maternal recovery occurs after removal of the placental products [28].

Preeclampsia might be the consequence of an unsuccessful attack of the maternal innate immune system towards the implanting blastocyst, eventually results in defective implantation which may lead to stimulation of the maternal inflammatory response [29]. In ED pregnancies there is only a short duration of exposure to non-maternal antigens, which could lead to an altered or inadequate immunoprotection of placentation, eventually resulting in preeclampsia. The incidence of preeclampsia in pregnancies conceived with assisted reproductive technologies and thus related with potentially less exposure of sperm, is indeed higher [30].

Uterine NK cells are supposed to play a pivotal role during implantation and preeclampsia. NK cells express killer immuno-globulin like receptors (KIR) to which HLA is able to bind. KIR receptors can be divided in inhibiting (AA) and activating (BB) KIRs. The combination of maternal KIR AA genotype especially with an HLA-C2 is associated with an increased risk of preeclampsia [31]. HLA-C2 has a much stronger binding with inhibiting KIRs than with activating KIRs. The interaction of maternal uNK cells with an AA genotype with a fetal C2 allele expressed on placental trophoblast tissue will possibly result in an inhibition of uterine NK cells. It is thought that the inhibition of uterine NK cells results in inadequate trophoblast invasion into the spiral arteries, which will eventually lead to preeclampsia. Such a KIR AA – HLA-C2 combination is supposed to have an evolutionary disadvantage. In populations with a high frequency of KIR AA, a low frequency of HLA-C2 and vice versa was found [31]. If the fetus has a HLA-C2 gene, the risk of getting preeclampsia is two times higher (OR 2.09, 95% CI: 1.24-3.58, p=0.007) [32]. Of course, preeclampsia not based on this KIR AA – HLA-C2 combination should not be excluded and other mechanisms as well play a role in the pathogenesis of preeclampsia.

Immunology in complicated ED pregnancies

Success of egg donation procedures

The European Society of Human Reproduction and Embryology (ESRHE) publishes annual data on assisted reproductive technology. After ED, 5516 clinical pregnancies were reported resulting from 12685 embryo transfers, giving a clinical pregnancy rate of 43.5%. The mean birth rate of these embryo transfers was 27.2% (n=3448) [33]. This means that a total of 71.8% of all embryo transfers after ED do not result in a continuing pregnancy. For IVF the pregnancy rate was 32.4% (31665 pregnancies from 96572 embryo transfers). At a first glance this higher pregnancy rate in ED pregnancies compared to IVF pregnancies is surprising. However, the reason to perform ED is ovarian failure and, as there are no uterine abnormalities, ED might be more successful

compared to IVF pregnancies, in which there may be an underlying and unknown mechanism responsible for implantation failure. Unsuccessful embryo transfers in ED procedures, resulting in miscarriage may be related to a non optimal HLA-match between the egg donor, sperm and gestational carrier. Surprisingly, nearly 30% of all embryo transfers in ED pregnancies result in a continuing pregnancy, resulting in a mother who carries a completely allogeneic fetal allograft. A number of complications have been described, of which some might be due to the allogeneic nature of the fetus.

Taking the more vigorous immune response in ED into account, it could be of importance to perform HLA-typing of the egg donor and recipient in order to select haplo-identical combinations which would be more similar to spontaneously conceived pregnancies. However, this suggestion has to be evaluated in well designed studies. HLA-typing could then be performed before fertilization of the donated egg, whereby the combination of maternal KIR AA – fetal HLA-C2 and sperm donors with the C2/C2 genotype should be avoided in order to decrease the incidence of preeclampsia. In the Netherlands commercial and anonymous egg donations are forbidden by law. ED based on a non-commercial basis is allowed, but infertile women should find their egg donor by themselves. In several cases this might be a family member who is donating an oocyte, but many women, who want to make use of the opportunity of ED to get pregnant, go abroad for the treatment. It might be useful to perform an international study on the relevance of HLA/KIR matching and the success rate of ED pregnancies. The sperm of donors with a C1/C1 genotype is predicted to be safer than C2/C2 males, since this certain results in a fetus expressing C2 [32].

The underlying immunogenetic differences between donor and recipient in solid organ transplantation and ED pregnancies are similar and form the basis of their most important complications (graft rejection and preeclampsia). However, the medical regimes for women pregnant via ED or for transplantation patients are totally different. For solid organ transplantation the donor requires an extensive screening and the patient receives immunosuppressive therapy besides a comprehensive medical follow up. In contrast, an ED pregnancy occurs mostly via an unknown donor, the pregnant women does not receive extra medical care, and does not use any additional medication (Figure 1). ED pregnancies results in an immunologically unique situation and until now the immunological mechanism behind the success of these pregnancies remains unclear. It remains to be established whether immunological principles additional to those present in spontaneously conceived pregnancies are operating in the ED fully allogeneic pregnancies.

Uncomplicated pregnancy and immunology

Placental development in an allogeneic environment

The development of the placenta is essential for fetal development and growth during uncomplicated pregnancy as it prevents rejection of the fetal allograft, and exerts metabolic and endocrine functions. The placenta develops from fetal derived cells and is able to anchor in the maternal myometrium. Several immune escape mechanisms are necessary to enable growth of the immunogenetically foreign fetal cells into the maternal uterine lining. The blastocyst consists of the inner cell mass, which will form the embryo, and the trophoblast, which will form the placenta and fetal membranes. During implantation the blastocyst invades the maternal uterine epithelium (Figure 2). Placental progenitor stem cells develop into invasive extravillous trophoblast or into non-invasive trophoblast cells covering placental villi. Villous trophoblast can be classified in two types; the mononuclear cytotrophoblast and, after fusing, the multinuclear syncytiotrophoblast layer overlying the villi is formed. The syncytiotrophoblast, surrounded by maternal blood, is in direct contact with maternal immune cells. Extravillous trophoblast invades

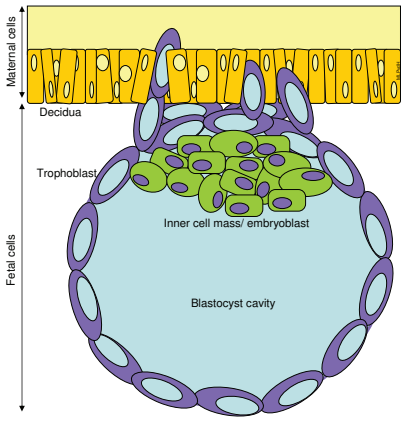

Figure 2 Implantation of the blastocyst in to the maternal endometrium. The genetically different blastocyst invades in to the maternal endometrium. Several immunological escape mechanisms are necessary for the fetal cells to be tolerated by the maternal immune cells, as depicted in detail in figure 3.

the maternal decidua and myometrium and is thereby responsible for anchoring the placenta. Maternal endothelial cells in the spiral arteries are replaced by endovascular trophoblast cells originating from extravillous trophoblast. It is also crucial for the supply of oxygen and nutrients to the fetus by changing the maternal vascular system. A balance of this invasion is very important; the cells need to invade enough for the anchoring and for receiving nutrients; on the other hand over-invasion of trophoblast cells has to be limited to protect the mother.

Fetal defense mechanisms

Fetal trophoblast cells are the crucial cell population in the placenta which protects the fetus from destruction by the maternal immune system. Villous cytotrophoblast is the inner layer of the villous surface epithelium. Villous syncytiotrophoblast is the superficial layer facing the intervillous space. Villous trophoblasts lack HLA expression and do not provoke an allogeneic immune response by circulating maternal T cells. Until now it is unexplained how these cells circumvent a maternal immune attack by e.g. peripheral NK cells, which normally would destroy cells without any HLA expression. The remaining trophoblast cells, the extravillous trophoblast, migrate into the maternal decidua and are the dominant cell type needed for the development of all nonvillous parts of the placenta. Extravillous trophoblast does not express HLA-A or -B, but does express HLA-E, -F, -G and -C [34], which serve as ligands for leukocyte inhibitory receptors. The consequences of these interactions include activation of pathways in natural killer (NK) cells and macrophages that interfere with the killer functions of these cells [34-36]. HLA-G has potent immunomodulatory functions [37], whereas HLA-C and -E have shown to elicit an allogeneic immunomodulatory response by maternal NK and T cells [38]. Several other immunomodulatory mechanisms have been postulated to contribute to successful pregnancy. Antigen presenting cells express a membrane bound or soluble form of HLA-G, which can activate the Fas/Fas ligand pathway resulting in destruction of activated T cells [39]. HLA class II molecules are not presented by trophoblast cells as the HLA class II transactivator (CIITA) is not expressed [40]. Furthermore, the B7H1 protein, a co-stimulatory molecule of the B7 family, is expressed on syncytiotrophoblast, which leads to inhibition of lymphocytes circulating in maternal blood [41]. In addition trophoblast cells contain indoleamine 2,3-dioygenase (IDO), an inhibitor of tryptophan metabolism; this may inactivate T cells since they reduce tryptophan, required for T cell activation [42]. TNFα, Fas ligand and TRAIL are ligands identified in or on human trophoblast cells which are able to support the pregnancy host defense by supporting maternal and/or fetal antibody production [43-45]. Th2 cytokines, produced at the maternal-fetal interface, can inhibit Th1 responses, improving fetal survival but impairing responses against some pathogens [46].

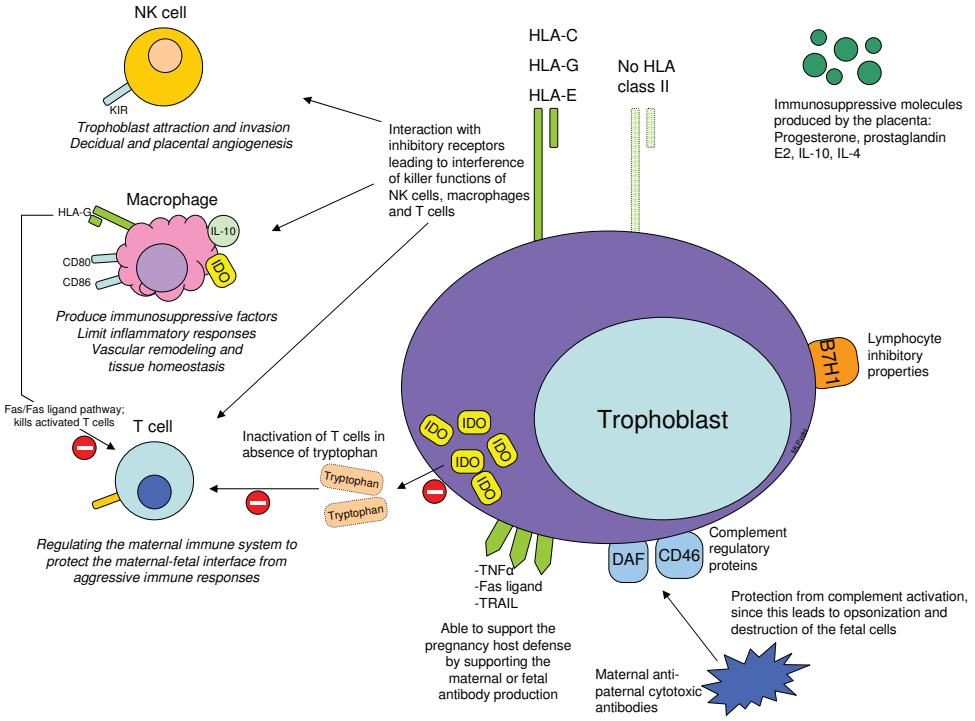

Figure 3 Defense mechanisms of trophoblast and maternal cells. Schematic overview of defense mechanisms of a trophoblast cell and maternal leukocytes during pregnancy at the fetal-maternal interface. Abbreviations: natural killer (NK), killer immuno-globulin like receptor (KIR), indoleamine 2,3-dioygenase (IDO), human leukocyte antigen (HLA), decay accelerating factor (DAF), tumor necrosis factor (TNF) and TNF-related apoptosis-inducing ligand (TRAIL).

The human placenta produces immunosuppressive molecules as progesterone, prostaglandin E2, and anti-inflammatory cytokines as IL-10 and IL-4 [35,47]. Finally, trophoblast cells express complement regulatory proteins, which are important to protect the fetal cells from complement dependent destruction [48]. Complement inhibition is required in normal pregnancy and uncontrolled activation at the maternal-fetal interface that leads to bad pregnancy outcomes [49]. All these mechanisms, summarized in Figure 3, maintain the immunosuppressive environment in the pregnant uterus and in this way, and possibly by other still undefined mechanisms, the (semi-)allogeneic fetus is capable to survive during its nine months housing in the uterus.

Maternal defense mechanisms

Multiple strategies are used by trophoblast cells, including altered HLA expression, synthesis of immunosuppressive molecules, and expression of high levels of complement regulatory proteins that may protect the embryonic tissues from destruction by maternal anti-paternal alloantibodies and T cells. Nevertheless, maternal leukocytes are potentially capable to elicit an alloimmune response since syncytiotrophoblasts and circulating syncytiotrophoblasts micro particles may come directly in contact with maternal immune cells [50].

During early pregnancy, the uterus seems to be immune compromised as T and B cells are hardly present. Macrophages and NK cells interact with the trophoblast cells. Decidual NK cells are different compared to peripheral NK cells. Decidual NK cells express perforin, granzyme A and

B but, unlike peripheral NK cells, they have a reduced cytolytic activity to HLA class I negative targets [51], secrete proteins with immunomodulatory potentials [52] and produce angiogenic factors like vascular endothelial growth factor and placental growth factor [53]. Decidual NK cells may recognize fetus HLA-C1 and HLA-C2 by the expression of KIR. Macrophages are antigen presenting cells which are the second most numerous type of leukocytes in the human decidua [54]. Macrophages are involved in both the innate and the adaptive immune system and consist of different functional subpopulations. Some macrophages promote inflammation by production of inflammatory molecules during an innate immune response and, as part of the adaptive immune system are able to present antigens to T cells. Others may have a role in immunosuppression by the expression low levels of costimulatory molecules CD80 and CD86 and the expression of indoleamine 2,3-dioxygenase, both preventing T lymphocyte activation [55]. The number of decidual T cells increases during pregnancy, starting with 5-20% of all CD45+ decidual lymphocytes in early pregnancy, till 40-80% at term [56]. Decidual T cells encompass a very heterogenic subset of T cells that include activated CD4+ and effector memory type CD8+ T cells. These activated T cells are found together with T cells subsets that are capable to suppress the decidual lymphocyte response [57]. T cells are in close contact with fetal trophoblast cells in the decidua; however they do not attack the non villous trophoblast cells, since trophoblast lack HLA class Ia expression. Fetus specific regulatory CD4+CD25bright T cells are present in human decidua in higher numbers compared to peripheral maternal blood [57], suggesting an important role for these cells at the fetal-maternal interface. It has been shown that fetus specific CD4+CD25bright T cells are recruited to the maternal decidua where they are able to suppress the local immune response [58]. T cells are able to produce a variety of type 1 and type 2 cytokines and thereby may contribute to the local regulation of the fetus-specific responses within the decidua. Also specific CD8+ T cell subsets, which do not express perforin and have a reduced expression of granzyme B, are more present in decidual tissue [59]. These cells also express KIR receptors which are then able to communicate with HLA-C expressed on trophoblast. The properties of these CD8+ T cells suggest that they may play a role in immune regulation at the fetal-maternal interface. Fetus specific immunological tolerance during pregnancy depends on a very complex network of cytokines, complement, hormones, immune and non-immune cells. Acceptance is not simply based on the consequence of a balance between the type 1 (associated with abortion) and type 2 (associated with successful pregnancy) cytokines, since many cytokines are pluripotent. However, in an uncomplicated pregnancy the child is able to survive in the semi-allogeneic environment and the mother accepts the semi-allograft. ED pregnancies reflect an extreme immunologic challenge, in which the fetal genome is immunogenetically fully allogeneic to the mother.

Conclusion

In ED pregnancies the fetus is allogeneic to the gestational carrier. This creates an interesting immunological paradox. The fetus is accepted by the mother although being immunogenetically completely unrelated to the mother (unless the egg is donated by a relative). In solid organ transplantation the same immunogenetic dissimilarity is present; however immunosuppressive drugs are unavoidable to maintain the graft. Resemblances between graft rejection and pregnancy complicated by preeclampsia are clearly present. Multiple immunomodulatory strategies are used by trophoblast cells in the placenta to avoid rejection, including altered HLA expression, synthesis of immunosuppressive molecules, and expression of high levels of complement regulatory proteins. We hypothesize that in ED pregnancies these immunomodulatory strategies lead to an active downregulation of the alloimmune response and as a consequence to acceptance of the fetal allograft. Knowledge of the immune mechanism, leading to successful ED pregnancy might be useful for future strategies to induce immune tolerance in solid organ transplantation.

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Clinical and immunologic aspects of egg donation pregnancies: a systematic review

6

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Abstract

Background: Egg donation (ED) makes it possible for subfertile women to conceive. Pregnancies achieved using ED with unrelated donors are unique, since the entire fetal genome is allogeneic to the mother. The aims of this review were to evaluate the consequences of ED pregnancies and to place them in the special context of their atypical immunologic relationships.

Methods: This review comprised an online search of English language publications listed in Pubmed/Medline, up to January 29th 2010. Seventy-nine papers met inclusion criteria. Using the literature and the authors' own experience, the relevant data on pregnancy outcome and complications, placental pathology and immunology were evaluated.

Results: Multiple studies document that ED pregnancies are associated with a higher incidence of pregnancy-induced hypertension and placental pathology. The incidence of other perinatal complications, such as intrauterine growth restriction, prematurity, and congenital malformations, is comparable to conventional *in vitro* fertilisation. During pregnancy, both local and systemic immunologic changes occur. In ED pregnancies these changes are more pronounced. There is almost no information in the literature on the long-term complications of ED pregnancies for the mother.

Conclusion: ED pregnancies have a higher risk of maternal morbidity. Due to the high degree of antigenic dissimilarity, ED pregnancies represent an interesting model to study complex immunologic interactions, as the fully allogeneic fetus is not rejected but tolerated by the pregnant woman. Knowledge of the immune system in ED pregnancies has broader significance, as it may also give insight into immunologic aspects of tolerance in solid organ transplantation.

Introduction

The first successful pregnancy achieved after egg donation (ED) was reported in 1984 [1]. Since then, thousands of pregnancies after ED have occurred worldwide. The original indication was premature ovarian failure [2,3]. More recent indications include advanced maternal age, diminished ovarian reserve, secondary infertility following treatment of childhood malignancies [4], multiple failed *in vitro* fertilisation (IVF) attempts [5], and maternally inherited genetic abnormalities [6]. Infertile women who do not produce euploid embryos also depend upon ED to achieve a successful pregnancy.

Eggs obtained from a suitable donor, either provided by relatives or via independent, sometimes for profit organizations [7], are fertilised with sperm of the recipient's partner or donor and the resulting embryos are transferred into the recipient's uterus. Some pregnancies achieved using ED are unique, since the entire fetal genome is allogeneic to the mother. Therefore, ED pregnancies represent an interesting model to study complex immunologic interactions between the fetus and the pregnant woman. Despite a continued increase in the number of ED pregnancies, relatively little is known about the underlying biology and long-term complications of this approach. Similar immunologic interactions exist in surrogate gestations, in which biological motherhood is achieved without pregnancy by transferring fertilised eggs to the uterus of a second woman. This treatment is used for women without a functioning uterus, or in women for whom pregnancy would be life-threatening [8].

Delaying childbirth and the resulting demand for infertility treatment have resulted in ~1% of United States (US) infants being conceived through assisted reproductive technologies [9]. Currently about 10% of the IVF cases in the US use ED [10]. This has increased the demand for the availability of oocyte donors; in the US more than 100,000 women have donated their oocytes [11]. In Europe, a recent report showed a total of 11491 egg donations [12].

ED was initially developed as a therapy for young women with premature ovarian failure, rather than as a means of overcoming the age-related decline in fertility. However, age-related infertility is now one of the most common reasons to use ED, especially in women over 40 [13]. The data suggest that fertility depends on oocyte age and quality and less on uterine age [14-16]. Some studies report that ED in women of advanced maternal age is as successful in establishing pregnancy as in younger recipients [17-20]. This would suggest that endometrial receptivity is unaltered by age [13,17]. However, in the late 40s and beyond, the success rate of ED starts to decline, so there are likely to be as-yet undiscovered factors that are affected by maternal age [14,15,21]. Advanced maternal age is almost always inherent to ED; thus it will therefore be a confounding factor in research studies of ED.

Obesity [22], an endometrial thickness of < 8mm, and the need for the use of GnRH analogue to down-regulate the pituitary before endometrial priming negatively influences pregnancy rates [23]. In contrast, high birth rates have been observed in frozen-thawed embryo replacement cycles in which embryos are derived from cycles that used GnRH analogues [24]. Besides the recipient's mid-cycle endometrial thickness, the quality of the transferred embryos is also important for a successful pregnancy [25-27].

Methods

The aims of this systematic review were to evaluate the consequences of ED pregnancies and to place the findings in the literature in the special context of their atypical immunologic relationships in ED pregnancies.

A search in PubMed, using the Medical Subject Headings (MeSH) terms 'oocyte donation' and 'egg donation', in combination with 'pregnancy outcome' or 'pregnancy complications' or 'immunology' or 'placenta' was performed. English language was used as a limit. Time was not limited but the search was completed on January 29th 2010. The titles and abstracts of the resulting articles were scanned and evaluated by the first, second and last authors (M.L.P.H, E.E.L.O.L. and S.A.S). Inclusion criteria were original and review articles that focused on current knowledge in ED pregnancies regarding pregnancy outcome and complications, placental pathology, and immunologic aspects. In addition, some background articles on reproductive and transplantation immunology were included. Exclusion criteria were: case reports, letters, and articles with an exclusive focus on ethics of ED. The main search identified 505 potentially relevant studies. Figure 1 shows the flow chart, which led to the final 79 references included in the review.

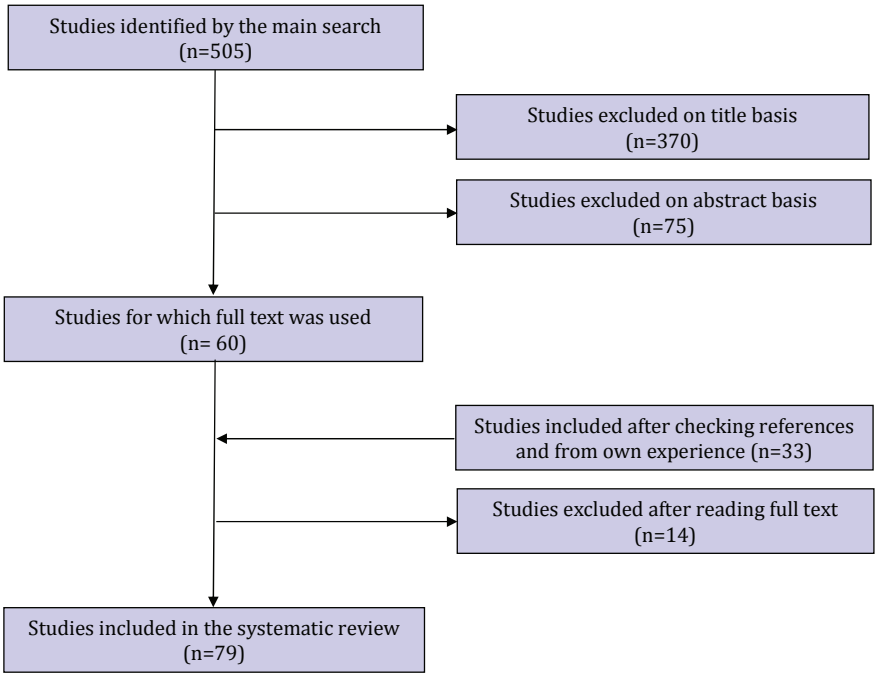

Figure 1 Flow chart depicting selection of articles for systematic review.

Consequences of egg donation pregnancies

Many studies of ED pregnancies have focused on perinatal complications, such as preeclampsia, the mode of delivery, and immediate neonatal problems, such as prematurity. In addition, ethical and medical concerns have been raised regarding the effects of treatment on the donor [11]. With regard to the recipient, most of the emphasis has been on short-term complications of pregnancy, because of the higher incidence of both early and late obstetrical problems. The reason for the higher incidence of complications in ED pregnancies is unclear from the literature reviewed.

Maternal complications

ED enables women of advanced age to achieve successful pregnancies. However, advanced maternal age leads to potential medical and obstetric complications. Pregnant recipients above the age of 40 are at an increased risk for gestational diabetes, preeclampsia and thrombophlebitis [28]; above the age of 45 they are at an increased risk of hypertension, proteinuria, premature rupture of membranes, second- and third trimester haemorrhage, preterm delivery and lower mean infant birth weights [15,29]. One study that corrected for maternal age and multiple gestation concluded that women who conceived with donor oocytes remain at high risk for preterm labor, preeclampsia, and protracted labor, requiring caesarean section delivery [30]. The rate of caesarean section deliveries in ED pregnancies is increased compared to spontaneous conceptions, and is reported to range from 40-76% of cases [4,5,18,31-35].

Pregnancy-induced hypertension

ED pregnancies are associated with a higher than expected incidence of pregnancy-induced hypertension (PIH), ranging from 16-40% of cases [5,31-34,36-39]. This is most likely due to a higher incidence of placental pathology [6]. It has been suggested that the increased rate of hypertension in ED pregnancies is related to advanced maternal age, nulliparity and ovarian failure [6], since these factors are associated with multiple obstetric complications [40]. However, a study by Sheffer-Mimouni *et al.* found that these factors were not independent risk factors for PIH [33]. They concluded that the higher incidence of PIH in ED pregnancies is due to an altered immune response. In another report, an increased risk for PIH was observed in women with ED pregnancies in women < 35 years or > 40 years of age [41].

In the studies above the control groups were spontaneously conceived pregnancies. Since IVF pregnancies are associated with more obstetric complications than naturally conceived pregnancies [42], they represent a more appropriate control group to examine the consequences of ED. Wiggins *et al*. found a 3-fold increased incidence of hypertensive complications in ED compared to standard IVF pregnancies (26% vs. 8%, respectively, p=0.02) [39]. For nulliparous women this difference was even more significant, with 37% of the ED group and 8% of the standard IVF group affected by hypertension (p<0.003). Multiple logistic regression analysis in nulliparous patients showed an odds ratio of 7.1 (p=0.019). In singleton and twin pregnancies the same effect was found (OR: 4.9, p=0.017). Maternal age was not an added risk factor for the development of PIH (OR: 1.0) [39]. Interestingly, the incidence of PIH appears to be significantly higher if the oocyte donor is unrelated to the recipient (20% vs. 3.7% for standard IVF, p=0.03), versus a related, sibling donor (8% vs. 3.7% for standard IVF, p=0.31) [43]. This study retrospectively analyzed 61 ED pregnancies that were classified into two subgroups according to the relationship between the ED and recipient, and 127 non donor IVF pregnancies. The groups were matched for age, parity and number of fetuses. This study is the only one that has specifically examined the immunogenetic origin of the egg and its relationship to complications of pregnancy. These data suggest that PIH is more frequent with an immunologically unrelated donor.

Bleeding

A possible result of the unique, non-physiological immunologic relationship between the fertilised oocyte and the maternal decidua is shallower placental invasion [44,45]. The higher incidence of bleeding complications in the first trimester could be related to this insufficient placentation. On the other hand, excessive invasion might result in more postpartum haemorrhage in ED pregnancies as a result of placenta praevia or abnormal placentation [33].

The incidence of first trimester vaginal bleeding is increased in ED pregnancies, ranging from 12-53% of cases [6,31,34]. Significant blood loss is estimated to occur in 43-53% of first trimester cases [33,34] and 6% of second trimester cases [6,33]. The incidence of first trimester bleeding is

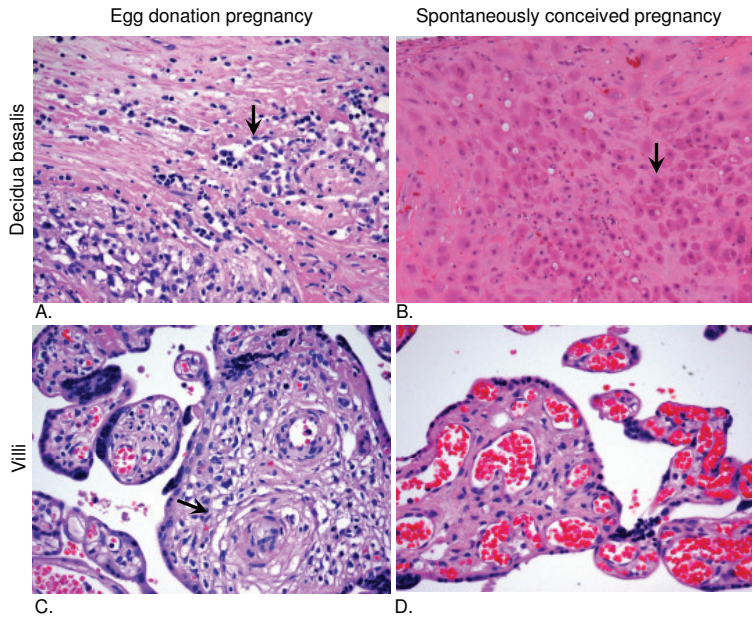

Figure 2 Photomicroscopic images from ED and spontaneously conceived pregnancies placentas. (H&E stained sections, original magnification 400X). A. Decidua basalis of ED pregnancy placenta with deciduitis illustrated by the infiltration of mononuclear cells (arrow). **B.** Normal decidua basalis from a spontaneously conceived pregnancy with normal decidual cells (arrow). **C.** Villi from an ED pregnancy placenta. The stromal cellularity is increased by an infiltrate of mononuclear cells (arrow). **D.** Villi of a spontaneously conceived pregnancy placenta.

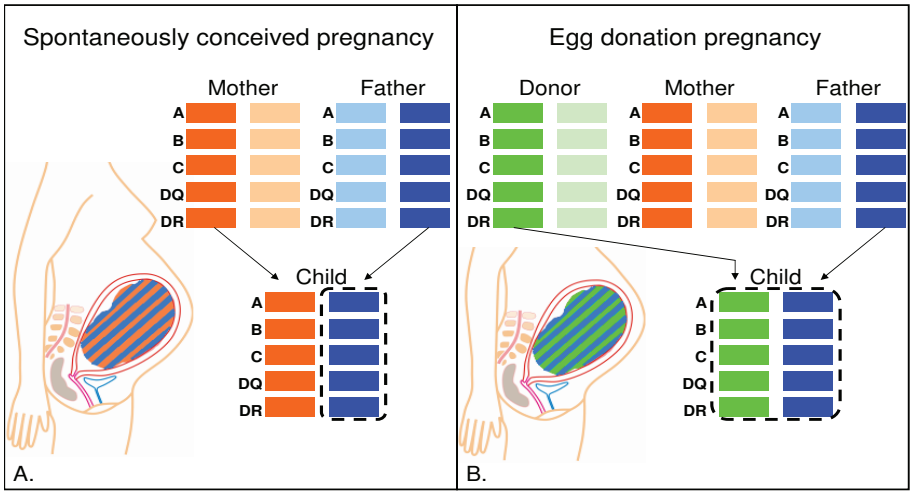

Figure 3 Schematic drawing of the inheritance of the most immunogenic HLA-antigens in a spontaneously conceived and an ED pregnancy. A. In a spontaneously conceived (or non donor IVF) pregnancy the child inherits antigens of the father and antigens of the mother. The 5 most immunogenic HLA antigens (HLA-A, -B, -C, -DR and -DQ) are depicted in orange for the mother and in blue for the father. The child inherits one set from the mother and one set from the father. Comparing the antigens of the child with the mother a maximum of 5 mismatches is possible (dashed line). **B.** In an unrelated ED pregnancy no antigens from the mother are present in the fetus. The antigens of the donor are depicted in green and the antigens from the father in blue. The set of genes inherited by the child contains no antigens of the mother, therefore, a maximum of 10 mismatches is possible between the mother and the child in an ED pregnancy (dashed line).

substantially higher if compared to standard IVF pregnancies [34] and second trimester bleeding is higher if compared to the spontaneously conceived population (< 1%) [46]. It has been assumed that more bleeding complications are associated with multiple implantation sites and early fetal loss [47]. However, in ED cases in which only two oocytes per cycle are transferred, the frequency of bleeding still remains high [34]. Other explanations, such as endometrial preparation therapy, have been suggested, but a possible relationship between various steroid replacement regimens and first trimester bleeding is difficult to assess.

Long-term consequences

The study of the trafficking of intact fetal cells into the maternal circulation (fetal cell microchimerism) is relevant to ED pregnancies, because it is not yet known if these circulating fetal cells play a role in establishing or maintaining tolerance to the conceptus. This merits further investigation. Furthermore, the consequences of the persistence of foreign circulating fetal cells for the mother's long-term health are currently unknown. In one study, however, allogeneic male fetal cells were shown to persist for up to 9 years in the circulation of healthy post-partum women who conceived using egg donors and delivered male infants [48]. The implications of becoming microchimeric with an unmatched population of fetal progenitor cells are an area for future research.

ED conception is often hidden from the mother's and the baby's medical records, so correlations between ED and specific adverse outcomes are difficult to make. In approximately 40-50% of the cases the fact that it was an ED pregnancy is never disclosed to the child or other family members [49]. The literature search revealed no studies evaluating long-term effects of ED for the mother. Long-term outcome studies are therefore warranted [50].

Fetal and neonatal complications

In most studies that assessed the obstetrical outcome after ED relatively little has been reported on fetal and/or neonatal complications. Elevated risks (relative to the general population) are primarily related to the higher incidence of multiple gestation [6,51]. The incidence of intrauterine growth restriction is also not increased compared to the general population [34]. The incidence of preterm deliveries in ED singleton pregnancies (10.6%) is not increased if compared to the general population [34,35]. Significantly, there appears to be no effect of ED pregnancy (with or without PIH) on neonatal birth weight [18,34]. The general health status of children under 5 years old who were conceived using ED is at least as good as that of children conceived using standard IVF procedures [52]. There is also no increase in the incidence of congenital malformations in infants resulting from ED pregnancies [33,35].

Placental pathology

At the fetal-maternal interface significant histological and immunohistochemical differences are present when comparing ED and non donor IVF pregnancies. Characteristic pathologic findings in ED cases include a higher incidence of villitis of unknown etiology, chronic deciduitis, massive chronic intervillositis, maternal floor infarction, and ischemic changes, as seen with preeclampsia [53-55] (Figure 2). The chronic deciduitis observed in ED placentas is characterized by its severity and the presence of a dense, fibrinoid deposition in the basal plate. Furthermore, an increased infiltration of CD4+ T helper cells and CD56+ NK cells is present in the basal plate of ED placentas [55]. It is in the basal plate where extravillous trophoblast (of fetal origin) interfaces with and invades the maternal tissue. The extravillous trophoblast cells do not express classical Human Leukocyte Antigen (HLA) -A and HLA-B molecules, thereby preventing interaction with cytotoxic

T cells. However, they do express a unique combination of HLA antigens (HLA-C and the non-classical HLA-E and HLA-G) that interact with KIR receptors on uterine natural killer cells [56-58], although HLA-C can also serve as a target molecule for CD8+ T cells [59]. The striking findings of a dense fibroid deposition and mononuclear cell infiltration in the basal plate suggest that the placental abnormalities are related to an immune-mediated response that is more pronounced in ED pregnancies. The placental damage may be the consequence of a type of graft-versus-host disease and/or organ rejection type of reaction [55].

Immunologic aspects of egg donation pregnancies

Normal fetal-maternal immunology

A successful pregnancy is an interesting immunologic paradox. The fetus carries paternal and maternal genes but is not rejected by the maternal immune system, over a period of nine months (Figure 3). In spontaneously conceived gestations, several specific protective mechanisms have been postulated to explain the maternal tolerance of the fetus. Since the fetal tissue is directly exposed to the maternal blood, it is at risk of being attacked by components of both the innate and acquired immune system, with the potential risk of death. Therefore, to develop tolerance to the fetus, humans need an immune privileged site at the fetal-maternal interface in order to reproduce [60]. In spontaneously conceived pregnancies, immune recognition of the semi-allogeneic fetus takes place, but the soluble and cellular components of the maternal immune system are kept under control (or are locally down-regulated), leading to a maternal immune system that favours implantation of the embryo [61]. The currently accepted view is that a successful pregnancy depends on an appropriate balance of the different components of the maternal immune system, with predominance of T helper 2 immunity [62-65]. At the human fetal-maternal interface, maternal recognition of fetal antigens presented by trophoblast cells or by fetal cells trafficking into the maternal circulation, is essential for the induction of immunoregulatory mechanisms [66]. It is apparent that activated T cells at the maternal interface include regulatory T cells [66,67]. These regulatory T cells have an important role in the local down-regulation of human fetal-specific allogeneic T cell responses [68]. In studies of peripheral blood only minor differences in systemic immunoregulation were found between pregnant women and non-pregnant female controls (unpublished data). All of these protective mechanisms maintain the immunosuppressive environment in the pregnant uterus, and in this way the semi-allogeneic fetus is capable of surviving in the uterus.

Parallels with blood transfusions

The mechanism(s) involved in the effective down-regulation of the maternal immune response to the semi-allogeneic fetus can be compared to the ones involved in the tolerizing effect of pre-transplant blood transfusions. Blood transfusions have an immunomodulating effect, as demonstrated by the positive association of kidney graft survival and the number of allogeneic transfusions [69]. In addition, a beneficial effect of HLA-DR matched transfusions has been shown in kidney [70] and heart [71] transplantation. Furthermore, more HLA alloantibodies are formed after HLA mismatched transfusions compared with HLA-DR shared transfusions [72]. Down-regulation of the immune response may occur by the induction of regulatory CD4+ T cells, which are induced when the donor and recipient share at least one HLA-class II molecule [73]. This immunomodulating effect only occurs in case of semi-allogeneic or one HLA-DR shared blood transfusions. Blood transfusions that are fully HLA mismatched with the recipient lead to immunization, rather than tolerization of the patient.

Immune studies in egg donation

Although other mechanisms can be involved, it is likely that down-regulation of the maternal alloimmune response to the fetus in an ED pregnancy is far more difficult than in spontaneously conceived pregnancies with semi-allogeneic fetuses. Compared with spontaneously conceived pregnancies, there is a higher degree of antigenic dissimilarity in ED cases. If the 5 most immunogenic HLA antigens (HLA-A, -B, -C, -DR, and -DQ) are taken into consideration, the maximal number of mismatches in spontaneous conceived pregnancies would be 5. In ED pregnancies this could reach a maximum of 10 mismatches (Figure 3). Since ED pregnancies are characterized by more HLA mismatches, it is to be expected that a possible relationship between aspects of immune regulation and the number of HLA mismatches will become more apparent in ED pregnancies. In pregnant women who conceived by ED, an increased percentage of intracellular IFN-γ (Th1) and IL-4 (Th2) positive CD4+ T lymphocytes was found in peripheral blood compared with pregnant women after spontaneous conception [74]. This hyperactivation of Th1 and Th2 cells, induced by the allogeneic fetus, is specific for ED pregnancies. IFN-γ is also involved in spiral artery formation. Furthermore, the Th2 effect was more pronounced in ED pregnancies than in spontaneously conceived pregnancies [74]. This suggests that the additional mechanism of Th2 immunity in ED pregnancies leads to a successful pregnancy, even with a completely allogeneic fetus. Although this study investigated immune cells in the peripheral blood, the widely accepted view is that the active immune mechanisms take place at the fetal-maternal interface; therefore, it is possible that an effect will be even more prominent at this location. Recently, a statistically significant correlation between the extent of HLA mismatches and the percentage of CD4+CD25dim activated T cells in the decidua parietalis of uncomplicated pregnancies was described [75].

In spontaneously conceived pregnancies, the correlation between the number of amino acid triplet sequence (HLA epitope) mismatches between pregnant women and their children, and antibody production in the pregnant woman against the paternal antigens inherited by the child has been studied [76]. A positive correlation was found between the number of triplet mismatches (0-22) and the percentage of women producing HLA antibodies ($p<0.0001$). If 0 triplet mismatches were present, no antibodies were formed, even in the case of 1 or 2 classical HLA antigen mismatches. It remains to be established whether the actual number of HLA mismatches or epitope mismatches is more important in establishing tolerance to the fetus. However, it is likely that in ED pregnancies, the number of both HLA antigen and epitope mismatches will be even higher than in spontaneously conceived pregnancies. Therefore, the percentage of women producing antibodies will be higher, and this may have clinical implications. Although the clinical relevance of specific anti-fetal HLA antibodies is controversial, a recent study clearly showed that the presence of these antibodies in early pregnancy is associated with a reduced chance of a live birth (Nielsen *et al.*, 2010 unpublished).

The immune system clearly plays an important role in ED pregnancies. Unfortunately, there is a lack of information from the mother's perspective about the long-term effects of exposure to foreign cells and antigens in the recipient, since the usual clinical endpoint is the chance of having a take-home baby. From the literature it is unknown at present whether, later in life, the consequences of having conceived using ED may be harmful or not. In addition, when investigating immunologic aspects of ED pregnancies it is important to analyze the underlying reason why ED was necessary. For example, it is accepted that premature ovarian failure is a heterogeneous disorder in which some of the idiopathic forms are based on abnormal self-recognition by the immune system [77]. It is possible that the preexisting immunologic mechanisms involved in premature ovarian failure may contribute to the immunologic differences between ED and spontaneously conceived pregnancies.

Discussion

Although ED gives infertile women the opportunity to conceive, it may lead to harmful consequences during pregnancy if compared with spontaneously conceived pregnancies. This review gave an overview of the consequences of ED pregnancies with respect to their atypical fetal-maternal immunologic relationships. Review of the literature showed that women who conceived by ED have an increased risk of PIH [5,31-34,36-39,41], an increased rate of caesarean section deliveries [4,5,18,31-35], an increased risk of postpartum haemorrhage [33], and an increased risk of first trimester vaginal bleeding [6,31,34]. All of these complications can be the consequence of ED pregnancies; however other factors that correlate with infertility and age could also be an underlying cause. For example, women conceiving through ED are more often primigravidas, and more frequently have ovarian failure compared with women who conceive spontaneously. These factors are all associated with obstetrical complications [40]. More studies that correct for these confounding variables (e.g. maternal age, nulliparity, and ovarian failure) are needed to determine the specific role that ED plays in these important obstetrical complications. The higher risk of maternal morbidity in women who conceived through ED is a limitation of this form of treatment for infertility. For the benefits to outweigh the risks it might be important to select low risk donor-recipient combinations. The egg donors should be less than 35 years old [78] and unaffected by infectious diseases or hereditary syndromes [5,79]. Considering the immunologic mechanisms in ED, it might be worthwhile to perform HLA-typing of donor and recipient in order to select haplo-identical combinations that would be more comparable to spontaneously conceived pregnancies than fully HLA mismatched combinations.

Although the literature conclusively demonstrates an increased risk of ED-related pregnancy complications for the mother, it does not show an increased complication rate for the fetus or newborn [33,35,52]. Since there is a general lack of studies on the long-term outcome of ED pregnancies, it is currently unknown whether the child or mother experiences any consequences later in life. It is therefore important to document ED conception in the medical record to evaluate the subsequent consequences of carrying an allogeneic fetus. In ED pregnancy, the mother is exposed to foreign cells and antigens, a situation that is comparable to blood transfusions and organ transplantation. ED pregnancy leads to a hyperactivation of Th1 and Th2 cells compared to spontaneously conceived pregnancies [74]. This suggests that the allogeneic fetus induces an additional mechanism that leads to a successful pregnancy. It is possible that these mechanisms may have its consequences later in life. Therefore, long-term follow-up studies are strongly recommended.

Conclusions

ED provides a valuable addition to the list of treatment options for women who require assisted reproductive therapy. The benefits of having a take-home baby are counter-balanced by the higher risk of maternal morbidity. The increased rate of complications may be related to the allogeneic nature of the fetus. To understand the underlying mechanism(s) of acceptance of the allogeneic fetus, more research regarding the unique immunologic aspects of ED pregnancies is warranted. Understanding the role of the immune system in successful ED pregnancies also has broader biomedical significance in that it may also give insight into immune mechanisms leading to immunologic tolerance for HLA mismatched solid organ transplants.

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Differential immunoregulation in successful oocyte donation pregnancies compared with naturally conceived pregnancies

7

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Abstract

Background: In oocyte donation (OD) pregnancies, there is a higher level of antigenic dissimilarity between mother and fetus compared to naturally conceived (NC) pregnancies. This might leads to a higher degree and/or a different type of immunoregulation to maintain an uncomplicated pregnancy.

Methods: To test the hypothesis, different immunological aspects of OD (n=28) were compared with those of NC (n=51), and as an additional control non-donor IVF (n=20) pregnancies. The expression of IL-10, IL-6, galectin-1, pSMAD2 and Flt-1 was studied immunohistochemically in decidua and in maternal serum. Maternal peripheral blood mononuclear cells (mPBMCs) were characterized by flowcytometry and correlated with the number of HLA mismatches. mPBMCs were stimulated with umbilical cord blood or control PBMCs in a mixed lymphocyte culture.

Results: Compared to NC, OD pregnancies expressed less IL-10, IL-6, galectin-1, pSMAD2 and Flt-1 in the decidua and more IL-10 and IL-6 in serum. The percentages of CD4+CD25bright and CD4+CD25dim cells were higher in mPBMCs of OD and IVF pregnancies compared to NC. The number of HLA mismatches was positively correlated with the percentage of activated CD4+CD25dim cells in mPBMCs of OD. Functional studies showed a lower proliferative response in OD pregnancies.

Conclusion: Immunoregulation in OD is different than in NC pregnancies. A higher degree of peripheral immunoregulation and a different cytokine profile in the decidua was found in OD and IVF pregnancies compared to NC pregnancies. More HLA mismatches in OD pregnancies leads to higher percentages of activated T cells in peripheral blood, but their reactivity is effectively compensated by regulatory T cells.

Introduction

In pregnancy, genetically different or semi-allogeneic fetal tissue invades the maternal decidua and is directly exposed to the maternal blood. This causes a risk of being attacked by components of the immune system of the mother. However, the fetus not only escapes maternal immune rejection, but induces tolerance, creating an immune privileged site at the fetal-maternal interface [1,2]. The most accepted view is that a successful pregnancy depends on an appropriate balance of the maternal immune system with a predominance of T helper 2 immunity [3-5]. In oocyte donation (OD) pregnancies, there is a higher degree of antigenic dissimilarity compared to naturally conceived (NC) pregnancies. In OD pregnancies, adaptation of the maternal immune system is probably even more necessary to maintain uncomplicated acceptance of the allogeneic fetus [6]. Indeed, an increased percentage of intracellular IFN-γ (Th1) and IL-4 (Th2) positive $CD4^+$ T lymphocytes was found in the peripheral blood of pregnant women who conceived by OD compared with pregnant women after natural conception [7]. This hyperactivation of Th1 and Th2 by the allogeneic fetus is specific for OD pregnancies [7]. Histological findings in OD placentas often show a host versus graft rejection phenomenon similar to that seen with solid organ transplantation [8]. Severe chronic deciduitis admixed with fibrinoid deposition has been observed in OD placentas compared with non-donor in vitro fertilization (IVF) placentas [8]. Chronic deciduitis is found in the basal plate of the placenta, the site where extravillous cytotrophoblast interfaces with the maternal decidua. This pathology is thought to have an immune basis. Although OD pregnancies represent a very interesting model to investigate immunological interactions, most research has focused on the medical maternal and fetal complications rather than on the basic immunology. Although much research has been performed on the immunology of normal pregnancy, the immunology of OD pregnancies has not been studied intensively. The current study focused on immunological aspects of OD pregnancies compared to NC pregnancies. We hypothesized that there are differential immunoregulatory mechanisms that govern OD pregnancies compared to NC and IVF pregnancies at both local (fetal-maternal interface) and peripheral (peripheral blood) levels.

Maternal immune adaptations to the developing embryo are necessary in order to guarantee pregnancy success. Cytokines play an important role in promoting immune tolerance. Interleukin-(IL)-10 is seen as a facilitator of successful pregnancy; alterations of its levels may be related to adverse pregnancy conditions [9,10]. In addition, increased concentrations of IL-6 and other pro-inflammatory (IL-1, TNF-α, and IL-8) cytokines are found in the placentas of pregnancies complicated by pre-term premature rupture of the membranes [11]. During pregnancy, the expression of gal-1 is upregulated during implantation. Supplemental administration of gal-1 rescues the pregnancy in a mouse model of spontaneous abortion by inducing expansion of regulatory T cells that produce IL-10 [12]. Decreased expression of gal-1 in trophoblasts may partly explain disturbed differentiation during early placentation that leads to early pregnancy loss [13]. Another important immunoregulatory molecule is TGF-β, which is involved in blastocyst implantation by inducing apoptosis of endometrial cells within the uterus [14]. Decidual TGF-β is proposed to act on uterine natural killer (NK) cells to downregulate their cytotoxicity, resulting in the uterine-specific phenotype [15]. Binding of TGF-β to its receptor leads to activation of the TGF-β/ALK5 signaling pathway, which results in a cascade of reactions that eventually lead to the phosphorylation of SMAD2 (pSMAD2). TGF-β is a repressor of cytotrophoblast outgrowth [15], and it plays a role in angiogenesis. Angiogenesis also forms part of the maternal adaptation during embryo implantation. Regulation of vascular endothelial growth factor (VEGF) levels is a highly regulated process. Flt-1 is a receptor for VEGF and placental growth factor (PLGF). A splice variant of Flt-1 is sFlt1 (also known as sVEGFR-1), which antagonizes the VEGF and PLGF receptors. This soluble form prevents interactions of VEGF and PLGF with the functional membrane bound Flt-1, which thereby leads to endothelial dysfunction [16]. In the peripheral blood of preeclampsia patients, soluble Flt-1 (sFlt-1) is expressed in excessive amounts [17]. Since OD pregnancies are

associated with a higher incidence of preeclampsia, the levels of sFlt-1 in serum and decidua were tested as well.

The maternal blood is present in the intervillous space and is in direct contact with the outer syncytiotrophoblast layer of the placenta. This layer undergoes turnover, which precipitates shedding of microparticles into maternal blood. Therefore, in addition to the analysis of cytokine expression in the decidua, we also studied cytokine levels in maternal serum and the phenotype of peripheral blood mononuclear cells (PBMCs) by flow cytometry. CD4CD25bright regulatory T cells are believed to have a crucial role in maintaining pregnancy [18]. Transfer of CD4+CD25+ T cells from normal pregnant mice in to abortion prone mice prevents fetal rejection in the abortion prone mice [19]. In human an increased percentage of CD4+CD25+ T cells is present during pregnancy [20], however this study does not distinguish between CD4+CD25bright and CD4+CD25dim cells. Previously, it has been shown that the CD4+CD25+ cells can be divided in three fractions [18]. The percentage of FoxP3+ cells within the CD25bright T cells in peripheral blood of pregnant women is around 53% [21]. FoxP3 is an additional marker which may help to distinguish between effector and regulatory cells. However, this phenotypic distinction remains controversial [22] and therefore functional tests remains to be established until a specific marker for regulatory T cells is found. Since OD pregnancies are characterized by a higher number of HLA mismatches compared with NC pregnancies, the role of the number HLA mismatches was studied by correlating phenotypic results with the degree of antigenic dissimilarity. Furthermore, functional assays were performed to demonstrate the immune reaction of maternal PBMCs against fetal cells. We hypothesized that differences in the immunoregulatory mechanisms are present between OD, non-donor IVF, and NC pregnancies.

Material and Methods

Patient selection

Pregnancies conceived by OD (n=28), non-donor IVF (n=20), and NC (n=51) were studied. The non donor IVF group consisted of cases that conceived by IVF with the woman's own oocytes. Medical records were reviewed and clinical data were summarized. Placentas, peripheral blood and umbilical cord blood (UCB) samples were collected at delivery from women after uncomplicated pregnancies at 37-42 weeks' gestational age. Exclusion criteria were complications such as preeclampsia, preterm birth, immunological diseases, and infections. Placental tissue samples were collected within five hours after delivery. The study protocol was approved by the ethics committee of the Leiden University Medical Center (LUMC), and informed consent of every patient was obtained.

Blood samples

Peripheral blood samples from the mothers and the UCB were obtained at term and collected in heparinized tubes. Blood was layered on a Ficoll Hypaque (LUMC pharmacy; Leiden, The Netherlands) gradient for density gradient centrifugation at room temperature (20min/800g). After centrifugation PBMCs were collected from the interface, washed twice and counted. The cells were frozen in fetal calf serum with 10% dimethyl sulfoxide and stored in liquid nitrogen until proliferation studies and flow cytometry analyses were performed.

HLA typing

For every mother and child the HLA-types were determined in the typing laboratory of the Leiden University Medical Center. DNA was typed for the loci HLA-A, -B, -C, -DRB1 and -DQB1 using sequence specific oligonucleotides (SSO) PCR. The number and types of HLA matches and mismatches for every mother and child combination was calculated (Calculation by Microsoft Access 2003).

Cytokine determination in serum and supernatant

The levels of IL-10 and IL-6 in maternal serum were tested using a Th1/Th2 Bio-Plex Luminex™ system assay (Bio-Rad Laboratories, Veenendaal, The Netherlands) following the manufacturer's instructions. TGF-β1 was tested with the Milliplex™MAP single plex assay (Millipore Corporation, Billerica, MA, USA). Samples were analyzed using a Bio-Plex™ Array Reader with Bio-Plex software. An enzyme-linked immunosorbent assay (ELISA) for the presence of galectin-1 was performed as described [23] and sFlt-1 in maternal serum was performed by following the manufacturer's operating instructions (R&D Systems, Minneapolis, Minnesota).

Immunohistochemistry

For every study group (OD, NC and non donor IVF pregnancies) ten cases were selected randomly for immunohistochemical staining. These selected cases were representative of the patient characteristics as shown for the whole cohort in Table I. The mode of delivery was not significantly different between the three groups. Tissue samples of the placenta and rolls of fetal membranes were fixed in 4% formalin and processed for immunohistochemistry as previously described [24]. Briefly, sequential serial sections (4μm-thick) were cut. Tissue sections were deparaffinized and endogenous peroxidase was blocked. Antigen retrieval was performed by boiling the sections for 10 minutes in citrate buffer (pH 6.0). The optimal dilution for each primary antibody was determined in positive decidual tissue selected on the basis of maximal specific reactivity and minimal background staining; IL-10 1:100 (HP9016, Hycult Biotech Inc, Plymouth meeting, PA), IL-6 1:20 (AF-206-NA R&D Systems Europe Ltd.), pSMAD2 1:1000, Flt-1 1:250 (SC-316, Santa Cruz Biotechnology, Inc, Heidelberg, Germany), gal-1 1:500 (sc-28248, Santa Cruz Biotechnology). The primary antibodies were incubated for one hour (IL-10 and IL-6 overnight) at room temperature at the appropriate dilutions in PBS with 1% BSA. As a negative control the primary antibody was replaced with PBS with 1% BSA. Slides were incubated for 30 minutes with Envision (DAKO, North America Inc, USA) or for IL-10 with Powervision (Immunologic, Duiven, the Netherlands). For IL-6, a secondary goat antibody was labeled with HRP, (DAKO, North America Inc, USA, 1:200), and for gal-1 a secondary goat anti-rabbit antibody was labeled with HRP, followed by incubation with diaminobenzidine (DAB, DAKO Cytomation). The tissue sections were subsequently counterstained with haematoxylin (SIGMA, Switzerland, Steinheim), except for the pSMAD2 slides, since this staining is positive in the nuclei. The slides were mounted in mounting medium (Surgipath Medical Ind., Inc. Richmond) and covered. Images of all immunohistochemical staining results were captured using a microscope (Carl Zeiss Inc., Oberkochen, Germany) and digitally analyzed (Zeiss Axioskop 40, magnification 200x, Zeiss Axiocam MRc 5 camera, 150x150dpi). For every staining of one placenta a total of 5 pictures of the decidua parietalis and 5 of the decidua basalis was taken, blinded for the study group. Only the decidua was selected; blood vessels and shadows were digitally removed. Using Image-J software [25], the number of positive pixels per area was measured, indicating the level of expression for each immunohistochemical staining. This program is able to identify and measure positive cells by setting a threshold. For every staining experiment, an automatically running function was made, predefining the threshold of a positive cell. This threshold was independently defined by two observers.

Phenotypic characterization of maternal peripheral blood cells

Flow cytometric analysis was performed with a standardized protocol, using gating strategies as previously described [18]. In short, a four-color immunofluorescence staining was performed with directly conjugated mouse-anti-human monoclonal antibodies. CD45-APC, CD14-PE, CD25-PE, CD3-PerCP and CD4-APC were used in concentrations according to manufacturer's instructions (Becton Dickinson). The maternal PBMCs used for the functional assays were phenotyped. Only spontaneously deliveries were included for this analysis. These values were compared with a previously run control panel of 39 NC spontaneously delivered pregnancies that were obtained using the same standardized protocol [26]. Flow cytometry was performed on a Calibur flow cytometer using Cellquest Pro software (Becton Dickinson) and all samples were analyzed using the same template. Percentages were calculated within the lymphogate set around the viable lymphocytes based on the expression of CD45, CD14 and CD3. The percentages of CD4+CD25dim and CD4+CD25bright cells were calculated within the CD3+CD4+ cell population.

Functional analysis

Functional assays were performed to demonstrate the reaction of mPBMCs against mother's paired umbilical cord blood (UCB), a third party UCB (3p UCB) and third party peripheral blood leukocytes (3p PBL). Responders (mPBMCs) and stimulators (UCB, 3p UCB and 3p PBL) for the mixed lymphocyte cultures (MLC) were selected on the basis of the number of HLA-DR mismatches. As a reflection of the normal genetics in pregnancy, responders of the OD, non donor IVF and NC pregnancies were selected to have 1 HLA-DR mismatch with their own UCB (as is usually the situation in non donor pregnancies). In addition, a fully allogeneic OD group was studied, which had 2 HLA-DR mismatches with own UCB (indicated in the figures as OD*). As controls, the maternal responder cells were stimulated with 2 HLA-DR mismatched 3p UCB and 3p PBL. In each group, a total of five mother-UCB combinations were tested. MLCs were set up with 50 µl of $1x10^6$ mPBMCs in culture medium added in triplicate wells in a round-bottom 96-well plate (Greiner Bio-one) to 50 µl with $1x10^6$ irradiated (30 Gy/3000 Rad) stimulators or culture medium. Proliferation was measured on day 7 by incorporation of ^{3}H-thymidine added during the last 16 hours of culture. The results were expressed as the median counts per minute (cpm) for each triplicate culture.

Statistical analysis

Descriptive statistical analyses were performed using Graph Pad Prism (Graph Pad Software Inc.) and SPSS (SPSS Inc 17). The non parametric one-way ANOVA Kruskal-Wallis test was performed, and when significant, the post test Dunns was used to analyze between more than two independent groups. The non-parametric Mann Whitney test was used to identify differences between two independent groups. Linear data was analyzed with the linear regression analysis. Data were considered significant at $p<0.05$.

Results

Clinical data

The patient characteristics are shown in Table I. No significant differences between the three groups were present with respect to gestational age. Maternal age was significantly higher and

gravidity was significantly lower in the OD group compared with the NC pregnancies. The mode of delivery differed significantly between the three groups. Therefore the influence of the mode of delivery (primary section, secondary section and spontaneous) and the pregnancy group was analyzed in a logistic regression model backward-stepwise (Wald). Pregnancy group and mode of delivery were used as a predictor for high (above median) or low (below median) serum levels. Using the backward model, pregnancy groups seemed to be the predictor for the levels of IL-10, IL-6, TGF-β, and the mode of delivery had no influence. For Gal-1 the mode of delivery

	Oocyte donation (OD) N=26	Naturally conceived (NC) N=51	Non donor IVF (IVF) N=20	P value ANOVA	Post test
Gestational age (days)	278.0 (257-293)	275.5 (264-293)	273.0 (257-294)	ns	
Maternal age (years)	37.5 (30-45)	33.0 (28-40)	36.0 (28-41)	0.0003	ED vs NC: *** ED vs IVF: ns IVF vs NC: ns
Gravidity (number)	2 (1-5)	3 (1-7)	2 (1-5)	0.023	ED vs NC: * ED vs IVF: ns IVF vs NC: ns
Total HLA mismatches (A, B, C, DR, DQ)	6 (2-10)	4 (0-5)	3 (0-4)	<0.0001	ED vs NC: *** ED vs IVF: *** IVF vs NC: ns
Mode of delivery				0.0012	ED vs NC: ns ED vs IVF: * IVF vs NC: **
- Primary section	7	35	4		
- Secundary section	5	1	2		
- Spontaneous	14	15	14		
- Vacuum extraction	2	1	1		
HLA class I mismatches	4.0 (1-6)	2.5 (0-3)	2.0 (0-3)	<0.0001	ED vs NC: *** ED vs IVF: *** IVF vs NC: ns
HLA class II mismatches	2 (0-4)	1 (0-2)	1 (0-2)	0.0001	ED vs NC: *** ED vs IVF: * IVF vs NC: ns

Table I Patient characteristics. Values are medians with the minimum and maximum. The one-way ANOVA Kruskal-Wallis test was performed. When significant, the post test Dunns was used to analyze differences between groups.

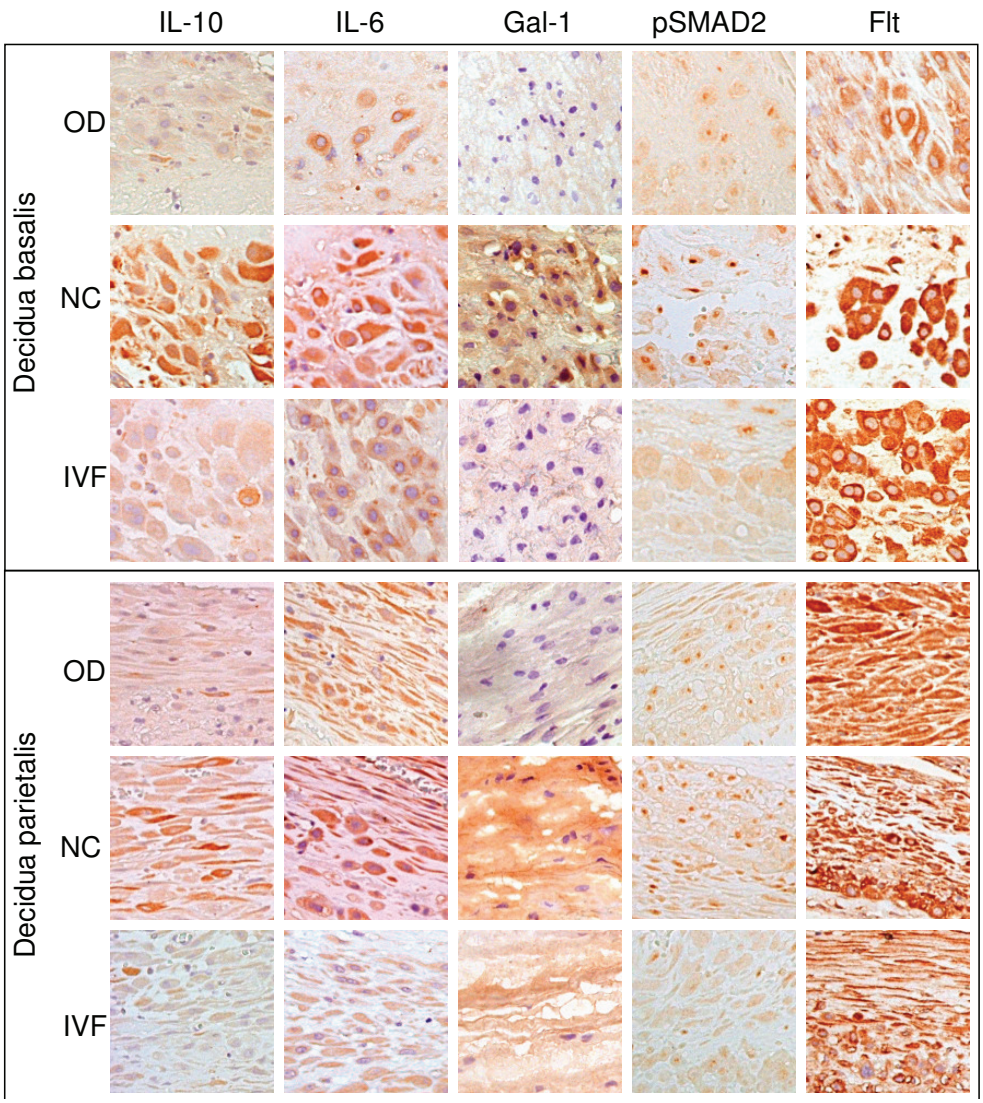

Figure 1 Immunohistochemical analysis. Photomicrographs of sections stained for IL-10, IL-6, galectin-1, pSMAD2 and Flt of the decidua basalis (upper panel) and decidua parietalis (lower panel). Original magnification x200. For every group, oocyte donation (OD), naturally conceived (NC) and non donor IVF, a representative example per staining is given. Positive cells are stained brown. Nuclei are stained blue (except for pSMAD2). The results of the digital image analysis of the immunohischochemical staining are depicted in Figure 2.

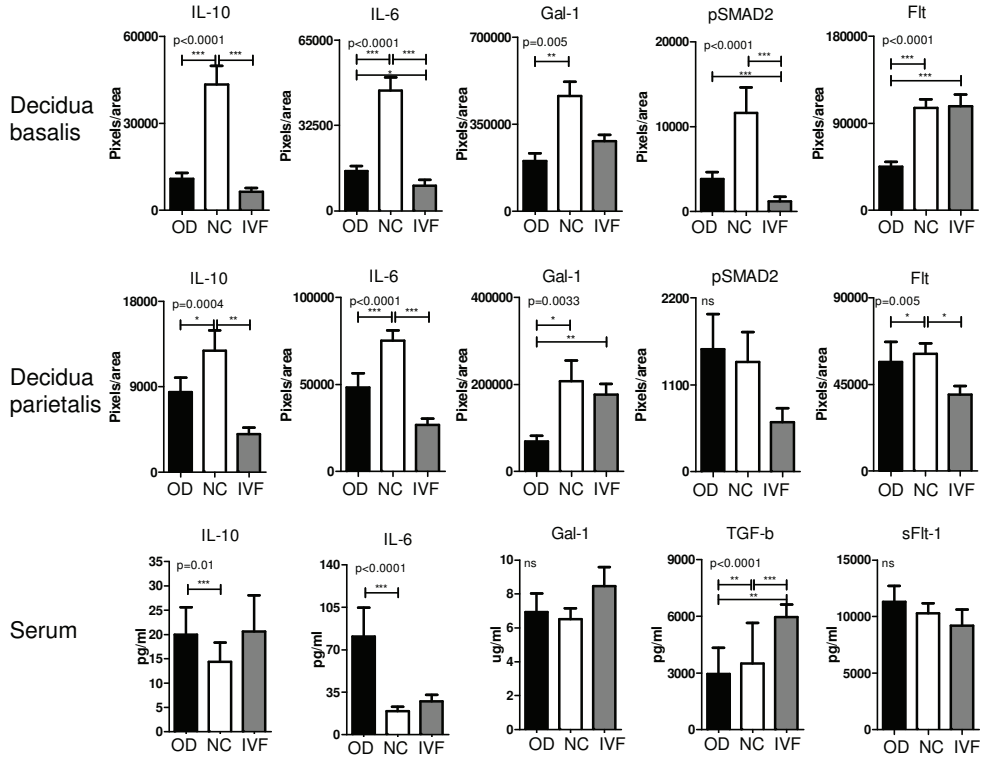

Figure 2 Placental and serum cytokine levels. Upper two rows show graphs illustrating the amount of positive pixels per standardized area in the decidua basalis and decidua parietalis of oocyte donation (OD, n=10), naturally conceived (NC, n=10) and non donor IVF pregnancies (IVF, n=10) tested by immunohistochemical staining. Lowest row shows results of serum cytokine levels defined by ELISA compared between OD (n=26), NC (n=51) and IVF (n=20). Level of significance indicated with asterisk, *** = p<0.001, **= p=0.001-0.01, *= p=0.01-0.05, ns = not significant. Values presented as means, error bars indicate the SEM.

seemed to be a predictor for higher serum levels. For sFlt, neither mode of delivery nor pregnancy group had an influence on the serum levels. This statistical test was not performed for the immunohistochemistry analysis since in those selected cases there was no significant difference between the groups in mode of delivery. For the flow cytometry analysis only spontaneously delivered pregnancies were used. Inherent to OD pregnancies the number of HLA mismatches was significantly higher compared to NC and non donor IVF pregnancies (6, 4, 3 respectively, p=<0.0001). When analyzed separately, both the number of HLA class I and class II mismatches were significantly higher in OD compared to NC and non donor IVF pregnancies (p=<0.0001 and p=0.0001 respectively).

Immunohistochemical studies in placenta

The decidua of OD, NC and IVF pregnancies all showed cells that were positively stained by the antibodies used (Figure 1). Figure 1 shows representative pictures of the immunohistochemical staining by location for every antibody used. The results of the digital image analysis of the immunohischochemical staining are depicted in Figure 2. Significant differences in the decidua basalis and decidua parietalis of OD, NC and IVF pregnancies were found for all the tested

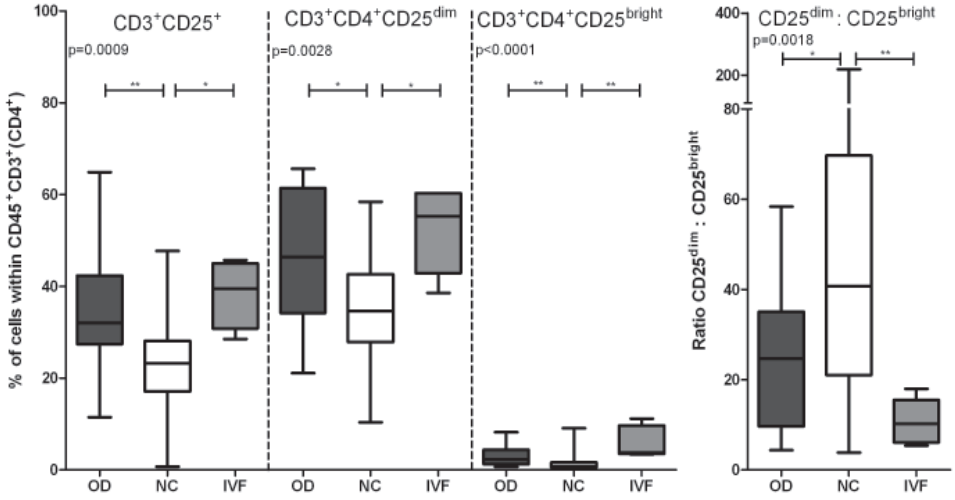

Figure 3 CD25+ cells in peripheral blood samples of oocyte donation (OD, n=13), naturally conceived (NC, n=39) and non donor IVF pregnancies (IVF, n=5). Level of significance indicated with asterisk, *** = p<0.001, **= p=0.001-0.01, *= p=0.01-0.05, ns = not significant. All values are of spontaneously delivered pregnancies. Values presented as means, error bars indicate the SEM. The horizontal line within the box indicates the median, the ends of the box correspond to the upper and lower quartiles of the data and the whiskers indicate minimum and maximum values.

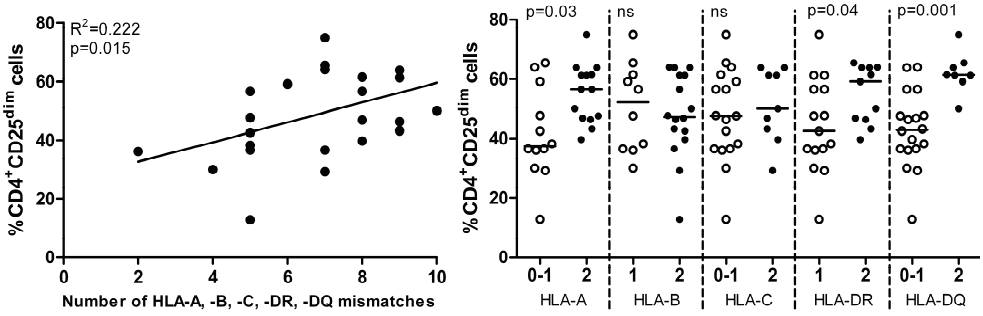

Figure 4 Correlation between the number of HLA mismatches and the percentage of CD4+CD25dim cells in oocyte donation (OD) pregnancies (n=26) in peripheral blood. A. Considering HLA-A, -B, -C, -DR and -DQ the maximal number of HLA mismatches is 10 in OD pregnancies. A positive correlation between the number of mismatches and more CD4+CD25dim cells in peripheral blood of OD pregnancies is found. B. The number of mismatches of the HLA-A, HLA-DR and HLA-DQ antigens relates with more CD4+CD25dim cells in peripheral blood of OD pregnancies (p=0.03, p=0.04 and p=0.001 respectively). The horizontal lines represent the median of the data. The non parametric Mann-Whitney test was performed to analyze values between the groups (ns = non significant).

cytokines except for pSMAD2 in the decidua parietalis. The level of expression in the decidua basalis and parietalis was significantly lower in OD pregnancies compared with NC pregnancies for IL-10, IL-6, gal-1 and Flt (Figure 2). IVF showed similar results at both locations compared to OD for IL-10, IL-6 and Flt (only in the decidua parietalis, Figure 2). The level of expression of IL-6 and pSMAD2 in the decidua basalis was significantly higher in OD pregnancies compared with IVF pregnancies (Figure 2). The level of expression of Flt in the decidua basalis and gal-1 in the decidua parietalis is significantly lower in OD pregnancies compared with IVF pregnancies (Figure 2).

Maternal serum analysis

The ANOVA test showed significant differences between OD, NC and IVF pregnancies with respect to serum levels of IL-10, IL-6 and TGF-β1 ($p=0.01$, $p<0.0001$ and $p<0.0001$ respectively, Figure 2). The levels of sFlt-1 and galectin-1 did not significantly differ between the three groups. Serum levels of IL-6 and IL-10 are significantly higher in OD pregnancies compared with NC pregnancies. TGF-β1 serum levels were statistically significantly lowest in OD pregnancies, followed by NC pregnancies, and highest in IVF pregnancies.

Correlation between the number of HLA mismatches and the percentage of CD4CD25dim cells in the peripheral blood

The percentage of CD3+CD25+ cells was higher in OD and IVF pregnancies compared to NC pregnancies ($p=0.0009$, Figure 3). Within the CD25 fraction the percentage of CD25dim cells was also higher in OD and IVF pregnancies compared to NC pregnancies ($p=0.0028$, Figure 3). The same was found for CD4+CD25bright cells; the percentage of CD4+CD25bright cells was higher in OD and IVF pregnancies compared to NC pregnancies ($p<0.0001$, Figure 3). The ratio of CD4+CD25dim:CD4+CD25bright cells was lower in OD and IVF pregnancies compared to NC

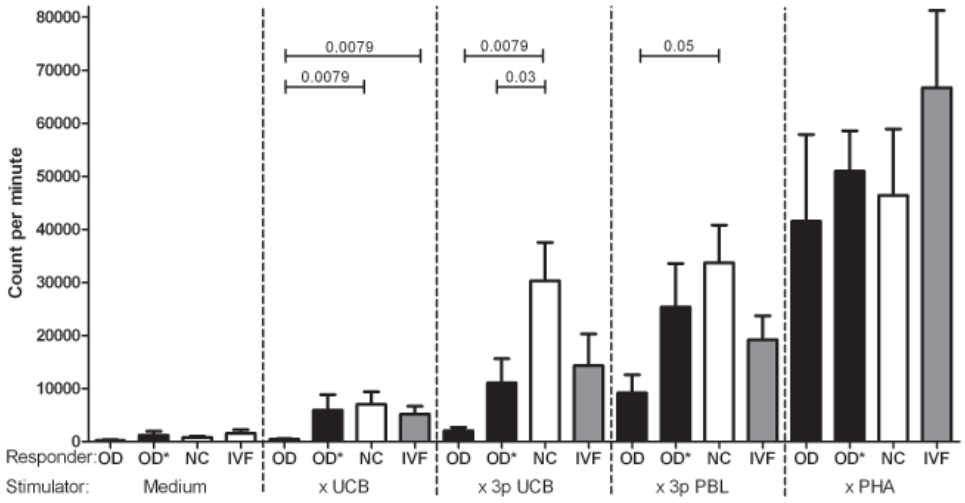

Figure 5 Mixed lymphocyte culture. Results in counts per minute. The responders were the maternal PBMCs of OD with one HLA-DR mismatch (n=5), OD with two HLA-DR mismatch (OD*, n=5), normal conceived (NC, n=5) and non donor IVF pregnancies (IVF, n=5). They were stimulated with their own UCB, 3p UCB or 3p PBL. Medium and PHA were used as a control. Values are presented as means, with error bars indicating the SEM. The non parametric T test was performed to analyze values between the groups. A p value of <0.05 was considered significant.

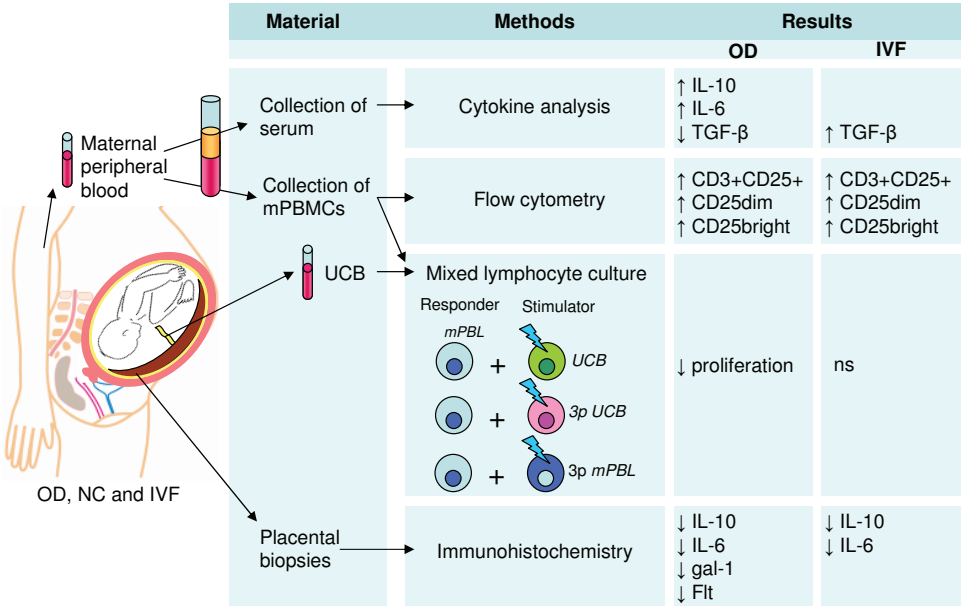

Figure 6 Overview of materials, methods and results. Maternal blood was used to collect serum for the performance of cytokine analysis by ELISA. mPBMCs were collected and analyzed by flow cytometry. Together with irradiated UCB, 3p UCB and 3p mPBL mPBMCs were cultured. The placenta and fetal membranes were collected after delivery to perform immunohistochemical staining. The main results for OD and IVF compared to NC are shown in the last two rows. Abbreviations: oocyte donation (OD), naturally conceived (NC) in vitro fertilization (IVF), maternal peripheral blood mononuclear cells (mPBMCs), maternal peripheral blood lymphocytes (mPBL) umbilical cord blood (UCB), third party (3p), non significant (ns).

pregnancies (p=0.0018, Figure 3). A significant positive correlation between the percentage of CD4+CD25dim in peripheral blood and the number of HLA mismatches was found in OD pregnancies (R^2:0.022 p=0.015, Figure 4). No correlation was found between the number of HLA mismatches and CD4+CD25bright or CD4+CD25dim cells in NC pregnancies (data not shown). However, the number of HLA mismatches did not affect the ratio of CD4+CD25dim:CD4+CD25bright cells in OD and NC pregnancies (data not shown). To determine whether mismatches of a specific HLA locus were responsible for the increased percentage of CD4+CD25dim cells, the HLA mismatches were analyzed separately by locus. HLA-A, HLA-DR and HLA-DQ mismatches significantly correlated with the increased percentage of CD4+CD25dim in peripheral OD blood (p=0.03, p=0.04 and p=0.001, respectively, Figure 4).

Mixed lymphocyte cultures

To investigate the fetus-specific immune response of mPBMCs collected after OD, NC, and non donor IVF pregnancies, these cells were stimulated with their own UCB, control UCB, and PBL. The OD group was subdivided in a group with one HLA-DR mismatch and a group with two HLA-DR mismatches (OD*). To determine the proliferative capacity of mPBMCs, the cells were stimulated with PHA. No significant differences between the groups were observed (Figure 5). As a negative control, cells were cultured with medium for 6 days. No significant differences were found here either between the groups (Figure 5). In the case of one HLA-DR mismatch, the mPBMCs of the OD pregnancy group showed significantly lower proliferation against their own UCB compared with

NC pregnancies (p=0.0079, Figure 5), and also compared with IVF pregnancies (p=0.0079, Figure 5). Also the response to 3p UCB and 3p PBL by OD pregnancies showed a significantly lower proliferation compared with NC pregnancies (p=0.0079 and p=0.05 respectively, Figure 5). Even when OD with two HLA mismatches were compared to NC, with a single HLA-DR mismatch, the response by OD pregnancies was still significantly lower compared with NC pregnancies (p=0.03, Figure 5).

An overview of the major differences observed between the different types of pregnancies is given in Figure 6.

Discussion

OD pregnancies are a result of in vitro fertilization of a donated oocyte by a relative, or more commonly an unrelated donor. In the latter case, neither of the fetal haplotypes matches with the gestational carrier. This creates a unique immunological situation to study the immune mechanisms that underlie these pregnancies. A better understanding of the maternal immune adaptations during OD pregnancy could increase the chance of successful gestation in patients with a history of infertility. In this work, we found strong peripheral regulation in OD and IVF pregnancies compared to NC pregnancies, and a differential maternal adaptation is in the decidua of OD and IVF pregnancies (Figure 6).

The results of this study showed that several immunological aspects are different in OD pregnancies compared with NC pregnancies. OD pregnancies express less IL-10, IL-6, gal-1, pSMAD2 and Flt-1 in the decidua (except for pSMAD2 in the decidua parietalis) compared to NC pregnancies. Although less immunoregulation in the decidua of OD and IVF pregnancies was found, we found more immunoregulation in the peripheral blood compared with NC pregnancies. In serum, OD pregnancies express more IL-10 and IL-6 but less TGF-β compared with NC pregnancies. Although these results suggests that IL-10 is plays an important role in uncomplicated pregnancy, recent data in mice show that a defect in IL-10 is not harmful for the pregnancy [27]. Immunohistochemical staining of pSMAD2 was used in this study to define TGF-β signaling. TGF-β is a repressor of cytotrophoblast outgrowth [15], possibly resulting in a proper balance for optimal placental growth. An imbalance in TGF-β signaling may lead to placental pathologies. We found less pSMAD2 placental expression in uncomplicated OD and IVF pregnancies, without placental pathology. Generally, in IVF pregnancies, placenta accreta occurs more frequently [28]. It is possible that the lower amount of pSMAD2 is counterbalanced by an altered mechanism, preventing placental pathologies. Gal-1 can be present intracellular and extracellular and thereby may elicit different functions [29]. Immunohistochemical staining of Gal-1 in our study shows that it is expressed in cytoplasm compartment in decidua basalis and extracellularly in decidua parietalis. It is possible that this different location is a result of trophoblast invasion. In the decidua parietalis non-invading trophoblast contacts the chorion and in the decidua basalis there is interaction between decidual cells and invading villous trophoblast.

The cytokines analyzed in this study have an immunological regulatory role during pregnancy. We therefore hypothesized that we would find increased IL-10, IL-6 and gal-1 expression in the decidua of OD pregnancies compared with NC. However, we found the opposite. Overall, we found that these cytokines were lower in OD and IVF pregnancies compared to NC pregnancies at the fetal-maternal interface. It is possible that maternal adaptation of the fetal allograft is more prominent during the first trimester. Therefore, it would be worthwhile investigating the immunological alterations of cytokines in decidua of first trimester samples. There are, however, limitations to the access of human first trimester samples. Furthermore, it is possible that immunoregulation in OD pregnancies also takes place on the fetal side of the placenta, as

suggested by preliminary observations which shows an OD-specific lesion at the fetal side of the placenta, containing cells involved in the immunomodulation (Schonkeren et al, submitted). More studies are necessary to unravel the underlying mechanisms in the immunologic differences between OD and NC pregnancies.

The percentages of CD4+CD25bright and CD4+CD25dim cells were higher in mPBMCs of OD and IVF pregnancies compared to NC. In OD, the number of HLA mismatches was positively correlated with the percentage of CD4+CD25dim cells (activated T cells) in mPBMCs of OD. However, there was no correlation with the number of HLA mismatches and the ratio CD4+CD25dim:CD4+CD25bright in OD pregnancies, suggesting that a higher number of CD4+CD25bright cells is necessary to regulate the immune system peripherally in OD pregnancies. That seems to be the case, as we demonstrated by our functional analyses. In OD pregnancies, more peripheral immunoregulation is present. The ratio of CD4+CD25dim:CD4+CD25bright was significantly lower in OD and IVF peripheral blood samples compared to NC. This suggests that relatively more CD4+CD25bright regulatory cells are present compared to NC pregnancies with the same number of CD4+CD25dim cells. A previous study showed hyperactivation of T helper 1 and T helper 2 cells in peripheral blood of OD compared with NC pregnancies [7]. The authors stated that the activation of T helper 2 cells and the relative suppression of T helper 1 chemokine expression reflected an additional regulatory counteractive mechanism. In agreement with this finding, we found a higher percentage of blood CD4+CD25dim activated T cells in OD compared with NC pregnancies, and the percentage of blood CD4+CD25bright regulatory T cells was higher compared to the activated T cells, suggesting a counteractive response in OD pregnancies.

The number of HLA mismatches also appears to play a crucial role, as a positive correlation was found between with the percentage of CD4+CD25dim cells in peripheral blood of OD pregnancies. This correlation was not present in the peripheral blood of NC pregnancies. A higher number of HLA-A, -DR and -DQ mismatches leads to more CD4+CD25dim cells in maternal peripheral blood in OD pregnancy. No correlation was found between the ratio CD4+CD25dim:CD4+CD25bright and number of HLA mismatches for OD and NC pregnancies (data not shown), suggesting that the higher number of activated cells is controlled by a higher number of regulatory cells. The percentage of CD4+CD25bright and CD4+CD25dim cells in peripheral blood of term NC pregnancies is comparable with previous studies [18]. Previously, we showed a central role of HLA-C mismatches in the induction of the decidual lymphocyte response to fetal cells by CD4+CD25dim cells [26]. In contrast, the number of CD4+CD25dim cells in the peripheral blood of OD pregnancies was not mediated by the number of HLA-C mismatches, or by the presence of HLA-C1 or HLA-C2 mismatches (data not shown). This shows that for decidual regulation HLA-C, the only classical HLA antigen expressed on trophoblast, plays an important role. In contrast, for peripheral responses, HLA-A, -DR and -DQ but not HLA-C are essential, since the presence of more CD4+CD25dim cells in the periphery of OD pregnancies was associated with a higher number of mismatches on the HLA-A, HLA-DR and HLA-DQ antigens. To explain these results, we postulate that the impact of fetal microchimerism in maternal blood plays a role in the immunological response, as seen in the OD pregnancies. Antigens on fetal cells migrate in to the maternal blood, and fetal (and thus partly paternal) antigens may be able to modulate the maternal immune response during pregnancy, which persists in maternal circulation for decades after delivery [30]. The presence of fully allogeneic fetal cells in maternal circulation has been demonstrated in after OD [31]. Strong peripheral immunoregulation in OD might therefore be beneficial for the persistence of microchimerism.

Our functional assays confirm the presence of a stronger peripheral immunoregulation in OD and IVF pregnancies. mPBMCs of OD pregnancies with one HLA-DR mismatch showed less proliferation upon stimulation with own UCB compared to NC and IVF pregnancies. This indicates that in IVF and NC pregnancies less peripheral regulation of immunological response towards the

woman's infant is present. Even in OD pregnancies with two HLA-DR mismatches the alloimmune response to the UCB is lower than in NC pregnancies. The mechanisms behind the differences in response to fetus-specific stimulation in an OD pregnancy remain to be established. In a previous study in NC pregnancies, we found no significant differences between the responses of mPBMCs to her own child or a control child compared to non-pregnant controls [32]. This indicates that peripheral immune regulation in NC pregnant women is not very different from that of non-pregnant controls. Here, we show that in OD pregnancies the situation is different. The peripheral immune response is significantly altered compared to NC pregnant controls.

A limitation of this study is that it is difficult to define a proper control group for OD pregnancies. Woman who undergo non donor IVF receive two hormonal treatments; one to retrieve the oocytes and a second one for the induction of a proper milieu before embryo transfer. Woman who undergo OD only receive the hormonal treatment necessary for embryo transfer. Although upon implantation the embryo consists of only a few cells, and alterations in the immune system during decidualization by hormonal treatment used in assisted reproductive techniques may affect those cells in the first trimester, it is unlikely that this treatment is responsible for immunological disturbances in the last trimester. However, vulnerability to changes in the hormonal surroundings of the blastocyst in the periconceptional period might results in peri-implantation programming and explains long term effects via changing of phenotype of fetal cells. This remains to be further elucidated and indeed it has been shown that in vitro culture of embryos is associated with changes in fetal outcomes [33]. It even has been proposed that the composition of culture medium is of more influence than the procedure of in vitro culture itself [34].

In conclusion, in this study we provide evidence that the immunoregulation in OD pregnancies is different compared with NC pregnancies, both with regard to the peripheral and the local immune responses. The number of HLA mismatches in OD pregnancies affects the number of activated T cells in the periphery. The mechanisms by which this altered immune response is evoked remain to be established. Future studies are necessary to investigate the immunoregulatory mechanisms involved in successful, but also in pathological, OD pregnancies.

Acknowledgements

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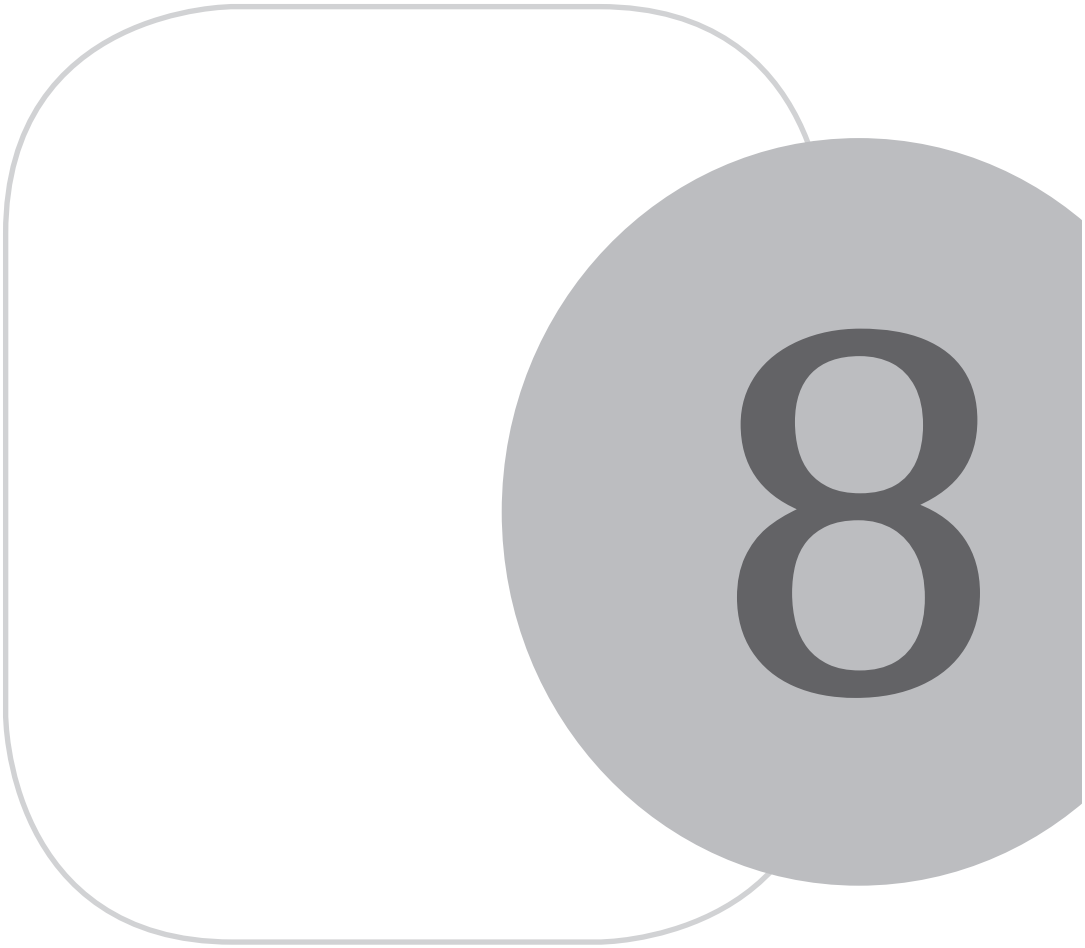
Summary and general discussion



Summary and general discussion

This thesis investigated immunological factors during pregnancy at the fetal-maternal interface. Uncomplicated naturally conceived, preeclamptic, uncomplicated egg donation (ED), and uncomplicated IVF pregnancies were included to define possible alterations in immunological mechanisms. The fetus consists of paternal and maternal genes, and is thereby semi-allogeneic towards the mother. Despite of these immunogenetic differences the fetus escapes from immune rejection by the maternal immune system and is tolerated for the duration of the pregnancy. This thesis stated that changes in immune regulation locally in the placenta contribute to the pathogenesis of preeclampsia, and therefore preeclampsia was seen as an immunological challenge during pregnancy. Another immunological challenge is reflected by ED pregnancies. Even in ED pregnancies, where the fetus is totally allogeneic towards the mother, the fetus escapes from immune rejection by the maternal immune system. This thesis hypothesized that differential immunoregulation during pregnancy is necessary to accept the allogeneic fetus in ED pregnancies. Table 1 gives an overview of the pregnancies, materials, methods, results and conclusions of every chapter discussed in this thesis.

A general introduction on pregnancy was given in **chapter 1**. The biological basis of placental development, reproductive immunology, preeclampsia and ED is reviewed.

No systematic study on specific and non-specific maternal immune responses during normal pregnancy compared with non-pregnant controls was performed so far. We analyzed the phenotype of cells of peripheral blood samples of both groups and studied the proliferative capacity of these cells upon specific or non-specific stimulation (**chapter 2**). Non pregnant control females were compared with women carrying an uncomplicated pregnancy. We found no differences between the response of maternal peripheral blood mononuclear cells (mPBMCs) and control PBMCs (cPBMCs) upon specific stimulation. Although this was not reflected in the proliferative immune response upon specific stimulation, a different composition of leukocyte subsets was found in peripheral blood samples. Pregnant women contained a higher percentage of CD14+ cells and CD25dim cells, and a lower percentage of CD16+CD56bright and CD16+CD56+ NK cells. Furthermore, serum of pregnant women contained more IL-6, IL-10 and IL-17. Stimulation of mPBMCs and cPBMCs with allogeneic stimuli resulted in different amounts of cytokine production between the two groups. These data indicate that a pregnant woman is capable of creating a fine-tuned environment, optimal for the growth and survival of the fetus, but as well optimal for the mother to maintain adequate immune responses to infections or diseases.

Preeclampsia is a disease of pregnancy caused by multiple factors. The etiology is not fully understood. Preeclampsia is thought to be an immunologically driven disease because there is an association with primigravida, while subsequent pregnancies with the same father are protected. Also the possibility of preeclampsia occurring in subsequent pregnancies with a different father, the protective role of blood transfusions, previous abortions and prolonged semen or seminal fluid exposure are in line with this reasoning [1,2]. Macrophages are an abundant cell population in the human decidua, alterations within this cell population may lead to immunological disturbance, possibly involved in preeclampsia. Decidual macrophages play an important role in promoting immune tolerance via the production of anti-inflammatory substances, like IL-10 and indoleamine 2,3-diogygenase (IDO). Trophoblast may interact with decidua macrophages via HLA-G and thereby stimulate the production of anti-inflammatory cytokines. Macrophages appear to disrupt vascular smooth muscle in the spiral arteries, prior to trophoblast invasion, and activated macrophages have been shown to inhibit trophoblast invasion [3]. These data suggest that inadequate production of the cytokines by macrophages, and their roles in spiral artery remodeling, potentially contribute to the pathogenesis of preeclampsia. We therefore investigated the distribution and phenotype in decidual macrophages in preeclamptic and control pregnancies (**chapter 3**) [4]. Macrophages polarize into different phenotypes. Pro-inflammatory macrophages

Pregnancies	Materials	Methods	Results	Conclusion
Chapter 2				
Uncomplicated spontaneously concieved (mPBMC) Non pregnant women (cPBMC)	UCB mPBMC cPBMC	FACS: MLC: Luminex:	**mPBMC vs cPBMC:** ↓ NK cells, ↑ monocytes, ↑ CD3+CD25+ T cells ↑ CD3+CD25dim T cells No difference in proliferation upon stimulation with aCD3, PHA, fetus specific UCB and 3rd party UCB between mPBMCs and cPBMCs **mPBMC vc cPBMC:** ↑ IL-6 (after stimulation with UCB or 3rd party UCB) ↑ IL-10 (after stimulation with 3rd party UCB), ↑ IL-17 (after a-specific stimulation)	The maternal peripheral immune response is altered during pregnancy, though these differences do not result in quantitative changes in proliferative responses during pregnancy compared to non-pregnant controls.
Chapter 3				
Preterm preeclamptic Preterm control Term control	Placentas Fetal membranes	IHC:	**Preterm preeclampsia vs preterm controls** ↑ CD14 and CD163 expression in the decidua basalis ↓ IL-10 expression in the decidua parietalis ↓CD163/CD14 in the decidua basalis ↑DC SIGN/CD14 in the decidua basalis and parietalis CD14+ macrophages did express Flt-1	Alterations in distribution and phenotype of macrophages in the decidua of preterm preeclamptic pregnancies compared to control pregnancies may contribute to the pathogenesis of preeclampsia.
Chapter 4				
Non donor IVF ED	Clinical data Placenta	Comparison of clinical data	IVF: dizygotic twin pregnancy was complicated by preeclampsia and intra uterine growth retardation. ED: dizygotic twin pregnancy was complicated by severe preeclampsia, both fetuses had a normal fetal birth weight.	We suggest a different pathophysiological mechanism of preeclampsia after ED compared with preeclampsia in non-donor IVF conception.
Chapter 5				
ED	79 publications	Review of literature	ED pregnancies are associated with a higher incidence of pregnancy-induced hypertension and placental pathology. Perinatal complications, such as intrauterine growth restriction, prematurity, and congenital malformations, is comparable to conventional *in vitro* fertilisation.	ED pregnancies have a higher risk of maternal morbidity. Due to the high degree of antigenic dissimilarity, ED pregnancies represent an interesting model to study complex immunologic interactions.
Chapter 6				
ED		Test hypothesis	During ED pregnancies the mother has to cope with a higher degree of antigenic dissimilarity compared with spontaneously conceived pregnancies. Maternal cells and fetal cells come in close contact. Understanding the immune mechanisms gives more insight into the question why the (semi) allogeneic fetus is accepted and not rejected by the mother.	The immunologic interactions between mother and child in successful ED pregnancies may be relevant for the induction of immunological tolerance in solid organ transplantation.
Chapter 7				
ED IVF NC	Placenta Fetal membranes UCB Maternal blood	IHC: Luminex: FACS: HLA relation: MLC:	**ED vs NC:** ↓IL-10, IL-6, galectin-1, pSMAD2 and Flt **ED vs NC:** ↑ IL-10, IL-6 , ↓TGF-β **ED/IVF vs NC:** ↑ CD4+CD25bright and CD4+CD25dim cells were higher in mPBMCs The number of HLA mismatches was positively correlated with the percentage of activated CD4+CD25dim T cells in mPBMCs of ED. **ED/IVF vs NC:** ↑ immunoregulation	Immunoregulation in ED is different than in NC pregnancies, partly due to procedure considering similarities with IVF pregnancies. An altered peripheral and local regulation was found in ED and IVF pregnancies compared to NC pregnancies, shown by the level of cytokines present in serum and placenta tissue, the phenotype of the cells in the periphery and the response of mPBMCs upon stimulation.

Table 1 Main results of the chapters discussed in this thesis. For abbreviations, see the abbreviation list.

(type 1) have pro-inflammatory and cytotoxic properties and are able to eradicate intracellular pathogens. In contrast, anti-inflammatory macrophages (type 2) display anti-inflammatory properties and are able to secrete IL-10. CD163 is a marker present on type 2 macrophages. The human decidua contains type 2 macrophages contributing to the immune suppression necessary to maintain the semi-allogeneic fetus whereas type 1 macrophages are able to defend against utero-placental infection, but possibly do not contribute to tolerance of the fetus. We found a decreased expression of type 2 macrophages in the decidua basalis of preeclamptic pregnancies compared to preterm control pregnancies. Furthermore, the level of expression of IL-10 was lower in the decidua parietalis of preeclamptic pregnancies compared to preterm control pregnancies. The distribution of decidual macrophages is altered in preeclamptic pregnancies and we showed that the macrophages contain fmslike tyrosine kinase 1 (Flt-1). The soluble form of this cytokine (sFlt-1) is produced in excessive forms in preeclampsia. Macrophages may be responsible for this increased sFlt-1 production, and by an alteration of distribution they may contribute to the pathogenesis of preeclampsia.

Immunological tolerance between mother and fetus is needed for successful reproduction. Sharing of too many HLA antigens between mother and father has been shown to form a disadvantage on pregnancy outcome, sharing of too few HLA antigens may as well alter pregnancy outcome [5]. Since preeclampsia is a disorder of the immunological mechanisms involved in the normal fetal-maternal responses, possibly based on HLA (mis)matching, we hypothesized that the immunological mechanism of preeclampsia is different in normal pregnancies and ED pregnancies (**chapter 4**) [6,7]. Two dizygotic pregnancies are described. Both pregnancies resulted from assisted reproductive techniques. Interestingly, the non donor IVF pregnancy developed preeclampsia with severe growth retardation and the ED pregnancy developed preeclampsia without fetal growth retardation. Although only two cases are investigated, these nicely illustrate the hypothesis why preeclampsia in ED pregnancies might have another pathophysiological mechanism.

ED pregnancies represent a very interesting model to investigate immunological interactions. So far most research is focused on the medical complications in mother and fetus rather than basic immunology. The immunological dissimilarity in ED pregnancies is comparable to the immunological dissimilarity in solid organ transplantations. The acceptance of a fetal allograft in normal pregnancies requires the avoidance of rejection by altered HLA expression on trophoblast cells, production of immunosuppressive cytokines and other immunomodulatory strategies. These mechanisms are most probably as well present in ED pregnancies. However, since the potentially higher degree of immunological dissimilarity compared with naturally conceived pregnancies, additional mechanisms are possibly needed for the acceptance of the fetal allograft (**chapter 5**). Expanding knowledge of immunological mechanisms in successful and failed ED pregnancies is potential viable in understanding the immunological interactions involved in acceptance or rejection of solid organ transplantations.

ED gives infertile women the opportunity to conceive, however, it has a higher incidence of harmful maternal consequences, compared with naturally conceived pregnancies (**chapter 6**) [8]. Women who conceived by ED have an increased risk of pregnancy induced hypertension, increased rate of cesarean section deliveries, increased risk of post partum hemorrhage and an increased risk of first trimester vaginal bleeding. However, it is not associated with an increased complication rate for the fetus or newborn.

The immunological mechanism in ED, naturally conceived and non donor IVF pregnancies has been studied in **chapter 7**. We found an altered immune reaction, locally as well as peripherally in ED and non donor IVF pregnancies compared with naturally conceived pregnancies. We tested this by the analysis of several cytokines in serum and placental tissue. Furthermore, mPBMCs were phenotyped by flow cytometry and correlated with the number of HLA mismatches.

mPBMCs were stimulated with (allogeneic) UCB or control PBMCs in a mixed lymphocyte reaction. Differences in cytokine expression in decidua and maternal serum between ED, IVF and naturally conceived pregnancies were found. The percentage CD4+CD25bright and CD4+CD25dim were higher in mPBMCs of ED and IVF pregnancies compared to naturally conceived pregnancies. In ED pregnancies, the number of HLA mismatches is positively correlated with the percentage of CD4+CD25dim in mPBMCs. Although the exact mechanism remains to be elucidated, we found that the immunology in ED and non donor IVF pregnancies is altered compared to naturally conceived pregnancies. The presence of fetal cells in maternal blood (fetal microchimerism) possibly plays a role. Antigens of fetal cells migrate into the maternal blood, and fetal (and thus partly parternal) antigens may be able to modulate the maternal immune response during pregnancy. In ED pregnancies the impact of microchimerism might be altered, and thereby leading to a positive correlation between the percentage of CD4+CD25dim and the number of HLA mismatches in ED pregnancies.

Experimental considerations

To identify possible immunologic mechanisms contributing to aberrant immunology in pregnancy, it is essential to validate factors potentially influencing the outcome. In our studies we cautiously selected the control groups. In chapter 3, in addition to a term control group, a preterm control group was selected. In this way the gestational age between the preterm preeclamptic group and the preterm control group did not significantly differ. As the gestational age might affect the immunological outcome, this influence of time was ruled out. In chapter 7 a non donor IVF group was included to serve as a control group for ED pregnancies, both had comparable hormonal treatment before pregnancy. Furthermore, for all our tissue included, we delicately defined the location of immunological importance. On a protocol basis we collect placentas of pregnancies with our interest. Biopsies at three locations of the placenta and fetal membranes are taken. If there would be a difference in immune regulation at different locations, this potential bias is ruled out by this approach.

We used mixed lymphocyte cultures (MLC or mixed lymphocyte reactions) to measure T cell alloimmune responses. When allogeneic leukocytes are cultured together, T cell populations expand. The total proliferation of lymphocytes is measured by monitoring the uptake of 3H thymidine, during cell division. In this test the stimulators are inactivated by radiation, and are not able to proliferate. CD4+ T cells are critical for this reaction, and therefore the HLA class II mismatch plays an important role. In our studies, HLA typings of all mothers and children were performed and the number of mismatches between mother and child were calculated. For the performance of the MLCs, control cells were selected on the basis of the number of HLA-DR mismatches.

The immunohistochemical protocols were extensively tested for every antigen described in this thesis. For every test a negative control was used, to test for the specificity of the antibody involved. We also reduced non-specific background staining. The main cause of non-specific background staining is non-immunological binding of the specific immune sera by hydrophobic and electrostatic forces to certain sites within tissue sections. Since some antibodies showed non-specific background staining, we reduced this by blocking with serum. Furthermore, tissue may show endogenous peroxidase activity, resulting in non-specific staining. The solution commonly used for eliminating endogenous peroxidase activity is by the pretreatment of the tissue section with hydrogen peroxide prior to incubation of primary antibody. The precision, by which we tested our stainings, resulted in optimal staining for every antibody used. We used digital analysis methods to analyze our staining intensity, as a consequence objective results where gathered. Only the relevant areas were selected for analysis. We focused the analysis of the immunohistochemical

staining on the decidua basalis and the decidua parietalis. These are the maternal parts of the placenta and fetal membranes respectively; hereby the immune interactions at the fetal-maternal interface were studied. Maternal blood floats through the placenta in the intervillous space. The analysis of maternal peripheral blood provides information on the systemic maternal immune responses. It contains trophoblast micro particles, shed from the syncytiotrophoblast layer of the villi. By the analysis of the decidua basalis, decidua parietalis and the maternal peripheral blood, the three different fetal-maternal interfaces were studied. To better characterize the relative proportion of the studied cell populations of maternal peripheral blood, the results of the flow cytometry analysis were presented as the fraction of cells in the CD45+ and CD3+ fraction. The separate fractions did not significantly differ. Therefore, we expressed the results as a percentage of the absolute number of CD45+CD3+ cells.

The term fetal allograft is widely used. The mother accepts the immunologically foreign fetus during uncomplicated pregnancy, like an engrafted organ is tolerated [9]. The consequence of this understanding is that immunopathological recognition of fetal antigens might be viewed as an alloimmune reaction like graft rejection [10]. Despite the resemblances there are arguments against this model. The fetal derived cells, which invade the maternal decidua, use several immunological escape mechanisms. These mechanisms are not present during organ transplantation. For example, the villous trophoblast expresses no HLA antigens on its surface, and extravillous trophoblast expresses a very particular set of HLA antigens. Therefore, the use of the term allograft should not be used carelessly. However, many common mechanisms determining graft and fetal outcome exist. Pregnancy and organ transplantation both reflects a precise balance between pro acceptance and anti rejection stimuli. Analysis of immune reactions shows that graft rejection shares many similar mechanisms with recurrent spontaneously abortions and preeclampsia [9]. Decreased graft rejection and successful pregnancy outcome is associated with the presence of unique suppressor cells producing elevated levels of type 2 cytokines. It has been shown that the rejection of allograft and spontaneous abortions are associated with elevated type 1 cytokines [9]. However, acceptance solely based on a type 2 phenomenon is oversimplified since type 1 cytokines are as well necessary to avoid rejection of the (fetal) allograft [10].

Future perspective

In this thesis uncomplicated ED pregnancies were investigated, and considered the fetus as fully allogeneic. The placentas were collected after nine months uncomplicated pregnancy. It would be valuable to study also the immunological interactions upon embryo transfer. After transfer the maternal cells meet the allogeneic fetal cells for the first time. This is an interesting time point to investigate the maternal immunological response. Collection of local tissue is limited at this time point, but the peripheral immune response might as well be altered. Furthermore, it would be very interesting to analyze those pregnancies which fail to succeed in the beginning. Only 30% of embryo transfers after ED succeed, thus 70% of all pregnancies after ED, are not continuing [11]. The embryo transfers which result in miscarriage might have a very interesting immunological basis, possibly playing a role in the pathology of a miscarriage. Tissue of these pregnancies is therefore viable to study, and might give more insights in the immunological interactions, already during the implantation phase of pregnancy. The pathological mechanism of preeclampsia during ED pregnancies might be different compared with naturally conceived pregnancies. This thesis focused on uncomplicated ED pregnancies. However, investigation of preeclamptic ED placentas is essential to confirm this hypothesis.

In addition to ED pregnancies, mole pregnancies (hydatidiform mole) are as well fully allogeneic towards the mother. A complete mole pregnancy is entirely derived from the paternal genome. It is caused by a single sperm combining with an egg which has lost its DNA. The genotype is

typically 46,XX (diploid), which is due to subsequent mitosis of the fertilizing sperm. It is also possible that an empty egg is fertilized by two sperms, which may result in the 46,XY genotype. Immunological interactions between mother and the mole pregnancy are possible comparable with the immunology in ED pregnancies.

Recurrent miscarriage represents a complication in pregnancy, is also supposed to have an immunological pathophysiology. There must be a reason why in those maternal and paternal combinations the couple is not able to get pregnant. Immune cells at the fetal-maternal interface of recurrent miscarriage pregnancies might reveal the underlying immunological mechanism.

Conclusion

In conclusion, this thesis studied uncomplicated pregnancies and two immunological challenges during pregnancy, preeclampsia and ED. First, uncomplicated pregnancies were studied and we found that pregnancy is characterized by changes in cytokine production and composition of peripheral blood leukocytes, which is not reflected in the proliferative response to the fetus. There seems to be an optimal balance between maternal protection against infections and fetal tolerance. Second, pregnancies complicated by preeclampsia were investigated. We found a differential distribution and phenotype of decidual macrophages in preterm preeclamptic pregnancies, which may explain why less immunoregulation takes place in preeclampsia placentas. Finally, ED pregnancies were analyzed leading to the hypothesis that preeclampsia might have different pathophysiological mechanism compared with normal and non donor IVF pregnancies. Furthermore, the resemblances between solid organ transplantation were discussed and we showed that ED pregnancies lead to more maternal complications and the immunoregulation in ED and IVF pregnancies is altered compared with normal pregnancies. We found differential immunological interactions in successful ED pregnancies compared with naturally conceived pregnancies.

These results indicate that preeclampsia and ED pregnancies are indeed immunological challenges during pregnancy. It is a scientific challenge to further reveal the immunological mechanism of preeclampsia and ED pregnancies, contributing to precious information for the fields of immunology, transplantation and obstetrics.

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Nederlandse samenvatting
Publications
Curriculum Vitae
Dankwoord
Abbreviations

Ontwikkeling van de placenta

Zwangerschap is een interessante immunologische situatie. Gedurende negen maanden is de moeder zwanger van een foetus die zowel maternale (moederlijke) als paternale (vaderlijke) genen bij zich draagt, zonder dat er een immunologische afstoting plaatsvindt door de moeder. De herkomst van de foetus en placenta ligt in een klompje cellen, de blastocyste, dat in de baarmoeder groeit. De foetus komt niet direct in aanraking met maternale cellen, maar de placenta groeit wel in de uterus. Gedurende de eerste weken van de zwangerschap begint het complexe proces van de placenta ontwikkeling. De placenta wordt opgebouwd door trofoblastcellen, deze cellen vormen de barrière tussen de foetus en de moeder. Twee belangrijke subtypes van trofoblast bestaan: cytotrofoblast stamcellen en syncytiotrofoblast. Het syncytiotrofoblast is een dikke laag van cellen samengevloeid, en dus uit meerdere celkernen bestaat. Cytotrofoblast stamcellen zijn in staat om zich te vermenigvuldigen in nieuwe trofoblastcellen, die de basis vormen van syncytiotrofoblast of welke differentiëren in extra villeuze trofoblastcellen. Extra villeuze trofoblastcellen kunnen ingroeien in de decidua, spierweefsel en de spiraal arteriën van de uterus. In de arteriën zorgen trofoblastcellen voor een vergroting van de diameter, resulterend in bloed toevoer van de moeder richting de placenta. Op deze manier krijgt de groeiende foetus voldoende voedingsstoffen en zuurstof aangeleverd. De invasie van foetale cellen in de moederlijke decidua is verder verantwoordelijk voor het verankeren van de placenta aan de uterus. In gezonde zwangerschappen wordt dit proces strikt gereguleerd, over-invasie kan namelijk schadelijk zijn voor de moeder, en inadequate invasie zal leiden tot zwangerschapscomplicaties zoals pre-eclampsie (zwangerschapsvergiftiging).

Immunologie en het foetale-maternale grensgebied

De locatie waar de placenta ingroeit in de uterus, de decidua basalis, vormt één van de drie foetale-maternale grensgebieden. Het tweede gebied bevindt zich meer richting de foetale kant van de placenta, waar het syncytiotrofoblast een laag over de villi vormt. Het maternale bloed stroomt in de intervilleuze ruimtes en komt hier dus in direct contact met de foetale syncytiotrofoblast. Het laatste grensgebied ligt in de foetale vliezen. Deze vliezen bestaan uit vier lagen. De eerste laag, vanuit de foetus gezien, is het amnion gevolgd door het chorion, de trofoblast en aan de maternale zijde de decidua parietalis. De trofoblastcellen in de vliezen zijn een populatie van extravilleuze trofoblastcellen en komen direct in contact met de maternale zijde van de vliezen, de decidua parietalis.

Deze drie foetale-maternale grensgebieden worden in dit proefschrift onderzocht. In deze gebieden zorgen verschillende immunologische mechanismen ervoor, dat de foetus niet wordt afgestoten door de moeder. Voor het ontstaan van een immuunreactie is het onderscheid tussen lichaamseigen en niet-lichaamseigen van belang. Dit onderscheid is ook van belang bij de acceptatie van getransplanteerde organen. Humane Leukocyten Antigenen (HLA) van de donor zijn niet-lichaamseigen antigenen waartegen de ontvanger na een orgaantransplantatie een afstotingsreactie kan ontwikkelen. T cellen zijn cellen van het immuunsysteem die de vreemde HLA moleculen kunnen herkennen en een afstotingsreactie kunnen veroorzaken. De T staat voor thymus (zwezerik). T cellen vormen een belangrijk onderdeel van het specifieke immuunsysteem van de mens. De macrofaag is deel van de niet-specifieke immuniteit. Macrofagen zijn in staat antigenen te presenteren op het celoppervlak. De T cel kan deze antigenen herkennen en hierop weer vermenigvuldigen en differentiëren, wat uiteindelijk kan leiden tot een immuunreactie. Het uiteindelijke doel van een immuunreactie is indringers of veranderde lichaamseigen cellen (bijvoorbeeld bij tumoren) te verwijderen. Onder indringers vallen bacteriën, parasieten en virussen. Maar cellen van een getransplanteerd orgaan, of de niet-lichaamseigen foetus

kunnen ook als indringer worden gezien, en daardoor dus een immuunreactie uitlokken. De trofoblastcellen hebben verschillende mechanismen ontwikkeld die ervoor zorgen dat zij kunnen ontsnappen aan een aanval door het moederlijke immuunsysteem. Tijdens de zwangerschap zorgt het moederlijke immuunsysteem voor een onderdrukking van deze reactie tegen de foetus, zodat de zwangerschap voldragen kan worden. Deze onderdrukking vindt voornamelijk lokaal in de baarmoeder plaats, zodat het immuunsysteem in de rest van het lichaam intact blijft om daar bescherming te bieden tegen indringers.

Verschillen in het immuunsysteem tussen zwangere en niet-zwangere vrouwen worden besproken in **hoofdstuk 2**. In het perifere bloed van deze vrouwen wordt gekeken of er verschillen zijn tussen het fenotype van de T cellen, en hoe zij reageren wanneer deze cellen in een laboratorische setting samen worden gebracht met cellen van haar eigen kind, een vreemd kind of een vreemde volwassene. In deze test wordt de mate van vermenigvuldiging van de moederlijke T cellen gemeten. Wij vonden dat de T cellen tussen zwangeren en niet-zwangeren niet in hogere mate vermenigvuldigen na specifieke stimulatie, alhoewel er wel fenotypische verschillen van T cellen werden gevonden. Deze resultaten suggereren dat zwangere vrouwen een precieze balans vormen tussen de zorg voor optimale groei van de foetus, en ook voor handhaving van een adequate immuun reactie tegen infecties.

In dit proefschrift worden twee immunologische uitdagingen beschreven welke mogelijk veel vragen van het maternale immuunsysteem om de zwangerschap niet af te stoten. Dit zijn pre-eclampsie en eiceldonatie zwangerschappen.

Pre-eclampsie

Pre-eclampsie (zwangerschapsvergiftiging) is een zwangerschapscomplicatie die gekarakteriseerd wordt door hypertensie en eiwitverlies. In ongeveer 5% van alle zwangerschappen komt pre-eclampsie voor en het is een belangrijke oorzaak van de maternale morbiditeit en mortaliteit in de westerse wereld. Ondanks uitgebreid onderzoek blijft de precieze oorzaak van pre-eclampsie onduidelijk. Pre-eclampsie kan leiden tot levensbedreigende ziektes; eclampsie en HELLP. Wanneer er convulsies optreden tijdens de pre-eclamptische zwangerschap spreekt men van eclampsie. Het HELLP syndroom kenmerkt zich door multi orgaan falen. De foetus is vaak groei-vertraagd en het leidt vaker tot, al dan niet geïnduceerde, vroeggeboorte. De klinische symptomen van pre-eclampsie komen vaak pas tot uiting na de 20^{e} zwangerschapsweek. Veel onderzoek richt zich momenteel op het bepalen van een marker welke het ontstaan van pre-eclampsie kan voorspellen, echter deze markers zijn nog niet klinisch toepasbaar. Behalve symptoombestrijding is de enige effectieve behandeling van pre-eclampsie het verwijderen van de placenta. Dit wijst op het feit dat de placenta een belangrijke rol speelt in de ontstaanswijze van pre-eclampsie. De spiraalarteriën in de placenta zijn niet goed gevormd. De extravilleuze cytotrofoblastcellen infiltreren wel het deciduale gedeelte van de uterus, maar reiken niet tot het myometrium. Hierdoor ontwikkelen de spiraalarteriën een onvoldoende diametergrootte hetgeen resulteert in placentaire hypoperfusie. Deze verslechterde placentatie, samen met ischemie zorgen voor productie van factoren welke verantwoordelijk zijn voor systemische endotheel schade.

Pre-eclampsie wordt gezien als een immunologische aandoening. Het komt vaker voor bij eerste zwangerschappen, bij bepaalde families, en wanneer sperma-expositie voor de zwangerschap laag was. Mede hierdoor wordt verondersteld dat eerdere blootstelling aan paternale antigenen beschermend werkt tegen het ontstaan van pre-eclampsie. In **hoofdstuk 3** worden zwangerschappen gecompliceerd door pre-eclampsie besproken. Macrofagen zijn witte bloedcellen welke in vergelijking met andere witte bloedcellen in hoge mate voorkomen in de maternale decidua. Verstoring van het type en aantal macrofagen kan een rol spelen in de pathofysiologie

van pre-eclampsie. Placenta's van ongecompliceerde, pre-eclamptische zwangerschappen zijn verzameld en vervolgens door middel van immunologische kleuringen is gekeken naar het type macrofagen in de decidua. Wij vonden dat in de decidua van pre-eclamptische zwangerschappen, naar verhouding, minder immuunregulerende macrofagen voorkwamen. Mogelijk leidt deze verstoring in vergelijking met ongecompliceerde zwangerschappen tot het ontstaan van pre-eclampsie.

Eiceldonatie

Pre-eclampsie wordt in de literatuur vergeleken met de immunologische reactie verantwoordelijk voor de afstoting van een transplantatie orgaan. Bij een orgaantransplantatie wordt een lichaamsvreemd orgaan in het lichaam van de ontvanger geplaatst. Deze situatie is vergelijkbaar met de zwangerschap tot stand gekomen door eiceldonatie. De moeder is zwanger van een totaal lichaamsvreemd kind, het bevat genen van de vader en van de eiceldonor. Immunologische kennis betreffende de acceptatie van de zwangerschap kan in een breder perspectief worden toegepast bij de acceptatie van transplantatie organen en auto-immuunziekten.

In **hoofdstuk 4** wordt het voorkomen van pre-eclampsie bij een eiceldonatie zwangerschap besproken. Twee zwangerschappen worden met elkaar vergeleken: een tweeling zwangerschap tot stand gekomen na in vitro fertilisatie (IVF) met de eicel van de moeder zelf en een tweeling zwangerschap ook tot stand gekomen door IVF, maar met donor eicellen. In de IVF zwangerschap ontstaat pre-eclampsie en is er ernstige groeivertraging van beide kinderen, en in de eiceldonatie zwangerschap ontstaat eveneens pre-eclampsie, maar hebben de kinderen een normaal geboortegewicht. Mogelijk is de pathogenese van pre-eclampsie anders in eiceldonatie zwangerschappen, de totale genetische mismatch tussen moeder en kind kan hier een rol in spelen.

De hypothese dat het immunologische mechanisme bij eiceldonatie zwangerschappen en de acceptatie van orgaantransplantaties vergelijkbaar zijn, wordt onderbouwd in **hoofdstuk 5**. Bij de acceptatie van een totaal lichaamsvreemde foetus, zoals bij de eiceldonatie zwangerschappen, spelen mogelijk immunologische mechanismen een rol, die ook van toepassing zijn bij de acceptatie van een lichaamsvreemd orgaan. Kennis van deze mechanismen kan mogelijk toegepast worden in het veld van de transplantatie geneeskunde.

Ook al biedt eiceldonatie de mogelijkheid aan onvruchtbare vrouwen toch zwanger te worden, deze zwangerschappen gaan in vergelijking met spontane zwangerschappen gepaard met meer maternale complicaties. Deze klinische en immunologische aspecten van de eiceldonatie zwangerschappen worden uiteengezet in **hoofdstuk 6**. De moeder heeft vaker hypertensieve aandoeningen (zoals pre-eclampsie), keizersneden en vaginale bloedingen. De moeder leidt dus aan meer complicaties in eiceldonatie zwangerschappen, maar de klinische uitkomst van de foetus is net zo goed als in spontane zwangerschappen.

De immunologische regulatie in eiceldonatie en IVF zwangerschappen is anders dan in spontane zwangerschappen (**hoofdstuk 7**). De hormonale behandeling in deze zwangerschappen beïnvloedt het klompje cellen, dat later de foetus wordt, waarschijnlijk op een zodanige wijze, dat het terug te zien is in de placenta na geboorte. Ook in het moederbloed vonden wij verschil in het fenotype van de cellen van eiceldonatie en IVF zwangerschappen vergeleken met spontane zwangerschappen. Opvallend was dat bij een toename van het genetisch aantal verschillen tussen moeder en kind bij een eiceldonatie zwangerschap, de moeder meer geactiveerde T cellen in haar bloed had. Er lijkt een optimale balans te zijn tussen de afstoting en acceptatie van de foetus.

Dit proefschrift heeft twee immunologische uitdagingen tijdens de zwangerschap onderzocht, de resultaten onderbouwen dat eiceldonatie en pre-eclamptische zwangerschappen inderdaad een provocatie van het moederlijke immuunsysteem vormen. Nu is het een uitdaging om het immunologische mechanisme van pre-eclampsie en eiceldonatie zwangerschappen verder te ontrafelen, deze kennis zorgt voor waardevolle informatie voor zowel de immunologie, transplantatie en obstetrie.

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Curriculum Vitae

Marie-Louise van der Hoorn werd geboren op 10 januari 1983 te Langeraar. In 2001 behaalde zij haar VWO diploma aan het Teylingen College locatie Leeuwenhorst te Noordwijkerhout. In het zelfde jaar begon zij aan haar studie Biomedische Wetenschappen, welke zij van 2003-2004 onderbrak voor een jaar M.F.L.S. bestuur (Medische Faculteit der Leidse Studenten). Zij haalde haar Bachelor diploma in 2005 waarna zij via de zij-instroom in het derde jaar van Geneeskunde startte. In 2009 studeerde zij af voor Geneeskunde en Biomedische Wetenschappen (cum laude). Voor Geneeskunde bezocht zij het St. Lukes Hospital in Malta voor een klinische stage op de afdeling cardiothoracale chirurgie. Tijdens haar Bachelor fase van Biomedische Wetenschappen heeft zij een half jaar aan het Karolinska Instituut in Zweden gestudeerd en tijdens haar Master fase is zij nog eens terug gegaan voor het vak immunologie. De afstudeerstage van de studie Biomedische Wetenschappen betrof het onderzoek naar de rol van macrofagen in pre-eclampsie, waar zij de prijs beste student Biomedische Wetenschappen 2009 voor ontving. Deze stage werd verricht in het lab Verloskunde in het Leids Universitair Medisch Centrum, te Leiden. De stage werd uitgebreid tot een promotie onderzoek op de afdeling Immunohematologie en Bloedtransfusie en de afdeling Verloskunde van het LUMC, onder begeleiding van Prof. dr. F.H.J. Claas, en dr. S.A. Scherjon. Dit onderzoek heeft geleid tot dit proefschrift. Tijdens deze onderzoeksperiode heeft ze tijdelijk als arts-assistent niet in opleiding gewerkt, in het Reinier de Graaf ziekenhuis in Delft, waarna zij in 2011 haar promotie onderzoek heeft afgerond. In juli 2011 begon zij haar opleiding tot gynaecoloog in Delft (Opleiders: H.A. Bremer en W.A. ter Harmsel) en het LUMC (Opleider: J.M.M. van Lith).

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Marie-Louise van der Hoorn

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3p	third party
APC	antigen presenting cell
BDCA	blood dendritic cell antigen
CD	cluster of differentiation
CPM	counts per minute
DAF	decay accelerating factor
DC SIGN	dendritic cell-specific intercellular adhesion molecule-3 grabbin nonintegrin
DC	dendritic cell
ED	egg donation
ELISA	enzyme linked immunosorbent assay
Eng	endoglin
FACS	fluorescent activated cell sorting
Flt	fmslike tyrosine kinase
FM	fetal membranes
Gal-1	galectin-1
HELLP	hemolysis elevated liver enzymes low platelets
HLA	human leukocyte antigen
IDO	indoleamine 2,3-dioygenase
IFN-γ	interferon-γ
IL	interleukin
IVF	in vitro fertilization
KIR	killer immuno-globulin like receptor
LUMC	leiden university medical center
M1	macrophages type 1
M2	macrophages type 2
MACS	magnetic activated cell sorting
MHC	major histocompatibility complex
MLC	mixed lymphocyte culture
MLR	mixed lymphocyte reaction
NK	natural killer
OD	oocyte donation
PBL	peripheral blood leukocyte
PBMC	peripheral blood mononuclear cell
PHA	phytohaemagglutinin
PLGF	placental growth factor
pSMAD	phosphorylated SMAD
RNA	ribo nucleic acid
sEng	soluble endoglin
sFlt	soluble fmslike tyrosine kinase 1
SI	stimulation index
TGF	transforming growth factor
Th	T helper
TNF	tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
UCB	umbilical cord blood
VEGF	vascular endothelial growth factor

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