Maternal recognition of the (semi) allogeneic fetus during implantation and pregnancy

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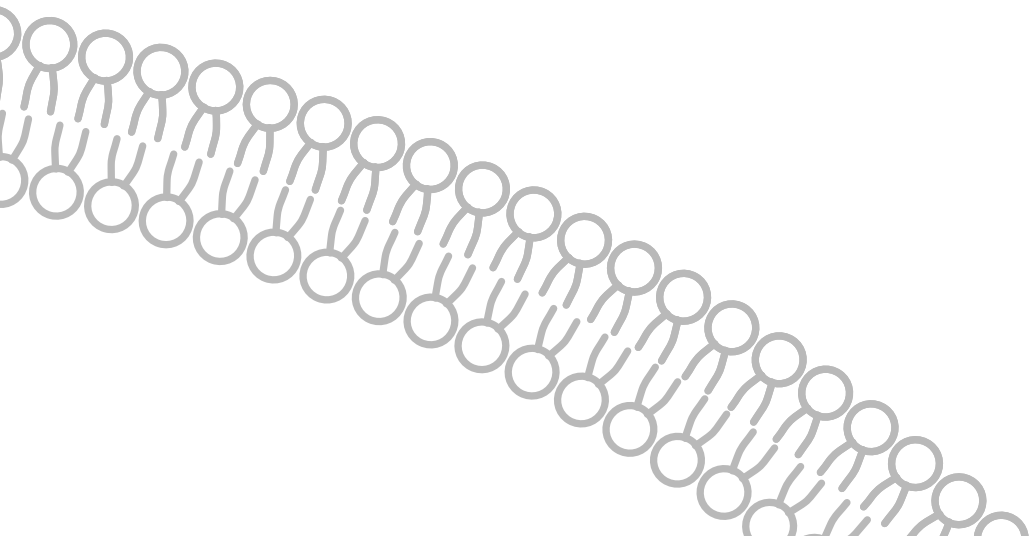
Chapter 1

General Introduction



Contents

Pregnancy is an immunological paradox

Pregnancy is a great challenge for the maternal immune system. The fetus has a genetic makeup equally derived from both the father and mother but escapes maternal rejection and is tolerated by several maternal and fetal mechanisms. The immunologist Medawar was in 1953 the first who proposed possible mechanisms to explain this 'immunological paradox of pregnancy' [1]; anatomical separation between mother and child to establish immune privilege of the uterus, immaturity of the fetal antigens and a diminished maternal responsiveness to pregnancy [1]. Nowadays it is clear that almost all aspects of his hypothesis are actually incorrect. The placenta is not a cell impermeable barrier, as there is evidence of entry of fetal material into the maternal circulation consisting of fetal cells (microchimerism), syncytiothrophoblast fragments, fetal DNA [2], and debris from apoptotic cells. Furthermore, fetal trophoblasts are in direct contact with maternal cells at a location known as the fetal-maternal interface and the uterus is not an immunologically privileged site as ectopic pregnancies, even at term age, occur.
There is no immaturity of the fetal antigens; the fetus is recognized by the maternal immune system by the expression of specific human leukocyte antigens (HLA) [3] and in fact this recognition seems to be essential for normal pregnancy [4].
And finally, though there is remission of several, mainly T cell mediated, auto-immune diseases and pregnant women are more vulnerable to certain viral infections and parasites [5], the maternal immune system is not inert during pregnancy and plays an active role in the success of pregnancy. Still, the actual mechanisms by which the maternal innate and adaptive immune system accepts the semi-allogeneic fetus during pregnancy are not completely understood. The aim of this chapter is to introduce some general aspects of immunology and describe certain aspects of the immune modulation during pregnancy- and pregnancy complications.

1 General Immunology

Immunology reflects the physiological mechanisms that humans and other animals use to defend their bodies from invasion by other organisms. In order to function properly immune cells must distinguish between the own healthy cells and pathogens like viruses, bacteria and parasites. Traditionally the immune system is divided into two components, the innate immunity and adaptive immunity, though several immune cells and cytokines are involved in both the innate and adaptive immune response. An immune response to infection starts with innate immune mechanisms that are fast, fixed in their mode and effective in stopping most infections at an early stage [6]. In vertebrates the adaptive system develops after a few days when the first two lines of defence, the physical barriers and innate immunity, fail to stop the invading pathogen. Unique features of the adaptive

or acquired immunity are its ability to distinguish one microorganism from another and to create immunological memory [6].

1.1 Innate immunity

As stated earlier, the first line of defence against pathogens that penetrate the epithelial surface is the innate immune response. This immunity is always available and does not improve with repeated exposure to the same pathogen.

The innate immune system consists of a cellular component, natural killer (NK) cells, mast cells, basophils, eosinophils, macrophages, neutrophils and dendritic cells, and a humoral component, chemokines, cytokines and the complement system. The macrophage and neutrophil act by engulfing and intracellular killing of microorganisms in a process called phagocytosis. Neutrophils, member of the polymorphonuclear cell family, are short living cells that are recruited from the circulation to the site of infection by the release of inflammatory mediators. Macrophages are on the other hand long-living cells that function in phagocytosis and in addition are capable of extracellular killing, contribute to tissue remodelling and act as antigen-presenting cells.

The natural killer cells can recognize infected- or tumour cells and are able to kill these cells directly by proteins facilitating entry into the cytoplasm and induction of apoptosis i.e., perforin and granzyme. These cells were named 'natural killers' because they do not require activation in order to kill cells that are missing 'self' major histocompatibility complex (MHC) class I molecules [7]. They express the killer immunoglobulin-like receptor (KIR), with an immunoreceptor tyrosine-based inhibition motif and a tyrosine-based activation motif. By interaction with 'self' ligands the inhibitory KIRs are inhibited and reactive to cells that miss the self MHC class I ligand. Furthermore, activating KIRS may cause NK cell activation after interaction with 'non self' MHC class I ligands.

All cells of the innate immunity can interact with cells of the adaptive immunity. An important cell in activating naïve T cells and modulating B cells is the dendritic cell which acts by antigen presenting [8]. Dendritic cells are distributed to almost all tissues, but are particularly prominent at mucosal surfaces.

As a major effector mechanism, the complement system plays a central role in innate immunity. It consists of more than 30 soluble- and membrane-bound proteins that are produced constitutively by the liver [6]. Activation of the complement system can occur via three pathways and although these pathways differ in how they are triggered, they all lead to C3 activation and deposition of C3b. The alternative pathway is triggered by changes in the physicochemical environment caused by constituents of some bacterial surfaces. The lectin mediated pathway is initiated by the mannose-binding lectin (MBL),

which binds to carbohydrates on pathogens. The classical pathway is part of both the innate and adaptive immunity and is activated by binding of C1q to IgG-or IgM immune complexes or to C-reactive protein on the pathogen's surface. The three complement activation pathways all result in the formation of the membrane attack complex (MAC), a tube-like pore inserted into the membrane which causes osmotic lysis of the target cell. Another important action of complement is the opsonisation of pathogens, which facilitates the uptake and killing by phagocytes. Finally, complement receptors clear immune complexes from the circulation, and the anaphylatoxic peptides C3a and C5a can recruit neutrophils and monocytes to the site of infection.

1.2 Adaptive immunity

In the innate immune response, pathogens are recognized by a fixed repertoire of cell-surface receptors and soluble effector molecules; genes encoding these immune molecules are stable and only occasional new variants arise. This is in great contrast with adaptive immunity, with almost an infinite number of different versions of ligand-specific T- and B cell receptors [6]. Moreover, during an immune reaction memory immune cells are generated that remain in lymphoid organs or mucosal tissue to respond more vigorously to a recurrent infection by the same pathogen.

1.2.1 B cell activation and antibodies

In coincidence with the innate immune system, the adaptive immune system has a cellular and a humoral component. The humoral response occurs after uptake of antigen by the B cell receptor (BCR), followed by interaction with primed T cells and costimulation through CD40L-CD40 and specific cytokines, producing antigen-specific antibodies a soluble form of the cell surface immunoglobulins (Figure 1) [6]. Essential for this antibody response is that the interacting T- and B cell are specific for the same, or different parts of the antigen. Antibodies are glycoproteins consisting of four polypeptide chains, two identical heavy chains and two identical light chains. The variable region (V region) is the location with hyper variable amino acid sequences and forms the antigen binding site. The remaining part of the heavy and light chains has limited variation and is called the constant region (C region). This C region specifies the immunoglobulin class and subclass; there are five classes or isotypes called IgA, IgD, IgE, IgG and IgM, 4 subclasses of IgG and two subclasses of IgA. Of the five isotypes, IgM is always the first antibody to be secreted in an immune response, as this is the BCR on naïve B cells. During differentiation isotype switching takes place, which means that some B cells start to produce antibodies of a different class that mediate other effector functions at different locations [9].

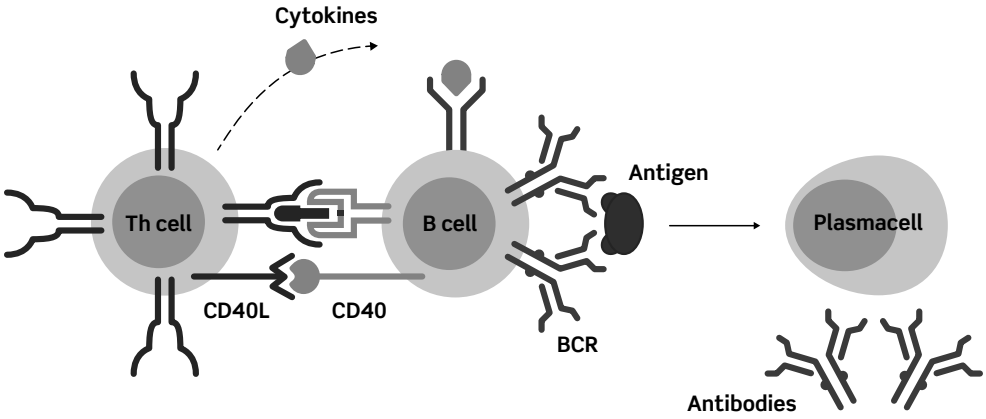


Figure 1. B cell activation and antibody production. Adjusted from [6]

The main functions of antibodies are neutralization and opsonisation. With neutralization antibodies bind tightly to a site of the pathogen, thereby neutralizing its toxic activity and preventing interaction with human cells. Opsonisation is the process in which IgG antibodies coat the cell surface of a pathogen. The constant regions can bind to receptors on a phagocyte and promote the ingestion and destruction by these phagocytes. Furthermore, IgM, IgG1 and IgG3 can activate complement through the classical pathway; C3b fragments then provide ligands for the complement receptor CR1 on macrophage and phagocytosis occurs. The initiated complement cascade can also lead to the formation of MAC and as a consequence lysis of the cell. Finally, antibodies bound to infected cells allow NK cells to kill them through antibody-dependent cellular cytotoxicity.

A fraction of B cells do not differentiate into antibody secreting plasma cells but become B memory cells that are able to respond rapidly when the antigen is encountered again [10].

1.2.2 T cell activation

Whereas B cells recognize whole molecules and intact pathogens, the T cell receptor (TCR) binds only to short antigens in the binding groove of the major histocompatibility complex (MHC), presented on the cell surface. T cell activation requires the interaction of the TCR with the appropriate MHC/ peptide complex and interaction of co-stimulatory molecule CD28 with CD80/CD86 on antigen presenting cells. Upon T cell activation, T cells express the high affinity IL-2 receptor and produce IL-2, which drives clonal expansion. After this expansion, $CD8^+$ T cells may differentiate into cytotoxic effector cells, whereas $CD4^+$ T cells develop into T helper 1 (Th1), T helper 2 (Th2), regulatory T cells or T helper 17 (Th17) cells [11], depending on the types of cytokines present in the location where T cell activation occurs (Figure 2).

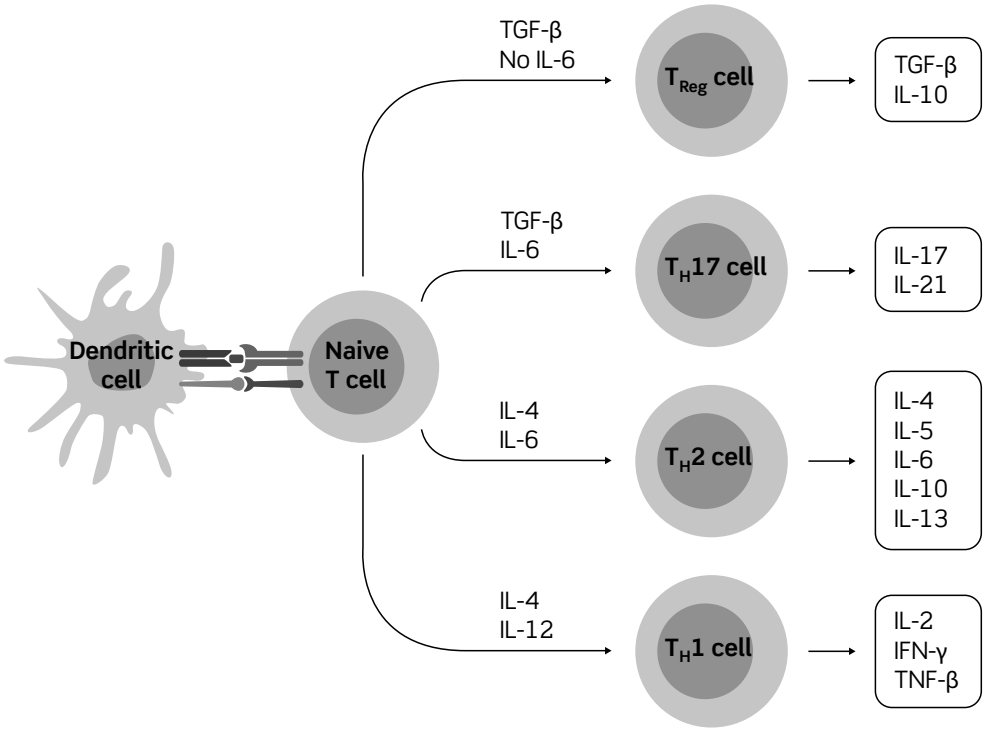


Figure 2. T cell activation and differentiation of CD4⁺ T helper subsets

In the presence of pro-inflammatory cytokines such as IL-4 and IL-12, CD4⁺ T cells develop into a Th1 or Th2 effector phenotypes [12]. A milieu dominated by TGF-β and devoid of pro-inflammatory cytokines directs CD4⁺ T cells into the tissue-protective T regulatory phenotype, whereas this regulatory cell is inhibited and Th17 cells are generated if inflammatory cytokines IL-6 and /or IL-21 are present along with TGF-β [13]. The Th1 cells synthesize interleukin (IL)-2, interferon (IFN)-γ and tumor necrosis factor (TNF)-β and induce cellular immunity, whereas Th2 cells synthesize IL-4, IL-5, IL-6, IL-10 and IL-13 and induce humoral immunity and thus stimulate B cells to produce antibodies [14]. Th17 cells mediate mainly immune responses against extracellular bacteria and fungi and play an important role in the induction of autoimmune diseases [15]. They are named after their production of IL-17, but also produce IL-21 and IL-22. After infection both CD4⁺ and CD8⁺ cells replicate and develop into memory cells that will elicit a stronger immune response in case of a reinfection with the same pathogen.

A specialized subset of T cells distinguished from the other classes by their role in tolerance as opposed to immunity is the regulatory T cell. This term actually refers to a family of T lymphocytes with suppressive/ regulatory properties: the naturally occurring CD4⁺CD25bright regulatory T cell (Treg) derived from the thymus and the peripherally

induced type 1 regulatory T cells (Tr1) and T helper 3 (Th3) regulatory cells. When mentioning regulatory T cells in this thesis we aim at the naturally occurring regulatory T cell. Regulatory T cells are characterized by the surface expression of CD25, although activated $CD4^+$ T cells also express this marker. Recent studies have shown that $CD4^+$ T cells expressing high levels of CD25 ($CD25^{bright}$) have regulatory capacity, while cells expressing intermediate levels of CD25 ($CD25^{dim}$) are activated T cells [16]. $CD4^+CD25^{bright}$ regulatory T cells are able to control immune responsiveness to self- and allo-antigens and are able to suppress autoimmunity[17]. Isolated peripheral $CD4^+CD25^+$ regulatory T cells furthermore express FoxP3, the gene encoding the transcription factor Scurfin. Scurfin deficient mice lack regulatory T cells and suffer from autoimmunity, conversely mice with overexpression of FoxP3 display increased immunosuppressive activity when compared to wild type mice [18,19]. To distinguish further between regulatory and activated T cells additional markers have been introduced, like the cytotoxic T lymphocyte associate protein 4 (CTLA-4), glucocorticoid-induced tumor necrosis factor receptor (GITR) and CD95 [20]. Low expression of CD127 is further used to identify regulatory T cells that is inversely correlated with FoxP3 expression [21]. However, all these surface markers can be dynamically expressed on other cell populations and functional tests remain necessary to distinguish Treg cells from related T cells.

Upon encounter of specific peptide in context of HLA class I and in presence of co-stimulatory signals, naïve $CD8^+$ T cells, characterized by the surface expression of CD45RA, CCR7 and CD27 and CD28, differentiate into cytotoxic effector $CD8^+$ T cells. These effector cells are capable of elicit a cytotoxic response due to their expression of cytolytic molecules perforin, granzymes and FAS ligand [22]. Perforin is a membrane perturbing protein that delivers granzymes in the target cell [23]. Granzymes consist of different subtypes inducing DNA fragmentation and apoptosis or alternative mechanisms to kill the target cell [23,24]. FAS ligand, as will be discussed further on, induces apoptosis in cells expressing FAS. $CD8^+$ T cells may also differentiate into regulatory or suppressor T cells, though the phenotypic markers and dynamics of these processes remain controversial [25]. In vitro, a $CD8^+$ suppressor T cell has been generated, characterized by the $CD8^+CD28^-$ phenotype, which is believed to suppress immune responses by induction of inhibitory receptors on antigen presenting cells by direct cell-cell contact. The in vivo relevance of this subset remains however unclear [26].

1.3 HLA molecules

Whether or not to mediate a response by the adaptive immune system is dependent on the recognition of 'self' and 'not-self'. The MHC antigens are highly polymorphic glycoproteins, meaning that a single locus may have numerous alternative forms. The main function of MHC antigens is peptide presentation to T cells. In humans, the MHC is called the human leukocyte antigen (HLA) complex, located on the short arm of chromosome 6 (Figure 3).

HLA molecules are co-dominantly expressed, both alleles are expressed at similar extent, and every person inherits one allele from the father and one from the mother. The chance that two siblings have an identical HLA genotype is therefore 25%, although meiotic recombination of genes within the HLA complex can lower this percentage.

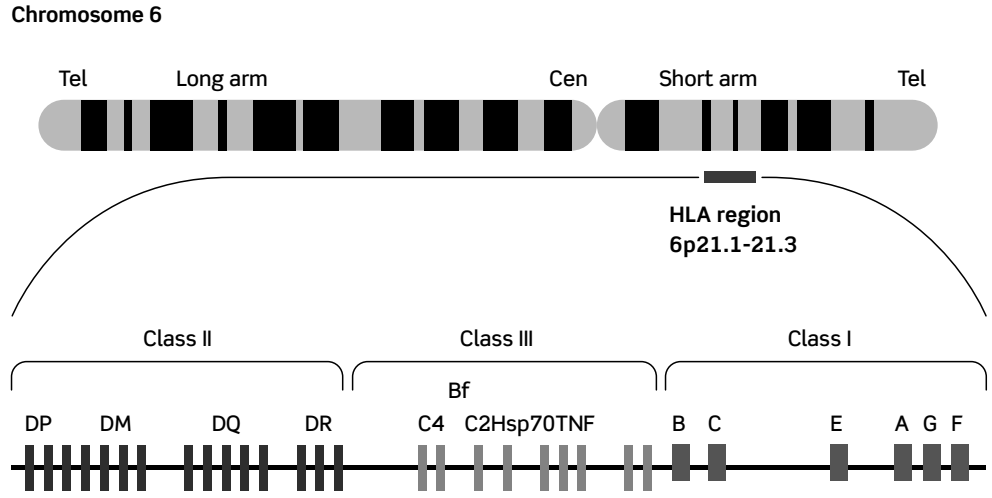


Figure 3. Localisation of the HLA complex on chromosome 6. Adjusted from [27]

The MHC is divided into three regions; class I, II and III. The class I region includes the classical or class Ia genes, HLA-A, HLA-B, HLA-C, and the non-classical or Ib genes HLA-E, HLA-F and HLA-G. The molecules consist of a transmembrane α chain, made up of three extracellular domains α1, α2 and α3, non-covalently complexed with the non-MHC encoded protein β2-microglobulin. HLA class I molecules are presented on all nucleated cells and on platelets. Their function is to present antigens to $CD8^+$ T cells and form ligands for receptors on natural killer (NK) cells. The function of HLA-F is however unknown; it seems monomorphic and remains intracellular [6]. Furthermore, five MHC class II isotypes exist; HLA-DM, HLA-DO, HLA-DP, HLA-DQ and HLA-DR. These molecules consist of two transmembrane chains (α and β), each with a domain for peptide binding and an immunoglobulin-like supporting domain. The HLA class II molecules are constitutively expressed on antigen presenting cells (APC), like dendritic cells, macrophages and B cells, though HLA-DM en -DO are intracellular and regulate the loading of other HLA class II isotypes, which present peptides to CD4 T cells [6].

The class III region, positioned on the chromosome between class I and II, also contains genes involved in immune response, though not directly involved in antigen processing or T cell presentation [6]. This high density of non-HLA genes includes genes for complement components (C2, C4), heat shock protein (Hsp70) and cytokines (TNF).

1.4 Cytokines

Cytokines are glycoproteins produced by T cells and macrophages (a.o.) and are involved in signaling between cells during immune responses. They act by binding to specific receptors, setting off a cascade that leads to induction, enhancement or inhibition of a number of cytokine-regulated genes in the nucleus [28]. Today, over 100 different human cytokines have been discovered in immunology, virology and hematology explaining their wide variety in nomenclature. We highlight only the cytokines relevant for this thesis.

As already mentioned, proliferation and differentiation of T cells is driven by the cytokine interleukin (IL)-2, which is synthesized by the activated T cell itself. Besides its potent T cell growth factor activity, IL-2 induces proliferation of natural killer (NK) cells and augments their cytolytic activity [29], is essential for activation-induced cell death (AICD) [30] and drives the development of regulatory T cells [31]. IL-2 signals through a receptor complex consisting of IL-2 receptor alpha (CD25), IL-2 receptor beta (CD122) and IL-2 receptor gamma (CD132), which is now known to be shared by IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 [32]. Normal pregnancy is characterized by inhibition of a IL-2 mediated type 1 immune response, whereas preeclampsia was found to be associated with elevated production of IL-2 [33].

IL-6 is a multifunctional cytokine that has important roles in inflammatory response and in directing T cell differentiation in adaptive immunity. It is produced by various cells, including macrophages, dendritic cells, lymphocytes and placental trophoblasts [34]. Elevated IL-6 is frequently evident in the altered cytokine profiles of women with unexplained infertility, recurrent spontaneous miscarriages, and preeclampsia as this excessive bioavailability possibly inhibits generation of CD4+ regulatory T cells required for pregnancy tolerance [35].

IL-10 is a cytokine produced by T regulatory cells and Th2 cells. It inhibits T cell activation and production of cytotoxic cytokines (IL-12 and IFN-γ) but stimulates induction of regulatory T cells [36], suppressing a Th1 response [11]. IL-10 can also be produced by Th1 cells, NK cells, macrophages, B cells and dendritic cells.

As mentioned, IL-17, a pro-inflammatory cytokine IL-1 that induces the expression of many mediators of inflammation, is produced by Th17, a recently discovered T cell population involved in the maternal immunomodulation [37,38]. These cells are closely related to regulatory T cells and differentiate upon inflammatory signals whereas conditions that promote tolerance favor generation of Treg [39]. 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.

Transforming growth factor beta (TGF-β) is a protein with well-described immunosuppressive effects that controls cell proliferation and differentiation. It is secreted by many cell types, including macrophages, in a latent form; serum proteinases such as plasmin catalyze the formation of active TGF-β. TGF-β is part of a superfamily of proteins known as the transforming growth factor beta superfamily, which are closely associated with tissue remodeling events and reproductive processes [40]. TGF-β superfamily members are expressed in the endometrium and are supposed to have role in preparation events for implantation, particularly in promoting decidualisation of endometrial stroma, possibly by acting on uterine NK cells to downregulate their cytotoxicity leading to an uterine-specific phenotype [40]. Furthermore, TGF-β is required for the development of regulatory T cells and this specialized T cell subset in turn synthesizes (a.o.) TGF-β to mediate their suppressive action.

Tumor necrosis factor alpha (TNF-α) is a cytokine involved in systemic inflammation mainly produced by activated macrophages and other cell types such as $CD4^+$ lymphocytes and NK cells. In response to TNF-α, vascular endothelial cells make platelet-activating factor (PAF), triggering blood clotting. This prevents leakage of blood and prevents further pathogens from entering the blood. In the pre- and peri-implantation uterus TNF-α is overexpressed, though it has limited expression during established pregnancy [41] suggesting an important role of this cytokine during implantation.

2 Immunology during implantation and placentation

2.1 Leukocytes during the menstrual cycle

Leukocyte subsets change dynamically during the menstrual cycling. Each cycle starts with shedding of decidua when implantation has not occurred; the menstrual phase. After menstruation, during the follicular phase, estrogen levels increase which induces the regeneration of endometrium. Neutrophils, eosinophils and mast cells are recruited to the endometrium, probably to protect the regenerating endometrium [42]. Meanwhile, from a cohort of ovarian follicles one dominant follicle, or Graffian follicle, will mature. While estrogen suppresses production of LH from the anterior pituitary gland in the follicular phase, continuing high level of estrogen increases LH production exponentially. The surge in LH secretion is followed by extrusion of the dominant follicle through the capsule of the ovary, an event called ovulation [43]. Directly after ovulation the endometrium starts decidualizing and preparing for implantation. A limited period during the midluteal phase of a normo-ovulatory cycle is referred to as the 'window of implantation' [44]. This is the most receptive period of the endometrium for implantation of the embryo. In preparation of this event, leukocytes accumulate in the uterus. uNK cells are present at low level pre-ovulation but 6-7 days after the LH surge increase in number to peak in the late luteal

phase [45]. The most abundant leukocyte population during the proliferative phase are T cells. Regulatory T cells accumulate before ovulation and decrease in number during the luteal phase [46]. The cell population that does not fluctuate is the macrophage; numbers remain constant throughout menstrual cycle and pregnancy [47].

2.2 Placentation

Upon fertilization, the dividing ovum travels down the fallopian tube to reach the decidualized endometrium around day 6 or 7 post conception. The potential embryo undergoes several mitotic divisions and at the 128-cell stage the blastocyst is formed, which consists of an inner cell mass, destined to become the embryo and an outer cell mass, destined to form the placenta [48]. The human blastocyst implants with the trophectoderm cells overlying the inner cell mass establishing the initial contact with the uterine epithelial and maternal blood cells. The blastocyst attaches and penetrates into the decidua and uterine capillaries. Next two layers of the cytotrophoblasts (CTB) cells differentiate from the outer cell mass. The first forms a continuous syncytiotrophoblast (sTB) layer that covers the villous placenta [49]. The second subset of CTB cells consist of an underlying layer of mononuclear cytotrophoblast progenitor cells; the extravillous trophoblasts (EVT). These cells migrate into the decidua as far as the inner myometrium, attaching the placenta to the mother. By destroying the smooth muscle walls of the spiral arteries and replacing the endothelium, the blood flow to the intervillous space is increased which is required for normal fetal growth and development [50].

By day 8 post conception spaces begin to appear within the sTB layer, forming a series of lacunae separated by trabeculae of syncytiotrophoblasts. These lacunae fuse to form the utero-placental circulation, after 12 weeks the foetus depends on the placenta for its demand for oxygen and nutrients [51]. At term pregnancy, the fetal- maternal interface is composed of the amnion and chorion, of fetal origin, while the decidua is of maternal origin. Three distinct locations of fetal-maternal contact can be identified at this stage. Extravillous trophoblasts interact with maternal cells at the decidua basalis, non-invading syncytiotrophoblasts of the chorion contact the maternal part of the decidua parietalis and, as discussed earlier, fetal material is released into the maternal circulation.

Compared with the majority of other mammals, placentation in human is complex and highly invasive [51]. It was stated that such extensive invasion is possibly the result of the most obvious differences between humans and apes; bipedalism and enlarged brain size. Upright gait alters the redistributed blood flow to the uterus because of the pull of gravity. Moreover, to support the development of the large fetal brain, 60% of total nutrients are directed to this organ, compared with only 20% in other mammalian pregnancies [52].

2.3 Immune modulation in pregnancy

Many mechanisms are suggested to be involved in maternal immune modulation and acceptance of the allogeneic fetus during pregnancy. In subsequent paragraphs we will give an overview of the known fetal and maternal mechanisms.

2.3.1 HLA-expression

The human trophoblast shows a remarkable expression of HLA on its cell membrane. The syncytiothrophoblast layer lacks both MHC class I and class II molecules [50], although it is reported that the syncytium produces a soluble isoform of the HLA-class Ib antigen [53]. HLA class II genes, the main target for alloreactive T cells are not translated in human trophoblast cells. The extravillous invading trophoblast cells do express one HLA class Ia molecule (HLA-C) and the non-classical antigens HLA-E, and -G, to avoid NK cell mediated cytotoxicity [50].

HLA-G was the first identified HLA class Ib molecule [3,54] and is unusual in its characteristics. Only a few alleles have been identified and its mRNA has undergone multiple splicing, resulting in 4 membrane bound (HLA-G1, G2, G3 and G4) and 3 soluble isoforms; sHLA-G5, G6 and G7 [55,56]. HLA-G has a restricted tissue expression, including the placenta and membranes, the thymus, eyes, and various types of tumour and stromal cells during inflammation and malignancy [57]. From the genomic structure it was postulated that the primary function of HLA-G is not antigen presentation but acting as an inhibitory ligand for NK cells macrophages, T cells and possibly B cells, by interacting with leukocyte immunoglobulin-like receptor (LIR), immunoglobulin-like transcript (ILT) and killer immunoglobulin-like receptor [3,55,58]. Trophoblast HLA-G binding to ILT expressed by uterine dendritic cells influences regulatory T cells to down regulate the adaptive immune response in the uterus [59]. The ILT2 receptor is also found on a small percentage of uterine NK (uNK) cells, though it binds HLA-G at low affinity [60]. Furthermore, by interacting with the KIR2DL4 receptor on uNK cells it stimulates the production of a range of cytokines, chemokines and angiogenic factors, which in turn promotes the trophoblast invasion and blood vessel development associated with normal implantation. Throughout pregnancy, the trophoblast expression of HLA-G is decreasing as gestation progresses [49,61]. The soluble isoforms of HLA-G, especially sHLA-G5 and sHLA-G6, are reported to possess immunosuppressive functions by an apoptotic effect on activated $CD8^+$ T cells [53] and suppression of an allo-proliferative response [62]. Furthermore, experimental data suggests that the development of a Th2 cytokine response (see paragraph 2.3.3) is associated with high concentration of sHLA-G and maintenance of pregnancy [63]. The level of soluble HLA-G was found to be associated with a specific HLA-G allele [64]; insertion of a 14 basepair segment in exon 8 of the 3'UTR affects HLA-G mRNA stability [65] resulting in lower levels of soluble HLA-G [66]. In addition, HLA-G gene expression and protein level regulation is associated with polymorphism in the promotor region or

5'upstream regulatory region [67]. A single nucleotide polymorphism (SNP) at position -725C>G may alter the methylation profile resulting in a modification of gene expression.

HLA-E is expressed by EVT and binds to CD94/NKG2A^{bright} cells on NK cells. It was shown that the binding affinity of the inhibitory receptor CD94/NKG2A for HLA-E was higher than that of the activating CD94/NKG2C receptor. This binding activity is also dependent on the sequence of the bound HLA-derived signal peptide. An HLA-E molecule loaded with HLA-G leader sequence peptide binds both CD94/NKG2A and CD94/NKG2C with the highest affinity of all class I derived signal peptides[50,60,68]. In this way, HLA-G could influence the maternal response indirectly through presentation by HLA-E.

The only classical HLA molecule expressed on trophoblasts is HLA-C, which can be recognized by KIR on both NK- and T cells [69]. In addition, intact HLA-C on allogeneic fetal cells can be recognized directly by CD8$^+$ T cells and indirectly by CD4$^+$ T cells [70]. Trophoblast HLA-C is highly polymorphic, expressing a paternal allele on the cell surface and leading to cytokine and angiogenic factor production [59,61]. HLA-C is divided into two subtypes based on the amino acid at position 80 of the α1 domain, namely HLA-C1^{asn80} or HLA-C2^{lys80}. These two subtypes represent different KIR epitopes. The KIR can be divided into activating (B haplotype) and inhibiting (A haplotype) [71] and nowadays much research is focusing on the interaction between these two HLA-C types and two KIR haplotypes resulting in a balance of inhibiting and activating NK cells in (un)complicated pregnancy [72-75].

2.3.2 Decidual NK cells

The EVT encounter a wide range of maternal tissue leukocytes: CD56^{dim} CD16$^+$ and CD56^{bright} CD16$^-$ NK cells [76], CD14$^+$ macrophages [77], CD4$^+$/CD25^{bright} - and CD8$^+$/CD28$^-$ regulatory T cells [78,79] among others. The predominant cell population of lymphoid cells early in pregnancy consists of the uterine NK cell (70%), these numbers decrease during pregnancy untill 3% at term [80]. This suggest that NK cells play a crucial role in the development of the placenta [81]. The early decidua is further comprised of T cells (10%), macrophages (20%) and dendritic cells (2%). B cells are virtually absent [50,61]. There is a massive influx of macrophages, neutrophils and T cells with the onset of labor at term [82].

The NK cells in decidua have a phenotype that differs from most peripheral blood NK cells; they express the NK cell marker CD56 at high levels and lack the marker CD16. Hereby they regulate immunological response mechanisms rather by cytokine supply than by their cytotoxic potential [83]. NK cells in the decidua play an important role in angiogenesis via the production of angiogenic factors such as TGF-β, angiopoietin (Ang) 1 and 2, vascular endothelial growth factor (VEGF) and placental growth factor (PlGF), since these factors enhance invasion of EVT [50].

$CD56^{bright}$ NK cell receptors which bind HLA class I molecules are CD94/NKG2 heterodimers, ILT (immunoglobulin-like transcript) and KIR [60]. The interactions with their ligands can be either activating or inhibitory. Uterine NK cells have, although in close contact with extravillous trophoblasts, no cytolytic activity against these cells, possibly because of constant stimulation of, for example NKG2D [84]. The killer-cell immunoglobulin-like receptors are encoded by a family of about 10 genes and exhibit an extensive degree of diversity, meaning that unrelated individuals often have a different KIR genotype [85]. Nevertheless, two distinct KIR haplotypes can be distinguished: haplotype A and B. The A haplotypes has seven KIR loci with only one activating receptor; KIR2DS4. The gene for this receptor may have a deletion resulting in no activating receptors on the haplotype A. Most of the activating receptors (KIR2DS1, 2, 3, 5 and KIR3DS1) are present on haplotype B, as well as two inhibitory receptors (KIR2DL2 and KIR2DL5)[85]. KIR mediated NK responses in individuals with 2 copies of the haplotype A (AA) are thus mainly inhibitory [86]. The phenotype of uterine cells is skewed toward a higher proportion of NK cells expressing KIR's with specificities for HLA-C allotypes than found in peripheral blood from the same individual[87].

2.3.3 Th1/Th2 balance

Wegmann et al [88] were the first to show that normal pregnancy in mice is characterized by a dominance of the Th2 over the Th1 type immune response. Indeed, many studies show that a Th1 reaction, with mainly inflammatory response is often correlated with pregnancy complications whereas a Th2 cell induction generates non-inflammatory responses compatible with survival of the fetus [14,88-91].

Nowadays however, little consensus on this Th1/Th2 shift in peripheral blood in normal human pregnancy exists [91-93] since mice lacking Th2 cytokines undergo normal pregnancies with unaffected litter size [94]. Moreover, T cells are not the only producers of cytokines and Th1 cytokines actually seem to play an essential role very early in embryonic development [91]. More candidate mechanisms have been proposed to describe the balance between immunostimulation and immunoregulation during pregnancy. Saito et al [11] state that while the Th1/Th2 balance is shifted, Th3 and Tr1 cells, which produce immunosuppressive cytokines TGF-β and IL-10 respectively, regulate the Th1 cell-induced rejection.

Another important type of T cell in immunosuppression is the $CD4^{+}CD^{25}$bright regulatory T cells, playing an important role in induction of tolerance to paternal antigens during pregnancy. Indeed it was shown that regulatory T cells prevent rejection and are needed for successful pregnancy in mice [95]. The decidua of an abortion prone mouse model was shown to contain IFN-γ producing T cells (Th1 cells), Tregs from normal pregnant mice could inhibit proliferation and cytokine production of these Th1 cells [96]. Moreover,

transfer of Tregs was shown to prevent fetal resorption of mice [96,97], most probably by enhancing Th2 cytokine production (IL-4 and IL-10) [98]. As in mice, reduced levels of Tregs were found in the decidua of recurrent miscarriage patients [99], as in the decidua of patients with preeclampsia [100]. There are three possible mechanisms by which CD4+CD25bright Treg induce immunoregulation. The first mechanism is by cell-to-cell interaction via membrane-bound TGF-β, LAG-3, Galectin 1, and CTLA-4, though exact mechanisms have not been fully clarified [11]. Secondly, cytokines such as IL-10 and TGF-β produced by Treg cells inhibit T cell activation and the third mechanism is when CTLA-4 expressed on Treg induces indolamine 2,3-dioxygenase (IDO) expression by dendritic cells and macrophages [11].

Closely related to regulatory T cells are the newly discovered Th17 cells, producing selectively IL-17. This cell population plays a critical role for the induction of inflammation and pathogenesis of autoimmune diseases and rejection. Th17 and regulatory T cells appear to share a common lineage; upon stimulation with IL-6 or IL-1 the differentiation of naive T cells is towards the Th17 cell pathway, whereas in the absence of IL-6 the conversion of Th17 is suppressed and the expression of Foxp3 is induced [20,101]. The above has led to the proposal of a new paradigm: the Th1/Th2/Th17 and Treg paradigm [101].

In addition, besides $CD4^{+}CD25^{bright}$ regulatory T cells also $CD8^{+}CD28^{-}$ T cells have been shown to have a suppressive capacity. Our group has recently identified higher percentages of $CD4^{+}CD25^{bright}$ and $CD8^{+}CD28^{-}$ T cells in decidua compared to peripheral blood suggesting an important role for these T cell subsets locally at the fetal maternal interface [78].

2.3.4 Decidual macrophages

The second largest subset of leukocytes in the early pregnant decidua are the macrophages. It is suggested that this density of macrophages in uterine tissue remains high during pregnancy and increases significantly during labor [80]. Upon fertilization, circulating monocytes migrate in the decidua and differentiate into tissue resident macrophages. Macrophages stimulate the differentiation of other leukocyte subpopulations and induce the expression of factors that activate leukocytes. In the decidua, two types of macrophages exist; pro-inflammatory $CD163^{-}$ type 1 macrophages which produces IL-12 and immune modulatory $CD163^{+}$ type 2 macrophages which produce high levels of IL-10. Decidual macrophages constitute mainly of the M2 type [102] and express low levels of co-stimulatory molecules CD80 and CD86, implying that they may induce tolerance of maternal T cells [103].

2.3.5 IDO

Indolamine 2,3-dioxygenase (IDO) is an enzymatic protein that is synthesized by syncytiotrophoblast, decidual macrophages and possibly dendritic cells. IDO catabolizes tryptophan and supposedly inhibits T cell proliferation by tryptophan depletion or production of toxic metabolites [104]. Removal of this T cell suppression by inhibiting IDO led to extensive inflammation, complement deposition and hemorrhagic necrosis at the feto-maternal interface [105].

2.3.6 Complement regulatory proteins

In transplantation, complement activation is an important effector of graft rejection [106]. In order to sustain the 'fetal graft', complement inhibition is an absolutely requirement. Indeed, uncontrolled complement activation is prevented by three membrane bound regulatory proteins: decay accelerating factor (DAF), membrane cofactor protein (MCP) and CD59 [105] and soluble regulators. DAF accelerates the destruction of the C3 convertase enzymes that activate C3 and amplify the classical and alternative complement pathways. MCP is a cofactor for Factor I-mediated degradation and inactivation of C3b and C4b, thereby preventing further activation and amplification of the complement cascade. Finally CD59 prevents the assembly of MAC, thereby blocking the lysis effect of this complex [107]. Furthermore, several soluble regulators as factor I, factor H, and C4 binding protein, affect enzymes involved in the alternative pathway.

2.3.7 FAS ligand

The expression of FAS-ligand (FASL or CD95L) on the trophoblast induces apoptosis of FAS expressing CD4+ T cells or CD8+ T cells and NK cells via their FAS receptors [108]. The role of this FASL mediated apoptosis is however still controversial. FASL was observed in syncytiotrophoblasts, on the cytotrophoblast and on decidual leukocytes [109], suggesting to be a mechanism protecting the trophoblast against activated leukocytes. The expression of FASL however does not appear to be mandatory for pregnancy success [110].

3 Pregnancy complications and oocyte donation pregnancy

In this thesis we focus on the maternal recognition of the semi-allogeneic fetus in uncomplicated pregnancies and in complicated pregnancies. We hypothesize that no, or incomplete adjustment of the immune response may result in pregnancy complications. The reproductive disorders studied are recurrent implantation failure, recurrent spontaneous miscarriages and preeclampsia. It is postulated that the primary pathogenesis of these pregnancy complications are shared [111], though the clinical

manifestations of this process present at different time-points in pregnancy (Figure 4). These poor pregnancy outcomes are frequently occurring clinical problems and until now no preventive strategy or effective treatment can be given.

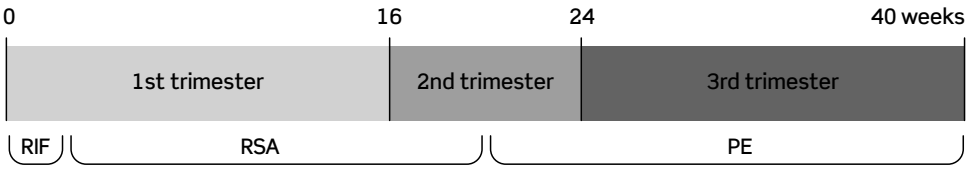


Figure 4. Timeline of manifestation of the pregnancy complications recurrent implantation failure (RIF) recurrent spontaneous miscarriages (RSA) and preeclampsia (PE)

In addition we examine oocyte donation pregnancies in which the maternal immune system must tolerate an often completely allogeneic fetus. These pregnancies represent an interesting model to study immunological interactions relevant for solid organ transplantation [112].

3.1 Recurrent implantation failure

Embryo implantation is a process that is not easily achieved in nature; 30% of human conceptions are lost due to failure of implantation [113]. In human in vitro fertilization (IVF), although apparently viable embryos are transferred into an adequately prepared endometrium, even a higher percentage of implantation failure is suggested [114]. The implantation of the embryo after transfer indeed is the major success rate-limiting step in IVF. Since 1978, the technique of IVF has been adjusted to improve outcomes, and nowadays it is easy to achieve an average of 15 oocytes and fertilization of up to 70% of the eggs. However the average live birth rate remains under 30% and this percentage has not increased in the last ten years [115]. Therefore, to improve IVF success a better understanding of the mechanisms responsible for implantation failure is necessary.

In contrast to natural cycles, IVF treatment subjects the ovaries to hormonal and mechanical manipulation, which results in the production of numerous oocytes, but in turn may negatively affect the normal process of cytoplasmic and nuclear maturation [116]. Moreover, the embryos and gametes are subjected to an artificial milieu of culture media which may also reduce the chance for successful implantation [116]. The complexity and abundance of the process of implantation makes a single definition of implantation failure difficult to determine [117]; the most frequently used criteria of implantation failure are repeated, consecutive failure of high quality embryos to implant following three attempts at IVF or the transfer of more than ten embryos in multiple transfers [118]. Unfortunately, because of the varied definitions used, the exact prevalence of recurrent implantation failure is difficult to determine. Furthermore, many variables may interfere with successful

implantation, including woman's age, indication for IVF, treatment protocol employed, embryo quality, number of embryos transferred, sperm quality, transfer technique and the primed endometrium. When studying implantation failure therefore these factors should be taken into account.

3.2 Recurrent miscarriages

Spontaneous miscarriage, the loss of pregnancy before the fetus has reached viability, is the most common complication in human pregnancy as 10-15% of recognized pregnancies will end in miscarriage [119]. The recurrence of this condition is obviously named recurrent spontaneous miscarriages, defined as the loss of three or more consecutive pregnancies prior to the 20th week of gestation and affecting around 1-2% of couples. Several eventual causes have been proposed, such as chromosomal or anatomical abnormalities, endocrine factors, infection, maternal thrombophilia or exposure to environmental factors (smoking, alcohol, radiation) [120]. Disappointingly, in about 50-75% of the couples no underlying cause for the recurrent pregnancy losses can be identified [121].

It was suggested that dysregulation of the bi-directional immunological relationship between mother and child may also play a role [122] and that recurrent spontaneous miscarriages could be considered as "graft-rejection-like" alloimmune reaction [123].

3.3 Preeclampsia

Trophoblast invasion is essential for establishing the utero-placental circulation; the invasion is a highly delicate balance. Excessive invasion at the surface of the myometrium, into or trough this muscle layer is a condition respectively known as placenta accreta, increta and percreta [124]. Inadequate trophoblast invasion and remodeling of the spiral artery leads to an inadequate blood supply in the placenta and ultimately to pre-eclampsia. Preeclampsia is a relatively common but potentially dangerous disorder in human pregnancy, significantly contributing to maternal and neonatal morbidity and mortality. It affects 1-7% of nulliparous women, who have a 3 fold higher risk than multiparous women [125,126]. It is a pregnancy-specific disease, characterized by endothelial dysfunction and presenting as maternal new-onset hypertension (diastolic blood pressure of ≥90 mmHg) or worsening of pre-existent hypertension and substantial proteinuria (≥300 mg in 24h) after 20 weeks of gestation [127]. Additional symptoms are headache, visual disturbances, oedema and epigastric pain [127,128]. A more severe form of preeclampsia is the HELLP syndrome, characterized by hemolysis, elevated liver enzymes, and thrombocytopenia. Furthermore, the syndrome can be accompanied with grand mal seizures; the life-threatening eclampsia [129].

Various factors have been described increasing the risk of a woman developing preeclampsia: maternal age >40 years, nulliparity, a history of preeclampsia, a family

history of preeclampsia, multiple pregnancy, obesity and several underlying medical conditions, such as pre-existent hypertension, renal disease, insulin dependent diabetes and antiphospholipid syndrome [130]. Strikingly, though it is referred to as a pregnancy-specific disorder which diminishes after delivery, women with a history of preeclampsia are at increased risk of future cardiovascular diseases [131].

The exact pathophysiological mechanism of preeclampsia is largely unknown, though it is believed that the pathological process originates in the placenta. A useful model for understanding the pathophysiology of preeclampsia is considering it as a two-stage model [132]. Stage 1 or early onset preeclampsia is characterized by the inadequate placental perfusion, resulting in reduced placenta volume and adverse perinatal outcomes as prematurity, intrauterine growth restriction and fetal distress [133]. In stage 2, or late onset preeclampsia, placentation is usually normal which results in normal fetal growth and better maternal and fetal outcome [134]. The question remains however what links these two stages.

As normal placentation requires development of immune tolerance between mother and fetus, it has been postulated that the immune system plays an important role in the aetiology of preeclampsia [71]. The immune maladaptation hypothesis of preeclampsia explains why preeclampsia is more common in women that have inadequate time to develop immune tolerance to paternally-derived antigens, such as first pregnancies, after change of paternity or prolonged interval between partners and among women using barrier contraceptives [135-137]. In addition, women who conceive via donor sperm [138,139] or via surgically obtained sperm [140] have an increased risk of developing preeclampsia. Multiple cycles with the same sperm on the other hand appears to elicit a protective effect [138] on preeclampsia.

3.4 Oocyte donation pregnancy

Oocyte donation (OD) is a specific method of artificial reproductive technology that resembles *in vitro* fertilization, with the exception that an (un)related donor is subjected to the hormone treatment and oocyte retrieval instead of the recipient. The first successful pregnancy achieved after OD was reported in 1983 [141], this pregnancy however ended in a miscarriage. A year later Lutjen et al reported the first birth after OD [142]. Since, worldwide thousands of oocyte donations have occurred to date. The original indication was premature ovarian failure [143], nowadays the indication has been extended to other forms of infertility including menopausal women [144], diminished ovarian reserve to multiple failed IVF attempts [145] and patients with a genetic trait precluding use of their own oocytes [146]. In their annual report on assisted reproductive therapies, the European Society of Human Reproduction and Embryology (ESHRE) publishes data of 22 countries performing oocyte donation [147]. In most of the countries with no data OD is

illegal, though in the Netherlands, showing no data in the ESHRE report, only commercial and anonymous donation is forbidden by law [148]. This obliges women to altruistic donation from family or friends, or to go abroad to find a suitable donor, paying between 3000 to 30000 euro per treatment [149]. Delaying childbirth and the increasing demand for infertility treatment have resulted in ~1% of US infants being conceived through assisted reproduction technologies [150]. In Europe approximately 5% of all IVF cycles are performed with donated oocytes; in 2009 a total of 21449 OD cycles were performed, existing from 16463 fresh and 5036 thawed transfers [147]. For fresh transfers, this resulted in 7507 pregnancies, giving a pregnancy rate of 45.7%. This pregnancy rate number is not associated with recipient diagnosis of oocyte donation [151]. In comparison, the pregnancy rate after fresh IVF aspirations is only 28.9% [147]. This lower pregnancy rate is surprising, as the techniques are to a large extent similar. However, contrary to the known cause of reproductive failure and reason for OD, for IVF a large portion of patients have unexplained infertility and an unknown mechanism responsible for the failure of implantation. Furthermore, although female age is a large predictor for success in IVF [152] and age-related infertility is nowadays the most common indication to perform OD, the pregnancy rate seems to depend on quality and age of the donor [153], rather than age of the uterus [154,155]. Remarkably, the actual delivery rates after OD and IVF are quite comparable; 5891 deliveries from a total of 21499 transfers after OD (fresh and thawed), resulting in a delivery rate of 27% and 25078 deliveries from a total of 110940 embryo transfers after IVF, resulting in a delivery rate of 23% [147].

Despite the continued increase in the number of oocyte donation pregnancies, relatively little is known about the underlying biology and long-term complications of this approach. Most of the attention in the literature regarding outcome in oocyte donor pregnancies has been paid to perinatal complications, such as preeclampsia, the type of delivery, and immediate neonatal problems, such as prematurity. Indeed, OD pregnancies are accompanied with an increase in early and late obstetrical problems [156], compared to spontaneous and in vitro fertilization pregnancies. There is a higher risk for pregnancy induced hypertension, caesarean section deliveries and bleeding complications in first trimester as well as postpartum [157-161]. The reasons for this higher incidence of complications remain unclear, though it is suggested that the incidence of pregnancy induced hypertension is higher if the oocyte donor is not genetically related to the recipient [162]. Indeed, pregnancies achieved from oocyte or embryo donation are unique since they have resulted from donor gametes whose entire fetal genome could be allogeneic to the mother (Figure 5) and therefore, OD pregnancies represent an interesting model to study complex immunological interactions.

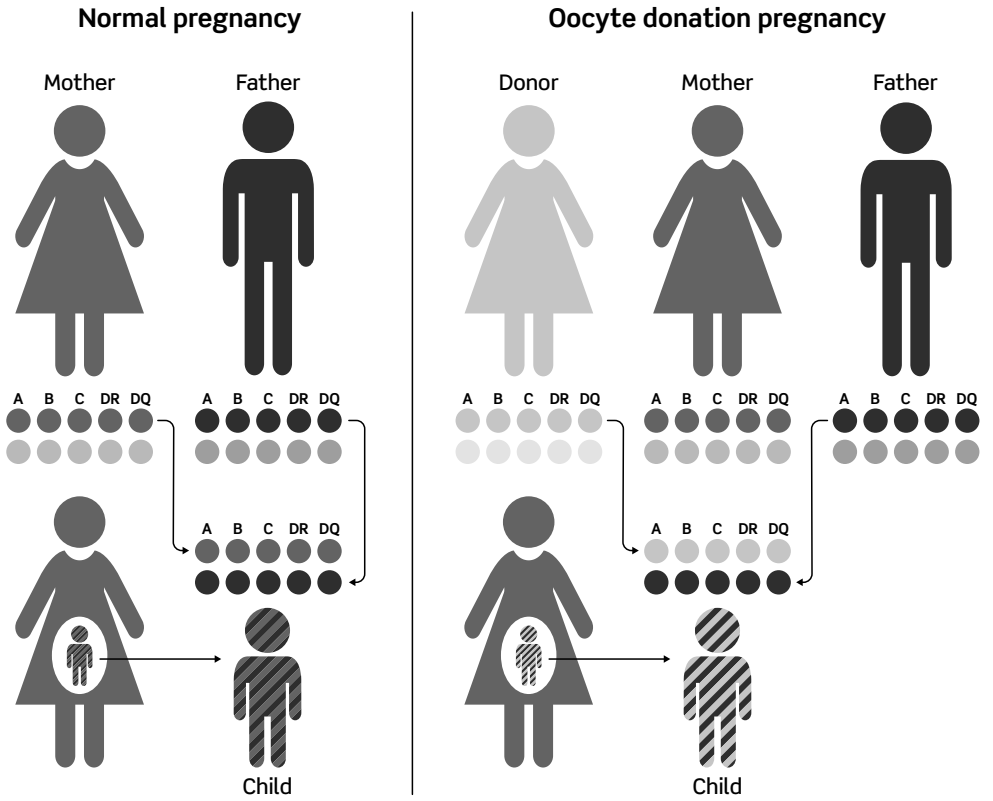


Figure 5. Inheritance of the most immunogenic HLA antigens (-A, -B, -C, -DR and -DQ) in a spontaneously conceived, normal, pregnancy and oocyte donation pregnancy. Adjusted from [163]

4 Outline of this thesis

In his attempt of explaining the immunological paradox of pregnancy, one of the proposals of Sir Peter Medawar was an impaired maternal response to fetal cells [1]; a diminished innate and cell-mediated or humoral adaptive immune response should lead to the acceptance of the semi-allogeneic fetus. Though this proposal has not stood the test of time, exact mechanisms explaining the maternal-fetal tolerance remain to be clarified.

In this project we focus on the maternal recognition by adaptive and innate immune system of the allogeneic antigen on fetal cells in uncomplicated pregnancies and reproductive disorders such as implantation failure, recurrent spontaneous miscarriages and preeclampsia. It is postulated that the primary pathogenesis of these complications are shared [111], though the clinical presentation is at different time-points in pregnancy. Unraveling the mechanisms of immunomodulation could help to elucidate these poor pregnancy outcomes, may affect diagnostic tools and suggestions for possibilities of

new immunomodulatory strategies and therapy. Furthermore, it may help understanding important concepts in transplantation, autoimmunity and possibly, neoplasia control.

First we investigate the maternal response and recognition of fetal cells in uncomplicated pregnancies. The peripheral proliferative response to fetal antigens in uncomplicated pregnancies is compared with non-pregnant controls in **Chapter 2**. As a result of maternal recognition during pregnancy of paternal alloantigens, 10-30% of women produce antibodies to fetal HLA [164]. The effect of these HLA antibodies on pregnancy complications is reviewed in **Chapter 3**. **Chapter 4** examines the formation of HLA antibodies in relation to the degree of immunogenetic differences between the fetus and the pregnant woman in uncomplicated oocyte donation pregnancies. As the fetal genome could be complete allogeneic to the mother OD pregnancies represent an interesting model to study immunological interactions relevant for transplantation. OD pregnancies complicated by preeclamspia are studied in **Chapter 5**. In **Chapter 6** we hypothesize that successful, continuing OD pregnancies show fewer immunogenetic mismatches as expected by chance, indicating maternal recognition of some HLA matches as being essential for pregnancy success.

Such a high degree of HLA-incompatibility normally does not occur in natural conceived pregnancies. On the other hand is too much histocompatibility among couples thought to be associated with certain pregnancy complications, as recognition of paternal antigens by the maternal immune system is essential for normal pregnancy. In **Chapter 7** we systematically review whether HLA sharing between partners, specific HLA allele frequencies or insertion into the HLA-G allele is more prevalent in patients with recurrent spontaneous miscarriages. These immunogenetic determinants are also prospectively determined in patients with recurrent implantation failure after IVF (**Chapter 8**). We furthermore determine the immunophenotypic profile and function of peripheral blood T lymphocytes in women with recurrent implantation failure after IVF in **Chapter 9**.

Chapter 10 provides a summary of the chapters and a general conclusion of this thesis. Moreover, implications for clinical practice and future research are discussed.