General Introduction

Tell me and I will forget
Teach me and I will learn
Involve me and I will remember
Chapter 1

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‘Self’ and ‘non-self’ recognition is one of the most important mechanisms for the immune system whether or not to mediate an immune response (1). During pregnancy the maternal immune system has to tolerate the persistence of ‘non-self’ (allogeneic) fetal cells in maternal tissue. Although many mechanisms have shown to contribute to the prevention of a destructive immune response to fetal cells mediated by maternal alloreactive lymphocytes, the immune acceptance of the ‘non-self’ (allogeneic) fetus in pregnancy is an immunologic paradox (2). The aim of this thesis is to determine whether maternal T cells contribute to fetus specific immune recognition and if mechanisms of fetus specific immune regulation exist in human pregnancy. A special emphasis is given to fetus specific immune recognition and immune regulation by maternal T cells at the fetal-maternal interface. This general introduction contains two parts. The first part on general immunology gives a brief overview on histocompatibility antigens and the immunologic mechanisms of alloimmune recognition. In addition, the mechanisms of T cell activation and T cell differentiation are described in more detail. The second part on immune regulation in pregnancy starts with a brief description of implantation and the formation of the fetal-maternal interfaces in human pregnancy. Furthermore, the different fetal trophoblast cell subsets and the mechanism of immune evasion by trophoblasts are described. The last part describes which maternal leukocytes are present at the fetal maternal interface and summarizes the current knowledge on fetus specific immune regulation by regulatory T cells during pregnancy.

1.1 GENERAL IMMUNOLOGY

Antigen (Ag) presentation by the Major Histocompatibility Complex (MHC) initiates an antigen specific immune response by T lymphocytes. MHC molecules are highly polymorphic complexes and in humans known as Human Leukocyte Antigens (HLA). HLA plays an important role in organ and tissue transplantation where the polymorphic residues serve as the main targets for allogeneic lymphocyte responses that can lead to allograft rejection. During pregnancy the HLA differences between mother and child are a potential target for immunologic rejection, however in uncomplicated pregnancy these HLA differences lead to immune acceptance. This introduction on general immunology will describe the different MHC molecules, provides an overview of the different pathways of allorecognition by different lymphocyte subsets and describes the mechanisms of Ag specific T cell activation and T cell regulation in more detail.

1.1.1 Histocompatibility antigens

Histocompatibility antigens are polymorphic proteins that play a major role in organ and tissue transplant rejection. The major histocompatibility complex (MHC) antigens are highly polymorphic proteins of which the main function is peptide presentation to antigen specific T cells. MHC can be divided in class I and class II molecules that can bind CD8+ and CD4+ T cells respectively (1). Minor histocompatibility antigens are polymorphic proteins that can serve as allopeptides in organ and tissue transplantation and cause transplant rejection.
MHC class I molecules are expressed on all nucleated cells and consist of 1 transmembrane α-chain and a β2-microglobulin (Figure 1a). The α-chain consists of 3 domains. The α1 and α2-domains form the peptide binding site and the α3 and β2-microglobulin stabilize the MHC complex. The α3 domain also contains a CD8 binding site so that CD8+ cells can adhere to the MHC class I molecules. Peptides presented by MHC class I molecules are mainly derived from intracellular self proteins and proteins derived from intracellular pathogens like viral proteins (1). Besides the function of peptide presentation to CD8+ T cells, a subpopulation of MHC class I molecules play a major role in Natural Killer (NK) cell activation and deactivation trough MHC class I receptors expressed on NK cells (3). In humans a group of classical MHC class I molecules (HLA-A, HLA-B, HLA-C) is found next to a group of non-classical MHC class I molecules (HLA-E, HLA-F and HLA-G). In contrast to the classical MHC class I molecules, the non-classical molecules contain limited polymorphisms, exhibit a limited peptide presentation function and are expressed on specific cell types. The non-classical MHC class I molecules play an important role in NK cell activation (HLA-E, HLA-G), induce regulatory T cells (HLA-G) (4) and may modulate APC function (HLA-G) (5). In contrast, HLA-E may also contain properties that activate allogeneic T cells and can bind viral as well as bacterial peptides (6,7), while the function of HLA-F is largely unknown (8).

MHC class II molecules are constitutively expressed on professional antigen presenting cells (APCs), like dendritic cells (DCs), macrophages and B cells. However, the presence of inflammatory cytokines can induce MHC class II expression on other cell types like T cells and endothelial cells. MHC class II molecules consist of 2 transmembrane chains that both contribute to the peptide binding site (Figure 1b). MHC class II molecules contain a CD4 binding domain so that CD4+ cells can adhere to the MHC class II molecules. Peptides presented in MHC class II are generally derived from extracellular agents. Here fore self proteins as well as proteins derived from extracellular pathogens like bacteria are taken up by endosomes and are processed and presented in MHC class II molecules. In Humans 3 types MHC class II molecules are found: HLA-DR, HLA-DQ and HLA-DP. The main function of MHC class II molecules is peptide presentation to antigen specific CD4+ T cells (1).

Minor histocompatibility antigens (mHag) are polymorphic proteins that can serve as allopeptides in organ and tissue transplantation and cause transplant rejection. In contrast to MHC that is highly polymorphic and contains a peptide presenting function, mHags are normal proteins that contain limited polymorphisms between donor and recipient. However, mHag can play role in graft versus host disease (GvHD) in bone marrow transplantation and may contribute to solid organ rejection. Hereby mHag negative T cells can recognize and respond to mHag positive cells. An example of a mHag is the male Y chromosome (HY) that can be recognized in female recipients. Hereby male HY peptides that are presented in MHC class I molecules on male donor cells can be recognized by female CD8+ T cells. This can occur when the male and female cells share the MHC class I allotype in which the HY peptide is presented. In addition, male HY proteins can be processed and presented in MHC class II molecules on female APCs. Subsequently, HY specific CD4+ T cells can be activated and initiate an immune response. Pregnancy of male fetuses can induce minor histocompatibility antigen-specific cytotoxic T cells to HY (9,10).
1.1.2 HLA recognition and the allogeneic response

The immune response made by an individual that is directed to antigens from another genetically different individual form the same species is called the allogeneic response. An allogeneic response includes the response to HLA matched as well as HLA mismatched organs and tissues and is the main cause of allograft rejection during transplantation. The allogeneic response is heterogenic and mediated by different immune cells using different immunologic pathways. The possible ways of HLA recognition and subsequent allogeneic response are schematically depicted in figure 2-4 and are explained in the next paragraphs.

**Direct HLA recognition** involves T cells that recognize intact allogeneic HLA-peptide complexes presented on the surface of donor cells. Direct HLA recognition includes CD8+ T cell activation by MHC class I/peptide complexes and CD4+ T cell activation of MHC class II/peptide complexes (Figure 2a). The frequency of T cells directly recognizing donor HLA is extremely high and includes T cell clones recognizing donor HLA irrespective of the bound peptide. In addition, direct allorecognition may also include primed T cell clones, originally designed to respond to viral peptides in self HLA, that recognize a similar conformation in donor HLA and peptide (cross reactivity) (11). In organ transplantation direct allorecognition contributes to initiation of acute rejection (12). During pregnancy maternal peripheral blood lymphocytes have shown to be able to elicit a direct cytotoxic T cell response to fetal cells (13). In addition, the proliferative response of maternal lymphocytes to fetal umbilical cord blood cells is comparable to the response to unrelated umbilical cord blood cells with a similar HLA-DR difference (14).

**Indirect HLA recognition** involves donor antigen uptake by recipient APCs. After processing and peptide presentation in context of MHC class II, antigen specific CD4+ T cells can be activated and initiate an immune response (Figure 2b). Allopeptides can be derived from allogeneic HLA molecules or minor histocompatibility antigens that differ between donor and recipient. The frequency of T cell clones involved in the indirect pathway is a ~100 fold lower than in the direct pathway and may play a role in chronic transplant rejection (15,16). During human pregnancy fetus specific CTL are found in maternal peripheral blood (10,13). Although the mechanisms by which these fetus specific T cells are induced are highly speculative, these cytotoxic T cells may be induced via the direct and/or the indirect allo recognition pathway.
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NK cells express killer immuno-globulin like receptors (KIRs) that can specifically recognize subtypes of classical and non-classical MHC class I molecules. KIRs comprise receptors with an immunoreceptor tyrosine-based inhibition motif (ITIM) and immunoreceptor tyrosine-based activation motif (ITAM). Inhibitory KIRs are inhibited by interaction with their specific self ligand (self recognition) and are alloreactive to cells that miss the specific self MHC class I ligand (missing self) (17) (Figure 3). Of most activating KIRs the MHC class I ligand is unknown. Although there is no experimental evidence supporting this, activating KIRs may cause NK cell activation upon encounter of the non-self (allogeneic) MHC class I ligands (non-self recognition) (Figure 3). NK cell alloreactivity can prevent engraftment in non-identical haematopoietic stem cell transplantation. In solid organ transplantation there is no evidence for a direct role of NK cell alloreactivity in acute graft rejection. However, interaction between NK cells and T cells may enhance a detrimental alloresponse in organ transplantation (18). In pregnancy NK cell activation through KIR plays a major role during implantation and facilitates infiltration of trophoblasts into maternal tissue. Furthermore, incompatibility of maternal KIR genotype and the fetal HLA-C KIR epitope leads to increased risk of pregnancy complications like pre-eclampsia (19) and may induce spontaneous abortions (20). However, the contribution of self-, missing self and non self recognition in decidual NK cell activation remains to be elucidated.

B cells can take up donor antigens via their B cell receptor (BCR). However, BCR-antigen interaction is not sufficient to cause B cell activation and antibody production. Interaction of B cells with primed T cells with similar Ag specificity is required and provides costimulation through CD40L-CD40 interaction (Figure 4). Thereafter clonal expansion, isotype switching, antibody secretion and memory B cell development occurs. The presence of donor specific HLA antibodies in the circulation of transplant recipients has a negative impact on transplantation outcome (21). During pregnancy fetus specific HLA antibodies are present in maternal serum of approximately 30% of the cases (22,23). Although the role of HLA antibody formation in pregnancy is unclear, there is no evidence for a negative effect pregnancy outcome. In contrast, the presence of anti-paternal antibodies is suggested to be associated with a higher live birth rate, whereas the absence of anti-paternal antibodies is associated with recurrent spontaneous abortions (24).
1.1.3 T cell activation and T cell differentiation

Binding of the T cell receptor (TCR) to the appropriate MHC/peptide complex alone is not sufficient to induce T cell activation. T cell activation also requires interaction of the costimulatory molecule CD28 with CD80/CD86 on APCs. The binding strength of the TCR-MHC/peptide complex determines whether or not unprimed (naïve) T cells are activated and differentiate. At low antigenic strength and in the absence of co stimulation, T cells die by neglect whereas too high antigenic strength and over stimulation leads to Antigen Induced Cell Dead (AICD). Upon the initial T cell activation, activated T cells express the high-affinity IL-2 receptor alpha (CD25), and produce IL-2. The secreted IL-2 binds the surface CD25 and drives clonal expansion of the activated T cells. To stop clonal expansion of T cells, the T cells express CTLA-4 (CD152) that similar to CD28 binds CD80/CD86. However, in contrast to binding of CD28, CTLA-4 inhibits further T cell proliferation. After the expansion phase, CD8+ T cells may differentiate into cytotoxic effector cells whereas CD4+ T cells develop into T helper 1 (Th1), T helper 2 (Th2) or regulatory T cells. Whether or not CD8+ T cells can also differentiate...
into regulatory or suppressor T cells is controversial. After clearance of the infection both CD4+ and CD8+ T cells form memory T cells that can elicit a rapid response in a subsequent infection with the same pathogen (1). The divergent differentiation pathways of CD8+ and CD4+ T cells are discussed in the next paragraph and depicted in figure 5 and figure 6 respectively.

**CD8 T cell differentiation and characterization**

studies have identified 4 phenotypically and functionally distinct CD8+ T cell subsets: naïve, effector, central-memory (CM) and effector-memory (EM) CD8+ T cells (Figure 5). **Naïve CD8+ T cells** have not yet encountered their specific antigen and are characterized by the surface expression of the CD45RA, the lymph node homing receptor CCR7 and presence of the costimulatory molecules CD28 and CD27. Upon encounter of the specific peptide in context of MHC class I and in the presence of correct costimulatory signals, CD8+ T cells differentiate into cytotoxic effector T cells. During **CD8+ effector T cell** differentiation CD8+ T cells lose expression of the lymph node homing receptor CCR7 and costimulatory molecules CD27 and CD28. For this reason CD8+ effector T cells do not contain a lymph node homing potential or potential to receive further costimulation. During CD8+ differentiation, effector cells gain expression of the cytolytic molecules perforin, granzymes and FAS ligand (FASL). Perforin is a membrane perturbing protein that delivers granzymes in target cells (25). In humans granzymes consist of different subtypes of which Granzyme B is essential to induce DNA fragmentation and apoptosis in target cells (25) whereas, granzyme A and granzyme K may provide alternative mechanisms to kill target cells (26,27). FASL induces apoptosis in cells expressing FAS. Upon clearance of the infection by CD8+ cytotoxic T cells and when no antigens remain, a small fraction of CD8+ T cells differentiate into Central-Memory (CM) T cells. **CM CD8+ T cells** express the T cell memory marker CD45RO and thereby the cells gain an increased survival potential. In addition, CM cells recuperate the lymph node recirculation potential by expressing CCR7. Hereby CM CD8+ cells can scan APCs in lymph nodes and can elicit a rapid response upon encounter of recall antigen during a re-infection with the same pathogen. When an acute infection is cleared but antigens remain present, possibly in the form of a latent viral infection, a small fraction of CD8+ T cells differentiate into Effector-Memory (EM) cells. **EM CD8+ T cells** express the T cell memory marker CD45RO but do not regain lymph node homing receptors and are therefore believed to stay at the infection site. Subtypes of EM cells maintain their cytotoxic capacity and contain an increased survival potential so they can directly elicit a cytotoxic response upon encounter of infected cells. EM subtypes can be identified using the combination of the costimulatory molecules CD28 and CD27. The phenotypes and main functions of the different EM subsets are depicted in Figure 5 (28). Although the CD8+ T cell differentiation process has been extensively studied and is depicted here as a step by step process, many controversies exist among additional marker that can be used to identify the CD8 T cell subsets. In addition, CD8+ differentiation is a dynamic process were also CM cells and EM cells may further differentiate and switch phenotype and function. CM and EM CD8+ T cells may differentiate back into effector cells. However the additional phenotypic markers and the dynamics of these differentiation processes are controversial (29,30).
Naive (N)
- unprimed cells
- IL-2 production
- survival potential

Effector (E)
- cytotoxicity
- perforin & granzyme
- IFNγ & TNFα production
- short lived (FAS+)

Effector-Memory (EM)
- proliferate to recall Ag
- survival potential
  EM-1 (CD27+CD28+)
  -> CM like cells
  EM-2 (CD27+CD28-)
  -> partial effector cells
  EM-3 (CD27-CD28-)
  -> effector like cells
  EM-4 (CD27-CD28+)
  -> CM like cells

Central-Memory (CM)
- proliferate to recall Ag
- IL-2, IL-4 production
- IFNγ & TNFα production
- survival potential

Neglect cell death due to low antigenic stimulation or absence of cytokines

AICD Antigen Induced Cell Death due to over stimulation by too high antigenic strength.

CD8+ Treg cells
- Existence is controversial.
- Expression of CD103.
- LAG-3 and CCL-4 are suggested CD8+ Treg markers. Cells possibly function via IL-10 secretion.

Figure 5. CD8+ T cell differentiation and characterization
**CD8+ suppressor T cells** have recently been identified by several groups and are believed to suppress immune responses by modulation of APCs or cytokine secretion. The first CD8+ Ts cell subset is characterized by the CD8+CD28- phenotype and induces the inhibitory receptors (ILT-3 and ILT-4) on APCs by direct cell-cell contact. Subsequently the APC inhibits CD4+ T cell proliferation (31). These CD8+CD28- Ts cells are generated after multiple rounds of allogeneic and xenogeneic stimulation in vitro, and therefore the in vivo relevance of this CD8+ Ts subset remains uncertain. Another CD8+ Ts subset is characterized by the expression of LAG-3 and mediates suppression through secretion of the CC chemokine ligand 4 (CCL-4). These CD8+ Ts cells are generated by in vitro expansion of CD8+ cells from in vivo primed donors, but can also be found in pathogen infected tissue (32). In addition, other markers like CD103+ have been suggested to identify alloantigen induced CD8+ Ts that may work via IL-10 secretion or cell-cell contact (33). Recently it has been shown that trophoblasts isolated from first trimester pregnancies can induce CD8+CD103+ suppressor T cells when co cultured with peripheral blood CD8+ T cells (34). For each of these CD8+ suppressor subsets additional studies are necessary to reveal their in vivo relevance and function.

**CD4+ T cell differentiation** occurs after CD4+ T cell activation with the appropriate peptide in context of MHC class II and in the presence of the correct costimulatory signals. CD4+ T cells can differentiate into T helper 1 (Th1), T helper 2 (Th2) or regulatory T cells (Treg) (Figure 6). Generation of Th1 cell requires the presence of IFN-γ and IL-12 production by APCs whereas in the absence IFN-γ and in the presence of IL-4, CD4+ T cells differentiate into Th2 cells (35). Subsequently Th1 cells produce high levels of IFN-γ and TNF-α and induce cellular immune responses where macrophages and cytotoxic CD8+ T cells are activated. Th2 cells produce IL-4 and IL-5 and provide B cell help that may result in antibody production. Upon clearance of the infection Th1 and Th2 cells can differentiate into Effector-Memory (EM) cells or Central Memory (CM) cells. Upon activation of CD4+ T cells with DCs that display a distinct activation status to DCs that induce Th1 or Th2 cells, CD4+ T cells can differentiate CD4+ regulatory T cells. Although research on Treg subsets is impaired by the lack of true functional Treg markers, 2 distinct types of induced Treg subsets, Tr1 and Th3 cells, have been identified based on their cytokine profile. Tr1 cells produce high levels of IL-10 that inhibits production of IL-12 and TNF by DCs and macrophages (36) whereas, Th3 cells secrete high levels of TGF-β and can inhibit Th1 (37) and NK cell responses (38).

**Naturally occurring CD4+ regulatory T cells** (Tregs) are thymic derived cells that are different to the peripheral induced Tr1 and Th3 cells (Figure 6). Naturally occurring Tregs are thought to play a major role in self tolerance. How natural Tregs are induced in the thymus is controversial, the TCR-MHC/peptide binding strength and presence of cytokines in the thymus may play a role in natural Treg generation. Natural Tregs are found within the CD25+ T cell population expressing high levels of CD25 (CD25bright), although, expression of CD25 is not exclusive for regulatory T cells. Effector T cells can also express high levels of CD25 while regulatory T cells can be found in the CD25- or CD25dim fraction (39,40). Additional markers like FOXP3, CTLA-4, GITR and activation markers like HLA-DR and CD69 can help to distinguish effector from regulatory cells. However, conclusions based on phenotypic characterization remain controversial (41,42). Until a specific marker for regulatory T cells is found, functional tests are required to identify and study Treg cells.
1.2 IMMUNE REGULATION AT THE FETAL-MATERNAL INTERFACE

Many mechanisms are suggested to be involved in maternal immune tolerance and immune acceptance of the allogeneic fetus during pregnancy. Fetal trophoblasts play an essential role in circumventing a maternal immune attack by altered HLA expression profiles and expression of inhibitory proteins like IDO and FAS. Nevertheless, maternal leukocytes potentially capable of an alloimmune response are present in decidual tissue but together with immune regulatory leukocyte subsets. This introduction on immune regulation at the fetal-maternal interface will therefore give an overview on the different fetal trophoblasts populations, the role of maternal leukocyte subsets present at the fetal maternal interface and will summarize current knowledge of CD4+CD25+ T regulatory cells in fetus specific immune regulation in mice and human pregnancy.

Figure 6. CD4+ T cell differentiation and Treg subsets
1.2.1 Implantation and formation of the fetal-maternal interfaces

Implantation occurs five or six days after fertilization when attachment of the blastocyst to the maternal endometrium takes place. In response to the blastocyst attachment and in the presence of progesterone, cells of the maternal endometrium enlarge and the number of spiral arteries increase. The cellular changes and endometrial vascularisation is called the decidual reaction. Within a few days after attachment the entire endometrium is transformed in decidual tissue. In early pregnancy 3 distinct decidual compartments can be identified; decidua basalis (d.basalis), decidua capsularis (d.capsularis) and decidua parietalis (d.parietalis) (Figure 7a). D.basalis is the maternal part of the placenta, d.capsularis lines the superficial part of the embryo and d.parietalis lines the remainder of the uterine cavity. During gestation as the embryo grows the d.capsularis merges with d.parietalis so that at term pregnancy only d.basalis and d.parietalis can be identified (Figure 7b). All decidual tissue is from maternal origin, whereas the extra-embryonic cells give rise to the fetal part of the placenta (villi) and the fetal membranes amnion and chorion (Figure 7a-b). Embryo development requires exchange of nutrients, waste products and gases between the maternal blood flow and the fetus. Development of the utero-placental circulation involves deep infiltration of fetal cells (trophoblasts) in the uterus and removal of the smooth muscle layer around spiral arteries to increase the maternal blood flow. In addition, fetal trophoblasts cells anchor in the uterus and give rise to extensive branched villi. Blood vessels develop inside the villi and via the umbilical cord vessels connect to the embryonic blood flow. After week 12 the space surrounding the villi (intervillous space) is filled with maternal blood for optimal nutrient exchange. Until the end of pregnancy, placental development continues with increased villi growth and branching to support the increasing demand for oxygen and nutrients from the fetus (43-45).

Three distinct fetal-maternal interfaces where fetal and maternal tissues connect can be identified in human pregnancy. 1. Maternal peripheral blood contacts the syncytiotrophoblast layer during utero-placental circulation. In addition maternal peripheral blood contains microparticles derived from the syncytiotrophoblast layer (46,47). 2. The decidua basalis, the maternal part of the placenta interacts with invading villous trophoblasts and 3. The decidua parietalis the maternal part of the membranes contacts the non-invading trophoblasts of the chorion. Each interface contains specialized fetal trophoblast cells with distinct HLA expression profiles and unique immune modulatory and immune stimulatory capacities (46,48-50). In addition, many differences in presence of maternal leukocyte subsets exist between maternal peripheral blood, decidua basalis and decidua parietalis (51,52).

1.2.2 Fetal trophoblasts and HLA expression

Upon the formation of the blastocyst and blastocyst implantation, the extra embryonic cells differentiate into several types of trophoblasts with a distinct function and immunomodulatory potential. Hereby fetal trophoblast can infiltrate maternal tissue and open up spiral arteries, develop villi for optimal nutrient exchange and form the fetal membranes. The morphology of the different trophoblast populations in the placenta and the membranes are depicted in Figure 8 and Figure 9 respectively (45,48).
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Villous trophoblasts form the placental villi of which the main function is to transport nutrients and gases from maternal blood to fetal cord blood. The placental villi consist of fetal blood vessels that are surrounded by cytotrophoblasts and covered by a syncytiotrophoblast layer. The inner cytotrophoblast layer grows out from the anchoring villi that attach the villi to maternal decidua. Cytotrophoblasts provide villous renewal by dividing and continuous replacement of the syncytiotrophoblast layer. The syncytiotrophoblast cell layer does not divide and sheds syncytiotrophoblast micro particles (STBM) in the maternal blood circulation (46). Villous trophoblasts and STBM lack HLA expression and do thereby not provoke an allogeneic response by circulating maternal T cells. However, syncytiotrophoblasts and circulation STBM may cause a systemic inflammatory response whereby especially the maternal innate immune system is activated (47).

Extra villous trophoblasts migrate from the anchoring villi into decidual tissue were they are called interstitial trophoblasts. With help from decidual NK cells, interstitial trophoblast move into spiral arteries were they destroy the arterial smooth muscle cell layer and replace the maternal endothelial cells. Inside the spiral arteries trophoblasts are called endovascular trophoblasts. Other types of interstitial trophoblast cells move deep into the maternal myometrium and fuse to become multinucleated trophoblast giant cells. Extra villous trophoblasts express HLA-C, HLA-E and HLA-G (8,53,54). HLA-G has shown to have potent immunomodulatory functions (4,5), whereas HLA-C and HLA-E may induce an allogeneic response by maternal NK cells and T cells (48).

Fetal membrane trophoblasts consist of anionic and chorionic trophoblasts, as well as multinucleated trophoblast giant cells. The amnion consists of 1 layer of amniotic trophoblasts surrounded by an impermeable collagen layer. The chorion contains chorionic trophoblast as well as multinucleated giant cells. In contrast to the trophoblast giant cells at the implantation site, giant cells in the fetal membranes seem less invasive. Amniotic and chorionic trophoblasts as well as trophoblasts trophoblast giant cells express HLA-C, HLA-E and HLA-G (8,53).

Figure 7. Development of the placenta, fetal membranes and deciduae at ~12 weeks (a) and at human term pregnancy (b)
1.2.3 Immune evasion by trophoblasts

Fetal trophoblasts circumvent a maternal immune attack by not expressing HLA-A, HLA-B, HLA-DR, HLA-DQ and HLA-DP molecules. However, as described in the previous paragraphs, specific trophoblast subsets do express HLA-C and the non-classical MHC class I molecules HLA-E, HLA-F, and HLA-G, whose functions are described in paragraph 1.2.1. Besides an altered HLA expression, trophoblasts also express a wide range of molecules that may play a role in immune evasion from maternal immune cells (55). In the next paragraph the most important immune modulating molecules are described. Although many functional studies on these molecules have been performed in mice models, their importance in the human system remains to be elucidated.

**B7 family members** (CD80/CD86) bind to CD28 molecules and provide co-stimulatory signals, which, in combination with TCR-MHC peptide recognition, activate T cells. Recently, new subtypes of B7 molecules have been identified, that are expressed on extra villous trophoblast cells (B7-H1, B7-H2, B7-H3) and syncytiotrophoblast cells (B7-H1, B7-DC). B7-H2 and B7-H3 stimulate Th2 responses and inhibit Th1 responses, whereas B7-H1 and B7-DC bind the CD28 family member PD-1 and are critically important to induce self-tolerance (49, 55). The absence of B7-H1 in mice results in loss of allogeneic but not of syngeneic fetuses. Showing that B7 molecules are critically important in maintenance of immune tolerance in murine pregnancy (56).

**Indoleamine-2,3-dioxygenase (IDO)** is a tryptophan-catabolising enzyme that inhibits T cell proliferation by tryptophan depletion or production of toxic metabolites (57). IDO is expressed on trophoblast cells but is also induced on APCs by IFN-γ. Chemical inhibition of IDO leads to gestation failure in allogeneic mice, however genetic elimination of IDO does not prevent allogeneic pregnancy (58, 59).

**FAS Ligand (FASL)** can induce apoptosis in FAS expressing cells, like activated CD4+ and CD8+ T cells. FASL is expressed on fetal trophoblasts as well as maternal decidual cells, however the role of FASL mediated apoptosis is still controversial. FASL+ fetal trophoblasts may induce deletion of fetus specific effector cells in murine pregnancy (60) whereas FASL+ decidual cells may limit infiltration of FAS+ maternal leucocytes (61, 62).

1.2.4 Maternal leucocytes at the fetal maternal interface

Decidual tissue is populated by many types of leukocytes, like NK cells, macrophages, dendritic cells, and T cells, whereas B cells are virtually absent. NK cells mainly consist of the uterine CD56+CD16- subset but also CD56+CD16+ NK cells are found. Decidual macrophages are abundant and may include pro-inflammatory CD163− type 1 macrophages (mf1) and immune modulatory CD163+ type 2 macrophages (mf2). Dendritic cells are rarely found and their function remains to be elucidated. Decidual T cells are very heterogenic containing CD4+ and CD8+ activated T cells and T cells with a merely regulatory function. In addition, minor subpopulations of T cells like γδTCR+ T cells, αβTCR+ CD4-CD8- T cells and NKT cells are found in decidual tissue.

**Decidual CD56+CD16− NK cells** constitute 50-90% of lymphocytes in early pregnancy decidua and although the number of decidual NK cells significantly decrease during pregnancy, the cells remain present until the end of pregnancy (52). Decidual NK cells
Figure 8. Cells at the fetal-maternal interface at the implantation site a) shows a trophoblast specific cytokeratin staining using immunohistochemistry, b) shows a schematic illustration of the cell types in placenta.
are a unique NK cell subset with many differences to peripheral blood NK cells. Like peripheral NK cells, decidual NK cells express perforin, granzyme A and B and many NK cell activating receptors like NKp30, NKp44, NKp46 and NKG2D. However, decidual NK cells contain reduced cytolytic activity to MHC class I negative targets (63) and secrete proteins with immunomodulatory potential (64). Decidual NK cells have also shown to express KIRs specific for HLA-C1 and HLA-C2 by which they may specifically recognize allogeneic fetal HLA-C. The combination of a maternal inhibitory KIR genotype in the presence of a fetal HLA-C2 ligand is associated with an increased risk for pre-eclampsia (19). However, the experimental basis for this observation remains to be defined. Furthermore, in vitro and in vivo migration experiments by Hanna et al. show that decidual NK cells but not peripheral blood NK cells can direct trophoblasts invasion by secretion of IL-8 and IP-10 (65). In addition, decidual NK cells but not peripheral blood NK cells produce angiogenic factors like vascular endothelial growth factor (VEGF) and placental growth factor (PLGF) that induced endothelial cell migration and formation of endothelial cell networks (65). Hereby decidual NK cells may regulate key developmental processes and at the fetal-maternal interface.

Decidual macrophages are the most abundant antigen presenting cells (APCs) at the fetal maternal interface and consist of >15% of CD45+ decidual leucocytes. Decidual macrophages have high expression of the activation markers HLA-DR and CD86 whereas CD80 expression is reduced. Many differences have been observed in decidual macrophage phenotype during gestation and also between d.basalis and d.parietalis macrophages. For example CD105, DC-SIGN and MMR expression is increased on d.basalis macrophages compared to d.parietalis macrophages. CD105 is an endoglin that binds TGF-β1, TGF-β3 and forms complexes with the TGF-β receptor, this data may indicate that TGF-β signalling is more predominant in d.basalis than d.parietalis. Furthermore, DC-SIGN+ macrophages are unusual and some classify DC-SIGN+ cells as dendritic cells. However, MMR and DC-SIGN expression may play an important role in tissue homeostasis by clearance of proteases and degraded extra cellular matrix products that may be released during trophoblast invasion and spiral artery remodelling (66). Furthermore, preliminary unpublished analysis using CD163 as a marker to distinguish pro-inflammatory type 1 macrophages (mf1) and anti-inflammatory type 2 macrophages (mf2) show that d.basalis contain both CD163+ and CD163- macrophages whereas d.parietalis contains only CD163+ macrophages. Mf1 cells are CD163- pro inflammatory macrophages that produce high levels of IL-12 and contain T cell stimulating potential. Mf2 cells are CD163+ anti-inflammatory macrophages that produce high levels of IL-10, contain high phagocytic potential but do not activate T cells (67,68). Although activated macrophages have an increased ability to inhibit trophoblast migration (69), experiments analyzing the functional capacities of decidual macrophages subsets are hampered by technical limitations described in chapter 7. Furthermore, CD4+CD25+FOXP3+ Treg cells have shown to induce alternatively activated macrophages expressing MMR and CD163+ (70). Interaction between decidual T cells and macrophages can therefore be crucial in determining differences between d.basalis and d.parietalis.
Figure 9. Fetal-maternal membranes a) shows a trophoblast specific cytokeratin staining using immunohistochemistry, b) shows a schematic illustration of the cell types in the membranes.

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**Dendritic cells (DCs)** are antigen presenting cells with a unique ability to initiate antigen specific immune activation or immune tolerance. In humans CD83+ DCs are increased in endometrial biopsies just before ovulation in comparison to other endometrial phases (71). In addition, DCSIGN+ DCs with poor T cell activation potential have been identified in early human decidua (72,73) whereas CD83+ DCs with potent T cell stimulatory capacity are sparsely found in early decidua (74,75,76) An immune regulatory potential of decidual DCs in pregnancy tolerance is often suggested but the experimental evidence is lacking and hampered by extremely low cell counts and many phenotypic similarities to decidual macrophages.

**Decidual T cells** comprise a very heterogenic subset of cells with major differences to peripheral blood T cells. Decidual T cells constitute ~10% of lymphocytes in early pregnancy decidual lymphocytes. The lymphocyte composition between individuals is highly variable however the percentage of T cells increases during pregnancy, so that at term pregnancy 40-90% of all lymphocytes are T cells. Decidual T cells consist of CD4+ and CD8+ T cells with an activated phenotype as well as cells with a regulatory phenotype. Many differences are found between d.basalis and d.parietalis T cells subsets (77). CD4+CD25+FOXP3+ regulatory T cells (Tregs) is the most extensively studied T regulatory cell subset in pregnancy and will be described in paragraph 1.3.3. CD8+ T cells comprise 50-60% of decidual T cells and although studies have shown that trophoblasts can induce CD8+ regulatory T cells (34). The in vivo function and Ag specificity of decidual CD8+ cells is not yet understood. Besides CD4+ and CD8+ T cells also atypical T cell subsets like γδTCR+ T cells (78), CD4-CD8- αβTCR+ and NKT cells (79) are found among decidual T cells.

**γδTCR+ T cells** in peripheral blood are mainly CD4-CD8- T cells and have been shown to recognize non-peptide antigens derived from microbes and plants. In addition, γδTCR+ T cells may recognize and respond to alloantigens (80,81). In contrast, γδTCR+ T may also contain immune-regulatory properties and may provide mechanisms of self tolerance in peripheral tissues (82,83). Decidual γδTCR+ T cells are mainly CD4-CD8- T cells that contain cytolytic potential by expressing perforin, granzyme A and B and FASL (84). However, decidual γδTCR+ T cells also express IL-10 and TGF-β providing them with an immuno modulatory potential. (85).

**αβTCR+ CD4-CD8- double negative (DN) T cells** have shown to specifically down regulate immune responses to allo antigens in vitro and in vivo. Furthermore, DN T cells can specifically kill activated CD4+ and CD8+ T cells with a similar TCR specificity. In addition, studies in mice have shown that infusion of DN T cells prolong donor specific graft survival (86). Human decidua contains an increased proportion of αβTCR+ DN T cells compared to peripheral blood. Decidual αβTCR+ DN T cells express increased levels of HLA-C specific KIRs however functional analysis should further elucidate their function in alloimmune regulation at the fetal maternal interface (chapter 6).

**NKT cells** have shown to be key regulators in immune tolerance. Decidual NKT cells comprise 0.48% of decidual CD3+ cells, a frequency 10 times greater than found in peripheral blood. A like peripheral NKT cells, decidual NKT cells contain the invariant Vα24+Vβ11+ TCR and are CD1d restricted. CD1d is expressed on extra villos trophoblasts and although additional studies are required, activation NKT cells may play a role in immune regulation at the fetal-maternal interface (79).
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1.2.5 Fetus specific immune regulation

Although many mechanisms of maternal immune regulation have been suggested to play a role in fetal-maternal tolerance, limited functional data is present on fetus specific immune regulation in human pregnancy. CD4+CD25+ Treg cells are the most extensively studied regulatory cell subset in human and in mice pregnancy. Although NK cells, dendritic cells, macrophages and particularly CD8+ T cells may contain a role in fetus specific immune regulation, thus far no functional data is available to support this. Therefore this paragraph focuses on the role of CD4+CD25+ T cells in murine models and human pregnancy.

CD4+CD25+ T cells in murine models have shown to be important in fetus specific immune tolerance. Depletion of peripheral CD4+CD25+ T cells leads to gestation failure in allogeneic but not in syngeneic pregnancy (87). In addition, adoptive transfer of CD4+CD25+ T cells from normal pregnant mice to abortion prone mice, prevents fetal rejection in the abortion prone mouse model (88). Other studies with chemokine receptor CCR5 deficient mice have identified a highly suppressive CCR5+ Treg subset and show that CCR5 may play a role in regulatory T cell migration to the pregnant uterus (89). Murine pregnancy is in many aspects different to human pregnancy such as placental structure, trophoblast invasion and duration of gestation. More importantly mice lack HLA-C like molecules and have more limited MHC polymorphisms compared to humans. Thereby experimental murine models contain inbred strains that are raised in pathogen free environments and thus lack both the genetic and environmental complexity experienced by humans. Although, murine models may identify novel pathways for fetal-maternal tolerance, studies using murine models for pregnancy pathology like recurrent spontaneous abortions (RSA) and pre-eclampsia can therefore not directly be translated to humans.

CD4+CD25+FOXP3+ Treg cells in human pregnancy are studied extensively and several groups have shown the dynamics of peripheral blood CD4+CD25+ cells compartment before and during pregnancy. In non-pregnant but fertile woman, a significant expansion of the peripheral blood CD4+CD25+FOXP3+ Treg cells is observed just before ovulation (90). In addition, studies have shown an increased percentage CD4+CD25+ T cells during pregnancy (91,92), although these studies do not distinguish CD4+CD25dim activated T cells (Tact) and CD4+CD25bright Treg cells. The hormone estrogen which increases during ovulation and pregnancy has shown to promote expansion of CD4+CD25+FOXP3+Tregs and increases the suppressive potential (93,94). Hereby estrogens may enhance Treg development just before and during pregnancy. In women who experienced recurrent spontaneous abortions (RSA) and pre-eclampsia, the peripheral blood levels of CD4+CD25+FOXP3+ Treg cells do not increase before ovulation and contain functional deficiencies (90,95). In addition, the proportion of decidual CD4+CD25+FOXP3+ Treg cells is significantly lower in RSA cases compared to elective abortions (96). These studies suggest that reduction or functional impairment of CD4+CD25+ T regulatory cells play a role in the development of pregnancy pathology.
1.3 The aim of this thesis is to define mechanisms of fetus-specific immune recognition and immune regulation by T cells at the fetal maternal interface during uncomplicated human pregnancy. In order to perform phenotypic and functional analysis of decidual T cells we developed methods for isolation and purification of decidual leucocytes suitable for functional analysis (chapter 7). Chapter 2 describes the distribution of CD4+CD25+ and CD8+CD28- T cell subsets in maternal peripheral blood, decidua basalis and decidua parietalis in early and at term pregnancy. Chapter 3 addresses the phenotype of CD4+CD25dim and CD4+CD25bright T cells in peripheral blood and decidua and more importantly the functional fetus specific and non-specific immune regulatory capacity of peripheral and decidual CD4+CD25bright T cells. In order to study factors that contribute to maternal T cell activation or induction of regulatory T cells we designed a database using Microsoft Access software to correlate clinical parameters, HLA typing and FACS data. The database analysis revealed that HLA-C is crucial for T cell activation and induction of functional Tregs in decidua parietalis whereas clinical parameters are not (chapter 4). Chapter 5 addresses the differentiation status of peripheral and decidual CD8+ T cells and reveals that decidual CD8+ T cells display an effector-memory phenotype but without the acquisition of the cytolytic molecule perforin. KIR expression on decidual T cells may provide alternative mechanisms of fetal HLA-C recognition at the fetal maternal interface and is described in chapter 6. Chapter 8 provides a general conclusion and discussion to put all data into (clinical) perspective. Finally chapter 9 summarizes the most important findings in Dutch to provide an overview of this thesis for non-expert readers.
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