Fetus specific immune recognition and regulation by T cells at the fetal-maternal inferface in human pregnancy

Tilburgs, T.

FETUS SPECIFIC IMMUNE RECOGNITION AND REGULATION BY T CELLS AT THE FETAL-MATERNAL INTERFACE IN HUMAN PREGNANCY

TAMARA TILBURGS

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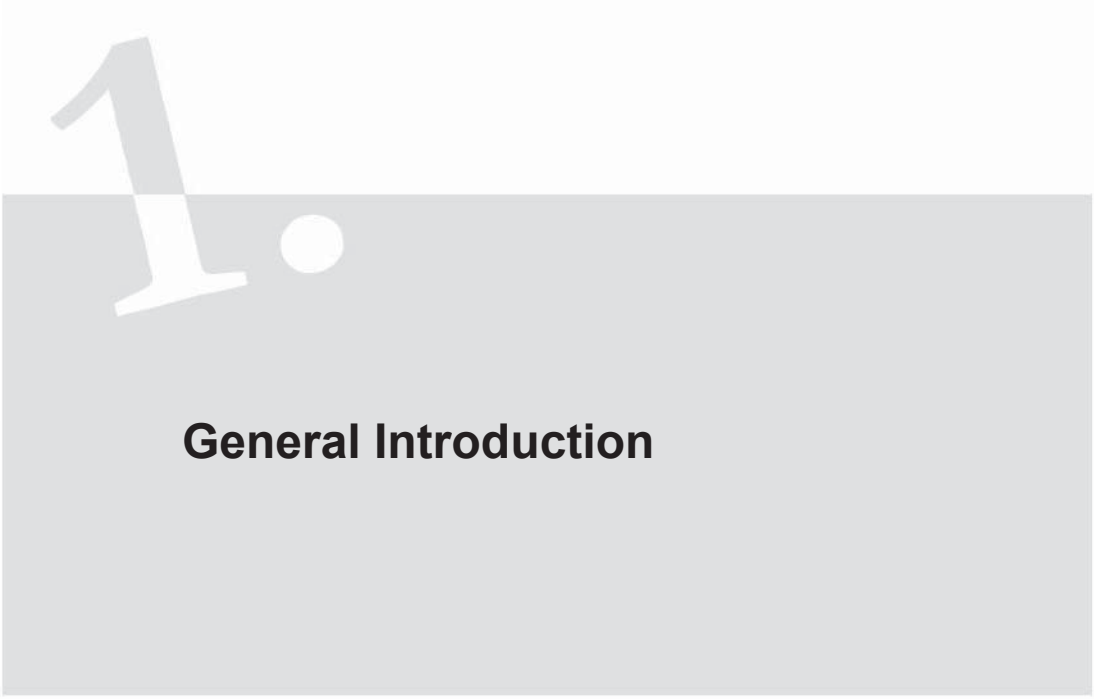

General Introduction

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

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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 extra cellular agents. Here fore self proteins as well as proteins derived from extra cellular 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).

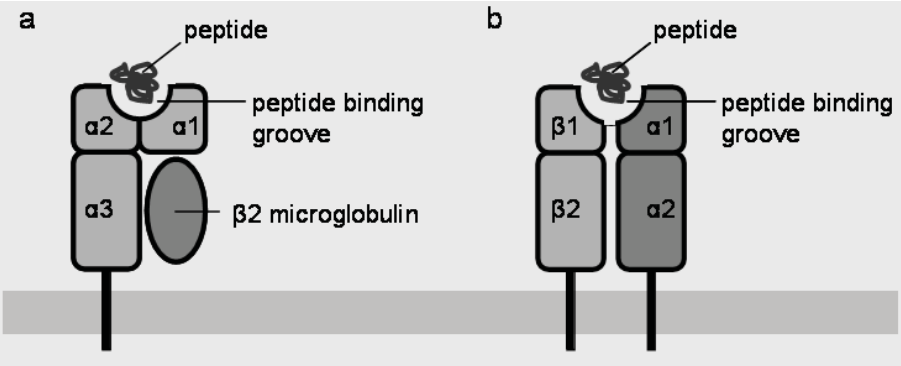


Figure 1. MHC class I (a) and MHC class II (b) molecules

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.

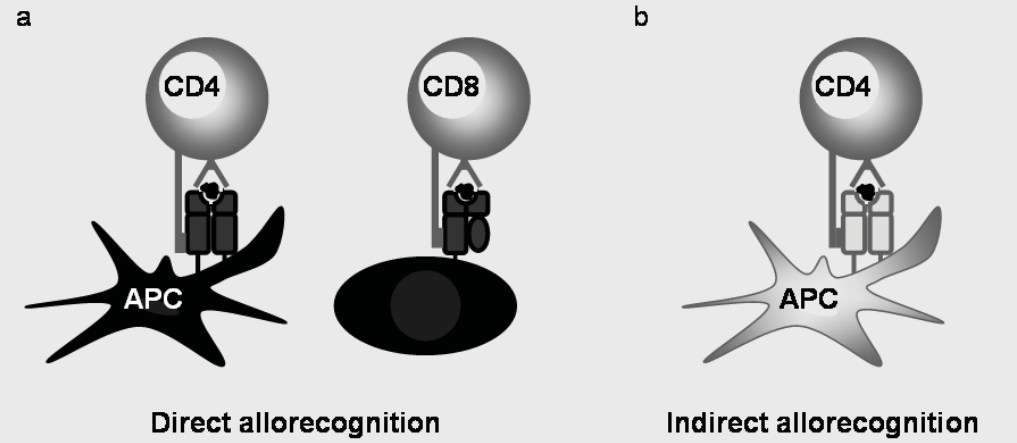


Figure 2. Direct (a) and Indirect (b) allorecognition by T cells

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

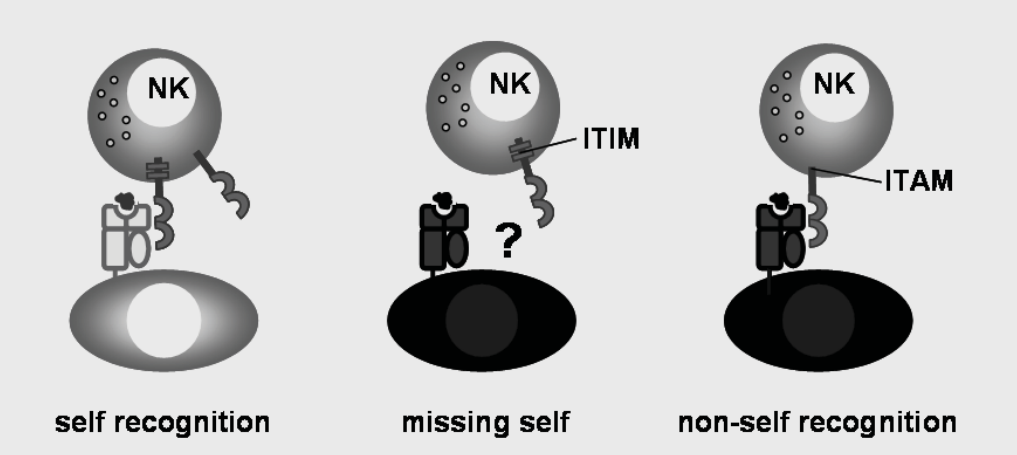


Figure 3. Self-, missing self and non-self recognition by NK cells

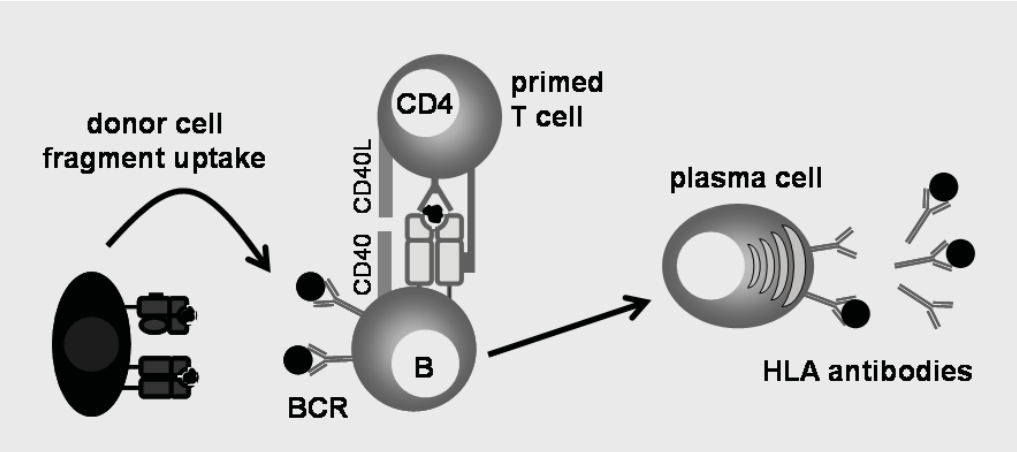
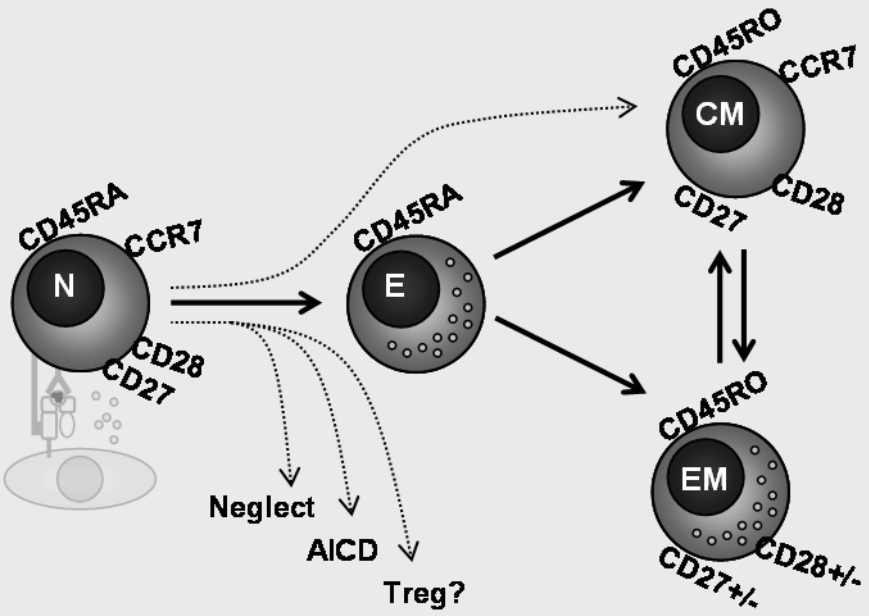


Figure 4. B cell activation and HLA antibody production

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 Dead due to over stimulation by too high antigenic strength.

CD8+ Treg cells
Existence is controversial. Expression of CD103, LAG-3 and CCL-4 are a 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 cell 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.

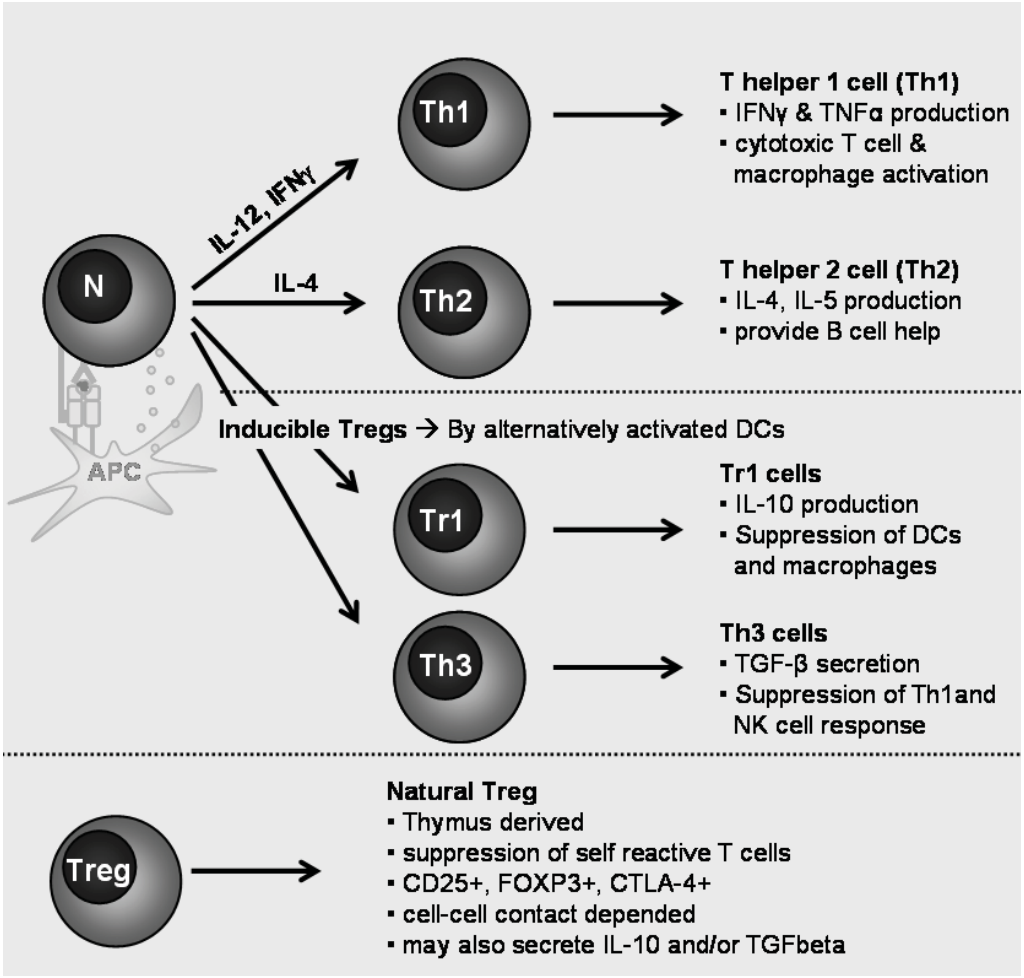


Figure 6. CD4+ T cell differentiation and Treg subsets

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).

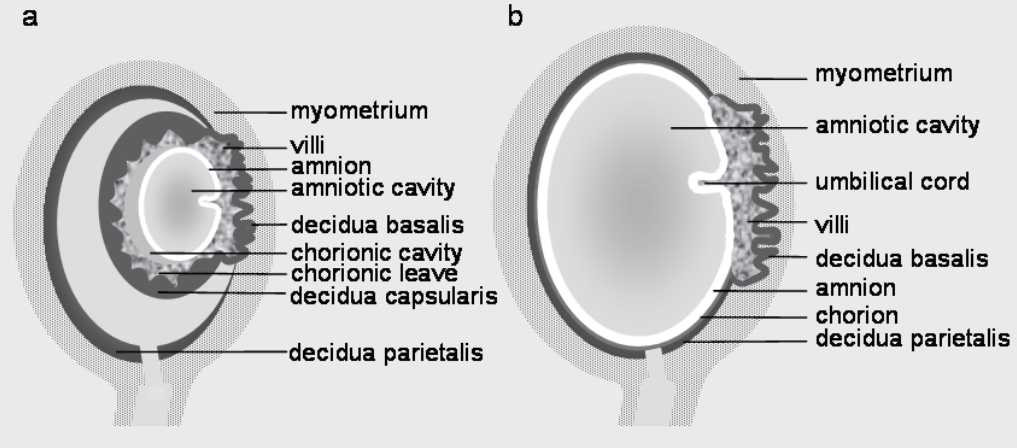


Figure 7. Development of the placenta, fetal membranes and deciduae at ~12 weeks (a) and at human term pregnancy (b)

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).

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 trophoblasts subsets do express HLA-C and the non-classical MHC class I molecules HLA-E, HLA-F, HLA-G of which the 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 costimulatory 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

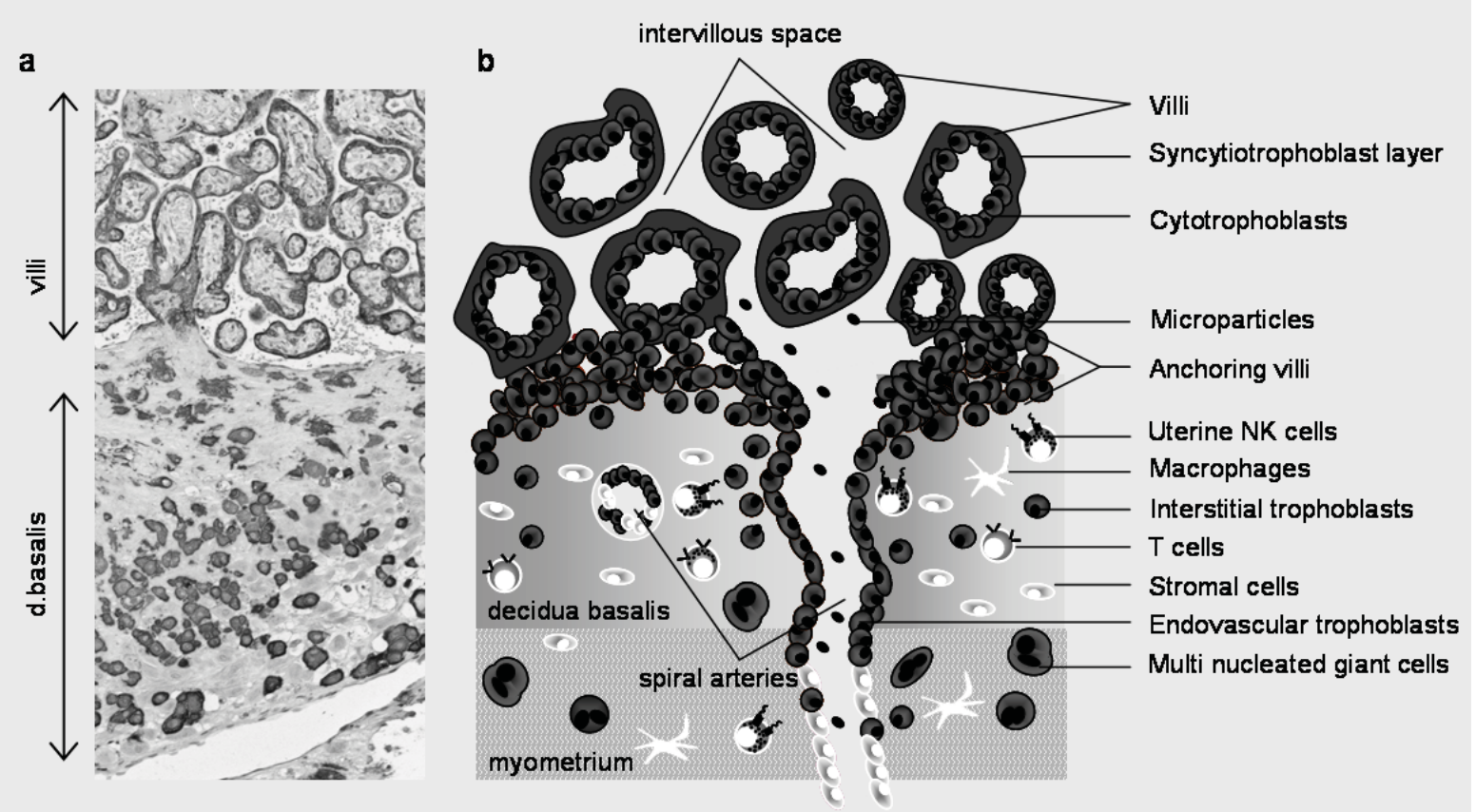


Figure 8. Cells at the fetal-maternal interface at the implantation site a) shows a trophoblast specific cytokeratin staining using immunohistochemistry, b) shows a scematic illustration of the cell types in placenta

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.

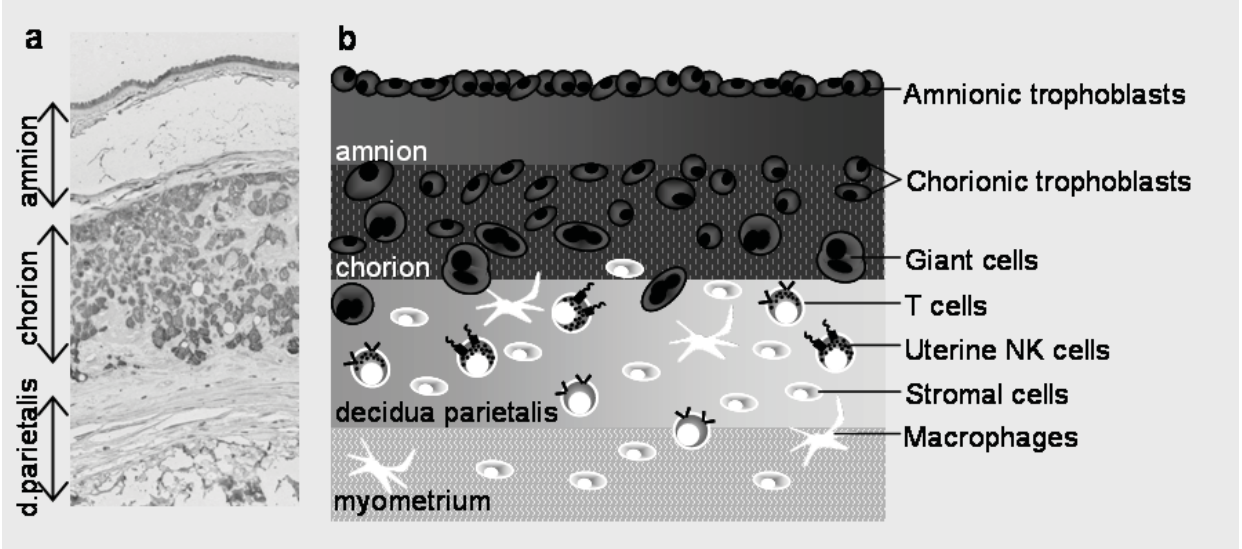


Figure 9. Fetal-maternal membranes a) shows a trophoblast specific cytokeratin staining using immunohistochemistry, b) shows a scematic illustration of the cell types in the membranes

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 villous trophoblasts and although additional studies are required, activation NKT cells may play a role in immune regulation at the fetal-maternal interface (79).

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+CD25bright T 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

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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Differential distribution of CD4+CD25bright and CD8+CD28- T cells in decidua and maternal blood during human pregnancy

Placenta 2006

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Ik kwam, ik zag en ik schreef alles op

Abstract

During pregnancy several maternal and fetal mechanisms are established to prevent a destructive immune response against the allogeneic fetus. Despite these mechanisms, fetus specific T-cells persist throughout gestation but little is known about the regulation of these T-cells. Recently CD4+CD25+ regulatory T-cells have been identified in human decidua. Human decidua forms the maternal part of the fetal-maternal interface and is subdivided in two distinct regions: the decidua (d.) basalis and the decidua (d.) parietalis. The aim of this study was to determine the distribution of specific T-cell subsets in d.basalis and d.parietalis in early and term pregnancy, with a special emphasis on the presence of CD4+CD25bright (regulatory) T-cells and CD8+CD28- (suppressor) T-cells. In addition, we compared phenotypic characteristics of decidua derived T-cell subsets with maternal peripheral blood (mPBL) T-cells and T-cells from non-pregnant controls. We identified significantly higher percentages of CD4+CD25bright 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. The major differences in T-cell subset distribution and the presence of additional phenotypic differences between T-cells in d.basalis, d.parietalis and mPBL may reflect specific immunomodulatory functions of these T-cell subsets at these different sites during pregnancy.

Introduction

During pregnancy several maternal and fetal mechanisms are established to prevent a destructive immune response to the allogeneic fetus. Although some of these mechanisms can lead to the deletion of fetus specific effector cells, maternal lymphocytes capable of attacking the fetus do persist throughout gestation (1,2). Besides effector cells, also regulatory T-cells are present at the fetal-maternal interface which have been shown to be important in the acceptance of the allogeneic fetus in murine pregnancy (3) and the maintenance of early pregnancy in human (4). In early pregnancy maternal tolerance is important to allow invasion of fetal trophoblasts to anchor the placenta. Later in pregnancy maternal allo-reactivity might play a role in initiation of parturition5. However, little is known about the regulation of maternal allo-reactivity and maternal tolerance at the fetal-maternal interface during pregnancy.

Human decidua form the maternal part of the fetal-maternal interface and is subdivided in two distinct regions: the decidua (d.) basalis, the maternal part of the placenta at the implantation site which directly contacts the invading interstitial fetal trophoblasts; and the decidua (d.) parietalis, the maternal part of the membranes connected to the fetal trophoblasts of chorion. Previous studies have shown a differential expression of HLA (6) and differences in production of specific prostaglandins (7) by trophoblasts at the implantation site compared to trophoblasts in the chorion. These differences might directly lead to differences in influx, expansion or maturation of specific lymphocyte subsets. In fact, previous studies by our group have shown a differential distribution of NK-cell subsets in d.basalis compared to d.parietalis (8), and an increased activation of T-cells in d.parietalis compared to d.basalis (9).

The aim of this study is to determine the distribution of regulatory T-cell subsets locally at the fetal-maternal interface. Recent studies have shown that CD4+ T-cells expressing high levels of CD25 (CD25bright) have regulatory capacity, whereas cells expressing intermediate levels of CD25 (CD25dim) are activated T-cells (10,11). Besides CD4+CD25bright regulatory T-cells also CD8+CD28- T-cells have been shown to have a suppressive capacity, (12) however this T-cell subset has not been studied in pregnancy yet. To determine the distribution of regulatory T-cells during pregnancy we separately examined d.basalis and d.parietalis for the presence of CD4+CD25dim (activated) T-cells, CD4+CD25bright (regulatory) T-cells and CD8+CD28- (suppressor) T-cells. In addition we compared phenotypic characteristics of decidua derived T-cell subsets with maternal peripheral blood (mPBL) T-cells and T-cells from healthy non-pregnant control donors (cPBL). Fluctuations in lymphocyte subsets throughout pregnancy have been described (13) therefore we examined lymphocyte isolates of early pregnancy subjects (17-23weeks) and after term pregnancy (>37weeks).

Materials and methods

Blood and tissue samples

Paired samples of d.basalis, d.parietalis and heparinized maternal peripheral blood (mPBL) were obtained from 14 healthy women after uncomplicated term pregnancy (gestational age range: 37-42 weeks). All tissue samples were collected between May 2004 and May 2005 and were not used in previous publications. Tissue samples were obtained after delivery by elective cesarean section or uncomplicated spontaneous vaginal delivery. Early pregnancy samples were obtained from healthy women undergoing surgical termination of pregnancy for social reasons (gestational age range: 17–23 weeks; n=13). Not in all cases paired d.basalis and d.parietalis could be obtained from early decidua samples. mPBL samples were obtained either directly before or directly after delivery or surgical curettage. Control PBL (cPBL) samples were obtained from healthy non-pregnant female volunteer donors (n=14; age range: 22-43 years). Signed informed consent was obtained from all women, and the study received approval by the LUMC Ethics Committee.

Lymphocyte isolation

Thin slices of d.basalis were macroscopically dissected from the maternal-facing surface of the placenta. For early pregnancy d.basalis tissue, only d.basalis connected to villous tissue was used and processed similar to term placenta. After dissection the remains of villous tissue were cut of d.basalis with a scissor. D.parietalis was collected by removing the amnion and delicately scraping the d.parietalis from the chorion. The obtained tissue was washed thoroughly with firm shaking and thereafter finely minced between two scalpel blades in PBS. After mincing, decidual tissue was washed again until the supernatant was completely transparent to further minimize contamination with blood and villi. In addition, analysis of villous tissue samples, umbilical cord blood and mPBL samples do not show presence of the decidua specific Treg subsets. Decidual fragments were incubated with 0.2% collagenase I (Gibco-BRL, Grand Island, NY) and 0.2% DNAse I (Gibco) in RPMI-1640 medium, gently shaking in a waterbath at 37ºC for 60 min and thereafter washed once with RPMI. As a control PBL samples were incubated with the enzyme preparations, this did not induce changes of CD25 and CD28 expression in these samples (data not shown). In addition similar percentages of Treg subsets were observed in decidua samples isolated with a mechanical isolation protocol (data not shown). Showing that enzymatic treatment does not affect or induce Treg subsets. The resultant suspensions were filtered through a 70µm sieve (Becton Dickinson, Labware; Franklin Lakes New Jersey), washed in RPMI, and layered on Ficoll Hypaque (LUMC pharmacy; Leiden, The Netherlands) for density gradient centrifugation (20min/800g). PBL samples were directly layered on a Ficoll Hypaque gradient. Mononuclear cells were collected, washed twice with PBS containing 1% FCS and all cells were fixed with 1% paraformaldehyde and stored at 4˚C until the time of cell staining and flow cytometric analysis.

Flow cytometry

The following directly conjugated mouse-anti-human mAbs were used for four-color immunofluorescence staining: CD45-APC, CD14-PE, CD25-PE, CD3-PerCP, CD4-APC, CD8-PE and CD28-APC (Becton Dickinson) and used in concentrations according to manufactures instructions. Flow cytometry was performed on a FACS Calibur using Cellquest-pro Software (BD). Analysis of all decidua and PBL samples was done at the

same time, using the same Cellquest-pro template. Calculations were done within the lymphocyte gate, set around the viable lymphocytes based on expression of CD45, CD14 and CD3 in a separate acquisition (14). Gating strategies are shown in figure 1. Shortly: CD45+CD14- cells are selected within a density plot, CD45+CD14- cells are back gated in a scatter plot and the lymphocyte gate (R2) is set around the viable lymphocytes. Within the lymphocyte gate (R2) CD3+ T-cells are selected by R3. A combination of R2 and R3 are shown in figure 2a and 3a. A minimum of 10.000 CD3+ cells within the lymphocyte gate (R2+R3), were acquired in flow cytometric analysis. The percentage of CD4+CD25bright T-cells was calculated within the CD3+CD4+ cell fraction and the percentage of CD8+CD28- cells was calculated within the CD3+CD8+ cell fraction. For these calculations an extra gate around CD3+CD4+ cells and CD3+CD8+ respectively within the lymphocyte gate is selected. The Mean Fluorescence Intensity (MFI) was used to determine relative expression of CD25 and CD28 within the CD3+CD4+CD25bright cells and the CD3+CD8+CD28+ cell fractions respectively.

Statistical analysis

Lymphocyte subset distribution among d.basalis, d.parietalis and maternal PBL, were compared using the paired Wilcoxon signed rank test for paired lymphocyte isolates. The Mann-Whitney U test was used to compare non-paired lymphocyte isolates. P values <0.05 are considered to denote significant differences.

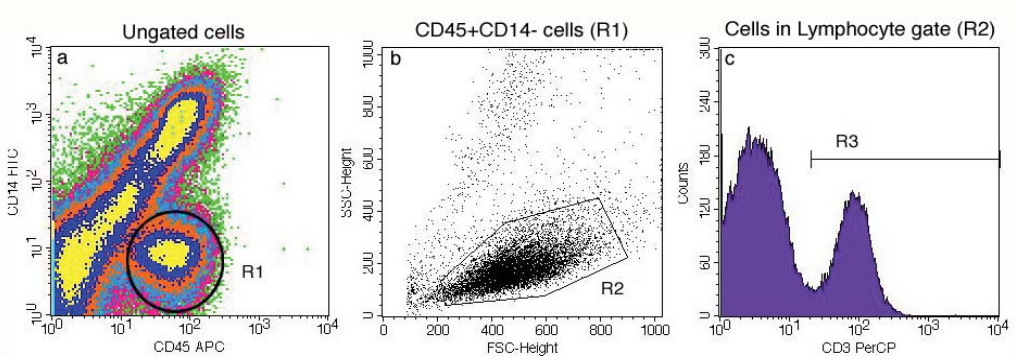

Figure 1. Gating strategy for decidual CD3+ cells
(a) A representative density plot of CD45 and CD14 expression within the total acquired events in d.parietalis and the selection of CD45+CD14- cells with gate R1, (b) shows CD45+CD14- cells back gated in a FSC/SSC dotplot. Gate R2 shows selection of viable lymphocytes avoiding other decidual cell types. (c) Shows a histogram plot of CD3 expression within the lymphocyte gate R2, and subsequent selection of CD3+ cells by gate R3.

Results

Distribution of CD4+CD25dim and CD4+CD25bright T-cells

In all lymphocyte isolates derived from control PBL, maternal PBL, d. basalis and d. parietalis, 3 distinct fractions of CD4+ T-cells can be identified, a CD25-, a CD25dim and a CD25bright fraction (Figure 1a). A significantly higher percentage of CD4+CD25bright T-cells is observed in d.parietalis compared to d.basalis both in early pregnancy (p=0.044) and term pregnancy (p=0.011) (Figure 1a-b). In addition, a significantly higher percentage of CD4+CD25bright T-cells is observed in all decidua samples compared to maternal PBL samples and PBL samples of non-pregnant control donors (Figure 1b) (all p-values <0.05). Furthermore, CD4+CD25bright T-cells derived from mPBL show a significantly lower expression of CD25 (median MFI=151; range: 105-234; n=14) in comparison with d.basalis (median MFI=289; range: 173-395; n=14; p=<0.001) and d.parietalis (median MFI=471; range: 293-1052; n=14; p=<0.001) after term pregnancy (Figure 1a) and in early pregnancy subjects (data not shown). Further analysis of the CD4+CD25bright T-cell fraction shows a significant increase in percentage of CD4+CD25bright T-cells in d.parietalis at term pregnancy compared to d.parietalis of early pregnancy subjects (p=0.049). The percentage of CD4+CD25bright T-cells in d.basalis and mPBL do not change during pregnancy. No significant differences are found between percentage of CD4+CD25bright T-cells in PBL samples of early pregnancy, of term pregnancy and of non-pregnant controls (Figure 1b).

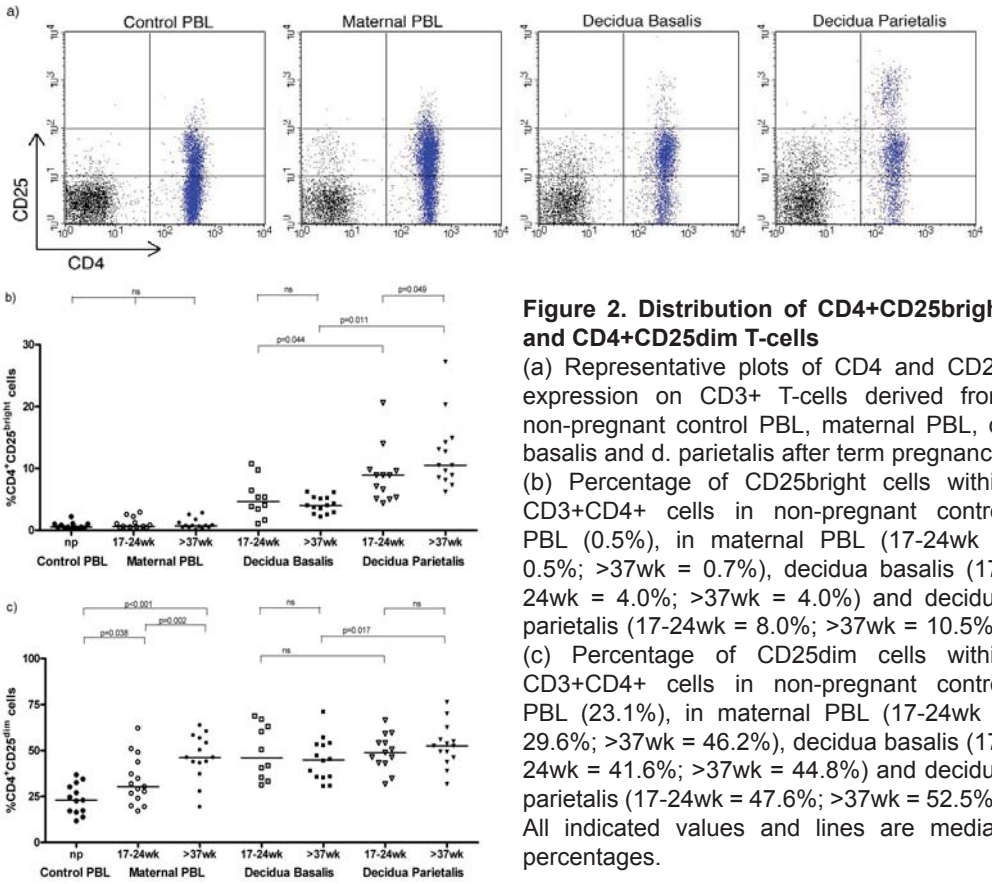

Figure 2. Distribution of CD4+CD25bright and CD4+CD25dim T-cells

(a) Representative plots of CD4 and CD25 expression on CD3+ T-cells derived from non-pregnant control PBL, maternal PBL, d. basalis and d. parietalis after term pregnancy. (b) Percentage of CD25bright cells within CD3+CD4+ cells in non-pregnant control PBL (0.5%), in maternal PBL (17-24wk = 0.5%; >37wk = 0.7%), decidua basalis (17-24wk = 4.0%; >37wk = 4.0%) and decidua parietalis (17-24wk = 8.0%; >37wk = 10.5%). (c) Percentage of CD25dim cells within CD3+CD4+ cells in non-pregnant control PBL (23.1%), in maternal PBL (17-24wk = 29.6%; >37wk = 46.2%), decidua basalis (17-24wk = 41.6%; >37wk = 44.8%) and decidua parietalis (17-24wk = 47.6%; >37wk = 52.5%). All indicated values and lines are median percentages.

In line with a previous report by our group (9), at term pregnancy an increased percentage of CD4+CD25dim T-cells is found in d.parietalis compared to d.basalis (p=0.017) (Figure 1c). However in early pregnancy no significant differences in the CD4+CD25dim T-cell fraction in d.parietalis compared to d.basalis is observed. Further analysis of the CD4+CD25dim T-cell fraction shows a significant increase in percentage of CD4+CD25dim T-cells in mPBL at term pregnancy compared to mPBL of early pregnancy subjects (p=0.002) and to cPBL samples of non-pregnant controls (p=0.038) (Figure 1c). No significant increase in the CD4+CD25dim fraction is observed in d.basalis and d.parietalis at term pregnancy compared to early pregnancy subjects (Figure 1c).

Distribution of CD8+CD28- T-cells

In all lymphocyte isolates derived from control PBL, maternal PBL, d. basalis and d. parietalis a clear population of CD3+CD8+CD28- T-cells can be identified (Figure 2a). A significantly higher percentage of CD8+CD28- T-cells is observed in d.parietalis compared to d.basalis in early pregnancy (p<0.001) and after term pregnancy (p=0.004) (Figure 2a-b). In addition, a significantly higher percentage of CD8+CD28- T-cells are observed in all decidua samples compared to maternal PBL samples and PBL samples of non-pregnant control donors (all p- values <0.05) (Figure 2b). No significant differences are found in percentage of CD8+CD28- T-cells in PBL samples derived from early pregnancy subjects, term pregnancy subjects and non-pregnant volunteers (Figure 2b). Further analysis of the CD8+CD28- T-cell fraction does not show significant changes of the percentage CD8+CD28- T-cells in d.basalis, d.parietalis and mPBL between early and term pregnancy subjects.

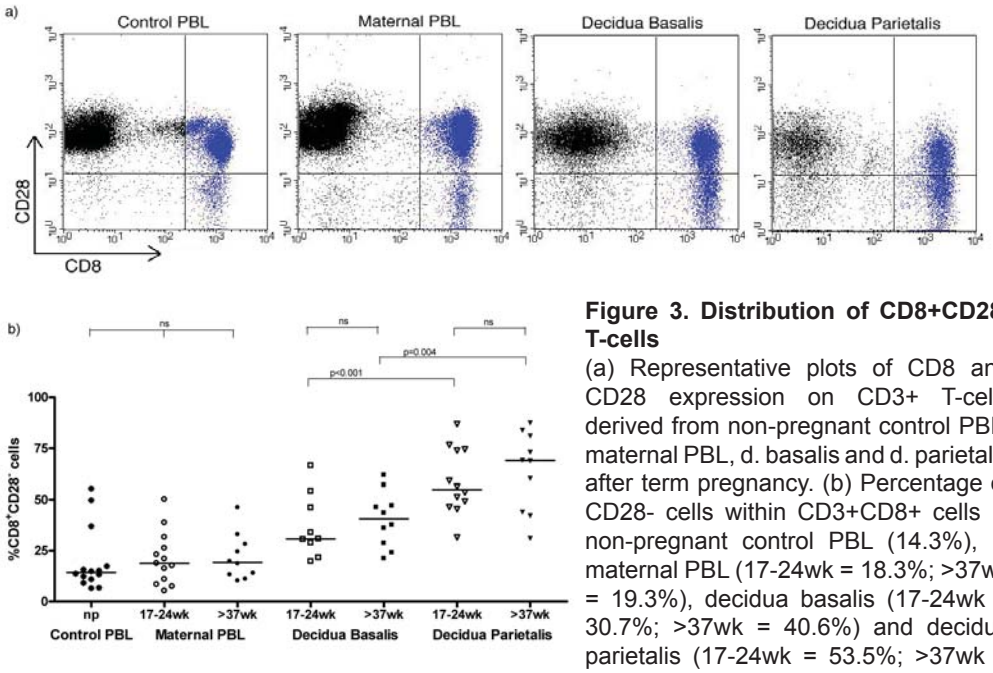

Figure 3. Distribution of CD8+CD28- T-cells

(a) Representative plots of CD8 and CD28 expression on CD3+ T-cells derived from non-pregnant control PBL, maternal PBL, d. basalis and d. parietalis after term pregnancy. (b) Percentage of CD28- cells within CD3+CD8+ cells in non-pregnant control PBL (14.3%), in maternal PBL (17-24wk = 18.3%; >37wk = 19.3%), decidua basalis (17-24wk = 30.7%; >37wk = 40.6%) and decidua parietalis (17-24wk = 53.5%; >37wk = 68.9%). All indicated values and lines are median percentages.

Expression of CD28 on CD8+CD28+ T-cells

Analysis of CD8+CD28+ T-cells shows a significant reduction in CD28 on d.basalis and d.parietalis derived CD8+CD28+ T-cells at term pregnancy compared to mPBL derived CD8+CD28+ T-cells (p=0.002 and p=0.006 respectively) (figure 4). In addition a significantly lower expression of CD28 is found on mPBL derived CD8+CD28+ T-cells in early pregnancy subjects compared to term pregnancy subjects (p=0.003) and non-pregnant volunteers (p=0.005). However CD28 expression on CD8+CD28+ T-cells in d.basalis and d.parietalis do not differ significantly in early pregnancy compared to term pregnancy. Similar results of CD28 expression were observed in decidua samples isolated with a mechanical isolation protocol (data not shown), showing that the reduction of CD28 expression on the decidual samples is not an effect of the enzymatic treatment.

Discussion

In the present study, we identified major differences in T-cell subset distribution and phenotypic characteristics between d.basalis, d.parietalis and maternal PBL, in early pregnancy and term pregnancy. We identified a higher percentage of CD4+CD25bright and CD8+CD28- T-cells in d.basalis and d.parietalis in comparison with maternal PBL samples and PBL of healthy non-pregnant control donors. The high proportion of CD4+CD25bright and CD8+CD28- T-cells in decidua suggests either an active recruitment or local expansion of these T-cell subsets at the fetal-maternal interface. The relative increase in percentage of CD4+CD25bright T-cells in the d.parietalis during pregnancy also suggests an active recruitment to or local expansion of CD4+CD25bright T-cells in the d.parietalis as pregnancy progresses.

Decidual CD4+CD25bright T-cells have significant higher expression of CD25 and CD152 (CTLA-4) (unpublished data by Tilburgs et al.) compared to mPBL and cPBL derived CD4+CD25bright T-cells. In addition the CD28 expression on CD8+CD28+ T-

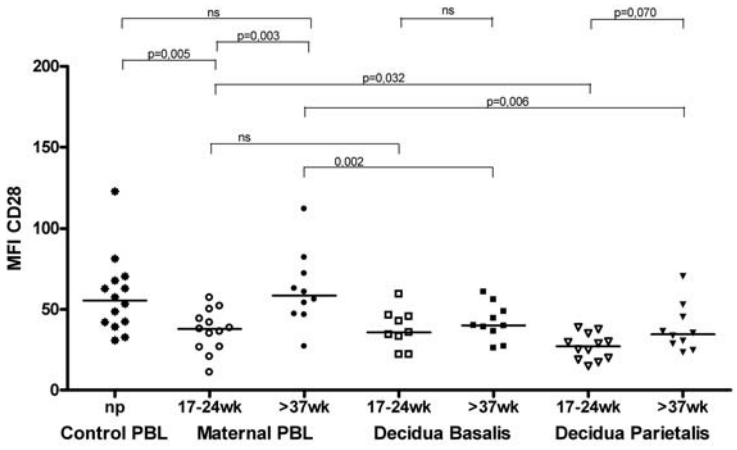

Figure 4. Expression of CD28 on CD8+CD28+ T-cells
Mean Fluorescence Intensity of CD28 on CD8+CD28+ T-cells in non-pregnant control PBL (MFI=55) in maternal PBL (17-24wk MFI=37; >37wk MFI= 59), decidua basalis (17-24wk MFI=39; >37wk MFI= 40) and decidua parietalis (17-24wk MFI=29; >37wk MFI=35). All indicated values and lines are median percentages.

cells is significantly reduced in d.basalis and d.parietalis compared to mPBL and cPBL. CD28 and CD152 both act as receptors for B7 ligands (CD80/CD86) on APCs. CD152, which is highly expressed on the CD4+CD25bright T-cells, is considered to be a negative regulator of T-cell activation. Although the mechanisms underlying its inhibitory activity are still not fully understood, competition with CD28 for binding to B7 molecules and the induction of a direct inhibitory signal are among the suggested mechanisms. In contrast CD28 is considered as an effective co-stimulatory molecule, which enhances T-cell activation. The low expression of CD28 on the decidual CD8+ T-cell may reduce the effectiveness of the CD28-B7 co-stimulatory pathway. The expression of CD152 and the reduction of CD28 on decidual T-cells might enable these cells to down regulate detrimental (allogeneic) T-cell responses at the fetal-maternal interface. In addition, Petroff et al. show that the newly discovered immunomodulatory variants of the B7 molecules B7-DC, B7-H2 and B7H3 are differentially expressed by trophoblasts and stromal cells in the human placenta (15). Thereby trophoblasts and stromal cells can regulate maternal lymphocytes responses at the fetal-maternal interface by expressing immunomodulatory B7 molecules.

Recent reports by Heikkinen et al. and Somerset et al. have suggested a decrease of CD4+CD25+ Treg cells in maternal PBL in 3rd trimester pregnancy compared to 1st and 2nd trimester (16,17). In these studies no distinction between CD4+CD25dim and CD4+CD25bright T-cells is made. In contrast to this, we clearly identified 3 distinct subsets of CD4+ T-cells and did not observe a significant change of the percentage of CD4+CD25bright T-cells in mPBL in early pregnancy (2nd trimester) compared term pregnancy. In addition, a significant increase in the CD4+CD25dim T-cell fraction in mPBL was observed at term pregnancy. The differences in CD4+CD25+ T-cell subsets found by these previous studies might be explained by a different time point of maternal PBL sampling or differences in gating strategies of CD25 expression. The increase in CD4+CD25dim activated T-cells in the late phase of pregnancy might be due to the continuous exposure of antigens of the developing fetus and may also play a role in the initiation of parturition [5]. In contrast to our results Sasaki et al. describe higher percentages of CD4+CD25bright cells in peripheral blood compared to decidual tissue (4). These percentages are calculated within total lymphocytes defined by scatter plot profile. If however the percentages of CD4+CD25bright cells are recalculated within total population of CD4+ cells, an increased percentage of CD4+CD25bright cells in decidua (22,24%) compared to peripheral blood (8,5%)is found which confirms our results. These percentages of CD4+CD25bright cells are substantially higher in comparison with our results, but are due to gating of CD25bright cells based on lower fluorescence intensity threshold of CD25.

Previous reports by our group have shown differences in NK-cell distribution in d.basalis and d.parietalis and an increased activation of T-cells in d.parietalis compared to d.basalis. In the present study, besides activated T-cells a significantly higher percentage of T-cells with a regulatory phenotype (CD4+CD25bright and CD8+CD28- T-cells), are found in the d.parietalis compared to the d.basalis. These findings suggest a differential regulation or influx of T-cells in d.parietalis compared to the d.basalis. This might be due to a differential expression of HLA (6) or the production of specific prostaglandins by trophoblasts in the chorion (connected to the d.parietalis) compared to the extra-villous trophoblasts at the implantation site (connected to the d.basalis) (7). A report by Polanczyk et al. showed that the female sex hormone estrogen can drive expansion of the CD4+CD25+ regulatory T-cell compartment (18). This or other pregnancy specific

hormones or decidua specific prostaglandins may determine the influx or expansion of CD4+CD25bright T-cells at the fetal-maternal interface. Besides regulatory T-cells also the decidua specific CD56bright NK cells have been shown to have specific immunomodulatory functions (19). In addition, NK cells can by interaction of their Killer Ig-like Receptors (KIRs) with the fetal HLA-C antigens on the trophoblast influence the decidual environment and regulate the development of the placenta (20). By interaction of NK-cells with stromal cells or by direct interaction of NK-cells with T-cells in decidua, NK cells can affect the influx, maturation or expansion of specific T-cell subsets.

Recently the expression of the transcription factor Foxp3 has been suggested to discriminate regulatory T-cells from activated T-cells (21). However, these results remain controversial and thus far no specific (surface-) marker has been ascribed to Treg cells (22). Investigation of additional Treg markers such as Foxp3 and CTLA-4 and function of the decidual and peripheral CD4+CD25bright and CD8+CD28- Treg subsets are in progress. The unusually high percentage of CD4+CD25bright and CD8+CD28- T-cells in decidua indicates an important role for these T-cell subsets locally at the fetal-maternal interface. In addition, the major differences in T-cell subset distribution among d.basalis and d.parietalis may reflect specific immunomodulatory functions of these T-cell subsets at the site of implantation compared to the fetal-maternal interface of the membranes. Future functional studies with isolated decidual T-cell subsets may identify novel mechanisms involved in influx, expansion or maturation of T-cells and increase our understanding of the delicate balance between maternal tolerance and maternal allo-reactivity.

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3.

Evidence for a selective migration of fetus specific CD4+CD25bright regulatory T cells from the peripheral blood to the decidua in human pregnancy

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Elke beslissing is een experiment met onbekende uitkomst

Abstract

During pregnancy the maternal immune system has to tolerate the persistence of fetal alloantigens. Many mechanisms contribute to the prevention of a destructive immune response mediated by maternal alloreactive lymphocytes directed against the allogeneic fetus. Murine studies suggest that CD4+CD25+ T cells provide mechanisms of specific immune tolerance to fetal alloantigens during pregnancy. Previous studies by our group demonstrate that a significantly higher percentage of activated T cells and CD4+CD25bright T cells are present in decidual tissue in comparison with maternal peripheral blood in human pregnancy. In this study we examined the phenotypic and functional properties of CD4+CD25bright T cells derived from maternal peripheral blood and decidual tissue. Depletion of CD4+CD25bright T cells from maternal peripheral blood demonstrates regulation to 3rd party umbilical cord blood cells comparable to non-pregnant controls, whereas the suppressive capacity to umbilical cord blood cells of her own child is absent. Furthermore, maternal peripheral blood shows a reduced percentage of CD4+CD25brightFOXP3+ and CD4+CD25brightHLA-DR+ cells compared to peripheral blood of non-pregnant controls. In contrast, decidual lymphocyte isolates contain high percentages of CD4+CD25bright T cells with a regulatory phenotype that are able to down regulate fetus-specific and non-specific immune responses. These data suggest a preferential recruitment of fetus-specific regulatory T cells from maternal peripheral blood to the fetal-maternal interface where they may contribute to the local regulation of fetus specific responses.

Introduction

Many mechanisms are suggested to be involved in maternal immune tolerance and immunologic acceptance of the allogeneic fetus during pregnancy. Fetal trophoblasts play a crucial role in circumventing a destructive maternal immune response in different ways. Fetal tissue can inhibit allogeneic immune responses by expressing IDO (that inhibits rapid proliferation of cells) (1,2) FAS ligand (that can cause apoptosis of activated cells that express FAS) (3) and complement inhibitory proteins to prevent complement activation (4). These mechanisms can inhibit immune responses at the fetal maternal interface in an antigen non-specific manner (5). Trophoblasts do not express the classical HLA-A, HLA-B and HLA-DR, -DQ and -DP molecules that are the main targets for alloreactive T cells in transplantation. However, trophoblasts do express HLA-C, HLA-E and HLA-G molecules by which they can avoid NK cell mediated cytotoxicity. HLA-G expressing cells have shown to induce regulatory T cells (6). In contrast, the highly polymorphic HLA-C can induce NK cell tolerance but can also be a target for allogeneic T cells. Data from bone marrow transplantation patients has shown that a single HLA-C allele-mismatch can elicit a cytotoxic T cell response (7) and is associated with a lower patient survival. In addition, HLA-E can decrease NK and CTL cytotoxicity (8) but has also shown to exhibit alloantigenic properties that are indistinguishable from classical MHC class I molecules (9). Neutralization of possible cytotoxic T cells with direct specificity for HLA-C, HLA-E or indirectly presented minor histocompatibility antigens seems essential for the immunologic acceptance of the allogeneic fetus. Maternal leucocytes present at the fetal-maternal interface include decidua-specific CD16-CD56bright NK cells and T cells whereas B cells are virtually absent. Decidual NK cells have shown to regulate trophoblast invasion by expression of NK cell receptors and the secretion of cytokines (10). Incompatibility of maternal killer immunoglobulin-like receptor (KIR)2 genotype and the fetal HLA-C allotype leads to increased risk of pregnancy complications like pre-eclampsia (11), suggesting that NK cells play a role in fetus-specific immune regulation. Murine studies have shown that depletion of peripheral blood CD4+CD25+ cells leads to gestation failure in allogeneic but not in syngeneic pregnancy (12). These data suggest that T cells play a role in specific immune tolerance to fetal alloantigens in murine pregnancy. Recent studies have shown that high percentages of T cells are present in decidual tissue in human term pregnancy and that peripheral blood T cell subsets change during pregnancy (13-16). In addition, a significantly higher percentage of CD4+CD25bright T cells is present in decidual tissue compared to maternal peripheral blood (13,17). CD4+CD25+ T cells are extensively studied by many groups for their regulatory capacities. 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 (18,19). Additional markers like CTLA-4, FOXP3 and activation markers like HLA-DR and CD69 can help to distinguish effector from regulatory cells. However, conclusions based on phenotypic characterization remain controversial (20,21). Until a specific marker for regulatory T cells is found, functional tests are required to identify and study regulatory T cells. The aim of this study is to analyze phenotypic and functional properties of CD4+CD25bright T cells during pregnancy in tissue isolates from decidua basalis (d.basalis), the maternal part of the placenta at the implantation site connected to invading fetal trophoblasts, the decidua parietalis (d.parietalis) the maternal part of the membranes connected to the fetal trophoblasts of the chorion and maternal peripheral blood (mPBL)2 samples.

Materials & Methods

Blood and tissue samples

Paired samples of d.basalis, d.parietalis, heparinised maternal peripheral blood (mPBL) and heparinised umbilical cord blood (UCB)2 were obtained from healthy women after uncomplicated term pregnancy (gestational age range: 37-42 weeks). Tissue samples were obtained after delivery by elective caesarean section or uncomplicated spontaneous vaginal delivery. Early pregnancy samples were obtained from healthy women undergoing surgical termination of pregnancy for social reasons (gestational age range: 17–23 weeks). From the early decidua samples in not all cases paired d.basalis and d.parietalis could be obtained. mPBL samples were obtained either directly before or directly after delivery or surgical curettage. UCB cells were obtained directly after delivery from the umbilical cord veins. Control PBL (cPBL)2 samples were obtained from healthy non-pregnant female volunteer donors (age range: 22-43 years). Tissue samples used for phenotypic analysis are partly similar to those described previously (13). Signed informed consent was obtained from all women, and the study received approval by the LUMC Ethics Committee (P02-200).

Lymphocyte isolation

Lymphocyte isolation from decidua was done as described previously (13). Shortly: d.basalis was macroscopically dissected from the maternal side of the placenta. D.parietalis was collected by removing the amnion and delicately scraping the d.parietalis from the chorion. The obtained tissue was washed thoroughly with PBS and thereafter finely minced between two scalpel blades in PBS. Decidual fragments were incubated with 0.2% collagenase I (Gibco-BRL, Grand Island, NY) and 0.02% DNAse I (Gibco) in RPMI-1640 medium, gently shaking in a waterbath at 37ºC for 60 min and thereafter washed once with RPMI. The resultant suspensions were filtered through a 70µm sieve (BD, Labware; NJ) and washed once in RPMI. For phenotypic analysis the isolates were layered on Ficoll Hypaque (LUMC pharmacy; Leiden, The Netherlands) for density gradient centrifugation (20min/800g). PBL and UCB samples were directly layered on a Ficoll Hypaque gradient. Mononuclear cells were collected, washed twice with PBS containing 1% FCS and all cells were fixed with 1% paraformaldehyde and stored at 4˚C until cell staining and flow cytometric analysis. For functional analysis the decidual lymphocyte isolates were layered on a Percoll gradient of (7.5ml 1.08g/ml; 12.5ml 1.053g/ml; 20ml 1.034g/ml) for density gradient centrifugation (30min/800g) to minimize contaminating cell debris and non-lymphocyte cell types. Lymphocytes were isolated from the 1.08g/ml – 1.053g/ml interface. Comparison of the cell suspension obtained after Ficoll gradient and Percoll gradient isolation did not show any significant difference in composition of lymphocyte and T cell subsets (data not shown).

Flow cytometry

The following directly conjugated mouse-anti-human mAb were used for four-color immunofluorescence surface staining: CD45-APC, CD14-PE, CD25-PE, CD3-PerCP, CD4-APC, CD69-FITC and HLA-DR-FITC (Becton Dickinson) and used in concentrations according to manufactures instructions. For intracellular expression of CTLA-4, cells were stained for surface expression of CD3, CD4 and CD25, treated with permeabilizing solution buffer (containing: 0.1% saponine, 5% FCS and 0.05% sodium-azide in PBS) for 10 min and thereafter stained with anti-CTLA-4-APC mAb (Becton Dickinson). Intracellular expression of FOXP3 was determined using an APC anti-human FOXP3

Staining set (eBioscience; San Diego; CA) according to manufactures instructions. Flowcytometry was performed on a FACS Calibur using Cellquest-pro Software (Becton Dickinson) as described previously (13). The percentages of CD4+CD25dim and CD4+CD25bright T cells were calculated within the CD3+CD4+ cell fraction and the percentages of FOXP3, CTLA-4, CD69 and HLA-DR positive cells were calculated within the CD3+CD4+CD25dim or CD3+CD4+CD25bright cell fractions. FACS analysis of all decidua and PBL samples was done using the same Cellquest-pro template, the fluorescence intensity to distinguish CD4+CD25dim and CD4+CD25bright cells was determined on decidual samples and exactly copied to PBL samples.

Functional assays

For functional analysis the decidual and peripheral blood isolates were FACS sorted on a Flow sorter ARIA (Becton Dickinson) with DIVA software. Isolates were stained for surface CD4-FITC, CD25-PE, CD45-APC and thereafter sorted for viable CD45+ cells or CD45+ cells without CD4+CD25bright cells. All cells were sorted within the lymphocyte gate set around the viable lymphocytes avoiding granulocytes, macrophages and other contaminating cell types. Cells were washed once in RPMI and thereafter incubated in RPMI supplemented with L-glutamine 2 mM, penicillin 50 units/ml en streptomycin 50 µg/ml (all obtained from Gibco Laboratories) and 10% human serum in a round-bottomed 96 well plate (Costar Cambridge, MA, USA) at a density of 50.000 cells per well in triplicate. For anti-CD3 stimulation wells were pre-coated with 10 µg/ml, 2 µg/ml OKT-3 (Orthoclone) or 5 µg/ml, 1 µg/ml UCHT-1 (BD Pharmingen) for 2 hours at 37ºC. For stimulation with UCB, 50.000 irradiated (3000 Rad) UCB cells were added. All responders and stimulator cells were DNA typed for HLA-A, -B, -C, -DR and -DQ. Cells were incubated at 37ºC with 5% CO2. At day 4 50 µl of supernatant was collected and stored at -20ºC until the time of analysis. Supernatants were analyzed with a Th1-Th2 Bio-plex premixed human cytokine panel Th1/Th2 (containing IL-2, IL-4, IL-5, IL-10, IL-13, GM-CSF, IFN-γ and TNF-α) (Biorad Laboratories, Veenendaal, The Netherlands) according to manufactures description. After the collection of the supernatants, proliferation was measured as [3H]thymidine (1µCi) incorporation for another 16 hours by liquid scintillation spectroscopy using a betaplate (Perkin Elmer-Wallac, Turku, Finland). Results were expressed as the median counts per minute (cpm) for each triplicate culture. The suppression index (S.I.)2 of CD4+CD25bright T cells is depicted as the ratio between paired proliferation (cpm) or cytokine production (pg/ml) of the CD45+ fraction depleted for CD4+CD25bright cells and the CD45+ fraction. All samples below the background of 700 cpm or 7 pg/ml IFN-γ are excluded to calculate a S.I. and samples with a negative S.I. are depicted as 0.

Immuno Histochemistry

Paired d.basalis and d.parietalis isolates of early and term pregnancies were embedded in paraffin for immunohistochemical analysis. Serial 4µm thick tissue sections were deparaffinized using xylene and 100% ethanol and rehydrated with 70% and 50% ethanol. Endogenous peroxidase activity was blocked with methanol containing 0.3% H2O2. Antigen retrieval was performed by microwaving the sections for 12 minutes in boiling citrate buffer (10 mMol/L; pH 6.0). The tissue sections were incubated with the primary antibody diluted in PBS containing 1% BSA overnight in a moist chamber. Sections were washed three times and incubated with secondary antibody for 60 minutes in a moist chamber. Following three washes in PBS the sections were embedded in Mowiol (Calbiochem, La Jolla, CA). The antibodies used are: Rabbit polyclonal CD4 (Santa

Cruz Biotechnology); rabbit polyclonal CD3 (Abcam); goat anti rabbit IgG TexasRed (Abcam); mouse monoclonal to FOXP3 (236A/E7) (Abcam) and goat anti mouse IgG1-FITC (BD). The localization of CD4+FOXP3+ or CD3+FOXP3+ cells was determined using fluorescence microscopy.

Statistical analysis

To determine differences between more than 2 groups, a non-parametric Kruskal-Wallis one way ANOVA was performed. If $p<0.05$ a Dunn's multiple comparison post test was performed to compare all pairs of columns. The Wilcoxon signed rank test was performed to determine differences between paired groups. The Mann-Whitney test was used to determine differences between non-paired groups. P-values <0.05 are considered to denote significant differences.

3

Results

Characterization of decidual CD4+CD25dim and CD4+CD25bright T cells

Consistent with a previous report by our group (13) we observed a significantly higher percentage of CD4+CD25bright T cells in all decidual samples compared to non pregnant control PBL samples and maternal PBL samples. In addition, a significantly higher percentage of CD4+CD25bright T cells is observed in d.parietalis compared to d.basalis both in early (17-24 wk) and term pregnancy (>37 wk) (data not shown). To further characterize decidual CD4+CD25dim and CD4+CD25bright T cells we performed flowcytometric analysis for the Treg markers FOXP3 and CTLA-4 (both intracellular), and surface expression of the T cell activation markers CD69 and HLA-DR. Representative FACS plots and the gating strategy for determining CD4+CD25dim and CD4+CD25bright T cells and FOXP3+, CTLA-4+, CD69+ and HLA-DR+ cells are shown in Figure 1a-b.

The decidual CD4+CD25dim and CD4+CD25bright T cell populations are two clearly distinctive cell populations. Decidual CD4+CD25bright T cells show a regulatory phenotype with high percentages of FOXP3+, CTLA-4+, HLA-DR+ cells and low percentages of CD69+ cells. In contrast, the CD4+CD25dim T cell fraction of all decidual isolates show an activated phenotype containing low percentages FOXP3+, CTLA-4+, HLA-DR+ cells and high percentages of CD69+ cells. The decidual CD4+CD25bright T cell population is a small but homogeneous cell population with no significant differences in percentage FOXP3+, CTLA-4+, CD69+ and HLA-DR+ cells between d.basalis and d.parietalis samples and no differences between early (17-24 weeks) and term (>37 weeks) pregnancy samples (Figure 1c-f). The decidual CD4+CD25dim T cell population contain minor differences in percentages of CTLA-4+ cells and CD69+ cells between d.basalis and d.parietalis samples and early (17-24 weeks) and term (>37 weeks) pregnancy samples (Figure 1c-f).

Different phenotype of CD4+CD25bright T cells in decidua compared to peripheral blood

To compare the phenotype of decidual and peripheral blood CD4+CD25bright T cells, analysis of peripheral blood samples from healthy non-pregnant female donors and the maternal peripheral blood samples from early and term pregnancy were analyzed similar to the decidual isolates. All decidual CD4+CD25bright T cell fractions contain a significantly higher percentage of CTLA-4+, CD69+ and HLA-DR+ cells compared to non pregnant control PBL ($p<0.0001$; $p<0.0001$; $p<0.008$ respectively), and maternal

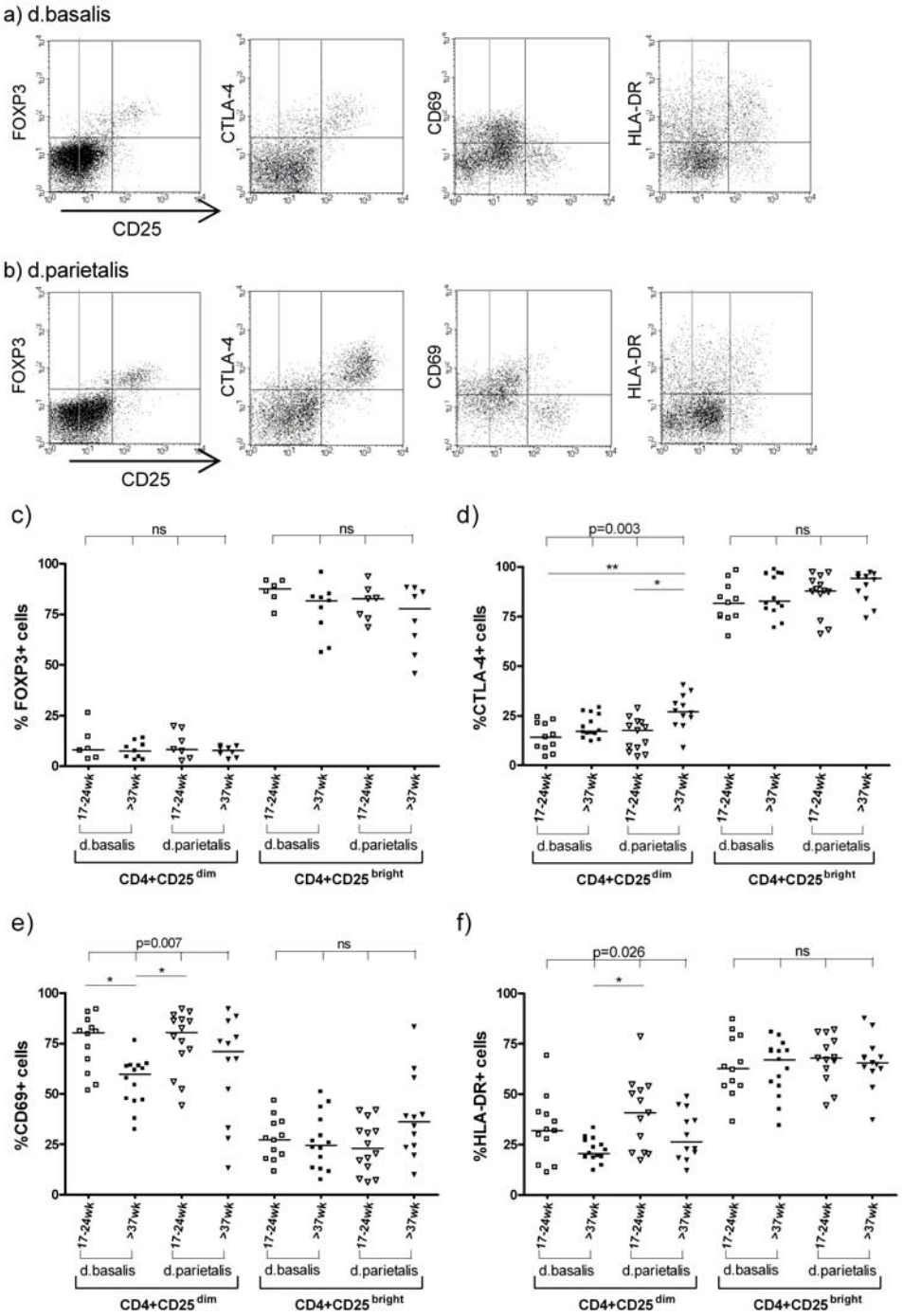


Figure 1. Characteristics of decidual CD4+CD25dim and CD4+CD25bright T cells
Representative dotplots of CD25 and intracellular FOXP3, intracellular CTLA-4, CD69 and HLA-DR expression in d. basalis (a) and d. parietalis (b) after term pregnancy. All plots are gated for CD3+CD4+ T cells within the lymphocyte gate. Percentage of FOXP3+ (c), CTLA-4+ (d), CD69+ (e) and HLA-DR + (f) cells within CD4+CD25dim or CD4+CD25bright T cell fraction of d.basalis and d.parietalis in early pregnancy (17-24 weeks) and after term (>37 weeks) pregnancy. Lines indicate median percentages.

PBL both in early and term pregnancy (all p-values <0.0001) (Figure 2b-d). In addition, a significantly higher percentage of FOXP3+ cells is observed in decidual CD4+CD25bright T cells compared to CD4+CD25bright T cells from maternal PBL (p<0.0001). However, no significant difference in percentage FOXP3+ cells in the decidual CD4+CD25bright T cell fractions compared to the CD4+CD25bright T cell fractions non-pregnant control PBL is observed (Figure 2a).

Comparison of the CD4+CD25bright T cell fraction from maternal PBL and non-pregnant control PBL shows a significantly lower percentage of FOXP3+ in the CD4+CD25bright T cell fraction in maternal PBL in early (52%) and term (53%) pregnancy, compared to the CD4+CD25bright T cell fractions of non-pregnant controls (79%) (p<0.05; p<0.05). In addition, the CD4+CD25bright T cell fraction in maternal peripheral blood in early (35%) and term (37%) pregnancy contains significantly less HLA-DR+ cells compared to non pregnant controls (50%) (p<0.01; p<0.05).

Functional analysis of CD4+CD25bright T cells

To examine the suppressive capacity of decidual and peripheral blood CD4+CD25bright T cells, we isolated a lymphocyte fraction containing all CD45+ cells and a CD45+ fraction depleted for CD4+CD25bright T cells by FACSsort. Representative FACS plots and the gating strategy are shown in Figure 3a-b. Both fractions were stimulated with plate bound OKT-3 (10 µg/ml and 2 µg/ml) and plate bound UCHT-1 (5 µg/ml and 1 µg/ml) and examined for proliferation capacity by tritium incorporation while the supernatants were examined for cytokine production by a Bio-plex bead array.

The proliferative capacity and IFN-γ production of peripheral blood isolates was not affected by depletion of the CD4+CD25bright cells using OKT-3 or UCHT-1 stimulation. In contrast, the d.basalis isolate shows a significant increase in IFN-γ production after depletion of the CD4+CD25bright cells (p=0.027) and a slight but not significant increase in proliferation (p=0.064) using OKT-3 stimulation. UCHT-1 stimulation induces high proliferative responses (range 61.000 – 240.000 cpm) and IFN-γ production (range 175 – 6400 pg/ml) in all decidual isolates. However proliferation and IFN- γ production after UCHT-1 stimulation was not affected by depletion of CD4+CD25bright cells in all isolates. In d.parietalis a group of high responders (proliferation > 30.000 cpm and IFN-γ > 400 pg/ml) and group of low responders (proliferation < 10.000 cpm and IFN-γ < 100 pg/ml) can be identified (Figure 3c, 3e). Both groups were checked for differences in clinical parameters (birth order, time of membrane rupture, maternal age etc.) that could have led to an increased immune activation. There was no difference in any of these parameters except for gender of the child, the high responders carried all female children (n=5) and the low responders all male children except for 1 female (n=5+1). To compare the suppressive capacity of the four different isolates a Suppression Index (S.I.) was determined but no significant differences were observed with regard to proliferation (Figure 3d) and IFN-γ production (Figure 3f). Besides IFN-γ all other cytokines (IL-2, IL-4, IL-5, IL-10, IL-13, GM-CSF, and TNF-α) were analyzed but no significant differences in these cytokine concentrations were observed in the CD45+ fraction compared to the CD4+CD25bright depleted fraction .

Fetus specific suppression capacity of CD4+CD25bright T cells

In order to determine whether there is a fetus specific component in the suppressive capacity of maternal peripheral blood and decidual CD4+CD25bright T cells, CD45+ cells and a CD45+ fraction depleted for CD4+CD25bright T cells were stimulated with

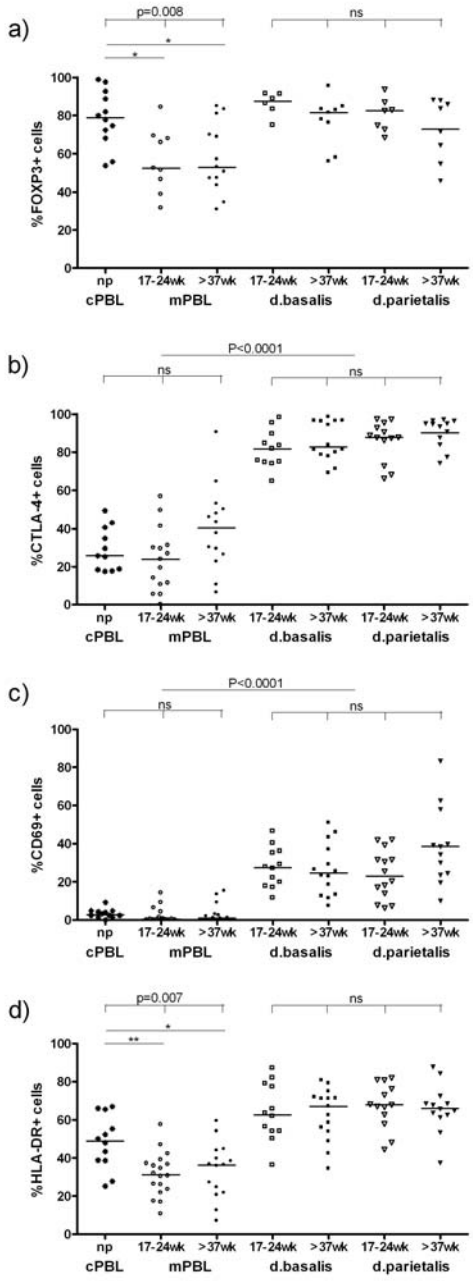


Figure 2. Characteristics of decidual and peripheral blood CD4+CD25bright cells
Percentage of FOXP3+ (a), CTLA-4+ (b), CD69+ (c) and HLA-DR + (d) cells within CD4+CD25bright T cell fraction of non-pregnant (np) control PBL (cPBL) and maternal PBL, d.basalis and d.parietalis in early (17-24wk) and after term (>37wk) pregnancy. Lines indicate median percentages.

umbilical cord blood (UCB) cells of the fetus and with a 3rd party UCB. In both d. basalis and d.parietalis isolates the depletion of CD4+CD25bright T cells leads to a significant increase in proliferation to UCB cells (p=0.034; p=0.027) and a 3rd party UCB (p=0.001; p=0.039) (Figure 4a). To compare the suppressive capacity of maternal peripheral blood and the decidual isolates a Suppression Index (S.I.) was determined. CD4+CD25bright T cells from d.basalis and d.parietalis contain a significant higher suppressive capacity to regulate fetus specific UCB cells compared to maternal

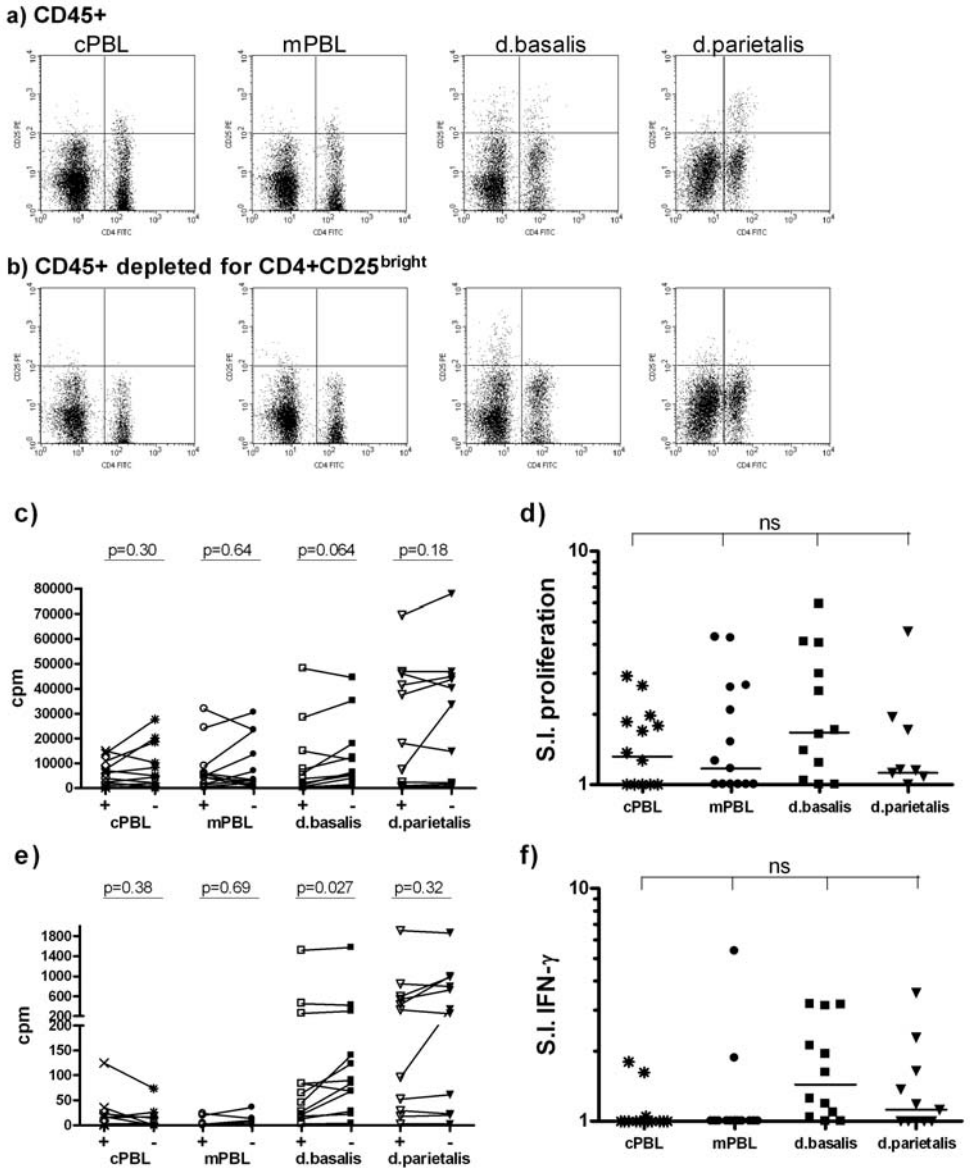


Figure 3. Function of CD4+CD25bright cells to OKT-3 stimulation
Representative dotplots of CD4 and CD25 expression within the CD45+ fraction (a) and the CD45+ fraction depleted for CD4+CD25bright cells (b) of cPBL, mPBL, d.basalis and d.parietalis isolates after FACS sorting. All plots are gated for CD45+ cells within the lymphocyte gate. Proliferation (c) and IFNγ production (e) of CD45+ cells (+) and CD45+ cell depleted for CD4+CD25bright cells (-) after OKT-3 stimulation. Isolates of cPBL, mPBL, d.basalis and d.parietalis are shown. The suppression index (S.I.) of proliferation (d) and IFN-γ production (f) of all samples is depicted.

peripheral blood CD4+CD25bright T cells ($p<0.05$; $p<.0.05$) (Figure 4b). However no difference in suppressive capacity between decidual and maternal peripheral blood CD4+CD25bright T cells to 3rd party UCB cells is observed (Figure 4b). Interestingly, maternal peripheral blood shows a reduced suppressive capacity to UCB of her own fetus (median SI=1.0) compared to UCB of a 3rd party fetus (median SI= 1.29) ($p=0.052$) (data not shown). All mother-child combinations are haplo- identical for HLA-A, -B, -C, -DR and -DQ. No difference is observed between fully mismatched or haplo-identical 3rd party UCB stimulator cells using maternal and non-pregnant control responder cells. The capacity of maternal peripheral blood to suppress 3rd party UCB is similar to the capacity of non-pregnant controls to suppress UCB (data not shown).

Percentage of CD4+CD25 bright T cells

In order to investigate whether the observed difference in suppressive capacity are caused by differences in percentages of CD4+CD25bright cells is the isolates, all fractions obtained after FACS sorting were reanalyzed on a FACS Calibur. The percentage of CD4+CD25bright T cells within the CD4+ T cell population and within the CD45+ populations were determined. In line with previous studies d.basalis and d.parietalis lymphocyte isolates contain higher percentages of CD4+CD25bright T cells within the CD4+ T cell population compared to peripheral blood isolates (Figure 5a-c). Within the CD45+ fraction the cPBL, mPBL and d.basalis contain similar percentages of CD4+CD25bright T cells (median percentages: 1.0%; 1.1%; 1.4% respectively), resulting in Treg-lymphocyte ratio of ~1:100. D.parietalis contains a higher percentage of CD4+CD25bright T cells (2.8%) within the CD45+ fraction resulting in a ratio of 1:36. No correlation between the Treg-lymphocyte ratios and the suppression index of all individual experiments was observed. The percentage CD4+CD25dim T cells did not differ in the CD45+ fraction and the CD4+CD25bright depleted fraction (data not shown).

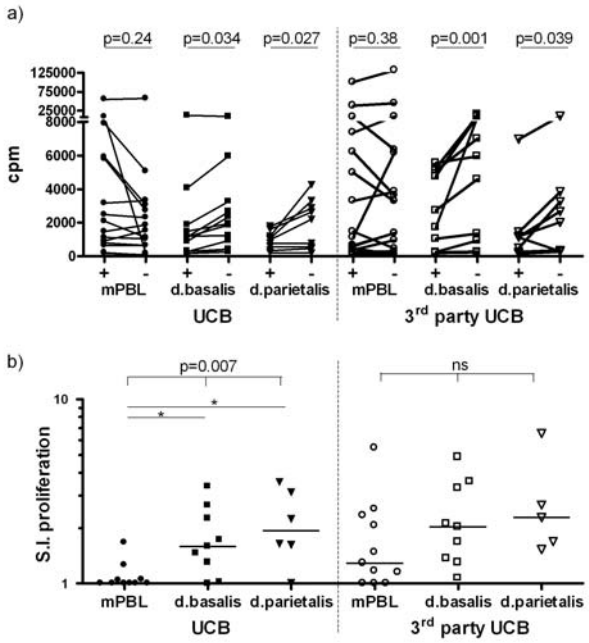


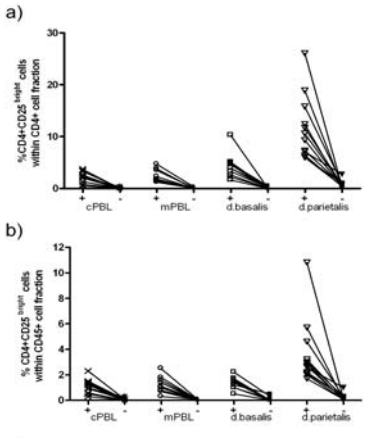
Figure 4. Fetus specific and fetus non-specific suppression by decidual CD4+CD25bright T cells
a) Shows proliferation of CD45+ cells (+) and CD45+ cell depleted for CD4+CD25bright cells (-) after fetus specific UCB (left) and 3rd party UCB stimulation (right). Isolates of mPBL, d.basalis and d.parietalis are shown. b) shows the suppression index (S.I.) of proliferation of mPBL, d.basalis and d.parietalis after fetus specific UCB (left) and 3rd party UCB stimulation (right).

Localization of decidual CD3+FOXP3+ cells at the fetal-maternal interface.

In order confirm the localization of CD3+ regulatory T cells in decidual tissue, we analyzed paraffin embedded tissue sections of the placenta (containing d.basalis and villi) and the membranes (containing amnion, chorion and d.parietalis) in early and term pregnancy. The sections were stained for CD4 in combination with FOXP3 or CD3 in combination with FOXP3. All sections show a preferential localization of CD4+FOXP3+ and CD3+FOXP3+ cells in maternal tissue (i.e. present in d.basalis but not in villous tissue (Figure 6a) and in d.parietalis but not in chorion and amnion (Figure 6b). In addition, a high variation in numbers of CD3+FOXP3- and CD3+FOXP3+ is observed between individual patients (Figure 6b-c).

Discussion

In this study we investigated the phenotypic and functional properties of decidual and peripheral blood CD4+CD25bright T cells. Two clearly distinguished populations of CD4+CD25dim and CD4+CD25bright T cell subsets were found in all decidual isolates. CD4+CD25dim T cells show an activated phenotype containing high percentages of HLA-DR+ and CD69+ cells and low percentages of FOXP3+ and CTLA-4+ cells. In contrast, decidual CD4+CD25bright T cells show a regulatory phenotype containing high percentages of FOXP3+, CTLA-4+ and HLA-DR+ cells. Decidual CD4+CD25bright T cells are a homogeneous cell population with no significant differences in phenotype between d.basalis and d.parietalis isolates or between 2nd and 3rd trimester pregnancies. Decidual CD4+CD25bright T cells show an increased expression of CTLA-4, HLA-DR, CD69 and CD25 compared with peripheral blood CD4+CD25bright T cells. Understanding the functional significance of the phenotypic differences in peripheral and decidual CD4+CD25bright Treg cells is limited by the lack of true Treg specific surface markers and therefore the inability to define mechanisms of suppression. Identification of regulatory T cells based upon their phenotypic characterization is



c)

%CD4+CD25^{bright} cells		CD45+	CD45+ depleted for CD4+CD25^{bright}
cPBL	within CD4+	2.1%	0.1%
	within CD45+	1.0%	0.1%
mPBL	within CD4+	1.8%	0.1%
	within CD45+	1.1%	0.1%
d.basalis	within CD4+	3.9%	0.1%
	within CD45+	1.4%	0.0%
d.parietalis	within CD4+	10.0%	0.6%
	within CD45+	2.8%	0.2%

Figure 5. Percentage of CD4+CD25 bright T cells
Percentage of CD4+CD25 bright T cells in CD45+ fraction (+) and CD45+ fraction depleted for CD4+CD25bright T cells (-) of cPBL, mPBL, d.basalis and d.parietalis isolates after FACS sorting. Percentages of CD4+CD25 bright T cells within the CD45+ T cell population (a) and within the CD4+ population (b) are depicted. Table 5c indicates the median percentages of CD4+CD25 bright T cells of all samples.

controversial (20,21) and functional tests are required to identify regulatory T cells. The identification of novel Treg specific markers CD39 and CD73 that are functionally involved in immunosuppressive activity in mice (22) is promising for future studies but their relevance remains to be confirmed in the human system. In addition, mechanistic studies on FOXP3 function or signalling of immunoregulatory molecules like TGF-β show the dynamics of Treg generation (23,24) and may eventually lead to elucidation of the differences between peripheral and decidual CD4+CD25bright T cells.

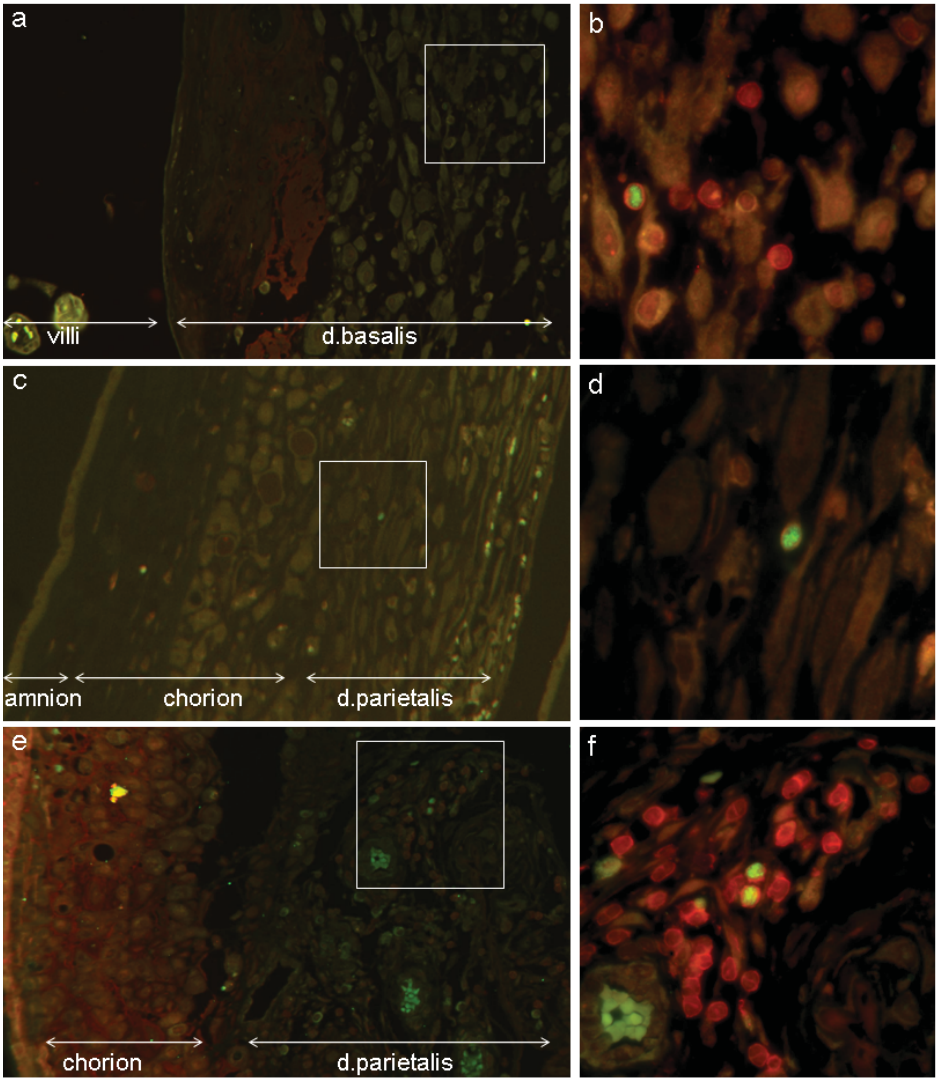


Figure 6. Localization of CD3+ FOXP3+ cells at the fetal maternal interface
Immuno histochemical staining of CD3-TexasRED and FOXP3-alexa488 in placenta sections (a-b) and sections of the membranes (c-f). a) shows an overview of placental tissue containing villi and d.basalis and b) the localization of CD3+FOXP3+ and CD3+FOXP3- cells in d.basalis. c) shows an overview of membranes containing amnion, chorion and d.parietalis tissue and d) the localization of a CD3+FOXP3+ cell in d.parietalis. e) shows an overview of membranes from a second individual containing chorion and d.parietalis tissue and f) shows the localization of CD3+FOXP3+ and CD3+FOXP3- cells in d.parietalis.

Many studies have shown that CD4+CD25bright T cells can suppress specific and non-specific immune responses in a dose dependent manner. Similar results were obtained in functional assays, which differed with regard to experimental setup, effector cell populations (total CD3+ cells, CD4+CD25- T cells), Treg-Teffector ratios, sources of APC, and readout systems (proliferation, cytokines) (25,26). The aim of our study was to compare the contribution of CD4+CD25bright T cells in regulating maternal lymphocyte responses at the fetal-maternal interface and in maternal peripheral blood. For this we isolated the complete lymphocyte fraction and compared proliferation and cytokine responses of the total lymphocyte fraction with the CD4+CD25bright depleted lymphocyte fraction. In contrast to other studies where the modulating effect of an isolated subpopulation of responder cells is tested, we measure the potential of CD4+CD25bright cells to suppress the reactivity of the different lymphocyte populations including CD4+CD25+, CD8+ T cells and NK cells, present in the blood or in the decidua which is compatible to the in vivo situation. In addition we used CD3 stimulation and stimulation with UCB cells to determine whether there is a fetus-specific component in CD4+CD25bright T cells mediated suppression

Upon CD3 stimulation, we found a variable increase in proliferation or IFN-γ production after depletion of CD4+CD25bright T cells. In the d.basalis a significant increase in IFN-γ production after depletion of CD4+CD25bright T cells was observed in all individuals. In the d.parietalis a group of high responders with a clear increase in proliferation and IFN-γ production after depletion of CD4+CD25bright T cells was found next to a group of low responders without a clear regulatory capacity of the CD4+CD25bright cells. Between these two groups the gender of the child differed, the high responders being all female (n=5) and the low responders all male except for 1 female (n=5+1). The numbers in this group are too small to state significance but in further studies this difference should be further elucidated. These data are suggestive for an individual variation in the contribution of CD4+CD25bright T cells in the regulation of the local immune response. In this study we did not observe differences in the suppression capacity of peripheral blood lymphocyte isolates and decidual lymphocyte isolates to CD3 stimulation using the OKT-3 and UCHT-1 clone. The type of suppression assay we used, lacking antigen presenting cells (APCs) and the low ratio CD4+CD25bright cells that are depleted from the total lymphocyte isolate, might lead to a low sensitivity to detect regulation. It does however provide the best reflection of the in vivo activation status of all lymphocytes and capacity of Treg cells to regulate their response. Nevertheless, future experiments should elucidate possible differences in regulatory capacity of decidual and peripheral CD4+CD25bright T cells by mixing Tregs and lymphocytes in higher ratios and test the influence of APCs.

The dynamics of immune regulation during pregnancy is shown by the fact that depletion of CD4+CD25bright T cells from maternal peripheral blood does not affect the immune response to her own child whereas immune regulation to a 3rd party UCB is comparable to non-pregnant controls. In addition, mPBL samples show a reduced percentage of CD4+CD25brightFOXP3+ and CD4+CD25brightHLA-DR+ cells compared to peripheral blood of non-pregnant controls. In contrast, decidual tissue contains a high proportion of CD4+CD25bright T cells with a regulatory phenotype and despite the individual variation between the patients; the decidual CD4+CD25bright T cells contain the capacity to regulate fetus-specific and fetus non-specific responses. These data suggest that fetus-specific regulatory T cells are specifically recruited from the periphery to the fetal-maternal interface. A recent study examining the dynamics of

CD4+CD25bright T cells during the menstrual cycle has demonstrated an expansion of CD4+CD25brightFOXP3+ T cells just before ovulation (27). In addition, reduced numbers of Treg cells and a diminished suppressive capacity of these cells was observed in woman with recurrent spontaneous abortions (17). Besides the impairment of expansion of functional Treg populations, defects in recruitment of CD4+CD25bright Treg cells to the fetal-maternal interface may play a role in development of pathology during pregnancy.

The leukocyte composition of decidual isolates is highly variable among individuals (data not shown). Analysis of 14 uncomplicated term deliveries show an average T cell percentage of 51±13% in d.basalis, 64±11% in d.parietalis and 71±11% in mPBL (all calculated within the CD45+ lymphocyte fraction), compared to 75±3% in peripheral blood of non-pregnant controls. In addition there is high variation in percentage CD4+CD25bright T cells in d.basalis and d.parietalis isolates (13). We did not find a correlation between the percentage of depleted CD4+CD25bright T cells and the observed suppression capacity. However, the variation in suppression capacity between the samples might be due to a different leukocyte composition of the isolates. Decidual T cells comprise a very heterogeneous subset of T cells containing CD4+ and CD8+ cells with highly activated phenotypes as well as cells with a merely regulatory phenotype (13,16). The activated decidual T cells might be more difficult to suppress in comparison to peripheral blood T cells, resulting in similar suppression indexes. The decidual isolates also contain variable percentages of decidual NK cells and although studies have shown that CD4+CD25+ T cells can inhibit natural killer cell functions (28), future studies should examine the potential inhibitory effect of CD4+CD25bright T cells on decidual NK cells.

Based on the high variation between lymphocyte properties in individual pregnancies, including lymphocyte gain, lymphocyte composition and the variable contribution of CD4+CD25bright T cells to suppress decidual lymphocyte responses, we hypothesize that each pregnancy generates a unique combination of regulatory mechanisms to result in a successful pregnancy. These regulatory mechanisms can include non-specific suppression mechanisms mediated by the expression of IDO, FAS, complement inhibitor proteins or more specific mechanisms mediated by HLA-expression patterns (1-5), NK-cell – trophoblast interactions (10,11), decidual macrophages or regulatory T cells (1,13,14). Maternal genotype (like HLA genotype, KIR genotype, or cytokine polymorphisms), or maternal history (regarding birth order, infection history) and the combination of fetal HLA matches and mismatches may determine which regulatory mechanisms are most predominant.

The mechanisms by which regulatory T cells can inhibit fetus-specific responses at the fetal maternal interface remain to be elucidated. Examining the functional differences between decidual and peripheral blood CD4+CD25bright T cells might identify factors that can induce regulatory CD4+CD25bright T cells at the fetal-maternal interface and may help to understand conditions of placental pathology where regulatory T cells are reduced (17,29). In this study we demonstrate that fetus-specific regulatory T cells are absent in maternal peripheral blood at term pregnancy. In addition, we demonstrate that decidual CD4+CD25bright T cells suppress fetus specific and non-specific responses. Our data suggest a preferential recruitment of fetus-specific regulatory T cells from maternal peripheral blood to the fetal-maternal interface and suggest that CD4+CD25bright T cells contribute to the regulation of fetus-specific responses in human decidua.

Acknowledgments

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4.

A fetal-maternal HLA-C mismatch induces decidual T cell activation and is required for induction of functional Tregs

Submitted for Publication

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Planning zonder actie is vruchteloos, actie zonder planning fataal

Abstract

HLA-C is the only polymorphic classical histocompatibility antigen expressed by fetal trophoblasts at the fetal-maternal interface. HLA-C ligands engage members of the killer immunoglobulin-like receptor family expressed on decidual NK cells and may facilitate trophoblast invasion into maternal tissue. Thus far no evidence has been provided that decidual T cells specifically recognize and respond to fetal alloantigens at the fetal-maternal interface. In this study, we show that pregnancies containing a HLA-C mismatched child induce an increased percentage of CD4+CD25dim activated T cells in decidual tissue. In addition, HLA-C mismatched pregnancies exhibit a decidual lymphocyte response to fetal cells and contain functional CD4+CD25bright regulatory T cells in decidual tissue, whereas HLA-C matched pregnancies do not. This suggests that decidual T cells specifically recognize fetal HLA-C at the fetal-maternal interface but are prevented to induce a destructive immune response in uncomplicated pregnancies.

Introduction

Formation of the human placenta involves deep infiltration of fetal trophoblasts in the uterus and includes the persistence of fetal alloantigens in maternal tissue. Fetal trophoblast tissue does not express HLA-A, -B, -DR, -DQ and -DP molecules that are the main targets for alloreactive T cells (1). However, trophoblast cells do express HLA-C, -E and -G molecules by which NK cell mediated cytotoxicity is avoided. HLA-G has been shown to induce regulatory T cells (2) while HLA-C is a highly polymorphic histocompatibility antigen that can elicit an allogeneic T cell response (3,4). The importance of HLA-C in human pregnancy has been demonstrated on basis of its interaction with the killer cell immunoglobulin-like receptors (KIRs) expressed by decidual NK cells (5,6). KIRs contain activating and inhibitory receptors that can inhibit NK cell function when 'self' HLA-C is recognized and activate NK cells in the absence of self 'missing self' (7). Decidual NK cells have been shown to facilitate infiltration of trophoblasts into maternal tissue (8). Thereby, incompatibility of maternal KIR genotype and the fetal HLA-C KIR epitope leads to an increased risk of pregnancy complications like pre-eclampsia (9) and may induce spontaneous abortions (10). The maternal immune system is capable to form antibodies to fetal HLA and induce CTLs to fetal HLA and minor histocompatibility antigens (11-13). Thus far no evidence has been provided that decidual T cells can specifically recognize and respond to fetal alloantigens at the fetal-maternal interface.

CD4+CD25brightFOXP3+ regulatory T cells (Treg) and CD4+CD25dim activated T cells (Tact) are concentrated in decidual tissue during pregnancy (14,15). In addition, CD4+CD25bright Treg cells have shown to regulate fetus-specific and fetus non-specific immune responses in humans (16) and in mice (17,18). However, a high variation in decidual lymphocyte responses and lymphocyte composition is observed in uncomplicated term pregnancies. Each pregnancy consists of a unique mother-child combination that may generate different levels of lymphocyte activation and may require different types of immune regulatory mechanisms. Maternal genotype (i.e. HLA and KIR genotype), maternal history (i.e. maternal age, number of previous pregnancies) or fetal characteristics (i.e. gender, fetal weight) and the combination of fetal HLA matches and mismatches may determine which regulatory mechanisms are most predominant (16).

The aim of this study was to determine factors that contribute to maternal T cell activation and generation of regulatory T cells in uncomplicated human pregnancy. A significant correlation was found between a HLA-C mismatched pregnancy and an increase in the percentage of CD4+CD25dim activated T cells and the presence of functional CD4+CD25bright Tregs in decidua parietalis.

Materials & Methods

Blood and tissue samples and patient selection

Samples of d.basalis (n=41), d.parietalis (n=41) and heparinised maternal peripheral blood samples (n=80) were obtained from healthy women after uncomplicated term pregnancy. From all pregnancies heparinised fetal blood was obtained from the umbilical cord directly after delivery. Uncomplicated term pregnancies were selected based on the following clinical parameters: Gestational age ≥37 weeks; No signs of pre-eclampsia (highest diastole ≤90 mm/hg and no proteinuria); no signs of placental insufficiency (birthweight > 10th centile and/or normal umbilical artery Doppler studies during pregnancy); non smokers; no medication during pregnancy, except supplements like irontablets or folic acid. Only singleton pregnancies were included. Clinical parameters are depicted in Table 1. Non pregnant control peripheral blood samples (n=27) were obtained from healthy volunteer blood donors. Signed informed consent was obtained from all women and blood donors, and the study received medical ethical approval by the LUMC Ethics Committee (P02-200).

Lymphocyte isolation

Lymphocyte isolation from decidua was done as described previously (14). In brief, d.basalis was macroscopically dissected from the maternal side of the placenta. D.parietalis was collected by removing the amnion and delicately scraping the d.parietalis from the chorion. The obtained tissue was washed thoroughly with PBS and thereafter finely minced between two scalpel blades in PBS. Decidual fragments were incubated with 0.2% collagenase I (Gibco-BRL, Grand Island, NY) and 0.02% DNAse I (Gibco) in RPMI-1640 medium, gently shaking in a water bath at 37ºC for 60 min and thereafter washed once with RPMI-1640. The resultant suspensions were filtered through a 70µm sieve (BD, Labware; NJ) and washed once in RPMI-1640 medium. The decidual isolates were layered on a Percoll gradient of (7.5ml 1.08g/ml; 12.5ml 1.053g/ml; 20ml 1.034g/ml) for density gradient centrifugation (30min/800g), lymphocytes were isolated from the 1.08g/ml – 1.053g/ml interface. Peripheral blood and umbilical cord blood (UCB) samples were directly layered on a Ficoll Hypaque gradient (LUMC pharmacy; Leiden, The Netherlands) for density gradient centrifugation (20min/800g). Mononuclear cells were collected, washed twice with PBS containing 1% FCS and all cells were fixed with 1% paraformaldehyde and stored at 4˚C until cell staining and flow cytometric analysis.

Flow cytometry

The following directly conjugated mouse-anti-human MoAbs were used for immunofluorescence staining: CD3-PerCP, CD4-APC, CD8-PE, CD25-PE (Becton Dickinson), CD28-APC and CD45-APC (BD Pharmingen) and used in concentrations according to the manufacturer's instructions. Flow cytometry was performed on a FACS Calibur using Cellquest-pro software. Analysis of all decidua and PBL samples was done using the same Cellquest-pro template. Calculations were done within the lymphocyte gate, set around the viable lymphocytes as previously described (14). The percentages of CD4+CD25dim and CD4+CD25bright T-cells were calculated within the CD3+CD4+ cell fraction whereas the percentage of CD8+CD28- T cells were determined within the CD3+CD8+ cell fraction.

Functional assays

The functional analyses of decidual and peripheral blood isolates are described previously (16). In brief, decidual and peripheral blood isolates were FACS sorted on a Flow sorter ARIA (Becton Dickinson). Isolates were sorted for viable CD45+ cells and a CD45+ cell fraction without CD4+CD25bright cells. All cells were sorted within the lymphocyte gate set around the viable lymphocytes avoiding granulocytes, macrophages and other contaminating cell types. Cells were incubated in RPMI supplemented with L-glutamine 2 mM, penicillin 50 units/ml en streptomycin 50 µg/ml (all obtained from Gibco Laboratories) and 10% human serum in a round-bottomed 96 well plate (Costar Cambridge, MA, USA) at a density of 50.000 cells per well in triplicate. All fractions were stimulated with 50.000 irradiated (3000 Rad) UCB cells from the own child and incubated at 37ºC with 5% CO2 for 5 days. Proliferation was measured by [3H]thymidine (1µCi) incorporation for the last 16 hours and measured by liquid scintillation spectroscopy using a betaplate (Perkin Elmer-Wallac, Turku, Finland). Results are expressed as the median counts per minute (cpm) for each triplicate culture. The suppression index (S.I.) of CD4+CD25bright T cells is depicted as the ratio of the proliferation (cpm) of the CD45+ depleted for CD4+CD25bright fraction and proliferation (cpm) of the CD45+ fraction.

HLA typing

All mothers and children were DNA typed at low resolution for the loci HLA-A, -B, -C, -DRB1 and -DQB1 using the Sequence Specific Oligonucleotides (SSO) PCR technique. HLA typing was performed at the national reference laboratory for histocompatibility testing (Leiden University Medical Center, The Netherlands). The number of fetal-maternal HLA mismatches was determined. Hereby a haplo-identical mother-child combination contains the maximum of 5 HLA mismatches, whereas more identical mother-child combinations contain less HLA mismatches. The HLA-C1 and C2 group of both mother and child was established on basis of the presence of SER77ASN80 (C1) and ASN77LYS80 (C2) in the DNA sequence (19) and the number of C1 and C2 alleles present in the mother but not in the child (‘missing self’) was determined.

Statistical analysis

All statistical analyses were performed using SPSS 12.0 software. To determine differences between 2 independent groups a non-parametric Mann-Whitney U test was performed and for >2 independent groups a non-parametric Kruskal-Wallis H test. For linear parameters linear regression analysis was performed. Multivariate analyses were performed with a Backward Regression model. All p-values <0.05 are considered to denote significant differences.

Results

Positive association between the number of fetal-maternal HLA mismatches and the percentage of CD4+CD25dim T cells in decidua parietalis

To examine the hypothesis that HLA mismatches between mother and child may induce T cell activation or induce regulatory T cells in maternal blood and decidual tissue, we determined the percentages of CD4+CD25dim T cells and CD4+CD25bright T cells in maternal peripheral blood (mPBL) (n=80), decidua basalis (n=41) and decidua parietalis (n=41) isolates (Figure 1). Linear regression analysis shows that with an increased number of HLA-A, -B, -C, -DR and -DQ mismatches between mother and child a significant increase in percentage of CD4+CD25dim T cells in d.parietalis (p=0.0035, r2=0.21) is observed (Figure 2a). Although decidua basalis and maternal PBL (mPBL) contain significantly increased percentages of CD4+CD25dim T cells in comparison to non-pregnant control PBL (Figure 1a), no correlation between the percentage of CD4+CD25dim T cells and the number of HLA mismatches is observed in decidua basalis and mPBL. In addition no correlations are observed between the percentage of CD4+CD25bright T cells and the number of HLA mismatches (data not shown).

HLA-C mismatch is crucial for decidual T cell activation

To determine which HLA locus mismatch is responsible for the increase in percentage of CD4+CD25dim T cells in decidua parietalis, the HLA-A, -B, -C, -DRB1, -DQB1 mismatches were analyzed separately in a multivariate regression model. The model shows a significant correlation between the presence of a HLA-C mismatch and the increased percentage of CD4+CD25dim T cells in d.parietalis (p=0.03). In contrast HLA-A, -B, -DRB1 or -DQB1 mismatches do not correlate with the percentage of CD4+CD25dim T cells (Figure 2b, 3a).

Table 1. Clinical parameters

	Parameter	Range	Mean	st.dev	
Mother	Age	23-42	33	5	(year)
	Gravidity	1-9	2.8	1.5	
	Parity	0-4	1.5	1.1	
	Miscarriages[1]	0-7	0.6	1.1	
	Induced abortions	0.2	0.1	0.4	
	EUG[2]/MOLA[3]	0	0	0	
	Highest diastole	60-90	76	8	(mm/Hg)
Child	Gender	59% Female	41% Male		
	Birth weight	2570-5285	3585	515	(gram)
	Placenta weight	350-1100	632	149	(gram)
Delivery	Gestational age	37.1-42.4	39.5	1.1	(week)
	Delivery type	40% CS[4]	60% SVD[5]		
	Interval of membrane rupture at delivery	1-3000	362	627	(minute)

[1]) Miscarriages in maternal history; [2]) Extra Uterine Gravidity; [3]) Mola Hydratiform Pregnancy;
[4]) Ceserean Section without contractions; [5]) Spontaneous Vaginal Delivery with contractions

A fetal-maternal HLA-C mismatch induces lymphocyte proliferation and functional CD4+CD25bright Treg cells

The effect of a fetal HLA-C mismatch on the fetus specific lymphocyte proliferative responses and the regulatory role of CD4+CD25bright T cells was analyzed. In order to obtain sufficient cells for functional analysis of the decidual CD4+CD25bright T cells we purified all CD45+ lymphocytes by FACS sort from decidua parietalis isolates. In addition we compared the proliferative response of CD45+ lymphocytes with a CD45+ lymphocyte fraction depleted for CD4+CD25bright T cells to determine the suppressive capacity of CD4+CD25bright T cells. Both fractions were stimulated with fetal umbilical cord blood (UCB) cells. Decidual lymphocytes derived from pregnancies without a HLA-C mismatch do not respond to fetal UCB cells (Figure 3b). In contrast decidual lymphocyte fractions from pregnancies with a HLA-C mismatch do proliferate to fetal UCB cells. Depletion of the CD4+CD25bright T cells results in an increased proliferative response in HLA-C mismatched but not in HLA-C matched pregnancies (Figure 3b). To compare the suppressive capacity of CD4+CD25bright T cells in HLA-C matched and HLA-C mismatched pregnancies a Suppression Index (S.I.) was determined and a significant increase in suppression capacity is found in HLA-C mismatched pregnancies (p=0.048) (Figure 3c).

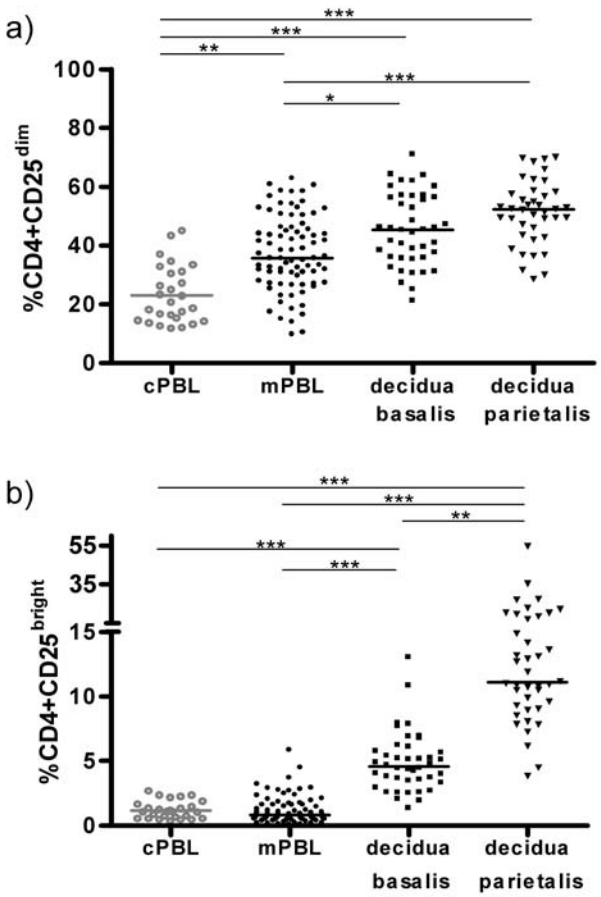


Figure 1. Distribution of CD4+CD25dim and CD4+CD25bright T cells
The percentage of CD4+CD25dim (a) and CD4+CD25bright (b) in non pregnant control PBL (cPBL), maternal PBL (mPBL), decidua basalis and decidua parietalis (lines indicate median percentages; *p<0.01; **p<0.01; ***p<0.001).

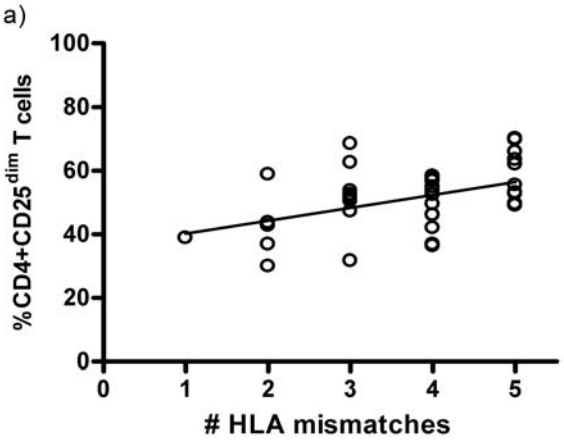
HLA-C KIR differences

To eliminate the possibility that the increase in CD4+CD25dim T cells is caused by a HLA-C KIR epitope difference rather than a HLA-C allele mismatch, the HLA-C1 and C2 groups of both mother and child were determined based on the presence of SER77ASN80 (C1) and ASN77LYS80 (C2) in the DNA sequence (19). NK cell activation through KIR is inhibited in the presence of self MHC class I but can be activated by the absence of self (missing-self) MHC class I molecules 7. The number of HLA-C1/C2 KIR epitope matches and 'missing self' epitopes was analyzed with the HLA-C allele mismatch in a multivariate regression model. No correlation is observed in the presence of KIR differences and the percentage of CD4+CD25dim activated T cells in decidua parietalis, decidua basalis and maternal PBL.

Clinical parameters are not associated with the induction of regulatory T cells or T cell activation

Besides HLA mismatches between mother and child, clinical parameters can potentially influence T cell activation or quantity of regulatory T cells during pregnancy. All clinical parameters (Table 1) were analyzed for a possible correlation with the percentage of CD4+CD25dim or CD4+CD25bright cells. However, no correlation between any of the clinical parameters and the percentage of CD4+CD25dim or CD4+CD25bright T cells was observed in mPBL, decidua basalis and decidua parietalis (data not shown). Including the HLA-C allele mismatch, clinical parameters and the percentage CD4+CD25dim T cells in multivariate regression models did not show a significant correlation between clinical parameters and a HLA-C mismatch or between clinical parameters and the percentage of CD4+CD25dim T cells.

a)



b)

	Significance
HLA-A mismatch	ns
HLA-B mismatch	ns
HLA-C mismatch	0.030
HLA-DRB1 mismatch	ns
HLA-DQB1 mismatch	ns

Figure 2. Fetal-maternal HLA mismatches correlate with decidual CD4+CD25dim T cells
a) Total HLA-A, -B, -C, -DR and -DQ mismatches significantly correlate with an increased percentage of CD4+CD25dim T cells in decidua parietalis (p=0.0035; r2=0.21; n=39); b) HLA-C but not HLA-A, -B, -DR and –DQ correlates with the percentage of CD4+CD25dim T cells in decidua parietalis (p=0.030).

Discussion

In order to investigate which factors are crucial for T cell activation and induction of regulatory T cells during human pregnancy, we determined the percentage CD4+CD25dim activated T cells (Tact) and CD4+CD25bright regulatory T cells (Treg) in maternal peripheral blood and decidual tissue. We analyzed the results in relation to the number of HLA-A, -B, -C, -DRB1, -DQB1 matches and mismatches and clinical data of mother, child and pregnancy. Our data provide the first evidence that a fetal HLA-C mismatch leads to maternal T cell activation in decidual tissue. Besides the increase in the percentage CD4+CD25dim Tact cells, lymphocytes from pregnancies with a HLA-C mismatch proliferate upon fetus specific stimulation and contain functional CD4+CD25bright Treg cells. In contrast lymphocyte fractions from HLA-C matched pregnancies do not proliferate and do not contain functional CD4+CD25bright Treg cells.

The increased activation of CD4+ T cells in the presence of a HLA-C allele mismatch, suggests a T cell receptor (TCR) mediated recognition of fetal HLA-C in decidual tissue. As CD4+ T cells are involved, the HLA-C alleles are probably not directly recognized as intact allogeneic MHC-class I molecules. More likely, maternal CD4+ T cells recognize fetal HLA-C through indirect allo-recognition, where fetal HLA-C antigens are processed and presented as peptides in the context of self MHC class II on Antigen Presenting Cells (APC) (20). The increase in T cell activation is not associated with HLA-C KIR differences, eliminating a possible indirect effect of NK cells on decidual T cell activation. Besides CD4+CD25dim and CD4+CD25bright T cells we analyzed the percentage of CD8+CD28- effector T cells in decidual isolates. CD8+ T cells are MHC class I restricted and may directly recognize allogeneic HLA-C. However, we did not observe correlations in the percentage of CD8+CD28- T cells and fetal-maternal HLA-C mismatches. Previous studies have shown that particular CD4+ and CD8+ T cell subsets can express KIR by which they can directly recognize MHC class I molecules

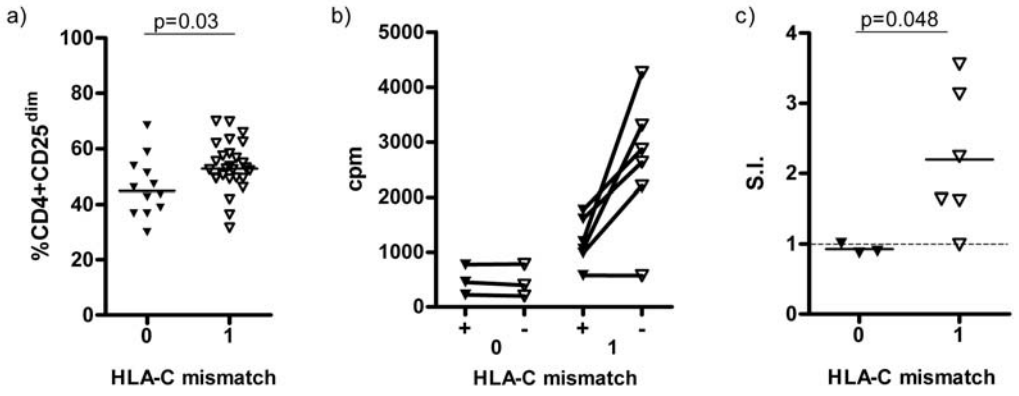
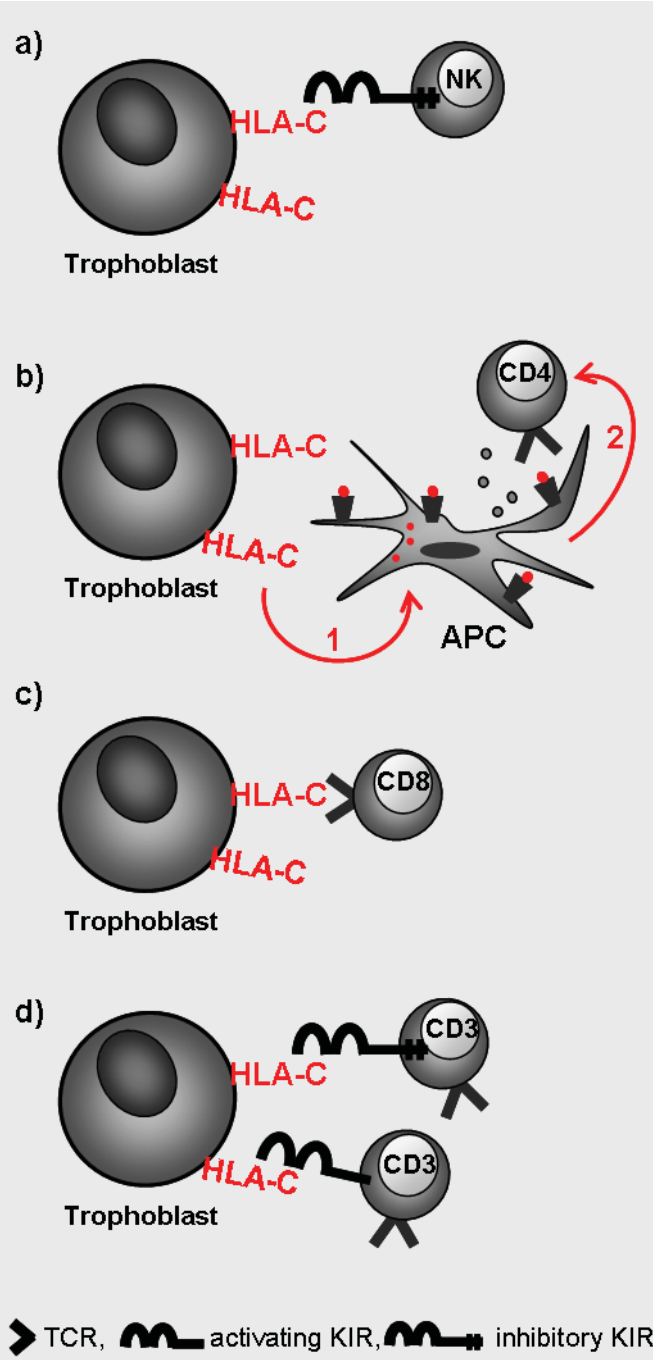


Figure 3. Differences between HLA-C matched and HLA-C mismatched pregnancies
a) HLA-C mismatched pregnancies contain a significantly increased percentage of CD4+CD25dim activated T cells; b) HLA-C mismatched pregnancies (1) but not HLA-C matched pregnancies (0) induce a decidual lymphocyte response to fetal cells and c) HLA-C mismatched pregnancies (1) but not HLA-C matched pregnancies (0) contain functional CD4+CD25bright regulatory T cells in decidua parietalis (+ = CD45+ fraction; – = CD25bright depleted CD45+ fraction; Suppression Index (S.I.) is the ratio CD45+ fraction / CD25bright depleted CD45+ fraction (cpm)).

(21). However, our ongoing studies do not indicate that decidual CD4+ or CD8+ T cells express KIRs in high frequencies. The possible mechanisms of HLA-C recognition at the fetal-maternal interface are depicted in figure 4.



a) NK cells can specifically recognize HLA-C1 and HLA-C2 using inhibitory (KIR2DL1/2/3) KIRs. The inhibitory receptors contain a long ITIM motif and are inhibited by self HLA-C and are reactive to cells lacking self HLA-C *(ref 3)*

b) Indirect all-recognition of fetal HLA-C by CD4+ T cells. 1) HLA-C antigens are processed and 2) presented to CD4+ T cells as peptides in context of self MHC class II on Antigen Presenting Cells (APCs) *(This paper)*

c) CD8+ T cells may directly recognize intact HLA-C on allogeneic fetal cells *(specificity of decidual CD8+ T cells remains to be determined)*

d) Specific T cell subsets have shown to express KIR receptors by which they may recognize their HLA-C KIR ligand. Decidual T cells express KIR in low frequencies *(Chapter 6)*

Figure 4. Mechanisms of HLA-C recognition at the fetal-maternal interface

The role of decidual NK cells in pregnancy is extensively studied and shows that NK cells can recognize allogeneic fetal HLA-C through KIR (1,6,8). In addition, unfavourable combinations of maternal KIR genotype and fetal HLA-C ligands lead to a higher incidence of pre-eclampsia (9) and unexplained spontaneous abortions (10). Pre-eclampsia is mainly a disease of the primiparous woman and subsequent pregnancies with the same partner are at lower risk (22,23). It is tempting to speculate that induction of T cell tolerance may prevent a detrimental response in subsequent pregnancies.

The differences in immune reactivity induced by a fetal HLA-C matched or HLA-C mismatched pregnancy may induce alterations in the maternal and fetal immune system. Hereby a HLA-C matched or HLA-C mismatched pregnancy in the maternal and fetal history may contain divergent implications for autoimmune disease, allograft tolerance and induction of microchimerism (24-26). However analysis of the presence of HLA antibodies in maternal serum did not show correlations with the presence or absence of a fetal HLA-C (data not shown). Therefore the induced T cell activation and regulatory T cells seem merely a local effect at the fetal-maternal interface rather then inducing peripheral immune alterations.

The increase in CD4+CD25dim Tact cells and functional CD4+CD25bright Tregs in HLA-C mismatched pregnancies is observed in decidua parietalis but not in decidua basalis or mPBL. Decidua parietalis is the maternal part of the membranes connected to the non-invasive trophoblasts of the chorion, whereas d.basalis is the maternal part of the placenta at the implantation site, connected to invading fetal trophoblasts. Besides differences in T cells subsets (14,27) different NK cell (28) and macrophage subsets (29) are found in decidua basalis and decidua parietalis. NK cells and macrophages can influence the immunologic environment in decidua basalis and decidua parietalis and thereby may differently affect the influx, expansion or maturation of T cells in decidua basalis and decidua parietalis (5). In addition, differential expression of molecules like TGF-β (30), IDO and FAS (31) or differences in HLA expression (32) may induce divergent mechanisms of T cell activation and regulation in decidua basalis and decidua parietalis. Maternal peripheral blood contacts the syncytiotrophoblast layer during utero-placental circulation. Syncytiotrophoblasts do not express HLA-A, -B, -C, -DRB1, DQB1 molecules and therefore cannot induce allogeneic T cell activation. However, in case of placental lesions, fetal blood may directly enter the maternal circulation and induce an allogeneic T cell response. Such a response would imply recognition of fetal HLA-A, -B, -C -DRB1 and -DQB1 alloantigens and can not be compared with the observed decidual response. Nevertheless, we did not detect an increased T cell activation in mPBL with more HLA mismatches.

In conclusion, a fetal HLA-C mismatch leads to increased decidual T cell activation in uncomplicated human pregnancy, showing that decidual T cells specifically recognize fetal HLA-C. However, HLA-C recognition does not lead to a detrimental immune response in decidual tissue. Furthermore, HLA-C mismatched pregnancies exhibit a decidual lymphocyte response to fetal cells and contain functional CD4+CD25bright regulatory T cells in decidual tissue, whereas HLA-C matched pregnancies do not. The absence of additional ‘danger signals’ in healthy decidua may explain why maternal T cells specifically recognize fetal alloantigens but do not reject the fetal allograft (33,34). Further unravelling of the mechanisms of fetus specific immune recognition and immune regulation by decidual T cells may be crucial to understand why some pregnancies are successful whereas others are not.

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Human decidua contains differentiated CD8+ Effector-Memory T cells

Manuscript in Preparation

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Je hebt kleine leugens, je hebt grote leugens en je hebt statistiek

Abstract

During pregnancy maternal lymphocytes at the fetal-maternal interface play a key role in the immune acceptance of the allogeneic fetus. Decidual NK cells contain immune modulatory properties and facilitate trophoblast invasion into maternal tissue. More recently, CD4+CD25bright regulatory T cells have shown to be concentrated in decidual tissue where they are able to suppress fetus-specific and non-specific responses. However, decidual CD8+ T cells form the largest fraction of T cells at the fetal-maternal interface but limited data is present on the characteristics of these cells. Therefore we performed phenotypic analysis of the decidual and peripheral CD8+ T cell pool with CD45RA, CCR7, CD28 and CD27 expression using nine-colour flowcytometry. In addition, we examined expression of the cytolytic molecules perforin, granzyme B and granzyme K to determine the cytotoxic potential of the decidual CD8+ T cell subsets. Our data demonstrate that decidual CD8+ T cells mainly consist of differentiated Effector-Memory cells while unprimed naïve cells are almost absent. Unlike peripheral blood Effector-Memory CD8+ T cells, the decidual Effector-Memory CD8+ T cells do not express perforin and have a reduced expression of granzyme B. Apparently, the functional features of decidual CD8+ T cells do not correspond their matching phenotype in peripheral blood. These data show that decidual CD8+ T cells may pursue alternative means of effector cell differentiation and indicate that decidual CD8+ T cell differentiation and regulation may play a crucial role in maternal immune tolerance to the fetus.

Introduction

Maternal lymphocytes at the fetal-maternal interface play a key role in the immune acceptance of the allogeneic fetus. Many studies have shown that decidual NK cells contain immune modulatory properties and facilitate trophoblast invasion in to maternal tissue (1,2). More recently, CD4+CD25bright regulatory T cells have shown to be concentrated in decidual tissue and are able to suppress fetus-specific and non-specific responses (3). These cells are suggested to prevent a destructive immune response to the allogeneic fetus. But so far the mechanisms for fetus specific immune recognition and the possible effector cell functions of decidual T cells remain poorly defined. Previous studies have shown that the maternal immune system is capable to form antibodies and to induce specific CTLs to fetal HLA and minor histocompatibility antigens (mHags) (4-6). Recent data by our group indicate that a fetal-maternal HLA-C mismatch correlates with an increased decidual CD4+ T cell activation and regulation (T.Tilburgs et.al submitted document). Decidual CD8+ T cells form the largest fraction of T cells at the fetal-maternal interface and are main candidates to recognize and respond to fetal HLA-C at the fetal-maternal interface. However limited data is available on the phenotype and function of decidual CD8+ T cells during human pregnancy.

In healthy individuals and during viral infections, the CD8 effector T cell differentiation process has been studied extensively (7-9). These studies have identified particular CD8+ T cell subsets capable to elicit a cytotoxic response and identified many phenotypic markers to categorize these cells. In this study we use the cell surface markers CD45RA and CCR7 to discriminate Naïve (RA+CCR7+), Effector (RA+/CCR7-), Effector-Memory (EM) (RA-CCR7-) and Central-Memory (CM) (RA-CCR7+) CD8+ T cells (10-13) in decidual tissue. In addition we analyzed the expression of the co-stimulatory molecules CD28 and CD27 to determine additional the heterogeneity in CD8+ Effector-Memory cells (EM-1 28+27+; EM-2 28-27+; EM-3 28-27- and EM-4 28+27-) (12). Furthermore, the expression of the cytotolytic molecules perforin, granzyme B and granzyme K was studied to examine the cytolytic capacity of the decidual CD8+ T cell subsets. Perforin is a membrane perturbing protein that delivers granzymes in the target cell (14). Among all known granzymes, granzyme B is essential to induce DNA fragmentation and apoptosis in target cells (14) whereas granzyme K may provide alternative mechanisms to kill target cells (15,16). We examined the CD8+ T cell pool at 2 different sites of the fetal maternal interface; the decidua basalis, the maternal part of the placenta at the implantation site, and the decidua parietalis the maternal part of the membranes connected to the fetal trophoblasts of the chorion. We compared the CD8+ T cell subset distribution in decidual tissue to the maternal peripheral blood (mPBL). As a control, we analyzed peripheral blood CD8+ T cells of age matched healthy volunteer donors.

Materials and Methods

Blood and tissue samples

Samples of decidua basalis, decidua parietalis and heparinised maternal peripheral blood (mPBL) were obtained from healthy women after uncomplicated term pregnancy (gestational age range: 37 – 42 weeks). Tissue samples were obtained after delivery by elective caesarean section or uncomplicated spontaneous vaginal delivery. mPBL samples were obtained either directly before or directly after delivery. Control PBL (cPBL)2 samples were obtained from healthy non-pregnant volunteer female donors. Signed informed consent was obtained from all women, and the study received approval by the LUMC Ethics Committee (P02-200).

Lymphocyte isolation

Lymphocyte isolation from decidua was done as described previously (17). In brief: decidua basalis was macroscopically dissected from the maternal side of the placenta. Decidua parietalis was collected by removing the amnion and delicately scraping the decidua parietalis from the chorion. The obtained tissue was washed thoroughly with PBS and thereafter finely minced between two scalpel blades in PBS. Decidual fragments were incubated with 0.2% collagenase I (Gibco-BRL, Grand Island, NY) and 0.02% DNAse I (Gibco) in RPMI-1640 medium, gently shaking in a waterbath at 37ºC for 60 min and thereafter washed once with RPMI. The resultant suspensions were filtered through a 70µm sieve (BD, Labware; NJ) and washed once in RPMI. The decidual isolates were layered on a Percoll gradient of (7.5ml 1.080g/ml; 12.5ml 1.053g/ml; 20ml 1.023g/ml) for density gradient centrifugation (30min/800g), lymphocytes were isolated from the 1.080g/ml – 1.053g/ml interface. PBL samples were directly layered on a Ficoll Hypaque gradient (LUMC pharmacy; Leiden, The Netherlands) for density gradient centrifugation (20min/800g). Mononuclear cells were collected, washed twice with PBS containing 1% FCS and all cells were fixed with 1% paraformaldehyde and stored at 4˚C until cell staining and flow cytometric analysis.

Flow cytometry

A nine-colour FACS panel was analyzed on a LSR-II Flowcytometer (Becton Dickinson) using FACS DIVA software. The LSR-II configuration and MoAbs used are listed in Table 1. All MoAbs were titrated to determine optimal dilutions. All CD8+ T cells are analyzed within the lymphocyte gate and are selected within the CD45+CD3+CD14-CD8+ phenotype. The gating strategy to determine the proportion of CD8+ T cell subsets is shown in figure 1A-C and depicted as percentage within CD8+ T cell fraction (figure 1D). To determine intracellular expression of perforin, granzyme B, and granzyme K, the cells were first stained for surface expression of CD45, CD3, CD14, CD8, CD45RA, CCR7, CD28 and CD27 and thereafter treated with permeabilizing solution buffer (containing: 0.1% saponine, 5% FCS and 0.05% sodium-azide in PBS) for 10 min and stained with perforin, granzyme B, granzyme K and all matching isotype controls in separate tubes. Perforin, granzyme B and granzyme K expression is depicted as Mean Fluorescence Intensity (MFI) within the Naïve, Effector, CM, EM-1, EM-2, EM-3 and EM-4 CD8+ T cell subsets. All matching isotypes controls were analyzed in parallel and did not show positive MFIs. Data acquisition of decidua and PBL samples was done using the same LSR-II settings and data analysis was done using the same FACS DIVA analysis template.

Statistical analysis

To determine differences between more than 2 groups, a non-parametric Kruskal-Wallis one way ANOVA was performed. If $p<0.05$ a Dunn's multiple comparison post test was performed to compare all pairs of columns. P-values <0.05 are considered to reflect significant differences.

Laser	Filter	Conjugates	MoAbs
488 nm	530/30	FITC	CCR7[1]; Granzyme K[2]; IgG1[2]
	575/26	PE	CCR7[4]; CD27[3]; Perforin[4]; IgG2b[4]
	610/20	PE-TxRED	CD45RA[1]
	695/40	PerCP	CD8[3]
	780/60	PE-Cy7	CD14[4]
405 nm	440/40	Pacific Blue	CD3[3]
	560/20	Cascade Yellow	CD45[5]
633 nm	660/20	APC	CD28[4]
	730/45	Alexa700	CD27[6]; GranzymeB[3]; IgG1[3]

[1]Caltag; [2]ImmunoTools GMBH; [3]BD; [4]BD Pharmingen; [5]DAKO; [6]e-biosciences

Table 1. LSR-II configuration and MoAbs used

Results

Decidual CD8+ T cells mainly consist of differentiated Effector-Memory cells

Human peripheral blood CD8+ T cells can be separated into four functionally distinct cell populations based on CD45RA and CCR7 expression: Naïve (RA+CCR7+), Effector (RA+CCR7-), Central-Memory (CM) (RA-CCR7+) and Effector-Memory (EM) (RA-CCR7-) cells (Figure 1A, B). Subsequent analysis of CD28 and CD27 identifies 4 subsets of EM cells: EM-1 (28+27+); EM-2 (28-27+); EM-3 (28-27-) and EM-4 (28+27-) and 3 subsets of Effector cells: pre-Effector-1 (pE-1) (28+27+); pE-2 (28-27+) and Effector cells (28-27-) (Figure 1A, B). Analysis of peripheral blood of non-pregnant controls (cPBL) and maternal peripheral blood (mPBL) shows that Naïve, Effector and EM-1 cells are the most abundant cell types in peripheral blood, whereas CM, EM-2, EM-3 and EM-4 cells are minor cell populations (Figure 1D). In contrast, analysis of lymphocyte isolates from decidua basalis and decidua parietalis shows that EM-1, EM-2 and EM-3 cells form the largest fractions of CD8+ T cells in the decidua (Figure 1C, D). Thereby, the EM-2 and EM-3 cell fractions in both decidua basalis and decidua parietalis are significantly increased compared to the PBL samples whereas the proportion of Naïve cells in both decidua basalis and decidua parietalis is significantly reduced in comparison to the PBL samples (Figure 1C, D).

Reduced expression of perforin in decidual CD8+ T cells

CD8+ T cell subsets from peripheral blood and decidua were analyzed for intracellular expression of perforin. Consistent with a previous report by Romero et.al (12), peripheral blood CD8+ Effector and EM-3 cells express high levels of perforin, whereas the expression of perforin in EM-2 cells is detectable but reduced in comparison with Effector and EM-3 cells. Naïve, CM and EM-1 CD8+ T cells in peripheral blood do not express perforin (Figure 2A, B). Analysis of decidual T cell subsets shows significantly reduced levels of perforin in the CD8+ Effector, EM-2 and EM-3 cell populations in comparison with the peripheral blood CD8+ T cell subsets with the matching phenotype (Figure 2A, B). Alike peripheral blood Naïve and CM CD8+ T cells, decidual Naïve and CM CD8+ T cells do not express perforin. As a positive control for the lymphocyte isolation procedure and flowcytometric analysis, decidual and peripheral blood CD45+CD3-CD56+ NK cells were analyzed for expression of perforin. High proportions of CD45+CD3-CD56+ NK cells from decidua and peripheral blood do express perforin (Figure 2C).

Reduced expression of granzyme B in decidual CD8+ T cells

Confirming previous studies, our data show that peripheral blood CD8+ Effector and EM-3 T cells were found to express high levels of granzyme B whereas EM-2 cells contain intermediate levels of granzyme B (Figure 3A, B). Comparison of control PBL and maternal PBL shows a slight but not significantly increased MFI of granzyme B in Effector and EM-3 cell in maternal PBL compared to control PBL. In contrast, Effector, EM-2 and EM-3 CD8+ T cells from decidua basalis display significantly reduced levels of granzyme B compared to the peripheral blood CD8+ T cell subsets with the matching phenotype (Figure 3A, B). In decidua parietalis the EM-3 cell fraction contain significantly reduced levels of granzyme B, whereas the Effector and EM-2 cells contain similar levels of granzyme B compared to their matching phenotype from peripheral blood. As a positive control for the lymphocyte isolation procedure and flowcytometric analysis, we analyzed CD45+CD3-CD56+ NK cells from decidua and peripheral blood for expression of granzyme B. High proportions of CD45+CD3-CD56+ NK cells from decidua and peripheral blood express granzyme B (Figure 3C).

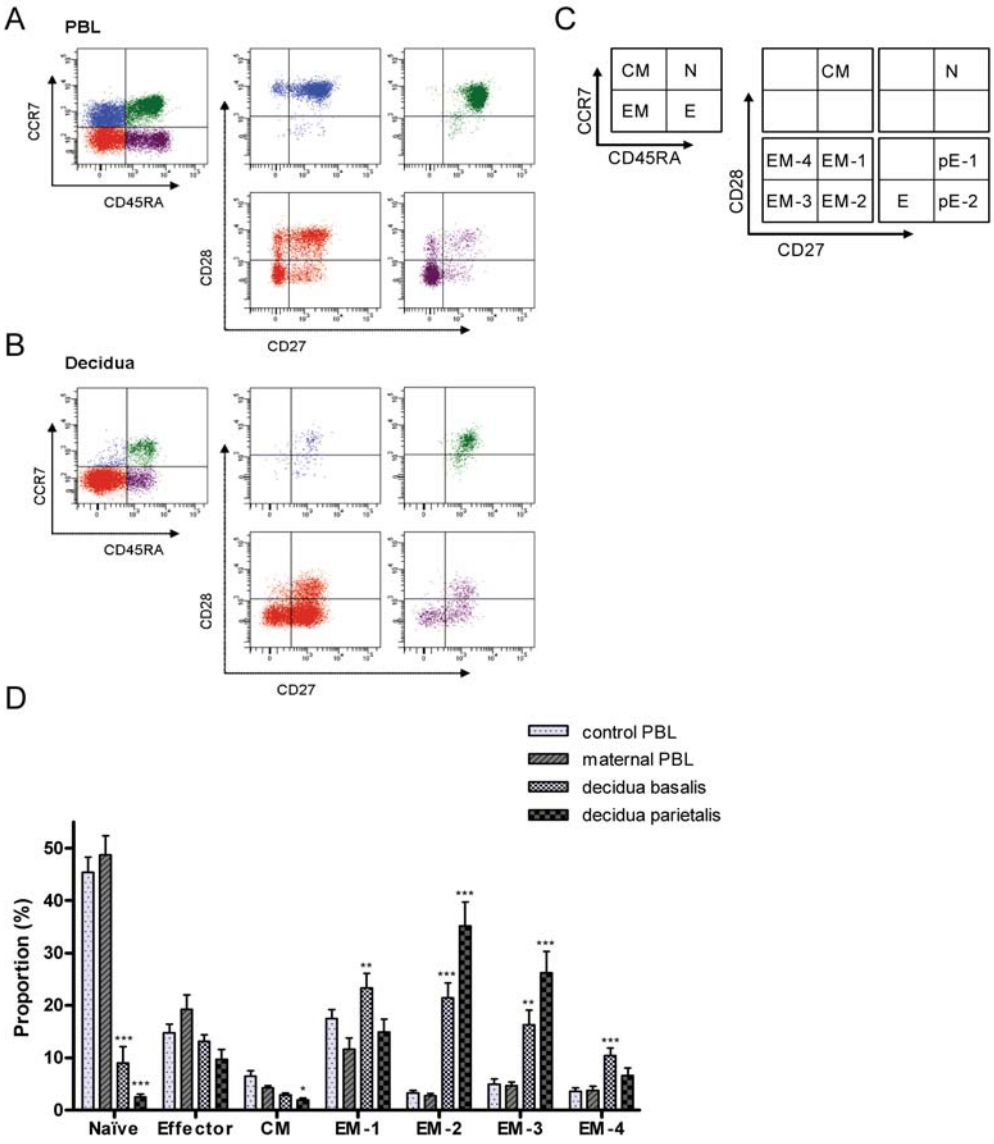

Figure 1. CD8+ T cell subsets in peripheral blood and decidua
CD8+ T cells from (A) peripheral blood and (B) decidua are separated into 4 subsets based on CD45RA and CCR7 expression. Each of these subsets are analysed for CD27 and CD28 expression. C) Nine subpopulations of CD8+ T cells can be distinguished Naïve (N), Effector (E), pre-effector-1 (pE-1) and pE-2, Central Memory (CM), Effector Memory-1 (EM-1), EM-2, EM-3 and EM-4 cells. D) The proportion of Naïve, Effector (E, pE-1 and pE-2), CM, EM-1, EM-2, EM-3 and EM-4 cells are determined in PBL from non-pregnant control PBL (n=22), maternal PBL (n=20), decidua basalis (n=15) and decidua parietalis (n=14) isolates. Bars indicate average percentage and st.dev. *p<0.05, **p<0.01, ***p<0.001

Granzyme K is expressed in decidual and peripheral blood EM-1 and EM-4 CD8+ T cells

Granzyme K is one of the least studied granzymes in humans. Although granzyme B is essential to induce DNA fragmentation and apoptosis of target cells, expression of Granzyme K may provide alternative means to induce cell death. Examining all different CD8+ T cell subsets from control PBL, maternal PBL and decidua shows that EM-1 and EM-4 cells express the highest levels of Granzyme K whereas effector cells express intermediate levels of granzyme K. No significant difference in granzyme K expression in all CD8+ T cells subsets is observed between peripheral blood and decidual samples (Figure 4).

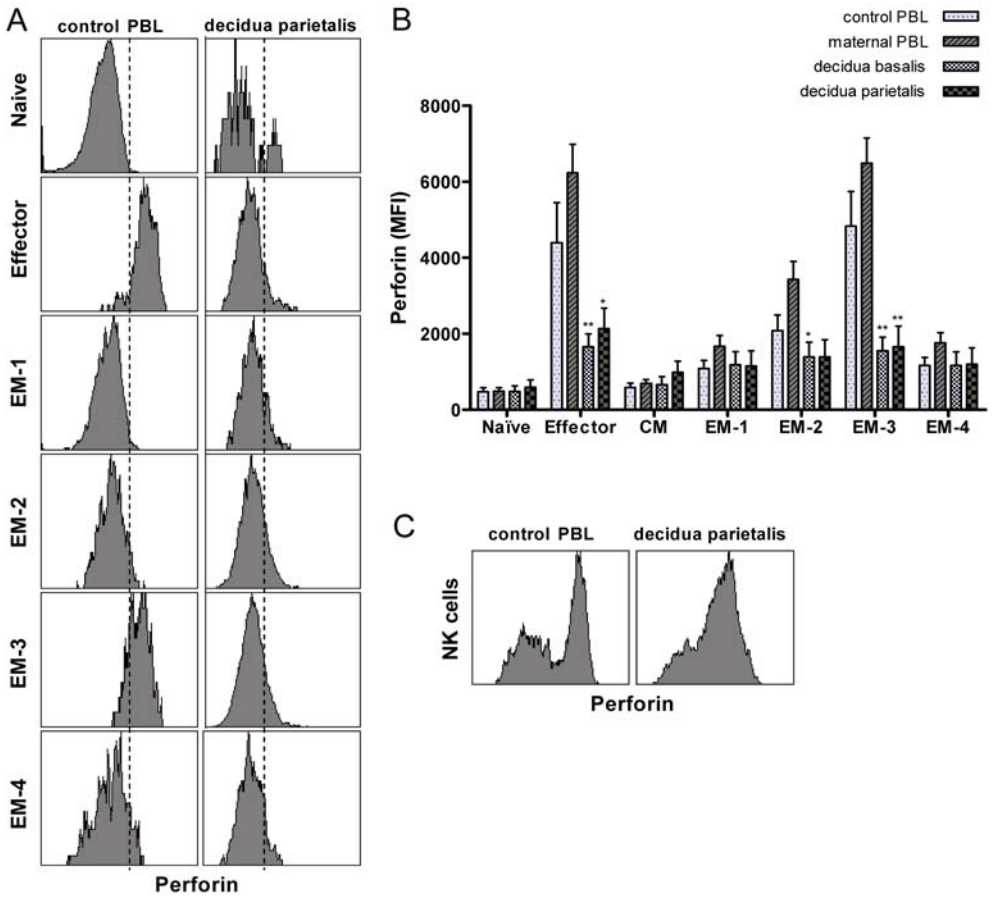

Figure 2. Perforin expression in CD8+ T cell subsets in decidua and PBL
A) Representative histograms of intracellular perforin expression in CD8+ T cell subsets from PBL and decidua. B) Shows average MFI of perforin and st.dev within control PBL (n=9), maternal PBL (n=10) decidua basalis (n=6) and decidua parietalis (n=6) CD8+ T cell subsets. Bars indicate average percentage and st.dev. *p<0.05, **p<0.01, ***p<0.001. C) Representative histograms of perforin expression in CD45+CD3-CD56+ NK cells from control PBL and decidua parietalis.

Decidual lymphocyte isolation procedure has no effect on decidual CD8+ T cell phenotype and intracellular expression of perforin and granzyme B

As decidual lymphocyte isolation procedures may alter the lymphocyte phenotype and functional activity of the isolated cells we included several controls to confirm the flowcytometrc analysis. Firstly as a positive control for the FACS analysis we analyzed CD45+CD3-CD56+ NK cells for perforin and granzyme B expression. Data show that a high proportion of decidual NK cells express perforin and granzyme B (figure 2C and figure 3C respectively). Furthermore we treated peripheral blood samples with enzymes and percoll gradient centrifugation similar to the decidual lymphocyte isolation procedure. Subsequently we analysed CCR7, CD45RA, CD28 and CD27 expression and included analysis of perforin and granzyme B expression. No differences were observed between the CD8+ T cells subsets between the treated and untreated samples. In addition no differences were observed in perforin and granzyme B expression within the CD8+ T cell subsets (data not shown).

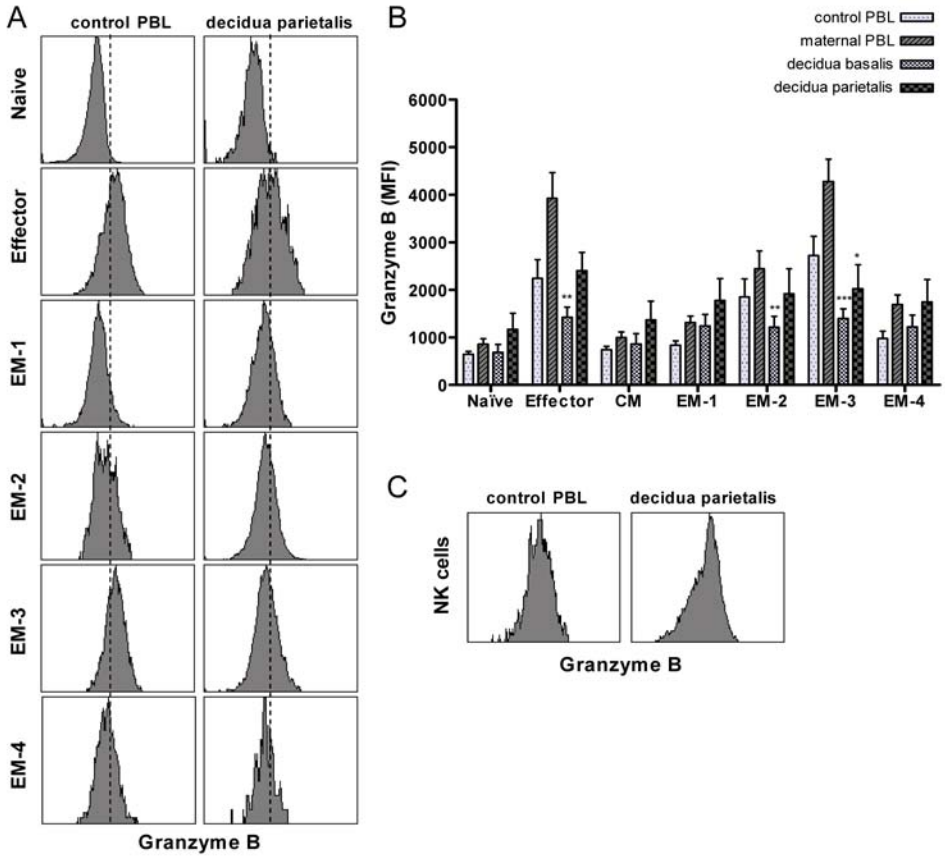

Figure 3. Granzyme B expression in CD8+ T cell subsets in decidua and PBL
A) Shows representative histograms of intracellular granzyme B expression in CD8 T cell subsets from PBL and decidua. B) shows average MFI of granzyme B and st.dev within control PBL (n=11), maternal PBL (n=11), decidua basalis (n=8) and decidua parietalis (n=7) CD8+ T cell subsets. Bars indicate average percentage and st.dev. *p<0.05, **p<0.01, ***p<0.001 C) Representative histograms of granzyme B expression in CD45+CD3-CD56+ NK cells from control PBL and decidua parietalis.

Discussion

This study demonstrates that decidual CD8+ T cells mainly consist of highly differentiated Effector-Memory (EM) and Effector T cells, whereas unprimed naïve cells are almost absent. In contrast to peripheral blood CD8+ EM and Effector cells, decidual CD8+ EM and Effector cells do not express the cytolytic molecule perforin. In addition, decidual CD8+ EM and Effector cells do express granzyme B and granzyme K, but the expression of granzyme B is reduced in comparison with the peripheral blood CD8+ T cells of the matching phenotype. These data suggest that local regulation of CD8+ T cell differentiation may play a crucial role in maintenance of maternal immune tolerance to the fetus during human pregnancy.

The functional features of decidual CD8+ T cells do not correspond with the functional features of their matching phenotype in peripheral blood. Peripheral blood Effector cells, EM-2 and EM-3 cells highly express the effector molecules perforin and granzyme B,

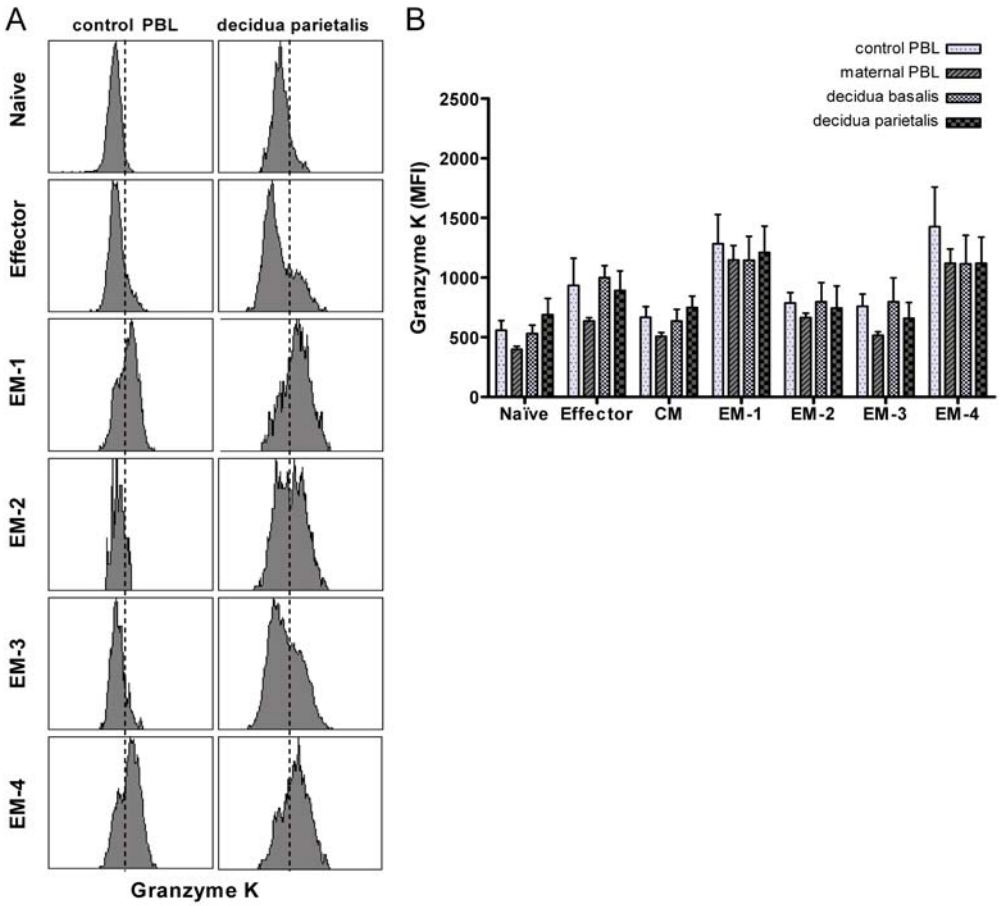

Figure 4. Granzyme K expression in CD8+ T cell subsets in decidua and PBL
A) Shows representative histograms of intracellular granzyme K expression in CD8 T cell subsets from control PBL (n=4), maternal PBL (n=5) and decidua basalis (n=4) and decidua parietalis (n=4). B) shows average MFI of granzyme K and st.dev within cPBL, mPBL d.basalis and d.parietalis CD8+ T cell subsets. Bars indicate average percentage and st.dev.

whereas in decidual CD8+ T cells perforin expression is absent. Furthermore, granzyme B expression is reduced in decidual Effector, EM-2 and EM-3 cells whereas granzyme K is normally expressed in EM-1, EM-4 and Effector cells. Uptake of granzyme B by target cells is essential for DNA fragmentation and apoptosis of the target cells. Although granzyme B can be taken up by target cells trough endocytosis independently of perforin, apoptosis of target cells by internalized granzyme B may not be induced until perforin is added (18-20). Besides perforin human CTLs may express a second membrane disrupting protein known as granulysin. Granulysin causes membrane-lipid degradation but whether high enough concentrations can be reached in the immunological synapse to facilitate granzyme entry or target cell death is unclear (21). Although our flowcytometric analysis may be confirmed with experimental analysis of the isolated CD8+ T cell subsets, by not expressing perforin decidual CD8+ Effector and EM T cells may not be able to elicit a full cytotoxic response. Besides perforin and granzyme mediated cytotoxicity CTLs can also induce target cell death by engaging with cell surface death receptors, such as FAS that interacts with FASL on the CTL but FASL is not expressed on any of the CD8+ T cell subsets in decidua. From this we conclude that decidual CD8+ T cells are highly differentiated cells that do not express conventional cytolytic mediators to induce target cell death.

Fetal trophoblasts at the fetal-maternal interface do not express HLA-A, -B, -DR, -DQ and DP molecules. Therefore HLA-C and possibly indirectly presented minor histocompatiblity antigens (mHags) are the main targets to which a decidual CD8+ T cells response may be directed. However no correlation was found between the percentage of CD8+CD28- T cells and the presence or absence of a fetal-maternal HLA-C mismatch. Previously mHag specific CTLs have been shown to be induced in maternal peripheral blood during pregnancy (5). Our preliminary data do show the presence of HY specific CD8+ T cells in term pregnancy decidual tissue (data not shown). It is difficult to establish which factors attract CD8+ T cells to the decidual tissue and cause CD8+ T cell differentiation at the fetal-maternal interface. Whether the differentiated decidual CD8+ T cells originate from an influx from the periphery or are the result of a local expansion and maturation is also unknown. However the presence of highly differentiated CD8+ T cells does imply that fetal alloantigens are present at the fetal-maternal interface and attract a CD8+ T cell response.

Many factors can influence the CD8+ T cell differentiation process. Studies have shown that at optimal antigen strength CD8+ T cells differentiate to full effector cells. If the signal strength is too high or too weak CD8+ T cells die by antigen induced cell death (AICD), death by neglect or differentiate but do not obtain effector functions (11). In addition, cytokines are highly important in the induction of T cell activation and may determine the outcome of a T cell differentiation process. At the fetal maternal interface both these mechanisms may explain the lack of effector function in the differentiated CD8+ T cells. Other mechanisms like the expression of HLA-G (22) and IDO (23) and the presence of a high proportion of CD4+CD25bright regulatory T cells (3,17), alternatively activated macrophages (24,25) and CD56bright NK cells (26) in decidual tissue can also influence the CD8+ T cell differentiation process at the fetal-maternal interface. Further functional studies with isolated CD8+ T cell subsets from decidua and peripheral blood may clarify alternative mechanisms of CD8+ cytotoxicity or CD8+ T cell regulation. In addition, defects in decidual CD8+ T cell regulation or differentiation may play a role developing conditions of placental pathology where placental and fetal growth are impaired.

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Expression of NK receptors on decidual T cells in human pregnancy

Submitted for Publication

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Succes is going from failure to failure without loss of enthousiasm
Winston Churchill

Abstract

Specific receptors enable NK cells to discriminate between self cells with normal expression of MHC class I and cells that have a low or no expression of MHC class I molecules. Besides on NK cells, these receptors can be expressed on T cell subsets, mainly on CD8+ T cells but also on γδTCR+ T cells and CD4+ T cells. Although, the function of NK receptor expression on T cells is not completely understood, various studies have shown that they are involved down regulation of T cell receptor (TCR) mediated activation and affect effector functions, like cytotoxicity and cytokine production. The aim of this study was to analyze expression of NK receptors on the peripheral and decidual T cells during human pregnancy using flowcytometry. We demonstrate that a proportion of decidual T cells express HLA-C specific killer immunoglobulin like receptors (KIRs). Furthermore, a small proportion of decidual T cells express the HLA-E specific CD94-NKG2A inhibitory and CD94-NKG2C activating receptors. Decidual KIR+ and CD94-NKG2+ T cells mainly display a CD3+CD4-CD8- phenotype. However, decidual tissue also contains higher percentages of KIR and CD94-NKG2 expressing CD4+ and CD8+ T cells compared to peripheral blood. So far, the functional capacities of decidual T cells expressing the NK receptors are unknown but NK receptor expression on decidual T cells may provide an alternative way for T cells to distinguish self cells from allogeneic fetal cells and to modulate the decidual immune response.

Introduction

During pregnancy the maternal immune system has to tolerate the persistence of allogeneic fetal cells in maternal tissue. Fetal trophoblasts circumvent a destructive maternal immune response by not expressing the classical HLA-A, HLA-B, HLA-DR, HLA-DQ and HLA-DP molecules that are the main targets for allogeneic T cells. Trophoblasts do express HLA-C and the non-classical HLA-E, HLA-F and HLA-G molecules (1). Hereby NK cell mediated cytotoxicity is avoided but HLA-C is a highly polymorphic histocompatibility antigen that can also elicit a cytotoxic T cell response (2,3). Recent data by our group show that a fetal-maternal HLA-C mismatch correlates with an increased decidual T cell activation (T.Tilburgs et.al submitted). Besides T cell and TCR mediated allorecognition, NK cells and T cells can express NK receptors that can specifically recognize subtypes of MHC class I molecules.

In humans the NK receptors include killer immunoglobulin-like receptors (KIRs) that can recognize specific types of HLA-C and HLA-G molecules and the CD94-NKG2 heterodimers of the C type lectin family that recognize HLA-E. These receptors were originally described in NK cells and enable them to discriminate between self cells with normal expression of MHC class I and cells that have lost or under express MHC class I molecules (4-6). Subsequently these receptors were also found to be expressed on T cell subsets, primarily on CD8+ T cells but also on γδTCR+ T cells and CD4+ T cells (7,8). Although, the function of NK receptors on T cells is not completely understood, various studies have shown that engagement of NK receptors on T cells can result in down regulation of TCR mediated activation and affect their effector function, like cytotoxicity and cytokine production (9-12). During pregnancy NK cell activation through KIR has been shown to be important in placental development (13). In addition, incompatibility of maternal KIR genotype and fetal HLA-C ligands lead to a higher incidence of pre-eclampsia (14) and unexplained spontaneous abortions (15). Expression of NK receptors on decidual T cells has not been shown so far, however it may provide an alternative way for recognition of fetal cells and modulation of the decidual lymphocyte response.

The aim of this study is to asses the expression of NK receptors on the T cell pool during human pregnancy using flowcytometry. We examine the expression of the HLA-C specific KIRs CD158a/h that recognizes HLA-C molecules containing a SER77ASN80 (HLA-C2) and CD158b/j that recognizes HLA-C containing an ASN77LYS80 (HLA-C1) in the sequence. Furthermore we examine the expression of the HLA-E specific CD94-NKG2A inhibitory and CD94-NKG2C activating receptors. All analyses were performed both on T cells derived from maternal peripheral blood and T cells derived from the two fetal-maternal interfaces at human term pregnancy; the decidua basalis (d.basalis) and the decidua parietalis (d.parietalis).

Materials and Methods

Blood and tissue samples

Paired samples of d.basalis, d.parietalis and heparinised maternal peripheral blood (mPBL) were obtained from healthy women after uncomplicated term pregnancy (gestational age range: 37 – 42 weeks). Tissue samples were obtained after delivery by elective caesarean section or uncomplicated spontaneous vaginal delivery. Control PBL (cPBL) samples were obtained from healthy non-pregnant volunteer donors. Signed informed consent was obtained from all women, and the study received approval by the LUMC Ethics Committee (P02-200).

Lymphocyte isolation

Lymphocyte isolation from decidua was done as described previously (16). In brief: d.basalis was macroscopically dissected from the maternal side of the placenta. D.parietalis was collected by removing the amnion and delicately scraping the d.parietalis from the chorion. The obtained tissue was washed thoroughly with PBS and thereafter finely minced between two scalpel blades in PBS. Decidual fragments were incubated with 0.2% collagenase I (Gibco-BRL, Grand Island, NY) and 0.02% DNAse I (Gibco) in RPMI-1640 medium, gently shaking in a waterbath at 37ºC for 60 min and thereafter washed once with RPMI. The resultant suspensions were filtered through a 70µm sieve (BD, Labware; NJ) and washed once in RPMI. The decidual isolates were layered on a Percoll gradient of (7.5ml 1.080g/ml; 12.5ml 1.053g/ml; 20ml 1.034g/ml) for density gradient centrifugation (30min/800g), lymphocytes were isolated from the 1.080g/ml – 1.053g/ml interface. PBL samples were directly layered on a Ficoll Hypaque gradient (LUMC pharmacy; Leiden, The Netherlands) for density gradient centrifugation (20min/800g). Mononuclear cells were collected, washed twice with PBS containing 1% FCS and all cells were fixed with 1% paraformaldehyde and stored at 4˚C until cell staining and flow cytometric analysis.

Flow cytometry

A nine-color FACS panel was analyzed on a LSR-II Flowcytometer (Becton Dickinson) using FACS DIVA software. The characteristics of the receptors analyzed are depicted in Table 1 and the LSR-II configuration and MoAbs used are listed in Table 2. All MoAbs were titrated to determine optimal dilutions. All T cells are analyzed within the lymphocyte gate and are selected within the CD45+CD3+CD14- gate. Data acquisition of all decidua and PBL samples was done using the same LSR-II settings and data analysis was done using the same FACS DIVA analysis template.

Table 1. NK receptors and their ligands

Receptor	Alias	Ligand	Motif
CD158a/h	KIR2DL1; KIR2DS1	HLA-C2	Inhibitory/Activating
CD158b/j	KIR2DL2/3; KIR2DS2	HLA-C1	Inhibitory/Activating
CD94-NKG2A		HLA-E	Inhibitory
CD94-NKG2C		HLA-E	Activating

Table 1. NK receptors and their ligands

HLA typing

All mothers and children were HLA typed at low resolution for HLA-C using the Sequence Specific Oligonucleotides (SSO) PCR technique. HLA typing was performed at the national reference laboratory for histocompatibility testing (Leiden University Medical Center, The Netherlands). The HLA-C1 and HLA-C2 group of both mother and child was established on basis of the presence of SER77ASN80 (C1) and ASN77LYS80 (C2) in the HLA-C sequence (17). In addition, the number of C1 and C2 alleles present in the mother but not in the child ('missing self') were determined.

Statistical analysis

To determine differences between more than 2 groups, a non-parametric Kruskal-Wallis one way ANOVA was performed. If $p<0.05$ a Dunn's multiple comparison post test was performed to compare all pairs of columns. The Mann-Whitney U test was used to determine differences between non-paired groups. P-values <0.05 are considered to denote significant differences.

Table 2. LSR-II configuration and used monoclonal antibodies

Laser	Filter	MoAbs and Conjugates
488nm	530/30	CD158b/j FITC[1]; CD94 FITC[2] TCR-aß FITC[3];
	575/26	CD158a/h PE[1]; NKG2A PE[2]; TCR-?d PE[3]
	610/20	CD4 PE-TxRED[5]
	695/40	CD8 Percp[3]
	780/60	CD14 PE-Cy7[1]
405nm	440/40	CD3 Pacific Blue[3]
	560/20	CD45 Cascade Yellow[6]
633nm	660/20	CD158b/j APC[4]; NKG2C APC[2]; CD28 APC[1];
	730/45	CD56 Alexa700[3]

[1]BD Pharmingen; [2]R&D Systems; [3]BD; [4]Beckman Coulter; [5]Caltag; [6]DAKO

Table 2. LSR-II configuration and used monoclonal antibodies

Results

Decidual tissue has a distinct T cell composition in comparison to peripheral blood T cells

The T cell composition of d.basalis and d.parietalis was compared with that from control PBL (cPBL) and maternal PBL (mPBL). The proportions of CD4+, CD8+ and CD4-CD8- T cells are similar in cPBL and mPBL samples (Figure 1a). In contrast, d.basalis and d.parietalis tissue isolates contain significantly lower percentages of CD4+ T cells and a significantly higher percentage of CD8+ and CD4-CD8- T cells (Figure 1a). In addition, d.parietalis CD3+ cells contain a significantly increased proportion of γδTCR+ T cells in comparison to d.basalis and the cPBL and mPBL samples (Figure 1b). The γδTCR+ T cells are mainly found within the CD4-CD8- T cells phenotype (Figure 1b). However in d.parietalis also a small but not significantly increased percentage of CD8+ γδTCR+ T cells are found in comparison with d.basalis and the cPBL and mPBL samples.

KIR expression on T cell subsets in human pregnancy

KIR expression on cPBL, mPBL, d.basalis and d.parietalis T cells was analyzed using antibodies against the HLA-C2 specific KIR, CD158a/h and the HLA-C1 specific KIR, CD158b/j. The percentage of CD158a/h+ cells and CD158b/j+ cells was determined within the CD4-CD8-, CD8+ and CD4+ T cell subsets (Figure 2). As a positive control, analysis of CD158a/h and CD158b/j expression on CD3-CD56+ NK cells was included. In all samples the frequency of CD158a/h+ and CD158b/j+ cells is higher in CD4-CD8-

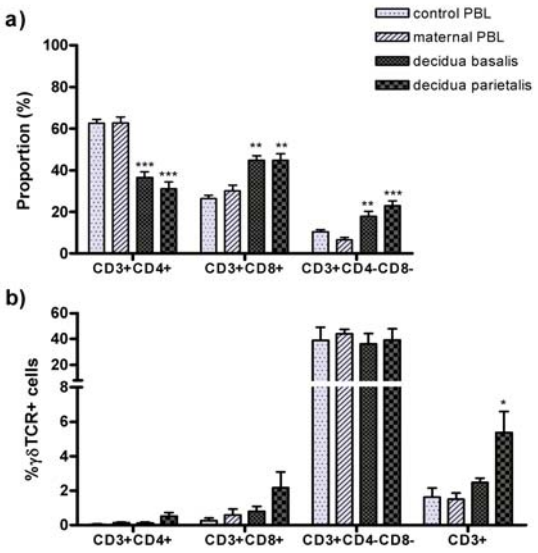

Figure 1. T cell composition in control PBL, maternal PBL, decidua basalis and decidua parietalis

a) the proportion of CD4+, CD8+ and CD4-CD8- populations within the CD3+ T cell subset and in cPBL (n=21), mPBL (n=15), d.basalis (n=13) and d.parietalis (n=13). b) the proportion of γδ-TCR+ cells within CD3+CD4+, CD3+CD8+ and CD3+CD4-CD8- and total CD3+ cell populations and in cPBL (n=4), mPBL (n=4), d.basalis (n=4) and d.parietalis (n=4). All cell fractions are gated for CD45+CD3+CD14- lymphocytes (bars indicate mean percentages and standard deviation; *p<0.05; ** p<0.01; ***p<0.001).

T cells compared to CD8+ T cells. The lowest frequency of CD158a/h+ and CD158b/j+ is observed within the CD4+ T cells. Both d.basalis and d.parietalis CD4+ T cells contain a significantly higher proportion of CD158a/h+ cells and CD158b/j+ cells compared to CD4+ T cells in cPBL and mPBL. In addition, CD8+ T cells in d.parietalis contain a significantly higher proportion of CD158a/h+ cells compared to mPBL and cPBL CD8+ T cells. Phenotypic analysis shows that CD4+KIR+ and CD8+KIR+ T cells mainly express the αβTCR, whereas CD3+CD4-CD8- KIR+ T cells contain both αβTCR+ and γδTCR+ T cells (Figure 3). In addition, the CD4+KIR+ and CD8+KIR+ T cells are mainly found within the CD28- T cell population (Figure 3).

Fetal HLA-C2 type influences CD158a/h expression on T cells in decidua basalis

To determine whether HLA-C differences between mother and child influence the expression of KIR on maternal T cells, both mother and child were DNA typed for HLA-C. The HLA-C1 and HLA-C2 group of both mother and child was established on basis of the presence of SER77ASN80 (C1) and ASN77LYS80 (C2) in the HLA-C sequence (17). All but one child expressed HLA-C1. Therefore only the presence or absence of a HLA-C2+ child was analyzed for a possible correlation with the expression of the HLA-C2 specific KIR (CD158a/h) on T cells. In d.basalis a significantly lower percentage of CD8+CD158a/h+ T cells and a slightly lower percentage of CD4+CD158a/h+ T cells is observed in pregnancies containing a HLA-C2+ child (Figure 4). The lower percentage

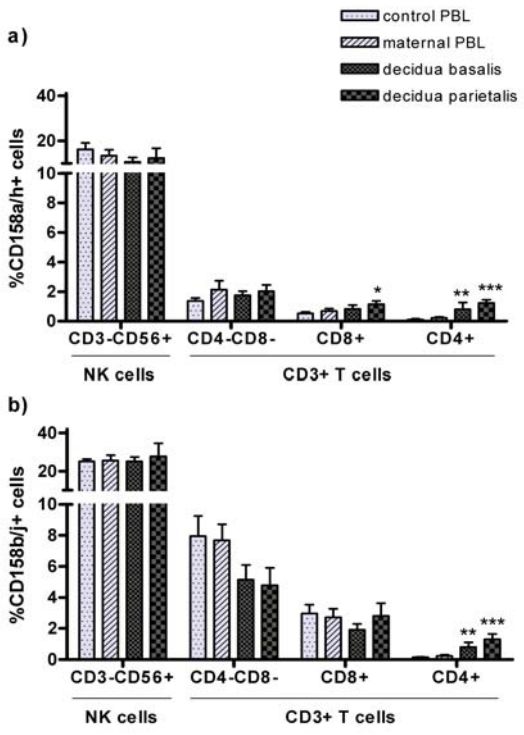

Figure 2. KIR expression on decidual and peripheral blood T cells
Proportion of CD158a/h+ (a) and CD158b/j+ (b) within CD4+, CD8+ and CD4-CD8- T cells and NK cells in cPBL (n=30), mPBL (n=21), d.basalis (n=16) and d.parietalis (n=16) (bars indicate mean percentages and standard deviation; *p<0.05; ** p<0.01; ***p<0.001)

of CD8+CD158a/h+ and CD4+CD158a/h+ T cells in the presence of a HLA-C2+ child is irrespective of the presence or absence of maternal HLA-C2. In mPBL and d.parietalis no significant differences in CD158a/h expression on T cell subsets were observed between pregnancies with or without a HLA-C2+ child (data not shown). In addition, no correlation were observed between the number of HLA-C1 and HLA-C2 epitopes present in the mother but not in the child ('missing self') and the proportion of KIR+ T cells.

CD94, NKG2A and NKG2C expression on T cell subsets in human pregnancy

In all PBL and decidual samples the proportion of CD94+ and CD94+NKG2A+ cells is higher in CD3+CD4-CD8- T cells compared to CD8+ and CD4+ T cells (Figure 5a,b). The proportion of CD94+NKG2C+ cells is not different in CD4-CD8-, CD8+ and CD4+ T cells (Figure 5c). D.parietalis CD4+ T cells contain a significantly higher proportion of CD94+, CD94+NKG2A+ and CD94+NKG2C+ cells compared to peripheral CD4+ T cells. No significant differences between cPBL, mPBL and d.basalis are observed with respect to CD94+, CD94+NKG2A+ and CD94+NKG2C+ expression on CD8+ and CD4-CD8- T cells.

Discussion

Decidual tissue contains T cells that are phenotypically and functionally different from peripheral blood T cells. Furthermore, decidual T cells are heterogenic and contain CD4+ and CD8+ T cells with an activated as well as a regulatory phenotype and function (18,19). Increased proportions of atypical T cell populations like CD4-CD8-αβTCR+ T cells, CD3+γδTCR+ T cells and NKT cells are found in decidual tissue in comparison to peripheral blood. For each of these T cell subsets specific immuno-modulatory functions have been described (20-22). In this study we demonstrate that KIR+ T cells are present in d.basalis and d.parietalis tissue. Decidual KIR+ T cells mainly display a CD4-CD8- phenotype, however CD8+KIR+ T cells and CD4+KIR+ T cells are also present in higher proportions than in peripheral blood samples. In addition, CD4+ T cells in d.parietalis contain a higher proportion of CD94+, CD94+NKG2A+ and CD94+NKG2C+ cells compared to peripheral CD4+ T cells. This suggests that decidual T cells, besides their T cell receptor (TCR), may use additional receptors to recognize fetal cells which can affect the subsequent immune response.

The mechanisms by which KIR expression on T cells is induced are largely unknown. KIR expression on T cells can not be induced by cytokines or TCR activation in vitro (23). The majority of CD8+KIR+ cells have been shown to express inhibitory rather than activating KIRs. In this study we did not examine the presence or absence of the intracytoplasmic ITIM or ITAM to distinguish inhibitory from activating KIRs. Various studies have shown that engagement of NK receptors on T cells can result in inhibition of cytotoxicity and cytokine production (9-12). Recently, HLA-E restricted CD8+αβTCR+KIR+ T cell clones have been shown to display cytotoxicity against target cells that are negative for classical MHC class I molecules (6). Hereby CD8+KIR+ T cells contain NK cell like functions and immune-regulatory mechanisms. Previous studies have shown that CD4+KIR+ T cells recognize viral recall antigens in a MHC class II dependent manner. In addition, CD4+KIR+ T cells are enriched for the effector memory phenotype and produce IFNγ. It is postulated that engagement of inhibitory KIRs on T cells may enhance survival of memory T cells, possibly via increased resistance to

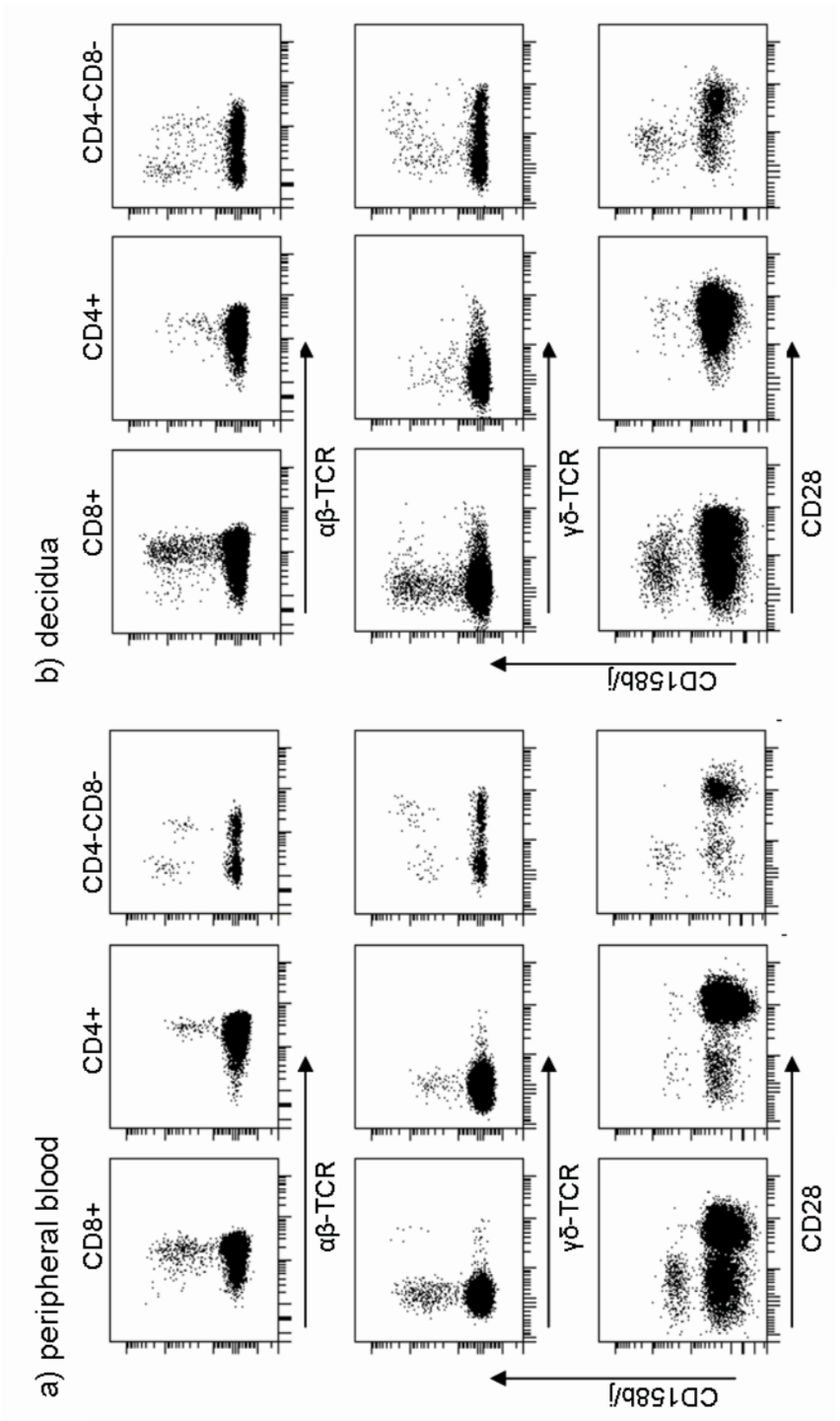

Figure 3. Phenotype of KIR+ T cells in peripheral blood and decidua
Representative dotplots of CD158b/j expression combined with αβTCR, γδTCR and CD28 expression in CD8+, CD4+ and CD4-CD8- T cells in peripheral blood (a) and decidua (b)

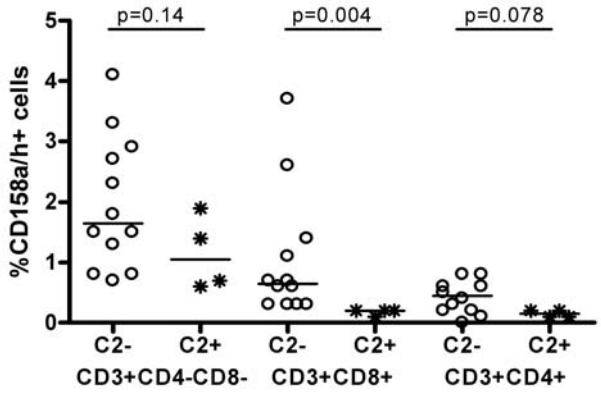

Figure 4. Fetal HLA-C type influences CD158a/h expression on d.basalis T cells
Proportion of CD158a/h+ cells within CD4-CD8-, CD8+ and CD4+ T cells in d.basalis (n=16) from pregnancies with an HLA-C2 negative (C2-) and HLA-C2 positive (C2+) child (lines indicate median percentages).

Activation Induced Cell Death (AICD) (8). Although no experimental data is present to demonstrate the functional capacities of decidual KIR+ T cells, expression of these receptors may serve as an additional tool to distinguish self cells from allogeneic fetal cells.

In contrast to KIR expression on T cells, CD94-NKG2A expression on CD8+ T cells is easily induced by cytokines and T cell activation (23). Alike KIR expression, ligation of CD94-NKG2A can lead to specific inhibition of TCR mediated cytotoxicity and cytokine production in specific T cells clones as well as inhibiting allogeneic cytotoxicity (24). CD94-NKG2 receptors are not recognizing HLA-E specific polymorphisms but may contain specificity for the peptides presented in HLA-E. Peptides presented in HLA-E comprise viral, bacterial as well as cellular stress proteins such as heat shock protein 60 (HSP60) (25-27). Although not supported by experimental data, the expression of CD94-NKG2 receptors may down regulate the decidual T cell response in case of oxidative stress or other types of cellular stress in placental tissue.

A decrease of the HLA-C2 specific KIR on CD4+ and CD8+ T cells was observed in d.basalis in pregnancies containing a HLA-C2+ child irrespective of the presence or absence of maternal HLA-C2. Although this observation needs to be confirmed, this suggests that the fetal HLA-C type can influence KIR expression on maternal T cells present at the fetal maternal interface.

In conclusion, this study shows that decidual T cells comprise a very heterogenic subset of cells, with higher proportions of atypical T cells compared to peripheral blood. We demonstrate that decidual CD4+ and CD8+ T cells contain increased proportions of KIR+ cells and CD94-NKG2+ cells. The percentage of CD4-CD8- T cells is increased in decidual tissue and CD4-CD8- T cells contain comparable percentages of KIR+ cells and CD94-NKG2+ cells to peripheral blood T cells. Decidual KIR+ T cells and CD94-NKG2+ T cells may contribute to the regulation of the decidual immune response, where they may distinguish allogeneic fetal cells from self, but at the same time prevent a detrimental immune response.

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Do’s and don’ts in isolation and purification of decidual lymphocytes and macrophages

Submitted for Publication

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Een notitieboek is als een woordcamera

Abstract

Human decidual tissue contains a significant number of leukocytes, phenotypically and functionally different from peripheral blood leukocytes. In vitro studies using purified human decidual leukocytes subsets will improve our understanding of the role of these cells in acceptance of the allogeneic fetus and their possible role in development of pregnancy pathology. However, decidual leukocyte isolation and purification procedures are complex and may alter leukocyte phenotype and functional activity. In this study we describe an optimized decidual leukocyte isolation procedure using enzymatic digestion, followed by percoll gradient centrifugation. Subsequently we purified leukocyte subsets using immuno-magnetic beads and autoMACS separation or immuno-fluorescence labelling and FACS sort. The procedure is suitable for isolation and purification of leukocytes from decidua basalis and decidua parietalis tissue. With this protocol highly purified, viable and functionally active decidual lymphocytes and macrophages can be obtained. The isolation and purification procedure has minimal effect on lymphocyte phenotype and functional activity. In contrast, isolation and purification may alter macrophage cell surface markers and cytokine secretion profiles. The potential pitfalls of the decidual leukocyte isolation and purification procedure are discussed including the proper controls to be included in future studies using decidual leukocyte isolates.

Introduction

During pregnancy the maternal immune system has to tolerate the persistence of fetal cells in maternal tissue. Many mechanisms including various fetal and maternal cell types have shown to contribute to the prevention of a destructive immune response at the fetal-maternal interface. However, the immune acceptance of the allogeneic fetus remains an immunologic paradox (1,2). Decidual tissue is populated by many types of maternal leukocytes like NK cells, macrophages, dendritic cells and T cells that contain many phenotypic and functional differences from circulating peripheral blood leukocytes (3-5). In vitro studies using purified leukocyte subsets from the fetal-maternal interfaces will improve our understanding of the role of these cells in acceptance of the allogeneic fetus and development of pregnancy pathology.

In human pregnancy three fetal-maternal interfaces where fetal and maternal tissues connect can be identified; 1 maternal peripheral blood that contacts the fetal syncytiotrophoblast layer during utero-placental circulation, 2 decidua basalis the maternal part of the placenta at the implantation site that connects with the invading extra villous trophoblasts and 3 decidua parietalis the maternal part of the membranes contacts the non-invading trophoblast of the chorion. Each interface contains specialized fetal trophoblast cells with distinct HLA expression profiles and unique immune modulatory and immune stimulatory capacities (6-9). In addition, many differences in presence of leukocyte subsets exist between maternal peripheral blood, decidua basalis and decidua parietalis (4,10,11). Decidual leukocyte isolation and purification procedures may alter leukocyte phenotype and functional activity 12,13. Therefore isolation protocols require constant improvement and appropriate controls must be included in all studies. We describe an optimized technique to obtain purified, viable and functionally active leucocytes from decidua basalis and decidua parietalis that is suitable for phenotypic and functional analysis. In addition, we discuss potential pitfalls during isolation and purification of decidual leukocytes and suggest controls that can be included in future studies using decidual leukocyte isolates.

Materials & Methods

Blood and tissue samples

Samples of decidua basalis, decidua parietalis and heparinised maternal peripheral blood were obtained from healthy women after uncomplicated term pregnancy (gestational age ≥37 weeks). Tissue samples were obtained after delivery by elective caesarean section or spontaneous vaginal delivery. Non pregnant control peripheral blood samples were obtained from healthy volunteer blood donors. Signed informed consent was obtained from all women, and the study received medical ethical approval by the LUMC Ethics Committee (P02-200).

Lymphocyte isolation

Lymphocyte isolation from decidua was done as described previously (4,5). Decidua basalis was macroscopically dissected from the maternal side of the placenta. After dissection, the remains of villous tissue were cut from decidua basalis with scissors. Decidua parietalis was collected by removing the amnion and delicately scraping the decidua parietalis from the chorion. The obtained tissue was washed thoroughly with firm shaking (without centrifugation) in PBS. Thereafter the tissue was finely minced between two scalpel blades. To minimize contamination with blood and villi decidual tissue fragments were washed several times in PBS (without centrifugation) until the supernatant was completely transparent. Decidual fragments were incubated with 0.2% collagenase I (Gibco-BRL, Grand Island, NY) and 0.02% DNAse I (Gibco) in RPMI-1640 medium, gently shaking in a waterbath at 37ºC for 60 min and thereafter washed once with RPMI. The resultant suspensions were filtered through a 70µm sieve (Becton Dickinson Labware; New Jersey, USA) under negative pressure and thereafter washed once in RPMI-1640. The decidual isolates were resuspended in 20 ml of Percoll (1.023 g/ml) (Amersham Biosciences, Amersham, UK) and layered on a Percoll gradient of (10ml 1.080g/ml and 12.5ml 1.053g/ml) for density gradient centrifugation for 30 min at 2000 rpm (Figure 1b). Lymphocytes were isolated from the 1.080g/ml – 1.053g/ml interface whereas macrophages were isolated from the 1.053g/ml – 1.034g/ml interface. Maternal peripheral blood and umbilical cord blood (UCB) samples were directly layered on a Ficoll Hypaque gradient (LUMC pharmacy; Leiden, The Netherlands) for density gradient centrifugation for 20 min at 2000 rpm. Mononuclear cells were collected, washed twice with PBS containing 1% FCS. All cells were fixed with 1% paraformaldehyde and stored at 4˚C until cell staining and flow cytometric analysis.

Flowcytometry

The following directly conjugated mouse-anti-human MoAbs were used for four-color immunofluorescence staining: CD14-FITC, CD27-FITC, CD56-FITC, CD69-FITC, HLA-DR-FITC, CD8-PE, CD14-PE, CD19-PE, CD25-PE, CD80-PE, CD86-PE, CD3-PerCP, CD4-APC (Becton Dickinson, Franklin Lakes, NJ, USA), CD163-PE, CD28-APC, CD45-APC (BD Pharmingen, San Diego, CA, USA) and CD66b-FITC (Serotech, UK). MoAbs are used in concentrations according to manufactures instructions. Flowcytometry was performed on a FACS Calibur using Cellquest-pro Software (Becton Dickinson). Analysis of the lymphocytes were done within the lymphocyte gate, set around the viable lymphocytes as previously described 4. The percentage of lymphocytes were analyzed as CD45+ cells within the lymphocyte gate and depicted as percentage of total events. The percentages of CD3+, CD56+ and CD19+ cells were calculated within the CD45+ cell fraction. The percentage of macrophages (CD45+CD14+) and

granulocytes (CD45+CD66b+) were calculated within total events and without using the lymphocyte gate. Expression of HLA-DR, CD80, CD86 and CD163 was analyzed within the CD45+CD14+ gate.

Lymphocyte purification and functional analysis

Lymphocyte isolates were purified using immuno-magnetic labelling and autoMACS (Miltenyi Biotec) separation or immuno-fluorescence labelling and FACS sorting. For immuno-magnetic labelling decidual lymphocytes isolates were resuspended in autoMACS buffer containing 0.2% EDTA and 0.5% FCS. Thereafter lymphocytes were stained with directly labelled CD14 microbeads (Miltenyi Biotec), CD66b (Serotech), HLA-G (Serotech) and HAI-1 (kindly provide by Professor Hiroaki Kataoka, University of Miyazaki, Japan) MoAbs for 30 min. The cells were washed once and Goat-anti-Mouse labelled microbeads (Miltenyi Biotec) were added for 30 min. Thereafter the cells were washed again and filtered through a 40µm sieve (BD Labware) and separated using the negative selection protocol in sensitive mode (depleteS) on autoMACS. Cells were washed once with PBS 1% FCS (Greiner, Bio-one BV Alphen ad Rijn, The Netherlands) and all fractions were analyzed with flowcytometry for cell purity and counted with Türk and Eosine for cell viability. For Immuno-fluorescence labelling the lymphocyte fraction was stained with CD45-APC (BD Pharmingen) for 30 min in RPMI-1640 1%FCS (Greiner, Bio-one BV). Thereafter the cells were washed in RPMI-1640 1%FCS and filtered through a 40µm sieve (BD Labware). All lymphocyte fractions were FACS sorted on a Flow sorter ARIA (Becton Dickinson) for viable CD45+ lymphocytes. The cells were sorted within the lymphocyte gate set around the viable lymphocytes avoiding granulocytes, macrophages and other contaminating cell types. After cell sorting the

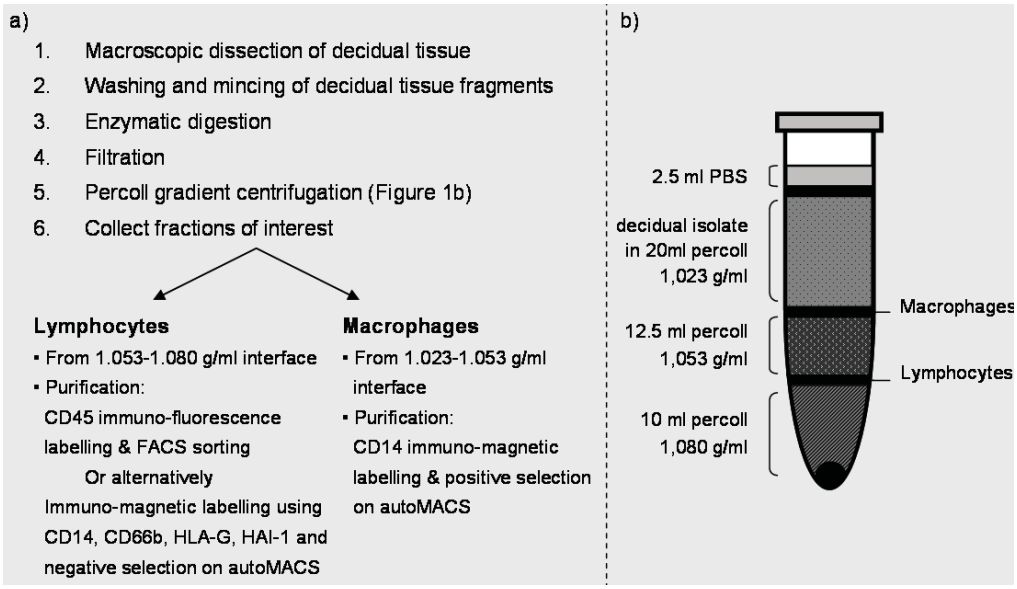

Figure 1. decidual leukocyte isolation and purification procedure
a) Shows a schematic diagram of the decidual leukocyte isolation and purification procedure and b) the properties of the Percoll gradient for separation of decidual lymphocyte and decidual macrophage fractions.

lymphocytes were incubated in RPMI-1640 supplemented with L-glutamine 2 mM, penicillin 50 units/ml en streptomycin 50 µg/ml (all obtained from Gibco Laboratories) and 10% human serum in a round-bottomed 96 well plate (Costar Cambridge, MA, USA) at a density of 50.000 cells per well in triplicate. All fractions were stimulated with pre-coated anti-CD3 using UCHT-1 (BD Pharmingen) at 5 µg/ml for 2 hours or OKT-3 (Orthoclone) at 10 µg/ml for 4 hours. Cultures were incubated at 37ºC with 5% CO2. At day 4 supernatants were collected and stored at -20 ºC until the time of analysis. Supernatants were analyzed with a Th1-Th2 Bio-plex pre mixed human cytokine panel (containing IL-2, IL-4, IL-5, IL-10, IL-12(p70), IL-13, GM-CSF, IFN-γ and TNF-α) (Biorad Laboratories; Veenendaal; The Netherlands) according to manufactures description. Proliferation was measured by [3H]thymidine (1µCi) incorporation for the last 16 hours and measured by liquid scintillation spectroscopy using a betaplate (Perkin Elmer-Wallac, Turku, Finland). Results are expressed as the median counts per minute (cpm) for each triplicate culture.

Macrophage purification and functional analysis

Macrophage fractions were purified using immuno-magnetic labelling and autoMACS (Miltenyi Biotec) separation. Herefore macrophage isolates were resuspended in MACS buffer containing 0.2% EDTA and 0.5% FCS (Greiner, Bio-one BV) and stained with directly labelled CD14 microbeads (Miltenyi Biotec) for 30 min. The cells were washed once, filtered through a 40µm sieve (BD Labware) and separated using a double positive selection protocol (Posseld2) on autoMACS. After selection macrophages were incubated in RPMI-1640 supplemented with L-glutamine 2 mM, penicillin 50 units/ml en streptomycin 50 µg/ml (all obtained from Gibco Laboratories) and 10% FCS in a 24 well plate (Costar Cambridge, MA, USA) at a density of 0.5 x106 cells per well. Macrophages were stimulated with LPS (100 ng/ml) alone, LPS (100 ng/ml) + IFN-γ (500 units) or cultured with medium alone. After 16 hours supernatants were collected and stored at -20 ºC until the time of analysis. Supernatants were analyzed with Bio-plex cytokine panel for IL-10, IL-12(p70), IL-12(p40), IL-1β and TNF-α (BioRad Laboratories; Veenendaal; The Netherlands) according to manufactures description.

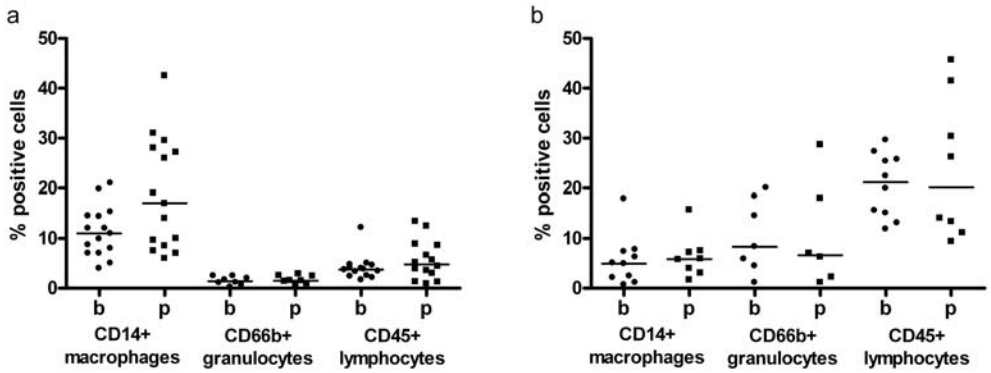

Figure 2. Leukocyte composition of decidual macrophage and decidual lymphocyte enriched fractions

a) The percentage of CD14+ macrophages, CD66b+ granulocytes and CD45+ lymphocytes in the (a) macrophage enriched fraction from the 1.023-1.053 interface and (b) the lymphocyte enriched fraction from the 1.053-1.080 interface in decidua basalis (b) and decidua parietalis (p) samples. Percentage is depicted as percentage in total events and lines indicate median percentages.

Results

Isolation of decidual macrophages and lymphocytes

The isolation procedure as depicted in figure 1a contains the following important steps: Macroscopic dissection of decidual tissue and extensive washing and mincing of decidual tissue fragments is followed by enzymatic digestion, filtration and percoll gradient centrifugation. Following percoll gradient centrifugation, the gradient contains 3 interfaces that contain different cell types (Figure 1b). The macrophage and the lymphocyte enriched fractions are harvested from the 1.023-1.053 g/ml interface and 1.053-1.080 g/ml interface respectively. FACS analysis of the macrophage enriched fraction shows 11.3% ± 5.0% and 18.9% ± 11.2% CD14+ macrophages in decidua basalis and decidua parietalis respectively whereas the percentage of granulocytes and lymphocytes are significantly less (Figure 2a). FACS analysis of the lymphocyte enriched fraction shows 20.6% ± 6.4% and 24.0% ± 14.2% CD45+ lymphocytes in decidua basalis and decidua parietalis respectively whereas the percentage of macrophages and granulocytes are significantly less (Figure 2b).

Percoll gradient optimisation

For optimisation of the percoll gradient we analysed the use of several combinations of percoll layers with different densities. Increase of the bottom layer density (1.080 g/ml) to 1.085 g/ml results in an increased percentage of contaminating granulocytes in the lymphocyte fraction whereas a decrease to 1.075 g/ml leads to an increase loss of lymphocytes to the cell pellet. Increase in the middle percoll layer density (1.053 g/ml) to 1.058 g/ml results in an increased number of macrophages in the macrophage fraction but also to a high loss of lymphocytes to the macrophage fraction. Reduction of the middle percoll layer density (1.053 g/ml) to 1.047 g/ml results in loss of macrophages from the macrophage fraction to the lymphocyte fraction. A small increase in number of lymphocytes in the lymphocyte fraction is observed although the percentage of lymphocytes in the lymphocyte fraction is reduced in comparison with the 1.053 g/ml layer, which may complicate FACS analysis and further purification steps (data not

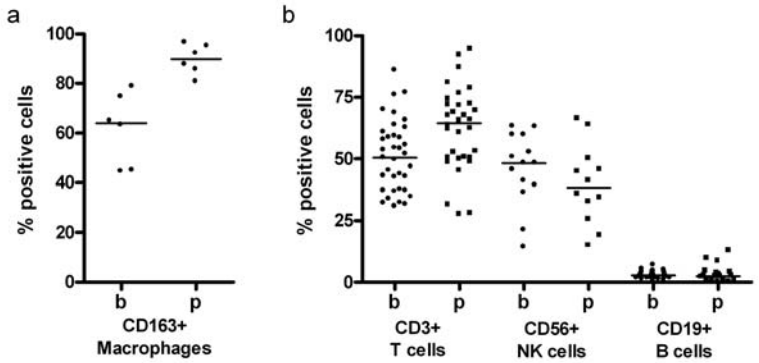

Figure 3. Different macrophage and lymphocyte subsets in decidua basalis and decidua parietalis

a) The percentage of CD163+ cells within the CD45+CD14+ macrophage fraction in decidua basalis (b) and decidua parietalis (p) samples. b) Shows the percentage of CD3+ T cells, CD56+ NK cells and CD19+ B cells within the CD45+ lymphocyte fraction in d.basalis (b) and d.parietalis (p) samples. (Lines indicate median percentages)

shown). Furthermore, the lymphocyte and T cell composition from 1.023-1.053 g/ml interface and 1.053-1.080 g/ml interface were compared and showed no differences indicating that no specific selection of particular lymphocyte and T cell subsets to the 1.023-1.053 g/ml interface and 1.053-1.080 g/ml interface occurs during percoll gradient centrifugation (data not shown).

Different macrophage and lymphocyte subsets in decidua basalis and decidua parietalis

Although a high variation in leukocyte composition is found in different isolates, no significant differences are present in the percentages of macrophages, granulocytes and lymphocyte between decidua basalis and decidua parietalis isolates (Figure 2). However, analysis of the macrophage and lymphocyte subsets, show significant differences between decidua basalis and decidua parietalis. Decidua parietalis contains mainly CD163+ macrophage type 2 cells, whereas decidua basalis contains both CD163- type 1 and CD163+ type 2 macrophages (Figure 3a). In addition, decidua parietalis contains an increased percentage of CD3+ T cells compared to the percentage of CD56+ NK cells whereas decidua basalis contains equal percentages of CD3+ T cells and CD56+ NK cells (Figure 3b).

As enzymatic digestion may influence expression of cell surface markers, we analyzed the influence of the enzymatic digestion protocol on lymphocyte and monocyte markers by adding enzymes to peripheral blood samples and incubating the samples for 1 hour at 37°C similar to the decidual isolation protocol. Enzymatic digestion does not influence cell surface expression of CD45, CD19, CD3, CD25, CD69, HLA-DR, CD8, CD27 and CD28 on peripheral blood lymphocytes (Figure 4a, b). Although the expression of CD4 and CD56 was reduced after enzymatic treatment, the CD4+ and CD56+ population were clearly visible and percentages CD4+ and CD56+ cells remained unchanged (figure 4a, b). In contrast, analysis of enzyme treated monocytes and percoll gradient centrifugation modifies expression of monocyte activation markers like HLA-DR and CD80 while expression of CD14 and CD163 is not affected (Figure 4c).

Lymphocyte purification and functional analysis

In order to perform functional tests with decidual lymphocytes, further purification of the decidual lymphocyte enriched fraction was performed using FACS sort or autoMACS separation. For the FACS sort purification CD45+ fluorescence labelling was used and lymphocytes were selected for viable CD45+ cells within the lymphocyte gate. For autoMACS separation a negative selection protocol to deplete contaminating CD14+ macrophages, CD66b+ granulocytes, HLA-G+ trophoblasts and HAI-1+ trophoblasts was used. FACS analysis of the purified lymphocyte isolates shows that after FACS sort the highest lymphocyte purity is achieved in decidua basalis and decidua parietalis isolates (figure 5a). In addition cell counting using either Türk in a Burker-Türk chamber or MGG stained cell smears shows that contamination in lymphocyte isolates after FACSsort is mainly cell debris, whereas after autoMACS purification also non-lymphocyte cell types are visible (data not shown). The number of lymphocytes was determined in the unsorted, autoMACS and FACS sorted fractions. There are no significant differences in lymphocyte gain (figure 5b) or sorting efficiency between autoMACS and FACS sorting (data not shown). In addition, of all fractions the lymphocyte viability was determined using Eosine staining and shows >98% viability of lymphocytes in both purification protocols (data not shown).

To analyze the functional capacity of the purified lymphocytes FACS sorted lymphocyte fractions of decidua basalis and decidua parietalis, were stimulated with plate bound anti-CD3 using the OKT-3 and UCTH-1 clones. In addition, CD45+ lymphocyte isolates from maternal PBL and non-pregnant control PBL were included. For control of the isolation procedure, control PBL samples were incubated with enzymes, enriched for lymphocytes using a percoll gradient and thereafter FACS sorted for CD45+ lymphocytes similar to the decidual lymphocyte isolation protocol. Upon stimulation with OKT-3 and UCTH-1 all CD45+ lymphocyte fractions show high proliferative responses (Figure 6a). No significant differences in proliferative capacity are observed between peripheral blood and decidual lymphocytes.

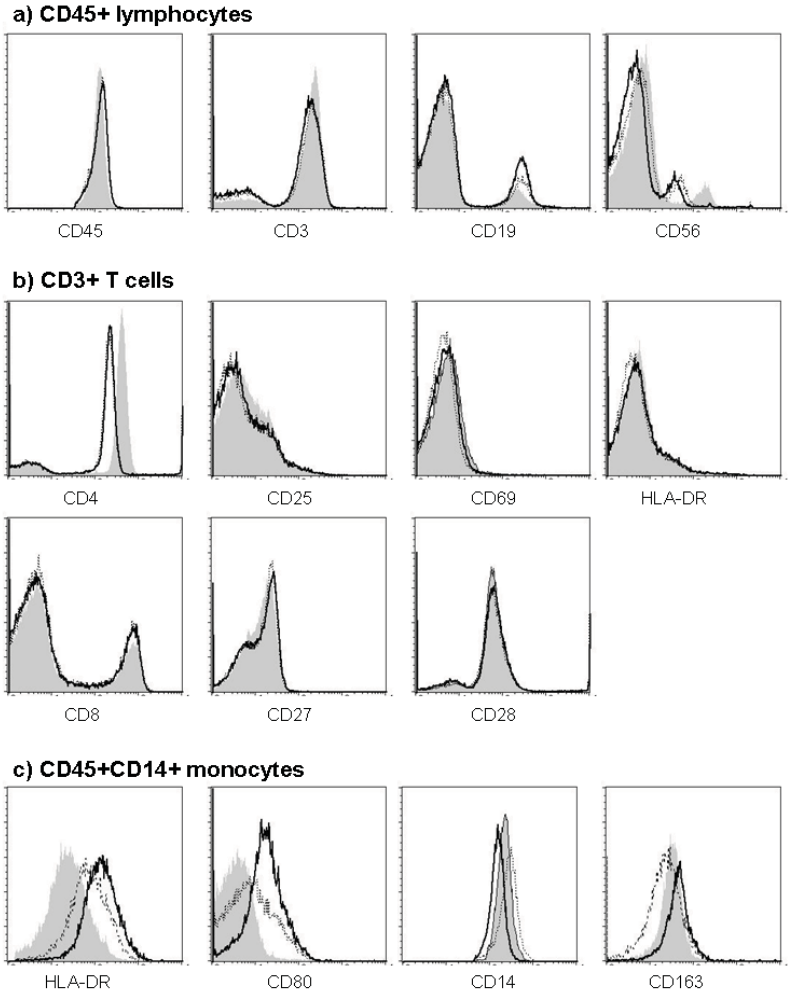

Figure 4. Influence of enzymatic digestion and percoll gradient centrifugation on leukocyte cell surface marker expression
Representative histograms showing Mean Fluorescence Intensity of a) CD45, CD3, CD19 and CD56 in CD45+ lymphocytes, b) CD4, CD25, CD69, HLA-DR, CD8, CD27 and CD28 on CD3+ T cells and c) HLA-DR, CD80, CD14 and CD163 in CD45+CD14+ monocytes from untreated PBL combined with a ficoll gradient (grey histogram), enzymatic digestion and ficoll gradient (dotted line) and enzymatic digestion with percoll gradient (black line)

Furthermore no significant differences in proliferative capacity are observed between the control PBL and the control PBL treated with the decidual lymphocyte isolation protocol (Figure 6a). Upon stimulation with OKT-3 a significant increase of IFNγ, IL-10 and CM-CSF is observed in decidual lymphocyte cultures in comparison with peripheral blood lymphocytes. However lymphocytes from control PBL samples treated with the decidual isolation protocol also show a comparable increase in IFNγ, IL-10 and CM-CSF. Upon stimulation with UCTH-1 a significant decrease of IL-2 is observed in decidual lymphocyte cultures in comparison with peripheral blood lymphocytes (Figure 6d). However lymphocytes from control PBL samples treated with the decidual isolation protocol also show a comparable decrease in IL-2. Upon UCTH-1 stimulation no significant differences in IFNγ, IL-10, IL-4, IL-5, IL-13, TNFα and GM-CSF production are observed between peripheral blood and decidual lymphocytes or between the control PBL and control PBL treated with the isolation procedure (Figure 6b-i).

Macrophage purification and functional analysis

In order to perform functional tests with decidual macrophages further purification of the decidual macrophage enriched fraction was required and performed using CD14 double positive selection on autoMACS. The macrophage purity of all sorted and unsorted fractions were assessed using flowcytometry and show a high macrophage purity in decidua basalis (83.2% ± 8.6%) and decidua parietalis (86.0% ± 24.2%) fractions after autoMACS sorting (Figure 7a). The macrophage yield in unsorted fractions could not be determined due to high contamination with other non macrophage cell types and cell debris. After autoMACS separation the number of macrophages were determined and show high macrophage yield in decidua basalis (median: 3.6x106 ± 2.0 x106; n=9) and decidua parietalis (median: 2.6x106 ± 1.7 x106; n=9) fractions (Figure 7b).

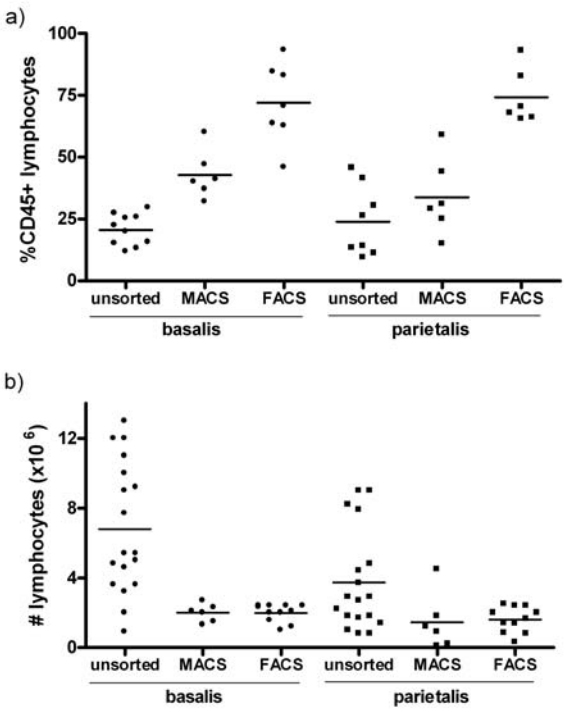

Figure 5. Decidual lymphocyte purity (a) and lymphocyte yield (b) before and after autoMACS or FACSsort purification in decidua basalis and decidua parietalis isolates. Lines indicate median percentages

To determine the functional capacity of decidual macrophages after the isolation and purification procedure, macrophages were stimulated with medium, LPS or LPS and IFN-γ and incubated for 16 hours at 37°C at 5% CO2. In addition, CD14+ monocytes isolated from control PBL and maternal PBL were included. For control of the isolation procedure, control PBL samples were incubated with enzymes and enriched for monocytes using a percoll gradient similar to the decidual isolation protocol. Supernatants were collected from all fractions and analyzed for IL-10, IL-1β, TNF-α, IL-12(p70) and IL-12(p40). LPS and LPS+IFN-γ stimulation induces IL-10 production in all peripheral blood and decidual macrophage fractions. IL-10 production in decidua basalis macrophages stimulated with medium, LPS and LPS+IFN-γ is significantly increased in comparison to peripheral blood monocytes stimulated with medium, LPS and LPS+IFN-γ. However monocytes from control PBL samples treated with the decidual isolation protocol also produce substantially increased levels of IL-10 after stimulation with medium, LPS and LPS+IFN-γ (Figure 8a). Upon LPS and LPS+IFN-γ stimulation monocytes from cPBL and mPBL produce increasing levels of IL-1β, TNF-α, IL-12(p70) and IL-12(p40). Macrophages derived from decidua basalis and decidua parietalis produce significantly lower levels of IL-1β, TNF-α, IL-12(p70) and IL-12(p40) upon LPS and LPS+IFN-γ stimulation in comparison to monocytes from cPBL and mPBL (Figure 9b-e). Monocytes from peripheral blood samples treated with the decidual isolation protocol produce similar or higher levels of IL-1β and IL-12(p70) and lower levels of TNF-α and IL-12(p40) compared to untreated PBL samples (Figure 8b-e).

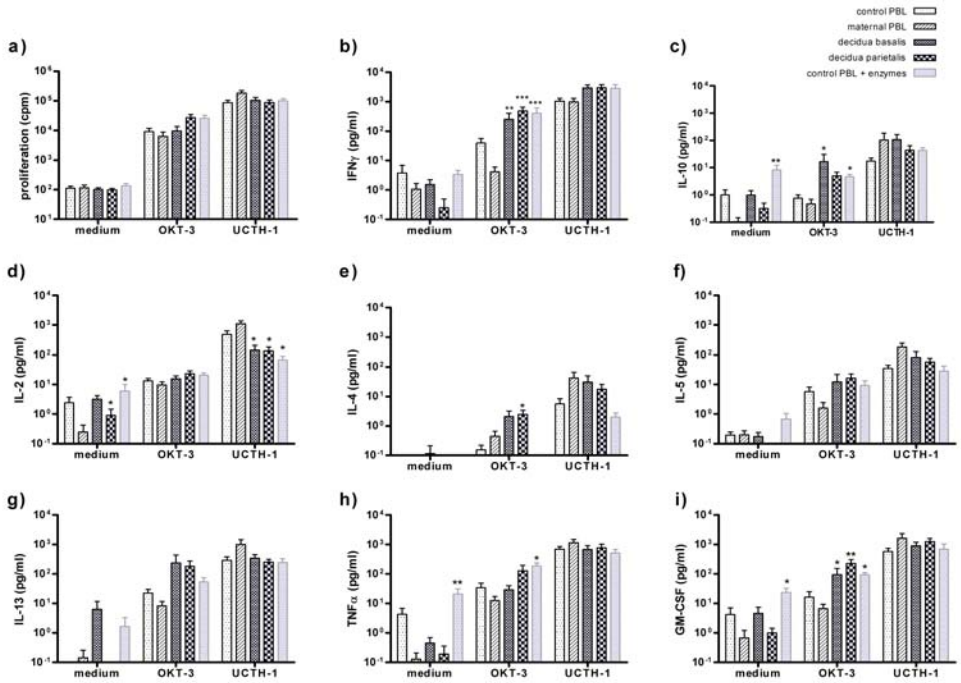

Figure 6. Proliferation and cytokine production by decidual lymphocytes
a) proliferation, b) IFNγ, c) IL-10, d) IL-2, e) IL-4, f) IL-5, g) IL-13, h) TNFα and i) GM-CSF production by purified CD45+ lymphocytes from control PBL (n=16), maternal PBL (n=11), decidua basalis (n=10), decidua parietalis (n=10) and enzyme treated control PBL (n=6) upon medium, OKT-3 and UTCH-1 stimulation. Bars indicate mean and standard deviation (* $p<0.05$; **$p<0.01$; *$p<0.001$).

Discussion

Modifications and disparity in decidual leukocyte isolation and purification procedures can induce controversial results of experiments performed by individual laboratories. In addition, decidual leukocyte isolation and purification procedures can also modify decidual leukocyte phenotype and functional capacity. Therefore decidual cell isolation procedures require constant improvement and the use of appropriate controls for proper interpretation of experiments using decidual leukocyte isolates. Previous studies have already suggested improvements at different phases of decidual leukocyte isolation procedure. The use of enzymatic digestion above mechanical disaggregation has shown to increase leukocyte viability and cell yield (14,15). However, enzymatic treatment may disrupt cell surface molecules and different combinations of enzymes may be favoured for specific cell subsets (12,14-16). In addition, the use of percoll gradient centrifugation as a replacement of ficoll gradient centrifugation has shown to increase cell viability and lymphocyte purity (13). However, optimization of the decidual leukocyte isolation protocol may be influenced by gestational age, decidual type and cells of interest. The aim of this study was to optimize the decidual leukocyte isolation and purification procedure so that highly purified, viable decidual lymphocytes and macrophages can be obtained from decidua basalis and decidua parietalis tissue which can be used in phenotypic and functional studies. The protocol is developed using term pregnancy samples, however the protocol has previously shown to be applicable for use in 1st and 2nd trimester samples (4,11).

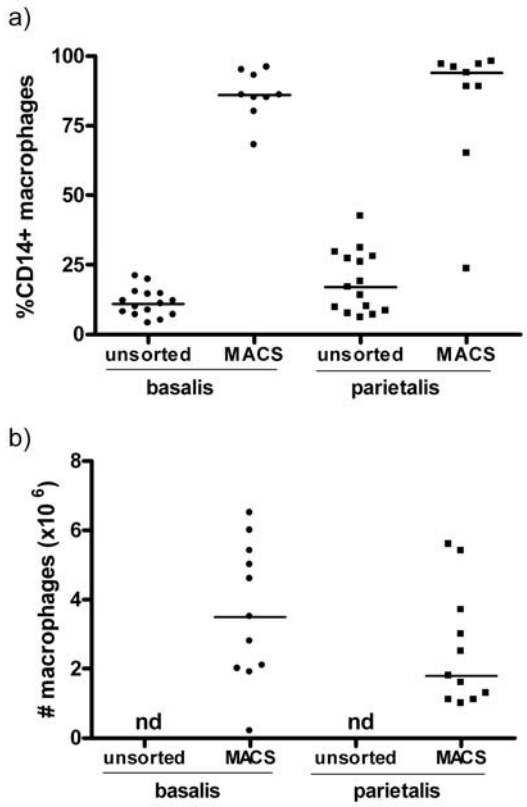

Figure 7. Decidual macrophage purity (a) and macrophage yield (b) before and after autoMACS separation in decidua basalis and decidua parietalis isolates. Lines indicate median percentages.

As previously described (15) phenotypic analysis of lymphocyte subsets shows a minimal influence of enzymatic digestion and percoll gradient centrifugation procedures on lymphocyte cell surface molecules. In addition, purified decidual lymphocytes contain comparable capacity to proliferate upon anti-CD3 stimulation to peripheral lymphocytes. In addition, treatment of peripheral blood lymphocytes with the decidual lymphocyte isolation procedure does not influence the proliferative capacity. However the increase in IFNγ, IL-10 and CM-CSF upon OKT-3 stimulation and the decrease of IL-2 upon UCTH-1 stimulation by decidual lymphocytes is also observed in the treated peripheral blood lymphocytes cultures. Therefore the changes in production by decidual lymphocytes can be a factor of decidual lymphocytes themselves or may be related to the isolation procedure. Analysis of monocyte cell surface molecules upon enzymatic digestion and percoll gradient centrifugation induces modification of the monocyte cell surface molecules HLA-DR, CD80 and CD86 whereas CD163 expression remains unchanged. In addition, enzymatic digestion and percoll gradient centrifugation alters cytokine secretion profiles of peripheral monocytes by significantly increasing IL-10 and IL-1β secretion while TNF-α and IL-12(p40) secretion is reduced. Therefore the observed increase in IL-10 production by decidua basalis macrophages can be either intrinsic factor of these macrophages or may be related to the isolation

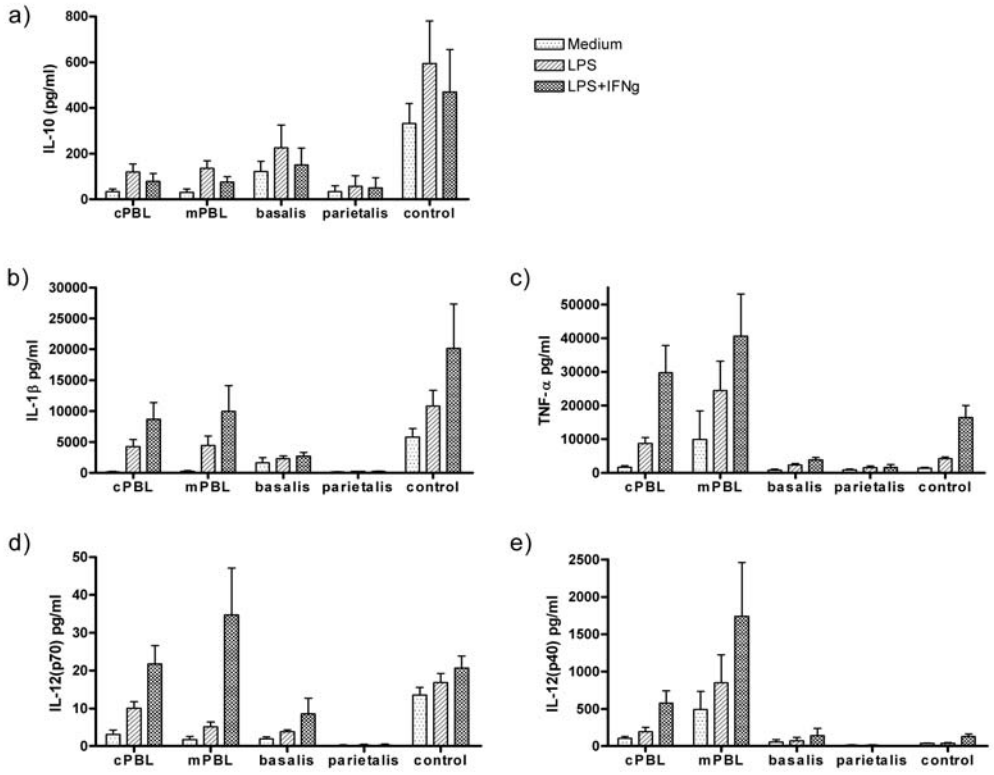

Figure 8. Cytokine production by purified decidual macrophages
a) IL-10, b) IL-1β, c) TNF-α, d) IL-12(p70) and e) IL-12(p40) production by purified CD14+ monocytes/macrophages from control PBL (cPBL) (n=14) maternal PBL (mPBL) (n=11), decidua basalis (n=10), decidua parietalis (n=9) and enzyme treated PBLs (control) (n=8) upon medium, LPS and LPS+IFN-γ stimulation. Bars indicate means and standard deviation.

procedure. Similarly the reduced secretion of IL-1β, TNF-α, IL-12(p70) and IL-12(p40) in both decidua basalis and decidua parietalis macrophages can be a feature of decidual macrophages or a side effect of the isolation procedure. Further refinements in the macrophage isolation protocol are required to perform reliable functional experiments and exclude the influence of the isolation protocol on decidual macrophages. The use of other enzyme cocktails may diminish macrophage modifications. In addition, the use of Immuno Histochemistry (IHC) can provide a good control for confirmation of phenotypic analysis of the macrophage subsets done by flowcytometry.

For purification of lymphocytes we isolated CD45+ lymphocytes by FACS sort and used a negative selection protocol on autoMACS to deplete contaminating cell types (CD14+ macrophages, CD66b+ granulocytes, HLA-G+ trophoblast and HAI-1+ trophoblasts), from lymphocyte isolates. By FACS sort the highest lymphocyte purity was obtained. Thereby the FACS sort enables the use of multicolour immune fluorescence labelling whereby multiple marker can be used for purification of particular cell subsets. However logistics to use the FACS sort facility is limited and therefore not all freshly delivered placenta's can be purified using FACS sort. In contrast, autoMACS separation contains more limited separation possibilities, but the use of autoMACS separation is easily accessible and provides the flexibility that is required to purify freshly delivered placentas at any time. Positive autoMACS selection for CD45+ cells will not only select CD45+ lymphocytes, but also CD45+ macrophages and CD45+ granulocytes whereas positive selection for CD3 may inhibit T cell function. Therefore we used a negative selection protocol to deplete CD14+ macrophages, CD66b+ granulocytes, HLA-G+ trophoblast and HAI-1+ trophoblasts from the lymphocyte fractions (17). However this selection did not result in high lymphocyte purity and non lymphocyte cell types remain present after separation. Additional MoAbs to deplete more contaminating cell types, like endothelial cells (CD31) trophoblast subsets (CD9) and fibroblasts (CD9, FSA) can be included in the depletion cocktail to further enhance lymphocyte purity (18).

Decidual leukocyte isolation protocols are crucial to investigate the dynamics and functional capacities of human decidual leukocytes subsets. Thereby, in vitro experiments using purified human decidual leukocytes subsets will improve our understanding of the role of these cells in acceptance of the allogeneic fetus and their possible role in development of pathology during pregnancy. However, we show that decidual macrophage isolation and purification procedures can alter macrophage phenotype and functional activity. In contrast the isolation and purification procedure has minimal effect on lymphocyte phenotype and proliferative capacity whereas changes in cytokine production by lymphocytes are also observed. For this reason the isolation protocols require constant improvement and appropriate controls must be included in all studies. For phenotypic analysis, flowcytometry can be used next to IHC controls. In addition, controls assessing the influence of enzymatic treatment and percoll gradient selection on the leukocyte subsets of interest should be included in all future studies assessing phenotypic and functional characteristics of decidual leukocyte subsets.

Acknowledgements

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8.

General Conclusions and Discussion

Er zijn meerdere aanvliegroutes naar je uiteindelijke doel

In order to identify the possible immunologic mechanisms contributing to aberrant pregnancy it is essential to clarify first the mechanisms of fetus specific immune recognition and immune regulation at the fetal-maternal interface during normal pregnancy. To accomplish this, one has to develop and validate methods to assess functional characteristics of isolated leukocyte subsets from the fetal-maternal interface (chapter 7). Besides the analysis of decidual leukocytes, also fetal-maternal HLA differences, clinical pregnancy data, fetal characteristics and maternal genotyping are factors that are crucial for decidual leukocyte activation and induction of regulatory cell subsets.

In this thesis, we demonstrate that CD4+CD25bright T cells which are concentrated in decidual tissue have the capacity to down regulate fetus specific and 3rd party (non-specific) responses. In contrast, CD4+CD25bright T cells in maternal peripheral blood can regulate 3rd party (non-specific) responses, comparable to non-pregnant controls, while the capacity to regulate the fetus specific response is absent. These data suggest a preferential recruitment of fetus specific regulatory T cells from the peripheral blood to the fetal maternal interface (chapter 2 and 3) (1,2). Analysis of the CD8+ T cell pool during pregnancy shows that decidual CD8+ T cells mainly consist of differentiated Effector-Memory T cells, while unprimed Naïve cells are almost absent. Decidual Effector-Memory CD8+ T cells contain significantly reduced levels of the cytolytic molecule perforin. These data are suggestive for an alternative CD8+ T cell differentiation and regulation process that may play a crucial role in maintenance of maternal immune tolerance to the fetus (chapter 5). Database analysis of clinical pregnancy data, fetal-maternal HLA mismatches and decidual lymphocyte responses led to the conclusion that a fetal-maternal HLA-C mismatch is crucial for decidual CD4+ T cell activation and required for induction of functional CD4+CD25bright regulatory T cells in decidua. Hereby we provide the first evidence that decidual T cells specifically recognize a fetal HLA-C mismatch at the fetal-maternal interface, possibly using the indirect allorecognition pathway. However HLA-C recognition does not induce a destructive immune response in uncomplicated pregnancies (chapter 4). Besides TCR mediated allorecognition, low frequencies of decidual T cells express NK receptors that can specifically recognize HLA-C allotypes. Engagement of NK receptors on T cells can result in down regulation of TCR mediated T cell activation. Although, no experimental evidence is present so far, NK receptor expression on decidual T cells may provide an alternative way for decidual T cells to recognize allogeneic fetal cells and modulate the decidual immune response (chapter 6). In conclusion, this thesis shows that decidual T cells comprise a very heterogenic subset of T cells that include activated CD4+ and Effector-Memory type CD8+ T cells. However, these highly activated T cells are found together with T cell subsets that are capable to suppress the decidual lymphocyte response. Furthermore, we show that decidual T cells can specifically recognize a fetal-maternal HLA-C mismatch. Hereby we demonstrate that mechanisms of fetus specific allorecognition and T cell regulation are present at the fetal-maternal interface in uncomplicated human pregnancy. Further unravelling of the mechanisms of fetus specific immune recognition and immune regulation may be crucial to understand why some pregnancies are successful whereas others are not.

All experiments in this thesis were performed using lymphocyte isolates from decidua basalis and decidua parietalis tissue as well as maternal PBL samples and control PBL

samples of non-pregnant donors. Hereby we studied maternal leukocyte responses from 3 important fetal-maternal interfaces and compared the maternal response to the immune response in non-pregnant controls. By analyzing decidua basalis and decidua parietalis tissue we examine fetal-maternal immune interactions locally in the uterus. Decidua basalis forms the maternal part of the placenta at the implantation site and connects with the invading fetal trophoblasts. Decidua parietalis forms the maternal part of the membranes that contacts the non-invasive trophoblasts of the chorion. Comparison of decidua basalis and decidua parietalis leukocytes shows many differences in leukocyte composition, phenotype and lymphocyte responses. Decidua basalis lymphocytes at term pregnancy contain approximately equal percentages of T cells (50%) and NK cells (48%) while decidua parietalis lymphocytes contain an increased percentage of T cells (65%) compared to NK cells (38%) (Chapter 7). In addition, the proportions of CD4+CD25bright Treg cells and highly differentiated CD8+ EM-2 and EM-3 T cells are increased in decidua parietalis in comparison to decidua basalis. These data show an increased T cells activation and T cell differentiation in decidua parietalis in comparison with decidua basalis. Analysis of lymphocyte function shows comparable proliferation and IFN-γ production by decidua basalis and decidua parietalis lymphocytes. In addition, the CD4+CD25bright Treg cells from both decidua basalis and decidua parietalis are capable of regulating fetus specific UCB and 3rd party UCB responses. This data indicates that the functional capacity of decidua basalis and decidua parietalis to elicit an immune response is comparable. Furthermore, in decidua parietalis but not in decidua basalis a fetal-maternal HLA-C mismatch is correlated with an increased CD4+ T cell activation and the induction of functional CD4+CD25bright Treg cells. This indicates that in TCR mediated recognition of allogeneic HLA-C occurs in decidua parietalis tissue. Whether or not T cells in decidua basalis can specifically recognize HLA-C remains unclear, however as HLA-C is expressed on the invading extra villous trophoblasts it is likely that HLA-C specific T cells are present in decidua basalis but they may not be visible within a more complex array of immune responses or diminished by decidua basalis specific immune regulatory mechanisms. Besides differences in T cell and NK cell responses also differences in macrophage subsets and a differential expression of molecules like TGF-β, FAS and HLA expression profiles may induce divergent immune responses in decidua basalis and decidua parietalis (3-6). In conclusion, all data suggests that decidua parietalis lymphocyte responses consists of a more T cell dominated response while decidua basalis responses may include a more complex immune reaction including both NK cell and T cell responses. Decidual NK cells play a key role in placental development and facilitate trophoblasts invasion (7) whereas decidual T cells play an important role in fetus specific immune recognition and immune regulation. Immune regulation of fetus specific T cells seems essential to prevent a detrimental response at the fetal-maternal interface and may be a requirement for successful pregnancy.

Maternal peripheral blood analysis provides information about the systemic maternal immune response and can be used as a diagnostic tool for monitoring peripheral immune responses during pregnancy. Maternal peripheral blood contacts the syncytiotrophoblast layer during utero-placental circulation and contains trophoblast micro particles that are shed from the syncytiotrophoblast surface. Syncytiotrophoblasts and the trophoblast micro particles do not express MHC class I molecules and therefore they can not directly elicit an alloimmune response by T cells. Previous studies show that maternal

peripheral blood monocytes are primed to produce more TNF-α, IL-12(p70) and IL-18 in comparison to non-pregnant individuals. In addition, trophoblast micro particles induce TNF-α, IL-12(p70) and IL-18 and suppress IFN-γ responses indicative for a systemic activation of the maternal innate immune system during uncomplicated pregnancy (8-10). In this thesis we demonstrate a significant increase of CD4+CD25dim activated T cells and a small but not significant increase in CD8+ Effector T cells in maternal peripheral blood. It is not clear whether T cell activation in maternal peripheral blood is induced by indirect Ag presentation by antologous APCs or direct recognition of fetal cells in maternal circulation. Furthermore, CD4+CD25brightFOXP3+ and CD4+CD25brightHLA-DR+ Treg cells are reduced in maternal PBL whereas maternal PBL CD4+CD25bright T cells are not able to suppress the response to umbilical cord cells of the fetus. In contrast depletion of CD4+CD25bright T cells in maternal peripheral blood decreases the fetus specific response indicating that maternal peripheral blood CD4+CD25bright T cells are activated fetus specific cells. In conclusion, our data shows that fetus specific immune recognition and T cell activation in maternal peripheral blood takes place in uncomplicated pregnancy. Peripheral T cell activation may contribute to the systemic inflammation in normal pregnancy and intensify the maternal inflammatory response during conditions of pregnancy pathology like pre-eclampsia.

In this thesis all experiments and analysis of maternal leukocytes concerned uncomplicated pregnancies. Nevertheless, major variations were observed between individual pregnancies regarding decidual leukocyte yield, leukocyte composition and lymphocyte responses suggesting that each pregnancy concerns a unique mother-child combination that may generate distinct levels of immune activation and requires a distinct combination of immune regulatory mechanisms in order to result in a successful pregnancy. These regulatory mechanisms may include expression of non-specific immune regulatory molecules like IDO, FAS, TGF-β and complement inhibitors or more immune specific mechanisms like HLA expression profiles, NK cell - trophoblast interactions, decidual macrophages, or regulatory T cells. Maternal genotype (like HLA genotype, KIR genotype and cytokine polymorphisms), maternal history (number of previous pregnancies, infection history) and the combination of fetal-maternal HLA matches and mismatches may determine which regulatory mechanisms are most predominant.

It is clear from this thesis that fetal-maternal HLA-C differences significantly influence decidual T cell activation, the decidual lymphocyte response and induction of functional regulatory T cells. Pregnancies containing a HLA-C mismatched child imply a decidual lymphocyte response to fetal cells and the need of functional CD4+CD25bright regulatory T cells in decidual tissue whereas HLA-C matched pregnancies do not. Previous studies have shown that incompatibility of maternal KIR genotype and fetal HLA-C allotype increases the risk for pre-eclampsia and spontaneous abortions (11,12). Although limited experimental data is present on how KIR incompatibilities may influence the decidual lymphocyte response, KIR+ T cells or T cell – NK cell interactions may be involved in the decidual lymphocyte response. The major challenge for future studies is to examine the mechanisms of fetus specific immune recognition and immune regulation by decidual T cells and NK cells in aberrant pregnancy. In conclusion, fetus specific immune recognition and immune activation by T cells takes place in uncomplicated pregnancy and does not lead to a detrimental immune response. The presence of

immune regulatory cells and/or the absence of additional 'danger signals' in healthy decidua may explain why maternal T cells which are able to specifically recognize fetal alloantigens do not reject the fetal allograft (13,14).

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9.

Nederlandse Samenvatting

Little by little one travels far J.R.R. Tolkien

Tolerantie in de baarmoeder

Tijdens de zwangerschap ontstaat een unieke immuun-tolerantie van de moeder voor haar ongeboren kind. Hierdoor kan de foetus die zowel moederlijke als vaderlijke genen heeft normaal groeien en wordt deze niet afgestoten zoals wel bij een orgaan transplantatie kan gebeuren. Dit proefschrift beschrijft het onderzoek naar de immunologische mechanismen van deze moederlijke tolerantie. In dit onderzoek beschrijven we de immuunreactie in het perifeer bloed van de moeder als wel de lokale immuunreactie in de baarmoeder op het grens gebied van moederlijk en foetaal weefsel. In de toekomst kan dit onderzoek bijdragen aan het begrijpen van het ontstaan van gecompliceerde zwangerschappen waar zwangerschapsvergiftiging, groeivertraging van het kind, vroeggeboorten of miskramen optreden.

Moeder-kind verschillen

Zelf en niet-zelf (vreemd) herkenning is een van de belangrijkste mechanismen voor het immuun systeem om wel of geen afweerreactie te beginnen. In een afstotingsreactie na orgaan transplantatie zijn de Humane Leukocyten Antigenen (HLA) van de donor de belangrijkste vreemde moleculen waartegen de ontvanger een afweerreactie ontwikkeld. De T cellen van de ontvanger zijn de belangrijkste immuun cellen die de vreemde HLA moleculen van de donor herkennen en daar tegen een afstotingsreactie kunnen veroorzaken. In dit proefschrift beschrijven wij een type T cellen ‘de regulatoire T cellen’ die de afstotingsreactie van moeder tegen het ‘vreemde kind’ onderdrukken. Deze regulatoire T cellen zijn in hoge concentratie aanwezig op het grensgebied van moederlijk en foetaal weefsel in de moederkoek en de vliezen rond het vruchtwater. Doordat deze regulatoire T cellen verminderd aanwezig zijn in het moederbloed ten opzichte van de gezonde niet zwangere controle groep, lijkt het erop dat deze nuttige regulatoire T cellen zich tijdens de zwangerschap verplaatsen vanuit het moederbloed naar de baarmoeder. Het moederlijke immuunsysteem zorgt er zo voor dat lokaal in de baarmoeder de afweerreactie wordt onderdrukt en de foetus niet wordt afgestoten. Dit terwijl het immuunsysteem in de rest van het lichaam intact blijft om ook tijdens de zwangerschap bescherming te bieden tegen bacteriën en virussen.

Gezonde zwangerschappen

Om meer te leren over de regulatie en veranderingen van het immuunsysteem tijdens de normale zwangerschap hebben we een database onderzoek opgezet waarmee 81 zwangerschappen in detail zijn onderzocht. Hierbij zijn de klinische gegevens van de moeder (zoals leeftijd, aantal eerdere zwangerschappen en bloeddruk), het kind (zoals geboorte gewicht en geslacht) en de zwangerschap (zoals zwangerschapsduur, type bevalling) opgeslagen en gecombineerd met de analyse van de percentages nuttige ‘regulatoire T cellen’ en potentieel gevaarlijke ‘geactiveerde T cellen’ in de moederkoek en de vliezen rond het vruchtwater. Daarnaast hebben we ook door middel van DNA analyse de HLA typen van moeder en kind bepaald en de verschillen in HLA tussen moeder en kind berekend. Uit dit database onderzoek is naar voren gekomen dat bij een HLA verschil, van het type HLA-C, een verhoogde concentratie ‘geactiveerde T cellen’ voorkomt in de vruchtwatervliezen. Uit eerdere literatuur is gebleken dat dit type HLA-C aanwezig is op foetale trophoblast cellen waarbij men aangetoond heeft dat het HLA-C tolerantie van andere typen moederlijke immuun cellen (NK cellen) induceert. Met ons onderzoek hebben wij echter aangetoond dat het HLA-C ook activatie van

potentieel schadelijke T cellen veroorzaakt. De moederlijke T cellen herkennen dus de vreemde HLA moleculen van het kind, maar toch ontstaat er geen afstotingsreactie. Dit komt mogelijk door de aanwezigheid van een hoge concentratie aan nuttige 'regulatoire T cellen' in de baarmoeder. In het database onderzoek is verder in deze populatie van gezonde zwangerschappen geen verband gevonden tussen de aanwezigheid van de verschillende T cellen en klinische gegevens zoals de leeftijd van de moeder, het aantal eerdere zwangerschappen of het geboorte gewicht van het kind.

Toekomst van het onderzoek

In dit proefschrift is onderzoek gedaan naar de immunologische veranderingen tijdens gezonde zwangerschappen. Samenvattend laat ons onderzoek zien dat moederlijke immuun cellen in de baarmoeder het 'lichaamsvreemde kind' herkennen maar dat hiertegen, door de aanwezigheid van nuttige 'regulatoire T cellen' geen schadelijke afweerreactie wordt opwekt. In toekomstig onderzoek is het belangrijk onze bevindingen te vertalen naar zwangerschappen waarbij complicaties zoals zwangerschapsverg iftiging, foetale groeivertraging, vroeggeboorten of miskramen optreden. Hierbij is het belangrijk om te bepalen of de nuttige 'regulatoire T cellen' aanwezig zijn in de baarmoeder van gecompliceerde zwangerschappen en ook of deze goed werken in het onderdrukken van de afstotingsreactie. Daarnaast is het van groot belang om verder fundamenteel onderzoek te doen naar het ontstaan van deze bijzonder nuttige regulatoire T cellen. Factoren zoals eiwitten of andere cel typen uit de placenta, kunnen bijdragen aan het vormen van regulatoire T cellen. Het identificeren van deze factoren geeft verder inzicht in de mechanismen van deze natuurlijke vorm van immuun tolerantie. Dit fundamentele onderzoek kan daarmee een belangrijke bijdrage leveren aan verder begrip van zwangerschap complicaties, maar ook een toepassing hebben in andere onderzoeksgebieden zoals de transplantatie immunologie of onderzoek naar autoimmuun ziekten zoals Diabetes, Multiple Sclerosis en de Ziekte van Crohn. In deze onderzoeken is het induceren van tolerantie tegen het vreemde transplantaat of lichaamseigen cellen het ultieme doel. Zwangerschap is een fascinerende gebeurtenis waar met de geboorte van een kind een nieuw leven begint en waarbij de geboorte van de placenta een unieke mogelijkheid schept voor wetenschappelijk onderzoek naar de natuurlijke mechanismen van moederlijke immuun tolerantie.

Meer informatie in de media over dit onderzoek kunt u vinden in:

1. Elsevier Video 28 juni 2008 'Dwarse Rozendaal / Het kind als de ultieme parasiet'. http://video.reedbusiness.nl/elsevier/index.php?id=2196&category=elsevier;&publisher =elsevier Door Simon Rozendaal

2. Leids Universitair Medisch Centrum, CICERO, nr. 8, 21 juni 2008 pag 4-5 'Welkom in de baarmoeder'. Door Raymon Heemskerk

Abbrevriations

AICD	Antigen Induced Cell Death
APC	Antigen Presenting Cell
BCR	B cell receptor
CCL-4	CC Chemokine Ligand-4
CCR7	CC Chemokine Receptor-7
CM cell	Central-Memory cell
cPBL	control Peripheral Blood Leukocyte
CPM	Counts Per Minute
CTLA-4 (CD152)	Cytotoxic T Lymphocyte Asociated Antigen-4
DC	Dendritic Cell
d.basalis	Decidua Basalis
d.parietalis	Decidua Parietalis
EM cell	Effector-Memory cell
FOXP3	Forkheadbox protein-3
HLA	Human Leukocyte Antigen
IDO	Indoleamine-2,3-dioxygenase
IFN-γ	Interferon-γ
IL	Interleukin
ITAM	Immunoreceptor Tyrosine-based Activation Motif
ITIM	Immunoreceptor Tyrosine-based Inhibition Motif
KIR	Killer Immunoglobulin-like Receptor
MHC	Major Histocompatibitlity Complex
mPBL	maternal Peripheral Blood Leukocyte
NK cell	Natural Killer cell
RSA	Recurrent Spontaneous Abortions
SI	Suppression Index
STBM	Syncytiotrophoblast microparticles
TCR	T cell receptor
TGF-β	Tumor Growth Factor-β
Tact	activated T cell
Treg	regulatory T cell
TNF-α	Tumor Necrosis Factor-α
UCB	Umbilical Cord Blood

Curriculum Vitae

Tamara Tilburgs werd geboren op 1 augustus 1976 te Den Helder. Na het behalen van het MAVO diploma aan de Antonius MAVO te Wijk bij Duurstede werd in 1992 begonnen aan de opleiding tot laboratorium medewerker aan het Versfelt-Gijssen College te Utrecht. In 1995 werd deze opleiding vervolgd met de studie Hoger Laboratorium Onderwijs aan de Hogeschool van Utrecht. De stage bij deze studie betrof een onderzoek, onder begeleiding van Dr. Cécilia Söderberg-Nauclér en Prof. Erna Möller, naar de infectie van humaan cytomegalovirus in monocyten en macrofagen op de afdeling Klinische Immunologie van het Karolinska Instituut te Stockholm, Zweden. Voor dit onderzoek werd een Leonardo da Vinci beurs van de Europese Unie verkregen. Na het behalen van het HLO diploma in 1999 werd begonnen aan een studie Medische Biologie aan de Vrije Universiteit te Amsterdam. De afstudeerstage betrof een onderzoek, onder begeleiding van Prof. Ineke ten Berge, naar de proliferatie en differentiatie van allo-reactieve lymfocyten bij niertransplantatie patiënten op de afdeling Experimentele Immunologie & Interne Geneeskunde van het Academisch Medisch Centrum, Amsterdam. Na het behalen van het doctoraal diploma in oktober 2002 werd koers gezet naar Zuid-Amerika om gedurende 1 jaar Spaans te leren, te reizen en als vrijwilliger te werken in onder meer een dierenopvang in Bolivia en een Faziënda in Brazilië waar het gidsen van toeristen en vertalen van Portugees en Spaans tot de werkzaamheden behoorden. Na terugkeer werd in januari 2004 begonnen met het promotie onderzoek op de afdeling Immunohematologie en Bloedtransfusie en de afdeling Verloskunde van het Leids Universitair Medisch Centrum, te Leiden onder begeleiding van Prof. Frans Claas en Dr. Sicco Scherjon. Dit onderzoek heeft geleidt tot dit proefschrift. In januari 2009 hoop ik te beginnen met een post doctoraal fellowship, onder begeleiding van Prof. Jack Strominger, op de afdeling Molecular and Cellular Biology, Harvard University, Cambridge, Verenigde Staten.

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