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Leukocytes at the maternal-fetal interface in human pregnancy

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**LEUKOCYTES AT THE MATERNAL-FETAL INTERFACE
IN HUMAN PREGNANCY**

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LEUKOCYTES AT THE MATERNAL-FETAL INTERFACE IN HUMAN PREGNANCY

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For David

For my family — the core

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CHAPTER 1

INTRODUCTION: IMMUNOLOGY AT THE MATERNAL-FETAL INTERFACE: AN OVERVIEW OF CURRENT KNOWLEDGE

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THE PARADOX OF PREGNANCY

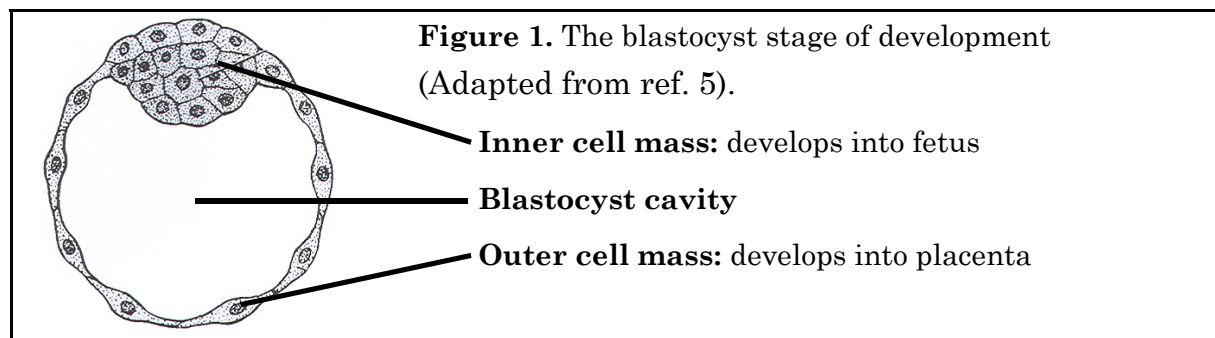
A fetus has a genetic makeup equally derived from both the father and the mother, comprised of both paternal and maternal genes. During pregnancy, the maternal immune system is functionally intact, capable of fighting infection and protecting from foreign pathogens. The fetus, however, escapes immune rejection from the maternal immune system and is tolerated for the duration of pregnancy. Historically, the fetus has been termed a successful “semi-allograft,” even a tumor or parasite. This phenomenon by which the fetus is tolerated by the maternal immune system, known as the paradox of pregnancy, was first described in 1953 by Sir Medawar (1). The mechanism by which this paradox occurs is the central question in reproductive immunology and has been the focus of extensive research for the last fifty years. The field of reproductive immunology has progressed to gain a better understanding of not only infertility and obstetrics, but also transplantation and immunology. The solution to the paradox of pregnancy has many implications in the management and treatment of complicated pregnancy, infertility, assisted reproduction and allogeneic graft survival.

IMPLANTATION

The endometrium is the mucosal lining of the uterus. Composed of glandular, stromal and immune cells and blood vessels, the endometrium essentially undergoes cyclical changes in order to support a potential pregnancy. After ovulation during the secretory phase of the menstrual cycle, this tissue begins to transform into the decidua under the influence of progesterone and estrogen in preparation for implantation. During this process, known as decidualization, stromal cells adjacent to spiral arteries enlarge accumulating glycogen and lipid and produce growth factors and matrix components to support implantation and placental growth, glandular hyperplasia occurs, and leukocytes infiltrate (2).

Implantation is the process by which an embryo attaches to the uterine wall and by which the placenta is established. The process depends on a delicate series of

events. Upon fertilization, the dividing fertilized egg or ovum travels down the fallopian tube to implant on the decidualized endometrium around day 6 or 7 post-fertilization. At the blastocyst stage of the development, the fertilized ovum consists of an inner cell mass, destined to become the embryo, and an outer cell mass formed by placental cells, known as trophoblasts, destined to become the placenta (Figure 1) (3). Trophoblasts differentiate into several lineages, such as cytotrophoblasts, syncytiotrophoblasts and extravillous trophoblasts, which are distributed in specific locations in the developing placenta and serve specialized functions (4).



Human placentation is described as hemochorial. Maternal blood bathes the outer surface of the fetal villi, within which contain fetal capillaries carrying fetal blood (6). Essentially, in the hemochorial arrangement, the maternal and fetal circulations are separated by a placental barrier, made up of a continuous layer of trophoblasts covering the villi (Figure 2). Throughout pregnancy, the two circulatory systems remain entirely separate, while the placental barrier undergoes quantitative changes.

As the blastocyst attaches and penetrates into the decidua and uterine capillaries, two layers of trophoblasts differentiate from the outer cell mass. The penetrating outer layer is comprised of multinucleated syncytiotrophoblasts that form a syncytium, while mononuclear cytotrophoblasts develop from the inner trophoblast layer. Pools of maternal blood, known as lacunae, surround and develop within the trophoblastic fetal syncytium. As the lacunae fuse, uteroplacental circulation is created. Fetal villi and the intervillous space are derived from the penetrating trophoblasts and developing lacunae (3, 7).

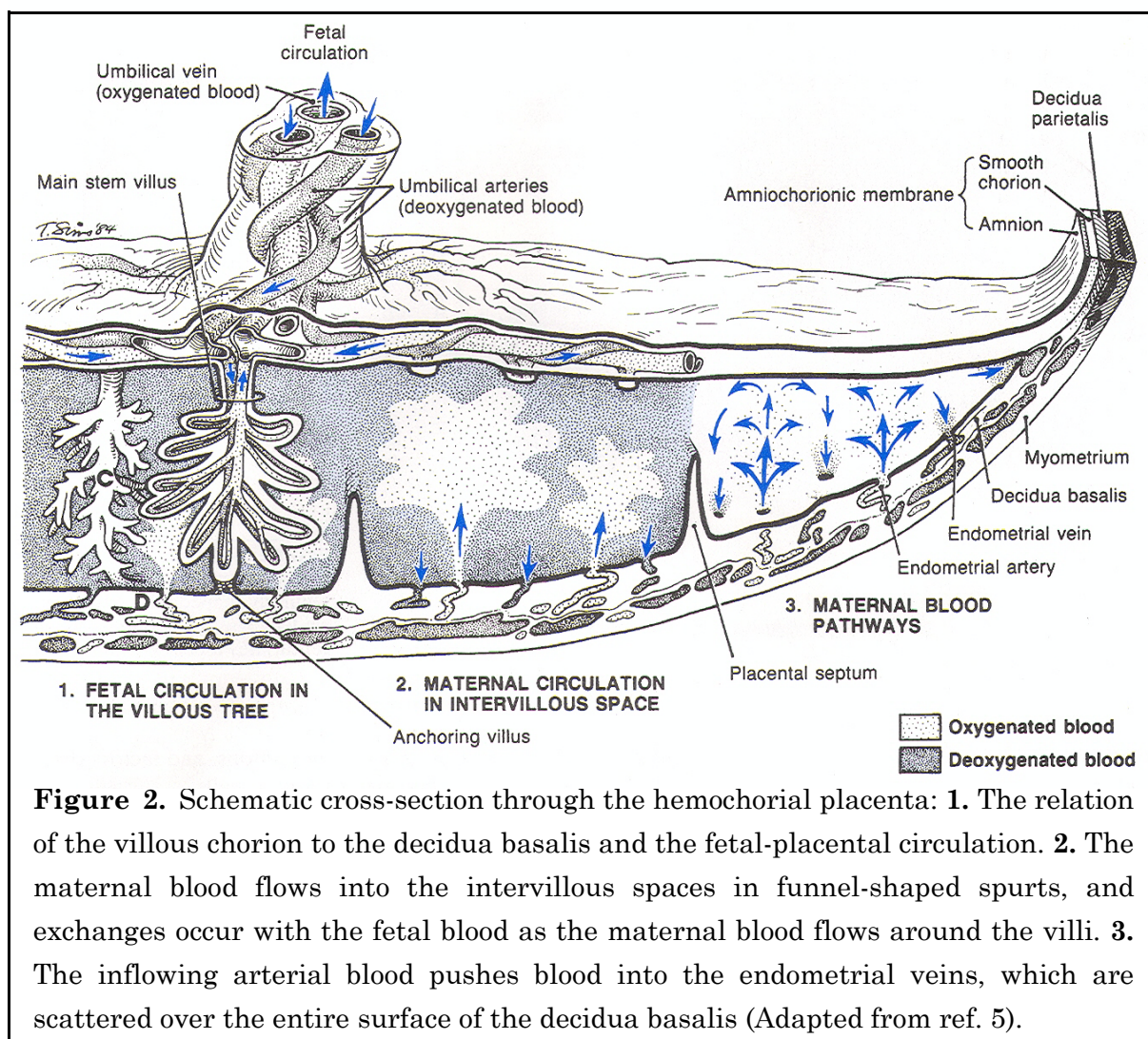
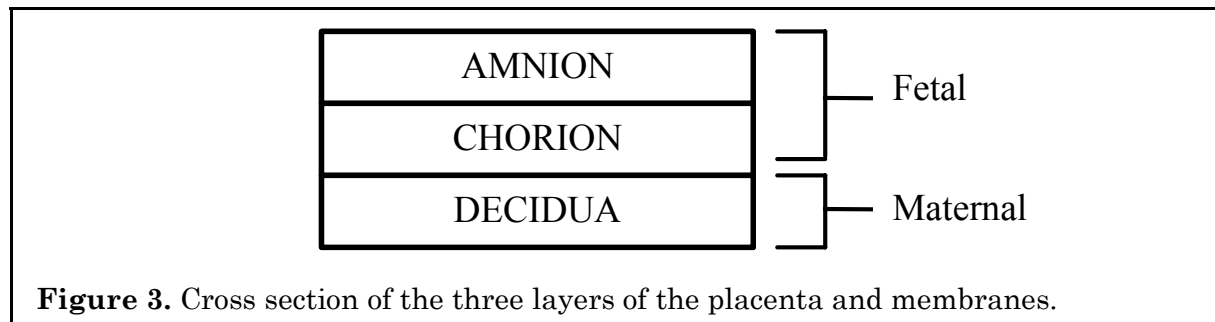


Figure 2. Schematic cross-section through the hemochorial placenta: **1.** The relation of the villous chorion to the decidua basalis and the fetal-placental circulation. **2.** The maternal blood flows into the intervillous spaces in funnel-shaped spurts, and exchanges occur with the fetal blood as the maternal blood flows around the villi. **3.** The inflowing arterial blood pushes blood into the endometrial veins, which are scattered over the entire surface of the decidua basalis (Adapted from ref. 5).

THE PLACENTA AND FETAL MEMBRANES

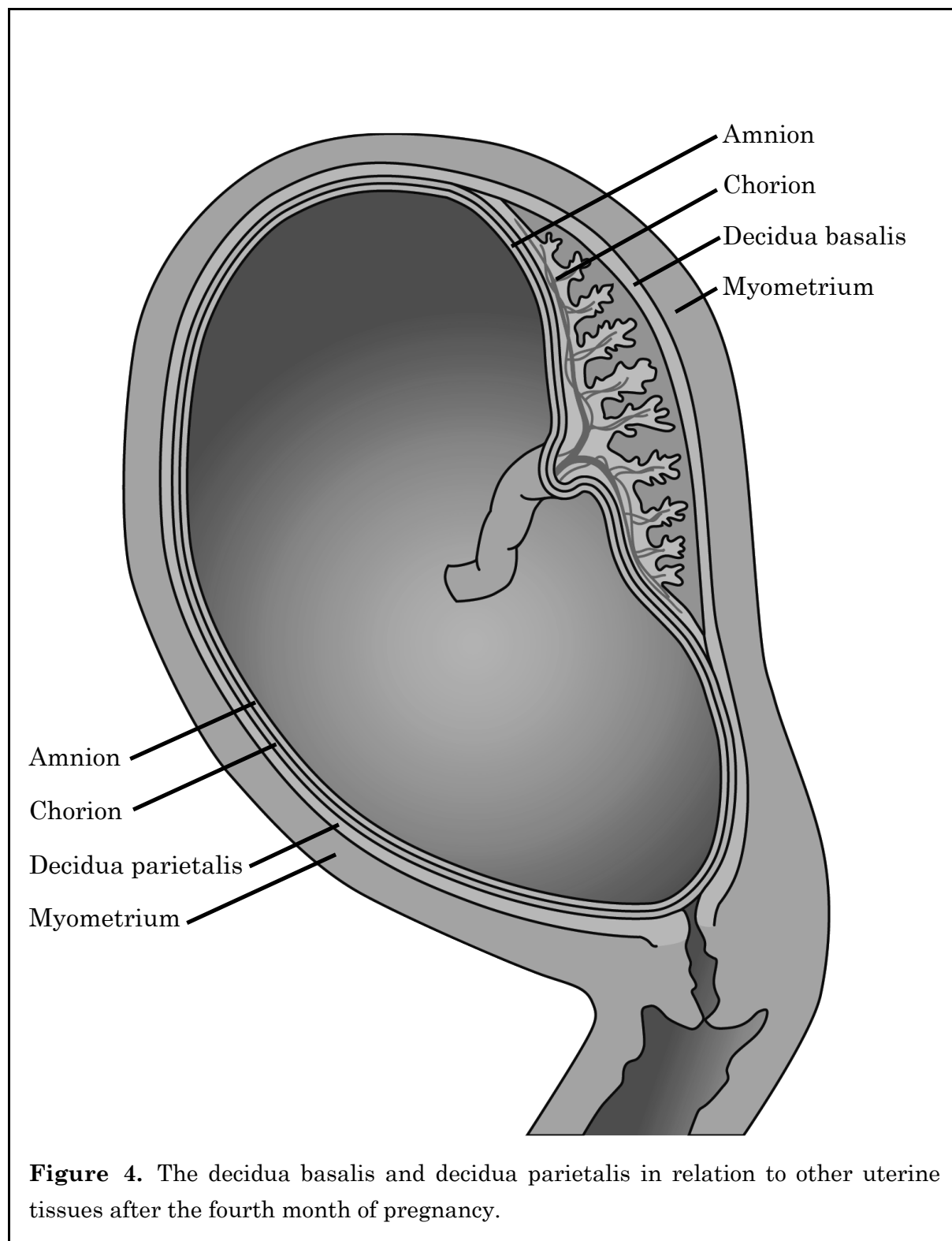
The human placenta serves many functions throughout pregnancy, including the transport of fetal nutrients and metabolic products such as oxygen and carbon dioxide as well as the release of numerous hormones and enzymes into the maternal bloodstream. Importantly, the human placenta and membranes are the site of direct contact between the maternal and fetal tissue, thus establishing the maternal-fetal interface. The placenta and membranes are composed of three basic structures or layers, seen in cross-section in Figure 3. The amnion and chorion are of fetal origin, while the decidua is of maternal origin. Within these layers, there are several cell types present (7). Notably, maternal decidua and maternal blood are in contact with extraembryonic cells and not with embryonic cells or fetal blood.



Decidua

The decidua, the maternal tissue layer of the placenta and fetal membranes, is derived from the endometrium. It was first described by William Hunter, a British gynecologist from the 19th century (8). The tissue was considered analogous to the leaves of a deciduous tree since the tissue is shed from the uterus after delivery just as the leaves on a tree are shed.

The decidua can be divided into three types defined by the anatomic location: decidua basalis, decidua parietalis and decidua capsularis. The decidua basalis is the site of implantation and the location of the developing placenta. The first site of direct contact between maternal and fetal tissue is between the decidua basalis and invading chorionic trophoblasts. The decidua capsularis covers the developing embryo, and the decidua parietalis lines the remainder of the uterine cavity (5). By the end of the first trimester as the fetus develops, the space between the decidua capsularis and decidua parietalis is obliterated and the two layers fuse, forming the second site of contact between the maternal and fetal tissue. Thus, by the second trimester, there are two distinct regions of the maternal-fetal interface where maternal decidua comes into direct contact with fetal extraembryonic tissue (Figure 4) (5). The focus of most immunologic studies has centered primarily on the decidua basalis, where immune interaction is expected to take place. Remarkably, the decidua parietalis has not been as well studied as the decidua basalis, yet it is an important site of the maternal-fetal interface.



Amnion

The amnion is a translucent membranous tissue adjacent to the amniotic fluid, which provides necessary nutrients to the amnion cells. It is composed of an epithelial cell layer, a mesenchymal cell layer and an outer intermediate layer adjacent to the chorion that can swell to facilitate sliding of the amnion across the chorion (9, 10).

Chorion

The chorion, a more opaque layer, is adjacent to the decidua. The chorion is composed of two layers, an outer reticular adjacent to the amnion that is structurally similar to the amniotic mesenchymal layer and predominately made up of fibroblasts and macrophages, and more importantly, an inner epithelial cellular layer composed of trophoblast cells. The primary cells of the chorion are trophoblast cells (9, 10).

In the fetal membranes, a remnant space of the early gestational sac separates the amnion and chorion prior to approximately 12 weeks gestation after which they adhere to one another. In the fetal membranes, the chorion or chorion laeve is thin (Figure 5), whereas in the placenta, the chorion forms a thick parenchymal layer (Figure 6). Fetal membranes contain many cell types, but are avascular and without nerve cells. Notably, the amnion and chorion laeve of the fetal membranes form the paracrine arm of maternal-fetal communication by transporting a wide variety of hormones, enzymes, and essential molecules between the maternal and fetal compartments.

GENERAL IMMUNOLOGY***Immune Response***

An immune response involves recognition and elimination of a pathogen or foreign material. It is divided into two categories, the innate or non-adaptive immune response and the acquired or adaptive immune response. Essentially, the innate response is non-specific forming the first line of defense against infection; the acquired response is highly specific for a particular pathogen improving with successive encounters via memory. Importantly, the immune response is primarily produced by leukocytes (11). There are various

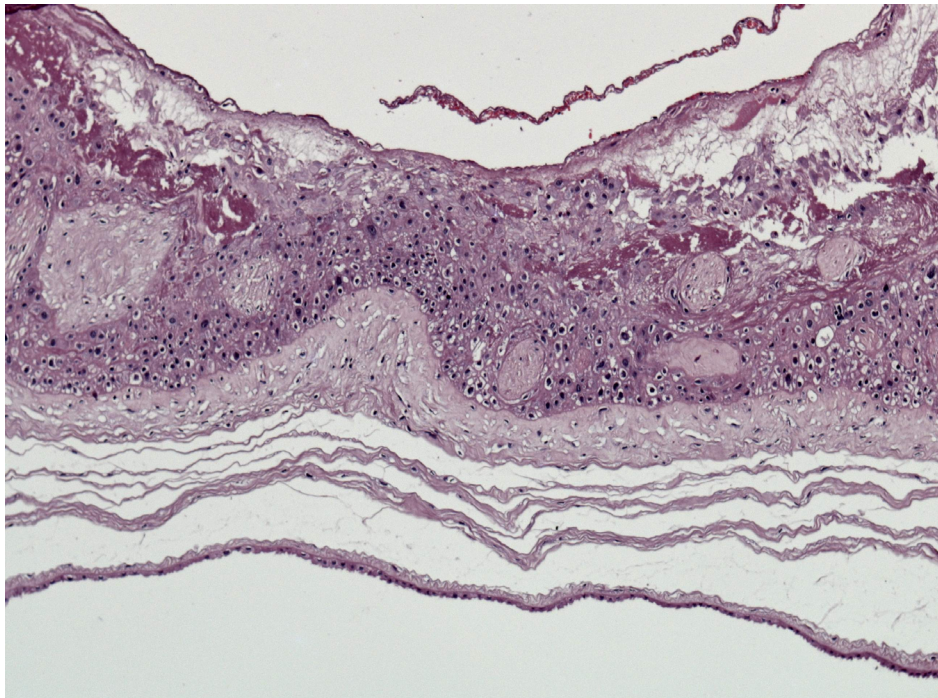


Figure 5. Cross-section of the fetal membranes (provided by Margriet de Jong).

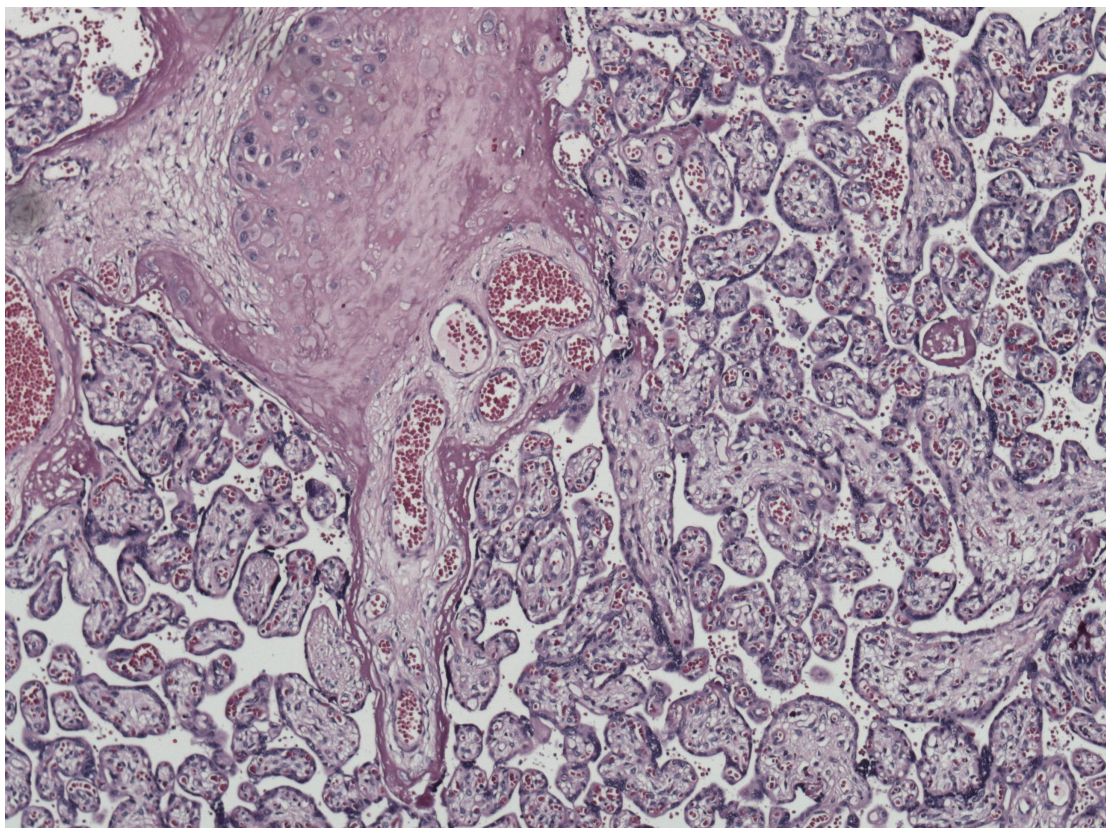


Figure 6. Cross-section of the placenta (provided by Margriet de Jong).

subpopulations of leukocytes, including natural killer cells, macrophages, dendritic cells, T lymphocytes and B lymphocytes.

Immune Recognition

The Major Histocompatibility Complex (MHC) encodes genes that are responsible for the antigens involved in immune recognition in the human immune system known as Human Leukocyte Antigens (HLA). These highly polymorphic genes, located on chromosome 6, are organized into regions including Class I and Class II HLA molecules (12). Class I molecules are expressed on nearly all nucleated cells and platelets, while Class II molecules are expressed mainly on antigen presenting cells, including dendritic cells and macrophages, and also on activated T cells. Class I molecules are encoded by three distinct loci, known as HLA-A, HLA-B and HLA-C. Class II molecules are encoded by three different loci, known as HLA-DR, HLA-DQ and HLA-DP. Class I and Class II molecules are involved in the presentation of foreign material to the cells of the immune system by different pathways. Generally, endogenous peptides are synthesized from self proteins or viral antigens and presented by MHC Class I molecules (13), while exogenous peptides are processed from extracellular proteins and bound to MHC Class II molecules (14, 15). The recognition of MHC alloantigens by T cells occurs via two distinct pathways, a direct and indirect pathway (16). In the direct pathway, T cells recognize alloantigens as intact molecules on allogeneic stimulator cells. In the indirect pathway, T cells recognize alloantigens presented by self MHC molecules. Peptides presented by Class I molecules are mainly recognized by antigen specific T cells of the CD8 phenotype (17), while peptides presented by Class II molecules are recognized by T cells of the CD4 phenotype (18). Incompatibility for these highly polymorphic genes between donor and recipient is the basis of graft rejection (19). Interestingly, HLA molecules were first recognized over fifty years ago by Dausset, Payne and van Rood who demonstrated their presence with agglutinating antibodies in the sera of multiparous women and of patients following multiple blood transfusions (20-22).

Cells of the Immune System

Cells of the immune system are derived from primitive hematopoietic stem cells. The development and differentiation of these cells is dependent on the micro-environment and on cell interactions. Each cell type expresses characteristic cell

surface molecules that allow for identification.

Natural Killer (NK) cells and phagocytes, including monocytes, macrophages and polymorphonuclear granulocytes, are the primary cells of the innate immune system. NK cells are able to produce immunoregulatory cytokines and to recognize and kill target cells with low or aberrant expression of HLA Class I molecules via killer-cell immunoglobulin-like receptors (KIR) (23, 24). KIR are receptors for specific HLA class I molecules and are expressed by both NK and T cells. The KIR locus consists of multiple genes, which form numerous haplotypes that differ in both gene content and allele combination. KIR haplotypes are classified into two groups defined by the relative content of genes encoding inhibitory and activating KIR (25). Upon binding, KIR either activate NK cell responses or abort activating signals and inhibit NK cell functions. Cells lacking Class I molecules are promptly killed by NK cells resulting from the predominant effect of activating NK receptors (24, 26). Phenotypically characterized by the expression of CD56 and the lack of expression of CD3, NK cells are divided into two distinct subsets defined as CD56^{dim}CD16⁺CD3⁻ and CD56^{bright}CD16⁻CD3⁻. Ninety percent of NK cells in normal circulating peripheral blood have the classical phenotype of CD56^{dim}CD16⁺CD3⁻, and the remaining 10% are CD56^{bright}CD16⁻CD3⁻ (27).

Macrophages are a diverse group of resident and recruited antigen presenting cells of both the innate and acquired immune system. Widely distributed throughout the body, these cells are involved in tissue homeostasis and host defense. These cells can produce a variety of molecules including growth factors, chemokines, pro-and anti-inflammatory cytokines, proteolytic enzymes and adhesion molecules (28). They also express HLA Class II and co-stimulatory molecules allowing for antigen presentation and immune activation. Macrophages can be activated via the classical or alternative pathway. In the classical pathway, cytokines such as interferon (IFN)- γ enhance inflammation and anti-microbial activity, while cytokines such as interleukin (IL)-4 and IL-13 induce alternative activation and enhance clearance, antigen presentation to B cells and repair allergic and parasitic immune reactions (29).

The adaptive immune system is comprised of antigen presenting cells and

lymphocytes. Dendritic cells (DCs) are an essential population of potent antigen presenting cells and capable of activating naïve T cells and modulating B and NK cells (30, 31). Distributed to almost all tissues, DCs are particularly prominent at mucosal surfaces. DCs are defined by the high expression of HLA-DR in the absence of expression of lineage specific markers for NK cells, macrophages, B cells and T cells and further identified based on maturation or lineage. During the maturation process, DCs upregulate the expression of different co-stimulatory molecules, such as CD40, CD58, CD80 and CD86 as well as CD83 and MHC molecules, most of which are involved in the subsequent interaction between DCs and T cells. Presentation of antigens by immature DCs to T cells induces antigen-specific T cell tolerance, whereas antigen capture and presentation by DCs after maturation results in activation of naïve, memory and cytotoxic T cells and B cells (32, 33).

T cells are an essential part of adaptive immunity. There are several different types of T cells with a variety of functions. T helper 1 (T_H1) cells interact with phagocytes and help them destroy intracellular pathogens. T_H2 cells interact with B cells and help them differentiate and produce antibody. Cytotoxic T cells directly destroy infected cells. Importantly, T cells recognize antigen, but only when presented on the cell surface of other cells by MHC molecules. T cells exert their effect by either cytokine production and release or direct cell-cell interaction. In fact, T helper cells can be further subdivided according to the cytokines that they produce. T_H1 type cytokines lead to the induction of cells such as cytotoxic T cells and macrophages and include IFN- γ , tumor necrosis factor (TNF)- α , IL-1, IL-2, IL-12 and IL-15 while T_H2 type cytokines mediate allergic and antibody responses and include IL-4, IL-5, IL-6, IL-10 and granulocyte-macrophage colony stimulating factor (GM-CSF) (34-36). Regulatory T cells represent another important population of T cells. These naturally occurring cells are able to control immune responsiveness to self- and allo-antigens and are essential for the active suppression of autoimmunity and for transplantation tolerance (37, 38). Recent studies have demonstrated that CD4⁺CD25⁺ regulatory T cells express CD25 at higher levels and more persistently than activated T cells (39, 40). These CD4⁺CD25^{bright} regulatory T cells are capable of inhibiting proliferation and cytokine secretion induced by T-cell receptor cross-linking of CD4⁺CD25⁻ responder T cells in a contact-dependent manner (40). Essentially,

regulatory T cells inhibit inappropriate activation of cell- and antibody-mediated immune responses as well as innate immune responses (37, 41).

IMMUNOLOGY OF THE MATERNAL-FETAL INTERFACE

FETAL CELLS

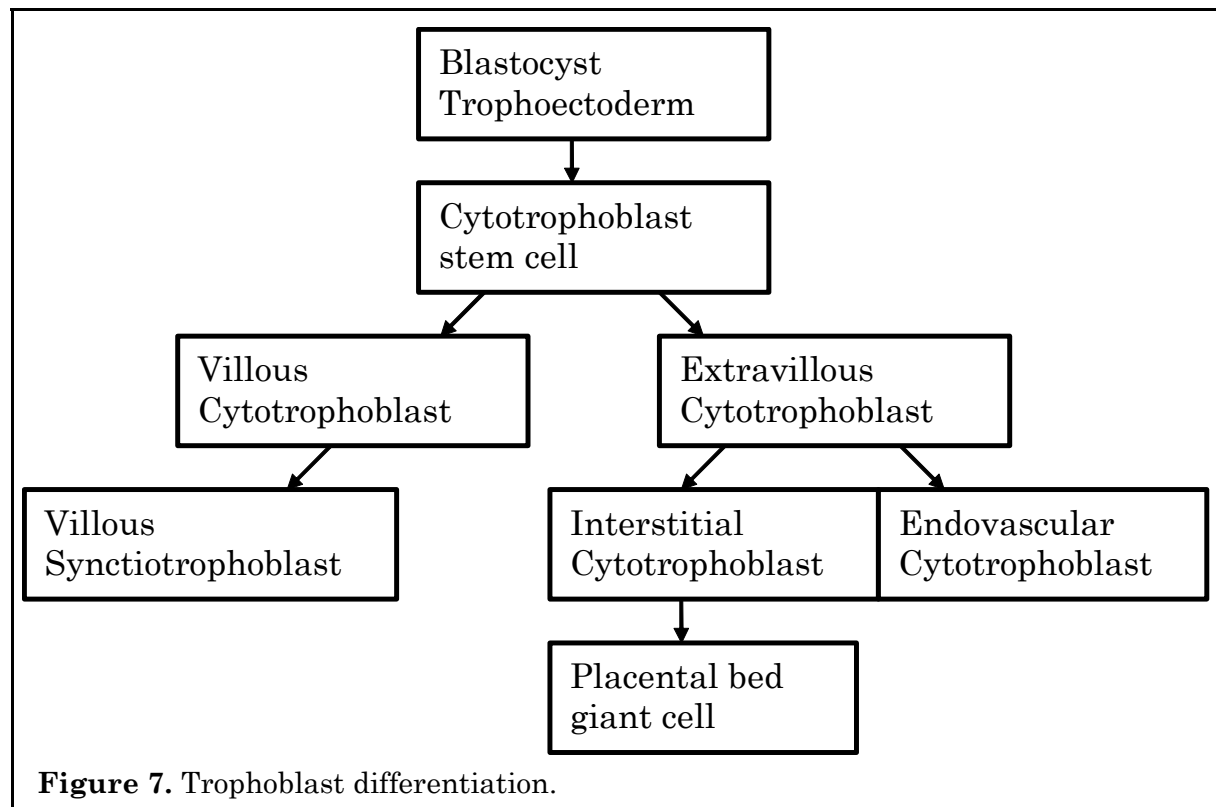
Trophoblasts

Trophoblasts are specialized fetal cells that serve an essential role in implantation and formation of the maternal-fetal interface. These cells are derived from trophoectodermal cells of the outer cell mass of the blastocyst and are extraembryonic. Both the placenta and fetal membranes contain trophoblasts. Through differentiation, several types of trophoblasts are derived including cytotrophoblasts and syncytiotrophoblasts (Figure 7). The cytotrophoblast is a small germative cell from which other trophoblast lineages derive, including the villous and extravillous cytotrophoblast and syncytiotrophoblast. The syncytiotrophoblast is a terminally differentiated, multinucleated cell with abundant cytoplasm. Essentially, trophoblast differentiation is divided into two pathways, villous and extravillous pathways, depending on location and function. The process of differentiation is controlled by fetal as well as maternal factors of the placental bed (42, 43).

In the hemochorial placenta of humans, a lacunar network of pooled maternal blood from uterine spiral arteries surrounding fetal villi (as seen in Figure 2). There are two types of fetal villi: floating and anchoring villi. In essence, the floating villi float in the pool of maternal blood, and the anchoring villi anchor into the maternal decidua. Both types of villi have the basic same structure with an outer layer of syncytiotrophoblast and an inner layer of cytotrophoblasts. In anchoring villi, however, there are two types of cytotrophoblasts, villous and extravillous cytotrophoblasts. Unlike villous cytotrophoblasts, extravillous cytotrophoblasts are capable of detaching from the proliferating cytotrophoblast cell column, invading into the decidua, migrating toward spiral arteries and differentiating further to become interstitial or endovascular trophoblasts depending on location (4). Via differentiation, trophoblasts can acquire properties of leukocytes or endothelial cells that enable many of their specialized functions (44). For example, endovascular trophoblasts invade uterine spiral arteries,

acquire a vascular adhesion phenotype, and replace the maternal endothelium to accommodate for the increased blood flow, allowing these cells to line maternal vessels (45-47). Trophoblast migration into decidua is dependent on adhesion molecule expression and the binding to extracellular matrix proteins (48). Invasion of cytotrophoblasts is also associated with an increase in production of proteinases, specifically matrix metalloproteinase-9, thus improving invasion (49). Essentially, villous trophoblasts interact with maternal blood and function to transport oxygen and nutrients to the fetus. Extravillous trophoblasts interact with maternal uterine tissue and function to establish placental blood supply.

All fetal trophoblasts lack Class I HLA-A and HLA-B as well as Class II molecules. Only the population of extravillous trophoblasts selectively expresses the classical Class Ia HLA-C and non-classical Class Ib molecules HLA-E and HLA-G (50-54). Through this unique HLA expression, extravillous trophoblasts are capable of interacting with receptors, such as CD94/NKG2A, KIRs and those of the immunoglobulin-like transcript (ILT) family expressed by uterine NK cells, T cell subsets and macrophages leading to activation or inhibition (55-60). The ligand-receptor pairs provide an interface between maternal immune cells and fetal trophoblasts and are possible candidates for controlling their interaction and protecting trophoblasts from attack by maternal cells (61). HLA-G is the



ligand for inhibitory receptors of members of the ILT family, ILT2 and ILT4, and also inhibitory KIR2DL4 (62-65). Additionally, HLA-G has also been shown to recognize the CD8 molecule in humans (66). HLA-E binds CD94/NKG2A/C (54), and HLA-C binds KIRs (67).

While HLA-C and HLA-E have ubiquitous expression, HLA-G has limited expression, including the placenta and membranes, the thymus and eye, and on various types of tumor and stromal cells during inflammation and malignancy (68-70). HLA-G has several important features. Via alternative splicing, multiple isoforms of the HLA-G gene can be generated, including four membrane-bound isoforms and three soluble isoforms (71, 72). Unlike classical HLA, HLA-G has few alleles and exhibits limited polymorphism across widely different ethnic origins (73, 74). Like classical HLA, HLA-G can bind and present peptides to CD8 specific T cells (75, 76). Interestingly, HLA-G also is a regulator of HLA-E expression (77). HLA-G has been shown to inhibit allogeneic proliferation of T cells (78), NK cell cytotoxicity (79) and antigen specific T cell cytotoxicity (80). Specifically, HLA-G has been detected in first trimester and term tissue, extravillous and villous cytotrophoblasts, including in the chorionic membrane (81, 82), in term amnion cells (83), in amniotic fluid (84) and on endothelial cells of fetal vessels rather than maternal spiral arteries. There are generally fewer studies on MHC expression in the fetal membranes. HLA-E and HLA-G can be detected on the amniochorion (81, 83), while HLA-C has not been detected (85). Throughout pregnancy, trophoblast expression of HLA-G is differential and decreases as gestation progresses (51).

Trophoblasts also produce or express immunologically relevant molecules such as IL-10 (86, 87), GM-CSF (88) and Fas Ligand (89, 90), and therefore may play an active immunological role in regulating the immune response to the fetal allograft.

MATERNAL CELLS

The decidua is populated by a variety of cell types, including leukocytes, stromal cells and mesenchymal stem cells. With regard to leukocytes, there are several subpopulations present. In non-pregnant and pregnant decidualized endometrium, the three primary leukocyte subpopulations are uterine NK cells,

macrophages and T cells (91-97). B cells are virtually absent. Small unique subpopulations include natural killer T (NKT) cells, T-cell receptor (TCR) $\gamma\delta$ cells and DCs (98-100).

Natural Killer Cells

In contrast to peripheral blood, CD56^{bright}CD16⁻CD3⁻ cells represent the major subset of NK cells in the uterus and are termed large granular lymphocytes or uterine NK cells (93, 94, 96, 101-104). Representing a unique population of cells in the nonpregnant and pregnant endometrium, these CD56^{bright}CD16⁻ cells have prominent cytoplasmic granules (105, 106), are minimally cytotoxic yet highly proliferative (102, 107) and express a wide variety of NK and cytokine receptors and adhesion molecules (108). The CD56^{bright}CD16⁻ uterine NK cells also produce a variety of cytokines, such as GM-CSF, IFN- γ , TNF- α , IL-2, IL-10 and leukemia inhibitory factor (109-113). The proportion of NK cells changes throughout the menstrual cycle and pregnancy. Interestingly, NK cells populate the endometrium prior to pregnancy, implying that the fetus does not play a direct role in their distribution (93, 94, 96, 101, 103). Trafficking, homing, or *in situ* proliferation have been implicated as possible mechanisms in the variation of uterine NK cells in the endometrium and decidua (114). Although the specific role of uterine NK cells is still unknown, it is suggested that they regulate placental development by mediating maternal mucosal and arterial function and fetal extraembryonic trophoblast invasion (115).

Macrophages

Widely distributed throughout the body, macrophages constitute 20-30% of the leukocytes in the decidua and are a stable population of cells in this tissue (95, 116). It is suggested that during pregnancy, these cells may play a role not only in host protection to infections, but also in implantation, placental growth, vascular transformation and maternal-fetal immune tolerance (117-119). Decidual macrophages express HLA-DR and also low levels of the co-stimulatory molecules CD80 and CD86, implying that they may induce tolerance of maternal T cells by failing to give a sufficient co-stimulatory signal (120). Decidual macrophages produce spontaneously high levels of IL-10, a immunoregulatory cytokine known to inhibit the expression of HLA-DR and co-stimulatory molecules on antigen presenting cells (121) and to modulate DCs and T cells

(122), thus possibly reducing potential T-cell activation against fetal antigens. Unlike inflammatory macrophages, macrophages populating the decidua produce lower levels of proinflammatory IL-1b possibly resulting in less inflammation in the setting of intrauterine infection (120). By establishing an adequate microenvironment via cytokine production, macrophages promote cell growth and inhibit harmful inflammatory immune reactions.

T Cells

In general, cells of the innate immune system seem to dominate the decidua during pregnancy (123); however, T cells represent an important population in this tissue that are less well studied in comparison to uterine NK cells. The decidual T cell population changes throughout the menstrual cycle and pregnancy, progressively declining in early pregnancy and then rising to become the most abundant leukocyte population in term pregnancy (124, 125). Furthermore, it is also shown that T cells in term pregnancy decidua basalis express cell surface markers indicating activation, such as CD25, CD69 and HLA-DR (125). The reappearance of T cells during term pregnancy and their expression of activation markers could imply that these cells play a more important role in the maintenance of pregnancy. Recently, tryptophan catabolizing enzyme, indoleamine 2,3-dioxygenase (IDO) was shown to block the maternal T cell activation against the murine fetus (126-128). Human placental and decidual cells have been shown to express IDO (129-130), further influencing immunoregulation at the maternal-fetal interface

Regulatory T Cells

Regulatory T cells represent another important population of T cells. These naturally occurring cells are able to control immune responsiveness to self- and alloantigens and are essential for the active suppression of autoimmunity and for transplantation tolerance (37, 38). CD4⁺CD25^{bright} regulatory T cells recently have been implicated as important cells at the maternal-fetal interface. Recently, in a mouse model, it has been shown that the absence of CD4⁺CD25⁺ regulatory T cells led to a failure of gestation due to immunological rejection of the fetus, suggesting that these cells may mediate maternal tolerance to the fetus (131). Decidual and peripheral blood CD4⁺CD25^{high} T cells are present during early pregnancy, representing approximately 22% of CD4⁺ cells in early

pregnancy decidua and 9% in peripheral blood (132). The proportion of regulatory T cells in the decidua of normal term pregnancy has yet to be fully studied. With regards to complicated pregnancy, it has been suggested that the function of these cells may be impaired in pathological conditions such as recurrent spontaneous abortion, preterm labor and preeclampsia (133). Although the factors regulating these cells is unknown, regulatory T cells appear to be crucial in the maintenance of tolerance in pregnancy.

TCR $\gamma\delta$ Cells

TCR $\gamma\delta$ cells are an essential part of the innate immune system recognizing antigen upon cell infection, stress or transformation. T cells are divided into two lineages defined by their expression of TCR: TCR $\alpha\beta$ and TCR $\gamma\delta$ cells. Approximately, 90 to 95% of circulating T cells use TCR α and β chains, while 5–10% use TCR γ and δ chains. While the TCR $\alpha\beta$ cells are a part of the adaptive immune system, TCR $\gamma\delta$ cells are part of the innate immune system and are generated in the thymus and in an extrathymic compartment (134). In adult animals and humans TCR $\gamma\delta$ cells can be further divided into two groups: circulating cells and resident cells of the mucosal surfaces, such as in the digestive, respiratory and urogenital tracts. Unlike TCR $\alpha\beta$ cells, TCR $\gamma\delta$ cells are not MHC restricted, allowing them to recognize antigens in a fundamentally different way than that of $\alpha\beta$ T cells, more similar to antibodies, able to directly recognize molecules without antigen processing and presentation (135).

TCR $\gamma\delta$ cells can interact and modulate the activity of other immune cells directly or by cytokine production and thus function as regulatory cells (135). TCR $\gamma\delta$ cells are present in non-pregnant and pregnant decidualized human endometrium (99, 136, 137). Hormonally controlled, decidual TCR $\gamma\delta$ cells are large granular lymphocytes with cytoplasmic granules, expressing CD2, CD69, NK receptors CD94 and NKG2D and CTLA 4, a marker associated with regulatory T cells and lacking expression of both CD4 and CD8 (138, 139). Interestingly, it is suggested that extrathymic T-cell maturation occurs in the decidua of early pregnancy and that CD56⁺CD16⁻ decidual NK cells are immature progenitors for T cells (140). Similar to resident TCR $\gamma\delta$ cells at other mucosal sites, decidual TCR $\gamma\delta$ cells are activated but resting, possessing a cytotoxic potency and capable of expressing and storing cytolytic molecules such as granzyme Fas ligand (FasL), which

rapidly available upon stimulation (140).

Natural Killer T Cells

Natural Killer T cells represent a conserved T cell sublineage and appear to play an important immunoregulatory role in the immune system (141). Functionally different from conventional T cells, NKT cells possess an invariant TCR α chain associated with a heterogenous TCR β chain (142). While an endogenous activating ligand is unknown, a synthetic glycolipid is capable of activating NKT cells when presented by the class I-like molecule, CD1d (143-145). Activated NKT cells rapidly produce a variety of cytokines including IL-4, TNF, IFN and GM-CSF (146, 147) and are capable of interacting with DCs, NK cells, T and B cells (141). Interestingly, NKT cells are associated with the onset of diabetes in humans, tumor clearance, allograft survival and as mediators of anterior chamber acquired immune deviation (ACAID) in which specific tolerance is generated to antigens introduced into the anterior chamber of the eye (98).

NKT cells comprise 0.48% of the CD3⁺ T cells population in first trimester decidua (98). While comprising a small leukocyte population in the decidua, they are present at a frequency ten times greater than that in peripheral blood. These cells also exhibit a marked T_H1-like cytokine bias with IFN- γ but also can produce IL-4 and GM-CSF (98, 148). Fetal trophoblasts express CD1d, suggesting that decidual NKT cell interactions with CD1d-expressing trophoblasts play a specific role at the maternal-fetal interface, possibly in the acceptance of the fetal allograft and/or in placental development (98).

Dendritic cells

Dendritic cells present in decidua may play a pivotal role in sampling, processing and presenting fetally derived trophoblast antigens to the maternal immune system (100). These cells comprise approximately 1.7% of CD45⁺ cells in the isolates of first trimester decidual tissue and have the phenotype of myeloid immature DCs (100). First trimester decidua also contains DCs with morphological and functional features of typical mature DCs including co-stimulatory molecule expression of CD40, CD45, CD83 and HLA-DR (149). Cells with dendritic processes are found scattered through both the decidua basalis and decidua parietalis as well as in endothelial-lined spaces (100). It is suggested that

a bi-directional cross-talk between decidual CD56^{bright} uterine NK cells and decidual CD83⁺ DCs results in activation and proliferation of autologous NK cells (150, 151). Furthermore, it is suggested that immature DCs in the decidua have the potential to capture fetal antigens from extravillous trophoblasts and present to local maternal T cells, thus inducing tolerance to those antigen peptides (118).

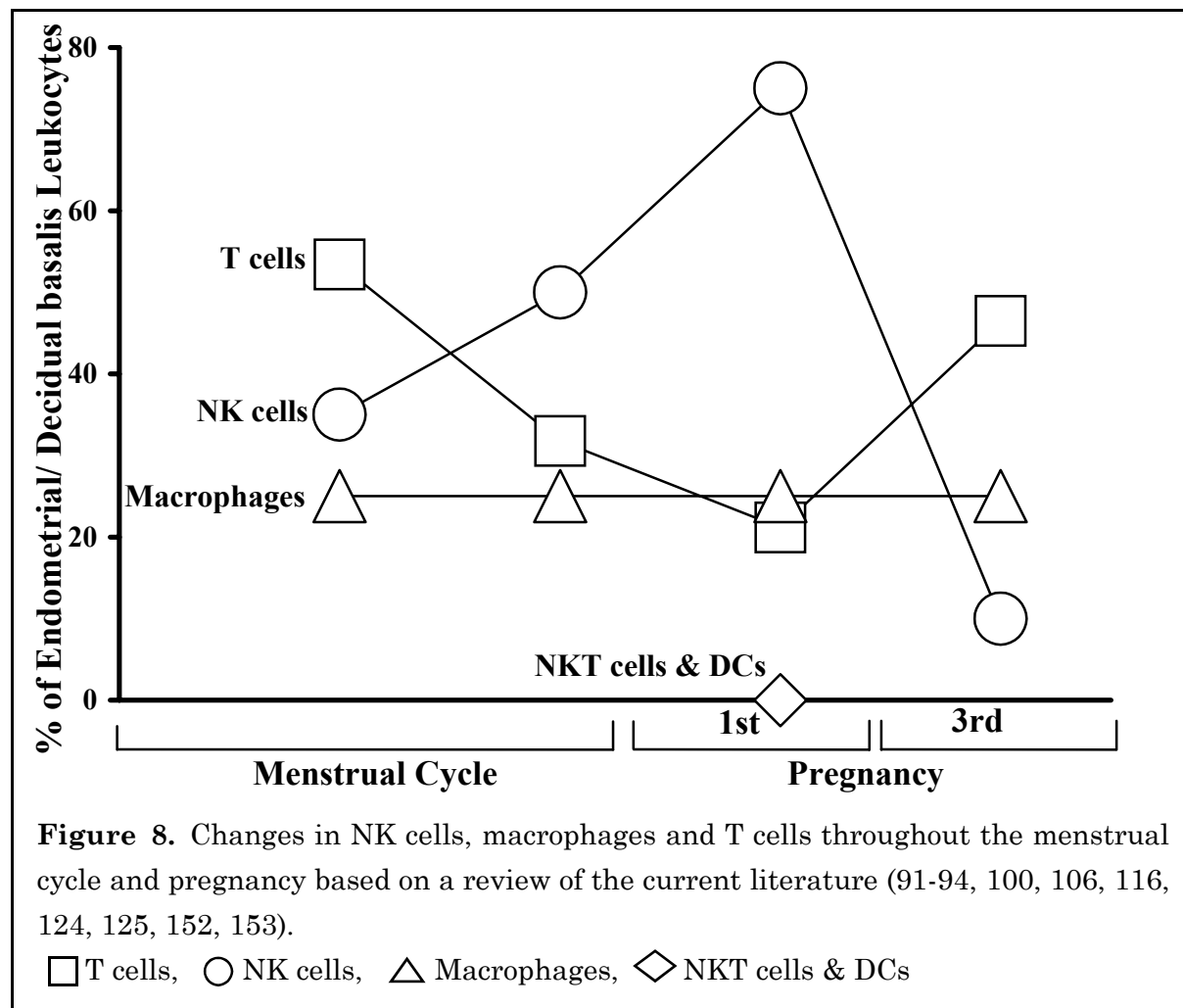
Changes in cells during the menstrual cycle and pregnancy

The cellular composition changes throughout the menstrual cycle and pregnancy. Changes in CD56^{bright}CD16⁻ NK cells occur throughout the menstrual cycle and pregnancy. In the late secretory phase of the menstrual cycle, these cells become a prominent population of endometrial leukocytes in preparation for a possible conception (94, 96, 101, 103). When pregnancy occurs, CD56^{bright}CD16⁻ NK cells account for up to 70% of the leukocytes at the decidua basalis (93, 152). The characteristic accumulation and predominance of these cells at the maternal-fetal interface at the beginning of the first trimester implies a putative role in the control of implantation and placentation. It is often stated that as pregnancy progresses, the number of uterine NK cells in the decidua basalis gradually declines or becomes virtually absent by term pregnancy (114, 153). Histologic studies in mice also indicate that the high proportion of uterine NK cells is limited only to early pregnancy (154, 155).

T cells, on the other hand, show a reciprocal distribution during the menstrual cycle and pregnancy. In the early proliferative phase of the menstrual cycle, T cells are an abundant leukocyte population accounting for approximately 45-60% of the leukocytes in the endometrium (96, 124). As the endometrium prepares for the possible reception of a fertilized ovum during the secretory phase, the percentage of T cells progressively decreases. An additional decline characterizes the first trimester transformation of endometrium into decidua, in which the total T-cell population represents only 6-30% of the decidual leukocytes (93, 95, 96, 116, 124). However, by term pregnancy, 45-50% of the leukocytes in the decidua basalis are CD3⁺, accounting for the most abundant leukocyte population (116, 125).

Lastly, macrophages remain a stable population of cells throughout the menstrual cycle and pregnancy. Macrophages account for 20-30% of the decidual

leukocyte population and do not fluctuate in proportion like NK and T cells (95, 116). Studies of NKT cells and DCs cells have focused on early first trimester decidua; little is known about the change in these cells during the menstrual cycle and pregnancy.



CYTOKINES

Cytokines play a significant role in the immunological mechanisms involving placental growth and the maintenance of pregnancy (156). It was originally proposed by Wegmann that pregnancy requires a dominance of T_H2 cytokines and that an established successful pregnancy is characterized by low levels of T_H1 cytokines and by a shift in the cytokine pattern from T_H1 to T_H2 (157). This T_H1/T_H2 paradigm of reproduction suggests that pregnancy rejection is mediated by T_H1 cytokines, while maintenance and acceptance is mediated by T_H2

cytokines (35, 158). Current research has altered this paradigm to suggest that the T_H1/T_H2 paradigm may be an oversimplification of role of cytokines in reproduction (159). For example, mice lacking T_H2 cytokines, including IL-4, IL-5, IL-9, IL-10 and IL-13 undergo normal allogeneic pregnancy with unaffected litter size (160, 161). T_H1 cytokines may in fact serve an essential role very early in embryonic development and then again during labor (124). Regardless of the precise balance of cytokines, there is a complex network of cytokines at the maternal-fetal interface involved in regulation of immune responsiveness.

MOUSE MODEL IN REPRODUCTIVE IMMUNOLOGY

The mouse model has proven to be essential for the characterization of human decidual leukocytes by allowing manipulative studies that are not possible in humans. Mice and humans both have hemochorial placentas in which there is direct contact between maternal blood and trophoblasts. Importantly, analogous cell types, such as trophoblasts and NK cells, as well as correlate surface markers and receptors have been identified. There are differences, however, in implantation, placental morphogenesis and endocrine function (115). In mice, the trophoectoderm of the blastocyst develops into an ectoplacental cone to become the placenta. NK cells are not present in non-pregnant murine endometrium, with decidualization occurring only after implantation. In pregnancy, NK cells are confined to the central decidua basalis and mesometrial region of the uterine musculature where there is minimal trophoblast invasion (115, 155). Although human and mice placentas differ, common features allow for valuable studies.

IMMUNE MECHANISMS AT THE MATERNAL-FETAL INTERFACE

When the paradox of pregnancy first was proposed, four hypotheses were presented by Medawar and Billingham to explain maternal tolerance of the fetus (1, 162). It was suggested that the fetus lacks immunogenicity; that the maternal immune system is suppressed during pregnancy; that there is the elaboration of an immune barrier by the placenta; and that the uterus is an immunoprivileged site. Since 1953, many advances in the field of reproductive immunology have changed the opinion on and reliability of these hypotheses. With the research amassed throughout the last fifty years, several new mechanisms have emerged to explain the paradox of pregnancy and maternal tolerance toward the fetus.

The direct contact between fetal trophoblasts that invade the decidua and maternal immunocompetent cells in the decidua suggests that mechanisms of immunosuppression or tolerance must exist to avoid of rejection the fetus. According to the classic laws of transplantation immunology, foreign fetal antigens should be recognized and induce a response by the maternal immune system. It therefore would seem logical that nonrecognition of the fetus is advantageous. It is now proposed, however, that immune recognition of pregnancy is important for the maintenance of pregnancy. In fact, inadequate recognition may result in failed pregnancy (139). This is in contrast to the successful maintenance of an organ transplant. It has been noted that the comparison of pregnancy with tissue transplantation is inaccurate and a misleading framework (123, 163). Unlike pregnancy, a blood or organ allograft requires medical or surgical placement, acutely exposing the recipient to a significant amount of foreign antigen and generally will be rejected following antigen presentation by antigen presenting cells (123). The immunological relationship between the mother and the fetus is a delicate balance between fetal antigen presentation and recognition and response by functionally intact maternal immune system (139).

There is evidence that fetal recognition occurs during pregnancy. Beginning at 4-6 weeks gestation, the transfer of fetal cells into the maternal circulation occurs in most pregnancies and continues until delivery, leading to microchimerism (164). Interestingly, fetal cells in maternal circulation have been shown to persist 27 years after pregnancy (165). Conversely, maternal cells also have been detected in human fetal umbilical cord blood at birth (166) and within primary lymphoid organs in the murine system (167). Anti-paternal antibodies are detectable in sera of women with successful pregnancies (168). Studies investigating the presence of anti-paternal antibodies and the outcome of subsequent pregnancies demonstrate that these antibodies are positively associated with a higher live birth rate, whether induced by immunization or previous pregnancies. Decreased anti-paternal alloantibody production is associated with recurrent spontaneous abortion (168). Semen also may play an essential role in recognition. Studies in mice indicate that factors in seminal fluid can initiate a maternal immune response by providing the necessary antigenic

and environmental signals (169). Furthermore, HLA matching between couples is associated with spontaneous abortion (170, 171), suggesting that the lack of immune recognition is deleterious to pregnancy. Maternal recognition does not appear to compromise pregnancy, but rather promote pregnancy.

There is evidence that the maternal immune system is altered during pregnancy. Systemically, the innate immune system is enhanced and activated (123), while acquired cell-mediated immunity is diminished (172). Beginning at the first trimester, there is a progressive relative lymphopenia and neutrophilia (173, 174). Specifically, there is an increase in the percentage of circulating monocytes and granulocytes. By the third trimester these cells express an activated phenotype, with cell surface marker expression similar to those during systemic sepsis and also display increased phagocytosis and intracellular reactive oxygen species (175-177). Resulting from the alteration of the maternal immune system, the risk of specific intracellular infections in pregnancy, such as malaria, pneumonia and influenza may be increased, while certain cell-mediated autoimmune diseases, such as rheumatoid arthritis and multiple sclerosis improve (178).

Immune tolerance is the mechanism by which the host is protected against self-reactive T cells that escape the thymus. Although tolerance is normally defined in terms of the prevention of responses against self-antigens, there is also tolerance induced to foreign antigen as in the setting of transplantation. In the setting of pregnancy, there is recent evidence in the murine model that there is systemic tolerance of the fetus. It has been shown that maternal T cells acquire a transient state of tolerance to paternal antigens during pregnancy. Fetal antigen-specific T cells downregulate CD8 co-receptors during pregnancy, resulting in reversible functional unresponsiveness to paternal antigens (179). T cells specific for fetal antigens decrease in an antigen-specific manner during pregnancy and remain low postpartum (180). Furthermore, maternal regulatory T cells are expanded systemically during pregnancy in alloantigen-independent manner, suggesting that this population of cells is essential for the suppression of maternal immune aggression directed against the fetus (131). Importantly, maternal recognition of fetal antigens can lead to tolerance.

OUTLINE OF THESIS

To gain a better understanding of the immunoregulatory mechanisms of pregnancy, studies of leukocytes in human decidua generally have focused on early first trimester decidua, the stage in which implantation and placentation are established. Leukocytes in human term decidua have been the focus of fewer studies. The third trimester, however, represents the stage in which pregnancy and placentation are maintained. Furthermore, those studies of term decidua mainly have focused on leukocytes derived from decidua basalis, the site of implantation. The decidua parietalis, lining the remainder of the uterine cavity is another important region of the maternal-fetal interface which forms contact with fetal tissue at the end of the first trimester.

The aim of this thesis was to study the distribution of decidual leukocytes, specifically uterine NK cells and T cells, in the decidua basalis and parietalis in the setting of normal term pregnancy, following elective cesarean section or spontaneous vaginal delivery, in the setting of complicated pregnancy, and in the setting of assisted reproduction, and to study the function of term decidual leukocytes, specifically proliferation and cytokine production, in response to fetal and allogeneic blood leukocytes. Techniques involving flow cytometry, immunohistochemistry and mixed lymphocyte cultures were used to perform these studies.

In **Chapter 2**, the expression of CD16 and CD56 on leukocytes by flow cytometry and immunohistochemistry in normal term decidua basalis and parietalis is compared and quantified. The focus of **Chapter 3** is on T cells, specifically the expression of activation markers, in term decidua basalis and decidua parietalis. Importantly, it must be noted that the twenty placentas from women undergoing elective cesarean section in Chapter 3 were also included in part of the subject groups in Chapters 2, 4 and 7.

In **Chapter 4**, the effect of labor on decidual leukocytes is determined by quantifying leukocyte subpopulations and the activation state of T cells in term decidua basalis and decidua parietalis obtained from spontaneous vaginal delivery and comparing the results with those of elective cesarean section.

The function of leukocytes in the decidua basalis and parietalis, including their ability to proliferate in response to fetal or allogeneic cells and their ability to produce cytokines is an important focus in order to understand the role of these cells in the maintenance of pregnancy. The aim of **Chapter 5** was to examine the function of leukocytes isolated from term decidua basalis and parietalis by measuring their proliferation and cytokine production in response to various stimuli including fetal and allogeneic blood leukocytes in a one-way mixed lymphocyte culture and by comparing it to the responses from maternal and allogeneic peripheral blood leukocytes.

In **Chapters 6 and 7**, decidual leukocytes were studied in the setting of complicated pregnancy and of assisted reproduction to provide new insight into the immunology of the maternal-fetal interface. Preeclampsia (PE) and intrauterine growth restriction (IUGR) are significant complications of pregnancy associated with high perinatal morbidity and mortality and with varying morphologic placental development. In assisted reproduction, specifically gestational surrogacy, the surrogate typically has no genetic link to the fetus and is presented with two sets of mismatched histocompatibility antigens. It provides a unique model to investigate the field of reproductive immunology and seems to more accurately characterize the setting of organ transplantation.

In **Chapter 8**, future plans are suggested, conclusions are summarized and a discussion is presented. In conclusion, the human placenta and fetal membranes are of elegant design, comprised of a multitude of cells, containing a variety of molecular messengers, allowing for the maintenance of pregnancy and directing the complex physiology of the mother and fetus. While research in this field has impacted immunology, obstetrics and transplantation, future research is essential to further promote translation into the clinical setting.

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CHAPTER 2

DIFFERENTIAL DISTRIBUTION OF NK CELLS IN DECIDUA BASALIS AND PARIETALIS AFTER UNCOMPLICATED HUMAN TERM PREGNANCY

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ABSTRACT

As pregnancy progresses, a characteristic decline in the percentage of CD56^{bright}CD16⁻ uterine NK cells occurs. Studies of term decidua, however, have focused only on leukocytes derived from decidua basalis, the site of implantation. The decidua parietalis, lining the remainder of the uterine cavity is another important region of the maternal-fetal interface which forms contact with fetal tissue at the end of the first trimester. The aim of this study was to evaluate possible differences in expression of CD16 and CD56 on leukocytes from normal term decidua basalis and decidua parietalis.

Decidua basalis and parietalis samples were obtained from 30 placentas collected after elective cesarean section. Percentages of leukocyte subpopulations and NK cell subsets within the CD45⁺ cell fraction were determined by flow cytometry. In six decidual samples, concurrent immunohistochemical staining was performed.

Higher percentages of CD56^{dim}CD16⁺ NK cells and CD56⁻CD16⁺ cells were found in decidua basalis in comparison to decidua parietalis. In contrast, the percentage of CD56^{bright}CD16⁻ uterine NK cells was significantly higher in decidua parietalis. Immunohistochemical quantification supported flow cytometric results.

We conclude that significant differences exist with respect to the distribution of NK cells in term decidua basalis and parietalis. Future functional studies may improve our understanding of their role at the maternal-fetal interface.

INTRODUCTION

Natural killer (NK) cells represent an important effector component of the innate immune system, able to produce immunoregulatory cytokines and to recognize and kill target cells (1). Phenotypically characterized by the expression of CD56 and the lack of expression of CD3, there are two distinct subsets of NK cells defined as CD56^{dim}CD16⁺CD3⁻ and CD56^{bright}CD16⁻CD3⁻. Ninety percent of NK cells in normal circulating peripheral blood have the classical phenotype of CD56^{dim}CD16⁺CD3⁻, and the remaining 10% are CD56^{bright}CD16⁻CD3⁻ (2).

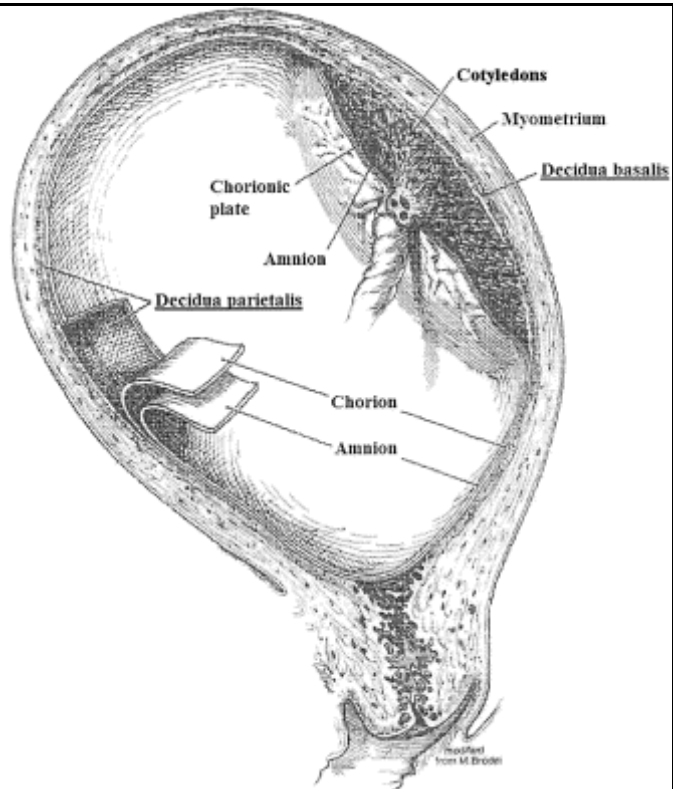
In contrast to peripheral blood, CD56^{bright}CD16⁻CD3⁻ cells represent the major subset of NK cells in the uterus and are termed large granular lymphocytes or uterine NK cells (3-8). Representing a unique population of cells in the non-pregnant and pregnant endometrium, these CD56^{bright}CD16⁻ cells have prominent cytoplasmic granules (9, 10), are minimally cytotoxic yet highly proliferative (11, 12), produce a number of cytokines (13-16) and express a wide variety of NK and cytokine receptors and adhesion molecules (17). Although the specific role of these cells is still unknown, it is suggested that they regulate placental development by mediating maternal mucosal and arterial function and fetal extraembryonic trophoblast invasion (18).

Changes in CD56^{bright}CD16⁻ NK cells occur throughout the menstrual cycle and pregnancy. In the late secretory phase of the menstrual cycle, these cells become a prominent population of endometrial leukocytes in preparation for a possible conception (4-7). Concurrently, the uterine mucosa begins to undergo decidualization, a hormone-dependent process in which endometrial stromal cells enlarge and become capable of producing growth factors and matrix components to support implantation and placental growth (19). When pregnancy occurs, CD56^{bright}CD16⁻ NK cells account for up to 70% of the leukocytes at the site of implantation, identified as the decidua basalis (3, 20). The characteristic accumulation and predominance of these cells at the maternal-fetal interface at the beginning of the first trimester implies a putative role in the control of implantation and placentation. It is often stated that as pregnancy progresses, the number of uterine NK cells in the decidua basalis gradually declines or becomes virtually absent by term pregnancy (21). Histological studies in mice

also indicate that the high proportion of uterine NK cells is limited only to early pregnancy (22, 23).

There are two distinct regions of the maternal-fetal interface where maternal decidua comes into direct contact with fetal extraembryonic tissue – one between decidua basalis and the invading interstitial trophoblasts and the other between tissue lining the remainder of uterine cavity, identified as the decidua parietalis and the amniochorion (Figure 1). While the decidua basalis encounters fetal tissue on the first day of implantation, contact between the decidua parietalis and fetal tissue does not occur until the end of the first trimester when fetal growth obliterates the uterine cavity. The focus of most immunological studies has centered primarily on the decidua basalis, where immune interaction is expected to take place. Remarkably, the decidua parietalis has not been as well studied as the decidua basalis, yet it is an important site of the maternal-fetal interface. Our recent studies revealed that there are, in fact, significant differences in term decidua basalis and parietalis. Flow cytometric quantification of term decidual leukocytes showed a significantly higher percentage of T cells expressing CD25, HLA-DR, CD45RO and CD69 activation markers in decidua parietalis as compared to decidua basalis (24).

Figure 1. The decidua basalis and decidua parietalis in relation to other uterine tissues after the fourth month of pregnancy (Adapted from Cunningham FG, Gant NF, Leveno KJ, Gilstrap LC, Hauth JC, Wenstrom KD: Williams Obstetrics 21st edition. New York, McGraw-Hill, 98, 1997).



The aim of the present study was to quantify and compare the expression of CD16 and CD56 on leukocytes by flow cytometry and immunohistochemistry in normal term decidua basalis and parietalis.

MATERIALS AND METHODS

Tissue specimens

Paired normal term decidua basalis and parietalis samples were obtained from 30 placentas following uncomplicated pregnancy. To exclude the effects of labor or vaginal delivery on the decidual cells, only placentas from pregnant women delivered by elective cesarean section prior to the onset of labor were selected. All women received spinal anesthesia prior to cesarean section. All cesarean sections were performed for breech presentation and resulted in the birth of a non-growth restricted healthy neonate. Term decidua basalis tissue was macroscopically dissected from the central region of the maternal-facing surface, and term decidua parietalis tissue was collected by removing the amnion and delicately scraping the tissue from the chorion. Areas of ischemia or necrosis were avoided. Data from 20 of the 30 placentas were used in our concurrent study previously published (24). Additionally, 10 ml of maternal peripheral blood were collected from six of the patients prior to the cesarean section into heparinized tubes. Informed consent was obtained from all patients.

Cell isolation for flow cytometric analysis

For flow cytometric analysis, decidual tissue was prepared into a single cell suspension. To avoid selective cell death or selective loss of surface proteins, mechanical disaggregation rather than enzymatic digestion was used to process the decidual tissue and to isolate the decidual leukocytes. The obtained tissue was washed and finely minced between two scalpel blades in a small volume of PBS. Minced decidual fragments were gently worked through an open filter chamber (NPBI International BV; Emmer Compascuum, the Netherlands, and Beldico; Marche-en-Famenne, Belgium) with PBS. The resultant suspension was filtered through a 70µm sieve (Becton Dickinson Labware; Franklin Lakes, New Jersey), washed in PBS and layered on an equal volume of ficoll hypaque (Leiden University Medical Center Pharmacy; Leiden, the Netherlands) at room temperature for density gradient centrifugation for 20 minutes at 800g.

Mononuclear cells were collected from the interface, washed twice and resuspended in PBS for cell staining. Maternal peripheral blood mononuclear cells were similarly isolated by density gradient centrifugation and collected.

Flow cytometric staining of leukocytes

Directly conjugated fluorescent antibodies directed against a variety of leukocyte antigens (Table 1) were used for two- and three-color immunofluorescence staining to determine leukocyte subpopulations within decidua basalis and decidua parietalis. Cells were stained with monoclonal antibodies at a density of 10^6 cells/ml in PBS (100 μ l/ sample) and incubated for 30 minutes at 4°C in the dark. Following two washes with PBS, cells were resuspended and fixed with PBS containing 1% paraformaldehyde. Cells were then analyzed on a Becton-Dickinson FACScan flow cytometer. The double-labeled CD45 and CD14 sample was used to establish the optimal lymphocyte gate based on light scattering profile and immunofluorescence (25). Directly labeled isotype-control antibodies were used for the exclusion of non-specific binding. Approximately 5,000 events were acquired in the gating window. The acquired data were then analyzed using Becton-Dickinson CellQuest software™ (San Jose, CA, USA).

The percentages of CD16⁺, CD56⁺, CD14⁺, CD19⁺, CD3⁺, CD4⁺ and CD8⁺ and cells in decidua basalis and decidua parietalis were determined and calculated as a percentage of the CD45⁺ cell fraction. To precisely characterize the expression of CD16 and CD56, the percentages of CD56^{dim}CD16⁺ NK cells, CD56^{bright}CD16⁻ uterine NK cells and CD56⁻CD16⁺ cells were established by delineating regions around these various populations set based on maternal peripheral blood samples.

Methodological control

As a control, one 19 week decidual sample was analyzed. This tissue was obtained after an elective abortion on social grounds of a normally developing pregnancy. From this sample, decidua basalis tissue was microscopically separated from fetal tissue, leukocytes were isolated, and flow cytometric analysis was performed as previously described. In comparison to previous studies (3, 20), similar percentages of leukocyte subpopulations were found, specifically a high percentage (66.7%) of CD56^{bright}CD16⁻ cells (data not shown).

Table 1. Monoclonal antibodies for flow cytometry.

Antigen Specificity	Antibody	Cell Type	Fluorochrome	Type of Labeling	Source ^a
CD45	HLe-1	Leukocyte	FITC	Direct	BD
CD14	Leu-M3	Macrophage/ Monocyte Granulocyte	PE	Direct	BD
CD19	Leu-12	B cell	FITC	Direct	BD
CD3	Leu-4	T cell	APC PerCP-Cy5	Direct	BD
CD4	Leu-3a	T cell subset	FITC PE	Direct	BD
CD8	Leu-2a	T cell subset	FITC PE	Direct	BD
CD16	Leu-11a Leu-11c	NK cell Macrophage/ Monocyte Granulocyte	FITC PE	Direct	BD
CD56	Leu-19	NK cell	FITC	Direct	BD

^aBecton-Dickinson (BD) Immunocytometry Systems (San Jose, CA, USA)

Immunohistochemical staining

Six of the tissue specimens of decidua basalis and parietalis were concurrently embedded in paraffin for immunohistochemical labeling of leukocytes using antibodies listed in Table 2. Serial 3 µm thick tissue sections were mounted on slides coated with 3-aminopropyltriethoxysilane and glutaraldehyde (Sigma; Zwijndrecht, Netherlands). Tissue sections were deparaffinized using xylene and 100% ethanol. Endogenous peroxidase activity was blocked with methanol containing 0.3% H₂O₂ (Sigma; Zwijndrecht, Netherlands) for 20 minutes. Sections were then rehydrated with 70% and 50% ethanol. Antigen retrieval pre-treatment was performed by two different methods depending on the primary antibody. Tissue sections were pre-treated by microwaving the sections for 10 minutes in boiling citrate buffer (10 mmol/L, pH 6.0) or in boiling Tris/EDTA

buffer (1 mmol/L, pH 9.0). Following two washes in PBS, tissue sections were incubated with primary antibodies at the appropriate dilutions in PBS with 1% BSA (Sigma; Zwijndrecht, Netherlands) overnight in a moist chamber. Sections were washed three times in PBS and further incubated for 30 minutes with the secondary biotinylated anti-mouse IgG immunoglobulin (Dako; Glostrup, Denmark) diluted 1:100 in PBS with 1% BSA. Following three washes in PBS, sections were incubated for 30 minutes with a biotinylated horse radish peroxidase/ streptavidin complex (Dako; Glostrup, Denmark) diluted 1:100 in PBS with 1% BSA. The reaction was developed in a 10-minute incubation period with 0.5 mg/ml diaminobenzidine tetrahydrochloride (Sigma; Zwijndrecht, Netherlands), 0.3% H₂O₂ in 0.05M Tris-HCL (pH 7.6). Sections were then counterstained with Mayer's hematoxylin (Klinipath; Duiven, Netherlands) for 15 seconds and then washed for 5 minutes with demiwater.

Table 2. Monoclonal antibodies for immunohistochemistry.

Antigen Specificity	Antibody	Cell Type	Pre-treatment	Dilution	Source ^a
CD16	16CO2	NK cell Macrophage/ Monocyte Granulocyte	Microwave - Citrate	1:10	Lab Vison Neomarkers
CD56	NCAM	NK cell	Microwave - EDTA	1:200	Sanbio

^aLab Vison Neomarkers (Duiven, the Netherlands)

Quantification of immunohistochemistry

The number of positive cells in 10 high-power fields (400x magnification) was quantified for each specimen by two independent observers (A.P.S.-T. and J.J.S.). Fields were selected to contain only decidua, and cells within blood vessels were not counted. The mean number of CD16⁺ and CD56⁺ cells per high power field was determined.

Statistical Analysis

To compare differences in decidual leukocyte subsets in the decidua basalis and decidua parietalis, the Wilcoxon signed rank test, a non-parametric paired t-test, was performed. The value of $p < 0.05$ was defined as significant.

RESULTS

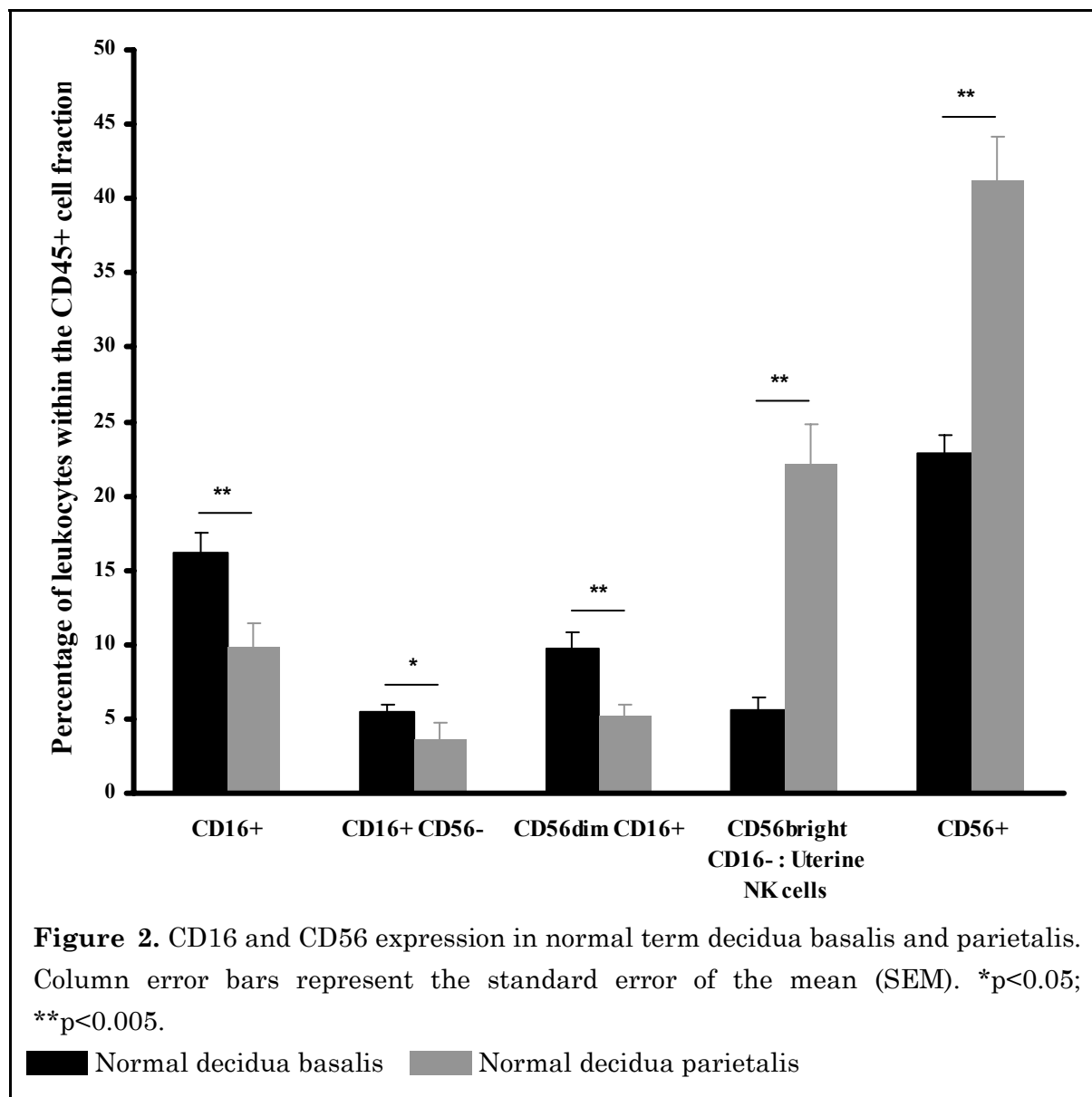
Expression of CD16 and CD56 in normal term decidua basalis and parietalis

The percentage of several leukocyte subpopulations was examined in normal term decidua basalis and parietalis by flow cytometry (Figure 2) and summarized in Table 3. Comparison of the expression of CD16 and CD56 on leukocytes in these two tissue sites showed a differential distribution (Figure 2). In decidua basalis, the percentage of CD56⁺ cells was significantly lower (22.8%, $p < 0.0001$) in comparison to that of decidua parietalis (41.2%). In contrast, the percentage of CD16⁺ cells was significantly higher (16.1%, $p = 0.0015$) in decidua basalis in comparison to that of decidua parietalis (9.8%). To characterize NK cells subsets, regions were defined around populations of CD56^{dim}CD16⁺ NK cells, CD56^{bright}CD16⁻ uterine NK cells and CD56⁻CD16⁺ cells based on maternal peripheral blood samples (Figure 3). Analysis of the decidua basalis and parietalis showed that there was a significant increase in the percentage of CD56⁻CD16⁺ cells and CD56^{dim}CD16⁺ cells in decidua basalis ($p = 0.0069$ and $p = 0.0009$, respectively). Most importantly, the percentage of CD56^{bright}CD16⁻ uterine NK cells was 22.1% in decidua parietalis, significantly higher in comparison to 5.6% found in decidua basalis ($p < 0.0001$).

Quantification of CD16 and CD56 expression (Figure 4) in decidua tissue sections stained by immunohistochemistry confirmed results between decidua basalis and parietalis found by flow cytometric analysis (Table 4). In decidua basalis, the mean number of CD56⁺ cells was significantly lower (1.6; $p = 0.0005$) in comparison to that of decidua parietalis (6.0). In contrast, the mean number of CD16⁺ cells per high power field was significantly higher (8.0; $p = 0.01$) in decidua basalis in comparison to that of decidua parietalis (5.5).

Expression of additional markers in normal term decidua basalis and parietalis

The percentages of CD45⁺ leukocytes, CD14⁺ macrophages, CD19⁺ B cells, CD3⁺ T cells and CD8⁺ cells were similar in decidua basalis and parietalis (Figure 1 and Table 3). A higher percentage of CD4⁺ cells was found in the decidua basalis in comparison to that of the decidua basalis ($p = 0.023$).



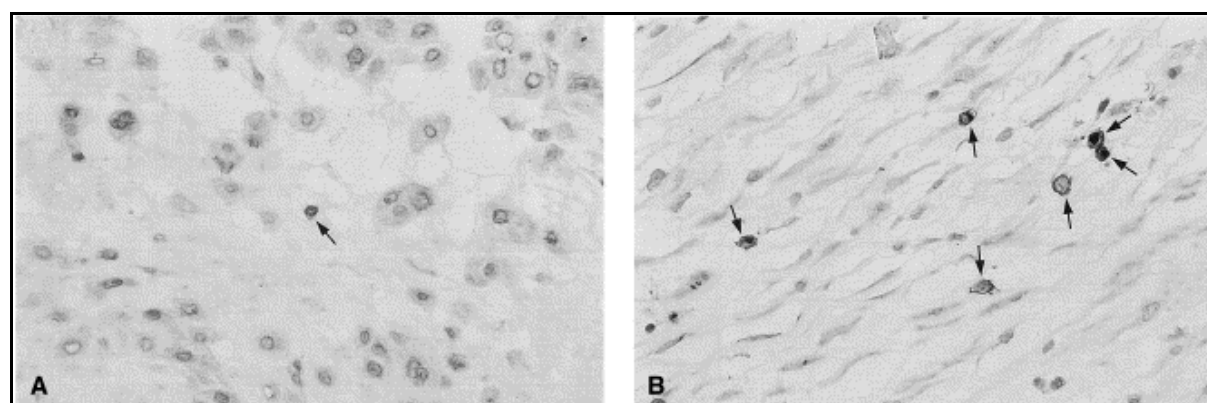
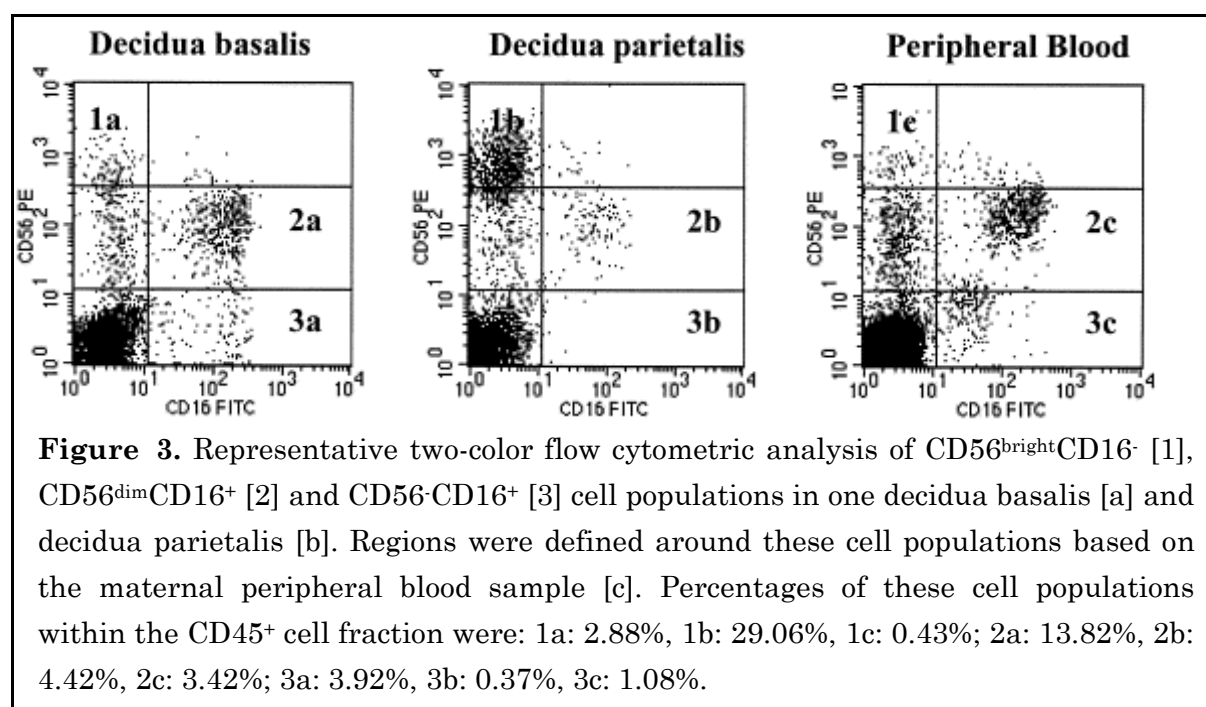


Table 3. Mean percentage of leukocytes in normal term decidua basalis and parietalis determined by flow cytometry.

Leukocyte Markers	Decidua basalis (n=30)	Decidua parietalis (n=30)	Significance
CD45⁺	36.0	31.5	NS
CD14⁺ /CD45⁺	14.4	17.4	NS
CD19⁺/CD45⁺	5.5	3.8	NS
CD3⁺/CD45⁺	56.5	52.7	NS
CD4⁺/CD45⁺	27.4	18.6	p=0.023
CD8⁺/CD45⁺	31.6	31.5	NS
CD16⁺/CD45⁺	16.1	9.8	p=0.0015
CD16⁺CD56⁻/CD45⁺	5.5	3.7	p=0.0069
CD56^{dim}CD16⁺/CD45⁺	9.8	5.3	p=0.0009
Uterine NK cells: CD56^{bright}CD16⁻/CD45⁺	5.6	22.1	p<0.0001
CD56⁺/CD45⁺	22.8	41.2	p<0.0001

Table 4. Mean number of positive leukocytes per high power field in normal term decidua basalis and parietalis determined by immunohistochemistry.

Leukocyte Markers	Decidua basalis (n=6) [range]	Decidua parietalis (n=6) [range]	Significance
CD16⁺	8.0 [2.0-11.9]	5.5 [3.0-7.5]	p=0.01
CD56⁺	1.6 [0-7.7]	6.0 [0.5-13.2]	p=0.0005

DISCUSSION

This study indicates that there are regional differences in the expression of CD16 and CD56 on leukocytes in term decidua basalis and parietalis. By precisely defining NK cell subsets, an analysis of NK cells in both term decidua basalis and term decidua parietalis was performed using flow cytometry and confirmed by immunohistochemistry. A higher percentage of CD56^{bright}CD16⁻ uterine NK cells was found in decidua parietalis in comparison to decidua basalis. In contrast, the percentage of CD56^{dim}CD16⁺ NK cells was significantly increased in decidua basalis. These results show that uterine NK cells account for a substantial proportion of leukocytes in term decidua parietalis, thus indicating that the presence of uterine NK cells, contrary to current belief, is not only restricted to early pregnancy.

The factors leading to the unequal distribution of NK cells in the decidua are unknown. A possible mechanism to explain the lower number of uterine NK cells in the decidua basalis may involve the classical and non-classical MHC class I molecules expressed in the placenta. HLA-C, HLA-E and HLA-G are expressed by the population of extravillous trophoblast that invade the uterus (26-30) and interact with receptors, such as CD94/NKG2A and killer-cell immunoglobulin-like receptors, expressed by uterine NK cells leading to activation or inhibition (31-35). The abundant presence of HLA-C, HLA-E and HLA-G in tissue adjacent to decidua basalis may account for the decreased percentage of uterine NK cells in that region compared to that of decidua parietalis.

Another mechanism to account for the higher number of CD56^{bright}CD16⁻ uterine NK cells in the decidua parietalis and the increased percentage of CD56^{dim}CD16⁺ in the decidua basalis may involve homing of CD56^{bright} cells from the maternal circulation to the decidua or may involve maturation from a uterine NK cell precursor. A recent study on trafficking of NK cells shows that cells from secondary lymphoid tissues of pregnant mice can reconstitute uterine NK cells in NK-cell-deficient mice and that human peripheral blood CD56^{bright} cells can adhere selectively to mouse uteri (36). Homing and recruitment of uterine NK cells to the maternal-fetal interface may be mediated by adhesion molecules (36-38), by molecules expressed by trophoblasts, such as monocyte inflammatory protein (39), and by molecules expressed by uterine NK cells, such as very late

antigen-1 or CD49a (40). There is perhaps increased homing of uterine NK cells to term decidua parietalis. Future studies are necessary to determine mechanisms involved in the regional difference of uterine NK cells in term decidua.

Although it is often stated that there is a progressive decrease in or even an absence of uterine NK cells in term decidua, current references that clearly illustrate such data are lacking. In general, studies on human term decidual leukocytes are limited. A few publications report substantial reductions or low percentages in CD56⁺ cells isolated from term decidua, depending on the tissue examined and the techniques used (42-44). However, these studies do not clearly distinguish CD56^{dim}CD16⁻ and CD56^{bright}CD16⁻ NK cell subsets, but rather simply evaluate CD56⁺ cells. In studies on term decidua basalis, the percentage of CD56⁺ cells, as determined by flow cytometry, was generally similar, ranging between 12% and approximately 24%, to that of the current study. In a study by Vince *et al.* (42), leukocytes in term decidua parietalis were analyzed. The percentage of CD56⁺ cells, however, was substantially lower compared to that of the current study. There are several important differences between the two studies that may account for this disparity, including the use of enzymatic digestion to isolate decidual cells, the CD56 monoclonal antibody specificity, the use of indirect immunofluorescence staining, and the method to calculate the percentage of positive cells.

The aim of the current study was to compare differences in decidua basalis and parietalis from term pregnancy. In a study by Haller *et al.* (45), decidua basalis and parietalis from first trimester pregnancy obtained under ultrasonic guidance was compared and analyzed by immunohistochemistry. The mean number of CD56⁺ cells was higher in first trimester decidua parietalis compared to early decidua basalis, analogous to findings of the present study. The difference, however, was not significant and may have been a result of the small sample size (n=6) or the gestational age of the decidua.

In the present study, a significantly increased percentage of CD56⁺CD16⁺ cells was found in decidua basalis in comparison to decidua parietalis. Although further phenotypic analysis is required, these cells are most likely granulocytes.

It is suggested, however, that the precise phenotype of the CD16⁺ cells in term decidua is unclear (43). By flow cytometric analysis, fewer CD16⁺ cells as compared to CD56⁺ cells were found in decidua basalis and parietalis. On the other hand, more CD16⁺ cells as compared to CD56⁺ cells were found by immunohistochemical analysis, similar to results reported by Vargas. Several studies indicate that trophoblasts in early and term placental tissue express CD16 (46-48). Recognizing the IgG Fc receptor III, CD16 is suggested to be involved in the transport of IgG across the placenta. Some CD16⁺ trophoblasts were possibly included in immunohistochemical quantification, but by flow cytometric analysis, these cells were excluded by gating. Furthermore, the difference also may be due to variation in monoclonal antibodies used for flow cytometry or immunohistochemistry.

Our findings also indicate that the percentages of CD45⁺ leukocytes, CD14⁺ macrophages, CD19⁺ B cells, CD3⁺ T cells and CD8⁺ cells are similar in decidua basalis and parietalis. While there are similar percentages of CD8⁺ cells in decidua basalis and parietalis, a significantly higher percentage of CD4⁺ cells is found in decidua basalis. Including both CD3⁺ and CD3⁻ cells, the CD8⁺ population likely includes both cytotoxic T cells and NK cells. Our study on CD3⁺ cells in term decidua further characterizes CD4 and CD8 expression on and the activation state of T cells (24). To confirm that our method for quantification of the various leukocyte subpopulations calculated within the CD45⁺ cell fraction was accurate, these percentages were also determined by concurrent three-color staining with CD45 in APC in combination with the various leukocyte markers in FITC and PE in two of the decidua basalis and parietalis samples and compared. Equivalent percentages of CD14⁺, CD19⁺, CD3⁺, CD4⁺, CD8⁺, CD16⁺ and CD56⁺ cells were obtained by both methods of flow cytometric staining and analysis (data not shown).

It is unlikely that contamination of decidual samples by maternal peripheral blood or umbilical cord blood accounts for the regional differences in the two decidual sites. Primarily, tissue samples were carefully collected and thoroughly washed with PBS prior to cell isolation. This is the described method to remove possibly contaminating maternal or fetal blood from the tissue (44). Secondly, flow cytometric results were confirmed by immunohistochemical analysis, in

which only cells located in the tissue and not in vessels were quantified. Using immunohistochemistry, differences between the decidua basalis and parietalis were similar to those found by flow cytometry. Additionally, the minor percentage of B cells found in both decidua basalis and parietalis and the lack of a significant difference between the percentages of B cells in the decidual sites suggests that circulating blood, which contains a higher percentage of B cells, did not appreciably contaminate decidual samples.

In summary, this study establishes that there are significant differences in the distribution of NK cells in term decidua basalis and decidua parietalis. Although decidual leukocytes have been well characterized, their role in the maintenance of pregnancy and in the development of obstetrical pathologies, such as fetal growth restriction and pre-eclampsia, remains unknown. To improve our understanding, future studies should focus on the mechanism involved in the regional distribution of NK cells and on the function of these cells in term decidua.

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CHAPTER 3

INCREASED T-CELL ACTIVATION IN DECIDUA PARIETALIS COMPARED TO DECIDUA BASALIS IN UNCOMPLICATED HUMAN TERM PREGNANCY

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ABSTRACT

PROBLEM: The aim of this study was to quantify and compare activated T cells in term decidua basalis and parietalis using flow cytometry.

METHOD OF STUDY: Term decidua basalis and parietalis samples were obtained from 20 placentas collected after elective caesarean section. Percentages of leukocyte subclasses within the CD45⁺ cell fraction and activated T cells were determined by flow cytometry.

RESULTS: There was no significant difference in CD45⁺, CD14⁺, CD19⁺, and CD3⁺ cell percentages. However, within the CD3⁺ population, there were significantly more TCR $\gamma\delta$ ⁺ and CD8⁺ cells in decidua parietalis compared to decidua basalis. More importantly, percentages of T cells expressing CD25, HLA-DR, CD45RO, and CD69 markers were significantly increased in decidua parietalis.

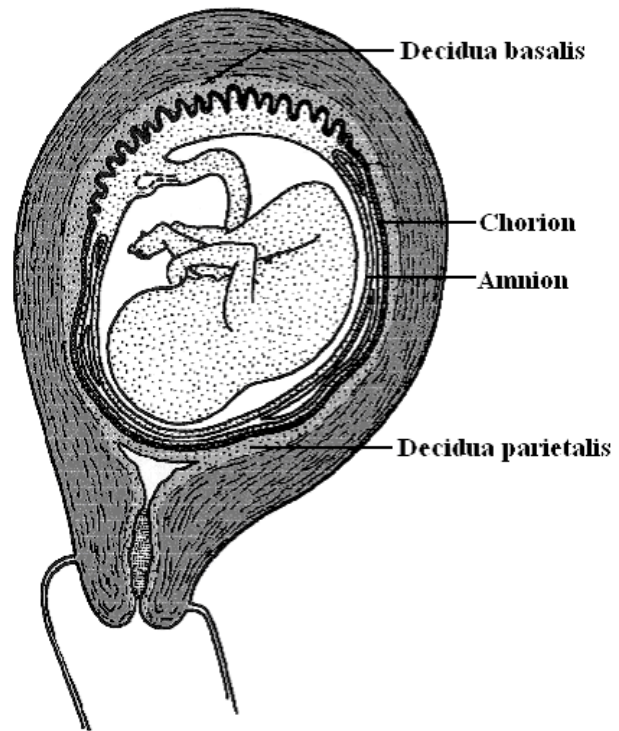
CONCLUSION: These findings suggest that there are more activated T cells in decidua parietalis than in decidua basalis. Further investigation into differences between the two decidual sites may expand our understanding of the immunology of the maternal-fetal interface.

INTRODUCTION

T cells, macrophages, and CD56⁺CD16⁻ decidual NK cells are the three primary leukocyte subclasses in non-pregnant and pregnant decidualized endometrium (1-8). In the early proliferative phase of the menstrual cycle, T cells are an abundant leukocyte population accounting for approximately 45% to 60% of the leukocytes in the endometrium (6, 9). As the endometrium prepares for the possible reception of a fertilized ovum during the secretory phase, the percentage of T cells progressively decreases (6, 9). An additional decline characterizes the first trimester transformation of endometrium into decidua, in which the total T-cell population represents only 6% to 30% of the decidual leukocytes (5, 6, 9-11). This progressive decline in T cells in early pregnancy suggests that T cells may not be critical to trophoblast invasion (12). However, in term pregnancy, 45% to 50% of the leukocytes in the decidua basalis, the site of trophoblast invasion, are CD3⁺, accounting for the most abundant leukocyte population (11, 13). Furthermore, it is also shown that T cells in term pregnancy decidua basalis express cell surface markers indicating activation, such as CD25, CD69, and HLA-DR (13). The reappearance of T cells during term pregnancy and their expression of activation markers imply that these cells play a more important role in the maintenance of pregnancy.

There are two distinct regions of direct contact between the maternal decidua and tissue of fetal origin – one between decidua basalis and the invading interstitial trophoblasts and the other between the tissue lining the remainder of uterine cavity, identified as the decidua parietalis, and the amniochorion (Figure 1). The decidua parietalis is the focus of far fewer studies as compared to the decidua basalis. Our earlier data revealed that there are, in fact, significant differences in the proportion of NK cell populations in the decidua basalis and parietalis. Our flow cytometric quantification of term decidual leukocytes showed a significantly increased percentage of CD56⁺CD16⁻ NK cells in the decidua parietalis as well as a significantly increased percentage of CD16⁺ cells, which included the CD56⁺CD16⁺ NK cell population, in the decidua basalis (14). To our knowledge, no study has examined the differential expression of activation markers in the decidua parietalis. The aim of this study was to quantify and compare the percentage of activated T cells in term decidua basalis and parietalis using flow cytometry.

Figure 1. Relationship of the fetus and the decidua basalis and parietalis after the fourth month of pregnancy (Adapted from Tuchmann-Duplessis H, David G, Haegel P: Illustrated Human Embryology. Vol 1. Paris, Masson and Company Paris, 1971, p.71).



MATERIALS AND METHODS

Isolation of decidua basalis and parietalis cells

Normal term decidua basalis and parietalis samples were obtained from 20 placentas collected after elective caesarean section following uncomplicated pregnancy. All caesarean sections were performed for breach presentation and resulted in the birth of a non-growth restricted healthy neonate. Term decidua basalis tissue was macroscopically dissected from the maternal-facing surface, and term decidua parietalis tissue was collected by removing the amnion and delicately scraping the tissue from the chorion. Areas of ischemia or necrosis were avoided. The obtained tissue was washed and finely minced between two scalpel blades in a small volume of phosphate buffered saline (PBS). Minced decidual fragments were gently worked through an open filter chamber (NPBI International BV; Emmer Compascuum, the Netherlands) with PBS. The resultant suspension was filtered through a 70 μ m sieve (Becton Dickinson Labware; Franklin Lakes, New Jersey), washed in PBS, and layered on an equal volume of ficoll hypaque (Leiden University Medical Center Pharmacy; Leiden,

the Netherlands) at room temperature for density gradient centrifugation (for 20min at 800g). Mononuclear cells were collected from the interface, washed twice, and resuspended in PBS for cell staining.

Cell Staining

Directly conjugated fluorescent antibodies directed against the following antigens were used for two- and three-color immunofluorescence staining: CD45 [HLe-1; Fluorescein isothiocyanate (FITC)], CD14 [Leu-M3; Phycoerythrin (PE)], CD3 [Leu-4; Allophycocyanin (APC) or Peridinin Chlorophyll-a Protein-Cytochrome 5.5 (PerCP-Cy5)], CD8 (Leu-2a; FITC), CD4 (Leu-3a; PE), CD19 (Leu-12; FITC), CD56 (Leu-19; FITC), CD16 (Leu-11a; PE), CD25 (Anti-IL-2R; FITC), HLA-DR (Anti-HLA-DR; PE), CD45RA (Leu-18; FITC), CD45RO (UCHL1; PE), CD69 (Leu-23; FITC), and TCR $\alpha\beta$ (WT31; FITC) all from Becton-Dickinson Immunocytometry Systems (San Jose, CA, USA) and TCR $\gamma\delta$ (5A6.E9; FITC, Serotec, Oxford, England). Sixteen to 20 of the decidual samples were analyzed for expression of all abovementioned except for CD69, which was only analyzed in 7 samples.

All cells were stained at densities of 10^6 cells/ ml in PBS and incubated for 30 minutes at 4°C in the dark. Following two washes with PBS, cells were resuspended and fixed with PBS containing 1% paraformaldehyde. Cells were then analyzed on a Becton-Dickinson FACScan flow cytometer. The double-labeled CD45 and CD14 sample was used to establish the optimal lymphocyte gate based on light scattering profile and immunofluorescence (15). Approximately 3,000 events were acquired in the gating window. The acquired data were then analyzed using CellQuest™ software (Becton-Dickinson, San Jose, CA, USA). The percentages of CD14⁺, CD19⁺, CD3⁺, CD56⁺CD16⁻, and CD16⁺ cells were calculated within and represented as a percentage of the CD45⁺ cell fraction. To determine the expression of activation markers on T cells, cells that were within the leukocyte gate and that were also positive for CD3 were gated based on these two parameters and analyzed for the expression of the activation marker of interest.

Effect of maternal peripheral blood

To confirm that maternal peripheral blood was not preferentially contaminating

the decidua basalis or parietalis samples or possibly affecting the results, a peripheral blood sample drawn from the mother prior to collection of four of the 20 placental samples was compared to its corresponding decidual basalis and parietalis samples. Peripheral blood mononuclear cells were isolated by density gradient centrifugation, stained at a density of 10^6 cells/ ml and analyzed for the expression of the same markers simultaneously with isolated decidual cells.

Statistical Analysis

To compare differences in decidual leukocyte subsets in the decidua basalis and decidua parietalis, the Wilcoxon signed rank test was performed. The conventional value of $p < 0.05$ was defined as significant.

RESULTS

The isolated leukocytes from normal term decidua basalis and parietalis were analyzed by flow cytometry and compared. The results of this comparison are summarized in Table 1. As established in our previous study (14), the percentage of decidual NK cells, defined as $CD56^+CD16^-$ cells, was significantly increased in the decidua parietalis in comparison with the decidua basalis, 28.2% and 11.0%, respectively ($p < 0.001$). On the other hand, the percentage of $CD16^+$ cells, which included $CD56^+CD16^+$ and $CD56^-CD16^+$ cells, was significantly increased in the decidua basalis in comparison with the decidua parietalis. There was no significant difference in the percentages of $CD45^+$, $CD14^+$, and $CD3^+$ cells in the decidua basalis and parietalis. In the present study, we also confirmed that there was no significant difference in the percentage of $CD19^+$ B cells in the two decidual sites. Furthermore, the $CD19^+$ cells in the decidua basalis and parietalis composed only a minor percentage of cells within the total $CD45^+$ fraction, 6.1% and 3.8%, respectively ($p = 0.057$). This data is summarized in Figure 2.

Although the percentage of $CD3^+$ T cells was similar in the decidua basalis and parietalis, there were slightly but significantly more $TCR\gamma\delta^+$ cells within the $CD3^+$ cell population in the decidua parietalis than in the decidua basalis, 13.9% and 8.2%, respectively ($p = 0.001$). Furthermore, the percentage of $CD3^+CD8^+$ cells was slightly but significantly greater in the decidua parietalis than in the decidua basalis, 43.2% and 53.8%, respectively ($p = 0.001$). Conversely, the

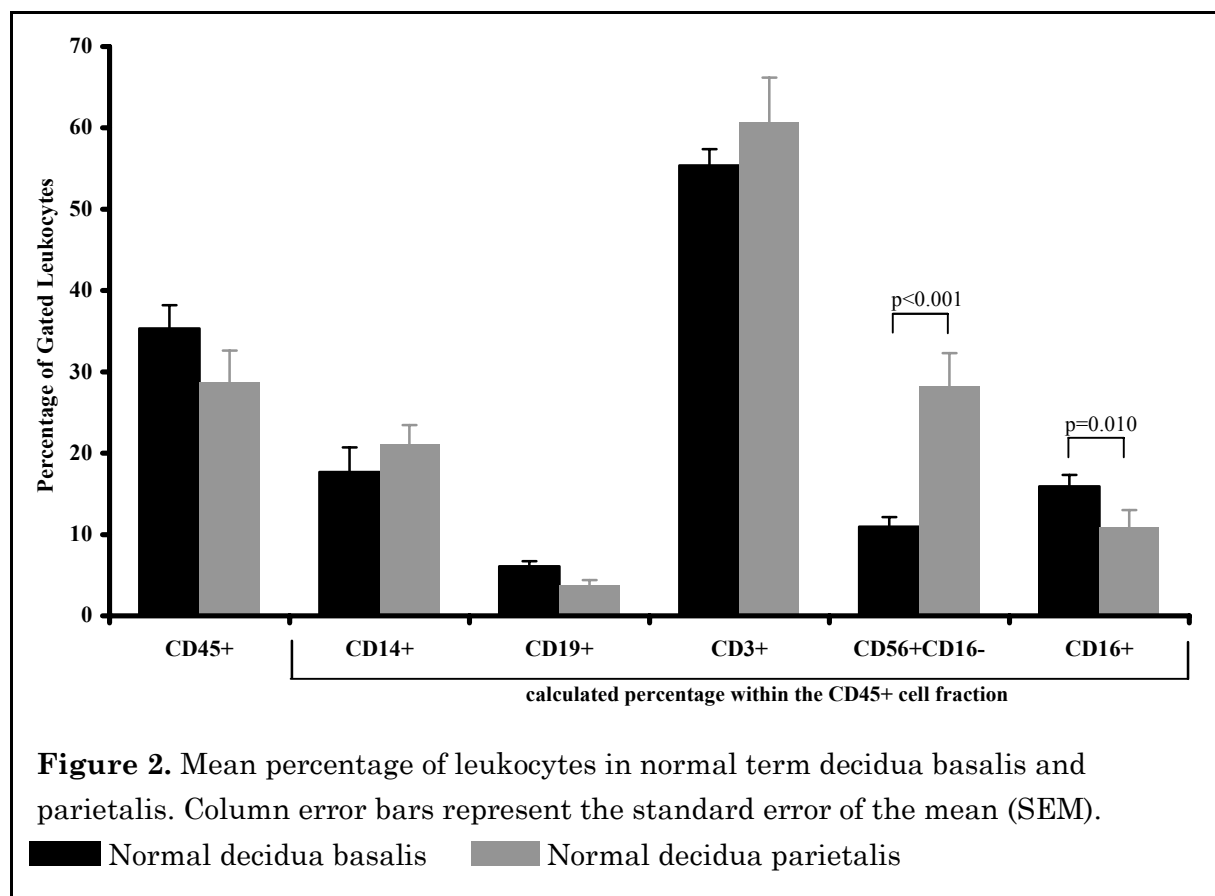
percentage of CD3⁺CD4⁺ cells was greater in the decidua basalis than in the parietalis, 52.6% and 42.2%, respectively (p=0.003). These results are graphically summarized in Figure 3.

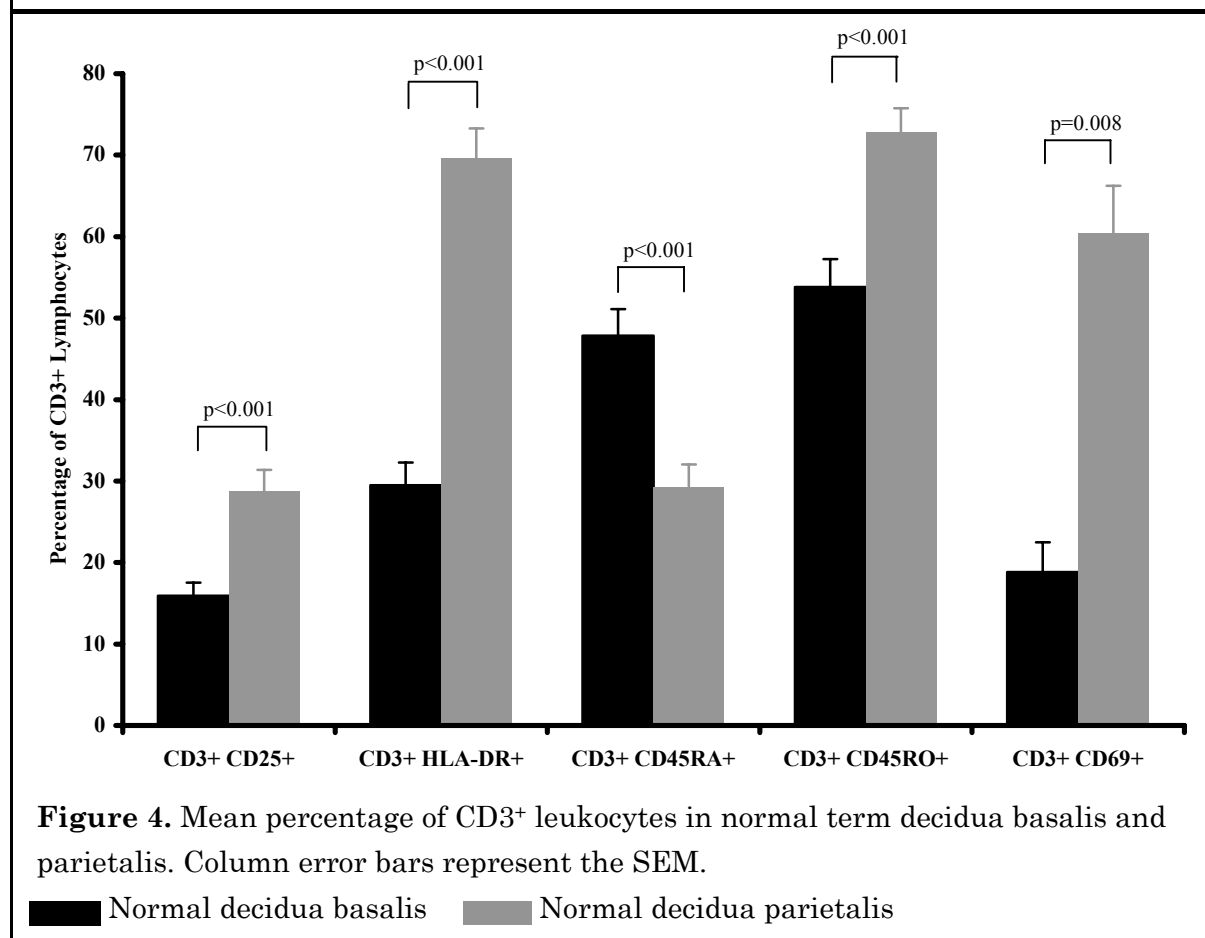
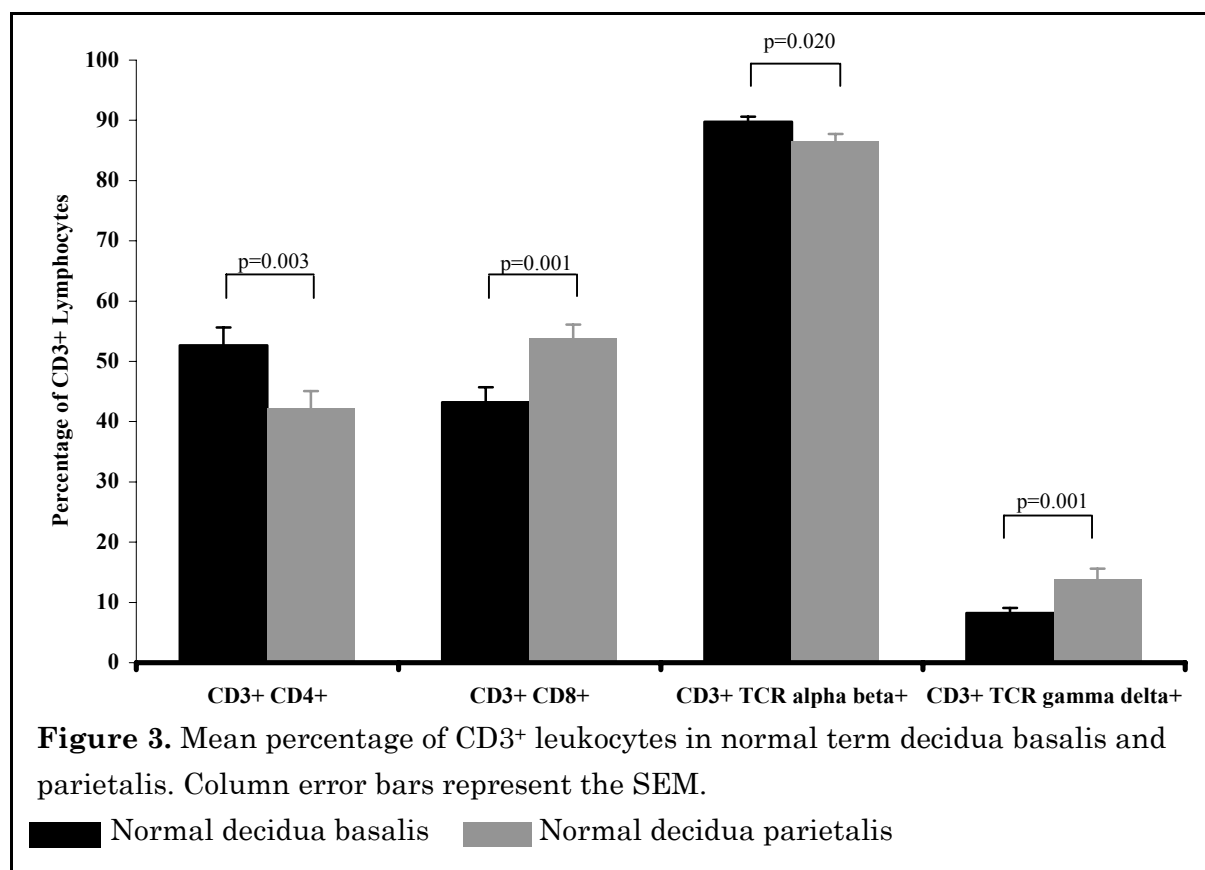
Table I. Mean percentage of leukocytes in normal term decidua basalis and parietalis.

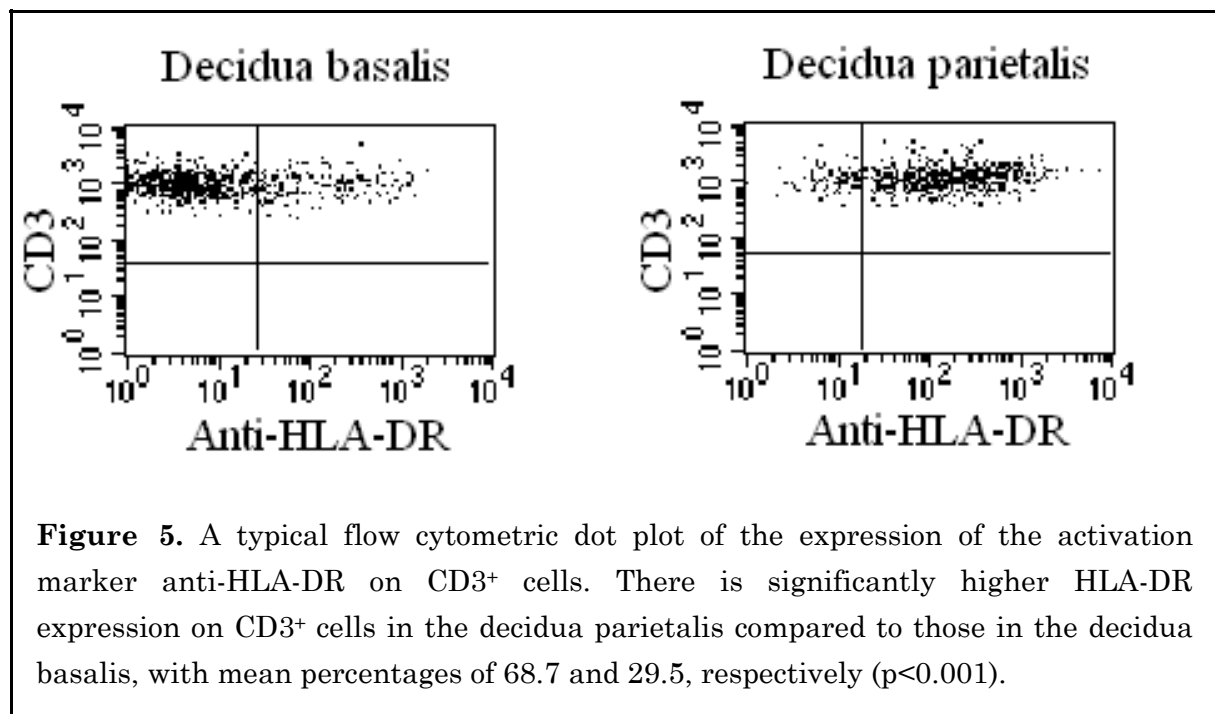
Leukocyte Markers	Term decidua basalis	Term decidua parietalis	p value
CD45⁺	35.3	28.8	0.103
CD14⁺/CD45⁺	17.7	21.0	0.301
CD3⁺/CD45⁺	55.4	60.6	0.195
CD19⁺/CD45⁺	6.1	3.8	0.057
CD16⁺/CD45⁺	15.9	10.9	0.010
Decidual NK cells CD56⁺CD16⁺/CD45⁺	11.0	28.2	<0.001
CD3⁺CD4⁺	52.6	42.2	0.003
CD3⁺CD8⁺	43.2	53.8	0.001
CD3⁺CD4⁺ / CD3⁺CD8⁺ ratio	1.4	0.9	0.001
CD3⁺TCR$\alpha\beta$⁺	89.8	86.4	0.020
CD3⁺TCR$\gamma\delta$⁺	8.2	13.9	0.001
CD3⁺TCR$\alpha\beta$⁺ / CD3⁺TCR$\gamma\delta$⁺ ratio	14.0	8.0	0.002
CD3⁺CD25⁺	15.9	28.7	<0.001
CD3⁺HLA-DR⁺	29.5	69.7	<0.001
CD3⁺CD45RA⁺	47.9	29.3	<0.001
CD3⁺CD45RO⁺	53.8	72.9	<0.001
CD3⁺CD45RA⁺ / CD3⁺CD45RO⁺ ratio	1.1	0.4	<0.001
CD3⁺CD69⁺	18.8	60.5	0.008

More remarkably, the percentage of activated T cells, expressing four different markers implying T-cell activation, CD25, HLA-DR, CD45RO, or CD69 within the CD3⁺ cell population, was consistently and significantly increased in the decidua parietalis when compared to that of the decidua basalis (Figure 4). An illustrative flow cytometric profile of the activation marker, HLA-DR, expressed on CD3⁺ cells is shown in Figure 5. Furthermore, the percentage of naïve T cells, as defined by co-expression of CD3 and CD45RA, was significantly greater in the decidua basalis than in the decidua parietalis, 47.9% and 29.3%, respectively ($p < 0.001$).

Results of the comparison between maternal peripheral blood and decidua basalis and parietalis samples did not suggest that peripheral blood was preferentially contaminating the decidua basalis.







DISCUSSION

Flow cytometric quantification and comparison of leukocytes of term decidual basalis and parietalis confirms that there are distributional differences between the two decidual sites. The significantly higher percentage of cells expressing CD25, CD69, HLA-DR, and CD45RO on CD3⁺ cells in the decidua parietalis indicates that T cells in this site are activated in term pregnancy. Cells in term decidua basalis also have been shown to express CD25, CD69, and HLA-DR activation markers (13). In the present study, we confirm the finding, but also extend the finding to show that there are more activated T cells in term decidua parietalis than in term decidua basalis.

The markers, CD25, CD69, HLA-DR, CD45RO, and CD45RA were applied in this study to evaluate T-cell activation. CD25, the alpha chain of interleukin-2 receptor, is an early universal T-cell marker that increases upon activation (16). CD69, another early activation marker, is present on activated T, B and NK cells (17). Although the function of CD69 is unknown, the induction of CD69 expression on T cells is shown to be very rapid (18, 19). HLA-DR is a relatively

late activation marker, expressed by B cells, monocytes, macrophages, and activated T and NK cells (20, 21). CD45RA, an isoform of the leukocyte common antigen, is expressed by naïve T lymphocytes, and upon activation, antigen density decreases (22). On the contrary, CD45RO is present at low density early in T lymphocyte maturation cycle. Upon activation, naïve T lymphocytes first acquire CD45RO and then lose CD45RA. CD45RO⁺ cells are considered to be a primed population of memory T cells (23-25). Thus, the phenotype of cells in the term decidua parietalis determined using these markers indicates that these cells are in an activated state.

Immunohistological and flow cytometric studies focusing on decidua of the first trimester have shown that various types of lymphocytes are activated and produce cytokines (26-35). Previous studies of decidua basalis samples from first trimester pregnancy demonstrate that T cells express CD25, CD69, HLA-DR, and CD45RO activation markers (29-31, 33-35). CD25 surface antigen was detected on 6 to 27% of first trimester decidual T cells. CD69 expression was found on 58 to 73% of these cells, and 32% to 70% expressed HLA-DR (29, 31, 34, 35). Approximately, 85% of CD4⁺ T cells and 59% of CD8⁺ T cells expressed CD45RO antigen (33). The percentages of activated T cells in first trimester decidua basalis measured in these studies appear to be higher than those found in term decidua basalis, and yet similar to those found in our term decidua parietalis, suggesting that the proportion of activated T cells declines in the decidua basalis during pregnancy. In general, immune activation appears paradoxical at a site in which immunosuppression should predominate in order to guarantee maternal tolerance toward the fetus. It is suggested, however, that maternal T cells promote and contribute positively to placental growth and trophoblast invasion (36). These studies imply that regionally activated T cells in the decidua basalis of early pregnancy may play an important role and that their balance with other leukocyte subsets may be essential to the maintenance of pregnancy.

Factors leading to the unequal distribution and activation of T cells in the decidua are unknown. Mediators, such as prolactin or indoleamine 2,3-dioxygenase (IDO), may be plausible candidates involved in the activation process. Prolactin, known to be produced in the decidualized human endometrium, transported through fetal membranes, and secreted into amniotic

fluid (37-39), induces cell proliferation and activation and is involved in the maintenance of immunocompetence (40, 41). Furthermore, prolactin receptors have been identified in the placenta and amnion (42). It is suggested, yet not fully clear, that there are regional and periodic differences in prolactin gene expression in the decidual cells during pregnancy (43). Perhaps there is increased prolactin expression in the decidua parietalis leading to T-cell activation. IDO, on the other hand, is synthesized and secreted by syncytiotrophoblasts and catabolizes and degrades tryptophan (44). Present in human placental villous explants, IDO has been shown to inhibit lymphocyte proliferation via tryptophan depletion (45-47). Increased IDO enzymatic levels or activity or lower levels of tryptophan in the decidual basalis are possible mechanisms for the detected differences in T-cell activation. In a study by Sedlmayr, IDO was irregularly localized in term placental samples to the mesenchymal core and found in isolated areas of the syncytiotrophoblast (48). Further investigation into prolactin, IDO, and tryptophan localization in the decidua basalis and parietalis seems warranted.

Activation by fetal trophoblasts followed by selective Fas mediated apoptosis is another plausible mechanism to explain the decreased percentage of activated T cells in the decidua basalis. Fas, a cell surface receptor of the tumor necrosis factor family (CD95), mediates apoptosis by binding FasL (49, 50). In a study by Hammer et al, apoptotic nuclei arising from CD45⁺ leukocytes were detected scattered within the whole functional stroma of the non-invaded decidua parietalis. In the decidua basalis, the apoptotic leukocytes were irregularly distributed and concentrated in the area around necrotic nuclei and especially in the primary fetal-maternal contact zone where trophoblasts from the cell columns began to invade the maternal tissue (51). In light of this study, our study suggests that there are less activated cells in the basalis because these cells have been activated and then selectively programmed to die by the invading trophoblasts. In the decidua parietalis, where there is less invasion by trophoblasts, but simply contact, the activated cells are not killed to the same extent by trophoblastic FasL. It is also plausible that there is perhaps a lower expression of Fas on T cells in the decidua parietalis or higher expression of FasL in the trophoblasts of the decidua.

Another conceivable explanation is that the T-cell activation is related to inflammation and infection in a fraction of the decidua parietalis samples. Ascending infection into the lower uterine segment can occur in term pregnancy prior to labor, and such an infection could potentially affect the decidua parietalis. However, the increased percentage of activated T cells in the decidua parietalis was consistently detected in every sample, not in just a few outlying samples, implying a highly improbable situation in which infection and inflammation was present in every pregnancy. Furthermore, the percentage of cells expressing CD16, a marker associated not only with classical NK cells but also with neutrophils and inflammation, was significantly increased in the decidua basalis and not the decidua parietalis. Therefore, it is highly unlikely that the significantly increased percentage of activated T cells in the decidua parietalis was due to an inflammatory response in a number of cases.

Additionally in this study, we confirm our previous findings that there are significantly increased CD56⁺CD16⁻ decidual NK cells in the decidual parietalis, while there are significantly increased CD16⁺ cells, which includes CD56⁺CD16⁺ and CD56⁻CD16⁺ populations, in the decidua basalis. We also confirm that there is no difference in CD14⁺, CD45⁺, and CD3⁺ cell populations in the two decidual sites.¹⁴ In this study, the finding is extended to show that similar percentages of CD19⁺ B cells are found in both decidual sites. The minor percentage of B cells and the lack of a significant difference between the percentages of B cells detected in the decidua basalis and parietalis also indicates that maternal peripheral blood, which has a substantially higher percentage of B cells, did not appreciably contaminate our samples.

The results of our present study also indicate that there are significantly more CD3⁺CD4⁺ cells in the decidua basalis than CD3⁺CD8⁺ cells, while the opposite is true for the decidua parietalis. The proportion of CD4⁺ and CD8⁺ T cells may reflect an inherent difference in activity of the two decidual sites, but both CD4⁺ and CD8⁺ T cells can be involved in a destructive immune response and in regulation. It is important to indicate that comparable to other previous studies (11-14), the total percentage of CD8⁺ cells, including those that are CD3⁺ and

CD3⁺, however, was higher than the total percentage of CD4⁺ cells in both the decidua basalis and parietalis (data not shown). Furthermore, in agreement with the study by Abadia-Molina *et al.*, CD3⁺ lymphocytes were the most abundant lymphocyte in the term decidua basalis (13). We showed that this finding also applies to the decidua parietalis.

In this study, slightly yet significantly more $\gamma\delta$ T cells were found in the decidua parietalis than in the decidua basalis. There has been considerable controversy concerning the expression of the $\gamma\delta$ T-cell receptor on decidual T cells. Although the function of these cells is not clearly identified, they may have a regulatory role in the decidua (52, 53). The study by Mincheva-Nilsson *et al.* suggests that the extrathymic T-cell maturation occurs in the decidua of early pregnancy and that CD56⁺CD16⁻ decidual NK cells are immature progenitors for T cells (54). In consideration of these studies, the significant increase in TCR $\gamma\delta$ ⁺ and CD56⁺CD16⁻ decidual NK cells further suggests the potential and unique immunologic role of these cells in the decidua parietalis.

The findings of this study extend our understanding of the many events occurring at the maternal-fetal interface and suggest that there may be particular inherent differences in the nature and function of the decidua basalis and decidua parietalis that control specific cells in these tissues.

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CHAPTER 4

COMPARISON OF DECIDUAL LEUKOCYTES FOLLOWING SPONTANEOUS VAGINAL DELIVERY AND ELECTIVE CESAREAN SECTION IN HUMAN TERM PREGNANCY

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ABSTRACT

The aim of this study was to quantify and compare leukocyte populations in term decidua basalis and parietalis obtained after spontaneous vaginal delivery (SVD) or elective cesarean section (CS) without labor. Decidua basalis and parietalis samples were obtained from placentas after SVD (n=20) and after CS (n=30). Following mechanical disaggregation, leukocytes were purified and stained with monoclonal antibodies. Percentages of leukocyte subclasses within the CD45⁺ cell fraction and activated T cells were determined by flow cytometry. No differences were found in the percentages of CD45⁺ cells or CD56^{bright}CD16⁺ uterine natural killer (NK) cells between decidua basalis from SVD and CS or between decidua parietalis from SVD and CS. In decidua basalis and parietalis from SVD, a significantly higher number of CD56^{dim}CD16⁺ NK cells was found compared to CS. In decidua basalis from SVD, there was a significantly lower percentage of CD14⁺ cells and higher percentage of CD19⁺ cells compared to CS. The percentage of CD3⁺T cells expressing CD25 or human leukocyte antigen (HLA)-DR was significantly decreased in decidua basalis and parietalis from SVD compared to CS. Comparison of decidua collected after SVD or CS suggests that labor is associated with dynamic changes in the distribution of decidual leukocytes, specifically NK and T cell subpopulations. In particular, the disappearance of the CD4⁺CD25⁺ T cell population, which possibly contains a subpopulation of regulatory T cells, may contribute to the initiation of labor. Further investigation into factors affecting decidual leukocytes may expand our understanding of the immunological events at the maternal-fetal interface.

INTRODUCTION

In the late secretory phase of the menstrual cycle, the mucosal lining of the uterus transforms from the endometrium into the decidua in preparation for pregnancy. During this hormone-dependent process, endometrial glands enlarge, spiral arteries become tortuous, and stromal cells differentiate into large glycogen-filled decidual cells to support placental implantation and growth (1, 2). Importantly, leukocytes become a prominent cell population in the decidua, including CD56^{bright}CD16⁻ uterine natural killer (NK) cells, macrophages and T cells (3-5). The cellular composition of the decidua, however, is dynamic and changes in proportion and distribution during the menstrual cycle and throughout pregnancy. (4, 6-14). Serving as an immunologically specialized tissue, the decidua and its components carry out multiple functions and play an essential role in implantation and in the maintenance of pregnancy (1, 15). Decidual leukocytes may contribute both directly and indirectly by expressing receptors that potentially mediate fetal trophoblast recognition and invasion and by producing cytokines that modulate and regulate maternal immune and vascular function (16, 17).

Upon implantation, the decidua is in direct contact with fetal extraembryonic tissue, thus establishing the maternal-fetal interface. By term pregnancy, there are two distinct regions of the maternal-fetal interface: one in the placenta between the decidua basalis and the invading interstitial trophoblasts, and the other at the fetal membranes between the decidua parietalis and the amniochorion. While direct cell-to-cell contact between decidua basalis and fetal tissue occurs just six days after fertilization, direct contact between decidua parietalis and fetal tissue occurs only in the second trimester of pregnancy as fetal growth obliterates the chorionic and uterine cavities (1). Our recent studies on decidua collected after elective cesarean section revealed that there are significant differences between term decidua basalis and decidua parietalis. Flow cytometric quantification of decidual leukocyte subpopulations showed a significantly higher percentage of CD56^{bright}CD16⁻ NK cells and of CD3⁺ T cells expressing activation markers CD25, human leukocyte antigen (HLA)-DR, CD45RO, or CD69 in the decidua parietalis in comparison to the decidua basalis indicative for a differential immunoregulation in these two regions of the

maternal-fetal interface (18, 19).

Spontaneous labor at term is associated with specific changes in the distribution and proportion of leukocyte subpopulations and in various immunologic mediators found in gestational tissues, including cervix, myometrium, and decidua, and also in umbilical cord blood and maternal peripheral blood when compared to cesarean section without labor (20-35). The changes suggest that the stress of labor possibly has an important effect on immunoregulatory processes in gestational tissues. The aim of this study, therefore, was to determine the effect of labor on decidual leukocytes by quantifying decidual leukocyte subpopulations and the activation state of T cells in term decidua basalis and decidua parietalis obtained from spontaneous vaginal delivery (SVD) and comparing the results with those of elective cesarean section (CS).

MATERIALS AND METHODS

Subjects

The study population consisted of 50 pregnant women from the Leiden University Medical Center in Leiden, the Netherlands undergoing delivery in a one and a half year period between October 2001 and April 2003. Paired normal term decidua basalis and parietalis samples were obtained from two groups of term deliveries: term SVD (gestational age range: 38-42.1 weeks; maternal age range: 20.5-40.4 years; n=20) and term elective CS before the onset of labor indicated for breech presentation (gestational age range: 38-41.6 weeks; maternal age range: 21.4-42 years; n=30). All women in the CS group received combined spinal epidural anesthesia prior to the procedure. Although such anesthesia can cause minimal changes in systemic maternal blood pressure, a significant effect caused by a difference in the use of anesthesia on decidual leukocytes is unlikely due to, firstly its regional use and secondly to the low dose of the anesthetic used. No general anesthesia was administered to this group. The incidence of the use of epidurals in the SVD group is 15%. Some samples from the CS study group were used in previous publications (18, 19). Women in which a caesarean was performed after having labour were excluded from the study as the proportion of lymphocyte subsets in decidua obtained could have

potentially affected by other factors as prolonged duration of labor or fetal distress, for which we could not control. All deliveries followed uncomplicated pregnancies and resulted in the birth of a non-growth restricted healthy neonate. Informed consent was obtained from all women, and the study received the appropriate ethical approval by the Leiden University Medical Ethics Committee.

Isolation of decidua basalis and parietalis cells

Term decidua basalis tissue was macroscopically dissected from the central region of the maternal-facing surface, and term decidua parietalis tissue was collected by removing the amnion and delicately scraping the tissue from the chorion. Areas of ischemia or necrosis were avoided. The obtained tissue was thoroughly washed and finely minced between two scalpel blades in phosphate buffered saline (PBS). Minced decidual fragments were gently worked through an open filter chamber (NPBI International BV, Emmer-Compascuum, the Netherlands, and Beldico, Marche-en-Famenne, Belgium) with PBS. The resultant suspension was filtered through a 70µm sieve (Becton Dickinson, Labware; Franklin Lakes, New Jersey), washed in PBS, and layered on an equal volume of ficoll hypaque (Leiden University Medical Center Pharmacy; Leiden, the Netherlands) at room temperature for density gradient centrifugation (for 20 minutes at 800g). Mononuclear cells were collected from the interface, washed twice, and resuspended in PBS for cell staining.

Cell Staining

Directly conjugated fluorescent antibodies directed against the following antigens were used for two- and three-color immunofluorescence staining: CD45 [HLe-1; Fluorescein isothiocyanate (FITC)], CD14 [Leu-M3; Phycoerythrin (PE)], CD3 [Leu-4; Allophycocyanin (APC) or Peridinin Chlorophyll-a Protein-Cytochrome 5.5 (PerCP-Cy5)], CD8 (Leu-2a; FITC), CD4 (Leu-3a; PE), CD19 (Leu-12; FITC), CD56 (Leu-19; FITC, Leu-19; PE), CD16 (Leu-11a; FITC, Leu-11c; PE), CD25 (Anti-IL-2R; FITC), HLA-DR (Anti-HLA-DR; PE), CD45RA (Leu-18; FITC), CD45RO (UCHL1; PE), CD69 (Leu-23; FITC), and TCRαβ (WT31; FITC) all from Becton-Dickinson Immunocytometry Systems (San Jose, CA, USA) and TCRγδ (5A6.E9; FITC, Serotec, Oxford, England and Biocarta,

Hamburg, Germany).

Cells were stained with monoclonal antibodies at a density of 10^6 cells/ ml in PBS (100 μ l/ sample) and incubated for 30 minutes at 4°C in the dark. Following two washes with PBS, cells were resuspended and fixed with PBS containing 1% paraformaldehyde. Cells were then analyzed on a Becton-Dickinson FACScan flow cytometer. The double-labeled CD45 and CD14 sample was used to establish the optimal lymphocyte gate based on the light scattering profile and immunofluorescence (36). Although a leukocyte gate was established, the percentage of CD45⁺ cells was often not high in this gate presumably due to red blood cells or dead cells that were also present in this gate. The purpose of this gate was simply to better define the leukocyte population and to exclude most of the cells with high forward and side scatter from the flow cytometric analysis. Approximately 5,000 events were acquired in the gating window. The acquired data were then analyzed using Becton-Dickinson CellQuest™ software (San Jose, CA, USA). The percentages of CD14⁺, CD19⁺ and CD3⁺ cells were calculated within and represented as a percentage of the CD45⁺ cell fraction. To characterize the expression of CD16 and CD56, the percentages of CD56⁺CD16⁺ cells, CD56^{dim}CD16⁺ NK cells and CD56^{bright}CD16⁻ uterine NK cells were established by delineating regions around these various populations. To determine the expression of activation markers on T cells, cells that were within the leukocyte gate and that were also positive for CD3 were gated based on these two parameters and analyzed for the expression of the activation marker of interest. For 10 samples in each study group, the co-expression of CD4 or CD8 on CD3⁺CD25⁺ and CD3⁺HLA-DR⁺ cells was analyzed using three-color immunofluorescence.

Statistical Analysis

The Mann-Whitney test, a non-parametric non-paired t-test, was performed to identify differences in decidual leukocyte subsets between the two study groups. The two-tailed p value of $p < 0.05$ was defined as significant.

RESULTS

Leukocytes subpopulations in term decidua basalis and parietalis after SVD or CS

Leukocytes isolated from normal term decidua basalis and parietalis obtained following SVD and CS were analyzed by flow cytometry. No significant differences were found between CD45⁺ cell percentages in decidua basalis or decidua parietalis from SVD and CS. Specific subpopulations of leukocytes, however, significantly differed within the CD45⁺ cell fraction. In decidua basalis from SVD, there was a significantly decreased percentage of CD14⁺ macrophages and increased percentage of CD19⁺ B cells in comparison to decidua basalis from CS (Fig 1a). In decidua parietalis from SVD, the percentage of CD14⁺ cells was also significantly decreased in comparison to decidua parietalis from CS, while there was no significant difference in the percentage of CD19⁺ cells in the decidua parietalis from SVD and CS (Fig. 1b).

CD56 and CD16 expression on term decidua basalis and parietalis leukocytes after SVD or CS

In decidua parietalis from SVD and CS, there were similarly high percentages of CD56^{bright}CD16⁻ uterine NK cells (17.6% and 22.1%), while in decidua basalis from SVD and CS, there were equally low percentages of uterine NK cells (4.5% and 5.6%). The differential distribution of CD56^{bright}CD16⁻ uterine NK cells in decidua parietalis and basalis was not affected by labor. No significant differences were found in the percentage of CD56⁻CD16⁺ cells in decidua basalis from SVD and CS or decidua parietalis from SVD and CS. In contrast, decidua basalis and decidua parietalis from SVD contained significantly higher percentages of CD56^{dim}CD16⁺ NK cells (13.8% and 10.4%) respectively compared to decidua basalis (9.8%; $p=0.0415$) and decidua parietalis from CS (5.3%; $p=0.0203$) (Fig. 1a; Fig. 1b).

T cells in term decidua basalis and parietalis after SVD or CS

Within the CD3⁺ T cell population (Table 1), there were no significant differences in the percentages of CD4⁺, CD8⁺, TCR $\alpha\beta$ ⁺ and TCR $\gamma\delta$ ⁺ cells in decidua basalis from SVD and CS. In decidua parietalis from SVD, there was a

significant increase in the percentage of CD3⁺CD4⁺ cells and a significant decrease in the percentage of CD3⁺CD8⁺ cells in comparison to that of decidua parietalis from CS ($p=0.0066$ and $p=0.0420$, respectively). T cells expressing activation markers, CD25, HLA-DR, CD45RO and CD69, and expressing a naïve T cell marker, CD45RA, were determined in decidua basalis and parietalis from SVD and CS. There were no significant differences in the percentages of CD3⁺CD45RA⁺, CD3⁺CD45RO⁺ and CD3⁺CD69⁺ cells when comparing decidua basalis from SVD and CS and decidua parietalis from SVD and CS. Importantly, the percentage of CD3⁺ cells expressing the markers of activation, CD25 and HLA-DR, was significantly lower in the decidua basalis and parietalis from SVD when compared to decidua basalis and parietalis from CS (Fig. 2a; Fig. 2b).

CD3⁺CD25⁺ and CD3⁺HLA-DR⁺ cells in term decidua basalis and parietalis after SVD or CS

To characterize the CD3⁺ T cells expressing CD25 and HLA-DR, these populations were analyzed for their expression of CD4 and CD8 using three-color immunofluorescence. The majority of CD3⁺CD25⁺ cells in decidua basalis and parietalis from SVD and CS expressed CD4, while the generally more CD3⁺HLA-DR⁺ cells in decidua basalis and parietalis from SVD and CS expressed CD8. Notably, the lower number of CD3⁺CD25⁺ cells in decidua from SVD was due to a lower number of CD3⁺CD4⁺CD25⁺ cells, while the lower number of CD3⁺HLA-DR⁺ cells was due to a lower number of both CD3⁺CD4⁺HLA-DR⁺ cells and CD3⁺CD8⁺HLA-DR⁺ cells (Fig. 2a; Fig. 2b).

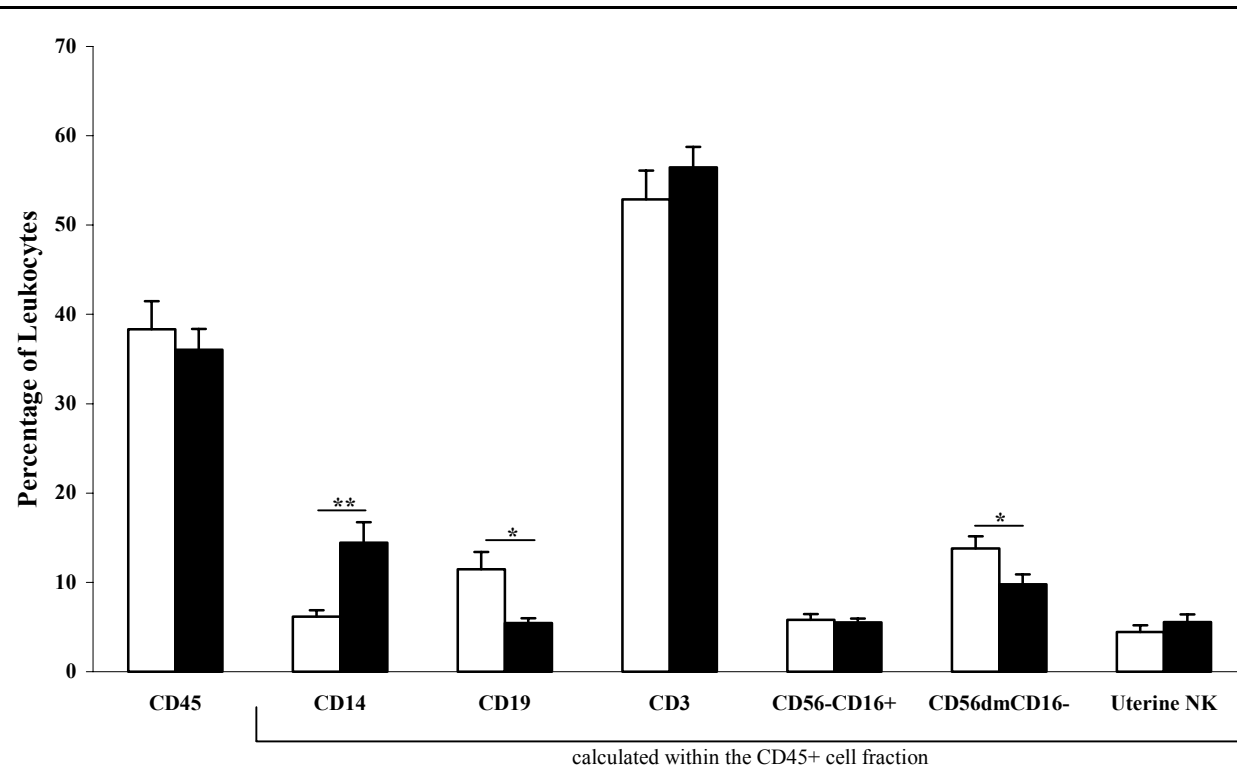


Figure 1a. Mean percentage of leukocytes in normal term decidua basalis collected from spontaneous vaginal delivery (SVD) or elective cesarean section (CS). Column error bars represent the standard error of the mean (SEM). * $p < 0.05$, ** $p < 0.005$, Mann-Whitney test. Decidua basalis from SVD Decidua basalis from CS

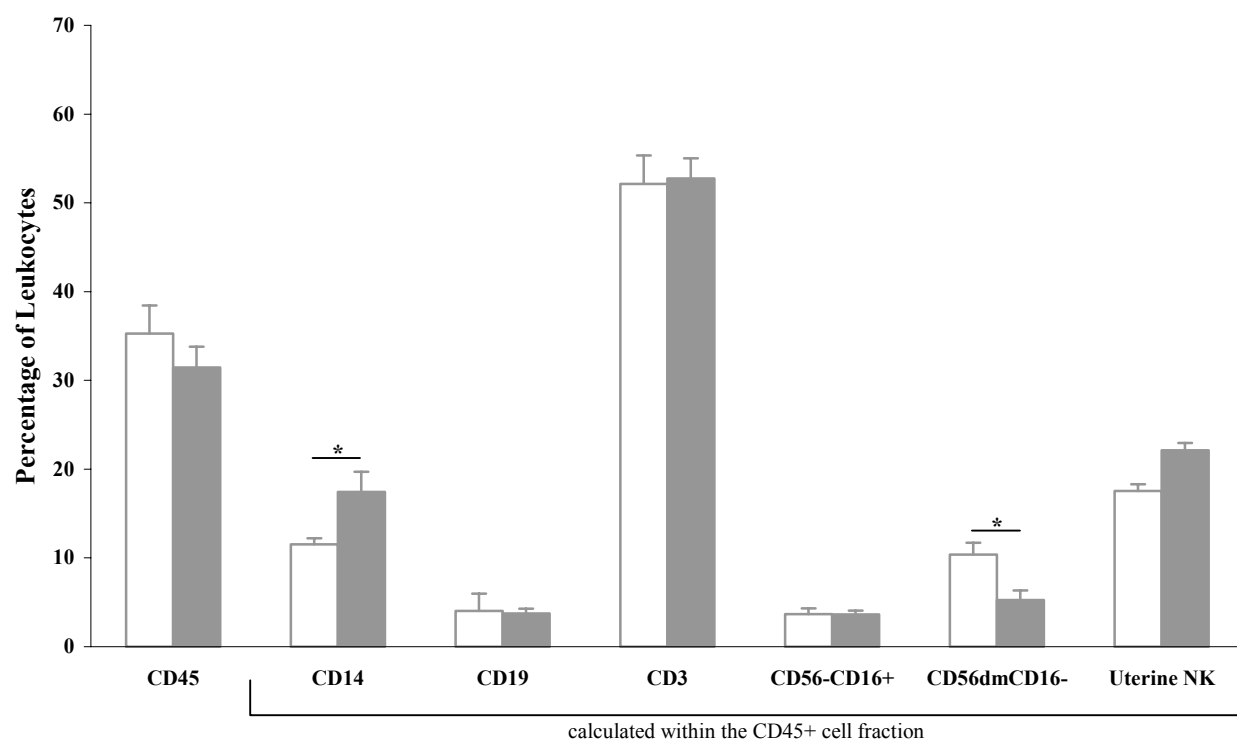


Figure 1b. Mean percentage of leukocytes in normal term decidua parietalis collected from SVD or CS. Column error bars represent the SEM. * $p < 0.05$, ** $p < 0.005$, Mann-Whitney test. Decidua parietalis from SVD Decidua parietalis from CS

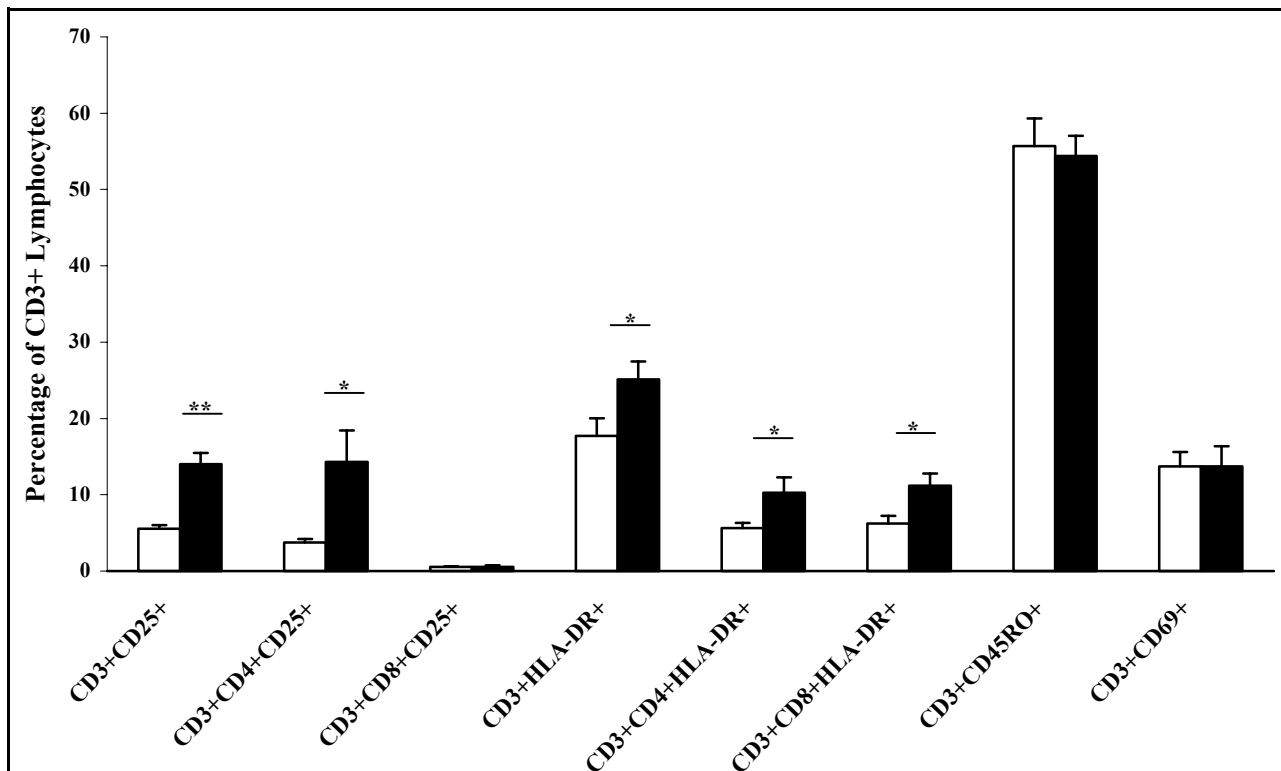


Figure 2a. Mean percentage of CD3⁺ lymphocytes expressing activation markers, CD25, HLA-DR, CD45RO and CD69 in normal term decidua basalis collected from SVD or CS. Column error bars represent the SEM. * p<0.05, ** p<0.005, Mann-Whitney test. Decidua basalis from SVD Decidua basalis from CS

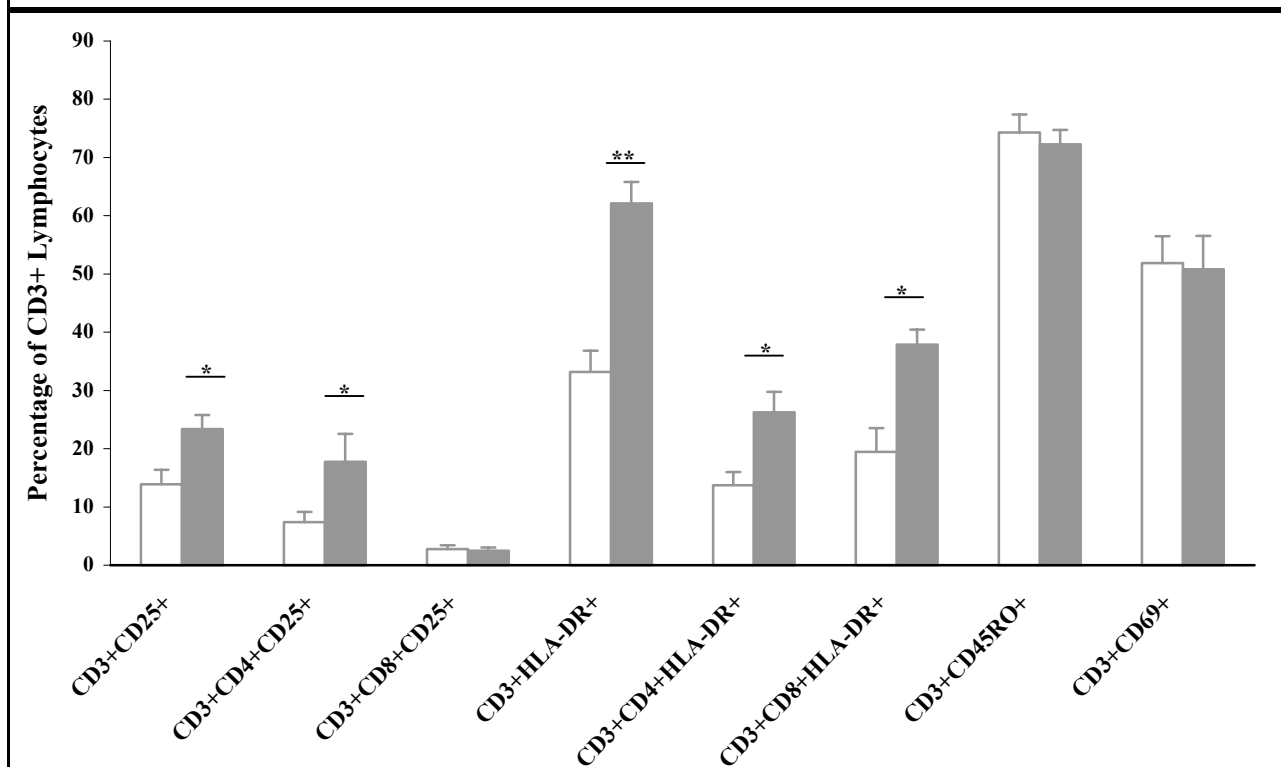


Figure 2b. Mean percentage of CD3⁺ lymphocytes expressing activation markers, CD25, HLA-DR, CD45RO and CD69 in normal term decidua parietalis collected from SVD or CS. Column error bars represent the SEM. * p<0.05, ** p<0.005, Mann-Whitney test. Decidua parietalis from SVD Decidua parietalis from CS

Table 1. Comparison of the mean percentage of CD3⁺ lymphocytes expressing various T cell markers in normal term decidua basalis or decidua parietalis between spontaneous vaginal delivery (SVD) and elective cesarean section (CS) determined by flow cytometry.

Leukocyte Markers	SVD Decidua basalis (n=20)	CS Decidua basalis (n=30)	Significance ^a	SVD Decidua parietalis (n=20)	CS Decidua parietalis (n=30)	Significance ^a
CD3 ⁺ CD4 ⁺	53.9	52.5	NS	52.5	44.1	p=0.0066
CD3 ⁺ CD8 ⁺	46.4	43.3	NS	48.3	52.8	p=0.0420
CD3 ⁺ TCR- $\alpha\beta$ ⁺	89.8	90.2	NS	90.4	87.2	NS
CD3 ⁺ TCR- $\gamma\delta$ ⁺	8.8	7.6	NS	9.9	12.5	NS
CD3 ⁺ CD25 ⁺	5.5	14.0	p<0.0001	13.9	23.4	p=0.0123
CD3 ⁺ CD4 ⁺ CD25 ⁺	3.7	14.3	p=0.0260	7.4	17.7	p=0.0303
CD3 ⁺ CD8 ⁺ CD25 ⁺	0.6	0.6	NS	2.8	2.5	NS
CD3 ⁺ HLA-DR ⁺	17.7	25.1	p=0.0367	33.2	62.1	p<0.0001
CD3 ⁺ CD4 ⁺ HLA-DR ⁺	5.6	10.3	p=0.0400	13.7	26.2	p=0.0193
CD3 ⁺ CD8 ⁺ HLA-DR ⁺	6.2	11.2	p=0.0303	19.5	37.9	p=0.0101
CD3 ⁺ CD45RA ⁺	38.7	44.4	NS	24.4	30.1	NS
CD3 ⁺ CD45RO ⁺	55.7	54.4	NS	74.3	72.3	NS
CD3 ⁺ CD69 ⁺	13.7	13.7	NS	51.9	50.8	NS

^aMann-Whitney test

DISCUSSION

Comparison of decidua collected after SVD and CS suggests that labor is associated with significant differences in the distribution and proportion of specific subpopulations within the CD45⁺ cell fraction, including CD14⁺ macrophages, CD19⁺ B cells, CD56^{dim}CD16⁺ NK cells, CD3⁺CD4⁺ cells, CD3⁺CD8⁺ cells and CD3⁺ T cells expressing CD25 or HLA-DR.

Spontaneous labor at term can be described as a cascade of events that is associated with specific changes in leukocyte subpopulations, cell adhesion molecule expression and various immunologic mediators. In umbilical cord blood, neutrophils, monocytes, and NK cells are significantly increased following labor when compared to that after CS, while T cells are significantly decreased (33, 37). Enumeration of leukocyte subpopulations in maternal peripheral blood shows an increase in the total white blood cell count following labor (22). In the cervix, leukocytes, predominantly macrophages and neutrophils infiltrate concurrent with the process of cervical ripening and labor (20, 24, 29). In the myometrium, there is similarly a massive influx of macrophages and neutrophils as well as T cells in the lower uterine segment at the onset of labor at term (34). In the current study, it is established that the percentages of macrophages and CD56^{dim}CD16⁺ NK cells increase and the percentages of CD3⁺CD4⁺CD25⁺ cells and CD3⁺CD4⁺HLA-DR⁺ and CD3⁺CD8⁺HLA-DR⁺ cells decrease in the decidua basalis and parietalis after SVD.

A possible mechanism to account for the changes in decidual leukocytes following labor may involve migration of these cells to or from other tissue sites, such as to the cervix or myometrium or from peripheral blood. The decreased percentage of macrophages found in the decidua basalis and parietalis following labor in the current study may account for the presence of these cells in the cervix or myometrium. It has been suggested that cell adhesion molecules are involved in the control of leukocyte infiltration in the cervix and myometrium during late pregnancy, cervical dilation and labor (34, 38, 39). Cytokines such as Interleukin (IL)-1b, IL-6, IL-8, and Tumor Necrosis Factor- α are expressed in various gestational tissue sites and increase following spontaneous labor (29, 35). The increased percentage of CD56^{dim}CD16⁺ NK cells found in the decidua

basalis and parietalis following labor may be mediated by specific signaling mediators, such as cytokines or chemokines. CD56^{dim}CD16⁺ NK cells express chemokine receptors CXCR1, CXCR2, CXCR3, CXCR4 and CX3CR1 (40). It is plausible that these cells are recruited from the peripheral blood where they represent the major NK cell subclass to the decidua during labor by chemokines. In the current study, there was a significantly higher percentage of CD19⁺ B cells in decidua basalis from SVD compared to that from CS. B cells, however, are virtually absent or found in very low percentages in decidua basalis and parietalis from term pregnancy following CS (6, 13, 14, 18). It is possible that the process of labor preferentially could attract and activate B cells in the decidua basalis.

In several previous studies, placentas from uncomplicated pregnancies were examined for acute inflammation in the chorion-decidua parietalis by analyzing and grading histologic samples for the presence of polymorphonuclear leukocytes. Following the onset of labor, there was, in fact, a significant increase in polymorphonuclear leukocytes (26, 31). It is difficult, however, to clearly define leukocyte subpopulations by routine histology. In a study by Osman *et al.* (29), several leukocyte subpopulations in decidua parietalis were quantified and compared before and after labor using immunohistochemistry. Histological analysis demonstrated that the median density of CD45⁺ leukocytes, CD68⁺ macrophages, neutrophils, CD3⁺ T cells, CD20⁺ B cells was similar in decidua parietalis following CS without labor and CS with labor. Our findings in decidua parietalis are in general agreement with those of Osman *et al.* (29). We extend the findings by quantifying NK and T-cell subpopulations in decidua basalis and parietalis. Contrary to the study of Osman *et al.* (29), a decreased percentage of CD14⁺ in decidua parietalis was found following labor in the current study, the small disparity between the two studies could be due to differences in the labor study group (CS with labor and SVD), the antibody used to identify macrophages (CD68 and CD14), or the method of leukocyte quantification (immunohistochemistry and flow cytometry).

Our previous studies on decidua collected after elective CS revealed that there are significant differences between term decidua basalis and decidua parietalis,

including a higher percentage of CD56^{bright}CD16⁻ NK cells and CD3⁺CD25⁺ cells in the decidua parietalis (18, 19). The current study extends our previous findings to establish that decidua parietalis contains a significantly higher percentage of CD56^{bright}CD16⁻ uterine NK cells regardless of the effect of labor. Importantly, the majority of CD3⁺CD25⁺ cells in decidua basalis and parietalis express CD4 and may represent a small population of regulatory T cells. Regulatory T cells are a unique cell population able to control the immune responsiveness to alloantigens via cell-to-cell contact and are essential for the active suppression of autoimmunity and for transplantation tolerance (41, 42). Recent studies have demonstrated that CD4⁺CD25⁺ regulatory T cells express CD25 at higher levels and more persistently than activated T cells (43, 44). These CD4⁺CD25^{bright} regulatory T cells are capable of inhibiting proliferation and cytokine secretion induced by TCR cross-linking of CD4⁺CD25⁻ responder T cells in a contact-dependent manner (44). In a pilot study, we tried to distinguish CD4⁺CD25⁺ regulatory T cells from CD4⁺CD25⁺ activated T cells by the level of CD25 expression. These preliminary experiments showed a higher percentage of CD4⁺CD25^{bright} T cells in the CD4⁺CD25⁺ T cell population in the decidua parietalis in comparison to the decidua basalis. A reduction of this population of CD4⁺CD25^{bright} T cells was observed when SVD samples were compared to CS samples. While regulatory T cells have not been described in the decidua, they could potentially serve as mediators in maternal tolerance of the fetal compartment by potently suppressing the activation and proliferation of CD4⁺CD25⁻ T cells and also CD8⁺ T cells. The decrease or disappearance of regulatory T cells from the decidua potentially enables the activated alloreactive CD4⁺CD25⁻ and CD8⁺ T cells to reach their effector status thus initiating the process of labor and delivery. To characterize these cells as regulatory T cells, determination of their expression of Foxp3, a unique and critical regulator in regulatory T cell development and function (45), or their expression of GITR, a member of the Tumor Necrosis Factor receptor superfamily that plays a key role in immunological self-tolerance (46, 47), is necessary for future studies.

Identification and comparison of the effects of labor on leukocytes in the decidua basalis and parietalis suggests that labor significantly affects the distribution and proportion of certain leukocyte subpopulations. Further investigation into

the nature and function and into the regulation of these leukocytes at the maternal-fetal interface is necessary to understand the events occurring during spontaneous labor and in the maintenance of pregnancy.

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CHAPTER 5

MATERNAL DECIDUAL LEUKOCYTES PROLIFERATE IN RESPONSE TO FETAL UMBILICAL CORD BLOOD AND ALLOGENEIC BLOOD LEUKOCYTES IN UNCOMPLICATED HUMAN TERM PREGNANCY

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ABSTRACT

In term placenta, contact between maternal and fetal tissue occurs between decidua basalis and invading trophoblasts and between decidua parietalis and amniochorion. In earlier studies, we found a higher percentage of uterine NK cells and activated T cells in decidua parietalis compared to decidua basalis. Our aim was to study the capacity of these maternal cells to produce an alloimmune response to the fetus. Samples of decidua basalis and parietalis, maternal peripheral blood (PB), and umbilical cord blood (UCB) were obtained from 10 maternal-fetal pairs after elective cesarean section. Allogeneic PB was obtained from HLA-typed volunteers. Purified responder leukocyte populations were co-cultured for 4 days in a mixed lymphocyte culture (MLC) with autologous maternal serum and irradiated stimulators including UCB, maternal, and allogeneic PB leukocytes (PBL). Furthermore, the cytokine production induced by this interaction with allogeneic cells was measured. A proliferative alloimmune response was observed in term decidual leukocytes both against UCB and allogeneic cells as stimulators. This proliferation was associated with the production of IFN- γ and IL-2. We conclude that maternal leukocytes present in term decidua basalis and parietalis are capable of producing an alloimmune response to UCB including the production of T helper 1 cytokines.

INTRODUCTION

The mechanism by which the fetus avoids rejection and by which the functionally intact maternal immune system tolerates the fetus is the central question in reproductive immunology posed over fifty years ago by Medawar and Billingham (1, 2). The decidua, the maternal tissue in direct contact with fetal extraembryonic tissue, has been a focus of much investigation in an attempt to elucidate mechanisms involved in the delicate immunologic balance of pregnancy. This tissue originates from the mucosal lining of the uterus and establishes the maternal-fetal interface upon fertilization. By term pregnancy, there are two distinct regions of the maternal-fetal interface where maternal decidua contacts fetal tissue – one is at the placenta between the decidua basalis, the site of implantation, and the invading interstitial trophoblasts; and the other is at the fetal membranes between the decidua parietalis, the tissue lining the remainder of uterine cavity and the amniochorion. Focusing primarily on the decidua basalis, immunohistological and flow cytometric studies of first trimester and term pregnancy decidua have identified numerous cells of bone marrow origin, including macrophages, T lymphocytes, and uterine NK cells with a unique CD56^{bright}CD16⁻CD3⁻ phenotype (3-9). The proportions of these cell populations change throughout pregnancy (10); however, the role of these cells in implantation, placentation, and the maintenance of pregnancy is unclear. Our recent studies on decidual leukocytes revealed that there are significant differences in the distribution and proportion of these cells when comparing decidua basalis and decidua parietalis derived from placentas of uncomplicated term pregnancies following elective cesarean section. Flow cytometric quantification showed a significantly higher percentage of CD56^{bright}CD16⁻ NK cells and of CD3⁺ T cells expressing activation markers CD25, Human Leukocyte Antigen (HLA)-DR, CD45RO, or CD69 in the decidua parietalis in comparison to the decidua basalis (11, 12). This difference suggests that the regulation of leukocytes in these two regions of the maternal-fetal interface and their *in vivo* function may differ.

The aim of this study was to examine the function of leukocytes isolated from term decidua basalis and parietalis by measuring their proliferation and cytokine production in response to various stimuli including fetal and allogeneic

blood leukocytes in a one-way mixed lymphocyte culture (MLC) and by comparing it to the responses from maternal and allogeneic peripheral blood leukocytes.

MATERIALS AND METHODS

Samples

Samples of maternal peripheral blood (PB), decidua basalis and parietalis and umbilical cord blood (UCB) were obtained at the time of delivery from ten maternal-fetal pairs following uncomplicated term pregnancies which were selected on the basis that they had an HLA-DR mismatch. Because vaginal delivery is associated with changes in decidual leukocytes (13), only samples from pregnant women delivering by elective cesarean section prior to the onset of labor were selected. All cesarean sections were performed for breech presentation and resulted in the birth of a non-growth restricted healthy neonate. Informed consent was obtained from all women, and ethical approval was received from the Leiden University Medical Ethics Committee. Molecular typing was performed on DNA obtained from all maternal and fetal blood samples by polymerase chain reaction /sequence-specific oligonucleotide using a reverse dot-blot method (14).

Decidua

Term decidua basalis tissue was macroscopically dissected from the maternal-facing surface, and term decidua parietalis tissue was collected by removing the amnion and delicately peeling the tissue from the chorion. Any areas of ischemia or necrosis were avoided. To avoid selective cell death or selective loss of surface proteins, mechanical disaggregation rather than enzymatic digestion was used to process decidual tissue and to isolate decidual leukocytes. The tissue was thoroughly washed and finely minced between two scalpel blades in phosphate buffered saline (PBS) (Leiden University Medical Center Pharmacy, Leiden, the Netherlands). Minced decidual fragments were gently worked through an open filter chamber (Beldico, Marche-en- Famenne, Belgium) with PBS. The resultant suspension was filtered through a 70µm sieve (Becton Dickinson Labware, Franklin Lakes, New Jersey, USA), washed in PBS and layered on an

equal volume of ficoll hypaque (Leiden University Medical Center Pharmacy, Leiden, the Netherlands) at room temperature for density gradient centrifugation for 20 minutes at 800g. Mononuclear cells were collected from the interface, washed twice, counted and resuspended for culture.

Maternal Peripheral Blood and Umbilical Cord Blood

For each maternal-fetal pair, maternal PB (20ml) was drawn before the cesarean section and collected into a heparinized tube for leukocyte isolation and also into a glass tube for isolation of maternal serum for later use in cell culture. UCB (10ml) was obtained from the umbilical vein after cord clamping and collected into a heparinized tube. Mononuclear cells were similarly isolated by density gradient centrifugation as abovementioned and resuspended for culture.

Allogeneic Peripheral Blood

Allogeneic PB was obtained from three random HLA-typed volunteers. PBL were isolated from heparinized blood by density gradient centrifugation and frozen in (RPMI) 1640 medium (Gibco, Paisley, Scotland) with 20% fetal calf serum (Greiner, Frickenhausen, Germany) and 10% dimethylsulfoxide (Fluka, Buchs, Switzerland) at -70°C and stored in liquid nitrogen until use. For use in cell culture, cells were resuspended in 2ml of FCS, washed twice in RPMI 1640 medium supplemented with 2mM L-glutamine (Gibco, Paisley, Scotland), 100U/ml penicillin and 100 µg/ml streptomycin (Gibco, Paisley, Scotland), counted and resuspended at the appropriate cell concentration.

One-way Mixed Lymphocyte Culture

Cultures were established in triplicate in 96 well v-bottomed plates (Costar, Cambridge, MA, USA) in a total volume of 150µl of RPMI 1640 medium supplemented with 2mM L-glutamine, 100U/ml penicillin and 100 µg/ml streptomycin and 10% autologous maternal serum. Each well contained 1×10^5 responder cells in 100µl of culture medium and 1×10^5 irradiated (3000 rad) stimulator cells in 50µl of culture medium. Combinations of responder and stimulator cells from each maternal-fetal pair were established. Responder cells included decidua basalis leukocytes, decidua parietalis leukocytes, maternal PB

leukocytes (PBL), and allogeneic (HLA-mismatched) PBL. Medium alone and anti-CD3 stimulation (see below) were used as controls for the responder cells. Stimulator cells included corresponding umbilical cord blood lymphocytes from the maternal-fetal pair and allogeneic PBL. Stimulator controls included decidua basalis leukocytes, decidua parietalis leukocytes, maternal PBL, and medium alone. Stimulator cells were irradiated at 3000 rads before culture to prevent their proliferation. Cultures were incubated for 96 hours at 37°C in a humidified atmosphere of 5% CO₂. Fifty µl of supernatant was removed from each triplicate well and pooled for cytokine analysis. Cultures were pulsed with 1µCi/ well ³H thymidine diluted in RPMI 1640 medium, incubated for another 18 hours at 37°C in a humidified atmosphere of 5% CO₂, and then harvested. Thymidine incorporation was measured by liquid scintillation counter (Wallac, Turku, Finland). Results were expressed as the median counts per minute (cpm) for each triplicate culture.

Cytokine Analysis

To analyze cytokine concentration in the supernatant, T_H1 and T_H2 Human Cytokine Becton-Dickinson Cytometric Bead Array (CBA) Kit II (San Jose, CA, USA) was used. The kit included six bead populations with distinct fluorescence intensities coated with capture antibodies specific for IL-2, IL-4, IL-6, IL-10, Tumor Necrosis Factor (TNF)-α, Interferon (IFN)-γ proteins. The CBA beads were then mixed with the phycoerythrin (PE)-conjugated detection antibodies and then incubated with recombinant standards or test samples to form sandwich complexes. Following acquisition of sample data using the Becton-Dickinson FACScan flow cytometer, the sample results were generated in a graphical and tabular format using the Becton-Dickinson CBA Analysis Software (San Jose, CA, USA).

Anti-CD3 Stimulation

Anti-CD3 stimulation of responder cells was performed in triplicate in a 96 well flat-bottom plate (Costar, Cambridge, MA, USA). Fifty µl of anti-CD3 (OKT3, Orthoclone, Leiden University Medical Center Pharmacy, Leiden, Netherlands) diluted in PBS or RPMI at 1µg/ml concentration was added to coat each test well and incubated for 2 hours at 37°C. The OKT3 suspension was pipetted out of the

wells, and wells were washed twice with PBS. Five $\times 10^4$ responder cells in 200 μ l of culture medium were then added. Responder cells included leukocytes from decidua basalis, decidua parietalis, maternal PB and allogeneic PB; culture medium was used as a control. The plate was incubated for 4 days at 37°C in a humidified atmosphere of 5% CO₂, and 100 μ l of supernatant was removed from each triplicate well and pooled for cytokine analysis. Cultures were pulsed with 1 μ Ci/ well ³H thymidine diluted in RPMI 1640 medium, incubated for another 18 hours at 37°C in a humidified atmosphere of 5% CO₂, and then harvested. ³H thymidine incorporation was measured by liquid scintillation counting. Results were expressed as the median cpm for each triplicate culture.

Statistical Analysis

To compare proliferative responses of decidual leukocytes and maternal and allogeneic PB with paired controls, the Wilcoxon signed rank test, a non-parametric paired t-test, was performed. The value of $p < 0.05$ was defined as significant.

RESULTS

Proliferation Assays

The cultures of term leukocytes from decidua basalis produced significant proliferative response to anti-CD3 ($p = 0.031$), as did leukocytes from decidua parietalis (0.031). Furthermore, in response to anti-CD3, significant proliferation was seen in cultures of maternal PBL ($p = 0.008$).

The proliferative responses of term decidua leukocytes and maternal PBL are summarized in Table I, which depict the analysis performed in the 10 maternal-fetal pairs. A significantly higher proliferative response was seen in leukocytes from term decidua basalis and parietalis against both UCB cells ($p = 0.039$ and $p = 0.014$, respectively) and against allogeneic PBL as stimulators ($p = 0.004$ and $p = 0.002$, respectively). The response of decidual leukocytes against stimulator controls, including autologous cells from decidua and from maternal PB, was not significantly different from responses to medium alone. Comparing the response of decidua basalis leukocytes against UCB to that of decidua parietalis against

UCB revealed no statistically significant difference ($p=0.301$). There was, however, a significantly higher proliferation by decidua basalis leukocytes in response to allogeneic PBL ($p=0.020$) in comparison to proliferation by decidua parietalis leukocytes.

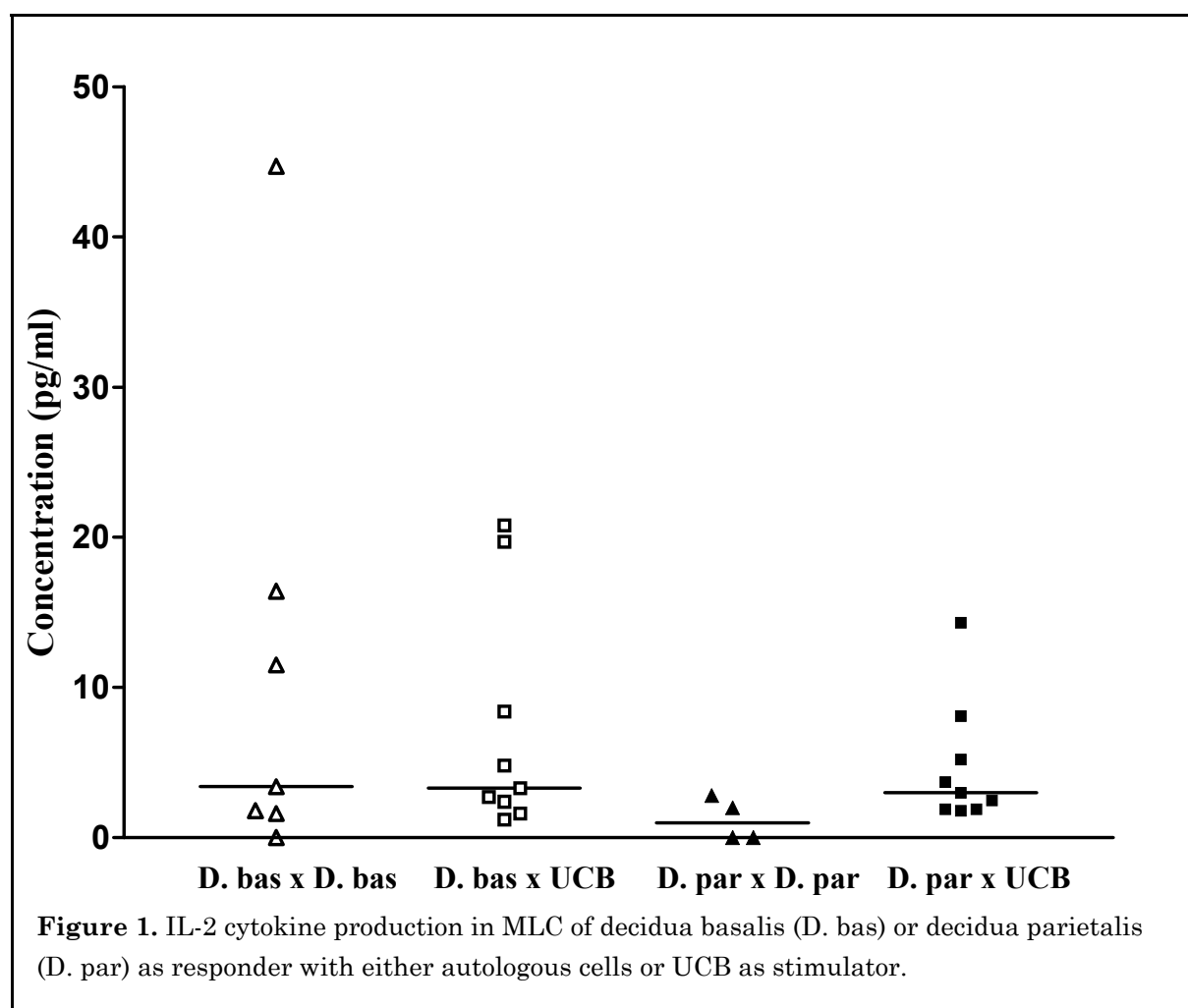
Table I also depicts the responses of maternal and allogeneic PBL to the various stimuli. Predictably, maternal PBL showed a significant response to UCB cells ($p=0.012$) and to allogeneic PBL cells ($p<0.0001$). Furthermore, leukocytes from allogeneic PB proliferated in response to stimulation by decidua basalis ($p=0.008$) and decidua parietalis ($p=0.016$), and similarly in response to controls of UCB ($p=0.002$) and of maternal PB ($p=0.003$).

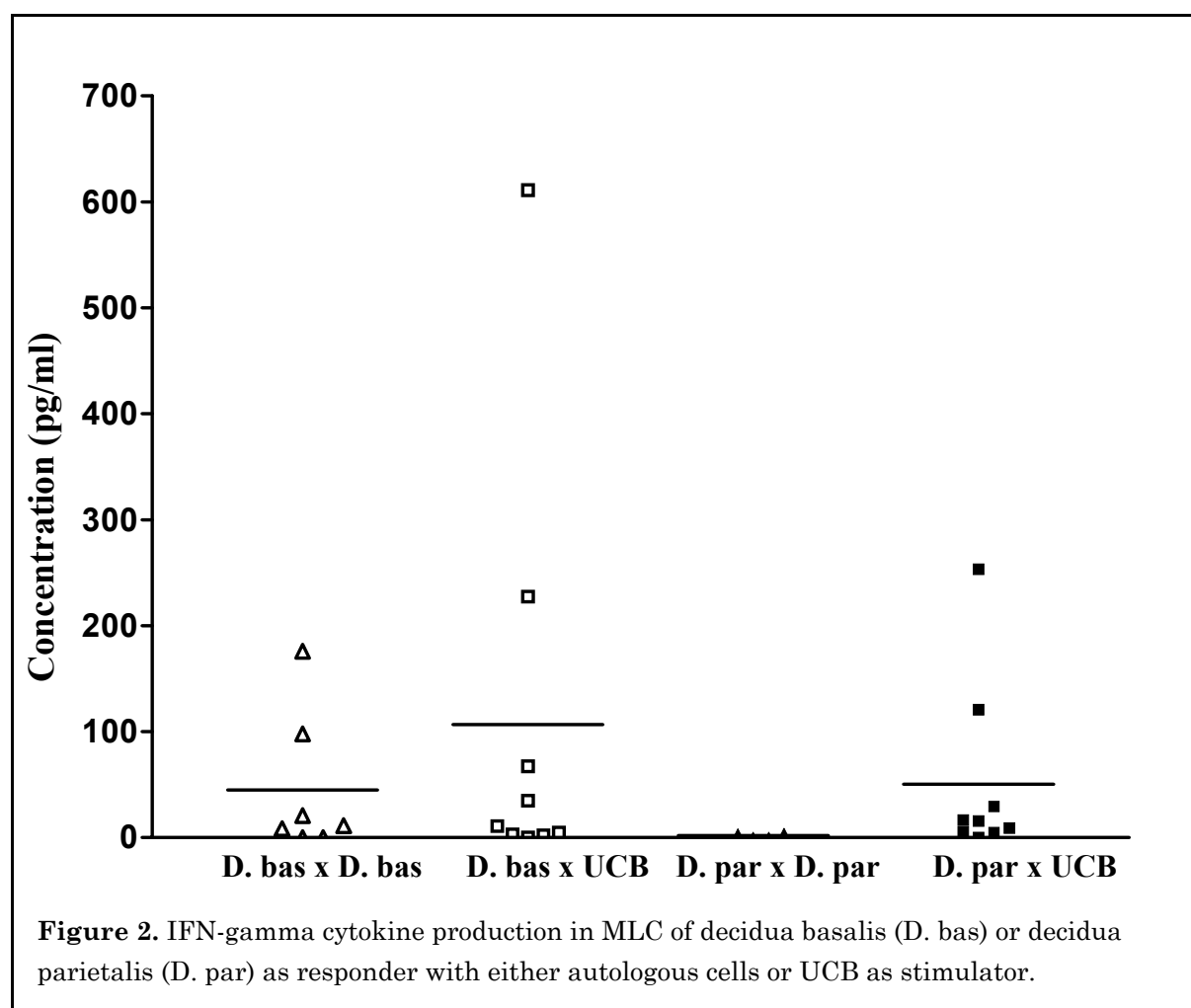
Cytokine Analysis

The proliferative response of leukocytes from decidua basalis, decidua parietalis or allogeneic PB when stimulated with UCB and allogeneic PB cell populations were associated with production of IFN- γ and IL-2 (Figure 1 and 2). There was a higher production of IFN- γ and IL-2 in cultures of decidua basalis or parietalis when these cells were stimulated with UCB in comparison to control autologous stimulation with decidua basalis or parietalis, respectively. Furthermore, there was a significantly higher production of IFN- γ ($p=0.016$) in cultures of decidua basalis with allogeneic PB as stimulator as compared to the control. IFN- γ and IL-2 production was also higher in cultures of decidua parietalis with allogeneic PB as stimulator. No significant production of TNF- α , IL-10 or IL-4 was observed in these experiments, whereas IL-6 was found in all cases (data not shown). In accordance with the proliferation data, a higher production of IFN- γ and IL-2 was found in cultures of maternal PBL as responder with allogeneic PBL or UCB as stimulator. There was no production of TNF- α , IL-10 or IL-4 above background, whereas IL-6 was found in all cases (data not shown).

Table I. Proliferation measured in counts per minute (cpm) of decidua basalis, decidua parietalis, maternal PB, and allogeneic PB in response to decidua basalis, decidua parietalis, maternal PB, UCB, allogeneic PB, and anti-CD3 statistically compared to the proliferation when cultured with medium alone (n=10).

Responder	Stimulator	cpm^a	(Range)	P Value^b
D. basalis	Medium	1585	(133.8- 5341)	
	D. parietalis	1695	(160.7-6517)	0.438 (NS)
	Maternal PB	1724	(411.8-4693)	0.910 (NS)
	UCB	3584	(89.50- 9482)	0.039
	Allogeneic PB	7522	(2347-14320)	0.004
	Anti-CD3	87770	(22390-157600)	0.031
D. parietalis	Medium	1009	(143.4-3461)	
	D. basalis	1028	(266.1-2867)	0.578 (NS)
	Maternal PB	980.8	(181.1-1877)	0.846 (NS)
	UCB	1931	(130.6-6038)	0.014
	Allogeneic PB	3154	(573.5-8360)	0.002
	Anti-CD3	61090	(4197-111200)	0.031
Maternal PB	Medium	280.6	(102.1-908.8)	
	D. basalis	756.9	(102.1-2183)	0.156 (NS)
	D. parietalis	205.7	(50.30-406.6)	0.938 (NS)
	UCB	3057	(140.8-6113)	0.012
	Allogeneic PB	8450	(992.1-18910)	<0.0001
	Anti-CD3	116300	(85060-157200)	0.008
Allogeneic PB	Medium	302.5	(55.70-1137)	
	D. basalis	10510	(131.6-27660)	0.008
	D. parietalis	9645	(168.4-27460)	0.016
	UCB	6619	(1444-20760)	0.002
	Maternal PB	8000	(352.3-15690)	0.003
	Anti-CD3	49900	(3335-115400)	0.008





DISCUSSION

The function of leukocytes in the decidua basalis and parietalis, including their ability to proliferate in response to fetal or allogeneic cells and their ability to produce cytokines is an important focus in order to understand the role of these cells in the maintenance of pregnancy. In the current study, we used a one-way MLC to assess these functions and compared it to maternal and allogeneic peripheral blood at term pregnancy. Furthermore, it was found that these decidual leukocytes proliferated and produced cytokines in response to fetal and allogeneic cells. These findings indicate that decidual leukocytes are capable of initiating and stimulating a classical alloimmune response.

It has been well established that decidual leukocytes from both first trimester

and term pregnancy decidua do not proliferate in response to extravillous trophoblasts from the chorion or chorionic laeve (9, 15, 16). Three MHC class I molecules have been detected on extravillous trophoblast, including HLA-C, HLA-G and HLA-E (17-22). These molecules interact with specific receptors, such as CD94/NKG2A and killer-cell immunoglobulin-like receptors (KIR) that are expressed by subpopulations of NK and $\gamma\delta$ T cells and also by subpopulations of memory $\alpha\beta$ T cells (23-28). It is suggested that the expression of KIR by decidual cells leads to the protection of fetal trophoblasts (29, 30) and that the stimulation of lymphocytes is essential at the placental interface (31, 32).

To our knowledge, there are no previous studies that examine the proliferation of decidual leukocytes from early or term pregnancy in response to fetal UCB cells. In the current study, a significantly higher proliferative response was seen when leukocytes from term decidua basalis and parietalis were cultured with UCB cells, cells of fetal origin, as compared to controls. It has been shown that UCB cells are capable of eliciting a proliferative response as stimulators in a MLC comparable to that of irradiated adult PBL (33). Although UCB cells essentially do not come into direct physical contact with decidual leukocytes due to anatomical separation, fetal cells of hematopoietic origin such as nucleated red blood cells, lymphocytes, and stem cells enter the maternal circulation during all pregnancies (34-36). Furthermore, it is of scientific interest to confirm that term decidual leukocytes are capable of eliciting an immune response against cells of fetal origin, a finding not previously reported. In this study, we show that in the decidua (basalis and parietalis) alloreactive cells respond to HLA mismatched allogeneic PBL and also towards UCB of the fetus. The recognition of MHC alloantigens by T cells occurs via two distinct pathways, a direct and indirect pathway (37). In the direct pathway, T cells recognize alloantigens as intact molecules on allogeneic stimulator cells. In the indirect pathway, T cells recognize alloantigens presented by self MHC molecules. In the current study, it is plausible that the mechanism by which decidual leukocytes recognize and mount an immune response may occur via the indirect pathway of allorecognition. Although it is suggested that the fetus escapes rejection from the maternal immune system by mechanisms involving HLA, KIR, and cytokines, these data suggest that there is a local role for regulatory mechanisms

controlling the specific immune responsiveness to the fetus in which (CD4⁺CD25^{hi/bright}) regulatory T cells may be involved. In a previous study, we have shown that the majority of CD3⁺CD25⁺ cells in decidua basalis and parietalis express CD4 (13). Our preliminary analysis confirms the presence of this population (38). CD4⁺CD25⁺ cells also have been described in term decidua parietalis, this population was not further characterized, however, as CD25^{hi/bright} nor proven to be functional by assay (39). Alternatively, it has been shown that early human pregnancy decidua contains functional CD4⁺CD25^{bright} T cells (40). Although regulatory T cells have not been fully characterized in term decidua, they could potentially serve as mediators in maternal tolerance of the fetal compartment by potentially suppressing the activation and proliferation of CD4⁺CD25⁻ T cells, CD4⁺CD25⁺ T cells, and also CD8⁺ T cells.

Interestingly, it has been shown that decidual leukocytes derived from first trimester decidua are not capable of proliferating in response to allogeneic PBL (15, 41, 42). In the study by King *et al.*, no proliferative response was observed in decidual leukocytes from first trimester tissue cultured with irradiated allogeneic PBL as stimulators in a one-way MLC (16). In the present study, it is shown that decidual leukocytes from uncomplicated term pregnancy, in contrast to that described from early pregnancy, significantly proliferate in response to allogeneic PBL. The difference in the proliferative response of first trimester and term pregnancy decidua derived leukocytes against allogeneic PBL is likely due to a change in the distribution and proportion of the responsible immune cells. Although first trimester and term pregnancy decidua both contain macrophages, T cells and uterine NK cells, the proportions of these cell populations indeed change throughout pregnancy (10). During early pregnancy, uterine CD56^{bright}CD16⁻ NK cells account for up to 70% of the leukocytes in the deciduas basalis (4, 43), however, as pregnancy progresses, the number of uterine NK cells gradually declines (44). Conversely, during first trimester pregnancy, the total T cell population represents only 6% to 30% of the decidual leukocytes (4, 6, 45, 46); while in term pregnancy, T cells comprise the most abundant leukocyte population (9, 10). Furthermore, results from our previous studies of term pregnancy decidua indicated that there is a higher percentage of CD56^{bright} CD16⁻ NK cells and of CD3⁺ T cells expressing several activation

markers in the decidua parietalis in comparison to the decidua basalis (11, 12). The varying proportion of cell types throughout pregnancy and their function could account for the difference in proliferation in response to allogeneic cells when comparing early and term pregnancy. Our findings warrant future investigation using purified decidual cell populations to further understand the proliferative response against allogeneic and UCB cells.

Although there is a significantly higher percentage of uterine NK cells and of T cells expressing activation markers in the decidua parietalis than in the decidua basalis, unexpectedly, in this study, a significantly higher proliferation was found when decidua basalis leukocytes were stimulated by allogeneic PBL in comparison to the response by decidua parietalis leukocytes. The significant differences in the proportion of decidual leukocytes in these two regions may explain this difference in proliferation. We also found in our previous studies that there is no significant difference in the percentage of CD45⁺ leukocytes or CD14⁺ macrophages when comparing the two regions of the maternal-fetal interface to account for the difference. Interestingly, no significant difference in response against UCB was found when comparing decidua basalis and decidua parietalis indicating a possible biological significance.

It is suggested that cytokines play a significant role in the immunological mechanisms involving placental growth and the maintenance of pregnancy (47). T helper cells are subdivided according to the cytokines that they produce. T_H1 type cytokines lead to the induction of cytotoxic T cells and include IFN- γ , IL-2, TNF- α and IL-12. T_H2 type cytokines mediate allergic and antibody responses and include IL-4, IL-5 and IL-6. Generally, in reproduction, there is a dichotomous T helper response. Established successful pregnancy is characterized by low levels of T_H1 cytokines and by a shift in the cytokine pattern from T_H1 to T_H2 ; T_H1/T_H2 paradigm of reproduction suggests that pregnancy rejection is mediated by T_H1 cytokines, while maintenance and acceptance is mediated by T_H2 cytokines (48,49). While the T_H1/T_H2 paradigm may be an oversimplification of role of cytokines in reproduction (50), there is a complex network of cytokines at the maternal-fetal interface involved in regulation of immune responsiveness. In the current study, it is shown that T_H1

cytokines, such as IFN- γ predominate in *in vitro* cultures of decidual basalis or decidua parietalis with UCB or allogeneic PB cells as stimulators. In the *in vivo* setting, decidual leukocytes do not come into direct physical contact with UCB or allogeneic PBL. However, decidual leukocytes can proliferate in response to HLA antigens on UCB, minor peptides via the indirect pathway, and allogeneic PB and are capable of maintaining a cytokine microenvironment that may lead to a cytotoxic immune response and be deleterious to these cells.

The function of leukocytes in the decidua basalis and parietalis, including their ability to proliferate in response to fetal or allogeneic cells and their ability to produce cytokines, is important for our understanding of the dynamic balance occurring at the maternal-fetal interface. Future studies are necessary to elucidate the roles of these cells in the maintenance of pregnancy.

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CHAPTER 6

ABBERANT COMPOSITION OF DECIDUAL LEUKOCYTES IN HUMAN PREGNANCY ASSOCIATED WITH INTRAUTERINE GROWTH RESTRICTION WITH AND WITHOUT PREECLAMPSIA

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ABSTRACT**OBJECTIVE**

The aim of this study was to determine the role potential of decidual leukocytes in intrauterine growth restriction (IUGR) and preeclampsia by quantifying the percentage of leukocytes in decidua basalis and parietalis associated with IUGR with and without preeclampsia in relation to the situation in normal pregnancy.

STUDY DESIGN

Paired decidua basalis and parietalis samples were obtained from pregnancy with IUGR alone (n=10), with preeclampsia and IUGR (n=11), and normal term pregnancy (n=30). Following mechanical isolation and staining, percentages of leukocytes were determined by flow cytometry.

RESULTS

The percentage of $\text{TCR}\gamma\delta^+\text{CD}3^+$ and $\text{CD}4^+\text{CD}25^+$ cells was lower in decidua basalis and parietalis associated with IUGR with and without preeclampsia compared to normal pregnancy. Furthermore, a higher percentage of uterine $\text{CD}56^+\text{CD}16^-$ NK cells was found in decidua basalis of IUGR alone compared to normal control pregnancies.

CONCLUSION

Significant differences in decidual leukocytes exist between IUGR with and without preeclampsia, compared to normal control pregnancies, suggestive for an aberrant immunoregulation in both diseases.

INTRODUCTION

Since the paradox of pregnancy was first proposed by Medawar in 1953 (1), the complex interaction between maternal and fetal cells in the placenta of normal pregnancy has been the focus of much research. Invasion of uterine spiral arteries and decidua by fetal trophoblasts accommodates for the increased blood flow of the fetoplacental unit. Multiple mechanisms contribute to the balance of invasion at the maternal-fetal interface, including the expression of classical HLA-C and non-classical class I HLA-G and HLA-E on fetal trophoblasts, the production of cytokines affecting angiogenesis and vascular stability, and the presence of maternal leukocytes within the decidua (2). Immune cells form a major component of the decidua, and throughout pregnancy, the percentage of CD56^{bright}CD16⁻ uterine cells and T cells varies in decidua basalis, the site of implantation, and in decidua parietalis, the tissue lining the remainder of the uterine cavity (3, 4).

The field of reproductive immunology in part progresses in hope of providing clinical treatment for and improving the outcome of complicated pregnancies. The role of the placental bed in the pathogenesis of a variety of pregnancy disorders, such as preeclampsia (PE) and intrauterine growth restriction (IUGR), is clinically relevant, as both are associated with increased perinatal morbidity and mortality. PE is a significant complication of pregnancy defined by hypertension, proteinuria, and edema, presenting as early as the second trimester and affecting approximately 5-8% of pregnancies (5). IUGR is characterized as a syndrome marked by failure to reach growth potential, affecting 3-7% of pregnancies and can present alone as idiopathic IUGR or in association with PE (6).

It is hypothesized that abnormal maternal-fetal cellular interactions within the placenta contribute to the pathogenesis of both IUGR and PE, but differ in IUGR with and without PE. To our knowledge, no previous study has investigated and specifically compared decidual leukocytes in IUGR with and without PE. Our previous studies indicate that there are significant differences in the distribution and proportion of leukocytes when comparing decidua basalis and decidua parietalis of normal term pregnancy (7, 8). The aim of this study

was to determine the role of decidual leukocytes in IUGR and PE by quantifying the distribution and percentage of leukocytes in decidua basalis and parietalis from placentas associated with IUGR alone or with IUGR and PE in comparison with that of normal term deciduas using flow cytometric analysis.

MATERIALS AND METHODS

Subjects

Paired decidua basalis and parietalis samples were obtained from three groups: pregnancy associated with IUGR alone (n=10), pregnancy associated with PE and IUGR (PE/ IUGR) (n=11), and normal term pregnancy (n=30). All women were delivered by elective cesarean section. Clinical characteristics of the study and control groups are summarized in Table I. PE was defined according to the criteria of the American College of Obstetricians and Gynecologists (5). Women with a systolic blood pressure of 140 mmHg or higher or a diastolic blood pressure of 90 mmHg or higher occurring after 20 weeks of gestation in a woman whose blood pressure has previously been normal and proteinuria with excretion of 0.3 g or more of protein in a 24-hour urine specimen were selected. IUGR was defined by Doppler abnormalities in the umbilical artery and as fetal weight below the 10th percentile for appropriate population-based cross-sectional growth charts (9). Because vaginal delivery is associated with changes in decidual leukocytes (10), only samples from pregnant women delivering by elective cesarean section prior to the onset of labor were selected.

For normal term pregnancy samples, paired tissue from decidua basalis and parietalis was obtained from placentas following elective cesarean section before the onset of labor indicated for breech presentation. These samples were used in previous publications (7, 8). All deliveries followed uncomplicated term pregnancies and resulted in the birth of a non-growth restricted healthy neonate. Informed consent was obtained from all women in the three groups, and the study received the appropriate ethical approval by the Leiden University Medical Ethics Committee in Leiden, the Netherlands.

Isolation of decidua basalis and parietalis cells

Term decidua basalis tissue was macroscopically dissected from several

different locations of the basal plate and placental bed of the maternal-facing surface, and term decidua parietalis tissue was collected by removing the amnion and delicately scraping the tissue from the chorion as previously described (8). Areas of ischemia or necrosis were avoided. The obtained tissue was thoroughly washed and finely minced between two scalpel blades in phosphate buffered saline (PBS). Minced decidual fragments were gently worked through an open filter chamber (NPBI International BV, Emmer-Compascuum, the Netherlands, and Beldico, Marche-en-Famenne, Belgium) with PBS. The resultant suspension was filtered through a 70µm sieve (Becton Dickinson, Labware; Franklin Lakes, New Jersey), washed in PBS, and layered on an equal volume of ficoll hypaque (Leiden University Medical Center Pharmacy; Leiden, the Netherlands) at room temperature for density gradient centrifugation (for 20 minutes at 800g). Mononuclear cells were collected from the interface, washed twice, and resuspended in PBS for cell staining.

Cell Staining

Directly conjugated fluorescent antibodies directed against the following antigens were used for two- and three-color immunofluorescence staining: CD45 [HLe-1; Fluorescein isothiocyanate (FITC)], CD3 [Leu-4; Allophycocyanin (APC) or Peridinin Chlorophyll-a Protein-Cytochrome 5.5 (PerCP-Cy5)], CD8 (Leu-2a; FITC), CD4 [Leu-3a; Phycoerythrin (PE)], CD19 (Leu-12; FITC), CD56 (Leu-19; FITC, Leu-19; PE), CD16 (Leu-11a; FITC, Leu-11c; PE), CD25 (Anti-IL-2R; FITC), HLA-DR (Anti-HLA-DR; PE) and CD45RO (UCHL1; PE) all from Becton-Dickinson Immunocytometry Systems (San Jose, CA, USA) and TCRγδ (5A6.E9; FITC, Serotec, Oxford, England and Biocarta, Hamburg, Germany).

Cells were stained with monoclonal antibodies at a density of 10⁶ cells/ ml in PBS (100µl/ sample) and incubated for 30 minutes at 4°C in the dark. Following two PBS washes, cells were resuspended and fixed with PBS containing 1% paraformaldehyde. Cells were then analyzed on a Becton-Dickinson FACScan flow cytometer. Approximately 5,000 events were acquired in the CD45⁺ leukocyte gating window. The acquired data were then analyzed using Becton-Dickinson CellQuest™ software (San Jose, CA, USA). The percentages of CD16⁺, CD56⁺, CD19⁺, CD3⁺, CD4⁺ and CD8⁺ cells in decidua basalis and

decidua parietalis were determined and calculated as a percentage of the CD45⁺ cell fraction. To further characterize the expression of CD16 and CD56, the percentages of CD56^{dim}CD16⁺ NK cells and CD56^{bright}CD16⁻ uterine NK cells were established by delineating regions around these various populations. To determine T cell marker expression, cells that were within the leukocyte gate and also CD3⁺ were gated based on these two parameters and analyzed for the expression of the activation marker of interest.

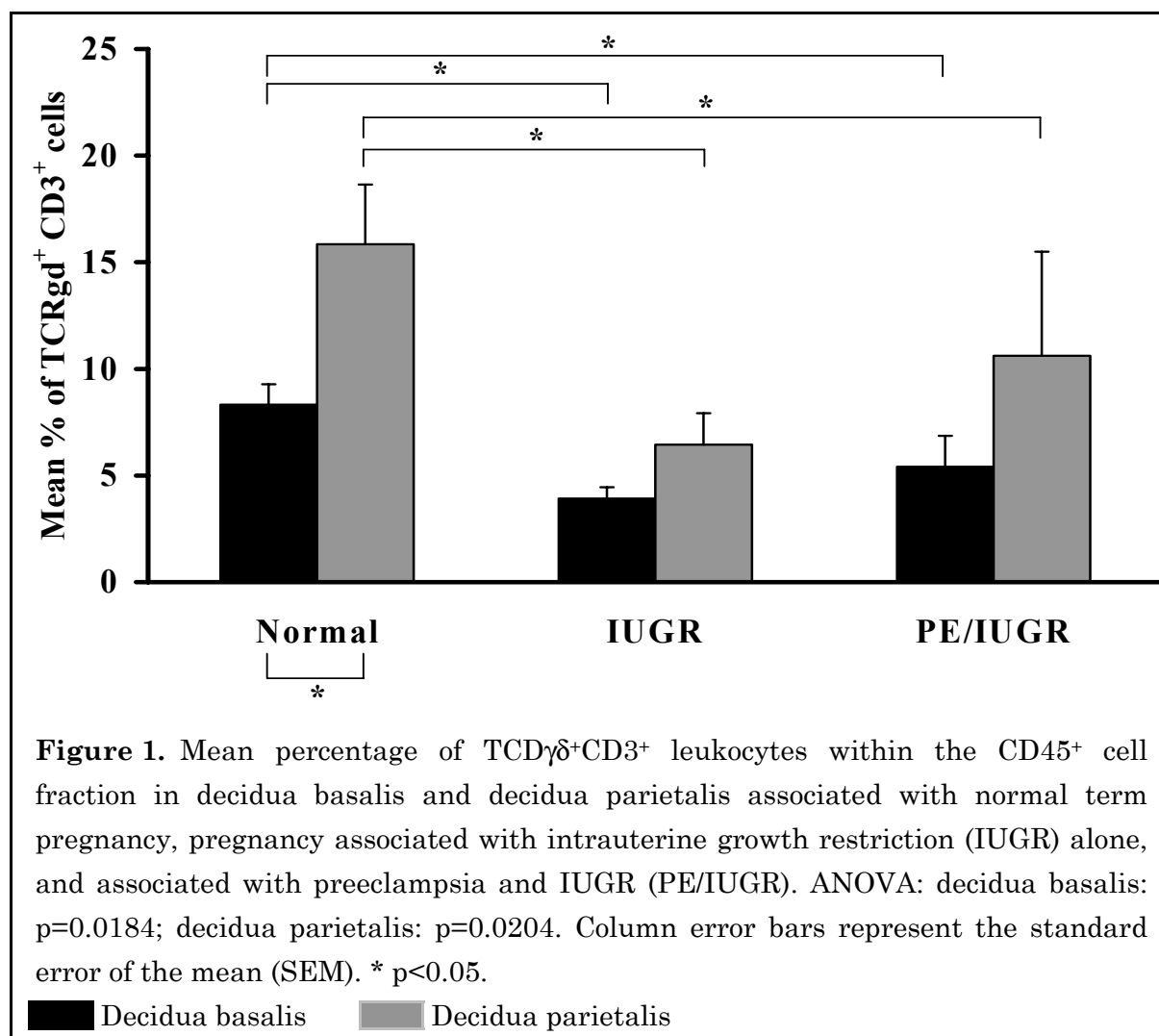
Statistical Analysis

To compare differences in leukocyte subsets in the decidua basalis or in the decidua parietalis between two study groups, the Mann-Whitney test was performed. The Wilcoxon signed rank test was used to compare differences between decidua basalis and parietalis within each group. The non-parametric Kruskal-Wallis analysis of variance (ANOVA) test was performed to compare the mean percentages of the three groups. The conventional value of $p < 0.05$ was defined as significant.

RESULTS

Comparison of leukocytes from decidua basalis and decidua parietalis from placentas associated with IUGR alone and with PE/IUGR and normal control pregnancies are shown in Figures 1-5. Using ANOVA analysis to compare IUGR, PE/IUGR and term groups, no significant difference was seen in the mean percentage of leukocytes in decidua basalis and in decidua parietalis expressing CD45, CD19, CD3, CD4, and CD8. As Figure 1 depicts, the percentage of TCR $\gamma\delta$ ⁺ cells within the CD3⁺ cell population however was significantly lower in the decidua basalis associated with IUGR alone (3.93%) and also PE/IUGR (5.42%) when compared with the control group (8.32%; ANOVA: $p = 0.0184$; Mann-Whitney: $p = 0.0124$ and $p = 0.0480$, respectively). A similar difference also was found when comparing the percentage of TCR $\gamma\delta$ ⁺ cells within the decidua parietalis with IUGR (6.44%) and PE/IUGR (10.61%) to that of control group (15.85%; ANOVA: $p = 0.0204$; Mann-Whitney: $p = 0.0204$ and $p = 0.0363$). No significant difference was found in the mean percentage of TCR $\gamma\delta$ ⁺ cells when comparing IUGR and PE/IUGR groups in decidua basalis or parietalis (Mann-Whitney: $p = 0.9623$ and $p = 0.9623$, respectively). When

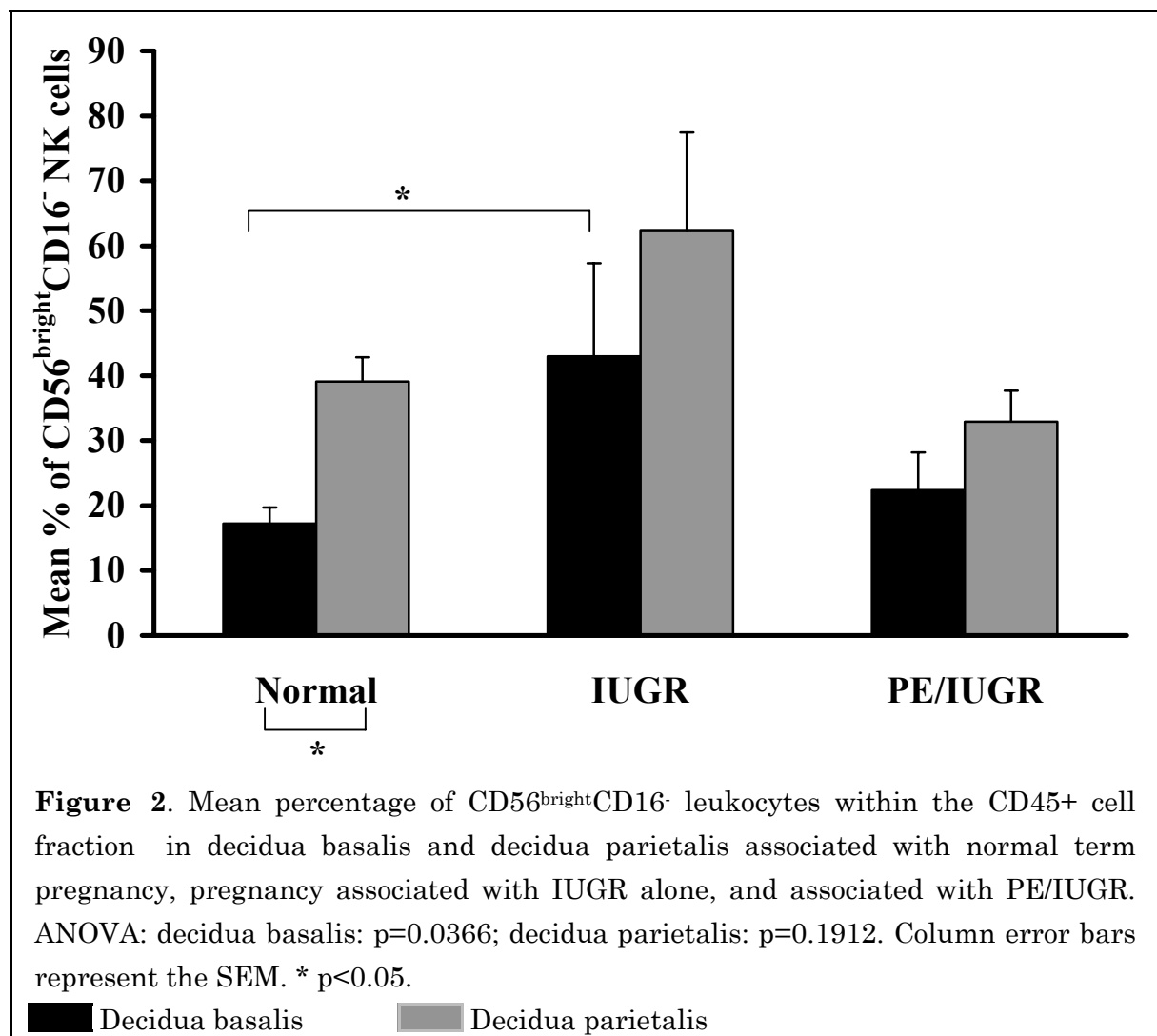
comparing $\text{TCR}\gamma\delta^+$ expression between decidua basalis and decidua parietalis, a significant difference was only found in normal term pregnancy with a higher percentage in decidua parietalis (Wilcoxon: $p=0.0007$), not in IUGR with or without PE.

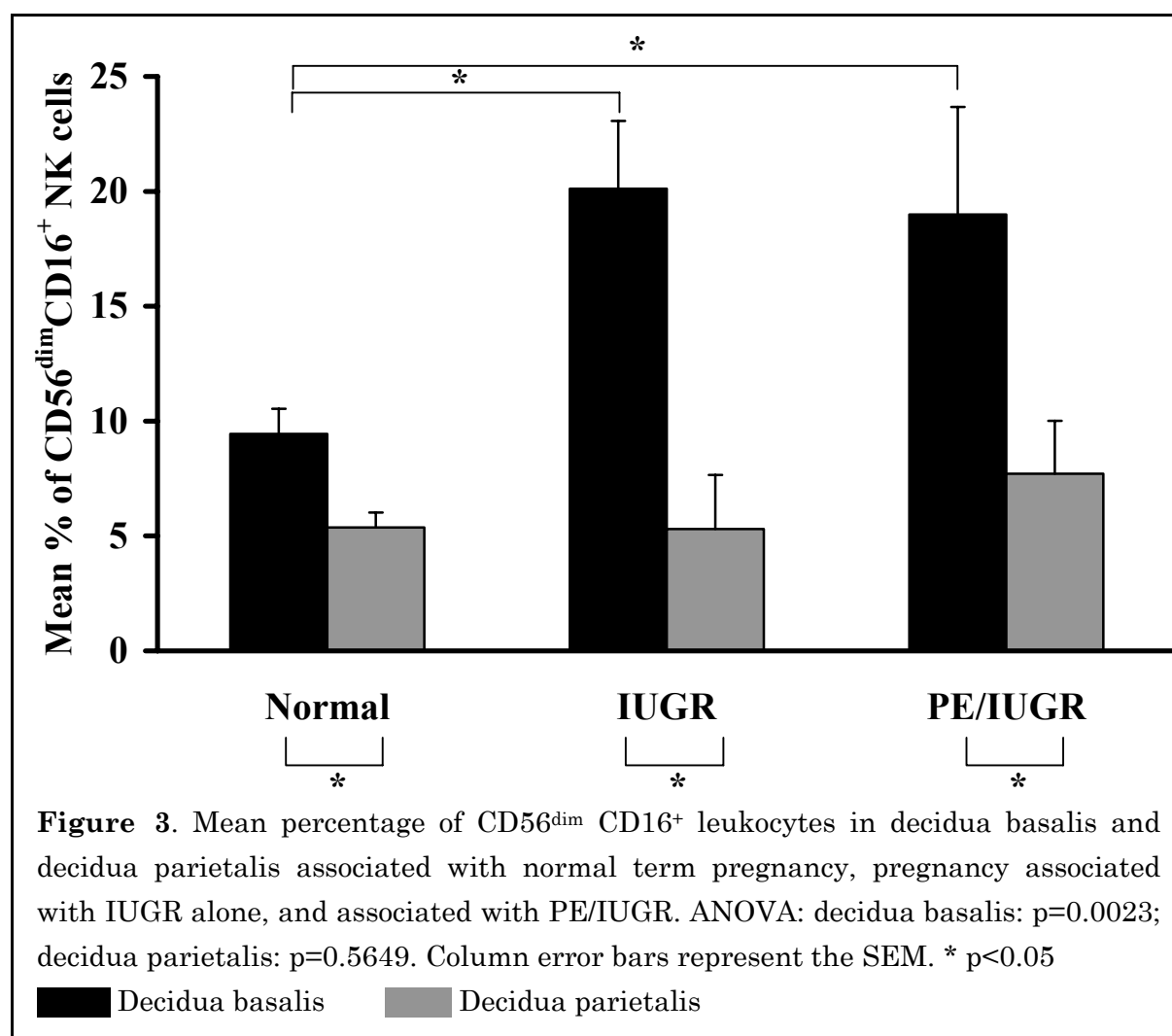


Expression of NK cell markers

Figures 2 and 3 depict the expression of CD56 and CD16, specifically the uterine $\text{CD}56^{\text{bright}}\text{CD}16^-$ NK cell population and the cytotoxic $\text{CD}56^{\text{dim}}\text{CD}16^+$ NK cell population. In the decidua basalis, the mean percentage of uterine NK cells was 42.98% in the IUGR group, 22.36% in the PE/IUGR group, and 17.24% in the control group, indicating a significant difference when comparing the three groups (ANOVA: $p=0.0366$). Specifically, there was a significantly higher

percentage of uterine NK cells in the decidua basalis in IUGR alone when compared to normal term pregnancy (Mann-Whitney: $p=0.0100$). This difference was not found in PE/IUGR (Mann-Whitney: $p=0.6509$). Interestingly, there was no significant difference in the percentage of uterine NK cells in the decidua parietalis when comparing the three groups (ANOVA: $p=0.1912$). With regard to $CD56^{\text{dim}}CD16^+$ NK cells, there was a significant difference in the decidua basalis when comparing all three groups (figure 3; ANOVA: $p=0.0023$). Specifically, there was a significantly higher percentage of cytotoxic NK cells in the decidua basalis in IUGR alone and in PE/IUGR when compared to normal term pregnancy (Mann-Whitney: $p=0.0027$; $p=0.0152$, respectively). No significant difference in the percentage of $CD56^{\text{dim}}CD16^+$ cells in the decidua parietalis was found when comparing the three groups (ANOVA: $p=0.5649$). When comparing NK cell subsets between decidua basalis and decidua parietalis within each

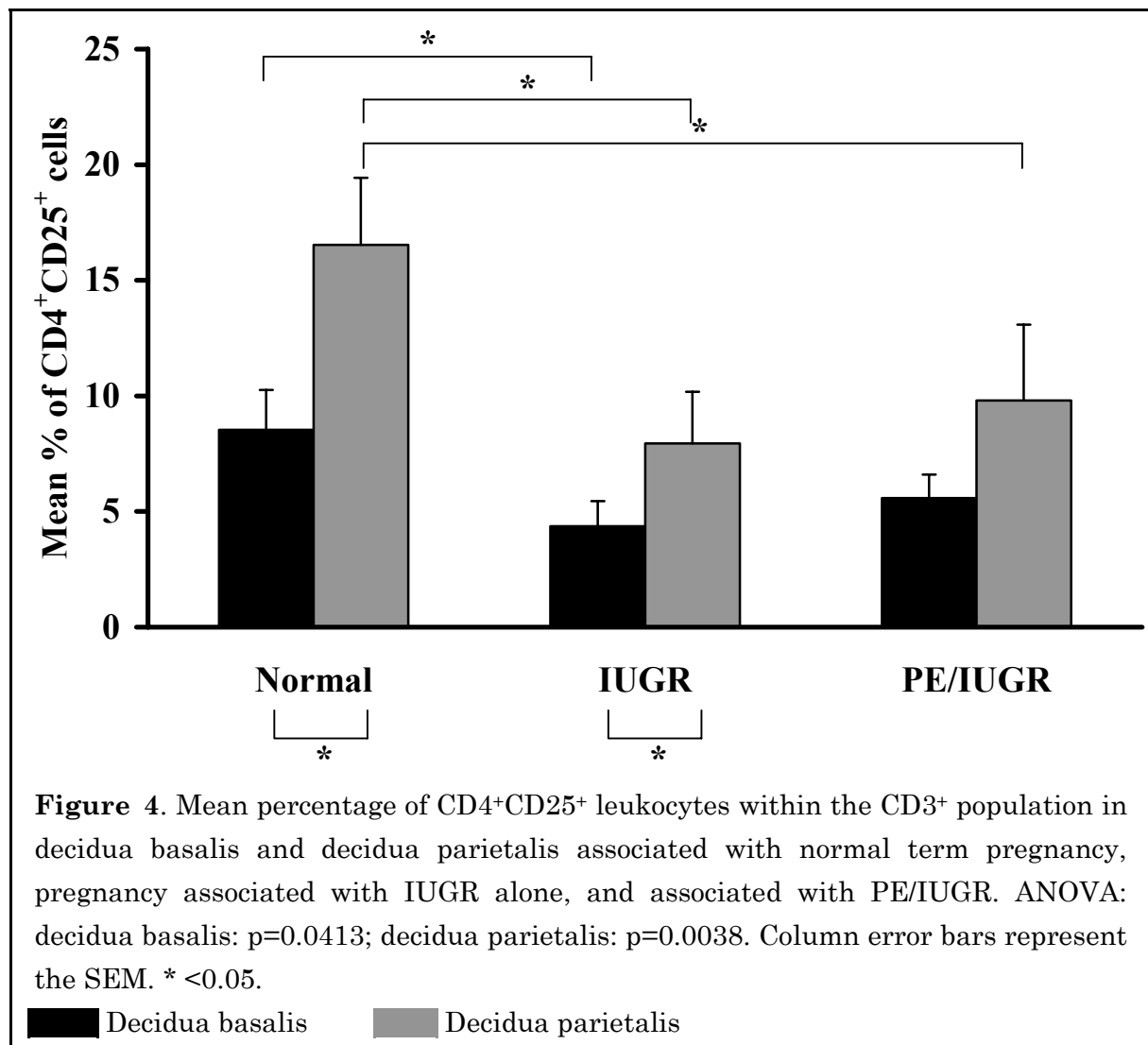




group, a significant difference was found only in normal term pregnancy, with a higher percentage of CD56^{bright}CD16⁻ NK cells in the decidua parietalis (Wilcoxon: normal p<0.0001; IUGR p=0.4688; PE/IUGR p=0.2402), while a higher percentage of CD56^{dim}CD16⁺ NK cells in the decidua basalis of all three groups (Wilcoxon: normal p=0.0024; IUGR p=0.0078; PE/IUGR p=0.0273).

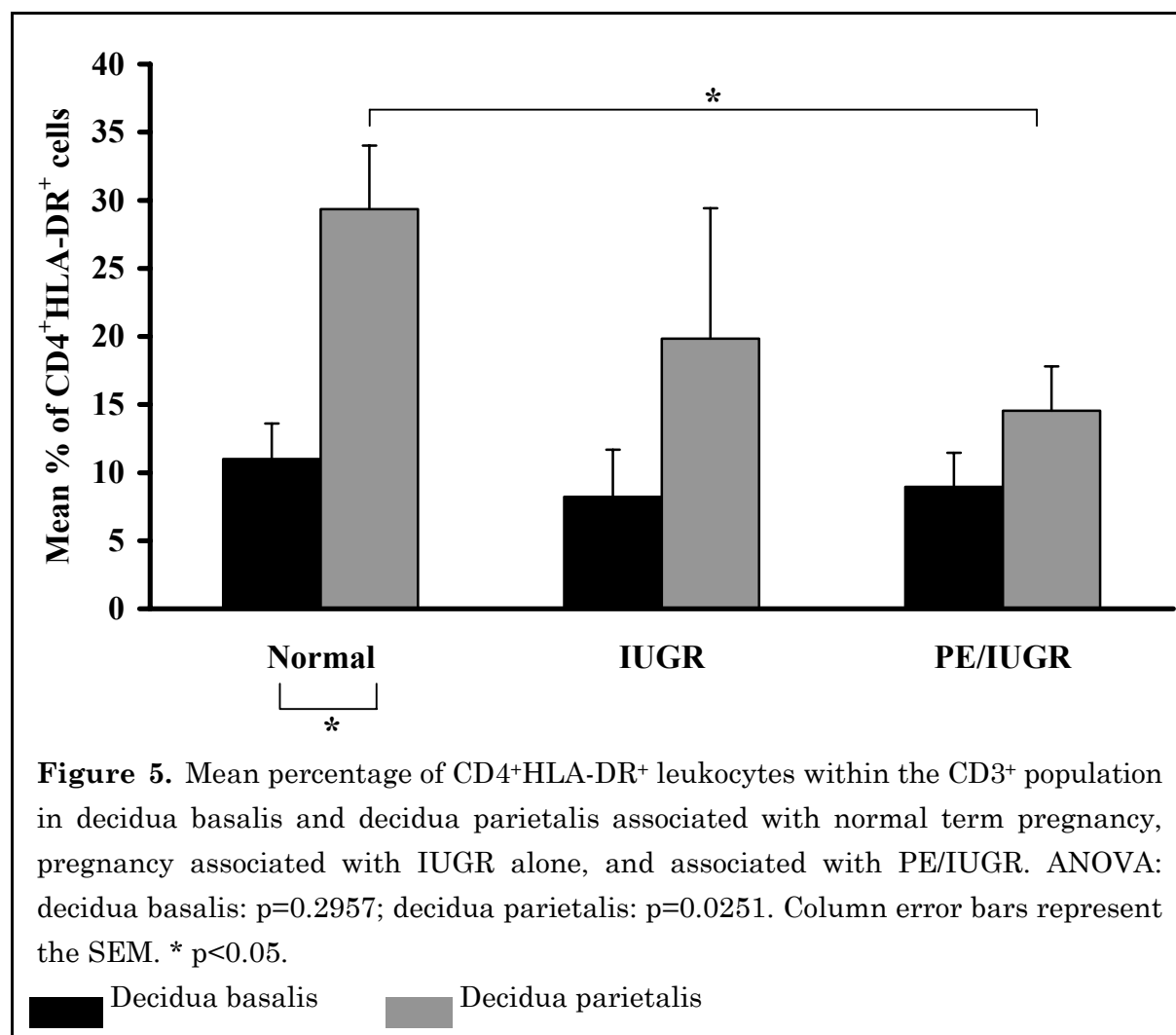
Expression of activation markers by CD3⁺ T cells

When comparing the three groups within the CD3⁺ population, there was a significant difference in the percentage of CD4⁺CD25⁺ cells in the decidua basalis (ANOVA: p=0.0413) and in the decidua parietalis (ANOVA: p=0.0038) (Figure 4). Specifically, analysis showed a lower percentage of CD4⁺CD25⁺ cells in IUGR decidua basalis (4.37%; Mann-Whitney: p=0.0250) and in IUGR



decidua parietalis (7.95%; Mann-Whitney: p=0.0068) when compared to normal pregnancy decidua basalis (8.53%) and parietalis (16.53%). Also, a lower percentage of CD4⁺CD25⁺ cells was found in PE/IUGR decidua parietalis (9.81%) when compared to normal term pregnancy (Mann-Whitney: p=0.0035), but not in IUGR/PE decidua basalis (5.59%; Mann-Whitney: p=0.0529). Comparing decidua basalis and parietalis within each group showed a significantly lower percentage of CD4⁺CD25⁺ T cells in the decidua basalis in IUGR and normal pregnancy (Wilcoxon: p=0.0039 and p=0.0156, respectively). In the decidua basalis, no significant difference was found when comparing presence of CD8⁺CD25⁺, CD4⁺HLA-DR⁺, CD8⁺HLA-DR⁺, and CD45RO⁺ within the CD3⁺ population between all three groups (ANOVA: p>0.05) (data not

shown). Conversely, in the decidua parietalis, while no difference was found in the percentage of CD8⁺HLA-DR⁺, CD8⁺CD25⁺ or CD45RO⁺ cells, there was a significantly lower percentage of CD4⁺HLA-DR⁺ cells within the T cell population (ANOVA: $p=0.0251$). Specifically, the percentage of CD4⁺HLA-DR⁺ cells was lower in decidua parietalis associated with PE/IUGR ($p=0.0068$) when compared to that in normal term pregnancy (Figure 5).



Leukocyte subclasses within overlapping gestational ages

To exclude that differences between normal and IUGR pregnancies (with and without preeclampsia) are explained by differences in gestational age we compared in these two groups the leukocyte subclasses in overlapping gestational ages (Table I). To increase the number of cases with a comparable gestational age, we combined IUGR pregnancies with and without PE, as no differences in leukocyte subclasses were apparent in the original cohort. All leukocyte subclasses that were different between the total cohort of IUGR and controls were also different in pregnancies with overlapping gestational age.

Table I. Comparison of leukocyte subsets in control and study groups with an overlap in gestational age.

	Control (n=6)		IUGR with and without PE (n=6)	
Gestational Age (weeks) mean (range)	37.0 (35.6-37.4)		35.1 (34.0-36.5) *	
Decidua	basalis	parietalis	basalis	parietalis
TCR$\gamma\delta^+$CD3$^+$	5.6 $\pm$ 1.6	6.2 $\pm$ 1.1	3.1 $\pm$ 0.9	3.5 $\pm$ 1.0
uNK cells	19.4 $\pm$ 8.9	45.2 $\pm$ 19.6	47.8 $\pm$ 19.4	25.1 $\pm$ 4.1
CD4$^+$CD25$^+$	9.7 $\pm$ 4.0	13.9 $\pm$ 3.3	6.2 $\pm$ 1.4	8.5 $\pm$ 3.9

* Mann Whitney U test: p = 0.004

Subjects

Clinical characteristics of the IUGR group and PE/IUGR are summarized in Table II. No significant difference was found between IUGR and PE/IUGR groups when comparing maternal age, gestational age, fetal weight or placental weight (Mann-Whitney: p=0.3722; p=0.872; p=0.4704; p=0.7577, respectively). Maternal age was significantly higher in the normal term pregnancy group and differed when comparing all three groups (ANOVA: p=0.0403). As expected, gestational age, fetal weight and placental weight also were significantly higher in the normal term pregnancy group when comparing all three groups (ANOVA: p<0.0001; p<0.0001; p<0.0001, respectively).

Table II. Clinical characteristics of control and study groups.

Clinical Characteristic	Control [n=30] mean (range)	IUGR [n= 9] mean (range)	PE/IUGR [n=11] mean (range)	P value
Maternal age (yrs)	34.2 (21.4-42.3)*	31.7 (21.2-37.0)	29.8 (17.0-38.2)	0.0403
Gestational age (wks)	38.4 (35.6-41.6)*	31.2 (27.0-36.1)	30.8 (26.6-36.5)	<0.001
Fetal weight (gm)	3355 (1880-4975)*	1044 (577-1710)	1170 (660-2180)	<0.001
Placental weight (gm)	591 (315-960)*	219 (90-310)	230 (150-273)	<0.001

*ANOVA analysis comparing all three groups: $p < 0.05$

COMMENT

Recent studies investigating variations in morphology of placentas associated with PE and IUGR suggest that idiopathic IUGR and IUGR in PE have a separate etiology (11). IUGR alone is associated with a reduction in villous area alone, while IUGR in PE is associated with changes in syncytiotrophoblast quantity, more specifically the amount of syncytiotrophoblast cytoplasm (12). However, in our study, flow cytometric quantification and comparison of decidual leukocyte subsets in IUGR with and without PE demonstrates that between these two pathologies of pregnancy, no significant differences are evident. Both IUGR alone and PE associated with IUGR are likely to represent a similar pathology, as we could not find a clear distinction in the immune regulation between these two disease entities. Interestingly, both IUGR and PE are associated with shallow extravillous trophoblast invasion, the clinical symptoms manifested in these diseases, however, are quite different. In comparison to normal pregnancy, placental weight and size are lower in both PE (13) and in IUGR (14). In conjunction with these findings, we find substantial differences in decidual leukocyte subsets between, on the one hand, these two pathologies of pregnancies and on the other hand normal control pregnancies. The significantly lower percentage of $\text{TCR}\gamma\delta^+\text{CD}3^+$ cells and $\text{CD}4^+\text{CD}25^+$ T cells in decidua basalis and parietalis associated with IUGR with and without PE is

suggestive for a regulatory role of these cells in normal pregnancy. The increase of uterine CD56^{bright}CD16⁻ NK cells in decidua basalis associated with IUGR without PE may indicate that their contribution to the local immune response is different in the setting of complicated pregnancy.

Because IUGR and PE often are associated with iatrogenic preterm delivery, the differences seen in decidual leukocytes in IUGR with and without PE may be gestational age dependent or may result from the pathogenesises of idiopathic IUGR and IUGR developing secondary to PE. To exclude the possibility that our findings are caused by differences in gestational age between the groups, we compared the findings within overlapping gestational age cohorts. Similar differences were observed in the groups with overlapping gestational ages, supporting a differential immunological environment in the maternal decidua in IUGR and PE/IUGR pregnancies as compared to control pregnancies, not explained by differences in gestational age.

In previous studies, significant changes in decidual leukocytes, including macrophages and NK cells, were found to be associated with PE and IUGR (15,16). While the etiology of PE and IUGR is not fully understood, it is suggested that immune dysfunction or maladaptation contributes to the pathogenesis of PE (17) and IUGR (18). Uterine NK cells express both activating and inhibitory killer cell immunoglobulin-like receptors (KIR), some of which interact with and recognize HLA-E, HLA-C, or HLA-G antigen (19). These ligand-receptor pairs suggest a role of these cells in controlling extravillous trophoblast invasion. In a recent study by Hiby et al., HLA-C ligands on fetal trophoblasts and KIR on uterine NK cells are implicated as factors associated with PE (20). PE is significantly more prevalent in women who are homozygous for inhibitory KIR and is associated with fetal expression of HLA-C ligands specific for inhibitory KIR. The absence of activating KIR likely favors PE, and an overly inhibited uterine NK cell may cause trophoblasts to prematurely cease remodelling of uterine vessels, increasing the likelihood of PE (20). It is suggested that cooperation between maternal NK cells and fetal trophoblasts remodels the blood supply. In the current study where only PE in association with IUGR was studied, not PE alone, we did not see a significantly lower

percentage of uterine NK cells in pregnancy associated with PE/IUGR. However, in the setting of idiopathic IUGR we find a significantly increased percentage of uterine NK cells in the decidua basalis, while such a trend is also apparent for the decidua parietalis.

In the current study, the percentage of $\text{TCR}\gamma\delta^+\text{CD}3^+$ cells was significantly lower in decidua basalis and parietalis associated with IUGR with and without PE/IUGR when compared with that of normal pregnancy. Considerable research and controversy on the expression of the $\gamma\delta$ T-cell receptor on decidual cells has developed in recent years. Although the function of these cells is not clearly identified, they may have a regulatory role in the decidua. It is suggested that the extrathymic T-cell maturation occurs in the decidua of early pregnancy and that $\text{CD}56^{\text{dim}}\text{CD}16^-$ decidual NK cells are immature progenitors for T cells (21). Furthermore, unlike $\text{TCR}\alpha\beta^+$ cells, $\text{TCR}\gamma\delta^+$ cells are not MHC-restricted. A significantly lower percentage of $\text{TCR}\gamma\delta^+\text{CD}3^+$ in IUGR and PE/IUGR may occur as a result of placental maldevelopment or may cause aberrant maldevelopment as a result of the lack of the necessary immune recognition of fetal antigen or cytokine production for proper placental development. The findings of the current study further suggest the potential and unique immunologic role of these cells in normal placental development.

In the current study, also a lower percentage of $\text{CD}4^+\text{CD}25^+$ cells was found in IUGR decidua basalis and parietalis and in PE/IUGR decidua parietalis. Our previous data has shown that the majority of $\text{CD}3^+\text{CD}25^+$ cells in decidua basalis and parietalis express CD4 (7) and that there is a higher percentage of $\text{CD}4^+\text{CD}25^{\text{bright}}$ T cells in the $\text{CD}4^+\text{CD}25^+$ T cell population in the decidua parietalis in comparison to the decidua basalis (22). It has been suggested that $\text{CD}4^+\text{CD}25^{\text{high}}$ regulatory T cells play an important role in the maintenance of pregnancy controlling the specific immune responsiveness to the fetus and that their recruitment or function may be impaired in pathological conditions (23). It is plausible that $\text{CD}4^+\text{CD}25^+$ cells are necessary for adequate fetoplacental development and that overly inhibited $\text{CD}4^+\text{CD}25^+$ T cells result in an immune response preventing such development, ultimately leading to IUGR.

The function of leukocytes in the decidua basalis and parietalis is important for our understanding of the dynamic balance occurring at the maternal-fetal interface. The current study does not indicate a potential distinction in immune regulation in the pathogenesis of IUGR with and without PE, although considerable differences are found compared to normal pregnancies. It is important to note that leukocytes at term pregnancy are remote from the events at early pregnancy when the pathology of IUGR and PE are believed to develop. Future studies are necessary to elucidate the functional roles of decidual leukocytes in the maintenance of pregnancy. Importantly, future research may establish effective preventive, diagnostic, and therapeutic strategies for IUGR and PE in hopes of improving future pregnancy outcomes.

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CHAPTER 7

INCREASED IMMUNE ACTIVATION OF DECIDUAL LEUKOCYTES IN A SURROGATE GESTATIONAL CARRIER

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ABSTRACT

OBJECTIVE: To compare leukocyte populations in term decidua basalis and parietalis from normal pregnancy with the pregnancy of a surrogate gestational carrier (SGC) who was mismatched for both human leukocyte antigen (HLA)-DR alleles and by chance, shared one set of HLA class I alleles with the fetus.

DESIGN: Case-control study.

SETTING: Academic research laboratory.

PATIENT(S): A SGC who received embryos conceived by in vitro fertilization and delivered a term singleton by elective cesarean section and 20 women who delivered term singletons by elective cesarean section.

INTERVENTION(S): Decidual samples were collected immediately following elective cesarean section. Decidual leukocytes were isolated by mechanical dispersion. Percentages of leukocyte populations were determined using flow cytometry.

RESULT(S): In SGC decidua basalis and parietalis, there were higher percentages of CD45⁺ leukocytes, CD56⁺CD16⁺ uterine NK cells, and CD3⁺HLA-DR⁺ and CD3⁺CD25⁺ T cells in comparison to that of normal term decidua basalis and parietalis. A higher percentage of CD4⁺ T cells also was found in SGC decidual tissues, possibly directly related to the presence of two HLA Class II DR mismatches in the fetus.

CONCLUSION(S): These findings suggest that during this SGC pregnancy, a more active immune response is present, possibly related the more profound presence of HLA class II mismatches and thus a greater requirement for immunomodulation. Further studies in SGC pregnancies are required to substantiate and extend these findings also to fully HLA class I and class II mismatched cases.

INTRODUCTION

The fetus with a genetic makeup derived from both the mother and father is often described as a semiallograft to the maternal immune system. The mechanism by which the semiallogeneic fetus avoids rejection despite a functionally intact maternal immune system is the central question in reproductive immunology.

The decidua, the maternal tissue in direct contact with fetal tissue, has been the focus of much investigation in an attempt to elucidate contributing factors involved in the delicate immunologic balance of pregnancy. There are two distinct regions of contact between the tissue of fetal origin and the maternal decidua – one between the invading interstitial trophoblasts and the decidua basalis, the site of implantation, and the other between the amniochorion and the decidua parietalis, the tissue lining the remainder of uterine cavity. Focusing primarily on the decidua basalis, immunohistological and flow cytometric studies of first trimester and term pregnancy decidua have identified numerous leukocytes, including macrophages, T lymphocytes, and large granular lymphocytes of the NK cell lineage with a unique CD56^{bright}CD16⁺CD3⁺ phenotype (1-8). Our previous studies on decidual leukocytes revealed significant differences in the distribution and proportion of these cells when comparing decidua basalis and decidua parietalis derived from placentas of uncomplicated term pregnancies following elective cesarean section with a higher percentage of CD56^{bright}CD16⁺ NK cells and of CD3⁺ T cells expressing activation markers CD25, Human Leukocyte Antigen (HLA)-DR, CD45RO, and CD69 in the decidua parietalis (9, 10).

The field of reproductive immunology in part has progressed in hope of gaining a better understanding of not only infertility and pregnancy complications, but also tolerance induction and the improvement of graft survival in the setting of allogeneic organ transplantation. Gestational surrogacy provides a unique model to investigate these fields and seems to more accurately represent the setting of organ transplantation. In this model, the surrogate typically has no genetic link to the fetus and is presented with not one, but two sets of allogeneic histocompatibility antigens. Serving as an invaluable therapy for a woman with

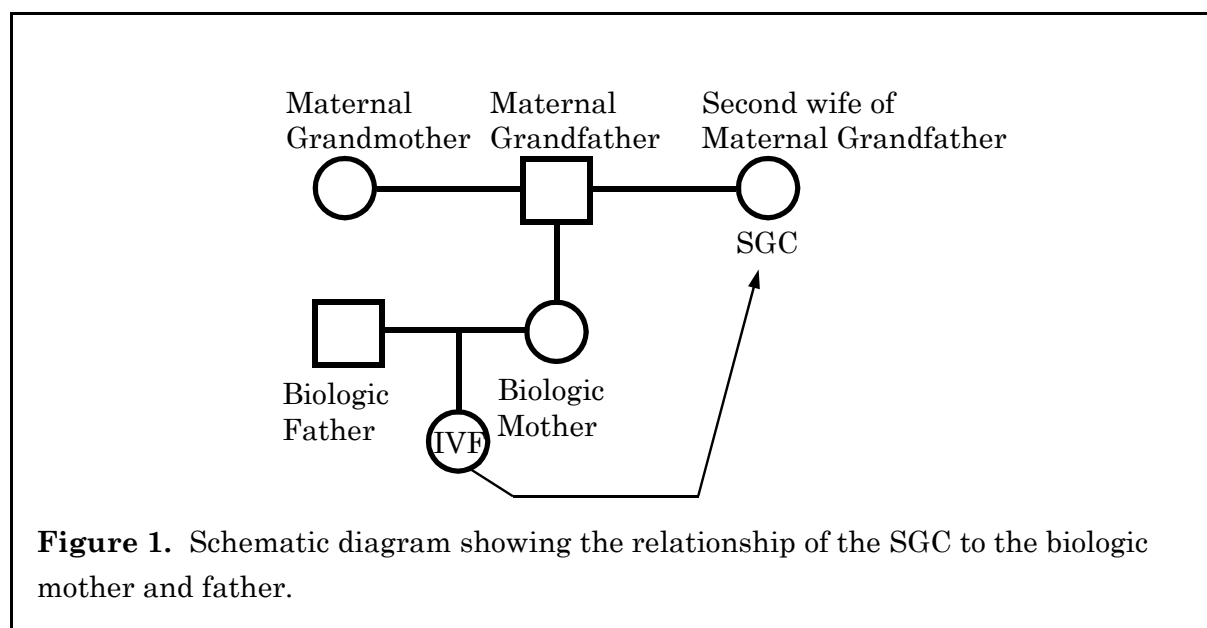
no uterus, a nonfunctioning uterus, or a contraindication to pregnancy, gestational surrogacy achieves fetal conception by using the gametes of the couple in which pregnancy is impossible or contraindicated. The resulting embryo is then transferred to the hormonally primed uterus of a surrogate gestational carrier (SGC). The fetus, thus, could be referred to as an “allograft” rather than a “semiallograft.” To our knowledge, no study has yet determined the decidual cell populations in the setting of gestational surrogacy.

Our aim in this study was to compare the proportion of different cell populations in the term decidua basalis and parietalis of a SGC with those in normal term decidua basalis and parietalis using flow cytometry and to relate these findings to the specific HLA mismatches between the SGC and the fetus.

MATERIALS AND METHODS

Subjects

The pregnancy of a SGC was conceived by *in vitro* fertilization (IVF) using donor ova and sperm from the biologic mother (age 25) and biologic father (age 28). The biologic mother had severe chronic kidney disease contraindicating pregnancy and necessitating the use of a SGC. The relationship of the SGC to the biologic mother and father is shown in Figure 1. Two embryos at the four to six cell stage were transferred into the hormonally primed uterus of the SGC (age 38) on day 17 of her cycle. The artificial cycle was initiated using Estradiol Valerate (Progynova, Schering, Weesp, the Netherlands) and Progesterone, and ovulation was suppressed using Triptorelin acetate (Decapeptyl, Ferring, Hoofddorp, the Netherlands). Pregnancy was confirmed 12 days following embryo transfer. Fetal heart activity was seen, and the crown-rump-length was 11mm in accordance with the gestational age of 55 days. Throughout the pregnancy, the growth of the fetus was monitored and progressed normally. The pregnancy was uncomplicated. At 41 weeks, an elective cesarean section was performed by maternal request without complications and resulted in the birth of an apparently healthy 3850 gram baby girl. A few days after birth, it was determined that the infant had a severe congenital heart malformation, which was successfully reconstructed in two subsequent operations.



Control samples from twenty uncomplicated normal term singleton pregnancies spontaneously conceived were obtained from placentas collected after elective cesarean section. Some samples were also used in previous studies (9, 10). All cesarean sections were performed for breech presentation and resulted in the birth of a non-growth restricted healthy neonate. Informed consent was obtained from all women, and ethical approval was received from the Leiden University Medical Ethics Committee. The decidual cells from these samples were isolated as abovementioned and used to compare results with those of the SGC.

HLA Typing

To determine the specific HLA mismatches between the SGC and the fetus, HLA Class I and II typings of the SGC, biologic mother, biologic father, and baby were performed. Blood samples were obtained, and molecular typing was performed on DNA by polymerase chain reaction/sequence-specific oligonucleotide using a reverse dot-blot method (11).

Isolation of decidua basalis and parietalis cells

Decidual samples were collected from the placentas immediately following elective cesarean section. Decidua basalis tissue samples were macroscopically dissected from the maternal-facing surface, and decidua parietalis samples were collected by removing the amnion and delicately scraping the tissue from the

chorion. Areas of ischemia or necrosis were avoided. The obtained tissue was finely minced between two scalpel blades in a small volume of phosphate buffered saline (PBS). Minced decidual fragments were gently worked through an open filter chamber (NPBI International BV; Emmer Compascuum, the Netherlands) with PBS. The resultant suspension was filtered through a 70µm sieve (Becton Dickinson Labware; Franklin Lakes, New Jersey), washed in PBS, and layered on an equal volume of ficoll (Leiden University Medical Center Pharmacy; Leiden, the Netherlands) at room temperature for density gradient centrifugation (for 20 min at 800g). Mononuclear cells were collected from the interface, washed twice, and resuspended in PBS for cell staining.

Cell Staining

Directly conjugated fluorescent antibodies directed against the following antigens were used for two-or three-color immunofluorescence staining: CD45 [HLe-1; Fluorescein isothiocyanate (FITC)], CD14 [Leu-M3; Phycoerythrin (PE)], CD3 [Leu-4; Allophycocyanin (APC)], CD8 (Leu-2a; FITC), CD4 (Leu-3a; PE), CD56 (Leu-19; FITC), CD16 (Leu-11a; PE), CD25 (Anti-IL-2R; FITC), and HLA-DR (Anti-HLA-DR; PE) all from Becton-Dickinson Immunocytometry Systems (San Jose, CA, USA).

All cells were stained at densities of 10^6 cells per ml in PBS and incubated for 30 minutes at 4°C in the dark. Following two washes with PBS, cells were resuspended in PBS and fixed with PBS containing 1% paraformaldehyde. Cells were then analyzed on a Becton-Dickinson FACScan flow cytometer. The double-labeled CD45 and CD14 sample was used to establish the optimal leukocyte gate based on light scatter profile and immunofluorescence (12). Approximately 3,000 events were acquired in the gating window. The acquired data were then analyzed using Cellquest software. The percentages of CD14⁺, CD19⁺, CD3⁺, CD56⁺CD16⁻, and CD16⁺ were enumerated within and represented as a percentage of the CD45⁺ cell fraction.

Statistical Analysis

For the normal term decidua basalis and parietalis samples (n=20), the 95% confidence interval (CI) was calculated to quantify the precision of the mean.

The results of SGC sample were then compared to this interval.

RESULTS

HLA mismatches between SGC and fetus

Although the SGC was the second wife of the father of the biological mother (Fig. 1.), by chance, she shared the HLA class I alleles which the fetus had inherited from the biological father (i.e. HLA A*0301, HLA B*0702 and HLA C*0702). The degree of HLA class I sharing between SGC and the fetus, therefore, was similar to a normal pregnancy (Table I). The fetus, however, was mismatched for both HLA class II DR alleles with the SGC, a fully allogeneic situation, never occurring in normal pregnancy.

Table I. Human Leukocyte Antigen (HLA) alleles of surrogate mother, biological parents and infant.

	HLA-A		HLA-B		HLA-Cw	
SGC	*0301	*2401	*0702	*0301	*2401	*0702
Biologic Father	*0201	*0301	*0702	*4101	*17	*0702
Biologic Mother	*3201	*6801	*3501	*4002	*0202	*0401
Infant	*3201	*0301	*0702	*4002	*0202	*0702
	HLA-DR		HLA-DP		HLA-DQ	
SGC	*0402	*1301	*0402	*0402	*0302	*0603
Biologic Father	*0401	*0701	*0401	*1401	*0302	*0303
Biologic Mother	*11	*11	*0301	*0402	*0301	*0301
Infant	*11	*0401	*0401	*0402	*0301	*0302

Note: Mismatches between the infant and SGC are in bold and italicized

Comparison of leukocyte subpopulations in the deciduas basalis of the SGC and of normal term pregnancy

The percentage of isolated leukocytes from the decidua basalis of the SGC and of normal term pregnancy were determined by flow cytometry for cell surface marker expression, compared and summarized in Table II. In the decidua basalis of the SGC, the percentage of CD45⁺ cells was higher in comparison with that of the decidua basalis of normal term pregnancy, 50.8% and 35.3% (95% Confidence Interval (CI)=29.3-41.4), respectively. This difference did not influence the subsequent analysis, as these were expressed as a percentage of CD45⁺ cells. The percentage of CD14⁺ cells was similar in the decidua basalis of the SGC and of normal term pregnancy, 12.7% and 17.7% (95% CI=11.4-24.0), respectively. Alternatively, there were apparent differences in the expression of CD16 and CD56 in the two groups. Importantly, the CD56⁺CD16⁻ uterine NK cell population was greater in the decidua basalis of the SGC, 31.8%, compared with normal term decidua basalis, 11.0% (95% CI=8.5-13.4), while the percentage of CD16⁺ cells was 32.2% in the SGC decidua basalis and 15.9% (95% CI=12.9-18.9) in normal term decidua basalis.

The percentage of T cells, as determined by the number of CD3⁺ cells, was higher in normal term decidua basalis than in the SGC decidua basalis, 55.4% (95% CI=51.1-59.6) and 40.6%, respectively. Of those CD3⁺ cells, the percentage of CD3⁺CD4⁺ cells was 68.7% in the SGC decidua basalis and 52.6% (95% CI=46.4-58.9) in normal term decidua basalis, while the percentage of CD3⁺CD8⁺ cells was 30.1% in the SGC decidua basalis and 43.2% (95% CI=38.0-48.4) in normal term decidua basalis. Additionally, the population of CD3⁺ cells expressing markers of activation was greater than that of normal term pregnancy. The percentage of cells expressing CD25, the IL-2 receptor, was greatly increased on the CD3⁺ cells in the SGC decidua basalis, 36.6%, in comparison with the normal term decidua basalis, 15.9% (95% CI=12.6-19.3). In the SGC decidua, the percentage of CD3⁺HLA-DR⁺ cells was markedly increased, 92.2%, in comparison to that of normal term decidua basalis, 29.5% (95% CI=23.7-35.3).

Table II. Percentage of leukocytes in term decidua basalis of SGC and normal term decidua basalis.

	SGC term decidua basalis (n=1)	Normal term decidua basalis (n=20)	95% CI
CD45⁺	76.7	28.8	20.7-36.9
CD14⁺	10.0	21.0	16.0-26.1
CD3⁺	62.2	60.6	49.0-72.3
CD16⁺	10.8	10.9	6.4-15.3
Uterine NK cells (CD56⁺CD16⁻)	42.9	28.2	19.6-36.8
CD3⁺CD4⁺	60.3	42.2	36.2-48.2
CD3⁺CD8⁺	36.2	53.8	48.8-58.7
CD3⁺CD25⁺	38.7	28.7	23.2-34.3
CD3⁺HLA-DR⁺	90.0	69.7	62.1-77.2

Note: Percentages of leukocytes in the SGC pregnancy that are outside the ranges of the control normal term pregnancies are in bold.

Comparison of leukocytes subpopulations in the decidua parietalis of the surrogate gestational carrier and of a normal term pregnancy

The leukocyte subpopulations in the decidua parietalis of the SGC were also compared to those of normal term decidua parietalis (Table III). Like decidua basalis, the expression of CD45 was greatly increased in the SGC decidua parietalis in comparison to normal term decidua parietalis, 76.7% and 28.8% (95% CI=20.7-36.9), respectively. Again, as the subsequent analysis were performed as a percentage of CD45⁺ cells, the following results are not influenced by this difference. The percentage of CD14⁺ cells was 10.0% in the SGC decidua parietalis and 21.0% (95% CI=16.0-26.1) in normal term decidua parietalis. In the decidua parietalis of the SGC, the percentage of CD16⁺ cells was 10.8% and 10.9% (95% CI=6.4-15.3) in normal term decidua parietalis. As in decidua basalis, the uterine NK cell population in the parietalis was greater in the SGC, 42.9% compared with normal term decidua parietalis, 28.2% (95% CI=19.6-36.8).

In both the decidua parietalis of the SGC and normal term pregnancy, a similar percentage of CD3⁺ cells was found, 62.2% and 60.6% (95% CI=49.0-72.3). Of those CD3⁺ cells, the percentage of CD3⁺CD4⁺ cells was higher in the decidua parietalis of the SGC, 60.3%, than in the decidua parietalis of normal term pregnancies, 42.2% (95% CI=36.2-48.2). The percentage of CD3⁺CD8⁺ cells, on the other hand, was higher in the decidua parietalis of normal term pregnancy, 53.8% (95% CI=48.8-58.7) than in the decidua parietalis of the SGC, 36.2%. Similar to the SGC decidua basalis, the percentage CD3⁺ expressing CD25 was increased in the SGC decidua parietalis, 38.7%, in comparison with that of normal term decidua parietalis, 28.7% (95% CI=23.2-34.3). Similarly, the percentage of CD3⁺HLA-DR⁺ cells was higher in the SGC decidua parietalis, 90.0%, than in normal term decidua parietalis, 67.9% (95% CI=62.1-77.2).

Table III. Percentage of leukocytes in term decidua basalis of SGC and normal term decidua basalis.

	SGC term decidua basalis (n=1)	Normal term decidua basalis (n=20)	95% CI
CD45⁺	50.8	35.3	29.3-41.4
CD14⁺	12.7	17.7	11.4-24.0
CD3⁺	40.6	55.4	51.1-59.6
CD16⁺	32.2	15.9	12.9-18.9
Uterine NK cells (CD56⁺CD16⁻)	31.8	11.0	8.5-13.4
CD3⁺CD4⁺	68.7	52.6	46.4-58.9
CD3⁺CD8⁺	30.1	43.2	38.0-48.4
CD3⁺CD25⁺	36.6	15.9	12.6-19.3
CD3⁺HLA-DR⁺	92.2	29.5	23.7-35.3

Note: Percentages of leukocytes in the SGC pregnancy that are outside the ranges of the control normal term pregnancies are in bold.

DISCUSSION

Results of this study show that in the setting of gestational surrogacy, there are greater percentages of CD56⁺CD16⁻ uterine NK cells and activated CD3⁺ T cell expressing HLA-DR or CD25 in the SGC decidua basalis and parietalis in comparison with that of normal term pregnancy. Differences in leukocyte populations in gestational surrogacy and normal pregnancy suggest that the regulation and function of leukocytes may differ in these two settings. Contributing factors leading to these differences are unknown; however, there are several potential mechanisms.

The difference in the percentage of T cells expressing HLA-DR or CD25 is most remarkable in the SGC decidua basalis, the site of implantation. This difference may be due to a strong immune response against fetal antigens or may serve as a mechanism to protect the allograft fetus. In normal pregnancy, there is recent evidence in the murine model that there is systemic tolerance of the fetus as maternal T cells acquire a transient state of tolerance to paternal antigens during pregnancy. Fetal antigen-specific T cells downregulate CD8 co receptors during pregnancy, resulting in reversible functional unresponsiveness to paternal antigens (23). T cells specific for fetal antigens decrease in an antigen-specific manner during pregnancy and remain low postpartum (24). Furthermore, maternal regulatory T cells are expanded systemically during pregnancy in alloantigen-independent manner, and it is suggested that this population of cells is essential for the suppression of maternal immune aggression directed against the fetus (25). Locally, decidual leukocytes may be involved in maternal tolerance of the fetus. The CD3⁺CD25⁺ population was not further defined in this study, but it might well be that these cells represent both activated T cells and regulatory T cells. This will be the topic of investigation in further cases of SGC.

The higher percentage of uterine NK and T cells expressing markers of activation in both the decidua basalis and parietalis in the SGC pregnancy could be due to the HLA differences between the SGC and fetus. In the current study, the SGC shares the paternal HLA-A and -B antigens (A3 B7) with the fetus, but has a different HLA-DR allele, resulting in a fetus differing for both HLA-DR

alleles with the SGC. This additional difference or the lack of sharing of HLA-DR allele with the mother could lead to a stronger maternal immune response, resulting in an increased percentage of activated CD4⁺ T cells. Despite this stronger immune response, pregnancy evolved uncomplicated.

Interestingly, several studies indicate that sharing of human leukocyte antigens may affect the success of IVF. There is a highly significant excess of HLA sharing in couples where IVF treatment fails (17-19). Furthermore, HLA matching between couples is associated with spontaneous abortion (20, 21). Apparently, immune recognition of pregnancy is important for the maintenance of pregnancy. In fact, lack of immune recognition is thought to be deleterious to pregnancy (22). The differences in HLA sharing and the more intense immune activation observed may have been beneficial for the outcome of the SGC pregnancy. However, it is unclear whether a certain degree of HLA sharing between mother and fetus is necessary for a successful pregnancy. More studies stemming from this case report are required to elucidate the effects of IVF and HLA mismatches on the decidual cell population.

Following allogeneic or autologous bone marrow transplantation, NK cells are among the first lymphocytes to repopulate the peripheral blood. In some patients, up to 40% of peripheral blood lymphocytes are CD56^{bright}CD16^{dim/-} (26, 27). It is suggested that these cells serve a unique functional role in the innate immune response as the primary source of NK cell-derived immunoregulatory cytokines (28). In pregnancy, CD56^{bright}CD16⁻CD3⁻ cells represent the major subset of NK cells in the uterus (2, 3). Trafficking, homing, or in situ proliferation have been implicated as possible mechanisms in the variation of uterine NK cells in the endometrium and decidua (29). Although the specific role of uterine NK cells is still unknown, it is suggested that they regulate placental development by mediating maternal mucosal and arterial function and fetal extraembryonic trophoblast invasion (30). It is plausible that there are important differences regulating uterine NK cell proliferation or function in the setting of gestational surrogacy compared to normal pregnancy.

Generally, the IVF procedure is associated with a significantly worse perinatal

outcome than that of spontaneously conceived pregnancies. In pregnancy conceived by IVF as compared to naturally conceived pregnancy, there is increased incidence of vaginal bleeding, hypertension requiring hospitalization, intrauterine growth retardation, placenta previa, and preterm delivery (13, 14). Patients undergoing IVF, however, are on average older, more frequently primiparous than the normal obstetric population and are infertile or subfertile, conferring an additional risk of maternal and perinatal complications (15, 16). The outcome of IVF pregnancies in surrogate gestational carriers, who are generally healthy young fertile women, is not included in the abovementioned studies. In light of the current study, the IVF procedure, however, could have contributed to the higher percentage of activated cells and uterine NK cells.

The study of the immunology of the uterus, an immune privileged site, has thrived in hopes of gaining a better understanding of tolerance induction in the setting of allogeneic organ transplantation. Over the last two decades, a wide array of studies has been performed on the immune components of normal early and term pregnancy with this goal in mind. While it has been noted that the comparison of pregnancy with tissue transplantation may be inaccurate and a misleading framework (31, 32), the setting of gestational surrogacy, nevertheless, is an interesting model in which to study the immunology of the maternal-fetal interface. The procedure, however, is relatively uncommon, making it difficult to obtain many samples of term decidual tissue. Although additional studies are necessary in order to confirm these findings, this study provides unique insight into the immunology and maintenance of pregnancy.

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CHAPTER 8

DISCUSSION AND SUMMARY

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DISCUSSION

During pregnancy, the maternal immune system is functionally intact, capable of fighting infection and protecting from foreign pathogens. The fetus with a genetic makeup derived from both the mother and father, escapes immune rejection from the maternal immune system and is tolerated for the duration of pregnancy. The mechanism by which this phenomenon, known as the paradox of pregnancy, occurs is the central question in reproductive immunology and has been the focus of extensive research for the last fifty years. The solution to the paradox of pregnancy has many implications in the management and treatment of complicated pregnancy, infertility, assisted reproduction and allogeneic graft survival. To gain a better understanding of the paradox of pregnancy and local immunoregulatory mechanisms, we focused our studies on leukocytes at the maternal-fetal interface. Locally, the decidua basalis and parietalis may provide a special environment to mediate the delicate balance between fetal antigen presentation and maternal recognition and response.

DIFFERENTIAL DISTRIBUTION OF LEUKOCYTES IN NORMAL TERM DECIDUA BASALIS AND PARIETALIS

Our studies revealed that there are significant differences in the distribution and proportion of leukocytes when comparing decidua basalis and decidua parietalis derived from placentas of uncomplicated term pregnancies. In **Chapters 2** and **3**, flow cytometric quantification showed significantly higher percentages of CD56^{bright}CD16⁻ NK cells, CD3⁺ T cells expressing CD25, HLA-DR, CD45RO, CD69 or TCR $\gamma\delta$ in the decidua parietalis in comparison to the decidua basalis. Importantly, these differences suggest that the regulation of leukocytes in these two regions of the maternal-fetal interface and their *in vivo* function may differ.

Mechanisms involved in differential distribution of decidual leukocytes

The factors leading to the unequal distribution of NK cells, T cells expressing

activation markers and $\gamma\delta$ T cells in the decidua basalis and parietalis are unknown. There are, however, several potential mechanisms.

Differences between the two sites of the maternal-fetal interface exist. When comparing the placenta and fetal membranes, there are inherent differences in development, anatomy and histology (1). The placenta is the site of implantation and trophoblast invasion. The decidua basalis comes into direct contact with trophoblasts on the first day of implantation. The fetal membranes develop from the gestational sac and are not associated with invasion of trophoblasts. The decidua parietalis only comes into contact with trophoblasts after the fourth month of pregnancy. Macroscopically, these tissues appear very different. The placenta is vascular with a thick chorionic parenchyma containing several lineages of trophoblasts, while the fetal membranes are avascular with a thin chorionic membrane containing only extravillous trophoblasts. Furthermore, when comparing the two sites of the maternal-fetal interface, there is differential chorionic HLA expression (2). There are also differences in the cytokine microenvironment (3) and in the production of mediators, such as hormones and prostaglandins (4-11). Such differences could lead to or result from the differential distribution of cell types when comparing the two sites. Interaction of trophoblasts with decidual leukocytes could lead to altered function of either cell type resulting in differences between the decidua basalis and parietalis, including altered cytokine production or cell surface molecule upregulation or downregulation. These differences have important consequences for our understanding of the maternal-fetal interface.

HLA expression

A difference in the expression of HLA-C, HLA-E and HLA-G in tissue adjacent to decidua may account for the differential distribution. Through HLA expression, extravillous trophoblasts are capable of interacting with receptors expressed by uterine NK cells, T cell subsets and macrophages leading to activation or inhibition (12-17). It is plausible that differential HLA expression in the chorion of the placenta and fetal membranes leads to the inhibition or activation of leukocytes in the decidua.

Homing and maturation

Another mechanism to account for the differential distribution of cells in the decidua, specifically the higher number of CD56^{bright}CD16⁻ uterine NK cells in the decidua parietalis may involve homing or maturation. It is plausible that there is homing of CD56^{bright} cells from the maternal circulation to the decidua. A study on trafficking of NK cells shows that cells from secondary lymphoid tissues of pregnant mice can reconstitute uterine NK cells in NK-cell-deficient mice and that human peripheral blood CD56^{bright} cells can adhere selectively to mouse uteri (18). Homing and recruitment of uterine NK cells to the maternal-fetal interface may be mediated by adhesion molecules (18, 19, 20), by molecules expressed by trophoblasts, such as monocyte inflammatory protein (21), or by molecules expressed by NK cells, such as very late antigen-1 or CD49a (22).

There is perhaps maturation from a uterine NK cell precursor in term decidua parietalis. Significantly more $\gamma\delta$ T cells are found in the decidua parietalis than in the decidua basalis. There has been considerable controversy concerning the expression of the $\gamma\delta$ T-cell receptor on decidual T cells. The study by Mincheva-Nilsson et al. suggests that extrathymic T-cell maturation occurs in the decidua of early pregnancy and that CD56⁺CD16⁻ decidual NK cells are immature progenitors for T cells (23). The significant increase in $\gamma\delta$ T cells and CD56⁺CD16⁻ NK cells further suggests the potential and unique immunologic role of these cells in the decidua parietalis.

Cytokine microenvironment and secreted mediators

Differences in the cytokine microenvironment or presence of other secreted messengers between the two sites are potential explanations for the differential distribution. It has been suggested, for example, that uterine NK cell function varies in different areas of the decidua basalis; those cells which are in perivascular position are exposed to a different microenvironment than distant cells (24). In first trimester decidua, increased IFN- γ levels have been reported in the decidua basalis compared to decidua parietalis (3). Lastly, there is also differential expression of hormones and mediators like human chorionic gonadotropin (HCG) and pregnancy-specific glycoprotein (PSG) (4). PSG, part of the immunoglobulin superfamily, is selectively expressed in the membranes and

placenta and has been suggested to regulate T cell function during pregnancy (25). Estrogen and progesterone are produced by the placenta. These hormones are modulators of the innate and acquired immune responses (26, 27). It is plausible that there is differential expression of these hormones at the maternal-fetal interface. Lastly, numerous other messengers and peptides (5-11) also have differential expression when comparing the fetal membranes and placenta and may serve a potential role in the regulation of immune cells at the maternal-fetal interface.

The significantly higher percentage of CD3⁺ T cells expressing CD25, CD69, HLA-DR, and CD45RO in the decidua parietalis indicated that T cells in this site may be activated in term pregnancy. Immunohistological and flow cytometric studies focusing on decidua of the first trimester have shown that various types of decidual lymphocytes are activated and produce cytokines (28-37). Maternal T cells may promote and contribute positively to placental growth and trophoblast invasion (38). We extended these findings to suggest that regionally activated T cells in the decidua parietalis may play an important role in the maintenance of term pregnancy. Additionally, we examined peripheral blood samples for comparison with corresponding decidual samples for many of our subjects and found that there were fewer CD3⁺ cells expressing markers of activation in peripheral blood, suggesting a possible difference in local and systemic immune regulation in pregnancy.

Factors leading to the unequal distribution and activation of T cells in the decidua are unknown. Mediators, such as prolactin or indoleamine 2,3-dioxygenase (IDO), are plausible candidates. Prolactin, known to be produced in the decidualized human endometrium, transported through fetal membranes, and secreted into amniotic fluid (39-41), induces cell proliferation and activation and is involved in the maintenance of immunocompetence (42, 43). Furthermore, prolactin receptors have been identified in the placenta and amnion (44). There are regional and periodic differences in prolactin gene expression in the decidual cells during pregnancy (45). Increased prolactin expression in the decidua parietalis could lead to T-cell activation. IDO, another important mediator, is synthesized and secreted by syncytiotrophoblasts and catabolizes and degrades

tryptophan (46). Present in human placental villous explants, IDO has been shown to inhibit lymphocyte proliferation via tryptophan depletion (47-49). Increased IDO enzymatic levels or activity or lower levels of tryptophan in the decidua basalis are possible mechanisms for differences in T cells. In a study by Sedlmayr, IDO was irregularly localized in term placental samples to the mesenchymal core and found in isolated areas of the syncytiotrophoblast (50).

Fas-mediated apoptosis

Selective Fas-mediated apoptosis is another plausible mechanism to explain lower percentage of activated T cells in the decidua basalis. Fas, a cell surface receptor of the tumor necrosis factor family (CD95), mediates apoptosis by binding FasL (51, 52). In a study by Hammer *et al.*, apoptotic nuclei arising from CD45⁺ leukocytes were detected scattered within the entire functional stroma of the decidua parietalis. In the decidua basalis, the apoptotic leukocytes were irregularly distributed and concentrated in the area around necrotic nuclei and especially in the primary fetal-maternal contact zone where trophoblasts from the cell columns began to invade the maternal tissue (53). A recent study of first trimester decidua basalis indicates the FasL expression by maternal decidual cells is inversely related to Fas-expressing leukocytes in the same region (54). Maternal decidual cells may be involved in preventing recruitment of leukocytes at the maternal-fetal interface via Fas-mediated apoptosis and thus promote trophoblast invasion (54). In light of these studies, our studies suggest that there are fewer activated cells in the decidua basalis because these cells have been activated and then selectively programmed to die to allow trophoblast invasion. In the decidua parietalis, where there is no invasion by trophoblasts, but simply contact, the activated cells are not killed to the same extent by trophoblastic FasL. It is also plausible that there is perhaps a lower expression of Fas on T cells in the decidua parietalis or higher expression of FasL in the decidua cells of the fetal chorionic membrane.

Contamination and technique

It is unlikely that contamination of decidual samples by maternal peripheral blood or umbilical cord blood accounts for the regional differences in the two decidual sites. Primarily, tissue samples used in our studies were carefully

collected and thoroughly washed prior to cell isolation. This is a commonly described method to remove possibly contaminating maternal or fetal blood from the tissue. Secondly, in one of our uterine NK cell study, flow cytometric results were confirmed by immunohistochemical analysis, in which only cells located in the tissue and not in vessels were quantified. Additionally, the minor percentage of B cells found in both decidua basalis and parietalis and the lack of a significant difference between the percentages of B cells in the decidual sites suggests that circulating blood, which contains a higher percentage of B cells, did not appreciably contaminate decidual samples. We examined peripheral blood samples for comparison with corresponding decidual samples for many of our subjects and find that there were fewer CD3⁺ cells expressing markers of activation in peripheral blood, suggesting a possible difference in local and systemic immune regulation in pregnancy.

In our studies, data on decidual leukocytes was presented as a percentage of the CD45⁺ cell fraction. No significant difference in the absolute number of CD45⁺ cells in decidua basalis and decidua parietalis was found in any of our studies of normal term pregnancy. Therefore, data was presented as a percentage of the absolute number of CD45⁺ cells acquired within the flow cytometric gating window in order to better characterize the relative proportion of the other cell populations. Follow-up studies on decidual leukocytes within our group now use four color staining techniques with CD45 as one of the markers in every sample and show comparable results.

Implications

The possibility of differences between decidual cells in distinct areas of the placenta and fetal membranes has important consequences for *in vitro* studies of decidual leukocytes. Numerous studies using decidua do not distinguish the difference between decidua basalis and decidua parietalis and do not indicate which tissue was used. Because of possible phenotypic and functional differences in the decidual leukocytes at the two sites, it will be important to clearly state the origin of decidua in future studies in the field.

PROPORTION OF LEUKOCYTES IN TERM DECIDUA BASALIS AND PARIETALIS

Our studies verify that leukocytes are present in both the decidua basalis and decidua parietalis in term pregnancy. Specifically, uterine NK cells account for a substantial proportion of leukocytes in term decidua parietalis as confirmed by flow cytometry and immunohistochemistry, thus indicating that these cells, contrary to current belief, are not only restricted to early pregnancy. It is known that CD3⁺ lymphocytes are the most abundant lymphocyte population in term decidua basalis (55). We extended this finding to show that CD3⁺ lymphocytes are also the most abundant lymphocyte population in term decidua parietalis, and that these cells express markers of activation.

Potential role of leukocytes in term decidua

While it is well accepted that decidual leukocytes play an essential role in implantation and early pregnancy, the role of these cells in term pregnancy is less clear. Uterine NK cells in term pregnancy may serve to protect the fetoplacental unit from infection. NK cells are an important component of the innate immune system and potentially may reduce susceptibility to infection from viral or other foreign pathogens during pregnancy. While placentation has already been established by term pregnancy, it is plausible that the control of trophoblast invasion remains essential even in the third trimester. Decidual leukocytes may continue to control such invasion through cell-cell interaction, by creating the appropriate cytokine environment at the maternal-fetal interface and by maintaining immune recognition in pregnancy.

FUNCTIONAL STUDY OF LEUKOCYTES IN TERM DECIDUA BASALIS AND PARIETALIS

The function of decidual leukocytes is an important focus in order to understand the role of these cells in the maintenance of pregnancy. Using a one-way mixed lymphocyte culture in **Chapter 5**, we showed that these cells proliferate and produce cytokines in response to fetal and allogeneic cells, indicating that decidual leukocytes are capable of initiating and stimulating a classical alloimmune response.

It has been well established that decidual leukocytes from both first trimester and term pregnancy decidua do not proliferate in response to extravillous trophoblasts from the chorion or chorionic laeve (55, 56). To our knowledge, there are no previous studies that examine the proliferation of decidual leukocytes from early or term pregnancy in response to fetal UCB cells. In our study, a significantly higher proliferative response was seen when leukocytes from term decidua basalis and parietalis were cultured with UCB cells, cells of fetal origin, as compared to controls. It has been shown that UCB cells are capable of eliciting a proliferative response as stimulators in a MLC comparable to that of irradiated adult PBL (57). Although UCB cells essentially do not come into direct physical contact with decidual leukocytes as a result of anatomical separation, fetal cells of hematopoietic origin such as nucleated red blood cells, lymphocytes, and stem cells enter the maternal circulation during all pregnancies (58, 59, 60). Furthermore, it is of scientific interest to confirm that term decidual leukocytes are capable of eliciting an immune response against cells of fetal origin, a finding not previously reported.

In our study, we also showed that in the decidua, alloreactive cells respond to HLA mismatched allogeneic PBL and towards UCB of the fetus. It is plausible that the mechanism by which decidual leukocytes recognize and mount an immune response may occur via the indirect pathway of allorecognition. Although it is suggested that the fetus escapes rejection from the maternal immune system by mechanisms involving HLA, KIR, and cytokines, these data suggest that there is a local role for regulatory mechanisms controlling the specific immune responsiveness to the fetus in which (CD4⁺CD25^{hi/bright}) regulatory T cells may be involved. In preliminary studies performed by our group, analysis confirms the presence of this population in the decidua basalis and parietalis (61). Although it has been shown by others that early human pregnancy decidua contains functional CD4⁺CD25^{bright} T cells (62), functional studies of term decidual regulatory T cells are currently ongoing. Term regulatory T cells could potentially serve as mediators in maternal tolerance of the fetal compartment by potentially suppressing the activation and proliferation of CD4⁺CD25⁻ T cells, CD4⁺CD25⁺ T cells, and also CD8⁺ T cells.

Interestingly, it has been shown that decidual leukocytes derived from first trimester decidua are not capable of proliferating in response to allogeneic PBL (32, 56, 63). In the study by King *et al.*, no proliferative response was observed in decidual leukocytes from first trimester tissue cultured with irradiated allogeneic PBL as stimulators in a one-way MLC (64). In our functional study, we shown that decidual leukocytes from uncomplicated term pregnancy, in contrast to that described from early pregnancy, significantly proliferate in response to allogeneic PBL. The difference in the proliferative response of first trimester and term pregnancy decidua derived leukocytes against allogeneic PBL is likely due to a change in the distribution and proportion of the responsible immune cells. The varying proportion of cell types throughout pregnancy and their function could account for the difference in proliferation in response to allogeneic cells when comparing early and term pregnancy.

ELECTIVE CESAREAN SECTION AND SPONTANEOUS VAGINAL DELIVERY

To pursue our studies further, we compared leukocytes from decidua basalis and parietalis collected after spontaneous vaginal delivery (SVD) and cesarean section (CS) as described in **Chapter 4**. Our findings showed that labor also is associated with significant differences in the distribution and proportion of decidual leukocyte subpopulations within the CD45⁺ cell fraction, including a higher percentage of macrophages, CD56^{dim}CD16⁺ NK cells and B cells and a lower percentage of CD3⁺CD4⁺CD25⁺ cells, CD3⁺CD4⁺HLA-DR⁺ and CD3⁺CD8⁺HLA-DR⁺ cells.

Spontaneous labor at term can be described as a cascade of events that is associated with specific changes in leukocyte subpopulations, cell adhesion molecule expression and various immunologic mediators (65-68). A possible mechanism to account for the changes in decidual leukocytes following labor may involve migration of these cells to or from other tissue sites, such as to the cervix or myometrium or from peripheral blood (66, 69, 70). The decreased percentage of macrophages found in the decidua basalis and parietalis following labor may account for the presence of these cells in the cervix or myometrium. It

has been suggested that cell adhesion molecules are involved in the control of leukocyte infiltration in the cervix and myometrium during late pregnancy, cervical dilation and labor (67, 70, 71).

The increased percentage of CD56^{dim}CD16⁺ NK cells found in the decidua basalis and parietalis following labor may be mediated by specific signaling mediators, such as cytokines or chemokines. CD56^{dim}CD16⁺ NK cells express chemokine receptors CXCR1, CXCR2, CXCR3, CXCR4 and CX3CR1 (72). It is also plausible that these cells are recruited from the peripheral blood where they represent the major NK cell subclass to the decidua during labor by chemokines. In the current study, there was a significantly higher percentage of CD19⁺ B cells in decidua basalis from SVD compared to that from CS. It is possible that labor preferentially attracts and activate B cells in decidua basalis.

Importantly, a reduction in CD4⁺CD25⁺ T cells was observed when SVD samples were compared to CS samples. While these cells may represent a small population of regulatory T cells, we did not specifically distinguish these cells as regulatory T cells based on high CD25 expression. Theoretically, in the setting of labor, a decrease or disappearance of regulatory T cells from the decidua may enable the activated alloreactive CD4⁺CD25⁻ and CD8⁺ T cells to reach their effector status thus initiating the process of labor and delivery.

CLINICAL IMPLICATIONS

Pathologic pregnancy

Normal placentation is critical for a successful pregnancy. Studies into the role of immune cells at the maternal-fetal interface may be beneficial for the management of pathologic pregnancies in which placentation is abnormal. Fetal trophoblasts must safely and accurately invade the maternal endometrium in order to establish the crucial uteroplacental circulation. This invasion is a highly delicate balance. Excessive invasion at the surface of the myometrium or muscle layer of the uterus is a condition known as placenta accreta. Excessive invasion into the myometrium is known as placenta increta, while through the myometrium is known as placenta percreta. Such disorders are associated with

maternal risks such as hemorrhage and even death. Inadequate invasion is associated with preeclampsia. Preeclampsia is a significant complication of pregnancy defined by hypertension, proteinuria, and edema, presenting during the second trimester, affecting approximately 5-8% of pregnancies (73). IUGR is also a clinically relevant complication of pregnancy associated with increased perinatal morbidity and mortality. It is characterized as a syndrome marked by failure to reach growth potential, affecting 3-7% of pregnancies and can present alone as idiopathic IUGR or in association with PE (74, 75).

Because complications such as preeclampsia, placenta accreta, increta and percreta and IUGR may reflect complications in placentation much earlier in pregnancy, it is important to study the potential immunoregulatory mechanisms involved at the maternal-fetal interface in both early and term decidual tissue. One of our studies focused on the role of decidual leukocytes in IUGR and PE by quantifying the distribution and percentage of leukocytes in decidua basalis and parietalis from placentas associated with IUGR with and without PE and by comparing with normal term decidua in **Chapter 6**. Results showed significant differences in decidual leukocytes exist between IUGR with and without PE indicating a potential distinction in the role of these cells in the immunoregulation of each disease.

While the etiology of PE and IUGR is not fully understood, it is suggested that immune dysfunction or maladaptation contributes to the pathogenesis of PE (76) and IUGR (77). Primipaternity (78), decreased sperm exposure (79), donor insemination (80), oocyte donation (81), family history (82) and genetic predisposition (83) confer an increased risk. Uterine NK cells also are implicated in the immune regulation of pathologic pregnancies. In a recent study by Hiby *et al.*, HLA-C ligands on fetal trophoblasts and KIR on uterine NK cells are implicated as factors associated with PE (83). PE is significantly more prevalent in women who are homozygous for inhibitory KIR genes than in those who are heterozygous or homozygous for activating KIR. The increased prevalence in women homozygous for inhibitory KIR was due to pregnancies where the fetus expressed HLA-C ligands that were specific for inhibitory KIR. The study suggests that the absence of activating KIR favors PE and that an overly

inhibited uterine NK cell causes trophoblasts to prematurely cease remodeling of uterine blood vessels, increasing the likelihood of PE.

Other pathologic complications associated with pregnancy and potentially or partially mediated by immune dysregulation include molar pregnancy and recurrent miscarriage. A molar pregnancy or hydatidiform mole is a gestational trophoblastic disease in which trophoblast cells proliferate abnormally. A complete hydatidiform mole is derived entirely from the paternal genome, is diploid with a 46,XX karyotype and characterized by generalized trophoblastic hyperplasia and no fetus. Because its chromosomes are entirely paternal in origin, a complete mole represents a fetal allograft rather than semiallograft and thus represents a unique setting in which to study the immunology of the maternal immune response. Studies have shown an increase in activated CD4⁺ T cells in decidua associated with complete hydatidiform moles compared with normal early pregnancy suggesting an altered maternal immune response against molar trophoblast (84). Recurrent miscarriage, usually defined as the loss of three or more consecutive pregnancies prior to 20 or even 28 weeks of pregnancy (85), also represents a complication in pregnancy and is another interesting model in which to study immune cells at the maternal-fetal interface. Studies on recurrent miscarriage or spontaneous abortion suggest that it is associated with an increase in CD56⁺ cells (86, 87) in peripheral blood and a concurrent decrease in CD56⁺ cells (88) and T cells (89) in the decidua.

Assisted reproduction

An important goal of understanding the immunology of the maternal-fetal interface is to improve the diagnosis and management of infertility with assisted reproduction. The setting of gestational surrogacy is an interesting model in which to study the topic and is described in **Chapter 7**. In this model, the surrogate typically has no genetic link to the fetus and is presented with not one, but two sets of mismatched histocompatibility antigens. Serving as an invaluable therapy for a woman with no uterus, a nonfunctioning uterus, or a contraindication to pregnancy, gestational surrogacy achieves fetal conception by using the gametes of the “infertile” couple, and the resulting embryo is then transferred to the hormonally primed uterus of the surrogate gestational carrier

(SGC). The fetus, thus, could be referred to as an “allograft” rather than a “semiallograft.” We reported the case of a surrogate gestational carrier and found that there are higher percentages of CD56⁺ CD16⁺ uterine NK cells, and activated CD3⁺ HLA-DR⁺ and CD3⁺ CD25⁺ T cells in comparison to that of normal term decidua basalis and parietalis. Although further studies using this model are required to elucidate the function of these cells, this case report provides new insight into the immunology of the maternal-fetal interface.

Ultimately, developing techniques to control mechanisms involved in maternal immune tolerance to the fetus would be highly beneficial in the clinical setting of obstetrics and transplantation. Such techniques may help overcome such complications as preterm labor, infertility, and preeclampsia. It appears that a specific balance of immune responsiveness is necessary to promote the normal growth of the fetus.

FUTURE STUDIES

In summary, our study establishes that there are significant differences in the distribution and function of leukocytes in term decidua basalis and decidua parietalis in the setting of normal term pregnancy, complicated pregnancy, and gestational surrogacy. To improve our understanding, it is important that future studies concentrate on mechanisms involved in the regional distribution, including a testable model to specifically study the interaction between leukocytes in decidua parietalis and amniochorion. An important focus may involve mediators in recruitment, proliferation and cell death at the maternal-fetal interface. Functional studies using purified decidual cell populations are warranted to better understand the proliferative response against allogeneic and UCB cells. There is also interest in further characterization of several cell populations, including NKT cells, dendritic cells, macrophages and $\gamma\delta$ T cells, but most importantly the CD3⁺CD4⁺CD25⁺ regulatory T cell population, both phenotypically and functionally. could be an important focus of future research. Expectantly, the next fifty years of research in the field of reproductive immunology will prove to be as fruitful as the last fifty years, ultimately resulting in powerful advances in immunology, obstetrics and transplantation.

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CHAPTER 9

SAMENVATTING: DUTCH SUMMARY

SAMENVATTING

Tijdens de zwangerschap is het maternale immuun systeem intact. Het blijft in staat het lichaam tegen infecties te verdedigen. Merkwaardig genoeg ontsnapt de foetus tijdens de zwangerschap aan deze immuunafweer, hoewel de foetus voor de helft uit vaderlijke genen bestaat. Het mechanisme achter deze paradox van de zwangerschap is de vraag waar het in de reproductieve immunologie om draait, en vormt de kern van uitgebreid onderzoek op dit gebied gedurende de laatste vijftig jaar. Het vinden van de oplossing van deze paradoxale puzzel kan belangrijke implicaties met zich mee brengen voor de begeleiding en behandeling van gecompliceerde zwangerschappen, als ook voor vruchtbaarheidsbehandelingen en zelfs voor orgaanoverleving na transplantatie. Onze studies richtten zich op de leukocyten op het grensgebied tussen moeder en kind in de placenta om zo een beter begrip te verkrijgen van de immuunregulatie die daar plaatsvindt. De decidua basalis en parietalis zouden een belangrijke omgeving kunnen vormen die de delicate balans tussen het aanbod van foetale antigenen aan de ene kant en maternale antigeen herkenning en reactie aan de andere kant medieert.

PROPORTIONELE VERDELING VAN LEUKOCYTEN IN DE NORMALE ÀTERME DECIDUA BASALIS EN PARIETALIS

Onze experimenten laten significante verschillen in de proportionele verdeling van leukocyten zien in de decidua basalis in vergelijking met de decidua parietalis verkregen uit placenta's na normale, ongecompliceerde àterme zwangerschappen. In **hoofdstuk 2 en 3** worden door middel van flow cytometrie leukocyten gekwantificeerd. Significant hogere percentages CD56^{bright}CD16⁻ NK cellen, CD3⁺ T cellen die tevens CD25 tot expressie brengen, HLA-DR, CD45RO, CD69, of TCR $\gamma\delta$ worden aangetroffen in de decidua parietalis dan in de decidua basalis. Dit is een belangrijke bevinding die suggereert dat de immuunregulatie en de *in vivo* functie in deze foeto-maternale grensgebieden verschilt.

De factoren die tot een onevenredige verdeling van NK cellen, geactiveerde T cellen en $\gamma\delta$ T cellen in de decidua basalis en parietalis leiden zijn onbekend. Er zijn echter verscheidene mechanismen mogelijk: Er bestaan belangrijke verschillen tussen de twee foeto-maternale grensgebieden. Zowel anatomische, histologische als ook verschillen in de ontwikkeling moeten in acht genomen worden wanneer de placenta en de foetale membranen worden vergeleken (1). De placenta ontstaat waar de foetus zich innestelt in de uterus door middel van ingroei van trophoblasten. De decidua basalis komt in direct contact met de trophoblasten op de eerste dag van implantatie. De foetale membranen ontwikkelen zich uit de dooierzak en nemen niet deel aan de ingroei zoals trophoblasten. De decidua parietalis komt pas in direct contact met trophoblasten na de vierde maand van de zwangerschap. Macroscopisch zien deze weefsels er dan ook heel verschillend uit. De placenta is zeer gevasculariseerd en heeft een dikke laag chorion parenchym dat vele lagen met verscheidene soorten trophoblasten bevat. De foetale membranen zijn juist avasculair, en bestaan uit een dunne laag chorion die alleen extravilleuzetrophoblasten bevat. In verdere vergelijking tussen de twee plaatsen waar foeto-maternaal contact plaatsvindt, komt naar voren dat verschillend chorion HLA tot expressie komt in deze gebieden (2), als ook verschillen in productie van cytokines (3), en andere mediators zoals hormonen en prostaglandines (4-11).

Onze bevindingen hebben belangrijke consequenties voor *in vitro* studies naar de rol van deciduale leukocyten. De talloze studies die de decidua onderzochten maakten geen onderscheid tussen decidua basalis en de decidua parietalis. Vanwege fenotypische en functionele verschillen is het voortaan in publicaties in dit vakgebied uiterst belangrijk om duidelijk de oorsprong van de decidua aan te geven.

Voorts bevestigt ons onderzoek dat uterine NK cellen een belangrijk deel van de leukocyten vormen in de à terme decidua parietalis en, in tegenstelling tot wat tot heden aangenomen werd, zeker niet alleen gelimiteerd zijn tot de vroege zwangerschap. Uterine NK cellen in à terme zwangerschap zouden een belangrijke rol kunnen spelen in de bescherming van de foeto-placentale

eenheid. NK cellen vormen een belangrijk deel van het aangeboren immuunsysteem en zouden mogelijk kwetsbaarheid voor infectie door virale en andere pathogenen kunnen verminderen tijdens de zwangerschap. Hoewel de placenta volgroeid is aan het eind van de zwangerschap, is het aannemelijk dat controle van de ingroei van trophoblasten essentieel blijft, zelfs in het derde trimester. Deciduale leukocyten zouden zeer wel de controle kunnen blijven uitvoeren op de ingroei door middel van directe cel-cel interactie of door het juiste cytokinemilieu te creëren in het foeto-maternale grensgebied, of mogelijk ook door de antigenherkenning tijdens de zwangerschap te onderhouden.

ONDERZOEK NAAR DE FUNCTIE VAN LEUKOCYTEN IN DE NORMALE ÀTERME DECIDUA BASALIS EN PARIETALIS

De functie van leukocyten in de decidua basalis en parietalis verdient belangrijke aandacht om de rol van deze cellen beter te leren begrijpen in het onderhouden van de zwangerschap. Door middel van het toepassen van de zogenaamde “one-way mixed lymphocyte culture” techniek in **hoofdstuk 5** hebben we aangetoond dat de deciduale leukocyten prolifereren en aangezet worden tot het produceren van cytokines wanneer zij in aanraking komen met foetale en allogene cellen.

Het is wel bekend dat deciduale leukocyten verkregen uit eerste trimester zwangerschappen als ook uit àterme decidua geen proliferatie respons vertonen wanneer zij in aanraking komen met extravillieuze trophoblasten uit het chorion of het chorion membraan (12, 13). Er zijn ons geen studies bekend die onderzochten of er proliferatie van deciduale leukocyten verkregen uit vroege of àterme zwangerschap plaatsvindt, in respons op foetale navelstreng bloedcellen (UCB). In ons onderzoek was er een significant sterkere proliferatierespons van leukocyten van zowel decidua basalis als ook parietalis wanneer zij in weefselcultuur werden gebracht met UCB cellen, van foetale oorsprong, in vergelijking met controles. Voorts is het van wetenschappelijk belang dat hier tevens bevestigd wordt dat àterme deciduale leukocyten in staat zijn een immuunreactie te vertonen tegen cellen van foetale oorsprong, een vinding die niet eerder gerapporteerd was.

ELECTIEVE KEIZERSNEDE EN SPONTANE BEVALLING

In vervolg op onze eerdere studies vergeleken we leukocyten uit de decidua basalis en parietalis verkregen na spontane bevalling en keizersnede zoals beschreven in **hoofdstuk 4**. Onze resultaten suggereren dat het bevallen als zodanig geassocieerd is met significante verschillen in de distributie en proportie van subpopulaties van deciduale leukocyten binnen de CD45⁺ celfractie, alsmede hogere percentages macrofagen, CD56^{dim}CD16⁺ NK cellen en B cellen en een lager percentage CD3⁺CD4⁺CD25⁺, CD3⁺CD4⁺HLA-DR⁺ en CD3⁺CD8⁺HLA-DR⁺ cellen.

De spontane àterm bevalling kan beschreven worden als een opeenvolging van gebeurtenissen die geassocieerd is met specifieke veranderingen in de subpopulaties van leukocyten, celadhesiemolecuul expressie, en verscheidene immunologische mediators (14-17). Een mogelijk mechanisme dat de veranderingen in de deciduale leukocyten na een bevalling kan verklaren zou kunnen zijn dat deciduale leukocyten migreren naar andere weefsels zoals de cervix, het myometrium of naar de perifere bloedsomloop (15, 18, 19).

KLINISCHE IMPLICATIES

De pathologische zwangerschap

Het vormen van een normale placenta is essentieel voor een succesvolle zwangerschap. Studies die de rol van immuuncellen op het foeto-maternale grensgebied onderzoeken kunnen belangrijk bijdragen aan het begrip en behandeling van pathologische zwangerschappen waarbij er verstoringen plaatsvinden in de vorming van een normale placenta, zoals het geval is bij preeclampsie (PE), en intrauterine groei vertraging (IUGR). Preeclampsie is een belangrijke complicatie van de zwangerschap die gedefinieerd wordt door hoge bloeddruk, proteïnurie en oedeem en presenteert zich meestal in het tweede trimester in ongeveer 5 tot 8% van de zwangerschappen (20). IUGR is ook een klinisch relevante complicatie van zwangerschap en is geassocieerd met verhoogde perinatale morbiditeit en mortaliteit. IUGR wordt gekarakteriseerd als een syndroom dat leidt tot de sterk verminderde groei van de foetus, en komt voor in 3 tot 7% van de zwangerschappen. Het kan geïsoleerd voorkomen of in associatie met PE (21, 22).

Een van onze studies richtte zich op de rol van deciduale leukocyten in IUGR en PE, door de distributie en percentages van de leukocyten in de decidua basalis en parietalis te kwantificeren in placenta's verkregen van zwangerschappen met IUGR met en zonder PE in hoofdstuk 6. De resultaten laten zien dat er belangrijke verschillen bestaan in de distributie en percentages van deciduale leukocyten in IUGR met of zonder PE in vergelijking met normale zwangerschappen. Dit suggereert een mogelijk belangrijke rol van deze cellen in de immuunregulatie van deze aandoeningen.

Vruchtbaarheidsbehandelingen

Een beter begrip van de immunologie van het foeto-maternale grensgebied is een belangrijk doel dat kan leiden tot het verbeteren van de diagnostiek en behandeling van infertiliteit en mogelijk vruchtbaarheidsbehandelingen. Een interessant model is draagmoederschap zoals beschreven in **hoofdstuk 7**. In dit model heeft de draagmoeder geen genetische overeenkomst met de foetus en wordt in de baarmoeder geconfronteerd met niet één maar twee histocompatibiliteits antigenen (of meer) . Zowel de vaderlijke genen alsook de genen van de moeder, de donor van de eicel, zijn vreemd voor de vrouw die de vrucht in haar baarmoeder huisvest. De foetus kan in deze situatie dus als een “allograft” beschouwd worden in plaats van een “semi-allograft.” Wij rapporteren hier een casus van een draagmoeder en vinden hogere percentages CD56⁺CD16⁻ uterine NK cellen en geactiveerde CD3⁺HLA-DR⁺ en CD3⁺CD25⁺ Tcellen in vergelijking met normale à terme decidua basalis en parietalis. Hoewel er meer onderzoek nodig is in dit model om de rol van deze cellen te begrijpen, geeft deze casus nieuw inzicht in de immunologie van het foeto-maternale grensgebied.

Uiteindelijk kunnen hopelijk technieken ontwikkeld worden waarmee controle uitgeoefend kan worden op de mechanismen die van belang zijn voor de tolerantie van de moeder voor de foetus. Deze technieken zouden kunnen leiden tot belangrijke verbeteringen in het de klinische obstetrie en mogelijk in de transplantatie geneeskunde.

TOEKOMSTIG ONDERZOEK

Samenvattend laat ons onderzoek zien dat er belangrijke verschillen bestaan in de functie en verdeling van leukocyten in de àterme decidua basalis en decidua parietalis. Dit is het geval zowel in normale zwangerschappen, gecompliceerde zwangerschappen en bij draagmoederschap. Om een beter begrip te verkrijgen zullen toekomstige studies zich moeten richten op de mechanismen die deze verschillen kunnen verklaren. Vervolgonderzoek zal de aandacht moeten vestigen op mediators, mechanismen die tot het rekruteren, of prolifereren of zelfs celdood van de leukocyten op het foeto-maternaal grensgebied leiden. Het lijkt belangrijk met betrekking tot het doen van functionele studies om met gezuiverde, geïsoleerde celpopulaties uit te zoeken hoe de proliferatie reactie op allogeen en UCB cellen tot stand komt. Naast deze functionele studies zal ook het bestuderen van de cellulaire interactie op weefselniveau, met behulp van recent ontwikkelde, meerkleuren immunohistochemische technieken van belang zijn. Confocale microscopische technieken, waarmee verschillende cellulaire subpopulaties, in onderlinge interactie met elkaar, in beeld kunnen worden gebracht zijn wat dit betreft belovend. Wij hebben ook interesse in het karakteriseren van de CD3⁺CD4⁺CD25⁺ regulatoire Tcel populatie, zowel fenotypisch als functioneel. Verder onderzoek naar andere subpopulaties zoals NKT cellen, dendritische cellen, macrofagen en $\gamma\delta$ T cellen in de decidua basalis en parietalis tijdens zowel normale als gecompliceerde zwangerschap is nodig. Hopelijk zijn de volgende vijftig jaar onderzoek op het gebied van de foetale en maternale immunologie net zo vruchtbaar als de afgelopen vijftig jaar.

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Aliana Patricia Sindram-Trujillo was born on August 31, 1976 in Cleveland, Ohio, United States of America. In 1995, she received her high school diploma from Forsyth Country Day School in Lewisville, North Carolina. Following high school, Aliana attended Duke University in Durham, North Carolina from 1995 to 1998 and received a Bachelor of Science degree with a major in Biology and a minor in Chemistry. During her undergraduate education, Aliana worked in the Center of Genetic and Cellular Therapies in the laboratory of Dr. Clay Smith under the direct supervision of Dr. Robert Storms and completed research on the identification of hematopoietic stem cells in umbilical cord blood using a novel flow cytometric technique. In 1999, she began her medical training at the University of North Carolina School of Medicine in Chapel Hill, North Carolina. In June 2001 after completing the basic medical science studies, she traveled to the Netherlands. In collaboration with the Department of Immunohematology and Blood Transfusion and the Department of Obstetrics at the Leiden University Medical Center, she began her graduate study in the field of Reproductive Immunology under the supervision of Prof. Dr. Frans Claas and Prof. Dr. Humphrey Kanhai. In August 2003, Aliana returned to Chapel Hill to complete her medical training and received her medical degree in May 2005 from the University of North Carolina School of Medicine. Aliana is currently living in Durham, North Carolina, completing her residency training in the Department of Anesthesiology at Duke University.

