IMPLANTATION DISORDERS: IMMUNOLOGICAL BACKGROUND

Ph.D. Thesis

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2008.

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LIST OF ABBREVIATIONS

APC	allophycocyanin
BSA	bovine serum albumin
CTLA	cytotoxic T lymphocyte-antigen
EVTC	endovascular trophoblast cells
FACS	fluorescence activated cell sorter
Fas	apoptosis stimulating fragment
FasL	apoptosis stimulating fragment ligand
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
HELLP	haemolytic anaemia, elevated liver enzymes, low platelet count
HLA	human leukocyte antigen
IFN	interferon
Ig	immunoglobulin
IL	interleukin
iNKT	invariant natural killer T
ITAM	immunoreceptor tyrosine–based activation motif
ITIM	immunoreceptor tyrosine–based inhibition motif
IUGR	intrauterin growth retardation
KIR	killer immunoglobulin (Ig)-like receptor
LH	luteinizing hormone

mAb	monoclonal antibody
MACS	magnetic-activated cell separation
MHC	major histocompatibilty antigen
MIC	MHC class I chain-related
NK	natural killer
PBS	phosphate buffered saline
PE	phycoerythrin
Pi	propidium iodide
PIBF	progesterone induced blocking factor
RM	recurrent miscarriage
RPMI	Roswell Park Memorial Institute
SEM	standard error of mean
TCR	T cell receptor
TGF	transforming growth factor
Th	T helper
TIM	T cell immunoglobulin mucin
TIMP	tissue inhibitors of matrix metalloproteinases
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
Treg	regulatory T
VEGF	vascular endothelial growth factor

INTRODUCTION

IMPLANTATION

Pregnancy is a natural model of an optimal immune regulation in a graft-host relation. Though 50% of fetal antigens are of paternal origin, and there is ample evidence that these antigens are recognized, the immune system of the mother tolerates the semi-allogeneic fetus. However, while creating a favorable environment for the fetus, the maternal immune system must be prepared to control possible emerging infections. Therefore, a delicate balance is established to satisfy contradictory interests of mother and fetus.

Five-ten days after the LH surge there is a short period of time available for successful implantation. This is called the implantation window (1). During this interval cyclic secretion of estrogens and progesterone triggers morphological and physiological changes of the endometrium, and creates a suitable endometrial environment for embryo implantation and maintenance of early pregnancy.

Blastocyst formation usually begins at day 5 after conception and implantation takes place approximately at day 7. The blastocyst is made up of an inner cell mass which forms the embryo later on, and of an outer cell mass which forms the trophoblast. Since the embryo does not come into direct contact with the maternal organism the trophoblast represents the fetal compartment of the placenta. The trophoblast consists of an inner layer called cytotrophoblast and of an outer layer called syncytiotrophoblast. Multinucleated syncytiotrophoblast cells coat the chorionic villi and are constantly bathed in maternal

blood flowing through the intervillous space. The extravillous cytotrophoblast is an invasive subpopulation that gets in direct contact with decidual cells (2, 3, 4).

Fetal tissues are semiallogenic and paternal antigens can be recognized as foreign which leads to immunoactivation. While the syncytiotrophoblast and villous cytotrophoblast are devoid of HLA molecules (5) the extravillous cytotrophoblast expresses a special combination of HLA class I antigens: HLA-G, HLA-E and a small amount of HLA-C (6, 7, 8, 9). HLA-G and HLA-E are non-classical class I molecules, with a limited polymorphism and low cell surface expression (10). HLA-C is polymorphic, therefore, paternal specificity is also expressed at the fetomaternal interface.

Decidualization is characterized by endometrial expansion and storage of nutrients. For establishing an appropriate blood supply uterine arteries develop new branches called spiral arteries. Trophoblast invasion of the decidua, as well as the development of spiral arteries are, - at least partly - controlled by the influx of maternal immune cells which recognize the semiallogenic fetus and contribute to the establishment of pregnancy.

Immune cells and factors affecting decidualization and implantation

Three relatively minor lymphocyte subpopulations are significantly enriched in the decidua, and play a major role in creating a favorable environment for implantation and the early development of the fetus.

NK cells play diverse roles in the reproductive process. CD 56^{bright} $CD16^{neg}$ and $CD3^{neg}$ granulated decidual NK cells, which constitute a dominant lymphocyte population in the early decidua (11) are - despite their high perforin content - not cytotoxic (12), but

secrete an array of angiogenic factors and cytokines (13). The dynamics of the appearance of uterine NK cells suggests that one of their functions might be the control of placentation.

Potential cytotoxic mechanisms exerted by NK cells can damage the trophoblast, induce ablation of placenta, on the other hand, TNF-α - produced by NK cells in response to intrauterine infections - via facilitating prostaglandin synthesis may induce uterine contractions and initiate preterm labor. Decreased expression of HLA-G on the trophoblast may result in inadequate trophoblast invasion leading to an abnormal interaction with decidual natural killer (NK) cells, which are believed to play a major role in these processes through the production of immunoregulatory cytokines and angiogenic factors (14).

In mice, high peripheral NK activity was shown to be associated with deleterious effects on fetal development (15). Transfer of high NK activity spleen cells from poly (I) poly (C) -treated mice to pregnant Balb/c mice induces abortion (16). Normal human pregnancy is characterized by low peripheral NK activity (17), whereas increased NK activity seems to be an attribute of spontaneous abortions of unknown etiology (18, 19, 20, 21, 22).

γ/δ T cells represent a minor subpopulation of peripheral T cells. They constitute 5 percent of the peripheral T cell population and show unique features in structure, distribution and function. Ninety five % of peripheral blood γ/δ T cells express the Vδ2 chain in combination with Vγ9 (23). γ/δ T cells are enriched in mucosal surfaces of the respiratory, digestive and urogenital tracts, as well as in the placenta. In contrast to their circulating counterparts, resident γ/δ T cells preferentially express the Vδ1 chain (24, 25). γ/δ T cells play an important role in the elimination of several bacterial and viral infections and tumor-surveillance (24-26). Cytokine production by γ/δ T cells can either facilitate the

adaptive immune response or contribute to immunoregulation and immunosuppression (27).

During pregnancy an accumulation of γ/δ T cells can be observed both in the peripheral blood and in the decidua (28-30) suggesting that these cells have an important immunomodulatory role during gestation.

Invariant NKT (iNKT) cells carry - in addition to natural killer cell receptors- the strongly restricted Vα24JαQ TCRα chain combined with limited TCRβ chains, usually with Vβ11 (31*)*. iNKT cells recognize antigens presented by the monomorphic CD1d MHC class I like molecule (32-34). Although their natural ligand has yet to be identified, they can be activated with a glycolipid, α-galactosylceramide (α-GalCer) derived from a marine sponge (35, 36). Once they get activated, iNKT cells can act cytolytic via the perforin/granzyme B pathway or by expression of the Fas ligand inducing apoptosis of target cells (37- 39). On the other hand, upon CD1d restricted activation, iNKT cells quickly release cytokines of both Th1 and Th2 type leading to stimulation, activation and proliferation of T cells, B cells, or NK cells (40-42).

Fetal extravillous cytotrophoblast express CD1d and maternal iNKT cells are enriched in decidual tissue, indicating the possible role of iNKT cells in the immunological changes observed during implantation and later on during the time of pregnancy (34).

NK cells, γ/δ T cells and iNKT cells have many features in common:

- All of them are able to recognize antigens in a non-MHC restricted way.
- They represent a link between the innate and the acquired immune system since they are capable of carrying out both immunoregulatory and cytotoxic functions (43-45).

- All three cell types show a specific tissue distribution profile, with significant accumulation of NK cells in the early decidua, of γ/δ T cells on mucosal surfaces as well as the decidua, and of iNKT cells in the liver, the bone marrow and the decidua (34, 46, 47).
- They kill target cells by using secretory (perforin/granzyme mediated) and non-secretory mechanisms (Fas-ligand) (48, 49).
- All of them express NK inhibitory and activating receptors.

Cytotoxic activity is the result of inhibitory and activating signals, due to the interaction of cell surface activating and inhibitory receptors with ligands expressed on the surface of the target cell (50-52). Three major superfamilies of NK receptors have been described: the killer immunoglobulin (Ig)-like receptor (KIR) superfamily which recognizes classical MHC class I molecules, the C-type lectin superfamily recognizing non-classical MHC class I or class I-like molecules, and the natural cytotoxicity receptors (53).

Killer immunoglobulin-like receptors (KIR) are classified by the length of their cytoplasmic tails. The long tail KIRs (KIR2DL and KIR3DL) mediate an inhibitory signal via their immunoreceptor tyrosine-based inhibition motifs (ITIM), while the short tail receptors (KIR2DS and KIR3DS) are associated with adaptor proteins bearing immunoreceptor tyrosine-based activating motifs (ITAM) and mediate activating signals. KIR2DL1 and KIR2DL2 as well as their activating counterpart KIR2DS1, and KIR2DS2 recognize epitopes shared by alleles of the group 1 or group 2 HLA-C allotypes respectively 54). KIR2DL4 (a receptor without a short counterpart) recognizes the nonclassical MHC class I allele HLA G (55). Ligation of this receptor induces IFN-γ production, but no lytic activity.

C-type lectin receptors are composed of a common subunit (CD94) and a C-type lectin NKG2, which determines the functional specificity of the receptor (56). The inhibitory receptor CD94/NKG2A/B specifically binds the nonclassical class I molecule HLA-E (57-60). HLA-E is also bound by the activating receptor CD94/NKG2C, although with a lower affinity (61). NKG2D is an activating C-type lectin receptor, which does not associate with CD94 but is expressed as a homodimer, and signals through association with an adaptor protein DAP10 and phosphatidylinositol (PI)-3 kinase (62). Ligands for NKG2D include the polymorphic MHC class I chain-related (MIC) peptides, MICA and MICB (63, 64) and the human cytomegalovirus UL16 binding proteins (ULBPs) (65). These are not expressed on normal cells, but are induced by “stress” or neoplastic transformation. The expression of these ligands may, therefore, be signals of “danger” to the NK cells.

Natural cytotoxicity receptors are Ig-like activating receptors that have been implicated in the recognition and lysis of tumor cells by human NK cells (66, 67). Three natural cytotoxicity receptors have been described, of which two (NKp46 and NKp30) are constitutively expressed on all peripheral blood NK cells but not other immune cells, while the third, NKp44, is expressed only on IL-2-activated NK cells. The ligands for these receptors are unknown.

Activated lymphocytes from healthy pregnant women express progesterone receptors and produce a mediator protein named the **Progesterone Induced Blocking Factor (PIBF)** (68-70). PIBF plays a role in the maintenance of pregnancy by inducing a Th2 dominant cytokine production and by inhibiting NK activity (71). The immunomodulatory functions of PIBF appear to be essential for successful pregnancy, since neutralizing endogenous PIBF in pregnant mice results in fetal resorptions (72).

IMPLANTATION DISORDERS

After apposition of the blastocyst, the trophoblast starts to produce metalloproteinases and invades the decidua. Trophoblast invasion is strictly controlled both in space and time. The decidua secretes extracellular matrix molecules, e.g., collagen IV, laminin, heparan sulphate, and hydrates the tissue during the implantation window, which also promotes invasiveness of trophoblast. At the same time factors that limit trophoblast invasion are also produced. Among others, transforming growth factor (TGF) β inhibits trophoblast cell proliferation and induces the expression of TIMPs (tissue inhibitors of matrix metalloproteinases).

Decidual recognition of fetal HLA antigens expressed by the trophoblast contributes to the control of invasion. Interaction of uterine NK cells, iNKT cells and γ/δ T cells with the non-polymorphic HLA-G and HLA-E usually induces the secretion of Th-2 type cytokines (e. g.: IL-10, TGF-β). On the other hand, recognition of paternal HLA-C molecules expressed on the trophoblast results in a classical inflammatory response (73-75), which by loosening the tissue facilitates trophoblast invasion. Additionally, IFN-γ produced during inflammation promotes vascular remodeling (76).

During the first trimester, trophoblast cells penetrate the maternal decidual spiral arteries (endovascular trophoblast cells, EVTC). Until the 9th week of gestation, the decidual capillaries remain obstructed by trophoblast plugs which act like filters enabling maternal blood plasma diffusion and controlling oxygen tension (77). This process is called the first vascular invasion by the cytotrophoblast.

After the 9th week, fetal growth and development accelerate requiring a better supply of oxygen and nutrients. This is warranted by recanalization and remodelling of the

uteroplacental arteries. Endovascular trophoblast cells are built in the wall and in the interstitium of the maternal arteries resulting in dilated vessels (78). The second vascular invasion by the cytotrophoblast is completed by the 13th week of gestation.

Disturbances of implantation may manifest as early asymptomatic fetal loss, or they can also result in the development of obstetric syndromes, where pregnancy is normally established, but later on clinical symptoms develop. Impaired implantation has been identified as the underlying pathology of recurrent spontaneous abortion, pre-eclampsia and intrauterine growth retardation.

Recurrent miscarriage

Recurrent miscarriage (RM) (defined as three or more consecutive pregnancy losses) affects 0.5 to 1% of couples (79). The pathophysiology of RM is complex. The suggested causes include anatomical, genetic and molecular abnormalities, endocrine disorders, thrombophilias and anti-phospholipid syndrome. In approximately 50% of the cases neither of the above can be identified (79-81).

Most of the latter are thought to have had implantation disorders caused by inappropriate maternal immune response. During the luteal phase of the ovarian cycle, the immune response is shifted toward the Th2-type (82). Several studies confirmed a pathological activation of decidual lymphocytes in RM (83-85). Women with RM have been shown to have a Th1-dominant cytokine profile (86-90). In a prospective study, Kruse et al. (2003) found generally lower Th1/Th2 cytokine ratios in RM patients with high serum progesterone levels than in those with low serum progesterone levels, suggesting that serum progesterone might have an influence on cytokine production (91). It has been recently demonstrated that endometrial interleukin-18, -15, and -12 levels correlate negatively with

uterine receptivity (92). Potential cytotoxic mechanisms exerted by NK cells can damage the trophoblast, and induce ablation of placenta. In spite of their high perforin content, decidual NK cells show low spontaneous cytotoxic activity during normal pregnancy (93).

RM is associated with an increased number of endometrial NK cells (94), and decidual lymphocytes from failed pregnancies contain less perforin than those from normal pregnancy deciduas (95), suggesting that an increased rate of degranulation takes place in the former case. Furthermore, a dominant population of TGF-β-producing NK3 type cells in normal decidua is significantly reduced in deciduas from women with RM (96). Immunological alterations also affect the acquired immunity: the percentage of systemic CD4+ T lymphocytes, CD8+ T cytotoxic cells and CD5+ B lymphocytes is significantly higher in patients with recurrent spontaneous abortion compared to healthy women (79).

Pre-eclampsia

Pre-eclampsia which affects approximately 10% of pregnancies (97), is a severe and dangerous obstetrical disease characterized by high blood pressure (>140/90 Hgmm) and proteinuria (>100 mg/dl), which might result in edema (98). Several organic complications (e.g. hepatic involvement, renal failure) may associate with the syndrome and contribute to the development of eclampsia, characterized by convulsions. HELLP (*h*aemolytic anaemia, *e*levated *l*iver enzymes, *l*ow *p*latelet count) syndrome is also considered to be a potential manifestation of pre-eclampsia. Since there is no prevention and effective therapy of the syndrome, screening of pregnant women and - in case of need - induction of the delivery, are the only possibilities managing pre-eclampsia.

The precipitating factor in pre-eclampsia is the pathological development of the placenta. Clinical symptoms develop after the 20th week and are present as long the placenta persists in the uterus.

It is generally agreed that pre-eclampsia develops in two stages. Pre-clinical stage 1 occurs at time of implantation when insufficient trophoblast invasion leads to poor placentation resulting in placental hypoxia. Recent data suggest that fetal antigens fail to properly activate the decidual lymphocytes, resulting in insufficient production of angiogenetic factors and the lack of a local inflammatory reaction (78, 99). The absence of the above – owing to insufficient invasion of endovascular trophoblast - leads to impaired placental development (100). Since there are no symptoms of pre-eclampsia during stage-1, it is impossible to predict and thoroughly investigate the disease at that early timepoint.

The limited uteroplacental circulation becomes functionally insufficient at the 20th week when fetal growth accelerates. Stage 2 pre-eclampsia is a systemic disease. An oxidatively stressed placenta releases factors (e.g. soluble receptor for vascular endothelial growth factor (VEGF) 1, neurokinin B, syncytiotrophoblast membrane microparticles) into the maternal circulation, which cause a systemic inflammatory response and endothelial dysfunction that manifest in the clinical signs of pre-eclampsia (101).

The two stages of pre-eclampsia involve the innate immune system in different ways. In the first stage, there is an important element localized to the placental bed. In the second stage, a diffuse systemic response predominates similarly to rejection mechanisms.

Intrauterine growth retardation (IUGR)

The clinical findings of stage 1 of pre-eclampsia can manifest as either a maternal- or a fetal syndrome. Intrauterine growth retardation occurs when placental deficiency decelerates fetal development leading to births of neonates with small size for gestational age (102).

AIMS OF THE STUDY AND RESULTS

The successful implantation of the embryo into the uterus is a pivotal event in human development which defines not only the future of the pregnancy but of the individual itself. Implantation failure (infertility) and miscarriage affect one in six couples (103), with physical, emotional and financial consequences. Poor implantation may result in intrauterine growth retardation and/or pre-eclampsia which are the major causes of fetal handicap and fetal and maternal death in Europe, and have long-term economic costs. Furthermore, it is now becoming apparent that both babies and mothers from pre-eclamptic pregnancies are much more likely to develop cardiovascular disease and diabetes in later life (104, 105), imposing a heavy burden on health care systems. It is therefore vital to identify the mechanisms that affect implantation for the diagnosis of implantation failure, miscarriage and pre-eclampsia.

The study of normal and pathological implantation raises several problems. The availability of normal human placentae is restricted. Pregnant mice have proved themselves to be excellent models for studying implantation failures by allowing the use of manipulative techniques (76, 106). However, the mouse model has its limitations. Because of the anatomical differences from the human, and a lower level of complexity in the murine implantation process (106, 107), data from animal experiments cannot be directly extrapolated to the human situation. The analysis of isolated decidual lymphocytes from elective abortion material of healthy pregnant women allows comparative studies of decidual and peripheral immunological processes at 6-12 weeks of gestation. However, the

study of late consequences of human implantation disorders is restricted to the examination of peripheral blood lymphocytes.

The aim of this study was to compare peripheral immune responses of women with different clinical manifestations of implantation disorders to those of healthy pregnant women and non-pregnant individuals.

Altogether 251 subjects were involved in the study. These included 124 healthy pregnant women, 24 women at risk for premature pregnancy termination, 41 women with severe pre-eclampsia and 62 non pregnant women. Clinical data of the patients are summarized in Table 1.

Table 1.

Clinical data of patients included in the study

Groups	**Normal pregnancy (n=124)**	**Pre-eclampsia (n=41)**	**Premature labor (n=24)**	**Non-pregnant women (n=62)**
Age (mean)	28.16	26.3	27.8	29,6
Gestational age (mean±SEM)	32.43 (24-41)	35.2 (29-41)	30.33 (24-37)	-
Parity (mean±SEM)	0.85 (0-3)	0.30 (0-2)	0.21 (0-1)	-
No. of previous miscarriages (mean±SEM)	0.30 (0-3)	0.11 (0-1)	0.47 (0-4)	-

In the first study we determined the ratio of different γ/δ T cell subsets in peripheral blood of pregnant women with or without the risk of premature pregnancy termination. Furthermore, in a longitudinal study we determined the V chain usage of circulating γ/δ T

cells from 23 healthy pregnant women at the 33–39th week of gestation, and that in the same women during labor. The interval between the two samplings was 4–6 weeks on the average.

Due to the lack of classical HLA antigens on most classes of the trophoblast, the majority of fetal antigens are presented in a non-MHC-restricted way. Therefore, decidual γ/δ T cells are the likely candidates for recognizing these antigens. There is an increased presence of activated γ/δ T cells in the decidua (108) and these cells preferentially use the Vδ1 chain (109). Findings by Mincheva-Nilsson et al. (110) suggest that the human early decidua is a transient site for extrathymic maturation. Earlier data from our laboratory revealed an increased rate of activated γ/δ T cells in peripheral blood of healthy pregnant women (111). These cells similarly to decidual γ/δ T cells preferentially use the Vδ1 chain (111, 112), which allows the hypothesis that these lymphocytes are of decidual origin. In healthy non-pregnant individuals 95 % of circulating γ/δ T cells express the Vγ9/Vδ2 chain combination (113). These cells are cytotoxic and play an important role in the elimination of several bacterial and viral infections and tumor-surveillance (24-26), while normal pregnancy is characterized by an expansion of the circulating non-cytotoxic Vδ1+ chain expressing subpopulation.

Our study revealed a significant increase of the potentially cytotoxic Vγ9/Vδ2 positive T cell population during labor, together with a decrease of the non-cytotoxic Vγ4/Vδ1 T-cell population.

Next we analyzed the ratio of circulating Vδ2+ and Vδ1+ T cells in normal pregnancy, in women at risk for premature pregnancy termination and in non-pregnant

controls. Compared to the controls, both γ/δ T cell subsets were significantly increased in the peripheral blood of pregnant women. The percentage of Vδ2+ T cells was significantly higher, whereas the ratio of Vδ1+ T cells was significantly lower in pregnant women at risk for premature pregnancy termination than in normal pregnancy. These findings confirm our earlier data on accumulation of γ/δ T cells in the peripheral blood of pregnant women. The increased ratio of Vδ2+/Vδ1+ T cells observed in women at risk for premature pregnancy termination may contribute to the unfavorable immunological conditions leading to the cessation of pregnancy. .

Cytotoxic activity depends on the balance of inhibitory and activating signals, following the interaction of cell surface activating and inhibitory receptors with ligands expressed by the target cell (50-52).

NKG2A, the inhibitory NK cell receptor belongs to the C-type lectin superfamily. The ligand of NKG2A is the non-classical Class I MHC product; HLA-E, expressed by the trophoblast. The majority of decidual γ/δ T cells express the CD94/NKG2A complex (114, 115). Analyzing the NKG2A expression on peripheral blood Vδ2+ T cells, we found a significantly lower expression on those of women at risk of premature pregnancy termination and of non-pregnant subjects than on Vδ2+ T cells from healthy pregnant women. This data suggest that Vδ2+ T cells of women at risk for premature pregnancy termination are less capable of recognizing HLA-E, thus they are less likely to receive the inhibitory signals that would prevent cell activation than Vδ2+ T cells of healthy pregnant women.

Viability of lymphocytes determines the duration of effector mechanisms. When expressed on the cell surface, annexins promote pro-apoptotic mechanisms, or alternatively,

the removal of cells that have undergone apoptosis. Therefore, we determined the expression of Annexin V on circulating Vδ2+ T cells. The percentage of Annexin V+ Vδ2+ T cells was significantly lower in patients at risk for premature pregnancy termination than in healthy pregnant women indicating reduced tendency for apoptosis and a longer lifespan of cytotoxic Vδ2+ T cells in this condition. Annexin is also known as lipocortin. Lipocortins suppress phospholipase A2. This is the mechanism by which glucocorticoids inhibit inflammation, but since activation of phospholipase A2 is needed for the liberation of arachidonic acid, annexins also interfere with prostaglandin production. Increased production of annexin by inhibiting the activity of phospholipase A2, will block prostaglandin production, thus delay the induction of labor. (116)

Taken together the above data suggest the possible role of γ/δ T cells in the pathogenesis of threatened premature pregnancy termination. The dominance of the potentially cytotoxic Vδ2+ subset, with decreased NKG2A expression and reduced apoptotic capacity might contribute to the lack of a Th2 shift and to the pathophysiology of high risk pregnancy. **(Paper 1)**

Improper implantation and poor placentation usually result in early pregnancy loss, yet in a part of the cases pregnancy proceeds normally, till the beginning of the third trimester, when the limited utero-placental circulation cannot further comply with the requirements of the fetus. Th1-type responses turn to be dominant; the mild inflammatory response associated with normal implantation becomes systemic and exaggerated, and finally clinical symptoms appear.

Next we focused on the role γ/δ T cells and immunoregulatory mechanisms in stage 2 pre-eclampsia.

Cell-mediated cytotoxicity takes place either via the perforin/granzyme-mediated secretory/necrotic killing or the TNF family ligand-mediated apoptotic killing. In the first case; following target-cell recognition, the contents of the cytotoxic granule are released into the immunological synapse formed between the killer cell and its target. Cytotoxic granules contain two membrane-perturbing proteins, perforin and granulysin and a group of serine proteases called granzymes (117-120). Perforin, a Ca2+-dependent pore-forming protein shows homology with complement components. Perforin monomers are inserted into the plasma membrane of target cells and polymerize into pore-forming aggregates (121), which leads to granzyme entry and lysis. In the cytoplasm granzymes activate a cascade of caspases, leading to activation of DNAse, and resulting in apoptosis. Granulysin inserts into cell membranes, inducing ion fluxes and the induction of apoptosis (122, 123).

The intracellular expression of perforin and IFN-γ by Vδ2+ T cells was significantly higher both in pre-eclamptic patients and in non-pregnant individuals than in healthy pregnant women, suggesting an enhanced cytotoxic capacity of the Vδ2+ cell population from pre-eclamptic women.

Apoptosis is an important immunoregulatory mechanism that occurs in a wide variety of physiological and pathological situations, since the viability of lymphocytes determines the duration time of effector mechanisms. Two important apoptosis-inducing pathways include the TNF-induced and the Fas-Fas ligand -mediated mechanisms, both involving members of the TNF receptor (TNFR) family. The Fas/FasL pathway is important in many cellular events including the induction of apoptotic cell death and inflammation. Fas ligand (FasL), a Type II transmembrane protein is a member of the TNF

family. FasL is stored in specialized secretory lysosomes of the cytotoxic cells and is only delivered to the cell surface upon recognition of Fas on the target cell. Membrane bound FasL triggers apoptosis of the Fas-expressing cell (124). We found a decreased Fas (CD95) expression on Vδ2+ cells of pre-eclamptic women, which suggests an impaired apoptotic potential of these cells.

Analyzing inhibitory and activating NK cell receptor expression we found that the expression of the inhibitory receptor NKG2A by Vδ2+ T cells of pre-eclamptic patients was significantly lower, than by those of healthy pregnant- or non-pregnant women. CD94/NKG2C is the activating counterpart of CD95/NKG2A. Vδ2+ T cells of pre-eclamptic women expressed significantly more NKG2C than those of women with normal pregnancy or those of non-pregnant controls. The rate of co-expression of the two receptors (NKG2A/NKG2C) on Vδ2+ T cells of pregnant women with pre-eclampsia was significantly lower than on those of women with normal pregnancies or of non-pregnant women.

The expansion and differentiation of Th precursor populations into Th1 or Th2 pathways regulate immune responses to bacteria, viruses, autoantigens and alloantigens. The magnitude of T cell responses is influenced by cytokines and a group of accessory molecules including immunoglobulin superfamily members. TIM-3 (T cell immunoglobulin mucin 3) - an immunoglobulin superfamily member - has recently been identified as a negative regulator of tissue destructive immune responses. TIM-3 is preferentially expressed by Th1 cells (125, 126). Expression of this molecule by Th1 cells provides a key checkpoint that serves to dampen pro-inflammatory Th1-dependent T cell responses and limit the associated tissue injury. We found significantly decreased

expression of TIM-3 by Vδ2+T cells of pre-eclamptic women, which could account for the increased inflammatory property of these cells. TIM-3 exerts a direct inhibitory signal to the Th1 cells, thus increased frequency of perforin and IFN-γ producing Vδ2+ T cells in pre-eclamptic patients could simply be due to the lack of the TIM-3 mediated regulatory effect.

Regulatory T cells (CD4+CD25^{bright} T cells) play a central role in the maintenance of self tolerance as well as in the long-term acceptance of allogenic transplants (127, 128). Sanches-Fueyo et al. demonstrated that the TIM-3-dependent pathway is involved in the CD4+CD25+ T cell-dependent immunoregulation (129). Among CD4+CD25+T cells those with high fluorescence intensity of CD25 have been identified as the ones that exert regulatory functions (130), while expression of low levels of CD25 by CD4+ T cells appears to indicate T cell activation. Inadequate tolerance induction increases the risk of pre-eclampsia. Recent data have shown that CD4+CD25+ Treg cells are essential in the maintenance of allo-pregnancy in mice (131). Furthermore, decreased levels of Treg cells have been observed in the peripheral blood of patients with spontaneous abortion (132). In our expreiments, the percentage of CD4+CD25^{bright} T cells of gated lymphocytes was significantly lower in pre-eclamptic pregnant women than in non-pregnant controls, but the difference from healthy pregnant women did not reach the level of statistical significance.

Following activation, T cells begin to induce the expression of Cytotoxic T lymphocyte antigen-4 (CTLA-4), which shows a high sequence homology with CD28, and competes with CD28 for B7 molecules (127). CD28 is one of the molecules expressed on T cells that provide the co-stimulatory signals, which are required for T cell activation. CD28 is constitutively expressed on naïve T cells and serves as the receptor for B7 molecules

expressed by antigen presenting cells. B7 expression is upregulated in activated antigen presenting cells. Stimulation through CD28 in addition to the TCR can provide a potent co-stimulatory signal to T cells for the production of various cytokines. The percentage of CD4+CD25^{bright} T cells among gated lymphocytes was significantly lower, while the percentage of CTLA-4 expressing cells among CD4+CD25^{bright} T cells was significantly higher in pre-eclamptic pregnant women than in non-pregnant controls, and healthy pregnant women. This observation indicates that though present at a lower frequency, a higher percentage of CD4+CD25^{bright} T cells is activated in pre-eclamptic patients than in healthy individuals.

Our data indicate that Vδ2+T cells of pre-eclamptic patients demonstrate an increased perforin and IFN-γ expression, which could be explained by dysregulation of NK cell receptor expression. These Th1 polarized cells were less susceptible to apoptosis than Vδ2+ T cells from healthy pregnant women. Activated Vδ2+ T cells of pre-eclamptic women have an increased cytotoxic potential, which may be explained by the altered expression of NK cell inhibitory and activating receptors. Taken together, this series of observations suggest the role of multiple pathways in generating an exaggerated systemic inflammatory response observed in the 2nd, clinical phase of pre-eclampsia. **(Paper 2)**

Invariant NKT (iNKT) cells serve as a link between the innate and the acquired immune system. Depending upon the circumstances, they are able both to exert cytotoxicity and to regulate the function of other cells by secreting cytokines. iNKT cells are significantly enriched in the decidua (34), thus these cells might also have a role in regulating local immune responses during pregnancy, and if so, an altered function of iNKT cells could also play a part in the development of pathologies. In order to test whether

altered iNKT cell function might contribute to the pathogenesis of pre-eclampsia, we compared the percentage of activated-, perforin containing- as well as IFN-γ-producing- and apoptotic iNKT cells of pre-eclamptic patients to those of healthy pregnant women and non-pregnant individuals.

First, we determined the activation rate of iNKT cells by measuring the expression of CD69. The percentage of CD69 positive activated iNKT cells was significantly higher in pre-eclamptic patients than in healthy pregnant or non-pregnant women.

In pre-eclamptic patients, the percentage of potentially cytotoxic, perforin expressing iNKT cells was found to be significantly higher compared to healthy pregnant women. Similar elevation of perforin expression by iNKT cells was found in non-pregnant individuals, compared to healthy pregnant women. In line with this, in pre-eclamptic patients and in non pregnant women the percentage of circulating IFN-γ expressing iNKT cells was significantly higher than in healthy pregnant woman. These data suggest, that activated iNKT cells functionally contribute to the enhanced inflammatory immune response observed in pre-eclampsia.

Apoptotic potential of iNKT cells was determined by measuring Fas (CD95) expression. We found that in healthy pregnant women significantly more iNKT cells express CD95, than in either pre-eclamptic patients or non-pregnant individuals predicting a longer lifespan of activated, cytotoxic iNKT cells in pre-eclampsia.

The observed activation and Th1 profile of iNKT cells in pre-eclamptic patients may be induced by signals transmitted by different NK cell receptors on their surface. Hence, we investigated the distribution of NK cell activating and inhibitory receptors on these cells. There was no difference in the expression of the activating NKG2D receptor by

iNKT cells among the groups, whereas the percentages of iNKT cells expressing the inhibitory receptor NKG2A as well as of those co-expressing NKG2A and NKG2D were significantly lower in pre-eclamptic patients than in healthy pregnant women. The percentage of NKG2A expression on NKG2D+ iNKT cells was also significantly lower in pre-eclamptic patients than in healthy pregnant women or in non-pregnant women. Altered NK cell receptor expression may lead to restricted inhibitory signal transduction, and contribute to the development of activated, Th1 type iNKT cells, seen in pre-eclamptic patients. **(Paper 3)**

These data suggest that among others, several minor lymphocyte subsets, including γ/δ T cells, regulatory T cells and invariant NKT cells play a role in the development of poor implantation-related pregnancy pathologies.

In addition to the relative dominance of the cytotoxic population of γ/δ T cells, owing to an imbalance of NK activating and inhibitory receptors, all three lymphocyte populations gain a cytotoxic function, and contribute to exaggerated systemic inflammatory responses, that are characteristic of pregnancy termination as well as of the late stage of pre-eclampsia.

THESES

In women at risk for premature pregnancy termination:

1. Circulating Vδ2+ T lymphocytes are increased in number indicating their possible role in this pathological condition.
2. The percentage of Vδ1+ T cells is significantly lower than in healthy pregnant women resulting in reduced production of γ/δ T cell-derived Th2 type cytokines.
3. The ratio of Vδ2+ / Vδ1+ T cells is significantly higher than in healthy pregnant women suggesting the dominance of the potentially cytotoxic Vδ2+ T cell population over Vδ1+ T lymphocytes.
4. Circulating Vδ2 TCR + cells express less NKG2A than those of healthy pregnant women resulting in a reduced capacity of transducing inhibitory signals.
5. The number of apoptotic Vδ2+ T cells is significantly decreased compared to healthy pregnant controls. This phenomenon may explain the increased number of Vδ2+ T cells observed in the pathological condition.

In peripheral blood of women with pre-eclampsia:

6. Intracellular expression of perforin by Vδ2+ T cells is significantly higher than in those with normal pregnancy or non-pregnant individuals.
7. The expression of the IFN-γ by Vδ2+ T cells is significantly higher than in healthy pregnant women.

8. CD95 expression by Vδ2+ T cells is significantly lower than in non-pregnant women indicating reduced apoptotic potential.
9. The expression of the NK inhibitory receptor NKG2A by Vδ2+ T cells is found to be significantly lower in than in those with normal pregnancy or non-pregnant women.
10. NKG2C expression by Vδ2+ T cells is significantly higher than in normal pregnancy or non-pregnant women. Therefore, activating signals could be transmitted more efficiently in these cells.
11. In pre-eclampsia, the NKG2A/NKG2C co-expression by Vδ2+ T cells is significantly reduced compared to those with normal pregnancy or non-pregnant women.
12. The percentage of Vδ2+ cells expressing NKG2A together with NKG2C is significantly lower than in healthy pregnant women, indicating a reduced inhibitory capacity of the NK activating receptor positive cells.
13. TIM-3 expression on Vδ2+T cells is significantly decreased compared to healthy pregnant women. These finding suggest a failure to control the tissue destructive immune responses observed in pre-eclampsia.
14. The frequency of peripheral blood CD4+CD25^{bright} cells is lower than in healthy pregnant or non-pregnant women contributing to an impaired management of systemic inflammation.
15. The percentage of CTLA-4 expressing cells among CD4+CD25^{bright} T cells is significantly higher than that in healthy pregnant or in non-pregnant women, suggesting an enhanced activation of this cell population in pre-eclampsia.

16. There is a significantly higher percentage of activated iNKT cells than in healthy pregnant women.
17. The number of peripheral perforin producing iNKT cells is significantly increased compared to healthy pregnant controls suggesting a potential cytolytic activity of these cells.
18. IFN-γ expression by iNKT cells is significantly higher than in healthy pregnant woman, indicating a Th1 profile of iNKT cells.
19. Apoptotic capacity of peripheral iNKT cells is significantly reduced compared to healthy pregnant women, suggesting a longer lifespan of these cells.
20. The percentage of iNKT cells expressing the inhibitory receptor NKG2A as well as of those co-expressing NKG2A and NKG2D are significantly reduced. These results confirm that iNKT cells show an altered NK cell receptor expression pattern compared to those in normal pregnancy, and consequently inhibitory signals might be transmitted less efficiently.

MATERIALS AND METHODS

Separation of peripheral blood lymphocytes

Lymphocytes were isolated from heparinized peripheral blood on Ficoll Paque gradient, washed twice with RPMI 1640 medium and adjusted to a cell count of $1x10^6$/ml. The cells were incubated with appropriate concentrations of different monoclonal antibodies or conjugated to target cells.

Monoclonal antibodies

The following mAbs were used:

Origin	Specificity of antibody	Clone	Manufacturer
Surface staining			
mouse	anti-human TCR Vδ2	clone 15D	T-cell Diagnostic Inc., Woburn, MA, USA
mouse	anti-human TCR Vδ2 – FITC	clone B6.1	BD Pharmingen, Soft Flow Hungary Kft., Hungary
mouse	anti-human TCR Vδ1-FITC	clone TS8.2	T-cell Diagnostic Inc., Woburn, MA, USA
mouse	anti-human CD 69-APC	clone FN50	BD Pharmingen, Soft Flow Hungary Kft., Hungary
mouse	anti-human invariant NKT-FITC	clone 6B11	BD Pharmingen, Soft Flow Hungary Kft., Hungary
mouse	anti-human CD95-PE	clone DX2	BD Pharmingen, Soft Flow

			Hungary Kft., Hungary
mouse	anti-human NKG2A-PE	clone Z199	Immunotech, Csertex Kft, Hungary
mouse	anti-human NKG2A-APC	clone 131411	R&D Systems, Biomedica Hungária Kft.
mouse	anti-human NKG2D-APC	clone 149810	R&D Systems, Biomedica Hungária Kft.
mouse	anti-human NKG2C-PE	clone 134591	R&D Systems, Biomedica Hungária Kft.
mouse	anti-human CD4 -FITC	clone-RPA-T4	BD Pharmingen, Soft Flow Hungary Kft., Hungary
mouse	anti-human CD25-FITC	clone-M-A251	BD Pharmingen, Soft Flow Hungary Kft., Hungary
mouse	anti-human TIM-3	clone 344823	R&D Systems, Biomedica Hungária Kft.
mouse	anti-human CTLA-4 - PECy5	clone-BNI3	BD Pharmingen, Soft Flow Hungary Kft., Hungary
goat	anti-FITC IgG Microbeads		Miltenyi Biotec, Frank Diagnosztika, Hungary
goat	anti-mouse biotin		Amersham-Pharmacia Biotech, Hungary

Intracellular staining			
mouse	anti-human perforin-PE	clone 27-35	BD Pharmingen, Soft Flow Hungary Kft., Hungary
mouse	anti-human IL-10-APC	clone JES3-19F1	BD Pharmingen, Soft Flow Hungary Kft., Hungary
mouse	anti-human IFN-γ-APC	clone B27	BD Pharmingen, Soft Flow Hungary Kft., Hungary

Control antibodies included isotype-matched unlabeled, furthermore, PE-conjugated, APC-conjugated, FITC-conjugated and PECy5-conjugated mouse antibodies (BD Pharmingen, Soft Flow Hungary Kft. Hungary).

MiniMACS γδ T cell separation

Vδ2+ T cells were separated using MiniMACS immunomagnetic beads, following the instructions of the manufacturer (Miltenyi Biotec, Frank Diagnosztika, Hungary). Briefly, cells were washed with PBS and resuspended at a cell count of $1x10^7$/ml in PBS containing 0,5% BSA (Sigma Aldrich Kft., Hungary) and 2 mM of EDTA. Ten million cells were incubated for 10 minutes at 4°C with 10 μg anti-Vδ2 mAb. After incubation, lymphocytes were washed twice and resuspended in 80 μl buffer and 20 μl of Goat anti-Mouse IgG Microbeads (Miltenyi Biotec, Frank Diagnosztika, Hungary) were added. Cells were incubated for 15 minutes at 4°C, and then washed. Pelleted cells were resuspended in 500 μl buffer and applied to a MiniMACS column fitted to a magnet. The column was washed six times and then removed from the magnetic separator. The magnetic adherent

cells were flushed out of the column into a fresh tube using a plunger included in the MiniMACS Kit. Magnetic adherent cells were washed and 10^5 cells were incubated with an appropriate dilution of FITC conjugated anti-Vδ2 mAb for 30 minutes. During incubation, samples were protected from light. After washing, the cells were resuspended in 250 µl FACS buffer containing 1% paraformaldehyde and stored at 4°C, in dark, to be processed for FACS analysis the following day. The purity of the resulting cell suspension was checked by FACS analysis. Usually a 75 to 80 % enrichment of Vδ2 TCR positive cells was obtained.

Labeling of lymphocytes and flow cytometric analysis

50 µl heparinized venous blood; diluted with an equal volume of 10% FCS containing RPMI 1640 was incubated for 30 minutes at room temperature with the fluorochrome-labeled monoclonal antibodies.

After surface staining, the cells were incubated with 1:10 diluted FACS Lysing Solution (BD Pharmingen, Soft Flow Hungary Kft. Hungary) for 10 minutes and washed twice with PBS buffer. Finally the cells were resuspended in 300 µl PBS containing 1% paraformaldehyde, and stored at 4 ^{o}C in dark until FACS analysis.

For detecting perforin positive cells, after surface labeling the cells were incubated with 1:10 diluted FACS Permeabilizing Solution (BD Pharmingen, Soft Flow Hungary Kft. Hungary) for 10 minutes and then washed with PBS. The cells were then incubated with PE-conjugated mouse anti-human perforin for 30 minutes at room temperature, washed with PBS and fixed with PBS containing 1% paraformaldehyde.

For detecting cytokine positive cells, 500 µl heparinized venous blood was diluted 1:1 with RPMI1640 containing 10% FCS and 10 µg brefeldin A, 25 ng phorbol myristyl acetate and 1 µg ionomycin (all from Sigma-Aldrich Kft., Hungary). The samples were incubated for 4 hours at 37 ^{o}C in 5% CO_2. After stimulation the cells were labeled by surface staining. After surface labeling the cells were incubated with 1:10 diluted FACS Permeabilizing Solution (BD Pharmingen, Soft Flow Hungary Kft. Hungary) for 10 minutes and then washed with PBS. The cells were then incubated with the labeled anti-human cytokine antibody for 30 minutes at room temperature, washed with PBS and fixed with PBS containing 1% paraformaldehyde.

Labeled cells were analyzed with a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems) equipped with the CellQuest software program (Becton Dickinson) for data acquisition and analysis.

Apoptosis of Vδ2+ T cells by annexin V staining

Magnetic bead-separated Vδ2+ lymphocytes were adjusted to a concentration of $1x10^5$/mL of annexin-binding buffer, and 1-ml aliquots were centrifuged in FACS tubes. The cells were labeled for 15 minutes at room temperature in 100 µl annexin binding buffer with 10 µl biotinilated annexin V (BD-Pharmingen, Soft Flow Hungary Kft., Pécs, Hungary). Cells were washed with annexin-binding buffer, resuspended in 100 µl annexin-binding buffer, and stained with 10 µl Streptavidin-APC (BD Pharmingen, Soft Flow Hungary Kft. Hungary) and 10 µl propidium iodide (PI, Sigma-Aldrich Kft., Hungary) by gently mixing for 15 minutes at room temperature in the dark. Binding buffer was added to each tube to restore the volume to 300 µl, and the samples were analyzed by FACSort. In

setting compensation, cells stained with annexin V alone or PI alone were used. Apoptotic cells stain with annexin V, while necrotic cells stain with both annexin V and PI.

Statistics

In paper1, the two-tailed Student's t-test and the paired t-test were used for statistical evaluation of the data. Differences were considered significant if the *P* value was equal to or less than 0.05.

In paper 2 and paper 3, data were expressed as median. Statistical analysis was performed using statistical software SPSS version 11.0 package (SPSS, Inc. Chicago, IL). Group comparisons were made using non-parametric Mann-Whitney U-test. Differences were accepted as significant at a level of $p<0.05$.

SUMMARY

The process of implantation and placentation are immunologically unique phenomena during which, immunocompetent fetal tissue invades the endometrium creating a successful collaboration between the two different sites . Fetal antigens are presented on the invading trophoblast, partly in context with HLA-C, a limited amount of which is expressed on certain trophoblast populations. The non-classical Class I molecules; HLA-G and HLA-E are highly expressed by the trophoblast. Recognition of these molecules by resident lymphocytes results in a mild local inflammatory response that is needed for implantation. NK cells, iNKT cells, γ/δ T cells are highly enriched in the decidua. All of them possess activating and inhibitory NK cell receptors for MHC molecules, which enable them to distinguish maternal self from fetal non-self.

Successful implantation is controlled by a precisely tuned balance of activating and inhibitory mechanisms. The establishment of an invasive phenotype in the trophoblast involves a host of cellular processes and an array of expression and/or repression of several genes involved in cell adhesion, composition of the extra-cellular matrix, matrix digestion, angiogenesis, apoptosis or cell cycle arrest.

Activation of NK cells, iNKT cells, γ/δ T cells leads to local inflammatory processes, which results in extracellular matrix digestion, (thus facilitating trophoblast invasion) and enhances angiogenesis of the developing placenta. At the same time, non-classical HLA molecules convey immunotolerance by moderating the inflammatory process. The local immunological changes are reflected in the maternal peripheral blood.

Disturbances of implantation and placentation constitute the pathological background of recurrent miscarriage, pre-eclampsia or intrauterine growth retardation.

Maladaptation of both local and systemic immune responses can be observed in women suffering from the above disorders.

In recurrent aborters local and systemic inflammatory processes become exaggerated with dominating Th1 responses, particularly by lymphocytes of the innate immune system. Due to poor vascularization of the placenta at the time of implantation, an enhanced systemic inflammation presents in the third trimester resulting in pre-eclampsia. Because of an increased decidual resistance to trophoblast invasion, placental development and remodeling of the spiral arteries is not supported. These pathological changes manifest in clinical symptoms when fetal growth accelerates and placental blood supply becomes insufficient. Subsequently, the placenta becomes oxidatively stressed and releases factors in the maternal circulation that elicit a strong activation of the immune system. Activated lymphocytes secrete pro-inflammatory cytokines, show increased cytotoxic potential and reduced apoptotic capacity. The generalized inflammation affects several maternal organs and persists as long as the placenta is attached to the uterus.

Successful implantation and placentation requires a close cooperation and active communication between fetal and maternal tissues. Initially, disorders of the process manifest in local pathological phenomena, however this is usually followed by systemic changes. Early diagnosis of these conditions is essential for targeted health care and management. Identification of circulating markers of fetal/placental and maternal pathology in patients will enable researchers to understand the underlying mechanisms and translate their findings to new therapies, and ultimately prevention of these devastating diseases.

PAPERS

Paper 1

Szereday L., Barakonyi A., Miko E., Varga P., Szekeres-Bartho J.: γδ T cell subsets, NKG2A expression and apoptosis of Vδ2+ T cells in pregnant women with or without risk for premature pregnancy termination. Am. J. Reprod. Immunol. 2003; 6: 490-496.

Paper 2

Miko E., Szereday L., Barakonyi A., Jarkovich A., Varga P., Szekeres-Bartho J.: Immunoactivation in pre-eclampsia: Vδ2+ and regulatory T cells during the inflammatory stage. J. Reprod. Immunol.(in press)

Paper 3

Miko E., Szereday L., Barakonyi A., Jarkovich A., Varga P., Szekeres-Bartho J.: The role of invariant NKT cells in pre-eclampsia. Am. J. Reprod. Immunol. 2008;60:118-126.

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