IMPLANTATION DISORDERS: IMMUNOLOGICAL BACKGROUND

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PhD Theses

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INTRODUCTION

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.

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 and expresses a special combination of HLA class I antigens: HLA-G, HLA-E and a small amount of HLA-C. HLA-G and HLA-E are non-classical class I molecules, with a limited polymorphism and low cell surface expression. HLA-C is polymorphic, therefore, paternal specificity is also expressed at the fetomaternal interface. 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. 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.

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, 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 (cytokine production) and cytotoxic functions.
- 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.

- They kill target cells by using secretory (perforin/granzyme mediated) and non-secretory mechanisms (Fas-ligand).
- 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. 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 with unknown ligands.

Decidual recognition of fetal HLA antigens expressed by the trophoblast contributes to the control of invasion and embryo implantation. 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, which by loosening the tissue facilitates trophoblast invasion. Additionally, IFN-γ produced during inflammation promotes vascular remodeling .

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. In the case of recurrent spontaneous abortion, several studies confirmed a pathological activation of decidual lymphocytes and these women have been shown to have a Th1-dominant cytokine profile. In pre-eclampsia, insufficient trophoblast invasion leads to poor placentation resulting in placental hypoxia and later on in systemic maternal symptoms.

AIMS OF THE STUDY

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.

1. Investigation of peripheral γ/δ T cell characteristics in women at risk for premature pregnancy termination,n in normal pregnancy, and in non-pregnant controls.

2. Investigation of peripheral γ/δ T cell and regulatory T cell characteristics in pre-eclamptic women, in normal pregnancy, and in non-pregnant controls.
3. Investigation of iNKT cell characteristics in pre-eclamptic women, in normal pregnancy, and in non-pregnant controls.

Most of all we were interested in the expression of the cytotoxic perforin molecule, in the expression of the strong pro-inflammatory cytokine IFN-γ, in the apoptotic capacity (Fas/CD95 expression, positivity for annexin V), in the activation rate (CD69+) and in the NK cell receptor expression pattern of the peripheral lymphocytes.

MATERIALS AND METHODS

Patients

Altogether 251 subjects were involved in the study. Clinical data of the patients are summarized in the following table:

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)	-

Separation of peripheralvenous 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.

MiniMACS γδ T cell separation

Vδ2+ T cells were separated using MiniMACS immunomagnetic beads, following the instructions of the manufacturer. Briefly, cells were washed with PBS. Ten million cells were labeled with anti-Vδ2 monoclonal antibody. After incubation, lymphocytes were washed and goat anti-mouse IgG microbeads were added to the cells. Immunomagnetic labeled cells were applied to a MiniMACS column fitted to a magnet. The column was washed more 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. 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 red blood cells were lysed and fixed with paraformaldehyde.

For detecting perforin positive cells, after surface labeling the cells were permeabilized and labeled with fluorochrome-conjugated mouse anti-human perforin. After labeling, cells were washed, fixed with paraformaldehyde.

For detecting cytokine positive cells, 500 µl heparinized venous blood was diluted 1:1 with RPMI1640 containing 10% FCS. Stimulation of cytokine synthesis was achieved with the brefeldin A, phorbol myristyl acetate and ionomycin. The samples were incubated for 4 hours at 37 ^{o}C in 5% CO_2. After incubation of the cells were labeled by surface staining as mentioned above. After labeling the cells were permeabilized and labeled with fluorochrome-conjugated mouse anti-human cytokine antibody, washed and fixed with paraformaldehyde.

All samples were stored at 4 ^{o}C in dark until FACS analysis. Labeled cells were analyzed with a FACSCalibur flow cytometer 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 resuspended in annexin-binding buffer. The cells were labeled with biotinilated annexin V. Cells were washed and stained with Streptavidin-APC and propidium iodide. The samples were analyzed with a

FACSCalibur flow cytometer. 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.

RESULTS

1/a: We determined 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.

1/b: 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.

1/c: The percentage of annexin V+ Vδ2+ T cells was significantly lower in patients at risk for premature pregnancy termination than in healthy pregnant women.

2/a: 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.

2/b: We found a decreased Fas (CD95) expression on Vδ2+ cells of pre-eclamptic women.

2/c: 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.

2/d: We found significantly decreased expression of TIM-3 by Vδ2+T cells of pre-eclamptic women. TIM-3 (T cell immunoglobulin mucin 3) has recently been identified as a negative regulator of tissue destructive immune responses.

2/e: In our experiments, the percentage of regulatory T cells (CD4+CD25bright) of gated lymphocytes was significantly lower in pre-eclamptic pregnant women than in non-pregnant controls. Following activation, T cells begin to induce the expression of Cytotoxic T lymphocyte antigen-4 (CTLA-4). The percentage of CTLA-4 expressing cells among CD4+CD25bright T cells was significantly higher in pre-eclamptic pregnant women than in non-pregnant controls, and healthy pregnant women.

3/a: 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.

3/b: 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.

3/c: We found that in healthy pregnant women significantly more iNKT cells express CD95, than in either pre-eclamptic patients or non-pregnant individuals.

3/d: 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.

THESES AND CONCLUSIONS

1. Our data suggest the 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 contribute to the lack of

a Th2 shift and to the pathophysiology of high risk pregnancy. In our hands, the immunological changes observed in the periphery correlate with and reflect the local decidual processes known from the literature. Data from the peripheral immune responses may have diagnostic and predictive significance.

2. We demonstrated an enhanced cytotoxic capacity and Th1 polarization of the Vδ2+T cell population in pre-eclampsia. This could be explained by the altered expression of NK cell inhibitory and activating receptors.on the cell surface. These cells were less susceptible to apoptosis than Vδ2+ T cells from healthy pregnant women predicting a longer lifespan of pre-eclamptic Vδ2+ T cells. 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.
3. The reduced percentage of regulatory T cells in pre-eclamptic pregnant women suggests inadequate tolerance induction. Enhanced CTLA-4 expression by these cells indicates that though present at a lower frequency, a higher percentage of regulatory T cells is activated in pre-eclamptic patients than in healthy individuals.
4. We demonstrated that in pre-eclampsia, iNKT cells show a pathological activation rate and - similar to the characteristics of pre-eclamptic Vδ2+ T cells – a polarization towards the Th1 type immunity. The co-directional changes of these cell populations indicate a common triggering factor and central regulatory mechanisms in the background.

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