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Immunological challenges during pregnancy : preeclampsia and egg donation

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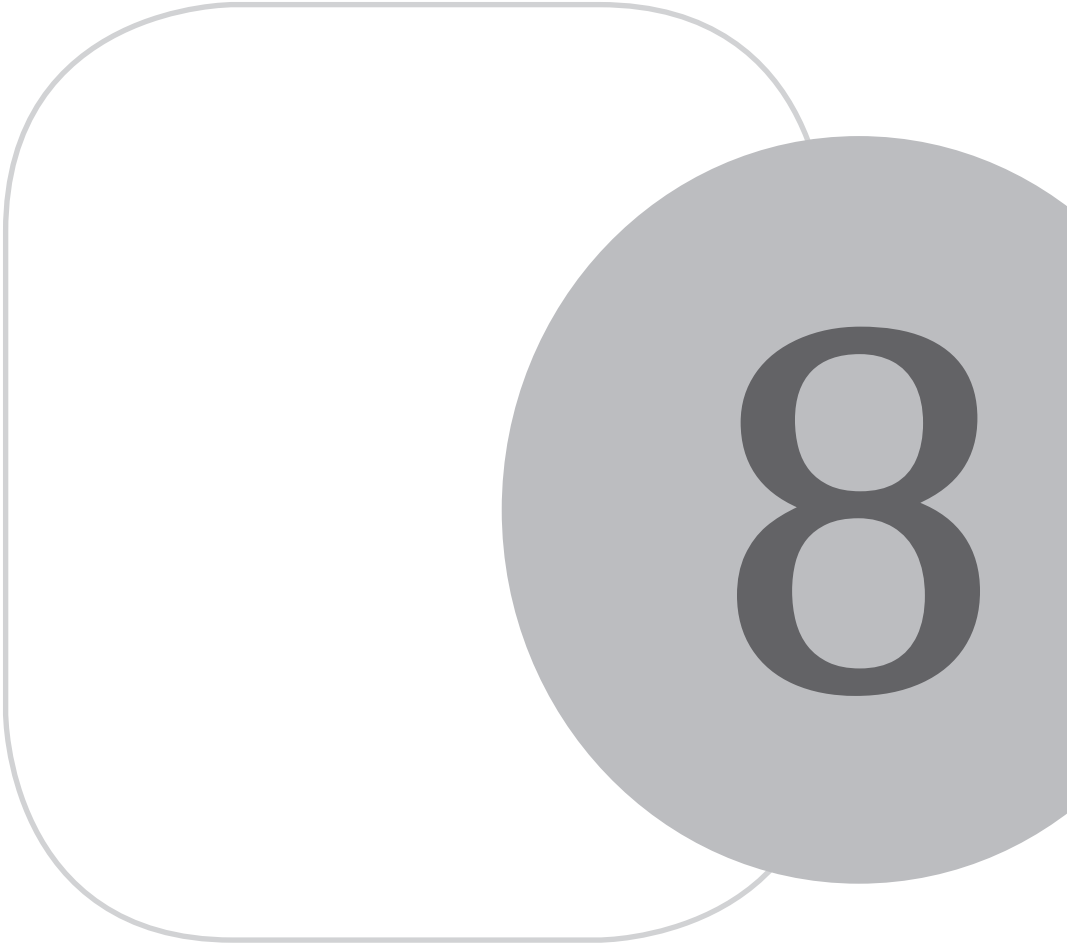
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Summary and general discussion



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This thesis investigated immunological factors during pregnancy at the fetal-maternal interface. Uncomplicated naturally conceived, preeclamptic, uncomplicated egg donation (ED), and uncomplicated IVF pregnancies were included to define possible alterations in immunological mechanisms. The fetus consists of paternal and maternal genes, and is thereby semi-allogeneic towards the mother. Despite of these immunogenetic differences the fetus escapes from immune rejection by the maternal immune system and is tolerated for the duration of the pregnancy. This thesis stated that changes in immune regulation locally in the placenta contribute to the pathogenesis of preeclampsia, and therefore preeclampsia was seen as an immunological challenge during pregnancy. Another immunological challenge is reflected by ED pregnancies. Even in ED pregnancies, where the fetus is totally allogeneic towards the mother, the fetus escapes from immune rejection by the maternal immune system. This thesis hypothesized that differential immunoregulation during pregnancy is necessary to accept the allogeneic fetus in ED pregnancies. Table 1 gives an overview of the pregnancies, materials, methods, results and conclusions of every chapter discussed in this thesis.

A general introduction on pregnancy was given in **chapter 1**. The biological basis of placental development, reproductive immunology, preeclampsia and ED is reviewed.

No systematic study on specific and non-specific maternal immune responses during normal pregnancy compared with non-pregnant controls was performed so far. We analyzed the phenotype of cells of peripheral blood samples of both groups and studied the proliferative capacity of these cells upon specific or non-specific stimulation (**chapter 2**). Non pregnant control females were compared with women carrying an uncomplicated pregnancy. We found no differences between the response of maternal peripheral blood mononuclear cells (mPBMCs) and control PBMCs (cPBMCs) upon specific stimulation. Although this was not reflected in the proliferative immune response upon specific stimulation, a different composition of leukocyte subsets was found in peripheral blood samples. Pregnant women contained a higher percentage of CD14+ cells and CD25dim cells, and a lower percentage of CD16+CD56bright and CD16+CD56+ NK cells. Furthermore, serum of pregnant women contained more IL-6, IL-10 and IL-17. Stimulation of mPBMCs and cPBMCs with allogeneic stimuli resulted in different amounts of cytokine production between the two groups. These data indicate that a pregnant woman is capable of creating a fine-tuned environment, optimal for the growth and survival of the fetus, but as well optimal for the mother to maintain adequate immune responses to infections or diseases.

Preeclampsia is a disease of pregnancy caused by multiple factors. The etiology is not fully understood. Preeclampsia is thought to be an immunologically driven disease because there is an association with primigravida, while subsequent pregnancies with the same father are protected. Also the possibility of preeclampsia occurring in subsequent pregnancies with a different father, the protective role of blood transfusions, previous abortions and prolonged semen or seminal fluid exposure are in line with this reasoning [1,2]. Macrophages are an abundant cell population in the human decidua, alterations within this cell population may lead to immunological disturbance, possibly involved in preeclampsia. Decidual macrophages play an important role in promoting immune tolerance via the production of anti-inflammatory substances, like IL-10 and indoleamine 2,3-dioxygenase (IDO). Trophoblast may interact with decidual macrophages via HLA-G and thereby stimulate the production of anti-inflammatory cytokines. Macrophages appear to disrupt vascular smooth muscle in the spiral arteries, prior to trophoblast invasion, and activated macrophages have been shown to inhibit trophoblast invasion [3]. These data suggest that inadequate production of the cytokines by macrophages, and their roles in spiral artery remodeling, potentially contribute to the pathogenesis of preeclampsia. We therefore investigated the distribution and phenotype in decidual macrophages in preeclamptic and control pregnancies (**chapter 3**) [4]. Macrophages polarize into different phenotypes. Pro-inflammatory macrophages

Pregnancies	Materials	Methods	Results	Conclusion
Chapter 2				
Uncomplicated spontaneously conceived (mPBMC)	UCB	FACS:	mPBMC vs cPBMC: ↓ NK cells, ↑ monocytes, ↑ CD3+CD25+ T cells ↑ CD3+CD25dim T cells	The maternal peripheral immune response is altered during pregnancy, though these differences do not result in quantitative changes in proliferative responses during pregnancy compared to non-pregnant controls.
		MLC:	No difference in proliferation upon stimulation with aCD3, PHA, fetus specific UCB and 3 rd party UCB between mPBMCs and cPBMCs	
Non pregnant women (cPBMC)	mPBMC	Luminex:	mPBMC vs cPBMC: ↑ IL-6 (after stimulation with UCB or 3 rd party UCB) ↑ IL-10 (after stimulation with 3 rd party UCB), ↑ IL-17 (after a-specific stimulation)	
		cPBMC		
Chapter 3				
Preterm preeclamptic	Placentas	IHC:	Preterm preeclampsia vs preterm controls ↑ CD14 and CD163 expression in the decidua basalis	Alterations in distribution and phenotype of macrophages in the decidua of preterm preeclamptic pregnancies compared to control pregnancies may contribute to the pathogenesis of preeclampsia.
			Preterm control	
Term control	Fetal membranes	↑ DC SIGN/CD14 in the decidua basalis and parietalis CD14+ macrophages did express Flt-1		
Chapter 4				
Non donor IVF	Clinical data	Comparison of clinical data	IVF: dizygotic twin pregnancy was complicated by preeclampsia and intra uterine growth retardation.	We suggest a different pathophysiological mechanism of preeclampsia after ED compared with preeclampsia in non-donor IVF conception.
ED	Placenta		ED: dizygotic twin pregnancy was complicated by severe preeclampsia, both fetuses had a normal fetal birth weight.	
Chapter 5				
ED	79 publications	Review of literature	ED pregnancies are associated with a higher incidence of pregnancy-induced hypertension and placental pathology. Perinatal complications, such as intrauterine growth restriction, prematurity, and congenital malformations, is comparable to conventional <i>in vitro</i> fertilisation.	ED pregnancies have a higher risk of maternal morbidity. Due to the high degree of antigenic dissimilarity, ED pregnancies represent an interesting model to study complex immunologic interactions.
Chapter 6				
ED		Test hypothesis	During ED pregnancies the mother has to cope with a higher degree of antigenic dissimilarity compared with spontaneously conceived pregnancies. Maternal cells and fetal cells come in close contact. Understanding the immune mechanisms gives more insight into the question why the (semi) allogeneic fetus is accepted and not rejected by the mother.	The immunologic interactions between mother and child in successful ED pregnancies may be relevant for the induction of immunologic tolerance in solid organ transplantation.
Chapter 7				
ED	Placenta	IHC:	ED vs NC: ↓ IL-10, IL-6, galectin-1, pSMAD2 and Flt	Immunoregulation in ED is different than in NC pregnancies, partly due to procedure considering similarities with IVF pregnancies. An altered peripheral and local regulation was found in ED and IVF pregnancies compared to NC pregnancies, shown by the level of cytokines present in serum and placenta tissue, the phenotype of the cells in the periphery and the response of mPBMCs upon stimulation.
		Luminex:	ED vs NC: ↑ IL-10, IL-6, ↓ TGF-β	
IVF	Fetal membranes	FACS:	ED/IVF vs NC: ↑ CD4+CD25bright and CD4+CD25dim cells were higher in mPBMCs	
NC	UCB	HLA relation:	The number of HLA mismatches was positively correlated with the percentage of activated CD4+CD25dim T cells in mPBMCs of ED.	
	Maternal blood	MLC:	ED/IVF vs NC: ↑ immunoregulation	

Table 1 Main results of the chapters discussed in this thesis. For abbreviations, see the abbreviation list.

(type 1) have pro-inflammatory and cytotoxic properties and are able to eradicate intracellular pathogens. In contrast, anti-inflammatory macrophages (type 2) display anti-inflammatory properties and are able to secrete IL-10. CD163 is a marker present on type 2 macrophages. The human decidua contains type 2 macrophages contributing to the immune suppression necessary to maintain the semi-allogeneic fetus whereas type 1 macrophages are able to defend against utero-placental infection, but possibly do not contribute to tolerance of the fetus. We found a decreased expression of type 2 macrophages in the decidua basalis of preeclamptic pregnancies compared to preterm control pregnancies. Furthermore, the level of expression of IL-10 was lower in the decidua parietalis of preeclamptic pregnancies compared to preterm control pregnancies. The distribution of decidual macrophages is altered in preeclamptic pregnancies and we showed that the macrophages contain fmslike tyrosine kinase 1 (Flt-1). The soluble form of this cytokine (sFlt-1) is produced in excessive forms in preeclampsia. Macrophages may be responsible for this increased sFlt-1 production, and by an alteration of distribution they may contribute to the pathogenesis of preeclampsia.

Immunological tolerance between mother and fetus is needed for successful reproduction. Sharing of too many HLA antigens between mother and father has been shown to form a disadvantage on pregnancy outcome, sharing of too few HLA antigens may as well alter pregnancy outcome [5]. Since preeclampsia is a disorder of the immunological mechanisms involved in the normal fetal-maternal responses, possibly based on HLA (mis)matching, we hypothesized that the immunological mechanism of preeclampsia is different in normal pregnancies and ED pregnancies (**chapter 4**) [6,7]. Two dizygotic pregnancies are described. Both pregnancies resulted from assisted reproductive techniques. Interestingly, the non donor IVF pregnancy developed preeclampsia with severe growth retardation and the ED pregnancy developed preeclampsia without fetal growth retardation. Although only two cases are investigated, these nicely illustrate the hypothesis why preeclampsia in ED pregnancies might have another pathophysiological mechanism.

ED pregnancies represent a very interesting model to investigate immunological interactions. So far most research is focused on the medical complications in mother and fetus rather than basic immunology. The immunological dissimilarity in ED pregnancies is comparable to the immunological dissimilarity in solid organ transplantations. The acceptance of a fetal allograft in normal pregnancies requires the avoidance of rejection by altered HLA expression on trophoblast cells, production of immunosuppressive cytokines and other immunomodulatory strategies. These mechanisms are most probably as well present in ED pregnancies. However, since the potentially higher degree of immunological dissimilarity compared with naturally conceived pregnancies, additional mechanisms are possibly needed for the acceptance of the fetal allograft (**chapter 5**). Expanding knowledge of immunological mechanisms in successful and failed ED pregnancies is potential viable in understanding the immunological interactions involved in acceptance or rejection of solid organ transplantations.

ED gives infertile women the opportunity to conceive, however, it has a higher incidence of harmful maternal consequences, compared with naturally conceived pregnancies (**chapter 6**) [8]. Women who conceived by ED have an increased risk of pregnancy induced hypertension, increased rate of cesarean section deliveries, increased risk of post partum hemorrhage and an increased risk of first trimester vaginal bleeding. However, it is not associated with an increased complication rate for the fetus or newborn.

The immunological mechanism in ED, naturally conceived and non donor IVF pregnancies has been studied in **chapter 7**. We found an altered immune reaction, locally as well as peripherally in ED and non donor IVF pregnancies compared with naturally conceived pregnancies. We tested this by the analysis of several cytokines in serum and placental tissue. Furthermore, mPBMCs were phenotyped by flow cytometry and correlated with the number of HLA mismatches.

mPBMCs were stimulated with (allogeneic) UCB or control PBMCs in a mixed lymphocyte reaction. Differences in cytokine expression in decidua and maternal serum between ED, IVF and naturally conceived pregnancies were found. The percentage CD4+CD25bright and CD4+CD25dim were higher in mPBMCs of ED and IVF pregnancies compared to naturally conceived pregnancies. In ED pregnancies, the number of HLA mismatches is positively correlated with the percentage of CD4+CD25dim in mPBMCs. Although the exact mechanism remains to be elucidated, we found that the immunology in ED and non donor IVF pregnancies is altered compared to naturally conceived pregnancies. The presence of fetal cells in maternal blood (fetal microchimerism) possibly plays a role. Antigens of fetal cells migrate into the maternal blood, and fetal (and thus partly parternal) antigens may be able to modulate the maternal immune response during pregnancy. In ED pregnancies the impact of microchimerism might be altered, and thereby leading to a positive correlation between the percentage of CD4+CD25dim and the number of HLA mismatches in ED pregnancies.

Experimental considerations

To identify possible immunologic mechanisms contributing to aberrant immunology in pregnancy, it is essential to validate factors potentially influencing the outcome. In our studies we cautiously selected the control groups. In chapter 3, in addition to a term control group, a preterm control group was selected. In this way the gestational age between the preterm preeclamptic group and the preterm control group did not significantly differ. As the gestational age might affect the immunological outcome, this influence of time was ruled out. In chapter 7 a non donor IVF group was included to serve as a control group for ED pregnancies, both had comparable hormonal treatment before pregnancy. Furthermore, for all our tissue included, we delicately defined the location of immunological importance. On a protocol basis we collect placentas of pregnancies with our interest. Biopsies at three locations of the placenta and fetal membranes are taken. If there would be a difference in immune regulation at different locations, this potential bias is ruled out by this approach.

We used mixed lymphocyte cultures (MLC or mixed lymphocyte reactions) to measure T cell alloimmune responses. When allogeneic leukocytes are cultured together, T cell populations expand. The total proliferation of lymphocytes is measured by monitoring the uptake of 3H thymidine, during cell division. In this test the stimulators are inactivated by radiation, and are not able to proliferate. CD4+ T cells are critical for this reaction, and therefore the HLA class II mismatch plays an important role. In our studies, HLA typings of all mothers and children were performed and the number of mismatches between mother and child were calculated. For the performance of the MLCs, control cells were selected on the basis of the number of HLA-DR mismatches.

The immunohistochemical protocols were extensively tested for every antigen described in this thesis. For every test a negative control was used, to test for the specificity of the antibody involved. We also reduced non-specific background staining. The main cause of non-specific background staining is non-immunological binding of the specific immune sera by hydrophobic and electrostatic forces to certain sites within tissue sections. Since some antibodies showed non-specific background staining, we reduced this by blocking with serum. Furthermore, tissue may show endogenous peroxidase activity, resulting in non-specific staining. The solution commonly used for eliminating endogenous peroxidase activity is by the pretreatment of the tissue section with hydrogen peroxide prior to incubation of primary antibody. The precision, by which we tested our stainings, resulted in optimal staining for every antibody used. We used digital analysis methods to analyze our staining intensity, as a consequence objective results were gathered. Only the relevant areas were selected for analysis. We focused the analysis of the immunohistochemical

staining on the decidua basalis and the decidua parietalis. These are the maternal parts of the placenta and fetal membranes respectively; hereby the immune interactions at the fetal-maternal interface were studied. Maternal blood floats through the placenta in the intervillous space. The analysis of maternal peripheral blood provides information on the systemic maternal immune responses. It contains trophoblast micro particles, shed from the syncytiotrophoblast layer of the villi. By the analysis of the decidua basalis, decidua parietalis and the maternal peripheral blood, the three different fetal-maternal interfaces were studied. To better characterize the relative proportion of the studied cell populations of maternal peripheral blood, the results of the flow cytometry analysis were presented as the fraction of cells in the CD45+ and CD3+ fraction. The separate fractions did not significantly differ. Therefore, we expressed the results as a percentage of the absolute number of CD45+CD3+ cells.

The term fetal allograft is widely used. The mother accepts the immunologically foreign fetus during uncomplicated pregnancy, like an engrafted organ is tolerated [9]. The consequence of this understanding is that immunopathological recognition of fetal antigens might be viewed as an alloimmune reaction like graft rejection [10]. Despite the resemblances there are arguments against this model. The fetal derived cells, which invade the maternal decidua, use several immunological escape mechanisms. These mechanisms are not present during organ transplantation. For example, the villous trophoblast expresses no HLA antigens on its surface, and extravillous trophoblast expresses a very particular set of HLA antigens. Therefore, the use of the term allograft should not be used carelessly. However, many common mechanisms determining graft and fetal outcome exist. Pregnancy and organ transplantation both reflects a precise balance between pro acceptance and anti rejection stimuli. Analysis of immune reactions shows that graft rejection shares many similar mechanisms with recurrent spontaneously abortions and preeclampsia [9]. Decreased graft rejection and successful pregnancy outcome is associated with the presence of unique suppressor cells producing elevated levels of type 2 cytokines. It has been shown that the rejection of allograft and spontaneous abortions are associated with elevated type 1 cytokines [9]. However, acceptance solely based on a type 2 phenomenon is oversimplified since type 1 cytokines are as well necessary to avoid rejection of the (fetal) allograft [10].

Future perspective

In this thesis uncomplicated ED pregnancies were investigated, and considered the fetus as fully allogeneic. The placentas were collected after nine months uncomplicated pregnancy. It would be valuable to study also the immunological interactions upon embryo transfer. After transfer the maternal cells meet the allogeneic fetal cells for the first time. This is an interesting time point to investigate the maternal immunological response. Collection of local tissue is limited at this time point, but the peripheral immune response might as well be altered. Furthermore, it would be very interesting to analyze those pregnancies which fail to succeed in the beginning. Only 30% of embryo transfers after ED succeed, thus 70% of all pregnancies after ED, are not continuing [11]. The embryo transfers which result in miscarriage might have a very interesting immunological basis, possibly playing a role in the pathology of a miscarriage. Tissue of these pregnancies is therefore viable to study, and might give more insights in the immunological interactions, already during the implantation phase of pregnancy. The pathological mechanism of preeclampsia during ED pregnancies might be different compared with naturally conceived pregnancies. This thesis focused on uncomplicated ED pregnancies. However, investigation of preeclamptic ED placentas is essential to confirm this hypothesis.

In addition to ED pregnancies, mole pregnancies (hydatidiform mole) are as well fully allogeneic towards the mother. A complete mole pregnancy is entirely derived from the paternal genome. It is caused by a single sperm combining with an egg which has lost its DNA. The genotype is

typically 46,XX (diploid), which is due to subsequent mitosis of the fertilizing sperm. It is also possible that an empty egg is fertilized by two sperms, which may result in the 46,XY genotype. Immunological interactions between mother and the mole pregnancy are possible comparable with the immunology in ED pregnancies.

Recurrent miscarriage represents a complication in pregnancy, is also supposed to have an immunological pathophysiology. There must be a reason why in those maternal and paternal combinations the couple is not able to get pregnant. Immune cells at the fetal-maternal interface of recurrent miscarriage pregnancies might reveal the underlying immunological mechanism.

Conclusion

In conclusion, this thesis studied uncomplicated pregnancies and two immunological challenges during pregnancy, preeclampsia and ED. First, uncomplicated pregnancies were studied and we found that pregnancy is characterized by changes in cytokine production and composition of peripheral blood leukocytes, which is not reflected in the proliferative response to the fetus. There seems to be an optimal balance between maternal protection against infections and fetal tolerance. Second, pregnancies complicated by preeclampsia were investigated. We found a differential distribution and phenotype of decidual macrophages in preterm preeclamptic pregnancies, which may explain why less immunoregulation takes place in preeclampsia placentas. Finally, ED pregnancies were analyzed leading to the hypothesis that preeclampsia might have different pathophysiological mechanism compared with normal and non donor IVF pregnancies. Furthermore, the resemblances between solid organ transplantation were discussed and we showed that ED pregnancies lead to more maternal complications and the immunoregulation in ED and IVF pregnancies is altered compared with normal pregnancies. We found differential immunological interactions in successful ED pregnancies compared with naturally conceived pregnancies.

These results indicate that preeclampsia and ED pregnancies are indeed immunological challenges during pregnancy. It is a scientific challenge to further reveal the immunological mechanism of preeclampsia and ED pregnancies, contributing to precious information for the fields of immunology, transplantation and obstetrics.

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