Maternal-fetal HLA compatibility and trophoblast-immune interactions in healthy and preeclamptic pregnancy: elegance in complexity

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Summary and general discussion

Summary of results

A healthy start of life requires a well-established and maintained 'immune balance', as a wide array of pregnancy complications are associated with dysregulated immune processes. Understanding these processes can help patients and their families with insight and support, assist healthcare providers in prevention, diagnosis and treatment, and offer scientists valuable comprehension for future care strategies.

In this dissertation, we aimed to contribute to understanding which pathways of maternal-fetal immune modulation are required for a healthy pregnancy and how these pathways are altered in the pathophysiology of preeclampsia. This thesis focused primarily on two immune processes: maternal-fetal HLA compatibility and factors by which trophoblasts may influence maternal immune cells, specifically $CD4^+$ T cells.

In **Chapter 2**, we analyzed the relationship between maternal-fetal HLA compatibility and the occurrence of either an uncomplicated pregnancy or preeclampsia. This analysis demonstrated that maternal-fetal HLA matching in uncomplicated pregnancies was not different than expected-by-chance. However, increased compatibility of total HLA and HLA class I, but especially HLA-C, is associated with preeclampsia. The distribution of the genotype-combinations of maternal killer-cell immunoglobulin-like receptors (KIR) and paternally inherited HLA-C differed in preeclamptic pregnancies, with an overall tendency towards a higher prevalence of HLA-C2, compared to uncomplicated pregnancies. These analyses were extended to a genetically isolated population, described in **Chapter 3**, where no significant difference was found between observed and expected maternal-fetal HLA (mis)matches in uncomplicated pregnancies, despite increased HLA homozygosity in the population. However, in pregnancies with hypertensive complications significantly more HLA-DQB1 mismatches were observed. This was reflected in PIRCHE-II scores, which predict $CD4^+$ T cell responses to mismatched HLA epitopes. In **Chapter 4**, we explored a non-invasive technique of fetal HLA screening in early pregnancy, with the aim to use it in preeclampsia risk management in the future. Our method of isolating extravillous trophoblasts (EVT) using fluorescence-activated cell sorting (FACS) resulted in a detectable fetal HLA genotype though the technique requires further optimization. In **Chapter 5**, we investigated whether the expression of certain co-inhibitory molecules was altered in term placenta of preeclamptic cases, particularly in oocyte donation (OD) pregnancies. The hypothesis was that in these pregnancies the increased immunogenetic dissimilarity may require a higher extent of immune regulation compared to naturally conceived pregnancies. We detected alterations of PD-L1, CD200, and IDO expression in the placenta of OD pregnancies, and found that placental CD200 protein expression is positively correlated with the number of HLA mismatches (total and HLA class I) in uncomplicated OD cases. Finally, in **Chapter 6**, we investigated the potential effect of

EVT on the phenotype of the decidual $CD4^+$ T cell population. Our in vitro study of first trimester primary differentiated EVT that were co-cultured with activated conventional $CD4^+$ T cells (Tconv) suggests that EVT can directly promote the acquisition of regulatory features in Tconv. By elucidating mechanisms of maternal-fetal immune interactions, this dissertation contributes to a deeper understanding of pregnancy, and helps to provide a foundation for targeted research aimed at improving outcomes in pregnancies that are at risk of preeclampsia.

Finding the right match

We have come a long way since Peter Medawar, who later on won the Nobel Prize for his work on transplantation immunology, first described the 'immunological paradox of pregnancy' in 1953 (1). He compared the fetal-placental unit to a 'simple' organ graft, suggesting that the fetus must evade maternal immune recognition to survive. However, research since then has revealed that several adjustments must be made to his theories (2). Unlike in organ transplantation, the maternal and fetal blood circulations do not mix, eliminating direct immune exposure. Additionally, pregnancy failure is not necessarily linked to an influx of maternal T cells in the placenta, nor does formation of fetus-specific HLA antibodies in the mother correlate with clinical outcomes (3, 4). Medawar proposed that the fetus might escape immune detection through antigenic immaturity and generalized immunosuppression, yet we now know that HLA expression by fetal extravillous trophoblast cells begins as early as six weeks of gestation, and while the maternal immune system is modulated during pregnancy, it is not broadly suppressed (5-7). Indeed, such a state would be highly detrimental, increasing susceptibility to infections. Furthermore, although HLA-A, B and HLA class II are not expressed on trophoblasts, other HLA molecules are expressed and facilitate spiral artery remodeling via innate immune cells during placentation and thereby a successful pregnancy (7). These findings challenge the comparison between pregnancy and transplantation. However, Medawar was correct in one important aspect: the placenta serves as an anatomical barrier, a concept that can now be expanded to include its role as an immunological barrier.

Additionally, it is important to recognize that the immune system predates both mammalian pregnancy and the placenta in evolutionary history. While it is often stated that the maternal immune system adapts to pregnancy, it remains an open question whether the mother tolerates the fetus or rather the fetus evading maternal immunity. Moreover, the concept of tolerance itself warrants reconsideration: should we speak of tolerance, implying passive acceptance, or rather of modulation, an active and dynamic interaction between maternal and fetal immune components? There are surely enough arguments for the latter.

Maternal-fetal HLA compatibility in uncomplicated versus preeclamptic pregnancies

Trophoblasts are unique cells with a highly specialized immune profile. While syncytiotrophoblasts (STB) do not express any classical HLA molecules (neither class I nor class II), EVT selectively express HLA-C alongside the non-classical molecules HLA-G, HLA-E, and HLA-F (5). Each of these molecules presents a distinct peptide repertoire and engages specific maternal receptors. HLA-C, the only highly polymorphic classical class I molecule on EVT, presents fetal peptides to and binds to KIR on decidual NK (dNK) cells and $CD8^+$ T cells. KIR/HLA-C interactions play a key role in EVT invasion, spiral artery remodeling and therefore placentation (8). HLA-E primarily presents leader peptides derived from other class I molecules and interacts with CD94/NKG2A (inhibitory) or CD94/NKG2C (activating) receptors, expressed on NK cells and subsets of $CD8^+$ T cells (9). HLA-F is recognized by KIR3DL2, KIR3DS1, and LILRB1 on NK and T cells, but its function in peptide presentation is not fully understood (5). HLA-G presents a restricted set of self-peptides and engages inhibitory receptors including LILRB1 and LILRB2 on NK cells, T cells, dendritic cells (DCs), and macrophages (10). This restricted expression of HLA molecules on the trophoblast membrane has led to the assumption that direct immune recognition is limited. However, next to direct HLA recognition, fetal HLA molecules, including HLA class II, can still be processed and presented as peptides by antigen presenting cells (APCs), especially DCs and macrophages. In theory, both direct and indirect recognition of fetal HLA can shape maternal immune responses. These responses may either support or disrupt fetal and placental development, influencing pregnancy outcomes. These immune recognition pathways are increasingly well characterized, but their clinical implications remain incompletely understood. Deeper understanding may ultimately help identify pregnancies at increased risk for preeclampsia and related complications, allowing for more tailored counselling and monitoring.

In **Chapter 2** and **Chapter 3**, we investigated the relationship between maternal-fetal HLA compatibility and the occurrence of hypertensive complications in naturally conceived pregnancies. To investigate whether there is a preferential selection for HLA compatibility and specific KIR/HLA-C combinations, observed frequencies of maternal-fetal HLA (mis)matches were compared to expected-by-chance frequencies based on a validated method of inter-cohort randomization. In **Chapter 2**, we found no significant deviation from expected maternal-fetal HLA matching frequencies in uncomplicated pregnancies. Similarly, no significant deviations were observed in KIR/HLA-C combinations, suggesting that specific KIR/HLA-C interactions do not appear to be selectively enriched in uncomplicated pregnancies. These findings support the notion that successful pregnancy does not rely on a shift from the Hardy-Weinberg equilibrium (the expected genetic variation in a stable population) concerning maternal-fetal HLA compatibility, reinforcing the idea

that the maternal immune system can accommodate a wide range of HLA mismatches without jeopardizing pregnancy success.

To further explore the effect of high maternal-fetal HLA compatibility on the development of hypertensive disorders in pregnancy, we expanded our analysis to a genetically isolated population in **Chapter 3**. We hypothesized that in a population where parents have increased antigenic similarity, a shift towards decreased maternal-fetal HLA compatibility might be beneficial for fertility and pregnancy outcomes. We would therefore expect to see a deviation from random HLA distributions favoring increased mismatching. However, in uncomplicated pregnancies, we again found no significant differences between observed and expected maternal-fetal HLA (mis)matches. These findings further support the absence of preferential selection for HLA allele frequencies in uncomplicated pregnancies, even within this genetically isolated population. Despite the presumed increased parental HLA similarity, the HLA haplotypes appear to be sufficiently polymorphic to sustain successful pregnancies without requiring selection for maternal-fetal HLA (in)compatibility.

Taken together, these findings suggest that a certain degree of HLA dissimilarity between mother and fetus is either not influential or is even necessary for a healthy pregnancy. Increasing evidence supports the latter, particularly in the context of placental development. Successful pregnancy requires extensive remodeling of uterine spiral arteries to increase their diameter and facilitate a slow, high-volume blood flow, ensuring adequate oxygen and nutrient supply to the fetus. This process of vascular remodeling that is highly dependent on interactions between maternal decidual immune cells and invasive EVT. Central to this crosstalk is fetal HLA-C, expressed on the surface of EVT from both maternal and paternal alleles, which is recognized by KIR on dNK cells (7). KIR receptors can either be activating or inhibitory, while the HLA-C1 and -C2 allotypes bind preferentially to specific KIR receptors (8). Therefore, the particular combination of HLA-C/KIR influences dNK function, which in turn can enhance or impair placentation (11). This association is reflected in clinical studies; pregnancies in mothers with a homozygous KIR haplotype A (KIR AA) are at an increased risk of preeclampsia, particularly when the fetus inherits more HLA-C group 2 (C2) alleles than the mother (12). The unfavorable combination of maternal KIR AA and a paternal HLA-C allele bearing a C2 epitope is also associated with fetal growth restriction (FGR) and recurrent pregnancy loss (RPL) (13).

A common underlying defect in preeclampsia and FGR is abnormal placentation, leading to reduced uteroplacental blood flow. Proper remodeling of the uterine spiral arteries is crucial for ensuring sufficient perfusion to the developing fetus, and failures in this process are a hallmark of these pregnancy disorders. The critical interactions between dNK and invasive EVT occur early in gestation within the decidua basalis, setting the foundation

for adequate placenta development (11). It is hypothesized that inadequate activation of dNK cells, due to less favorable KIR/HLA-C combinations, diminishes the secretion of key cytokines such as GM-CSF and VEGF, which are essential for promoting EVT migration and invasion and therefore artery remodeling (14). Shallow trophoblast invasion may be the root cause of poor placentation, ultimately contributing to preeclampsia, FGR, and RPL. As previously discussed, referring to 'immunosuppression' as a requirement for pregnancy would once again be incorrect and the exact opposite of the actual process.

HLA-C can also be recognized by decidual T cells, which is far less studied than the interaction with dNK cells. Although $CD8^+$ and $CD4^+$ T cells form a minority in the decidua, they increase in portion during gestation (15). Decidual regulatory T cells (Tregs) have been shown to suppress fetus-specific and non-specific responses, as they displayed a higher suppressive capacity to their umbilical cord blood cells of the fetus compared to fetal cells of a third party, or to their peripheral blood counterparts (16). Interestingly, HLA-C mismatched pregnancies showed higher levels of $CD4^+CD25^{dim}$ activated T cells and increased numbers of functional decidual $CD4^+CD25^{HI}$ Tregs, compared to HLA-C matched pregnancies (17). This suggests that fetal HLA-C may specifically promote Treg differentiation, either through direct or indirect recognition.

Our research described in **Chapter 2** showed that preeclampsia is linked to maternal-fetal HLA-C matching, which is in line with the potential beneficial effects of HLA-C mismatching on $CD4^+$ T cell activity. Furthermore, these findings underscore the pivotal role of HLA-C in placenta development, as HLA-C-dependent interactions shaped by inherited HLA-C genotypes influence dNK cell function in spiral artery remodeling and placentation (11). Preeclamptic pregnancies exhibited a higher degree of maternal-fetal HLA-C, HLA class I, and total HLA matching compared to both internal expected-by-chance values and to uncomplicated pregnancies. Additionally, the distribution of maternal KIR and paternally inherited HLA-C genotype combinations differed in preeclamptic pregnancies, showing a higher prevalence of HLA-C2 compared to uncomplicated pregnancies. Our study was the first to demonstrate the association of overall HLA-C matching with preeclampsia in a larger cohort than previously investigated. Previous studies did not take HLA-C or the full repertoire of HLA class I and class II loci into account (18, 19). Unlike earlier studies that focused on maternal-paternal HLA sharing, we specifically examined maternal-fetal HLA compatibility, allowing analysis of the inherited HLA haplotype (18). Furthermore, we extended the analysis beyond HLA-A and -B to include all classical HLA class I molecules and expanded the scope of HLA class II beyond HLA-DR.

The distribution of KIR and HLA-C genotype combinations in preeclamptic cases within our study of **Chapter 2** was similar to the findings of Hiby et al., with increased presence of HLA-

C2, though less pronounced in our cohort (12). As KIR alleles differ in binding affinities to individual HLA-C2 allotypes, future analyses should include typing of individual fetal HLA-C alleles in addition to the C1/C2 distinction (20). Furthermore, cases and controls were not matched on genetic admixture, highlighting the need for larger studies. This is important because genetic variations linked to ancestry can influence disease susceptibility and outcomes. Additionally, some populations might have had a stronger selection against the KIR AA/HLA-C2 combination than others, potentially due to factors unrelated to pregnancy. Activating KIR are shown to confer advantages in infectious diseases such as HIV, but are also linked to several autoimmune diseases, likely due to their role in enhancing NK cell activity (21). However, HLA-C2 and certain inhibitory KIR have been linked to protective immune responses against certain pathogens as well. Ultimately, larger studies across different populations will be needed to determine population-specific odds ratios for the association between KIR/HLA-C and preeclampsia.

The findings from **Chapter 2** and **Chapter 3** provide an important step toward assessing whether maternal-fetal HLA compatibility, and particularly specific KIR/HLA-C combinations, can be used to guide individualized counselling, preventive measures and therapeutic management during pregnancy. While linking HLA genotyping to pregnancy outcome will initially rely on parental genotyping, methods to determine fetal HLA in utero, as investigated in **Chapter 4**, may enable direct assessment of the maternal-fetal HLA combination and further personalize antenatal care. Clinical application will also require integration into multivariate models alongside established risk factors. Beyond prediction, this knowledge can also help to refine mechanistic subtyping of preeclampsia and inspire novel immunomodulatory therapies. Lastly, ethical considerations must remain in sight. Using HLA compatibility as a reproductive decision factor raises questions about genetic selection and reproductive autonomy. Ensuring equitable access to such sophisticated HLA-based risk stratification is essential, as it may otherwise be limited to high-resource settings and potentially widen disparities in maternal care. The risk of over-medicalization should also be carefully considered. Moreover, HLA typing could reveal additional genetic information (e.g., disease susceptibilities, paternity issues) that require careful consideration and informed consent procedures.

An indirect role for HLA class II

HLA-C matching is important, but it is not the only cause of preeclampsia, since not all combinations of maternal KIR AA and paternally inherited HLA-C2 pregnancies lead to preeclampsia. A second link between maternal-fetal HLA compatibility and preeclampsia was found in **Chapter 3**. We found a significant increase in observed versus expected maternal-fetal HLA-DQB1 mismatches in pregnancies with hypertensive complications. In line with this finding, higher PIRCHE-II scores (prediction of T cell epitopes; mismatched

fetal HLA-derived peptides that can be presented by maternal HLA class II) were observed for HLA-DQB1 and HLA class II in pregnancies with hypertensive complications compared to uncomplicated pregnancies. Furthermore, children of the uncomplicated pregnancies in this cohort had a significantly higher prevalence of homozygosity for HLA-DQB1. The group of pregnancies with hypertensive complications included 42% preeclamptic cases. HLA class II mismatching must contribute to the etiology of preeclampsia through a different mechanism than HLA-C matching, since trophoblasts do not normally express HLA class II. Therefore, fetal HLA class II antigens will be presented to maternal immune cells by other cell types, particularly DCs and macrophages. The activity of CD4+ T cells that recognize HLA class II will not be specifically directed at trophoblasts, since no cognate contact via TCR-HLA class II interaction can be established.

We suspect that HLA class II mismatches contribute to preeclampsia pathophysiology via T cell activation. In solid organ transplantation, HLA-DQ mismatching is the most immunogenic and potentially most pathogenic mismatch compared to other targets (22). We expect that maternal-fetal HLA-DQB1 mismatches can result in increased indirect $CD4^+$ T-cell allorecognition and can negatively affect the balance between effector and regulatory T cell populations. We hypothesize that this effect on T cells is more important for either sustaining or disrupting immune modulation than the generation of HLA antibodies through B cell activation, as HLA antibodies are not necessarily associated with adverse clinical outcomes (23). Only certain HLA antibodies have been implicated in pregnancy complications, while others do not appear to have an effect (4).

We hypothesize that the presentation of polymorphic fetal HLA antigens, particularly class II, has distinct effects depending on whether it occurs locally at the maternal-fetal interface or systemically in the peripheral blood. Locally, a highly specialized immune tolerogenic environment is maintained by uterine immune cells and trophoblasts, which play a pivotal role through the expression of immunomodulatory molecules. In contrast, fetal antigens entering the peripheral circulation encounter a different immune landscape, where immune cells lack the same level of specialization and are not within an immune tolerogenic environment (24). Although Treg populations are present in the periphery, immune cells there lack the specialized tolerogenic environment of the uterus, which may contribute to the imbalance between effector T cells and Tregs observed in preeclampsia (25). This suggests that desired immune response following fetal HLA and/or antigen recognition in (decidual) T cells is opposite from that of decidual NK cells. However, while excessive T cell activation may be detrimental, some degree of recognition is necessary for the induction of Tregs.

A two-step interplay of fetal antigens and maternal immune response

Fetal cells cross the placenta to the maternal blood flow and some can persist in maternal organs and circulation long after pregnancy, a phenomenon termed fetal microchimerism. In case of placental inflammation, such as that resulting from inadequate placentation, there may be increased trafficking of fetal particles into the maternal peripheral blood (26). This heightened fetal antigen exposure is expected to create an increased demand for systemic immune tolerance, which might not always be successfully established. Intrauterine inflammation, for instance, has been shown to increase maternal $CD8^+$ T cell infiltration into the placenta of mice, where these cells secrete pro-inflammatory cytokines such as IFN-γ and TNF-α (27). Inflammatory cytokines can also compromise the structural integrity of the placental barrier, further facilitating immune cell migration (26, 28). This pro-inflammatory shift can reduce the T cell activation threshold and strengthen activation of cytotoxic T cells, contributing to systemic inflammatory states associated with pregnancy complications (28). Moreover, inflammation has been linked to aberrant expression of HLA class II molecules in the placenta, as observed in cases of villitis and intervillositis marked by lymphocytic infiltration (29, 30). Preeclampsia has also been associated with abnormal HLA-DR expression on STB-derived extracellular vesicles in the maternal bloodstream (31).

These immune responses do not merely depend on fetal antigen presentation, which is inevitable, but rather on how the maternal immune system reacts to these antigens. In other words, the outcome is shaped by a two-step interplay: the compatibility of the fetal HLA haplotype determines which antigens are presented, while the maternal immune system directs the response. For this, oocyte donation (OD) pregnancies are a good example. OD pregnancy, a situation with increased immunogeneity due to the complete genetic mismatch between mother and child, is associated with increased hypertensive complications (32, 33). However, not all highly HLA incompatible OD pregnancies show complications. Uncomplicated 'fully allogeneic' OD pregnancies show increased immune regulatory features such as increased expression of IDO, as we showed in **Chapter 5**. In line with these findings, a higher degree of HLA mismatches within uncomplicated OD pregnancies has also been associated with an increased percentage of Tregs and $CD163^+$ type 2 macrophages in the decidua (34, 35). Interestingly, these pregnancies also showed an increased percentages of activated T cells in the peripheral blood, but not a higher alloreactivity to the fetus (36). These observations show that the maternal immune system can adapt to extreme HLA incompatibility with compensatory immune regulatory mechanisms.

It would be interesting to also investigate the KIR/HLA-C combinations in OD pregnancies with and without preeclampsia. In theory, in OD pregnancies, if both fetal HLA-C alleles would be HLA-C2 and therefore non-self, the inhibitory signal to dNK cells would potentially

be even stronger in mothers carrying the KIR AA genotype. If such a combination is strongly associated with preeclampsia in OD pregnancies as well, it would reinforce the role of this KIR/HLA-C interaction in the pathogenesis of preeclampsia. This could also specifically aid risk stratification for OD pregnancies, which are at higher risk of developing preeclampsia (32, 33). Taking it one step further, investigating which additional immunomodulating features are present in those cases will shed more light on the mechanisms on why some pregnancies with KIR AA/HLA-C are confronted with complications while other appear to have sufficient compensating mechanism.

HLA compatibility and preeclampsia: interpreting heterogeneous results

The identification of both HLA-C (**Chapter 2**) and HLA-DQB1 (**Chapter 3**) associations with preeclampsia likely reflects distinct but complementary immunological pathways. Differences in population selection, genetic background, study design, disease definition, and underlying pathophysiological mechanisms may determine which HLA-dependent pathway becomes most apparent. Our study in **Chapter 3** did not reveal an association between HLA-C matching and hypertensive complications, which may be due to several factors. First, hypertensive complications encompass a broader range of conditions, including not only preeclampsia, but also cases of gestational hypertension without preeclampsia. It is currently unclear whether gestational hypertension is a precursor to preeclampsia. Therefore, the broad definition "gestational hypertension" may mask associations that would be apparent in a more precisely defined disease groups. This point further highlights the need for larger cohorts. Second, a general HLA matching analysis may lack necessary details; a more in-depth screening is needed to assess not only alleles, but also the immunogenicity of specific (mis)matches. We employed the PIRCHE algorithm to evaluate the effect on $CD4^+$ T cells of fetal HLA-derived epitopes presented in maternal HLA class II. However, this approach does not account for fetal HLA-C interactions with maternal NK cells. Future algorithms, potentially based on studies like those from Hiby et al., may provide better understanding of (the predicted effects of) HLA-C/KIR interactions (12, 13). To fully capture their impact, these models should integrate additional immunological factors rather than relying solely on HLA matching information, since that is only part of the picture. Other elements, such as leader peptides and maternal CMV status, also influence immune responses and may vary across populations. Leader peptides derived from a variety of proteins play an important role in ensuring transport of certain HLA molecules to the cell membrane. In particular, HLA-E presents leader peptides derived from HLA class I molecules and interacts with NK cells via NKG2A. Since leader peptide sequences are HLA allele-dependent, their variation may influence pregnancy outcomes. Additionally, maternal CMV status could be relevant, as CMV encodes leader peptides similar to those of HLA-A and HLA-C (37). Based on this data, it is hypothesized that in CMV-seropositive women, fetal HLA-A/C-derived leader peptides might elicit a

T-cell-mediated response, linking prior infections to immune modulation in pregnancy. Given these factors, future studies could expand HLA typing to include class Ib molecules, such as HLA-E and HLA-G, as both exhibit polymorphisms and are associated with allo-immunization (38). HLA-E expression is promoted by HLA-G, which influences cytolytic NK cell activity, but also promotes an immune tolerant microenvironment by T cells and macrophages (10). Both altered HLA-G levels as genotype variations of HLA-G have been associated with preeclampsia (10).

The features discussed above highlight that the immune response to HLA (mis)matching can vary between individuals, influenced by factors such as NK and T cell education, prior antigen exposure, and genetic background (39). Even immune priming through paternal antigens in seminal fluid prior to conception has been proposed as a key factor in pregnancy outcomes (40, 41). Therefore, the precise role of maternal-fetal HLA compatibility within the complex immunological landscape during pregnancy remains to be fully elucidated. Along with larger cohort studies, a new systematic review and meta-analysis of existing research are needed to consolidate current knowledge on the role of maternal-fetal HLA compatibility in pregnancy. This is particularly important since the last comprehensive review was conducted 20 years ago (18). This would help identify remaining gaps and guide future study designs to determine the predictive value of specific HLA (mis)matches for preeclampsia risk. The many associative studies conducted to date differ in terms of population and patient selection, HLA typing methods, and statistical rigor.

Finally, fetal HLA recognition is not the only factor influencing spiral artery remodeling and proper placentation. In addition to the heterogeneity of HLA and immune-related genes, it is important to recognize that the measured outcome, preeclampsia, is itself heterogenous. This heterogeneity complicates research, leads to incomparable patient groups, and results in varying findings, each reflecting different aspects of a complex condition rather than one being definitively more accurate than the others. Potential heterogeneity makes it difficult to predict which women will develop preeclampsia, often resulting in overly cautious clinical care. Proteomic and transcriptomic studies have identified 3-5 pathophysiologic clusters in preeclampsia (42, 43). These studies generally converge on three main subtypes: 1) Placental/Canonical preeclampsia, characterized by strong (anti)angiogenic changes and oxidative stress. 2) Immunological preeclampsia, marked by an overrepresentation of immune and proinflammatory genes. 3) Maternal preeclampsia, with minimal placental gene expression changes. These subtypes may underlie the commonly used classifications of early vs. late and mild vs. severe preeclampsia. They might also explain why, for example, aspirin as a preventive measure is only effective in a subset of patients (44).

It is hypothesized that multiple pathways can lead to the same clinical presentation of preeclampsia, resulting in a similar end-stage condition that becomes indistinguishable once symptoms appear or when examined in third-trimester placental tissue (42, 43). Given that current treatment options are limited to symptomatic management and delivery of the fetus and placenta, the clinical relevance of subtyping preeclampsia remains an open question. However, subtyping could facilitate the identification of specific, targetable pathophysiological pathways, which opens the way for new therapies. Such approach would require that subtypes are both identifiable and preferably predictive and usable for prevention, but also for treatment. This is particularly important given that many studies rely on maternal or fetal tissues, including the placenta, obtained at the end of pregnancy.

A related issue worth addressing is the apparent division within preeclampsia research between vascular and immune-centered perspectives. This division warrants nuance, as these mechanisms are not mutually exclusive. Maternal cardiovascular maladaptation to pregnancy, leading to placental hypoperfusion, could cause secondary placental dysfunction in preeclampsia. Conversely, aberrant placentation due to immunological factors affecting spiral artery remodeling and EVT invasion depth could lead to reduced uteroplacental blood flow and, subsequently, systemic cardiovascular stress (45). One example, discussed in **Chapter 5**, is the role of co-inhibitory pathways. Inflammation in preeclampsia is related to cytokine release, which alters T cell function and behavior, contributing to vascular damage. The PD-1 pathway, for instance, inhibits pro-inflammatory $CD8^+$ T cell-driven damage to vascular endothelial cells (46). In preeclamptic cases, PD-1 expression is downregulated on Th17 cells and clonally expanded $CD8^+$ effector memory T cells, while it is upregulated on Tregs (47). The strict separation of vascular and immune research perspectives is counterproductive. In the true spirit of scientific inquiry, explanations should not be prematurely excluded when many aspects of preeclampsia pathophysiology remain unknown.

It is important to note that the studies of **Chapters 2** and **Chapter 3** included solely ongoing pregnancies that resulted in live birth, without accounting for the potential impact of HLA compatibility on conception or early pregnancy loss. Certain maternal-fetal HLA combinations may still confer an advantage for pregnancy continuation. Notably, we observed that 38.9% of the women included in the study had a history of one or more pregnancy losses, highlighting a remarkably high frequency of (recurrent) pregnancy loss. A future prospective cohort study among all couples in this specific population who are trying to conceive, incorporating fetal HLA analysis of miscarriage material, may provide crucial insights into how HLA compatibility influences implantation success and pregnancy viability at earlier stages.

Additionally, the incidence of pregnancy-related hypertensive complications remains unknown for the specific population studied in **Chapter 3**. Retrospective data collection on this aspect would help clarify the relationship between genetic diversity and the occurrence of these complications, further enhancing our understanding of maternal-fetal HLA interactions in pregnancy.

Moving towards clinical application of fetal HLA typing

The predictive value and clinical relevance of HLA genotypes in pregnancy outcomes must be further understood to integrate our findings into obstetric risk management and expand research on maternal-fetal HLA compatibility. Therefore, in **Chapter 4**, we aimed to achieve fetal HLA typing in early pregnancy through a non-invasive technique. Our results demonstrated that fetal HLA genotyping may be feasible using FACS-based isolation of HLA-G^+ EVT from cervical samples.

While HLA screening could potentially contribute to obstetric risk assessment, it is unlikely to serve as a standalone predictor. Instead, it should be incorporated into a multifactorial prediction model alongside other established risk factors for preeclampsia. For now, fetal HLA typing will remain limited to research applications due to insufficient evidence regarding the precise role of maternal-fetal HLA associations in pregnancy complications. For instance, contrary to the knowledge concerning HLA (mis)matching and outcome in organ transplantation, the odds ratio of specific HLA combinations in preeclampsia risk prediction is not yet determined, and neither is the exact (immunological) effect of different maternal-fetal HLA combinations.

Currently, fetal HLA typing has no place in routine prenatal screening. The accuracy of genetic inference needs to be improved as there is allelic variation at individual KIR loci and the binding affinity with the HLA-C2 epitopes may differ (48). Studies on the effect of allelic variation on pregnancy outcome and other characteristics are warranted. This will require large cohort studies. However, (pre-conceptional) maternal-fetal HLA profiling might prove useful in the reduction of pregnancy complications when applied to specific patients groups such as surrogacy, oocyte donation or sperm donation. Likewise, further research is needed to refine the potential applications of fetal HLA typing. Continued efforts in this area could enable its use in pregnancy losses, allowing comparisons between miscarriages and ongoing pregnancies from the same parents. Early fetal HLA screening would make it possible to prospectively follow pregnancies with specific HLA profiles, and their subsequent impact on pregnancy success.

Future advancements in EVT isolation from peripheral blood may eventually render cervical sampling unnecessary. However, cervical smears likely yield higher EVT numbers and,

given their proximity to the placenta, may better reflect placental EVT composition. This can also aid research on other pregnancy complications with placental-based pathophysiology such as (inter)villositis. Further research should focus on improving trophoblast subtype markers to distinguish them from maternal cells with the same lineage precursors and to investigate resemblance to the different placental EVTs. On a broader level, ensuring transparent publication of negative results is essential. Notably, other research groups in the Netherlands and Belgium have attempted to apply the TRIC technique for various purposes but without success, reinforcing the need for openly sharing both successes and challenges in EVT isolation methodologies. Our study also highlights the critical importance of protocol reproducibility and optimization. We observed that even minor adjustments to the protocol, such as shorter incubation times, increased wash cycles, and careful handling of the supernatant significantly impacted EVT isolation efficiency. However, despite our efforts, we were unable to fully replicate the TRIC protocol that was described earlier. (49, 50) This challenge is not unique to our study: other published protocols, such as those for isolating viable trophoblasts from term placenta, though not described in this dissertation, have similarly proven difficult to reproduce in our laboratory. Many protocols lack transparency, omitting crucial methodological details or assuming standardized procedures for steps such as centrifugation and washing, which can vary between laboratories. Greater openness in scientific reporting and concerted effort to counteract *publication bias* is essential to advancing the field.

A closer look at the multitalented trophoblast

Trophoblast cells are the earliest extra-embryonic cells to differentiate in the mammalian embryo and constitute the interface between the fetus and the mother throughout pregnancy. After implantation, the villous cytotrophoblast cells proliferate to branching villi that are covered by multinucleated STB, creating the site for nutrient and oxygen exchange with maternal blood. EVT cells arise from the villi and invade the decidua basalis to transform the uterine spiral arteries into low-resistance and high conductance vessels. This creates two main sites of contact between maternal leukocytes and fetal trophoblasts. The fetus itself is never in contact with maternal blood or decidua, with the placenta always functioning as a barrier between the two individuals.

To establish an immunomodulated environment that supports adequate trophoblast invasion and migration, the trophoblast must exhibit a versatile array of cellular characteristics, depending on its location and the maternal cells it encounters. In this dissertation, we explored several mechanisms through which the maternal immune system is influenced by the trophoblast. In general, the location and gestational timing are critical factors when investigating immune mechanisms during pregnancy.

In human biology, immune checkpoint molecules are referred to as a series of co-inhibitory receptors and ligands that are expressed on the immune cells that regulate the extent of immune activation. They play beneficial roles in promoting transplantation tolerance, enabling tumor immune escape, and preventing autoimmunity. These molecules are increasingly recognized as key regulators of immune function in pregnancy. However, their precise role remains unclear. Therefore, in **Chapter 5**, we investigated how co-inhibitory molecules may help constitute maternal immunomodulation in naturally conceived, but also in OD pregnancies. OD pregnancies were examined not only because they are associated with an increased risk of preeclampsia, but also because the heightened immunogenetic dissimilarity between mother and fetus is thought to necessitate enhanced immune regulation compared to naturally conceived pregnancies. This makes them a valuable model for studying maternal-fetal immune interactions. We found altered expression of immune checkpoint co-inhibitory ligands by trophoblasts in the placenta of OD pregnancies in comparison to naturally conceived pregnancies. Uncomplicated OD pregnancies showed decreased protein expression of PD-L1 and CD200, while mRNA expression of IDO (downstream of co-inhibitory CTLA-4 signaling (51)) was increased. In preeclamptic pregnancies, decreased placental CD200 protein expression compared to uncomplicated pregnancies supports existing evidence of a disturbed immune balance and T cell regulation (52, 53).

In the study presented in **Chapter 5**, we investigated placental villi at term. Previous research has shown that the expression of co-inhibitory molecules changes throughout gestation (54). For example, PD-L1 decreases towards term, while PD-1 increases and TIM-3 remains stable throughout gestation. Interestingly, at the RNA level we did not see significant differences between the study groups in other co-inhibitory ligands such as Galectin-9 and PVR, while previous studies showed high levels of these components in EVT or other placental cell interactions in the first trimester (55). These molecules may therefore play a more prominent role earlier in pregnancy, which could explain why differences between pre-eclampsia and healthy pregnancies or between oocyte donation and naturally conceived pregnancies are not apparent at term. Indeed, these findings confirm a specific role of the co-inhibitory pathways depending on time and location. Studies in mice show that CD200 expression could prevent inflammation-induced abortion and that PD-L1 blockade leads to increased fetal resorption while enhancing fetal-specific T cell responses (56-58). However, it remains unclear how the levels of PD-L1 and CD200 are regulated and whether they play a similar role in the establishment of maternal-fetal tolerance at placentation or primarily in its maintenance later in pregnancy. Additionally, most studies focus on EVT, with relatively less attention given to STB. This is notable, as changes in maternal peripheral blood leukocytes, which circulate past the STB, are frequently studied in relation

to pregnancy complications such as preeclampsia. These circulating immune cells differ significantly from their specialized decidual counterparts (24).

Studies have shown differences in STB function under hypoxic stress and release of angiogenic factors such as placental growth factor (PlGF) and soluble fms-like tyrosine kinase-1 (sFlt-1) (59). It would be interesting to explore how this relates to the expression of co-inhibitory molecules. While PlGF, a member of the VEGF family, promotes trophoblast growth, differentiation, and placental angiogenesis, sFlt-1 acts as a soluble receptor that antagonizes VEGF and PlGF signaling. Under stress conditions, the STB produces lower levels of PlGF and higher levels of sFlt-1, particularly in degenerative regions known as syncytial knots. Although direct mechanistic links between co-inhibitory molecules and sFlt-1/PlGF levels have not yet been established, it is plausible that dysregulation of immune checkpoints (such as reduced CD200 and PD-L1 expression) promotes an inflammatory environment that favors the production of anti-angiogenic factors, ultimately contributing to placental dysfunction. This has also been described for complement regulatory proteins by the trophoblast, where reduced expression dysregulates VEGF signaling (60). Once again, this highlights a potential bridge between the immune and vascular research hypotheses, as discussed above.

Chapter 5 provides additional support for the findings presented in **Chapter 2**. As we investigated the relationship between placental CD200 protein expression and maternal-fetal HLA compatibility, we found that CD200 expression was positively correlated with the degree of HLA mismatching in uncomplicated OD pregnancies, including HLA-C mismatches in particular. In **Chapter 2**, we had already observed an association between HLA-C matching and the occurrence of preeclampsia. While a certain level of HLA-C mismatching seems to be necessary for effective local spiral artery remodeling by EVT and dNK cells as described above, our findings suggest that this may be systemically counterbalanced by increased CD200 expression on the STB.

The role of co-inhibitory molecules investigated in **Chapter 5** prompted us to further explore the immunomodulating mechanisms by EVT, especially considering the key role of Tregs in pregnancy. Reduced Treg populations have been linked to preeclampsia, and co-inhibitory molecules are known to promote regulatory T cell functions, while EVT are expected to express co-inhibitory molecules. Therefore, in **Chapter 6**, processes initiating maternal-fetal immune tolerance establishment were investigated by conducting an in vitro study using primary first-trimester EVT co-cultured with activated Tconv to investigate their influence. This co-culture induced a regulatory phenotype in activated Tconv, characterized by increased expression of CD25, FOXP3, and HELIOS, alongside decreased CCR7 expression. This phenotypic shift depended on CD3, and especially CD28 co-stimulation, and was

accompanied by enhanced cell proliferation and increased secretion of immunomodulatory factors, including IL-10 and IDO. These results indicate that, when APCs provide CD3/CD28 stimulation in the local microenvironment, EVT may serve as important inducers of Tregs within the decidua.

As described in **Chapter 6**, our findings contribute to the ongoing debate on whether decidual regulatory T cells are thymus-derived (tTregs) with self-antigen specificity or peripherally induced (pTregs) with specificity for paternal antigens. Based on recent TCR sequencing studies, the majority of decidual Tregs appear to be thymus-derived, with clonal expansion suggesting pre-existing Treg populations that become activated and adapt to the decidual environment (61, 62). Peripherally induced Tregs, likely fewer in number, also play an important role as shown by a mouse study demonstrating the necessity of pregnancy-specific pTreg induction for proper placental morphogenesis (63). Thus, the decidual Treg compartment likely represents a multilayered system in which both tTregs and pTregs contribute in complementary ways to maternal–fetal tolerance. Understanding whether and how local interactions with EVT are associated to systemic increases in Tregs remains a question for future research. Clarifying this mechanism could help explain the shifts in T cell populations that are characteristic of preeclampsia.

The main limitation of this study is the absence of the complex cellular microenvironment that is normally seen in the decidua in vivo, particularly key players such as macrophages and DCs. As discussed in **Chapter 6**, EVT are unlikely to directly activate $CD4^+$ T cells via TCR engagement as they generally do not express HLA class II molecules. Instead, antigen presentation is likely mediated by macrophages and DCs. These cells can also provide CD28 co-stimulation through CD80/CD86, a requirement of the observed effect of EVT on Tconv. Decidual macrophages, for instance, are known to promote FOXP3 expression in $CD4^+$ T cells, contributing to a regulatory phenotype (64). Conversely, EVT can shape the macrophage compartment by inducing anti-inflammatory M2-like macrophages (65, 66). Interestingly, decidual macrophages also express PD-L1 on their surface, which binds to PD-1 on decidual T cells to suppress their IFN-γ production. Moreover, macrophages can promote proliferation, migration, and invasion of EVT through different pathways. In addition, the in vitro co-culture system may not fully replicate the in vivo maternal-fetal interface, particularly in terms of cytokine gradients, oxygen tension, and three-dimensional cellular architecture. A ‘placenta-on-a-chip’ model could provide a more physiologically relevant system to study these interactions. Multiple models have been developed to study barrier functions, implantation, but also preeclampsia (67). Such designs incorporate different cell types, such as endothelial cells with trophoblasts, on microfluidic chips to mimic flow and extracellular matrix. Extensive designs further include immune cells and decidualized stromal cells to recreate the cellular complexity of the maternal-fetal

interface (68). This enables real-time visualization and quantitative assessment of immune-trophoblast crosstalk, dynamic recruitment or inhibition of immune cell subsets and key events like endothelial remodeling, barrier disruption, and immune-stromal modulation of invasion. It would offer a more comprehensive pathway analysis, for example of downstream signaling of IDO, PD-L2, CD200 and HLA-G, and bringing forth insights that are less reliant on deduction and more reflective of the in vivo situation. This can be achieved through approaches such as longitudinal secretome profiling, single-cell transcriptomics, and spatial proteomics, which capture dynamic molecular responses within the engineered interface.

Such approaches could also further define the relationship between immune regulation and other mechanisms that are likely essential, though beyond the scope of this dissertation. One example is the involvement of decidual $CD8^+$ T cells (dT). These $CD8^+$ dT may directly recognize non-self paternal HLA-C expressed on EVT through classical TCR-peptide engagement, with their responsiveness potentially further modulated by HLA-C specific KIR expressed on a subset of decidual T cells (69). Yet, EVT appear capable of suppressing $CD8^+$ dT activity (70). Although these $CD8^+$ dT show increased expression of co-inhibitory molecules, they can still respond to proinflammatory events, such as infections (71). Another relevant mechanism by the first trimester trophoblast involves Fas ligand, which induces apoptosis in maternal immune cells to prevent immune-mediated damage (72). In addition, placental hormones such as progesterone, estrogen, and human chorionic gonadotropin play an important role in regulating Tregs (73). Finally, extracellular vesicles contribute to immune modulation by transporting not only fetal antigens, but also immunoregulatory molecules, both locally and systemically (74).

For co-culture models that incorporate immune cells, advances in pathway analysis can be achieved by using blocking antibodies. However, gene silencing techniques such as siRNA or CRISPR-based knockout studies may offer a more robust and precise approach. Conducting such experiments within the co-culture system of **Chapter 6** could help identify which immunomodulatory molecules expressed by trophoblasts are essential for T cell direction, for eventually establishing fetal tolerance. As a next step, transcriptomic analysis both cell types could reveal patterns of gene expression changes. Together, these approaches can help further validate the relevance of immune alterations observed in preeclampsia, as described in **Chapter 5**.

In general, the link between Treg induction and maternal-fetal HLA-matching remains one of the most important underlying questions. Previous studies have shown that maternal-fetal HLA-C-mismatched pregnancies are associated with higher numbers of decidual $CD4^+CD25^{HI}$ T cells, which also exhibited increased suppressive function compared to HLA-C-matched pregnancies (17). This aligns with our previous findings that maternal-

fetal HLA-C compatibility is associated with preeclampsia (**Chapter 2**). While the effect of specific HLA-C-KIR receptor combinations on uterine NK cell function is well established (12, 13), the impact of maternal-fetal HLA matching on Treg populations remains less explored. Therefore, an important next step would be to assess different levels of allogenicity or match-mismatch scenarios between Tconv and EVT to determine which combinations most significantly influence Treg induction, especially given the considerable variation in immunogenicity across different HLA loci. This will require the inclusion of APCs to investigate responses to HLA loci beyond HLA-C and to incorporate indirect allorecognition.

Another important challenge in studying immune interactions at the maternal-fetal interface is the significant difference between humans and animal models in various aspects of pregnancy. These include differences in pregnancy duration, placental development and the extent of trophoblast invasion (8, 75). Moreover, most animals (except apes in rare cases), do not develop preeclampsia, limiting their usefulness in investigation this condition (76). Interestingly, mice can be completely syngeneic or consanguineous without developing pregnancy complications, which further highlights the limitations of translating (HLA compatibility) findings from animal models to humans.

The beautiful dance of pregnancy

It is evident that maternal-fetal immune interactions are governed by a remarkably intricate system. Reflecting on the discussion of Medawar's comparison between pregnancy and transplantation in paragraph 2, the last two chapters indicate that a more fitting parallel may be found in tumor immunology. Both the placenta and tumor environment involve spatially and temporally regulated immune adaptations to allow for survival and growth while maintaining immune equilibrium. There are numerous additional mutual characteristics such as the initiation from a single cell, extensive proliferation, invasion and migration into surrounding tissue, initiation of angiogenesis, and lack of cell-contact inhibition (77).

This complexity underscores the importance of collaborative research between these fields, which would undoubtedly yield insights beneficial to both. From an evolutionary perspective, the coexistence of an invasive placenta with a functional immune system suggests a long-standing, finely tuned co-evolution; one that allowed for the emergence of successful viviparity without compromising immune defense. (78) To capture the beauty and complexity of pregnancy on a more abstract level, one might compare it to a dance: a dynamic interplay of carefully choreographed movements, where each partner adjusts to the other with precision and elegance. Though the steps are familiar and often repeated, the variations are endless; and when performed well, it appears effortless, despite the immense strength and coordination it requires.

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