Immune parameters affecting maternal tolerance towards the fetus in normal and aberrant pregnancies

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The research presented in this thesis was performed at the Department of Immunohematology and Blood Transfusion at Leiden University Medical Center.

Financial support for the publication of this thesis was kindly provided by: National Reference Center for Histocompatibility Testing, NVLE, GenDx, ICT Healthcare Technology Solutions BV, ChipSoft, CleanAir by Baker, VPS diagnositics.

Cover design and layout:	Judith Berden
Printed by:	GVO drukkers & vormgevers B.V.
ISBN:	978-94-6332-626-1

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Proefschrift

ter verkrijging van

de graad van Doctor aan de Universiteit Leiden,

op gezag van Rector Magnificus prof. mr. C.J.J.M. Stolker,

volgens besluit van het College voor Promoties

te verdedigen op dinsdag 16 juni 2020,

klokke 15.00 uur

door

Moniek Henriëtte Catharina Craenmehr

geboren te Horst, Nederland

in 1988

Nothing in life is to be feared, it is only to be understood.
Now is the time to understand more, so that we may fear less.

- Marie Curie (7 November 1867 – 4 July 1934)

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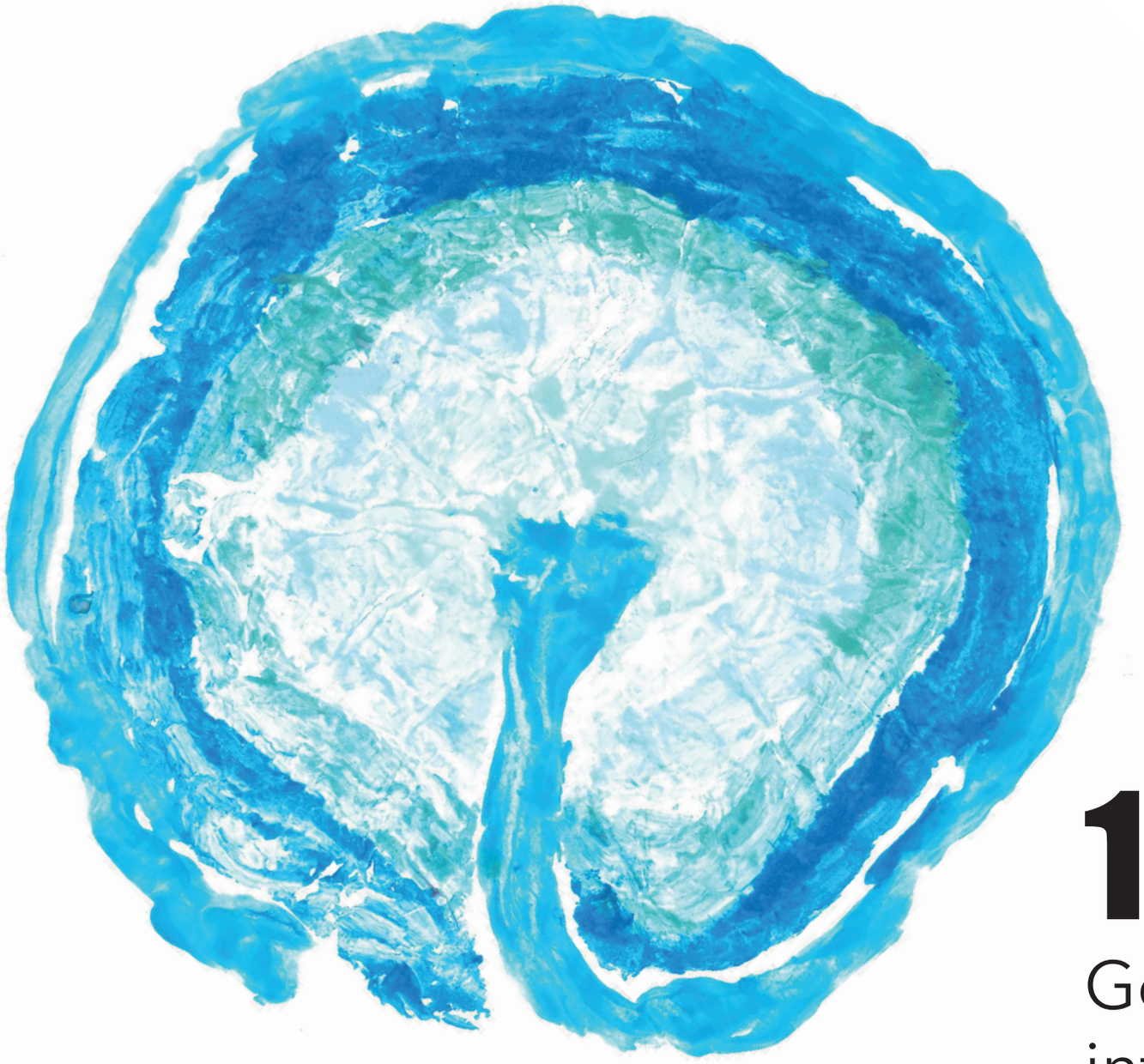

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General introduction

Basics of the immune system

The immune system is a collection of organs, tissues, cells and molecules that protects against microorganisms trying to infect the human body. Microorganisms can reproduce and evolve very rapidly during the course of an infection, and they can cause disease if not controlled by the human immune system. Defense against these microorganisms is mediated by early reactions of the innate immune system and later responses of the adaptive immune system.

When pathogens gain entry to the human body the innate immune system can react quickly, because it consists of defense mechanisms that are in place even before infection. These mechanisms are specific for structures that are common to groups of related microbes and remain essentially the same in repeated infections. When cells of the innate immune system sense the presence of pathogens or products from an injured cell, they start to secrete proteins called cytokines that interact with other cells to trigger the innate immune response. Cells that can engulf the invading microorganism or kill infected cells are brought rapidly and in large numbers into the infected tissue. This induces a state of inflammation in the infected tissue, causing symptoms like heat, pain, redness, and swelling.

Sometimes pathogens are able to withstand innate immunity and their elimination requires the more powerful and more specific mechanisms of adaptive immunity. When this happens, the innate immune response helps to slow the spread of the infection while lymphocytes become activated that vastly increase the power and focus of the immune response. Lymphocytes and their secreted products are the main components of the adaptive immune response. They express membrane receptors that have an extraordinary capacity to distinguish between different microbes and molecules. When a pathogen is recognized, lymphocytes start to proliferate and differentiate, producing large numbers of effector cells specific for that pathogen. Some of the lymphocytes that recognize the pathogen persist

in the body and provide long-term immunological memory. These memory cells respond more rapidly and vigorously upon a subsequent encounter with the same pathogen.

Cells of the immune system

The cells of the immune system consist mainly of white blood cells or leukocytes. Different types of cells with different characteristic morphological features and functions exist. The main immune cells covered in this thesis are explained here:

Innate immune system:

- **Monocytes** circulate in the blood and travel to tissues, where they mature into macrophages or dendritic cells and take up residence.
- **Macrophages** are large, irregularly shaped cells, which can capture, engulf, and kill microorganisms. Macrophages are characterized by an extensive cytoplasm with numerous vacuoles often containing engulfed material.
- **Dendritic cells (DC)** are resident in the body's tissues and have a distinctive star shaped morphology. They can act as cellular messengers mediating an adaptive immune response when it is needed. For this, they will capture microbial antigens, transport these antigens to lymphoid organs, and present the antigens to naïve T lymphocytes to initiate immune responses.
- **Natural killer (NK) cells** are the killer lymphocytes of the innate immune response. They migrate from the blood into infected tissues, where they prevent the spread of infection by killing virus-infected cells and secrete cytokines that slow the progress of viral replication in infected cells.

Adaptive immune system:

- **B lymphocytes or B cells**: small lymphocytes with cell-surface receptors called immunoglobulins. B cells can be activated to become plasma cells, which are effector cells that secrete soluble forms of immunoglobulin called antibodies that bind to pathogens.
- **T lymphocytes or T cells**: small lymphocytes that have membrane-bound receptors for the recognition of peptides derived from foreign proteins. T cells can be subdivided into CD8+ cytotoxic T cells and CD4+ helper T cells according to their effector functions. Cytotoxic T cells kill cells that produce foreign antigens, such as virus infected cells, whereas helper T cells secrete cytokines that help other cells of the immune system to become fully activated effector cells. CD4+ cells can be subdivided again into Th1, Th2, Th17 or regulatory T cells.

These immune cells can produce cytokines, which mediate and regulate aspects of the immune response. One cell can synthesize different kinds of cytokines and one cytokine can be produced by different kinds of cells. These cytokines can have multiple biologic effects, thereby stimulating or inhibiting the production of others. Therefore, the function of a cytokine can be greatly influenced by other cytokines secreted together with it. These are the main cytokines covered in this thesis:

- **IL-2** drives the growth, survival and differentiation of T cells and is involved in the maintenance of regulatory T cells. IL-2 is mainly produced by activated CD4+ T cells, but also by activated CD8+ T cells , NK cells and DC.
- **IL-12** is a pro-inflammatory cytokine that promotes the differentiation of Th1 cells. It is produced by DC, macrophages and B cells. IL-12 induces the

production of IFN-γ by NK cells and T cells, which stimulates additional antigen presenting cells (APC) to produce IL-12.

- **IFN-γ** is a major pro-inflammatory cytokine, functioning mainly as an activator of effector cells of the immune system. It is produced by CD4+ Th1 cells, CD8+ T cells and NK cells and it is an important mediator of macrophage activation and effector function, resulting in increased ingestion of microbes and the destruction of the ingested pathogens.
- **TNF-α** is a powerful inducer of inflammation. It is mainly produced by macrophages, but can also be produced by many other cell types, such as T cells, NK cells and DC. TNF-α helps recruiting immune cells to the inflammation site and promotes macrophage differentiation.
- **IL-10** is involved in controlling the immune response. It is produced by many immune cell populations, including activated macrophages and DC, B cells, regulatory T cells, and Th1 and Th2 cells. IL-10 inhibits the expression of co-stimulatory molecules and class II major histocompatibility complex (MHC) molecules on DC and macrophages, and it inhibits their production of IL-12.
- **TGF-β** inhibits proliferation and effector functions of T cells to provide regulation of cellular immunity. It is produced by various cell types, including T cells and monocytes. It can inhibit the development of Th1 and Th2 subsets and is involved in the development of regulatory T cells.

Development of lymphocytes

Lymphocytes develop and mature to the stage at which they are able to respond to a pathogen in the primary lymphoid tissues, i.e. the bone marrow and the thymus. B and T lymphocytes both arise from stem cells in the bone marrow, but

whereas B cells complete their maturation here, T cells leave the bone marrow at an immature stage to mature in the thymus. After maturation, these naïve lymphocytes migrate to the secondary lymphoid tissues, where they may respond to invading pathogens. Here, the few B and T cells expressing receptors that bind to the foreign antigen will be activated to proliferate and differentiate into effector cells and memory cells. DC are the most effective APC for activating naïve T cells and initiating T cell responses. They are specialized in the uptake and breakdown of pathogens. Resting DC capture microbial antigens and transform into mobile cells, migrating from the infected tissue to the secondary lymphoid tissue that drains the infected site. Here they present the antigens to the T cells and activate them. Following activation, effector cells then migrate to the infected tissues, where they collaborate with cells of the innate immune system to control the infection.

Generation of T cell subsets

Precursors that express both CD4 and CD8 differentiate into either CD4+ or CD8+ T cells within the thymus. CD8+ T cells can differentiate into cytotoxic T lymphocytes whose major effector function is to kill infected target cells. Naïve CD4+ T cells can be activated by antigens to differentiate into helper T cells, synthesizing cell surface molecules and soluble cytokines that activate and help other types of cells – mostly macrophages and B cells – to participate in the immune response.

The differentiation pathway that an activated naïve T cell will take is decided at an early stage of activation. T cell activation requires signals provided by molecules on APC, called co-stimulatory molecules, in addition to antigen-induced signals. Co-stimulation is called the second signal for T cell activation, because it functions together with antigen, the first signal, to stimulate T cells. The best characterized co-stimulatory pathway involves the T cell surface receptor CD28 and the co-stimulatory molecules CD80 (B7-1) and CD86 (B7-2) expressed on APC. Interaction

of these receptor-ligand pairs mainly results in T cell proliferation and the secretion of cytokines, such as IL-2. Receptors homologous to CD28 and their ligands homologous to B7 have been identified. Some of these, e.g. CTLA-4 and PD1, have inhibitory effects, whereas others provide activating signals. The balance between stimulation of activating and inhibitory receptors of the CD28 family influences the outcome of T cell activation. The third signal for T cell activation is provided by the cytokines produced by APC and other immune cells present at the site of the immune response. These cytokines make the differentiating T cell become gradually committed to one specific pathway (Figure 1). Naïve T cells activated in the presence of IL-12 and IFN-γ will become Th1 effector T cells, which induce macrophage activation and inflammation. T cells that become activated in the presence of IL-4 will differentiate into Th2 effector cells, which induce B cell differentiation and eosinophil activation by the production of IL-4 and IL-5. Naïve T cells activated in the presence of IL-23 will differentiate into a third subset of effector cells: Th17 cells. These cells secrete cytokines, such as IL-17, that lead to the recruitment of neutrophils to the site of infection. All three subsets produce cytokines that promote the development of this subset and inhibit differentiation toward other CD4+ subpopulations.

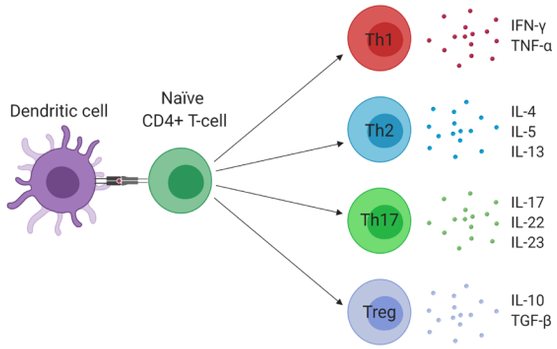

Figure 1. Naïve T cells get activated and differentiate into one of several T helper cell lineages, including Th1, T2, Th17, and regulatory T cells, as defined by the secretion of a specific set of cytokines and function, that subsequently modulates the immune response. Created with BioRender.com

Regulatory T cells are another distinct population of T cells. These can be subdivided into natural and induced regulatory T cells: natural regulatory T cells develop in the thymus and then migrate elsewhere, whereas inducible regulatory T cells are generated from naïve T cells in the periphery under various tolerogenic conditions. Regulatory T cells are involved in preventing an active immune response by restricting the function of effector T cells, immunoglobulin production by B cells, cytotoxic activity of NK cells and maturation of DC. They do so by secreting cytokines, such as IL-10 and TGF-β, and expressing molecules, such as CTLA-4, all of which are hallmark mediators of regulatory T cell suppression. In this way, they modulate the immune system and maintain immune homeostasis in the body. This active form of tolerance mediated by regulatory T cells is very important for maintaining self tolerance and protecting the integrity of the body's tissues and organs.

Major histocompatibility complex (MHC)

T cells can only recognize antigen peptides when they are bound by major histocompatibility complex (MHC) molecules. There are two types of MHC molecules, MHC class I and MHC class II. MHC class I is expressed on virtually all nucleated cells and presents antigens from intracellular pathogens to CD8+ T cells, whereas MHC class II is expressed by professional APC that present antigens from extracellular pathogens to CD4+ helper T cells.

To increase the efficiency of antigen presentation, APC express several forms of MHC class I and II molecules, each with a different peptide-binding specificity. In addition, there are many different genetic variants, or alleles, for each of these genes within the human population. Each individual expresses the alleles that are inherited as haplotypes from each of the two parents. This means that most people carry two different alleles of each MHC gene, being heterozygous. This

maximizes the number of MHC molecules available to bind pathogen-derived peptides for presentation to T cells and enables greater variety than would be possible in homozygous individuals, who carry two identical alleles of a given gene. Another word for this allelic variation is polymorphism, and MHC genes are known to be highly polymorphic genes. This variability is maintained in human populations through the need to successfully display a wide range of processed foreign peptides to the T cell antigen receptor. MHC genes that have little or no genetic variation are described as monomorphic and genes having a few alleles are described as oligomorphic.

The human MHC is called the human leukocyte antigen (HLA) complex. There are six HLA class I genes, namely the classical, highly polymorphic HLA-A, HLA-B and HLA-C, and the non-classical HLA-E, HLA-F and HLA-G, which exhibit limited polymorphism. The highly polymorphic HLA-DR, HLA-DQ, and HLA-DP genes reside within the class II region.

Allogeneic immune response

T cells should only recognize foreign peptides presented by that individual's HLA molecules. During thymic T cell development, immature T cells that recognize and bind to HLA molecules will get a survival signal. If these immature cells do not interact strongly enough they will not get the survival signal and die. This is called positive selection. Any cells having T cell receptors that bind with high affinity to HLA molecules with self peptide are eliminated, which is called negative selection. This mechanism prevents a person's T cells from attacking their own healthy tissue and triggering autoimmunity. An extraordinary situation in which foreign HLA molecules are introduced in an individual is transplantation. In the case of organ transplantation it is important that the graft is accepted by the immune system of the receiving party. Transplants of most tissues between any pair of individuals in

the absence of pharmacological immunosuppression, except identical twins, will be rejected due to HLA disparity. Alloreactive T cells in the recipient's circulation can be activated by these allogeneic HLA molecules expressed by the graft. This is called direct allorecognition and would lead to a potent T cell response that attacks the graft. Donor HLA molecules can also be captured and processed by recipient APC that enter grafts. This is called indirect allorecognition. Peptides derived from the allogeneic HLA molecules are presented in association with self MHC molecules and recognized by the host's T cells.

To reduce the probability of graft rejection, donor and recipient are matched for HLA. However, even fully HLA matched individuals undergoing transplantation can experience rejection of the graft, indicating that non-HLA immunity can also contribute substantially to transplant failure. Above all, the recipient needs to be on lifelong immunosuppressants, to prevent that the immune system will mount an immune response and reject the graft.

Immunological paradox of pregnancy

A situation in which there is natural tolerance against a foreign tissue is pregnancy. In case of a successful pregnancy, the maternal immune system does not reject the semi-allogeneic fetus, but lets it peacefully exist in the uterus. In the 1950s, Medawar was already intrigued by this phenomenon and came up with three possible explanations for this immunological paradox: (1) the fetus is physically separated from the maternal periphery, therefore the maternal immune system does not detect the fetus and will not react to it; (2) fetal antigens are not mature and therefore cannot be recognized by the maternal immune system; (3) the maternal immune system is inactive at the time of pregnancy and therefore it will not mount an immune response against the fetus [1]. In the past few decades it became clear that all three hypotheses were incorrect. In contrast to Medawar's first hypothesis,

there is direct contact between maternal blood and fetal trophoblast tissue during pregnancy, and fetal cells can persist in the maternal circulation for decades after pregnancy [2], which is called microchimerism. Furthermore, we know that the maternal immune system can recognize and react to fetal cells. The mother can develop antibodies directed against fetal HLA antigens [3], and *in vitro* tests show that maternal CD4+ and CD8+ T cells can respond to fetal cells [4, 5], ruling out Medawar's second and third hypotheses. However, all of these observations were done with peripheral blood cells of the (pregnant) woman. More locally, at the fetal-maternal interface, cells of the maternal immune system were shown to have a more tolerant phenotype [6, 7].

Mechanisms supporting acceptation of the semi-allogeneic fetus

Increasing evidence suggests that the maternal immune response towards the fetus plays a determinative role in the success of pregnancy. Several mechanisms are involved in the induction of maternal tolerance and immunologic acceptance of the semi-allogeneic fetus during pregnancy (Figure 2). Mechanisms for the evasion of the maternal immune response by the fetus include the absence of the HLA class I antigens A and B and HLA class II on fetal trophoblast cells, preventing allorecognition by T cells and B cells. The fetal trophoblasts do contain HLA-C, HLA-E, HLA-F, and HLA-G to control maternal immune responses, by modulating the activity of decidual natural killer (NK) cells, macrophages, and T cells [8-11].

HLA-G acts on multiple immune subsets by interaction with immunoglobulin-like transcript (ILT) receptors. ILT2/LILRB1 is expressed on monocytes, DC, B cells, and subsets of NK and T cells [12], whereas ILT4/LILRB2 is almost exclusively expressed by cells of the myelomonocytic lineage [13]. Through interaction with ILT receptors, HLA-G can inhibit proliferation and activation of different immune subsets [14-16], preventing a maternal immune response against paternal antigens. By alternative

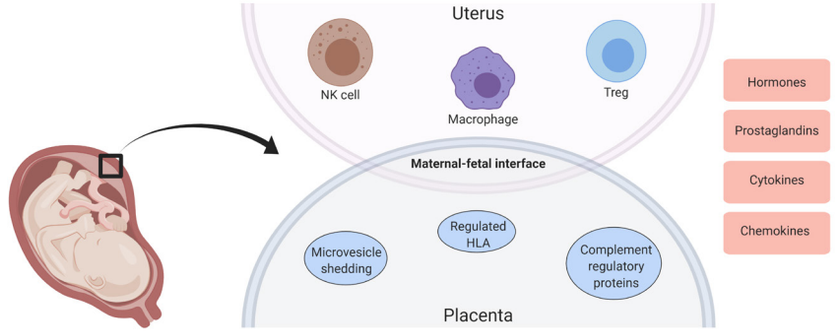

Figure 2. Several mechanisms at both maternal and fetal side are involved to prevent the maternal immune system from rejecting the fetus. Fetal cells express and produce immune regulatory molecules to prevent an attack by maternal immune cells. Maternal immune cells interact to suppress an active immune response towards fetal antigens. Created with BioRender.com

splicing, HLA-G pre-mRNA can give rise to seven different isoforms, of which four are membrane-bound (HLA-G1, -G2, -G3 and -G4) and three are soluble (HLA-G5, -G6 and -G7) [17]. Whereas in healthy tissue membrane-bound HLA-G is only expressed on trophoblasts, the soluble form of HLA-G can be detected in various body fluids, such as amniotic fluid, blood and seminal plasma [18, 19]. Several polymorphisms are present in the 3 prime untranslated region (3'UTR) of the HLA-G gene. Since the 3'UTR is targeted by miRNA that can negatively influence expression, polymorphisms in this region may have an influence on the efficiency of miRNA binding, and consequently on the level of HLA-G expression and on pregnancy outcome.

Another mechanism by which the trophoblast cells may escape attack from maternal immune cells is via the expression of apoptosis-inducing ligands, such as Fas Ligand (FasL) and tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) [20, 21]. Expression of Fas is found on decidual leukocytes, suggesting that FasL expression and production by trophoblast cells may be a mechanism protecting the trophoblast against activated leukocytes [22, 23]. Also the programmed death/programmed death ligand (PD1/PDL1) coinhibitory pathway plays a role

in fetomaternal tolerance, by limiting the expansion of alloreactive T cells [24]. Furthermore, trophoblasts display high levels of complement regulatory proteins, such as decay-accelerating factor (DAF, CD55), membrane cofactor protein (MCP, CD46) and MAC-inhibitory protein (MAC-IP, membrane inhibitor of reactive lysis (MIRL), CD59) [25, 26]. These proteins are important for protecting the fetal cells from potential destruction by complement components.

In addition, soluble immunomodulators are present at the maternal-fetal interface. Trophoblasts synthesize indoleamine 2,3-dioxygenase (IDO) [27, 28], a tryptophan catabolizing immunomodulatory enzyme that prevents maternal T cell activation. Also transforming growth factor beta (TGF-β), prostaglandin E2 (PGE2), galectin-1, and IL-10 are produced by the human placenta [29-32], all of which can promote the generation of tolerogenic immune cells.

Trophoblast cells can also produce and secrete extracellular vesicles of different size, morphology, and function, which may participate in the maternal–fetal cross-talk during pregnancy [33]. These placenta-derived microparticles, such as nanovesicles and exosomes, can also enter the maternal circulation [33]. The concentration of placenta-derived exosomes increases with gestational age and they are thought to play a role in regulating the maternal immune system during pregnancy [34]. Their composition comprises of placental proteins, mRNA, and microRNAs and reflects the cell type from which the vesicle originates. Previous studies have shown that trophoblast cells secrete functional FasL and TRAIL via exosomes [35]. Also PDL1 and HLA-G can be released from the placenta via exosomes [36].

Taken together, these findings suggest there are multiple mechanisms to prevent maternal immune rejection of the semi-allogeneic fetus. It is possible that a disbalance in the immunological environment in the placenta can lead to pregnancy related problems, such as pregnancy loss.

Recurrent miscarriage

Approximately 15% of pregnant women experience spontaneous loss of a clinically recognized pregnancy. About 1–2% of couples trying to conceive are confronted with recurrent miscarriage (RM) [Unpublished data][37]. Various definitions of RM are being used. Some consider RM as two or more failed clinical pregnancies defined by ultrasonography or histopathologic examination [38]. Others define RM as three consecutive pregnancy losses within the first 24 weeks of gestation, including biochemical pregnancies and non-visualized pregnancies [38]. This discrepancy makes it hard to study underlying causes for this phenomenon and to compare the outcome of different studies.

Several factors influence the risk of miscarriage such as maternal age and previous pregnancy loss. Etiological categories for RM include chromosomal abnormalities, uterine anatomic abnormalities, and antiphospholipid syndrome. However, in a significant proportion of the couples trying to conceive the underlying cause for this recurring problem is unknown (Figure 3) [unpublished data][39]. This burden of continuous uncertainty has a major impact on the lives of these women and their partners.

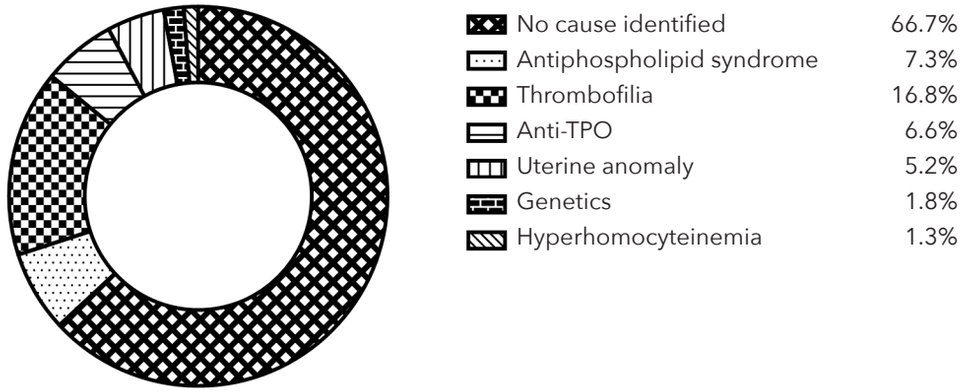

Figure 3. Several etiological factors for recurrent miscarriage have been identified. However, more than half of the couples experiencing recurrent miscarriage do not know the underlying cause.

It has been postulated that immunologic aberrations may be the cause in many of these unexplained cases of RM. Several immune factors have been investigated in women with RM. Defects in complement-inhibitory proteins, maternal regulatory T-cells, tryptophan catabolizing enzymes, and immunoregulatory cytokines at the fetomaternal interface have been implicated to play a role in RM [40-43]. In order to prepare the immune system of a woman against the 'foreign' cells of a future pregnancy, several immunologic treatments have been suggested to induce a proper immunomodulation such as transfusion of paternal leukocytes prior to conception or passive immunization with intravenous immunoglobulin (IVIG) during pregnancy. In 2014 the effect of these immunological treatments on the chance of live births in women with a history of RM was determined in a Cochrane review [44]. It was concluded that immunotherapy did not lower the risk of future miscarriage in women who repeatedly miscarry, and that these therapies should no longer be offered as a treatment.

Paternal factors

Programming of the uterine environment for successful implantation in a semi-allogeneic pregnancy may be effectuated by the presence of semen in the woman's genital tract. Semen contains not only paternal HLA antigen but also immunomodulatory factors, such as chemokines, cytokines and prostaglandins [45, 46]. The introduction of seminal plasma at intercourse elicits recruitment of macrophages, DC, and, memory T cells in the female reproductive tract [47].

Besides the classical HLA antigens, seminal plasma contains soluble HLA-G (sHLA-G) [48, 49]. Additionally, seminal plasma contains immunomodulatory factors TGF-β and PGE2. Seminal TGF-β has been shown to be a principal stimulating agent in the post-coital inflammatory response, and could be essential for induction of immune tolerance to paternal antigens [46]. Removal of seminal prostaglandins resulted in a dramatic decrease in immune suppressive activity [50].

Immune recognition of paternal antigens may play a role in pregnancy complications: change of partner is a risk factor for intrauterine growth restriction, preterm birth, low birth weight and infant mortality, and it counteracts the protective effect of multiparity against preeclampsia [51-53]. Additionally, the length of unprotected sexual cohabitation affects the incidence of pregnancy-induced hypertensive disorders [54, 55], and oral exposure to semen is correlated with a diminished occurrence of preeclampsia [45]. Furthermore, preeclampsia occurs more frequently in pregnancies induced by artificial insemination with donor semen [56]. Combined, these findings indicate that exposure to paternal antigens prior to gestation may have a beneficial effect on pregnancy outcome.

Study of reproductive immunology in mice

In mouse models it has been shown that the lack of certain immune cell subsets, e.g. Tregs specific for paternal antigens, leads to a higher incidence of failed pregnancies [57]. Furthermore, injection of interferon (IFN)-γ or IL-2 in mice results in increased abortion rates, whereas injection of IL-10 results in decreased abortion rates [58, 59]. However, murine pregnancies are very different from the human situation [60]. The most remarkable difference is a second placenta type in mice, the inverted yolk sac placenta, which is completely absent in humans. Furthermore, human trophoblast cells show deep interstitial and endovascular invasion, reaching the human myometrium (Figure 4), whereas the murine labyrinth only shows shallow trophoblast invasion.

Additionally, the time of gestation in mice is only three weeks and many of the developmental processes that occur in humans during intrauterine life are

postnatal events in mice. Direct extrapolation from animal models to humans has led to assumptions of mechanisms for which the evidence is incomplete. Therefore, in our research we only use human pregnancies to study parameters, which affect the induction of maternal tolerance towards the fetus.

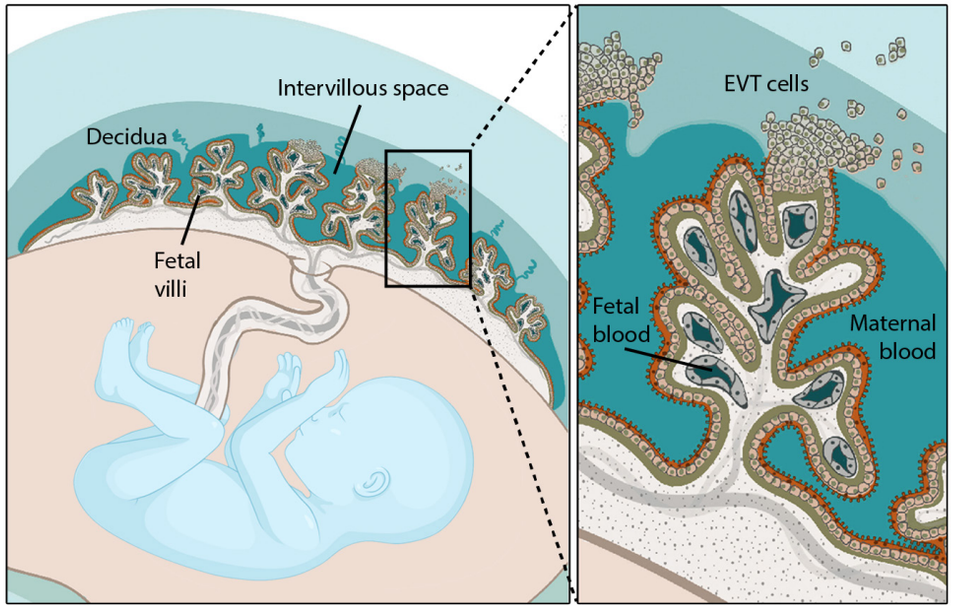

Figure 4. Immediately after implantation, cells forming the outermost layer of the blastocyst give rise to diverse trophoblast cell types. Invasive trophoblasts migrate into the maternal endometrium. Fetal villi will be generated by proliferation and invasion, and throughout pregnancy there will be villous branching and vascularization. Through these villi, nutrients and oxygen can be exchanged ensuring appropriate fetal development and growth. Adapted from V.B. Zeldovich. PLOS Pathogens. 2011.

Aims of this thesis

With the studies described in this thesis, we want to get more insight in the immunologic mechanisms that play a role in pregnancy. The results of this research can help to identify underlying etiologies in patients with unexplained pregnancy complications, such as recurrent miscarriage. Identifying these causes is important for providing answers and taking away anxiety in these couples, and eventually for the development of effective therapies. Furthermore, elucidating the mechanism

leading to survival or rejection of the fetal allograft is not only essential for our understanding of processes leading to normal and abnormal pregnancies, but may also result in important concepts in the field of transplantation and autoimmunity.

We start with a literature study to answer the question: what is wrong with regulatory T cells in recurrent miscarriage (**Chapter 2**)? Regulatory T cells play a pivotal role in controlling adaptive immune responses and maintaining self-tolerance. This unique subpopulation of T cells has shown to be involved in preventing autoimmunity, and tolerance to allogeneic organ grafts after transplantation [61, 62]. The suppressive activity of Tregs is mediated either in a cell-cell contact-mediated fashion via cytotoxic T lymphocyte-associated protein 4 (CTLA-4) or by the secretion of cytokines such as TGF-β or IL-10. Dynamic changes in circulating Treg frequencies during pregnancy have been found: a marked increase during early pregnancy, peaking during the second trimester, and a progressive decrease to levels comparable with non-pregnant conditions at term [63-66]. Because data suggest that regulatory T cells (Treg) are involved in the maternal acceptance of the allogeneic foetus, RM could possibly be explained by a disturbance of the Treg network.

In a retrospective observational study, we investigated the role of HLA-DR sharing between mother and child in pregnancy outcome. Children inherit one HLA haplotype from each parent (Figure 5), so a mother will always share one HLA haplotype with her child. Paternally-inherited fetal HLA antigens can induce maternal immune activation to secure and promote the pregnancy. Does HLA-DR incompatibility between mother and child have a positive influence on pregnancy outcome parameters (**Chapter 3**)?

Next, we study HLA-G expression in placentas of women with a history of RM and controls. We study whether HLA-G expression in term placenta is different in women with a history of RM compared to healthy controls

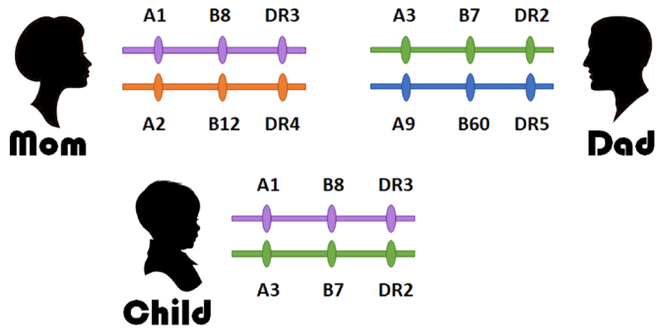

Figure 5. HLA is inherited as a set, which is known as a haplotype. A child inherits one HLA haplotype from each parent. Therefore, there is a 25% chance siblings inherit the same set of HLA.

and analyze whether this is related to HLA-G genotype (**Chapter 4**). In soluble form, HLA-G is also present body fluids. We investigate the role of the man by analyzing their HLA-G genotype and examine whether there is an association with sHLA-G levels in seminal plasma (**Chapter 5**).

To analyze the effect of seminal plasma on the phenotype and function of certain immune cell subsets *in vitro* tests with human cells are commonly used. However, it is very important to take into account under which circumstances these experiments are performed. In **Chapter 6** we study the effect of seminal plasma on human DC, which we culture in the presence of different protein sources (fetal calf serum/human serum). Previous studies suggestive for an immune modulating role of seminal plasma had been performed in the presence of FCS, which is known to affect the vitality of human immune cells in the presence of seminal fluid. We questioned whether the presence of seminal plasma indeed leads to the differentiation of anti-inflammatory DC, when these are cultured with human serum instead of fetal calf serum?

Finally, **Chapter 7** provides a summary of and general discussion of the results found in this thesis.

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Craenmehr, M. H. C.; Heidt, S.; Eikmans, M.; Claas, F. H. J., HLA 2016, 87, (2), 69-78.

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what is wrong with regulatory t cells and foetomaternal tolerance in women with recurrent miscarriages

Abstract

Couples of whom the woman has had a miscarriage have two major concerns: the cause and possible risk of recurrence. Unfortunately, a significant proportion of cases of recurrent miscarriage (RM) remain unexplained despite detailed investigation. Since data suggest that regulatory T cells (Treg) are involved in the maternal acceptance of the allogeneic foetus, RM could possibly be explained by a disturbance of the Treg network. The possible role of Tregs in RM is described in this review, as well as their potential application in diagnostics and therapeutic intervention trials.

Introduction

Approximately 15% of pregnant women experience spontaneous loss of a clinically recognized pregnancy. About 1-2% of couples trying to conceive are confronted with recurrent miscarriage (RM), which is defined as three or more consecutive miscarriages before the 20th week of gestation [1-8]. Several factors influence the risk of miscarriage such as maternal age and previous pregnancy loss. The major known causes include antiphospholipid syndrome, abnormal parental karyotype, endocrine disorders and uterine anomalies [1-7, 9, 10]. However, the cause of RM can only be determined in half of the patients. This burden of continuous uncertainty has a major impact on the lives of women and their partners.

Increasing evidence suggests that the maternal immune response towards the foetus plays a determinative role in the success of pregnancy [9, 11, 12]. Several mechanisms are involved in the induction of maternal tolerance and immunologic acceptance of the semi-allogeneic foetus during pregnancy. Besides the immunological changes occurring locally at the foetal-maternal interface, peripheral immune responses are also altered during pregnancy [9, 13]. Mechanisms for the evasion of the maternal immune response by the foetus include the absence of the classical major histocompatibility complex (MHC) class I antigens human leukocyte antigen (HLA)-A and HLA-B and MHC class II on foetal trophoblast cells preventing allorecognition by T cells and the presence of HLA-C, HLA-E, HLA-F and HLA-G [14-18], preventing allorecognition by natural killer (NK) cells. Furthermore, HLA-G facilitates semi-allogeneic pregnancy by inhibiting maternal immune responses to foreign (paternal) antigens [19]. Another mechanism contributing to immune protection of the foetus is complement inhibition by regulatory proteins decay-accelerating factor (DAF, CD55), membrane cofactor protein (MCP, CD46) and MAC-inhibitory protein (MAC-IP, CD59), and soluble regulators. In addition, trophoblast tissue synthesizes indoleamine 2,3-dioxygenase (IDO), a tryptophan catabolizing enzyme that prevents maternal T cell activation, while galectin-1

(Gal-1) is also expressed at implantation sites, which promotes the generation of tolerogenic dendritic cells [20]. Also the PD1/PDL1 coinhibitory pathway plays a role in foetomaternal tolerance, by limiting the expansion of alloreactive T cells [21]. Other tolerance-inducing cell types highly prevalent in the decidua are CD163+ M2 type macrophages and CD56brightCD16- dNK cells [22]. Another important player in this field is the regulatory T cell (Treg). This heterogeneous subset of T cells suppresses the induction and proliferation of effector T cells and plays an essential role in the sustainability of peripheral immune tolerance [23-26]. The mother's acceptance of the foetus, which can be seen as an allograft expressing paternally inherited alloantigens, during pregnancy is a unique example of how the immune system reshapes a destructive alloimmune response to a state of tolerance. Therefore, knowledge on the role of Tregs in successful and aberrant pregnancy may also be relevant for cell and organ transplantation as acceptance of the allograft is a desirable goal in both reproductive immunology and transplantation.

In this review, the role of Tregs in foetal-maternal immune tolerance as well as in recurrent miscarriage will be discussed. The subject of preeclampsia will not be addressed in this review, because of the difference in pathophysiology. Understanding the complex mechanisms of foetomaternal tolerance has important implications for developing novel strategies to induce immunologic tolerance in humans in general and for prevention of spontaneous abortion in high-risk populations in particular.

Regulatory T cells: Phenotype and function

Regulatory T cells play a pivotal role in controlling adaptive immune responses and maintaining self-tolerance. This unique subpopulation of T cells has shown to be involved in preventing autoimmunity, and tolerating allogeneic organ grafts in rodent models. The suppressive activity of Tregs is mediated either in a cell-cell contact mediated fashion via cytotoxic T lymphocyte-associated protein 4 (CTLA-4) or by the secretion of cytokines such as transforming growth factor beta (TGF-β) or interleukin (IL)-10 [27-31]. In 1995, Sakaguchi characterized a subpopulation of T cells with suppressive capacity [32]. These regulatory T cells were then described as being CD4+CD25+ T cells, a phenotype definitely not unique to Tregs. Ever since, a major obstacle to the study and application of Tregs in the human setting has been the lack of specific cell surface markers to define Tregs and separate them from other T cell subsets [33, 34]. The transcription factor forkhead box P3 (FoxP3) was considered as a specific marker for Tregs essential for their thymic development, phenotype, and function [35-37]. Although Foxp3 is expressed exclusively by Tregs in mice, Foxp3 expression in humans occurs in immunosuppressive Tregs as well as in recently activated effector T cells, and thus does not specifically identify human regulatory T cells [38-40]. To address this limitation, high expression of CD25 and downregulation of the IL-7 receptor (CD127), along with intracellular Foxp3 expression, have been used as phenotypic markers for regulatory T cells. Several investigators confirmed that isolation of T cells with high expression of CD25 and low expression of CD127 will result in a highly purified population of Tregs with suppressive capacities in functional assays [33, 35, 41-43]. Other markers that have been associated with (certain subsets of) Tregs are Helios, CTLA-4, CD45RA/RO, CD62L, C-C chemokine receptor type 6 (CCR6) and CD39 [28, 43, 44]. Tregs are comprised of two main populations: thymus-derived natural Tregs and peripherally generated induced Tregs [45]. However, in most studies concerning Tregs in recurrent miscarriages no distinction was made between both populations.

Tregs in normal pregnancy

Tregs in rodent models of pregnancy

In 2004, Aluvihare and colleagues were the first to report that Tregs are required for the maternal immune system to tolerate a foetal allograft in mice [46]. They showed an unusually high proportion of CD4+CD25+ Tregs in almost all tissues of pregnant mice compared to non-pregnant mice, independent of the presence of a paternal MHC difference. Treg frequencies in blood of mice increased during early pregnancy, progressively decreased from mid-gestation onwards, and at term returned to levels that are comparable to non-pregnant conditions [47]. This indicates that the maternal immune system undergoes a systemic change during pregnancy. In addition to the expansion of Tregs in pregnant compared to non-pregnant mice, a diminished number and function of Tregs was found in abortion-prone animals [48-50]. These animals expressed even lower levels of CD4+CD25+ Tregs than age-matched non-pregnant control mice. The abovementioned results suggest a crucial role for Tregs in avoiding immunological rejection of the foetus.

To test whether Tregs are indispensable for maternal immune tolerance toward the foetus, adoptive transfer experiments in mice were performed [46, 51]. Transfer of lymphocytes depleted of CD4+CD25+ Tregs into pregnant T-cell-deficient mice led to gestation failure. Additionally, the adoptive transfer of pregnancy-induced Tregs into abortion-prone mice prior to mating significantly increased IL-10 and TGF-β mRNA expression in decidua and lowered the foetal resorption rates [48, 51]. This suggests an active and essential role for Tregs in mediating maternal tolerance to the foetus. Importantly, this treatment was only successful if applied at an early stage of pregnancy, and transfer of Tregs from non-pregnant mice to the abortion-prone mice was ineffective [50, 52]. Blocking regulatory T cell function by an anti-CD25 monoclonal antibody (mAb) on day 0 of pregnancy in normal pregnant mice inhibited implantation, while anti-CD25 mAb treatment later in pregnancy reduced Treg cell numbers, but did not induce any parameters reflecting abnormal pregnancy [48, 50, 53]. These findings suggest that Tregs are important to mediate maternal tolerance to the allogeneic foetus in the implantation phase and early stages of pregnancy, while Tregs may not be required for maintenance of the late stage of allogeneic pregnancy.

Although Aluvihare and colleagues argued that expansion of Tregs during pregnancy is alloantigen-independent [46], Zhao and colleagues reported that frequencies of CD4+CD25+ Tregs increase to greater extent in allogeneic than in syngeneic pregnancies in mice [47]. In addition, Shima *et al*. showed that administration of anti-CD25 mAb early in pregnancy induced implantation failure in allogeneic pregnant mice, but not in syngeneic pregnant mice [53]. These results suggest an involvement of paternal antigens in Treg expansion.

Treg induction by seminal plasma

The stages wherein Tregs specific for paternal antigens develop are not yet fully defined. There is evidence that seminal plasma may already induce a tolerogenic environment.In mice, paternal antigens and maternal MHC class II cells can be found in the vaginal mucus already within the first hours of pregnancy [54], indicating the possibility of local antigen presentation at very early stages. Exposure of the mouse female genital mucosa to seminal plasma induced the expansion of CD4+CD25+FoxP3+ Tregs in the lymph nodes draining the uterus, promoting tolerance to paternal alloantigens [55-57]. The increase in CD4+CD25+ cells was abrogated when seminal vesicles were excised before mating [55, 56]. Immediately after insemination, paternal antigens were found in several organs of the female mice [54, 57]. This emphasizes the possibility that Tregs proliferate after encountering semen-derived paternal antigens presented on antigen-presenting cells (APCs) in secondary lymphoid organs. More specifically, a soluble form of

CD38 (sCD38) released from seminal vesicles to the seminal plasma might play a role in this process. Soluble CD38 in seminal plasma was shown to be crucial for the induction of uterine tolerogenic dendritic cells (DCs) and CD4+Foxp3+ Tregs [58]. Deficiency of sCD38 in seminal fluid increased the loss rate of allogeneic foetuses, which could be rescued by a direct injection of recombinant sCD38 into the uterus. The immunoregulatory role of seminal plasma is not exclusive to rodents. Exposure of human peripheral blood T cells to seminal plasma *in vitro* led to increased mRNA expression of CD25, IL-10 and FoxP3, which was partly dependent on the presence of APCs [59]. These results suggest that seminal plasma contains immunomodulatory factors that may contribute to the formation of a tolerogenic environment at the embryo implantation site and that exposure to seminal fluid at mating promotes a state of functional tolerance mediated by expansion of the local antigen-specific Treg pool. One of these immune modulating aspects in semen could be soluble HLA (sHLA), as human seminal plasma contains sHLA-G and sHLA class I [59, 60]. HLA-G inhibits the proliferation and cytotoxic functions of T cells and induces immunosuppressive T cells [15-17, 61]. Peptides derived from the paternal HLA class I antigens in the seminal plasma may be presented by maternal APCs in the endometrium and when the proper cytokines are present in the seminal fluid this may lead to the induction of regulatory T cells (Figure 1). Several prostaglandins, cytokines and chemokines have been described to be present in seminal plasma, such as pro-inflammatory IL-1, IL-6, IL-8, tumor necrosis factor (TNF)-α, interferon (IFN)-γ, granulocyte macrophage colony-stimulating factor (GM-CSF) and chemokine C-X-C motif ligand (CXCL)1, and anti-inflammatory prostaglandin E2 (PGE2), TGF-β, CXCL10, chemokine C-C motif ligand 17 (CCL17), MCP-1 and macrophage colony-stimulating factor (M-CSF) [62-66]. Increasing evidence suggests that proteins in seminal fluid are able to interact with the vaginal, cervical and uterine epithelium to elicit a series of changes in the immune responsiveness of the female [67-69]. However, seminal plasma shows great variety between men in the concentrations of cytokines and in the strength and quality of the cytokine response elicited [68]. This diversity might influence the maternal immune response. A profile with high levels of regulatory proteins, such as TGF-β and PGE2, can contribute to the secretion of inhibitory cytokines TGF-β, IL-10 and IL-35 by maternal APCs. The secretion of these cytokines can lead to the suppression of activation and expansion of conventional T lymphocytes and the induction of maternal regulatory T cells and tolerogenic DCs (Figure 1; cytokine profile A), whereas a cytokine profile with high levels of pro-inflammatory cytokines, such as GM-CSF and IL-8, might induce a response eliciting the expression of pro-inflammatory cytokines and chemokines and the recruitment of macrophages, dendritic cells, and lymphocytes (Figure 1; cytokine profile B). This inflammatory response might lead to pregnancy complications or even pregnancy loss.

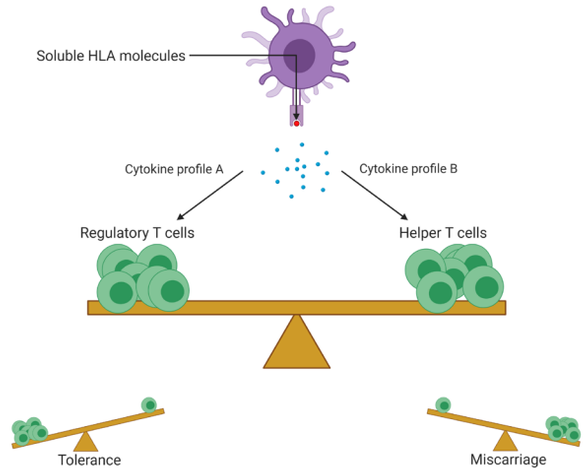

Figure 1. Soluble HLA molecules and cytokines may affect the local immune response during implantation. Paternal HLA antigens, present in seminal plasma in the form of sHLA, might be taken up and presented by maternal APCs. These APCs present the allogeneic peptides to naïve T cells. The cytokine environment present at the time the paternal antigens are first encountered is pivotal in controlling differentiation of APCs, which can determine the strength and quality of the ensuing T cell response. Many cytokines are present in seminal plasma. The specific cytokine profile in seminal plasma varies between semen samples. When regulatory proteins, e.g. TGF-β and PGE2, are present in the seminal fluid, this can contribute to the secretion of inhibitory cytokines TGF-β, IL-10 and IL-35, which can lead to the induction of specific regulatory T cells and a tolerogenic environment (cytokine profile A). On the other hand, the cytokine profile in the seminal plasma can contribute to the promotion of a Th1 like response, which can lead to activation and expansion of conventional T lymphocytes and pregnancy complications (cytokine profile B). Adapted due to poor quality of original figure. Created with BioRender.com.

Tregs in human pregnancy

In humans, dynamic changes in circulating CD4+CD25+ Treg frequencies during pregnancy have been found, similar to what was seen in mice: a marked increase during early pregnancy, peaking during the second trimester, and a progressive decrease to levels comparable to non-pregnant conditions at term [47, 70-72]. Svensson-Arvelund *et al*. showed that human foetally derived placental tissue promotes the induction of suppressive CD25highCD127lowFoxp3+ Tregs within tissue *in vitro*, in parallel with increased IL-10 production [22]. The expansion of Tregs was mediated, in part, by TGF-β and IL-10, produced particularly by trophoblast cells. Galectin-1, a progesterone regulated protein expressed at the foetomaternal interface, also induces the expansion of CD4+CD25+FoxP3+ Tregs[73]. In addition, the PD1/PDL1 pathway promotes both the induction and maintenance of CD4+Foxp3+ regulatory T cells, where PDL1 is expressed by foetal cells and PD1 is expressed by maternal cells [74-76]. A novel inhibitory cytokine identified to play a role in the regulation of maternal-foetal immune tolerance is IL-35. This cytokine is produced primarily by CD4+Foxp3+ Tregs and is required for maximal suppressive activity of Tregs *in vitro* and *in vivo* [77, 78]. It has been reported that first-trimester human trophoblast cells express and secrete IL-35, which might contribute to their suppressive capacity toward maternal immune cells [79]. Interestingly, it was found that the level of IL-35 was significantly higher in pregnant females compared to age-matched non-pregnant females, which may suggest that increased IL-35 in pregnancy provides immune protection for the foetus [77]. In this way, by several placental factors acting in concert, the foetal placenta is able to create a tolerant uterine environment.

The induction of labour in humans is associated with a decrease of peripheral CD4+CD25high Tregs and a sharp increase of peripheral CD4+CD25low T cells [47], the latter largely representing activated effector T cells. Tilburgs *et al*. also observed this significant increase in the CD4+CD25low T cell fraction in maternal peripheral blood lymphocytes at term pregnancy compared to peripheral blood of early pregnancy subjects and to peripheral blood of non-pregnant controls. However, they did not observe significant differences in the level of peripheral CD4+CD25high T cells in early pregnancy, term pregnancy, and non-pregnant controls [80]. When comparing the decidua to maternal peripheral blood and peripheral blood of non-pregnant controls, a significantly higher percentage of CD4+CD25high T cells was found [13, 80]. Furthermore, these CD4+CD25high T cells from the decidua contained a significantly higher suppressive capacity to regulate the maternal immune response to foetus-specific UCB cells compared to CD4+CD25high T cells in maternal blood [13]. These results suggest that foetus-specific Tregs are specifically recruited from the periphery to the foetal-maternal interface. Sindram-Trujillo *et al*. compared the immune cell composition of decidua collected after spontaneous vaginal delivery to elective caesarean section without labour. Labour appeared to be associated with dynamic changes in the distribution of decidual leukocytes, specifically NK and T cell subpopulations. The percentage of CD3+CD4+CD25+ cells in the decidua basalis and decidua parietalis after spontaneous vaginal delivery was lower than after caesarean section [81]. This down-regulation of Tregs might lead to an abnormal immune milieu, which confers susceptibility to pregnancy loss. Hence, low Treg levels may be associated with recurrent miscarriages.

Tregs in recurrent miscarriages

Before pregnancy

Compared to non-pregnant women, peripheral CD4+CD25high Tregs are increased in healthy women early during pregnancy. However, they are decreased in women with (recurrent) miscarriages compared to normal early pregnancy, at a level comparable with that of non-pregnant controls [70, 82, 83]. This difference in

Treg level can already be detected in non-pregnant women with RM. It has been shown that Treg frequencies undergo profound changes during the menstrual cycle [84]. Fertile women showed an expansion of Tregs in the late follicular phase followed by a dramatic decrease in Treg frequencies in the luteal phase of the menstrual cycle, whereas women with RM had similar Treg frequencies at both phases. At both the follicular and luteal phases, decreased frequencies of peripheral Tregs were observed in women with unexplained RM compared to fertile controls [85, 86]. These low levels of Tregs were similar to peripheral Treg numbers in postmenopausal women [86]. This may suggest that reproductive failure results from the inability of Tregs to sufficiently expand during the pre-implantation phase. Furthermore, infertile women have significantly reduced Foxp3 mRNA levels in the endometrium, supporting the concept that unsuccessful pregnancy is caused by the lack of sufficient Tregs [87]. In healthy women, Tregs are capable of regulating effector T cells that respond to paternal antigens. A lack of regulation thus may also be detected by high levels of paternal antigen-specific effector T cells. Indeed, when women with RM were compared to controls, the frequency of sperm antigen specific effector T cells was higher and accompanied by a lower frequency of sperm antigen specific Tregs [88]. Furthermore, these sperm specific Tregs in women with RM expressed less Ubc13, which is a critical molecule preventing Tregs from differentiating into effector T cells [88, 89]. Knockdown of Ubc13 from isolated Tregs converted the Tregs to effector T cells.

During pregnancy

Lower proportions of CD4+CD25high T cells with FoxP3 expression are found in peripheral blood and decidua from pregnant women with RM compared to those with normal early pregnancies [85, 90-93]. This suggests that women with unexplained RM are less capable to induce and maintain immune tolerance towards foetal alloantigens. Furthermore, it has been shown that the level of IL-17+ T cells and ratio of IL-17+ T cells/Tregs was significantly increased in peripheral blood from non-pregnant women with unexplained RM when compared with fertile controls [85, 93]. Th17 cells can exert a rapid response at sites of inflammation and may play a role in allograft rejection in solid organ transplantation [94, 95]. Likewise, trophoblast invasion from the allogeneic foetus and the shedding of foetal antigens may stimulate a maternal systemic inflammatory response and may therefore cause the emergence of Th17 cells [94]. This suggests that an immunologic imbalance and subsequent immune dysregulation by the altered Th17/Treg cell populations influences pregnancy outcome.

When compared with specimens obtained from abortions on social indication, the proportion of decidual CD4+CD25high T cells in products of conception from miscarriages was significantly lower [96]. This confirms that decidual CD4+CD25high T cells are likely to contribute to the mechanisms mediating maternal immune tolerance and maintenance of pregnancy. In addition to the decreased frequency of CD4+CD25+CD127low Tregs in unexplained RM decidua compared to controls, the suppressive activity of CD4+CD25+CD127low cells on effector T cell proliferation was impaired in unexplained RM decidua [97]. Higher Treg numbers were required to exert a similar magnitude of *in vitro* suppression, mediated predominantly through TGF-β and IL-10, compared to CD4+CD25+FoxP3+ cells from fertile women [86, 97]. The expression of intracellular TGF-β and IL-10 in Tregs was lower in the RM group than in the control group [97]. As mentioned before, IL-35 is required for maximal suppressive activity of Tregs *in vitro* and *in vivo* [66, 67], and whereas this cytokine was increased in normal pregnancy, it was decreased in RM women [77]. Also galectin-1 expression was decreased in women with RM compared to healthy early pregnant women [98].

Women with RM having low CD4+CD25+Foxp3+ Treg levels in the first trimester

experienced a significantly lower ongoing pregnancy rate than those with a higher Treg level in the first trimester [99]. The decreased expansion of Tregs during pregnancy in the unexplained RM group may predispose to pregnancy loss, and Tregs might serve as a pregnancy marker to aid in predicting miscarriage risk in newly pregnant women. [99, 100]. Furthermore, this highlights the opportunity to use Treg therapy to increase the success rate in women who repeatedly experience pregnancy losses.

Tregs as a therapy for recurrent miscarriages

Trials on the use of Tregs to treat graft-versus-host disease (GvHD) in patients with a stem cell transplant showed acceptable safety and promising efficacy, e.g. reduced incidence of severe acute GvHD [101-104]. This has led to the use of Tregs in other fields as well. Whereas studies in solid organ transplantation are already focusing on safety [105], the administration of Tregs has not yet been applied to pregnancy. However, immunotherapeutic procedures that indirectly increase Tregs to prevent maternal rejection of the foetus have been introduced. These immunotherapies include boosting the maternal immune response by paternal (woman's partner) or third-party (donor) lymphocyte immunization. Alternative immunotherapies include products derived from early embryos (trophoblast membranes) or antibodies derived from blood (immunoglobulin therapy). Paternal or third-party lymphocyte immunization has been the most widely used treatment for alloimmune-mediated miscarriages. However, this therapy is still controversial in terms of effectiveness. The latest Cochrane review by Wong *et al*. showed that none of these treatments provided a significant beneficial effect over placebo in improving the live birth rate or reducing the risk of future miscarriage in women who had RM [106]. Nevertheless, some studies showed that the proportion of CD4+CD25high T cells in peripheral blood from women with unexplained RM was significantly increased after paternal or third-party lymphocyte immunization therapy [107-109], and 80-90% of patients who underwent immunotherapy successfully delivered a baby [109]. Furthermore, the proportion of Tregs was significantly higher in successfully pregnant women than in those with pregnancy loss after lymphocyte therapy [107-109]. In those who experienced an unsuccessful pregnancy, no significant change of the proportion of CD4+CD25+ T cell/PBMC and CD4+CD25+CD127-/CD4+ T cell was observed and the level of Tregs remained low. After successful immunotherapy, the percentage of Th17 cells was significantly lower and the Th17/Treg ratio significantly decreased to a level comparable to that before immunotherapy. Unfortunately, in these studies it is not uniformly described whether lymphocyte immunization was performed with cells of the partner or a third-party.

Other therapies that intend to induce Tregs in women with unexplained RM involve the administration of cytokines and hormones. Scarpellini and Sbracia tested the use of granulocyte colony-stimulating factor (G-CSF) in women with unexplained RM [110]. G-CSF is a cytokine that, amongst others, can recruit and activate tolerogenic dendritic cells, which can aid in the generation of Tregs [9, 111, 112]. G-CSF treatment showed an evident effect on the pregnancies of women with RM, with a remarkable increase in success rate and a consequent reduction of miscarriages. Currently, the RESPONSE trial is testing the effect of G-CSF administration in women with three or more unexplained miscarriages in a randomised, double-blind, placebo-controlled trial (NCT02156063). Also, progesterone is suggested to be an important regulator of systemic and local Treg development and function [113, 114]. For now, it is still unclear whether it is effective in women with RM [115, 116]. The report of a large multicentre study (PROMISE) of progesterone supplementation for RM is currently awaiting publication (ISRCTN92644181).

Conclusions

Tregs have a critical role in maintaining immune tolerance to self-antigens and to foreign antigens of the semi-allogeneic foetus: a deficiency in Tregs is associated with implantation rejection at early stages of pregnancy and abortion. Whether immunotherapy can play a role by preventing maternal rejection of the foetus has yet to be established, but modulation of the immune system as (part of) a therapeutic strategy is certainly a valid option to prevent recurrent miscarriages.

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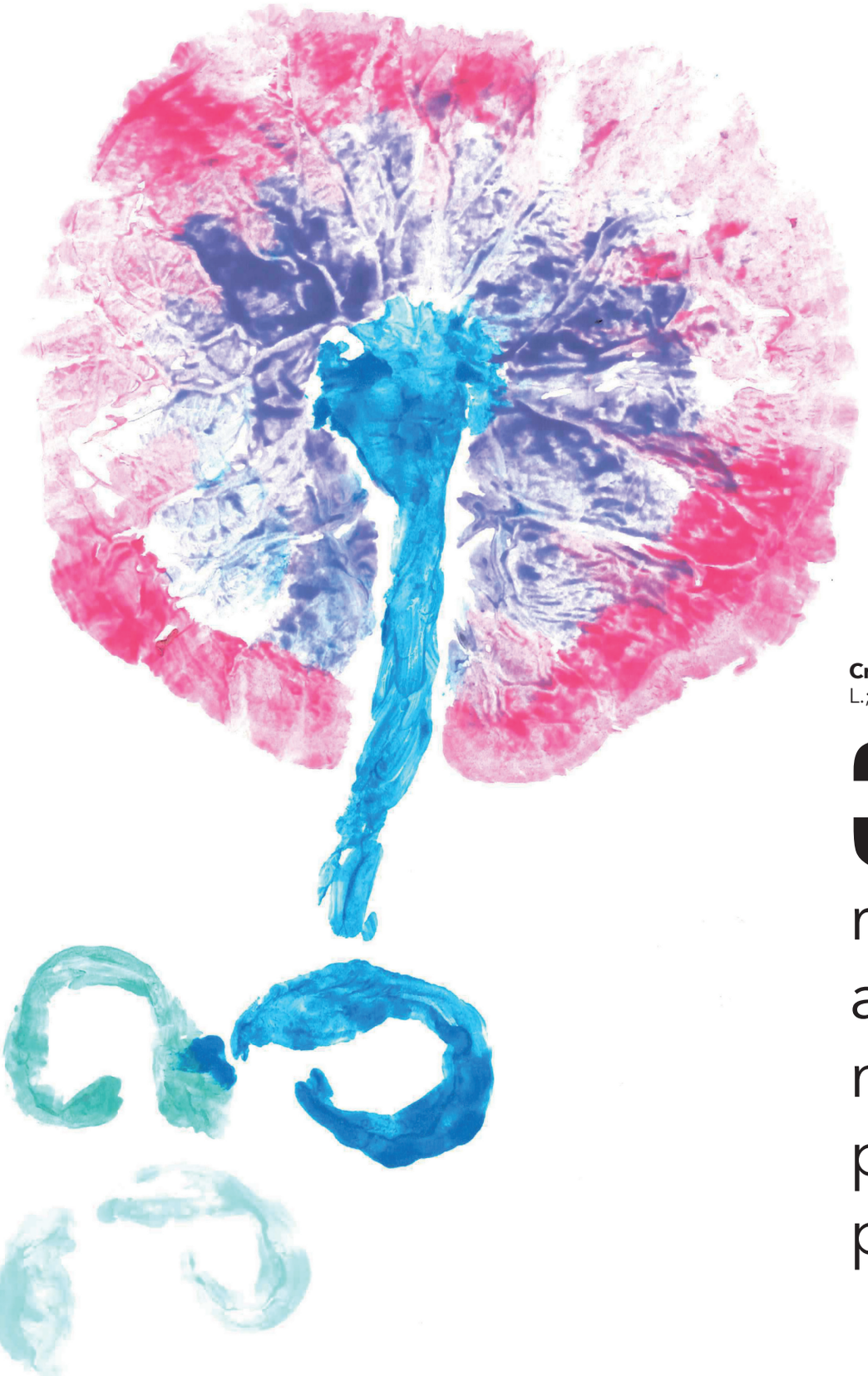


Craenmehr, M. H. C.; van Egmond, A.; Haasnoot, G. W.; Eikmans, M.; Roelen, D. L.; Heidt, S.; Scherjon, S. A.; Claas, F. H. J., J Reprod Immunol 2019, 133, 15-17.

3

reciprocal HLA-DR allogenicity between mother and child affects pregnancy outcome parameters

Abstract

Successful pregnancy outcome depends on local immunoregulatory mechanisms preventing a detrimental immune response towards the semi-allogeneic fetus. We investigated the influence of HLA-DR (in)compatibility on pregnancy outcome parameters in 480 women. The parameters tested were birth weight, individualized birthweight ratio (IBR), gestational age and maternal highest diastolic blood pressure. Irrespective of pregnancy complications, maternal-fetal HLA-DR incompatibility resulted in increased IBR. We conclude that reciprocal HLA-DR allogenicity between mother and child positively affect pregnancy outcome parameters.

Introduction

Successful pregnancy outcome depends on local immunoregulatory mechanisms preventing a detrimental maternal immune response towards the semi-allogeneic fetus. Paternally-inherited fetal HLA antigens can induce maternal immune activation and a variety of immune cells are recruited to the placental bed to secure and promote the pregnancy. Regulatory T cells (Tregs) play an important role in successful pregnancy. These Tregs are generally CD4+ and are thus HLA class II restricted. In organ transplantation, matching for HLA-DR leads to a better graft survival and function [1].

In the setting of pre-transplant blood transfusion it has been shown that at least one HLA-DR antigen has to be shared between donor and recipient in order to induce a tolerogenic effect on the course of a subsequent renal transplantation, while incompatibility for the second HLA-DR antigen enhances a stable, rejection-free, allograft function [2, 3].

In line with this blood transfusion concept, the pregnant mother has to accept the semi-allogeneic fetus. Trophoblast cells do not express HLA-DR, but fetal chimeric cells can cross the placenta and trigger a maternal immune response. Moreover, such transfer is bidirectional [4]. Both maternal and fetal cells can cross the placenta and fetal immune cells can also respond to maternal alloantigens.

Several studies have aimed at finding a correlation between pregnancy complications such as preeclampsia (PE) or recurrent miscarriage (RM) and the presence of certain HLA alleles, maternal homozygosity or sharing of HLA between mother and father or between mother and fetus. Recently, a systematic review showed that HLA-B sharing and HLA-DR sharing were both associated with the occurrence of recurrent miscarriage [5]. These results suggest that there is a negative correlation between HLA sharing and a favorable pregnancy outcome. This is in line with previous findings, suggesting that HLA sharing between mother

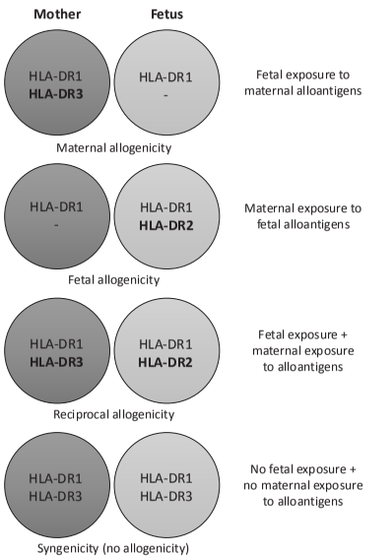
and child is associated with pregnancies complicated by PE [6]. These studies focused on pregnancy complications and do not necessarily represent the interaction of HLA molecules and immune cells during uncomplicated pregnancy. Therefore, we sought to take a different approach to examine the possible effect of HLA on pregnancy outcome with the use of objective parameters.

We conducted a retrospective, observational study to investigate the influence of fetal and maternal HLA-DR sharing on pregnancy outcome using objective outcome parameters as birth weight, gestational age and maternal highest diastolic blood pressure.

Materials & Methods

We retrospectively studied a cohort of 480 women who gave birth in the Leiden University Medical Center between 1992 and 2011, and their children. The majority of the pregnancies (59%) investigated were uncomplicated term pregnancies representing successful pregnancy. All women signed informed consent and the study was approved by the Ethics Committee of the Leiden University Medical Center. HLA-DRB1 typing of both mother and child was performed by SSO PCR technique using a reverse dot-blot method at the national reference laboratory for histocompatibility testing (Leiden University Medical Center, the Netherlands). We divided the woman-child pairs into four previously described groups [6] based on the degree of HLA-DR compatibility, as depicted in Figure 1. Maternal allogenicity was defined as the situation in which the mother expresses two distinct HLA-DR antigens and the fetus only expresses one allelic form. In the situation of fetal allogenicity the fetus expresses two distinct HLA-DR antigens, whereas the mother only expresses one allelic form. In the reciprocal allogenicity group both the mother and fetus express two distinct HLA-DR antigens of which one of the HLA-DR antigens is mismatched between mother and child. Syngenicity was

A



B

Variable	Allogenicity	N	Median	Min	Max	P-value
IBR	Maternal	54	1.00	0.51	1.39	**0.030***
	Fetal	50	0.97	0.62	1.43	
	Reciprocal	309	1.06	0.44	1.49	
	Syngenicity	53	1.03	0.53	1.49	
Birth weight (gram)	Maternal	55	3100	657	4975	**0.029***
	Fetal	51	3170	879	4550	
	Reciprocal	319	3365	625	5285	
	Syngenicity	55	3230	990	4155	
Highest diastole (mmHg)	Maternal	52	80	65	120	0.939
	Fetal	50	80	60	110	
	Reciprocal	317	80	60	160	
	Syngenicity	53	80	60	130	
Gestational age (days)	Maternal	55	269	202	292	0.898
	Fetal	51	270	209	295	
	Reciprocal	319	271	190	297	
	Syngenicity	55	270	210	294	

* P <0.05. The p-values were calculated using the Kruskal-Wallis test.

Figure 1. HLA-DR allogenicity and pregnancy outcome. (A) The different types of maternal-fetal HLA relationships and potential for maternal and/or fetal exposure to alloantigens. Maternal allogenicity: the mother expresses two distinct HLA-DR antigens and the fetus only expresses one allelic form. Fetal allogenicity: the fetus expresses two distinct HLA-DR antigens, whereas the mother only expresses one allelic form. Reciprocal allogenicity: both the mother and fetus express two distinct HLA-DR antigens of which one of the HLA-DR antigens is mismatched between mother and child. Syngenicity: the mother and child express the same HLA-DR antigens. (B) Results of pregnancy outcome parameters for different HLA-DR allogenicity groups.

defined as the situation in which the mother and child express the same HLA-DR antigens.

The parameters tested were birth weight, individualized birthweight ratio (IBR), gestational age and maternal highest diastolic blood pressure. The IBR is a ratio of the actual birthweight divided by the predicted birthweight [7]. It is calculated by dividing the actual birth weight by the mean birth weight of children of the same sex born after a pregnancy with equal parity and gestational age, as derived from the Kloosterman tables [8]. Supplementary Tables S1 and S2 show the characteristics of the study population.

All other statistical analyses were performed using SPSS Statistics 23 software (IBM SPSS Software, New York, USA). Non-parametric tests were used, since data were not normally distributed according to the Shapiro-Wilk normality test. The Kruskal-Wallis test was used to analyze the distribution of the pregnancy outcome parameters between the different HLA-DR groups. P-values lower than 0.05 were considered statistically significant. To test for independent effects of HLA-DR on pregnancy outcome parameters, we included covariates in a regression model. Inclusion criterion for inclusion in the multivariate analysis was a univariate P-value of <0.1.

Results and discussion

The present study showed that reciprocal allogenicity is significantly related to a higher IBR (Figure 1). The group in which both the mother and fetus express two distinct HLA-DR antigens, with one HLA-DR mismatch between mother and child, had the highest birth weight (P=0.029) and IBR (P=0.030). After correction for maternal age, gravidity, parity, spontaneous abortion, PE/HELLP and smoking, we found a trend for reciprocal HLA-DR allogenicity and birth weight (P=0.068). The association between reciprocal HLA-DR allogenicity and IBR was independent of these factors (P=0.042). The IBR is a superior measure for abnormal and normal growth, because this factor effectively controls for physiological birthweight determinants. These results indicate that the optimal situation for pregnancy is reciprocal allogenicity. Our results suggest that incompatibility for one HLA-DR antigen between mother and fetus leads to triggering and activation of the immune response, while the other HLA-DR antigen has to be shared in order to induce immune regulation. Since reciprocal allogenicity was the most optimal situation found in our study, both fetal and maternal immune responses seem to be important. Although trophoblast cells do not express HLA-DR, HLA-DR+ fetal chimeric cells can cross the placenta [4] and interact with the maternal immune system leading to a similar immune regulation as previously has been described for pretransplant blood transfusions [2]. During pregnancy, increased numbers of CD4+ Tregs are indeed present in the decidua and contribute to the regulation of fetus-specific responses [9].

Similarly, HLA-DR+ chimeric maternal cells in the fetus will interact with the developing fetal immune system, leading to the establishment of a large pool of fetal Tregs [10]. This T cell tolerance towards maternal alloantigens perceived in utero may even be maintained after birth through the establishment of long-lived Tregs, which play a crucial role in the clinical observations showing that mismatches for non-inherited maternal antigens (NIMAs) are better tolerated than non-inherited paternal alloantigens in the setting of adult solid organ transplantation [11].

The percentage of preterm births in this study (26%) is quite high. This is the direct result of collecting retrospective data from women who gave birth in a Dutch academic hospital. In the Netherlands it is still common to give birth at home under supervision of a midwife, which will have led to a relatively high percentage of deliveries with pregnancy complications in hospitals.

We did not collect any information on socioeconomic status, marital status, education, and race-ethnicity. Even though we think it is unlikely that these variables would have influenced the effect of HLA-DR allogenicity on pregnancy outcome parameters, we cannot fully exclude the effect of these factors.

In summary, we conclude that the most optimal situation for a successful pregnancy is that of reciprocal HLA-DR allogenicity. This suggests that active induction of immune tolerance from both maternal and fetal side is important.

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Supplementary Table S1. Maternal characteristics of the 480 women included in the study.

Mother		n
Age (years) *	33 (19-46)	480
Highest diastolic pressure (mmHg) *	81 (60-160)	472
Proteinuria (positive) #	56 (11.7%)	480
Gravidity*	3 (1-10)	480
Parity*	1 (0-6)	480
Previous spontaneous abortions*	1 (0-7)	480
Smoking#		480
- No smoking	409 (85.2%)	
- 1-10 cigarettes/day	18 (3.8%)	
- >10 cigarettes/day	10 (2.1%)	
- Unknown	43 (9.0%)	

* Mean value with the range between parentheses. # Number with the percentage of the total population.

Supplementary Table S2. Pregnancy characteristics of the 480 women included in the study.

Pregnancy		n
Gestational age (days) *	264 (190-297)	480
Mode of delivery#		480
- Spontaneous	200 (41.7%)	
- Caesarean section	280 (58.3%)	
Indication primary caesarean section (n= 246) #		246
- Breech presentation	70 (28.5%)	
- Caesarean previous pregnancy	59 (24.0%)	
- Obstetric medical history	23 (9.3%)	
- Maternal/Fetal indication	16 (6.5%)	
- Other	78 (31.7%)	
Indication secondary caesarean section (n= 34) #		34
- Failure 1st stage	5 (14.7%)	
- Failure 2nd stage	7 (20.6%)	
- Maternal indication	2 (5.9%)	
- Fetal indication	15 (44.1%)	
- Other	5 (14.7%)	
Child		
Birth weight (gram) *	3090 (625-5285)	480
Gender (male) #	236 (49.2%)	480
Placenta weight (gram) *	559 (100-1480)	381
Complications		
Pre-eclampsia#	47 (9.8%)	480
HELLP#	7 (1.5%)	480
IUGR (<5th percentile)#	22 (4.6%)	480
Preterm (<37 weeks)#	123 (26%)	480

* Mean value with the range between parentheses. # Number with the percentage of the total population.

Supplementary Table S3. Factors affecting the effect of HLA-DR reciprocal allogenicity on birth weight and IBR.

		Univariate regression				**Multivariate regression**			
			95% C.I.				**95% C.I.**		
		β	**Lower**	**Upper**	**P-value**	**β**	**Lower**	**Upper**	**P-value**
Birth weight (gram)	Maternal age	28.394	11.47	45.32	0.001*	19.671	4.97	34.38	0.009*
	Gravidity	88.767	36.47	141.07	0.001*	-23.598	-84.31	37.12	0.445
	Parity	168.622	89.87	247.38	0.000*	58.956	-32.97	150.89	0.208
	Spontaneous abortions	35.481	-45.59	119.56	0.407				
	PE/HELLP	-1799.130	-2018.50	-1579.76	0.000*	-1698.406	-1928.28	-1468.53	<0.001*
	Smoking	-419.493	-756.11	-82.87	0.015*	-264.964	-536.73	6.80	0.056
	DR reciprocal allogenicity	165.293	-10.40	340.99	0.065	130.951	-9.95	271.85	0.068
IBR	Maternal age	0.002	0.00	0.01	0.154				
	Gravidity	0.013	0.00	0.02	0.016	0.000	-0.01	0.01	0.974
	Parity	0.025	0.01	0.04	0.002*	0.011	-0.01	0.03	0.284
	Spontaneous abortions	0.004	-0.01	0.02	0.622				
	PE/HELLP	-0.217	-0.27	-0.17	0.000*	-0.205	-0.26	-0.15	<0.001*
	Smoking	-0.094	-0.16	-0.03	0.005*	-0.077	-0.14	-0.02	0.013*
	DR reciprocal allogenicity	0.045	0.01	0.08	0.010*	0.033	0.00	0.07	0.042*

β = regression coefficient. C.I. = Confidence interval. * P <0.05. The p-values were calculated using linear regression.

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4

increased HLA-G expression in term placenta of women with a history of recurrent miscarriage despite their genetic predisposition to decreased HLA-G levels

Abstract

HLA-G is an immune modulating molecule present on fetal extravillous trophoblasts at the fetal-maternal interface. Single nucleotide polymorphisms (SNPs) in the 3 prime untranslated region (3'UTR) of the HLA-G gene can affect the level of HLA-G expression, which may be altered in women with recurrent miscarriages (RM). This case-control study included 23 women with a medical history of three or more consecutive miscarriages who delivered a child after uncomplicated pregnancy, and 46 controls with uncomplicated pregnancy. Genomic DNA was isolated to sequence the 3'UTR of HLA-G. Tissue from term placentas was processed to quantify HLA-G protein and mRNA levels. The women with a history of RM had a lower frequency of the HLA-G 3'UTR 14-bp del/del genotype compared to controls (OR 0.28; P = 0.039), which has previously been related to higher soluble HLA-G levels. Yet, HLA-G protein (OR 6.67; P = 0.006) and mRNA (OR 6.33; P = 0.010) expression was increased in term placentas of women with a history of RM compared to controls. In conclusion, during a successful pregnancy HLA-G expression is elevated in term placentas from women with a history of RM compared to controls, despite a genetic predisposition associated with decreased HLA-G levels. These findings suggest that HLA-G upregulation could be a compensatory mechanism in the occurrence of RM to achieve an ongoing pregnancy.

Introduction

About 1 to 2% of couples trying to conceive experience recurrent miscarriages (RM) [1]. Accepted etiological categories for RM include chromosomal abnormalities, uterine anatomic abnormalities and antiphospholipid antibody syndrome. However, a significant proportion of the couples trying to conceive do not know the underlying cause for this recurring problem [2], leaving them with a burden of uncertainty.

During pregnancy, the maternal immune system needs to accept the semi-allogeneic fetal tissue. For this reason, several mechanisms are at play at the fetal-maternal interface. The absence of the human leukocyte antigen (HLA) class I antigens A and B and HLA class II on fetal trophoblast cells helps to prevent allorecognition by T and B cells, whereas the presence of HLA-C, HLA-E, HLA-F and HLA-G provide self-signals to control (natural killer) NK responses [3, 4]. Low levels of HLA-G have been associated with RM [5, 6]. By alternative splicing, the HLA-G pre-mRNA can give rise to seven different isoforms, of which four are membrane-bound (HLA-G1, -G2, -G3 and -G4) and three are soluble (HLA-G5, -G6 and -G7) [7]. Whereas in healthy tissue membrane-bound HLA-G is only expressed on trophoblasts, the soluble form of HLA-G can be detected in various body fluids, such as amniotic fluid, blood and seminal plasma [8-10]. One mechanism leading to the generation of soluble (s)HLA-G1 is the cleavage of membrane-bound HLA-G from the cell surface by the activity of metalloproteinases [11].

Several polymorphisms are present in the 3 prime untranslated region (3'UTR) of the HLA-G gene. Since the 3'UTR is targeted by microRNAs (miRNA) that can negatively influence expression, polymorphisms in this region may have an influence on the efficiency of miRNA binding, and consequently on the level of HLA-G expression and on pregnancy outcome. The 14 bp insertion/deletion polymorphism affects the stability of HLA-G mRNA and thereby the expression

of HLA-G [12]: the insertion is associated with low levels of sHLA-G [13]. Presence of the +3187A allele is associated with decreased mRNA stability and decreased HLA-G expression [14]. The presence of a guanine at position +3142 increases the affinity of miR-148a, miR-148b and miR-152, which leads to downregulation of HLA-G expression [15].

We analyzed the HLA-G 3'UTR genotype of women with a history of RM and of a control group of women with no history of RM. We also analyzed the HLA-G 3'UTR genotype of their offspring. The combination of multiple polymorphic sites was used to generate 3-UTR haplotypes. In addition, we studied HLA-G mRNA and protein expression levels in term placentas of women with successful pregnancies in both study groups.

Materials and Methods

Subjects and materials

This case control study included women with a medical history of RM who delivered a child after uncomplicated pregnancy. These women visited the Department of Obstetrics and Gynecology, Leiden University Medical Center (LUMC) between 2012 and 2015, and no underlying cause for RM was found after a full clinical workup according to the local guidelines, which are in line with the international ESHRE guideline. Twenty-three women with a history of at least three miscarriages and an uncomplicated singleton pregnancy were included in this study, of whom placental tissue was stored for research purposes. For the control group, 46 women were included with a history of ≤1 miscarriage, of whom placental tissue of a healthy singleton pregnancy was stored for research purposes after delivery at the Department of Obstetrics and Gynecology, LUMC.

For additional experiments we collected products of conception from eight first trimester miscarriages (GA: 6-10 weeks) and four first trimester elective abortions (GA: 5-10 weeks). The miscarriage material was obtained from women with a history of RM from the Department of Obstetrics and Gynecology in the LUMC. Elective abortion material was received anonymously from an abortion clinic [16].

The protocol was approved by the Ethical committee of the LUMC (P11.196), and all participants gave informed consent for inclusion in the study.

HLA-G polymorphisms and haplotypes

Peripheral blood and umbilical cord blood for both groups was processed to genotype HLA-G in the mothers and children, respectively. Genomic DNA was isolated to sequence a 699/713-bp fragment covering the 3'UTR of exon 8, starting just before the 14-bp insertion/deletion and ending 591-bp downstream of the insertion/deletion. To sequence the haplotype on each of the two alleles, amplification reactions were performed using the generic 3'-primer that was tailed with a M13 sequence to cover the 3'UTR region of HLA-G. The following polymorphisms were identified: the 14-bp insertion/deletion (rs371194629), +3003C/T (rs1707), +3010C/G (rs1710), +3027A/C (rs17179101), +3035C/T (rs17179108), +3142C/G (rs1063320), +3187A/G (rs9380142), +3196C/G (rs1610696), +3422C/T (rs17875408), +3496A/G (rs1233330), and +3509G/T (rs1611139).

UTR haplotypes were composed based on the combination of SNPs. Conversion of sequencing data to UTR haplotypes was carried out by using specialized HLA interpretation software (SBT Engine, GenDX, Utrecht, the Netherlands). The forward primer (GTGATGGGCTGTTTAAAGTGTCACC), the reverse primer (GACGTTGTAAAACGACGGCCAGTAGGGGAAGAGGTGTAGGGGTCTG) and an M13 universal primer (GACGTTGTAAAACGACGGCCAGT) were ordered from

Sigma (St. Louis, Missouri USA). The underlining represents the M13 sequence.

Immunohistochemistry

HLA-G and trophoblasts were detected by standard immunohistochemical procedures. After delivery, placental tissues were dissected and fixed in 4% neutral buffered formaldehyde and embedded in paraffin. Section slides of 6 µm were cut, mounted on Superfrost/Plus glass slides (Thermo Scientific, Waltham, Massachusetts, USA), and dried overnight at 37°C. Sections were deparaffinized in xylene and ethanol. Depending on the primary antibody used, unmasking of the antigens was achieved by enzyme digestion with trypsin or incubation with citrate buffer in a microwave. This was followed by endogenous peroxidase blocking in 3% H_2O_2 in methanol for enzymatic staining. All incubations were at room temperature and wash steps in between the incubations were performed in PBS. Slides were pre-incubated with PBS/1% BSA to reduce background staining. Excess buffer was removed and slides were incubated with mouse monoclonal primary antibodies overnight at room temperature. Antibodies were diluted in PBS containing 1% BSA.

For enzymatic and immunofluorescence staining, primary antibodies against the free heavy chain of all HLA-G isoforms (MEM-G2; EXBIO Praha, Czech Republic) and against cytokeratin 8 (CAM5.2; Becton Dickinson, Franklin Lakes, New Jersey, USA) were used. The next day, incubation with secondary antibody (EnVision solution, goat anti-mouse HRP, undiluted; DAKO, Agilent, Santa Clara, California, USA) for enzymatic staining; Goat-anti-mouse IgG1-AF488 A21121 and Goat-anti-mouse IgG2a-AF546 A21133 for immunofluorescence staining; Thermo Scientific) was performed, and substrate was visualized with diaminobenzidine (DAB metal Enhanced substrate kit; 34065; Thermo Scientific) for enzymatic staining. Specimens were counterstained with hematoxylin and mounted in Micromount Mounting Medium (Leica, Nussloch, Germany) for enzymatic staining and ProLong Gold Antifade Mountant with DAPI (P36931; Thermo Scientific) for immunofluorescence staining.

Quantification of immunohistochemical stainings

We set out to compare the extent of MEMG2 and CAM5.2 staining in term placentas between the study groups. All slides were scanned by a Pannoramic Midi scanner (3DHISTECH, Budapest, Hungary). The entire decidua basalis was quantitatively analyzed using the HistoQuant modus in Quant Center software (3DHISTECH). This was done by two investigators (JS and HK) independently for 10 placentas to analyze interobserver variability. For each staining, the same thresholds and training scenarios were used for patient and control slides. We corrected for the selected surface area when calculating the percentage positivity of a staining.

For first trimester material, we could not define the decidua. Therefore, we analyzed only the HLA-G positive parts of the slides. Scoring of the slides was performed by two investigators (JS and ME) independently, blinded for the cause of the abortion. Based on the extent of staining, cases were classified according to a semi-quantitative scoring system, i.e., (1) minimal, (2) moderate, or (3) intense staining. Examples of stainings are shown in Supplementary Fig. S1.

RNA isolation and qPCR

Tissue homogenates from term placentas were processed for mRNA quantification of HLA-G by real-time qPCR. Tissue sections were immersed in ML lysis buffer (Nucleospin miRNA isolation kit from Macherey-Nagel, Düren, Germany) and stored at -20°C until isolation. RNA was extracted using NucleoSpin columns (Macherey-Nagel) and tested for integrity by gel electrophoresis (Experion, Bio-

Rad). RNA quantity was determined on a NanoDrop 2000 Spectrophotometer (Thermo Scientific). RNA was combined with oligo DT (Promega; 0.5 µg), dNTP (Promega; 10 mM), and random nucleotide hexamers (0.5 µg; Promega, Fitchburg, Wisconsin, USA). This mixture was incubated at 65°C for 5 minutes and then put on ice. Complementary DNA synthesis from mRNA was carried out using Superscript III (40 µg/µL RNAseOUT, SuperScriptIII 200 µg/µL, 0.1M DTT; Promega). The reactions were proceeded at 25°C for 5 minutes and 50°C for 60 minutes. Reactions were terminated by increasing the temperature to 70°C for 5 minutes.

PCR assays were carried out using iQ™ SYBR® Green Supermix (Bio-Rad) on a Viia7 Real-time PCR system (Applied Biosystems, Foster City, California, USA). The PCR program consisted of 10 minutes at 95 °C, followed by 40 cycles of 15 seconds at 95 °C and 1 minute at 60 °C. Levels of mRNA transcripts for HLA-G were normalized to the geometric mean signal of reference genes GAPDH and β-actin. The forward (ACCCACTCCTCCACCTTTGAC) and reverse (TCCACCACCCTGTTGCTGTAG) primer for GAPDH; the forward (ACCACACCTTCTACAATGAG) and reverse (TAGCACAGCCTGGATAGC) primer for beta-actin; the forward (GACAGCGACTCGGCGT) and reverse (GTGTTCCGTGTCTCCTCT) primer for HLA-G were ordered from Sigma.

We also studied miRNA levels in the tissue homogenates. For this, RNA template was reverse transcribed into cDNA using the miRCURY LNA™ Universal RT miR PCR kit (Exiqon, Vedbaek, Denmark). LNA™ enhanced primer sets were used targeting the following miRNAs of interest: hsa-miR-148a (MIMAT0000243), hsa-miR-148b (MIMAT0000759), hsa-miR-152 (MIMAT0000438), and hsa-miR-365 (MIMAT0000710). Levels of these miRNAs were normalized to the geometric mean signal of previously described reference genes hsa-miR-16 (MIMAT0000069) and hsa-miR-103 (MIMAT0000101) [17, 18].

All PCR reactions were performed in duplicate. Signals were normalized using the ΔΔCq method. Quantitative PCR measurements were analyzed using QuantStudio Real-Time PCR System Software (Applied Biosystems). To verify the accuracy of amplification, melting curve analyses were performed at the end of each PCR run.

Statistical analysis

Spearman's correlation analysis and Bland–Altman plotting were performed for the assessments of validity and reproducibility [19]. Differences between groups were tested by Mann-Whitney U tests, chi-square tests or logistic regression analysis. Values of $P < 0.05$ were considered to indicate statistical significance. Association between HLA-G SNPs and RM was studied with binary logistic regression. Per HLA-G genotype the highest prevalence was defined as the reference group. Alleles with a frequency of <5% were excluded from analysis. For the calculations on the HLA-G genotypes Bonferroni adjustment was used to correct for multiple comparisons. Observed heterozygosity in both groups was computed by the direct counting method. Adherences of genotypic proportions to expectations under Hardy–Weinberg equilibrium were tested separately for each SNP using the PyPop 0.7.0 software (California, USA) [20]. Statistical analyses were performed using GraphPad Prism version 7.02 for Windows (GraphPad Software, California, USA) and SPSS Statistics 23 (IBM SPSS Software, New York, USA).

Results

Patient characteristics

Characteristics of the RM group and control group are listed in Table 1. Groups did not differ in maternal age and gestational age (GA) at delivery. As expected, the women in the RM group had fewer previous live born children compared to the

control women (P < 0.001). Of the RM group, 65.2% had no children, compared to 19.6% in the control group.

Table 1. Subject characteristics.

	Pregnancy after RM (n=23)	Uneventful pregnancy (n=46)	P-value*
Maternal age at time of index pregnancy in years	34 (22-39) #	33 (20-41) #	0.548
Gestational age at time of birth in weeks	39 (37-41) #	39 (37-42) #	0.109
Gravidity at time of index pregnancy	5 (4-9)#	3 (1-7) #	<0.001
Parity at time of index pregnancy	0 (0-2) #	1 (0-5) #	<0.001
Number of previous miscarriages	4 (3-7) #	0 (0-1) #	<0.001

*Mann-Whitney U Test; # median, min-max

HLA-G polymorphisms and haplotypes

We analyzed multiple SNPs to distinguish eight haplotypes of the 3-UTR in exon 8 of HLA-G. All genotyped SNPs fit the Hardy–Weinberg expected proportions in both groups of women and in their offspring (Supplementary Tables S1 and S2). No differences in frequency for individual SNPs or in haplotype distribution was found between groups (Supplementary Tables S3 and S4), except for the 14-bp indel polymorphism. We found a higher frequency of HLA-G 14-bp ins/del heterozygotes in RM women (65.2%) as compared to control women (36.4%) (OR 3.28; P = 0.026) and a lower del/del genotype (17.4% compared to 43.2%) (OR 0.28; P = 0.039), whereas the frequencies of ins/ins genotype are very similar in both groups (17.4% vs. 20.5%) (Table 2). Nevertheless the allelic frequencies of deletion and insertion do not differ significantly between RM and controls (Table 2). The 14-bp insertion is known to influence mRNA stability *[21]*, resulting in lower HLA-G expression [13]. The children in both groups did not differ in frequency of individual SNPs (Supplementary Table S6), haplotypes (Supplementary Table S5) and 14-bp indel (Table 3).

Table 2. The 14-bp insertion/deletion in the 3'UTR region of HLA-G in the women with a history of RM and the control groups.

	RM women (n=23)		Control women (n=44)*		OR	95% CI	P-value$
Genotype frequency							
Del/Del	4	17.4%	19	43.2%	**0.28**	**0.08-0.95**	**0.039**
Ins/Del	15	65.2%	16	36.4%	**3.28**	**1.14-9.43**	**0.026**
Ins/Ins	4	17.4%	9	20.5%	0.82	0.22-3.01	0.810
Phenotype frequency							
Ins phenotype	19	82.6%	25	56.8%	**3.61**	**1.05-12.38**	**0.039**
Del phenotype	19	82.6%	35	79.6%	1.22	0.33-4.50	0.810
Allele frequency							
Insertion	23	50.0%	54	38.6%	1.59	0.77-3.26	0.205
Deletion	23	50.0%	54	61.4%	0.63	0.31-1.29	0.205

*In 2 control subjects the 14bp ins/del could not be defined (4%).
$Chi-square.
OR, odds ratio; 95% CI, 95% confidence interval; del, deletion; ins, insertion.

Table 3. The 14-bp insertion/deletion in the 3'UTR region of HLA-G in the offspring of the group with a history of RM and the control group.

	RM offspring (n=23)		Control offspring (n=45)*		OR	95% CI	P-value$
Genotype frequency							
Del/Del	8	34.8%	16	34.0%	0.97	1.33-2.77	0.969
Ins/Del	11	47.8%	22	46.8%	0.96	0.35-2.62	0.936
Ins/Ins	4	17.4%	7	14.9%	1.14	0.30-4.39	0.789
Phenotype frequency							
Ins phenotype	15	65.2%	29	61.7%	1.03	0.36-2.97	0.969
Del phenotype	19	82.6%	38	84.4%	0.88	0.23-3.36	0.789
Allele frequency							
Insertion	19	43.3%	36	40.0%	1.06	0.51-2.17	0.875
Deletion	27	58.7%	54	60.0%	0.95	0.46-1.95	0.875

*In 1 control subject the 14bp ins/del could not be defined (2%).
$Chi-square.
OR, odds ratio; 95% CI, 95% confidence interval; del, deletion; ins, insertion.

Placental HLA-G expression is elevated in women with history of RM

Trophoblasts were stained in the term placentas by means of immunohistochemistry with an anti-cytokeratin antibody (CAM5.2) (Fig. 1a). On the sequential slides an antibody recognizing the free heavy chain of all HLA-G isoforms (MEM-G2) was applied (Fig. 1b). Expression of HLA-G was confined to the cytokeratin positive cells in the decidua basalis, as determined by double staining experiments by immunofluorescence (Supplementary Fig. S2). We annotated the decidual part of the placental tissues and quantified the extent of staining within these annotations. Spearman's correlation coefficient of the inter-observer reproducibility for our approach of quantitation was r-0.79. In the Bland–Altman plot of inter-observer measurements (Supplementary Fig. S3), most of the values ranged within a mean ± two SD, meaning that the reproducibility of the measurement is acceptable *[19]*.

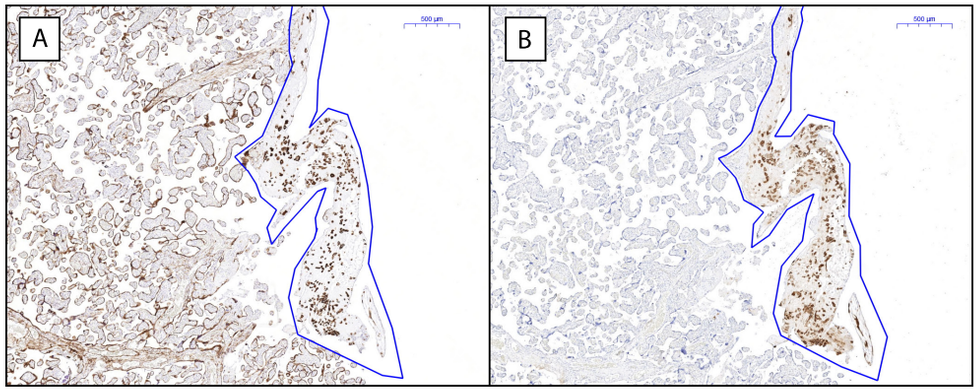

Figure 1. Expression of trophoblast cell marker and HLA-G in term placenta. Representative examples of staining for (a) trophoblasts with cytokeratin marker CAM5.2 and (b) all HLA-G iso forms with marker MEM-G2. Decidual parts of the placenta were annotated to specify the area for analysis.

No significant difference was observed in the extent of trophoblast staining between groups (Fig. 2a). However, the extent of decidual HLA-G protein expression was elevated in the placentas of women with a history of RM (median 32.6%) compared to the control group (median 21.9%, P < 0.0001) (Fig. 2b).

HLA-G expression was similar in placentas of women who gave birth to their firstborn compared to women who already had a successful previous pregnancy (Supplementary Fig. S4). Using the median expression in the controls, the RM subjects were divided into either low or high HLA-G protein expression groups (Table 4). From RM cases, 87.0% belonged to the high HLA-G protein expression group (OR 6.67, 95% CI: 1.74-25.57; P = 0.006).

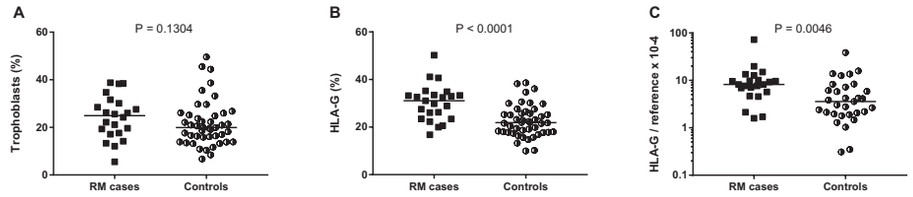

Figure 2. (a) Percentage positivity for trophoblast staining. No difference was observed in trophoblast staining between women with a history of RM and controls. (b) Percentage positivity for HLA-G staining. A higher HLA-G protein expression was observed in the decidual part of the placenta of women with a history of RM compared to controls. (c) HLA-G mRNA expression was measured in the placentas of women with a history of RM and controls. HLA-G mRNA expression was increased in term placenta of women with a history of RM compared to controls.

Table 4. HLA-G protein expression in the placentas of women with a history of RM and controls.

	RM women (n=23)		Control women (n=44)*		OR	95% CI	P-value
Low HLA-G protein expression	3	13.0%	23	50.0%	**6.67**	**1.74-25.57**	**0.006$**
High HLA-G protein expression	20	87.0%	23	50.0%			

$logistic regression. OR, odds ratio; 95% CI, 95% confidence interval.

To verify the differences observed at the protein level, we analyzed the mRNA expression of HLA-G in homogenates of term placentas from both groups. For this, we developed primers targeting exon 2 and 3 of the HLA-G gene, so all HLA-G isoforms were recognized. To verify that the primers only recognize HLA-G, and not HLA-C, their specificity was checked by sequencing of the amplicons. The mean Cq value for all placentas was 25.2 ± 2.0 (range 21-33), indicating expression well above background. Similar to what was observed for HLA-G protein expression,

the placentas of women with a history of RM had a 2.3-fold higher HLA-G mRNA expression than women without a history of RM (median relative level 8.2 versus 3.6, P < 0.005) (Fig. 2c). RM subjects were divided into either low or high HLA-G mRNA expression groups (Table 5). From RM cases, 86.4% belonged to the high HLA-G mRNA expression group (OR 6.33, 95% CI: 1.56-25.71; P = 0.010). No correlation was found between maternal and fetal HLA-G genotype with HLA-G expression.

Table 5. HLA-G mRNA expression in the placentas of women with a history of RM and controls.

	RM women (n=22)*		Control women (n=32)*		OR	95% CI	P-value
Low HLA-G mRNA expression	3	13.6%	16	50.0%	**6.33**	**1.56-25.71**	**0.010$**
High HLA-G mRNA expression	19	86.4%	16	50.0%			

*In 1 RM case (4%) and 12 control subjects (26%) mRNA expression could not be defined.
$logistic regression. OR, odds ratio; 95% CI, 95% confidence interval.

We wondered whether the higher placental HLA-G expression in the RM group was accompanied by a lower level of miRNAs. Members of the miR-148 family, and miR152 and miR-365 have been identified to target the 3'UTR of HLA-G *[22, 23]*. Cq values for miR-148a, miR148b and miR-152 ranged between 16 and 29. Cq values for miR-365 ranged between 25 and 39. After normalization for two reference miRNAs, no difference was observed between groups in the levels of miR-152 and miR-365 (Supplementary Fig. S5). Placental miR-148a (P = 0.0009) and miR-148b levels (P = 0.0154) were higher in the RM group compared to controls. Thus, increased HLA-G expression in the RM group was not accompanied by decreased miRNA levels.

HLA-G expression in first trimester miscarriage material and elective abortions is similar

Since HLA-G protein expression was elevated in term placentas after successful pregnancies, we additionally analyzed HLA-G expression in first trimester placentas, using the same antibody for immunohistochemistry. To this aim, we collected first trimester miscarriage of patients with a history of RM and elective abortion material and stained slides for HLA-G. Since the decidua could not be clearly defined in this early material, HLA-G positive region were selected to semi-quantitatively scored the extent of protein staining. The average score for each group is shown in Fig. 3. No difference in HLA-G protein expression was found between early miscarriages and elective abortions.

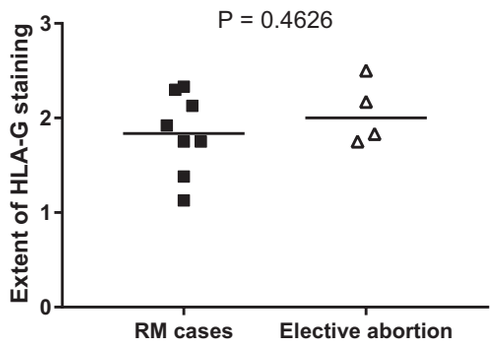

Figure 3. Amount of HLA-G staining in first trimester miscarriage and elective abortion material. HLA-G positive parts in the EVT regions of the placental slides were scored to be (1) minimally, (2) moderately or (3) intensely stained.

Discussion

In this study we investigated the HLA-G genotype and HLA-G mRNA and protein expression in term placentas of women with a history of RM and of women with no such history. A homogenous well-defined case group of women with at least three consecutive unexplained RM within 20 weeks of gestation was included. A

lower frequency of the HLA-G 3'UTR 14-bp deletion genotype was observed in the case group, suggesting that genetic predisposition to a low level of HLA-G played a role in the etiology of previous RM. In the current successful pregnancies, a significantly higher HLA-G protein and mRNA expression was found in the placenta of the RM group compared to the control group.

The most studied polymorphism in exon 8 of 3-UTR of the HLA-G gene is the 14 bp indel polymorphism, which has been associated with altered HLA-G expression. The insertion genotype is associated with low levels of sHLA-G [13]. In addition, the fetal 14-bp ins/ins genotype has been associated with lower surface expression of HLA-G on first trimester trophoblast cells than the 14-bp del/del genotype [24]. We did not find any differences in fetal HLA-G 3'UTR haplotypes or individual SNPs between both groups. However, we found a higher frequency of HLA-G 14-bp ins/del heterozygotes in RM women (65.2%) as compared with control women (39.1%), and a lower frequency of HLA-G 14 bp del/del homozygotes (17.4% and 43.2%, respectively). This is consistent with some studies [25-27], but not others [28, 29]. Since several studies have focused on the HLA-G 14-bp polymorphism in RM with controversial or inconclusive results, Wang *et al* performed a meta-analysis [30], which suggested that the HLA-G 14-bp insertion allele was associated with increased risk of RM. In 2014, yet another meta-analysis indicated that there was only an association between the HLA-G 14-bp indel polymorphism and RM in patients with three or more miscarriages [31]. In the present study, we have not addressed polymorphisms in the HLA-G promotor region, but they may be associated to RM, as recently shown [32].

Both the individual SNPs and the most common extended 3-UTR haplotypes of HLA-G were studied in the group of women with a history of RM and controls. HLA-G haplotype distribution and frequencies of individual SNPs in the 3-UTR region of HLA-G were neither significantly different between the groups of women, nor in their offspring (Supplementary Table S3-6). Studies have shown

that individual SNPs in the 3-UTR region of HLA-G are not significantly associated with RM, but that the UTR-4 haplotype seemed to be protective against RM [33, 34]. Similarly we observed a lower incidence of the HLA-G UTR-4 haplotype in women with RM (10.9% in RM women vs 15.9% in control women) (Supplementary Table S3). Remarkably, the HLA-G UTR-4 haplotype was more frequently present in the offspring of women with RM than in the offspring of controls (21.7% vs 13.3% respectively) (Supplementary Table S5) and less frequently in miscarriage material from women with RM (10%, data not shown). Even though these results were not statistically significant, possibly due to limited sample size, collectively they support the idea that this haplotype might have a protective effect in uncomplicated pregnancy.

HLA-G in the placenta is suggested to play a role in the induction of immunological tolerance at the fetal-maternal interface, by functioning as a trophoblast-restricted inhibitory ligand of maternal immune cells. Only a few studies have focused on HLA-G protein expression in the placentas of women with a history of RM, with contradicting results [35, 36]. Remarkably, the present immune-histochemical analysis of term placentas of successful pregnancies showed a significantly higher HLA-G protein expression in women with a history of RM compared to controls, although this RM group had a lower frequency of the 14 bp del/del genotype. This is not in line with results previously found in peripheral blood [25, 37] and suggests that local regulation is involved. HLA-G was mostly confined to the trophoblast areas at the fetal-maternal interface (decidua basalis), as determined by double label immunofluorescence experiments (Supplementary Fig. S2), and the level of HLA-G expression was independent of previous pregnancies.

Since the level of HLA-G expression can depend on the differentiation status of EVTs, as determined by *in vitro* studies using isolated primary trophoblasts [38], it is unclear whether the observed differences in HLA-G expression are a direct consequence of transcriptional regulation or a secondarily of an altered

differentiation status of the EVTs. Possibly, for a successful pregnancy to occur after previous RM, a compensatory mechanism resulting in high HLA-G protein expression is in place. When comparing first trimester miscarriage material of women with a history of RM and material of elective abortions, we did not observe a difference in HLA-G expression between both groups, suggesting that successful pregnancy in women with a history of RM is due to high fetal HLA-G expression in the current pregnancy. Besides HLA-G, other molecules and immune interactions may be involved in the immune-regulation leading to successful pregnancy.

We found a higher miR-148a and miR-148b expression in the term placentas of the RM group despite the elevated HLA-G expression. Apparently, either these miRNAs do not bind or binding does not result in post-transcriptional repression of HLA-G. This leads us to hypothesize that the higher HLA-G protein expression in the RM group may be the result of an epigenetically-regulated compensatory mechanism to achieve an ongoing pregnancy in patients with a history of RM. Alternatively, the higher HLA-G protein expression in the case group may be an epiphenomenon resulting from the previous miscarriages. It is possible that the elevated HLA-G in the term placentas of women with RM is the result of proteolytic cleavage of the membrane bound HLA-G1 isoform resulting from activity of metalloproteases, leading to elevated sHLA-G levels. The antibody recognizing MEMG2 in our immune-histochemical assays does not distinguish membrane bound HLA-G from soluble HLA-G. Previous miscarriages could lead to increased metalloprotease (MMP) levels [39], which in turn lead to increased proteolytic shedding of HLA-G1 [11]. MMP2 and MMP9 mRNA expression was not elevated in term placentas of women with a history of RM compared to controls (Supplementary Fig. S6), but this does not fully exclude the involvement of MMPs since their activity was not tested in the current setting.

In conclusion, whereas women with RM have a genetic predisposition to lower HLA-G levels, HLA-G expression is increased in the placenta of ongoing pregnancies after RM. This implies that HLA-G upregulation could be a compensatory mechanism in the occurrence of RM to achieve an ongoing pregnancy. Whether the higher HLA-G expression in the ongoing pregnancy after RM is a cause or a consequence of the successful pregnancy remains to be established. Future studies should be concentrated on further establishing the role of HLA-G in complicated pregnancies. Measurement of maternal sHLA-G may provide further insight on the prognosis of the outcome of pregnancies in women with a history of RM.

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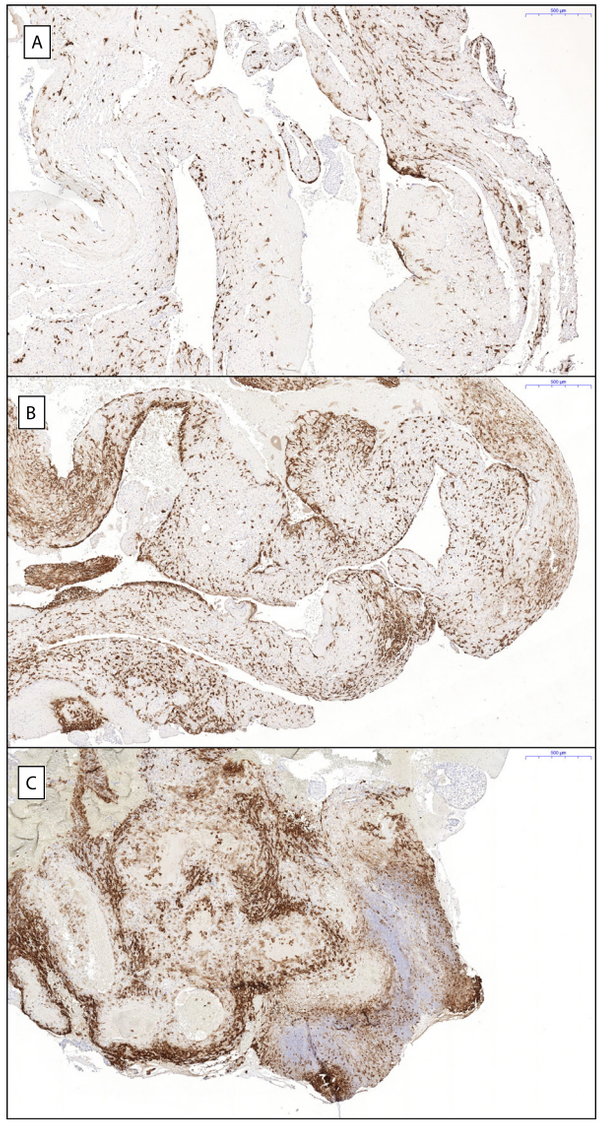
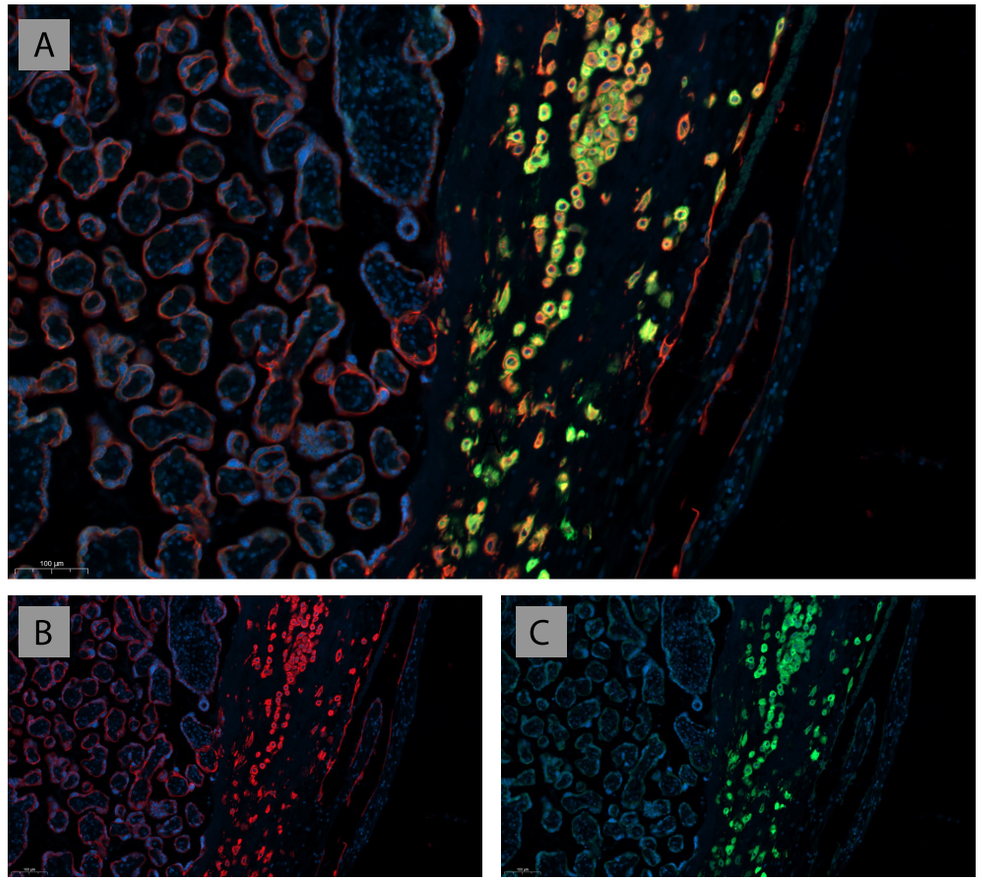
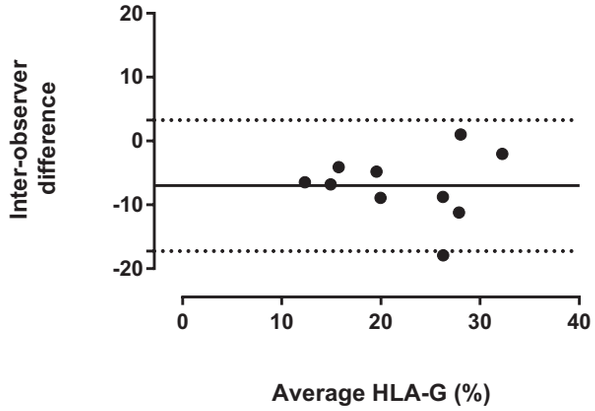


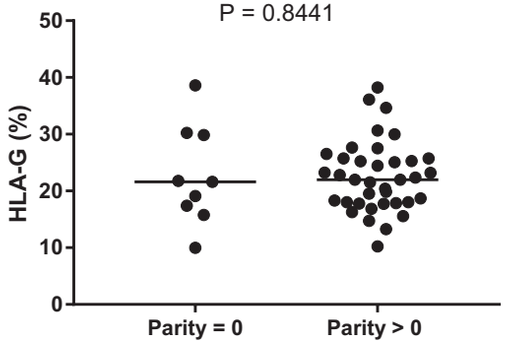
Supplementary Figure S1. Examples of minimal (a), moderate (b), and intense (c) HLA-G staining in first trimester placenta.



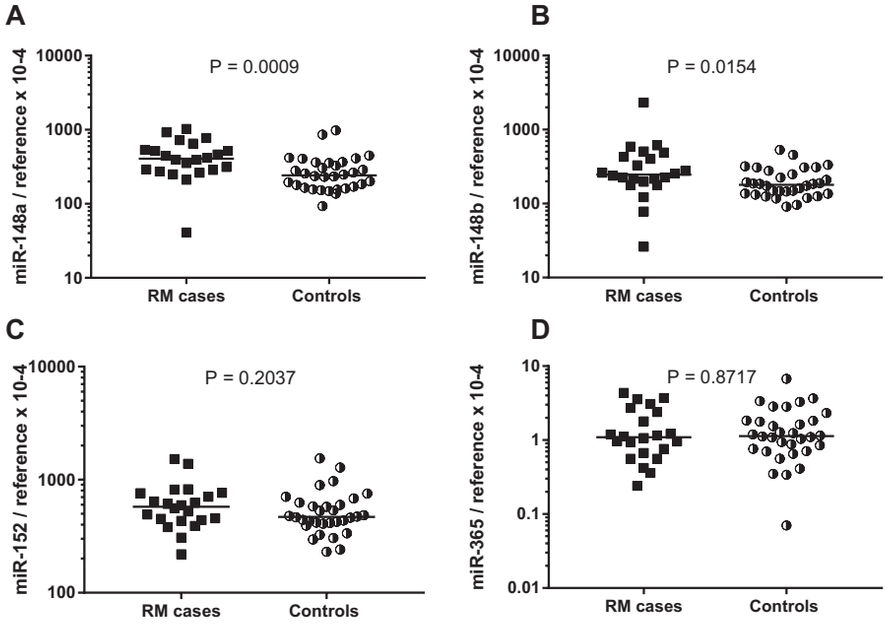
Supplementary Figure S2. (a) Cytokeratin 8 (CAM5.2, red) and HLA-G (MEM-G2, green) colocalize in the decidua; yellow in merged image indicates overlap of red and green labels. (b) CAM5.2 stains all trophoblasts in the placenta. (c) MEM-G2 staining is limited to the extravillous trophoblasts in the decidual part of the placenta.



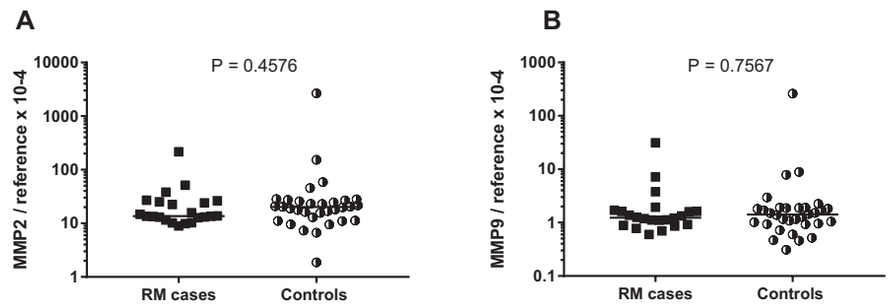
Supplementary Figure S3. Bland-Altman plot of interobserver measurements of HLA-G staining. Ninety percent of the values ranged within a mean ±2 SD deviations, indicating acceptable reproducibility.



Supplementary Figure S4. HLA-G expression in placentas of healthy first pregnancies compared to subsequent pregnancies. Previous pregnancies did not influence placental HLA-G expression in the control group with uncomplicated pregnancies.



Supplementary Figure S5. miRNA expression in term placentas of women with a history of RM and controls. (a-b) miR-148a and miR-148b expression was elevated in placenta of women with a history of RM compared to controls. No difference in miRNA expression was seen for (c) miR-152 and (d) miR-365 (statistics: Mann-Whitney test).



Supplementary Figure S6. MMP2 and MMP9 mRNA expression in term placentas of women with a history of RM and controls. (a) MMP2 mRNA expression was similar between women with a history of RM and controls. (b) MMP9 mRNA expression was similar between women with a history of RM and controls (statistics: Mann-Whitney test).

Supplementary Table S1. Hardy-Weinberg analyses for HLA-G 3'UTR genotypes in women with RM and controls.

SNP	*Recurrent miscarriage (n=23)*			*Controls (n=46)*		
	common *P*	homozygote *P*	heterozygot *P*	common *P*	homozygote *P*	heterozygot *P*
14-bp	0.1444	0.3020	0.3020	0.1220	0.3119	0.2870
+3003	#	0.9370	&	0.5888	0.7548	0.6054
+3010	0.7171	0.7637	0.7526	0.1379	0.2975	0.2953
+3027	#	0.9653	&	0.8602	0.9474	0.8626
+3035	0.6481	0.7965	0.6619	0.7823	0.9045	0.7874
+3142	0.7171	0.7637	0.7526	0.2322	0.3997	0.3987
+3187	0.5487	0.6508	0.5836	0.3077	0.3077	0.2440
+3196	0.4238	0.5347	0.4688	0.2315	0.3908	0.2679
+3422	0.9250	0.9520	0.9292	0.5149	0.7170	0.5321
+3496	#	0.8996	&	0.5888	0.7548	0.6054
+3509	0.5855	0.6588	0.6280	0.2938	0.4428	0.3332

All Hardy-Weinberg analyses. *P*, p value. # Too many parameters for chi-square test. & Too few cases to calculate p-value.

Supplementary Table S2. Hardy-Weinberg analyses for HLA-G 3'UTR genotypes in the RM and control offspring.

SNP	*Recurrent miscarriage (n=23)*			*Controls (n=46)*		
	common *P*	homozygote *P*	heterozygot *P*	common *P*	homozygote *P*	heterozygot *P*
14-bp	0.9577	0.9647	0.9637	0.9011	0.9341	0.9314
+3003	0.9471	0.9644	0.9504	0.8976	0.9458	0.9013
+3010	0.2058	0.2920	0.2883	0.3181	0.4854	0.4820
+3027	#	0.9653	&	#	0.9877	&
+3035	0.7224	0.8528	0.7319	0.7009	0.8533	0.7094
+3142	0.2921	0.4575	0.4567	0.8841	0.9179	0.9179
+3187	0.8147	0.8691	0.8275	0.7358	0.7894	0.7617
+3196	0.5487	0.6508	0.5836	0.7862	0.8389	0.8038
+3422	0.4882	0.6574	0.5128	0.9110	0.9424	0.9161
+3496	0.9471	0.9644	0.9504	0.8976	0.9458	0.9013
+3509	0.5487	0.6508	0.5836	#	0.8150	0.7861

All Hardy-Weinberg analyses. *P*, p value. # Too many parameters for chi-square test. & Too few cases to calculate p-value.

Supplementary Table S3. Haplotypes of women in the RM group and the control group.

	RM women 2n=46		***Controls 2n=88****		**OR**	**95% C.I.**		**P**
						Lower	**Upper**	
UTR-1	13	28.3%	28	31.8%	0.90	0.413	1.965	0.792
UTR-2	16	34.8%	23	26.1%	1.60	0.742	3.451	0.231
UTR-3	5	10.9%	8	9.1%	1.28	0.394	4.159	0.681
UTR-4	5	10.9%	14	15.9%	0.68	0.229	2.019	0.487
UTR-5	4	8.7%	4	4.5%	2.10	0.499	8.789	0.312
UTR-7	3	6.5%	7	8.0%	0.85	0.209	3.440	0.817
UTR-8	0	0.0%	0	0.0%	x	x	x	x
UTR-18	0	0.0%	3	3.4%	0.00	0.000		0.999
UTR-N	0	0.0%	1	1.1%	0.00	0.000		1.000
	46	100%	88	100%				

All univariate logistic regression analysis. *P*, *p* value; *OR*, odds ratio; *95% CI*, 95% confidence interval; n.a, not applicable. *In 2 control subjects the UTR haplotype could not be defined (4%). The 3'UTR haplotype nomenclature is consistent with publication by Castelli *et al*.

Supplementary Table S4. HLA-G 3'UTR genotypic polymorphisms in women with recurrent miscarriage and uneventful pregnancy.

		RM women (n=23)		*Controls (n=44)*		*OR*	*95% CI*	*P*	*Pc*
3003	**CC**	0	0.0%	2	4.5%	n.c.			
	CT	4	17.4%	10	22.7%	0.67	0.19-2.45	0.549	1.000
	TT	19	82.6%	32	72.7%	ref.			
3010	**CC**	8	34.8%	12	27.3%	0.94	0.30-3.01	0.923	1.000
	CG	12	52.2%	17	38.6%	ref.			
	GG	3	13.0%	15	34.1%	0.28	0.07-1.20	0.087	1.000
3027	**AA**	0	0.0%	0	0.0%	n.c.			
	AC	3	13.0%	6	13.6%	0.95	0.22-4.21	0.946	1.000
	CC	20	87.0%	38	86.4%	ref.			
3035	**CC**	16	69.6%	36	81.8%	ref.			
	CT	7	30.4%	8	18.2%	1.97	0.61-6.36	0.258	1.000
	TT	0	0.0%	0	0.0%	n.c.			
3142	**CC**	3	13.0%	14	31.8%	0.32	0.08-1.36	0.124	1.000
	CG	12	52.2%	18	40.9%	ref.			
	GG	8	34.8%	12	27.3%	1.00	0.32-3.17	1.000	1.000
3187	**AA**	11	47.8%	23	52.3%	ref.			
	AG	11	47.8%	14	31.8%	1.64	0.57-4.48	0.362	1.000
	GG	1	4.3%	7	15.9%	0.30	0.03-2.74	0.285	1.000
3196	**CC**	10	43.5%	27	61.4%	ref.			
	CG	12	52.2%	12	27.3%	2.70	0.92-7.95	0.071	1.000
	GG	1	4.3%	5	11.4%	n.c.			
3422	**CC**	15	65.2%	33	75.0%	ref.			
	CT	7	30.4%	9	20.5%	1.71	0.54-5.46	0.365	1.000
	TT	1	4.3%	2	4.5%	n.c.			
3496	**AA**	0	0.0%	2	4.5%	n.c.			
	AG	5	21.7%	10	22.7%	0.89	0.26-3.01	0.850	1.000
	GG	18	78.3%	32	72.7%	ref.			
3509	**GG**	9	39.1%	26	59.1%	ref.			
	GT	12	52.2%	13	29.5%	2.67	0.90-7.94	0.078	1.000
	TT	2	8.7%	5	11.4%	1.16	0.19-7.04	0.875	1.000

Data are all n (%). All univariate logistic regression analysis. Per HLA-G genotype the highest prevalence was defined as the reference group. If percentages in a group were below 5%, no calculations were performed. *P*, *p* value; *Pc*, *p* value corrected for multiple comparisons; *OR*, odds ratio; *95% CI*, 95% confidence interval; n.c, not calculated; ref, reference group.

Supplementary Table S5. Haplotypes of the offspring in the RM group and the control group.

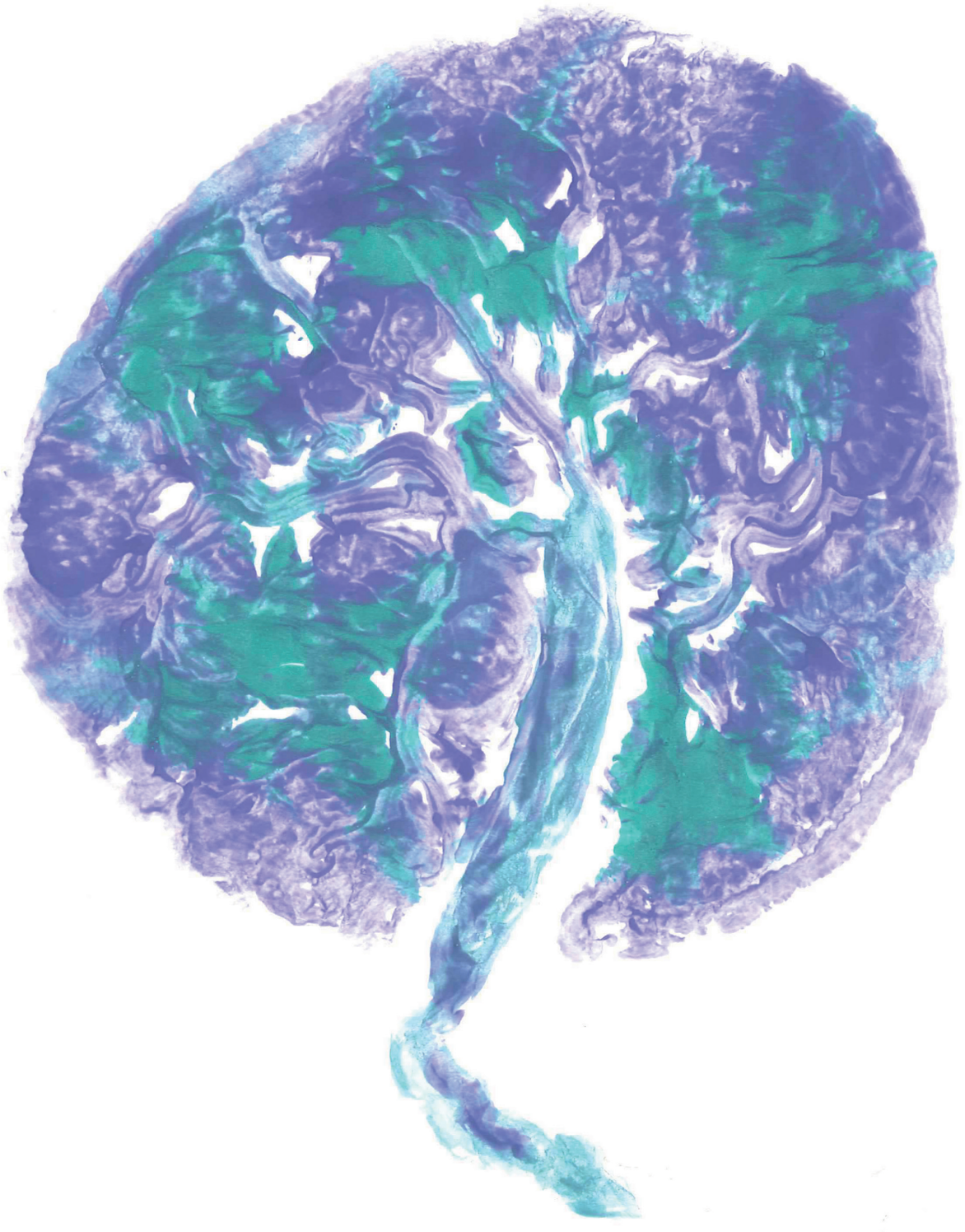
	RM offspring 2n=46		*Controls 2n=90**		OR	95% C.I.		P
						Lower	Upper	
UTR-1	11	23.9%	28	31.1%	0.70	0.309	1.566	0.381
UTR-2	13	28.3%	26	28.9%	0.97	0.441	2.131	0.939
UTR-3	6	13.0%	11	12.2%	1.08	0.371	3.125	0.891
UTR-4	10	21.7%	12	13.3%	1.81	0.714	4.565	0.212
UTR-5	3	6.5%	7	7.8%	0.83	0.204	3.360	0.791
UTR-7	3	6.5%	3	3.3%	2.02	0.392	10.446	0.400
UTR-8	0	0.0%	1	1.1%	0.00	0.000		1.000
UTR-18	0	0.0%	2	2.2%	0.00	0.000		0.999
UTR-N	0	0.0%	0	0.0%	x	x	x	x
	46	100%	90	100%				

All univariate logistic regression analysis. *P*, *p* value; *OR*, odds ratio; *95% CI*, 95% confidence interval; n.a, not applicable. *In 1 control subject the UTR haplotype could not be defined (2%). The 3'UTR haplotype nomenclature is consistent with publication by Castelli *et al*.

Supplementary Table S6. HLA-G 3'UTR genotypic polymorphisms in the offspring of women with recurrent miscarriage and uneventful pregnancy.

		RM offspring (n=23)		*Controls (n=45)*		*OR*	*95% CI*	*P*	*Pc*
3003	**CC**	1	4.3%	1	2.2%	n.c.			
	CT	8	34.8%	10	22.2%	1.94	0.63-5.95	0.245	1.000
	TT	14	60.9%	34	75.6%	ref.			
3010	**CC**	5	21.7%	11	24.4%	0.58	0.16-2.02	0.389	1.000
	CG	15	65.2%	19	42.2%	ref.			
	GG	3	13.0%	15	33.3%	0.25	0.06-1.04	0.057	0.969
3027	**AA**	0	0.0%	0	0.0%	n.c.			
	AC	3	13.0%	3	6.7%	2.10	0.39-11.34	0.389	1.000
	CC	20	87.0%	42	93.3%	ref.			
3035	**CC**	17	73.9%	35	77.8%	ref.			
	CT	6	26.1%	10	22.2%	1.24	0.38-3.97	0.722	1.000
	TT	0	0.0%	0	0.0%	n.c.			
3142	**CC**	5	21.7%	11	24.4%	0.71	0.20-2.50	0.598	1.000
	CG	14	60.9%	22	48.9%	ref.			
	GG	4	17.4%	12	26.7%	0.52	0.14-1.95	0.335	1.000
3187	**AA**	13	56.5%	20	44.4%	ref.			
	AG	9	39.1%	21	46.7%	0.66	0.23-1.88	0.436	1.000
	GG	1	4.3%	4	8.9%	n.c.			
3196	**CC**	11	47.8%	24	53.3%	ref.			
	CG	11	47.8%	17	37.8%	1.41	0.50-4.00	0.516	1.000
	GG	1	4.3%	4	8.9%	n.c.			
3422	**CC**	14	60.9%	29	64.4%	ref.			
	CT	9	39.1%	14	31.1%	1.33	0.47-3.82	0.594	1.000
	TT	0	0.0%	2	4.4%	n.c.			
3496	**AA**	1	4.3%	1	2.2%	n.c.			
	AG	8	34.8%	10	22.2%	1.94	0.63-5.95	0.245	1.000
	GG	14	60.9%	34	75.6%	ref.			
3509	**GG**	11	47.8%	23	51.1%	ref.			
	GT	11	47.8%	17	37.8%	1.35	0.48-3.85	0.571	1.000
	TT	1	4.3%	4	8.9%	0.52	0.05-5.25	0.581	1.000
	CT	0	0.0%	1	2.2%	n.c.			

Data are all n (%). All univariate logistic regression analysis. Per HLA-G genotype the highest prevalence was defined as the reference group. If percentages in a group were below 5%, no calculations were performed. *P*, *p* value; *Pc*, *p* value corrected for multiple comparisons; *OR*, odds ratio; *95% CI*, 95% confidence interval; n.c, not calculated; ref, reference group.



Craenmehr, M. H. C.; Haasnoot, G. W.; Drabbels, J. J. M.; Spruyt, M. J.; Cao, M.; van der Keur, C.; Kapsenberg, J. M.; Uyar-Mercankaya, M.; van Beelen, E.; Meuleman, T.; van der Hoorn, M. L. P.; Heidt, S.; Claas, F. H. J.; Eikmans, M., HLA 2019, 94, (4), 339-346.

5

soluble human leukocyte antigen (HLA)-G levels in seminal plasma are associated with HLA-G 3'UTR genotypes and haplotypes

Abstract

Soluble HLA-G (sHLA-G) levels in human seminal plasma (SP) can be diverse and may affect the establishment of maternal-fetal tolerance and thereby the outcome of pregnancy. We investigated whether sHLA-G levels in SP are associated with polymorphisms in the 3'-untranslated region (UTR) and UTR haplotypes of the *HLA-G* gene. Furthermore, we compared the *HLA-G* genotype distribution and sHLA-G levels between men, whose partner experienced unexplained recurrent miscarriage (RM), and controls.

Soluble HLA-G levels (n=156) and *HLA-G* genotyping (n=176) were determined in SP samples. The concentration of sHLA-G was significantly associated with several single nucleotide polymorphisms (SNPs): the 14 base pair (bp) insertion/deletion (indel), +3010, +3142, +3187, +3196, and +3509. High levels of sHLA-G were associated with UTR-1 and low levels with UTR-2, UTR-4 , and UTR-7 ($P<0.0001$).

HLA-G genotype distribution and sHLA-G levels in SP were not significantly different between the RM group (n=44) and controls (n=31).

In conclusion, seminal sHLA-G levels are associated with both singular SNPs and 3'UTR haplotypes. *HLA-G* genotype and sHLA-G levels in SP are not different between men whose partner experienced RM and controls, indicating that miscarriages are not solely the result of low sHLA-G levels in SP. Instead it is more likely that these miscarriages are the result of a multifactorial immunologic mechanism, whereby the *HLA-G* 3'UTR 14 bp ins/ins genotype plays a role in a proportion of the cases. Future studies should look into the functions of sHLA-G in SP and the consequences of low or high levels on the chance to conceive.

Introduction

Semen contains various immunomodulatory factors, such as chemokines and cytokines [1], but also soluble human leukocyte antigens (sHLA), which together can induce a local immune response in an immune regulatory environment [2]. The presence of seminal plasma (SP) in the female reproductive tract after coitus can lead to an influx of immune cells, e.g. the number of CD14+ macrophages and CD1a+ dendritic cells were shown to be approximately two-fold increased upon semen exposure [3]. Immune recognition of paternal antigens may play a role in pregnancy complications: change of partner is a risk factor for intrauterine growth restriction, preterm birth, low birth weight and infant mortality, and it counteracts the protective effect of multiparity against preeclampsia [4-6]. Additionally, the length of unprotected sexual cohabitation affects the incidence of pregnancy-induced hypertensive disorders [7, 8], and oral exposure to semen is correlated with a diminished occurrence of preeclampsia [2]. Furthermore, preeclampsia occurs more frequently in pregnancies induced by artificial insemination with donor semen [9]. Combined, these findings indicate that exposure to paternal antigens prior to gestation has a beneficial effect on pregnancy outcome. Besides the classical HLA antigens, SP contains soluble HLA-G (sHLA-G) [10]. Compared to classical HLA, *HLA-G* shows a low level of polymorphism, and does not have a major role in antigen presentation. The primary function of HLA-G lies most probably in regulating immune functions through interaction with receptors on various immune cell subsets [11]. Whereas HLA-G can inhibit the cytotoxic function of both NK cells and $CD8^+$ T cells, it is also involved in the induction of immunoregulatory antigen presenting cells and $CD4^+$ T cells [12-14]. Furthermore, the presence of sHLA-G has been shown to be beneficial for the success rate of assisted reproduction techniques [15].

The level of sHLA-G in body fluids appears to be related to specific polymorphisms in the 3'-untranslated region (3'UTR) of the *HLA-G* gene. The 14 base pair (bp)

insertion/deletion (indel) polymorphism has shown to be associated with sHLA-G levels in blood and semen [16, 17]. Furthermore, the G/C at position +3142 and the G/A at position +3187, which are involved in microRNA (miRNA) mediated post-transcriptional regulation, seem to influence sHLA-G levels in blood [18, 19]. Other SNPs, such as +3003 T/C, +3010 G/C, +3027 C/A and +3035 C/T have been proposed as potential miRNA binding sites [20], but they have not been studied extensively in relation to sHLA-G levels. At least eight polymorphisms together make up UTR haplotypes [21]. UTR haplotypes containing the 14 bp deletion (i.e., UTR-1) are associated with high sHLA-G levels in blood plasma, whereas those with the 14 bp insertion (i.e., UTR-7) are associated with low sHLA-G levels [22].

Although several studies have demonstrated associations between *HLA-G* 3'UTR polymorphic sites and sHLA-G concentration, these were solely focused on sHLA-G concentrations in blood plasma [22]. The association between the 14 bp indel polymorphic site and sHLA-G levels was previously evaluated in SP [17], but the full 3'UTR region was not included. Here we assess for the first time the correlation between sHLA-G levels in semen samples with the sequence of multiple *HLA-G* 3'UTR variation sites determining extensive haplotypes. Low levels of HLA-G in women have been associated with recurrent miscarriage (RM) [23, 24], but the effect of sHLA-G in semen on RM has not been studied. Additionally, we studied sHLA-G levels in SP of couples with a history of RM, with the aim to determine whether aberrant sHLA-G levels in SP could be an explanation for these couples experiencing RM.

Materials and Methods

Study samples

Semen samples were obtained from 156 men visiting the reproductive medicine clinic at the Leiden University Medical Center (LUMC). Of these, 101 semen samples were obtained from men visiting the *in vitro* fertilization (IVF) clinic. SP samples were collected via masturbation and samples containing leukocytes, as a marker for infection, were excluded from this study. Forty-four samples were collected from men enrolled in a study of couples with a history of RM. These couples had experienced at least three miscarriages, for which the cause remained unknown after a full clinical work-up at the reproductive medicine clinic at the LUMC. Blood collected from men of RM couples was used for *HLA-G* genotyping. As a control group, we collected blood and semen samples from men, who fathered at least one live birth and did not have a history of RM. We obtained 31 unique blood samples from these controls and 11 unique semen samples. Within four hours after collection, semen samples were centrifuged at 2,000 rpm for 10 min, sperm cells were discarded and aliquots of SP were stored at −80°C.

HLA-G genotype determination

HLA-G genotype determination has previously been described [25]. In short, genomic DNA was isolated from blood or from SP, when blood was not available. The 699/713-bp fragment covering the 3'UTR of exon 8 was sequenced, starting just before the 14 bp insertion/deletion and ending 591 bp downstream of the insertion/deletion. To sequence the haplotype on each of the two alleles, amplification reactions were performed using the generic 3'-primer that was tailed with a M13 sequence to cover the 3'UTR region of *HLA-G*. The following polymorphisms were identified: the 14 bp insertion/deletion (rs371194629), +3003C>T (rs1707), +3010G>C (rs1710), +3027C>A (rs17179101), +3035C>T (rs17179108), +3142C>G (rs1063320), +3187A>G (rs9380142), +3196C>G (rs1610696), +3422C>T (rs17875408), +3496A>G (rs1233330), and +3509G>T (rs1611139).

UTR haplotypes were composed based on the combination of eight SNPs. Nomenclature was used according to Castelli *et al.* [21]. In case the combination of SNPs could not fit any of the established UTR haplotypes, these samples were categorized as UTR-N. Conversion of sequencing data to UTR haplotypes was carried out by using a specialized HLA interpretation software tool (SBT Engine, GenDX, Utrecht, the Netherlands). The forward primer (GTGATGGGCTGTTTAAAGTGTCACC), the reverse primer (GACGTTGTAAAACGACGGCCAGTAGGGGAAGAGGTGTAGGGGTCTG) and the M13 universal primer (GACGTTGTAAAACGACGGCCAGT) were ordered from Sigma (St. Louis, Missouri, USA). The underlining represents the M13 sequence.

Soluble HLA-G determination

For sHLA-G determination, samples were thawed at room temperature and centrifuged at 14,000 rpm for 4 min. The level of soluble HLA-G1/HLA-G5 molecules in the plasma samples was determined by a commercially available sandwich enzyme linked immunosorbent assay (ELISA) (EXBIO, Praha, Czech Republic) according to the manufacturer's instructions. This ELISA specifically detects soluble HLA-G1 and HLA-G5 in a β2-microglobulin-associated form. The limit of detection was 0.6 units/mL. The standard curve ranged from 3.9 to 125 units/ml. Samples were tested in the assay at 1:5 and 1:10 dilution, using dilution buffer 1 of the kit. Subsequently, samples were measured at different dilutions to remain in the linear part of the standard curve (ranging from 1:2 to 1:100).

Samples were run in duplicate and mean absorbance was measured at 450 nm wavelength using a BIO-RAD Microplate Reader and Microplate Manager 6 software (Hercules, California, USA). Calculations were done according to the manufacturer's guidelines. Standard curves based on the absorbance of calibrators of known concentrations were used for the determination of sHLA-G concentration in the samples of interest. Results were expressed as units/mL.

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 7.02 for Windows (GraphPad Software, California, USA) and SPSS Statistics 23 (IBM SPSS Software, New York, USA). Normality of distribution was examined with D'Agostino & Pearson normality test. Differences between groups were tested by Mann-Whitney U tests or Chi-square tests. P-values of <0.05 were considered to indicate statistical significance. Spearman's correlation coefficient (r) was used to demonstrate the relationship between the volume of the ejaculate and the sHLA-G concentration. Distribution of genotype frequencies among groups was tested by a Kruskal-Wallis test. The association between the presence of specific *HLA-G* genotypes in RM or healthy controls semen samples was studied with binary logistic regression. For each *HLA-G* genotype the highest prevalence was defined as the reference group. If percentages in a group were below 5%, no calculations were performed. For the calculations on the *HLA-G* genotypes, Dunn's post hoc test was used to correct for multiple comparisons. Observed heterozygosity in both groups was computed by the direct counting method. Adherences of genotypic proportions to expectations under Hardy–Weinberg equilibrium were tested separately for each SNP using PyPop 0.7.0 software (California, USA) [26].

Results

HLA-G genotype and distributions

We analyzed multiple SNPs to distinguish *HLA-G* 3-UTR haplotypes. All genotyped SNPs fitted the Hardy–Weinberg (HWE) expected proportions, except for +3003 and +3010 (Supplementary Table 1). When these HWE analyses were performed

for the three groups separately, only the IVF group did not fit the HWE analysis for the +3010, whereas the other two groups did (Supplementary Table 2).

sHLA-G levels in seminal plasma are associated with HLA-G 3'UTR haplotype and HLA-G 3'UTR polymorphisms

Soluble HLA-G levels did not fit a Gaussian distribution (P<0.0001) and therefore we used non-parametric statistical tests. Median sHLA-G levels for all *HLA-G* 3'UTR haplotypes can be found in Table 1. For some controls, only the *HLA-G* 3'UTR genotype was determined, but we did not have SP samples to determine sHLA-G concentrations ("missing" in Table 1). The level of sHLA-G was not influenced by the volume of the ejaculate (Supplementary Figure 2).

Table 1. sHLA-G in seminal plasma per haplotype.

	n	Missing	%	Median	Minimum	Maximum
UTR-01	93	13	26.4	639.4	93.0	4988.0
UTR-02	92	10	26.1	102.5	23.6	1799.1
UTR-03	43	7	12.2	255.9	62.6	2408.0
UTR-04	64	7	18.8	132.4	23.0	3917.3
UTR-05	11	0	3.1	256.8	108.0	1838.8
UTR-06	2	0	0.6	1582.6	909.6	2255.6
UTR-07	23	2	6.5	159.0	58.2	1769.5
UTR-08	1	0	0.3	206.5	206.5	206.5
UTR-10	2	0	0.6	202.8	108.0	297.7
UTR-18	4	0	1.1	2578.6	1848.0	4642.7
UTR-N	17	1	4.8	425.8	23.0	4642.7
Total	312	40	100%			

Since with heterozygous 3'UTR haplotypes combinations (diplotypes) it is unclear which haplotypes has the most dominant influence on sHLA-G levels, we analyzed homozygous samples. Homozygous haplotypes showed significant differences between UTR-1, UTR-2, UTR-3, UTR-4, and UTR-7 (P<0.0001) (Figure 1). Dunn's post hoc test showed that sHLA-G levels between UTR-1 (median: 639.4 units/mL) and UTR-2 (median: 102.5 units/mL; P<0.0001) and between UTR-1 and UTR-4 (median: 132.4 units/mL; P=0.0377) were significantly different after correction for multiple comparisons.

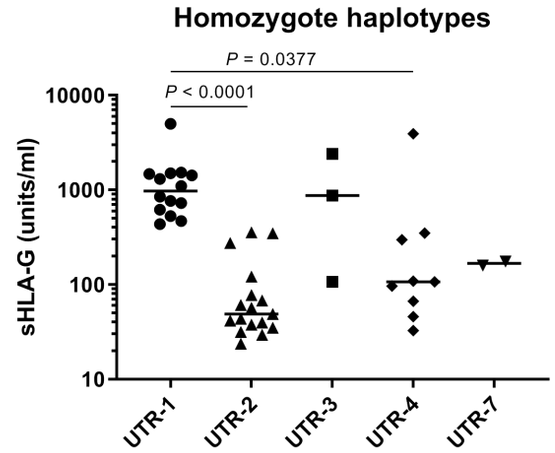


Figure 1. sHLA-G levels in SP for separate homozygous haplotypes. sHLA-G levels in SP are significantly different for the homozygous *HLA-G* 3'UTR haplotypes UTR-1, UTR-2, UTR-3, UTR-4 and UTR-7 (P<0.0001). Dunn's post hoc test showed that sHLA-G levels in UTR-1 and UTR-2 (P<0.0001) and UTR-1 and UTR-4 (P=0.0377) were significantly different after correcting for multiple comparisons.

To evaluate whether specific SNPs are involved in differences in sHLA-G levels per haplotype, we analyzed sHLA-G levels for SNPs separately. The concentration of sHLA-G in SP samples was significantly associated with the 14 bp ins/del, +3003 C/T, +3010 C/G, +3142 C/G, +3187 A/G, +3196 C/G, +3496 A/G and +3509 G/T polymorphic sites in the 3'UTR part of the *HLA-G* gene (Figure 2A-F and Supplementary Table 3).

The 14 bp del/del genotype showed the highest level of sHLA-G, and the 14 bp ins/ins genotype showed the lowest sHLA-G level (P<0.0001). Furthermore, individuals

with +3142 CC (median: 776.7 units/mL), +3196 CC (median: 443.0 units/mL), +3010 GG (median: 619.5 units/mL), +3187 GG (median: 973.1 units/mL), +3496 GG (median: 359.7 units/mL) and +3509 GG (median: 436 units/mL) showed higher sHLA-G levels than individuals with +3142 GG (median: 153.5 units/mL, P<0.0001), +3196 GG (median: 56.74 units/mL, P<0.0001), +3010 CC (median: 182.9 units/mL, P=0.0013), +3187 AA (median: 121.6 units/mL, P<0.0001), +3496 AA (median: 81.39 units/mL, P=0.0095), and +3509 TT genotypes (median: 56.74 units/mL, P<0.0001), respectively. Dunn's post hoc test for multiple comparisons showed significant differences for all these polymorphisms, except for +3003 and +3496 (Figure 2A-F).

Analysis of the IVF group and the RM group separately showed similar associations between *HLA-G* genotype and sHLA-G levels, although significance for several SNPs was lost after multiple comparisons due to small samples sizes (data not shown). The group with fertile controls was too small for separate analysis.

sHLA-G levels in seminal plasma of RM group and controls

To evaluate whether differences in *HLA-G* genotype and sHLA-G levels could be found for semen samples of men, whose partner experienced RM, we analyzed groups separately. No differences in frequency for individual SNPs (Supplementary Table 4) or in haplotype distribution (Table 2) were found between semen samples from the RM group and semen samples from controls. However, although not significant, the frequency of the 14 bp ins/ins genotype, which was associated with low levels of sHLA-G, was three times higher in the RM group than in controls (18% vs. 6%, P=0.137). The median concentration of sHLA-G was 269.7 units/mL in all SP samples compared to 233.8 units/mL in SP samples from men with a history of RM and 297.3 units/mL in SP samples of healthy controls (Table 3). The levels of sHLA-G were not significantly different between the RM group and controls (Supplementary Figure 1).

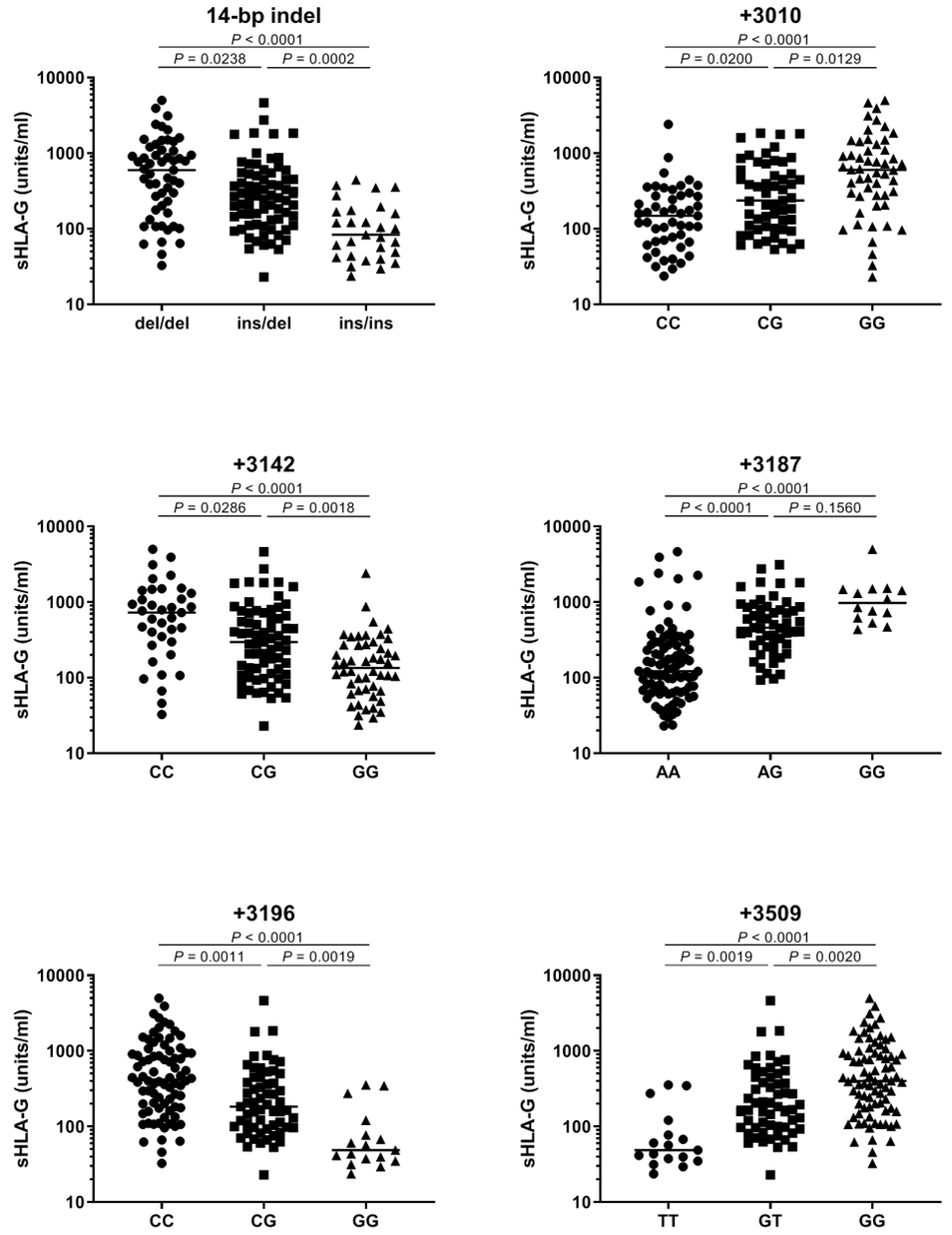


Figure 2. The sHLA-G concentration in SP samples is associated with several SNPs of the 3'UTR part *HLA-G* gene. The concentration of sHLA-G in seminal plasma samples is significantly associated with (a) 14 bp ins/del, (b) +3010 C/G, (c) +3142 C/G, (d) +3187 A/G, (e) +3196 C/G and (f) +3509 G/T polymorphic sites in the *HLA-G* 3'UTR after correction for multiple comparisons.

Table 2. HLA-G 3'UTR haplotype frequencies in RM semen samples and samples of fertile controls.

	RM (2n=88)	%	Fertile controls (2n=62)	%	OR	95% C.I. Lower	Upper	P-value
UTR-1	23	26.1%	17	27.4%	0.937	0.450	1.950	0.861
UTR-2	25	28.4%	17	27.4%	1.050	0.509	2.169	0.894
UTR-3	12	13.6%	8	12.9%	1.066	0.408	2.784	0.897
UTR-4	18	20.5%	15	24.2%	0.806	0.370	1.755	0.587
UTR-5	4	4.5%	0	0%	*Inf.*	0.000	*Inf.*	*0.999*
UTR-7	3	3.4%	3	4.8%	0.694	0.135	3.558	0.662
UTR-N	3	3.4%	2	3.2%	1.059	0.172	6.531	0.951

All univariate logistic regression analyses. *P*, *p* value; *OR*, odds ratio; *95% CI*, 95% confidence interval; The 3'UTR haplotype nomenclature is consistent with publication by Castelli et al.

Table 3. sHLA-G levels in semen samples.

	All (n=176)	IVF (n=101)	RM (n=44)	Fertile controls (n=11)
Missing	20	0	0	20
Median	269.67	271.38	233.77	297.26
Mean	546.93	600.59	477.67	331.18
Std. deviation	794.02	872.29	684.92	211.53
Minimum	23.03	23.03	27.43	100.50
Maximum	4988.79	4988.79	3917.29	851.32

Discussion

In this study, we showed an association of sHLA-G levels with HLA-G 3'UTR haplotypes, as well as with singular SNPs. Furthermore, there was no significant difference in HLA-G genotype and sHLA-G levels in semen between men whose partner had a history of RM and controls.

When comparing genotype frequencies to expected HWE frequencies for each group, we observed that the IVF group deviates from HWE for the +3010 polymorphism, whereas the other two groups fit. Since the samples in this study group are not from healthy controls, this could indicate that this SNP may play a role in conception, but additional research is required to draw any conclusions.

Regarding HLA-G 3'UTR haplotypes we found five haplotypes exhibiting frequencies higher than 5% (UTR-1, UTR-2, UTR-3, UTR-4, UTR-7) and five others with lower frequencies (UTR-5, UTR-6, UTR-8, UTR-10, UTR-18). Some combinations of SNPS did not fit any of the established UTR haplotypes and were therefore categorized as UTR-N. In line with previous data [27], UTR-1 and UTR-2 were the most frequently observed haplotypes. We reported the frequencies of eleven polymorphic sites: 14 bp ins/del, +3003C/T, +3010C/G, +3027A/C, +3035C/T, +3142C/G, +3187A/G, +3196C/G, +3422C/T, +3496A/G and +3509G/T. The most studied polymorphism of the 3-UTR of the HLA-G gene is the 14 bp indel polymorphism, which has been associated with altered HLA-G expression. We observed that individuals exhibiting the 14 bp del/del genotype indeed exhibited higher sHLA-G levels in SP compared to the 14 bp ins/ins genotype. This is in line with other studies, describing the association between the 14 bp insertion allele and decreased levels of sHLA-G in blood plasma and serum [16, 28, 29]. It is suggested that the insertion of 14 bases leads to removal of 92 bases from the start of exon 8, affecting mRNA stability and degradation rate [30].

Other SNPs at the 3-UTR, which are associated with HLA-G expression levels, are represented by the presence of guanine in the position +3142, which increses the affinity of specific miRNA for HLA-G mRNA, leading to decreased HLA-G expression [19]. Another SNP is represented by the presence of an adenine at position +3187, decreasing the stability of HLA-G mRNA [18]. Indeed, we did observe lower sHLA-G levels in the semen of individuals with +3142 GG or +3187 AA genotype. Of all haplotypes found in this study, the only haplotype presenting a guanine at position +3187 is UTR-1. Moreover, taking the possible effect of each of the known variation sites that may influence HLA-G production together, UTR-1

is theoretically the most suitable to produce high HLA-G amounts, because it is the only UTR that harbours the +3187 G allele, as well as the +3142 C and the 14 bp del. Indeed, in the present study UTR-1 was clearly associated with higher levels of sHLA-G. UTR-2 and UTR-7 both harbour the +3187 A allele, as well as the +3142 G and the 14 bp ins. In line with our expectations, these UTR haplotypes were associated with low HLA-G levels. Remarkably, the UTR-4 was generally associated with low sHLA-G levels, even though this haplotype harbours the 14 bp del and the +3142 C. It appears that the influence of adenine at position +3187 on sHLA-G levels is very strong or that another yet unknown factor influences the level of sHLA-G in these cases.

We found a higher incidence of the 14 bp ins/ins in men whose partner experienced RM (18%) compared to controls (6%), although this difference was not significant. Taking into account that this genotype is associated with lower sHLA-G levels, this may underline the concept that rather a multifactorial process accounts for miscarriage.

We were restricted to collecting one semen sample per man. Therefore, we were not able to analyze sHLA-G concentrations over time and we cannot exclude the possibility that sHLA-G levels in SP fluctuate over time.

In summary, we provided data on the impact of the most frequent HLA-G 3'UTR variation sites on sHLA-G levels in SP, and conclude that sHLA-G levels in SP are influenced by HLA-G haplotypes and separate SNPs. On the population level, we did not find differences in sHLA-G levels between SP samples from RM and controls, indicating that miscarriages cannot solely be explained by HLA-G genes and low sHLA-G levels in SP. Instead it is more likely that these miscarriages are the result of a multifactorial immunologic mechanism, in which the HLA-G 3'UTR 14 bp ins/ins genotype plays a role in a proportion of the cases. Future studies should look into the functions of sHLA-G in SP and the consequences of low or high levels on the chance to conceive.

Acknowledgments

We thank the fertility clinic of the Leiden University Medical Center for providing the SP samples.

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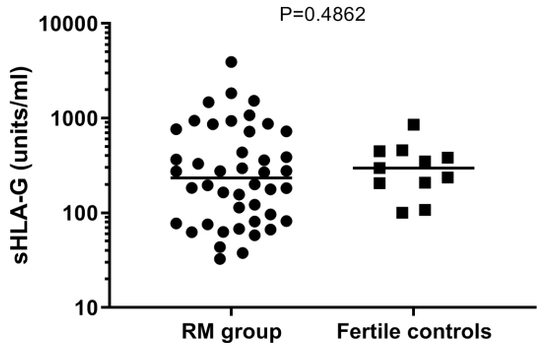
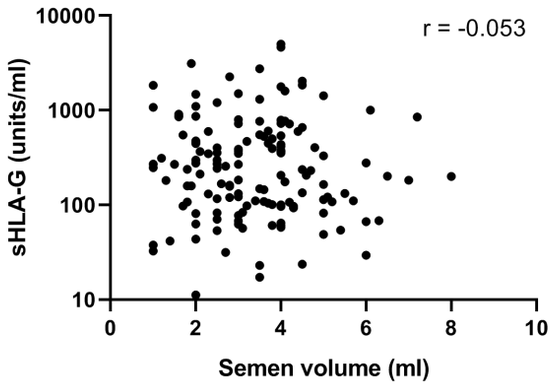


Supplementary Figure 1. sHLA-G concentrations in SP samples from RM cases and controls. sHLA-G concentrations in SP samples were not significantly different between men whose partner experienced RM (median 233.8 units/mL) and controls (median 297.3 units/mL).



Supplementary Figure 2. sHLA-G concentrations in relation to semen volumes (n=177). sHLA-G concentrations are not associated with semen volume (Spearman's r=-0.0533).

Supplementary Table 1. Hardy-Weinberg analyses for HLA-G 3'UTR genotypes in all semen samples.

	All (n=176)		
	common *P*	homozygotes *P*	heterozygotes *P*
14-bp	0.7658	0.8417	0.8361
+3003	**0.0252***	0.4339	0.2251
+3010	**0.0003***	**0.0107***	**0.0106***
+3027	0.6934	0.8753	0.6992
+3035	0.8336	0.9175	0.8388
+3142	0.1965	0.3651	0.3624
+3187	0.1841	0.5051	0.4060
+3196	0.2859	0.5639	0.4923
+3422	0.6747	0.8068	0.6886
+3496	**0.0341***	0.4519	0.2478
+3509	0.1960	0.4912	0.4081

All Hardy-Weinberg analyses. *P*, p value. * P < 0.05.

Supplementary Table 2. Hardy-Weinberg analyses for HLA-G 3'UTR genotypes in separate groups of semen samples.

	RM (n=44)			*Fertile controls (n=31)*			*IVF (n=101)*		
	common *P*	homozygotes *P*	heterozygotes *P*	common *P*	homozygotes *P*	heterozygotes *P*	common *P*	homozygotes *P*	heterozygotes *P*
14-bp	0.5129	0.6640	0.6506	0.3492	0.4518	0.4035	0.6077	0.7244	0.7193
+3003	0.2254	0.4280	0.2538	0.3560	0.5272	0.3886	0.8000	0.8857	0.8083
+3010	0.0704	0.2008	0.2008	0.8150	0.8704	0.8692	**0.0002**	**0.0093**	**0.0092**
+3027	#	0.9873	0.9936	#	0.9782	#	0.6666	0.8412	0.6753
+3035	0.7823	0.9045	0.7874	#	0.9606	#	0.7012	0.8333	0.7125
+3142	0.3801	0.5377	0.5358	0.5725	0.6917	0.6905	0.1707	0.3429	0.3361
+3187	0.6007	0.7020	0.6295	0.8374	0.8783	0.8507	0.1575	0.4778	0.3764
+3196	0.6611	0.7329	0.6916	0.4088	0.5379	0.4487	0.1035	0.3792	0.2950
+3422	0.8379	0.8965	0.8467	0.6237	0.7867	0.6376	0.5086	0.7076	0.5268
+3496	0.2254	0.4280	0.2538	0.4490	0.5925	0.482	0.8000	0.8857	0.8083
+3509	0.6611	0.7329	0.6916	0.4088	0.5379	0.4487	0.0519	0.3072	0.2159

All Hardy-Weinberg analyses. P, p value. # Too many parameters for chi-square test.

Supplementary Table 3. Comparisons of seminal plasma soluble HLA-G levels (units/ml) in the whole group, stratified according to the SNPs in the HLA-G 3' UTR.

Polymorphism		All		Soluble HLA-G levels	P-value
		(n=156)		Median	
14-bp indel	Del/Del	55	35%	597.6	**<0.0001[1]**
	Ins/del	72	46%	262.4	
	Ins/Ins	29	19%	83.24	
3003	C/C	9	6%	106.6	**0.0226[2]**
	C/T	39	25%	161.4	
	T/T	108	69%	351.9	
3010	C/C	49	31%	148.0	**<0.0001[3]**
	C/G	55	35%	235.9	
	G/G	52	33%	601.5	
3027	A/A	2	1%	167.4	0.8247
	A/C	21	13%	199.6	
	C/C	133	85%	274.0	
3035	C/C	121	78%	274.0	0.9849
	C/T	32	21%	250.3	
	T/T	3	2%	175.8	
3142	C/C	37	24%	725.3	**<0.0001[4]**
	C/G	69	44%	295.2	
	G/G	50	32%	134.9	
3187	A/A	89	57%	121.7	**<0.0001[5]**
	A/G	53	34%	458.0	
	G/G	14	9%	973.1	
3196	C/C	80	51%	420.2	**<0.0001[6]**
	C/G	59	38%	184	
	G/G	17	11%	48.87	
3422	C/C	112	72%	270.9	0.6918
	C/T	38	24%	269.7	
	T/T	6	4%	305.4	
3496	A/A	9	6%	106.6	**0.0226[7]**
	A/G	39	25%	161.4	
	G/G	108	69%	351.9	
3509	G/G	82	53%	402.6	**<0.0001[8]**
	G/T	57	37%	184.0	
	T/T	17	11%	48.87	

All Kruskal-Wallis tests.

[1-8] *P* values as determined by the Dunn's multiple comparisons test

[1] 14-pb

del/del vs. ins/del	**0.0238**
del/del vs. ins/ins	**<0.0001**
ins/del vs. ins/ins	**0.0002**

[2] +3003 C/T

CC vs. CT	>0.9999
CC vs. TT	0.1330
CT vs. TT	0.0950

[3] +3010 C/G

CC vs. CG	**0.0200**
CC vs. GG	**<0.0001**
CG vs. GG	**0.0129**

[4] +3142 C/G

CC vs. CG	**0.0286**
CC vs. GG	**<0.0001**
CG vs. GG	**0.0018**

[5] +3187 A/G

AA vs. AG	**<0.0001**
AA vs. GG	**<0.0001**
AG vs. GG	0.1560

[6] +3196 C/G

CC vs. CG	**0.0011**
CC vs. GG	**<0.0001**
CG vs. GG	**0.0019**

[7] +3496 A/G

AA vs. AG	>0.9999
AA vs. GG	0.1330
AG vs. GG	0.0950

[8] +3509 T/G

TT vs. GT	**0.0019**
TT vs. GG	**<0.0001**
GT vs. GG	**0.0020**

Supplementary Table 4. HLA-G 3'UTR SNPs in RM samples and fertile controls.

		RM (n=44)		Fertile controls (n=31)		OR	95% C.I. Lower	95% C.I. Upper	Sig.
14-bp indel	Ins	27	61%	19	61%				
	Del/Del	17	39%	12	39%	1.268	0.472	3.401	0.638
	Ins/del	19	43%	17	55%	ref			
	Ins/Ins	8	18%	2	6%	3.579	0.666	19.241	0.137
3003	C	14	32%	11	35%				
	C/C	4	9%	3	10%	0.889	0.179	4.404	0.885
	C/T	10	23%	8	26%	0.833	0.281	2.474	0.743
	T/T	30	68%	20	65%	ref			
3010	C	30	68%	22	71%				
	C/C	14	32%	6	19%	2.333	0.716	7.601	0.160
	C/G	16	36%	16	52%	ref			
	G/G	14	32%	9	29%	1.556	0.525	4.612	0.426
3027	A	3	7%	3	10%				
	A/A	0	0%	0	0%	n.c.			
	A/C	3	7%	3	10%	0.683	0.128	3.631	0.655
	C/C	41	93%	28	90%	ref			
3035	T	8	18%	4	13%				
	C/C	36	82%	27	87%	ref			
	C/T	8	18%	4	13%	1.500	0.409	5.503	0.541
	T/T	0	0%	0	0%	n.c.			
3142	C	30	68%	25	81%				
	C/C	11	25%	8	26%	1.230	0.401	3.776	0.717
	C/G	19	43%	17	55%	ref			
	G/G	14	32%	6	19%	2.088	0.655	6.652	0.213
3187	G	19	43%	15	48%				
	A/A	25	57%	16	52%	ref			
	A/G	15	34%	13	42%	0.738	0.279	1.952	0.541
	G/G	4	9%	2	6%				
3196	G	22	50%	16	52%				
	C/C	22	50%	15	48%	ref			
	C/G	17	39%	15	48%	0.773	0.297	2.009	0.597
	G/G	5	11%	1	3%	n.c.			
3422	T	15	34%	9	29%				
	C/C	29	66%	22	71%	ref			
	C/T	13	30%	9	29%	1.096	0.397	3.022	0.860
	T/T	2	5%	0	0%	n.c.			
3496	A	14	32%	12	39%				
	A/A	4	9%	3	10%	0.844	0.170	4.197	0.836
	A/G	10	23%	9	29%	0.704	0.242	2.048	0.519
	G/G	30	68%	19	61%	ref			
3509	T	22	50%	16	52%				
	G/G	22	50%	15	48%	ref			
	G/T	17	39%	15	48%	0.773	0.297	2.009	0.597
	T/T	5	11%	1	3%	n.c.			

All univariate logistic regression analyses. Per HLA-G genotype the highest prevalence was defined as the reference group. If percentages in a group were below 5%, no calculations were performed. OR, odds ratio; 95% C.I., 95% confidence interval; n.c., not calculated; ref, reference group.

Craenmehr, M. H. C.; van der Keur, C; Anholts, J. D. H.; Kapsenberg, H. M.; van der Westerlaken, L.A.J.; van Kooten, C; Claas, F. H. J.; Heidt, S.; Eikmans, M. Submitted to J Reprod Immunol 2020, 137, 103076.

6

effect of seminal plasma on dendritic cell differentiation *in vitro* depends on the serum source in culture medium

Abstract

Dendritic cells (DC) are key in shaping immune responses and are recruited to the human cervix after coitus by seminal plasma (SP). SP has been shown to skew the differentiation of monocyte-derived DC towards an anti-inflammatory profile when cultured in medium containing fetal calf serum (FCS). However, DC cultured in FCS show phenotypical differences when compared to those cultured in medium containing human serum (HS). Therefore, to create a setting more similar to the *in vivo* situations in humans, we tested the immune regulatory effect of SP on DC in cell cultures containing HS. We confirmed that SP skewed FBS-DC towards a tolerogenic profile. HS-DC cultured in the presence of SP showed increased CD14 and decreased CD1a gene expression, accompanied by an increased percentage of CD14+CD1a- cells. Both TGF-β and IL-10 gene expression were elevated in LPS matured SP-DC, the latter accompanied by increased protein expression. Whereas no effect on the pro-inflammatory cytokines IL-12b and TNF-α mRNA levels was found, IL-12p70 protein levels were decreased compared to control DC. Co-cultures of SP-DC or control DC with allogeneic PBMC did not show an effect of SP on proliferation or inflammatory cytokine production. SP can skew the differentiation of monocyte-derived DC cultured in HS towards alternatively activated DC. This immune regulatory phenotype appears to be less pronounced when compared to SP-treated DC cultured in FCS containing medium. These findings highlight the importance of the serum source used in SP treated cell cultures *in vitro*.

Introduction

Semen contains various immunomodulatory factors, such as chemokines, cytokines and prostaglandins, but also soluble HLA antigens, which can be recognized as foreign and evoke an immune response [1-4]. The presence of seminal plasma (SP) in the female reproductive tract after coitus can lead to an influx of immune cells [5]. Many studies have demonstrated that factors in human SP can suppress the function of several components of the immune system including T-cells, B-cells, natural killer (NK) cells, and the complement system [6-11]. Furthermore, Lenicov showed that SP can redirect the differentiation of human dendritic cells (DC) toward a regulatory phenotype [12]. DC are professional antigen presenting cells that have the ability to capture and present antigens to T cells, in particular for the stimulation of naïve T cells. They play a key role in inducing an active immune response as well as maintaining tolerance. DC differentiated from human monocytes in the presence of SP expressed low levels of CD1a and high levels of CD14, which are hallmarks of tolerogenic DC [13]. While these SP-DC showed increased expression of maturation markers HLA-DR and CD86, they were unable to develop a fully mature phenotype in response to lipopolysaccharides (LPS). Upon LPS treatment, SP-DC produced low amounts of the inflammatory cytokines IL-12p70, IL-1β, TNF-α, and IL-6 and elevated levels of the regulatory cytokines IL-10 and TGF-β compared to control DC.

Potent suppression of NK and T cell responses by SP components *in vitro* has been shown to be dependent on the addition of bovine serum factors [14]. NK cell mediated cytotoxicity against K562 targets was suppressed when the effectors were treated with SP in the presence of 10% fetal calf serum (FCS), but suppression was considerably less when the effectors were treated with SP in the presence of 10% autologous human plasma [14]. Furthermore, human SP has been shown to induce cytotoxic effects on lymphocytes in cultures containing FCS [10]. So far, the immunomodulatory effect of SP on human DC has only been studied in *in*

vitro cultures containing FCS. Previous studies showed that human DC cultured in medium containing FCS are different from those cultured in medium containing HS. HS cultured DC are described to be more granular and heterogeneous and have a decreased CD1a expression compared to FCS cultured DC [15-18]. Thus, studies showing an effect of SP on human DC in cultures with FCS may have resulted in physiologically less relevant conclusions. We therefore examined the effect of SP on human DC in cultures containing fetal bovine serum (FBS) or human serum (HS). A less profound effect on DC biology in HS containing cultures was found compared to cultures containing FBS .

Material and Methods

Semen samples

All semen samples were obtained from men visiting the fertility clinic at the Leiden University Medical Center (LUMC). SP samples were collected via masturbation. Sperm quality (semen volume, sperm density, motility, morphology and viscosity) was assessed the same day. Normozoospermic samples were selected using the WHO guidelines [19]. Within four hours after collection, semen samples were centrifuged at 2,000 rpm for 10 min, sperm cells were discarded and aliquots of SP were stored at −80°C. For addition to cell cultures, samples were thawed at room temperature and centrifuged at 14,000 rpm for 4 min.

In vitro generation of human dendritic cells

Human peripheral blood mononuclear cells (PBMC) were isolated by means of density gradient centrifugation (Ficoll separation solution, pharmacy LUMC) from buffy coats obtained from anonymous healthy donors (Sanquin Blood Supply, Amsterdam, the Netherlands) after informed consent. PBMC were washed three times with PBS and monocytes were purified using CD14-MicroBeads according to the manufacturer's protocol (Miltenyi Biotech, Bergisch Gladbach, Germany).

CD14+ cells were seeded in 12-well tissue culture plates (Corning Costar, Merck KGaA, Darmstadt, Germany) at a density of 1.5 x 10^6 cells per well in 1.5 ml. SP was added at the beginning of the culture at a final concentration of 1:1,000 (unless stated otherwise). Multiple semen samples were used in parallel for each experiment. CD14+ cells were cultured for 6 days in RPMI-1640 (Gibco, Thermo Fisher Scientific, Waltham, Massachusetts, United States) supplemented with 1% L-glutamine (Gibco, Thermo Fisher Scientific), 1% penicillin/streptomycin (Lonza, Basel, Switzerland) containing 500 U/ml recombinant human IL-4 (Gentaur, Kampenhout, Belgium), 800 U/mL recombinant human GM-CSF (Gibco, Thermo Fisher Scientific) and either 8% heat inactivated FBS (Merck) or 8% pooled inactivated HS. Whereas in most experiments CD14+ cells from one source were cultured in either FBS or HS, in a few experiments CD14+ cells from one donor were cultured in parallel in both FBS and HS. On day 3, culture medium including supplements was refreshed. On day 6, the resulting immature DC were either harvested or treated with 100 ng/mL LPS (Merck) for maturation. After 48 h, culture supernatants were harvested and frozen until further use, and activated DC were harvested for further analysis. Culture conditions were at 37°C in a humidified atmosphere in the presence of 5% CO_2.

Quantitative PCR

RNA was extracted using NucleoSpin® RNA spin columns (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. RNA quantity and integrity were determined on a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific). First strand cDNA was synthesized from 200 ng total RNA using Superscript III RT (Invitrogen, RT, 200 U/µl), dNTP (10 mM each; Promega, Madison, Wisconsin, USA), RNase OUT (40 U/ul; Invitrogen, Thermo Fisher Scientific), DTT (0,1

M; Invitrogen, Thermo Fisher Scientific), oligodT (OligodT 15, 0,5 ug/ul; Promega) and random nucleotide hexamers (0,5 ug/ul; Promega). Real-time quantitative PCR was performed using the Real Time PCR machine ViiA7 (Life Technologies, Carlsbad, California, USA) based on specific primers and general fluorescence detection with SYBR Green (BioRad). To control for sample loading and to allow for normalization between samples, *β-actin* and GAPDH were analysed. Primer sequences can be found in Table 1.

Table 1. Primer sequences.

	5'primer	3'primer
β-actin	ACCACACCTTCTACAATGAG	TAGCACAGCCTGGATAGC
GAPDH	ACCCACTCCTCCACCTTTGAC	TCCACCACCCTGTTGCTGTAG
CD14	AGCCTAGACCTCAGCCACAA	CTTGGCTGGCAGTCCTTTAG
CD1a	ATGGTATCTCCGCGCAAC	AAGCCCACGGAACTGTGAT
CD80	GAAGCAAGGGGCTGAAAAG	GGAAGTTCCCAGAAGAGGTCA
CD86	CGAGCAATATGACCATCTTCTG	CGCTTCTTCTTCTTCCATTTCC
HLA-DR	AATGGAGAGCACGGTCTG	TGTCCTTTCTGATTCCTGAAG
TGF-β	CCCAGCATCTGCAAAGCTC	GTCAATGTACAGCTGCCGCA
TNF-α	CCCCAGGGACCTCTCTCTAATC	TACAACATGGGCTACAGGCTTG
IL-10	GCGCTGTCATCGATTTCTTCC	GTAGATGCCTTTCTCTTGGAGCTTA
IL-12a	CCAGAGTCCCGGGAAAGTC	ACCAGGGTAGCCACAAGG
IL-12b	CCCTGACATTCTGCGTTCA	AGGTCTTGTCCGTGAAGACTCTA

Antibodies and flow cytometry

The following fluorochrome-conjugated antibodies (clone) for flow cytometry were used: HLA-DR (L243), CD1a (HI-149), CD14 (M5E2), CD80 (L307.4), and CD45 (HI30). All antibodies were obtained from BD Pharmingen (Becton Dickinson, Franklin Lakes, New Jersey, United States). Flow cytometric data was acquired on an LSR-II flow cytometer (Becton Dickinson) and analysed using FACS DIVA 8.0.2 (Becton Dickinson) and FlowJo 10.0.8 (Ashland, Oregon, United States) software.

Cell proliferation assay (T Cell Stimulation in vitro)

Mixed lymphocyte reactions (MLR) were performed in an allogeneic setting: PBMC (1 x 10^6 cells/ml) were co-cultured in triplicate wells with activated DC at a 1:10 ratio in RPMI supplemented with 8% FBS or HS. HLA typing of PBMC and DC was performed by SSO PCR technique using a reverse dot-blot method at the National Reference Laboratory for Histocompatibility Testing (Leiden University Medical Center, the Netherlands). The responders and stimulators were two HLA-DR antigen mismatched, in order to induce an alloantigen-specific immune response. PBMC were activated with 1 μg/ml phytohaemagglutinin (PHA) (Remel, San Diego, California, United States) as positive control. Cells were cultured in a 96-well round-bottomed plate for 5 days after which culture supernatants were harvested and frozen until further use. Cells were exposed to [3H]-thymidine (Pelkin Elmer, Waltham, Massachusetts, United States) during the last 18 h of culture after which [3H]-thymidine uptake was measured by using a liquid scintillation counter (Micro Beta Trilux 1450; Pelkin Elmer).

Cytokine analysis

Supernatants of DC cultures and MLR were analysed for the presence of cytokines using the Luminex-based Bio-Plex Pro™ Human Cytokine Th1/Th2 Assay (Bio-Rad Laboratories, Veenendaal, the Netherlands) following the manufacturer's instructions. Samples were analysed using a Bio-Plex™ Array Reader with Bio-Plex software (Bio-Rad). All samples were measured in duplicate. Additionally, we measured the cytokine levels in culture conditions without cells. These baseline cytokine values in the medium and/or SP were subtracted from the cytokine

production in the wells with cells.

Statistical analysis

Data are expressed as medians ± SD, unless stated otherwise. Data were analysed using the Wilcoxon matched-pairs signed rank test or the Mann-Whitney U test. Data were considered statistically significant when $p<0.05$.

Results

SP alters the phenotype and function of FBS cultured DC

In a first set of experiments, we confirmed that SP induced a change in gene expression in immature DC cultured in FBS containing medium. In these culture conditions, CD14 mRNA levels were increased and CD1a mRNA levels were decreased in SP-DC compared to control DC. Also, we observed that mRNA levels of CD86 and HLA-DR were upregulated in SP-DC compared to control DC (Figure 1A). Additionally, we confirmed that DC incubated with SP in FBS containing medium for six days showed an alternative phenotype by analysis of protein expression of CD14 and CD1a (Figure 1B-C) and that this effect of SP was dose-dependent (Figure 1D). Furthermore, HLA-DR surface expression was upregulated in SP-DC compared to control DC (Figure 1E).

Upon LPS maturation, gene expression levels of the anti-inflammatory cytokines IL-10 and TGF-β1 were increased in SP-DC compared to control DC, whereas gene expression of the pro-inflammatory cytokines IL-12b and TNF-α was decreased (Figure 1F). In line with these results and with published data, SP-DC showed increased IL-10 and decreased IL-12p70 protein production compared to control-DC (Figure 1G-H). Whereas TNF-α gene expression was marginally affected, we

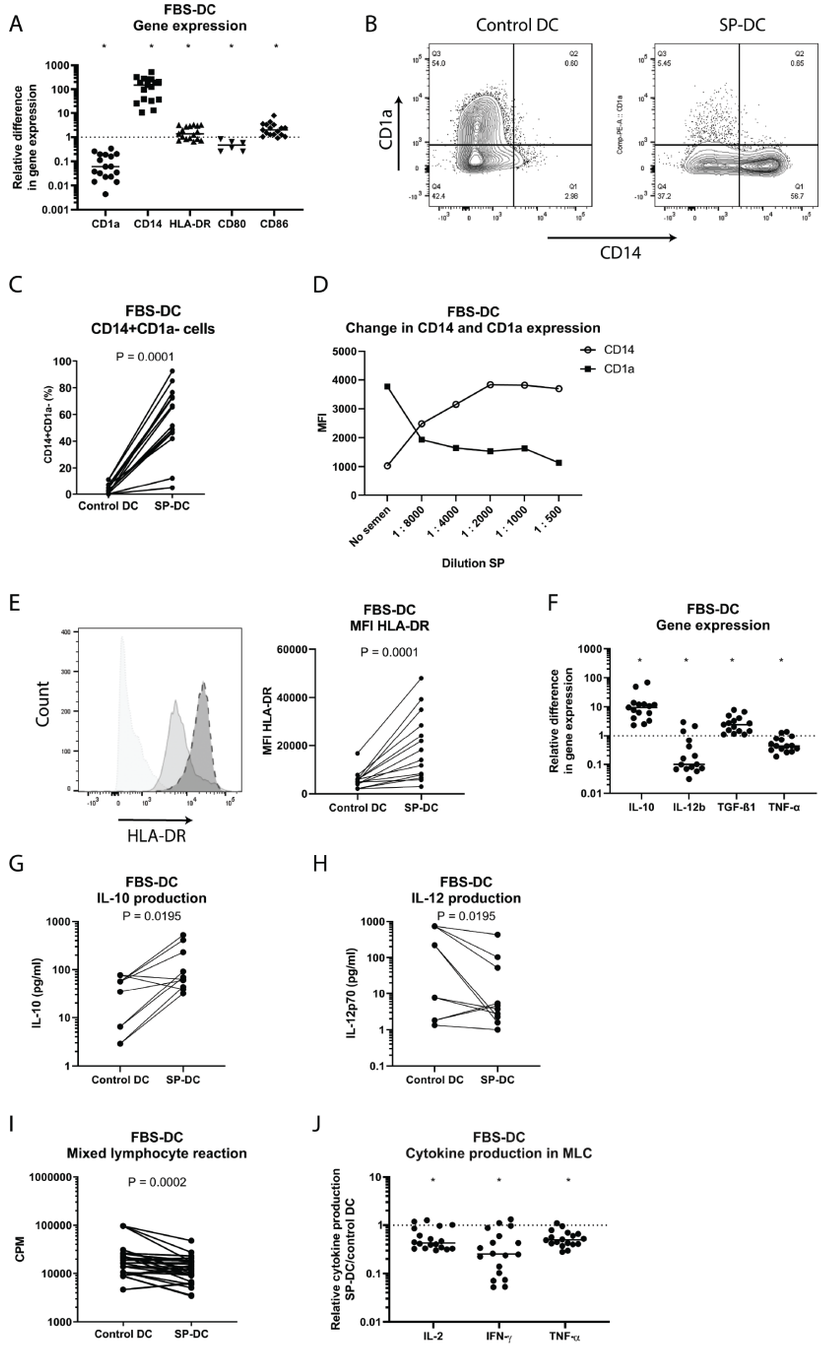


Figure 1. Phenotypical and functional characterization of dendritic cells (DC) cultured with fetal bovine serum (FBS) in the presence or absence of seminal plasma (SP).

(A) Difference in CD1a (median 0.06; P<0.0001), CD14 (median 150.80; P<0.0001), HLA-DR (median 1.40; P=0.0395), CD80 (median 0.47; P=0.0313) and CD86 (median 2.01; P<0.0001) mRNA expression between immature SP-DC and control DC (n=17; except CD80 n=6). Expression level in the control DC was set to 1 for each gene, and relative expression levels in SP-DC were compared with the control.

(B) CD14 and CD1a expression in immature DC cultured in the presence or absence of SP was analyzed by flow cytometry. Dot plots from a representative experiment (n=16) are shown.

(C) Percentage of CD14+CD1a- immature DC after culturing monocytes with or without SP (n=16).

(D) The effect of different concentrations of SP on CD14 and CD1a expression in immature DC. The more seminal plasma was added to the culture the more distinct the phenotype from control DC.

(E) Histogram from a representative experiment (n=14) is shown. HLA-DR mean fluorescence intensity (MFI) values in DC cultured with SP are compared to controls.

(F) Difference in IL-10 (median: 9.46; P<0.0001), IL-12b (median: 0.10; P=0.0015), TGF-β1 (median: 2.43; P<0.0001) and TNF-α (median: 0.43; P=0.0034) mRNA expression between mature SP-DC and control DC (n=15; except CD80 n=5). Expression level in the control DC was set as 1 for each gene, and relative expression levels in SP-DC were compared with the control.

(G) Cytokine production in DC culture upon stimulation with LPS (n=10). Mature SP-DC produced high levels of IL-10 (P=0.0195) compared to mature control DC (in pg/ml).

(H) Cytokine production in DC culture upon stimulation with LPS (n=10). Mature SP-DC produced low levels of IL-12p70 (P=0.0195) compared to mature control DC (in pg/ml).

(I) Proliferation of T cells stimulated in co-cultures with either control DC or SP-DC in counts per minute (CPM)(n=31). SP-DC had low stimulatory capacity compared to control DC (P=0.0002).

(J) Relative IL-2, IFN-γ and TNF-α production in the co-culture with SP-DC compared to the co-culture with control DC (n=28). Low amounts of IL-2 (median 0.51; P<0.0001), IFN-γ (median 0.42; P=0.0001) and TNF-α (median 0.54; P<0.0001) were found in co-cultures of PBMC with SP-DC compared to co-cultures with control DC. Cytokine level in the control DC was set to 1 for each cytokine, and relative cytokine levels in SP-DC were compared with the control. Absolute numbers of cytokine levels are depicted in Table 2.

did not observe a decreased TNF-α protein production in SP-DC compared to control DC (data not shown).

In line with these phenotypic and functional characteristics, SP-DC showed a decreased stimulatory capacity in co-cultures with allogeneic PBMCs, significantly affecting the proliferative potential op the responder cells compared to cultures with control DC (Figure 1I). Concomitantly, the production of pro-inflammatory cytokines IFN-γ, TNF-α, and IL-2 during co-culture was significantly decreased compared to control DC (Table 2; Figure 1J).

Table 2. IL-2, IFN-γ, and TNF-α production in co-cultures of PBMC with either SP-DC or control DC.

	IL-2			IFN-γ			TNF-α		
	Control DC	SP-DC		Control DC	SP-DC		Control DC	SP-DC	
FCS									
Median (pg/ml)	195	88	P<0.0001	311	76	P=0.0006	184	89	P<0.0001
Minimum (pg/ml)	44	38		29	12		43	25	
Maximum (pg/ml)	535	369		9975	12018		3381	1655	
Nr of values	28	28		28	28		28	28	
HS									
Median (pg/ml)	46	38	P=0.9697	83	63	P=0.2036	1154	1209	P=0.4697
Minimum (pg/ml)	19	21		18	9		262	199	
Maximum (pg/ml)	63	99		504	404		1724	1918	
Nr of values	12	12		12	12		12	12	

SP alters the phenotype of HS cultured immature DC

To determine whether SP had similar effects on DC in HS containing cultures, we analysed CD14, CD1a, CD86, and HLA-DR gene expression in the treated cells. Similar to FBS cultured SP-DC, CD14 and HLA-DR mRNA expression was increased and CD1a mRNA expression was decreased in immature SP-DC compared to control DC, albeit to a lesser extent. In contrast, SP did not affect CD80 or CD86 mRNA in this culture condition (Figure 2A).

By means of flow cytometry, we confirmed the generation of predominantly CD1a negative DC that were cultured in the presence of HS, as was shown in previous

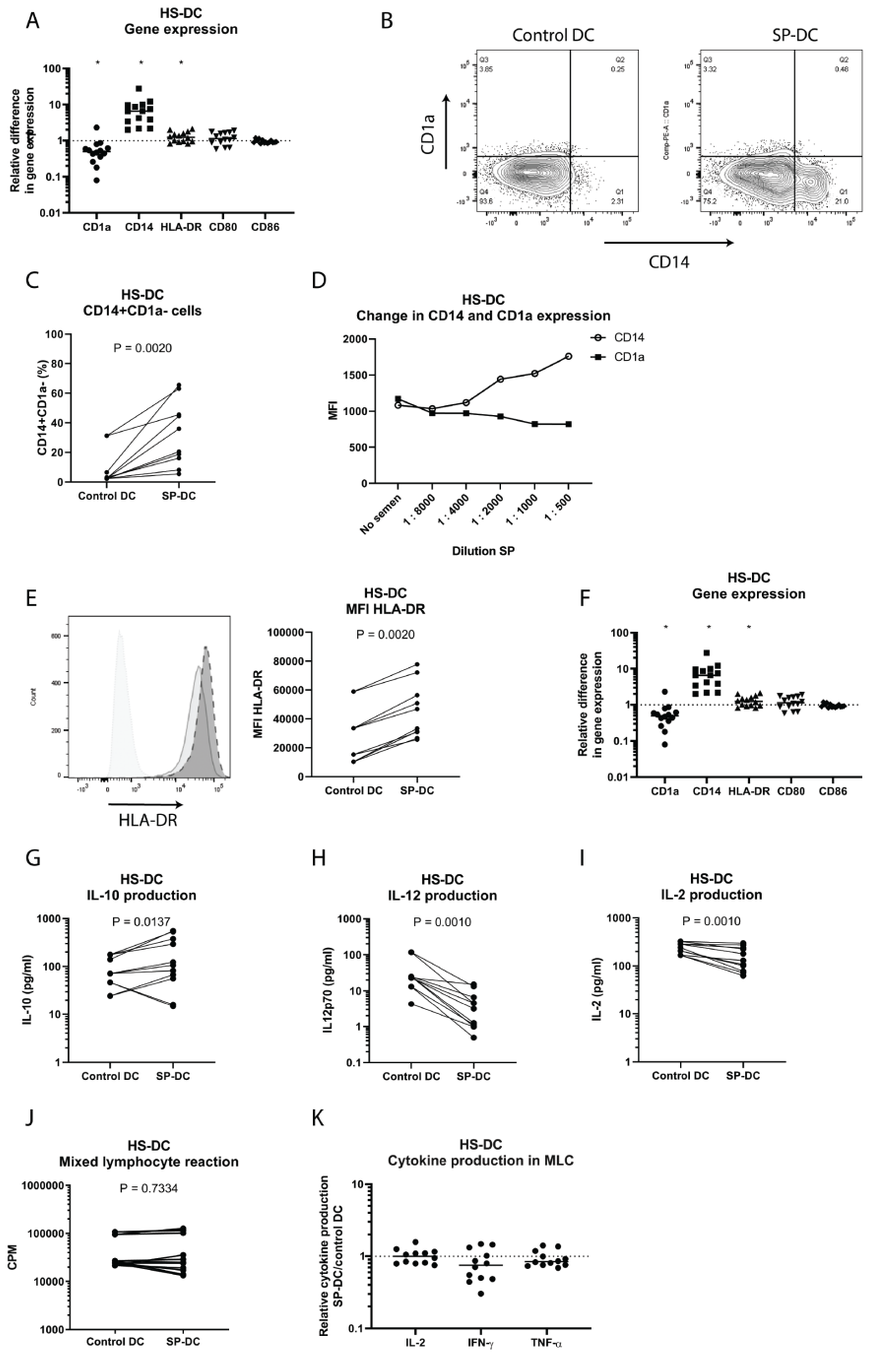


Figure 2. Phenotypical and functional characterization of dendritic cells (DC) cultured with human serum (HS) in the presence or absence of seminal plasma (SP).

(A) Difference in CD1a (median 0.50; P=0.0023), CD14 (median 6.61; P=0.0001), HLA-DR (median 1.25; P=0.0353), CD80 (median 1.14; P=0.0906), CD86 (median 0.90; P=0.2412) mRNA expression between immature SP-DC and control DC (n=14). Expression level in the control DC was set as 1 for each gene, and relative expression levels in SP-DC were compared with the control.

(B) CD14 and CD1a expression in immature DC cultured in the presence or absence of SP was analyzed by flow cytometry. Dot plots from a representative experiment (n=12) are shown.

(C) Percentage of CD14+CD1a- immature DC after culturing monocytes with or without SP (n=12).

(D) The effect of different concentrations SP on CD14 and CD1a expression in immature DC. The more seminal plasma was added to the culture the more distinct the phenotype from control DC.

(E) Difference in IL-10 (median 4.57; P=0.0002), TGF- β1 (median 1.36; P=0.0017), COX-2 (median 2.82; P=0.0105), S100A8 (median 3.54; P=0.0005) and S100A9 (median 4.56; P=0.0002) mRNA expression between mature SP-DC and control DC (n=13). Expression level in the control DC was set as 1 for each gene, and relative expression levels in SP-DC were compared with the control.

(F) Histogram from a representative experiment (n=12) is shown. HLA-DR mean fluorescence intensity (MFI) values in DC cultured with SP are compared to controls.

(G) Cytokine production in DC culture upon stimulation with LPS (n=11). Mature SP-DC produced high levels of IL-10 (P=0.0137) compared to mature control DC (in pg/ml).

(H) Cytokine production in DC culture upon stimulation with LPS (n=11). Mature SP-DC produced low levels of IL-12p70 (P=0.0010) compared to mature control DC (in pg/ml).

(I) Cytokine production in DC culture upon stimulation with LPS (n=11). Mature SP-DC produced low levels of IL-2 (P=0.0010) compared to mature control DC (in pg/ml).

(J) Proliferation of T cells stimulated in co-cultures with either control DC or SP-DC in counts per minute (CPM) (n=12). SP-DC had similar stimulatory capacity compared to control DC (P=0.733).

(K) Relative IL-2, IFN-γ and TNF-α production in the co-culture with SP-DC compared to the co-culture with control DC (n=12). Similar amounts of IL-2 (median 1.00; P=0.970), IFN-γ (median 0.75; P=0.204) and TNF-α (median 0.84; P=0.470) were found in co-cultures of PBMC with SP-DC compared to co-cultures with control DC. Cytokine level in the control DC was set to 1 for each cytokine, and relative cytokine levels in SP-DC were compared with the control. Absolute numbers of cytokine levels are depicted in Table 2.

studies [15, 16]. A representative example of this CD1a negative population is shown in Figure 2B. Whereas HS cultured DC did not upregulate CD1a, we did observe that SP led to a higher percentage of CD14+CD1a- cells, and that this change in CD14/CD1a was dose dependent (Figure 2C-D). Additionally, we analysed the expression of HLA-DR in immature DC, but we did not observe a difference between SP-DC and control DC (data not shown).

SP leads to an increased IL-10 and decreased IL-12 production, but does not affect the stimulatory capacity of HS cultured DC

On day 6 we added LPS to the culture for DC maturation and 48 hours later we analysed expression levels of several genes. CD14 and HLA-DR mRNA expression was still increased in SP-DC compared to control DC, whereas CD1a mRNA expression was still decreased (data not shown). No difference was found for mRNA expression of pro-inflammatory markers IL-12b and TNF-α (data not shown). On the other hand, mRNA expression of anti-inflammatory markers IL-10, TGF-β, COX-2, S100A8, and S100A9 was increased in mature SP-DC compared to control DC (Figure 2E).

Next, we analysed surface marker expression. The increase of CD14+CD1a- cells we observed in immature SP-DC compared to control DC persisted, although not as strong as before LPS maturation (data not shown). Additionally, we observed an upregulated HLA-DR expression in mature SP-DC compared to control DC (Figure 2F).

To determine whether the changes in gene expression and phenotype of DC cultured in the presence of SP also resulted in an altered cytokine production, we analysed the cytokine profile produced by mature SP-DC and control DC. We observed higher IL-10 and lower IL-12 cytokine levels in supernatants of LPS activated SP-DC compared to control-DC (Figure 2G-H), consistent with the data obtained in FBS containing cultures. Additionally, we observed a lower IL-2 production for SP-DC compared to control-DC (Figure 2I).

In a next set of experiments, we analysed the stimulatory capacity of mature DC and the profile of cytokines produced in co-cultures of these cells with allogeneic PBMC. These co-cultures did not show a decreased T cell stimulatory capacity toward SP-DC compared to control DC (Figure 2J). Additionally, we did not find any differences in the production of pro-inflammatory cytokines IFN-γ, TNF-α, and IL-2 between co-cultures of PBMC with SP-DC and co-cultures with control DC (Table 2; Figure 2K).

Discussion

In this study, we showed that SP-DC cultured in HS containing medium were resembling anti-inflammatory DC with regards to phenotype, gene expression patterns, and cytokine production. However, the effect of SP on DC differentiation was less pronounced in HS cultured DC than in FBS cultured DC, and it did not result in an altered T cell stimulatory capacity.

It has been widely reported that factors in human SP are capable of affecting lymphocyte function *in vitro* [6-11]. However, the majority of studies demonstrating SP suppression have been carried out using culture medium containing bovine serum in the assays, and its presence may have influenced the immune responses, as was shown in studies that compared different serum sources [10, 14]. It was previously shown that SP promotes the differentiation of tolerogenic DC [12], but these *in vitro* experiments were performed with SP in the presence of FCS. Previous studies showed that human DC cultured in medium containing FCS are different from those cultured in medium containing HS. Therefore, to create a setting that is more similar to the *in vivo* situation in humans, we set out to study the effect of seminal plasma on DC differentiation in the absence of xenoproteins.

Culturing monocytes in the presence of SP in HS containing medium led to a change in gene expression. CD14, IL-10, and TGF-β mRNA levels were upregulated, whereas CD1a levels were downregulated in mature SP-DC, all of these are hallmarks of tolerogenic DC [13, 20]. Additionally, we found higher mRNA levels for anti-inflammatory markers S100A8, S100A9, and COX-2. Previous studies have shown that S100A8 and S100A9 were upregulated in IL-10 treated tolerogenic

DC [21].S100A9 deficient DC have pro-inflammatory characteristics, such as an increase in IL-12p40 secretion and T-cell proliferation [22], and the addition of exogenous S100A8/S100A9 to the culture reduced T-cell proliferation [22]. COX2 can enhance the production of IL-10 in DC and has cytotoxic T cell suppressive function, which can be reversed by COX-2 inhibition. The induction of COX2 in SP-DC may promote a stable tolerogenic phenotype of SP-DC via a positive feedback loop between prostaglandin E2 (PGE2) and COX2 [23].

Analysis of surface markers showed an increase in CD14+CD1a- cells in cultures with SP. This is a similar phenotype as that of other modulated DC, such as those cultured in the presence of vitamin D3, dexamethasone or IL-10, which all have reduced T cell stimulatory capacity [24-27]. Upon LPS maturation, SP-DC showed an increased HLA-DR expression, a molecule typically associated with DC maturation, and necessary for binding the T cell receptor [28]. In addition, SP-DC produced higher levels of IL-10 and lower levels of IL-12 than control DC, which is characteristic for tolerogenic DC [20]. All these results hint towards the induction of tolerogenic DC by SP in the presence of HS. However, culturing of SP-treated DC in HS did not translate to a functional effect of these cells on T-cell stimulation. In contrast to what we observed in co-cultures with FBS cultured DC, we showed that there was no difference in stimulatory capacity between SP-DC and control-DC, nor in the production of Th1 cytokines in co-cultures with allogeneic PBMC. The MLR is classically affected when using tolerogenic DC instead of mature DC [29, 30], but it is possible that the effect of SP in HS cultured DC is not prominent enough to have a functional effect.

In mice, within hours after mating, macrophages, DC, and granulocytes are recruited into the reproductive tract [31-33]. SP antigens are presented by female DC in lymph nodes draining the genital tract, thereby activating and expanding inducible regulatory T cell populations [1, 34]. Subsequently, these populations migrate to the implantation site and facilitate maternal immune tolerance towards the semi-allogeneic conceptus [35]. In humans, it was shown that the presence of SP in the female reproductive tract after coitus can lead to an influx of immune cells [5] and possibly modulate the local immune response. Extrapolation of *in vitro* data to the situation *in vivo* in humans remains a challenge, and in this study we showed that even the serum source can influence the results of an *in vitro* experiment.

Acknowledgments

We thank the fertility clinic of the Leiden University Medical Center for providing the SP samples and the National Reference Laboratory for Histocompatibility Testing for HLA typing. Furthermore, we thank Mieke Versluis-Lops, Merve Uyar-Mercankaya and Els van Beelen for Luminex tests.

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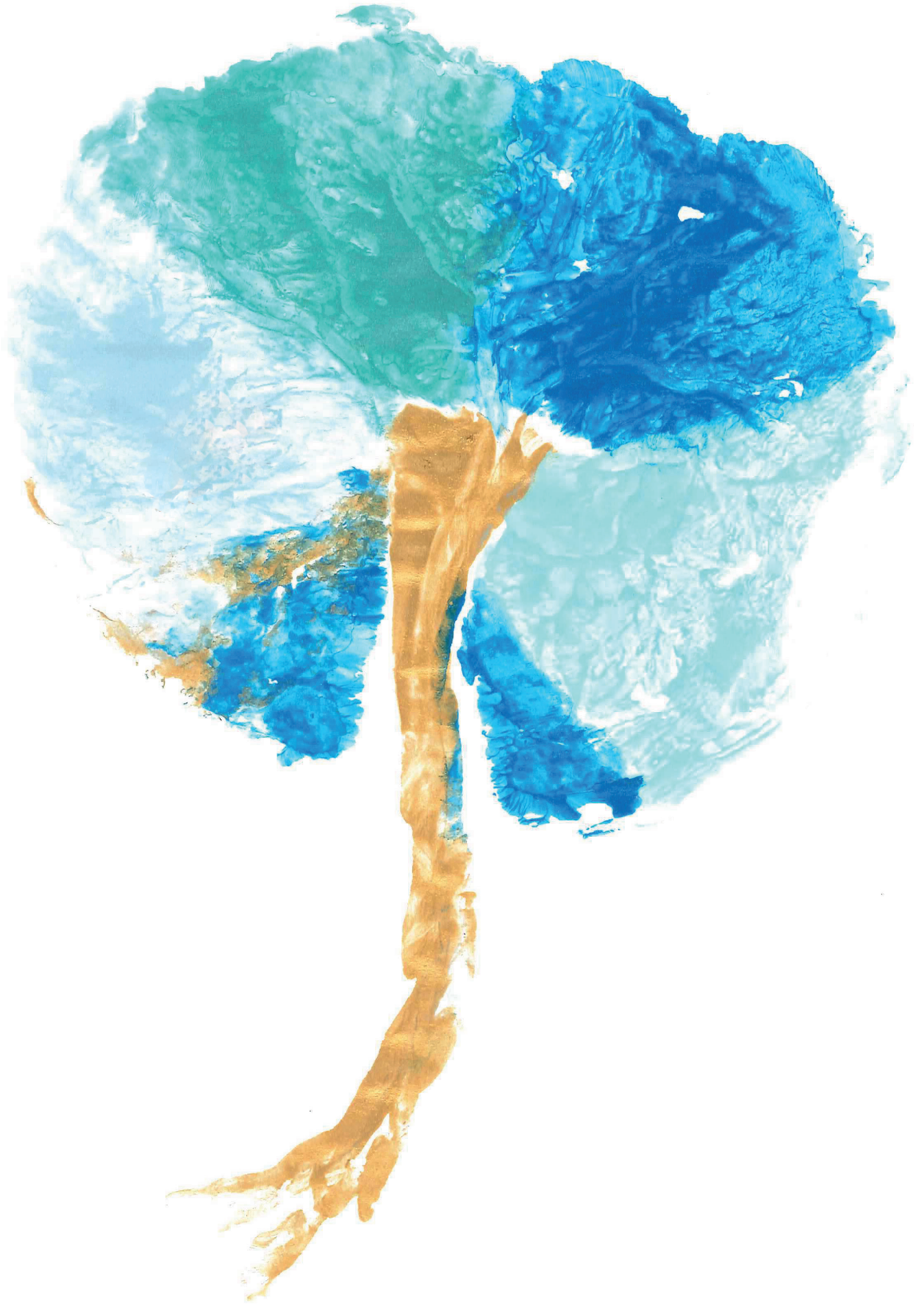

7

summary and general discussion

The mother's acceptance of the foetus, which can be seen as an allograft expressing paternally inherited alloantigens, is a unique example of how the immune system reshapes a destructive alloimmune response to a state of tolerance.

In **Chapter 2**, we discussed the role of regulatory T cells (Tregs) in foetal–maternal immune tolerance as well as in recurrent miscarriage (RM). Tregs have a critical role in maintaining immune tolerance to self-antigens and to foreign antigens of the semi-allogeneic foetus: a deficiency in Tregs is associated with implantation rejection at early stages of pregnancy and abortion . Lower proportions of Tregs are found in peripheral blood from pregnant women with RM during pregnancy and products of conception from miscarriages compared with peripheral blood specimens obtained from controls and abortions on social indication, respectively [1-5]. Even in non-pregnant women with a history of RM frequencies of peripheral Tregs are decreased compared to fertile controls [1, 6]. This suggests an important role for Tregs in pregnancy.

Tregs are generally CD4+ and thus restricted by HLA class II. In organ transplantation, matching for HLA-DR results in a better graft survival and function [7] and in the setting of pre-transplant blood transfusion sharing of at least one HLA-DR antigen leads to a tolerogenic effect on the course of a subsequent renal transplantation, while incompatibility for the second HLA-DR antigen enhances a stable, rejection-free, allograft function [8, 9]. We investigated the influence of HLA-DR (in)compatibility on pregnancy outcome parameters (**Chapter 3**), and observed that mutual maternal-fetal HLA-DR incompatibility resulted in increased birth weight and individualized birth rate ratio (IBR), irrespective of pregnancy complications. A limitation of this study is the low resolution typing, which leads to an underestimation of the number of HLA-DR mismatches between mother and child. However, all cases in the group of reciprocal allogenicity, the situation in which both the mother and fetus express two distinct HLA-DR antigens of which one of the HLA-DR antigens is mismatched between mother and child. It seems that the optimal situation for a successful pregnancy is that of reciprocal HLA-DR allogenicity, which may be explained by an active induction of immune tolerance from both maternal and fetal side. Further research on the immune mechanisms leading to this balance will reveal whether this is indeed the case.

Immune tolerance at the foetal-maternal interface can also be induced by HLA-G. HLA-G facilitates semi-allogeneic pregnancy by inhibiting maternal immune responses to foreign (paternal) antigens [10]. In **Chapter 4** we determined that women with RM have a genetic predisposition to lower HLA-G levels. In that perspective, it is remarkable that HLA-G expression is increased in the placenta of successful pregnancies in women with a history of RM. HLA-G was mostly confined to the trophoblast areas at the fetal-maternal interface (decidua basalis). Since the level of HLA-G expression can depend on the differentiation status of EVTs [11], it is unclear whether the observed differences in HLA-G expression are a direct consequence of transcriptional regulation or a secondarily of an altered differentiation status of the EVTs. We hypothesize that for a successful pregnancy to occur after previous RM, a compensatory mechanism resulting in high HLA-G protein expression is in place. Whether the higher HLA-G expression in the ongoing pregnancy after RM is a cause or a consequence of the successful pregnancy remains to be established. Future studies should be concentrated on further establishing the role of HLA-G in complicated pregnancies. Measurement of maternal sHLA-G may provide further insight on the prognosis of the outcome of pregnancies in women with a history of RM.

HLA-G may already play a role before implantation by creating a tolerant environment. We determined the presence of sHLA-G in seminal plasma (SP) and studied the impact of the most frequent HLA-G 3'UTR variation sites on sHLA-G levels in SP (**Chapter 5**). These studies showed that sHLA-G levels in SP are influenced by HLA-G haplotypes and separate SNPs. We found a higher incidence of the 14 bp ins/ins, a genotype associated with lower sHLA-G levels, in men of whom the

partner experienced RM compared to controls, although this difference was not significant. We did not find differences in sHLA-G levels between SP samples from RM and controls on the population level, indicating that not all miscarriages can be explained by HLA-G genes and low sHLA-G levels in SP. It is more likely that different immune mechanisms contribute to the occurrence of so far unexplained recurrent miscarriages. Other factors that have been associated with RM are high levels of circulating TNF-α and low levels of galectin-1 and mannose-binding lectin (MBL) [12-14]. These are thought to lead to pro-inflammatory processes in women with RM, and thereby contributing to an active maternal immune response toward the fetus. TNF-α is an inflammatory cytokine that promotes inflammation. Galectin-1 can induce differentiation of tolerogenic DC that promote the expansion of IL-10 secreting Tregs. MBL is a complement activating protein that enhances phagocytosis. Women with low MBL may have impaired clearance of apoptotic cells, leading to inflammation. However, these factors may have different functions in the circulation compared to the decidua, and these functions might also change during the course of pregnancy. This makes research on the role of such factors in RM very difficult.

SP contains not only soluble HLA-G, but also sHLA class I. Peptides derived from the paternal HLA class I antigens in the SP may be presented by maternal antigen presenting cells (APC) in the endometrium and when the proper cytokines are present in the seminal fluid this may lead to the induction of Tregs. The effect of SP on dendritic cells (DC) has been tested before *in vitro*. It was shown that SP can skew the differentiation of monocyte-derived DC towards an anti-inflammatory profile when cultured in medium containing fetal calf serum (FCS). However, FCS contains xenoproteins, which can also influence human DC. In **Chapter 6**, we showed that SP can skew the differentiation of monocyte-derived DC cultured in HS towards alternatively activated DC. However, this phenotype appears to be less immune regulatory when compared to SP-treated DC cultured in FCS containing medium. These findings highlight the importance of the serum source used in SP treated cell cultures *in vitro*. Furthermore, it would be interesting to study the effect of SP on DC in samples from couples with RM in comparison to SP and DC from healthy donors.

Clinical relevance

Successful pregnancy can be regarded as a biologic example of graft acceptance, in which the semi-allogeneic foetus is protected from rejection by a proper regulation of the maternal immune system. Different mechanisms play a role in establishing tolerance towards the fetus, some of which already before conception takes place. The presence of SP in the female reproductive tract after coitus can lead to an influx of immune cells [15]. Semen contains various immunomodulatory factors, such as chemokines, cytokines and prostaglandins, but also soluble HLA antigens, which can be recognized as foreign and evoke an immune response [16-19]. There is great variety in the content of SP between men. The variety in cytokine concentration in SP can lead to a difference in the strength and quality of the cytokine response elicited and could be involved in a differential regulation of the immune response to seminal antigens [20]. Paternal HLA antigens in SP might be taken up and presented by maternal APC, mostly DC (Figure 1). These APC migrate to the draining lymph node and present the allogeneic peptides to naïve T cells. The cytokine environment present at the time the paternal antigens are first encountered is pivotal in controlling differentiation of APC, which can determine the strength and quality of the ensuing T cell response. By using *in vitro* experiments, we showed that SP can skew the differentiation of monocyte-derived DC cultured in HS towards alternatively activated DC with anti-inflammatory characteristics (**Chapter 6**). In the *in vivo* situation, when regulatory proteins, e.g. transforming growth factor beta (TGF-β), prostaglandin E2 (PGE2)

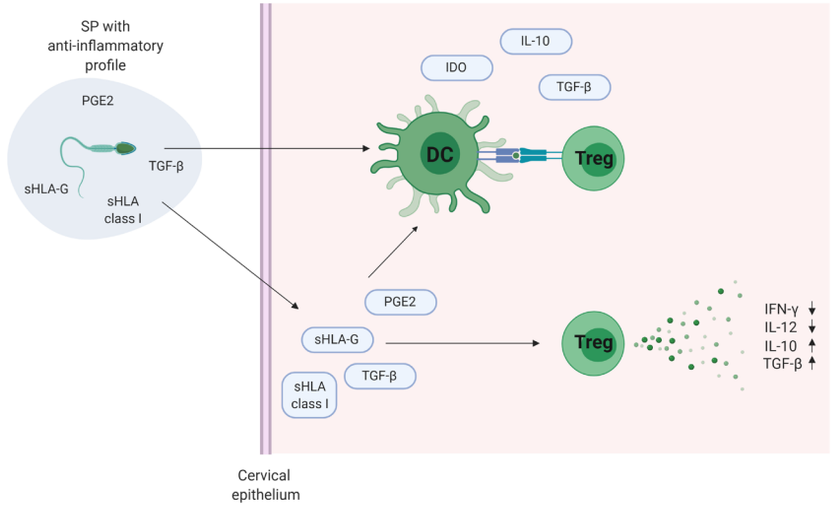

Figure 1. Seminal plasma (SP) contains inflammatory chemokines that induce accumulation and differentiation of dendritic cells (DC) and monocytes. PGE2 and TGF-β in SP promote the differentiationof tolerogenic DCs (TolDC). TolDC take paternal antigens in SP and present these to naive T cells, resulting in induction of paternal antigen-specific regulatory T cells (Treg) cells in uterine draining lymph nodes. Created with BioRender.com.

and sHLA-G, are present in SP, this could lead to DC with a tolerogenic profile and subsequently the induction of antigen specific Tregs. In this way, antigens in the SP elicit T-cell activation to initiate the female immune response during pregnancy, when these paternal antigen specific Treg may be expanded. During pregnancy, fetal trophoblast cells come into contact with maternal immune cells in the decidua (Figure 2). The expanding paternal antigen-specific Treg block effector T cell proliferation and induce apoptosis. Furthermore, they produce cytokines, such as IL-10 and TGF-β, which skew DC to a tolerogenic profile. These tolerogenic DC inhibit effector T cell proliferation and differentiation. Maternal T cells and DC also express receptors that can recognize HLA-G on trophoblasts. *In vitro* tests showed that HLA-G can inhibit cytotoxicity and cytokine production by T cells, and that it can skew DC toward a tolerogenic phenotype, contributing to downregulation of a possible allogeneic immune response [21-23]. Other immune cells that come into contact with trophoblast cells are uterine NK (uNK) cells.

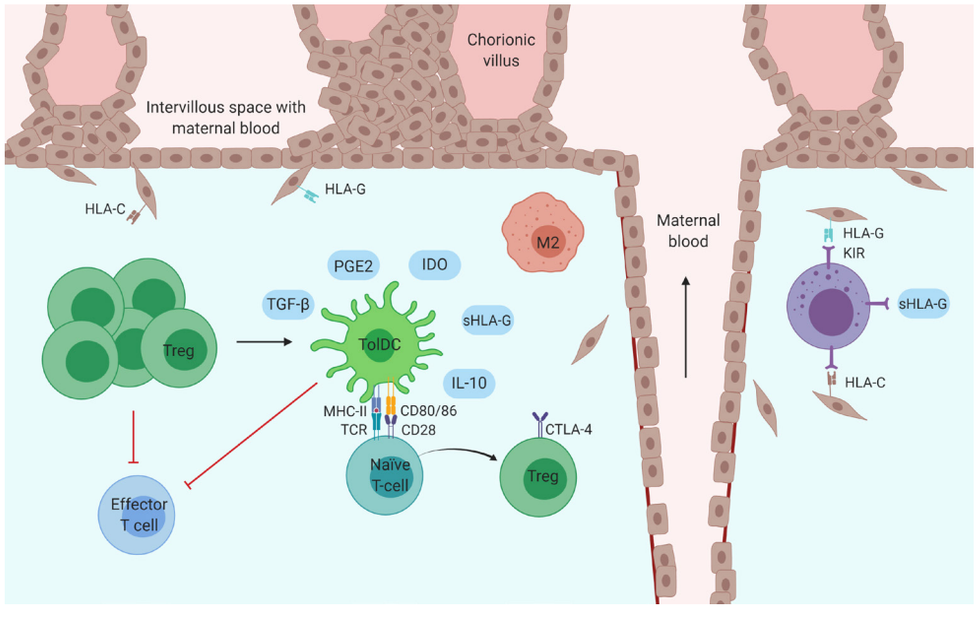

Figure 2. After implantation, the regulatory T (Treg) cells quickly move to the uterus resulting in successful pregnancy. Treg cells block effector T cell proliferation and induce apoptosis. By secreting cytokines, such as IL-10 and TGF-β, they induce tolerogenic DC (TolDC). These TolDC inhibit effector T cell proliferation and differentiation. Created with BioRender.com.

These uNK cells can recognize HLA-C and HLA-G on the fetal trophoblast cells. They express receptors of the killer cell immunoglobulin-like receptor (KIR) family, which seem to be important in balancing the NK activating and inhibitory signalling. It is thought that the main function of uNK cells is to produce cytokines, growth factors, angiogenic factors and other factors for trophoblast migration and spiral artery remodeling. For this, inhibitory and activating signalling need to be in balance.

In addition, it appears that immune regulation towards the fetus needs to be actively induced. We showed that reciprocal HLA-DR allogenicity between mother and child is the optimal situation for pregnancy (**Chapter 3**). In this situation, both the mother and fetus express two distinct HLA-DR antigens of which one of the HLA-DR antigens is mismatched between mother and child. This suggests

that incompatibility for one HLA-DR antigen between mother and fetus leads to triggering and activation of the immune response, while the other HLA-DR antigen has to be shared in order to induce immune regulation (Figure 3). Even though trophoblast cells do not express HLA-DR during normal pregnancy, both maternal and fetal cells can cross the placenta and trigger an immune response [24, 25]. That HLA mismatches can be beneficial for pregnancy was also shown by Tilburgs et al., who showed an increased percentage of decidual activated T cells in HLA-C mismatched pregnancies compared to HLA-C matched pregnancies [26]. Additionally, they reported that decidual Tregs had an increased suppressive capacity in HLA-C mismatched pregnancies compared to HLA-C matched pregnancies. Since reciprocal allogenicity was the most optimal situation found in our study, both fetal and maternal immune responses seem to be important.

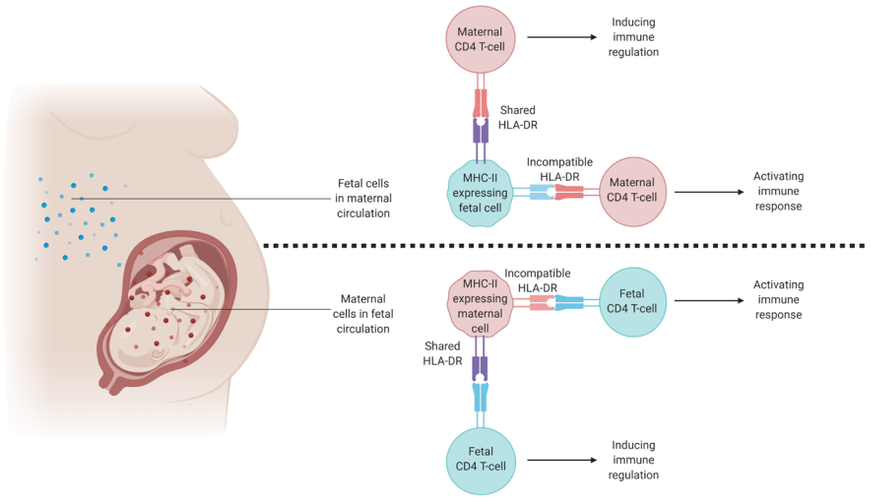

Figure 3. Incompatibility for one HLA-DR antigen between mother and fetus leads to triggering and activation of the immune response, while the other HLA-DR antigen has to be shared in order to induce immune regulation. Created with BioRender.com.

Recurrent miscarriage

On the other hand, a cytokine profile in SP with high levels of pro-inflammatory cytokines and low levels of PGE2 and TGF-β might contribute to the promotion of a Th1-like response, which can lead to activation and expansion of conventional T lymphocytes instead of Tregs (Figure 4).

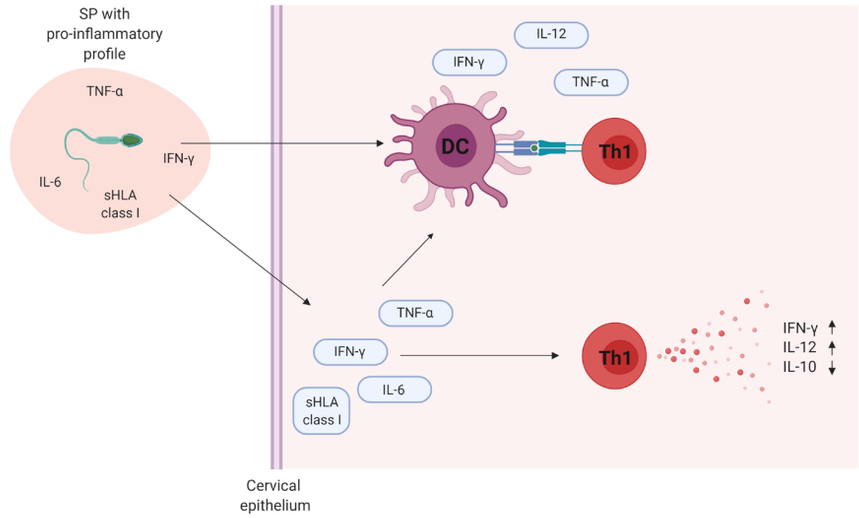

Figure 4. When seminal plasma (SP) contains only low amounts of factors, such as PGE2 and TGF-β, that can skew dendritic cell towards a tolerogenic phenotype, it is possible that paternal antigens are taken up by dendritic cells and that the subsequent immune response results mainly in the induction of Th1 cells instead of regulator T cells. Created with BioRender.com.

We showed that sHLA-G levels in SP vary between men, and that these levels are associated with HLA-G 3'UTR haplotypes, as well as with singular SNPs (**Chapter 5**). However, we did not find a difference in sHLA-G levels between SP samples from men from couples with RM compared to controls. There are probably multiple factors at play that can have similar effects or that can compensate, synergize or antagonize with other factors in SP. It is still possible that if SP samples contain low amounts of cytokines that are involved in the induction of tolerogenic DC, such as TGF-β and PGE2, a Th1 response is promoted instead of a regulatory

response. In our review, we discussed the lower proportions of Tregs in women with RM (**Chapter 2**). These decreased amounts of Tregs might be the result of a pro-inflammatory profile of SP. During pregnancy these paternal antigen specific Th1 cells could expand and might lead to an increased amount of effector cells. Increased amounts of CD8 cytotoxic T cells could recognize HLA-C on trophoblast cells and attack (Figure 5). In the end, this inflammatory response might lead to pregnancy complications or even pregnancy loss.

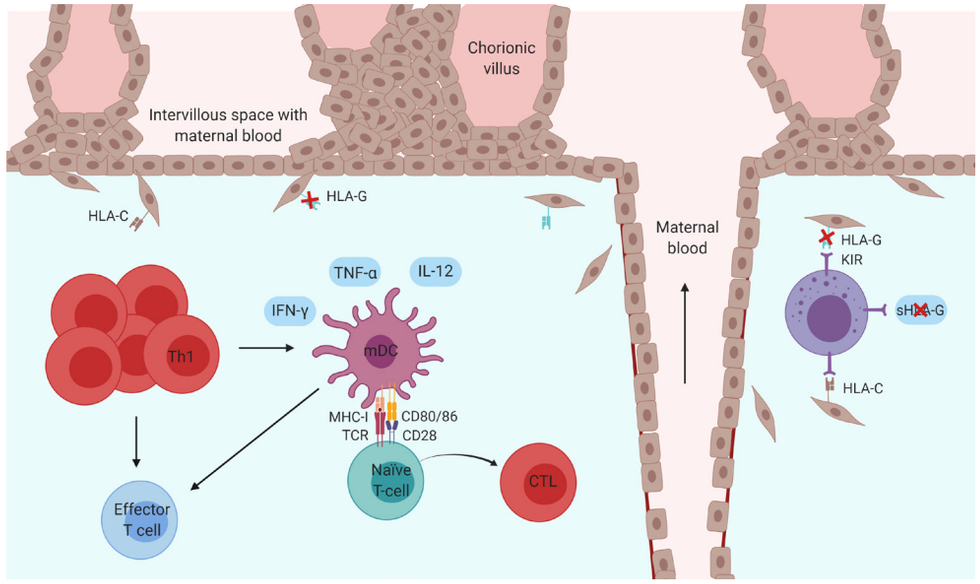

Figure 5. After implantation, the Th1 cells move to the uterus and expand. Th1 cell induce effector T cell proliferation. By the secretion of cytokines, such as IFN-γ and TNF-α they create a pro-inflammatory environment, stimulating the allogeneic maternal immune response. This might lead to rejection of the fetus and a miscarriage. Created with BioRender.com.

Besides sHLA-G levels in SP, we studied the expression of HLA-G in term placentas of women with a history of RM. Remarkably, these placentas showed elevated HLA-G expression compared to term placentas of controls (**Chapter 4**), suggesting that HLA-G upregulation could be a compensatory mechanism in the occurrence of RM to achieve an ongoing pregnancy. It is possible that earlier pregnancies with lower HLA-G expressing trophoblasts ended in pregnancy loss. A decreased expression of HLA-G might have led to a disbalance in inhibitory and activating signals in uNK cells, and subsequently to abnormal placentation and finally a miscarriage.

Conclusion

All together, immunology seems to play an important role in pregnancy, and the composition of immune cells at the fetomaternal interface appears to be involved in pregnancy success. However, whether immunotherapy can play a role by preventing maternal rejection of the foetus has yet to be established, but modulation of the immune system as (part of) a therapeutic strategy could be a valid option to prevent RM.

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8

dutch summary,
list of publications,
acknowledgements,
curriculum vitae

Nederlandse samenvatting

Zwangerschap kan gezien worden als een immunologische paradox. Ook al is het kind gedeeltelijk lichaamsvreemd, het wordt niet afgestoten door het immuunsysteem van de moeder. Dit is opmerkelijk: als de man een orgaan zou doneren aan zijn partner, zou dit orgaan hoogstwaarschijnlijk afgestoten worden of zou de vrouw levenslang immunosuppressiva moeten nemen om afstoting te voorkomen.

Het immuunsysteem onderscheidt lichaamseigen van lichaamsvreemd. Dat wat herkend wordt als lichaamsvreemd wordt afgestoten. Hierbij spelen humaan Leukocyt Antigenen (HLA) een belangrijke rol. HLA zijn eiwitten op cellen die verschillen per individu en die herkend kunnen worden als lichaamsvreemd. HLA wordt per set overgeërfd (zie Figuur 5 op pagina 152): het kind erft één set van de moeder en één van de vader. De eiwitten die het kind erft van de vader kunnen herkend worden als lichaamsvreemd door immuuncellen van de moeder.

Na innesteling van de bevruchte eicel in de baarmoederwand, groeit een deel van de cellen uit tot de foetus en het andere deel tot de placenta. De cellen die de placenta vormen dringen de baarmoederwand in en hechten zich daar vast. De placenta is de plek waar foetale en maternale cellen met elkaar in contact komen, en zuurstof en nutriënten uitgewisseld worden tussen moeder en kind. Hier vindt op verschillende manieren ook immuunregulatie plaats, zodat het maternale immuunsysteem het kind niet actief afstoot. Onvoldoende regulatie zou er toe kunnen leiden dat het maternale immuunsysteem toch het kind afstoot, met een miskraam als gevolg.

Door middel van dit onderzoek willen we meer duidelijkheid over onderliggende oorzaken kunnen geven aan patiënten met zwangerschapscomplicaties, zoals herhaalde miskramen. Verder hopen we te kunnen leren van de immunologische processen tijdens de zwangerschap en deze kennis toe te passen in onderzoek naar de immuunrespons na transplantatie. Hierbij speelt de verschuiving van rejectie naar tolerantie natuurlijk ook een belangrijke rol.

In **hoofdstuk 2** evalueren we eerder onderzoek naar de rol van regulatoire T cellen (Tregs) in zwangerschap en in het geval van herhaalde miskramen (RM). Tregs hebben een belangrijke rol in het reguleren van de maternale immuunrespons tegen eigen antigenen en vreemde antigenen van de gedeeltelijk lichaamsvreemde foetus. Een verlaagd aantal Tregs is geassocieerd met implantatiefalen vroeg tijdens de zwangerschap en met miskramen. Lagere proporties Tregs zijn gevonden in perifeer bloed van zwangere vrouwen met RM tijdens de zwangerschap ten opzichten van controles, en ook in producten van miskramen ten opzichte van gekozen abortussen. Zelfs in niet-zwangere vrouwen met RM in de voorgeschiedenis werden lageren aantallen Tregs in het bloed gevonden ten opzichte van controles. Dit suggereert een belangrijke rol voor Tregs tijdens de zwangerschap.

Tregs zijn meestal CD4+ T cellen en daardoor alleen compatibel met HLA klasse II. In orgaantransplantatie leidt het matchen voor HLA-DR tot beter functioneren en hogere overlevingskans van het orgaan. In het geval van een bloedtransfusie voorafgaand aan de transplantatie leidt het delen van ten minste één HLA-DR antigen tot een tolerogeen effect tijdens de transplantatie, terwijl het niet-delen van het tweede HLA-DR antigen een stabiele, rejectie-vrije orgaanfunctie verbetert. We bestudeerden de invloed van HLA-DR incompatibiliteit op zwangerschapsuitkomstparameters (**hoofdstuk 3**), en we zagen dat het wel of niet delen van HLA-DR (moeder en kind) invloed had op het geboortegewicht en IBR, onafhankelijk van zwangerschapscomplicaties. Het lijkt er op dat reciprocal HLA-DR allogenicity het meest optimaal is voor succesvolle zwangerschap. Dit kan verklaard worden door actieve aanzetting tot immuunregulatie in moeder en kind. Verder onderzoek naar het immuunmechanisme dat leidt tot deze regulatie is nodig om uit te wijzen of dit werkelijk het geval is.

Immuunregulatie tussen moeder en kind kan ook aangedreven worden door HLA-G. HLA-G faciliteert semi-allogene zwangerschap door de maternale immuunrespons tegen vreemde (paternale) antigenen te verhinderen. In **hoofdstuk 4** toonden we aan dat vrouwen met RM een genetische aanleg hebben voor lagere HLA-G waardes. In dat perspectief is het opmerkelijk dat HLA-G expressie verhoogd is in de placenta van succesvolle zwangerschappen in vrouwen met RM in hun voorgeschiedenis. HLA-G kwam het meest tot expressie in de foetale cellen in de placenta. Aangezien de waardes van HLA-G expressie ook kunnen afhangen van de differentiatiestatus van de foetale cel, is het nog onduidelijk of de bevonden verschillen in HLA-G expressie een direct gevolg zijn van transcriptionele regulatie of een later gevolg van een veranderde differentiatiestatus van de foetale cel. We denken dat in een succesvolle zwangerschap na RM een compensatiemechanisme resulteert in een hogere HLA-G eiwit expressie. Of deze hogere HLA-G expressie in de doorgaande zwangerschap na RM een oorzaak of een gevolg is van de succesvolle zwangerschap moet nog bekeken worden. Toekomstig onderzoek zou zich moeten concentreren op het verder uitzoeken van de rol van HLA-G in zwangerschapscomplicaties. Het meten van maternaal sHLA-G zou verder inzicht kunnen verschaffen in de prognose van de uitkomst van zwangerschappen in vrouwen met RM in hun voorgeschiedenis.

HLA-G zou ook al een rol kunnen spelen in het creëren van een tolerante omgeving vóór de implantatie. We bepaalden de aanwezigheid van oplosbaar HLA (sHLA)-G in seminaal plasma (SP) en bestudeerden de impact van de meest frequente HLA-G 3 'UTR variatieplekken op sHLA-G in SP (**hoofdstuk 5**). Deze studies toonden aan dat sHLA-G waardes in SP beïnvloed worden door HLA-G haplotypes en aparte SNPs. We vonden een hogere incidentie van de 14 bp ins/ins, een genotype geassocieerd met lagere sHLA-G waarden, in mannen van koppels met RM ten opzichte van controles, al was dit verschil niet significant. We vonden geen verschillen in sHLA-G waarden tussen SP samples van mannen van koppels met RM ten opzichte van controles op populatieniveau, wat impliceert dat niet alle miskramen verklaard kunnen worden door HLA-G genen en lage sHLA-G waarden in SP. Het is meer waarschijnlijk dat verschillende immuunmechanismes bijdragen aan de tot nu toe onverklaarde RM.

SP bevat niet alleen sHLA-G, maar ook sHLA klasse I. Gedeeltes van dit paternaal sHLA kunnen gepresenteerd worden door maternale antigen presenterende cellen (APC), met name dendritische cellen (DC), in het endometrium. In combinatie met regulerende cytokines in SP zou dit kunnen leiden tot de inductie van Tregs. Het effect van SP op DC is eerder getest *in vitro*. Het is aangetoond dat SP de differentiatie van gekweekte DC kan trekken naar een anti-inflammatoir profiel. Deze testopzet bevatte medium met foetaal kalf serum (FCS), en FCS bevat eiwitten die niet in mensen voorkomen. Deze vreemde eiwitten kunnen de humane DC beïnvloeden. In **hoofdstuk 6** tonen we aan dat SP de differentiatie van DC gekweekt met humaan serum kan trekken naar een alternatief geactiveerde DC, maar dit fenotype lijkt minder immuunregulerend te zijn wanneer het vergeleken wordt met SP behandelde DC gekweekt met humaan serum. Deze bevindingen benadrukken het belang van de serumbron gebruikt in SP behandelde celkweken *in vitro*. Het zou interessant zijn om het effect van SP te bestuderen op DC in samples van koppels met RM en deze te vergelijken met SP en DC van gezonde donoren.

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J Reprod Immunol 2020, 137, 103076.

Acknowledgements

Een proefschrift maak je niet alleen en op deze plek wil ik iedereen bedanken die mij hierbij geholpen heeft. Zoals velen van jullie weten is mijn PhD niet altijd de gemakkelijkste tijd voor mij geweest en is dit gepaard gegaan met vele ups en downs. Alleen had ik dit nooit tot een goed einde kunnen brengen, maar gelukkig had ik veel mensen om mij heen die mij hierbij geholpen hebben. Een paar mensen wil ik hier in het bijzonder bedanken.

Frans, zonder jou was ik natuurlijk nergens geweest. Jouw hart voor en jouw kennis over de reproductieve immunologie maakte dat ik altijd bij je terecht kon met vragen. Waar ik problemen zag, kwam jij met oplossingen. Ik heb ontzettend veel bewondering voor je talent om mensen te blijven motiveren.

Michael en Sebas, bedankt voor het altijd kritisch meedenken en het ter discussie stellen van de resultaten. Jullie opbouwende kritiek ten aanzien van mijn drafts voor een paper zorgde altijd weer voor verbetering. Bedankt dat jullie mijn onderzoek telkens weer een stap verder hielpen.

Iedereen van de reproductieve immunologie en in het bijzonder Carin, Hanneke en Godelieve, zonder jullie hulp had ik het nooit gered. Vele uren hebben we samen op het lab gespendeerd. Bedankt dat jullie mij vanaf het begin opgevangen hebben, voor alle prakische tips, alle hulp en het meedenken bij elk experiment, maar ook daarbuiten.

Medewerkers van de groep Claas, typ- en screeninglab, research en ICT, de complement-groep, bedankt voor alle hulp, getoonde interesse en gezelligheid. In het bijzonder Jos, mijn mede noord-Limburger, door jou voelde ik me meteen thuis in het verre Leiden. Ook Jacqy, Paul en Geert, bedankt voor alle hulp met mijn papers, de techniek en statistiek. Buiten alle hulp met werkgerelateerde zaken, was er ook altijd tijd voor een praatje over andere dingen. Dave, Els, Yvonne de

Vaal, Manon, Marijke, Janine, jullie hebben me geholpen met experimenten voor papers die het al dan niet gehaald hebben, bedankt voor alle hulp. Anouk, het liefst kwam ik altijd even bij je langs om een afspraak met een van de heren in te plannen, ondanks hun volle agenda's leidde jij alles in goede banen.

Mede "PhD'ers" Anita, Cynthia, Gonca, Caroline, Heleen, May, Juliette, Douwe, Michelle, Helena, een aantal van jullie zijn inmiddels al klaar, de rest gaat snel volgen. Bedankt voor het sparren over werkgerelateerde en niet-werkgererelateerde zaken tijdens borrels, etentjes, congressen of gewoon in onze kamer. "Despite the company" was het toch vaak wel heel gezellig. Ook studenten Kaveri, Iris, Milo, Angelos en Kim, bedankt voor hulp en gezelligheid.

Marie-Louise, bedankt voor jouw enthousiasme en het aansporen tot doorzetten. Na een gesprek met jou kon ik altijd vol goede moed weer verder. Ook alle andere personen van het geboortehuis die geholpen hebben bij het verzamelen van materiaal en alle vrouwen en mannen die materiaal hebben afgestaan, bedankt.

Alle collega's van de IHB en andere afdelingen en in het bijzonder Cees, Tanja en Ruben, bedankt voor het kritisch meedenken, nieuwe ideeën en het tonen van interesse in de gang van zaken.

Alle voetbalchicks en trainers, met jullie kon ik mijn gedachten even verzetten tijdens onze wekelijkse trainingen en wedstrijden, movie nights, etentjes en borrels. Bij jullie kon en kan ik altijd alles kwijt. Geblesseerd of niet, ik kan jullie helemaal niet missen en pak graag een avondje gezelligheid mee waar dat kan.

Mijn vrienden en vriendinnen uit Limburg, mijn nichtjes en de rest van de familie, mijn jaarclubgenoten en voedingsmiepen door de rest van het land en ver daarbuiten, bedankt voor alle afleiding en gezelligheid. Ik zie jullie nooit genoeg, maar ondanks dat kan ik altijd bij jullie terecht. Wanneer we elkaar zien is het altijd gezellig en voelt het als vanouds. Bedankt dat jullie er altijd voor me zijn.

En dan natuurlijk mijn paranimfen. Anita, ik was blij dat ik een PhD maatje erbij kreeg binnen de RI. Ik genoot van onze hardloopmomenten 's ochtends vroeg voor werk. Jij hebt het promoveren natuurlijk net zelf meegemaakt, ik ben blij dat je deze bijzondere dag nu aan mijn zijde mee wilt maken. Bedankt voor alle hulp, tips en tricks tijdens de vele koffiemomenten, maar ook dat we kunnen delen wat er minder goed gaat.

Judith, we zijn natuurlijk al jaren vriendinnen en vanaf het begin wist ik dat jij degene was die de lay-out van mijn boekje moest gaan doen. Je hebt echt prachtwerk afgeleverd! Ik ben blij dat de afstand tussen Venlo en Leiden nooit te groot is geweest. Bedankt voor de weekendjes die je naar Leiden kwam om samen te genieten van zon en GT's; bedankt voor het blijven vragen naar updates over mijn onderzoek, al ging alles altijd te langzaam; bedankt voor het verzorgen van mijn boekje en de dag van mijn verdediging.

Giel, Thijs en Olyne, ik ben heel blij dat ik jullie om me heen heb en dat we elkaar ondanks de afstand toch regelmatig zien en spreken. Bedankt voor alle hulp, steun en betrokkenheid en dat ik altijd op jullie kan rekenen.

Lieve pap en mam, ik weet dat jullie ontzettend trots op mij zijn. Ik weet ook dat ik dit zonder jullie steun en vertrouwen in mij nooit had gekund. Jullie staan altijd voor mij klaar als ik iets nodig heb en ik kan altijd bij jullie terecht. Jullie beseffen niet hoeveel jullie voor mij gedaan hebben en nog steeds altijd doen. Ondanks tegenslagen, gaan jullie onverminderd door. Weet dat ik ook ontzettend trots op jullie ben.

Curriculum vitae

Moniek Henriëtte Catharina Craenmehr werd geboren op 25 maart 1988 te Horst. In 2006 behaalde zij haar VWO diploma aan het Dendron College te Horst. In datzelfde jaar begon zij de studie Pedagogische Wetenschappen en Onderwijskunde aan de Radboud Universiteit Nijmegen. Halverwege het eerste leerjaar besloot zij dat dit niet het juiste pad voor haar was en leerde zij wiskunde en scheikunde bij, zodat ze in 2007 toegelaten werd tot de bachelor Voeding & Gezondheid aan de Wageningen Universiteit. Deze studie onderbrak zij vier maanden voor de Almanakredactie van de Wageningse Studentenvereniging Ceres. Haar bachelor thesis bestond uit een literatuurstudie naar het effect van probiotica op de darmpermeabiliteit in topsporters. Moniek haalde haar bachelor diploma in 2011 en startte daarna met een master Biomedische Wetenschappen aan de Radboud Universiteit Nijmegen. Tijdens haar master deed zij twee stages. Haar eerste stage vond plaats aan het Charité in Berlijn. Zij bestudeerde het T-cel repertoire in enteropathie geassocieerd T cel lymfoom, refractaire coeliakie en chronische coeliakie. Haar tweede stage in het RadboudUMC betrof de rol van B-cellen en T-cellen in EBV-gerelateerde lymfoproliferatieve aandoeningen. Na het afronden van haar master, ging zij aan de slag op de pathologieafdeling van het RadboudUMC als researchanalist. In augustus 2014 startte zij met haar PhD in Leiden onder begeleiding van professor Frans Claas. In november 2019 startte zij als Business Development Manager bij BD. Hier is zij verantwoordelijk voor BD's Cell and Biomarker Preservation (CBP) portfolio in de Benelux.