Human leucocyte antigen class Ib molecules in pregnancy success and early pregnancy loss

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TABLE OF CONTENTS

- Introduction
- Search method and HLA nomenclature
- HLA-G expression at the materno-fetal interface
- Functions of HLA-G during pregnancy
- HLA-G polymorphism and pregnancy success
- Soluble HLA-G: A possible biomarker in human reproduction
- HLA-E alleles and expression in relation to pregnancy
- HLA-E in regulating NK cells during pregnancy
- Ambiguity of the basic role of HLA-F in reproduction
- New aspects of HLA-F function
- Significance of HLA-C
- Conclusions and future perspectives

BACKGROUND: The human leucocyte antigen (HLA) class Ib molecules, HLA-E, -F and -G, are expressed at the materno-fetal interface. Because of the apparent immunoregulatory functions of these proteins, they may be involved in successful acceptance of the semi-allogenic fetus during pregnancy.

METHODS: The literature on polymorphisms of the three genes, expression patterns of the proteins, and interactions with immune cell receptors have been evaluated to elucidate whether HLA-E, -F and -G are involved in the pathogenesis of some cases of recurrent miscarriages and unexplained infertility.

RESULTS AND CONCLUSIONS: The HLA class Ib molecules seem to induce suppression of the maternal immune system, but are not necessarily fundamental factors for pregnancy success. However, evidence points towards low expression of these proteins, especially HLA-G, being associated with reduced fertility. To clarify the functions of HLA-E, -F and -G future studies need to link investigations of the polymorphisms in these genes to measurements of protein levels, and examine the role of these proteins in the complex interplay of immune cells and cytokines at the materno-fetal interface.

Key words: MHC / HLA class Ib / pregnancy / recurrent miscarriages / assisted reproduction

Introduction

The human leucocyte antigen (HLA) system has functions in antigen processing and presentation. The genes encoding these molecules are located on the short arm of chromosome 6 and can be divided into HLA class I and class II.

The HLA class I genes are expressed on the surface of all nucleated cells, and are responsible for displaying foreign peptides to the CD8+ T cells. This will typically elicit a specific T cell response with cytotoxic immunological processes killing all cells displaying foreign peptide on their surfaces. The class I molecules consist of six different proteins,
of which the classical HLA-A, -B and -C are highly polymorphic and have central functions in antigen presentation and processing as described, whereas the non-classical HLA-E, -F and -G show decreased polymorphism, restricted tissue distribution and have other functions than HLA class Ia, as outlined in this review.

The class II genes encode five different molecules, of which HLA-DR, -DP, -DQ and -DO are polymorphic and function directly in antigen presentation, whereas the HLA-DM and HLA-DO show decreased polymorphism and function in regulating the peptide load of the other HLA class II molecules. The HLA class II molecules are mainly expressed on dendritic cells, B lymphocytes and macrophages, which express foreign peptides on the cell surface, giving the CD4+ T cells the possibility of initiating either a Th helper I (Th1) or a Th2 response if pathogen recognition occurs. This means that, depending on the cytokines present, and the type of cell activating the T cell, a Th1 response characterized by a cell-mediated immune response, or a Th2 response characterized by the production of antibodies, will be initiated (Parham, 2005a, b). Balancing the Th1 and Th2 immune responses seems to be an important role during pregnancy, as it is believed that the maternal immune system shifts from a Th1 profile to a less cytotoxic Th2 profile in response to fertilization (Wegmann et al., 1993; Billington, 2003). However, the discovery that some cytokines, such as IL-10, are secreted by both Th1 and Th2 cells indicates that a simple Th1/Th2 shift is a simplified concept, not sufficient to explain how tolerance by the maternal immune system is necessary for fetal survival. The increased complexity has recently drawn focus towards regulatory T cells, suggesting that this cell population is important in balancing the Th1 and Th2 responses.

Furthermore, the discovery that fetal cells are devoid of the highly polymorphic HLA class Ia molecules, except for a low expression of HLA-C, is believed to play a dominant role for the induction of tolerance to the semi-allogenic fetus (Moffett-King et al., 2003). Interestingly, the fetal-derived tissue in placenta do express the less polymorphic HLA class Ib molecules, HLA-E, -F and -G, which has led to increased interest in the immunological role of these three proteins during pregnancy (Fig. 1; Favier et al., 2007). Originally, HLA-G was thought to be expressed only in fetal tissues, but recently the molecule has also been found in healthy human tissue such as thymic medulla, cornea and pancreas. In addition, expression of HLA-G seems to be either induced or increased in a number of pathological conditions such as cancer, autoimmune diseases and viral infections (Favier et al., 2007).

HLA-E is expressed in all human tissues where HLA class Ia molecules are expressed (Heinrichs and Orr, 1990). HLA-F expression seems to be limited to the tonsils, spleen, thymic tissue, and placenta, and overall transcription of this gene appears to be higher in lymphoid cells compared with non-lymphoid cells (Lepin et al., 2000).

In addition, a part of the explanation of how the fetus avoids immunological attack from the maternal immune system, might lie in the fact that immune cells that are in close contact with fetal tissues, display altered functions compared with peripheral immune cells. In this regard, around 90% of uterine natural killer cells (uNK cells) have an unusual phenotype, (CD56bright) expressing high levels of CD56, displaying decreased cytotoxicity and an altered receptor expression with higher levels of both CD94/NKG2 and IL2Rα/βγ, and essentially absence of Killer-cell immunoglobulin-like receptor (KIR; Starkey et al., 1991; Jacobs et al., 2001). These cells accumulate in large numbers at the implantation site (Ashkar et al., 2003), and have been shown to be the main cytokine-producing NK cells (Cooper et al., 2001), secreting cytokines, which might be important during implantation and subsequent angiogenesis and development of the placenta (Saito et al., 1993; Trundle and Moffett, 2004; Higuma-Myojo et al., 2005).

Decidual macrophages might also have immunoregulatory functions as they express indolamine-2,3-dioxygenase (IDO), which metabolizes the amino acid tryptophan needed for T cell activation and displays decreased amounts of the T lymphocyte co-stimulatory molecules CD80 and CD86. Concerning T cells, approximately three times as many CD3+CD8+ cells as CD3+CD4+ cells are found in the decidua compared with peripheral blood (Bulmer et al., 1991). In women experiencing recurrent miscarriages (RMs) and unexplained infertility, disturbances of this ratio might be involved in the pathogenesis (Klentzeris et al., 1992; Lachapelle et al., 1996). Besides these T cells, a small subgroup of the CD3+CD56+ NKT cells called invariant Natural Killer T (iNKT) cells are found in decidua, and might have a role in the development of tolerance as they are able to suppress antifetal recognition through interactions with CD1d on the surface of trophoblast cells when IL-10 is present (Scaife et al., 2006; Uemura et al., 2008). A reduced number of these cells has been correlated with RM (Yahata et al., 1998; Yamamoto et al., 1999). Recently, a new population of T cells has been identified as regulatory T cells. These cells are characterized by the CD4+CD25+ phenotype, and express high levels of FoxP3, which is known as a transcriptional regulator important in development and function of cells. Among other functions, these cells inhibit the proliferation and cytokine secretion of CD3+ T cells, reduce the cytotoxicity of NK cells and prevent dendritic cell maturation (Sakaguchi et al., 2006). The regulatory T cells make up 10–15% of all CD4+ T lymphocytes and they seem to be most abundant during early pregnancy (Heikkinen et al., 2004; Sasaki et al., 2004). The exact mechanisms of how tolerance is induced by these cells are not yet clarified, but the cells have been shown to express IDO preventing T cell activation, thereby abolishing the immunological processes carried out by these cells (Heikkinen et al., 2004).

In this review, the possible role of the HLA class Ib molecules in the pathogenesis of infertility and RM is discussed in an immunological context. Infertility is defined as the inability of a couple to conceive after 1 year, and 10–15% of couples in the reproductive age can be categorized as infertile. Interestingly, 15–20% of infertility cases cannot be accounted for by any known factor (Schorge, 2008). RM is defined as three or more consecutive pregnancy losses before the 20th gestational week, and is estimated to affect 1–2% of all couples (Coulam, 1991). Interestingly, in about 50% of RM cases no etiological factor can be identified (Lee and Silver, 2000). It can be speculated that disturbances in the processes generating the characteristic immune tolerance during pregnancy might have a role in the pathogenesis of some cases of RM and unexplained infertility.
The HLA nomenclature system has recently been revised and in this review, both the nomenclature in use at the time where the specific studies were performed and the corresponding new nomenclature are used because the literature does not always offer a description of the specific alleles and polymorphisms, as we know them currently. If any doubt exists on the transformation, all possible alleles corresponding to the new nomenclature are indicated.

**HLA-G expression at the materno-fetal interface**

Of the HLA class Ib molecules, HLA-G is the most intensively studied, and much effort has been put into specifying the role of this protein in developing tolerance during pregnancy.

![Figure 1](https://academic.oup.com/humupd/article-abstract/18/1/92/856055/18102566555)

**Figure 1** A schematic representation of the fetus, placenta, decidua and functional significance of the three HLA class Ib molecules: HLA-F, -E and -G. The rectangular insert shows a magnification of the materno-fetal interface with localization of the trophoblast cell populations. The cytotrophoblast (CT) cells serve as progenitors for the differentiated trophoblast cell populations; the extravillous trophoblast (EVT) cells, which express HLA-E, HLA-G and probably HLA-F on their surfaces; and the syncytiotrophoblast (ST) cells, which along with the villous trophoblast (VT) cells might express soluble HLA-G and possibly intracellularly located HLA-E and HLA-F. Arrows from the round insert display the interactions of HLA-E, -F and -G with different receptors on the surface of uNK cells, B19 monocytes, regulatory T cells (Treg) and CD4+/-CD8+ T cells. See text for details.

At present, seven alternatively spliced variants of HLA-G have been identified, counting four membrane-bound and three soluble isoforms, as represented in Fig. 2A (Ishitani and Geraghty, 1992; Pascale et al., 2000a). Of these variants, only HLA-G1 and -G5 associate with β2-microglobulin (β2m). This is an important fact when designing assays that detects different HLA-G isoforms, as some antibodies only bind to HLA-G molecules when these are complexed with β2m.

The expression of splice variants in different trophoblast cell populations of placenta is an area of controversy. It is well-documented that invasive extravillous trophoblast (EVT) cells express a membrane-anchored HLA-G protein at the cell surface, as first shown by Ellis et al. (1986) and subsequently by several groups. Whether or not other trophoblast cell populations, such as syncytiotrophoblast (ST) cells and VT cells express HLA-G, is still a matter of discussion, and this disagreement is—probably—primarily caused by technical differences, such as selection of antibodies, between different
Figure 2 (A) HLA-G gene structure showing the extracellular, transmembrane and intracellular regions. The localization of gene polymorphisms important in pathological conditions of pregnancy is marked as well. Alternative splicing of HLA-G results in production of four membrane-bound and three soluble isoforms of the protein. In addition to the seven isoforms, alternative HLA-G molecules have been demonstrated in transfection studies, however at present, there is not sufficient evidence that these molecules are found at the materno-fetal interface. No alternative splicing is known for HLA-E and HLA-F. (B) Schematic representations of HLA-G as a disulfide-linked β2m-associated dimer, a β2m-free disulfide dimer and a β2m-free heavy chain. (C) Posttranslational modifications specific for HLA-E and HLA-F, respectively. A nonamer sequence is needed for the surface expression of HLA-E, whereas HLA-F has been found to include a cytoplasmic tail for export from the ER, and a RxR motif specific for HLA-F for localization to the Golgiia complex, where HLA-F protein has been shown to accumulate. Which route HLA-F takes to a possible surface expression is still unknown.
Functions of HLA-G during pregnancy

Of special interest, regarding the functions of HLA-G, is the interaction with the inhibitory receptors, immunoglobulin-like transcript-2 and -4 (ILT-2 and ILT-4), which is expressed by a wide variety of immune cells and selectively on the surface of monocytes, macrophages and dendritic cells, respectively (Colonna et al., 1997, 1998). It has been shown that the binding affinity of these two receptors for HLA-G is higher than for other HLA class I molecules, indicating specific functions as a result of the interactions (Fig. 1; Shiroishi et al., 2003). Both these receptors have been shown to compete with CD8 in binding HLA-G, which could prevent activation of cytotoxic CD8+ T cells by preventing an interaction between HLA-G and CD8, thereby contributing to the induction of tolerance (Shiroishi et al., 2003). Furthermore, HLA-G has been shown to up-regulate both ILT-2 and ILT-4, along with the killer-cell immunoglobulin-like receptor-2DL4 (KIR2DL4), on the surface of both antigen presenting cells, NK cells, and CD4+ T cells without preceding antigenic co-stimulation. This provides an interesting possibility that these receptors function at the materno-fetal interface by raising the threshold of the maternal immune system activation, thereby serving to induce tolerance (LeMaucoult et al., 2005). In continuation of this, both soluble and membrane-bound HLA-G proteins have been shown to induce inhibition of T cell alloproliferation through both ILT-2 and ILT-4. This was validated by the abolishment of inhibition when adding antibodies against these receptors (Naji et al., 2007).

The significance of HLA-G dimerization for receptor interactions

Experiments have shown that the HLA-G dimer has higher affinity for ILT-2 and ILT-4 than the HLA-G monomer has, and this has drawn focus towards which effects dimerization might have (Shiroishi et al., 2006). A transfection study stated that sHLA-G dimers inhibited allo-recognition by reducing proliferation of both CD4+ and CD8+ T cells, and that this was accompanied by an up-regulation of ILT2 on the CD8+ cells, probably leading to increased inhibition (Zhang et al., 2009). Apps et al. recently found that homodimeric complexes of HLA-G associated with β2m binds to ILT-2 expressed on all decidual dendritic cells and macrophages. This interaction results in altered cytokine expression with increased levels of IL-6 and IL-10, indicating a shift towards the Th2 cytokine profile. In addition, they found that the interaction resulted in an inhibition of the indirect T cell proliferation carried out by the antigen presenting cells, as these encountered non-self tissue antigens (Apps et al., 2007). As free heavy chains of HLA-G have been detected on the surface of trophoblast cells, these have also been examined, and the necessity of β2m association for the interaction with ILT-2 has been confirmed (Gonen-Gross et al., 2005). Conversely, ILT-4 has been shown to interact with both β2m-associated and free HLA-G (Shiroishi et al., 2006).

Specific interactions of HLA-G with ILT-2 and ILT-4

In addition, transfection studies have shown that ILT-2 interact with HLA-G1 and prevent lysis by NK cells. By adding antibodies against the ILT-2 receptor, lysis was restored, providing evidence that a
specific interaction between these two molecules can inhibit cell lysis (Navarro et al., 1999). Recently, it has also been shown that HLA-G down-regulates IFN-γ produced by NK cells as a result of interaction with ILT-2, but under certain conditions, up-regulation of this cytokine is observed as well, indicating the necessity of tight regulation (Morel and Bellón, 2008). A recent study proved that the interaction between HLA-G and ILT-2 resulted in inhibition of NK cell cytolytic functions and impairment of the release of IFN-γ (Favier et al., 2010). However, it is still unclear whether uNK cells actually do display cytotoxicity in vivo, which makes the significance of these findings difficult to evaluate. At this point it is more likely that uNK cells are cytokine-producing upon activation rather than cytotoxic. Whether cytotoxicity to trophoblast cells carried out by peripheral NK cells during the invasion of the trophoblast into the decidua could interfere with the generation of tolerance is still unknown.

The effects of HLA-G interaction with ILT-4 on the surface of dendritic cells have also been an area of focus. A study by Gros et al. showed that HLA-DR and CD80 were reduced in dendritic cells in the presence of sHLA-G, and in addition, a reduction of IL-12 secretion was observed. This resulted in a decreased activation of NK cells, pointing towards a role for HLA-G in inhibiting dendritic cells and preventing activation of effector cells in the immune system (Gros et al., 2008). Recently, IL-10 dependent dendritic cells DC-10 has been shown to induce differentiation of naive CD4+ T cells into type 1 regulatory T cells, as a result of the interaction between ILT-4 and HLA-G (Gregori et al., 2010). These Tr1 cells produce high levels of IL-10, transforming growth factor (TGF)-β and IL-5, low levels of IFN-γ and IL-2, and no IL-4, and have shown to actively suppress immune responses mainly via IL-10 and TGF-β (Groux et al., 1997).

Specific interactions between HLA-G and KIR2DL4

An additional receptor for HLA-G is KIR2DL4, which has been shown to bind HLA-G both in transfection studies and when expressed on the surface of JEG-3 cells (Rajagopalan and Long, 1999; Yu et al., 2006). The receptor is present on a wide subset of NK cells, especially in decidua, and seems to be HLA-G specific, indicating that the interaction is immunologically important during pregnancy (Ponte et al., 1999). Giving this specificity, determination of the fundamental signalling during pregnancy seemed one step closer, but one study found that a woman with a history of several successful pregnancies was homozygous for a genotype not encoding KIR2DL4 (Gomez-Lozano et al., 2003). Evidence indicates that this receptor might not only result in inhibition of NK cells but has the ability to provide activating signals as well (Lanier et al., 1998). However, currently there is no knowledge on whether these activating signals take place in uNK cells when KIR2DL4 encounters HLA-G, and what the possible outcome of this activating signal might be, although secretion of cytokines has been suggested. Supporting this is a study showing that a cell line transfected with HLA-G could not reverse lysis carried out by uNK cells but did induce a release of cytokines. In addition, it was shown that the protein level of KIR2DL4 in uNK cells isolated from fertile controls was much higher than in uNK cells from women experiencing RM (Wei-hua et al., 2007).

Validation of the receptor interaction studies

Most of the studies have been carried out on peripheral immune cells, and therefore, it will be interesting to perform the same studies on T cells from decidua and isolated trophoblast cells expressing HLA-G, to assess whether the interactions and functions described here occur at the materno-fetal interface. Studies of receptor interactions in vivo are sparse, but a recent, remarkable study managed to knock down HLA-G in human first trimester EVT cells by RNA interference. The results were intriguing, since NK cells showed considerable cytotoxicity to the HLA-G-knock-downed cells compared with the non-knock-downed controls, indicating that the interactions between HLA-G and the receptors ILT-2, ILT-4 and in addition KIR2DL4 on the surface of uNK cells are important for fetal survival (Chen et al., 2010).

HLA-G polymorphism and pregnancy success

In the HLA-G gene, several polymorphisms have been identified, which might have a role in the pathogenesis of infertility and RM. Studies have focused on polymorphisms in the coding region, in the 5′ upstream regulatory region (5′URR) and in the 3′ untranslated region (3′UTR), respectively (Fig. 2B).

To date, 15 polymorphisms at the protein level and two null alleles have been identified, and most studies have focused on the null allele G*01:05N, which do not encode the full-length HLA-G1 or HLA-G5 (http://www.HLA-alleles.org). Discovery of this allele led to speculations on whether fetal homozygosity of G*01:05N could be an etiological factor in couples experiencing RM and in unexplained infertility, but this was partially ruled out by the detection of G*01:05N homozygous adults (Suarez et al., 1997; Ober et al., 1998). Despite this, several studies have found an increased frequency of this allele in couples experiencing RM (Aldrich et al., 2001; Pfeiffer et al., 2001), defining it as a risk factor. Surprisingly, another study failed to show this correlation, and conversely found an increased frequency of this allele in the population, challenging the postulation that full-length HLA-G is crucial for successful pregnancy (Abbas et al., 2004).

The explanation behind this paradox might be found in the unique splice pattern of HLA-G, where other isoforms might compensate for the lack of HLA-G1 and HLA-G5. In support of this hypothesis, it has been shown that in cells transfected with G*01:05N, full-length primary transcript was detected, but at a decreased level compared with cells transfected with G*01:01 (G*01:01:02:01/02). Furthermore, protein expression of the full-length HLA-G1 and HLA-G5 could not be detected in the G*01:05N transfected cells and neither could HLA-G4. In contrast, expression of the membrane-bound HLA-G2 and -G3 and/or soluble HLA-G6 and G7 was detected, but could not be distinguished due to the lack of specific antibodies. Because inhibition of NK cell-mediated lysis was observed in these cells lacking full-length protein, evidence pointed towards the fact that other HLA-G isoforms than HLA-G1 and -G5 could provide sufficient immunological protection (Le Discordre et al., 2005).

Another HLA-G null allele G*01:13N has been described, in which expression of all splice variants is compromised. This allele has only been found in heterozygous individuals and the frequency in the population seems to be limited (Mendes-Junior et al., 2010).

An overview of the studies examining the association of HLA-G alleles and RM is shown in Table I. Some groups find no association
Table I Association between HLA-G polymorphisms in the coding regions and RM.

<table>
<thead>
<tr>
<th>Study</th>
<th>Number of cases</th>
<th>Inclusion criteria for cases</th>
<th>Number of controls</th>
<th>Inclusion criteria for controls</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penzes et al. (1999)</td>
<td>21 couples</td>
<td>RM - not specified</td>
<td>72 individuals</td>
<td>Healthy individuals</td>
<td>No association</td>
</tr>
<tr>
<td>Yamashita et al. (1999)</td>
<td>20 couples</td>
<td>RM - not specified</td>
<td>54 individuals</td>
<td>Not informed</td>
<td>No association</td>
</tr>
<tr>
<td>Pfeiffer et al. (2001)</td>
<td>78 couples</td>
<td>≥ 3 RM0s</td>
<td>52 couples</td>
<td>≥ 1 normal pregnancy</td>
<td>G<em>010103 (G</em>01:01:03:01:02)</td>
</tr>
<tr>
<td>Aldrich et al. (2001)</td>
<td>113 couples</td>
<td>≥ 3 RM0s</td>
<td>No controls</td>
<td>No history of fertility problems</td>
<td>G<em>0104 (G</em>01:04:01:02:03:04:05) G<em>0105N (G</em>01:05N)</td>
</tr>
<tr>
<td>Hvid et al. (2002)</td>
<td>61 couples</td>
<td>≥ 3 RM0s</td>
<td>47 fertile couples</td>
<td>≥ 1 normal pregnancy</td>
<td>Trend for G<em>0106 (G</em>01:06)</td>
</tr>
<tr>
<td>Abbas et al. (2004)</td>
<td>120 women</td>
<td>≥ 3 RM0s</td>
<td>120 women</td>
<td>≥ 3 normal pregnancy</td>
<td>Trend for G<em>0103 (G</em>01:01:03:01:02)</td>
</tr>
<tr>
<td>Yan et al. (2006a, b)</td>
<td>69 women</td>
<td>≥ 3 RM0s</td>
<td>146 women</td>
<td>≥ 1 normal pregnancy</td>
<td>No association</td>
</tr>
</tbody>
</table>

Soluble HLA-G: a possible biomarker in human reproduction

Soluble HLA-G during pregnancy

Soluble HLA-G has been detected in blood plasma and amniotic fluids and as a consequence, much effort has been put into developing methods to determine the concentration of sHLA-G in various body fluids, and to understand what kind of functions this immunomodulatory molecule might have in these compartments (Rebmann et al., 1999).

An overview of studies linking sHLA-G in blood plasma to pregnancy success is listed in Table III. Early studies used the antibody W6/32, and could not distinguish sHLA-G from other soluble HLA class I proteins, such as HLA-A, -B, -C and -E, making evaluations of these studies difficult. Yet, sera from 20 women experiencing fetal loss of unknown aetiology were shown to contain lower levels of sHLA-G than sera from women in first trimester of intact pregnancies (Athanassakis et al., 1999). By using an antibody specific for the sHLA-G isofoms, 16G1, Hunt et al. (2000) tried to eliminate the cross reactivity, and showed that levels of sHLA-G were approximately twice as high in pregnant women as in non-pregnant women. To elucidate the possible immunological functions of sHLA-G in blood plasma during pregnancy, Pfeiffer et al. examined infertile women correlated with reduced levels of HLA-G mRNA, possibly providing an explanation for the increased frequency in women with RM (Hvid et al., 2003). This study also demonstrated an association of the 14 bp insertion sequence with a deletion of 92 bp of exon 8. It has been shown that deletion of the 92 bp is associated with higher stability of HLA-G mRNA than the +14 bp transcripts (Rousseau et al., 2003). To date it is unknown whether deletion of the 92 bp results in a lower stability of the transcript, than deletion of the 14 bp sequence.
### Table II  Association between HLA-G polymorphisms in the non-coding regions and RM

<table>
<thead>
<tr>
<th>Study</th>
<th>Number of cases</th>
<th>Inclusion criteria for cases</th>
<th>Number of controls</th>
<th>Inclusion criteria for controls</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ober et al. (2003)</td>
<td>42 hutereite couples</td>
<td>≥ 3 RMs</td>
<td>58</td>
<td>≥ 2 normal pregnancies</td>
<td>Association of sporadic fetal loss with 725G/−725G genotype</td>
</tr>
<tr>
<td>Spak-Szmigiel et al. (2008)</td>
<td>58 couples</td>
<td></td>
<td>58</td>
<td></td>
<td>No association</td>
</tr>
<tr>
<td>Roussev and Coulam (2007)</td>
<td>25 couples</td>
<td>History of RM</td>
<td>Not informed</td>
<td>Not informed</td>
<td>−725G mutation</td>
</tr>
<tr>
<td>14 bp ins/del polymorphism</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hvid et al. (2002, 2004a, b)</td>
<td>61 women</td>
<td>≥ 3 RMs</td>
<td>93</td>
<td>≥ 2 normal pregnancies</td>
<td>+14 bp/+14 bp genotype</td>
</tr>
<tr>
<td>Tripathi et al. (2004)</td>
<td>120 women</td>
<td>≥ 3 RMs</td>
<td>120</td>
<td>≥ 3 normal pregnancies</td>
<td>−14 bp/+14 bp genotype</td>
</tr>
<tr>
<td>Yan et al. (2006a, b)</td>
<td>79 women</td>
<td>≥ 3 RMs</td>
<td>109</td>
<td>≥ 2 normal pregnancies</td>
<td>Trend for +14 bp allele</td>
</tr>
<tr>
<td>Xue et al. (2007)</td>
<td>24 women</td>
<td>≥ 3 RMs</td>
<td>88</td>
<td>≥ One normal pregnancy</td>
<td>−14 bp/+14 bp genotype</td>
</tr>
<tr>
<td>Spak-Szmigiel et al. (2008)</td>
<td>58 couples</td>
<td>≥ 3 RMs</td>
<td>58</td>
<td>≥ 2 normal pregnancies</td>
<td>No association.</td>
</tr>
<tr>
<td>Suryanarayana et al. (2008)</td>
<td>169 women</td>
<td>≥ 3 RMs</td>
<td>92</td>
<td>≥ One normal pregnancy</td>
<td>Association with genotypes including the +14 bp allele and a 1570T → C mutation</td>
</tr>
<tr>
<td>Berger et al. (2010)</td>
<td>238 women</td>
<td>≥ 2 RMs</td>
<td>233</td>
<td>≥ One normal pregnancy</td>
<td>Trend for +14 bp/+14 bp genotype Association with two SNP defined haplotypes</td>
</tr>
<tr>
<td>Zhu et al. (2010)</td>
<td>188 women</td>
<td>≥ 3 RMs (Additionally 138 women with ≥ 2 RMs)</td>
<td>251</td>
<td>≥ 1 normal pregnancy</td>
<td>Trend for +14 bp/+14 bp genotype Association only in women ≥ 4 RMs</td>
</tr>
</tbody>
</table>

### Table III  Association between soluble HLA-G levels in blood plasma and pregnancy success.

<table>
<thead>
<tr>
<th>Study</th>
<th>Method</th>
<th>Number of cases</th>
<th>Inclusion criteria for cases</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Athanassakis et al. (1999)</td>
<td>Detection of HLA-G with W6/32</td>
<td>20 women</td>
<td>Fetal loss of unknown aetiology</td>
<td>Decreased level of sHLA-G in women with fetal loss compared with controls</td>
</tr>
<tr>
<td>Pfeiffer et al. (2000)</td>
<td>Depletion of HLA class Ia and HLA-E with mAb TP25.99 Detection of HLA-G with W6/32 and anti-β2m</td>
<td>65 women</td>
<td>Undergoing IVF treatment</td>
<td>Pre-ovulatory sHLA-G levels and sHLA-G levels in early pregnancy lower in women with fetal loss, than in women with intact pregnancies</td>
</tr>
<tr>
<td>Hvid et al. (2004a, b)</td>
<td>Detection with MEM-G/9 and W6/32</td>
<td>85 individuals</td>
<td>Undergoing IVF treatment</td>
<td>No detectable sHLA-G in individuals with:</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• +14 bp/+14 bp genotype</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• 14 bp/−14 bp genotype and −725G mutation</td>
</tr>
<tr>
<td>Spak-Szmigiel et al. (2007)</td>
<td>Not described</td>
<td>20 women</td>
<td>≥ 3 consecutive IVF failures</td>
<td>Decreased level of sHLA-G in women with IVF failure compared with controls</td>
</tr>
</tbody>
</table>
undergoing IVF treatment in a pilot study. They showed that pre-ovulatory levels of sHLA-G in 20 IVF treated women with subsequent miscarriages were much lower than in 45 women with intact pregnancies (Pfeiffer et al., 2000). A difference in sHLA-G levels between the women with intact pregnancies and those experiencing early miscarriages was also observed during the following 9 weeks of gestation, indicating that higher levels of sHLA-G might be correlated with successful treatment in couples with fertility problems. Another study found that in subjects homozygous for the +14 bp insertion sequence, which has also been associated with RM, no sHLA-G in blood plasma could be detected. In addition, the −725G polymorphism in the 5’ URR accompanied by two 14 bp deletion alleles also resulted in lack of detectable sHLA-G levels in blood plasma (Hviid et al., 2004b). Here, MEM-G/9 and W6/32 were used. These antibodies have been stated as the most specific combination in detecting sHLA-G, when a possibility of cross reactivity with other HLA class I molecules is present (Sageshima et al., 2007). Finally, another study reported that levels of sHLA-G in peripheral blood were significantly lower in women having experienced at least three IVF failures than in fertile controls. No significant reduction was seen in women experiencing RM, although absolute numbers pointed towards a lower concentration of sHLA-G in this group as well (Sipak-Szmigiel et al., 2007).

The concentrations of sHLA-G in maternal blood has been shown to increase significantly during the first trimester of pregnancy, and from this point, levels decline during second and especially third trimester based upon single measurements on blood samples from different groups of pregnant women (Steinborn et al., 2007). Yet, this expression pattern is similar to that of other biomarkers during pregnancy, and therefore does not necessarily reflect specific functions of sHLA-G during different stages of pregnancy. In contrast to this study, Rizzo et al. managed to discern detection of the genuine HLA-G5 and the sHLA-G1, which can be shed from the cell membrane. This was obtained by detecting both isoforms with the specific MEM-G/9 antibody and selectively capturing HLA-G5 with the intron-4 specific 5A6G7 antibody, and subsequently determine the amount of sHLA-G1 as the difference between these two values (Rizzo et al., 2009). The study found that high or detectable values of sHLA-G are not fundamental for pregnancy, since sHLA-G could not be detected in maternal plasma from all women with normal, healthy pregnancies. However, a lower level of sHLA-G1 in late pregnancy was linked to an increased risk of pre-eclampsia, while high levels of HLA-G5 seemed to be associated with more severe pre-eclampsia and other pregnancy complications such as intrauterine growth retardation. This will not be discussed further in this review, but provides the basis for carrying out similar experiments in women with a history of RM and women undergoing infertility treatment.

The detection of sHLA-G in plasma also led to the question of whether antibodies against HLA-G were secreted into the blood of pregnant women, and whether these could provide immunological challenges in subsequent pregnancies if preformed antibodies bound to sHLA-G prevent the protein from carrying out fundamental immunological functions in the peripheral blood of these pregnant women. Hunt et al. showed that antibodies against sHLA-G could be detected in sera from some multigravid women, but not from women who never had children, or from men. Yet, the six women, who were found to secrete anti-HLA-G antibodies all delivered healthy babies, leading to the conclusion that any production of anti-HLA-G antibodies cannot provide explanation for any of the pregnancy complications discussed here (Hunt et al., 2003).

### The use of soluble HLA-G in assisted reproduction

As it has become evident that at least some trophoblast cell populations secrete sHLA-G, much effort has been put into determining how early in fetal development it is possible to detect this protein. In particular, many studies have tried to link the detection of sHLA-G to obtaining of clinical pregnancy in assisted reproductive treatments (ARTs), as summarized in Table IV. From this, it is clear that several studies have correlated detection of sHLA-G in oocyte culture supernatant to successful pregnancies in ART procedures such as ICSI and IVF. While some studies report that no clinical pregnancies were achieved from negative sHLA-G cultures at all (Fuzzi et al., 2002; Noci et al., 2005), others simply find that pregnancy rate is decreased when using these embryos compared with embryos from sHLA-G positive cultures (Sher et al., 2004; Yie et al., 2005; Desai et al., 2006). Despite the great number of reports, questions have been raised on whether the detection of sHLA-G is an artefact (Ménézo et al., 2006). A problem posed here, is that no standardized recombinant sHLA-G protein has been available, and therefore, studies are forced to rely on estimations of protein levels detected. In a study by Rebmann et al., a purified sHLA-G standard for measuring protein levels was used, and it was only possible to detect sHLA-G in ~20% of the embryo cultures. In addition, it was found that sHLA-G correlated well with attainment of clinical pregnancy after IVF treatment and especially after ICSI (Rebmann et al., 2007). In the search for optimal experimental conditions, including the best combination of antibodies for ELISA, a study by Sageshima et al. (2007) failed to detect sHLA-G in any culture supernatants of fertilized eggs at Day 2–3 and Day 4–6. However, it was reported that some of the supernatants showed intensities between the background and the cut-off value of 1 ng/ml, and therefore it was concluded that a far more sensitive ELISA method is needed to confirm production of sHLA-G in early embryos. Another study found that only in one of three fertility treatment centres, sHLA-G could be associated with better implantation rates (Tabiasco et al., 2009). At this centre, only ICSI and not IVF treatment was carried out, supporting the work by Rebmann et al. described above. Since a higher number of sHLA-G positive embryo culture supernatants were found after IVF than after ICSI, and implantation rates were generally higher after ICSI than after IVF the study concluded that sHLA-G cannot predict pregnancy success alone, but multiple parameters such as type of ART procedure and oocyte culture medium also plays an important role. However, the detection level of this assay was evaluated to be 7 ± 2 ng/ml, and evidence indicates that lower detection levels are needed in order for sHLA-G to be a good predictor of pregnancy success. In addition, this study only measured implantation rates, whereas other studies examined the obtainment of clinical pregnancies, making comparison difficult. Overall, it is unlikely, that sHLA-G alone could be responsible for the obtainment and maintenance of pregnancy but it is more likely a factor among several that might be important. A study by Shaiky et al. analysed the sHLA-G expression in early embryos and showed that sHLA-G expression was variable.
but mainly localized to the trophoectoderm layer and projections from this, indicating a role during implantation, where fetal and maternal tissues are in close apposition (Shaikly et al., 2008). Evidence points towards the fact that sHLA-G can be one of the useful tools when selecting embryos for transfer in fertility procedures, and for this reason resources are put into developing ELISA methods of higher specificity and sensitivity for the future goal of sHLA-G serving as a non-invasive biomarker, increasing the chances of successful pregnancies in couples undergoing ART procedures. A recent meta-analysis states that in samples of good embryo quality, detection of sHLA-G is a much better prognostic factor for obtaining clinical pregnancy than is the case when examining embryos of mixed quality (Vercammen et al., 2008). A German multicenter study supports this finding, showing that morphological grading is the most powerful predictor of pregnancy, but that detection of sHLA-G is also a powerful tool, with better prognostic value than factors such as age, prior ARTs and number of embryos transferred (Rebmann et al., 2010).

### Table IV Association between soluble HLA-G levels in early embryo cultures and pregnancy success.

<table>
<thead>
<tr>
<th>Study</th>
<th>Antibodies for detection of sHLA-G</th>
<th>Number of patients</th>
<th>Procedure</th>
<th>Overall pregnancy rate (%)</th>
<th>Pregnancy rate in sHLA-G positive embryo cultures</th>
<th>Pregnancy rate in sHLA-G negative embryo cultures</th>
<th>Significant association</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fuzzi et al. (2002)</td>
<td>MEM-G/9 and W6/32</td>
<td>101</td>
<td>IVF/ICSI</td>
<td>18</td>
<td>24%</td>
<td>0%</td>
<td>Yes (P = 0.0026)</td>
</tr>
<tr>
<td>Sher et al. (2004)</td>
<td>MEM-G/I and W6/32</td>
<td>201</td>
<td>ICSI</td>
<td>51</td>
<td>Women &lt; 39 years: 71%; Women &gt; 39 years: 52%</td>
<td>Women &lt; 39 years: 22%; Women &gt; 39 years: 15%</td>
<td>Yes; (&lt;39 years: P &lt; 0.0001); (&gt;39 years: P &lt; 0.003)</td>
</tr>
<tr>
<td>Noci et al. (2005)</td>
<td>MEM-G/9 and W6/32</td>
<td>66</td>
<td>IVF/ICSI</td>
<td>14</td>
<td>23%</td>
<td>0%</td>
<td>Slight (P = 0.045)</td>
</tr>
<tr>
<td>Yie et al. (2005)</td>
<td>4H84 and 3C/G4</td>
<td>137</td>
<td>IVF</td>
<td>37</td>
<td>48%</td>
<td>17%</td>
<td>Yes (P = 0.0026)</td>
</tr>
<tr>
<td>Desai et al. (2006)</td>
<td>Not informed</td>
<td>83</td>
<td>ICSI</td>
<td>55</td>
<td>64%</td>
<td>36%</td>
<td>Yes (P &lt; 0.05)</td>
</tr>
<tr>
<td>Rebmann et al. (2007)</td>
<td>MEM-G/9 and anti-β2m</td>
<td>313</td>
<td>IVF/ICSI</td>
<td>25</td>
<td>40% After ICSI: 42%; After ICSI: 18%</td>
<td>(P &lt; 0.001). Yet, after ICSI (P = 0.0001)</td>
<td>Not overall, implantation rate in one centre (P = 0.034) (Only ICSI performed here)</td>
</tr>
<tr>
<td>Tabiasco et al. (2009)</td>
<td>MEM-G/9 and W6/32</td>
<td>355</td>
<td>IVF/ICSI</td>
<td>33</td>
<td>34%</td>
<td>19%</td>
<td></td>
</tr>
<tr>
<td>Rebmann et al. (2010)</td>
<td>MEM-G/9 and anti-β2m</td>
<td>2364</td>
<td>IVF/ICSI (36 TESE/96 not specified)</td>
<td>31</td>
<td>41%</td>
<td>26%</td>
<td>Yes (P &lt; 0.001)</td>
</tr>
</tbody>
</table>

IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection; TESE, testicular sperm extraction.

**The presence of HLA-G in the male reproductive system**

Due to the findings that sHLA-G can be detected in early embryos, studies have been performed to examine whether HLA-G could have paternal origin and serve important functions during fertilization and implantation. HLA-G has been detected in human prostatic tissue in one study, and even though HLA-G1 mRNA was the most prominent transcript found, immunohistochemistry only revealed expression of HLA-G5 using the antibody 1-2C3 (Langat et al., 2006). On this basis, interest has also been directed towards whether seminal plasma could contain sHLA-G protein. There is evidence that sHLA-G is found in seminal plasma and that large individual variation in sHLA-G levels exist (Larsen et al., 2011). This study also found HLA-G expression in normal testis and epididymal tissues, but it is still unclear where in the male reproductive system the sHLA-G detected in seminal plasma originates from. However, detection of the protein in this fluid indicates that sHLA-G may serve immunoregulatory roles during fertilization and implantation. In the future, it will be interesting to determine sHLA-G levels in seminal plasma from men with fertility problems, to observe whether there is an association between sHLA-G concentrations in seminal plasma and pregnancy outcome in couples with unexplained infertility and RM.

Regarding possible functions carried out by sHLA-G, a study by Selmani et al. showed that HLA-G5 was required to expand the population of CD25+ CD4+ FoxP3 cells, and that lymphocytes subsequently exhibited hypo-responsiveness to allogenic stimuli, supporting the hypothesis that HLA-G5 induces tolerance through these regulatory T cells (Selmani et al., 2008). In addition, it has been observed that recombiant HLA-G5 and HLA-G6 molecules are able to induce a reduction in IL-10 secretion and a significant increase in TGF-β1 production in myelomonocytic cells (McIntire et al., 2004). This supports that sHLA-G present in seminal plasma could alter the immunological functions carried out by macrophages, leading to a decreased
immunological response. In addition, TGF-β has been shown to induce differentiation of naive CD25+CD4+ T cells into regulatory T cells expressing FoxP3 molecules, to induce proliferation of these regulatory T cells and to inhibit target cell lysis carried out by NK cells through these regulatory T cells, leading to the development of tolerance (Chen et al., 2003; Ghiringhelli et al., 2005a, b). In clinical studies, it has been shown that women experiencing RM have a reduced number of CD25+CD4+FoxP3 cells in peripheral blood, and in addition, these cells were shown to be functionally deficient, as a higher number of cells from these women were required to induce suppression of the immune response (Arruvito et al., 2007).

In addition, expression of FoxP3 transcripts in endometrial tissue was reduced in a group of women with unexplained primary infertility (Jasper et al., 2003). In a recent study, RM women were shown to have lower amounts of IL-2-inducible FoxP3 regulatory T cells in peripheral blood (Arruvito et al., 2010). Furthermore, RM women proved to have reduced amounts of IL-2 and TGF-β in sera compared with controls, but elevated levels of IL-6. The study showed that induction of these regulatory T cells were carried out through IL-2 signalling and STAT-5 leading to expression of FoxP3, and stimulation with IL-2 resulted in a much lower FoxP3 expression in RM women than in fertile controls, indicating a deficiency in this signalling pathway. A reduced immune suppression, in agreement with previous observations, was also detected (Arruvito et al., 2010). In summary, evidence point towards a complex interplay between both decidual and peripheral immune cells and the cytokines secreted, and further studies are needed to obtain a more concise understanding of these interactions.

**HLA-E alleles and expression in relation to pregnancy**

To assess whether HLA-E is important during pregnancy, studies have focused on the expression of this molecule at the materno-fetal interface. Both mRNA and protein products have been identified in a variety of cells, but surface expression of the protein is restricted to cells that express other HLA class I molecules able to provide a nonamer signalling sequence needed for directing the protein to the membrane, as shown in Fig. 2C (Lee et al., 1998a). Using first trimester placental and decidual tissues, King et al. (2000) found that trophoblast cells express HLA-E on the cell surface. Subsequently, Ishitani et al. (2003) found that HLA-E protein could be detected in all cell populations expressing either membrane-bound or soluble HLA-G including EVT, VT and ST cells. Another group only found HLA-E located intracellularly in VT cells, and only sporadic cytoplasmic expression of the protein in ST. In addition, HLA-E was only detected in some of the EVT cells, and this expression also seemed to be limited to the cytoplasm (Bhalla et al., 2006). This study also tried to identify a difference between placental expression of HLA-E in women with RM and fertile controls, but failed to show a significant correlation. Recent work has confirmed the expression of HLA-E on the surface of EVT cells but has failed to identify the protein in any other subpopulation at the materno-fetal interface (Apps et al., 2009).

Lately, a study by Shaikly et al. (2010) has shown that HLA-E is co-expressed with HLA-G at the surface of the trophoectoderm of Day 6 blastocyst preimplantation embryos, suggesting that both these molecules are important during implantation. However, the surface expression of HLA-E needs to be verified by other groups to validate this finding.

A few studies have also focused on the expression of HLA-E in the male reproductive system, but have only managed to identify transcripts in spermatoocytes, spermato.des and most recently in ejaculated sperm, whereas no HLA-E protein could be detected (Guillaudeux et al., 1996; Avendano et al., 2009). The latter study showed that the level of HLA-E mRNA was higher in a group of fertile men than in a group of men undergoing consultation for infertility. Furthermore, the mRNA products were detectable 24 h after ICSI, possibly indicating that the mRNA products may have a function during early embryo development.

To date, nine different alleles of the HLA-E gene have been described (http://www.HLA.allels.org). Only three variants can be distinguished at the protein level, and several groups have examined the frequency of these in different populations. This has led to the conclusion that the E*0104 (E*01:04) allele is very rare, and therefore, only two alleles are of practical importance: E*0101 (E*01:01:01:01/02:03) and E*0103 (E*01:03:01:01/02 or E*01:03:02:01 or E*01:03:03:04). These two alleles are separated on the basis of a non-synonymous substitution at codon 107 from arginine (HLA-ER) to glycine (HLA-EG) (Ohya et al., 1990; Geraghty et al., 1992; Gomez-Casado et al., 1997; Grimsley and Ober, 1997). Several studies have focused on the distribution of these two alleles in women experiencing RM (Table V). Only one study reported that the frequency of HLA-ER was significantly higher in women with RM than in fertile controls. The correlation was particularly strong in HLA-EG homozygous RM women (Tripathi et al., 2006). Pfeiffer et al. (2001) also reported an increased frequency of the HLA-ER variant in RM women, but the difference was not significant. Other studies however, failed to show this correlation but differed in selection of control individuals and by inclusion criteria for RM women, as seen in Table V (Steffensen et al., 1998; Kanai et al., 2001).

Providing a possible explanation for the increased frequency of HLA-ER in RM women is the functional differences at the protein level between this allele and HLA-EG. Strong et al. (2003) found, using flow cytometry, that the surface expression of HLA-E was much lower in cells transfected with HLA-EB than in cells transfected with HLA-EG, while the overall protein level did not differ. Because surface expression of HLA-E depends on a nonamer sequence, as described previously, peptide affinities of HLA-EG and HLA-ER have been compared. Using ELISA, it was shown that peptide affinity for a nonamer sequence derived from HLA-G was higher for HLA-EG than for HLA-ER. This correlated with the finding that thermal stability was higher in HLA-EG, and supported the hypothesis of a decreased surface expression on cells from individuals expressing the HLA-ER allele (Strong et al., 2003). Overall, this shows that decreased surface expression of HLA-E in persons carrying the HLA-ER variant could be a risk factor in the aetiology of RM, indicating that HLA-E has an important role in the maintenance of pregnancy.

However, at present there is no clear evidence that HLA-E is involved in the pathogenesis of RM, and further studies that include a higher number of patients are needed to clarify whether or not the HLA-ER variant occurs with an increased frequency in RM women. Furthermore, a consensus needs to be reached about the inclusion criteria used in these studies and the selection of control individuals.
**Table V** Association between HLA-E allelic variants and RM.

<table>
<thead>
<tr>
<th>Study</th>
<th>Number of cases</th>
<th>Inclusion criteria for cases</th>
<th>Number of controls</th>
<th>Inclusion criteria for controls</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steffensen et al. (1998)</td>
<td>82 women</td>
<td>≥ 5 RMs</td>
<td>150 healthy controls</td>
<td>None specified</td>
<td>No association</td>
</tr>
<tr>
<td>Kani et al. (2001)</td>
<td>30 couples</td>
<td>≥ 3 RMs</td>
<td>38 couples</td>
<td>≥ One normal pregnancy</td>
<td>No association</td>
</tr>
<tr>
<td>Pfeffer et al. (2001)</td>
<td>78 couples</td>
<td>≥ 3 RMs</td>
<td>52 healthy controls</td>
<td>≥ One normal pregnancy (No history of fertility problems)</td>
<td>No association, but in women with ≥ 5 RMs: Trend for E<em>0101 and E</em>0102 (E*01:01:01:01/02/03)</td>
</tr>
<tr>
<td>Tripathi et al. (2006)</td>
<td>120 women</td>
<td>≥ 3 RMs</td>
<td>120 women</td>
<td>≥ 3 normal pregnancies</td>
<td>E<em>0101 (E</em>01:01:01:01/02/03) (P = 0.043)</td>
</tr>
<tr>
<td>Bhalla et al. (2006)</td>
<td>45 placentas from RM women</td>
<td>Not informed</td>
<td>17 gestation matched placenta</td>
<td>From women undergoing elective terminations of pregnancy</td>
<td>No association in EVT</td>
</tr>
</tbody>
</table>

E*0102 has been shown to be identical with E*01:01:01:01.

**HLA-E in regulating NK cells during pregnancy**

The possible role of HLA-E as an immunomodulator during pregnancy came into view, as it was shown that cells transfected with HLA-E were able to inhibit cytolysis carried out by NK cells through the CD94/NKG2A receptor (Fig. 1; Lee et al., 1998; Borrego et al., 2002). This receptor is expressed by the CD56<sup>bright</sup> uNK cells and the inhibition requires surface expression of HLA-E and therefore also expression of another HLA class I molecule (Verma et al., 1997; Ashkar et al., 2003). Because HLA-G, and to a lesser extent HLA-C, are expressed on trophoblast cells, studies have focused on the co-expression of HLA-E with these two molecules (Moffett-King et al., 2002).

Performing cytotoxicity experiments, King et al. showed that antibodies against HLA-G could partially restore lysis, while this was not the case with antibodies against HLA-C, indicating that HLA-G is the most important molecule in providing the nonamer sequence needed for surface expression of HLA-E. Yet, these results were obtained from transfecant cell lines, and the study failed to demonstrate lysis of trophoblast cells carried out by uNK cells (King et al., 2000). While the CD94/NKG2A receptor was clearly shown to be involved in inhibition of cytolysis, it was shown that the HLA-E/G-nonamer peptide induced cytotoxicity very efficiently through the CD94/NKG2C receptor (Llano et al., 1998). However, whether this interaction takes place between HLA-E on trophoblast cells and the NKG2C receptor on uNK cells is still unknown. The induction of cytotoxicity through NKG2C might seem conflicting with the idea of HLA-E as an immunosuppressive molecule during pregnancy, and because no specific antibodies for the receptors have been available until recently, this paradox remains to be elucidated. Yet, several studies provide possible explanations. First, it has been reported that the inhibitory NKG2A receptor has much higher affinity for HLA-E than the activating NKG2C receptor, which could account for the overall response being inhibitory (Vales-Gomez et al., 1999).

Second, it has been shown that 98% of CD56<sup>bright</sup> uNK cells express the NKG2A receptor, while only 20% express the NKG2C receptor. In addition, all CD56<sup>bright</sup> uNK cells in the uterus, that express NKG2C also express NKG2A, in contrast to peripheral NK cells, where no simultaneous expression is observed, indicating that NK cells in decidua might have special regulatory functions on this basis (Kusumi et al., 2006). Third, it has been shown that NKG2A and NKG2C compete for binding to CD94 (Labonte and Letvin, 2004), and therefore also for surface expression, and this balance could be a reflection of the total lytic activity. In addition, the function of CD56<sup>bright</sup> uNK cells in general might be cytokine secretion and regulation rather than cytotoxicity, since these cells have been shown to be the main cytokine-producing cells in the decidua (Cooper et al., 2001).

Peptide substitutions at P5, P6 and P8 of the HLA-G nonamer sequence have been shown to impair recognition by HLA-E and therefore also impair surface expression (Miller et al., 2003; Hoare et al., 2008). On this basis, it would be interesting to try to identify possible amino acid substitutions in the nonamer sequence provided by HLA-G in couples experiencing fertility problems, to assess whether the inhibition of cytotoxicity through the interaction of CD94/NKG2A on uNK cells and HLA-E on trophoblast cells is a fundamental factor in obtaining pregnancy.

**Ambiguity of the basic role of HLA-F in reproduction**

HLA-F was discovered in 1990 and remains the least studied of the HLA class Ib molecules (Geraghty et al., 1990). This gene also exhibits low polymorphism, and to date only four variants at the protein level have been identified (http://www.HLA.alleles.org). In addition, a nonsense mutation in exon 3, resulting in a HLA-F null allele has been found, but only in heterozygous individuals, and no functional importance of this allele has yet been reported (Uchigiri et al., 1997). Furthermore, a synonymous mutation has been found in exon 2 but since this
The HLA-F unique RxR motif is responsible for localizing the protein to the endoplasmic reticulum (ER), and that this is different from those of other HLA molecules. As shown by Shobu et al. (2003), the synthesis and posttranslational modifications of HLA-F have been examined first trimester placenta for surface expression, and not tissues from later stages of gestation (Nagamatsu et al., 2006). Subsequently, Nagamatsu et al. found that HLA-F was only expressed in the cytoplasm of first trimester EVT cells at low levels, but during the second, and particularly the third trimester, the protein level was increased and surface expression observed. Furthermore, they found a weak expression of HLA-F in VT and ST cells with no differences in expression levels during the course of gestation. By flow cytometry, it was shown that only ~16% of the cells expressed HLA-F on their surface (Shobu et al., 2006). Unexpectedly, Apps et al. examined decidual and placental tissue at term and from elective terminations of normal pregnancies, and detected HLA-F mRNA, but failed to confirm any protein expression. They used a different antibody against HLA-F than the other groups, and they suggest that the weak expression of HLA-F that has been observed in previous studies was in fact not expression by trophoblast cells, but possibly uNK cells (Apps et al., 2008b). It is apparent that the confusion about the expression of HLA-F at the materno-fetal interface could be due to insufficient characterization of the cells, or cross reactivity of some of the antibodies with other (non-classical) HLA molecules.

A novel role of HLA-F: possible implications for pregnancy

In conclusion, the data on HLA-F are contradictory and while it seems likely that the protein is expressed on the surface of cells at the materno-fetal interface, a possible role in developing tolerance during pregnancy is still in question. A recent important study by Lee et al. (2010) used a different approach in trying to elucidate the functions of this protein. They examined the distribution of HLA-F in lymphocytes and found that all resting B cells, T cells, NK cells and monocytes express HLA-F within the cells, but upon activation, surface expression is induced, as shown by flow cytometry. An intriguing fact is that surface expression is not up-regulated in the regulatory T cells. This led the authors to hypothesize that HLA-F might be involved in regulating the immune response during pregnancy by serving as a marker of maternal activated lymphocytes, interacting with the regulatory T cells making them secrete inhibitory cytokines and induce suppressive signalling, which will result in the generation of tolerance (Fig. 1). In this regard, studies have shown that the number of regulatory T cells might be reduced in women experiencing spontaneous miscarriage compared with women with successful pregnancies (Sasaki et al., 2004). It is mandatory to examine whether HLA-F might bind to any receptors on the surface of these regulatory T cells.

New aspects of the function of HLA-F

Characterization of possible receptor interactions of HLA-F have been sparse, but the protein has been demonstrated to interact with ILT-2 and ILT-4 on the surface of monocytes and CD19+ B cells, but not on CD56 NK cells or CD3+ T cells (Fig. 1). The interaction could only be inhibited partially by adding antibodies against ILT-2 and ILT-4, suggesting that HLA-F might also interact with other receptors on the surface of these cells. The tetramers used in this study were prepared without peptide ligands, and at present there is no knowledge to whether specific peptides binding to HLA-F may induce surface expression or may result in interactions with different sets of receptors, as it is the case for HLA-E (Lepin et al., 2000).

Possible interactions of HLA-F with relevant receptors

The synthesis and posttranslational modifications of HLA-F have been shown to be different from those of other HLA molecules. As shown in Fig. 2C, it has been found that the cytoplasmic tail of HLA-F is essential for exportation from the endoplasmic reticulum (ER), and that the HLA-F unique RxR motif is responsible for localizing the protein to the Golgi complex where it seems to accumulate (Boyle et al., 2006). Contrary to other HLA-class I molecules, HLA-F surface expression in vitro seems to be independent of TAP and partially independent of tapasin, even though the molecules have the ability to bind peptide in the ER (Lepin et al., 2000; Wainwright et al., 2000; Lee and Geraghty, 2003). This indicates that surface expression of HLA-F might take alternative routes compared with other HLA class I molecules.

Significance of HLA-C

Although HLA-C belongs to the classical HLA class Ia molecules it has similarities with the non-classical HLA molecules in relation to pregnancy success. HLA-C is a highly polymorphic molecule, which is expressed ubiquitously like HLA-E. HLA-C is expressed on the surface of EVT cells both as β2m-associated molecules and as free heavy chains. The expression of HLA-C on EVT generally seems to be low, but can be up-regulated by IFN-γ (King et al., 1996, 2000). To date, no expression of the polymorphic HLA-C in VT or ST cells has been reported.

Regarding receptor interactions, HLA-C has been shown to interact with KIR2D receptors, which are expressed on the surface of uNK cells. Given the high degree of polymorphism of both KIR and HLA-C, many different receptor interactions are possible and will vary with each pregnancy. Basically, HLA-C molecules can be divided into two groups regarding interaction with KIR receptors. HLA-C1 molecules have serine at codon 77 and asparagine at codon 80 and include HLA-Cw1, -Cw3, -Cw7, -Cw8, -Cw12, -Cw14 and...
of serving as an important diagnostic tool when examining couples explained infertility. On this basis, it is clear that HLA-G has the potential negative results in ART procedures in couples experiencing unexplained miscarriage, and that there is a correlation between these gene variants and medias available, this can be carried out as a routine procedure serving to optimize the results in ART treatments. Newfound interest also lies in determining which role paternal sHLA-G levels in seminal plasma might have for the development of pregnancy complications, and in the future it will be interesting to examine both male and female levels of sHLA-G in couples with fertility problems to try to clarify the importance of this factor in the pathogenesis of these conditions.

A challenge posed in determining the role of HLA-G and the other class lb molecules in the pathogenesis of RSA is the two parallel lines of studies in this field. On one hand, basic research on immune cells and cytokines try to clarify the receptor interactions and mechanisms resulting from these proteins, and on the other hand, clinical studies examine HLA alleles, polymorphisms and protein levels in couples experiencing RSA and unexplained infertility, to try to determine a correlation. Although it is not possible to quantify immune cells and cytokines in decidua in these patients to obtain a more integrated image of the complex immunological interplay during pregnancy, one must always remember to use the knowledge from basic research in clinical studies and vice versa.

Conclusions and future perspectives
At this point it is clear that the HLA class Ib molecules along with HLA-C are expressed at the materno-fetal interface. At the same time many transfection studies and studies on interactions with peripheral immune cells show that these three proteins and HLA-C interact with different receptors on immune cells resulting in immunomodulation. Because of this, it is apposite to believe that these proteins carry out immunoregulatory functions that serve to induce tolerance to the semi-allogenic fetus during pregnancy. However, at present, studies examining how isolated trophoblast cells and immune cells from decidua interact are sparse, and more experiments need to be carried out to clarify the specific underlying immunoregulatory mechanisms that take place during pregnancy.

Clinical studies have examined the different HLA alleles and polymorphisms and how these might be linked to pathological disturbances in pregnancy such as RM and unexplained infertility. At present, no consensus has been reached in this area, but results indicate that some HLA-G alleles and polymorphisms are risk factors in RM, and that there is a correlation between these gene variants and negative results in ART procedures in couples experiencing unexplained infertility. On this basis, it is clear that HLA-G has the potential of serving as an important diagnostic tool when examining couples undergoing infertility treatment. Today, different experimental studies agree that presence of sHLA-G is a positive prognostic factor when transferring embryos in IVF treatments. As soon as a good quantitative method for measuring sHLA-G levels in IVF

Authors’ roles
M.D. conceived and designed the study, identified the articles, acquired and analysed the data, drafted the first version of the manuscript and revised the manuscript. T.V.F.H. conceived and designed the study, identified the articles, acquired and analysed the data and revised the manuscript.

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### Appendix

#### Box 1  Mechanisms to avoid immunological recognition and rejection of the fetus by the maternal immune response.

<table>
<thead>
<tr>
<th>Altered expression of HLA:</th>
<th>Absence of the polymorphic HLA-A and HLA-B, and low expression of HLA-C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Th1/Th2 shift:</td>
<td>Although a simplified concept, it seems that the maternal immune system shifts to a less cytotoxic profile during pregnancy</td>
</tr>
<tr>
<td>Altered characteristics of immune cells in decidua:</td>
<td>CD56bright uNK cells with low cytotoxicity and high secretion of regulatory cytokines</td>
</tr>
<tr>
<td>Decidual iNKT cells:</td>
<td>Decidual macrophages displaying decreased amounts of the T lymphocyte co-stimulatory molecules CD80 and CD86</td>
</tr>
<tr>
<td>Expression of CD4+CD25+FoxP3 Treg cells:</td>
<td>Induce tolerance by inhibiting proliferation and function of T cells, reduce NK cell cytotoxicity and prevent dendritic cell maturation</td>
</tr>
<tr>
<td>IDO:</td>
<td>Expressed by T regulatory cells and decidual macrophages, metabolizing tryptophan needed for T cell activation</td>
</tr>
</tbody>
</table>

#### Box 2  Cell types in decidua and their functions.

<table>
<thead>
<tr>
<th>Cell type:</th>
<th>Amount of total leukocyte count (%)</th>
<th>Function and pathology:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uterine NK cells</td>
<td>~70</td>
<td>Reduce cytotoxicity through the receptors ILT2 and -4, KIR2DL4 and CD94/NKG2A that interacts with HLA-E, -F and -G</td>
</tr>
<tr>
<td>Macrophages</td>
<td>~20</td>
<td>Expression of IDO to decrease T-cell activation</td>
</tr>
<tr>
<td>T cells:</td>
<td>~10</td>
<td>Low expression of T cell co-stimulatory molecules</td>
</tr>
<tr>
<td>CD3+CD8+</td>
<td></td>
<td>Disturbances of a CD3+/CD8+/CD3−/CD8− ratio correlated with RM and unexplained infertility</td>
</tr>
<tr>
<td>CD3+CD4+</td>
<td></td>
<td>Reduced number of iNKT cells correlated with RM</td>
</tr>
<tr>
<td>CD3+CD56+ (iNKT)</td>
<td></td>
<td>Reduced number of CD25+ CD4+FoxP3 cells in peripheral blood, and functional deficiency of these cells correlated with RM</td>
</tr>
<tr>
<td>CD25+CD4+FoxP3 cells</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>