Why natural killer cells are not enough: a further understanding of killer immunoglobulin-like receptor and human leukocyte antigen

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The immune system's role in recurrent reproductive failure is a controversial issue in assisted reproduction. Most studies into immune system implication in reproduction have focused on finding markers of peripheral blood and less on the uterine environment. Peripheral blood natural killer cells have become an "immune study core" for women with recurrent miscarriage or recurrent implantation failure, based on the mistaken notion that they cause reproductive failure by killing or "rejecting" the embryo. Maternal-fetal tolerance begins at the uterine level, so successful adaptation to the fetus occurs after a complicated process. Insufficient uterine lining invasion by an invading extravillous trophoblast is the primary defect in pregnancy disorders such as recurrent miscarriage. This process is regulated by the interaction between maternal killer immunoglobulin-like receptors (KIRs), expressed by uterine natural killer cells (uNK), and their ligand human leukocyte antigen (HLA) C, expressed by the extravillous trophoblast. Pregnancies are an increased risk of disorders in mothers with KIR AA when the fetus has paternal HLA-C2. A recent report has indicated that the expression of more than one paternal HLA-C by the extravillous trophoblast in assisted reproduction may affect placentation in mothers with KIR AA. This review provides insight into the immune system's role in assisted reproductive treatments. These insights can have an impact on the selection of single-embryo transfer and/or oocyte/sperm donor according to HLA-C in patients with recurrent implantation failure and recurrent miscarriage depending on their KIR haplotype. (Fertil Steril® 2017;107:1273–8. ©2017 by American Society for Reproductive Medicine.)
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In recent decades, substantial progress has been made to improve the outcome of assisted reproductive technologies (ART). Our knowledge about folliculogenesis, in vitro embryo culture and their chromosomal composition, and endometrial receptivity has vastly improved in recent years. Nevertheless, a high percentage of embryos (50%) are still lost immediately after implantation or shortly after as clinical miscarriage. One study (1) has reported a 52% cumulative live birth rate (LBR) after transferring up to five embryos, and another an LBR of 79% after transferring 15 embryos. Chromosome screening and fresh embryo transfer significantly increases in vitro fertilization (IVF) implantation and delivery rates, but we still lose euploid embryos (2, 3).

What happens to those embryos? Other factors, not just embryo aneuploidies—by far, the main contributor to embryo losses—might contribute to implantation failure or miscarriage, such as endometrial factors, hydrosalpinges, infections, or immune maternal tolerance to pregnancy. At the same time, owing to repeated failed cycles even after gamete donation, we all have witnessed increasing patient demand for immune tests and "immune treatments." Although this patient demand may be unjustified, we need to understand if there is a rationale behind using it or not.

The role of the immune system in recurrent miscarriage (RM) and recurrent implantation failure (RIF) is one of the most controversial issues in ART (4). Controversy is partly due to the fact that most studies of the immune system in reproduction have focused on finding markers of peripheral blood (5, 6) and quick solutions using different immunomodulation lines (7, 8). The main reason why immune treatments have failed so far, and why immune tests (peripheral blood natural killer [pbNK] or uterine natural killer [uNK] cell testing) have

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shown very weak or no predictive values, is poor study design and great patient heterogeneity (4, 9).

As we know, maternal tolerance begins at the uterine level, and successful adaptation to the semiallogeneic fetus is more complicated than the initially suggested concept led us to believe.

Peripheral blood natural killer (NK) cells have become an "immune study core" for women with RM or RIF, based on the mistaken notion that they cause reproductive failure by killing or "rejecting" the embryo.

Some reports have presented an overview (5), e.g., that pbNK and uNK cells merge together with the simple "NK cells" marker as the "main immune cells at the maternal-fetal interface." From an immunologic point of view, this is an erroneous judgment, because pbNK cells and uNK are completely different types of immune cells (10, 11). pbNK cells are cytotoxic, represent the first line of defense against viruses, tumors, and damaged cells, and are not trained to "reject" or kill healthy embryos.

In peripheral blood, NK cells are considered to be a heterogeneous population, formed by various subsets with a differing function, surface phenotype (90% are $CD56^{dim}CD16+$ and 10% are $CD56^{bright}CD16-$), and anatomic localization (12–14).

Killer immunoglobulin-like receptors (KIRs) determine the NK cell function in the context of other receptor-ligand interactions and permutations of 28 NK cell receptors, which result in at least 10,000 different NK cell subsets in a given individual (11, 15, 16). Furthermore, the KIR repertoires of uNK and pbNK differ when taken from the same woman at the same time.

Uterine NK cells show a prevalence of the $CD56^{bright}CD16-$ NK cell subset, whose activity is influenced by the KIR repertoires, and differ absolutely from pbNKs in phenotype markers and functional activity (10, 17).

The uNK killing function is very weak compared with pbNK (18). With infections, this can change as the CMV infection elicits a different uNK effector function. uNK cells are capable of controlling cytomegalovirus (CMV) infection and acquiring the cytotoxic phenotype against CMV-infected decidual fibroblasts by means of receptor repertoire modulation (19), but this process has not been shown against a healthy embryo.

Uterine NKs acquire their functional properties in utero, are predominant in the nonpregnant endometrium, and increase and change their morphology during the secretory phase of the menstrual cycle. uNKs taken at 8–10 weeks of gestation more frequently present receptors that bind extravillous trophoblast (EVT) human leukocyte antigen (HLA) C (KIR2DL1/S1+ and KIR2DL2/3/S2+) than pbNK cells (15, 20). This dominance in the decidua of uNKs that express HLA-C–binding KIR is not so obvious in the endometrial uNK isolated from nonpregnant women, which is suggestive of repertoire adaptation to pregnancy (15).

Uterine NKs proliferate and differentiate in the specialized progesterone-dominated microenvironment and under endometrial-derived interleukin-15 (16). Early in pregnancy, uNKs infiltrate the trophoblast by controlling trophoblast invasion and remodeling uterine spiral arteries, which increases the contact area between maternal blood and trophoblast cells, the main process for healthy placenta development (12). Thus, the increased number of uNKs in the secretory phase of the menstrual cycle and pregnancy (90% of local immune cells in the first trimester of pregnancy) is a physiologic process that focuses on helping embryo implantation and is not a marker of "embryo rejection."

The number and function of pbNK and uNK cells show wide variability depending on the patient's clinical condition, e.g., infections, autoimmunity or tumor, day of menstrual cycle, treatment condition (ovarian stimulation), stress, time of day, exercise, etc. Studies of NK cells and reproductive issues have not taken this NK-cell physiologic variation into account, nor differences in pbNK and uNK cell receptors, whose activation is essential for its function: So, which of the 10,000 different NK cell subsets have been studied?

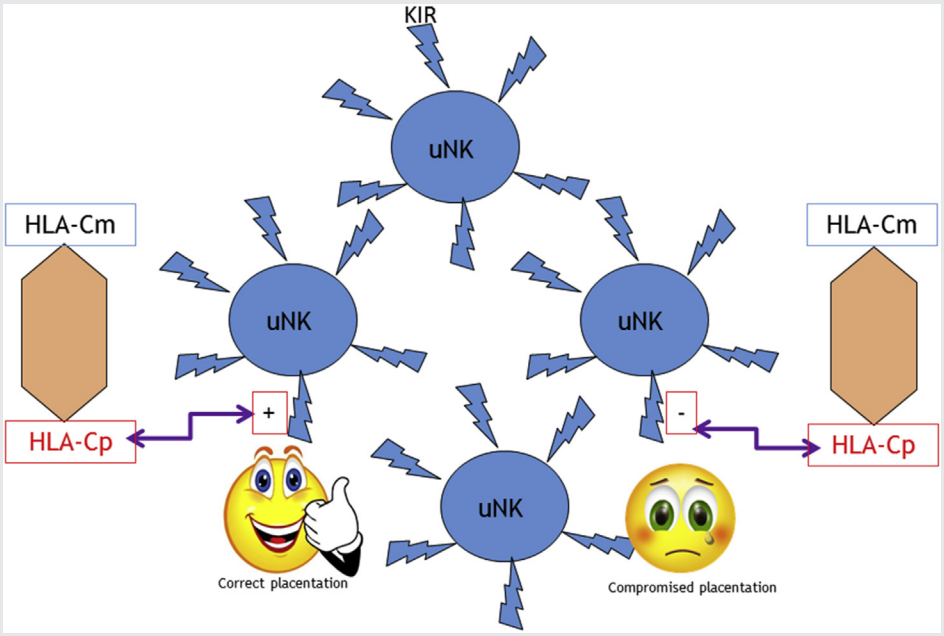
Using "NK cells" to describe and use these contrasting subsets of NK cells at a random time, characterized only by their surface phenotype, as a unique immune marker in women with infertility or disorders of pregnancy only adds more confusion about the immune role and tests in ART.

NEVERTHELESS, WHAT IS THE REAL IMMUNE SYSTEM FUNCTION IN REPRODUCTION?

The fetal cells that come into direct contact with the mother's immune system in the uterus are trophoblast cells, the layer that surrounds the blastocyst (21, 22), and the mother's uterine immune system is dominated by uNK cells (23), $CD56^{bright}CD16-$, the most abundant leukocyte population during the first trimester of human pregnancy (24).

Maternal and fetal circulations do not mix, although a transient exchange of cells occurs, particularly during the trauma of delivery (12). Successful maternal adaptation to the semiallogenic fetus occurs in the uterus at the site of placentation. The key to the maternofetal tolerance process is the remodeling of the spiral arteries, with destruction of the media by invading EVT cells. EVT cells express class I HLA-C and nonclassic HLA-G and HLA-E antigens, whereas class I antigens HLA-A and HLA-B and class II antigens are absent (25, 26). The villous trophoblast bathed in maternal blood is entirely HLA-null. Nevertheless, HLA-E and HLA-G are oligomorphic, the HLA-C molecules expressed by EVT cells are polymorphic, and ligands for KIRs are expressed by uNK cells (27). The EVTs that invade the maternal decidua are of fetal origin, and express high levels of HLA-C that is recognized by uNK KIRs. Maternal and paternal HLA-C allotypes, are expressed at the same time and at high levels on the EVT cell surface. KIRs are the most variable receptors in uNKs, with diversity in gene number between individuals and allelic diversity at individual KIR loci (27). Both polymorphic maternal KIRs and fetal HLA-C molecules are variable and specific to a particular pregnancy (12). In any pregnancy, the maternal KIR genotype could be AA (nonactivating KIRs), AB, or BB (one to ten activating KIRs) (28). The HLA-C ligands for KIRs are divided into two groups: HLA-C1 and HLA-C2. Of the two, C2 is a stronger ligand than C1 (29). Haplotypes A contain mainly genes for inhibitory KIR, and haplotypes B have additional genes that encode activating KIR. Presence of activating KIR2DS1 (B haplotype) confers protection from pregnancy disorders (30), and its absence (A

FIGURE 1



Placentation is regulated by interactions between maternal killer immunoglobulin-like receptors (KIRs), expressed by uterine natural killer cells (uNKs), and fetal human leukocyte antigen (HLA) C molecules, expressed by extravillous trophoblasts. Maternal and paternal HLA-C (HLA-Cm and HLA-Cp, respectively) are non-self ("foreign"); + = activating KIR; − = inhibitory KIR.

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haplotype) increases the risk of pregnancy complications (12, 20, 31). A similar protective effect, has been recently described for another activating receptor, KIR2DS4, when carried together with KIR2DS1 (32). Similarly to KIR2DS1, the triggering of KIR2DS4 to uNK cells leads to the secretion of granulocyte-macrophage colony-stimulating factor and other chemokines known to promote placental trophoblast invasion (32).

Placentation is regulated by interactions between maternal KIRs expressed by uNKs, and fetal HLA-C molecules, expressed by EVTs (33, 34) (Fig. 1). Hiby et al. showed that invading EVTs are the principal site of HLA-C expression in the decidua basalis and that both maternal and paternal HLA-C allotypes are presented to KIRs (30, 35). Insufficient invasion of the uterine lining by trophoblasts and vascular conversion in the decidua are thought to be the primary defect in disorders such as RM, preeclampsia, and fetal growth restriction (FGR) (29). This process is regulated by the interaction between maternal KIRs, expressed by the uNKs, and their ligand HLA-C, expressed by EVTs (Fig. 2).

Pregnancies are at increased risk of RM, preeclampsia, or FGR in mothers who are homozygous for KIR haplotype A (KIR AA) when the fetus has more HLA-C2 genes than the mother and when additional fetal HLA-C2 alleles are of paternal origin (30). Protection from preeclampsia is likely mediated by activating KIR2DS1 (B haplotype), which also binds HLA-C2. Thus, depending on the particular KIR–HLA-C interaction, is trophoblast cell invasion regulated.

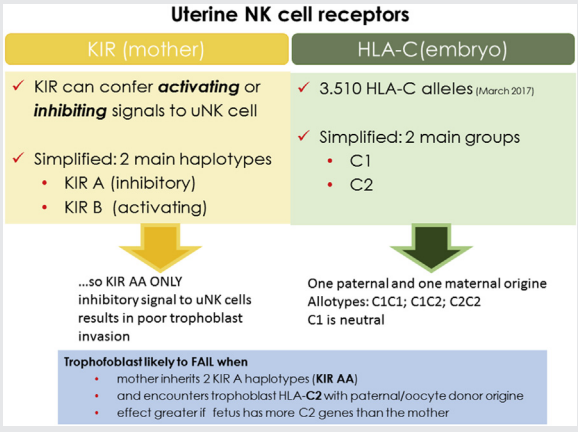
Hiby et al. (34) and Faridi and Agrawal (36) have reported differences in the outcomes of medically unassisted pregnancies, increased risk of RM, preeclampsia, and FGR, in mothers with KIR AA who carry a fetus with paternal HLA-C2. These findings suggest that placentation is deficient when a very strong inhibitory signal to uNK cells mediated by the KIR A haplotype gene exists. Hiby et al. (30,33–35,37) performed larger cohort studies that analyzed both maternal and paternal genotypes along with a large control group. They demonstrated a clear difference between the KIR and HLA-C genotypes in patients with disorders such as RM, preeclampsia, and FGR. Epidemiologic studies have provided clear evidence that selection for human reproductive success has adapted to KIR and HLA-C genes and could be responsible for maintaining balanced polymorphisms between the HLA-C1 and HLA-C2 groups and the A and B KIR haplotypes (23, 35, 38, 39).

HOWEVER, WHAT HAPPENS IN ART?

Assisted pregnancies differ from medically unassisted pregnancies because the patients often receive more than one embryo per transfer and donor oocytes, sperm, or embryos also are often used.

After double-embryo transfer (DET), the expression of more than one paternal HLA-C per trophoblast cell is induced. In oocyte-donation cycles, which are increasingly demanded owing to advanced maternal age, oocyte-maternal HLA-C, which is genetically different from the mother's receptor, behaves as a paternal HLA-C. This implies that more non-self

FIGURE 2



KIR–HLA-C system. Abbreviations as in Figure 1.

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HLA antigens are presented to the maternal KIR per transfer compared with "normal" pregnancies.

After DET in an oocyte-donation cycle, the expression of two non-self or "paternal" HLA-Cs in the EVTs per embryo is present in the decidua basalis. The presentation of trophoblast antigens (HLA-C) to uNK KIRs happens much more frequently than in natural pregnancies, because embryo transfer (ET) can be performed monthly in RIF patients.

In human populations, pregnancy disorders are predicted to reduce the frequency of KIR A, HLA-C2, or both, and this selection is thought to have originated during human evolution (27, 38, 39). An inverse correlation between frequencies of KIR AA and HLA-C2 has observed. Populations with the highest KIR AA frequency (Japanese and Koreans) present the lowest HLA-C2 frequencies, whereas populations with the lowest KIR AA frequency (Aboriginal Australians and Asian Indians) have the highest HLA-C2 frequencies. Natural selection seems to have driven an allele-level KIR A haplotype and HLA-C1 ligand to an unusually high frequency in Japanese and Koreans, because the detrimental KIR AA–HLA-C2 combination does not significantly affect pregnancy outcomes in those populations (40).

This correlation provides evidence that selection for human reproductive success has adapted to the KIR and HLA-C genes and could be responsible for maintaining balanced polymorphisms between the HLA-C1 and HLA-C2 groups and the A and B KIR haplotypes (27, 35, 38, 39).

However, this natural human evolution is not currently taken into account during ART. Furthermore, donor oocytes are often used in ART, and the literature describes higher maternal morbidity (pregnancy-induced hypertension, 25% for preeclampsia, FGR) (41) and preterm birth in oocyte-donation pregnancies compared with ART pregnancies with own oocytes (42, 43). Although part of this increased frequency of complications may be due to the main indication for oocyte donation, which is advanced maternal age, recent age-matched data have confirmed this higher frequency of undesired events in young patients, and immunology maladaptation could be the reason (42, 44, 45).

Increased paternal HLA-C expression after DET could be associated with more pregnancy disorders than single-embryo transfer (SET) in mothers with an inhibitory KIR haplotype (AA). A recent study (46) has analyzed pregnancy, miscarriage, and the LBR per cycle according to KIR haplotype, categorized by DET or SET. A higher early miscarriage rate after DET when the patient's own oocytes were used occurred in those with KIR AA (22.8%), followed by those with KIR AB (16.7%), compared with mothers with KIR BB (11.1%; $P<.03$). A significantly decreased LBR per cycle after DET with the use of donated oocytes was observed in mothers with KIR AA (7.5%) compared with those with KIR AB (26.4%) and KIR BB (21.5%; $P<.006$) (46).

The lower LBR after DET in donor-oocyte cycles in KIR AA mothers may be due to an increased expression of non-self HLA-C (paternal and oocyte donor HLA-C). In this case, four "paternal" HLA-Cs per trophoblast cell per DET: one paternal and one oocyte-donor HLA-C per trophoblast cell and embryo because oocyte-donor HLA-C behaves as "paternal" non-self HLA-C. Expressing four "paternal" HLA-Cs is more likely to find at least one paternal or oocyte-donor HLA-C2 (by allelic frequency) than in own oocytes and SET, and implantation or placentation failure is more likely to occur in KIR AA mothers.

No other report has studied the impact of KIR–HLA-C on donor-oocyte cycles. The authors speculated that completing a normal pregnancy was possible only for mothers with the KIR AA haplotype who carried a baby with a least one non-self HLA-C1. Recently, they performed a prospective study (47) that included 30 women with unknown etiology of RIF and RM who underwent oocyte-donation assisted reproductive cycles. All of the women had a KIR AA genotype and their partners had HLA-C2 genes. They underwent 54 ET cycles (82.76% DET, 17.24% SET) with unknown HLA-C oocyte donors and 28 cycles with HLA-C1C1 donors (21.05% DET, 78.95% SET). Pregnancy, miscarriage, and LBR per cycle after ET have been studied with unknown oocyte donor HLA-C and after transfers with HLA-C1C1 oocyte donors.

A higher pregnancy rate per cycle after HLA-C1C1 oocyte donor transfer (85.71%), compared with unknown HLA-C oocyte donor cycles (31.48%), was observed in the same KIR AA patients with the same HLA-C2 partners ($P<.0001$). A higher miscarriage rate per cycle after unknown HLA-C oocyte donor transfer (94.44%) was observed compared with HLA-C1C1 oocyte donor transfer (8.33%; $P<.0001$). A significantly increased LBR per cycle was noted after ET with an HLA-C1C1 oocyte donor (82.14%) compared with the LBR in the same KIR AA patients and HLA-C2 partners after cycles with unknown HLA-C oocyte donors (0%; $P<.0001$). Another recent study (48) has reported that the proportion of pregnancies that end in loss is significantly higher in KIR AA patients when a C2C2 euploid embryo is transferred than when C1C1 or C1C2 embryos are transferred (33% vs. 12.3% vs. 14.1%, respectively; $P<.01$).

These new findings show that maternal KIR haplotype and fetal HLA-C have an impact on LBR after IVF cycles, especially when donor oocytes and DET are used. Expressing four paternal

HLA-Cs in EVT cells after DET with the use of donor oocytes is more likely to result in at least one non-self HLA-C2 (even with the HLA-C2 allelic frequency in a white population) than with the use of one's own oocytes after SET, and implantation or placentation failure is more likely to occur in mothers with the KIR AA haplotype. Therefore, selecting HLA-C1 among oocyte and/or sperm donors for patients who undergo oocyte donation and who express inhibitory KIR haplotypes could be more efficient and safer. The authors assume that it is a small sample and that it is the first report to observe differences in LBR according to oocyte donor HLA-C in KIR AA mothers with HLA-C2 partners. Apart from statistical significance, the association strength is noticeably high, which confers greater confidence to the findings. However, larger studies are needed and should be replicated by other groups before finally accepting and applying this theory to routine clinical practice (49).

A new concept is emerging as evidence indicates important physiologic roles for uNK cells in healthy placentation as well as for abnormal uNK cell function in pregnancy disorders. The combination of maternal KIR haplotype and parental/donor HLA-C could predict which couples can benefit from SET versus DET or from donor selection according to HLA-C to increase the LBR per cycle and would help to reduce the number of embryos transferred by facilitating the increase in elective SET. Therefore, selecting HLA-C1 among oocyte and/or sperm donors for patients who undergo oocyte donation ART and inhibitory KIR could be more efficient and safer, as epidemiologic studies have identified (12, 30, 50).

CONCLUSION

A new concept is emerging in that the uterine immune system uses NK cell allorecognition to regulate placentation and to control the maternofetal interface. In ART, these new insights (46, 47) could have an impact on elective SET selection in patients with RM or RIF and a KIR AA haplotype. Although data are still premature and need to be validated (49), they could be of clinical significance. They could help with oocyte and/or sperm donor selection according to HLA-C in patients with RM or RIF and a KIR AA haplotype, because HLA-C1C1 donors are predicted to be safer, and C2C2 sperm or oocyte donors may be more "dangerous," according to epidemiologic studies (30, 50).

This is a new concept and, based on it, it is reasonable to think that the use of different lines of immune therapies (e.g., intralipid, "antipaternal immunization," intravenous immunoglobulin, prednisolone, tumor necrosis factor α blockers), to reduce NK cell activity in infertile women has to be reconsidered (51) because the scientific principle of maternofetal tolerance has been misunderstood.

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