CHAPTER I

INTRODUCTION

1.1 Introduction:

Pregnancy is a complex phenomenon where the maternal immune system accepts the allogenic fetus, while retaining the ability to mount an immune response against potential pathogens (1-3). Immune dysfunction at the maternal-fetal interface has been linked to early pregnancy failure (4-6). Spontaneous abortion (SAB), defined by the loss of a fetus before twenty weeks of gestation is one of the most crucial early pregnancy complication affecting 10% of clinically confirmed pregnancies (7,8). Repeated occurrence of two or more SAB is termed as recurrent spontaneous abortion (RSAB) which is observed in 2-3% of all early pregnancy losses (9-11). While factors such as chromosomal and hormonal anomalies, uterine abnormalities, autoantibodies, and immune dysfunction are known causes (12-16), the etiology of approximately 40% of these cases remains unidentified (16-18).

The maternal decidua is the interface between maternal immune cells and fetal cells (2,19- 21). The decidua during early pregnancy consists primarily of trophoblast cells of fetal origin and maternal decidual immune cells (DICs), majority (40-60%) of which comprise of a special subset of natural killer cells referred to as decidual natural killer (dNK), macrophages and some T cells. Decidual changes in the endometrial stroma play a crucial role in facilitating implantation and placentation (23-24). After implantation, specialized cells known as cytotrophoblasts infiltrate the uterine decidua and undergo transformation into extravillous trophoblasts (EVTs). By the eighth week of pregnancy, these EVTs invade the decidua, establishes direct communication with the maternal uterine spiral arteries, providing blood supply to the surrounding tissue. The EVTs also breaks down the muscular walls and replacing the endothelial cells that line them. (11,17-21). The maternal spiral arteries which are small, adrenergic-sensitive, high-resistance vessels are modified into wider, adrenergic- insensitive, low-resistance conduits, enabling increased blood flow to meet the demands of the developing fetus. Disrupted communication between EVTs and DICs results in uncontrolled immune responses and an imbalance in maternal-fetal immune tolerance (24-25)(Figure 1).

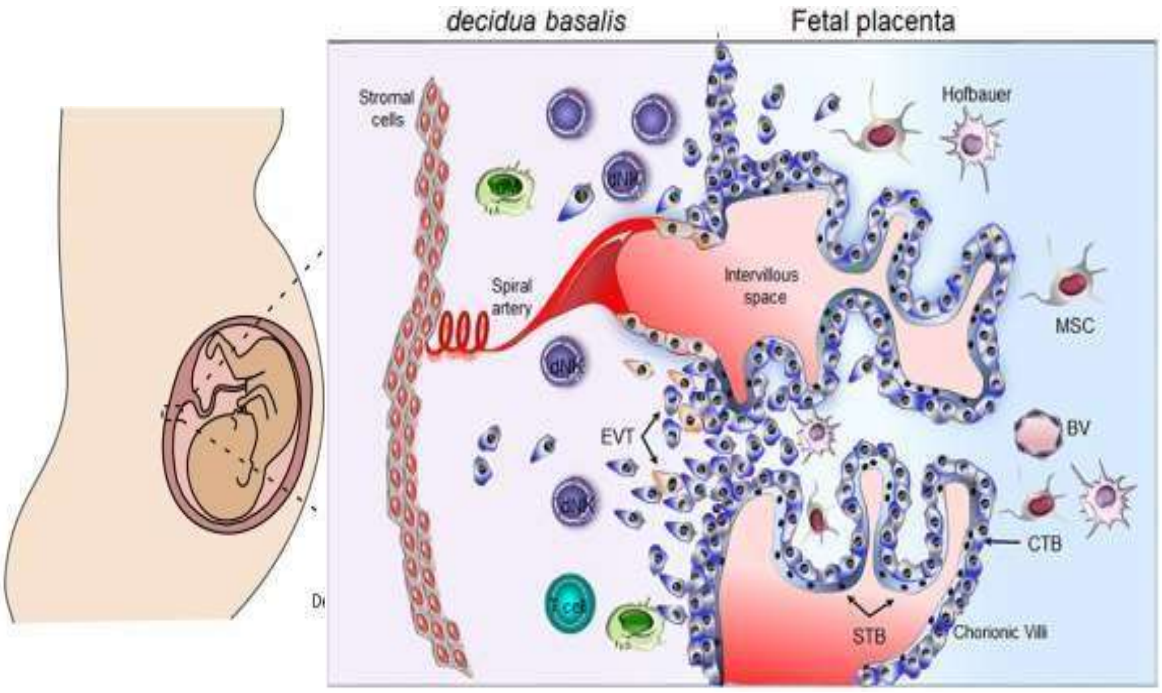

Figure 1: The schematic representation of Local microenvironment of the decidua, maternal- fetal interface, where floating chorionic villi are immersed in maternal blood within the intervillous space. The outer layer consists of a multinucleated syncytiotrophoblast (STB) responsible for nutrient transport and acting as a barrier. Underneath the STB layer, there is a layer of cytotrophoblast cells (CTBs), which differentiate into invasive EVTs that penetrate the maternal decidua. Maternal dNK) cells play an active role in attracting invasive EVTs and facilitating the remodeling of spiral arteries through the release of soluble factors such as cytokines, chemokines, and proangiogenic factors. Invasive EVTs also interact with decidual macrophages (dM) and T cells. Other components present include fetal blood vessels (BV), mesenchymal stem cells (MSC), and Hofbauer cells (fetal macrophages).

Image courtesy Adapted from *Frontiers in immunology*, Features of human decidual NK cells in healthy pregnancy and during viral infection, Ferrat et. Al., 2019, 1397.

The balanced interaction between predominant DICs, dNK cells and EVTs which is crucial for early placentation is mediated by receptors expressed on surface of dNK cells(25-26). NK cells carry an array of activating and inhibitory receptors that recognize the ligands on target cells and control the cytolytic function (25). Activating receptors, NKG2D and members of natural cytotoxicity receptors (NCR) group facilitates allorecognition and cytotoxicity (26- 28). While, inhibitory receptors-KIRs, CD94-NKG2A and co-receptors like CD96, TIGIT and PD1 complex mediate inhibition of effector function of NK cells (28,29). Unlike the periphery, dNK exhibit CD56 bright CD16-KIR+ phenotype, lower cytotoxicity, and higher cytokine secretion. dNK is minimal cytotoxic and instead produce cytokines, growth factors, and angiogenic factors needed to appropriately remodel the maternal spiral arteries, promoting angiogenesis and attracting invasive trophoblasts to the decidua(25-28). Interestingly, it has also been observed that as deeper and earlier endovascular trophoblast invasion with enhanced vascularization and angiogenesis, observed in repeated pregnancies, conducive to healthy placentation as compared to first pregnancies where it's a struggle. The concept of Pregnancy Trained dNK cells" (PTdNKs) suggests that these cells remember the first pregnancy and better assist future gestations. (24-26). The origin of dNK cells in the uterus during pregnancy is still debated with two main hypotheses proposing their origin recruitment from peripheral NK cells or differentiation within the uterus from local progenitor cells (30-32). Evidence from recent findings suggest that trophoblast cells expressing various chemokines such as CXCL10, CXCL12, CCL3, and CX3CL1 might facilitate the recruitment and migration of NK cells from the peripheral blood towards the decidua, favoring pregnancy (33-36). During the process of placental development, cytotrophoblasts release a chemokine called CCL3/MIP-1 alpha, while decidual trophoblast cells lining the maternal blood vessels secrete another chemokine called CXCL12/SDF-1(34- 35). These chemokines attract specific types of natural killer cells, characterized by the presence of CCR5 and CXCR4 receptors respectively from the maternal circulation. In response, dNK cells release CXCL8/IL-8 and CXCL10/IP-10 chemokines, which guide trophoblast cells expressing CXCR1 and CXCR3 receptors towards the process of endovascular invasion and vascular remodelling (31-35) (Figure 2).

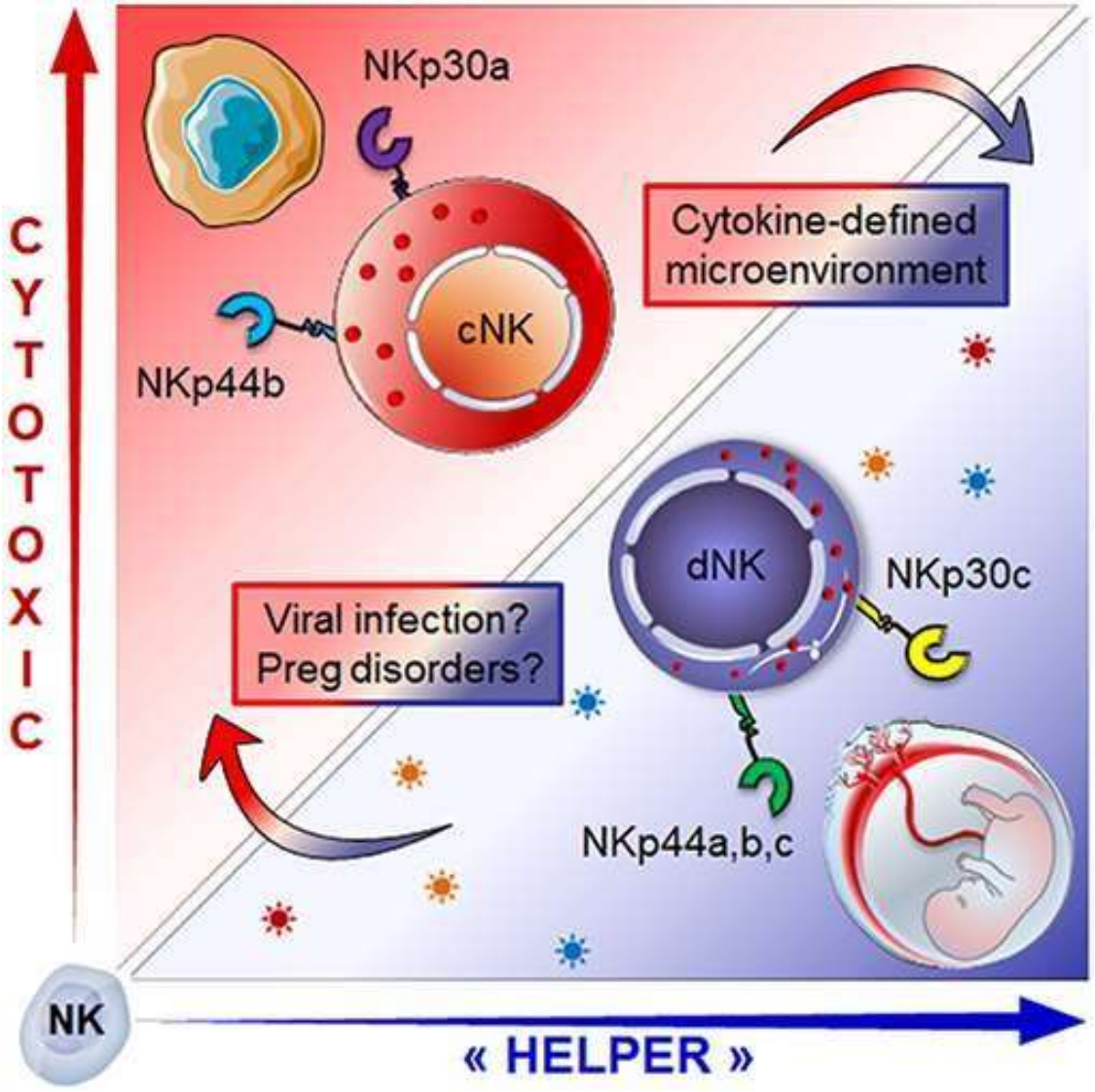

Figure 2: The schematic representation of transformation of NK Cell Function during Placentation. The dNK cells transition from Cytotoxic to "Helper-like" or Tolerogenic Functions during early pregnancy These dNK cells secrete cytokines, chemokines, and growth factors which orchestrates complex molecular signalling networks within the maternal-fetal interface, supporting placentation. Image courtesy Adapted from *Frontiers in immunology*, Features of human decidual NK cells in healthy pregnancy and during viral infection, Ferrat et. al., 2019, 1397.

KIR receptors expressed on surface of NK belong to a multigene family which shows high diversity with respect to gene content (genotype), allelic polymorphism and copy number variations (CNVs) (39, 40). Encoded in the leukocyte receptor complex (LRC) on human chromosome 19q13.4, KIRs consist of fourteen genes [KIR2DL1–5, KIR3DL1–3, KIR2DS1– 5, KIR3DS1] and two 2 pseudogenes (39). Their inhibitory receptors have long cytoplasmic tails (L) containing immunoreceptor tyrosine-based inhibition motifs (ITIMs) which gives inhibitory signals (41, 42). However, they also bear immune-receptor tyrosine-based activating motifs (ITAMs) in short tail (S) receptors, resulting in transmission of an activating signal(43). Based on their gene content, KIRs are categorized into haplogroup-A and haplogroup-B. While haplogroup A is conserved and has higher inhibitory gene content, haplogroup B has variation with different combination of inhibitory and activating genes(44). KIR2DL4 is different from the other KIR family members as it only contains one ITIM instead of two and possesses an arginine in its transmembrane domain (45), suggesting its potential activating downstream signalling (46). KIR2DL4 expressed on dNK cells has both an activating and inhibitory signaling (46, 47). It is present in endosomal compartment as well as on cell surface and upon interacting with HLAG, KIR2DL4 mediates endosomal signaling for the secretion of numerous cytokines and chemokines and growth factors essential for early placentation (48, 49) (Figure 3).

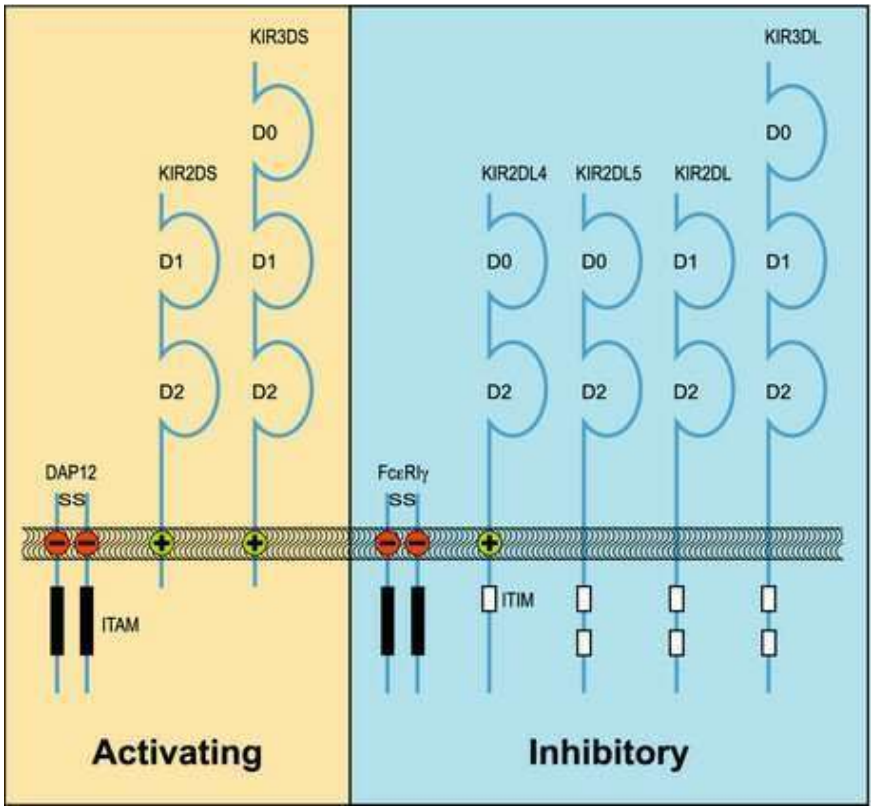

Figure 3: Schematic representation of diverse types of killer immunoglobulin receptor (KIR) gene products in humans and their functional characteristics. KIR molecules are transmembrane proteins, with inhibitory receptors (denoted as "L") possessing immune receptor tyrosine-based inhibitory motifs (ITIMs). Activating receptors (denoted as "S") require adapter molecules with immune receptor tyrosine-based activation motifs (ITAMs) to transmit their signalling. Image courtesy: Adapted from *Immunological reviews*, Co-evolution of the MHC class I and KIR gene families in rhesus macaques: ancestry and plasticity, Groot et al . 2015, 228-245.

EVT expresses both HLA-C and non-classical HLA class I molecules, including HLA-E, HLA-F, and HLA-G (50-52) . HLA-G ligand expression is tissue restricted, however it is abundantly expressed in trophoblast cells. HLA-G is the only known ligand for KIR 2DL4 receptors expressed on dNK cells (53, 54). It mediates tolerance to the semi allogenic fetus and promotes angiogenesis and vascularization (55).

HLA-G levels in the maternal serum as well as in early decidua and term placenta correlated positively with pregnancy outcome in study reports by us and others. HLA-G stands out from other contemporary HLAs due to its unique feature of high polymorphism in non-coding regions, as opposed to the highly polymorphic coding regions observed in other HLAs.

And mutations in these non-coding regions have been associated with its expression in cancers and other viral diseases. An interesting thing about HLA-G is its alternate splicing of primary transcript, HLA-G exhibits seven distinct isoforms which are both membrane- bound (HLA-G1, G2, G3, G4) and soluble (sHLA-G: sHLA-G5, G6, G7) (60). HLA-G1 is the complete molecule with classical alpha chain structure, which is non covalently associated with the β-2-microglobulin chain, HLA-G5 isoforms closely resembles HLA-G1 in having the three extracellular domains of alpha chain but lacks the transmembrane domain (61). All the other isoforms are shorter and lack one or two domains of the heavy chain, either in extracellular (HLA-G1/G2/G3/G4) or cytosolic domain (HLA-G5/G6/G7) (62) (Figure 4).

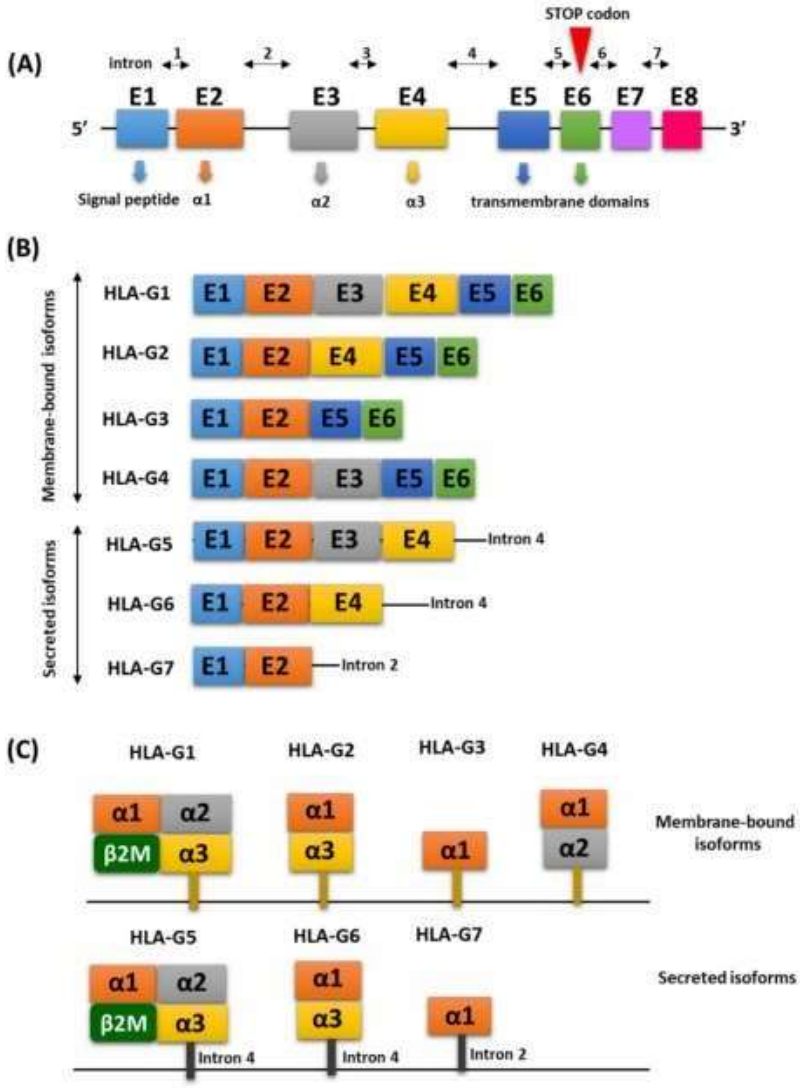

Figure 4: HLA-G genetic organization based on (A) genetic organization of the region responsible for HLA-G coding; (B) types of HLA-G isoforms with a particular emphasis on membrane-associated and secreted isoforms; and (C) spatial arrangement of the domains of individual HLA-G isoforms.

Image courtesy: Adapted from Int. J. Mol. Sci, The HLA-G Immune Checkpoint Plays a Pivotal Role in the Regulation of Immune Response in Autoimmune Diseases, Zaborek et. Al., 2021, 22.

Apart from HLA-G mediated NK activation ,a balance of activating and inhibitory signalling by maternal KIRs, on interaction with EVT expressed HLA-C determines NK activation status that is crucial for establishment of healthy pregnancy (63-65). HLA-C molecule shows two allotypes- HLA-C1 and HLA-C2, depending on the presence of asparagine (in HLA-C1) or lysine (in HLA-C2) at position 80 in its alpha 2 domain (65) (Figure 5). The C2 ligand is cognate ligand for the inhibitory receptor KIR2DL1 and is reported to deliver the strongest inhibitory signal, while it also binds to the activating allele KIR2DS1(66). This inhibition by KIR2DL1 and HLA-C2 is balanced by activating signal delivered by HLA-C2 & KIR2DS1(67). HLA-C1 is the cognate ligand for inhibitory receptors KIR2DL2 and KIR2DL3, but with less effective inhibitory signaling as compared to KIR2DL1. In addition, KIR2DL2 and to a lesser extent KIR2DL3 show some cross-reactivity with C2 ligands (40) (Figure 6).

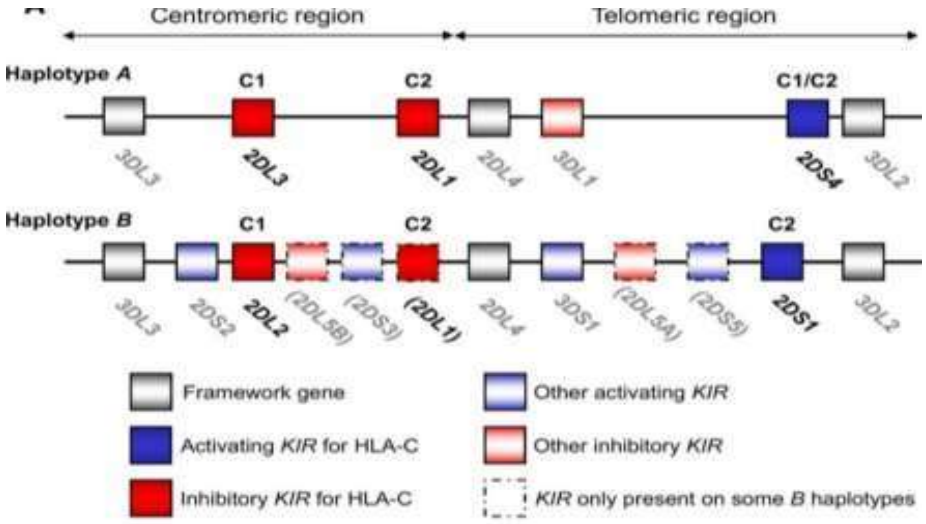

Figure 5: This diagram depicts the genetic composition of KIR haplotypes and their relationship with HLA-C receptors. Gray boxes represent framework KIR genes found in all haplotypes. Blue boxes represent KIR genes encoding activating receptors for HLA-C, while red boxes represent KIR genes encoding inhibitory receptors for HLA-C. Shaded boxes indicate genes for activating or inhibitory receptors for other ligands. Dotted-line outlined boxes signify KIR genes present specific B haplotypes. Image courtesy: Adapted from *Journal of leukocyte biology*, Maternal KIR and fetal HLA-C: a fine balance, Chazara, et. al., 2011, 703-716.

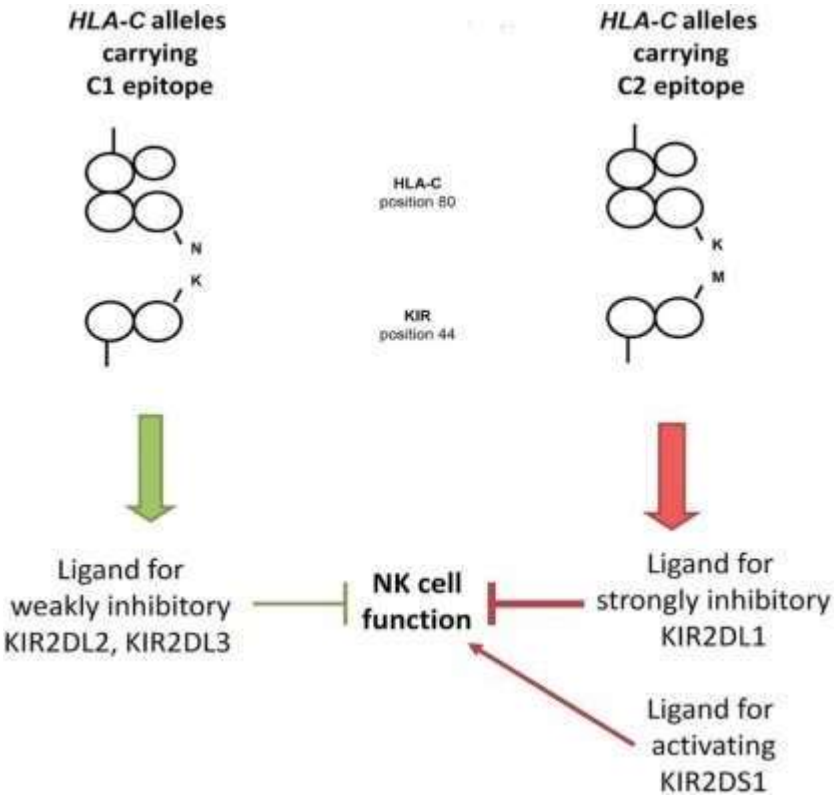

Figure 6: Schematic representation of *HLA-C* variation. *HLA-C* alleles are divided into two groups according to a dimorphism at position 80 the α1 domain (C1: $Asn_{80,}$ C2: Lys_{80}). Different KIRs bind to the C1 and the C2 epitope depending on a dimorphism at position 44. Image courtesy: Adapted from, Reproductive BioMedicine Online Variation of maternal KIR and fetal HLA-C genes in reproductive failure: too early for clinical intervention, Moffett et al et. al., Volume 33, Issue 6,2016.

In line with earlier study reports of different KIR-HLA combined genotype in different ethnicities it was observed that Japanese population had AA genotype with HLA C1 allotypes and conversely, the Asian populations had higher activating gene content with KIR B haplogroup and HLA C2 allotypes (68, 69), favoring pregnancy, emphasizing on the co- evolution of KIRs with HLA class I molecules. KIR2DS1 on dNK by HLA-C interaction has been reported to secrete beneficial cytokines and growth factors, especially granulocyte– monocyte colony stimulation factor (GM-CSF) and facilitate trophoblast invasion (70). While few groups of researchers showed that KIR2DS1 in conjunction with paternal HLA-C2 was beneficial for pregnancy the others showed it as a risk factor (70). Also, lower KIR2DL1 levels in peripheral NK cells, as well as lower levels of both KIR2DL1 and KIR2DS1 correlated with adverse pregnancy outcome in other studies (71,72).

Immune dysfunction, caused by presence of autoantibodies also has an adverse role in early placentation. Autoantibodies in pathological pregnancies are implicated in NK cell imbalance and in obstetrical antiphospholipid syndrome (OAPS), characterized by persistent antiphospholipid antibodies (APLAs) (73-75). The primary antibodies responsible for the thrombotic symptoms of are β2GPI-dependent antiphospholipid antibodies and anticardiolipin autoantibodies. β2GPI, a protein that is consistently present on the surface of placental cells (76-78). Abnormal expression of APLAs can induce inflammatory reactions and vascular endothelial damage, disturbing the homeostasis in blood flow from mother to fetus, leading to microvascular thrombus formation and defect in placentation (77-78). Anti- β2GPI antibodies inhibit trophoblastic cell autophagy and activate inflammasomes, amplifying the inflammatory response and potentially resulting in fetal rejection (76-77). Additionally, the presence of antinuclear autoantibodies (ANAs) targeting cell nucleus components is associated with increased risks of complications like preeclampsia and fetal growth restriction (79-82). ANAs may impact embryo quality and development, reducing pregnancy and implantation rates (79-84). Understanding the mechanisms underlying these

immune dysregulations in conjunction with KIR-HLA system is important for elucidating the factors contributing to adverse pregnancy outcomes.

A balanced activation of NK cells via maternal KIR2DL1/S1 with fetal HLA-C is well documented for mother-neonate pairs, however studies on early abortus are limited (73, 74). HLA-C expression study in abortus tissue would provide an insight from the perspective of KIR-HLA interaction and NK cell activation in early pregnancy loss. Besides, there is limited data on HLA-G isoforms, KIR2D-HLA-C genotype in early abortus, it was thus pertinent to investigate HLA-G isoforms, KIR-HLA-C combined genotypes, KIR CNVs and their expression in early pregnancy loss. This study is particularly novel considering the distinct genetic makeup of individuals from the north-eastern part of India, where no previous data on HLA-G isoforms in the context of early pregnancy exists. Considering the distinct genetic makeup of individuals in the north-eastern region of India, it was crucial to investigate the HLA-G isoforms in early pregnancy loss, as to the best of our knowledge no data has been reported from this population so far.

The existing knowledge on HLA content in early spontaneous abortion (SAB) is limited, emphasizing the importance of exploring NK cell activation and its regulation through KIR- HLA interactions in relation to pregnancy failure, including both early and full-term pregnancies. With these considerations in mind, the study aimed to address the following objectives.

Objective:

1. Determination of autoantibody profile (ANA and APLA) and its association with SAB.

2a. Investigation into KIR-HLA combined genotype, KIR copy number variations (CNV)and HLA-G isoforms and KIR-HLA expression in relation to SAB.

2b. Study on 3'UTR mediated regulation of HLA-G in pregnancy .

3. Study on NK phenotype and activation status in relation to SAB.

4. Characterization of early abortus and term placenta with respect to histology and vascularization in SAB and in health pregnancy.

We observed higher proportion of HLA-G4 isoforms in term placenta of the healthy participants of study cohort. In contrast, SAB early abortus showed higher frequency of HLA- G3 isoforms suggesting the difference between proportions of HLA-G4 and HLA-G3 isoforms in our study cohort. Our data on mother-neonate pair belonging to healthy group was consistent with a balanced activation by KIR2DL1+/S1+ with HLA-C2 allotype in contrast to SAB where iKIR2DL1+ was more frequent (chi-square, p=0.003) with low HLA- C2 allotype occurrence. In concordance with this, in SAB early abortus tissue, higher iKIR2DL1+/ S1- with low HLA-C content indicates poor activation of NK cells. Predominance of $CD56^{dim}$ $CD16^{+}$ NK cells, increased $CXCR4^{+}$ cells with elevated IFN-γ levels and IL-15 levels in the decidua of the SAB abortus provided evidence of imbalanced NK cell activation and immune dysregulation in early pregnancy failure.

Furthermore, the histological examination of the placental tissue revealed the presence of inflammatory cells, confirming the occurrence of inflammation in SAB abortus when compared to the healthy group. Higher HLA-C, KIR2D content in abortus than in term placenta showed its larger requirement in early pregnancy, in addition to soluble HLA-G isoforms for favouring placentation. Additionally, our study found no association between autoantibodies, such as anticardiolipin and antinuclear autoantibodies, and SAB. Although B2GP1 IgM positivity was only observed in participants with a history of SAB, the frequency was too low to draw meaningful conclusions. The study supports differential expression of KIRs in early and term phase of pregnancy, emphasizing on necessity of NK expressed KIRs for EVT invasion and tissue remodeling in early decidua.

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