ABSTRACT

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. Immune dysfunction at the maternal-fetal interface has been linked to early pregnancy failure. 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. 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. While factors such as chromosomal and hormonal anomalies, uterine abnormalities, autoantibodies and immune dysfunction are known causes the etiology of approximately 40% of these cases remains unidentified. Our study did not find any association between autoantibodies, such as anticardiolipin (aCL) and antinuclear autoantibodies (ANA), and SAB. However, we found that B2GP1 IgM positivity was observed only in participants with a history of SAB; the frequency was too low to draw meaningful conclusions. Nonetheless, we observed a higher incidence of aCL antibodies compared to other autoantibodies in both the healthy history group and the SAB history group. This finding emphasizes the predominant presence of aCL in the general population.

The maternal decidua, which is the interface between maternal immune cells and fetal cell consists primarily of trophoblast cells of fetal origin, decidual stromal cells which comprises majority of maternal NK cells (40-60%), macrophages and some T cells. Decidual changes in the endometrial stroma play a crucial role in facilitating implantation and placentation. NK cells carry an array of activating and inhibitory receptors that recognize the ligands on target cells and control the cytolytic function. Activating receptors, NKG2D and members of natural cytotoxicity receptors (NCR) group facilitates allo-recognition and cytotoxicity. While, inhibitory receptors-KIRs,CD94-NKG2A and co-receptors like CD96,TIGIT and PD1 complex mediate inhibition of effector function of NK cells. Unlike the periphery, decidua is however dominated (40-60%) by a special subset of NK cells referred to as decidual natural Killer (dNK) cells.

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. 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, favouring pregnancy. Notably, most dNK exhibit CD56 bright CD16- KIR+ phenotype, lower cytotoxicity, and higher cytokine secretion.

KIRs expressed on surface of dNK are a multigene which family cells show high diversity with respect to gene content (genotype), allelic polymorphism and copy number variations (CNVs). Encoded in the leukocyte receptor complex (LRC) on human chromosome 19q13.4, KIRs consist of fourteen genes [KIR2DL1–5, KIR3DL1–3, KIR2DS1–5, and KIR3DS1] and two pseudogenes. The inhibitory receptors have long cytoplasmic tails (L) containing immunoreceptor tyrosine-based inhibition motifs (ITIMs) which gives inhibitory signals. However, they also bear immune-receptor tyrosine-based activating motifs (ITAMs) in short tail (S) receptors, resulting in transmission of an activating signal. 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. 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, suggesting its potential activating downstream signalling.

The decidua invading extra villous trophoblast cells (EVTs) expresses both HLA-C and non-classical HLA class I molecules, including HLA-E, HLA-F, and HLA-G. Although HLA-G expression is tissue restricted, it is abundantly expressed in trophoblast cells. HLA-G is the only known ligand for KIR 2DL4 receptors expressed on dNK cells. KIR2DL4 expressed on dNK cells, having both an activating and inhibitory signaling is present in both endosomal compartment and on cell surface. And upon interacting with HLAG, KIR2DL4 mediates endosomal signalling for the secretion of numerous cytokines and chemokines and growth factors essential for early placentation. Studies, including our own and those conducted by other researchers have shown that HLA- G levels in the maternal serum as well as in early decidua and term placenta correlated positively with pregnancy outcome. Due to 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). 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. 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).

Our study showed higher proportion of HLA-G4 isoforms in healthy placenta (Z test, p=0.003). In contrast, SAB abortus showed higher proportion of HLA-G3 isoforms (Z test, p=0.001) suggesting the difference between proportions of HLA-G4 and HLA-G3 isoforms in our study cohort. Additionally, considering the noted difference in HLA-G between healthy term placent and SAB history placenta we examined the miRNA profile (miR-14a and miR-152) in the 3' untranslated region (3'UTR) of the HLA-G gene with emphasis on SNP + 3142 C > G (rs1063320) and 14 bp DEL/IN polymorphism (rs66554220) at position + 2960, heat shock factors (HSF) and DNA methyltransferase (DNMT1) in 5'URR in healthy placenta vs term placenta. Our data on predominance of "GC" heterozygotes among the study participants suggests balancing selection in our population. Despite the upregulation of miR-148a and miR-152 in placenta samples, we found no correlation between these miRNAs in the placenta. Interestingly, we observed that HLA-G expression is influenced by the combination of the 14 bp DEL allele, which is in linkage disequilibrium with the SNP +3142 C > G, and the 5' URR HLA-G genes-HSF1 and DNMT1. The upregulation of DNMT1 in the placenta of individuals with history of SAB could be a compensatory response aimed at reducing the aberrant expression of HLA-G transcripts, as higher levels of DNMT1 were found to be positively associated with HLA-G transcript levels in SAB. Further investigation into the methylation state of the HLA-G promoter is required because we did not examine the promoter-specific methylation state of HLA-G, which is a crucial mechanism for HLA-G expression.

A balance of activating and inhibitory signalling by maternal KIRs, on interaction with EVT expressed HLA-C determines NK activation status, crucial for establishment of healthy pregnancy. HLA-C molecule shows two allotype- 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 . 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.

This inhibition by KIR2DL1 HLA-C2 is balanced by activating signalling delivered by HLA-C2 &KIR2DS1.HLA-C1 is the cognate ligand for inhibitory receptors KIR2DL2 and KIR2DL3, but with less effective inhibitory signalling as compared to KIR2DL1. In addition, KIR2DL2 and to a lesser extent KIR2DL3 show some cross- reactivity with C2 ligands.

Different KIR-HLA combined genotype exists in different ethnicities, and it was observed that Japanese population had AA genotype with HLA C1 allotype and conversely, the Asian populations had higher activating gene content with KIR B haplogroup and HLA C2 allotypes, favouring 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. A 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 in pregnancy. Also, in some other studies lower KIR2DL1 levels in peripheral NK cells, as well as lower levels of both KIR2DL1 and KIR2S1 has been correlated with adverse pregnancy outcome.While 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. 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.

Our data on mother-neonate pair belonging to healthy group is 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 CD56dim CD16+ NK cells (p=0.003), low CXCR4+expression and elevated IFN-gamma (t test,p=0.016) and IL-15 levels in the decidua of the SAB abortus provides evidence of imbalanced NK cell activation and immune dysregulation in early pregnancy failure. In contrast, upregulation of key chemokine CXCL2, increased VEGFA levels, inhibition of IFN-gamma upregulation promoted dNK migration while CCL5, CXCL3 maintained an optimal EVT invasion, favouring a healthy pregnancy. The histological examination of the placental tissue revealed the presence of inflammatory cells, confirming the occurrence of inflammation in SAB abortus but not in healthy abortus. This was validated

by protein study which showed is a transition from an anti-inflammatory to a proinflammatory cytokine environment in healthy term placenta, which is essential for a healthy term delivery, by reducing the levels of SOCS. The study suggests an imbalance in the activation of natural killer (NK) cells and immune dysregulation in early pregnancy failure.