CHAPTER VII

SUMMARY AND CONCLUSION

7.1. Summary

SAB miscarriage is a significant event that occurs in approximately 10% of clinically recognized pregnancies during the early stages. RSAB is observed in 2-3 % of early pregnancy loss. Immune dysfunction between maternal fetal cells at decidua has been linked to early pregnancy loss. The interaction between decidua-dominating CD56 bright CD16- dNK cells and EVTs through their KIR receptors interacting with HLA-C ligands is crucial for trophoblast invasion and facilitating increased blood flow for fetal development. dNK also plays also interact with HLA-G for mediating fetal tolerance. The interplay of dNK and stromal cells with chemokines and cytokines creates an environment conducive to healthy pregnancy. However, due to differencial expression of dNK cells population and their changing receptor expression during pregnancy, any imbalance can disrupt the maintenance of pregnancy. Predisposition to autoantibodies further dampens placentation by inhibiting EVT proliferation and their invasion to decidua.

The objective of this study was to investigate the placental microenvironment in relation to SAB by analyzing early decidua and term pregnancy samples. Specifically, we examined the isoform profile of HLA-G in early abortus and term placenta. Additionally, we assessed the expression of HLA-G in abortus with SAB miscarriage and in term placenta tissue of participants with a history of SAB. The regulation of HLA-G was studied in terms of 3'UTR region mutations and miRs, including miR-148a, miR-152, as well as 5'URR regulatory genes DNMT1 and HSF1. The combined genotype of KIR-2DL1/2DS1-HLA-C was investigated in 46 mother-neonate pairs and further validated in a larger cohort. To understand the discrepancy in KIR distribution, we also explored copy number variations (CNVs) of the KIR2DL1 and KIR2DS1 genes in the study cohort. Furthermore, we analyzed the protein expression of KIR2D and HLAC in SAB abortus and placenta with a history of SAB, comparing them to control samples. Considering the importance of optimal KIR-HLA signalling for balanced downstream NK cell activation, we examined the NK cell phenotype in early decidua, as well as NK cell activation and the interplay of cytokines and chemokines in abortus and term placenta, in relation to adverse pregnancy outcomes. Histological analysis of both abortus and placenta samples was conducted to validate the presence of inflammation related to SAB. Furthermore, autoantibody profile was examined for study participants in relation to SAB.

The salient findings of the study are summarized as follows-

1)The proportion of HLA-G3 isoforms was significantly higher in SAB compared to healthy abortus. Soluble isoforms (HLA-G5, HLA-G6, and HLA-G7) were detected in healthy abortus, while only HLA-G5 was found in SAB abortus. Significant difference in the proportion of HLA-G4 (z-test for two proportions / Two-tailed test, p=0.001) between the healthy and SAB history placenta samples. Minimal presence of HLA-G soluble isoforms was noted in placenta.

2) We observed comparable levels of HLA-G protein between the healthy and SAB abortus and also between the two placentas. Nonetheless, logistic regression analysis predicted HLA-G as predictive marker for a healthy outcome of pregnancy (AUC=0.8).

3) In 3'UTR of HLA-G, despite high G alleles, miR-148a and miR-152 was upregulated in placenta samples. miR-148a levels were comparable between SAB and healthy group. However, miR-152 was downregulated in healthy as compared to SAB where it showed base level expression. 14 bp DEL alleles were predominant in healthy group. Both DNMT1 transcript levels were higher in SAB placenta than healthy placenta. However, HSF1 mRNA levels were higher in SAB compared to control.

4) In paired mother-neonates, the healthy history mothers had KIR2DL1+/S1+ genotype in contrast. SAB history mothers had KIR2DL1+/S1+ as well as KIR2SDL1+/S1- This indicated towards higher prevalence of activating KIR2DS1 in healthy mothers (chi-square, p=0.003) as compared to SAB history mothers. On examining the HLA-C allotype of neonates it was observed that HLA-C2 allele predominated in neonate belonging to healthy mothers than SAB mothers (chi-square , p=0.001). In line with this ,C2C2 genotype was predominant in healthy neonates while C1C2 and C1C1 were predominant in neonates of SAB group mothers. Combined maternal KIR and neonate HLA-C data suggests that in case of SAB group, the maternal KIR2DL1/S1 get minimal cognate ligand HLA-C2 from neonates, attributing to suboptimal NK signalling .In case of healthy group higher maternal KIR2DS1 gets adequate cognate ligand HLA-C2 in neonates to activate NK cells and trigger downstream signalling.

5) Higher iKIR2DL1 content in conjunction with low HLA-C levels suggested suboptimal interaction between KIR -HLA and poor activation of NK in SAB abortus.

In addition, KIR2D protein levels were higher in healthy history placenta than SAB history placenta(p=0.001) HLA- C was higher in SAB as compared to healthy placenta (p=0.001)

6) The proportion of the CD56+CD16- population was found to be higher in healthy abortus compared to SAB abortus. Subsequently, we observed that the proportion of CD56+ CD9+ cells was similar between the two groups.The mean fluorescent intensity of CD9 in the CD56+ population was found to be consistent with the population data. IFNγ+ population independently of CD56 expression, we found that IFNγ+ cells were more abundant in SAB (Mann-Whitney U test, $p \leq 0.02$). We observed that the CD56+CXCR4 was more prevalent in healthy abortus compared to SAB.

7) IFN-γ (t test, p = 0.016) and IL10 (t test, p = 0.002) were high in SAB abortus than in healthy abortus.In contrast, term placenta showed that most cytokines IL1B,IL2, ,IL18,TGFB,IFNG,IL10 exhibited elevated levels in the healthy group. We performed Multivariate cox analysis taking in all the variables vs survival of the fetus where IL15 showed significant hazard ratio of 1.13 (p value= 0.00805).Interestingly , SOCS were comparable in SAB abortus and healthy abortus . Wherein, in placenta the healthy group had higher SOCS levels as compared to SAB.

8) Higher expression of CCL5,CCL2,CXCL8 was seen in healthy abortus as compared to SAB, the data in placenta showed higher CCL5 in SAB than in healthy, while CCL2 and CXCL8 was higher in healthy than in SAB. CXCL9 and CXCL10 levels did not show any difference between the groups. We further compared the levels of chemokines between placenta and abortus and observed that levels of CXCL9 (p=0.032), CCL2 (p=0.0001) and CXCL10 (p=0.00012) were significantly higher in abortus than in placenta in healthy group, signifying their significance in early placentation.

9) Expression of VEGFA was higher in healthy abortus than SAB. The levels were comparable in placenta. Furthermore, histology study by H& E staining where the mean degree of decidua, which represents the extent of specialized endometrial tissue, was comparable between healthy abortus and SAB abortus samples, indicating similar levels of decidua development in both groups. However, inflammatory cells were present in three SAB cases, while no inflammation was observed in healthy abortus

samples. Necrotic areas were identified in two SAB placenta samples, indicating tissue damage and cell death but was not observed in the healthy placenta samples.

7.2. Conclusion

Our study findings indicate that the presence of membrane-bound HLA-G4, higher copy numbers of KIR2DS1 and KIR2DL1/KIR2DS1-HLAC2 combined genotype in mother-neonate pairs are linked to balanced activation of NK cells and positive pregnancy outcomes. Conversely, the presence of HLA-G3 isoforms along with higher KIR2DL1 or KIR2DL1+/S1+-HLAC2 combinations compromise maternal tolerance and may contribute to insufficient NK activation, potentially leading to pregnancy complications such as SAB. Additionally, we observed that chemokines CXCL2,CCL5,CXCL3 regulate dNK migration, EVT invasion and increases VEGF A levels promote a healthy pregnancy. Abnormal expression of IL1B, IFNγ, and IL10, was associated with immune imbalances contributing to early pregnancy loss. Furthermore, there is a transition from an anti-inflammatory to a proinflammatory cytokine environment in late pregnancy, which is essential for a healthy term delivery by reducing the levels of SOCS

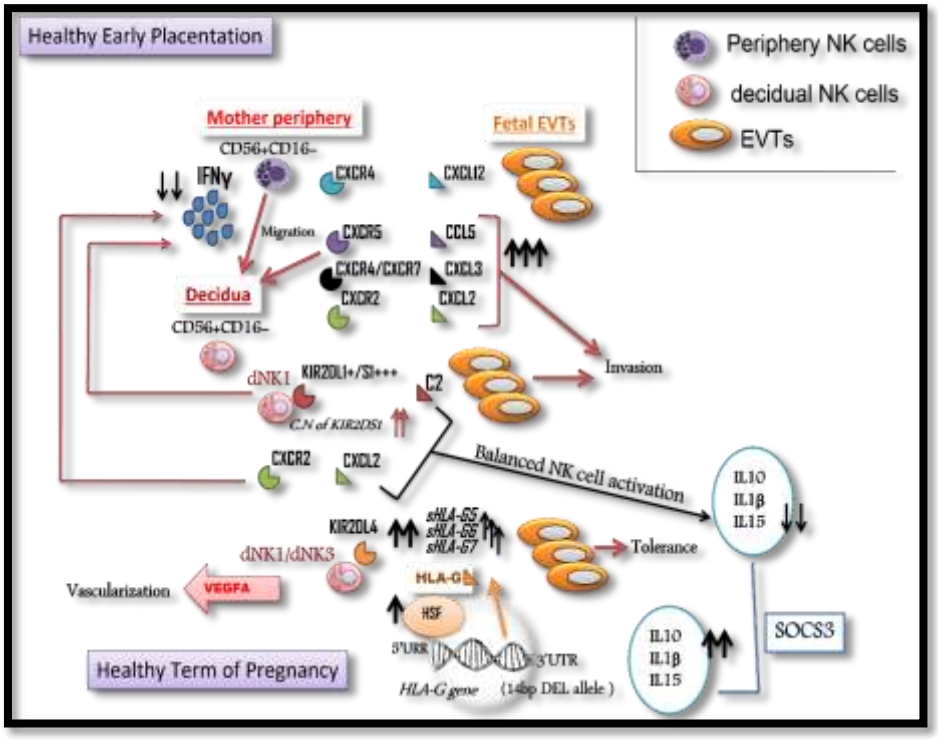

Figure 48 a: A hypothetical model summarizing the findings of the study in healthy scenario.

In a healthy pregnancy, the early stage of placentation involves the migration of $CD56^+$ $CD16^-$ cells from the periphery to the decidua. This migration is facilitated by the expression of CXCR4 receptors on these cells. The balanced signaling of low INFγ levels by KIR2DL1-S1 with C2, along with a higher copy number of KIR2DS1, plays a role in spiral artery remodeling. Additionally, low levels of CCL2 contribute to a reduction in INFγ, allowing for an elevation of CXCR4 and promoting decidual NK cell migration. The upregulation of CCL5 and CXCL3 further maintains optimal invasion. Furthermore, the increase in dNK cells induces the production of VEGF A and soluble HLA-G, with the latter being predominantly expressed in the decidua. HLA-G levels are upregulated by a high frequency of DEL allele at position in + 2960 UTR region of HLA-G and increased expression of *HSF1* in the 5' URR region. The balanced NK cell signalling also maintains an anti-inflammatory environment in the decidua, which later shifts to a proinflammatory state conducive for term delivery of the fetus.

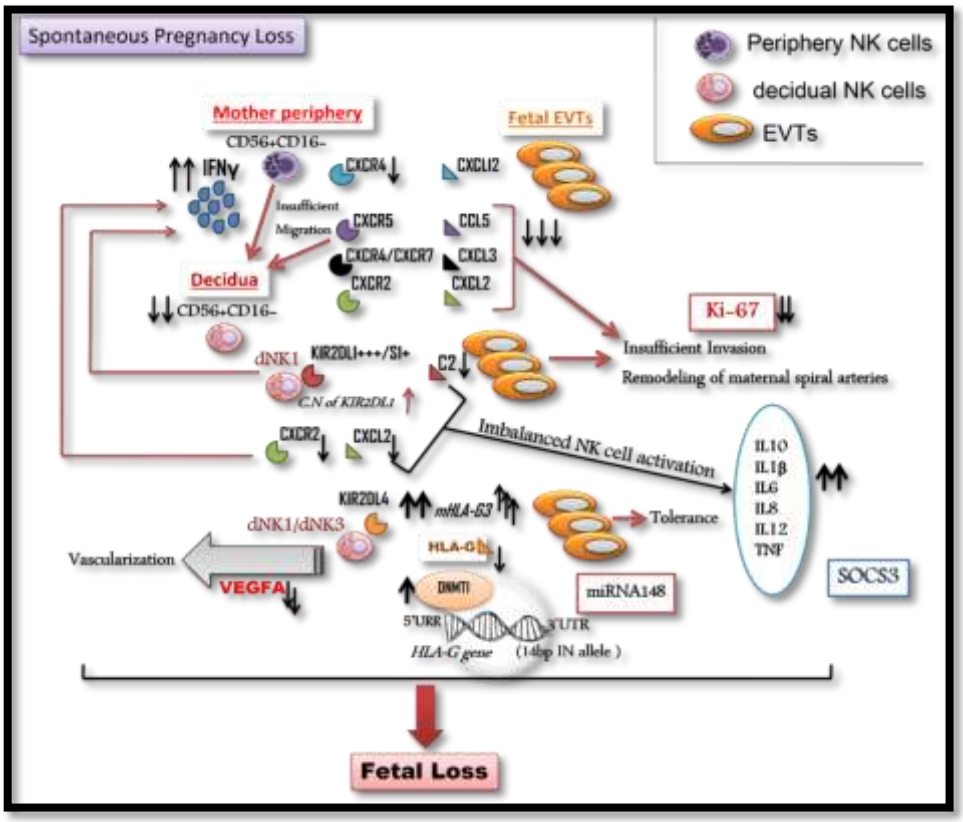

Figure 48 b: A hypothetical model summarizing the findings of the study in SAB miscarriage.

The migration of $CD56^+$ $CD16^-$ cells from the periphery to the decidua is hindered due to limited expression of $CXCR4^+$ NK cells . Insufficient levels of CCL2 lead to increased INFγ, resulting in the inhibition of $CXCR4^+$ cell migration. The presence of KIR2DL1 with low C2 contributes to imbalanced signaling, affecting spiral artery remodeling and leading to elevated INFγ levels. Additionally, reduced levels of CCL5 and CXCL3 contribute to insufficient invasion, as indicated by decreased ki67 and

VEGFA levels. Moreover, lower levels of HLA-G are observed due to increased *DNMT1* levels and the presence of IN alleles in + 2960 UTR region of HLA-G. While miR-148 levels increase, no significant correlation is found with HLA-G levels. Notably, an inflammatory cytokine milieu persists in the decidua, with IL15 demonstrating a significant hazard ratio associated with fetal death.