CHAPTER V

STUDY ON NK PHENOTYPE AND NK ACTIVATION STATUS IN RELATION TO SPONTANEOUS ABORTIONS.

5.1 Introduction

The maternal decidua is the interface between maternal immune cells and fetus (1-3). It is home to decidual stromal cells which comprise of majorly (40-60%) maternal NK cells, macrophages and some T cells together with Extravillous trophoblast (EVT) cells of fetal origin(5-9).

NK cells are cytotoxic innate lymphoid cells, and were first discovered thanks to their ability to kill tumor cells, and later found to also kill pathogen-infected cells (10-12). In humans, conventional NK cells are present in peripheral blood (pNK cells) and are distributed widely throughout the body(13-14). However, in addition to pNK cells, in humans NK cells are also found in peripheral tissues, such as the liver, lungs, skin and uterus, and are termed tissue-resident NK (trNK) cells (14-17). Most trNK cells are the subset of $CD56^{bright}$ NK cells. Decidual NK (dNK) cells are a specialized type of trNK cells found at endometrial decidual tissue (18-23).They display a CD56 bright CD16- KIR+ phenotype, lower cytotoxicity, and higher cytokine secretion capabilities(23-28).

Different subsets of dNK have been identified, spanning from early placentation to the term of pregnancy. dNK1 cells express killer cell immunoglobulin-like receptors (KIRs) and Leukocyte Immunoglobulin-Like Receptor B1 (ILT2), which play a role in trophoblast invasion and establishing an immune-tolerant microenvironment by interacting with HLA molecules expressed by EVTs, such as HLA-C and HLA-G(24-27). These cells also possess cytoplasmic granule proteins involved in immunity against placental infections and enzymes related to glycolysis(28-29). On the other hand, dNK2 cells express the chemokine receptor CXCR1, which mediate the recruitment of EVTs and dendritic cells at the fetal-maternal interface, specifically recruiting cDC1 cells through CCL5-CCR1 interactions(30-35). However, the frequency of dNK 3 cells remains relatively low in the decidua. The origin of dNK cells, in the uterus during pregnancy is still debated with two main hypotheses proposing their recruitment from peripheral NK cells while others support differentiation within the uterus from local progenitor cells (35-39). 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 (40-42).

Chemokines are multifunctional molecules involved in intercellular communication and signal transduction (43-46). Chemokine/chemokine receptor interactions dominate the trafficking of leukocytes, the mechanisms underlying the recruitment and maintenance of DICs most likely involve the expression and secretion of chemokines at the maternal–fetal interface(45-48). Decidual cells produce various types of chemokines, such as CCL2, CXCL8, CX3CL1, CXCL10 and CXCL12, at significant levels (47-50).These chemokines are differently involved in the migration of peripheral NK (pNK) and decidual NK (dNK) cells into the decidua (51-53).Interestingly, CXCL12 and CX3CL1 preferentially attract CD161 pNK cells, while CXCL10 is essential for the recruitment of $CD56^{+}CD16^{-}$ pNK cells. Uterine expression of CXCL14 may also play a role in uterine NK-cell recruitment during the early pregnancy (54-55). However, a clear picture of the effects of CXCL14 on uterine NK-cell recruitment in the context of the uterus is still undecided (54-55).

dNK cells are potent source of cytokines in the endometrium (56-58). The regulation of the endometrial immune system is crucial for successful implantation and maintenance of pregnancy, and this is achieved through the synchronized secretion of specific types of cytokines (59).The pro-inflammatory Th1 cell subtype secretes cytokines such as interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), and interleukins IL-1, IL-2, IL-12, IL-15, and IL-18. Conversely, the Th2 subtype secretes anti-inflammatory cytokines, including IL-4, IL-5, IL-10, IL-13, and granulocyte-macrophage colony-stimulating factor (GM-CSF) (60). The balance between these cytokines, represented by the Th1/Th2 ratio, undergoes periodic changes throughout pregnancy, reflecting different stages and processes(61-62). Cytokines, such as IL-1, IL-6, IL-8, TNF, matrix metalloproteinases (MMPs), and prostaglandins, also contribute to cervical effacement/dilatation and rupture of the membranes, leading to labor and delivery(63-64).

Considering that dNK cells is crucial for modulating innate immune responses and preserving a balanced pro- and anti-inflammatory environment in the decidua throughout gestation(65), our study aims to investigate the NK cell phenotypes and their activation status and their regulation in early decidua and in term placenta, specifically in relation to SAB.

5.2. Materials and methods

5.2.1. Study site, study design, and participants

A hospital-based case-control study was designed with 97obstetric participants as the study cohort. The study was conducted at Tezpur Medical College and Hospital (TMCH) and Gogoi Nursing Home Complex (GNHC) in Tezpur, Assam. The SAB history group (N=16) included age-matched obstetric participants with one or more fetal losses before 15 weeks of gestation, while the control group (N=40) included women with at least one live birth.

We included an additional 24 women who experienced spontaneous miscarriage between 12-15 weeks of pregnancy. Samples were collected from the products of conception, specifically from the spontaneous abortus, from these participants after obtaining written informed consent. Additionally, we included 17 women who underwent voluntary medically induced termination of pregnancy at the same gestational age. Samples from the conceptus obtained from these women served as control abortus samples. The conceptus product were collected by the clinical staff only after obtaining written informed consent of the participants/their guardians .

Ethical permission was obtained from the Institutional Ethics Committee of Tezpur Medical College and Hospital (TMCH) with sanction numbers IEC/14 dated 08-08-2017. All participants were provided with a detailed patient information sheet and a patient consent form as per the guidelines of Indian Council of Medical Research (ICMR). The forms were provided in both English and the local language. Participants were recruited for the study only after obtaining their written informed consent and or that of their guardians. The following were the exclusion and inclusion criteria of the study-

Inclusion Criteria

1. Female participants of reproductive age group of median age, irrespective of pregnancy status
2. History of SAB /RSAB (case)
3. For Control group history with minimum one childbirth

Exclusion Criteria

1. Uterine anomalies
2. Hormonal imbalance .
3. History of neonatal death/ any debilitating diseases .

The information of obstetric history of the participants, ethnicity, demographic and other characteristics were collected and recorded in the form of proformas by the research staff of both the hospitals.

5.2.2. Isolation of RNA, cDNA and Protein from tissue samples

Placenta and abortus tissue samples were collected in 1.5 ml RNA later solution (Ambion, United States) and homogenized using a hand-held tissue grinder (G-Biosciences, United States). DNA, RNA, and protein were extracted using Allprep DNA/RNA/protein kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA was converted to cDNA using the High capacity cDNA reverse transcription kit (Invitrogen, Applied Biosystems, Foster City, United States) according to the manufacturer's instructions. The quantity and quality of the isolated genomic DNA, cDNA and protein were assessed by Nano-VueTM plus spectrophotometer (GE Healthcare, Little Chalfont, United Kingdom). Quality assessment was done by checking the expression of the ß-actin protein in ELISA using the β-actin primary antibody (Invitrogen, Applied Biosystems, Foster City, United States).

5.2.2. Gene Expression Profiles: IL1B, IL2, IL15, IL18, IL21, TGFB, IFNG, TNF, IL10, VEGFA, Cyclin D, Ki-67, Keratin 18 in abortus and placenta

Expression of the genes were studied in term placenta and abortus samples. Gene expression study was performed using SYBR green-based assays (Applied Biosystems, USA) on QuantStudio 3 Time PCR System (Applied Biosystems, USA). *GAPDH* was used as the endogenous control for normalization of expression levels.10 term placenta from women with history of minimum two live births and without any medical anomalies were used as the calibrators for mRNA quantification by the comparative CT method. Primer sequences to amplify the above mentioned genes have been tabulated in Table 26. RT^2 qPCR Primer Assays (Qiagen, Hilden, Germany) were used to study the expression of the marker genes as given in Real-time PCR was carried out in 10µL of reaction volume consisting of 1X Sybr green PCR master mix, 0.4 µM of each primer, 100ng of cDNA and water to adjust the volume. Cycling conditions for Real-

Time PCR are presented in Table 1. The relative expression of the target genes was determined using the formula $2^{-\Delta\Delta Ct}$ method which was derived as follows-

$\Delta\Delta C_t = \Delta C_t$of patient – $\Delta C_t$of the calibrator

where $\Delta C_t = C_t$ of target gene – C_t of endogenous control (GAPDH)

RQ (Relative quantification) = $2^{-\Delta\Delta Ct}$ which represents the fold change in expression of the target gene relative to the calibrator.

Targetted genes were successfully amplified without any nonspecific amplification as presented in AppendixI.

Table 26 : Primers of RT^2 qPCR Primer Assays

S. No	Gene	Primer
1	*MKI67*	PPH01024E, RT^2 qPCR Primer Assays (Qiagen, Hilden, Germany)
2	*KRT18*	PPH00452F, RT^2 qPCR Primer Assays (Qiagen, Hilden, Germany)
3	*VEGF A*	PPH00251C, RT^2 qPCR Primer Assays (Qiagen, Hilden, Germany)
4	*CCND1*	PPH00128F, RT^2 qPCR Primer Assays, (Qiagen, Hilden, Germany)

S. No	Gene	Primer
5	*IL-10*	FORWARD - GTGATGCCCCAAGCTGAGA REVERSE - CCCCCAGGGAGTTCACATG
6	*TGF-β*	FORWARD- CTATTCAAGACCACCCACCTTCTG REVERSE- CTCCCGGCAAAAGGTAGGA
7	*IFN-γ*	PPH00380C, RT^2 qPCR Primer Assays (Qiagen, Hilden, Germany)
8	*GAPDH*	PPH00150F, RT^2 qPCR Primer Assays (Qiagen, Hilden, Germany)
9	*HLA -G*	PPH23053B, RT^2 qPCR Primer Assays (Qiagen, Hilden, Germany

5.2.2.2 Protein expression study of cytokine ,chemokines and cell markers .

5.2.2.2.1 Expression of NK surface marker CD56, growth factor: VEGFA, Ki-67, keratin 18 and SOCS 3 proteins by ELISA in abortus and placenta

Indirect ELISA was performed to quantify CD56, Ki-67, Keratin 18 ,VEGFA and SOCS 3 in in 97 tissue samples . The anitbodies used were CD56 (BD Biosciences, United States) , Ki-67 (Sigma-Aldrich, Merck, Germany), keratin 18 (Invitrogen, Applied Biosystems, United States), VEGF (Invitrogen, Applied Biosystems, United States), SOCS 3 (Invitrogen, Applied Biosystems, Foster City, CA, USA). Goat anti-mouse HRP conjugated antibody was used as the secondary antibody (BD Biosciences, United States). Twenty adjacent normal tissues were used as negative controls. The optical density (O.D.) was determined in duplicate readings in Varioskan Lux multimode microplate reader (Thermo Fisher Scientific, United States). The mean (O.D.) plus three standard deviations of the ten controls was used as the cut off reading. The total protein levels were expressed as an arbitrary unit or AU which was calculated as described in section 4a.2.5.

5.2.2.2.2 Cytokine and chemokine study in tissue samples by Cytometric Bead Array in flow cytometry platform.

Protein expression of cytokines (IL-10, IL-12p70, TNF-, IL-1β, IL-6, and IL-8) and chemokines (CCL2, CCL5, CXCL8, IP-10, and CXCL9) was determined in abortus (15 SAB, 15 healthy) and placenta (5 healthy and 5 SAB history). CBA kit for inflammatory cytokines and for chemokines (BD Biosciences, United States) was used for the same according to manufacturer's instructions. Both the kits were used to determine the quantity of the sets of inflammatory cytokines and chemokines in individual samples. Mean Fluorescence Intensity (MFI) was recorded and the quantity of cytokine and chemokine protein (pg/mL) in each sample was determined by FCAP array software (BD Biosciences, United States).

5.2.3 . Single cell preparation , phenotyping and NK activation study

5.2.3.1 Single cell suspension preparation

Media preparation

The RPMI-1640 media was sterilized by passing it through a 0.2 μm membrane filter using a 10 ml syringe. This filtered media was then mixed with 1% antimycotic-antibiotic to create incomplete media. The required amount of incomplete media was transferred to a new 15 ml Falcon tube, and 10% Fetal Bovine Serum (FBS) was added to make it complete media.

Tissue processing

1. To process the tissue, a fresh tissue sample obtained from the hospital was suspended in 2 ml of RPMI-1640 incomplete media. The media was discarded, and the tissue was washed by resuspending it in 5-7 ml of PBS (Phosphate-Buffered Saline). This washing step was repeated three times until a clearer mass of tissue was obtained.

2. Next, the tissue mass was transferred onto a petri dish containing 1-2 ml of 1% antimycotic-antibiotic solution to prevent dehydration. A portion of the tissue was set aside for explant culture, while the remaining tissue mass was further processed.

3. The remaining tissue mass was finely minced using a lancet or blade. The minced fragments, along with the antibiotic solution, were transferred to a 15 ml Falcon tube containing 5 ml of PBS. The tube was then centrifuged at 600g for 5 minutes at 4°C.

The supernatant was discarded, and the minced tissue fragments were resuspended in 5-7 ml of PBS. This step was followed by another centrifugation at 600g for 5 minutes at 4°C. Again, the supernatant was discarded, and the tissue fragments were resuspended in RPMI-1640 incomplete media. A subsequent centrifugation at 600g for 5-7 minutes at 4°C was performed, and the supernatant was discarded.

4. Next, 3 ml of an enzyme cocktail containing trypsin, elastase, hyaluronidase, and collagenase was added to the Falcon tube containing the minced tissue fragments. The tube was placed on a thermoblock and incubated for 45 minutes at 450 rpm and 37°C. After this incubation, a short centrifugation step was performed.

5. The resulting supernatant was transferred to a new 15 ml Falcon tube, and 200 µl of trypsin inhibitor was added to it. The tube was then centrifuged at 600g for 10 minutes at 4°C. The cell pellet obtained after centrifugation was resuspended in 2-4 ml of RPMI-1640 complete media.

6. To obtain a single-cell suspension, the resuspended cells were strained into a 50 ml Falcon tube using a cell strainer. A portion of the cell suspension was plated in a 12 or 24 well plate and placed in a CO2 incubator for further culture. The remaining cell suspension was transferred into a 2 ml microcentrifuge tube (MCT) for further analysis using a flow cytometer.

5.2.3.1.1 NK phenotyping and activation status

Preparation of reagents

Staining buffer: 5% FBS in 1X PBS + 0.1% sodium azide (added if needed for long storage)

Wash Buffer : 1X PBS + 0.1% Sodium azide (added if needed for long storage)

Fixative : 1% parafolmaldehyde (stock of 4%) dissolved in 1X PBS

Staining of Samples for NK Phenotype (CD56, CD16, CD9) and activation Status by CXCR4 and IFN-gamma

1. Cells suspended in 200 µl of media were transferred to a 2 ml microcentrifuge tube (MCT) and centrifuged at 600g for 10 minutes at 4°C.

2. The supernatant was discarded, and 400 µl of staining buffer was added to the tube. The cells were resuspended in the buffer by gently flushing with a pipette.

3. Fluorophore-conjugated surface marker antibodies: CD45,CD56,CD16,CD9 (BD, Biosciences, United States) and CXCR4 (Invitrogen, Applied Biosystems, United States) were added, followed by a brief centrifugation step. The cells were mixed by pulse vortexing and incubated in the dark for 20 minutes at room temperature. The information on antibodies.

5. 1 ml of wash buffer was added to the MCT and centrifuged at 500g for 5 minutes. The supernatant was discarded, leaving a small amount at the bottom.

6. RBC lysis was performed to obtain a white pellet containing the white blood cells (WBCs). A sample of 15 µl was taken aside for cell counting.

7. 2 ml of fixative buffer was added to the MCT containing WBCs bound to antibodies. The mixture was mixed by pulse vortexing and incubated in the dark for 10 minutes at room temperature.

8. After incubation, the MCT was centrifuged at 500g for 5 minutes at room temperature. The supernatant was discarded, leaving a small amount at the bottom to resuspend the pellet.

9. 500 µl of permeabilizing buffer was added and mixed by pulse vortexing. The MCT was then incubated in the dark for 20-30 minutes at room temperature.

10. After incubation, 1 ml of wash buffer was added to the MCT and centrifuged at 300g for 5 minutes at room temperature. The supernatant was discarded, leaving approximately 150-200 µl at the bottom of the MCT to resuspend the pellet.

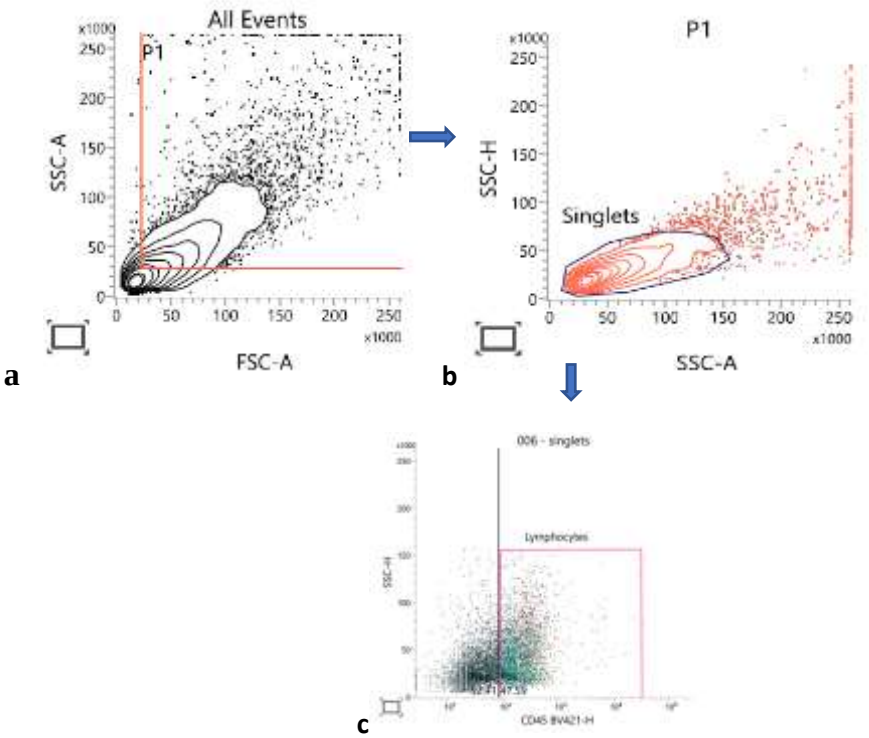
11. IFN gamma antibody was added to the pellet, followed by a brief centrifugation step. Pulse vortexing was performed, followed by an incubation in the dark for 30 minutes at room temperature and Final washing was done by adding 700 µl of wash buffer.

12. The supernatant was discarded, leaving approximately 200 µl of wash buffer at the bottom of the MCT.300 µl of wash buffer was added to bring the volume to approximately 500 µl for resuspending the cells.

13.The MCT with cells bound to fluorophore-conjugated antibodies was then used for flow cytometry.

5.2.3.2 Gating strategy used

Cells that were gated in all events and identified as singlets were selected for further analysis. Among these cells, those positive for CD45 were considered lymphocytes. The population positive for CD56 was quantified. A scatter plot of CD56 versus CD16 was generated, and the positive population for CD56 and negative for CD16 was designated as decidual cells, while the population with dim CD56 and positive CD16 was identified as cytotoxic cells. In context of CD56+ population, we also examined the presence of CD9-positive cells. For signaling analysis, we gated on the CD56-positive population and assessed the presence of CXCR4. Furthermore, we examined the production of IFN-gamma in these CD56-positive, CXCR4-positive cells. We also compared the overall production of IFN-gamma with the production in the CD56-positive, CXCR4-positive population. Figure 30 provides a graphical depiction of the steps involved in selecting specific cell populations for analysis.



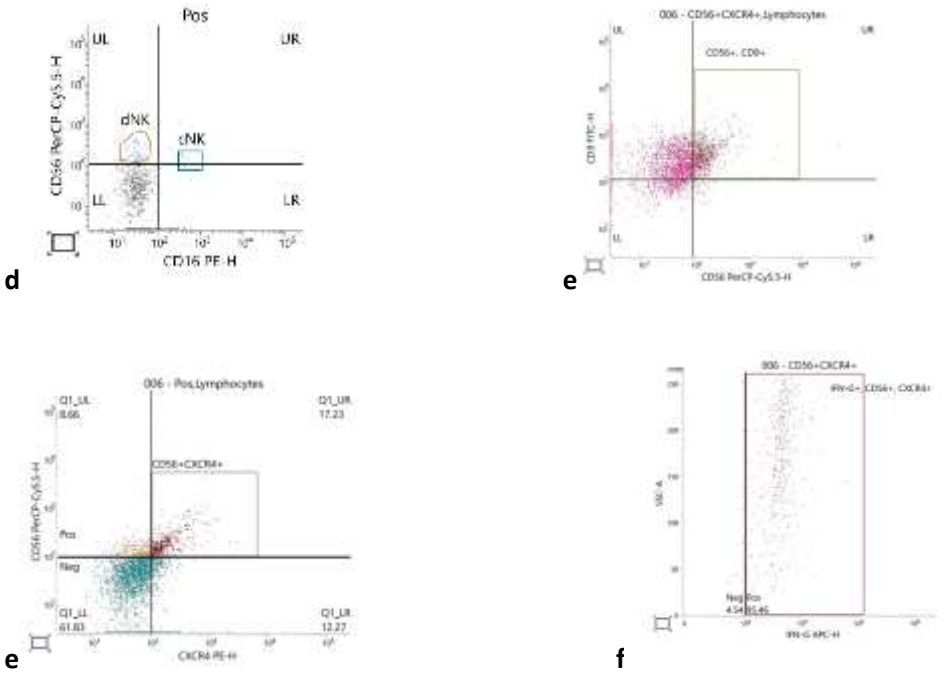


Figure 30: CD45-positive cells were gated in singlets. The expression of CD56 and CD16 was then analyzed using specific markers (a-c, d, e), allowing the identification of distinct subsets of NK cells, including $CD56^{dim}CD16^{+}$ and $CD56^{+}CD16^{-}$ populations.In order to study IFN-gamma signalling, the focus was on the $CD56^{+}CXCR4^{+}$ population (a-c, f, g). Within this specific subset, the presence of cells producing IFN-γ was examined.

5.2.4. Statistical analysis

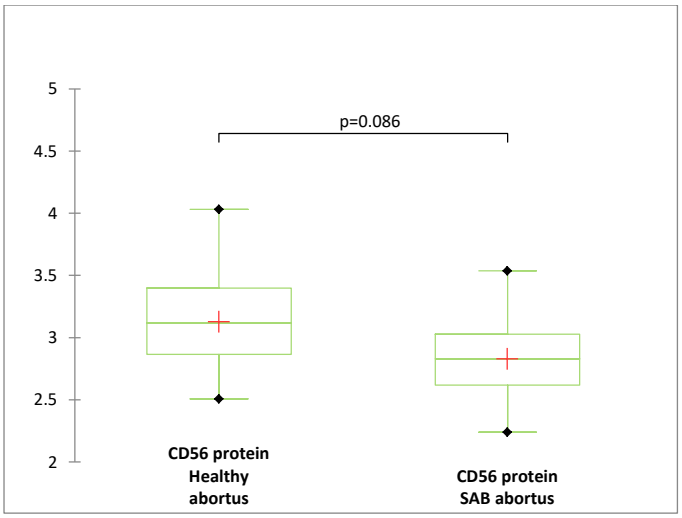
Participants with incomplete data or incomplete investigations were excluded from the analysis. Statistical analysis of the data was performed using XLSTAT 2015 and XLSTAT student versions. Correlation analysis was performed between the expression of cytokines, chemokines with NK signalling markers using the Pearson's Correlation test. Student's *t*-test was used for comparison between the mean values. A p-value < 0.05 was considered statistically significant.

5.3 Results

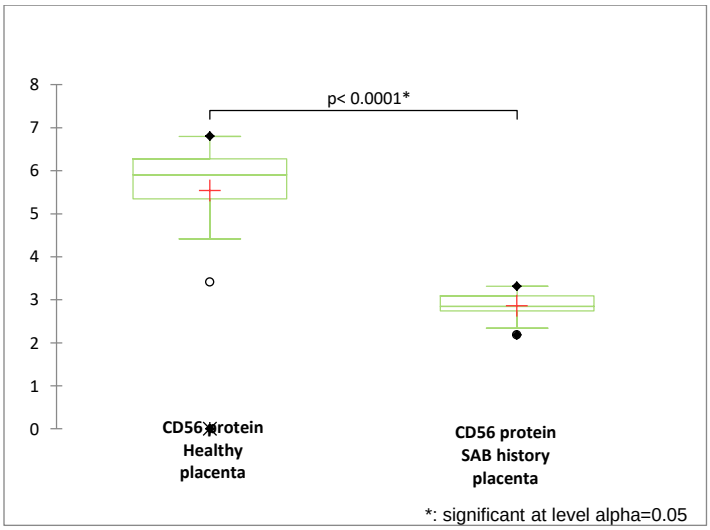
5.3.1 Expression of CD56 protein in tissue samples

The analysis of CD56 levels by ELISA ,in abortus revealed significantly higher protein levels in healthy abortus compared to SAB (Mann-Whitney U test, $p < 0.0001$) (Figure 31a). CD56 levels were comparable between SAB and healthy placenta although the difference did not reach statistical significance (Figure 31b). Interestingly, when comparing healthy abortus samples with term healthy placenta, it was found that

healthy abortus samples exhibited higher CD56 protein levels (student t test ,p= < 0.002).



(a)



(b)

Figure 31 : Expression of CD56 protein in (a)healthy vs SAB abortus tissue and (b) healthy vs SAB placenta tissues. Values indicate protein expression in AU and error bars represent standard deviation from the mean. CD56 levels were higher (3.1±0.4) in healthy abortus than in (2.8±0.3) SAB abortus. Healthy placenta (5.5±1.3)had higher CD56 levels as compared to SAB history placenta(2.8±0.3) .

5.3.2 NK phenotyping and activation status in abortus in relation to early pregnancy loss.

As the expression of the CD56 surface marker was significantly higher in abortus samples (healthy=15, SAB=15), flow cytometric approach was employed to investigate decidual NK cells using CD56 as the marker. Additionally, CD16, a marker of cytotoxic NK cells, and CD9, a marker of decidual NK cells, were also utilized in conjunction with CD56.

The proportion of CD56+ population among lymphocytes was higher in healthy abortus compared to SAB abortus, as shown in the Figure 32 a. MFI of CD56 was in concordance with it and showed higher expression in healthy abortus than SAB abortus (student t test , p=0.05) Figure 32 b.

Furthermore, the proportion of the CD56+CD16- population was found to be higher in healthy abortus compared to SAB abortus, as depicted in the Figure 33. Subsequently, we examined the CD9 population and observed that the proportion of CD56+ CD9+ cells was similar between SAB and healthy abortus, as shown in the Figure 34

We observed that the CD56+CXCR4+IFNγ+ population was more prevalent in healthy abortus compared to SAB Figure 35. However, when examining the IFNγ^{+} population independently of CD56 expression, we found that IFNγ^{+} cells were more abundant in SAB (Mann-Whitney U test, $p \leq 0.02$). The MFI results were consistent with the percentage of positive cells obtained from the flow cytometry data Figure 35.

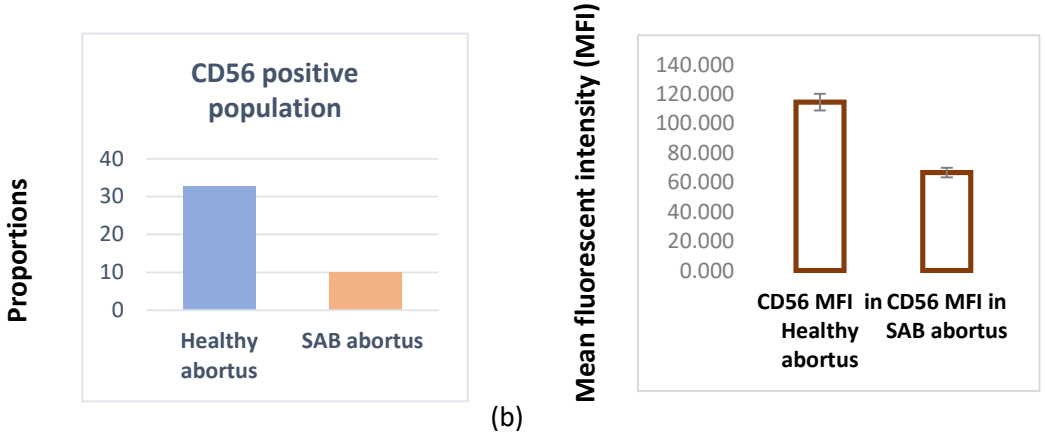


Figure 32 : Proportion of CD56^{+} Cell population in healthy vs SAB abortus (a). Mean flouroscent Intensity of CD56 $^{+}$ cells in (MFI± SD) (114.5±1.2) healthy vs in (66.5±1) SAB abortus (b).

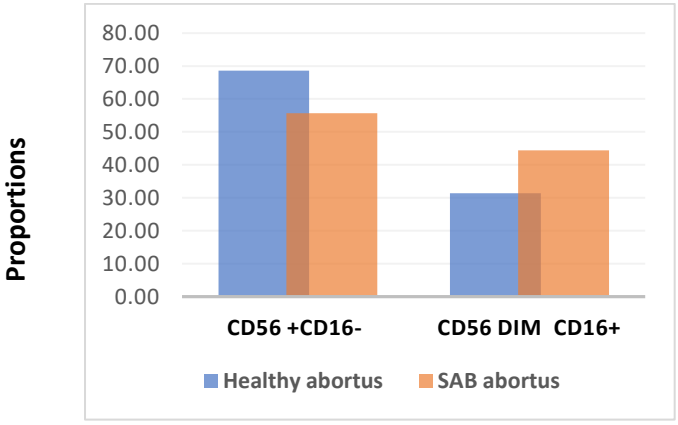
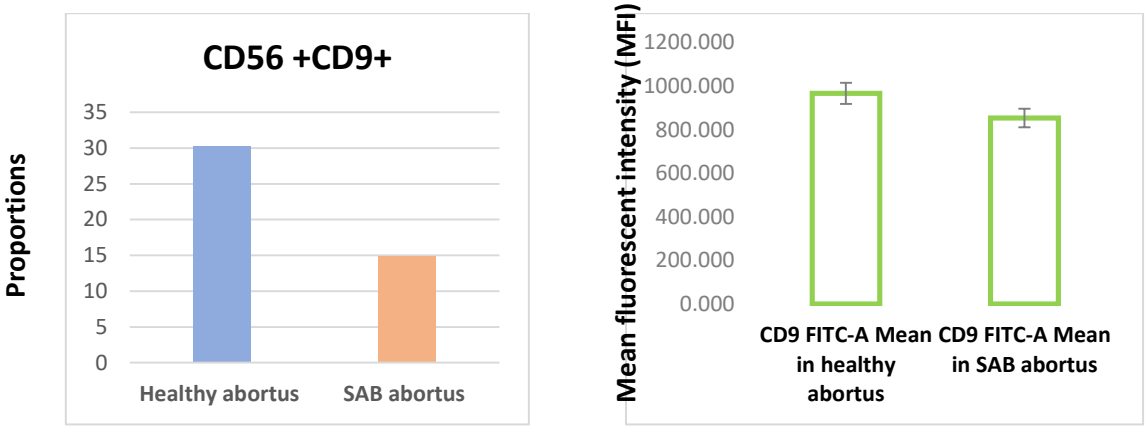
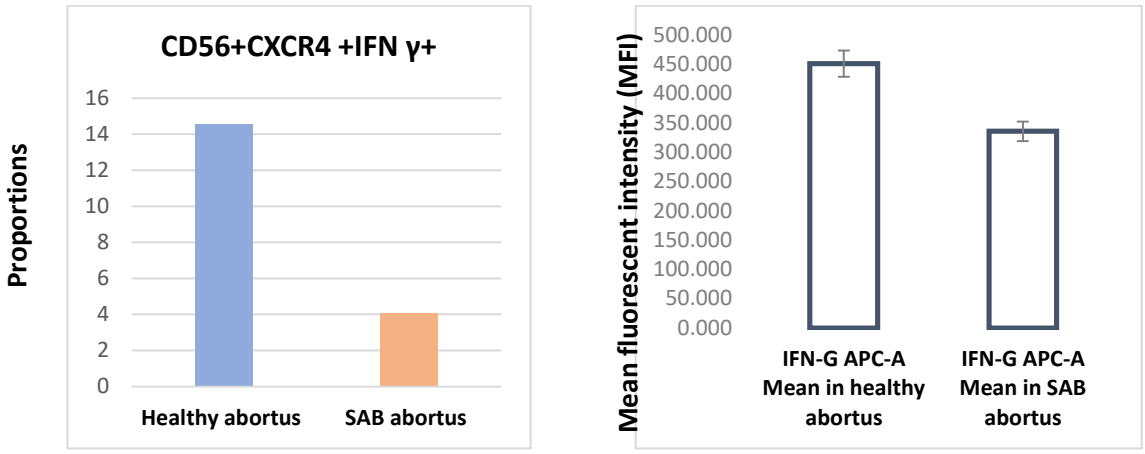


Figure 33 : Proportion of $CD56^{+}$ $CD16^{-}$ and $CD56^{dim}$ CD16+ cell population in healthy vs SAB abortus



(a) (b)

Figure 34: Proportion of $CD56^{+}$ $CD9^{+}$ cell population in healthy vs SAB abortus (a). Mean flouroscent Intensity of $CD9^{+}$in (MFI± SD) (967±1) healthy vs in (854±1.2)SAB abortus (b).



(a) **(b)**

Figure 35 : Proportion of $CD56^{+}$ $CXCR4^{+}$ $IFN\gamma^{+}$ cell population in healthy vs SAB abortus (a). Mean flouroscent Intensity of $IFN\gamma^{+}$ in (MFI± SD)(451.06±1) healthy vs in (335±.8) SAB abortus (b).

5.3.3 Cytokine and Chemokine environment in abortus and placenta .

5.3.3.1 Cytokine expression study

IL1B, IL2, IL15, IL18, IL21, TGFβ, IFNγ, TNF, IL10 transcript level studied in 97 abortus and placenta samples . In abortus ,it was observed that levels of IL15 and TNF were elevated in healthy as compared to SAB abortus. Conversely, in SAB abortus, transcript levels of *IL1B, IL2, IL18, IL21, TGFβ, IFNγ, and IL10* collectively showed higher expression. In the specific case of *IFNγ* (t test, p = 0.016) and *IL10* (t test, p=0.002), significant p-values were observed, highlighting a notable difference between the two groups abortus Figure 36a . Protein levels of cytokines IL1B, IL8, IL10, TNF, IL12, and IL6 were observed to be elevated in SAB abortus Figure 36b. However, these elevations were not statistically significant. The protein levels of IL10 and IL1B also correlated with the transcript expression in SAB Figure 36 c

In the placenta, most cytokines IL1B, IL2, IL15, IL18, TGFβ, IFNγ, IL10 exhibited elevated levels in the healthy group. However, the levels of IL21 and TNF were found to be different, showing no significant elevation in the healthy group compared to other cytokines placenta (Figure 37a) .The protein levels of IL1B, IL10, TNF, IL12, and IL6 were also higher in SAB placenta than healthy placenta. IL8 levels were higher in healthy placenta (Figure 37b). 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) (Table 27). Our data collectively suggests negative correlation of IL15 , IFNγ and IL10 with healthy status of pregnancy.

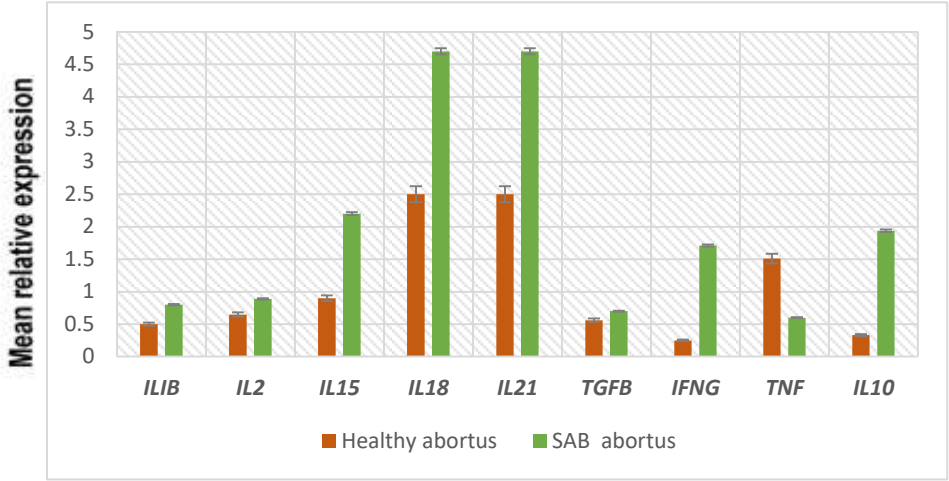


Figure 36 a: Mean relative expression of cytokines in healthy vs SAB abortus . Mean relative expression was calculated as fold change. Error bars represented the standard deviation from the mean.

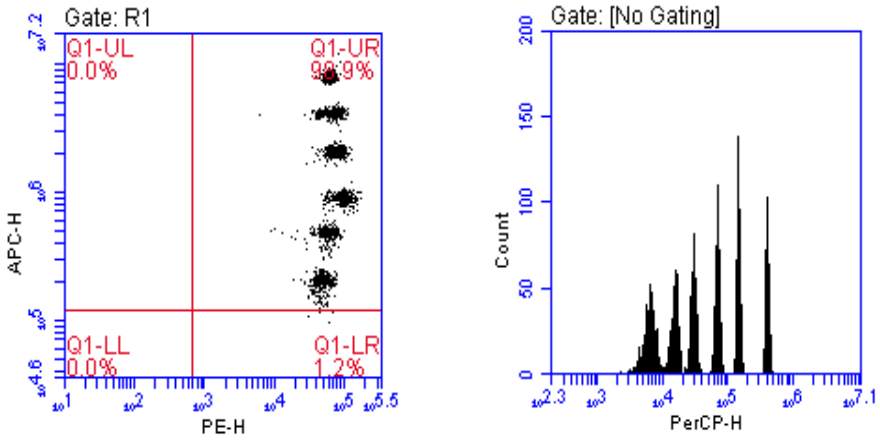


Figure 36 b: Quantification of inflammatory cytokine proteins in in healthy abortus tissue. Flow cytometry image showing results of bead-based capture of all the six cytokine proteins (IL-10, IL-12p70, TNF, IL-1β, IL-6, and IL-8) in an individual sample (A) and Fluorescence intensity of the proteins in that sample (B).

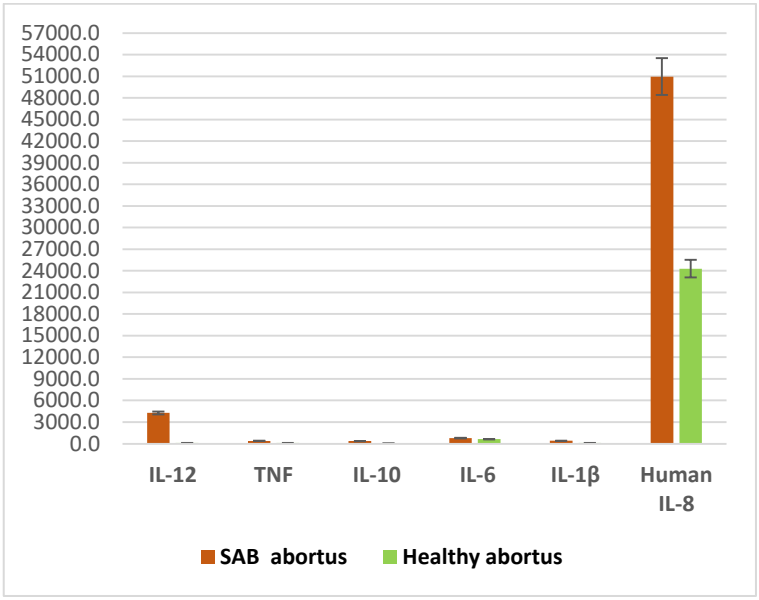


Figure 36 c : Expression of cytokines in healthy vs SAB abortus. Values indicate protein expression in **pg/mL** and error bars represent standard deviation from the mean.

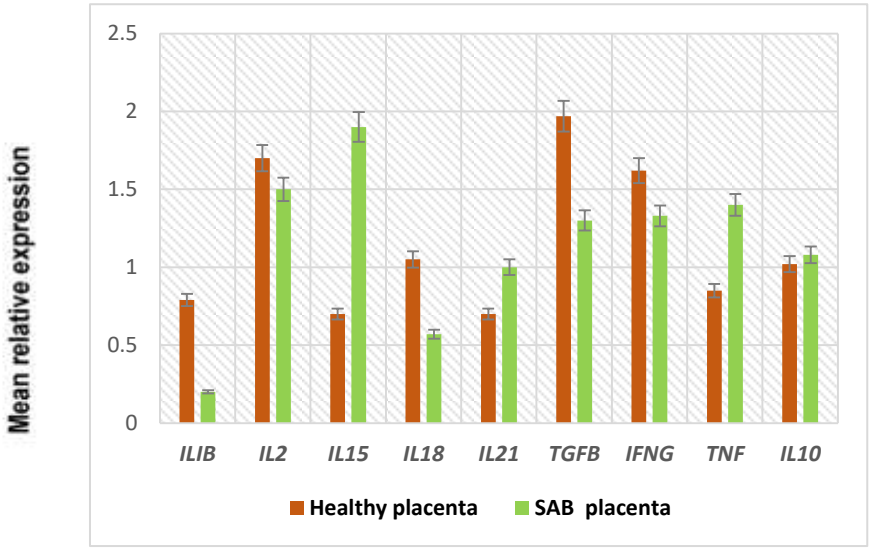


Figure 37 a : Mean relative expression of cytokines in healthy vs SAB history placenta . Mean relative expression was calculated as fold change. Error bars represented the standard deviation from the mean.

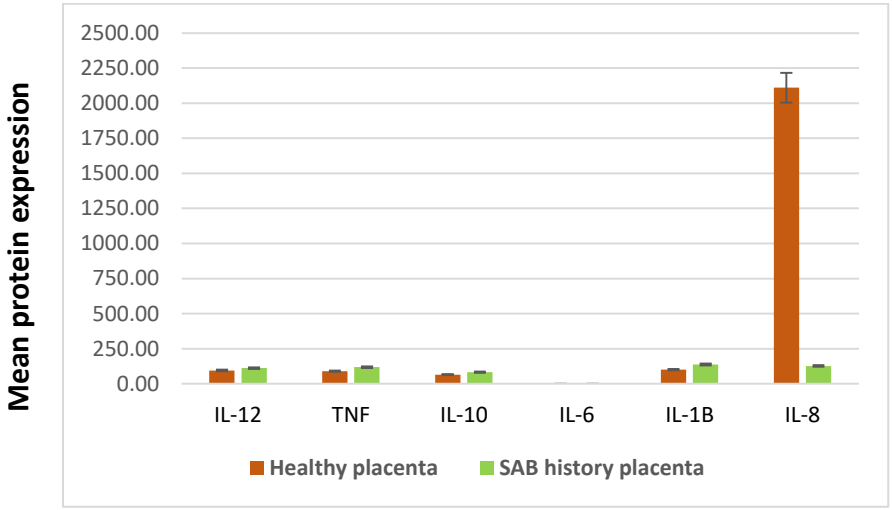


Figure 37b : Expression of cytokines in healthy vs SAB history placenta . Values indicate protein expression in pg/mL and error bars represent standard deviation from the mean.

Table 27 : Multivariate cox analysis reveals that IL15 shows significant hazard ratio and p values of cytokines with obs history.

Cytokines	Hazard ratio	P-value
IL1B	1.023602	0.70841
IL2	1.077944	0.26707
IL15	1.138448	0.00805
IL18	0.987964	0.60216
IL21	0.993185	0.41849
TGF-β	1.049232	0.35767
IFNγ	0.903050	0.13433
P-value for the multivariate regression model		
Likelihood ratio test		0.1
Wald test		0.06
Score (logrank) test		0.05

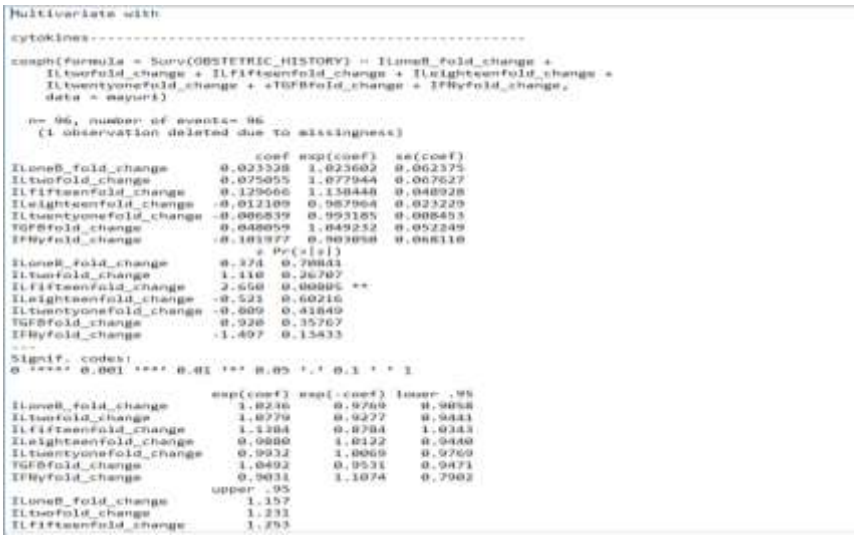
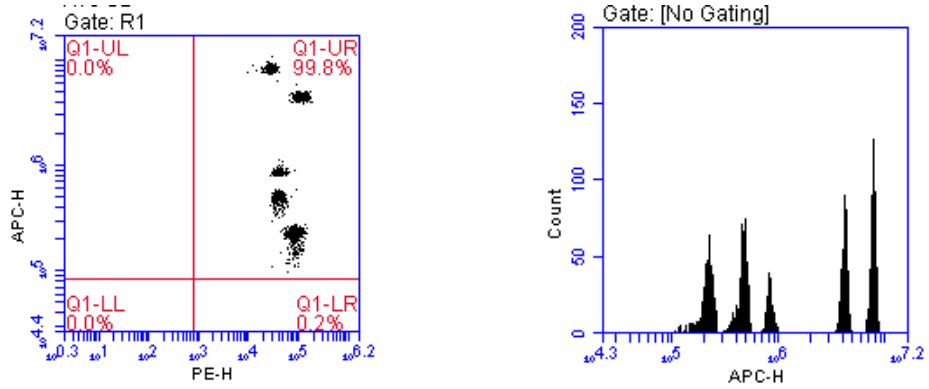


Figure 38 : Pictorial representation of Multivariate cox analysis done for determining correlation of cytokines on survival of the fetus .

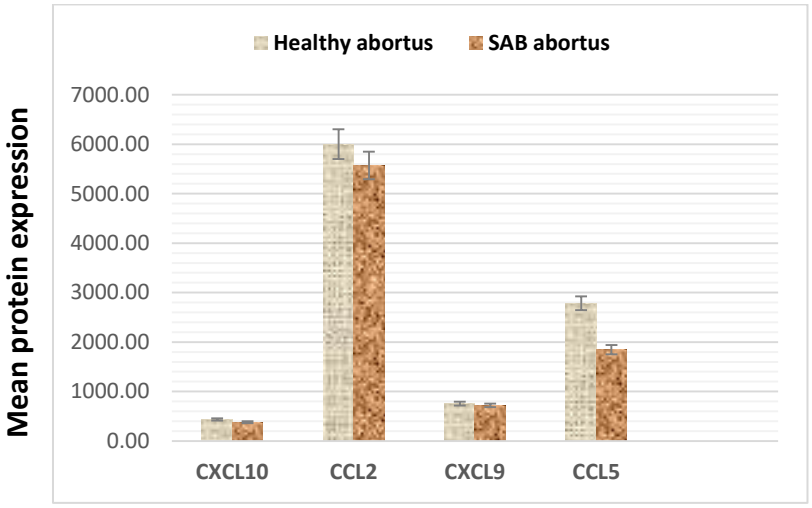
5.3.3.1 Chemokine expression study

Expression level of key chemokines - CCL5 , CCL2, CXCL8, CXCL9 and CXCL10 were studied to understand the NK activation and migration in decidua vs placenta. While our data showed higher expression of CCL5, CCL2, CXCL8 in healthy abortus as compared to SAB (Figure 39 a). This data was consistent with our data on high IFNγ level in SAB abortus .Placenta showed higher CCL5 in SAB history than healthy, while CCL2 and CXCL8 was higher in healthy than SAB. (Figure 39 b).

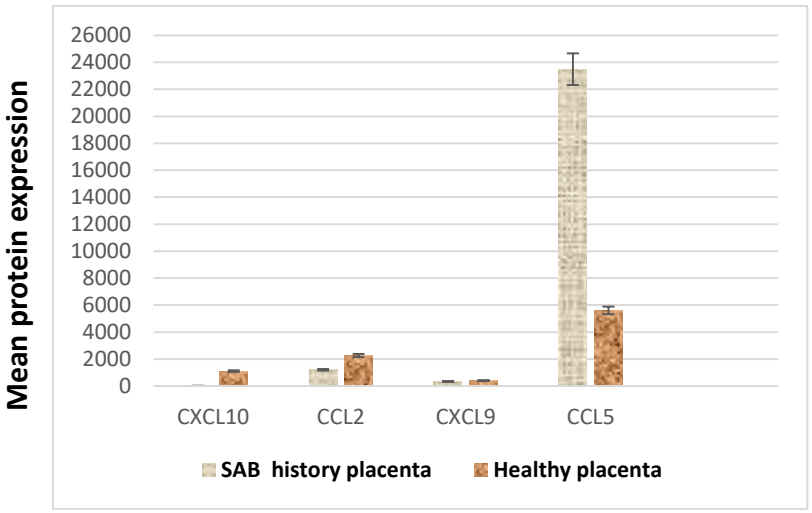


(a) (b)

Figure 39 a : Quantification of chemokine proteins in healthy abortus tissue . Flow cytometry image showing results of bead-based capture of all the five chemokines (CCL2, CCL5, CXCL8, IP-10, and CXCL9) in an individual sample (a) and Fluorescence intensity of the proteins in that sample (b).



(a)

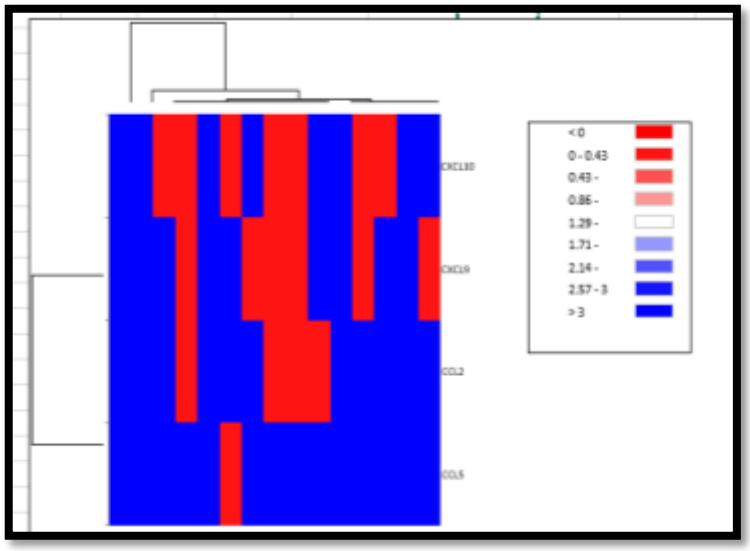


(b)

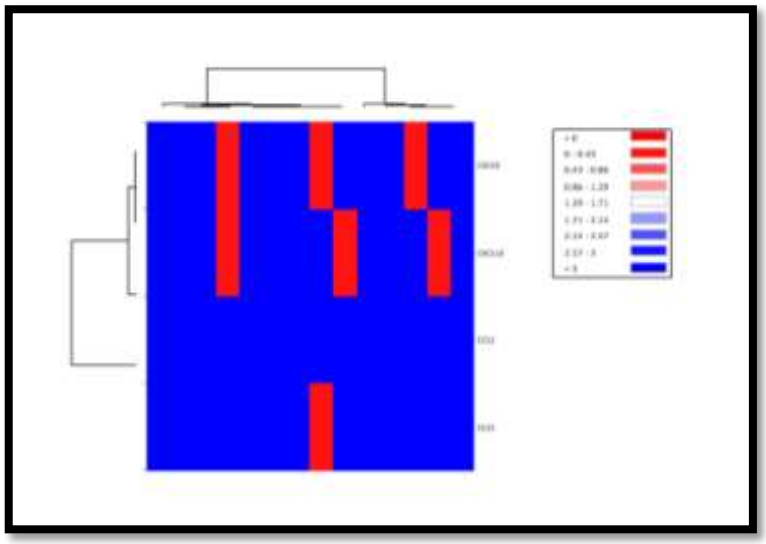
Figure 39 b : Expression of chemokines in(a) healthy vs SAB abortus and in (b)healthy vs SAB history placenta . Values indicate protein expression in pg/mL and error bars represent standard deviation from the mean.

5.3.3.2 Heat Map Analysis of key chemokines

Heat map analysis revealed that there was a distinct profile of chemokines between SAB abortus and healthy abortus samples as presented in Figure 40.



(a)



(b)

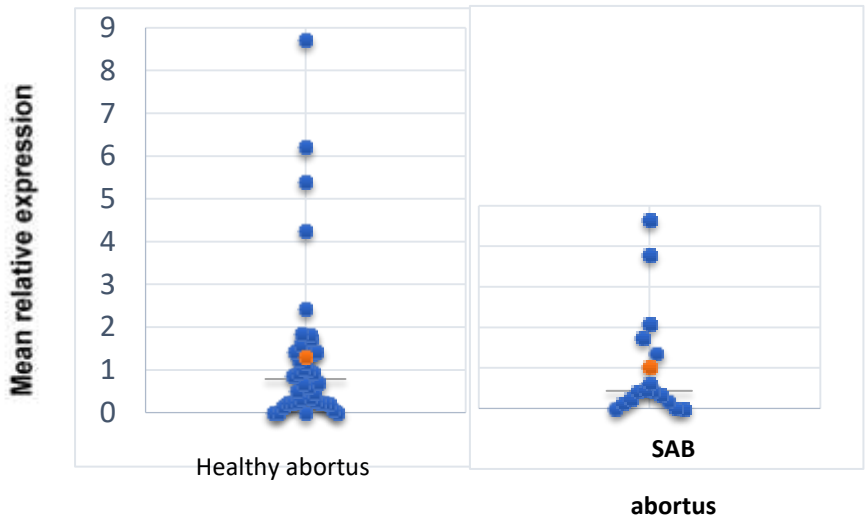
Figure 40 : Heatmap showing expression of key chemokines of migration and EVT invasion (a)SAB abortus and in (b) healthy abortus . Blue color represents very high expression, light blue color represents intermediate expression and red color represents low expression. Expression profile similarities are depicted by the branch lengths of the dendrogram.

5.3.4 Expression study of growth factor : VEGF-A, cell proliferation and differentiation markers : Keratin 18, and Ki-67 in abortus and placenta

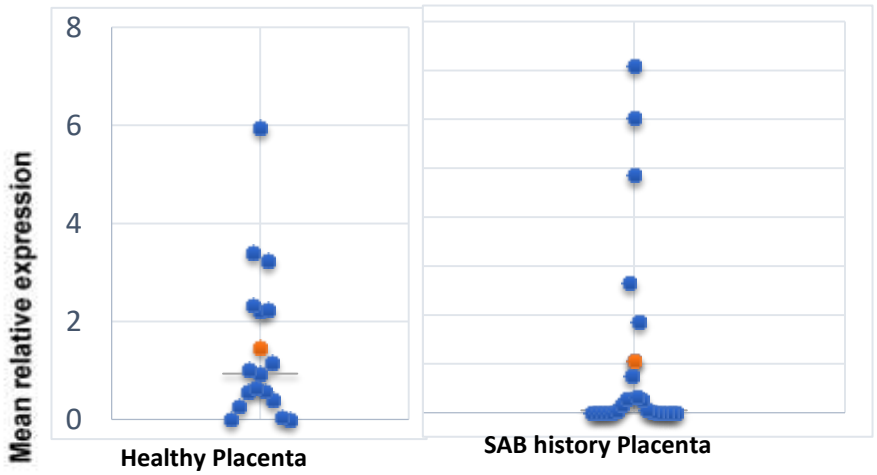
VEGFA transcript levels as well as protein levels were higher in healthy abortus than SAB group. (Figure 41a). Placental data showed comparable levels of VEGFA transcript between SAB and healthy, but protein levels were higher in healthy placenta as compared to SAB placenta (Figure 41b) .

Keratin-18 transcript levels were higher in SAB abortus as compared to healthy group and its protein levels were in concordance with transcript levels. Despite of comparable transcript levels between both groups, Keratin 18 protein levels were higher in SAB placental tissue as compared to healthy placenta (p value=0.001). A negative correlation was observed between VEGFA and Keratin-18 in healthy abortus /placenta (p= 0.03). (Figure 42a, Figure 42b)

Furthermore, the protein levels of Ki-67 were studied in tissue samples, Ki-67 levels were higher in healthy abortus as compared to SAB abortus. However, SAB placenta showed higher ki-67 levels than healthy placenta (Figure 43).

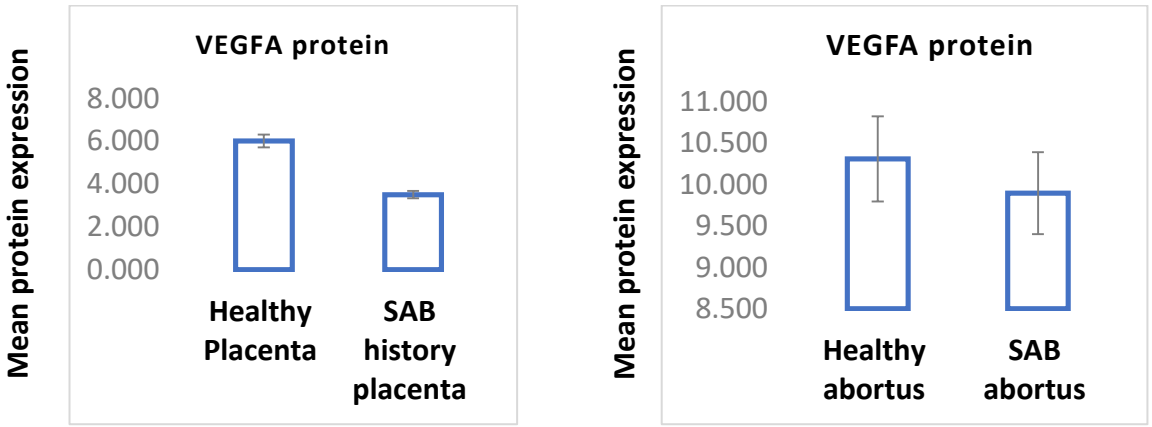


(a)



(b)

Figure 41a **:** Transcript expression of VEGFA in abortus (a) and protein expression in (b) abortus . Mean relative expression was calculated as fold change. Error bars represented the standard deviation from the mean.



(a) **(b)**

Figure 41 b: Protein expression of VEGFA in placenta (a) and protein expression in (b) abortus.The expression was determined as the arbitrary unit or AU and error bars represent standard deviation from the mean.

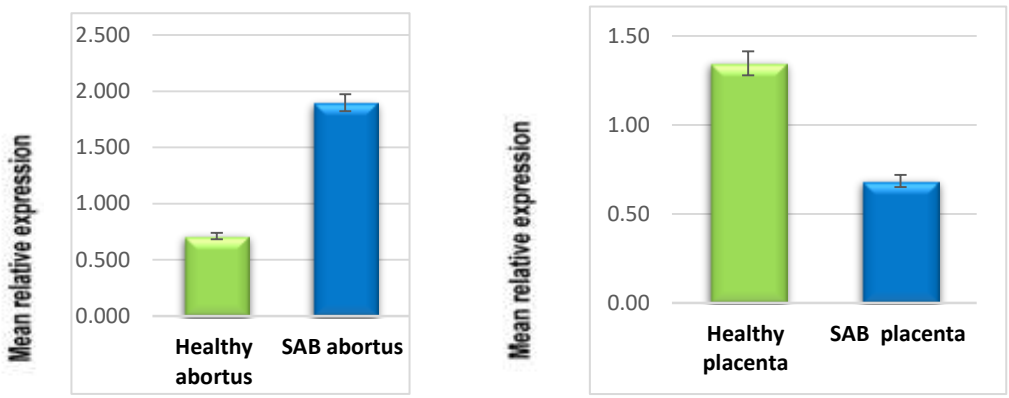
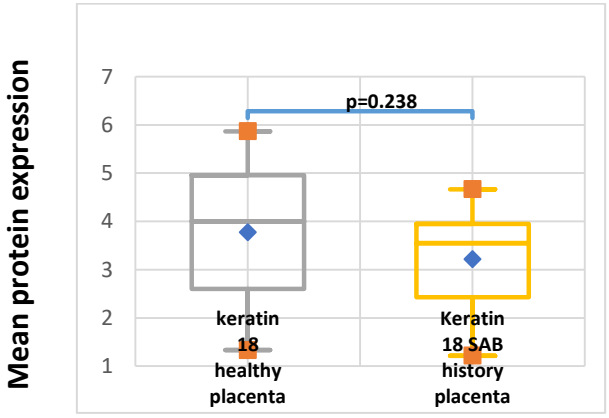
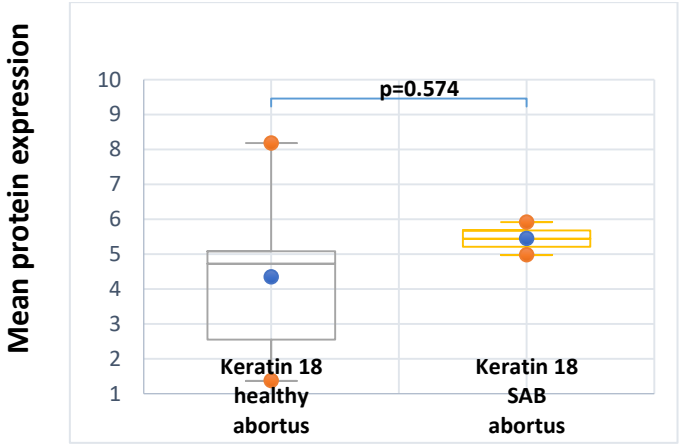


Figure 42a: Transcript expression of Keratin-18 in abortus (a) and expression in (b) placenta. Mean relative expression was calculated as fold change. Error bars represented the standard deviation from the mean.

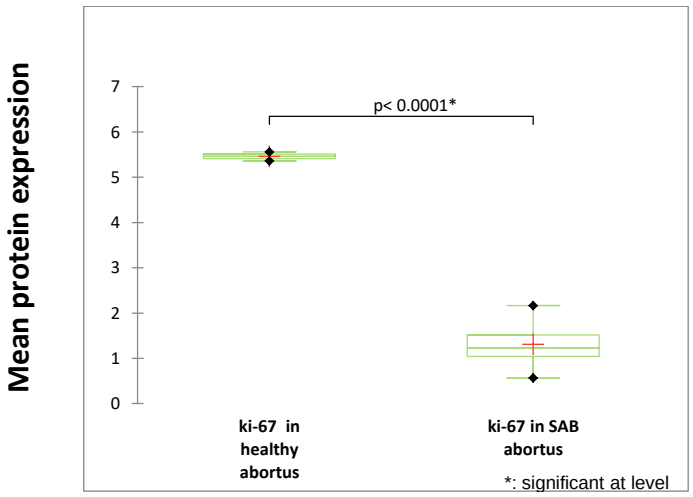


(a)

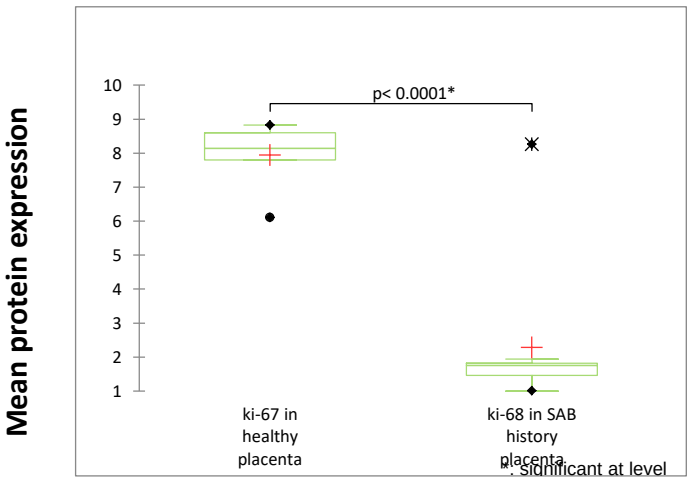


(b)

Figure 42b: Protein expression of Keratin 18 in abortus (a) and protein expression in (b) placenta . The expression was determined as the arbitrary unit or AU and error bars represent standard deviation from the mean.



(a)

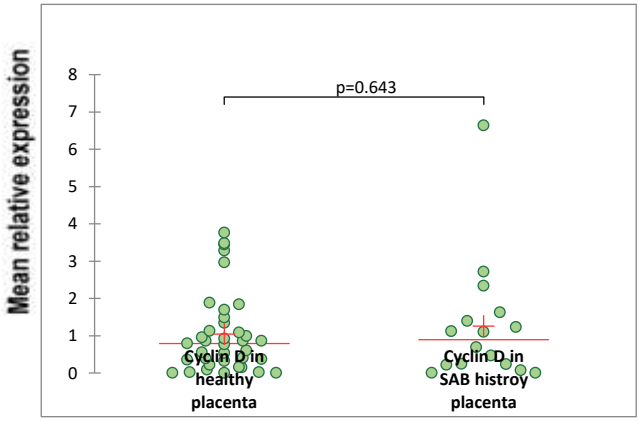


(b)

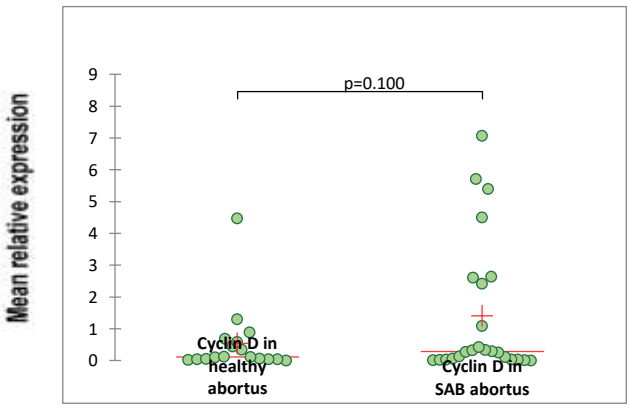
Figure 43: Protein expression of Ki-67 in abortus (a) and in (b) placenta.The expression was determined as the arbitrary unit or AU and error bars represent standard deviation from the mean.

5.3.5. Expression study cell cycle marker-Cyclin D in abortus and placenta

The transcript levels of Cyclin D were higher in SAB abortus compared to healthy group. In addition, the healthy placenta had higher Cyclin D levels compared to SAB group, although it did not reach statistical significance (Figure 44)



(a)

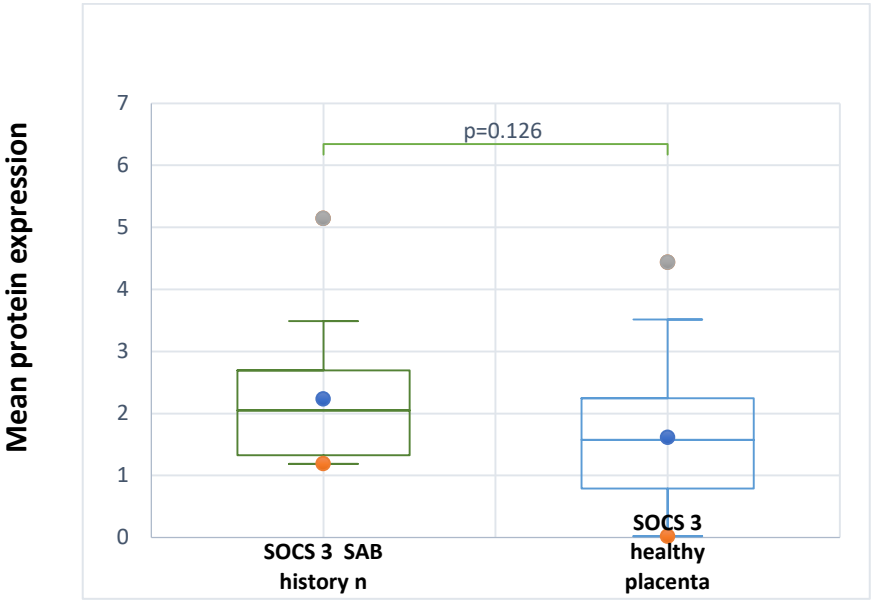


(b)

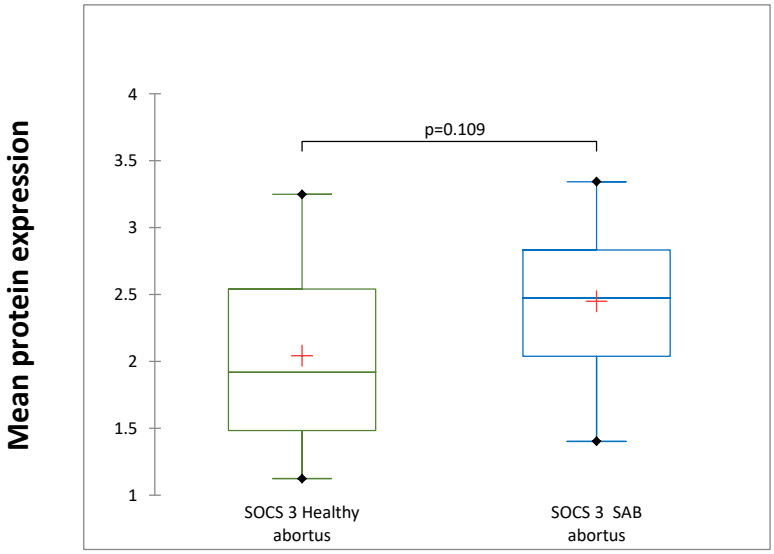
Figure 44: Transcript expression of cyclin D in abortus (a) and in (b) placenta

5.3.6 SOCs 3 expression in abortus and placenta

The levels of SOCs were comparable in SAB abortus and healthy abortus. However, in term placenta the healthy group had higher SOCs levels compared to SAB. (Figure 45). Our data is further supported by Logistic regression analysis which showed that high levels of SOCS3 were associated with the healthy pregnancy. The ROC curve with an AUC of 0.85 indicates the strength of the model.



(a)



(b)

Figure 45: Protein expression of SOCS 3 in abortus (a) and in (b) placenta. The expression was determined as the arbitrary unit or AU and error bars represent standard deviation from the mean.

5.4 Discussion

The central role of dNK cells in early placentation, particularly in the remodeling of placental arteries and trophoblastic invasion, has been widely reported (1-3). Our study findings, demonstrating the predominance of $CD56^{+}$ $CD16^{-}$ $CD9^{+}$ dNK cells in healthy early abortus compared to SAB abortus are consistent with previous studies that showed a higher percentage of $CD56^{+}$ $CD16^{-}$ cells in luteal phase endometrial biopsies of healthy fertile women (66-68). However, our findings differ from studies that have observed a higher proportion of $CD56^{+}$ dNK cells in cases of early pregnancy failures (69-73).

Our findings showing a higher proportion of $CD56^{+}$ $CD16^{-}$ cells in healthy individuals along with an increased proportion of $CXCR4^{+}$ $CD56^{+}$ cells, provide support that CXCR4 is mediating the migration of $CD56^{+}$ $CD16^{-}$ cells from the periphery to the decidua (74-76). These findings were in concordance with earlier studies which showed that CXCR4/CXCL12 signaling supports trophoblast invasiveness by increasing the secretion of MMP-9 and MMP-2 in normal pregnancy(74-78). The expression of the CXCR4 receptor is unique compared to other chemokine receptors, as it is equally expressed on both $CD56^{dim}$ $CD16^{+}$ and $CD56^{+}$ $CD16^{-}$ NK cell subsets(73-75). This balanced expression suggests the crucial role of CXCR4 in localization of dNK cells towards decidua.

Elevated levels of IFN-γ with low expression of $CXCR4^{+}$ showed a strong negative correlation between IFN-γ and CXCR4 in SAB, and prompts towards imbalance in migration of dNK due to IFN-γ (75-77,82). Furthermore, the elevated levels of CCL2 in healthy abortus is in sync with studies where it was shown that Estrogens regulate uterine natural killer (uNK)-cell migration directly and promote secretion of CCL2 from uNK cells, which facilitates uNK cell-mediated angiogenesis(83,84). It may be noted that inflammatory reaction is essential for implantation however a balanced cytokine-chemokine environment with a transient proinflammatory environment supporting pregnancy(85-90). During the window of implantation, the uterus is "primed" under the action of ovarian hormones to release proinflammatory cytokines and chemokines, including IL-8, IL-15, IL-6, CXCL10 and CXCL11 (90-93), which activate and recruit large populations of decidual immune cells to the endometrium at the time of implantation. Interestingly, while EVT invasion and interaction with dNK are crucial for placentation, It is known that excess EVT invasion can endanger

placenta and mother dNK can also secrete a range of cytokines, TNF-α, TGF-β and IFN-γ, inhibit EVT excessive invasion in later stages (94-95).

Our findings on high IFN-γ in SAB abortus is in consistent with earlier study that showed IFN-γ inhibits cell migration and proliferation mediated by SDF-1 in NK cells. Some reserchers have also suggested that dNK produced hormones progesterone could inhibit the IFN-γ production of uNK cells *via* GR (96,97). However, it is in contradictory with studies that showed that the combined exposure of decidual cells to IFN-γ and TNF-α synergistically enhances the expression of chemokines involved in recruiting NK cells (98,99).

Elevated VEGF-A levels in healthy abortus are in agreement with previous studies suggesting that CXCR4/CXCL12 expression in the decidua is not only involved in cell migration but also mediates angiogenesis and vascularization processes (98,99). Interestingly, it has been documented that CXCL12-CXCR4 signaling axis stimulates vascular endothelial growth factor (VEGF) synthesis, resulting in increased production of both CXCR4 and CXCL12, favoring placentation (100-102). Furthermore, our study revealed elevated levels of CCL5, CCL2, and CXCL8 in healthy abortus, indicating a skewed chemokine profile that supports healthy placentation. Previous studies have suggested the involvement of these chemokines in regulating excessive trophoblast invasiveness(102-107). Specifically, CCL2 through its receptor CCR2, promotes the proliferation and growth of primary human decidual stromal cells (DSCs) (103,104). Estrogens directly control the migration of (uNK) cells and stimulate the release of CCL2 by uNK cells, which supports uNK cell-driven angiogenesis (108,109). Upregulated CCL2/CCR2 enhanced primary human decidual stromal cell (DSC) invasion, while CCL5 and CCL3 has been reported to promote EVTs migration from in vitro explant cultures study, with EVT cells expressing high levels of CCR1(110-111). Furthermore, it has been reported that maternal platelets release CCL5 and primary EVTs can capture them to enhance their invasion capabilities (112). CXCL8, an interleukin-8 identified as a major chemotactic factor involved in regulating trophoblast invasion(113)). It along its receptors are observed in interstitial EVTs, vascular smooth muscle cells, and endothelial cells in patients with sporadic miscarriage.

Collectively, these findings underscore the regulatory function of chemokines, including CCL2, CCL5, and CXCL8 in controlling trophoblast invasion, ensuring a

balance between proper invasion and preventing excessive invasion by EVTs Interestingly, in addition to chemokines elevated levels of key proinflammatory cytokines IL15, IL10,IL1B in SAB abortus indicates proinflammatory biasness in SAB, disturbing placentation and contributing to SAB (114-115). While IL-10 is a pleiotropic anti-inflammatory cytokine (116-17) acting in the down-regulation of the immune response by inhibiting the IL-1β, IL-6 and PGE2 production by choriodecidua (118). Nevertheless, IL-10 enhances production of cytokines and matrix metalloproteinases within the amnion(119-120). The effects of IL-10 in fetal membranes depend on the precise tissue site. Mid-trimester Amniotic Fluid Interleukins (IL-1β, IL-10 and IL-18) as possible predictors of Preterm Delivery (121-123).

Our findings demonstrated elevated levels of proinflammatory cytokines, including IL15, IL10, and IL1B in SAB abortus, indicating a proinflammatory bias in SAB. While some amount of inflammatory response is essential for trophoblast invasion, excessive proinflammatory environment is unfavorable. Notably, previous studies have shown that IL-15 strongly induces the secretion of IL-10 in human NK cells, and IL-10, in turn, has an additive effect on the signaling cascade in NK cells (124-125). While others have showed that IL-15 plays a crucial role in eliminating senescent cells. During the proliferative phase, the growth of the endometrium leads to replication stress, causing senescence and the release of inflammatory mediators by specific EnSCs (126-127). Decidual cells secrete IL-15, activating uNK cells and facilitating the removal of senescent cells(126-127). This clearance process remodels and rejuvenates the endometrium, creating an optimal environment for embryo implantation while maintaining a balanced proinflammatory milieu (126).

It is important to note that IL-10, an anti-inflammatory cytokine with pleiotropic effects, functions by down-regulating the immune response, inhibiting the production of IL-1β, IL-6, and PGE2 by choriodecidua (127,128). However, there is evidence suggesting that IL-10 can also enhance the production of cytokines and matrix metalloproteinases within the amnion (129). IL-10 has the capacity to suppress immune function while simultaneously up-regulating the initial stages of the innate immune response mediated by NK cells, with its effects being influenced by the specific tissue site (130).

Our contradictory findings on term placenta than abortus , with higher levels of IFNγ and IL-8 suggest in healthy shows a shift from anti inflammatory to proinflammatory which is necessary for cervical dilation and preparing the uterus for delivery, an environment conducive to successful pregnancy outcome (131). Our observation of low levels of SOCS-3 in healthy placentas further supports the speculation that downregulation of SOCS allows for the expression of pro-inflammatory cytokines, facilitating successful delivery(132-133).

In terms of the temporal variation in cytokine and chemokine profiles, we observed significant differences in the expression of chemokines CXCL9, CCL2, and CXCL10 between the healthy early abortus and healthy term placenta . Although we couldn't study the early and later decidua tissue from the same participants, our findings from age-matched women experiencing fetal loss at the same gestational age support the importance of chemokines primarily in migration during the early phase of pregnancy, while also indicating the differential expression of cytokines by dNK cells occurs throughout gestation of pregnancy. In addition ,we noted higher levels of Ki-67 in healthy abortus compared to healthy term placenta is in line with earlier reports where highest expression of Ki-67 in villous cytotrophoblasts during the first trimester was observed, which gradually decreased in term placenta(132-134). Proliferation is essential for trophoblastic cells, as well as villous stromal cells and blood vessels, for the maturation and branching of villi and crucial for early placentation (134-135).

To conclude, our study demonstrates differential expression patterns of cytokines and chemokines in early and late pregnancy. Chemokine-driven migration of decidual NK cells and optimal EVT invasion play crucial roles in supporting a healthy pregnancy. Aberrant cytokine expression, such as IL1B, IFNG, and IL10, is associated with immune imbalances contributing to early pregnancy loss. Additionally, a transition towards a proinflammatory cytokine environment in late pregnancy is essential for successful pregnancy outcomes. These findings highlight the dynamic nature of cytokine regulation throughout pregnancy and provide insights into potential interventions to improve pregnancy health.

5.5 References

1. Murata, H., Tanaka, S., & Okada, H. (2021). Immune tolerance of the human decidua. *Journal of clinical medicine*, *10*(2), 351.

2. Wynn, R. M. (1974). Ultrastructural development of the human decidua. *American journal of obstetrics and gynecology*, *118*(5), 652-670.

3. ROMERO, R., WU, Y. K., BRODY, D. T., OYARZUN, E., DUFF, G. W., & DURUM, S. K. (1989). Human decidua: a source of interleukin-1. *Obstetrics & Gynecology*, *73*(1), 31-34.

4. Tabiasco, J., Rabot, M., Aguerre-Girr, M., El Costa, H., Berrebi, A., Parant, O., ... & Le Bouteiller, P. (2006). Human decidual NK cells: unique phenotype and functional properties–a review. *Placenta*, *27*, 34-39.

5. Vacca, P., Montaldo, E., Croxatto, D., Loiacono, F., Canegallo, F., Venturini, P. L., ... & Mingari, M. C. (2015). Identification of diverse innate lymphoid cells in human decidua. *Mucosal immunology*, *8*(2), 254-264.

6. Bulmer, J. N., Denise, P. A. C. E., & Ritson, A. (1988). Immunoregulatory cells in human decidua: morphology, immunohistochemistry and function. *Reproduction Nutrition Développement*, *28*(6B), 1599-1614.

7. Liu, S., Diao, L., Huang, C., Li, Y., Zeng, Y., & Kwak-Kim, J. Y. (2017). The role of decidual immune cells on human pregnancy. *Journal of reproductive immunology*, *124*, 44-53.

8. Lash, G. E., Robson, S. C., & Bulmer, J. N. (2010). Functional role of uterine natural killer (uNK) cells in human early pregnancy decidua. *Placenta*, *31*, S87-S92.

9. Kämmerer, U., Schoppet, M., McLellan, A. D., Kapp, M., Huppertz, H. I., Kämpgen, E., & Dietl, J. (2000). Human decidua contains potent immunostimulatory CD83+ dendritic cells. *The American journal of pathology*, *157*(1), 159-169.

10. Smyth, M. J., Cretney, E., Kelly, J. M., Westwood, J. A., Street, S. E., Yagita, H., ... & Hayakawa, Y. (2005). Activation of NK cell cytotoxicity. *Molecular immunology*, *42*(4), 501-510.

11. Poli, A., Michel, T., Thérésine, M., Andrès, E., Hentges, F., & Zimmer, J. (2009). CD56bright natural killer (NK) cells: an important NK cell subset. *Immunology*, *126*(4), 458-465.

12. Mamessier, E., Pradel, L. C., Thibult, M. L., Drevet, C., Zouine, A., Jacquemier, J., ... & Olive, D. (2013). Peripheral blood NK cells from breast cancer patients are tumor-induced composite subsets. *The Journal of Immunology*, *190*(5), 2424-2436.

13. Trinchieri, G. M. S. J. L. B., Matsumoto-Kobayashi, M., Clark, S. C., Seehra, J., London, L., & Perussia, B. (1984). Response of resting human peripheral blood natural killer cells to interleukin 2. *The Journal of experimental medicine*, *160*(4), 1147-1169.

14. Peng, H., & Tian, Z. (2017, June). Diversity of tissue-resident NK cells. In *Seminars in immunology* (Vol. 31, pp. 3-10). Academic Press.

15. Sojka, D. K., Tian, Z., & Yokoyama, W. M. (2014, April). Tissue-resident natural killer cells and their potential diversity. In *Seminars in immunology* (Vol. 26, No. 2, pp. 127-131). Academic Press.

16. Marquardt, N., Kekäläinen, E., Chen, P., Lourda, M., Wilson, J. N., Scharenberg, M., ... & Michaëlsson, J. (2019). Unique transcriptional and protein-expression signature in human lung tissue-resident NK cells. *Nature communications*, *10*(1), 3841.

17. Zhou, J., Tian, Z., & Peng, H. (2020). Tissue-resident NK cells and other innate lymphoid cells. *Advances in Immunology*, *145*, 37-53.

18. Fraser, R., & Zenclussen, A. C. (2022). Killer timing: the temporal uterine natural killer cell differentiation pathway and implications for female reproductive health. *Frontiers in Endocrinology*, *13*, 904744.

19. Zhang, J., Dunk, C. E., & Lye, S. J. (2013). Sphingosine signalling regulates decidual NK cell angiogenic phenotype and trophoblast migration. *Human Reproduction*, *28*(11), 3026-3037.

20. Cerdeira, A. S., Rajakumar, A., Royle, C. M., Lo, A., Husain, Z., Thadhani, R. I., ... & Kopcow, H. D. (2013). Conversion of peripheral blood NK cells to a decidual NK-like phenotype by a cocktail of defined factors. *The Journal of Immunology*, *190*(8), 3939-3948.

21. Tao, Y., Li, Y. H., Piao, H. L., Zhou, W. J., Zhang, D., Fu, Q., ... & Du, M. R. (2015). CD56brightCD25+ NK cells are preferentially recruited to the maternal/fetal interface in early human pregnancy. *Cellular & molecular immunology*, *12*(1), 77-86.

22. Tao, Y., Li, Y. H., Piao, H. L., Zhou, W. J., Zhang, D., Fu, Q., ... & Du, M. R. (2015). CD56brightCD25+ NK cells are preferentially recruited to the maternal/fetal interface in early human pregnancy. *Cellular & molecular immunology*, *12*(1), 77-86.

23. Lu, H., Jin, L. P., Huang, H. L., Ha, S. Y., Yang, H. L., Chang, R. Q., ... & Li, M. Q. (2020). Trophoblast-derived CXCL12 promotes CD56brightCD82− CD29+ NK cell enrichment in the decidua. *American Journal of Reproductive Immunology*, *83*(2).

24. Plaisier, M. (2011). Decidualisation and angiogenesis. *Best practice & research Clinical obstetrics & gynaecology*, *25*(3), 259-271.

25. Lima, P. D., Zhang, J., Dunk, C., Lye, S. J., & Anne Croy, B. (2014). Leukocyte driven-decidual angiogenesis in early pregnancy. *Cellular & molecular immunology*, *11*(6), 522-537.

26. Lima, P. D., Zhang, J., Dunk, C., Lye, S. J., & Anne Croy, B. (2014). Leukocyte driven-decidual angiogenesis in early pregnancy. *Cellular & molecular immunology*, *11*(6), 522-537.

27. Li, G., Huang, W., Xia, Q., Yang, K., Liu, R., Zhu, H., & Jiang, W. (2008). Role of uterine natural killer cells in angiogenesis of human decidua of the first-trimester pregnancy. *Science in China Series C: Life Sciences*, *51*, 111-119.

28. Kopcow, H. D., & Karumanchi, S. A. (2007). Angiogenic factors and natural killer (NK) cells in the pathogenesis of preeclampsia. *Journal of reproductive immunology*, *76*(1-2), 23-29.

29. Fu, B., Tian, Z., & Wei, H. (2014). Subsets of human natural killer cells and their regulatory effects. *Immunology*, *141*(4), 483-489.

30. Montaldo, E., Vacca, P., Chiossone, L., Croxatto, D., Loiacono, F., Martini, S., ... & Mingari, M. C. (2016). Unique Eomes+ NK cell subsets are present in uterus and decidua during early pregnancy. *Frontiers in immunology*, *6*, 646.

31. Santoni, A., Carlino, C., Stabile, H., & Gismondi, A. (2008). Mechanisms underlying recruitment and accumulation of decidual NK cells in uterus during pregnancy. *American Journal of Reproductive Immunology*, *59*(5), 417-424.

32. Santoni, A., Carlino, C., Stabile, H., & Gismondi, A. (2008). Mechanisms underlying recruitment and accumulation of decidual NK cells in uterus during pregnancy. *American Journal of Reproductive Immunology*, *59*(5), 417-424.

33. Hanna, J., Wald, O., Goldman-Wohl, D., Prus, D., Markel, G., Gazit, R., ... & Mandelboim, O. (2003). CXCL12 expression by invasive trophoblasts induces the specific migration of CD16–human natural killer cells. *Blood*, *102*(5), 1569-1577.

34. Keskin, D. B., Allan, D. S., Rybalov, B., Andzelm, M. M., Stern, J. N., Kopcow, H. D., ... & Strominger, J. L. (2007). TGFβ promotes conversion of CD16+ peripheral blood NK cells into CD16− NK cells with similarities to decidual NK cells. *Proceedings of the National Academy of Sciences*, *104*(9), 3378-3383.

35. Wang, F., Zhou, Y., Fu, B., Wu, Y., Zhang, R., Sun, R., ... & Wei, H. (2014). Molecular signatures and transcriptional regulatory networks of human immature decidual NK and mature peripheral NK cells. *European journal of immunology*, *44*(9), 2771-2784.

36. Bulmer, J. N., & Lash, G. E. (2019). Uterine natural killer cells: time for a re-appraisal?. *F1000Research*, *8*.

37. Vacca, P., Moretta, L., Moretta, A., & Mingari, M. C. (2011). Origin, phenotype and function of human natural killer cells in pregnancy. *Trends in immunology*, *32*(11), 517-523.

38. Chiossone, L., Vacca, P., Orecchia, P., Croxatto, D., Damonte, P., Astigiano, S., ... & Mingari, M. C. (2014). In vivo generation of decidual natural killer cells from resident hematopoietic progenitors. *Haematologica*, *99*(3), 448.

39. Santoni, A., Zingoni, A., Cerboni, C., & Gismondi, A. (2007). Natural killer (NK) cells from killers to regulators: distinct features between peripheral blood and decidual NK cells. *American Journal of Reproductive Immunology*, *58*(3), 280-288.

40. Nancy, P., Tagliani, E., Tay, C. S., Asp, P., Levy, D. E., & Erlebacher, A. (2012). Chemokine gene silencing in decidual stromal cells limits T cell access to the maternal-fetal interface. *Science*, *336*(6086), 1317-1321.

41. Huang, S. J., Schatz, F., Masch, R., Rahman, M., Buchwalder, L., Niven-Fairchild, T., ... & Lockwood, C. J. (2006). Regulation of chemokine production in response to pro-inflammatory cytokines in first trimester decidual cells. *Journal of reproductive immunology*, *72*(1-2), 60-73.

42. Sato, Y. (2020). Endovascular trophoblast and spiral artery remodeling. *Molecular and cellular endocrinology*, *503*, 110699.

43. Zlotnik, A., Yoshie, O., & Nomiyama, H. (2006). The chemokine and chemokine receptor superfamilies and their molecular evolution. *Genome biology*, *7*(12), 1-11.

44. Zlotnik, A., & Yoshie, O. (2012). The chemokine superfamily revisited. *Immunity*, *36*(5), 705-716.

45. Allen, S. J., Crown, S. E., & Handel, T. M. (2007). Chemokine: receptor structure, interactions, and antagonism. *Annu. Rev. Immunol.*, *25*, 787-820.

46. Nancy, P., Tagliani, E., Tay, C. S., Asp, P., Levy, D. E., & Erlebacher, A. (2012). Chemokine gene silencing in decidual stromal cells limits T cell access to the maternal-fetal interface. *Science*, *336*(6086), 1317-1321.

47. Huang, S. J., Schatz, F., Masch, R., Rahman, M., Buchwalder, L., Niven-Fairchild, T., ... & Lockwood, C. J. (2006). Regulation of chemokine production in response to pro-inflammatory cytokines in first trimester decidual cells. *Journal of reproductive immunology*, *72*(1-2), 60-73.

48. Huang, Y., Zhu, X. Y., Du, M. R., & Li, D. J. (2008). Human trophoblasts recruited T lymphocytes and monocytes into decidua by secretion of chemokine CXCL16 and interaction with CXCR6 in the first-trimester pregnancy. *The Journal of Immunology*, *180*(4), 2367-2375.

49. Shynlova, O., Boros-Rausch, A., Farine, T., Adams Waldorf, K. M., Dunk, C., & Lye, S. J. (2021). Decidual inflammation drives chemokine-mediated immune infiltration contributing to term labor. *The Journal of Immunology*, *207*(8), 2015-2026.

50. Shynlova, O., Boros-Rausch, A., Farine, T., Adams Waldorf, K. M., Dunk, C., & Lye, S. J. (2021). Decidual inflammation drives chemokine-mediated immune infiltration contributing to term labor. *The Journal of Immunology*, *207*(8), 2015-2026.

51. Shynlova, O., Boros-Rausch, A., Farine, T., Adams Waldorf, K. M., Dunk, C., & Lye, S. J. (2021). Decidual inflammation drives chemokine-mediated immune infiltration contributing to term labor. *The Journal of Immunology*, *207*(8), 2015-2026.

52. Santoni, A., Carlino, C., Stabile, H., & Gismondi, A. (2008). Mechanisms underlying recruitment and accumulation of decidual NK cells in uterus during pregnancy. *American Journal of Reproductive Immunology*, *59*(5), 417-424.

53. Red-Horse, K., Drake, P. M., & Fisher, S. J. (2004). Human pregnancy: the role of chemokine networks at the fetal–maternal interface. *Expert reviews in molecular medicine*, *6*(11), 1-14.

54. Iida, Y., Xu, B., Xuan, H., Glover, K. J., Tanaka, H., Hu, X., ... & Dalman, R. L. (2013). Peptide inhibitor of CXCL4–CCL5 heterodimer formation, MKEY, inhibits experimental aortic aneurysm initiation and progression. *Arteriosclerosis, thrombosis, and vascular biology*, *33*(4), 718-726.

55. Maes, M. The uterine-chemokine-brain axis: menstrual cycle-associated symptoms (MCAS) are in part mediated by CCL2, CCL5, CCL11, CXCL8 and CXCL10.

56. Raghupathy, R., & Kalinka, J. (2008). Cytokine imbalance in pregnancy complications and its modulation. *Front Biosci*, *13*(1), 985-94.

57. SHIMAOKA, Y., Hidaka, Y. O. H., TADA, H., AMINO, N., NAKAMURA, T., MURATA, Y., ... & MORIMOTO, Y. (2000). Changes in cytokine production during and after normal pregnancy. *American Journal of Reproductive Immunology*, *44*(3), 143-147.

58. Szereday, L., & Szekeres-Bartho, J. (1997). Cytokine production by lymphocytes in pregnancy. *American Journal of Reproductive Immunology*, *38*(6), 418-422.

59. Raghupathy, R. (2001, August). Pregnancy: success and failure within the Th1/Th2/Th3 paradigm. In *Seminars in immunology* (Vol. 13, No. 4, pp. 219-227). Academic Press.

60. Chaouat, G. (2007, June). The Th1/Th2 paradigm: still important in pregnancy?. In *Seminars in immunopathology* (Vol. 29, pp. 95-113). Springer-Verlag.

61. Shurin, M. R., Lu, L., Kalinski, P., Stewart-Akers, A. M., & Lotze, M. T. (1999, December). Th1/Th2 balance in cancer, transplantation and pregnancy. In *Springer seminars in immunopathology* (Vol. 21, pp. 339-359). Springer-Verlag.

62. Chaouat, G., Diallo, J. T., Volumenie, J. L., Menu, E., Gras, G., Delage, G., & Mognetti, B. (1997). Immune suppression and Th1/Th2 balance in pregnancy revisited: a (very) personal tribute to Tom Wegmann. *American journal of reproductive immunology*, *37*(6), 427-434.

63. Mauviel, A. (1993). Cytokine regulation of metalloproteinase gene expression. *Journal of cellular biochemistry*, *53*(4), 288-295.

64. Van Lint, P., & Libert, C. (2007). Chemokine and cytokine processing by matrix metalloproteinases and its effect on leukocyte migration and inflammation. *Journal of Leucocyte Biology*, *82*(6), 1375-1381.

65. Wallace, A. E., Fraser, R., & Cartwright, J. E. (2012). Extravillous trophoblast and decidual natural killer cells: a remodelling partnership. *Human reproduction update*, *18*(4), 458-471.

66. Sotnikova, N., Voronin, D., Antsiferova, Y., & Bukina, E. (2014). Interaction of Decidual CD 56+ NK with Trophoblast Cells during Normal Pregnancy and Recurrent Spontaneous Abortion at Early Term of Gestation. *Scandinavian journal of immunology*, *80*(3), 198-208.

67. Bachmayer, N., Rafik Hamad, R., Liszka, L., Bremme, K., & Sverremark-Ekström, E. (2006). Aberrant uterine natural killer (NK)-cell expression and altered placental and serum levels of the NK-cell promoting cytokine interleukin-12 in pre-eclampsia. *American Journal of Reproductive Immunology*, *56*(5-6), 292-301.

68. Mikhailova, V. A., Belyakova, K. L., Selkov, S. A., & Sokolov, D. I. (2017). Peculiarities of NK cells differentiation: CD56dim and CD56bright NK cells at pregnancy and in non-pregnant state. *Medical Immunology (Russia)*, *19*(1), 19-26.

69. Melsen, J. E., Lugthart, G., Lankester, A. C., & Schilham, M. W. (2016). Human circulating and tissue-resident CD56bright natural killer cell populations. *Frontiers in immunology*, *7*, 262.

70. Taima, A., Fukui, A., Yamaya, A., Yokota, M., Fukuhara, R., & Yokoyama, Y. (2020). A semen-based stimulation method to analyze cytokine production by uterine CD56bright natural killer cells in women with recurrent pregnancy loss. *Journal of Reproductive Immunology*, *142*, 103206.

71. Fukui, A., Funamizu, A., Yokota, M., Yamada, K., Nakamua, R., Fukuhara, R., ... & Mizunuma, H. (2011). Uterine and circulating natural killer cells and their roles in women with recurrent pregnancy loss, implantation failure and preeclampsia. *Journal of reproductive immunology*, *90*(1), 105-110.

72. Fukui, K., Yoshimoto, I., Matsubara, K., Hori, R., Ochi, H., & Ito, M. (1999). Leukocyte function-associated antigen-1 expression on decidual natural killer

cells in patients with early pregnancy loss. *Molecular human reproduction*, *5*(11), 1083-1088.

73. Grimstad, F., & Krieg, S. (2016). Immunogenetic contributions to recurrent pregnancy loss. *Journal of assisted reproduction and genetics*, *33*, 833-847.

74. Tao, Y., Li, Y. H., Zhang, D., Xu, L., Chen, J. J., Sang, Y. F., ... & Du, M. R. (2021). Decidual [CXCR4. sup.+][CD56. sup. bright] NK cells as a novel NK subset in maternal-foetal immune tolerance to alleviate early pregnancy failure. *Clinical and Translational Medicine*, *11*(10), NA-NA.

75. Tao, Y., Li, Y. H., Piao, H. L., Zhou, W. J., Zhang, D., Fu, Q., ... & Du, M. R. (2015). CD56brightCD25+ NK cells are preferentially recruited to the maternal/fetal interface in early human pregnancy. *Cellular & molecular immunology*, *12*(1), 77-86.

76. Lu, H., Jin, L. P., Huang, H. L., Ha, S. Y., Yang, H. L., Chang, R. Q., ... & Li, M. Q. (2020). Trophoblast-derived CXCL12 promotes CD56brightCD82− CD29+ NK cell enrichment in the decidua. *American Journal of Reproductive Immunology*, *83*(2).

77. Li, Z. Y., Song, Z. H., Meng, C. Y., Yang, D. D., Yang, Y., & Peng, J. P. (2015). IFN-γ modulates Ly-49 receptors on NK cells in IFN-γ-induced pregnancy failure. *Scientific reports*, *5*(1), 18159.

78. Chao, H. H., Li, L., Gao, X., Wang, C., & Yue, W. (2019). CXCL12 expression in aborted mouse uteri induced by IFN-γ: Potential anti-inflammatory effect involves in endometrial restoration after abortion in mice. *Gene*, *700*, 38-46.

79. Gokce, S., Herkiloglu, D., Cevik, O., & Turan, V. (2022). Role of chemokines in early pregnancy loss. *Experimental and Therapeutic Medicine*, *23*(6), 1-7.

80. Li, Z. Y., Song, Z. H., Meng, C. Y., Yang, D. D., Yang, Y., & Peng, J. P. (2015). IFN-γ modulates Ly-49 receptors on NK cells in IFN-γ-induced pregnancy failure. *Scientific reports*, *5*(1), 18159.

81. Han, J., Jeong, W., Gu, M. J., Yoo, I., Yun, C. H., Kim, J., & Ka, H. (2018). Cysteine-X-cysteine motif chemokine ligand 12 and its receptor CXCR4: expression, regulation, and possible function at the maternal–conceptus interface during early pregnancy in pigs. *Biology of Reproduction*, *99*(6), 1137-1148.

82. Murphy, S. P., Tayade, C., Ashkar, A. A., Hatta, K., Zhang, J., & Croy, B. A. (2009). Interferon gamma in successful pregnancies. *Biology of reproduction*, *80*(5), 848-859.

83. Carlino, C., Stabile, H., Morrone, S., Bulla, R., Soriani, A., Agostinis, C., ... & Gismondi, A. (2008). Recruitment of circulating NK cells through decidual tissues: a possible mechanism controlling NK cell accumulation in the uterus during early pregnancy. *Blood, The Journal of the American Society of Hematology*, *111*(6), 3108-3115.

84. Gibson, D. A., Greaves, E., Critchley, H. O. D., & Saunders, P. T. K. (2015). Estrogen-dependent regulation of human uterine natural killer cells promotes vascular remodelling via secretion of CCL2. *Human reproduction*, *30*(6), 1290-1301.

85. Vishnyakova, P., Poltavets, A., Nikitina, M., Midiber, K., Mikhaleva, L., Muminova, K., ... & Sukhikh, G. (2021). Expression of estrogen receptor α by decidual macrophages in preeclampsia. *Biomedicines*, *9*(2), 191.

86. Wilder, R. L. (1998). Hormones, pregnancy, and autoimmune diseases. *Annals of the New York Academy of Sciences*, *840*(1), 45-50.

87. Meyyazhagan, A., Kuchi Bhotla, H., Pappuswamy, M., Tsibizova, V., Al Qasem, M., & Di Renzo, G. C. (2023). Cytokine see-saw across pregnancy, its related complexities and consequences. *International Journal of Gynecology & Obstetrics*, *160*(2), 516-525.

88. SHIMAOKA, Y., Hidaka, Y. O. H., TADA, H., AMINO, N., NAKAMURA, T., MURATA, Y., ... & MORIMOTO, Y. (2000). Changes in cytokine production during and after normal pregnancy. *American Journal of Reproductive Immunology*, *44*(3), 143-147.

89. Carp, H. J. A. (2018). Progestogens and pregnancy loss. *Climacteric*, *21*(4), 380-384.

90. Reinhard, G., Noll, A., Schlebusch, H., Mallmann, P., & Ruecker, A. V. (1998). Shifts in the TH1/TH2 balance during human pregnancy correlate with apoptotic changes. *Biochemical and biophysical research communications*, *245*(3), 933-938.

91. Red-Horse, K., Drake, P. M., & Fisher, S. J. (2004). Human pregnancy: the role of chemokine networks at the fetal–maternal interface. *Expert reviews in molecular medicine*, *6*(11), 1-14.

92. Kheshtchin, N., Gharagozloo, M., Andalib, A., Ghahiri, A., Maracy, M. R., & Rezaei, A. (2010). The expression of Th1-and Th2-related chemokine receptors in women with recurrent miscarriage: the impact of lymphocyte immunotherapy. *American Journal of Reproductive Immunology*, *64*(2), 104-112.

93. Reinhard, G., Noll, A., Schlebusch, H., Mallmann, P., & Ruecker, A. V. (1998). Shifts in the TH1/TH2 balance during human pregnancy correlate with apoptotic changes. *Biochemical and biophysical research communications*, *245*(3), 933-938.

94. Orsi, N. M., & Tribe, R. M. (2008). Cytokine networks and the regulation of uterine function in pregnancy and parturition. *Journal of neuroendocrinology*, *20*(4), 462-469.

95. Hebisch, G., Neumaier-Wagner, P. M., Huch, R., & von Mandach, U. (2004). Maternal serum interleukin-1β,-6 and-8 levels and potential determinants in pregnancy and peripartum.

96. Hughes, G. C. (2012). Progesterone and autoimmune disease. *Autoimmunity reviews*, *11*(6-7), A502-A514.

97. Sentman, C. L., Meadows, S. K., Wira, C. R., & Eriksson, M. (2004). Recruitment of uterine NK cells: induction of CXC chemokine ligands 10 and 11 in human endometrium by estradiol and progesterone. *The Journal of Immunology*, *173*(11), 6760-6766.

98. Jo, T., Terada, N., Takauchi, Y., Saji, F., Nishizawa, Y., Tanaka, S., & Kosaka, H. (1995). Cytotoxic actions of cytokines on cultured mouse luteal cells are independent of nitric oxide. *The Journal of Steroid Biochemistry and Molecular Biology*, *55*(3-4), 291-296.

99. Ma, C., Liu, G., Liu, W., Xu, W., Li, H., Piao, S., ... & Feng, W. (2021). CXCL1 stimulates decidual angiogenesis via the VEGF-A pathway during the first trimester of pregnancy. *Molecular and cellular biochemistry*, *476*, 2989-2998.

100. Segerer, S., Kämmerer, U., Kapp, M., Dietl, J., & Rieger, L. (2009). Upregulation of chemokine and cytokine production during pregnancy. *Gynecologic and obstetric investigation*, *67*(3), 145-150.

101. Wang, L., Li, X., Zhao, Y., Fang, C., Lian, Y., Gou, W., ... & Zhu, X. (2015). Insights into the mechanism of CXCL12-mediated signaling in trophoblast

functions and placental angiogenesis. *Acta Biochimica et Biophysica Sinica*, *47*(9), 663-672.

102. Quinn, K. E., Ashley, A. K., Reynolds, L. P., Grazul-Bilska, A. T., & Ashley, R. L. (2014). Activation of the CXCL12/CXCR4 signaling axis may drive vascularization of the ovine placenta. *Domestic animal endocrinology*, *47*, 11-21.

103. Hannan, N. J., Jones, R. L., White, C. A., & Salamonsen, L. A. (2006). The chemokines, CX3CL1, CCL14, and CCL4, promote human trophoblast migration at the feto-maternal interface. *Biology of reproduction*, *74*(5), 896-904.

104. Huang, Y., Zhu, X. Y., Du, M. R., Wu, X., Wang, M. Y., & Li, D. J. (2006). Chemokine CXCL16, a scavenger receptor, induces proliferation and invasion of first-trimester human trophoblast cells in an autocrine manner. *Human reproduction*, *21*(4), 1083-1091.

105. Fujiwara, H., Sato, Y., Nishioka, Y., Yoshioka, S., Kosaka, K., Fujii, H., ... & Higuchi, T. (2005). New regulatory mechanisms for human extravillous trophoblast invasion. *Reproductive Medicine and Biology*, *4*, 189-195.

106. Check, J. H., Aly, J., & Chang, E. (2016). Improving the chance of successful implantation–part I–embryo attachment to the endometrium and adequate trophoblast invasion. *Clinical and experimental obstetrics & gynecology*, *43*(6), 787-791.

107. Zhou, W. H., Du, M. R., Dong, L., Yu, J., & Li, D. J. (2008). Chemokine CXCL12 promotes the cross-talk between trophoblasts and decidual stromal cells in human first-trimester pregnancy. *Human reproduction*, *23*(12), 2669-2679.

108. Boudot, A., Kerdivel, G., Habauzit, D., Eeckhoute, J., Le Dily, F., Flouriot, G., ... & Pakdel, F. (2011). Differential estrogen-regulation of CXCL12 chemokine receptors, CXCR4 and CXCR7, contributes to the growth effect of estrogens in breast cancer cells. *PloS one*, *6*(6), e20898.

109. Janis, K., Hoeltke, J., Nazareth, M., Fanti, P., Poppenberg, K., & Aronica, S. M. (2004). Estrogen decreases expression of chemokine receptors, and suppresses chemokine bioactivity in murine monocytes. *American journal of reproductive immunology*, *51*(1), 22-31.

110. Fujiwara, H., Sato, Y., Nishioka, Y., Yoshioka, S., Kosaka, K., Fujii, H., ... & Higuchi, T. (2005). New regulatory mechanisms for human extravillous trophoblast invasion. *Reproductive Medicine and Biology*, *4*, 189-195.

111. Fujiwara, H., Matsumoto, H., Sato, Y., Horie, A., Ono, M., Nakamura, M., ... & Araki, Y. (2018). Factors regulating human extravillous trophoblast invasion: chemokine-peptidase and CD9-integrin systems. *Current Pharmaceutical Biotechnology*, *19*(10), 764-770.

112. Wei, X., Zhang, Y., Wu, F., Tian, F., & Lin, Y. (2022). The role of extravillous trophoblasts and uterine NK cells in vascular remodeling during pregnancy. *Frontiers in Immunology*, *13*, 951482.

113. Dimitriadis, E., Nie, G., Hannan, N. J., Paiva, P., & Salamonsen, L. A. (2009). Local regulation of implantation at the human fetal-maternal interface. *The International journal of developmental biology*, *54*(2-3), 313-322.

114. Yockey, L. J., & Iwasaki, A. (2018). Interferons and proinflammatory cytokines in pregnancy and fetal development. *Immunity*, *49*(3), 397-412.

115. Challis, J. R., Lockwood, C. J., Myatt, L., Norman, J. E., Strauss, J. F., & Petraglia, F. (2009). Inflammation and pregnancy. *Reproductive sciences*, *16*, 206-215.

116. Rallis, K. S., Corrigan, A. E., Dadah, H., George, A. M., Keshwara, S. M., Sideris, M., & Szabados, B. (2021). Cytokine-based cancer immunotherapy: Challenges and opportunities for IL-10. *Anticancer research*, *41*(7), 3247-3252.

117. Chomarat, P., Vannier, E., Dechanet, J., Rissoan, M. C., Banchereau, J., Dinarello, C. A., & Miossec, P. (1995). Balance of IL-1 receptor antagonist/IL-1 beta in rheumatoid synovium and its regulation by IL-4 and IL-10. *Journal of immunology (Baltimore, Md.: 1950)*, *154*(3), 1432-1439.

118. Lacraz, S., Nicod, L. P., Chicheportiche, R., Welgus, H. G., & Dayer, J. M. (1995). IL-10 inhibits metalloproteinase and stimulates TIMP-1 production in human mononuclear phagocytes. *The Journal of clinical investigation*, *96*(5), 2304-2310.

119. Reitamo, S., Remitz, A., Tamai, K., & Uitto, J. (1994). Interleukin-10 modulates type I collagen and matrix metalloprotease gene expression in cultured human skin fibroblasts. *The Journal of clinical investigation*, *94*(6), 2489-2492.

120. Wagner, S., Stegen, C., Bouterfa, H., Huettner, C., Kerkau, S., Roggendorf, W., ... & Tonn, J. C. (1998). Expression of matrix metalloproteinases in human glioma cell lines in the presence of IL-10. *Journal of neuro-oncology*, *40*, 113-122.

121. Vogel, I., Thorsen, P., Curry, A., Sandager, P., & Uldbjerg, N. (2005). Biomarkers for the prediction of preterm delivery. *Acta obstetricia et gynecologica Scandinavica*, *84*(6), 516-525.

122. Jung, E. Y., Park, J. W., Ryu, A., Lee, S. Y., Cho, S. H., & Park, K. H. (2016). Prediction of impending preterm delivery based on sonographic cervical length and different cytokine levels in cervicovaginal fluid in preterm labor. *Journal of Obstetrics and Gynaecology Research*, *42*(2), 158-165.

123. Lockwood, C. J., & Kuczynski, E. (1999). Markers of risk for preterm delivery.

124. Fawaz, L. M., Sharif-Askari, E., & Menezes, J. (1999). Up-regulation of NK cytotoxic activity via IL-15 induction by different viruses: a comparative study. *The Journal of Immunology*, *163*(8), 4473-4480.

125. Park, J. Y., Lee, S. H., Yoon, S. R., Park, Y. J., Jung, H., Kim, T. D., & Choi, I. (2011). IL-15-induced IL-10 increases the cytolytic activity of human natural killer cells. *Molecules and cells*, *32*, 265-272.

126. Murata, H., Tanaka, S., & Okada, H. (2022). The regulators of human endometrial stromal cell decidualization. *Biomolecules*, *12*(9), 1275.

127. Gong, G. S., Muyayalo, K. P., Zhang, Y. J., Lin, X. X., & Liao, A. H. (2023). Flip a coin: Cell senescence at the maternal-fetal interface. *Biology of Reproduction*, ioad071.

128. Lauw, F. N., Pajkrt, D., Hack, C. E., Kurimoto, M., van Deventer, S. J., & van der Poll, T. (2000). Proinflammatory effects of IL-10 during human endotoxemia. *The Journal of Immunology*, *165*(5), 2783-2789.

129. Hutchins, A. P., Diez, D., & Miranda-Saavedra, D. (2013). The IL-10/STAT3-mediated anti-inflammatory response: recent developments and future challenges. *Briefings in functional genomics*, *12*(6), 489-498.

130. Steinborn, A., Von Gall, C., Hildenbrand, R., Stutte, H. J., & Kaufmann, M. (1998). Identification of placental cytokine-producing cells in term and preterm labor. *Obstetrics & Gynecology*, *91*(3), 329-335.

131. Shouda, T., Yoshida, T., Hanada, T., Wakioka, T., Oishi, M., Miyoshi, K., ... & Yoshimura, A. (2001). Induction of the cytokine signal regulator SOCS3/CIS3

as a therapeutic strategy for treating inflammatory arthritis. *The Journal of clinical investigation*, *108*(12), 1781-1788.

132. Fitzgerald, J. S., Toth, B., Jeschke, U., Schleussner, E., & Markert, U. R. (2009). Knocking off the suppressors of cytokine signaling (SOCS): their roles in mammalian pregnancy. *Journal of reproductive immunology*, *83*(1-2), 117-123.

133. Ozkan, Z. S., Devecı, D., Sımsek, M., Ilhan, F., Rısvanlı, A., & Sapmaz, E. (2015). What is the impact of SOCS3, IL-35 and IL17 in immune pathogenesis of recurrent pregnancy loss?. *The Journal of Maternal-Fetal & Neonatal Medicine*, *28*(3), 324-328.

134. Bulmer, J. N., Morrison, L., & Johnson, P. M. (1988). Expression of the proliferation markers Ki67 and transferrin receptor by human trophoblast populations. *Journal of reproductive immunology*, *14*(3), 291-302.

135. Hasanzadeh, M., Sharifi, N., Esmaieli, H., Daloee, M. S., & Tabari, A. (2013). Immunohistochemical expression of the proliferative marker Ki67 in hydatidiform moles and its diagnostic value in the progression to gestational trophoblastic neoplasia. *Journal of Obstetrics and Gynaecology Research*, *39*(2), 572-577.

Salient findings of ChapterV

The predominance of CD56dim CD16+ NK cells (p=0.003), low CXCR4 expression, and elevated levels of IFN-gamma (t test, p=0.016) and IL-15 in the decidua of SAB abortus provide evidence of imbalanced NK cell activation and immune dysregulation in early pregnancy failure. It was observed that chemokines CXCL2, CCL5, and CXCL3 regulate decidual NK migration, extravillous trophoblast (EVT) invasion, and increase VEGF A levels, promoting a healthy pregnancy. Abnormal expression of IL1B, IFNγ, and IL10 was associated with immune imbalances contributing to early pregnancy loss. Furthermore, our data prompted towards a transition from an anti-inflammatory to a pro-inflammatory cytokine environment in late pregnancy, which is essential for a healthy term delivery by reducing the levels of SOCS.