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ORIGINAL ARTICLE

Isolation and characterization of uterine leukocytes collected using a uterine swab technique

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Abstract

Problem: Leukocytes from the maternal-fetal interface are a valuable tool to study local changes in immune function during pregnancy; however, sampling can be challenging due to inadequate tissue availability and the invasive nature of placental bed biopsy. Here, we aim to purify and characterize leukocytes from paired peripheral and uterine blood samples to assess whether a less invasive method of uterine blood collection could yield a population of enriched uterine leukocytes suitable for ex vivo and in vitro analyses.

Method of Study: Human peripheral blood mononuclear cells (PBMC) and uterine blood mononuclear cells (UBMC) expressed from surgical gauze post C-section were isolated, and immunophenotypic information was acquired by multi-parameter flow cytometry. PBMC and UBMC were stained for markers used to define T and B lymphocytes, macrophages, regulatory T (T_{Reg}) cells, and natural killer (NK) cells. Prime flow was performed to check expression and analysis of $CD16^{-}CD56^{++}$ and CD16⁻CD56++ NK transcripts in PBMC and UBMC samples.

Results: Immunophenotyping revealed that over 95% of both live PBMC and UBMC consisted of $CD45^{+}$ leukocytes. Higher percentages of $CD16^{-}CD56^{++}$, characterized as uterine NK (uNK) cells, were observed in UBMC samples as compared to PBMC samples (18.41% of $CD45^{+}CD3^{-}$ vs. 2.73%, respectively), suggesting that $CD16^{-}CD56^{++}$ cells were enriched in these samples. In UBMC, 49.64% of CD3-negative cells were of peripheral NK phenotype ($CD16^{+}CD56^{++}$), suggesting infiltration of maternal peripheral NK (pNK) cell in the uterine interface.

Conclusion: Intrauterine leukocytes, especially $CD16^{-}CD56^{++}$ NK cells, can be collected in sufficient numbers with increased purity by sampling the uterine cavity postdelivery with surgical gauze. Our results suggest that this non-invasive protocol is a useful sampling technique for isolating $CD16^{-}CD56^{++}$ cells, however, due to peripheral blood contamination, the NK cell yield could be lower compared to actual decidual or endometrial samples *post-partum* which is more invasive.

KEYWORDS
cesarean section, immunophenotyping, maternal, peripheral, PrimeFlow RNA, uterine leukocytes

1 | INTRODUCTION

The maternal-fetal interface is an active immunological site during pregnancy.[1,2] This intimacy of contact between fetal and maternal tissues provides ample fetal antigenic exposure to the maternal immune system.[3,4] Maternal immune cells play an important role in maintaining balance within the uterus by promoting a proinflammatory state during implantation and early placentation , an anti-inflammatory environment during the second and third trimesters, and finally a pro-inflammatory setting during parturition.[5–11] Maternal immune cells and fetal trophoblasts are important regulators of inflammatory and immunomodulatory responses at the maternal-fetal interface, contributing to the immunological environment within the uterus.[12] Extravillous trophoblast cells express non-classical human leukocyte antigen-G (*HLA-G*), which provides inhibitory signals to NK cells, macrophages, monocytes, and lymphocytes through inhibitory leukocyte immunoglobulin-like receptors 1 and 2, CD94/NKG2A,[13–18] and killer-immunoglobulin-like receptor, KIR2DL4.[19,20] These receptors are likely critical to promote immunosuppression, vascular remodeling, placental development, and thus fetal survival.[21]

In 1954, Peter Medawar first raised the question of how maternal-fetal immunological tolerance is established to protect the fetal allograft.[1,2] In successful pregnancy, immunological tolerance is achieved through the involvement of various innate and adaptive immune cells, including NK cells, B cells, T cells ($CD4^+$, $CD8^+$, T regulatory), monocytes, and others.[3,11,12,22–24] Uterine NK (uNK) cells, macrophages, and T lymphocytes comprise ~40% of the cells at the maternal-fetal interface,[22,25] and regulate many cellular and physiological processes during pregnancy. Uterine NK cells facilitate invasion of trophoblast into maternal tissues, spiral artery remodeling,[26–28] maternal-fetal tolerance,[3,29] and initiation of labor.[30] $CD4^+CD25^+$ T_{Reg} cells suppress fetus-specific and non-specific immune responses in human decidual tissues.[31] Alterations in activation status, proportion, and functions of T_{Reg} cells may result in pregnancy-related disorders such as spontaneous abortion, preterm labor, preeclampsia, and intrauterine growth restriction.[32,33] Therefore, phenotypic and functional characterization of decidual leukocytes provide immense insight into immunological mechanisms of normal pregnancy and dysregulated immune-mediated pregnancy disorders.

To gain insight into the functions of decidual leukocytes, it is important to obtain an adequate samples of these cells. The first illustration of the "placental bed" and direct macroscopic evidence for spiral arteries therein was revealed by Hunter in his book published in the year 1774, which revolutionized the study of pregnancy.[34] Trophoblast cells were identified in the second half of the 19th century owing to the discovery of the microscope and its rapid advances. Early placental bed research relied mostly on hysterectomies and postmortem specimens of uteri with in situ placentas.[35] The largest collections of early pregnancy specimens reside in the Carnegie Collection at the Human Developmental Anatomy Center, Washington DC, [36] and the Boyd collection at the Centre for Trophoblast Research, University of Cambridge;[37] both have been fundamental to placental study. Intact uteri with pregnancies ranging from 8 to 18 weeks gestational age and pregnant hysterectomies were source of spiral remodeling work performed by Brosens and Pijnenborg.[38–41] However, hysterectomy during pregnancy has become limited and rare procedure due to more advanced techniques as well as ethical issues. Still, for placental complications such as preeclampsia, a representative sample is needed to study the placental bed.[42] An ultrasound-guided transcervical punch biopsy to sample uterine spiral arteries[43] focusing leucocytes in the first trimester was used by Michel et al. in 1990. Currently, this is the largest collection of early placental bed biopsies reported. Sampling techniques such as unguided punch biopsy to collect myometrial sections of the spiral arteries[44] and curved scissors or scalpel to collect spiral artery and trophoblasts from the placental bed were used during Caesarean section (third trimester). In 1996, Schafer et al. employed wiping of the uterine cavity post-C section with a sterile tampon to collect decidual tissue samples.[46] Despite the development of these techniques, obtaining precise and adequate quantity of samples is challenging due to limited amounts of tissue available, increasing difficulty of first trimester samples, and the invasive nature of placental bed biopsy.

Recently, Kahn *et al.* employed a technique to collect leukocytes from the placental bed whereby surgical sponges are used to wipe the uterine cavity following C-section delivery.[47] This method is routinely used to remove excess blood and ensure complete removal of the placenta from the uterine cavity, and thus does not pose added risk to the patient. We employed this method to analyze the phenotypic properties of placental bed NK cells, T cells, B cells, and monocytes in comparison to those in the peripheral blood.

2 | MATERIAL AND METHODS

2.1 | Human subjects and ethics statement

Our study was approved by the Institutional Review Board of Michigan State University and all patients signed an informed consent form prior to enrolling in the study (MSU, East Lansing, Office of Human Subjects Protection Program, Biomedical and Health Sciences (IRB # 15484)). Healthy normal reproductive age women ($N = 10$) with uncomplicated term Cesarean sections were recruited between July 2017 and July 2018. Table 1 summarizes the demographic characteristics of the subjects.

TABLE 1 Clinical and demographic characteristics of the patients used in the study

Characteristic	Value
Number of samples	10
Maternal age (Mean ± SD)	27.5 ± 4.9
BMI (Mean ± SD)	31.6 ± 7.0
Gravidity (Median, Range)	2(1–4)
Parity (Median, Range)	2 (1–3)
Ethnicity	
Caucasian	5
Black	3
Unknown	1
Fetal sex	
Male newborn	3
Female	6
Mode of delivery	C-section
Gestational age (Mean ± SD; weeks)	38.75 ± 1.04

2.2 | Specimen collection

Peripheral blood leukocytes were obtained in collection tubes containing heparin using standard aseptic venipuncture (8–20 ml/patient). From the same patients, blood from the uterine cavity (placental bed blood) was collected after the delivery of the fetus and placenta. After C-section, a sterile surgical gauze pad was used to wipe the cavity of excess blood and placental tissue. This process was repeated using a second sterile pad; for our study, the second gauze pad from each patient was placed in a container with 50 ml of sterile phosphate buffered saline (PBS) for collection of placental bed leukocytes.

2.3 | Mononuclear cell isolation

To collect placental bed leukocytes, the surgical gauze in PBS was squeezed into a sterile container, re-moistened with an additional 50 ml PBS and squeezed again to collect more cells. Peripheral blood was diluted with equal volume of 1× PBS. Both samples were layered over Ficoll-Paque (GE Healthcare, Chicago, IL) and centrifuged at 400 x *g* for 45 min at room temperature (RT). Cells from the Ficoll/PBS interface were harvested, avoiding mixture of the interface with Ficoll, and washed with thrice the volume of complete RPMI media (RPMI with 10% Fetal Bovine Serum (FBS), 2 mM Penicillin-Streptomycin, 100 mM β-mercaptoethanol). Red blood cells were lysed in ammonium-chloride-potassium (ACK) lysis buffer for 3 min at RT, and leukocytes were washed twice with 10 ml RPMI media. Finally, the cells were resuspended in 1–2 ml of complete RPMI and counted using a hemacytometer, and viability was determined using trypan blue dye. Viability of both peripheral blood and placental bed leukocytes were routinely >95%. Cells were cryopreserved at −80°C until downstream analysis.

2.4 | Multiparameter flow cytometry

Cryopreserved peripheral and placental bed leukocytes, paired for each patient, were stained with markers for NK cells, T cells, B cells, and monocytes (Table 2). For staining, 1×10^6 cells were stained with Live/Dead fixable yellow fluorescent dye (Thermo Fisher Scientific, Waltham, MA) on ice for 30 min. Cells were washed with PBS and resuspended in flow staining buffer (5% FBS and 0.1% sodium azide in PBS) and transferred to round-bottom 96-well plates. Previously titrated antibodies (Table 2) were added to the corresponding wells at the indicated concentration for a final volume of 100 µl/well. The cells were stained on ice for 30 min, washed, and fixed in 1% paraformaldehyde. For intracellular staining, cells were pelleted after surface staining and permeabilized using True Nuclear Transcription Buffer (BioLegend, San Diego, CA) according to the manufacturer's protocol. Anti-FoxP3 antibody (5 µg per test) was added to the cells, and the cells were incubated for 30 min at ambient temperature. The cells were washed and resuspended for analysis.Intracellular RNA analysis was performed using the PrimeFlow RNA Assay (TermoFisher) according to the manufacturer's instructions, using probes to detect C-C Motif Chemokine Ligand 5 (CCL5), interleukin-8 (IL-8, and Placenta Growth Factor (PGF). For all analyses, fluorescence minus one (FMO) controls and unstained negative controls were stained in parallel using the same conditions.

2.5 | Flow cytometry data acquisition and analysis

Flow cytometry data were acquired using a BD LSRII flow cytometer (BD Biosciences) together with FACSDiva 8.0 acquisition software located in the MSU Flow Cytometry Core. Acquired data were analyzed on Kaluza Flow Analysis Software version 1.5a (Beckman Coulter, Indianapolis, IN). The gating strategy is illustrated in Figure 1.

The PrimeFlow results were analyzed using a Cytek™ Aurora spectral cytometer (Cytek Biosciences, CA, USA).

2.6 | Statistical analysis

Sample size was determined based on the effect size derived from data at 5% significance level and 90% power using software G*Power 3.1 (https://stats.idre.ucla.edu/other/gpower/; date of last access, 08/30/2022). Results are expressed as mean ± standard error of the mean (SEM). Shapiro's test was used to assess normality distribution of the dataset. Statistical differences in percent expression of cell surface markers between PBMC and UBMC were determined by Welch's paired t-test for normally distributed data and Mann-Whitney U test for non-normally distributed data ($\alpha = 0.05$). Statistical analysis and graphical representations were performed using RStudio (Boston, MA). For boxplots, the middle line represents median percent gated cells, upper and lower box regions indicate third and first quartiles (75th and 25th percentiles) and whiskers represent 1.5 times the interquartile range. Dotted lines represent the paired samples.

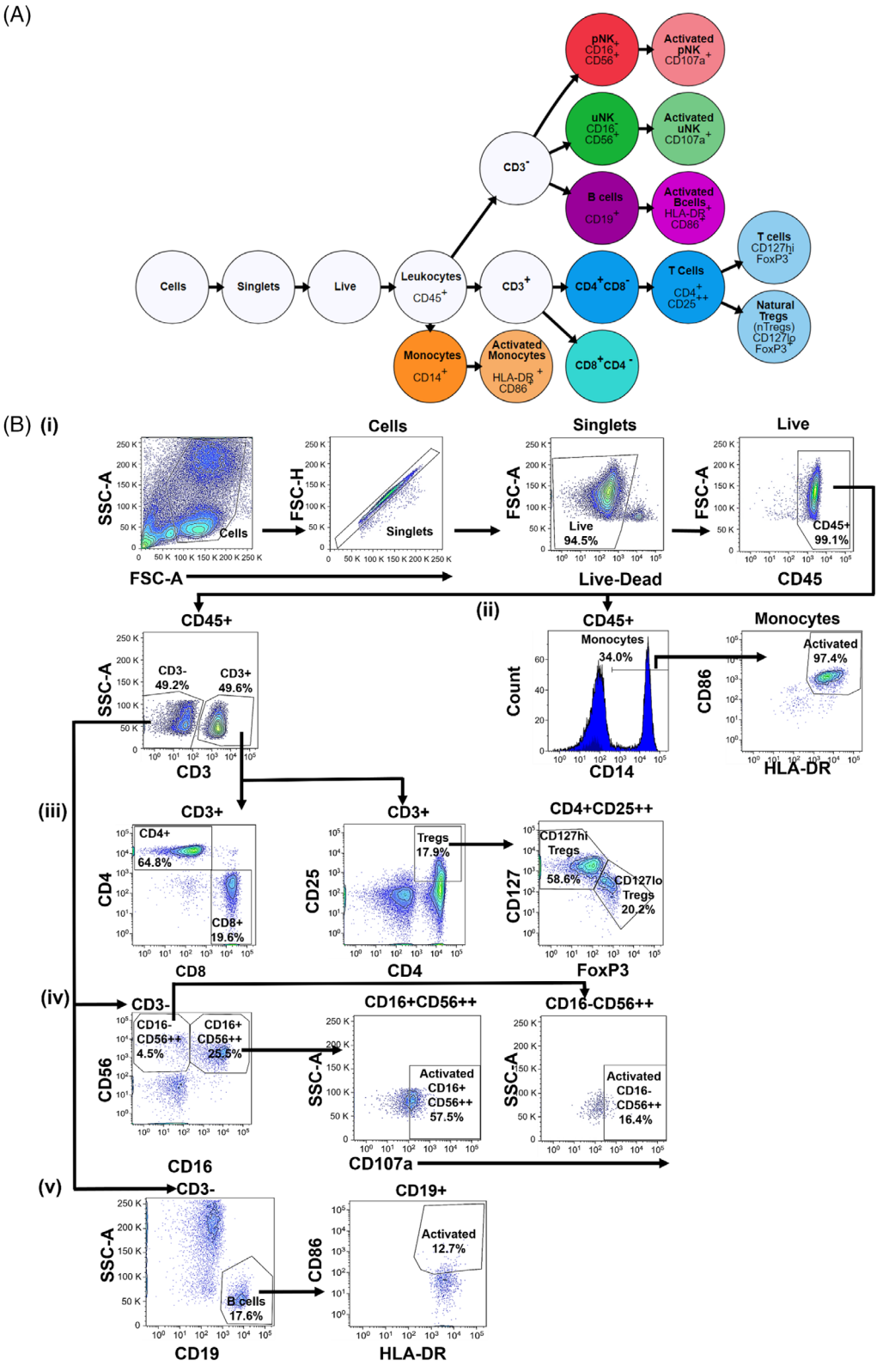

FIGURE 1 Leukocytes identified in the PBMC and UBMC samples from term human C-section deliveries. (A) Gating strategy for identification of major leukocytes subsets from PBMC and UBMC samples. (B) Gating strategy to identify live cells and live CD45$^+$ (i); Monocytes [CD45$^+$ CD14$^+$] (ii); CD4 T cells [CD45$^+$ CD3$^+$CD4$^+$CD8$^-$], CD8 T cells [CD45$^+$ CD3$^+$CD4$^-$CD8$^+$], CD127high [CD45$^+$ CD4$^+$CD25^{++}CD127^{high}FoxP3$^-$] T cells, and T regulatory cells [CD45$^+$ CD3$^+$CD4$^+$CD8$^-$CD25$^+$FoxP3$^+$CD127^{low} (iii); CD3$^-$CD16$^+$CD56$^+$ and CD3$^-$CD16$^-$CD56^{++} NK cell subsets (iv); and B cells [CD3$^-$CD19$^+$] and activated [CD3$^-$CD19$^+$CD86$^+$HLA-DR$^+$] B cells (v). The gating strategy displayed for a representative patient peripheral blood sample was applied for flow cytometric analysis of all placental bed and peripheral blood patient samples

TABLE 2 Antibodies used for flow cytometric analysis

Antigen	Clone	Fluorochrome	Isotype	Supplier	Cat. No.	Concentration (μg/100 μl)
CD45	2D1	APC-H7	Ms IgG1k	BDBiosciences	560274	2
CD3	HIT3a	AF488	Ms IgG2ak	BioLegend	300319	0.25
CD4	RPA-T4	PerCp-Cy5.5	Ms IgG1k	BioLegend	300530	0.25
CD8	HIT8a	PE-Cy7	Ms IgG1k	BioLegend	300913	0.25
CD25	3G8	PE	Ms IgG1k	BioLegend	356103	0.5
CD127	A019D5	BV421	Ms IgG1k	BioLegend	351309	0.25
CD19	HIB19	PE-Cy7	Ms IgG1k	BioLegend	302215	0.25
CD14	HCD14	PE	Ms IgG1k	BioLegend	325605	1.0
CD86	IT2.2	BV421	Ms IgG2bκ	BioLegend	305425	0.125
HLA-DR	L243	APC	Ms IgG2aκ	BioLegend	307609	0.125
CD56	5.1H11	APC	Ms IgG1k	BioLegend	362503	0.35
CD16	3G8	PE	Ms IgG1k	BioLegend	302007	0.0625
CD107a	H4A3	BV421	Ms IgG1k	BioLegend	328625	0.25
FoxP3	206D	AF647	Ms IgG1k	BioLegend	320113	0.15

3 | RESULTS

The age, body mass index, and obstetric history of the women who participated in the study are summarized in Table 1. The gating strategy for leukocyte analysis is depicted in Figure 1. Percentage of the live cells identified as dimly stained with Live/Dead fixable yellow fluorescent dye, as illustrated Figure 1, ranged from 87.3% to 98.9% in PBMC samples (mean, 95.18 ± 1.35%) and 64.9% to 99.16% in UBMC (mean, 89.34 ± 3.75%). The percentage of live cells identified as $CD45^+$ leukocytes in PBMC samples ($N = 10$) ranged from 86.1% to 99.7% (mean, 96.36 ± 1.57%) . In UBMC samples, $CD45^+$ leukocytes ranged from 77.3 to 99.7% (mean 96.52 ± 2.14%) of live cells ($N = 10$).

3.1 | Uterine swab samples are enriched in uterine NK (uNK) cells

Uterine NK cells have a characteristic phenotype of $CD45^+CD3^-CD16^-CD56^{++}$,[6] and a small proportion of cells with the same phenotype is found in the periphery. Likewise, conventional NK cells have a phenotype of $CD3^-CD16^+CD56^{++}$ and these cells comprise the majority of peripheral NK cells. $CD16^-CD56^{++}$ NK cells were enriched among placental bed cells as compared to peripheral blood cells (18.41 ± 3.44% vs. 2.73 ± 0.29% of $CD45^+CD3^-$ cells, respectively; $N = 10$) (Figure 2). In contrast, $CD16^+CD56^{++}$ NK cells represented 18.69 ± 3.67% of $CD45^+CD3^-$ cells in peripheral blood, and 49.64 ± 7.31% of the placental bed cells (Figure 2A, B). The mean ratio of $CD16^-CD56^{++}$ to $CD16^+CD56^{++}$ NK cells was higher in UBMC compared to PBMC (1.14 ± 0.72 vs. 0.37 ± 0.18, respectively) (Figure 2C). To assess the activation status of $CD16^-CD56^{++}$and $CD16^+CD56^{++}$ cells in placental bed and peripheral blood, we evaluated expression of the degranulation marker, CD107a, on their surface. The percentage of activated $CD16^-CD56^{++}$ cells in placental bed blood was comparable to those in the peripheral blood (35.87 ± 5.84% and 47.84 ± 5.91%, respectively) (Figure 3A). Similarly, the percentage of activated $CD16^+CD56^+$ cells within placental bed blood was similar to that in the peripheral blood (52.86 ± 4.74% and 52.76 ± 5.29%, respectively) (Figure 3B).

To determine the expression of genes in NK cells in the placental bed and peripheral blood cells, we conducted PrimeFlow RNA analysis, which allows concomitant analysis of intracellular mRNA transcripts and surface protein via flow cytometry. We screened the samples for C-C Motif Chemokine Ligand 5 (CCL5, or RANTES), interleukin-8 (IL-8), and placenta growth factor (PGF) expression in matched placental bed and peripheral blood leukocytes . These cytokines were chosen based on reports of their strong expression in uNK cells as compared to peripheral blood NK cells.[28,48]

$CD16^-CD56^{++}$ NK cells in placental bed samples expressed significantly more CCL5 mRNA compared to peripheral blood leukocytes (Figure 4). On the other hand, $CD16^+CD56^{++}$ cells in placental bed and peripheral blood expressed similar levels of *RANTES* mRNA. There was no difference between *IL8* and *PGF* mRNA levels in either type of NK cells between placental bed and peripheral blood samples.

3.2 | Monocytes predominate in peripheral blood

Monocytes ($CD45^+CD14^+$) ranged from 12.47% to 44.69% ($N = 10$) in peripheral blood, with a mean percentage of 27.42 ± 3.31%. Mean percentage of $CD45^+CD14^+$ monocytes in placental bed samples was lower (6.38 ± 1.45% of live $CD45^+$) compared to PBMC (27.42 ± 3.31; Figure 5A). Furthermore, a lower percentage of placental bed monocytes expressedthe activation markers CD86, and HLA-DR compared to peripheral blood monocytes (50.24 ± 7.53% of $CD14^+$ vs. 90.63 ± 2.60%, respectively) (Figure 5B).

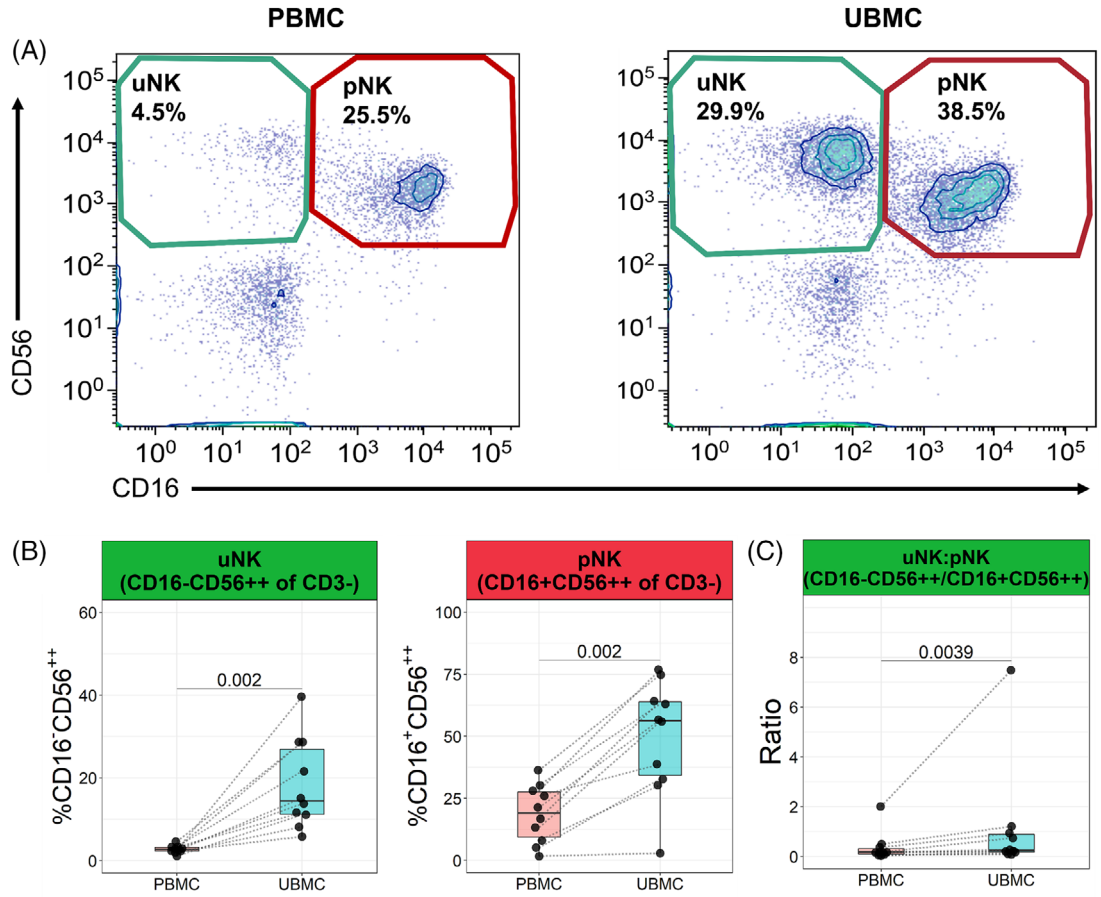

FIGURE 2 $CD16^{+}CD56^{+}$ and $CD16^{+}CD56^{+}$ NK cell subsets assessed at term C-section delivery. Gates for NK cell subsets in paired placental bed and peripheral blood samples of a representative patient is shown (A); box plots show elevated mean percentages of $CD16^{-}CD56^{++}$ NK cell subset (Paired Mann Whitney *U* test; $p = 0.0002$) and $CD16^{+}CD56^{++}$ cell subset (Paired t-test; $p = 0.002$) in placental bed samples compared to peripheral blood samples (B); Ratio of $CD16^{-}CD56^{++}$ to $CD16^{+}CD56^{++}$ NK cells is significantly increased in placental bed samples compared to peripheral blood (Paired Mann Whitney *U* test; $p = 0.0039$) (C).

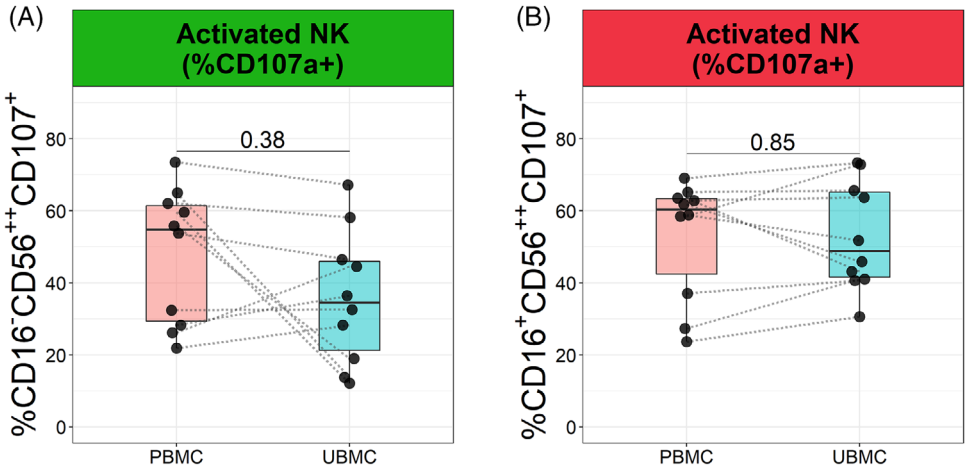

FIGURE 3 Activated (CD107a+) $CD16^{-}CD56^{++}$ and activated $CD16^{+}CD56^{++}$ cell subsets assessed at term C-section delivery. Percent of activated $CD16^{-}CD56^{++}$ and $CD16^{+}CD56^{++}$ cells was similar in placental bed and peripheral blood (Paired Mann Whitney *U* test; $p = 0.38$ and $p = 0.85$, respectively) (A and B).

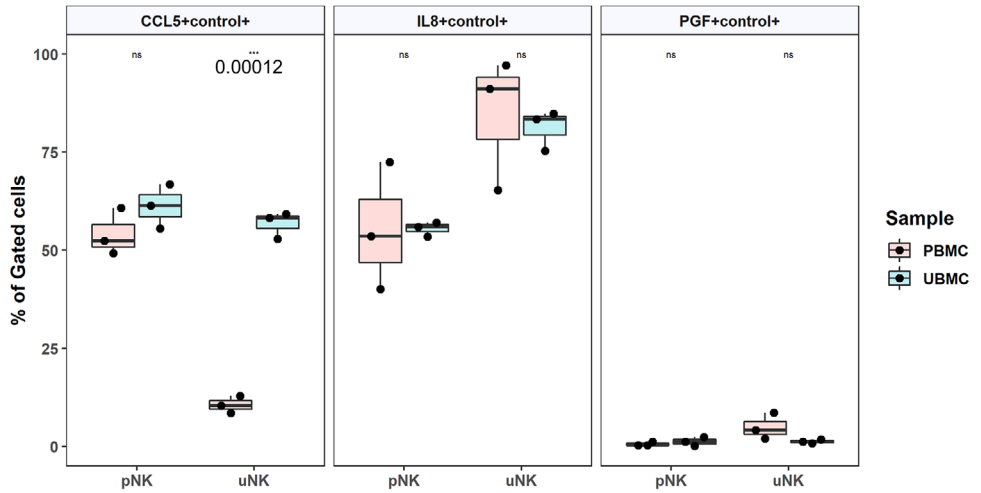

FIGURE 4 PrimeFlow RNA assay: CD16^{-}CD56^{++} NK cells in placental bed samples had a significantly higher expression of CCL5 mRNA compared to that of peripheral blood samples (paired t-test; $p = 0.00012$). NK subsets did not show significantly different expression of IL8 and PGF mRNA

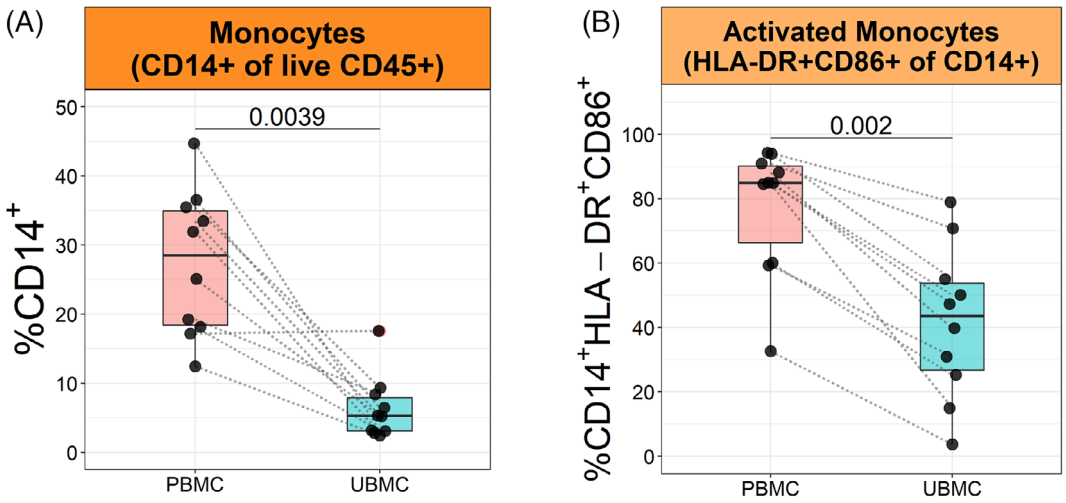

FIGURE 5 Percent of CD14^{+} monocytes of total leukocytes (live CD45^{+}) (Paired Mann-Whitney *U* test; $p = 0.0039$) (A) and activated monocytes (HLA-DR^{+}CD86^{+}) of total monocytes (live CD45^{+}CD14^{+}) (Paired Mann-Whitney *U* test; $p = 0.002$) (B) were significantly higher in peripheral blood than in placental bed samples

3.3 | Uterine leukocytes contain reduced proportion of B cells than in peripheral blood

The percentage of B cells (CD45^{+}CD3^{-}CD19^{+}) of the total live CD45^{+} leukocytes was higher in peripheral blood compared to placental bed samples (10.34 ± 0.94% vs. 5.36 ± 1.33%, respectively) (Figure 6A). To investigate the activation status of B cells, we also evaluated the expression of CD86 and human leukocyte antigen (HLA) class-II molecule HLA-DR.[49,50] The proportions of B cells expressing both receptors were similar between peripheral blood and placental bed samples (Figure 6B).

3.4 | Regulatory T cell abundance is similar in placental bed and peripheral blood

The mean percentages of CD4^{+} T lymphocytes of live CD45^{+} leukocytes in the placental bed and peripheral blood were similar (Figure 7A). However, there was a higher percentage of CD8^{+} T cells in placental bed compared to peripheral blood samples (Figure 7B).

T_{Reg} cells are defined based on the expression of CD4 and CD25 surface markers and the intracellular transcription factor FoxP3.[51,52] CD25,the α chain of the interleukin 2 (IL-2) receptor,[53] low expression of the surface marker CD127, and high expression of CD25 have also

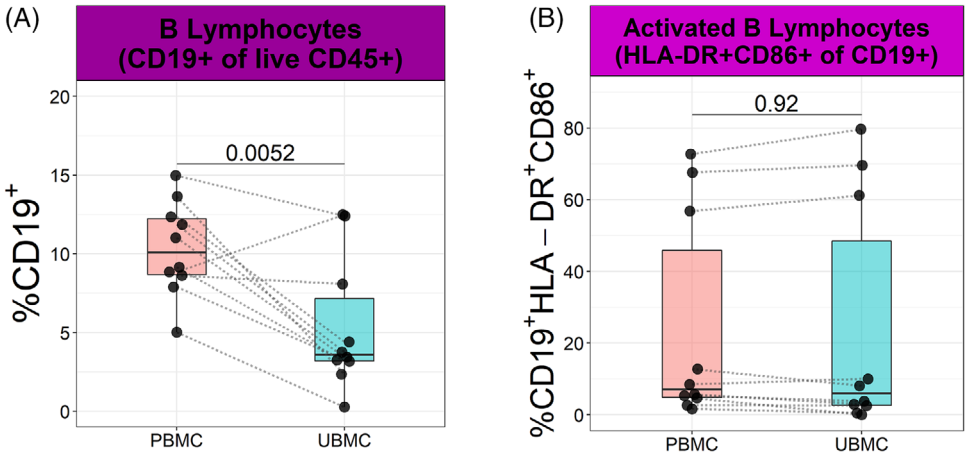

FIGURE 6 Term uterine leukocytes contained a reduced proportion of B cells compared to peripheral blood leukocytes. Uterine blood mononuclear cells (UBMC) contained significantly lowers percentages of $CD19^+$ B cells when compared to that of PBMC (paired t-test; $p = 0.0052$) (A); Activated ($HLA\text{-}DR^+CD86^+$) percentages of B cells were not significantly different in PBMC and UBMC (Paired Mann-Whitney *U* test; $p = 0.92$) (B)

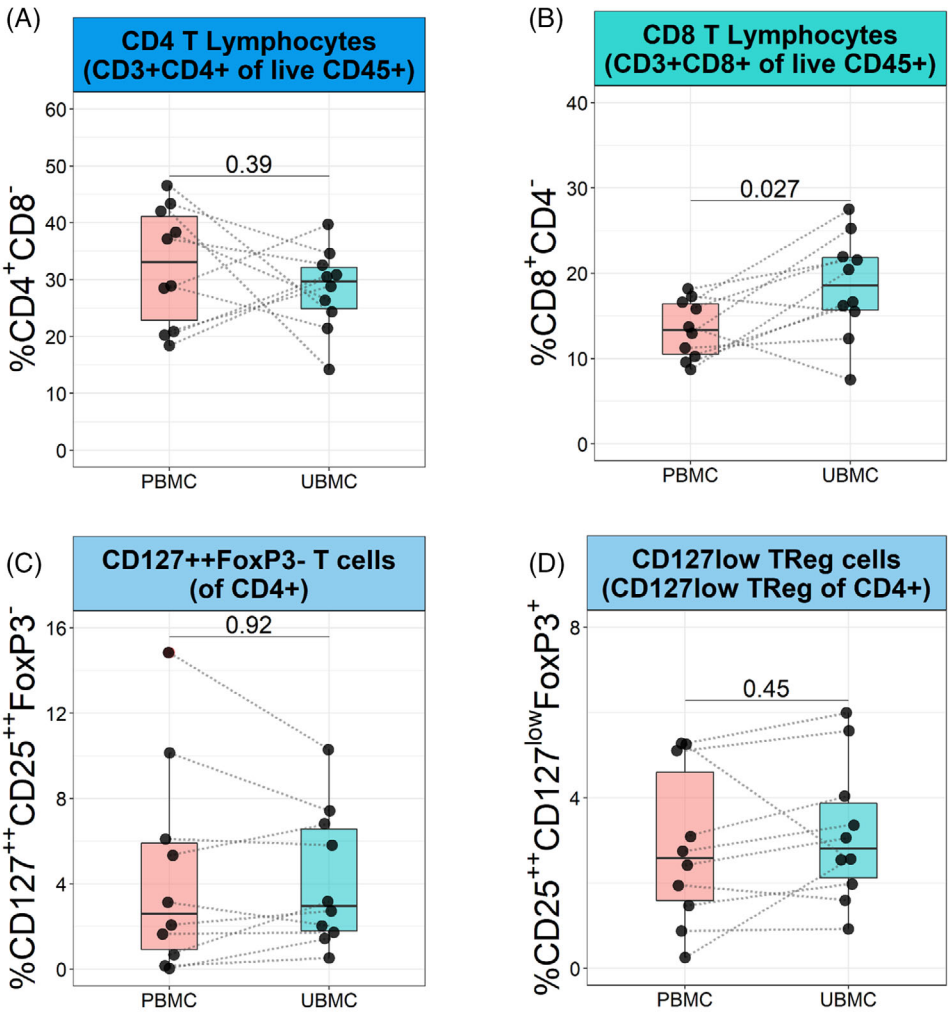

FIGURE 7 Assessment of T cell populations within PBMC and UBMC leukocyte populations. CD4 T cells ($CD45^+CD3^+CD4^+CD8^-$) were comparable in peripheral blood and placental bed samples (paired t-test; $p = 0.39$) (A); CD8 T lymphocytes ($CD45^+CD3^+CD4^-CD8^+$) were observed at significantly higher percentages in placental bed blood compared to peripheral blood (paired t-test; $p = 0.027$) (B); Percentages of $CD4^+CD25^{++}CD127^{high}FoxP3^-$ T_{Reg} cells were not significantly different between peripheral and placental bed samples (Paired Mann-Whitney *U* test; $p = 0.92$) (C). $CD4^+CD25^{++}CD127^{low}FoxP3^+$ T regs were not significantly different (paired t-test; $p = 0.45$) (D)

been used to delineate FoxP3$^+$ natural T_{Reg} cells.[54–57] Therefore, in this study we used CD25, CD127, and FoxP3 to distinguish T_{Reg} cells, as defined in previous studies.[58,59] CD4$^+$CD25$^+$CD127^{high}FoxP3$^-$ subset of cells were not significantly different between peripheral blood cells (4.41 ± 1.53%) and placental bed cells (4.19 ± 1.01%) (Figure 7C). Similarly, the CD4$^+$CD25$^+$CD127^{low}FoxP3$^+$ T_{Reg} proportions in peripheral and placental bed cells were not significantly different (Figure 7D).

4 | DISCUSSION

Isolation of pure and substantial numbers of uterine leukocytes and their phenotypic characterization is challenging due to limited amount of decidual tissue available and invasive nature of techniques available. Various techniques to collect uterine leukocytes and placental bed samples have been reported with varying success rates.[35,43–46] To combat these limitations and to improve yield, we employed a non-invasive method to obtain leukocytes from surgical gauze used to wipe the uterine cavity clean after Caesarean section delivery. We report that sampling uterine leukocytes with a surgical swab yields enriched uNK cells in placental bed samples. With this technique, obtaining live leukocytes including uNK cells, B and T cells, and monocytes is feasible, although an accurate and critical evaluation and interpretation must be practiced to characterize these immune cells . Leukocytes (CD45$^+$ cells) with >95% viability were obtained from both PBMC and UBMC matched samples. Our data demonstrate that uterine leukocytes, especially CD16$^-$CD56^{++} NK cells, are enriched by sampling with the surgical gauze method . This is consistent with the previous work[47] where they identified higher proportion of CD56^{hi}CD3- at the uteroplacental interface relative to peripheral blood; however, these authors did not use CD16 (FcγRIII) surface receptor in their NK cell analysis. Moreover, we show that there is infiltration of maternal pNK cells (CD16+CD56++CD3-) in UBMC samples (Figure 2B) suggesting a potential mixing of pNK cells with the uNK cells at the interface. This method is effective for isolating uterine leukocytes , is much less invasive, and yields greater numbers of leukocytes compared to the uterine biopsy technique.

Higher pNK cells in our UBMC analyses may be due to increased blood volume during pregnancy, which possibly diverted to the uterine site. There is hypervolemia during pregnancy and this increased blood flowing into the uterus will be of constituents different than that in peripheral venous blood. nAalysis between CD16$^+$CD56^{++} pNK cells in PBMC and UBMC samples showed a positive correlation (R = 0.81). Similarly, CD127-low T_{Reg} cells show a positive correlation (0.74) in PBMC and UBMC samples. Our findings thus reveal a unique population of cells in UBMC compared to PBMC, and therefore need further functional characterization. Likewise, an intriguing finding is that UBMC samples contained decreased proportions of blood- (and not tissue resident) B cells and monocytes compared to PBMC samples. Monocytes could become activated, and release inflammatory cytokines, and B cells could activate to secrete reactive antibodies by the decidual T cells, therefore, lower density of both of these populations in UBMC samples may be helpful in immune protection of the fetus . Designing a way to evaluate whether CD16$^+$CD56^{++} cells are resident decidual or infiltrating immune cells will be critical, as this could provide valuable insight about possible interactions of CD16$^+$CD56$^+$ and CD16$^-$CD56$^+$ cells in the uterine cavity that may regulate local immune tolerance mechanisms.

These findings beg further investigation of functional differences between intrauterine and peripheral CD16$^+$CD56$^+$ NK cells. Previously, flow cytometric quantification of term decidual leukocytes has shown that both CD16$^-$CD56^{++} uNK and CD16$^+$CD56^{++} pNK cells are present at the maternal-fetal interface.[24,60,61] CD107a (also known as Lysosomal-associated membrane protein-1(LAMP-1)) has been described as a marker of CD8$^+$ T cell degranulation following stimulation; this protein is also upregulated on the surface of CD16$^-$CD56^{++} uNK cells following stimulation by MHC-negative target cells.[62] We found that the percent of activated (CD107a$^+$) CD16$^+$CD56^{++} cells in the peripheral blood was comparable to that of uterine blood. A previous study reported the presence of activated and cytotoxic CD16$^+$CD56^{++} pNK cells in normal term peripheral blood[63] compared to non-pregnant controls; however, the investigators did not define activation status in uterine blood. Furthermore, the PrimeFlow assay in our study shows increased expression of CCL5 (*RANTES*) mRNA in CD16$^-$CD56^{++} NK cells in UBMC samples compared to that of PBMC samples. . Our results support previous reports of uNK cells' ability to express CCL5 at a higher level than pNK cells, and demonstrate feasibility of capturing uNK cells from conducting non-invasive collection of uterine blood at the time of birth.[28]

Monocytes are another innate immune cell subset present at the maternal-fetal interface. Direct and indirect contact with the placenta may activate monocytes and other leukocytes resulting in upregulation of activation markers.[64] Therefore, we assessed CD86 and HLA-DR expression on monocytes from peripheral and uterine blood as activation markers. Flow cytometric analysis of monocytes showed that the percent of CD45$^+$CD14$^+$ monocytes as well as percent of activated monocytes (HLA-DR$^+$CD86$^+$) are present at significantly lower levels in uterine blood than peripheral blood, suggesting that most of the uterine monocytes were not activated in the uterus in these C-section deliveries. The mean percentage of CD14$^+$ PBMC monocytes (27.42 ± 3.31% of CD45$^+$ cells) from term pregnancies in our study was comparable to the previous study by Bartmann et al., 2014.[65]

cyB cells are major regulators of adaptive immunity and endowed with pleiotropic functional abilities such as antibody production, antigen presentation, and immunomodulatory cytokine production. Here, we show that the CD19$^+$ B cell population is reduced in the uterine blood compared to peripheral blood. A modest increase in B cells at the maternal-fetal interface occurs between 27 and 33 weeks of gestation and slightly declines at term.[65] The lower percentage of B cells in UBMC samples in our study further suggests an enriched population of other uterine leukocytes. Certain maternal antibodies migrate across placenta to confer passive immunity to the fetus and presence of tissue-resident B cells and IL-10 producing regulatory B cells (B10 cells) at the feto-maternal interface may support pregnancy .[66–69] The reduction in the proportion of B cells in the uterine blood shown in

our study potentially minimizes exposure of the fetus to potentially harmful antibodies that would otherwise be produced against fetal antigens.[70] Activated B cells express CD80/CD86 and can receive costimulatory signals to and from T cells. CD86, through T cell-expressed CD28, signals T cell activation in response to antigen engagement via HLA-DR expressed on antigen-presenting cells (APCs).[71,72] HLA-DR is primarily expressed on APCs and is a transmembrane *HLA* class II glycoprotein responsible for antigen presentation to T lymphocytes. This interaction leads to generation of an antigen-specific adaptive immune response.[73] However, our study shows that HLA-DR and CD86 expression by $CD19^+$ B cells in peripheral and uterine blood are similar. Furthermore, other investigators have demonstrated that $CD19^+$B cells and B-1 cells, which constitutively secrete "natural" antibody in human choriodecidual tissue, are increased in spontaneous preterm compared to term labor patients.[74] Those studies showed elevated expression of activated memory and plasma cell associated molecules on B cells, including CD86, compared to non-pregnant control samples.[74,75] A further investigation of tissue resident B cell markers, B-1 cells, and regulatory B cells in PBMC and UBMC from C-section control and abnormal deliveries would be useful in order to shed light on regulatory role of B cells in pregnancy.

We also evaluated the proportions of $CD4^+$, $CD8^+$, and T_{Reg} cells in uterine blood in comparison to those in peripheral blood of C-section deliveries at term. We demonstrated that at term, $CD4^+$ T lymphocytes, as well as $CD4^+CD25^+CD127^{low}FoxP3^+$ T_{Reg} cells did not differ significantly between PBMC and UBMC in matched donor samples. Alternatively, we identified a slightly higher percentage of $CD8^+$ T cells in UBMC compared to PBMC. Regulatory phenotypes, $CD4^+CD25^+$ and $CD8^+CD28^+$, have been identified in decidua from early and term pregnancy suggesting potential immunomodulatory role of CD4 and CD8 T cells in the feto-maternal interface.[76] We did not investigate how many of these $CD8^+$ T lymphocytes are of regulatory phenotype. However, we did explore the expression level of $CD127^{high}$ T cells ($CD4^+CD25^+CD127^{high}FoxP3^-$), which arise during the effector phase of immune responses and acquire functionality and characteristics of memory T cells.[77] We observed similar expression of these cells in peripheral and uterine blood samples.

In conclusion, we demonstrate a method for successfully isolating viable decidual cells using surgical gauze for the characterization of immune cells using flow cytometry. Based on viability of uterine leukocytes from this method (>95%), in addition to flow cytometric analysis, cell sorting and functional assays could also be performed on the cells collected using this protocol. Increased purity of target populations of cells may be isolated by flow cytometric assessment of immune cell subtype-specific markers. Importantly, an enriched fraction of $CD16^-CD56^+$ NK can be isolated using this surgical gauze method. Our results demonstrate that this method may be useful for retrieving uterine leukocytes for research including in cases of pregnancy complications.

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CONFLICT OF INTEREST

All authors disclose no potential sources of conflict of interest.

DATA AVAILABILITY STATEMENT

The author has provided the required Data Availability Statement, and if applicable, included functional and accurate links to said data therein.

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