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Cellular immune responses in amniotic fluid of women with preterm prelabor rupture of membranes

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Abstract

Background: Preterm birth is the leading cause of perinatal morbidity and mortality. Preterm prelabor rupture of membranes (pPROM) occurs in 30% of preterm births, and is thus a major contributor to maternal and neonatal morbidity. However, the cellular immune responses in amniotic fluid of women with pPROM have not been investigated.

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Declaration of Interests Statement

We have no conflicts of interest to declare.

Methods: Amniotic fluid samples were obtained from women with pPROM and a positive (n=7) or negative (n=10) microbiological culture. Flow cytometry was performed to evaluate the phenotype and number of amniotic fluid leukocytes. The correlation between amniotic fluid immune cells and interleukin-6 concentration or white blood cell count in amniotic fluid was calculated.

Results: Women with pPROM and a positive amniotic fluid culture had: 1) a greater number of total leukocytes in amniotic fluid, including neutrophils and monocytes/macrophages, and 2) increased numbers of total T cells in amniotic fluid, namely CD4+ T cells and CD8+ T cells, but not B cells. The numbers of neutrophils and monocytes/macrophages were positively correlated with IL-6 concentrations and WBC counts in amniotic fluid of women with pPROM.

Conclusion: Women with pPROM and a positive amniotic fluid culture exhibit a more severe cellular immune response than those with a negative culture, which is associated with well-known markers of intra-amniotic inflammation.

Keywords

Acute chorioamnionitis; Clinical chorioamnionitis; Fetal inflammatory response; Funisitis; Innate immune cells; Interleukin-6; Labor; Microbial invasion of the amniotic cavity; Pregnancy; Preterm labor; Prematurity

INTRODUCTION

Preterm birth is the leading cause of perinatal morbidity and mortality worldwide (1–3). Approximately 30% of preterm births are preceded by preterm prelabor rupture of membranes (pPROM) (4–12), defined as the rupture of the chorioamniotic membranes before the onset of preterm labor (i.e. <37 weeks) (4, 6–12), and thus pPROM is a major contributor to maternal and neonatal morbidity (13–31). This clinical condition is considered a great obstetrical syndrome (32–36), which has recently been subcategorized using microbiological techniques coupled with amniotic fluid concentrations of interleukin-6 (IL-6) (37–39). Hence, it is now established that pPROM can occur in the presence of either intra-amniotic infection [detectable microorganisms in amniotic fluid in the presence of elevated concentrations of IL-6 (≥2.6 ng/mL)], sterile intra-amniotic inflammation (elevated concentrations of IL-6 in the absence of detectable microorganisms), or in the absence of intra-amniotic inflammation (IL-6 concentration <2.6 ng/mL) (38). Although the clinical management of pPROM includes antibiotic administration (40–45), it is well documented that the intensity of the intra-amniotic inflammatory response is associated with the severity of fetal inflammatory responses (37, 38, 46–48). Indeed, neonates born to women with pPROM and intra-amniotic infection are at a higher risk of neonatal morbidity than those born to women with intra-amniotic inflammation (37, 38, 47). Therefore, the characterization of the immune response in amniotic fluid of women with pPROM is warranted.

The amniotic fluid contains a diverse array of innate and adaptive immune cells that varies as normal gestation progresses (49). Specifically, neutrophils, monocytes/macrophages, T cells, innate lymphoid cells, natural killer (NK) cells, and B cells are present in the amniotic

cavity of women with a normal pregnancy (49–51). These cellular immune responses are augmented in women with pregnancy complications such as preterm labor with intact membranes (52), clinical chorioamnionitis at term (53), and preterm clinical chorioamnionitis (54). However, the cellular immune responses in amniotic fluid of women with pPROM have not been investigated.

Herein, we utilized multi-color flow cytometry to investigate the cellular composition of amniotic fluid from women who underwent pPROM with or without positive microbiological cultures. Moreover, we correlated the number of amniotic fluid immune cells with well-known markers of intra-amniotic inflammation (white blood cell count and IL-6 concentration).

METHODS

Study design and population:

This retrospective cross-sectional study was conducted by searching our clinical database and bank of biological samples. The collection of samples was approved by the Institutional Review Boards of the Detroit Medical Center (Detroit, MI, USA), Wayne State University, and the Perinatology Research Branch, an intramural program of the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, National Institutes of Health, U.S. Department of Health and Human Services. All women provided written informed consent prior to the collection of amniotic fluid.

This study included 17 amniotic fluid samples collected from patients with pPROM, and a reachable amniotic fluid pocket, and either a positive amniotic fluid microbiological culture (n=7) or a negative amniotic fluid microbiological culture (n=10) (see clinical definitions and amniotic fluid sample collection below) (Table 1). For all patients, the amniocentesis was performed after the diagnosis of pPROM and the time between the collection of the amniotic fluid sample and rupture of membranes was ≤2 days (this criterion was used to preserve a meaningful relationship between amniotic fluid studies and pPROM). The demographic and clinical characteristics of the study population are shown in Table 1. Multiple pregnancies, fetal malformations and genetic disorders were excluded from this study.

Clinical definitions

Gestational age was determined by the date of the last menstrual period and confirmed by ultrasound examination. The gestational age derived from sonographic fetal biometry was used if the estimation was inconsistent with menstrual dating. Preterm PROM was defined as amniorrhexis confirmed by vaginal pooling, ferning, or a positive nitrazine test prior to the onset of labor before 37 weeks of gestation (55–57).

Placental histopathological examination

Placentas were examined histologically by perinatal pathologists blinded to clinical diagnoses and obstetrical outcomes according to standardized Perinatology Research Branch protocols (58, 59). Briefly, three to nine sections of the placenta were examined, and at least

one full-thickness section was taken from the center of the placenta; others were taken randomly from the placental disc. Acute inflammatory lesions of the placenta (maternal inflammatory response and fetal inflammatory response) were diagnosed according to established criteria, including staging and grading (58, 60). The proportions of patients whose placentas presented acute maternal and/or fetal inflammatory responses are displayed in Table 1.

Amniotic fluid sample collection

Amniotic fluid samples were obtained by transabdominal amniocentesis under antiseptic conditions and monitored by ultrasound in order to detect intra-amniotic inflammation and/or infection in patients with pPROM. Samples of amniotic fluid were transported to the laboratory in a sterile, capped syringe and immunophenotyping was performed immediately. The rest of the sample was centrifuged at 1300 x *g* for 10 minutes at 4°C, and the supernatant was stored at −80°C until use. Additionally, an aliquot of amniotic fluid was transported to the clinical laboratory for culture of aerobic/anaerobic bacteria and genital mycoplasmas. The clinical laboratory also performed tests to determine an amniotic fluid white blood cell (WBC) count (61), a Gram stain examination (62), and a glucose concentration (63).

Determination of IL-6 concentration in amniotic fluid

Amniotic fluid concentrations of IL-6 were determined, as previously established (64) using a sensitive and specific enzyme immunoassay obtained from R&D systems (Minneapolis, MN, USA). The IL-6 concentrations were determined by interpolation from the standard curves. The inter- and intra-assay coefficients of variation for IL-6 were 8.7% and 4.6%, respectively. The sensitivity of the IL-6 assay was 0.09 pg/mL.

Immunophenotyping by flow cytometry

Amniotic fluid samples (0.5–1 mL) were centrifuged at 300 x *g* for 5 minutes at room temperature. The resulting amniotic fluid pellet was resuspended in 1 mL of 1X phosphate-buffered saline (PBS) (Life Technologies, Grand Island, NY, USA) and stained with BD Horizon Fixable Viability Stain 510 dye (BD Biosciences, San Jose, CA, USA). Cells were washed in 1X PBS and incubated with 20 μL of human FcR blocking reagent (Miltenyi Biotec, San Diego, CA, USA) in 80 μL of stain buffer (BD Biosciences) for 10 minutes at 4°C. Next, cells were incubated with extracellular fluorochrome-conjugated anti-human monoclonal antibodies for 30 minutes at 4°C in the dark (Supplementary Table 1). Stained cells were then washed with 1X PBS, resuspended in 0.5 mL of stain buffer, and acquired using the BD LSR II or LSRFortessa Flow Cytometer (BD Bioscience) and BD FACSDiva 6.0 software (BD Bioscience). The analysis was performed, and the figures were generated using the FlowJo version 10 software (FlowJo, Ashland, OR, USA). The absolute number of cells was determined using CountBright absolute counting beads (Molecular Probes, Eugene, OR, USA).

Statistical analysis

Statistical analyses were conducted using GraphPad Prism version 8.0.1 for Windows (GraphPad Software, San Diego, California, USA, www.graphpad.com). For patient demographics, the Mann-Whitney *U*-test was used to compare continuous variables and the Fisher's exact test was used for nominal variables. The Mann-Whitney *U*-test was performed to compare the number of amniotic fluid immune cells between the two study groups: two-tailed p-values are reported. The Spearman correlation between amniotic fluid immune cells and IL-6 concentration or white blood cell count in amniotic fluid was calculated. A p-value <0.05 was considered statistically significant for all tests.

RESULTS

Characteristics of the study population

The clinical and demographic characteristics of the study population are shown in Table 1. A total of 17 amniotic fluid samples were collected from women with pPROM. Ten women had amniotic fluid microbiological cultures that were negative for bacterial growth and seven women had positive amniotic fluid microbiological cultures. Women with pPROM and positive cultures had significantly higher amniotic fluid concentrations of IL-6 and white blood cells, and lower glucose concentrations, compared to those with negative cultures (Table 1). There were no significant differences in maternal age, primiparity, pre-pregnancy body mass index, race, gestational age at amniocentesis and delivery, or birthweight between the two study groups, which may be due to the number of samples included in this study (Table 1). The most common microorganism found in amniotic fluid from women with pPROM and a positive microbiological culture was *Ureaplasma urealyticum*. Out of the seven patients with a positive microbial culture: three patients had only Ureaplasma spp; one patient had only *Actinomyces odontoliticus*; one patient had *Bacteroides*, *Gardnerella vaginalis*, and *Ureaplasma urealyticum*; one patient had Candida spp., Group B *Streptococcus*, and *Ureaplasma urealyticum*; and one patient had both Mycoplasma spp. and Ureaplasma spp. Patients with a positive culture had a greater prevalence of necrotizing chorioamnionitis compared to those with a negative culture (Table 1).

Immune cell populations in amniotic fluid of women with pPROM

A representative image of the flow cytometry gating strategy used to detect leukocytes in amniotic fluid from women with pPROM is shown in Figure 1A. Briefly, viable cells were gated within the single cell population (i.e. singlets), and then further identified as total leukocytes (CD45+ cells), neutrophils (CD45+CD15+CD14− cells), monocytes/macrophages (CD45+CD15−CD14+ cells), B cells (CD45+CD15−CD14−CD3−CD19+ cells), and T cells (CD45+CD15−CD14−CD3+CD19− cells) (Figure 1A). T cells were further subdivided into CD4+ T cells (CD3+CD4+CD8− cells) and CD8+ T cells (CD3+CD4−CD8+ cells) (Figure 1A).

The overall number of amniotic fluid leukocytes was significantly increased in women with pPROM who had a positive amniotic fluid culture compared to those with a negative culture (Figure 1B). Additionally, quantification of neutrophils and monocytes/macrophages

revealed that these innate immune cells were present in higher abundance in amniotic fluid of women with pPROM and a positive culture compared to those with a negative culture (Figure 1C&D).

In addition to innate immune cells, adaptive immune cells (T cells and B cells) are also found in amniotic fluid during normal pregnancy (49). Therefore, we next determined whether the numbers of these adaptive immune cells were altered in amniotic fluid of women from our two study groups. The total T-cell population, CD4+ T cells (i.e. helper T cells), and CD8+ T cells (i.e. cytotoxic T cells) were all significantly increased in amniotic fluid of women with pPROM and a positive culture compared to those with pPROM and a negative culture (Figure 2A–C). The numbers of amniotic fluid B cells in women with pPROM and a positive culture tended to be higher than in women with a negative amniotic fluid culture; however, this increase did not reach statistical significance (Figure 2D).

Taken together, the results of the flow cytometric analysis reveal that total leukocytes, and more specific leukocyte subsets such as neutrophils, monocytes/macrophages, and T cells (CD4+ and CD8+ T cells), are present in higher abundance in amniotic fluid of women with pPROM and a positive amniotic fluid culture compared to those with a negative culture.

Correlation between the number of innate immune cells and clinical inflammatory markers in amniotic fluid of women with pPROM

Next, we determined whether the numbers of the most abundant subsets of amniotic fluid leukocytes (neutrophils and monocytes/macrophages) in women with pPROM were correlated with the IL-6 concentrations and WBC counts measured in amniotic fluid. We found that increasing concentrations of the pro-inflammatory cytokine IL-6 were positively correlated with increasing abundance of neutrophils in amniotic fluid (Figure 3A, $r=0.7$, $p=0.002$). Similarly, increasing IL-6 concentrations were positively correlated with the presence of elevated numbers of amniotic fluid monocytes/macrophages, though this relationship was slightly less strong than that with neutrophils (Figure 3B, $r=0.63$, $p=0.007$). Positive correlations between WBC counts and neutrophils (Figure 3C, $r=0.66$, $p=0.005$) and monocytes/macrophages (Figure 3D, $r=0.68$, $p=0.003$) were also found in amniotic fluid. These results showed that the number of innate immune cells in amniotic fluid positively correlate with well-known markers of intra-amniotic inflammation in women with pPROM.

DISCUSSION

Principle Findings

Herein, we report that women with pPROM and a positive amniotic fluid culture had: 1) a greater number of total leukocytes in amniotic fluid including neutrophils and monocytes/macrophages, and 2) increased numbers of total T cells in amniotic fluid, namely CD4+ T cells and CD8+ T cells, but not B cells. Further, we report that the number of neutrophils and monocytes/macrophages were positively correlated with IL-6 concentrations and WBC counts in amniotic fluid of women with pPROM. Collectively, these findings indicate that women with pPROM and a positive amniotic fluid culture exhibit a more severe cellular

immune response than those with a negative culture, which is associated with well-known markers of intra-amniotic inflammation.

Amniotic fluid neutrophils in women with pPROM

It is well established that neutrophils are the most abundant immune cell type in the amniotic cavity of women with intra-amniotic infection (52–54, 61, 65, 66). These innate immune cells can be predominantly of fetal origin in preterm gestations and of maternal origin at term (66). However, whether the number of amniotic fluid neutrophils differs in the context of pPROM with and without culturable microorganisms had not yet been shown. In the current study, we showed that the number of neutrophils in amniotic fluid is significantly higher in women with pPROM and a positive microbiological culture than in those without culturable microorganisms. Studies investigating the functions of amniotic fluid neutrophils have shown that these innate immune cells can phagocytize bacteria invading the amniotic cavity (67), form neutrophil extracellular traps (NETs) (68) and may degranulate, releasing anti-microbial molecules such as myeloperoxidase (69–71), alpha-defensins (70, 72–74), elastase (70, 75, 76), cathepsin G (70, 77), lactoferrin (78), pentraxin-3 (79), and cathelicidin (69, 70) as well as reactive oxygen species (80) into the amniotic cavity. In addition to participating in the host defense response to microbes, neutrophils can also release pro-inflammatory cytokines such as IL-8, TNF-α, MIP-1α, MIP-1β, IL-1α, and IL-1β, which are implicated in the mechanisms leading to premature labor in the context of intra-amniotic infection (52, 53, 81–94). Furthermore, amniotic fluid neutrophils may also participate in the pathogenesis of pPROM by releasing neutrophil elastase and metalloproteinases (76, 95–102); yet, this hypothesis has not been mechanistically investigated.

It is worth mentioning that amniotic fluid neutrophils are increased in all reported pregnancy complications associated with intra-amniotic inflammation (52–54), suggesting that these innate immune cells participate in the common pathway of parturition and host defense mechanisms taking place in the amniotic cavity regardless of the obstetrical syndrome. Further studies may be required to investigate whether amniotic fluid neutrophils diverge in signaling pathways among the different pregnancy complications associated with prematurity and/or adverse neonatal outcomes.

Amniotic fluid monocytes/macrophages in women with pPROM

In the current study, flow cytometric analysis also revealed that monocyte/macrophages, which are the second most abundant leukocyte population in amniotic fluid (49, 52–54, 61), are increased in women with pPROM and a positive amniotic fluid culture. This is in line with previous studies showing that amniotic fluid monocytes/macrophages are elevated in women with preterm labor and intact membranes (52), clinical chorioamnionitis at term (53), and preterm clinical chorioamnionitis (54) with a positive microbiological culture.

Recently, the origin of the amniotic fluid monocytes/macrophages in women with intra-amniotic inflammation or infection was described (103). Specifically, amniotic fluid monocytes/macrophages were found to be predominantly of maternal origin in women with intra-amniotic inflammation/infection who delivered late preterm or term neonates, whereas

most samples with predominantly fetal monocytes/macrophages in amniotic fluid were from women who delivered early preterm neonates (103).

The classical function of monocytes is to produce and release pro-inflammatory mediators such as cytokines (104); however, their functions can vary according to the microenvironment (104–107). Indeed, a recent study demonstrated that placental macrophages can respond to microbes by releasing extracellular traps (METs) (108), providing further evidence that these cells can have additional functions in the amniotic cavity beyond cytokine release. Yet, we have shown that monocytes/macrophages release different cytokines than those released by neutrophils, indicating that both cells have distinct and specific functions in the amniotic cavity (52, 53). For example, we previously showed that IL-1β is expressed in greater amounts by monocytes/macrophages than by neutrophils in women with clinical chorioamnionitis at term and a positive microbiological culture (52, 53). Nonetheless, additional research is required to investigate the diverse signaling pathways between neutrophils and monocytes in the context of intra-amniotic inflammation and/or infection.

Correlation between flow cytometric analysis of amniotic fluid and classical biomarkers of intra-amniotic inflammation

The traditional biomarker of intra-amniotic inflammation has been the amniotic fluid WBC count (27, 61, 65, 109, 110). We have previously shown that amniotic fluid WBC counts obtained using the traditional method (hemocytometer) correlate well with counts obtained using flow cytometry in women with clinical chorioamnionitis at term (53). In line with this finding, we report herein that a significant correlation exists between the numbers of amniotic fluid neutrophils or monocytes/macrophages acquired by flow cytometry and WBC counts obtained by hemocytometer in patients with pPROM.

More recently, the concentration of IL-6 in amniotic fluid was demonstrated to be a reliable biomarker for intra-amniotic inflammation (64, 111). Amniotic fluid IL-6 concentrations were also shown to correlate with the numbers of total leukocytes, neutrophils, or monocytes/macrophages obtained using flow cytometry in women with clinical chorioamnionitis at term (53). Similarly, in the current study we show that IL-6 concentrations are significantly correlated with the numbers of neutrophils or monocytes/macrophages in amniotic fluid of women with pPROM.

Together, these results indicate that flow cytometric analysis of amniotic fluid immune cell composition can provide a reliable overview of the intra-amniotic inflammatory response in women with pPROM.

Conclusion

In the current study, we show that women with pPROM and a positive amniotic fluid microbiological culture have increased numbers of neutrophils, monocytes/macrophages, and T cells (CD4+ and CD8+ T cells) compared to patients with a negative culture. Such an increase was correlated with the concomitant rise in amniotic fluid IL-6 concentrations, and immune cell numbers obtained by flow cytometry were also correlated with traditional WBC

counts. These results indicate that the intra-amniotic cellular immune response in patients with pPROM is more severe in the presence of invading microorganisms, and that such a response is primarily driven by neutrophils, monocytes/macrophages, and T cells. These data provide insights into the cellular immune responses taking place in the amniotic cavity of women with pPROM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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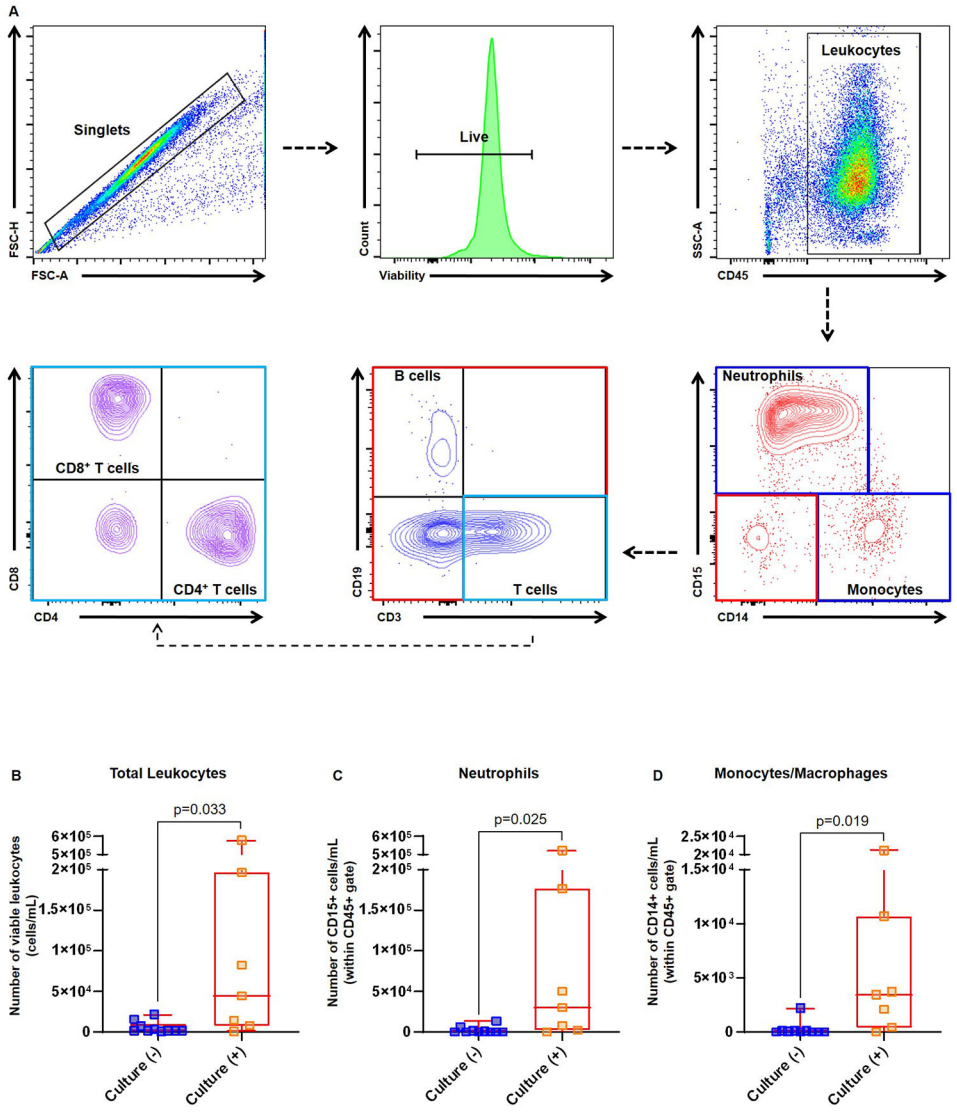

Figure 1. Total leukocytes and innate immune cells in amniotic fluid.

(A) Representative flow cytometry gating strategy showing leukocyte populations in amniotic fluid from women with preterm prelabor rupture of membranes (pPROM). Immune cells were initially gated within the viability gate and CD45+ gate followed by lineage gating for neutrophils (CD45+CD15+CD14−cells), monocytes/macrophages (CD45+CD15−CD14+ cells), T cells (CD45+CD15−CD14−CD3+CD19− cells) and B cells (CD45+CD15−CD14−CD3−CD19+ cells). T cells were subsequently gated for CD4+ T cells (CD3+CD4+CD8− cells) and CD8+ T cells (CD3+CD4−CD8+ cells). Numbers of **(B)** total leukocytes (CD45+ cells/mL), **(C)** neutrophils (CD15+ cells/mL), and **(D)** monocytes/macrophages (CD14+ cells/mL) in amniotic fluid from women with pPROM who had either a negative or positive amniotic fluid culture. N = 7–10 per group. Midlines = median, boxes = interquartile ranges, and whiskers = minimum/maximum ranges. Forward Scatter Height (FSC-H), Forward Scatter Area (FSC-A), and Side Scatter Area (SSC-A).

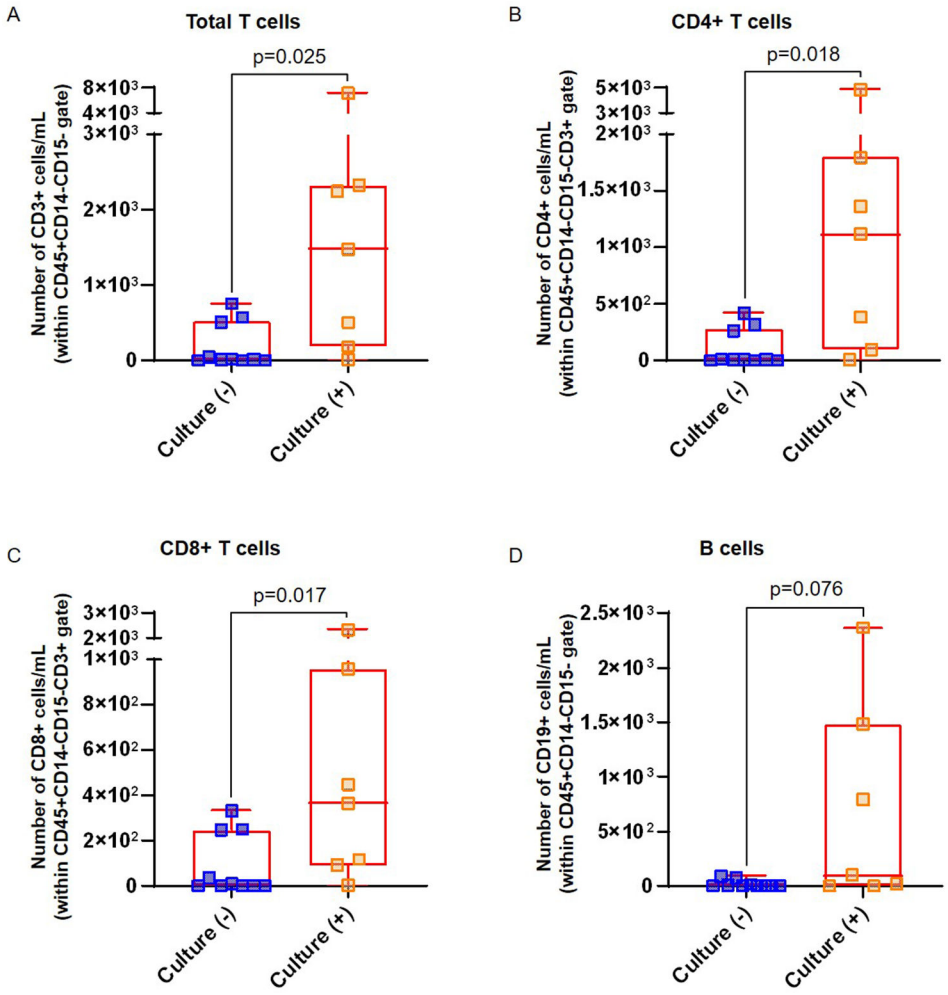

Figure 2. Adaptive immune cells in amniotic fluid.

Numbers of **(A)** total T cells (cells/mL), **(B)** CD4+ T cells (cells/mL), **(C)** CD8+ T cells (cells/mL), and **(D)** B cells (CD19+ cells/mL) in amniotic fluid from women with pPROM who had either a negative or positive amniotic fluid culture. Lymphocyte populations were gated as shown in Figure 1A. N = 7–10 per group. Midlines = median, boxes = interquartile ranges, and whiskers = minimum/maximum ranges.

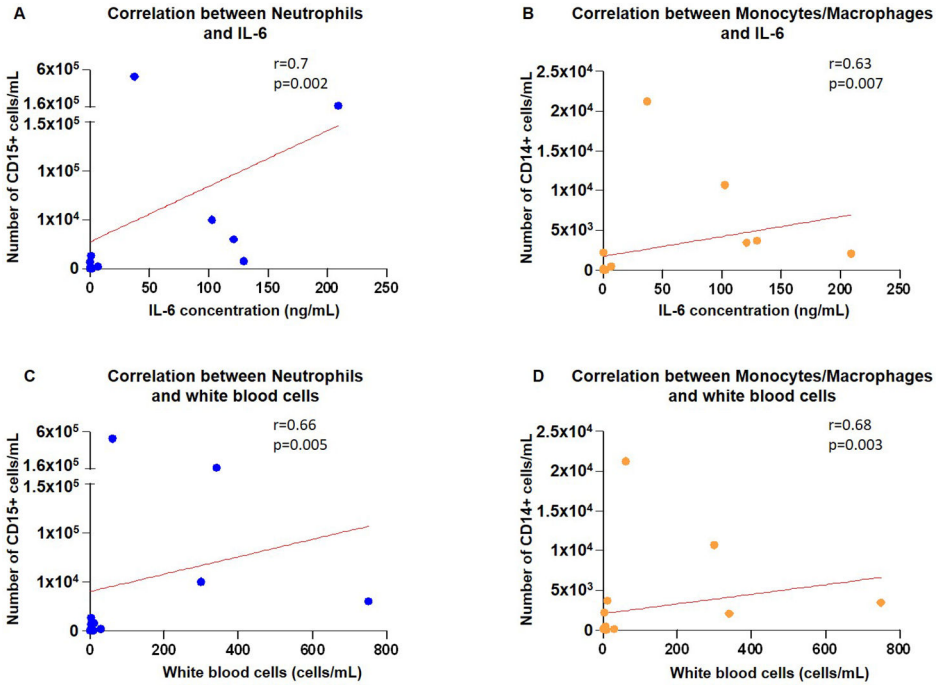

Figure 3. Correlation between neutrophils or monocytes/macrophages and IL-6 concentrations or white blood cell counts in amniotic fluid.

The relationship between amniotic fluid concentrations of IL-6 and the number of neutrophils (CD15+ cells) **(A)** or monocytes/macrophages (CD14+ cells) **(B)** in amniotic fluid of women with pPROM. The correlation between amniotic fluid white blood cell counts obtained by hemocytometer and the number of neutrophils (CD15+ cells) **(C)** or monocytes/macrophages (CD14+ cells) **(D)** in amniotic fluid of women with pPROM. Correlations were assessed using a Spearman's test. Correlation coefficients and *p* values are shown for each plot. The regression line is indicated. N = 17.

Table 1.

Clinical and Demographic characteristics of women with pPROM.

	Negative culture (n=10)	Positive culture (n=7)	p-value
Maternal age (years; median [IQR])[a]	26.5 (25.3–31.3)	33 (28.5–33.5)	0.46
Body mass index (kg/m^2; median [IQR])[a]	29.1 (23.8–33.7)	32 (24.6–36.6)[c]	0.85
Primiparity[b]	30% (3/10)	0% (0/7)	0.23
Race/Ethnicity[b]			0.74
African-American	80% (8/10)	71.4% (5/7)	
White	10% (1/10)	28.6% (2/7)	
Other	10% (1/10)	0% (0/7)	
Gestational age at amniocentesis (weeks; median [IQR])[a]	30.6 (26–33)	25.6 (22.6–31.9)	0.65
IL-6 (ng/mL; median [IQR])[a]	0.2 (0.1–0.4)	102.8 (22–125.3)	0.003
Amniotic Fluid Glucose (mg/dl; median [IQR])[a]	33.5 (23–41)	10 (1–15)	0.003
Amniotic Fluid WBC (cells/mm^3; median [IQR])[a]	1.5 (0–3.5)	60 (7.5–319.5)	0.007
Gestational age at membrane rupture (weeks; median [IQR])[a]	30.5 (26–32.9)	25.4 (22.5–31.9)	0.67
Gestational age at delivery (weeks; median [IQR])[a]	33 (28.4–33.9)	25.7 (23.1–32.1)	0.38
Cesarean section[b]	10% (1/10)	14.3% (1/7)	1.0
Birthweight (grams; median [IQR])[a]	1725 (1127.5–1850)	840 (527–2025)	0.88
Acute maternal inflammatory response[b]			
Stage 1 (Early acute subchorionitis or chorionitis)	40% (4/10)	0% (0/6)[d]	0.23
Stage 2 (Acute chorioamnionitis)	30% (3/10)	16.7% (1/6)[d]	1.0
Stage 3 (Necrotizing chorioamnionitis)	0% (0/10)	50% (3/6)[d]	0.036
Acute fetal inflammatory response[b]			
Stage 1 (Chorionic vasculitis or umbilical phlebitis)	20% (2/10)	50% (3/6)[d]	0.3
Stage 2 (Umbilical arteritis)	10%(1/10)	16.7 % (1/6)[d]	1.0
Stage 3 (Necrotizing funisitis)	10% (1/10)	0% (0/6)[d]	1.0

Data are given as median (interquartile range, IQR) and percentage (n/N).

[a]Mann-Whitney test.

[b]Fisher's exact test

[c]Two missing data.

[d]One missing data.

Abbreviations: pPROM, preterm prelabor rupture of membranes; WBC, white blood cells