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Cellular immune responses in amniotic fluid of women with preterm labor and intra-amniotic infection or intra-amniotic inflammation

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Abstract

Problem: Preterm birth is commonly preceded by preterm labor, a syndrome that is causally linked to both intra-amniotic infection and intra-amniotic inflammation. However, the stereotypical cellular immune responses in these two clinical conditions are poorly understood.

Method of study: Amniotic fluid samples (n = 26) were collected from women diagnosed with preterm labor and intra-amniotic infection (amniotic fluid IL-6 concentrations ≥2.6 ng/mL and culturable microorganisms, n = 10) or intra-amniotic inflammation (amniotic fluid IL-6 concentrations ≥2.6 ng/mL without culturable microorganisms, n = 16). Flow cytometry was performed to evaluate the phenotype and number of amniotic fluid leukocytes. Amniotic fluid concentrations of classical pro-inflammatory cytokines, type 1 and type 2 cytokines, and T-cell chemokines were determined using immunoassays.

Results: Women with spontaneous preterm labor and intra-amniotic infection had (a) a greater number of total leukocytes, including neutrophils and monocytes/macrophages, in amniotic fluid; (b) a higher number of total T cells and $CD4^+$ T cells, but not $CD8^+$ T cells or B cells, in amniotic fluid; and (c) increased amniotic fluid concentrations of IL-6, IL-1β, and IL-10, compared to those with intra-amniotic inflammation. However, no differences in amniotic fluid concentrations of T-cell cytokines and chemokines were observed between these two clinical conditions.

Conclusion: The cellular immune responses observed in women with preterm labor and intra-amniotic infection are more severe than in those with intra-amniotic inflammation, and neutrophils, monocytes/macrophages, and $CD4^+$ T cells are the main immune cells responding to microorganisms that invade the amniotic cavity. These findings provide insights into the intra-amniotic immune mechanisms underlying the human syndrome of preterm labor.

KEYWORDS
chorioamnionitis, fetal inflammatory response syndrome, funisitis, microbial invasion of the amniotic cavity, placental inflammation, pregnancy

1 | INTRODUCTION

Preterm birth remains one of the most common obstetrical syndromes today and is a primary cause of perinatal morbidity and mortality worldwide.[1-5] On average, two-thirds of all preterm births are preceded by spontaneous preterm labor,[6] a syndrome of multiple etiologies.[7] Of all the proposed causes of preterm labor, intra-amniotic inflammation/infection has been causally linked to preterm birth.[8-17] Intra-amniotic inflammation can result from microbial invasion of the amniotic cavity, which is referred to as intra-amniotic infection.[9,10,12,18-29] Yet, inflammation in the amniotic cavity can also occur in the absence of culturable microorganisms, which is simply known as intra-amniotic inflammation.[16,27,30] Recently, we showed using molecular microbiological techniques that a subset of patients with preterm labor and intra-amniotic inflammation do not have detectable bacteria in the amniotic cavity, which we termed sterile intra-amniotic inflammation.[31-34] This condition is associated with elevated concentrations of endogenous danger signals, or alarmins (molecules released upon cellular stress or damage[35-37]), in amniotic fluid;[38-43] although of interest, it is not yet a clinical diagnosis as the use of molecular microbiological techniques is not common practice in obstetrics. Therefore, patients with preterm labor are either diagnosed with intra-amniotic infection or intra-amniotic inflammation. Although both clinical conditions are associated with preterm labor and adverse neonatal outcomes,[32,44] their management is different (intra-amniotic infection is treated with antibiotics[45]), and only intra-amniotic infection is linked to maternal morbidity and mortality.[46] Therefore, elucidating the stereotypical immune responses in intra-amniotic infection and intra-amniotic inflammation is essential for understanding these clinical conditions.

Flow cytometry has emerged as a cutting-edge technique for the evaluation of immune cells in small volumes of biological fluids such as cerebrospinal fluid,[47,48] urine,[49-51] ascitic fluid,[52] and sputum[53] in the clinical setting. Indeed, flow cytometry has been utilized to identify specific immune cell types, as well as their expressed mediators, in amniotic fluid of women with intra-amniotic inflammation/infection and clinical chorioamnionitis at term.[54,55] This technique also allowed for the identification of the newly described innate lymphoid cells in the amniotic cavity of women during the second trimester.[55,56] Herein, we utilized flow cytometry to characterize the cellular immune responses in the amniotic cavity of women diagnosed with preterm labor and intra-amniotic infection or intra-amniotic inflammation.

2 | MATERIALS AND METHODS

2.1 | Study population and characteristics

This cross-sectional study included patients who underwent transabdominal amniocentesis due to clinical indications. 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 Institutes of Health, National Institutes of Health,US Department of Health and Human Services (NICHD/NIH/DHHS). All women provided written informed consent prior to the collection of amniotic fluid. This study included 26 amniotic fluid samples (collected from 2013 to 2016) from women classified into the following groups: (a) women with spontaneous preterm labor who delivered preterm with intra-amniotic inflammation (n = 16) and (b) women with spontaneous preterm labor who delivered preterm with intra-amniotic infection (n = 10) (see diagnostic criteria below). For all patients who delivered preterm, the time between the collection of the amniotic fluid sample and delivery was ≤7 days. Demographic and clinical characteristics of the study population are shown in Table 1.

2.2 | 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. Spontaneous preterm labor was diagnosed by the presence of regular uterine contractions (at least two contractions every 10 minutes) associated with cervical changes in patients <37 weeks of gestation. Microbial invasion of the amniotic cavity (MIAC) was defined as a positive amniotic fluid culture, including genital mycoplasmas.[9,10,22,57,58] Intra-amniotic inflammation was defined as an amniotic fluid IL-6 concentration ≥2.6 ng/mL in the absence of culturable bacteria.[27,59-64] Intra-amniotic infection was defined as the presence of MIAC together with intra-amniotic inflammation.[31-33,65-74]

TABLE 1 Clinical and demographic characteristics of women who underwent spontaneous preterm labor

	Preterm labor and birth with intra-amniotic infection (n = 10)	Preterm labor and birth with intra-amniotic inflammation (n = 16)	*P*-value
Maternal age (y; median [IQR])[a]	27.5 (22.8-35.8)	25.5 (22-28.3)	.3
Body mass index (kg/m^2; median [IQR])[a]	28.3 (27-36.5)[d]	26.3 (24.2-30.7)	.1
Primiparity[b]	0% (0/10)	12.5% (2/16)	.5
Race[b]			1
African American	80% (8/10)	81.3% (13/16)	
Caucasian	20% (2/10)	12.5% (2/16)	
Other	0% (0/10)	6.3% (1/16)	
Gestational age at amniocentesis (wk; median [IQR])[a]	25.9 (22.5-29.8)	27 (22.9-31.2)	.4
IL-6 (ng/mL; median [IQR])[a]	128.7 (78-186.2)	47.8 (16.7-122.9)	.04
White blood cell count, cells/mm^3,[a]	307 (89-1254.5)	2 (0-11.8)	.003
Amniotic fluid glucose, mg/dL[a]	3.5 (1-9.8)	12.5 (6-26)	.053
Gestational age at delivery (wk; median [IQR])[a]	25.9 (23-30)	27.6 (23.4-31.3)	.4
Cesarean section[b]	0% (0/10)	31.3% (5/16)	.1
Birthweight (g)[a]	935 (533.3-1428.8)	1037.5 (697.5-1778.8)	.6
Acute maternal inflammatory response			
Stage 1 (acute subchorionitis)[b]	0% (0/8)[c]	23.1% (3/13)[d]	.2
Stage 2 (acute chorioamnionitis)[b]	37.5% (3/8)[c]	30.8% (4/13)[d]	1
Stage 3 (acute necrotizing chorioamnionitis)[b]	50% (4/8)[c]	30.8% (4/13)[d]	.6
Acute fetal inflammatory response			
Stage 1 (acute phlebitis/chronic vasculitis)[b]	50% (4/8)[c]	53.8% (7/13)[d]	1
Stage 2 (acute arteritis)[b]	25% (2/8)[c]	0% (0/13)[d]	.1
Stage 3 (necrotizing funisitis)[b]	12.5% (1/8)[c]	0% (0/13)[d]	.3

Note: Data are given as median (interquartile range, IQR) and percentage (n/N).
[a]Mann–Whitney U test.
[b]Fisher's exact test.
[c]Two missing data.
[d]Three missing data.

2.3 | Placental histopathological examination

Placentas were examined histologically by perinatal pathologists blinded to clinical diagnoses and obstetrical outcomes according to standardized Perinatology Research Branch protocols.[75,76] 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 disk. Acute inflammatory lesions of the placenta (maternal inflammatory response and fetal inflammatory response) were diagnosed according to established criteria, including staging and grading.[75,77] The proportions of patients whose placentas presented acute maternal and/or fetal inflammatory responses are displayed in Table 1.

2.4 | 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 preterm labor. 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 *g* for 10 minutes at 4°C, and the supernatant was stored at −80°C until use. Also, an aliquot of amniotic fluid was transported to the clinical laboratory for culture of aerobic/anaerobic bacteria and genital mycoplasmas. The clinical tests also included the determination of an amniotic fluid white blood cell count,[78] a Gram stain examination,[79] a glucose concentration,[80] and an IL-6 concentration.[27]

2.5 | Determination of IL-6 in amniotic fluid

Amniotic fluid concentrations of IL-6 were determined by 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 curve. The inter- and intra-assay coefficients of variation for IL-6 were 8.7% and 4.6%, respectively. The detection limit of the IL-6 assay was 0.09 pg/mL. The IL-6 concentrations in amniotic fluid were determined for clinical purposes.

2.6 | Immunophenotyping by flow cytometry

Amniotic fluid samples (0.5-1 mL) were centrifuged at 300 *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 (Table S1). 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).

2.7 | Amniotic fluid cytokine/chemokine concentrations

Amniotic fluid samples were assessed using the V-PLEX Proinflammatory Panel 1 kit (Meso Scale Discovery, Rockville, MD, USA) to measure amniotic fluid concentrations of IFN-γ, TNF-α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, and IL-13, according to the manufacturer's instructions. Plates were read using the SECTOR 2400 Imager (Meso Scale Discovery). Standard curves were generated, and the assay values of the samples were interpolated from the curves. The detection limits of the assays were 0.37 pg/mL (IFN-γ), 0.04 pg/mL (TNF-α), 0.05 pg/mL (IL-1β), 0.09 pg/mL (IL-2), 0.02 pg/mL (IL-4), 0.06 pg/mL (IL-6), 0.07 pg/mL (IL-8), 0.04 pg/mL (IL-10), 0.11 pg/mL (IL-12p70), and 0.24 pg/mL (IL-13). Inter-assay and intra-assay coefficients of variation were <10.5%.

Amniotic fluid concentrations of CXCL10 (Cat#DIP100, R&D Systems) and CXCL11 (Cat#K151UWK-1, Meso Scale Discovery) were determined using individual sensitive and specific immunoassays, according to the manufacturers' instructions. The concentrations of CXCL10 and CXCL11 were determined by interpolation from the standard curve. The detection limits of the assays were 1.67 pg/mL (CXCL10) and 1.5 pg/mL (CXCL11). The inter-assay and intra-assay coefficients of variation were less than 9.8% for CXCL10 and less than 16.8% for CXCL11.

2.8 | Statistical analysis

Statistical analyses were conducted using SPSS software version 19.0 (IBM Corporation, Armonk, NY, USA). For patient demographics, the Mann-Whitney U test was performed for continuous variables and Fisher's exact test for nominal variables. The Mann–Whitney U test was also performed when comparing cell numbers and cytokine/chemokine concentrations between the study groups. A *P*-value <.05 was considered statistically significant.

3 | RESULTS

3.1 | Characteristics of the study population

The demographic and clinical characteristics of the study population are shown in Table 1. A total of 26 amniotic fluid samples were collected from women who underwent spontaneous preterm labor and birth either with intra-amniotic infection (n = 10) or with intra-amniotic inflammation (n = 16). Amniotic fluid concentrations of IL-6 and white blood cell counts were higher in women with preterm labor and intra-amniotic infection compared to those with intra-amniotic inflammation (Table 1). Glucose concentrations tended to be lower in women with intra-amniotic infection compared to those with intra-amniotic inflammation (Table 1). Women with intra-amniotic inflammation and those with intra-amniotic infection presented acute maternal and fetal inflammatory responses in the placenta (Table 1). The following microorganisms were detected in women diagnosed with intra-amniotic infection: *Ureaplasma urealyticum*, *Mycoplasma hominis*, *Fusobacterium* spp., *Candida* spp., *Gardnerella vaginalis*, *Peptostreptococcus*, *Streptococcus* serogroup C, and *Enterococcus faecalis*.

3.2 | Leukocyte populations in amniotic fluid of women with preterm labor and intra-amniotic inflammation or intra-amniotic infection

Figure 1A,C shows representative images of the flow cytometry gating strategy used to detect leukocytes in amniotic fluid from women with preterm labor and intra-amniotic inflammation (Figure 1A) or intra-amniotic infection (Figure 1C). Representative t-SNE plots illustrate the amniotic fluid leukocyte populations found in the two study groups (Figure 1B,D). Notably, more women with preterm labor and intra-amniotic infection (80%) displayed a high proportion of neutrophils and monocytes/macrophages in amniotic fluid compared to those with intra-amniotic inflammation without detectable microorganisms (50%; Figure 1D vs 1B). Indeed, the abundant neutrophils and monocytes/macrophages in amniotic fluid of women with intra-amniotic infection masked the other immune cell types (eg, T cells and B cells) that are clearly identified in women with intra-amniotic inflammation (Figure 1D vs 1B).

We then quantified the numbers of total leukocytes in amniotic fluid from the two study groups. Women with preterm labor and intra-amniotic infection had greater total numbers of leukocytes in amniotic fluid compared to those with intra-amniotic inflammation (Figure 2A). Quantification of neutrophils ($CD15^+$ leukocytes) and monocytes/macrophages ($CD14^+$ leukocytes) in amniotic fluid showed that the numbers of these innate immune cells were greater in women with intra-amniotic infection compared to those with intra-amniotic inflammation (Figure 2B,C), mirroring the relative differences observed between the t-SNE plots in Figure 1.

We previously demonstrated that adaptive immune cells (ie, T cells and B cells) are also present in amniotic fluid during normal

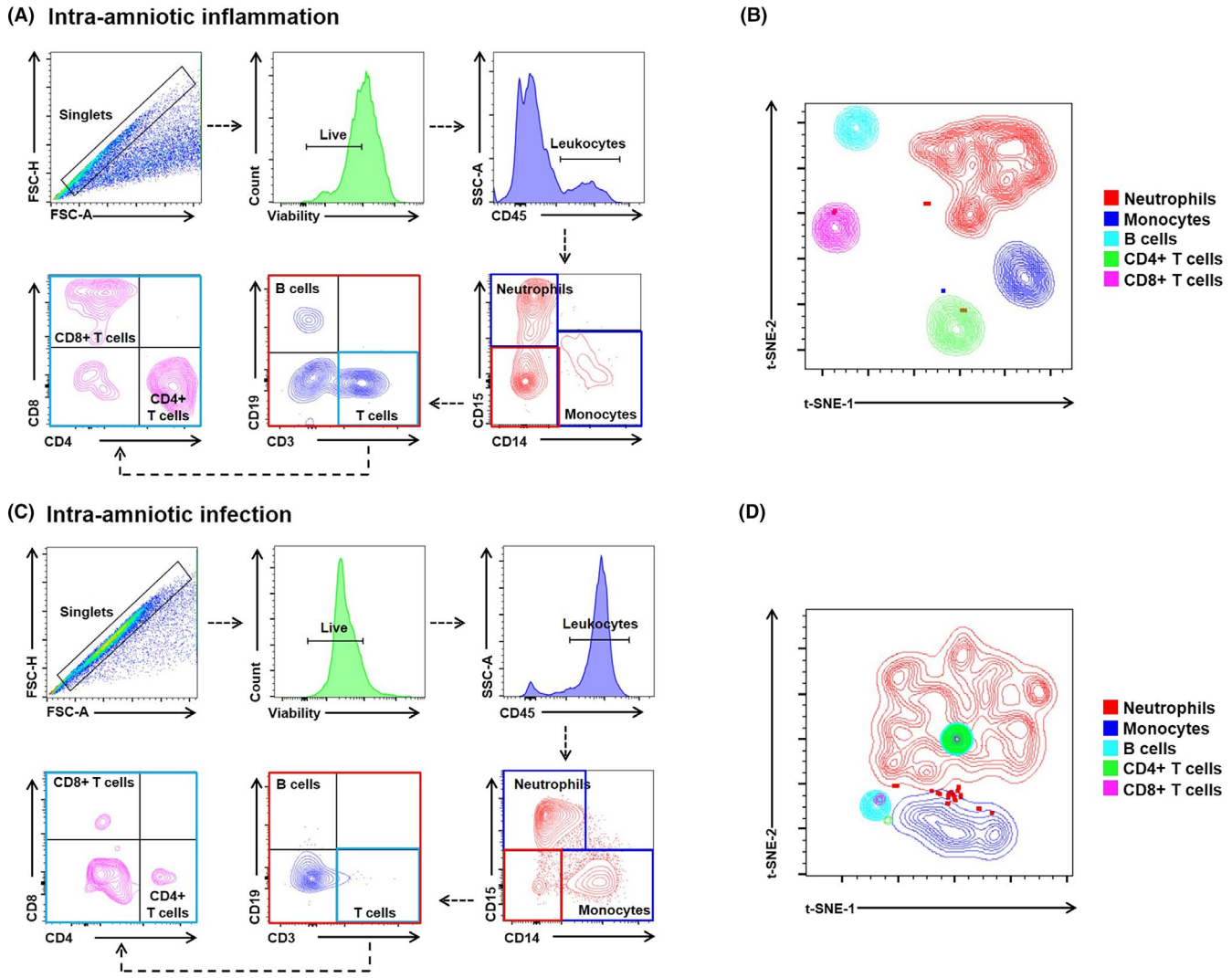

FIGURE 1 Leukocyte populations in amniotic fluid. Representative flow cytometry gating strategies and t-distributed stochastic neighbor embedding (t-SNE) plots showing leukocyte populations in amniotic fluid from women who underwent spontaneous preterm labor and birth with intra-amniotic inflammation (A-B) or with intra-amniotic infection (C-D). Immune cells were initially gated within the viability gate and $CD45^+$ gate, followed by lineage gating for neutrophils ($CD45^+$ $CD15^+$ cells), monocytes/macrophages ($CD45^+$ $CD14^+$ cells), T cells ($CD45^+$ $CD3^+$ cells), which were subsequently gated for $CD4^+$ T cells ($CD45^+$ $CD3^+$ $CD4^+$ cells) and $CD8^+$ T cells ($CD45^+$ $CD3^+$ $CD8^+$ cells), and B cells ($CD45^+$ $CD19^+$ cells). Plots are representative of 10-16 samples per group

pregnancy.[55] Therefore, we then determined whether the numbers of such cells were altered in amniotic fluid from our two study groups. We found that the total T-cell population ($CD3^+$ leukocytes) and $CD4^+$ T cells were significantly increased in women with preterm labor and intra-amniotic infection compared to those with intra-amniotic inflammation (Figure 3A,B). The numbers of $CD8^+$ T cells were also higher in women with preterm labor and intra-amniotic infection compared to those with intra-amniotic inflammation, although this was not significant (Figure 3C). Lastly, we determined the numbers of B cells ($CD19^+$ leukocytes) and found no statistical differences between the two study groups (Figure 3D).

Together, these results indicate that total leukocytes, as well as specific leukocyte subsets, namely neutrophils, monocytes/macrophages, and $CD4^+$ T cells, are increased in amniotic fluid of women with preterm labor and intra-amniotic infection compared to those with intra-amniotic inflammation.

3.3 | Cytokine and chemokine concentrations in amniotic fluid of women with preterm labor and intra-amniotic inflammation or intra-amniotic infection

Next, we investigated whether the increased numbers of amniotic fluid leukocytes in women with preterm labor and intra-amniotic infection were associated with an increase in cytokine concentrations. We found that the concentrations of the pro-inflammatory cytokines IL-6 and IL-1β were both significantly elevated in amniotic fluid of women with preterm labor and intra-amniotic infection compared to those with intra-amniotic inflammation (Figure 4A,B). In contrast,

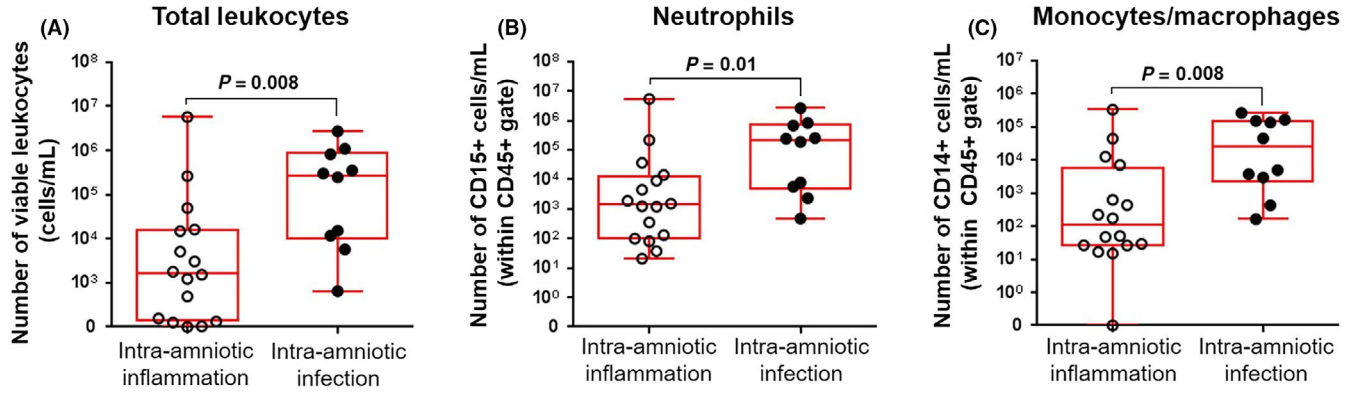

FIGURE 2 Total leukocytes and innate immune cells in amniotic fluid. Numbers of (A) total leukocytes ($CD45^+$ cells/mL), (B) neutrophils ($CD15^+$ cells/mL), and (C) monocytes/macrophages ($CD14^+$ cells/mL) in amniotic fluid from women who underwent spontaneous preterm labor and birth with intra-amniotic inflammation or with intra-amniotic infection. N = 10-16 per group. Midlines = medians, boxes = interquartile ranges, and whiskers = minimum/maximum ranges

concentrations of IL-8 and IL-12p70 were not significantly different between women with preterm labor and intra-amniotic infection and those with intra-amniotic inflammation (Figure 4C,D).

As the numbers of amniotic fluid total T cells and $CD4^+$ T cells were increased in women with intra-amniotic infection, the concentrations of type 1 and type 2 cytokines, as well as T-cell chemokines, were determined. The type 1 cytokines IL-2, IFN-γ, and TNF-α were not significantly different between women with intra-amniotic infection and those with intra-amniotic inflammation (Figure 5A-C). While IL-4 and IL-13 did not change, the amniotic fluid concentration of the type 2 cytokine IL-10 tended to increase in women with intra-amniotic infection compared to those with intra-amniotic inflammation (Figure 5D-F). No differences were observed in amniotic fluid concentrations of the T-cell chemokines CXCL10 and CXCL11 between the study groups (Figure 5G,H).

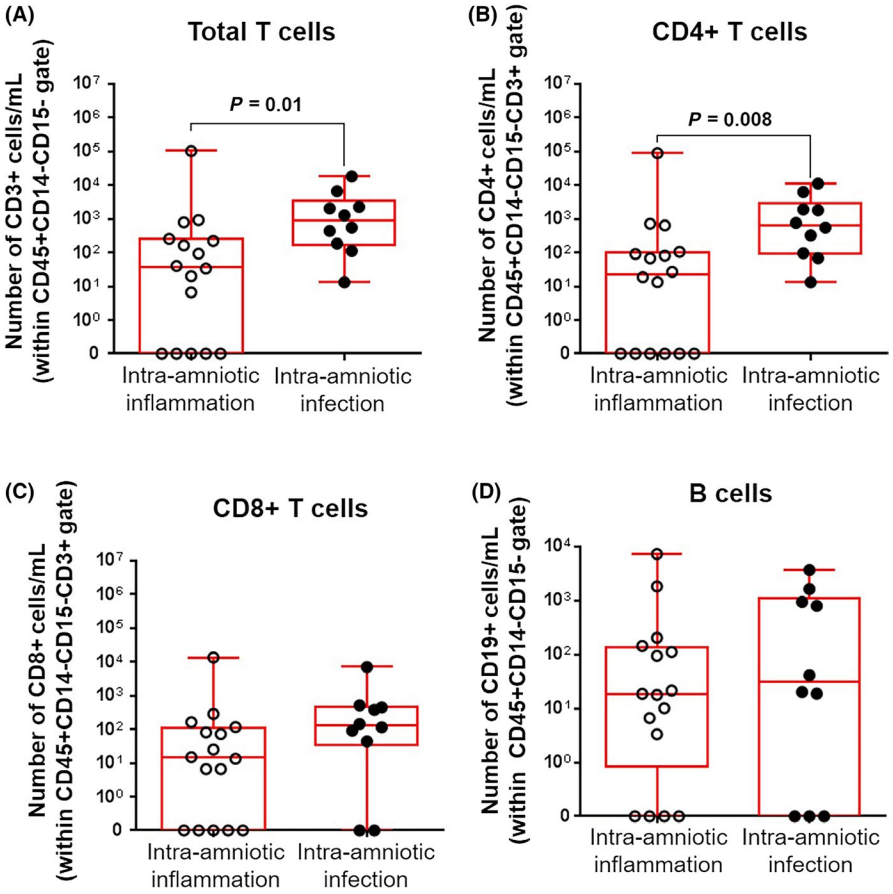

FIGURE 3 Adaptive immune cells in amniotic fluid. Numbers of (A) total T cells ($CD3^+$ cells/mL), (B) $CD4^+$ T cells ($CD3^+$ $CD4^+$ cells/mL), (C) $CD8^+$ T cells ($CD3^+$ $CD8^+$ cells/mL), and (D) B cells ($CD19^+$ cells/mL) in amniotic fluid from women who underwent spontaneous preterm labor and birth with intra-amniotic inflammation or with intra-amniotic infection. N = 10-16 per group. Midlines = medians, boxes = interquartile ranges, and whiskers = minimum/maximum ranges

4 | DISCUSSION

4.1 | Principal findings

Herein, we report that women with spontaneous preterm labor and intra-amniotic infection had (a) a greater number of total leukocytes including neutrophils and monocytes/macrophages in amniotic fluid; (b) a higher number of total T cells and $CD4^+$ T cells, but not $CD8^+$ T cells or B cells, in amniotic fluid; and (c) increased amniotic fluid concentrations of IL-6, IL-1β, and IL-10, compared to those with intra-amniotic inflammation. However, no differences in amniotic fluid concentrations of T-cell cytokines and chemokines were observed between these two clinical conditions. Collectively, these results indicate that the cellular immune responses observed in women with preterm labor and intra-amniotic infection are more severe than in those with intra-amniotic inflammation and are characterized by an increased number of neutrophils, monocytes/macrophages, and $CD4^+$ T cells.

4.2 | Amniotic fluid neutrophils in women with preterm labor and intra-amniotic infection or intra-amniotic inflammation

It is well known that neutrophils are the most abundant immune cell type in the amniotic cavity of women with intra-amniotic infection and/or intra-amniotic inflammation.[54,78,81,82] Yet, whether the number of amniotic fluid neutrophils differs between intra-amniotic inflammatory processes with and without culturable microorganisms has not been shown until now. Herein, we showed that the number of amniotic fluid neutrophils is higher in women with preterm labor and intra-amniotic infection than in those with intra-amniotic inflammation without culturable microorganisms, indicating that different thresholds in the number of these immune cells may allow for the differentiation of these two clinical conditions.

In women with preterm labor and intra-amniotic infection, as well as other pathogen-mediated immune responses,[83,84] neutrophils participate in the main mechanisms of microbial killing: degranulation, phagocytosis, and neutrophil extracellular trap (NET) formation. For example, amniotic fluid neutrophils can produce reactive oxygen species[85] and release antimicrobial molecules such as alpha-defensins,[86-89] myeloperoxidase,[29,89,90] cathepsin G,[89,91] elastase,[89,92,93] lactoferrin,[94] pentraxin-3,[95] and cathelicidin,[29,89] all of which are found in the intra-amniotic space. Amniotic fluid neutrophils of fetal or maternal origin[82] can also actively participate in killing microbes invading the amniotic cavity by performing phagocytosis[96] and forming NETs.[97] Indeed, NETs are also formed by maternal neutrophils invading the amniotic cavity[97] and chorioamniotic membranes[98,99] in cases with intra-amniotic infection. In addition to killing microbes, amniotic fluid neutrophils can release pro-inflammatory cytokines such as IL-8, TNF-α, MIP-1α, MIP-1β, IL-1α, and IL-1β into the intra-amniotic space in cases with MIAC and clinical chorioamnionitis at term.[54] These cytokines have been implicated in the pathogenesis of preterm labor in the context of intra-amniotic infection.[38,39,43,81,100-109] Specifically, IL-1β is a central mediator in the pathogenesis of preterm labor because the systemic[110,111] and intra-amniotic[11,112-118] administration of this cytokine in pregnant animals induces preterm birth. The mechanisms whereby IL-1β induces preterm labor and birth, in the context of intra-amniotic infection, involve the nucleotide-binding oligomerization domain, leucine-rich repeat, and pyrin domain containing 3 (NLRP3) inflammasome, an intracellular multiprotein complex that can be activated in the fetal membranes by microbial products in mice (eg, lipopolysaccharide; LPS).[119] Consistently, women with preterm labor and acute histologic chorioamnionitis (a placental lesion associated with intra-amniotic infection[120-122]) also display inflammasome activation in the amniotic fluid[34] and chorioamniotic membranes.[123] Together, these data indicate that amniotic fluid neutrophils participate in both the host defense and inflammatory mechanisms implicated in the pathogenesis of preterm labor and birth in women with intra-amniotic infection.

Women with intra-amniotic inflammation also had neutrophils in their amniotic fluid; however, the numbers were lower than in those with intra-amniotic infection. This finding shows that the mechanisms of inflammation occurring in women without culturable microorganisms in amniotic fluid are distinct from and less severe than in those with intra-amniotic infection. One possibility is that a subset

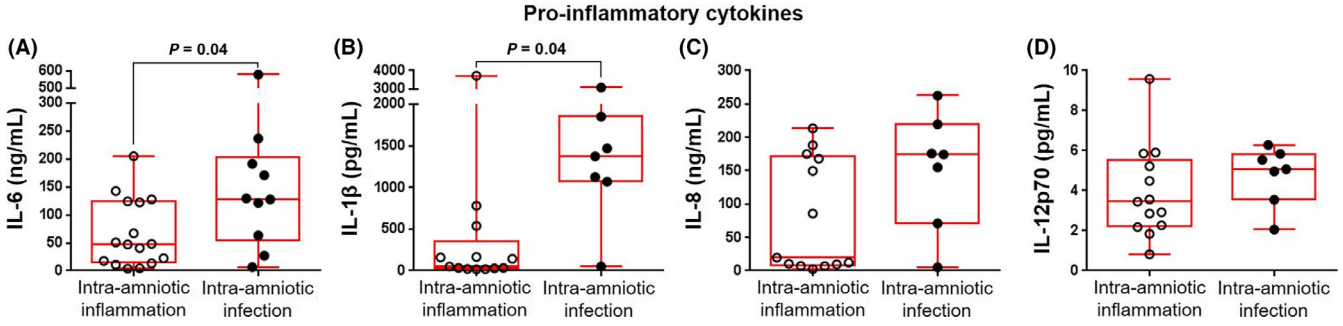

FIGURE 4 Pro-inflammatory cytokines in amniotic fluid. Concentrations of (A) IL-6 (ng/mL), (B) IL-1β (pg/mL), (C) IL-8 (ng/mL), and (D) IL-12p70 (pg/mL) in amniotic fluid from women who underwent spontaneous preterm labor and birth with intra-amniotic inflammation or with intra-amniotic infection. N = 7-16 per group. Midlines = medians, boxes = interquartile ranges, and whiskers = minimum/maximum ranges

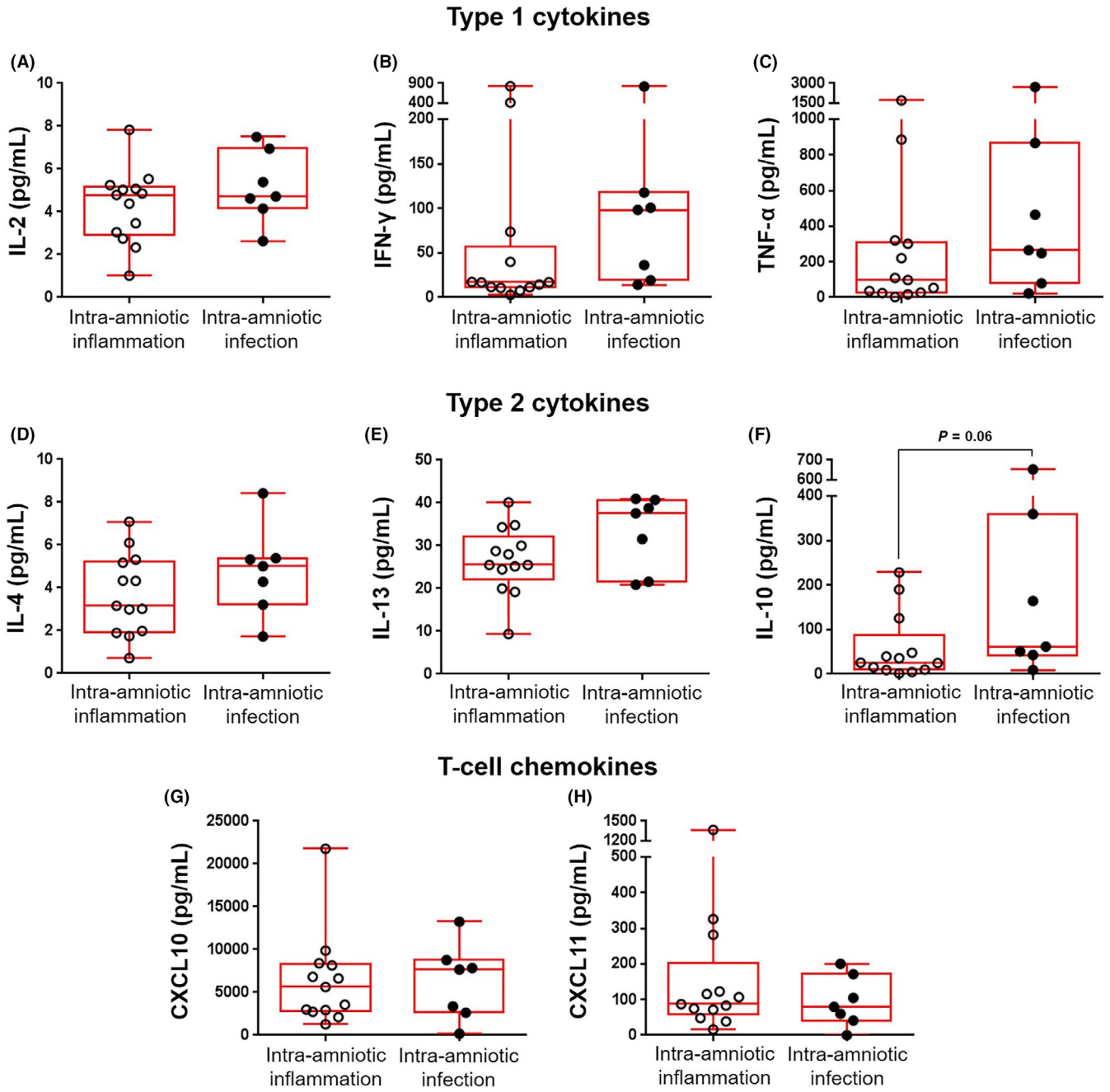

FIGURE 5 Type 1 and type 2 cytokines and T-cell chemokines in amniotic fluid. Concentrations of the type 1 cytokines (A) IL-2 (pg/mL), (B) IFN-γ (pg/mL), and (C) TNF-α (pg/mL); type 2 cytokines (D) IL-4 (pg/mL), (E) IL-13 (pg/mL), and (F) IL-10 (pg/mL); and T-cell chemokines (G) CXCL10 (pg/mL) and (H) CXCL11 (pg/mL) in amniotic fluid from women who underwent spontaneous preterm labor and birth with intra-amniotic inflammation or with intra-amniotic infection. N = 7-13 per group. Midlines = medians, boxes = interquartile ranges, and whiskers = minimum/maximum ranges

of women who underwent preterm labor with intra-amniotic inflammation had elevated amniotic fluid concentrations of alarmins[43] (eg, IL-1α,[39] HMGB1,[42,124] HSP70,[41] and S100B[40]), referred to as sterile intra-amniotic inflammation.[32-34] Human studies have provided evidence that preterm labor with sterile intra-amniotic inflammation is less severe than preterm labor with microbial-induced intra-amniotic inflammation.[34,43,125] Indeed, only ~50% of pregnant mice intra-amniotically injected with physiologically relevant concentrations of alarmins undergo preterm birth,[126,127] whereas almost all of those injected with a microbial product (LPS) deliver preterm.[128,129] The mechanisms whereby alarmins induced preterm labor and birth also involved the NLRP3 inflammasome;[127,130] yet, these will be discussed below as sterile inflammation is mainly mediated by monocytes/macrophages.[131,132] Taken together, these data consistently show that women with preterm labor and intra-amniotic inflammation without culturable microorganisms had a milder intra-amniotic

inflammatory response, including a lower number of amniotic fluid neutrophils, than those with intra-amniotic infection.

It is worth mentioning that women with preterm labor and intra-amniotic inflammation may have been infected by non-culturable microorganisms. Microorganisms such as *Sneathia* spp.,[31,33,133] *Neisseria* spp.,[33,133] and *Fusobacterium nucleatum*[31,134] have proven difficult to culture from amniotic fluid using traditional clinical methods. However, whether such non-culturable microorganisms can lead to a stronger intra-amniotic inflammatory response than that mediated by alarmins requires further investigation.

4.3 | Amniotic fluid monocytes/macrophages in women with preterm labor and intra-amniotic infection or intra-amniotic inflammation

We also demonstrated that the number of monocytes/macrophages is increased in women with preterm labor and intra-amniotic infection compared to those with intra-amniotic inflammation. Monocytes/macrophages are commonly found together with neutrophils in amniotic fluid of women with intra-amniotic infection and/or inflammation.[54,78] As neutrophils typically represent the dominant immune cell population in such women,[54,78] the functions of monocytes/macrophages in the context of intra-amniotic infection or inflammation have been less investigated.

Monocytes are chemo-attracted to sites of inflammation, where they attain one of several different activation states depending on the microenvironment.[135] Stimulation of innate sensors such as Toll-like receptors through detection of bacterial products activates the production of reactive oxygen species and pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-12 by monocytes,[135] which are mediators found in amniotic fluid of women with preterm labor and intra-amniotic infection.[38,39,54,104-106,136] A recent study demonstrated that placental macrophages can respond to microbes by releasing extracellular traps (METs),[137] suggesting that monocytes/macrophages in the amniotic cavity may have other functions in addition to cytokine release. Yet, the question of whether amniotic fluid monocytes/macrophages are predominantly of maternal or fetal origin, especially in the context of intra-amniotic infection, remains unanswered.

Monocytes/macrophages were present, albeit in lesser numbers, in amniotic fluid of women with preterm labor and intra-amniotic inflammation, providing further confirmation that the intra-amniotic inflammatory process is less severe in the absence of culturable microorganisms.[34,43,125] In tissues, resident macrophages act as sentinels, orchestrating the clearance of damaged cells to maintain homeostasis.[138] This sentinel-like function relies, in part, on the variety of pattern recognition receptors (eg, Toll-like receptors) and cytosolic receptors (eg, NLRP3) expressed by macrophages.[139-141] Macrophages, therefore, are considered to be the first to detect danger signals or alarmins released by damaged cells.[132] This concept was demonstrated by a murine study showing that sterile inflammation in response to cell death is driven by macrophages through the release of IL-1α and IL-1β.[131] Moreover, neutrophilic influx to the site of injury was dependent on macrophage-released cytokines, as deficient mice lacked such infiltration.[131] These findings provide a model in which macrophages are the initiators of sterile inflammation that is followed by neutrophil recruitment.[132] Similarly, amniotic fluid monocytes/macrophages may be acting as sentinels, responding to alarmins released upon cellular senescence of placental/fetal tissues[142] which, in turn, will induce pro-inflammatory immune responses and recruit more immune cells (eg, neutrophils) into the amniotic cavity (ie, sterile intra-amniotic inflammation), inducing preterm labor and birth. Yet, further research is required to test this hypothesis. In support of this concept, it has been suggested that fetal macrophages may initiate parturition.[143,144]

The molecular mechanisms of sterile inflammation in the amniotic cavity[32-34] are thought to involve the NLRP3 inflammasome.[127,130] Inflammasome complexes assemble to provide a scaffold for activation of caspase-1,[145-163] which, in turn, cleaves pro-IL-1β and pro-IL-18 into their mature and active forms.[164-171] Inflammasome activation can result in an inflammatory type of cell death, referred to as pyroptosis,[172-175] in which the molecule gasdermin D forms pores in the host cell membrane, [173,176-179] allowing for the release of cytosolic proteins such as IL-1β.[166,169] Pyroptosis was originally described in macrophages,[172,173,180] and we recently demonstrated that the effector molecule of pyroptosis, gasdermin D, is present in amniotic fluid of women with preterm labor and sterile intra-amniotic inflammation.[125] Herein, we propose that amniotic fluid monocytes/macrophages undergo inflammasome-mediated pyroptosis, a potential mechanism for sterile intra-amniotic inflammation in women with preterm labor.

Collectively, these findings indicate that amniotic fluid monocytes/macrophages play different roles in subsets of women with preterm labor: While cytokine release and MET formation are central to intra-amniotic infection, inflammasome-mediated pyroptosis occurs in the setting of sterile intra-amniotic inflammation.

4.4 | Amniotic fluid $CD4^+$ T cells in women with preterm labor and intra-amniotic infection or intra-amniotic inflammation

In the current study, we found that $CD4^+$ T cells, but not $CD8^+$ T cells, are increased in women with preterm labor and intra-amniotic infection compared to those with intra-amniotic inflammation. This is consistent with our previous report showing that T cells are one of the dominant immune cell populations present in the amniotic fluid of women in preterm gestation.[55] These adaptive cells are likely derived from the fetus, as amniotic fluid neutrophils in preterm gestation are predominantly of fetal origin; [82] however, their origin has yet to be elucidated. Our findings are in line with previous reports showing that fetal immune activation occurs in preterm labor[181,182] and that a population of central memory $CD4^+$ T cells is increased in the cord blood of preterm neonates born to women with preterm labor.[183] Such a fetal T-cell response could be initiated by *in utero* exposure to pathogens[184-186] and/or maternal antigens.[183,187] The mechanisms whereby fetal T cells could initiate

preterm parturition involve the secretion of pro-inflammatory mediators, such as IFN-γ and TNF-α, and the activation of myometrial contractility.[183] Whether fetal T-cell activation is implicated in the mechanisms leading to preterm labor and birth in the absence of intra-amniotic infection/inflammation is still unknown.

5 | CONCLUSION

In the current study, we report that women with spontaneous preterm labor and intra-amniotic infection had increased numbers of amniotic fluid neutrophils, monocytes/macrophages, and $CD4^+$ T cells compared to those with intra-amniotic inflammation. Such cellular immune responses were accompanied by elevated amniotic fluid concentrations of IL-6, IL-1β, and IL-10. These results provide evidence that the cellular immune responses observed in women with preterm labor and intra-amniotic infection are more severe than in those with intra-amniotic inflammation, and that neutrophils, monocytes/macrophages, and $CD4^+$ T cells are the main immune cells responding to microorganisms invading the amniotic cavity.

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CONFLICT OF INTEREST

The authors report no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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