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Inflammasome activation during spontaneous preterm labor with intra-amniotic infection or sterile intra-amniotic inflammation

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Problem: The inflammasome is implicated in the mechanisms that lead to spontaneous preterm labor (PTL). However, whether there is inflammasome activation in the amniotic cavity of women with PTL and intra-amniotic infection (IAI) or sterile intra-amniotic inflammation (SIAI) is unknown.

Method of study: Amniotic fluid samples were collected from women with PTL who delivered at term (n = 31) or preterm without IAI or SIAI (n = 35), with SIAI (n = 27), or with IAI (n = 17). As a readout of inflammasome activation, extracellular ASC (apoptosis-associated speck-like protein containing a CARD) was measured in amniotic fluid by ELISA and the expression of ASC, caspase-1, and interleukin (IL)-1β was detected in the chorioamniotic membranes by multiplex immunofluorescence. Acute inflammatory responses in amniotic fluid and the placenta were also evaluated.

Results: (a) Amniotic fluid concentrations of ASC and IL-6 were higher in women with PTL and IAI or SIAI than in those who delivered preterm or at term without intra-amniotic inflammation; (b) amniotic fluid concentrations of ASC and IL-6 were lower in women with PTL and SIAI than in those with IAI; (c) there was a significant nonlinear correlation between ASC and IL-6 amniotic fluid concentrations; (d) the expression of inflammasome-related proteins (ASC, caspase-1, and IL-1β) in the chorioamniotic membranes was increased in women with PTL and IAI or SIAI than in those who delivered preterm or at term without intra-amniotic inflammation; (e) inflammasome activation in the chorioamniotic membranes was weaker in women with

PTL and SIAI than in those with IAI; (f) women with PTL and IAI had elevated amniotic fluid white blood cell counts compared to those without this clinical condition; and (g) severe acute placental inflammatory lesions were observed in women with PTL and IAI and in a subset of women with PTL and SIAI.

Conclusion: Inflammasome activation occurs in the settings of intra-amniotic infection and sterile intra-amniotic inflammation during spontaneous preterm labor.

1 | INTRODUCTION

Preterm birth is a leading cause of perinatal morbidity and mortality worldwide,[1-3] which is commonly preceded by spontaneous preterm labor.[4-8] Among the known etiologies, intra-amniotic infection/inflammation is the most studied causal link to spontaneous preterm labor.[9-11] Intra-amniotic inflammation can be initiated as a result of microbial invasion of the amniotic cavity (ie, intra-amniotic infection or IAI) or by damage-associated molecular patterns (DAMPs) or alarmins (ie, sterile intra-amniotic inflammation or SIAI).[12-21] Sterile intra-amniotic inflammation is an inflammatory process in which microorganisms cannot be detected using both cultivation and molecular microbiology techniques.[12-21] This clinical condition is frequently observed in women: (a) with preterm labor and intact membranes,[13] (b) with an asymptomatic short cervix,[14] (c) with preterm prelabor rupture of membranes,[15] and (d) with clinical chorioamnionitis at term.[16] Given that sterile inflammation is induced by alarmins[22-24] and that such molecules are increased in the amniotic fluid of women who deliver preterm, we have proposed and shown that alarmins can initiate the mechanisms that lead to spontaneous preterm labor.[25-32]

The mechanisms that lead to spontaneous preterm labor in the context of IAI or SIAI are thought to involve the inflammasome.[32-39] There are several types of inflammasomes that are named based on their sensor molecule.[40-43] Nucleotide-binding domain-like receptor (NLR) inflammasomes are cytoplasmic multiprotein complexes composed of (a) the sensor molecule (eg, NLR family pyrin domain-containing protein 3 or NLRP3), (b) the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC) or PYD and CARD domain-containing protein (PYCARD), and (c) pro-caspase-1.[44-58] Once activated, the assembled inflammasome complex induces the autocatalytic cleavage of pro-caspase-1 into its active form which, in turn, can cleave the inflammatory cytokines pro-interleukin (IL)-1β and pro-IL-18 into their mature and secreted bioactive forms,[59-69] inducing a specific form of inflammatory cell death termed pyroptosis.[70-72] During inflammasome activation, the ASC adaptor protein assembles into a large, microscopically visible intracellular complex (commonly referred to as a "speck") that consists of multimers of ASC dimers.[73,74] ASC specks can serve as danger signals through release into the extracellular space, where they can amplify the inflammatory response.[75,76] Therefore, the detection of ASC specks and/or their extracellular release provides a readout of *in vivo* inflammasome activation.[77] Recently, we provided evidence showing that, in the context of IAI or SIAI, there is inflammasome activation in the chorioamniotic membranes of women who deliver at term[78-80] or preterm.[81] In addition, inflammasome activation in the amniotic cavity was demonstrated by detecting elevated concentrations of extracellular ASC in women who underwent spontaneous labor at term.[82] However, whether there is inflammasome activation in the amniotic cavity of women with spontaneous preterm labor in the context of IAI or SIAI is unknown.

Although both IAI and SIAI are associated with adverse pregnancy and neonatal outcomes,[13,83] there is evidence that the intra-amniotic inflammatory responses are different between these two clinical conditions.[84] Therefore, besides determining inflammasome activation in amniotic fluid, the acute inflammatory responses in the amniotic cavity and placenta were evaluated in women with spontaneous preterm labor with IAI or SIAI.

2 | MATERIALS AND METHODS

2.1 | Study design and population

This was a retrospective cross-sectional study 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 110 amniotic fluid samples collected from women classified into the following groups (Table 1): (a) women who presented with signs of spontaneous preterm labor but delivered at term with a negative amniotic fluid culture and an IL-6 concentration <2.6 ng/mL (n = 31); (b) women with spontaneous preterm labor who delivered preterm without IAI or SIAI (n = 35); (c) women with spontaneous preterm labor who delivered preterm with SIAI (n = 27); and (d) women with spontaneous preterm labor who delivered preterm with IAI (n = 17) (see diagnostic criteria below).

TABLE 1 Clinical and demographic characteristics of the study population

		Preterm Delivery			
	Preterm labor which delivered at term (n = 31)	Preterm labor without sterile intra-amniotic inflammation or infection (n = 35)	Preterm labor with sterile intra-amniotic inflammation (n = 27)	Preterm labor with intra-amniotic infection (n = 17)	*P*-value
Maternal age (years)[a]	23 (20-25.5)	23 (19.5-25.5)	24 (20.5-27)	23 (20-26)	0.7
Prepregnancy body mass index (kg/m^2)[a]	21.6 (19.8-29.3)[d]	23.5 (20.7-27.8)[e]	28.2 (23.2-33.4)[e]	24.4 (21.7-31.9)[d]	0.05
Race[b]					0.6
African American	96.8 (30/31)	82.9 (29/35)	88.9 (24/27)	94.1 (16/17)	
Caucasian	0 (0/31)	8.6 (3/35)	7.4 (2/27)	5.9 (1/17)	
Hispanic	0 (0/31)	5.7 (2/35)	0 (0/27)	0 (0/17)	
Other	3.2 (1/31)	2.9 (1/35)	3.7 (1/27)	0 (0/17)	
Gestational age at amniocentesis (weeks)[a]	31.3 (30.6-32.7)	31.4 (28.5-32.4)	26.4 (23.8-30.2)	26.7 (22.6-31)	0.001
Delivery route[b]					0.1
Vaginal	96.8 (30/31)	91.2 (31/34)[c]	77.8 (21/27)	88.2 (15/17)	
Cesarean section	3.2 (1/31)	8.8 (3/34)[c]	22.2 (6/27)	11.8 (2/17)	
Gestational age at delivery (weeks)[a]	38.7 (37.4-39.4)	34.1 (32.4-35.9)	26.7 (24.5-31.3)	26.7 (22.6-31)	<0.001
Birthweight	3080 (2952.5-3362.5)	2277.5 (1631.3-2457.5)[c]	917 (592.5-1545)	1040 (471-1370)	<0.001

Data are given as median (interquartile range) and percentage (n/N).
[a]Kruskal-Wallis test with multiple comparisons.
[b]Fisher's exact test.
[c]One missing data.
[d]Two missing data.
[e]Three missing data.

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 with a gestational age between 20 and 36 (6/7) weeks. Microbial invasion of the amniotic cavity (MIAC) was defined as a positive amniotic fluid culture and/or a polymerase chain reaction with electrospray ionization mass spectrometry (PCR/ESI-MS) (Ibis® Technology—Athogen, Carlsbad, CA, USA) test result.[85-88] Intra-amniotic inflammation was defined as an amniotic fluid IL-6 concentration ≥2.6 ng/mL.[89-92] SIAI was defined as an amniotic fluid IL-6 concentration ≥2.6 ng/mL[89] without microorganisms detected by culture or PCR/ESI-MS.[12-21,93] IAI (or microbial-associated intra-amniotic inflammation) was defined as the presence of MIAC with intra-amniotic inflammation.[12-21,94,95]

2.3 | Amniotic fluid sample collection

Amniotic fluid samples were obtained by transabdominal amniocentesis under antiseptic conditions and monitored by ultrasound. Transabdominal amniocentesis was performed for the detection of intra-amniotic inflammation and/or infection. Samples of amniotic fluid were transported to the laboratory in a sterile capped syringe and centrifuged at 1,300×*g* for 10 min at 4°C, and the supernatant was stored at −80°C until use. A portion of this amniotic fluid was also transported to the clinical laboratory for culture of aerobic/anaerobic bacteria and genital mycoplasmas. The clinical tests also included the determination of amniotic fluid white blood cell count,[96] glucose concentration,[97] Gram stain,[98] and IL-6 concentration.[89]

2.4 | 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). 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.

2.5 | Determination of extracellular ASC in amniotic fluid

Concentrations of extracellular ASC in the amniotic fluid were determined by using a sensitive and specific enzyme-linked

immunosorbent assay (ELISA) kit obtained from LifeSpan Biosciences (Seattle, WA). This ELISA kit was initially validated in our laboratory prior to the execution of this study. Amniotic fluid concentrations of ASC were obtained by interpolation from the standard curve. The inter- and intra-assay coefficients of variation were 5.0% and 8.6%, respectively. The sensitivity of the assay was 0.131 ng/mL.

2.6 | Placental histopathological examination

Sampling of the placentas was conducted according to protocols established by the Perinatology Research Branch. Five-μm-thick sections of formalin-fixed, paraffin-embedded tissue specimens were cut and mounted on SuperFrost™ Plus microscope slides (Erie Scientific LLC, Portsmouth, NH). After deparaffinization, slides were rehydrated and stained with hematoxylin and eosin. A minimum of 5 full-thickness sections of chorionic plate, 3 sections of umbilical cord, and 3 chorioamniotic membrane rolls from each case were examined by placental pathologists who were blinded to the clinical histories and additional testing results. Acute inflammatory lesions of the placenta (maternal inflammatory response and fetal inflammatory response) were diagnosed according to established criteria, including staging and grading.[99-104]

2.7 | Multiplex immunofluorescence and phenoptics (ie, multispectral imaging)

Tissue sections (5-μm-thick) were prepared from the chorioamniotic membranes (amnion and choriodecidua) of women who underwent spontaneous preterm labor. Multiplex immunofluorescence staining was performed using the Opal 7 kit (Cat#NEL811001KT; PerkinElmer, Waltham, MA), according to the manufacturer's instructions. Prior to multiplex immunofluorescence staining, each analyte was individually optimized with single antibody staining combined with different fluorescent TSA® reagents (PerkinElmer). After deparaffinization, slides were placed in antigen retrieval (AR) buffer and boiled using a microwave oven. Following blocking to eliminate nonspecific binding, slides were incubated with antibodies against ASC (PYCARD) (Cat#AG-25B-0006-C100; AdipoGen, San Diego, CA), caspase-1 (CAT#MA5-16215; Invitrogen, Rockford, IL), or IL-1β (Cat#NBP1-19775; Novus Biologicals, Littleton, CO) at room temperature. The slides were then washed and incubated with Opal Polymer HRP Ms+Rb (Cat#ARH1001EA; PerkinElmer). Next, the slides were incubated with one of the following fluorescent TSA® reagents included in the Opal 7 kit to detect each antibody staining: Opal 520, Opal 570, or Opal 690 (dilution 1:100). After washing, the slides were counterstained with Spectral DAPI (Cat#FP1490; PerkinElmer) and mounted using ProLong Diamond Antifade Mountant (Life Technologies, Eugene, OR). Autofluorescence slides as well as slides stained with isotype (negative controls) were included. Multiplex staining was performed by consecutively staining slide-mounted tissues using the same antibody concentrations and conditions validated through singleplex staining. Each previous primary and secondary antibody was removed by boiling in AR buffer before the application of the next primary antibody. After multiplex staining, the slides were imaged using the Vectra Polaris Multispectral Imaging System (PerkinElmer) and images were analyzed using the InForm 2.4.1 image analysis software (PerkinElmer).

2.8 | Statistical Analysis

Statistical analysis was performed using the R statistical language and environment (www.r-project.org). Data were compared between groups using unpaired Wilcoxon tests, and *P*-values were adjusted across comparisons and the two analytes (IL-6 and ASC) to control the false discovery rate. Adjustment for gestational age at sampling was performed using a linear regression model. An adjusted *P*-value (ie, *q*-value) <0.05 was considered a significant result. The magnitude of differences was expressed as the difference in the means, after $\log_2$ transformation of the data, to obtain $\log_2$ fold changes in the concentrations. The correlation between ASC and IL-6 levels was assessed via Spearman correlation tests and was inspected using locally weighted scatter plot smoothing (LOESS).

3 | RESULTS

3.1 | Characteristics of the study population

The demographic and clinical characteristics of the study population are shown in Table 1. There were no differences in maternal age, body mass index, or race between the study groups (Table 1). The majority of women included in this study were African American (Table 1). Gestational age at amniocentesis and delivery was different among the study groups; therefore, statistical analysis included adjustments for gestational age at sampling (Table 1). Birthweights were also significantly different among the study groups (Table 1).

3.2 | ASC amniotic fluid concentrations in women with spontaneous preterm labor

Upon inflammasome activation, the ASC protein is released into the extracellular space.[75,76] As a readout of inflammasome activation, we determined whether extracellular ASC could be detected in amniotic fluid of women who underwent spontaneous preterm labor with IAI or SIAI. Amniotic fluid concentrations of ASC were significantly higher in women who underwent spontaneous preterm labor with IAI than in those who delivered preterm or at term without intra-amniotic inflammation [spontaneous preterm labor with IAI: median 365.6 ng/mL (IQR: 186.7-1160 ng/mL) *vs.* spontaneous preterm labor without intra-amniotic inflammation: median 12.8 ng/mL (IQR: 9.8-16.9 ng/mL); $P < 0.001$ or *vs.* spontaneous preterm labor who delivered at term: median 8.9 ng/mL (IQR: 7.5-11.8 ng/mL); $P < 0.001$] (Figure 1). Moreover, amniotic fluid concentrations of ASC were elevated in women who underwent spontaneous preterm labor with SIAI compared to those who delivered preterm or at term without intra-amniotic inflammation [spontaneous preterm labor with SIAI: median 50.6 ng/mL (IQR:

39.7-162.7 ng/mL) *vs.* spontaneous preterm labor without intra-amniotic inflammation: median 12.8 ng/mL (IQR: 9.8-16.9 ng/mL); $P < 0.001$ or vs spontaneous preterm labor who delivered at term: median 8.9 ng/mL (IQR: 7.5-11.8 ng/mL); $P < 0.001$] (Figure 1). However, women who underwent spontaneous preterm labor with IAI had higher amniotic fluid concentrations of ASC than those with SIAI [spontaneous preterm labor with IAI: median 365.6 ng/mL (IQR: 186.7-1160 ng/mL) vs spontaneous preterm labor with SIAI: median 50.6 ng/mL (IQR: 39.7-162.7 ng/mL); $P < 0.001$] (Figure 1). Women who underwent spontaneous preterm labor and delivered preterm without intra-amniotic inflammation tended to have greater amniotic fluid concentrations of ASC than those who delivered at term [spontaneous preterm labor without intra-amniotic inflammation: median 12.8 ng/mL (IQR: 9.8-16.9 ng/mL) *vs.* spontaneous preterm labor who delivered at term: median 8.9 ng/mL (IQR: 7.5-11.8 ng/mL); $P = 0.1$], but such an increase did not reach statistical significance (Figure 1).

3.3 | IL-6 amniotic fluid concentration in women with spontaneous preterm labor

In order to correlate the ASC amniotic fluid concentrations with the degree of intra-amniotic inflammation, amniotic fluid concentrations of IL-6 were determined as previously reported.[12-15,89,105] Women who underwent spontaneous preterm labor with IAI had higher amniotic fluid concentrations of IL-6 than those who delivered preterm or at term without intra-amniotic inflammation [spontaneous preterm labor with IAI: median 97 800 pg/mL (IQR 15 651-134 950 pg/mL) *vs.* spontaneous preterm labor without intra-amniotic inflammation: median 507 pg/mL (IQR: 187.5-934 pg/mL); $P < 0.001$ or *vs.* spontaneous preterm labor who delivered at term: median 322 pg/mL (IQR: 185-455 pg/mL); $P < 0.001$] (Figure 2). Women who underwent spontaneous preterm labor with SIAI also had increased amniotic fluid concentrations of IL-6 compared to those who delivered preterm or at term without intra-amniotic inflammation [spontaneous preterm labor with SIAI: median 11 247 pg/mL (IQR: 5303-23 354 pg/mL) *vs.* spontaneous preterm labor without intra-amniotic inflammation: median 507 pg/mL (IQR: 187.5-934 pg/mL); $P < 0.001$ or *vs.* spontaneous preterm labor who delivered at term: median 322 pg/mL (IQR: 185-455 pg/mL); $P < 0.001$] (Figure 2). Yet, women who underwent spontaneous preterm labor with IAI had higher amniotic fluid concentrations of IL-6 than those with SIAI [spontaneous preterm labor with IAI: median 97 800 pg/mL (IQR 15 651-134 950 pg/mL) *vs.* spontaneous preterm labor with SIAI: median 11 247 pg/mL (IQR

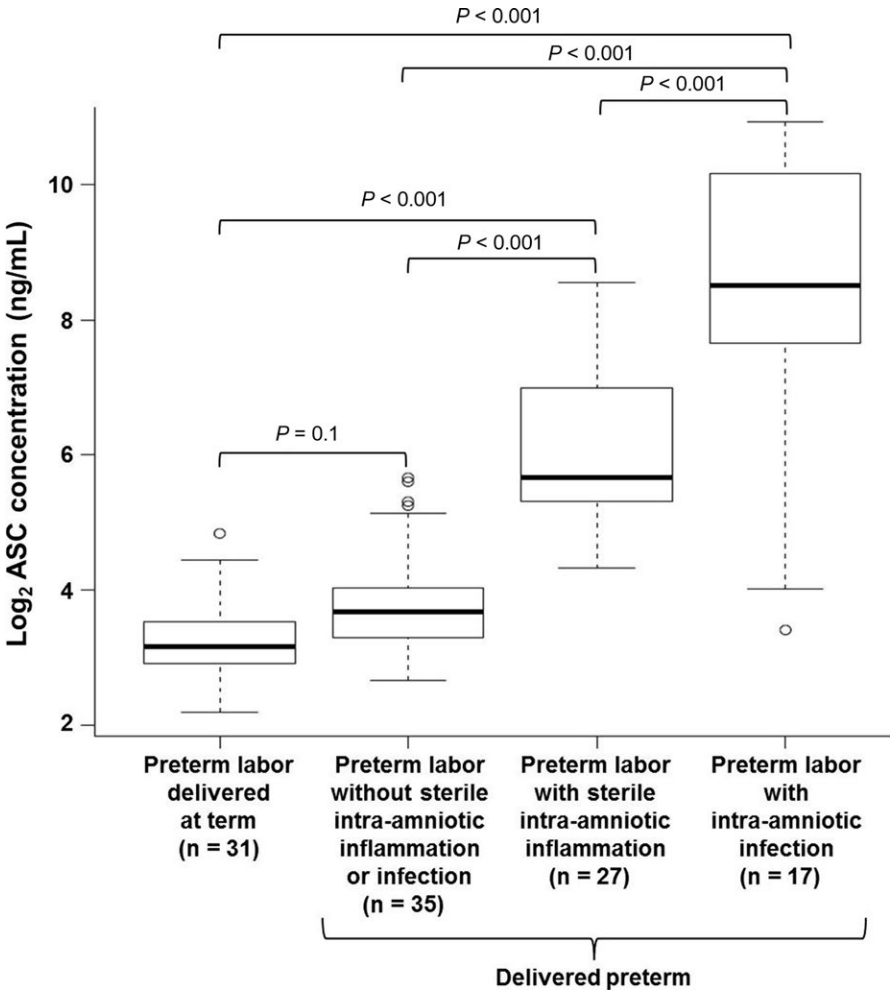

FIGURE 1 Amniotic fluid ASC concentrations in women who underwent spontaneous preterm labor. Extracellular ASC (ng/mL) was measured in amniotic fluid of women who underwent spontaneous preterm labor but delivered at term (n = 31) and those who delivered preterm without sterile intra-amniotic inflammation or intra-amniotic infection (n = 35), with sterile intra-amniotic inflammation (n = 27), or with intra-amniotic infection (n = 17). Data are shown as $\log_2$ concentration (ng/mL)

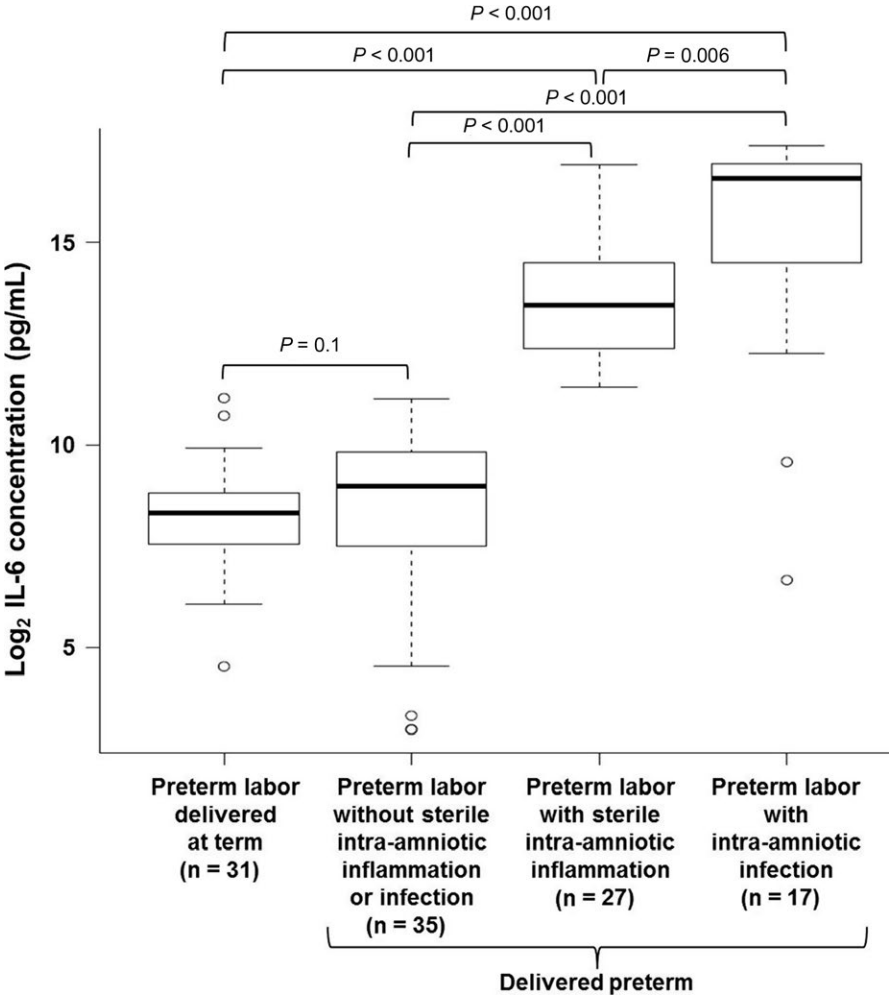

FIGURE 2 Amniotic fluid IL-6 concentrations in women who underwent spontaneous preterm labor. IL-6 (pg/mL) was measured in amniotic fluid of women who underwent spontaneous preterm labor but delivered at term (n = 31) and those who delivered preterm without sterile intra-amniotic inflammation or intra-amniotic infection (n = 35), with sterile intra-amniotic inflammation (n = 27), or with intra-amniotic infection (n = 17). Data are shown as $\log_2$ concentration (pg/mL)

5303-23 354 pg/mL); $P = 0.006$] (Figure 2). No differences were observed between women who underwent spontaneous preterm labor and delivered preterm without intra-amniotic inflammation and those who delivered at term (Figure 2).

3.4 | Correlation between ASC and IL-6 amniotic fluid concentrations

There was a significant nonlinear correlation between ASC and IL-6 amniotic fluid concentrations (Figure 3) (Spearman correlation 0.61, $P < 0.0001$). ASC amniotic fluid concentrations started to increase when IL-6 concentrations surpassed 1000 pg/mL (~10 units on the $\log_2$ scale in Figure 3). The nonlinear (quadratic) relation between $\log_2$ IL-6 and ASC amniotic fluid concentrations was significantly better than a linear fit (ANOVA $P < 0.001$).

3.5 | Are ASC amniotic fluid concentrations associated with inflammasome activation in the chorioamniotic membranes?

Given that ASC amniotic fluid concentrations were significantly higher in women with intra-amniotic inflammation regardless of the presence of microorganisms, we next evaluated whether both IAI and SIAI were associated with inflammasome activation in the chorioamniotic membranes. Multiplex immunofluorescence staining followed by phenoptics (ie, multispectral imaging) was performed in the chorioamniotic membranes from women who underwent spontaneous preterm labor to colocalize the expression of ASC, caspase-1, and IL-1β. Figures 4 and 5 include the detection of ASC, caspase-1, and IL-1β in the chorioamniotic membranes from women in the different study groups. In order to represent cellular components and layers of the chorioamniotic membranes, we show a cell map created by using the function of cell segmentation (left column in Figures 4 and 5). In each image of Figure 4, the amnion epithelium is at the top and the decidua is at the bottom. The expression of ASC, caspase-1, and IL-1β was observed in the chorioamniotic membranes of women with IAI or SIAI, being higher in those with IAI (Figure 4). A magnification of the amnion-chorion interface is shown in Figure 5, illustrating that the three proteins are elevated in tissues from women with IAI compared to those with SIAI. The expression of ASC and caspase-1 was also minimally detected in the chorioamniotic membranes from women who underwent spontaneous preterm labor and delivered preterm without intra-amniotic inflammation, but was absent in those who delivered at term (Figures 4, 5).

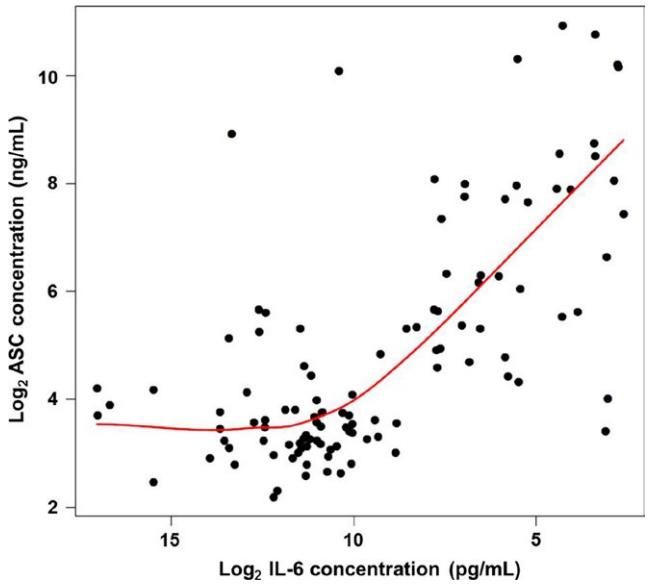

FIGURE 3 Correlation between ASC and IL-6 amniotic fluid concentrations in women who underwent spontaneous preterm labor. Data are shown as $\log_2$ concentration. The red line represents a locally weighted scatter plot smoothing (LOESS) estimating the average $\log_2$ ASC concentration as a function of $\log_2$ IL-6 concentration

3.6 | Acute inflammatory responses in the amniotic cavity and placenta of women with spontaneous preterm labor

In order to complement our observations in amniotic fluid, other indicators of intra-amniotic inflammation (eg, amniotic fluid white blood cell count and glucose concentration) were evaluated in our study population (Table 2). The number of white blood cells in amniotic fluid was higher in women who underwent spontaneous preterm labor with IAI compared to other groups (Table 2). Women who underwent spontaneous preterm labor with SIAI had a modest increase in the number of white blood cells found in amniotic fluid compared to those who delivered preterm without intra-amniotic inflammation and those who delivered at term (Table 2). As expected, women who underwent spontaneous preterm labor with IAI had a reduced amniotic fluid glucose concentration compared to other study groups (Table 2). Women who underwent spontaneous preterm labor with SIAI had comparable amniotic fluid glucose concentrations to those who delivered preterm or at term without intra-amniotic inflammation (Table 2).

Acute maternal and fetal inflammatory responses in the placenta were also evaluated among the study groups. Mild and moderate acute maternal (stages 1 and 2) and fetal (stage 1) inflammatory

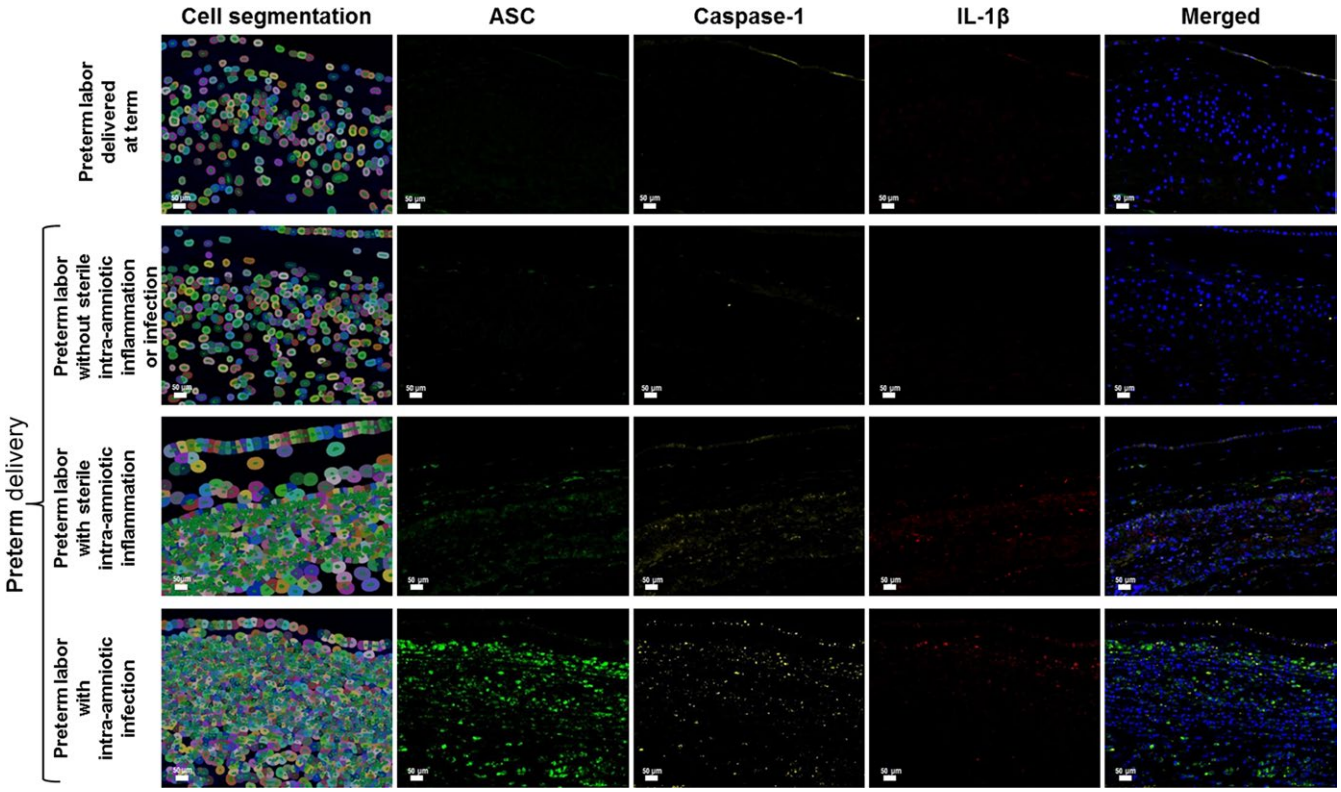

FIGURE 4 Expression and colocalization of inflammasome components in the chorioamniotic membranes of women who underwent spontaneous preterm labor. Multiplex immunofluorescence staining of ASC (green), caspase-1 (yellow), and IL-1β (red) was performed in the chorioamniotic membranes of women who underwent spontaneous preterm labor but delivered at term and those who delivered preterm without sterile intra-amniotic inflammation or intra-amniotic infection, with sterile intra-amniotic inflammation alone, or with intra-amniotic infection. Phenoptics was performed to generate cell segmentation images as well as separate and merged immunofluorescence images. Images are representative of 3 experiments per group. Images were taken at 400× magnification, and scale bars represent 50 μm

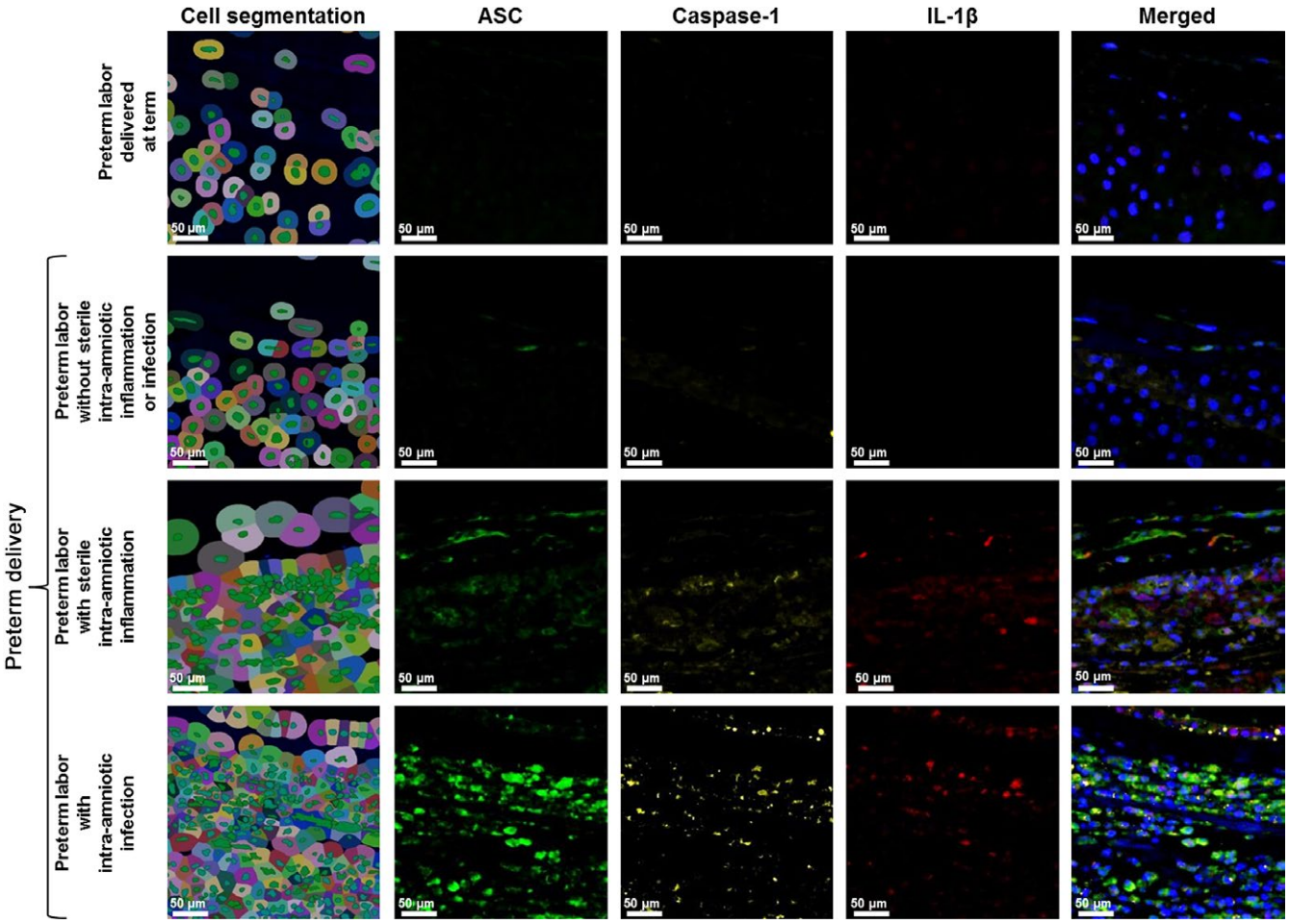

FIGURE 5 Magnified view of inflammasome component expression at the amnion-chorion interface of women who underwent spontaneous preterm labor. Multiplex immunofluorescence staining of ASC (green), caspase-1 (yellow), and IL-1β (red) was performed in the chorioamniotic membranes of women who underwent spontaneous preterm labor but delivered at term and those who delivered preterm without sterile intra-amniotic inflammation or intra-amniotic infection, with sterile intra-amniotic inflammation alone, or with intra-amniotic infection. Phenoptics was performed to generate cell segmentation images as well as separate and merged immunofluorescence images. Images are representative of 3 experiments per group. Images were taken at 400× magnification, and a close-up of the amnion-chorion interface is shown. Scale bars represent 50 μm

responses were similarly observed among the study groups (Table 2). However, severe acute maternal (stage 3) and fetal (stage 2) inflammatory responses were more frequently observed in women who underwent spontaneous preterm labor with IAI (Table 2). A subset of women who underwent spontaneous preterm labor with SIAI presented acute necrotizing chorioamnionitis (acute maternal inflammatory response stage 3); yet, this placental lesion was not as commonly observed as in women with IAI (Table 2). Women who underwent spontaneous preterm labor with SIAI presented comparable rates of acute arteritis (acute fetal inflammatory response stage 2) to those who delivered at term or preterm without IAI or SIAI (Table 2).

4 | DISCUSSION

4.1 | Principal findings

The principal findings of the study are as follows: (a) Amniotic fluid concentrations of ASC (extracellular ASC indicative of inflammasome activation) and IL-6 were higher in women who underwent spontaneous preterm labor with IAI or SIAI than in those who delivered preterm or at term without intra-amniotic inflammation; (b) amniotic fluid concentrations of ASC and IL-6 were lower in women with PTL and SIAI than in those with IAI; (c) there was a significant nonlinear correlation between ASC and IL-6 amniotic fluid concentrations; (d) the expression of inflammasome-related proteins (ASC, caspase-1, and IL-1β) in the chorioamniotic membranes was increased in women who underwent spontaneous preterm labor with IAI or SIAI than in those who delivered preterm or at term without intra-amniotic inflammation; (e) inflammasome activation in the chorioamniotic membranes was weaker in women who underwent spontaneous preterm labor with SIAI than in those with IAI; (f) women who underwent spontaneous preterm labor with IAI had elevated white blood cell counts and reduced glucose levels in amniotic fluid compared to the other 3 study groups; (g) women who underwent spontaneous preterm labor with SIAI had a modest increase in the number of white

TABLE 2 White blood cell count and glucose concentration in amniotic fluid and placental histopathology in the study population

		Preterm Delivery			
	Preterm labor which delivered at term (n = 31)	Preterm labor without sterile intra-amniotic inflammation or infection (n = 35)	Preterm labor with sterile intra-amniotic inflammation (n = 27)	Preterm labor with intra-amniotic infection (n = 17)	*P*-value
White blood cell count (cells/mm^3)[a]	0 (0-2.5)	1 (0-3)	2.5 (0.8-13.3)[e]	295 (23-420)	<0.001
Amniotic fluid glucose (mg/dL)[a]	30 (24-34.5)	29 (20.5-33)	21 (19-26)[d]	10 (1-17)[c]	<0.001
Acute maternal inflammatory response					
Stage 1 (acute subchorionitis)[b]	13.8% (4/29)[d]	12.9% (4/31)[f]	29.2% (7/24)[e]	12.5% (2/16)[c]	0.3
Stage 2 (acute chorioamnionitis)[b]	17.2% (5/29)[d]	16.1% (5/31)[f]	12.5% (3/24)[e]	18.8% (3/16)[c]	0.9
Stage 3 (acute necrotizing chorioamnionitis)[b]	0% (0/29)[d]	0% (0/31)[f]	16.7% (4/24)[e]	68.8% (11/16)[c]	<0.001
Acute fetal inflammatory response					
Stage 1 (acute phlebitis/chorionic vasculitis)[b]	13.8% (4/29)[d]	9.7% (3/31)[f]	20.8% (5/24)[e]	31.3% (5/16)[c]	0.2
Stage 2 (acute arteritis)[b]	3.4% (1/29)[d]	3.2% (1/31)[f]	4.2% (1/24)[e]	50% (8/16)[c]	<0.001

Data are given as median (interquartile range) and percentage (n/N).
[a]Kruskal-Wallis test with multiple comparisons.
[b]Fisher's exact test.
[c]One missing data.
[d]Two missing data.
[e]Three missing data.
[f]Four missing data.

blood cells in amniotic fluid and comparable glucose levels to those who delivered preterm or at term without intra-amniotic inflammation; (h) severe acute maternal and fetal inflammatory responses in the placenta were frequently observed in women who underwent spontaneous preterm labor with IAI; and (i) a subset of women with spontaneous preterm labor and SIAI had severe acute maternal inflammatory responses in the placenta.

4.2 | Inflammasome activation in spontaneous preterm labor with intra-amniotic infection

Herein, we showed that women who underwent spontaneous preterm labor with IAI had the highest amniotic fluid concentrations of extracellular ASC, which coincides with most elevated concentrations of IL-6 (ie, intra-amniotic inflammation). These results are consistent with previous studies, which demonstrated that amniotic fluid concentrations of caspase-1[33] (the predominant inflammasome-activated caspase[46]), IL-1β,[26] and IL-18[106] (inflammasome-processed cytokines[56]) are greater in women with intra-amniotic infection/inflammation than in those without this clinical condition. More recently, it was reported that the chorioamniotic membranes from women who underwent spontaneous preterm labor with acute histologic chorioamnionitis (a placental lesion strongly associated with IAI[99,100,102,107–116]) displayed the following: (a) elevated mRNA and protein levels of NLRP3 (ie, inflammasome sensor molecule); (b) increased expression and amounts of active caspase-1; (c) high concentrations of mature IL-1β and IL-18; and (d) enhanced inflammasome assembly (ie, ASC/caspase-1 complexes), compared to those without this placental lesion.[81] Furthermore, in vitro studies have found that microbial products (eg, lipopolysaccharide) induce the activation of caspase-1 and release of IL-1β in the chorioamniotic membranes[35,37,117] and that *Ureaplasma* species (genital mycoplasmas are the most common microorganisms found in women with IAI[12,16,118–123]) are capable of activating the inflammasome pathway in murine macrophages.[124] Together, these findings indicate that the inflammasome is involved in the mechanisms that lead to microbial-associated preterm labor and birth.

Women with IAI have numerous amniotic fluid immune cells,[96,125–128] which could be of fetal and/or maternal origin,[92] and commonly present severe acute inflammatory lesions in the placenta.[99,129] This suggests that, besides the chorioamniotic membranes, maternal and fetal leukocytes are a source of extracellular ASC in the amniotic cavity. Further research is needed to investigate whether the different microorganisms invading the amniotic cavity can differentially activate the inflammasome in the chorioamniotic membranes and amniotic fluid immune cells.

4.3 | Inflammasome activation in spontaneous preterm labor with sterile intra-amniotic inflammation

We found that women who underwent spontaneous preterm labor with SIAI had higher amniotic fluid concentrations of extracellular

ASC than those who delivered preterm or at term in the absence of intra-amniotic inflammation. Women with SIAI harbor a unique environment in the amniotic cavity in which the module of IL-1α is enriched compared to those without intra-amniotic inflammation.[84] IL-1α is a potent alarmin[130] that is increased in the amniotic fluid of women with intra-amniotic inflammation[26] and induces preterm delivery in mice,[131] an effect that can be abrogated by pretreatment with the IL-1 receptor antagonist.[132] Importantly, the amniotic fluid IL-1α module contained high mobility group box (HMGB)1,[84] a prototypic alarmin,[133,134] whose intra-amniotic concentrations are a predictor of a shorter interval to delivery[13] and whose administration induces preterm labor and birth in mice.[31] We proposed that the mechanisms whereby alarmins induce preterm birth involve the inflammasome since the incubation of the chorioamniotic membranes with HMGB1 induced the upregulation of inflammasome components (eg, NLRP3), activation of caspase-1, and release of mature IL-1β.[32] Taken together, these data suggest that inflammasome activation in the intra-amniotic space can occur in the setting of SIAI, a process that could be initiated by alarmins.

Amniotic fluid ASC concentrations were lower in women with SIAI than in those with IAI. It is well established that the NLRP3 inflammasome can be activated by both microbes[45,135–145] and alarmins;[146–154] yet, it was recently shown that sterile signals generate weaker and delayed NLRP3 inflammasome-dependent inflammatory responses compared to those triggered by microbial signals.[155] In line with this concept, women with SIAI had a lower number of amniotic fluid leukocytes and presented a reduced frequency of acute placental lesions compared to those with IAI. Furthermore, a protein network analysis of women who underwent spontaneous preterm labor showed that inflammatory responses in SIAI are distinct and not as severe as in IAI.[84] Collectively, these data suggest that the intra-amniotic inflammatory process initiated by alarmins is milder than that triggered by microbes. A potential source of alarmins in the context of SIAI is the choriodecidua, which undergoes cellular senescence during spontaneous preterm labor.[156]

4.4 | Do all preterm births involve inflammasome activation?

In the current study, we also showed that, in the absence of intra-amniotic inflammation, women who underwent spontaneous preterm labor and delivered preterm tended to have higher amniotic fluid concentrations of extracellular ASC and IL-6 than those who delivered at term. The chorioamniotic membranes from women who delivered preterm in the absence of intra-amniotic inflammation also displayed low expression of ASC and caspase-1. These findings are consistent with previous observations showing that the chorioamniotic membranes from women who underwent spontaneous preterm labor without acute histologic chorioamnionitis exhibit signs of inflammasome assembly (ie, ASC/caspase-1 complexes); yet, these complexes were not as abundant as in those with this placental lesion.[81] These results suggest that, in the absence of high concentrations of IL-6, preterm labor is associated with a mild intra-amniotic inflammatory response, which is only partially mediated by the inflammasome. We propose that the adaptive immune system may participate in such an inflammatory process. This concept is supported by the following observations: (a) Effector T cells can activate the NLRP3 inflammasome in antigen-presenting cells, amplifying adaptive immune responses;[157] (b) T cells are present in the amniotic fluid[128] and chorioamniotic membranes;[158,159] and (c) maternal and fetal T-cell activation is associated with preterm labor and birth.[160–162]

5 | CONCLUSION

The data presented herein showed that the process of premature labor in the context of IAI and SIAI is characterized by the activation of the inflammasome as evidenced by elevated concentrations of extracellular ASC and expression of inflammasome components in the chorioamniotic membranes. Such an inflammatory process is weaker in women with SIAI compared to those with IAI. Collectively, these results suggest that inflammasome activation, either driven by microbes or alarmins, is a common pathway implicated in the pathogenesis of preterm labor and birth.

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CONFLICT OF INTEREST

The authors declared no potential conflict of interests.

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