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HHS Public Access
Author manuscript
J Matern Fetal Neonatal Med. Author manuscript; available in PMC 2020 June 01.

Published in final edited form as:
J Matern Fetal Neonatal Med. 2019 June ; 32(12): 1978–1991. doi:10.1080/14767058.2017.1422714.

IN VIVO EVIDENCE OF INFLAMMASOME ACTIVATION DURING SPONTANEOUS LABOR AT TERM

Bogdan Panaitescu, MD, PhD[1,2], **Roberto Romero, MD, D. Med. Sci.**[1,3,4,5], **Nardhy Gomez-Lopez, MSc, PhD**[1,2,6], **Yi Xu, PhD**[1,2], **Yaozhu Leng, MSc**[1,2], **Eli Maymon, MD**[1,2], **Percy Pacora, MD**[1,2], **Offer Erez, MD**[1,2], **Lami Yeo, MD**[1,2], **Sonia S. Hassan, MD**[1,2], and **Chaur-Dong Hsu, MD, MPH**[2]

[1]Perinatology Research Branch, NICHD/NIH/DHHS, Bethesda, MD, & Detroit, MI, USA

[2]Department of Obstetrics & Gynecology, Wayne State University School of Medicine, Detroit, MI, USA

[3]Department of Obstetrics & Gynecology, University of Michigan, Ann Arbor, MI, USA

[4]Department of Epidemiology & Biostatistics, Michigan State University, East Lansing, MI, USA

[5]Center for Molecular Medicine & Genetics, Wayne State University, Detroit, MI, USA

[6]Department of Immunology, Microbiology & Biochemistry, Wayne State University School of Medicine, Detroit, MI, USA

Abstract

Objective—Upon inflammasome activation, the adaptor protein of the inflammasome apoptosis-associated speck-like protein containing a CARD (ASC) forms intracellular specks, which can be released into the extracellular space. The objectives of this study were to investigate whether: 1) extracellular ASC is present in the amniotic fluid of women who delivered at term; 2) amniotic fluid ASC concentrations are greater in women who underwent spontaneous labor at term than in those who delivered at term in the absence of labor; and 3) amniotic epithelial and mesenchymal cells can form intracellular ASC specks *in vitro.*

Methods—This retrospective cross-sectional study included amniotic fluid samples from 41 women who delivered at term in the absence of labor (n=24) or underwent spontaneous labor at term (n=17). Amniotic epithelial and mesenchymal cells were also isolated from the chorioamniotic membranes from a separate group of women delivered at term (n=3), in which ASC speck formation was assessed by confocal microscopy. Monocytes from healthy individuals were used as positive controls for ASC speck formation (n=3).

Address correspondence to: Roberto Romero, MD, D. Med. Sci., Perinatology Research Branch, NICHD/NIH/DHHS, Wayne State University/Hutzel Women's Hospital, 3990 John R, Box 4, Detroit, MI 48201, USA, Telephone: (313) 993-2700, Fax: (313) 993-2694, prbchiefstaff@med.wayne.edu; Bogdan Panaitescu, MD, PhD, Department of Obstetrics and Gynecology, Wayne State University School of Medicine, Perinatology Research Branch, NICHD/NIH/DHHS, Detroit, Michigan 48201, USA, Tel (313) 577-3922, bogdanp@wayne.edu.

DECLARATION OF INTEREST STATEMENT
The authors report no conflicts of interest.

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Results—1) The adaptor protein of the inflammasome ASC is detectable in the amniotic fluid of women who delivered at term; 2) Amniotic fluid ASC concentration was higher in women who underwent spontaneous labor at term than in those who delivered at term without labor; and 3) Amniotic epithelial and mesenchymal cells are capable of forming ASC specks and filaments *in vitro*.

Conclusion—Amniotic fluid ASC concentrations are increased in women who underwent spontaneous labor at term. Amniotic epithelial and mesenchymal cells are capable of forming ASC specks, suggesting that these cells are a source of extracellular ASC in the amniotic fluid. These findings provide *in vivo* evidence that there is inflammasome activation in the amniotic cavity during the physiological process of labor at term.

Keywords

Apoptosis-associated speck-like protein containing a CARD (ASC); ASC speck; amnion; caspase-1; chorion; chorioamniotic membranes; cytokine; cryopyrin; labor; interleukin-1 beta; IL-18; intra-amniotic inflammation; biomarker; NOD-like receptor (NLR); NLR Family Pyrin Domain Containing 3 protein (NLRP3); normal pregnancy; parturition; preterm labor; pattern recognition receptor (PRR); PYD And CARD Domain-Containing Protein (PYCARD); sterile inflammation

INTRODUCTION

In most women, spontaneous labor at term occurs in the absence of intra-amniotic infection [1, 2] and, therefore, is considered a sterile inflammatory process [3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13]. This process is characterized by the increased bioavailability of cytokines [14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28] and chemokines [29, 30, 31, 32, 33, 34] in the amniotic fluid, maternal circulation [35, 36, 37], and reproductive tissues [27, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52]. In addition, labor is accompanied by the infiltration of innate (e.g. neutrophils and macrophages) inflammatory cells into the cervix [45, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62], myometrium [55, 63, 64, 65, 66, 67, 68, 69], and chorioamniotic membranes [41, 70, 71, 72, 73]. The latter tissues also contain infiltrating adaptive effector immune cells such as T cells [72, 74] and, to a lesser extent, B cells [75]. The mechanisms responsible for the sterile inflammatory process of labor at term are not fully understood.

Sterile inflammation is induced by danger signals derived from necrosis or cellular stress [76], termed damage-associated molecular patterns (DAMPs) [77, 78] or alarmins [79], which can activate the inflammasome [80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91]. Inflammasomes are cytoplasmic multi-protein complexes composed of: 1) a sensor molecule or pattern recognition receptor (PRR) [e.g. nucleotide-binding oligomerization domain-like receptors (NLRs)], 2) the adaptor protein [apoptosis-associated speck-like protein (ASC) or PYD and CARD domain containing protein (PYCARD)], and 3) pro-caspase-1 [92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126]. Upon activation, the inflammasome complex induces the auto-catalytic cleavage of pro-caspase-1 into its active form [92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110,

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111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126] which, in turn, can cleave pro-IL-1β and pro-IL-18 into their mature and secreted bioactive forms [127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137]. Active forms of caspase-1 are also required to induce a specific type of pro-inflammatory programmed cell death termed pyroptosis [138, 139, 140].

Inflammasomes are implicated in the mechanisms that lead to the sterile inflammatory process of labor at term [141, 142, 143, 144, 145] and preterm [146, 147]. Inflammasome activation includes two steps: initiation of the nuclear factor kappa B (NF-κB) pathway [148, 149] and assembly of the inflammasome complex [149]. Upon inflammasome activation, the ASC protein assembles into a large intracellular complex termed “speck” that consists of multimers of ASC dimers [150, 151]. ASC specks, however, can also be released to the extracellular space and remain stable for extended periods of time [152], where they can serve as danger signals amplifying the inflammatory response [153, 154]. Recently, we showed that intracellular ASC specks are abundant in the chorioamniotic membranes and choriodecidual leukocytes from women who underwent spontaneous labor at term [145]. However, whether amniotic cells can form ASC specks and release them into the amniotic fluid during the physiological process of labor (i.e. *in vivo* evidence of inflammasome activation) is unknown.

In the study herein, we investigated whether: 1) extracellular ASC is present in the amniotic fluid of women who delivered at term; 2) amniotic fluid ASC concentrations are greater in women who underwent spontaneous labor at term than in those who delivered at term in the absence of labor; and 3) amniotic epithelial and mesenchymal cells can form intracellular ASC specks *in vitro*.

MATERIALS AND METHODS

Study population

This is a retrospective cross-sectional study conducted by searching our clinical database and bank of biological samples which included 41 patients who had amniotic fluid samples obtained by trans-abdominal amniocentesis. Women were grouped into the following: 1) subjects who delivered at term in the absence of labor (n=24); and 2) subjects who underwent spontaneous labor at term (n=17). The chorioamniotic membranes from a separate group of women who delivered at term (n=3) were also collected for primary cell culture isolation of amniotic epithelial and mesenchymal cells. As positive controls, monocytes were isolated from healthy individuals (n=3). Both amniotic primary cells and monocytes were used for the *in vitro* determination of intra- and extracellular ASC specks. Women who had positive cultures for microorganisms and high concentrations of IL-6 (2.6 ng/mL) [1, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182] were excluded. Women with multiple gestations, or those who had fetuses affected with chromosomal and/or sonographic abnormalities were also excluded. Maternal and neonatal data were obtained by retrospective clinical chart review.

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All patients provided written informed consent to donate additional amniotic fluid or chorioamniotic membrane samples for research purposes, according to protocols 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 (NICHD/NIH/DHHS).

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 term labor was defined as the presence of regular uterine contractions with a frequency of at least 1 every 10 mins, and cervical change after 37 weeks of gestation. Acute histologic chorioamnionitis was diagnosed based on the presence of inflammatory cells in the chorionic plate and/or chorioamniotic membranes [183, 184, 185, 186], while acute funisitis was diagnosed by the presence of neutrophils in the wall of umbilical vessels and/or Wharton's jelly, using previously described criteria [183, 184, 187, 188, 189].

Amniotic fluid sample collection

Amniotic fluid samples were obtained by trans-abdominal amniocentesis under ultrasound guidance. Women at term (37 weeks) not in labor underwent amniocentesis to either assess fetal lung maturity prior to cesarean delivery or for research purposes. Women who underwent spontaneous labor at term underwent amniocentesis to assess fetal lung maturity or to detect the presence or absence of intra-amniotic infection/inflammation, as described above. Samples of amniotic fluid were transported to the laboratory in a sterile capped syringe, centrifuged at 1300 × g for 10 min at 4 °C, and the supernatant was stored at −70 °C until use.

Determination of ASC concentrations in the amniotic fluid

Concentrations of ASC (also termed PYCARD) in the amniotic fluid were determined by using a sensitive and specific enzyme-linked immunosorbent assay (ELISA) kit obtained from LifeSpan BioSciences (Seattle, WA, USA). This ELISA kit was initially validated in our laboratory prior 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 5.004% and 8.614%, respectively. The sensitivity of the assay was 0.131 ng/mL.

Primary cell culture of amniotic epithelial and mesenchymal cells

Immediately after collection, the amnion membrane was manually peeled from the underlying chorion layer of chorioamniotic membranes and then cut into 2×2cm pieces. Amniotic mesenchymal cells (AMCs) were obtained by digesting the amnion fragments in 1mg/mL collagenase A (Sigma-Aldrich, St. Louis, MO) at 37°C with gentle shaking for 3 hours. The tissues were then filtered through a 100μm cell strainer (Falcon; Corning Life Sciences, Durham, NC) and centrifuged at 200 × g for 10 min. AMCs were suspended in

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Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Grand Island, NY) containing 10% fetal bovine serum (FBS, Life Technologies) and 100U/mL penicillin and streptomycin antibiotics (Life Technologies). Amniotic epithelial cells (AECs) were obtained by rinsing the amnion fragments in 0.05% (w/v) trypsin/EDTA (Life Technologies) and incubating such fragments with 15mL of fresh trypsin/EDTA at 37°C with gentle shaking for 10 min. The trypsin digestion supernatant was discarded and the amnion fragments were placed into fresh trypsin/EDTA solution and further digested at 37°C for 40 min with gentle shaking. The total digestion/incubation process was repeated twice. In order to stop digestion, FBS was added to the supernatant between each incubation period. Finally, the supernatant was centrifuged for 10 min at 200 × g. Isolated AECs and AMCs were then cultured in DMEM containing 10% FBS and 100U/mL penicillin and streptomycin at 37°C with 5% CO_2. All experiments were performed with cells from passage 2-3.

Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated using the density gradient reagent Histopaque 1077 (Sigma-Aldrich), according to the manufacturer's instructions. Briefly, 6mL of peripheral blood were layered on top of 6mL of Histopaque 1077 and centrifuged at 450 × g for 30 min with no break at room temperature. PBMCs were collected from the interphase of the plasma and gradient and washed with 1X phosphate-buffered saline (1X PBS; Life Technologies). PBMCs were then resuspended in RPMI 1640 medium (Life Technologies) supplemented with 10% FBS and 1% of penicillin/streptomycin at a density of 1×10^6 cells/mL.

ASC speck formation and immunofluorescence

PBMCs were seeded into a four-well Lab-Tek chamber slide (Thermo Fisher Scientific, Rochester, NY) at 5×10^5 cells/well and cultured at 37°C with 5% CO_2 for one hour to allow the monocytes to attach to the slide. Following incubation, the culture medium was gently aspirated and monocytes were incubated with 1μg/mL of lipopolysaccharide (LPS; Escherichia coli 0111:B4; Sigma-Aldrich) alone for 2 hours followed by addition of 20μM of nigericin (catalog number N7142, Sigma-Aldrich) for an additional hour. Non-treated monocytes were used as a negative control.

Amniotic epithelial and mesenchymal cells were plated into four-well chamber slides (BD Falcon, Bedford, MA) at 1×10^4 cells per well. Amniotic epithelial and mesenchymal cells were cultured overnight (approximately 18 hours) with 1μg/mL of LPS (Escherichia coli 0111:B4) followed by addition of 20μM of nigericin for an additional hour.

Next, all of the cells were fixed using 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 20 min at room temperature, rinsed with 1X PBS, and permeabilized using 0.25% Triton X-100 (Promega, Madison, WI) for 5 min at room temperature. Prior to staining, non-specific antibody interactions were blocked using serum-free protein blocker (Cat#X09090; DAKO North America, Carpinteria, CA) for 30 min at room temperature. Cells were then stained with a rabbit anti-human ASC (Cat#AG-25B-0006-C100; Adipogen, San Diego, CA) antibody at room temperature for one hour. Rabbit IgG was used as a negative control. Following staining, cells were washed with 1X PBS containing 0.1%

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Tween 20 (PBST) (Sigma-Aldrich). After blocking for 10 min with 10% goat serum (KPL, Gaithersburg, MD), secondary goat anti-rabbit IgG–Alexa Fluor 594 (Life Technologies) was added and cells were incubated for one hour at room temperature in the dark. Finally, cells were washed with 1X PBST and mounted using ProLong Diamond Antifade Mountant with DAPI (Life Technologies). Immunofluorescence was visualized using a Zeiss LSM 780 laser scanning confocal microscope (Carl Zeiss Microscopy, Jena, Germany) at the Microscopy, Imaging, and Cytometry Resources Core at the Wayne State University School of Medicine (http://micr.med.wayne.edu/).

Statistical Analysis

Statistical analysis was performed using SPSS version 19 (IBM Corp, Armonk, NY, USA) or GraphPad Prism (La Jolla, CA, USA) software. Normality tests were used to determine whether the data were normally distributed. Mann-Whitney *U* tests were used to compare continuous non-parametric variables between groups. Fisher's exact tests were used to compare proportions between groups. Unpaired *t* tests were used to compare continuous parametric variables. A p value of 0.05 was considered statistically significant.

RESULTS

Characteristics of the study population

A total of 41 amniotic fluid samples from women who underwent transabdominal amniocentesis with (n=17) or without (n=24) spontaneous labor at term were included in this study. Demographic and clinical characteristics of the study population are displayed in Table 1. There were no significant differences in the median maternal age, body mass index (BMI), and gestational age at delivery between the groups. Most of the women included in this study were African-American and delivered healthy neonates (Apgar score 8 [190, 191, 192]). All of the women who underwent spontaneous labor at term delivered vaginally (59%, 10/17) and all of the women who delivered at term in the absence of labor underwent a cesarean section (100%, 24/24). The median gestational age at amniocentesis was modestly shorter in the term no labor group [38.35 weeks (37.15-39)] than in the spontaneous labor at term group [39.3 (37.8-39.9) weeks] (p=0.044). Acute histologic chorioamnionitis was more frequent in women who underwent spontaneous labor at term (29.4%, 5/17) than in those who delivered at term in the absence of labor (0%, 0/24) (p=0.008).

Amniotic fluid ASC concentration is increased in women with spontaneous labor at term

ASC is a cytoplasmic protein; however, upon inflammasome activation, specks are formed and can be released to the extracellular space and remain stable for extended periods of time [152]. As readout for inflammasome activation, we determined the presence of extracellular ASC protein in the amniotic fluid. All of the amniotic fluid samples from women who delivered at term (41/41) had detectable levels of the extracellular ASC protein (Figure 1). Yet, the amniotic fluid concentration of ASC was greater in women who underwent spontaneous labor at term than in those who delivered at term in the absence of labor (Figure 1, p=0.01).

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Amniotic epithelial and mesenchymal cells form intra- and extracellular ASC specks *in vitro*

First, we evaluated whether primary cultures of amniotic epithelial and mesenchymal cells could form intracellular ASC specks *in vitro*. Upon inflammasome stimulation (LPS+ nigericin), ASC speck formation was observed in monocytes (positive controls, Figure 2A–B) as well as in amniotic epithelial and mesenchymal cells (Figure 2C–D). In monocytes, ASC specks were observed in the cytoplasm as a large red dot (arrows, Figure 2B). Isotype controls did not show any signal, as expected (Figure 2A). In amniotic mesenchymal cells, the ASC protein was observed in the cytoplasm and nucleus (yellow arrow, Figure 2D) and forming ASC filaments (white arrow, Figure 2D), which are created prior to ASC speck formation and inflammasome activation [193, 194, 195, 196, 197]. Isotype controls did not show any signal, as expected (Figure 2C). In the same fashion, the ASC protein in amniotic epithelial cells was observed in the cytoplasm and nucleus (yellow arrow, Figure 2F) and forming ASC speck-like structures (white arrow, Figure 2F). Isotype controls did not show any signal, as expected (Figure 2E). These results show that amniotic epithelial and mesenchymal cells are capable of forming intracellular ASC specks upon inflammasome activation.

DISCUSSION

Principal findings of the study

In the current study, we report that: 1) extracellular ASC was present in the amniotic fluid of women who delivered at term; 2) amniotic fluid ASC concentrations were greater in women who underwent spontaneous labor at term than in those who delivered at term in the absence of labor; and 3) amniotic epithelial and mesenchymal cells formed intracellular ASC specks and filaments *in vitro*.

A role for the inflammasome in spontaneous labor at term

Evidence supporting the participation of the inflammasome in the mechanisms of spontaneous labor at term [141] includes the following: 1) caspase 1, the predominant inflammasome-activated caspase [98, 198], is present in the amniotic fluid and its concentrations are higher in women with spontaneous labor at term than in those without labor [142]; 2) amniotic fluid concentrations of IL-1β are increased in women who underwent spontaneous labor at term compared to those who delivered at term in the absence of labor [14, 15, 16, 42]; 3) amniotic fluid concentrations of IL-18 are greater in term pregnancies than in the second trimester [199]; 4) the chorioamniotic membranes from women who underwent spontaneous labor at term display increased expression of the inflammasome sensor molecule NLRP3 (NLR Family Pyrin Domain Containing 3 protein or cryopyrin), contain high amounts of active forms of caspase-1, and release elevated concentrations of mature IL-1β compared to those from women who delivered at term in the absence of labor [143]; and 5) ASC/caspase-1 complexes (i.e. inflammasome assembly) are greater in the chorioamniotic membranes and choriodecidual leukocytes from women who underwent spontaneous labor at term than in those without labor. Herein, we provide further *in vivo* evidence that there is inflammasome activation in the amniotic cavity of women who underwent spontaneous labor at term. Specifically, we showed that amniotic epithelial and

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mesenchymal cells can form ASC speck-like structures and filaments, which are created prior to ASC speck formation upon inflammasome activation [193, 194, 195, 196, 197]. Such ASC specks can then be released and found extracellularly in the amniotic cavity, where they are elevated in women who underwent spontaneous labor at term. In the amniotic cavity, extracellular ASC can function as a danger signal to amplify the sterile inflammatory process that accompanies the process of labor [3, 4, 5, 6, 7, 8, 9, 10]. Collectively, these findings provide solid evidence that there is inflammasome activation during the physiological process of spontaneous labor at term.

A role for inflammasome-processed IL-1β in spontaneous labor at term

The biochemical function of the inflammasome is to activate caspase-1 [127, 128, 129, 133, 134, 137] which, in turn, will lead to the maturation of IL-1β [92, 94, 98, 99, 102, 113, 118, 125, 126, 200]. In line with the findings reported herein, we previously showed that the chorioamniotic membranes express *IL1B* and release the mature protein form, and that such a process was increased in the chorioamniotic membranes from women who had undergone spontaneous labor at term than in those without labor [143]. The biological functions of bioactive IL-1β during the process of labor comprise: 1) stimulation of prostaglandin biosynthesis by the human amnion [201], decidual cells [202], chorioamniotic membranes [203], and myometrial cells [204, 205], 2) induction of the expression of cyclooxygenase-2 by human myometrial cells [206], 3) upregulation of the expression of matrix metalloproteinases (e.g. MMP-9) by human cervical smooth muscle cells [207] and the amnion [208]; and 4) induction of pro-inflammatory cytokines by decidual cells [209]. Indeed, systemic administration of IL-1β induces preterm labor and birth in mice [17, 210] and monkeys [211, 212, 213, 214, 215, 216, 217, 218], confirming the central role of this cytokine in the mechanisms responsible for labor at term.

CONCLUSION

The adaptor protein of the inflammasome ASC, which is released upon inflammasome activation, is detectable in the amniotic fluid of women who delivered at term and increased in those who underwent spontaneous labor at term. Amniotic epithelial and mesenchymal cells are capable of forming ASC specks and filaments *in vitro*, suggesting that these cells are a source of extracellular ASC in the amniotic fluid. These findings provide *in vivo* evidence that there is inflammasome activation in the amniotic cavity during the physiological process of labor at term.

Acknowledgments

This research was supported, in part, by the Perinatology Research Branch (PRB), Division of Intramural Research, *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, National Institutes of Health, U. S. Department of Health and Human Services (NICHD/NIH/DHHS), and, in part, with federal funds from the NICHD/NIH/DHHS under Contract No. HHSN275201300006C. This research was also supported by the Wayne State University Perinatal Initiative in Maternal, Perinatal and Child Health. We gratefully acknowledge the PRB Translational Research laboratory for their contributions to the execution of this study. We thank the physicians and nurses from the Center for Advanced Obstetrical Care and Research and the Intrapartum Unit for their help in collecting human samples. We also thank staff members of the PRB Clinical Laboratory and the PRB Histology/ Pathology Unit for the processing and examination of the pathological sections.

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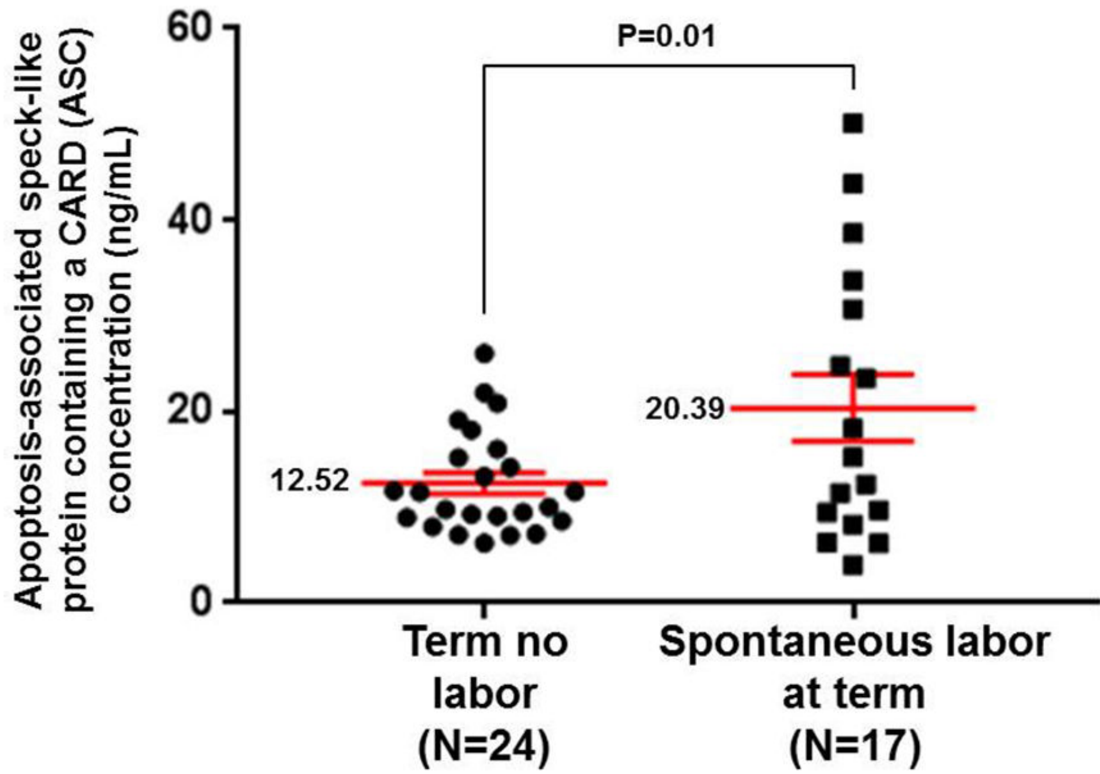

Figure 1.

Amniotic fluid ASC concentrations (ng/mL) in women who delivered at term in the absence of labor (n= 24) and women who underwent spontaneous labor at term (n=17). Red line represents the mean ± SEM.

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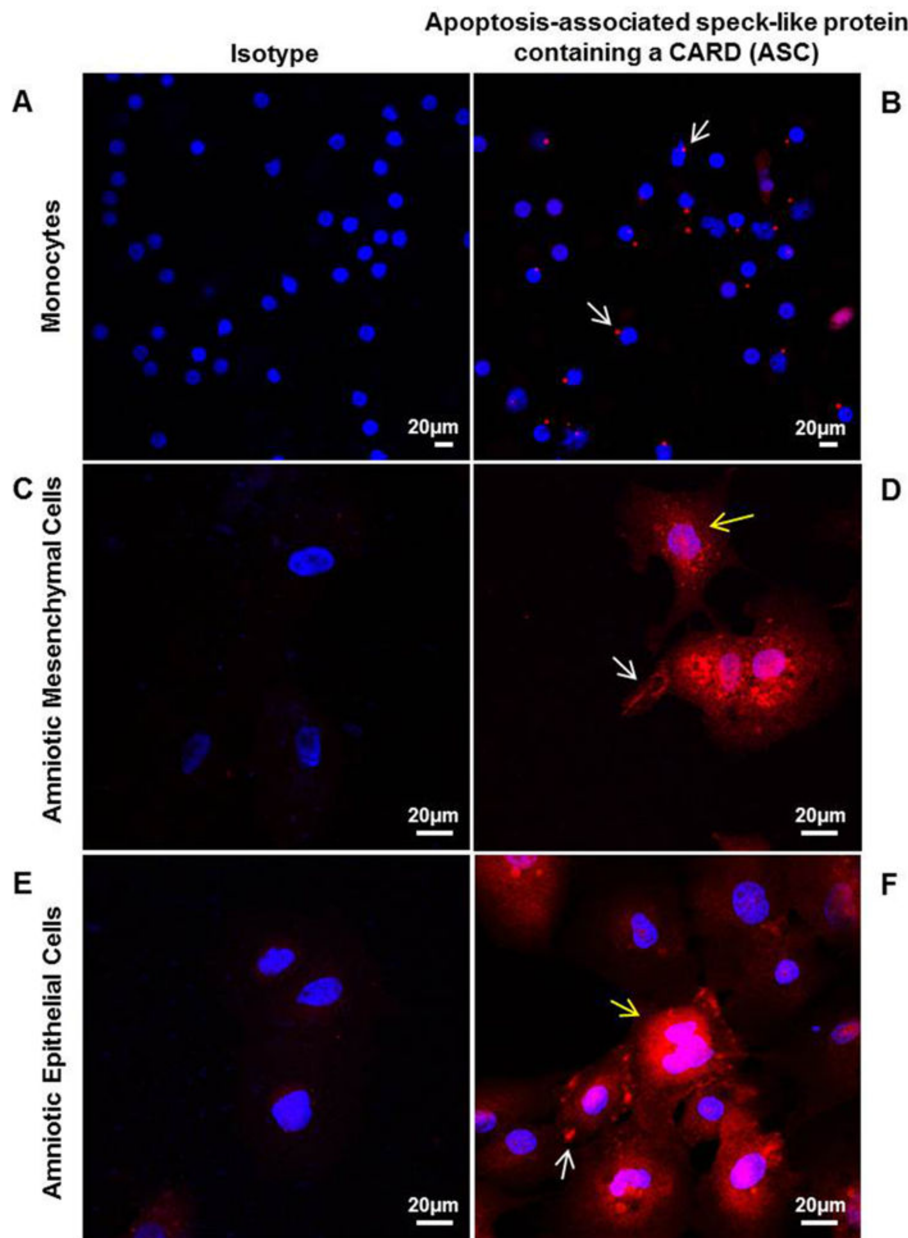

Figure 2.

ASC speck formation in amniotic epithelial and mesenchymal primary cells. Monocytes and amniotic cells were incubated with lipopolysaccharide (1μg/mL) and nigericin (20μM) and ASC speck formation was assessed by confocal microscopy. (A-B) Monocytes isolated from the peripheral blood of healthy individuals were used as positive controls (n=3). (C-F) Amniotic epithelial and mesenchymal cells were isolated from the chorioamniotic membranes from women who delivered at term in the absence of labor (n=3). Right panel: ASC signal is shown in red. Left panel: isotype controls. The blue signal is DAPI (nuclei). White arrows show ASC specks/filaments and yellow arrows show cytoplasmic/nuclear

ASC. 400× magnification. ASC, apoptosis-associated speck-like protein containing a CARD.

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Table 1

Clinical and demographic characteristics of the study population

	Term no labor (N=24)	Spontaneous labor at term (N=17)	P-value
Maternal age (years)[a]	26.5 (22.5-29.75)	24 (22-28.5)	0.338
Body mass index (kg/m^2)[a]	33.3 (25.3-36.6)	27.3 (20.4-34)	0.075
Race[b]			
African-American	70.8% (17/24)	94% (16/17)	0.11
Caucasian	20.8% (5/24)	6% (1/17)	0.372
Hispanic	4.2% (1/24)	0	1
Other	4.2% (1/24)	0	1
Gestational age at amniocentesis (weeks)[a]	38.35 (37.15-39)	39.3 (37.8-39.9)	0.044
Delivery route[b]			
Vaginal	0	59% (10/17)	<0.0001
Cesarean section	100% (24/24)	41% (7/17)	<0.0001
Gestational age at delivery (weeks)[a]	38.5 (37.4-39.08)	39.3 (37.9-39.9)	0.051
Birthweight[a]	3158 (2991-3445)	3080 (2845-3378)	0.121
Apgar score at 1 min[a]	9 (8-9)	8 (8-9)	0.597
Apgar score at 5 min[a]	9 (9-9)	9 (9-9)	0.831
Histopathological placental findings[b]			
Acute histologic chorioamnionitis	0	29.4% (5/17)	0.008
Acute funisitis	4.2% (1/24)	29.4% (5/17)	0.065

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

[a]Mann-Whitney U test.

[b]Fisher's exact test.