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# A Role for the Inflammasome in Spontaneous Preterm Labor With Acute Histologic Chorioamnionitis

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## Abstract

Inflammasomes are cytosolic multiprotein complexes that orchestrate inflammation in response to pathogens and endogenous danger signals. Herein, we determined whether the chorioamniotic membranes from women in spontaneous preterm labor with acute histologic chorioamnionitis (1) express major inflammasome components; (2) express caspase (CASP)-1 and CASP-4 as well as their active forms; (3) exhibit apoptosis-associated speck-like protein containing a CARD (ASC)/CASP-1 complex formation; and (4) release the mature forms of interleukin (IL)-1β and IL-18. We utilized quantitative reverse transcription polymerase chain reaction, enzyme-linked immunosorbent assay, immunoblotting, and immunohistochemistry to determine the messenger RNA (mRNA) and protein expression of major inflammasome components, nucleotide-binding oligomerization domain (NOD) proteins, and the pro- and mature/active forms of CASP-1, CASP-4, IL-1β, and IL-18. The ASC/CASP-1 complex formation was determined using an in situ proximity ligation assay. When comparing the chorioamniotic membranes from women in spontaneous preterm labor with acute histologic chorioamnionitis to those without this placental lesion, we found that (1) the mRNA of NLR family pyrin domain-containing protein (*NLRP*)*1, NLRP3*, NLR family CARD domain-containing protein 4 (*NLRC4*), and *NOD2* were higher; (2) the NLRP3 protein was increased; (3) the mRNA and active form (p10) of CASP-1 were greater; (4) the mRNA and active form of CASP-4 were increased; (5) the mRNA and mature form of IL-1β were higher; (6) the mature form of IL-18 was elevated; and (7) ASC/CASP-1 complex formation was increased. In conclusion, spontaneous preterm labor with acute histologic chorioamnionitis is characterized by an upregulation of NLRP3 and the active form of CASP-4, as well as increased ASC/CASP-1 complex formation, which may participate in the activation of CASP-1 and the maturation of IL-1β and IL-18 in the chorioamniotic membranes. These findings provide the first evidence that supports a role for the inflammasome in the pathological inflammation implicated in spontaneous preterm labor with acute histologic chorioamnionitis.

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## Introduction

Preterm birth is one of the most common, yet detrimental, obstetrical syndromes[1-6] and the leading cause of perinatal morbidity and mortality worldwide.[7-10] Approximately 70% of all preterm births are preceded by spontaneous preterm labor,[1,5,11-13] a syndrome of multiple pathological processes.[14] Of all the putative causes associated with spontaneous preterm labor, only intraamniotic inflammation/infection has been causally linked to preterm birth.[15-38] Intraamniotic inflammation/infection generally results in acute histologic chorioamnionitis;[39-48] thus, this placental lesion is strongly associated with preterm labor and birth.[40,49-53] Acute histologic chorioamnionitis can also occur in the setting of sterile intraamniotic inflammation,[54-58] an inflammatory process in which microorganisms cannot be detected using both cultivation and molecular microbiology techniques.[54-63] Sterile inflammation is induced by danger signals, termed damage-associated molecular patterns (DAMPs)[64,65] or alarmins,[66] derived from necrotic cells or cellular stress.[67] Therefore, acute histologic chorioamnionitis is evidence of intraamniotic inflammation regardless of the presence of infection.[68]

Acute histologic chorioamnionitis is defined by the infiltration of neutrophils and monocytes/macrophages into the chorioamniotic membranes,[45,48,68-72] which is mediated by a gradient of potent chemokines, including interleukin (IL)-8,[68,73,74] C-X-C motif chemokine ligand 6 (CXCL6),[68,75] and C-C motif chemokine ligand 2 (CCL2)[76] (also known as monocyte chemoattractant protein or MCP-1). This pathological activation of the innate immune system is observed in spontaneous preterm labor and includes high concentrations of pro-inflammatory cytokines, such as IL-1α, IL-1β, tumor necrosis factor α, and IL-6 in the amniotic fluid,[77-89] decidua,[90,91] and umbilical cord blood.[92-94] Elevated concentrations of these cytokines are linked to adverse neonatal outcomes.[80,82,83,92,95-111] Therefore, the study herein focuses on the mechanisms implicated in the pathophysiology of acute histologic chorioamnionitis in spontaneous preterm labor.

The inflammasome is implicated in physiological[112-114] and pathological inflammation (ie, acute histologic chorioamnionitis[115]) during human parturition at term; however, its role in spontaneous preterm labor is unknown. Inflammasomes are cytoplasmic multiprotein complexes that promote an inflammatory response through the release of the mature forms of IL-1β and IL-18.[116-150] The inflammasome complex contains: (1) a pattern recognition receptor (PRR or sensor molecule), (2) the adaptor protein ASC (apoptosis-associated speck-like protein containing a CARD), and (3) pro-caspase-1 (pro-CASP-1).[116-152] Inflammasome activation prompts the release of active CASP-1 which, in turn, can participate in the processing of mature IL-1β and IL-18.[153-163] These events induce a pro-inflammatory programmed cell death termed pyroptosis.[164-166] In addition, active CASP-4 participates in the noncanonical activation of the inflammasome by activating CASP-1[149,167-171], which can lead to pyroptosis and the release of alarmins (eg, high mobility group box-1 or HMGB1) in a CASP-1-independent manner.[172]

Herein, we propose that the inflammasome is implicated in the pathological inflammation (acute histologic chorioamnionitis) associated with spontaneous preterm labor. Such a hypothesis is supported by the fact that amniotic fluid concentrations of CASP-1,[113] IL-1β,[78] and IL-18[173] are greater in women who undergo spontaneous preterm labor with intraamniotic infection/inflammation than in those without this clinical condition. The aims of this study were to determine whether the chorioamniotic membranes from women in spontaneous preterm labor with acute histologic chorioamnionitis: (1) express major inflammasome components; (2) express CASP-1 and CASP-4 as well as their active forms; (3) exhibit ASC/CASP-1 complex formation; and (4) release the mature forms of IL-1β and IL-18.

## Materials and Methods

### Human Participants, Clinical Specimens, and Definitions

In order to conduct this case–control study, chorioamniotic membrane samples from women who underwent spontaneous preterm labor with (PTL-ACA) or without (PTL) acute histologic chorioamnionitis were collected from the Bank of Biological Specimens of the Detroit Medical Center, Wayne State University, and the Perinatology Research Branch (Detroit, Michigan), an intramural program of the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, National Institutes of Health, US Department of Health and Human Services. The Institutional Review Boards of these institutions approved the collection and use of biological materials for research purposes. Participants provided written informed consent and samples were collected within 0.5 hour after delivery. Table 1 includes the demographic and clinical characteristics of the study population. Multiparous women or women with neonates having congenital or chromosomal abnormalities were excluded. Preterm labor was diagnosed by the presence of regular uterine contractions (at least 3 in 30 minutes) and documented cervical changes in patients with a gestational age between 20 and 36 6/7 weeks. Preterm delivery was defined as birth prior to week 37 of gestation.

### Placental Histopathological Examinations

Five-μm-thick sections of formalin-fixed, paraffin-embedded tissue specimens were cut and mounted on SuperFrost Plus

**Table** 1. Demographic and Clinical Characteristics of the Study Groups.

| | PTL (n = 33) | PTL-ACA (n = 37) | *P* Value |
|---|---|---|---|
| Maternal age, years, median (IQR)[a] | 24.5 (19.8-32.3) | 26 (21-31.3) | NS |
| Race, n (%)[b] | | | NS |
| African American | 24 (72.7) | 30 (81.1) | |
| Caucasian | 4 (12.1) | 5 (13.5) | |
| Other | 5 (15.2) | 2 (5.4) | |
| Maternal weight, kg, median (IQR)[a] | 65.5 (54-75.3) | 69.5 (54-85) | NS |
| Body mass index, kg/m$^2$, median (IQR)[a] | 24.6 (20.6-29) | 24.4 (20.2-32.9) | NS |
| Primiparity, n (%)[b] | 5 (15.2) | 5 (13.5) | NS |
| Gestational age at delivery, weeks, median (IQR)[a] | 31.7 (30-33) | 31.8 (30.3-32.9) | NS |
| Birth weight, g, median (IQR)[a] | 1571.5 (1193.8-1928.8) | 1560 (1218.8-1906.3) | NS |
| Cesarean section, n (%)[b] | 8 (24.2) | 7 (18.9) | NS |
| Acute histologic chorioamnionitis, n (%)[b] | 0 (0) | 37 (100) | <.0001 |

Abbreviations: PTL, preterm labor without acute histologic chorioamnionitis; PTL-ACA, preterm labor with acute histologic chorioamnionitis; IQR, interquartile range; NS, non-significant.
[a]Mann-Whitney *U* test.
[b]Fisher's exact test.

microscope slides (Erie Scientific LLC, Portsmouth, New Hampshire). In each case, several tissue sections of the chorioamniotic membranes, umbilical cord, and placental disk were examined. After deparaffinization, slides were rehydrated, stained with hematoxylin–eosin, and evaluated by pathologists who were blinded to the clinical outcome, according to published criteria.[68,69] The diagnosis of acute histologic chorioamnionitis was made when the infiltration of neutrophils into the chorionic trophoblast layer or chorioamniotic connective tissue was observed.[68,69]

## *RNA Isolation, cDNA Generation, and Quantitative Reverse Transcription Polymerase Chain Reaction Analysis*

Total RNA was extracted from snap-frozen chorioamniotic membrane samples (PTL, n = 33 and PTL-ACA, n = 37) using TRIzol (Invitrogen, Life Technologies Corporation, Grand Island, New York) and Qiagen RNeasy kits (Qiagen, Gaithersburg, Maryland). The purity, concentration, and integrity of the RNA were assessed using the NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA) and the Bioanalyzer 2100 (Agilent Technologies, Wilmington, Delaware). cDNA was generated using the SuperScript III First-Strand Synthesis System (Invitrogen) and oligo(dT)20 primers (Invitrogen). Gene expression profiling was performed on the BioMark System for high-throughput quantitative reverse transcription polymerase chain reaction (Fluidigm, San Francisco, California) and the ABI 7500 FAST Real-Time PCR System (Applied Biosystems, Life Technologies Corporation, Foster City, California) with TaqMan gene expression assays (Applied Biosystems) listed in Supplementary Table 1.

## *Chorioamniotic Membrane Tissue Lysates*

Tissue lysates were prepared by homogenizing snap-frozen chorioamniotic membranes (PTL and PTL-ACA, n = 10 each) in 2 mL of 1X phosphate-buffered saline (PBS; Invitrogen) containing a complete protease inhibitor cocktail (Cat. No. 11697498001; Roche Applied Science, Mannheim, Germany). Next, lysates were centrifuged at 15700×g for 5 minutes at 4°C, and the supernatant was collected and stored at −80°C. Prior to enzyme-linked immunosorbent assay (ELISA) or immunoblotting, total protein determination was determined using the Quick Start Bradford Protein Assay Kit (Bio-Rad, Hercules, California).

## *Enzyme-Linked Immunosorbent Assays*

Protein concentrations of NLR family pyrin domain-containing protein (NLRP)1, NLRP3, absent in melanoma 2 (AIM2), nucleotide-binding oligomerization domain (NOD) 2 protein, CASP-1, CASP-4, IL-18, pro-IL-1β, and mature IL-1β were determined in chorioamniotic membrane tissue lysates by immunoassays (NLRP1, NLRP3, and NOD2 ELISA kits from Cusabio, Wuhan, Hubei, P.R. China; AIM2, CASP-1, and CASP-4 ELISA kits from Cloud Clone, Houston, Texas; pro-IL-1β and IL-1β ELISA kits from R&D Systems, Minneapolis, Minnesota; and IL-18 ELISA kits from MBL International Corporation, Woburn, Massachusetts), as previously described.[114] The sensitivities of the assays were <4.67 pg/mL for NLRP1, <0.039 ng/mL for NLRP3, <0.056 ng/mL for AIM2, <6.25 pg/mL for NOD2, <0.112 ng/mL for CASP-1, <0.053 ng/mL for CASP-4, 3.3 pg/mL for pro-IL-1β, <1 pg/mL for mature IL-1β, and <12.5 pg/mL for IL-18. The IL-1β ELISA kit measured about 10% of the pro-IL-1β. The immunoassays for NLR family CARD domain-containing protein 4 (NLRC4) and NOD1 did not meet our criteria for validation; instead, immunoblotting was performed.

## *Immunohistochemistry*

Tissue sections (5-μm thick) were prepared from the chorioamniotic membranes (PTL and PTL-ACA, n = 10 each).

Immunohistochemistry for NLRP1, NLRP3, NLRC4, AIM2, NOD1, NOD2, CASP-1, CASP-4, IL-1β, and IL-18 was performed, as previously described.[114] Supplementary Table 2 includes the utilized primary antibodies and immunostaining conditions. Quantification of the intensity was performed using a PerkinElmer Panoramic MIDI slide scanner (PerkinElmer, Waltham, Massachusetts).

## Immunoblotting

The protein quantity of NLRC4 and NOD1, as well as the active/mature forms of CASP-1, CASP-4, and IL-18, was determined by immunoblotting. Tissue lysates (50 μg per well) were subjected to 4% to 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (Invitrogen). Separated proteins were then transferred onto nitrocellulose membranes (Bio-Rad). Next, membranes were submerged in blocking solution (5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween-20, Bio-Rad or StartingBlock T20 Block Buffer, ThermoFisher Scientific, Inc, Rockford, Illinois) and probed overnight at 4°C with the following human antibodies: mouse anti-NLRC4 antibody (BioLegend, San Diego, California), rabbit anti-NOD1 polyclonal antibody (Enzo Life Sciences, Farmingdale, New York[174]), mouse anti-CASP-1 monoclonal antibody (R&D Systems), rabbit anti-CASP-4 polyclonal antibody (Abcam, Cambridge, Massachusetts), or rabbit anti-IL-18 polyclonal antibody (Santa Cruz Biotechnology, Dallas, Texas). A horseradish peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulin G (IgG; Cell Signaling, Boston, Massachusetts) was used as a secondary antibody. Chemiluminescence signals were detected with ChemiGlow West Reagents (Protein Simple, Santa Clara, California), and images were acquired using the FUJIFILM LAS-4000 Imaging System (FUJIFILM North America Corporation, Valhalla, New York). Finally, nitrocellulose membranes were then stripped with Restore Plus Western Blot Stripping Buffer (Pierce Biotechnology, ThermoFisher Scientific Inc) for 15 minutes, washed with 1X PBS, blocked, and reprobed for 1 hour at room temperature with a mouse anti-beta-actin (ACTB) monoclonal antibody (Sigma-Aldrich). Chemiluminescence signals were again detected with ChemiGlow West Reagents, and images were acquired using the FUJIFILM LAS-4000 Imaging System.

## In Situ Proximity Ligation Assay

ASC/CASP-1 complex formation was detected by identifying protein interactions between ASC and CASP-1 using a Duolink in situ proximity ligation assay kit (Olink Bioscience, Uppsala, Sweden), following the manufacturer's instructions. Briefly, chorioamniotic membrane tissues (PTL and PTL-ACA, n = 7 each) were frozen in Tissue-Plus O.C.T. compound (Fisher HealthCare, Houston, Texas) immediately after collection. Cryogenic sections were cut to 10 μm and placed on glass microscope slides (Fisherbrand Superfrost Plus slides; Thermo Scientific, Waltham, Massachusetts). The sections were fixed using 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, Pennsylvania) for 20 minutes at room temperature, rinsed with 1X PBS, and permeabilized using 0.25% Triton X-100 (Promega, Madison, Wisconsin) for 5 minutes at room temperature. Prior to staining, nonspecific antibody interactions were blocked using serum-free protein blocker (Cat#X09090; DAKO North America, Carpinteria, California) for 30 minutes at room temperature. The sections were then stained at 4°C overnight with the following antibodies: rabbit anti-human ASC (Cat#AG-25B-0006-C100; Adipogen, San Diego, California) and mouse anti-human CASP-1 (Cat#MAB6251, clone 661228; R&D Systems, Minneapolis, Minnesota). Rabbit IgG and mouse IgG2A were used as negative controls, respectively. Following staining, slides were briefly washed with 1X Wash Buffer A from the Duolink kit and incubated with the provided proximity ligation assay probes for 1 hour at 37°C, followed by a second wash with Wash Buffer A. The slides were then incubated with Duolink ligase solution for 30 minutes at 37°C, washed with Wash Buffer A, and incubated for 100 minutes with Duolink polymerase solution. The slides were washed with 1X Wash Buffer B and mounted with Duolink mounting media with DAPI (4',6-diamidino-2-phenylindole). Immunofluorescence was visualized using an Olympus BX60 fluorescence microscope (Olympus, Tokyo, Japan) at 400× magnification. Images were acquired using an Olympus DP71 camera and DP Controller software (Olympus). Semiquantification was performed using the Duolink image analysis software. Images and a video were acquired using a Zeiss LSM 800 laser scanning confocal microscope (Carl Zeiss Microscopy, Jena, Germany) at the Microscopy, Imaging, and Cytometry Resources Core at Wayne State University School of Medicine (http://micr.med.wayne.edu/). ASC/CASP-1 complex formation was calculated by dividing the number of signals over the area of the tissue, which was expressed as pixels.

## Statistical Analyses

The SPSS version19.0 software (SPSS Inc, Chicago, Illinois) was used to analyze demographic and clinical data. Comparisons between the 2 groups (PTL vs PTL-ACA) were performed using the Fisher's exact test for proportions as well as the Mann-Whitney *U* test for nonnormally distributed continuous variables. Gene expressions relative to *ACTB*/*GAPDH*/*RPLP0* were calculated as $-\Delta Ct$ values, where $\Delta Ct$ ($\Delta Ct = Ct_{target} - Ct_{reference}$) was computed for each sample after averaging the Ct values over the technical replicates. Group means of gene expression were then compared using *t* tests from an analysis of variance linear model and the resulting *P* values were adjusted using the Benjamini-Hochberg procedure. Spearman correlations were used to examine the relationship between inflammasome components and products. An adjusted *P* value of $\leq .05$ was considered statistically significant.

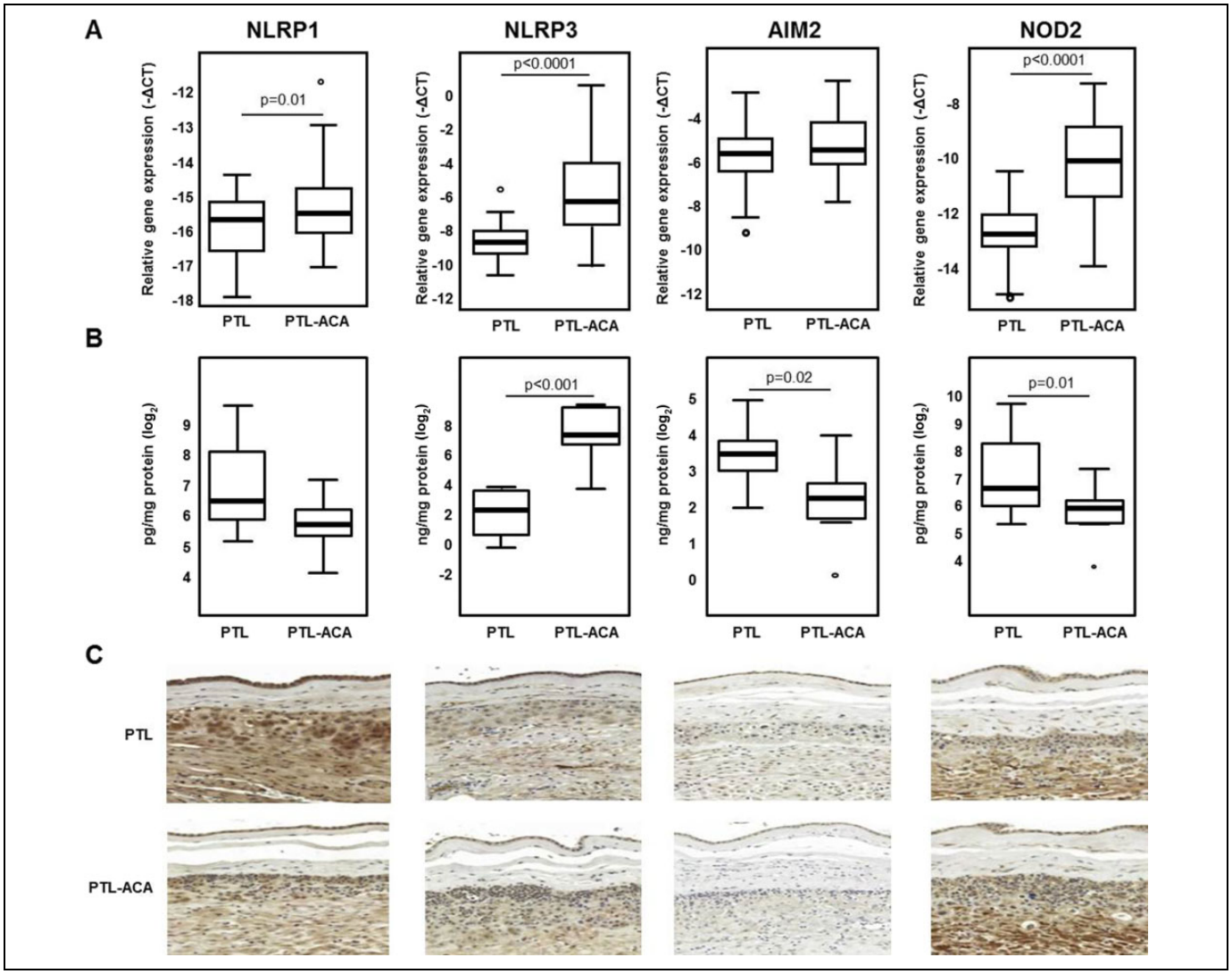


**Figure 1.** Inflammasome components and NOD2 protein in the chorioamniotic membranes. A, mRNA abundance of inflammasome components and *NOD2* protein in the chorioamniotic membranes from women in spontaneous preterm labor with (PTL-ACA, n = 37) or without (PTL, n = 33) acute histologic chorioamnionitis. Relative gene expressions are presented as $-\Delta$Ct values. *T* tests from an analysis of variance (ANOVA) linear model and the resulting *P* values were adjusted using the Benjamini-Hochberg procedure. B, Protein concentrations of inflammasome components and NOD2 in chorioamniotic membrane tissue lysates (n = 10 each). Mann-Whitney *U* tests. C, Representative immunostainings for inflammasome components and NOD2 in the chorioamniotic membranes (n = 10 each), 200× magnification. Circles denote outlier values. mRNA indicates messenger RNA; NOD, nucleotide-binding oligomerization domain; PTL, preterm labor without acute histologic chorioamnionitis; PTL-ACA, preterm labor with acute histologic chorioamnionitis.

## Results

### *mRNA Abundance and Protein Expression of Inflammasome Components and NOD Proteins in the Chorioamniotic Membranes in Spontaneous Preterm Labor with and without Acute Histologic Chorioamnionitis*

First, the expression of major inflammasome components (NLRP1, NLRP3, AIM2, and NLRC4) and NOD proteins (NOD1 and NOD2) was determined in the chorioamniotic membranes from women who had undergone spontaneous preterm labor. The chorioamniotic membranes from women who had undergone spontaneous preterm labor with and without acute histologic chorioamnionitis expressed all of the inflammasome components. The mRNA abundance of *NLRP1*, *NLRP3*, *NLRC4*, and *NOD2* was higher in the chorioamniotic membranes from women who underwent spontaneous preterm labor with acute histologic chorioamnionitis than in those without this placental lesion (Figures 1A and 2A). The protein concentrations of NLRP3 were greater in the chorioamniotic membranes from women who underwent spontaneous preterm labor with acute histologic chorioamnionitis than in those without this placental lesion (Figure 1B). Whereas the protein concentrations of AIM2 and NOD2 were lower in the chorioamniotic membranes from women who underwent spontaneous

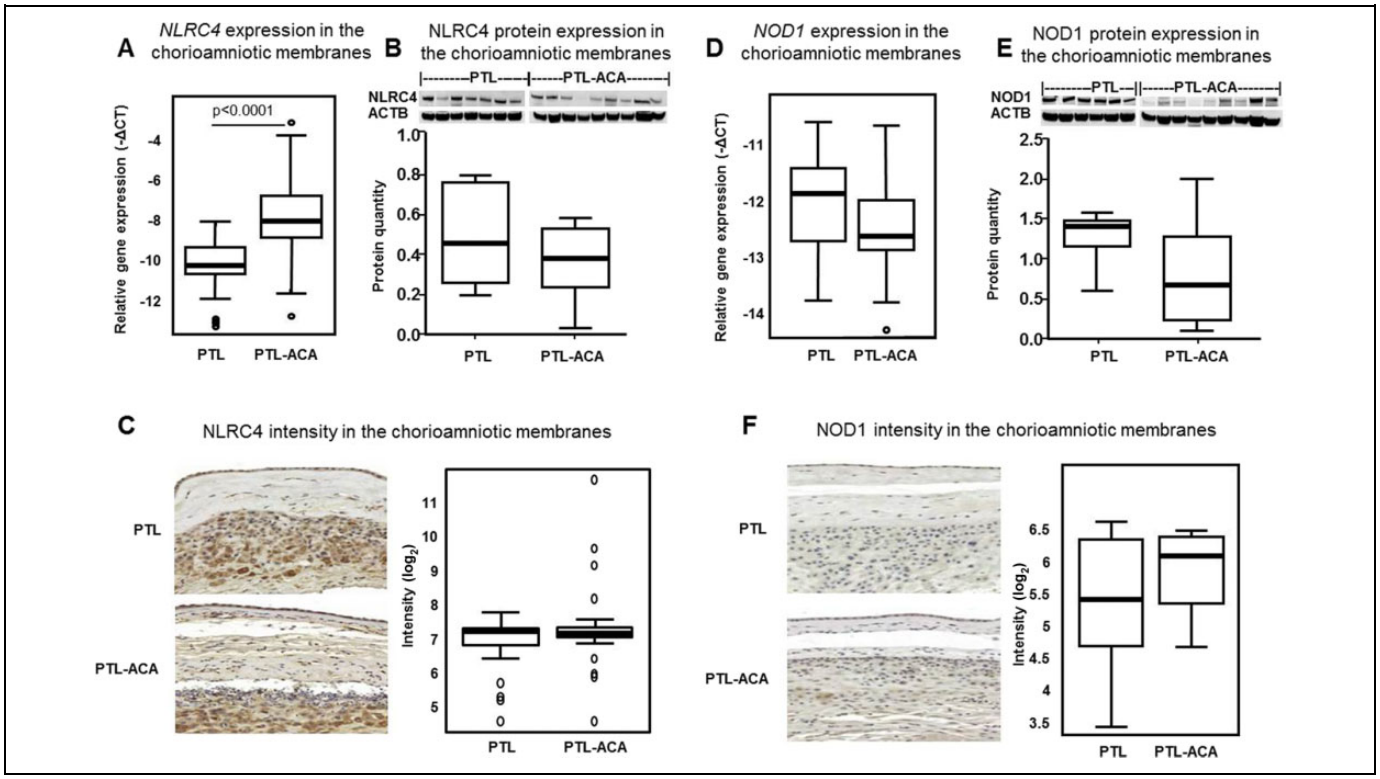


**Figure 2.** NLRC4 and NOD1 in the chorioamniotic membranes. (A and D) mRNA abundance of *NLRC4* and *NOD1* in the chorioamniotic membranes from women in spontaneous preterm labor with (PTL-ACA, n = 37) or without (PTL, n = 33) acute histologic chorioamnionitis. Relative gene expressions are presented as $-\Delta$Ct values. *T* tests from an analysis of variance (ANOVA) linear model and the resulting *P* values were adjusted using the Benjamini-Hochberg procedure. (B and E) Protein quantity of NLRC4 and NOD1 in chorioamniotic membrane tissue lysates (n = 6-9 each). Mann-Whitney *U* tests. (C and F) Intensity of the immunostainings for NLRC4 and NOD1 in the chorioamniotic membranes (n = 10 each) and representative immunostainings, 200× magnifications. Mann-Whitney *U* tests. Circles denote outlier values. mRNA indicates messenger RNA; NLRC4, NLR family CARD domain-containing protein 4; NOD, nucleotide-binding oligomerization domain; PTL, preterm labor without acute histologic chorioamnionitis; PTL-ACA, preterm labor with acute histologic chorioamnionitis.

preterm labor with acute histologic chorioamnionitis than in those without this placental lesion (Figure 1B). The protein concentrations of NLRP1, NLRC4, and NOD1 were similar between these 2 groups (Figures 1B, 2B and E).

Immunohistochemistry analysis revealed that all of the inflammasome components and NOD proteins were expressed in the chorioamniotic membranes from women who had undergone spontaneous preterm labor with and without acute histologic chorioamnionitis. The NLRP1, NLRP3, NLRC4, and NOD2 proteins showed strong immunoreactivity in the chorionic trophoblast cells, decidual stromal cells, and amniotic mesodermal and epithelial cells (Figures 1C and 2C). However, AIM2 and NOD1 showed weak immunoreactivity in the chorionic trophoblast cells, decidual stromal cells, and amniotic mesodermal and epithelial cells (Figures 1C and 2F).

The combined increase in the mRNA abundance and protein expression of NLRP3 in the chorioamniotic membranes from women who underwent spontaneous preterm labor with acute histologic chorioamnionitis suggests that the inflammasome may participate in the pathological activation of the innate immune system, leading to preterm labor in the setting of intraamniotic inflammation.

## *Activation of CASP-1 and CASP-4 in the Chorioamniotic Membranes in Spontaneous Preterm Labor with Acute Histologic Chorioamnionitis*

Next, we examined whether the increased concentration of NLRP3 was linked to the activation of CASP-1 and CASP-4 in the chorioamniotic membranes from women who had undergone spontaneous preterm labor with acute histologic chorioamnionitis. The mRNA abundance of *CASP1* and *CASP4* was greater in the chorioamniotic membranes from women who underwent spontaneous preterm labor with acute histologic chorioamnionitis than in those without this placental lesion (Figure 3A). Although the protein concentrations of CASP-1 and CASP-4 were not significantly different between the 2 groups (Figure 3B), semiquantitative analysis of immunostaining revealed that CASP-1 was increased in the chorioamniotic membranes from women who underwent spontaneous preterm labor with acute histologic chorioamnionitis (Figure 3C). Also, the protein quantities of one of the active forms of CASP-1 (p10) and the active form of CASP-4 (p20) were increased in the chorioamniotic membranes from women who had undergone spontaneous preterm labor with acute histologic

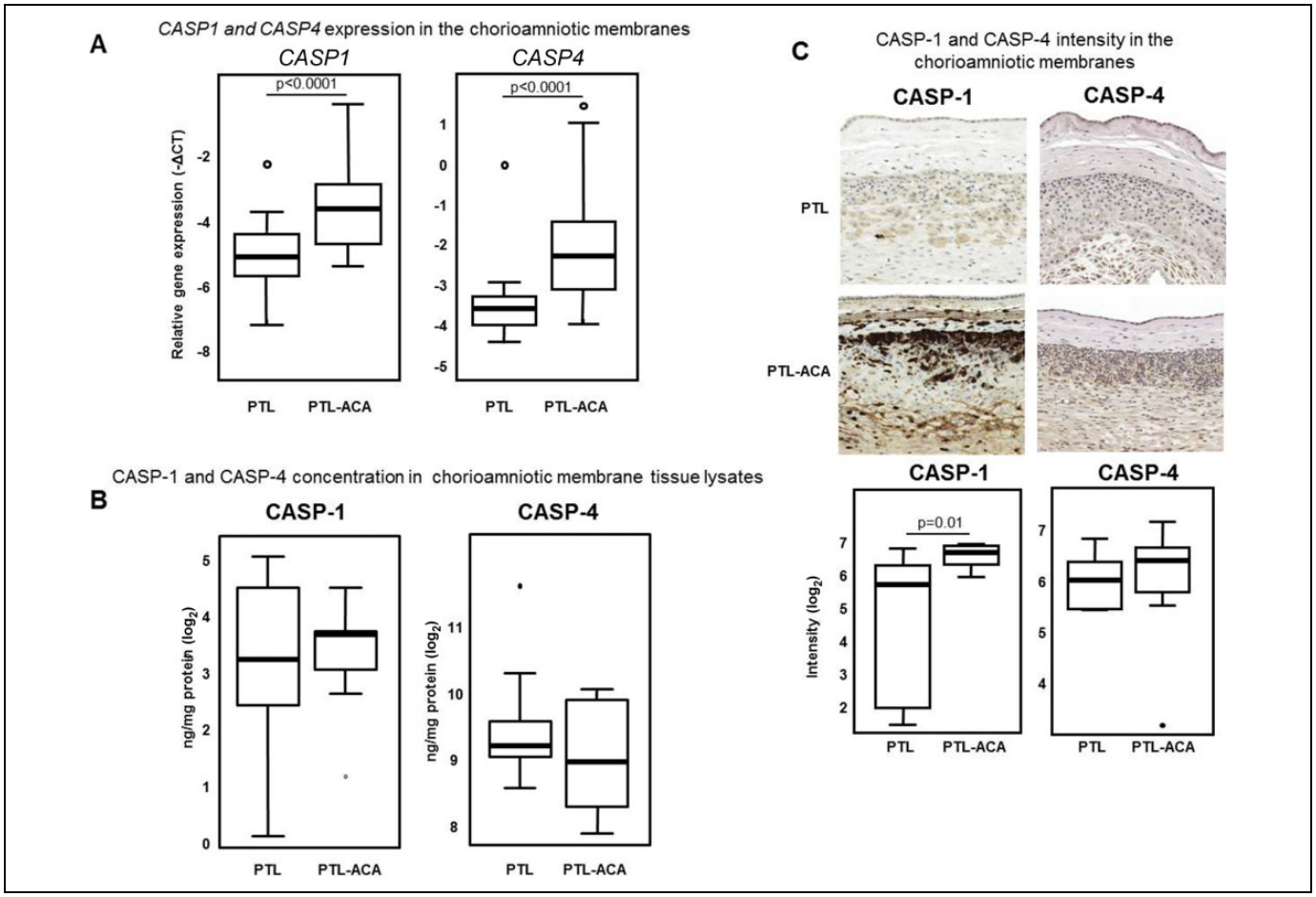


**Figure 3.** mRNA and protein expression of inflammatory caspases in the chorioamniotic membranes. A, mRNA abundance of caspase (*CASP*1) and *CASP4* in the chorioamniotic membranes from women in spontaneous preterm labor with (PTL-ACA, n = 37) or without (PTL, n = 33) acute histologic chorioamnionitis. Relative gene expressions are presented as $-\Delta$Ct values. *T* tests from an analysis of variance (ANOVA) linear model and the resulting *P* values were adjusted using the Benjamini-Hochberg procedure. B, Protein concentrations of CASP-1 and CASP-4 in the chorioamniotic membrane tissue lysates (n = 10 each). Mann-Whitney *U* tests. C, Intensity of the immunostainings for CASP-1 and CASP-4 in the chorioamniotic membranes (n = 10 each) and representative immunostainings, 200× magnifications. Mann-Whitney *U* tests. Circles denote outlier values. mRNA indicates messenger RNA; PTL, preterm labor without acute histologic chorioamnionitis; PTL-ACA, preterm labor with acute histologic chorioamnionitis.

chorioamnionitis (Figure 4A, D, E, and G). In contrast, pro-CASP-1 was reduced in the chorioamniotic membranes from women who had undergone spontaneous preterm labor with acute histologic chorioamnionitis (Figure 4B). The active form of CASP-1 (p20) in the chorioamniotic membranes from women who had undergone spontaneous preterm labor with acute histologic chorioamnionitis seemed to be lower than in those without this placental lesion; yet, this reduction did not reach statistical significance ($P = .07$, Figure 4C). The quantity of the pro-CASP-4 was not different between these 2 groups (Figure 4F). Altogether, these findings provide evidence that there is activation of CASP-1 in the chorioamniotic membranes, which is likely mediated by the inflammasome and active CASP-4 during spontaneous preterm labor with acute histologic chorioamnionitis.

### *Increased mRNA Abundance and Protein Expression of IL-1β in the Chorioamniotic Membranes in Spontaneous Preterm Labor with Acute Histologic Chorioamnionitis*

Active forms of CASP-1 can convert pro-IL-1β into its mature form.[154-156,175,176] Active CASP-4 can also induce the noncanonical activation of IL-1β.[171,177-181] Hence, we determined whether the activation of CASP-1 and CASP-4 was correlated with the abundance of the mature form of IL-1β in the chorioamniotic membranes from women who had undergone spontaneous preterm labor with acute histologic chorioamnionitis. The mRNA abundance of *IL1B* in the chorioamniotic membranes from women who underwent spontaneous preterm labor with acute histologic chorioamnionitis was increased compared to those without this placental lesion (Figure 5A). The

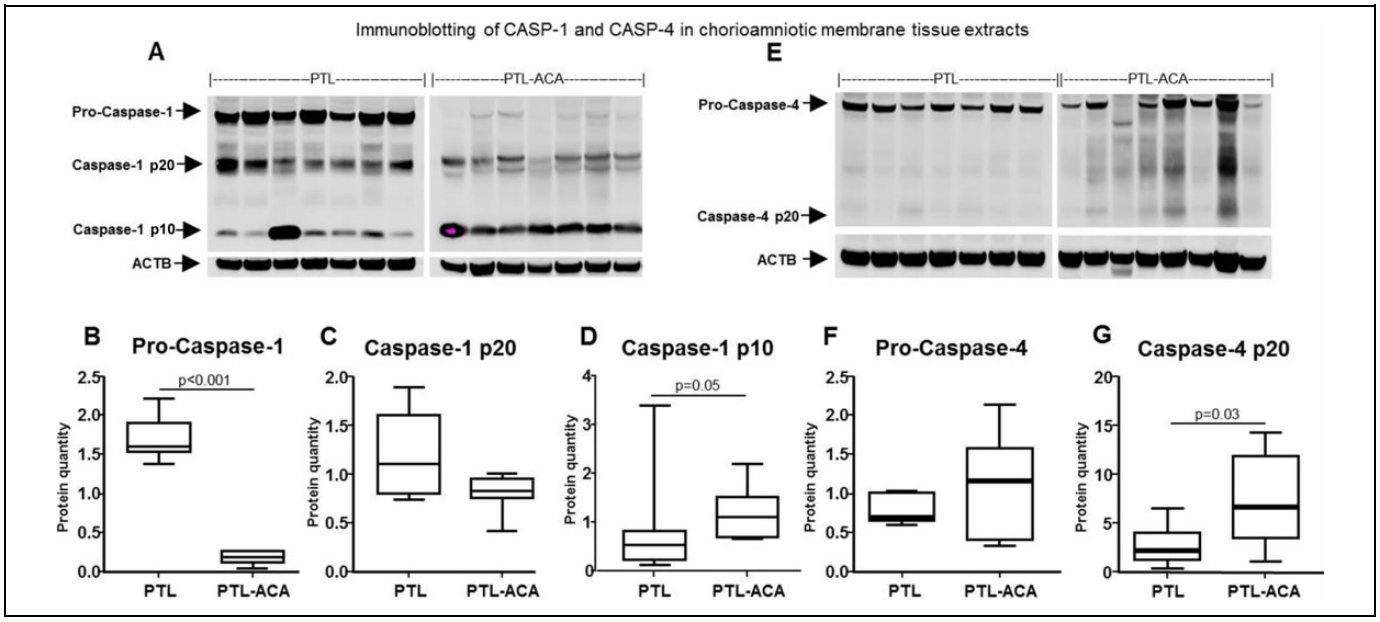


**Figure 4.** Inflammatory caspases in the chorioamniotic membranes. Immunoblotting of caspase (CASP)-1 (A) in the chorioamniotic membranes from women in spontaneous preterm labor with (PTL-ACA, n = 7) or without (PTL, n = 7) acute histologic chorioamnionitis. Quantification of the zymogen (B) and its active forms (C and D). Immunoblotting of CASP-4 (E) in the chorioamniotic membranes from women in spontaneous preterm labor with (PTL-ACA, n = 7) or without (PTL, n = 7) acute histologic chorioamnionitis. Quantification of the zymogen (F) and its active form (G). ACTB was used as an internal control and for quantifications. Mann-Whitney *U* tests; PTL, preterm labor without acute histologic chorioamnionitis; PTL-ACA, preterm labor with acute histologic chorioamnionitis.

concentration of mature IL-1β was greater in the chorioamniotic membranes from women who had undergone spontaneous preterm labor with acute histologic chorioamnionitis than in those without this placental lesion (Figure 5B). In contrast, the concentration of pro-IL-1β was reduced in preterm cases with acute histologic chorioamnionitis (Figure 5B). Furthermore, IL-1β intensity was stronger in the chorioamniotic membranes from women who underwent spontaneous preterm labor with acute histologic chorioamnionitis than in those without this placental lesion (Figure 5C). These data suggest that the active forms of CASP-1 and CASP-4 may be involved in the processing of mature IL-1β in the chorioamniotic membranes during spontaneous preterm labor with acute histologic chorioamnionitis.

## *An Increase in the Protein Expression and Mature Form of IL-18 in the Chorioamniotic Membranes in Spontaneous Preterm Labor with Acute Histologic Chorioamnionitis*

Active forms of CASP-1 can convert pro-IL-18 into its mature form.[157] Also, CASP-4 plays a role in the activation and release of IL-18 in response to enteric pathogens (eg, *Salmonella typhimurium* and enteropathogenic *Escherichia coli*) through intracellular endotoxin sensing.[179] Next, we examined whether the activation of CASP-1 and CASP-4 was linked to the release of mature IL-18 by the chorioamniotic membranes from women who underwent spontaneous preterm labor with acute histologic chorioamnionitis. In line with previously published data,[173] we found that the chorioamniotic membranes from women who had undergone spontaneous preterm labor with acute histologic chorioamnionitis had a greater protein concentration of IL-18 (Figure 6B) and a higher quantity of the mature form of this cytokine (Figure 6D) than those without this placental lesion. No significant differences were observed in the mRNA abundance (Figure 6A) and immunoreactivity (Figure 6C) of IL-18 in the chorioamniotic membranes between these 2 groups. These data demonstrate that the activation of CASP-1 and CASP-4 is associated with an increase of the protein concentration and mature form of IL-18 in the chorioamniotic membranes from women who had undergone spontaneous preterm labor with acute histologic chorioamnionitis.

A subanalysis of the protein data demonstrated positive and significant correlations between (1) active CASP-1 and mature IL-1β (Spearman $\rho = .58$, $P = .032$, Supplementary Figure 1A); (2) NLRP3 and mature IL-1β (Spearman $\rho = .76$, $P < .001$, Supplementary Figure 1B); (3) active CASP-1 and NLRP3 (Spearman $\rho = .69$, $P = .008$, Supplementary Figure 1C); (4) mature IL-18 and NLRP3 (Spearman $\rho = .53$, $P = .036$, Supplementary Figure 1D); and (5) active CASP-4 and mature IL-1β (Spearman $\rho = .61$, $P = .019$, Supplementary Figure 1E). These results suggest that all of these proteins are increased in chorioamniotic membrane samples from women who underwent spontaneous preterm labor with acute histologic chorioamnionitis compared to those without this placental lesion.

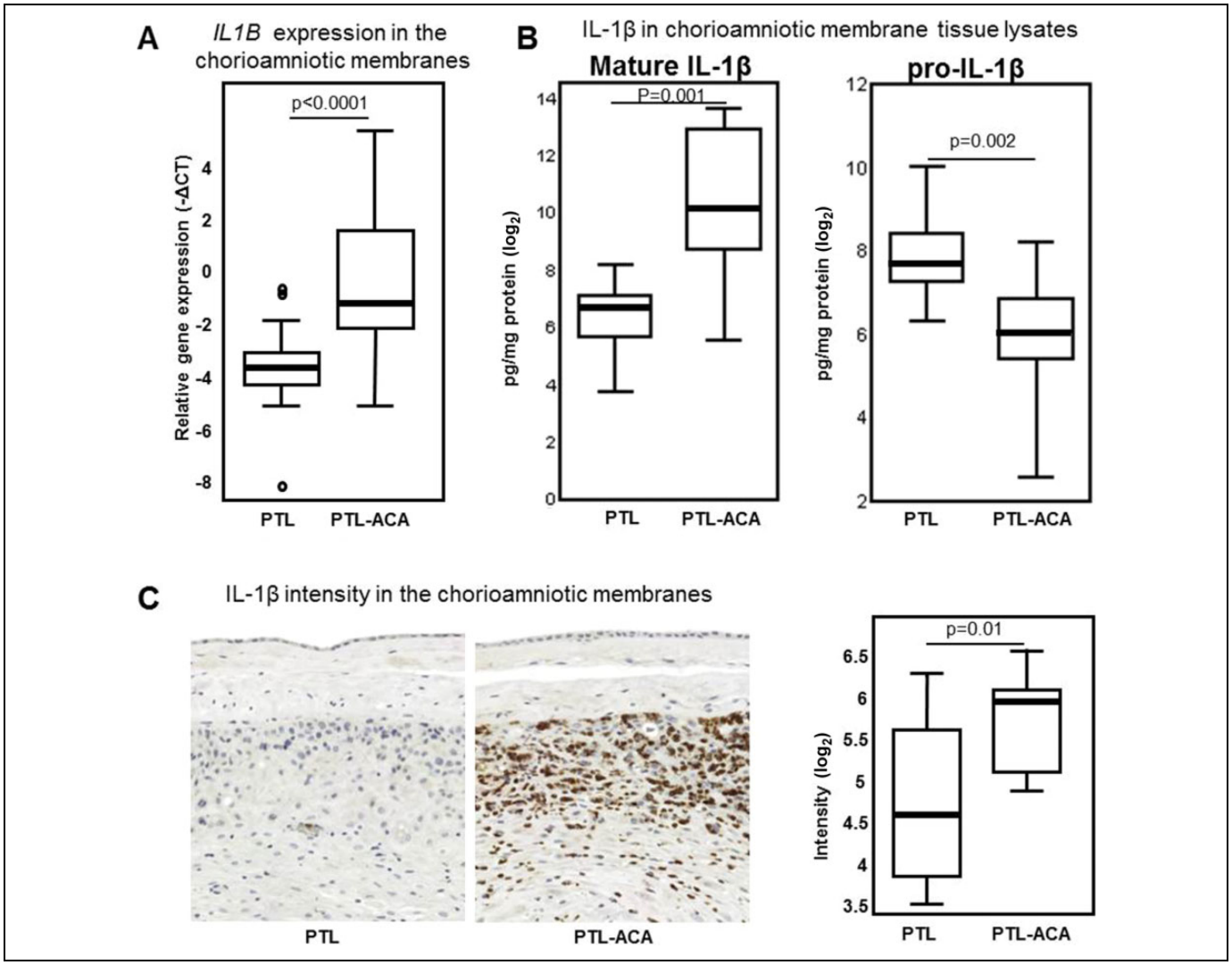


**Figure 5.** Interleukin (IL)-1β in the chorioamniotic membranes. A, mRNA abundance of *IL1B* in the chorioamniotic membranes from women in spontaneous preterm labor with (PTL-ACA, n = 37) or without (PTL, n = 33) acute histologic chorioamnionitis. Relative gene expressions are presented as $-\Delta$Ct values. *T* tests from an analysis of variance (ANOVA) linear model and the resulting *P* values were adjusted using the Benjamini-Hochberg procedure. Circles denote outlier values. B, Protein concentrations of the pro- and mature form of IL-1β in the chorioamniotic membrane lysates (n = 10 each). Mann-Whitney *U* tests. C, Intensity of the immunostainings for IL-1β in the chorioamniotic membranes (n = 10 each) and representative immunostainings, 200× magnifications. Mann-Whitney *U* tests. mRNA indicates messenger RNA; PTL, preterm labor without acute histologic chorioamnionitis; PTL-ACA, preterm labor with acute histologic chorioamnionitis.

### *Increased ASC/CASP-1 Complex Formation in the Chorioamniotic Membranes in Spontaneous Preterm Labor with Acute Histologic Chorioamnionitis*

Since there were positive correlations between the NLRP3 protein, the active form of CASP-1, and the mature forms of IL-1β and IL-18, we next investigated whether there was inflammasome assembly in the chorioamniotic membranes from women who underwent spontaneous preterm labor with acute histologic chorioamnionitis. Inflammasome assembly includes the oligomerization of the NLR protein, adaptor protein ASC, and CASP-1;[149,182] therefore, we used an in situ proximity ligation assay in order to determine the formation of ASC/CASP-1 complexes. ASC/CASP-1 complexes were identified in the chorioamniotic membranes from women who underwent spontaneous preterm labor with and without acute histologic chorioamnionitis (Figure 7A and B). However, ASC/CASP-1 complexes were greater in the chorioamniotic membranes from women who had undergone spontaneous preterm labor with acute histologic chorioamnionitis than in those without this placental lesion (Figure 7C). ASC/CASP-1 complexes were not detected in isotype controls (Figure 7D and E). A 3D reconstruction shows that there are ASC/CASP-1 complexes in all of the layers of the chorioamniotic membranes from women who underwent spontaneous preterm labor with acute histologic chorioamnionitis (Supplementary Video 1). These findings provide evidence that there is inflammasome assembly in the chorioamniotic

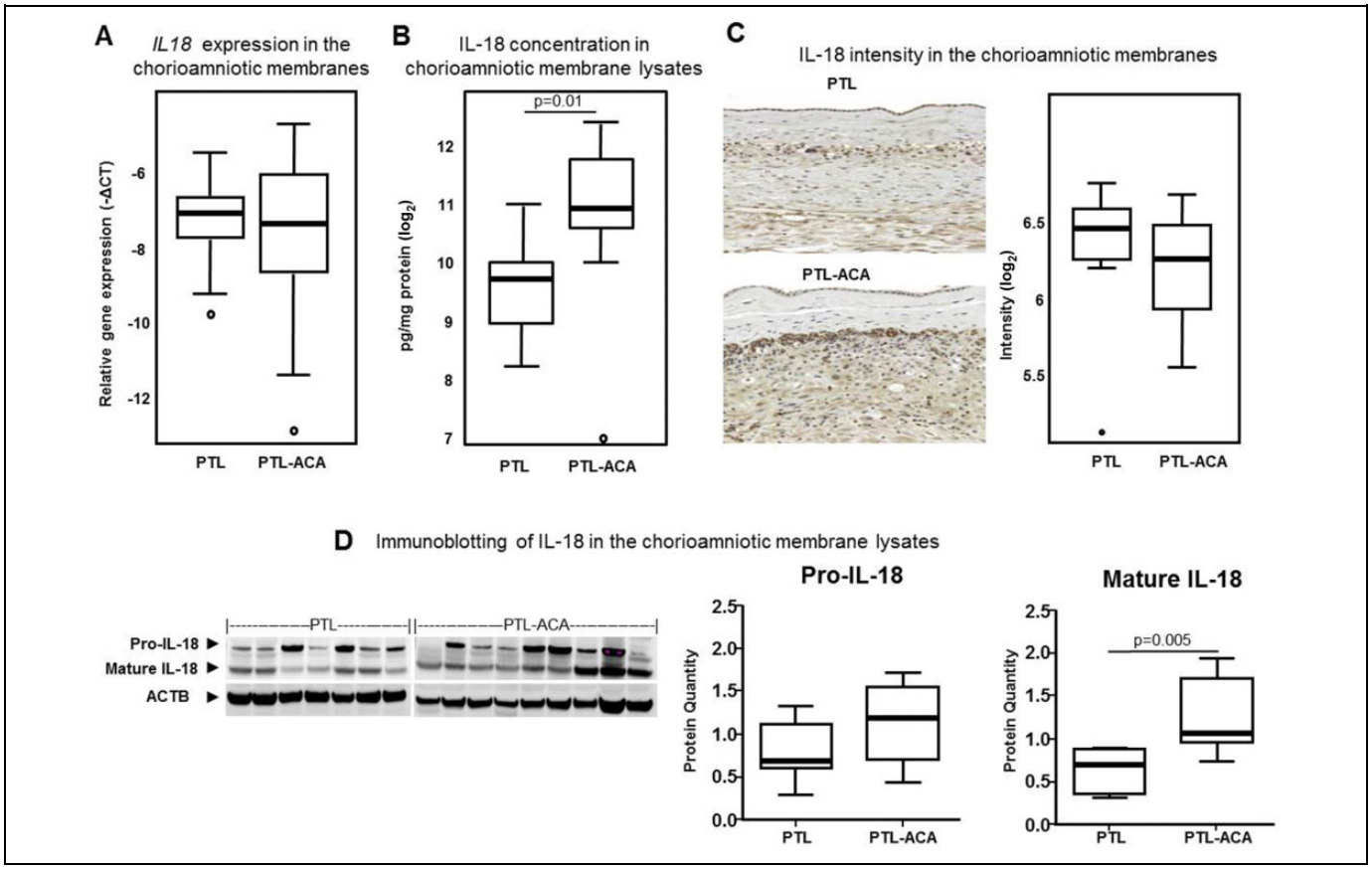


**Figure 6.** Interleukin (IL)-18 in the chorioamniotic membranes. A, mRNA abundance of *IL18* in the chorioamniotic membranes from women in spontaneous preterm labor with (PTL-ACA, n = 37) or without (PTL, n = 33) acute histologic chorioamnionitis. Relative gene expressions are presented as $-\Delta$Ct values. *T* tests from an analysis of variance (ANOVA) linear model and the resulting *P* values were adjusted using the Benjamini-Hochberg procedure. B, Protein concentrations of IL-18 in the chorioamniotic membrane tissue lysates (n = 10 each). Mann-Whitney *U* tests. C, Intensity of the immunostainings for IL-18 in the chorioamniotic membranes (n = 10 each) and representative immunostainings, 200× magnifications. Mann-Whitney *U* tests. D, Immunoblotting of IL-18 and its mature form in the chorioamniotic membranes and their quantifications (n = 7-9 each). ACTB was used as an internal control and for quantifications. Mann-Whitney *U* tests. Circles denote outlier values. mRNA indicates messenger RNA; PTL, preterm labor without acute histologic chorioamnionitis; PTL-ACA, preterm labor with acute histologic chorioamnionitis.

membranes during spontaneous preterm labor with acute histologic chorioamnionitis.

## Discussion

### *Principal Findings of the Study*

When comparing the chorioamniotic membranes from women in spontaneous preterm labor with acute histologic chorioamnionitis to those without this placental lesion, we found that (1) the mRNA expression of *NLRP1*, *NLRP3*, *NLRC4*, and *NOD2* was higher; (2) the NLRP3 protein was increased; (3) the mRNA and active form of CASP-1 were greater; (4) the mRNA and active form of CASP-4 were increased; (5) the mRNA and mature form of IL-1β were higher; (6) the mature form of IL-18 was elevated; and (7) ASC/CASP-1 complexes were increased. Altogether, these findings provide the first evidence that supports a role for the inflammasome in the pathological inflammatory process in the chorioamniotic membranes from women who had undergone spontaneous preterm labor with acute histologic chorioamnionitis.

A role for the inflammasome in the physiological[112-114] and pathological[113,114] inflammatory process of term parturition has been previously reported. Herein, we provided the first evidence that the NLRP3 inflammasome is involved in the pathological pro-inflammatory process of preterm parturition. The NLRP3 inflammasome includes the NLRP3 protein, the adaptor molecule ASC with 2 death-fold domains (1 pyrin domain and 1 CARD), and pro-CASP-1.[144,151,183,184] Several stimuli[185-191] including crystalline material,[187,192] necrosis-derived extracellular adenosine triphosphate,[193] vaccine adjuvants,[194-198] phospholipid cardiolipin and mitochondrial DNA,[199-201] bacterial toxins,[193,202,203] and other DAMPs[189,190] and pathogen-associated molecular patterns (PAMPs)[120,204-214] can induce the activation of the NLRP3 inflammasome. The process of activation of this inflammasome includes 2 steps—the priming and the oligomerization or assembly of the multiprotein complex.[182,215] The first step includes the sensing of

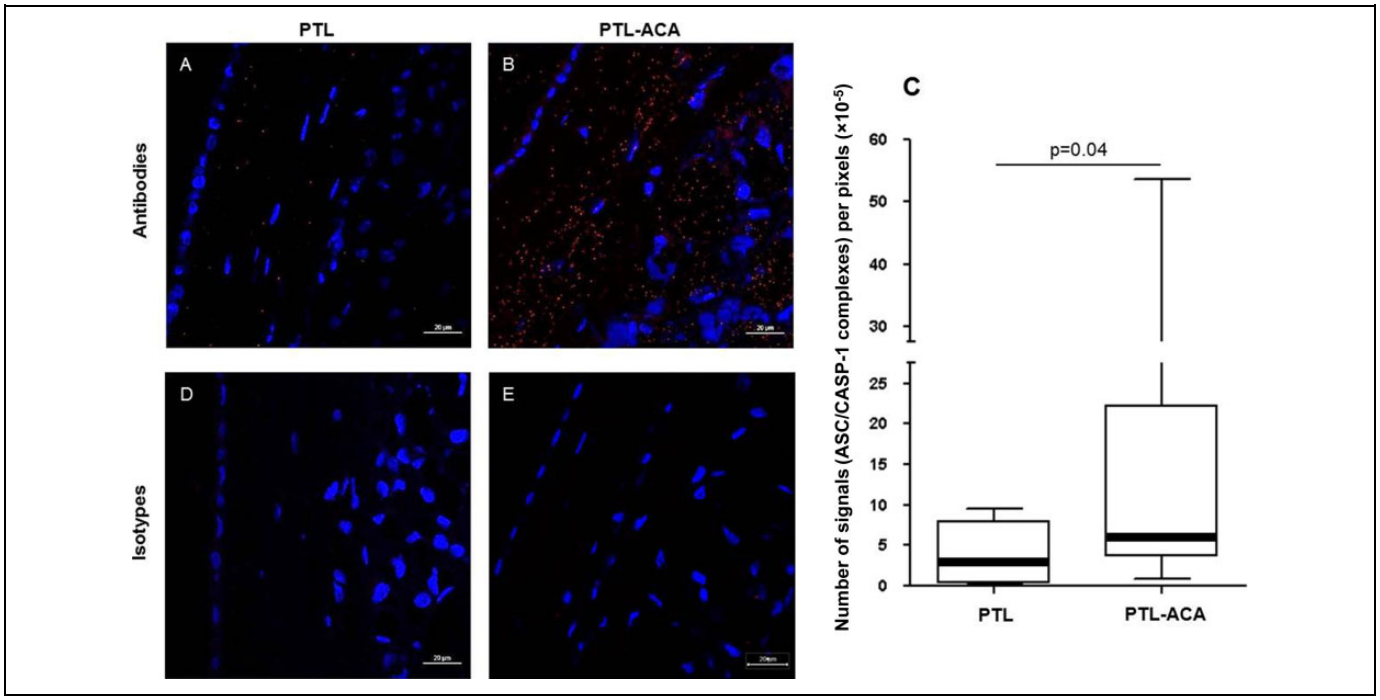


**Figure 7.** ASC/caspase (CASP)-1 complex formation in the chorioamniotic membranes. In situ proximity ligation assays for ASC and CASP-1 in the chorioamniotic membranes from women in spontaneous preterm labor with (PTL-ACA) or without (PTL) acute histologic chorioamnionitis. (A and B) Representative images of ASC/CASP-1 complexes (red signal) in PTL and PTL-ACA groups. (D and E) Representative images of isotype controls in PTL and PTL-ACA groups. The blue signal is DAPI (nuclei). 400× magnifications. C, Semi-quantification of the number of ASC/CASP-1 complexes in the chorioamniotic membranes (PTL and PTL-ACA, n = 7 each). ASC/CASP-1 complexes were calculated by dividing the number of signals over the area of the tissue, which was expressed as pixels. Mann-Whitney *U* tests.

the DAMP and/or PAMP via the PRR which, in turn, induces the activation of the nuclear factor kappa B pathway, resulting in the upregulation of the NLRP3 protein and the expression of pro-IL-1β.[182,215,216] The second step permits the oligomerization or assembly of the inflammasome complex, which includes the NLRP3, ASC, and CASP-1 proteins.[182,215] The current study provides evidence that there is priming and assembly of the NLRP3 inflammasome complex in the chorioamniotic membranes during spontaneous preterm labor with acute histologic chorioamnionitis. It is worth mentioning that there were some ASC/CASP-1 complexes (ie, inflammasome assembly) in the chorioamniotic membranes from women who underwent spontaneous preterm labor without acute chorioamnionitis. These data suggest that the inflammasome is also involved in the process of preterm parturition in the absence of acute histologic chorioamnionitis.

Oligomerization of the inflammasome leads to the recruitment of ASC, which binds and activates pro-CASP-1 via its CARD.[144,217] In the study herein, we found that the mRNA abundance, immunoreactivity, and the active form of CASP-1 (p10) are increased in the chorioamniotic membranes in spontaneous preterm labor with acute histologic chorioamnionitis. This finding is consistent with previous studies demonstrating that: (1) amniotic fluid CASP-1 concentrations are greater in women who underwent spontaneous preterm labor with intraamniotic infection/inflammation than in those who underwent preterm labor without this clinical condition;[113] and (2) CASP-1 concentration and the abundance of its mature form are increased in the uteri of mice prior to inflammation-induced preterm birth (mice injected with peptidoglycan and polyinosinic-polycytidylic acid) compared to term controls.[218] Together, these data suggest that during spontaneous preterm labor with acute histologic chorioamnionitis, the chorioamniotic membranes release abundant quantities of the active form of CASP-1, which is most likely mediated by the inflammasome.

CASP-4 (murine homologue CASP-11) is implicated in the activation of CASP-1 and participates in the non-cannonical activation of the inflammasome.[149,172,213,219] In the current study, we found that the mRNA abundance of CASP-4 and its mature form (p20) were increased in the chorioamniotic membranes from women who underwent spontaneous preterm labor with acute histologic chorioamnionitis. These data indicate that CASP-4 could be mediating the noncanonical activation of the inflammasome in spontaneous preterm labor with acute histologic chorioamnionitis. We suggest that the activation of CASP-4 is also implicated in the induction of preterm parturition in the setting of sterile intraamniotic inflammation. This concept is based on 2 observations—(1) CASP-4 can induce the release of alarmins in a CASP-1–independent manner[172] and (2) the intraamniotic administration of HMGB1 (a classic alarmin[220]) can induce preterm labor and birth.[221]

Following activation, CASP-1 converts inactive pro-IL-1β into its mature and secreted form.[155,222-228] IL-1β induces the expression and release of different mediators implicated in the

process of labor.[229-235] Indeed, the administration of IL-1β causes preterm birth in mice [236,237] and monkeys,[238-245] confirming an essential role for this cytokine in the pathological process of labor. This effect can be abrogated by the administration of the IL-1β receptor antagonist.[237] In the study herein, we demonstrated that the chorioamniotic membranes from women in spontaneous preterm labor with acute histologic chorioamnionitis released high amounts of mature IL-1β, which is most likely mediated by the active forms of CASP-1. This mature form of IL-1β will then participate in the pathological pro-inflammatory milieu that accompanies the premature process of labor. It is worth mentioning that pro-IL-1β was reduced in the chorioamniotic membranes from women in spontaneous preterm labor with acute histologic chorioamnionitis compared to those without this placental lesion, which is most likely due to the processing of this zymogen into its mature form.

The active forms of CASP-1[157-160,163] and CASP-4[179] can also convert pro-IL-18 into its mature form. IL-18 is present in the amniotic fluid, and its concentration is greater in women who underwent spontaneous preterm labor with microbial invasion of the amniotic cavity than in those without this clinical condition.[173,246] In the present study, the total concentration and mature form of IL-18 was increased in the chorioamniotic membranes from women who underwent spontaneous preterm labor with acute histologic chorioamnionitis. IL-18 is a major interferon γ inducing factor that activates Th-1 responses in T cells and natural killer cells.[247-254] Therefore, it is likely that IL-18 participates in the cellular pro-inflammatory milieu in the chorioamniotic membranes during spontaneous preterm labor with acute histologic chorioamnionitis.

## Conclusion

Herein, we provide the first evidence that supports a role for the NLRP3 inflammasome and CASP-4 in the activation of CASP-1 and the consequent release of mature IL-1β and IL-18 by the chorioamniotic membranes from women who underwent spontaneous preterm labor with acute histologic chorioamnionitis. These findings provide insight into the mechanisms that are implicated in the pathological pro-inflammatory process of spontaneous preterm labor in acute inflammation of the chorioamniotic membranes.

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### Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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### Supplementary Material

Supplementary material is available for this article online.

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