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A Role for the Inflammasome in Spontaneous Labor at Term

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Running head: The inflammasome in spontaneous labor at term

ABSTRACT

Objectives: Inflammasomes are signaling platforms that upon sensing pathogens and sterile stressors mediate the release of mature forms of interleukin (IL)-1β and IL-18. The aims of this study were to determine: 1) the expression of major inflammasome components in the chorioamniotic membranes in spontaneous labor at term; 2) whether there are changes in the inflammasome components associated with activation of caspase-1 and caspase-4; and 3) whether these events are associated with the release of the mature forms of IL-1β and IL-18.

Methods: Chorioamniotic membranes were collected from women at term with and without spontaneous labor. mRNA abundance and protein concentrations of inflammasome components, nucleotide-binding oligomerization domain-containing (NOD)1 and NOD2 proteins, caspase-1 and caspase-4, IL-1β, and IL-18 were quantified by qRT-PCR (n=28-29 each), ELISA (n=10 each) or immunoblotting (n=8 each), and immunohistochemistry (n=10 each). Active caspase-1 and caspase-4, as well as mature IL-18, were determined by immunoblotting (n=4 each), and pro- and mature forms of IL-1β were determined by ELISA (n=4-7 each).

Results: Inflammasome components and NOD proteins were expressed in the chorioamniotic membranes obtained from women at term. The chorioamniotic membranes from women who had undergone labor had: 1) higher NLRP3 (NOD-like receptor family, pyrin domain-containing protein 3) and NOD1 protein concentrations; 2) greater immunoreactivity for caspase-1 and caspase-4; 3) higher quantity of the active form of caspase 1 (p20); and 4) higher mRNA abundance and protein concentrations of pro- and mature IL-1β. However, mRNA abundance and protein concentrations of the mature form of IL-18 were not increased in tissues from women who underwent labor at term.

Conclusions: Spontaneous labor at term is characterized by the expression of inflammasome components , which may participate in the activation of caspase-1 leading to the consequent cleavage and release of mature IL-1β by the chorioamniotic membranes. These results support the participation of the inflammasome in the mechanisms responsible for spontaneous parturition at term.

Keywords: amnion, caspase-1, caspase-4, chorion, cytokine, labor, IL-1β, IL-18, NOD1, NOD2, normal pregnancy, parturition, sterile inflammation, preterm labor, biomarker, chorioamniotic membranes, NLRP1, NLRP3, NLRC4, AIM2

INTRODUCTION

Spontaneous term labor is a state of physiologic sterile inflammation.[1-6] The evidence in support of this concept includes the increased bioavailability of cytokines[7-21] and chemokines[22-27] in the amniotic fluid, maternal circulation,[28-31] and reproductive tissues.[20, 32-46] Moreover, parturition is accompanied by an influx of inflammatory cells, e.g., neutrophils and macrophages, into the cervix,[39, 47-56] myometrium,[49, 57-63] and chorioamniotic membranes.[35, 64-67] T cells are also present in the chorioamniotic membranes.[66, 68] The inflammatory response associated with normal spontaneous labor is considered sterile since intra-amniotic infection is absent in most women.[1, 2, 69-72]

The mechanisms responsible for sterile inflammation in parturition have not been elucidated, but are thought to involve inflammasomes,[73-75] which are high-molecular-weight multi-subunit protein complexes found in the cytoplasm capable of inducing an inflammatory response through the production of interleukin (IL)-1β and IL-18.[76-110] Their basic structure consists of: 1) an inflammasome sensor molecule, 2) the adaptor protein ASC (an apoptosis-associated speck-like protein), and 3) pro-caspase-1(pro-CASP-1).[76-110] Once activated, the inflammasome complex induces auto-catalytic cleavage of pro-CASP-1 into its mature/active form. Caspase-1 (CASP-1) can then cleave pro-IL-1β and pro-IL-18, and the newly described pro-IL-33, into the mature, secreted forms of the cytokines.[111-124] In addition, CASP-1 is required for a specific type of programmed cell death induced by inflammation: pyroptosis.[125-127] Recently, it was demonstrated that CASP-4 expression is required for activation of CASP-1 in ultraviolet B-irradiated keratinocytes and activated macrophages,[128] suggesting that caspase-4 acts upstream of caspase-1 and the inflammasome.[129]

Several inflammasomes have been identified and named after their respective pattern recognition receptors (PRRs):[130] NLR family pyrin domain (NLRP)1,[76] NLRP3,[131] NLR family caspase activation and recruitment domain (CARD) (NLRC)4 (also known as IPAF),[132, 133] interferon gamma-inducible protein 16 (IFI16),[134] or absent in melanoma (AIM2).[135-143] Inflammasome specificity depends on which ligand(s) the PRR recognizes, and once PRR-ligand binding occurs, the inflammasomes oligomerize with other components of the multi-protein complexes and become activated.[86, 94, 104, 110, 144, 145] Two additional PRRs belonging to the NLR family – the nucleotide-binding oligomerization domain-containing proteins 1 and 2 (NOD1 and NOD2) – recognize bacterial peptidoglycan segments, but do not recruit inflammasome

components.[90, 146-153] Instead, the NODs directly activate nuclear factor kappa B (NF-κB) proinflammatory signaling, which can induce the expression of pro-IL-1β[76, 87, 131, 146, 154-158] and pro-IL-18.[115] The functional combination of NOD proteins and inflammasome components (e.g., NOD2 and NLRP3) improves immune responses in murine dendritic cells.[159] Moreover, inflammasome activation is associated with an increased production of eicosanoids (prostaglandins and leukotrienes), which leads to further inflammation.[160]

We proposed that the inflammasome participates in labor at term[73, 74] and in pregnancy complications.[161-171] Indeed, we demonstrated that CASP-1, the predominant inflammasome-activated caspase,[82, 172] is present in the amniotic fluid and that its concentration increases as a function of gestational age.[75] In addition, we found that amniotic fluid CASP-1 concentration is higher in women in spontaneous labor at term than in those without labor.[75] This is mirrored by increased amniotic fluid IL-1β bioactivity and immunoreactivity in women in spontaneous labor at term.[8, 9, 36] IL-18 concentration in the amniotic fluid is also higher in term pregnancies than in the second trimester.[173] Collectively, this evidence supports the hypothesis that inflammasomes are involved in the physiologic sterile inflammatory process associated with spontaneous labor at term. The aims of this study were to determine whether: 1) inflammasomes are expressed in the chorioamniotic membranes from women who underwent spontaneous labor at term; 2) changes in inflammasome components are associated with activation of CASP-1 and CASP-4; and 3) these events are associated with the release of the mature forms of IL-1β and IL-18.

MATERIALS AND METHODS

Human subjects, clinical specimens, and definitions

A case control study was conducted including patients who delivered at term without labor (TNL) or at term after labor (TIL). Chorioamniotic membrane samples were collected from the Bank of Biological Specimens of the Perinatology Research Branch, NICHD/NIH/DHHS, Wayne State University, and The Detroit Medical Center (Detroit, MI, USA). The Institutional Review Boards of these institutions approved the collection and use of biological materials for research purposes. All participating women provided written informed consent, and samples were collected within 30 minutes after delivery. Demographic and clinical characteristics of these study groups are represented in **Table I**. Patients with multiple births or with neonates

having congenital or chromosomal abnormalities were excluded. Labor was defined by the presence of regular uterine contractions at a frequency of at least two contractions every 10 minutes with cervical changes resulting in delivery.[174] In each case, tissue sections of the chorioamniotic membranes were evaluated for acute histologic chorioamnionitis, according to published criteria,[175, 176] by pathologists who had been blinded to the clinical outcome. Samples collected from women with acute histologic chorioamnionitis were excluded from this study.

RNA isolation, cDNA generation, and qRT-PCR analysis

TRIzol® (Invitrogen™, Life Technologies Corporation, Grand Island, NY, USA) and Qiagen RNeasy® Kits (Qiagen, Gaithersburg, MD, USA) were used to extract total RNA from snap-frozen chorioamniotic membrane tissues (TNL, n=29, and TIL, n=28). RNA purity and concentration were assessed with the NanoDrop® 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA), and RNA integrity was evaluated with the Bioanalyzer 2100 (Agilent Technologies, Wilmington, DE, USA). The SuperScript® III First-Strand Synthesis System (Invitrogen) and oligo(dT)20 primers (Invitrogen) were utilized to generate cDNA. Gene expression profiling was performed on the BioMark™ System for high-throughput qRT-PCR (Fluidigm, San Francisco, CA, USA) and on the ABI 7500 FAST Real-Time PCR System (Applied Biosystems®, Life Technologies Corporation, Foster City, CA, USA) with TaqMan® gene expression assays (Applied Biosystems) listed in **Table II**.

Chorioamniotic membrane tissue lysates

Fragments of snap-frozen chorioamniotic membranes (TNL and TIL; n=10 each) were homogenized using a mechanical tissue homogenizer (T-25 Ultra-Turrax®, IKA® Works, Inc., Wilmington, NC, USA) in 2ml of 1X PBS containing a complete protease inhibitor cocktail (Cat. No. 11697498001; Roche Applied Science, Mannheim, Germany). Tissue lysates were centrifuged at 15700 x g for 5 min at 4°C, and the supernatant was collected and stored at -80°C. The protein concentration of the lysates was determined using the Quick Start™ Bradford Protein Assay Kit (Bio-Rad, Hercules, CA, USA). Triplicate cell lysates were obtained from the membranes of each patient.

Chorioamniotic membrane tissue supernatants

Chorioamniotic membrane samples were collected from each group of women (TNL, n=7, and TIL, n=4) and processed the same day. Tissue samples were washed with 1X PBS (Invitrogen) and cut into 2cm x 2cm pieces. These tissue explants were transferred into 6-well tissue culture plates containing 2ml of Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) per well supplemented with 10% Fetal Bovine Serum (FBS) (Invitrogen) and 1% Penicillin/Streptomycin (P/S) (Invitrogen). Tissue samples were placed in a humidified 5% CO_2 incubator at 37ºC overnight, and then tissue culture supernatants were collected and stored at -80°C. Triplicate supernatants were obtained from the membranes of each patient.

Enzyme-linked immunosorbent assays

The concentrations of NLRP1, NLRP3, AIM2, NOD2, CASP-1, CASP-4, IL18, pro-IL-1β, and IL-1β were measured in the chorioamniotic membrane tissue lysates or supernatants using specific and sensitive 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, TX, USA; pro-IL-1β and IL-1β ELISA kits from R&D Systems, Minneapolis, MN, USA; IL-18 ELISA kits from MBL International Corporation, Woburn, MA, USA), following the manufacturers' instructions. Briefly, recombinant human standards and the samples were incubated in duplicate wells of the 96-well microplates pre-coated with monoclonal antibodies specific for target analytes. During incubation, immobilized antibodies in the microplates bound to the target proteins present in the standard and sample groups. After washing the unbound substances, enzyme-conjugated antibodies bound to the target analytes were added to the wells. After the incubation, assay plates were washed to remove the unbound antibodies, followed by the addition of a substrate solution that developed color proportional to the amount of target protein bound in the initial step. Finally, the color development was stopped by the addition of a sulfuric acid solution, and the microplates were read using a programmable spectrophotometer (SpectraMax M5 Multi-Mode Microplate Reader, Molecular Devices, Sunnyvale, CA, USA). The sensitivities of the assays were <4.68 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 measures approximately 10% of the pro-IL-1β. The immunoassays for NLRC4 and NOD1 ELISA did not meet our criteria for validation; instead, immunoblotting was performed.

Immunohistochemistry

Samples of chorioamniotic membranes collected from each study group (TNL and TIL; n=10 each) were included. Five-µm-thick sections of formalin-fixed, paraffin-embedded chorioamniotic membrane tissues were placed on silanized slides. Immunostainings for NLRP1, NLRP3, NLRC4, AIM2, NOD1, NOD2, CASP-1, CASP-4, IL-1β, and IL-18 were performed using a Leica Bond Max automatic staining system (Leica Microsystems, Wetzlar, Germany), and the Bond™ Polymer Refine Detection Kit (Leica Microsystems) was used to detect the chromogenic reaction of horseradish peroxidase. Primary antibodies and a description of the immunostaining conditions are presented in **Table III**. Mouse IgG (Invitrogen) and rabbit IgG (Invitrogen) were used as negative controls. A PerkinElmer Pannoramic MIDI slide scanner (PerkinElmer, Waltham, MA, USA) was used to assess the intensity of staining (a semi-quantitative method of analysis).

Chorioamniotic membrane tissue extracts

Chorioamniotic membrane samples from the two study groups (TNL and TIL; n=4 each) were collected and processed on the same day. Ten or 12 tissue explants were obtained from each membrane using a dermatological punch (12mm Acu-Punch, Acuderm Inc., Fort Lauderdale, FL, USA). Tissue explants were placed at 37ºC in a humidified 5% CO_2 incubator for 24h in 500µL of DMEM (4.5 g/L glucose, L-glutamine, sodium pyruvate, and 1% antibiotics; Gibco®, Life Technologies) in a 24-well plate. Following incubation, tissue explants were homogenized in their conditioned medium using a mechanical tissue homogenizer (T-25 Ultra-Turrax, IKA Works, Inc.). Tissue extracts were centrifuged at 14,000 g for 3-5 min at 4°C, and the supernatant was collected and filtered using a syringe filter (Millex-GV Syringe Filter Unit, 0.22µm, PVDF, 33mm, gamma-sterilized, EMD Millipore, Billerica, MA, USA). Tissue extracts were stored at -80°C until use.

Immunoblotting

Chorioamniotic membrane tissue extracts (40µg for the caspases and 100µg for IL-18 per well) or tissue lysates (20µg for NOD1 and NLRC4 per well) were subjected to 4-12% SDS-polyacrylamide gel electrophoresis (Invitrogen). After electrophoresis, separated proteins were transferred onto nitrocellulose membranes (Bio-Rad), and the membranes were blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween-20 (BioRad) and probed overnight at 4°C with specific human antibodies (mouse anti-CASP-1 monoclonal antibody [R&D Systems], rabbit anti-CASP-4 polyclonal antibody [Abcam, Cambridge, MA, USA], rabbit anti-IL-18 polyclonal antibody [Santa Cruz Biotechnology, Dallas, TX, USA], rabbit anti-NOD1 polyclonal antibody [Enzo Life Sciences, Farmingdale, NY, USA],[165] or mouse anti-NLRC4 (IPAF) antibody [BioLegend, San Diego, CA, USA]). Nitrocellulose membranes were then stripped with Restore™ Plus Western Blot Stripping Buffer (Pierce Biotechnology, Thermo Fisher Scientific Inc., Rockford, IL, USA) for 15 min, washed with PBS, blocked, and probed for 1h at room temperature with a mouse anti-GAPDH monoclonal antibody (Santa Cruz Biotechnology) or a mouse anti-ACTB monoclonal antibody (Sigma-Aldrich Co., Saint Louis, MO, USA). A horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Cell Signaling, Boston, MA, USA) was used as a secondary antibody. Signals were detected by chemiluminescence with ChemiGlow West Reagents (Protein Simple, Santa Clara, CA, USA). Images were acquired using the FUJIFILM LAS-4000 Imaging System (FUJIFILM North America Corporation, Valhalla, NY, USA).

Statistical analyses

Demographic and clinical data were analyzed using SPSS v.19.0 (SPSS Inc., Chicago, IL, USA). Comparisons among the groups were performed using the Chi-square and Fisher's exact tests for proportions, as well as the Mann-Whitney U-test for non-normally distributed continuous variables. All other data were analyzed in the R statistical language and environment (www.r-project.org). The qRT-PCR dataset, gene expressions relative to *ACTB*/*GAPDH/RPLP0* were calculated, and the fold-changes between the groups were estimated using a linear model in which the -ΔCt value of a gene (surrogate for $\log_2$ gene expression) was the dependent variable and the group was the independent variable. ELISA, IHC, and immunoblotting data were

analyzed using linear models in which the protein concentration or intensity was the dependent variable. A fold-change of expression >1.5 and a *P* value of <0.05 were regarded as statistically significant.

RESULTS

NLRP3 and NOD2 concentrations increase in the chorioamniotic membranes in spontaneous labor at term

We first investigated whether major inflammasome components (*NLRP1*, *NLRP3*, *NLRC4*, and *AIM2*), and NOD proteins (*NOD1* and *NOD2*) were expressed by the chorioamniotic membranes from women who had undergone spontaneous labor at term. All genes encoding for these proteins were expressed at a detectable level in the chorioamniotic membranes from women with or without spontaneous labor at term. However, no differences were observed in the expression of the evaluated genes, except *NOD1*, between these two groups of women (Figure 1A,Figures 2A and 2D).

NLRP3 and NOD2 concentrations were higher in the chorioamniotic membrane tissue lysates from women who underwent spontaneous labor at term than in those without labor (Figure 1B). However, no differences between these two groups of women were observed in the protein concentrations of the other evaluated NLRs (Figure 1B, Figures 2B and 2E). Immunostaining on formalin-fixed, paraffin-embedded tissue sections collected from the same samples showed that all these proteins are expressed in the chorioamniotic membranes. In general, most of the proteins showed immunoreactivity in the chorionic trophoblast cells as well as in the decidual stromal cells, while some showed weak immunoreactivity in the amniotic mesodermal and epithelial cells (Figure 1C, Figures 2C and 2F).

Collectively, these results suggest that inflammasomes may participate in the physiologic sterile inflammatory process of spontaneous parturition at term. Additionally, the constitutive expression of the investigated inflammasome components and NOD proteins in the chorioamniotic membranes suggests that inflammasomes may participate in pathological innate immune activation leading to preterm labor and term labor in the setting of intra-amniotic infection or sterile intra-amniotic inflammation.

Activation of CASP-1 in the chorioamniotic membranes in spontaneous labor at term

Activation of the inflammasome leads to the activation of the inflammatory caspases, i.e., CASP-1 and CASP-4.[76, 110, 128, 177, 178] We previously demonstrated that amniotic fluid concentration of CASP-1 is higher in women in spontaneous labor at term than in those wihout labor at term.[75] Therefore, we investigated whether the higher NLRP3 and NOD2 concentrations were associated with the activation of CASP-1 and CASP-4 in the chorioamniotic membranes in spontaneous labor at term. Genes encoding for *CASP-1* and *CASP-4* were expressed at detectable levels in the chorioamniotic membranes from women with or without spontaneous labor at term; however, the mRNA abundance and protein concentration were not significantly different between these two groups (Figures 3A and 3B). Semi-quantitative analysis of immunostaining indicated that the intensity of both CASP-1 and CASP-4 was higher in the chorioamniotic membranes from women who underwent spontaneous labor at term than in those without labor (Figure 3C). We also found that the immunoreactivity of the active form of CASP-1 (p20) was greater in the chorioamniotic membranes from women who underwent spontaneous labor at term than in women who did not undergo labor**.** The active form of CASP-4 was undetectable by immunoblotting (Figure 3D). These data suggest that spontaneous labor at term involves the participation of inflammasomes which, in turn, could activate CASP-1 in the chorioamniotic membranes.

Increased mRNA abundance and protein concentration of IL-1β in the chorioamniotic membranes in spontaneous labor at term

Activated CASP-1 subunits (p10 and p20) are able to convert inactive pro-IL-1β into its bioactive and secreted form.[112-114] We previously reported that the IL-1β concentration in amniotic fluid (and IL-1 bioactivity) was higher among women in labor than in those wihout labor.[7-9] Therefore, we investigated whether the activation of CASP-1 was associated with the release of mature IL-1β in the chorioamniotic membranes in women who underwent spontaneous labor at term. mRNA abundance of *IL1B* in the chorioamniotic membranes was higher in women who had undergone spontaneous labor at term than in those from women without labor (Figure 4A). The concentrations of mature IL-1β and its pro-form were also higher in the chorioamniotic membranes from women who underwent labor than in those from women without labor (Figure

4B). IL-1β immunoreactivity appeared to be greater in the chorioamniotic membranes from women with labor than in those without labor (Figure 4C). These data suggest that the active forms of CASP-1 may participate in the release of the mature form of IL-1β by the chorioamniotic membranes in spontaneous labor at term.

IL-18 expression does not increase in the chorioamniotic membranes in spontaneous labor at term

Activated CASP-1 subunits (p10 and p20) are also able to convert inactive pro-IL-18 into its bioactive and secreted form.[115] We previously demonstrated that during term pregnancies the IL-18 amniotic fluid concentration tends to be higher in women who undergo spontaneous labor than in women without labor; yet, this increase was not statistically significant.[173] We therefore investigated whether activation of CASP-1 was associated with the release of mature IL-18 in the chorioamniotic membranes of women who underwent spontaneous labor at term. Consistent with our published findings,[173] we found that the mRNA abundance and protein concentration of IL-18 in the chorioamniotic membranes were not different between women with and without spontaneous labor at term (Figure 5A-C). Indeed, mRNA expression of IL-18 was lower in the chorioamniotic membranes from women who underwent labor at term than in those without labor (Figure 5A)**.** No differences were observed in the abundance of the mature form of IL-18 in the chorioamniotic membranes between women with and without spontaneous labor at term (Figure 5D). These data suggest that IL-18 does not participate in the physiologic sterile inflammatory process of spontaneous parturition at term.

DISCUSSION

Principal findings of the study: 1) Inflammasome components and NOD proteins were expressed in the chorioamniotic membranes from women with normal term pregnancies; 2) NLRP3 and NOD2 protein concentrations were greater in the chorioamniotic membrane tissue lysates from women who underwent labor at term than in those from women who did not undergo labor; 3) the immunoreactivity of CASP-1 and CASP-4 in the chorioamniotic membranes was higher among women in labor at term than in those without labor; 4) the active form of CASP-1 (p20) was higher in the chorioamniotic membranes from women in labor at

term than in women without labor; 5) mRNA abundance and protein expression profiles of IL-1β were greater in the chorioamniotic membranes from women in spontaneous labor at term than in those without labor; and 6) mRNA abundance and protein expression profiles of IL-18 did not increase in the chorioamniotic membranes from women who underwent spontaneous labor at term. Collectively, these data support a role for the inflammasome (NLRP1, NLRP3, AIM2 or NLRC4) in the activation of CASP-1 and the consequent release of mature IL-1β by the chorioamniotic membranes of patients who underwent spontaneous labor at term.

The expression of inflammasome components and NOD proteins in the chorioamniotic membranes at term

We first demonstrated that inflammasome components NLRP1, NLRP3, NLRC4, and AIM2, as well as NOD1 and NOD2 proteins, were expressed at the mRNA and protein levels by the chorioamniotic membranes from women at term. This is consistent with previous studies showing that the human placenta constitutively expresses inflammasome components including NOD1, NOD2, NOD3, NOD4, NALP1, NALP2, NALP4, NALP7, NALP10, NALP12, and NAIP, as well as CASP-1, CASP-4, and CASP-5,[179] and that the chorioamniotic membranes express NOD1, NOD2, NLRP1, NLRP3, and ASC.[165] The expression of inflammasome components and inflammatory caspases in the chorioamniotic membranes may be an important feature of innate immune mechanisms at the maternal-fetal interface, which will ensure the rapid activation of an immune response when exogenous and/or endogenous signal(s) are recognized.

Increased concentrations of NLRP3 and NOD2 in the chorioamniotic membranes in spontaneous labor at term

A role for the inflammasomein the physiological proinflammatory processes of spontaneous labor at term was initially suggested by our group.[73, 75] Herein, we provide evidence to support this hypothesis, and demonstrate for the first time that NLRP3 (also known as cryopyrin), the PRR component of the NLRP3 inflammasome, is increased in the chorioamniotic membranes during spontaneous labor at term. Besides cryopyrin, the NLRP3 inflammasome contains the adaptor molecule ASC containing two death-fold domains, one pyrin domain and one CARD, and pro-caspase-1.[104, 131, 180, 181] Activation of the NLRP3 inflammasome can be

triggered by several stimuli,[182-188] chemically and structurally different, including crystalline material,[184, 189] extracellular ATP released from dying cells,[190] peptide aggregates such as vaccine adjuvant,[191-195] phospholipid cardiolipin and mitochondrial DNA,[196-198] bacterial toxins [190, 199, 200] [i.e., nigericin (*Streptomyces hygroscopicus*), listeriolysin O (*Listeria monoccytogenes)*, aerolysin (*Aeromonas*), β-and-γ hemolysins (*Staphylococcus aureus*)], DAMPs (damage-associated molecular patterns),[186, 187] and PAMPs (pathogen-associated molecular patterns) [80, 201-211]. Activation of the NLRP3 inflammasome requires two steps: priming and assembly of the inflammasome complex.[212, 213] The priming step is initiated by PRRs, cytokine receptors, or any other factor able to induce activation of NF-κB, which results in the up-regulation of NLRP3 to a functional level and pro-IL-1β expression.[212-214] The second step is post-transcriptional and allows the assembly of the NLRP3 inflammasome complex.[212, 213] Taken together, these data suggest that during spontaneous labor at term the chorioamniotic membranes increase the production of NLRP3 as an initial step for inflammasome activation. Whether there is assembly of the NLRP3 inflammasome complex in the chorioamniotic membranes during spontaneous labor at term requires further investigation.

We also found that the chorioamniotic membranes from women who underwent spontaneous labor had greater concentrations of NOD2 than those from women without labor, and such an increase was not associated with an up-regulation of *NOD2*. Recently, it was demonstrated that the mRNA expression of *NOD1* and *NOD2* in the myometrium is higher in women at term with labor than in those at term without labor.[215] NOD2 is an intracellular receptor that recognizes bacterial muramyl dipeptide (MDP) and activates NF-κB and MAPK pathways.[216, 217] In dendritic cells, NOD2 can act synergistically with the NLRP3 inflammasome in response to MDP and uric acid.[159] These results led us to suggest that the chorioamniotic membranes overproduce NOD2 protein which, in turn, could synergistically participate with the NLRP3 inflammasome in the physiological proinflammatory processes of spontaneous labor at term.

Activation of CASP-1, but not CASP-4, in the chorioamniotic membranes in spontaneous labor at term

Oligomerization of the inflammasome leads to the recruitment of ASC, which binds and activates pro-caspase-1 via its CARD.[104, 218] We therefore hypothesized that the active forms of CASP-1, p10 and p20, would be increased in the chorioamniotic membranes from women who underwent spontaneous labor at term. We previously demonstrated that CASP-1 concentration in the amniotic fluid from women in spontaneous labor at term is higher than in women wihout labor.[75] In the current study, we provide evidence to support our initial observation: CASP-1 immunoreactivity and its active form p20 are increased in the chorioamniotic membranes from women who underwent spontaneous labor at term. Recently, it was also found that the mRNA expression and active form p10 of CASP-1 are increased in the zone of rupture of the chorioamniotic membranes from women who undergo spontaneous labor at term.[219] Together, these data suggest that during spontaneous labor at term, the chorioamniotic membranes release active forms of CASP1.

Unlike Lappas,[219] we did not find differences between the mRNA expression of *CASP-1* in the chorioamniotic membranes from women who underwent labor and non-labor deliveries. This discrepancy could be attributed to differences in sampling, number of observations, and inclusion of decidua in our experiments. Specifically, we sampled the middle portion of the chorioamniotic membranes and did not restrict ourselves to sampling the zone of rupture.[66] Moreover, we utilized full-thickness, unaltered chorioamniotic membranes containing decidua parietalis since this type of sample includes immune cells that participate in labor;[220, 221] and our sample size (n=28-29) was considerably larger than that included in the aforementioned study (n=8).

CASP-11 (human homologue CASP-4) is necessary for the activation of CASP-1, which results in the non-canonical NLRP3 inflammasome activation in response to Gram-negative bacteria such as *Citrobacter rodentium*, *Escherichia coli*, *Vibrio cholerae*, and *Salmonella typhimurium*.[210, 222, 223] Although we found that CASP-4 immunoreactivity was higher in the chorioamniotic membranes from women who underwent spontaneous labor at term than in those without labor, we did not find the active form of CASP-4 in these tissues. These results demonstrate that the activation of CASP-4 is not implicated in the physiological proinflammatory processes of spontaneous labor at term. Our results also suggest that the

chorioamniotic membranes can synthesize CASP-4 in the event of infection, where Gram-negative bacteria may cause non-canonical activation of inflammasomes.

Increased mRNA abundance and protein concentration of IL-1β in the chorioamniotic membranes in spontaneous labor at term

The produced active forms of CASP-1 (p10 and p20) assemble to form hetero-tetramers that convert inactive pro-IL-1β into its bioactive and secreted form.[113, 224-230] Therefore, we investigated whether the chorioamniotic membranes express *IL1B* and release its bioactive form. We found that the mRNA abundance and release of the pro- and mature forms of IL-1β were higher in the chorioamniotic membranes from women who had undergone spontaneous labor at term than in those without labor. Elevated amniotic fluid IL-1β in spontaneous labor at term was demonstrated more than two decades ago.[7-9] IL-1β actively participates in the process of labor by inducing: 1) the biosynthesis of prostaglandin E2 by the human amnion[231] and myometrial cells[232, 233], 2) the expression of cyclooxygenase-2 in human myometrial cells,[234] and 3) the expression of matrix-metabolizing enzymes (MMP-1, -3, -9, and cathepsin S) in human cervical smooth muscle cells.[235] Indeed, systemic administration of IL-1β causes preterm birth in mice[10, 236] and monkeys,[237-244] confirming a central role for IL-1β in the process of labor, and this effect can be abrogated by the administration of the IL-1 receptor antagonist.[10] In the study herein, we demonstrated that the chorioamniotic membranes release mature IL-1β, which is most likely mediated by active CASP-1. This mature form of IL-1β will then participate in the physiological process of spontaneous labor at term.

mRNA abundance and protein concentration of IL-18 in the chorioamniotic membranes do not increase in spontaneous labor at term

In addition to cleaving IL-1β, active CASP-1 converts pro-IL-18 into its mature form.[115-118, 124] Yet, contrary to IL-1β, IL-18 concentration in the amniotic fluid does not significantly increase during term and preterm parturition.[173] This is consistent with our results since no increase was observed in the concentration of IL-18 in the chorioamniotic membrane extracts from women who underwent spontaneous labor at term when compared to those women without labor. Indeed, the mRNA abundance of *IL-18* was lower in the chorioamniotic membranes from

women who underwent labor than in those without labor, and the mature form of IL-18 was observed mainly in non-laboring tissues at term. IL-18 is a major IFN-γ inducing factor that activates Th-1 responses in T cells and NK cells,[245-252] and its concentration in the amniotic fluid increased in response to intra-amniotic infection.[173, 253] It is possible that IL-18 participates in host defense against pathogens in the chorioamniotic membranes rather than in the physiologic inflammatory process of spontaneous labor at term.

In summary, the study herein provides evidence that supports a role for the inflammasome (NLRP1, NLRP3, AIM2, or NLRC4) in the activation of CASP-1 and the consequent release of mature IL-1β by the chorioamniotic membranes in spontaneous labor at term. Up-regulation of NLRP3 and NOD2 proteins suggests that these NLRs are implicated in the physiological proinflammatory process of spontaneous labor at term; yet, further research is needed in order to prove inflammasome assembly and activation in the chorioamniotic membranes.

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DISCLOSURE/CONFLICT OF INTEREST

The authors disclose no conflicts of interest.

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FIGURE LEGENDS

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 at term with (TIL, n=28) or without labor (TNL, n=29). Relative gene expressions are presented as -ΔCt values. (B) Protein concentrations of inflammasome components and NOD2 in chorioamniotic membrane tissue lysates (n=10 each). (C) Representative immunostainings for inflammasome components and NOD2 in the chorioamniotic membranes (n=10 each), 200× magnification.

Figure 2. NOD1 and NLRC4 in the chorioamniotic membranes. (A and D) mRNA abundance of *NOD1* and *NLRC4* in the chorioamniotic membranes from women at term with (TIL, n=28) or without labor (TNL, n=29). Relative gene expressions are presented as -ΔCt values. (B and E) Protein quantity of NOD1 and NLRC4 in chorioamniotic membrane tissue lysates (n=8 each). (C and F) Intensity of the immunostainings for NOD1 and NLRC4 in the chorioamniotic membranes (n=10 each) and representative immunostainings, 200× magnification.

Figure 3. Inflammatory caspases in the chorioamniotic membranes. (A) mRNA abundance of *CASP-1* and *CASP-4* in the chorioamniotic membranes from women at term with (TIL, n=28) or without labor (TNL, n=29). Relative gene expressions are presented as -ΔCt values. (B) Protein concentrations of CASP-1 and CASP-4 in chorioamniotic membrane tissue lysates (n=10 each). (C) Intensity of the immunostainings for CASP-1 and CASP-4 in the chorioamniotic membranes (n=10 each) and representative immunostainings, 200× magnification. (D) Immunoblotting of CASP-1, CASP-4, and GAPDH in the chorioamniotic membranes and their quantifications (n=4 each).

Figure 4. IL-1β in the chorioamniotic membranes. (A) mRNA abundance of *IL1β* in the chorioamniotic membranes from women at term with (TIL, n=28) or without labor (TNL, n=29).

Relative gene expressions are presented as -ΔCt values. (B) Protein concentrations of mature and pro-form IL-1β in chorioamniotic membrane supernatants (TNL, n=7, and TIL, n=4). (C) Intensity of the immunostainings for IL-1β in the chorioamniotic membranes (n=10 each) and representative immunostainings, 200× magnification.

Figure 5. IL-18 in the chorioamniotic membranes. (A) mRNA abundance of *IL-18* in the chorioamniotic membranes from women at term with (TIL, n=28) or without labor (TNL, n=29). Relative gene expressions are presented as -ΔCt values. (B) Protein concentrations of IL-18 in chorioamniotic membrane tissue lysates (n=10 each). (C) Intensity of the immunostainings for IL-18 in the chorioamniotic membranes (n=10 each) and representative immunostainings, 200× magnification. (D) Immunoblotting of IL-18 and GAPDH in the chorioamniotic membranes (n=4 each).

Table I. Demographic and clinical characteristics of the study populations

	TNL (n=29)	TIL (n=28)	P value
Maternal age (years)*	24.0 (19-35.0)	21.5 (16.0-31.0)	0.01
Race**			
African-American	25 (89.3%)	24 (88.9%)	
Caucasian	1 (3.6%)	2 (7.4%)	NS
Hispanic	1 (3.6%)	0 (0.0%)	
Other	1 (3.6%)	1 (3.7%)	
Maternal weight (kg)*	86 (44-136)	66 (49-109)	0.0002
Body mass index (kg/m^2)*	33.9 (19.80-48.40)	24.5 (18.30-41.20)	0.001
Gestational age at delivery (weeks)*	39.4 (37.1-40.9)	39.7 (37.1-41.7)	NS
Birth weight (grams)*	3385 (2960-4010)	3270 (2750-3870)	NS
Cesarean section**	100%	32.1%	0.01
Acute chorioamnionitis	0%	0%	NA

*Mann-Whitney test

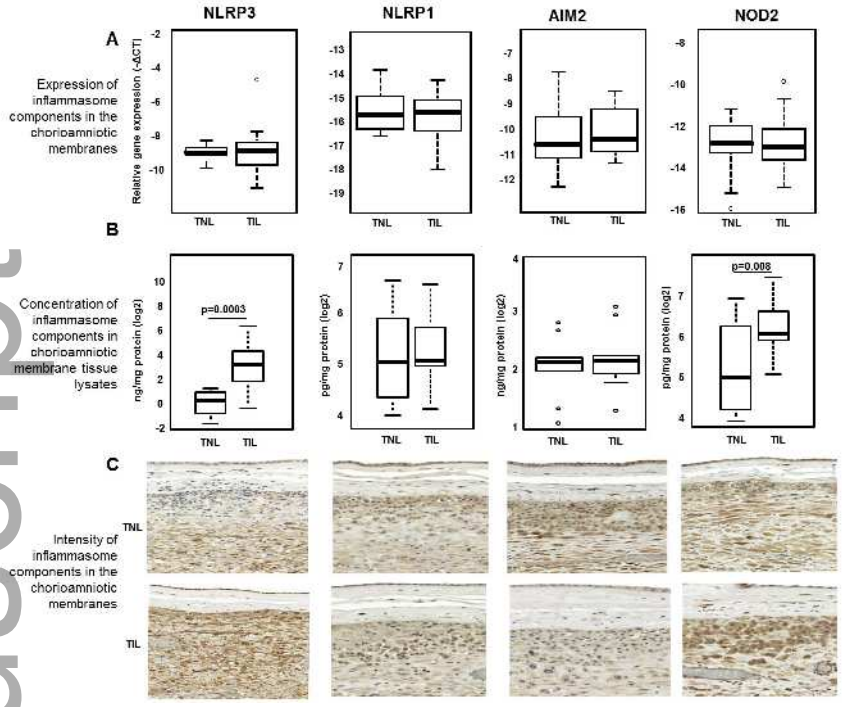
**Chi-square test

Table II. TaqMan® assays used for gene expression profiling

Gene Symbol	Protein Name	Assay ID
ACTB	Actin beta	Hs99999903_m1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Hs99999905_m1
RPLP0	Ribosomal protein, large, P0	Hs99999902_m1
NLRP1	NACHT, LRR and PYD domains-containing protein 1	Hs00248187_m1
NLRP3	NACHT, LRR and PYD domains-containing protein 3	Hs00918082_m1
NLRC4	NLR family CARD domain-containing protein 4	Hs00368367_m1
NOD1	Nucleotide-binding oligomerization domain-containing protein 1	Hs00196075_m1
NOD2	Nucleotide-binding oligomerization domain-containing protein 2	Hs00223394_m1
AIM2	Absent in melanoma 2	Hs00915710_m1
CASP1	Caspase-1 / Interleukin-1 converting enzyme	Hs00354836_m1
CASP4	Caspase-4	Hs01031947_m1
IL1B	Interleukin-1 beta	Hs00174097_m1
IL18	Interleukin-18	Hs01038788_m1

Table III. Primary antibodies and immunostaining conditions

Primary antibody	Vendor	Host species / clonality	Dilution	Incubation time
Anti-AIM2	Santa Cruz Biotechnology (Dallas, TX, USA)	Rabbit / polyclonal	(1:100)	15 min
Anti-CARD7 (NLRP1)	Acris Antibodies (Herford, Germany)	Rabbit / polyclonal	(1:500)	15 min
Anti-CARD12 (NLRC4)	Abcam Inc. (Cambridge, MA, USA)	Rabbit / polyclonal	(1:1500)	15 min
Anti-CARD15 (NOD2)	Abcam Inc. (Cambridge, MA, USA)	Rabbit / polyclonal	(1:200)	15 min
Anti-CASP1	R&D Systems, Inc. (Minneapolis, MN, USA)	Mouse / monoclonal	(1:2000)	15 min
Anti-CASP4	Abcam Inc. (Cambridge, MA, USA)	Rabbit / polyclonal	(1:100)	15 min
Anti-CIAS/NALP3 (NLRP3)	Millipore Corporation (Temecula, CA, USA)	Rabbit / polyclonal	(1:100)	15 min
Anti-NOD1	Lifespan Bio (Seattle, WA, USA)	Rabbit / polyclonal	(1:200)	15 min
IL-1β	Novus Biologicals (Littleton, CO, USA)	Rabbit / polyclonal	(1:50)	15 min
IL-18	Lifespan Bio (Seattle, WA, USA)	Rabbit / polyclonal	(1:10)	15 min



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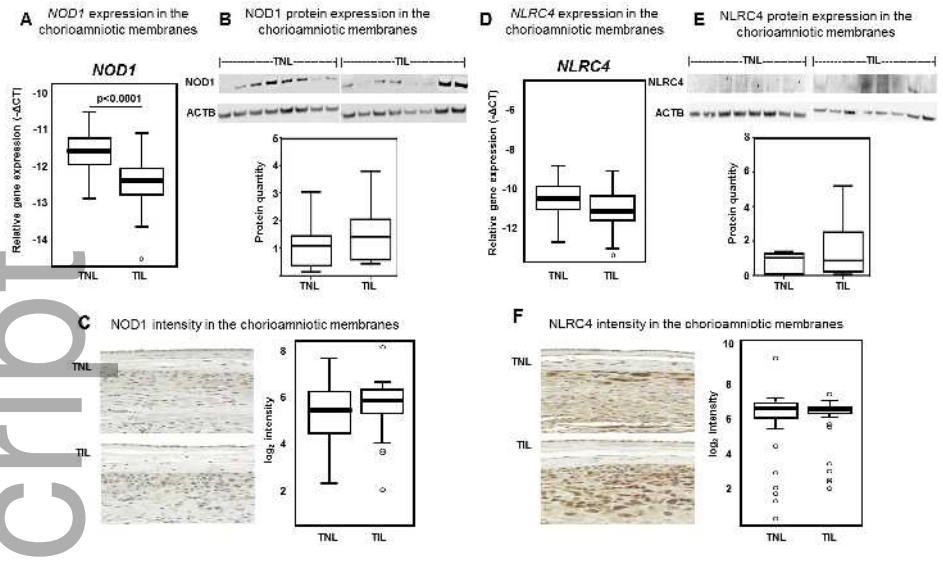

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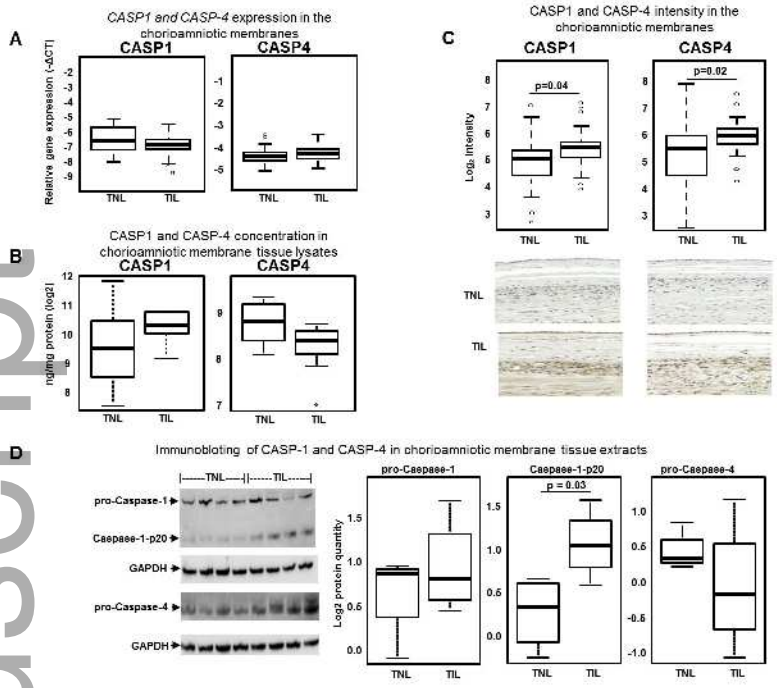

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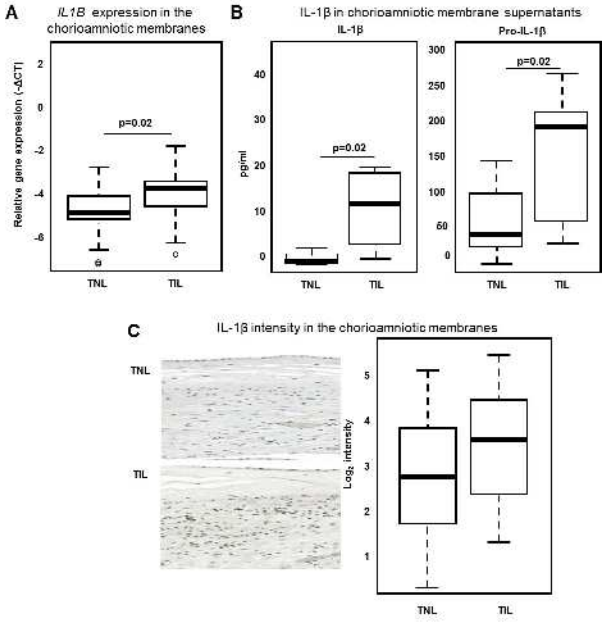

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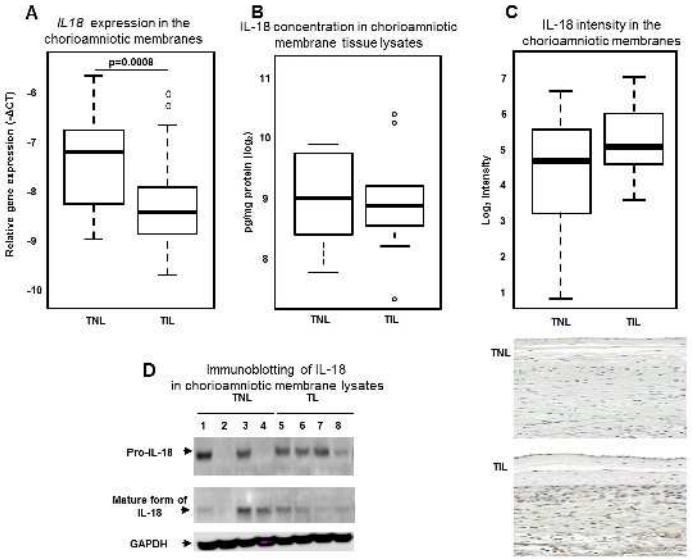

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