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A Role for the Inflammasome in Spontaneous Labor at Term with Acute Histologic Chorioamnionitis

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Abstract

Inflammasomes are cytosolic signaling platforms that regulate the activation of caspase (CASP)-1, which induces the maturation of interleukin (IL)-1β and IL-18. Herein, we determined whether the chorioamniotic membranes from women in spontaneous labor at term with acute histologic chorioamnionitis express major inflammasome components and whether these changes are associated with the activation of CASP-1 and CASP-4 and the release of mature IL-1β and IL-18. When comparing the chorioamniotic membranes from women in spontaneous labor at term with acute histologic chorioamnionitis to those without this placental lesion, we found that (1) the messenger RNA (mRNA) abundance of NLR family pyrin domain containing 3 (*NLRP3*), NLR family CARD domain containing 4 (*NLRC4*), absent in melanoma 2 (*AIM2*), and nucleotide binding oligomerization domain 2 (*NOD2*) was higher; (2) the NLRP3 and NLRC4 protein quantities were increased; (3) the mRNA and protein expressions of CASP-1 and its active forms were greater; (4) CASP-4 was increased at the mRNA level only; (5) the mRNA and protein expressions of IL-1β and its mature form were higher; and (6) a modest increase in the total protein concentration and abundance of the mature form of IL-18 was observed. *In vitro* incubation of the chorioamniotic membranes with the CASP-1 inhibitor, VX765, decreased the release of endotoxin-induced IL-1β and IL-18 (2-fold) but not IL-6 or tumor necrosis factor α. In conclusion, spontaneous labor at term with acute histologic chorioamnionitis is characterized by an upregulation of inflammasome components which, in turn, may participate in the activation of CASP-1 and lead to the release of mature IL-1β by the chorioamniotic membranes. These results support a role for the inflammasome in the mechanisms responsible for spontaneous labor at term with acute histologic chorioamnionitis.

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Introduction

Acute histologic chorioamnionitis is the most common placental lesion and represents the presence of intra-amniotic infection.[1,2] Yet, acute histologic chorioamnionitis can also occur as a result of sterile intra-amniotic inflammation, which is induced by danger signals released under cellular stress, injury, or death.[3-6] These signals are termed damage-associated molecular pattern molecules[7] or alarmins.[8] Acute histologic chorioamnionitis is strongly associated with preterm labor/birth[9-12]; however, it is frequently observed in the placentas of women who delivered after spontaneous labor at term.[13,14] The presence of this placental lesion at term is strongly associated with intrapartum fever and elevated levels of maternal white blood cell counts, interleukin (IL)-8, and IL-6 at admission for delivery.[15] Furthermore, acute histologic chorioamnionitis at term is associated with elevated concentrations of cytokines such as IL-1α, IL-1β, tumor necrosis factor (TNF)-α, IL-6, and IL-8 in the amniotic fluid,[16] umbilical cord blood,[15,17-20] and decidual tissues.[21-26] Elevated concentrations of cytokines in these compartments are linked to adverse neonatal outcomes.[27-46] Therefore, our investigation focused on the mechanisms implicated in spontaneous labor at term with acute histologic chorioamnionitis.

Acute inflammation of the chorioamniotic membranes is characterized by the infiltration of neutrophils.[1,47] The mechanisms implicated in the pathogenesis of this placental lesion involve chemotactic signals including those derived from IL-8, C-X-C chemokine ligand (CXCL) 6 and growth regulated oncogene (GRO)α,[1] all of which are found in the amniotic fluid.[48-61] Acute histologic chorioamnionitis is also associated with elevated concentrations of cytokines, such as IL-1β in the amniotic fluid[29] and chorioamniotic membranes (including the decidua).[21] The IL-1β cytokine is synthesized as a zymogen (or pro-form), which requires cleavage in order to be biologically active and released extracellularly.[62-65] The cleavage of IL-1β can be performed by intracellular protein caspase (CASP)-1[62-68] which, in turn, could be activated by cytoplasmic complexes termed inflammasomes.[69-97] Active CASP-1 can also cleave pro-IL-18 into its mature form.[66-68,98,99] Inflammasome complexes contain (1) a pattern recognition receptor (a sensor molecule), (2) the adaptor protein ASC (an apoptosis-associated speck-like protein), and (3) pro-CASP-1.[100-109] In addition to inflammasomes, active CASP-4 is also required, but not necessary, for the activation of CASP-1.[110,111]

Inflammasomes are implicated in physiological[112-114] and pathological[113,115-124] inflammations during pregnancy. Recently, we provided evidence that supports a role for the inflammasome in the physiological inflammatory response that accompanies human spontaneous labor at term.[114] Since amniotic fluid concentrations of CASP-1,[113] IL-1β,[125] and IL-18[126] are greater in women with intra-amniotic infection/inflammation (i.e. pathological inflammation) than in those without intra-amniotic infection/inflammation, we proposed that the inflammasome is also implicated in the pathological inflammatory process in acute histologic chorioamnionitis during spontaneous labor at term.

In this study, we aimed to determine (1) whether inflammasome components are expressed in the chorioamniotic membranes from women who have undergone spontaneous labor at term with histological signs of acute chorioamnionitis and (2) whether these changes are linked to the activation of CASP-1 and CASP-4 and to the release of the mature forms of IL-1β and IL-18. In addition, we investigated whether *in vitro* incubation of term nonlabor chorioamniotic membrane explants with the CASP-1 inhibitor, VX765, decreases the release of endotoxin-induced IL-1β and IL-18.

Materials and Methods

Human Participants, Clinical Specimens, and Definitions

This case-control study included patients who delivered at term after labor with (TIL-ACA) and without (TIL) acute histologic chorioamnionitis. Samples from the chorioamniotic membranes were collected from the Bank of Biological Specimens of the Detroit Medical Center, Wayne State University, and the Perinatology Research Branch, NICHD/NIH/DHHS (Detroit, Michigan). The review boards of these institutions approved the collection and the use of biological materials for research purposes. Participants provided written informed consent, and samples were collected within 0.5 hours after delivery. Table 1 includes the demographic and clinical characteristics of the study groups. Multiparous women or women 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 2 contractions every 10 minutes with cervical changes resulting in delivery.[127]

For the *in vitro* experiments, chorioamniotic membrane samples were collected within 30 minutes after delivery from healthy pregnant women at term without labor. Women included in this study had no medical complications and delivered neonates of appropriate weight for gestational age.[128]

Placental Histopathological Examinations

Five-micrometer-thick sections of formalin-fixed, paraffin-embedded tissue specimens were cut and mounted on Superfrost Plus microscope slides (Erie Scientific LLC, Portsmouth, New Hampshire). In each case, several tissue sections of the chorioamniotic membranes, umbilical cord, and placental disc were examined. After deparaffinization, slides were rehydrated, stained with hematoxylin–eosin, and evaluated by

Table 1. Demographic and Clinical Characteristics of the Study Population.

	Spontaneous Term Labor (TIL, n = 28)	Spontaneous Term Labor with Acute Chorioamnionitis (TIL-ACA, n = 32)	*P* Value
Maternal age, years[a]	21.0 (18.5-26.0)	21.0 (19.0-26.5)	NS
Race[b]			
African American	24 (88.9%)	29 (90.6%)	NS
Caucasian	2 (7.4%)	1 (3.1%)	
Hispanic	0 (0.0%)	0 (0.0%)	
Other	1 (3.7%)	2 (6.3%)	
Maternal weight[a], kg	66 (59-75)	68 (60-77)	NS
Body mass index[a], kg/m^2	24.5 (22.1-29.1)	26.4 (22.5-28.3)	NS
Primiparity[b]	11 (39.3%)	14 (43.8%)	NS
Gestational age at delivery, weeks[a]	39.7 (38.7-40.5)	40.1 (38.6-40.6)	NS
Birth weight, g[a]	3252 (3125-3420)	3487 (3085-3765)	NS
Cesarean section[b]	35.7%	50%	NS
Acute chorioamnionitis[b]	0%	100%	<.0001

Abbreviation: NS, not significant.
[a]Mann-Whitney *U* test.
[b]Fisher's exact test.

pathologists who had been blinded to the clinical outcome, according to the published criteria.[1,129] The diagnosis of acute histologic chorioamnionitis was made when the infiltration of neutrophils into the chorionic trophoblast layer or chorioamniotic connective tissue was observed.[1,129]

RNA Isolation, Complementary DNA Synthesis, and Quantitative Reverse Transcription Polymerase Chain Reaction Analysis

Total RNA was extracted from snap-frozen chorioamniotic membrane samples (TIL, n = 28 and TIL-ACA, n = 32) and from term nonlabor tissue explants (n = 6) 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 by using the NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, Delaware), and the Bioanalyzer 2100 (Agilent Technologies, Wilmington, Delaware). Complementary DNA was synthesized 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 (qRT-PCR; 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 I.

Chorioamniotic Membrane Tissue Lysates

Tissue lysates were prepared by homogenizing snap-frozen chorioamniotic membranes (TIL and TIL-ACA; n = 10 each) in 2 mL of 1× 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 the NLR family pyrin domain containing (NLRP)1, NLRP3, absent in melanoma 2 (AIM2), nucleotide-binding oligomerization domain (NOD)2, 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, 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 <4.68 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 measured about 10% of pro-IL-1β. The immunoassays for NLRC4 and NOD1 did not meet our criteria for validation; instead, immunoblotting was performed.

Concentrations of human IL-1β, IL-18, IL-6, and TNF-α in cell culture supernatants from *in vitro* experiments were measured with sensitive and specific immunoassays (Meso Scale Discovery, Gaithersburg, Maryland), according to the manufacturer's instructions. Briefly, 150 µL of 1% (w/v) Blocker B Solution were dispensed into each well of the precoated plates and incubated for 1 hour with vigorous shaking at room

temperature. Plates were then washed 3 times with 1× PBS and 0.05% Tween-20 (Sigma-Aldrich, St Louis, Missouri). Twenty-five microliters of each sample or calibrator were dispensed into separate wells of the plates and incubated for 2 hours with vigorous shaking at room temperature. The samples and calibrators were discarded, and the plates were washed 3 times with 1× PBS and 0.05% Tween-20 (Sigma-Aldrich), followed by an addition of 25 µL of the 1× detection antibody solution into each well. Plates were incubated for 2 hours with vigorous shaking at room temperature. The detection antibody was removed and the plates were washed 3 times with 1× PBS and 0.05% Tween-20 (Sigma-Aldrich). One hundred fifty microliters of 2× Read Buffer T (Meso Scale Discovery) were added to each well, and the signals were read by the Sector 2400 Imager (Meso Scale Discovery). Standard curves were generated, and the assay values of the samples were extrapolated from the curves. The sensitivities of the assays were 0.268 pg/mL for IL-1β, 0.216 pg/mL for IL-18, 0.206 pg/mL for IL-6, and 0.417 pg/mL for TNF-α.

Immunohistochemistry

Tissue sections (5 µm thick) were prepared from the chorioamniotic membranes (TIL and TIL-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 II includes the utilized primary antibodies and immunostaining conditions. Quantification of the intensity was performed using a Perkin-Elmer Panoramic MIDI slide scanner (PerkinElmer, Waltham, Massachusetts).

Immunoblotting

The protein quantity of NLRC4 and NOD1 and the active/mature forms of CASP-1, CASP-4, and IL-18 were determined by immunoblotting. Tissue lysates (50 µg for NLRC4, NOD1, and IL-18; 20 µg for CASP-1; and 40 µg for CASP-4 per well) were subjected to 4% to 12% sodium dodecyl sulfate 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),[130] 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 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). Nitrocellulose membranes were then stripped with Restore Plus Western Blot Stripping Buffer (Pierce Biotechnology, ThermoFisher Scientific Inc) for 15 minutes, washed with 1× PBS, blocked, and reprobed for 1 hour at room temperature with a mouse anti-beta actin (ACTB) monoclonal antibody (Sigma-Aldrich) or a mouse anti-glyceraldehyde 3-phosphate dehydrogenase monoclonal antibody (Santa Cruz Biotechnology). Chemiluminescence signals were again detected with ChemiGlow West Reagents, and images were acquired using the Fujifilm LAS-4000 Imaging System.

In Vitro *Experiments in Chorioamniotic Membrane Explants*

Chorioamniotic membrane samples were collected from women undergoing elective cesarean delivery (n = 6) and processed within 30 minutes. Tissue samples were washed with 1× PBS and cut into 2 cm × 2 cm pieces. These tissue explants were transferred into 6-well tissue culture plates containing 2 mL of Dulbecco's Modified Eagle Medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin (Invitrogen). Tissue explants were incubated in a humidified 5% CO_2 incubator at 37°C with or without the CASP-1 inhibitor VX765 (10 µM in 0.1% DMSO; MedKoo Biosciences, Chapel Hill, North Carolina) for 30 minutes before an endotoxin or lipopolysaccharide (10 ng/mL; LPS, *Escherichia coli* 0111: B4, Sigma-Aldrich) was added into the culture medium. Incubations were continued for 16 hours; then tissue culture supernatants were collected for ELISA, and tissue samples were homogenized in TRIzol reagent (Invitrogen) for qRT-PCR. Triplicate supernatants were obtained from the membranes of each patient.

Statistical Analyses

The SPSS v.19.0 software (SPSS Inc, Chicago, Illinois) was used to analyze demographic and clinical data. Comparisons between the 2 groups (TIL vs TIL-ACA) were performed using 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* (snap-frozen samples) or *RPLPO* (*in vitro* experiments) were calculated as $-\Delta Ct$ values, where ΔCt ($\Delta Ct = Ct_{target} - Ct_{reference}$) 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 the variance linear model and the resulting *P* values were adjusted using the Benjamini-Hochberg procedure. An adjusted *P* value of $\leq .05$ was considered statistically significant.

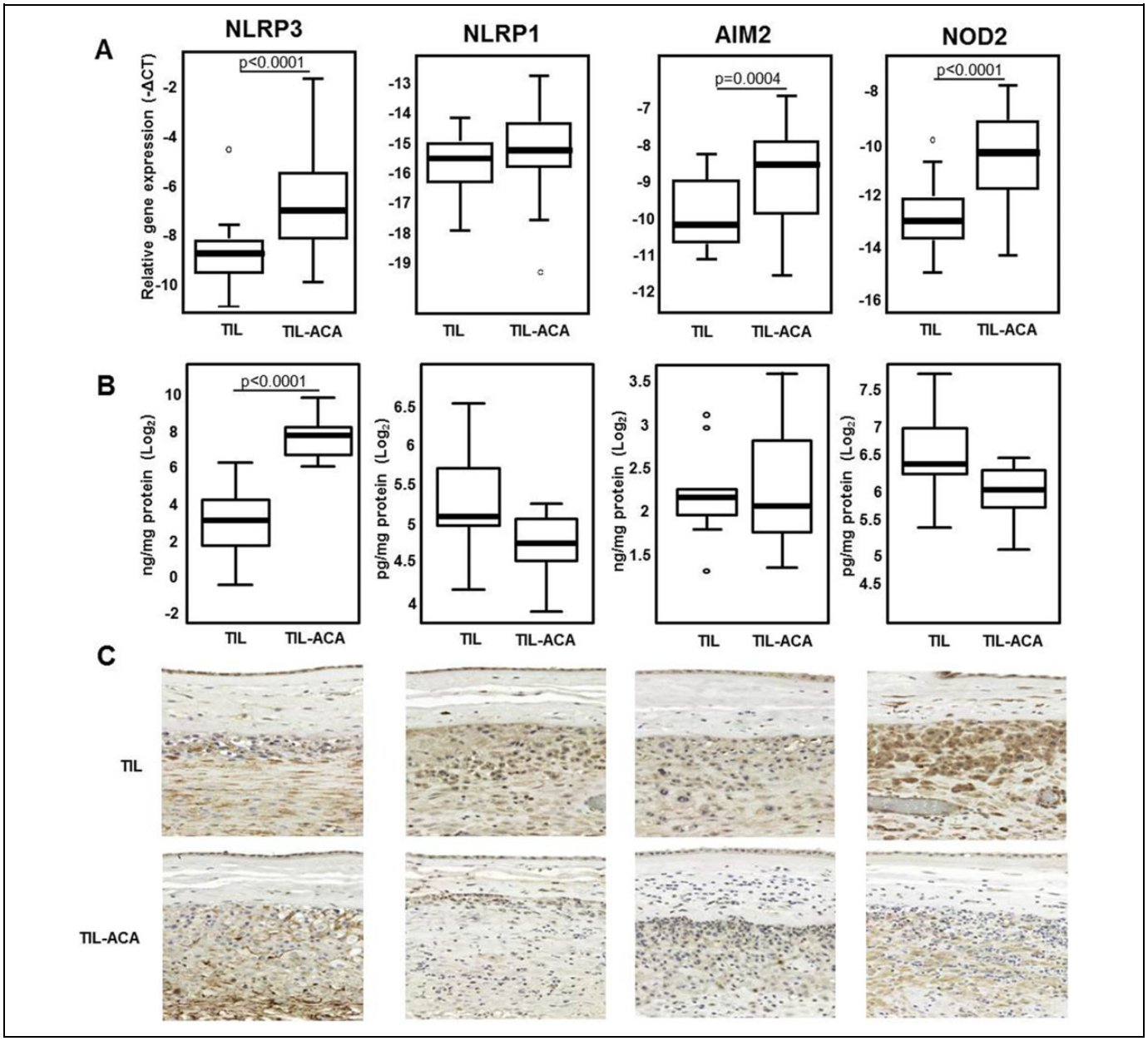

Figure 1. Inflammasome components and NOD2 protein in the chorioamniotic membranes. A, Messenger RNA abundance of inflammasome components and *NOD2* in the chorioamniotic membranes from women in spontaneous labor at term with (TIL-ACA, n = 32) or without (TIL, n = 28) acute histologic chorioamnionitis. Relative gene expressions are presented as $-\Delta$Ct 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. NOD indicates nucleotide-binding oligomerization domain.

Results

Messenger RNA Abundance and Protein Expression of NLRP3 and NLRC4 Increase in the Chorioamniotic Membranes in Spontaneous Labor at Term with Acute Histologic Chorioamnionitis

The messenger RNA (mRNA) abundance of *NLRP3*, *NLRC4*, *AIM2*, and *NOD2* was greater in the chorioamniotic membranes from women who underwent spontaneous labor at term with acute histologic chorioamnionitis than in those without this placental lesion (Figures 1A and 2A). However, no differences were observed in the mRNA abundance of *NLRP1* and *NOD1* between these groups (Figures 1A and 2D). The NLRP3 and NLRC4 protein concentrations were increased in the chorioamniotic membranes from women who underwent spontaneous labor at term with acute histologic chorioamnionitis compared to those without this placental lesion (Figures 1B and 2B). No differences were observed in the protein

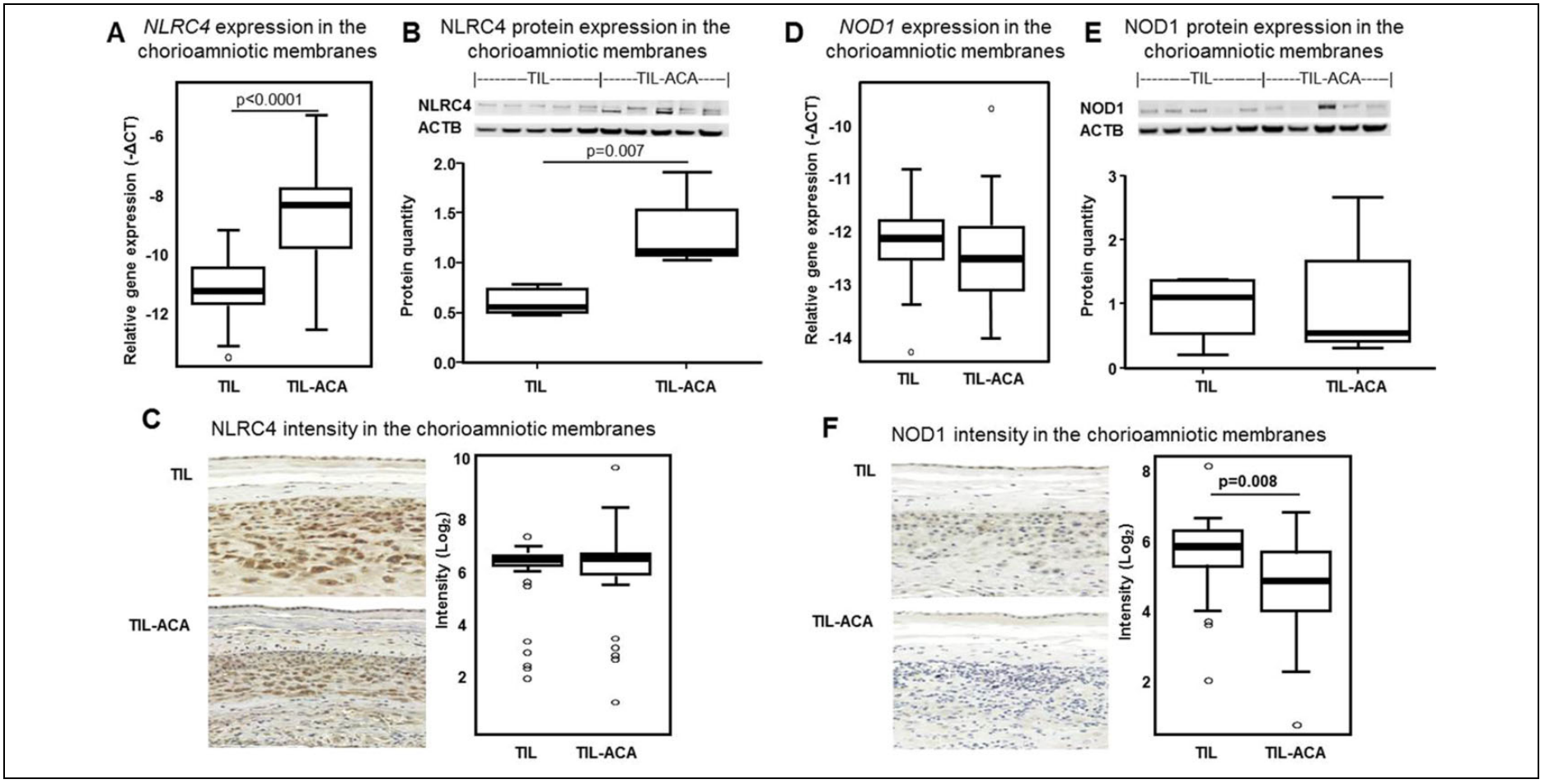

Figure 2. NLRC4 and NOD1 in the chorioamniotic membranes. A and D, Messenger RNA abundance of *NLRC4* and *NOD1* in the chorioamniotic membranes from women in spontaneous labor at term with (TIL-ACA, n = 32) or without (TIL, n = 28) acute histologic chorioamnionitis. Relative gene expressions are presented as $-\Delta$Ct values. B and E, Protein quantity of NLRC4 and NOD1 in chorioamniotic membrane tissue lysates (n = 5 each). C and F, Intensity of the immunostainings for NLRC4 and NOD1 in the chorioamniotic membranes (n = 10 each) and representative immunostainings, 200× magnifications. NOD indicates nucleotide-binding oligomerization domain.

concentrations of NLRP1, AIM2, NOD2, and NOD1 between these 2 groups of women (Figures 1B and 2E).

Immunohistochemistry revealed that all of the inflammasome components and NOD proteins were expressed by the chorioamniotic membranes from women who underwent spontaneous labor at term with and without acute histologic chorioamnionitis. These proteins were detected in the chorionic trophoblast cells, decidual stromal cells, and amniotic mesodermal and epithelial cells (Figures 1C, 2C, and 2F). However, NOD1 immunoreactivity seemed to be lower in the chorioamniotic membranes from women who underwent spontaneous labor at term with acute histologic chorioamnionitis than in those without this placental lesion (Figure 2F).

The collective increase in the mRNA abundance and protein expression of NLRP3 and NLRC4 in the chorioamniotic membranes from women who underwent spontaneous labor at term with acute histologic chorioamnionitis provides evidence that the inflammasome may participate in the pathological activation of the innate immune system in the setting of intraamniotic infection/inflammation.

Activation of CASP-1 *in the Chorioamniotic Membranes in Spontaneous Labor at Term with Acute Histologic Chorioamnionitis*

We then investigated whether the increased protein concentrations of NLRP3 and NLRC4 in the chorioamniotic membranes from women who underwent spontaneous labor at term with acute histologic chorioamnionitis were linked to the activation of CASP-1 and CASP-4. The mRNA abundance of *CASP1* and *CASP4* in the chorioamniotic membranes from women who underwent spontaneous labor at term with acute histologic chorioamnionitis was greater than in those without this placental lesion (Figure 3A). The protein concentrations of CASP-1 and CASP-4 were not significantly different between the 2 groups (Figure 3B). Immunohistochemistry analysis revealed that CASP-1, but not CASP-4, was greater in the chorioamniotic membranes from women who had undergone spontaneous labor at term with acute histologic chorioamnionitis than in those without this placental lesion (Figures 3C and 3D). Also, the active forms of CASP-1 (p10 and p20) were increased in the chorioamniotic membranes from women who had undergone spontaneous labor at term with acute histologic chorioamnionitis compared to those without this placental lesion (Figures 3E and 3G). In contrast, the pro-form of CASP-1 was reduced in the chorioamniotic membranes from women who had undergone spontaneous labor at term with acute histologic chorioamnionitis (Figure 3G). The active form of CASP-4 was undetectable by immunoblotting (Figure 3E). However, the pro-form of CASP-4 was detected but no differences were observed between the chorioamniotic membranes from women who had undergone spontaneous labor at term with acute histologic chorioamnionitis and those without this placental lesion (Figure 3F). Altogether, these data suggest that pathological inflammation in spontaneous labor at term with acute histologic chorioamnionitis involves the participation of NLRP3 and NLRC4 inflammasomes which, in turn, may participate in the activation of CASP-1 in the chorioamniotic membranes.

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

Active forms of CASP-1 convert inactive pro-IL-1β into its mature form.[62-68] We then determined whether the chorioamniotic membranes from women who had undergone spontaneous labor at term with acute histologic chorioamnionitis release the mature form of IL-1β. The mRNA abundance of *IL1*β was greater in the chorioamniotic membranes from women who underwent spontaneous labor at term with acute histologic chorioamnionitis than in those without this placental lesion (Figure 4A). Also, the protein concentration of mature IL-1β was greater in the chorioamniotic membranes from women who had undergone spontaneous labor at term with acute histologic chorioamnionitis than in those without this placental lesion (Figure 4B). In contrast, the concentration of pro-IL-1β was reduced in cases with acute histologic chorioamnionitis (Figure 4B). Immunohistochemistry analysis revealed that the immunoreactivity of IL-1β was stronger in the chorioamniotic membranes from women who underwent spontaneous labor at term with acute histologic chorioamnionitis than in those without this placental lesion (Figure 4C). These findings suggest that pathological inflammation in spontaneous labor at term with acute histologic chorioamnionitis involves the release of mature IL-1β, which is likely processed by the active form of CASP-1 in the chorioamniotic membranes.

A Modest Increase in the Total Protein Concentration and Abundance of the Mature Form of IL-18 *in the Chorioamniotic Membranes in Spontaneous Labor at Term with Acute Histologic Chorioamnionitis*

Active forms of CASP-1 also convert inactive pro-IL-18 into its mature form.[98] Therefore, we investigated whether the chorioamniotic membranes from women who had undergone spontaneous labor at term with acute histologic chorioamnionitis release the mature form of IL-18. No differences were observed in the mRNA abundance (Figure 5A) and immunoreactivity (Figure 5C) of IL-18 between the chorioamniotic membranes from women who had undergone spontaneous labor at term with acute histologic chorioamnionitis and those without this placental lesion. A modest but not significant increase in the total protein concentration (Figure 5B) and the abundance of the mature form (Figure 5D) of IL-18 was observed in the chorioamniotic membranes from women who underwent spontaneous labor at term with acute histologic chorioamnionitis compared to those without this placental lesion. These data suggest that IL-18 may be involved in the pathological inflammation of the chorioamniotic membranes

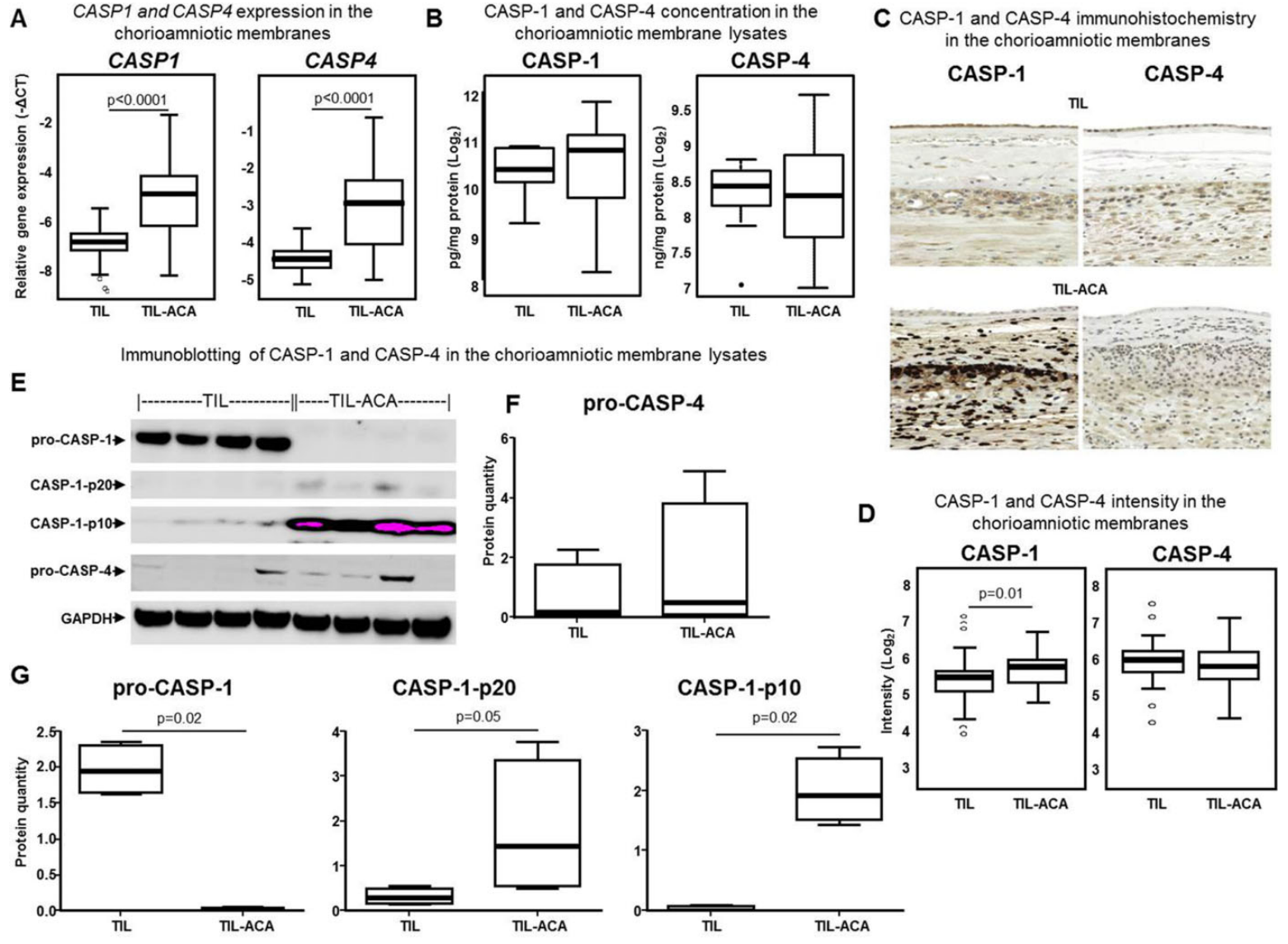

Figure 3. Inflammatory caspases in the chorioamniotic membranes. A, Messenger RNA abundance of *CASP1* and *CASP4* in the chorioamniotic membranes from women in spontaneous labor at term with (TIL-ACA, n = 32) or without (TIL, n = 28) acute histologic chorioamnionitis. Relative gene expressions are presented as $-\Delta$Ct values. B, Protein concentrations of CASP-1 and CASP-4 in chorioamniotic membrane tissue lysates (n = 10 each). C and D, Intensity of the immunostainings for CASP-1 and CASP-4 in the chorioamniotic membranes (n = 10 each) and representative immunostainings, 200× magnifications. E and F, Immunoblotting of CASP-1, CASP-4, its active forms, and GAPDH in the chorioamniotic membranes and their quantifications (n = 4 each). CASP indicates caspase.

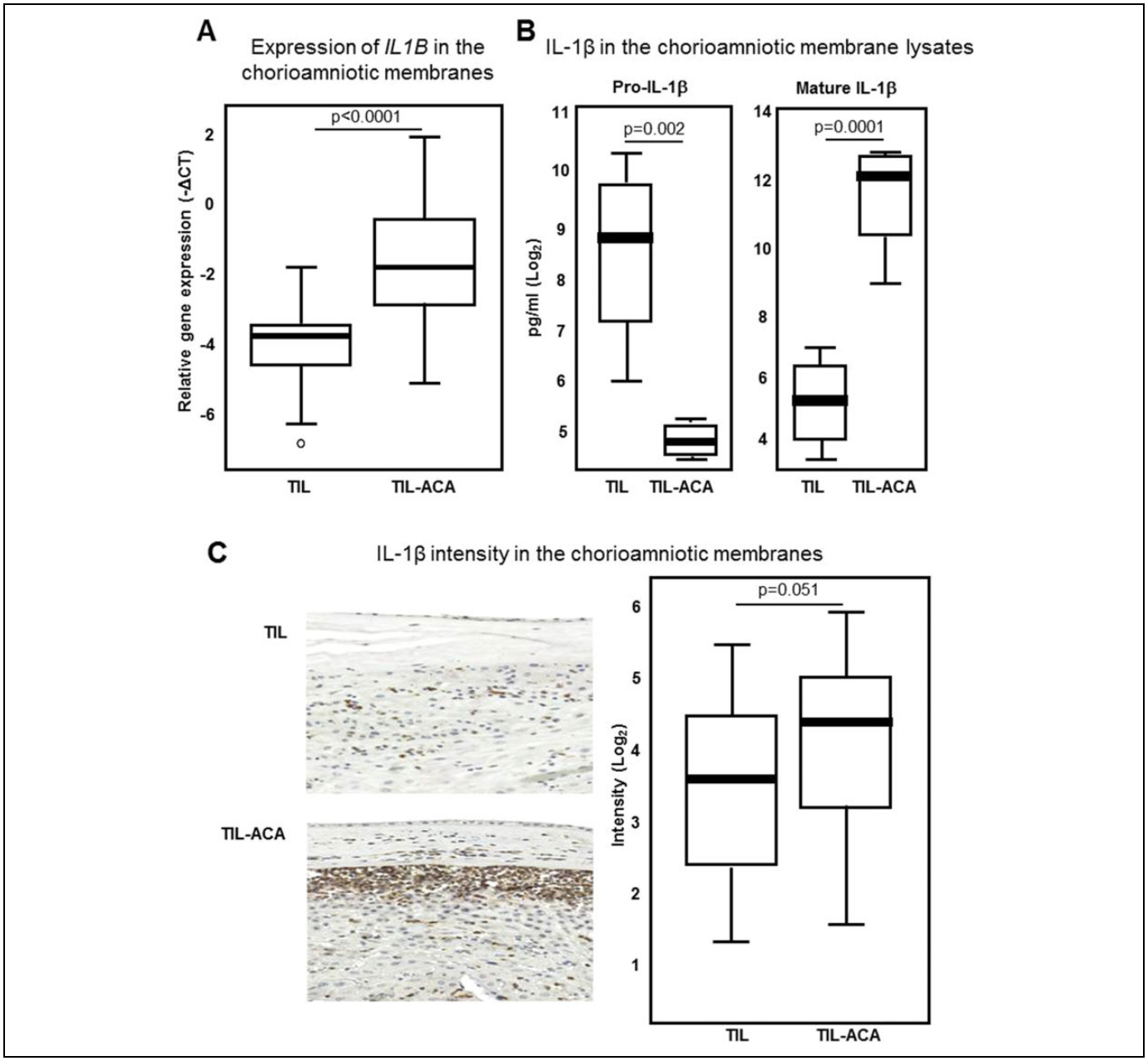

Figure 4. Interleukin (IL) 1β in the chorioamniotic membranes. A, Messenger RNA abundance of *IL1*β in the chorioamniotic membranes from women in spontaneous labor at term with (TIL-ACA, n = 32) or without (TIL, n = 28) acute histologic chorioamnionitis. Relative gene expressions are presented as −ΔCt values. B, Protein concentrations of the pro- and mature form of IL-1β in chorioamniotic membrane lysates (n = 10 each). C, Intensity of the immunostainings for IL-1β in the chorioamniotic membranes (n = 10 each) and representative immunostainings, 200× magnifications.

during spontaneous labor at term with acute histologic chorioamnionitis.

In Vitro *Incubation of the Chorioamniotic Membranes with a* CASP-1 *Inhibitor, VX765, Reduces the LPS-Induced Release of* IL-1β and IL-18

Finally, we examined whether the CASP-1 inhibitor, VX765, would reduce the LPS-induced mRNA abundance of pro-inflammatory cytokines in the chorioamniotic membranes. Incubation with LPS increased the mRNA abundance of *NLRP3*, *AIM2*, *NOD2*, *CASP1* (2-fold; $P = 0.058$), *CASP4*, *IL1*β, *IL6*, and *TNF* in the chorioamniotic membranes (Figure 6A and Supplementary Table III). *In vitro* incubation with LPS also increased the release of IL-1β, IL-18, IL-6, and TNF-α by the chorioamniotic membranes (Figure 6B). However, incubation with the CASP-1 inhibitor, VX765, reduced the LPS-induced release of inflammasome-dependent IL-1β and IL-18 (2-fold, $P = .058$) but did not diminish the release of

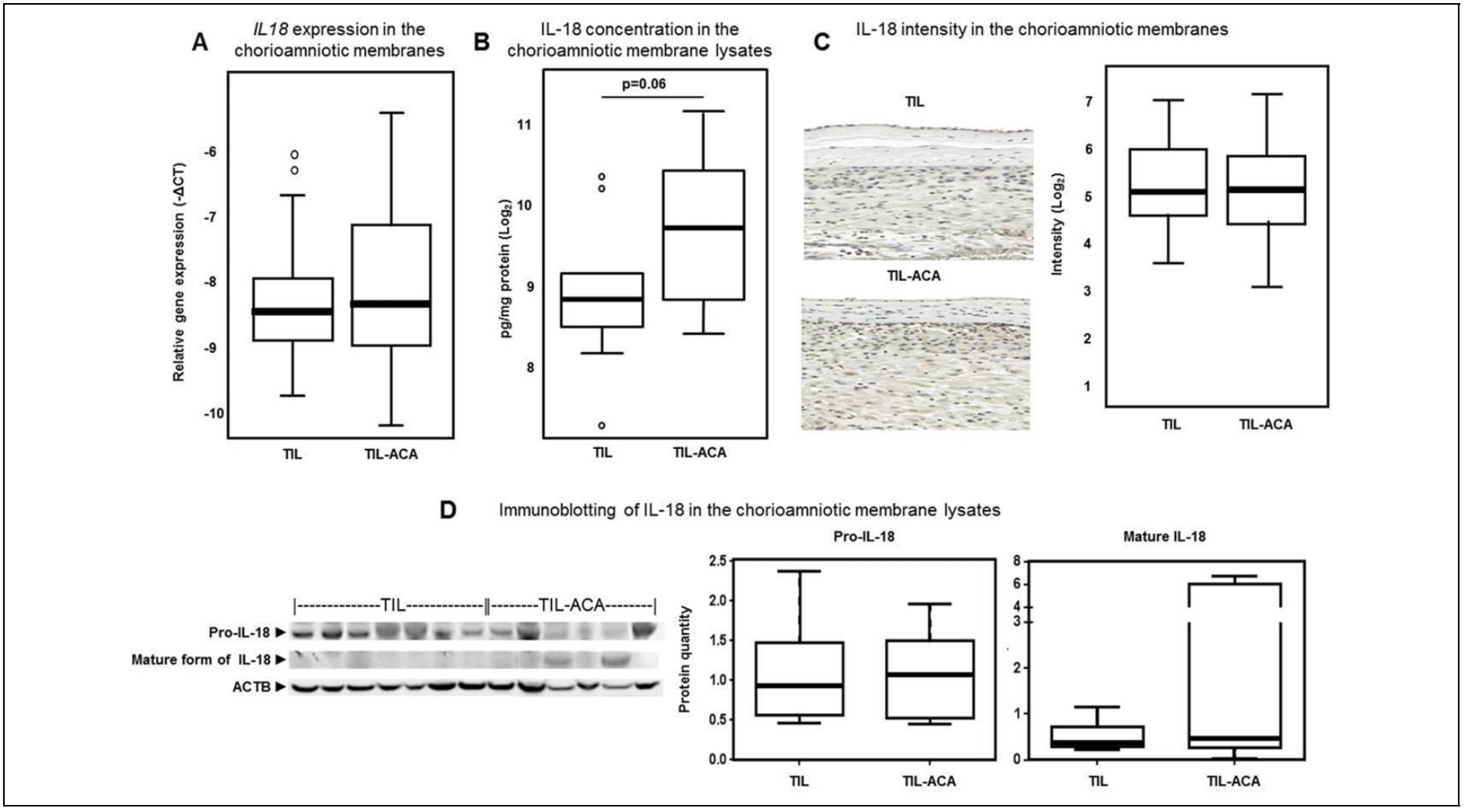

Figure 5. Interleukin (IL)-8 in the chorioamniotic membranes. A, Messenger RNA abundance of *IL18* in the chorioamniotic membranes from women in spontaneous labor at term with (TIL-ACA, n = 32) or without (TIL, n = 28) acute histologic chorioamnionitis. Relative gene expressions are presented as $-\Delta$Ct 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× magnifications. D, Immunoblotting of IL-18, its mature form, and ACTB in the chorioamniotic membranes and their quantifications (n = 6-7 each).

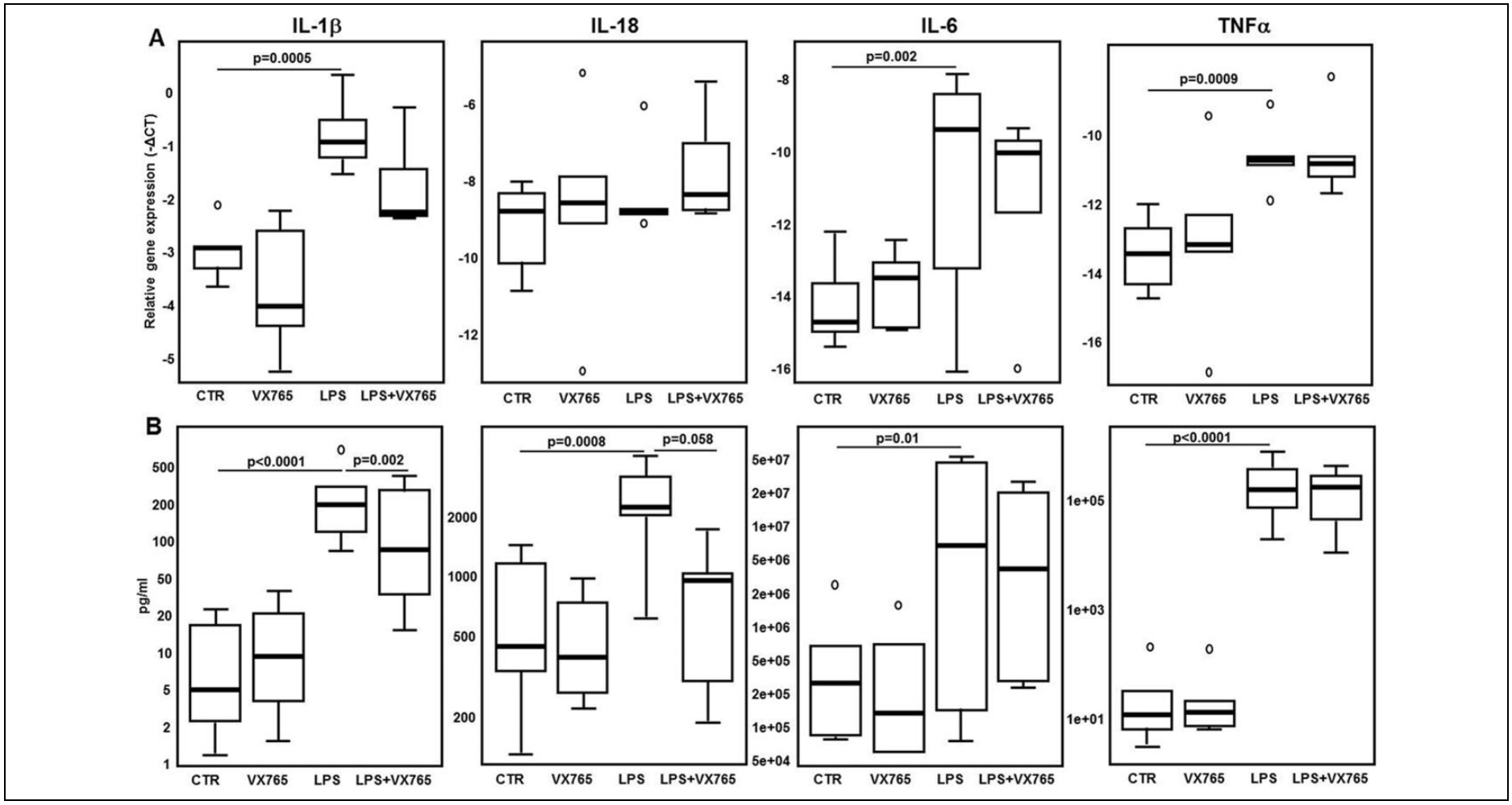

Figure 6. *In vitro* incubation of the chorioamniotic membranes with the CASP-1 inhibitor, VX765, reduces the LPS-induced release of IL-1β and IL-18. A, Messenger RNA abundance of *IL1β*, *IL18*, *IL6*, and *TNF* in chorioamniotic membrane explants from women who delivered at term without labor (n = 6) that were incubated with LPS, the CASP-1 inhibitor VX765, or both. Relative gene expressions are presented as −ΔCt values. B, Protein concentrations of IL-1β, IL-18, IL-6, and TNF-α in chorioamniotic membrane tissue lysates (n = 6). Experiments were performed in triplicate. CASP-1 indicates caspase 1; IL, interleukin; LPS, lipopolysaccharide; TNF, tumor necrosis factor.

inflammasome-independent IL-6 and TNF-α (Figure 6B). These data demonstrate that the release of LPS-induced IL-1β, and most likely IL-18, is mediated by CASP-1 in the chorioamniotic membranes.

Discussion

Principal findings of the study: (1) mRNA abundance of *NLRP3*, *NLRC4*, *AIM2*, and *NOD2* in the chorioamniotic membranes was higher in women in spontaneous labor at term with acute histologic chorioamnionitis than in those without this placental lesion, (2) NLRP3 and NLRC4 protein concentrations were higher in the chorioamniotic membranes from women who underwent spontaneous labor at term with acute histologic chorioamnionitis than in those without this placental lesion, (3) mRNA abundance of *CASP1* and *CASP4* was greater in the chorioamniotic membranes from women who underwent spontaneous labor at term with acute histologic chorioamnionitis than in those without this placental lesion, (4) the immunoreactivity of CASP-1 and the abundance of its active forms (p10 and p20) were increased among women who had undergone spontaneous labor at term with acute histologic chorioamnionitis compared to those without this placental lesion, (5) the mRNA abundance, protein concentration, immunoreactivity, and mature form of IL-1β were greater in the chorioamniotic membranes from women who underwent spontaneous labor at term with acute histologic chorioamnionitis than in those without this placental lesion, (6) a modest increase in the total protein concentration and abundance of the mature form of IL-18 was observed in the chorioamniotic membranes from women who underwent spontaneous labor at term with acute histologic chorioamnionitis compared to those without this placental lesion, and (7) *in vitro* incubation of the chorioamniotic membranes with the CASP-1 inhibitor, VX765, reduced the LPS-induced release of inflammasome-dependent IL-1β and IL-18 (2-fold) but did not diminish the release of inflammasome-independent IL-6 and TNF-α. Taken together, these findings support a role for the inflammasome in the pathological inflammation of the chorioamniotic membranes during spontaneous labor at term with acute histologic chorioamnionitis.

Previously, we proposed a role for the inflammasome in the physiological pro-inflammatory process of spontaneous labor at term.[112-114] Herein, we demonstrated that the mRNA and protein expressions of NLRP3 are increased in the chorioamniotic membranes from women who underwent spontaneous labor at term with acute histologic chorioamnionitis. The NLRP3 inflammasome complex includes the NLRP3 protein, the adaptor molecule ASC, and pro-CASP-1.[70,106,131,132] The activation of the NLRP3 inflammasome can be induced by microorganisms and danger signals[75,133-161] and requires 2 steps—priming and the assembly of the inflammasome complex.[162,163] Although the first step includes the activation of the nuclear factor kappa B (NF-κB) pathway, which induces the upregulation of the NLR protein and IL-1β, the second step permits the assembly of the inflammasome complex.[162-164] The data presented herein provide evidence that there is priming of the NLRP3 inflammasome in the chorioamniotic membranes during spontaneous labor at term with acute histologic chorioamnionitis. Further studies are required to demonstrate that there is assembly of the NLRP3 inflammasome complex in the chorioamniotic membranes.

We also found that the NLRC4 inflammasome may be involved in the pathological pro-inflammatory process of spontaneous labor at term with acute histologic chorioamnionitis. NLRC4 is mostly expressed in myeloid cells and can also regulate CASP-1 activation and IL-1β processing.[165] NLRC4 contains an *N*-terminal caspase activation and recruitment domain (CARD), a central NACHT (NACHT is an acronym for neuronal apoptosis inhibitor protein), C2TA (major histocompatibility complex (MHC) class 2 transcription activator), HET-E (incompatibility locus protein from *Podospora anserina*) and TP1 (telomerase-associated protein 1) domain, and *C*-terminal leucine-rich repeat (LRR).[165] The activation of the NLRC4 inflammasome is mediated by flagellins[166,167] and requires the deletion of the LRR domain.[168] Comparable to NLRP3, NLRC4 activation also requires the chaperone protein, heat-shock protein 90 and the ubiquitin ligase-associated protein SGT1.[169] However, there are striking differences between NLRC4 and other NLR proteins. For example, activation of CASP-1 by NLRC4 primarily leads to cell death (i.e. necrosis).[165] However, the activation of CASP-1 by NLRP3 largely results in IL-1β and IL-18 processing without leading to cell death (i.e. pyroptosis).[165] Also, activation of CASP-1 by NLRC4 does not involve potassium.[170] Therefore, NLRP3 and NLRC4 inflammasomes activate CASP-1 with certain specificity.[165] Altogether, these data suggest that the pathological pro-inflammatory process in spontaneous labor at term with acute histologic chorioamnionitis includes the participation of both NLRP3 and NLRC4, which most likely results in pyroptosis and necrosis in the chorioamniotic membranes.

Inflammasome assembly/activation leads to the processing of pro-CASP-1 into its active form.[106,171] Herein, we found that the active forms of CASP-1 are increased in the chorioamniotic membranes from women in spontaneous labor at term with acute histologic chorioamnionitis. This finding is in line with a previous study demonstrating that amniotic fluid CASP-1 concentrations are greater in women with intra-amniotic infection/inflammation who delivered preterm than in cases without this clinical condition.[113] This finding suggests that during spontaneous labor at term with acute histologic chorioamnionitis, the chorioamniotic membranes release abundant active forms of CASP-1, which is likely mediated by the inflammasome.

Activation of the noncanonical NLRP3 inflammasome pathway is induced by gram-negative bacteria and includes the participation of CASP-4 (murine homologue CASP-11) in the activation of CASP-1.[158,172,173] Although the chorioamniotic membranes from women who underwent spontaneous labor at term with acute histologic chorioamnionitis expressed high

levels of CASP-4 at the RNA level, its protein and mature form were not different between tissues with and without acute inflammation. These data do not support a role of CASP-4 in the pathological pro-inflammatory process of spontaneous labor at term with acute histologic chorioamnionitis.

The active forms of CASP-1 can convert inactive pro-IL-1β[62-68] and pro-IL18[66-68,98,99] into their mature forms. Herein, we found that the chorioamniotic membranes from women in spontaneous labor at term with acute histologic chorioamnionitis release high amounts of mature IL-1β. To determine whether the release of mature IL-1β from the chorioamniotic membranes was driven by the activation of CASP-1, we used VX765 in an *in vitro* model of inflammation induced by LPS. This model is widely used *in vitro*[174-177] and *in vivo*.[178-184] VX765 is a potent, selective, and competitive inhibitor of CASP-1, which inhibits the CASP-1-dependent release of IL-1β and IL-18 from peripheral blood mononuclear cells[185] and whole blood.[186] In this study, VX765 inhibited the LPS-induced release of inflammasome-dependent IL-1β and IL-18 (2-fold) by the chorioamniotic membranes without affecting the release of inflammasome-independent IL-6 or TNF-α. The IL-1β cytokine participates in several processes of parturition,[187-193] and its causal role was demonstrated by the fact that injection of this cytokine in mice[194,195] and monkeys[196-203] induces preterm labor and birth. Collectively, these data demonstrate that the chorioamniotic membranes release the mature form of IL-1β, which is likely mediated by the inflammasome-derived active forms of CASP-1 during spontaneous labor at term with acute histologic chorioamnionitis.

We observed a modest increase in the total protein concentration and abundance of the mature form of IL-18 in the chorioamniotic membranes from women who underwent spontaneous labor at term with acute histologic chorioamnionitis compared to those without this placental lesion. The lack of statistical significance between these 2 study groups may be due to the sample size. Regardless, this finding is in line with a previous report demonstrating that amniotic fluid concentrations of IL-18 are higher in women who underwent spontaneous labor at term with microbial invasion of the amniotic cavity (MIAC) than in those who delivered at term without this clinical condition.[126] These data suggest that IL-18 may be involved in the pathological inflammation of the chorioamniotic membranes during spontaneous labor at term with acute histologic chorioamnionitis.

Conclusion

This study provides evidence supporting a role for the inflammasome in the activation of CASP-1 and the release of mature IL-1β by the chorioamniotic membranes from women in spontaneous labor at term with acute histologic chorioamnionitis. The selective upregulation of NLRP3 and NLRC4 suggests that these inflammasomes are implicated in the mechanisms responsible for pathological inflammation (i.e. acute histologic chorioamnionitis) in term parturition.

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Supplemental Material

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