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Research Article

Intra-amniotic inflammation induces preterm birth by activating the NLRP3 inflammasome[†]

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Abstract

Intra-amniotic inflammation is strongly associated with spontaneous preterm labor and birth, the leading cause of perinatal mortality and morbidity worldwide. Previous studies have suggested a role for the NLRP3 (NLR family pyrin domain-containing protein 3) inflammasome in the mechanisms that lead to preterm labor and birth. However, a causal link between the NLRP3 inflammasome and preterm labor/birth induced by intra-amniotic inflammation has not been established. Herein, using an animal model of lipopolysaccharide-induced intra-amniotic inflammation (IAI), we demonstrated that there was priming of the NLRP3 inflammasome (1) at the transcriptional level, indicated by enhanced mRNA expression of inflammasome-related genes (*Nlrp3*, *Casp1*, *Il1b*); and (2) at the protein level, indicated by greater protein concentrations of NLRP3, in both the fetal membranes and decidua basalis prior to preterm birth. Additionally, we showed that there was canonical activation of the NLRP3 inflammasome in the fetal membranes, but not in the decidua basalis, prior to IAI-induced preterm birth as evidenced by increased protein levels of active caspase-1. Protein concentrations of released IL1β were also increased in both the fetal membranes and decidua basalis, as well as in the amniotic fluid, prior to IAI-induced preterm

birth. Finally, using the specific NLRP3 inhibitor, MCC950, we showed that in vivo inhibition of the NLRP3 inflammasome reduced IAI-induced preterm birth and neonatal mortality. Collectively, these results provide a causal link between NLRP3 inflammasome activation and spontaneous preterm labor and birth in the context of intra-amniotic inflammation. We also showed that, by targeting the NLRP3 inflammasome, adverse pregnancy and neonatal outcomes can be significantly reduced.

Summary Sentence

Intra-amniotic inflammation induces the activation of the NLRP3 inflammasome in the fetal membranes and decidua basalis prior to preterm birth, which is significantly reduced by inhibiting such a pathway.

Key words: amniotic fluid, fetal inflammatory response syndrome, intra-amniotic infection, acute chorioamnionitis, funisitis, clinical chorioamnionitis, neutrophils, interleukin-1-beta, cytokines, caspase-1, NLRP3 inhibitor, fetal membranes, decidua, LPS.

Introduction

Intra-amniotic inflammation is a causal link to spontaneous preterm birth [1–8], the leading cause of perinatal mortality and morbidity worldwide [9–12]. Such inflammation can occur as the result of microbial invasion of the amniotic cavity (MIAC), referred to as intra-amniotic infection, or from danger signals or alarmins, known as sterile intra-amniotic inflammation [13–16]. Intra-amniotic infection is characterized by an increased white blood cell count [17–22] and elevated concentrations of cytokines [23–35] and lipid mediators (e.g. prostaglandins) [36–43] in the amniotic fluid. This intra-amniotic inflammatory response can result in deleterious effects on the offspring [27, 28, 44–49]. Therefore, the elucidation of the mechanisms involved in intra-amniotic inflammation leading to preterm birth is critical for the development of novel treatment strategies to reduce adverse neonatal outcomes [50, 51].

Recently, we provided evidence supporting a role for the inflammasome in the mechanisms leading to intra-amniotic inflammation associated with preterm labor [52–55]. Inflammasomes are cytoplasmic multi-protein complexes that are mainly expressed by innate immune cells [56–74]. Inflammasome activation includes two steps: the priming and the assembly of the multi-protein complex [75, 76]. In the first step, microbial products or alarmins are sensed by pattern recognition receptors, inducing the activation of the NF-κB pathway, which results in the upregulation (mRNA and protein) of the inflammasome sensor molecule (e.g. NLR family pyrin domain-containing protein or NLRP3) and other related proteins [75–77]. In the second step, the inflammasome complex is assembled [75, 76], inducing the activation of caspase-1 (CASP-1) [56, 61, 63, 71, 72, 74]. Active forms of CASP-1 can then lead to the maturation of pro-interleukin (IL)1β and pro-IL18 into their bioactive forms [78–88]. Both the priming and activation of the inflammasome have been described in the chorioamniotic membranes and amniotic cavity of women with term [89–92] or preterm [52, 55] labor. However, a causal link between inflammasome activation and spontaneous preterm labor in the context of intra-amniotic inflammation has not been established.

Herein, using a previously established model of intra-amniotic inflammation that resembles the subclinical syndrome of preterm labor associated with MIAC [93] (hereafter referred to as IAI-induced preterm birth model), we investigated whether a microbial product (lipopolysaccharide [LPS]) could induce the activation of the inflammasome at the maternal-fetal interface (fetal membranes and decidua basalis) prior to preterm birth. In addition, we investigated whether inhibition of such a pathway could prevent preterm birth and reduce adverse neonatal outcomes.

Methods

Mice

C57BL/6 mice were purchased from The Jackson Laboratory in Bar Harbor, ME, USA, and bred in the animal care facility at the C.S. Mott Center for Human Growth and Development at Wayne State University, Detroit, MI, USA. All mice were housed under a circadian cycle (12 h light/12 h dark). Females 8–12 weeks old were mated with males of the same background and proven fertility. Female mice were checked daily between 8:00 a.m. and 9:00 a.m. for the appearance of a vaginal plug, which indicated 0.5 days post coitum (dpc). Females were then placed into new cages, and their weights were monitored daily. A gain of two or more grams by 12.5 dpc confirmed pregnancy. All procedures were approved by the Institutional Animal Care and Use Committee at Wayne State University (Protocol No. A-07–03-15).

Murine model of intra-amniotic inflammation-induced preterm birth

Intra-amniotic administration of LPS [93] (IAI-induced preterm birth): dams were anesthetized on 16.5 dpc by inhalation of 2–3% isoflurane (Aerrane, Baxter Healthcare Corporation, Deerfield, IL, USA) and 1–2 L/min of oxygen in an induction chamber. Anesthesia was maintained with a mixture of 1.5–2% isoflurane and 1.5–2 L/min of oxygen. Dams were positioned on a heating pad and stabilized with adhesive tape. Fur removal from the abdomen was achieved by applying Nair cream (Church & Dwight Co., Inc., Ewing, NJ, USA) to this area. Body temperature was maintained in the range of $37 \pm 1°$C and detected with a rectal probe (VisualSonics, Toronto, ON, Canada), and respiratory and heart rates were monitored by electrodes embedded in the heating pad. An ultrasound probe was fixed and mobilized with a mechanical holder, and the transducer was slowly moved toward the abdomen. Ultrasound-guided intra-amniotic injection of LPS (*Escherichia coli* O111: B4; Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 100 ng dissolved in 25 μL of sterile 1X phosphate-buffered saline (PBS; Fisher Scientific Bioreagents, Fair Lawn, NJ, USA) was performed in each amniotic sac using a 30G needle (BD PrecisionGlide Needle, Becton Dickinson, Franklin Lakes, NJ, USA). Controls were injected with 25 μL of sterile 1X PBS. The syringe was stabilized by a mechanical holder (VisualSonics Inc., Toronto, ON, Canada). Following the ultrasound, mice were placed under a heat lamp for recovery (defined as when the mouse resumes normal activity, such as walking and responding), which typically occurred 10–20 min after removal from anesthesia.

RNA isolation, cDNA synthesis, and quantitative reverse transcription polymerase chain reaction analysis

Dams were intra-amniotically injected with either LPS or PBS on 16.5 dpc. Mice were euthanized on 17.5 dpc (15–17 h post-injection) and dissection to obtain the fetal membranes and decidua basalis was performed (Figure 1A and B, n = 12 per group). Tissues were placed in RNA*later*^{TM} Stabilization Solution (Invitrogen by Thermo Fisher Scientific, Carlsbad, CA, USA), according to the manufacturer's instructions. The samples were kept at 4°C overnight, after which the RNA*later*^{TM} solution was removed, and the tissues were stored at −80°C until analysis. Total RNA was isolated from the fetal membranes and decidua basalis using QIAshredders, RNase-Free DNase Sets, and RNeasy Mini Kits (all from Qiagen, Hilden, Germany), according to the manufacturer's instructions. RNA concentrations and purity 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). Complementary (c)DNA was synthesized using SuperScript III First-Strand Synthesis SuperMix (Invitrogen by Thermo Fisher Scientific). Gene expression profiling was performed on the BioMark System (Fluidigm, San Francisco, CA, USA) for high-throughput qRT-PCR with the TaqMan gene expression assays (Applied Biosystems, Life Technologies Corporation, Foster City, CA, USA) listed in Supplemental Table S1.

Western blots for inflammasome-related proteins

For analysis of the inflammasome-related proteins in the fetal membranes and decidua basalis, dams were intra-amniotically injected with either LPS or PBS on 16.5 dpc. Mice were euthanized on 17.5 dpc and dissection to obtain the fetal membranes and decidua basalis was performed (Figure 1A and B, n = 6 per group). Tissues were snap-frozen in liquid nitrogen and stored at −80°C until analysis. Tissue lysates were prepared by mechanically homogenizing snap-frozen fetal membranes and decidua basalis in 1X PBS containing a complete protease inhibitor cocktail (Roche Applied Sciences, Mannheim, Germany). Lysates were centrifuged at 15 700 x g for 5 min at 4°C and the supernatants were stored at −80°C until use.

For analysis of inflammasome-related proteins in murine macrophages, bone marrow was collected from C57BL/6 mice and the cells were differentiated in IMDM medium (Thermo Scientific) with 10% FBS (Invitrogen) and 10ng/mL of M-CSF (Cat#576402; BioLegend, San Diego, CA, USA) at 37°C and 5% CO_2 for 7 days. Bone marrow-derived macrophages (BMDMs) were seeded into 6-well tissue culture plates (Fisher Scientific) at 5×10^5 cells/well and cultured at 37°C with 5% CO_2 overnight. Following incubation, the culture medium was gently aspirated and replaced with fresh medium. For MCC950 pretreatment, 10 μM MCC950 (Cat#PZ0280; Sigma-Aldrich) was added to the culture media and the BMDMs were incubated at 37°C with 5% CO_2 for 30 min. Following treatment with MCC950, the BMDMs were incubated with 0.5 μg/mL of LPS (*Escherichia coli* 0111: B4; Sigma-Aldrich) at 37°C with 5% CO_2 for 4 h, followed by the addition of 10 μM of nigericin (Cat#N7143; Sigma-Aldrich) for an additional hour. Non-treated BMDMs were used as a negative control. The cell supernatants were collected and centrifuged at 1300 x g for 5 min to remove floating cells and debris. The cell-free supernatants were then concentrated to 10X with the Amicon Ultra Centrifuge filter (Cat#UFC800324, Ultracel 3K, EMD Millipore, Darmstadt, Germany) and stored at −20°C until use. Cultured BMDMs were then collected and lysed

with RIPA buffer (Sigma-Aldrich) containing a complete protease inhibitor cocktail (Roche Applied Sciences). Lysates were centrifuged at 15 700 x g for 5 min at 4°C and the supernatants were collected and stored at −20°C until use.

Prior to immunoblotting, total protein concentration was determined using the Pierce BCA Protein Assay Kit (Cat#23225; Pierce Biotechnology, Thermo Fisher Scientific, Inc., Rockford, IL). Fetal membrane and decidua basalis tissue lysates (50 μg per well), cell lysates (10 μg per well), and concentrated cell supernatants (40 μl) were subjected to electrophoresis in 4%–12% sodium dodecyl sulphate-polyacrylamide gels (Cat#NP0336BOX, Invitrogen by Thermo Fisher Scientific, Carlsbad, CA). Separated proteins were then transferred onto nitrocellulose membranes (Cat#1620145, Bio-Rad, Hercules, CA). Next, the nitrocellulose membranes were submerged in blocking solution (StartingBlock T20 Blocking Buffer, Thermo Fisher Scientific, Inc.) for 30 min at room temperature and then probed overnight at 4°C with the following mouse antibodies: mouse anti-NLRP3 (Cat#AG-20B-0014-C100, 1μg/mL, Adipogen Life Sciences, San Diego, CA), rat anti-CASP-1 (Cat#14–9832-82, 5μg/mL, Invitrogen), and rat anti-IL1β (Cat#MAB4011, 1μg/mL, R&D Systems, Inc., Minneapolis, MN). Finally, nitrocellulose membranes were then stripped with Restore PLUS Western Blot Stripping Buffer (Pierce Biotechnology, Thermo Fisher Scientific, Inc.) for 15 min, washed with 1X PBS, blocked, and re-probed for 1 h at room temperature with a mouse anti-β-actin (ACTB) monoclonal antibody (Cat#A5441, Sigma-Aldrich). Chemiluminescence signals were detected with the ChemiGlow West Substrate Kit (ProteinSimple, San Jose, CA) and images were acquired using the Fujifilm ImageQuant LAS-4000 Imaging System (GE Life Sciences, Pittsburgh, PA). Quantification was performed using ImageJ.

ELISA determination of IL1β concentrations in the fetal membranes and decidua basalis

Dams were intra-amniotically injected with either LPS or PBS on 16.5 dpc. Mice were euthanized on 17.5 dpc and dissection to obtain the fetal membranes and decidua basalis was performed (Figure 1A and B, n = 10 per group). Tissues were snap-frozen in liquid nitrogen and stored at −80°C until analysis. Tissue lysates were prepared by mechanically homogenizing snap-frozen fetal membranes and decidua basalis in the Cell Lysis Buffer 2 (Cat#895347; R&D Systems, Inc.). Lysates were centrifuged at 15 700 x g for 5 min at 4°C and the supernatants were stored at −80°C until use. Prior to ELISA, total protein concentration was determined using the Pierce BCA Protein Assay Kit. Concentrations of IL1β in the fetal membranes and decidua basalis were determined by using a sensitive and specific ELISA assay kit (Cat#MLB00C; R&D Systems, Inc.). This ELISA kit was initially validated in our laboratory prior to the execution of this study. Tissue concentrations of IL1β were obtained by interpolation from the standard curve. The sensitivity of the assay was 2.31 pg/mL. The inter- and intra-assay coefficients of variation were less than 10%.

Amniotic fluid concentrations of IL1β were also determined, as previously reported [94].

MCC950 treatment

Dams were intra-peritoneally injected on 16.5 dpc with 50mg/kg of the NLRP3 inhibitor MCC950 dissolved in 200 μL of sterile 1X PBS (n = 8–10 per group). This dosage of MCC950 has been shown to inhibit the NLRP3 inflammasome in vivo [95]. Shortly after (1–2 h), dams received the intra-amniotic administration of LPS or PBS as
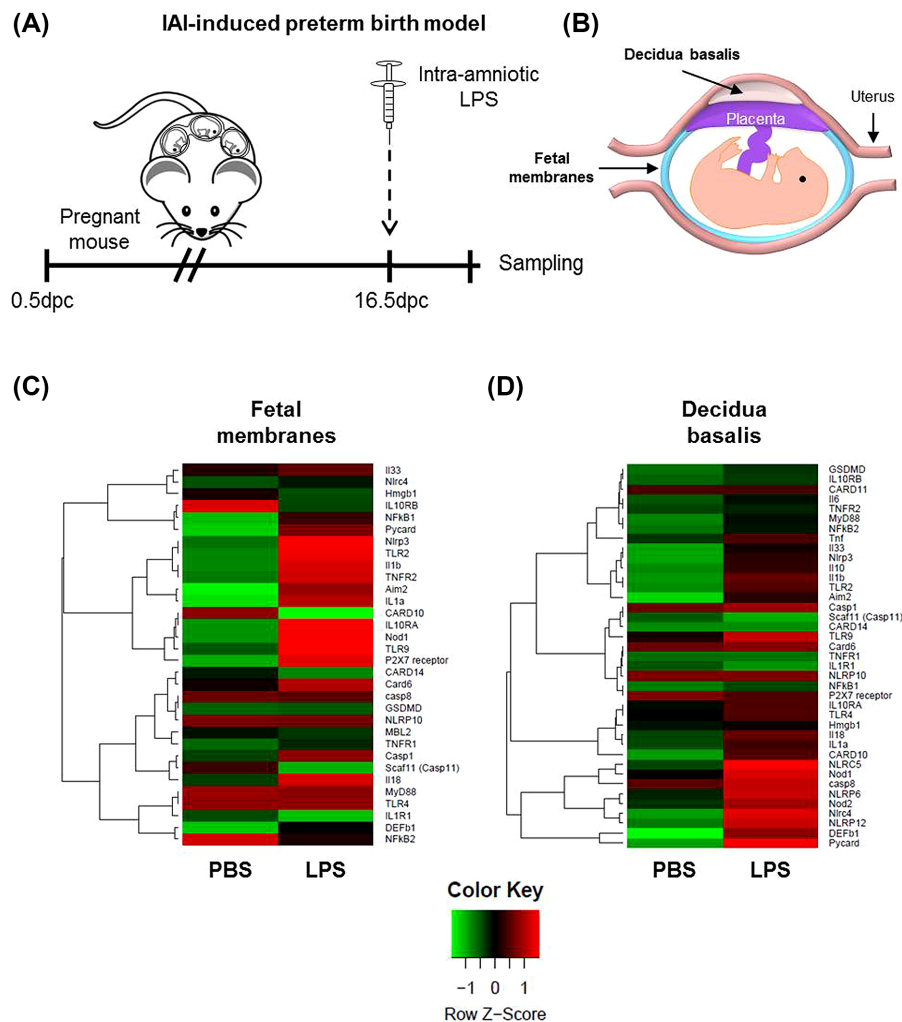
Figure 1. Inflammasome-related gene profiles are upregulated in the fetal membranes and decidua basalis prior to intra-amniotic inflammation (IAI)-induced preterm birth. **A**) Animal model of IAI-induced preterm labor and birth. **B**) Spatial localization of the fetal membranes and decidua basalis in the murine uterus. Heatmaps showing regulation of inflammasome-related genes in the **C**) fetal membranes and **D**) decidua basalis. LPS = lipopolysaccharide; PBS = phosphate-buffered saline (vehicle control). Green = downregulation, red = upregulation. N = 12 per group.

described above. Controls were injected with LPS or PBS alone. The rates of preterm labor/birth and neonatal mortality were obtained for each group.

Video monitoring and definition of preterm labor/birth and neonatal mortality

Dams were monitored via video camera (Sony Corporation, Tokyo, Japan) until delivery to obtain the rates of preterm birth and neonatal mortality. Preterm birth was defined as delivery occurring before 18.5 dpc, and its rate was represented by the percentage of dams delivering preterm among the total number of mice injected. The rate of neonatal mortality for each litter was defined as the proportion of delivered pups found dead among the total litter size.

Immunofluorescence microscopy to detect ASC specks

BMDMs were obtained as described above and seeded into a four-well Lab-Tek chamber slide (Thermo Fisher Scientific, Rochester, NY, USA) at 1.25×10^5 cells/well and cultured at $37°C$ with 5%

CO_2 overnight. Following incubation, the BMDMs were treated with MCC950 and LPS + nigericin as described above. Next, the BMDMs were fixed using 4% paraformaldehyde (Electron Microscopy Sciences Hatfield, PA, USA) for 20 min at room temperature, rinsed with 1X PBS, and permeabilized using 0.25% Triton X-100 (Promega, Madison, WI, USA) for 5 min at room temperature. Prior to staining, non-specific antibody interactions were blocked using serum-free protein blocker (Cat#X09090; DAKO, Carpinteria, CA, USA) for 30 min at room temperature. BMDMs were then stained with a rabbit anti-ASC (Cat#AG-25B-0006-C100; Adipogen, San Diego, CA) antibody at room temperature for 1 h. Rabbit IgG was used as a negative control. Following staining, the BMDMs were washed with 1X PBS containing 0.1% Tween 20 (PBS-T) (Sigma-Aldrich). After blocking for 10 min with 10% goat serum (KPL, Gaithersburg, MD, USA), secondary goat anti-rabbit IgG–Alexa Fluor 594 (Life Technologies) was added and BMDMs were incubated for 1 h at room temperature in the dark. Finally, the BMDMs were washed with 1X PBS-T and mounted using Pro-Long Diamond Antifade Mountant with DAPI (Life Technologies).

Immunofluorescence was visualized using an Olympus BX60 fluorescence microscope (Olympus, Tokyo, Japan) at 400X original magnification. The pictures were taken using an Olympus DP71 camera and DP Controller Software (Olympus).

Statistical analysis

Data were analyzed using IBM SPSS v19.0 (IBM Corporation, Armonk, NY) and the R statistical language and environment (www.r-project.org). Negative ΔCt values were determined using multiple reference genes (*Gusb*, *Hsp90ab1*, *Gapdh*, and *Actb*) averaged within each sample to determine gene expression levels. A heat map was created for the group mean expression matrix (gene x group mean), with individual gene expression level being standardized first. The heat map represents the Z-scores of the mean ($-\Delta$Ct) and the hierarchical clustering using correlation distance. Relative fold changes for mRNA expression of *Nlrp3*, *Casp1*, *Il1b*, and *Il18* were calculated using the $2^{-\Delta\Delta CT}$ method [96]. For gene expression, protein expression, and protein concentrations, the statistical significance of group comparisons was assessed using the Mann–Whitney *U*-test. For rates of preterm birth and neonatal mortality, the statistical significance of group comparisons was assessed using the Fisher's exact test. A *P*-value < 0.05 was considered significant.

Results

Inflammasome-related genes are upregulated in the fetal membranes and decidua basalis prior to IAI-induced preterm birth

First, we determined whether the expression of inflammasome-related genes was altered in the fetal membranes and decidua basalis prior to IAI-induced preterm birth. Figure 1A shows the timeline of intra-amniotic injection of LPS and sampling of the fetal membranes and decidua basalis. Figure 1B is a representation of the spatial localization of the fetal membranes and decidua basalis in the murine uterus. The fetal membranes from dams injected with LPS showed upregulation of inflammasome-related genes compared to their PBS controls (Figure 1C). Likewise, the decidua basalis from dams injected with LPS had higher expression of inflammasome-related genes than PBS controls (Figure 1D). Yet, the inflammasome-related gene profiles between the fetal membranes and the decidua basalis were different prior to IAI-induced preterm birth (Figure 1C vs. 1D).

Given that the human chorioamniotic membranes from women with spontaneous preterm labor expressed high mRNA levels of *NLRP3*, *CASP1*, and *IL1B* [52], we investigated the expression of these molecules prior to IAI-induced preterm birth. In addition, we determined the expression of *Il18* since its processing is also mediated by the inflammasome [57, 71, 82, 97]. The fetal membranes from dams injected with LPS had greater expression of *Nlrp3*, *Casp1*, and *Il1b* than those from PBS controls (Figure 2A). In the decidua basalis from dams injected with LPS, however, only *Nlrp3* and *Il1b* were upregulated (Figure 2B). The expression of *Il18* was not upregulated in either the fetal membranes or the decidua basalis prior to IAI-induced preterm birth (Figure 2A and B).

Collectively, these findings indicate that, at the transcriptional level, there is priming of the NLRP3 inflammasome (i.e. upregulation of the inflammasome-related genes [75, 76]) in both the fetal membranes and decidua basalis prior to IAI-induced preterm birth.

The NLRP3 protein is increased in the fetal membranes and decidua basalis prior to IAI-induced preterm birth

The chorioamniotic membranes from women with spontaneous preterm labor displayed increased amounts of the NLRP3 inflammasome sensor molecule [52]. Therefore, we next investigated whether this protein was overexpressed prior to IAI-induced preterm birth. Consistent with our human findings, the protein quantities of NLRP3 in the fetal membranes and decidua basalis were greater in dams injected with LPS than in PBS controls (Figure 3). This finding confirms that, at the protein level, there is priming of the NLRP3 inflammasome in both the fetal membranes and decidua basalis prior to IAI-induced preterm birth.

The activation of caspase-1 is increased in the fetal membranes, but not in the decidua basalis, prior to IAI-induced preterm birth

Following priming, the NLRP3 inflammasome is assembled, inducing the activation of CASP-1 [56, 61, 63, 71, 72, 74]. Thus, we next investigated whether there is activation of CASP-1 prior to IAI-induced preterm birth. Different forms of partially processed CASP-1 (e.g. p35) were detected by Western blot (Figure 4A and B); yet we focused on quantifying the p20 active form that has biological function [78]. The protein quantity of active CASP-1 (p20 form) in the fetal membranes was greater in dams injected with LPS than in PBS controls (Figure 4A). However, the active form of CASP-1 in the decidua basalis was not different between dams injected with LPS and PBS controls (Figure 4B). The protein quantity of pro-CASP-1 in the fetal membranes and decidua basalis was not different between dams injected with LPS and PBS controls (Figure 4A and B). These data show that in the fetal membranes, but not in the decidua basalis, there is canonical activation of the NLRP3 inflammasome prior to IAI-induced preterm birth.

The concentration of IL1β is increased in the fetal membranes and decidua basalis prior to IAI-induced preterm birth

Following activation of the NLRP3 inflammasome, the active forms of CASP-1 can induce the release of mature IL1β into the extracellular space [78–88]. Hence, we determined the concentration of IL1β in the fetal membranes and decidua basalis prior to IAI-induced preterm birth. The concentration of released IL1β in the fetal membranes and decidua basalis from dams injected with LPS was greater than in those from PBS controls (Figure 5). In addition, we determined the concentration of released IL1β in the amniotic fluid. Similar to the protein extracts of the fetal membranes and decidua basalis, amniotic fluid concentrations of released IL1β were increased in dams injected with LPS (Supplementary Figure 1).

It is worth mentioning that the mature form of IL18, another cytokine processed by the inflammasome [83], was neither detected in the fetal membranes nor in the decidua basalis of dams from any group (data not shown).
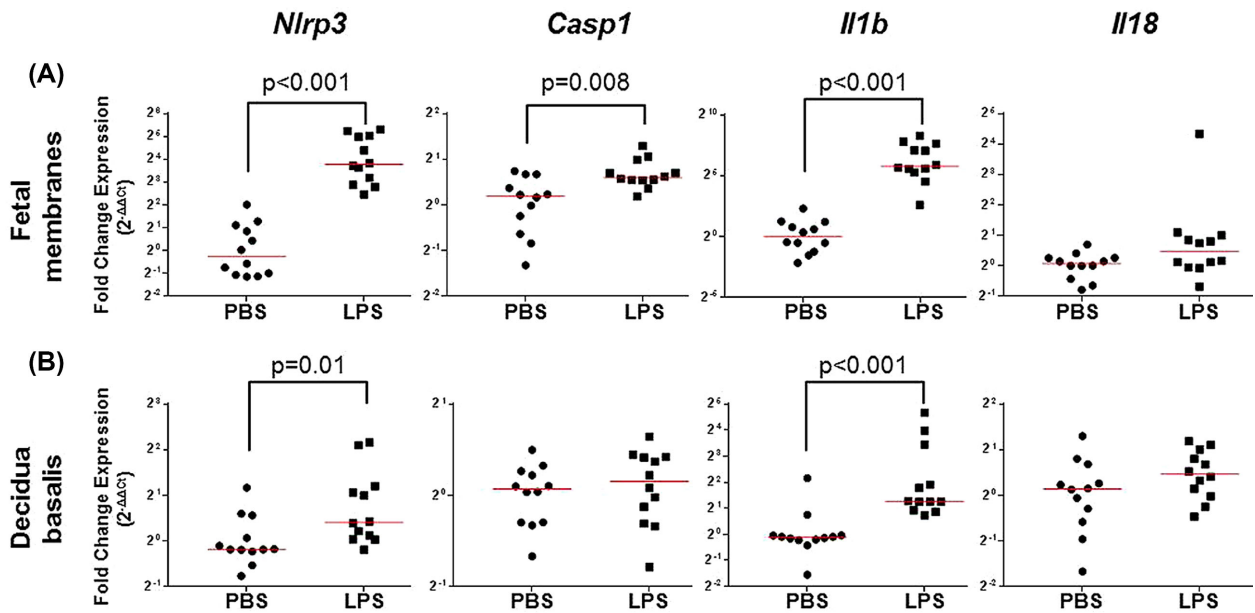
Figure 2. NLRP3 inflammasome-related genes are upregulated in the fetal membranes and decidua basalis prior to intra-amniotic inflammation (IAI)-induced preterm birth. Expression of *Nlrp3*, *Casp1*, *Il1b*, and *Il18* in the **A**) fetal membranes and **B**) decidua basalis prior to IAI-induced preterm birth. LPS = lipopolysaccharide; PBS = phosphate-buffered saline (vehicle control). Middle lines indicate medians. N = 12 per group.
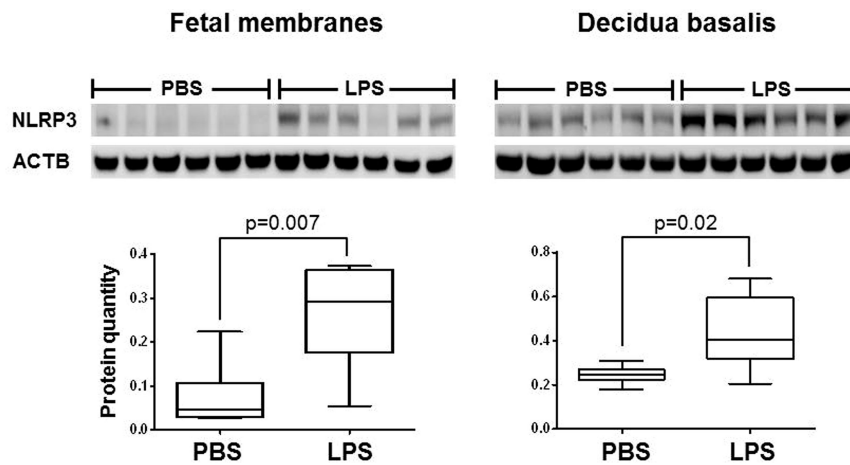

Figure 3. NLRP3 protein concentrations are increased in the fetal membranes and decidua basalis prior to intra-amniotic inflammation (IAI)-induced preterm birth. LPS = lipopolysaccharide; PBS = phosphate-buffered saline (vehicle control). ACTB = β-actin. Midlines indicate medians, boxes show interquartile range, and whiskers indicate min-max range. N = 6 per group.

Inhibition of the NLRP3 inflammasome prevents IAI-induced preterm birth and reduces adverse neonatal outcomes

Up to this point, we have shown that the fetal membranes and decidua basalis released IL1β prior to IAI-induced preterm birth. Next, we tested whether inhibition of the NLRP3 inflammasome could prevent preterm birth and, more importantly, its adverse neonatal outcomes. In vivo inhibition of the NLRP3 inflammasome is achieved by systemic administration of the specific NLRP3 inhibitor, MCC950 [95]. Dams were randomized to be treated or untreated with MCC950 (Figure 6A). As expected [93, 94], most of the animals intra-amniotically injected with LPS had a shorter time interval from injection to delivery compared to PBS controls (Figure 6B) and delivered preterm (Figure 6C), whereas

PBS controls delivered at term (Figure 6C). Treatment with MCC950 restored the time interval between LPS injection and delivery (Figure 6B) and reduced the rate of preterm birth by 30% (Figure 6C). Importantly, treating dams with MCC950 reduced the rate of neonatal mortality by approximately 30%, and such a reduction was statistically significant (Figure 6D). To test whether the activation of the NLRP3 inflammasome was inhibited in our model, murine BMDMs were incubated with LPS + nigericin with and without MCC950 treatment. BMDMs incubated with LPS + nigericin contained high amounts of active CASP-1 and mature IL1β, indicating inflammasome activation (Figure 7A). These effects were inhibited by treatment with MCC950 (Figure 7A). In addition, we proved that treatment with MCC950 inhibited ASC speck formation (red dots indicated by yellow arrows) induced by LPS + nigericin (Figure 7B).
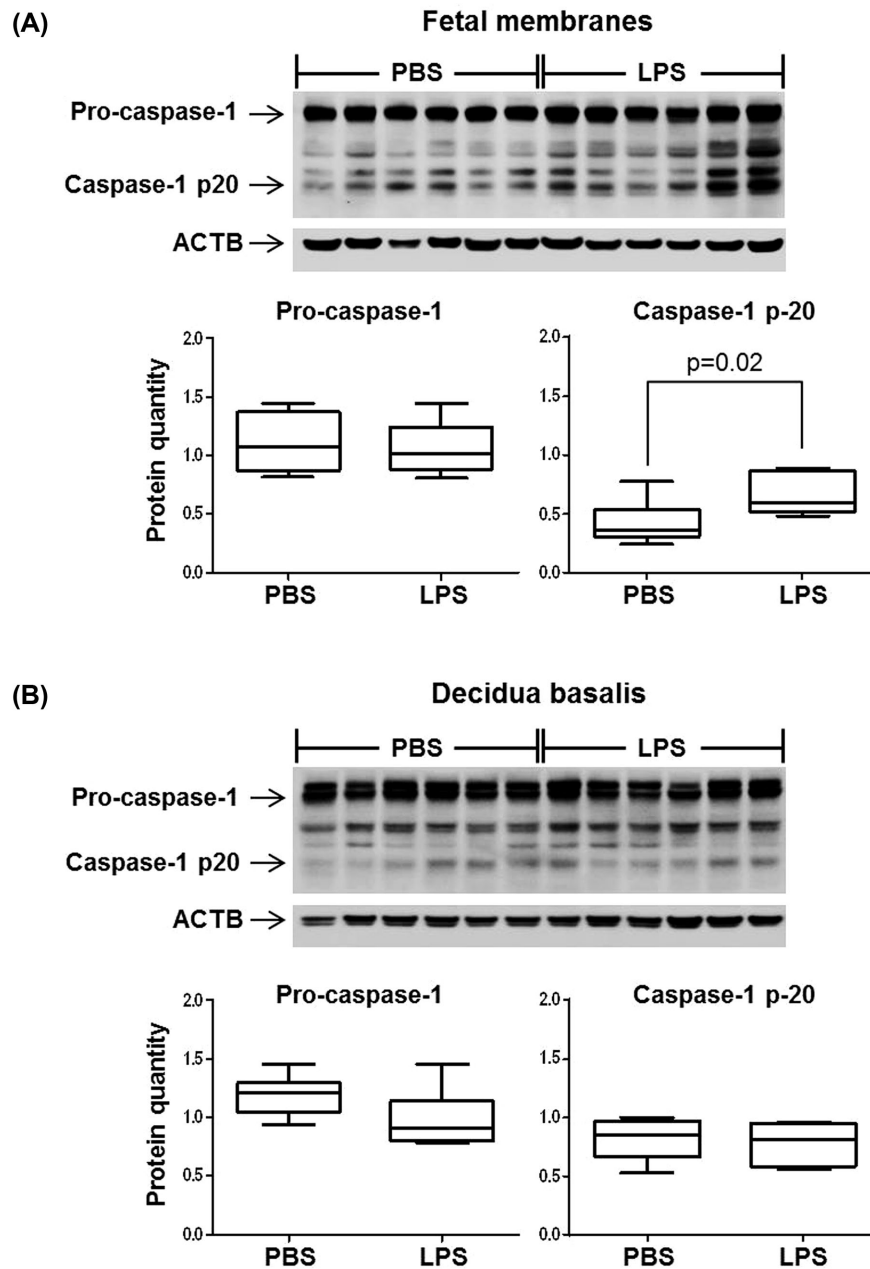
(A)

Fetal membranes

Figure 4. Active caspase-1 is increased in the fetal membranes prior to intra-amniotic inflammation (IAI)-induced preterm birth. Protein concentrations of pro-caspase-1 and active caspase-1 p20 in the **A**) fetal membranes and **B**) decidua basalis prior to IAI-induced preterm birth. LPS = lipopolysaccharide; PBS = phosphate-buffered saline (vehicle control). ACTB = β-actin. Midlines indicate medians, boxes show interquartile range, and whiskers indicate min-max range. N = 6 per group.

These results show that the NLRP3 inflammasome is implicated in the mechanisms that lead to spontaneous preterm labor in the context of intra-amniotic infection, and that by inhibiting this pathway adverse neonatal outcomes can be significantly reduced.

Discussion

Principle findings

In this study, we demonstrated that there was priming of the NLRP3 inflammasome (1) at the transcriptional level, indicated by enhanced mRNA expression of inflammasome-related genes (*Nlrp3*, *Casp1*,

Il1b); and (2) at the protein level, indicated by greater protein concentrations of NLRP3, in both the fetal membranes and decidua basalis prior to IAI-induced preterm birth. Additionally, we showed that there was canonical activation of the NLRP3 inflammasome in the fetal membranes, but not in the decidua basalis, prior to IAI-induced preterm birth as evidenced by increased protein levels of active caspase-1. Protein concentrations of released IL1β were also increased in both the fetal membranes and decidua basalis, as well as in the amniotic fluid, prior to IAI-induced preterm birth. Finally, using the specific NLRP3 inhibitor, MCC950, we showed that in vivo inhibition of the NLRP3 inflammasome extended gestational length
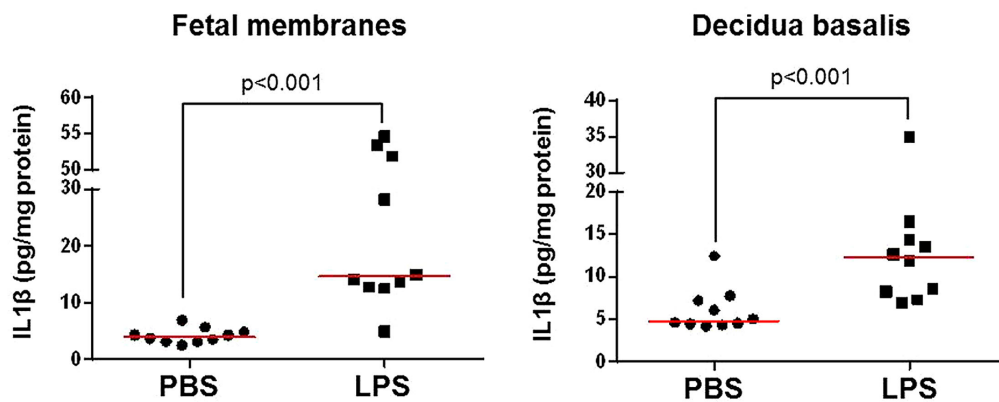
Fetal membranes

Decidua basalis

Figure 5. Interleukin (IL)1β is increased in the fetal membranes and decidua basalis prior to intra-amniotic inflammation (IAI)-induced preterm birth. LPS = lipopolysaccharide; PBS = phosphate-buffered saline (vehicle control). Middle lines indicate medians. N = 10 per group.
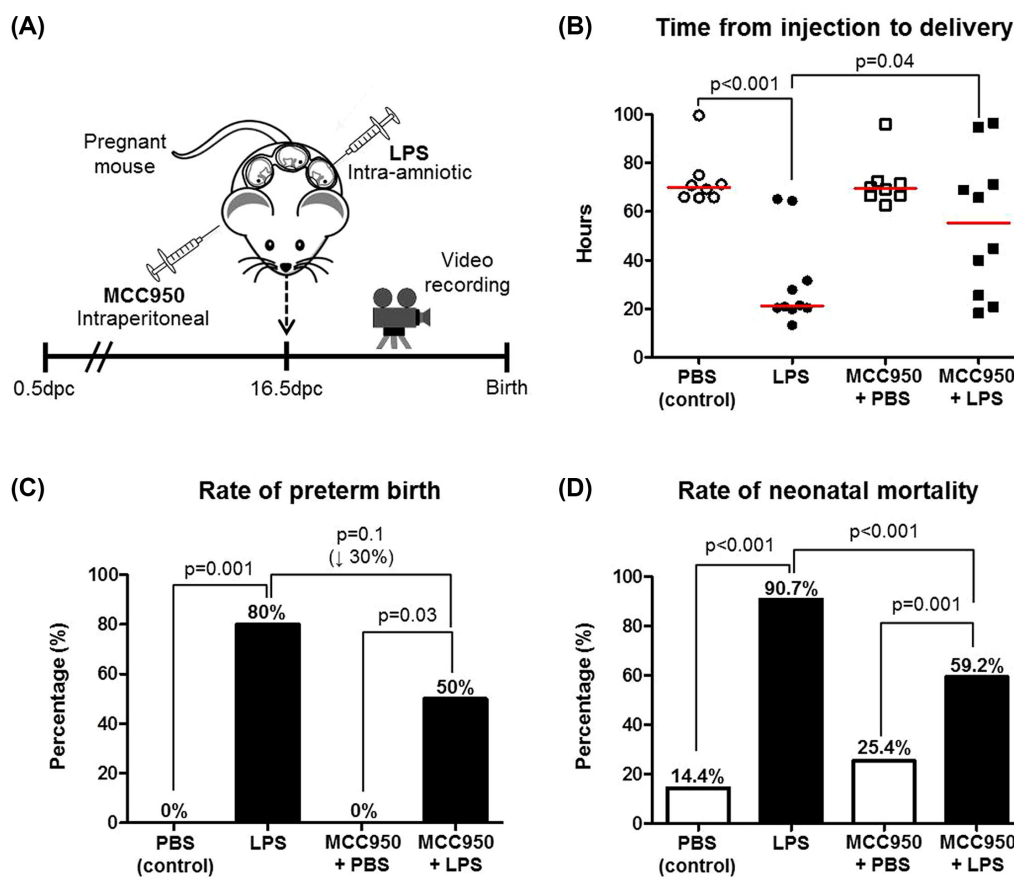
(A)

(B) **Time from injection to delivery**

(C) **Rate of preterm birth**

(D) **Rate of neonatal mortality**

Figure 6. Inhibition of the NLRP3 inflammasome via MCC950 reduces intra-amniotic inflammation (IAI)-induced preterm birth and neonatal mortality. **A**) Animal model of IAI-induced preterm labor and birth with MCC950 treatment. **B**) Time intervals (hours) from intra-amniotic injection to delivery Midlines indicate medians. **C**) Rates of preterm birth. **D**) Rates of neonatal mortality. Data are shown as percentages of the total group size. N = 8–10 per group.

and significantly reduced IAI-induced preterm birth and neonatal mortality.

The NLRP3 inflammasome in the intra-amniotic space during spontaneous preterm labor

Inflammasome activation and assembly in the chorioamniotic membranes are associated with spontaneous preterm labor, either in the context of intra-amniotic infection [52, 55] or sterile intra-amniotic inflammation [55]. Expression of inflammasome-related genes (e.g. *NLRP3*, *CASP1*, *CASP4*, and *IL1B*) is upregulated in the chorioamniotic membranes of women who underwent spontaneous preterm labor with acute histologic chorioamnionitis (a placental lesion characterized by infiltration of neutrophils into the chorioamniotic membranes [98–104] and associated with both intra-amniotic infection and sterile intra-amniotic inflammation [13–16, 55]) compared to women who delivered preterm without this placental lesion [52].
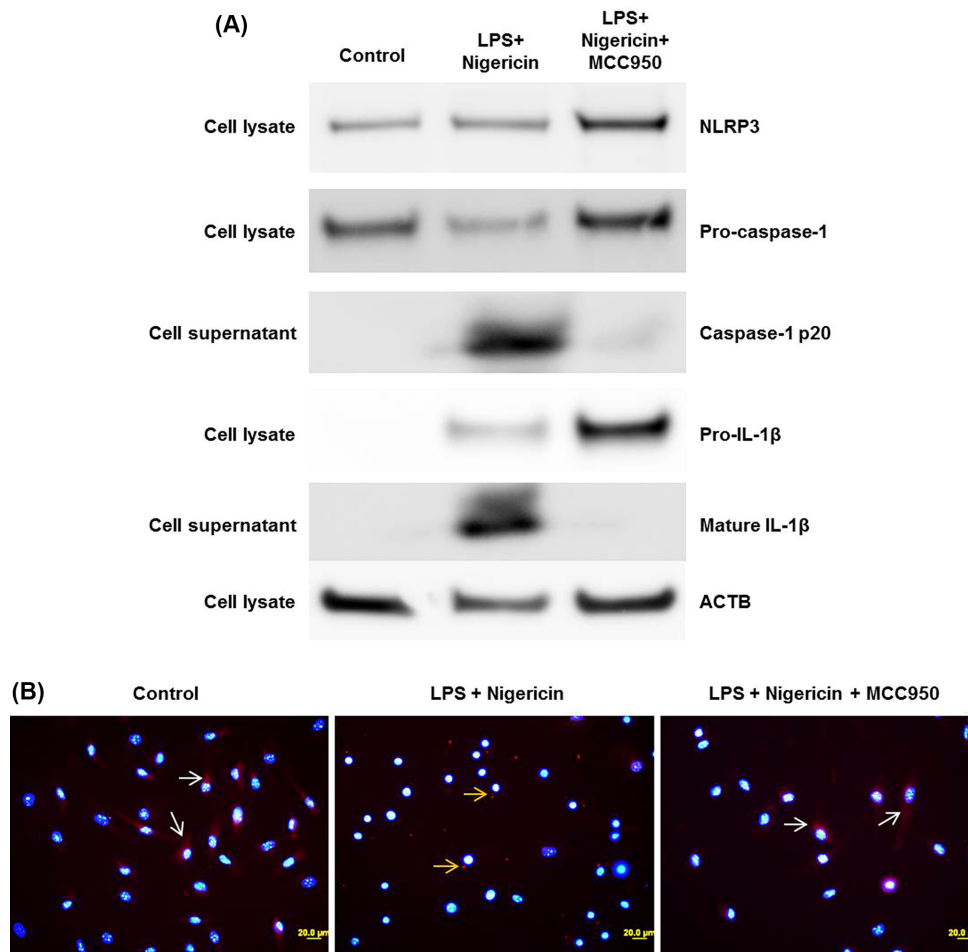
Figure 7. In vitro inhibition of NLRP3 inflammasome activation by MCC950. Bone marrow-derived macrophages were incubated with LPS and nigericin, with and without treatment with MCC950. **A**) Western blots showing the NLRP3 protein, pro-caspase-1, active caspase-1, pro-IL1β, and mature IL1β in the cell lysates or supernatants. Beta-actin (ACTB) was used as an internal control. **B**) ASC speck formation by bone marrow-derived macrophages incubated with LPS and nigericin, with and without treatment with MCC950. White arrows indicate cytoplasmic ASC, yellow arrows indicate ASC specks (red dots). Magnification 400X.

The formation of ASC (apoptosis-associated speck-like protein containing a CARD; adaptor protein of the inflammasome)/CASP-1 complexes and the levels of active CASP-1, IL1β, and IL18 are also increased in the chorioamniotic membranes from these women [52]. Moreover, women with intra-amniotic infection or sterile intra-amniotic inflammation had increased protein expression of ASC, CASP-1, and IL1β in the chorioamniotic membranes compared to women who underwent spontaneous preterm labor without intra-amniotic inflammation/infection [55]. Such findings are in line with the current study in which we showed that the fetal membranes from mice intra-amniotically injected with LPS, a model of intra-amniotic inflammation [93], have greater mRNA and protein expression of NLRP3, active CASP-1, and IL1β, indicating that both priming and activation of the NLRP3 inflammasome [75, 76] occur in the chorioamniotic membranes during intra-amniotic inflammation-induced preterm labor and birth. Yet, dams intra-amniotically injected with LPS did not have higher mRNA expression or detectable mature IL18 in the fetal membranes, which contrasts with what is observed in the chorioamniotic membranes of women with acute histologic chorioamnionitis [52]. This discrepancy may be due to the fact that the murine fetal membranes are not attached to the decidua

(i.e. decidua parietalis), which is the source of maternal neutrophils infiltrating the human chorioamniotic membranes [21, 103].

The amniotic fluid from women with spontaneous preterm labor and intra-amniotic inflammation/infection contains high concentrations of inflammasome-related proteins such as CASP-1 [105], IL1β [24, 106], IL18 [107, 108], and ASC [55]. A possible source for these proteins is the immune cells that invade the amniotic cavity in response to microbial invasion [17–22]. For example, neutrophils, monocytes/macrophages, T cells, NK cells, and B cells are abundant in the amniotic fluid of women with intra-amniotic inflammation/infection [17, 20–22], some of which can express inflammasome components [109–111]. Yet, further research is required to demonstrate that amniotic fluid immune cells express inflammasome components in the context of intra-amniotic infection and/or sterile intra-amniotic inflammation.

The NLRP3 inflammasome responds to both microbial [60, 112–119] and non-infectious or endogenous [120–128] signals. Thus, we and others have proposed that microbial products [94, 129–132] and alarmins [133] can activate the inflammasome pathway in the context of intra-amniotic inflammation-associated preterm labor. Recent in vitro studies showed that *Ureaplasma*

spp [134], *Gardnerella vaginalis* [135], and group B streptococcus (GBS) [136], microorganisms commonly found in women with intra-amniotic infection [13, 35, 137–142], induce activation of the NLRP3 inflammasome in macrophages, further implicating this pathway in the mechanisms leading to preterm labor. Moreover, the herpes virus MHV-68 enhanced LPS-induced secretion of IL1β by chorioamniotic membranes, a process likely mediated through the NLRP3 inflammasome [131]. This finding provides evidence for the potential of viruses to prime and/or activate the NLRP3 inflammasome, especially in the context of an active polymicrobial infection [131].

The NLRP3 inflammasome in the decidua basalis during spontaneous preterm labor

Several studies showed that the decidua is a source of IL1β [143–146], suggesting that the inflammasome is implicated in the processing of this cytokine at the maternal-fetal interface. Recent studies have supported such a concept. For example, decidual stromal cells expressed high levels of inflammasome-related genes (*NLRP3*, *CASP1*, and *IL1B*) upon in vitro LPS stimulation [147]. Furthermore, choriodecidual leukocytes from women who underwent spontaneous labor at term had increased formation of ASC "specks" (intracellular aggregates of ASC protein), an indicator of inflammasome activation [148, 149], compared to those who

delivered at term without labor [150]. In this study, we provided evidence demonstrating that, in the context of intra-amniotic inflammation induced by an endotoxin, the processing of IL1β in the decidua basalis could be mediated by an active caspase-1-independent mechanism such as the non-canonical pathway of the inflammasome. In this pathway, cytoplasmic CASP-4/5 (human) or CASP-11 (mouse) directly recognizes microbial products (e.g. LPS) in the intracellular space which can initiate activation of the inflammasome in a CASP-1-independent manner [118, 151, 152]. Alternatively, decidual IL1β could have been processed by inflammasome-independent mechanisms such as neutrophil elastase, cathepsin G, collagenase [153], mast cell chymase [154], matrix metalloproteinases (MMP) 2, 3, and 9 [155], neutrophil proteinase 3 [156], and cathepsin C [157]. The presence of additional caspase-1-independent mechanisms for IL1β cleavage was confirmed by a study in which CASP-1 and ASC-deficient mice had unimpaired IL1β production [158].

In this study, we observed that the decidua basalis contained high amounts of the NLRP3 protein, but this was not accompanied by an increase in the active form of CASP-1. These data suggest that, in the decidua basalis, the NLRP3 inflammasome may be implicated in different processes that are not related to pyroptosis (i.e. processing of CASP-1). Recent reports have shown that the NLRP3 inflammasome is expressed in neutrophils and involved in the generation of neutrophil extracellular traps (NETs) [159, 160]. In line with this

Figure 8. Conceptual framework. Intra-amniotic inflammation triggered by a microbial product (e.g. LPS) induced the canonical activation of the NLRP3 inflammasome in the fetal membranes, as evidenced by the high amounts of the NLRP3 protein, active CASP-1, and released IL1β. In the decidua basalis, however, the increased amounts of the NLRP3 protein did not coincide with an increase in active form of CASP-1, suggesting that CASP-1-independent mechanisms (e.g. non-canonical activation of the NLRP3 inflammasome) participate in the processing of IL1β in this compartment. Other pathways implicated in the processing of IL1β in the decidual tissues are also displayed.

concept, we have shown that NETs are formed in the chorioamniotic membranes with acute histologic chorioamnionitis [161], suggesting that the NLRP3 inflammasome is expressed by decidual neutrophils infiltrating the chorioamniotic space during intra-amniotic infection and participates in NET formation.

A novel strategy to prevent intra-amniotic inflammation-induced preterm birth

The NLRP3 inflammasome has previously been proposed as a potential therapeutic target to treat multiple inflammatory diseases [162–169]. Several synthetic [162–165, 167] and natural [166, 168] compounds with varying specificity for the NLRP3 pathway have been tested. One promising treatment is MCC950, a specific small-molecule inhibitor of the NLRP3 inflammasome [95]. The exact mechanisms whereby MCC950 prevents NLRP3 inflammasome activation are unknown; however, it was established that this compound does not target other inflammasome sensor molecules such as NLRP1, AIM2, or NLRC4 [95]. This specific inhibitor was used in multiple mouse models of inflammatory disease [170–179]; moreover, a preliminary study provided evidence that MCC950 could be safe for clinical use in humans [180]. In addition, several recent studies have indicated that MCC950 can be used to treat neonatal [95, 181] and infant [182] mice in models of cryopyrin-associated periodic syndrome (CAPS) [95], Muckle–Wells syndrome [181], and influenza A viral infection [182]. In this study, we demonstrated that inhibition of the NLRP3 inflammasome via MCC950 extends gestational length and can not only reduce the rate of IAI-induced preterm birth by 30%, but can significantly improve neonatal survival as well. It is worth mentioning that treatment with MCC950 did not completely prevent preterm birth and adverse neonatal outcomes, suggesting that LPS triggered NLRP3 inflammasome-independent mechanisms (e.g. ras/raf-1/MEK/MAPK [183] and p38 MAPK [184]) that were not blocked by this inhibitor. Together with the above evidence, our results support a role for the NLRP3 inflammasome in the inflammatory mechanisms leading to preterm labor/birth and adverse neonatal outcomes.

Conclusion

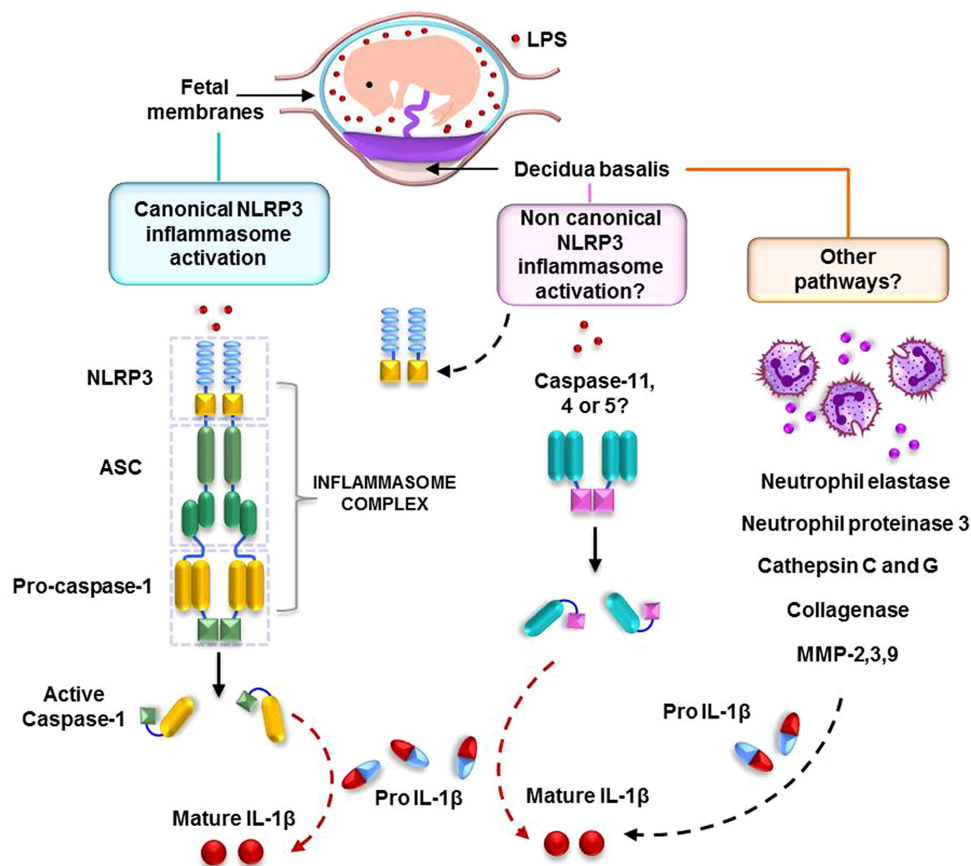This study provides evidence showing that intra-amniotic inflammation triggered by a microbial product (e.g. LPS) induced the canonical activation of the NLRP3 inflammasome in the fetal membranes, as evidenced by the high amounts of the NLRP3 protein, active CASP-1, and released IL1β (Figure 8). In the decidua basalis, however, the increased amounts of the NLRP3 protein did not coincide with an increase in active form of CASP-1, suggesting that CASP-1-independent mechanisms (e.g. non-canonical activation of the NLRP3 inflammasome) participate in the processing of IL1β in this compartment (Figure 8).

Supplementary data

Supplementary data are available at *BIOLRE* online.

Supplemental Figure 1. Concentrations of IL1β in the amniotic fluid of mice intra-amniotically injected with LPS [94]. Dams were intra-amniotically injected with either LPS or PBS (n = 5–6 each) on 16.5 dpc. Mice were euthanized on 17.5 dpc and the amniotic fluid was collected. The ProcartaPlex Mouse Cytokine & Chemokine Panel 1A 36-plex (Invitrogen) was used to measure the concentrations of IL1β in amniotic fluid samples, according to the manufacturer's

instructions. Plates were read using the Luminex 100 SystemFill (Luminex, Austin, TX, USA), and analyte concentrations were calculated with ProcartaPlex Analyst 1.0 Software from Affymetrix, San Diego, CA, USA. The sensitivity of the assay was 0.14 pg/mL (IL1β). The inter- and intra-assay coefficients of variation were less than 10%.

Supplementary Table S1. TaqMan® gene expression assays.

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