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Research Article
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Research Article

Inhibition of the NLRP3 inflammasome can prevent sterile intra-amniotic inflammation, preterm labor/birth, and adverse neonatal outcomes†

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Abstract

Sterile intra-amniotic inflammation is commonly observed in patients with spontaneous preterm labor, a syndrome that commonly precedes preterm birth, the leading cause of perinatal morbidity and mortality worldwide. However, the mechanisms leading to sterile intra-amniotic inflammation are poorly understood and no treatment exists for this clinical condition. Herein, we investigated whether the alarmin S100B could induce sterile intra-amniotic inflammation by activating the NLRP3 inflammasome, and whether the inhibition of this pathway could prevent preterm labor/birth and adverse neonatal outcomes. We found that the ultrasound-guided intra-amniotic administration of S100B induced a 50% rate of preterm labor/birth and a high rate of neonatal mortality (59.7%) without altering the fetal and placental weights. Using a multiplex cytokine array and immunoblotting, we reported that S100B caused a proinflammatory response in the amniotic cavity and induced the activation of the NLRP3 inflammasome in the fetal membranes, indicated by the upregulation of the NLRP3 protein and increased release of active caspase-1 and mature IL-1β.

Inhibition of the NLRP3 inflammasome via the specific inhibitor MCC950 prevented preterm labor/birth by 35.7% and reduced neonatal mortality by 26.7%. Yet, inhibition of the NLRP3 inflammasome at term did not drastically obstruct the physiological process of parturition. In conclusion, the data presented herein indicate that the alarmin S100B can induce sterile intra-amniotic inflammation, preterm labor/birth, and adverse neonatal outcomes by activating the NLRP3 inflammasome, which can be prevented by inhibiting such a pathway. These findings provide evidence that sterile intra-amniotic inflammation could be treated by targeting the NLRP3 inflammasome.

Summary Sentence

Intra-amniotic administration of the alarmin S100B, at clinically relevant concentrations, induces preterm labor/birth and adverse neonatal outcomes by activating the NLRP3 inflammasome, which can be prevented by targeting this pathway.

Key words: acute chorioamnionitis, alarmins, amniotic fluid, caspase-1, cytokines, damage-associated molecular patterns, danger signals, funisitis, interleukin-1β, mice, inhibitor, S100B.

Introduction

Preterm birth is the leading cause of perinatal morbidity and mortality worldwide [1, 2]. Approximately 70% of all preterm births are preceded by spontaneous preterm labor [3], a syndrome of multiple etiologies [4]. The current view is that most cases of spontaneous preterm labor with intra-amniotic inflammation occur in the absence of microorganisms and therefore represent a sterile inflammatory process [5–9]. We have coined the term "sterile intra-amniotic inflammation" to define the inflammatory process in which microorganisms cannot be detected by using both cultivation and molecular microbiology techniques in amniotic fluid [7, 10–20]. Sterile intra-amniotic inflammation is also observed in women with an asymptomatic short cervix [10], preterm prelabor rupture of membranes [11], or clinical chorioamnionitis at term [12]. The importance of sterile intra-amniotic inflammation is highlighted by the observations that patients affected by this condition have a similar rate of preterm birth and adverse neonatal outcomes to patients with proven intra-amniotic infection [7]. In addition, women with sterile intra-amniotic inflammation also present acute histologic chorioamnionitis and funisitis in their placentas [7], inflammatory lesions strongly associated with intra-amniotic infection [21–33]. Taken together, these findings suggest that, similar to intra-amniotic infection, sterile intra-amniotic inflammation has deleterious effects on the offspring. Hence, elucidation of the mechanisms that lead to sterile intra-amniotic inflammation will help to identify novel therapeutic strategies to prevent prematurity and its adverse neonatal outcomes.

Sterile inflammation can be initiated by endogenous danger signals derived from cellular necrosis, senescence, or stress, referred to as damage-associated molecular patterns [34, 35] or alarmins [36]. In line with this concept, women with spontaneous preterm labor and sterile intra-amniotic inflammation harbor an alarmin-enriched cytokine network in the amniotic cavity, containing interleukin (IL)-1α and high mobility group box 1 (HMGB1) [37], both of which are danger signals [38]. Administration of IL-1α and HMGB1 induces preterm birth in mice [39–42], which suggested to us that other alarmins can trigger the mechanisms that lead to preterm parturition in the context of sterile intra-amniotic inflammation. Given that S100 proteins can act as alarmins [38, 43, 44] and women with spontaneous preterm labor and intra-amniotic inflammation have elevated concentrations of the S100B protein [45], we first investigated whether the ultrasound-guided intra-amniotic administration of S100B will induce preterm labor/birth and adverse neonatal outcomes.

The mechanisms that lead to sterile intra-amniotic inflammation in preterm [46–50] and term [46, 51–54] labor implicate inflammasomes, which are cytoplasmic high-molecular-weight multisubunit protein complexes that upon assembly induce the activation of caspase-1 [55–73] and the consequent release of mature IL-1β and/or IL-18 [74–82]. Accordingly, alarmins (e.g. HMGB1) can trigger the activation of the nucleotide-binding oligomerization domain, leucine-rich repeat, and pyrin domain-containing 3 (NLRP3) inflammasome in the chorioamniotic membranes, inducing the release of active/mature forms of caspase-1 and IL-1β [83]. Therefore, we next investigated whether the alarmin S100B could induce the activation of the NLRP3 inflammasome and hypothesized that preterm labor/birth and adverse neonatal outcomes could be prevented by inhibiting this pathway.

Materials and methods

Mice

C57BL/6 (B6) mice were purchased from The Jackson Laboratory (Bar Harbor, ME), and bred in the animal care facility at C.S. Mott Center for Human Growth and Development at Wayne State University (Detroit, MI). All mice were kept under a circadian cycle (light: dark = 12:12 h). Females, 8–12 weeks old, were bred with males of proven fertility. Female mice were checked daily in the morning for the appearance of a vaginal plug, which indicated 0.5 days post coitum (dpc). Females were then housed separately from the males, their weights were monitored daily, and a gain of 2 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).

Intra-amniotic administration of S100B

Pregnant B6 mice (16.5 dpc) were anesthetized by inhalation of 2–3% isoflurane (Aerrane, Baxter Healthcare Corporation, Deerfield, IL) and 1–2 L/min of oxygen in an induction chamber. Anesthesia was maintained with a mixture of 1.75–2% isoflurane and 2 L/min of oxygen during the ultrasound procedure, which was performed using the Vevo® 2100 Imaging System (VisualSonics Inc., Toronto, Ontario, Canada). Mice were positioned on the heating pad included in the ultrasound system, and stabilized with an adhesive tape. Fur

removal from the abdomen was accomplished by applying Nair depilatory cream (Church & Dwight Co., Inc., Ewing, NJ) to that area. Body temperature was maintained at 37 ± 1°C and detected by using a rectal probe included in the ultrasound system. Respiratory and heart rates were monitored through 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 S100B (Cat#1820-SB; R&D Systems, Inc., Minneapolis, MN) at a concentration of 0.1 ng (n = 5), 1.2 ng (n = 5), 30 ng (n = 5), or 60 ng (n = 10) in 25 μL of sterile 1X phosphate-buffered saline (PBS; Fisher Scientific Bioreagents, Fair Lawn, NJ) was performed in each amniotic sac using a 30-gauge needle (BD PrecisionGlide Needle, Becton Dickinson, Franklin Lakes, NJ). These dosages were chosen based on the pathophysiologic concentrations of S100B in the amniotic fluid of women with intra-amniotic inflammation and/or infection who delivered preterm (100–30 000 pg/mL) [45]. Controls were injected with 25 μL of PBS per amniotic sac (n = 9). Following the ultrasound, mice were placed under a heat lamp until they regained full motor function which occurred 5–10 min after heating.

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

Immediately after intra-amniotic injection of S100B or PBS, dams were monitored until delivery using a video camera and infrared light (Sony Corporation, Tokyo, Japan). Gestational length was defined as the time elapsed from the detection of the vaginal plug (0.5 dpc) through the delivery of the first pup. Preterm labor/birth was defined as delivery occurring before 18.5 dpc, and its rate was represented by the percentage of females delivering preterm among the total number of mice. Dystocia was defined as disturbed progression of labor (duration of labor ≥ 6 h), and its rate was represented by the percentage of females in dystocia among those delivering successfully. The rate of neonatal mortality at birth was defined as the proportion of born pups found dead among the total litter size.

Fetal and placental weights

Pregnant B6 mice were injected with 60 ng/25 μL of S100B or 25 μL of PBS in each amniotic sac on 16.5 dpc (n = 4–16 dams). After 16–18 h, dams were euthanized, amniotic fluid was collected, and fetal & placental weights were determined using a scale (DIA-20; American Weight Scales, Norcross, GA). Photographs of fetuses and placentas were also recorded.

Analysis of amniotic fluid cytokine concentrations

Concentrations of the proinflammatory cytokines IFN-γ, IL-10, IL-12p70, IL-1β, IL-2, IL-4, IL-5, IL-6, KC/GRO, and TNF-α in amniotic fluid were determined by using a sensitive and specific multiplex assay kit obtained from Meso Scale Discovery (Cat#K15048D; Rockville, MD). This multiplex kit was initially validated in our laboratory prior to the execution of this study. Amniotic fluid concentrations of cytokines were obtained by interpolation from the standard curve. The sensitivities of the assays were 0.04 pg/mL (IFN-γ), 0.95 pg/mL (IL-10), 9.95 pg/mL (IL-12p70), 0.11 pg/mL (IL-1β), 0.22 pg/mL (IL-2), 0.14 pg/mL (IL-4), 0.07 pg/mL (IL-5), 0.61 pg/mL (IL-6), 0.24 pg/mL (KC/GRO), and 0.13 pg/mL (TNF-α). The mean coefficients of variation ranged from 5.097 to 18.135%.

Immunoblotting

Pregnant B6 mice underwent ultrasound-guided intra-amniotic injection of S100B (60 ng/25 μL per sac) (n = 6) or PBS (25 μL per sac) as controls on 16.5 dpc (n = 6 each). Dams were sacrificed on 17.5 dpc and two to four fetal membrane samples were obtained from each dam. Tissue lysates were prepared by homogenizing snap-frozen fetal membranes in 1X PBS containing a complete protease inhibitor cocktail (Roche Applied Sciences, Mannheim, Germany). Lysates were centrifuged at 15 700 × *g* for 5 min at 4°C and the supernatants were stored at –80°C until use. Prior to immunoblotting, total protein concentration was determined using the Quick Start Bradford Protein Assay Kit (Bio-Rad, Hercules, CA). Fetal membrane lysates (50 μg per well) were subjected to electrophoresis in 4–12% sodium dodecyl sulfate-polyacrylamide gels (Cat#NP0336BOX, Invitrogen, Carlsbad, CA). Separated proteins were then transferred onto nitrocellulose membranes (Cat#1620145, Bio-Rad). Next, the nitrocellulose membranes were submerged in blocking solution (Starting-Block T20 Blocking Buffer, ThermoFisher Scientific, Inc., Rockford, IL) and probed overnight at 4°C with the following mouse antibodies: mouse anti-NLRP3 (Cat#AG-20B-0014-C100, 1 μg/mL, Adipogen Life Sciences, San Diego, CA), rat anti-Caspase-1 (Cat# 14-9832-82, 5 μg/mL, Invitrogen), and rat anti-IL-1β (Cat#MAB4011, 1 μg/mL, R&D Systems, Inc., Minneapolis, MN) (Supplementary Table S1). Finally, nitrocellulose membranes were then stripped with Restore PLUS Western Blot Stripping Buffer (Pierce Biotechnology, ThermoFisher 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, St. Louis, MO) (Supplementary Table S1). 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.

Lysates of murine bone marrow-derived macrophages served as positive controls for NLRP3, pro-caspase-1, and caspase-1 p35 protein expression, and culture supernatants from the same cells served as positive controls for active IL-1β and caspase-1 p20. Briefly, the murine bone marrow-derived macrophages were incubated with 0.5 μg/mL of lipopolysaccharide (LPS; *Escherichia coli* 0111: B4; Sigma-Aldrich) for 4 h followed by 10 μM of nigericin (Cat#N7143; Sigma-Aldrich) for an additional hour. After treatment, cells were lysed and culture supernatants were concentrated 10X by centrifugation using Amicon Ultra Centrifugal Filters (Cat#UFC800324; Millipore Sigma, Burlington, MA).

Inhibition of the NLRP3 inflammasome via MCC950 to prevent S100B-induced preterm labor/birth

Pregnant B6 mice were intraperitoneally injected on 16.5 dpc with 50 mg/kg of the NLRP3 inhibitor MCC950 (Cat#PZ0280; Sigma-Aldrich) dissolved in 200 μL of sterile PBS. This dosage of MCC950 has been shown to inhibit the NLRP3 inflammasome in vivo [84]. Shortly after, mice received the intra-amniotic administration of S100B (60 ng/25 μL per sac, n = 14) or PBS (25 μL per sac, n = 9). Positive controls were injected with S100B alone (60 ng/25 μL per sac, n = 16). The rates of preterm labor/birth and neonatal mortality as well as gestational length were recorded in each group, as previously mentioned. The effect of NLRP3 inflammasome inhibition was also tested at term parturition, and in this case, mice were injected with MCC950 on 18.5 dpc and/or 19.5 dpc (until delivery).

Statistical analysis

Statistical analyses were performed using the SPSS software, Version 19.0 (IBM Corporation, Armonk, NY). The Shapiro–Wilk test was used to evaluate the distribution of the data. The Fisher exact test was used to compare the rates of preterm labor/birth and neonatal mortality between groups. The Mann–Whitney *U*-test was used to compare the rest of the variables. Survival curves were used to plot and compare the gestational length data (Mantel–Cox test). A P-value ≤ 0.05 was considered statistically significant.

Results

Intra-amniotic administration of S100B induces preterm labor/birth and neonatal mortality

We first investigated whether pathophysiological concentrations of the alarmin S100B could induce preterm labor/birth in mice. Validation of this murine model included dams that were intra-amniotically injected with 0.1 ng (n = 5; lowest pathological concentration [45]), 1.2 ng (n = 5), or 30 ng (n = 5; highest pathological concentration [45]) of S100B (Figure 1A and B) based on the range of amniotic fluid concentrations observed in women who delivered preterm with intra-amniotic inflammation [45]. No preterm labor/birth was observed in these mice (data not shown). Given that mice required double the pathological amniotic fluid concentration of LPS [85] to deliver preterm [86], we tested whether the intra-amniotic administration of 60 ng of S100B would induce preterm labor/birth (Figure 1A and B). Intra-amniotic administration of this concentration of S100B induced a 50% (5/10) rate of preterm birth (Figure 1C) compared to PBS-injected controls, which all delivered at term (9/9). Consequently, the gestational length of mice injected with S100B was lower than that of PBS-injected controls (Figure 1D). In addition, the rate of neonatal mortality was increased among pups born to dams injected with S100B (59.7%, 47/79 pups from 10 litters) compared to those born to PBS-injected controls (14.4%, 8/56 pups from 8 litters) (Figure 1E).

The effects of the intra-amniotic administration of the alarmin S100B on the fetus prior to preterm birth

A large proportion (59.7%) of the preterm neonates born to dams injected with S100B failed to thrive (Figure 1E). Therefore, we next evaluated whether the intra-amniotic administration of S100B could alter fetal and placental growth parameters (e.g. height and weight) and/or induce a proinflammatory response in the amniotic cavity. Fetuses from dams injected with S100B appeared similar in size to those from PBS-injected controls (Figure 2A). In addition, the placental and fetal weights were not different between these two groups of mice (Figure 2B and C). However, fetuses looked dehydrated (Figure 2A) and the concentration of several proinflammatory cytokines (IL-1β, IFN-γ, IL-12p70, IL-10, IL-2, IL-4, and IL-5) in the amniotic fluid were higher in dams injected with S100B than in

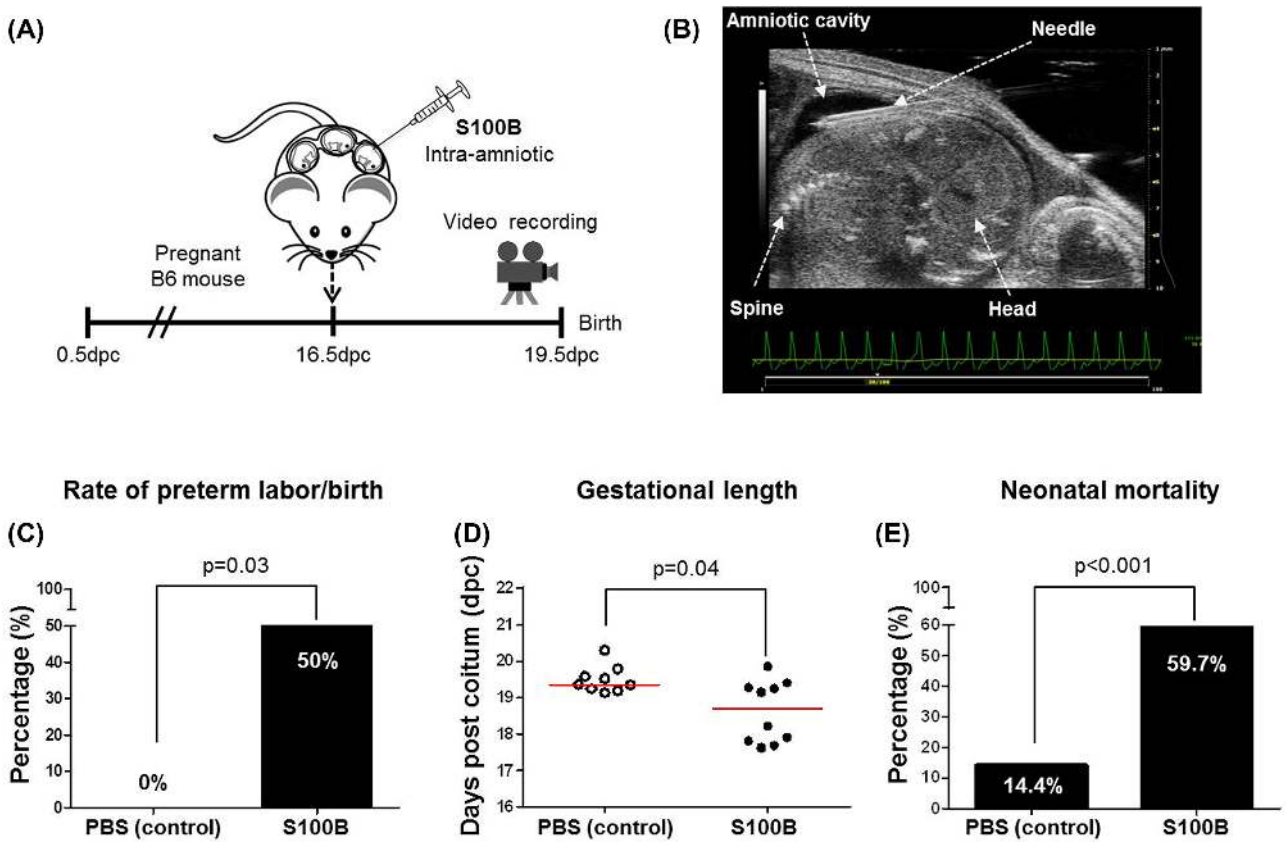

Figure 1. Intra-amniotic administration of the alarmin S100B. (A) Pregnant C57BL/6 dams were intra-amniotically injected on 16.5 days post coitum (dpc) with 60 ng/25 μL of S100B or saline (1X phosphate-buffered saline; PBS). (B) Intra-amniotic injections were performed using a high-frequency ultrasound system. After injection, mice were monitored by camera until delivery and the (C) rate of preterm labor/birth, (D) gestational length, and (E) rate of neonatal mortality were recorded. Black lines represent medians. n = 8–10 dams per group.

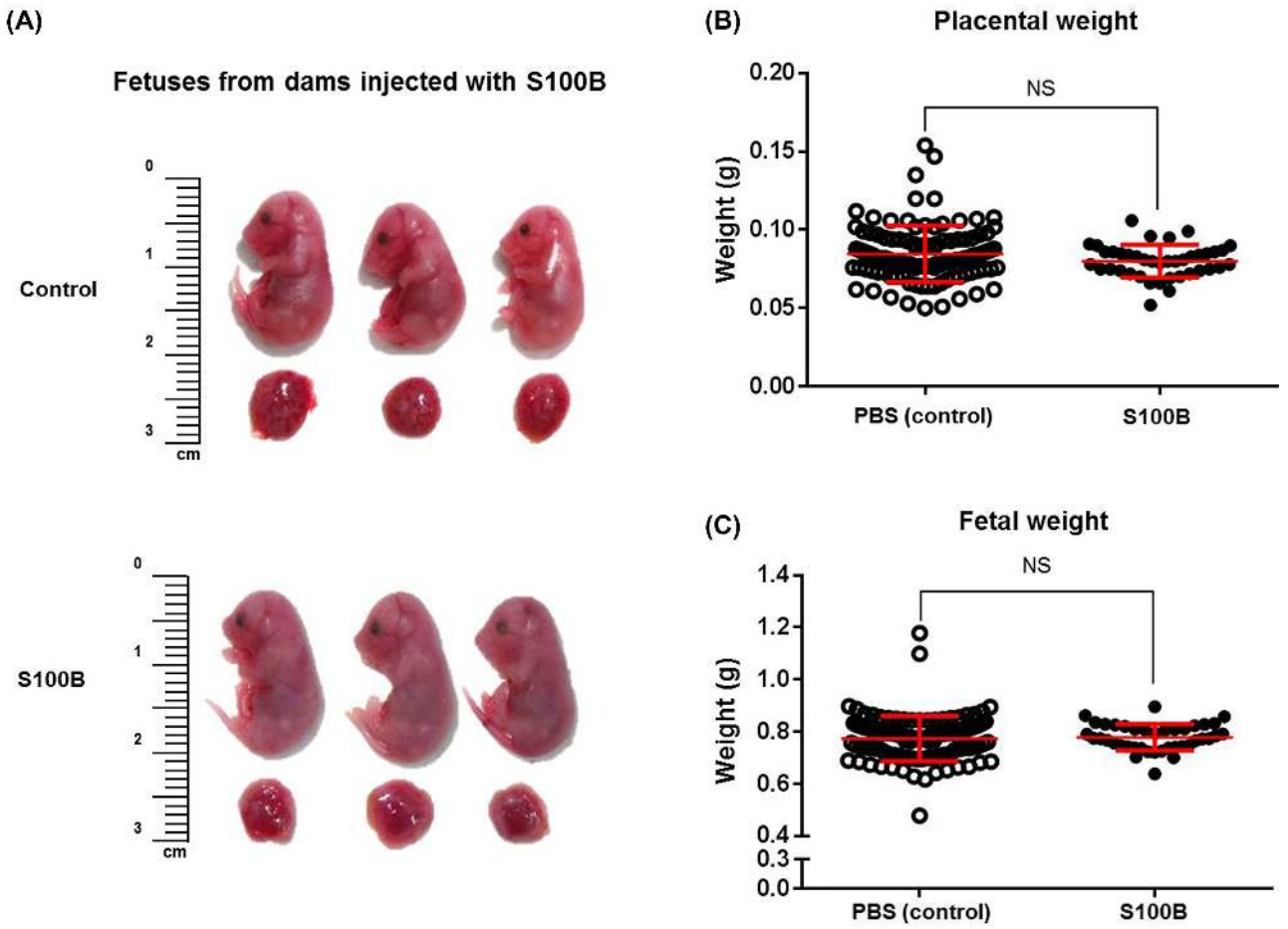

Figure 2. Fetal and placental growth parameters after intra-amniotic administration of the alarmin S100B. (A) Sizes of fetuses and placentas from dams intra-amniotically injected with S100B. Ruler lines indicate centimeters. The (B) placental and (C) fetal weights were recorded. Gray lines represent means ± standard deviation. n = 4–16 litters per group.

controls (Figure 3). Amniotic fluid concentrations of IL-6 also tended to be elevated in dams injected with S100B (Figure 3). No differences were observed in amniotic fluid concentrations of KC/GRO and TNF-α between these two groups of mice (data not shown). These results show that although the intra-amniotic administration of S100B did not apparently affect the size and weight of the fetus and placenta, elevated concentrations of this danger signal induced an intra-amniotic proinflammatory response.

Intra-amniotic administration of the alarmin S100B induces NLRP3 inflammasome activation

Our previous studies suggested that the alarmin HMGB1 induces preterm labor/birth by activating the NLRP3 inflammasome [41, 83]. In addition, we reported evidence indicating that there is activation of the NLRP3 inflammasome in the chorioamniotic membranes from women with spontaneous preterm labor [47]. Therefore, we next investigated whether the intra-amniotic administration of S100B could induce the activation of the NLRP3 inflammasome in the fetal membranes prior to preterm birth. As readouts of NLRP3 inflammasome activation, we determined the protein quantity of the NLRP3 sensor molecule of the inflammasome, the active forms of caspase-1, and the mature form of IL-1β (Figure 4A and B). The protein quantities of NLRP3 and mature IL-1β were higher in the fetal membranes of dams injected with S100B than in those from controls (Figure 4C and D). The protein quantities of caspase-1 p20 and p35 were greater in the fetal membranes of dams injected with S100B than in those of controls (Figure 4E); yet, this increase did not reach statistical significance for p20 (Figure 4F). The protein quantity of pro-caspase-1 did not increase upon intra-amniotic injection of S100B (Figure 4B). These findings indicate that the intra-amniotic administration of S100B induces the NLRP3 inflammasome activation in the fetal membranes prior to preterm birth.

Inhibition of the NLRP3 inflammasome prevents S100B-induced preterm labor/birth and adverse neonatal outcomes

Next, we investigated whether the in vivo inhibition of the NLRP3 inflammasome via MCC950 [84] could prevent alarmin-induced preterm labor/birth and reduce adverse neonatal outcomes (Figure 5A). Administration of MCC950 prevented S100B-induced preterm labor/birth by 35.7% (50% of preterm birth rate with S100B alone [8/16] vs 14.3% of preterm birth rate with MCC950 + S100B [2/14]) (Figure 5B). Control mice injected with MCC950 + PBS delivered at term (10/10) (Figure 5B). Consequently, in dams injected with MCC950 + S100B the gestational length was extended, similar to that of controls (Figure 5C). Importantly, treating

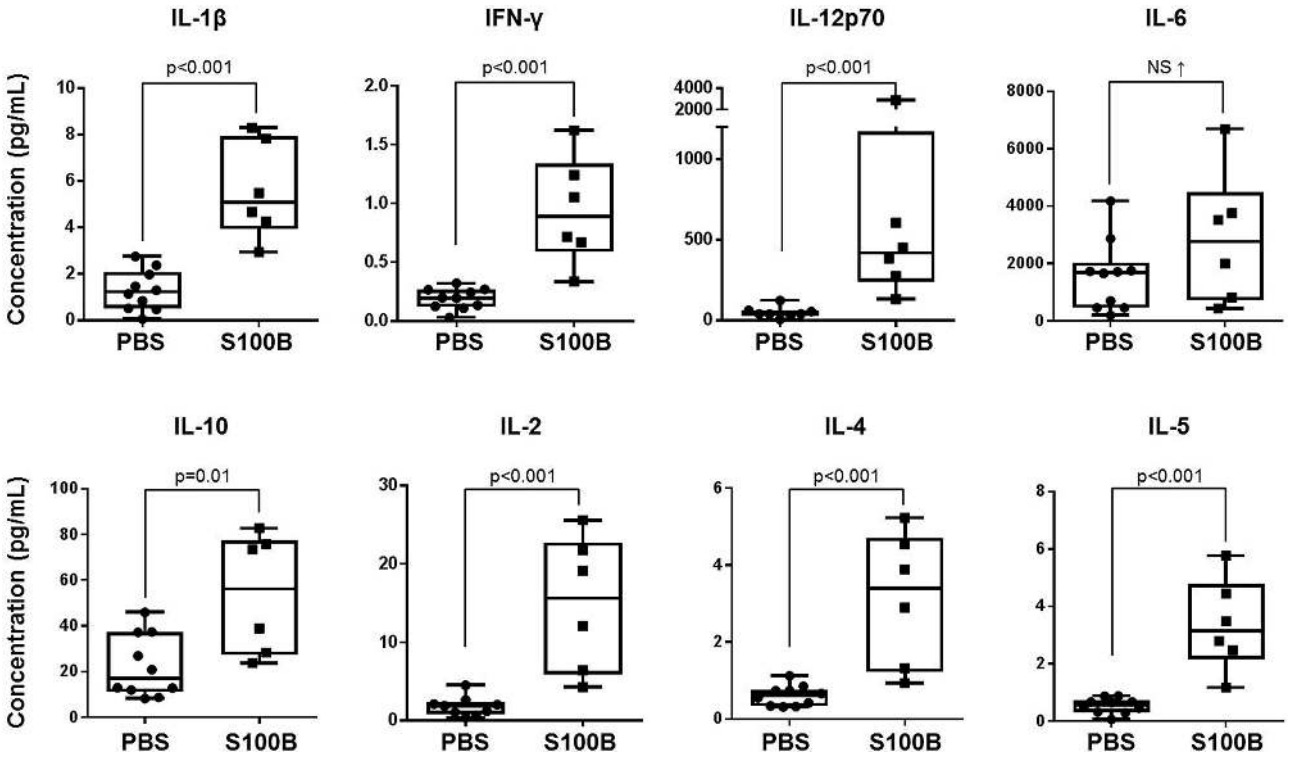

Figure 3. Amniotic fluid cytokines in dams intra-amniotically injected with the alarmin S100B. Amniotic fluid concentrations of IL-1β, IFN-γ, IL-12p70, IL-6, IL-10, IL-2, IL-4, and IL-5 are shown. Lines represent medians. n = 6–10 dams per group.

S100B-injected dams with MCC950 reduced the rate of neonatal mortality (S100B [55.9%, 71/125 from 15 litters] vs MCC950 + S100B [29.2%, 27/90 from 12 litters]), which was similar to that observed in MCC950 + PBS controls (25.4%, 15/57 from 6 litters) (Figure 5D). These data indicate that inhibition of the NLRP3 inflammasome could dampen sterile intra-amniotic inflammation, preventing preterm labor/birth and adverse neonatal outcomes.

Inhibition of the NLRP3 inflammasome in term parturition

Activation of the NLRP3 inflammasome has also been implicated in the mechanisms that lead to the term parturition [51–54]. Therefore, we also investigated whether the in vivo inhibition of the NLRP3 inflammasome via MCC950 [84] could obstruct the normal process of term parturition (i.e. dystocia) or extend the gestational length. Administration of MCC950 to pregnant mice on 18.5 dpc (Figure 6A) resulted in a modest increase in dystocia (20%, 2/10) compared to PBS controls (0%, 0/10). Yet, most of the MCC950-injected mice delivered at similar gestational lengths to controls (Figure 6B). In addition, MCC950-injected dams delivered healthy term neonates, similar to those from control dams (data not shown). These findings suggest that, in mice, the activation of the NLRP3 inflammasome is not required for the onset of term parturition.

Discussion

Principal findings

The intra-amniotic administration of the alarmin S100B (1) induced a 50% rate of preterm labor/birth; (2) a high rate (59.7%) of neonatal mortality; (3) did not alter the fetal and placental weights but caused a proinflammatory response in the amniotic cavity; and (4) induced the activation of the NLRP3 inflammasome in the fetal membranes, as evidenced by the upregulation of the NLRP3 protein and increased release of active caspase-1 and mature IL-1β. Inhibition of the NLRP3 inflammasome via the specific inhibitor MCC950 prevented preterm labor/birth by 35.7% and reduced the rate of neonatal mortality by 26.7%. Interestingly, inhibition of the NLRP3 inflammasome at term did not drastically obstruct the normal process of parturition.

The alarmin S100B induces preterm labor/birth

Herein, we first showed that the intra-amniotic administration of the alarmin S100B, at amniotic fluid concentrations based on those observed in women with intra-amniotic inflammation [45], induced preterm labor/birth. Previous studies have shown that the systemic administration of the alarmin IL-1α can result in preterm birth in mice [39, 40]. A more recent study showed that the intra-amniotic administration of the prototypical alarmin HMGB1 at pathophysiological amniotic fluid concentrations also induces preterm labor/birth [41]. In addition, IL-33 (a classical alarmin [38, 87]) is expressed in the decidual tissues and upregulated in cases with acute histologic chorioamnionitis [88], a placental lesion associated with preterm labor [31]. Together, these findings strengthen our hypothesis that alarmins can induce intra-amniotic inflammation and preterm labor and birth.

It is worth mentioning that the concentration of S100B required for inducing preterm birth in mice is higher than what is observed in the clinical setting of intra-amniotic inflammation [45]. This could be explained by the fact that several alarmins are present in the amniotic cavity of women with sterile intra-amniotic inflammation [37] and, therefore, a single alarmin may not be enough to recapitulate the

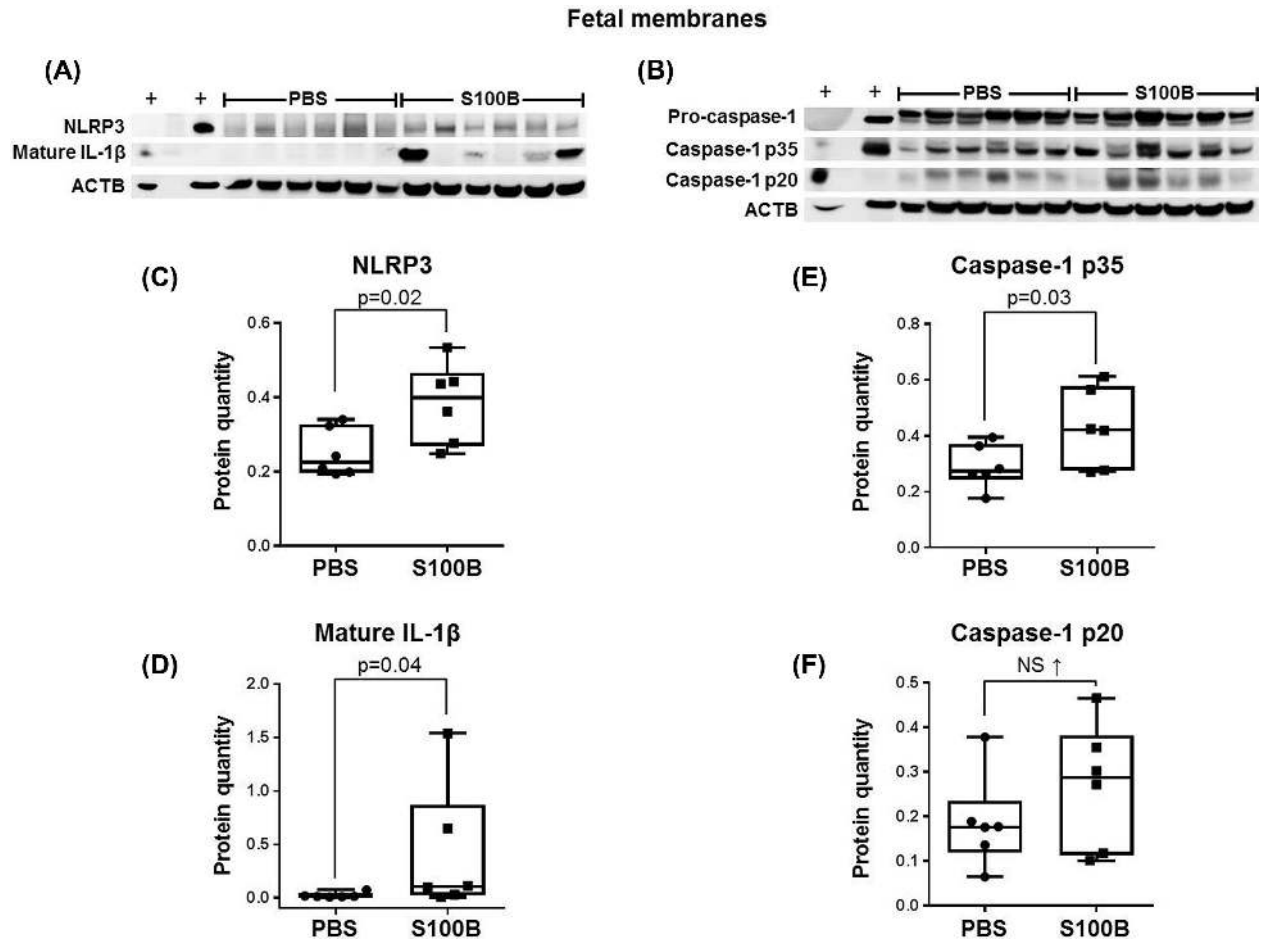

Figure 4. Protein quantities of the NLRP3 inflammasome components in the fetal membranes from dams intra-amniotically injected with the alarmin S100B. Images showing immunoblotting of (A) NLRP3 and mature IL-1β or (B) pro-caspase-1, caspase-1 p35, and caspase-1 p20 in fetal membranes lysates. Bone marrow-derived macrophages served as positive controls for NLRP3, pro-caspase-1, and caspase-1 p35, and culture supernatants from the same cells were positive controls for active IL-1β and caspase-1 p20. Lines represent medians. n = 6 dams per group.

clinical condition. In addition, mouse–human differences should be acknowledged [89].

A central question is: What are the sources of alarmins in the amniotic cavity? We have previously provided evidence suggesting that the chorioamniotic membranes from women with spontaneous preterm labor and birth display signs of cellular senescence [8]. Such a process is associated with the release of alarmins [90]; therefore, the chorioamniotic membranes may be a source of alarmins in the context of sterile intra-amniotic inflammation.

The alarmin S100B induces a proinflammatory response in the amniotic cavity and adverse neonatal outcomes

The intra-amniotic administration of S100B induced a proinflammatory response in the amniotic cavity. S100 proteins can activate the MAPK and NF-κB pathways via receptor for advanced glycation endproducts (RAGE) and/or Toll-like receptor (TLR)-4, leading to the release of multiple inflammatory mediators such as cytokines [38]. In line with this evidence, the chorioamniotic membranes upregulate the expression of RAGE upon incubation with alarmins (e.g. HMGB1) [83]. Other fetal tissues such as the lung can also express this receptor [91, 92], indicating that the conceptus can respond to danger signals (e.g. S100B) initiating a cytokine storm in the amniotic cavity. This inflammatory response could cause deleterious effects on the fetus, inducing adverse neonatal outcomes [93–102]. The relationship between alarmins and intra-amniotic inflammation is such that the concentration of S100B in the cerebrospinal fluid, peripheral blood, and umbilical cord blood is used as a biomarker for neonatal injury [103]. Consistent with these findings, approximately 60% of neonates born to dams intra-amniotically injected with S100B died shortly after delivery; yet, 40% of these neonates continue to thrive. This is in contrast to what is observed in neonates born to dams injected with microbial products (e.g. LPS), which all die shortly after delivery [104], suggesting that the inflammatory process initiated by alarmins is weaker than that initiated by microbes invading the amniotic cavity. This observation has promising implications since pregnancy-approved drugs with anti-inflammatory properties (e.g. progesterone [105–112], steroids [113–115], human chorionic gonadotrophin [116]) could be used for the treatment of sterile intra-amniotic inflammation.

The alarmin S100B induces the activation of the inflammasome prior to preterm birth

In the current study, we found that the intra-amniotic administration of S100B induced the activation of the NLRP3 inflammasome in the

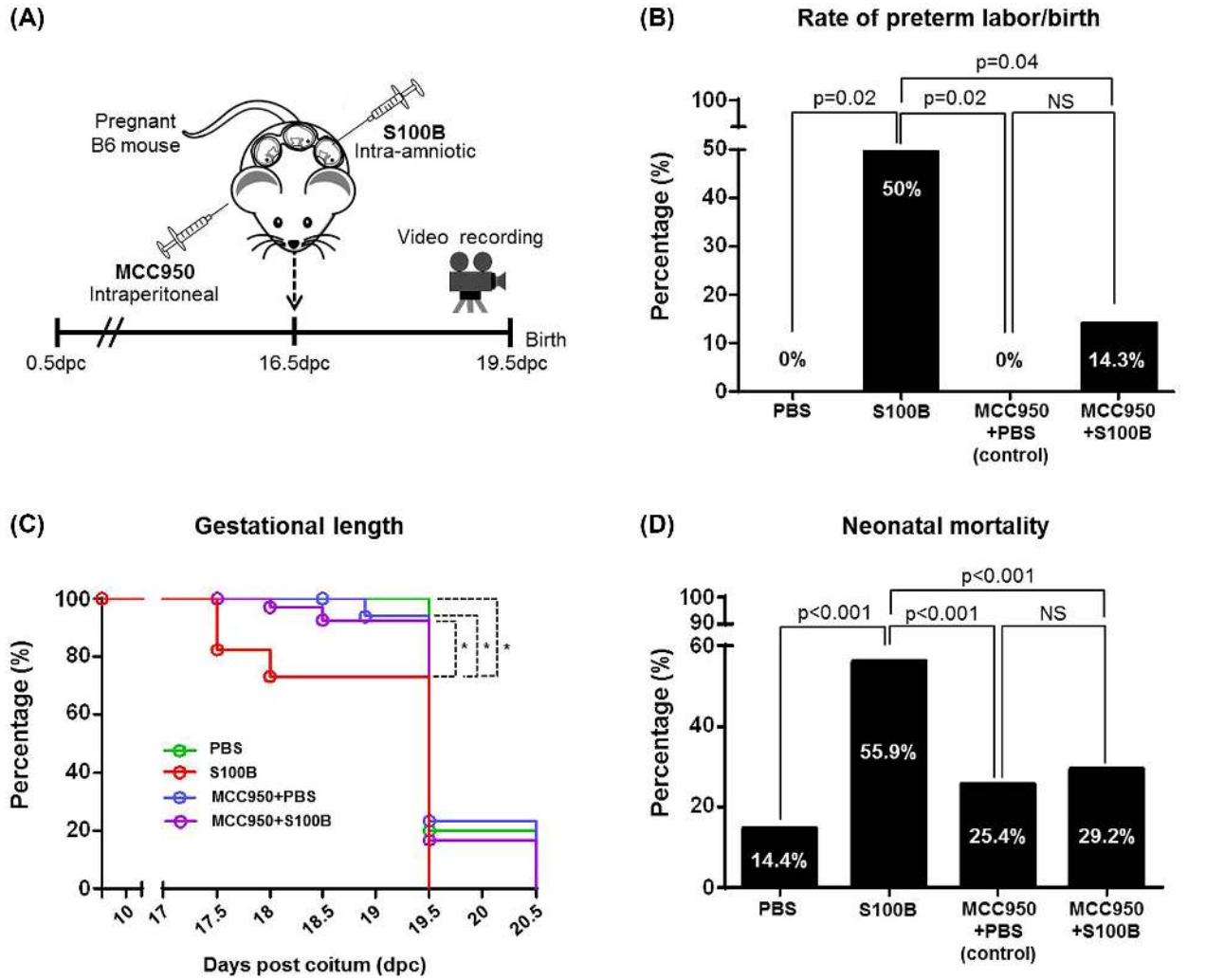

Figure 5. Inhibition of the NLRP3 inflammasome via MCC950 in dams intra-amniotically injected with S100B. (A) Pregnant C57BL/6 dams were intraperitoneally injected on 16.5 days post coitum(dpc) with 50 mg/kg of the NLRP3 inhibitor MCC950 followed by intra-amniotic administration of S100B (60 ng/25 μL) or saline (1X phosphate-buffered saline; PBS). After injection, mice were monitored by camera until delivery and the (B) rate of preterm labor/birth, (C) gestational length, and (D) rate of neonatal mortality were recorded. $^{*}P < 0.004$. n = 6–16 dams per group.

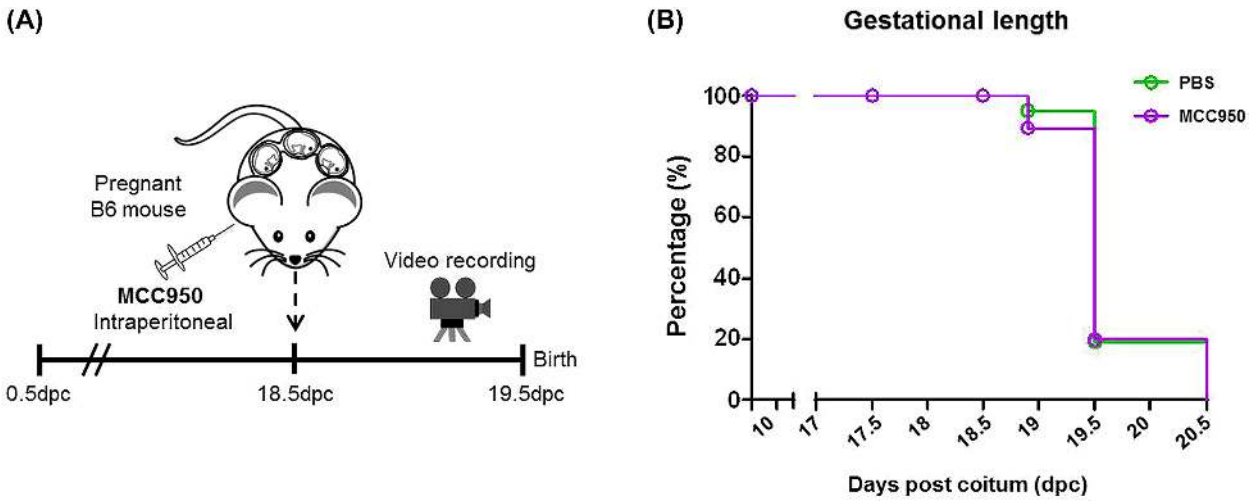

Figure 6. Administration of MCC950 at term gestation. (A) Pregnant C57BL/6 dams were intraperitoneally injected from 18.5 days post coitum (dpc) until delivery with 50 mg/kg of the NLRP3 inhibitor MCC950. After injection, mice were monitored by camera until delivery and the (B) gestational length was recorded. n = 10 dams per group.

fetal membranes, as evidenced by increased quantities of NLRP3, active caspase-1, and mature IL-1β. These findings are consistent with previous in vitro and in vivo studies showing that S100 proteins (e.g. S100A8/A9/A12) can induce the activation of the NLRP3 inflammasome [117–119]. In our prior studies, we surmised that the prototypical alarmin HMGB1 also induces preterm labor/birth by activating the NLRP3 inflammasome in the chorioamniotic membranes [41, 83]. Importantly, the current S100B-induced preterm labor/birth model resembles what is observed in the human syndrome: (1) the chorioamniotic membranes of women with spontaneous preterm labor and acute histologic chorioamnionitis (a placental lesion observed in patients with preterm labor and sterile intra-amniotic inflammation [5, 7]) had higher expression/quantity of NLRP3, active forms of caspase-1 and mature IL-1β, as well as increased assembly of the inflammasome complex than those without this placental lesion [47]; and (2) there is enhanced inflammasome activation (amniotic fluid concentrations of extracellular ASC, the adaptor protein of the inflammasome that is released upon activation [120, 121]) in the amniotic cavity of women with spontaneous preterm labor and sterile intra-amniotic inflammation than in those without this clinical condition who delivered preterm or at term [49]. Collectively, these findings support the concept that alarmins induce sterile intra-amniotic inflammation by activating the NLRP3 inflammasome in the fetal membranes, which can lead to preterm labor and birth. It is worth mentioning that, besides the fetal membranes, alarmins could also be sensed by pattern recognition receptors such as RAGE expressed by the placental [122, 123], fetal [92], and decidual [123] tissues. Therefore, both the fetal and maternal tissues participate in the mechanisms leading to sterile intra-amniotic inflammation.

Treatment with the NLRP3 inhibitor MCC950 prevents alarmin-induced preterm labor/birth and adverse neonatal outcomes

Up to this point, our results show that the alarmin S100B induces intra-amniotic inflammation by activating the NLRP3 inflammasome, which leads to preterm labor/birth and adverse neonatal outcomes. Therefore, we next focused on inhibiting the NLRP3 inflammasome, which has been targeted for treating inflammatory diseases [124–126]. MCC950 is a specific small-molecule inhibitor of the NLRP3 inflammasome [84]. The mechanism whereby MCC950 inhibits the NLRP3 inflammasome is still unclear. It was proposed that this molecule targets the interaction between NLRP3 and NEK7, a serine-threonine kinase which is upstream of NLRP3 [127, 128], preventing the activation and assembly of the inflammasome complex [129]. However, a recent report showed that MCC950 suppressed the NLRP3 inflammasome in a NEK7-independent manner [130]. Importantly, MCC950 specifically targets the NLRP3 inflammasome, but not the NLRP1, AIM2, or NLRC4 inflammasomes, in a murine model of experimental autoimmune encephalitis [84]. This specific NLRP3 inhibitor has been widely used to reduce inflammasome-mediated inflammatory processes such as diabetic encephalopathy [131], colitis [130], traumatic brain injury [132, 133], atherosclerosis [134], and stroke [135] in mice. Indeed, a recent study provided evidence that the treatment of inflammatory diseases with NLRP3 inhibitors is safe in humans [136]. More recently, we demonstrated that inhibiting the activation of the NLRP3 inflammasome via MCC950 prevents microbial-induced intra-amniotic inflammation, preterm labor/birth, and adverse neonatal outcomes [50]. Herein, we showed that MCC950 can prevent S100B-induced preterm labor/birth, suggesting that blocking the NLRP3 inflammasome can also offer a novel strategy to prevent sterile intra-amniotic inflammation, a condition that currently lacks treatment.

A question raised by these findings is whether the administration of MCC950 is safe for the mother and, more importantly, the fetus. Recent studies have shown that MCC950 can be used to treat inflammatory disorders and diseases in the neonatal [84, 137] and infant [138] periods. However, further investigation is required to evaluate the safety of inhibiting the NLRP3 inflammasome during pregnancy.

Inhibition of the NLRP3 inflammasome at term does not obstruct the normal process of parturition

Accumulating evidence supports a role for the inflammasome in the mechanisms that lead to spontaneous labor at term. Such evidence includes the demonstration that amniotic fluid concentrations of caspase-1 [46], IL-1β [139–142], and extracellular ASC [54] are higher in women with spontaneous labor at term than in those without labor. In addition, the chorioamniotic membranes from women with spontaneous labor at term had greater expression/amounts of NLRP3, active caspase-1, mature IL-1β, and ASC/caspase-1 complexes (i.e. inflammasome assembly) than those without labor [51, 53, 143]. Therefore, we hypothesized that inhibiting the NLRP3 inflammasome could obstruct the normal process of term parturition. Contrary to our hypothesis, most of the animals injected

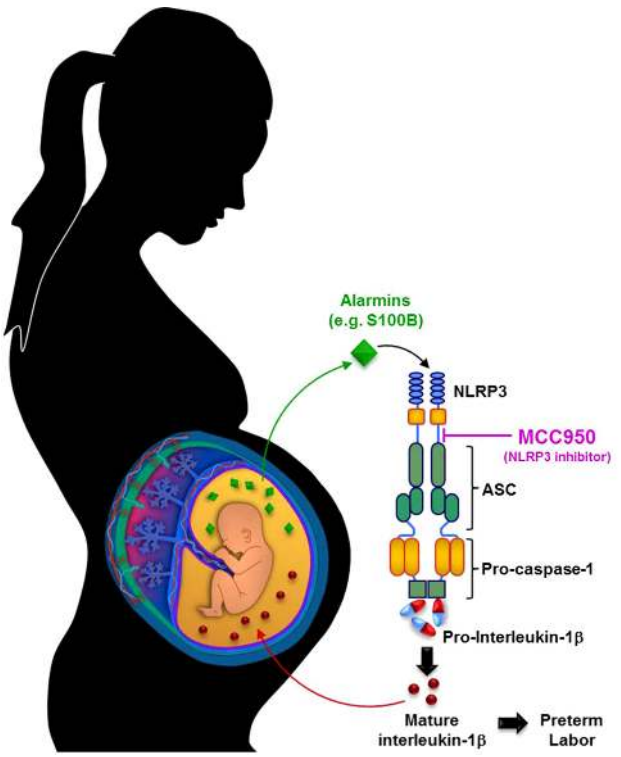

Figure 7. Conceptual framework. The alarmin S100B can initiate an intra-amniotic inflammatory response characterized by the activation of the NLRP3 inflammasome (complex including the NLRP3 sensor molecule, the ASC adaptor protein, and pro-caspase-1). This pathway leads to the processing and release of caspase-1 and the subsequent maturation of IL-1β which, in turn, can lead to preterm labor and birth. Inhibiting the NLRP3 inflammasome (e.g. MCC950) represents a novel strategy to prevent sterile intra-amniotic inflammation, preterm labor and birth, and adverse neonatal outcomes.

with the NLRP3 inhibitor, MCC950, delivered at similar gestational lengths to controls. Yet, a small proportion (20%) of these underwent dystocia. These data suggest that, although the NLRP3 inflammasome is implicated in human labor at term, such a pathway is not necessary for the onset of term parturition in mice. In addition, these findings indicate that targeting the NLRP3 inflammasome does not interfere with the physiological process of term parturition, but is effective for tackling the pathological process of preterm labor.

Conclusion

The data presented herein showed that the intra-amniotic administration of the alarmin S100B at clinically relevant concentrations induces preterm labor/birth and adverse neonatal outcomes by initiating intra-amniotic inflammatory responses. Specifically, the alarmin S100B activated the NLRP3 inflammasome in the fetal membranes, as evidenced by the increased protein quantities of the NLRP3 sensor molecule, active caspase-1, and mature IL-1β (Figure 7). Importantly, alarmin-induced preterm labor/birth and neonatal mortality were reduced by treating dams with the NLRP3 inhibitor, MCC950 (Figure 7). Yet, the inhibition of the NLRP3 inflammasome at term does not obstruct the physiological process of parturition. Collectively, these findings suggest that sterile intra-amniotic inflammation could be treated by targeting the NLRP3 inflammasome.

Supplementary data

Supplementary data are available at *BIOLRE* online.

Supplementary Table S1. Antibodies used for immunoblotting.

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Conflict of Interest: The authors declare no potential conflicts of interest.

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